Diversitat del gènere *Loxosceles* Heineken & Lowe, 1832 a la Mediterrània i les Illes Canàries: sistemàtica, biogeografia i loxoscelisme

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TESI DOCTORAL

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TESI DOCTORAL

Memòria presentada per

Enric Planas Figueras

per optar al grau de

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Als meus pares

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CONTINGUT

INTRODUCCIÓ GENERAL

LA BIODIVERSITAT

Tot i ser àmpliament generalitzat, el terme **biodiversitat** és relativament recent. Va ser publicat per primer cop el 1986 pel funcionari nord-americà W. Rosen, encara que la seva popularització es deu en gran mesura al famós entomòleg E. O. Wilson. Com passa amb altres conceptes biològics, la definició de biodiversitat és complexa. Biodiversitat prové de la contracció de "diversitat biològica", que primerament s'havia utilitzat per a referir-se a diversitat o riquesa específica (Hamilton, 2005). Tot i això, en les definicions actuals del terme, s'abracen també escales tant per sota com per sobre del nivell d'espècie, com es veu en la definició proposada en el Conveni de les Nacions Unides per la Diversitat Biològica (2012; www.biodiv.org):

> *" Per diversitat biològica s'entén la variabilitat d'organismes vius de qualsevol origen, inclosos, entre d'altres, els ecosistemes terrestres, els marins i altres ecosistemes aquàtics i els complexos ecològics dels quals en formen part; comprèn la diversitat dins de cada espècie, entre les espècies i dels ecosistemes"*

Independentment de quina definició concreta de biodiversitat agafem, una de les qüestions fonamentals en la Biologia és conèixer la biodiversitat del Planeta. Des de 1758, amb l'adveniment del sistema de **nomenclatura binomial** o Linneana, s'han documentat menys de dos milions d'espècies, i s'estima que, com a mínim, en queden per descriure entre 3 i 8 milions, encara que algunes prediccions superen aquestes xifres en un ordre de magnitud (May, 2010; Mora *et al*., 2011; 2013; Scheffers *et al*., 2012; Costello *et al*., 2013). Per tant, seguint amb el mateix ritme actual de descripcions (entre el període 1990 i 2000 fou aproximadament de 8000 descripcions per any; Mora *et al*., 2013), ens trobaríem a mig camí de completar aquesta tasca. De fet, això no seria el problema més greu, si no fos que ens trobem en el que s'ha anomenat la "**sisena extinció massiva**" (Barnosky *et al*., 2011). A diferència de les cinc anteriors, aquesta està bàsicament provocada directament o indirectament per la pressió antropogènica que assola el planeta. La causa més evident n'és l'augment de la temperatura per sobre dels nivells típics en èpoques interglacials, provocat per l'increment en l'emissió de CO_2 derivada d'activitats humanes. Per compensar aquest canvi i donat prou temps, els organismes poden o bé desplaçar-se a nous territoris climàticament més favorables, o adaptar-se a les noves condicions (Gibbons *et al*., 2000;

Walther *et al*., 2002). Actualment diversos factors com la fragmentació de l'hàbitat, la sobreexplotació dels recursos pesquers i cinegètics, la contaminació i les espècies invasores estan actuant en sinergia amb l'escalfament global, i per tant, accelerant les taxes d'extinció (Mooney i Cleland, 2001; Pimm *et al*., 2006; Sinervo *et al*., 2010). Una de les conseqüències d'aquesta **crisi de biodiversitat**, serà per tant, que una part de les espècies del Planeta s'acabarà extingint sense haver estat mai descobertes abans (Mora *et al*., 2013).

L'ESTUDI DE LA BIODIVERSITAT

La unitat d'estudi

L'estudi de la biodiversitat va més enllà de recomptar espècies, però en canvi, les espècies s'erigeixen com a unitats fonamentals en l'organització biològica i en l'estudi de la biodiversitat. El **concepte d'espècie,** és a dir, què és una espècie, s'ha reconegut com la pregunta més fonamental en la sistemàtica (Cracraft, 2002) i per això, la seva resposta ha generat innumerables debats i definicions (Mayr, 1942; Mayden, 1997; de Queiroz, 1998; Cracraft, 2002; Wheeler i Meier, 2000; Wilkins, 2009, entre molts altres). El problema del **concepte d'espècie** o la incapacitat d'acord entorn del concepte d'espècie, va intrínsecament lligat a la **delimitació d'espècies**, o com determinar els límits de les espècies (de Queiroz, 2007). Per tant, l'aplicació d'un concepte o altre d'espècie, es tradueix en una delimitació diferent que tindrà conseqüències en àrees més enllà de les dedicades a aquesta problemàtica, com poden ser la biogeografia, la biologia de la conservació, l'ecologia o l'etologia. Separant la part teòrica comuna dels diferents conceptes, respecte dels criteris operatius que les diferenciaven, de Queiroz (1998, 2005, 2007) va proposar el **concepte unificat d'espècie** (*unified species concept* - USC). Segons l'USC, les espècies equivalen a la separació evolutiva de llinatges metapoblacionals, entenent com a llinatge una successió ancestre-descendent i com a metapoblació, una població inclusiva formada per subpoblacions connectades entre elles. Segons aquest concepte, les propietats que diferencien els anteriors conceptes d'espècie, passen a ser propietats contingents de les espècies en l'USC, i per tant, propietats que les espècies podran, o no, adquirir al llarg del temps, deixant de ser imprescindibles i passant a ser utilitzades com a línies d'evidència en la delimitació de les espècies (Fig. 1). En aquest sentit, una espècie nominal (tàxon) representa una hipòtesi de l'existència d'un llinatge proposada sobre la base d'almenys una línia d'evidència que ho suggereixi, mentre que altres línies d'evidència poden o no sostenir aquesta hipòtesi (de Queiroz, 2007; Padial i de la Riva, 2010).

Conceptes, disciplines i principals metodologies

A causa de l'amplitud en la definició de biodiversitat, diverses disciplines l'estudien des de diferents punts de vista, però una de les principals és sens dubte la **sistemàtica**. Com en altres conceptes, la definició d'aquesta disciplina ha anat variant al llarg de la història i segons les escoles de pensament, però en termes generals es pot definir com la disciplina

dedicada en el seu conjunt a descobrir i anomenar la biodiversitat, a classificar-la i a estudiar-ne les relacions evolutives i la seva distribució (Cracraft, 2002) i inclou la **taxonomia** que se centra a descobrir, delimitar, descriure, anomenar i classificar els organismes o tàxons, tant des d'un punt teòric com pràctic. Els postulats de Willi Hennig (1950, 1966) van assentar les bases de la **sistemàtica filogenètica** o cladística, que promulgava obtenir una classificació que reflecteixi les relacions genealògiques entre els organismes. De forma simplificadora, aquest marc conceptual advoca per la utilització d'estats de caràcters **sinapomòrfics** (derivats i compartits), per tal de reconstruir-ne les relacions evolutives (**filogènies**) i definir grups **monofilètics,** que han de servir de base per a la classificació (Hennig, 1966, 1975). La sistemàtica va viure un canvi de paradigma gràcies a la invenció de la **PCR** (Reacció en Cadena de la Polimerasa), que va generalitzar l'adquisició i l'ús de seqüències d'ADN, permetent la seva utilització en aquest camp i donant a lloc a la **sistemàtica molecular**. Per acomodar aquest nou tipus de caràcters - fins al moment s'havien utilitzat majoritàriament caràcters morfològics - va ser necessària una forçada renovació conceptual, que encara segueix en diversos punts.

Per tal d'inferir les relacions filogenètiques són necessaris un seguit de passos, que s'exemplifiquen a continuació pel cas concret de la reconstrucció filogenètica basada en dades moleculars. El primer pas, consistent en establir l'homologia dels caràcters, rep el nom d'alineament quan els caràcters són moleculars, i identifica les posicions homòlogues en seqüències homòlogues (ortòlogues) de diferents individus. Segons la naturalesa de la seqüència d'ADN, l'alineament pot ser més o menys problemàtic, i per això és necessària la utilització de diferents algoritmes que tinguin en compte les particularitats dels marcadors moleculars utilitzats (Castresana, 2000). Una vegada s'ha obtingut l'alineament, s'escull el model evolutiu que s'ajusta millor a les seqüències utilitzades, que serà depenent de cada matriu (Posada, 2008). S'ha proposat una gran varietat de mètodes de reconstrucció filogenètica, però els més influents són els mètodes basats en l'optimització de caràcters de la **màxima parsimònia**, la **màxima versemblança** i la **inferència bayesiana** (Wiley i Lieberman, 2011). La màxima parsimònia considera com a millor arbre aquell que implica menys nombre de canvis, mentre que la màxima versemblança tracta de buscar l'arbre més versemblant a partir de les dades observades i d'un model evolutiu. A diferència de la primera metodologia, la màxima versemblança calcula la longitud de les branques, que seran proporcionals al nombre de canvis calculats a partir del model evolutiu especificat.

De forma semblant, la inferència bayesiana utilitza un model evolutiu determinat, i en aquest cas, el millor arbre és aquell amb una major probabilitat posterior donades les dades observades i el model d'evolució. Un dels avantatges d'aquesta metodologia és que té en compte la incertesa filogenètica, i se sol presentar l'arbre consens d'aquells amb probabilitats posteriors més elevades. Per la seva flexibilitat i eficàcia en la filogenètica molecular, els dos últims mètodes han desbancat a la màxima parsimònia, que havia estat la metodologia prevalent (Page i Holmes, 1998).

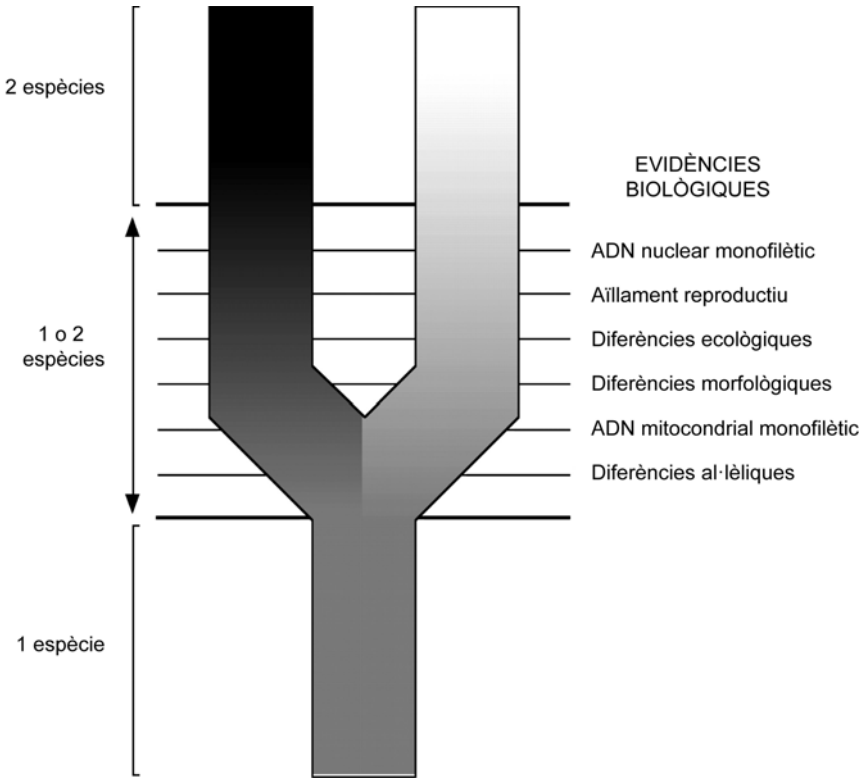

Figura 1 Representació molt simplificadora del procés d'especiació en un llinatge metapoblacional. L'enfosquiment o aclariment de les dues branques simbolitza el procés progressiu de divergència al llarg del temps (de baix a dalt) dels dos llinatges germans. A mesura que se separen, aquests adquireixen diferents propietats. L'asincronia en l'adquisició d'aquestes propietats implica un període d'incertesa on diferents conceptes d'espècie poden entrar en conflicte, mentre que abans de l'adquisició de la primera propietat els diferents conceptes coincidiran en reconèixer una sola espècie i després de l'última en reconeixeran dues. Adaptat de: de Queiroz (2005) i Leliaert *et al*. (2014).

La tendència preponderant en la reconstrucció filogenètica ha estat la utilització de matrius provinents de la concatenació de múltiples marcadors moleculars basant-se en la filosofia de l'**evidència total** (Kluge, 1998). Tot i això, aquesta pràctica no està exempta de

problemes, com els previstos segons la teoria de coalescència i que distingeixen el binomi **arbre gènic - arbre d'espècies**. Aquesta distinció, tot i que ja s'havia apuntat en el passat (Maddison, 1997), s'ha mantingut poc considerada (Edwards, 2009). Una de les causes d'aquest conflicte és la **retenció de polimorfismes ancestrals** ("*incomplete lineage sorting"*), on el polimorfisme ancestral persisteix al llarg d'esdeveniments d'especiació, fent que la genealogia inferida tingui una topologia potencialment diferent de la de l'arbre d'espècies. Aquesta causa de conflicte, també anomenada de **coalescència profunda** ("*deep coalescence"*), serà més accentuada com major sigui la població efectiva i menor el temps entre esdeveniments d'especiació (Maddison, 1997; Edwards, 2009). Tot i això, la implementació pràctica de models (**models coalescents de múltiples espècies**, "*multispecies coalescent models"*) que consideren els efectes de la retenció de polimorfismes ancestrals en la reconstrucció filogenètica és recent i tot just està en ple desenvolupament (Liu *et al*. 2009; Degnan i Rosenberg, 2009; Heled i Drummond, 2010). Amb aquest paradigma es permet connectar la inferència filogenètica, preocupada principalment per les relacions entre espècies, amb la biologia de poblacions, centrada en els processos evolutius intraespecífics, i les seves aplicacions s'han estès en diversos camps com veurem més endavant. Un element limitant per a l'aplicació d'aquestes models ha estat la disponibilitat de múltiples marcadors independents, especialment en organismes no model. En aquest sentit, les **noves tècniques de seqüenciació** ("*next-generation sequencing"*) estan permetent l'adquisició de quantitats de dades moleculars fins fa poc inimaginables, permetent superar aquesta limitació.

La utilització de l'ADN també ha revolucionat el camp de la taxonomia. La limitació que suposa el gran desconeixement taxonòmic per altres disciplines dedicades a l'estudi de la biodiversitat, s'ha anomenat **impediment taxonòmic** (La Salle *et al*., 2009). Aquest reconeixement ha comportat una anàlisi de la conjuntura actual de la taxonomia per tal de buscar-ne les causes i les solucions (Rodman i Cody, 2003; Wilson, 2003; Agnarsson i Kuntner, 2007). Entre altres accions i propostes, es va veure que la integració de les dades moleculars en la taxonomia podria ajudar a superar aquest impediment (Hebert *et al*., 2003; Padial i de la Riva, 2007). En un primer moment es va arribar a propugnar l'ús únic de l'ADN en taxonomia, substituint per complet la taxonomia "clàssica" basada en la morfologia (Tautz *et al*., 2003), encara que, i després d'intensos debats, a la pràctica s'està arribant cap a un consens, la **taxonomia integrativa** (Dayrat, 2005; Padial *et al*., 2010;

Schlick-Steiner *et al*., 2010; Edwards i Knowles, 2014). Aquesta aproximació sosté que més que valorar la superioritat d'unes evidències enfront d'unes altres, se'n pot treure redit de la seva complementarietat (Dayrat, 2005; Schlick-Steiner *et al*., 2010; Padial *et al*., 2010). Al seu torn, aquest debat al voltant de l'ús de les dades moleculars en la taxonomia ha reactivat l'interès en la delimitació d'espècies. En els últims anys s'han proposat noves metodologies expressament encarades en aquesta fi, principalment utilitzant caràcters moleculars (revisats a Fujita *et al*., 2012) però també combinant-se amb caràcters morfològics (Edwards i Knowles, 2014).

Una de les aplicacions que ha creat més expectatives alhora que debats en l'aplicació de dades moleculars en la taxonomia, és l'anomenat **codi de barres genètic o d'ADN** (Hebert *et al*., 2003). Aquest se sosté en l'ús d'un fragment estàndard d'ADN, en el cas dels animals la subunitat I del gen mitocondrial citocrom oxidasa, popularment conegut com a ***coi***, com a identificador universal (Hebert *et al*., 2003). El raonament és construir una base de dades pública gestionada per especialistes en els diferents grups d'organismes, principalment la *Barcoding of Life Database* (BOLD; Ratnasingham i Hebert, 2007), amb el principal objectiu de tenir representada la màxima diversitat taxonòmica del mateix fragment de *coi*, i que aquesta serveixi com a referència per a poder identificar totes les espècies, en qualsevol dels seus estadis (Hebert *et al*., 2003). A més de la seva utilitat per a la identificació taxonòmica d'exemplars desconeguts (Dinca *et al*., 2011), diversos autors van proposar que la metodologia podria servir per a la delimitació o descobriment d'espècies, ja sigui basant-se en llindars de distàncies genètiques (Herbert *et al*., 2003) o utilitzant l'anomenat "barcode gap" (Puillandre *et al*., 2012), interval entre les distàncies genètiques intraespecífiques i les interespecífiques. Encara que la delimitació de llinatges basats en el paradigma del codi de barres genètic és de gran utilitat, i ha servit en nombrosos grups per posar al descobert una enorme biodiversitat (Hebert *et al*., 2004), la utilització d'un llindar universal podria sobreestimar o subestimar el nombre d'espècies (Meyer i Paulay, 2005) i com s'ha comentat anteriorment, és preferible la utilització integrada de diverses evidències per a delimitar i descriure les espècies de forma rigorosa.

En conjunt, tots aquests avenços han situat la sistemàtica com a un pilar essencial en la Biologia, i a més, han servit perquè en surti reforçada pel que fa a la velocitat, l'eficiència, i en la precisió per acomplir els seus objectius en l'estudi de la biodiversitat. De forma paral·lela, l'ús de les seqüències d'ADN també ha afectat de forma rellevant altres

disciplines.

Un exemple el trobem en el cas de la **biogeografia**. Aquesta és una disciplina sintètica enfocada en documentar i entendre els patrons de distribució de la biodiversitat (Wiley i Lieberman, 2011). Una de les aportacions que més impacte ha tingut en la biogeografia, i especialment en la **biogeografia d'illes**, ha estat l'ús del **rellotge molecular**. Aquest concepte es basa en l'observació que la taxa d'evolució molecular és relativament constant al llarg del temps i entre diferents organismes (Zuckerkandl i Pauling, 1965), i que per tant, pot existir una relació lineal entre distància genètica i temps evolutiu, permetent estimar un marc temporal si es coneix la taxa d'evolució (Bromhan i Woolfit, 2004). No obstant això, la premissa de l'existència d'una taxa d'evolució constant entre organismes, o **rellotge molecular estricte** s'ha demostrat ser molt simplificadora, per la qual cosa s'estan implementant metodologies més realistes, que permeten un cert "relaxament" del rellotge molecular. Segurament, el punt més sensible en l'ús del rellotge molecular rau en el seu calibratge, necessari per a convertir les distàncies evolutives en distàncies temporals absolutes. Per fer-ho, és indispensable assumir la correspondència entre un node d'un arbre filogenètic a un esdeveniment del qual en tinguem una constància temporal, com poden ser els registres fòssils o els processos geològics (Bromham i Penny, 2003; Ho, 2007; Weir i Schulter, 2008; Hipsley *et al*., 2009).

La **filogeografia**, entesa com l'estudi dels principis i processos que governen la distribució geogràfica de llinatges genealògics (Avise, 2000), és una disciplina recent on la utilització de dades moleculars hi té un pes molt important. Aquesta disciplina actua de pont entre la biologia de poblacions i la filogenètica (Hickerson *et al*., 2010), com un continu que travessa els límits d'espècie (Brito i Edwards, 2009). En els seus inicis, la filogeografia se centrava a estudiar des d'un punt de vista descriptiu, els patrons de variació a nivell d'espècie, utilitzant com a marcador genètic predilecte l'ADN mitocondrial, pels avantatges que presenta (facilitat d'amplificació, absència de recombinació, i una mida efectiva relativament petita; Avise, 2000). Malgrat aquests avantatges, l'ADN mitocondrial és d'exclusiva herència materna en la majoria d'organismes, i per tant, els resultats obtinguts a partir de la seva informació no són representatius de tot el genoma. De forma semblant a la filogenètica molecular, la tendència actual és la d'incorporar múltiples marcadors, i utilitzar models basats en la teoria de coalescència per a estimar els paràmetres i testar-ne les hipòtesis, anomenant-se **filogeografia estadística** (Knowles i Maddison, 2002; Hickerson

et al., 2010).

La **modelització de nínxols ecològics** ("*ecological niche modelling*" - ENM) és un camp que en els últims anys ha agafat molta rellevància, sobretot en relació a la biogeografia i la filogeografia, on aporta una perspectiva independent a les mateixes preguntes (Peterson i Nyari, 2008; Metcalf *et al*., 2014). A la vegada, el fet que es tracta d'una nova disciplina comporta que molts dels conceptes i metodologies no estiguin del tot assentats provocant un continu debat, començant pel mateix nom: modelització de nínxols ecològics (ENM) respecte modelització de distribucions d'espècies (SDM) (Peterson i Soberón, 2012).

En qualsevol cas, per entendre el procés de modelització, primer de tot és necessari aclarir la dualitat entre dos espais, l'**espai geogràfic** real (G) i l'**espai ambiental** abstracte (E). Aquesta dualitat implica que no hi ha un lligam directe entre la topologia de l'espai ambiental i el geogràfic (Soberón i Nakamura, 2009). G sol estar definit per dues dimensions, en canvi E és un espai multidimensional, amb una dimensió per cada un del conjunt d'atributs (variables escenopoètiques) que la conformen (Peterson *et al*., 2011). Una bona manera de contextualitzar els conceptes és a partir del diagrama biòtic - abiòtic - mobilitat o **BAM** (Soberón i Peterson, 2005), on es representen els tres factors principals que afecten la distribució d'una espècie en un temps particular (Fig. 2). La zona A representa aquella regió en un espai geogràfic (G) on es compleixen els requisits abiòtics (temperatura, precipitació, etc.) per un creixement positiu de l'espècie en qüestió. De forma anàloga, B representa la regió en un espai geogràfic (G) on les interaccions biològiques amb altres espècies (depredació, pol·linització, etc.) són favorables. Per últim, M representa la regió en l'espai geogràfic (G) que ha estat accessible per a l'espècie en un temps determinat (dispersió). Això ens permet distingir dues àrees més, per un costat la G_0, definida com l'**àrea ocupada**, i la G_I o **àrea envaïble**, on tot i donar-s'hi les condicions biòtiques i abiòtiques òptimes, l'espècie no hi ha pogut arribar (per exemple per barreres geogràfiques). Per tant, la unió d'aquestes dues àrees és equivalent a la **distribució potencial** de l'espècie G_p.

En l'espai ambiental, el **nínxol de l'espècie** (N_f) és un subconjunt d'E, que pot no estar totalment representat en G, mentre que el nínxol condicionat segons l'espai accessible (M) i l'espai mostrejat (S), és el que s'hi troba present (Peterson *et al*., 2011). Quan modelitzem, s'estableix un enllaç entre l'espai G (on es troben les localitats de l'espècie) i l'espai E (variables associades a G) i és en aquest espai on es comparen les variables associades a les

localitats respecte a les associades a on no es troben les localitats. L'última part de la modelització tractarà de classificar en l'espai G aquelles àrees que segons l'algoritme utilitzat són aptes per a l'espècie. Per tal que aquesta estimació correspongui a $G_{p,}$ és important establir de forma explícita la zona M i S, ja que tenen un efecte significatiu en el resultat de la modelització (Barve *et al*., 2011; Saupe *et al*., 2012).

Fins aquest punt, podríem considerar que tant els ENM com els SDM són equivalents, i les diferències rauen en l'ús i interpretació que se'n pugui fer dels resultats obtinguts (Peterson i Soberón, 2012), amb els SDM restringits en l'estudi de la distribució actual. En canvi, una de les potencialitats més interessants de l'ENM, és que si s'accepta la hipòtesi de **conservació de nínxol ecològic** (Peterson, 2011; Guisan *et al*., 2014), es poden utilitzar les tècniques de modelització juntament amb **reconstruccions paleoclimàtiques** per projectar les distribucions potencials en diferents punts del passat. En el camp de la filogeografia, aquestes projeccions poden servir de base per a formular hipòtesis filogeogràfiques que es podran testar amb les dades moleculars (Richards *et al*., 2007; Carstens i Richards, 2007; Peterson i Nyari, 2008; Metcalf *et al*., 2014).

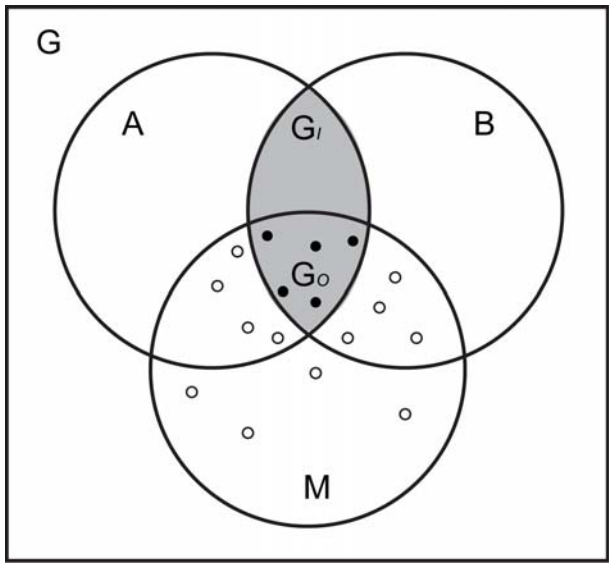

Figura 2 Representació esquemàtica del diagrama BAM, on s'il·lustra la interacció entre els factors abiòtics (A), biòtics (B) i relacionats amb la mobilitat (M). G representa l'espai geogràfic, G_0 l'àrea ocupada, G_1 l'àrea envaïble, i la unió d'aquestes dues (en gris) és l'àrea potencial (G_p). Els cercles petits indiquen localitats, els negres indiquen presències i els blancs absències. Adaptat de Soberón i Peterson, 2005.

MARC GEOGRÀFIC

La regió Mediterrània, entesa com la **conca Mediterrània** juntament amb les **Illes Canàries** i Madeira, es va reconèixer com un dels 25 **punts calents de biodiversitat** a nivell mundial pel seu elevat nombre d'endemismes i per l'alt grau d'amenaça en què es troben (Myers *et al*., 2000). Encara que aquest famós estudi només es basés en plantes vasculars i vertebrats (excepte peixos), segurament un coneixement més profund de la biodiversitat en grups mega-diversos com els artròpodes, no faria sinó consolidar-ne els resultats. L'elevada biodiversitat i a la vegada, l'alt grau d'amenaça en aquestes àrees es deuen a la combinació de diferents factors que han actuat a diferents escales, tant temporals com geogràfiques, i que es resumeixen a continuació per a les tres principals regions on s'ha treballat en aquesta tesi doctoral.

La Conca Mediterrània

La Conca Mediterrània és una de les regions més riques i complexes de la Terra, tant des del punt de vista geològic, biològic com cultural (Blondel *et al*., 2010). Aquesta complexitat fa que sigui difícil, fins i tot, definir-ne els límits d'una forma clara. Es pot considerar que la Conca Mediterrània abasta les zones contigües a la **Mar Mediterrània** que tenen actualment un **clima mediterrani** (Thompson, 2005; Fig. 3), cobrint uns 2.3 milions de km^2 repartits per més de 24 països (Blondel *et al*., 2010). Un dels factors més importants per entendre la biodiversitat a la Mediterrània és la seva complexa història geològica. Tot i així, aquest no ha estat el factor més determinant pel grup d'estudi distribuït per la Mediterrània en la present tesi, així que només apuntaré de forma resumida els esdeveniments més rellevants.

La Mar Mediterrània troba els seus orígens en l'antic mar de Tetis, quan per conseqüència dels moviments relatius de les plaques tectòniques Eurasiàtica, Africana i Aràbiga, es va acabar fragmentant durant l'Oligocè en una sèrie de conques tancades (Blondel *et al*., 2010; Thompson, 2005; Mansion *et al*., 2008; Rosenbaum, 2002). La configuració actual prové del tancament definitiu de la connexió entre l'antic Tetis i l'oceà Índic per la col·lisió entre les plaques Aràbiga i Eurasiàtica durant el Miocè mitjà (Krijgsman, 2002). Un dels esdeveniments geològics més importants és l'orogènesi Alpina i el subseqüent trencament de l'antic cinturó Hercinià en diverses microplaques, actualment formant part de Sardenya, Calàbria, Illes Balears, Cabília, Serralada Bètica i el Rif, durant l'Oligocè (Rosenbaum,

2002). Un dels exemples més paradigmàtics de l'afectació d'aquest trencament sobre la biota el trobem en les aranyes del gènere *Parachtes* (Bidegaray-Batista i Arnedo, 2011). Segurament, un dels períodes més dramàtics de la història geològica de la Mediterrània és l'anomenada **Crisi del Messinià**, quan a causa de la interrupció de la connexió marítima entre la Mediterrània i l'Atlàntic fa uns 5.96 Ma, i al balanç hídric negatiu de la Mediterrània, es va dessecar tota la Mar en molt poc temps (Krijgsman i Wilsonk, 1999; Krijgsman, 2002; Rouchy i Caruso, 2006). Se suposa que els efectes sobre la biota marina van ser catastròfics (Bianchi i Morri, 2000), però en canvi, van permetre l'intercanvi de fauna i flora terrestre entre Àfrica, Europa, i les múltiples illes mediterrànies. Aquests intercanvis es van interrompre de forma sobtada fa uns 5.33 Ma, quan en obrir-se l'Estret de Gibraltar, l'aigua de l'Atlàntic va reomplir la Mar Mediterrània (Garcia-Castellanos *et al*. 2009), provocant l'especiació vicariant en nombrosos grups (Palmer i Camberfort, 2000; Carranza *et al*. 2006; Sousa *et al*. 2012; Habel *et al*. 2012; Huseman *et al*. 2013 per més referències).

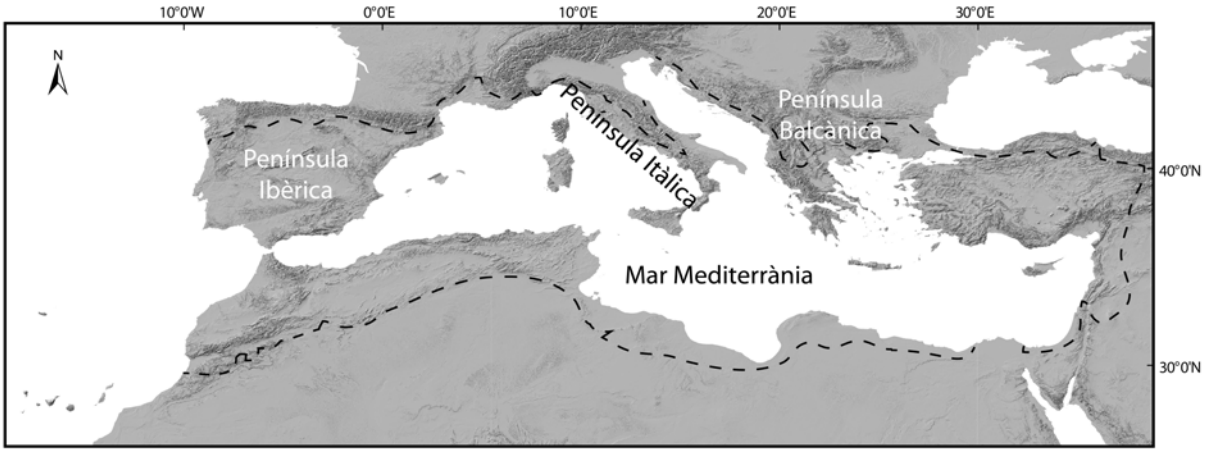

Figura 3 Mapa de la Conca Mediterrània on s'assenyalen les tres gran penínsules del sud d'Europa. L'àrea compresa en la regió biogeogràfica de la Mediterrània *sensu* Quézel i Médail (2003) està delimitada per la línia discontínua.

Si bé els esdeveniments geològics han tingut un paper destacat en la biodiversitat mediterrània, els factors climàtics també han estat rellevants, sobretot en períodes més recents, amb la consolidació del clima mediterrani i posteriorment amb les glaciacions del Quaternari. La transició gradual però profunda cap a un clima mediterrani va començar durant el Pliocè mitjà (~3.2 Ma), i es degué principalment a una fluctuació en el règim de pluges, més que no pas a un canvi en la temperatura (Suc, 1984). Aquest nou clima,

més càlides de l'any, va tenir un fort impacte sobretot en la flora, promovent que la vegetació esclerofílica esdevingués predominant. Però sens dubte, un dels factors climàtics amb més incidència per a la biota, no només a la conca Mediterrània sinó a tot Europa, i a nivell global, va ser les fluctuacions climàtiques que van començar al Quaternari (Hewitt, 1996). Aquesta època va estar dominada per unes oscil·lacions climàtiques d'uns 100 000 anys de durada, on els períodes freds (**glacials**) eren més prolongats que no pas els càlids (**interglacials**). La causa fonamental dels cicles s'explicaria per l'excentricitat de l'òrbita terrestre, encara que factors addicionals com moviments tectònics o canvis en els corrents oceànics, podrien modificar-ne la periodicitat i la intensitat (Hewitt, 1996). Les conseqüències per a la biota d'aquests cicles van dependre en part de la latitud i de la topografia, però de forma general a Europa, es podria acceptar que van forçar un desplaçament cap al sud de les distribucions. La configuració geogràfica del continent europeu, amb tres grans penínsules a les zones més temperades (Península Ibèrica, Itàlica i Balcànica, Fig. 3), va facilitar que aquestes actuessin com a refugis, el que es coneix com a **refugis glacials** del sud d'Europa (Hewitt, 1996, 2000) i que des d'aquests refugis recolonitzessin el territori continental durant els interglacials. Tot i això, diferents factors van impedir que nombrosos grups d'organismes s'adaptessin a les canviants condicions climàtiques, produint-ne l'extinció (Koch i Barnosky, 2006; Postigo-Mijarra *et al*., 2010). En canvi, en altres grups la reducció de la distribució o l'aïllament en refugis va facilitar la seva diversificació, amb nombrosos exemples d'especiació al·lopàtrica (Weiss i Ferrand, 2007 i referències). Malgrat tot, l'acumulació de dades i els esforços per delimitar els refugis han posat de manifest que la hipòtesi de les tres penínsules mediterrànies com els tres grans refugis del sud d'Europa és massa simplificadora per explicar els patrons d'endemismes i de diversitat trobats actualment. Això, ha reobert un debat al voltant del concepte de refugi, proposant-se noves hipòtesis per explicar per exemple, la possible existència de refugis al Nord d'Europa (Stewart i Lister, 2001; Schmitt i Varga, 2012; criticat per Tzedakis *et al*., 2013), o de refugis dins de refugis com en la Península Ibèrica (Gómez i Lunt, 2007).

Però sens dubte, el factor que ha modulat amb major intensitat el paisatge mediterrani en temps històrics ha estat l'acció continuada dels humans. Des de la revolució neolítica (fa uns 10 000 anys) a ençà, nombroses cultures han poblat les ribes de la Mar Mediterrània i navegat les seves aigües (Abulalfia, 2011), alterant primer les terres per a la pastura i

navegat les seves aigües (Abulalfia, 2011), alterant primer les terres per a la pastura i l'agricultura, i més recentment, per a la construcció desmesurada, modificant de formes diverses la fauna i flora que ara hi trobem, ja sigui per l'extinció de certs grups, per l'afavoriment d'uns altres o per la introducció d'espècies foranes.

La regió del Souss-Massa

Una regió que mereix una especial atenció és la situada al sud-oest del Marroc, delimitada per les conques dels rius Souss i Massa, entre els vessants sud de l'**Alt Atles** i incloent part de l'**Anti-Atles**. La història geològica d'aquesta zona és d'una enorme complexitat, incloses roques exposades d'origen Paleozoic a les àrees més muntanyoses, àrees d'origen volcànic com les del voltant d'Ouarzazate o de Sidi Ifni, i fins a sediments d'origen Quaternari a les mateixes valls dels rius (Ennih i Liégeois, 2008), i la seva revisió detallada va més enllà dels objectius d'aquesta introducció. La major part d'aquesta regió està inclosa dins la Reserva de la Biosfera Arganeraie, creada el 1998 per conservar el bioma dominant d'aquesta àrea, del qual l'**Argània** (*Argania spinosa* (L) Skeels), espècie endèmica del Marroc, n'és l'element més conspicu. Actualment, aquesta zona gaudeix d'un clima semi-àrid i àrid, amb una marcada estacionalitat pel que fa al règim de pluges (Bouchaou *et al.*, 2008), i les zones de menys altitud (< 250 m.) s'inclouen dins l'estatge inframediterrani, on la mitjana de la temperatura mínima del mes més fred no se situa per sota dels 7°C (Blondel *et al*., 2010). Aquest clima suau configura una ecorregió (la del bosc sec mediterrani i matollar suculent d'acàcies i d'argànies) només compartida per certes parts de la Macaronèsia, especialment Fuerteventura i Lanzarote, relativament properes geogràficament (Fig. 4). Aquesta unitat ecològica entre les dues àrees s'ha estudiat de forma majoritària basant-se en la flora, amb algunes espècies comunes o vicariants entre elles (Médail i Quézel, 1999), però certament diferents grups faunístics de les Canàries també tenen els seus respectius grups germans en aquesta zona com els rèptils del gènere *Chalcides* (Carranza *et al.*, 2008), els caragols del gènere *Theba* (Greve *et al.*, 2010), o les papallones del gènere *Gonepteryx* (Brunton i Hurst, 1998). Malgrat els pocs grups estudiats en aquesta regió, diferents estudis han subratllat l'elevada diversitat (ja sigui específica o genètica) existent, com en el gènere *Theba* (Greve *et al.*, 2010) o de forma més destacable en els patrons de microal·lopatria dels escorpins del gènere *Buthus* (Habel *et al.*, 2012; Husemann *et al.*, 2012; Sousa *et al.*, 2012).

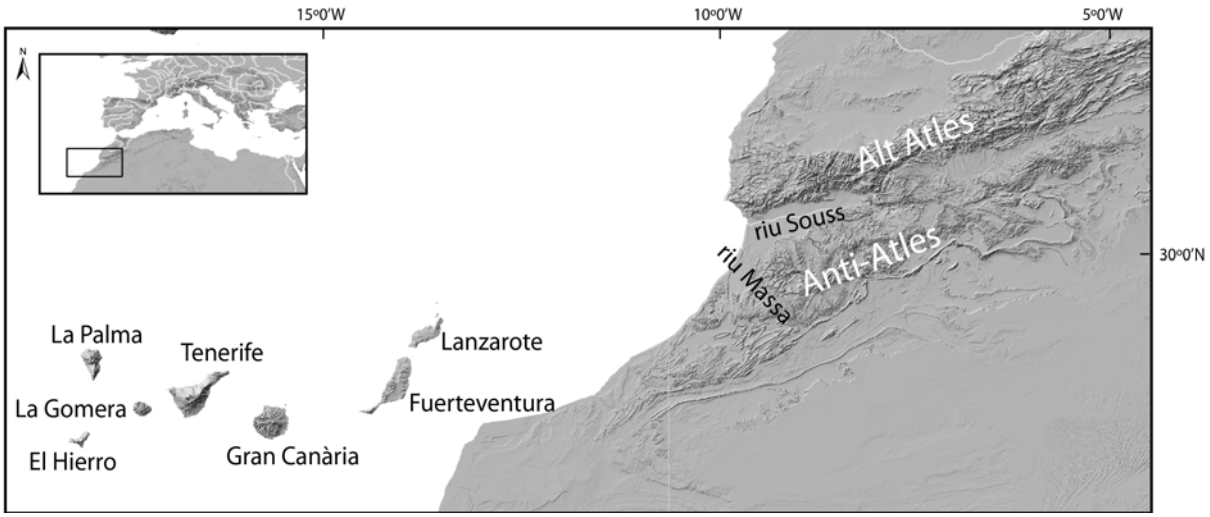

Figura 4 Mapa de la regió del Souss-Massa i les Illes Canàries.

Les Illes Canàries

Situades en aigües de l'Atlàntic, les Illes Canàries, juntament amb quatre arxipèlags d'origen volcànic (Cap Verd, Illes Salvatges, Madeira i Açores), formen la regió biogeogràfica de la **Macaronèsia** (del grec antic μακάρων [feliç] νῆσοι [illes]). Aquest terme va ser utilitzat per primera vegada per Engler (1879), incloent-hi en un primer moment els arxipèlags d'Açores, Madeira i Illes Canàries. La unitat d'aquesta regió s'ha reconegut basant-se majoritàriament en els elements endèmics de la flora vascular, sota la hipòtesi que són representants relictes d'una antiga flora subtropical abundant en el Terciari, encara que diferents autors han qüestionat que aquesta unitat sigui generalitzable a altres grups (Vanderpoorten *et al.*, 2007), o l'han eixamplat incloent-hi també la regió del Nord d'Àfrica comentada en l'anterior secció (Kim *et al*., 1996).

L'arxipèlag de les Illes Canàries el formen set illes principals, disposades en una orientació d'est a oest: Fuerteventura, Lanzarote, Gran Canària, Tenerife, La Gomera, La Palma i El Hierro, i diversos illots, la majoria situats prop de Lanzarote. L'illa més propera al continent és Fuerteventura, del qual n'està separada per tan sols uns 100 kilòmetres en el seu punt més proper, el Cap Jubuy, prop de Tarfaya, mentre que la més distant és La Palma, a 460 kilòmetres (Fig. 4).

Les illes volcàniques segueixen un procés en certa mesura ontogenètic al llarg de la seva existència, començant per una fase de naixement i construcció submarina, emergència (utilitzada com a edat de l'illa en estudis biogeogràfics) i construcció subaèria, erosió i dominància de desmantellament, i reducció fins al seu enfonsament (Fernández-Palacios *et al*., 2011). Actualment a les Canàries hi trobem exemples d'illes en la majoria d'aquestes

fases.

Les illes de El Hierro, La Palma i Tenerife són les que encara es troben amb una fase de construcció, amb etapes de vulcanisme recent i elevant-se fins a grans altituds (més de 3000 metres). Tot i això, **El Hierro** (1.1 Ma) i **La Palma** (3.5 Ma) són illes relativament joves, mentre que **Tenerife** té una història geològica més complexa. De fet, l'actual illa de Tenerife prové de la unió de tres illes que emergiren de forma independent, la més antiga, **Adeje**, data d'uns 11 Ma, i es va unir a les altres dues, **Teno** (8 Ma) i **Anaga** (6 Ma) durant els últims 3.5 Ma.

En canvi, tant **La Gomera** (12 Ma) com **Gran Canària** (14.5 Ma) es troben en la fase d'erosió i desmantellament, on són dominants els processos destructius (com grans esllavissades) i on els de construcció hi perden importància. Tot i això, en ambdues illes hi ha hagut períodes volcànics posterosius, dels quals cal destacar, per les seves repercussions sobre la biota, l'erupció del **Roque Nublo** (Anderson *et al*., 2009). Aquest període de vulcanisme va succeir en diferents etapes successives i de diferent intensitat, entre 5.3 i 3.7 Ma. Per la seva dimensió, s'ha proposat que va poder exterminar tota forma de vida de l'illa, per la qual cosa s'ha utilitzat l'edat post-Roque Nublo com a edat de l'illa (Whittaker *et al*., 2008), encara que diferents estudis apunten que tot i el seu efecte remarcable, va permetre la supervivència de nombrosos grups (Emerson, 2003; Anderson *et al*., 2009).

Per últim, les dues illes més orientals de l'arxipèlag, **Fuerteventura** i **Lanzarote**, pertanyents a la mateixa base volcànica també són les més antigues, amb edats de 21 i 15 Ma, respectivament, i actualment es troben en la fase anterior al seu enfonsament, amb una orografia relativament suau. Tot i això, ambdues illes no han estat del tot inactives, i especialment Lanzarote ha sofert importants períodes d'activitat volcànica, fins i tot en temps històrics, quan l'erupció del Timanfaya (1730 - 1736) va cobrir de lava i cendra una quarta part de l'illa (Criado *et al*., 2013). De fet, l'illa tal com la coneixem actualment prové de la unió de dues protoilles, **Los Ajaches** al sud i d'uns 15 Ma, i **Famara** al nord i d'uns 10 Ma, que va ocórrer fa uns 7 Ma. Tot i això, l'activitat volcànica va perdurar i diferents esdeveniments de vulcanisme s'han succeït en els últims 1.2 Ma (Carracedo *et al*., 2002; Ancochea *et al*., 2004). Lanzarote i Fuerteventura estan separades per una llengua d'aigua d'uns 15 kilòmetres d'amplada i menys de 200 metres de profunditat, la qual cosa ha permès que durant els períodes glacials on el nivell del mar era significativament inferior a l'actual, les dues illes i els diversos illots s'unissin formant una sola illa anomenada **Mahan**.

DIVERSITAT I DISTRIBUCIÓ DEL GÈNERE *LOXOSCELES*

Les aranyes del gènere *Loxosceles* Heineken & Lowe, 1832 (del grec antic λόξος [oblic] i σκέλος [potes], per la posició de les potes quan estan en repòs; Fig. 5A) es troben en una gran varietat d'hàbitats, encara que majoritàriament són pròpies d'ambients relativament secs, ja siguin més o menys càlids. Són aranyes nocturnes i lucífugues (fugen de la llum) i per això es troben en llocs obscurs, com per exemple sota pedres o escorces d'arbres, i especialment en les entrades de coves, encara que en molts pocs casos presenten adaptacions a ambients cavernícoles (Gertsch i Ennik, 1983; Planas i Ribera, obs. pers.). En aquests llocs tranquils i foscos és on fan la seva densa i blavosa teranyina aferrada a terra, i on resten per caçar les preses potencials, segurament detectades per vibracions en la teranyina (Coddington *et al*., 2002). Les femelles també utilitzen les teranyines per a realitzar les postes, que solen estar compostes per una mitjana de 25 - 65 ous segons l'estudi i l'espècie, encara que tant el nombre d'ous com el nombre de postes és variable (*L. reclusa* Gertsch i Mulaik, 1940: Hite *et al*., 1966, Horner i Stewart, 1968; *L. gaucho* Gertsch, 1967: Rinaldi, 1997). Tant mascles com femelles sovint realitzen les successives mudes sobre la teranyina, i el nombre i l'interval de temps entre mudes dependrà de les condicions ambientals i la disponibilitat d'aliment (Galiano, 1967; Rinaldi, 1997; Fischer i Vasconcellos-Neto, 2005). El fet de trobar-se diverses mudes en el mateix lloc suggereix que es tracta d'animals generalment sedentaris (Fig. 5B), encara que els mascles realitzen desplaçaments actius per aparellar-se. Dels escassos estudis on s'ha estudiat la seva mobilitat, s'ha vist que aquests poden variar des d'un màxim de 2 m en una setmana en *L. rufipes* (Lucas, 1834) a 40 m en *L. similis* (Moenkhaus, 1898) encara que els desplaçaments es veuen condicionats per l'ambient i la disponibilitat d'aliment (Ferreira *et al*., 2005).

El gènere *Loxosceles* està inclòs en la família Sicariidae Keyserling, 1880, juntament amb el gènere *Sicarius*, i està formada per 131 espècies, distribuïdes de forma desigual entre els gèneres *Loxosceles* (107 espècies) i *Sicarius* Walckenaer, 1847 (24 espècies). Les espècies del gènere *Loxosceles* són originàries de les zones temperades i tropicals d'Amèrica i d'Àfrica, i una sola espècie, *Loxosceles rufescens* (Dufour, 1820), és endèmica de la Mediterrània. La major part de les espècies tenen àrees de distribució relativament petites, amb algunes tan sols conegudes d'una localitat, com *L. francisca* Gertsch & Ennik, 1983 o *L. carmena* Gertsch & Ennik, 1983 (Gertsch i Ennik, 1983). Tot i això, existeixen algunes excepcions a aquest patró general, i algunes espècies amb hàbits antropòfils (espècies que

coexisteixen amb els humans) han estat transportades de forma passiva i introduïdes en diverses zones del planeta. Aquest és el cas, per exemple, de *Loxosceles laeta* (Nicolet, 1849) i *L. gaucho*, que, tot i ser originàries d'Amèrica del Sud, han estat citades en diverses localitats d'Amèrica del Nord, d'Europa i del Nord d'Àfrica. No obstant això, l'única espècie actualment considerada cosmopolita és *L. rufescens*, ja que s'ha establert puntualment en àrees tan distants com el Japó, Madagascar, Austràlia o els Estats Units (Platnick, 2014). Curiosament, tal com va observar Gertsch (1967), *L. rufescens* no ha estat mai citada a l'Amèrica del Sud, malgrat l'intens transport marítim que ha existit amb zones de la conca Mediterrània d'on n'és originària. El fet que una espècie es pugui introduir en una nova àrea depèn de diversos factors, com la competència interepecífica amb les espècies autòctones o unes condicions ambientals propícies, i probablement el primer factor és el que ha impedit l'establiment d'aquesta espècie a l'Amèrica del Sud, on com s'ha comentat, *L. laeta* i *L. gaucho* ocupen també els hàbitats antropòfils on se sol trobar *L. rufescens* en les àrees on ha estat introduïda.

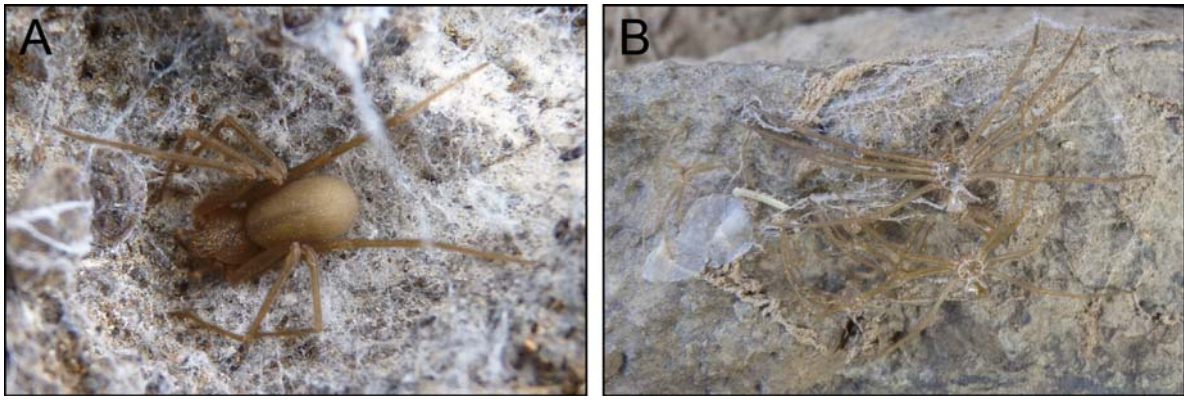

Figura 5 A) Fotografia de *Loxosceles* sp. FVLZ en posició de repòs sobre la teranyina, on es pot veure la disposició típica de les potes que dóna nom al gènere. **B)** Fotografia de mudes de *Loxosceles* sp. de diferents estadis.

Fins a les revisions taxonòmiques de Gertsch i col·laboradors (Gertsch 1958; 1967; 1973; Gertsch i Ennik, 1983), només s'havien descrit de forma puntual diverses espècies, i la mateixa tendència ha seguit posteriorment, amb només deu espècies descrites des del 1983 (Fig. 6A). Si hom es fixa en com està distribuïda actualment la diversitat dins del gènere, es podria pensar que aquesta és molt superior al continent americà, i que només unes poques espècies han arribat, o han sobreviscut com a relictes, al continent africà. Res més lluny de la realitat, doncs, el patró de distribució que observem actualment es deu sobretot a biaixos pel que fa als estudis realitzats, amb una gran diversitat en les àrees incloses en les revisions

de Gertsch i col·laboradors (Amèrica del Sud, Central i del Nord), i amb poques espècies en la resta de regions (Fig. 6B).

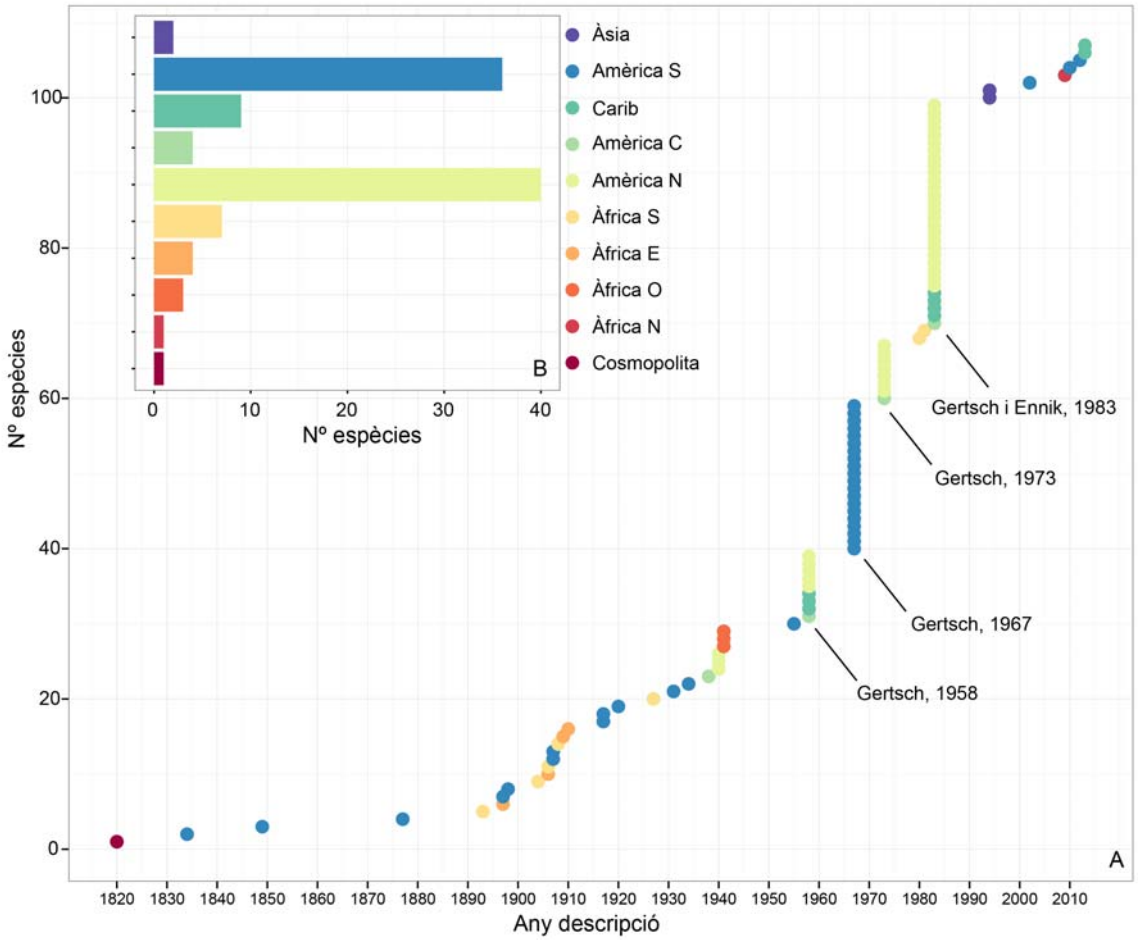

Figura 6 A) Corba d'acumulació de les espècies descrites del gènere *Loxosceles,* amb l'any de la descripció en l'eix horitzontal i el nombre d'espècies en l'eix vertical. Cada punt representa una nova espècie i el color correspon a la seva distribució. S'han assenyalat els treballs de Gertsch i Gertsch i Ennik. **B)** Nombre total d'espècies descrites per a cadascuna de les àrees geogràfiques principals on es distribueix el gènere *Loxosceles*. Elaborat a partir de Platnick, 2014.

Les primeres descripcions d'espècies de *Loxosceles*, de forma semblant que en altres grups d'aranyes, es basaven en caràcters somàtics com la mida i disposició dels ulls, la llargada de les potes o la coloració del cos. Entre mitjans i finals del segle XIX, es va veure que espècies superficialment semblants d'aranyes es podien distingir fàcilment a partir de les estructures reproductores, fent que tant els palps dels mascles com les parts internes i externes dels òrgans reproductors femenins guanyessin importància en la sistemàtica d'aranyes (Huber, 2005). Tot i això, com a aranyes araenomorfes haplogines, les estructures reproductores de *Loxosceles* són senzilles, la qual cosa ha dificultat el seu estudi, amb una

tendència a infravalorar la diversitat present en el grup (Gertsch, 1967). En les esmentades revisions, Gertsch va descriure i agrupar les diferents espècies basant-se en combinacions de caràcters, principalment de les estructures reproductores, tant masculines com femenines. Les diferències entre espècies properes són moltes vegades subtils, basant-se en les proporcions relatives dels diferents segments dels palps i sobretot en detalls de l'èmbol, mentre que les femelles concentren les seves diferències en la forma dels receptacles seminals. En resum, la delimitació d'espècies en *Loxosceles* és complicada sobretot per la simplicitat dels seus caràcters i tal com Gertsch i Ennik van sentenciar, "*els graus de diferència en caràcters genitàlics són menys grans, o almenys menys fàcils de descriure verbalment o gràficament que els d'altres espècies d'aranyes*" [*the degrees of difference in genitalic characters are less great or perhaps less easy to describe verbally or pictorially than those of some other spiders*] (Gertsch i Ennik, 1983: 273).

La poca variabilitat en els caràcters morfològics existents en *Loxosceles* ha estat una de les causes que ha impedit reconèixer la diversitat existent dins del gènere, i per tant, la utilització de caràcters moleculars pot ser de gran utilitat en aquest grup. Tot i això, la primera reconstrucció filogenètica basada en caràcters moleculars, i on s'inclogueren representants de tots els grups d'espècies del gènere, no aparegué fins al 2008 (Binford *et al*. 2008). Els resultats d'aquest estudi donaren suport a les agrupacions proposades per Gertsch, trobant que els grups d'espècies proposats per l'autor formaven clades ben recolzats en la filogènia molecular. Les espècies sud-africanes del grup *spinulosa* van resultar ser el grup germà de la resta de membres del gènere, mentre que el grup *vonwredei*, l'altre grup sud-africà inclòs en les anàlisis, era grup germà del clade format per espècies sud-americanes. Biogeogràficament, aquesta relació va ser interpretada com a efecte de la separació de Gondwana, encara que no totes les anàlisis recolzaven aquesta hipòtesi (Binford *et al*., 2008). La proximitat filogenètica entre *Loxosceles rufescens* i *L. amazonica* Gertsch, 1967 (descrita d'Amèrica del Sud) sí que estava ben suportada en les diferents anàlisis, relació que Gertsch ja havia reconegut basant-se en caràcters morfològics (Gertsch, 1967). Aquesta relació intercontinental va resultar ser massa recent (72 - 11 Ma) per a identificar-la com a conseqüència de la separació de Gondwana, i l'explicació alternativa donada pels autors, basant-se en les dates obtingudes i en la distribució dels tàxons, és la d'una dispersió facilitada per un pont terrestre entre ambdós continents (Binford *et al.*, 2008), encara que un origen africà de l'espècie i una posterior colonització americana

facilitada pel transport humà no pot ser descartada amb la informació actual.

Un segon estudi, també basat en caràcters moleculars, se centrà principalment en la diversitat del gènere *Loxosceles* al Nord d'Àfrica, i va posar al descobert l'existència d'una elevada diversitat dins de *L. rufescens*, amb diferents llinatges estesos per la Mediterrània (Duncan *et al*., 2010). En l'estudi, també es van incloure diversos exemplars de *L. rufescens* introduïts als Estats Units i a Austràlia i es va veure que genèticament eren indistingibles dels representants mediterranis. De forma semblant, els exemplars identificats com a *L. lacta* Wang, 1994 de Xina eren genèticament idèntics a alguns representants mediterranis de *L. rufescens*. En aquest estudi, a més de *L. rufescens* s'inclogueren algunes de les espècies filogenèticament més properes com *L. mrazig*, *L. amazonica* i *L. foutadjalloni*, i altres individus no identificats a nivell d'espècie i genèticament molt diferents a la resta d'espècies descrites fins al moment.

Rerefons taxonòmic del gènere *Loxosceles* al Nord d'Àfrica i a la Mediterrània

Loxosceles rufescens, **espècie tipus** del gènere, va ser descrita el 1820 pel metge militar napoleònic Léon Dufour a partir del material recol·lectat al seu pas per València durant la Guerra del Francés (1808). Primer es va descriure sota el nom de *Scytodes rufescens* (o *Scytodes blonde*), ja que malgrat que hi veié clares diferències amb els representants del gènere *Scytodes* Latreille, 1804 com *S. thoracica* (Latreille, 1802), no es va veure amb cor d'establir un nou gènere per aquesta espècie. En la descripció, Dufour esmenta que l'espècie és abundant en les muntanyes calcàries del Regne de València, principalment pels voltants de Sagunt, localitat que s'establí com a *terra típica* per a l'espècie. No va ser fins a l'any 1835 que es va formalitzar el gènere *Loxosceles*, amb la descripció de *L. citigrada* Heineken & Lowe, 1832, descrita com a endèmica de Madeira i sinonimitzada posteriorment amb *L. rufescens*. Des d'aquest moment, diferents espècies es van descriure progressivament de la Mediterrània i del Nord d'Àfrica, de les que cal destacar *L. erythrocephala* Koch, 1838 de Grècia, i *L. distincta* Lucas, 1846 i *Loxosceles compactilis* Simon, 1881, d'Algèria. La major part de les descripcions i il·lustracions d'aquestes espècies són curtes i superficials, cosa que impedeix una distinció clara entre les diferents espècies i va propiciar que cada autor interpretés de forma diferent els límits específics. Per exemple, el prolífic aracnòleg francès Èugene Simon, va sinonimitzar primer *L. distincta* amb *L. erytrocephala* (Simon, 1873), però més tard, després de descriure *L. compactilis* d'Algèria (Simon, 1881), ell mateix va sinonimitzar *L. erytrocephala* amb *L. rufescens* i va

admetre no poder diferenciar les femelles de *L. compactilis* (malgrat ser l'autor de la descripció a partir d'una femella) de les de *L. distincta*. Aquesta confusió taxonòmica no es va revisar fins a l'any 1969, quan l'aracnòleg italià Paolo Brignoli va portar a terme una revisió taxonòmica de les espècies italianes i malteses del gènere *Loxosceles*, i va concloure que la variabilitat trobada es devia a diversitat fenotípica dins d'una mateixa espècie i no pas a diversitat interespecífica. Aquests resultats però, no van tenir una implicació taxonòmica fins a un posterior estudi morfològic dels gèneres *Scytodes* i *Loxosceles* (Brignoli, 1976). En aquest, Brignoli va establir la sinonimització formal de *L. distincta* i *L. compactilis* amb *L. rufescens* (Brignoli, 1976), deixant per tant, aquesta última, com a única espècie vàlida a la Conca Mediterrània. Per a justificar-ho, a més de la variabilitat intraespecífica referida en l'anterior estudi, Brignoli també es basa en la incapacitat de poder trobar els exemplars tipus de cada espècie, i en la poca consistència en la identificació dels individus dipositats al *Muséum national d'Histoire naturelle* de Paris, on haurien d'estar dipositats aquests exemplars.

IMPORTÀNCIA MÈDICA DEL GÈNERE *LOXOSCELES*: EL LOXOSCELISME

Tot i que les aranyes (Araneae) són el clade d'organismes verinosos més nombrós, només en comptades espècies s'ha estudiat d'alguna forma el verí, majoritàriament en aquelles rellevants per la seva importància mèdica, com les dels gèneres *Latrodectus*, *Phoneutria* o *Atrax,* a més de *Loxosceles* (Vassilevski *et al*., 2009). El verí de *Loxosceles* està compost per un complex de proteïnes, pèptids i altres components de poc pes molecular (revisat a Gremski *et al*., 2014). De tot aquest complex, sens dubte, les molècules que han atret més interès són les de toxines amb activitat esfingomielinasa D (SMase D) expressades per la família gènica *SicTox*. S'ha demostrat que l'acció de les SMase D és suficient i necessària per a desencadenar les lesions dermonecròtiques provocades per l'acció del verí en humans (Silva *et al*., 2004), i que aquestes estan altament expressades en els transcriptomes de *L. laeta* i *L. intermedia* (Fernandes-Pedrosa *et al*., 2008; Gremski *et al*., 2010). Malgrat els greus efectes d'aquestes toxines sobre diferents grups de vertebrats, les aranyes del gènere *Loxosceles* s'alimenten bàsicament d'artròpodes, i recentment s'ha vist que la seva acció insecticida és molt elevada (Zobel-Thropp *et al*., 2012). Sorprenentment, les SMase D són absents en altres grups d'aranyes i només s'han identificat en els dos gèneres de la família Sicariidae, *Loxosceles* i *Sicarius* (Binford i Wells, 2003). Per l'elevat grau de conservació entre els motius aminoacídics de les SMase D dels sicàrids i dels bacteris *Corynebacteria*, es va proposar que l'origen d'aquesta família gènica podria deure's a una transferència lateral des dels bacteris cap a les aranyes (Cordes i Binford, 2006).

L'any 1934 per primera vegada es va poder relacionar inequívocament un tipus peculiar de necrosi cutània amb els efectes de les picades de les aranyes del gènere *Loxosceles* (Macchiavelo, 1937), coneguts com a **loxoscelisme**. Les aranyes del gènere *Loxosceles* no són agressives, i com en la majoria d'aranyes, les picades solen ocórrer de forma accidental, especialment durant la nit. En un primer moment, la picada de *Loxosceles* no és dolorosa i en alguns casos no té cap efecte posterior, mentre que en la majoria de casos els primers signes i símptomes clínics es comencen a fer evidents al cap d'unes hores, generalment provocant un edema i eritema en l'àrea de la picada, des d'on s'estén gravitacionalment. En alguns casos el quadre clínic es deté en aquest punt, mentre que en altres, la inflamació i el dolor local augmenten progressivament, acompanyant-se a vegades de marejos, nàusees, cefalees o febre, fins que al cap de pocs dies comença a desenvolupar-se una necrosi cutània local. Només en un nombre molt menor de casos, els efectes són sistèmics, podent

provocar hemòlisi intravascular, coagulació intravascular disseminada o insuficiència renal (Swanson i Vetter, 2006; Vetter i Isbister, 2008; Cabrerizo *et al*., 2009; Gremski *et al*., 2014). No hi ha una explicació definitiva als diferents efectes del loxoscelime, i sembla que diversos factors dispars hi poden col·laborar, com l'estat de salut o l'edat de la persona afectada, l'espècie causant de la picada, el sexe o l'estat de desenvolupament de l'aranya.

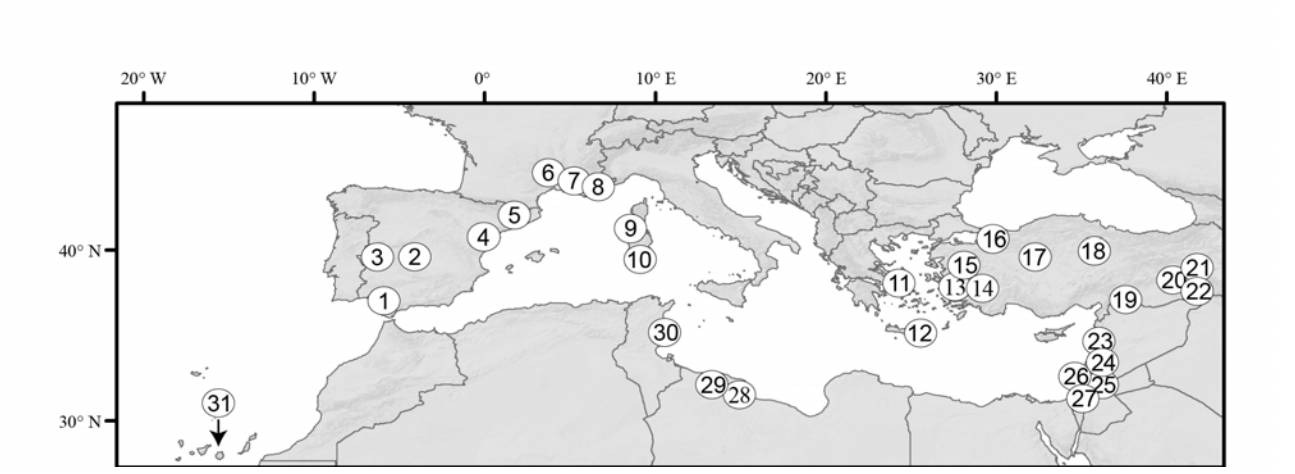

Figura 7 Mapa on s'indiquen els casos de loxoscelisme publicats en la regió Mediterrània. Els números corresponen a les següents referències: 1) Portilla Cuenca *et al*. 2005; 2) Hernández Pérez *et al*., 2012; 3) Zaragoza Fernández *et al*. 2008; 4) Entrambasaguas *et al*. 2007; 5) Garriga *et al*. 2003; 6) Pernet *et al*. 2010; 7) Lagier *et al*. 2012; 8) Hubiche *et al*., 2013; 9) Farace *et al*. 2005; 10) Ribuffo *et al*. 2012; 11) Makris *et al*. 2009; 12) Stefanidou *et al*. 2006; 13) Ermertcan *et al*. 2010; 14) Bajin *et al*. 2011; 15) Atilla *et al*. 2004; 16) Yigit *et al*. 2008; 17) Akyildiz *et al*. 2009; 18) Gulalp *et al*. 2011; 19) Kose *et al*. 2006; 20) Akdeniz *et al*. 2007; 21) Bilgili *et al*. 2012; 22) Taskesen *et al*. 2011; 23) Efrati, 1969; 24) Dyachenko *et al*. 2006; 25) Davidovici *et al*. 2006; 26) Cohen *et al*. 1999; 27) Borkan *et al*. 1995; 28) Elghblawi 2009; 29) Elghblawi, 2010; 30) Ben Said *et al*. 2010; 31) Conca, R. pers. comm.

Per tal que un cas de loxoscelime es pugui considerar com a segur, s'han de complir una sèrie de condicions en el seu diagnòstic, començant per a l'evidència que la picada ha estat provocada per una aranya, i que aquesta s'hagi pogut identificar com a membre del gènere *Loxosceles*, preferentment per un expert. Com que la picada en si no és dolorosa, se sol donar de forma accidental, i els primers símptomes no apareixen fins hores més tard de l'accident, aquesta condició no es compleix en la majoria dels casos i el diagnòstic es basa majoritàriament en el quadre clínic i les simptomatologies posteriors, i per tant, la majoria dels casos han de ser considerats com a probables o possibles (Isbister *et al*., 2002; Vetter i Swanson, 2007; però veure Davidovici i Halevy, 2008). A més, s'ha de tenir en compte que diferents factors poden donar uns símptomes similars, com les infeccions per *Staphylococcus aureus*, i per tant, la dificultat en obtenir diagnòstics fiable impedeix en

gran mesura establir l'abast epidemiològic real del loxoscelime. Per exemple, als Estats Units el reconeixement mediàtic de les aranyes *Loxosceles* és notable, i cada any diversos casos de necrosi cutània es relacionen amb suposats casos de loxoscelisme, tot i donar-se en àrees fora de la distribució de *Loxosceles*, augmentant aparentment el seu abast (Vetter i Barger, 2002; Vetter i Swanson, 2007).

A la Mediterrània, tenint en compte l'abundància de *L. rufescens*, els casos de loxoscelime són relativament poc freqüents (Fig. 7), amb per exemple 10-12 casos de mitjana anuals a l'Hospital Clínic de Barcelona (Dr. Santiago Nogué, comunicació personal). Tot i que el seu abast real no ha estat mai estudiat en profunditat, s'ha proposat en diverses ocasions que el verí d'aquesta espècie és de menor importància mèdica que el d'altres espècies del mateix gènere, com *L. laeta* a l'Amèrica del Sud i *L. reclusa* a l'Amèrica del Nord, responsables de nombrosos casos de loxoscelime (Vetter i Swanson, 2007; Cohen *et al*., 2009). Tot i això, fins ara no s'ha presentat evidències que confirmin aquesta hipòtesi i de fet, en els dos estudis on s'ha explorat el verí de *L. rufescens*, l'activitat esfingomielinasa D era elevada, comparable a la de les espècies americanes (Binford i Wells, 2003; Binford *et al*., 2009). La poca informació disponible, tant pel que fa a la biologia de *Loxosceles* en aquesta àrea com a la seva importància mèdica, sovint ha afavorit que els pocs casos apareguts s'acompanyin d'una notable repercussió mediàtica sensacionalista, confonent a l'opinió pública i als mateixos serveis sanitaris.

OBJECTIUS

OBJECTIUS

L'objectiu principal d'aquesta tesi doctoral és estudiar des d'un punt de vista evolutiu la diversitat d'aranyes del gènere *Loxosceles* a la Conca Mediterrània, al Nord d'Àfrica i a les Illes Canàries. Per tal d'acomplir aquest objectiu general s'han establert els següents objectius específics:

1) Obtenir un mostreig complet en tota l'àrea d'estudi, principalment en les Illes Canàries i el Marroc, on s'espera una major diversitat.

2) Situar la diversitat obtinguda en un context filogenètic mitjançant tècniques moleculars amb la utilització de múltiples marcadors.

3) Generar nous marcadors moleculars nuclears que ens permetin estudiar detalladament la diversitat genètica en escales geogràfiques reduïdes i contrastar els patrons obtinguts amb marcadors mitocondrials.

4) Investigar els factors que han contribuït més notablement en la generació de la diversitat existent, fent especial èmfasi als processos geològics i als canvis climàtics.

5) Delimitar les distribucions dels diferents llinatges evolutius existents i caracteritzar-ne els patrons biogeogràfics.

6) Revisar la taxonomia del gènere en la regió d'estudi mitjançant la integració de diverses fonts d'evidència, principalment molecular i morfològica.

7) Analitzar la variació del verí en diferents llinatges representatius de la diversitat genètica existent a la Mediterrània i a les Illes Canàries, i inferir la seva relació amb els casos de loxoscelisme d'aquestes àrees.

ESTRUCTURA DELS RESULTATS

Els resultats d'un o de més objectius es presenten en vuit articles diferents agrupats en quatre capítols.

El **Capítol 1** està centrat en l'estudi de la diversitat genètica existent dins de *Loxosceles rufescens* i inclou un sol article (Article 1). Per aquest estudi s'ha realitzat un ampli mostreig en tota la Conca Mediterrània (*Objectiu 1*). A partir de dades mitocondrials s'han delimitat diferents llinatges, s'han estudiat els patrons fil·logeogràfics i dilucidat els processos que els han generat, fent especial èmfasi als efectes climàtics de les glaciacions Pleistocèniques a partir de tècniques de modelització de nínxols ecològics (*Objectius 2, 4* i *5*).

El **Capítol 2** inclou quatre articles (Articles 2, 3, 4 i 5) centrats en la diversitat del gènere *Loxosceles* a les Illes Canàries i en el desenvolupament de nous marcadors moleculars. Concretament, en l'Article 2 es posa de manifest l'existència de diversos llinatges evolutius endèmics de l'arxipèlag Canari, a partir d'un ampli mostreig en totes les illes (*Objectiu 1*). Mitjançant anàlisis filogenètiques s'han delimitat set llinatges evolutius independents i s'ha pogut inferir la seva història evolutiva, incloent-hi el temps i el mode de colonització (*Objectius 2, 4* i *5*). En l'Article 3 s'ha realitzat la descripció formal de sis noves espècies a partir de l'estudi morfològic i molecular dels llinatges endèmics detectats a les Illes Canàries, explorant també la utilitat del codi de barres genètic per a la seva utilització en la identificació d'aquestes espècies (*Objectius 2* i *6*). L'Article 4 recull els resultats de l'estudi centrat en el desenvolupament de nous marcadors microsatèl·lits utilitzant les noves tecnologies de seqüenciació (*Objectiu 3*). En l'Article 5 s'estudien els patrons fil·logeogràfics de l'espècie endèmica de les Illes de Fuerteventura i Lanzarote, aplicant els marcadors moleculars específicament dissenyats per aquesta espècie (*Objectius 4* i *5*).

El **Capítol 3** inclou dos articles (Articles 6 i 7) enfocats en diversos aspectes taxonòmics i biogeogràfics de les *Loxosceles* del Nord d'Àfrica. En l'Article 6 es descriu una nova espècie de Tunísia, representant la segona espècie existent al Nord d'Àfrica (*Objectiu 6*). L'Article 7 se centra en l'estudi de la diversitat existent en la zona sud del Marroc, principalment en la regió delimitada per les conques dels rius Souss i Massa. A partir dels exemplars obtinguts en els mostreigs intensius realitzats en aquesta regió, i utilitzant

diversos marcadors moleculars, es detecten i circumscriuen diversos llinatges independents, es proposen diferents factors que han pogut generar l'enorme diversitat trobada i es realitza un estudi morfològic preliminar que recolza els resultats genètics (*Objectius 1, 2, 4, 5* i *6*).

El **Capítol 6** inclou un sol article (Article 8) on s'estudia la variació del verí de les aranyes del gènere *Loxosceles*. Concretament, s'estudia la variació qualitativa en la composició del verí, el grau d'activitat de l'esfingomielinasa D, l'enzim essencial pels efectes tòxics del verí i la diversitat filogenètica expressada d'aquest enzim, en diferents llinatges de *Loxosceles rufescens* i en dues noves espècies endèmiques de les Illes Canàries (*Objectius 2* i *7)*.

En els següents apartats (Discussió general i Conclusions) es realitza una discussió conjunta dels resultats de la tesi, amb l'objectiu d'integrar els resultats obtinguts i discutits de forma separada en cadascun dels Articles, per tal de contextualitzar-los i extreure'n les conclusions més rellevants.

RESULTATS

INFORME DEL DIRECTOR

El Dr. Carles Ribera Almerje, com a director de la tesi doctoral titulada "Diversitat del gènere *Loxosceles* Heineken & Lowe, 1832 a la Mediterrània i les Illes Canàries: sistemàtica, biogeografia i loxoscelisme", presenta a continuació el factor d'impacte dels articles publicats i l'estat de publicació de la resta d'articles que componen la present Tesi.

Article 1 Planas E, Saupe EE, Lima-Ribeiro MS, Peterson AT, Ribera C. Ecological niche and phylogeography elucidate complex biogeographic patterns in *Loxosceles rufescens* (Araneae, Sicariidae) in the Mediterranean Basin.
Pendent de decisió editorial a: *BMC Evolutionary Biology*
Impact factor 2012: 3.285 Q2: *Evolutionary Biology*

Article 2 Planas E, Ribera C. 2014. Uncovering overlooked island diversity: colonization and diversification of the medically important spider genus *Loxosceles* (Arachnida: Sicariidae) on the Canary Islands. *Journal of Biogeography* 41: 1255–1266
Impact factor 2012: 4.863 Q1: *Ecology*

Article 3 Planas E, Ribera C. Description of six new species of *Loxosceles* (Araneae: Sicariidae) endemic to the Canary Islands, and the utility of DNA barcoding for their fast and accurate identification.
En revisió a: *Zoological Journal of the Linnean Society*
Impact factor 2012: 2.583 Q1: *Zoology*

Article 4 Planas E, Bernaus L, Ribera C. Development of novel microsatellite markers for the spider genus *Loxosceles* (Sicariidae) using next-generation sequencing.
En revisió a: *The Journal of Arachnology*
Impact factor 2012: 0.729 Q3: Entomology

Article 5 Planas E, Bernaus L, Sánchez-Gracia A, Ribera C. Genetic diversity is affected by Pleistocenic sea-level changes and volcanic activity in a spider of the genus *Loxosceles* endemic to the eastern Canary Islands.
En preparació *

Article 6 Ribera C, Planas E. 2009. A new species of *Loxosceles* (Araneae, Sicariidae) from Tunisia. *ZooKeys* 16: 217-225.
Impact factor 2012: 0.864 Q3: *Zoology*

Article 7 Planas E, Ribera C. On the shoulders of Atlas: high genetic diversity of *Loxosceles* spiders (Araneae: Sicariidae) in the Souss-Massa and adjacent regions (NW Africa).
En preparació *

Article 8 Planas E, Zobel-Thropp PA, Ribera C, Binford G. Not as docile as it looks: *Loxosceles* venom variation and loxoscelism in the Mediterranean Basin and the Canary Islands.
En revisió a: *Toxicon*
Impact factor 201*2*: 2.924 Q2: *Toxicology*

* Els articles 5 i 7 estan en fase de preparació i es preveu enviar-los a revistes del primer quartil de les àrees d'*Evolutionary Biology* i *Zoology*, respectivament.

A més, certifico que la memòria d'aquesta Tesi ha estat elaborada per n'Enric Planas Figueras (EP) en la seva totalitat i que els articles que la componen no seran utilitzats en cap altra tesi doctoral. A continuació es detalla la contribució de cadascun dels coautors en els diferents articles presentats en aquesta Tesi.

Article 1 En CR i l'**EP** van dissenyar l'estudi i van participar en el treball de camp. L'**EP** va realitzar el treball de laboratori i les anàlisis filogenètiques. En MLR va preparar els models climàtics. L'ATP, l'EES i l'**EP** van dissenyar i realitzar les anàlisis de nínxol ecològic. L'EES i l'**EP** van escriure el primer esborrany del treball i la resta d'autors van contribuir en la seva millora.

Article 2 En CR i l'**EP** van dissenyar l'estudi. L'**EP** va realitzar el treball de camp, el treball de laboratori i les anàlisis filogenètiques. L'**EP** va escriure el primer esborrany del treball i en CR el va revisar.

Article 3 En CR i l'**EP** van dissenyar l'estudi. L'**EP** va realitzar el treball de camp, l'estudi morfològic, les anàlisis estadístiques i les moleculars. L'**EP** va escriure el primer esborrany del treball i en CR el va revisar.

Article 4 En CR i l'**EP** van dissenyar l'estudi. L'**EP** va realitzar el treball de camp. La LB va realitzar el treball de laboratori. La LB i l'**EP** van realitzar les anàlisis bioinformàtiques. L'**EP** va escriure el primer esborrany del treball i en CR el va revisar.

Article 5 En CR i l'**EP** van dissenyar l'estudi. L'**EP** va realitzar el treball de camp. La LB va realitzar el treball de laboratori. L'ASG i l'**EP** van realitzar les anàlisis. L'**EP** va escriure el primer esborrany del treball i en CR el va revisar.

Article 6 En CR va dissenyar l'estudi i va realitzar l'estudi morfològic i filogenètic. L'**EP** va realitzar les il·lustracions. En CR va escriure el primer esborrany del treball i l'**EP** el va revisar.

Article 7 En CR i l'**EP** van dissenyar l'estudi, van realitzar el treball de camp i van estudiar morfològicament el material. L'**EP** va realitzar el treball de laboratori i les anàlisis filogenètiques. L'**EP** va escriure el primer esborrany del treball i en CR el va revisar.

Article 8 En CR i l'**EP** van dissenyar l'estudi. La GB, la PZT i l'**EP** van realitzar el treball de laboratori. L'**EP** va realitzar les anàlisis estadístiques i filogenètiques. L'**EP** va escriure el primer esborrany i la resta dels autors van contribuir en la seva millora.

Barcelona, de juliol de 2014,

Carles Ribera Almerje

CAPÍTOL 1

Diversitat del gènere *Loxosceles* a la Conca Mediterrània

Article 1 Planas E, Saupe EE, Lima-Ribeiro MS, Peterson AT, Ribera C. Ecological niche and phylogeography elucidate complex biogeographic patterns in *Loxosceles rufescens* (Araneae, Sicariidae) in the Mediterranean Basin.

Patrons biogeogràfics complexos en *Loxosceles rufescens* (Araneae, Sicariidae) a la Conca Mediterrània elucidats per models de nínxol ecològic i filogeografia

RESUM

Comprendre la història evolutiva de complexos d'espècies morfològicament críptiques és dificultós, i esdevé més intrincat quan les distribucions geogràfiques han estat modificades per dispersions afavorides pels humans. Aquesta situació és comuna a la Conca Mediterrània, on a més de l'elevada heterogeneïtat ambiental característica de la regió, la perllongada presència humana ha enterbolit els processos biogeogràfics que han conformat la diversitat actual. *Loxosceles rufescens* (Araneae, Sicariidae) n'és un exemple paradigmàtic: essent nativa de la Mediterrània, aquesta espècie s'ha dispersat per tot el món gràcies a viure lligada als humans. Un estudi previ va revelar una considerable diversitat molecular en aquesta espècie, suggerint l'existència d'espècies críptiques, però les relacions entre els llinatges no es corresponien amb les localitats geogràfiques.

Amb les anàlisis de delimitació realitzades amb les dades de la subunitat I del citocrom oxidasa c, s'han identificat 11 llinatges evolutius amb dos patrons filogeogràfics contrastats: (1) uns llinatges amb poblacions ben estructurades al Marroc i la Península Ibèrica, i (2) uns llinatges que manquen d'estructura geogràfica al llarg de la Mediterrània. Les estimes d'edats han situat la major part de diversificacions en el Pleistocè, i la diferenciació al·lopàtrica dels llinatges és compatible amb els múltiples refugis Pleistocènics identificats amb la modelització de nínxol ecològics (ENM). Sembla que el transport indirecte pels humans ha complicat la biogeografia actual en aquest grup d'aranyes sinantròpiques i d'importància mèdica.

En aquest estudi hem integrat models de nínxol ecològic (ENM) amb anàlisis filogeogràfiques per tal d'elucidar la història evolutiva de *L. rufescens* a la Conca Mediterrània, amb especial èmfasi en l'origen de la diversitat de l'ADN mitocondrial. Hem trobat suport per a la hipòtesi que proposa el Nord d'Àfrica com a centre d'origen de *L. rufescens* i que la diversitat genètica actual es va originar en al·lopatria, segurament promoguda per l'acció successiva de les glaciacions Pleistocèniques. Aquest escenari amb múltiples refugis dins de la Conca Mediterrània, principalment al Nord d'Àfrica, el vam corroborar combinant els resultats de vuit models generals de circulació oceano-atmosfèrics (AOGCM) i utilitzant dues metodologies de delimitació de refugis diferents. Els resultats

dels ENM van resultar útils per obtenir una visió general dels possibles refugis, però la delimitació detallada d'aquests depenia del nivell d'astringència aplicat en la concordança entre models.

PARAULES CLAU: aràcnid, evolució, dispersió afavorida pels humans, glaciacions Plesitocèniques, refugis, aranya.

Ecological niche and phylogeography elucidate complex biogeographic patterns in *Loxosceles rufescens* (Araneae, Sicariidae) in the Mediterranean Basin

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ABSTRACT

Background

Understanding the evolutionary history of morphologically cryptic species complexes is difficult, and made even more challenging when geographic distributions have been modified by human-mediated dispersal. This situation is common in the Mediterranean Basin where, aside from the environmental heterogeneity of the region, protracted human presence obscures the biogeographic processes that shaped current diversity. *Loxosceles rufescens* (Araneae, Sicariidae) is an ideal example: native to the Mediterranean, the species has dispersed worldwide via cohabitation with humans. A previous study revealed considerable molecular diversity, suggesting cryptic species, but relationships among lineages did not correspond to geographic location.

Results

Delimitation analyses on cytochrome *c* oxidase subunit I identified 11 different evolutionary lineages, presenting two contrasting phylogeographic patterns: (1) lineages with well-structured populations in Morocco and Iberia, and (2) lineages lacking geographic structure across the Mediterranean Basin. Dating analyses placed main diversification events in the Pleistocene, and multiple Pleistocene refugia, identified using ecological niche modeling (ENM), are compatible with allopatric differentiation of lineages. Human-mediated transportation appears to have complicated the current biogeography of this medically important and synanthropic spider.

Conclusions

We integrated ecological niche models with phylogeographic analyses to elucidate the

evolutionary history of *L. rufescens* in the Mediterranean Basin, with emphasis on origins of mtDNA diversity. We found support for the hypothesis that northern Africa was the center of origin for *L. rufescens*, and that current genetic diversity originated in allopatry, likely promoted by successive glaciations during the Pleistocene. We corroborated the scenario of multiple refugia within the Mediterranean, principally in northern Africa, combining results from 8 atmosphere-ocean general circulation models (AOGCM) with two different refugium-delimitation methodologies. ENM results were useful for providing general views of putative refugia, with fine-scale details depending on the level of stringency applied for agreement among models.

KEYWORDS: Arachnid, evolution, human-mediated dispersal, Pleistocene glaciations, refugia, spider.

BACKGROUND

The Mediterranean Basin was placed among 25 world biodiversity 'hotspots for conservation priority' based on high levels of endemism and rapid loss of natural areas (Myers *et al*., 2000). Humans began transforming Mediterranean ecosystems >10,000 years ago (Abulalfia, 2011), such that today, only 4.7% of primary vegetation remains unaltered in the region Geri *et al*., 2010). Despite these long-standing impacts, the Mediterranean remains home to a diverse flora and fauna.

Several factors (e.g. climate, geology) promoted development of this diversity at different temporal and geographic scales, such as the Messinian Salinity Crisis and the onset of a Mediterranean-type climate ~3.2 Mya (Blondel *et al*., 2010). Glaciations during the Pleistocene (~2.6 – 0.02 Mya) also played a role in shaping current diversity patterns (Médail & Diadema, 2009): climatic fluctuations during this period caused regional extinctions (Koch & Barnosky, 2006; Postigo-Mijarra *et al*., 2010) and promoted range shifts and diversification via allopatric speciation (e.g. Hewitt, 2000). The three major southern Mediterranean peninsulas (Iberia, Italy, Balkans) were long thought to have served as major refugia for European flora and fauna during Pleistocene glaciations (Hewitt, 2000), but recent studies have challenged this paradigm as too simplistic to explain observed patterns (Weiss & Ferrand, 2007; Feliner, 2011). As a consequence, some authors have argued for refugia within refugia (Gómez & Lunt, 2007) or multiple northern refugia

(Stewart & Lister, 2001; Schmitt & Varga, 2011; but see Tzedakis *et al*., 2013).

Different approaches have been used to delimit glacial refugia in the Mediterranean. Traditionally, paleoecological evidence (Elenga *et al*., 2000; Tzedakis *et al*., 2002; Carrión *et al*., 2010) and concentrations of endemic taxa (Médial & Quézel, 1997) were used, but more recently, comparative phylogeographic studies have delineated "phylogeographic hotspots", or areas with unique genetic diversity (Médail & Diadema, 2009), while others have used ecological niche models (ENMs) in conjunction with paleoclimatic simulations (e.g. e.g. Besnard *et al*., 2013). The latter methodology projects environmental requirements of species onto past conditions, thus offering an approach that is independent and complementary (Carstens & Richards, 2007; Waltari *et al*., 2007; Peterson & Nyári, 2008). Each approach has drawbacks and merits, but identifying regions using multiple approaches offers increased confidence (Waltari *et al*., 2007; Peterson, 2009; Collevatti *et al*., 2013). Although other studies have successfully integrated phylogeographic and ENM approaches to uncover putative refugial areas (Peterson *et al*., 2004; Richards *et al*., 2007; Wilson & Pitts, 2012), few have treated the entire Mediterranean Basin (Jakob *et al*., 2007; Lozier & Mills, 2009; Besnard *et al*., 2013), and none have considered the added complexity of a human commensal.

Two *Loxosceles* spider species coexist in the Mediterranean: *L. rufescens* (Dufour 1820) and the Tunisian *L. mrazig* Ribera & Planas 2009. *Loxosceles rufescens* originated in the Mediterranean (Gertch, 1967; Binford *et al*., 2008; Duncan *et al*, 2010; Ribera & Planas, 2009) but has been transported worldwide by humans (Gertch, 1967; Binford *et al*., 2008; Duncan *et al*., 2010; Platnick, 2013). Duncan *et al*. (2010) documented diverse genetic lineages among individuals morphologically consistent with *L. rufescens*, suggesting cryptic speciation. The morphological simplicity within *Loxosceles* makes traditional species delimitation "singularly difficult" (Brignoli, 1969, p. 142), so genetically-based methodologies are key to illuminating the evolutionary history of this group. In addition, relationships between *L. rufescens* lineages are not predictable by geographic location (Duncan *et al*., 2010), in contrast with the high spatial structure of populations in other *Loxosceles* species (Binford *et al*., 2008; Planas & Ribera, 2014). Therefore, *Loxosceles rufescens* is an ideal model to unravel the role of climate change and human impacts on the evolutionary history of Mediterranean species.

In this contribution, we examine mtDNA diversity within *L. rufescens*, and elucidate

evolutionary processes that promoted this diversity via a combination of phylogeographic and ENM approaches. Our working hypothesis is that current mtDNA diversity was generated allopatrically in glacial refugia across the Mediterranean, and that *L. rufescens* biogeography was since obfuscated by human activity. This hypothesis offers three opportunities for testing: (1) divergence times should coincide with periods of repeated glaciations (~2.6 – 0.02 My), (2) multiple putative refugia should have existed to provide areas of origin for distinct evolutionary lineages, and (3) widespread lineages should show no spatial structure within the Mediterranean Basin.

METHODS

Taxonomic sampling

We sampled *L. rufescens* populations across the Mediterranean Basin to replicate and complement previous sampling (Duncan *et al*., 2010) and to increase the likelihood of discovering new lineages. In all, 158 localities were sampled across 8 countries (Figure 1 and Additional file 1). From these localities, 310 individuals were sequenced and included in our analyses.

Molecular data

We included at least one individual from each locality in molecular analyses. Total genomic DNA was extracted using SPEEDTOOLS Tissue DNA Extraction Kit (BIOTOOLS) following manufacturer's protocols. We amplified a portion of the cytochrome *c* oxidase subunit I (*cox1*) using LINF and GAYAR primers (Planas & Ribera, 2014), producing 1016-bp fragments; we used different combinations of C1-J-1718 and C1-J-2183 (Simon *et al*., 1994) and C1-N-2191 (Folmer *et al*., 1994) internal primers when the first set failed. PCR reactions were conducted at a final volume of 25 μL using either *Taq* polymerase (Promega) or Biotools *Pfu* DNA Polymerase (Biotools). PCR products were cycle-sequenced in both directions using the same PCR primers and the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were derived from these products in an ABI 3700 automated sequencer at the Serveis Científico-Tècnics of the Universitat de Barcelona and in Macrogen, Inc. (Seoul, Korea). Raw sequences were edited and assembled with GENEIOUS 4.6.5 (Drummond *et al.,* 2009). To avoid amplification of pseudogenes reported by Duncan *et al*. (2010) for *Loxosceles cox1*, we primarily used *Loxosceles*-specific primers to assure amplification of correct fragments; we also translated

sequences into amino-acids, and checked for stop-codons.

Sequences were aligned unambiguously in GENEIOUS using ClustalW (Thompson *et al*. 1994) with default parameters. We partitioned data by codon position and explored best partitioning schemes and substitution models simultaneously using PartitionFinder v.1.0.1 (Lanfear *et al*., 2012) under a Bayesian information criterion for the entire matrix. These steps were conducted independently for individuals employed in delimitation analyses, and for the reduced set used in dating analyses.

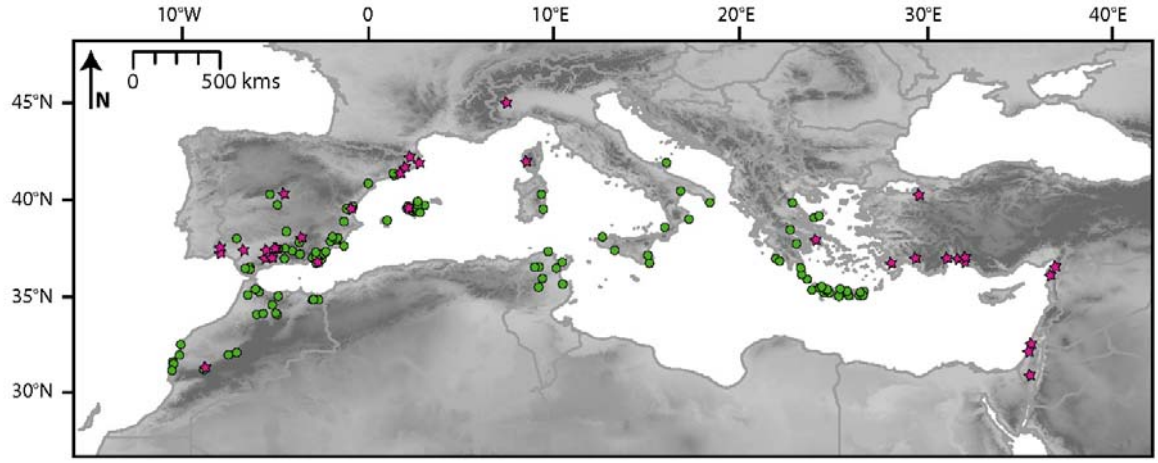


Figure1 Map of sampling localities. Green circles represent localities used in ENM analyses; pink stars indicate additional sampled localities used in the phylogenetic analyses.

Phylogenetic analyses

We used maximum likelihood (ML) and Bayesian inference (BI) to infer phylogenetic relationships from a dataset containing one representative of each *cox1* haplotype. ML analyses were conducted in RAxML 7.4.2 (Stamatakis, 2006) with the aid of the graphical front-end RAXML-GUI 1.3 (Silvestro & Michalak, 2011). We applied a rapid hill-climbing search algorithm, and conducted 1000 non-parametric bootstrap replicates. BI analyses were conducted in MrBayes 3.2 (Ronquist *et al*., 2012) with two independent runs of 2 million generations with four Markov chains (one cold, three heated), sampling every 1000 generations. We checked convergence of chains visually in Tracer (Rambaut & Drummond, 2007) until effective sample sizes (EES) were above 200, and the average standard deviations of split frequencies (ASDSF) of the two runs were below 0.01. The first 25% of trees in each run were discarded as burn-in, and a majority-rule consensus tree was generated from remaining trees. BI trees were also obtained with BEAST (Drummond *et*

al., 2012) using a coalescent tree prior with a constant population size and a relaxed lognormal clock (rate fixed arbitrarily at 1). Two independent runs of 20 million generations (sampling every 1000th generation) were used for each analysis. We assessed convergence and correct mixing of chains by inspecting the trace plots and ensuring EES > 200 in Tracer. The two runs were combined using LogCombiner and TreeAnotator (Drummond & Rambaut, 2007) after removing a 10% burn-in of the samples. Position of the root of the tree was estimated implicitly in BEAST (Drummond *et al*., 2006) and used for rooting RAxML and MrBayes trees.

Genetic p-distances between and within lineages (see *Molecular delimitation analyses*) were calculated using MEGA5 (Tamura *et al*., 2011). To study demographic history (only for lineages with $N > 10$), we applied two neutrality tests: Fu's F_S (Fu, 1997) and R_2 (Ramos-Onsins & Rozas, 2002) in DnaSP v5.10 (Librado & Rozas, 2009). We assessed statistical significance and confidence intervals using coalescence simulations in DnaSP with 1000 replicates and default parameters. We excluded 6 sequences from the A6 lineage that lacked the data for the 3' or 5' ends.

Molecular delimitation analyses

Morphological traits provide few means of distinguishing lineages of *L. rufescens*, but genetic distances between lineages are high and different lineages are often sympatric at micro-scales (Duncan *et al*., 2010; pers. obs.); consequently, assignment of individuals to lineages is not a simple function of geographic location. To account for this, we used two methodologies for objective delimitation of evolutionary lineages: (1) a General Mixed Yule Coalescent model (GMYC), and (2) phylogenetic network estimation using statistical parsimony (TCS). Although these methodologies have been successful at circumscribing species and often yield results congruent with alternative species delimitation methods (e.g. based on morphology, behavior; Esselstyn *et al*., 2012; Papadopoulou *et al*., 2013), their utility depends on different factors (e.g. effective population size, sampling scheme; Lohse, 2008; Esselstyn *et al*., 2012; Fujisawa & Barraclough, 2013), with results tending perhaps towards overestimation of species numbers (Salter *et al*., 2013).

GMYC.—GMYC is a species delimitation method that provides an objective way to delimit genetic clusters (Fujisawa & Barraclough, 2013). The method was developed to identify putative species in poorly-known groups based on single molecular markers (Pons *et al*., 2006). The model seeks transition points (thresholds) between inter-specific

relationships and intra-specific coalescent events, and subsequently tests the likelihood of the model against a null model that assumes a single branching process for the entire tree (Pons *et al*., 2006). Since GMYC requires identical sequences to be removed (Fujisawa & Barraclough, 2013), we included one representative of each haplotype. Because GMYC is sensitive to relative branch lengths and topology of the ultrametric tree (Papadopoulou *et al*., 2013), we explored effects of alternative input trees obtained from ML using RAxML, and BI using MrBayes and BEAST, as described below.

We generated a ML tree using RAxML 7.4.2 (Stamatakis, 2006) as outlined above. We converted this result to an ultrametric tree using PATHd8 (Britton *et al*., 2007), arbitrarily fixing the root at 100 units. Bayesian trees were derived using MrBayes 3.2 (Ronquist *et al.,* 2012), with two independent runs of 50 million generations with four Markov chains each (one cold, three heated), sampling every 1000 generations with a coalescent clock prior. The clock rate was arbitrarily fixed to 1, and we used a lognormal distribution as a prior for population size. We checked for chain convergence as described above. The ultrametric tree was imported into R (R Core Team, 2013) and made fully dichotomous with the multi2di function in the APE package v. 30-8 (Paradis *et al*., 2004). The ultrametric Bayesian tree from BEAST was obtained as outlined above.

TCS.—Separate haplotype networks in statistical parsimony analyses might provide a useful and objective method to delimit individuals into evolutionary significant units (Hart & Sunday, 2007). Networks delimited using a 95% parsimony connection limit, i.e. the probability that two DNA sequences share a parsimonious relationship without multiple substitutions underlying any single nucleotide difference (Templeton, 1992), generally correspond to single species (78% in 663 examples in Hart & Sunday, 2007). We obtained statistical parsimony networks in TCS 1.3 (Clement *et al*., 2000) using our complete dataset (310 individuals), applying 95% and 99% connection limits, and treating gaps as missing data.

Patterns of genetic diversity

We investigated how mtDNA diversity is distributed across geography within *L. rufescens* following Arrigo *et al*. (2010); this methodology is of particular use when localities have unequal sample sizes. Diversity statistics are computed by considering samples located within a perimeter around a grid point. We set grid points every 100 km in latitude and longitude, and computations were assessed across random sets of 5 individuals,

bootstrapping 1000 times. We calculated three diversity indices, total diversity (H_T), haplotype richness (H_R), and rarity index (R) (Besnard *et al*., 2013) in R (R Core Team, 2013) using custom scripts provided by N. Arrigo. High genetic diversity combined with rare haplotypes are characteristics of populations with a long *in situ* history, so identification of this pattern could indicate regions of origin of the different lineages (Ehrich *et al*., 2008). We explored effects of different parameters on diversity indices, varying grid point distances 50-450 km and numbers of individuals per analysis 3-10.

We tested the geographic structure of each lineage using a Mantel test implemented in the Isolation By Distance Web Service v. 3.23 (Jensen *et al*., 2005). Geographic distances among localities were calculated using the Geographic Distance Matrix Generator v. 1.2.3 (Ersts, http://biodiversityinformatics.amnh.org/open_source/gdmg), and genetic p-distances between localities were calculated in MEGA. We performed Mantel tests with 999 permutations to assess significance of correlations between genetic distances and log-transformed geographic distances. We excluded lineages B1 and B3 from Mantel tests owing to low numbers of localities (< 5). Lineages A1 to A5 were pooled for the analyses (see results), and remaining lineages were assessed independently.

Dating analysis

Since we lacked reliable calibration points within the *L. rufescens* lineage, we explored two divergent rates. First, we used a *Loxosceles*-specific molecular rate obtained using fossil and island ages as calibration points (Planas & Ribera, 2014). Second, we applied the substitution rate obtained for the same mtDNA gene in a closely-related spider family (Dysderidae; Bidegaray-Batista & Arnedo, 2012). As the two rates are fairly divergent, we suspect that actual divergence times for *L. rufescens* fall somewhere between these end points. Rates were incorporated as priors under a normal distribution with mean 0.095 ± 0.001 and 0.0199 ± 0.001, respectively. Dating analyses were conducted in BEAST v1.7.4 (Drummond *et al.,* 2012), using an uncorrelated lognormal relaxed clock (Drummond *et al*., 2006) and a Yule tree prior. One representative of each lineage was included in analyses, and we used two independent runs of 10 million generations, sampling every 1000th generation, for each analysis. We assessed convergence and correct mixing of chains by inspecting trace plots and ensuring EES > 200 using Tracer. Runs were combined using LogCombiner and TreeAnotator, after removing a 10% burn-in.

Ecological Niche Modeling (ENM)

Study area.—Niche models for *L. rufescens* were calibrated within a hypothesis of a region that was 'sampled' by the species over its relevant history; in other words, a region we hypothesized that the species had been able to deem suitable/unsuitable over its history (**M**; *sensu* Barve *et al*., 2011), intersected with regions that were sampled as part of this study (Peterson, 2011). To calculate **M**, we buffered *L. rufescens* records by the longest distance from the sea to a documented locality (~350 km), which provided an estimate of the dispersal capability of the species. We excluded areas that we were unable to sample, or where closely-related species occur (*L. mrazig* in southern parts of Tunisia and southeastern Morocco; an undescribed species group in the Sous Valley of Morocco). These steps left a calibration area comprising Morocco, the Iberian Peninsula, Balearic Islands, Sardinia, Sicily, continental Italy, Greece and adjacent islands, Crete, and Tunisia. After model calibration in this area, we projected results to the entirety of the Mediterranean Basin, within a bounding rectangle of 48.2–26.7° latitude and -14.8–41.2° longitude (see Fig 1).

Climatic data.—We obtained climatic data from 8 coupled atmosphere-ocean general circulation models (AOGCMs) simulations: Community Climate System Model (CCSM), Centre National de Recherches Météorologiques (CNRM), Consortium for Small-scale Modelling (COSMOS), Goddard Institute for Space Studies (GISS), Institute Pierre Simon Laplace (IPSL), Model for Interdisciplinary Research on Climate (MIROC), Max-Planck Institut für Meterologie (MPI), and Meteorological Research Institute (MRI). These AOGCMs were obtained from the multi-model ensemble in the Coupled Model Intercomparison Project Phase 5 (CMIP5: http://cmip-pcmdi.llnl.gov/) and the Paleoclimate Modelling Intercomparison Project Phase 3 (PMIP3: http://pmip3.lsce.ipsl.fr/); more details about the climate models are provided in Additional file 2 and in Taylor *et al*. (2012).

We downloaded monthly simulation outputs for annual precipitation and mean, maximum, and minimum temperatures from the pre-industrial experiment, which characterized current climatic conditions. To characterize past conditions, we used paleoclimatic simulations for the mid-Holocene (~6 Ka) and Last Glacial Maximum (LGM, ~21 Ka) from each AOGCM, except GISS and COSMOS, which lacked mid-Holocene outputs. To produce climate scenarios at resolutions relevant to the spatial scale of species' distributions, we downscaled climatic layers to 0.5º resolution using a standard change-factor approach (Wilby *et al*., 2004): (1) for each AOGCM, we computed the difference

among the past (mid-Holocene and LGM) and current simulations (i.e. pre-industrial), and the difference (i.e. climate change trends) and current climate were interpolated to 0.5° spatial resolution using kriging; (2) these differences were added to the interpolated current climate to obtain the interpolated past conditions. We used absolute differences for temperatures and relative differences for precipitation (see http://www.worldclim.org/downscaling, for more details). This procedure maintains higher-resolution topography in downscaled climates and ensures coherency of climate patterns over time (Hijmans & Graham, 2006). From these downscaled climatic scenarios, we computed 19 so-called 'bioclimatic' variables (http://www.worldclim.org/bioclim), but we excluded mean temperature of wettest and driest quarters, and precipitation of warmest and coldest quarters, owing to spatial artifacts that emerge in these four variables.

For each AOGCM, we performed a principal components analysis in R (R Core Team, 2013) on the 15 bioclimatic variables over the calibration area to create new axes that summarized variation in fewer, independent dimensions, and to reduce co-linearity among variables. We retained those principal components that explained cumulatively 99% of the overall variance in the dataset (i.e. the first 6 principal components for all AOGCMs except GISS, which required only the first 5) for model calibration. These principal components were used to calculate corresponding composite variables for mid-Holocene and LGM conditions. The PCA structure for current conditions was enforced for the past conditions using a script in R (R Core Team, 2013) written by A. Lira and N. Barve (U. Kansas).

Occurrence data.—We used a subset (130 records) of occurrence data associated with samples employed in the genetic analyses. These localities were obtained directly from fieldwork in natural areas by EP and others (see Acknowledgments), and have precise latitude/longitude coordinates derived from GPS measurements. To consider potential biasing effects introduced by spatial autocorrelation, such that spatially clumped points would over-represent certain environments, we calculated spatial lags in environmental data using Geostatistical Analyst in ArcMap 10 (ESRI, Redlands, CA), and subsampled the records to create 10 replicate datasets using a script in R (R Core Team, 2013) written by N. Barve (U. Kansas). Based on lag calculations, we enforced a minimum distance of 50 km between localities. Subsampling occurrence data to account for environmental lag ensures that suitable conditions are evenly weighted during model calibration. Each subset included 62-65 occurrence records.

Modeling algorithm.—ENMs were generated using Maxent v. 3.1.1 (Phillips *et al*., 2006), which can be monitored for extrapolation errors when projecting to past climates (Elith *et al*., 2011, Owens *et al*., 2013). Maxent minimizes the relative entropy between two probability densities—one from the distributional data and one from the background or study area—defined in covariate space (Elith *et al*., 2010). We used default parameters, but specified 100 bootstrap replicates per occurrence dataset and a minimum training presence threshold rule to avoid omission error. We took the median of the 100 runs per occurrence dataset multiplied by 1000, and converted to integer grids in ArcMap v.10. These grids were then used to calculate the median of the 10 subsets for each AOGCM. The resulting models were converted to binary grids based on all 130 localities using a minimum training present approach (Peterson *et al*., 2008). Use of multiple AOGCMs (Fordham *et al*., 2012) provides a broader estimate of suitable conditions for *L. rufescens*, but we acknowledge that ensemble-modeling approaches may shed additional light on model-dependent results (see Collevatti *et al*., 2013).

When transferring models temporally or spatially, conditions outside the range of climatic values in the calibration region (**M**) may be encountered, leading to situations of extrapolation. To identify these regions, we used a script in R (R Core Team, 2013) written by N. Barve (http://hdl.handle.net/1808/10122) to create Mobility Oriented Parity (MOP) maps (Owens *et al*., 2013). Areas identified as both suitable and extrapolative were removed from analyses, to avoid interpreting results outside of known climatic response conditions for *L. rufescens*.

Model evaluation.—To evaluate predictive power of models, we partitioned two of the replicate occurrence datasets at random: half of the data was used in model calibration, and the other half for model testing via partial Receiver Operator Characteristic (partial ROC) approaches (Peterson *et al*., 2008). Partial ROC avoids many of the problems associated with traditional ROC analyses, such as equal weighting of omission and commission errors, and consideration of model thresholds that yield irrelevant predictions. These tests were run using a Visual Basic routine developed by N. Barve (http://hdl.handle.net/1808/10059), with an expected error rate of $E = 1\%$ (Peterson *et al*., 2008). We performed 1000 bootstrap iterations by resampling 50% of test points with replacement.

Identifying refugial areas.—We identified possible refugia as areas that remained continuously suitable from the LGM to present. Because glaciations were common

throughout the Pleistocene, with recurrent glacial and interglacial conditions occurring in nearly-regular cycles and similar amplitude (at least since 800 ka) (Lisiecki & Raymo, 2005), we assume that the three time slices used here (LGM, mid-Holocene and present) capture, at least to some degree, the key climatic conditions across the entire Pleistocene (Tzedakis *et al*., 2004; Rodríguez-Sánchez & Arroyo, 2008). We acknowledge, however, that these reconstructions are merely broad estimates of potential refugial conditions for *L. rufescens* and lack constraining data for the earlier half of the epoch.

We identified refugia using two approaches: approach one (M1) required AOGCMs to agree on suitable area for each time slice, applying four different thresholds (8 of 8 to AOGCMs agree, 6/8, 4/8, and 1/8). This resulted in four different suitability maps for the LGM, the mid-Holocene, and the present-day. The intersection of the three time slices was taken as the final refugial area, which created four different possible refugial scenarios (Figure 3A). The second approach (M2) calculated refugial area across the three time slices for each individual AOGCM (Figure 3B); in other words, the intersection of suitable area was taken across the three time slices (LGM, mid-Holocene, and present-day) for each AOGCM independently. From the individual AOGCM maps of refugial areas, we applied the threshold criteria of M1 to identify consensus regions (8 of 8 AOGCMs agree, 6/8, 4/8 and 1/8). In effect, these two methods explored sensitivity to threshold choice, and resulted in 8 putative refugial maps. We repeated the two methods without COSMOS and IPSL, as these AOGCMs often exhibit anomalous climatic patterns compared to other AOGCMs. Using ENM to identify putative refugia can elucidate potential divergence mechanisms; however, considerable caution should be exercised in interpreting such analyses, particularly in light of the spatial grain of these data: coarse-resolution climate data cannot detect fine-scale phenomena (i.e. microrefugia *sensu* Rull, 2010).

RESULTS

Phylogenetic analyses

New sequences obtained during this study were deposited in GenBank with accession numbers KJ560560 - KJ560863 (Additional file 1); additional sequences were downloaded from GenBank (Additional file 1). In total, 310 sequences were used, containing 63 different haplotypes. PartitionFinder suggested a non-partitioned codon scheme with a HKY+G substitution model as best fit for these data under the Bayesian information

criterion; we used this partition scheme and substitution model in all phylogenetic analyses except RAxML, where only a GTR+G model was available.

Phylogenetic results were nearly identical between BI and ML approaches (available on TreeBase S15925). In both cases, *L. rufescens* was split in two main clades: A and B (Figure 2). Clade A included six well-supported lineages (all with bootstrap support >88% and posterior probabilities >0.76). Four lineages were composed exclusively of individuals from Morocco (termed lineages A1-A4). A1 placed as sister to a well-supported clade comprising A2-A6; the clade composed of A2-A4 was placed as sister to a clade composed of A5-A6. A5 included individuals from two Iberian Peninsula populations, and A6 included individuals from across the Mediterranean.

Clade B comprised 5 lineages (B1-B5), all well-supported (bootstrap support >97%, posterior probabilities 1.0). B5 placed as sister to the remaining lineages of clade B, and lineage B1 was sister to a well-supported clade (bootstrap support 99%, posterior probabilities 1.0) comprising B2-B4, but this latter relationship was not well supported (bootstrap support 54%, posterior probabilities 0.64). B2 placed as sister to B3 and B4 (bootstrap support 79%, posterior probabilities 0.98). B3 was composed exclusively of Iberian Peninsula individuals; the remaining lineages included individuals from different Mediterranean regions.

Genetic p-distances ranged from 1.5-7.8% between the various lineages (Additional file 3). The two major clades (A and B) were separated by a p-distance of 7.04% (Additional file 3). Neutrality tests for the lineages are presented in Table 1. Fu's *F*s test for demographic expansion was negative and significant in all cases except lineage B3. The R_2 test was low and significant for the two lineages with higher sample sizes (A6 and B5) in the left tail. As a whole, these results suggest a recent demographic expansion for all lineages except for lineage B3.

Molecular delimitation analyses

We used three methods to obtain the ultrametric trees required for GMYC analyses (Figure 2). In all three cases, the likelihood ratio test of the Yule model was significantly better than the null hypothesis (BEAST $p = 4.22 \times 10^{-6}$, RAxML and PATHd8 $p = 7.42 \times 10^{-7}$, and MrBayes $p = 1.28 \times 10^{-6}$, respectively). Clusters identified (i.e. GMYC groups composed of more than single individuals) were mostly congruent across methods; in all three, 7 clusters

were delimited. Slight differences between approaches appeared in terms of detecting singletons: in total, we recovered 14 entities (clusters plus singletons) using the ultrametric tree obtained with BEAST (confidence interval: 11-21), while the remaining two analyses delimited 11 entities (confidence interval: 11-14 with RAxML, 10-11 with MrBayes). Differences occurred regarding lineages B5, A4, and A6, wherein GMYC analyses using the BEAST tree split each of these lineages into two clusters (Figure 2).

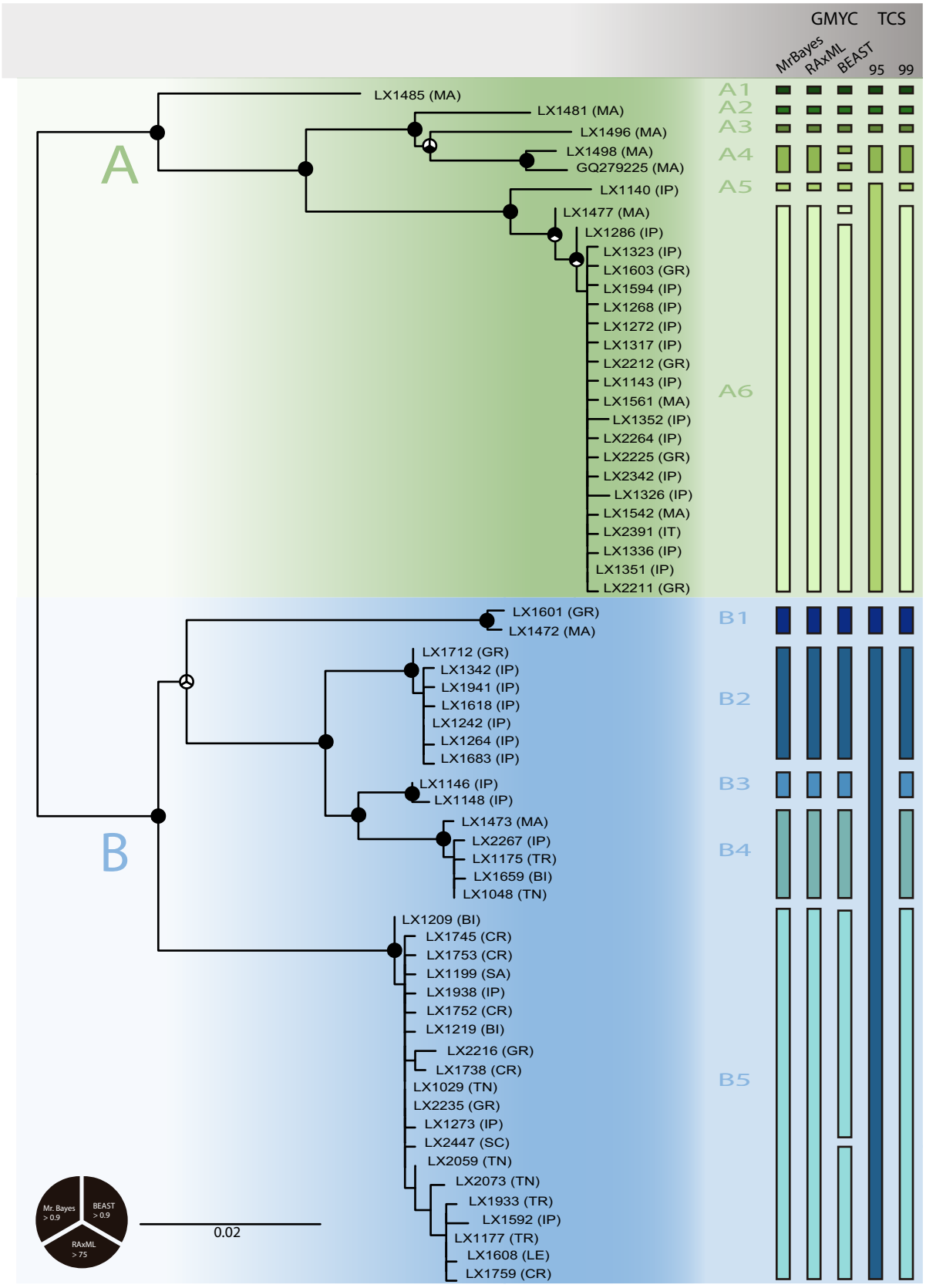
Table 1: Summary of the nucleotide diversity estimates and neutrality tests

Lineage	n	sites	H	H_D	π	F_S	95% CI	R_2	95% CI
A6	136	808	20	0.298	0.00045	-34.268***	-4.43468-3.29319	0.0185*	0.01460-0.23791
B2	36	480	5	0.260	0.00057	-4.111***	-1.89259-2.72200	0.0704 NS	0.05397-0.25
B3	10	565	2	0.2	0.00035	-0.339 NS	-0.59381-1.52347	0.3 NS	0.17778-0.3
B4	42	555	5	0.184	0.00043	-4.408***	-2.08853-2.63058	0.0781 NS	0.04646-0.25377
B5	61	407	12	0.572	0.00187	-9.883***	-3.39388-4.14765	0.0415*	0.04556-0.22971

Abreviations: * _ P<0.05; *** _ P<0.01; NS _ non significant; n _ number of sequences; H _ number of haplotypes; Hd _ Haplotype diversity; π _ nucleotide diversity; F_S _ Fu's Fs; R_2 _ Ramos-Onsins & Rozas R_2 test; CI _ Confidence interval.

TCS results varied depending on the connection limit (Figure 2): a 95% connection limit resulted in fewer independent networks compared to a 99% limit. In the former case, maximum number of calculated steps was 13, forming 7 independent networks; in the latter case, maximum number of calculated steps was 5, with 11 independent networks. The higher connection limit mirrored GMYC results. We found two main patterns in the haplotype networks: (1) several lineages composed of individuals restricted to one or a few localities that harbor only one or a few haplotypes, and conversely, (2) single haplotypes present in individuals from across the Mediterranean Basin, with closely related haplotypes forming a star-like network. (Figure 4).

Figure 2 Maximum likelihood tree based on single representatives of each *cox1* haplotype. Node circles represent maximum likelihood bootstrap and Bayesian posterior probabilities as shown in the legend. Green indicates the A clade, and blue the B clade. Each column on the right indicates a different delimitation method, and delimited lineages are represented with colored bars. Abbreviations: MA (Morocco), IP (Iberian Peninsula), GR (Greece), IT (Italian Peninsula), TR (Anatolian Peninsula), BI (Balearic Islands), TN (Tunisia), CR (Crete), SC (Sicily), LE (Levant).


GMYC
TCS
MrBayes
RAxML
BEAST
95
99
A
B
A1
A2
A3
A4
A5
A6
B1
B2
B3
B4
B5
LX1485 (MA)
LX1481 (MA)
LX1496 (MA)
LX1498 (MA)
GQ279225 (MA)
LX1140 (IP)
LX1477 (MA)
LX1286 (IP)
LX1323 (IP)
LX1603 (GR)
LX1594 (IP)
LX1268 (IP)
LX1272 (IP)
LX1317 (IP)
LX2212 (GR)
LX1143 (IP)
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LX1175 (TR)
LX1659 (BI)
LX1048 (TN)
LX1209 (BI)
LX1745 (CR)
LX1753 (CR)
LX1199 (SA)
LX1938 (IP)
LX1752 (CR)
LX1219 (BI)
LX2216 (GR)
LX1738 (CR)
LX1029 (TN)
LX2235 (GR)
LX1273 (IP)
LX2447 (SC)
LX2059 (TN)
LX2073 (TN)
LX1933 (TR)
LX1592 (IP)
LX1177 (TR)
LX1608 (LE)
LX1759 (CR)
Mr. Bayes > 0.9
BEAST > 0.9
RAxML > 75
0.02

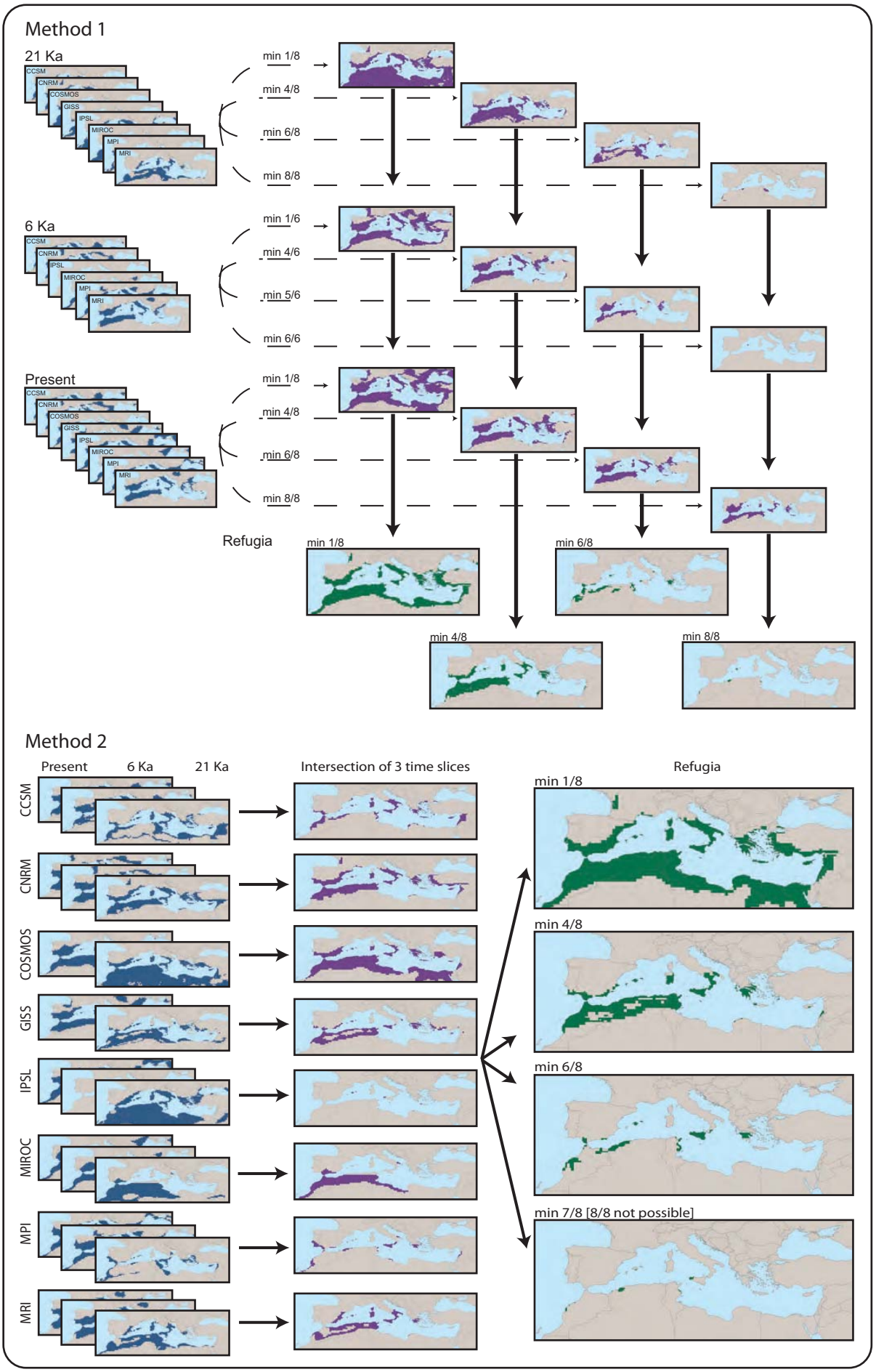
Patterns of genetic diversity

Analyses were conducted across the entire dataset and within lineages with wide distributions (i.e. A6, B2, B4, and B5; results presented in Additional file 4). Analyses across all lineages lacked clear geographic patterns for total diversity (H_T) and haplotype richness (H_R); however, they exhibited higher values for the rarity index (R) in southern Morocco. Analyses of individual lineages showed clear geographic patterns for lineage B2 and B4, wherein higher values for all diversity statistics were obtained in the Iberian Peninsula and Balearic Islands, respectively. Lineages A6 and B5 showed no clear geographic pattern, with highest values in various isolated regions. Different parametrizations for this test produced similar patterns (results not shown); decreasing the number of individuals to 3 (not recommended by the script authors, N. Arrigo pers. comm.) gave the same pattern, except within lineage B2, where high values for all statistics characterized the region around Greece. The Mantel test showed weak correlations between genetic and geographic distance matrices for lineages A6, B2, B4, and B5 ($r < 0.24$), with tests not significant except for lineage B5 (Table 2). Conversely, when lineages A1-A5 are pooled, we obtained a stronger and significant correlation ($r = 0.52$, $P < 0.05$; Table 2), suggesting that they are geographically structured.

Table 2: Results of Mantel test analyses performed with 10000 permutations to assess the significance of the correlation between genetic distances and log-transformed geographic distances.

Lineage	r	*P*
A1-A5	0.5235	0.0116
A6	0.0582	0.1762
B2	0.0024	0.4831
B4	0.1594	0.0593
B5	0.2424	<0.0001

Figure 3 Refugium delimitation methods. Refugial area were identified as the intersection of the three time slices. Method 1 sought consensus among the 8 AOGCMs in each time slice by requiring (a) all 8 to agree, (b) 6/8 to agree, (c) 4/8 to agree, or (d) at least one. Method 2 sought the intersection of the three time slices for each AOGCM independently, subsequently requiring the AOGCMs to agree in the same fashion as M1: (a) at least 7 (100% was not possible) to agree; (b) 6/8 to agree, (c) 4/8 to agree, and (d) at least one.


Method 1
21 Ka
CCSM
CNRM
COSMOS
GISS
IPSL
MIROC
MPI
MRI
min 1/8
min 4/8
min 6/8
min 8/8
6 Ka
min 1/6
min 4/6
min 5/6
min 6/6
Present
Refugia
Method 2
Present
6 Ka
21 Ka
Intersection of 3 time slices
Refugia
min 7/8 [8/8 not possible]

Dating analyses

Divergence time estimates differed depending on the rate used in calibration (see Additional file 5). Both analyses, however, dated major diversification events to the period of Pleistocene glaciations. The estimated split between the two major clades (A *versus* B, Figure 2) was dated at 0.356 Ma (95% HPD: 0.243-0.487) using the *Loxosceles*-specific rate, and at 1.968 Ma (1.322-2.698) with the *Parachtes* rate.

Niche models

Present.—Models from individual AOGCMs were predictive of independent suites of occurrence points, with all models statistically significant in partial ROC tests (all $P < 0.05$; Additional file 6). Predictions were consistent across AOGCMs (Additional file 7), with suitable areas identified in the southern Iberian Peninsula, Italy, Greece, western Turkey, northern Africa, and various Mediterranean islands. Minor differences were noted among models, for example with less suitable area identified in Turkey under MIROC and MRI. Regions environmentally outside those represented within **M** (highlighted by MOP analyses) were also consistent across AOGCMs, covering desert areas (e.g. the Sahara and Sinai Peninsula) and some regions around the Black Sea; these regions were not considered in our analyses.

Paleoprojections.—Most regions identified as suitable in the present were also identified as suitable during the mid-Holocene (Additional file 7). MOP results were similar to those in the present as well, with one exception: although IPSL identified potential distributions congruent with those in other AOGCMs, MOP analyses indicated most of these regions as environmentally novel. A similar situation occurred with the LGM projection for MIROC. Refugial area delimitation was not affected by removal of these two models with odd results.

Compared to present and mid-Holocene projections, LGM potential distributions shifted southward. The main Mediterranean peninsulas (i.e. Iberia, Italy, Balkans) retained suitable conditions, although reduced in extent, but extensive regions of the Sahara, which were largely unsuitable in the present and mid-Holocene, were identified as suitable during the LGM under most AOGCMs. Regions environmentally outside environments represented within M occurred across broad swaths of the northern and southeastern portions of our study area.

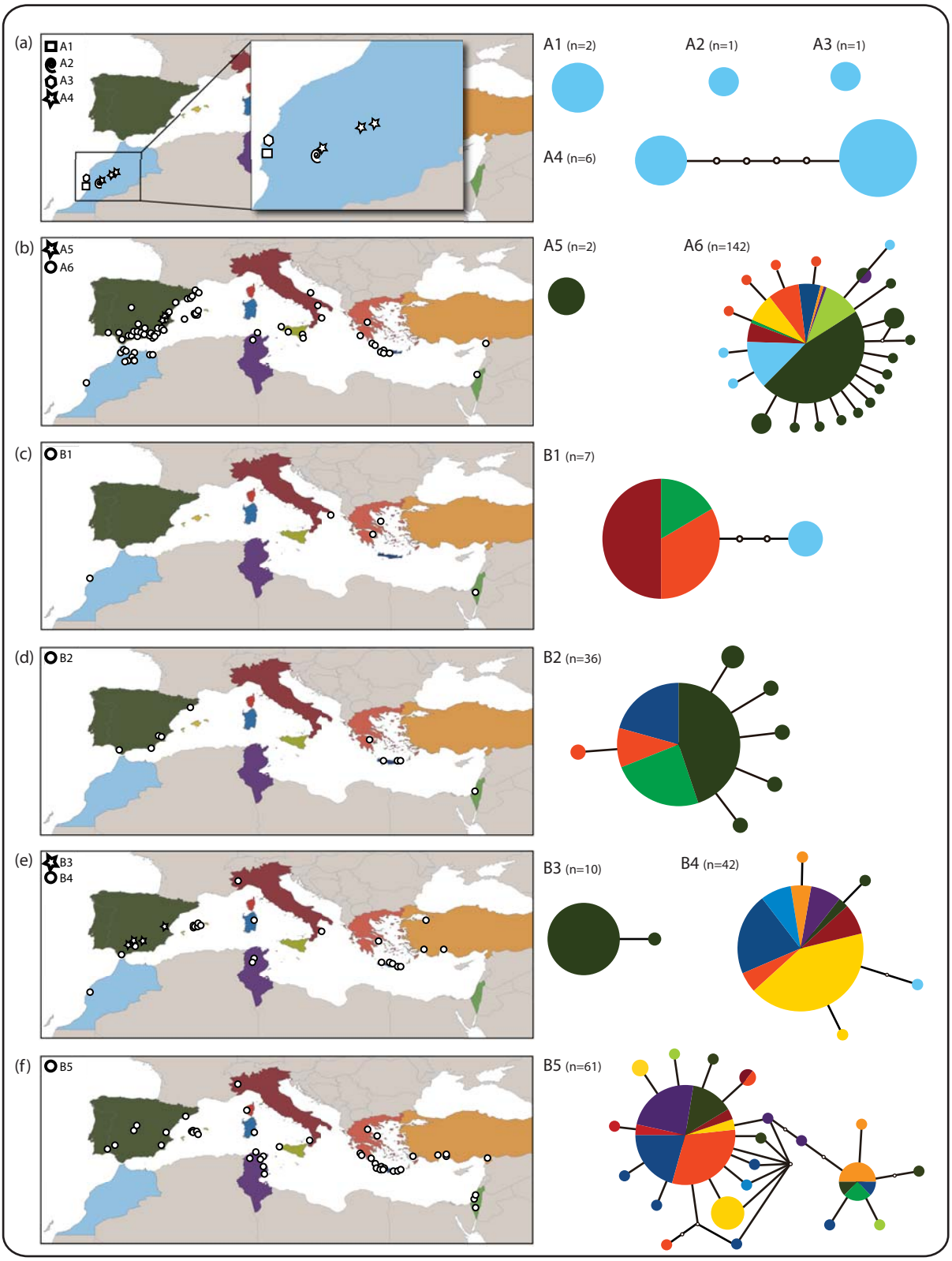


Figure 4 Distribution map and haplotype network for each lineage. Colors on haplotype networks correspond to colored areas on the maps to the left. Haplotype networks are not to the same scale among lineages. Each circle represents one haplotype, and colors correspond to frequencies of origin region of the haplotype (n = number of individuals). Note the two contrasting phylogeographic patterns, with lineages A1 to A5 restricted to one or a few well-structured populations, whereas the lineages distributed across the Mediterranean Basin generally lack geographic structure, likely a consequence of human-mediated dispersal.

Identifying refugial areas

Results from individual AOGCMs were similar, with the exception of IPSL. Because IPSL is based on a different vegetation model from other AOGCMs (for example, bare soil is considered a type of vegetation, whereas other AOGCMs ignore this factor), we ran refugial delimitation analyses with and without IPSL; putative refugia were congruent across the two methodologies and with and without IPSL (Figure 5; without IPSL not shown). Depending on the level of stringency enforced for AOGCM agreement, 4-14 major, independent, and isolated refugia were identified (Figure 5 a-e). When less stringent agreement thresholds were applied (Figure 5 a and e), the entire Mediterranean rim was identified as refugial, except for the northern and eastern coast of the Adriatic Sea and Gulf of Genoa. With intermediate levels of stringency (Figure 5 b, c, f, g), most refugia were situated in the western Mediterranean, primarily Morocco, Algeria, and parts of the Iberian Peninsula (Cabo de Gata, Cadiz/Algeciras region, Valencia region; sensu Médail & Diadema, 2009). The Balearic Islands, especially Mallorca, were identified as refugial, even under most stringent criteria (Figure 5 d and h); some parts of Sicily were recovered under most scenarios (except the strictest criterion in M1). Unlike the western Mediterranean, few parts of the eastern half of the Mediterranean were identified as refugia. For example, only some areas of the Peloponnese (Greece) were recovered consistently as putative refugia; broad areas of Anatolia and the Levant coast were recovered as refugia only under the least stringent AOGCM agreement levels.

DISCUSSION

Genetic diversity and biogeography

We document high mtDNA genetic diversity in *L. rufescens* across the Mediterranean Basin, as reported previously by Duncan *et al*. (2010), underscoring the importance of broad sampling efforts for accurate representation of diversity patterns. Most of the delimitation methods recover 11 distinct evolutionary lineages (Figure 2), but molecular delimitation analyses, particularly those using only one line of evidence (in this case, mtDNA), can be prone to over-delimitation (Carstens *et al*., 2013). Thus, identified lineages should be taken as a basis for further studies of taxonomic status using integrative approaches based on morphology and variable nuclear markers (Satler *et al*., 2013), and the phylogeographic patterns uncovered herein merit reexamination with additional nuclear

data. Nevertheless, even without further analyses, the existence of such divergent mitochondrial lineages deserves attention, and our main aim was to understand the factors promoting this diversity.

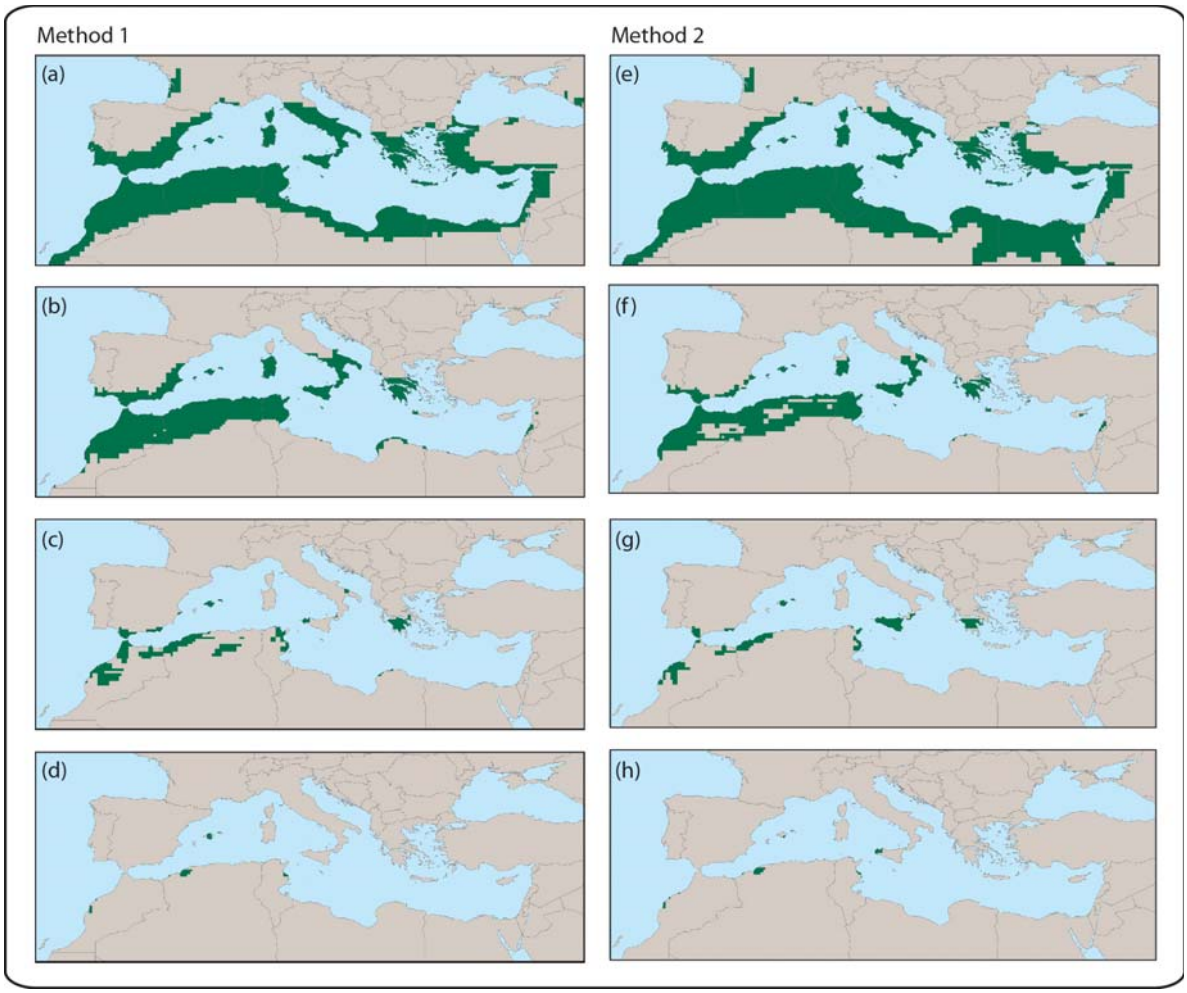


Figure 5 Refugium maps developed via two delimitation methods. Maps a, b, c and d were obtained with Method 1, and maps e, f, g and h were obtained using Method 2, as described in Figure 3.

Genetic diversity is not distributed uniformly among lineages within *L. rufescens*, and is, in fact, highly heterogeneous (Figures 3, 4 and S2). Broadly, we find two contrasting phylogeographic patterns: the mountainous region of Morocco harbors several lineages with well-structured populations, whereas lineages distributed across the broader Mediterranean Basin generally lack geographic structure.

Four lineages (A1-A4) are distributed along the western slopes of the Atlas Mountains (Figure 4), including individuals from A4 referred to as the "Asni clade" by Duncan *et al*.

(2010). Although lineages A1-A4 live in close proximity (within 50 - 200 km), they exhibit striking genetic divergence (>4.7%, Additional file 3). The Atlas Mountain region shows the highest rarity index values in analyses considering all lineages (Additional file 4). This pattern of deep genetic divergence and haplotype differentiation among populations in close proximity may be explained by long-term presence of this species in the region and low dispersal capacity under natural conditions. Together with A5, these lineages are spatially structured in that genetic diversity is positively correlated with distance between localities. Altogether, these patterns are consistent with spider species with similar dispersal capacities (e.g. mygalomorphs, see Satler *et al*., 2013), and with other *Loxoscel*es species, such as those from the Canary Island (Planas & Ribera, 2014), *Loxosceles mrazi*g, and a related group from the Sous Valley (Morocco; Planas unpublished data).

Most individuals, however, belong to lineages widespread across the Mediterranean (e.g. A6 and most B lineages, Figure 4). Haplotype networks for these lineages have star-like shapes, with one common haplotype shared among individuals distributed across the Mediterranean Basin, and fewer, less-common haplotypes, restricted to individuals from one or a few localities. For almost all lineages, no clear correspondence exists between haplotypes and geography, with weak correlations between genetic and geographic distances. For example, the most common haplotype from A6 is found across the entirety of the Mediterranean Basin (Figure 4). This lineage, named the "Iberian clade" by Duncan *et al*. (2010), appears to be the most common in the western Mediterranean, and contains individuals from Sagunt, the type locality of *L. rufescens*.

Biogeographic patterns within clade B are more complex. The exception is lineage B3, where all individuals are found in the Iberian Peninsula. In lineages B2, B4 and B5, individuals with the most common haplotype are widespread across the Mediterranean, as in A6, although no clear pattern emerges that links genetic diversity with geography (Additional file 4 and Table 2). Lineage B1 represents the most extreme example of the complex distributional patterns found within *L. rufescens*. Even given the extensive sampling we conducted, we found individuals of this lineage at only 5 localities, some separated by >4000 km (although more samples from this lineage might produce the typical star-like shape of the B clade lineages).

Discerning between natural and human-mediated dispersals can be difficult in the Mediterranean (Husemann *et al*., 2013, and references therein). Current genetic patterns for

Mediterranean lineages do not coincide with those expected as a result of secondary contact through natural processes. If naturally occurring, contact would be restricted to particular areas and/or occur between or among only a few lineages. Here, multiple lineages are distributed across the Mediterranean, including on islands, a pattern that is difficult to explain by natural processes in organisms with low dispersal abilities. The lack of geographic structure within most of the Mediterranean *Loxosceles* lineages contrasts with the highly structured patterns found for lineages A1-A4, distributed in the mountainous region of Morocco, with the former pattern a likely consequence of human-mediated dispersal. Although *L. rufescens* originated in the Mediterranean Basin (Gertch, 1967; Duncan *et al*., 2010, see below), the species has been introduced to many parts of the world, including Australia, Madagascar and North America (Gertch, 1967; Duncan *et al*., 2010; Green *et al*., 2009; Planas & Ribera, 2014). Human transportation seems a likely mechanism to explain how some haplotypes are distributed across the Mediterranean Basin, including on several islands and on both African and European shores. *Loxosceles rufescens* possesses two life traits that facilitate dispersal with human assistance: high starvation tolerance (Kobelt & Nentwig, 2008) and urban microhabitat preferences. Maritime commerce in this region has been active for >5000 years (Abulafia, 2011), and transportation of cultivated plants (Khadari *et al*., 2005; Delplancke *et al*., 2013), domesticated animals (Zeder *et al*., 2008), and wild animals such as reptiles (Carranza & Arnold, 2006), snails (Guiller & Madec, 2010; Jesse *et al*., 2011), mosquitoes (Porretta *et al*., 2011), and freshwater triclads (Solà *et al*., 2013) has been documented widely throughout the Mediterranean. Thus, the expected "natural" biogeographic patterns have been blurred for this region, and the complex phylogeographic pattern documented here for *L. rufescens* represents a clear example of human influence on species' distributional dynamics.

Refugia and origins of genetic diversity

Although human-mediated transportation of *L. rufescens* likely impacted current distributional patterns for this species within the Mediterranean Basin, the aim of this study was to assess when and how the distinct lineages originated. Combining divergence time and refugial estimates, we marshal two distinct data streams toward answering these questions (Peterson, 2009).

Our dating analyses place diversification events during the Pleistocene glaciations

(Additional file 5). Although we are not able to link diversification events to individual climatic events (i.e. a particular glacial-interglacial cycle) with any confidence, these dates provide a coarse-resolution estimate of diversification timing. The placement of key diversification events during the Pleistocene indicates that processes operating during this period (i.e. glacial/interglacial cycles) likely played an important role in shaping current diversity of the species, as with numerous other species in the Mediterranean Basin (e.g. Rokas *et al*., 2003; Hewitt, 2011; Jesse *et al*., 2011; Kindler *et al*., 2013; Planas *et al*., 2013; Salvi *et al*., 2013). The results from our ENM analyses largely corroborate this scenario of multiple refugia within the Mediterranean for *L. rufescens*, although details depended on the level of stringency applied for agreement among the 8 different AOGCM models (Figure 5). The shape and size of refugia differ markedly depending on the AOGCM used (Additional file 7). In other words, ENM is useful for providing general views of putative refugia, rather than for identifying actual borders. Combining results from different AOGCMs, we obtain a consensus view of general patterns for the latter half of the Pleistocene epoch (Nogués-Bravo, 2009), which, when an intermediate threshold (Figure 5) is considered, agrees with refugia obtained for plants using phylogeographic approaches (Médail & Diadema, 2009).

In both methods (Figure 5), major refugia are concentrated in the western Maghreb. Indeed, in phylogenetic terms, four evolutionary lineages (A1-A4) are found in this area, signifying a hot spot of lineage richness. This richness supports the hypothesis that northern Africa is the center of origin for *L. rufescens*, as previously hypothesized by Gertch (1967) and Duncan *et al*. (2010). Additionally, the sister group to *L. rufescens* is found south of this area, in the High and Anti-Atlas Mountains and the Sous Valley (Morocco; Planas unpublished data), which lends further support to a northern African origin for these lineages and for *L. rufescens* as a whole. This region has been postulated as a climatic refugium for various animals and plants in light of its complex orography (e.g. Anti-Atlas, High Atlas) and climatic stability (Husemann *et al*., 2013 and references therein).

More challenging is linking putative refugia to the origins of the Mediterranean linages (A5, A6, B), with current distributional and genetic patterns most likely the result of population mixing through human-mediated transportation. Although some of these divergent lineages now occur in sympatry, they likely originated in allopatry, given the dominance of this speciation mechanism for the genus (Binford *et al*., 2008; Planas &

Ribera, 2014) and the geographic results summarized above (Figure 5). Lineages A5 and B3 have small distributions and are endemic to the Iberian Peninsula, which may reflect refugial areas, especially along the southern and eastern Iberian Mediterranean coast. However, 5 other lineages are widely distributed across the Mediterranean.

Genetic diversity patterns should help in elucidating the origins of these lineages, assuming that refugial areas should harbor higher genetic diversity (but see Widmer & Lexer, 2001). Such is the case for lineage B4 (Additional file 4), where highest genetic diversity is found in the Balearic Islands, an area identified consistently as a refugium (Figures 4 and S3). However, lineages A6, B1, B2, and B5 are widespread across the Mediterranean and do not show any correspondence between genetic diversity and a single putative refugial area; these lineages may have originated in one of the remaining predicted refugia (e.g. Sicily, southern Italian Peninsula, the Peloponnese), but subsequent processes (e.g. human-mediated transportation) appear to have erased ancient biogeographic signals. More extensive sampling in the central and eastern Mediterranean may help to resolve this question.

CONCLUSIONS

In this study we delimited 11 evolutionary lineages within *Loxosceles rufescens* in the Mediterranean Basin based on mtDNA data. Genetic diversity was not distributed uniformly, and we found two contrasting phylogeographic patterns: (1) the southern region of Morocco holds several lineages with well-structured populations, (2) whereas lineages distributed across the broader Mediterranean Basin generally lack geographic structure. By combining results from 8 AOGCMs with two different refugium-delimitation methodologies, we corroborated the scenario of multiple refugia within the Mediterranean, principally in northern Africa. ENMs were useful for providing general views of putative refugia, with fine-scale details depending on the level of stringency applied for agreement among models. Although refugial delimitation remains challenging, by combining ENM with phylogeographic approaches, we found support for the hypothesis that northern Africa was the center of origin for *L. rufescens*, that current genetic diversity probably originated in allopatry and was promoted by successive glaciations during the Pleistocene, and that protracted human activities impacted the current distributional patterns of *L. rufescens* within the Mediterranean Basin.

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ADDITIONAL MATERIAL

Additional file 1 Information on geographic location, inclusion in ENM analysis (Y – yes; N – no), mtDNA lineage and Genbank accession number for the 310 individuals included in this study. In bold, individuals included in the phylogenetic analyses. Abbreviations: CS – Corsica; CR – Crete; GR - Greece, IB - Balearic Islands; IT – Italy; LE – Levant; MA - Morocco; PI – Iberian Peninsula; SA – Sardinia; SC – Sicily; TN – Tunisia; TR – Turkey.

ID	LONGITUDE	LATITUDE	ENM ANALYSIS	REGION	LINEAGE	*cox1* ACCESSION NUMBER
LX1485_MA	31.15739	-9.69767	Y	MA	A1	KJ560651
LX1486_MA	31.15739	-9.69767	Y	MA	A1	KJ560652
LX1481_MA	31.50096	-9.6158	Y	MA	A2	KJ560650
LX1496_MA	31.18955	-8.05766	Y	MA	A3	KJ560653
GQ279225	31.250088	-7.983352	Y	MA	A4	GQ279225
GQ279226	31.250088	-7.983352	N	MA	A4	GQ279226
LX1498_MA	31.95973	-6.76811	Y	MA	A4	KJ560654
LX1499_MA	31.95973	-6.76811	Y	MA	A4	KJ560655
LX1507_MA	32.08467	-6.33358	Y	MA	A4	KJ560656
LX1510_MA	32.08467	-6.33358	Y	MA	A4	KJ560657
LX1348_PI	38.86894	-0.7589	Y	PI	A5	KJ560634
LX1140_PI	39.549464	-0.41551	N	PI	A5	KJ560569
LX1762_CR	35.00651	24.94693	Y	CR	A6	KJ560747
LX1763_CR	35.00651	24.94693	Y	CR	A6	KJ560748
LX1764_CR	35.00651	24.94693	Y	CR	A6	KJ560749
LX1735_CR	35.1901167	24.44925	Y	CR	A6	KJ560733
LX1746_CR	35.2018667	24.16235	Y	CR	A6	KJ560739
LX1741_CR	35.32475	24.2812833	Y	CR	A6	KJ560736
LX1742_CR	35.32475	24.2812833	Y	CR	A6	KJ560737
LX2225_GR	35.881197	23.305596	Y	GR	A6	KJ560801
LX2226_GR	35.881197	23.305596	Y	GR	A6	KJ560802
LX2227_GR	35.88179	23.289468	Y	GR	A6	KJ560803
LX2221_GR	35.886535	23.295876	Y	GR	A6	KJ560799
LX2222_GR	35.886535	23.295876	Y	GR	A6	KJ560800
LX2236_GR	36.14744	23.018313	Y	GR	A6	KJ560807
LX2237_GR	36.14744	23.018313	Y	GR	A6	KJ560808
LX2238_GR	36.14744	23.018313	Y	GR	A6	KJ560809
LX2239_GR	36.14744	23.018313	Y	GR	A6	KJ560810
LX2241_GR	36.14744	23.018313	Y	GR	A6	KJ560812
LX2242_GR	36.14744	23.018313	Y	GR	A6	KJ560813
LX2211_GR	36.965	21.662	Y	GR	A6	KJ560796
LX2212_GR	36.965	21.662	Y	GR	A6	KJ560797
LX1603_GR	38.4523167	22.4213833	Y	GR	A6	KJ560686
LX1591_IB	38.94437	1.47775	Y	IB	A6	KJ560678

ID	LONGITUDE	LATITUDE	ENM ANALYSIS	REGION	LINEAGE	*cox1* ACCESSION NUMBER
LX1644_IB	39.3943833	2.84726667	Y	IB	A6	KJ560708
LX1633_IB	39.4582333	2.76845	Y	IB	A6	KJ560698
LX1638_IB	39.4582333	2.76845	Y	IB	A6	KJ560702
LX1640_IB	39.4582333	2.76845	Y	IB	A6	KJ560704
LX1650_IB	39.4709667	3.0209	Y	IB	A6	KJ560709
LX1673_IB	39.5120333	2.92628333	Y	IB	A6	KJ560715
LX1227_IB	39.5979639	2.64130278	Y	IB	A6	KJ560598
LX1390_IB	39.8860972	3.06191944	Y	IB	A6	KJ560646
LX2391_IT	39.01587	17.16621	Y	IT	A6	KJ560844
LX2383_IT	40.46077	16.72798	Y	IT	A6	KJ560840
LX2384_IT	40.46077	16.72798	Y	IT	A6	KJ560841
LX2385_IT	40.46077	16.72798	Y	IT	A6	KJ560842
LX2354_IT	41.937	15.98725	Y	IT	A6	KJ560834
LX2355_IT	41.937	15.98725	Y	IT	A6	KJ560835
LX2356_IT	41.937	15.98725	Y	IT	A6	KJ560836
LX1834_LE	32.535773	34.949127	N	LE	A6	KJ560767
LX1477_MA	31.60901	-9.65915	Y	MA	A6	KJ560649
LX1532_MA	34.0573	-4.23691	Y	MA	A6	KJ560661
LX1538_MA	34.0573	-4.23691	Y	MA	A6	KJ560662
LX1520_MA	34.05933	-5.30338	Y	MA	A6	KJ560658
LX1523_MA	34.10654	-4.96948	Y	MA	A6	KJ560659
LX1530_MA	34.12708	-4.30482	Y	MA	A6	KJ560660
LX1577_MA	34.5612	-4.49946	Y	MA	A6	KJ560669
LX1550_MA	34.80333	-2.39686	Y	MA	A6	KJ560664
LX1561_MA	34.80333	-2.39686	Y	MA	A6	KJ560665
LX1542_MA	34.82454	-2.08165	Y	MA	A6	KJ560663
LX1564_MA	34.83786	-2.35774	Y	MA	A6	KJ560666
LX1567_MA	34.83786	-2.35774	Y	MA	A6	KJ560667
LX1571_MA	35.00546	-4.17899	Y	MA	A6	KJ560668
LX1578_MA	35.00546	-4.17899	Y	MA	A6	KJ560670
LX1136_MA	35.0868738	-5.7641125	Y	MA	A6	KJ560568
LX1134_MA	35.2380333	-5.1740167	Y	MA	A6	KJ560566
LX1135_MA	35.2380333	-5.1740167	Y	MA	A6	KJ560567
LX1377_MA	35.3639073	-5.3715844	Y	MA	A6	KJ560643
LX1944_PI	36.51784	-5.65624	Y	PI	A6	KJ560782
LX1334_PI	36.80214	-2.14298	Y	PI	A6	KJ560630
LX1335_PI	36.80214	-2.14298	Y	PI	A6	KJ560631
LX1336_PI	36.80214	-2.14298	Y	PI	A6	KJ560632
LX1326_PI	36.84535	-2.01746	Y	PI	A6	KJ560627
LX1327_PI	36.84535	-2.01746	Y	PI	A6	KJ560628
LX1317_PI	36.84788	-2.0249	Y	PI	A6	KJ560623

ID	LONGITUDE	LATITUDE	ENM ANALYSIS	REGION	LINEAGE	*cox1* ACCESSION NUMBER
LX1319_PI	36.84788	-2.0249	Y	PI	A6	KJ560624
LX1323_PI	36.84788	-2.0249	Y	PI	A6	KJ560625
LX1325_PI	36.84788	-2.0249	Y	PI	A6	KJ560626
LX1315_PI	36.94333	-1.90926	Y	PI	A6	KJ560622
LX2279_PI	36.9617	-3.8662	Y	PI	A6	KJ560825
LX2456_PI	36.970732	-4.832509	Y	PI	A6	KJ560860
LX1305_PI	36.98014	-2.17114	Y	PI	A6	KJ560619
LX1306_PI	36.98014	-2.17114	Y	PI	A6	KJ560620
LX1313_PI	36.98014	-2.17114	Y	PI	A6	KJ560621
LX1014_PI	36.99055	-2.399166	Y	PI	A6	KJ560560
LX2283_PI	37.0361522	-4.4987515	N	PI	A6	KJ560828
LX2287_PI	37.0361522	-4.4987515	N	PI	A6	KJ560829
LX1299_PI	37.04982	-2.20497	Y	PI	A6	KJ560617
LX1300_PI	37.04982	-2.20497	Y	PI	A6	KJ560618
LX2277_PI	37.17809	-3.05636	Y	PI	A6	KJ560823
LX2278_PI	37.17809	-3.05636	Y	PI	A6	KJ560824
LX1286_PI	37.21327	-1.82724	Y	PI	A6	KJ560613
LX1288_PI	37.21327	-1.82724	Y	PI	A6	KJ560614
LX1940_PI	37.25949	-7.2	Y	PI	A6	KJ560780
LX1290_PI	37.29408	-2.22548	Y	PI	A6	KJ560615
LX1291_PI	37.29408	-2.22548	Y	PI	A6	KJ560616
LX1279_PI	37.32173	-1.70208	Y	PI	A6	KJ560612
LX1606_PI	37.36468	-3.47183	Y	PI	A6	KJ560689
LX1196_PI	37.3658056	-3.46925	Y	PI	A6	KJ560586
LX2263_PI	37.385707	-5.981608	N	PI	A6	KJ560818
LX1594_PI	37.4763427	-4.2792721	Y	PI	A6	KJ560680
LX1605_PI	37.49108	-3.82442	Y	PI	A6	KJ560688
LX1276_PI	37.6079	-0.75988	Y	PI	A6	KJ560610
LX1277_PI	37.60821	-0.75619	Y	PI	A6	KJ560611
LX2282_PI	37.85253	-1.47114	Y	PI	A6	KJ560827
LX1267_PI	37.92965	-1.13036	Y	PI	A6	KJ560604
LX1268_PI	37.92965	-1.13036	Y	PI	A6	KJ560605
LX1270_PI	37.92965	-1.13036	Y	PI	A6	KJ560606
LX1272_PI	37.92965	-1.13036	Y	PI	A6	KJ560607
LX1275_PI	37.92965	-1.13036	Y	PI	A6	KJ560609
LX1190_PI	37.9984214	-2.9874861	N	PI	A6	KJ560584
LX2269_PI	38.36102	-3.75513	Y	PI	A6	KJ560821
LX1356_PI	39.54699	-0.5102	Y	PI	A6	KJ560637
LX1359_PI	39.54699	-0.5102	Y	PI	A6	KJ560638
LX1141_PI	39.549464	-0.41551	N	PI	A6	KJ560570
LX1142_PI	39.549464	-0.41551	N	PI	A6	KJ560571

ID	LONGITUDE	LATITUDE	ENM ANALYSIS	REGION	LINEAGE	*cox1* ACCESSION NUMBER
LX1143_PI	39.549464	-0.41551	N	PI	A6	KJ560572
LX1144_PI	39.549464	-0.41551	N	PI	A6	KJ560573
LX1145_PI	39.549464	-0.41551	N	PI	A6	KJ560574
LX1874_PI	39.6678667	-0.2886833	Y	PI	A6	KJ560774
LX1351_PI	39.68481	-0.30005	Y	PI	A6	KF717003
LX1352_PI	39.68481	-0.30005	Y	PI	A6	KJ560635
LX1354_PI	39.68481	-0.30005	Y	PI	A6	KJ560636
LX2454_PI	40.30466	-4.61335	Y	PI	A6	KJ560858
LX2455_PI	40.30466	-4.61335	Y	PI	A6	KJ560859
LX1822_PI	40.86461	0.501121	Y	PI	A6	KJ560760
LX2342_PI	40.86461	0.501121	Y	PI	A6	KJ560830
LX2347_PI	40.86461	0.501121	Y	PI	A6	KJ560831
LX2348_PI	40.86461	0.501121	Y	PI	A6	KJ560832
LX2349_PI	40.86461	0.501121	Y	PI	A6	KJ560833
LX2280_PI	41.282	1.835	Y	PI	A6	KJ560826
LX2151_PI	41.38605	2.1639	N	PI	A6	KJ560793
LX2152_PI	41.38605	2.1639	N	PI	A6	KJ560794
LX2264_PI	41.38605	2.1639	N	PI	A6	KJ560819
LX2458_PI	41.640269	2.403385	Y	PI	A6	KJ560862
LX2459_PI	41.640269	2.403385	Y	PI	A6	KJ560863
LX1191_PI	41.9181333	3.1639	Y	PI	A6	KJ560585
LX1823_PI	41.9181333	3.1639	Y	PI	A6	KJ560761
LX1590_PI	42.2439458	2.694298	Y	PI	A6	KJ560677
LX1819_SC	36.72157	15.11847	Y	SC	A6	KJ560757
LX1820_SC	36.72157	15.11847	Y	SC	A6	KJ560758
LX1821_SC	36.72157	15.11847	Y	SC	A6	KJ560759
LX2411_SC	36.72166	15.11794	Y	SC	A6	KJ560847
LX2412_SC	36.72166	15.11794	Y	SC	A6	KJ560848
LX2413_SC	36.72166	15.11794	Y	SC	A6	KJ560849
LX2420_SC	37.13928	15.03244	Y	SC	A6	KJ560850
LX2423_SC	37.13928	15.03244	Y	SC	A6	KJ560851
LX2424_SC	37.13928	15.03244	Y	SC	A6	KJ560852
LX2434_SC	37.39064	13.29294	Y	SC	A6	KJ560853
LX2436_SC	37.39064	13.29294	Y	SC	A6	KJ560854
LX2448_SC	38.08555	12.67299	Y	SC	A6	KJ560856
LX1046_TN	36.5300442	9.36006146	Y	TN	A6	FJ986186
LX2084_TN	37.329	9.84616	Y	TN	A6	KJ560788
LX1178_TR	36.1113889	35.9458333	Y	TR	A6	KJ560583
LX1714_GR	37.7224667	22.7517	Y	GR	B1	KJ560726
LX1601_GR	39.1923333	23.9233333	Y	GR	B1	KJ560684
LX2369_IT	39.86921	18.24284	Y	IT	B1	KJ560837

ID	LONGITUDE	LATITUDE	ENM ANALYSIS	REGION	LINEAGE	*cox1* ACCESSION NUMBER
LX2370_IT	39.86921	18.24284	Y	IT	B1	KJ560838
LX2371_IT	39.86921	18.24284	Y	IT	B1	KJ560839
LX1609_LE	30.897868	34.894342	Y	LE	B1	KJ560692
LX1472_MA	32.50405	-9.25307	Y	MA	B1	KJ560647
LX1584_CR	35.215925	26.0701278	Y	CR	B2	KJ560675
LX1582_CR	35.2188889	26.0690722	Y	CR	B2	KJ560673
LX1583_CR	35.2188889	26.0690722	Y	CR	B2	KJ560674
LX1770_CR	35.25165	26.25206	Y	CR	B2	KJ560754
LX1771_CR	35.25165	26.25206	Y	CR	B2	KJ560755
LX1737_CR	35.3508167	24.35395	Y	CR	B2	KJ560734
LX1712_GR	37.7224667	22.7517	Y	GR	B2	KJ560724
LX1713_GR	37.7224667	22.7517	Y	GR	B2	KJ560725
LX1715_GR	37.7224667	22.7517	Y	GR	B2	KJ560727
LX1716_GR	37.7224667	22.7517	Y	GR	B2	KJ560728
LX1829_LE	32.116533	34.799977	N	LE	B2	KJ560762
LX1830_LE	32.116533	34.799977	N	LE	B2	KJ560763
LX1831_LE	32.116533	34.799977	N	LE	B2	KJ560764
LX1832_LE	32.116533	34.799977	N	LE	B2	KJ560765
LX1835_LE	32.116533	34.799977	N	LE	B2	KJ560768
LX1837_LE	32.116533	34.799977	N	LE	B2	KJ560770
LX1838_LE	32.116533	34.799977	N	LE	B2	KJ560771
LX1941_PI	36.44859	-5.88949	Y	PI	B2	KJ560781
LX1330_PI	36.7285	-2.19096	Y	PI	B2	KF717002
LX1331_PI	36.7285	-2.19096	Y	PI	B2	KJ560629
LX1342_PI	36.80214	-2.14298	Y	PI	B2	KJ560633
LX1242_PI	38.0362	-1.09445	Y	PI	B2	KJ560600
LX1244_PI	38.0362	-1.09445	Y	PI	B2	KJ560601
LX1264_PI	38.0362	-1.09445	Y	PI	B2	KJ560602
LX1618_PI	38.0362	-1.09445	Y	PI	B2	KJ560695
LX1620_PI	38.0362	-1.09445	Y	PI	B2	KJ560696
LX1630_PI	38.0362	-1.09445	Y	PI	B2	KJ560697
LX1680_PI	38.0362	-1.09445	Y	PI	B2	KJ560717
LX1681_PI	38.0362	-1.09445	Y	PI	B2	KJ560718
LX1682_PI	38.0362	-1.09445	Y	PI	B2	KJ560719
LX1683_PI	38.0362	-1.09445	Y	PI	B2	KJ560720
LX1684_PI	38.0362	-1.09445	Y	PI	B2	KJ560721
LX1685_PI	38.0362	-1.09445	Y	PI	B2	KJ560722
LX1686_PI	38.0362	-1.09445	Y	PI	B2	KJ560723
LX1607_PI	38.08524	-1.36514	Y	PI	B2	KJ560690
LX2457_PI	41.640269	2.403385	Y	PI	B2	KJ560861
LX1146_PI	37.383717	-4.786571	N	PI	B3	KJ560575

ID	LONGITUDE	LATITUDE	ENM ANALYSIS	REGION	LINEAGE	*cox1* ACCESSION NUMBER
LX1596_PI	37.4958902	-4.2559177	Y	PI	B3	KJ560681
LX2271_PI	37.79839	-3.08411	Y	PI	B3	KJ560822
LX1361_PI	39.53622	-0.62619	Y	PI	B3	KJ560639
LX1365_PI	39.53622	-0.62619	Y	PI	B3	KJ560640
LX1369_PI	39.53622	-0.62619	Y	PI	B3	KJ560641
LX1375_PI	39.53622	-0.62619	Y	PI	B3	KJ560642
LX1376_PI	39.53622	-0.62619	Y	PI	B3	KF717004
LX1860_PI	39.53622	-0.62619	Y	PI	B3	KJ560772
LX1148_PI	39.536261	-0.6263104	Y	PI	B3	KJ560576
LX1767_CR	35.04143	26.19772	Y	CR	B4	KJ560751
LX1768_CR	35.04143	26.19772	Y	CR	B4	KJ560752
LX1747_CR	35.2018667	24.16235	Y	CR	B4	KJ560740
LX1769_CR	35.25165	26.25206	Y	CR	B4	KJ560753
LX1766_CR	35.26239	25.38262	Y	CR	B4	KJ560750
LX1728_CR	35.3917333	25.0268833	Y	CR	B4	KJ560729
LX1729_CR	35.3917333	25.0268833	Y	CR	B4	KJ560730
LX1730_CR	35.3917333	25.0268833	Y	CR	B4	KJ560731
LX1585_GR	37.9254833	23.7584667	Y	GR	B4	KJ560676
LX1604_GR	37.9254833	23.7584667	Y	GR	B4	KJ560687
LX1210_IB	39.3468472	3.18578889	Y	IB	B4	KJ560592
LX1635_IB	39.4582333	2.76845	Y	IB	B4	KJ560699
LX1636_IB	39.4582333	2.76845	Y	IB	B4	KJ560700
LX1637_IB	39.4582333	2.76845	Y	IB	B4	KJ560701
LX1639_IB	39.4582333	2.76845	Y	IB	B4	KJ560703
LX1641_IB	39.4582333	2.76845	Y	IB	B4	KJ560705
LX1642_IB	39.4582333	2.76845	Y	IB	B4	KJ560706
LX1643_IB	39.4582333	2.76845	Y	IB	B4	KJ560707
LX1659_IB	39.5333	2.53353333	Y	IB	B4	KJ560711
LX1612_IB	39.5642139	2.54599444	Y	IB	B4	KJ560693
LX1229_IB	39.5643778	2.54600556	Y	IB	B4	KJ560599
LX1212_IB	39.6059808	2.59872944	Y	IB	B4	KJ560594
LX1213_IB	39.6059808	2.59872944	Y	IB	B4	KJ560595
LX1669_IB	39.68175	2.55013333	Y	IB	B4	KJ560713
LX1655_IB	39.7392667	3.42635	Y	IB	B4	KJ560710
LX1671_IB	39.7480833	3.07003333	Y	IB	B4	KJ560714
LX1388_IB	39.9156694	3.07403333	Y	IB	B4	KJ560645
LX2390_IT	39.01587	17.16621	Y	IT	B4	KJ560843
LX2395_IT	39.01587	17.16621	Y	IT	B4	KJ560845
LX1131_IT	45.054061	7.702051	N	IT	B4	KJ560565
LX1473_MA	31.9564	-9.31604	Y	MA	B4	KJ560648
LX2267_PI	36.38845	-5.65146	Y	PI	B4	KJ560820

ID	LONGITUDE	LATITUDE	ENM ANALYSIS	REGION	LINEAGE	*cox1* ACCESSION NUMBER
LX1597_PI	37.476554	-4.1728106	Y	PI	B4	KJ560682
LX1200_SA	40.2886111	9.50725	Y	SA	B4	KJ560588
LX1202_SA	40.2886111	9.50725	Y	SA	B4	KJ560589
LX1207_SA	40.2886111	9.50725	Y	SA	B4	KJ560590
LX2095_TN	35.47567	9.34092	Y	TN	B4	KJ560789
LX2097_TN	35.47567	9.34092	Y	TN	B4	KJ560790
LX1048_TN	35.9165506	9.55883798	Y	TN	B4	KJ560562
LX1175_TR	36.938501	31.169117	N	TR	B4	KJ560580
LX1174_TR	36.973232	28.916784	N	TR	B4	KJ560579
LX1173_TR	40.265756	29.121893	N	TR	B4	KJ560578
LX1580_CR	35.0381306	25.4583583	Y	CR	B5	KJ560671
LX1581_CR	35.0479444	26.0101694	Y	CR	B5	KJ560672
LX1734_CR	35.1901167	24.44925	Y	CR	B5	KJ560732
LX1745_CR	35.2018667	24.16235	Y	CR	B5	KJ560738
LX1772_CR	35.25165	26.25206	Y	CR	B5	KJ560756
LX1759_CR	35.3273167	23.5537833	Y	CR	B5	KJ560745
LX1760_CR	35.3273167	23.5537833	Y	CR	B5	KJ560746
LX1738_CR	35.3508167	24.35395	Y	CR	B5	KJ560735
LX1749_CR	35.43195	23.94625	Y	CR	B5	KJ560741
LX1750_CR	35.43195	23.94625	Y	CR	B5	KJ560742
LX1752_CR	35.51075	24.06835	Y	CR	B5	KJ560743
LX1753_CR	35.51075	24.06835	Y	CR	B5	KJ560744
LX1920_CS	42.01999	8.72525	Y	CS	B5	KJ560775
LX2232_GR	36.14744	23.018313	Y	GR	B5	KJ560804
LX2233_GR	36.14744	23.018313	Y	GR	B5	KJ560805
LX2235_GR	36.14744	23.018313	Y	GR	B5	KJ560806
LX2240_GR	36.14744	23.018313	Y	GR	B5	KJ560811
LX2246_GR	36.14744	23.018313	Y	GR	B5	KJ560814
LX2247_GR	36.14744	23.018313	Y	GR	B5	KJ560815
LX2260_GR	36.480069	22.967564	Y	GR	B5	KJ560816
LX2261_GR	36.480069	22.967564	Y	GR	B5	KJ560817
LX2200_GR	36.82893	21.87662	Y	GR	B5	KJ560795
LX2216_GR	36.965	21.662	Y	GR	B5	KJ560798
LX1602_GR	39.0907861	23.6605722	Y	GR	B5	KJ560685
LX1600_GR	39.85435	22.5398333	Y	GR	B5	KJ560683
LX1209_IB	39.3468472	3.18578889	Y	IB	B5	KJ560591
LX1211_IB	39.3468472	3.18578889	Y	IB	B5	KJ560593
LX1660_IB	39.5333	2.53353333	Y	IB	B5	KJ560712
LX1676_IB	39.5826167	3.01868333	Y	IB	B5	KJ560716
LX1613_IB	39.597975	2.64130278	Y	IB	B5	KJ560694
LX1219_IB	39.609295	2.5991	Y	IB	B5	KJ560596

ID	LONGITUDE	LATITUDE	ENM ANALYSIS	REGION	LINEAGE	*cox1* ACCESSION NUMBER
LX1220_IB	39.609295	2.5991	Y	IB	B5	KJ560597
LX2404_IT	38.58894	15.89409	Y	IT	B5	KJ560846
LX1129_IT	45.054061	7.702051	N	IT	B5	KJ560564
LX1608_LE	30.897868	34.894342	Y	LE	B5	KJ560691
LX1836_LE	32.116533	34.799977	N	LE	B5	KJ560769
LX1833_LE	32.535773	34.949127	N	LE	B5	KJ560766
LX1938_PI	37.61826	-7.2	Y	PI	B5	KJ560779
LX1266_PI	37.92965	-1.13036	Y	PI	B5	KJ560603
LX1273_PI	37.92965	-1.13036	Y	PI	B5	KJ560608
LX1592_PI	37.99675	-6.3422	Y	PI	B5	KJ560679
LX1862_PI	39.5660667	-0.4582333	Y	PI	B5	KJ560773
LX2453_PI	39.75239	-4.21469	Y	PI	B5	KJ560857
LX1058_PI	40.318501	-3.880352	N	PI	B5	KJ560563
LX1385_PI	41.3922222	1.80777778	Y	PI	B5	KJ560644
LX1199_SA	39.5305	9.59586111	Y	SA	B5	KJ560587
LX2447_SC	38.08555	12.67299	Y	SC	B5	KJ560855
LX1151_TN	35.6201759	10.6005232	Y	TN	B5	KJ560577
LX2142_TN	36.46527	10.27901	Y	TN	B5	KJ560791
LX2143_TN	36.46527	10.27901	Y	TN	B5	KJ560792
LX1029_TN	36.5101533	9.13655918	Y	TN	B5	KJ560561
LX2058_TN	36.77661	10.58439	Y	TN	B5	KJ560783
LX2059_TN	36.77661	10.58439	Y	TN	B5	KJ560784
LX2067_TN	36.77661	10.58439	Y	TN	B5	KJ560785
LX2073_TN	37.329	9.84616	Y	TN	B5	KJ560786
LX2081_TN	37.329	9.84616	Y	TN	B5	KJ560787
LX1176_TR	36.508913	36.186102	N	TR	B5	KJ560581
LX1935_TR	36.726286	27.689188	N	TR	B5	KJ560778
LX1933_TR	36.777248	31.475971	N	TR	B5	KJ560776
LX1177_TR	36.9716667	31.5333333	Y	TR	B5	KJ560582
LX1934_TR	36.974331	30.573129	N	TR	B5	KJ560777

Additional file 2 Details on climatic models (AOGCMs) used for Ecological Niche Modeling.

Model ID	Modeling Center	Resolution*	Source	Year
CCSM4	National Center for Atmospheric Research, USA	0.9° × 1.25°	CMIP5/PMIP3	2012
CNRM-CM5	Centre National de Recherches Meteorologiques / Centre Europeen de Recherche et Formation Avancees en Calcul Scientifique, France	1.4° x 1.4°	CMIP5/PMIP3	2012
COSMOS-ASO (FUB)	Freie Universität Berlin, Germany	3.75 x 3.7	PMIP3	2012
GISS-E2-R	NASA Goddard Institute for Space Studies, USA	2.5° x 2.0°	CMIP5/PMIP3	2012
MIROC-ESM	Atmosphere and Ocean Research Institute (University of Tokyo), National Institute for Environmental Studies, and Japan Agency for Marine-Earth Science and Technology, Japan	2.8° × 2.8°	CMIP5/PMIP3	2012
IPSL-CM5A-LR	Institut Pierre Simon Laplace, France	3.75 x 1.9	CMIP5/PMIP3	2012
MPI-ESM-P	Max Planck Institute for Meteorology, Germany	1.9 x 1.9	CMIP5/PMIP3	2011
MRI-CGCM3	Meteorological Research Institute, Japan	1.1° x 1.1°	CMIP5/PMIP3	2012

* Longitude × latitude

CMIP5 – Coupled Model Intercomparison Project, Phase 5 (http://cmip-pcmdi.llnl.gov/)

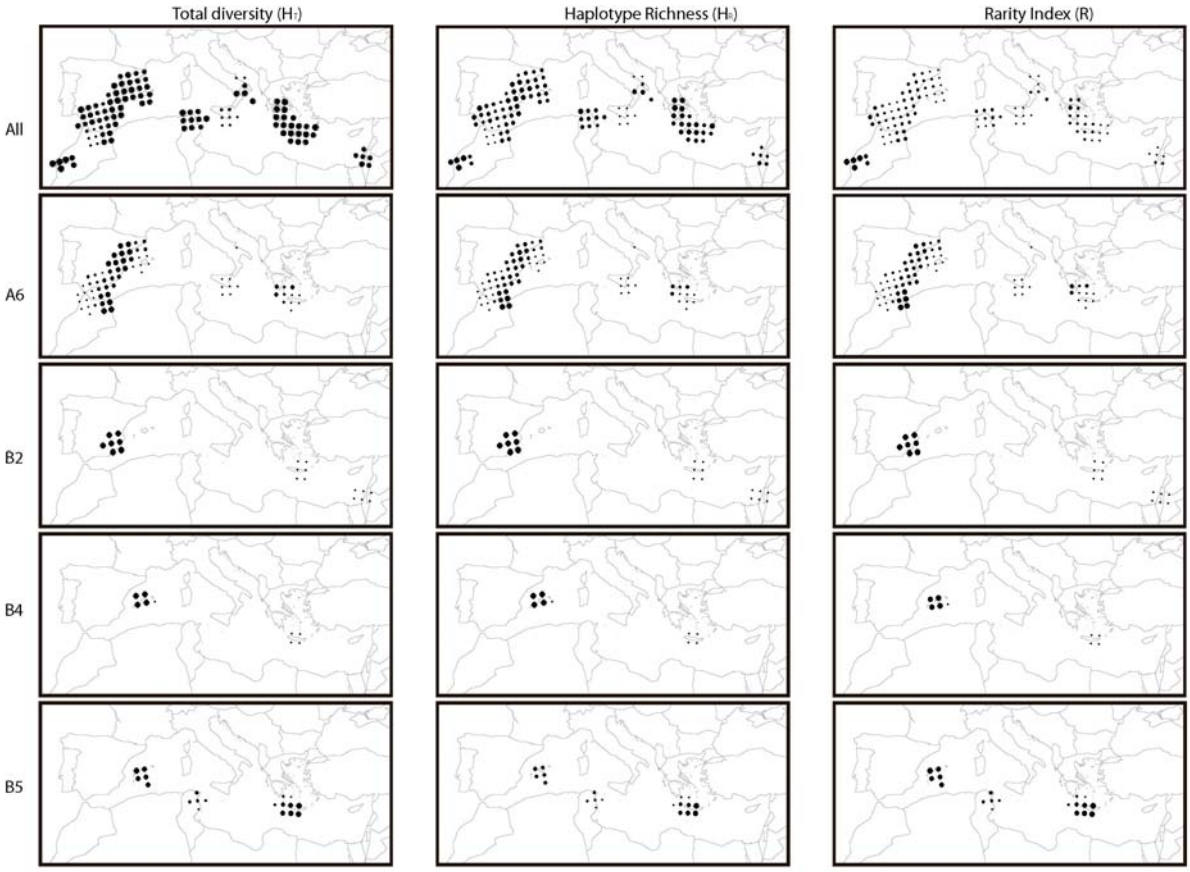
PMIP3 – Paleoclimate Modelling Intercomparison Project, Phase 3 (http://pmip3.lsce.ipsl.fr/

Additional file 3 Estimates of genetic p-distance between (above the diagonal) and within (in bold) lineages. In the inset genetic p-distance between the two clades.

	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5
A1	**0.000**										
A2	0.047	-									
A3	0.055	0.026	-								
A4	0.051	0.024	0.025	**0.004**							
A5	0.047	0.041	0.047	0.042	**0.000**						
A6	0.048	0.041	0.048	0.042	0.015	**0.000**					
B1	0.059	0.071	0.078	0.069	0.075	0.076	**0.001**				
B2	0.056	0.062	0.065	0.064	0.072	0.073	0.045	**0.001**			
B3	0.049	0.060	0.063	0.062	0.067	0.068	0.046	0.018	**0.000**		
B4	0.054	0.061	0.064	0.060	0.070	0.071	0.044	0.022	0.015	**0.000**	
B5	0.060	0.066	0.069	0.068	0.071	0.069	0.052	0.043	0.043	0.042	**0.002**

Clade	B
A	0.07

Additional file 4 Genetic diversity of *Loxosceles rufescens* in the Mediterranean. Total diversity (H_T), haplotype richness (H_R) and rarity index (R) are represented for *L. rufescens* (all) and separately for lineages A6, B2, B4 and B5. Diversity statistics are computed by considering samples located within a perimeter around a grid point. We set grid points every 100 km in latitude and longitude, and computations were conducted across random sets of 5 individuals, bootstrapping 1000 times.

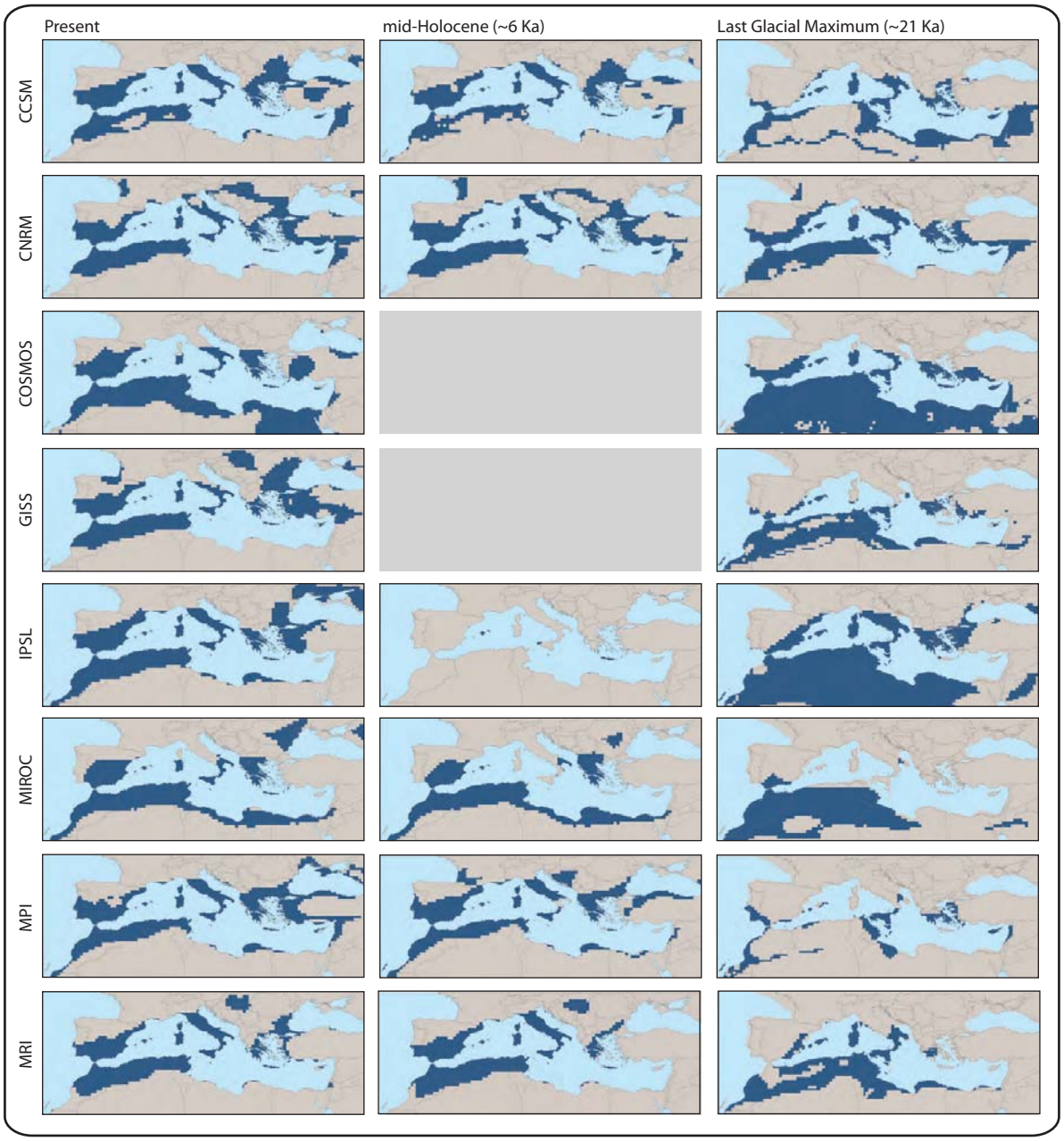


Additional file 5 Dating analysis results in Mya obtained from two different *cox1* especific rates; (1) using *Parachtes* rate and (2) using mean rate obtained for *Loxosceles*.

mrca	1	2
A-B	1.968 (1.322-2.698)	0.356 (0.243-0.487)
A	1.606 (1.021-2.248)	0.291 (0.192-0.413)
A6-A5-A4-A3-A2	0.936 (0.588-1.334)	0.169 (0.107-0.238)
A6-A5-A4-A3	0.857 (0.547-1.218)	0.155 (0.099-0.216)
A6-A5-A4	0.75 (0.47-1.074)	0.136 (0.859-0.191)
A6-A5	0.25 (0.099-0.423)	0.046 (0.184-0.078)
B	1.2631 (0.76-1.885)	0.228 (0.136-0.334)
B4-B3-B2-B1	1.096 (0.613-1.618)	0.197 (0.113-0.29)
B4-B3-B2	0.499 (0.235-0.798)	0.09 (0.045-0.145)
B4-B3	0.313 (0.125-0.537)	0.057 (0.021-0.096)

Additional file 6 Partial ROC analyses. Partial ROC tests ($P < 0.05$) using two data subsets with 1000 replicates.

AOGCM	Data subset	0.99
CCSM	Set 1	0.0047
	Set 2	0.0002
CNRM	Set 1	0.0023
	Set 2	0.0002
COSMOS	Set 1	0.0001
	Set 2	0.0080
GISS	Set 1	0.0048
	Set 2	0.0032
IPSL	Set 1	0.0000
	Set 2	0.0001
MIROC	Set 1	0.0000
	Set 2	0.0001
MPI	Set 1	0.1526
	Set 2	0.0211
MRI	Set 1	0.0029
	Set 2	0.0073



Additional file 7 Ecological niche modelling results for each general circulation model. Ecological niche modelling results for each general circulation model in each time slice (Last Glacial Maximum, mid-Holocene, and present).

CAPÍTOL 2

Diversitat del gènere *Loxosceles* a les Illes Canàries

Article 2 Planas E, Ribera C. 2014. Uncovering overlooked island diversity: colonization and diversification of the medically important spider genus *Loxosceles* (Arachnida: Sicariidae) on the Canary Islands.

Article 3 Planas E, Ribera C. Description of six new species of *Loxosceles* (Araneae: Sicariidae) endemic to the Canary Islands, and the utility of DNA barcoding for their fast and accurate identification.

Article 4 Planas E, Bernaus L, Ribera C. Development of novel microsatellite markers for the spider genus *Loxosceles* (Sicariidae) using next-generation sequencing.

Article 5 Planas E, Bernaus L, Sánchez-Gracia A, Ribera C. Genetic diversity is affected by Pleistocenic sea-level changes and volcanic activity in a spider of the genus *Loxosceles* endemic to the eastern Canary Islands.

Descobrint diversitat desapercebuda en illes: colonització i diversificació de les aranyes amb importància mèdica del gènere *Loxosceles* (Arachnida: Sicariidae) a les Illes Canàries

RESUM

El nostre objectiu en aquest estudi ha estat entendre la història evolutiva del gènere d'aranyes *Loxosceles* a les Illes Canàries. Hem desentranyat la diversitat existent en l'arxipèlag canari, i investigat el seu origen, mode, i tempo de colonització cap i entre les illes en un marc filogenètic.

Hem portat a terme un extens mostreig a les Illes Canàries, i examinat les relacions filogenètiques entre els representants del gènere *Loxosceles* d'aquestes illes respecte als representants de l'oest d'Àfrica i la Conca Mediterrània. Per tal de delimitar els llinatges evolutius hem utilitzat el criteri evolutiu GMYC (*general mixed Yule coalescent*), i hem aplicat punts de calibratge (fòssils i biogeogràfics) per tal d'estimar les edats dels principals esdeveniments cladogenètics dins les Illes Canàries sota un marc Bayesià.

Les anàlisis filogenètiques han posat al descobert l'existència d'un clade ben recolzat endèmic de les Illes Canàries, format per set llinatges evolutius distribuïts de forma al·lopàtrica. La major part de dispersions entre Illes van succeir durant el Miocè superior. A més, també s'han trobat representants de l'espècie cosmopolita *Loxosceles rufescens* en l'arxipèlag.

En aquest estudi hem revelat l'existència d'un grup endèmic d'aranyes amb importància mèdica. El patró de diversitat en aquest grup es correspon amb el predit per la *dynamic theory of oceanic island biogeography*, on el màxim de diversitat es troba en les illes d'edats intermèdies. La via de colonització d'aquest grup és compatible amb un model de colonització per *stepping-stone*, seguint una progressió des de les illes més antigues, cap a les més modernes. Les dispersions entre illes han actuat com el major catalitzador per la diversificació dins del grup, però en alguns casos la diversificació ha estat dins d'una mateixa illa, com en Gran Canària, on el vulcanisme del Roque Nublo va actuar com a agent vicariant, promovent la separació dels dos llinatges. L'espècie recentment introduïda *L. rufescens* cohabita en aquest arxipèlag amb els llinatges endèmics.

PARAULES CLAU: biogeografia, espècies introduïdes, endemismes d'illes, evolució en illes, Macaronèsia, Conca Mediterrània, filogeografia, Roque Nublo.

Journal of Biogeography (*J. Biogeogr.*) (2014) **41**, 1255–1266

ORIGINAL ARTICLE

Uncovering overlooked island diversity: colonization and diversification of the medically important spider genus *Loxosceles* (Arachnida: Sicariidae) on the Canary Islands

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ABSTRACT

Aim Our aim was to assess the evolutionary history of the spider genus *Loxosceles* on the Canary Islands. We unravelled its present diversity within the archipelago, and investigated its origin, mode and tempo of colonization to and between the islands using a phylogenetic framework.

Location Canary Islands, Madeira, Iberian Peninsula, North Africa, Mediterranean region, Guinea.

Methods We conducted extensive sampling across the Canary Islands, and examined the phylogenetic relationships among the Canary Island representatives of the genus *Loxosceles* and with regard to species from western Africa and the Mediterranean Basin. We used an evolutionary criterion (general mixed Yule coalescent) to delimit the evolutionary lineages, and applied fossil and biogeographical calibration points to estimate dates for major cladogenetic events within the Canary Islands using a Bayesian framework.

Results Phylogenetic analyses revealed the existence of a well-supported clade formed exclusively by Canarian *Loxosceles* specimens, comprising seven allopatrically distributed evolutionary lineages. Major dispersal events between the islands occurred during the late Miocene. Representatives of the cosmopolitan *Loxosceles rufescens* were also found on the archipelago.

Main conclusions We have revealed the existence of an overlooked endemic group of medically important spiders. The pattern of diversity of this group fits well with the general dynamic theory of oceanic island biogeography, where maximum diversity is found on islands of intermediate age. The colonization pathway of the group is compatible with a stepping-stone model. Between-islands dispersal was the major driving force for diversification in the group, but a few within-island speciation events were also inferred, such as on Gran Canaria, where the Roque Nublo volcanic event acted as a vicariant agent, promoting the split between the two Gran Canarian lineages. The recently introduced *L. rufescens* is cohabiting with the endemic lineages.

Keywords

Biogeography, introduced species, island endemics, island evolution, *Loxosceles*, Macaronesia, Mediterranean Basin, phylogeography, Roque Nublo.

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INTRODUCTION

Oceanic islands are excellent natural laboratories for revealing the evolutionary mechanisms involved in generating diversity (Emerson, 2002; Gillespie & Roderick, 2002; Whittaker & Fernández-Palacios, 2007). Reliable documentation of this diversity, however, is a necessary step in conducting studies of such islands, and understanding the origin of an island group of taxa is of primary interest (Emerson, 2002). As oceanic islands, the Canary Islands have never been in contact with a continental landmass; consequently, all the terrestrial biota encountered there can be traced back to an

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original dispersal event, either active or passive. Two principal areas have acted as a source for the Canary Island biota: the neighbouring North Africa landmass and the Iberian Peninsula (Juan *et al.*, 2000). However, in some cases, more closely related groups are found on other islands of the Macaronesian archipelago, i.e. the Azores, Madeira, Selvagens and Cape Verde, or even in more distant regions, such as the New World (Carine, 2005).

Diversification patterns and colonization pathways are also a central subject in the study of the evolutionary history of island taxa. The use of neutral markers for phylogenetic inference has greatly improved our present understanding of these island biogeographical patterns (Juan *et al.*, 2000; Emerson, 2002). In addition, molecular data provide information on the time frame of the diversification of island taxa. Oceanic islands have been widely used as calibration points for divergence time estimation, given that the age of subaerial volcanism that formed the original island provides a maximum time limit for the evolutionary history of its biota (but see Heads, 2011).

Volcanism also plays a key role in shaping within-island diversification. Several studies have shown that phylogeographical patterns in organisms such as spiders, beetles and lizards closely match well-dated pulses of volcanic activity. In the Canary Islands, these effects are most evident for recent volcanism events (e.g. Emerson *et al.*, 2006; Bidegaray-Batista *et al.*, 2007) but effects of older volcanic activity (e.g. the eruption of Roque Nublo on the island of Gran Canaria) can still be found (e.g. Contreras-Díaz *et al.*, 2003).

Spiders are among the most diverse of the arthropod orders, and have been shown to be a good model for the study of island biogeography (Hormiga *et al.*, 2003; Gillespie, 2004; De Busschere *et al.*, 2010). The present-day knowledge of spider diversity on the Canary Islands is reasonably complete (Cardoso *et al.*, 2010), including an updated checklist (Macías, 2010) and several taxonomic revisions and phylogeographical studies (Wunderlich, 1994; Arnedo *et al.*, 2001, 2007; Platnick *et al.*, 2001; López-Mercader, 2005; Bidegaray-Batista *et al.*, 2007; Dimitrov & Ribera, 2007; Dimitrov *et al.*, 2008; Macías-Hernandez *et al.*, 2010, 2013a, 2013b). The information gathered for these studies, along with research conducted on other terrestrial invertebrates, has led some authors to propose emerging evolutionary and biogeographical patterns (Juan *et al.*, 2000; Cardoso *et al.*, 2010; Cameron *et al.*, 2013), generating greater interest in such research.

The spider genus *Loxosceles* Heineken and Lowe, 1832 is one of the two genera that form the family Sicariidae Keyserling, 1880. *Loxosceles* consists of 107 species (Platnick, 2014), mostly distributed across the Americas and Africa (Gertsch, 1967; Gertsch & Ennik, 1983; Binford *et al.*, 2008). Most of the species show a narrow, circumscribed, distribution, although a few exceptions exist, for example *Loxosceles laeta* (Nicolet, 1849), *Loxosceles reclusa* Gertsch & Mulaik, 1940 and *Loxosceles rufescens* (Dufour, 1820). *Loxosceles* spiders are well known for their medical relevance; their bites can cause severe dermonecrotic lesions in mammals (see da Silva *et al.*, 2004, and Swanson & Vetter, 2006, for reviews), mostly caused by the action of the enzyme sphingomyelinase D (Binford *et al.*, 2009), which causes the clinical condition of loxoscelism.

In the Canary Islands, the only representative of the genus presently recorded is *L. rufescens*, which is considered as 'probably introduced' in the Canarian species checklist (Macías, 2010). *Loxosceles rufescens* is considered to be cosmopolitan because it has probably been transported worldwide by humans, but its native distribution appears to be the Mediterranean region (Gertsch, 1967; Duncan *et al.*, 2009). However, Duncan *et al.* (2009) included two distinct *Loxosceles* representatives from Gran Canaria in their analyses: one that was molecularly indistinguishable from the Iberian *L. rufescens*, and another that was morphologically and molecularly divergent not only from *L. rufescens* but also from any other *Loxosceles* species.

In the present study, our aim was to (1) investigate the diversity of the genus *Loxosceles* in the Canary Islands archipelago, and (2) infer a phylogenetic framework for investigating the biogeographical patterns and evolutionary history of *Loxosceles*. We conducted a thorough sampling of *Loxosceles* in the Canary Islands, and obtained DNA sequence data from those specimens plus representatives from related geographical regions, to infer a phylogeny and to delimit distinct evolutionary lineages. Molecular data were used further to propose a time frame for the diversification of the group, using fossil and biogeographical calibration points. Because of the medical importance of *Loxosceles* spiders, the results of this study could have far-reaching implications for the islands' epidemiology and treatment of spider bites.

MATERIALS AND METHODS

Sampling and molecular data collection

Loxosceles specimens were collected between 2009 and 2012 and complemented with material provided by colleagues. To assess their relationships with continental representatives of the genus, we included known species present in the Mediterranean Basin, namely two individuals of *Loxosceles mrazig* Ribera & Planas, 2009 from Tunisia (Ribera & Planas, 2009), and three of *L. rufescens* from the Iberian Peninsula, illustrating the main evolutionary lineages found across the entire Mediterranean (Duncan *et al.*, 2009; E. Planas *et al.*, unpublished data). In addition, we included two individuals belonging to *Loxosceles foutadjalloni* Millot, 1941 from Guinea (Duncan *et al.*, 2009), two *L. rufescens* from Madeira and three *Loxosceles* aff. *rufescens* individuals from the Anti-Atlas region of Morocco (E.P. & C.R., unpublished data). Phylogenetic trees were rooted with one representative of *Loxosceles vonwredei* Newlands, 1980 from Namibia (Binford *et al.*, 2008).

For all the individuals, we amplified a partial fragment of the cytochrome *c* oxidase subunit I (*COI*). Additionally, one individual per locality was genotyped for three more gene

regions, namely one mitochondrial fragment spanning the 3′ half of the large ribosomal (r) unit (16S rRNA), the transfer (t)RNA leucine (*L1*) and the 5′ end of NADH dehydrogenase (*nad1*), and two nuclear genes, histone 3 (*H3*) and the internal transcribed spacer 2 (ITS2). Primers are listed in Table 1 and polymerase chain reactions (PCR) were conducted following Planas *et al.* (2013). Raw sequences were edited and assembled with Geneious 4.6.5 (Drummond *et al.*, 2009). All new sequences obtained were deposited in GenBank (see Appendix S1 in Supporting Information).

Alignment, partitioning analyses and evolutionary model selection

We performed sequence alignments using the online version of mafft (Katoh *et al.*, 2005). We applied the G-INS-i algorithm (Katoh *et al.*, 2005) for protein-coding fragments and the Q-INS-i algorithm (Katoh & Toh, 2008) for the ribosomal gene fragments. Both algorithms were applied using the default options.

We explored 11 alternative partitioning schemes using the 'user-specific schemes' option in PartitionFinder 1.0.1 (Lanfear *et al.*, 2012). Partitioning schemes ranged from considering all the gene fragments together as one partition, to a highly partitioned scheme with each gene fragment considered as an independent partition and the third codon position separated from the first and second in coding regions (see Appendix S2). We used the Bayesian information criterion to select among the partition schemes and evolutionary models (Lanfear *et al.*, 2012).

Phylogenetic analyses and delimitation of evolutionary lineages

We conducted phylogenetic analyses of the concatenated matrix using maximum likelihood (ML) and Bayesian inference (BI). ML analyses were conducted using RAxML 7.4.2 (Stamatakis, 2006) through the graphical front-end RAxML-GUI 1.3 (Silvestro & Michalak, 2011) in rapid-hill climbing mode. A GTR+G+I nucleotide substitution model was applied to each of the partitions corresponding to the best partition scheme selected, and a nonparametric bootstrap support analysis of 1000 pseudoreplicates was conducted. BI analyses were conducted with MrBayes 3.2 (Ronquist *et al.*, 2012). The best-fitting model was applied to each partition, and all parameters were unlinked across partitions. Two independent runs were performed to check for convergence. For each analysis, four Markov chains were run (one cold, three heated) for 2 million iterations, saving trees each 1000th generation. The first 25% of trees in each run were discarded as burn-in following visual inspection in Tracer (Rambaut & Drummond, 2007), after ensuring that the Markov chains had reached a stationary (average standard deviation of the split frequencies of the two runs < 0.01), and a majority-rule consensus tree was generated from the remaining trees.

We used the tree-based general mixed Yule coalescent (GMYC) model (Pons *et al.*, 2006) to define species boundaries. This method has been applied successfully for DNA-based species delimitation in different organisms, including spiders (e.g. Hendrixson *et al.*, 2012; Planas *et al.*, 2013). Although GMYC relies exclusively on one line of evidence, i.e. mitochondrial (mt)DNA, for species delimitation, it has been found to be accurate and conservative in groups with small effective populations and high divergence times between species (Fujisawa & Barraclough, 2013), as is the case in this study.

We generated an ultrametric tree with beast 1.7.4 (Drummond *et al.*, 2012) based on the *COI* DNA sequences. We used a coalescent tree prior with a constant population size (Monaghan *et al.*, 2009) and an uncorrelated relaxed molecular clock with a molecular rate set to 1, as we were only interested in the relative branch lengths. We performed two independent runs of 10 million generations each. The results of the two analyses were inspected in Tracer to assess the correct mixing of the chains (effective sample size,

Table 1 The primers used for the study of the spider genus *Loxosceles* (Arachnida: Sicariidae) from the Canary Islands.

Gene fragment*	Primer	PO†	Sequence (5′–3′)	Reference
COI	LINF	F	GGGCNTGRTCWGGNATRATAGG	This study
	CANF	F	GCDGGDGCTTCTTCDATTATRGG	This study
	LJEF	F	TCARCATYTRTTTTGRTTTTTTGG	This study
	GAYAR	R	GAAAATGHGCHACHACRTAATAAGTRTC	This study
	CANR	R	GCNCCYATAATHGAAGAAGC	This study
	LJER	R	TGTTGAAAYAAAATHGGRTCHCC	This study
16S+L1+nad1	Lox16SF	F	CGCCCTGTTTAACAAAAACATCAC	This study
	16SR	R	CCTTTAACGAATTTGAATATA	Hedin & Maddison (2001)
H3	H3aF	F	ATGGCTCGTACCAAGCAGACVGC	Colgan *et al.* (1998)
	H3aR	R	ATATCCTTRGGCATRATRGTGAC	Colgan *et al.* (1998)
ITS2	CAS28sB1d	R	TTCTTTTCCTCCSCTTAYTRATATGCTTAA	Ji *et al.* (2003)
	5.8SF	F	CACGGGTCGATGAAGAACGC	This study

**COI*, partial fragment of the cytochrome *c* oxidase subunit I; *16S*, mitochondrial fragment spanning the 3′ half of the large ribosomal unit; *L1*, the transfer (t)RNA leucine; *nad1*, the 5′ end of NADH dehydrogenase; *H3*, histone 3; ITS2, internal transcribed spacer 2.
†Primer orientation: F, forward; R, reverse.

ESS > 200). We then conducted a 10% burn-in of each analysis and tree combination in LogCombiner and TreeAnnotator (Drummond & Rambaut, 2007). The GMYC delimitation was conducted with the R package 'SPLITS' (Ezard *et al.*, 2009). Both single-threshold (Pons *et al.*, 2006) and multiple-threshold (Monaghan *et al.*, 2009) models were applied to the tree, and a likelihood ratio test (LRT) was applied to select the best model.

We calculated the genetic divergence of *COI* sequences [uncorrected pairwise (*p*)-distance] between and within the different Canarian evolutionary lineages (i.e. GMYC clusters) using mega 5 (Tamura *et al.*, 2011), removing alignment gaps in pairwise comparisons.

Molecular dating analyses

We estimated absolute divergence times and substitution rates using a Bayesian approach as implemented in beast 1.7.4 (Drummond *et al.*, 2012), applying an uncorrelated lognormal relaxed clock (Drummond *et al.*, 2006). We combined one representative of each evolutionary lineage of our ingroup (Canarian and Mediterranean) with individuals belonging to the *Loxosceles amazonica* Gertsch, 1967; *Loxosceles gaucho* Gertsch, 1967; *Loxosceles hirsuta* Mello-Leitao, 1931, *L. laeta*, *L. reclusa* and *L. vonwredei* species groups. We estimated absolute divergence times by incorporating fossil and biogeographical calibration points. Three *Loxosceles* species are described from Dominican amber (Wunderlich, 1988, 2004). We thus constrained the ancestral node of the *L. reclusa* group (including Caribbean and North American *Loxosceles*; Binford *et al.*, 2008) to a minimum bound. The age of the Dominican amber is estimated to be 16 Ma (Iturralde-Vinent, 2001), although some authors have used older ages (e.g. 20 Ma; Binford *et al.*, 2008). To account for age uncertainty, we used an exponential distribution with a 'hard' minimum bound at 16 Ma and 95% of the posterior density within 21.99 Ma (10% of the older age 20 Ma), following Hipsley *et al.* (2009). In oceanic islands, geological ages represent the time of the earliest possible colonization and they can be incorporated as maximum bounds assuming that species formation was subsequent to island emergence. However, further major assumptions are also implied in this case, which when violated would lead to incorrect divergence dates and molecular rates (Emerson, 2007). We used uniform priors from 0 to the time of emergence of the oldest available island for the internal Canary Island dispersal events: (1) age of Gran Canaria (14.5 Ma) for the most recent common ancestor (MRCA) of Canarian endemic lineages; (2) age of the oldest emerged part of Tenerife (11.6 Ma) for the subsequent node; (3) age of La Gomera (10.5 Ma) for the MRCA of Tenerife and La Gomera endemic lineages. Well-supported clades recovered in the ML and BI analyses of the concatenated matrix and those well supported in Binford *et al.* (2008) were constrained in the beast analyses. Two independent runs of 30 million generations (sampling every 1000th generation) were performed for each analysis. Convergence of the chains was assessed and burn-in was conducted as above.

RESULTS

Appendix S1 lists the locality information for all 134 *Loxosceles* individuals used in the study, 125 of which correspond to 46 localities in the Canary Islands (Fig. 1).

The concatenated matrix used in the phylogenetic analyses consisted of 2269 bp. The total length of the mtDNA gene

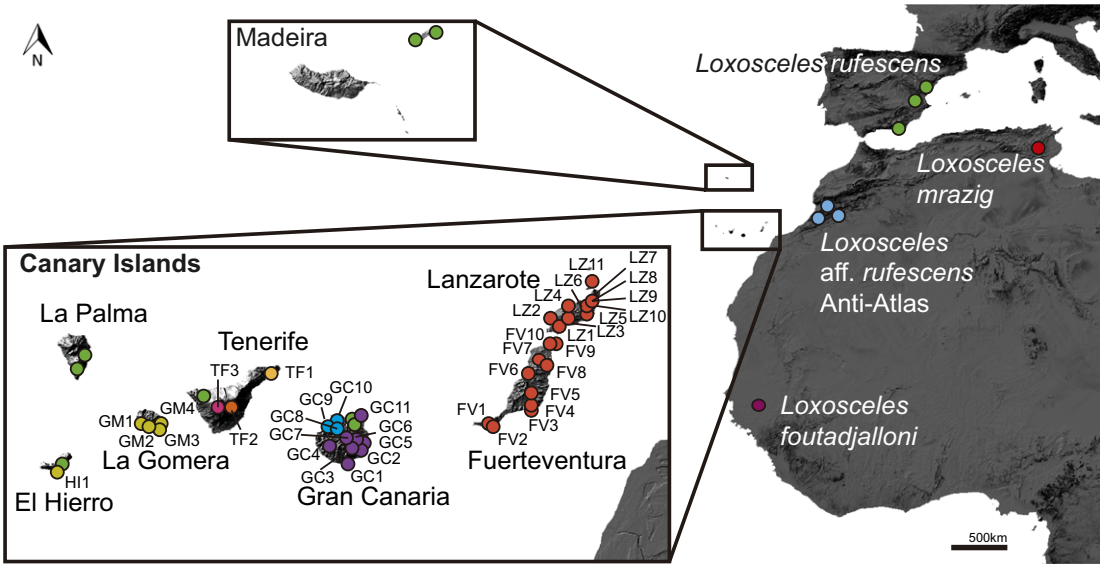

Figure 1 Sampling localities of *Loxosceles* specimens. Colours correspond to those used in Fig. 2. Locality numbers are given for the Canarian endemic clade and correspond to those used in Appendix S1. FV, Fuerteventura; LZ, Lanzarote; GC, Gran Canaria; TF, Tenerife; GM, La Gomera; HI, El Hierro.

fragments was 1600 bp (*COI* 942 bp, *16S* 547 bp, *L1* 57 bp and *nad1* 54 bp) and of the nuclear (nu)DNA gene fragments 669 bp (*H3* 327 bp and ITS2 342 bp).

The preferred partitioning scheme included three partitions: the mtDNA gene fragments, except the third codon position of the *COI* gene; the third codon position of the *COI* gene; and the concatenation of the two nuDNA gene regions. Information on length, variable sites and the selected evolutionary model for each of these three partitions is presented in Appendix S2.

Delimitation of evolutionary lineages and phylogenetic analyses

In the GMYC analyses, both the single- and multiple-threshold models fitted to the data significantly better than the null

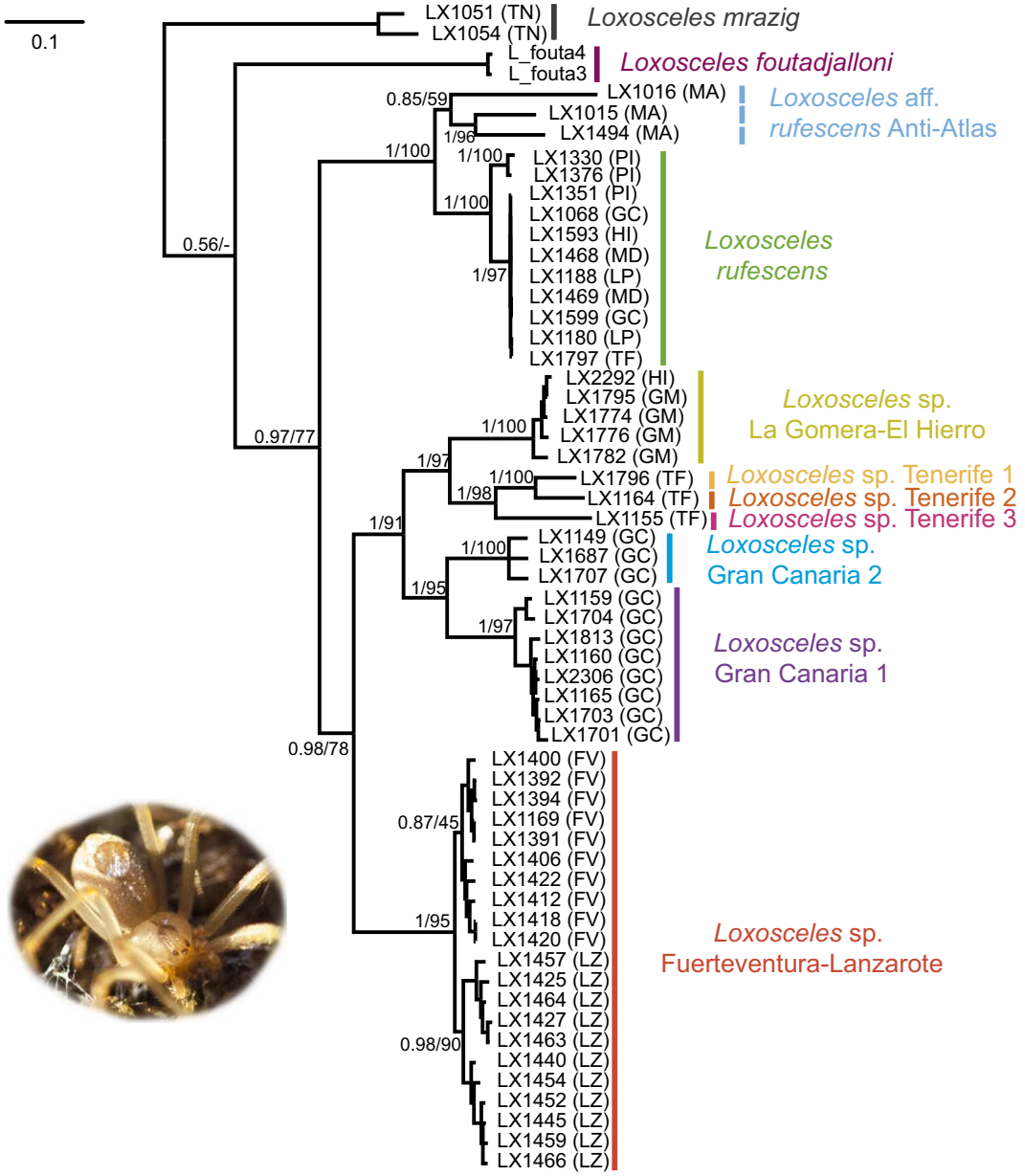

Figure 2 Bayesian inference (BI) tree inferred based on a concatenated matrix [*COI*, partial fragment of the cytochrome *c* oxidase subunit I; *16S*, mitochondrial fragment spanning the 3′ half of the large ribosomal unit; *L1*, the transfer (t)RNA leucine; *nad1*, the 5′ end of NADH dehydrogenase; *H3*, histone 3; ITS2, internal transcribed spacer 2] of Canary Islands' endemic *Loxosceles* and close relatives. Numbers next to nodes correspond to posterior probability values in the BI analysis and to bootstrap support in the maximum likelihood (ML) analysis. Next to each specimen code the geographical region is provided: MD, Madeira; TN, Tunisia; MA, Morocco; GC, Gran Canaria; TF, Tenerife; HI, El Hierro; GM, La Gomera; FV, Fuerteventura; LZ, Lanzarote; LP, La Palma; PI, Iberian Peninsula). Evolutionary lineages delimited with a general mixed Yule coalescent (GMYC) model are named and coloured. The tree was rooted with *L. vonwredei* (not shown).

model (single, $P = 0.0113$; multiple, $P = 0.0185$) but they did not differ significantly from each other ($\chi^2 = 0.7728$, d.f. = 3, $P = 0.8560$). Thus the simplest model (single) was favoured. Seven clusters and seven entities were retrieved, from which four clusters and three entities were formed exclusively by Canarian individuals (Fig. 2).

The topologies of the analyses conducted with BI and ML were almost identical. Differences between the two methods included the position of *L. mrazig* and *L. foutadjalloni* with regard to the clade grouping the remaining representatives (i.e. *L. rufescens* s.l. and the Canarian clade). However, these relationships were not well supported in any of the analyses (Fig. 2). The sister relationship between the Canarian clade and the *L. rufescens* s.l. clade was well supported in both analyses [posterior probability (PP) = 0.97, ML bootstrap support BS) = 77].

The clade formed exclusively by Canarian representatives was supported in both the BI and ML analyses (PP = 0.98, BS = 78; Fig. 2). Within the Canarian clade, all the representatives from Fuerteventura and Lanzarote clustered together with strong support (PP = 0.9, BS = 96) and constituted a single GMYC cluster. Similarly, the Gran Canarian (GC) specimens formed a clade (PP = 1, BS = 95) that included two GMYC clusters (GC1 and GC2), both well supported (PP = 1, BS = 97, and PP = 1, BS = 100, for GC1 and GC2, respectively). These two evolutionary lineages from Gran Canaria showed a 12.5% uncorrected divergence for *COI* and were distributed allopatrically (see Appendix S3). In Tenerife, the three sampled representatives formed a monophyletic group (PP = 1, BS = 98) and were delimited as three independent GMYC entities, with the *COI* uncorrected genetic distances among them being greater than 11.4% (Table 2). The representatives of La Gomera and the single specimen from El Hierro formed a clade (PP = 1, BS = 100) and a single GMYC cluster. Relationships between the main Canarian lineages were all well supported. The Gomera–El Hierro clade and the Tenerife clade formed a clade sister to the Gran Canaria clade, and all of them were in turn sister to the Fuerteventura–Lanzarote clade.

Within the *L. rufescens* s.l. clade (PP = 1, BS = 100), two different clades were recovered in both analyses: one corresponded to specimens from the Anti-Atlas region (*Loxosceles* aff. *rufescens* Anti-Atlas) and the other to the remaining *L. rufescens* specimens sampled (*L. rufescens* s.s.). In the *L. rufescens s.s.* clade, the representatives from the Iberian Peninsula, specifically from the type locality of the species (Sagunt), formed a clade together with several individuals from the Canary Islands (Gran Canaria, El Hierro, La Palma and Tenerife) and the two specimens from Madeira (PP = 1, BS = 100), with a very low within-clade *COI* uncorrected genetic distance (0.1%). The sister group of this clade included two additional specimens from the Iberian Peninsula, and its relationship with the former clade was well supported in both analyses (PP = 1, BS = 100).

Molecular dating analyses

The dataset consisted of 37 terminals and 1107 bp from sequences of two mtDNA genes, *COI* and *16S*, common to this study and Binford *et al.* (2008). The estimated chronogram is shown in Fig. 3. The age of the oldest split within the Canary Islands' endemic *Loxosceles*, which separated the Fuerteventura–Lanzarote lineage from the rest of the Canary Islands group, was estimated to be 8.4 Ma (95% highest posterior density, HPD: 6.03–10.98 Ma). The split between the Gran Canaria species and the westernmost lineages was estimated to be 7.1 Ma (HPD: 5.09–9.26 Ma), and the MRCA of the two species from Gran Canaria was dated as 4.42 Ma (HPD: 2.65–6.41 Ma). The split between the Tenerife representatives and the lineage from La Gomera–El Hierro was estimated to be 5.33 Ma (HPD: 3.73–7.14 Ma). The first split within Tenerife was dated as 3.67 Ma (HPD: 2.45–5.12 Ma) and the second split as 1.99 Ma (HPD: 1.1–2.96 Ma). The estimated mean mtDNA substitution rate (12.686% divergence per Myr) was comparable with other rates calculated specifically for spiders in oceanic islands (5.3–9.8% divergence Myr^{-1}; Arnedo & Gillespie, 2006; Dimitrov *et al.*, 2008; Macías-Hernandez *et al.*, 2010) and five times higher than the 'standard' 2.3 divergence Myr^{-1} (Brower, 1994).

Table 2 Mean uncorrected distances of the partial fragment of the cytochrome *c* oxidase subunit I (*COI*) between (below the diagonal) and within (diagonal, in italics) *Loxosceles* endemic lineages from the Canary Islands. The final column and row shows the mean uncorrected distance between individuals from La Gomera (GM) and El Hierro (HI).

	FV–LZ	GC1	GC2	TF1	TF2	TF3	GM–HI	HI
FV–LZ	*0.044*							
GC1	0.152	*0.028*						
GC2	0.148	0.125	*0.046*					
TF1	0.153	0.134	0.146	–				
TF2	0.150	0.140	0.141	0.114	–			
TF3	0.172	0.145	0.154	0.126	0.123	*0.001*		
GM–HI	0.148	0.130	0.132	0.106	0.120	0.136	*0.017*	
GM								0.014

Abbreviations: FV–LZ, *Loxosceles* sp. Fuerteventura–Lanzarote; GC1, *Loxosceles* sp. Gran Canaria 1; GC2, *Loxosceles* sp. Gran Canaria 2; TF1; *Loxosceles* sp. Tenerife 1; TF2, *Loxosceles* sp. Tenerife 2; TF3, *Loxosceles* sp. Tenerife 3, GM-HI; *Loxosceles* sp. La Gomera–El Hierro.

DISCUSSION

An overlooked *Loxosceles* lineage endemic to the Canary Islands

The results of ML and BI phylogenetic analyses (Fig. 2) revealed the presence of a new *Loxosceles* lineage endemic to the archipelago. The GMYC species delimitation method identified seven evolutionary lineages within the archipelago. All of the islands, except La Palma, harbour at least one lineage. The distribution of the endemic *Loxosceles* diversity fits well with the 'general dynamic model of oceanic island biogeography' (Whittaker *et al.*, 2008, 2010), with maximum diversity found on islands of intermediate age. This diversity hump-shaped pattern has also been found in other spider genera (Cardoso *et al.*, 2010).

Fuerteventura and Lanzarote together with the two neighbouring islets of Lobos and La Graciosa harbour a monophyletic lineage, recovered as an independent GMYC cluster and separated by a 14% *COI* uncorrected genetic distance (Table 2) from the rest of the endemic Canarian lineages. Within this lineage, two monophyletic groups were recognized, one distributed across Fuerteventura and Lobos and the other across Lanzarote and La Graciosa.

The two putative *Loxosceles* species of Gran Canaria are distributed allopatrically. Their distribution ranges roughly match the two parts of the island that were less affected by the Roque Nublo eruptive period (Appendix S3). A similar distribution pattern has been found in other organisms, including the gecko *Tarentola boettgeri*, the skink *Chalcides sexlineatus* and the beetle genus *Pimelia* (Pestano & Brown, 1999; Contreras-Díaz *et al.*, 2003; Gübitz *et al.*, 2005). It has been postulated that a total extinction of the biota occurred in Gran Canaria as a result of the Roque Nublo volcanic event (see Anderson *et al.*, 2009), although most of the current evidence obtained from molecular data and fossils does not support such a mass extinction (Emerson, 2003; Anderson *et al.*, 2009). There is no doubt, however, that this volcanic event had severe consequences on the island biota, especially for species associated with forest communities (Emerson, 2003). Divergence time estimates place this split (4.42 Ma) within the temporal framework of the volcanic pulse (5–3 Ma) (Anderson *et al.*, 2009) and, therefore, it appears reasonable to associate the Roque Nublo eruption with the vicariant origin of the two local *Loxosceles* species of Gran Canaria.

Tenerife originated as three isolated massifs (Anaga, Teno and Roque del Conde) that emerged as a result of independent volcanic cycles. These three proto-islands were later joined as a consequence of the Las Cañadas emergence, forming the current island (see Marrero & Francisco-Ortega, 2001, and Carracedo *et al.*, 2002, for reviews). This complex volcanic history has shaped the diversity and distribution patterns of several organisms, including the two well-studied spider genera *Dysdera* and *Pholcus* (Dimitrov *et al.*, 2008; Macías-Hernandez *et al.*, 2013a). Similarly, this could be the main explanation for the current relationships between the three putative *Loxosceles* species found in Tenerife.

Another putative species of *Loxosceles* was found on the islands of La Gomera and El Hierro. The individual from El Hierro falls among the representatives of La Gomera, which in combination with the low genetic distances (1.4%) suggests a recent colonization from La Gomera to El Hierro. El Hierro is the youngest island in the archipelago (1 Ma) and its endemic fauna usually shows close affinity with that of La Gomera, e.g. *Calathus spretus* (Emerson *et al.*, 1999), *Pholcus bimbache* (Dimitrov *et al.*, 2008) and *Dysdera gomerensis* (Macías-Hernandez *et al.*, 2013a).

Origin and colonization of the Canary Islands

The most confident strategy for testing the monophyly of an island group is to include all the closely related species from continental areas and neighbouring archipelagos in the analyses (Emerson, 2002). We therefore included specimens from Madeira, all the currently accepted *Loxosceles* species in the Mediterranean (i.e. *L. rufescens* and *L. mrazig*) and *L. foutadjalloni* from Guinea in our phylogenetic analyses. Special effort was taken to include representatives of different lineages distributed across the Anti-Atlas region of Morocco (E.P. & C.R., unpublished data). This region has been shown to be a continental source for many groups on the Canary Islands, because of its proximity with the eastern-most islands (Juan *et al.*, 2000, and references therein). However, omission of lineages because of incomplete sampling or extinction of continental relatives is a common caveat, which should be kept in mind when studying island biota. Nevertheless, the monophyly of the endemic Canarian *Loxosceles* is clear from the results presented here, obtained from molecular analyses, and is also supported by the morphological synapomorphies shared by all of the Canarian representatives (E.P. & C.R., unpublished data). This endemic group is a sister to the clade formed by *L. rufescens* and some divergent lineages from the Anti-Atlas region, and thus it could be hypothesized that the colonization occurred from the north-western part of Africa between 11.3 and 8.4 Ma (Fig. 3). However, for conclusive evidence, it would be essential to sample the region between the Anti-Atlas and Guinea intensively, as overlooked lineages could still be found and shed further light on the biogeographical history of the group.

In the Canary Islands, the stepping-stone model of colonization has been postulated as the most common pattern of colonization shown by several groups of organisms (Juan *et al.*, 2000; Sanmartín *et al.*, 2008). The model predicts that the colonization of an archipelago begins with a dispersal event from the continent to the geographically closest island. Then a succession of dispersal events leads to the colonization of the remaining islands. In this archipelago, where the islands closer to the mainland are also the older islands (Fuerteventura and Lanzarote), this pattern also corresponds

to the progression rule, which states that younger islands are colonized from nearby, older ones (Funk & Wagner, 1995). Given the inferred relationships within *Loxosceles* representatives in the Canary Islands, we do not have any evidence for rejecting the stepping-stone model as the pattern followed by these spiders for the colonization of the archipelago, in succession from the easternmost to the westernmost islands (Fig. 3).

Loxosceles rufescens as an introduced species

Before the present study, the genus *Loxosceles* in the Canary Islands was represented exclusively by *L. rufescens* (see Introduction). In our analyses, some of the *Loxosceles* individuals collected in the Canaries were closely related to the *L. rufescens* Iberian lineage from the type locality (Fig. 2). Additionally, the two individuals from Madeira fell within the same lineage. The genetic distance of *COI* within this group is 0.1%, which is much lower than the intraspecific divergences found in any other group. Altogether, this evidence points towards a recent introduction of *L. rufescens* to the archipelago. Most Canarian *L. rufescens* were collected in human-disturbed areas; they were never collected together with any of the Canarian endemic *Loxosceles*. La Palma is the only island where we failed to obtain endemic *Loxosceles*, and only *L. rufescens* was collected, even from well-preserved habitats

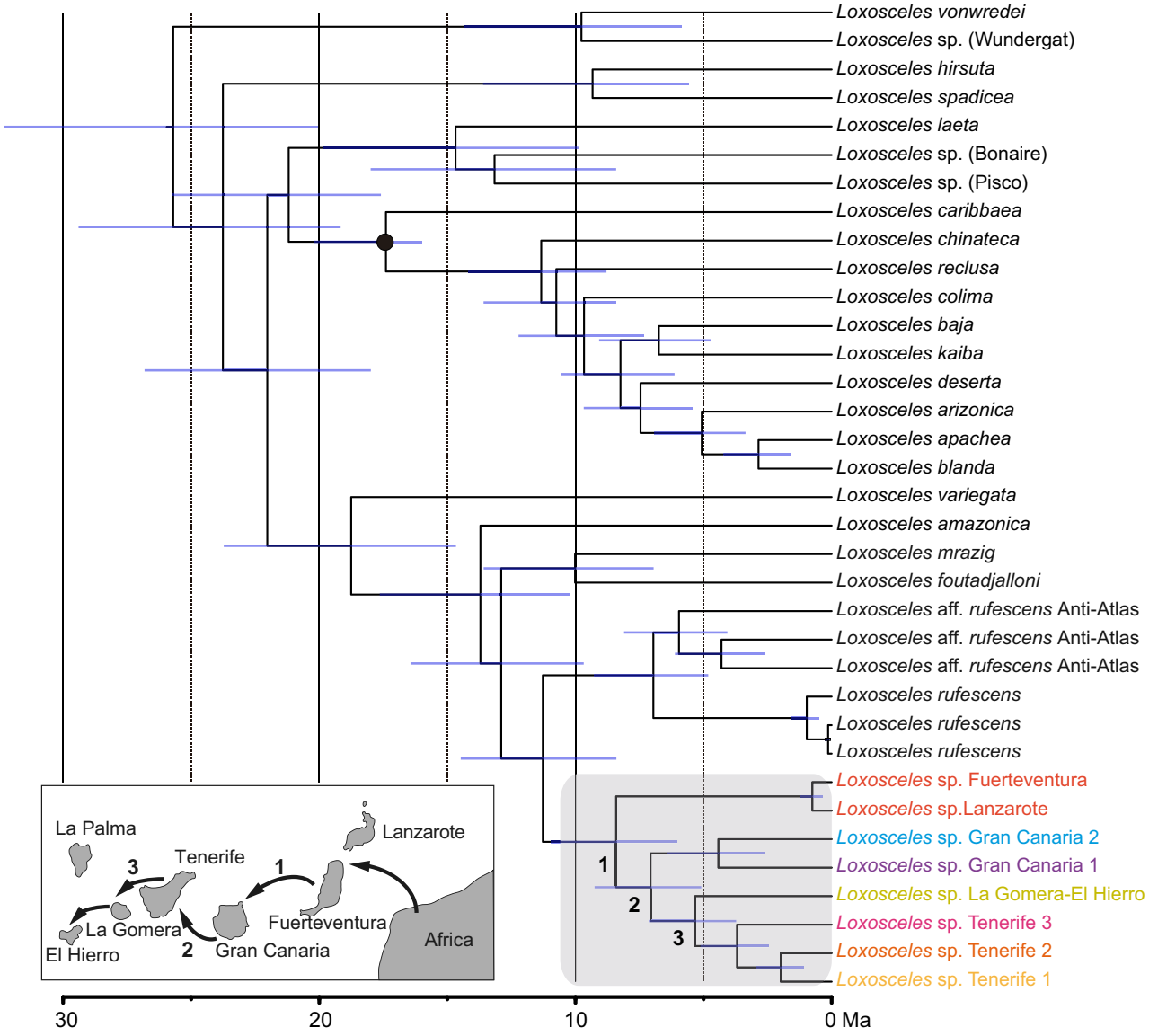

Figure 3 Chronogram of *Loxosceles* specimens obtained using BEAST calibrated with fossil and island ages (see Materials and Methods). The black dot indicates the node calibrated as a minimum bound with a *Loxosceles* fossil from Dominican amber. The Canarian endemic lineage is highlighted in grey. Bars at internodes correspond to the 95% highest posterior density (HPD). The inset figure shows the Canary Islands, with arrows representing dispersal events using the stepping-stone model of colonization. Numbers in the inset figure correspond to those used in the chronogram.

such as caves. Conversely, Fuerteventura, Lanzarote and La Gomera are the only islands where we did not collect *L. rufescens*.

In the Canary Islands, as in the other Macaronesian archipelagos and on oceanic islands in general, there are few, if any, venomous animals. Therefore the introduction of *L. rufescens* to the Canary Islands is of medical relevance. Although loxoscelism is rare, even in areas with high *Loxosceles* density (Vetter, 2003), the synathropic lifestyle of *L. rufescens* facilitates human contact, making envenomation by this species more likely than by the endemic ones. The existence of two distinct evolutionary lineages of *Loxosceles* in the Canaries, the endemic lineage and *L. rufescens*, should be taken into consideration in future health management policies.

CONCLUSIONS

In this study we conducted a thorough sampling across the Canary Islands that revealed the existence of an overlooked endemic group of *Loxosceles*. Each Canary island, with the exception of La Palma, holds at least one endemic lineage. In total seven GMYC delimited lineages were retrieved, the diversity and distribution of which follow the general dynamic theory of oceanic island biogeography. The colonization of the Canary Islands by the endemic *Loxosceles* is compatible with a stepping-stone dispersal pattern from the easternmost island closest to the continent towards the western islands. This successive colonization and subsequent differentiation has acted as the main diversification driver, but a few within-island speciation events are also inferred, as in Gran Canaria, where the Roque Nublo volcanic event acted as a vicariant agent, promoting the split between the two Gran Canarian lineages. Finally, we found that the cosmopolitan *L. rufescens* is also present on most of the islands, where it should be considered to be an introduced species. Both lineages should be considered in future health management policies.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Study material.
Appendix S2 Partition schemes.
Appendix S3 Distribution of *Loxosceles* species from Gran Canaria.

BIOSKETCHES

Enric Planas is a PhD student at the University of Barcelona, studying the biogeography and systematics of *Loxosceles* spiders in the Mediterranean and the Canary Islands.

Carles Ribera is an associate professor at the University of Barcelona. His research focuses on the taxonomy and biogeography of spiders of the Mediterranean Basin and oceanic islands.

Editor: Brent Emerson

Appendix S1 Study material. A list of the specimens used for the study of the spider genus *Loxosceles* (Arachnida: Sicariidae) from the Canary Islands, with their identification code, locality name, locality number (LN), coordinates and GenBank accession numbers. Individuals included in maximum likelihood (ML) and Bayesian inference (BI) phylogenetic analyses are in bold, and those used for dating analyses are underlined. *COI*, partial fragment of the cytochrome *c* oxidase subunit I; *16S*, mitochondrial fragment spanning the 3′ half of the large ribosomal unit; *L1*, the transfer (t)RNA leucine; *nad1*, the 5′ end of NADH dehydrogenase; *H3*, histone 3; ITS2, internal transcribed spacer 2. Next to each specimen code the geographical region is provided: MD, Madeira; TN, Tunisia; MA, Morocco; GC, Gran Canaria; TF, Tenerife; HI, El Hierro; GM, La Gomera; FV, Fuerteventura; LZ, Lanzarote; LP, La Palma; PI, Iberian Peninsula. Further information on the individuals of the *Loxosceles foutadjalloni*, *L. amazonica*, *L. gaucho*, *L. spadicea*, *L. laeta*, *L. reclusa* and *L. vonwredei* species group can be found in Binford *et al.* (2008).

CODE	LOCALITY	LATITUDE	LONGITUDE	LN	*COI*	*16S*	*H3*	ITS2
***Loxosceles* sp. Fuerteventura-Lanzarote**								
LX1069_FV	Cofete	28.10265	-14.38232	FV1	KF669916			
LX1394_FV	Cofete	28.10265	-14.38232	FV1	KF669931	KF670037	KF670079	KF670114
LX1392_FV	Península Jandía	28.0854	-14.37196	FV2	KF669929	KF670036	KF670078	KF670113
LX1393_FV	Península Jandía	28.0854	-14.37196	FV2	KF669930			
LX1391_FV	Tequital	28.26339	-13.94874	FV3	KF669928	KF670035	KF670077	KF670112
LX1169_FV	Tuineje	28.277677	-13.950876	FV4	KF669927	KF670034	KF670076	KF670111
LX1395_FV	Cueva Tiscamanitas	28.33501	-13.99525	FV5	KF669932			
LX1397_FV	Cueva Tiscamanitas	28.33501	-13.99525	FV5	KF669933			
LX1398_FV	Cueva Tiscamanitas	28.33501	-13.99525	FV5	KF669934			
LX1399_FV	Cueva Tiscamanitas	28.33501	-13.99525	FV5	KF669935			
LX1400_FV	Cueva Tiscamanitas	28.33501	-13.99525	FV5	KF669936	KF670038	KF670080	KF670115
LX1403_FV	Cueva Tiscamanitas	28.33501	-13.99525	FV5	KF669937			
LX1404_FV	Cueva Tiscamanitas	28.33501	-13.99525	FV5	KF669938			
LX1406_FV	Aguas Verdes	28.47845	-14.05128	FV6	KF669939	KF670043	KF670081	
LX1407_FV	Aguas Verdes	28.47845	-14.05128	FV6	KF669940			
LX1408_FV	Aguas Verdes	28.47845	-14.05128	FV6	KF669941			
LX1409_FV	Aguas Verdes	28.47845	-14.05128	FV6	KF669942			
LX1421_FV	Calderilla de Roja	28.62996	-13.83421	FV7	KF669951			
LX1422_FV	Calderilla de Roja	28.62996	-13.83421	FV7	KF669952	KF670042	KF670085	
LX1423_FV	Calderilla de Roja	28.62996	-13.83421	FV7	KF669953			
LX1424_FV	Calderilla de Roja	28.62996	-13.83421	FV7	KF669954			
LX1410_FV	Villaverde	28.65061	-13.91518	FV8	KF669943			
LX1411_FV	Villaverde	28.65061	-13.91518	FV8	KF669944			
LX1412_FV	Villaverde	28.65061	-13.91518	FV8	KF669945	KF670041	KF670082	
LX1415_FV	Lobos	28.7439	-13.82542	FV9	KF669946			
LX1416_FV	Lobos	28.7439	-13.82542	FV9	KF669947			
LX1417_FV	Lobos	28.7439	-13.82542	FV9	KF669948			
LX1418_FV	Lobos	28.7439	-13.82542	FV9	KF669949	KF670039	KF670083	KF670116
LX1037_FV	Lobos	28.75205	-13.83010	FV10	KF669915			
LX1420_FV	Caldera Lobos	28.75205	-13.83010	FV10	KF669950	KF670040	KF670084	KF670117
LX1426_LZ	Costa Papagayo	28.86038	-13.77626	LA1	KF669956			
LX1427_LZ	Costa Papagayo	28.86038	-13.77626	LA1	KF669957	KF670050	KF670087	KF670119

CODE	LOCALITY	LATITUDE	LONGITUDE	LN	*COI*	*16S*	*H3*	ITS2
LX1429_LZ	Costa Papagayo	28.86038	-13.77626	LA1	KF669958			
LX1425_LZ	Salinas de Janubio	28.94288	-13.81886	LA2	KF669955	KF670053	KF670086	KF670118
LX1460_LZ	Puerto Calero	28.93313	-13.70168	LA3	KF669981			
LX1461_LZ	Puerto Calero	28.93313	-13.70168	LA3	KF669982			
LX1462_LZ	Puerto Calero	28.93313	-13.70168	LA3	KF669983			
LX1463_LZ	Puerto Calero	28.93313	-13.70168	LA3	KF669984	KF670051	KF670094	KF670125
LX1464_LZ	Tinajo	29.0614	-13.6777	LA4	KF669985	KF670052	KF670095	
LX1457_LZ	Costa Teguise	29.02955	-13.51675	LA5	KF669978	KF670054	KF670092	
LX1458_LZ	Costa Teguise	29.02955	-13.51675	LA5	KF669979			
LX1432_LZ	Los Valles	29.07914	-13.52484	LA6	KF669959			
LX1433_LZ	Los Valles	29.07914	-13.52484	LA6	KF669960			
LX1434_LZ	Los Valles	29.07914	-13.52484	LA6	KF669961			
LX1435_LZ	Los Valles	29.07914	-13.52484	LA6	KF669962			
LX1436_LZ	Los Valles	29.07914	-13.52484	LA6	KF669963			
LX1437_LZ	Los Valles	29.07914	-13.52484	LA6	KF669964			
LX1438_LZ	Los Valles	29.07914	-13.52484	LA6	KF669965			
LX1440_LZ	Los Valles	29.07914	-13.52484	LA6	KF669966	KF670049	KF670088	KF670120
LX1466_LZ	Teguise	29.09475	-13.55653	LA7	KF669986	KF670045	KF670096	
LX1467_LZ	Teguise	29.09475	-13.55653	LA7	KF669987			
LX1453_LZ	Barranco Hondo del Valle	29.14139	-13.48203	LA8	KF669974			
LX1454_LZ	Barranco Hondo del Valle	29.14139	-13.48203	LA8	KF669975	KF670047	KF670091	KF670123
LX1455_LZ	Barranco Hondo del Valle	29.14139	-13.48203	LA8	KF669976			
LX1456_LZ	Barranco Hondo del Valle	29.14139	-13.48203	LA8	KF669977			
LX1451_LZ	Guinate	29.17536	-13.50576	LA9	KF669972			
LX1452_LZ	Guinate	29.17536	-13.50576	LA9	KF669973	KF670046	KF670090	KF670122
LX1443_LZ	Mirador del Río	29.21336	-13.48111	LA10	KF669967			
LX1444_LZ	Mirador del Río	29.21336	-13.48111	LA10	KF669968			
LX1445_LZ	Mirador del Río	29.21336	-13.48111	LA10	KF669969	KF670044	KF670089	KF670121
LX1446_LZ	Mirador del Río	29.21336	-13.48111	LA10	KF669970			
LX1448_LZ	Mirador del Río	29.21336	-13.48111	LA10	KF669971			
LX1459_LZ	La Graciosa	29.2472	-13.518	LA11	KF669980	KF670048	KF670093	KF670124
***Loxosceles* sp. Gran Canaria 1**								
LX1704_GC	Ladera de los Pinos	27.8064	-15.58276	GC1	KF669996	KF670063		
LX1813_GC	Los Corralillos	27.883766	-15.45515	GC2	KF670023	KF670062	KF670104	KF670132
LX1814_GC	Los Corralillos	27.883766	-15.45515	GC2	KF670024			
LX1815_GC	Los Corralillos	27.883766	-15.45515	GC2	KF670025			
LX1816_GC	Los Corralillos	27.883766	-15.45515	GC2	KF670026			
LX1817_GC	Los Corralillos	27.883766	-15.45515	GC2	KF670027			
LX1160_GC	Cueva Santa Lucía	27.91022	-15.52989	GC3	KF669921		KF670072	KF670108
LX1161_GC	Cueva Santa Lucía	27.91022	-15.52989	GC3	KF669922			
LX1162_GC	Cueva Santa Lucía	27.91022	-15.52989	GC3	KF669923			
LX1163_GC	Cueva Santa Lucía	27.91022	-15.52989	GC3	KF669924			
LX1159_GC	Cueva de las Niñas	27.92462	-15.67237	GC4	KF669920		KF670073	
LX1689_GC	Cueva de las Niñas	27.92462	-15.67237	GC4	KF669989			
LX1690_GC	Cueva de las Niñas	27.92462	-15.67237	GC4	KF669990			
LX1165_GC (4992)	Barranco del Draguillo	27.945994	-15.446264	GC5	KF669926	KF670058	KF670075	KF670110
LX2303_GC	Barranco del Draguillo	27.945994	-15.446264	GC5	KF670029			
LX1702_GC	Montaña Togüela	27.962383	-15.46426	GC6	KF669994			
LX1703_GC	Montaña Togüela	27.962383	-15.46426	GC6	KF669995	KF670059	KF670098	
LX2304_GC	Llanos de la Pez	27.964311	-15.585547	GC7	KF670030			

LX2306_GC	Llanos de la Pez	27.964311	-15.585547	GC7	KF670031	KF670060	KF670105	
LX2307_GC	Llanos de la Pez	27.964311	-15.585547	GC7	KF670032			
LX2308_GC	Llanos de la Pez	27.964311	-15.585547	GC7	KF670033			
LX1696_GC	La Isleta	28.17405	-15.418816	GC11	KF669991			
LX1697_GC	La Isleta	28.17405	-15.418816	GC11	KF669992			
LX1701_GC	La Isleta	28.17405	-15.418816	GC11	KF669993	KF670061	KF670097	KF670126
LX1800_GC	La Isleta	28.17405	-15.418816	GC11	KF670018			
LX1801_GC	La Isleta	28.17405	-15.418816	GC11	KF670019			
LX1802_GC	La Isleta	28.17405	-15.418816	GC11	KF670020			
LX1804_GC	La Isleta	28.17405	-15.418816	GC11	KF670021			
LX1806_GC	La Isleta	28.17405	-15.418816	GC11	KF670022			
***Loxosceles* sp. Gran Canaria 2**								
LX1687_GC	Acusa Seca	28.013833	-15.674466	GC8	KF669988	KF670055		
LX1705_GC	El Risco	28.0601	-15.7296	GC9	KF669997			
LX1706_GC	El Risco	28.0601	-15.7296	GC9	KF669998			
LX1707_GC	El Risco	28.0601	-15.7296	GC9	KF669999	KF670056	KF670099	KF670127
LX1710_GC	El Risco	28.0601	-15.7296	GC9	KF670000			
LX1149_GC	Agaete	28.067428	-15.660415	GC10	KF669917	KF670057	KF670070	KF670106
***Loxosceles* sp. Tenerife 1**								
LX1796_TF	Barranco de las Huertas	28.521333	-16.167816	TF1	KF670017	KF670065		
***Loxosceles* sp. Tenerife 2**								
LX1164_TF	Cumbre Arico	28.249216	-16.528747	TF2	KF669925	KF670064	KF670074	KF670109
***Loxosceles* sp. Tenerife 3**								
LX1155_TF	Los Roques	28.236254	-16.642505	TF3	KF669918	KF670066	KF670071	KF670107
LX1157_TF	Los Roques	28.236254	-16.642505	TF3	KF669919			
***Loxosceles* sp. La Gomera**								
LX1782_GM	Tapagache	28.084133	-17.289016	GM1	KF670007		KF670102	KF670130
LX1784_GM	Tapagache	28.084133	-17.289016	GM1	KF670008			
LX1785_GM	Tapagache	28.084133	-17.289016	GM1	KF670009			
LX1776_GM	Igualero	28.087616	-17.256866	GM2	KF670003	KF670069	KF670101	KF670129
LX1778_GM	Igualero	28.087616	-17.256866	GM2	KF670004			
LX1779_GM	Igualero	28.087616	-17.256866	GM2	KF670005			
LX1781_GM	Igualero	28.087616	-17.256866	GM2	KF670006			
LX1773_GM	Barranco del Paijén	28.08845	-17.19955	GM3	KF670001			
LX1774_GM	Barranco del Paijén	28.08845	-17.19955	GM3	KF670002	KF670067	KF670100	KF670128
LX1789_GM	Playa de Ávolo	28.114683	-17.113166	GM4	KF670010			
LX1790_GM	Playa de Ávolo	28.114683	-17.113166	GM4	KF670011			
LX1791_GM	Playa de Ávolo	28.114683	-17.113166	GM4	KF670012			
LX1792_GM	Playa de Ávolo	28.114683	-17.113166	GM4	KF670013			
LX1793_GM	Playa de Ávolo	28.114683	-17.113166	GM4	KF670014			
LX1794_GM	Playa de Ávolo	28.114683	-17.113166	GM4	KF670015			
LX1795_GM	Playa de Ávolo	28.114683	-17.113166	GM4	KF670016	KF670068	KF670103	KF670131
LX2292_HI	Cueva del Linke	27.653012	-17.981626	HI1	KF670028			
Loxosceles rufescens								
LX1068_GC	Firgas	28.107403	-15.563147		KF716999	KF717016	KF716986	KF717028
LX1599_GC	Subida San Felipe	28.137199	-15.58452		KF717009		KF716994	KF717036
LX1797_TF	Buenavista	28.376966	-16.848883		KF717010	KF717019	KF716995	KF717037
LX1593_HI	Cueva de la Curva	27.692348	-17.972877		KF717008	KF717018	KF716993	KF717035
LX1180_LP (2462/AN)	Cueva del Ratón	28.468916	-17.850893		KF717000	KF717015	KF716987	KF717029
LX1188_LP (2460/AN)	Cueva del Diablo				KF717001			
LX1468_MD	Porto Santo, Moledo				KF717005	KF717013		
LX1469_MD	Porto Santo, Golf				KF717006	KF717014	KF716991	KF717033

CODE	LOCALITY	LATITUDE	LONGITUDE	LN	*COI*	*16S*	*H3*	ITS2
LX1351_PI	Sagunt	39.68481	-0.30005		KF717003	KF717017	KF716989	KF717031
LX1330_PI	Cabo de Gata	36.7285	-2.19096		KF717002	KF717020	KF716988	KF717030
LX1376_PI	Vilamarxant	39.53622	-0.62619		KF717004	KF717021	KF716990	KF717032
***Loxosceles* aff. *rufescens* Anti-Atlas**								
LX1015_MA	Aguerka, Meraveilles Cave	30.296733	-8.4744		KF716996	KF717011	KF716982	KF717024
LX1016_MA	Bouizakarne, Tleta-Akhssas Cave	29.248983	-9.74345		KF716997	KF717022	KF716983	KF717025
LX1494_MA	Tizergzaouine	29.84582	-8.93754		KF717007	KF717012	KF716992	KF717034
Loxosceles mrazig								
LX1051_TN	Bou Onvane, near Gafsa	34.343363	9.073509		KF716998	KF717023	KF716984	KF717026
LX1054_TN	Douz	33.407436	9.044977		FJ986179	GQ279173	KF716985	KF717027
Loxosceles foutadjalloni								
07050101L01					GQ279238	GQ279179		
07050201L03					GQ279242	GQ279177		
***Loxosceles amazonica* group**								
Loxosceles amazonica					EU817674	EU817813		
***Loxosceles gaucho* group**								
Loxosceles variegata					EU817675	EU817797		
***Loxosceles spadicea* group**								
Loxosceles hirsuta					EU817678	EU817805		
Loxosceles spadicea					EU817677	EU817804		
***Loxosceles laeta* group**								
Loxosceles laeta					EU817680	EU817794		
Loxosceles sp. 3 GJB-2008					EU817658	EU817809		
Loxosceles sp. 4 GJB-2008					EU817679	EU817811		
***Loxosceles reclusa* group**								
Loxosceles apachea					EU817665	EU817793		
Loxosceles arizonica					EU817663	EU817798		
Loxosceles baja					EU817661	EU817792		
Loxosceles blanda					EU817664	EU817818		
Loxosceles caribbaea					EU817659	EU817819		
Loxosceles chinateca					EU817670	EU817802		
Loxosceles colima					EU817668	EU817800		
Loxosceles deserta					EU817667	EU817799		
Loxosceles kaiba					EU817662	EU817808		
Loxosceles reclusa					EU817669	EU817801		
***Loxosceles vonwredei* group**								
Loxosceles sp. 5 GJB-2008					EU817682	EU817806		
L. vonwredei					EU817681	EU817814		

Appendix S2 Partition schemes used for the analysis of the spider genus *Loxosceles* (Arachnida: Sicariidae) from the Canary Islands. (a) Data blocks and (b) partition schemes specified in PARTITIONFINDER 1.0.1 (Lanfear *et al.*, 2012). The selected partition scheme under the Bayesian information criterion is shown in bold. (c) Best-fit substitution model for each partition in the selected partition scheme used in the phylogenetic analyses. COI_c1-c2, first and second codon position of the cytochrome *c* oxidase subunit I (*COI*); COI_c3, third codon position of the cytochrome *c* oxidase subunit I (*COI*); 16S, fragment spanning the 3′ half of the large ribosomal (r) unit (16S rRNA); tRNA, the transfer (t)RNA leucine (*L1*); NADH_c1-c2, first and second codon position of the 5′ end of NADH dehydrogenase (*nad1)*; NADH_c3, third codon position of the 5′ end of NADH dehydrogenase (*nad1)*; H3_c1-c2, first and second codon position of the nuclear gene histone 3 (*H3*); H3_c3, third codon position of the nuclear gene histone 3 (*H3*); ITS2, the internal transcribed spacer 2 (ITS2).

(a) DATA BLOCKS

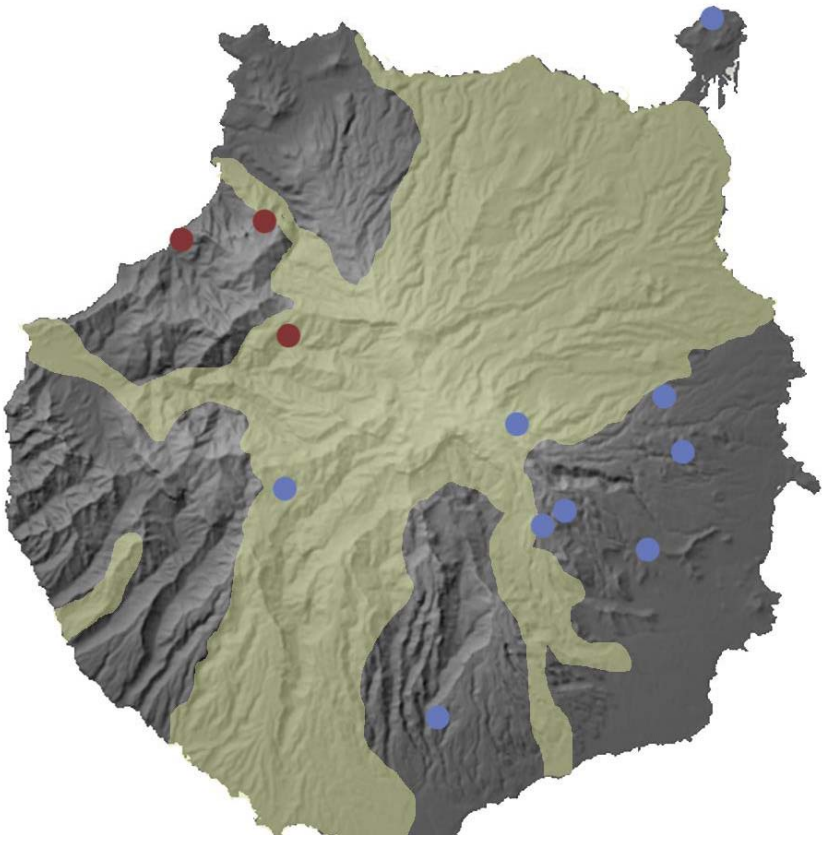
COI_c1-c2 = 1-942\3 2-942\3;
COI_c3 = 3-942\3;
16S = 943-1489;
tRNA = 1490-1546;
NADH_c1-c2 = 1547-1600\3 1548-1600\3;
NADH_c3 = 1549-1600\3;
H3_c1-c2 = 1601-1927\3 1602-1927\3;
H3_c3 = 1603-1927\3;
ITS2 = 1928-2269;

(b) SCHEMES
one = (COI_c1-c2, COI_c3, 16S, tRNA, NADH_c1-c2, NADH_c3, H3_c1-c2, H3_c3, ITS2);
two = (COI_c1-c2, COI_c3, 16S, tRNA, NADH_c1-c2, NADH_c3) (H3_c1-c2, H3_c3, ITS2);
three = (COI_c1-c2, COI_c3, NADH_c1-c2, NADH_c3) (16S, tRNA) (H3_c1-c2, H3_c3) (ITS2);
four = (COI_c1-c2, NADH_c1-c2) (COI_c3, NADH_c3) (16S, tRNA) (H3_c1-c2) (H3_c3) (ITS2);
five = (COI_c1-c2) (NADH_c1-c2) (COI_c3) (NADH_c3) (16S, tRNA) (H3_c1-c2) (H3_c3) (ITS2);
six = (COI_c1-c2, NADH_c1-c2) (COI_c3, NADH_c3) (16S, tRNA) (H3_c1-c2, H3_c3) (ITS2);
seven = (COI_c1-c2) (COI_c3) (NADH_c1-c2, NADH_c3) (16S, tRNA) (H3_c1-c2, H3_c3) (ITS2);
eight = (COI_c1-c2, NADH_c1-c2, NADH_c3) (COI_c3) (16S, tRNA) (H3_c1-c2, H3_c3) (ITS2);
nine = (COI_c1-c2, NADH_c1-c2, NADH_c3, 16S, tRNA) (COI_c3) (H3_c1-c2, H3_c3) (ITS2);
ten = (COI_c1-c2, NADH_c1-c2, NADH_c3, 16S, tRNA) (COI_c3) (H3_c1-c2, H3_c3, ITS2);
eleven = (COI_c1-c2, COI_c3) (NADH_c1-c2, NADH_c3) (16S, tRNA) (H3_c1-c2, H3_c3) (ITS2);

(c)

Partition (scheme ten)	Length (bp)	Substitution model (RAxML)	Substitution model (MrBayes)
p1	1286	GTR + I + G	GTR + I + G
p2	314	GTR + G	GTR + G
p3	669	GTR + I + G	K80 + I + G

Appendix S3 Map of Gran Canaria showing the lava flows (in yellow) of the Roque Nublo volcanic phase (5–3 Ma; modified from Fig. 14.2 of Marrero & Francisco-Ortega, 2002, and Fig. 1b of Anderson *et al.*, 2009). Coloured circles represent the localities of the two endemic lineages: purple, *Loxosceles* sp. Gran Canaria 1; blue, *Loxosceles* sp. Gran Canaria 2.



Descripció de sis noves espècies de *Loxosceles* (Araneae: Sicariidae) endèmiques de les Illes Canàries, i la utilitat del codi de barres genètic per la seva ràpida i acurada identificació

RESUM

Hem realitzat un estudi taxonòmic integratiu de la radiació d'aranyes del gènere *Loxosceles* endèmiques de les Illes Canàries combinant dades moleculars (ADN mitocondrial i nuclear) i morfològiques, i hem procedit a la descripció formal de sis noves espècies: ***Loxosceles mahan* sp. nov.** endèmica de Fuerteventura, Lanzarote, i illots adjacents; ***Loxosceles bentejui* sp. nov.** i ***Loxosceles tazarte* sp. nov.** ambdues endèmiques de Gran Canària; ***Loxosceles guayota* sp. nov.** i ***Loxosceles tibicena* sp. nov.** ambdues endèmiques de Tenerife; i ***Loxosceles hupalupa* sp. nov.** endèmica de La Gomera i El Hierro. Aquestes noves espècies s'inclouen en el grup d'espècies *rufescens*, i es distingeixen clarament de *L. rufescens* per una conspícua marca negra amb forma de V a la part posterior de la pars cefàlica, per la llargada de l'èmbol i per la forma dels receptacles seminals. Ja que un pas crucial en el desenvolupament d'una òptima gestió mèdica en accidents provocats per picades d'aranyes és la correcta identificació de l'aranya involucrada, hem testat l'eficàcia del codi de barres genètic com una eina ràpida i fiable per a la identificació de les espècies d'aranyes *Loxosceles* que es troben a les Illes Canàries, incloent-hi l'espècie introduïda pels humans *L. rufescens*

PARAULES CLAU: aràcnids, artròpodes, elongació d'extremitats, loxoscelisme, Macaronèsia, aranyes d'importància mèdica, delimitació d'espècies, aranya, taxonomia.

Description of six new species of *Loxosceles* (Araneae: Sicariidae) endemic to the Canary Islands, and the utility of DNA barcoding for their fast and accurate identification

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ABSTRACT

We conducted an integrative taxonomic study of a radiation of *Loxosceles* spiders endemic to the Canary Islands combining molecular (mtDNA and nDNA) and morphological data, and proceed to the formal description of six new species: ***Loxosceles mahan* sp. nov.** endemic to Fuerteventura, Lanzarote, and adjacent islets; ***Loxosceles bentejui* sp. nov.** and ***Loxosceles tazarte* sp. nov.** both endemic to Gran Canaria; ***Loxosceles guayota* sp. nov.** and ***Loxosceles tibicena* sp. nov.** both endemic to Tenerife; and ***Loxosceles hupalupa* sp. nov.** endemic to La Gomera and El Hierro. These new species are included in the *rufescens* group, and are clearly distinguished from *L. rufescens* by a conspicuous dark V-mark posteriorly on pars cephalica, by the embolus length and by the shape of seminal receptacles. Given that a crucial step for the development of proper health management is the correct identification of the species involved in bite accidents, we additionally tested the efficacy of DNA barcoding as a fast and reliable tool for identifying the *Loxosceles* species found in the Canary Islands, including the human-introduced *L. rufescens*.

KEYWORDS: arachnids, arthropods, leg elongation, loxoscelism, Macaronesia, medically important spiders, species delimitation, spider, taxonomy.

INTRODUCTION

The importance of oceanic islands as natural laboratories for evolutionary studies has been acknowledged by Darwin and Wallace (Wallace, 1855; Darwin, 1859). Since then, numerous evolutionary studies conducted in islands, especially in oceanic islands such as the Galapagos, the Hawaii archipelago or the Canary Islands, have served to uncover

fascinating processes and patterns that ultimately led to general theories such as "The theory of island biogeography" (MacArthur & Wilson, 1967; Wagner & Funk, 1995; Whittaker *et al.*, 2008). To achieve these objectives, biologists generally have relied on species as the basic units on which to conduct evolutionary studies and to elucidate general patterns (de Queiroz, 2005). Thus, a complete inventory of rigorously delineated species is an essential first step (Dayrat, 2005).

With more than 500 spp in the Canary Islands (Macías, 2010), spiders are among the most diverse and well-known groups of arthropods in the archipelago (Cardoso *et al*., 2010). Thus, the recent discovery of an endemic Canarian clade of *Loxosceles* Heineken & Lowe, 1832 spiders, distributed across the archipelago was an exceptional case (Fig. 1; Planas & Ribera, 2014). *Loxosceles* comprises 107 species (Platnick, 2014) distributed predominantly in temperate and tropical areas in North, Central and South America (Gertsch, 1967; Gertsch & Ennik, 1983; Binford *et al*., 2008), although some species are located in Africa (Lotz, 2012) and two in the Mediterranean basin (Ribera & Planas, 2009; Duncan *et al*., 2010). This diversity is strongly biased towards those areas where extensive taxonomic revisions have been conducted (e.g., most of the American *Loxosceles* species have been described in three monographs; Gertsch 1958; 1967; Gertsch & Ennik, 1983), and recent studies based on molecular evidence have suggested the existent diversity could be highly underestimated (Binford *et al.*, 2008; Duncan *et al*., 2010; Planas & Ribera, 2014). One of the factors that has hampered a comprehensive view of the present diversity is the subtle variation in genitalia characters. This difficulty was noted by Brignoli in the Italian and Maltan revision of the genus (Brignoli, 1969), when he stated that "*...è evidente che la tassonomia dei Loxosceles è di singolare difficoltà...*" [it is evident that the taxonomy of *Loxosceles* is singularly difficult] (Brignoli, 1969: 142), and by Gertsch and Ennik, who concluded that in *Loxosceles*, "*... degrees of difference in genitalic characters are less great or perhaps less easy to describe verbally or pictorially than those of some other spiders...*"(Gertsch & Ennik, 1983: 273), notwithstanding that they had described most of the currently accepted *Loxosceles* species.

Nevertheless, these difficulties are not exclusive to *Loxosceles*, and systematists working on morphologically homogeneous spider groups (e.g., mygalomorphs) have encountered similar obstacles (e.g., Hamilton *et al*., 2014 and references therein). Thus, it is especially in these "cryptic" species groups *sensu* Bickford *et al*. (2007) (i.e., two or more species

classified as a single nominal species because they are at least superficially indistinguishable morphologically) where the usage of DNA-based methods has been successful in uncovering the existent diversity, and in delimiting species boundaries. Currently, it is generally accepted that evidence from multiple and complementary disciplines (e.g., additional molecular markers, ecological information, morphological characters) should be integrated when possible to improve our level of confidence when formalising species hypothesis (Dayrat, 2005; Padial *et al*., 2010). In this study, we followed this reasoning and provided additional sets of data (i.e., morphological and nuclear molecular markers (nDNA)) to test the taxonomic status of the Canarian endemic *Loxosceles* evolutionary lineages previously delimited with mtDNA markers (Planas & Ribera, 2014).

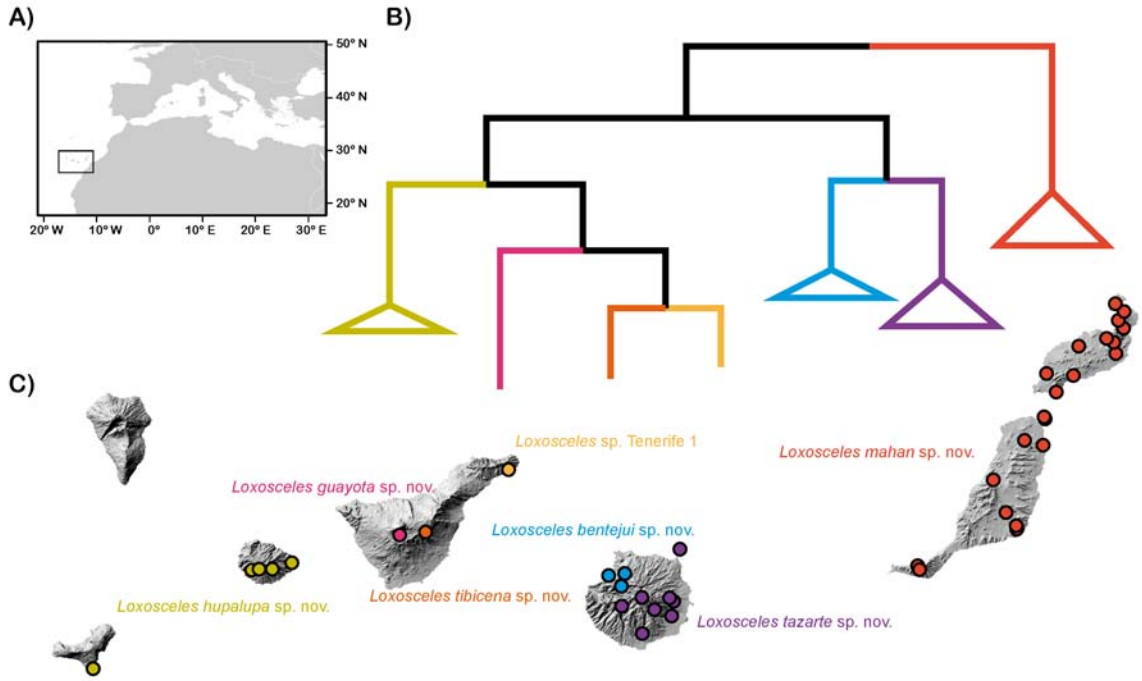

Figure 1 A) Geographical position of the Canary Islands, indicated by the inset box. **B)** Phylogenetic relationships between the Canary Islands endemic *Loxosceles* species; lineages with more than one individual are collapsed (see Planas and Ribera, 2014 for further information). **C)** Sampling localities of the Canary Islands endemic *Loxosceles*, colours correspond to B.

DNA barcoding was first presented as a fast and accurate method for species identification (Hebert *et al*., 2003), although additional goals, including species delimitation, were rapidly proposed (Hebert *et al*., 2004). Although these two aims (i.e.,

species identification and species delimitation or species discovery) are justifiable, the confusion between the objectives, methods and interpretations of them have provoked major criticism (Collins & Cruickshank, 2013 and references therein). Despite the limitations of this methodology, the effectiveness of DNA barcoding for identification was proven to be reliable in different groups, including spiders (Barret & Hebert, 2005; Astrin *et al*., 2006; Robinson *et al*., 2009; Huber, Fischer & Astrin, 2010; Hamilton, Formanowicz & Bond, 2011) and the potential identification capacity attracted attention from multiple and diverse fields such as border biosecurity (Armstrong & Ball, 2005), food consumption (Barbuto *et al*., 2010), forensics (Dawnay *et al*., 2007) and clinical diagnosis (Bruni *et al*., 2010). This methodology could also be a great aid in identifying spiders of medical relevance, such as *Loxosceles*. Although *Loxosceles* bites are scarce (Vetter & Barger, 2002), they can produce serious dermonecrotic lesions, a condition medically known as loxoscelism (Swanson & Vetter, 2005). However, misidentification of biting spiders could over-diagnose loxoscelism (Vetter, 2003) and complicate further understanding of its medical importance. Thus, providing a reliable DNA barcode reference library with tested efficacy would facilitate this first step in the correct management of loxoscelism.

The aim of this study was to conduct an integrative taxonomic revision of the Canarian endemic *Loxosceles* spiders. We examined nDNA sequences and morphology to evaluate the distinctiveness of the highly divergent evolutionary lineages delimited with mtDNA data in a previous study (Planas & Ribera, 2014). The results confirmed the existence of six new species that are formally described herein. Additionally, we evaluated the use of DNA barcoding for a reliable identification of the different *Loxosceles* species found in the Canary Islands, including the human-introduced *L. rufescens* (Dufour, 1882), and discussed the utility of this tool in the health management of medically important spider species.

MATERIAL AND METHODS

Studied material

Most of the *Loxosceles* specimens were collected during field trips to the Canary Islands between 2009 and 2012 and were complemented with material borrowed from the Arachnida collection of the Zoological Department of the University of La Laguna, Tenerife (ULL-DZUL) and from the Senckenberg Museum, Frankfurt am Main (SMF). All new material is currently deposited in the Arachnid Collection of the Centre de Recursos de

Biodiversitat Animal (CRBA) at the University of Barcelona (UB) but will later be partly transferred to the other two aforementioned institutions.

Morphological study

A total of 40 adult specimens were examined following Ribera & Planas (2009). All morphological measurements are given in millimetres. We measured the maximum carapace length (CL) and the maximum carapace width (CW) in dorsal position. Leg article (excluding coxa and trochanter) lengths were measured in lateral view along the dorsal margin for the four legs (i.e., Leg 1 to Leg 4). Leg formula represents the numbered legs arranged in a descending order, with legs differentiated by less than 0.5 mm underlined, following Gertsch (1967). We measured palps of all males in lateral view, except the tibia and tarsus, which were also measured in dorsal view. We took additional measurements from holotypes and female paratypes including pars cephalica length (PCL) and width (PCW); eye largest diameters (AME, anterior median eyes; ALE, anterior lateral eyes; APE, anterior posterior eyes), taken from the spans of the lens; lateral (LEL, LER; left and right, respectively) and central eyes dyads (CE) and the distance between them (LEL-CE, LER-CE); and clypeus height and abdomen length.

Statistical analyses

We graphically explored the sample variability of the ratio leg length / CL between species and sex. According to the statistical significance of correlations between leg lengths (Pearson $r > 0.97$, $p < 0.0001$) further analyses were conducted considering Leg 1. Sexual dimorphism and differences between species regarding Leg 1 and CW were investigated using an Analysis of Covariance (ANCOVA), treating CL as a covariate, to reduce the effect of the body size. The ANCOVA assumption of the homogeneity of slopes was corroborated by testing the interaction between the dependent variable and CL. Furthermore, assumptions of normality and homoscedasticity were evaluated with Shapiro-Wilk's and Levene's test, respectively. Post-hoc Tukey tests were conducted to compare species pairs adjusting for multiple comparison with the single-step method. Differences between species in male palp segments (femur length (PFL), patella length (PPL), tibia length (PTL) and tarsus length (PTAL)) were investigated using multiple Analyses of Covariance, with CL as a covariate, and testing for ANCOVA assumptions as above. All statistical analyses and plots were conducted in R (R core team, 2014).

Molecular analyses

Cytochrome oxidase 1 (*cox1*) sequences for the Canarian endemic *Loxosceles* species, including most of the DNA barcode region, were obtained from GenBank based on a previous study (Planas & Ribera, 2014). Similarly, the internal transcribed spacer 2 (its2) sequences were retrieved from Genbank and complemented with 35 additional sequences obtained by following the same procedures as in the aforementioned study and deposited in Genbank (accession number XXX to XXX; Supplementary Table 1). We performed sequence alignments as in Planas & Ribera (2014) and conducted statistical parsimony networks of the its2 dataset in TCS 1.3 (Clement, Posada & Crandall, 2000), applying the 95% threshold limit and treating gaps as missing data.

DNA Barcoding

We constructed a neighbor-joining (NJ) phenogram in MEGA v. 6 (Tamura *et al*., 2013) using the Kimura-2-Parameter (K2P) and pairwise deletion. We used this method as it is widely used in DNA Barcoding studies because it is fast, computationally simple and definitive (Goldstein & DeSalle, 2011) and we used it to graphically represent species divergences and not for phylogenetic inference (see Planas & Ribera, 2014). DNA barcoding efficacy should be tested using independent data against a reference library; to simulate this scenario, we created a reference library using all available information (i.e., all *cox1* sequences) and computed different identification success statistics (see below) treating each individual as an unknown query (Brown *et al*., 2012). Different metrics have been proposed for identifying species using DNA barcodes (Meier *et al*., 2006), and we focused on two widely used methods based on distance measures because they performed better than tree-based methods (van Velzen *et al*., 2012). We used (1) the nearest neighbour criterion (NN) to assign the query sequence to the same species as its closest sequence in the reference library, and (2) the Barcode of Life Data System (BOLD; www.barcodinglife.org) threshold criteria of 1%, which assigned the query sequence to the same species as the sequences with less than 1% divergence, as implemented in the R package SPIDER (Brown *et al*., 2012). We obtained the threshold distance that minimised the identification error rates (i.e., false negative, false positive) with the threshold optimization function *threshOpt* in SPIDER testing 50 thresholds from 0.1 % to 15 %. The presence of a "barcoding gap" was checked graphically by plotting the maximum intraspecific K2P genetic distance to the smallest interspecific K2P genetic distance. Finally

we depicted the *cox1* variation using the program Fingerprint (Lou & Golding, 2007) and obtained diagnostic nucleotides for the species described herein and *L. rufescens* using the function *nucDiag* in the R package SPIDER. The reference *cox1* aligned matrix and the NJ tree is available for download at TreeBASE (http://treebase.org; accession number: 15746).

RESULTS

Taxonomy

Family **Sicariidae** Keyserling, 1880

Genus ***Loxosceles*** Heineken & Lowe, 1832

***Loxosceles mahan* sp. nov.**

Figs. 2-3

Loxosceles sp. FV-LZ (Planas & Ribera, 2014)

Holotype: 1♂**,** CRBA-LX1428 (MorphoBank: M326598-M326603), Costa Papagayo, Playa Blanca - Barranco de los Pilos, Lanzarote, Spain, 28.86038 N 13.77626 W, 69 m asl., 06.III.2010, V. Opatova & E. Planas leg.

Paratypes: 2♀, CRBA-LX1430, -LX1431 (MorphoBank: M326604-M326608), same locality and data as holotype; 1♂, SMF-29348, Costa Papagayo, XI.1972, Schmidt leg.

Other material examined: Lanzarote: 1♂, CRBA-LX1425, Salinas de Janubio, 28.9429 N 13.8189 W, 28 m asl., 06.III.2010, Opatova, V. & Planas, E. leg.; 1♂, CRBA-LX1465, Peña Negra, Tinajo, 29.0614 N 13.6777 W, 237 m asl., 09.III.2010, Opatova, V. & Planas, E. leg.; 2♀, CRBA-LX1440, -LX1441, Lomo Guantesivi, Los Valles, Teomise, 29.0791 N 13.5249 W, 317 m asl., 07.III.2010, Opatova, V. & Planas, E. leg.; 3♀, CRBA-LX1448, -LX1449, -LX1450, Mirador del Río, Yé, 29.2134 N 13.4811 W, 417 m asl., 07.III.2010, Opatova, V. & Planas, E. leg.; 1♀, CRBA-LX1456; **Fuerteventura:** 1♂, ULL-DZUL-34172, Montaña de Vallebrón, 03.IV.2004, GIET leg.; 2♀, CRBA-LX1404, -LX1405, Cueva de Tiscamanita, Tiscamanita, 28.3350 N 13.9953 W, 178 m asl., 03.III.2010, Opatova, V. & Planas, E. leg.; 3♀, CRBA-LX1412, -LX1413, -LX1414, Malpais de Villaverde, Villaverde, 28.6506 N 13.9152 W, 174 m asl., 04.III.2010, Opatova, V. & Planas, E. leg.; **La Graciosa:** 2♂ CRBA-LX2300, -LX2301, 24.II.1995, C. Ribera leg.; **Lobos:** 2♀, CRBA-LX1037, -LX1037-2, Cresta de la Caldera, 30.III.2004; 2♀, CRBA-LX1418, -LX1419, Playa de la Concha, 28.7439 N 13.8254 W, 13m., 05.III.2010, Opatova,

V. & Planas, E. leg.

Etymology: The specific epithet is a noun in apposition that refers to the single volcanic edifice that includes the current islands of Lanzarote and Fuerteventura, and the surrounding islets of Lobos, La Graciosa, Montaña Clara and Alegranza.

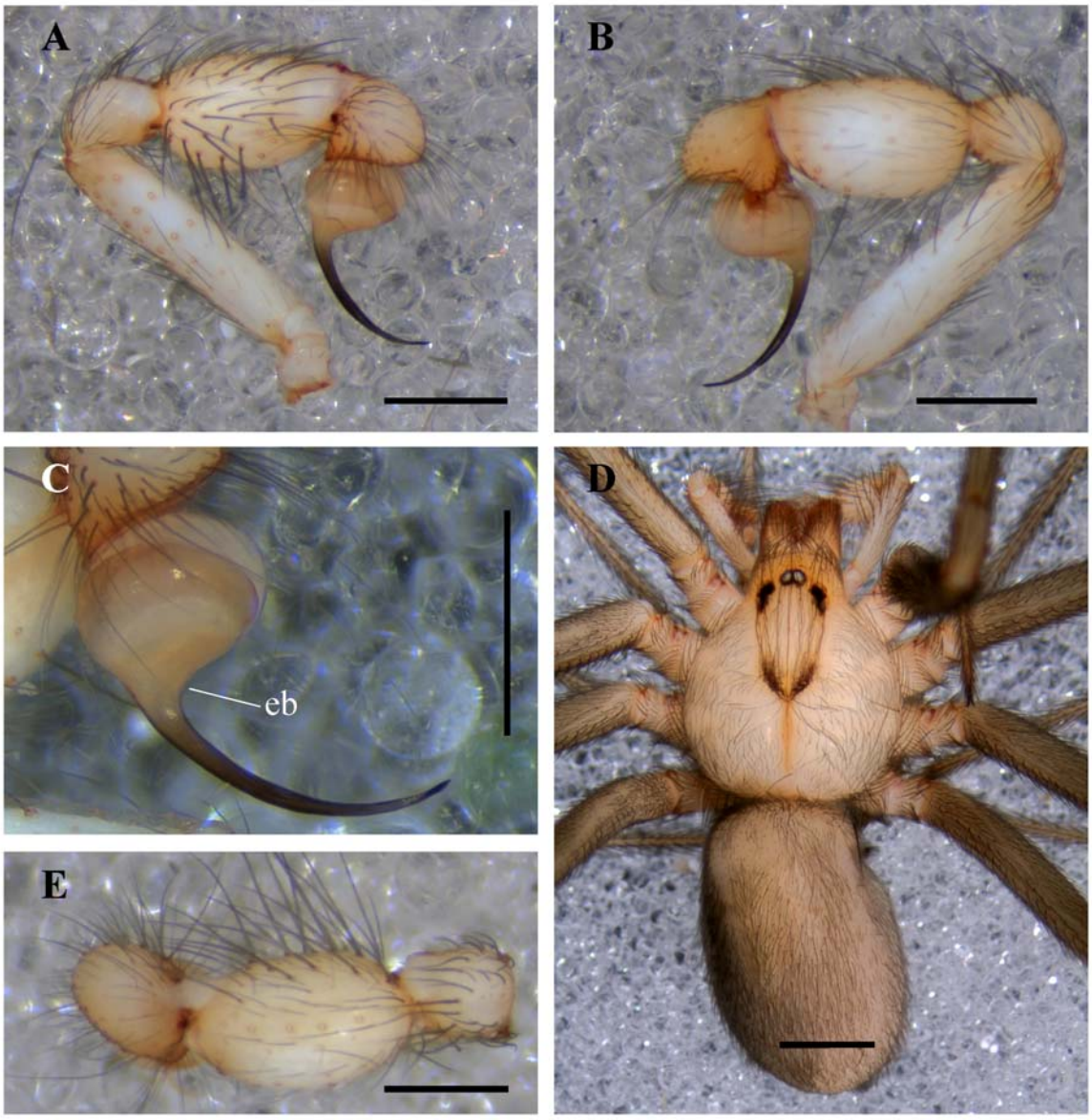

Figure 2 ♂ ***Loxosceles mahan* sp. nov. A)** palp, prolateral view. **B)** palp, retrolateral view. **C)** bulb and embolus. **D)** cephalothorax, dorsal view. E: palp, dorsal view. Abbreviation: eb, embolus base. Scale bars: A, B, C, E: 0.5 mm; D: 1 mm.

Diagnosis: Differs clearly from *L. rufescens* by conspicuous dark V-mark posteriorly on pars cephalica (Fig. 2 D), proportion of the embolus, markedly shorter in *L. rufescens* (Fig. 2; Gertsch, 1967: plates 3-4), and shape of seminal receptacles (Fig. 3). It is also easily

distinguished from *L. mrazig* by male palp tibia, which is distinctly oval, slightly longer than wide in *L. mrazig*. Males of ***Loxosceles mahan* sp. nov.** can be distinguished from the remaining Canary Island endemic species by the long, gently curved embolus that is straight at the tip (Fig. 2 A-C). Females differ from the remaining Canary Island endemic species by having seminal receptacles with a long medium part ended in a single lobe. Both sexes also can be distinguished by the leg formula (Supplementary Table 2) and relatively shorter legs (Fig. 13). ***Loxosceles mahan* sp. nov.** can be distinguished from the remaining Canary Island endemic species and from *L. rufescens* by five (465 (G/A); 466 (C); 468 (T); 774 (A); 852 (T)) *cox1* diagnostic nucleotide changes based on the alignment deposited in TreeBASE (accession number 15746) (Supplementary Fig. 1).

Description: Male (holotype). **Specimen preparation and condition:** Specimen collected alive and preserved in ethanol 96º. Left pedipalp removed and conserved in a vial with specimen. **Colouration:** Carapace pale yellowish, clypeus and median groove slightly darkened. Conspicuous dark V-mark posteriorly on pars cephalica (Fig. 2 D). Carapace with dispersed short black setae. Eye tubercles black. Chelicerae reddish brown, darkened in its distal part. Sternum bright yellowish, paler than carapace. Labium and gnathocoxae pale reddish brown. Legs pale yellowish with the apical segments slightly darkened. Sternum, labium, gnathocoxae and legs covered by long setae interspersed with shorter and thicker setae. Abdomen pale yellowish to greyish, densely coated by short setae. **Cephalothorax:** Carapace slightly longer (2.89) than wide (2.52), truncated behind, widely rounded on sides, narrowed in front. Carapace evenly convex, with median groove moderately deep, elongated, occupying roughly the posterior third. Pars cephalica elongated (1.72 long, 0.8 wide). Larger setae or bristles placed in a single row in the side margin of the carapace pointing anteriorly. Similar setae forming seven rows in the pars cephalica, interspersed with shorter setae. Clypeus height 0.29. Eye sizes: ALE 0.18 PME 0.12 PLE 0.18. LE separated from ME by narrow diameter of ME. Sternum longer (1.53) than wide (1.38). **Abdomen:** Elongate oval (3.47 long 2.24 wide) in dorsal view. **Legs:** Leg formula 2 4 1 3. Leg 1 (17.37): femur 4.81/patella 1/tibia 5.32/metatarsus 5.1/tarsus 1.14; Leg 2 (20.36): 5.4/1.04/6.24/6.54/1.14; Leg 3 (15.3): 4.35/0.92/4/4.93/1.1; Leg 4 (17.46): 4.84/0.85/4.61/5.86/1.3. **Male palp:** (Fig. 2 A-C, E) Femur cylindrical (1.32 long, 0.27 wide). Patella subglobular, roughly as long as wide (0.42). Tibia wider at the base (0.83 long, 0.52 wide), rounded more abruptly towards the patella than the tarsus, oval in dorsal

view. Tarsus short, as large as bulb (0.43). Bulb globular, slightly compressed dorsally. Maximum bulb width four times wider than embolus base. Embolus long and steadily curved, approximately 1.8 times longer than bulb width. Long, curved setae facing apically, distributed sparsely in femur, tibia and tarsus, denser on retrolateral side. **Variation:** Supplementary Table 2. Leg 1 (n = 6): 13.88-18.46 (mean: 16.52); leg 2 (n = 5): 16.07-20.85 (mean: 18.80); leg 3 (n = 6): 13.02-16.10 (mean: 14.86); leg 4 (n = 6): 14.91-18.22 (mean: 16.84); CL (n = 6): 2.38-3.05 (mean: 2.77); CW (n = 6): 2.14-2.77 (mean: 2.5). General colouration ranges from pale-yellow to darker yellow-orange, depending on the time from the last moult. The shape of the dark V-mark also presents some variation in intensity and extent.

Female (Paratype, CRBA-LX1431). **Specimen preparation and condition:** Specimen collected live and preserved in ethanol 96º. Genitalia removed and conserved in a vial with specimen. Legs 3 right and 1 and 4 left removed. **Colouration:** Similar as in male but slightly darker. Carapace yellow-orange, with pars cephalica slightly darkened. Palps and chelicerae reddish. **Cephalothorax:** Carapace longer (2.97) than wide (2.43), truncated behind, widely rounded on sides, less narrowed in front than males. Carapace evenly convex, with median groove moderately deep, elongated, occupying roughly the posterior third. Pars cephalica elongated (1.87 long, 0.9 wide) and wider than in males. Clypeus height 0.36. Eyes sizes: ALE 0.18 PME 0.13 PLE 0.18. LE separated from ME by 0.16. Sternum longer (1.54) than wide (1.16). **Abdomen:** Elongate oval (4.06 long 2.42 wide) in dorsal view. **Legs:** Leg formula 2 4 1 3. Leg 1 (12.97): femur 3.76/patella 0.93/tibia 3.72/metatarsus 3.56/tarsus 1; Leg 2 (14.06): 4.01/0.98/4.07/4.11/0.89; Leg 3 (12): 3.59/0.93/3.03/3.52/0.93; Leg 4 (13.94): 4/0.97/3.64/4.27/1.06. **Female genitalia:** (Fig. 3) Atriobursal orifices situated in two large, rounded and almost contiguous pouches, slightly sclerotised around. Base of seminal receptacles conical and wide, reinforced laterally with a curved, dark, sclerotised band. Medium part long, cylindrical and gently directed towards the centre. Apical part strongly curved, pointing dorsally. Seminal receptacles not touching each other, separated at their closer point by a distance of roughly the diameter of the apical part. **Variation:** Supplementary Table 2. Leg 1 (n = 6): 10.69-22.86 (mean: 14.81); leg 2 (n = 6): 12.27-25.19 (mean: 16.18); leg 3 (n = 6): 10.38-20.64 (mean: 13.61); leg 4 (n = 6): 12.17-24.03 (mean: 15.75); CL (n = 5): 2.87-4.29 (mean: 3.24); CW (n = 5): 2.43-3.58 (mean: 2.76). Similar range of general colouration variation as in males. Genital

morphology presents little variability in males, and females with some differences in the direction of the apical curvature of sperm receptacles, pointing dorsally.

Distribution and natural history: *Loxosceles mahan* sp. nov. is endemic and exclusive to the easternmost Canary Islands of Lanzarote and Fuerteventura, and islets of Lobos, La Graciosa and Montaña Clara (Fig. 1). Found in a variety of habitats, from humid localities, such as in Mirador del Río (Lanzarote), to xeric areas such as Península Jandía (Fuerteventura). Common under big stones, usually lying above a dense whitish-blue web.

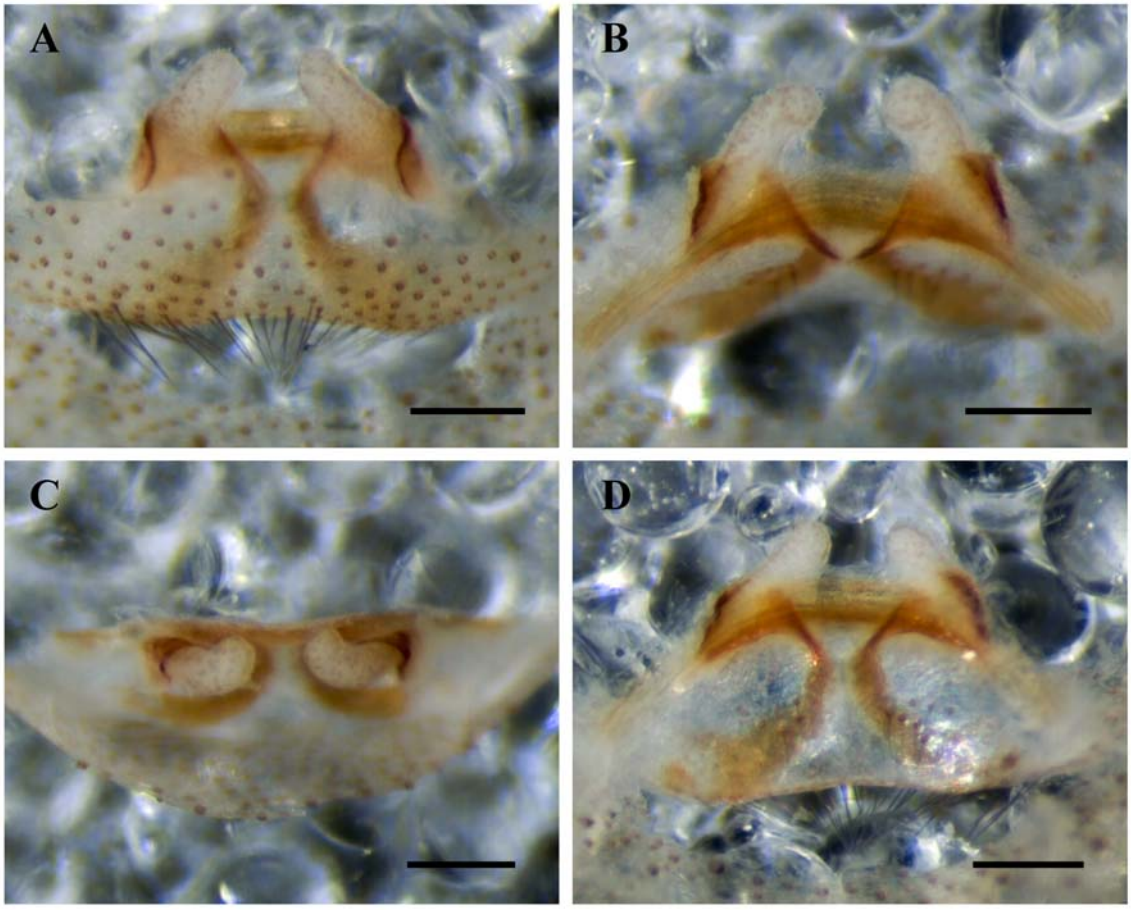

Figure 3 ♀ ***Loxosceles mahan* sp. nov.** Vulva, **A)** ventral view. **B)** dorsal view. **C)** anterior view. **D)** posterior view. Abbreviations: b, base of seminal receptacle. m, medium part of seminal receptacle. a, apical part of seminal receptacle. Scale bars: 0.2 mm.

***Loxosceles tazarte* sp. nov.**

Loxosceles sp. GC-1 (Planas & Ribera, 2014)

Figs 4-5

Holotype: 1♂, ULL-DZUL-34177 (LX1165) (MorphoBank: M326610-M326615), Barranco del Draguillo, Gran Canaria, 27.94599 N 15.44626 W, 28.XII.2008, Macías, N. leg.

Paratypes: 1♀, ULL-DZUL-34181 (LX2302) (MorphoBank: M326616-M326621), 1♂ ULL-DZUL-34182 (LX2303), same locality as holotype, 26.XII.2010, López, H. leg.

Other material examined: Gran Canaria: 1♂ ULL-DZUL-34180, Los Majaletes - Cazadores, 13.X.2003, López, H. leg.; 1♂, CRBA-LX1818, Los Corralillos, 27.88377 N 15.45515 W, 140 m asl, 27.II.2011, Opatova, V. & Planas, E.; 1♀, CRBA-LX1160, Cueva Santa Lucía, 27.91022 N 15.52989 W, 868 m asl, 25.IV.2009, Espluga, R. & Planas, E. leg.; 1♂, CRBA-LX1688, 1♀, CRBA-LX1689, Cueva de las Niñas, 27.92462 N 15.67237 W, 928 m asl., 12.XII.2012, Araya, M. & Planas, E. leg.; 4♀, ULL-DZUL-34184, -34185 (LX2306), -34186 (LX2307), -34187 (LX2308), Llanos de la Pez, Tejeda, 27.964311 N 15.58555 W, 31.III.2012, de la Cruz, S. & Macías, N.; 1♂, ULL-DZUL-34178, Cabecera Valle Temisa, 29.90928 N 15.51331 W, 28.XI.2004, Castro, R. leg.; 1♂, CRBA-LX1692, 1♀, CRBA-LX1693, La Isleta, 28.17405 N 15.41882 W, 251 m asl, 13.XII.2010, Araya, M. & Planas, E..

Etymology: The species epithet is a noun in apposition in honour of Tazarte, faycan (spiritual leader) of Telde, that actively participate in the resistance against the Castilian conquest of Gran Canaria, and who died jumping from a cliff before surrendering.

Diagnosis: Differs from *L. rufescens* and *L. mrazig* by the same morphological combination than ***L. mahan* sp. nov.** (see above). Males of ***L. tazarte* sp. nov.** can be distinguished from the remaining Canary Island species by the shape of the embolus (Fig 4 A-C), and females, by the long inner lobe of the apical part of seminal receptacles (Fig. 5). Morphologically similar to ***L. bentejui* sp. nov.** but males can be distinguished by the less oval shape of the tibia palp (Fig. 4 A-B, E), and females by the U-shaped inner lobe of the apical part of seminal receptacles (Fig. 5 A-B), although some variation exist regarding these characters. ***Loxosceles tazarte* sp. nov.** also can be distinguished from the remaining Canary Island endemic species and from *L. rufescens* by two (156 (A); 435 (C/T)) *cox1*

diagnostic nucleotide changes based on the alignment deposited in TreeBASE (accession number 15746) (Supplementary Fig. 1).

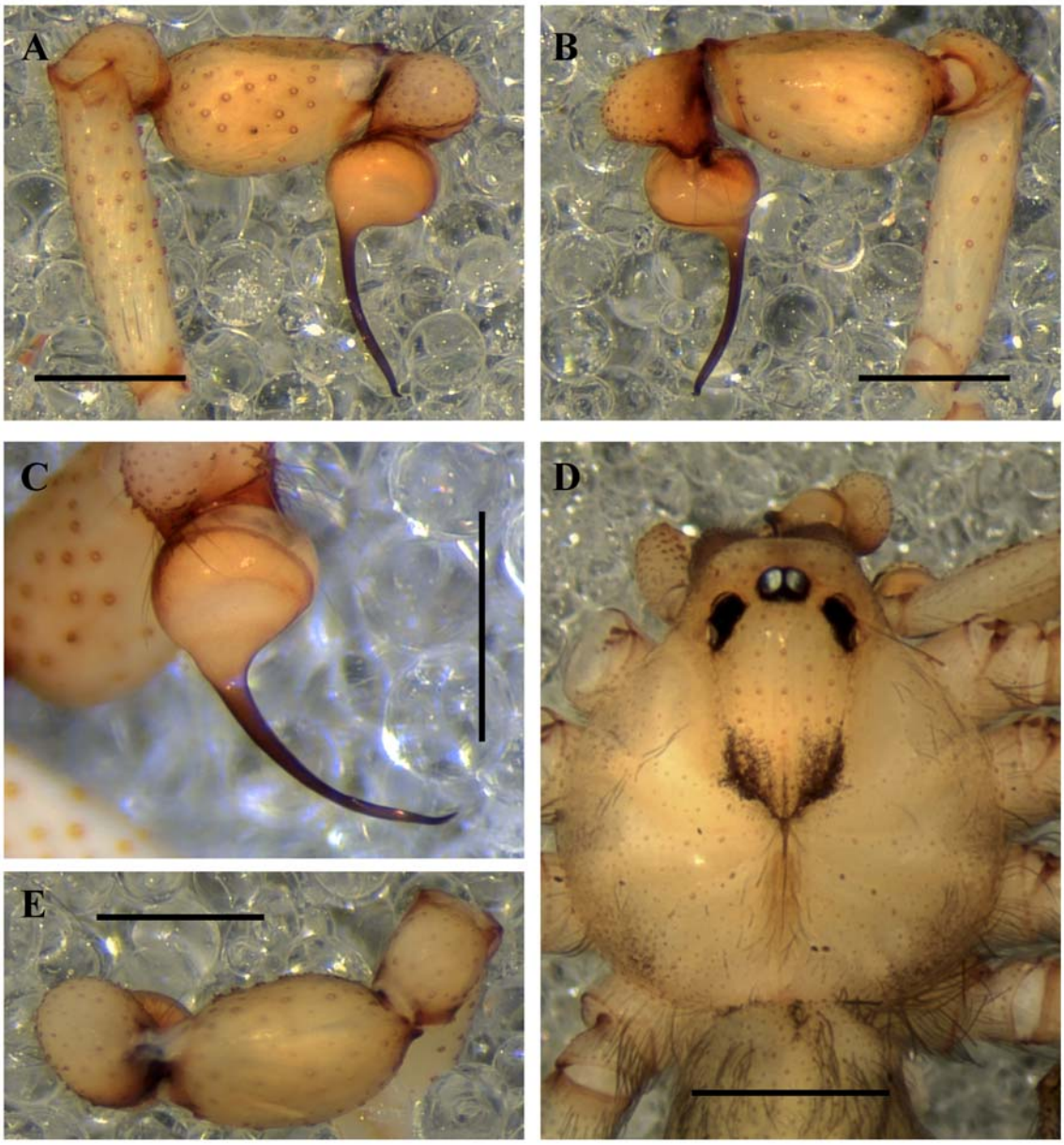

Figure 4 ♂ ***Loxosceles tazarte*** **sp. nov. A)** palp, prolateral view. **B)** palp, retrolateral view. **C)** bulb and embolus. **D)** cephalothorax, dorsal view. E: palp, dorsal view. Scale bars: A, B, C, E: 0.5 mm; D: 1 mm.

Description: Male (holotype). **Specimen preparation and condition:** Specimen collected in MSS traps and preserved in ethanol 96º. Pedipalps removed and conserved with specimen and left legs 1, 2 and 3 removed. Due to preservation, pedipalp tibia slightly compressed in its dorso-anterior part and most setae had fallen. **Colouration:** Carapace

yellowish, clypeus and median groove slightly darkened. Conspicuous dark V-mark posteriorly on pars cephalica, expanding finely towards the median groove (Fig. 4D). Carapace with few dispersed short black setae. Eye tubercles black. Chelicerae reddish brown, darkened in its distal part. Sternum bright yellowish, paler than carapace. Labium and gnathocoxae pale reddish brown. Legs pale yellowish with the apical segments slightly darkened. Abdomen pale yellowish to greyish, coated by short setae. **Cephalothorax:** Carapace slightly longer (2.34) than wide (2.23), truncated behind, widely rounded on sides, narrowed in front. Carapace evenly convex, with median groove moderately deep, elongated, occupying roughly the posterior third. Pars cephalica elongated (1.15 long, 0.76 wide). Most of larger setae rubbed off to leave integument with conspicuous alveoli, forming 7 rows in the pars cephalica, and six radial rows departing from its posterior part. Clypeus height 0.211. Eye sizes: ALE 0.16 PME 0.13 PLE 0.15. LE separated from ME by narrow diameter of ME. Sternum longer (1.4) than wide (1.25). **Abdomen:** Elongate oval (2.48 long 1.6 wide) in dorsal view. **Legs:** Leg formula 2 1 4 3. Leg 1 (15.42): femur 4.15/patella 0.87/tibia 4.59/metatarsus 4.65/tarsus 1.16; Leg 2 (17.44): 4.77/0.87/5.12/5.44/1.24; Leg 3 (13.51): 3.75/0.85/3.57/4.31/1.03; Leg 4 (15.11): 4.11/0.81/4.12/4.83/1.24. **Male palp:** (Fig. 4) Femur cylindrical (1.06 long, 0.26 wide). Patella subglobular, roughly as long as wide (0.4). Tibia wider at the base (0.77 long, 0.45 wide), paunchy ventrally, rounded more abruptly towards the patella than the tarsus. Tarsus longer than wide, as large as bulb (0.41). Bulb globular, slightly compressed dorsally. Maximum bulb width 4.5 times wider than embolus base. Embolus long, approximately 1.9 times longer than bulb width, gently curved with an apparent bending at mid-length and slightly sinuous towards the tip (Fig. 4 A-C). Long, curved setae facing apically, situated in tarsus. Conspicuous setae alveoli distributed sparsely in femur and tibia. **Variation:** Supplementary Table 2. Leg 1 (n = 7): 14.71-24.09 (mean: 18.3); leg 2 (n = 6): 17.01-28.68 (mean: 21.14); leg 3 (n = 6): 13.51-20.38 (mean: 16.46); leg 4 (n = 7): 15.09-22.34 (mean: 17.68); CL (n = 7): 2.34-3.21 (mean: 2.73); CW (n = 7): 2.2-2.88 (mean: 2.5). General colouration ranges from pale-yellow to darker yellow-orange, depending on the time from the last moult. The shape of the dark V-shaped patterns also presents some variation in intensity and extent.

Female (Paratype, ULL-DZUL-34181). **Specimen preparation and condition:** Specimen collected in MSS traps and preserved in ethanol 96º. Genitalia removed and conserved in a

vial with specimen. Legs 3 and 4 left removed. **Colouration:** Similar as in male but slightly darker. Carapace yellow-orange, with pars cephalica slightly darkened. Palps and chelicerae reddish. **Cephalothorax:** Carapace slightly longer (2.88) than wide (2.63). Carapace evenly convex, with median groove moderately deep, elongated, occupying roughly the posterior third. Pars cephalica elongated (1.98 long, 0.95 wide). Clypeus height 0.302 Eye sizes: ALE 0.18 PME 0.12 PLE 0.16. LE separated from ME by 0.17. Sternum longer (1.6) than wide (1.46). **Abdomen:** Elongate oval (4.06 long 2.7 wide) in dorsal view. **Legs:** Leg formula 2 4 1 3. Leg 1 (14.65): femur 4.28/patella 0.94/tibia 4.19/metatarsus 4.12/tarsus 1.12; Leg 2 (15.77): 4.55/0.92/4.56/4.59/1.15; Leg 3 (13.12): 3.89/0.98/3.4/3.77/1.08; Leg 4 (15.14): 4.31/1.02/3.98/4.6/1.23. **Female genitalia: (**Fig. 5) Atriobursal orifices situated in two large, rounded and almost contiguous pouches, slightly sclerotised around. Base of seminal receptacles conical and wide, reinforced laterally with a curved, dark, sclerotised band. Medium part very short, bifurcating towards the tip in two cilindrical lobes. External lobe short and tapered, while inner longer, more than three times the former, U-shapped, pointing ventrally or laterally. Seminal receptacles not touching each other, separated at their closer point by roughly the apical diameter of the inner lobes. **Variation:** Supplementary Table 2. Leg 1 (n = 4): 14.16-16.30 (mean: 15.32); leg 2 (n = 3): 15.45-17.84 (mean: 16.36); leg 3 (n = 4): 13.06-15.38 (mean: 14.03); leg 4 (n = 4): 14.83-16.68 (mean: 15.76); CL (n = 4): 2.6-3.14 (mean: 2.89); CW (n = 4): 2.43-2.78 (mean: 2.61). Similar range of general colouration variation as in males. Seminal receptacles variable, specially in the shape of the inner lobe.

Distribution and natural history: Endemic to Gran Canaria. Known from the central, south and eastern parts of the island, and from Peninsula de la Isleta. Distinguished from ***L. bentejui* sp. nov.**, also endemic to Gran Canaria, based on allopatric distribution (Fig. 1). Some specimens have been collected in MSS traps.

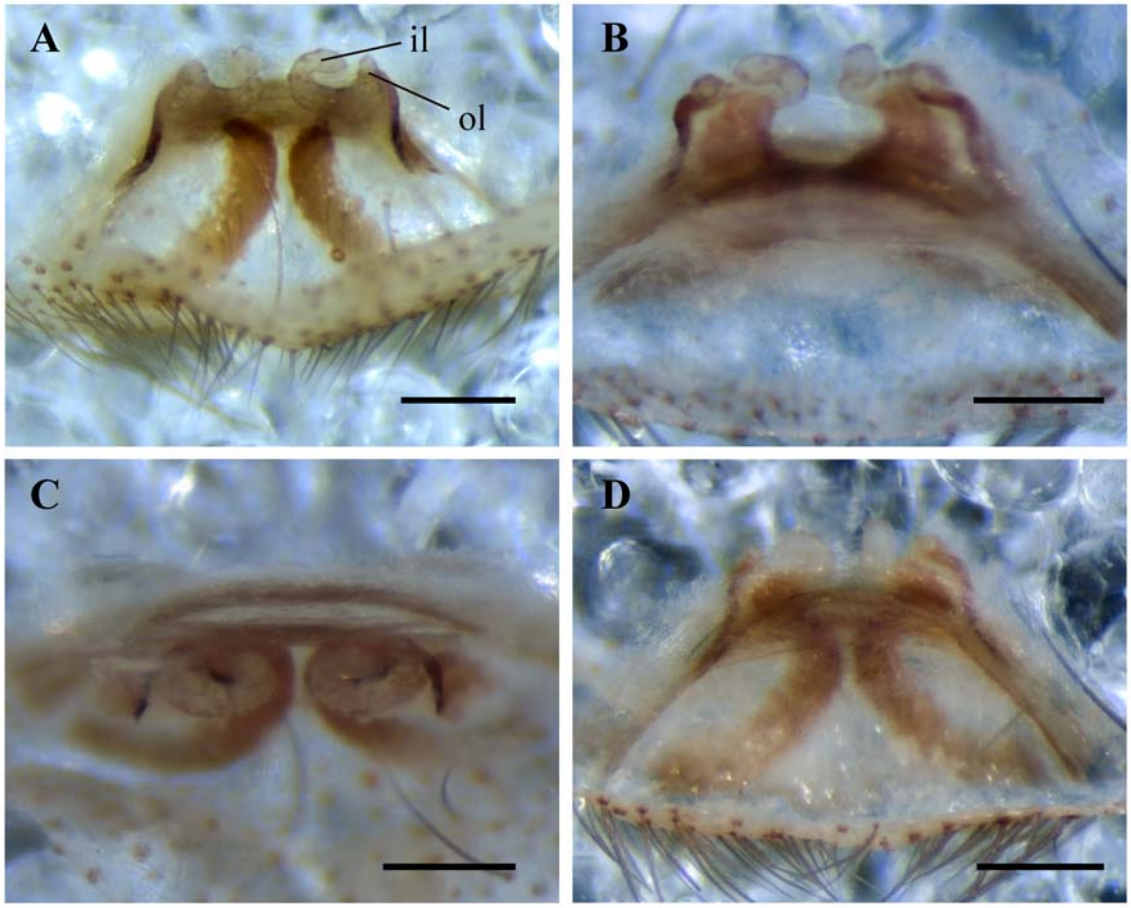

Figure 5 ♀ ***Loxosceles tazarte* sp. nov.** Vulva, **A)** ventral view. **B)** dorsal view. **C)** anterior view. **D)** posterior view. Abbreviations: il, inner lobe of seminal receptacle. ol, outer lobe of seminal receptacle. Scale bars: 0.2 mm.

***Loxosceles bentejui* sp. nov.**

Figs. 6-7

Loxosceles sp. GC-2 (Planas & Ribera, 2014)

Holotype: 1♂, CRBA-LX1711 (MorphoBank: M326622-M326627), El Risco, Gran Canaria, 28.0601 N 15.7296 W, 190 m asl., 24.XII.2010, Araya, M. & Planas, E.leg.

Paratypes: 2♀, CRBA-LX1708 (MorphoBank: M326628-M326634), -LX1709, same locality and date as holotype; 1♀ SMF-XXX (LX1126), Barranco de Guayedra, S Agaete, Gran Canaria, 28.08075 N 15.70475 W, 103 m asl., 1.XII.2003, Jäger, P. leg.

Etymology: The species epithet is a noun in apposition in honor of Bentejui, aboriginal warrior that actively participate in the resistance against the Castilian conquest of Gran Canaria, and who died jumping from a cliff before surrendering.

Diagnosis: Differs from *L. rufescens* and *L. mrazig* by the same morphological combination than ***L. mahan* sp. nov.** (see above) and from the Canary Island endemic species by the same combination than ***L. tazarte* sp. nov.** (see above). Males can be distinguished from its sister species ***L. tazarte* sp. nov.**, by the shape of the tibia palp (Fig. 6 A-B, E), and females, by the L-shaped inner lobe of the apical part of seminal receptacles (Fig. 7), although some variation exist regarding these characters. ***Loxosceles bentejui* sp. nov.** also can be distinguished from the remaining Canary Island endemic species and from *L. rufescens* by three (387 (T); 875 (C); 879 (C)) *cox1* diagnostic nucleotide changes based on the alignment deposited in TreeBASE (accession number 15746) (Supplementary Fig. 1).

Description: Male (holotype). **Specimen preparation and condition:** Specimen collected alive and preserved in ethanol 96º. Left pedipalp and leg 1 R removed and conserved in a vial with specimen. **Colouration:** Carapace pale yellowish, clypeus slightly darkened. Conspicuous dark V-mark posteriorly on pars cephalica (Fig. 6 D). Median groove slightly darkened. Carapace with dispersed short black setae. Eye tubercles black. Chelicerae reddish brown, darkened in its distal part. Sternum bright yellowish, paler than carapace. Labium and gnathocoxae pale reddish brown. Legs pale yellowish with the apical segments slightly darkened. Abdomen pale yellowish to greyish, coated by short black setae. **Cephalothorax:** Carapace slightly longer (2.91) than wide (2.6), truncated behind, widely rounded on sides, narrowed in front. Carapace evenly convex, with median groove moderately deep, elongated, occupying roughly the posterior third. Pars cephalica elongated (1.75 long, 0.84 wide). Long seate forming seven parallel rows in the pars cephalica, and six radial rows departing from its posterior part. Clypeus height 0.275. Eye sizes: ALE 0.17 PME 0.13 PLE 0.19. LE separated from ME by 0.1. Sternum longer (1.53) than wide (1.38). **Abdomen:** Elongate oval (3.63 long 2.5 wide) in dorsal view. **Legs:** Leg formula 2 1 4 3. Leg 1 (20.63): femur 5.66/patella 0.96/tibia 6.23/metatarsus 6.4/tarsus 1.38. Leg 2 (23.45): 6.22/0.99/7.08/7.71/1.45. Leg 3 (17.47): 4.91/0.99/4.67/5.73/1.17. Leg 4 (18.97): 5.09/0.97/5.16/6.28/1.47. **Male palp:** (Fig. 6 A-C, E) Femur cylindrical (1.3 long, 0.32 wide). Patella subglobular, roughly as long as wide (0.42). Tibia oval (0.84 long, 0.52 wide, lateral and dorsal). Tarsus short, as large as bulb (0.44). Long curved setae facing apically, situated sparsely in femur, tibia and tarsus, especially in retrolateral position. Bulb similar to ***Loxosceles tazarte* sp. nov.**, at least as large as the tarsus. Maximum bulb width 4.7

times wider than embolus base. Embolus long, approximately 1.6 times longer than bulb width and slightly curved in its medial part. Distal part rather more curved than the medial and slightly sinuous. **Variation:** Supplementary Table 2. Males known only from the type specimen.

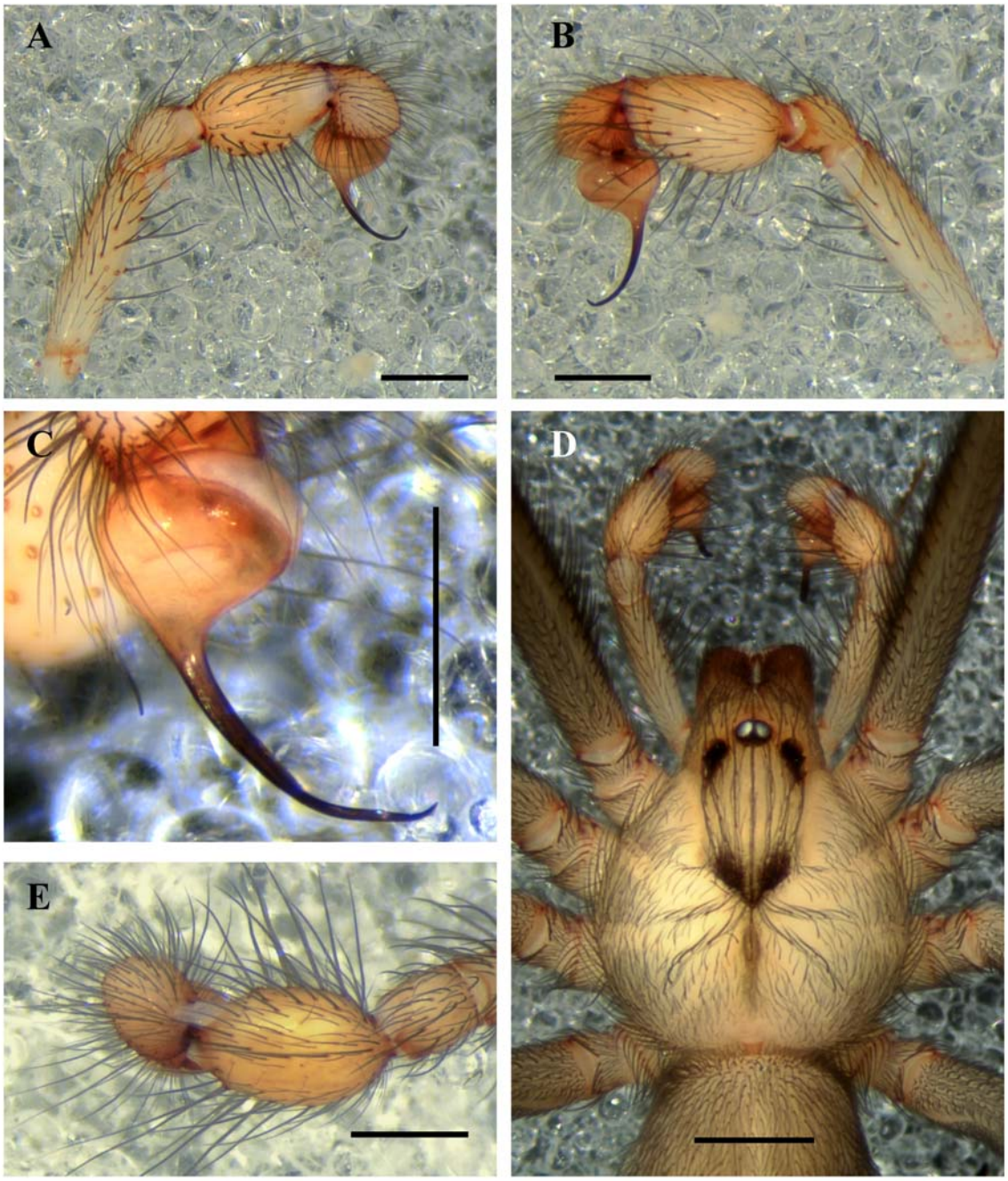

Figure 6 ♂ ***Loxosceles bentejui*** **sp. nov. A)** palp, prolateral view. **B)** palp, retrolateral view. **C)** bulb and embolus. **D)** cephalothorax, dorsal view. E: palp, dorsal view. Scale bars: A, B, C, E: 0.5 mm; D: 1 mm.

Female (Paratype, CRBA-LX1708). **Specimen preparation and condition:** Specimen collected alive and preserved in ethanol 96º. Genitalia removed and conserved in a vial with specimen. **Colouration:** Similar colouration as in male. Carapace yellow-orange, with pars cephalica slightly darkened. Palps and chelicerae reddish. Dark V-mark posteriorly on pars cephalica greater than in male. **Cephalothorax:** Carapace slightly longer (3.35) than wide (2.83). Pars cephalica elongated (2.04 long, 0.99 wide). Clypeus height 0.383. Eye sizes: ALE 0.17 PME 0.12 PLE 0.17. LE separated from ME by 0.16. Sternum longer (1.59) than wide (1.4). **Abdomen:** Elongate oval (3.15 long 2.08 wide) in dorsal view. **Legs:** Leg formula 2 4 1 3. Leg 1 (16.78): femur 4.73/patella 1.11/tibia 4.94/metatarsus 4.85/tarsus 1.15 Leg 2 (18.05): 4.95/1.14/5.19/5.51/1.26 Leg 3 (14.86): 4.26/1.04/3.97/4.52/1.07 Leg 4 (16.96): 4.78/1.01/4.56/5.35/1.26. **Female genitalia:** (Fig. 7) Atriobursal orifices situated in two large, rounded, almost contiguous pouches, slightly sclerotised around. Base of seminal receptacles conical and wide, reinforced laterally with a curved, dark, sclerotised band. Medium part very short, bifurcating towards the tip in two cilindrical lobes. The external short and tapered and the inner approximately twice as long as the former and L-shapped, pointing ventrally. Seminal receptacles without touching each other, separated at their closer point by slightly more than the apical diameter of the inner lobes. **Variation:** Supplementary Table 2. CRBA-LX1709: leg 1 16.09; leg 2 17.81; leg 3 14.54; leg 4 16.45; CL 3.22; CW 2.79. General colouration darker than CRBA-LX1708. Seminal receptacles nearly identical except that in this specimen the inner lobe is pointing anteriorly.

Distribution and natural history: Endemic to Gran Canaria. Known from three localities in the north-eastern part of the island, in the Barranco de Agaete, pinar de Acusa Seca and in the Barranco de El Risco, where it has been found more abundantly under big stones. Distinguished from ***L. tazarte*** **sp. nov.**, also endemic to Gran Canaria, based on allopatric distribution (Fig. 1).

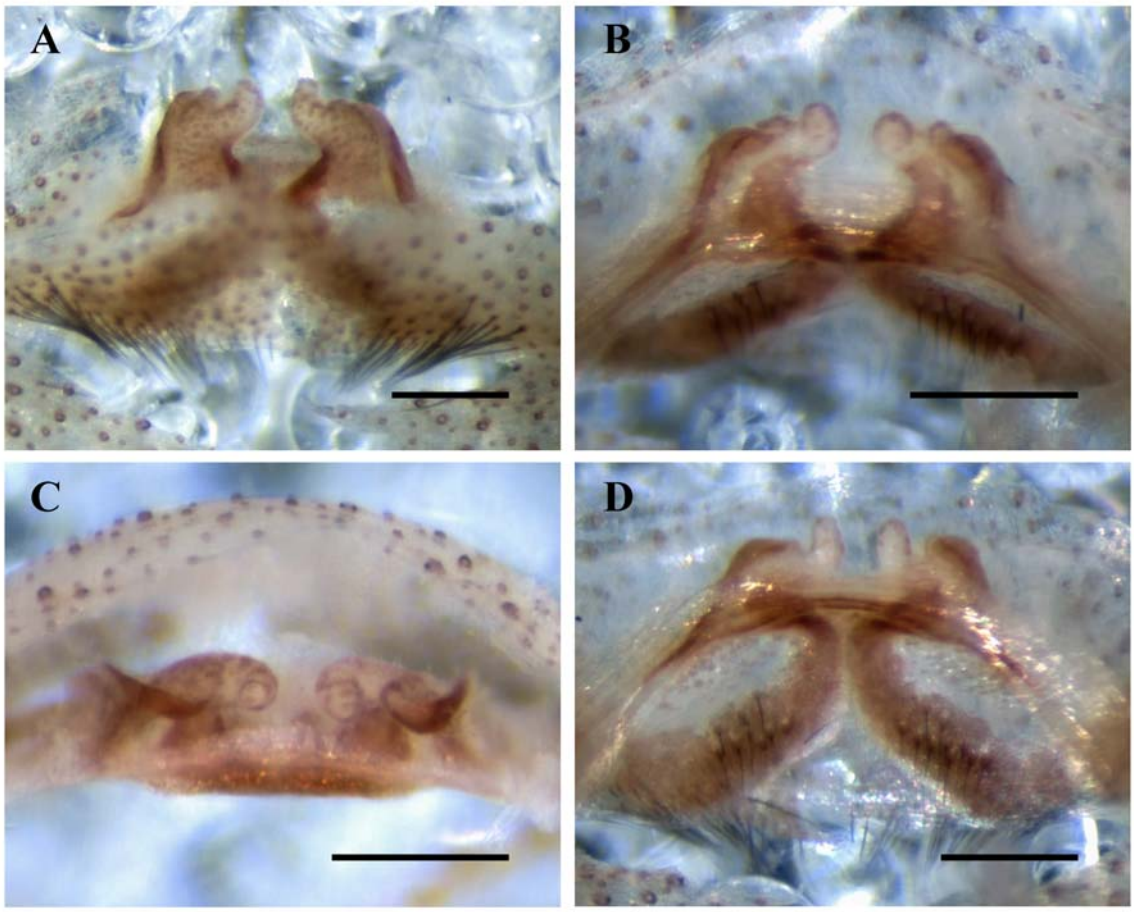

Figure 7 ♀ ***Loxosceles bentejui*** **sp. nov.** Vulva, **A)** ventral view. **B)** dorsal view. **C)** anterior view. **D)** posterior view. Scale bars: 0.2 mm.

Loxosceles tibicena **sp. nov.**

Figs. 8-9

Loxosceles sp. TF-2 (Planas & Ribera, 2014)

Holotype: 1♂, ULL-DZUL-34197 (MorphoBank: M326635-M326640), Cumbres Arico. Pista Izaña-Contador, Tenerife, 1975 m asl, 05.II.2007, GIET leg.

Paratype: 1♀, ULL-DZUL-34202 (LX1164) (MorphoBank: M326641-M326645), same locality and data as holotype.

Other material examined: Tenerife: 3♂, ULL-DZUL-34199, -34200, -34201, Barranco del Río, Lomo Largo (Arico/Granadilla de Aboba), pitfall trap, 28.18736 N 16.58227 W, 1700 m asl, 23.III.1985, Peraza, J.M. leg.

Etymology: The species epithet is a noun in apposition that refers to a mythological

creature of the Guanches (aboriginal inhabitants of the Canary Islands) that was thought to live deep in the caves.

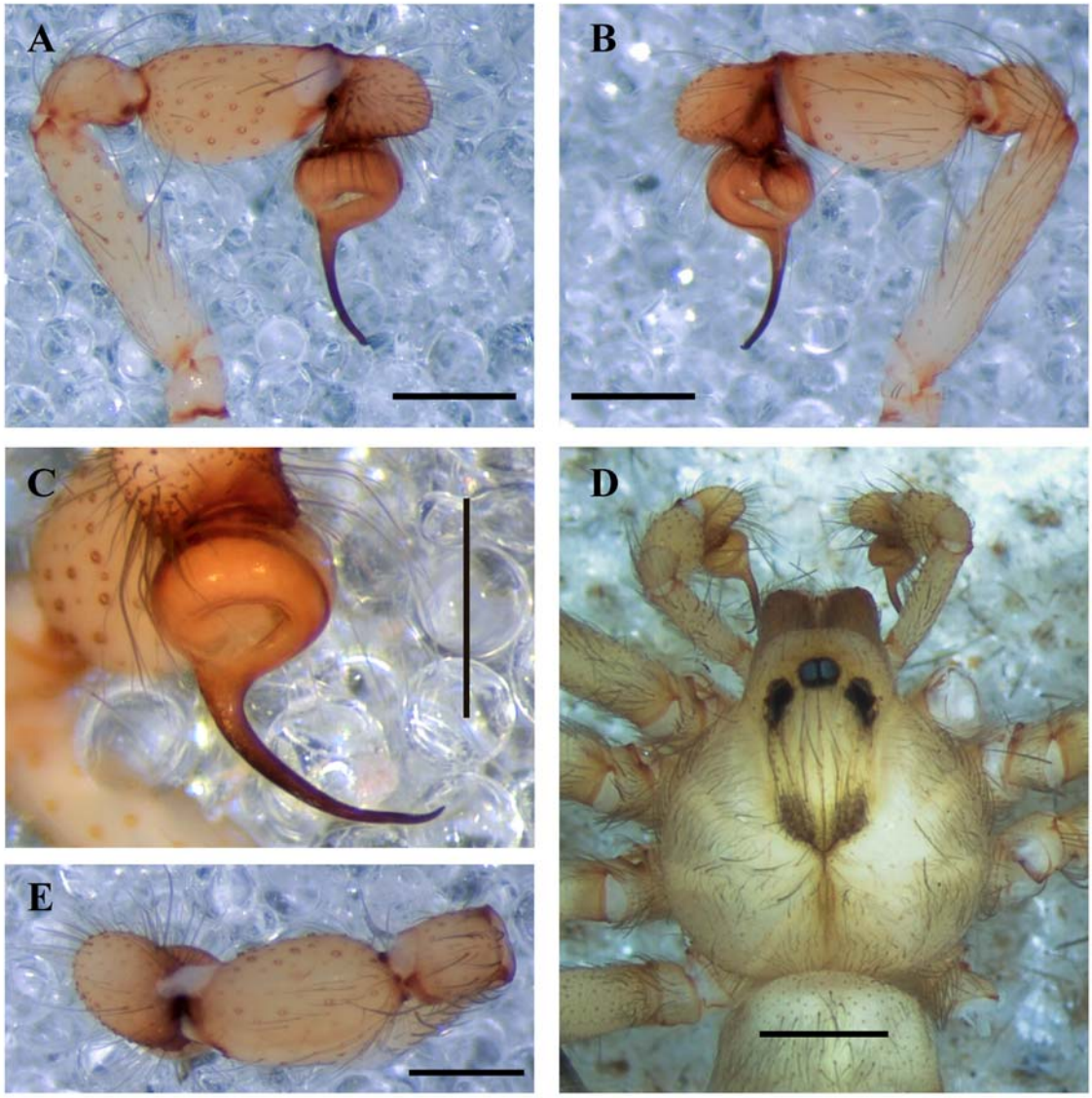

Figure 8 ♂ ***Loxosceles tibicena*** **sp. nov. A)** palp, prolateral view. **B)** palp, retrolateral view. **C)** bulb and embolus. **D)** cephalothorax, dorsal view. E: palp, dorsal view. Scale bars: A, B, C, E: 0.5 mm; D: 1 mm.

Diagnosis: Differ from *L. rufescens* and *L. mrazig* by the same morphological combination than ***L. mahan*** **sp. nov.** (see above). Males of ***L. tibicena*** **sp. nov.**, can be distinguished from the remaining Canary Island species by the shape of the embolus, which is strongly curved after the embolus base (Fig. 8 C), and females, by the wide, straight and directed towards the centre inner lobe of the apical part of seminal receptacles (Fig. 9). Differ from ***L. guayota*** **sp. nov.**, also endemic to Tenerife, by the size, by relative shorter leg lengths,

and by longer embolus. ***Loxosceles tibicena* sp. nov.** also can be distinguished from the remaining Canary Island endemic species and from *L. rufescens* by 12 (6 (T); 10 (A); 51 (G); 231 (C); 330 (A); 453 (G); 600 (C); 672 (G); 753 (A); 780 (A); 827 (G); 906 (A)) *cox1* diagnostic nucleotide changes based on the alignment deposited in TreeBASE (accession number 15746) (Supplementary Fig. 1).

Description: Male (holotype). **Specimen preparation and condition:** Specimen preserved in ethanol 96º. Left pedipalp removed and conserved in a vial with specimen. Some legs removed or broken. **Colouration:** Carapace pale yellowish, clypeus, pars cephalica and median groove slightly darkened. Conspicuous dark V-mark posteriorly on pars cephalica (Fig. 8D). Carapace with dispersed short black setae. Eye tubercles black. Chelicerae reddish brown, darkened in its distal part. Sternum bright yellowish, paler than carapace. Labium and gnathocoxae pale reddish brown. Legs pale yellowish with the apical segments slightly darkened. Sternum, labium, gnathocoxae and legs covered by long setae interspersed with shorter and thicker setae. Abdomen pale yellowish to greyish, densely coated by short setae. **Cephalothorax:** Carapace slightly longer (2.63) than wide (2.38), truncated behind, widely rounded on sides, narrowed in front. Carapace evenly convex, with median groove moderately deep, elongated, occupying roughly the posterior third. Pars cephalica elongated (1.73 long, 0.79 wide). Large setae or bristles placed in a single row in the side margin of the carapace pointing anteriorly. Similar setae forming seven rows in the pars cephalica, and six radial rows departing from its posterior part, interspersed with shorter setae. Clypeus height 0.21. Eye sizes: ALE 0.19 PME 0.13 PLE 0.12. LE separated from ME by narrow diameter of ME. Sternum longer (1.56) than wide (1.33). **Abdomen:** Elongate oval (3.13 long 2.1 wide) in dorsal view. **Legs:** Leg formula 2 1 4 3. Leg 1 (20.19): femur 5.59/patella 0.97/tibia 6.01/metatarsus 6.17/tarsus 1.45. Leg 2 (23.35): 6.3/1/6.92/7.57/1.56. Leg 3 (17.11): 5/0.86/4.58/5.51/1.16. Leg 4 (19.51): 5.33/1.03/5.11/6.48/1.56. **Male palp:** (Fig. 8 A-C, E) Femur cylindrical (1.23 long, 0.29 wide). Patella subglobular, roughly as long as wide (0.38). Tibia elongated, flattened dorsally (0.8 long, 0.48 wide). Tarsus short, as large as bulb (0.43). Bulb globular, slightly compressed dorsally. Maximum bulb width slightly less than four times wider than embolus base. Embolus long approximately 1.6 times longer than bulb width. Strongly curved after embolus base, medial part slightly straight, gently curved and slightly sinuous in its distal part. Long, curved setae facing apically, distributed sparsely in femur, tibia and tarsus,

denser on retrolateral side. **Variation:** Supplementary Table 2. Leg 1 (n = 2): 22.1-23.82 (mean: 22.96); leg 2 (n = 2): 26.26-28.49 (mean: 27.38); leg 3 (n = 2): 18.9-20.45 (mean: 19.67); leg 4 (n = 2): 20.84-22.09 (mean: 21.47); CL (n = 3): 2.58-3.12 (mean: 2.83), CW (n = 3): 2.72-2.75 (mean: 2.73).

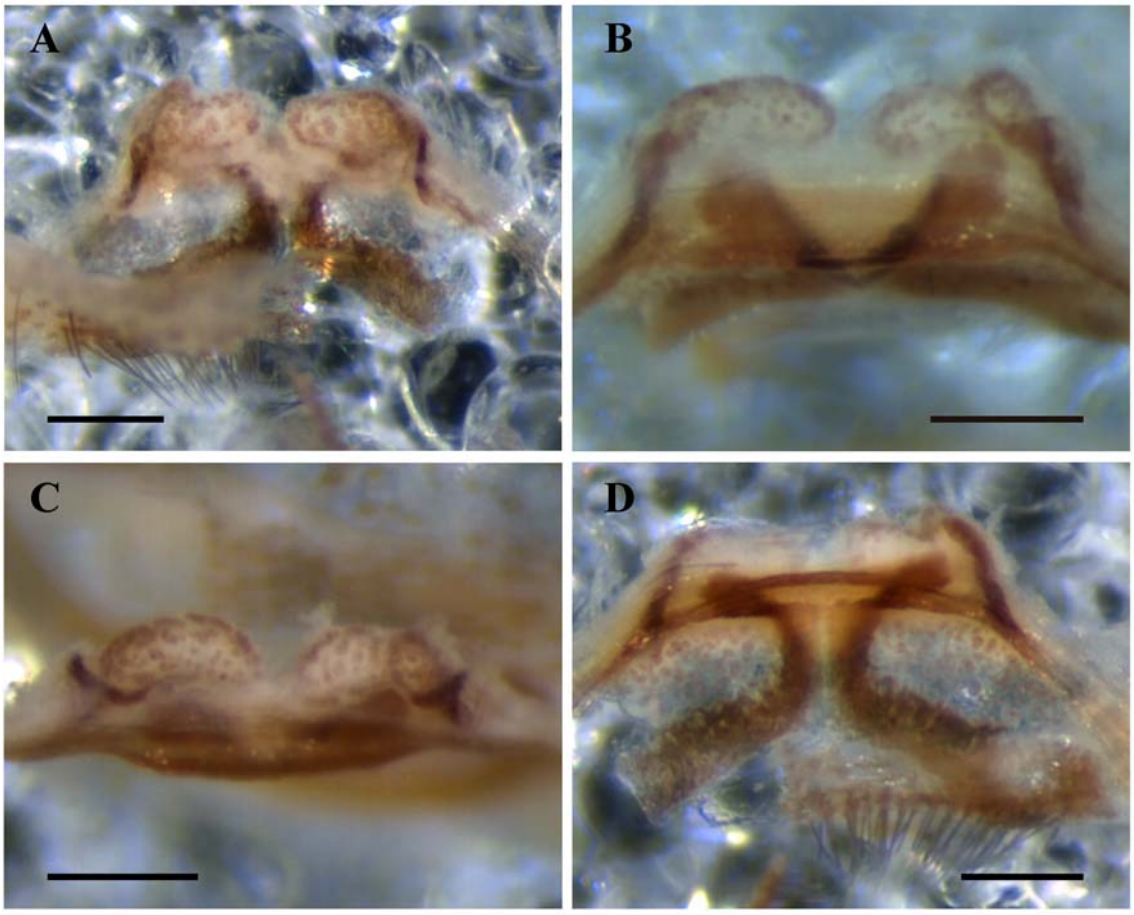

Figure 9 ♀ ***Loxosceles tibicena* sp. nov.** Vulva, **A)** ventral view. **B)** dorsal view. **C)** anterior view. **D)** posterior view. Scale bars: 0.2 mm.

Female (Paratype (ULL-DZUL-34202)). **Specimen preparation and condition:** Specimen preserved in ethanol 96º. Genitalia removed and conserved in a vial with specimen. Legs 4 R and 3 R removed for molecular study. **Colouration:** Darker than in male, with chelicerae and pedipalps brownish-red. **Cephalothorax:** Carapace slightly longer (3.35) than wide (3). Pars cephalica elongated (2.14 long, 1 wide). Clypeus height: 0.37. Eye sizes: ALE 0.23 PME 0.14 PLE 0.23. LE separated from ME by diameter of PME. Sternum longer (1.93) than wide (1.55). Labium 0.78 long, 0.64 wide at its base. **Abdomen:** Elongate oval (4.16 long 2.56 wide) in dorsal view. **Legs:** Leg formula 2 4 1 3. Leg 1 (18.43): femur

5.34/patella 1.11/tibia 5.38/metatarsus 5.34/tarsus 1.26 Leg 2 (19.92): 5.76/1.17/5.48/6.12/1.39 Leg 3 (16.59): 5.01/1.18/4.26/5.01/1.13 Leg 4 (18.6): 5.23/1.03/4.95/5.96/1.43. **Female genitalia:** (Fig. 9) Atriobursal orifices situated in two large, rounded, almost contiguous pouches, slightly sclerotised around. Base of seminal receptacles conical and wide, reinforced laterally with a curved, dark, sclerotised band. Medium part short, cylindrical and strongly curved towards the centre. Subtle lobe protruding on its extrernal side. Inner lobe wide, straight, directed towards the center. Seminal receptacles not touching each other, separated at their closer point by roughly the apical diameter of the inner lobes. **Variation:** Females known only from the paratype specimen.

Distribution and natural history: Endemic to Tenerife. Known from two localities in central-east Tenerife. Extensive direct sampling effort was conducted near these localities without finding further specimens.

***Loxosceles guayota* sp. nov.**

Fig. 10

Loxosceles cf. *rufescens* (Arechavaleta *et al*., 1998)

Loxosceles sp. TF-3 (Planas & Ribera, 2014)

Holotype: 1♂, CRBA-LX1156 (MorphoBank: M326646-M326651), small cave near Los Roques Cave, Tenerife, 28.23625 N 16.64251 W, 2272 m asl, 22.IV.2009, Espluga, R., Janowski, A. & Planas, E. leg.

Paratype: 1♂, CRBA-LX1155, same locality and data as holotype.

Etymology: The species epithet is a noun in apposition that refers to a malignant mythological deity of the Guanches that was thought to live inside the Teide Volcano, and to be responsible for its eruptions.

Diagnosis: Differ from *L. rufescens* and *L. mrazig* by the same morphological combination than ***L. mahan* sp. nov.** (see above). Males of ***L. guayota* sp. nov.** can be distinguished from the remaining Canary Island species, including its sister species ***L. tibicena* sp. nov.**, also endemic to Tenerife, by its wider embolus (Fig. 10 A-C, E), relatively longer legs (Fig. 13), and larger size. ***Loxosceles guayota* sp. nov.** also can be distinguished from the remaining Canary Island endemic species and from *L. rufescens* by 20 (9 (A); 141 (C); 192 (C); 208 (G): 211 (T); 213 (A); 220 (C); 240 (C); 454 (C); 465 (C); 487 (G); 588 (G); 621

(G); 753 (G); 777 (T); 795 (G); 807 (C); 810 (A); 882 (C); 939 (T)) *cox1* diagnostic nucleotide changes based on the alignment deposited in TreeBASE (accession number 15746) (Supplementary Fig. 1).

Figure 10 ♂ ***Loxosceles guayota*** **sp. nov. A)** palp, prolateral view. **B)** palp, retrolateral view. **C)** bulb and embolus. **D)** palp, dorsal view. **E)** cephalothorax, dorsal view. Scale bars: A, B, C, E: 0.5 mm; D: 1 mm.

Description: Male (holotype). **Specimen preparation and condition:** Specimen preserved in ethanol 70º. Left pedipalp removed and conserved in a vial with specimen. **Colouration:** Carapace pale yellowish, clypeus, pars cephalica and median groove slightly darkened. Conspicuous dark V-mark posteriorly on pars cephalica (Fig. 10D). Carapace with

dispersed short black setae. Eye tubercles black. Chelicerae reddish brown, darkened in its distal part. Sternum bright yellowish, paler than carapace. Labium and gnathocoxae pale reddish brown. Legs pale yellowish with the apical segments slightly darkened. Sternum, labium, gnathocoxae and legs covered by long setae interspersed with shorter and thicker setae. Abdomen pale yellowish to greyish, densely coated by short setae. **Cephalothorax:** Carapace slightly longer (3.62) than wide (3.27), truncated behind, widely rounded on sides, narrowed in front. Carapace evenly convex, with median groove moderately deep, elongated, occupying roughly the posterior third. Pars cephalica elongated (2.2 long, 1.11 wide). Large setae or bristles placed in a single row in the side margin of the carapace pointing anteriorly. Similar setae forming seven rows in the pars cephalica, perceived by the alveoli when setae had fallen. Six radial rows departing from posterior part of pars cephalica, interspersed with shorter setae. Clypeus height 0.435. Eyes sizes: ALE 0.23 PME 0.16 PLE 0.24. LE separated from ME by narrow diameter of ME. Sternum longer (2.06) than wide (1.93). **Abdomen:** Elongate oval (4.86 long 2.84 wide) in dorsal view. **Legs:** Leg formula 2 1 4 3. Leg 1 (34.78): femur 9.59/ patella 1.31/ tibia 10.86/ metatarsus 10.87/ tarsus 2.15. Leg 2 (42.95): 11.7/1.44/13.26/14.22/2.33. Leg 3 (29.18): 8.41/1.3/8.14/9.64/1.69. Leg 4 (32): 8.74/1.32/8.82/10.93/2.19. **Male palp** (Fig. 10 A-C, E) Femur cylindrical (1.62 long, 0.35 wide). Patella subglobular, roughly as long as wide (0.43). Tibia elongated, flattened dorsally (1 long, 0.58 wide). Tarsus short, as large as bulb (0.62). Bulb globular, slightly compressed dorsally, wider than tarsus. Maximum bulb width roughly three times wider than embolus base. Embolus long, approximately 1.5 times longer than bulb width. Medial part of embolus thick and nearly straight. Distal part clearly thinner, curved and slightly sinuous. Long, curved setae facing apically, distributed sparsely in femur, tibia and tarsus, denser on retrolateral side. **Variation:** Supplementary Table 2. CRBA-LX1155: leg 1: -; leg 2: 42.92; leg 3: 29.31; leg 4: 31.94; CL: 3.37; CW: 3.36.

Female: Unknown

Distribution and natural history: Endemic to Tenerife. Collected from a single, subaerial volcanic tube. Direct sampling effort was conducted in the same and nearby cavities, without finding further specimens.

***Loxosceles hupalupa* sp. nov.**

Figs. 11-12

Loxosceles rufescens (Schmidt, 1981)

Loxosceles sp. GM-HI (Planas & Ribera, 2014)

Holotype: 1♂, CRBA-LX1786 (MorphoBank: M326652-M326657), Playa de Ávolo, San Sebastián de la Gomera, La Gomera, 28.1147 N 17.11317 W, 40 m asl, 17.II.2011, Espluga, R. & Planas, E. leg.

Paratype: 1♂, CRBA-LX1787, 1♀, CRBA-LX1789 (MorphoBank: M326658-M326663), same locality and data as holotype.

Other material examined: La Gomera: 1♀, CRBA-LX1785, Tapagache, 28.08413 N 17.28902 W, 758 m asl, 16.II.2011,Espluga, R. & Planas, E. leg.; 2♀, CRBA-LX1778, -LX1781), Igualero, 28.08762 N 17.25687, 1161 m asl, 15.II.2011, Espluga, R. & Planas, E. leg.; 1♀, CRBA-LX1774, Barranco del Paijén, 28.08845 N 17.19955 W, 888 m asl, 15.II.2011, Espluga, R. & Planas, E. leg.; 1♂subad., SMF-29479, Santiago, 29.V.1905, Schmidt, G. leg. **El Hierro**: 4♀, ULL-DZUL-34165, -34166 (LX2292), -34167 (LX2293), -34168, Cueva del Linke, 27.65301 N 17.98163 W, 16.XI.1985, Medina leg.

Etymology: The specific epithet is a noun in apposition that refers to the name of an ancient aborigine of the island of La Gomera.

Diagnosis: Differ from *L. rufescens* and *L. mrazig* by the same morphological combination than ***L. mahan* sp. nov.** (see above). Males of ***Loxosceles hupalupa* sp. nov.** can de distinguished from the remaining endemic Canary Island species by the shorter palpal tibia (in relation to CL) (Fig. 11 A), and females, by the wide and curved inner lobe of the apical part of seminal receptacles. ***Loxosceles hupalupa* sp. nov.** also can be distinguished from the remaining Canary Island endemic species and from *L. rufescens* by two (450 (C); 531 (T)) *cox1* diagnostic nucleotide changes based on the alignment deposited in TreeBASE (accession number 15746) (Supplementary Fig. 1).

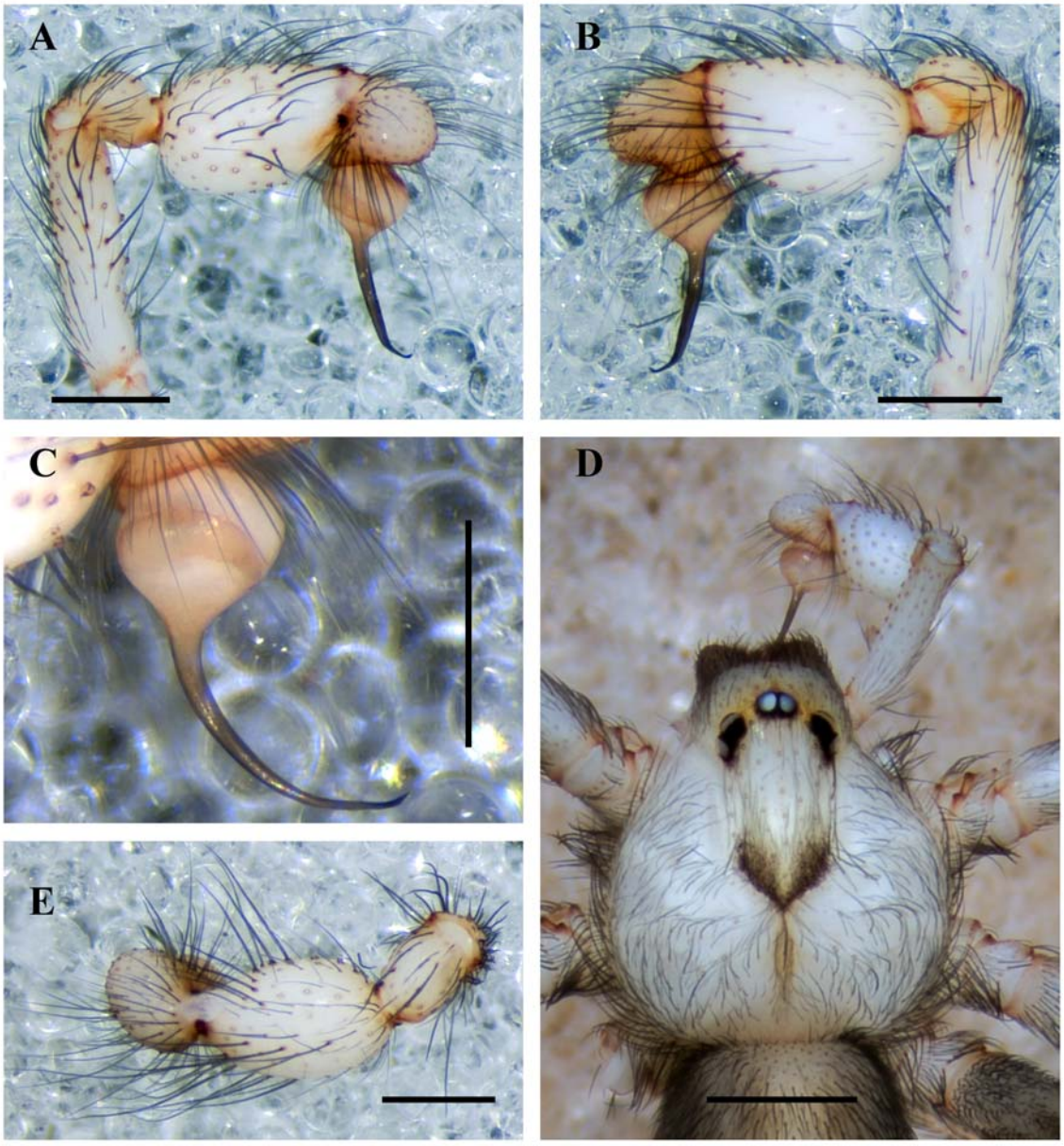

Figure 11 ♂ ***Loxosceles hupalupa*** **sp. nov. A)** palp, prolateral view. **B)** palp, retrolateral view. **C)** bulb and embolus. **D)** cephalothorax, dorsal view. **E)** palp, dorsal view. Scale bars: A, B, C, E: 0.5 mm; D: 1 mm.

Description: Male (holotype). **Specimen preparation and condition:** Specimen collected alive and preserved in ethanol 96º. Left pedipalp removed and conserved in a vial with specimen; leg 4 L separated and leg 1 L broken at patella. **Colouration:** Carapace yellowish white, clypeus, pars cephalica and median groove slightly darkened. Conspicuous dark V-mark posteriorly on pars cephalica (Fig. 11D). Carapace with dispersed short black setae. Eye tubercles black. Chelicerae reddish brown, darkened in its distal part. Sternum

bright yellowish, paler than carapace. Labium and gnathocoxae pale reddish brown. Legs yellowish white with the apical segments slightly darkened. Sternum, labium, gnathocoxae and legs covered by long setae interspersed with shorter and thicker setae. Abdomen pale yellowish to greyish, densely coated by short setae. **Cephalothorax:** Carapace slightly longer (2.51) than wide (2.36), truncated behind, widely rounded on sides, narrowed in front. Carapace evenly convex, with median groove moderately deep, elongated, occupying roughly the posterior third. Pars cephalica elongated (1.66 long, 0.76 wide). Large setae or bristles placed in a single row in the side margin of the carapace pointing anteriorly. Most of larger setae of pars cephalica rubbed off to leave integument with conspicuous alveoli, forming seven parallel rows and six radial rows departing from its posterior part, interspersed with shorter setae. Clypeus height 0.226. Eye sizes: ALE 0.19 PME 0.12 PLE 0.14. LE separated from ME by approximately the diameter of ME. Sternum longer (1.41) than wide (1.23). Labium 0.62 long 048 wide at its base, apically narrowed and rounded. Gnathocoxae distally convergent, enclosing the labium. **Abdomen:** Elongate oval (3.18 long 2.32 wide) in dorsal view. **Legs:** Leg formula 2 1 4 3. Leg 1 (19.51): femur 5.4/ patella 0.9/ tibia 5.94/ metatarsus 5.86/ tarsus 1.41. Leg 2 (21.53): 5.8/1.01/6.51/6.76/1.45. Leg 3 (16.56): 4.68/0.97/4.5/5.17/1.24 Leg 4 (17.88): 4.81/0.89/4.91/5.81/1.46. **Male palp:** (Fig. 11 A-C, E) Palpal femur cylindrical (1.29 long, 0.28 wide). Patella subglobular, roughly as long as wide (0.4). Tibia wider at the base (0.82 long, 0.52), paunchy ventrally, rounded more abruptly towards the patella than the tarsus, oval in dorsal view. Tarsus longer (0.5) than bulb (0.4). Bulb globular, slightly compressed dorsally. Maximum bulb width roughly five to six times wider than embolus base. Embolus long, approximately 1.9 times longer than bulb width, gently curved with an apparent bending at third-length and slightly sinuous towards the tip. Long, curved setae facing apically, distributed sparsely in femur, tibia and tarsus, denser on retrolateral side. **Variation:** Supplementary Table 2. CRBA-LX1787: leg 1: 20.61 ; leg 2: 23.72; leg 3: 18.67; leg 4: 19.87; CL: 2.7; CW: 2.7.

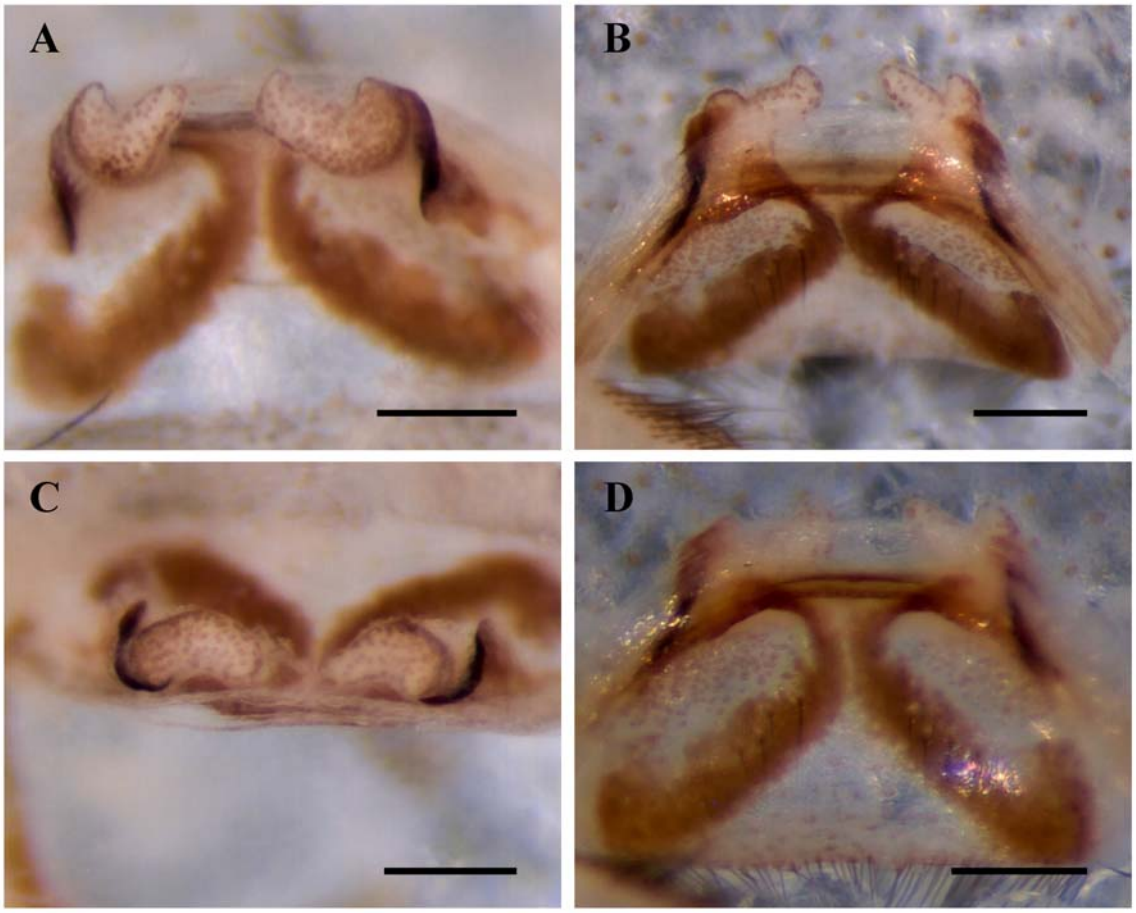

Figure 12 ♀ ***Loxosceles hupalupa* sp. nov.** Vulva, **A)** ventral view. **B)** dorsal view. **C)** anterior view. **D)** posterior view. Scale bars: 0.2 mm.

Female (Paratype (CRBA-LX1789)). **Specimen preparation and condition:** Specimen collected alive and preserved in 96º ethanol. Genitalia removed and conserved in a vial with specimen. Chelicerae, Leg 4 R and 4L removed and missing. **Colouration:** Similar as in male. Carapace yellow-orange, with pars cephalica slighty darkened. Palps and chelicerae reddish. Dark V-shaped pattern in the posterior part of pars cephalica greater than in males. **Cephalothorax:** Carapace slightly longer (3.71) than wide (3.35). Pars cephalica elongated (2.52 long, 1.15 wide). Eye sizes: ALE 0.27 PME 0.16 PLE 0.21. LE separated from ME by 0.17. Sternum longer (2) than wide (1.6). **Abdomen:** Elongate oval (5.42 long 3.46 wide) in dorsal view. **Legs:** Leg formula -. Leg 1(23.09): femur 6.57/patella 1.32/tibia 6.97/metatarsus 6.72/tarsus 1.51 Leg 2 (25.27): 7.15/1.35/7.72/7.6/1.45 Leg 3 (20.58): 6.01/1.39/5.5/6.31/1.37 Leg 4 (-): -/-/-/- **Female genitalia: (**Fig. 12) Atriobursal orifices situated in two large, rounded, almost contiguous pouches, slightly sclerotised around. Base of seminal receptacles strongly conical and wide, reinforced laterally with a curved, dark,

sclerotised band. Medium part short, cylindrical and strongly curved towards the centre. Subtle lobe protruding on its extrernal side. Inner lobe wide, curved, directed towards the center. Seminal receptacles without touching each other, separated at their closer point by more than the apical diameter of the inner lobes. **Variation:** Seminal receptacles may differ in the direction of the inner lobe curvature. Supplementary Table 2. Leg 1 (n = 4): 16.55-24.58 (mean: 21.22); leg 2 (n = 5): 16.41-27.4 (mean: 22.65); leg 3 (n = 4): 13.78-21.54 (mean: 17.75); leg 4 (n = 5): 15.71-24.82 (mean: 19.69); CL (n = 5): 2.89-3.79 (mean: 3.41); CW (n = 5): 2.63-3.38 (mean: 3.1). General colouration ranges from pale-yellow to darker yellow-orange, depending on the time from the last moult. The shape of the dark V-mark also presents some variation in intensity and extent.

Distribution and natural history: Endemic to La Gomera and El Hierro. Known from localities situated in the southern part of La Gomera, where they can be found under big stones, and from a single cave in El Hierro.

Statistical analyses

The graphical representation of leg length / CL illustrates a general tendency towards leg elongation, from ***Loxosceles mahan* sp. nov.** towards ***L. guayota* sp. nov.**, which is more pronounced in males than in females (Fig. 13). Results of the ANCOVA for the variable Leg 1 length showed significant sexual dimorphism ($F_{(1,29)}$ = 94.859, *P* = 1.197e-10) and significant differences between species ($F_{(5,29)}$ = 21.345, *P* = 6.430e-09). A post-hoc Tukey test detected significant differences between ***Loxosceles guayota* sp. nov.** and all the remaining species and between ***L. mahan* sp. nov** and the remaining species except ***L. bentejui* sp. nov.** (*P* = 0.375). Results of the ANCOVA for CW showed significant sexual dimorphism ($F_{1,32}$ = 3.066, *P* = 0.089) and a significant difference between species ($F_{5,32}$=4.743, *P* = 0.002). Post-hoc Tukey tests identified significant differences between ***L. mahan* sp. nov.** and ***L. hupalupa* sp. nov.** (*P* = 0.0172), ***L. mahan* sp. nov.** and ***L. tibicena* sp. nov.** (P = 0.070), ***L. mahan* sp. nov.** and ***L. guayota* sp. nov.** (*P* = 0.0179) and between ***L. guayota* sp. nov.** and ***L. bentejui* sp. nov.** (*P* = 0.0516). By contrast, results of the ANCOVA analysis for male palp segments, showed no significant differences in PFL ($F_{(5,15)}$ = 1.542, *P* = 0.236), PPL ($F_{(5,15)}$ = 0.907, *P* = 0.502) and PTAL ($F_{(5,15)}$ = 1.354, *P* = 0.296) and only slight significant differences in PTL between ***L. hupalupa* sp. nov.** and ***L. mahan* sp. nov.** (*P* = 0.025), and ***L. hupalupa* sp. nov.** and ***L. tazarte* sp. nov.** (*P* = 0.027).

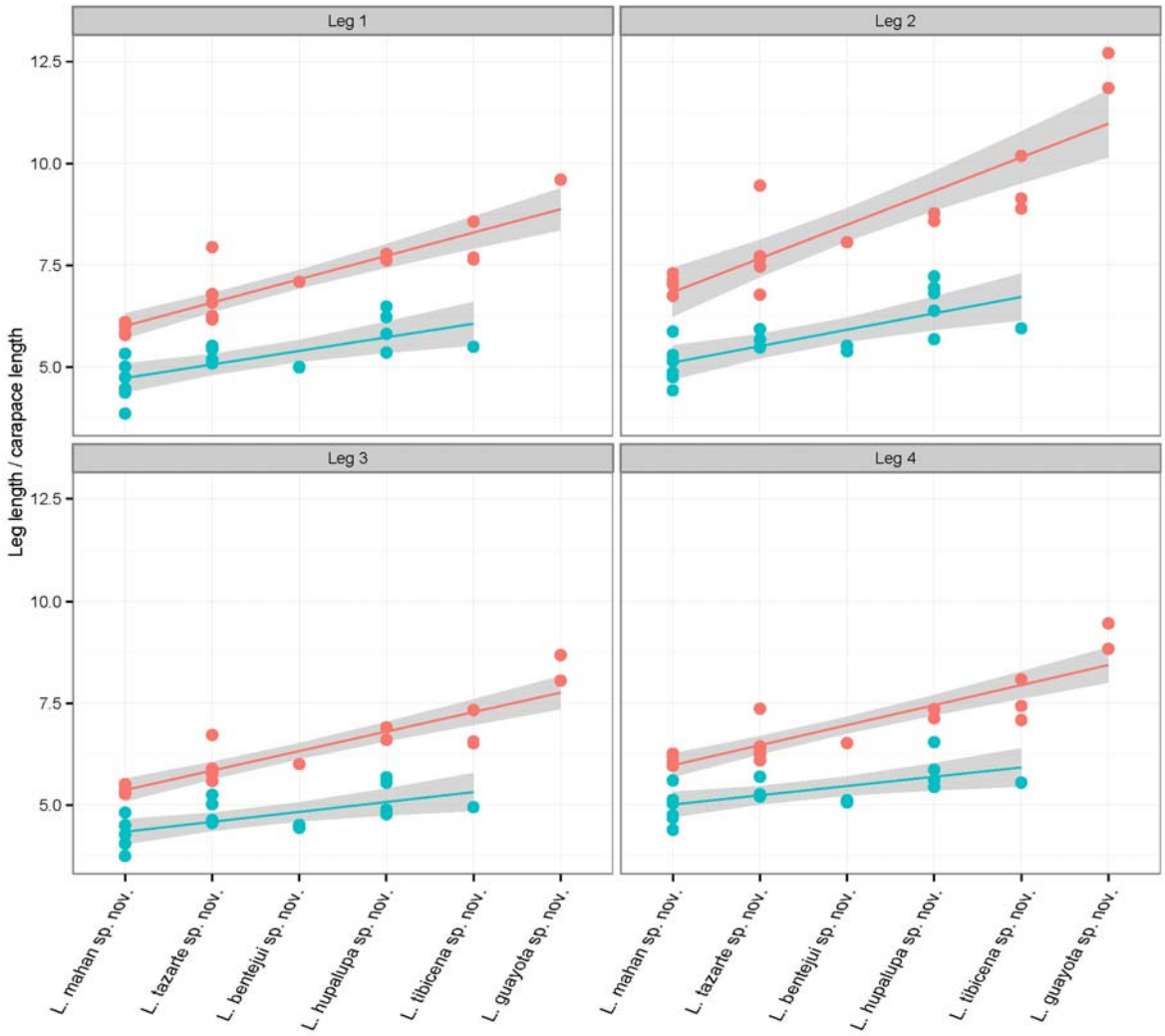

Figure 13 Graphical representation of the leg length / carapace length ratio variation for Leg 1 to Leg 4. Females are coloured in green and males in red. Continuous line corresponds to the linear regression for each sex and leg, with the 95% confidence interval in grey.

Its2 networks

The its2 dataset included 62 individuals; 40 belonging to ***L. mahanensis* sp. nov.**; 11 to ***L. tazarte* sp. nov.**; four to ***L. bentejui* sp. nov.**; one to ***L. tibicena* sp. nov.**; one to ***L. guayota* sp. nov.** and five to ***L. hupalupa* sp. nov.** Haplotype networks of the its2 sequences are presented in Fig. 14. Reconstruction of the haplotype network for each species resulted in one independent network, except for ***L. tazarte* sp. nov.**, which resulted in two independent networks. As in mtDNA, its2 showed a high level of genetic differentiation, with all the its2 haplotypes being private for each species. During manual editing of the sequences, we observed some cases that showed evidence of multiple copies of its2 due to insertion or

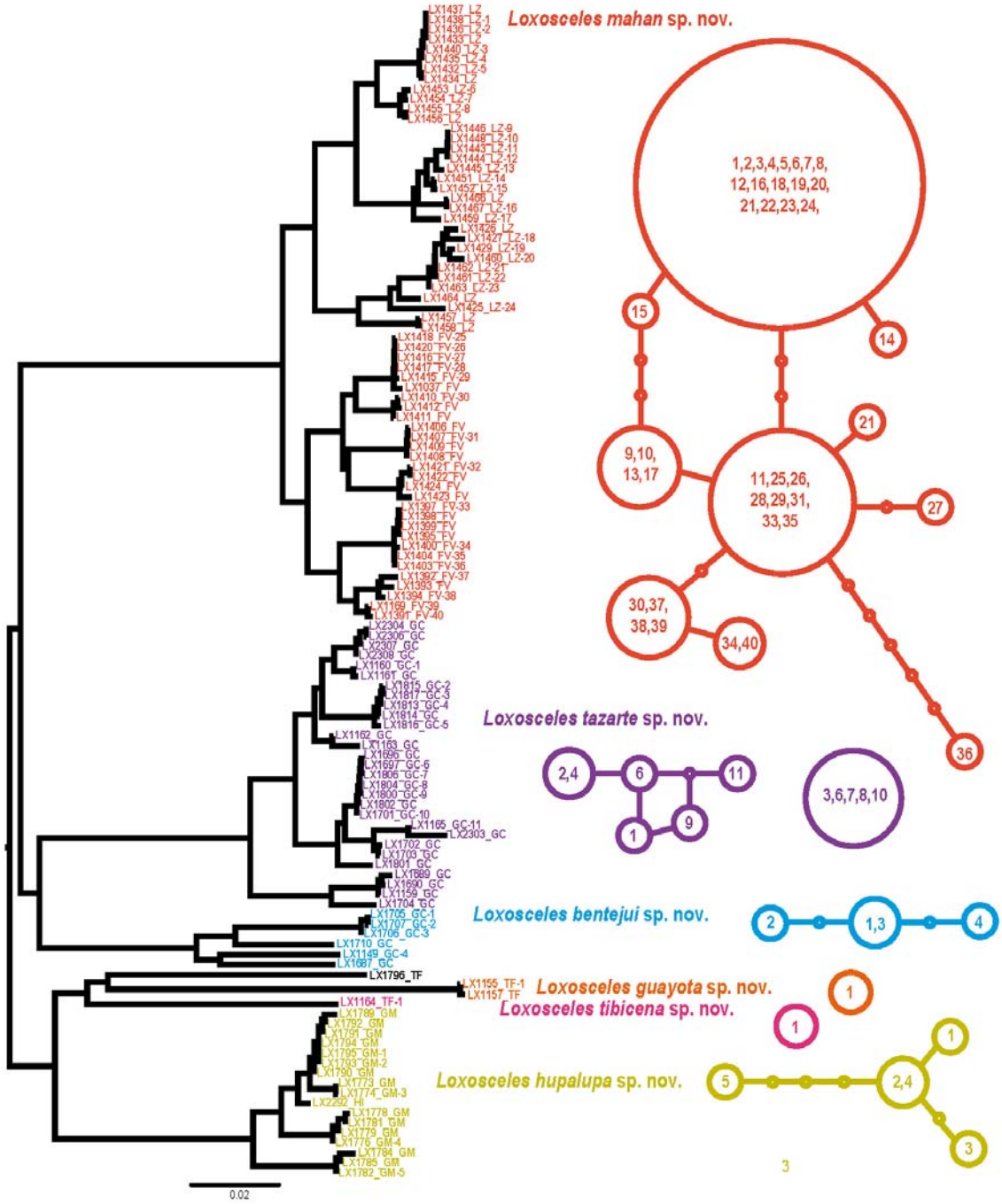

Figure 14 Neighbour-joining tree constructed with the *cox1* sequences of the Canary Islands endemic *Loxosceles* species. Codes at the tips correspond to specimen code, followed by island name abbreviation and number referring to the haplotype network. Haplotype networks are drawn using the its2 sequences. Each circle represents a haplotype, and numbers within the circles correspond to those in the NJ tree.

deletion of nucleotides, a phenomenon that has already been reported in other spider groups (e.g., Macías-Hernández *et al.*, 2013). To avoid confusion induced by this situation, we conducted the analyses by treating gaps as missing data, and thus only accounting for nucleotide substitutions; therefore, we most likely underestimated the existent diversity in its2.

DNA barcoding

The *cox1* dataset for DNA barcoding analyses included 124 individuals; 62 belonging to ***L. mahanensis* sp. nov.**, 29 to ***L. tazarte* sp. nov.**, six to ***L. bentejui* sp. nov.**, one to *L.* sp. TF1; one to ***L. tibicena* sp. nov.**, two to ***L. guayota* sp. nov.**, 17 to ***L. hupalupa* sp. nov.** and six to *L. rufescens*. The existence of the "barcoding gap", that is, a disjunction between the maximum intraspecific genetic distance against the nearest interspecific distance, was confirmed visually (Fig. 15). All dots fall above the 1:1 line, meaning that in all cases, the maximum intraspecific genetic distance is lower than the distance to the nearest neighbour. The optimum threshold for this dataset was between 5.5 and 9.7 % K2P distance, where the identification error was 0 %. Identification accuracy was tested with two different measures: the NN and the BOLD criteria (1% divergence threshold). The NN resulted in 100 % identification success in species represented by more than one sequence in the reference library. By contrast, the BOLD criterion performed worse, producing seven false positive errors (*sensu* Meyer & Paulay, 2005). Diagnostic nucleotide positions were found in all the new species, as well as for *L. rufescens* and are indicated in the diagnosis description section (see above) and in the Supplementary Fig. 1.

DISCUSSION

Systematics remarks

Delimitation of species boundaries in spiders, as in many other arthropods, is mainly based on genitalia. This approach is more complicated in spider groups such as haplogyne or mygalomorph spiders, which are characterised by relatively simple genitalic features (Hendrixson *et al*., 2012). *Loxosceles* spiders represent a good example of such simplicity, with only subtle variation in genitalic morphology among closely related species (Gertsch & Ennik, 1983). The six endemic species described herein are no exception, and despite being clearly distinct genetically, they are generally difficult to distinguish on the basis of morphology. In males, major differences, although scarce, were concentrated in embolus

shape, whereas females presented evident differences in the general shape of the seminal receptacles. These results are in accordance with differences found between closely related species in other *Loxosceles* species groups where, surprisingly, in many cases, females presented more readily diagnostic characters than males (Gertsch 1958; 1967; Gertsch & Ennik, 1983). By contrast, molecular data showed clear differences between species, with uncorrected genetic distances between the newly described species for *cox1* greater than 12 % (Planas & Ribera, 2014), the presence of a clear "barcoding gap" (Fig. 15) and nucleotide diagnostic positions in the *cox1* (Supplementary Fig. 1), and finally, divergent, private its2 haplotypes (Fig. 14). Thus, the species hypotheses formulated herein are supported by three alternative operational criteria under the unified species concept (de Queiroz, 2007).

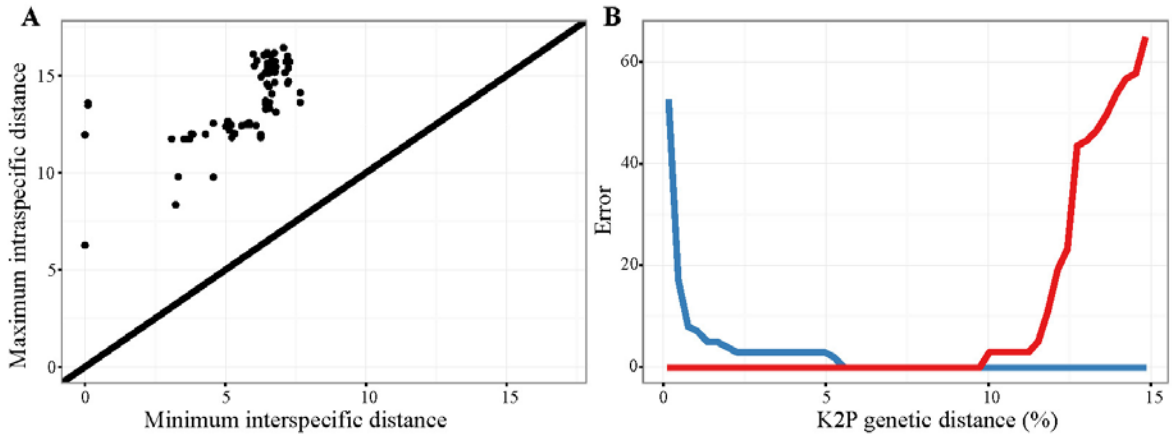

Figure 15 A) graphical representation of the barcode gap comparing the minimum interspecific K2P distance to the maximum intraspecific K2P distance. Values above the 1:1 line indicate the presence of a "barcode gap". **B)** graphical representation of the identification error. False positives in blue and false negatives in red. The optimum threshold value is between 5.5 and 9.8% KSP distance, where error is 0%.

We refrained from describing one evolutionary lineage, *Loxosceles* sp. TF-1 from Tenerife, delimited in the previous study (Planas & Ribera, 2014), as a nominal species. Only one juvenile from a single locality was collected from this lineage; therefore it was not possible to study its genitalia. Although this individual presented the characteristic dark V-shaped mark, in agreement with the morphology of the other endemic species, and its mtDNA sequence shows diagnostic nucleotide positions, thus fulfilling the requirements of the International Code of Zoological Nomenclature (ICZN) for a valid diagnosis and description of a new species (Brower, 2010; Cook *et al*., 2010), we decided not to formally

describe this putative species until additional adult specimens were collected from the Anaga region in Tenerife, where this individual was found. Not providing a formal description of this lineage, that is, leaving it as a "dark taxon" *sensu* Page (2011) is not without consequences. Species are the main unit in most biological fields, such as conservation biology or biogeography, and therefore, by failing to provide a description of this putative species, we are underestimating the current diversity found in Tenerife, affecting essentially related downstream studies.

The Canarian species described herein, together with the aforementioned *Loxosceles* sp. TF-1, formed a monophyletic group in phylogenetic analyses conducted with multiple molecular markers (Planas & Ribera, 2014). All these species were also clearly distinguished by morphological characters, with the presence of a dark V-shaped mark at the posterior part of the pars cephalica being the most conspicuous somatic one. This character is not present in any *L. rufescens* s.l. representatives, the sister group to the Canarian clade (Planas & Ribera, 2014); in contrast, *L. mrazig*, the other Mediterranean *Loxosceles* species from Tunisia, exhibits similar dark marks, although smaller and less conspicuous (Ribera & Planas, 2009). Based on genitalic characters, Gertsch (1958, 1967; Gertsch & Ennik, 1983) recognised different species groups, that resulted as monophyletic in a phylogenetic study conducted with molecular data (Binford *et al*., 2008). Given the molecular phylogeny in Planas & Ribera (2014) and the morphological features commented on above (e.g., genitalic characters), it seems reasonable to include the six new species described herein to the *L. rufescens* species group (Gertsch, 1983, Binford *et al*., 2008).

Insular gigantism or troglomorphism?

Many evolutionary studies have been conducted in islands, leading to the discovery of interesting patterns, such as the "island-rule" (Van Valen, 1973). This term was coined to describe the tendency towards gigantism of small island mammals, or towards dwarfism of large island mammals. Despite being an appealing general rule, phylogenetic comparative analyses showed that this hypothesis is not always supported (Meiri, Dayan & Simberloff, 2006; Meiri, Cooper & Purvis, 2008; McClain & Durst, 2013; Itescu *et al*., 2014). To our knowledge, the "island-rule" has never been explicitly tested in spiders, although several examples of insular gigantism exist, such as the *Orsonwelles* spiders in Hawaii (Hormiga, 2002) or the *Laminacauda gigas* Millidge, 1991 in the Juan Fernández archipelago (Berland, 1924). In the Canary Islands, the highly diverse genus *Dysdera* also presents

examples of gigantism, for example *D. arabisenen* Arnedo & Ribera, 1997, and *D. insulana* Simon, 1883, and of dwarfism, for example *D. minutisima* Wunderlich, 1992 and *D. andamanae* Arnedo & Ribera, 1997. The most outstanding example within the Canary Islands *Loxosceles* is ***L. guayota* sp. nov.**, endemic to Tenerife. This species has a larger body length, with a carapace width significantly higher than the other species, and it presents a pronounced leg elongation (Fig. 13). Interestingly, the tendency towards leg elongation, exemplified by ***L. mahan* sp. nov.** from Fuerteventura and Lanzarote, the species with the lowest Ll/CL proportion (Leg length/Carapace length) and ***L. guayota* sp. nov.** the species with the highest Ll/CL ratio, follows an old to young succession. If we could rigorously relate this tendency to an "island-rule" effect, then we probably should reject the hypothesised model of colonisation from Tenerife to La Gomera (Planas & Ribera, 2014) to an inverse direction, that is, from La Gomera to Tenerife. Nevertheless, given our limited data, it is premature to consider this trend as an example of insular gigantism, and further studies,which should include additional specimens and continental relatives, should be conducted to test this hypothesis. However, in interpreting these results, we should consider troglomorphism as a possible confounding factor. *Loxosceles* are generally considered troglophiles, because of their abundance in caves; in the Canary Islands, at least some individuals of all the endemic species were found in volcanic tubes, and several individuals were also collected in mesocavernous shallow substratum (MSS) traps. Interestingly, ***Loxosceles guayota* sp. nov.** is the only species exclusively collected in volcanic tubes; thus, the extraordinary leg elongation observed in this species could be related to troglomorphism rather to an effect of insular gigantism.

DNA barcoding as a tool for fast and reliable identification of medically relevant groups

Although *Loxosceles* bites are rare and accidental, loxoscelism can result in serious medical complications. A crucial step needed in order to develop proper health management is correct identification of the species involved in bite accidents. DNA barcoding has been shown to be a fast and reliable method for accurate species identification (Robinson *et al*., 2009), but importantly, its accuracy relies on previous taxonomic knowledge, necessary for providing a reference library. This knowledge is also useful to critically interpret automatic identification protocols provided by services as BOLD, which delivers a species identification if the query sequence shows a tight match, with less than 1% divergence, to a

reference sequence (Ratnasingham & Hebert, 2007). We showed that the threshold divergence used by default in BOLD (i.e., 1%) was significantly lower than the empirically optimized threshold of 5.5 % for an accurate species identification in Canary Island *Loxosceles*. This result is in accordance with previous studies (Puillandre *et al*., 2011; Virgilio *et al*., 2012) and should be considered when the objective of the DNA barcoding is "species discovery" rather than "species identification" (Collins & Cruickshank, 2013). Nevertheless, DNA-based identification is advantageous for many reasons, especially because it allows the identification of individuals independent of sex and age, a common limitation when identifying spiders. Additionally, mtDNA genes such as *cox1* are easy to amplify even in individuals collected under poor conservation conditions (Vink, Thomas & Paquin, 2005), as could be the case in spiders collected in situations related to bites. Consequently, the application of DNA barcoding in identifying *Loxosceles* spiders would certainly help to improve the current knowledge of their medical importance.

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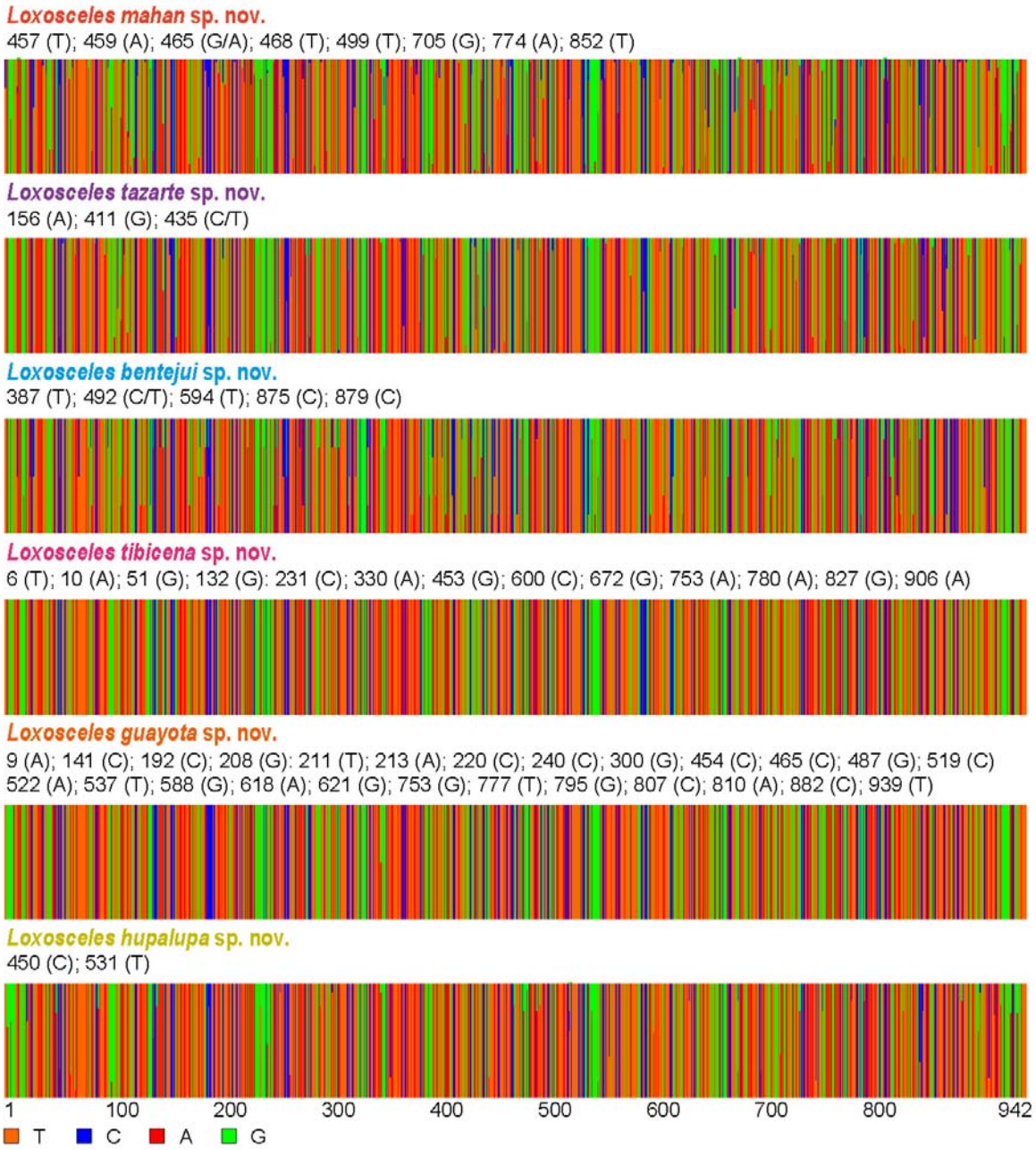

Supplementary Figure 1 Graphical representation of the DNA barcoding with diagnostic nucleotide positions for the endemic Canary Islands *Loxosceles* species described in this study.

Supplementary Table 1 Study material. A list of the specimens used for this study for the molecular analyses or listed in the taxonomic part, with their identification code, sex (M, male; F, female; J, juvenile), type status (H, holotype; P, paratype), date of collection, locality name, coordinates, and MorhoBank and GenBank accession numbers. Next to specimen code of the individuals included in the Arachnid Collection of the Centre de Recursos de Biodiversitat Animal (CRBA) at the University of Barcelona (UB) the geographical region is provided (GC, Gran Canaria; TF, Tenerife; HI, El Hierro; GM, La Gomera; FV, Fuerteventura; LZ, Lanzarote; LP, La Palma).

CODE	SEX	T.M.	DATE	LOCALITY	LATITUDE	LONGITUDE	MORPHOBANK	COI	ITS2
***Loxosceles mahan* sp. nov.**									
ULL-DZUL-34170 (LX1069_FV)			03/04/2004	Cofete	28.10265	-14.38232		KF669916	
ULL-DZUL-34172	M		01/04/2004	Montaña de Vallebrón					
LX1394_FV			03/02/2010	Cofete	28.10265	-14.38232		KF669931	KF670114
LX1392_FV			03/02/2010	Península Jandía	28.0854	-14.37196		KF669929	KF670113
LX1393_FV	Fj		03/02/2010	Península Jandía	28.0854	-14.37196		KF669930	
LX1391_FV	Mj		03/02/2010	Tequital	28.26339	-13.94874		KF669928	KF670112
LX1169_FV	Fj		05/12/2002	Tuineje	28.277677	-13.950876		KF669927	KF670111
LX1395_FV	J		03/03/2010	Cueva Tiscamanitas	28.33501	-13.99525		KF669932	
LX1397_FV	J		03/03/2010	Cueva Tiscamanitas	28.33501	-13.99525		KF669933	X
LX1398_FV	J		03/03/2010	Cueva Tiscamanitas	28.33501	-13.99525		KF669934	
LX1399_FV	J		03/03/2010	Cueva Tiscamanitas	28.33501	-13.99525		KF669935	
LX1400_FV	J		03/03/2010	Cueva Tiscamanitas	28.33501	-13.99525		KF669936	KF670115
LX1403_FV	Mj		03/03/2010	Cueva Tiscamanitas	28.33501	-13.99525		KF669937	X
LX1404_FV	F		03/03/2010	Cueva Tiscamanitas	28.33501	-13.99525		KF669938	X
LX1406_FV	J		03/04/2010	Aguas Verdes	28.47845	-14.05128		KF669939	
LX1407_FV	Fj		03/04/2010	Aguas Verdes	28.47845	-14.05128		KF669940	X
LX1408_FV	Fj		03/04/2010	Aguas Verdes	28.47845	-14.05128		KF669941	
LX1409_FV	Fj		03/04/2010	Aguas Verdes	28.47845	-14.05128		KF669942	
LX1421_FV	Mj		03/05/2010	Calderilla de Roja	28.62996	-13.83421		KF669951	X
LX1422_FV	Fj		03/05/2010	Calderilla de Roja	28.62996	-13.83421		KF669952	
LX1423_FV	Fj		03/05/2010	Calderilla de Roja	28.62996	-13.83421		KF669953	
LX1424_FV	Fj		03/05/2010	Calderilla de Roja	28.62996	-13.83421		KF669954	
LX1410_FV	J		03/04/2010	Villaverde	28.65061	-13.91518		KF669943	X
LX1411_FV	Fj		03/04/2010	Villaverde	28.65061	-13.91518		KF669944	
LX1412_FV	F		03/04/2010	Villaverde	28.65061	-13.91518		KF669945	
LX1413_FV	F		03/04/2010	Villaverde	28.65061	-13.91518			
LX1415_FV	J		03/05/2010	Lobos	28.7439	-13.82542		KF669946	X
LX1416_FV	J		03/05/2010	Lobos	28.7439	-13.82542		KF669947	X
LX1417_FV	Fj		03/05/2010	Lobos	28.7439	-13.82542		KF669948	X
LX1418_FV	F		03/05/2010	Lobos	28.7439	-13.82542		KF669949	KF670116
LX1037_FV	F		30/03/2004	Lobos	28.75205	-13.83010		KF669915	
LX1037-2_FV	F		30/03/2004	Lobos	28.75205	-13.83010			

LX1420_FV	Fj		03/05/2010	Caldera Lobos	28.75205	-13.83010		KF669950	KF670117
LX1426_LZ	J		06/03/2010	Costa Papagayo	28.86038	-13.77626		KF669956	
LX1427_LZ	J		06/03/2010	Costa Papagayo	28.86038	-13.77626		KF669957	KF670119
LX1428_LZ	M	H	06/03/2010	Costa Papagayo	28.86038	-13.77626	M326598-M326603		
LX1429_LZ	Fj		06/03/2010	Costa Papagayo	28.86038	-13.77626		KF669958	X
LX1430_LZ	F	P	06/03/2010	Costa Papagayo	28.86038	-13.77626			
LX1431_LZ	F	P	06/03/2010	Costa Papagayo	28.86038	-13.77626	M326604-M326608		
SMF-29348)	M	P	11/1972	Costa Papagayo					
LX1425_LZ	M		06/03/2010	Salinas de Janubio	28.94288	-13.81886		KF669955	KF670118
LX1460_LZ	J		09/03/2010	Puerto Calero	28.93313	-13.70168		KF669981	X
LX1461_LZ	J		09/03/2010	Puerto Calero	28.93313	-13.70168		KF669982	X
LX1462_LZ	J		09/03/2010	Puerto Calero	28.93313	-13.70168		KF669983	X
LX1463_LZ	J		09/03/2010	Puerto Calero	28.93313	-13.70168		KF669984	KF670125
LX1464_LZ	J		09/03/2010	Tinajo	29.0614	-13.6777		KF669985	
LX1465_LZ	M		09/03/2010	Tinajo	29.0614	-13.6777			
LX1457_LZ	J		07/03/2010	Costa Teguise	29.02955	-13.51675		KF669978	
LX1458_LZ	J		07/03/2010	Costa Teguise	29.02955	-13.51675		KF669979	
LX1432_LZ	J		07/03/2010	Los Valles	29.07914	-13.52484		KF669959	X
LX1433_LZ	J		07/03/2010	Los Valles	29.07914	-13.52484		KF669960	
LX1434_LZ	J		07/03/2010	Los Valles	29.07914	-13.52484		KF669961	
LX1435_LZ	J		07/03/2010	Los Valles	29.07914	-13.52484		KF669962	X
LX1436_LZ	J		07/03/2010	Los Valles	29.07914	-13.52484		KF669963	X
LX1437_LZ	J		07/03/2010	Los Valles	29.07914	-13.52484		KF669964	
LX1438_LZ	J		07/03/2010	Los Valles	29.07914	-13.52484		KF669965	X
LX1440_LZ	F		07/03/2010	Los Valles	29.07914	-13.52484		KF669966	KF670120
LX1466_LZ	J		09/03/2010	Teguise	29.09475	-13.55653		KF669986	
LX1467_LZ	J		09/03/2010	Teguise	29.09475	-13.55653		KF669987	X
LX1453_LZ	J		07/03/2010	Barranco Hondo del Valle	29.14139	-13.48203		KF669974	X
LX1454_LZ	J		07/03/2010	Barranco Hondo del Valle	29.14139	-13.48203		KF669975	KF670123
LX1455_LZ	J		07/03/2010	Barranco Hondo del Valle	29.14139	-13.48203		KF669976	X
LX1456_LZ	F		07/03/2010	Barranco Hondo del Valle	29.14139	-13.48203		KF669977	
LX1451_LZ	J		07/03/2010	Guinate	29.17536	-13.50576		KF669972	X
LX1452_LZ	J		07/03/2010	Guinate	29.17536	-13.50576		KF669973	KF670122
LX1443_LZ	J		07/03/2010	Mirador del Río	29.21336	-13.48111		KF669967	X

CODE	SEX	T.M.	DATE	LOCALITY	LATITUDE	LONGITUDE	MORPHOBANK	COI	ITS2
LX1444_LZ	J		07/03/2010	Mirador del Río	29.21336	-13.48111		KF669968	X
LX1445_LZ	J		07/03/2010	Mirador del Río	29.21336	-13.48111		KF669969	KF670121
LX1446_LZ	Mj		07/03/2010	Mirador del Río	29.21336	-13.48111		KF669970	X
LX1448_LZ	F		07/03/2010	Mirador del Río	29.21336	-13.48111		KF669971	X
LX1450_LZ	F		07/03/2010	Mirador del Río	29.21336	-13.48111			
LX1459_LZ	J		08/03/2010	La Graciosa	29.2472	-13.518		KF669980	KF670124
LX2300_LZ	M		24/02/1995	La Graciosa					
LX2301_LZ	M		24/02/1995	La Graciosa					
***Loxosceles tazarte* sp. nov.**									
ULL-DZUL-34180	M		13/04/2003	Los Majaletes - Cazadores					
LX1704_GC	J		13/12/2010	Ladera de los Pinos	27.8064	-15.58276		KF669996	
LX1813_GC	J		27/02/2011	Los Corralillos	27.883766	-15.45515		KF670023	KF670132
LX1814_GC	J		27/02/2011	Los Corralillos	27.883766	-15.45515		KF670024	
LX1815_GC	J		27/02/2011	Los Corralillos	27.883766	-15.45515		KF670025	X
LX1816_GC	J		27/02/2011	Los Corralillos	27.883766	-15.45515		KF670026	X
LX1817_GC	J		27/02/2011	Los Corralillos	27.883766	-15.45515		KF670027	X
LX1818_GC	M		27/02/2011	Los Corralillos	27.883766	-15.45515			
LX1160_GC	F		25/04/2009	Cueva Santa Lucía	27.91022	-15.52989		KF669921	KF670108
LX1161_GC	J		25/04/2009	Cueva Santa Lucía	27.91022	-15.52989		KF669922	
LX1162_GC	J		25/04/2009	Cueva Santa Lucía	27.91022	-15.52989		KF669923	
LX1163_GC	J		25/04/2009	Cueva Santa Lucía	27.91022	-15.52989		KF669924	
LX1159_GC	J		26/04/2009	Cueva de las Niñas	27.92462	-15.67237		KF669920	
LX1688_GC	M		12/12/2010	Cueva de las Niñas	27.92462	-15.67237			
LX1689_GC	F		12/12/2010	Cueva de las Niñas	27.92462	-15.67237		KF669989	
LX1690_GC	J		12/12/2010	Cueva de las Niñas	27.92462	-15.67237		KF669990	
ULL-DZUL-34177 (LX1165_GC)	M	H	28/12/2008	Barranco del Draguillo	27.945994	-15.446264	M326610-M326615	KF669926	KF670110
ULL-DZUL-34181 (LX2302_GC)	F	P	26/12/2010	Barranco del Draguillo	27.945994	-15.446264	M326616-M326621		
ULL-DZUL-34182 (LX2303_GC)	M	P	26/12/2010	Barranco del Draguillo	27.945994	-15.446264		KF670029	
LX1702_GC	J		13/12/2010	Montaña Togüela	27.962383	-15.46426		KF669994	
LX1703_GC	J		13/12/2010	Montaña Togüela	27.962383	-15.46426		KF669995	
ULL-DZUL-34183 (LX2304_GC)	J		31/03/2012	Llanos de la Pez	27.964311	-15.585547		KF670030	
ULL-DZUL-34184	F		31/03/2012	Llanos de la Pez	27.964311	-15.585547		KF670031	
ULL-DZUL-34185 (LX2306_GC)	F		31/03/2012	Llanos de la Pez	27.964311	-15.585547		KF670031	

ULL-DZUL-34186 (LX2307_GC)	F		31/03/2012	Llanos de la Pez	27.964311	-15.585547		KF670032	
ULL-DZUL-34187 (LX2308_GC)	F		31/03/2012	Llanos de la Pez	27.964311	-15.585547		KF670033	
ULL-DZUL-34178	M			Cabecera Valle Temisa	29.90928	-15.51331			
LX1692_GC	M		13/12/2010	La Isleta	28.17405	-15.418816			
LX1693_GC	F		13/12/2010	La Isleta	28.17405	-15.418816			
LX1696_GC	J		13/12/2010	La Isleta	28.17405	-15.418816		KF669991	
LX1697_GC	J		13/12/2010	La Isleta	28.17405	-15.418816		KF669992	X
LX1701_GC	J		13/12/2010	La Isleta	28.17405	-15.418816		KF669993	KF670126
LX1800_GC	J		13/12/2010	La Isleta	28.17405	-15.418816		KF670018	X
LX1801_GC	J		13/12/2010	La Isleta	28.17405	-15.418816		KF670019	
LX1802_GC	J		13/12/2010	La Isleta	28.17405	-15.418816		KF670020	
LX1804_GC	J		13/12/2010	La Isleta	28.17405	-15.418816		KF670021	X
LX1806_GC	J		13/12/2010	La Isleta	28.17405	-15.418816		KF670022	X
***Loxosceles bentejui* sp. nov**									
LX1687_GC	J		11/12/2010	Acusa Seca	28.013833	-15.674466		KF669988	
LX1705_GC	J		14/12/2010	El Risco	28.0601	-15.7296		KF669997	X
LX1706_GC	J		14/12/2010	El Risco	28.0601	-15.7296		KF669998	X
LX1707_GC	Fj		14/12/2010	El Risco	28.0601	-15.7296		KF669999	KF670127
LX1708_GC	F	P	14/12/2010	El Risco	28.0601	-15.7296	M326628-M326634		
LX1709_GC	F	P	14/12/2010	El Risco	28.0601	-15.7296			
LX1710_GC	J		14/12/2010	El Risco	28.0601	-15.7296		KF670000	
LX1711_GC	M	H	14/12/2010	El Risco	28.0601	-15.7296	M326622-M326627		
ULL-DZUL-34188 (LX1149_GC)	J		09/05/2004	Agaete	28.067428	-15.660415		KF669917	KF670106
SMF-XXX (LX1126_GC)	F		1/12/2003	Barranco de Guayedra	28.08075	-15.70475			
***Loxosceles* sp. Tenerife 1**									
LX1796_TF	J		20/02/11	Barranco de las Huertas	28.521333	-16.167816		KF670017	
***Loxosceles tibicena* sp. nov.**									
ULL-DZUL-34197 (LX1164_TF)	M	H	05/02/2007	Cumbre Arico	28.249216	-16.528747	M326635-M326640		
ULL-DZUL-34202 (LX1164_TF)	F	P	05/02/2007	Cumbre Arico	28.249216	-16.528747	M326641-M326645	KF669925	KF670109
ULL-DZUL-34199	M		23/03/1985	Barranco del Río, Lomo Largo, Arico	28.18736	-16.58227			
ULL-DZUL-34200	M		23/03/1985	Barranco del Río, Lomo Largo, Arico	28.18736	-16.58227			
ULL-DZUL-34201	M		23/03/1985	Barranco del Río, Lomo Largo, Arico	28.18736	-16.58227			
***Loxosceles guayota* sp. nov.**									

CODE	SEX	T.M.	DATE	LOCALITY	LATITUDE	LONGITUDE	MORPHOBANK	COI	ITS2
LX1155_TF	M	P	22/04/2009	Los Roques	28.236254	-16.642505		KF669918	KF670107
LX1156_TF	M	H	22/04/2009	Los Roques	28.236254	-16.642505	M326646-M326651		
LX1157_TF	Fj		22/04/2009	Los Roques	28.236254	-16.642505		KF669919	
***Loxoscele hupalupa* sp. nov.**									
SMF-29479	1Mj		29/5/1905	Santiago					
LX1782_GM	J		16/02/2011	Tapagache	28.084133	-17.289016		KF670007	KF670130
LX1784_GM	Fj		16/02/2011	Tapagache	28.084133	-17.289016		KF670008	
LX1785_GM	F		16/02/2011	Tapagache	28.084133	-17.289016		KF670009	
LX1776_GM	J		15/02/2011	Igualero	28.087616	-17.256866		KF670003	KF670129
LX1778_GM	F		15/02/2011	Igualero	28.087616	-17.256866		KF670004	
LX1779_GM	Mj		15/02/2011	Igualero	28.087616	-17.256866		KF670005	
LX1781_GM	F		15/02/2011	Igualero	28.087616	-17.256866		KF670006	
LX1773_GM	Fj		15/02/2011	Barranco del Paijén	28.08845	-17.19955		KF670001	
LX1774_GM	F		15/02/2011	Barranco del Paijén	28.08845	-17.19955		KF670002	KF670128
LX1786_GM	M	H	17/02/2011	Playa de Ávolo	28.114683	-17.113166	M326652-M326657		
LX1787_GM	M	P	17/02/2011	Playa de Ávolo	28.114683	-17.113166			
LX1789_GM	F	P	17/02/2011	Playa de Ávolo	28.114683	-17.113166	M326658-M326663	KF670010	
LX1790_GM	J		17/02/2011	Playa de Ávolo	28.114683	-17.113166		KF670011	
LX1791_GM	J		17/02/2011	Playa de Ávolo	28.114683	-17.113166		KF670012	
LX1792_GM	J		17/02/2011	Playa de Ávolo	28.114683	-17.113166		KF670013	
LX1793_GM	J		17/02/2011	Playa de Ávolo	28.114683	-17.113166		KF670014	X
LX1794_GM	J		17/02/2011	Playa de Ávolo	28.114683	-17.113166		KF670015	
LX1795_GM	J		17/02/2011	Playa de Ávolo	28.114683	-17.113166		KF670016	KF670131
ULL-DZUL-34165	F		16/11/1985	Cueva del Linke	27.653012	-17.981626			
ULL-DZUL-34166 (LX2292_HI)	F		16/11/1985	Cueva del Linke	27.653012	-17.981626		KF670028	
ULL-DZUL-34167 (LX2293_HI)	F		16/11/1985	Cueva del Linke	27.653012	-17.981626			
ULL-DZUL-34168	F		16/11/1985	Cueva del Linke	27.653012	-17.981626			
Loxosceles rufescens									
ULL-DZUL-34175 (LX1068_GC)				Firgas	28.107403	-15.563147		KF716999	KF717028
ULL-DZUL-34176 (LX1599_GC)				Subida San Felipe	28.137199	-15.58452		KF717009	KF717036
LX1797_TF				Buenavista	28.376966	-16.848883		KF717010	KF717037
LX1593_HI				Cueva de la Curva	27.692348	-17.972877		KF717008	KF717035
ULL-DZUL-34190 (LX1180_LP)				Cueva del Ratón	28.468916	-17.850893		KF717000	KF717029
ULL-DZUL-34192 (LX1188_LP)				Cueva del Diablo				KF717001	

Supplementary Table 2. Leg formula, leg length, carapace length (CL), and carapace width (CW) measurements of the six new species endemic to the Canary Islands. Mean (minimum - maximum). * n-1.

Species	Sex	Leg formula	Leg 1	Leg 2	Leg 3	Leg 4	CL	CW
***L. mahan* sp. nov.**	♂ n = 6	2 4 1 3	16.52 (13.88 - 18.46)	19.34 * (16.07 - 21.77)	14.86 (13.02 - 16.1)	16.84 (14.91 - 18.22)	2.767 (2.381 - 3.054)	2.5 (2.135 - 2.766)
	♀ n = 6	2 4 1 3	14.81 (10.69 - 22.86)	16.18 (12.27 - 25.19)	13.54 (10.38 - 20.64)	15.75 (12.17 - 24.03)	3.162 (2.772 - 4.290)	2.704 (2.428 - 3.576)
***L. tazarte* sp. nov.**	♂ n = 7	2 1 4 3	18.3 (14.71 - 24.09)	21.14 * (17.01 - 28.68)	16.46 (13.51 - 20.38)	17.68 * (15.09 - 22.34)	2.73 (2.343 - 3.208)	2.504 (2.202 - 2.881)
	♀ n = 4	2 4 1 3	15.32 (14.16 - 16.3)	16.36 * (15.45 - 17.84)	14.03 (13.06 - 15.38)	15.76 (14.83 - 16.68)	2.889 (2.604 - 3.143)	2.61 (2.427 - 2.783)
***L. bentejui* sp. nov.**	♂ n = 1	2 1 4 3	20.63	23.46	17.46	18.96	2.908	2.603
	♀ n = 2	2 4 1 3	16.43 (16.08 - 16.77)	17.93 (17.81 - 17.98)	14.7 (14.54 - 14.86)	16.73 (16.5 - 16.96)	3.287 (3.224 - 3.350)	2.810 (2.791 - 2.83)
***L. tibicena* sp. nov.**	♂ n = 4	2 1 4 3	22.04 * (20.18 - 23.82)	26.03 * (23.34 - 28.49)	18.82 (17.11 - 20.45)	20.81 * (19.5 - 22.09)	2.779 (2.578 - 3.118)	2.638 (2.375 - 2.745)
	♀ n = 1	2 4 1 3	18.41	19.93	17.75	19.69	3.407	3.054
***L. guayota* sp. nov.**	♂ n = 2	2 1 4 3	34.78 *	42.92 (42.92 - 42.93)	29.24 (29.17 - 29.31)	31.97 (31.94 - 32)	3.499 (3.376 - 3.622)	3.314 (3.272 - 3.355)
***L. hupalupa* sp. nov.**	♂ n = 2	2 1 4 3	20.06 (19.51 - 20.61)	22.62 (21.53 - 23.72)	17.61 (16.55 - 18.66)	18.87 (17.87 - 19.87)	2.605 (2.507 - 2.703)	2.529 (2.361 - 2.697)
	♀ n = 5	2 4 1 3	21.22 * (16.55 - 24.58)	22.65 (16.41 - 27.4)	17.75 * (13.78 - 21.54)	19.69 (15.71 - 24.82)	3.407 (2.887 - 3.792)	3.054 (2.632 - 3.383)

Desenvolupament de nous marcadors microsatèl·lits pel gènere d'aranyes *Loxosceles* (Sicariidae) utilitzant tecnologies de seqüenciació de nova generació

RESUM

En aquest estudi s'explica pas per pas el procés de desenvolupament *de novo* de marcadors microsatèl·lits (SSRs) en dues espècies d'aranyes *Loxosceles*. Hem utilitzat les seqüències obtingudes mitjançant la seqüenciació massiva de nova generació (Roche 454) per seleccionar centenars de SSRs potencialment amplificables. Després de testar la seva amplificació i amplificació creuada, hem caracteritzat 18 SSRs, 11 dels quals han estat polimòrfics en *L. rufescens* i set en *L.* sp. Fuerteventura-Lanzarote. Aquesta és per tant una metodologia relativament ràpida i econòmica per a desenvolupar marcadors nuclears d'evolució ràpida en aranyes.

PARAULES CLAU: 454, marcadors nuclears, Mediterrània, Illes Canàries

Development of novel microsatellite markers for the spider genus *Loxosceles* (Sicariidae) using next-generation sequencing

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ABSTRACT

We report the step-by-step process of developing *de novo* microsatellite (SSRs) loci in two *Loxosceles* spider species. We used reads obtained with next-generation sequencing (Roche 454) to select hundreds of potentially amplifiable SSRs. After testing amplification and cross-amplification, we characterized 18 SSRs, 11 of which were polymorphic in *L. rufescens* and 7 in *L. sp.* Fuerteventura - Lanzarote. This method is thus a relatively fast and economic procedure for the development of fast-evolving nuclear markers in spiders.

KEYWORDS: 454, nuclear markers, Mediterranean, Canary Islands.

Microsatellites (SSRs: simple sequence repeats) are popular codominant genetic markers used in many areas of research, including molecular ecology and population genetics. They consist of tandem repeats of very short nucleotide motifs (1-6 base long). One of the properties that make SSRs attractive for evolutionary studies is their high mutation rate (Guichoux *et al*., 2011). However, the technical and economic effort required for developing *de novo* SSRs in organisms for which no or few genomic resources are available (the so called non-model organisms) has made their use often prohibitive until recently. Nevertheless, the emergence of next-generation sequencing technologies has reduced the economic and technical difficulties of developing SSRs, and has boosted their use in a wide range of organisms, including spiders (Parmakelis *et al*., 2013).

In this study, we focused on spiders of the genus *Loxosceles* Heineken and Lowe, 1832 (Araneae: Sicariidae) from the Mediterranean Basin and the Canary Islands. *Loxosceles rufescens* (Dufour, 1820) is considered cosmopolitan (Platnick, 2013) but it is native from the Mediterranean (Gertsch, 1967, Duncan *et al*., 2010). In this region several deep mitochondrial lineages have been detected (Duncan *et al*., 2010, Planas *et al*., Paper 1) some of them lacking geographic structure, probably due to the blurring effects of

continuous human-mediated transportation. Recently, Planas and Ribera (2014) revealed the existence of an overlooked endemic group of *Loxosceles* spiders in the Canary Islands. Fuerteventura and Lanzarote, the easternmost islands in the archipelago, harbour one of the identified lineages. Despite the relatively impoverished fauna of Fuerteventura and Lanzarote, these two islands together with the surrounding islets have shown to be ideal places to study phylogeographical processes (i.e. Bidegaray-Batista *et al*., 2007; Macías-Hernández *et al*., 2013). We conducted this study to have fast-evolving nuclear loci with the aim to study fine-scale processes (e.g. recent volcanism) in the *Loxosceles* species endemic from Fuerteventura - Lanzarote (hereinafter *Loxosceles sp.* FV-LZ) and to be able to contrast the mitochondrial patterns currently found within *L. rufescens* (Planas *et al*., Paper 5)

Here we report on results using next-generation sequencing for obtaining SSRs and describe the step-by-step process from DNA extraction to the characterization of the of *Loxosceles,* two of which belong to two different evolutionary lineages (A6 and B3; Planas *et al*., Paper 1) within *L. rufescens* and a third individual belonging to *Loxosceles sp.* FV-LZ, using the SpeedTools Tissue DNA Extraction Kit (Biotools) following the manufacturer's protocol (Step 1 in Fig. 1). We conducted pyrosequencing on a Roche Life Science 454 GS-FLX System at the Scientific-Technical Services of University of Barcelona (Step 2 in Fig. 1). Roche 454 is the next-generation sequencing technology with larger average fragment size obtained, thus increasing the probability that the fragments containing SSRs have flanking regions enabling primer design. We pooled samples using individual multiplex identifiers (MIDs) together with an *Echinaster sepositus* sample (Garcia-Cisneros *et al.*, 2013) in half a plate since physical separation decreases the overall number of sequences obtained. We obtained a total of 143 708 reads with a mean length of 341.86 bp for *Loxosceles sp.* FV-LZ, 45 377 (mean length 313.91 bp) for *L. rufescens* A6 and 195 081 (mean length 346.24 bp) for *L. rufescens* B3. Raw data was processed with Roche's 454 pipeline using default settings for quality control and with seqclean (https://sourceforge.net/projects/seqclean/) to process all the sequences in order to remove low quality sequences and contaminants. Sequence reads from duplicated loci and mobile elements were identified in iQDD (Meglécz *et al*., 2010) using default parameters and excluded from further analyses. With iQDD we searched for reads that met several requirements, as suggested by Guichoux *et al*. (2011) (Step 3 in Fig. 1). Specifically, we

looked exclusively for SSRs with perfect motif repetition, thus improving the probabilities that the SSRs followed a stepwise mutation model. We searched for SSRs with a minimum of 11 repeats in dinucleotides and 8 repeats in tri-, tetra-, penta- and hexanucleotides, but no more than 16 repeats in both cases. Primers for selected SSRs were designed with the software PRIMER 3 (Rozen & Skaletsky, 2000) included in iQDD. We avoided designing primers in flanking regions containing short repeats (e.g. nanosatellites) and we selected putative PCR products between 90 and 500bp of length (Step 4 in Fig. 1). Among all the possible primer combinations for each SSRs, we kept those with better evaluation applying stringent parameters to ensure amplification (i.e. no primer-dimer interaction, similar annealing temperature, GC primer end content and primer end stability).

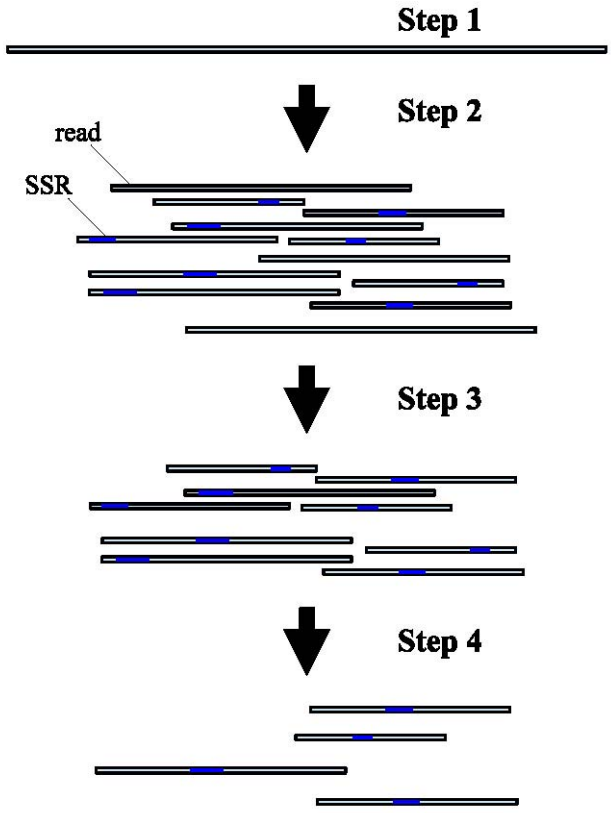

Figure 1 Work-flow of the next-generation sequencing approach to SSRs marker development. *Step 1*: extraction of genomic DNA; *Step 2*: thousands of reads produced using 454 pyrosequencing; *Step 3*: bioinformatic tools are used to select reads containing SSRs; *Step 4*: selection of reads where primers can be designed.

The number of reads containing SSRs and the numbers of these with suitable flanking PCR-primer sites are shown in Table 1. Dinucleotides were the most frequent SSRs followed by tri, tetra, penta- and hexanucleotides (Table 1). Even when applying very stringent parameters for SSRs selection, we obtained a total of 800 reads containing SSRs that met the requirements specified above. We should point out that decreasing the level of rigorousness when mining for SSR (e.g. decreasing to 8 the minimum number of tandem repeat) would have increased substantially the number of yielded SSRs. We selected 58 SSRs from the hundreds of candidate SSRs, considering the length of the expected PCR product. We then tested their amplification and cross-amplification success in eight individuals, four from *L. rufescens* and four from *L. sp.* FV-LZ (i.e. we tested SSRs obtained from *L. rufescens* reads for amplification in *L. sp.* FV-LZ individuals and viceversa). Of the 58 SSRs checked, 40 were rejected due to unsuccessful PCR amplification.

Table 1 Number reads containing SSRs and the numbers of these with suitable flanking PCR-primer sites. In each cell, the number of reads obtained from each individual is shown (*Loxosceles sp.* FV-LZ / *L. rufescens* A6 / *L. rufescens* B3).

	Dinucleotides	Trinucleotides	Tetranucleotides	Pentanucleotides	Hexanucleotides
Reads containing SSRs	3525/961/4261	334/141/673	38/34/181	3/0/6	0/0/1
Reads with potentially amplifiable SSRs	327/107/206	37/21/87	1/1/7	1/0/5	0/0/0

We kept the 18 SSRs loci with higher amplification success and labelled the forward primers with fluorescent dye. We tested for polymorphism using 38 *L. rufescens* individuals from four different localities and 16 *Loxosceles sp.* FV-LZ individuals from four different localities. We conducted PCR reactions in a final volume of 10 µL using Biotools *Pfu* DNA Polymerase (Biotools). Annealing temperatures ranged between 58 and 42 Cº in all primer pairs. We pooled PCR products according to dye type and expected allele size ranges and genotyped them in an ABI 3730XLs automated sequencer at Macrogen (Seoul) with the internal size standard 500 LIZ. We used the Microsatellite Plugin 1.3 in Geneious 6.1.6 (Biomatters) for allele calling. For each locus, primer sequences together with the number of alleles (N_a), observed (H_O) and expected (H_E) heterozygosity are listed in Table 2.

All but one SSR were polymorphic for at least one of the two tested species. One SSR (ME083) obtained from *L. rufescens* reads amplified successfully in *Loxosceles sp.* FV-LZ individuals although it was monomorphic in both species. Three SSRs obtained from *L. sp.* FV-LZ reads amplified successfully in *L. rufescens*, being monomorphic in one locus (CA238) and polymorphic in the other two loci (CA027 and CA243). In total 11 polymorphic SSRs were developed for *L. rufescens* and 7 for *L. sp.* FV-LZ.

The results from this study indicate that next-generation sequencing (e.g. Roche 454) is an efficient and cost-effective procedure for the fast development of microsatellite loci in non-model organisms. We obtained thousands of reads by sequencing three *Loxosceles* specimens in half a plate of Roche 454, and used a fast bioinformatic pipeline applying strict criteria to select hundreds of potentially amplifiable SSR. We then selected 58 candidate SSRs and tested their amplification and cross-amplification. Eighteen of these SSRs were then characterized using different populations from the two *Loxosceles* species leading to 11 polymorphic SSRs for *L. rufescens* and 7 for *L. sp.* FV-LZ.

Table 2 Characteristics of 18 microsatellite loci, tested with 38 samples of *Loxosceles rufescens* from four different localities and 16 samples of *Loxosceles sp.* FV-LZ from four different localities. Locus name, accession number, repeat motif and primer sequences (F: forward, R: reverse) are listed for each locus. In the right part of the table, the first row in each locus refers to the total number of alleles, allele size (bp), expected heterozygosity (He) and observed heterozygosity (Ho) for *L. rufescens* and in the second row for each locus the same information is given for *Loxosceles sp.* FV-LZ.

Locus	Accession number	Repeat motif	Primer sequence (5'–3')	Total number of alleles	Allele size (bp)	Ho	He
ME012	XXX	(AGAT)	F: GTGGGTGGTCCATTGATAGG	8	137-165	0.57	0.77
			R: TTTAACAAGACGCAGCGAAA	-	-	-	-
ME031	XXX	(AAAT)	F: AAACTTCGATTTATTTTGTTTCTTG	4	89-109	0.19	0.66
			R: AAATGTCTGGCGGATCAGAA	-	-	-	-
ME034	XXX	(AAAT)	F: CGTCTGCAGTGTGAACGG	6	93-149	0.47	0.71
			R: ATATGTGCTTTTGCGCCTGT	-	-	-	-
ME064	XXX	(AAAT)	F: TCTGTAAATGGATTCTCATCTGTTG	2	151-155	0.13	0.12
			R: TCGTCCAACCATCCTCTTTC	-	-	-	-
ME067	XXX	(AGAT)	F: TGTGATGTACCTGCGTTCGT	4	142-160	0.11	0.10
			R: GCAAGATCAACCCACAACCT	-	-	-	-
ME077	XXX	(AAACT)	F: TATGTAATCACCGGGGTTGG	3	152-177	0.21	0.55
			R: CGTGCAATCTGGTTAACTTCG	-	-	-	-
ME083	XXX	(ACACT)	F: TAGGGAATGGAATGGCAGAC	1	160-160	0	0
			R: TTTGCAGATTTGATCTGGGAC	1	163-163	0	0
ME088	XXX	(AAAT)	F: AGCGTTGATACAGGTGGTCC	3	208-254	0.10	0.59
			R: TCACTGCACAGTGTAAAGCCA	-	-	-	-
ME103	XXX	(AAT)	F: TTAGCGACCTTCCCTGTCAC	6	262-280	0.34	0.73

Locus	Accession number	Repeat motif	Primer sequence (5'–3')	Total number of alleles	Allele size (bp)	Ho	He
			R: TGGTAAACGGGAGGACTAGG	-	-	-	-
ME113	XXX	(AAT)	F: AACCTGAAGGGCTGATGAAT	6	75-96	0,37	0,78
			R: CAGGAGCAGGATGCCATATT	-	-	-	-
CA001	XXX	(AAT)	F: ATGTATCACGCGCCTTTTG	-	-	-	-
			R: GTTGTCTGGAGCAAACAGCA	5	75-93	0.60	0.72
CA003	XXX	(AAT)	F: TGTACCAGGGGCTGGTCTAA	-	-	-	-
			R: CATACGTGGTGGCAGCATAC	5	66-92	0.28	0.73
CA027	XXX	(AAGTG)	F: TACCACAAGGGGAGAATCCA	3	103-113	0.39	0.32
			R: AAGCCAGAGGTGCAATTGTT	10	132-182	0.40	0.79
CA030	XXX	(AAT)	F: AGGTGTGGCACTACCGTTTT	-	-	-	-
			R: CAAATGAGCATTCAACCTCG	7	133-157	0.46	0.70
CA038	XXX	(AAT)	F: ATGTTTGAGGGGTCTCGTTG	-	-	-	-
			R: ACATGATGCCCCACGATAAT	4	272-284	0.93	0.69
CA105	XXX	(AC)	F: TAAATAACCTGATATCGGATCTAT-GAC	-	-	-	-
			R: AAAGTATATCGGACAAA-CATCCAACC	5	255-267	0.75	0.75
CA238	XXX	(AG)	F: GGCACCCCAGACTAACAAGA	1	233-233	0.00	0.00
			R: ACCTCTGGCACGAATACACC	4	221-231	0.93	0.69
CA243	XXX	(AT)	F: AATAACGGAGACCGTGCAAC	5	225-279	0.68	0.64
			R: CCTCCAGTATCCGAAGACGA	-	-	-	-

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La diversitat genètica es veu afectada pels canvis en el nivell del mar durant el Pleistocè i per l'activitat volcànica en una espècie d'aranya del gènere *Loxosceles* endèmica de les illes orientals Canàries

RESUM

Les illes orientals de l'arxipèlag Canari (Fuerteventura, Lanzarote i els illots adjacents), varen estar connectades durant llargs períodes del Pleistocè, quan a conseqüència de baixades en el nivell del mar promogudes pels cicles glacials van deixar exposades superfícies de terra que actualment es troben submergides, formant una paleoilla anomenada Mahan. Aquesta connexió recurrent explica en part l'existència d'un alt nombre d'espècies endèmiques distribuïdes entre aquestes illes. *Loxosceles* sp. FV-LZ n'és un d'aquest exemples, i per la seva abundància i la poca capacitat de dispersió, representa un bon model per explorar els efectes dels canvis en el nivell del mar en la diversitat genètica d'espècies endèmiques de Mahan. En aquest treball hem estudiat la diversitat genètica espacial en *Loxosceles* sp. FV-LZ utilitzant múltiples marcadors genètics (seqüències d'ADN mitocondrial i nuclear, i microsatèl·lits) i aplicant diverses metodologies basades en assumpcions genètiques i geogràfiques diferents. Hem trobat que malgrat la contínua connexió entre les dues illes, els individus de cadascuna d'elles formen llinatges recíprocament monofilètics. Els individus de Lobos i de La Graciosa s'agrupen amb els de les localitats més properes de Fuerteventura i Lanzarote, respectivament. La diversitat genètica ha estat estructurada principalment per un patró d'aïllament per distància a Fuerteventura, i en canvi, a Lanzarote sembla que l'origen dels dos llinatges mitocondrials es deu als efectes del vulcanisme Pleistocènic. A més, hem trobat suport per a la importància biogeogràfica que representa la zona de Zonzamas al centre-est de Lanzarote.

PARAULES CLAU: Mahan, evolució en illes, aïllament per distància, nivell del mar, exclusió competitiva, microsatèl·lits.

Genetic diversity is affected by Pleistocenic sea-level changes and volcanic activity in a spider of the genus *Loxosceles* endemic to the eastern Canary Islands

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ABSTRACT

The eastern Canary Islands of Fuerteventura, Lanzarote and their adjacent islets were connected during long periods in the Pleistocene, when sea-level low-stands promoted by climatic cycles led to the exposure of currently submerged surface situated between them, forming the paleoisland of Mahan. This recurrent connection partially explains the high number of endemic species distributed across these islands. *Loxosceles* sp. FV-LZ is one of such examples, and due to its abundance and low dispersal capacities represents a good model to explore the effects of sea-level changes in shaping the genetic diversity of species endemic to Mahan. Herein, we study the spatial genetic diversity of *Loxosceles* sp FV-LZ with multiple genetic markers (mtDNA and nuDNA sequences and microsatellites) using an array of different methodologies that rely on different genetic and geographic assumptions. We found that despite the recurrent connection of the two islands, individuals from each island formed reciprocally monophyletic groups. Individuals from Lobos and La Graciosa were grouped with those from the closest localities in Fuerteventura and Lanzarote, respectively. Genetic diversity was mainly structured by an isolation-by-distance pattern in Fuerteventura, but in Lanzarote, the origin of two deep mitochondrial lineages is probably related to the effects of Pleistocenic volcanism. Furthermore, we found support for the biogeographical relevance of the Zonzamas area in central-eastern Lanzarote.

KEYWORDS: Mahan, island evolution, isolation-by-distance, sea-level, competitive exclusion.

INTRODUCTION

Due to their intrinsic significance, volcanism-related events intuitively emerge as a key factor in shaping the distribution of volcanic island diversity, and different studies carried

out in well-studied oceanic archipelagos, such as Hawaii, the Galapagos or the Canary Islands, have provided good examples of this fact (Juan *et al*., 2000; Beheragay *et al*., 2003; Vandergast *et al*., 2004; Emerson *et al*., 2006; Bloor *et al*., 2008). Besides volcanism, alternative factors such as climate shifts or sea level oscillations are increasingly gaining importance in island biogeography (Hooghiemstra *et al*., 2013; Ali & Aitchison, 2014; Rijsdijk *et al*., 2014). In fact, current distribution of biological diversity has been affected by a complex combination of independent factors acting at different times and geographic scales.

The Canarian archipelago is situated off the northwest African coast and comprises seven main volcanic islands and several islets. The islands exhibit an east-west arrangement, that also corresponds to an older to younger order, being Fuerteventura the easternmost and oldest island (~20.5 Mya) and El Hierro the westernmost and youngest one (~1.12 Mya). Lanzarote, the second oldest island in the archipelago (~15.5 Mya), is situated North of Fuerteventura, and both form part of the same insular shelf together with the islets of Lobos, La Graciosa, Montaña Clara and Alegranza. The unity of the eastern Canaries (i.e. Fuerteventura, Lanzarote and adjacent islets) has recurrently become evident during the Pleistocenic glacial cycles, when sea level globally changed, being higher during interglacials and lower during glacial maximums (Webb & Bartlein, 1992; Rijsdijk *et al*., 2014; Rohling *et al*., 2014). These eustatic changes exposed land-bridge connections between islands and islets, leading to the formation of a single greater island, known as Mahan (García-Talavera, 1999; Fernández-Palacios *et al*., 2011). Dispersion during these low sea-level periods has been proposed for two different spider species of the genus *Dysdera*, either from northern Lanzarote to northern islets or between different islets (Bidegaray-Batista *et al*. 2007; Macías-Hernández *et al*., 2013). Besides the recurrent eustatic changes, the complex and prolonged volcanic history of the eastern Canaries has also been a relevant factor in shaping the current diversity. For example, volcanic eruptions prompted the connection of the two precursors of Lanzarote (i.e. Los Ajaches (15.5 Ma) and Famara (13.2 Ma), the southern and northern parts of the island, respectively) during the Pleistocene, enabling a postcolonization expansion of *Gallotia atlantica* across the present-day island (Bloor *et al*., 2008). This species was also affected by subsequent eruptions promoting an east–west vicariance across the island (Bloor *et al*., 2008).

Until recently, the diversity of the spider genus *Loxosceles* was highly underestimated in

the Canary Islands, where only the introduced *L. rufescens* was recognized (Macías, 2010). This picture drastically changed after the discovery of the existence of a clade endemic to this archipelago (Planas & Ribera, 2014), composed by six new species (Planas & Ribera, Paper 3). The reconstruction of the phylogenetic relationships and the proximity of Fuerteventura and Lanzarote to the continent provided strong indications that these were the first of the Canary Islands colonized by the ancestor of the Canarian endemics (Planas & Ribera, 2014). Only one species, *Loxosceles sp.* FV-LZ*,* is presently distributed across Fuerteventura, Lanzarote and the adjacent islets of Lobos and La Graciosa. The estimated divergence time for the split between the populations of the two main islands was 0.86 Myr, a relatively recent divergence if we consider that they have been present on these islands for a relatively long period (> 10 Myr).

In this study, we seek to discern the key factors that acted in shaping the genetic diversity of the spider species *Loxosceles sp.* FV-LZ, distributed across a single insular shelf in the Canary Islands*,* with a special emphasis on investigating the effects of volcanism and sea level changes. We explored the phylogeographic patterns using different molecular markers [*cox1* and 16S (mtDNA), H3 and its2 (nDNA), and seven microsatellites (nDNA)], and implemented an array of different methodologies to decipher the mode and tempo of the processes that led to the distribution of the genetic diversity across these islands.

MATERIALS AND METHODS

Sampling and molecular data collection

Loxosceles sp. FV-LZ specimens were collected in 2009 from 21 localities (Fig. 1 A). Sequences of the mtDNA cytochrome oxidase I (*cox1*) and 16S rRNA and of the nDNA internal-transcript fragment 2 (its2) and H3, were produced in previous studies (Planas & Ribera, 2014, Planas & Ribera, Paper 3). Additionally, we genotyped the same individuals for seven newly developed microsatellite loci (CA001, CA003, CA027, CA030, CA038, CA105, CA238) following the same procedure as in Planas *et al*. (Paper 4). PCR products were pooled in two groups and analyzed in an ABI 3730XLs automated sequencer (Macrogen, Seoul) with the internal size standard 500 LIZ (Applied Biosystems). Allele sizes were retrieved with the Microsatellite Plugin 1.3 in Geneious 6.1.6 (Biomatters).

Alignment and haplotype networks

DNA sequences were aligned using MAFFT (Katoh & Toh, 2008) with default settings for *cox1* and H3 and using the Q-INS-i algorithm for its2 and 16S sequences. We investigated haplotype relationships of *cox1* and its2 (the best represented markers, see Results) by constructing haplotype networks following two different approaches: statistical parsimony and median-joining algorithms (Posada & Crandall, 2001). Haplotype analyses are sensitive to missing data and, because some sequences were shorter, we trimmed 5' and 3' ends of both alignments. The statistical parsimony algorithm connects haplotypes when the maximum number of differences among them is below a parsimony connection limit and splits in independent networks the haplotypes that exceed this threshold. We applied a 90% threshold in TCS (Clement *et al*. 2000) and treated gaps as 5th state in the analysis of its2. The median-joining networks combine the minimum-spanning trees within a single network and were constructed in Network (Fluxus Technology) with default parameters for both genes.

Genealogical trees and population tree

Gene trees were reconstructed with both Maximum Likelihood (ML) and Bayesian Inference (BI) using representatives of each of the remaining Canary Island endemic *Loxosceles* as outgroups. ML analyses were conducted in RAxML BlackBox (Stamatakis *et al*. 2008) applying a GTRGAMMA model, and BI analyses were conducted in BEAST v1.8 (Heled & Drummond, 2012) using the best-fit model of nucleotide evolution for each marker as selected in jModelTest (Posada, 2008), a strict molecular clock, with the rate fixed to 1, and a constant size coalescent tree prior. We used the multispecies coalescent approach as implemented in *BEAST v 1.8 (Heled & Drummond, 2012) to estimate the best population tree for *Loxosceles sp.* FV-LZ. This methodology requires an *a priori* grouping of individuals, and the groups considered were the three main lineages (i.e. FVL, LZ1 and LZ2) obtained in mtDNA gene trees and recovered in clustering analyses based on microsatellites (see below and Results). This way we were able to deal with expected gene tree incongruences due to incomplete lineage sorting. At the same time, the multispecies coalescent model was used to estimate the divergence times of the intraspecific events. We calibrated the root of the species tree with the date estimated (i.e. 0.86 Ma) in Planas & Ribera (2014) for the split between the two main lineages, the one composed by individuals from Fuerteventura and Lobos (FVL hereinafter) and the other by individuals from Lanzarote and La Graciosa (LZG hereinafter). We conducted separate analyses for the

nucleotide data (i.e., mtDNA and nuDNA) and for the microsatellite data, as they cannot be included in the same analysis in *BEAST. Each marker was treated as an independent partition, with unlinked substitution models, defining a GTR substitution model for both mtDNA markers, and a HKY for the nuDNA. MtDNA markers were linked under the same gene tree, while the two nuDNA were unlinked. A strict clock model was defined for each marker with a uniform prior from 0 to 0.2 for the mtDNA markers and 0 to 0.1 for the nuDNA. In the microsatellite analyses, the substitution model was linked across markers, while the other parameters were unlinked and default priors were applied, except for the root of the species tree as commented above. Two independent runs of 50 million generations each, sampling every 1000th generation, were performed remotely on the CIPRES Science Gateway V 3.3 cluster (Miller *et al*., 2010). We assessed convergence and correct mixing of the chains by inspecting the trace plots in Tracer and ensuring that EES > 200 for all parameters, and then we used LogCombiner and TreeAnotator for combining the results and producing the trees after a 10% burn-in of the samples obtained.

Clustering and spatial genetic analyses

Identification of non-*a priori* genetic clusters was performed using microsatellite data, with complementary methods that rely on different genetic assumptions. The first method is based on Bayesian clustering and it is implemented in the program STRUCTURE (Pritchard *et al*., 2000). Briefly, STRUCTURE seeks for genetic clusters that minimize departures from the Hardy-Weinberg equilibrium using allele frequencies (Prichard *et al*., 2000). Analyses were conducted considering the admixture model and uncorrelated allele frequencies among populations, performing 20 independent runs (100000 iterations and 50000 burn-in) for each K between K=1 and K=21 (number of localities). The most likely number of clusters was inferred based on Evanno's method (Evanno *et al*., 2005) implemented in Structure Harvester (Earl & vonHoldt, 2012). A second analysis was conducted on each island independently (considering Lobos together with Fuerteventura (FVL), and La Graciosa with Lanzarote (LZG); see Results), following the same procedure as described above. We used the software CLUMPP (Jakobsson & Rosenberg, 2007) to combine the results across the 20 independent runs for the selected K, and Distruct (Rosenberg, 2004) to graphically represent the results. The second method relies on less strong genetic assumptions (Jombart *et al*., 2010). It is based on the Discriminant Analysis of Principal Components (DAPC) to define a model that maximizes between-group and

minimizes within-group genetic variation, and it has been shown to perform similarly to STRUCTURE in simulated "island models" and better in "stepping-stone models" (Jombart *et al*., 2010). The optimal number of clusters was obtained with the find.cluster function in the adegenet R package (Jombart, 2008) conducting successive K-means clustering runs for K = 1 to K = 21. The best clustering model was selected under the Bayesian Information Criterion and was used in DAPC analyses. As in the STRUCTURE analyses, we conducted further independent DAPC analyses for each island (i.e. FVL and LZG). The last method, spatial principal component analysis (sPCA), is a multivariate method that combines spatial information with principal components, and it has been shown to be an efficient method to identify spatial genetic patterns (Jombart *et al*., 2008). As above, we conducted the analyses on the whole dataset and on datasets of each island (i.e. FVL and LZG) with the adegenet R package (Jombart, 2008). Due to the clustering of some localities, we used the "inverse distance" option to construct the spatial weights. To better detect visually putative barriers to gene flow, we interpolated principal components with the interp function in R package akima (Akima *et al*., 2009) and plotted with ggmap (Kahle & Wickham, 2013).

Genetic diversity and demographic analyses

The *cox1* and its2 trimmed alignments (see above) were used to calculate three descriptive parameters (number of haplotypes (*h*), haplotype (H_d) and nucleotide (π) diversity) for the whole dataset and for each of the two island groups independently (i.e. FVL and LZG). We tested for departures from the equilibrium model with Fu's F and Tajima's D test and for demographic expansion with Ramos-Rozas R_2 test, performing all the analyses in DnaSP (Librado & Rozas 2009).

Isolation by distance

We tested the relationship between genetic (*p*-distance for *cox1* and *F*st for microsatellites) and geographic distance with a Mantel test conducted in the Isolation By Distance web service (Jensen *et al*., 2005), performing 1000 permutations. Prior to the analyses with microsatellite data, localities with one individual were combined if they were separated less than 3 km (i.e. FV1 and FV2, FV3 and FV4, FV9 and FV10), or discarded (LZ2, LZ4 and LZ11).

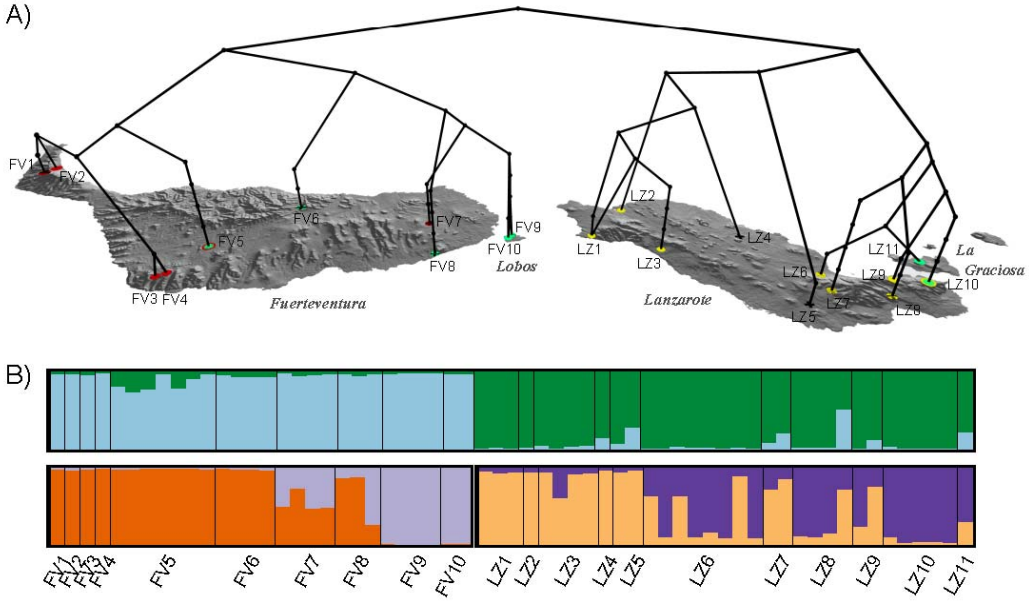

Figure 1 A) Bayesian Inference tree obtained with *cox1* sequences of 61 individuals (without outgroups) overlaid on a topographic map of the Eastern Canary Islands: Fuerteventura (localities FV1 to FV8), Lobos (FV9 and FV10), Lanzarote (LZ1 to LZ10) and La Graciosa (LZ11) (not shown in South-North direction). Colored circles correspond to the inclusion of the individual(s) of each locality to one of the three independent its2 networks obtained in TCS analyses (crosses represent missing data for this marker and locality). **B)** STRUCTURE results based on microsatellite data: K=2 results of the analysis conducted on the whole dataset and K=2 results of the analyses conducted on separate datasets of the previously delimited clusters (i.e. Fuerteventura and Lobos, Lanzarote and La Graciosa). Each vertical line represents the proportional membership assignment of one individual to each of the two clusters. Black lines delimit sampling localities and locality codes correspond to those on the map.

RESULTS

Haplotype networks, genealogical trees and population tree

The alignment of *cox1* sequences included 62 *Loxosceles sp.* FV-LZ individuals and was 942 bp long (512 bp for the trimmed alignment). The its2 alignment included 40 *L. sp.* FV-LZ individuals and was 324 bp long (264 bp for the trimmed alignment). 16S and H3 alignments included 21 *L. sp.* FV-LZ individuals (one from each locality) and were 547 and 288 bp long, respectively (Supplementary Table 1). Five individuals, one from each of the species endemic to the Canary Island were included in gene tree analyses as outgroups. Microsatellite markers were successfully amplified in most of the individuals, with missing

data less than 5%. The statistical parsimony *cox1* haplotype network constructed with TCS was split in four non-connected networks (90% homoplasy threshold) (Fig. 2). Two networks were composed exclusively by individuals from FVL, and the remaining two by individuals from LZG. None of the *cox1* haplotypes was shared across localities. The network of the nuclear its2 was split in three non-connected netwoks. One network was composed exclusively by individuals from Fuerteventura another by individuals from Lanzarote and the third one by individuals from both islands and the two islets (Fig. 2). Both statistical parsimony and median-joining algorithms resulted in the same connections when considering only closely related haplotypes (Fig. 2).

Gene trees obtained with BEAST were generally coincident with the ones reconstructed with ML (Supplementary Fig. 1). Some incongruences were found in *cox1* and H3 trees, where the two islands were reciprocally monophyletic in Bayesian analyses, while Fuerteventura was paraphyletic with respect to Lanzarote in the ML analyses. Gene trees of the 16S marker placed in all cases Lanzarote as paraphyletic with respect to Fuerteventura, while in its2 analyses both islands were paraphyletic.

The population tree reconstructed with nucleotide and microsatellite data resulted in the same topology, with the two lineages from Lanzarote forming a monophyletic group. Divergence time between the two groups from Lanzarote was estimated at 0.33 Ma (0.1 - 0.64 Ma) with nucleotide data and at 0.185 Ma (0 - 0.37 Ma) with microsatellite data.

Clustering and spatial genetic analyses

The optimal number of clusters obtained in STRUCTURE analyses following the Evanno method was K=2 (Fig. 1), assigning to one group all the individuals from Fuerteventura and Lobos (FVL), and to the other group the individuals from Lanzarote and La Graciosa (LZG). Subsequent analysis conducted considering each group individually (i.e. FVL and LZG) led to an optimal clustering at K=2 in each one of the groups (Fig. 1). In FVL, one homogeneous cluster included individuals from southern and central localities (FV1 to FV6), another homogeneous cluster was exclusively composed by the individuals from Lobos, while the remaining individuals from two localities (FV7 - FV8) exhibited an admixture origin (Fig. 1). Similarly, in Lanzarote, one cluster grouped individuals from southern to central-western localities (LZ1 to LZ5 and LZ7), while the northern and La Graciosa individuals were included in a second cluster (LZ10 - LZ11), and some individuals in-between (LZ6, LZ8 and LZ9) showed varying levels of admixture (Fig. 1).

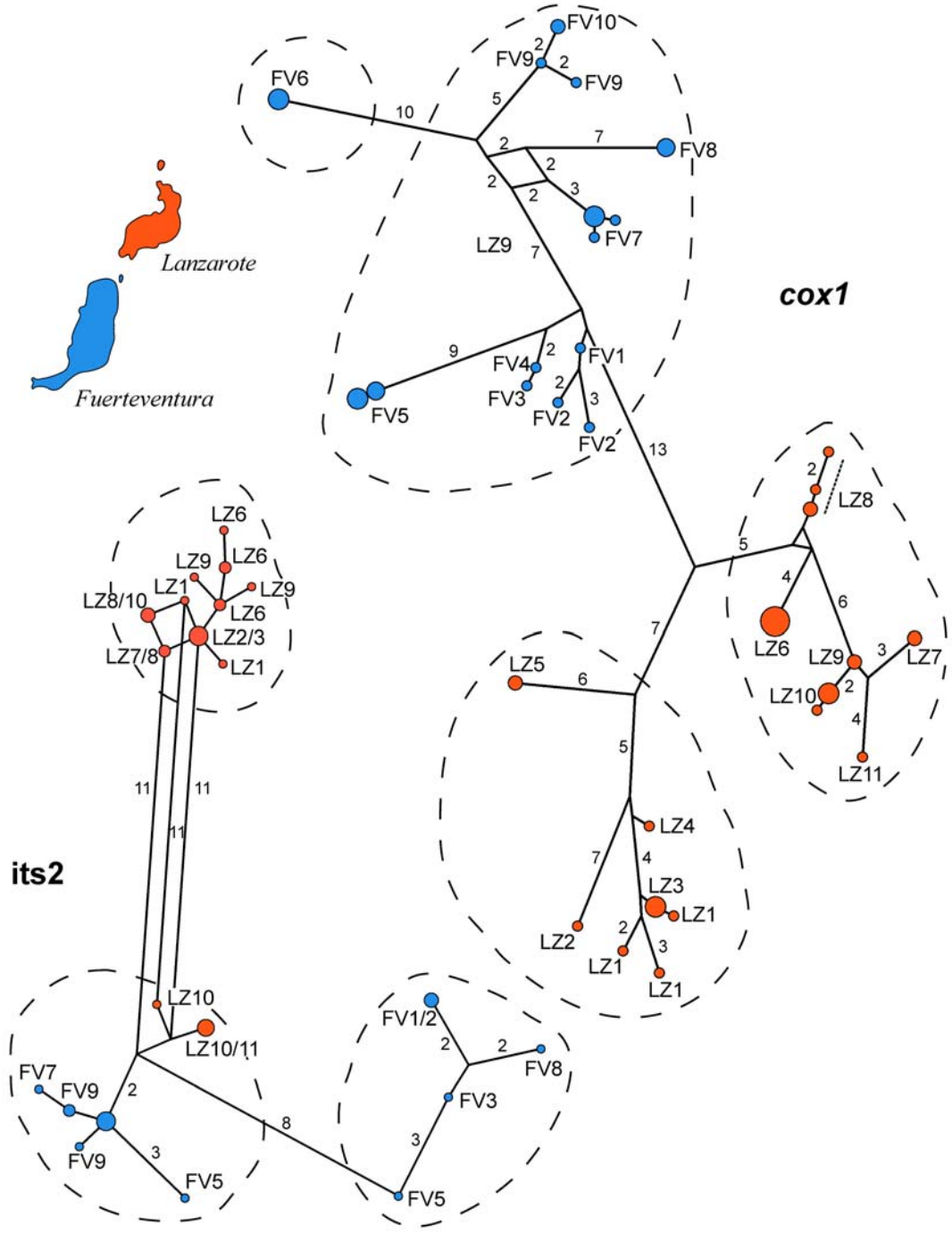

Figure 2 Haplotype networks calculated with the median-joining algorithm in Network for the *cox1* and its2 datasets. Independent networks obtained with statistical parsimony in TCS are marked with dashed lines. Circles represent different haplotypes and their size is proportional to the number of individuals that shared the haplotype. Colors correspond to colored map on the left: blue indicates Fuerteventura and Lobos localities and orange Lanzarote and La Graciosa ones. Numbers next to the lines represent mutation steps between haplotypes and each haplotype is labeled according to its locality as in Fig. 1.

DAPC analyses gave overall similar results to STRUCTURE. We retained 5 principal components (> 60 % variance) and one discriminant function in the DAPC analyses. In the first analysis, the optimal number of clusters was K=2 (highest BIC difference), although K=4 was the absolute minimum (Supplementary Fig. 2). The resulting groups when using K=2 coincided with FVL and LZG. Only one individual from Fuerteventura (LX1400-FV5) presented less than 90% of probability of membership in a single cluster (Supplementary Fig. 2). In the DAPC analysis conducted for FVL, K=3 was the absolute minimum, although as above, K=2 was selected considering that it represented the highest BIC difference (Supplementary Fig. 2). One group included the individuals from localities FV1 to FV6, while the second group included the remaining individuals except one individual from FV8 that presented less than 80% membership probability (LX1411). K=2 was the optimal number of clusters for the LZG dataset, and the DAPC analyses clearly separated the individuals from localities LZ1 to LZ5 with respect to those from LZ6 to LZ11. Only one individual (LX1461) from LZ3 presented nearly 100% membership probability for the group composed of northern localities (Supplementary Fig. 2).

As shown in Fig. 3B, the resulting pattern of the first sPCA score paralleled STRUCTURE and DAPC results (Fig. 1 and Supplementary Fig. 2), separating the individuals from the each main island together with its adjacent islet (i.e. FVL from LZG) in two distinct clusters. However, with the second score, individuals from Lobos were grouped with those from southern Lanzarote in one group, and all the remaining individuals in a second group (Supplementary Figure 3B). The first eigenvalue of the spatial principal component analysis (sPCA) was clearly higher than the remaining, although we retained the first two as both presented high spatial structuring (I_1 = 0.87, I_2 = 0.82) and high variance (var_1 = 0.55, var_2 = 0.24). The existence of a global pattern was further confirmed with the global test (P < 0.001) while local structure was not significant (P = 0.462). Similarly to STRUCTURE and DAPC analyses, we conducted two additional sPCA analyses considering FVL and LZG independently. For the sPCA analysis in FVL, we retained the first two scores, that showed strong spatial autocorrelation (I_1 = 0.87, I_2 = 0.91) and high variance (var_1 = 0.55, var_2 = 0.29). The permutation global test was significant (P < 0.001) while the local test was not (P = 0.54). The pattern obtained with the first score separates the four northern localities (FV7-FV10) from the southern ones (Fig. 3), while with the second score localities FV7 and FV8 appeared differentiated. The sPCA analysis conducted

with LZG individuals also presented a significant global pattern ($P < 0.001$) and no local one ($P = 0.98$). In this analysis, we retained the first three scores as they showed high spatial autocorrelation ($I_1 = 0.83$, $I_2 = 0.79$, $I_3 = 0.85$) and high variance ($var_1=0.39$, $var_2=0.22$, $var_3=0.19$). The pattern detected with the first score shows a clear separation between the five northern localities with respect to LZ5 and the remaining localities (Fig. 3). Differently, with the second sPCA score, LZ5 is also differentiated from the remaining localities, but less differentiated with respect to the northern localities. When considering the third score, localities situated in the eastern coast of Lanzarote are separated from those in the western coast (Supplementary Fig. 3).

Genetic diversity and demographic analyses

Summary statistics for *cox1* and its2 alignments are shown in Table 1. Haplotype and nucleotide diversity values were high for both markers, although higher in *cox1* than in its2. This pattern was also maintained when considering the two islands separately (Table 1). Neutrality tests (i.e. Fu's Fs, Tajima's D and Ramos-Onsins & Rozas R2) were not significant for any marker (i.e. *cox1* and its2) in any case (i.e. for all the data and separate analysis for each island) and thus the neutral model could not be rejected (Table 1).

Number of alleles in microsatellite markers ranged from 5 in CA105 to 14 in CA030, with a mean of 8.43 (Supplementary Table 2). Estimates of expected heterozygosity were generally higher than observed heterozygosity, which suggests population structuring.

Table 1 Summary of molecular diversity estimates and neutrality tests. Abbreviations: n, number of individuals; *h*, number of haplotypes; H_d, haplotype diversity; π, nucleotide diversity; ^{NS}, non significant.

	Lineage	n	*h*	H_d	π	Fu's *Fs*	Tajima's *D*	Ramos-Onsins & Rozas, R_2
cox1	FV-LZ	62	31	0.964	0.044	0.039 ^{NS}	0.25169 ^{NS}	0.1230 ^{NS}
	FVL	29	15	0.93	0.031	1.630 ^{NS}	0.56685 ^{NS}	0.1478 ^{NS}
	LZG	33	16	0.917	0.031	1.914 ^{NS}	0.25055 ^{NS}	0.1316 ^{NS}
	LZ1	11	7	0.873	0.018	1.249 ^{NS}	-0.61022 ^{NS}	0.126 ^{NS}
	LZ2	22	9	0.84	0.015	1.864 ^{NS}	0.54478 ^{NS}	0.1567 ^{NS}
its2	FV-LZ	40	10	0.773	0.017	0.436 ^{NS}	0.78881 ^{NS}	0.1414 ^{NS}
	FVL	16	6	0.767	0.007	-1.284 ^{NS}	-0.37834 ^{NS}	0.1216 ^{NS}
	LZG	24	5	0.486	0.01	1.670 ^{NS}	0.52484 ^{NS}	0.1526 ^{NS}

Isolation by distance

The relationship between genetic (*cox1* and microsatellites) and geographic distance was assessed in the two islands (FVLZ) and in each island separately (Table 2). Analyses performed with *cox1* showed a positive and significant correlation with geographic distance ($p < 0.05$), and the correlation was stronger in the analysis of individuals from Lanzarote alone. Analyses conducted with *F*st calculated with microsatellites between localities were also positively correlated with geographic distance but only significant ($p < 0.05$) in the analyses including the two islands. Mantel's r score remained almost identical when analyses were performed in each islands, although in that case, both analyses resulted non-significant. However, this lack of significance was probably affected by low statistical power due to small sample size.

Table 2 Summary results for the Mantel tests and the reduced major axis (RMA) regression of IBD slope. The significance was assessed with 1000 permutations. Analyses were conducted on the whole dataset (FV-LZ) and on each island independently (FVL and LZG). Abbreviations: r, IBD r statistic (correlation); p, P value; n, pairwise contrasts (number of populations).

	Lineage	r	p	RMA slope	n
cox1 (p-distance)	FV-LZ	0.4821	< 0.0010	3.819e-04	210 (21)
	FVL	0.4887	0.010	4.219e-04	45 (10)
	LZG	0.7035	< 0.001	1.278e-03	55 (9)
Microsatellites (*F*st)	FV-LZ	0.3393	0.0150	3.395e-03	105 (15)
	FVL	0.3117	0.153	5.744e-03	21 (7)
	LZG	0.3328	0.086	7.811e-03	28 (8)

DISCUSSION

United but separate

The most recent common ancestor of the Canary Island clade of *Loxosceles* dispersed from mainland Africa to Fuerteventura or Lanzarote more than 10 Mya, from where recurrent dispersal events following “the progression rule” led to the colonization of the remaining islands, with the only exception of La Palma (Planas & Ribera, 2014). While nowadays two or three different species of this genus share the same island in Gran Canaria and Tenerife, respectively (Planas & Ribera, 2014; Paper 3), it is surprising, given the

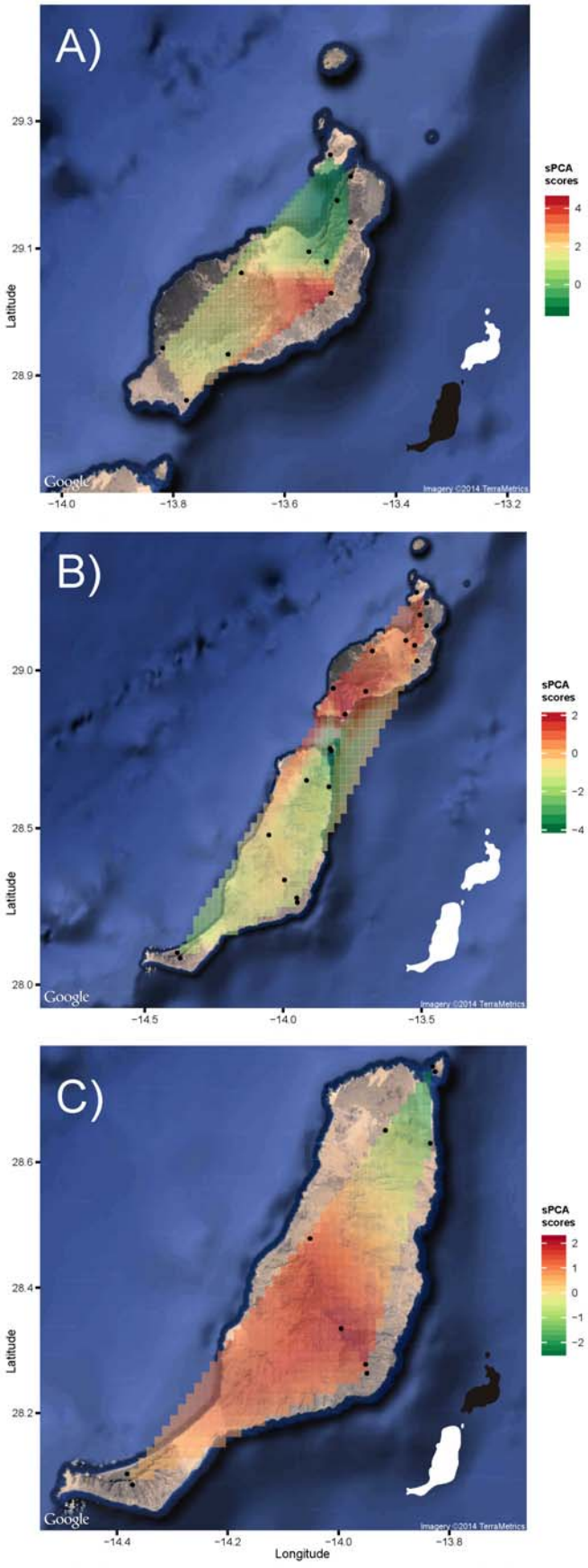

Figure 3 Projection of the first global components of the Spatial Principal Component Analysis (sPCA) conducted with microsatellites for **A)** Lanzarote and La Graciosa, **B)** Lanzarote, Fuerteventura and adjacent islets, **C)** Fuerteventura and Lobos. To better detect visually putative barriers to gene flow, values between sampled localities were interpolated with the interp function in R package akima (Akima *et al.*, 2009) and plotted with ggmap (Kahle & Wickham, 2013).

prolonged time since colonization, that in the Eastern Islands (i.e. Fuerteventura, Lanzarote and adjacent islets) only one species with recent intraspecific divergences is found. One explanation for the lack of older lineages within these islands is compatible with the occurrence of extinction events (Whittaker *et al*., 2008; Bidegaray-Batista *et al*., 2013). For several Canarian groups with lower diversity in the easternmost islands, these events have been related to the progressive dryness of the islands, promoting the extinction of groups with strong humid habitat requirements (Arnedo *et al*., 2000; Emerson & Oromí, 2005). However, similar to other *Loxosceles* species, *L. sp.* FV-LZ is well-adapted to arid or semi-arid habitats, where it is often found in abundance (Planas & Ribera, Paper 3), and it is only rarely found in humid environments. Therefore, the current climatic conditions of these islands seem to fit well with the ecological requirements of *L. sp.* FV-LZ, and thus, the extinction due to climatic changes is improbable for this species. On the other hand, due to their intrinsic stochasticity, volcanism-related extinction events cannot be discarded, and indeed, multiple examples of lineage extinction linked to volcanic events have been suggested in Macaronesian islands (Cardoso *et al*., 2010).

We found support for the reciprocal monophyly of the two main island groups (Fuerteventura and Lobos; Lanzarote and La Graciosa), in population tree analyses and in clustering analyses (Fig. 1, 3 and Supplementary Figure 2). The separation between the two groups is consistent with an evident geographical barrier, the Bocaina strait, that lies between the two islands and that appeared recurrently during the Quaternary separating Fuerteventura from Lanzarote. These two islands together with their adjacent islets formed a single, extensive island known as Mahan, during the greatest part of the last 0.9 Mya, when sea-level fell to as much as -120 m with respect to the present-time sea level (Rijsdijik *et al*., 2014). Thus, the current situation of two separate islands and several islets could be seen as a transient state during this period. In this situation, proposed scenarios of dispersal events from island to island would be flawed, and better explanations would assume recurrent vicariant events (sea barriers) breaking land connections of the populations inhabiting Mahan. Thus, the land surface connecting the two islands, currently below sea level, was likely colonized by individuals from both North Fuerteventura and South Lanzarote during sea-level lowstands, leading to a continuous distribution across Mahan. The isolation by distance pattern supports this view, with similar positive correlation between genetic and geographic distances in the analyses conducted with all

localities or grouped by island (but see below). Thus, sea level increases acted as a recurrent extinction factor for these populations, interrupting the genetic connectivity across Mahan, leading to the current reciprocal monophyly of the two main islands. Also, intraspecific competitive exclusion, a widespread and often obviated process that successfully excludes new dispersers (Waters, 2011), probably impeded the substantial advance of these lineages once they came in contact, explaining the lack of admixture found in clustering analyses, although gene-flow between them would have been sufficient to maintain genetic cohesion between the two lineages. The results of the second global score of sPCA analysis (Supplementary Figure B) corroborate the unity of individuals from across the Bocaina strait. Indeed, sPCA has been shown to retrieve genetic structures that could be overlooked by other methodologies (Jombart *et al*., 2008).

Fuerteventura

The few phylogeographic studies conducted in Fuerteventura coincide in highlighting the distinctiveness of the southernmost Península Jandía lineages (Juan *et al*., 1998; Bidegaray-Batista *et al*., 2007; Greve *et al*., 2012; López *et al*., 2013). In contrast, the individuals included in our analyses from Jandía (localities FV1 and FV2) were not distinguished by higher divergence, and were always closely related to those from the nearest localities in the main part of Fuerteventura (localities FV3 and FV4). Clustering analyses, conducted with microsatellite data, recovered two groups of individuals, a northern and a southern one (Fig. 1, 3 and Supplementary Figure 2). Although this separation does not coincide with any known barrier to gene flow, the same results were obtained with alternative methodologies that are differentially affected by possible biases (Rutledge *et al*., 2010). For example, sPCA explicitly accounts for spatial autocorrelation (Jombart *et al*., 2008) and its results exhibited the same two genetic clusters as the ones in STRUCTURE analyses. However, this pattern was not coincident with the groups obtained with mtDNA data. In *cox1* TCS analyses, individuals from FV6 were set apart from the remaining ones, although in gene trees these individuals were always closely related with those from the northern localities (Supplementary Fig. 1). In *Loxosceles* spiders, females show higher levels of philopatry than males, and this may be mirrored in a strong population structure as observed in mtDNA markers, for example with *cox1* haplotypes being exclusive from each locality (Fig. 2). Despite this population structure, the shallow c*ox1* divergences suggest that the IBD pattern, that predicts that genetic similarity between individuals would be

proportional to the geographic distance between them, shapes a gradual divergence across the island. Thus, additional sampling should be conducted in the area between localities FV6 and FV7 to further corroborate this putative barrier to gene flow recovered with microsatellite data and to test if this separation is related to the current physical geography of the island or to historical processes.

Although a sea barrier currently separates Lobos from Fuerteventura, individuals from Lobos appeared closely related to those from northern Fuerteventura localities (FV7 and FV8, Fig. 1 and 2). This pattern is in accordance with the IBD pattern and with the view that these islands should be interpreted as one unit, especially for the last 0.9 Myr (see above), considering that the mean sea level for this period was 60 m lower than at present (Rijsdijk *et al*., 2014), and that Lobos is only separated by shallow waters (~20 m) from Fuerteventura. Similar phylogeographic patterns have also been found in studies conducted with grasshoppers *Purpuraria erna* (López *et al*., 2013) and land snails *Theba* sp. (Greve *et al*., 2012).

Lanzarote: divided by volcanoes

Similarly to the pattern found in Fuerteventura, two groups exhibited a north-south structuring in Lanzarote, although a closer examination revealed interesting differences. While in Fuerteventura the divergences were relatively shallow and clearly geographically structured, in Lanzarote the two deep mitochondrial lineages almost coincided geographically in the area where localities LZ5 and LZ7 are found in close proximity (Fig. 1). The two groups recovered with DAPC and sPCA analyses coincide with the two main mitochondrial lineages (Fig. 1, 3 and Supplementary Fig. 1 and 2), while STRUCTURE analyses placed individuals from locality LZ7 with the southern group rather than with the northern one (Fig. 1). Even though, as above, differences between patterns obtained with nuclear or mitochondrial markers could be the result of different dispersal capacities between sexes, in this case the differentiation predicted with IBD is not so gradual and the separation appears in a narrow area. Thus, this area corresponds more likely to a secondary contact zone between two groups that had previously been separated. The separation between the two main lineages was estimated at between 0.165 and 0.3 Ma, a time coincident with extensive volcanism in the central part of Lanzarote that started at 0.75 Ma and that continued until very recently (Carracedo & Rodrígues-Badiola, 1993). Vicariance promoted by the extensive volcanic activity that occurred in central Lanzarote during the

upper Pleistocene and the Holocene also affected allopatric fragmentation in *Gallotia atlantica* lizards (Bloor *et al*., 2008), in the ground spiders *Dysdera lancerotensis* (Bidegaray-Batista *et al*., 2007) and in *Dysdera alegranzaensis* (Macías-Hernández *et al*., 2013). Although divergence time estimates were slightly older in these studies, the multispecies coalescent approach used in our dating analyses, has been shown to provide younger age estimates (McCormack *et al*., 2011), and thus, discrepancies could be related to this effect. Major catastrophic events such as volcanic eruptions probably prompted the extinction of the populations from this central area, providing new opportunities for colonization. Interestingly, in previous studies the area around Zonzamas, near locality LZ5 (Fig. 1), had been interpreted as a possible refugium during these volcanism phases (Macías-Hernández *et al*., 2013 and references herein). In *Loxosceles sp.* FV-LZ, the individuals from this locality seem to differ genetically from those from the remaining localities in sPCA analyses (Fig. 2), also when considering the second score of these analyses (Supplementary Fig. 3), supporting the biogeographical relevance of this area.

Individuals from La Graciosa are generally closely related to the ones from northern Lanzarote localities. This pattern has also been found in *Hegeter politus* snails (Juan *et al*., 1998), in the woodlouse-hounter spider *Dysdera alegranzaensis* (Macías-Hernández *et al*., 2013) and in the trap-door spider *Titanidiops canariensis* (Opatova & Arnedo, pers. comm.). As in the case between Lobos and Fuerteventura, the land connection between La Graciosa and Lanzarote is expected to be the rule rather the exception during the Pleistocene, and the pattern obtained in *Loxosceles* sp. FV-LZ strongly corroborates this hypothesis.

CONCLUSIONS

Low sea level during most of the last 0.9 Myr allowed the connection of Fuerteventura, Lanzarote and their neighboring islets in a single island, Mahan. *Loxosceles sp.* FV-LZ is endemic to Mahan, and due to its abundance and low dispersal capacity it represents a good model to test the effects of such sea-level changes in shaping phylogeographic patterns. Recurrent extinction events during sea-level rise in the area between the two main present-day islands reinforced the apparent separation in two reciprocal monophyletic groups, corroborated by nucleotide and microsatellite data. Although the phylogeographic pattern observed in Fuerteventura is hypothesized to have been mainly shaped by the effect of

isolation by distance, the pattern in Lanzarote is more complex, and volcanism-related events occurring during the Late Pleistocene in the central part of the island have probably led to the formation of two deep lineages. Furthermore, our results provide further support to the previously suggested Zonzamas volcanic refugium in central-eastern Lanzarote. Individuals from the islets of Lobos and La Graciosa were grouped with the neighboring localities of Fuerteventura and Lanzarote, respectively. This pattern is in accordance with the long periods of land connection given the lower sea level during the Pleistocene and the shallow water-barrier currently separating these islets and islands.

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SUPPLEMENTARY MATERIAL

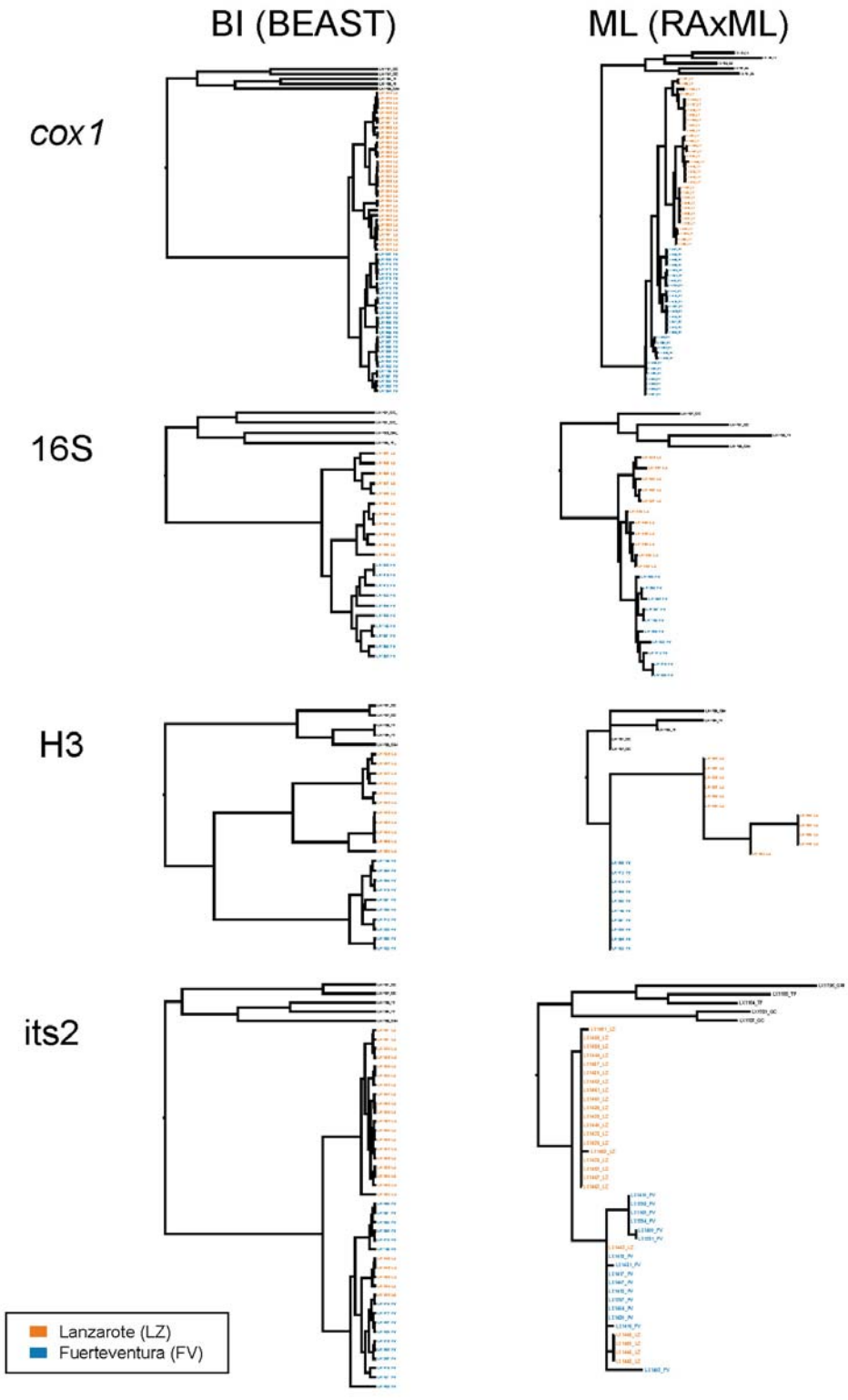
Supplementary Table 2 Microsatellite numbers of alleles (N), and observed (H_O) and expected (H_E) heterozigosity for each locus and mean for the whole dataset (FV-LZ) and for the two main groups (FVL and LZG).

	FV-LZ			FVL			LZG		
	N	H_O	H_E	N	H_O	H_E	N	H_O	H_E
CA003	7	0.268	0.801	6	0.360	0.815	2	0.194	0.508
CA038	6	0.232	0.646	4	0.346	0.669	5	0.133	0.515
CA001	10	0.554	0.765	8	0.375	0.815	8	0.169	0.718
CA105	5	0.776	0.751	4	0.539	0.553	4	0.969	0.668
CA027	10	0.417	0.830	8	0.222	0.816	9	0.576	0.815
CA030	14	0.491	0.887	10	0.607	0.828	14	0.380	0.893
CA238	7	0.607	0.709	6	0.625	0.694	7	0.594	0.484
mean	8.429	0.770	0.478	6.571	0.741	0.439	7	0.657	0.505
s.d.	3.101	0.191	0.080	2.225	0.152	0.106	3.916	0.292	0.162

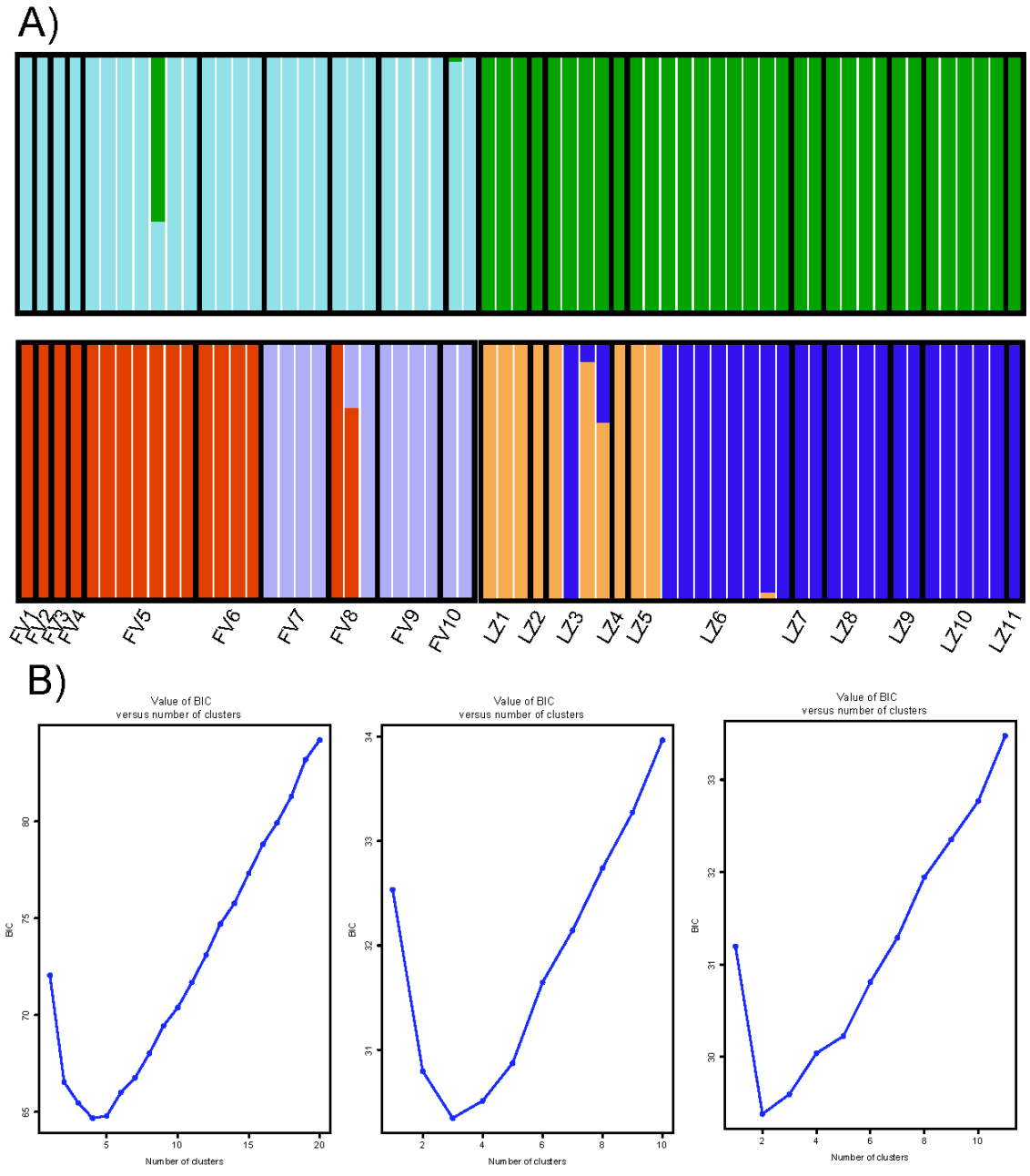
Supplementary Table 1 Study material. A list of the specimens used for the phylogeographic study of *Loxosceles* sp. FV-LZ, with their identification code, locality name, locality number (LN), coordinates and GenBank accession numbers.

CODE	LOCALITY	LATITUDE	LONGITUDE	LN	*COI*	*16S*	*H3*	ITS2
LX1394_FV	Cofete	28.10265	-14.38232	FV1	KF669931	KF670037	KF670079	KF670114
LX1392_FV	Península Jandía	28.0854	-14.37196	FV2	KF669929	KF670036	KF670078	KF670113
LX1391_FV	Tequital	28.26339	-13.94874	FV3	KF669928	KF670035	KF670077	KF670112
LX1169_FV	Tuineje	28.277677	-13.950876	FV4	KF669927	KF670034	KF670076	KF670111
LX1395_FV	Cueva Tiscamanitas	28.33501	-13.99525	FV5	KF669932			
LX1397_FV	Cueva Tiscamanitas	28.33501	-13.99525	FV5	KF669933			X
LX1398_FV	Cueva Tiscamanitas	28.33501	-13.99525	FV5	KF669934			
LX1399_FV	Cueva Tiscamanitas	28.33501	-13.99525	FV5	KF669935			
LX1400_FV	Cueva Tiscamanitas	28.33501	-13.99525	FV5	KF669936	KF670038	KF670080	KF670115
LX1403_FV	Cueva Tiscamanitas	28.33501	-13.99525	FV5	KF669937			X
LX1404_FV	Cueva Tiscamanitas	28.33501	-13.99525	FV5	KF669938			X
LX1406_FV	Aguas Verdes	28.47845	-14.05128	FV6	KF669939	KF670043	KF670081	
LX1407_FV	Aguas Verdes	28.47845	-14.05128	FV6	KF669940			X
LX1408_FV	Aguas Verdes	28.47845	-14.05128	FV6	KF669941			
LX1409_FV	Aguas Verdes	28.47845	-14.05128	FV6	KF669942			
LX1421_FV	Calderilla de Roja	28.62996	-13.83421	FV7	KF669951			X
LX1422_FV	Calderilla de Roja	28.62996	-13.83421	FV7	KF669952	KF670042	KF670085	
LX1423_FV	Calderilla de Roja	28.62996	-13.83421	FV7	KF669953			
LX1424_FV	Calderilla de Roja	28.62996	-13.83421	FV7	KF669954			
LX1410_FV	Villaverde	28.65061	-13.91518	FV8	KF669943			
LX1411_FV	Villaverde	28.65061	-13.91518	FV8	KF669944			
LX1412_FV	Villaverde	28.65061	-13.91518	FV8	KF669945	KF670041	KF670082	
LX1415_FV	Lobos	28.7439	-13.82542	FV9	KF669946			
LX1416_FV	Lobos	28.7439	-13.82542	FV9	KF669947			
LX1417_FV	Lobos	28.7439	-13.82542	FV9	KF669948			
LX1418_FV	Lobos	28.7439	-13.82542	FV9	KF669949	KF670039	KF670083	KF670116
LX1037_FV	Lobos	28.75205	-13.83010	FV10	KF669915			
LX1420_FV	Caldera Lobos	28.75205	-13.83010	FV10	KF669950	KF670040	KF670084	KF670117
LX1426_LZ	Costa Papagayo	28.86038	-13.77626	LA1	KF669956			
LX1427_LZ	Costa Papagayo	28.86038	-13.77626	LA1	KF669957	KF670050	KF670087	KF670119
LX1429_LZ	Costa Papagayo	28.86038	-13.77626	LA1	KF669958			X
LX1425_LZ	Salinas de Janubio	28.94288	-13.81886	LA2	KF669955	KF670053	KF670086	KF670118
LX1460_LZ	Puerto Calero	28.93313	-13.70168	LA3	KF669981			X
LX1461_LZ	Puerto Calero	28.93313	-13.70168	LA3	KF669982			X
LX1462_LZ	Puerto Calero	28.93313	-13.70168	LA3	KF669983			X
LX1463_LZ	Puerto Calero	28.93313	-13.70168	LA3	KF669984	KF670051	KF670094	KF670125
LX1464_LZ	Tinajo	29.0614	-13.6777	LA4	KF669985	KF670052	KF670095	
LX1457_LZ	Costa Teguise	29.02955	-13.51675	LA5	KF669978	KF670054	KF670092	
LX1458_LZ	Costa Teguise	29.02955	-13.51675	LA5	KF669979			
LX1432_LZ	Los Valles	29.07914	-13.52484	LA6	KF669959			X
LX1433_LZ	Los Valles	29.07914	-13.52484	LA6	KF669960			X
LX1434_LZ	Los Valles	29.07914	-13.52484	LA6	KF669961			
LX1435_LZ	Los Valles	29.07914	-13.52484	LA6	KF669962			X
LX1436_LZ	Los Valles	29.07914	-13.52484	LA6	KF669963			X
LX1437_LZ	Los Valles	29.07914	-13.52484	LA6	KF669964			
LX1438_LZ	Los Valles	29.07914	-13.52484	LA6	KF669965			X

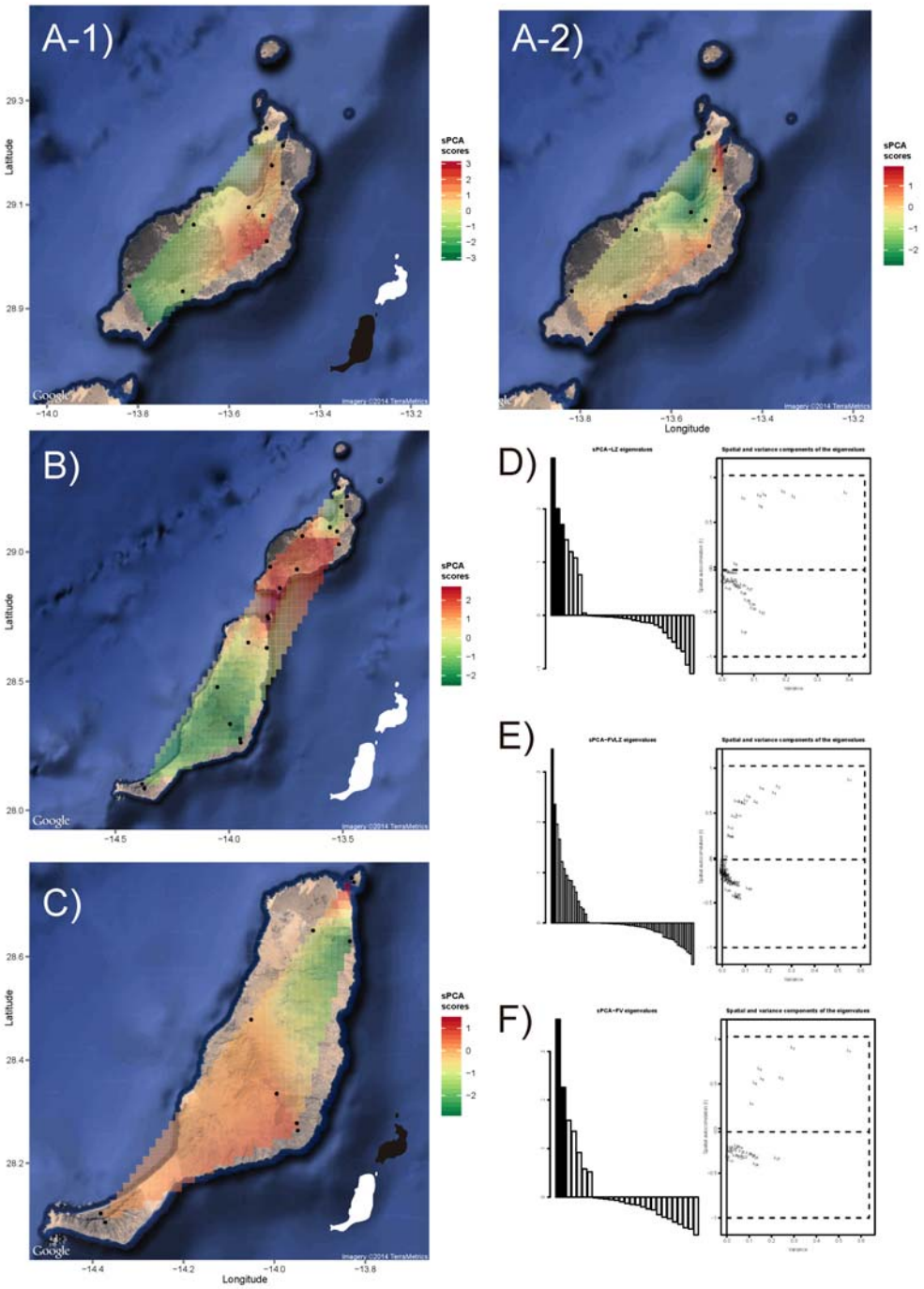
LX1440_LZ	Los Valles	29.07914	-13.52484	LA6	KF669966	KF670049	KF670088	KF670120
LX1466_LZ	Teguise	29.09475	-13.55653	LA7	KF669986	KF670045	KF670096	
LX1467_LZ	Teguise	29.09475	-13.55653	LA7	KF669987			
LX1453_LZ	Barranco Hondo del Valle	29.14139	-13.48203	LA8	KF669974			
LX1454_LZ	Barranco Hondo del Valle	29.14139	-13.48203	LA8	KF669975	KF670047	KF670091	KF670123
LX1455_LZ	Barranco Hondo del Valle	29.14139	-13.48203	LA8	KF669976			X
LX1456_LZ	Barranco Hondo del Valle	29.14139	-13.48203	LA8	KF669977			X
LX1451_LZ	Guinate	29.17536	-13.50576	LA9	KF669972			X
LX1452_LZ	Guinate	29.17536	-13.50576	LA9	KF669973	KF670046	KF670090	KF670122
LX1443_LZ	Mirador del Río	29.21336	-13.48111	LA10	KF669967			X
LX1444_LZ	Mirador del Río	29.21336	-13.48111	LA10	KF669968			
LX1445_LZ	Mirador del Río	29.21336	-13.48111	LA10	KF669969	KF670044	KF670089	KF670121
LX1446_LZ	Mirador del Río	29.21336	-13.48111	LA10	KF669970			X
LX1448_LZ	Mirador del Río	29.21336	-13.48111	LA10	KF669971			
LX1459_LZ	La Graciosa	29.2472	-13.518	LA11	KF669980	KF670048	KF670093	KF670124



Supplementary Figure 1 Gene trees for the *cox1*, 16S, H3 and its2 reconstructed with Bayesian Inference (BEAST) and Maximum Likelihood (RAxML). Colored codes of the individuals correspond to the two main clusters: blue indicates Fuerteventura and Lobos localities, and orange Lanzarote and La Graciosa ones, as in Figure 2. Outgroups are not colored.



Supplementary Figure 2 Results of the Discriminant Analysis of Principal Components (DAPC) conducted with the microsatellite dataset. **A)** DAPC plot of group membership. First diagram shows DAPC results for K = 2 of the analysis conducted on the whole dataset, second diagram shows DAPC results for K=2 on the analyses conducted on separate datasets of the previously delimited clusters (i.e. Fuerteventura and Lobos, Lanzarote and La Graciosa). Each vertical line represents the proportional membership assignment of one individual to each of the two clusters. Black lines delimit sampling localities and locality codes correspond to those on the map.**B)** graph of BIC values for increasing values of k for analysis conducted with Fuertventura and Lobos, on the whole dataset, and Lanzarote and La Graciosa, respectively.



Supplementary Figure 3 Results of the Spatial Principal Component Analysis (sPCA). **A1)** Projection of the second and **A2)** third global component of the sPCA conducted with the microsatellite dataset from individuals from Lanzarote and La Graciosa. **B)** Projection of the second global component of the sPCA conducted with the whole microsatellite dataset and **C)** the dataset of individuals from Fuerteventura and Lobos. As in Fig. 3, to better detect visually putative barriers to gene flow, values between sampled localities were interpolated with the interp function in R package akima (Akima et al., 2009) and plotted with ggmap (Kahle and Wickham, 2013). sPCA eigenvalues, and their decomposition into variance and spatial autocorrelation (*I*) components of sPCA eigenvalues (λ_n) for the analyses conducted with **D)** the dataset of individuals from Lanzarote and La Graciosa, E) the whole dataset and F) the dataset of individuals from Fuerteventura and Lobos. Eigenvalues retained in the analyses are highlighted in black. Positive eigenvalues reflect global patterns and negative eigenvalues reflect local patterns.

CAPÍTOL 3

Diversitat del gènere *Loxosceles* al Nord d'Àfrica

Article 6 Ribera C, Planas E. 2009. A new species of *Loxosceles* (Araneae, Sicariidae) from Tunisia.

Article 7 Planas E, Ribera C. On the shoulders of Atlas: high genetic diversity of *Loxosceles* spiders (Araneae: Sicariidae) in the Souss-Massa and adjacent regions (NW Africa).

Una nova espècie de *Loxosceles* (Araneae: Sicariidae) de Tunísia

RESUM

En el present treball es descriu i s'il·lustra una nova espècie del gènere d'aranyes *Loxosceles*, *L. mrazig* **sp. n.** trobada a Tunísia. El bulb dels mascles presenta una certa semblança amb el de *L. gaucho* de Brasil, però les proporcions dels segments del palp i la coloració general del cos revelen diferències significatives entre aquestes dues espècies. Les distàncies genètiques obtingudes amb el gen mitocondrial citocrom oxidasa I (*cox1*) revelen que l'espècimen de Tunísia presenta unes distàncies genètiques molt elevades respecte a *L. gaucho* (superiors al 20%). Les espècies americanes *L. gaucho* i *L. laeta* formen el grup germà del grup format per espècies mediterrànies (*L. rufescens* i l'individu de Tunísia).

PARAULES CLAU: taxonomia, Araneae, *Loxosceles*, nova espècie, Tunísia.

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RESEARCH ARTICLE

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A new species of *Loxosceles* (Araneae, Sicariidae) from Tunisia

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Abstract

A new species of the spider genus *Loxosceles*, *L. mrazig* **sp. n.**, found in Tunisia is described and illustrated. The male bulb shows a high degree of morphological similarity to that of *L. gaucho* from Brazil, but the proportions of the palpal segments and the general colouration of the body reveal significant differences between the two species. A distance analysis of the sequences of the mitochondrial gene *cox1* reveals that the specimen from Tunisia shows high genetic distance from *L. gaucho* (more than 20%). The American species *L. gaucho* and *L. laeta* form a sister group to the Mediterranean representatives (*L. rufescens* and the Tunisian specimen).

Keywords

Taxonomy, Araneae, *Loxosceles*, new species, Tunisia

Introduction

The genus *Loxosceles* Heineken et Lowe, 1832 is currently known to comprise 97 species (Platnick 2009), 82 of which occur in America, 12 in Africa and two in China. Following Brignoli's (1969, 1976) contributions with respect to the Mediterranean basin, only a single species is currently accepted as valid, *L. rufescens* (Dufour, 1820),

whose type locality is near Sagunt, Valencia (Spain). The other two (sub-) species are considered *nomina dubia*: *L. decemnotata* Franganillo, 1925 from Spain and *L. rufescens lucifuga* Simon, 1910 from Algeria. In the same paper Brignoli (1976) reported the South American species *L. gaucho* Gertsch, 1967 from Tunisia.

In 2007 colleagues from the Ecology Department at the University of Barcelona collected in Douz (Tunisia) a male of *Loxosceles* in a dune located several kilometres from the city. The morphology of the copulatory bulb is remarkably similar to that of *L. gaucho* from Brazil, although the differences of the proportions of the male palpal segments plus the general colouration of the body suggested that it could be a different species. In order to test this curious distribution, we used the cytochrome oxidase I gene (*cox1*) to compare this new record with *L. gaucho* (Sao Paulo, Brazil).

Material and methods

Taxonomy. Specimens were examined under a Zeiss Stereo Discovery V12 stereomicroscope equipped with an Infinity X DeltaPix digital camera. Digital microscopic images were edited using DeltaPix DpxWiew Pro AZ V. 13.6 software, using an enhanced focus function. Ink drawn digital illustrations were generated with the assistance of Photoshop CS3 software.

Measurements were taken using the enhanced focus function incorporated into the DeltaPix DpxWiew Pro AZ software. All morphological measurements are given in millimetres. Prosoma and opisthosoma measurements were taken in dorsal view. Total body length represents the sum of the lengths of the prosoma and opisthosoma, omitting the pedicel. Eye largest diameters were taken from the spans of the lens. The largest leg article lengths were measured in lateral view without detaching the legs from the specimen, by placing the article being measured in a perpendicular position. Holotype, and all other specimens are deposited in the Arachnid Collection of the CRBA (Centre de Recursos de Biodiversitat Animal) at the University of Barcelona; catalogue numbers are given in brackets.

Abbreviations used in the text. CRBA – Centre de Recursos de Biodiversitat Animal, Universitat de Barcelona, Spain. Eyes: **ME** = median eyes; **LE** = lateral eyes.

Molecular data. Taxonomic sampling. Taxa analyzed in the present study are listed in Table 2. *L. mrazig* sp. n. from Douz (Tunisia), *L. gaucho* from Sao Paulo (Brazil) and nine specimens of *L. rufescens* from different localities in the Iberian Peninsula and Tunisia were analyzed. In addition we included a representative of *Loxosceles laeta* (Nicolet, 1849) (Montevideo, Uruguay) in order to test the phylogenetic affinities of *L. gaucho* with other South American species, since *L. laeta* belongs to a different species group (Gertsch 1967; Binford et al. 2008). A sequence from *Dysdera crocata* C. L. Koch, 1838 from GenBank was also included to root the tree.

Sample Storage and DNA Extraction. Specimens were preserved in 95% or absolute ethanol and stored at 4°C. Total genomic DNA was extracted from legs of a single specimen using the QIamp® DNA Mini Kit (QIAGEN) following the manufacturer's

protocols. The approximate concentration and purity of the DNA obtained were verified using 1.5% agarose/TBE gel electrophoresis.

PCR Amplification and Sequencing. A total of 899 bp of the cytochrome oxidase I gene (*cox1*) was amplified from each individual using PCR with the following primer pairs: C1-J-1718 (Simon et al. 1994) with C1-N-2776 (Hedin and Maddison 2001). The PCR reaction mixture contained a final concentration of 0.2 µM of each primer, 0.2 mM of each dNTPs, 0.5 U Taq polymerase (Promega), with the supplied buffer, and 1.5-2.5 mM Mg Cl2 in a final volume of 25 µL.

A Perking-ElmerCetus Moldel 480 thermocycler was used to perform 35 iterations of the following cycle: 30s at 94°C, 45s at 44°C, and 1 min at 72°C, beginning with an additional step of 3 min at 94°C, and ending with another step of 5 min at 72°C. The PCR results were visualized by means of a 1.5% agarose/TBE gel. Amplified products were purified using MultiScreen 96 – well filter plates from Millipore. The purified products were directly cycle-sequenced from both strands using ABI BigDye (Applied Biosystems) chemistry and run out on ABI Prism 377 (Applied Biosystems) automated sequencers. Sequencing reactions were performed in our lab with the forward and reverse PCR primers and one additional pair of internal *cox1* primers, CI-J-2183 and C1-N-2191 (Simon et al. 1994). The resulting products were run and analyzed at the Serveis Científico-Tècnics of the Universitat de Barcelona.

Alignment. Raw sequences were compared against chromatograms and complementary contigs built and edited using the Geneious Pro 3.6.2 software (http://www.genious.com). Sequences were manipulated and preliminary manual alignments constructed using BioEdit V.7.0.5.3 (Hall 1999). Alignment of *cox1* was trivial, given that no evidence of insertions/deletions was observed.

Genetic distances and distance analyses. Uncorrected genetic distances between and within taxa were estimated with MEGA v.3.0 (Kumar et al. 2004). The Neighbour-joining algorithm was applied to the estimated genetic distances to build a phenogram (Saitou and Nei 1987) conducted with the same program. Clade support was assessed via Bootstrap (Felsenstein 1985) as implemented in MEGA, based on 1000 bootstrap replicates.

Results. Molecular data. The distance tree is shown in Fig. 1. The specimens identified as *L. rufescens* form a monophyletic clade with a high support value (100%). *L. mrazig* sp. n. is supported as more closely related to *L. rufescens* (75% bootstrap value) than to the two South American representatives included in this analysis: *L. gaucho* and *L. laeta*. The latter two species cluster together with moderate support (68%).

Averages between group genetic distances are presented in Table 1. The cluster formed by the nine specimens of *L. rufescens* from Spain and Tunisia shows scarce

Table 1. Average between group genetic distances of gene *cox1* from the four species analyzed.

	L. rufescens	***L. mrazig***	***L. gaucho***
L. mrazig	0.1991		
L. gaucho	0.1927	0.2063	
L. laeta	0.1973	0.2086	0.1635

within-group average genetic distances (0.26%) and suggests that this species shows a high genetic coherence. The deep genetic divergence between *L. mrazig* and *L. gaucho* (20.63%) together with the observation that both species belong to different clusters provide clear evidence that *L. mrazig* is an independent evolutionary lineage and should, therefore, be considered a different species.

Table 2. Species included in the phylogenetic analysis and GenBank accession numbers for *cox1*.

Species	Locality	GenBank Accession number
Loxosceles laeta	Montevideo, Uruguay	FJ986177
Loxosceles gaucho	Sao Paulo, Brazil	FJ986178
Loxosceles mrazig sp. n.	Douz, Tunisia	FJ986179
Loxosceles rufescens	Torrejon de Ardoz, Madrid, Spain	FJ986183
Loxosceles rufescens	Ciudad Real, Spain	FJ986185
Loxosceles rufescens	Denia, Alacant, Spain	FJ986187
Loxosceles rufescens	Chumilla, Murcia, Spain	FJ986181
Loxosceles rufescens	Barcelona, Spain	FJ986182
Loxosceles rufescens	Siles, Jaen, Spain	FJ986188
Loxosceles rufescens	Sierra Gorda, Cartagena, Murcia, Spain	FJ986180
Loxosceles rufescens	Alacant, Spain	FJ986184
Loxosceles rufescens	Testour, Tunisia	FJ986186
Dysdera crocata	Hoz de Pergrina, Guadalajara, Spain	EF458137

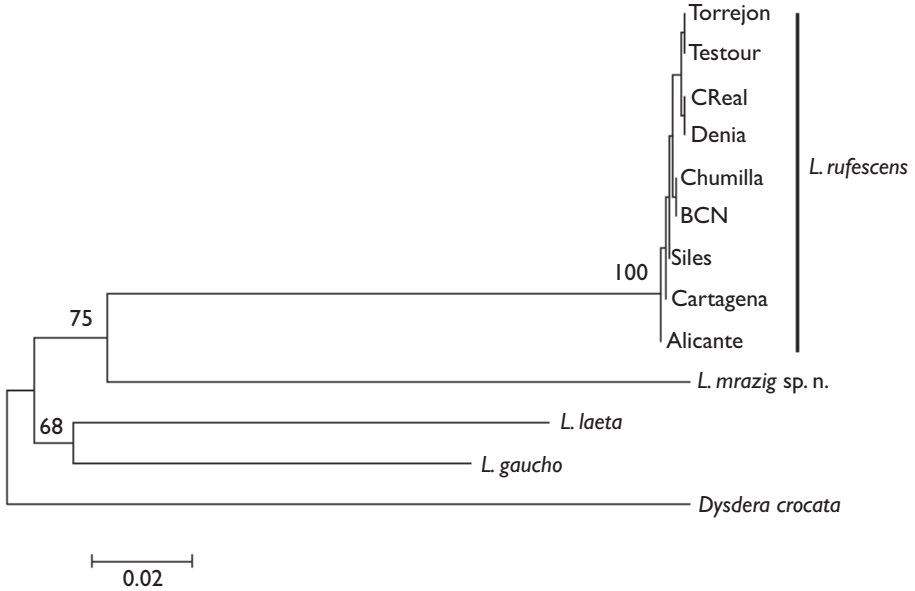


Figure 1. Neighbour-joining distance tree. Different representatives of *L. rufescens* from the western Mediterranean basin (Spain and Tunisia), *L. mrazig* sp. n., *L. gaucho* and *L. laeta* are included. Numbers on nodes represent bootstrap support values.

Taxonomy

Family Sicariidae
Genus *Loxosceles* Heineken et Lowe, 1832

***Loxosceles mrazig* sp. n.**
urn:lsid:zoobank.org:act:A8F75878-85CA-4567-874B-EBC8CC24CD02
Figs 2-7

Material examined. 1 male (Holotype) from Douz, Tunisia, 33° 24' 26.77" N, 09°02'41.92"E, 27 January 2007, Cesc Múrria leg. (CRBA-LX1054).

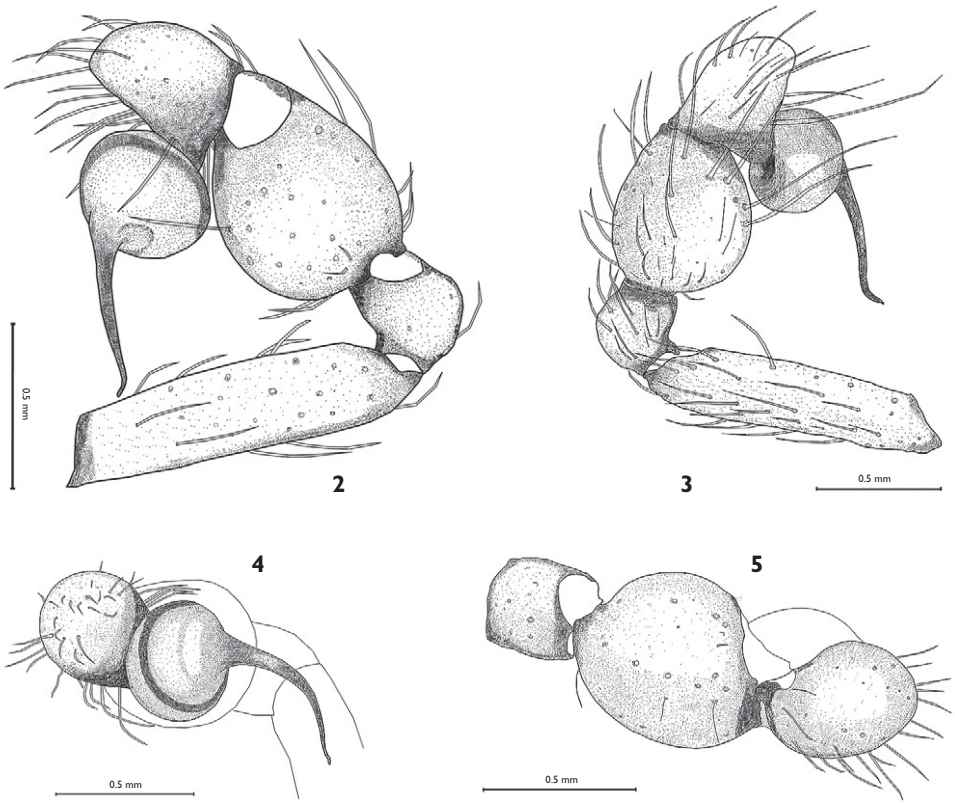
Material for comparison. 2 males, 2 females of *L. gaucho* (CRBA-LX1024) from Sao Paulo, Brazil, November 2007, A. Brescovit leg.; 2 males, 2 females of *L. laeta* (CRBA-LX1028) from Montevideo, Uruguay, L. Acosta leg.; 1 male, of *L. rufescens* (CRBA-LX1012) from Gavà, Barcelona, López-Pancorbo leg.

Etymology. The species' name honours the people called Mrazig, formerly nomadic, living in and around the city of Douz (Tunisia). The Mrazig are the descendants of the Banu Saleim tribe that fled the Arabian Peninsula in the seventh century and came to Tunisia in the thirteenth century. It is known that they practiced transhumance in the Great Sahara. Noun in apposition.

Diagnosis. Differs from *L. gaucho*, *L. rufescens* and its similar relatives in the proportion of male palp segments, mainly the tibia. In *L. mrazig* the tibia is markedly oval, slightly longer than wide (0.63 - 0.54) (Figs 2, 3, 5); in *L. gaucho* it is ¾ as wide as long (Gertsch 1967, plates 3-4), whereas, in *L. rufescens*, it is slightly oval, although dorsally almost straight (Gertsch 1967, plate 10). Also differs from *L. rufescens* by the size of the tegulum and the size and shape of the embolus (Figs 2-5). Body pigmentation yellowish-brown in *L. gaucho* and pale yellow in *L. mrazig*. In general, the morphological differences compared to *L. rufescens* are more conspicuous. The size of the tegulum and, especially, the shape and length of the embolus are clearly different.

Description. Colouration: Carapace pale yellowish with a fine, pale brown lateral stripe. Median groove and adjacent integuments darkened. Pars cephalica slightly darkened, brown coloured, and clearly demarcated by a lateral reddish brown line. Less conspicuous, but still important, diagnostic traits are the four thin longitudinal lines (lightly impressed when seen under higher magnification) located in the centre of the pars cephalica (Fig. 6). Eye tubercles black. Sternum pale yellowish, paler than carapace. Labium and gnathocoxae with slightly more pigmentation. Legs light yellow or somewhat shaded, with the apical segments slightly darkened. Opisthosoma yellowish-white.

Prosoma. Carapace (Fig. 6) slightly longer (2.39) than wide (2.15). Median groove deep, occupying the posterior third of carapace. Clypeal width slightly more than 2.5 diameters of ME. Eyes close together (Fig. 7); LE separated from ME by the diameter of ME. LE larger than ME (0.18 - 0.1 respectively). Sternum about ⅔ as wide as long, extended between the IV pair of coxae. Labium as long as wide at its base, apically narrowed and rounded. Gnathocoxae distally convergent, enclosing the labium.



Figures 2-5. Male palp of *Loxosceles mrazig* sp. n. **2** prolateral view **3** retrolateral view **4** apical view **5** dorsal view.

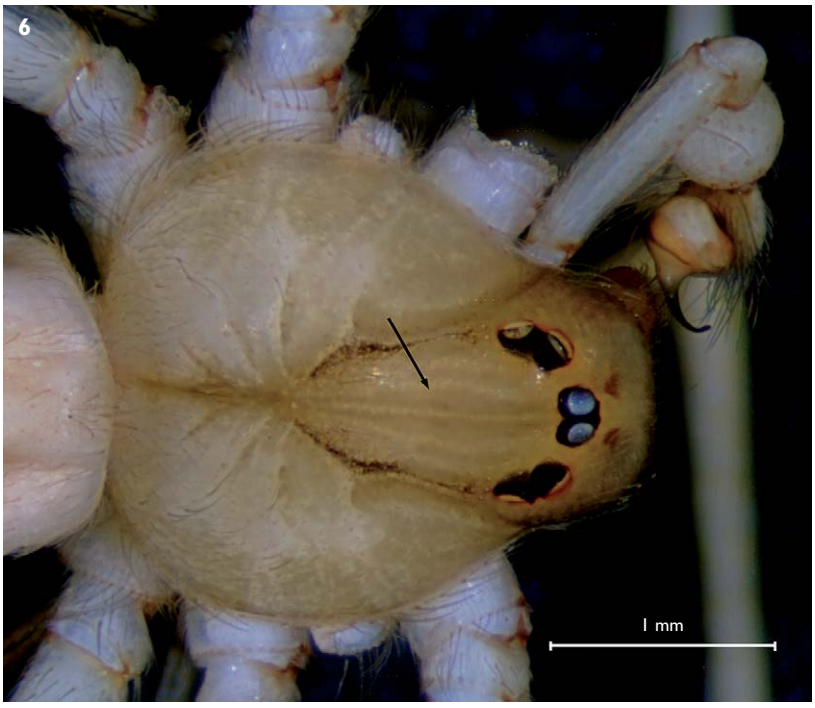
Opisthosoma elongate oval in dorsal view.

Male palp (Figs 2-5). Femur cylindrical, more than five times longer than wide. Tibia short, oval, slightly longer than wide. Tarsus flattened below, slightly shorter than tibia, rounded apically. Tegulum large, 4/5 as wide as tarsal length. Embolus enlarged at base, forming a sinuous curve, about 1.5 times longer than tegulum.

Measurements. Male (holotype): Prosoma 2.15 wide, 2.39 long: opisthosoma 3.22 long. Total body length 5.61. Legs: I: coxa 0.81, trochanter 0.23, femur 5.42, patella 0.84, tibia 5.70, metatarsus 5.76, tarsus 1.38, total length 20.14; II: coxa 0.58, rest of segments missing. III: coxa 0.81, trochanter 0.23, femur 4.94, patella 0.82, tibia 4.69, metatarsus 5.37, tarsus 1.12, total length 17.98; IV: coxa 0.81, trochanter 0.23, femur 5.26, patella 0.84, tibia 5.40, metatarsus 6.42, tarsus 1.33, total length 20.29; Palp: femur 1.19, patella 0.36, tibia 0.63, tarsus 0.56, total length 2.74.

Female unknown.

Distribution. So far, *L. mrazig* is known only from the type locality. The unique specimen was collected in a dune of sand near the city of Douz.



Figures 6-7. Prosoma of *Loxosceles mrazig* sp. n. **6** dorsal view, arrow indicates the four longitudinal lines located in the centre of the pars cephalica **7** frontal view.

Discussion

The possibility that this species should be assigned to *L. gaucho* can be ruled out due to the high genetic distance observed between both species (more than 20%) and especially because they do not form a sister group relationship, but belonging to different clades. The morphological similarity can be explained as a convergence phenomenon due to the simple morphological structures of the copulatory organs found in haplogyne spiders.

Determining the closest relatives is difficult for this species due to the lack of current knowledge on African *Loxosceles* species. Taking into account the shape of the male bulb, this species could be related to *L. foutadjalloni* Millot, 1941 from Guinea, in which the proportional palpal segments differs notably (mainly the tibia) and by the shape and size of the embolus. *L. mrazig* sp. n. – which could possibly be a member of a different group, or form a subgroup with the above mentioned *L. foutadjalloni*. *L. mrazig* sp. n. – is the second *Loxosceles* species known from the Mediterranean basin.

Acknowledgements

We would like to thank Luís Acosta from Uruguay and Antonio Brescovit from Brazil for their inestimable help and collaboration in providing us with the specimens of *L. gaucho* and *L. laeta* for this work. We also thank Cesc Múrria for collecting the new species and Miquel A. Arnedo for providing the sequence of *D. crocata*. This research was supported by the Spanish Ministry of Education and Science grants CGL2008-03385/BOS and CGL2006-13374/BOS.

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Sobre les espatlles d'Atles: una elevada diversitat genètica en les aranyes del gènere *Loxosceles* (Araneae: Sicariidae) a la regió del Souss-Massa i àrees adjacents (Nord Oest d'Àfrica)

RESUM

Aquest estudi documenta i caracteritza la diversitat d'aranyes del gènere *Loxosceles* en la regió del Souss-Massa, des dels vessants sud-oest de l'Alt Atles a les parts nord-oest de l'Anti-Atles. L'extens mostreig realitzat en aquesta regió biogeogràficament rellevant ha permès obtenir més de 100 individus de 19 localitats diferents. S'han utilitzat cinc marcadors moleculars (*cox1*, 16S, its2, 28S i H3) per tal de delimitar i inferir les relacions filogenètiques entre els diferents llinatges. A més, hem examinat qualitativament els caràcters genitàlics, tant masculins com femenins, en cadascun dels llinatges delimitats amb les dades moleculars. S'ha trobat una elevada diversitat d'aranyes del gènere *Loxosceles* a la regió del Souss-Massa, amb sis llinatges distribuïts de forma al·lopàtrica, afegint-se a la diversitat insuficientment estudiada d'aquest gènere a la regió Mediterrània i posant al descobert la rellevància biològica d'aquesta regió rica en endemismes.

PARAULES CLAU: muntanyes de l'Atles, vicariància, microal·lopatria, endemicitat, Marroc, aranyes.

On the shoulders of Atlas: high genetic diversity of *Loxosceles* spiders (Araneae: Sicariidae) in the Souss-Massa and adjacent regions (NW Africa)

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ABSTRACT

In this study, we focused on the diversity of the *Loxosceles* spiders in the biogeographically relevant region of the Souss-Massa (Morocco), across the south-western slopes of the High Atlas and the north-western part of the Anti-Atlas. We conducted a comprehensive sampling in this region collecting more than 100 individuals from 19 localities. We used five molecular markers (*cox1*, 16S, its2, 28S and H3) and carried out molecular analyses in order to investigate the phylogenetic placement of the lineages endemic to the Souss-Massa region and to delimit independent evolutionary lineages. Additionally, we qualitatively examined male and female genitalic characters of each lineage. Our study documents and characterizes the diversity of *Loxosceles* spiders in the Souss-Massa region where highly divergent lineages are distributed allopatrically, adding to the understudied diversity of the genus in the Mediterranean region and underlines the biological relevance of this endemism-rich area.

KEYWORDS: Atlas mountains, vicariance, microallopatry, endemicity, Morocco, spiders.

INTRODUCTION

The diversity of the spider genus *Loxosceles* Heineken & Lowe, 1832 is predominantly found in the temperate and tropical areas of North, Central and South America (Gertsch, 1967; Gertsch & Ennik, 1983; Binford *et al*., 2008). Successive comprehensive taxonomic revisions placed the numerous allopatric narrow-ranged species in different species groups on the basis of morphological characters, principally of the male palpi (Gertsch, 1967; Gertsch & Ennik, 1983). The systematic knowledge on this genus is less exhaustive in Africa and the Mediterranean Basin (Ribera & Planas, 2009; Duncan *et al*., 2010; Lotz, 2012), creating a biased image of higher specific diversity in the Americas. The conserved

morphology of somatic and genitalic features in the species of this haplogyne genus contrasts with the high divergences found with molecular data (Binford *et al*., 2008; Ribera & Planas, 2009; Duncan *et al*., 2010; Planas & Ribera, 2014; Planas *et al*., Paper 1; Planas & Ribera, Paper 3). For example, uncorrected *cox1 p*-distance between the recently discovered species endemic to the Canary Islands ranged from 10.6 % to 17.2% (Planas & Ribera, 2014).

According to current species accounts, the genus *Loxosceles* is represented by two species in North Africa: *L. mrazig* Ribera & Planas, 2009 and *L. rufescens* (Dufour, 1820). *Loxosceles mrazig* was described from a single locality in the arid environments of Douz, in Tunisia (Ribera & Planas, 2009). In contrast, *L. rufescens* is distributed across the Mediterranean Basin and has been introduced in different continents (Duncan *et al*., 2010; Planas *et al*., Paper 1). This latter species was described from the Iberian Peninsula and a recent study based on molecular and ecological niche modelling analyses revealed that Morocco harbours multiple intraspecific lineages exclusive to one or few localities in the the north-eastern foothills of the High Atlas, and that this area probably hosted refugia during Pleistocenic climatic oscillations (Planas *et al*., Paper 1). This result adds further support to a North African origin for this species, as was previously suggested by Duncan *et al*. (2010) based partially on the phylogenetic placement of a geographically close but genetically divergent Moroccan specimen (MA0101 in Duncan *et al*., 2010). Individuals from this and two other localities in the South of High Atlas were placed as sister to *L. rufescens* in Planas & Ribera (2014; i.e. "*Loxosceles* aff. *rufescens* Anti-Atlas clade"), together with which they formed a well-supported clade. Overall, these results suggested that this region could host higher diversity than that currently recognised.

Indeed, the Souss-Massa region, broadly delimited by the Souss and Massa river basins, including the Anti-Atlas mountains and the south-eastern foothills of the High Atlas, is known for its biogeographical importance. This region is dominated by the endemic tree *Argania spinosa*, and it is partially included in the Arganeraie Biosphere Reserve, declared a Biosphere Reserve by UNESCO in 1998 (Msanda *et al*., 2005; Charrouf & Guillaume, 2009). Despite the prevalence of this species, the vegetation landscape is also dominated by the gum tree *Acacia gummifera* and various *Euphorbia* shrubs (Médail & Quezel, 1999). This region is also distinguished by harbouring relict elements such as the dragon tree *Dracaena draco* subsp. *ajgal* (Benabid & Cuzin, 1997; Médail & Quezel, 1999) or the

Black cobra *Naja haje* (Bons & Geniez, 1996), and by high levels of endemicity in terrestrial land snails (Greve *et al*., 2010) and scorpions (Habel *et al*., 2012; Husemann *et al*., 2012; Sousa *et al*., 2012; Pedroso *et al*., 2013).

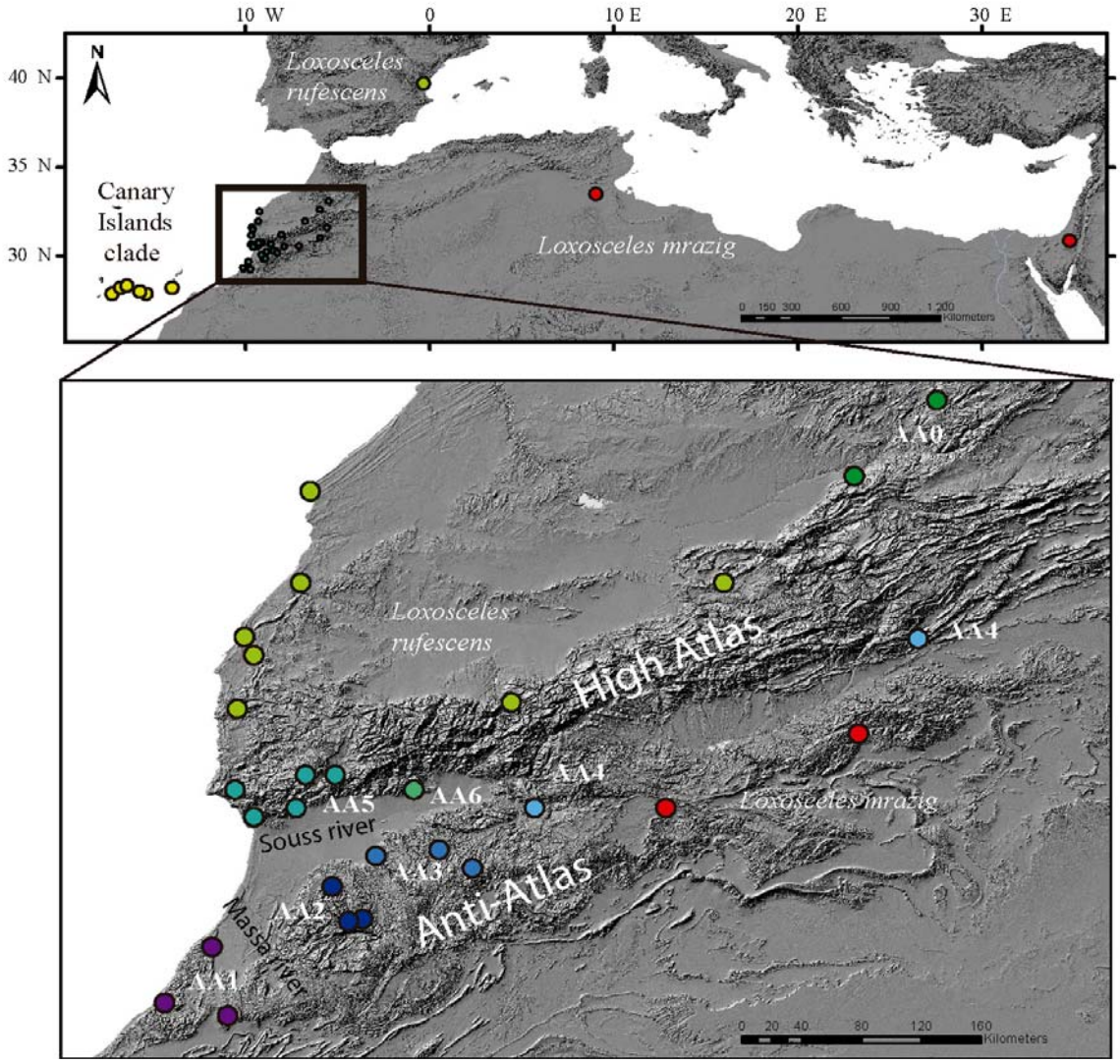

Figure 1 Sampling localities of the individuals included in this study are represented by circles and as in Fig. 2 and 3.

Given the preliminary data on high genetic diversity in *Loxosceles* and groups with similar ecological requirements in southern Morocco, in this study we focused on the Souss-Massa region to study the diversity of this spider genus. We conducted a comprehensive sampling across this area and used molecular data and phylogenetic analyses to discover, characterize, and delimit the existent genetic diversity in the region. This information was then used to delimit evolutionary lineages and to preliminary assess the morphological differences between them.

MATERIAL AND METHODS

Sampling

We included in this study a total of 56 *Loxosceles* specimens. Individuals from 19 localities from the Souss-Massa region and surrounding areas were collected during three sampling trips in 2007, 2010 and 2012 (Fig. 1). We also included one representative of each of the seven *Loxosceles rufescens* lineages distributed in Morocco, as identified in Planas *et al*. (Paper 1), that is lineages A1, A2, A3, A4, A6, B1 and B4, one individual from the type locality of the species, in Sagunt, Iberian Peninsula (lineage A6 in Planas *et al*., Paper 1),one representative of each Canary Island endemic species (Planas & Ribera, 2014) and four representatives of *L. mrazig*, including one individual from the type locality (Douz, Tunisia).

DNA extraction and sequencing

We extracted DNA from at least two individuals from each locality following the same procedure as in Planas *et al*. (Paper 1) and amplified a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I (*cox1*). We then selected one or two representatives from each locality based on the genetic variability observed in the *cox1* dataset, and amplified four additional markers: a fragment of the mitochondrial large ribosomal unit (16S); the nuclear gene histone 3 (H3); the internal transcribed spacer 2 (its2) and the nuclear large ribosomal unit (28S). Primers used are listed in Table 1. Selected sequences of *cox1*, H3 and its2 produced in previous studies were downloaded from GenBank and included in the dataset. Accession numbers from all the sequences used in the present study are listed in Supplementary Table 1.

Alignment, dataset partitioning and phylogenetic analyses

We performed alignments applying the G-INS-i algorithm for the protein-coding genes and the Q-INS-i algorithm for the ribosomal genes in the online version of MAFFT v. 7.157 (Katoh *et al*., 2005; Katoh & Toh, 2008).

Choosing an appropriate partitioning scheme is an important step in phylogenetic inference because it can affect the accuracy of the analyses (Lanfear *et al.*, 2012 and references therein). We selected the best partitioning scheme and the best-fit substitution model for each partition simultaneously using PartitionFinder 1.1.1 (Lanfear *et al*., 2012). The search for the best partitioning scheme among the nine possible data partitions (i.e. each ribosomal

gene and each codon position in protein-coding genes were defined as a putative partition) was conducted with the "greedy" heuristic algorithm. Partitioning schemes and substitution models selected with both the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) were employed in phylogenetic reconstructions to test for differences in the results.

We conducted preliminary analyses with *cox1* sequences using maximum likelihood (ML) in the RaxML-HPC BlackBox online server (Stamatakis *et al*., 2008). Phylogenetic analyses of the concatenated dataset applying the AIC and the BIC selected partition schemes were conducted using ML and Bayesian Inference (BI). ML analyses were conducted with the RAxML-HPC2 (Stamatakis, 2014) applying a GTRGAMMA nucleotide substitution model to each partition and conducting 1000 pseudoreplicates of the nonparametric bootstrap analysis. BI analyses were conducted in MrBayes 3.2.2 (Ronquist *et al*., 2012) applying the best-fit substitution model to each partition, and conducting two independent runs of five million generations each, saving trees each 1000th generation and discarding the first 25% of trees as burn-in after ensuring a correct mixing of the chains (EES > 200) in Tracer. ML and BI analyses were conducted on the CIPRES cluster (Miller *et al*., 2010).

Due to the lack of previous systematic information regarding either the morphological or genetic features of the study group, we attempted to objectively identify the evolutionary lineages within *Loxosceles* of the Souss-Massa region applying the General Mixed Yule Coalescent (GMYC) model (Pons *et al*., 2006). Because the method is sensitive to the input ultrametric tree, we used different approaches following Planas *et al*. (Paper 1). However, GMYC analyses grouped exclusively individuals from the same locality and therefore use of the method was discarded for this the study. Alternatively, we delimited major lineages in the group of interest based on the topology, branch length and support obtained in the phylogenetic analyses.

We calculated genetic *p*-distances between and within lineages using MEGA 6 (Tamura *et al*., 2013) with the complete *cox1* dataset that included all sequenced individuals of the *Loxosceles* collected in the Souss-Massa region and the selected *L. rufescen*s representatives included in the phylogenetic analyses.

Morphological variability

We selected adult specimens as representatives from each group for the qualitative study of

male palpi and female vulvae. Images were taken under a Zeiss Stereo Discovery V12 stereomicroscope equipped with an Infinity X DeltaPix digital camera.

Table 1 List of the primers used for this study. * Primer orientation: F, forward; R, reverse.

Gene fragment	Primer	PO*	Sequence (5′–3′)	Reference
cox1	LINF	F	GGGCNTGRTCWGGNATRATAGG	Planas & Ribera (2014)
	GAYAR	R	GAAAATGHGCHACHACRTAATAAGTRTC	Planas & Ribera (2014)
16S	Lox16SF	F	CGCCCTGTTTAACAAAAACATCAC	Planas & Ribera (2014)
	16SR	R	CCTTTAACGAATTTGAATATA	Hedin & Maddison (2001)
H3	H3aF	F	ATGGCTCGTACCAAGCAGACVGC	Colgan *et al*. (1998)
	H3aR	R	ATATCCTTRGGCATRATRGTGAC	Colgan *et al*. (1998)
28S	ZR1	F	GTCTTGAAACACGGACCAAGGAGTCT	Bond & Hedin (2006)
	A56	R	TCTTAGGACCGACTGACC	Bond & Hedin (2006)
	A53	F	CCGAAGTTTCCCTCAGGATAGC	Bond & Hedin (2006)
	A58	R	AGAGCCAATCCTTGTCCCGA	Bond & Hedin (2006)
its2	CAS28sB1d	R	TTCTTTTCCTCCSCTTAYTRATATGCTTAA	Ji *et al*. (2003)
	5.8SF	F	CACGGGTCGATGAAGAACGC	Planas & Ribera (2014)

RESULTS

Sampling

Locality altitudes in the Souss-Massa region ranged from 186 m near the Atlantic coast of Sidi Ifni to 1588 m near Tikki in the High Atlas Mountains. Sampling effort in different localities in the south-eastern foothills of the Anti-Atlas Mountains, roughly between Bouizakarne and Foum-Zguid villages, did not produce successful results. Individuals from two localities in the Jbel Sarhro Mountains were morphologically consistent with *L. mrazig*, substantially extending the known distribution for this species to the west (approximately by 1500 km). We additionally added one individual morphologically consistent with this species collected in the Negev Desert in Israel, being the easternmost known locality.

Molecular data

The preliminary analyses conducted with the *cox1* data set including 39 *Loxosceles* individuals from the Souss-Massa uncovered extensive geographic structure. All the haplotypes were exclusive from a single locality, and generally, the same haplotype was shared across all individuals of the same locality. Based on this information, we selected one or two individuals from each locality, leading to a total of 24 individuals, that together

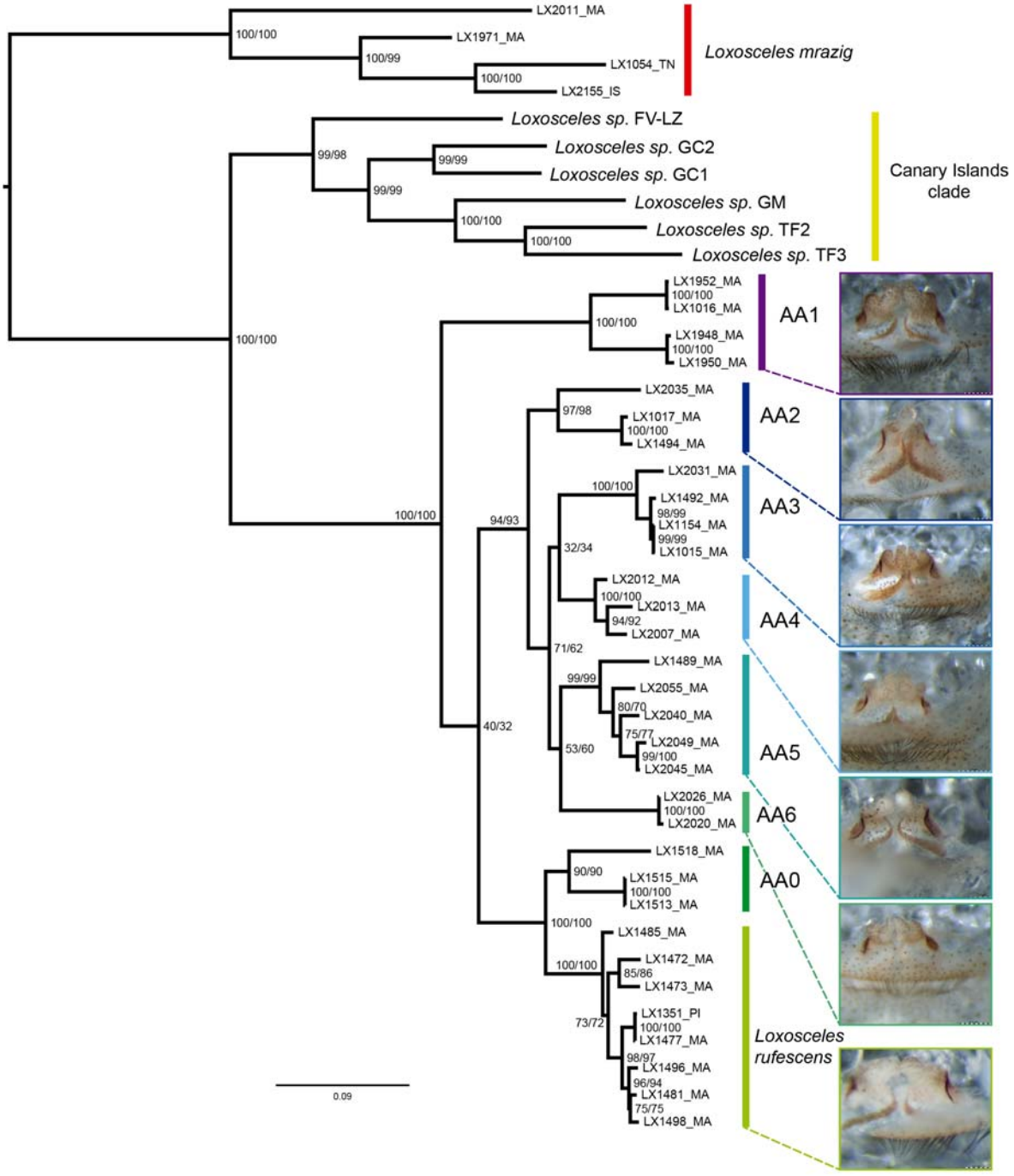

Figure 2 ML tree of the Mediterranean, North African and Canary Islands *Loxosceles* species obtained with the concatenated dataset of five molecular markers (i.e. *cox1*, 16S, its2, H3 and 28S) using the AIC-selected partition scheme. Numbers next to the nodes refer to bootstrap support values of the ML analyses conducted with the AIC- and BIC-selected partition scheme, respectively. Next to each specimen code the geographical region is indicated: MA (Morocco), PI (Iberian Peninsula), TN (Tunisia), IS (Israel). Vertical bars are as in Fig. 1, and photographs of genitalic morphology from females of lineages AA1 to AA6 and *L. rufescens* appear next to each lineage.

with the eight *L. rufescens* specimens, the six representatives of the Canary Islands endemic species and the four individuals of *L. mrazig*, composed a data set of 42 individuals. In total, the concatenated matrix was 3417 bp long, including 923 bp of the *cox1*, 587 bp of the 16S, 326 bp of the H3, 320 bp of the its2 and 1251 bp of the 28S markers.

The best partitioning scheme selected with the AIC differed from the one selected with the BIC, and both partitioning schemes were used in phylogenetic analyses. The AIC-selected scheme included five partitions: 1) the first and second *cox1* codon position together with the third codon position of H3; 2) the third *cox1* codon position; 3) the 16S; 4) the 28S together with the first and second H3 codon position; 5) the its2. Differently, the BIC-selected scheme included two partitions: 1) all the putative partitions except for the third *cox1* codon position; 2) the third *cox1* codon position.

The results of the ML and BI analyses conducted with the two partition schemes (i.e. AIC and BIC) are summarized in Fig. 2 and Fig. 3, respectively. ML results were identical among the two partition schemes, while the BI results of the analysis conducted with the BIC-selected scheme differed in the posterior probabilities of some clades with respect to the ones obtained with the AIC-selected scheme. In the former BI analyses, the clade formed by AA3 and AA4 had lower support, leading to a trichotomy with the also weakly supported clade formed by AA5 and AA6 lineages. Some differences were found between the results of ML and BI analyses. In ML, AA1 clade was placed sister to the remaining *Loxosceles* from Souss-Massa and *L. rufescens* representatives, although this latter group was not well-supported in any of the analyses (ML bootstrapp support < 50) and thus, should be interpreted as a polytomy. Differently, BI analyses placed AA1 together with the remaining Souss-Massa lineages (i.e. AA2 to AA6), while a second group was composed by representatives of AA0 and *L. rufescens*. This clade was well-supported in the two BI analyses (Posterior Probabilities (PP) > 0.99) and ML analyses (ML bootstrap = 100). The group composed by lineages AA1 to AA6 was less well-supported (PP = 0.87 and PP = 0.97 in BIC-scheme and AIC-scheme, respectively). The group composed by lineages AA2 to AA6 was well-supported in both BI and ML analyses (PP = 1; ML bootstrap > 93), and the group formed by lineages AA3 to AA6 received strong support in BI analyses (PP = 1) and weak support in ML analyses (ML bootstrap 71 and 62; AIC and BIC, respectively). AA3-AA4 was only well-supported in the BI analyses conducted with the AIC-selected scheme (PP = 1), as was the case in the AA5-AA6 group (PP = 0.97).

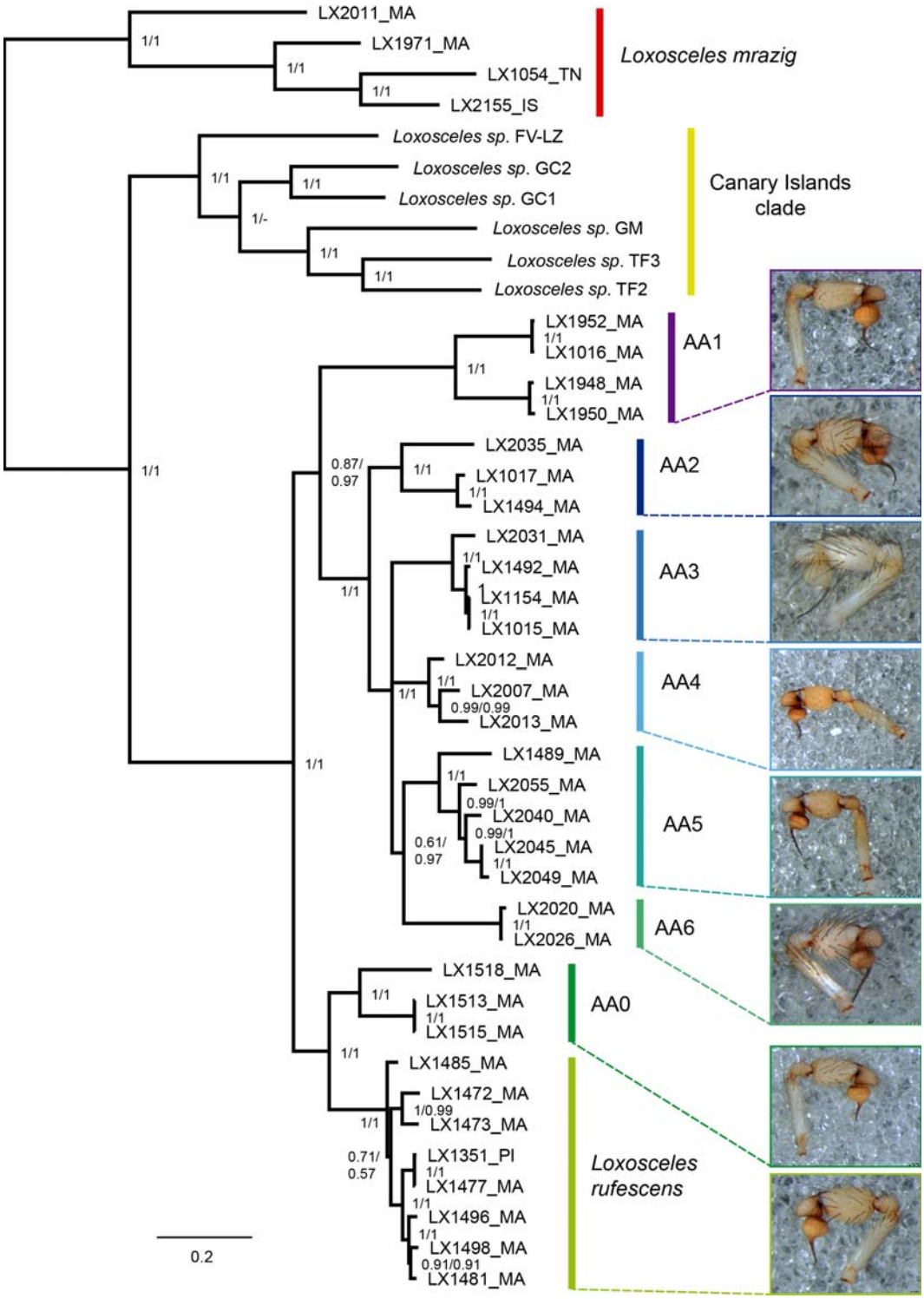

Figure 3 BI tree of the Mediterranean, North African and Canary Islands *Loxosceles* species obtained with the concatenated dataset of five molecular markers (i.e. *cox1*, 16S, its2, H3 and 28S) using the BIC-selected partition scheme. Numbers next to the nodes refer to posterior probability values of the BI analyses conducted with the BIC- and AIC-selected partition scheme, respectively. Next to each specimen code the geographical region is indicated: MA (Morocco), PI (Iberian Peninsula), TN (Tunisia), IS (Israel). Vertical bars are coloured as in Fig. 1, and photographs of genitalic morphology from males of lineages AA0 to AA6 and *L. rufescens* appear next to each lineage.

Genetic *p*-distances between lineages were high, and ranged from 11% between AA4 and AA5 to 15.6% between AA0 and AA1 (Table 2). Genetic distances within groups were also high, ranging from 1.3% between the four representatives from a single locality in lineage AA6, to 7.1% between individuals from three localities in lineages AA1.

Table 2 Mean uncorrected genetic distances calculated with *cox1* sequences between (below the diagonal) and within (diagonal, in italics) *Loxosceles* lineages from the Souss-Massa region (AA1 to AA6), AA0 and *L. rufescens*.

	L. rufescens	AA0	AA1	AA2	AA3	AA4	AA5	AA6
L. rufescens	*5.2*							
AA0	11.8	*5.5*						
AA1	14.7	15.6	*7.1*					
AA2	13.9	15.2	15.1	*7*				
AA3	13.8	14.3	15.5	12.7	*3*			
AA4	14.3	15.2	15.1	13.4	11.1	*3.8*		
AA5	14.5	15.1	14.5	13.1	11.8	11	*4.8*	
AA6	14	15	15.4	13.4	11.9	12.3	11.9	*1.3*

Morphological variability

Pictures of male palps are shown in Fig. 3 and female vulvae in Fig. 2. It should be noted that one individual per lineage was examined, and thus, we lack information representing the morphological variability existent within each lineage. The male from lineage AA0 was morphologically consistent with *L. rufescens*, although the tibia was slightly longer than in the latter. The male of lineage AA1 possessed a longer tibia and longer embolus than *L. rufescens*, although not as long as in *L. foutadjalloni* (Fig. 3 in Duncan *et al*., 2010; Fig. 9C in Lotz, 2012). The males of the remaining lineages were also morphologically distinct from *L. rufescens*, with shorter tibiae although longer than those of *L. mrazig*, from which they also could be distinguished by the embolus shape, being clearly longer in males of the lineages AA2 to AA6. Vulvae from the lineages AA1 to AA6 varied greatly, and none was morphologically consistent with *L. rufescens*. AA1 vulva resembled the one of *L. foutadjalloni* (Fig. 10B in Lotz, 2012). The vulva of lineage AA2 was clearly asymmetrical, with the inner lobe of the left seminal receptacle longer than the right one. Vulvae from the remaining lineages presented different modifications in the inner lobe, which were overall longer than in *L. rufescens*. The vulva of lineage AA0 was not examined due to lack of adult female material.

DISCUSSION

We revealed the existence of a diverse group of *Loxosceles* spiders from the Souss-Massa region of Morocco. The extensive fieldwork conducted in the area led to the discovery and delimitation of the distribution of six different lineages closely related to *L. rufescens*, together with which they form a well-supported clade in phylogenetic analyses.

General biogeographic patterns of North-African *Loxosceles*

Planas *et al*. (Paper 1) reported the existence of multiple allopatric lineages within *L. rufescens* in the south-west of Morocco. These lineages are situated in the north-western slopes of the High Atlas Mountains and while some of them are restricted to one or two localities (i.e. A1 to A4), others are found also in multiple Mediterranean sites (i.e. A6, B1 and B4) (Planas *et al*., Paper 1). In the present study, we found one lineage, AA0 from the western High Atlas, that was consistently recovered as sister to *L. rufescens*. Only one adult specimen (Fig. 3) was available from this lineage, and it was morphologically indistinguishable from *L. rufescens* although highly divergent genetically (11.8% *cox1 p*-distance). Pending further sampling in this area that would likely uncover additional related lineages and clarify the taxonomic status of this and expected new lineages, we considered the clade formed by *L. rufescens* and lineage AA0 as *L. rufescens sensu lato* (*s.l.*) as opposed to *L. rufescens sensu stricto* (*s. str.*) or *L. rufescens*.

The dense sampling conducted in the present study led to a clear geographic delimitation of the distribution of *L. rufescens s. l.* north of the High Atlas with respect to the lineages AA1 to AA6, distributed south of this mountain range (Fig. 1). Indeed, the biogeographical relevance of the High Atlas Mountains has been revealed in other studies, where similar patterns have been found for different taxa. For example, this mountain range acted as a geographical barrier separating north and south lineages in the freshwater turtle *Maremys leprosa* (Fritz *et al*., 2006), in the *Acanthodactylus* (Fonseca *et al*., 2009) and *Agama* lizards (Brown *et al*., 2002), in the scorpion genus *Buthus* (Sousa *et al*., 2012) and between the two wolf spider species *Lycosa suboculata* and *L. aff. suboculata* (Planas *et al*., 2013). Although in these examples the same biogeographical barrier has been invoked to explain current biogeographical patterns, estimated ages are highly divergent between them, and thus, they were probably affected by unrelated processes. In *Loxosceles*, representatives of lineages AA1, AA2 and AA3 were included in the dating analysis of Planas and Ribera

(2014; with specimen codes: LX1016, LX1494 and LX1015, respectively), and the split between these lineages with respect to *L. rufescens* was estimated at approximately 7 Ma (4.4-8.6 Ma). This time-frame is comparable, given the methodological differences, to the ages obtained in *Agama* (9 Ma) and *Acanthodactylus* (9.3 Ma), both cases where this separation was related to the Atlas uplift, while estimated ages for *Mauremys* and *Lycosa* placed this diversification during the Pleistocene, and thus the separation was probably promoted by climatic events.

Lineages AA1 to AA6 were distributed across different mountain massifs in the Souss-Massa region. Although this region is located at a Saharan latitude, and thus it is characterized by arid and semi-arid conditions, the climate is highly influenced by the high mountains which surround the area and affect the precipitation regime, and also by the disposition of the river valleys, especially the Souss valley, that permit the entrance of Atlantic Ocean humidity far inland (Elmouden *et al*., 2005). The combination of its complex orography and especially, the climatic idiosyncrasies of the region partially explain this exceptional biological richness of the region. The distribution of the *Loxosceles* lineages AA1 to AA6 is clearly confined to areas affected by relatively higher moisture and they were not found in surrounding more arid areas.

Interestingly, in some of these arid areas we found *L. mrazig* s. l. representatives. *Loxosceles mrazig* was described from an area dominated by arid environments (Ribera & Planas, 2009) and the extent of its distribution is currently unknown. Although the study of this species is not within the scope of the present work, it is worth highlighting the high genetic divergence between *L. mrazig* individuals (Fig. 2 and 3). To our knowledge, individuals morphologically consistent with *L. mrazig* are found in eastern areas of Morocco, in Algeria, Tunisia, Egypt, Jordan and Saudi Arabia (Duncan *et al*., 2010; Desouky & El-Hennawy, 2012; Planas & Ribera, unpublished) although in some cases they have been misidentified as *L. rufescens* (e.g. Desouky & El-Hennawy, 2012). A comprehensive sampling is needed across this broad area in order to study the extent of this species' distribution and to delimit the putative species that could arise in this likely species complex. Overall, these results suggest that while *L. rufescens* and related lineages (i.e. AA0 to AA6) are distributed in different Mediterranean climate regions, including the thermo- and infra-mediterranean gradients present in the Souss-Massa region, *L. mrazig* is distributed across the arid and semi-arid areas of North-Africa and Arabia. This ecological

distinction between mesic and dryer areas was also found in the gecko sister species *Stenodactylus mauritanicus* and *S. sthenodactylus* (Metallinou *et al*., 2012).

Emerging patterns of microallopatry in the Souss-Massa region

The genetic diversity existent within and between the *Loxosceles* lineages of the Souss-Massa region surpassed the already high genetic diversity existent within *L. rufescens s. str*. lineages commented above (Planas *et al*., Paper 1). For example, the three localities of the lineage AA2 are separated by less than 30 km one from another, and the genetic distances calculated with *cox1* exceed 7% within this lineage, and reach 12.7% divergence between this and the neighbouring lineage AA3, separated by roughly 30 km. Indeed, different phylogeographic studies conducted in this area on scorpions of the genus *Buthus* also uncovered the existence of multiple microallopatric endemic lineages (Habel *et al*., 2012; Husemann *et al*., 2012). Thus, in agreement with these studies, it seems plausible that the orographic features of the area in combination with the microhabitat preferences and low dispersal abilities of *Loxosceles* could have triggered this great diversity. It is worth noticing that the deep river gorges present in this region could represent natural barriers between lineages, as for example, the river Âït-Baha for the lineages AA2 and AA3.

As in the *Loxosceles* spiders, the *Buthus* scorpions showed a strong geographic structure, and a scenario of competitive exclusion between divergent lineages was proposed to explain the existence and maintenance of strictly geography-dependent lineages (Habel *et al*., 2012). In a recent study, differences in agonistic behaviour between two introduced and sympatric *Loxosceles* species (i.e. *L. intermedia* and *L. laeta*) were found to be one of the possible causal mechanisms underlying the segregation patterns that these two species show in Brazilian localities. Although these *Loxosceles* species displayed different behaviours, in resident-intruder disputes the residency status was the most decisive factor favouring residents (Fischer *et al*., 2014). Thus, if agonistic interactions between individuals of *Loxosceles* from the Souss-Massa region follow a similar pattern, it would be likely that competitive exclusion is the main factor preserving the strong geographic structure found within and between lineages, in addition to the orography. This pattern is found in all the lineages except in AA4. In this latter case, the two localities are separated by more than 250 km and surprisingly, the genetic distance within this lineage is as low as 3.8%. This pattern could be explained if the eastern locality, situated in the Todra gorge in the south-central foothills of the High Atlas was recently colonized. If competitive exclusion is a decisive

factor in shaping the current diversity, it is expected that this colonization would have been possible if the new areas were not previously inhabited by any competing *Loxosceles* populations. To our knowledge, the lineage AA4 is the easternmost of all Souss-Massa lineages, and thus, individuals from this lineage are more likely to be able to disperse eastwards, where according to the information at hand no other *Loxosceles* lineage is present. Thus, lineage AA4 should be further studied, and providing additional samples between the two localities currently known and exploring neighboring areas in order to detect distribution boundaries between this and the surrounding lineages or species, could shed light onto the importance of competitive exclusion in the existence and maintenance of the microallopatric patterns.

Considerations on species delimitation in *Loxosceles*

Delimiting species boundaries in *Loxosceles* based on morphology has been shown to be a difficult task due to their highly conserved morphology. As a result, most of the nominal species were described and diagnosed from closely related species using only subtle differences (Gertsch, 1967; Gertsch & Ennik, 1983) and it has been suggested that delimitation of species boundaries in this genus could benefit from molecular-based methods (Binford *et al*., 2008; Duncan *et al*., 2010; Planas & Ribera, 2014; Paper 3). We conducted preliminary analyses applying the GMYC method, which resulted useful in delimiting *Loxosceles* lineages in previous studies (Planas & Ribera, 2014; Planas *et al*., Paper 1). However, in the present study this method failed to group individuals from more than one locality. The extreme population structure that arises in organisms with low dispersal abilities, such as *Loxosceles* and other spider genera (Satler *et al*., 2013 and references herein), is challenging for molecular species-delimitation methods. Methods that rely on a single marker, usually mitochondrial (Carstens *et al*., 2013), could be even more affected by the higher philopatric tendency exhibited by females. Further analyses applying species delimitation methods that employ multiple markers should be conducted to clarify the taxonomic status of the lineages existent in this group. Most importantly, additional sampling effort in unexplored regions between lineages could help in delimiting the putative species boundaries.

Pending additional molecular analyses, different lineages within the *Loxosceles* from the Souss-Massa region were delimited *ad hoc*, based on the phylogenetic results and on their geographical distribution of the lineages. We found clear morphological differences, both in

male and female sexual characters, compared to the two currently accepted species in North Africa: *Loxosceles rufescens* and *L. mrazig*. Surprisingly, considerable differences also were found between the representatives of the different lineages. However, these analyses were based on a single representative from each lineage, and thus, the study of additional individuals is warranted in order to detect possible population variability and confirm the putative diagnostic characters.

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Supplementary Table 1 Study material. A list of the specimens used in the molecular analyses, with their identification code, lineage, locality coordinates, and GenBank accession numbers. Sequences that lack accession numbers are marked with X. Individuals selected for the phylogenetic analyses are in bold.

ID	SPECIES	LINEAGE	LATITUDE	LONGITUDE	COI	16S	Histona	28S	ITS2
LX1950_MA	*Loxosceles* aff. Anti-Atlas	AA1	29.32432	-10.13400	X	X	X	X	X
LX1948_MA	*Loxosceles* aff. Anti-Atlas	AA1	29.67976	-9.84589	X	X	X	X	X
LX1016_MA	*Loxosceles* aff. Anti-Atlas	AA1	29.248983	-9.74345	KF716997	KF717022	KF716983	X	KF717025
LX1952_MA	*Loxosceles* aff. Anti-Atlas	AA1	29.248983	-9.74345	X	X	X	X	X
LX1947_MA	*Loxosceles* aff. Anti-Atlas	AA1	29.67976	-9.84589	X				
LX1951_MA	*Loxosceles* aff. Anti-Atlas	AA1	29.248983	-9.74345	X				
LX2035_MA	*Loxosceles* aff. Anti-Atlas	AA2	30.06650	-9.13406	X	X	X	X	X
LX1017_MA	*Loxosceles* aff. Anti-Atlas	AA2	29.83503	-9.02465	X		X	X	X
LX1494_MA	*Loxosceles* aff. Anti-Atlas	AA2	29.84582	-8.93754	KF717007	KF717012	KF716985	X	KF717027
LX2034_MA	*Loxosceles* aff. Anti-Atlas	AA2	30.06650	-9.13406	X				
LX2031_MA	*Loxosceles* aff. Anti-Atlas	AA3	30.23587	-8.85828	X	X	X	X	X
LX1154_MA	*Loxosceles* aff. Anti-Atlas	AA3	30.28915	-8.49550	X	X	X	X	X
LX1015_MA	*Loxosceles* aff. Anti-Atlas	AA3	30.29673	-8.47440	KF716996	KF717011	KF716982	X	KF717024
LX1492_MA	*Loxosceles* aff. Anti-Atlas	AA3	30.17806	-8.28465	X	X	X	X	X
LX1493_MA	*Loxosceles* aff. Anti-Atlas	AA3	30.17806	-8.28465	X				
LX2032_MA	*Loxosceles* aff. Anti-Atlas	AA3	30.23587	-8.85828	X				
LX2033_MA	*Loxosceles* aff. Anti-Atlas	AA3	30.23587	-8.85828	X				
LX2012_MA	*Loxosceles* aff. Anti-Atlas	AA4	30.53616	-7.91073	X		X	X	X
LX2013_MA	*Loxosceles* aff. Anti-Atlas	AA4	30.53616	-7.91073	X		X	X	X
LX2007_MA	*Loxosceles* aff. Anti-Atlas	AA4	31.60135	-5.58666	X	X	X	X	X
LX1070_MA	*Loxosceles* aff. Anti-Atlas	AA4	31.583163	-5.591186	X				
LX2006_MA	*Loxosceles* aff. Anti-Atlas	AA4	31.60135	-5.58666	X				
LX1489_MA	*Loxosceles* aff. Anti-Atlas	AA5	30.64256	-9.70562	X		X	X	X
LX2055_MA	*Loxosceles* aff. Anti-Atlas	AA5	30.50577	-9.59384	X	X	X	X	
LX2040_MA	*Loxosceles* aff. Anti-Atlas	AA5	30.55540	-9.34718	X	X	X	X	X
LX2049_MA	*Loxosceles* aff. Anti-Atlas	AA5	30.74326	-9.28983	X	X	X	X	X
LX2045_MA	*Loxosceles* aff. Anti-Atlas	AA5	30.74694	-9.12111	X	X	X	X	X
LX2041_MA	*Loxosceles* aff. Anti-Atlas	AA5	30.55540	-9.34718	X				
LX2043_MA	*Loxosceles* aff. Anti-Atlas	AA5	30.74694	-9.12111	X				
LX2047_MA	*Loxosceles* aff. Anti-Atlas	AA5	30.74694	-9.12111	X				
LX2027_MA	*Loxosceles* aff. Anti-Atlas	AA6	30.65656	-8.63490	X				
LX2020_MA	*Loxosceles* aff. Anti-Atlas	AA6	30.65656	-8.63490	X	X	X	X	
LX2026_MA	*Loxosceles* aff. Anti-Atlas	AA6	30.65656	-8.63490	X		X	X	X
LX2024_MA	*Loxosceles* aff. Anti-Atlas	AA6	30.65656	-8.63490	X				
LX1513_MA	*Loxosceles rufescens*	AA0	32.62310	-5.97435	X	X	X	X	X
LX1515_MA	*Loxosceles rufescens*	AA0	32.62310	-5.97435	X	X	X	X	X
LX1518_MA	*Loxosceles rufescens*	AA0	33.08753	-5.48984	X	X	X	X	X
LX1514_MA	*Loxosceles rufescens*	AA0	32.62310	-5.97435	X				

ID	SPECIES	LINEAGE	LATITUDE	LONGITUDE	COI	16S	Histona	28S	ITS2
LX1485_MA	*Loxosceles rufescens*	A1	31.15739	-9.69767	X	X	X	X	X
LX1481_MA	*Loxosceles rufescens*	A2	31.50096	-9.6158	X	X	X	X	X
LX1496_MA	*Loxosceles rufescens*	A3	31.18955	-8.05766	X	X	X	X	X
LX1498_MA	*Loxosceles rufescens*	A4	31.95973	-6.76811	X	X	X	X	X
LX1477_MA	*Loxosceles rufescens*	A6	31.60901	-9.65915	X	X	X	X	X
LX1351_PI	*Loxosceles rufescens*	A6	39.68481	-0.30005	KF717003	KF717017	KF716989	X	KF717031
LX1472_MA	*Loxosceles rufescens*	B1	32.50405	-9.25307	X	X	X	X	X
LX1473_MA	*Loxosceles rufescens*	B4	31.9564	-9.31604	X	X	X	X	X
LX1054_TN	*Loxosceles mrazig*		33.45870	9.06461	FJ986179	GQ279173	KF716985	X	KF717027
LX2011_MA	*Loxosceles mrazig* complex		30.54749	-7.10936	X		X	X	X
LX1971_MA	*Loxosceles mrazig* complex		31.01699	-5.95061	X	X	X	X	X
LX2155_IS	*Loxosceles mrazig* complex		30.854186	34.7703366	X		X	X	X
LX1391_FV	*Loxosceles* sp. Fuerteventura-Lanzarote		28.26339	-13.94874	KF669928	KF670035	KF670077	X	KF670112
LX1160_GC	*Loxosceles* sp. Gran Canaria 1		27.91022	-15.52989	KF669921		KF670073	X	KF670108
LX1149_GC	*Loxosceles* sp. Gran Canaria 2		28.069994	-15.657899	KF669917	KF670057	KF670070	X	KF670106
LX1776_GM	*Loxosceles* sp. La Gomera		28.0876167	-17.25686	KF670003	KF670069	KF670101		KF670129
LX1164_TF	*Loxosceles* sp. Tenerife 2		28.2492167	-16.528747	KF669925	KF670064	KF670074	X	KF670109
LX1155_TF	*Loxosceles* sp. Tenerife 3		28.236255	-16.64251	KF669918	KF670066	KF670071	X	KF670107

CAPÍTOL 4

Diversitat del verí i loxoscelisme a la Conca Mediterrània i a les Illes Canàries

Article 8 Planas E, Zobel-Thropp PA, Ribera C, Binford G. Not as docile as it looks: *Loxosceles* venom variation and loxoscelism in the Mediterranean Basin and the Canary Islands.

No tan dòcils com semblen: variació en el verí de *Loxosceles* i loxoscelisme en la Conca Mediterrània i en les Illes Canàries

RESUM

La importància mèdica de les aranyes *Loxosceles* ha promogut una àmplia recerca en diferents aspectes dels seus verins. La majoria dels casos de loxoscelisme s'han documentat d'Amèrica del Sud i del Nord, i per tant, la major part dels treballs s'han centrat en les espècies de *Loxosceles* d'aquestes regions. Curiosament, tot i que l'espècie *L. rufescens* és endèmica de la Mediterrània, on és abundant fins i tot en àrees alterades pels humans, els casos de loxoscelisme són escassos en aquesta regió. Per això, s'ha suggerit que el verí de *L. rufescens* podria ser de menor importància mèdica que el d'altres espècies del mateix gènere. En aquest estudi posem a prova aquesta hipòtesi utilitzant múltiples aproximacions per estudiar la variació del verí en alguns llinatges i espècies de la Conca Mediterrània i les Illes Canàries. Hem trobat que l'activitat SMase D, el component clau en el verí de *Loxosceles*, és comparable a la d'altres espècies americanes, la importància mèdica de les quals ha estat comprovada. La composició proteica del verí utilitzant SDS-PAGE presenta algunes diferències entre els tàxons de *Loxosceles* en el patró de bandes i en la intensitat, principalment entre les espècies de Canàries respecte als llinatges de *L. rufescens*. També hi ha diferències entre aquestes espècies en l'expressió de diferents paràlegs de la família gènica *SicTox*, essent les espècies de Canàries menys diverses. En conclusió, els nostres resultats no recolzen la hipòtesis prèvia, i suggereixen que el verí d'aquestes espècies és de fet, tan potent com el d'altres espècies de *Loxosceles*, i per tant, aquestes s'haurien de considerar de rellevància mèdica.

PARAULES CLAU: aranyes, evolució, esfingomielinasa D (SMase D), SDS-PAGE, *SicTox*.

Not as docile as it looks: *Loxosceles* venom variation and loxoscelism in the Mediterranean Basin and the Canary Islands

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ABSTRACT

The medical importance of *Loxosceles* spiders has promoted extensive research on different aspects of their venoms. Most of the reported cases of loxoscelism have occurred in the Americas, and thus, much work has focused on North and South American *Loxosceles* species. Interestingly, loxoscelism cases are rare in the Mediterranean Basin although *L. rufescens*, endemic to the Mediterranean, is an abundant spider even in human-altered areas. Thus, it has been suggested that the venom of *L. rufescens* could be of less medical relevance than that of its congeners. In this study, we challenge this hypothesis by using multiple approaches to study venom variation in selected species and lineages from the Mediterranean Basin and the Canary Islands. We found that SMase D activity, the key bioactive component of *Loxosceles* venom, is comparable to American species that are confirmed to have medically relevant bites. The venom protein composition using SDS-PAGE presents some differences among regional *Loxosceles* taxa in banding pattern and intensity, mostly between the Canarian and *L. rufescens* lineages. Differences between these species also exist in the expression of different paralogs of the *SicTox* gene family, with the Canarian species being less diverse. In conclusion, our results do not support the challenged hypothesis, and suggest that venom of these species may indeed be as potent as other *Loxosceles* species, and should be considered medically relevant taxa.

KEYWORDS: Spider, evolution, Sphingomyelinase D (SMase D), SDS-PAGE, *SicTox*.

INTRODUCTION

Spider venoms have become increasingly popular as a research focus because of their potential for agricultural and pharmacological applications (Chaim *et al*., 2011; King & Hardy, 2013; Klint *et al*., 2012; Windley *et al*., 2012). Recent conservative estimates point to the existence of more than four million molecules that selectively target a variety of vital physiological functions, providing a nearly infinite "pharmacological space" to explore (Escoubas *et al*., 2006). The venom of a single spider is a complex mixture of proteins, peptides and low-molecular-weight components that have evolved with the primary function to immobilize and kill prey (Chippaux *et al*., 1991; Zobel-Thropp *et al*., 2012). Although venom composition varies between closely related species, between populations of the same species and among individuals as seen in spiders (Binford, 2001; Escoubas *et al*., 2002; Garb & Hayashi, 2013), snakes (see Chippaux *et al*., 1991 for a revision), cone snails (Duda & Remigio, 2008) and scorpions (Abdel-Rahman *et al*., 2009), contributing factors are not well defined. Studying patterns of variation in spider venoms can provide a better understanding of mechanisms of toxin evolution, which may give clues to evaluating potential risks associated with envenomation. It can also help with developing rational antivenom strategies or fast and specific diagnoses and treatments involving medically important groups, such as the ones included in this study.

The spider family Sicariidae comprises two genera (i.e. *Loxosceles* and *Sicarius*) widely distributed in North, Central and South America, Africa and the Mediterranean Basin. Studies of crude venoms in Sicariidae spiders, have identified several molecules of varying sizes, some of which are proposed to be toxic (reviewed in Gremski *et al*., 2014) such as hyaluronidase (Young & Pincus, 2001; da Silveira *et al*., 2007), phospholipases-D (Kalapothakis *et al*., 2007), metalloproteases, serine proteases (Young & Pincus, 2001; Barbaro *et al*., 2005; Feitosa *et al*., 1998; Veiga *et al*., 2000) and insecticidal neurotoxins (de Castro *et al*., 2004). Among these components, most of the attention has focused on a family of enzymes with sphingomyelinase D (SMase D) activity. SMase D proteins belong to a multi-gene family named *SicTox* (sicariid toxin), members of which are expressed in venom glands of both *Loxosceles* and *Sicarius* (Binford *et al*., 2009 and references therein). Transcriptome analyses of two species – *L. laeta* (Fernandez-Pedrosa *et al*., 2008) and *L.*

intermedia (Gremski *et al*., 2010) – have shown that Expression Sequence Tags (ESTs) of SMase D represent the most abundant component of the EST expression profiling of venom glands. Sicariid venom can cause dermonecrotic lesions in humans, known as loxoscelism (*Loxosceles* reviews in da Silva *et al*., 2004; Vetter, 2008; *Sicarius* review in Newlands & Atkinson, 1988) but individually expressed and purified SMase D proteins injected into rabbits trigger a cascade of physiological events that lead to dermonecrosis, massive inflammatory response and lysis of red-blood cells among other effects; they have also been shown to solely reproduce the major biological effects of the whole venom (Barbaro *et al*., 2005; de Oliveira *et al*., 2005; Pretel *et al*., 2005; da Silveira *et al*., 2007; Ribeiro *et al*., 2007).

While much work has been done on *Loxosceles* venom from North and South American species, very little has focused on those from Africa and the Mediterranean. *L. rufescens* is widely distributed across the entire Mediterranean Basin, where it is found commonly in human-altered habitats. This facility to cohabit with humans has expanded the distribution of the species due to human-mediated transportation and it is considered cosmopolitan (Platnick, 2014). *Loxosceles rufescens* harbors 11 different evolutionary lineages delimited using mtDNA (Planas *et al*., Paper 1) and it has been suggested that cryptic species could coexist in this area (Duncan *et al*., 2010). In the Canary Islands, an endemic lineage harboring several evolutionary lineages have been uncovered recently (Planas & Ribera, 2014), and in some islands, the endemic lineages coexist with *L. rufescens*, introduced by humans into the archipelago.

Although *L. rufescens* is a common synanthropic species in the Mediterranean Basin, reports on loxoscelism are scarce, but are likely more prevalent than can be inferred from the literature (Cohen *et al*., 1999). Published reports have been identified in the Eastern Mediterranean Basin (e.g. Cohen *et al*., 1999; Davidovici *et al*., 2006; Stefanidou *et al*., 2006; Yigit *et al*., 2008) while fewer come from the Western Mediterranean Basin (e.g. de Entrambasaguas *et al*., 2007; Pernet *et al*., 2010; Ribuffo *et al*., 2012). It has been widely repeated throughout literature that a bite from *L. rufescens* has far less dangerous consequences than that of its congeneric species from the Americas such as *L. laeta* or *L. reclusa*, that it is less toxic (Vetter & Swanson, 2007) or that it produces a more benign clinical picture (Cohen *et al*., 1999). Several lines of evidence suggest that this assumption

is erroneous (Greene *et al*., 2009), but no study has tested this hypothesis to date. Misidentification of *Loxosceles* bites is common in the literature (Vetter & Swanson, 2007; Vetter, 2008; Gremski *et al*., 2014) and most of the reported cases do not follow the recommended criteria to confirm a spider bite: confirmation or observation of the bite, capture of the spider and identification by a specialized taxonomist (Isbister, 2002), complicating understanding of the actual medical relevance. News of a suspicious bite in 2006 in Gran Canaria, based on weak evidence, was echoed by local media inducing a serious public concern, a common reaction often involved in loxoscelism diagnoses (Vetter, 2008) and recently another suspicious case (R. Conca, pers. comm.) was reported from the same island.

In this study, we are interested in testing if *L. rufescens* SMase D, the key bioactive component of the venom, is different from that of other identified members of this genus that contribute to loxoscelism in humans. We investigated differences in the composition of *SicTox* genes and SMase D activity levels between *L. rufescens* and two closely related *Loxosceles* species from the Canary Islands. We also analyzed the amount of venom variation that exists within *L. rufescens,* including representatives of different lineages comprising most phylogenetic diversity in this species. Specifically, we (1) compare whole venom protein composition using SDS-PAGE, (2) compare SMase D activity levels among venoms, (3) compare the expression patterns of *SicTox* genes, and (4) analyze patterns of phylogenetic relatedness among *SicTox* genes isolated from the *L. rufescens* lineage.

MATERIAL AND METHODS

Taxon sampling

We included two endemic *Loxosceles* species from the Canary Islands: *Loxosceles* sp. GM-HI from La Gomera (GM) and *Loxosceles* sp. GC1 from Gran Canaria (GC) (Planas & Ribera, 2014), and three representatives from both major clades of *L. rufescens* (A and B), specifically from three evolutionary lineages found in the Iberian Peninsula (A6, B2 and B3). Lineage A6 is commonly found in the Western part of the Mediterranean Basin but has been distributed worldwide due to human-mediated transportation. Lineage B2 is less common but it is also widespread across the Mediterranean, finally lineage B3 is endemic to the Iberian Peninsula (Planas *et al*., Paper 1). Detailed collecting information is available

from E. Planas by request. We used *L. arizonica* for comparison in SMase D activity assays (Binford lab).

Venom extraction and quantification

We used electrical stimulation to extract venom as described in Binford & Wells (2003). Only mature females were used and venom samples were pooled among individuals to standardize for possible differences (Table 1). We pooled venom from the following number of individuals from each locality: four (A6), eight (B2), five (B3), three (GC) and six (LG). We diluted all venom in 1X Amplex Red buffer (5 mM CaCl2, 50 mM Tris-Cl, pH 8) and quantified total venom protein using the Coomassie Plus (Bradford) Assay Reagent (ThermoScientific, Pierce).

SDS-PAGE

Crude venom proteins (5 μg) were separated from each species using one-dimensional SDS-PAGE in a 12% Criterion Precast Gel (Bio-Rad). A Broad Range Molecular Weight Standard (New England Biolabs) was loaded for size reference. Proteins were visualized using a standard silver stain protocol.

SMase D Activity Assay

We assayed SMase D activity using a modification of the Amplex Red Phospholipase D Assay Kit (Molecular Probes, Invitrogen) as described in Binford *et al*. (2009) with sphingomyelin as the substrate. Fluorescence emission was measured from reactions that contained 0.5 μg of crude venom and each reaction was conducted twice. We used *L. arizonica* venom as a positive control for activity comparisons. For negative controls we substituted 1x Amplex Red Buffer for venom and ran the reactions with and without sphingomyelin, and also conducted one reaction for each species without sphingomyelin. We used a Perkin Elmer LS55 Luminescence Spectrometer (Waltham) for all measurements where an increase of fluorescence emission directly correlates to SMase D activity.

SMase D Expression Patterns

cDNA synthesis, PCR amplification and cloning

Two days after venom extraction, we dissected and removed venom glands and immediately froze them in liquid nitrogen (Table 1). For RNA isolation and cDNA

synthesis we followed the same procedure as described in Binford *et al*. (2009) using the ChargeSwitch® Total RNA Cell kit (Invitrogen) and SuperScript™ III Reverse Transcriptase (Invitrogen). SMase D was amplified using a pair of primers ("sphing1f" 5' -TGGATHATGGGNCAYATGGT- 3' and "anch2" 5' -CCACGCGTCGACTAGTAC- 3') that were previously used to successfully amplify a diverse set of SMase D homologs from a wide range of *Loxosceles* and *Sicarius* species (Binford *et al*., 2009). These primers amplify a fragment of ~1000 bp, including most of the mature protein and the poly-A tail. For PCR amplification, we used 0.2 µl of each cDNA pool, 5 µl MasterAmp F PreMix (Epicentre Technologies, 1x final concentration), and recombinant Taq Polymerase (New England Biolabs, 0.05 U/µl) in a total volume of 10 µl. The annealing temperature for all reactions was 60° C.

We cloned PCR products (~1 kb) into pCR®4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen). We screened bacterial transformants using PCR under the same optimal conditions used to amplify the inserts. Colonies with positive products were grown in selective medium and purified using the QIAPrep spin mini-prep kit (Qiagen). Inserts were sequenced for both directions with T3 and T7 primers at the Genomic Analysis and Technology Core (University of Arizona).

Sequence analysis and phylogenetics

Contigs were assembled and edited in Geneious 4.6.5 (Drummond *et al*., 2009). We searched all sequences using tBLASTx against the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov/) to confirm SMase D homology. We aligned all sequences using Clustal X (Thompson *et al*., 1994) and translated them into amino acid sequences using Geneious. Only coding regions were used for further analysis and we removed from the data set sequences that had early stop codons or frame-shift indels.

To investigate phylogenetic relationships, we used a subset of our data that included sequences that were minimally 5% different in amino acid sequence divergence for each species or lineage. We aligned these sequences with those from Binford *et al*. (2009) that follow the same criteria, using the published alignment as a guide. We analyzed the data set in a Bayesian framework using MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003). We conducted two simultaneous runs, each with four Markov Chain Monte Carlo chains for 2 x

10^6 generations, sampling every 100 generations. We confirmed convergence by examining the standard deviation of the split frequencies between the two simultaneous runs and the Potential Scale Reduction Factor (PSRF) diagnostic. We performed the burn-in discarding the first 5000 trees of each run and we constructed a majority-rule consensus tree from the remaining trees.

We tested for significant difference in the set of expressed SMase D paralogs using a chi-squared test ($p < 0.05$). The significance value of the chi-squared test was obtained with a Monte-Carlo simulation using 10000 replicates due to the low number of paralogs expressed in some clades. Statistical analyses were conducted in R (R Core Team, 2014).

RESULTS AND DISCUSSION

Crude Venom Analysis

SDS-PAGE analysis of crude venom extracted from individuals belonging to different *L. rufescens* lineages (A6, B2, B3) and novel Canarian species (LG, GC) are shown in Figure 1. Protein clusters corresponding to the molecular weights of known *Loxosceles* venom components are evident with differences in composition, number and intensity of bands across all species (Fig. 1). Tentative identification of known components likely included in bands was based on molecular weight, although further proteomics analyses are needed to corroborate their identity with confidence.

Comparing venoms based on one-dimensional gel migration of proteins is challenged by issues such as differential loading of venoms resulting in slight differences in total protein abundance and visibility of specific bands, and bands being heterogenous mixtures of proteins and peptides. Nonetheless, banding patterns tend to be repeatable within sexes from individuals in the same population or species (Binford, pers. obs.), making them a good heuristic comparison. The patterns of protein migration across taxa included here are comparable to *L. rufescens* individuals collected in North America (Binford *et al*., 2009). The degree of difference is more acute between *L. rufescens* and the two Canarian *Loxosceles* species, but some differences also exist within *L. rufescens* lineages. In high molecular weight regions, differences exist between A6 and the other two *L. rufescens* lineages (B2 and B3), where the former lack many bands that are conspicuous in B2 and B3

(Fig. 1Ai). Differences in this band range are also clear between *L. rufescens* and the Canarian species. A remarkable difference is observed in a dense band of ~63 kDa that is present only in LG (Fig. 1Aii). The most conspicuous bands in all cases were those situated between 30 and 36 kDa (Fig. 1B), that correspond to the estimated molecular weight of expressed proteins of *SicTox* gene family (Binford & Wells, 2003; Binford *et al*., 2009 and references therein). The density of the band was higher in B2 and less dense in GC. GC also lacks the 35 kDa band found in the rest of the representatives. There are further differences in banding patterns of proteins ranging from 21 to 27 kDa (Fig. 1C). Here, all three *L. rufescens* lineages show a conspicuous band in the ~27 kDa which is not visible in the Canarian species. The two Canarian species also lack the tenuous ~21 kDa band found in all three *L. rufescens* lineages. Although we can only speculate about the identity of these bands, their sizes match well with the 23 kDa fibrinogeneolytic protease and the 27.5 kDa gelatinolytic protease identified by Young & Pincus (2001), both belonging to the *Loxosceles* astacin-like proteases (LALP). These proteins have been suggested to play an important role in the spreading of other noxious components of the venom by degrading extracellular matrix molecules and rendering tissues more permeable (da Silveira *et al*., 2007; Trevisan-Silva *et al*., 2010; Gremski *et al*. 2014). A varying pattern of low molecular weight bands (Fig. 1D) is found in all the *L. rufescens* lineages, GC is remarkably different spanning size and intensity of this region. These bands most likely correspond to venom peptides (3 kDa - 10 kDa) known to be insecticidal, neurotoxic and abundant in most spider venoms but poorly understood in *Loxosceles* (Chaim *et al*., 2011). While some differences in detectability of relatively faint bands could be influenced by subtle differences in total protein loaded, we are convinced that the differences in presence/absence and relative size are sufficient to be real.

SMase D Activity

All species tested show high levels of SMase D activity in crude venom with no significant differences among them, or from the positive control (Fig. 2). The amount of fluorescence emitted in assays from all *L. rufescens* lineages is consistent with *L. rufescens* and *L. arizonica* results reported by Binford *et al*. (2009) and equivalent to the Canarian species. If the venom from *L. rufescens* is less potent than other *Loxosceles* species as the result of reduced SMase D activity, then we would expect to see lower amount(s) of SMase D

activity, but we do not. This high enzyme activity, in conjunction with intense SMase D bands in the 30-35 kDa region on the protein gel in Figure 1 suggests that *L. rufescens*, and the two Canarian species, contain *SicTox* proteins and SMase D activity and have the potential to be as potent as other *Loxosceles* species. Further studies, especially bioassays, should be conducted to confirm this hypothesis.

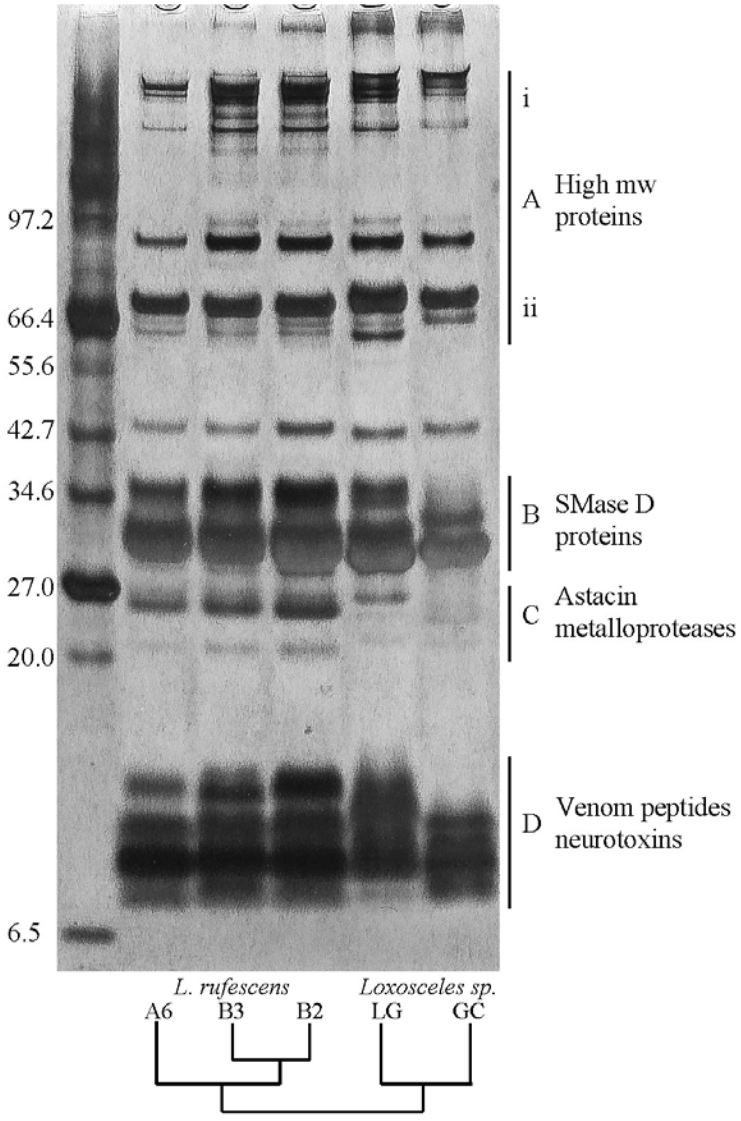

Figure 1 SDS-PAGE comparison of crude venoms from three lineages (A6, B2 and B5) of *Loxosceles rufescens* and two *Loxosceles* from the Canary Islands (GC and GM). Main banding clusters are labeled (A-D) for reference in the text. Schematic tree representing the phylogenetic relationship between *L. rufescens* lineages (A6, B2 and B5) and Canarian species. Broad range molecular weight markers are labeled for size reference.

Mase D phylogenetic diversity and expression patterns

We isolated a total of 325 SMase D homolog sequences. Of these, 301 were non-redundant at the nucleotide level and 262 were nonredundant at the amino acid level (Table 1). The phylogenetic analysis performed with a selection of sequences that were 5% divergent at the amino acid level (35) obtained in this study (GenBank accession numbers XXX - XXX), together with those of the alignment used in Binford *et al*. (2009), showed an almost identical topology compared to the aforementioned study (Fig. 3). In most of the lineages, the topology obtained within individual *SicTox* paralogs mirrored the phylogenetic relationships between *L. rufescens* and the Canarian species (Planas & Ribera, 2014).

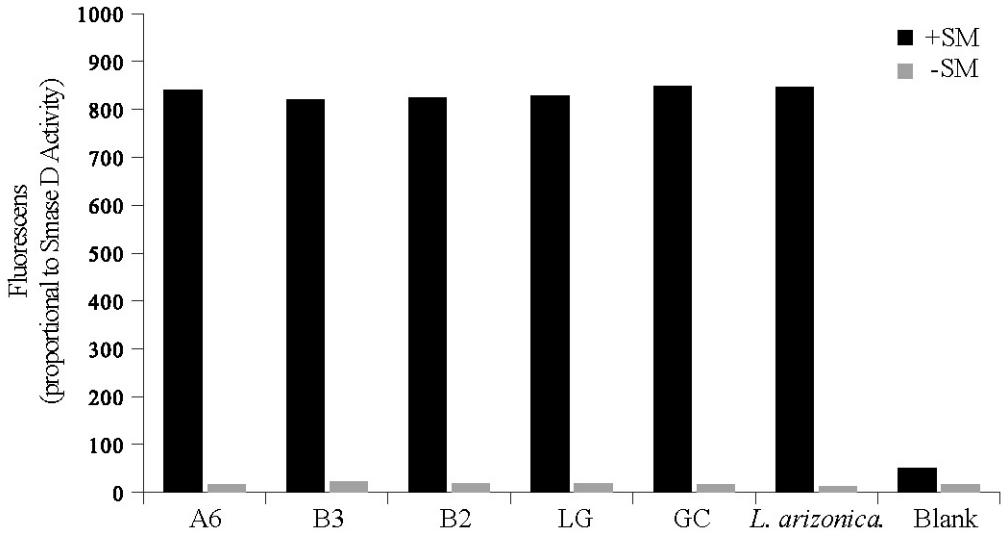

Figure 2 SMase D-specific activity of 0.5 mg of total protein from crude venom from each *Loxosceles rufescens* lineage (A6, B2 and B3) and from the two Canary Islands *Loxosceles* species (LG and GC). We used *L. arizonica* for positive control and substituted 1 x Amplex Red Buffer for venom in blank.

The SMase D paralogs that were most abundant in all studied species belong to clade αIC (Fig. 4). This clade was reported to exclusively include members of the closely related *gaucho*, *amazonica* and *rufescens* species groups (Binford *et al*., 2009). Here we also include two species endemic to the Canary Islands (Planas & Ribera, 2014). In both Canarian species, more than 90% of the sequences belong to this clade, while in the *L. rufescens* lineages, this clade represents a maximum of 79% in B3 and a minimum of 71% in A6. Within this clade, paralogs from the two main subclades were found: αIC1 and αIC2.

In all cases, the representatives of clade αIC1 were more common than the αIC2 clade. In LG and GC, αIC2 was roughly seven and four times less represented than αIC1, respectively. The difference was also high in B2 and B3, where representatives of αIC2 clade were 15 and five times less represented than αIC1 clade members, respectively. In A6, this difference was less marked, though αIC1 clade was only two times more represented than αIC2 clade. To date, only *L. rufescens* sequences reported in Binford *et al*. (2009) were included in the αIC2 clade; here, we have expanded this representation by adding members of the aforementioned Canarian species.

Table 1 Number of individuals used for SMase D expression analysis and total number of expressed SMase D homologs, and nonredundant sequences at nucleotide and aminoacid level.

Species	Individuals in mRNA pool	SMase D homologs recovered	Nonredundant nucleotide sequencies	Nonredundant aminoacid sequences
L. rufescens A6	3	63	60	53
L. rufescens B3	2	40	38	37
L. rufescens B2	4	62	54	51
Loxosceles sp. GC	3	72	65	55
Loxosceles sp. LG	4	88	84	66
Total	16	325	301	262

Sequences from species in this study are also represented in the αII *SicTox* clade; the Canarian species were less numerous than in the *L. rufescens* lineages. B2 was the only lineage that has one sequence belonging to αII2 clade, while all the other sequences belong to αII1 clade. Until now, the only αII2 clade sequence is from *Loxosceles intermedia* (LoxTox i5 in Kalapothakis *et al*., 2007) while αII1 clade is formed by representatives of *gaucho*, a*mazonica* and *rufescens* species groups, and by North American representatives of *spadicea* and *reclusa* groups. The last *SicTox* clade represented in our analysis is the αIV1 clade. In this case, no Canarian sequence is represented and only sequences from the *L. rufescens* lineages were included. In this study, we recovered *SicTox* α clade representatives, but not any from the β clade. There is, however, evidence that β clade homologs are present in *L. rufescens* venom from an introduced North American population that belongs to lineage A6, using MudPIT proteomic analysis (Binford *et al*., unpublished).

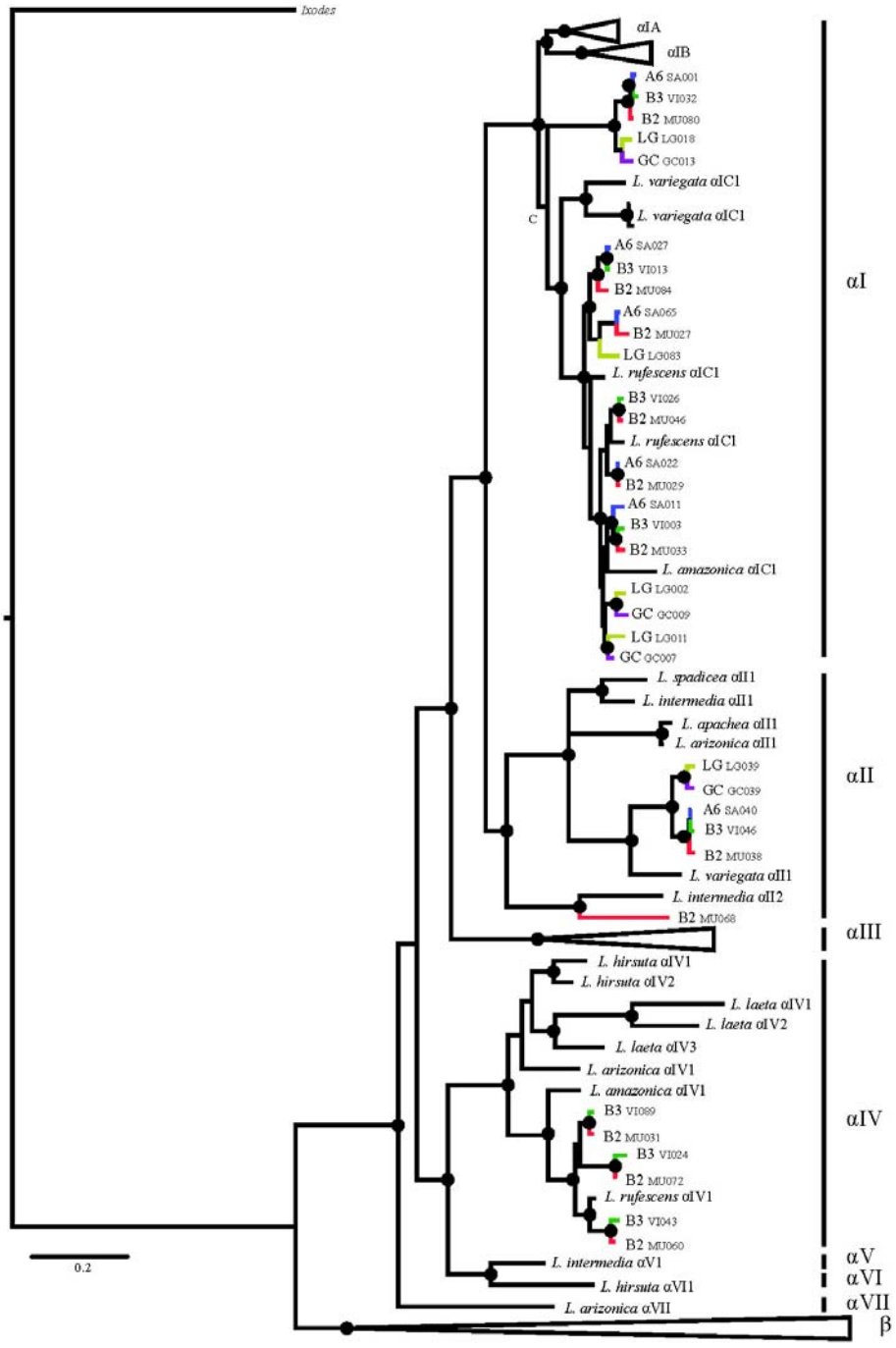

Figure 3 Phylogenetic tree obtained from Bayesian inference analysis of the combined dataset of SMase D sequences that were minimally 5% different in amino acid sequence divergence for each species or lineage (colored accordingly) obtained in this study and those from Binford *et al*. (2009) that follow the same criteria. Black dots in nodes indicate posterior probability support > 0.95.

In addition, cDNA library sequence analysis of A6 lineage has identified several *SicTo*x homologs from both α and β clades representing 7% of the whole venom gland transcriptome (Binford *et al*., unpublished).

We found significant differences among the SMase D expression patterns (i.e. different proportion of expressed paralogs) ($p < 1e^{-05}$). These differences are also significant when we compare the expression patterns among the three members of *L. rufescens* ($p = 0.0018$). Conversely, we haven't found differences between the two Canarian species ($p = 0.4456$) nor between A6 and B3 ($p = 0.3626$). Sequences were ascribed to known *SicTox* clades following the nomenclature proposed by Binford *et al*. (2009).

Patterns of venom variation

The Canarian species studied seem to show less complexity in venom than the Mediterranean ones. We found this pattern in the SDS-PAGE analysis of crude venom and also in the SMase D expression analysis, where evidence of a lack of expression of some paralogs of the *SicTox* gene family in the Canarian species was found (Fig. 1). Although we could not discard the possibility that we failed to sequence those paralogs, it seems rather improbable because we sequenced more clones from transcript pools of the two representatives of LG and GC, than from the *L. rufescens* lineages (Table 1).

It has been widely observed that when snakes and *Conus* snails change their diet, and thus prey preferences, changes in venom composition may also occur (Daltry *et al*., 1996; Duda, 2008; Duda & Lee, 2009). In spiders, there is less evidence of differences in venom composition related to diet preferences. However, work by Binford (2001) demonstrated a reduction on the relative proportion of low molecular weight components that was evolutionarily coincident with a shift from orb-weaving to wandering and an increase in diet breadth. Also, a recent study on myrmecophagous spiders of the genus *Zodarion*, (Pekár *et al*., 2012) reported that venom composition was markedly different and correlated with prey preference. In the same study, they reported that in a *Zodarion* specific biotype from the Macaronesian island of Madeira, the fact that different ant species inhabit the island has promoted changes in body size and probably in venom. Although specific studies in diet preferences of *Loxosceles* species are few (Zobel-Thropp *et al*., 2012), field and laboratory observations suggest that they are generalist predators. Since the venom

properties should be heavily dependent on the differences in diet and behaviors associated with the capture and digestion of the prey, the simplification of venom in the Canarian species could be due to a less diverse prey range found in the islands where they are distributed.

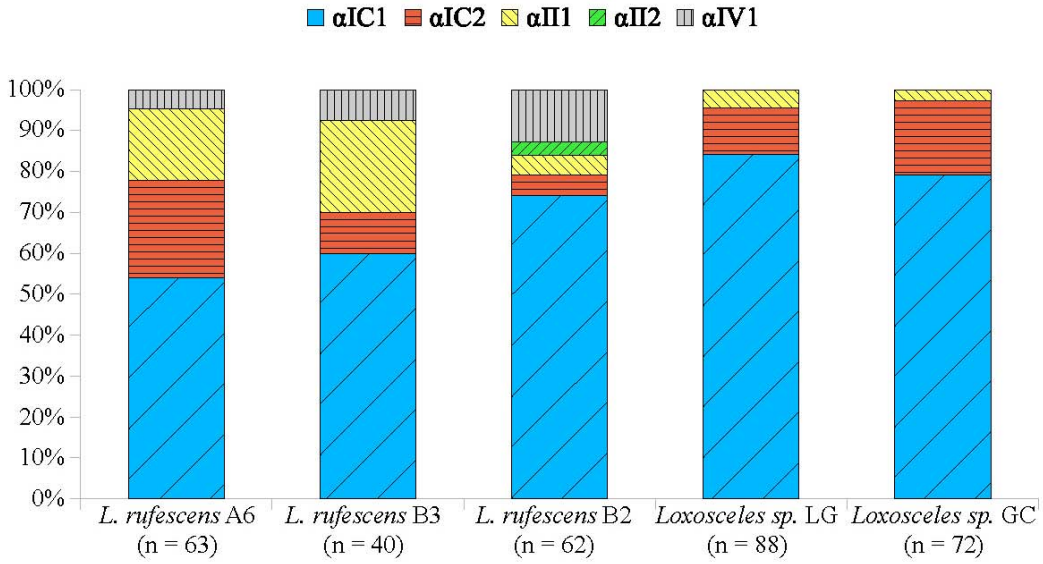

Figure 4 Relative proportions of SMase D paralogs expressed in each *Loxosceles rufescens* lineage (A6, B2 and B3) and from the two Canary Islands *Loxosceles* species (LG and GC). n = number of sequences.

Loxoscelism in the Mediterranean basin

In the Mediterranean basin, well-reported cases of loxoscelism are scarce. Among the published cases, the ones reported from the Eastern Mediterranean seem more abundant and acute than in the Western regions. Although this could be due to a bias in the medical prevalence of reporting these cases, it could also be explained by differences in the dermonecrotic consequences of the *Loxosceles* bite. Lineages A6 and B2 are distributed across the Mediterranean (Planas *et al.*, Paper 1) and we demonstrate evidence of differences in venom, especially in SMase D expression patterns. In order to relate these differences to a differential dermonecrotic activity, further studies need to assay the specific effects on vertebrates of every SMase D clade found in the venom of *L. rufescens* (i.e. αIC, αII and αIV), as they have never been studied in such detail.

We didn't find any difference between SMase D activity between *L. arizonica* and the

three species studied here. Some caution should be taken when relating SMase D activity to loxoscelism, as shown in *Sicarius ornatus*, where SMase D activity does not directly correlate with cytotoxic effects in rabbits (Lopes *et al*., 2013). Nonetheless, the dermonecrotic effects of a wide phylogenetic range of *Loxosceles* venom is firmly established. Also, the broad and consistent expression of multiple SMase D paralogs found in all the target species is reasonable evidence of their potential for causing loxoscelism. Thus, the venom of *L. rufescens* and close relatives should be conservatively considered to be potentially as active as other *Loxosceles* with know medically relevant bites.

Unfortunately, in neither of the two cases of loxoscelism that have been reported from the Canarian Islands were the necessary steps followed to unambiguously relate them to a spider bite. Envenomation by *Loxosceles* spiders most commonly takes place accidentally and in urban habitats. In the Canary Islands, *L. rufescens* has a tendency to live in human-altered areas while the endemic species are found in less disturbed areas (Planas & Ribera, 2014). Thus, if there are loxoscelism cases in the Canary Islands archipelago, the introduced *L. rufescens* is more likely to be the culprit than the endemic species. Meanwhile, caution should be taken with *Loxosceles* bites, both in the Mediterranean Basin and the Canary Islands, and more information should be given to medical personnel in order to gain reliable reports for further studies of loxoscelism in these areas.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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DISCUSSIÓ GENERAL

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Els resultats obtinguts en aquesta tesi doctoral han posat de manifest l'existència d'una elevada diversitat, tant des del punt de vista molecular com morfològic, en les aranyes del gènere *Loxosceles* a la Conca Mediterrània, a les Illes Canàries i al Nord d'Àfrica. Els resultats aportats corregeixen en gran mesura el dèficit de coneixement existent fins aquest moment, i han permès respondre les qüestions específiques proposades als objectius de la tesi. A més, aquest nou coneixement de ben segur permetrà plantejar i abordar noves hipòtesis rellevants des d'un punt de vista evolutiu, per a les quals, les aranyes del gènere *Loxosceles* s'erigeixen com a un excel·lent model d'estudi.

Els resultats de la present tesi han estat discutits en els respectius Articles, i per tant, a continuació es presenta, amb una vocació integradora, una discussió d'aquells resultats més rellevants per tal d'oferir una visió global de la seva significació, tant pel que fa a la sistemàtica del gènere, com per la biogeografia de l'àrea estudiada, i plantejar noves qüestions i futurs estudis.

Com estudiar el que sabem que no sabem

La diversitat específica del gènere *Loxosceles* es troba majoritàriament en les zones càlides i temperades d'Amèrica del Sud i del Nord, però tal com s'apuntava en la introducció, aquesta diversitat segurament està esbiaixada cap a les regions on s'ha treballat més extensament des d'un punt de vista taxonòmic (veure Introducció). En aquesta tesi s'ha constatat l'existència d'aquest biaix, especialment en el Nord d'Àfrica i en les Illes Canàries, però també en la Conca Mediterrània. Una de les limitacions principals en l'estudi de la biodiversitat és l'accés a la informació primària, és a dir, als organismes d'estudi. Els Museus d'Història Natural són les institucions que faciliten l'accés a aquesta informació, però la falta de recursos a diferents nivells fa que sovint no siguin del tot funcionals, o que la representació taxonòmica que alberguen sigui parcial. A més, els espècimens dipositats en aquests museus són moltes vegades inservibles per a estudis moleculars. Per tal de superar aquesta primera limitació és necessari i imprescindible la recol·lecció de nous individus. Així doncs, durant el transcurs d'aquesta tesi s'han realitzat nombroses campanyes de mostreig específicament dirigides en recol·lectar *Loxosceles* en les diferents regions d'interès. En total s'ha aconseguit reunir aproximadament 1500 individus de *Loxosceles*, del quals uns 1200 s'han recol·lectat en el transcurs de la tesi en més de 200

localitats, mentre que la resta han estat cedits generosament per col·legues. La importància d'aquest ampli mostreig ha estat evident per exemple en l'Article 1, on s'hi van incloure representants de 158 localitats, cosa que va permetre obtenir una visió més ajustada de la diversitat del gènere a la Mediterrània. Tot i això, en algunes àrees la consecució d'aquest objectiu no ha estat tan reeixida, i per exemple, malgrat un intens mostreig a Tenerife, només s'han pogut recol·lectar exemplars de les noves espècies endèmiques en dues localitats (Article 2), mentre que els de la tercera localitat coneguda foren cedits pels col·laboradors del Departament de Zoologia de la Universitat de la Laguna. Si bé en aquest cas s'haurien d'explorar les causes que provoquen l'escassetat de *Loxosceles* en aquesta illa, altres factors extrínsecs als organismes també poden afectar la seva obtenció i estudi. Un factor extern que sol afectar el mostreig en certes àrees és la seva inestabilitat política i la inseguretat que se'n genera (Brito *et al*., 2013). Així, tot i la potencial rellevància d'Algèria o Líbia en estudis enfocats en la biodiversitat Mediterrània, i per tant també en *Loxosceles*, el mostreig en aquestes àrees no s'ha pogut realitzar, i per tant, certes incògnites continuen sense resoldre. Cal recordar que es van descriure dues espècies de *Loxosceles* d'Algèria , *L. compactilis* i *L. distincta* que posteriorment foren sinonimitzades per Brignoli (1976). Així, tot i que s'ha aconseguit material d'aquest país gràcies a col·laboradors, no s'ha pogut aconseguir material fresc pels estudis moleculars.

Diversitat i biogeografia del gènere *Loxosceles* a la Conca Mediterrània, al Nord d'Àfrica i a les Illes Canàries

En l'article publicat de forma coetània a l'inici de la tesi per Duncan *et al*. (2010) s'apuntava l'existència d'una elevada diversitat genètica dins de *Loxosceles rufescens* en aquesta regió, i es feia evident la necessitat d'obtenir una major quantitat de dades per a poder separar els patrons deguts a processos "naturals" d'aquells afectats per l'acció humana. A partir del dens mostreig taxonòmic realitzat a la major part de la Conca Mediterrània i utilitzant la informació de les seqüències del gen mitocondrial del citocrom oxidasa 1 (*cox1*) es va constatar a l'Article 1 que en aquesta regió hi cohabiten 11 llinatges evolutius independents. La distribució dels diferents llinatges va permetre distingir la regió situada al sud del Marroc com la que presentava un nombre més elevat de llinatges i on alguns d'aquests es trobaven en només una o poques localitats, mentre que altres llinatges es trobaven repartits per tota la Conca Mediterrània, sense seguir un patró clar en cap de les anàlisis realitzades. Aquests patrons biogeogràfics contrastats van servir per a contestar la pregunta oberta en

Duncan *et al*. (2010) i suggerir que la distribució actual de les *Loxosceles rufescens* s'ha vist afectada pel transport indirecte facilitat pels humans en la regió Mediterrània, i que en canvi, en l'àrea nord dels vessants occidentals de l'Alt Atlas, l'estructura poblacional dels diferents llinatges es correspon amb la "típica" del gènere en condicions naturals, com s'ha trobat també en els articles 2, 5 i 7. Tot i que es evident que la pressió antròpica és més elevada a la Conca Mediterrània, i per tant, el transport indirecte hi és més probable, caldria estudiar si entre els representants dels llinatges "naturals" (és a dir, A1-A4) del Marroc i els representants dels llinatges Mediterranis, hi ha diferències en alguns dels trets de l'estratègia vital (Sol *et al*., 2012) que poguessin explicar les diferències observades respecte a la seva capacitat invasora.

Un dels objectius de l'Article 1 era entendre quins processos van promoure l'existència d'aquesta elevada diversitat en *L. rufescens*. Les datacions realitzades utilitzant dues taxes de substitució divergents, concordaren en situar l'origen dels múltiples llinatges durant el Pleistocè. Una de les característiques més rellevants d'aquest període, que s'estén des dels 2.6 Ma fins a 0.0117 Ma, foren els recurrents cicles glacials, on s'alternaren períodes llargs de clima fred amb períodes més càlids (veure Introducció i Article 1). Nombrosos grups d'organismes s'han vist afectats per aquests canvis climàtics, i una de les conseqüències en molts d'ells ha estat la diversificació al·lopàtrica promoguda per la supervivència durant els períodes més adversos en diferents refugis. En el cas d'Europa, aquests refugis s'han situat en les tres principals penínsules Mediterrànies (Hewitt *et al*., 2000), però sens dubte es tracta d'una simplificació donada l'heterogeneïtat orogràfica i climàtica d'aquestes àrees, i per tant, diferents estudis s'han centrat a delimitar de forma més precisa els possibles refugis (Gómez i Lunt, 2007; Médail i Diadema, 2009). Una de les tècniques utilitzades amb èxit per aquest objectiu és la combinació de la modelització de nínxols ecològics amb models paleoclimàtics, permetent obtenir estimes de les distribucions en diferents períodes del passat (de Lima *et al*., 2014). En l'Article 1 es va aplicar aquesta metodologia utilitzant vuit models generals de circulació oceano-atmosfèrica diferents en tres períodes temporals (present, Holocè mitjà (~6 000 anys) i l'Últim Màxim Glacial (~21 000 anys)). La utilització de dues metodologies per a la delimitació dels refugis potencials i l'aplicació de diferents graus d'astringència donaren diferents resultats, en gran mesura per la variació entre els models, encara que ambdues metodologies delimitaren zones coincidents amb algunes prèviament assenyalades mitjançant aproximacions independents. Per tant, es fa

evident la importància d'utilitzar diferents models paleoclimàtics per a tenir en consideració les diferències intrínseques d'aquests i la seva afectació en les interpretacions biològiques que se'n puguin derivar. A més, es posa en relleu la gran dificultat en delimitar àrees concretes amb aquestes metodologies, ja que s'ha de tenir en compte la informació utilitzada en la seva elaboració, i sobretot l'escala en la qual els resultats poden ser interpretats de forma realista. Tot i això, aquests resultats foren congruents amb els obtinguts amb les dades moleculars, i coincidiren en destacar principalment diferents àrees nord africanes com a possibles refugis.

Com s'ha vist a l'Article 7, als límits sud de la distribució de *L. rufescens* s'hi troben sis llinatges evolutius relacionats filogenèticament amb *L. rufescens*, però ben diferenciats des d'un punt de vista molecular i morfològic. Aquests llinatges, estan repartits per la regió influenciada per les conques dels rius Souss i Massa, incloent-hi els vessants Sud de l'Alt Atlas i grans parts de l'Anti-Atlas (Figura 1 de l'Article 7). La importància biogeogràfica d'aquesta regió ha estat assenyalada basant-se en patrons biogeogràfics de diferents grups i en els endemismes que hi habiten, però la diversificació del gènere *Loxosceles* en aquesta àrea sembla ser un exemple paradigmàtic. La separació nítida entre aquests sis llinatges i *L. rufescens* coincideix amb els massissos més occidentals de l'Alt Atlas, i encara que aquesta cadena muntanyosa delimita la separació entren llinatges d'altres grups d'organismes, en cap cas la separació entre els situats Nord i Sud d'aquesta barrera biogeogràfica són tan clares com en *Loxosceles* (veure referències a Husemann *et al*., 2013). La separació temporal entre aquests dos grups de *Loxosceles* segons les anàlisis obtingudes en l'Article 2, se situa com a mínim al voltant dels 7 Ma. La complexa història geològica d'aquesta regió impedeix relacionar de forma inequívoca els esdeveniments geològics amb els seus possibles efectes vicariants sobre la biota, però les edats estimades no són incompatibles amb els períodes més intensos en l'orogènesi de l'Alt Atlas ocorreguts fa uns 9 Ma i que possiblement també afectaren altres grups com els rèptils del gènere *Agama* o *Ptyodactylus* (Brown *et al*., 2002; M. Metallinou com. pers.). Tot i això, són necessàries noves estimacions dels temps de divergència ampliant el mostreig taxonòmic i genètic per establir un marc temporal més precís. De fet, durant els últims 10 Ma, les oscil·lacions climàtiques al Nord d'Àfrica han condicionat la biota, promovent en alguns casos canvis en la distribució o especiacions vicariants (Douady *et al*., 2003; Carranza *et al*., 2008, Planas *et al*., 2013). Malgrat això, les condicions climàtiques en la regió del Souss-Masssa semblen

haver estat menys rigoroses, com es pot interpretar de les distribucions obtingudes amb alguns models paleoclimàtiques de l'Article 1 (encara que el model va calibrar-se per a *L. rufescens*). En tot cas, l'orografia d'aquesta regió, amb canvis altitudinals pronunciats des de pocs metres sobre el nivell del mar fins a màxims de més de 2 000 metres, degué permetre també l'adaptació altitudinal de la distribució en funció de les condicions climàtiques, afavorint-hi la seva permanència. A més, aquesta orografia, amb muntanyes altes i vall profundes, s'ha assenyalat com a promotora en la diversificació dels llinatges microal·lopàtrics trobats en els escorpins del gènere *Buthus* (Husemann *et al*., 2012; Hable *et al*., 2012) i per tant, processos similars sembla que actuaren en els llinatges (AA1 a AA6) de *Loxosceles*.

A més de *Loxosceles rufescens* i els llinatges que acabem de veure de la regió del Souss-Massa, el gènere *Loxosceles* està representat per una segona espècie, *L. mrazig*, al Nord d'Àfrica. Aquesta es va descriure de Tunísia, però el mostreig realitzat durant la tesi ha permès estendre la seva àrea de distribució de forma notable, tal com s'explica en l'Article 7. Actualment, s'ha pogut mostrejar de forma òptima el Marroc, d'on es disposa d'individus de 7 localitats diferents, i Tunísia, amb unes 15, i dues més d'Israel. Com s'ha comentat al principi, tot i disposar també d'individus de 5 localitats algerianes, ni aquest país ni Líbia i Egipte s'han pogut mostrejar extensament durant el transcurs de la tesi. Per tant, tot i que la diversitat genètica existent no s'ha pogut estudiar en profunditat, en les anàlisis preliminars realitzades (Article 7 i resultats no presentats), els individus s'agrupen seguint un patró estrictament geogràfic diferenciats per distàncies genètiques comparables a les d'altres grups. Tot i això, les grans àrees no mostrejades dificulten la correcta interpretació d'aquests resultats. Per últim, cal apuntar que en cap cas s'han trobat en una mateixa localitat individus de les dues espècies o complexos d'espècies existents al Nord d'Àfrica, és a dir *L. rufescens* i *L. mrazig*, ni a Tunísia ni al Marroc, on el mostreig realitzat ha estat elevat. Segons la distribució d'aquests dos complexos d'espècies, sembla evident que presenten diferències ecològiques, amb *L. mrazig* ocupant hàbitats més àrids que els ambients mediterranis per on es distribueix *L. rufescens*.

Un dels resultats més rellevants d'aquesta tesi és el descobriment d'un nou clade format per 7 llinatges endèmics de les Illes Canàries. Aquest fet és rellevant, ja que la fauna aracnològica ha estat relativament ben estudiada en aquest arxipèlag. Actualment se'n coneixen 467 espècies, 420 de les quals endèmiques (Cardoso *et al.*,2010; Macías, 2010), i

malgrat que la descripció de noves espècies no és un fet excepcional, sí que ho és la descoberta d'una nova radiació. La diversitat d'aquest clade es correspon amb les prediccions de la "*general dynamic theory of oceanic island biogeography"* (Whittaker *et al*., 2008), amb una espècie distribuïda en les illes orientals de Fuerteventura i Lanzarote, dues espècies a l'illa de Gran Canària, tres llinatges (dues espècies nominals, veure Article 3 i més endavant) a Tenerife, i una espècie més distribuïda a La Gomera, però també trobada en una localitat de El Hierro. A més, per la topologia de la inferència filogenètica obtinguda i per la distribució comentada, el patró de colonització sembla haver seguit una progressió des de les illes més antigues, que en aquest arxipèlag també es corresponen amb les més properes al continent (Fuerteventura i Lanzarote) cap a les més noves i més allunyades del continent, a partir d'una sola dispersió ocorreguda durant el Miocè. La relativa simplicitat d'aquesta radiació contrasta amb la que presenten altres gèneres d'aranyes pels quals es disposa d'informació filogenètica i biogeogràfica, com *Dysdera* (Arnedo *et al*., 2001; Macías *et al*., 2008), *Pholcus* (Dimitrov *et al*., 2008) o *Spermophorides* (López-Mercader, 2005), i es pot deure a una menor capacitat dispersiva, una menor plasticitat adaptativa, o una major importància de processos denso-dependents com la competència interespecífica (Whittaker i Fernández-Palacios, 2007; Waters *et al*., 2012; Agnarsoon *et al*., 2014).

L'estudi filogeogràfic de l'espècie endèmica de Fuerteventura, Lanzarote i dels illots de Lobos i La Graciosa, ha servit per a conèixer detalladament els factors més rellevants que han afectat la diversitat genètica en aquesta espècie. De fet, tot i que actualment les esmentades illes i illots estan separats per mar, durant almenys bona part dels últims 900 mil anys han estat units formant una sola illa anomenada Mahan (Rijsdijk *et al*., 2014). Els resultats obtinguts en l'Article 5 posen de manifest que si bé l'actual separació entre les diferents illes afecta clarament la distribució de la diversitat genètica separant-la en dos grups monofilètics corresponents a les dues illes principals i als seus illots més propers, tant en marcadors mitocondrials com en els obtinguts amb els microsatèl·lits desenvolupats en l'Article 4, la unió reiterada entre les diferents illes ha permès la suficient cohesió genètica per a no diferenciar-se en dos llinatges més separats, com els que es troben a Gran Canària o a Tenerife. Aquesta hipòtesi també sembla recolzar-se pels patrons d'aïllament per distància trobats en les dues illes conjuntament o per separat. Així, és de suposar que durant els períodes glacials, poblacions d'aquesta espècie colonitzaven les zones recentment emergides, i la combinació de processos com la competència intraespecífica (Waters, 2011;

Waters *et al*., 2012) amb l'extinció produïda per la pujada recurrent del nivell del mar durant les èpoques interglacials, van acabar configurant l'estructuració genètica actual. Aquesta conjuntura proposada també és congruent amb les relacions entre els individus dels dos illots (Lobos i La Graciosa) amb les del Nord de Fuerteventura i Lanzarote, respectivament. Tot i això, per a testar estadísticament aquestes hipòtesis són necessàries anàlisis addicionals, actualment en preparació, ja sigui utilitzant els mètodes "*approximate Bayesian computation*" (ABC) (Csilléry *et al*., 2010) o nous mètodes especialment implementats per a testar hipòtesis d'exclusió interespecífica o intraespecífica (Ranjard *et al*., in press).

Tal com s'ha comentat en la introducció, les àrees en les quals s'han desenvolupat els estudis de la tesi, principalment la Conca Mediterrània i les Illes Canàries, estan assenyalades dins d'un dels punts calents de biodiversitat del planeta que presenta amenaces pel que fa a la seva conservació (Myers *et al*., 2000). Per tant, són àrees on *a priori*, és esperable trobar una alta biodiversitat, com s'ha demostrat en el grup d'estudi. Tot i això, les amenaces sobre el territori, ja sigui a nivell local o global, obliguen a accelerar les metodologies necessàries per a posar al descobert i conèixer aquesta diversitat. Com s'ha demostrat amb els resultats presentats i en els paràgrafs previs de la discussió, la informació genètica obtinguda ha estat de gran vàlua per a aquesta finalitat, i ha permès obtenir una visió completa de la diversitat filogenètica existent en *Loxosceles* a diferents nivells, i a entendre en certa mesura els principals processos que han actuat en la seva generació.

Concepte, delimitació i descripció d'espècies en el gènere *Loxosceles*

La informació genètica obtinguda, que ha servit per a ressaltar la diversitat i revelar els patrons biogeogràfics del gènere *Loxosceles*, també ha estat utilitzada en aquesta tesi com a informació rellevant per a poder delimitar aquells llinatges metapoblacionals independents, que per la seva independència evolutiva mereixen distingir-se en la categoria taxonòmica d'espècie. El concepte unificat d'espècie ha suposat un salt important en la distinció entre la part teòrica i pràctica, és a dir, entre el concepte i la delimitació de les espècies (de Queiroz, 2007, veure Introducció) i s'ha seguit com a marc conceptual en aquesta tesi. Històricament, les espècies del gènere *Loxosceles* han estat reconegudes a partir d'un concepte morfològic d'espècie, distingint a partir de combinacions de caràcters, el que es creia que eren espècies morfològiques distintes (Gertsch i Ennik, 1983). Tot i això, per la simplicitat de molts dels

caràcters de la genitalia utilitzats generalment en la distinció entre espècies properes, aquesta delimitació s'ha basat en diferències subtils i moltes vegades subjectives, provocant encesos debats entre diferents tradicions taxonòmiques (veure *The species problem in* Loxosceles, pàgines 271-276 a Gertsch i Ennik, 1983). És en aquest context, que la utilització de la informació obtinguda amb caràcters moleculars esdevé de gran importància per, almenys potencialment, poder superar d'una forma més ràpida i eficient part de les dificultats obtingudes amb la sola utilització de caràcters morfològics.

En la present tesi, la delimitació pràctica dels llinatges a partir de dades moleculars es va realitzar mitjançant diferents aproximacions, de les quals en destaca la del model *General Mixed Yule Coalescent* (GMYC) (Pons *et al*., 2006). En el cas concret de *L. rufescens* (Article 1), la informació prèvia disponible apuntava que diferents llinatges morfològicament indistingibles es trobessin repartits de forma no previsible per la Mediterrània, i per tant, la delimitació objectiva obtinguda mitjançant aquesta metodologia va ser de gran utilitat per a discretitzar la variabilitat genètica present. Tot i que els objectius d'aquest estudi no s'incloïen reobrir les qüestions taxonòmiques estudiades per Brignoli (1976), l'elevada diversitat genètica trobada és temptadora per a la reconsideració de l'estatus taxonòmic d'alguns dels llinatges o clades (per exemple clade A i B, Article 1). Per a fer-ho, s'ha plantejat en un futur pròxim la utilització dels marcadors nuclears (microsatèl·lits) desenvolupats en l'Article 4 per a l'estudi dels processos poblacionals i filogeogràfics dels diferents llinatges Mediterranis, amb especial atenció en aquelles àrees on diversos dels llinatges mitocondrials delimitats hi són simpàtrics, amb la hipòtesi taxonòmica que si la separació mitocondrial es mantingués també en aquests marcadors, es podrien tractar d'espècies diferents, i per tant, caldria reconsiderar el seu actual estatus taxonòmic.

El GMYC també s'ha utilitzat en l'Article 2 per a la delimitació dels diferents llinatges de *Loxosceles* de les Illes Canàries. En aquest cas, els llinatges delimitats a partir de la informació genètica mitocondrial van servir d'hipòtesi prèvia en l'Article 3. En aquest article es va poder testar la seva independència evolutiva amb evidències obtingudes de les dades de gens nuclears i de la morfologia, principalment dels caràcters reproductors (genitàlics i òrgans copuladors) però també dels somàtics. En conjunt, aquestes evidències independents van recolzar la validesa dels diferents llinatges i com a conseqüència es va actualitzar el seu estatus taxonòmic descrivint-les com a noves espècies. En canvi, en

l'estudi centrat en els llinatges de la regió del Souss-Massa, l'elevada estructuració poblacional evident per les grans distàncies genètiques obtingudes amb el gen mitocondrial *cox1*, juntament amb el mostreig obtingut, segurament afectaren de forma negativa la delimitació de diferents llinatges únicament amb aquest mètode, tal com s'ha trobat també en altres grups d'aranyes amb característiques poblacionals similars (Satler *et al*., 2013; Hamilton *et al*., 2014), per no acomplir algunes de les assumpcions en l'aplicació d'aquesta metodologia com l'absència d'estructuració poblacional (Papadopoulou *et al*., 2009). Per tal de delimitar, i per tant simplificar la diversitat obtinguda, es van reconèixer els diferents llinatges a partir de la topologia i la longitud relativa de les branques entre els diferents clades obtinguts en les anàlisis filogenètiques i de la distribució geogràfica dels diferents individus que componien els clades. Tot i la subjectivitat inherent en aquest procediment, els llinatges delimitats s'entenen com a hipòtesis, i per tant, cal contrastar-les amb evidències addicionals, per si fos adient, descriure-les com a espècies diferents. En casos similars, s'ha vist que és de gran utilitat, i fins i tot necessari, utilitzar múltiples marcadors nuclears i metodologies que tinguin en compte fonts d'errors previsibles des de la teoria de coalescència, per tal de delimitar d'una forma més ajustada aquells llinatges que veritablement han seguit una història evolutiva independent (Carstens *et al*., 2013). En aquest sentit, actualment s'està utilitzant la informació genètica disponible en els llinatges del Souss-Massa per portar a terme anàlisis utilitzant mètodes com el *Bayesian Phylogenetics and Phylogeography* o l'*spedeSTEM* tant per a "descobrir" llinatges independents com per contrastar aquells delimitats amb mètodes alternatius.

En organismes no model com les aranyes, la utilització d'aquestes metodologies s'ha vist enormement limitada per la disponibilitat d'encebadors per a marcadors nuclears utilitzables per aquesta funció. Tot i això, aquestes limitacions seran cada cop menys importants, principalment gràcies a les noves tecnologies de seqüenciació, que permeten obtenir grans quantitats de dades genètiques que potencialment es podran utilitzar o bé directament per a estudis de delimitacions d'espècies o per obtenir els marcadors necessàries per a aplicar les metodologies anteriorment descrites (Brewer *et al*., 2014).

En qualsevol cas, tot i que la delimitació d'espècies amb dades moleculars ofereix grans avantatges en grups on la morfologia s'ha demostrat, com a mínim, poc resolutiva, és recomanable utilitzar una aproximació integradora per a poder consensuar les possibles discrepàncies sorgides amb evidències moleculars i incorporar la informació morfològica,

ja que només a través de l'estudi de la morfologia es poden posar al descobert patrons evolutius de gran rellevància, com l'evolució de dimorfisme sexual (Kuntner i Elgar, 2014), dels caràcters troglomòrfics (Arnedo *et al*., 2007) o de diferents patrons biogeogràfics com l'"*island rule*" (Meiri, 2007), els dos últims discutits breument en l'Article 3. A més, la informació morfològica pot aportar evidències addicionals a la distinció dels diferents llinatges evolutius, i la seva incorporació en les descripcions i diagnòstic de les noves espècies és de gran importància, ja que és l'única informació disponible i utilitzada en la gran majoria d'espècies descrites fins ara.

Actualment es posseeix informació genètica de només una petita part de la biodiversitat de la Terra, i en el cas concret de *Loxosceles*, de 28 espècies nominals, malgrat l'augment d'estudis publicats en els últims anys. Tot i això, al GenBank hi ha 17 noms més sense categoria taxonòmica, entre els quals els 7 llinatges endèmics de les Illes Canàries obtinguts en l'Article 2. Aquestes seqüències manquen d'un nom binomial correcte segons els criteris de la Comissió Internacional de Nomenclatura Zoològica, i formen el que s'ha anomenat com a "*dark taxa*" (Page, 2011). Tot i que aquests tàxons (seqüències) poden ser utilitzats per a alguns estudis concrets, per exemple en l'Article 2, la no descripció formal dels mateixos els exclou de la majoria d'altres, i impedeix que la diversitat que representen sigui tinguda en compte en la gran majoria de camps de la biologia i altres ciències, que utilitzen el rang taxonòmic d'espècie com el principal identificador (Patterson *et al*., 2010). Conseqüentment, s'ha considerat del tot necessari proporcionar una descripció formal dels llinatges esmentats endèmics de les Illes Canàries i es treballarà en la mateixa direcció amb els llinatges del Souss-Massa.

Loxoscelisme a la Mediterrània i a les Illes Canàries

El desconeixement de la biodiversitat que ens envolta pot tenir conseqüències que s'escapen dels camps estrictament dedicats a la Biologia. L'any 2005, un suposat cas de picada per *Loxosceles* va ocórrer a Gran Canaria, amb conseqüències molt greus per a la persona afectada. Aquest seria el primer cas de loxoscelisme a les Illes Canàries, tot i això, la picada no fou mai confirmada i les causes que portaren a l'amputació de les cames de la persona afectada es relacionaren amb infecció per *Streptococcus pyogenes* (Dictamen 276/2010, Consejo Consultivo de Canarias, 2010). Malgrat els dubtes, la notícia va saltar als mitjans d'informació com a un cas greu de loxoscelisme, i de fet, s'apuntà que l'espècie causant va

ser *L. reclusa* o *L. laeta*. En part, això es devia al desconeixement de la diversitat de *Loxosceles* a les illes Canàries, d'on només *L. rufescens* havia estat citada i, per tant, assumint com a certes les hipòtesis que indiquen que el verí d'aquesta espècie és menys potent que el d'altres espècies del mateix gènere, la causant devia ser una altra espècie. La tendència de relacionar els casos més greus amb les espècies americanes també és corrent a la Conca Mediterrània, encara que aquestes no s'hi hagin citat mai (excepte una cita de *L. gaucho* de Tunisia per Brignoli, 1976). La falta de coneixement, de divulgació de l'existent i la manca de rigorositat periodística fa que aquests casos s'amplifiquin pels mitjans de comunicació, creant un clima d'alarma i misticisme al voltant de les aranyes (Vetter, 2000).

En l'Article 8 es mostren els resultats de l'estudi realitzat amb la intenció de pal·liar el desconeixement sobre el loxoscelisme en l'àrea d'estudi, principalment per contrastar la hipòtesi comentada anteriorment sobre la suposada menor gravetat del loxoscelisme provocat per *L. rufescens*, estudiant-ne la variació en tres llinatges de *L. rufescens* i en dues espècies endèmiques de Canàries, *L.* sp GC1 de Gran Canària i *L.* sp GM de La Gomera i El Hierro. Cal dir per avançat, que els efectes del verí depenen de múltiples factors, i que per tant, l'establiment de relacions causals entre els resultats del nostre estudi amb els efectes del loxoscelisme en humans són encara especulatius. Tot i això, els resultats globals de la tesi han ajudat a aclarir algunes incògnites pel que fa al loxoscelisme a la Mediterrània i les Illes Canàries. En primer lloc, s'ha vist que l'activitat esfingomielinasa D trobada en tots els llinatges de *L. rufescens* i en les dues espècies de Canàries és tan alta com en les espècies Americanes, confirmant així els resultats de Binford *et al*. (2009) per *L. rufescens*, i estenent-los a les espècies *L.* sp. GC1 i *L.* sp. LG-HI i als llinatges B2 i B3, que s'estudiaven per primera vegada. A més, també s'han trobat diferències significatives en l'expressió de diferents paràlegs de la família gènica *SicTox*, principalment entre *L. rufescens* i les dues espècies endèmiques de les Illes Canàries, però també dins dels llinatges de *L. rufescens*. Com que es desconeix l'acció concreta de cadascun d'aquests paràlegs, es fa difícil predir si tenen una rellevància pel que fa als seus efectes sobre els humans, encara que aquests resultats permeten plantejar algunes qüestions. Per exemple, l'explicació per les quals les dues espècies de les Illes Canàries tenen menys variabilitat en els paràlegs expressats, mancant completament l'expressió del clade αIV1 que sí que es troba en els diferents llinatges de *L. rufescens*. O bé, quina és la relació entre les diferències en l'expressió dels diferents paràlegs entre els llinatges de *L. rufescens*, principalment entre

el B2 i els altres dos (A6 i B3), tenint en compte que els casos de loxoscelisme reportats a la Mediterrània són més comuns a la part oriental que a l'occidental (veure Figura 7 de la Introducció), i que la distribució d'aquests llinatges és desigual (veure Figura 4, Article 1), encara que com s'ha mostrat en l'Article 1, no és possible predir la distribució geogràfica dels diferents llinatges repartits per la Mediterrània. En tot cas, amb els resultats obtinguts s'haurien de considerar tant *L. rufescens* com les dues espècies de les Illes Canàries, potencialment tan "perilloses" com les espècies americanes pel que fa a la seva picada. A més, donat el comportament antropogènic de *L. rufescens*, és previsible que aquesta espècie tingui més encontres accidentals amb humans. Aquest fet és rellevant a les Illes Canàries, on a més dels possibles efectes que pugui tenir aquesta introducció sobre la fauna autòctona, s'han de considerar els possibles efectes mèdics.

Per tal de millorar la comprensió del loxoscelisme a la Mediterrània i a les Illes Canàries, és necessari recollir més informació en diferents nivells. En els casos on l'afectat ha pogut capturar l'aranya involucrada en l'accident, és de gran importància que se'n faci una correcta identificació. Tot i que el coneixement sobre la diversitat i distribució de *Loxosceles* en aquestes àrees ha augmentat considerablement, la seva identificació continua essent difícil. En l'Article 3 es va testar l'eficàcia del mètode d'identificació basat en el codi de barres genètic i es va demostrar que és útil per a distingir les diferents espècies que es troben a les Illes Canàries, i per tant, aplicable per a la identificació a nivell específic. Sense aquest coneixement que permetria posar les bases necessàries per poder aconseguir una informació rigorosa sobre els casos de loxoscelisme i així, establir-ne d'una forma fiable la seva epidemiologia, no es podran portar a terme accions per a millorar-ne el diagnòstic o el tractament.

CONCLUSIONS

CONCLUSIONS GENERALS

1. S'han identificat 11 llinatges evolutius en *Loxosceles rufescens* amb les anàlisis de delimitació molecular realitzades amb dades d'ADN mitocondrial. Aquests llinatges presenten dos patrons filogeogràfics contrastats: (1) uns llinatges amb una estructuració poblacional molt marcada al Marroc i a la Península Ibèrica, i (2) uns llinatges que manquen d'estructuració geogràfica repartits per la Conca Mediterrània.

2. Les estimes d'edats han situat la majoria de diversificacions durant el Pleistocè, i a partir de models de nínxol ecològics (ENM) s'han identificat múltiples refugis Pleistocènics, essent per tant compatible la diferenciació al·lopàtrica dels múltiples llinatges, probablement promoguda per les successives glaciacions.

3. La biogeografia actual de *L. rufescens* a la Conca Mediterrània s'ha vist enterbolida pel transport indirecte facilitat pels humans.

4. Les anàlisis filogenètiques han posat al descobert l'existència d'un clade ben recolzat endèmic de les Illes Canàries, que compren set llinatges evolutius distribuïts al·lopàtricament.

5. La diversitat dins d'aquest grup s'ajusta a la *general dynamic theory of oceanic island biogeography*, amb el màxim de diversitat a Gran Canària i Tenerife, les illes amb edats intermèdies. El patró de colonització d'aquest grup és compatible amb un model *stepping-stone*, i la dispersió entre illes ha estat la major força diversificadora del grup. Tot i això, el vulcanisme del Roque Nublo ha actuat com a agent vicariant promovent la separació entre els dos llinatges de Gran Canària.

6. L'espècie *L. rufescens* ha estat recentment introduïda i cohabita amb els llinatges endèmics a l'arxipèlag Canari.

7. La integració de dades d'ADN nuclear i morfològiques han corroborat la distinció evolutiva dels llinatges prèviament delimitats a partir de seqüències d'ADN mitocondrial, i s'ha realitzat la descripció formal de sis noves espècies: *Loxosceles mahan* endèmica de Fuerteventura, Lanzarote, i dels illots adjacents; *Loxosceles bentejui* i *Loxosceles tazarte* ambdues endèmiques de Gran Canària; *Loxosceles guayota* i *Loxosceles tibicena*

ambdues endèmiques de Tenerife; i *Loxosceles hupalupa* endèmica de La Gomera i d'El Hierro. Totes les espècies s'inclouen dins del grup *rufescens.*

8. El codi de barres genètic és una eina ràpida i fiable per a la identificació de les espècies de *Loxosceles* de les Illes Canàries i es pot aplicar per a la correcta identificació de les aranyes involucrades en picades.

9. S'han desenvolupat set i onze nous marcadors microsatèl·lits per *L.* sp. Fuerteventura - Lanzarote i *L. rufescens*, respectivament utilitzant tecnologies de seqüenciació massiva, un mètode ràpid i econòmic per a obtenir microsatèl·lits en aranyes.

10. Malgrat que les illes de Fuerteventura i Lanzarote han estat connectades de forma recurrent a causa dels canvis en el nivell del mar succeïts durant el Pleistocè, els individus de cadascuna d'aquestes illes formen grups recíprocament monofilètics. En canvi, els individus de Lobos i de La Graciosa es van agrupar amb els de les localitats més properes de Fuerteventura i Lanzarote, respectivament.

11. La diversitat genètica s'estructura principalment segons un patró d'aïllament per distància a Fuerteventura, però a Lanzarote, l'origen dels dos llinatges mitocondrials es deu probablement als efectes del vulcanisme Pleistocènic.

12. A partir de caràcters morfològics i moleculars s'ha descrit una nova espècie de *Loxosceles* de Tunísia. *Loxosceles mrazig* representa la segona espècie d'aquest gènere a la Conca Mediterrània.

13. Les anàlisis filogenètiques amb les aranyes del gènere *Loxosceles* de la regió del Souss-Massa han posat de manifest sis llinatges molt divergents amb distribucions al·lopàtriques. Aquests llinatges es distingeixen morfològicament de *L. rufescens* i *L. mrazig*. Aquest estudi ressalta la rellevància biològica d'aquesta regió rica en endemismes.

14. S'ha ampliat la distribució coneguda de *Loxosceles mrazig*, anteriorment restringida a Tunísia, cap a l'est fins a Israel, i cap a l'oest fins al Marroc.

15. La composició proteica del verí utilitzant SDS-PAGE presenta algunes diferències en el patró de bandes i en la intensitat, principalment entre dues espècies de *Loxosceles*

endèmiques de les Illes Canàries i *L. rufescens*. També hi ha diferències entre aquestes espècies en l'expressió de diferents paràlegs de la família gènica *SicTox*, essent les espècies de Canàries menys diverses.

16. L'activitat Smase D de *L. rufescens* i les dues espècies de Canàries és comparable a la d'espècies americanes les quals s'ha confirmat que poden provocar picades rellevants des d'un punt de vista mèdic. El verí de *L. rufescens* i el de les dues espècies endèmiques de les Illes Canàries pot ser tan potent com el d'altres espècies de *Loxosceles*, i per tant, aquestes s'haurien de considerar rellevants des d'un punt de vista mèdic.

GENERAL CONCLUSIONS

1. Delimitation analyses conducted on mtDNA identified 11 different evolutionary lineages in *Loxosceles rufescens*, presenting two contrasting phylogeographic patterns: (1) lineages with well-structured populations in Morocco and Iberia, and (2) lineages lacking geographic structure across the Mediterranean Basin.

2. Dating analyses placed main diversification events in the Pleistocene, and multiple Pleistocene refugia, identified using ecological niche modelling (ENM), are compatible with allopatric differentiation of lineages, likely promoted by successive glaciations.

3. Human-mediated transportation appears to have complicated the current biogeography of *L. rufescens* in the Mediterranean Basin.

4. Phylogenetic analyses revealed the existence of a well-supported clade formed exclusively by Canarian *Loxosceles* specimens, comprising seven allopatrically distributed evolutionary lineages.

5. The pattern of diversity of this group fits well with the general dynamic theory of oceanic island biogeography, with the maximum diversity found in Gran Canaria and Tenerife, that are islands of intermediate age. The colonization pathway of the group is compatible with a stepping-stone model and between-island dispersal was the major driving force for diversification in the group. Furthermore, the Roque Nublo volcanic event acted as a vicariant agent promoting the split between the two Gran Canarian lineages.

6. The recently introduced *L. rufescens* is cohabiting with the endemic lineages in the Canarian archipelago.

7. The integration of nuclear DNA and morphological data corroborated the evolutionary distinctiveness of previously delimited lineages based on mtDNA sequences, and a formal description of six new species is provided: *Loxosceles mahan* endemic to Fuerteventura, Lanzarote, and adjacent islets; *Loxosceles bentejui* and *Loxosceles tazarte* both endemic to Gran Canaria; *Loxosceles guayota* and *Loxosceles tibicena* both endemic to Tenerife; and *Loxosceles hupalupa* endemic to La Gomera and El Hierro. All these

species are included in the *rufescens* group.

8. DNA barcoding is a fast and reliable tool for identifying the *Loxosceles* species found in the Canary Islands and could be applied for the correct identification of the species involved in bite accidents.

9. Seven and eleven polymorphic microsatellites markers were obtained for *L. sp.* Fuerteventura - Lanzarote and *L. rufescens*, respectively, using next-generation sequencing technology, a relatively fast and cost-effective procedure for the development of microsatellites in spiders.

10. Despite the recurrent connection between Fuerteventura and Lanzarote promoted by sea-level changes during the Pleistocene, individuals from each island formed reciprocally monophyletic groups. Differently, individuals from Lobos and La Graciosa were grouped with those from the closest localities in Fuerteventura and Lanzarote, respectively.

11. Genetic diversity was mainly structured by an isolation-by-distance pattern in Fuerteventura, but in Lanzarote, the origin of two deep mitochondrial lineages is probably related to the effects of Pleistocenic volcanism.

12. Based on morphological and molecular characters a new species of *Loxosceles* is described from Tunisia. *Loxosceles mrazig* represents the second species for the genus in the Mediterranean Basin.

13. Phylogenetic analyses of *Loxosceles* spiders in the Souss-Massa region uncovered six highly divergent lineages with allopatric distributions. These lineages are distinguished morphologically from *L. rufescens* and *L. mrazig*. Overall, this study underlines the biological relevance of this endemism-rich area.

14. The distribution of *Loxosceles mrazig*, previously known only from Tunisia, is extended eastwards to Israel and westwards to Morocco.

15. The venom protein composition using SDS-PAGE presents some differences in banding pattern and intensity, mostly between two *Loxosceles* species endemic to the Canary Islands and *L. rufescens*. Differences between these species also exist in the

expression of different paralogs of the *SicTox* gene family, with the Canarian species being less diverse.

16. The SMase D activity of *L. rufescens* and the two Canarian species is comparable to American species that are confirmed to have medically relevant bites. Thus, the venom of *L. rufescens* and of the two Canarian endemic species could be as potent as other *Loxosceles* species, and these taxa should be considered medically relevant.

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