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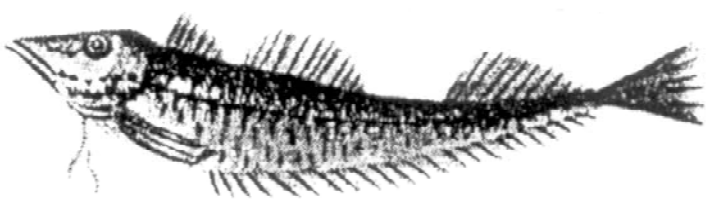

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Memòria presentada per Josep Carreras Carbonell, realitzada en el Centre d'Estudis Avançats de Blanes (CEAB-CSIC), per accedir al Títol de Doctor en Ciències Biològiques en el Departament de Genètica de la Facultat de Biologia, sota la direcció dels doctors Marta Pascual Berniola i Enrique Macpherson Mayol.

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Agraïments

Fa uns quants anys (tampoc gaires) vaig decidir que això de la pesca m'agradava i si volia treure alguna peça maca m'havia de mullar el cul.

Quan vaig començar anava una mica despistat, eren moltes coses noves de cop, que si les ulleres, el tub, les aletes… què utilitzo? Sort que des d'un bon començament he tingut dos barquers immillorables, coneixedors de les millors tècniques i llocs de pesca: la Marta i en Mac. Ells han sabut portar-me als millors punts i aconsellar-me sobre el material i la tècnica a utilitzar en cada ocasió i per a cada espècie. De ben segur que sense ells les captures que he fet durant aquests anys no haguessin estat possibles.

Recordo que els primers dies ni tan sols arribava al fons, les jornades eren infructuoses, o si més no això és el que jo pensava. Quan ja aconseguia arribar al fons no veia ni un peix, no els veig perquè no n'hi ha o perquè sóc dolent? em preguntava sovint. De mica en mica, i a base de molta, molta pràctica, vaig anar veient algun peix. Ep! només veient, no s'hem ficaven pas "a tiro". Amb el temps cada cop veia més peix i s'acostava més i més. Aleshores van començar a arribar les primeres captures, modestes però molt importants per a mi ja que et donen una injecció de moral molt important per continuar.

Ara doncs, era qüestió d'anar perfeccionant la tècnica, tenir bon material i sobretot d'anar molt i molt a l'aigua. Durant aquest temps he compartit experiències amb altres barquers i pescadors, que si fa o no fa, pescaven pels mateixos llocs que jo (tot i que sempre separats una certa distància, per allò de no molestar els peixos i que al final, l'un per l'altre no acabi pescant ningú…). A tots ells moltes gràcies ja que amb els seus coneixements i consells m'han ajudat molt a millorar i a superar-me. També crec que ha estat important la interacció amb pescadors i barquers d'altres mars i oceans, ja que les seves tècniques i punts de vista de la pesca m'han enriquit extraordinàriament, dotant-me de més recursos i ensenyant-me noves zones i tècniques de pesca.

Durant aquests anys m'he mogut principalment entre dos ports, el de Blanes i el de Barcelona. Sense el seu aixopluc les captures que he anat fent no haguessin estat possibles, ja que tant els mecànics, com els mariners i la gent de capitania m'han ajudat en tot moment i s'han ocupat de tenir sempre a punt tots els permisos, assegurances, revisions…

Doncs amb el temps i a base d'esforç les captures s'han anat succeint, un parell de llobarros macos, un mero "gordo" que es va enrocar i va donar una feinada brutal, un sarg soldat preciós, una serviola immensa que si no és pel carret no la trec, una orada un dia de primavera després d'una espera llarguíssima, un bonítol a poc fons o un anfós rosat molt fons, i també un parell de corvalls i alguna escórpora xula. Però hem faltava un peix, potser el més emblemàtic en el món de la pesca: un dèntol de bona mida. Finalment l'he fet. És una d'aquelles captures gairebé irrepetibles, que et fan sentir afortunat. Gràcies a tots aquells barquers, pescadors, mecànics, mariners… que d'una forma o altre hi heu contribuït.

Ara, ja penso amb el sopar que farem amb aquest "bitxo" al forn. Perquè, sens dubte, millor que pescar és tornar a port sabent que tens un munt d'amics i gent amb qui compartir les captures i que sempre et faran costat. Així doncs, el meu més "profund" agraïment a la persona que sempre m'ha esperat i ha confiat en mi, la Núria, i a tots els de casa per donar-me sempre suport incondicional, encara que sense saber gaire bé que coi hi feia allà pescant. De veritat moltes gràcies!

Ara suposo que s'obre una altra etapa de la meva vida com a pescador, ara sóc jo qui ha de portar la "zodiac" amunt i avall, i amb l'experiència adquirida buscar noves zones de pesca per afegir a la memòria del GPS, així com també omplir el dipòsit de benzina (cada dia més cara per cert) i buscar un port on amarrar. Espero sortir-me'n i mantenir la qualitat, més que la quantitat, de les captures.

A tots, moltes gràcies, per fer-m'ho passar tant bé pescant!

Índex

1.- Introducció General

En els últims anys, la utilització de tècniques moleculars per aproximar problemes en el camp de l'evolució, l'ecologia i la conservació ha experimentat un notable increment (Zane *et al*., 2002). Aquests estudis permeten des de la reconstrucció de les relacions filogenètiques existents entre espècies fins a l'estudi amb detall de l'estructura poblacional d'una determinada espècie. Els diferents marcadors moleculars tenen una determinada abundància en el genoma, una especificitat concreta, un nivell de polimorfisme, reproductibilitat, diferents requeriments tècnics... D'aquesta manera, per a respondre cada pregunta s'haurà d'escollir el marcador que millor s'adapti a cada situació.

Els estudis genètics en organismes marins realitzats en el Mediterrani són escassos i insuficients per prendre mesures de gestió adequades que assegurin la viabilitat de les espècies i que ens permetin estimar la biodiversitat en aquest medi. Aquest treball pretén analitzar, utilitzant diferents marcadors moleculars, aspectes filogenètics i poblacionals en diverses espècies de peixos litorals del Mediterrani amb la finalitat d'augmentar el coneixement sobre la seva ecologia i evolució.

1.1.- Filogènia i especiació

El Mediterrani és un mar càlid-temperat considerat com un "hot spot" pel que fa a la diversitat d'espècies de peixos (Blenniidae, Almada *et al*., 2001; Labridae, Hanel *et al*., 2002; Rajidae, Valsecchi *et al*., 2005). Presenta unes 540 espècies de peixos i aproximadament un 9.6% (52 espècies) són endèmiques (Briggs, 1974). Estudis recents, han constatat que algunes de les espècies inicialment considerades endèmiques també es troben en zones atlàntiques adjacents (Almada *et al*., 2001). Així mateix s'ha detectat una seixantena d'espècies migradores lessepsianes procedents del mar Roig (oceà Índic) a través del canal de Suez (Golani, 1999). La configuració actual del Mediterrani s'originà fa uns 40 milions d'anys. Durant aquest temps, el Mediterrani ha sofert almenys un episodi de dessecació i reompliment, és l'anomenada Crisis de Salinitat del Messinià (MSC) que va tenir lloc fa uns 5.6 – 5.2 milions d'anys (Hsü *et al*., 1977; Duggen *et al*., 2003). Durant aquest període de

dessecació, exceptuant un petit nombre d'espècies capaces de sobreviure en ambients salobres o hipersalins, les espècies marines que habitaven el Mediterrani es van extingir o van haver de migrar cap a l'Atlàntic. Després d'aquest període, el Mediterrani va experimentar un reompliment molt ràpid (aprox. 100 anys) degut a la reconnexió amb l'oceà Atlàntic a través de l'estret de Gibraltar (Hsü, 1972; Hsü *et al*., 1977).

La gran biodiversitat del Mediterrani es podria explicar, en part, per les especiacions ocasionades per la MSC, per les dràstiques fluctuacions climàtiques originades entre principis del Pleistocè (3.6 Ma) i finals del Pliocè (2.7 Ma) i per els períodes de glaciacions durant el Quaternari (1-2 Ma) (Sorice & Caputo, 1999). Aquestes fluctuacions impliquen variació en el nivell del mar, així com canvis en la temperatura superficial de les masses d'aigua, això dificulta la connexió entre masses d'aigua i conseqüentment entre les poblacions que les habiten (Avise, 2000a). D'aquesta manera l'aïllament de diferents conques o indrets geogràfics durant un període de temps suficient provoca una especiació al·lopàtrida entre les poblacions d'ambdós indrets. En el passat, l'Atlàntic i el Mediterrani han estat aïllats degut a canvis en el nivell del mar durant els períodes glacials i connectats durant els períodes interglacials, possiblement unes quantes vegades (Rögl, 1998; Taviani, 2002; Duggen *et al*., 2003). Això pot haver originat processos d'especiació al·lopàtrida entre les diferents zones (e.g. Borsa *et al*., 1997; Bargelloni *et al*., 2003). D'altra banda encara suposant que la connexió entre l'Atlàntic i el Mediterrani no s'hagi interromput durant els períodes glacials, la temperatura de l'aigua del Mediterrani ha estat més alta que la de les zones atlàntiques adjacents durant els períodes glacials (Thiede, 1978). Així moltes espècies presents en les zones càlides i temperades de l'Atlàntic han pogut sobreviure durant els períodes glacials dins del Mediterrani, recolonitzant l'Atlàntic durant els períodes interglacials amb temperatures més favorables (Almada *et al*., 2001).

Actualment ens trobem en un període interglacial i conseqüentment l'Atlàntic i el Mediterrani estan connectats. Tot i això, existeixen una sèrie de barreres hidrogràfiques que poden afectar la distribució de les espècies i la connexió entre les seves poblacions. Una de les barreres més estudiades és el front Almeria-Oran (AOF, Tintoré *et al*., 1988). Aquest front està associat amb un fort salt de temperatura

(1.4ºC) i salinitat (2 psu) en només 2 km de distància horitzontal i amb corrents d'uns 40 cm/s de direcció SE, de la costa espanyola cap al nord d'Àfrica (Tintoré *et al*., 1988). Molts estudis amb diferents organismes constaten un cert aïllament entre les dues regions; en mol·luscs (*Mytilus galloprovincialis*, Quesada *et al*., 1995, Sanjuan *et al*., 1996; *Sepia officinalis*, Pérez-Losada *et al*., 2002), crustacis (*Meganyctiphanes norvegica*, Zane *et al*., 2000) i peixos (*Dicentrarchus labrax*, Nacri *et al*., 1999, Lemaire *et al*., 2005; *Sparus aurata*, De Innocentiis *et al*., 2004; *Diplodus puntazzo*, Bargelloni *et al*., 2005) es troba una clara diferenciació entre poblacions atlàntiques i mediterrànies relacionada amb aquest front.

D'aquesta manera, la història geològica de la conca mediterrània, juntament amb l'anàlisi de determinades seqüències de DNA, tant mitocondrial com nuclear, ofereixen una oportunitat única per estudiar i entendre els processos de colonització, evolució, especiació i adaptació local que han tingut i tenen lloc en la majoria d'espècies mediterrànies de peixos.

1.2.- Estructura poblacional, autoreclutament i dispersió larvària

Aparentment, en el mar hom pot pensar que no hi ha barreres de cap mena, i que totes les poblacions de qualsevol espècies estan relativament interconnectades. D'aquesta manera, es creia que si degut a la pesca intensiva, a la construcció d'alguna infrastructura o a la destrucció d'una zona, les espècies presents en una determinada zona es veiessin afectades, aquestes es recuperarien de forma relativament ràpida per l'aportació d'individus de les zones veïnes més properes no afectades.

En el mar, la connexió entre poblacions és deguda principalment al moviment dels adults o a la dispersió de les larves en el plàncton (Palumbi, 2003). Aproximadament un 70% dels organismes marins tenen una fase planctònica, en la qual les larves poden dispersar-se, abans d'assentar-se en el que serà el seu hàbitat d'adult (Thorson, 1950). Sembla haver una marcada relació entre l'estructura poblacional d'una espècie i la seva capacitat de dispersió. Aquelles espècies que presenten una elevada mobilitat dels adults o que les larves passen un llarg període en el plàncton, poden tenir un major intercanvi d'individus entre poblacions (Broughton & Gold, 1997); en

canvi, les espècies amb larves amb un temps de vida planctònica reduït o amb adults poc mòbils mostraran una estructura poblacional molt evident (Doherty *et al*., 1995; Riginos & Victor, 2001). Encara hi ha pocs estudis d'aquesta mena, i no hi ha consens general sobre la importància de les barreres naturals i de la capacitat de dispersió en la configuració de l'estructura poblacional de les espècies (Bernardi *et al*., 2003; Taylor & Hellberg, 2003; Macpherson & Raventós, 2006). Un paràmetre essencial per tal d'estimar la connectivitat entre poblacions és la distància genètica, que està relacionada amb la taxa d'autoreclutament d'una determinada espècies en una població. Tot i que l'estima d'aquesta taxa té conseqüències importants en la biologia de la conservació de les espècies marines (Swearer *et al*., 2002; Thorrold *et al*., 2002), pocs treballs s'han dut a terme per tal de calcular-la en diferents espècies de peixos marins, i cap en el Mediterrani (Jones *et al*., 1999; Swearer *et al*., 1999; Thorrold *et al*., 2001; Miller & Shanks, 2004; Patterson *et al*., 2005; Jones *et al*., 2005).

Així doncs, un coneixement de la connectivitat entre poblacions, mitjançant marcadors moleculars i variables hidrogràfiques i geogràfiques, és fonamental per una adequada gestió dels recursos i per un adequat disseny de reserves marines que tinguin en compte els requeriments ecològics de cada espècie (Palumbi, 2003; Bell & Okamura, 2005).

1.3.- Les espècies escollides

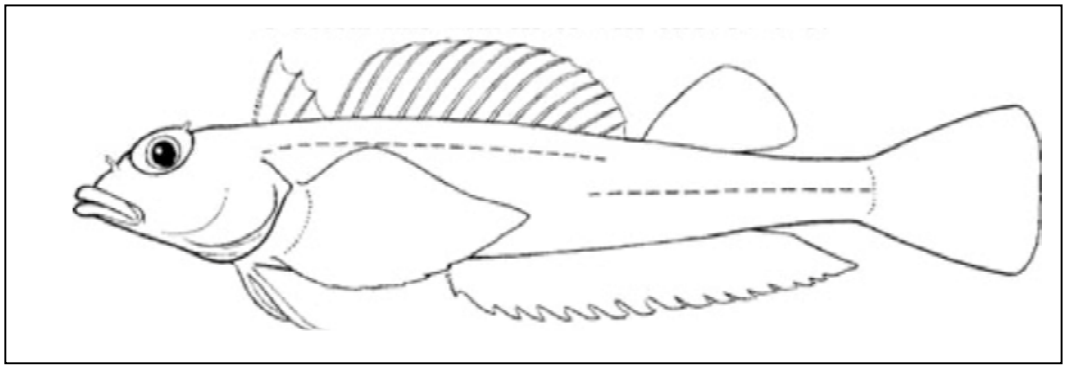
S'han seleccionat dues espècies litorals: *Tripterygion delaisi* Cadenat & Blache, 1971 i *Serranus cabrilla* L., amb una distribució geogràfica similar habitant el Mediterrani i l'Atlàntic est. Els adults són molt territorials, i no presenten moviments migratoris (Heymer, 1977; García-Rubies, 1999). Els ous de *S. cabrilla* són pelàgics mentre que els de *T. delaisi* són bentònics. A més, les larves de *S. cabrilla* estan entre 21 i 28 dies al plàncton en front dels 16-21 dies de les larves de *T. delaisi* (Raventós & Macpherson, 2001). Finalment, les larves de *T. delaisi* mostren una gran retenció no allunyant-se més de 100 metres de la línia de costa; al contrari de les larves de *S. cabrilla* que s'han trobat a l'altura del marge continental, a considerable distància de l'habitat dels adults (Sabatés *et al*., 2003). Així doncs, s'han seleccionat dues espècies de distribució i hàbitat similar, i amb una mobilitat de l'adult semblant; però

amb una capacitat potencial de dispersió larvària (CDL) teòricament molt diferent, de forma que constitueixen dos bons models per veure si la CDL té relació amb l'estructura poblacional de les espècies.

1.3.1.- Tripterygion delaisi

La família dels Tripterígids (Classe Osteïctis; Ordre Perciformes; Subordre Blennioidei) presenta 28 gèneres i 103 espècies, agrupats en dues tribus, la tribu Lepidoblenninae amb 9 gèneres i 31 espècies, i la tribu Tripterygiinae amb 19 gèneres i 72 espècies (Stepien *et al*., 1997). Com a característiques generals de la família, es pot dir que presenten una talla màxima d'uns 90 mm i són exclusivament bentònics, tenen un morro punxegut, el cos recobert d'escates ctenoidees (excepte el cap i el ventre) i presenten 3 aletes dorsals clarament separades (Zander, 1986) (Figura 1).

Figura 1. Dibuix representatiu de la família Tripterygiidae.



El gènere *Tripterygion* Risso, 1826, que pertany a la tribu Tripterygiinae, és l'únic representant de la família present al Mediterrani i a la costa nord-est atlàntica, i consta de 3 espècies; *T. delaisi* Cadenat & Blache, 1971, *T. melanurus* Guichenot, 1845 i *T. tripteronotus* Risso, 1810 (aquestes dues últimes endèmiques del Mediterrani). En *T. melanurus* s'han descrit dues subspècies: *T. m. melanurus* que habita les costes sud mediterrànies i té una taca negra molt visible en el peduncle caudal, absent en *T. m. minor* Kolombatovic, 1892, la distribució del qual sembla ser per la costa nord del Mediterrani (Zander, 1986) (Figura 2).

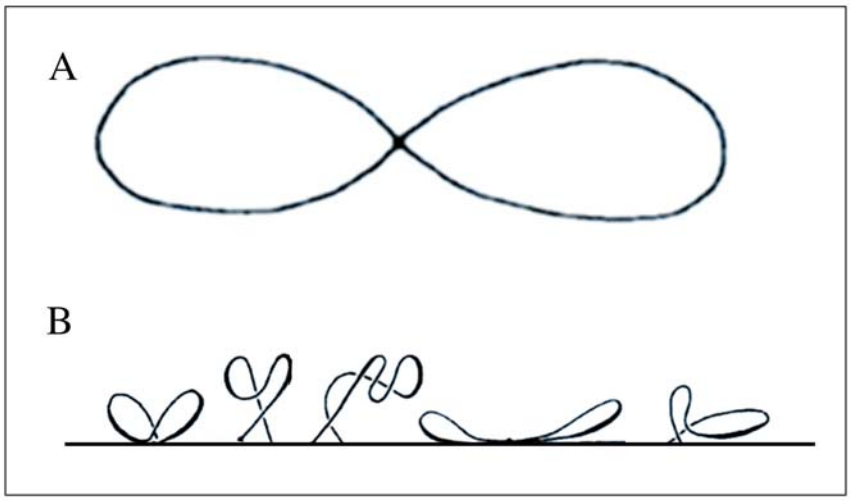
Figura 2. Espècies del gènere *Tripterygion*.

(A1): *T. delaisi* mascle en època de reproducció, (A2): *T. delaisi* femella o mascle no actiu, (B1): *T. tripteronotus* mascle en època de reproducció, (B2): *T. tripteronotus* femella o mascle no actiu, (C1): *T. m. melanurus*, (C2): *T. m. minor*.

A *Tripterygion delaisi* també s'han descrit dues subspècies: *T. d. xanthosoma* Zander & Heymer, 1976 s'estén per tot el Mediterrani i *T. d. delaisi* Cadenat & Blache, 1971 habita la costa atlàntica des del sud d'Anglaterra fins a Senegal, Madeira, Açores i Canàries (Zander, 1986); les diferències morfològiques entre les dues subspècies, mesurant diferents caràcters morfològics, són marginals i només són significatives comparant un gran nombre d'individus (Wirtz, 1980). Es pot dir, per tant, que morfològicament són indistingibles i únicament es diferencien en el fet que el mascle de la subspècie atlàntica realitza el ball nupcial en forma de 8 nedant perpendicularment respecte del fons, mentre que la subspècie mediterrània realitza la

mateixa figura però ho fa nedant paral·lelament sobre el fons (Wirtz, 1978) (Figura 3).

Figura 3. Esquema dels balls nupcial dels mascles de *T. d. xanthosoma* (A, vista en planta) i *T. d. delaisi* (B, vista lateral). Tret de Wirtz (1978)



T. delaisi és un del peixos d'aigües costaneres someres més comuns dins de la seva àrea de distribució. Es troba en un rang de profunditats que oscil·la entre 3 i 40 m, però presenta les densitats més elevades entre 6 i 12 m. Es troba en la majoria de substrats durs però prefereix les zones de grans blocs, extraploms i entrades de coves (zones relativament esciòfiles). Les dues subspècies també presenten diferències pel que fa el seu nínxol ecològic: *T. d. delaisi*, a l'Atlàntic, es troba freqüentment en zones que reben del 10% al 100% de la llum entrant i els mascles estableixen els seus territoris en zones altament fotòfiles però sempre a profunditats superiors a la de les marees baixes. D'altra banda, *T. d. xanthosoma*, en el Mediterrani, presenta un nínxol molt més reduït, i se situa preferentment en zones que reben del 1% al 10% de la llum entrant, això es deu a que aquí cohabita amb dues altres espècies del mateix gènere i rivalitza amb elles per l'espai. *T. tripteronotus* ocupa la zona superior, que rep entre un 10% i un 100% de la llum entrant, mentre que *T. melanurus* ocupa les zones més esciòfiles en les quals la llum entrant no supera l'1% (Wirtz, 1980). Malgrat aquesta diferenciació en l'espai es poden trobar individus de les tres espècies diferents cohabitant simpàtridament (Wirtz, 1978; observació personal).

Presenten sexes separats i durant el seu període reproductiu (d'abril a juliol) existeix dimorfisme sexual. Els mascles reproductors són territorials i presenten les aletes pèlviques, els primers radis de la primera aleta dorsal i tot el cap fins el punt d'inserció de les aletes pectorals negres, i tota la resta del cos d'un color groc molt vistós. A més, els primers radis de la segona dorsal s'allarguen extraordinàriament i agafen una tonalitat blavosa. D'altra banda les femelles i els mascles no territorials presenten una coloració críptica que mantenen tot l'any, són d'un color marronós clar amb 5 bandes transversals més fosques, l'última banda es situa a la base del peduncle caudal i s'estén cap a la base dels radis de l'aleta caudal (Zander, 1986; De Jonge & Videler, 1989).

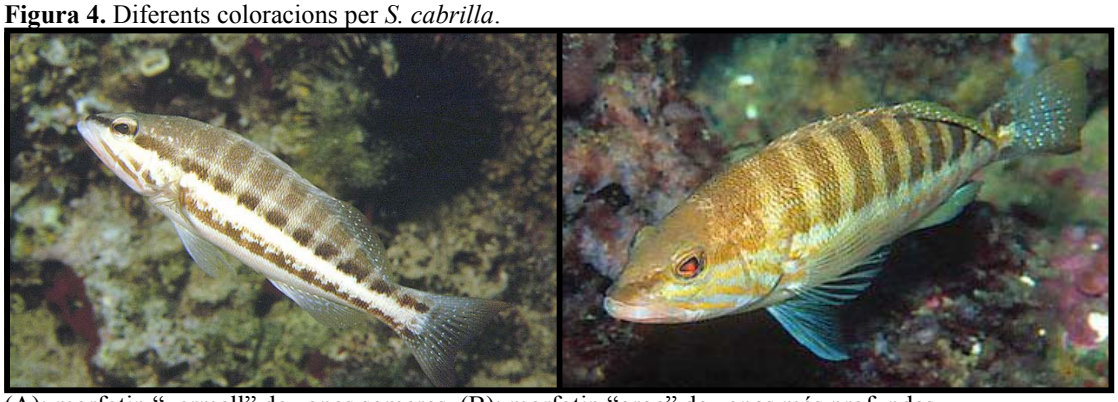
Cada mascle cuida i vigila un niu, d'uns 20 x 20 cm, situat en una zona relativament esciòfila, amb algues baixes (0.2 - 0.6 cm) i amb presència d'esponges incrustants. Les femelles són atretes cap al niu pel mascle reproductor degut a la seva coloració i al ball nupcial, un cop allà les femelles deposen els ous sobre el substrat (on queden fixats fins l'eclosió) i el mascle els fecunda. Així, un mascle pot fecundar ous de varies femelles. El mascle defensa les postes de les femelles que ell ha fecundat fins que els ous eclosionen (aproximadament entre 15 i 20 dies; T_{aigua} = 19ºC) i les larves s'alliberen al plàncton on hi estan entre 16 i 21 dies (Wirtz, 1980; De Jonge & Videler, 1989; Raventós & Macpherson, 2001).

1.3.2.- Serranus cabrilla

Els serrànids (Classe Osteïctis; Ordre Perciformes; Subordre Percoidei) són propis de mars tropicals o temperats i estan representats per més de 40 gèneres i unes 320 espècies. En el Mediterrani, la majoria dels serrànids són econòmicament importants i estan catalogats, segons la FAO, com espècies d'interès pesquer (Smith, 1981; Bauchot, 1987). Hi ha 14 espècies englobades en 6 gèneres dins de la família Serranidae en el Mediterrani: *Serranus* (*S. cabrilla*, *S. scriba*, *S. hepatus* i *S. atricauda*), *Epinephelus* (*E. marginatus*, *E. costae*, *E. caninus*, *E. anaeus*, *E. haifensis* i *E. malabaricus*), *Mycteroperca rubra*, *Polyprion americanus*, *Anthias anthias*, *Callanthias ruber*. Tots són de cos robust i una mica comprimit amb un cap gran, tot i que la seva mida oscil·la entre els 10 cm i els 1.5 m de longitud (Tortonese, 1986).

Serranus cabrilla es troba a tot el Mediterrani i a la costa atlàntica des del Canal de la Mànega fins a Sudàfrica. També es pot trobar ocasionalment al mar del Nord i com a migrador lessepsià també es troba al mar Roig. Li han estat descrites dues coloracions diferents, sense patrons intermedis, en funció de l'hàbitat en el que es troben. En les zones més someres trobem els exemplars de mida més petita i amb tonalitat vermellosa; en canvi els individus més grans i amb la tonalitat groguenca els trobem a les zones més profundes (Figura 4). Ambdues coloracions han estat considerades com variacions fenotípiques, relacionades amb l'edat o l'habitat (Cuvier & Valenciennes, 1828) o com subspècies (Dufossé, 1856; Dieuzède *et al*., 1954). Entre els individus amb diferents coloracions també s'han trobat variacions en el període reproductiu (Bruslé & Bruslé, 1975), en els paràsits que hostatgen (Oliver *et al*., 1980), en la proteïna del cristal·lí (Oliver *et al*., 1987) i en la proporció dels pigments de tunaxantina (Victor-Baptiste, 1980). D'altra banda, un estudi recent de Medioni *et al.* (2001) conclou que no hi ha diferències genètiques entre coloracions, i proposa que els individus amb coloració groguenca tenen més edat i provenen dels individus vermellosos més joves de les zones més someres.

Figura 4. Diferents coloracions per *S. cabrilla*.



(A): morfotip "vermell" de zones someres, (B): morfotip "groc" de zones més profundes

És una espècie demersal típicament litoral que habita tot tipus de fons (zones de *Posidonia oceanica*, altres fanerògames i algues, roques, fons de sorra i fang...) des de superfície fins als 500m. Comparteix hàbitat amb el seu congènere *S. scriba*, aquest però, té una distribució molt més superficial. Així les densitats màximes de *S. cabrilla* es troben en fons de coral·ligen entre 30 i 60m de fondària segons la transparència de l'aigua (Alcover *et al*., 1993).

El seu període reproductiu va d'abril a juliol en el Mediterrani i de juliol a agost a la zona del canal de la Mànega. Al igual que les altres espècies del gènere *Serranus* es tracta d'un dels pocs vertebrats hermafrodites simultanis o sincrònics, és a dir, que durant el període de posta poden actuar tant de mascles com de femelles. No adopten cap coloració especial durant aquest període i les seves pautes de reproducció no han estat descrites, encara que es creu que són molt similars a les del *S. scriba*, alliberant-se els ous a la columna d'aigua després d'una parada nupcial bastant complexa (Corbera *et al*., 1996). Els ous, però, són pelàgics i es troben en el plàncton superficial, no es coneix quan de temps triguen els ous a eclosionar però se suposa similar al d'altres serrànids; per *Epinephelus marginatus* (espècie de la mateixa família), els ous eclosionen entre el primer i el tercer dia després de la posta (Zabala, comunicació personal). Les larves de *S. cabrilla* estan entre 21 i 28 dies al plàncton (Raventós & Macpherson, 2001).

1.4.- Els marcadors utilitzats

A l'hora de plantejar-se resoldre qualsevol qüestió, el primer pas (i fonamental) és la correcta elecció del marcador a utilitzar. Per això s'han de tenir clars quins són els objectius a assolir per tal d'utilitzar en cada cas el marcador molecular més adient per a respondre les preguntes formulades.

1.4.1.- Seqüències d'ADN mitocondrial

L'ADNmt té varies característiques remarcables que fan que sigui un dels marcadors moleculars més utilitzats en estudis tant d'evolució com de fil·logeografia (Avise, 2000b; Féral, 2002). L'ADNmt citoplasmàtic presenta un elevat nombre de còpies, això i el fet que hi ha dissenyats una sèrie d'encebadors universals (Kocher *et al*., 1989; Palumbi *et al*., 1991; Folmer *et al*., 1994) fan que l'amplificació de fragments d'ADNmt utilitzant la PCR sigui molt fàcil i que es pugui dur a terme en la majoria de phila (Féral, 2002).

En la gran majoria d'eucariotes l'ADNmt té una herència materna (Birky, 1995), amb molt poques excepcions (*Mytilus galloprovincialis*, Zouros *et al*., 1994; *Musa*

acuminata, Faure *et al*., 1994), de totes maneres segons Hurst & Hoekstra (1994) aquestes excepcions no transgredeixen la regla general de la transmissió uniparental. Degut a aquest mecanisme de transmissió de l'ADNmt, aquest no presenta fenòmens de recombinació en animals, òbviament també existeixen algunes excepcions (*Mytilus galloprovincialis*, Ladoukakis & Zouros, 2001; *Platichthys flesus*, Hoarau *et al*., 2002).

Una altra característica important d'aquest tipus de seqüències és la seva ràpida taxa d'evolució (Brown *et al*., 1979), sobretot si es compara amb la de les seqüències nuclears (Avise, 1994). Una possible explicació per aquest fet pot ser la reduïda mida efectiva enfront dels gens nuclears, ja que els gens mitocondrials es transmeten únicament per herència materna en la majoria d'eucariotes (Birky, 1995). També pot influir el procés de fixació per selecció d'algun gen, arrossegant tots els altres degut a la manca de recombinació. D'altra banda, el que no hi hagi recombinació permet aplicar correctament la teoria de la coalescència ja que les relacions entre els haplotips són directes (Posada & Crandall, 2001). En la majoria d'espècies no hi ha heteroplàsmia (excepcions in Rokas *et al*., 2003), cada individu presenta un sol haplotip, i els gens mitocondrials són de còpia única, això fa que siguin de seqüenciació directa i per tant fàcilment analitzables.

Les remarcables característiques de l'ADNmt en combinació amb les facilitats i prestacions de la PCR, han potenciat les anàlisis evolutives i filogeogràfiques utilitzant aquests marcadors moleculars, permetent l'estudi de qualsevol metazou d'una forma ràpida i efectiva tant a nivell filogenètic com d'estructura poblacional a gran escala (Zhang & Hewitt, 1996; Féral, 2002). L'ADNmt ha estat tradicionalment utilitzat com una eina genealògica, permetent esclarir les relacions existents entre espècies a diferents nivells taxonòmics (Arnason *et al*., 1991; Apostolidis *et al*., 2001). Les seves característiques també han permès utilitzar aquest marcador en nombrosos estudis filogeogràfics. Segons Avise (2000b), un 70% dels estudis filogeogràfics han estat realitzats utilitzant l'ADNmt com a font d'informació. Encara que en alguns organismes la baixa variabilitat intraespecífica fa que no sigui gaire informatiu sobre l'estructura filogeogràfica (Duran *et al*., 2004)

1.4.2.- Microsatèl·lits

En els últims deu anys els microsatèl·lits (VNTR: variable number of tandem repeats, SSLP: simple sequence length polymorphism o SSR: single sequence repeats) s'han convertit en un dels marcadors moleculars més utilitzats en estudis poblacionals, desplaçant a la resta de marcadors en la majoria d'estudis genètics (DeWoody & Avise, 2000; Hutchinson *et al*., 2001), degut a que presenten elevats nivells de polimorfisme, són codominants, neutres, només es necessita una petita quantitat de teixit per realitzar les anàlisis, són fàcilment analitzables... (Jarne & Lagoda, 1996).

Els microsatèl·lits són seqüències d'ADN consistents en repeticions en tàndem d'1 a 6 bp (Queller *et al*., 1993). Aquestes seqüències estan distribuïdes a través del genoma de tots els eucariotes analitzats i també en el genoma cloroplàstic de plantes (Jarne & Lagoda, 1996), però gairebé sempre situades en regions no codificants, ja que el guany o la pèrdua de les repeticions provocaria canvis en la pauta de lectura (Hancock, 1999). Així doncs són considerats marcadors neutres, i no estan afectats ni per selecció ni per pressió ambiental. Malgrat això, els microsatèl·lits poden estar lligats a gens o regions que estiguin sotmeses a selecció i això pot fer que no siguin estrictament neutres (Estoup & Angers, 1998; Kashi & Soller, 1999). La seva densitat depèn de l'espècie i solen presentar elevats nivells de polimorfisme al·lèlic, ja que tenen una taxa de mutació molt elevada (probablement deguda al "slippage" durant la replicació o a entrecreuament desigual) (Bruford & Wayne, 1993; Eisen, 1999; Féral, 2002). Els microsatèl·lits més freqüents són els dinucleòtids (Kruglyak *et al*., 1998), i en aquests la classe més abundant és la $(CA)_n$, seguida de $(AT)_n$, $(GA)_n$ i finalment $(GC)_n$, malgrat això a les genoteques realitzades per a aïllar aquests loci s'ha vist que la freqüència de $(AT)_n$ i $(GC)_n$ és molt baixa, o fins i tot nul·la, probablement per culpa del biaix introduït per la autocomplementarietat d'aquestes sondes (Schug *et al*., 1998).

Els microsatèl·lits poden ser classificats segons la naturalesa de les repeticions que presenten en: perfectes, quan estan formats per un grup ininterromput de repeticions; imperfectes, formats per un grup de repeticions interromput per una o varies bases; i composts, quan dos o més grups de repeticions perfectes estan units entre si (Weber, 1990).

Estan considerats com uns bons marcadors moleculars, mendelians i neutres, essent un dels més indicats (sinó el que més) per estudiar la variabilitat i estructura de les espècies des d'un rang biogeogràfic a un nivell intrapoblacional o local (Ellegren, 1991). Segons Estoup (1998), els microsatèl·lits permeten detectar diferències genètiques allà on altres marcadors assumirien uniformitat. La majoria de les seves aplicacions es basen en la diferenciació i estructura inter i intrapoblacional, podent calcular la mida efectiva de les poblacions i el seu nivell de consanguinitat (Edwards *et al*., 1992). També es poden dur a terme estudis de variabilitat temporal en una mateixa població, tant a curt termini com utilitzant registres històrics, ja que aquests marcadors junt amb la PCR permeten amplificar fragments d'ADN a partir de molt petites quantitats de material i de mostres parcialment degradades (Schlötterer, 2000; Ellegren, 1991). En estudis intrapoblacionals i utilitzant uns 10 - 20 microsatèl·lits són perfectament viables estudis de parentiu que impliquin tests de paternitat (Queller *et al*., 1993; Estoup & Angers, 1998). D'aquesta manera també són àmpliament utilitzats en la majoria d'estudis forenses (Budowle *et al*., 1991) i de mapatge genètic (Weissenbach, 1992; Hearne *et al*., 1992).

Evidentment, també presenten problemes. Potser el principal apareix de bon començament degut a que s'han d'aïllar *de novo* per a la majoria d'espècies que són analitzades per primer cop (Zane *et al*., 2002), ja que al trobar-se en regions no codificadores, les seves regions flanquejants estan generalment poc conservades. Aquest és un procés lent i costós que actua com a obstacle a l'hora de decidir-se a utilitzar aquest marcador molecular (Primmer *et al*., 1996; Steinkellner *et al*., 1997). Així doncs una possible solució a aquest pas inicial és la utilització d'encebadors dissenyats per loci microsatèl·lits aïllats en espècies properes a la que es vol analitzar. Malauradament, l'èxit de l'amplificació de loci microsatèl·lits en una espècie utilitzant encebadors dissenyats per una altra espècie (el que en anglès es coneix com cross-species amplification, CSA) pot no ser gaire alt (Guillemaud *et al*., 2000). De la mateixa manera hom haurà de veure si els loci microsatèl·lits que amplifiquen en l'espècie objectiu són polimòrfics, i en quin grau, ja que sinó no seran útils per a cap dels estudis a plantejar (Primmer *et al*., 1996).

En analitzar les dades és important escollir un model mutacional que s'ajusti correctament a la variació dels loci microsatèl·lits per tal d'estimar de la forma més acurada possible mides i estructura poblacional (Estoup *et al*., 1995; O'Connell & Wright, 1997; Pascual *et al*., 2001). Malgrat s'han proposat múltiples models (Estoup *et al*., 2002; Li *et al*., 2002), tres són els utilitzats actualment de forma majoritària. L'IAM (infinite allele model, Kimura & Crow, 1964), el SMM (stepwise mutation model, Kimura & Ohta, 1978) i el TPM (two-phase model, DiRienzo *et al*., 1994). Segons l'IAM la mutació sempre dóna nous al·lels en la població i pot originar microsatèl·lits amb qualsevol nombre de repeticions; en canvi el SMM suposa que la mutació només es dóna perdent o guanyant una repetició i conseqüentment es poden donar al·lels ja presents en la població. Finalment, segons el TPM la mutació modifica la mida de l'al·lel en una repetició amb una probabilitat aproximada, P, encara que poden aparèixer al·lels nous que es diferencien en més d'una repetició amb una probabilitat 1-P.

El fenomen de l'homoplàsia es produeix quan hi ha una semblança estructural deguda a un paral·lelisme o a una evolució convergent, i no pas a un ancestre comú. En el cas dels microsatèl·lits, l'homoplàsia es produeix quan al·lels d'un mateix locus tenen mides iguals (Identical By State) però no són idèntics per ascendència (Identical By Descent). D'aquesta manera, dos al·lels, amb orígens diferents és considerarien iguals, fent disminuir la variabilitat del locus. Segons Estoup *et al*. (2002), però, l'homoplàsia dins d'una determinada espècie no sembla ser un problema important per la majoria de les anàlisis d'estructura poblacional, degut a què la gran quantitat de variabilitat observada pels loci microsatèl·lits compensa amb escreix, la seva evolució per homoplàsia. D'altra banda, quan s'utilitzen marcadors moleculars amb una elevada taxa de mutació entre diferents subspècies, l'homoplàsia podria ser present (Estoup *et al*., 1995).

En conclusió, els microsatèl·lits permeten analitzar l'estructura poblacional de les espècies i processos a nivell intrapoblacional degut a la seva elevada variabilitat (e.g. Burford & Wayne, 1993; O'Connell & Wright, 1997; Rico & Turner, 2002). D'altra banda, les seqüències de DNA permeten inferir les relacions filogenètiques entre diferents espècies, així com realitzar estudis de filogeografia (Avise, 1992, 2000b; Avise *et al*., 1987).

2.- Objectius

Aquesta tesi està centrada en conèixer la filogènia i l'estructura poblacional de dues espècies de peixos marins (*Tripterygion delaisi* i *Serranus cabrilla*) en el Mediterrani occidental. Les espècies escollides presenten una distribució i comportament de l'adult semblant (elevada territorialitat i escassa dispersió) però amb comportaments de les larves diferents (diferent capacitat de dispersió).

Per tal de situar ambdues espècies en la zona d'estudi i amb l'objectiu de reconstruir millor el context històric que les ha originat, i com les ha originat, s'ha realitzat una filogènia molecular, utilitzant diferents gens mitocondrials i nuclears. Així doncs, dues filogènies, una pels tripterígids i una altra pels serrànids que habiten el Mediterrani i la seva zona d'influència, han estat inferides. S'han utilitzat diferents mètodes de reconstrucció filogenètica i tots els gens han estat analitzats per separat i conjuntament. Una anàlisi d'aquest tipus ofereix una informació valuosa sobre la millor manera de tractar les dades quan es realitzen inferències filogenètiques.

També, s'ha donat un caire filogeogràfic a aquestes filogènies, utilitzant mostres de la mateixa espècie de diferents i distants llocs de la zona d'estudi, amb l'objectiu de definir, amb la màxima precisió, la distribució de les espècies així com poder detectar espècies críptiques i subspècies que poguessin donar problemes a l'hora de fer l'anàlisi de l'estructura poblacional.

Per tal d'obtenir l'estructura poblacional representativa de cada espècie i procedir a la posterior comparació s'han escollit uns marcadors moleculars altament variables: els microsatèl·lits. Per tal d'obtenir un nombre suficient d'aquests marcadors s'han realitzat dues genoteques enriquides (una per a cada espècie).

Un dels altres paràmetres importants que s'ha estimat ha estat el nivell d'autoreclutament d'una determinada població. L'estructura poblacional està fortament influenciada per l'autoreclutament. Els nivells d'autoreclutament s'han pogut estimar comparant els individus recent assentats (reclutes) amb els adults reproductors de la mateixa població i els de les altres poblacions adjacents. Aquest

estudi només s'ha realitzat per *T. delaisi*, degut a les dificultat de mostreig dels reclutes de *S. cabrilla*.

Finalment, per tal d'il·lustrar un dels principals problemes de la utilització dels microsatèl·lits com a marcador moleculars i predir l'èxit en la utilització d'encebadors dissenyats per una espècie en altres espècies (encara que siguin properes), s'ha establert la relació existent entre la divergència genètica (utilitzant dos gens mitocondrials) i l'èxit en l'amplificació de loci polimòrfics, entre l'espècie per la qual els encebadors han estat dissenyats (*T. delaisi* i *S. cabrilla*) i les espècies en les que han estat provats.

Així doncs, la tesi queda estructurada en els següents apartats:

- *Filogènia i especiació*

  Filogènia-filogeografia del gènere *Tripterygion* utilitzant 4 gens mitocondrials (12S, 16S, tRNA-valina i COI) i un nuclear (18S). Utilització de diferents mètodes d'inferència filogenètica i comparació entre ells. Anàlisi filogenètica de cada gen per separat i tots conjuntament. Redefinició de les espècies i subspècies del gènere *Tripterygion*, així com de les seves àrees de distribució. Descripció molecular i morfològica d'una nova espècie de tripterígid mediterrani: *T. tartessicum*, espècie críptica de *T. tripteronotus*. Definició de les seves àrees de distribució en el Mediterrani i hipòtesis sobre la seva aparició.

  Publicació 1: **Rapid radiation and cryptic speciation in Mediterranean triplefin blennies (Pisces: Tripterygiidae) combining multiple genes**

  Publicació 2: **Review of the *Tripterygion tripteronotus* (Risso, 1810) complex, with description of a new species from the Mediterranean Sea (Teleostei: Tripterygiidae)**

- *Aïllament de loci microsatèl·lits i les seves aplicacions*

Aïllament i caracterització dels loci microsatèl·lits per *T. delaisi* i *S. cabrilla*. I utilització dels encebadors dissenyats per *S. cabrilla* en les altres espècies de serrànids mediterranis obtenint la relació: divergència genètica respecte *S. cabrilla* (12S i 16S) *vs*. èxit d'amplificació i polimorfisme dels loci microsatèl·lits en les altres espècies de serrànids. Generalització d'aquesta relació en peixos obtenint totes les dades possibles de les bases de dades i d'altres articles. Filogènia-filogeografia dels serrànids mediterranis i de la seva àrea d'influència, utilitzant 4 gens mitocondrials (12S, 16S, tRNA-valina i COI).

Publicació 3: **Isolation and Characterization of microsatellite loci in *Tripterygion delaisi***

Publicació 4: **Characterization of twelve microsatellite markers in *Serranus cabrilla* (Pisces: Serranidae)**

Publicació 5: **Genetic divergence used to predict microsatellite cross-species amplification and maintenance of polymorphism in fishes**

- *Estructura poblacional, autoreclutament i dispersió larvària*

Estructuració poblacional, utilitzant microsatèl·lits, de *T. delaisi*, incloent les seves dues subspècies (*T. d. delaisi* a l'Atlàntic i *T. d. xanthosoma* al Mediterrani). Posteriorment, amb les poblacions ja definides, estima del grau d'autoreclutament en una població mediterrània de *T. delaisi* utilitzant microsatèl·lits com a marcadors moleculars entre els anys 2003 i 2005.

Estructura poblacional de *S. cabrilla* en el Mediterrani utilitzant microsatèl·lits i comparació amb l'estructura obtinguda per *T. delaisi* en el Mediterrani.

Publicació 6: **Population structure within and between subspecies of the Mediterranean triplefin fish *Tripterygion delaisi* revealed by highly polymorphic microsatellite loci**

Publicació 7: **High self-recruitment levels in a Mediterranean littoral fish population revealed by microsatellite markers**

Publicació 8: **Early life-history characteristics predict genetic differentiation in Mediterranean fishes**

- *Resum*

Resum global dels resultats obtinguts i de la discussió d'aquests.

- *Conclusions*

Conclusions finals que es desprenen d'aquest treball.

3.- Publicacions

3.1.- Filogènia i especiació

Publicació 1: Rapid radiation and cryptic speciation in Mediterranean triplefin blennies (Pisces: Tripterygiidae) combining multiple genes

Publicació 2: Review of the *Tripterygion tripteronotus* (Risso, 1810) complex, with description of a new species from the Mediterranean Sea (Teleostei: Tripterygiidae)

3.2.- Microsatèl·lits: aïllament i aplicabilitats

Publicació 3: Isolation and characterization of microsatellite loci in *Tripterygion delaisi*

Publicació 4: Characterization of 12 microsatellite markers in *Serranus cabrilla* (Pisces: Serranidae)

Publicació 5: Genetic divergence used to predict microsatellite cross-species amplification and maintenance of polymorphism in fishes

3.3.- Estructura poblacional, autoreclutament i dispersió larvària

Publicació 6: Population structure within and between subspecies of the Mediterranean triplefin fish *Tripterygion delaisi* revealed by highly polymorphic microsatellite loci

Publicació 7: High self-recruitment levels in a Mediterranean littoral fish population revealed by microsatellite markers

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Rapid radiation and cryptic speciation in mediterranean triplefin blennies (Pisces: Tripterygiidae) combining multiple genes

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Abstract

The genus *Tripterygion* is the unique genus of the family Tripterygiidae in the Mediterranean Sea and in the northeastern Atlantic coast. Three species and four subspecies had been described: *Tripterygion tripteronotus* and *Tripterygion melanurus* (*T. m. melanurus* and *T. m. minor*) are endemic of the Mediterranean, and *T. delaisi* (*T. d. delaisi* and *T. d. xanthosoma*) is found in both areas. We used five different genes (12S, 16S, tRNA-val, COI, and 18S) to elucidate their taxonomy status and their phylogenetic relationships. We employed different phylogenetic reconstructions that yielded different tree topologies. This discrepancy may be caused by the speciation process making difficult the reconstruction of a highly supported tree. All pair comparisons between these three species showed the same genetic divergence indicating that the speciation process could have been resolved by a rapid radiation event after the Messinian Salinity Crisis (5.2 Mya) leading to a trichotomy. Our molecular data revealed two clearly supported clades within *T. tripteronotus*, whose divergence largely exceeded that found between other fish species, consequently these two groups should be considered two cryptic species diverging 2.75–3.32 Mya along the Pliocene glaciations. On the contrary, none of the genes studied supported the existence of two subspecies of *T. melanurus*. Finally, the two subspecies of *T. delaisi* were validated and probably originated during the Quaternary climatic fluctuations (1.10–1.23 Mya), however their distribution ranges should be redefined.

Keywords: *Tripterygion*; Speciation; Rapid radiation; Cryptic species; Phylogeography; Multiple genes; Phylogeny

1. Introduction

The use of molecular tools to infer phylogenies has increased enormously in the last two decades. Molecular phylogenies are sometimes inferred by only one gene and in many cases this gene gives a robust tree topology with high bootstrap values (Allegrucci et al., 1999; Ballard et al., 1992). However, significantly different tree topologies can be obtained using different genes (Cristescu and Hebert, 2002; Mattern, 2004), therefore in order to get a closer approximation to the real phylogeny, relationships should be inferred from multiple molecular markers (Crow et al., 2004). Furthermore, different evolutionary rates have been described among genes, thus combining multiple genes may help to resolve the older and younger nodes in a phylogenetic tree (Apostolidis et al., 2001; Lin and Danforth, 2004). Nonetheless, some inconclusive results may arise from the use of several markers pointing to radiation events as responsible for the speciation processes. The marine fauna endemic of the Mediterranean Sea offers the opportunity to detect such events since the refilling of the Mediterranean basin after the Messinian Salinity Crisis, MSC, 5.2 Mya (Hsü et al., 1977) gives the baseline for contrasting this hypothesis.

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The genus *Tripterygion* Risso, 1826, is the only genus of the family Tripterygiidae in the Mediterranean Sea and northeastern Atlantic coast (Zander, 1986). Three species have been described: *Tripterygion tripteronotus*, Risso, 1810, and *Tripterygion melanurus* Guichenot, 1845, are endemic of the Mediterranean although they are also found in Atlantic waters near to the Gibraltar Straight (Zander, 1986), and *T. delaisi* Cadenat and Blache, 1971; is found in both areas (Wirtz, 1980). Two morphotypes nowadays considered two different subspecies (Zander, 1986) have been described in *T. melanurus*: *T. m. melanurus* is found along the southern Mediterranean coast and has a conspicuous dark spot on the caudal peduncle absent in *T. m. minor* Kolombatovic, 1892; which seems to be distributed along the northern Mediterranean coast. Nonetheless, individuals with dark spot, light spot, and no spot have been observed altogether in some populations (Zander, 1986; personal observation). Two subspecies with disjunctive distribution areas have also been described in *T. delaisi*: *T. d. delaisi* is found in the Atlantic coast from southern England to Senegal, Azores, Madeira, and Canary Is. and *T. d. xanthosoma* is present in the Mediterranean Sea (Zander, 1986). Morphological differences between specimens from different locations (Atlantic vs. Mediterranean) are marginal and only statistically different when large samples are compared (Wirtz, 1980). However, they can be easily differentiated during the courtship because *T. d. delaisi* males do a figure-8-swimming upwards into the water and *T. d. xanthosoma* do it only on the bottom (Zander, 1986). Individuals of the three species are common in shallow coastal waters (0–40 m), always living in rocky areas. *T. delaisi* prefers biotopes with reduced light such as under overhanging rocks or entrances of caves between 6 and 12 m. *T. tripteronotus* inhabits in light-exposed and shadowy biotopes preferably between 0 and 3 m. Finally, *T. melanurus* inhabits walls or ceilings of sea caves and other dimly lit biotopes (Macpherson, 1994; Wirtz, 1978).

Different scenarios have been hypothesized to explain the speciation process in this genus: (1) According to Zander (1973), a *Tripterygion* from west-african coastal waters diverged in the Atlantic into a more cold-resistant northern clade and a more thermophilous southern clade. After the last glaciation, the Mediterranean was colonized by the northern clade yielding to the present *T. delaisi*. When the water in the Mediterranean Sea warmed up the top few meters of the sublittoral were colonized by the southern clade, yielding to the present *T. tripteronotus*. Nothing was mentioned on the origin of *T. melanurus*. (2) Wirtz (1980) assumed that a primary West African *Tripterygion* invaded the Mediterranean Sea several times after the Mediterranean Salinity Crisis. The first group of invaders evolved to *T. melanurus*, the second one to *T. tripteronotus*, and the third one to the Mediterranean population of *T. delaisi*. (3) De Jonge and Videler (1989) suggested that a red morph of *T. delaisi* evolved into *T. tripteronotus* either in allopatry by isolation of individuals in shallow pools or in sympatry by the segregation of colour polymorphism linked to habitat use because red territorial males proved to be more successful breeders in shallow waters than yellow morphs. They also considered the existence of *T. melanurus* before this colonization event. Overall, all these hypotheses suggested that *T. delaisi* and *T. tripteronotus* were more closely related and more isolated to *T. melanurus*. Geertjes et al., 2001) reached similar conclusions working with allozymes, hypothesizing that divergence of these species started before the Pleistocene (1–2 Mya) and discussing about the possibility that *T. tripteronotus* and *T. delaisi* diverged sympatrically. (4) Zander (2004) suggested a possible new way of evolution. After MSC, an ancestral *T. delaisi* migrated from the Atlantic into Mediterranean where it diverged into *T. tripteronotus* and *T. melanurus* while adapting to two different light zones. A second migration event of *T. delaisi* from the Atlantic originated the Mediterranean subspecies *T. d. xanthosoma*.

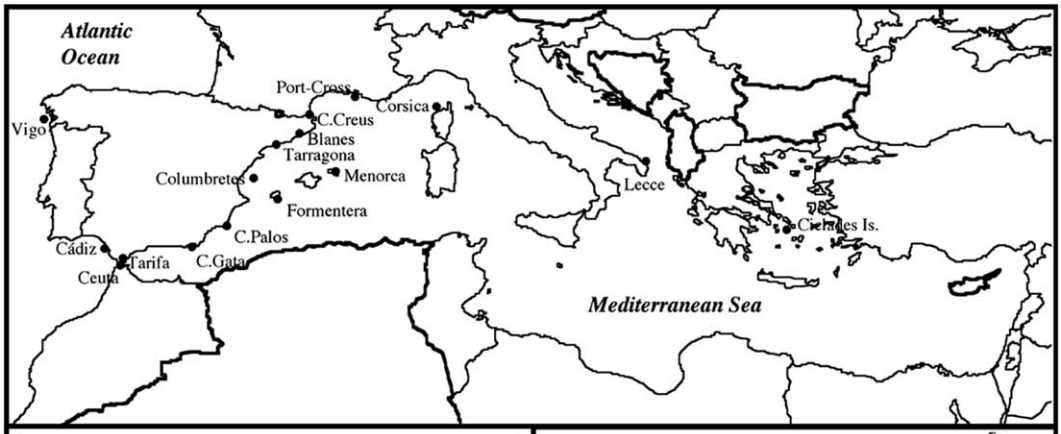
To elucidate the molecular taxonomic status of the genus *Tripterygion*, their speciation process and the phylogenetic relationships between species and subspecies we have used a nuclear gene (18S rRNA) and four mitochondrial genes: cytochrome oxidase I (COI), 12S rRNA, 16S rRNA, and the tRNA-Val lying between both ribosomal genes. With the use of these multiple markers we want to test whether the phylogeny inferred depends on the gene and methodology used in the phylogenetic reconstruction. The use of a phylogeographic approach has been useful to define the distribution areas of the species and at the same time can reveal undetected cryptic species.

2. Materials and methods

2.1. Samples

During 2002 and 2003 47 individuals were caught by SCUBA diving using hand nets from 18 localities (Fig. 1). Each specimen was preserved immediately in 100% ethanol. Individuals were classified by morphological characters as belonging to each species (Zander, 1986). Subspecies of *T. delaisi* were assigned according to their sampling localities, as *T. d. xanthosoma*, those from the Mediterranean Sea, or *T. d. delaisi*, those from the Atlantic Ocean. Subspecies of *T. melanurus* were assigned according to the presence of a dark spot on the caudal peduncle as *T. m. melanurus* or its absence as *T. m. minor*. When a weak spot was present no subspecies was assigned and spp. was used to design it.

Parablennius rouxi (family Blenniidae) from Blanes (BL) was used as the outgroup species since both



Sample locality	*T. delaisi*	*T. tripteronotus*	*T. melanurus*
Ciclades Is.	1(x)	2	2(mi)
Lecce	0	4	0
Port-Cross	0	0	1(mi)
Corsica	3(x)	2	1(mi)
Cap de Creus	1(x)	1	0
Blanes	2(x)	2	2(mi)
Tarragona	1(x)	1	0
Columbretes	1(x)	1	3(me, spp, mi)
Menorca	0	2	2(me)
Formentera	2(x)	2	0
Cabo de Palos	1(x)	1	1(me)
Cabo de Gata	1(x)	1	1(me)
Tarifa	2(x)	2	0
Ceuta	2(x)	3	0
Cádiz	0	2	1(me)
Vigo	1(d)	0	0
Faial	2(d)	0	0
Hierro	2(d)	0	0

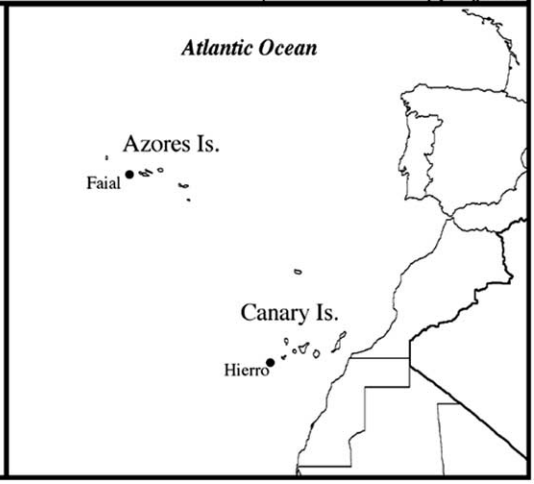


Fig. 1. Sample localities and number of individuals analysed of the three *Tripterygion* species. Subspecies are indicated in brackets and assigned according to Zander (1986): x, *xanthosoma*; d, *delaisi*; me, *melanurus*; mi, *minor*, and spp, no subspecies assigned.

families are closely related and belong to the same superfamily (Stepien et al., 1997). Previous analyses using other species of the Tripterygiidae family from Chile (*Girella laevifrons*, *Auchenionchus microchirris*, *Graus nigra*, and *Gobiesox marmoratus*) yielded more gaps in the alignments that those obtained with *P. rouxi*. Using 12S and 16S we observed that *P. rouxi* was phylogenetically closer to the Mediterranean species of the family Tripterygiidae (data not shown, EMBL Accession Nos.: AJ966656–62), indicating that a revision of the group is needed.

2.2. DNA extraction and sequencing

Total genomic DNA was extracted from fin or muscle tissue using the QIAamp DNA Minikit (Qiagen) or Chelex 10% protocol (Estoup et al., 1996). Fragments of 12S–16S rRNA, 16S rRNA, cytochrome oxidase I, and 18S rRNA genes were amplified by polymerase chain reaction (PCR) using previously published or newly designed primers (Table 1). Amplifications were carried out in 20 μL total volume with 1× reaction buffer (Genotek), 2 mM $MgCl_2$, 250 μM dNTPs, 0.25 μM of each primer, 1 U *Taq* polymerase (Genotek), and 20–30 ng genomic DNA. PCR was performed in a Primus 96 plus (MWG Biotech), and cycle parameters consisted of a first denaturing step at 94 °C for 2 min, followed by 35 cycles of 1 min at 94 °C, 1 min at the optimal annealing temperature for each locus (see Table 1) and 1 min at 72 °C, and a final extension at 72 °C for 7 min. PCR products were cleaned with the QIAquick PCR Purification Kit (Qiagen) or ethanol precipitation and sequenced with the BigDye Sequencing Kit ABI Prism. PCR products were purified by ethanol precipitation and analysed on an ABI 3700 automatic sequencer (Applied Biosystems) from the Scientific and Technical Services of the University of Barcelona. The sequences have been deposited in EMBL and their accession numbers are listed in Table 1.

2.3. Sequence analysis

DNA sequences were edited and aligned with SeqMan II (DNASTAR, Madison, WI) and ClustalX (Thompson et al., 1997) using default parameters and verified visually. The complete mitochondrial DNA sequences from two Blenniidae (*Petroscirtes breviceps* and *Salarias fasciatus*; Miya et al., 2003) were used to

Table 1
List of primers used in this study and accession numbers of the sequences deposited in EMBL

Gene	Primers	Primer sequence (5′–3′)	Annealing temperature (°C)	Reference	EMBL Accession Nos.
16S rRNA	16SAR	CGCCTGTTTATCAAAAACAT	56	Palumbi et al. (1991)	AJ868497–531
	16SBR	CCGGTCTGAACTCAGATCACGT		Palumbi et al. (1991)	AJ93766–74
12S–16S rRNA	12SF	AAAAAGCTTCAAACTGGGATTA GATACCCCACTAT	57	Kocher et al. (1989)	AJ872148–80
	16SBR	CCGGTCTGAACTCAGATCACGT		Palumbi et al. (1991)	AJ937975–83
Cytochrome oxidase I	LCO1490	GGTCAACAAATCATAAAGATATTGG	40	Folmer et al. (1994)	AJ872128–48
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA		Folmer et al. (1994)	AJ937964–65
Cytochrome oxidase I *T. delaisi*	COI-TdF	CTCCTTGGGGACGATCAAAT	55	This study	AJ872116–27
	COI-TdR	CAGAATAAGTGTTGATAAAGAATAGGG		This study	AJ937866–67
18S rRNA	18S-TF	AAACGGCTACCACATCCAAG	50	This study	AJ866980–83
	18S-TR	AACTAAGAACGGCCATGCAC		This study	

assign gene domains for the 12S and 16S fragments. The 16S sequence used came from two sources; the first 280 bp belong to the initial part of the gene and were sequenced from the 12S–16S rRNA fragment (Table 1). The last 419 bp belong to the mid-part of the gene and were sequenced from the 16S rRNA fragment. Both parts are separated by 702 bp, estimated by comparison to the complete mtDNA of *S. fasciatus*, and were joined and analysed simultaneously. Overall, we defined three gene domains: 12S rRNA, 16S rRNA, and tRNA-Val, all partial gene sequences except for tRNA-Val, which is complete. Ten indels for 12S rRNA, eight indels for 16S rRNA, and none indels for tRNA-Val, COI, and 18S rRNA were required for the correct alignment of each set of sequences. We used the program Gblocks to check the alignments (Castresana, 2000) and all positions with gaps were omitted for the phylogenetic reconstructions. Percentage sequence divergence (Dxy, Nei, 1987) was computed within and between subspecies and species respectively using the program DNAsp v4.0 (Rozas and Rozas, 1999).

The secondary structure of 12S and 16S rRNA genes may yield to unequal rates of nucleotide substitution between stems (paired sequence regions) and loops (unpaired sequence regions) (Wang and Lee, 2002). Consequently both regions were analysed separately in order to better resolve older nodes in the phylogeny (Medina and Walsh, 2000). The Vienna RNA package-RNAfold software (Hofacker et al., 1994; Zuker and Stiegler, 1981) was used to elucidate the secondary structure for our 12S and 16S rRNA sequences and define the stem and loop regions as independent data sets. The first, second, and third codon positions of the protein-encoding gene COI were also analysed as independent data sets. In order to eliminate all saturated regions from the phylogenetic analyses the degree of saturation was assessed by plotting Ts, Tv, and Ts + Tv versus uncorrected *p*-distances for all pairwise comparisons in each gene and independent data sets.

Each gene was analysed individually and all genes were joined creating a new data set. Phylogenetic trees were inferred using maximum-likelihood (ML) and maximum parsimony (MP) with PAUP* ver. 4.0b10 (Swofford, 2001), minimum evolution (ME) with MEGA ver. 3.0 (Kumar et al., 2004), and Bayesian inference (BI) using Mr Bayes 3.0b4 (Huelsenbeck and Ronquist, 2001). The computer program MODELTEST ver. 3.06 (Posada and Crandall, 1998) was used to choose the best-fit ML model under the Akaike Information Criterion (AIC) for each gene separately and for all genes combined and posteriorly applied in the ML and BI analyses. This criterion was chosen since it yields more reliable results (Posada and Buckley, 2004). Each analysis was subjected to 1000 bootstrap replicates. When all data were combined, the BI allowed considering each gene with its own evolution model. The Markov chain Monte Carlo (MCMC) algorithm with four Markov chains was run for 1,500,000 generations, sampled every 100 generations resulting in 15,000 trees. The first 1500 trees were eliminated since they did not reach the stationarity of the likelihood values and the rest were used to construct the consensus tree and obtain the posterior probabilities of the branches. The purpose of using all these different methodologies was to compare its resolution for solving the phylogenetic relationships in the genus *Tripterygion*.

The homogeneity of base composition across taxa was assessed using the goodness-of-fit (χ^2) test and the incongruence length difference test (ILD) (Farris et al., 1994) was computed to assess analytical differences between genes, both tests are implemented in PAUP*. In the latter test only parsimony informative characters were included and heuristic searches were performed with 10 random stepwise additions with TBR branch swapping and 1000 randomizations. However, ILD may be a poor indicator of data set combinability (Dowton and Austin, 2002; Yoder et al., 2001), thus we also used a similar method to the bootstrap combinable component criterion of De Queiroz (1993); trees were considered

significantly incongruent whenever different gene trees conflicted at nodes that were supported by BI posterior probabilities >95% or bootstrap values >80% (Moyer et al., 2004).

3. Results

3.1. Sequence analyses

We analysed a total of 2461 bp for all genes combined. For 18S rRNA (729 bp) only one haplotype was found within the genus *Tripterygion* which differed, only by transitional changes, 0.96% from the outgroup. For the other four genes the sequenced obtained was of 419 bp for 12S rRNA, 699 bp for 16S rRNA, 73 bp for tRNA-valina, and 541 bp for cytochrome oxidase I. All the mitochondrial genes used showed a similar percentage of variable sites ($\chi^2 = 4.08$, $P > 0.7$) ranging from 21.92 to 30.32%, however only the mtRNA genes had similar parsimony informative sites ($\chi^2 = 10.87$, $P > 0.05$) ranging from 13.12 to 17.88% being the percentage much larger for COI (22.92%). When loops and stems were considered separately for the 12S and 16S genes both variable and parsimony informative sites were similar ($\chi^2 = 4.85$, $P > 0.67$ and $\chi^2 = 5.45$, $P > 0.61$, respectively). For the COI protein coding gene, third codon positions were 81.56% variable, second codon positions were invariant and first codon positions were 6.14% variable. For each gene sequence the goodness-of-fit test showed homogeneous base composition across taxa ($P = 1.00$).

The Ts/Tv ratio ranged between 1.69 (tRNA-valina) and 4.26 (12S rRNA), with 3.72 for COI and 2.95 for 16S. There was no evidence of sequence saturation in our mtRNA genes neither in stem and loop regions when they were analysed independently, thus both regions were included in the phylogenetic analysis. However, saturation was found for transitions in COI when compared with the outgroup as well as comparing between species. Consequently we reconstructed the phylogeny of the group including and excluding transitions in order to asses its effect.

3.2. Phylogenetic analyses

We obtained a tree for each gene and phylogenetic methodology (Fig. 2). The models selected with the Akaike Information Criterion and applied to the tree reconstructions were the TVM + I + G ($\gamma = 0.39$) for the 12S rRNA, HKY + G ($\gamma = 0.22$) for 16S rRNA, TrN + I ($\gamma =$ equal) for tRNA-valina, HKY ($\gamma =$ equal) for 18S rRNA, KHY + G ($\gamma = 0.18$) and TVM + I ($\gamma = 0.21$) for COI with and without transitions, and finally TVM + I + G ($\gamma = 0.40$) and GTR + I + G ($\gamma = 0.53$) when all genes were combined including and excluding COI transitions, respectively. The tRNA-valina sequence was not used alone to reconstruct the phylogeny due to its small size, although it was included when all genes were combined.

The phylogenetic relationships among the three species of the genus *Tripterygion* varied depending on the method and gene used (Fig. 2). We recovered three tree topologies: (DEL (MEL, TRI)), (MEL (TRI, DEL)), and (MEL, TRI, and DEL) although node support values were in general low. The (DEL (MEL, TRI)) topology was recovered for all methodologies used with 12S. The alternative topology (MEL (TRI, DEL)) is obtained twice, with MP and ME on 16S. Finally, the trichotomy (MEL, TRI, and DEL) also was found twice, using ML and BI on 16S. For COI all three topologies were obtained depending on the method used, whereas the same tree topology (DEL (MEL, TRI)) was found for all methods when transitions were excluded (Fig. 2). Despite the tree differences recovered with each gene and

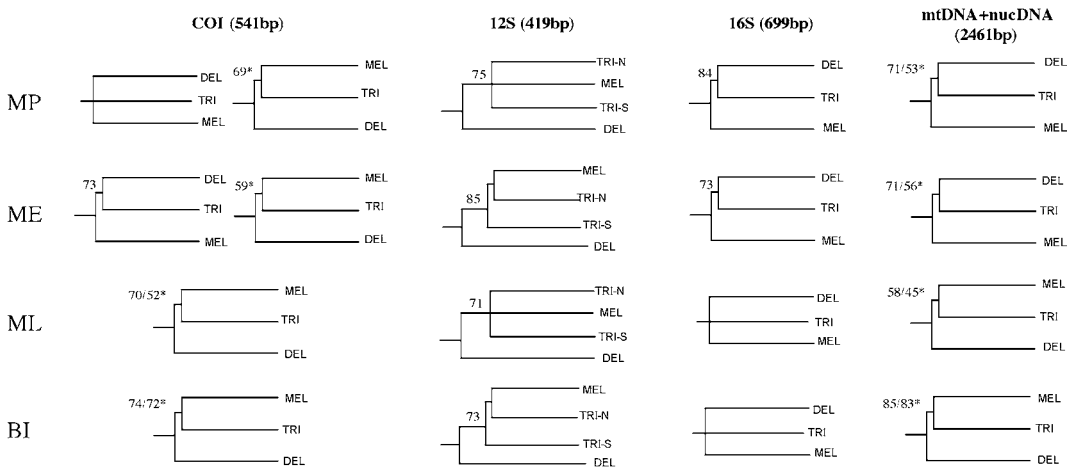


Fig. 2. MP, ME, ML, and BI trees for each gene individually (except for tRNA-val and 18S) and combining all genes, with multiple sample localities collapsed for clarity. DEL, *T. delaisi*; TRI, *T. tripteronotus* (N, Northern clade; S, Southern clade), and MEL, *T. melanurus*. Bootstrap (for MP, ME, and ML) and posterior probability (BI) values are shown. *Analyses excluding transitions in COI gene.

method, the partition homogeneity test showed no significant heterogeneity between genes (P_{ILD} range from 0.1 to 0.48) and although there is not a generally accepted *P*-value for a significant result, most authors agree to combine data when *P* values are greater than 0.05 (Cristescu and Hebert, 2002; Russello and Amato, 2004). Following the criterion of De Queiroz (1993) trees were also not considered incongruent. Two topologies were obtained when all genes were combined: *T. tripteronotus* and *T. delaisi* clustered together with MP and ME, whereas ML and BI methods grouped *T. tripteronotus* and *T. melanurus* although bootstrap values and posterior probabilities were lower than 80 and 95%, respectively. We have observed that MP and ME are strongly influenced by transitional changes (at third codon position) in COI, showing different topologies when including or excluding these changes, and resulting in a bootstrap decrease when all genes were combined, while ML was moderately affected. On the other hand, BI was not influenced at all by saturation in transitional changes being thus the most reliable method (Fig. 2).

All genes and methods clearly differentiated the three *Tripterygion* species with high supported values. In order to clarify the relationships within species we present in Fig. 3 the phylogenetic tree for all genes combined. Two main groups supported by high posterior probability were detected within *T. delaisi*; these same groups were obtained with all mtDNA genes except for tRNA-valina that only showed a unique haplotype for all *T. delaisi* specimens (data not shown). One group comprised Faial and Hierro specimens, from the Atlantic area; these two groups were as well differentiated due to the changes in COI and 12S rRNA genes. The other group included all Mediterranean specimens previously described as *T. d. xanthosoma* (Zander, 1986) and the Atlantic continental specimen from Vigo. Within *T. tripteronotus* two highly supported groups were found with all methods and genes used. One clade joined all specimens from the northern Mediterranean Spanish coast (Cap de Creus, Blanes, Tarragona, and Columbretes Is.), Corsica Island (France), Lecce (Italy), and Ciclades Islands (Greece) and the other included individuals from the southern Atlantic-Mediterranean Iberian coast (Cabo de Palos, Cabo de Gata, Tarifa, and Cádiz), Balearic Islands (Formentera and Menorca), and northern Africa (Ceuta) (Fig. 1). For tRNA-valina one haplotype was found for

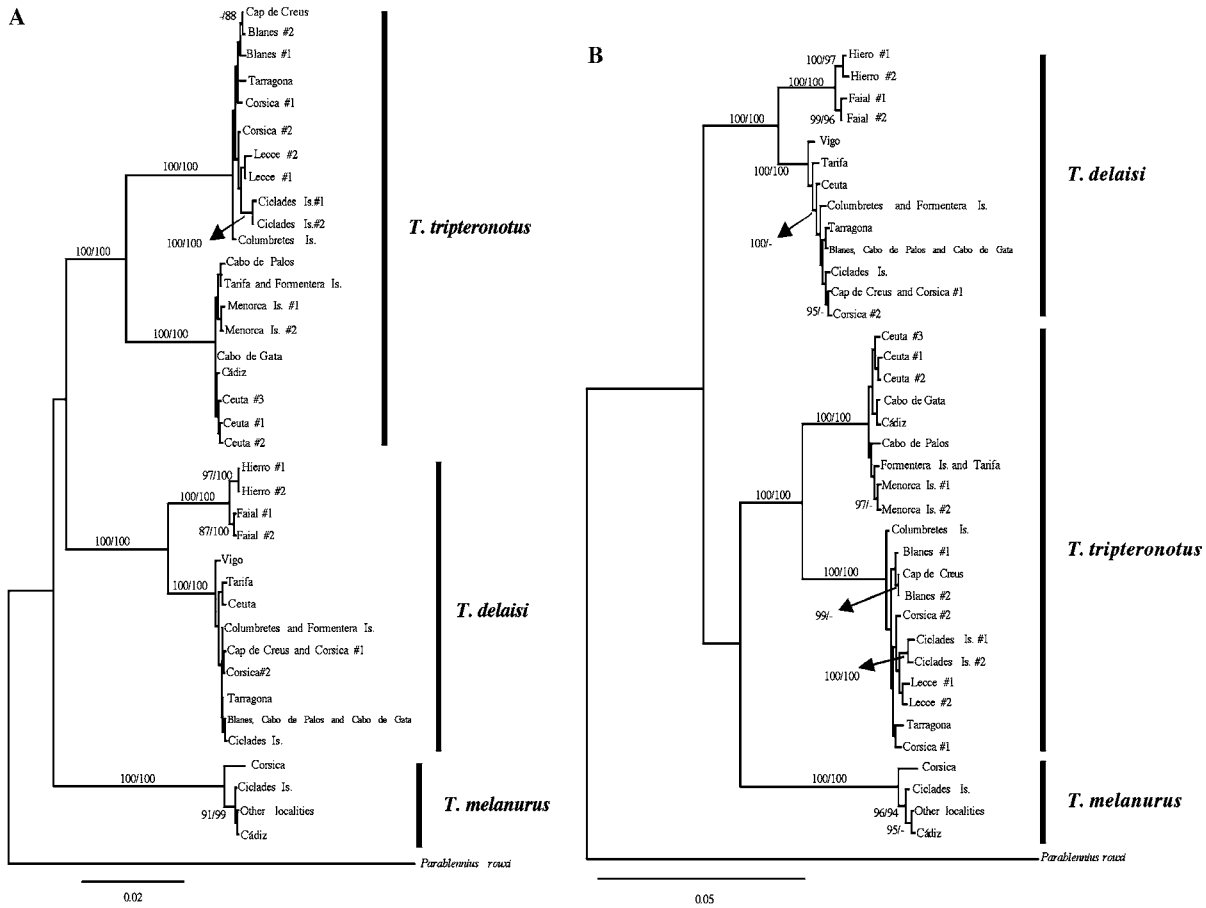


Fig. 3. Haplotype trees for the genus *Tripterygion* using all genes together. (A) Neighbor-joining tree with Maximum Parsimony and Minimum Evolution (MP/ME) bootstrap values. (B) Bayesian Inference tree with Bayesian inference probabilities and Maximum Likelihood bootstrap values (BI/ML). Only bootstrap values above 80% or probabilities above 95% are shown.

each group. Finally, in *T. melanurus* no differentiation between the two subspecies was detected.

3.3. Gene divergence

Mean percentage sequence divergence within and between subspecies and species, respectively are shown in Table 2. Considering all genes together the greatest variability was within *T. tripteronotus* (2.48 ± 0.17%), nearly twice that observed within *T. delaisi* (1.39 ± 0.29%) while the lowest was within *T. melanurus* (0.59 ± 0.18%) since only three haplotypes were found. For the latter species, one haplotype was widely distributed among all localities with the exception of Cádiz and Corsica, where each individual had a different haplotype. More reduced genetic variability was found inside groups within each species ranging from 0.10 to 0.28%. The mean divergence between northern and southern clades in *T. tripteronotus* was 4.56 ± 0.61% whereas between the two *T. delaisi* clades was only 2.79 ± 0.59%. Interspecific variability ranged between 7.23 ± 0.61% and 8.14 ± 1.46% (Table 2). Finally, 15.86 ± 2.57% mean sequence divergence was found between *Tripterygion* sp. and the outgroup (*P. rouxi*). When we analysed each gene individually, COI gene seemed to be generally more variable between and within species and subspecies than all other genes. However, COI gene presented a lower divergence than 12S and 16S genes between *Tripterygion* sp. and the outgroup, due to saturation in transitional changes.

4. Discussion

4.1. Phylogenetic reconstruction

In the present study we have obtained different tree topologies when using different genes and different phylogenetic reconstructions. However, if we consider that the different tree topologies are not incongruent if their posterior probabilities are not higher than 95% or the ML bootstrap values are not higher than 80% (Moyer et al., 2004), differences disappear and the most supported tree topology obtained with our data is a trichotomy. Hence, phylogenetic reconstructions with smaller bootstrap values than those mentioned above should not be considered reliable.

With MP all combinations had low bootstrap values except for 16S, where *T. delaisi* and *T. tripteronotus* grouped with a bootstrap value of 84% (Fig. 2). Furthermore, simulation studies comparing the performance of three phylogenetic confidence methods revealed that more correct monophyletic groups were supported by BI than by either MP or ML (Alfaro et al., 2003). Low bootstrap values were also obtained by ME with the exception of 12S where *T. melanurus* and *T. tripteronotus* clustered with a bootstrap value of 85%, however this methodology seems to be statistically inconsistent under a variety of model misspecifications (Susko et al., 2004). Takezaki et al. (2004) using 44 nuclear genes found an irresolvable trichotomy between Tetrapod, Coelacanth, and Lungfish explained by their divergence within a short interval of time. Therefore in our study increasing the number of genes would neither resolve the phylogeny since probably we are also facing with a process of rapid radiation. This divergence could have started after the Messinian Salinity Crisis (5.2 Mya) when the Straight of Gibraltar re-opened and the Mediterranean basin was refilled in a rather short period of time (ca. 100 years) (Hsü et al., 1977). The absence of variation for the 18S gene within the genus *Tripterygion* in comparison to the 0.96% divergence with the outgroup species, is also indicative of a relatively recent speciation process between these three species. Rapid radiation events have also been observed in reef fishes from the Pacific Ocean by Clements et al. (2003) and in haplochromine cichlids of Lake Tanganyika and Victoria (Strumbauer et al., 2003 and Verheyen et al., 2003, respectively) yielding similarly non-resolved phylogenetic reconstructions.

Table 2
Mean ± SD percentage sequence divergence value within and between subspecies and species

	COI	12S	16S	tRNA-valina	18S	All genes
T. d. delaisi	0.62 ± 0.24	0.38 ± 0.07	0.14 ± 0.07	0	0	0.25 ± 0.07
T. d. xanthosoma	0.44 ± 0.08	0.96 ± 0.48	0.35 ± 0.09	0	0	0.18 ± 0.03
T. tripteronotus north	0.53 ± 0.10	0.36 ± 0.08	0.50 ± 0.11	0	0	0.28 ± 0.03
T. tripteronotus south	0.24 ± 0.08	0.23 ± 0.12	0.29 ± 0.05	0	0	0.10 ± 0.01
T. d. delaisi vs. *T. d. xanthosoma*	8.47 ± 2.44	2.00 ± 0.73	2.43 ± 0.97	0	0	2.79 ± 0.59
T. tripteronotus north vs. *T. tripteronotus* south	8.90 ± 2.26	5.38 ± 2.13	6.06 ± 1.08	2.79 ± 0	0	4.56 ± 0.61
T. delaisi	4.23 ± 1.07	1.18 ± 0.31	1.45 ± 0.36	0	0	1.39 ± 0.29
T. tripteronotus	4.17 ± 1.11	3.03 ± 0.87	3.33 ± 0.38	2.79 ± 1.39	0	2.48 ± 0.17
T. melanurus	0.18 ± 0.09	0.23 ± 0.12	1.64 ± 0.73	0	0	0.59 ± 0.18
T. delaisi vs. *T. tripteronotus*	14.13 ± 1.83	7.64 ± 1.55	9.56 ± 1.37	10.27 ± 5.31	0	7.23 ± 0.61
T. delaisi vs. *T. melanurus*	13.98 ± 3.72	9.60 ± 2.99	11.66 ± 3.38	11.84 ± 0	0	8.14 ± 1.46
T. tripteronotus vs. *T. melanurus*	13.16 ± 3.33	7.83 ± 2.61	13.19 ± 2.51	7.19 ± 3.89	0	7.96 ± 1.16
Tripterygion sp. vs. outgroup	19.66 ± 4.02	24.19 ± 6.03	25.96 ± 5.19	18.14 ± 7.95	0.96 ± 0.13	15.86 ± 2.57

Tripterygion pronasus is the only fossil species found in the Mediterranean from the Miocene (5–20 Mya) of Oran (Arambourg, 1927). This species probably did not survive in that area during the Messinian Salinity Crisis, because the salinity in the basin was above 50‰ (Por and Dimentman, 1985). To explain the rapid speciation process leading to a trichotomy in the phylogenetic reconstructions, we hypothesize that when the Straight of Gibraltar re-opened 5.2 Mya, *T. pronasus* recolonized the Mediterranean basin as several other fish species (Hanel et al., 2002; Quignard, 1978). As observed in different taxa such as damselflies (Turgeon and McPeek, 2002) and gobies (Rüber et al., 2003) the lack of competition and new ecological niches can favour a rapid adaptive radiation. These factors may have played an important role during the speciation processes in the genus *Tripterygion*, right after the MSC, which yielded to the three species evolving in sympatry as a result of their adaptation to different habitats. Thus, into tideless Mediterranean Sea, *T. tripteronotus* appeared due to the adaptation to a new niche in the first water column metres. The colonization of caves and dimly lit biotopes resulted into *T. melanurus*, where its small size and permanent red colour is an excellent cryptic camouflage. Finally, *T. delaisi* inhabited shallow and deeper littoral zones. The low bootstrap values found in our phylogenetic reconstruction indicated rapid radiation and refused all previous hypothesis where *T. melanurus* was the basal species of the genus already present in the Mediterranean Sea. However, our results are closer to those precluded by Zander (2004), in which adaptation to a novel environment played an important role. According to Zander (2004), *T. delaisi* entered into the Mediterranean after MSC and by interspecific competition under the influence of the light gradient yielded to *T. tripteronotus* and *T. melanurus* species. A second migration of *T. delaisi* into the Mediterranean Sea would have displaced the other two species from the reflected light zone and evolved into the present *T. d. xanthosoma* subspecies. Further sampling of *T. delaisi* in the Atlantic waters is necessary to assess the direction of the migrants, in order to determine the subspeciation process.

4.2. Molecular clock: dating speciation

Using the relative rate test (MEGA version 3.0; Kumar et al., 2004), comparing each gene separately and combining all of them, we found a constant substitution rate among all *Tripterygion* species (P value >0.1). We also performed a likelihood ratio test (Goldman, 1993) to asses rate homogeneity for both 12S and 16S genes fragments. The log-likelihood values for both genes with and without assuming a molecular clock were calculated using PAUP* and likelihood ratio statistic was determined according Palkovacs et al., 2002) using the previous calculated evolutionary model for each gene individually. We obtained likelihood ratio statistic values of 1.08 for 12S and 0.98 for 16S genes with all taxa included. These values are much greater than the χ^2 critical value ($\alpha = 0.05$) of 7.26 (df = 15) and 13.85 (df = 24), respectively. So, the molecular clock hypothesis could be accepted and rate homogeneity among lineages is maintained within both genes. If we assume that the speciation process started when the Strait of Gibraltar re-opened we can estimate the evolutionary rate for each gene, using the mean distance found between *T. delaisi*, *T. tripteronotus*, and *T. melanurus* comparison pairs (Table 2). The estimated rate for 12S is 0.81 ± 0.23%/Myr and 1.10 ± 0.23%/Myr for 16S. The divergence rates that we have found for 12S and 16S genes are higher than those obtained in other studies: 0.4%/Myr for 12S and 16S in Notothenioid fishes (Stankovic et al., 2002), 0.35%/Myr for 16S in Labrid fishes (Hanel et al., 2002), and 0.38%/Myr for rRNA working in two genus (*Euproctus* and *Triturus*) of the family Salamandridae (Caccone et al., 1997). If we apply the evolutionary rate found in these studies, the divergence time between the three *Tripterygion* species would be 10–15 Mya. As a consequence these species should have survived in the Mediterranean basin during the Messinian Salinity Crisis, although this seems highly improbable since the salinity during the MSC in the Mediterranean was too high (>50‰) to allow survivorship of marine species. On the other hand, 1.4%/Myr divergence was estimated for 12S and 16S in the Mediterranean sand gobies resulting in a speciation burst at 3.9 Myr (Huyse et al., 2004). However when they apply the molecular clock of 0.86%/Myr estimated in the genus *Aphanius* by Hrbek and Meyer (2003) they found that some of the speciation processes in the marine sand gobies took place 5.3 Mya, closer to the re-opening of the Strait of Gibraltar in accordance to our results. When we applied the faster rate obtained by Huyse et al. (2004) using the mean genetic divergence among *Tripterygion*, species considering 12S and 16S (9.52%), we found that the speciation process took place 3.4 Mya and would be regarded as a post-messinian speciation event. However when we applied Hrbek and Meyer (2003) clock our speciation process was estimated to occur 4.96 Mya, in accordance to the major role of the MSC in the evolution of the *Tripterygion* species. Consequently the refilling of the Mediterranean basin after the MSC seems to trigger the adaptive radiation speciation of marine species.

The mean genetic divergence between the two well-differentiated *T. tripteronotus* clades (northern and southern) was 4.56 ± 0.61%. Thus, using our previously divergence rate for each gene we have calculated that the divergence time between these two clades was 3.32 ± 0.97 Myr for 12S and 2.75 ± 0.49 Myr for 16S. This divergence could be linked to the marine regressions during the Pliocene glaciations 2.7–3.6 Mya, which seem to have shaped the present distribution of moronid and

labrid Mediterranean fishes (Allegrucci et al., 1999; Hanel et al., 2002). The age concordance is almost perfect allowing the molecular clock calibration and supporting our speciation hypothesis.

The sharp phylogeographical break between *T. delaisi* was not between basins but between the Atlantic islands and the Iberian coast and western Mediterranean Islands. The mean genetic distance between these two clades was $2.79 \pm 0.59\%$, significantly less than the distance found between the two *T. tripteronotus* clades, indicating that other processes have shaped its present structure. Thus, when we applied the divergence rate found for each gene, the two *T. delaisi* clades were separated 1.23 ± 0.45 Myr for 12S and 1.10 ± 0.49 Myr for 16S. The climate fluctuations during the Quaternary (Nilsson, 1982) could be the cause of this isolation as suggested by Bargelloni et al. (2003) when comparing several sparid fishes.

The divergence rate among *Tripterygion* species estimated with COI was $1.32 \pm 0.28\%$/Myr however this value was not reliable partly due to the saturation effect in transitions and thus was not used to estimate divergence times. The differences with this gene between the clades within *T. tripteronotus* and *T. delaisi* were very similar although the other genes suggested that their divergence time was very different. When we considered a divergence time of 3.03 Myr between the *T. tripteronotus* clades (the mean value with 12S and 16S) the rate obtained was 1.46%/Myr, similar to the previous rate estimate. However the divergence rate was 3.61%/Myr when we considered the time of divergence between the two *T. delaisi* clades (1.17 Myr). When only transversions were considered, differences within *T. delaisi* even increased, indicating that besides saturation other factors such as the presence of an ancestral polymorphism in *T. delaisi*, probably maintained by balancing selection, should be taken into account.

4.3. Taxonomic implications

The species of the genus *Tripterygion* form a monophyletic group and each previously described species is well differentiated. However, according to our results, a taxonomic revision within them is needed (Fig. 3). The two morphotypes of *T. melanurus*, traditionally considered as two different subspecies (Zander, 1986), were not genetically different suggesting that no such subspecies exist (Fig. 3). Furthermore, the low variability and genetic homogeneity found among all sampled specimens suggest a recent expansion of the distribution range of the species. The analyses of individuals from other Mediterranean areas with the same markers and more polymorphic markers, such as microsatellites (Carreras-Carbonell et al., 2004), will allow us to test this hypothesis.

Two subspecies for *T. delaisi* are currently accepted, one from the Atlantic and the other from the Mediterranean Sea (Zander, 1986). Our molecular data divided the samples into two main groups with highly supported node values (>99%, Fig. 3); one clade included the Atlantic islands and the other clade included all individuals from the Iberian coast, Atlantic and Mediterranean, as well as the western Mediterranean Islands. Samples from the African Atlantic coast are necessary to define the distribution ranges of these two groups and elucidate the processes leading to their divergence. The mean distance for 12S was 2.00% while between different species of the genus *Coryphaenoides* was 3.31% (Morita, 1999) and 4% within the genus *Macullochella* (Jerry et al., 2001). Finally for 16S our mean distance was 2.43% while it ranges from 4.6 to 11.70% between congeneric species of the families Soleidae, Mullidae, and Apogonidae (Apostolidis et al., 2001; Mabuchi et al., 2003; Tinti et al., 2000). Therefore our molecular data support the existence of two well-differentiated clades within *T. delaisi*, which could be considered subspecies in the light of other fish studies at higher taxonomic level.

The two well-defined and highly supported clades in *T. tripteronotus* (north and south Mediterranean) showed greater divergence than between the two *T. delaisi* clades, which confirmed the existence of two cryptic species within *T. tripteronotus* (Fig. 3). Two different colour patterns and some different morphological characters had been described for individuals of *T. ripteronotus* from Northern and Southern Mediterranean, although no taxonomic differentiating status was assigned (Zander and Heymer, 1976). In the light of our studies these two morphs could probably belong to the northern and southern clades, respectively. Hence, morphological comparisons of different populations of these two cryptic species are needed to fully characterize them.

In this study, we have highlighted the importance of analysing multiple genes and only considering large node support values in phylogenetic reconstructions. Furthermore BI seems to be the most reliable methodology and proved to be less influenced by saturation in transitional changes. However, rapid radiation events are not easy to trace with a phylogenetic reconstruction. Using a phylogeographic approach we have revealed several speciation processes related to adaptive radiations and geological events. Comparisons with other taxa will be useful to elucidate how many cryptic species have been under detected and how relevant are the radiation processes after the MSC in the evolutionary history of the species of the Mediterranean Sea and adjacent waters.

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Radiació ràpida i especiació críptica en els tripterígids mediterranis (Pisces: Tripterygiidae): què ens diuen diferents gens?

El gènere *Tripterygion* és l'únic representant de la família Tripterygiidae present al Mediterrani i a la costa nordoriental atlàntica. Tres espècies i quatre subspècies han estat descrites: *Tripterygion tripteronotus* i *T. melanurus* (*T. m. melanurus* i *T. m. minor*) són endèmiques del Mediterrani, mentre que *T. delaisi* (*T. d. delaisi* i *T. d. xanthosoma*) també es troba a la zona atlàntica. S'han utilitzat cinc gens diferents (12S, 16S, tRNA-valina, COI i 18S) per tal de resoldre l'estat taxonòmic i les relacions filogenètiques existents dins d'aquest gènere. D'aquesta manera, utilitzant diferents gens i metodologies (MP, ML, ME i BI), s'han obtingut reconstruccions filogenètiques molt diferents i poc suportades. Aquestes discrepàncies poden ser degudes al propi procés d'especiació, el qual dificulta una reconstrucció fiable de les relacions dins del gènere. Les comparacions entre les tres espècies que formen el gènere mostren la mateixa divergència genètica, obtenint-se una tricotomia, la qual cosa indica que el procés d'especiació podria ser el resultat d'una ràpida radiació després de la crisis de salinitat durant el Messinià (fa 5.2Ma). Dins de l'espècie *T. tripteronotus* s'han trobat dos clades amb una divergència que supera àmpliament la distància interespecífica entre moltes espècies de peixos d'un mateix gènere, conseqüentment haurien de ser considerats dues espècies críptiques que van divergir ara fa 2.75-3.32 Ma, durant les glaciacions del Pliocè. En canvi, no hi ha diferenciació genètica entre les dues subspècies de *T. melanurus*. Finalment, les dues subspècies de *T. delaisi* són molecularment suportades i provablement originades durant les fluctuacions climàtiques del Quaternari (fa 1.10-1.23 Ma), encara que els seus rangs de distribució haurien de ser redefinits segons les dades moleculars.

Review of the *Tripterygion tripteronotus* (Risso, 1810) complex, with description of a new species from the Mediterranean Sea (Teleostei: Tripterygiidae)

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Running title: **description of a new species from *T. tripteronotus* complex.**

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Summary

We compared 52 localities of *Tripterygion tripteronotus* from the Mediterranean Sea and adjacent waters, using four gene sequences (12S rRNA, tRNA-valine, 16S rRNA and COI) and morphological characters. Two well-differentiated clades were found with molecular data, with a mean genetic divergence of 6.89±0.73%, foreseeing the existence of two different species. These two species have disjunctive geographic distribution areas without any molecular hybrid populations. Furthermore, subtle, but diagnostic morphological differences were also present between both species. *T. tripteronotus* is restricted to the northern Mediterranean basin, from the NE coast of Spain to Greece and Turkey, including the islands of Malta and Cyprus. *T. tartessicum* is geographically distributed along the southern coast of Spain, from Cape La Nao to Gulf of Cadiz, the Balearic Islands and northern Africa, from Morocco to Tunisia. According to molecular data, these two species could be diverged during the Pliocene glaciations 2.7-3.6 Mya.

KEYWORDS: *Tripterygion*, new species, molecular data, morphology, taxonomy, Mediterranean Sea.

Revisión del complejo *Tripterygion tripteronotus* (Risso, 1810), y descripción de una nueva especie en el mar Mediterráneo (Teleostei: Tripterygiidae).

Resumen

Se han estudiado 52 localidades mediterráneas y de aguas atlánticas adyacentes de *Tripterygion tripteronotus*, utilizando cuatro genes mitocondriales distintos (12S rRNA, tRNA-valina, 16S rRNA y COI) así como varios caracteres morfológicos. Se han encontrado dos grupos molecularmente bien diferenciados, la divergencia genética media presente entre ambos es de un 6.89±0.73%, indicando la posible presencia de dos especies distintas. Sus áreas de distribución están separadas y no se han encontrado poblaciones molecularmente híbridas. Además, se han encontrado pequeñas diferencias morfológicas que pueden ser utilizadas como caracteres diagnósticos entre las dos especies. *T. tripteronotus* se encuentra en la cuenca mediterránea norte, extendiéndose desde la costa NE de España hasta Grecia y Turquía, incluyendo las islas de Malta y Chipre. *T. tartessicum* se extiende por la costa sur de España, desde Cabo La Nao hasta el Golfo de Cádiz, las islas Baleares y el norte de África, desde Marruecos a Túnez. De acuerdo con los datos moleculares obtenidos, ambas especies pudieron divergir durante las glaciaciones ocurridas en el Plioceno hace unos 2.7-3.6 Ma.

PALABRAS CLAVE: *Tripterygion*, nueva especie, datos moleculares, morfología, taxonomía, Mar Mediterráneo.

Introduction

Molecular data provide a complementary approach to discriminate species separated by subtle morphological characters (Knowlton, 1993; Avise, 1994; Lima *et al*., 2005). In the last years, numerous authors have used molecular methods to detect cryptic species, either in fishes (Gilles *et al*., 2000; Chen *et al*., 2002; Gysels *et al*., 2004) or in other marine organisms (Tarjuelo *et al*., 2001).

The family Tripterygiidae contains species of bottom-living blennioid fishes, usually associated with rocky habitats, and inhabiting cold, temperate, subtropical and tropical areas (Fricke, 2002). The genus *Tripterygion* Risso, 1826, is the only genus of the family Tripterygiidae in the Mediterranean Sea and the northeastern Atlantic coast (Zander, 1986). Three species have been described: *T. tripteronotus*, Risso, 1810, and *T. melanurus*, Guichenot, 1845, are endemic of the Mediterranean, and *T. delaisi* Cadenat and Blache, 1971, is found in both areas (Wirtz, 1980). Individuals of the three species are common in shallow coastal waters (0-40 m), always living in rocky areas. *T. tripteronotus* inhabits in light-exposed and shadowy biotopes preferably between 0 and 3 m, whereas *T. delaisi* uses similar biotopes but in greater depth (between 3 and 40 m) and also biotopes with reduced light such as under overhanging rocks or entrances of caves. Finally, *T. melanurus* inhabits walls or ceilings of marine caves and other dimly lit biotopes (Wirtz, 1978; Macpherson, 1994; Zander, 2004).

The species of the genus *Tripterygion* form a monophyletic group and each previously described species is well genetically differentiated (Carreras-Carbonell *et al*., 2005). However, this recent phylogeographic study, using molecular data, indicated that: (1) the two morphotypes of *T. melanurus,* traditionally considered as two different subspecies by Zander (1986), were not genetically different suggesting that no such subspecies exist, (2) the two subspecies for *T. delaisi* currently accepted (Zander, 1986), were molecularly validated, and (3) *T. tripteronotus*, considered at present as a unique species, presented two well-defined and highly supported clades with greater divergence than that shown between the two *T. delaisi* subspecies, revealing the existence of two cryptic species within *T. tripteronotus* (Carreras-Carbonell *et al*., 2005). Zander and Heymer (1970) had already described two

different pattern bands in the caudal region for *T. tripteronotus* individuals from Banyuls-sur-Mer (France) and Mdiq (Morocco). Later, Zander and Heymer (1976) showed slight morphological differences in the dorsal fins between *T. tripteronotus* specimens from Israel and Lebanon in comparison to specimens from North-western Mediterranean. Although no taxonomic status was assigned, these morphological differences could be related to the two *T. tripteronotus* clades found by Carreras-Carbonell *et al*. (2005).

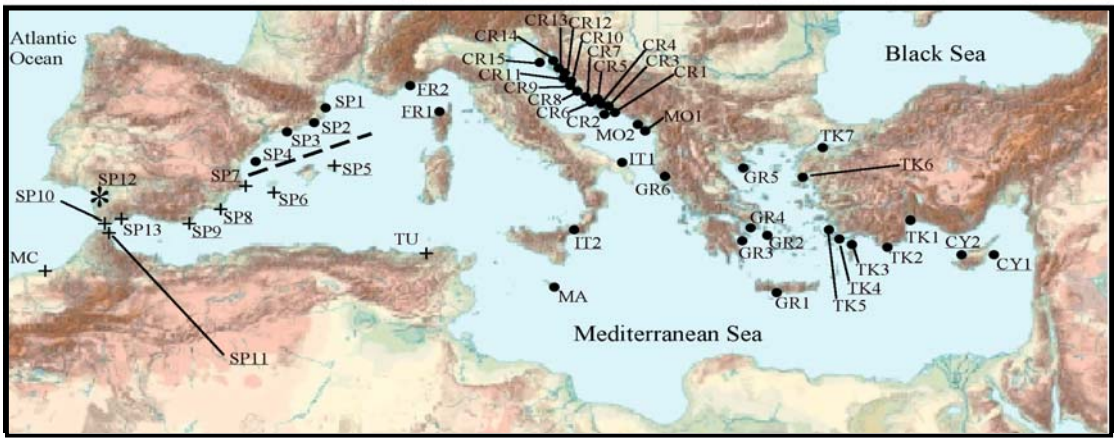
The aim of the present work was to describe the new species and search for morphological characters that allow differentiating both species using samples from 52 localities along the Mediterranean Sea and adjacent waters.

Materials and Methods

Sampling and repositories

Specimens of the two species of *Tripterygion* were collected at different localities of the Mediterranean Sea and Gulf of Cadiz; we also used specimens from Staatliches Museum fuer Naturkunde (Stuttgart, SMNS), totalizing individuals from 52 localities (Fig. 1). The number of individuals used for morphological and molecular analyses, as well as supplementary details about each sampling locality, are shown in Annex I.

Figure 1. Sampling localities for *T. tripteronotus* (●) and *T. tartessicum* (+). Dashed line shows the break zone between both species along the Mediterranean Spanish coast. (*): Holotype locality. Localities which individuals were molecularly analysed are underlined. See Annex I for locality abbreviations and further details.



The type series of the new species are deposited in the collections of the Instituto de Ciencias del Mar (Barcelona, IIPB), Museo Nacional de Ciencias Naturales (Madrid, MNCN) and Staatliches Museum fuer Naturkunde (Stuttgart, SMNS) (see Annex I).

Morphological analysis

In the description of the new species, the data of the paratypes follow those of the holotype, in parentheses. Lengths given and the terminology and other measurements used mainly follow Zander and Heymer (1970), Wheeler and Dunne (1975) and Fricke (1997). Lengths are explained below:

Predorsal length (PD)	distance between middle of upper lip and base of the 1st spine of the first dorsal fin.
Head length (HL)	distance between middle of upper lip and upper insertion of operculum.
Orbital diameter (OD)	maximum eye diameter.
Preorbital length (PO)	distance between middle of upper lip and anterior margin of eye.

The middle of the upper lip is used as the starting point for several lengths rather than the tip of the upper jaw, as the latter may be protractile.

Mandibular pore formula. This formula gives the number of pores under left dentary + number of median pore(s) + number of pores under right dentary.

Individuals were photographed alive in order to check their colour pattern; one or two right gills were removed and kept in absolute ethanol at room temperature. Specimens were individually fixed using buffered formol with 2% borax to maintain the colour pattern for further morphological analyses.

Key

The morphological taxonomic key only works for both sexes when morphometric measurements are used. Sometimes, males can also be distinguished

by discrete morphological characters, while females are identifiable only by their geographical distribution and accompanying males.

Molecular analysis

In order to analyse the genetic difference between *T. tripteronotus* and *T. tartessicum*, we used the sequences of the 12S, tRNA-valine and 16S (acc. num: AJ868510-23, AJ937970-74, AJ872149-60 and AJ937975-79), and COI (acc. num: AJ872128-40 and AJ937862-65) genes from Carreras-Carbonell *et al.* (2005). The same gene sequences were amplified from additional individuals from CY1 (AM260942 and AM260946), CY2 (AM260943 and AM260946), TK4 (AM260944 and AM260947), TK6 (AM260944 and AM260947), IT2 (AM260940-1 and AM260945), FR2 (AM086386-7), SP3 (AM086388-9), SP4 (AM086390-1), SP5 (AM086392-3), SP6 (AM086394-5), SP7 (AM086396-7) and SP8 (AM086398-9) (for location abbreviations and further sampling locality details see Annex I). We used *Tripterygion delaisi xanthosoma* (family Tripterygiidae) and *Parablennius rouxi* (family Blenniidae) from SP2 as internal and external outgroup species respectively (AJ868503, AJ872118 and AJ872164 for *T. d. xanthosoma* and AJ966656-62 for *P. rouxi*).

The homogeneity of base composition across taxa was assessed using the goodness-of-fit (χ^2) test and the incongruence length difference test (ILD) (Farris *et al*., 1994) was computed to assess analytical differences between genes, both tests are implemented in PAUP* ver. 4.0b10 (Swofford, 2001). In the latter test only parsimony informative characters were included and heuristic searches were performed with 10 random stepwise additions with TBR branch swapping and 1000 randomizations. Furthermore, trees were considered significantly incongruent whenever different gene trees conflicted at nodes that were supported by BI posterior probabilities >95% (Moyer *et al*., 2004).

Phylogenetic trees were inferred by Bayesian inference (BI) using Mr Bayes 3.0b4 (Huelsenbeck and Ronquist, 2001) because it seems to be the better methodology to infer phylogenetic relationships between species (Alfaro *et al.*, 2003) and its reconstruction seems to be not affected by saturated positions (Carreras-

Carbonell *et al*., 2005). The computer program MODELTEST ver. 3.06 (Posada and Crandall, 1998) was used to choose the best-fit ML model under the Akaike Information Criterion (AIC) for each gene separately and posteriorly applied in the BI analyses. The MCMC (Markov chain Monte Carlo) algorithm with four Markov chains was run for 1,500,000 generations, sampled every 100 generations resulting in 15,000 trees. The first 1500 trees were eliminated since they did not reach the stationarity of the likelihood values and the rest were used to construct the consensus tree and obtain the posterior probabilities of the branches.

Results

SYSTEMATIC ACCOUNT

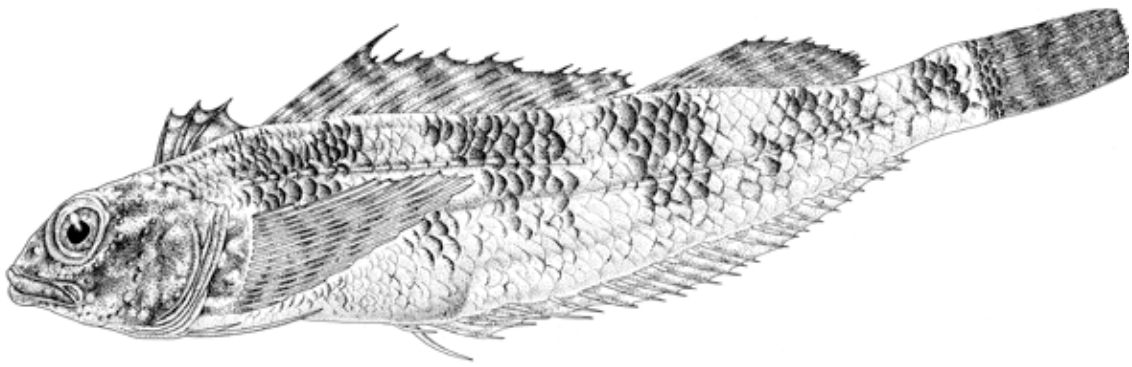
Tripterygion tartessicum new species

(Figures 2 and 3a)

Etymology

The name *tartessicum* referred to the old Spanish culture (*Tartessos*, at least dating from 1000 BC**)** located on the south coast of the Iberian peninsula (in modern Andalusia, Spain), where the new species is partially distributed.

Figure 2. *Tripterygion tartessicum* Holotype, IIPB 15/2005, male, 67mm TL, from SP12.



Morphological description

Body elongate and compressed, greatest height at base of anal fin, being about one-sixth total length. Scales ctenoid, covering entire body except base of pectoral fin and ventral abdominal region back to vent. Lateral line having two sections: anterior section with 20 (19-22) pored scales, posterior section with 22 (21-24) notched scales, having 42 (40-46) in total. Upper, anterior, section commencing at upper angle of opercular opening, slightly curving up over pectoral fin base and running parallel to dorsal profile to point below last 1-3 rays of second dorsal fin; canal running across exposed width of each scale. Lower, posterior, section commencing below, and in front of last scale or two of upper section, running along the mid-line of tail to caudal fin base; each scale with shallow notch in free-edge tip.

Three dorsal fins with III + XVI + 13 (III + XVI-XVIII + 12-13) rays. First dorsal fin lower than second and second higher than third. First just above pre-operculum, being rays of equal height. Second separated by short interspaces, origin slightly behind base of the pectoral fin; first ray longest, being in mature males nearly twice as long as rays in middle region, with distal half not united by membrane with following ray. Base of third fin about 0.6 length of second dorsal fin base.

Caudal fin truncate, with X (IX-X) principal branched rays, and 2 (2-3) procurrent lower and upper.

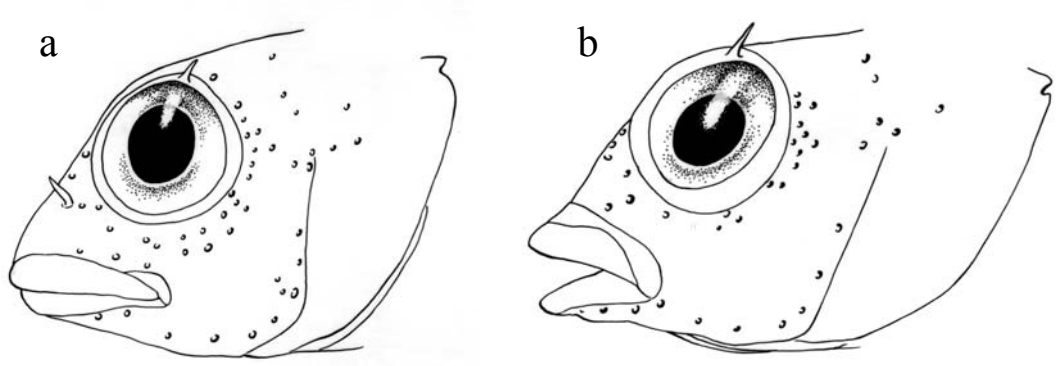
Anal fin elongate and of uniform height, with II + 23 (II + 22-24) rays. Anteriorly, 2 weak, slender, unsegmented rays, first shorter than second, which is slightly longer first segmented ray; succeeding rays united by membrane and decreasing in length posteriorly.

Pectorals long and broad, slightly overreaching midlength of second dorsal fin and base of anal fin; with 16 (15-16) rays, upper three rays short and simple, remainder branched; ninth ray, counted from upper edge, longest than others.

Pelvic fins with one short spine and two slender and segmented rays; longest ray reaching midlength of pectoral fin.

Head broad, scale less, profile acute, lips prominent. Head length 0.19 (0.16-0.22) times total length (TL). Orbit large, almost circular, diameter 0.32 (0.28-0.51) times head length, upper edge forming ridge along upper head profile. Pre-orbital and pre-dorsal lengths 0.05 and 0.14 times total length, respectively (0.06 and 0.18). Interorbital region concave. Mouth nearly horizontal, maxilla extending to level of front of pupil. Gill membrane continuous across throat. Teeth conical, in band in upper and lower jaws. Anterior nostril tubular, posterior nostril close to orbit edge. Cephalic canal pores as illustrated in Figure 3a, with preopercular-dentary series complete. The mandibular pore formula (Fricke 1997) was 3+2+3 (3-4+2+3-4), basically depending on the fish TL, suggesting that an increase in length could be associated with the apparition of a new pore in both dentaries. However, no significant relationship was found between this formula and TL, as well as no differences were found between both species. The interorbital series 2 (2-4) opened singly from the upper interorbital region to the upper lip. The preopercular series opened singly along the lower side of the preopercular canal, opening in pairs on the posterior pre-opercular edge. The nasal and suborbital canals usually opened in pairs, running along the lower and the posterior margins of the orbit; nasal pores 3 (1 to 3) placed in front of the anterior border of the eye; outer branch of suborbital pores ending as a cluster of pores in the postorbital region. Some nuchal pores running from the upper part of the operculum across the nape to the opposite side. Postocular canal with single pores (Fig. 3a).

Figure 3. Variation in the cephalic pore system between *T. tartessicum* (a, female 55mm from SP9) and *T. tripteronotus* (b, female 58mm from SP1).



Colouration in life

Mature males during reproduction period (March-August): black head, extending posteriorly to first dorsal, laterally to operculum edge, and ventrally including branchiostegal membranes across throat, base of pectoral fins and pelvic rays. Red body. Caudal fin with 4 red bars (dark brown in preserved specimens). First dorsal fin rays and membrane heavily pigmented. Anal fin with dusky marks, membrane hyaline. Pectoral fins hyaline, median rays with dusky margins on basal third. The rest of the year their colouration is as females or immature males (sneakers).

Females and immature males (sneakers): head and body with light brown with dark bars across flanks, last bar not forming extension onto base of caudal fin. First dorsal fin heavily pigmented both on rays and membrane, second and third dorsal fins with brownish bars. Caudal fin with 4 distinct brownish bars.

Habitat

The new species inhabits similar habitats than *T. tripteronotus*: shallow rocky shores to 6 m, preferably between 0 and 3 m; in light-exposed and shadowy biotopes, dominated by algal communities (e.g. *Corallina elongata*, *Cladophora spp*., *Litophyllum spp.*, *Enteromorpha spp*.). Nests are usually situated in sciaphyl habitats, dominated by steep rocky zones,without arborescent algae.

Comparison between T. tripteronotus and T. tartessicum

Background

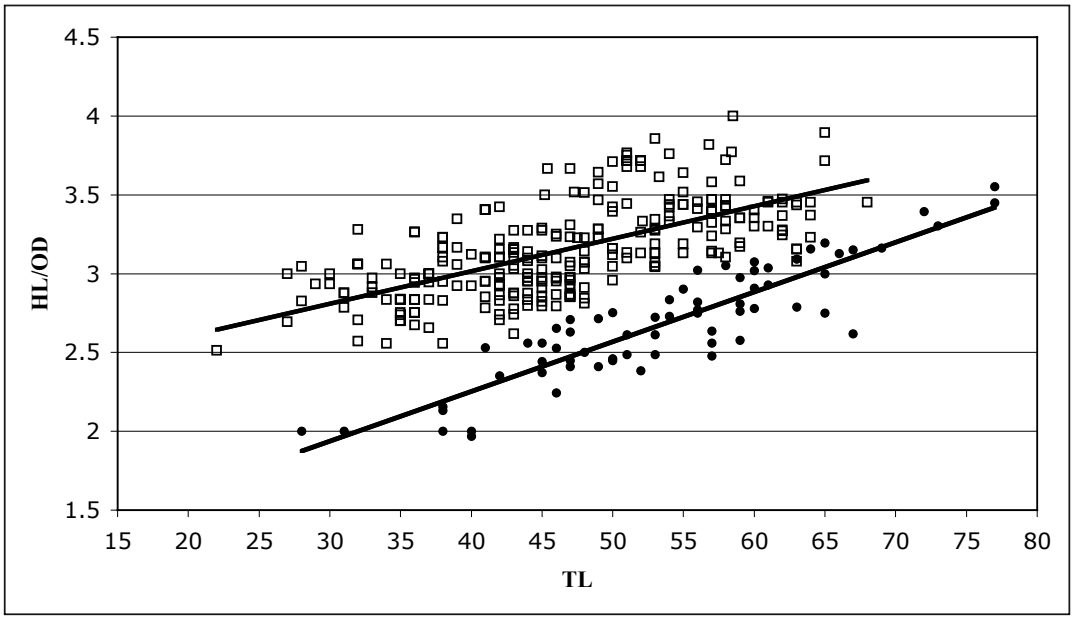
Tripterygion tripteronotus was described by Risso (1810) as *Blennius tripteronotus*, from specimens collected in Nice (France; FR2). Unfortunately the types seem to be lost. Subsequently, the species was named as *T. nasus* (Risso, 1826) from material collected in Nice (France; FR2), *T. melaenocephalus* (Cocco, 1829) from specimens collected in Messina (Italy; IT2), and *Tripterygium nikolskii* (Maksimov, 1909) described from Crimea (Ukraine, Black Sea). These names were

considered as junior synonyms of *T. tripteronotus* (see Hureau and Monod, 1973; Zander, 1986). Zander and Heymer (1970, 1976) mentioned some slight morphological differences between specimens from different localities (NW Mediterranean and Mediterranean coasts of Morocco and Israel), although they were considered as intraspecific variations.

Morphological Data

The morphological comparison of the present material of *T. tartessicum* with specimens of *T. tripteronotus* from different Mediterranean and adjacent waters localities showed that only slight differences exist between both species. The two species can be differentiated by a morphometric measurement: the orbital diameter (OD) is significantly longer in the new species (mean ratio head length/orbital diameter = 2.69±0.36) than in *T. tripteronotus* (3.16±0.29; Mann-Whitney U-test, $p<0.05$). When HL/OD was represented in front of TL, two well-differentiated and almost non-overlapping groups were found, corresponding to both species (Figure 4). In order to assure this differentiation, a multivariate analysis of covariance (MANCOVA) was implemented using TL as covariate and HL/OD as the dependent variable. The results showed highly significant differentiation between both groups ($F = 415.72$, $p<0.001$).

Figure 4. Plotted relationship between TL and HL/OD for all measured individuals from both species. (□): *T. tripteronotus*, (●): *T. tartessicum.* Regression equations are HL/OD = 0.0207TL + 2.1884 (R^2=0.42) and HL/OD = 0.0315TL + 0.991 (R^2 = 0.80), respectively.



Furthermore, the first ray of the second dorsal fin of the mature males has the distal half not united by a membrane with the following ray in *T. tripteronotus*, whereas, the first two rays can be united by a membrane from their respective tips in *T. tartessicum*. Additionally, the caudal fin usually has four red or brownish bars (black in preserved specimens) in the new species, whereas these bars are usually not distinct in *T. tripteronotus*. These two differences are similar to the ones described by Zander and Heymer (1976) although they may be considered with caution since they were not always observable in all the individuals collected.

We have also observed that the mating season seems to start slightly later in the new species. In fact, all mature males of *T. tripteronotus* are active in the Catalan coast (NE of Spain) at the beginning of May, whereas most mature males of the new species are not active at these dates in the coast of Murcia and Almeria (SE of Spain).

Molecular Data

We analysed a total of 1732bp for all genes combined in 55 individuals (18 *T. tartessicum* and 37 *T. tripteronontus*). A total of 10 haplotypes were found for *T. tartessicum*, whereas 17 were shown for *T. tripteronotus* (Fig. 5). Generally, all individuals from one locality shared the same haplotype, with the exception of TK4-TK6 and SP6-SP10 that shared the same haplotype as well. However, in some localities (SP2, FR1, IT1, IT2, GR2, SP5 and SP11) more than one haplotype was found. For each of the four mitochondrial genes the sequence obtained was of 419bp for 12S rRNA, 699bp for 16S rRNA, 73bp for tRNA-valine and 541bp for Cytochrome Oxidase I. All genes used showed a similar percentage of parsimony informative sites (Chi-square = 7.57 p = 0.36) ranging from 2.74% to 10.35%, however only the RNA genes had similar variable sites (Chi-square = 7.52 p = 0.18) ranging from 10.50% to 14.59%, being the percentage larger for COI (19.41%). For the COI protein coding gene, third codon positions were 54.19% variable, second codon positions were invariant and first codon positions were 4.47% variable. The Ts/Tv ratio ranged between 2.61 (16S) and 6.00 (COI) with 4.13 for 12S, and 2.63 for tRNA-valine. There was no evidence of sequence saturation in the analysed genes. For each gene sequence the goodness-of-fit test showed homogeneous base

composition across taxa (P = 1.00) and the partition homogeneity test showed no significant heterogeneity between genes (P_{ILD} range from 0.15 to 1.00) and although there is not a generally accepted P-value for a significant results, most authors agree to combine data when P-values are greater than 0.05 (Cristescu and Hebert, 2002; Russello and Amato, 2004).

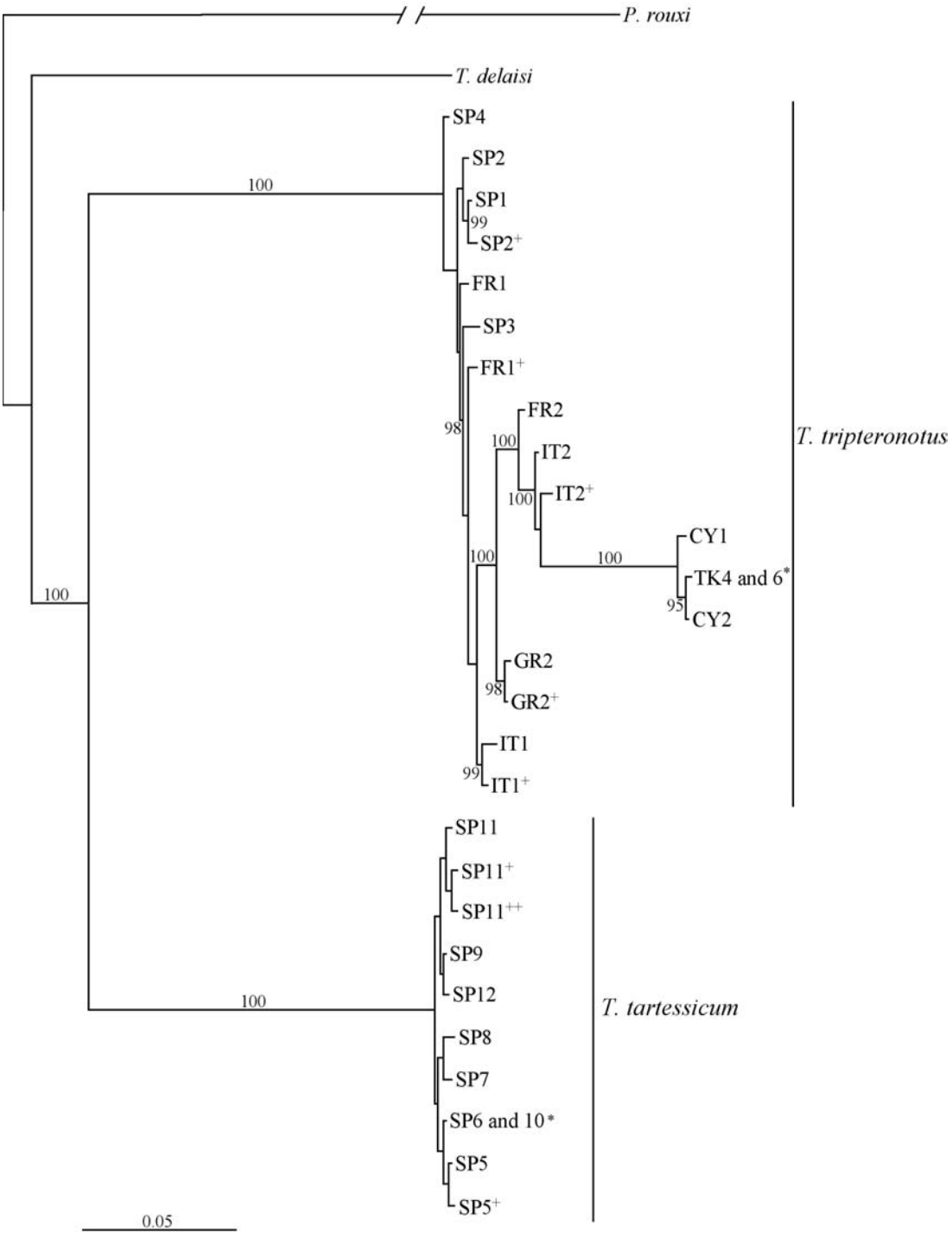
As assessed in Carreras-Carbonell *et al*. (2005), two well-supported clades for *T. tripteronotus* (northern and southern) were found with posterior probabilities of 100%. The southern clade, belonging to *T. tartessicum* presented no well-supported structure pattern between different localities. However, the northern clade (*T. tripteronotus*) showed several well-supported subclades that could be related to defined geographical areas (e.g. Cyprus and Turkey), indicating some degree of isolation between different populations (Fig. 5).

Molecular divergence between *T. tripteronotus* and *T. tartessicum* range between 9.14 % (COI) and 2.79% (tRNA-valine) with a mean value combining all genes of 6.89% (Table 1). No genetically and/or morphologically hybrid populations or individuals were found.

Table 1. Polymorphism and divergence within and between species, for each gene separately and all genes together (mean ± SD percentage).

	COI	**12S**	**16S**	**tRNA-valine**	**All genes**
T. tripteronotus	1.37±0.40	1.24±0.30	1.86±0.42	0	1.33±0.03
T. tartessicum	0.25±0.08	0.24±0.12	0.29±0.05	0	0.15±0.02
T. tripteronotus vs. *T. tartessicum*	9.14±2.01	5.32±1.78	6.72±0.95	2.79±0	6.89±0.73
T. tripteronotus vs. *T. delaisi*	13.58±3.92	8.23±3.09	10.85±2.81	11.84±0	11.03±2.60
T. tartessicum vs. *T. delaisi*	14.80±6.98	6.87±3.44	8.22±2.88	8.70±0	9.90±2.97
T. tripteronotus vs. *P. rouxi*	16.08±4.64	24.97±9.31	26.25±7.25	18.56±0	22.61±5.32
T. tartessicum vs. *P. rouxi*	18.49±8.72	24.64±12.32	24.59±8.61	16.44±0	22.35±6.71

Figure 5. Haplotype tree inferred from Bayesian Inference for *T. tartessicum* and *T. tripteronotus* species using all genes together. Only probabilities above 95% are shown. ($^{+}$ and $^{++}$): different haplotypes found in the same locality, (*): the same haplotype found in different localities. See Annex I for locality abbreviations and further details or Figure 1 for a quick geographical location.



Discussion

The new species is geographically distributed along the southern coast of Spain, from Cape La Nao (SP7) to Gulf of Cadiz (SP12), Balearic Islands (SP5 and SP6), and northern Africa, from Plage David (MC; Morocco, Atlantic Ocean) to Tunisia (TU1) (see Fig. 1). The eastern boundary in the distribution of the new species is unfortunately unknown. Some morphological characteristics (e.g. rays of the second dorsal fin and caudal bands) of the specimens collected in Israel by Zander and Heymer (1970, 1976) are closely related to those observed in the new species, suggesting the presence of *T. tartessicum* in that area. However, as we have mentioned above these morphological characters are not constant, and unfortunately we could not analyzed specimens from this locality, recommending future studies to confirm the taxonomic position of that material.

T. tripteronotus is restricted to the northern Mediterranean basin, including the NE coast of Spain (from SP4 to SP1), France (FR2 and also Corsica Is., FR1), Italy (IT1 and also Sicily Is., IT2), Adriatic Sea (CR1-15 and MO1-2), Malta Is. (MA1), Aegean Sea, including the coasts of Greece (GR3-5) and Turkey (TK3-6), as well as the Ciclades Islands (GR2) and Crete (GR1), Marmara Sea (TK7), as well as Mediterranean Turkish coast and Cyprus (TK1-2 and CY1-2) (see Fig. 1).

The individuals from Nice (FR2) and Messina (IT2) were grouped within *T. tripteronotus* northern clade, suggesting that all specimens from theses localities belonged to the species described by Risso (1810). Therefore, *T. melaenocephalus,* described by Cocco (1829), can be considered as a junior synonym of *T. tripteronotus,* in agreement with previous studies (e.g. Zander, 1986). The specimens from the Black Sea, originally identified as *T. nikolskii* (Maksimov, 1909) and synonymized with *T. tripteronotus,* could not be analysed. However, the presence of *T. tripteronotus* in the Aegean coasts of Greece and Turkey, as well as in the Marmara Sea, suggests that the specimens from the Black Sea may belong to *T. tripteronotus* or *T. nikolskii*, but not to the new species.

Our results confirm the validity of subtle morphological characters to distinguish species of the genus *Tripterygion*, and the existence of a cryptic species,

as occurs in other fish taxa (Gleeson *et al*., 1999; Henriques *et al*., 2002; Lima *et al*., 2005; Yamazaki *et al*., 2003). Nevertheless, the criteria used to designate distinct species based on molecular data are always controversial (Cracraft, 1989; Avise, 1994). The genetic divergence between *T. tripteronotus* and *T. tartessicum* is 9.14% for COI, 5.32% for 12S and 6.72% for 16S, similar to the divergence observed between other fish taxa. Yamazaki *et al.* (2003), using COI, found a sequence difference of 9.10±0.36% between two cryptic species of brook lamprey. For 16S, genetic distances between congeneric species of the families Soleidae, Mullidae and Apogonidae range between 4.6 and 11.70% (Tinti *et al.*, 2000; Apostolidis *et al.*, 2001; Mabuchi *et al.*, 2003). Finally, for 12S the mean genetic distance between congeneric species of the genus *Coryphaenoides* was 3.31% (Morita, 1999), 4% within the genus *Macullochella* (Jerry *et al.*, 2001) and a mean of 6.5% within different blenniidae genera (Stepien *et al.*, 1997). Henriques *et al.* (2002), in a revision of the genus *Lepadogaster* (Teleostei: Gobiesocidae), observed that the minimum distance between valid species was 3% at 12S rRNA. Furthermore, Almada *et al.* (2005) using 12S and 16S genes showed that the genetic differences between clearly morphologically differentiated European blenniids species of the genus *Parablennius* and *Lipophrys* were even smaller (1.3-1.6%). Within the genus *Tripterygion*, *T. tripteronotus* and *T. tartessicum* showed the smallest divergence indicating a more recent speciation event (Carreras-Carbonell *et al*., 2005).

The estimated divergence time found between both species was approximately 3.17 Myr when applying the evolutionary rates of 0.81±0.23%/Myr for 12S and 1.10±0.23%/Myr for 16S inferred for the genus *Tripterygion* (Carreras-Carbonell *et al*., 2005). This divergence could be originated by the marine regressions during the Pliocene glaciations (2.7-3.6 Mya), when the sea level dropped out several meters. During the glaciations, a barrier could be formed between Cape La Nao (SP7) and Balearic Islands (SP5 and SP6) actuating as a separation between both basins and allowing diversification between both clades. However, we cannot discard the existence of a barrier elsewhere (e.g. Gibraltar Strait) and a posterior expansion, being the boundaries the results of secondary contacts. Nowadays, the low larval and adult dispersal capabilities of *Tripterygion* species (Heymer, 1977; Wirtz, 1978; Sabatés *et al*., 2003; Carreras-Carbonell *et al*., 2006) and the circulation regime that separate the northern from the southern basins

(Send *et al.*, 1999) could be maintaining the distribution areas of both species non-overlapping.

Key to the Mediterranean tripterygiids

Modified from Zander (1986).

1a. Profile of head acute with an arch of about 60º; lips protruding; head mask of territorial males extending to breast; females and non-territorial males with marbled head; body permanently red. – *Tripterygion melanurus*.

1b. Profile of head more obtuse with an arch of about 70º; lips not protruding; head mask not extending to breast; females and non-territorial males without marbled head. – 2.

2a. Last dark bar of body forms a distinct black spot on caudal peduncle with an extension onto base of caudal finrays; body of territorial males yellow, head mask not extending to tip of pectoral fins. – 3.

2b. Last dark bar of body not forming an extension onto base of caudal fin; body of territorial males red; head mask extending to tip of pectoral fins. – 4.

3a. During the courtship males draw a figure-8-swimming upwards into the water. Current distribution: Macaronesia. – *Tripterygion delaisi delaisi*.

3b. During the courtship males draw a figure-8-swimming only on the bottom. Current distribution: Mediterranean Sea and Atlantic European coasts. – *Tripterygion delaisi xanthosoma*.

4a. Eyes large, head length less than 2.5 times orbit diameter (in individuals between 2 and 5 cm) (Fig. 4). – *Tripterygion tartessicum*.

4b. Eyes moderately large, head length more than 2.5 times orbit diameter (in individuals between 2 and 5 cm) (Fig. 4). – *Tripterygion tripteronotus*.

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Annex I. Specimens of the two *Tripterygion* species collected at different localities of the Mediterranean and Atlantic adjacent waters. The number of individuals used for morphological (Nm) and molecular (Ng) analyses, for each locality, are detailed.

	Map code	Country	Locality	Latitude Longitude	Depth (m)	Collection date	Nm/Ng	LT range (mm)	Catalogue number
Tripterygion tripteronotus	**CY1**	Cyprus	Akrotirion Gatas/Cape Greco, southeastern corner	34°32'N 33°00'E	0-1	May 2002	6/4	42-47	SMNS 23059
	CY2a	Cyprus	Karavas Alsavcak Bay, small island on eastern side of bay, 9 km west of Kyrenia/Kyreneia/Girne	35°21'13"N 33°13'15"E	0-1	20 May 1997	1/0	38	SMNS 19066
	CY2b	Cyprus	Karavas Alsavcak Bay, small island on eastern side of bay, 9 km west of Kyrenia/Kyreneia/Girne	35°21'13"N 33°13'06"E	0-1	23 May 1997	1/0	41	SMNS 19085
	CY2c	Cyprus	Karavas Alsavcak Bay, small island on eastern side of bay, 9 km west of Kyrenia/Kyreneia/Girne	35°21'13"N 33°13'06"E	0-1	24 May 1997	5/1	34-43	SMNS 19091
	CY2d	Cyprus	Karavas Alsavcak Bay, small island on eastern side of bay, 9 km west of Kyrenia/Kyreneia/Girne	35°21'13"N 33°13'06"E	0.6-1.5	27 May 1997	6/2	36-43	SMNS 19098
	CY2e	Cyprus	Karavas Alsavcak Bay, small island on eastern side of bay, 9 km west of Kyrenia/Kyreneia/Girne	35°21'13"N 33°13'06"E	3-5.5	27 May 1997	1/0	37	SMNS 19106
	CY2f	Cyprus	Karavas Alsavcak Bay, rocky shore and cave on western side of bay, 9 km west of Kyrenia/Kyreneia/Girne	35°21'13"N 33°13'06"E	0-1	23 May 1997	2/1	36-41	SMNS 19089
	CY2g	Cyprus	Karavas Alsavcak Bay, 9 km west of Kyrenia/Kyreneia/Girne	35°21'12"N 33°13'07"E	0-1	22 May 1997	2/0	38-42	SMNS 19089
	CY2h	Cyprus	Karavas Alsavcak Bay, small island on eastern side of bay, 9 km west of Kyrenia/Kyreneia/Girne	35°21'13"N 33°13'15"E	0-1	18 May 1997	2/0	34-37	SMNS 19054
	CY2i	Cyprus	Karavas Alsavcak Bay, small island on eastern side of bay, 9 km west of Kyrenia/Kyreneia/Girne	35°21'13"N 33°13'15"E	0-1	19 May 1997	4/1	37-44	SMNS 19059
	TK1	Turkey	Side, Pamphylia	36°45'58"N 31°23'04"E	n.a.	5 June 1988	1/0	43	SMNS 8402
	TK2a	Turkey	Kas, Lycia, Antalya Province	36°11'30"N 29°38'33"E	n.a.	9 June 1988	3/0	36-42	SMNS 8408
	TK2b	Turkey	Kas, southern harbour jetty, Lycia, Antalya Province	36°11'46"N 29°38'33"E	n.a.	7 June 1988	2/0	40-42	SMNS 8406

TK2c	Turkey	Kas, Lycia, Antalya Province	36°11'30"N 29°38'33"E	n.a.	10 June 1988	2/0	42-45	SMNS 8407
TK2d	Turkey	Kas, Lycia, Antalya Province	36°11'30"N 29°38'33"E	n.a.	11 June 1988	6/0	38-47	SMNS 8389
TK3	Turkey	Torba, ca. 12 km north of Bodrum, Karia	37°07'24"N 27°23'47"E	n.a.	19 June 1988	7/0	35-50	SMNS 8373
TK4a	Turkey	Bodrum, Karia, Egean Sea	37°01'53"N 27°25'38"E	n.a.	16 June 1988	5/1	39-58	SMNS 8392
TK4b	Turkey	Bodrum, Karia, Egean Sea	37°01'53"N 27°25'38"E	n.a.	17 June 1988	8/2	35-50	SMNS 8390
TK5	Turkey	Orag Island, Karia	36°58'35"N 27°35'39"E	n.a.	29 June 1988	6/0	35-52	SMNS 8375
TK6	Turkey	Bay south of Ayvalik, Province Balikesir, Egean Sea	39°14'N 26°38'E	n.a.	3 June 1969	1/1	58	SMNS 13607
TK7	Turkey	Erdek, west of Bandirma, Marmara Sea	40°24'N 27°48'E	n.a.	28 May 1969	10/-	40-59	SMNS 14326
GR1a	Greece	Elounda, north of Aghios Nikolaos, Kreta/Crete Island	35°24'N 24°40'E	n.a.	12 Aug. 1971	4/-	42-50	SMNS 14371
GR1b	Greece	Elounda, north of Aghios Nikolaos, Kreta/Crete Island	35°24'N 24°40'E	n.a.	10 Aug. 1971	3/-	42-50	SMNS 14369
GR2	Greece	Ciclades Is. Kythnos Is.	36º43'35"N 25º16'35"E	0-2	24 Oct. 2004	2/2	42-55	
GR3	Greece	Kyra Island, Gulf of Epidavros	37°37'30"N 23°12'00"E	n.a.	20 July 1970	7/-	28-36	SMNS 14361
GR4	Greece	Aiyina Island, southern tip, Saronian Gulf	37°41'N 23°24'E	n.a.	20 Aug. 1969	1/-	47	SMNS 14366
GR5	Greece	Porto Zografou, 24 km southeast of Nikiti, east coast, Sithonia, Chalkidiki	40°06'N 23°54'E	n.a.	18 Aug. 1994	2/-	59-62	SMNS 15737
GR6	Greece	Palaiokastrizza, Korfu/Corfu Island	39°43'N 19°38'E	n.a.	11 July 1977	2/-	57-64	SMNS 8395
MO1	Montenegro	Bay north of Budva, right side of river mouth	42°16'30"N 18°50'30"E	n.a.	7 May 1977	1/-	55	SMNS 13609
MO2	Montenegro	Bay of Kotor, at Bijela	42°27'N 18°41'E	n.a.	20 May 1969	1/-	45	SMNS 14327
CR1	Croatia	Lokrum Island, west shore, Dubrovnik	42°37'36"N 18°07'07"E	n.a.	21 Sep. 1987	6/0	32-53	SMNS 8391
CR2a	Croatia	Tatinica, northwest coast, Mljet Island	42°46'34"N 17°27'59"E	n.a.	18 Sep. 1987	1/0	46	SMNS 8400
CR2b	Croatia	southwest coast, Mljet Island	42°45'52"N 17°21'51"E	n.a.	17 Sep. 1987	3/0	54-68	SMNS 8404
CR3	Croatia	Sestrice Island, near Orebic, Peljesac	42°56'N 17°08'E	n.a.	9 Aug. 1963	2/-	62-63	SMNS 14325
CR4	Croatia	Orebic, Peljesac	42°56'N 17°08'E	n.a.	7 Aug. 1963	1/0	52	SMNS 14370
CR5	Croatia	Gojak Island, 12 km southeast of Kardeljevo	42°57'N 17°27'E	n.a.	20 Aug. 1963	5/0	42-47	SMNS 14362
CR6	Croatia	Badija Islet, beach at north coast of islet, east of Korçula city, Korçula Island	42°57'28"N 17°09'43"E	n.a.	9 Sep. 1987	4/0	41-51	SMNS 8399
CR7	Croatia	Podaca, 12 km northwest of Ploce	43°09'N 17°15'E	n.a.	6 Aug. 1963	1/0	51	SMNS 14368

CR8a	Croatia	Hvar City, Hvar Island	43°10'09"N 16°26'31"E	n.a.	26 Sep. 1987	6/0	39-54	SMNS 8401
CR8b	Croatia	Jerolim Islet, near Hvar, Hvar Island Group	43°09'28"N 16°23'31"E	n.a.	25 Sep. 1987	4/0	27-49	SMNS 8393
CR8c	Croatia	Lesina/Hvar City, Hvar Island	43°10'N 16°27'E	n.a.	June 1854	2/0	53-57	SMNS 420
CR9	Croatia	Bay of Rogoznica, at Rogoznica	43°31'10"N 15°59'00"E	n.a.	8 Sep. 1987	1/0	22	SMNS 25179
CR10a	Croatia	Biograd	43°55'N 15°23'E	n.a.	7 Aug. 1959	2/0	38-45	SMNS 13365
CR10b	Croatia	Biograd	43°55'N 15°23'E	n.a.	4-6 Aug. 1961	5/0	38-57	SMNS 13364
CR11	Croatia	Karlobag, coast at northern entrance into town	44°32'N 15°04'E	n.a.	5 Aug. 1963	2/0	50-55	SMNS 13605
CR12	Croatia	Gavza Bay, 3 km northwest of Cres City, Cres Island	44°59'24"N 14°23'24"E	n.a.	2 May 1989	2/0	61-64	SMNS 8664
CR13a	Croatia	Osor, Cres Island	44°42'N 14°23'E	0.7	12 Sep. 1989	2/0	36-43	SMNS 9428
CR13b	Croatia	Osor, Cres Island	44°42'N 14°23'E	n.a.	Sep. 1989	3/0	35-61	SMNS 9425
CR13c	Croatia	Osor, Cres Island	44°42'N 14°23'E	0.2	27 Sep. 1989	3/0	32-55	SMNS 9423
CR13d	Croatia	Osor, Cres Island	44°42'N 14°23'E	n.a.	Sep. 1990	4/0	35-63	SMNS 11236
CR14	Croatia	Cres City, Cres Island	44°57'24"N 14°24'21"E	n.a.	3 May 1989	3/0	43-63	SMNS 9214
CR15a	Croatia	Zlatne Stijene, 5 km south of Pula, Istria	44°50'30"N 13°50'30"E	n.a.	10 June 1978	2/0	60-63	SMNS 8410
CR15b	Croatia	Zlatne Stijene, 5 km south of Pula, Istria	44°50'30"N 13°50'30"E	n.a.	9 June 1978	5/0	47-60	SMNS 8396
IT1	Italy	Lecce Harbour	40º13'45"N 18º06'30"E	0-2	5 June 2004	5/4	36-59	
IT2	Italy	Sicily Is. Messina Harbour	38º11'N 15º33'E	0-2	4 Jan. 2006	5/5	49-63	
FR1	France	Corsica Is. Ile Rousse	42º37'39"N 8º55'37"E	0-2	24 Aug. 2004	2/2	28-31	
FR2	France	Nice Harbour	43º25'16"N 7º08'24"E	0-2	13 Mar. 2005	2/2	49-58	
SP1	Spain	Port de la Selva Harbour	42º42'38"N 3º19'50"E	0-2	12 Aug. 2004	12/1	43-63	
SP2	Spain	Blanes St.Francesc Bay	41º40'09"N 2º48'15"E	0-2	30 Jul. 2002	9/2	45-65	
SP3	Spain	Tarragona Altafulla	41º05'35"N 1º13'45"E	0-2	20 Jul. 2003	19/3	51-62	
SP4	Spain	Columbretes Is. La Foradada	39º53'50"N 0º41'15"E	0-2	4 Aug. 2002	4/3	38-47	
MA1a	Malta	Cirkewwa/Paradise Bay, southwest corner of bay, northwest coast, Malta Island	36°58'56"N 14°19'56"E	n.a.	10 Apr. 1974	1/-	58	SMNS 13045
MA1b	Malta	Cirkewwa/Paradise Bay, southwest corner of bay, northwest coast, Malta Island	36°58'56"N 14°19'56"E	0-1.5	10 Aug. 2005	7/-	30-44	SMNS 24888
MA1c	Malta	Cirkewwa/Paradise Bay, southwest corner of bay, Malta Island	36°58'56"N 14°19'56"E	0-1.2	12 Aug. 2005	4/-	29-47	SMNS 24911

	MA1d	Malta	Cirkewwa/Paradise Bay, southwest corner of bay, northwest coast, Malta Island	36°58'56"N 14°19'56"E	0-1.5	11 Aug. 2005	1/-	25	SMNS 24899
Tripterygion tartessicum	**SP5**	Spain	Menorca Is. Fornells Bay	40º04'23"N 4º08'31"E	0-2	5 Jul. 2002	2/2	50-60	
PARATYPES	**SP6**	Spain	Formentera Is. Punta Prima	38º44'N 1º25'14"E	0-2	7 May 2003	2/2	59-60	
PARATYPES	**SP7**	Spain	Cabo La Nao Dènia - Les Rotes	38º51'N 0º07'E	0-2	5 May 2005	4/4	48-63	
PARATYPES	**SP8**	Spain	Cabo de Palos Phare	37º37'57"N 0º41'56"W	0-2	30 Oct. 2002	12/2	41-60	
PARATYPES	**SP9**	Spain	Cabo de Gata Aguamarga - Almeria	36º59'43"N 1º53'41"W	0-2	26 Oct. 2002	16/1	38-67	
PARATYPES	**SP10**	Spain	Tarifa Las Palomas Is.	36º00'15"N 5º36'30"W	0-2	20 Oct 2003	2/2	42-65	
PARATYPES	**SP11**	Spain (Africa)	Ceuta Harbour	35º53'N 5º18'W	0-2	12 Feb. 2005	3/3	28-53	
HOLOTYPE Male 67mm	**SP12**	Spain	Cadiz Puercas Phare	36º18'N 6º12'W	0-2	22 Oct. 2003	12/2	31-67	IIPB 15/2005
PARATYPES	**SP13a**	Spain	1 km southwest of Punta de la Chullera, at Torreguadiaro (150 m northeast), Province Cadiz, Andalucia	36°18'23"N 5°15'39"W	0-1.5	14 Aug. 2004	2/0	72-73	SMNS 24307
PARATYPES	**SP13b**	Spain	1 km southwest of Punta de la Chullera, at Torreguadiaro (150 m northeast), Province Cadiz, Andalucia	36°18'23"N 5°15'39"W	0-1.5	19 Aug. 2004	2/0	63-77	SMNS 24327
PARATYPES	**MC**	Morocco	Plage David	n.a.	n.a.	June 1985	11/-	42-69	SMNS 13516
PARATYPE	**TU1a**	Tunisia	Rocky cape, 4 km east of Tabarca, 66 km east of Bone/Annaba (Algeria)	36°57'33"N 8°47'54"E	0.1-2	2 June 1998	1/-	58	SMNS 20366
PARATYPE	**TU1b**	Tunisia	Rocky cape, 4 km east of Tabarca, 66 km east of Bone/Annaba (Algeria)	36°57'33"N 8°47'54"E	0-1.8	27 May 1998	1/-	46	SMNS 20356
PARATYPES	**TU1c**	Tunisia	Rocky cape, 4 km east of Tabarca, 66 km east of Bone/Annaba (Algeria)	36°57'33"N 8°47'54"E	0-0.6	23 May 1998	2/-	52-61	SMNS 20342

(0): no amplifications were done, (-): amplifications were done but they did not succeed, (n.a.): no available data. The holotype and paratypes are labelled; the catalogue number for each individual is shown. (IIPB): Instituto de Ciencias del Mar de Barcelona, (SMNS): Staatliches Museum fuer Naturkunde Stuttgart. The first two letters in the map code identify each country, the number identifies the locality and the lower case letter identifies different collection dates.

Revisió del complex *Tripterygion tripteronotus* (Risso, 1810), i descripció d'una nova espècie en el mar Mediterrani (Teleostei: Tripterygiidae)

S'han estudiat 52 localitats mediterrànies i de zones atlàntiques adjacents de *Tripterygion tripteronotus*, utilitzant quatre gens mitocondrials diferents (12S rRNA, tRNA-valina, 16S rRNA, i COI) així com també diversos caràcters morfològics. S'han trobat dos grups molecularment molt ben diferenciats, essent la divergència genètica mitjana present entre ambdós grups d'un 6.89±0.73%, indicant la possible existència de dues espècies diferents. A més, s'han trobat petites diferències morfològiques que poden ser utilitzades com a caràcters diagnòstics entre les dues espècies. Les seves àrees de distribució estan separades i no s'han trobat poblacions molecularment híbrides. *T. tripteronotus* es troba en la conca mediterrània nord, des de la costa NE d'Espanya fins Grècia i Turquia, incloent les illes de Malta i Xipre. *T. tartessicum* s'estén per la costa sud d'Espanya, des de Cabo la Nao fins el Golf de Cadis, les illes Balears i el nord d'Àfrica, des de Marroc fins a Tunísia. D'acord amb les dades moleculars obtingudes, ambdues espècies van poder divergir durant les glaciacions del Pliocè, que van tenir lloc fa uns 2.7-3.6 Ma.

3.2.- Microsatèl·lits: aïllament i aplicabilitats

Publicació 3: Isolation and characterization of microsatellite loci in *Tripterygion delaisi*

Publicació 4: Characterization of 12 microsatellite markers in *Serranus cabrilla* (Pisces: Serranidae)

Publicació 5: Genetic divergence used to predict microsatellite cross-species amplification and maintenance of polymorphism in fishes

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PRIMER NOTE

Isolation and characterization of microsatellite loci in *Tripterygion delaisi*

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Abstract

We isolated 49 microsatellite loci from a genomic library of *Tripterygion delaisi* × *anthosoma* enriched for CA and GA repeats. Ten loci were screened in 30 individuals with high numbers of alleles per locus (averaging 15.5 ± 2.86) and observed heterozygosity (averaging 0.765 ± 0.052). No deviations from Hardy–Weinberg expectations were detected. These highly polymorphic markers will be useful in determining the spatial patterns of genetic diversity between and within subspecies of *Tripterygion delaisi*.

Keywords: enrichment, microsatellites, *Tripterygion delaisi*

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Dispersal capabilities may depend on the type of reproduction, the habitat of the species and the length of time individuals disperse. Although some fish pelagic larvae remain in the plankton for several days, they present high levels of larval retention which may cause genetic differentiation between groups separated only by a few hundreds of meters. This may be the case of the blackfaced blenny *Tripterygion delaisi*, one of the most common fishes in shallow Eastern Atlantic coastal waters. Two subspecies have been described: *T. d. xanthosoma* inhabiting the Mediterranean Sea, and *T. d. delaisi* inhabiting the Atlantic coast from south England to Senegal and Azores, Madeira and Canary Islands (Zander 1986). The dispersal capability of the species might be low since the larvae do not move more than 100 meters away from the coast (Sabatés *et al*. 2003) although they remain in the plankton for 17 days (Raventós & Macpherson 2001) thus, it constitutes a good model for investigating differentiation in marine fishes between and within subspecies. Herein, we report microsatellite markers isolated from the subspecies *T. d. xanthosoma*.

We constructed an enriched and partial genomic library following the FIASCO protocol (Zane *et al*. 2002). DNA of two individuals from Blanes (Spain) was simultaneously digested with *Mse*I and ligated to *Mse*I-adapters for three hours. Enrichment was performed using the Streptavidin Magnesphere Paramagnetic Particles Kit (Promega) with two biotinylated probes [$(CA)_{15}$ and $(GA)_{15}$]. Recovered DNA was amplified via polymerase chain reaction (PCR) and subsequently cloned using the P-GEM®-T Easy Vector System II (Promega). Positive clones were detected using digoxigenin-end-labelled probes following the protocol described in Estoup & Turgeon (http://www.inapg.inra.fr/dsa/microsat/microsat.htm). Approximately 1500 colonies were screened for microsatellites yielding 216 positive clones, 51 of which were sequenced.

We isolated 49 microsatellite loci (56% perfect, 38% imperfect and 6% compound) with a mean repeat length of 18.6 ± 1.38 and the insert size ranging from 166 to 450 bp. Among the clones, AC repeats were more frequent (75.5%) than AG repeats (24.5%) and 18.8% had flanking regions too short to design primers. Nineteen pairs of primers were designed using PRIMER 3 (Rozen & Skaletsky 2000). The utility of microsatellite primers and optimal annealing temperatures were determined by screening six individuals of the same population. PCR amplifications were carried out in 20 μL reactions, containing 1X reaction buffer (Genotek), 2 mM $MgCl_2$, 250 μM dNTPs, 0.25 μM of each primer, 1 U *Taq* polymerase (Genotek) and 20–30 ng genomic DNA. PCR was performed in a Primus 96 plus (MWG Biotech) with an initial 5 min denaturation at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at the locus-specific annealing temperature (Table 1) and 1 min at 72 °C, followed by a final 7 min extension at 72 °C. Ten primer pairs successfully amplified genomic DNA and their forward primer was fluorescently labelled (Table 1) to enable allele sizing in an ABI 3700 automatic sequencer from the Scientific and Technical Services of the University of Barcelona. The primers

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Table 1 Characterization of 10 microsatellite loci of *Tripterygion delaisi*

Locus	T_a (°C)	Repeat motif	Clone size (bp)	Size range (bp)	n	No. alleles	H_O	H_E	F_{IS}	Forward and reverse primer sequences (5′–3′)*
Td1	57	$(GA)_{20}$	183	158–184	30	13	0.867	0.904	0.038	NED-CACTTTATGACTAAATGACCACTGC ATCAGCGCTGCATTAGTGTC
Td2	57	$(TC)_{13}$	398	404–420	30	8	0.767	0.677	−0.135	NED-GCGCTTATTGAGCAACTGTG AGCCTCATGCAGGGTCTACT
Td3	57	$(TG)_8$	125	122–153	28	14	0.536	0.88	0.396	HEX-TGAATGGTAGAGCCAGTCAAAA TCAGGCAGATCTGTTTTCCA
Td4	55	$(CA)_{13}C(CA)_3[C(CA)_6]_2$	250	219–295	30	17	0.867	0.884	0.020	HEX-GCACGGGAACAGACTGATG GTGCTCCTGCGAGGAATAGA
Td6	55	$(AC)_{15}$	123	113–201	29	26	0.966	0.926	−0.044	6FAM-GGTCCTCCTGGTTTTTACCTG GACCAGTTGGTTGTGACTGG
Td7	55	$(CA)_{12}$	120	107–119	29	4	0.586	0.508	−0.161	HEX-TCTTGGAAACACGCTTGTAA GCACGTCTATTTGTCGTCCTC
Td8	55	$(CA)_4CG(CA)_2CG(CA)_4$	323	312–406	30	31	0.867	0.954	0.092	HEX-AGCGGATTTGACTGAGGAAA GGCTGTTTCTGAGCCAGTTT
Td9	55	$(CA)_{12}$	306	290–600	30	9	0.567	0.781	0.278	6FAM-AGGTACTTCGGGCAGGGTA CAATTGGAAACATGGAGTGG
Td10	55	$(AG)_{12}$	139	132–200	30	25	0.967	0.951	−0.017	6FAM-GACAAGACCGGCACATTTTC GGGACAAGAGGCAGAAGTTG
Td11	55	$(AC)_4AA(AC)_6$	283	271–301	30	8	0.667	0.663	−0.006	NED-TCTGAAATGCATTGAAGGAGAA TCCTGTCGGTCTGAGTTTCC
Mean ± SE							15.5 ± 2.86	0.765 ± 0.052	0.813 ± 0.048	

T_a, annealing temperature; n, number of individuals genotyped; H_O, observed heterozygosity; H_E, expected heterozygosity under Hardy–Weinberg equilibrium; F_{IS}, inbreeding coefficient.
*, GenBank Accession nos AY490907–AY490916.

that failed to amplify were designed in order to obtain either big (300–450 bp) or small (90–190 bp) bands. Probably this restriction in the design caused its failure and thus, less stringent design could result in a higher amplification success.

Microsatellite variability was analysed using 30 individuals from Columbretes Islands, Spain (39°53.9′ N, 0°41.2′ E) of the subspecies *T. d. xanthosoma*. DNA was isolated from single specimens kept in pure ethanol with the Chelex®100 resin following the protocol in Estoup *et al*. (1996); 3 μL of this rapid extraction were used in the amplification reaction. All loci were polymorphic with four to 31 alleles (mean 15.5 ± 2.86 alleles per locus, Table 1). The mean expected heterozygosity (0.813 ± 0.048) and the mean observed heterozygosity (0.765 ± 0.052) were high and not significantly different (Wilcoxon test, $Z = 0.56$, $P > 0.5$). The values of F_{IS} computed for each locus ranged from — 0.161 (Td07) to 0.396 (Td3) (Table 1). None of the loci showed significant departure from Hardy–Weinberg equilibrium after sequential Bonferroni corrections. No significant linkage disequilibrium was detected between loci pairs, although it was impossible to estimate linkage disequilibrium for Td10 since all analysed specimens had a different genotype. All analyses were carried out using GENEPOP version 3.3 (Raymond & Rousset 1995).

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Aïllament i caracterització de loci microsatèl·lits a *Tripterygion delaisi*

Tripterygion delaisi és un peix molt comú i exclusivament litoral amb un elevat grau de fidelitat al territori pel que fa als adults i amb una capacitat de dispersió larvària molt reduïda, ja que els ous són bentònics i les larves, tot i estar uns 17 dies al plàncton, no s'allunyen més de 100 metres de la costa. Presenta dues subspècies, *T. d. xanthosoma* al Mediterrani i *T. d. delaisi* a la costa atlàntica. Per tal de realitzar diversos estudis d'estructura i dinàmica poblacional, entre i dins de subspècies, amb el major grau de precisió possible, s'han escollit els microsatèl·lits com a marcadors moleculars. Aquests presenten una variabilitat molt més elevada que qualsevol altre marcador molecular, són codominants, neutres i fàcilment analitzables, per contra s'han d'aïllar *de novo* en aquelles espècies per les quals no es disposa de seqüències que els continguin. D'aquesta manera s'han aïllat 49 loci microsatèl·lits per *Tripterygion delaisi* a partir d'una genoteca enriquida amb repeticions CA i GA. D'aquests, se n'han escollit 10 de polimòrfics i amb bona amplificació. Posteriorment, s'han utilitzat per analitzar una població de 30 individus (Illes Columbretes, Espanya), observant-se de mitjana un nombre d'al·lels per locus elevat (15.5±2.86) així com una elevada heterozigositat observada (0.765±0.052). Tos els loci estaven en equilibri Hardy-Weinberg i no s'ha trobat desequilibri de lligament entre cap parella de loci. D'aquesta manera s'ha obtingut un conjunt de 10 loci microsatèl·lits, altament polimòrfics, indispensables per tal de reconstruir els patrons de diversitat genètica, així com l'estructura i dinàmica poblacional de *Tripterygion delaisi* a totes les escales.

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PRIMER NOTE

Characterization of 12 microsatellite markers in *Serranus cabrilla* (Pisces: Serranidae)

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Abstract

The commercial comber *Serranus cabrilla* is widely distributed in the Atlanto-Mediterranean region, inhabiting a great variety of habitats and depths. We developed primers for 12 polymorphic microsatellite loci to analyse the genetic structure between comber populations and between their colour morphs in order to establish correct fisheries management. Characterization of 25 individuals from Columbretes Islands (Spain) showed an average large number of alleles (9.5 ± 1.3) and observed heterozygosity (0.657 ± 0.06). Only two loci showed significant departure from Hardy–Weinberg equilibrium. We found no evidence of linkage disequilibrium between pairs of loci. We rejected for primer design one clone with a microsatellite within the transposable element TX_FR2.

Keywords: colour morphs, enrichment, microsatellites, multiplex, Serranidae, transposable element

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The comber *Serranus cabrilla* is a common demersal species inhabiting the eastern Atlantic Ocean and Mediterranean Sea, and it is found in sea grass beds, rocky, sandy and muddy bottoms, with a wide bathymetric range (5–500 m). It displays two different colorations (red and yellow) with no intermediate patterns; red morphs are smaller and found in shallow waters, whereas yellow morphs are bigger and inhabit deeper waters. These morphs have been considered as different subspecies, as variants during the biological cycle or changes due to phenotypic plasticity (Medioni *et al*. 2001 and references therein). The comber is considered one of the most important predators of early stage of fish and vagile invertebrates (Guidetti & Cattaneo-Vietti 2002). Combers are economically relevant and included in FAO catalogues as species of interest to fisheries in the central-eastern Atlantic, the Mediterranean and the Black Sea. Much is known about its ecology and biology (Torcu-Koc *et al*. 2004); however, there is no information about its populational structure and degree of connectivity between populations. Their larvae remain in the plankton stage for 21–28 days (Raventós & Macpherson 2001) and have been collected not only inshore but also over the continental shelf at a considerable distance from the habitats of the adults (Sabatés *et al*. 2003). Therefore, the larvae have a large potential dispersal to maintain connectivity between populations (Planes 2002). The use of microsatellite markers can help us to understand the relationships between comber populations and between both colour morphs.

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An enriched and partial genomic library was constructed following the FIASCO (fast isolation by AFLP of sequences containing repeats) protocol (Zane *et al*. 2002). DNA of two red morph individuals from Blanes, Spain (41°40.4'N, 2°48.2'E) was simultaneously digested with *Mse*I and ligated to *Mse*I-adapters for 3 h. Enrichment was performed using the Streptavidin MagneSphere Paramagnetic Particles Kit (Promega) with two biotinylated probes ($(CA)_{15}$ and $(GA)_{15}$). Recovered DNA was amplified via polymerase chain reaction (PCR) and subsequently cloned using the pGEM-T Easy Vector System II (Promega). Positive clones were detected using digoxigenin-end-labelled probes following the protocol described in Estoup & Turgeon (1996). Approximately 1000 colonies were screened for microsatellites resulting in 98 positive clones, 39 of which were sequenced. A total of 34 different sequenced clones (EMBL Accession nos: AM049405–38) were obtained, containing 43 microsatellite loci (79.56% perfect, 13.63% imperfect and 6.81% compound) with a mean repeat length of 14.5 ± 1.4 and a mean insert size of 298 ± 30 bp. AC dinucleotides were very abundant, the relationship between AC/AG repeats motif was 2/1. Microsatellite loci within transposable elements (TEs) are more prone to cause

Table 1 Characterization of 12 microsatellite loci of *Serranus cabrilla* from Columbretes Islands (n = 25)

Locus	Repeat motif	Clone size (bp)	Size range (bp)	No. of alleles	H_O	H_E	F_{IS}	Forward and reverse primer sequences (5′–3′)†
Sc05	$(AG)_{14}$	241	227–287	21	0.760	0.958	0.210*	NED-GACCCCTGGAGAGAGTTCAA CAGCTGCCACTCTTAGTAGTGAA
Sc08	$(CT)_{13}$	138	130–182	11	0.520	0.771	0.330*	NED-TCCGCCACAGTTTTCTATCC TCCATTTGGTGTCTGCATGT
Sc03	$(AC)_{10}$	147	127–173	7	0.680	0.716	0.051	HEX-GGGCGGAGAAGTGACATTTA GGATGAACATCACACGTTCTTT
Sc04	$(CA)_{10}$	242	234–252	8	0.840	0.780	−0.079	HEX-GTGCACAGCATAGCCAGAGA AAGTGAACATTCCCTGAGACG
Sc07	$(GT)_{16}$	236	228–242	6	0.800	0.805	0.006	6FAM-CACTTGGCTCGTGTCATTCT CCAACTGTCTCACCTGTGCT
Sc11	$(CT)_9GT(CT)_4$	127	115–127	5	0.240	0.226	−0.063	6FAM-AGTTGTTGCAGGGCTTTAGG TTTGGGACGTAACCTGATCC
Sc12	$(GT)_8$	214	212–226	6	0.640	0.666	0.040	NED-CCTGACATGAAACAAGATTTGC ACATGCAGCAGCGGTGAG
Sc15	$(CT)_5TT(CT)_5$	117	111–129	5	0.360	0.431	0.168	NED-GCAGCACATGAGTGTTGGTT TTGACTGAACACTAGGGATGGA
Sc06	$(CA)_{12}$	141	135–163	12	0.880	0.875	−0.006	HEX-AAAAGAGGCAGTGAAGAATTGG TCATCCATTTCCCTGTTTCA
Sc14	$(TG)_{24}$	261	229–281	14	0.840	0.833	−0.008	HEX-ACCTGTCTGCATGTGATCAGT GCATAAAGGGAAGCGAGTCA
Sc02	$(GT)_9$	228	226–284	11	0.600	0.628	0.045	6FAM-TGAGCTTAGTGTGGGTGCTG GTCCCATACTGGCTGAGTGC
Sc13	$(TG)_{13}$	125	125–145	8	0.720	0.822	0.126	6FAM-CAAAACACACTCGACCAACAA CGGTTTCTGTGGTGCTGAT
Mean ± SE				9.5 ± 1.3	0.657 ± 0.06	0.709 ± 0.06		

n, number of individuals genotyped; H_O, observed heterozygosity; H_E, expected heterozygosity under Hardy–Weinberg equilibrium; F_{IS}, inbreeding coefficient, *$P < 0.05$; †EMBL Accession nos AM049406–17. The horizontal dashed bar separates the two loci groups analysed in two different wells.

problems in posterior amplification and sizing processes (unpublished data). Thus, we have checked if the inserts contained TEs by comparison to reference collection of vertebrate repeats through the Censor web server (http://www.girinst.org). Clone Sc20 (AM049405) contained one TE (TX1_FR2) and was rejected for primer design. Also, 20.6% of the insert sequences could not be used due to the proximity of the microsatellite to the ends of the insert. Finally, 15 pairs of primers were designed using PRIMER 3 software (Rozen & Skaletsky 2000), of which 12 were amplifiable and polymorphic (5–21 alleles; Table 1). Four primer pairs (Sc06–14, Sc03–04, Sc02–13 and Sc12–15) can be amplified simultaneously. Multiplex PCR amplifications were performed in a final volume of 25 μL containing 1× reaction buffer (Genotek), 2 mM $MgCl_2$, 250 μM dNTPs, 0.1 μM of each primer pair, 1 U *Taq* polymerase (Genotek) and 20–30 ng genomic DNA. For loci Sc05, Sc07, Sc08 and Sc11, which have to be amplified individually, 0.2 μM of each primer pair was used. All PCRs were performed as described previously in Carreras-Carbonell *et al*. (2004) and with an annealing temperature of 55 °C. Two groups of six amplified loci of different colours and sizes were combined into two single wells (Table 1) and run with Ecogen 70-400 as internal size standard on ABI 3700 automated sequencer. Allele scoring was performed using GENESCAN and GENOTYPER software.

Loci variability was tested using 25 red morph individuals from Columbretes Islands, Spain (39°53.9′N, 0°41.2′E). The allele number is suitable to detect population differentiation and not prone to heterozygosity saturation or homoplasy (unpublished data). Expected heterozygosities were not significantly higher than observed (Wilcoxon test, $Z = 1.76$, $P = 0.08$), only two loci presented significant departure from Hardy–Weinberg equilibrium after sequential Bonferroni corrections (Table 1). There was no evidence of linkage disequilibrium between loci pairs. All analyses were carried out using GENEPOP (Raymond & Rousset 1995).

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Caracterització de 12 loci microsatèl·lits per *Serranus cabrilla (Pisces: Serranidae)*

El serrà (*Serranus cabrilla*) és una espècie demersal molt comú present en la gran majoria d'hàbitats i fondàries tant de l'Atlàntic oriental com del Mediterrani. Els adults són molts territorials i sedentaris, i pel que fa el seu període planctònic, tant els ous com les larves resten en la columna d'aigua entre 1-3, i 21-28 dies respectivament. A més, s'han trobat larves en aigües exteriors a la plataforma continental. Li han estat descrites dues coloracions diferents (groga i vermella) sense patrons intermedis; hom associa els morfotips vermells amb els individus més petits i d'aigües someres, mentre que el morfotip groc és típic dels individus més grans i que viuen en hàbitats més profunds. És una espècie catalogada, segons la FAO, com d'interès pesquer. Es coneix molt de la seva biologia i ecologia; però gairebé res de la seva estructura poblacional, indispensable, d'altra banda, per la correcta gestió de l'espècie. Així doncs, s'ha realitzat una genoteca enriquida amb repeticions CA i GA, a partir de la qual s'han desenvolupat encebadors específics per 12 loci microsatèl·lits polimòrfics, per a utilitzar-los com a marcadors moleculars. Aquests permetrien analitzar l'estructura genètica entre les poblacions de serrà i entre els seus dos morfotips. Una primera caracterització d'una població de 25 individus (Illes Columbretes, Espanya) utilitzant aquests nous marcadors, mostrà un nombre mitjà d'al·lels per locus elevat (9.5±1.3) així com una elevada heterozigositat observada (0.657±0.06). Només dos loci no estaven en equilibri Hardy-Weinberg i no es va trobar desequilibri de lligament entre cap parella de loci.

Genetic divergence used to predict microsatellite cross-species amplification and maintenance of polymorphism in fishes

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RUNNING HEADLINE: fish microsatellite cross-species amplification

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Abstract

Microsatellites are considered the most suitable markers for use in a wide variety of genetic, evolutionary and ecological studies due to their high polymorphism. However, their specificity is an obstacle to their widespread use, since amplification success across species is limited. In many studies involving microsatellites cross-species amplification, primers designed for one (source) species are used to amplify homologous loci in related (target) species. However, it is not clear how closely related the species must be. Genetic divergence is a clear and easy way in which to assess similarity between species and provides an accurate measure of their evolutionary distance. To assess the genetic divergence between species, two genes (12S rRNA and 16S rRNA) were chosen on the basis of their extensive use in phylogenetic and evolutionary analyses. Eight Mediterranean target species of the family Serranidae were analysed using twelve primers developed for *Serranus cabrilla.* Significant negative correlations were found between genetic divergence and both cross-species amplification and maintained polymorphism of microsatellite markers. The information gathered from other fish studies allowed quantifying the success of using microsatellites across fish species by computing regression equations that displayed the best fit for each correlation. Cross-species amplification success of 50% is expected when genetic divergence to source species is 7.30% for 12S or 9.03% for 16S. However, 50% of cross-species polymorphic loci are attained only if genetic divergence to the source species is not more than 4.35% for 12S and 6.39% for 16S.

Keywords: *cross-species amplification*, *genetic divergence*, *microsatellites, polymorphism, 12S rRNA, 16S rRNA*

INTRODUCTION

In the last twenty years, microsatellite markers have become a powerful tool with which to address a number of ecological and evolutionary questions (for review, see Queller *et al.,* 1993; Wright & Bentzen, 1994). They consist of tandemly repeated short nucleotide motifs (1-6 bp long) that are generally selectively neutral and widely distributed throughout eukaryotic genomes (Jarne & Lagoda, 1996). The usefulness of microsatellites as genetic markers is based on their inherent variability. Thus, due to their extreme polymorphism, microsatellite loci are considered the most suitable markers for forensic identification, parentage testing, gene mapping, conservation biology and population genetics (Jarne & Lagoda, 1996; Peakall *et al.,* 1998). They are also used to infer the evolution of species (Schlötterer, 2001 and references therein). However, amplification success across species is limited and microsatellite markers usually have to be isolated *de novo* for each new species, a process that is both expensive and time consuming. This specificity is an obstacle to the widespread use of microsatellites (Primmer *et al.,* 1996; Steinkellner *et al.,* 1997). Thus, one way to enhance the usefulness of microsatellites, once they have been isolated and sequenced in a source species, is to transfer these markers to related species, which has been done in numerous publications (Primmer *et al.,* 1996 and references therein). However, once cross-species amplifications have been successfully performed, locus polymorphism has to be confirmed, because monomorphic loci cannot be used in subsequent analyses. Unfortunately, many studies only report amplification success in target species (e.g. Guillemaud *et al.,* 2000; Farias *et al.,* 2003).

The success of cross-species microsatellite amplification is inversely correlated with the evolutionary distance between the source and the target species (Primmer *et al.,* 1996; Steinkellner *et al.,* 1997). In almost all studies involving cross-species amplification, some primers designed for one species can be used to amplify homologous loci in "related" species (e.g. Moore *et al.,* 1991; Primmer *et al.,* 1996; Martinez-Cruz *et al.,* 2002). However, it is not clear how closely related the species must be. The term "related species" is used in a wide range of comparisons; some studies performed in fish have attempted cross-species amplifications across congeneric species (within *Symphodus*, Arigoni & Largiader, 2000; within *Lipophrys*, Guillemaud *et al.,* 2000), confamilial species (within Cyprinidae, Holmen

et al., 2005; within Sparidae, Brown *et al.,* 2005), or even more distantly "related" species (Das *et al.,* 2005), with variable success. Genetic divergence is a clear and easy way to determine similarity between species and provides an accurate measure of their evolutionary distance. Despite this apparent utility, no previous studies have correlated genetic divergence with the success of cross-species amplification and maintenance of polymorphism between a source and a target species.

Serranids inhabit littoral and sublittoral areas and they are found over a wide bathymetric range (5-500m) in sea-grass beds and on rocky, sandy, and muddy bottoms (Fasola *et al.,* 1997). The majority of Mediterranean Serranids are economically relevant and included in the United Nations Food and Agriculture Organization catalogues as species of interest to fisheries (Smith, 1981; Bauchot, 1987). Despite their economic importance, only two microsatellite libraries are currently available: one containing 12 polymorphic microsatellite loci for *Serranus cabrilla* L. (Carreras-Carbonell *et al.,* 2006) and one containing 6 polymorphic loci for *Polyprion americanus* (Schneider) (Ball *et al.,* 2000). In the present work, *S. cabrilla* was used as the source species from which microsatellites were isolated and 8 Mediterranean species of the family Serranidae belonging to 4 different genera as well as *Apogon imberbis* (Lacepède) (outgroup) as the target species.

To assess the genetic divergence between species, two genes (12S rRNA and 16S rRNA) were chosen on the basis of their extensive use in phylogenetic and evolutionary analyses. Furthermore, those genes do not generally exhibit saturation between congeneric and confamilial species that could bias genetic divergence (Carreras-Carbonell *et al.,* 2005). The relationships between genetic divergence and microsatellite cross-species amplification and polymorphism were assessed between *S. cabrilla* and each target species. Finally, data from other fish studies were gathered to assess whether genetic divergence can reveal a general trend in the success of microsatellite cross-species amplification and maintenance of polymorphism that can be used in future studies.

MATERIALS AND METHODS

SAMPLE COLLECTION AND DNA EXTRACTION

Nine Serranidae species of the genera *Serranus*, *Epinephelus*, *Mycteroperca* and *Polyprion* from the Mediterranean Sea were analysed. *Apogon imberbis* (Apogonidae) was used as the outgroup species (Table I). Specimens were collected in the field by hook and line or spear gun, or purchased from commercial fish markets. Pectoral fin clips were removed and preserved in absolute ethanol. The number of individuals analysed from each species and their exact capture location are detailed in Table I. Total genomic DNA was extracted from fin tissue using the Chelex 10% protocol (Estoup *et al.,* 1996) or the QIAamp DNA Minikit (Qiagen) according to the manufacturer's instructions.

MITOCHONDRIAL DNA ANALYSIS

A fragment containing the 12S-16S rRNA genes was amplified by polymerase chain reaction (PCR) using the previously published primers 12SF (5'-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3'; Kocher *et al.,* 1989) and 16BR (5'-CCGGTCTGAACTCAGATCACGT-3'; Palumbi *et al.,* 1991). Amplifications were carried out in a total volume of 20μL containing 1X reaction buffer (Genotek), 2mM $MgCl_2$, 250μM dNTPs, 0.25μM of each primer, 1U Taq polymerase (Genotek), and 20-30ng genomic DNA. PCR was performed in a Primus 96 plus (MWG Biotech) and cycle parameters consisted of an initial denaturing step at 94ºC for 2 min followed by 35 cycles of 1 min at 94ºC, 1 min at 55ºC, and 1min at 72ºC, and a final extension at 72ºC for 7 min. PCR products were cleaned with the QIAquick PCR Purification Kit (Qiagen) and sequenced with the ABI Prism Big Dye Sequencing Kit. PCR products were purified by ethanol precipitation and analysed on an ABI 3700 automatic sequencer (Applied Biosystems) at the Scientific and Technical Services of the University of Barcelona. The different haplotype sequences found for each species have been deposited in the EMBL database and their accession numbers are listed in Table I.

Table I. Number of individuals (N) and sampling localities in mitochondrial DNA and microsatellite analyses for each species.

Species	mtDNA analyses			Microsatellite – CSA analyses														H_E
	N	Locality (N)	EMBL Accession numbers*	N	Locality (N)	Primer annealing T^a for each microsatellite locus (Number of alleles)												
						Sc02	Sc03	Sc04	Sc05	Sc06	Sc07	Sc08	Sc11	Sc12	Sc13	Sc14	Sc15	
Serranus cabrilla	8	CG; BL (3) (5)	AM158283-85	22	CO (22)	55 (10)	55 (6)	55 (7)	55 (21)	55 (12)	55 (6)	55 (10)	55 (4)	55 (5)	55 (8)	55 (12)	55 (4)	0.706
S. atricauda	4	CG; CA (1) (3)	AM158286-87	6	CG; CA (1) (5)	55 (6)	55 (5)	55 (6)	55 (6)	55 (9)	55 (4)	55 (1)	55 (4)	55 (4)	55 (4)	55 (6)	55 (3)	0.697
S. scriba	4	BL (4)	AM158288	22	BL (22)	55 (3)	-	55 (3)	-	55 (5)	55 (2)	55 (11)	55 (1)	55 (2)	-	55 (13)	55 (2)	0.351
S. hepatus	4	BL (4)	AM158289-90	6	BL (6)	55 (6)	-	55 (5)	-	55 (3)	55 (1)	55 (2)	55 (3)	55 (2)	-	-	55 (4)	0.567
Polyprion americanus	1	BL (1)	AM158291	1	BL (1)	-	-	50 (2)	50 (1)	55 (1)	55 (2)	-	55 (1)	55 (2)	-	-	55 (1)	0.429
Mycteroperca rubra	5	BA; CR (4) (1)	AM158292-93	8	BA; CR; MA (4) (1) (3)	55 (2)	-	50 (2)	-	55 (8)	55 (1)	-	55 (1)	55 (1)	-	-	55 (1)	0.204
Epinephelus caninus	1	BA (1)	AM158294	1	BA (1)	55 (1)	-	50 (2)	-	55 (2)	-	-	55 (1)	55 (1)	-	-	55 (1)	0.333
E. costae	5	CG; MA (3) (2)	AM158295-97	13	CI; BA; CG; MA (2) (5) (3) (3)	55 (8)	-	50 (5)	-	55 (8)	-	-	55 (1)	55 (1)	-	-	55 (4)	0.482
E. marginatus	5	BA; CG (3) (2)	AM158298-300	22	CI (22)	-	-	50 (3)	-	55 (6)	-	-	55 (1)	55 (1)	-	-	55 (3)	0.372
Apogon imberbis	2	BL (2)	AM158282	2	BL (2)	-	-	-	-		-	-	50 (2)	50 (2)	-	-	50 (3)	0.667

Sampling localities abbreviations: (CI) Cyclades Is., Greece; (CR) Corsica Is., France; (BL) Blanes, Spain; (MA) Mataró, Spain; (BA) Balearic Is., Spain; (CG) Cabo de Gata, Spain; (CO) Columbretes Is., Spain; (CA) Canary Is., Spain. (T^a) annealing temperature in ºC; (-) failed amplifications at 50ºC; (H_E) expected heterozygosity; (*) only different haplotype sequences for each species have been submitted.

DNA sequences were edited and aligned with SeqMan II (DNASTAR Inc., Madison, Wis.) and ClustalX (Thompson *et al.,* 1997) using default parameters and verified visually. The complete sequence of the tRNA-valine gene from *Epinephelus adscensionis* (Osbeck), which lies between the 12S and 16S genes (Smith & Wheeler, 2004), was used to assign gene domains for the coamplified 12S and 16S fragments. Gblocks software was used to check the alignments (Castresana, 2000), since regions that are not well conserved may not be homologous or may have been saturated by multiple substitutions, and the exclusion of poorly aligned positions and highly divergent regions aids phylogenetic reconstruction. The method makes the final alignment more suitable for phylogenetic analysis by selecting blocks of positions that meet a simple set of requirements regarding the number of contiguous conserved positions, lack of gaps, and the degree of conservation of flanking positions. Genetic divergence estimated as the percentage of haplotype sequence differences between species was calculated using the program PAUP* version 4.0b10 (Swofford, 2001).

The homogeneity of base composition across taxa was assessed using the goodness-of-fit (α^2) test and the incongruence length difference (ILD) test (Farris *et al.,* 1994) was used to assess analytical differences between genes; both tests are implemented in PAUP* ver. 4.0b10. In the latter test, only parsimony-informative characters were included and heuristic searches were performed with 10 random stepwise additions with TBR branch swapping and 1000 randomizations. In order to assess the degree of saturation, Ts, Tv and Ts+Tv *versus* genetic divergence for all pairwise comparisons in each gene independently were plotted.

Phylogenetic trees were inferred by Bayesian inference (BI) using Mr Bayes 3.0b4 (Huelsenbeck & Ronquist, 2001), since this method appears to be the best for inferring phylogenetic relationships between species (Alfaro *et al.,* 2003; Carreras-Carbonell *et al.,* 2005). The computer program MODELTEST version 3.06 (Posada & Crandall, 1998) was used to choose the best-fit evolution model under the Akaike information criterion (AIC) for each gene separately and then subsequently used in the BI analyses. The Markov chain Monte Carlo (MCMC) algorithm with four Markov chains was run for 1,500,000 generations, sampled every 100 generations, resulting in 15,000 trees. The first 1500 trees were eliminated since they did not

reach stationarity for the likelihood values and the rest were used to construct the consensus tree and obtain the posterior probabilities of the branches.

MICROSATELLITE DNA ANALYSIS

Twelve polymorphic microsatellite loci isolated from *Serranus cabrilla* (Carreras-Carbonell *et al.,* 2006) were tested in all sampled species (Table I). Primers were fluorescently end-labelled and PCR was carried out under the conditions described in Carreras-Carbonell *et al.* (2006). Whenever amplifications failed, the primer annealing temperature was reduced to 50ºC to increase amplification success (Table I). Amplified products were scored for polymorphism using an ABI 3700 automatic sequencer from the Scientific and Technical Services of the University of Barcelona. Alleles were sized with GENESCAN™ and GENOTYPER™ software against an internal size marker CST Rox 70-500 (BioVentures Inc.).

MITOCHONDRIAL DNA AND MICROSATELLITE COMPARISONS

Microsatellite and mitochondrial DNA results were compared in order to identify relationships between them. Genetic divergence between the target species and *S. cabrilla* (source species) was compared for 12S and 16S genes independently with the degree of successful microsatellite amplification and maintenance of polymorphism.

To investigate whether these results could be generalized to other fish species, information on amplification and polymorphism of microsatellite loci were gathered from other cross-species studies (Annex I). In parallel, mtDNA sequences for 12S and/or 16S genes were downloaded from GenBank in order to estimate the genetic divergence between the source and the target species for which microsatellite information was available (Annex I).

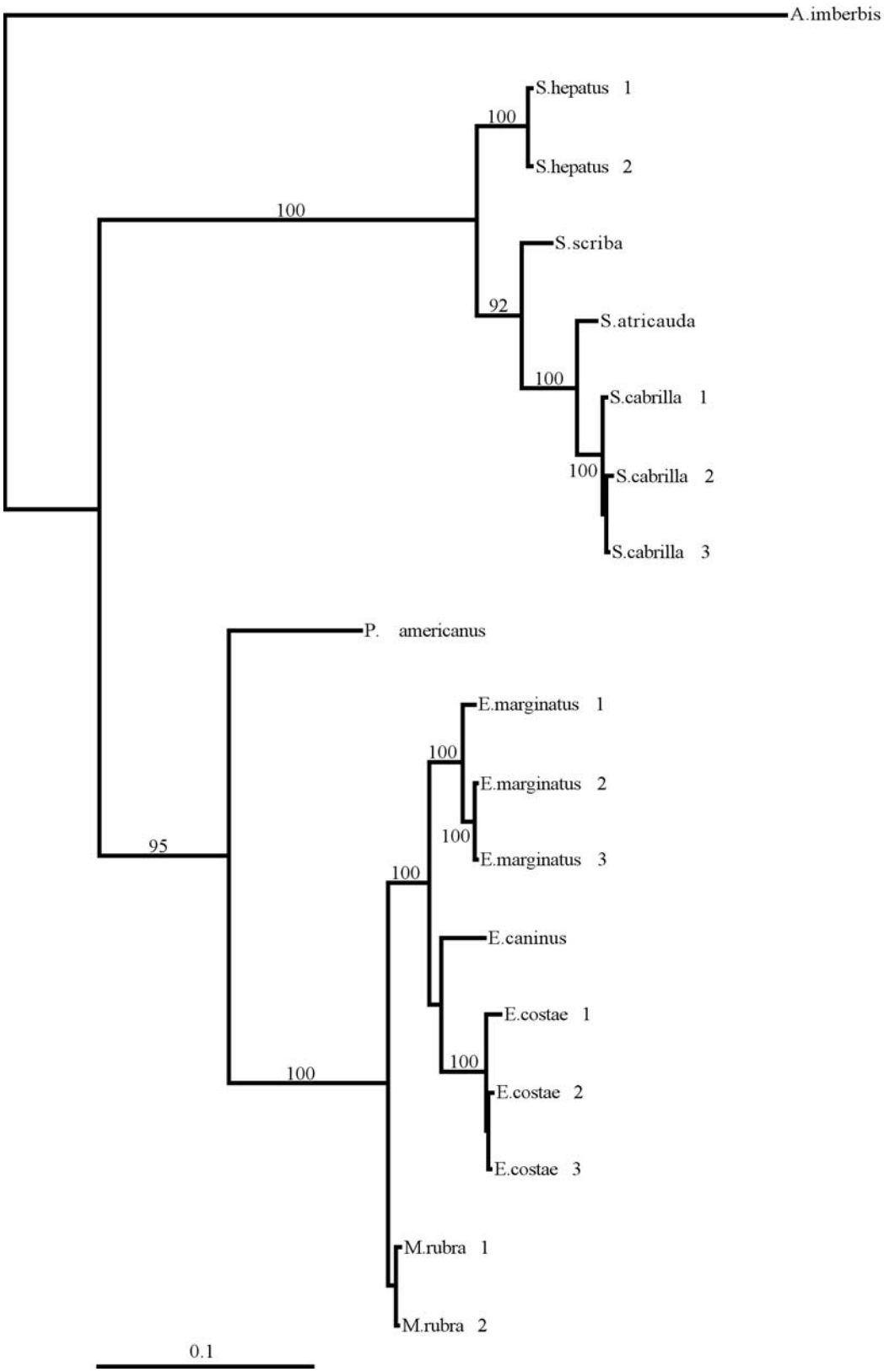
RESULTS

PHYLOGENETIC RECONSTRUCTION BASED ON MTDNA DATA

A total of 1162 bp was analysed for all genes combined: 354bp for 12S rRNA, 740bp for 16S rRNA, and 68bp for tRNA-valine. All genes showed a similar percentage of variable and parsimony-informative sites ranging from 27.68% to 44.11% (Chi-squared test, d.f. = 5, $P>0.05$) and from 18.64% to 39.71% (Chi-squared test, d.f. = 5, $P>0.05$), respectively. The Ts/Tv was 1.87 for 12S rRNA, 2.09 for 16S rRNA, and 7.39 for tRNA-valine. Saturation tests carried out for each gene independently showed no evidence of sequence saturation in these genes (data not shown). The goodness-of-fit test for each gene showed homogeneous base composition across taxa ($P = 1.00$) and the partition homogeneity test showed no significant heterogeneity between genes (P_{ILD} range from 0.10 to 0.59). Although there is no generally accepted P value for significant results, most authors agree data should be combined when P values are greater than 0.05 (Cristescu & Hebert, 2002; Russello & Amato, 2004). Thus, a phylogenetic tree with all three genes combined was reconstructed and this yielded high node-support values (Fig. 1). The models selected according to the AIC and applied to the tree reconstruction were as follows: TrN+I+G (I=0.47, α=0.41) for the 12S rRNA, TVM+I+G (I=0.44, α=0.56) for 16S rRNA, and K80+I (I=0.46, α=equal) for tRNA-valine. The Serranidae species analysed formed two main clades, one containing all *Serranus* species and the other joining *Polyprion americanus*, *Mycteroperca rubra* (Bloch), and all *Epinephelus* species (Fig. 1).

Genetic divergence between *S. cabrilla* and each target species is listed for 12S and 16S genes separately in Annex I. Genetic divergences were on average larger for 16S than for 12S (two sample t test, d.f.=8, $P<0.05$). Sequence divergence between *S. cabrilla* and congeneric species ranged between 1.41% and 6.21%, while comparison with confamilial species of the other main clade ranged from 14.69% to 17.91%. The largest genetic divergence (23.47%) was found between *S. cabrilla* and the outgroup (*A. imberbis*).

Figure 1. Bayesian haplotype tree for Mediterranean Serranidae species analysed using all genes together. Only Bayesian inference probabilities above 90% are shown.



CROSS-SPECIES AMPLIFICATION AND MAINTENANCE OF POLYMORPHISM OF MICROSATELLITE LOCI

Cross-species amplification performance with the 12 primer pairs isolated from *S. cabrilla* was tested in the 9 target species. For each species, the number of individuals analysed varied between one and 22 individuals (Table I). All microsatellite loci were successfully amplified in *Serranus atricauda* (Günther). However, only nine were successful amplified in *Serranus scriba* L. and eight in *Serranus hepatus* L. The amplification success decreased in confamilial species. Seven microsatellite loci were amplified in *P. americanus* and *M. rubra*, six in *Epinephelus caninus* (Valenciennes) and *Epinephelus costae* (Steindachner) and five in *Epinephelus Marginatus* L. When these primers were tested in the outgroup (*A. imberbis*) only three loci amplified.

In summary, loci Sc11, Sc12, and Sc15 amplified in all analysed species (including the outgroup) but all of them displayed a low degree of polymorphism (mean of 2.20 ± 0.23 alleles per species and locus). Loci Sc02, Sc04, and Sc06 amplified in most analysed Serranidae, while loci Sc08 and Sc14 only amplified in most analysed *Serranus* species. Finally, loci Sc03, Sc05, and Sc13 generally only amplified in the most closely related species, *S. atricauda* (Table I). All amplified fragments for each locus and species were of a similar length to that found in the source species (*S. cabrilla*). Although annealing temperature was lowered to 50ºC whenever amplifications failed, some loci still failed to amplify (Table I). No differences were found between expected (H_E) and observed (H_O) heterozygosity in any of the analysed species (Wilcoxon test, $P>0.05$, for all pairwise species comparisons).

The number of polymorphic loci was high within *Serranus* species, with only one amplifiable monomorphic locus in each species: Sc08 for *S. atricauda*, Sc11 for *S. scriba*, and Sc07 for *S. hepatus*. Expected heterozygosity did not show significant differences between *Serranus* species (Wilcoxon test, $P>0.05$, for all pairwise species comparisons) and the mean H_E within *Serranus* was 0.580 ± 0.08. Within confamilial species, polymorphic loci ranged from 2 to 4, and H_E ranged from 0.204 to 0.482, with a mean of 0.364 ± 0.05 (Table I). Although only single specimens of *P. americanus* and *E. caninus* were genotyped, three polymorphic loci were

identified in *P. americanus* and two in *E. caninus*, since these individuals were heterozygous at some loci. When these species were removed from the analyses, similar results were obtained within confamilial species for both the number of polymorphic loci (ranging from 3 to 4) and the mean H_E (0.353 ± 0.08).

Since polymorphism is sensitive to sample size, the number of alleles and heterozygosity were compared among three species with the same number of individuals (n=22): the source species (*S. cabrilla*), a congeneric target species (*S. scriba*), and a confamilial target species from the genus *Epinephelus* (*E. marginatus*), covering the whole range of genetic divergence from the source species. A mean number of 8.75 ± 1.38 alleles per polymorphic locus was found for *S. cabrilla*. This diversity decreased when analysing the congeneric species *S. scriba* (5.12 ± 1.55 alleles) and was even lower for the confamilial species *E. marginatus* (4.0 ± 1.00 alleles). However, no significant difference in H_E was found between the two target species (Wilcoxon test, n = 5, $P>0.5$) and H_E was higher in the source species (Wilcoxon test, n = 12, $P<0.05$, Table I).

When other studies involving cross-species microsatellite amplification were considered (see references in Annex I), the mean amplification success among congeneric species was 82.15 ± 4.51%. These values are similar to those found within *Serranus* species, in which a mean value of 80.55 ± 10.01% was obtained. However, not all amplifiable loci were polymorphic: within *Serranus* species the mean percentage of polymorphic loci was 72.23 ± 10.03%, and similar values were found among congeneric species in the other fish studies (see references in Annex I), where the mean percentage of polymorphic loci was 76.49 ± 9.42%.

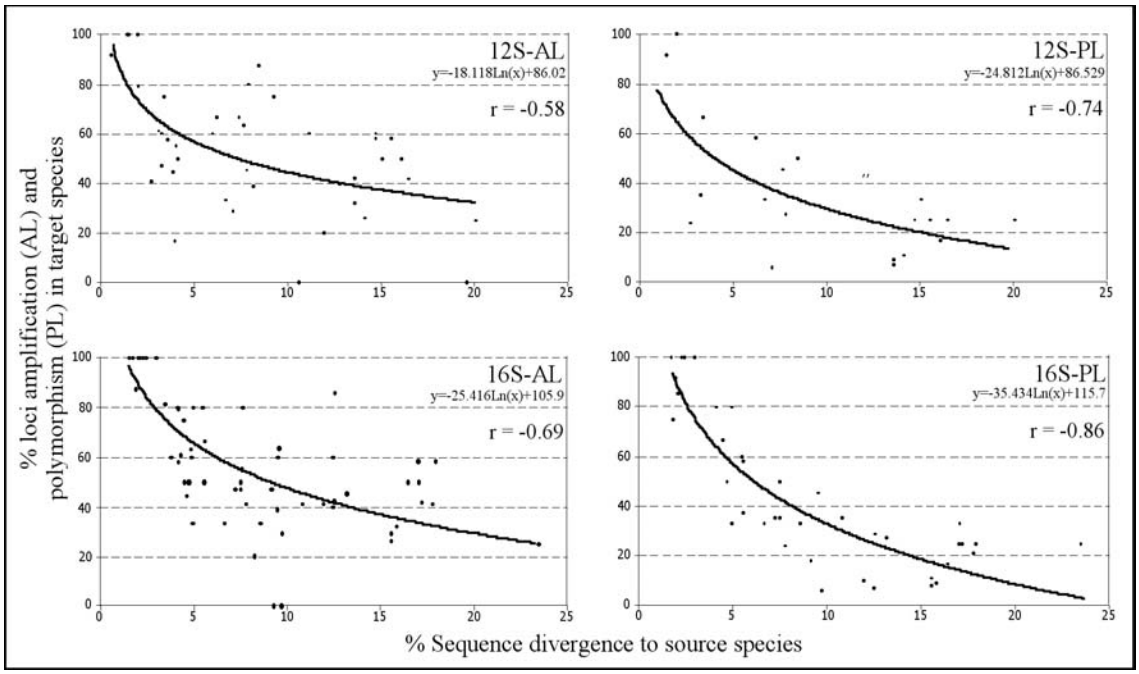
When *S. cabrilla* primers were tested in confamilial species (*Epinephelus*, *Mycteroperca* and *Polyprion*) the amplification success decreased (mean of 51.67 ± 3.12%) along with the polymorphism (mean of 25 ± 2.63%). Other confamilial fish studies have yielded similar results (see references in Annex I): the mean percentage of cross-species amplifiability was 48.12 ± 3.51%, while the mean percentage of polymorphic loci was 34.21 ± 4.72%.

GENETIC DIVERGENCE FROM SOURCE SPECIES *VS.* SUCCESS OF CROSS-SPECIES AMPLIFICATION AND MAINTENANCE OF POLYMORPHISM OF MICROSATELLITE LOCI

A clear relationship was found for both 12S and 16S genes independently between the genetic divergence from *S. cabrilla* and the number of amplifiable microsatellite loci. These data were better fitted to a logarithmic rather than a linear correlation. Genetic divergence from the source species displayed a significant negative correlation with success of microsatellite amplification ($r = -0.92$, $P<0.001$ for 12S and $r = -0.91$, $P<0.001$ for 16S). Since both amplification success and polymorphism are important in studies involving microsatellite loci, the percentage of polymorphic loci in target species over all analysed loci was estimated. When locus polymorphism was considered, significant negative correlations were found between the percentage of amplifiable polymorphic loci and the genetic divergence for both 12S ($r = -0.98$, $P<0.001$) and 16S ($r = -0.98$, $P<0.001$) genes.

As previously found in the family Serranidae, when other fish studies were included in the comparison (Fig. 2) significant negative correlations between genetic divergence and successful microsatellite amplification were detected across taxa ($r = -0.58$, $P<0.001$ for 12S and $r = -0.69$, $P<0.001$ for 16S). Significant correlations were also found when percentage of polymorphic loci *vs* genetic divergence was considered for 12S ($r = -0.74$, $P<0.001$) and 16S ($r = -0.86$, $P<0.001$). The regression equations that displayed the best fit for each logarithmic correlation are shown in Figure 2.

Figure 2. Relationships between genetic divergence to source species (using 12S and 16S genes independently) and percentage of microsatellite loci displaying cross-species amplification (AL) and polymorphism (PL) in target species. These graphics were constructed using sequence and microsatellite DNA data from Annex I.



DISCUSSION

PHYLOGENETIC RECONSTRUCTION

Each of the Mediterranean serranid fish genera formed a monophyletic group, and each species was well differentiated. The analysed genera clustered into two main clades: one clade including *Epinephelus*, *Mycteroperca*, and *Polyprion*, and the other clade containing *Serranus* (Fig. 1). Previous studies that used the 16S gene to reconstruct the phylogeny of serranid fishes by maximum-parsimony showed that the genera *Mycteroperca* and *Epinephelus* were paraphyletic (Craig *et al.,* 2001; Maggio *et al.,* 2005). However, the low bootstrap values that they obtained for the tree reconstruction and the use of only one gene, suggest that the results should be treated with caution. Similarly, when 12S and 16S were used independently, the phylogenetic reconstructions were less conclusive and yielded lower node-support values (data not shown), indicating that combinations of more than one gene should be used to infer phylogenetic reconstructions (see also Cristescu & Hebert, 2002;

Mattern, 2004; Carreras-Carbonell *et al.,* 2005). Pondella *et al.* (2003) analysed three Atlantic species of the genus *Serranus* and concluded that this genus contains an artificial assemblage of taxa, since they formed a paraphyletic group. In the present work all the Mediterranean species of this genus cluster in a monophyletic group.

SUCCESS OF CROSS-SPECIES AMPLIFICATION AND MAINTENANCE OF POLYMORPHISM OF MICROSATELLITE LOCI

A decrease in amplification success was observed when comparing a source species with either congeneric or confamilial target species. However, not all loci yielded the same results. Surprisingly, three loci (Sc11, Sc12 and Sc15) were amplified in all analysed species, including the outgroup. This indicates that the flanking regions were conserved across taxa. Similarly, Rico *et al.* (1996) reported conservation of some microsatellite flanking regions in fishes over a period of about 470 Myr. Therefore, these loci may be located in a less variable region of the genome or in a region suitable for selection, since these sequences, although generally considered neutral, may play an important role in eukaryotic genomes (Kashi & Soller, 1999).

The results of the present study along with data from previous studies (Annex I) indicated that, generally, 81.88 ± 3.99% of the loci characterized in a given fish species could be expected to amplify across congeneric species (with a mean genetic divergence of 4.60 ± 0.91% and 4.63 ± 0.77% for 12S and 16S, respectively), and that only 75.42 ± 7.30% would be polymorphic. Likewise, among confamilial species (with a mean genetic divergence of 9.53 ± 0.94% and 9.84 ± 0.77% for 12S and 16S, respectively), the mean percentage of amplifiable loci was 48.51 ± 3.14%, whereas it decreased to 32.64 ± 3.97% when only polymorphic loci were considered. Thus, a more marked reduction of maintained polymorphism in confamilial than congeneric species was seen, rendering cross-species amplification between confamilial species less effective.

GENETIC DIVERGENCE FROM SOURCE SPECIES *VS.* SUCCESS OF CROSS-SPECIES AMPLIFICATION AND MAINTENANCE OF POLYMORPHISM OF MICROSATELLITE LOCI

The comparisons between genetic divergence, using 12S and 16S genes, and success of microsatellite cross-species amplification and maintenance of polymorphism revealed a highly significant correlation. The same relationship for amplification success was obtained between Blenniidae species using the 12S gene and four microsatellite loci isolated from *Lipophrys pholis* L. (Guillemaud *et al.,* 2000), suggesting a relationship between genetic divergence and conservation of microsatellite loci. However the authors of that study did not test maintenance of polymorphism. Similar results have been reported for birds (Primmer *et al.,* 1996) and pinnipeds (Gemmell *et al.,* 1997), where a significant negative relationship was found between cross-species amplification and evolutionary distance to the source species, measured as the DNA-DNA hybridization ΔT_mH value. Furthermore, those authors showed that for species with a divergence time ranging between 10 and 20 Mya, 40-50% of primer sets would amplify and only 20-25% of those initially tested would be polymorphic. The divergence time between the two main clades obtained in the present work (*Serranus* vs. *Epinephelus-Mycteroperca-Polyprion*) was calculated using the rates (0.81%/Myr for 12S and 1.10%/Myr for 16S) estimated from *Tripterygion delaisi* (Cadenat & Blache) (Carreras-Carbonell *et al.,* 2005). Both clades diverged 8.70 ± 0.32 Mya and the mean percentage of amplifiable loci was 51.67 ± 3.12% and 25 ± 2.64% for polymorphic loci. Thus, these results are in agreement with the values proposed by Primmer *et al.* (1996) and Gemmell *et al.,* (1997). Nonetheless, divergence time can be biased since a constant rate is assumed among different taxa. Therefore, considering genetic divergence through the use of unsaturated genes is a better, more conservative approach.

All the information gathered on genetic divergence and the success of microsatellite amplification and maintenance of polymorphism support the results found in that study and allow to quantify the success of using microsatellites across fish species. Whenever genetic divergence between source and target species is 7.30% and 9.03% for 12S and 16S respectively, a cross-species amplification success of 50% is expected. However, to obtain the same percentage for cross-species amplifiable

polymorphic loci, the genetic divergence between source and target species will be no more than 4.35% for 12S and 6.39% for 16S (Fig. 2). Consequently, for any given pair of source and target species the percentage of amplifying and even polymorphic loci in the target species can be inferred using the equations in Figure 2. For this purpose the only prerequisite is to have the sequences for either 12S or 16S, which can be sequenced or even obtained in GenBank, since they are the most widely used genes in phylogenetic reconstruction.

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Annex I. Genetic divergence, for 12S and 16S gene sequences separately, and microsatellite cross-species amplification and polymorphism between each source species and its target species.

Source Species	Target Species	12S-%GD to Source species	16S-%GD to Source species	CSA %AL	CSA %PL	References	
						mtDNA	Microsatellites
Serranus cabrilla (F. Serranidae)	*S. atricauda*	1.41	1.98	100	91.7	Present study	Present study
	S. scriba	3.39	4.46	75	66.7		
	S. hepatus	6.21	5.56	66.7	58.3		
	Polyprion americanus	14.69	17.03	58.3	25		
	Mycteroperca rubra	15.54	17.91	58.3	25		
	Epinephelus caninus	16.10	16.44	50	16.7		
	E. costae	15.07	17.07	50	33.3		
	E. marginatus	16.48	17.16	41.7	25		
	Apogon imberbis	20.06	23.47	25	25		
Symphodus Ocellatus (F. Labridae)	*S. cinereus*	-	1.74	100	100	Hanel *et al.* (2000)	Arigoni & Largiader (2000) M=7
	S. roissali	-	2.09	100	85.7		
	S. tinca	-	2.96	100	100		
	S. rostratus	-	2.27	100	100		
	Coris julis	-	12.56	85.7	28.6		
Mycteroperca Bonaci (F. Serranidae)	*M. phenax*	-	4.10	80	80	Craig *et al.* (2001)	Zatcoff *et al.* (2002) M=5
	Epinephelus itajara	-	5.47	80	60		
	E. morio	-	4.96	80	80		
Sparus aurata (F. Sparidae)	*Dentex dentex*	-	8.56	33.4	33.4	Orrell *et al.* (2004)	Brown *et al.* (2005) M=6
	Spondyliosoma cantharus	-	6.65	33.4	33.4		
	Pagrus pagrus	-	7.51	50	50		
	Diplodus sargus	-	4.94	33.4	33.4		
	Lithognathus mormyrus	-	4.72	50	50		
Campylomormyrus numenius (F. Mormyridae)	*Petrocephalus soudanensis*	8.21	9.47	38.9	-	Sullivan *et al.* (2000)	Feulner *et al.* (2005) M=18
	Brienomyrus niger	4.11	4.47	50	-		
	Hippopotamyrus pictus	3.08	4.31	61.1	-		
	Mormyrus rume	3.85	4.65	44.4	-		
	Gnathonemus petersii	1.54	1.55	100	-		
Danio rerio (F. Cyprinidae)	*Scardinius erythrophthalmus*	13.59	15.83	32	9	Gilles & Lecointre (2000), Ludwig & Wolter (unpublished), Guo *et al.* (2005), Broughton *et al.* (2001)	Holmen *et al.* (2005) M=103
	Crassius crassius	13.59	12.50	42	7		
	Phoxinus phoxinus	-	17.78	41	21		
	Abramis brama	-	15.56	29	8		
	Rutilus rutilus	14.13	15.56	26	11		
	Gobio gobio	-	11.94	41	10		
Campostoma anomalum (F. Cyprinidae)	*Scardinius erythrophthalmus*	2.72	7.78	41	24	Simons & Mayden (1999), Guo *et al.* (2005), Ludwig & Wolter (unpublished), Gilles & Lecointre (2000)	Holmen *et al.* (2005) M=17
	Crassius crassius	7.06	9.72	29	6		
	Phoxinus phoxinus	-	10.83	41	35		
	Abramis brama	-	7.22	47	35		
	Rutilus rutilus	3.26	7.50	47	35		
	Gobio gobio	-	9.17	47	18		
Piaractus mesopotamicus (F. Serrasalminae)	*Colossoma macropomum*	-	1.85	87.5	75	Calcagnotto *et al.* (2005)	Calcagnotto *et al.* (2001) M=8
	Pygocentrus nattereri	-	5.56	50	37.5		
Tripterygion delaisi xanthosoma (F. Tripterygiidae)	*T. d. delaisi*	2.00	2.43	100	100	Carreras-Carbonell *et al.* (2006)	Carreras-Carbonell *et al.* (unpublished) M=11
	T. tripteronotus	7.64	9.56	63.6	45.4		
	T. melanurus	7.83	13.19	45.4	27.3		

Annex I. Continued

Coris julis (F. Labridae)	*C. atlantica*	8.47	-	87.5	50	Guillemaud *et al.* (2000)	Aurelle *et al.* (2003) M=8
Cyprinus carpio (F. Cyprinidae)	*Aristichthys nobilis*	6.70	-	33.3	33.3	Liu *et al.* (unpublished)	Tong *et al.* (2002) M=7
Labeo rohita (F. Cyprinidae)	*L. calbasu*	0.53	-	91.7	-	Shukla *et al.* (unpublished), Liao *et al.* (unpublished) and Liu *et al.* (unpublished)	Das *et al.* (2005) M=12
	Cirrhinus mrigala	9.26	-	75	-		
	Ctenopharyngodon idella	7.41	-	66.7	-		
	Cyprinus carpio	3.97	-	16.7	-		
Lipophrys pholis (F. Blenniidae)	*L. trigloides*	3.27	3.80	60	-	Almada *et al.* (2005)	Guillemaud *et al.* (2000) M=5
	L. canevai	7.90	7.60	80	-		
	Parablennius gattorugine	10.63	9.29	0	-		
	P. pilicornis	11.99	8.23	20	-		
	P. ruber	10.63	9.70	0	-		
	Salaria fluviatilis	11.17	9.49	60	-		
	S. pavo	14.71	12.45	60	-		
	Ophioblennius atlanticus	12.81	12.45	40	-		
	Coryphoblennius galerita	5.99	4.85	60	-		
Oncorhynchus mykiss (F. Salmonidae)	*O. clarki*	-	3.45	81.6	-	Bernales *et al.* (unpublished), Doiron *et al.* (2002), Arnason *et al.* (unpublished) and Zardoya *et al.* (1995)	Palti *et al.* (2002) M=38
	O. tshawytscha	2.02	4.14	79.4	-		
	O. nerka	-	4.83	63.1	-		
	Salmo salar	4.03	7.59	55.3	-		
	Salvelinus alpinus	3.61	4.14	57.9	-		
Mycteroperca microlepis (F. Serranidae)	*Epinephelus marginatus*	-	7.57	87.5	87.5	Craig *et al.* (2001) and present study	Chapman *et al.* (1999) and De Innocentiis *et al.* (2001) M=8

(CSA AL) percentage of cross-species amplifiable microsatellite loci; (CSA PL) percentage of cross-species polymorphic microsatellite loci; (M) number of microsatellite loci analysed in each CSA species group; (-) no data available. All target species belong to the same genus or family as their source species (except *Apogon imberbis* in Serranidae).

Divergència genètica com a indicador de l'amplificació creuada de loci microsatèl·lits entre espècies i el manteniment del seu polimorfisme en peixos.

Els microsatèl·lits, degut al seu elevat polimorfisme, són considerats com els marcadors més indicats per a utilitzar en una ampla varietat d'estudis genètics, evolucionaris i ecològics. D'altra banda, la seva elevada especificitat esdevé un obstacle al seu ús generalitzat, ja que l'èxit de l'amplificació creuada de loci microsatèl·lits entre espècies és limitat. En molt estudis que impliquen aquest tipus d'amplificació entre espècies, els encebadors dissenyats per una espècie (origen) són utilitzats per amplificar loci homòlegs en espècies properes (objectiu). Però no sembla gaire clar com de properes han de ser aquestes espècies. La divergència genètica és una via clara i fàcil per tal d'assignar el grau de similitud entre espècies, procurant una mesura acurada de la seva distància evolutiva. Així doncs, per tal d'assignar la divergència genètica entre espècies, s'han escollit dos gens, 12S rRNA i 16S rRNA, degut al seu ús extensiu en filogènies i anàlisis evolucionaries. Es van testar dotze parelles d'encebadors dissenyats per *Serranus cabrilla* (Pisces: Serranidae) en vuit espècies objectiu també mediterrànies i de la mateixa família. S'han trobat correlacions negatives i altament significatives entre la divergència genètica i l'amplificació creuada de loci microsatèl·lits entre espècies, així com del manteniment del seu polimorfisme. L'informació obtinguda a partir d'altres estudis realitzats també en peixos va permetre quantificar l'èxit de l'amplificació creuada de loci microsatèl·lits entre espècies de peixos i el manteniment del seu polimorfisme, calculant les equacions de regressió que millor s'ajustaven a cada correlació pels dos gens escollits independentment. L'èxit de l'amplificació creuada de loci microsatèl·lits entre espècies és del 50% quan la divergència genètica entre les espècies origen i objectiu és d'un 7.30% pel 12S o 9.03% pel 16S. A més, si es vol obtenir un èxit del 50% de loci amplificables i polimòrfics la divergència genètica entre espècies no ha de ser superior al 4.35% pel 12S o al 6.39% pel 16S.

3.3.- Estructura poblacional, autoreclutament i dispersió larvària

Publicació 6: Population structure within and between subspecies of the Mediterranean triplefin fish *Tripterygion delaisi* revealed by highly polymorphic microsatellite loci

Publicació 7: High self-recruitment levels in a Mediterranean littoral fish population revealed by microsatellite markers

Publicació 8: Early life-history characteristics predict genetic differentiation in Mediterranean fishes

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Population structure within and between subspecies of the Mediterranean triplefin fish *Tripterygion delaisi* revealed by highly polymorphic microsatellite loci

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Abstract

Although F_{ST} values are widely used to elucidate population relationships, in some cases, when employing highly polymorphic loci, they should be regarded with caution, particularly when subspecies are under consideration. *Tripterygion delaisi* presents two subspecies that were investigated here, using 10 microsatellite loci. A Bayesian approach allowed us to clearly identify both subspecies as two different evolutionary significant units. However, low F_{ST} values were found between subspecies as a consequence of the large number of alleles per locus, while homoplasy could be disregarded as indicated by the standardized genetic distance G'_{ST}. Heterozygosity saturation was observed in highly polymorphic loci containing more than 15 alleles, and this threshold was used to define two loci pools. The less variable loci pool revealed higher genetic variance between subspecies, while the more variable pool showed higher genetic variance between populations. Furthermore, higher differentiation was also observed between populations using G'_{ST} with the more variable loci. Nonetheless, a more reliable population structure within subspecies was obtained when all loci were included in the analyses. In *T. d. xanthosoma*, isolation by distance was detected between the eight analysed populations, and six genetically homogeneous clusters were inferred by Bayesian analyses that are in accordance with F_{ST} values. The neighbourhood-size method also indicated rather small dispersal capabilities. In conclusion, in fish with limited adult and larval dispersal capabilities, continuous rocky habitat seems to allow contact between populations and prevent genetic differentiation, while large discontinuities of sand or deep-water channels seems to reduce gene flow.

Keywords: Atlantic–Mediterranean transition, high polymorphism, homoplasy, microsatellites, subspecies, *Tripterygion*

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Introduction

In marine environments, connectivity between populations occurs mainly throughout adult movements and/or larval dispersion (Palumbi 2003). Approximately 70% of the marine organisms show a planktonic stage in which larvae can widely disperse before recruiting into the adult habitat (Thorson 1950). However, other factors such as currents, larval retention and the type of reproduction or habitat preferences of the species also affect population dynamics. Hence, population structure results from many factors and the importance of each factor changes, depending on the species (Muss *et al*. 2001; Stockley *et al*. 2005).

In fishes, species with high adult mobility or long larval periods tend to have significant gene flow between populations (Broughton & Gold 1997). On the other hand, those species with low adult/larval dispersion may show a higher isolation structure (Doherty *et al*. 1995; Riginos & Victor 2001). However, these studies are still scarce and there is no general consensus about the importance of environmental barriers and limited dispersal on genetic differentiation between populations (Bernardi *et al*. 2003; Taylor & Hellberg 2003). Furthermore, the knowledge of gene flow between populations is fundamental for either accurate management

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of resources or for designing more sustainable marine reserves (Palumbi 2003; Bell & Okamura 2005).

The western Mediterranean is an interesting geographical area characterized by a particular geomorphology and associated oceanography mostly conditioned by the inflow/outflow of water throughout the Gibraltar Strait (Hopkins 1985). The particular water circulation generates several oceanographic fronts and barriers for dispersal located from Oran (Morocco) to Almeria (Spain) and the Balearic Islands (Spain) (Tintoré *et al*. 1988; García-Ladona *et al*. 1996; Font *et al*. 1998). These fronts, as well as the influence of the Mediterranean and Atlantic waters, determine the distribution of numerous species (Abelló *et al*. 2002) and the gene flow between populations (Quesada *et al*. 1995; Naciri *et al*. 1999; Bargelloni *et al*. 2005).

In the Mediterranean Sea, numerous littoral species are highly territorial and are restricted to very specific habitats (Macpherson 1994; Guidetti *et al*. 2004). Among these species, those belonging to the genus *Tripterygion* (Family Tripterygiidae) are representatives of rocky shores and, although they have no commercial importance, they can serve as a model species for the study of population structuring in littoral rocky habitats.

Tripterygion delaisi is a common littoral fish in the Mediterranean Sea, always living in rocky habitats, preferentially in biotopes of reduced light, between 6 and 12 m (Zander 1986). Adult individuals are highly territorial, showing high levels of homing behaviour and parental care of the eggs, and they cannot swim even short distances (tens of metres) in open water or on sandy bottoms (Heymer 1977; Wirtz 1978). Larvae of *T. delaisi* remain in plankton for 16–21 days (Raventós & Macpherson 2001), although they are present almost exclusively in coastal waters (Sabatés *et al*. 2003). This suggests that larvae remain close to adult habitats, as reported in species belonging to this family native to reef areas (Leis 1982; Kingsford & Choat 1989; see also Hickford & Schiel 2003). Thus, the dispersal capability of *T. delaisi* might be low, consequently constituting an interesting model for investigating differentiation in marine fishes.

Two subspecies are currently accepted in *T. delaisi*: *T. d. xanthosoma* and *T. d. delaisi*. Morphological differences between subspecies are marginal and only statistically different when large samples are compared (Wirtz 1980). However, they can be differentiated easily during courtship because *T. d. delaisi* males do a figure-of-eight-swim upward toward the surface, while *T. d. xanthosoma* do this only on the bottom (Zander 1986). According to mitochondrial DNA (mtDNA) sequences, the two subspecies can be clearly identified and diverged *c.* 1.2 million years ago (Carreras-Carbonell *et al*. 2005). *T. d. delaisi* has been found on the Atlantic coast from southern England to Senegal, including the Macaronesian Islands, whereas *T. d. xanthosoma* is distributed along the Mediterranean Sea (Zander 1986). Recent studies, using mtDNA, have detected *T. d. xanthosoma* individuals in Atlantic populations (Carreras-Carbonell *et al*. 2005). However, the number of individuals analysed was small and the presence of mixed subspecies populations could not be discarded as a hypothesis.

Microsatellites are highly polymorphic nuclear loci that have been successfully used to infer population differentiation at different geographical scales. It is known that microsatellite markers show great variability within fish species, and particularly within marine ones (DeWoody & Avise 2000). They have been used to elucidate the degree of introgression between different subspecies (Pérez *et al*. 2002; Hille *et al*. 2003; Lorenzen & Siegismund 2004). They have also been widely employed to solve population structuring on a wide range of geographical levels (Appleyard *et al*. 2001; Rico & Turner 2002; Carlsson *et al*. 2004). Different markers, such as microsatellites and allozymes, sometimes yield different levels of genetic differentiation between populations, resulting in a higher power of statistical differentiation of microsatellites due to their higher polymorphism (Estoup *et al*. 1998). However, microsatellite loci with moderate or high polymorphism might yield different population structuring. Estimates of F_{ST} seem to decline with locus polymorphism, and this loss has been attributed to the effect of size homoplasy (O'Reilly *et al*. 2004). Nonetheless, high polymorphism can also produce small F_{ST} values even when alleles are not shared between populations (Balloux & Lugon-Moulin 2002). Consequently, the use of low and high polymorphic loci should be treated with caution and deserves some attention.

The aim of this study is to assess the population structure of *T. delaisi* using 10 *T. d. xanthosoma* polymorphic microsatellite loci. The sampling design will allow us to detect the level and pattern of differentiation within populations, within subspecies and between subspecies, and to define barriers for dispersal. Finally, the use of loci with different levels of polymorphism will allow us to explore the effect of locus polymorphism in estimating population differentiation, and the sequencing of different microsatellite alleles will contribute to better understand microsatellite evolution.

Materials and methods

Sampling and DNA extraction

A total of 283 individual specimens were collected by scuba divers using hand nets from eight coastal or island localities in the Spanish Mediterranean and two groups of Atlantic islands (Canaries and Azores) (Fig. 1). Populations were assigned to *Tripterygion delaisi xanthosoma* and *T. d. delaisi* subspecies according to mtDNA sequences (Carreras-Carbonell *et al*. 2005). Geographical distances between populations can be found in Table S1 (Supplementary material). In two Mediterranean island localities, Columbretes Island and Formentera Island, we sampled two locations (referred

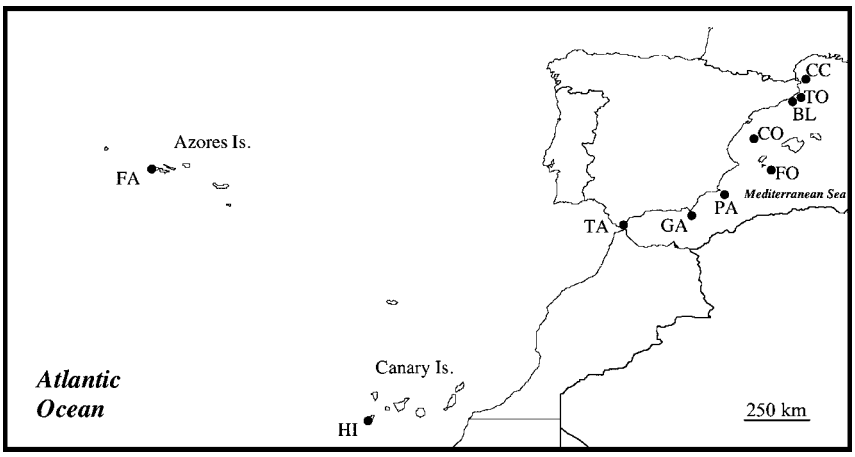

Fig. 1 Sampling stations of *Tripterygion delaisi xanthosoma*: Cap de Creus (CC), Tossa (TO), Blanes (BL), Columbretes Is. (CO), Formentera Is. (FO), Cabo de Palos (PA), Cabo de Gata (GA) and Tarifa (TA), and *T. d. delaisi*: Canary Is.-Hierro (HI) and Azores Is.-Faial (FA).

to as subpopulations) separated by a sandy bottom-water channel of 300 and 50 m deep, respectively, and *c*. 400 m wide for both localities. These channels cannot be crossed by adult individuals, since they always swim near the bottom and cannot reach this depth (Zander 1986).

A small portion of the anal fin was removed from living fish and preserved in absolute ethanol at room temperature; fishes were then measured and returned to the sea. In order to avoid temporal variability within and between locations, we only caught adult reproductive individuals of similar size and sampled all populations during the same year (2003). Total genomic DNA was extracted from fin tissue using the Chelex 10% protocol (Estoup *et al*. 1996).

PCR amplification and screening

We used the polymorphic microsatellite loci isolated from *T. delaisi* with the exception of locus Td03, which was excluded because amplifications were poor and allele-sizing misleading (Carreras-Carbonell *et al*. 2004). An extra locus (Td05) was included (EMBL Accession no. AJ971942) that can be amplified with the forward primer 5′-AATCGGACCAGCCGTAATCT-3′ and the reverse primer 5′-CCGAACTGTCACCCAAAAG-3′ at 55 °C annealing temperature. Polymerase chain reactions (PCRs) were carried out under conditions described in Carreras-Carbonell *et al*. (2004). Amplified products were scored using an ABI 3700 automatic sequencer from the Scientific and Technical Services of the University of Barcelona. Alleles were sized by GENESCAN™ and GENOTYPER™ software, with an internal size marker CST Rox 70–500 (BioVentures Inc.).

Statistical analyses

Allele frequencies, mean allelic richness, expected (H_E) and observed (H_O) heterozygosity per locus, and population were calculated using the POP100GENE computer program (available at http://www.ensam.inra.fr/URLB/pop100gene/pop100gene.html). Departures from the Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium were tested for each locus–population combination using GENEPOP version 3.4 (Raymond & Rousset 1995), which employ a Markov chain method with 5000 iterations, following the algorithm of Guo & Thompson (1992). These results were adjusted for multiple tests using the sequential Bonferroni procedure with $\alpha = 0.05$ (Rice 1989). In instances where the observed genotype frequencies deviated significantly from HWE, the program MICRO-CHECKER (Van Oosterhout *et al*. 2004) was used to infer the most probable cause of such HWE departures.

Genetic divergence between subspecies, populations and subpopulations was estimated by computing the classical F_{ST} approach (Wright 1951; Weir & Cockerham 1984). A structured analysis of molecular variance (AMOVA) was carried out to assess the component of genetic diversity attributable to (i) variance among subspecies; (ii) variance among populations within subspecies; and (iii) variance within populations. The program ARLEQUIN version 2.0 (Schneider *et al*. 2000) was used to carry out all the analyses mentioned above. In order to assess the effect of highly variable microsatellite loci, we independently plotted the expected heterozygosity vs. the number of alleles per locus for each population and locus to classify loci by their variability. Two pools of loci were created as a function of their variability, and the analysis of variance and F_{ST} values were estimated for each pool independently. Furthermore, additional analyses, also for these two loci pools independently and for all loci together, were performed in order to calculate the standardized genetic differentiation measure (G'_{ST}) proposed by Hedrick (2005), since the interpretation of genetic differentiation values could be problematic because of their dependence on the level of genetic variation. With this measure, the magnitude is the proportion of the maximum differentiation possible for the level of subpopulation homozygosity observed. Therefore, this

standardized measure allows comparison between loci pools with different levels of genetic variation.

The program STRUCTURE version 2.0 (Pritchard & Wen 2003) was used to detect the number of genetically homogeneous populations (*K*). The population structure was considered without prior information of the number of locations at which the individuals were sampled and into which location each individual belongs. We performed the analyses twice; once with all data available (*T. d. xanthosoma* and *T. d. delaisi* individuals) and once including only *T. d. xanthosoma* individuals. Following the recommendations of Evanno *et al*. (2005), we calculated an ad hoc statistic ΔK based on the rate of change in the log probability of data between successive *K*-values, since the height of these model values seems to accurately detect a correct estimation of the number of populations. For each data set, 20 runs were carried out in order to quantify the SD of the likelihood of each *K*. We tested a range of *K* values between 1 and 14.

The chord distance (Cavalli-Sforza & Edwards 1967) was used to reconstruct a population tree using the neighbour-joining algorithm implemented in the program POPULATIONS version 1.2.28 (Langella 2002). Branch node support was estimated over 1000 bootstraps performed with the re-sampling locus.

The correlation between pairwise multilocus distances ($X_{ST}/1 - X_{ST}$) and geographical distance (Ln distance) was assessed in *T. d. xanthosoma* populations using the Mantel permutation test (10 000 permutations; Mantel 1967) implemented in GENEPOP, where X_{ST} can be F_{ST} or G'_{ST}. The geographical distance in kilometres was computed as the coastline distance between continental sample locations, and as the straight geographical distance for island populations. In order to estimate the average dispersal distance of individuals during one generation time or the radius of the spatial area within which the population can be considered panmictic (2σ), we used the formula introduced by Wright (1946, 1969) for a continuously distributed population of randomly mating individuals distributed in a two-dimensional habitat: $N_s = 4\pi\sigma^2 D$. The neighbourhood size (N_s) is the number of mating individuals and can be calculated as the inverse of the regression slope between ($F_{ST}/1 - F_{ST}$) and geographical distance (Rousset 1997), and *D* is the density of breeding individuals. A generation time of 1 year was assumed for *T. delaisi*, since they reach maturity when they are 1 year old (De Jonge & Videler 1989).

Recent bottlenecks, either by founding events or by selection, can be detected by the depletion of allele number and heterozygosity excess. To determine whether a population exhibits a significant number of loci with heterozygosity excess, we used the Wilcoxon test implemented in the program BOTTLENECK version 1.2.02 (Piry *et al*. 1999). This seems the most appropriate since it provides the highest power for the number of loci involved (Piry *et al*. 1999; Maudet *et al*. 2002). Computations were based on the infinite allele model (IAM), the stepwise-mutation model (SMM) and the two-phase mutation model (TPM) using default settings.

Results

Genetic variability

High genetic variability has been found in *Tripterygion delaisi* both in terms of extensive polymorphism per population and locus (mean allelic richness = 14.17 ± 0.74) and of high expected (0.814 ± 0.018) and observed (0.772 ± 0.019) heterozygosities (Table S2, Supplementary material-). The mean number of alleles and the expected heterozygosity per population were significantly greater (Wilcoxon test, $Z = 2.40$ and $Z = 2.39$, respectively, $P < 0.05$) for *T. d. xanthosoma* (15.09 and 0.841) than for *T. d. delaisi* (10.50 and 0.707). No linkage disequilibrium between loci was observed in any of the populations, thus the 10 loci were considered statistically independent. Private alleles were present in all populations, indicating that the percentage in *T. d. delaisi* populations (mean = 19.28%) was significantly larger than in *T. d. xanthosoma* populations (mean = 4.35%), as assessed with the Mann–Whitney *U*-test ($Z = 2.09$, $P < 0.05$).

Significant departures from HWE were observed in most localities, with the exception of PA and GA in the Mediterranean, and FA in the Atlantic. When all loci where analysed separately, we observed that the departure was due to loci Td08 and Td09 in Mediterranean localities, and to locus Td05 in the Atlantic. The MICRO-CHECKER software detected the presence of null alleles in locus Td05 in HI, in locus Td08 in CC, BL, CO, FO and TA (with its frequency ranging from 0.13 to 0.49), and also in locus Td09 (with frequencies ranging from 0.10 to 0.17 in TO, BL, CO, FO and PA). Null alleles appear when one allele is unamplified due to mutations in the sequence where one of the primers was designed, and/or when technical problems associated with amplification and scoring arise (Hoarau *et al*. 2002). Technical issue could be ruled out because all homozygous individuals and failed amplifications for loci Td05, Td08 and Td09 were re-amplified, twice lowering the annealing temperature to 50 °C, and because accurate scoring of larger alleles with poor amplification was carried out.

In spite of the lower genetic variability in *T. d. delaisi* populations, no recent bottlenecks were detected under the TPM or SMM models in any of the populations analysed. No bottlenecks were detected, either, in *T. d. xanthosoma* populations under the same mutation models. Under the IAM, recent bottlenecks were detected in four Mediterranean (BL, FO, PA and GA) populations and in one Atlantic (FA) population. When the least polymorphic and the most polymorphic loci were used independently, no recent bottlenecks were detected under the TPM or SMM models in any of the populations analysed. However, recent bottlenecks

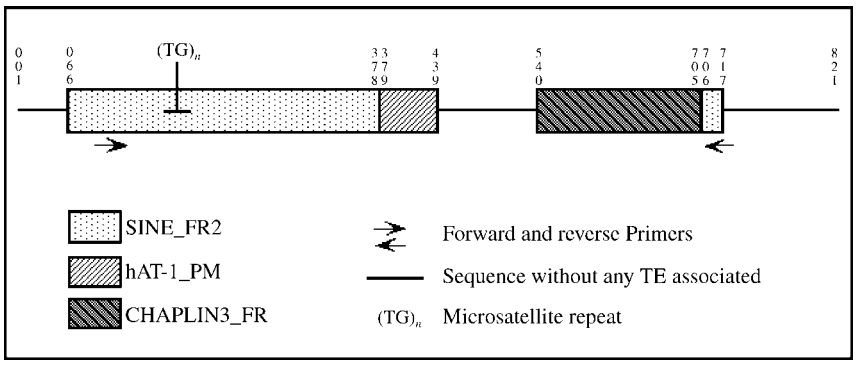

Fig. 2 Distribution of transposable elements (TEs) inserted in the 'large allele' from locus Td09.

were observed under IAM in five Mediterranean (BL, FO, PA, GA and TA) populations and one Atlantic (FA) population for the high polymorphic loci pool, whereas only the GA population showed significant evidence of a recent bottleneck under the IAM model when using only the least polymorphic loci. Given that the same results were obtained with the TPM and SMM models, this indicated that these mutation models better fitted the microsatellite evolution in *T. delaisi* and, consequently, that no recent bottleneck had occurred in any of the sampled localities.

Diagnostic loci between subspecies

Locus Td06 can be considered diagnostic between subspecies, since only odd alleles were found in *T. d. xanthosoma*, whereas only even alleles were present in *T. d. delaisi* (see Table S3, Supplementary material). In order to reject an erroneous allele size designation, and to demonstrate the reliability of these results, we cloned and sequenced three different Td06 alleles from *T. d. xanthosoma* (accession nos AM087633–35) and two from *T. d. delaisi* (accession nos AM087636–37), since direct sequencing was impossible due to the large number of repeats and reduced flanking sequence. Differences in allele size mainly resulted from the number of repeats in the microsatellites, although differences in the flanking regions were observed between the Mediterranean and Atlantic sequences. A TC insertion was detected in the Atlantic sequences at the end of the microsatellite repeat, while a C insertion was observed in the Mediterranean sequences close to the reverse primer, thus resulting in a base-pair difference (Table S4, Supplementary material).

We also sequenced several alleles for locus Td05 (3 alleles) and Td07 (2 alleles), since these two loci showed different size ranges between both subspecies (Table S2), which could be attributed to indels in the flanking region. In locus Td05, allele sizes ranged from 233 to 301 for *T. d. xanthosoma*, whereas a higher and wider range was found for *T. d. delaisi* (253–463 bp). In locus Td07, two nearly nonoverlapping ranges were observed between subspecies, *T. d. xanthosoma* ranging from 107 to 123 bp and *T. d. delaisi* ranging between 121 and 141 bp. No indels were found in those alleles sequenced consequently, with size differences in both loci only attributable to variations in the number of repeats.

Microsatellite loci within transposable elements

Locus Td09 featured a large allele that could be visualized in the agarose gel to an approximate size of 600 bp, but which could not be further identified since the internal standard used only permitted sizing between 70 and 500 bp. This large allele was sequenced for some individuals in both subspecies: *T. d. xanthosoma* [three individuals from BL (accession nos AM087638–40), two from GA (accession nos AM087641–42), and one from TA (accession no. AM087643)]; *T. d. delaisi* [two individuals from FA (accession nos AM087644–45) and one from HI (accession no. AM087646)]. These were then compared to the reference collection of vertebrate repeats on the Censor Web server (http://www.girinst.org; Jurka *et al*. 1996). We found that normal alleles from locus Td09 matched the repetitive element SINE_FR2 (75%) described in the tetraodontidae fish *Takifugu rubripes*. The large allele had two additional transposable elements (TEs) inserted, CHAPLIN3_FR (90%) and hAT-1_PM (91%), as well as a nonautonomous DNA transposon linked to CHAPLIN3_FR (Fig. 2). All sequenced alleles always presented these two TEs inserted in the same place regardless of population and subspecies. However, differences in the number of repeats were observed between these sequences. The two individuals from GA and the two from BL showed $(TG)_{11}$, while the other specimen from BL only had $(TG)_9$ repeats, and the two individuals from FA presented $(TG)_8$ repeats. Finally, imperfect microsatellite repeats were observed in the specimen from TA $[(TG)_5AAG(TG)_3]$ and HI $[(TG)_4TAGTGA(TG)_2]$. Furthermore, point mutations in the flanking region were also detected with a mean nucleotide diversity of 1.41 ± 0.29%. Nonetheless, all large alleles visualized in agarose gel were sized at 600 bp and included in the analysis. The remaining loci showed no similarity with known TEs.

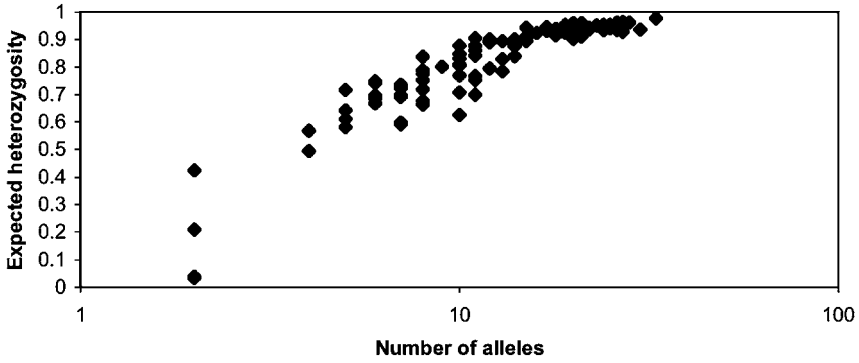

Fig. 3 Scatter plot of expected heterozygosity v. number of alleles across 10 loci in eight *Tripterygion delaisi xanthosoma* and two *T. d. delaisi* populations. The arrow indicates the heterozygosity value when the number of alleles is 15.

Low variability loci in Tripterygion delaisi delaisi

In *T. d. delaisi* populations, low polymorphism was found for Td09 and Td11 loci (seeTables S2 and S3). To infer the evolutionary processes affecting the low variability in this area, a few alleles for each locus were sequenced. Locus Td11 showed only two alleles per population: a highly common one (287; 0.983 in HI and 0.981 in FA) and a low frequency one (283, 0.017 in HI and 289, 0.019 in FA). However, *T. d. xanthosoma* populations showed a mean of 9.1 alleles for this locus. We sequenced two 287-alleles (one from HI and one from FA); both gave the same sequence with a perfect number of repeats (13 repeats; accsession nos AM087647–48). On the other hand, locus Td09 also presented only two alleles, one with a greater proportion than the other (298, 0.883 in HI and 0.704 in FA; 600, 0.117 in HI and 0.296 in FA), whereas *T. d. xanthosoma* populations presented a mean of seven alleles. We sequenced the 298-allele (one from HI and one from FA); the sequences of the flanking regions were identical (accession nos AM087649–50) between them, as well as with the sequence of the *T. d. xanthosoma* clone. However, an insertion was found in *T. d. delaisi* individuals that disrupted the repeat sequence [$(TG)_5$AGTA$(TG)_2$].

Locus variability and population differentiation

The scatter plot of the expected heterozygosity vs. the number of alleles per locus showed that there was a significant positive relationship between N_A and H_E (R^2 = 0.81, $P < 0.05$), although H_E remained constant when the number of alleles per locus was greater than 15 (Fig. 3). Due to heterozygosity saturation, we regarded 15 alleles as the separation value between more polymorphic and less polymorphic loci pools. Three loci pools were established, (i) one with the most variable loci (Td04, Td05, Td06, Td08 and Td10), having a mean across all populations of more than 15 alleles per locus (19.9 alleles per locus and mean H_E = 0.921); (ii) another with the least variable (Td01, Td02, Td07, Td09 and Td11), having less than 15 alleles per locus (8.4 alleles per locus and mean H_E = 0.708); and (iii) finally all loci together. We analysed each one separately in order to assess how locus variability affects the interpretation of the population structure in *T. delaisi*. The number of repeats in the least variable loci based on an average of all populations inferred from comparisons to the clone size ranged from 6.2 ± 2.5 to 26.2 ± 2.6, whereas in the most variable loci the number of repeats ranged from 7.6 ± 2.9 to 72.6 ± 13.1. The longest allele was estimated to have 124 repeats (locus Td05), with no repeats occurring in the smallest allele of loci Td10 and Td11, although indels in the flanking region cannot be fully discarded since not all alleles were sequenced.

Hierarchical analysis of molecular variance (Excoffier *et al*. 1992) revealed that most of the genetic variance was attributable to variations within sampled populations. Moreover, only a small amount of variance was explained by the differences among populations within subspecies (Table 1). We did observe, however that the source of variation was highest within populations with the most variable loci, while much larger variations were explained by differences between populations within subspecies with less variable loci. Nonetheless, all differences were statistically significant regardless of the pool used.

Multilocus F_{ST} estimates were calculated for each pairwise population comparison thrice, using a different loci pool each time (Table 2 and Table S5, Supplementary material). A high degree of differentiation between subspecies was found for all three loci pools. F_{ST} values between subspecies ranged from 0.053 to 0.096 for the most polymorphic loci pool, whereas values ranged from 0.191 to 0.326 when the least polymorphic loci pool was used. On the other hand, when the standardized genetic differentiation measure (G'_{ST}) was estimated, we found that the most variable loci pool showed greater G'_{ST} values than when using all loci together and even more when only the least variable loci pool was used. The two *T. d. delaisi* populations (HI and FA) were significantly differentiated independently of the loci pool used; the F_{ST} value was 0.056 for the most variable loci pool, 0.078 for the least variable loci pool, and 0.066 for all loci together (Table 2 and Table S5). Within *T. d. xanthosoma*, the two samples collected 400 m apart

Table 1 Analysis of molecular variance for *Tripterygion delaisi* sp.

	All loci		Loci with > 15 alleles		Loci with < 15 alleles	
Source of variation	Ss	%	Ss	%	Ss	%
Among subspecies	110.13	12.86*	17.31	3.65*	92.70	20.63*
Among populations/within subspecies	85.64	3.00**	34.68	2.30**	50.32	3.52**
Within populations	1982.77	84.14**	1014.75	94.04**	968.89	75.85**

Ss, sum of squares; %, percentage of variation; significance levels, *$P < 0.05$ and **$P < 0.005$.

Table 2 Multilocus F_{ST} (below diagonal) and G'_{ST} (above diagonal) values between population pairs using all loci together

All loci	CC	TO	BL	CO	FO	PA	GA	TA	HI	FA
CC		0.249	0.187	0.351	0.291	0.290	0.277	0.368	0.741	0.670
TO	0.004		0.185	0.340	0.253	0.331	0.283	0.383	0.797	0.660
BL	0.002	–0.001		0.285	0.255	0.257	0.265	0.322	0.747	0.655
CO	**0.043**	**0.033**	**0.029**		0.274	0.270	0.299	0.341	0.769	0.722
FO	**0.025**	**0.014**	**0.015**	**0.010**		0.261	0.286	0.302	0.781	0.701
PA	**0.028**	**0.028**	**0.021**	**0.036**	**0.028**		0.259	0.393	0.803	0.721
GA	**0.016**	**0.009**	**0.010**	**0.037**	**0.028**	**0.022**		0.338	0.763	0.783
TA	**0.045**	**0.043**	**0.041**	**0.066**	**0.043**	**0.066**	**0.046**		0.746	0.687
HI	**0.163**	**0.171**	**0.171**	**0.205**	**0.183**	**0.211**	**0.186**	**0.181**		0.375
FA	**0.139**	**0.131**	**0.138**	**0.170**	**0.150**	**0.176**	**0.151**	**0.153**	**0.066**	

Bold F_{ST} values are significantly greater than zero ($P < 0.05$). Population abbreviations are as in Fig. 1.

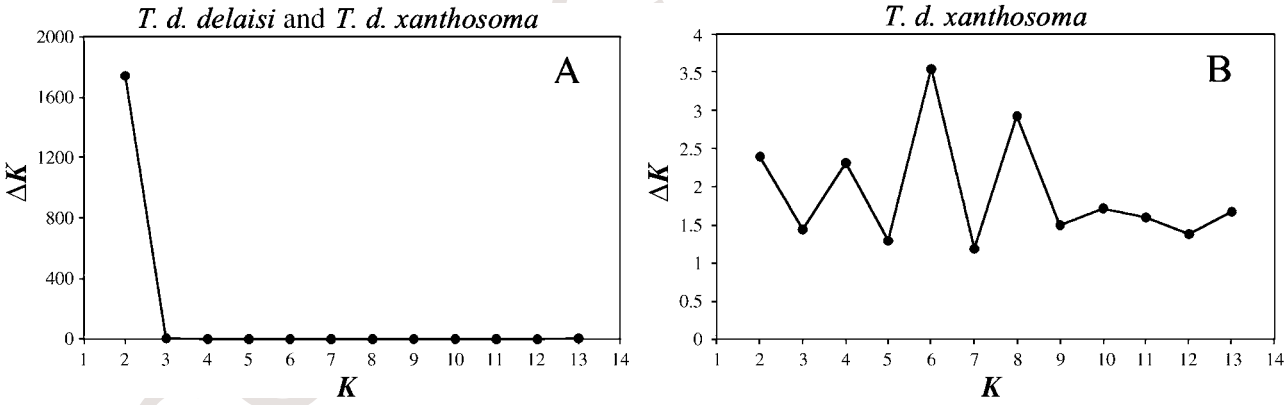

Fig. 4 Values of Δ*K* calculated as in Evanno *et al.* (2005) for each number of genetically homogeneous populations assumed (*K*), including all *Tripterygion delaisi delaisi* and *T. d. xanthosoma* individuals (A) and only including *T. d. xanthosoma* individuals (B).

in CO (F_{ST} = 0.006; $P > 0.05$) and FO (F_{ST} = –0.001; $P > 0.05$) could be considered homogeneous since genetic differentiation was found neither for all loci together, nor for the other two loci pools. Similar values for deviations from Hardy–Weinberg proportions were obtained when both subsamples were grouped or analysed independently. Furthermore, the populations of CC, TO and BL were not significantly differentiated using any of the three loci pools. In fact, no significant differentiation was observed between CC, TO, BL and GA; between CO and FO; or between PA, TO and BL when only the least variable loci were used.

To estimate the number of genetically homogeneous populations sampled in our study, we used the program STRUCTURE without prior information of the number of locations at which the individuals were sampled. When individuals of both subspecies were analysed together, only two genetically homogeneous clusters were detected (Fig. 4A), since a peak in Δ*K* was shown for *K* = 2, grouping

T. d. delaisi and *T. d. xanthosoma* individuals separately. The height of ΔK was used as an indicator of the strength of the signal detected by STRUCTURE (Evanno *et al*. 2005). When only *T. d. xanthosoma* individuals were included in the analyses the signal was smaller with bigger variances among replicates. Nonetheless, ΔK showed the largest peak at $K = 6$ (Fig. 4B), detecting six genetically homogeneous clusters in *T. d. xanthosoma*.

Significant associations between genetic differentiation (F_{ST}) and geographical distance in the *T. d. xanthosoma* samples was revealed by a Mantel test (Spearman's $R = 0.47$, $P = 0.047$), although only when using all loci, whereas it failed to detect isolation by distance when the least polymorphic and the most polymorphic loci were used independently. However, when using G'_{ST} as genetic distance, isolation by distance was detected by the Mantel test using all loci together ($R = 0.59$, $P = 0.005$) and both more ($R = 0.53$, $P = 0.01$) and less ($R = 0.42$, $P = 0.043$) variable loci pools. The regression of genetic distance (F_{ST}) against the log of distance between *T. d. xanthosoma* populations, using all loci together, gave a slope of 0.0097, corresponding to a neighbourhood size (N_s) of 103 individuals. The mean density of breeding adults in the area of study is 0.0192 individuals/m² (Macpherson *et al*. 2002) Thus, using 1 year as the generation time and substituting in the equation presented in the Material and methods section, we estimated that the average dispersal distance of individuals was 41.3 m/year.

The neighbour-joining tree clearly showed a high degree of genetic differentiation between *T. d. delaisi* and *T. d. xanthosoma* populations, with a 100% bootstrap value support between both clades. Within *T. d. xanthosoma*, CO–FO- and PA–GA-pairs formed two well-supported clusters separated from the other Mediterranean populations by 77% and 86% bootstrap values, respectively (Fig. 5).

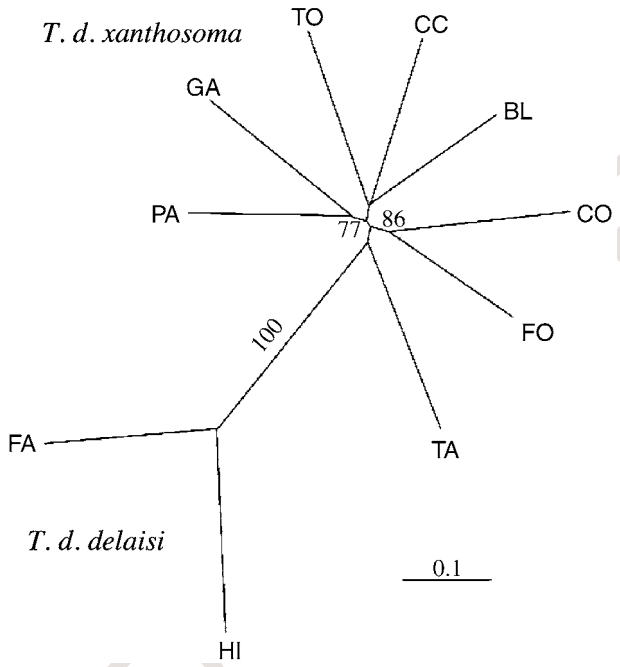

Fig. 5 Neighbour-joining phylogenetic tree (unrooted) for the 10 populations of *Tripterygion delaisi* sp. based on the data from 10 microsatellite loci using chord distance from Cavalli-Sforza & Edwards (1967). Only bootstrap values above 60% are shown. See Fig. 1 for the population abbreviations.

Discussion

Effects of locus polymorphism on population differentiation

Tripterygion delaisi shows a high degree of polymorphism (mean allelic richness = 14.17 ± 0.74) consistent with the mean number of alleles observed in other fish species (DeWoody & Avise 2000). We have found a negative and highly significant correlation between F_{ST} and both H_E ($R^2 = 0.82$, $P < 0.05$) and N_A ($R^2 = 0.72$, $P < 0.05$), showing an inverse relationship between locus polymorphism and F_{ST} values. Recent studies have reported similar results with less polymorphic allozymes and moderately polymorphic microsatellite loci, showing significantly greater estimates of F_{ST} values than highly polymorphic microsatellite loci (Freville *et al*. 2001; Olsen *et al*. 2004; O'Reilly *et al*. 2004).

Highly polymorphic loci, with high mutation rates, may reduce the degree of genetic divergence between populations when using typical measures of differentiation such as F_{ST}. According to Estoup *et al*. (2002), size homoplasy within a determined species does not pose a significant problem for many types of population genetic analyses, while the large amount of variability observed in microsatellite loci often largely compensates for their homoplasious evolution. However, when using molecular markers with a high mutation rate between distantly related subspecies, size homoplasy has been extensively noted (Estoup *et al*. 1995). O'Reilly *et al*. (2004) suggest that size homoplasy, rather than the effects of locus polymorphism per se, limits the resolution in populations with weak genetic structures. In the *T. delaisi* locus Td06, no allele was shared between subspecies, although it was the most polymorphic locus, with a mean richness of 22.2 alleles per population (see Results and Table S4). The small but significant F_{ST} value (0.0548; $P < 0.05$), when comparing both subspecies with this locus, indicates that the large number of alleles, and not homoplasy, is responsible for the low F_{ST} value. When estimating G'_{ST} for that locus between subspecies, the maximum difference value was obtained ($G'_{ST\text{-}td06} = 1$, data not shown). However, for other loci, in which alleles were shared between subspecies, size homoplasy could also be a complementary explanation for the low F_{ST} values

when highly polymorphic loci are used, as they provide a false sense of similarity between populations. To ascertain whether homoplasy was responsible for this similarity, we established two groups of loci; one with shared alleles (loci sharing was more than 20% of alleles between subspecies) and the other with nonshared alleles (loci sharing was less than 20% of alleles between subspecies). When we compared highly polymorphic loci between subspecies sharing (Td04 and Td10; mean $F_{ST} = 0.05$) and nonsharing alleles (Td05, Td06 and Td08; mean $F_{ST} = 0.05$), we found that mean F_{ST} values were both low and identical ($Z = 0$, $P = 1$), indicating that homoplasy does not explain this similarity. However, low polymorphic loci between subspecies showed large differences between loci-sharing alleles (Td01 and Td02; mean F_{ST} 0.08) and nonsharing alleles (Td07, Td09 and Td11; mean F_{ST} 0.29), although this significance was marginal due to the low number of comparisons ($Z = 1.73$, $P = 0.08$). On the other hand, differentiation measures cannot exceed the level of within-subpopulation homozygosity (Hedrick 1999, 2005); consequently the reduction in genetic differentiation for highly polymorphic markers may be a result of the low homozygosity. The standardized G'_{ST} values increased with loci variability; however, differences were not significant between high and low variable loci pools when comparing both subspecies ($Z = 1.3$, $P = 0.17$). Nonetheless, when loci sharing and nonsharing alleles were compared between subspecies, significant differences in G'_{ST} were obtained for both highly variable ($Z = 6.6$, $P < 0.001$) and low variable loci ($Z = 3.1$, $P < 0.001$). To summarize, the similarity in G'_{ST} values between loci with different allele richness indicated that homoplasy would not produce genetic similarity of divergent populations. F_{ST} values decreased with locus polymorphism, while G'_{ST} decreased with the existence of shared alleles between populations. Therefore, genetic differences observed between and within subspecies are not due to size homoplasy.

Hardy–Weinberg equilibrium and null alleles

Deviations from HWE that could be attributed to the presence of null alleles in some loci (Td05, Td08 and Td09) were observed in some populations. Other factors, such as inbreeding or the Wahlund effect, could also explain these deviations, although in this case we would expect many loci to have a heterozygote deficiency, and our results did not match this outcome.

Null alleles are a common problem with microsatellite loci that can yield high heterozygote deficiencies (Callen *et al*. 1993; O'Connell & Wright 1997). In locus Td09, null alleles might be generated by an insertion of one or more TEs in the primer-annealing region, rendering primer annealing and the subsequent allele amplification impossible. The fact that the remaining loci did not present any similarities to known TEs makes it less likely that TEs in the primer annealing regions generated null alleles in locus Td05 and Td08. There have been only a few reports concerning the association of minisatellites with TEs in animals (Eden 1985; Armour *et al*. 1989). The TE (SINE_FR2), found in all Td09 alleles (normal and large) belongs to a superfamily of short interspersed repetitive elements in vertebrates (V-SINEs) and is widespread in fishes and frogs. Each V-SINE includes a central conserved domain, preceded by a 5′-end tRNA-related region and followed by a potentially recombinogenic $(TG)_n$ tract, which is our microsatellite repeat. This domain is strongly conserved as it might be subjected to some form of positive selection, although its functional significance has yet to be confirmed in fishes (Ogiwara *et al*. 2002).

Although microsatellites are neutral markers, they can be linked to a locus under selection, which would result in a decrease in allele richness. The low variability found in the *T. d. delaisi* locus Td11 suggests that the 287-allele might be associated with a region that has recently been positively selected. In spite of the large number of repeats in that allele, low variability was observed in the Atlantic but not in the Mediterranean populations (see Table S3). However, the low variability found for locus Td09 in *T. d. delaisi* populations can also be explained by the disruption of the microsatellite by an insertion in the repeat region, which was present in the sample of colonizers of the Atlantic islands, thereby avoiding slippage. Thus, although selection could also be causing this effect, the reduction in the mutation rate seems to be the most parsimonious explanation of the observed pattern, given that allele number in *T. d. xanthosoma* was high.

Genetic differentiation between Tripterygion delaisi *subspecies*

Microsatellites are widely used to identify hybridization and separation between subspecies. In fishes, Ambali *et al*. (2000) utilized polymorphic microsatellite loci for characterizing *Oreochromis shiranus* subspecies in the Malawi region, which is difficult to distinguish morphologically. In other taxa, Bensch *et al*. (2002) employed microsatellite data to characterize hybrid individuals and hybrid zones between two subspecies of warblers. Using similar methods, Lorenzen & Siegismund (2004) found no hybridization between impala subspecies. The two *T. delaisi* subspecies can be easily differentiated with microsatellite loci, and have been clearly identified as two different genetic units using a Bayesian approach (Pritchard *et al*. 2000) implemented in STRUCTURE . Furthermore, the absence of shared alleles in locus Td06 indicates the lack of gene flow at the nuclear level. When we removed locus Td06 from the STRUCTURE analyses while comparing populations of both subspecies we obtained the same results, indicating a strong structuring and demonstrating the existence of two clearly differentiated

groups that coincide with both subspecies, independently of using or not using this diagnostic locus (data not shown). For locus Td09, the large allele with the same TEs inserted in the same position was observed in the two subspecies, conforming to an ancestral polymorphism generated before subspecies separation. No hybrid populations between subspecies were found, either at the nuclear or at the mtDNA level, and they should thus be treated as two different evolutionary significant units (Hedrick *et al*. 2001). However, more accurate sampling along the continental Atlantic coast should be carried out to discard the hypothesis of the existence of hybrid populations.

4

Genetic structure within subspecies

The significantly lower variability found for *T. d. delaisi* populations (N_A = 10.5 and H_E = 0.707) compared to *T. d. xanthosoma* populations (N_A = 15.1 and H_E = 0.841) both comparing the number of alleles (Z = 2.40, P = 0.016) and heterozygosity (Z = 2.39, P = 0.017) is not explained by a recent bottleneck; nonetheless, it could be due to the founder event during the colonization of the Atlantic islands. A more plausible hypothesis would be consistent with the decrease in loci variability for nonfocal species (Hutter *et al*. 1998), since the primers used were isolated from *T. d. xanthosoma* (Carreras-Carbonell *et al*. 2004).

The two Atlantic populations, belonging to the Azores and Canary Archipelagos, are separated by a great distance (1566 km) and, although now isolated, could have been linked somehow in the past (e.g. during the last glaciation, Ruddiman & McIntyre 1981). Carreras-Carbonell *et al*. (2005) analysed specimens belonging to both populations and found that FA and HI diverged 0.95% for the 12S rRNA gene. According to its evolutionary rate (0.81 ± 0.23%/million years), we established that the divergence time between the two *T. d. delaisi* populations was *c*. 11790 years. This divergence could be linked to the last glaciation, which started 21 000 years ago when sea levels dropped 130 m, and ended 13 000 years ago when sea levels rose again (Ruddiman & McIntyre 1981; Mix *et al*. 2001). The F_{ST} and G'_{ST} values between these two populations might reflect their common ancestry rather than the current gene flow (Table 2).

For *T. d. xanthosoma*, isolation by distance was detected using F_{ST} only when all loci were analysed together (P = 0.04), indicating that all loci might be used to infer population structure using this estimator. Isolation by distance was always found with G'_{ST} regardless of using different loci pools. However, when comparing G'_{ST} values from the most and the least variable loci between populations, highly significant differences were observed (Z = 10.89, P < 0.001) having larger values those comparisons using the most variable loci. These differences were also observed when comparing low and high in loci sharing (Z = 7.06, P < 0.001) and nonsharing alleles (Z = 8.06, P < 0.001). Therefore, homoplasy is not affecting genetic differentiation between populations. Using F_{ST} values, each population of *T. d. xanthosoma* remained isolated from the others with the exception of CC, TO and BL (Table 2). Therefore, we cannot consider these three sampled locations as different and isolated populations, even when using the most variable loci pool. This result is in accordance with the number of inferred populations for this subspecies using a Bayesian approach, since six genetically homogeneous clusters were obtained when all loci were used. However, when excluding one, two or three loci different K values were obtained, ranging from 2 to 9 with lower values of ΔK, resulting in a decrease of the statistical power (data not shown). The same reduced statistical power was attained whether or not there was indication of the presence of null alleles in the excluded loci. Nevertheless, F_{ST} values estimated excluding one, two or three loci (independently of holding null alleles or not) gave the same results as all loci together and indicated no genetic differentiation between CC, TO and BL populations (data not shown). Therefore, for estimating the number of groups with STRUCTURE, in agreement with F_{ST} values, all 10 loci had to be used since genetic differentiation between populations was low.

Genetic estimates of connectivity among marine populations and the determination of dispersal distances and origins of larvae and adults are still hard to establish (Bohonak 1999; Largier 2003). Larvae of *Tripterygion* species are situated inshore, usually between the coastline and *c*. 100 m offshore; consequently they are not observed along large sand beaches or between the continent and offshore islands (Sabatés *et al*. 2003). On the other hand, adults are always in rocky shores, they are highly territorial and cannot swim even short distances (tens of metres) in open water or on sandy bottoms (Heymer 1977; Wirtz 1978). As a consequence, their dispersal capabilities are very reduced, in agreement with the small dispersal distance estimated from the neighbourhood size. These characteristics would support the existence of the high genetic structure among populations of *T. d. xanthosoma*. These genetic breaks, as in other marine organisms, are associated with the presence of barriers to dispersal (Barber *et al*. 2002) that control the gene flow between populations. In the present study, CC and BL populations are separated by 125 km. TO is located between them; 112 km and 13 km apart from CC and BL, respectively. The coastline between these populations is a continuous rocky shore with only small sand gaps (< 15 km). Therefore high levels of gene flow between these populations that prevent genetic differentiation indicate that these sand gaps are not effective barriers for the dispersal of this species. However, significant genetic differentiation exists between populations involving similar geographical distances, such as the CO and FO islands, which are separated by 144 km of deep water; or the PA and GA coastal populations,

5

which are separated by 163 km with a wide zone of sand (32 km) between them. Therefore, large discontinuities (> 30 km) of sand or deep-water channels could be acting as effective barriers, preventing larval and adult exchange between *T. d. xanthosoma* populations. The existence of gene flow barriers has been reported from species with limited (e.g. Rico & Turner 2002 in cichlids of Lake Malawi) and large larval dispersal capabilities (e.g. Barber *et al*. 2002 in stomatopod crustaceans), emphasizing the importance of habitat discontinuities in the population structure of marine organisms.

The inshore distribution of adults and larvae suggests that coastal barriers could mainly shape the population structure of *T. d. xanthosoma*. Nevertheless, oceanographic discontinuities (e.g. currents, upwellings) can also play an important role in structuring marine populations (Bahri-Sfar *et al*. 2000). In the studied area, an oceanographic discontinuity separating Atlantic from Mediterranean waters, and situated southwest of the Cabo de Gata locality, GA (the Oran-Almería Front, OAF; Tintoré *et al*. 1988), seems to play an important role in separating populations of different taxa with larger dispersal capabilities, for example fishes (*Dicentrarchus labrax* in Naciri *et al*. 1999; *Diplodus puntazzo* and *Diplodus sargus* in Bargelloni *et al*. 2005) and molluscs (*Mytilus galloprovincialis* in Quesada *et al*. 1995). This discontinuity could represent the boundary between the northeast Atlantic and Mediterranean biogeographical regions (Quignard 1978). In this study, unfortunately, we only have one population on the western side of this demarcation. Nonetheless, this western population (TA) shows a higher and nearly constant genetic differentiation compared to all other Mediterranean populations, which may have resulted from such oceanographic discontinuity. Further sampling along the western side of OAF should be conducted to determine its role in the population structure of this species.

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6 Supplementary material

The supplementary material is available from http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC3003/MEC3003sm.htm

Table S1 XXXXXXXXXX

Table S2 XXXXXXXXXX

Table S3 XXXXXXXXXX

Table S4 XXXXXXXXXX

Table S5 XXXXXXXXXX

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Supplementary material

Table S1. Geographic distance, in kilometres, between *T. d. xanthosoma* populations.

	CC	TO	BL	CO	FO	PA	GA
TO	112						
BL	125	13					
CO	349	277	266				
FO	430	355	347	144			
PA	882	770	757	279	222		
GA	1045	933	920	394	350	163	
TA	1493	1381	1368	702	692	611	448

Table S2. Summary of genetic variation at ten microsatellite loci in *T. delaisi* populations. Population abbreviations are as in Figure 1.

		Locus									
Location		Td01	Td02	Td04	Td05	Td06	Td07	Td08	Td09	Td10	Td11
CC	n	40	40	40	40	38	40	32	40	40	40
	a	11	11	15	19	20	7	15	7	20	8
	H_E	0.905	0.877	0.894	0.932	0.957	0.691	0.942	0.697	0.960	0.719
	H_O	0.950	0.700	0.750	1	0.842	0.500	0.438	0.600	0.900	0.700
	HW	ns	*	ns	ns	ns	ns	**	**	ns	ns
TO	n	40	40	44	44	42	44	32	44	44	44
	a	13	13	17	18	21	5	17	8	20	9
	H_E	0.895	0.829	0.932	0.938	0.958	0.716	0.935	0.788	0.928	0.801
	H_O	0.800	0.900	0.955	0.909	0.952	0.727	0.813	0.591	0.818	0.864
	HW	ns	ns	ns	ns	ns	ns	ns	**	ns	ns
BL	n	66	66	68	68	66	68	58	68	68	68
	a	12	12	16	21	26	5	22	10	26	13
	H_E	0.890	0.795	0.924	0.946	0.962	0.642	0.936	0.709	0.951	0.785
	H_O	0.758	0.788	0.912	0.971	1	0.588	0.750	0.529	0.941	0.794
	HW	*	ns	ns	ns	ns	ns	**	*	ns	ns
CO	n	60	60	60	60	60	60	58	60	60	60
	a	12	8	14	24	27	4	26	8	25	8
	H_E	0.900	0.677	0.875	0.953	0.929	0.495	0.935	0.775	0.951	0.663
	H_O	0.867	0.767	0.867	1	0.967	0.567	0.793	0.567	0.967	0.667
	HW	ns	ns	ns	ns	ns	ns	**	*	ns	ns
FO	n	74	74	74	74	74	74	62	74	74	74
	a	15	11	19	23	25	6	26	6	27	11
	H_E	0.908	0.768	0.932	0.952	0.940	0.684	0.952	0.742	0.951	0.700
	H_O	0.892	0.676	1	0.892	0.973	0.730	0.645	0.432	0.919	0.676
	HW	ns	ns	ns	ns	ns	ns	**	**	ns	ns
PA	n	60	60	60	60	60	60	58	60	60	60
	a	12	10	14	18	22	5	25	6	22	7
	H_E	0.901	0.810	0.840	0.915	0.938	0.611	0.953	0.668	0.942	0.599
	H_O	0.967	0.900	0.800	0.867	0.967	0.733	0.862	0.400	0.967	0.600
	HW	ns	ns	ns	ns	ns	ns	ns	*	ns	ns
GA	n	40	40	40	40	40	40	38	40	40	40
	a	12	10	14	19	19	4	17	6	21	7
	H_E	0.895	0.847	0.882	0.938	0.954	0.568	0.945	0.749	0.959	0.736
	H_O	0.800	0.850	0.950	0.900	0.850	0.450	0.895	0.650	0.900	0.750
	HW	ns	ns	ns	ns	*	ns	ns	ns	ns	ns
TA	n	66	66	66	66	66	66	64	66	66	66
	a	14	7	24	27	28	6	21	5	33	10
	H_E	0.901	0.591	0.934	0.965	0.963	0.697	0.911	0.582	0.977	0.625
	H_O	0.879	0.606	0.970	0.909	0.909	0.848	0.531	0.667	0.909	0.515
	HW	ns	ns	ns	ns	ns	ns	**	ns	ns	ns

n, number of analysed chromosomes; a, number of alleles; H_E and H_O, expected and observed heterozygosity and HW, significance for deviation from Hardy-Weinberg proportions (ns=P>0.05; *=P<0.05 and **=P<0.005).

Table S2. Continued

Location		Locus									
		Td01	Td02	Td04	Td05	Td06	Td07	Td08	Td09	Td10	Td11
HI	n	60	60	60	58	60	60	60	60	60	60
	a	10	11	14	30	19	7	10	2	11	2
	H_E	0.769	0.842	0.877	0.936	0.924	0.722	0.830	0.210	0.753	0.033
	H_O	0.900	0.900	0.800	0.517	0.900	0.700	0.800	0.233	0.767	0.033
	HW	ns	ns	ns	**	ns	ns	ns	ns	ns	-
FA	n	54	54	54	54	50	54	54	54	54	54
	a	10	11	8	20	15	10	8	2	8	2
	H_E	0.877	0.860	0.837	0.902	0.911	0.806	0.838	0.425	0.753	0.037
	H_O	0.889	0.815	0.852	0.741	1	0.852	0.778	0.593	0.667	0.037
	HW	ns	ns	ns	ns	ns	ns	ns	ns	ns	-

Table S3. Microsatellite allele frequency in *T. delaisi* populations. Alleles are in base pairs. Private alleles are shown in bold type. See Figure 1 for the population abbreviations.

Locus	Allele	CC	TO	BL	CO	FO	PA	GA	TA	HI	FA
Td01	156	0.05	0	0	0	0	0	0	0.03	0	0
	158	0	0.075	0.015	0.05	0.108	0.033	0	0.061	0	0
	160	0	0	0.015	0.033	0.014	0	0	0	0	0
	162	0.1	0.025	0.03	0	0.014	0.05	0.05	0.061	0.4	0.204
	164	0.125	0.05	0.106	0.1	0.108	0.167	0.1	0.045	0.017	0
	166	0.2	0.225	0.182	0.133	0.162	0.067	0.25	0.242	0	0
	168	0.125	0.15	0.106	0.167	0.108	0.167	0.125	0.091	0.033	0.074
	170	0.1	0.1	0.136	0.183	0.108	0.15	0.05	0.091	0	0
	172	0.05	0.15	0.121	0.033	0.095	0.117	0.1	0.061	0.067	0.167
	174	0.125	0.025	0.167	0.1	0.014	0.067	0.1	0.091	0.083	0.093
	176	0.05	0.025	0.03	0.033	0.027	0.083	0.025	0	0.033	0.074
	178	0	0.025	0.061	0.067	0.095	0.033	0.025	0.091	0.25	0.037
	180	0.05	0.1	0.03	0.067	0.108	0.033	0.1	0.076	0.083	0.037
	182	0	0.025	0	0	0.014	0	0.05	0.015	0.017	0.204
	184	0	0	0	0.033	0.014	0.033	0.025	0	0.017	0
	186	0	0	0	0	0.014	0	0	0.015	0	0.056
	188	0	0	0	0	0	0	0	**0.03**	0	0
	200	0.025	0.025	0	0	0	0	0	0	0	0
	208	0	0	0	0	0	0	0	0	0	0.056
Td02	402	0	0	0	0	0	0	0.025	0	0.333	0
	404	0	0	0	0.067	0	0.017	0	0	0	0.019
	406	0	0.025	0	0	0	0	0.025	0	0.083	0
	408	0.075	0	0	0.017	0.054	0	0	0.03	0.033	0.037
	410	0.05	0.025	0.015	0.067	0.027	0.183	0.2	0.091	0	0.019
	412	0.25	0.1	0.167	0.183	0.081	0.117	0.025	0	0.117	0.111
	414	0.1	0.15	0.136	0.017	0.081	0.2	0.15	0.045	0.15	0.259
	416	0.2	0.375	0.379	0.533	0.432	0.333	0.3	0.621	0	0.222
	418	0.025	0.075	0.152	0.05	0.176	0.033	0.075	0.121	0.033	0.074
	420	0.125	0.025	0.015	0.067	0.081	0.033	0	0.076	0.033	0.056
	422	0.05	0	0.03	0	0.027	0	0	0	0.05	0.056
	424	0	0.05	0.015	0	0.014	0	0	0	0.083	0.111
	426	0.05	0.025	0	0	0.014	0	0	0	0.067	0.037
	428	0.025	0	0.015	0	0	0	0	0	0.017	0
	430	0.05	0.075	0.03	0	0	0.033	0.075	0.015	0	0
	432	0	0.025	0.03	0	0	0.033	0.075	0	0	0
	434	0	0	0	0	0	**0.017**	0	0	0	0
	436	0	0.025	0	0	0.014	0	0	0	0	0
	438	0	**0.025**	0	0	0	0	0	0	0	0
	440	0	0	0	0	0	0	**0.05**	0	0	0
	442	0	0	**0.015**	0	0	0	0	0	0	0
Td04	199	**0.025**	0	0	0	0	0	0	0	0	0
	209	0	0	0	0	0	0	0	0.015	0.05	0
	217	0	0	0	0	**0.014**	0	0	0	0	0
	219	0.025	0.068	0.074	0.1	0.108	0.017	0.025	0.03	0	0
	221	0	0.045	0	0	0.027	0	0	0.03	0.017	0

	223	0.1	0.091	0.103	0.017	0.068	0	0.075	0.076	0.233	0.278
	225	0.05	0.068	0.132	0.117	0.041	0.05	0	0.03	0.1	0.167
	227	0.1	0.068	0.103	0.05	0.041	0.017	0.025	0.061	0.117	0.111
	229	0	0	0	0	0	0.067	0.075	0.091	0.05	0.111
	231	0.025	0.023	0.044	0.283	0.081	0.05	0.3	0.091	0.183	0.204
	233	0	0	0	0	0.027	0	0	0.015	0.133	0.074
	235	0	0	0	0.083	0.027	0	0.025	0	0.017	0.037
	237	0.025	0.045	0.015	0.1	0.122	0.067	0.1	0.197	0	0
	239	0.025	0.068	0.044	0	0.041	0.083	0	0	0	0
	241	0	0	0	0	0.014	0.083	0.1	0.015	0.033	0
	243	0.125	0.159	0.132	0.017	0.149	0.033	0.05	0.03	0.017	0
	245	0.05	0.045	0.103	0.1	0.081	0.083	0.1	0.045	0	0.019
	247	0	0	0.015	0	0.014	0	0	0	0	0
	249	0.075	0.023	0.059	0	0.041	0.367	0	0.045	0.017	0
	251	0	0	0.015	0	0.054	0	0.025	0.015	0.017	0
	253	0.025	0	0	0.067	0.041	0.017	0.05	0.015	0.017	0
	255	0.05	0	0.029	0	0.014	0	0	0	0	0
	257	0.275	0.159	0.088	0	0	0	0	0.015	0	0
	259	0	0	0	0	0	0	**0.025**	0	0	0
	261	0	0.023	0	0	0	0.05	0.025	0.03	0	0
	263	0	0	0	0.017	0	0	0	0.015	0	0
	265	0	0.045	0.029	0.017	0	0	0	0.045	0	0
	267	0	0	0	0	0	0	0	0.015	0	0
	269	0	0	0	0	0	0	0	0.015	0	0
	271	0	0	0.015	0.017	0	0	0	0.045	0	0
	273	0	0	0	0	0	0.017	0	0.015	0	0
	279	0	**0.023**	0	0	0	0	0	0	0	0
	281	**0.025**	0	0	0	0	0	0	0	0	0
	283	0	**0.023**	0	0	0	0	0	0	0	0
	291	0	**0.023**	0	0	0	0	0	0	0	0
	295	0	0	0	**0.017**	0	0	0	0	0	0
Td05	233	0	0	0	0.017	0.014	0	0	0	0	0
	235	0	0.023	0	0.083	0.068	0.033	0	0.03	0	0
	237	0.025	0.023	0.044	0	0	0	0	0.015	0	0
	239	0.175	0.023	0.029	0	0.041	0.117	0.2	0.03	0	0
	241	0.025	0.068	0.074	0	0.108	0.033	0.025	0.03	0	0
	243	0	0	0.015	0	0.014	0.017	0.025	0.076	0	0
	245	0.025	0	0.059	0	0.068	0	0	0	0	0
	247	0.075	0	0.015	0	0	0.017	0	0	0	0
	249	0.025	0	0.015	0.05	0.054	0	0	0.015	0	0
	251	0.025	0.114	0.015	0.033	0.068	0	0	0	0	0
	253	0	0.068	0.074	0.017	0.041	0	0	0.106	0.017	0
	255	0.025	0.023	0.044	0.033	0.041	0.067	0.025	0.061	0	0.278
	257	0	0.023	0	0.017	0	0	0.05	0.015	0	0
	259	0	0.023	0	0	0.014	0	0	0.015	0	0
	261	0.05	0.045	0.059	0.017	0.054	0.2	0.025	0.015	0.017	0.019
	263	0	0	0.015	0	0	0	0.1	0.03	0	0
	265	0.05	0	0	0	0.108	0.017	0.025	0.015	0	0
	267	0	0	0	0.033	0	0.017	0	0	0	0
	269	0.05	0.114	0.059	0.05	0.014	0.05	0.025	0.061	0	0.074

	271	0.075	0	0.029	0.017	0.027	0.033	0	0.03	0	0.056
	273	0.05	0.159	0.132	0.017	0.027	0.067	0.075	0.03	0	0.056
	275	0.025	0.068	0	0.017	0.027	0	0	0.061	0	0
	277	0	0.023	0	0.05	0.014	0	0.025	0	0	0
	279	0.025	0	0	0.1	0	0.05	0.025	0.015	0	0
	281	0.05	0.023	0.044	0.017	0.054	0	0.05	0.045	0	0
	283	0	0	0.029	0.05	0.068	0.017	0.05	0.045	0	0
	285	0.025	0	0.059	0.1	0.041	0.15	0.075	0.061	0.224	0
	287	0.175	0.091	0.118	0.1	0.027	0.017	0.075	0.03	0.017	0
	289	0	0.045	0.029	0.083	0	0.05	0.05	0.045	0.034	0
	291	0	0.045	0.044	0.05	0.014	0	0.025	0.03	0.017	0
	293	0.025	0	0	0.017	0	0.05	0.05	0.045	0	0
	295	0	0	0	0.017	0	0	0	0.03	0	0
	297	0	0	0	0	0	0	0	0.015	0.017	0
	299	0	0	0	0	0	0	0	0	0.017	0.056
	301	0	0	0	**0.017**	0	0	0	0	0	0
	305	0	0	0	0	0	0	0	0	0	**0.037**
	311	0	0	0	0	0	0	0	0	**0.017**	0
	313	0	0	0	0	0	0	0	0	**0.017**	0
	315	0	0	0	0	0	0	0	0	0	**0.037**
	331	0	0	0	0	0	0	0	0	**0.017**	0
	333	0	0	0	0	0	0	0	0	0	**0.074**
	335	0	0	0	0	0	0	0	0	**0.017**	0
	341	0	0	0	0	0	0	0	0	**0.034**	0
	343	0	0	0	0	0	0	0	0	**0.034**	0
	351	0	0	0	0	0	0	0	0	0	**0.019**
	353	0	0	0	0	0	0	0	0	**0.017**	0
	357	0	0	0	0	0	0	0	0	**0.052**	0
	369	0	0	0	0	0	0	0	0	0.017	0.019
	371	0	0	0	0	0	0	0	0	0.034	0.037
	373	0	0	0	0	0	0	0	0	**0.017**	0
	375	0	0	0	0	0	0	0	0	0.017	0.037
	377	0	0	0	0	0	0	0	0	**0.034**	0
	379	0	0	0	0	0	0	0	0	**0.103**	0
	381	0	0	0	0	0	0	0	0	**0.034**	0
	383	0	0	0	0	0	0	0	0	**0.034**	0
	385	0	0	0	0	0	0	0	0	**0.034**	0
	387	0	0	0	0	0	0	0	0	0	**0.093**
	393	0	0	0	0	0	0	0	0	0	**0.019**
	395	0	0	0	0	0	0	0	0	0	**0.019**
	401	0	0	0	0	0	0	0	0	0	**0.019**
	403	0	0	0	0	0	0	0	0	0.017	0.019
	417	0	0	0	0	0	0	0	0	0.017	0.019
	419	0	0	0	0	0	0	0	0	0	**0.019**
	423	0	0	0	0	0	0	0	0	**0.017**	0
	447	0	0	0	0	0	0	0	0	**0.017**	0
	463	0	0	0	0	0	0	0	0	**0.034**	0
Td06	107	0	0	0	0	0	0	0	**0.015**	0	0
	111	0	0	**0.015**	0	0	0	0	0	0	0
	113	0.026	0	0	0.017	0	0.017	0	0.045	0	0

115	0.053	0.048	0.03	0.017	0.014	0.017	0.125	0	0	0
117	0.079	0.024	0.015	0.05	0.054	0	0.075	0.061	0	0
118	0	0	0	0	0	0	0	0	0	**0.06**
119	0.053	0.095	0.061	0.067	0.068	0.017	0	0.03	0	0
121	0.132	0.071	0.076	0.233	0.149	0.15	0.075	0.091	0	0
123	0.026	0.024	0.045	0.017	0.135	0.067	0.05	0.076	0	0
125	0	0.095	0.045	0.05	0.027	0.083	0.075	0.03	0	0
127	0.079	0	0.03	0.033	0.054	0.133	0.05	0.045	0	0
129	0	0	0.03	0.017	0.014	0	0.025	0.015	0	0
131	0.105	0.048	0.076	0	0.014	0.05	0.025	0.045	0	0
133	0.026	0	0	0.05	0.081	0.067	0	0.03	0	0
135	0	0.071	0.015	0.083	0	0.017	0	0.03	0	0
137	0.026	0.095	0.03	0.017	0.027	0	0.05	0.03	0	0
138	0	0	0	0	0	0	0	0	0.2	0.04
139	0.026	0.024	0.061	0	0	0.1	0.05	0.015	0	0
140	0	0	0	0	0	0	0	0	**0.017**	0
141	0.026	0.024	0.03	0	0.014	0.033	0.025	0	0	0
142	0	0	0	0	0	0	0	0	0.1	0.02
143	0.053	0.024	0.045	0.017	0.054	0.033	0.025	0.03	0	0
144	0	0	0	0	0	0	0	0	0.067	0.08
145	0	0	0.03	0.017	0.014	0	0	0	0	0
146	0	0	0	0	0	0	0	0	0.033	0.16
147	0	0.024	0.03	0	0.054	0	0	0.015	0	0
148	0	0	0	0	0	0	0	0	**0.017**	0
149	0	0.024	0	0.017	0.027	0	0	0	0	0
150	0	0	0	0	0	0	0	0	0.067	0.04
151	0.053	0.095	0.015	0.017	0	0.033	0.05	0.061	0	0
152	0	0	0	0	0	0	0	0	**0.017**	0
153	0.079	0.024	0.045	0	0.014	0	0.125	0.045	0	0
154	0	0	0	0	0	0	0	0	0.017	0.02
155	0.026	0	0	0	0	0	0	0.03	0	0
156	0	0	0	0	0	0	0	0	0.033	0.18
157	0.053	0	0	0.017	0.027	0.033	0	0.015	0	0
158	0	0	0	0	0	0	0	0	0.033	0.04
159	0	0	0	0	0.054	0.017	0.025	0	0	0
160	0	0	0	0	0	0	0	0	0.067	0.02
161	0	0.071	0.061	0.017	0.014	0.033	0	0	0	0
162	0	0	0	0	0	0	0	0	0.117	0.12
163	0.026	0.048	0.015	0.05	0.014	0.033	0.05	0	0	0
164	0	0	0	0	0	0	0	0	0.067	0.06
165	0	0	0	0.033	0.014	0.017	0	0	0	0
166	0	0	0	0	0	0	0	0	0.033	0.12
167	0	0	0.106	0	0.027	0.017	0	0.106	0	0
168	0	0	0	0	0	0	0	0	**0.017**	0
169	0	0.024	0.015	0.017	0	0	0	0.015	0	0
170	0	0	0	0	0	0	0	0	**0.017**	0
171	0	0.024	0	0	0.027	0	0.05	0	0	0
172	0	0	0	0	0	0	0	0	**0.05**	0
173	0	0	0.015	0.05	0	0	0	0.03	0	0
174	0	0	0	0	0	0	0	0	0	**0.02**
175	0	0	0	0	0	0.017	0.025	0	0	0

	176	0	0	0	0	0	0	0	0	**0.033**	0
	177	0	0	0.045	0	0	0	0.025	0	0	0
	180	0	0	0	0	0	0	0	0	0	**0.02**
	181	0.026	0	0	0.017	0	0.017	0	0	0	0
	183	0.026	0.024	0	0.017	0.014	0	0	0.015	0	0
	185	0	0	0	0	0	0	0	**0.015**	0	0
	189	0	0	0	0	0	0	0	**0.03**	0	0
	191	0	0	0	0	0	0	0	**0.015**	0	0
	193	0	0	0	**0.017**	0	0	0	0	0	0
	195	0	0	0	0	0	0	0	**0.015**	0	0
	197	0	0	0.015	0.017	0	0	0	0	0	0
	201	0	0	0	**0.033**	0	0	0	0	0	0
Td07	107	0.125	0.182	0.088	0.167	0.162	0.083	0.1	0.015	0	0
	109	0.45	0.432	0.471	0.133	0.149	0.467	0.6	0.258	0	0
	111	0	0.045	0	0	0	0	0	0.227	0.017	0.037
	113	0.025	0	0.044	0	0.081	0.017	0	0.439	0	0
	117	0.325	0.273	0.368	0.683	0.514	0.417	0.275	0.03	0	0
	119	0.025	0.068	0.029	0.017	0.081	0.017	0.025	0.03	0	0
	121	0.025	0	0	0	0.014	0	0	0	0.167	0.315
	123	0.025	0	0	0	0	0	0	0	0.367	0.259
	125	0	0	0	0	0	0	0	0	0.067	0.093
	127	0	0	0	0	0	0	0	0	0	**0.056**
	129	0	0	0	0	0	0	0	0	0	**0.167**
	131	0	0	0	0	0	0	0	0	0.35	0.019
	133	0	0	0	0	0	0	0	0	**0.017**	0
	135	0	0	0	0	0	0	0	0	0	**0.019**
	139	0	0	0	0	0	0	0	0	0	**0.019**
	141	0	0	0	0	0	0	0	0	0.017	0.019
Td08	311	0	0.031	0.071	0.086	0.032	0.052	0.079	0.031	0	0
	313	0.031	0.063	0.054	0	0.032	0	0	0.047	0	0
	315	0	0	0	**0.017**	0	0	0	0.016	0	0
	319	0	0	0	0	0	0.017	0	0	0	0
	321	0.031	0.094	0	0	0.048	0.034	0.053	0.016	0	0
	323	0.156	0.125	0.107	0.052	0.129	0.103	0.105	0.063	0	0
	325	0	0.031	0	0.034	0.016	0	0	0.063	0	0
	327	0	0	0	0	0	0	0	0	0.233	0.259
	329	0.094	0.031	0.143	0.034	0.048	0.069	0.053	0.078	0	0
	331	0.094	0.031	0.036	0.138	0.113	0.103	0.132	0.25	0.033	0.093
	333	0.031	0	0.018	0	0.048	0.052	0.079	0.016	0	0
	335	0.125	0.188	0.161	0.19	0.097	0.121	0.132	0.109	0	0
	337	0	0.031	0	0.017	0.048	0.034	0	0	0	0
	339	0	0.031	0	0.017	0	0	0	0.047	0	0
	341	0.063	0.063	0	0	0.016	0.017	0	0.047	0.15	0.167
	343	0	0	0	0.052	0.016	0	0.026	0.031	0.283	0.056
	345	0	0	0	0	0	0.017	0	0	0.067	0.241
	347	0	0	0.054	0.052	0.032	0.069	0.053	0.031	0.133	0.056
	349	0.063	0	0	0.034	0.048	0.052	0.026	0.031	0.017	0
	351	0	0	0	0.052	0	0.017	0	0	0.05	0.093
	353	0.094	0.031	0.036	0.017	0.032	0	0.079	0.016	0.017	0

	355	0	0.031	0	0	0	0	0	0.016	0	0
	357	0.063	0	0.018	0.017	0.016	0	0.053	0	0	0
	359	0	0	0.036	0.017	0.016	0.017	0	0	0	0
	361	0	0	0.036	0.017	0.032	0.017	0	0	0	0
	363	0	0	0.018	0.017	0	0	0.026	0.016	0	0
	365	0	0.125	0	0	0.016	0.017	0.026	0.016	0	0
	369	0	**0.031**	0	0	0	0	0	0	0	0
	371	0.031	0	0.036	0.017	0.065	0	0	0.047	0	0
	373	0	0	0.018	0	0.016	0	0.026	0	0	0
	375	0.031	0	0.054	0.017	0.016	0	0	0	0	0
	377	0	0	0	**0.017**	0	0	0	0	0	0
	379	0	0	0	0.017	0.016	0	0	0.016	0	0
	381	0.031	0	0.018	0.017	0	0	0	0	0	0
	383	0.063	0	0	0	0.016	0	0	0	0	0
	385	0	0	0	0	0	0	**0.026**	0	0	0
	387	0	0	0	0.017	0	0.017	0	0	0.017	0
	389	0	**0.031**	0	0	0	0	0	0	0	0
	391	0	0	0	0.017	0	0.017	0	0	0	0
	393	0	**0.031**	0	0	0	0	0	0	0	0
	395	0	0	0	0	**0.016**	0	0	0	0	0
	397	0	0	0.018	0	0	0.017	0	0	0	0.037
	399	0	0	0	0	0.016	0.017	0	0	0	0
	407	0	0	0	0	0	**0.034**	0	0	0	0
	409	0	0	0.018	0.017	0	0.017	0.026	0	0	0
	413	0	0	0.018	0	0	0	0	0	0	0
	417	0	0	0.018	0	0	0	0	0	0	0
	423	0	0	0.018	0	0	0.034	0	0	0	0
	431	0	0	0	0	0	**0.034**	0	0	0	0
Td09	290	0	0.023	0	0.017	0	0	0	0	0	0
	294	0.075	0.045	0.029	0.1	0.068	0.05	0.075	0.136	0	0
	296	0	0	**0.029**	0	0	0	0	0	0	0
	298	0.5	0.295	0.382	0.233	0.284	0.25	0.3	0.606	0.883	0.704
	300	0.025	0.023	0.015	0	0	0	0.025	0	0	0
	302	0.025	0.114	0.074	0.183	0.189	0.167	0.175	0.03	0	0
	304	0.05	0.023	0.029	0.017	0.027	0.017	0.05	0	0	0
	306	0	0	0	0.017	0.054	0	0	0	0	0
	310	0	0	**0.015**	0	0	0	0	0	0	0
	314	0	0	**0.029**	0	0	0	0	0	0	0
	316	0.1	0.159	0	0.067	0	0.017	0	0.03	0	0
	320	0	0	**0.015**	0	0	0	0	0	0	0
	600	0.225	0.318	0.382	0.367	0.378	0.5	0.375	0.197	0.117	0.296
Td10	114	0	0	0	0	0	0	0	**0.045**	0	0
	128	0.025	0.068	0	0	0.027	0	0.025	0.03	0	0
	130	0	**0.045**	0	0	0	0	0	0	0	0
	132	0	0	0	**0.033**	0	0	0	0	0	0
	134	0.05	0.023	0.029	0.017	0.054	0.033	0.025	0.015	0.033	0
	136	0.05	0.023	0.015	0.05	0.027	0.017	0	0.015	0.017	0
	138	0.025	0.045	0.029	0.033	0.027	0.05	0.05	0.015	0	0
	140	0.05	0.023	0.029	0.133	0.068	0.117	0.025	0.03	0	0

	142	0.025	0.023	0	0.017	0.081	0.1	0.05	0.045	0.033	0
	144	0	0	0	0	0	0	0	**0.015**	0	0
	146	0.025	0	0.029	0.017	0.014	0.017	0	0	0	0
	148	0.1	0.023	0.044	0.05	0.014	0.1	0.025	0.03	0.067	0.093
	150	0.1	0.023	0.132	0.017	0.027	0.033	0	0.03	0.067	0.037
	152	0.05	0	0.059	0.033	0.014	0.017	0.025	0.03	0.1	0.056
	154	0	0.023	0.015	0	0.027	0.033	0	0.015	0.133	0.056
	156	0.1	0.114	0.118	0.1	0.135	0.05	0.075	0.045	0.467	0.204
	158	0.075	0.159	0.074	0.033	0.095	0.017	0.075	0.015	0.05	0.444
	160	0.05	0.182	0.059	0.05	0.081	0.117	0.125	0.03	0.017	0.056
	162	0.05	0.023	0.074	0.117	0.027	0.1	0.075	0.045	0	0.056
	164	0.025	0.068	0.044	0.067	0.041	0.067	0.025	0.061	0	0
	166	0	0	0.015	0.017	0.041	0.017	0.05	0.015	0	0
	168	0.05	0	0.015	0.033	0.014	0	0.075	0.076	0	0
	170	0	0	0	0.033	0	0.017	0.025	0.045	0	0
	172	0.075	0.023	0.015	0.017	0.027	0.017	0	0.015	0	0
	174	0	0	0.029	0.017	0	0	0.025	0.015	0	0
	176	0	0	0.029	0	0.014	0	0.025	0.03	0.017	0
	178	0.025	0.023	0.044	0	0	0	0	0	0	0
	180	0	0	0	0	0	0	0	**0.015**	0	0
	182	0.025	0	0	0.033	0.041	0	0.05	0	0	0
	184	0	0	0	0	0	0	0.025	0.045	0	0
	186	0	0	0.015	0	0.014	0.017	0	0.03	0	0
	188	0	0.045	0	0	0	0	0.025	0	0	0
	190	0	0	0	0.033	0.014	0	0	0.045	0	0
	192	0	0.023	0	0	0	0.017	0	0.015	0	0
	194	0	0	0	**0.017**	0	0	0	0	0	0
	196	0	0	0	0.017	0.014	0.017	0	0.045	0	0
	198	0.025	0	0.029	0	0	0	0	0	0	0
	200	0	0	0	**0.017**	0	0	0	0	0	0
	202	0	0.023	0.015	0	0	0	0	0	0	0
	204	0	0	0.015	0	0.014	0	0	0	0	0
	208	0	0	0	0	0	0	0	**0.015**	0	0
	212	0	0	0.015	0	0.014	0	0.1	0	0	0
	214	0	0	0	0	0	0	0	**0.03**	0	0
	216	0	0	0	0	0.041	0	0	0.015	0	0
	218	0	0	0.015	0	0	0.033	0	0	0	0
	220	0	0	0	0	0	0	0	**0.03**	0	0
Td11	271	0.025	0.023	0.044	0.05	0.054	0.017	0.05	0.015	0	0
	279	0	0	**0.029**	0	0	0	0	0	0	0
	281	0.5	0.386	0.426	0.55	0.514	0.617	0.475	0.591	0	0
	283	0	0	0	0.017	0.014	0	0	0	0.017	0
	285	0	0.045	0.015	0	0	0	0.025	0	0	0
	287	0.125	0.182	0.103	0.167	0.108	0.117	0.15	0.121	0.983	0.981
	289	0.1	0.114	0.088	0.083	0.162	0.067	0	0.121	0	0.019
	291	0.125	0.091	0.088	0	0.027	0.083	0.1	0.061	0	0
	293	0.025	0.045	0.029	0.017	0.041	0.033	0.075	0.015	0	0
	295	0	0	0.015	0	0.014	0	0	0.015	0	0
	297	0.075	0.091	0.118	0.083	0.027	0.067	0.125	0.03	0	0
	299	0	0	0.015	0	0.027	0	0	0.015	0	0

301	0.025	0.023	0.015	0.033	0.014	0	0	0	0	0
305	0	0	**0.015**	0	0	0	0	0	0	0
325	0	0	0	0	0	0	0	**0.015**	0	0

Table S4. *T. d. xanthosoma* (*T. d. x.*) and *T. d. delaisi* (*T. d. d.*) sequence alignment of Td06 microsatellite allele sequences. Numbers beside the subspecies name indicate the total allele size; primer sequences have not been included. The TC and C insertions in *T. d. d*. and *T. d. x*. respectively, are framed.

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T. d. x.-121: CAGAATATATCCTAGCATGTCAATACAA(AC)14--CCCACCCCCCACTCCTCTATCTTC
T. d. x.-123: ............................(AC)15..........................
T. d. x.-143: ............................(AC)25.......A..................
T. d. d.-144: ............................(AC)25TC........................-
T. d. d.-156: ............................(AC)31TC........................-
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Table S5. Multilocus F_{ST} (below diagonal) and G'_{ST} (above diagonal) values between population pairs from the most and the least polymorphic loci pools

The most polymorphic loci (Td04, Td05, Td06, Td08 and Td10)

	CC	TO	BL	CO	FO	PA	GA	TA	HI	FA
CC		0.365	0.279	0.475	0.369	0.420	0.399	0.468	0.803	0.785
TO	0.007		0.293	0.504	0.381	0.529	0.474	0.508	0.819	0.744
BL	0.003	0.002		0.424	0.328	0.420	0.438	0.407	0.737	0.738
CO	**0.030**	**0.028**	**0.022**		0.356	0.403	0.376	0.376	0.704	0.762
FO	**0.016**	**0.012**	**0.010**	**0.015**		0.383	0.383	0.347	0.758	0.768
PA	**0.030**	**0.039**	**0.029**	**0.036**	**0.028**		0.418	0.454	0.801	0.829
GA	**0.021**	**0.023**	**0.021**	**0.014**	**0.017**	**0.033**		0.404	0.748	0.760
TA	**0.018**	**0.017**	**0.011**	**0.015**	**0.009**	**0.027**	**0.011**		0.765	0.767
HI	**0.066**	**0.066**	**0.053**	**0.062**	**0.058**	**0.084**	**0.062**	**0.058**		0.457
FA	**0.074**	**0.063**	**0.059**	**0.073**	**0.066**	**0.096**	**0.068**	**0.066**	**0.056**	

The least polymorphic loci (Td01, Td02, Td07, Td09 and Td11)

	CC	TO	BL	CO	FO	PA	GA	TA	HI	FA
CC		0.133	0.096	0.226	0.213	0.160	0.156	0.268	0.680	0.555
TO	0.002		0.078	0.176	0.125	0.133	0.091	0.258	0.774	0.576
BL	0.002	-0.002		0.146	0.122	0.094	0.092	0.237	0.757	0.573
CO	**0.052**	**0.038**	**0.036**		0.071	0.136	0.221	0.307	0.833	0.682
FO	**0.036**	**0.016**	**0.019**	0.005		0.139	0.188	0.257	0.803	0.634
PA	**0.022**	0.012	0.008	**0.032**	**0.024**		0.100	0.331	0.806	0.612
GA	0.012	-0.004	0.002	**0.061**	**0.039**	0.003		0.272	0.779	0.605
TA	**0.072**	**0.070**	**0.071**	**0.116**	**0.078**	**0.101**	**0.085**		0.727	0.608
HI	**0.252**	**0.266**	**0.274**	**0.326**	**0.288**	**0.316**	**0.293**	**0.293**		0.293
FA	**0.197**	**0.191**	**0.207**	**0.254**	**0.220**	**0.242**	**0.222**	**0.232**	**0.078**	

Bold F_{ST} values are significantly greater than zero ($P<0.05$). Population abbreviations are as in Figure 1.

Inferència de l'estructura poblacional de *Tripterygion delaisi*, dins i entre subspècies, a partir de loci microsatèl·lits altament polimòrfics.

Un dels estadístics més utilitzats per a determinar les relacions de connectivitat existents entre poblacions és el F_{ST}, tot i que en certes ocasions quan s'utilitzen loci altament polimòrfics els seus valors s'han de tractar amb cura, i especialment quan es tracta amb poblacions que pertanyen a diferents subspècies. *Tripterygion delaisi* presenta dues subspècies, poblacions d'ambdues subspècies han estat mostrejades i analitzades utilitzant 10 loci microsatèl·lits. Una aproximació Bayesiana ha permès identificar, d'una forma molt clara, aquestes subspècies com dues unitats evolutivament diferents. Malgrat això, els baixos valors de F_{ST} que s'han trobat entre subspècies, tot i que significatius, són conseqüència de la presència d'un gran nombre d'al·lels per locus. L'existència d'homoplàsia entre subspècies s'ha pogut descartar degut als valors obtinguts per la distància genètica estandarditzada G'_{ST}. Els loci altament polimòrfics, amb més de 15 al·lels, presentaven una heterozigositat totalment saturada, aquest valor de polimorfisme al·lèlic s'ha utilitzat per dividir els loci en dos grups segons el seu nombre d'al·lels mitjà. D'aquesta manera, s'observà que el grup de loci menys variables presentava una major variància genètica entre subspècies, mentre el grup format pels més variables mostrava una major variància genètica entre poblacions. A més, s'ha observat que hi ha molta més diferenciació entre poblacions utilitzant els G'_{ST} amb els loci més variables. Malgrat aquestes diferències, és quan s'inclouen tots el loci en l'anàlisi que s'obté un resultat més acurat de l'estructura poblacional. Entre les vuit poblacions de *T. d. xanthosoma* s'ha trobat aïllament per distància, i segons una anàlisi bayesiana, s'han definit sis unitats genèticament homogènies, aquest resultat concorda amb l'estructura obtinguda a partir dels valors de F_{ST}. Finalment, s'ha calculat una molt petita capacitat de dispersió a partir de la mida estimada de la població. En conclusió, en peixos amb una capacitat de dispersió limitada tant pel que fa a la fase adulta com a la larvària, un hàbitat continu de roca sembla permetre el contacte entre poblacions prevenint la diferenciació genètica. En canvi, llargues discontinuïtats de sorra o canals d'aigua profunda podrien estar reduint el flux gènic entre poblacions.

High self-recruitment levels in a Mediterranean littoral fish population revealed by microsatellite markers

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Abstract

Self-recruitment rates are essential parameters in the estimation of connectivity among populations, having important consequences in marine conservation biology. Using ten highly polymorphic microsatellite loci, we estimate, over three years, the self-recruitment in a population of *Tripterygion delaisi* in the NW Mediterranean. Six previously described source populations were used for the assignment (Costa Brava, Columbretes, Formentera, Cabo de Palos, Cabo de Gata and Tarifa). Even though this species has a 16-21 day larval duration, over three years a mean of 76.4±1.6% of the recruits settled had returned to their natal population. Furthermore, the rest of the recruits were mainly assigned to the geographically nearest populations. When refining in a more local scale the origin of individuals self-recruited to Costa Brava, using as source the three sampling localities that conform this population (Cap de Creus, Tossa and Blanes), the highest percentage was usually self-assigned to the adult source locality (Blanes) where recruits were sampled each year, with a mean percentage across three years of 42.6±6.0%. Our results suggest that a high proportion of the larvae of *T. delaisi* remained close to, or never leave, their natal spawning area. This observation can be extrapolated to other species with similar early life-history traits and low adult mobility and can have important implications for the conservation and management of Mediterranean littoral fishes.

Keywords: *Tripterygion delaisi*; microsatellite; self-recruitment; early life history; larval dispersal; Mediterranean; littoral fish; Bayesian assignment; temporal variation

Introduction

One of the main objectives of research on fish populations is to identify the factors that determine the number of new individuals recruited into the adult population (Cushing 1996). The majority of shallow-water marine species have a two-phase life cycle, in which quite sedentary, demersal adults (no mobile phase) produce pelagic larvae (mobile phase) (Leis 1991; Leis and Carson-Ewart 2000). These larvae disperse and their settlement processes can be influenced by different environmental factors, e.g. currents, winds, that determine the settlement strength (Wilson and Meekan 2001; Cowen 2002; Raventós and Macpherson 2005). For many years, it was assumed that these larvae disperse away from the parental population operating as an open system (Sale 1991; Caley et al. 1996). These initial studies considered larvae as passive particles and focused on hydrodynamic features to explain their distribution, predicting that larvae are flushed away from their natal locality in the predominant current direction (Roberts 1997). However, larvae of many fishes have been found capable to maintain strong and sustained swimming activity, as well as to use their sensory abilities to regulate their distribution and dispersion (Cowen 2002; Kingsford et al. 2002; Leis and McCormick 2002). Accordingly, some recent studies (e.g. Jones et al. 1999; Swearer et al. 1999; Jones et al. 2005) have demonstrated that populations are not always open and that the proportion of larvae that may return to their natal population (self-recruitment) is very high. These studies, therefore, suggest that the extent of dispersal between populations is lower than currently assumed, affecting the connectivity among populations and having important implications in marine conservation policies.

Unfortunately, at present, the number of studies is still scarce. Marked otoliths and trace-element concentrations in otoliths have been used to estimate the self-recruitment rate in different fish populations (Jones et al. 1999; Swearer et al. 1999; Thorrold et al. 2001; Miller and Shanks 2004; Patterson et al. 2005). Furthermore, Jones et al. (2005) using two different methods (marked larvae and parentage analyses using microsatellites) in a population of *Amphiprion polymnus* concluded that most settled juveniles had returned to a 2-hectare natal area. Knutsen et al. (2004) using microsatellites demonstrated an extensive but temporally variable drift of offshore cod larvae into coastal populations.

Microsatellites are highly polymorphic nuclear loci that have been successfully used to describe population structuring on a wide range of geographical levels (Appleyard et al. 2001; Rico and Turner 2002; Carlsson et al. 2004). Therefore, microsatellites seem to be a powerful tool to estimate population isolation and self-recruitment levels in fishes (Knutsen et al. 2004; Jones et al. 2005).

Tripterygion delaisi is a common littoral fish in the Mediterranean Sea, living always in rocky habitats, preferentially in biotopes of reduced light, between 6 and 12 m (Zander 1986). Adult individuals are highly territorial, showing high levels of homing behaviour (Heymer 1977), parental care of the eggs (Wirtz 1978) and cannot swim even short distances (tens of metres) in open water or on sandy bottoms. Larvae of *T. delaisi* remain in plankton for 16-21 days (Raventós and Macpherson 2001), although they are present almost exclusively in coastal waters (Sabatés et al. 2003).

The Mediterranean populations of *Tripterygion delaisi* show a clear genetic structure and a significant isolation by distance (Carreras-Carbonell et al. 2006), suggesting the existence of a potential high degree of self-recruitment in each population. The present study estimates, over three years, the self-recruitment in a population of *T. delaisi* in the NW Mediterranean. This could help to classify the population as genetically unconnected (closed) or connected (open), with a wide range of intermediate status depending on the percentage of recruits received from distant sources. Using ten highly polymorphic microsatellites (Carreras-Carbonell et al. 2004) we compare the new recruits of each year, with the adult reproductive specimens from the same locality and with adults from other seven adjacent localities separated by tens to hundreds of kilometres.

Materials and methods

Sampling and DNA extraction

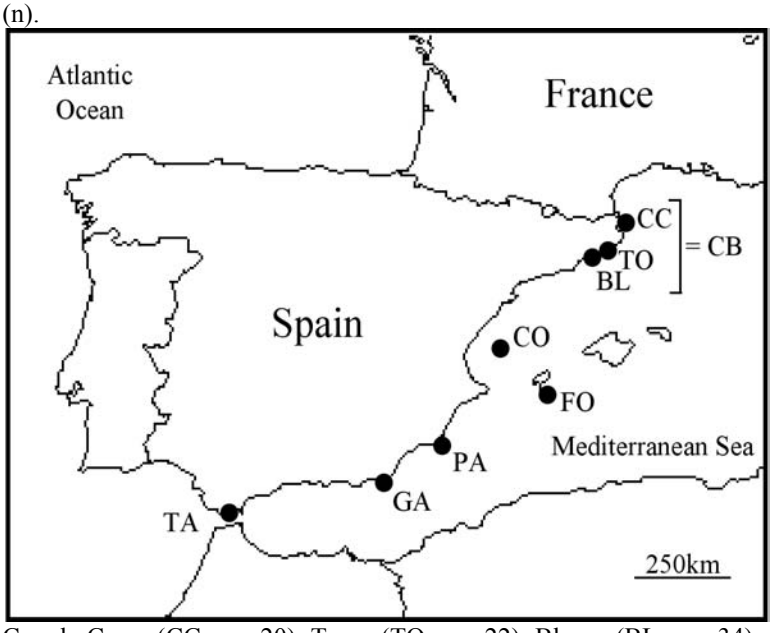
We studied recruits of triplefin blenny (*Tripterygion delaisi*) from Blanes locality (North-western Mediterranean; Spain). A total of 113 specimens were collected

during 2003 (n = 35; RBL03), 2004 (n = 47; RBL04) and 2005 (n = 31; RBL05) by SCUBA divers using hand nets. Each year, individuals were sampled from the same shallow rocky bay (St. Francesc – 41º 40.4'N, 2º 48.2'E).

A small portion of the anal fin was removed from living fish, which were then measured and released into the same sample site. All fins were preserved individually in absolute ethanol at room temperature. Total genomic DNA was extracted from fin tissue using the Chelex 10% protocol (Estoup et al. 1996).

The triplefin recruits from Blanes were compared to eight adult localities previously analysed from the western Mediterranean: Cap de Creus (CC), Tossa (TO), Blanes (BL), Columbretes Is. (CO), Formentera Is. (FO), Cabo de Palos (PA), Cabo de Gata (GA) and Tarifa (TA). Three of these localities (CC, TO and BL) presented no genetic differentiation and could not be considered isolated populations; furthermore, the existence of six populations was inferred using a Bayesian approach (Carreras-Carbonell et al. 2006). In accordance to this, we have grouped CC, TO and BL in a single population that in the present study we will refer to as Costa Brava (CB) (see Fig. 1).

Figure 1. Source localities of *Tripterygion delaisi* used in the assignment test and number of individuals analysed in each locality (n).



Cap de Creus (CC, n = 20), Tossa (TO, n = 22), Blanes (BL, n = 34), Columbretes Is. (CO, n = 30), Formentera Is. (FO, n = 37), Cabo de Palos (PA, n = 30), Cabo de Gata (GA, n = 20) and Tarifa (TA, n = 33). Data obtained from Carreras-Carbonell et al. (2006). Costa Brava (CB) groups three sampling locations (CC, TO and BL), see text for details.

PCR amplification and screening

We used the ten polymorphic microsatellite loci and polymerase chain reactions conditions described in Carreras-Carbonell et al. (2006). Amplified products were scored using an ABI 3700 automatic sequencer from the Scientific and Technical Services of the University of Barcelona. Alleles were sized by GENESCAN™ and GENOTYPER™ software, with an internal size marker CST Rox 70-500 (BioVentures Inc.).

Statistical analyses

Allele frequencies, mean allelic richness, expected (H_E) and observed (H_O) heterozygosity per locus, for each recruit year pools were calculated using GENECLASS2 program (Piry et al. 2004). The inbreeding coefficient (F_{IS}) in each generation was computed with GENETIX version 4.05 (Belkhir et al. 2004) and its confidence interval was estimated with 10000 bootstrapping values. Linkage disequilibrium between pairs of loci were tested for each recruit year using GENEPOP version 3.4 (Raymond and Rousset 1995), which employs a Markov chain method, with 5000 iterations, following the algorithm of Guo and Thompson (1992). These results were adjusted for multiple tests using the sequential Bonferroni procedure with $\chi = 0.05$ (Rice 1989).

Genetic differentiation between the three samples of recruits of BL and the six adult source populations was estimated using the classical F_{ST} approach (Wright 1951; Weir and Cockerham 1984). Significance between each pair comparison was tested using the Fisher's exact test implemented in GENEPOP program.

Population assignment test

Assignment tests were carried out using GENECLASS2 program (Piry et al. 2004) under the Bayesian assignment method of Rannala and Mountain (1997), since according to Cornuet et al. (1999) performed better in assigning individuals to their correct sampling populations than other likelihood-based and distance-based methods. The simulation algorithm of Paetkau et al. (2004) was used with 10^5 simulations and a

threshold of 0.05. First of all, the six previously differentiated populations described above were used as the source populations of the recruits. Afterwards, in order to estimate the self-recruitment in a smaller geographical scale, only the recruits assigned to Costa Brava population were reassigned using as source the three localities (Cap de Creus, Tossa and Blanes) that were grouped in this population.

Results

Genetic variability

An extensive polymorphism per generation and locus was found among recruit samples with high mean number of alleles (17.6±1.46) and high expected (0.855±0.020) and observed (0.778±0.030) heterozygosities. No differences were found between the three generations sampled in the mean number of alleles (Friedman ANOVA, $\chi^2 = 1.81$, P>0.4) and the expected heterozygosity ($\chi^2 = 0.97$, P>0.6) (Table 1). All loci were considered statistically independent since no linkage disequilibrium between loci pairs was observed in any *Tripterygion delaisi* generation sampled. Alleles not previously detected in any adult locality were found in each generation: five within 2003 recruits, two within 2004 recruits and seven within 2005 recruits; all of them in very low frequency.

Global F_{IS} values for the recruits of each year were statistically significant. We observed that these departures were mainly due to loci Td08 and Td09 for the three generations. Moreover, loci Td01 and Td02 in 2003 recruits and Td02 in 2004 recruits also showed significant F_{IS} values (Table 1). Loci Td08 and Td09 also presented deviations in all source populations (Carreras-Carbonell et al. 2006) that could be explained by the presence of null-alleles in these loci. Null-alleles appear when one allele is unamplified due to mutations in the sequence where one of the primers was designed, and/or when technical problems associated with amplification and scoring arise (Hoarau et al. 2002). Technical issue could be ruled out since all homozygous individuals and failed amplifications for loci Td01, Td02, Td08 and Td09 were re-amplified twice lowering the annealing temperature to 50ºC and accurate scoring of larger alleles with poor amplification was also carried out.

Table 1. Summary of genetic variation at ten microsatellite loci in recruits of the year of *Tripterygion delaisi* from Blanes across years: 2003 (RBL03), 2004 (RBL04) and 2005 (RBL05).

		Locus									
Year		Td01	Td02	Td04	Td05	Td06	Td07	Td08	Td09	Td10	Td11
RBL03	n	70	66	70	70	70	70	64	70	70	70
	a	16	13	20	25	24	5	26	7	24	11
	H_E	0.893	0.763	0.926	0.953	0.924	0.668	0.956	0.701	0.919	0.809
	H_O	0.743	0.545	0.800	0.914	0.971	0.714	0.813	0.429	0.914	0.714
	F_{IS}	0.170*	0.288*	0.138	0.041	-0.052	-0.70	0.152*	0.393*	0.005	0.119
RBL04	n	94	94	94	92	94	94	94	94	94	94
	a	12	16	20	25	31	8	29	9	27	10
	H_E	0.877	0.911	0.917	0.936	0.959	0.674	0.945	0.738	0.953	0.725
	H_O	0.872	0.787	0.915	0.978	0.915	0.702	0.745	0.362	0.894	0.681
	F_{IS}	0.005	0.137*	0.003	-0.046	0.046	-0.042	0.213*	0.513*	0.063	0.062
RBL05	n	60	56	62	60	62	60	52	58	62	58
	a	12	15	20	26	21	5	22	9	30	10
	H_E	0.885	0.823	0.936	0.961	0.948	0.589	0.956	0.714	0.963	0.737
	H_O	0.900	0.893	0.839	0.933	0.935	0.467	0.692	0.552	0.968	0.759
	F_{IS}	-0.017	-0.086	0.105	0.029	0.014	0.210	0.279*	0.230*	-0.005	-0.030

(n): number of analysed chromosomes, (a): number of alleles, (H_E) and (H_O): expected and observed heterozygosity respectively, and (F_{IS}): inbreeding coefficient and significance (*=$P<0.05$).

Significant genetic differentiation was found between the three samples of recruits of Blanes and all adult source populations, with the exception of Costa Brava (Table 2). The distances of the recruits of the different years were always smaller when compared to Costa Brava; however, significant genetic differentiation was found when recruits of 2003 were compared. Nevertheless the comparison of the distances between the recruits from the three years to each source population always yielded a strong correlation ($r \geq 0.95$, $P<0.005$) indicating that in spite of the differences found between recruits of the year their relative distances to adult populations were maintained through time. Similar results were obtained when locus Td08 and Td09, showing null alleles, were excluded from the analyses.

Table 2. Pairwise multilocus F_{ST} values between source populations and recruits of Blanes (RBL) collected yearly in 2003-2005.

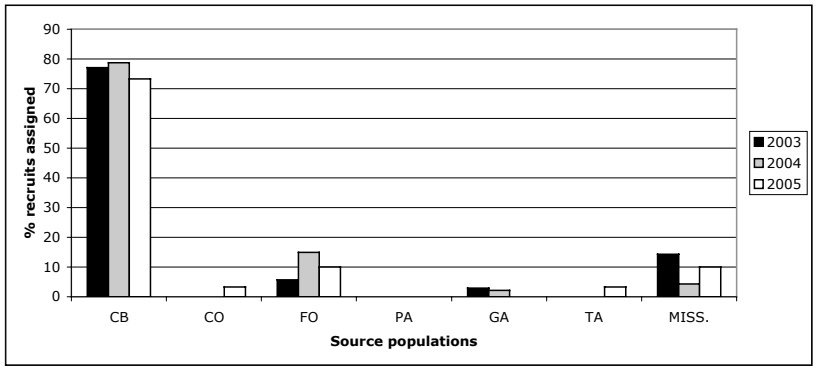
	Costa Brava	Columbretes	Formentera	Cabo de Palos	Cabo de Gata	Tarifa
RBL03	0.006*	0.037*	0.019*	0.027*	0.014*	0.043*
RBL04	0.003	0.029*	0.016*	0.018*	0.006*	0.047*
RBL05	0.002	0.028*	0.014*	0.015*	0.006*	0.045*

(*=P<0.05).

Self-recruitment estimation

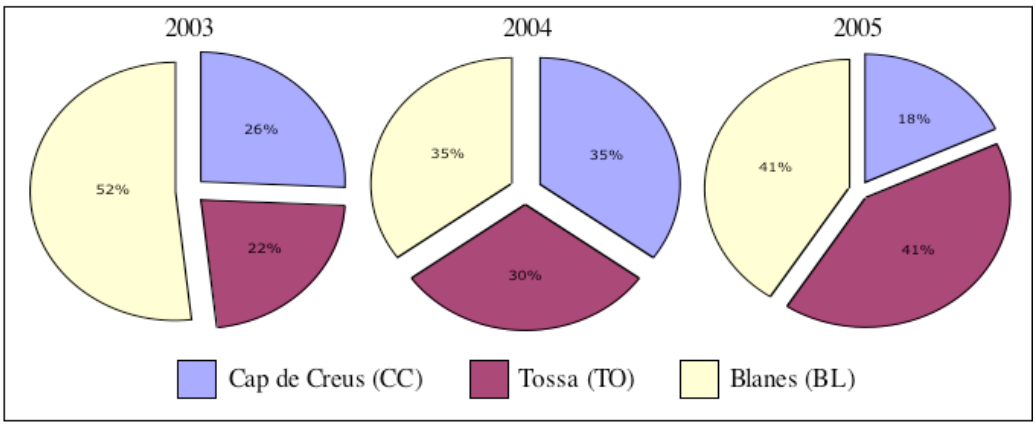
Control assignment test placed recruits in their expected source population using the method of Rannala and Mountain (1997) (Fig. 2). For 2003, the 77.1% of the recruits were assigned to Costa Brava; whereas 8.6% of the specimens were assigned to a more distant and genetically differentiated populations (Formentera Is., Cabo de Gata) as well as a 14.3% were unassigned. For 2004, the 78.7% of the recruits were assigned to Costa Brava and 17% of the specimens to a more distant and genetically differentiated populations (Formentera Is., Cabo de Gata); individuals without assigned population represented 4.3%. Finally, for 2005, the assigned percentage of recruits found for Costa Brava was 73.3%. The individuals assigned to a more distant and genetically differentiated populations represented the 16.7% (Columbretes Is., Formentera Is., Tarifa) and the percentage of miss-assigned individuals was 10%. Similar percentage of self-recruitment was obtained when loci having null alleles were excluded (mean percentage across three years = 78.7±4.0%). Furthermore, the rest of the individuals were assigned to the other source populations with similar frequencies as detected when all loci were used, with Formentera Is. being the greatest contributor of recruits (mean percentage across three years = 8.1±1.5%).

Figure 2. Percentage of recruits of the year of *Tripterygion delaisi* assigned to each source populations over three years (2003, 2004 and 2005). Population abbreviations as in Figure 1, (MISS.): miss assigned individuals.



In order to refine in a more local scale the origin of the recruits, the individuals assigned to Costa Brava were reassigned using as source the three sampling localities grouped in the Costa Brava population (Cap de Creus, Tossa and Blanes). All individuals were strongly assigned to one of these three localities. The highest percentage of the recruits was usually self-assigned to Blanes with mean percentage across three years of 42.6±6.0%, and smaller percentages were found for Tossa (31.0±6.6%) and Cap de Creus (26.4±6.0%) (Fig. 3). When loci with null alleles were excluded, the mean percentage of assigned recruits became more similar among the three localities (BL=33.7±5.4%, TO=32.5±3.6%, CC=33.8±1.9%).

Figure 3. Percentage of recruits assigned to Costa Brava over 2003, 2004 and 2005 reassigned using the three localities that were grouped in this population (CC, TO and BL).



Discussion

Self-recruitment rates are essential parameters in the estimation of connectivity among populations, having important consequences in marine conservation biology (Swearer et al. 2002; Thorrold et al. 2002). Self-recruitment studies in marine fishes are scarce, and they have used different methodologies, e.g. chemical marking (Jones et al. 1999; Jones et al. 2005), otolith microstructure and/or microchemistry (Swearer et al. 1999; Thorrold et al. 2001; Miller and Shanks 2004; Patterson et al. 2005), and more recently adding molecular techniques (Jones et al. 2005). Molecular markers have demonstrated their utility in assigning the origin of colonizers invading new areas, mainly in continental habitats (Genton et al. 2005). Assignment tests and paternity analyses have been used to establish the origin of cod (Knutsen et al. 2004) and clownfish (Jones et al. 2005) recruits, respectively, demonstrating the utility of

this methodology in the estimation of self-recruitment rates. In the present work, assignment tests were very robust, since similar results were obtained with and without loci having null alleles. Nevertheless, we used all loci to identify the origin of recruits since increasing the number of loci seems to yield higher statistical power when estimating the number of populations (Carreras-Carbonell et al. 2006).

In *Tripterygion delaisi*, self-recruitment was very high as revealed by the assignment tests. During the three years studied, the self-recruitment in the Costa Brava population ranged between 73.3 and 78.7 %. However, among the three years there is a mean percentage of 14.1±2.8 % of recruits assigned to other populations, mainly belonging to the geographically nearest ones (Columbretes Is. and Formentera Is.). Furthermore, detection of first generation migrants among the adult populations, using GENECLASS2, assigned 14 individuals to different localities, the majority of which (12) involved Formentera Is. This is in agreement with the isolation by distance observed among these western Mediterranean populations (Carreras-Carbonell et al. 2006), indicating that, although the populations were genetically differentiated, a small connexion between them could exist, allowing the interchange of individuals (via larvae) between populations.

When the recruits from Costa Brava were reassigned to a finer scale using the three localities that conformed this population (Cap de Creus, Tossa and Blanes), we observed that, on average, the highest proportion of the recruits settled in their natal locality (mean percentage across three years of 42.6±6.0%, Blanes) (Fig. 3). However, the recruit contribution of the other two localities was also high, reinforcing the idea that these three localities conformed a homogeneous population (Costa Brava) as suggested in Carreras-Carbonell et al. (2006). Therefore, we can conclude that the vast majority of larvae remain close to, or never leave, their population. Our results are in agreement with the high self-recruitment levels obtained in other studies (Jones et al. 1999; Jones et al. 2005; Swearer et al. 1999; Thorrold et al. 2001; Miller and Shanks 2004; Patterson et al. 2005), suggesting that the extent of dispersal between populations is lower than currently assumed.

Self-recruitment rate and, in general, gene flow among populations can be related with spawning characteristics and larval and adult strategies, e.g. Riginos and Victor

2001, Planes 2002 (however, see Shulman and Bermingham 1995). Eggs of *Tripterygion delaisi* are demersal and larvae remain in plankton for 16-21 days (Raventós and Macpherson 2001); however, these larvae are present almost exclusively in waters close to adult habitats during the spawning season (Sabatés et al. 2003). Thus, some retention mechanisms must be acting in these larvae during the mobile phase, since self-recruitment results imply that a significant percentage of spawned larvae come back to, or never leave, their natal population.

The inshore larval distribution of *Tripterygion delaisi* would determine that these species have lower dispersal possibilities than species with larvae situated offshore (Shanks and Eckert 2005). These differential dispersal capabilities could be due to stronger transport currents offshore than inshore (Tintoré et al. 1995; Largier 2003). Furthermore, larvae from benthic eggs, as those of *T. delaisi*, are larger, better swimmers, and have more developed sensory systems than larvae from pelagic eggs (Blaxter 1986). The combination of these characteristics may make retention more likely for larvae from benthic spawners than for larvae from pelagic spawners. Additionally, *T. delaisi* have planktonic larvae in spring-summer, when the wind regime (inshore winds) (Lloret et al. 2004) prevents dispersal of larvae promoting high self-recruitment rate. As Shanks and Eckert (2005) pointed out, the early life traits of many species may show an adaptation to the local oceanography, to avoid the alongshore loss of larvae. This promotes the settlement of larvae into their parental habitats.

The high self-recruitment rate observed in *Tripterygion delaisi* can be extrapolated to other species with short planktonic larval duration, larvae situated inshore and low adult mobility, e.g. Gobiesocidae, Syngnathidae (Macpherson and Raventós 2006) and can have important implications for the conservation and management of Mediterranean littoral fishes. Furthermore, *T. delaisi* populations are genetically isolated when large discontinuities of sand or deep-water channels (>30km) are present among them, preventing larval and adult exchange (Carreras-Carbonell et al. 2006). The results observed in the present paper and works from other authors (see references cited above) suggest that larval retention and current population isolation can be more elevated than presently assumed. These parameters are essential in the estimation of the population connectivity among areas, and are critical for sizing and

spacing marine protected areas (Sala et al. 2002; Cowen et al. 2006). Therefore, in order to maintain the connectivity among marine reserves in the western Mediterranean, the location of these protected zones may consider the degree of genetic isolation among populations, and the existence of geographic and ecological discontinuities that prevent gene flow among areas.

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Loci microsatèl·lits demostren un alt nivell d'autoreclutament en una espècie de peix mediterrani

Els nivells d'autoreclutament són paràmetres essencials per tal d'estimar el grau de connectivitat entre poblacions d'espècies marines, amb importants conseqüències per la biologia de la conservació. Utilitzant deu loci microsatèl·lits altament variables, s'ha estimat, durant tres anys, el nivell d'autoreclutament en una població de *Tripterygion delaisi* en el Mediterrani nordoccidental. S'han utilitzat sis poblacions, prèviament definides, com a referència per a realitzar els assignaments dels reclutes (Costa Brava, Columbretes, Formentera, Cabo de Palos, Cabo de Gata i Tarifa). Tot i que les larves d'aquesta espècie estan entre 16 i 21 dies al plàncton, durant els tres anys s'ha estimat que una mitja del 76.4±1.6% dels reclutes assentats han tornat a la seva població d'origen. A més, la resta de reclutes han sigut assignats, principalment, a les poblacions geogràficament més properes. Quan s'ha refinat l'anàlisi d'assignació, a una escala més petita, pels individus assignats a Costa Brava, utilitzant com a referència les tres localitats que la conformen (Cap de Creus, Tossa i Blanes), el percentatge més elevat de reclutes assignats ha sigut a la localitat d'origen (Blanes), on els reclutes han sigut mostrejats cada any, amb una mitja pels tres anys d'un 42.6±6.0%. El present estudi suggereix que un percentatge molt elevat de les larves de *T. delaisi* es queden molt a prop, o mai marxen, de la zona on han eclosionat. Aquests resultats poden ser transportats a altres espècies amb unes característiques en la primera fase del desenvolupament semblants i amb una mobilitat dels adults reduïda, i poden tenir implicacions importants per a la conservació i gestió de les espècies mediterrànies de peixos litorals.

Early life-history characteristics predict genetic differentiation in Mediterranean fishes

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Aquest article està pendent de submissió.

Abstract

The extent of dispersal by pelagic larvae in marine environments is central for understanding local population dynamics and designing sustainable marine reserves. Here we test the hypothesis that early life-history characteristics affect the rates of dispersal and, therefore, the levels of genetic partitioning between two Mediterranean littoral fishes: *Tripterygion delaisi* and *Serranus cabrilla*. These two species have similar sedentary adult behaviour, however they have markedly different early life histories: *T.delaisi* has benthic eggs, their larvae remain in the plankton between 16 and 21 days and develops inshore, whereas *S. cabrilla* has pelagic eggs, their larvae remain in the plankton between 21 and 28 days and develops offshore. We have found a clear distinction between the genetic population structure patterns of both species using highly variable microsatellite markers, showing a higher population structure for *T. delaisi* than for *S. cabrilla*. Our results suggest that large (>200 km) deep-water channels can be acting as effective barriers preventing larval and adult exchange between populations in both species, although smaller discontinuities (>30 km) would be affecting only *T. delaisi* due to their early life-history traits. Consequently a correspondence between population genetic structure and larval dispersal ability can be assessed combining several early life-history characteristics including larval duration (PLD), egg type and, spatial and temporal distributions of larvae relative to the coast.

Keywords: *Tripterygion delaisi*; *Serranus cabrilla*; microsatellite; early life history; larval dispersal; Mediterranean; littoral fish; larval behaviour; pelagic larval duration; egg type; genetic population structure

Introduction

Larvae of most shallow-water marine fish species have a pelagic phase, in which they are exposed to hydrodynamic transport processes that may disperse them to new places and then metamorphose into sedentary adults (Sale, 1980; Purcell *et al.,* 2006; exceptions in Leis, 1991). Larval exchange is assumed for most species to be the main mechanism uniting spatially discrete populations (Ehrlich, 1975), since demersal adults used to be more sedentary. The duration of this "mobile" phase depends on the species, ranging from 9 (*Symphodus ocellatus*) to 71 (*Lipophrys trigloides*) days for Mediterranean fishes (Raventós & Macpherson, 2001) while environmental factors, such as currents and winds, can also influence the settlement processes (Wilson & Meekan, 2001; Cowen, 2002; Raventós & Macpherson, 2005).

Some studies in marine fishes showed a strong relationship between PLD (pelagic larval duration) and the species population structure pattern. Riginos & Victor (2001), analyzing three blennioid species with different PLDs from the Californian Gulf, observed that the larval strategy gives an accurate approximation about the level of species population structure. Similarly, Purcell *et al.* (2006) found the same trend between two coral reef fish species around the Caribbean basin. Moreover, Doherty *et al.* (1995) established a negative, and highly significative, correlation between the PLD and the level of species population structure inferred from allozyme data using seven species from the Great Barrier Reef. On the contrary, other studies found that neither egg type (benthic versus planktonic) nor PLD has been shown to be a strong determinant of population structure (Shulman & Bermingham, 1995; Bohonak, 1999). More recently, Bay *et al.* (2006) detected a relationship between PLD and genetic structure using both, mitochondrial and nuclear, molecular markers in eight Pomacentridae species. These relationships, however, were caused by a single species (*Acanthochromis polyacanthus*), which differs from all the other species examined in lacking a larval phase. With this species excluded there was no relationship between PLD and genetic structure using either marker.

Other mechanisms than PDL, such as: inshore or offshore larval distributions and benthic or pelagic spawning strategies, can be influencing species dispersal potential.

The stronger currents offshore than inshore (Tintoré *et al.,* 1995; Largier, 2003) may determine that larvae located near the coastline would have lower dispersal potential than those located along the continental shelf and slope (Shanks & Eckert, 2005). Moreover, larvae from benthic eggs are larger, better swimmers, and have more developed sensory systems than larvae from pelagic spawners (Blaxter, 1986). The combination of these features may make retention more likely for larvae from benthic spawners than for larvae from pelagic eggs, thereby affecting their dispersal capabilities (Hickford & Schiel, 2003; Macpherson & Raventós, 2006).

Therefore, the pelagic phase seems to be the key to understand the dispersal pattern and the connectivity between populations of fish species. Unfortunately, direct observation of dispersal in larvae is unfeasible for most species, and thus, indirect estimations become the only form to approach dispersal in population structure studies. Indirect estimates of larval dispersal can be obtained using neutral genetic markers (Smith, 1990). Microsatellites are neutral highly polymorphic nuclear loci that have been successfully used to infer population differentiation at different geographical scales. It is known that microsatellite markers show great variability within fish species, and particularly within marine ones (DeWoody & Avise, 2000). They have been widely employed to solve population structuring on a wide range of geographical levels (Appleyard *et al*., 2001; Rico & Turner, 2002; Carlsson *et al*., 2004; Carreras-Carbonell *et al*., 2006a).

Tripterygion delaisi and *Serranus cabrilla* are two littoral Mediterranean species. The biology of both species is well known, specially their larval dispersal distribution pattern and their PLD inferred from otolith marks. Both species present similar geographic distribution, inhabiting the Mediterranean Sea and eastern Atlantic. Adults are highly territorial and no migratory movements have been described (Heymer, 1977; García-Rubies, 1999). *T. delaisi* has benthic eggs with parental care and hatching after 15 to 20 days (Wirtz, 1980), whereas *S. cabrilla* spawns in the water column having planktonic eggs, and their hatching time may be similar to other serranid species between 1 and 3 days (Zabala, personal communication). The larvae of *T. delaisi* remain in the plankton between 16 and 21 days, whereas larvae of *S. cabrilla* spent between 21 and 28 days in the water column (Raventós & Macpherson, 2001). *T. delaisi* larvae showed a great retention since

their larvae are found exclusively in near shore waters (<100 m to the adult habitats); on the contrary, *S. cabrilla* larvae have been collected along the continental shelf at a considerable distance from the habitats of the adults (Sabatés *et al*., 2003).

Consequently, both species will be used as model organisms to test the hypothesis that larval dispersal ability, as estimated from a comprehensive set of early life-history characteristics, including egg type, larval spatial distributions and PLD, can be used to predict the adult population's genetic structure. The extent of dispersal by pelagic larvae in marine environments is central for understanding local population dynamics and designing sustainable marine reserves (Palumbi, 2003; Bell & Okamura, 2005; Purcell *et al*., 2006).

Materials and Methods

Sampling and DNA extraction

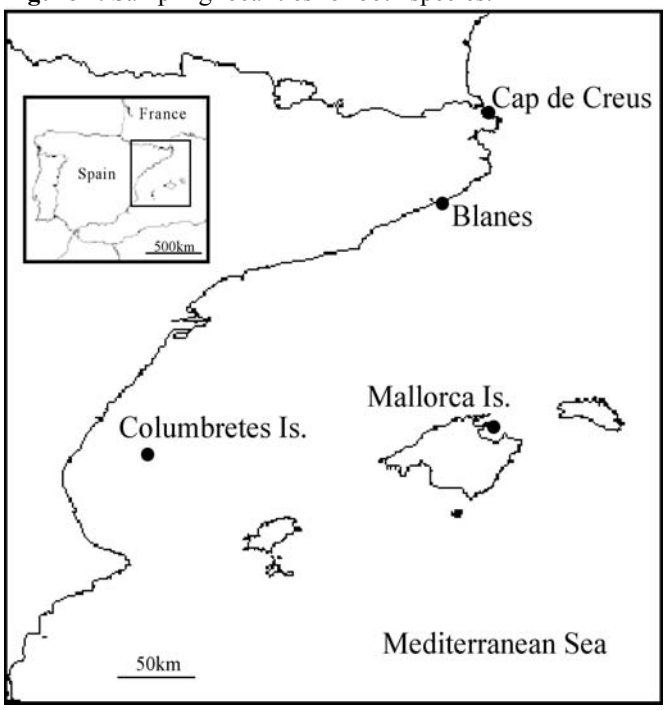
Both species were sampled at the same four localities: Cap de Creus (CC, n_{Td}=20, n_{Sc}=30), Blanes (BL, n_{Td}=36, n_{Sc}=30), Mallorca Is. (MA, n_{Td}=42, n_{Sc}=30) and Columbretes Is. (CO, n_{Td}=30, n_{Sc}=25) (Fig. 1). Geographical distances between populations can be found in Annex I. *Tripterygion delaisi* were collected by SCUBA divers using hand nets, a small portion of the anal fin was removed from living fish and preserved in absolute ethanol at room temperature; fishes were then returned to the sea. *Serranus cabrilla* specimens were sampled in the field by hook and line or spear gun, or purchased from commercial fish markets. Pectoral fin clips were removed and preserved also in absolute ethanol. Total genomic DNA was extracted from fin tissue using the Chelex 10% protocol (Estoup *et al*., 1996).

PCR amplification and screening

Amplifications of the 11 microsatellite loci isolated from *Serranus cabrilla* (Carreras-Carbonell *et al*. 2006b) and the 10 from *Tripterygion delaisi* (Carreras-Carbonell *et al*., 2004; 2006a) were carried out under the conditions previously described. Locus Sc02 for *S. cabrilla* was excluded because amplifications were poor and allele sizing misleading. Amplified products were scored using an ABI 3700

automatic sequencer from the Scientific and Technical Services of the University of Barcelona. Alleles were sized by GENESCAN™ and GENOTYPER™ software, with an internal size marker CST Rox 70-500 (BioVentures Inc.).

Figure 1. Sampling localities for both species.



Cap de Creus (n_{Td}=20, n_{Sc}=30), Blanes (n_{Td}=36, n_{Sc}=30), Mallorca Is. (n_{Td}=42, n_{Sc}=30) and Columbretes Is. (n_{Td}=30, n_{Sc}=25), (n): number of individuals analysed for *Tripterygion delaisi* ($_{Td}$) and *Serranus cabrilla* ($_{Sc}$).

Statistical analyses

Allele frequencies, mean allelic richness, expected (H_E) and observed (H_O) heterozygosity per locus, for each locality for both species were calculated using the GENECLASS2 program (Piry *et al.,* 2004). The inbreeding coefficient (F_{IS}) for each locus-locality combination was computed with GENETIX version 4.05 (Belkhir *et al.*, 2004) and its confidence interval was estimated with 10000 bootstrapping values. Linkage disequilibrium were tested within species for each locus-locality combination using GENEPOP version 3.4 (Raymond & Rousset, 1995), which employs a Markov chain method, with 5000 iterations, following the algorithm of Guo & Thompson (1992). These results were adjusted for multiple tests using the sequential Bonferroni procedure with $\Delta = 0.05$ (Rice, 1989). In instances where the observed genotype frequencies deviated significantly from HWE, the program MICRO-CHECKER

(Van Oosterhout *et al*., 2004) was used to infer the most probable cause of such HWE departures.

Within each species, genetic differentiation was estimated using the classical F_{ST} approach (Wright, 1951; Weir & Cockerham, 1984), and their significance between each pair of comparisons computed in ARLEQUIN v. 2.000 (Schneider *et al*., 2000).

The program STRUCTURE 2.0 (Pritchard & Wen, 2003) was used to detect the number of genetically homogeneous populations (K) for each species. The population structure was considered without prior information of the number of locations at which the individuals were sampled and into which location each individual belongs and considering frequencies independent. We performed the analyses following the recommendations of Evanno *et al*. (2005), we calculated an *ad hoc* statistic ΔK based in the rate of change in the log probability of data between successive K values, since the height of this model values seems to accurately detect a correct estimation of the number of populations. For each data set 20 runs were carried out in order to quantify the standard deviation (SD) of the likelihood of each K. We tested a range of Ks between 1 and 7.

The correlation between pairwise multilocus distances ($F_{ST}/(1- F_{ST})$) and geographical distance (Ln distance) was assessed for populations of both species using the Mantel permutation test (10000 permutations; Mantel, 1967) implemented in GENEPOP. The geographical distance in kilometres was computed as the coastline distance between continental sample locations and as the straight geographical distance for island populations (see Annex I).

Results

Genetic variability

High genetic variability has been found in both species in terms of extensive polymorphism per locality and locus. Mean allelic richness was 15.30±1.14 and 9.59±0.77 for *T. delaisi* and *S. cabrilla*, respectively. High expected and observed

heterozygosities were found in both *T. delaisi* (0.835±0.021 and 0.789±0.025) and *S. cabrilla* (0.702±0.031 and 0.659±0.031) (Table 1 and 2). However, values were always significantly greater for *T. delaisi* than for *S. cabrilla* either for N_A (Wilcoxon test, Z=3.02, P<0.005), H_E (Z=3.17, P<0.005) and H_O (Z=2.93, P<0.005). All loci were considered statistically independent since no linkage disequilibrium between them has been observed in any of both species locations sampled. Private alleles were present in all populations and loci; between both species no significant differences were found (13.54±0.18% for *T. delaisi* and 10.52±0.16% for *S. cabrilla*, Z=1.12 and P>0.3).

Table 1 Summary of genetic variation at ten microsatellite loci in *Tripterygion delaisi* localities.

		Locus									
		Td01	Td02	Td04	Td05	Td06	Td07	Td08	Td09	Td10	Td11
Cap de Creus	n	40	40	40	40	38	40	32	40	40	40
	a	11	11	15	19	20	7	15	7	20	8
	H_E	0.905	0.877	0.894	0.932	0.957	0.691	0.942	0.697	0.960	0.719
	H_O	0.950	0.700	0.750	1.000	0.842	0.500	0.438	0.600	0.900	0.700
	F_{IS}	-0.051	0.206	0.164	-0.075	0.123	0.282	0.543*	0.143	0.064	0.027
Blanes	n	66	66	68	68	66	68	58	68	68	68
	a	12	12	16	21	26	5	22	10	26	13
	H_E	0.890	0.795	0.924	0.946	0.962	0.642	0.936	0.709	0.951	0.785
	H_O	0.758	0.788	0.912	0.971	1.000	0.588	0.750	0.529	0.941	0.794
	F_{IS}	0.151	0.009	0.013	-0.027	-0.040	0.085	0.201*	0.256*	0.010	-0.012
Mallorca Is.	n	84	82	84	84	84	84	84	84	84	84
	a	16	12	18	25	24	7	20	5	26	7
	H_E	0.900	0.734	0.891	0.951	0.937	0.601	0.914	0.549	0.946	0.711
	H_O	0.881	0.854	0.881	0.952	0.881	0.548	0.738	0.643	1.000	0.738
	F_{IS}	0.022	-0.165	0.012	-0.002	0.060	0.090	0.194*	-0.173	-0.058	-0.039
Columbretes Is.	n	60	60	60	60	60	60	58	60	60	60
	a	12	8	14	24	27	4	26	8	25	8
	H_E	0.900	0.677	0.875	0.953	0.929	0.495	0.935	0.775	0.951	0.663
	H_O	0.867	0.767	0.867	1.000	0.967	0.567	0.793	0.567	0.967	0.667
	F_{IS}	0.038	-0.135	0.010	-0.050	-0.041	-0.146	0.154*	0.272*	-0.017	-0.006

(n): number of analysed chromosomes, (a): number of alleles, (H_E) and (H_O): expected and observed heterozygosity respectively, and (F_{IS}): inbreeding coefficient and significance (*=P<0.05).

For *Tripterygion delaisi*, F_{IS} values were statistically significant for most localities regarding loci Td08 and Td09 (Table 1). The MICRO-CHECKER software attributed deviations presented for both loci to the presence of null-alleles. For *Serranus cabrilla*, significant F_{IS} values were observed for different loci in different populations: Sc08 for Cap de Creus, Sc06 for Blanes, Sc05 for Mallorca Is. and Sc05 and Sc08 for Columbretes Is. (Table 2). These deviations could be explained by the presence of null alleles. Null-alleles appear when one allele is unamplified due to mutations in the sequence where one of the primers was designed, and/or when technical problems associated with amplification and scoring arise (Hoarau *et al.*, 2002). Technical issue could be ruled out since, for both species, all homozygous individuals and failed amplifications for the challenging loci were re-amplified twice lowering the annealing temperature to 50ºC and accurate scoring of larger alleles with poor amplification was also carried out.

Table 2. Summary of genetic variation at ten microsatellite loci in *Serranus cabrilla* localities.

		Locus										
		Sc03	Sc04	Sc05	Sc06	Sc07	Sc08	Sc11	Sc12	Sc13	Sc14	Sc15
Cap de Creus	n	60	60	60	60	58	60	60	60	60	60	60
	a	9	10	25	13	8	11	3	4	8	13	4
	H_E	0.686	0.784	0.964	0.845	0.806	0.741	0.158	0.680	0.754	0.855	0.441
	H_O	0.667	0.767	0.867	0.700	0.862	0.567	0.133	0.633	0.867	0.900	0.400
	F_{IS}	0.028	0.022	0.103	0.174	-0.071	0.238*	0.156	0.069	-0.153	-0.054	0.095
Blanes	n	60	60	56	60	60	58	60	60	60	60	60
	a	7	8	19	12	7	8	3	5	9	12	4
	H_E	0.554	0.766	0.949	0.829	0.764	0.656	0.215	0.621	0.792	0.822	0.372
	H_O	0.553	0.700	0.857	0.633	0.767	0.553	0.200	0.500	0.767	0.733	0.333
	F_{IS}	0.038	0.094	0.099	0.239*	-0.004	0.162	0.072	0.198	0.033	0.109	0.105
Mallorca Is.	n	60	58	60	60	60	60	60	58	60	60	58
	a	7	9	21	14	9	9	5	6	13	19	5
	H_E	0.688	0.842	0.953	0.889	0.808	0.728	0.351	0.545	0.831	0.893	0.449
	H_O	0.700	0.828	0.767	0.933	0.800	0.700	0.333	0.586	0.867	0.867	0.414
	F_{IS}	-0.017	0.017	0.198*	-0.051	0.010	0.039	0.051	-0.077	-0.044	0.030	0.081
Columbretes Is.	n	50	50	50	50	50	50	50	50	50	50	50
	a	7	8	21	12	6	11	5	6	8	14	5
	H_E	0.716	0.780	0.958	0.875	0.805	0.771	0.226	0.666	0.822	0.833	0.431
	H_O	0.680	0.840	0.760	0.880	0.800	0.520	0.240	0.640	0.720	0.840	0.360
	F_{IS}	0.051	-0.079	0.210*	-0.006	0.006	0.330*	-0.063	0.040	0.126	-0.008	0.168

(n): number of analysed chromosomes, (a): number of alleles, (H_E) and (H_O): expected and observed heterozygosity respectively, and (F_{IS}): inbreeding coefficient and significance (*=P<0.05).

Population differentiation

- *Tripterygion delaisi*

A high degree of differentiation between localities was found; only between the two most closely geographical localities (Cap de Creus and Blanes) no significant genetic differentiation was present (F_{ST}=0.002). Significant F_{ST} values among the other populations ranged between 0.026 and 0.044 (Table 3). Furthermore, to estimate the number of genetically homogeneous populations sampled in our study, we used the program STRUCTURE without prior information of the number of locations at which the individuals were sampled. Three genetically homogeneous clusters were detected, since a peak in ΔK was showed for K=3 (Fig. 2).

Table 3. Multilocus F_{ST} values among each population pair for both species, including (below) and excluding (above) loci with null alleles (Td08 and Td09 for *T. delaisi*, and Sc05 and Sc08 for *S. cabrilla*).

	Cap de Creus		**Blanes**		**Mallorca Is.**		**Columbretes Is.**	
	T.delaisi	*S.cabrilla*	*T.delaisi*	*S.cabrilla*	*T.delaisi*	*S.cabrilla*	*T.delaisi*	*S.cabrilla*
Cap de Creus			0.001	-0.001	0.028*	0.012*	0.039*	0.004
Blanes	0.002	-0.002			0.022*	0.013*	0.031*	0.004
Mallorca Is.	0.044*	0.009*	0.027*	0.008*			0.023*	0.011*
Columbretes Is.	0.041*	0.003	0.029*	0.002	0.026*	0.009*		

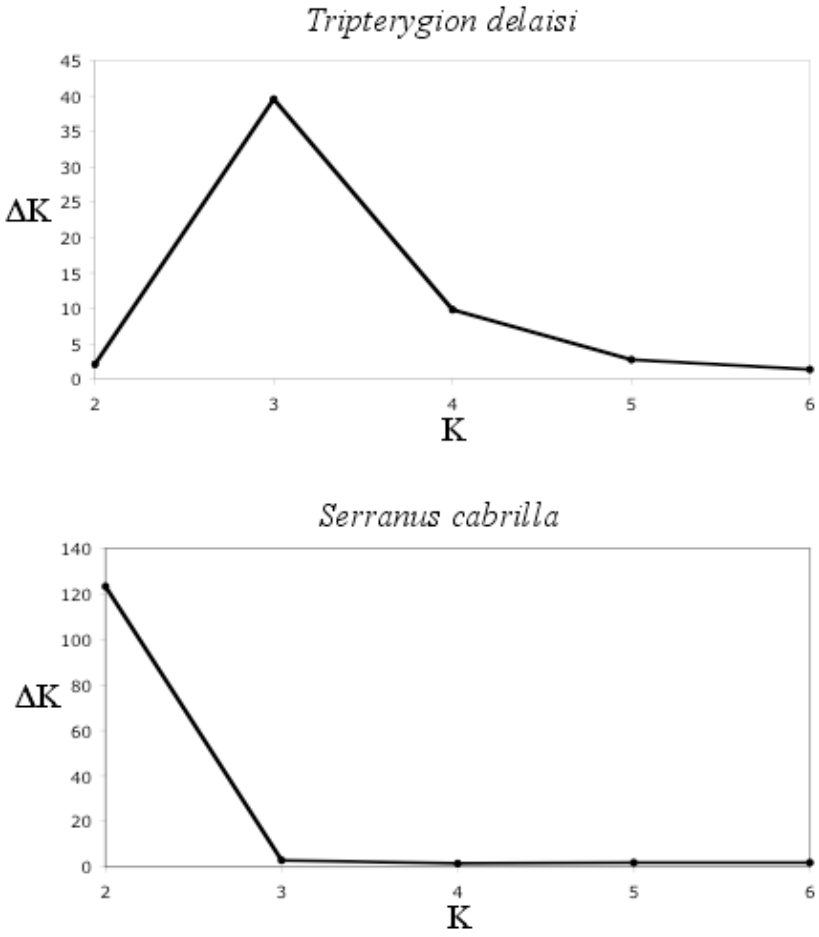
(*=P<0.05)

- *Serranus cabrilla*

Localities within this species showed weak structure pattern, Mallorca Is. locality was significantly different from the other three localities, which presented no significant genetic differentiation between them. F_{ST} values ranged from 0.008 to 0.009 between Mallorca Is. and the other localities, whereas these values ranged between -0.002 and 0.003 when comparing Cap de Creus, Blanes and Columbretes Is. localities (Table 3). Similarly, two genetically homogeneous populations were found, since a peak in ΔK was

showed for K=2 (Fig. 2). The height of ΔK was used as an indicator of the strength of the signal detected by STRUCTURE (Evanno *et al*., 2005).

Figure 2. Values of ΔK calculated as in Evanno *et al*. (2005) for each number of genetically homogeneous populations for each species independently.

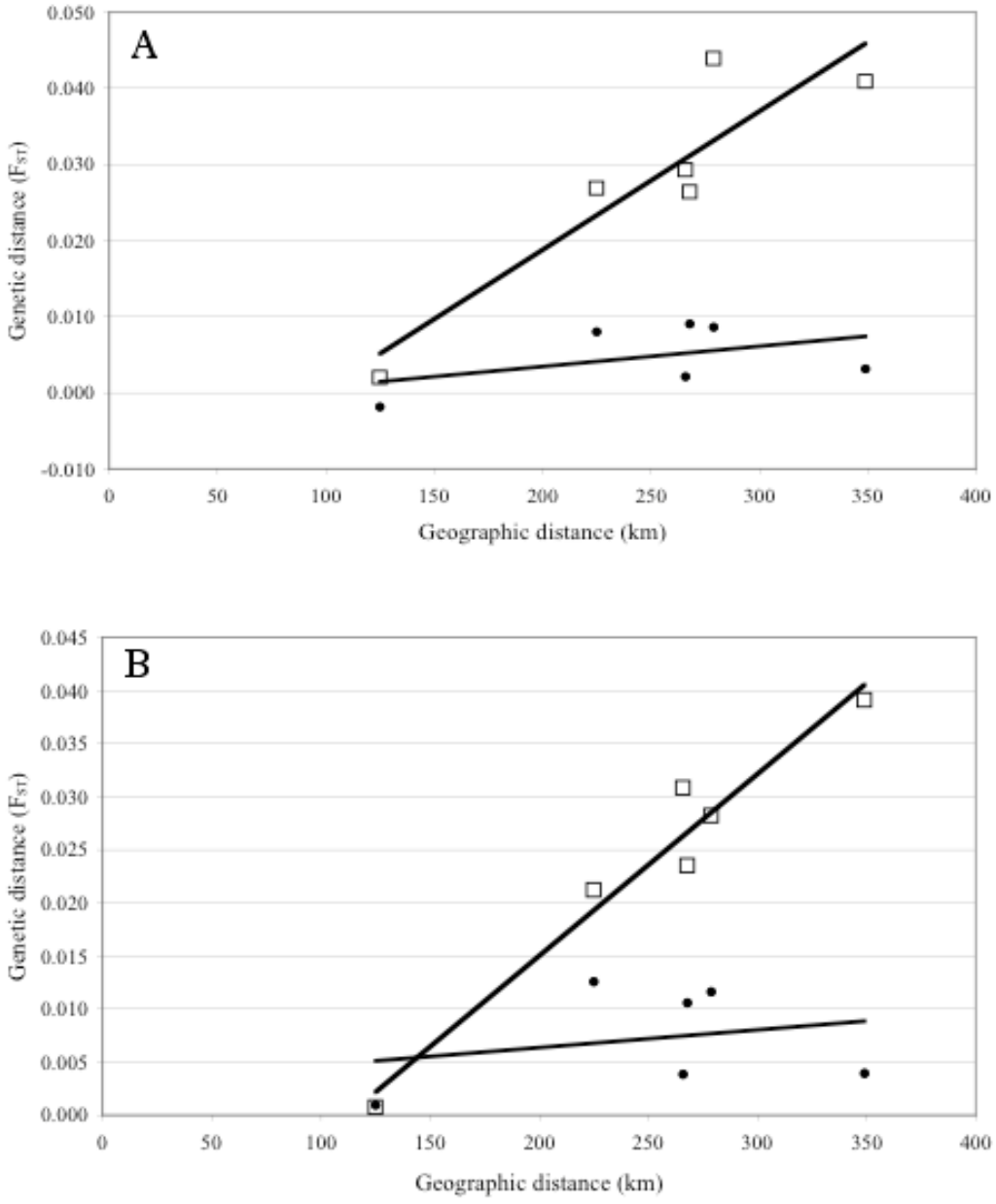


Significant differences were found when comparing F_{ST} values between both species (Wilcoxon test, Z=2.20, P<0.05), being larger for *Tripterygion delaisi* than for *Serranus cabrilla*. When removing individual loci with the highest proportion of null alleles (Td08 and Td09 for *T. delaisi* and Sc05 and Sc08 for *S. cabrilla*), the significance between population pairs did not change and F_{ST} values still remained larger for *T. delaisi* (Z=2.20, P<0.05) (Table 3).

No significant associations between genetic differentiation (F_{ST}) and geographic distance in both species were revealed by a Mantel test (Spearman's R=0.77, P=0.086 for *T. delaisi* and R=0.49, P=0.249 for *S. cabrilla*) (Fig. 3a). However, when

analyses were performed excluding loci with null alleles isolation-by-distance was found for *T. delaisi* (R=0.83, P=0.040), whereas for *S. cabrilla* populations no isolation-by-distance appeared (R=0.17, P=0.322) (Fig. 3b).

Figure 3. Relationship between geographic distance (km) and genetic distance (F_{ST}) for *T. delaisi* (□) and *S. cabrilla* (●) populations. (A) Including all loci and (B) excluding loci with null alleles (Td08 and Td09 for *T. delaisi*, and Sc05 and Sc08 for *S. cabrilla*).



Discussion

There was a clear distinction between the genetic population structure patterns of *T. delaisi* and *S. cabrilla*. Although F_{ST} values were low for both species, significantly greater values were found for *T. delaisi* than for *S. cabrilla* when comparing the same population pairs. And according to F_{ST} significance, three and two genetically homogeneous clusters were inferred by Bayesian analyses for *T. delaisi* and *S. cabrilla* species respectively. Furthermore, isolation-by-distance was found for *T. delaisi* populations when loci with null alleles were excluded. These results suggested that *T. delaisi* shows a higher population structure than *S. cabrilla*.

Differentiation in population structure of each species can be related with differences in their dispersal capabilities. The adult phases in both species are quite sedentary (Heymer, 1977; García-Rubies, 1999), therefore, their potential dispersal should be related with larval dispersal capabilities. These capabilities, measured as planktonic larval duration (PLD), are slightly larger for *S. cabrilla* (21-28 days) than for *T. delaisi* (16-21 days) (Raventós & Macpherson, 2001). However, dispersal potential can also be influenced by egg type and larval distribution (Shanks & Eckert, 2005). *S. cabrilla* is a pelagic spawner and their eggs remain in the plankton until the larvae hatch, furthermore, their larvae have been collected along the continental shelf, at a considerable distance from the habitats of the adults. Contrary, *T. delaisi* has benthic eggs with restricted larval distribution, showing a great retention since their larvae are found exclusively in inshore waters (Sabatés *et al.*, 2003). Several studies document stronger transport currents offshore than inshore (Tintore *et al.,* 1995; Largier, 2003). Thus, larvae situated near the coastline (*T. delaisi*) would have lower dispersal possibilities than those situated along the continental shelf and slope (*S. cabrilla*) (Shanks & Eckert, 2005; Macpherson & Raventós, 2006). Thus, the estimates of genetic differentiation in both species are consistent with their predicted dispersal abilities.

T. delaisi showed a strong genetic structure, with isolation-by-distance, when loci with null alleles were excluded. Carreras-Carbonell *et al*. (2006a), analysing eight Mediterranean *T. delaisi* populations have also found a strong population structure pattern. This previous work supported the idea that the genetic breaks between

populations or zones are associated with the presence of physical barriers to dispersal. Therefore, discontinuities (>30 km) of sand or deep-water channels could be acting as effective barriers, preventing larval and adult exchange between *T. delaisi* populations (Carreras-Carbonell *et al.*, 2006a). Cap de Creus and Blanes populations were genetically homogeneous since any barrier like previously described was present between them. However, the Ebro river delta, which constitutes a sand and fresh water barrier of *ca.* 50 km, could reduce gene flow between those localities and Columbretes Is.

On the other hand, *S. cabrilla* showed a weak population structure, without isolation-by-distance, even excluding loci with null alleles. More geographically distant populations should be analysed in order to be able to detect isolation-by-distance, since genetic differentiation between populations is smaller due to the greater dispersal ability of the individuals of this species. Cap de Creus, Blanes and Columbretes Is. populations were genetically homogeneous and only Mallorca Is. population remained isolated. Due to its wide bathymetric range (0-500m) and to its capacity to survive in almost all hard-bottom habitats, associated to its dispersal abilities, small rocks, wrecks or hard structures can act as suitable habitats, allowing the connection, by stepping stone system, between populations separated by sand or fresh water gaps. However Mallorca Is. is separated from the other populations by a deep-water channel (1000-2000m) of *ca.* 200 km that adults and larvae are not able to cross. Nevertheless, the larvae of *S. cabrilla* distributed along the continental shelf can easily colonize Columbretes Is. situated on the continental shelf, near to the coast (*ca.* 30 km).

Therefore, we can conclude that large (>200 km) deep-water channels can be acting as effective barriers preventing larval and adult exchange between populations in both *T. delaisi* and *S. cabrilla* although smaller discontinuities (>30 km) would be affecting only the former due their early life-history characteristics. Consequently a correspondence between population genetic structure and larval dispersal ability can be assessed combining several early life-history characteristics including larval duration (PLD), egg type and, spatial and temporal distributions of larvae relative to the coast. Although this study only examined two species, the large range of

differences in microsatellite partitioning pointed out the importance of the early life-history characteristics of species in determining their dispersal capabilities.

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ANNEX

Annex I. Geographic distance, in kilometres, between sampling localities.

	Cap de Creus	Blanes	Mallorca Is.
Blanes	125		
Mallorca Is.	297	225	
Columbretes Is.	349	266	268

Característiques de les primeres fases del desenvolupament com a indicadors de la diferenciació genètica en dues espècies de peixos mediterranis.

En la majoria d'espècies marines, el grau de dispersió degut a la seva fase pelàgica és fonamental per tal d'entendre llur dinàmica poblacional així com per dissenyar reserves marines molt més sostenibles. Aquí s'ha testat l'hipòtesi que les característiques de les primeres fases del desenvolupament de les espècies afecten el seu grau de dispersió, i com a conseqüència els nivells d'estructura poblacional, entre dues espècies de peixos mediterranis litorals: *Tripterygion delaisi* i *Serranus cabrilla*. Aquestes dues espècies tenen un comportament sedentari de l'adult similar; però presenten unes característiques, en les primeres fases del desenvolupament, molt diferents. Els ous de *T. delaisi* són bentònics i les larves estan en el plàncton entre 16 i 21 dies allunyant-se molt poc de la línia de costa; d'altra banda, *S. cabrilla* té uns ous pelàgics i les larves s'allunyen molt de la línia de costa, estant en el plàncton entre 21 i 28 dies. S'ha trobat una clara diferenciació entre les estructures poblacionals de les dues espècies utilitzant loci microsatèl·lits altament variables, mostrant una major estructura poblacional per *T. delaisi* que per *S. cabrilla*. Els resultats obtinguts suggereixen que canals amples d'aigua profunda (>200 km) poden estar actuant com importants barreres prevenint l'intercanvi de larves i adults entre poblacions en ambdues espècies, i que discontinuïtats petites (>30 km) poden estar afectant únicament a *T. delaisi* degut a les seves característiques en les primeres fases del desenvolupament. D'aquesta manera, existeix una clara correspondència entre l'estructura poblacional i la capacitat de dispersió larvària, la qual pot ser inferida combinant múltiples característiques de les primeres fases del desenvolupament de les espècies, incloent la duració larvària (PLD), el tipus d'ous i, la distribució espacial i temporal de la larva en relació a la costa.

4.- Resum

4.1.- Filogènies moleculars i especiació

4.1.1.- Processos d'especiació i la seva resolució

S'ha realitzat una filogènia molecular pel gènere de peixos *Tripterygion* a partir de cinc gens diferents (12S, 16S, tRNA-valina, COI i 18S), obtenint-se pels diferents gens, reconstruccions filogenètiques oposades i valors de suport del nodes no gaire elevats. Quan aquests es tracten tots de forma conjunta s'obté una tricotomia entre les tres espècies de tripterígids. De la mateixa manera, el percentatge de divergència genètica entre aquestes espècies, tractant tots els gens conjuntament, és molt semblant per les tres comparacions (aproximadament d'un 8%). Així doncs, es va poder inferir que les actuals espècies d'aquest gènere s'havien de veure condicionades per una ràpida radiació que propiciés l'especiació d'una espècie ancestral inicial. El procés més provable capaç de generar aquesta radiació havia de ser la dessecació del Mediterrani i el seu posterior reompliment, de forma molt ràpida (*c.a.* 100 anys), ara fa uns 5.2 Ma (Crisis de Salinitat del Messinià, MSC). Això va suposar, per l'espècie ancestral, la colonització d'un nou hàbitat amb nous nínxols, i d'aquesta manera la ràpida radiació adaptativa que va donar lloc a les actuals espècies de tripterígids mediterranis. Igualment, la utilització de diferents mètodes de reconstrucció filogenètica (Màxima Parsimònia: MP, Maximum Likelihood: ML, Minimum Evolution: ME i Inferència Bayesiana: BI) donaven, aparentment, resultats molt diferents quan s'utilitzaven els mateixos gens de forma independent o tots conjuntament. De totes maneres podem considerar que les topologies no són incongruents (Moyer *et al*., 2004) donat que els valors de bootstrap són inferiors al 80% i les probabilitats posteriors inferiors al 95%. Aquestes diferències es poden atribuir al propi procés d'especiació, el qual fa difícil una reconstrucció filogenètica fiable. Degut a que, per a la majoria de les reconstruccions, els valors de suport dels nodes eren molt baixos, la topologia més suportada va resultar ser una tricotomia, posant de manifest, una altra vegada, la rapidesa del procés d'especiació.

Les reconstruccions filogenètiques estimades a través d'Inferència Bayesiana semblen ser les més correctes, ja que no mostren un gran biaix quan el gen utilitzat presenta posicions saturades (Carreras-Carbonell *et al*., 2005).

A partir de les dades moleculars obtingudes i suposant que el ràpid procés d'especiació que va tenir lloc fa aproximadament uns 5.2 Ma, es van poder calibrar els rellotges moleculars i d'aquesta manera obtenir les taxes d'evolució pels gens 12S (0.81±0.23%/Ma) i 16S (1.10±0.23%/Ma), podent-se utilitzar per datar altres especiacions i divergències dins del gènere *Tripterygion*.

Molt freqüentment les filogènies moleculars són inferides a partir d'un sol gen. Tot i que en molts casos s'obté una reconstrucció altament suportada (Allegrucci *et al*., 1999; Ballard *et al*., 1992); reconstruccions significativament diferents es poden obtenir utilitzant diferents gens, generant certa controvèrsia a l'hora d'esclarir les relacions entre les espècies que s'analitzen (Cristescu & Hebert, 2002; Mattern, 2004). D'aquesta manera, per tal d'obtenir una reconstrucció filogenètica el més fiable possible, les relacions haurien de ser inferides a partir de l'anàlisi de múltiples gens (Crow *et al*., 2004). Així doncs, la majoria dels estudis filogenètic fets només amb una única seqüència de DNA quedarien en entredit. De la mateixa manera, quan el procés d'especiació és degut a una ràpida radiació, la reconstrucció és fa difícil, donat que el senyal és feble. Aleshores, l'utilització de diferents gens s'entreveu com la millor manera d'assegurar la correcta reconstrucció filogenètica, tot i que el nombre de gens utilitzats tampoc fa falta que sigui molt gran, ja que l'informació procedent d'un nombre molt elevat de gens no millora la reconstrucció filogenètica quan ens trobem davant d'una ràpida radiació com a procés d'especiació (Takezaki *et al*., 2004).

La conca mediterrània ha sofert una sèrie de processos eustàtics i geològics, els quals han propiciat una excepcional diversitat d'organismes amb moltes espècies endèmiques en la majoria de phila. Segons Briggs (1974) un 9.6% de les 540 espècies de peixos litorals presents en el Mediterrani són endèmiques. Així doncs, la conca mediterrània està considerada com un "hot spot" de diversitat a nivell mundial. Els esdeveniments passats, especialment els geològics i eustàtics, han pogut jugar un paper molt important en els processos d'especiació. Com s'ha vist, els tripterígids en

són un clar exemple (Carreras-Carbonell *et al*., 2005). Els processos d'especiació en els làbrids mediterranis també semblen ser el resultat dels nombrosos processos geològics que han tingut lloc a la conca mediterrània (Hanel *et al*., 2002). El mateix podem dir dels ràjids (Valsecchi *et al*., 2005), espàrids (Bargelloni *et al*., 2003), blènids (Almada *et al*., 2001) o moròmids (Allegrucci *et al*., 1999) presents al mar Mediterrani, dels ciprínids presents a les conques fluvials mediterrànies (Hrbek & Meyer, 2003) i dels gòbids amb espècies tant d'aigua dolça com salada (Penzo *et al*., 1998). Tots ells han sofert processos d'especiació associats a esdeveniments geològics o eustàtics que han tingut lloc a la conca mediterrània.

De forma similar, també es va realitzar una filogènia utilitzant tres gens (12S, 16S i tRNA-val) i amb un cert caràcter filogeogràfic, per les espècies de serrànids mediterranis per tal de situar l'altra espècie objectiu: *Serranus cabrilla*. Al igual que per la filogènia del tripterígids, utilitzant tot els gens conjuntament s'obtingué una reconstrucció molt més suportada que quan cada gen era utilitzat independentment. Cada gènere forma un grup monofilètic altament suportat, i dins de cada gènere cada espècie està ben diferenciada i sense clades interns. Així doncs, no es van trobar espècies críptiques per *S. cabrilla* ni per cap altra espècie de serrànid mediterrani. Podent-se tractar, les espècies prèviament definides, com unitats homogènies i independents.

El temps de divergència inferit utilitzant les taxes d'evolució estimades per *T. delaisi* (Carreras-Carbonell *et al*., 2005) ens situa la divergència entre *S. cabrilla* i *S. atricauda* fa aproximadament uns 1.1 Ma, aquest valor és similar al trobat entre les subspècies de *T. delaisi* i que s'ha relacionat amb les glaciacions del Quaternari. Alhora aquestes dues espècies van divergir fa uns 2.4 Ma de *S. scriba* i el grup format per aquestes tres espècies va divergir de *S. hepatus* fa uns 3.3 Ma. Aquesta última data coincidiria amb les dràstiques glaciacions d'entre principis del Pleistocè (3.6 Ma) i finals del Pliocè (2.7 Ma), obtenint-se el mateix temps de divergència que l'estimat entre les dues espècies abans considerades com *T. tripteronotus* (*T. tripteronotus* i *T. tartessicum*).

4.1.2.- Espècies críptiques

En la majoria de grups i hàbitats marins són comunes les espècies críptiques (per una extensa revisió veure Knowlton, 1993). En el Mediterrani ja hi ha descrites algunes espècies críptiques en peixos (e.g. *Pomatoschistus microps vs. P. marmoratus*, Berrebi *et al*., 2005; *Gobius auratus* species complex, Herler *et al*., 2005), i també en altres grups com ascidis (*Clavelina lepadiformis*, Tarjuelo *et al*., 2001; *Pseudodistoma crucigaster*, Tarjuelo *et al*., 2004), equinoderms (*Ophiothrix* spp., Baric & Sturmbauer, 1999) o esponges (*Scopalina* spp., Blanquer & Uriz, comunicació personal). Es troben noves espècies críptiques, fins i tot en gèneres econòmicament importants i extremadament ben estudiats, com per exemple en crustacis (Machordom & Macpherson, 2004). Moltes d'aquestes espècies críptiques han pogut ser demostrades únicament a través d'estudis moleculars (Colborn *et al*., 2001).

Així doncs, és molt important, abans de realitzar qualsevol estudi de dinàmica i estructura poblacional, realitzar un estudi previ per tal d'obtenir informació filogeogràfica sobre l'espècie o espècies d'interès. D'aquesta manera es comproven els seus rangs de distribució, assegurant que totes les poblacions que es pretenen analitzar *a posteriori* pertanyen a les espècies inicials escollides. D'aquesta manera, fent referència a Avise *et al*. (1987), la filogeografia intraespecífica constitueix el pont entre la sistemàtica i la genètica de poblacions.

Així doncs, la filogènia pel gènere *Tripterygion* es va realitzar amb un marcat caràcter filogeogràfic per les tres espècies del gènere. Es va observar que els dos morfotips de *T. melanurus*, tradicionalment considerats dues subspècies diferents (Zander, 1986), no presentaven diferenciació genètica, invalidant l'hipòtesi inicial de les subspècies. Per *T. delaisi*, els resultats moleculars obtinguts suporten l'existència de dos clades altament diferenciats corresponents a les dues subspècies descrites prèviament, tot i que els seus rangs de distribució haurien de ser redefinits, així doncs *T. d. delaisi* és present a la Macaronèsia i *T. d. xanthosoma* al Mediterrani i a l'Atlàntic continental. Utilitzant les divergències moleculars pels gens 12S i 16S, entre aquests dos clades, es va poder inferir que aquestes dues subspècies van divergir durant les fluctuacions climàtiques del Quaternari, ara fa uns 1.10-1.23 Ma.

Finalment, per *T. tripteronotus*, al analitzar individus de poblacions diferents es van trobar dos clades molt ben suportats amb una divergència que superava àmpliament la distància interespecífica entre moltes espècies de peixos d'un mateix gènere, conseqüentment van ser considerades com dues espècies críptiques que van divergir, segons les dades moleculars, ara fa 2.75-3.32 Ma, durant les glaciacions del Pliocè.

En un estudi posterior, s'ha ampliat l'àrea de mostreig per tal de delimitar les zones de distribució d'ambdues espècies. A més, es van analitzar els potencials caràcters morfològics capaços de diferenciar-les. D'aquesta manera, es va veure que les seves àrees de distribució estan separades: *T. tripteronotus* es troba a la conca mediterrània nord, de la costa NE espanyola fins Grècia i Turquia, incloent les illes de Malta i Xipre, mentre que la nova espècie descrita, anomenada *T. tartessicum*, habita la costa sud d'Espanya, des de Cabo la Nao fins al Golf de Cadis, les Illes Balears i el nord d'Àfrica, des de Marroc a Tunísia. A més, no es van trobar poblacions molecularment híbrides. D'altra banda, es van trobar petites diferències morfològiques entre les dues espècies, podent ser diferenciades únicament per el diàmetre orbital (OD). El qual és significativament més gran en la nova espècie que en *T. tripteronotus*. D'aquesta manera, *T. tartessicum* presenta una mida del cap (HL) 2.5 vegades menor que el OD (en individus de 2 a 5 cm), mentre que per *T. tripteronotus* la HL és més gran que 2.5 vegades el OD (també en individus de 2 a 5 cm). En estudis d'aquesta mena és on es pot veure la rellevància dels marcadors moleculars per a detectar espècies críptiques, incapaces de ser diferenciades, *a priori*, per característiques morfològiques.

Estudis d'aquesta mena reforcen l'importància del bon coneixement dels límits de distribució de les espècies, especialment dins de la zona que es pretén analitzar, abans de realitzar estudis d'estructura intraespecífica amb marcadors moleculars altament polimòrfics. Només d'aquesta manera es pot estar segur que l'aïllament genètic entre poblacions representa diferenciació intraespecífica i no interespecífica.

4.2.- Estima de l'estructura poblacional de diferents espècies de peixos

4.2.1.- Microsatèl·lis: marcadors moleculars altament polimòrfics

En vertebrats, especialment en peixos, sembla haver una freqüència de microsatèl·lits molt elevada en el genoma de les espècies; pel contrari ocells i plantes són els organismes que menys en tenen (revisió en Zane *et al*., 2002). Els microsatèl·lits són molt útils en estudis d'ecologia molecular. Com a pas previ s'han d'aïllar de les espècies que es volen analitzar, donat que l'utilització d'encebadors dissenyats en altres espècies no sembla donar bons resultats.

Així doncs, es van realitzar genoteques enriquides per ambdues espècies amb l'objectiu d'aconseguir uns 10 loci microsatèl·lits polimòrfics per cada una. Per *T. delaisi*, unes 1500 colònies van ser analitzades, resultant en 216 clons positius (un 14%), i d'aquests se'n van seqüenciar 51. Per *S. cabrilla*, es van obtenir 98 clons positius (9.8%) d'aproximadament 1000 colònies inicial, posteriorment se'n van seqüenciar 39. A partir d'aquestes seqüències amb microsatèl·lits, per ambdues espècies, es van dissenyar els primers per tal d'amplificar els loci microsatèl·lits a cada espècie.

Aquests dos treballs, han permès optimitzar aquests marcadors, podent-se utilitzar en estudis posteriors, per a les dues espècies. Un aspecte remarcable és el de les "multiplex", és a dir, l'amplificació de més d'un locus microsatèl·lit a la vegada, això fa que els costos posteriors, tant en termes de temps com de diners, es redueixin considerablement. D'aquesta manera, per *S. cabrilla* quatre parelles de loci microsatèl·lits han pogut ser amplificats en multiplex, mentre que només s'ha pogut optimitzar una parella de loci en multiplex per *T. delaisi*.

El procés de realització d'una genoteca, des de l'obtenció de la mostra fins a tenir perfectament optimitzades les amplificacions dels diferents loci microsatèl·lits obtinguts, va d'uns 3 a 10 mesos en funció de l'espècie. Aquest pas previ, únicament podria ser obviat si per alguna espècie propera a la que ens interessa estudiar ja hi ha realitzada una genoteca i els encebadors dissenyats per l'amplificació dels diferents loci microsatèl·lits aïllats en aquesta també funcionen, i són polimòrfics, per

l'espècie que es pretén estudiar (el que s'anomena cross-species amplification en anglès, CSA). És, per tant, molt important poder predir l'èxit abans d'esmerçar forces i diners en provar encebadors dissenyats per loci microsatèl·lits aïllats en altres espècies.

S'ha estudiat la relació entre l'èxit del CSA i la proximitat de les espècies, utilitzant dos gens mitocondrials (12S i 16S) per tal de quantificar de forma objectiva la relació existent entre les espècies. S'han integrat totes les dades possibles per peixos trobades a la bibliografia i s'ha vist que hi ha una correlació altament significativa entre l'èxit de l'amplificació, i del polimorfisme, dels loci i la divergència genètica entre les espècies. S'ha trobat que quan la divergència genètica entre l'espècie per a la qual s'han dissenyat els encebadors (source) i l'espècie objectiu (target) és del 7.30% i 9.03% pels gens 12S i 16S respectivament, l'èxit de la CSA és d'un 50%. Si a més, aquest mateix percentatge de loci han de ser polimòrfics, la distància entre les dues espècies no ha de ser superior al 4.35% per el 12S o al 6.39% per el 16S.

D'aquests resultats se'n desprèn la idea que aquesta relació és més o menys generalitzable a altres grups d'organismes. De fet, hi ha treballs realitzats en ocells (Primmer *et al*., 1996) i en pinnípeds (Gemmell *et al*., 1997) on s'han trobat resultats molt similars, utilitzant però, el valor de ΔT_mH d'hibridació DNA-DNA, com a mesura de la distància entre les espècies. El coneixement d'aquesta relació pot resultar molt útil per estudis posteriors, ja que permet estimar d'una forma ràpida l'èxit de la CSA entre dues espècies (source i target), únicament coneixent la seva divergència genètica per un d'aquests dos gens, que d'altra banda són els més àmpliament utilitzats en estudis de reconstrucció filogenètica.

4.2.2.- Influència del grau de polimorfisme dels marcadors utilitzats en l'estima del grau d'estructura poblacional de les espècies: homoplàsia?

Per tal d'inferir l'estructura poblacional de *Tripterygion delaisi*, a través de 10 loci microsatèl·lits altament polimòrfics, s'han utilitzat dos estimadors de la diferenciació genètica entre poblacions: el clàssic F_{ST} (Weir & Cockerham, 1984) i la diferenciació genètica estandarditzada, G'_{ST}, proposada, molt recentment, per Hedrick (2005).

S'ha trobat una correlació inversa, altament significativa, entre el valor de F_{ST} i l'heterozigositat esperada (H_E) així com també amb el nombre d'al·lels per locus (Na), indicant una relació inversa entre el polimorfisme del locus i els valors de F_{ST}.

De forma similar, estudis recents han demostrat que marcadors molt menys polimòrfics que els microsatèl·lits (al·lozims) i microsatèl·lits moderadament polimòrfics, mostraven valors de F_{ST} molt més grans que els obtinguts a partir de loci microsatèl·lits altament polimòrfics (Freville *et al*., 2001; Olsen *et al*., 2004; O'Reilly *et al*., 2004).

D'aquesta manera, quan s'utilitzen mesures de diferenciació clàssiques com els F_{ST}, loci altament polimòrfics, amb elevades taxes de mutació, poden disminuir els valors de F_{ST} encara que es mantenen els nivells de significació de diferenciació genètica amb altres mesures, com el test exacte de Fisher o fins i tot calculant la significació dels F_{ST} a partir dels intervals de confiança. Segons Estoup *et al*. (2002), l'homoplàsia dins d'una determinada espècie no sembla ser un problema important per a la majoria de les anàlisis d'estructura poblacional, degut a que la gran quantitat de variabilitat observada pels loci microsatèl·lits compensa amb escreix, la seva evolució per homoplàsia. D'altra banda, quan s'utilitzen marcadors moleculars amb una elevada taxa de mutació entre diferents subspècies, l'homoplàsia sí és present (Estoup *et al*., 1995). O'Reilly *et al*. (2004) suggereix que l'homoplàsia, més que els efectes de l'elevat polimorfisme *per se*, limita la resolució en espècies amb poca estructura poblacional.

Per *T. delaisi*, el locus Td06, tot i ser el més polimòrfic, no presentava cap al·lel compartit entre les dues subspècies (*T. d. delaisi* i *T. d. xanthosoma*). El petit, però significatiu, valor de F_{ST} obtingut comparant ambdues subspècies utilitzant únicament aquest locus, indica que l'elevada variabilitat i no l'homoplàsia és la responsable dels baixos valors de F_{ST}. El valor de G'_{ST}, per aquest locus i entre les dues subspècies és 1, o sigui la màxima diferència possible.

Per tal d'esclarir si l'homoplàsia realment és la responsable d'aquesta similitud, es van establir dos grups, un amb els loci compartits (loci amb més d'un 20% dels al·lels compartits entre subspècies) i l'altre amb els loci no compartits (loci amb

menys d'un 20% dels al·lels compartits entre subspècies). Quan s'han comparat únicament els loci altament polimòrfics entre els grups compartits i no compartits, els valors de F_{ST} van resultar idèntics i molt baixos, indicant que l'homoplàsia no era la causant d'aquesta similitud. Els valors de G'_{ST} s'incrementen al augmentar la variabilitat dels loci, tot i que les diferències entre els grups de loci més i menys variables no són significatives quan comparem les dues subspècies. D'altra banda, es van obtenir diferències significants pels valors de G'_{ST} en les comparacions entre els loci compartits i els no compartits entre les subspècies, tant pels altament polimòrfics com pels poc polimòrfics. En definitiva, la similitud en els valors de G'_{ST} entre els loci amb diferent nivell de polimorfisme indica que l'homoplàsia no podria produir la similitud genètica entre subspècies.

Així doncs, els valors de F_{ST} disminueixen a mesura que augmenta el polimorfisme, mentre que els valors de G'_{ST} augmenten amb la presència d'al·lels compartits entre poblacions o subspècies, d'aquesta manera, hem observat que les diferències genètiques observades entre i dins de subspècies no són degudes a homoplàsia.

4.2.3.- Estructura poblacional

Els microsatèl·lits s'han utilitzat de forma freqüent per tal d'identificar hibridació i separació entre subspècies (Ambali *et al*., 2000; Bensch *et al*., 2002; Lorenzen & Siegismund, 2004). *Tripterygion delaisi* presenta dues subspècies i aquestes són fàcilment diferenciables utilitzant loci microsatèl·lits. A través d'una aproximació bayesiana, s'han identificat clarament les seves dues subspècies com dues unitats genèticament diferents. A més, la manca d'al·lels compartits entre les dues subspècies pel locus Td06 indica la inexistència de flux gènic, al menys a nivell nuclear. De totes maneres, si aquest locus no s'inclou en l'anàlisi bayesiana, les dues subspècies segueixen essent dos dues unitats clarament diferenciades, reforçant l'existència de dos grups separats. No s'han trobat poblacions híbrides entre les dues subspècies, ni a nivell nuclear ni mitocondrial, així doncs aquestes han de ser tractades com dues unitats evolutives significativament diferents.

Dins de subspècies, per *T. d. delaisi*, present a la Macaronèsia, només s'han analitzat dues poblacions, una a l'arxipèlag de Canàries i l'altra al d'Açores (separades més de

1500 km), segons dades moleculars aquestes dues poblacions van divergir fa uns 12000 anys, possiblement durant l'última glaciació. Així doncs, els actuals valors de F_{ST} i G'_{ST} entre ambdues poblacions poden estar reflectint el seu avantpassat comú més que el flux gènic actual.

Per l'altra subspècie, *T. d. xanthosoma* (Mediterrani i Atlàntic continental) s'han analitzat vuit poblacions (Cap de Creus, Tossa, Blanes, Columbretes, Formentera, Cabo de Palos, Cabo de Gata i Tarifa) entre les quals s'ha trobat aïllament per distància. Utilitzant una anàlisi bayesiana s'han obtingut sis unitats genèticament diferenciades, aquests resultats són similars als obtinguts pels valor de F_{ST}, pels quals les tres localitats més properes (Cap de Creus, Tossa i Blanes) conformen una única població (definida com Costa Brava). D'aquesta manera, a través de l'anàlisi bayesiana i dels valors de F_{ST}, les sis poblacions queden perfectament definides. S'ha utilitzat la mida estimada de la població (neighbourhood size) per tal d'inferir la distància mitjana de dispersió dels individus adults durant una generació, obtenint-se un valor d'uns 40 metres per generació. Tots aquests resultats, sumats a les característiques dispersives de les seves larves, suporten l'existència d'una elevada estructura entre les poblacions de *T. d. xanthosoma*. De forma que d'acord amb les distàncies genètiques entre les poblacions analitzades i les característiques geogràfiques que les separen, es pot suggerir que discontinuïtats de sorra o aigua profunda de més de 30 km, estarien actuant com a barreres, reduint d'una forma molt dràstica, l'intercanvi d'adults i larves entre les poblacions de *T. delaisi*.

Finalment, un cop definides les poblacions de *T. delaisi*, s'ha estimat el grau d'autoreclutament per una població d'aquesta espècie en el Mediterrani nord-oest durant tres anys, constatant-se un elevat grau d'autoreclutament. Tot i que aquesta espècie té una PLD d'entre 16 i 21 dies, s'ha trobat que, de mitjana durant aquests tres anys, el 76.4±1.6% dels reclutes tornen a la seva població d'origen, mentre que la resta s'assignen, de forma majoritària, a les poblacions més properes. Aquest a estat el primer estudi en estimar l'autoreclutament d'una espècie de peix en el Mediterrani, i els resultats han estat semblants als realitzats en peixos, majoritàriament d'esculls coral·lins i amb altres marcadors no moleculars (Jones *et al*., 1999; Swearer *et al*., 1999; Thorrold *et al*., 2001; Miller & Shanks, 2004; Patterson *et al*., 2005). Tant sols Jones *et al*. (2005) han utilitzat microsatèl·lits per

realitzar estimes del nivell d'autoreclutament. Així doncs, aquests resultats demostren que un elevat percentatge de larves de *T. delaisi* es queden molt aprop, o mai arriben a marxar, de les zones on han estat alliberades, conferint un grau d'estructura poblacional molt elevat per aquesta espècie.

4.2.4.- Capacitat de dispersió larvària (CDL): un bon indicador del grau d'estructura de les poblacions?

La capacitat de dispersió larvària d'una espècie ve determinada per molts factors, tant intrínsecs de la pròpia larva o espècie com externs, és a dir factors ambientals (vents, corrents...) (Blaxter, 1986; Hickford & Schiel, 2003; Shanks & Eckert, 2005).

Com a factors intrínsecs s'ha de tenir en compte, primerament, el fet de si els ous són pelàgics o bentònics. Moltes espècies de peixos litorals són "pelagic spawners", i el vent i les corrents dispersen de forma passiva els seus ous (Black *et al*., 1991; Black, 1993). Els ous pelàgics són generalment més petits que els bentònics i solen produir larves més petites (de 3 a 5mm) (Thresher, 1984) amb uns sistemes sensorials i unes habilitats natatòries molt menors (Blaxter, 1986; Miller *et al*., 1988). Així doncs, la larva recent eclosionada pot sofrir una important dispersió passiva abans de tornar-se funcionalment competent. D'altra banda, els "non-pelagic spawners" (ja sigui perquè són vivípars o perquè deposen els ous al bentos) incuben els ous a les zones litorals i, en molts casos, retarden l'eclosió fins que les larves no tenen una mida comparativament més gran (de 5 a 10mm) (Thresher, 1984) amb aletes, ulls i estómacs funcionals (Barlow, 1981; Hunter, 1981; Thresher, 1984). Aquesta combinació de millors capacitats natatòries i sistemes sensorials més desenvolupats pot fer que hi hagi un grau de retenció més elevat per les larves de les espècies amb ous bentònics, especialment per aquelles que quan eclosionen són molt grans i estan ja molt desenvolupades. El temps d'incubació dels ous també jugarà un paper fonamental, essent més susceptibles de dispersió aquelles espècies que són "pelagic spawners", ja que l'ou es comporta com una partícula totalment passiva, podent ser transportada fàcilment pels vents i les corrents dominants (Roberts, 1997).

D'altra banda, les larves de les espècies amb ous bentònics (*T. delaisi*) són generalment més abundants a les zones costeres, mentre que les larves de les

espècies que tenen una posta pelàgica (*S. cabrilla*) és troben principalment en zones més allunyades de la costa (Sabatés, 1990; Suthers & Frank, 1991; veure però Hickford & Schiel, 2003). Segons Tintoré *et al*. (1995), la intensitat dels processos capaços de transportar les larves va creixent de forma gradual a mesura que ens allunyem de la línia de costa. Així doncs, com més allunyades estiguin les larves de la línia de costa més probabilitats de dispersió tindran. El patró de distribució temporal també té molta importància, ja que el règim de vents i corrents varia segons l'època de l'any, essent molt més probable la dispersió de les larves d'espècies que ponen a la tardor-hivern que no pas les que ho fan a la primavera-estiu, degut al sentit i intensitat dels vents dominants en cada estació (Shanks & Eckert, 2005; Macpherson & Raventós, 2006).

Un altre factor important a considerar és el temps que la larva resta al plàncton (PLD, pelagic larval duration). Alguns estudis que relacionen la PLD de les espècies amb la seva estructura poblacional. Doherty *et al*. (1995) van establir una correlació negativa i altament significativa entre la PLD i el grau d'estructura poblacional de les espècies, utilitzant al·lozims ($\log F_{ST} = -0.043(PLD) - 0.315$, $R^2 = 0.85$), en set espècies de peixos de la Gran Barrera de Corall. De la mateixa manera, Riginos & Victor (2001), utilitzant tres espècies de blennioids amb diferents PLDs en la zona del golf de Califòrnia, van constatar que l'estratègia de la larva ens dóna una idea molt aproximada del nivell d'estructura poblacional de les espècies. Més recentment, Purcell *et al*. (2006) han trobat una relació semblant entre dues espècies de peixos d'escull en el mar del Carib.

D'altra banda, hi ha estudis que mostren que ni el tipus d'ou de les espècies (pelàgic *vs*. bentònic) ni la PLD són determinants a l'hora de predir l'estructura poblacional de les espècies (Shulman & Bermingham, 1995; Bohonak, 1999). Més recentment, Bay *et al*. (2006) van detectar una relació significativa entre la PLD i el grau d'estructura genètica en vuit espècies de pomacèntrids, utilitzant marcadors moleculars tant mitocondrials com nuclears. La significança d'aquesta relació era causada per una sola espècie (*Acanthochromis polyacanthus*), la qual diferia de les altres en el fet que la seva larva no té fase platònica. Quan aquesta espècie s'exclou de l'anàlisi desapareix la relació entre la PLD i el grau d'estructura poblacional per les set espècies restants. Tot això suggereix que la PLD per si sola no és sempre un

bon estimador del grau d'estructura poblacional de les espècies, indicant que altres mecanismes han d'estar influenciant els patrons d'estructura poblacional de les diferents espècies de peixos. I per tant, aquest factor s'ha de complementar amb els altres explicats anteriorment per tal d'obtenir una idea fiable de la CDL de les espècies (Armsworth *et al*., 2001; Shanks *et al*., 2003).

De les dues espècies analitzades, *Tripterygion delaisi* presenta ous bentònics i molt grans (Wirtz, 1980), una distribució de les larves molt propera a la línia de costa (<100m, Sabatés *et al*., 2003) i una duració de la vida larvària d'entre 16 i 21 dies (Raventós & Macpherson, 2001), a més d'un adult molt territorial per al qual no estat descrits moviments migratoris i amb un elevat grau de fidelitat al territori (Heymer, 1977). Això fa entreveure que les capacitats de dispersió seran més aviat reduïdes. Així doncs, aquesta espècie tindria, *a priori*, una CDL molt reduïda. L'altra espècie, *Serranus cabrilla*, presenta un comportament de l'adult molt semblant (García-Rubies, 1999); però els seus ous són pelàgics i més petits, i les larves tenen una PLD d'entre 21 i 28 dies (Raventós & Macpherson, 2001). A més, segons Sabatés *et al*. (2003) les seves larves s'han trobat a l'altura del marge continental, a considerable distància de l'habitat dels adults. Per aquesta espècie, la CDL semblaria ser teòricament més gran que per *T. delaisi*.

Es van analitzar les mateixes quatre poblacions per les dues espècies (Cap de Creus, Blanes, Columbretes i Mallorca) i posteriorment es va realitzar la comparació de les seves estructures poblacionals, estimades a partir de loci microsatèl·lits. Els nivells d'estructura poblacional van resultar ser molt diferent entre les dues espècies. Per *T. delaisi* es va observar un elevat grau d'estructura poblacional amb un flux gènic reduït entre poblacions i amb aïllament per distància. A més, a partir de les quatre poblacions es van detectar tres grups genèticament homogenis, corresponents, segons els valors de F_{ST}, a Cap de Creus-Blanes, Columbretes i Mallorca. D'altra banda per *S. cabrilla*, entre les mateixes poblacions, els valors de les estimes de F_{ST} eren un ordre de magnitud inferior en comparació amb els de *T. delaisi*, mostrant una major connexió entre poblacions al llarg de la costa. Es van detectar, mitjançant inferència bayesiana, dos grups genèticament homogenis, corresponents, segons els valors de F_{ST}, a Cap de Creus-Blanes-Columbretes i Mallorca.

Els resultats obtinguts semblen indicar que llargues discontinuïtats de canals d'aigua profunda (>200 km) poden estar actuant com a barreres, reduint l'intercanvi de larves i d'adults entre poblacions d'ambdues espècies. A més, en *T. delaisi* discontinuïtats de sorra o canals d'aigua profunda de més de 30 km també redueixen de forma significativa el flux gènic entre poblacions.

Aquests resultats demostren l'existència d'una certa relació entre la CDL i l'estructura poblacional de les espècies. Aquesta és una relació inversa, de forma que si s'augmenta la CDL hi ha més connexió entre les poblacions d'aquella espècie i per tant el grau d'estructura poblacional disminueix. Aquesta relació, pot esdevenir molt útil per tal de dissenyar mostrejos eficients que permetin conèixer el grau de connectivitat entre poblacions el qual és essencial per dur a terme una gestió eficient dels recursos marins, així com per a projectar el disseny de reserves marines molt més efectives (Palumbi, 2003; Bell & Okamura, 2005).

5.- Conclusions

1. Les espècies que actualment formen el gènere *Tripterygion* es van originar per un procés ràpid de radiació adaptativa, degut a la utilització de nínxols diferents, durant el reompliment de la conca mediterrània després de la MSC, ara fa uns 5.2 Ma.

2. S'han trobat dos clades molt diferenciats per *T. tripteronotus*, els quals van divergir fa uns 2.7-3.6 Ma, durant les glaciacions del Pliocè, i que corresponen a dues espècies diferents *T. tripteronotus* i *T. tartessicum* n. sp.

3. Totes les espècies de serrànids mediterranis formen un grup monofilètic. Cada espècie està ben diferenciada i no s'han detectat espècies críptiques.

4. Hi ha una correlació negativa significativa entre l'èxit en l'amplificació i polimorfisme dels loci microsatèl.lits i la divergència genètica entre diferents espècies.

5. En *T. delaisi*, s'ha trobat una correlació inversa entre el polimorfisme del locus i el valor de F_{ST}. S'ha descartat que l'homoplàsia sigui la causant d'aquesta relació.

6. Les poblacions mediterrànies de *T. delaisi* (*T. d. xanthosoma*) presenten una marcada estructura genètica entre poblacions i aïllament per distància.

7. Un elevat percentatge de les larves de *T. delaisi* es queden a prop, o mai abandonen, la zona on han eclosionat. Això origina una taxa d'autoreclutament molt elevada.

8. La diferenciació genètica entre les poblacions de *Serranus cabrilla* és un ordre de magnitud inferior a la de *T. delaisi*.

9. Els resultats obtinguts per ambdues espècies demostren que el grau de diferenciació poblacional d'una espècie, amb una fase adulta sedentària, ve determinat de forma inversa per la seva capacitat de dispersió larvària.

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