UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

DEPARTAMENT DE PRODUCTES NATURALS, BIOLOGIA VEGETAL I EDAFOLOGIA

SECCIÓ DE BOTÀNICA

POLIPLOÏDIA, FILOGÈNIA I BIOGEOGRAFIA EN *CENTAUREA* L. SECCIÓ *ACROCENTRON* (Cass.) DC.

Mònica Font Garcia

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PROGRAMA DE DOCTORAT BIOLOGIA VEGETAL

POLIPLOÏDIA, FILOGÈNIA I BIOGEOGRAFIA EN *CENTAUREA* L. SECCIÓ *ACROCENTRON* (Cass.) DC.

Memòria presentada per Mònica Font Garcia per optar al títol de doctor per la Universitat de Barcelona

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ÍNDEX

1- INTRODUCCIÓ GENERAL

INTRODUCCIÓ GENERAL

La sistemàtica de les *Compositae* està marcada per tres grans fites i cadascuna d'elles ha implicat canvis profunds en la classificació de la família. La història d'aquesta classificació ha estat revisada a fons per Funk *et al.* (2005) i en presentarem una síntesi molt breu. Cassini (1819) va fer el primer intent de classificació de les *Compositae* i en va definir 20 tribus. Una classificació més sintètica va ser la proposada per Bentham (1873) i, molt poc temps després, per Hoffmann (1894). Ambdós autors van proposar una classificació en dues subfamílies i 16 tribus que ha estat seguida fins fa molt poc. Les dues últimes revisions de la família es van produir cap a finals del segle XX (Dittrich, 1977; Bremer, 1994), seguint la classificació feta per Hoffmann. El tercer conjunt de canvis a gran escala va ser produït per la introducció de mètodes basats en les anàlisis de DNA. Els primers a iniciar l'estudi van ser Jansen & Palmer (1987) utilitzant enzims de restricció en el DNA cloroplàstic, fet que va donar lloc a la descripció d'una tercera subfamília, Barnadesioideae (Benth. & Hook. f.) K. Bremer & R. K. Jansen; aquesta proposta va ser reflectida en Bremer (1994). I per últim, l'estudi més revolucionari fet per Panero & Funk (2002) on es van analitzar seqüències de nou regions cloroplàstiques de la família i es va proposar una nova classificació en 11 subfamílies i 35 tribus; aquest estudi és molt proper al punt de vista analític de Cassini. Les diferències entre les classificacions de Bremer (1994) i Panero & Funk (2002) es mostren a la Taula 1 (només s'hi representen els grups basals). L'alt suport estadístic de l'últim estudi i la seva correlació amb la morfologia ens porta a creure que la nova classificació de les *Compositae* és propera a ser definitiva.

Taula 1. Dues de les propostes més recents de classificació de les *Compositae* (únicament es mostren les branques basals)

BREMER (1994)	PANERO & FUNK (2002)
BARNADESIOIDEAE	BARNADESIOIDEAE
• BARNADESIEAE	• BARNADESIEAE
CICHORIOIDEAE	Stifftioideae clade (provisional)
• MUTISIEAE	• Stifftieae (prov.; S Amèrica, Àfrica)
• CICHORIEAE	MUTISIOIDEAE
• CARDUEAE	• MUTISIEAE (S Amèrica)
• VERNONIEAE	GOCHNATIOIDEAE
• LIABEAE	HECASTOCLEIOIDEAE
• ARCTOTEAE	• HECASTOCLEIDEAE
ASTEROIDEAE	CARDUOIDEAE
…… (3 subfamílies, 17 tribus)	• DICOMEAE
	• TARCHONANTHEAE
	• CARDUEAE
	…… (11 subfamílies, 36 tribus)

1.1- LA TRIBU *CARDUEAE*

La tribu *Cardueae* Cass. és una de les tribus més grans de les *Compositae*, amb prop de 2500 espècies. Estudis previs, basats en anàlisis de DNA, tant nuclear (Susanna *et al.*, 1995) com cloroplàstic combinat amb el nuclear (Garcia-Jacas *et al.*, 2002, 2006), confirmen que les *Cardueae* formen un grup monofilètic.

En la primera classificació de Cassini (1819), les *Cardueae* van ser dividides en tres tribus: *Echinopeae*, *Carlineae* i *Cardueae*, aquesta última amb dues subtribus: *Carduinae* i *Centaureinae*. Bentham (1873) i Hoffmann (1894) van proposar agrupar les tres tribus en una de sola, *Cardueae*, que es subdividia a l'hora en quatre subtribus: *Echinopinae* (Cass.) Dumort., *Carlininae* (Cass.) Dumort., *Carduinae* Cass. i *Centaureinae* (Cass.) Dumort.; aquesta subdivisió conservadora va ser acceptada durant molt temps. Tanmateix, la discussió de l'estatus de les *Echinopinae* es va reprendre quan Wagenitz (1976) va proposar la segregació de la subtribu com a una tribu separada,

Echinopeae. Dittrich (1977) va tornar al punt de vista de Cassini i va proposar que es tornessin a restablir les tribus *Echinopeae* i *Carlineae*. Finalment, Bremer (1994) va reintroduir una altra vegada la hipòtesi més conservadora, una única tribu, *Cardueae*, que, d'acord amb els últims estudis moleculars del nostre grup, és la millor solució (Susanna *et al.*, 1995, 2006; Garcia-Jacas *et al.*, 2002).

1.1.1-Classificació subtribal

Dins de *Cardueae*, hi ha l'acord general d'acceptar quatre grups, independentment del rang (tribu o subtribu). Tres de les subtribus són naturals (*Carlininae*, *Echinopinae*, i *Centaureinae*) i el quart grup, *Carduinae*, és una unió parafilètica (Garcia-Jacas *et al.*, 2002).

La subtribu *Centaureinae* és el grup més derivat i està caracteritzat per aquenis amb l'arèola d'inserció lateral-adaxial, un doble vil·là i, amb poques excepcions, fulles inermes. De tota manera, els límits entre *Carduinae* i *Centaureinae* són un desafiament perquè les diferències es donen en microcaràcters de l'aqueni i del vil·là que són difícils d'observar en exemplars d'herbari incomplerts o immadurs. Els exemples de *Nikitinia* Iljin i *Syreitschikovia* Pavlov il·lustren aquestes dificultats (Susanna *et al.*, 2002) i l'adscripció a una o altra subtribu ha estat comprovada amb estudis de filogènia molecular. Garcia-Jacas *et al.* (2002) van suggerir la possibilitat de reconèixer una cinquena subtribu, Cardopatiinae Less., amb dos gèneres: *Cardopatium* Juss. i *Cousiniopsis* Nevski. Aquesta subtribu ocuparia una posició intermèdia entre *Carlininae* i la resta de la tribu (Susanna *et al.*, 2006; Susanna & Garcia-Jacas, 2007).

1.1.2-Límits genèrics de la tribu Cardueae

La tribu de les *Cardueae*, a més de ser una de les més àmplies de la família, inclou també alguns dels gèneres més grans. Pel que fa als límits genèrics, basats en la morfologia i en estudis moleculars parcials, en l'última

revisió de les *Cardueae* (Susanna & Garcia-Jacas, 2007) es va adoptar un concepte genèric ampli per *Cousinia* Cass. (600 espècies), *Jurinea* Cass. (200 espècies) i *Saussurea* DC. (400 espècies), a causa de la manca de revisions sistemàtiques recents per a tots tres.

El gènere *Centaurea* L., un del més amplis, comprèn, segons diferents autors, entre 400 i 700 espècies (Dittrich, 1977; Bremer, 1994; Wagenitz & Hellwig, 1996; Hellwig, 2004) en gran part concentrades al sud-oest asiàtic i en la regió mediterrània. La concentració més gran d'espècies es troba a l'est d'Anatòlia i a la zona transcaucàsica (Wagenitz,1986; Hellwig, 2004).

Centaurea inclou herbes anuals o perennes, de vegades amb port subarbustiu, amb fulles normalment inermes, senceres o, de forma més comú, dividides. Els capítols poden ser solitaris o amb comflorescències corimbiformes, generalment amb aparença disciforme o radiada i heteròg ams, de manera més rara discoïdeus i homògams; les bràctees de l'involucre tenen, generalment, un apèndix fimbriat, lacerat o espinós; les flors poden ser de color rosat, vermelles, porpres, liles, blaves, grogues o blanques; aquenis oblongo-ovoidals o obcònics, una mica comprimits, glabres o escassament pilosos, amb l'hil·lum basal amb eleosoma; vil·là generalment doble, tot i que de vegades l'intern pot estar absent i molt poques vegades pot arribar a desaparèixer completament.

El gènere *Centaurea* ha estat tradicionalment considerat com un gènere problemàtic, a causa d'una gran diversitat morfològica, cariològica i pol·línica que presenta, i no ha estat absent d'intents de subdivisió que es van iniciar l'any 1819 quan Cassini el va subdividir en 40 gèneres independents que més tard van ser considerats com a seccions per Bentham (1873), Hoffmann (1894) i Hayek (1901). La gran diversitat existent en aquest gènere ha portat molts autors a dividir-lo de diverses maneres, però la falta de consens ha propiciat que estudis més recents sobre *Cardueae* (Ditrtrich, 1977; Bremer, 1994; Wagenitz & Hellwig, 1996) hagin adoptat una delimitació més àmplia i conservadora del gènere seguint les indicacions de Bentham (1873), Hoffmann (1894) i Hayek (1901).

Les últimes dades filogenètiques obtingudes a partir d'anàlisis moleculars demostren que la delimitació clàssica del gènere *Centaurea* en sentit ampli no és sostenible ja que es tracta d'un grup polifilètic (Susanna *et al.*, 1995; Garcia-Jacas *et al.*, 2001). Les dades moleculars assenyalen una posició basal i sense relació amb la resta de les *Centaurea* a aquelles que pertanyen a la secció *Centaurea.* Aquesta secció presenta un tipus de pol·len exclusiu, tipus *Centaurea*, que es troba prop de la base de l'evolució pol·línica (Wagenitz, 1955) i, a més, el número cromosòmic base de la secció és *x*= 15 (García-Jacas *et al.*, 1996, 2000) que també correspon a grups poc evolucionats. Estudis morfològics d'aquesta secció (Agababian, 1997) ja van concloure la necessitat de segregar-la com a gènere independent, tot i que dins d'aquesta secció hi ha l'espècie tipus del gènere *Centaurea*, *C. centaurium* L., Garcia-Jacas *et al.* (2000, 2001) van suggerir que s'adoptés una nova espècie tipus pel gènere *Centaurea*, seleccionada a partir del nucli del grup monofilètic i no de l'actual secció *Centaurea*. Adoptant aquesta solució, només era necessari canviar el nom de 17 espècies de la secció *Centaurea*. En línia amb aquesta opció, Greuter *et al.* (2001) van proposar conservar el nom de *Centaurea* amb una nova espècie tipus del gènere, *C. paniculata* L. pertanyent al grup *Jacea*. Sota aquesta proposta, l'antiga secció *Centaurea* adquiriria el rang de gènere i passaria a anomenar-se *Rhaponticoides* Vaillant (Greuter, 2003; Greuter *et al.*, 2005). Les antigues seccions *Acrocentron* i *Cyanus* també han estat considerades per Greuter *et al.* (2001) com a gèneres separats, anomenats respectivament *Colymbada* Hill i *Cyanus* Mill. De tota manera Garcia-Jacas *et al.* (2006) creuen que aquesta separació no està plenament sostinguda per les evidències morfològiques, cariològiques i moleculars que es disposen. Tenint en compte les connexions evidents entre *Acrocentron*, *Cyanus* i les seccions del grup *Jacea*, es suggereix (Susanna & Garcia-Jacas, 2007) que totes aquestes seccions s'haurien de mantenir dins del gènere *Centaurea* com a mínim fins que no hi hagi una ferma evidència del contrari. Coincidint en part amb aquesta afirmació, Greuter (2003) va tornar a incloure *Acrocentron* dins del gènere *Centaurea* amb un rang seccional, deixant *Cyanus* com a gènere diferent.

1.1.3-El grup Acrocentron

Cassini (1826) estableix una primera divisió dins de *Centaurea* i situa el gènere *Lopholoma* (amb el tipus *Centaurea scabiosa* L.) i el gènere *Acrocentron* (amb el tipus *Centaurea collina* L.) com a diferents per la presència d'una espina terminal en l'apèndix del gènere *Acrocentron*. Uns anys més tard, De Candolle (1838) va passar els gèneres proposats per Cassini a seccions: sect. *Acrocentron* (Cass.) DC. i dins d'aquesta va fer dues divisions segons la morfologia de les bràctees i el color dels flòsculs: *Lopholomoides* i *Euacrocentron.*

Hayek (1901), basant-se en representants orientals de la secció, va fer una nova proposta dividint *Acrocentron* en les següents divisions: *Centauroideae, Chysolepides, Collinae, Lopholomae, Macrocephalae*, *Orientales, Rhizanthae* i *Rupestres,* una classificació no aplicable a espècies occidentals (Garcia-Jacas, 1992). Més recentment Tzvelev (1963) va proposar un altre ordre sobre la sect. *Acrocentron* i va passar les subseccions fetes per Hayek dins de la sect. *Acrocentron* com a seccions; aquesta classificació ha estat seguida per Dostál (1976).

Routsi & Georgiadis (1999) divideixen la sect. *Acrocentron* en tres subseccions: subsect. *Graeca* amb *x*= 10 i les subsect. *Atropurpureae* i *Achaiae* amb *x*= 11, cadascuna d'elles caracteritzada per trets morfològics especials. De tota manera, cal dir que en aquesta divisió, igual que en la feta per Hayek gairebé un segle abans, només es tenen en compte les espècies orientals i caldria comprovar si aquesta classificació és extrapolable a la resta d'espècies de la secció.

Per a Gardou (1975), hi ha enormes dificultats per arribar a integrar dins d'una classificació moderna les bases biològiques, cariològiques, morfològiques o bioquímiques que podrien explicar la diversificació de la sect. *Acrocentron*.

Garcia-Jacas & Susanna (1992) coincideixen amb Wagenitz (1975) i Wagenitz & Gamal-Eldin (1985) en la dificultat d'establir una classificació intraseccional de la sect. *Acrocentron*; també creuen que l'estudi d'aquesta

secció ha d'incloure la sect. *Chamaecyanus* Willk. per raons palinològiques i per la fàcil hibridació que existeix entre els dos grups.

En un estudi de l'evolució del caràcters i la filogènia de les *Centaureinae*, Wagenitz & Hellwig (1996) van dividir el gènere *Centaurea* en grups informals. Dins del grup *Acrocentron* hi estarien incloses les següents seccions: sect. *Acrocentron,* sect. *Chamaecyanus*, sect. *Stephanochilus,* sect. *Crocodylium* i sect. *Aegialophila,* totes elles unides per caràcters morfològics, anatòmics i cariològics, però s'ha vist que les dues últimes seccions haurien de ser excloses i agrupades en un gènere diferent, *Crocodylium* Cass. (Garcia-Jacas *et al.*, 2001; Vilatersana *et al.*, 2001; Font *et al.*, 2002).

Finalment, en l'última definició d'*Acrocentron* suggerida per Font *et al.* (2002), es limita el grup a les seccions *Acrocentron, Chamaecyanus* i *Stephanochilus*.

La sect. *Acrocentron* forma un gran grup de *Centaurea* perimediterrànies amb un nombre molt elevat de tàxons (al voltant d'unes 50 espècies europees, 45 espècies orientals i, com a mínim, 14 espècies d'Àfrica del Nord). A més, aquesta secció presenta una distribució geogràfica reduïda, amb espècies endèmiques de massissos muntanyosos i sembla ser que no existeixen més de 10 espècies que puguin no ser considerades endèmiques. Tots aquests fets explicarien les dificultats trobades per la identificació precisa d'un tàxon i per integrar els diferents caràcters del grup.

La sect. *Chamaecyanus* va ser descrita a la base d'un petit grup d'espècies de la Península Ibèrica (Wilkomm & Lange, 1870). Per altra banda, també s'haurien d'incloure en els estudis de la sect. *Acrocentron* espècies de l'anomenada secció *Borjae* Valdés-Bermejo & Rivas Goday, una secció que segons Fernández Casas & Susanna (1986) és *Acrocentron.*

1.1.4-Evolució cariològica, poliploïdia i hibridació

Des de fa temps, s'ha vist la tendència de les Centaurees cap a una reducció del número cromosòmic bàsic i un increment de l'asimetria del cariotip (Fernández Casas & Fernández Morales, 1979; Siljak-Yakovlev, 1986). A la

subtribu en general, el número cromosòmic bàsic està correlacionat amb el tipus de pol·len; gèneres o seccions amb un tipus de pol·len "primitiu" tenen, en general, números cromosòmics alts, mentre que d'altres amb un tipus de pol·len més evolucionat tenen números cromosòmics més baixos (García-Jacas & Susanna, 1992; Susanna *et al.*, 1995; Garcia-Jacas *et al.*, 2001). Aquesta correlació també es dóna en altres caràcters morfològics (Wagenitz, 1955, Tonian,1980 entre d'altres).

De tota manera, el pas de *x*= 11 cap a *x*= 10 no implica grans canvis en el genoma, ja que es coneixen híbrids fèrtils entre espècies amb ambdós números cromosòmics (Fernández Casas & Susanna, 1986; Garcia-Jacas & Susanna, 1992). La direcció del canvi de *x*= 11 cap a *x*= 10 també està recolzada per la distribució geogràfica d'aquests números bàsics (Garcia-Jacas & Susanna, 1992). Segons aquests autors, l'àrea de distribució d'espècies amb *x*= 11 a l'est mediterrani és, en general, més petita i relictual que les de *x*= 10. Aquest patró de distribució també suggereix que és més arcaic el caràcter de número bàsic *x*= 11. A més, a l'est de la mediterrània, centre més antic i important de diversificació (Garcia-Jacas & Susanna, 1992, Routsi & Georgiadis, 1999), el número bàsic *x*= 11 és més freqüent que a l'oest mediterrani. Totes les espècies poliploides amb *x*= 10 són neopoliploides, mentre que els poliploides amb *x*= 11, que només existeixen al sud-oest mediterrani, són paleopoliploides amb àrees de distribució relictuals.

En un estudi recent sobre aquesta secció a Grècia, Routsi & Georgiadis (1999) suggereixen que des de Grècia es juga un paper important en la diferenciació de les espècies del grup *Acrocentron*. Basant-se en dades cariològiques donen una relació de quasi 3:1 entre espècies amb *x*= 11 i *x*= 10 per l'àrea de l'est mediterrani. Els taxons amb *x*= 11 de l'est de la mediterrània tenen diferent morfologia, estan taxonòmicament aïllats, tenen una àrea de distribució geogràfica restringida i són diploides.

Seguint la hipòtesi d'un origen oriental de la secció *Acrocentron*, sembla clar que l'expansió d'aquesta secció ha seguit la ruta de l'est cap a l'oest.

Un altre tret important dins *Acrocentron* és la poliploïdia. Fins fa molt poc, aquest fenomen havia estat considerat un fet poc comú, i les espècies

poliploides eren considerades com a genèticament uniformes amb una baixa capacitat adaptativa i amb tendència a la desaparició (Stebbins, 1971). Actualment, però, se sap que la poliploïdia és un procés comú i que juga un paper molt important en l'evolució de les plantes (Soltis & Soltis, 2000; Wendel 2000; Soltis *et al*., 2004a, 2004b). En els darrers 20 anys, els estudis moleculars en plantes han canviat la manera de veure el procés evolutiu de les espècies poliploides (Soltis *et al*., 2004a).

Tradicionalment, la poliploïdia ha estat vista com el resultat de la duplicació d'un únic genoma (autopoliploïdia) o de la combinació de dos o més genomes diferents (al·lopoliploïdia) (Stebbins, 1947, 1950; Grant, 1981); de tota manera, Grant (1981) afirma que els dos conceptes són els extrems d'una sèrie graduada. Tot i que històricament el fenomen d'autopoliploïdia ha estat considerat poc comú i que donava com a resultat tàxons maladaptatius (Wendel & Doyle, 2005) s'ha vist que, tot i probablement ser menys comú en la natura que l'al·lopoliploïdia, no és tant estranya com s'havia pensat (Ramsey & Schemske, 1998; Wendel & Doyle, 2005; Soltis *et al*., 2004a, 2004b).

Així doncs, els fenòmens de diploïdia i poliploïdia estan ben documentats al gènere *Centaurea*. Com a disploïdia trobem una sèrie ben correlacionada filogenèticament que va des de x= 7 fins a x= 12 (Garcia Jacas *et al*., 2001). Com a poliploïdia tenim exemples especialment freqüents a la Península Ibèrica dins el grup *Acrocentron* i *Chamaecyanus*. El cas més extrem i interessant de poliploïdia al grup *Chamaecyanus* és el de *C. toletana* Boiss. & Reuter, que presenta tres nivells de ploïdia ($2n$ = $2x$ = 20, $2n$ = $4x$ = 40, $2n$ = $6x$ = 60). El tetraploide va ser descrit com a espècie diferent, *C. cavanillesiana* Graells. De tota manera, moltes vegades s'han considerat com a espècies diferents aquelles que només presenten diferències en el seu número cromosòmic, però en aquest cas s'ha vist, examinant curosament el tipus i les localitats existents, que les diferències morfològiques entre aquestes espècies no existeixen (Fernández Casas & Susanna, 1986). La Figura 1 mostra l'extrema similaritat entre les poblacions diploides i tetraploides. Al contrari, sí és possible caracteritzar morfològicament l'espècie hexaploide, que també va ser descrita com a espècie diferent, *C. argecillensis* Gredilla, i que més tard es

Figura 1. Hàbit de *Centaurea toletana* diploide (Toledo: Risco de las Paradas, *Garcia-Jacas & Susanna 2634*) i *Centaurea toletana* tetraploide (Cuenca: Las Torcas, *Garcia-Jacas & Susanna 2644*).

va fusionar a *C. toletana* com a varietat per l'existència de moltes formes intermèdies entre el tipus tetraploide i l'hexaploide (Fernández Casas & Susanna, 1986). Aquests mateixos autors van concloure que l'espècie tetraploide era un autopoliploide ja que era del tot impossible diferenciar el diploide del tetraploide sense una mesura acuradíssima de la mida del pol·len o dels estigmes (Lewis, 1980; Ramsey & Schemske, 1998), però no van arribar a cap conclusió amb l'espècie hexaploide.

L'altre cas similar de poliploïdia el trobem en espècies de la secció *Acrocentron*, on trobem tres nivells de ploïdia: *Centaurea gabrielis-blancae* Fern. Casas (*C. ornata* var. *microcephala* Willk.), espècie diploide, *C. ornata* Willd., tetraploide; i *C. saxicola* Lag. hexaploide. Dostál (1976) va subordinar *C. saxicola* a *C. ornata* però una anàlisis filogenètica de la sect. *Acrocentron* (Font *et al.*, 2002) dóna suport al seu estatus com a espècie diferent; la Figura 2 mostra en aquest cas les diferències entre *C. ornata* i *C. saxicola*. En aquest grup, també podríem parlar de *C. kunkelii,* espècie descrita recentment per Garcia-Jacas (1998) localitzada al sud de la Península Ibèrica i que havia estat confosa molt sovint amb *C. ornata*. Garcia-Jacas (1998) va sospitar un origen híbrid per a aquesta espècie i va suggerir la hipòtesi que un del possibles pares era *C. saxicola*. L'altre possible progenitor és una espècie pertanyent a la sect. *Chamaecyanus*, *C. haenseleri* Boiss.

Figura 2. Hàbit de *Centaurea ornata* (Sòria, Ucero, Cuesta Galiana, *Garcia-Jacas & Susanna s. n.*) i *Centaurea saxicola* (Múrcia: La Azohia, *Garcia-Jacas, Susanna 1616 & Vilatersana)*.

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2- OBJECTIUS GENERALS

OBJECTIUS GENERALS

2.1-*Objectius evolutius: hibridació i poliploïdia*

- Aclarir la gènesi de la forma tetraploide de *Centaurea toletana* per comprovar si es tracta o no d'un autopoliploide, tal i com s'havia suggerit, o d'un al·lopoliploide.
- Aclarir l'origen de *Centaurea argecillensis* (6*x*) i verificar l'hipòtesi anterior de que es tracta d'un al·lopoliploide originat per hibridació de *C. toletana* i una altra espècie, o si pel contrari s'ha originat a partir de *C. toletana* 2*x* i 4*x*.
- Aclarir el tipus de poliploïdia del complex de *Centaurea ornata* mitjançant l'estudi de poblacions de *C. gabrielis-blancae*, *C. ornata* i *C. saxicola*, diploide, tetraploide i hexaploide respectivament.
- Intentar establir la relació de l'espècie hendecaploide *Centaurea kunkelii* amb el complex de *C. ornata* i establir quines podrien ser les espècies parentals.

2.2-*Objectius biogeogràfics, filogenètics i sistemàtics*

- Examinar amb dades moleculars els límits del grup *Acrocentron* i establir la relació i la posició de les seccions *Aegialophila*, *Acrocentron*, *Chamaecyanus* i *Stephanochilus* dins del grup.
- Proposar una filogènia molecular de la secció *Acrocentron* i verificar la correlació entre l'evolució i el nombre cromosòmic base.
- Aclarir, a partir de l'establiment de l'origen geogràfic de la secció *Acrocentron*, quins han estat els centres de radiació i dispersió del grup.
- Establir el patró de distribució de les espècies del grup *Acrocentron* dins de la Península Ibèrica i el nord d'Àfrica.

3- MATERIAL I MÈTODES

MATERIAL I MÈTODES

Aquesta memòria s'estructura en un compendi de quatre publicacions, dividides en dos grans blocs: el primer bloc ha estat agrupat com a hibridació i poliploïdia, i el segon bloc com a biogeografia i filogènia; un dels capítols està ja publicat, un altre està acceptat i pendent de publicació, un altre està en revisió i l'últim encara no ha estat enviat a cap revista. En aquest apartat recollim el conjunt de material i mètodes que ha estat utilitzat en aquest treball.

3.1- CARIOLOGIA I CITOGENÈTICA

3.1.1- RECOMPTES CROMOSÒMICS

S'han realitzat recomptes cromosòmics de totes les espècies estudiades pertanyents als complexes *Centaurea ornata* i *C. toletana*, per tal de verificar el nivell de ploïdia.

Material i mètode

El material emprat han estat meristems radiculars de plantes silvestres cultivades en testos a la zona experimental del Jardí Botànic de Barcelona, i els obtinguts de la germinació de granes recollides de poblacions silvestres que van estar sembrades en germinadors o en plaques de Petri al mateix Jardí Botànic.

Es van utilitzar dos tipus de pretractament. Inicialment, es va pretractar amb 8-hidroxiquinoleïna al 0.002% a 4ºC durant unes vuit hores aproximadament ja que havia estat el mètode més satisfactori per realitzar els recomptes d'espècies properes. Més endavant, vàrem utilitzar per realitzar el pretractament una solució de colquicina al 0.05% a temperatura ambient, durant un temps que oscil·lava entre les tres i les quatre hores, guardant relació amb el nivell de ploïdia de l'espècie a estudiar. Aquest canvi en el

pretractament va ser degut al posterior estudi cromosòmic amb tinció per fluorocroms, cromomicina i bisbenzamida, en el dos complexos poliploïdies.

Una vegada pretractat, tot el material es va fixar amb el reactiu de Carnoy (3:1 etanol absolut/àcid acètic glacial) i es va posar al congelador durant 24 hores, posteriorment es va passar el material a un tub que contenia alcohol de 70º i es guardà al congelador a -20ºC.

Abans de realitzar la tinció, el material va ser hidrolitzat durant una hora a temperatura ambient amb HCl 5N. Seguidament es varen realitzar dos rentats amb aigua destil·lada i un rentat amb àcid acètic al 45% de dos minuts cada un, la tinció es va fer amb orceïna acètica al 2%.

Una vegada separats els meristems, es va procedir al muntatge que es va fer en àcid acètic al 45%. Les preparacions es van conservar de forma permanent per congelació amb CO_2 per tal de separar el cobreobjectes i dos rentats de 10 minuts amb alcohol absolut i muntatge amb bàlsam del Canadà.

Les fotografies de les mitosis es van realitzar amb un microscopi òptic Zeiss Standard. Totes les preparacions permanents i els negatius de les fotografies són al Laboratori de Sistemàtica Molecular de l'Institut Botànic de Barcelona.

Per poder realitzar amb fiabilitat els estudis cariològics es van observar i mesurar un total de 10 plaques corresponents a les espècies diploides i tetraploides i 5 per les hexaploides. De l'única espècie hendecaploide que ha format part d'aquest estudi, només va estar possible obtenir una placa mitòtica que permetés ser comptada i mesurada amb fiabilitat.

3.1.2- TINCIÓ PER FLUOROCROMS

La tinció per fluorocroms ha estat la tècnica aplicada per tal de conèixer el patró de bandes d'heterocromatina constitutiva i les regions riques en AT i en CG dels cromosomes (Schweizer & Ehrendorfer, 1983). Els mètodes de marcatge per bandeig que de manera més comú s'han utilitzat en plantes (bandeig amb Giemsa C i cromomicina i bisbenzamida) permeten la definició d'un patró de bandes característic d'un grup d'organismes.

Material i mètode

El material emprat ha estat el mateix que l'utilitzat per als recomptes cromosòmics.

Es va utilitzar el pretractament amb colquicina al 0.05% i la fixació amb el reactiu de Carnoy.

La hidròlisi es va realitzar amb àcid acètic al 45% acabat de preparar a una temperatura de 60ºC durant un període de temps que oscil·lava entre els set i els 15 minuts, depenent de l'espècie.

Una vegada completada la hidròlisi es procedí a l'esclafament de la mostra entre el portaobjectes i el cobreobjectes amb una gota d'àcid acètic al 45% i es va buscar al microscopi si hi havia un nombre suficient de plaques bones que permetés, una vegada realitzada la tinció, observar un bona quantitat de cromosomes tenyits. Posteriorment es congelà amb CO_2 el portaobjectes i se'n separà el cobreobjectes, es va rentar la preparació amb un raig d'alcohol etílic absolut. Tot seguit, es va procedir a l'assecament a l'aire o en alcohol etílic absolut com a mínim durant 24 hores, podent-lo guardar així fins a un mes, sempre a les fosques.

Taula 1: Reactius pel bandeig

Cromomicina A_3

Solució A: Na_2HPO_4 . $2H_2O$ 0,2M

Solució B: àcid cítric 0,1M

McIlvaine pH= 7: 16,47 ml sol. A/ 100 ml H_2O
3,53 ml sol. B/ 100 ml H_2O
Verificar i ajustar el pH amb les solucions A o B segons s'escaigui.

McIlvaine pH= 7 + $MgSO_4$ 0,05M

McIlvaine pH= 5,5: 11,15 ml sol. A/ 100 ml H_2O
8,85 ml sol. B/ 100 ml H_2O
Verificar i ajustar el pH amb les solucions A o B segons s'escaigui.

Verd de metil 0,1%: 1 g de verd de metil/ 100 ml de McIlvaine pH= 5,5

Cromomicina: 0,2 mg cromomicina/ ml tampó McIlvaine pH= 7 + $MgSO_4$

Distamicina: 0,1 mg/ ml de tampó McIlvaine pH= 7
Cal preparar-la el mateix dia

HOECHST Bisbenzimida H33258

McIlvaine pH= 5,5

Sacarosa 60%

Hoechst: 1 mg/ 100 ml H_2O solució mare (es pot guardar al congelador)
Diluir 5 vegades amb tampó McIlvaine 5,5

Tinció amb cromomicina (A_3): per realitzar aquesta tinció s'han utilitzat les tècniques de Schweizer (1976), Kondo & Hizume (1982), Coulaud *et al*. (1995), Cerbah *et al.* (1995) i Vallès & Siljak-Yakovlev (1997) amb petites modificacions. Primer de tot es va procedir la incubació amb tampó McIlvaine pH= 7 durant 10 minuts, seguidament es decantà i es va incubar amb distamicina, passats 10 minuts es rentà amb tampó McIlvaine pH= 7 + $MgSO_4$ i s'incubà amb cromomicina durant 7 minuts. Es realitzà un segon rentat amb tampó McIlvaine pH= 7 + $MgSO_4$ i es va fer una contracoloració amb verd de metil durant 10 minuts. Totes aquestes passes van ser realitzades a les fosques. Seguidament, es va procedir a realitzar un rentat amb tampó McIlvaine pH= 5,5 on calia prendre la precaució d'escórrer bé la preparació però no deixar-la assecar, llavors es procedí al muntatge amb glicerol per

epifluorescència (Citifluor AF1) i tampó McIlvaine pH= 7 + $MgSO_4$ a parts iguals.

El tancament de les preparacions es va realitzar amb DPX (*Di-n-butylphthalate in xylene*) i les preparacions es conservaren a les fosques unes hores a temperatura ambient i després a 4ºC com a mínim un mes abans de ser observades.

Tinció amb bisbenzimida (Hoescht H33258): aquesta tinció es dugué a terme d'acord amb les tècniques procedimentals descrites per Martin & Hesemann (1988), Coulaud *et al.* (1995), Cerbah *et al.* (1995) i Vallès & Siljak-Yakovlev (1997) amb petites modificacions. El pas previ a la tinció va consistir en una hidratació de les mostres amb el seu pas per alcohols cada vegada més diluïts (70% v:v, 50% v:v i 30% v:v) per acabar en aigua destil·lada; cada passa va tenir una durada de 5 minuts.

La mostra hidratada va ser incubada amb tampó McIlvaine pH= 5,5 durant 10 minuts i, després d'escorrugut bé el tampó per decantació, es va incubar amb Hoescht durant un minut. Posteriorment, es va rentar amb tampó McIlvaine pH= 5,5 i es va posar a incubar amb aquest mateix tampó durant 15 minuts. Tots aquests procediments van tenir lloc a les fosques. Seguidament, es rentaren les mostres en aigua destil·lada i es va fer el muntatge amb sacarosa.

El tancament i la conservació de les mostres es va realitzar de la mateixa manera que per la cromomicina.

L'observació de les preparacions es va dur a terme amb un microscopi d'epifluorescència Zeiss Axioplan amb filtres 07 (excitació 457 nm, emissió 530 nm) pel que fa a les preparacions tenyides amb cromomicina i amb filtres 01 (excitació 365 nm, emissió 480 nm) per les tenyides amb bisbenzamida. Per obtenir les fotografies dels cromosomes i dels nuclis interfàsics, es van utilitzar pel·lícules ISO 400 Fujichrome, ISO 200 Kodachrome i ISO 100 Kodak Tmax.

3.2- SEQÜENCIACIÓ DE DNA

3.2.1- REGIONS DEL DNA RIBOSÒMIC NUCLEAR (nrDNA): ITS I ETS

L'anàlisi de les regions ITS (*Internal Transcribed Spacer*) del DNA ribosòmic nuclear ha estat molt utilitzada en els darrers anys per inferir la filogènia dins de les famílies d'Angiospermes i, concretament, dins les Compostes ha demostrat ser útil per a la reconstrucció de relacions filogenètiques dins i entre gèneres que estan molt relacionats (Kim & Jansen, 1994; Baldwin *et al.*, 1995; Susanna *et al.*, 1995; Garcia-Jacas *et al.*, 2000).

Els espaiadors interns transcrits (ITS1 i ITS2) són regions del DNA ribosòmic nuclear (nrDNA) no codificants. L'ITS1 està localitzat entre les regions codificants 18S i 5.8S, mentre que l'ITS2 està localitzat entre les regions codificants 5.8S i 26S (Fig. 1). Les regions ITS són les que presenten més variabilitat, en canvi, les regions codificants són les més conservades. Els espaiadors ITS estan involucrats en el procés d'acoblament de molècules precursores durant la maduració de les diferents subunitats del RNA ribosòmic (Baldwin *et al.*, 1995).

L'espaiador extern transcrit (ETS, *External Transcribed Spacer*) de les subunitats 18S-26S del DNA ribosòmic ha demostrat tenir un gran potencial per augmentar les dades obtingudes en l'estudi filogenètic fet a partir de la seqüenciació de la regió ITS en angiospermes (Baldwin & Markos, 1998). La regió ETS (Fig. 1) en angiospermes és més llarga que les regions ITS1 i ITS2 juntes i sembla evolucionar com a mínim tant ràpidament com l'ITS si parlem de seqüenciació de nucleòtids. Diversos estudis han demostrat que les dades obtingudes a partir de les anàlisis de la regió ITS i de la regió ETS són totalment congruents i combinables, ja que augmenta el suport de les anàlisis si es combinen les dues fraccions (Markos & Baldwin, 2001, 2002)

L'inici de la regió ETS està marcada pel lloc d'inici de la transcripció TIS (*Transcription Initation Site*) i la seva mida va des de 750 pb fins a 3170 pb (Bennet & Smith, 1991; Bathia *et al.*, 1996). És força freqüent que presenti seqüències repetides en el seu extrem 5', tal i com s'ha demostrat en diversos

gèneres de Compostes (Baldwin & Markos, 1998; Linder *et al.*, 2000; Markos & Baldwin, 2002), tot això ha propiciat que la seva llargada sigui molt variable i també ho és la seva composició. Aquesta circumstància ha fet dificultós el disseny d'un encebador universal que pugui ser utilitzat com a combinació amb el de l'extrem 3', zona relativament conservada. La part més utilitzada per a l'estudi filogenètic ha estat l'extrem 3' on no hi ha les repeticions.

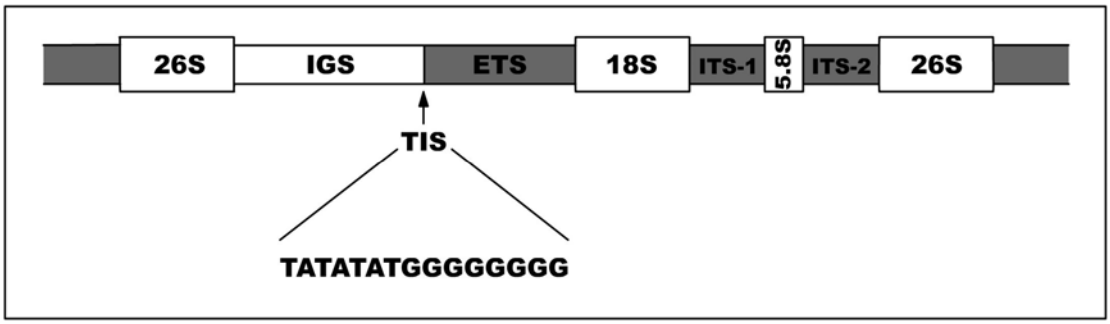

Fig. 1: Esquema de l'estructura i components adjacents de les regions ITS i ETS

3.2.2- REGIONS DEL DNA CLOROPLÀSTIC (cpDNA): *trn*H-*psb*A, *rp*S4-*trn*T-*trn*L, *ycf3*-*trn*S i *rp*L16

El genoma cloroplàstic de les angiospermes presenta normalment una herència materna.

Les seqüències de DNA cloroplàstic es poden considerar com a la primera font de dades per inferir estudis de filogènia en plantes i que només rivalitza amb les regions del DNA ribosòmic nuclear (Shaw *et al.*, 2005). El DNA cloroplàstic (cpDNA) és relativament estable, des del punt de vista evolutiu, tant a nivell poblacional com a nivell específic, i és per això que ha estat utilitzat en nombrosos estudis filogenètics, mitjançant dianes de restricció o seqüències nucleotídiques. Cal destacar que presenta diverses regions amb diferents taxes d'evolució, les quals permeten fer estudis en diferents rangs taxonòmics.

Les regions no codificants cloroplàstiques van començar a ser utilitzades per inferir relacions filogenètiques a nivells taxonòmics baixos (Sang *et al.*,1997), sota l'assumpció que aquestes regions haurien de ser menys

funcionals que les regions codificants i donarien nivells més grans de variació per a les anàlisis filogenètiques (Shaw *et al.*, 2005). Entre les primeres regions que es van començar a utilitzar trobem la regió *trn*T-*trn*L-*trn*L-*trn*F (Taberlet *et al.*, 1991), que es troba al costat del *rp*S4 i, des de fa relativament poc, s'ha notat un increment en la utilització d'altres regions no codificants com per exemple la *trn*H-*psb*A i *trn*S-*trn*G. A part, hi ha d'altres regions que han incrementat el seu estudi, ja sigui soles o amb combinació amb altres regions com l'intró *rp*L16 (Shaw *et al.*, 2005). Per últim, l'espaiador intergènic *ycf*3-*trn*S separa una pauta oberta de lectura (ORF *open reading frame*) de funció desconeguda (*ycf*3) i el RNA de transferència cloroplàstic per a la serina que porta l'anticodó GGA (*trn*S-GGA) (Hershkovitz, 2006) veure Figura 2.

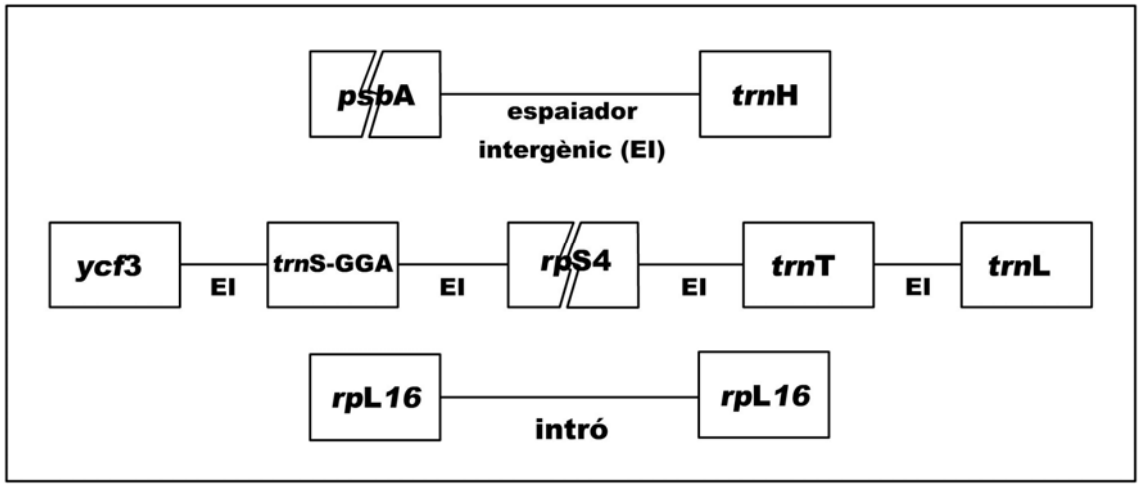

Fig. 2: Esquema de l'estructura i components adjacents de les regions cloroplàstiques estudiades

Material i mètode

Procés d'extracció del DNA genòmic: per tal de poder obtenir les seqüències de les regions estudiades es va procedir a l'extracció de DNA genòmic. Es van utilitzat tres tècniques diferents, però sempre a partir de fulles fresques o assecades amb gel de sílice (Chase & Hills, 1991) d'un o més individus d'una mateixa població, tot i que en alguns casos es va haver d'utilitzar material d'herbari.

a) Extracció amb CTAB de Doyle & Doyle (1987) i modificada per Soltis *et al.* (1991):

- Per a cada població estudiada es van pesar aproximadament 0,8 g de material fresc o bé 0,2 g de material assecat amb gel de sílice. Per a la lisi dels teixits vegetals, es van col·locar les fulles en un morter on s'hi va afegir 4 ml de tampó CTAB a 55ºC {2%(p:v) CTAB, NaCl 5M, EDTA.Na_2 (pH 8) 0,25M, 4% PVP-40, 0,5% 2-mercaptoetanol en 1M Tris-base, pH8} i, amb l'ajut de CO_2 es va triturar fins a aconseguir una pasta homogènia que es va transvasar a un tub de centrifugadora i es va deixar al bany a 55ºC durant uns 30 minuts.

- Per netejar el DNA, es van utilitzar 2,5 ml d'una solució 24:1 cloroform/alcohol isoamílic, es va barrejar per inversió i es va centrifugar a 4000 rpm durant 10 minuts. La fase aquosa es va traspassar a un tub cònic graduat i, per tal que el DNA precipités, s'afegiren dos terços del volum de la fase aquosa d'isopropanol fred, es barrejà per inversió i es guardà al congelador a –20ºC durant tota una nit.

- L'endemà es va passar aquesta solució a tubs *eppendorf* d'1,5 ml i es va centrifugar durant un minut a 14000 rpm, es va llençar el sobrenedant per decantació i es repetí tot aquest procediment fins a acabar la mostra i obtenir únicament el sediment. Aquest sediment es va deixar assecar al buit durant 30 minuts com a mínim i posteriorment es va resusprendre en 200 ml de TE estèril (10 mM Tris-base, 1mM EDTA.Na_2). Per finalitzar el procés es varen incubar els tubs al bany a 35ºC durant un temps que oscil·lava entre 1 hora i 24 hores i es van guardar al congelador a –20ºC.

b) Extracció amb CTAB de Doyle & Doyle (1987) i modificada per Cullings (1992):

Aquest procediment també es va utilitzar per a l'extracció de DNA de mostres fresques o conservades en silica-gel obtingudes en recol·leccions de camp, i també d'alguns plecs d'herbari recents.

El protocol d'extracció de DNA corresponent a aquest mètode va ser el següent:

- Per a cada individu es pesà 0,1 g de material fresc, o bé 0,02 g si es tractava de material conservat en sílica-gel o procedent de plecs d'herbari, i es posà en un tub *eppendorf.* Per a la lisi dels teixits vegetals, el material es va triturar amb una vareta de plàstic afegint-hi 500 µl de tampó CTAB {2%(p:v) CTAB, NaCl 5M, EDTA.Na_2 (pH 8) 0,25M, 4% PVP-40, 0,5% 2-mercaptoetanol en 1M Tris-base, pH8} en dues parts. Un cop obtinguda una massa homogènia, s'incubaren els tubs durant quatre hores a 65ºC.

- Passat aquest temps, es van col·locar els tubs en gel picat durant uns cinc minuts, per refredar-los. A continuació, es centrifugaren durant un minut a 14000 rpm. S'hi afegiren 500 µl de cloroform i es van agitar per inversió fins que el material presentà un aspecte lletós. Llavors es van centrifugar els tubs a 10000 rpm i es va transferir el sobrenedant a una altre tub *eppendorf*. Es van repetir els mateixos passos tornant a afegir 500 µl més de cloroform. Es tornà a recuperar el sobrenedant amb el DNA i s'abocà en un altre tub *eppendorf*. Aquestes dues transferències de fase a un tub *eppendorf* es realitzaren mitjançant una punta de pipeta tallada al biaix, per tal de no trencar el DNA genòmic per forces de cisalla.

- Es va afegir a la mostra una mescla d'acetat amònic 7,5 M (en una proporció igual a 0,08 vegades el volum de la fase aquosa) i isopropanol al 100% (en una proporció igual a 0,54 el volum de la mescla anterior). S'agitaren els tub donant petits copets i es deixaren reposar durant tota la nit a 4ºC.

- L'endemà es centrifugaren les mostres a 10000 rpm durant tres minuts. S'eliminà el sobrenedant per decantació. Es va afegir 1 ml d'etanol al 70%, invertint el tub dues o tres vegades per a rentar la superfície interna del tub, i es tornà a centrifugar durant tres minuts més. Es va treure el sobrenedant amb una pipeta *Pasteur* i es secà l'interior del tub amb un paper.

- Es van col·locar els tubs al buit durant 30 minuts i es resuspengué el pelet en 50 µl del tampó TE 1x estèril (10 mM Tris-base, 1mM EDTA.Na_2). Es van incubar els tubs al bany a 65ºC durant 15 minuts per assegurar-nos la resuspensió del pelet.

- Finalment, el DNA es guardà al congelador a -20ºC, per tal de conservar-lo, o a la nevera a 4ºC per a l'ús.

c) Extracció amb kit comercial:

En alguns casos en que el material d'herbari era molt antic o presentava mal aspecte, i també en algun cas puntual de material conservat en sílica-gel en que cap dels dos procediments anteriors havia funcionat, es va realitzar l'extracció mitjançant el kit "DNeasy® Mini Kit" (Qiagen Inc., Hilden, Germany), seguint les instruccions del fabricant. Consisteix en els passos següents:

- Es van triturar 0,02 g de fulles seques dins d'un *eppendorf*, i es van afegir 400 µl de tampó AP1 (*lysis buffer*) acabant el procés de trituració fins que no quedaren restes de teixits sencers. Es col·locà el tub *eppendorf* al congelador a -80ºC durant 10 minuts. A continuació es posà en un bany a 65ºC durant uns 10 minuts, homogeneïtzant la mostra per inversió de dues a tres vegades.

- Es centrifugà la mostra a 10000 rpm durant 30 segons. Passat aquest temps es van afegir 4 µl de Rnasa i es barrejà bé, seguidament s'afegiren 130 µl de tampó AP2 (*precipitation buffer*) i s'incubà 5 minuts en gel. Aquest pas és el que dóna lloc a la precipitació dels detergents, proteïnes i polisacàrids.

- Es centrifugà la mostra a 13000 rpm durant 5 minuts. S'agafà el sobrenedant i es filtrà, centrifugant-lo en una columneta del kit durant 2 minuts, també a 13000 rpm.

- Es va transferir el sobrenedant filtrat a un tub *eppendorf* juntament amb 1,5 volums de tampó AP3/E (*binding buffer* amb etanol). S'homogeneïtzà per inversió i es va procedir al filtrat d'aquesta barreja en una columneta del kit amb tub col·lector, centrifugant-la 1 minut a 8000 rpm per tal que el DNA quedés atrapat en el filtre. Es rentà el DNA del filtre afegint-hi 500 µl de tampó AW (*wash buffer*), amb alcohol, i centrifugant la columneta 1 minut a 8000 rpm. Es tornaren a afegir 500 µl més del mateix tampó i es centrifugà 2 minuts a 13000 rpm.

- Per recuperar el DNA del filtre es va posar la columneta sobre un *eppendorf* i s'hi va afegir 50 µl de tampó AE (*elution buffer*) que s'havia escalfat prèviament a 65ºC. S'incubà 5 minuts a temperatura ambient i es centrifugà 1 minut a 8000 rpm. Aquest últim pas es va repetir afegint 50 µl més de tampó AE i es guardà el DNA que havia quedat dipositat a l'*eppendorf*.

Comprovació de l'existència de DNA al material extret: per tal de verificar la presència de DNA de les mostres extretes, es va analitzar la mostra electroforèticament en un gel d'agarosa al 0,7% en 1x TBE (134 mM Tris-base, 0,25 mM EDTA.Na_2, 40mM àcid bòric, pH8). Es van carregar 5 µl de DNA extret i 2 µl de blau de brom fenol (20mM EDTA.Na_2, 8% glicerol, 1% laurilsarcosina i una punta d'espàtula de blau de brom fenol), a més es va afegir un marcador de DNA no tallat per estimar visualment la quantitat de DNA obtinguda en el procés d'extracció. Es va introduir aquest gel dins d'una cubeta d'electroforesi coberta amb tampó 1x TBE, el procés es va realitzar sota un voltatge constant de 100V durant mitja hora aproximadament. Una vegada migrades les mostres, el gel es va tenyir amb 100 ml d'aigua i 5 µl de bromur d'etidi (10 mg/ml) durant 10 minuts, a continuació, per tal d'eliminar l'excés de bromur, es va rentar el gel durant 5 minuts amb aigua destil·lada i es va examinar el resultat en un transil·luminador de llum UV. Es va fer una fotografia

del gel amb una càmera *Polaroid CU-5* i més endavant amb una *Canon Powershot G5* i el programa informàtic *Remote Capture.*

Amplificació del DNA: la regió ITS completa (espaiador ITS-1, gen 5.8S i espaiador ITS-2) va ser amplificada mitjançant la PCR, utilitzant els encebadors 1406F (Nickrent *et al.*, 1994) i ITS4 (White *et al.*, 1990). En alguns casos es va substituir l'encebador 1406F per l'ITS1 (White *et al.*, 1990). Les reaccions d'amplificació van ser dutes a terme en un volum final de 100 µl amb:

- 10 µl de tampó {160 mM $(NH_4)_2SO_4$, 670 mM Tris-Cl (pH 8,8 a 25ºC), 0,1% Tween-20 (Ecogen S.R.L., Barcelona)}.
- 16 µl d'una barreja 0,4 mM de cada nucleòtid: dATP, dCTP, dGTP i dTTP (Promega Corporation, Madison, WI).
- 5 µl de 50mM $MgCl_2$ (Ecogen S.R.L., Barcelona).
- 2 µl de 5 pmol/µl de cada un dels dos encebadors: el directe i l'invers.
- 55 µl d'aigua destil·lada estèril.
- 10 µl del DNA procedent de l'extracció.

Les reaccions es van posar en tubs de 0,2 ml i s'hi va afegir una gota d'oli mineral per tal d'evitar l'evaporació dels productes de la reacció durant l'amplificació del DNA. Els tubs es van col·locar en un termociclador MJ Research PTC 100 utilitzant el següent programa:

- Pas 1: 2 minuts a 94ºC, per tal de desnaturalitzar el DNA.
- Pas 2: 5 minuts a 80ºC, durant els quals s'afegeixen 0,1 µl de 5 U/ µl de *Taq* polimerasa (Ecotaq, Ecogen S. R. L., Barcelona).
- Pas 3: 30 cicles, cadascun format per les següents fases:
  - 1 minut i 30 segons a 94ºC per desnaturalitzar el DNA.
  - 2 minuts a 55ºC per a l'aparellament dels encebadors a el DNA.
  - 3 minuts a 72ºC per permetre l'extensió dels encebadors.
- Pas 4: 1 període d'extensió final de 15 minuts a 72ºC.

La regió ETS va ser amplificada i seqüenciada utilitzant l'encebador directe ETS1f i el 18S-2L com a encebador invers (Linder *et al.*, 2000), tot i que

en algunes ocasions, quan el 18S-2L no donava una bona amplificació, es va substituir pel 18SETS (Baldwin & Markos, 1998). Les reaccions d'amplificació van ser dutes a terme en un volum final de 25 µl amb:

- 2,5 µl de tampó {100 mM KCl, 100 mM Tris-Cl (pH 8,3 a 25ºC), (Applied Biosystems, Foster City, CA)}.
- 2,5 µl d'una barreja 0,4 mM de cada nucleòtid: dATP, dCTP, dGTP i dTTP (Applied Biosystems, Foster City, CA).
- 2,5 µl de 25mM $MgCl_2$ (Applied Biosystems, Foster City, CA).
- 0,5 µl de DMSO (Dimetil sulfòxid, Sigma-Aldrich, Schneillidorf,Germany).
- 1 µl de 5 pmol/µl de cada un dels dos encebadors: el directe i l'invers.
- 9,8 µl d'aigua destil·lada estèril.
- 5 µl d'una dilució de DNA (la concentració prèvia no es va determinar).
- 0,2 µl de *Taq* polimerasa (Applied Biosystems, Foster City, CA).

Els paràmetres de la reacció van ser els següents:

- Pas 1: 5 minuts a 94ºC, per tal de desnaturalitzar el DNA.
- Pas 2: 29 cicles, cadascun format per les següents fases:
  - 45 segons a 94ºC per desnaturalitzar el DNA.
  - 45 segons a 48ºC per a l'anellament dels encebadors a el DNA.
  - 40 segons a 72ºC per permetre l'extensió dels encebadors.
- Pas 3: 1 període d'extensió final de 7 minuts a 72ºC.

L'espaiador intergènic *trn*H-*psb*A va estar amplificat utilitzant l'encebador trnH (GUG) com a directe i psbA com a encebador invers (Hamilton, 1999). Les reaccions d'amplificació van ser dutes a terme en un volum final de 100 µl amb:

- 10 µl de tampó {160 mM $(NH_4)_2SO_4$, 670 mM Tris-Cl (pH 8,8 a 25ºC), 0,1% Tween-20 (Ecogen S.R.L., Barcelona)}.
- 16 µl d'una barreja 0,4 mM de cada nucleòtid: dATP, dCTP, dGTP i dTTP (Promega Corporation, Madison, WI).
- 5 µl de 25 mM $MgCl_2$ (Ecogen S.R.L., Barcelona).
- 5 µl de 5 pmol/µl de cada un dels dos encebadors: el directe i l'invers.
- 49 µl d'aigua destil·lada estèril.

- 10 µl d'una dilució de DNA (la concentració prèvia no es va determinar).
- 0,2 µl de *Taq* polimerasa (Ecotaq, Ecogen S.R.L., Barcelona).

Les condicions de PCR van estar descrites per Hamilton (1999) i són les següents:

- Pas 1: 5 minuts a 96ºC, per tal de desnaturalitzar el DNA.
- Pas 2: 35 cicles, cadascun format per les següents fases:
  45 segons a 96ºC per desnaturalitzar el DNA.
  1 minut a 53ºC per a l'aparellament dels encebadors.
  30 segons a 72ºC per permetre l'extensió dels encebadors.
- Pas 3: 1 període d'extensió final de 7 minuts a 72ºC.

L'espaiador intergènic *rp*S4-*trn*T-*trn*L va ser amplificat utilitzant els encebadors rpS4R2 (Shaw *et al.*, 2005), trnT^{UGU} 2F (*trn*A2 de Cronn *et al.*, 2002) com a directes i 5' trnL^{UAA}B (Taberlet *et al.*, 1991) i trnT^{UGU} R (Shaw *et al.*, 2005) com a encebadors inversos. Cal dir que per a algunes espècies en que aquests encebadors no donaven una bona amplificació se'n van dissenyar de nous a partir de les seqüències d'algunes de les espècies del grup que sí s'havien amplificat, són els següents: rpS4G (5' TTT CTA AGA TCA CTT CCA GC 3') com a encebador directe i trnT-B2R (5' AGC CTG CTT AGC TCA GAG GTT 3') com a invers. L'espaiador intergènic *ycf*3-*trn*S va ser amplificat utilitzant els encebadors SP43122F com a directe i SP44097R com a invers (Hershkovitz, 2006). L'intró *rp*L*16* va ser amplificat amb l'encebador rpL16F71 (Jordan *et al.*, 1996) com a directe i Rex2 (R. T. Kimball, University of Florida, pers. com.) com a encebador invers. Les reaccions d'amplificació van ser dutes a terme en un volum final de 25 µl amb:

- 2,5 µl de tampó {100 mM KCl, 100 mM Tris-Cl (pH 8,3 a 25ºC), (Applied Biosystems, Foster City, CA)}.
- 2,5 µl d'una barreja 0,4 mM de cada nucleòtid: dATP, dCTP, dGTP i dTTP (Applied Biosystems, Foster City, CA).
- 2,5 µl de 25 mM $MgCl_2$ (Applied Biosystems, Foster City, CA).
- 1 µl de 5 pmol/µl de cada un dels dos encebadors: el directe i l'invers.

- 5 µl d'una dilució de DNA (la concentració prèvia no es va determinar).
- 10,3 µl d'aigua destil·lada estèril.
- 0,2 µl de *Taq* polimerasa (Applied Biosystems, Foster City, CA).

La seqüència utilitzada per l'amplificació és la següent:

- Pas 1: 1 minut i 35 segons a 95ºC, per tal de desnaturalitzar el DNA.
- Pas 2: 35 cicles, cadascun format per les següents fases:
  1 minut i 30 segons a 95ºC per desnaturalitzar el DNA.
  1 minut i 30 segons a 52ºC per a l'aparellament dels encebadors a el DNA.
  2 minuts a 72ºC per permetre l'extensió dels encebadors.
- Pas 3: 1 període d'extensió final de 10 minuts a 72ºC.

Les reaccions de PCR de les regions ETS, *trn*H-*psb*A, *rp*S4-*trn*T-*trn*L, *ycf*3-*trn*S i *rp*L*16* es realitzaren en el termociclador PTC-200 Peltier Thermal Cycler (MJ Research, Inc.).

Per a la comprovació dels productes procedents de l'amplificació, es van carregar 5 µl de DNA procedents de la PCR, 4 µl d'aigua destil·lada i 1 µl de blau de brom fenol en un gel d'agarosa a l'1,2% en 1x TBE (134 mM Tris-base, 0,25 mM EDTA.Na_2, 40mM àcid bòric, pH8) i es va procedir de manera similar a la comprovació del material extret.

Productes de PCR clonats: en algunes mostres, es va observar més d'una banda en la comprovació dels productes amplificats de la regió ITS o bé de la regió ETS, i també en alguns casos que, posteriorment a la seqüenciació d'aquestes regions, es va observar l'amplificació de més d'un senyal o la presència de molt "soroll de fons" que impedia la correcta lectura de la seqüència, es va haver de clonar. Per als productes de PCR de totes les espècies que es van haver de clonar, es va utilitzar TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) seguint sempre les instruccions del fabricant, excepte que només es va utilitzar la meitat de les reaccions que marcaven les

instruccions del kit. Sempre que va ser possible es van escollir 16 colònies de cada reacció de PCR utilitzant els primers universals T7 i M13 sota les condicions següents:

- Pas 1: 10 minuts a 94ºC, per tal de desnaturalitzar el DNA.
- Pas 2: 30 cicles, cadascun format per les següents fases:
  30 segons a 94ºC per desnaturalitzar el DNA.
  1 minut a 55ºC per a l'aparellament dels encebadors.
  2 minuts a 72ºC per permetre l'extensió dels encebadors.
- Pas 3: 1 període d'extensió final de 10 minuts a 72ºC.

A partir d'aquí, se seleccionen de 8 a 10 productes del PCR per seqüenciar-los en les dues direccions utilitzant els mateixos primers.

Purificació dels productes d'amplificació: per purificar la majoria dels productes de la PCR, es van col·locar en minicolumnes "QIAquick PCR purification kit" (Qiagen Inc., Valencia, California) que utilitzen la propietat de les membranes de gel de sílice d'absorbir el DNA i es va procedir segons les instruccions del fabricant, que van ser les següents:

- En un tub de microcentrifugadora s'hi va posar tot el volum obtingut en la PCR, s'hi van afegir 5 volums de tampó PB (*binding buffer)* i es va barrejar. Aquesta solució es va col·locar en una columneta "QIAquik" disposada en un tub col·lector d'1,5 ml i se centrifugà tot el conjunt durant 1 minut a 14000 rpm. Es descartà el líquid del tub col·lector.

- Per tal de rentar el DNA es van afegir a la columneta 750 µl d'una solució formada en una proporció 1:4 de tampó PE (*wash buffer)* i alcohol absolut i es va centrifugar durant un minut a 14000 rpm. Es descartà el líquid del tub col·lector i es tornà a fer una segona centrifugació d'un minut per tal d'eliminar tot el tampó i l'alcohol.

- Per recuperar el DNA, es van afegir 30 µl de tampó EB (*elution buffer*) (10mM Tris-Cl, pH 8,5) al centre de la columneta col·locada dins d'un tub *eppendorf* estèril, es deixà reposar un minut i se centrifugà també durant un minut a 14000 rpm i el DNA purificat queda dins l'*eppendorf*.

En alguns casos en què el DNA no es va poder purificar utilitzant "QIAquick PCR purification kit" perquè la quantitat de DNA que s'obtenia no era la suficient per a la posterior seqüenciació, s'utilitzà un segon kit, "DNA Clean & Concentrator^{TM}-5" (Zymo Research, Orange, CA) i es va procedir segons les instruccions del fabricant, però modificant en algun cas el temps de centrifugació, de la següent manera:

- En un tub de microcentrifugadora es va posar tot el volum obtingut en la PCR, s'hi van afegir 2 volums de tampó *DNA Binding* (per a mostres amb menys de 50 µl s'afegien 100 µl de tampó) i es va barrejar. Aquesta solució es va col·locar en una columneta *Zymo-spin* disposada en un tub col·lector de 2 ml i es centrifugà tot el conjunt durant un minut a màxima velocitat (14000 rpm). Es descartà el líquid del tub col·lector.

- Per tal de rentar el DNA, es van afegir a la columneta 200 µl de *wash buffer* (tampó de neteja) i es va centrifugar durant un minut a 14000 rpm. Es tornaren a afegir 200 µl més de *wash buffer* i es tornà a fer una segona centrifugació d'un minut.

- Per recuperar el DNA, es van col·locar la columneta *Zymo-Spin* en un nou tub col·lector de 1,5 ml i s'afegiren de 6 a 10 µl d'aigua destil·lada estèril directament a la matriu de la columneta i se centrifugà, es tornà a repetir aquest procés una segona vegada i el DNA purificat quedà dins l'*eppendorf*.

Per a la comprovació dels productes procedents de la purificació es van carregar 1 µl de DNA, 4 µl d'aigua destil·lada i 2 µl de blau de brom fenol en un gel d'agarosa a l'1,2% en 1x TBE (134 mM Tris-base, 0,25 mM EDTA.Na_2, 40mM àcid bòric, pH8) i es va procedir de manera similar a la comprovació del material extret.

Reaccions de seqüenciació: per poder realitzar les reaccions de seqüenciació es va utilitzar el producte comercial *BigDye terminator Cycle Sequencing kit*

v3.1 (Applied Biosystems, Foster City, CA). Per a cada regió de DNA a estudiar, es va preparar un volum total de 20 µl que contenien:

- 1-2 µl de DNA procedent de la purificació (en funció de la intensitat comprovada en l'amplificació).
- 1 µl de 5 pmol/ µl de l'encebador (depenent de la regió)
- 2 µl de 5x tampó {(250 nM Tris-HCl, pH 9,0; 10mM $MgCl_2$) (Applied Biosystems, Foster City, CA)}.
- 2 µl de la barreja commercial que conté els quatre dNTP's marcats i la *Taq* polimerasa. (Applied Biosystems, Foster City, CA).
- 13-14 µl d'aigua destil·lada estèril (fins a obtenir els 20 µl totals).

Les reaccions es van dur a terme en un termociclador (PTC-200 Peltier Thermal Cycler, MJ Research, Inc.) seguint les passes següents:

- Pas 1: 1 minut a 96ºC, per tal de desnaturalitzar el DNA.
- Pas 2: 25 cicles cadascun d'ells amb els passos següents:
  - 10 segons a 96ºC per desnaturalitzar el DNA.
  - 5 segons a 50ºC per a l'aparellament dels encebadors.
  - 4 minuts a 60ºC per permetre l'extensió dels encebadors.

A continuació, els productes de les reaccions de seqüenciació es van passar a un *eppendorf* i s'hi va afegir 17 µl d'aigua destil·lada i 63 µl d'alcohol de 96º, es va agitar per homogeneïtzar la barreja i es va deixar reposar 15 minuts. Passat el temps de repòs es va centrifugar durant 20 minuts. A continuació, es van fer quatre rentats del sediment afegint cada vegada 200 µl d'alcohol de 70º als *eppendorf*, centrifugant-los durant 2 minuts i traient després el líquid amb una micropipeta *Pasteur*. Finalment, es van assecar els sediments al buit durant 30 minuts com a mínim.

Totes aquestes mostres van ser seqüenciades mitjançant el seqüenciador automàtic multicapilar ABI Prism® 3730 PE Biosystems/Hitachi de la Unitat de Genòmica dels Serveis Cientificotècnics de la Universitat de Barcelona.

3.3- ANÀLISIS FILOGENÈTIQUES

3.3.1- MÈTODES D'INFERÈNCIA FILOGENÈTICA

Una vegada obtingudes les seqüències d'una regió concreta del DNA del grup de tàxons que volem estudiar, caldrà alinear-les entre elles, o bé de forma manual, o bé amb l'ajuda d'algun programa que realitzi alineaments múltiples (ex: ClustalX, Thompson *et al.*, 1997; MAFFT, Katoh *et al.*, 2002), tenint en compte, en qualsevol cas, que no és convenient acceptar alineacions generades per un programa informàtic sense comprovar-les manualment (Soltis & Soltis, 2003). D'aquesta manera doncs, es generarà una matriu de seqüències.

Al nostre cas, les seqüències van estar alineades visualment per comparació de les seqüències dues a dues (Swofford & Olsen, 1990). En l'alineació convé maximitzar el nombre de residus homòlegs introduint *gaps* (forats) en una o altra seqüència. Els *gaps*, biològicament, simbolitzen insercions o deleccions que han donat en divergir les seqüències a partir d'un ancestre comú. En el moment d'afegir els *gaps* cal anar en compte de no introduir-los de forma indiscriminada, d'altra manera podríem estar alineant seqüències sense homologies reals. Així doncs, caldrà minimitzar els *gaps* per tal que l'alineació no perdi el seu sentit biològic. La qualitat de l'alineació és un pas clau en l'obtenció d'un arbre filogenètic fiable.

Una vegada s'ha obtingut l'alineació que suposem la millor possible, cal triar els tipus de mètodes d'inferència que aplicarem a les nostres dades per a la reconstrucció filogenètica. Tots els mètodes presenten avantatges i desavantatges, l'elecció dependrà dels objectius del treball i del volum i complexitat de les dades.

Actualment existeixen quatre tipus bàsics d'anàlisis filogenètiques: Màxima parsimònia (MP), Distància màxima, Màxima versemblança (ML) i Inferència bayesiana (IB). Desenvoluparem els dos tipus d'anàlisis que hem utilitzat.

Màxima parsimònia (MP): el criteri en què es basa és el següent: l'explicació més simple sempre és preferible a altres explicacions més

complexes, és a dir, que impliquin un nombre més gran d'assumpcions. Si apliquem aquest criteri a la filogènia, els millors arbres seran aquells que requereixin el menor nombre d'esdeveniments evolutius per poder explicar les dades observades (Fitch, 1971); de tota manera, cal tenir present que en determinats casos l'explicació més simple pot no correspondre's amb la realitat. Per estimar la fiabilitat de cada clade, s'utilitza el mètode de remostreig *bootstrap* (BP) (Felsenstein, 1985), que es basa a prendre submostres de l'alineament complet per crear arbres basats en aquestes. El procés iteratiu de BP (es considera una bona anàlisi a partir de 100 rèpliques) permet una estimació de la fiabilitat de cada clade.

Inferència bayesiana (IB): aquest mètode és una variant del de màxima versemblança i es basa en el criteri probabilístic, amb la diferència que a IB el que es calcula és la "probabilitat posterior", és a dir, la probabilitat de l'arbre donats un model evolutiu i una matriu de dades. Concretament, IB cerca el grup d'arbres que presenti una probabilitat posterior més elevada. Aquest mètode presenta les propietats estadístiques robustes de ML, amb l'avantatge suplementari d'ésser molt més ràpid i, per tant, ens permet analitzar matrius de gran volum de dades. A més, els resultats d'una anàlisi bayesiana són fàcilment interpretables, ja que la freqüència d'un clade en el conjunt d'arbres més probables és virtualment idèntica a la probabilitat posterior d'aquest clade i, per tant, no caldrà fer una anàlisi de BP per assessorar la fiabilitat de la topologia, a diferència dels altres mètodes citats (Huelsenbeck & Ronquist, 2001). De tota manera però, cal tenir en compte que les probabilitats posteriors poden ser sobreestimades respecte els valors de BP (Suzuki *et al.*, 2002) .

Les revisions i els estudis sobre els diferents tipus d'anàlisis filogenètiques són nombroses (Felsenstein, 1978; Hall, 2001; Huelsenbeck *et al.*, 2002; Archibald *et al.*, 2003; Soltis & Soltis, 2003; Nylander, 2004a). Molts

autors es decanten per un o altre mètode, però cal tenir present que totes i cadascuna d'aquestes anàlisis presenten alguna o altra limitació que s'ha de valorar a l'hora d'extreure conclusions sobre les relacions filogenètiques entre els tàxons estudiats. De tota manera, si el conjunt de dades amb el qual treballem és coherent (hem triat un marcador adequat, amb una taxa evolutiva que ens permet detectar les regions homòlogues i, alhora, proporciona caràcters informatius, l'alineament és acurat i s'han eliminat les regions ambigües, el conjunt de tàxons es troben relacionats i han estat adequadament mostrejats, i el grup extern escollit no és excessivament distant), les diferències en utilitzar un mètode o altre seran mínimes i de poca importància.

Així doncs, una vegada construïda la matriu s'han aplicat les anàlisis filogenètiques següents:

Màxima parsimònia: En les anàlisis de MP es van fer cerques heurístiques amb el programa PAUP versió 4.0b10 (Swofford, 2002). Els *gaps* es van codificar com a *missing data*. Una vegada es comença la cerca, el programa busca un arbre inicial i, a partir d'aquest arbre, es produeix una reorganització de les seves branques (*branch swapping*) per tal de trobar arbres més curts que l'inicial. Com a algoritme de reorganització de les branques, s'ha utilitzat el *tree-bisection-conection* (TBR). Per localitzar altres possibles "illes" d'arbres més parsimoniosos (Maddison, 1991), es van sotmetre les matrius a 100 rèpliques amb addició de tàxons a l'atzar (*random taxon addition)*, utilitzant també l'algoritme TBR. Per obtenir índexs de recolzament de les branques, es va calcular el *bootstrap* (Felsenstein, 1985), es van fer 100 processos de remostreig de la matriu amb l'addicció de tàxons simples i utilitzant també l'algoritme TBR. El percentatge de vegades que cada clade apareixia en els cladogrames més parsimoniosos obtinguts, és el valor de *bootstrap.*

Inferència bayesiana: l'estimació d'aquest paràmetre s'ha calculat utilitzant MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). El millor model disponible d'evolució molecular que es requereix per a l'estimació bayesiana de la filogènia va estar seleccionat

utilitzant l'estadístic *hierarchical likelihood ratio test* (hLTR) i l'*Akiake information criteria* (AIC) (Akaike, 1973) mitjançant el programari MrModeltest 2.2b (Nylander, 2004b), que considera únicament els models de substitució nucleòtida que són utilitzats per PAUP i MrBayes. Una vegada seleccionat el model de substitució nucleòtida, es van córrer simultàniament quatre cadenes *Markov Chain Monte Carlo*, començant per arbres a l'atzar, i guardant una generació de cada cent. Els primers 1000 arbres es van excloure (*burn in)* per evitar aquells que podien haver estat mostrejats abans de la convergència de les cadenes Markov.

3.3.2- ANÀLISIS RDP2 (detecció de recombinacions i anàlisis de seqüències alineades)

Per tal de verificar si les seqüències obtingudes dels clons eren o no recombinants vàrem utilitzar el programa d'anàlisis RDP2, Es tracta d'un programa de l'entorn Windows que examina les alineacions de les seqüències de nucleòtids, utilitzant 10 mètodes de detecció de recombinacions publicats que inclouen entre d'altres *geneconv, bootscan, maximum χ2, chimaera* i *sister Scanning*..., que identifiquen recombinants, seqüències parentals i donen una estimació del punt de recombinació, calculant la probabilitat potencial de recombinacions (Martin *et al.*, 2005).

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4- COMPENDI DE PUBLICACIONS

HIBRIDACIÓ I POLIPLOÏDIA

4.1 **Font, M.**, Vallès, J., Susanna, A. & Garcia-Jacas, N. En premsa. **Auto- and allopolyploidy in the *Centaurea toletana* complex (Asteraceae, Cardueae): karyotype and fluorochrome banding pattern analyses.** Collectanea Botanica

Auto- and allopolyploidy in *Centaurea* sect. *Acrocentron s. l.* (Asteraceae, Cardueae): karyotype and fluorochrome banding pattern analyses

Resum

La disploïdia i la poliploïdia són fenòmens que estan ben documentats dins del gènere *Centaurea*, especialment en la sect. *Acrocentron* i en un petit grup d'espècies de la Península Ibèrica descrit com a secció *Chamaecyanus*, estretament relacionada amb *Acrocentron*. Hem estudiat dos casos interessants de sèries poliploides d'ambdues seccions: la sèrie poliploide de *Centaurea toletana* de la sect. *Chamaecyanus* i la sèrie de *C. ornata* de la sect. *Acrocentron*. Hem realitzat un estudi cariològic utilitzant les anàlisis de cariotip clàssiques i el bandeig de cromosomes amb fluorocroms per tal de veure si és possible esbrinar a partir d'aquestes dades el tipus de ploïdia que presenten aquestes dues sèries.

El complex de *C. toletana* presenta tres nivells diferents de ploïdia, diploide, tetraploide i hexaploide, aquest últim ha estat normalment considerat com una espècie diferent, *C. argecillensis.* Els resultats obtinguts no contradiuen la hipòtesi original que l'espècie tetraploide *C. toletana* sigui un autopoliploide, però suggereixen que aquest cas d'autopoliploïdia, com molts altres, és el resultat de múltiples esdeveniments de poliploïdització. Pel que fa a *C. argecillensis,* els nostres resultats confirmen que és un al·lopoliploide que es va originar per hibridació de *C. toletana* i una espècie desconeguda també pertanyent a la sect. *Chamaecyanus*. A més, tractant-se d'una espècie colonitzadora, igual que *C. toletana* tetraploide, es suggereix que l'al·lopoliploïdia té orígens múltiples.

El complex de *C. ornata* presenta quatre nivells de ploïdia, diploide, tetraploide, hexaploide i hendecaploide. En aquesta secció però, els patrons de les bandes no ens han ajudat a determinar la relació entre la sèrie poliploide i

tampoc no ens han permès confirmar la hipòtesi dels possibles progenitors de *C. kunkelii.*

Auto- and allopolyploidy in *Centaurea* sect. *Acrocentron s. l.* (Asteraceae, Cardueae): karyotype and fluorochrome banding pattern analyses

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RESUM

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Paraules clau: *Acrocentron*, bandeig de cromosomes, cariologia, *Centaurea*, *Chamaecyanus*, citogenètica, fluorocroms, hibridació, poliploïdia.

ABSTRACT

FONT, M., J. VALLÈS, A. SUSANNA & N. GARCIA-JACAS (2008). Auto- and allopolyploidy in *Centaurea* sect. *Acrocentron s. l.* (Asteraceae, Cardueae): karyotype and fluorochrome banding pattern analyses. *Collect. Bot. (Barcelona)* **00**: 00-00.

Dysploidy and polyploidy are well documented in the large genus *Centaurea*, especially in sect. *Acrocentron* and in a small group of species from the Iberian Peninsula described as sect. *Chamaecyanus*, closely related to *Acrocentron*. We have explored two interesting cases of polyploid series in both sections: the polyploid series of *Centaurea toletana* in sect. *Chamaecyanus* and the series of *C. ornata* group in sect. *Acrocentron*. We have carried out a karyological study using both classic karyotype analyses and chromosome banding with fluorochromes.

The *C. toletana* complex exhibits three different ploidy levels, diploid, tetraploid and hexaploid, the latter usually considered a different species, *C. argecillensis*. Our results do not contradict the original hypothesis of tetraploid *C. toletana* being an autopolyploid, but they suggest that this case of autopolyploidy, as many others, is the result of multiple events of polyploidization.

Regarding *C. argecillensis*, our results confirm that it is an allopolyploid originated by hybridization of *C. toletana* and an unknown species. The colonizing habit of *C. argecillensis*, as it was the case of tetraploid *C. toletana*, suggests that the allopolyploid has multiple origins.

The *C. ornata* complex exhibits four ploidy levels, diploid, tetraploid, hexaploid and endecaploid. In this section, however, the banding patterns have not helped us to determine the relation between the polyploid series and neither have allowed us to confirm the hypothesis of the possible progenitors of *C. kunkelii*.

Key words: *Acrocentron*, *Centaurea*, *Chamaecyanus*, cytogenetics, fluorochrome banding, hybridization, karyology, polyploidy.

INTRODUCTION

Until recently, polyploidy has been considered as an uncommon and isolated fact and polyploids have been thought genetically uniform, with low adaptive capacity and tending to disappear (STEBBINS, 1971). Currently, polyploidy is regarded as a prominent force in plant evolution (SOLTIS & SOLTIS, 2000; WENDEL, 2000; SOLTIS *et al.*, 2003, 2004). In the last 20 years, molecular studies in plants have changed the understanding of polyploidy evolution. The estimated frequency of polyploidy has increased, and it is now recognised that multiple origins are the rule of most polyploids (SOLTIS *et al.*, 2003).

Traditionally, polyploidy has been thought to result from either duplication of a single genome (autopolyploidy) or from the combination of two or more differentiated genomes (allopolyploidy) (STEBBINS, 1947, 1950; GRANT, 1981). However, GRANT (1981) also noted that both concepts are the extremes of a graded series. Autopolyploidy has historically been considered maladaptive and relatively uncommon compared to allopolyploidy (WENDEL & DOYLE, 2005). Whereas it is clear now that allopolyploidy is probably more common than autopolyploidy in nature, the latter is far more prevalent than was once thought. In addition, it is necessary to say that the autopolyploids never are maladaptive (RAMSEY & SCHEMSKE, 1998; WENDEL & DOYLE, 2005; SOLTIS *et al.*, 2003, 2004).

An interesting case of study of polyploids is found in the genus *Centaurea.* Karyological features of *Centaurea* include both dysploidy and polyploidy. As to dysploidy, chromosome numbers in *Centaurea* range from $x = 12$ to $x = 7$ in a series well-correlated to phylogeny (GARCIA-JACAS *et al.*, 2001). As to polyploidy, it is especially frequent in sects. *Acrocentron* (Cass.) DC. and *Chamaecyanus* Willk. (GARCIA-JACAS, 1998).

Centaurea sect. *Chamaecyanus* was described on the basis of a small group of species from the Iberian Peninsula (WILLKOMM & LANGE, 1870). *Chamaecyanus* is related to sect. *Acrocentron*, one of the largest of *Centaurea* (FONT *et al.*, 2002), with about 100 species and a high percentage of endemics (GARDOU, 1975; WAGENITZ, 1975). The Ibero-North African region stands as the second most important centre of diversification after Turkey and the Balkans

(GARCIA-JACAS & SUSANNA, 1992). Differences between two sections rely only on the combination of acaulescent habit and reduced pappus in *Chamaecyanus*. Even though the boundaries are clear-cut, both sections are extremely close as demonstrated by the many fertile hybrids described between species of both sections (FERNÁNDEZ CASAS & SUSANNA, 1986) and subsectional rank looks certainly more appropriate for *Chamaecyanus*. Anyway, no matter the rank adopted (section or subsection), species of *Chamaecyanus* are a natural, monophyletic group as confirmed by DNA sequence analyses (FONT *et al.*, 2002).

The most extreme and interesting case of polyploidy in *Chamaecyanus* is *C. toletana* Boiss. & Reuter, which exhibits three different ploidy levels ($2n = 2x = 20$, $2n = 4x = 40$, and $2n = 6x = 60$). Tetraploid *C. toletana* was described as a different species, *C. cavanillesiana* Graells. However, careful examination of the type and new collections from the type localities in central Spain demonstrated that morphological differences between *C. toletana* and *C. cavanillesiana* were inexistent (FERNÁNDEZ CASAS & SUSANNA, 1986). In contrast, it is possible to characterize morphologically the hexaploid, which was also described as a different species, *C. argecillensis* Gredilla, later merged into *C. toletana* as a variety because many intermediate forms between the diploid type variety and the hexaploid exist (FERNÁNDEZ CASAS & SUSANNA, 1986). These authors concluded that the tetraploid was a true autopolyploid because it was impossible to differentiate the diploid from the tetraploid without carefully measuring pollen size or stigmas (LEWIS, 1980; RAMSEY & SCHENKE, 1998). However, FERNÁNDEZ CASAS & SUSANNA (1986) did not reach any conclusion on the hexaploid.

There is another case of similar polyploid series in sect. *Acrocentron* formed by three species *C. gabrielis-blancae* Fern. Casas (*C. ornata* var. *microcephala* Willk.), a diploid species, *C. ornata* Willd. a tetraploid and *C. saxicola* Lag. a hexaploid. DOSTÁL (1976) subordinated *C. saxicola* to *C. ornata* but a phylogenetic analysis of sect. *Acrocentron* (FONT *et al.*, 2002) supports its status as a different species. In addition *C. kunkelii*, described recently by GARCIA-JACAS (1998) on the basis of a plant from south Spain that had been

much confused with *C. ornata*, is also member of this group. Curiously GARCIA-JACAS (1998) suspected a hybridogenic origin for this endecaploid species, and one of the hypothesized parental species was *C. saxicola*, the other putative progenitor belonging to sect. *Chamaecyanus*, *C. haenseleri,* a tetraploid species (FERNÁNDEZ CASAS & SUSANNA, 1986).

Interest for polyploidy has increased lately with the emergence of new molecular techniques (extensively revised in SOLTIS *et al.*, 2004). Traditional views on meiotic pairing cannot be considered a valid criterion to distinguish between auto- and allopolyploids (QU *et al.*, 1998). Instead, karyotype analysis may be very helpful in establishing systematic and evolutionary relationships within polyploid complexes, particularly those data which constitute what SCHWEIZER & EHRENDORFER (1983) named banding style, i.e. the distribution pattern of different chromosome regions, such as constitutive heterochromatin (revealed with Giemsa C-banding method) or AT- and GC-rich DNA portions (revealed with fluorochrome banding methods). We have used banding methodology a) to verify whether autopolyploidy of *C. toletana* is reflected in the banding pattern, b) to explore the relationships of hexaploid *C. argecillensis* to the diploid and tetraploid levels of *C. toletana*, c) to establish the relationship between *C. gabrielis-blancae*, *C. ornata* and *C. saxicola* and verify if they constitute a polyploid series and d) to look after a confirmation of the hypothesis that *C. saxicola* and *C. haenseleri* are the progenitors of *C. kunkelii*.

MATERIALS AND METHODS

Adult plants were collected from wild populations of each taxon. The species studied are listed in Table 1, with indication of their origins.

Chromosome counts were made on somatic metaphases using the squash technique. Root meristems from germinating seeds collected in the wild or from wild plants cultivated in pots were used.

Samples were pretreated with 0.05% aqueous colchicine at room temperature for 3 h as a minimum. The material was fixed with Carnoy for 24 h at -20 ºC. Before staining, the material was hydrolyzed with 5N HCl for 1 h at room temperature. It was stained with 1% acetic orcein and mounted in 45%

acetic acid. For all the counts, a minimum of ten plates (diploid and tetraploid taxa) or five plates (hexaploid taxa) from different individuals was examined. Preparations were made permanent by CO2 freezing, dehydrating in ethanol and mounting in Canada balsam. The majority of photographs were taken using a Zeiss Standard microscope, and digital photographs were also taken using an Olympus 3030 camera mounted on an Olympus microscope U-TV1 X. The preparations, the negatives and the herbarium vouchers are preserved in the Botanical Institute of Barcelona (BC).

Numerical parameters of the karyotypes were calculated and the idiograms were obtained with software running on Lotus 1-2-3 prepared by J. Simon (Universitat de Barcelona).

For fluorochrome banding, root tips were hydrolysed with 45% acetic acid for 5-10 min at 60 ºC, and squashed in a drop of 45% acetic acid. After metaphase plate selection and cover slip removal by CO2 freezing, slides were rinsed with absolute ethanol and dried at room temperature in covered containers until use. Two different fluorochrome banding protocols were applied to reveal GC-rich (with chromomycin A3, abridged CMA) and AT-rich (with bisbenzimide Hoechst 33258, abridged Hoechst) regions. For chromomycin A3 staining the techniques of SCHWEIZER (1976), KONDO & HIZUME (1982), COULAUD *et al.* (1995), CERBAH *et al.* (1995) and VALLÈS & SILJAK-YAKOVLEV (1997) were used with minor modifications as follows: the slides were incubated in McIlvaine buffer pH 7; treated with distamycin A (0.1 g/l in McIlvaine buffer pH 7) for 10 min; stained with chromomycin A3 (0.1 g/l in McIlvaine buffer pH 7 + 0.005 M MgSO4) for 10 min; rinsed in the same buffer; counterstained with methyl green (0.1% in McIlvaine buffer pH 5.5) for 10 min; rinsed in McIlvaine buffer pH 5.5; and mounted in antifade glycerol solution (Citifluor AF1) - McIlvaine buffer pH 7 1:1. Bisbenzimide staining was carried out according to the techniques of MARTIN & HESEMANN (1988), COULAUD *et al.* (1995), CERBAH *et al.* (1995) and VALLÈS & SILJAK-YAKOVLEV (1997) with minor modifications as follows: the slides were successively rehydrated in 70, 50, and 30% ethanol and distilled water, each for 5 min; incubated in McIlvaine buffer pH 5.5 for 10 min; stained with bisbenzimide (2 x 10-3 g/l in McIlvaine buffer pH 5.5) for 1 min; and mounted in

60% sucrose. The observations were made with an epifluorescence Zeiss Axioplan microscope with filter sets 07 (excitation 457, emission 530 nm long pass) for chromomycin stained slides and 01 (excitation 365, emission 480 nm long pass) for bisbenzimide stained slides. ISO 400 Fujichrome, ISO 200 Kodachrome and ISO 100 TMax films were used to photograph the chromosomes and the interphase nuclei.

Table 1. Origin of the studied populations

Taxon	Collection data
Centaurea argecillensis Gredilla	Spain, Guadalajara: Argecilla, mountain slopes on the road to Ledanca, *Font & Susanna 1811* (BC).
Centaurea gabrielis-blancae Fern. Casas	Spain, Navarra: Lumbier, Foz de Lumbier, *Garcia-Jacas & Susanna 1592* (BC).
Centaurea haenseleri Boiss.	Spain, Málaga: 10 km to Jubrique, *Garcia-Jacas & Susanna 1888* (BC).
Centaurea kunkelii N.Garcia.	Spain, Almería: road AL-411 between Roquetas and Canjáyar, *García-Jacas, Susanna 1612 & Vilatersana* (BC).
Centaurea ornata Willd.	Spain, Soria: near San Esteban de Gormaz, *Garcia-Jacas & Susanna 1823* (BC). Spain, Huesca: between Salinas and de la Peña dam (A-132), *Vilatersana 58* (BC).
Centaurea saxicola Lag.	Spain, Murcia: La Azohía, near the watchtower, *Garcia-Jacas, Susanna 1616 & Vilatersana* (BC).
Centaurea toletana Boiss. & Reut. (2*x*)	Spain, Toledo: Risco de las Paradas, *Font & Susanna 1817* (BC). Spain, Toledo: mountains above San Pablo on the track to Baños del Robledillo, *Font & Susanna 1818* (BC).
Centaurea toletana Boiss. & Reut. (4*x*)	Spain, Madrid: Redueña, road N-320, 4 km to Torrelaguna, *Font & Susanna 1819* (BC).

RESULTS AND DISCUSSION

A summary of cytogenetic results, including chromosome number, chromosomal formula, karyotype symmetry data and the number of GC- and AT-rich regions, is presented in Table 2, except for the endecaploid species *C. kunkelii*, for which it was only possible to obtain a single metaphasic plate and we have not been able to make the calculations. In this table we can appreciate the different chromosome sizes between the species belonging to sect. *Acrocentron* and those from sect. *Chamaecyanus*.

Figure 1 (*C. toletana* series) and Fig. 2 (*C. ornata* series) show orcein-stained metaphase plates and chromosome morphology of the taxa studied. Karyotypes are very symmetrical in all the populations, and most chromosomes

Table 2. Summary of cytogenetic results. The superscripts indicate: [1] chromosomal formula according to LEVAN *et al*. (1964); [2] mean chromosome length; [3] chromosome length range; [4] total karyotype length; [5] centromeric index (I or index in LEVAN *et al*., 1964); [6] length ratio of long and short chromosome arms (LEVAN *et al.,* 1964); [7] intrachromosomal asymmetry index (ROMERO, 1986); [8] interchromosomal asymmetry index (ROMERO, 1986); [9] symmetry class according to STEBBINS (1971). CMA= chromomycin positive/negative marks; BB= bisbenzimide positive/negative marks. A= *Acrocentron*; Ch= *Chamaecyanus*.

Taxon	2*n*	Ploidy level	Section	Chromosomal formula[1]	MCL[2]	CLR[3]	TKL[4]	CI[5]	R[6]	A1[7]	A2[8]	Stebbins class[9]	CMA +/-	BB +/-
***Centaurea toletana* complex**														
C. toletana	20	2*x*	Ch	6m+2m^{sat}+2sm	5.89	3.93-8.68	58.85	41.75	1.41	0.29	0.24	2B	3/0	1/2
C. toletana	40	4*x*	Ch	16m+4m^{sat}	11	7.02-16.09	220.07	42.76	1.35	0.25	0.22	2A	5/0	2/1
C. argecillensis	60	6*x*	Ch	3M+22m+1m^{sat}+3sm+1sm^{sat}	7.28	4.93-11.25	218.49	42.67	1.37	0.24	0.22	2A	5/0	2/2
***Centaurea ornata* complex**														
C. gabrielis-blancae	20	2*x*	A	7m+1m^{sat}+1sm + 1sm^{sat}	2.82	1.99-4.28	28.16	42.01	1.41	0.27	0.22	2B	2/2	1/0
C. ornata	40	4*x*	A	1M+15m+3sm+1sm^{sat}	2.15	1.39-3.50	42.92	41.29	1.44	0.29	0.25	2A	4/1	3/0
C. haenseleri	40	4*x*	Ch	1M+18m+1m^{sat}	4.20	3.96-15.70	339.6	41.85	1.43	0.26	0.21	2A	4/1	3/0
C. saxicola	60	6*x*	A	25m+3sm+2sm^{sat}	2.54	1.51-4.11	76.17	42.05	1.41	0.26	0.24	2A	10/0	10/1
C. kunkelii	110	11*x*	A										16-18	6

are metacentric, with some submetacentric ones present. All the species have satellites, not always in the same position.

In all the taxa, GC- and AT-rich zones were detected. Fluorochrome banded metaphase plates are shown in Fig. 3. The haploid idiograms of each population with the location of GC- and AT-rich regions are presented in Fig. 4. For the hexaploid population (*C. argecillensis)*, the exact assignation of chromomycin and bisbenzimide bands in the idiogram was not performed (Fig. 4C), because of the extreme similarity in length and symmetry of all the chromosomes. We have not included either the idiograms of *C. saxicola* and *C. kunkelii*, since it was impossible to assign a banding patterns adjusted to the reality in these species.

***Centaurea toletana* complex**

In *C. toletana* series, GC- and AT-rich regions are predominantly terminal. This banding pattern agrees with those of other Cardueae, such as that of the *Xeranthemum* group (GARNATJE *et al.*, 2004), relatively close to the genus considered here. Chromomycin-positive bands are often coincidental with bisbenzimide-negative zones.

In diploid *C. toletana* three telomeric bands are present in the chromomycin staining, while there is only one in the bisbenzimide staining, together with two negative satellites (Figs. 3A, 3D, 4A).

In the tetraploid population, there are also three telomeric bands in the chromomycin staining with two more signals in two satellites (Figs. 3B, 4B). In the bisbenzimide staining, however, there are two positive signals, one telomeric and another one in a satellite, together with a negative satellite (Figs. 3E, 4B). Regarding *C. argecillensis*, we have already pointed out the impossibility of determining exactly the chromosomes where the signals are located, but there are five chromomicyn-positive signals, three of them telomeric, one centromeric and the last one in a satellite (Fig. 3C). As for bisbenzimide, there are two telomeric bands and two negative satellites (Fig. 3F).

According to these results, the fluorescent banding pattern of the tetraploid population is not a perfect duplicate of the diploid one: even though diploid and tetraploid *C. toletana* studied populations share at least three of the signals, there are obvious differences between an ideal duplication of the 2*x* pattern and our results.

In our opinion, this does not contradict the hypothesis of autopolyploidy, which, as we have seen, has very sound basis. In fact, we could not expect the tetraploid to be a perfect duplication of the genome of the diploid, which would be possible only in an ideal case that we would seldom find in nature. Many factors are responsible for these differences, but we can stress upon two obvious ones.

First factor is relative age of polyploidy. If tetraploid populations have long evolved without genetic exchange with the diploid ones, we can expect some differences, and diploid and tetraploid *C. toletana* do not coexist because their soil requirements are not compatible (FERNÁNDEZ CASAS & SUSANNA, 1986).

Second, and probably the most important factor, tetraploid *C. toletana* is a typical colonizing polyploid (STEBBINS, 1950; LEVIN, 1983). While diploid populations of *C. toletana* grow only on siliceous soils, tetraploid populations have a wider ecological tolerance and have colonized a very large extension in central Spain on clay soils, occupying frequently somewhat disturbed habitats (FERNÁNDEZ CASAS & SUSANNA, 1986). Multiple origins of these successful, colonizing polyploids are now accepted as a rule (SOLTIS & SOLTIS, 1993 and 1995; VAN DIJK & BAKX-SCHOTMAN, 1997). The model of distribution of diploid *C. toletana* also supports a multiple origin of the tetraploid race: the diploid cytotype grows in small and disjunct populations in which genetic drift could easily lead to partial reproductive isolation, favouring the occurrence of polyploidy, as was suggested for *Centaurea* and related genera by VILATERSANA *et al.* (2000) and GARNATJE *et al.* (2001). Our preliminary tests using RAPDs (FONT, unpublished data) suggest that there are differences between diploid populations of *C. toletana*. Thus, the origin of the tetraploid would be in multiple events of intraspecific hybridization between partly genetically isolated diploid individuals, rather than a single duplication in a population (intraspecific

hybridization has been suggested for many other autoployploids by SOLTIS *et al.* (2004). This would justify the differences found between a simple duplication of the 2*x* genome and our results.

Regarding hexaploid *C. argecillensis*, we have found no cytogenetic connections to the two other cytotypes of the polyploid series. An outstanding difference is the presence in the hexaploid species of a centromeric chromomycin-positive region, absent from both ploidy levels (diploid and tetraploid) of *C. toletana*, making it difficult to point out autopolyploidy as the mechanism of origin for the 6*x* taxon from the 2*x* and 4x one. Morphological relationships to *C. toletana* are so obvious that cannot be disregarded, and populations of *C. toletana* with tetraploid cytotype are common in the area of distribution of *C. argecillensis*. However, *C. argecillensis* is very probably an allopolyploid: the extreme morphological variability pointed out by FERNÁNDEZ CASAS & SUSANNA (1986) also suggests a hybrid origin. As was the case for the tetraploid, a multiple origin of the hexaploid is very probable, not only because of its variability, but also because the species is a very active colonizer (FERNÁNDEZ CASAS & SUSANNA, 1986). The question that remains open is the identity of the other species involved in the origin of *C. argecillensis*. Achene morphology is very reliable here: the wanting parental species must be probably a species of sect. *Chamaecyanus* because hybridization with species of sect. *Acrocentron* is reflected in the length of the pappus and in the frequent loss of the caulescent habit (revised in depth by GARCIA-JACAS, 1998). The identity of the second species that has originated *C. argecillensis* is a question that further studies using molecular markers and fluorescent and genomic *in situ* hybridization will try to make clear.

Centaurea ornata complex

Regarding the species belonging to the *C. ornata* series, we can observe in the diploid *C. gabrielis-blancae* the presence of four markings with the chromomycin staining, a positive centromeric band and another telomeric, and the presence of two negative satellites. However, we have found a single

centromeric marking in the larger chromosome in the case of the bisbenzimide staining (Figs. 3G, 4D).

In the two tetraploid species we observe four positive markings, three telomeric bands and a centromeric one, and a negative satellite marking with CMA in *C. ornata*, while with Hoechst staining we can see three telomeric markings. In *C. haenseleri* we also observe four positive CMA bands, three telomeric and one centromeric, together with a negative satellite. We must recall that the exact position of marks in two tetraploid species are different (Figs. 3H, 3I, 4E, 4F).

With respect to the hexaploid and endecaploid species, we can only point out the number of signals observed, since the high similarity in symmetry of the whole karyotype does not allow us to check which chromosomes bear the marks. In *C. saxicola* we can see with CMA staining a minimum of 10 markings, and also 10 with Hoechst, of which some are centromeric, and the presence of some negative satellite is also shown.

In *C. kunkelii* we observe 16 to 18 CMA markings and a minimum of six Hoechst bands, with two positive satellites.

In accordance with the results obtained, species from the *C. ornata* complex do not present a banding pattern allowing us to determine which has been the origin of the polyploid series. The bands present in the diploid species do not coincide with those revealed in the tetraploid and hexaploid species. A possible explanation to this fact could be the intra- and intergenomic reorganization of polyploid genomes, which can be extensive and may occur rapidly (SOLTIS *et al.*, 2003). In addition, tetraploid *C. ornata* does not share the same habitat that *C. gabrielis-blancae* (a diploid species) and *C. saxicola* (hexaploid). We must hypothesize that the formation of *C. saxicola* is an ancient event, and the long time ellapsed allowed extensive genomic reorganization.

The banding patterns obtained make it difficult to point out any relationship with *C. kunkelii* (endecaploid species); since it is not possible to determine to which chromosomes the bands belong. Anyway, the number of bands found in *C. kunkelii* does not agree with the number obtained in the other species. *Centaurea kunkelii* is known from a single population, which probably implies a

long isolation. This isolation could also be the reason for a deep restructuring of the genome that has evolved in a different way.

In later analyses using nuclear-ribosomal DNA sequences of the ETS region (FONT *et al.*, unpublished data) we have verified that the diploid and tetraploid species are grouped in the Iberian clade, whereas *C. saxicola* and *C. kunkelii* belong to a clade with the African species and some Iberian ones like *C. haenseleri*, *C. prolongi* and *C. lainzii* with which they share the area of distribution in South Spain. These results do not contradict our starting hypothesis that the possible progenitors of *C. kunkelii* are *C. saxicola* and *C. haenseleri*. Conversely, the ETS-based phylogeny groups *C. gabrielis-blancae* and *C. ornata*, a relationship that does not agree with the very different banding patterns obtained in the present work. In the case of *C. ornata*, this lack of correlation probably is caused by the multiple origins of polyploid species as previously stated.

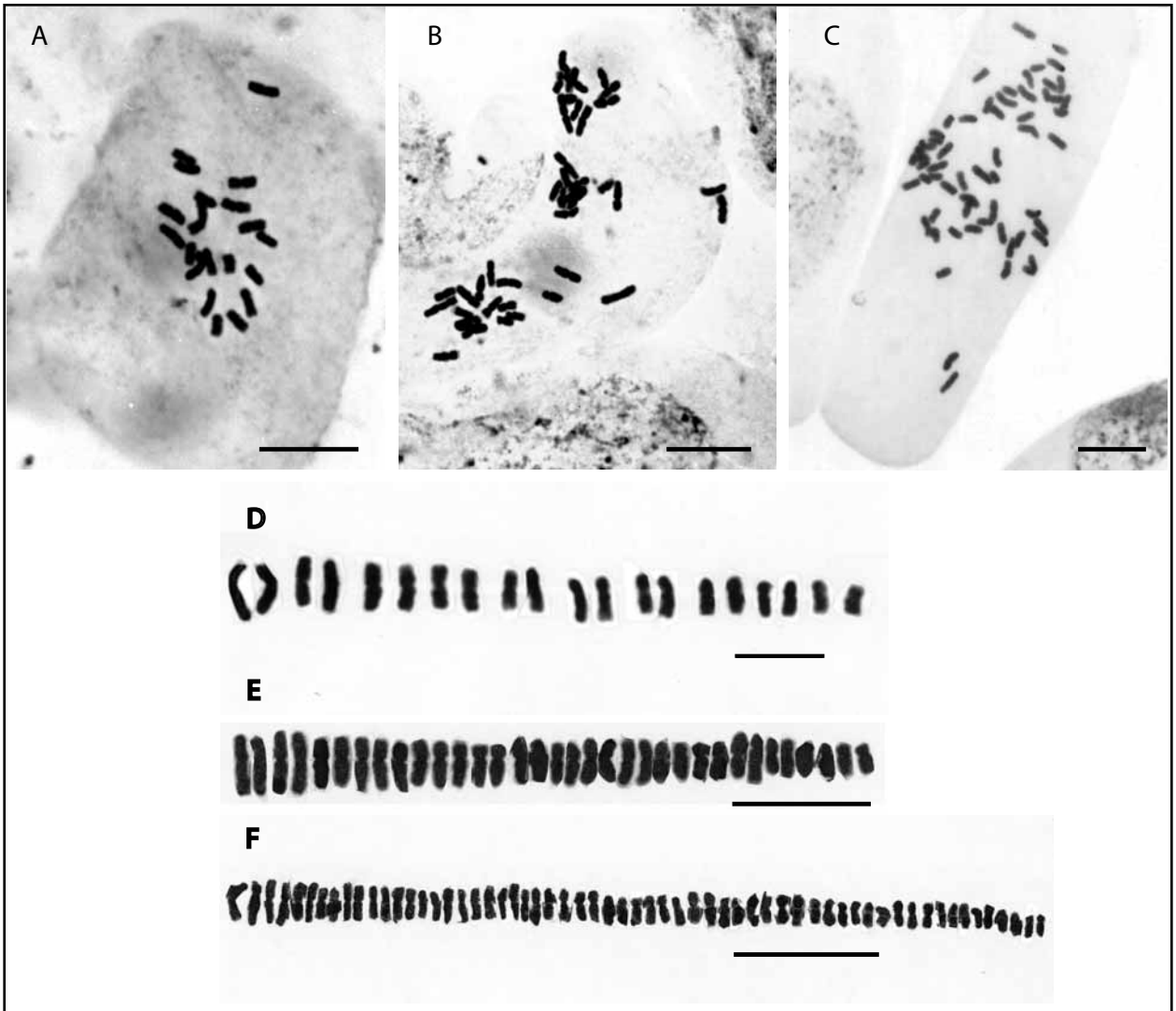

Fig. 1 Orcein-stained metaphase plates (A-C) and karyograms (D-F) of the studied populations. (A) and (D), *Centaurea toletana* (2*x*); (B) and (E), *Centaurea toletana* (4*x*); (C) and (F), *Centaurea argecillensis*. Scale bars: 10 μm.

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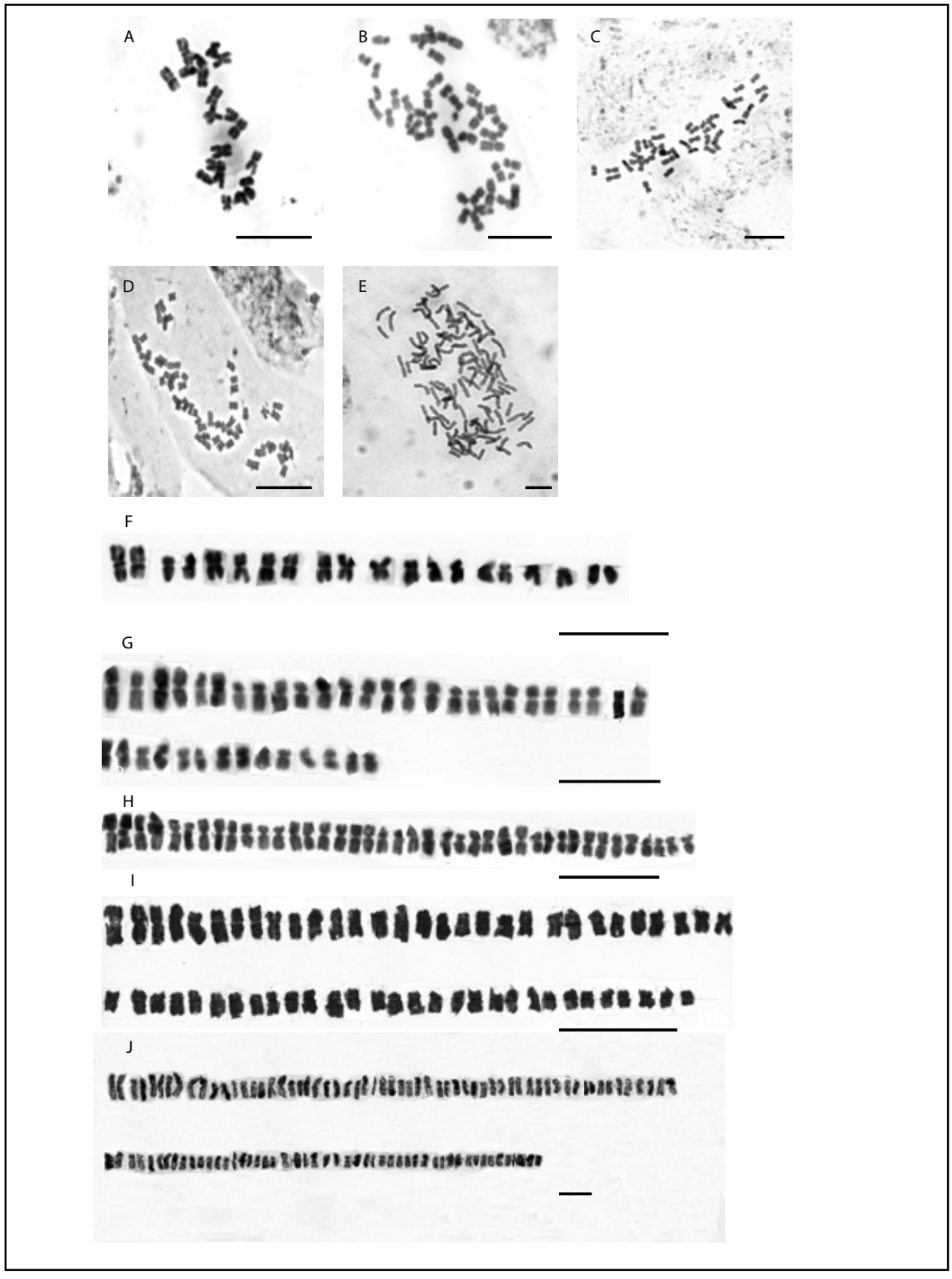

Fig. 2 Orcein-stained metaphase plates (A-E) and karyograms (F-J) of the studied populations. (A) and (F), *Centaurea gabrielis-blancae*; (B) and (G), *Centaurea ornata*; (C) and (H), *Centaurea haenseleri*; (D) and (I), *Centaurea saxicola*; (E) and (J), *Centaurea kunkelii*. Scale bars: 10 μm.

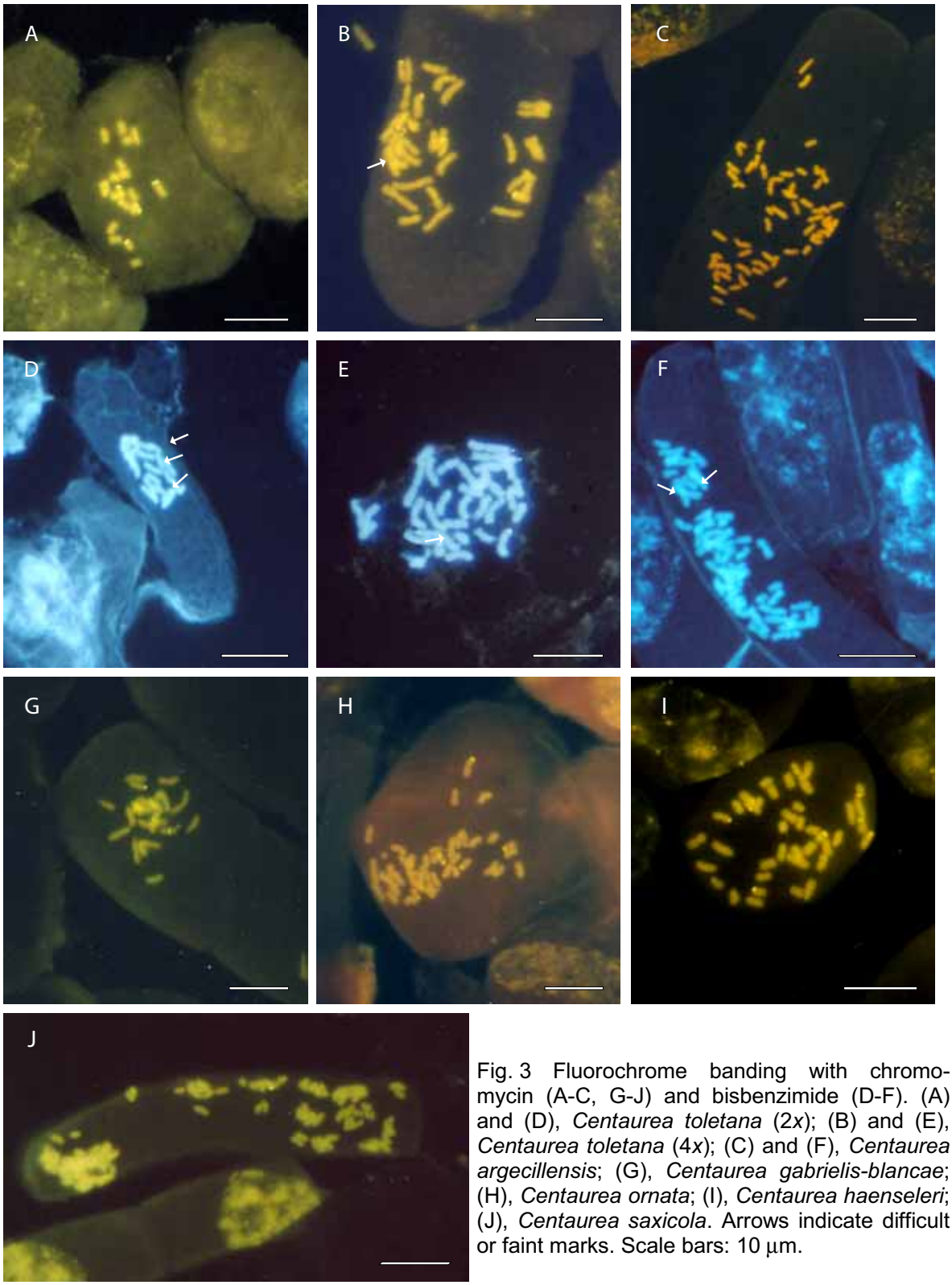

Fig. 3 Fluorochrome banding with chromomycin (A-C, G-J) and bisbenzimide (D-F). (A) and (D), *Centaurea toletana* (2*x*); (B) and (E), *Centaurea toletana* (4*x*); (C) and (F), *Centaurea argecillensis*; (G), *Centaurea gabrielis-blancae*; (H), *Centaurea ornata*; (I), *Centaurea haenseleri*; (J), *Centaurea saxicola*. Arrows indicate difficult or faint marks. Scale bars: 10 μm.

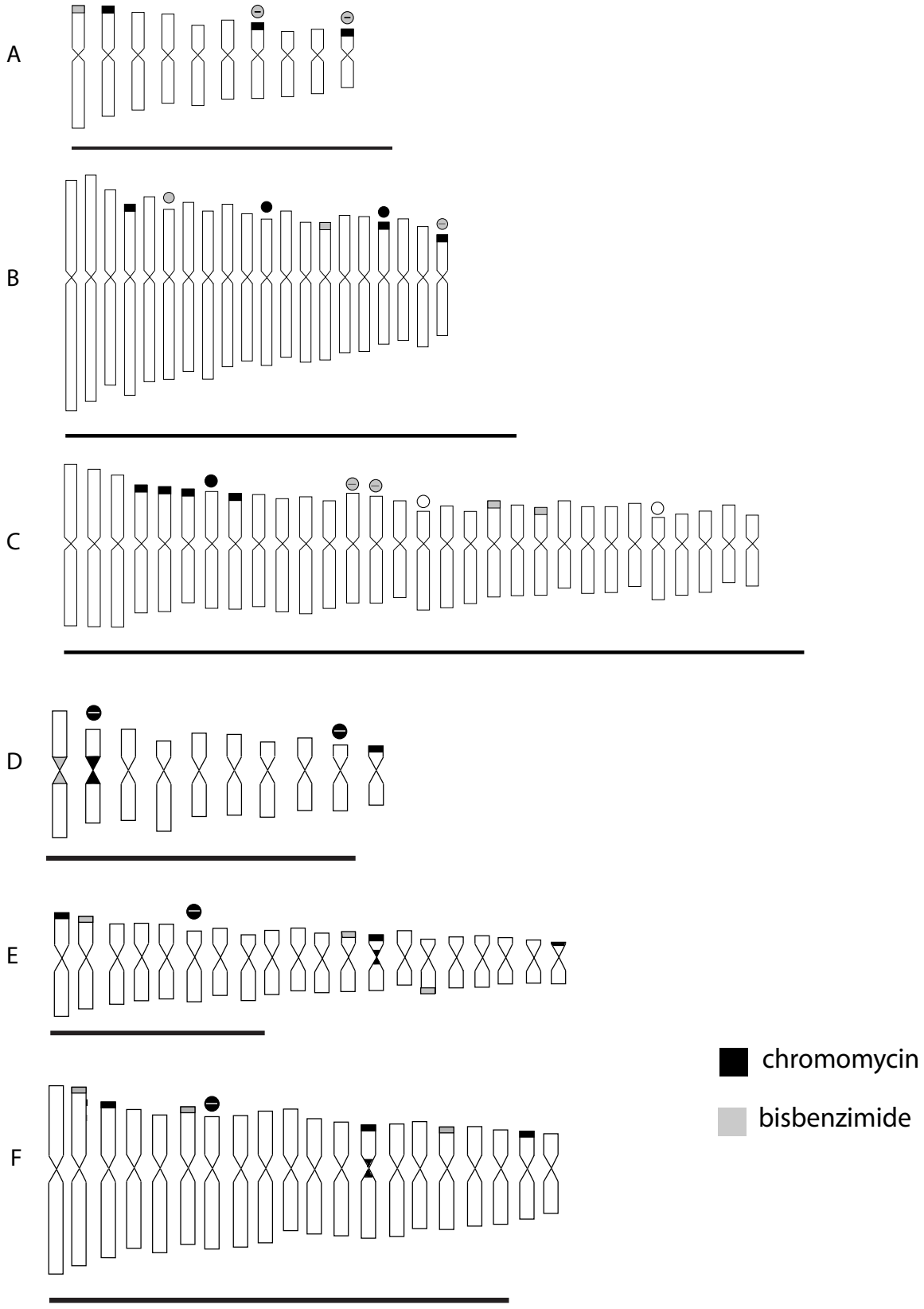

Fig. 4 Haploid idiograms of the studied populations with the location of GC-rich and AT-rich regions. Round dots indicate satellite markers. Chromomycin positive: solid black; bisbenzimide positive: grey. (A), *Centaurea toletana* (2*x*); (B), *Centaurea toletana* (4*x*); (C), *Centaurea argecillensis* (estimated position of the satellites); (D), *Centaurea gabrielis-blancae*; (E), *Centaurea ornata*; (F), *Centaurea haenseleri*. Scale bars: 10 μm.

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4.2 **Font, M.**, Garcia-Jacas, N., Soltis, P. S. & Susanna, A. Encara no enviat a cap revista. **The polyploid series of *Centaurea toletana*: glacial migrations and introgression evidenced by nrDNA and cpDNA sequence analyses**

The polyploid series of *Centaurea toletana*: glacial migrations and introgression evidenced by nrDNA and cpDNA sequence analyses

Resum

L'emergència de les noves tècniques moleculars ha incrementat l'interès per l'especiació híbrida i la poliploïdia. Hi ha molts casos documentats de poliploïdia en *Centaurea*, un dels millors exemples és el complex poliploide de *C. toletana* (2*x,* 4*x* i 6*x*). Hem inclòs en l'estudi una àmplia representació dels tres nivells de ploïdia de *C. toletana*, juntament amb espècies representatives del complex *Acrocentron-Chamaecyanus*, sobretot aquelles que comparteixen la seva àrea de distribució amb *C. toletana.*

A causa de la intensa hibridació que han patit les espècies d'aquest grup, cap dels marcadors utilitzats mostra una correlació amb la sistemàtica de les espècies *Acrocentron-Chamaecyanus.*

A les poblacions diploides de *C. toletana* es detecta la presència de quatre genomes: el seu propi, els de *C. ornata* ibèric i africà i el de *C. podospermifolia* aliens. Les diferències morfològiques entre aquestes tres espècies són òbvies, en canvi, en cap cas aquest creuament ha deixat cap tret morfològic. El desplaçament dels híbrids cap a la morfologia d'un dels parentals (*C. toletana*) per introgressió està confirmat en dos casos d'híbrids antics entre les seccions *Acrocentron* i *Chamaecyanus*.

La forma tetraploide de *C. toletana*, és taxonòmicament un autopoliploide, però presenta, en una de les poblacions, un ribotip més dels quatre presents en l'espècie diploide. Això reflecteix que hi ha hagut una hibridació posterior, una altra vegada sense senyals morfològiques, seguida d'introgressió. Per la seva part, el polimorfisme detectat en la regió ITS ja assenyala com a mínim dos orígens per a les espècies tetraploides.

Els resultats obtinguts en les poblacions hexaploides ens indiquen que un dels pares és *C. toletana* (4*x*) i l'altre és, probablement, *C. podospermifolia*

(2*x*). La hibridació explicaria molts trets morfològics que separen les espècies tetraploides de les hexaploides, i la presència del ribotip de *C. podospermifolila* en les poblacions diploides demostra que l'ancestre diploide de l'espècie actual se solapava amb l'àrea de distribució de *C. toletana*. L'origen híbrid de *C. argecillensis* s'explicaria per una especiació poliploide simpàtrica.

Centaurea ornata mostra una gran varietat de genomes. Si hi afegim el seu ampli rang de distribució i la seva capacitat per creuar-se, fa sospitar que aquesta espècie pugui haver estat vector de genomes aliens al llarg de la seva àrea de distribució.

El patró de distribució dels genomes de la Península Ibèrica i del nord d'Àfrica ens indica que les espècies d'aquestes àrees, que actualment no són simpàtriques, han passat llargs i freqüents períodes de contacte, fet que s'explicaria per les oscil·lacions altitudinals i latitudinals que han patit aquestes espècies durant els canvis climàtics del neogen.

The polyploid series of *Centaurea toletana*: glacial migrations and introgression evidenced by nrDNA and cpDNA sequence analyses

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Summary

- The polyploid series of *Centaurea toletana* comprises three levels (diploid, tetraploid and hexaploid). Previous studies, both morphologic and karyologic suggested that the tetraploid was a true autopolyploid, while the hexaploid was an allopolyploid and should be considered a different species.
- Sequencing of the ITS and *rps*4-*trn*T-*trn*L, *ycf*3-*trn*S and *rp*L16 regions, and extensive cloning of the ETS region, have evidenced that many diploid populations show different ribotypes from old hybridization events.
- The diverse ribotypes from the diploid populations are also present in the tetraploid ones. The extreme difficulties in classifying the tetraploid in one of the classic categories, auto- or allopolyploid, are discussed. The hexaploid *C. argecillensis* show many different ribotypes too, but it also shows an exclusive ribotype, which discards an autopolyploid origin. The pattern of introgression and gene flow implies several species from the Iberian Peninsula, but also some others as far as from the High Atlas in Morocco.
- The reasons for this extensive and long-reaching network of hybridization are also discussed in terms of the convulse climatic history of the western Mediterranean during the Neogene.

Introduction

Interest for hybrid speciation and polyploidy has increased lately with the emergence of new molecular techniques (extensively revised in Soltis *et al.*, 2004b). Because of the revival of the interest for polyploidy and the subsequent research carried out in the field, many of the paradigms on polyploid evolution were questioned. Some of the most dramatic changes in our views on polyploidy relate to autopolyploidy.

Classic studies presented autopolyploidy as maladaptive, and autopolyploids were considered an evolutionary dead end (Stebbins, 1971; Lewis, 1980; Grant, 1981). Recent research suggest, to the contrary, that many autopolyploids share with allopolyploids a very important trait: they have arisen recurrently from separate hybridization events (Soltis & Soltis, 1993, 1999; Soltis *et al.*, 2004a). This fact implies that, contrarily to being a cul-de-sac, autopolyploids have the benefits usually associated to allopolyploids in terms of increased adaptability.

Another paradigm on autopolyploidy that has changed because of new molecular evidence is the relative frequency of autopolyploidy, which was considered extremely infrequent (Stebbins, 1971; Grant, 1981) when compared to allopolyploidy. Actually, autopolyploidy is much more frequent than expected and, in most of the cases, it is the result of a cross between two individuals rather than the self-duplication of one single genome (Soltis & Soltis, 2003; Wendel & Doyle, 2005). An interesting hypothesis by Ramsey & Schemske (1998) is that interspecific hybridization should be often too low to generate many polyploids, and in this case autopolyploidy could be more frequent than allopolyploidy. Even though this could be considered an exaggeration, it is evident that current data confirm that autopolyploidy is not exceptional but frequent. In fact, following Soltis *et al.* (2004a), current research on polyploidy should be focused in autopolyploids, which have been somewhat neglected.

Along our studies in the genus *Centaurea* sensu lato (Compositae, Cardueae), we have encountered many cases of polyploidy, both auto- and allopolyploidy, which are worth studying with the new molecular tools. One of

the best examples is the polyploid complex of *C. toletana* Boiss. & Reuter (sect. *Chamaecyanus* Willk.). This section was described based on a small group of species from the Iberian Peninsula and is closely related to sect. *Acrocentron* (Cass.) DC., one of the largest of *Centaurea* (Font *et al.*, 2002): differences rely only on the combination of acaulescent habit and reduced pappus in *Chamaecyanus*. Even though the boundaries between *Acrocentron* and *Chamaecyanus* are clear-cut, both sections are extremely close as demonstrated by the many fertile hybrids described between species of both sections (Fernández Casas & Susanna, 1986) and subsectional rank looks certainly more appropriate for *Chamaecyanus*. Anyway, no matter the rank adopted (section or subsection), species of *Chamaecyanus* constitute a natural, monophyletic group as confirmed by DNA sequence analyses of the ITS region (Font *et al.*, 2002). There is, however, an important caveat emerging from previous studies: all the evidence (both morphologic and molecular) points towards intense introgression and reticulate evolution in the group, which shows virtually no barriers against hybridization. This was the reason adduced by Font *et al.* (2002) for explaining the very low resolution found in molecular survey of sect. *Acrocentron*.

Karyological features of *Centaurea* are very interesting and include both dysploidy and polyploidy. As to dysploidy, chromosome numbers in *Centaurea* range from $x = 12$ to $x = 7$ in a series well correlated to phylogeny (Garcia-Jacas *et al.*, 1996). A more limited but very interesting dysploidy series is present in sections *Acrocentron* and *Chamaecyanus* which show two numbers, $x = 11$ and $x = 10$. The sense of this dysploid series was studied by Garcia-Jacas & Susanna (1992), who concluded that is was a descending series with $x = 11$ as the ancestral basic number.

As to polyploidy, it is especially frequent in sects. *Acrocentron* and *Chamaecyanus* (Garcia-Jacas, 1998). Our case of study, *C. toletana* group, exhibits three different ploidy levels: $2n = 2x = 20$, $2n = 4x = 40$ and $2n = 6x = 60$.

Centaurea toletana is a robust acaulescent perennial with pinnatisect grey-green leaves and pectinate-fimbriate involucral bracts, and, as is usual in

the species of the group, morphological variability of leaves and bracts is very wide (Fernández Casas & Susanna, 1986).

Diploid populations of *C. toletana* constitute the type of the species. They are mid-mountain plants growing at 700-1200 m in the siliceous ranges of western-central Spain (Montes de Toledo and adjacent sierras in Extremadura, Fig. 1; Fernández-Casas & Susanna, 1986).

Tetraploid populations of *C. toletana* grow in limestone and clay soils in central Spain (Fig. 1) and were described as a different species, *C. cavanillesiana* Graells. However, careful examination of the type and new collections from the type localities in central Spain demonstrated that morphological differences between diploid *C. toletana* and tetraploid *C. cavanillesiana* were inexistent (Fernández Casas & Susanna, 1986); they concluded that the tetraploid was a true autopolyploid because it was impossible to differentiate the diploid from the tetraploid without carefully measuring pollen size or stigmas (Lewis, 1980; Ramsey & Schemske, 1998). However, no genetic studies were made.

As to the hexaploid, also described originally as a different species, *C. argecillensis* Gredilla, it shows some good morphological characters that segregate it from the diploid or the tetraploid: leaves are usually entire instead of pinnatisect; the appendages of the involucral bracts are reduced to a strong spine scarcely fimbriate instead of being long pectinate and weakly spinescent. However, there are many morphological intermediates between 4*x* and 6*x* and the status of the hexaploid was considered uncertain (Fernández Casas & Susanna, 1986). Hexaploid *C. argecillensis* grows on limestone and clay soils like the tetraploid race and both ploidy levels even share part of their range (Fig. 1). The extreme morphologic variability and colonizing behavior suggest an allopolyploid origin.

For our study, we have included a wide representation of the three ploidy levels of *C. toletana*. In view of the possibilities of reticulation and introgression that previous studies have revealed, we have included too a representation of species of the *Acrocentron-Chamaecyanus* complex, both Iberian and North

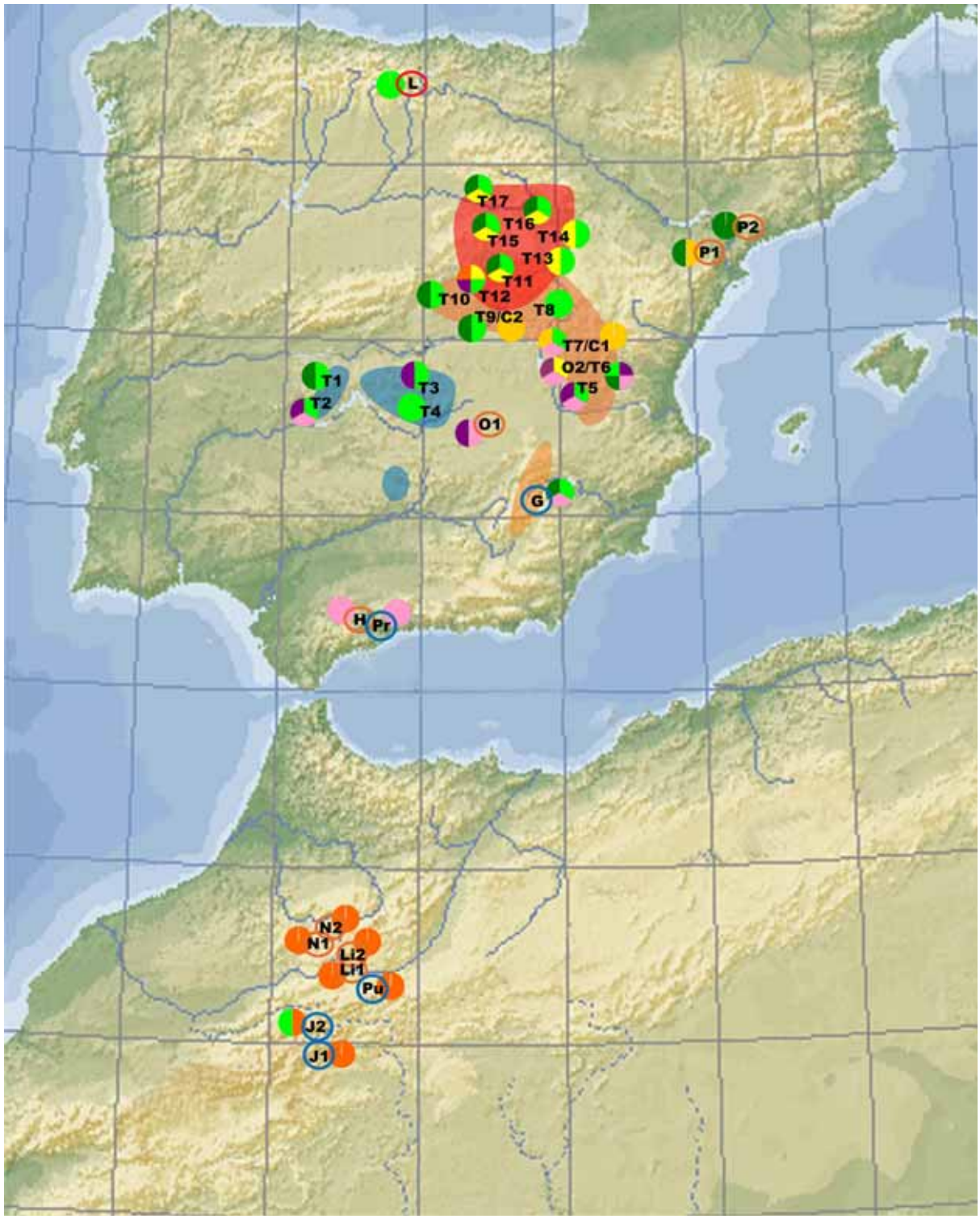

Figure 1. Map of the Iberian Peninsula and Northern Morocco with the distribution area of the polyploid levels of the *Centaurea toletana* complex: diploid *C. toletana* (blue), tetraploid *C. toletana* (orange), hexaploid *C. argecillensis* (red). Acronyms indicate the studied populations: populations T1-T4 (*C. toletana* 2x), T5-T10 (*C. toletana* 4x), T11 (*C. toletana* 4x-5x), T12-T17 (*C. argecillensis* 6x), C1-C2 (*C. cephalariifolia* 4x), O1-O2 (*C. ornata* 4x), P1-P2 (*C. podospermifolia* 4x), J1-J2 (*C. josiae* 2x), Li1-Li2 (*C. litardierei* 4x), N1-N2 (*C. nana* 4x), Pu (*C. pubescens* 2x), H (*C. haenseleri* 4x), Pr (*C. prolongi* 2x), G (*C. granatensis* 2x), L (*C. lagascana* 6x). Color charts indicate the ribotypes for each population: light green (I_T –Iberian toletana subtype), dark green (I_P -Iberian podospermifolia subtype), light yellow (R –Recombinant type), dark yellow (C – Cephalariifolia type), orange (A –African type), purple (O_I -Ornata Iberian subtype) and pink (O_A -Ornata African subtype).

African, with special emphasis in the species which presently share their area of distribution with *C. toletana*.

The objectives of this work are, using DNA sequences of three noncoding plastid regions (intron *rp*L16, the intergenic spacers *rp*S4-*trn*T-*trn*L and *ycf*3-*trn*S) and nuclear ribosomal internal and external transcribed spacers (ITS and ETS) regions, a) to elucidate the origin (allo- or auto-) of the tetraploid populations of *C. toletana*, b) to look for the origin of hexaploid *C. argecillensis*; and c) to examine the presence of gene flow, reticulation and introgression within the species of the complex.

Material and Methods

Plant material

Sampling focused on all the ploidy levels of the complex *C. toletana* (four diploid populations and six tetraploid and hexaploid populations). For each level, we included several populations representing geographical distribution. As potential parental species, most of the *Acrocentron* taxa growing in the Iberian Peninsula and North Africa were included in the study. In view of the difficult scenario in our preliminary screening, we included only one individual from each population. Outgroup was chosen among Eastern Mediterranean representatives of the *Acrocentron* group, on the basis of the results by Font *et al.* (2002). Voucher data and source are given in Table 1.

DNA Extraction, Amplification and Sequencing

Total genomic DNA was extracted following the CTAB method of Doyle & Doyle (1987) and Cullings (1992) from silica gel dried leaves collected in the field. In some cases, herbarium material was used.

Table 1. Origin of the material and herbaria where the vouchers are deposited.

Species and ploidy level	Acronym	Origin and herbaria
C. argecillensis 6*x*	T12	Spain, Guadalajara: between Ledanca and Argecilla, Garcia-Jacas & Susanna 2655, 7.06.06 (BC).
C. argecillensis 6*x*	T13	Spain, Soria: Velilla de Medinaceli, Garcia-Jacas & Susanna 2658, 7.06.06 (BC).
C. argecillensis 6*x*	T14	Spain, Soria: between Maján and Momblona, Garcia-Jacas & Susanna 2661, 7.06.06 (BC).
C. argecillensis 6*x*	T15	Spain, Soria: between Barahona and Marazovel, Garcia-Jacas & Susanna 2662, 8.06.06 (BC).
C. argecillensis 6*x*	T16	Spain, Soria: Rioseco de Soria, Garcia-Jacas & Susanna 2664, 8.06.06 (BC).
C. argecillensis 6*x*	T17	Spain, Soria: Santa María de la Hoyas, Garcia-Jacas & Susanna 2665, 8.06.06 (BC).
C. cephalariifolia 4*x*	C1	Spain, Cuenca: Tragacete, Garcia-Jacas & Susanna 2648, 7.06.06 (BC).
C. cephalariifolia 4*x*	C2	Spain, Guadalajara: between Brihuega and Torija, Garcia-Jacas & Susanna 2654, 7.06.06 (BC).
C. granatensis 2*x*	G	Spain, Granada: Sierra Guillimona, 1400-1500 m, Valdés et al. (SEV).
C. haenseleri 4*x*	H	Spain, Málaga: 10 km to Jubrique, Garcia-Jacas & Susanna 1888, 20.12.98 (BC).
C. josiae 2*x*	J1	Morocco: between the Gorges of the Dades and Agoudal, 2642 m, Romo 13878 & Vilatersana, 10.06.2006 (BC).
C. josiae 2*x*	J2	Morocco: Agoudal, 2368 m, Romo 13881 & Vilatersana, 10.06.2006 (BC).
C. lagascana 6*x*	L	Spain, Palencia: 500 m to Alba de los Cardaños, Font & Susanna 1822, 31.07.97 (BC).
C. litardierei 4*x*	Li1	Morocco: Oualegh, 1688 m, Romo 14049 & Vilatersana, 17.06.2006 (BC).
C. litardierei 4*x*	Li2	Morocco: between Oualegh and the Zad pass, 1889 m; Romo 14053 & Vilatersana, 1706.2006 (BC).
C. nana 4*x*	N1	Morocco: North of Aïn Leuh, 1499 m, Romo 14070 & Vilatersana, 19.06.2006 (BC).
C. nana 4*x*	N2	Morocco: South of Aïn Leuh, 1712 m, Romo 14074 & Vilatersana, 19.06.2006 (BC).
C. ornata 4*x*	O1	Spain, Toledo: Los Yébenes, Garcia-Jacas & Susanna 2638, 5.06.06 (BC).
C. ornata 4*x*	O2	Spain, Cuenca: Las Torcas de los Palancares, Garcia-Jacas & Susanna 2645, 6.06.06 (BC).
C. podospermifolia 4*x*	P1	Spain, Tarragona: Monte Caro, 1200 m, Susanna 2669 & Vilatersana, 3.10.06 (BC).
C. podospermifolia 4*x*	P2	Spain, Tarragona: Sierra de Cardó, 1000 m, Susanna 2671 & Vilatersana, 10.11.06 (BC).
C. prolongi 2*x*	Pr	Spain, Málaga: Sierra of Almijara, Frigiliana, 400-500 m Galbany & Arrabal, 4.07.04 (BC).
C. pubescens 2*x*	Pu	Morocco, Ksar es Souk: 2 km from Oued Amesheguir, Garnatje, Susanna 1795 & Vilatersana, 17.06.97 (BC).
C. toletana 2*x*	T1	Spain, Cáceres: Navalmoral, El Humilladero, Garcia-Jacas & Susanna 2630 4.06.06 (BC).
C. toletana 2*x*	T2	Spain, Cáceres: Cañamero mountain pass, 1061 m, Garcia-Jacas & Susanna 2631, 4.06.06 (BC).
C. toletana 2*x*	T3	Spain, Toledo: Risco de las Paradas, Garcia-Jacas & Susanna 2634, 5.06.06 (BC).
C. toletana 2*x*	T4	Spain, Toledo: San Pablo de los Montes, Garcia-Jacas & Susanna 2635, 5.06.06 (BC).
C. toletana 4*x*	T5	Spain, Cuenca: between Gabaldón and Almodóvar, Garcia-Jacas & Susanna 2642, 6.06.06 (BC).
C. toletana 4*x*	T6	Spain, Cuenca: Las Torcas de los Palancares, Garcia-Jacas & Susanna 2644, 6.06.06 (BC).
C. toletana 4*x*	T7	Spain, Cuenca: Tragacete, 1100 m, Garcia-Jacas & Susanna 2647, 6.06.06 (BC).

C. toletana 4*x*	T8	Spain, Cuenca: near Villanueva de Alcorón, Garcia-Jacas & Susanna 2650, 7.06.06 (BC).
C. toletana 4*x*	T9	Spain, Guadalajara: between Brihuega and Torija, Garcia-Jacas & Susanna 2653, 7.06.06 (BC).
C. toletana 4*x*	T10	Spain, Madrid: Redueña, 4 km to Torrelaguna, Font & Susanna 1819, 30.07.97 (BC).
C. toletana 4*x*-5*x*	T11	Spain, Guadalajara: Alcolea del Pinar, Garcia-Jacas & Susanna 2656, 7.06.06 (BC).
Outgroup		
C. aetholica 2*x*		Greece, Achaia: Antirion-Gavrolimni, Damboldt, 16.07.1976 (B).
C. kandevanensis 2*x*		Iran, Mazandaran: Chalus road, Baladeh, Garcia-Jacas, Mozaffarian, Susanna 1621 & Vallés, 1.07.96 (BC).
C. raphanina 2*x*		Greece, Chanion: Kydonias, mounts Levka, Strid 15093 & Papanicolau (B).

nrDNA ITS and ETS region amplification strategies: Double-stranded DNA of the ITS region was amplified using ITS1 as the forward primer and ITS4 as the reverse primer (White *et al*., 1990). The profile used for PCR amplification follows the protocol described in Susanna *et al*. (2006). The PCR products of ITS region were not cloned because we did not dectected double bands in any PCR products. The ETS region was amplified with ETS1F as the forward primer (Linder *et al*., 2000) and 18SETS as the reverse primer (Baldwin & Markos, 1998). The PCR was executed with the following conditions: 5 min denaturing at 95 ºC, followed by 30 cycles of 94 ºC denaturing for 45 s, 48 ºC annealing for 45 s and 72 ºC extension for 40 s, with an additional 7 min at 72 ºC. Contrarily to the ITS, many of the ETS PCR products exhibited double or even triple (pop. T12) bands. To verify whether the bands corresponded to different ETS copies, the PCR products of all the studied populations were cloned using TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, except that only half reactions were used. When possible, from eight to fifteen positive colonies from each reaction were screened with direct PCR using T7 and M13 universal primers following the protocol of Vilatersana *et al.* (2007). Eight to ten PCR products were selected for sequencing in both directions using the same primers.

cpDNA amplification strategies: Three noncoding regions were used. The intergenic spacer *rps*4-*trn*T-*trn*L was amplified using the primers rps4R2 as forward (Shaw *et al.*, 2005) and trnL-b as reverse (Taberlet *et al.*, 1991). The intergenic spacer *ycf*3-*trn*S was amplified with the primers SP43122F as

forward and the SP44097R as reverse (Hershkovitz, 2006).The *rp*L16 intron was amplified with the primers rpL16F71 (Jordan *et al.*, 1996) as forward and Rex2 (R. T. Kimball, University of Florida, pers. comm.) as reverse. The profile was the same for all of them and included a pre-heat step (95º for 1 min 35 s). Thirty-five cycles of amplification were carried out under the following conditions: 95 ºC for 1 min 30 s, 52 ºC for 1 min 30 s and 72 ºC for 2 min with an additional extension step of 10 min at 72 ºC.

nrDNA and cpDNA sequencing strategies: Chloroplast and nuclear PCR products were purified with the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Direct sequencing of the amplified DNA segments was performed using a BigDye Terminator Cycle Sequencing v3.1 (Applied Biosystems, Foster City, CA), following the manufacturer's the protocol. Direct sequencing of the amplified DNA segments was performed using a BigDye Terminator Cycle Sequencing v3.1 (Applied Biosystems, Foster City, CA), following the manufacturer's protocol. Nucleotide sequencing was performed on an ABI PRISM 3100 DNA Analyzer (Applied Biosystems, Foster City, CA). Unique substitutions in clones from one accession were excluded. Consensus sequences were generated for each accession and region, condensing the single base-pair differences among clones. This reduced the size of the matrices as well as the impact of PCR artifacts (chimerical sequences and Taq errors; Cline *et al.*, 1996; Popp & Oxelman, 2004).

Phylogenetic analyses

Sequences were aligned visually by sequential pairwise comparison (Swofford & Olsen, 1990). The data matrices are available on request from the corresponding author.

Parsimony analysis involved heuristic searches conducted with PAUP version 4.0b10 (Swofford, 2002) using tree-bisection-reconnection (TBR) branch swapping with character states specified as unordered and unweighted.

The indels were coded as presence/absence and added to the matrix. Preliminary analyses of the ETS clones matrix revealed that the tree limit of

PAUP was reached very quickly and thereafter we conducted a heuristic search with 1000 replicates and random taxon addition, saving only 500 trees per replicate. The tree limit of PAUP was not reached during the analysis of the combined plastid matrix and thus we carried a heuristic search with 100 replicates and random taxon addition without limiting the number of saved trees. Bootstrap analysis (Felsenstein, 1985) was performed using heuristic search with 100 replicates and the default options. For the ETS dataset, bootstrap analysis (BS) followed the approach by Lidén *et al.* (1997) using 1000 replicates, random taxon addition with 10 replicates, and no branch swapping.

RDP2 analysis

For verifying whether sequences of the clones were recombinant, we performed a RDP2 analysis (Martin *et al.*, 2005) using the default settings of the package.

Results

ITS sequences

Table 2 shows the variable nucleotide sites for the ITS sequences. The only sequences with polymorphic positions (which usually indicate hybridization) are tetraploid populations of *C. toletana* T5, T6, T7 and T8, and the population O2 of *C. ornata*. Diploid populations of *C. toletana* show two types of sequences, one in populations T1 and T2 from the western extreme of its range, and another one in populations T3 and T4 from the eastern range.

Tetraploid populations of *C. toletana* show most of detected polymorphic sites, indicating genetic interchange. Polymorphisms in tetraploid populations T5, T6 and T7 mark the two ribotypes of the diploid populations (positions 26 and 599, Table 2). Other polymorphic positions correspond to additivity between *C. toletana* and *C. ornata/prolongi/haenseleri* (positions 28 and 123).

All the populations of hexaploid *C. argecillensis* have identical sequences, with a unique insertion at the position 463. *Centaurea granatensis*, *C. lagascana* and *C. podospermifolia* have only one change regarding the ITS sequence of *C. toletana*. *Centaurea ornata*, *C. prolongi* and *C. haenseleri* also share the ITS sequences, with the same changes. *Centaurea cephalariifolia*

Table 2. Variable polymorphic positions of the ITS region of all studied taxa. A+G= R, A+C= M, A+T= W, G+T= K, G+C= S and C+T= Y.

Populations	Polymorphic position																											
	13	26	28	42	62	88	91	113	116	122	123	190	258	267	301	337	424	456	458	462	463	477	479	483	507	591	529	599
C. toletana T1 2x	C	C	T	C	A	T	T	A	T	C	G	A	A	C	G	G	C	A	T	T	—	G	C	G	T	A	G	C
C. toletana T2 2x	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	—	·	·	·	·	·	·	·
C. toletana T3 2x	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	—	·	·	·	·	·	·	A
C. toletana T4 2x	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	—	·	·	·	·	·	·	A
C. toletana T5 4x	·	Y	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	—	·	·	·	·	·	R	M
C. toletana T6 4x	·	·	Y	·	R	·	·	·	·	·	R	·	·	·	·	·	·	·	W	·	—	·	·	·	·	·	·	M
C. toletana T7 4x	·	·	Y	·	R	·	Y	·	·	·	·	·	·	·	·	·	·	·	·	·	—	·	·	·	·	·	R	M
C. toletana T8 4*x*	·	·	·	·	R	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	—	·	·	·	·	·	R	·
C. toletana T9 4*x*	·	Y	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	—	·	·	·	·	·	·	·
C. toletana T10 4*x*	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	—	·	·	·	·	·	·	G
C. toletana T11 4*x*—5*x* ?	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·
C. toletana T12 6*x*	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	K	·	·	·
C. toletana T13 6*x*	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·
C. toletana T14 6*x*	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·
C. toletana T15 6*x*	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·
C. toletana T16 6*x*	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·
C. toletana T17 6*x*	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·
C. podospermifolia P1 4*x*	A	·	·	·	·	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	—	·	·	·	·	·	·	·
C. podospermifolia P2 4*x*	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	—	·	·	·	·	·	·	·
C. granatensis G 2*x*	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	—	·	·	·	·	·	·	·
C. lagascana L 6*x*	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·	·	·	—	·	·	·	·	·	·	·
C. haenseleri H 4*x*	·	·	·	·	G	·	·	·	·	·	A	·	·	Y	·	·	·	·	·	·	—	·	·	·	·	·	·	·
C. prolongi Pr 2*x*	·	·	C	·	G	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	—	·	·	·	·	·	·	·
C. ornata O1 4*x*	·	·	C	·	G	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	—	·	·	·	·	·	·	·
C. ornata O2 4*x*	·	·	Y	·	R	·	·	·	·	·	R	·	·	·	R	R	·	·	·	·	—	·	·	·	·	·	·	·
C. josiae J1 2*x*	·	·	·	·	G	·	·	·	·	·	·	T	·	·	·	N	·	·	·	—	—	·	·	·	G	·	·	·
C. josiae J2 2*x*	·	·	·	·	G	·	·	·	·	·	·	T	·	·	·	·	·	·	·	—	—	·	·	·	G	·	·	·
C. litardierei Li1 4*x*	·	·	·	·	G	·	·	·	·	·	·	T	·	·	·	A	·	·	·	—	—	·	·	S	G	·	·	·
C. litardierei Li2 4*x*	·	·	·	·	G	·	·	·	·	·	·	T	·	·	·	·	·	·	·	—	—	·	·	S	G	·	·	·
C. nana N1 4*x*	·	·	·	·	G	·	·	·	·	Y	·	T	·	·	R	·	·	·	·	—	—	·	·	·	G	G	·	·
C. nana N2 4*x*	·	·	·	·	G	·	·	·	·	Y	·	T	·	·	R	·	·	·	·	—	—	·	·	·	G	·	·	·
C. pubescens Pu 2*x*	·	·	·	·	G	·	·	·	·	·	·	T	·	·	·	·	·	·	·	—	—	·	·	·	G	G	·	·
C. cephalariifolia C1 4*x*	·	·	·	·	G	·	C	·	A	·	·	·	·	·	·	·	·	G	G	—	—	T	Y	·	G	·	·	·
C. cephalariifolia C2 4*x*	·	·	·	·	G	·	C	·	A	·	·	·	·	·	·	·	·	G	G	—	—	T	Y	·	G	·	·	·
C. aetholica	·	·	·	T	G	C	C	G	·	·	·	·	T	·	·	·	·	G	G	—	—	·	·	A	G	·	·	·
C. raphanina	·	·	·	T	G	C	C	G	·	·	·	·	T	·	·	·	·	G	G	—	—	T	·	·	G	·	·	·
C. kandavanensis	·	·	·	·	G	C	C	·	·	·	·	·	·	·	·	·	M	·	G	—	—	·	A	·	G	·	·	·

shows changes that relate it to the east Mediterranean species used as outgroup (*C. aetholica*, *C. kandavanensis* and *C. raphanina*). African *C. josiae*, *C. litardierei*, *C. nana* and *C. pubescens* form a group with a shared change (position 190).

Phylogenetic analysis of the ETS cloned sequences

Figure 2 shows the strict consensus of 266,500 most parsimonious trees 468 steps long resulting from the analysis of the ETS matrix (1341 characters –192 informative characters- and 128 clones). Support for the nodes of the tree is very low and we shall comment only a few supported clades and the general topology of the tree. The Iberian-North-African group is monophyletic with high bootstrap support (BS= 97%). There is a group with moderate support (BS= 78%) formed by most of the clones of the north African species, with the addition of one clone of hexaploid *C. argecillensis* from population T12. The two populations of *C. cephalariifolia* are grouped in strongly supported clade (BS= 96%) with all the clones of *C. podospermifolia* from population P1 and one clone of population T7 of tetraploid *C. toletana*. The rest of the tree shows a very large polytomy moderately supported (BS= 80%) encompassing the Iberian clones, with the inclusion of the consensus clone of African *C. josiae* (pop. J2).

Based on the polymorphic positions of the cloned sequences (Table 3), the results of the phylogenetic analysis (Fig. 2) and the topology of the ETS sequences (Fig. 3), seven different ribotypes can be defined (Table 4).

The first two types are the very similar ribotypes Iberian-*toletana* (I_T) and Iberian-*podospermifolia* (I_P), which appear almost identical in Table 3. Ribotype I_T is present in all the populations of *C. toletana*, *C. lagascana* and *C. granatensis*. Ribotype I_p characterizes *C. podospermifolia* (I_P). The third type, very similar to the previous ones (Table 3), is one of the types found in *C. ornata* (O_I). The similarities of these three types are confirmed by the phylogenetic analysis that groups all of them in a polytomy (Figs. 2 and 3a).

The fourth ribotype is the recombinant type R that is defined based on the topology of the ETS region (Fig. 3d). Type R characterizes all the studied

Table 3. Variable polymorphic positions of the ETS region of some representative taxa from each ribotype. Populations are: T1b, T2b, T1a = *C. toletana*, P2 = *C. podospermifolia*, O1b = *C. ornata*, Li1 = *C. litardierei*, Pr = *C. prolongi*, C2 = *C. cephalariifolia* and *C. kandavan*. = *C. kandavanensis* . Ribotypes are: O_I = *C. ornata*, I_P = Iberian *C. podospermifolia*, I_T = Iberian *C. toletana*, C = *C. cephalariifolia*, O_A = African *C. ornata*, A = African.

Populations and individuals	Ribotype	Polymorphic positions																																										
		4	5	6	7	9	11	13	15	16	17	18	19	20	23	27	31	33	34	37	38	41	46	52	53	59	62	63	67	68	69	70	71	72	74	80	82	85	86	89	90	99	101	111	113
T1b	I_T	C	C	C	T	A	T	G	C	C	A	T	A	C	C	A	G	C	A	G	C	C	T	G	C	A	T	T	—	C	T	C	T	C	C	A	G	T	A	A	T	A	C	—	C
T2b	I_T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	T	.	.	.	A	.	T	.	.	.	.	.	.	.	.	—	.
T1a	I_T	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	—	.	.	.	.	.	.	.	.	.	.	.	.	.	.	—	.
P2	I_P	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	—	.
O1b	O_I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	—	.
Li1	A	G	.	A	.	G	C	—	Y	T	G	C	T	T	.	.	.	.	C	A	.	.	.	.	.	T	.	C	—	—	—	—	.	T	.	C	A	A	T	.	.	G	T	T	.
Pr	O_A	.	.	.	.	A	.	G	T	.	G	C	.	.	.	.	.	G	.	A	T	A	.	.	T	T	.	C	—	—	—	—	.	T	.	C	A	A	T	.	.	G	T	T	.
C2	C	G	A	T	A	A	.	—	T	T	G	A	C	.	.	.	.	.	.	A	.	.	C	.	.	T	C	G	—	—	.	.	.	.	.	T	G	A	T	T	C	G	T	—	T
C. raphanina OUT.		G	.	A	.	A	.	G	T	T	.	.	.	.	.	.	.	.	.	A	.	Y	.	.	.	T	.	C	—	—	—	—	.	.	.	C	A	A	T	.	.	G	T	—	.
C. kandavan. OUT.		.	.	A	.	A	.	G	T	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	.	C	—	—	—	—	.	A	.	C	A	A	T	.	C	G	T	—	.

Populations and individuals	Ribotype	Polymorphic positions																																									
		559	560	562	563	564	565	566	569	570	571	572	573	576	578	581	582	591	594	607	613	620	624	625	633	634	641	642	643	658	659	661	666	671	673	674	676	679	680	681	684	687	697	704	705
T1b	I_T	T	G	G	T	G	T	G	G	C	C	C	G	C	T	T	T	C	T	T	C	C	G	T	T	G	T	G	C	T	T	C	T	C	T	A	C	G	C	T	T	C	G	G	G
T2b	I_T	.	T	C	A	.	C	.	.	.	.	.	.	T	—	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	G	C	.	.	.	.
T1a	I_T	.	.	.	.	.	C	.	.	.	.	.	.	.	—	.	.	.	—	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	.	.
P2	I_P	.	.	.	.	.	C	.	.	.	.	.	.	.	—	.	.	.	.	.	.	.	.	.	.	.	C	.	T	.	.	.	.	.	.	G	.	.	.	G	.	T	.	.	.
O1b	O_I	A	.	.	.	.	C	C	.	.	.	.	.	.	—	.	.	.	.	C	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	G	.	.	.	C	C
Li1	A	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	.	.	.	.	A	T	.	.	.	.	A	.	G	G	T	.	T	G	G	.	.	.	G	.	.	.	.	.
Pr	O_A	A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	T	.	.	.	A	T	.	.	.	.	A	.	G	G	T	.	T	G	G	.	.	.	G	.	.	.	.	.
C2	C	C	.	.	.	.	C	A	.	T	G	G	.	.	—	G	C	.	.	.	.	.	.	A	.	C	.	.	.	.	G	.	.	.	.	G	.	.	.	G	C	.	.	.	.
C. raphanina OUT.		—	.	.	.	T	C	A	.	.	.	.	T	.	—	G	C	.	.	.	.	.	C	.	.	.	.	.	.	.	S	.	C	T	G	G	T	T	T	.	C	.	.	.	.
C. kandavan. OUT.		.	.	.	.	T	C	A	T	.	.	.	T	.	—	G	C	.	.	.	.	.	T	.	.	.	.	.	.	G	G	.	.	T	G	G	T	.	T	G	.	.	.	.	.

Populations and individuals	Ribotype	Polymorphic positions																																	
		748	755	769	794	801	802	803	815	834	850	852	861	876	888	895	899	902	906	915	919	929	940	941	945	951	965	968	986	1007	1027	1038	1040	1043	1069
T1b	I_T	T	T	C	G	T	T	G	G	T	G	T	G	T	T	G	T	T	G	A	T	T	T	C	C	C	A	T	T	C	T	C	T	C	C
T2b	I_T	.	.	.	.	.	.	.	.	C	A	A	.	.	.	.	.	C	A	G	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.
T1a	I_T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	C	.	A	.	.	G	.	.	.	.	.	.	.	T
P2	I_P	.	.	.	.	—	—	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
O1b	O_I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	A	C	.	.	.	.	.	.
Li1	A	.	C	.	.	.	.	.	.	.	.	A	.	.	.	.	.	C	A	G	.	.	.	.	.	T	.	.	.	.	C	.	.	T	T
Pr	O_A	.	C	T	.	.	.	.	.	.	.	A	.	.	.	A	.	C	A	G	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.
C2	C	.	C	.	.	.	.	T	C	.	.	.	A	.	C	.	.	C	A	G	.	.	C	.	.	.	.	.	.	.	.	.	.	T	.
C. raphanina OUT.		G	C	.	A	.	.	.	.	.	.	.	.	A	.	.	G	.	.	.	.	.	G	.	.	.	.	.	.	.	.	T	C	T	T
C. kandavan. OUT.		.	C	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.	.	T	.

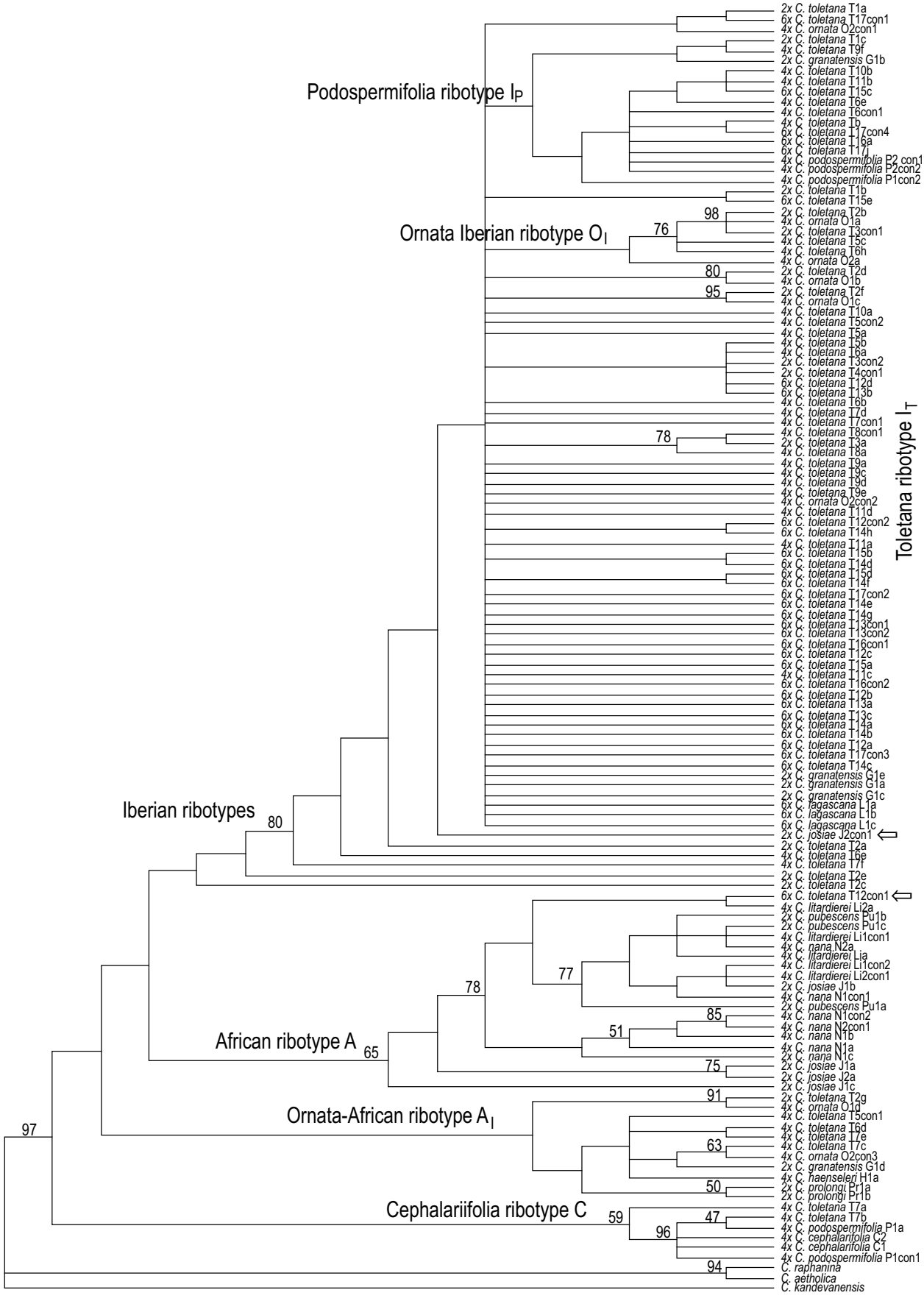

Figure 2. Majority rule consensus tree of 266500 most parsimonius trees. Numbers above branches are bootstrap values. Arrows indicate Iberian and African populations nested in the African and the Iberian clades respectively.

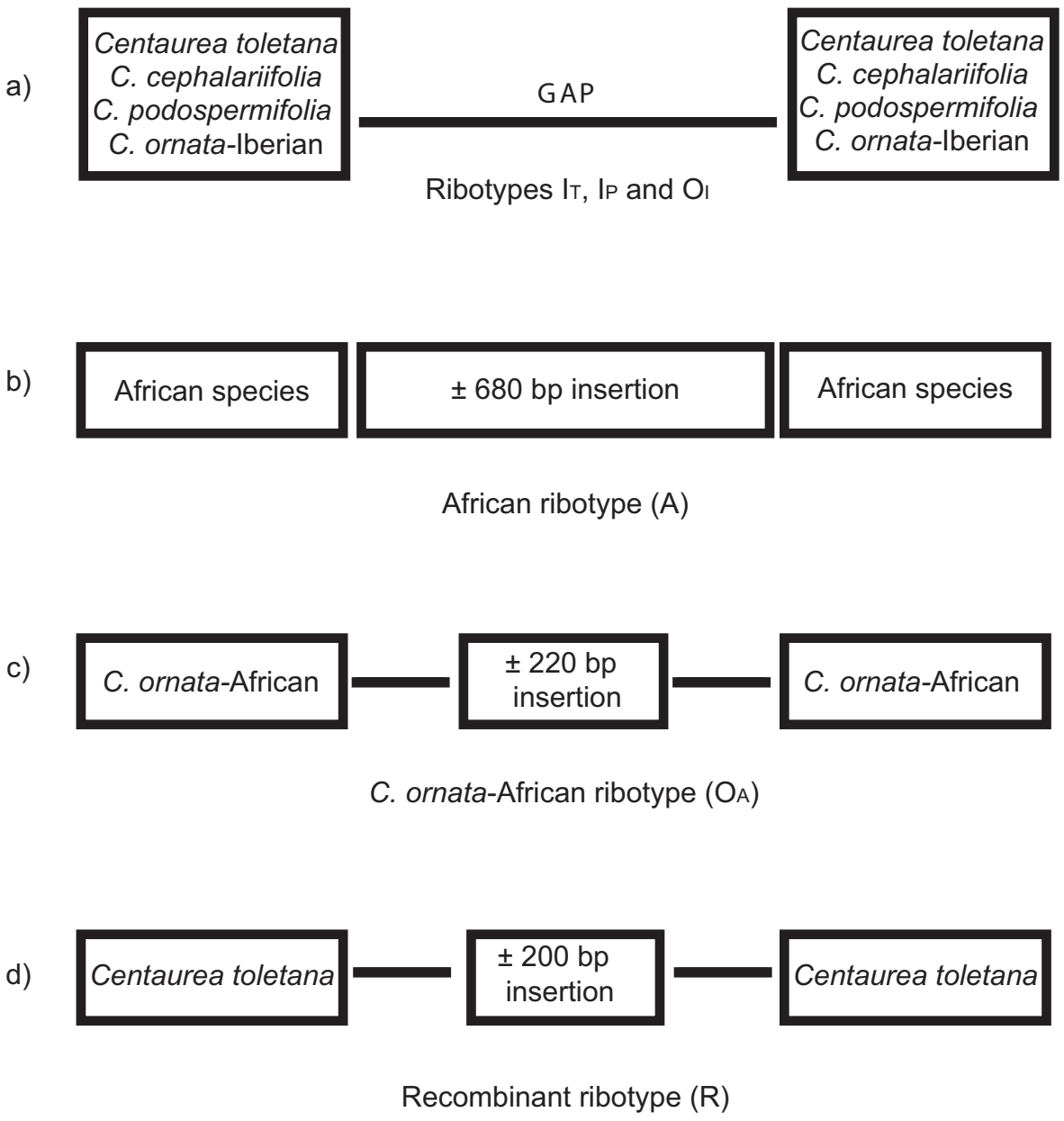

Figure 3. Topology of the ETS ribotypes.

populations of hexaploid *C. argecillensis* (T11-17) and population O2 of *C. ornata*. As shown in the Fig. 3d, the 5' and 3' ends are very similar to the type I_T of *C. toletana*, but the centre of the sequence has a large insertion that relates it to the ribotypes of the African species (Fig. 3b).

The fifth ribotype is the African version of the ribotype of *C. ornata* (O_A). As it was the case with the R ribotype, O_A type shows a large insertion in the middle of the ETS region that relates it to the African type (Fig. 3c). The sixth ribotype is the African type (A), which is shared by all the studied North-African species (Fig. 3b). In the phylogenetic analysis, ribotypes A and O_A form a clade well segregated from the polytomy of the Iberian ribotypes (Fig. 2). Finally, the seventh ribotype (C) is exclusive of *C. cephalariifolia* (Table 3).

The distribution of the different ribotypes is shown in Fig. 1. Most of the populations show more than one ribotype, even the diploid ones.

Recombinant sequences in the ETS region: RDP2 analysis detected some recombinant sequences, most of them involving clones of *C. toletana*. One of the sequences of hexaploid *C. argecillensis* (pops. T12 and T13) were identified as potential recombinants of one sequence from *C. toletana* 4*x* (pop. T7) and another from *C. podospermifolia* (pop. P2).

Plastid sequences

The results of the sequences of the three plastid markers are shown in Table 5. We have detected very few nucleotide sites for defining haplotypes. Maybe the most interesting result is that populations of the *C. toletana* complex do not share the same haplotype, because there are slight differences. The same occur for the rest of species in which we have studied more than one population (with the only exception of *C. podospermifolia*).

Population J2 of north African *C. josiae* shares its haplotype with two populations of *C. toletana*, one diploid (T4) and one tetraploid (T5). The second studied population of *C. josiae* (J1) has the same haplotype as two populations of the other African taxa, *C. nana* (N2) and *C. litardierei* (Li1); populations Li2 of *C. litardierei* and N2 of *C. nana* share another haplotype (Table 5). Phylogenetic analysis (not shown) does not offer any resolution, and even eastern

Mediterranean species that we have used as outgroups show great similarity to the ingroup.

Table 4. Ribotypes of all the studied taxa based on the topology of the ETS sequences and the results of the parsimony analysis. Ribotypes are: O_I = *C. ornata*, I_P = Iberian *C. podospermifolia*, I_T = Iberian *C. toletana*, C = *C. cephalariifolia*, O_A = African *C. ornata*, A = African and R = recombinant

Population	Ribotypes						
	O_I	**I_P**	**I_T**	**C**	**O_A**	**A**	**R**
C. toletana T1 **2*x***		I_P	I_T				
C. toletana T2 **2*x***	O_I		I_T		O_A		
C. toletana T3 **2*x***	O_I		I_T				
C. toletana T4 **2*x***			I_T				
C. toletana T5 **4*x***	O_I		I_T		O_A		
C. toletana T6 **4*x***	O_I	I_P	I_T		O_A		
C. toletana T7 **4*x***			I_T	C	O_A		
C. toletana T8 **4*x***			I_T				
C. toletana T9 **4*x***		I_P	I_T				
C. toletana T10 **4*x***		I_P	I_T				
C. toletana T11 **4*x*-5*x***		I_P	I_T				R
C. toletana T12 **6*x***	O_I		I_T			A	R
C. toletana T13 **6*x***			I_T				R
C. toletana T14 **6*x***			I_T				R
C. toletana T15 **6*x***		I_P	I_T				R
C. toletana T16 **6*x***		I_P	I_T				R
C. toletana T17 **6*x***		I_P	I_T				R
C. podospermifolia P1 **4*x***		I_P		C			
C. podospermifolia P2 **4*x***		I_P					
C. lagascana L **6*x***	O_I		I_T				
C. granatensis G **2*x***		I_P	I_T		O_A		
C. haenseleri H **4*x***					O_A		
C. prolongi Pr **2*x***					O_A		
C. ornata O1 **4*x***	O_I				O_A		
C. ornata O2 **4*x***	O_I				O_A		R
C. cephlariifolia C1 **4*x***				C			
C. cephlariifolia C2 **4*x***				C			
C. litardierei Li1**4*x***						A	
C. litardierei Li2 **4*x***						A	
C. nana N1 **4*x***						A	
C. nana N2 **4*x***						A	
C. josiae J1 **2*x***						A	
C. josiae J2 **2*x***	O_I					A	
C. pubescens Pu **2*x***						A	

Table 5. Variable polymorphic positions of the three plastid regions of all studied taxa.

Populations	Polymorphic position																																		
	73	129	357	455	670	762	763	764	889	913	1119	1144	1145	1147	1253	1371	1372	1441	1629	1631	1632	1637	1653	1656	1667	1671	1706	1790	1937	1972	2063	2134	2173	2250	2441	2442
C. toletana T1 2*x*	G	T	G	T	G	T	—	—	T	C	T	T	A	A	—	—	—	A	—	C	T	T	—	—	G	A	T	T	C	A	C	A	A	C	T	G
C. toletana T2 2*x*	·	·	·	·	·	·	—	—	·	·	·	·	·	·	—	—	—	·	—	·	·	·	—	—	·	·	·	·	·	·	·	·	·	·	·	·
C. toletana T3 2*x*	·	·	·	·	·	·	T	—	·	·	·	·	·	·	—	—	—	·	—	·	·	A	A	—	·	·	·	·	·	·	·	·	·	·	·	·
C. toletana T4 2*x*	A	·	·	·	·	—	—	—	·	·	G	·	·	·	—	—	—	·	T	·	·	·	A	—	·	·	C	·	·	·	·	·	G	·	·	·
C. toletana T5 4*x*	A	·	·	·	·	—	—	—	·	·	G	·	·	·	—	—	—	·	T	·	·	·	A	—	·	·	C	·	·	·	·	·	G	·	·	·
C. toletana T6 4*x*	·	·	·	·	·	·	—	—	·	·	·	·	·	·	T	—	—	·	—	·	·	·	A	—	·	·	·	·	·	·	·	·	G	·	·	·
C. toletana T7 4*x*	·	·	·	·	T	·	—	—	N	·	·	·	·	·	—	—	—	·	—	·	·	·	A	—	·	·	·	·	·	C	·	·	·	·	·	·
C. toletana T8 4*x*	·	·	·	·	·	—	—	—	·	·	·	·	·	·	T	—	—	·	—	·	·	·	A	—	·	·	·	·	·	·	·	·	·	·	·	A
C. toletana T9 4*x*	·	·	·	·	T	·	—	—	C	·	·	·	·	·	—	—	—	·	—	·	·	·	A	—	·	·	·	·	·	·	·	·	·	·	·	·
C. toletana T11 4*x*—5*x* ?	·	·	·	·	·	—	—	—	·	·	·	·	·	·	T	—	—	·	—	·	·	·	A	—	·	·	·	·	T	·	T	·	·	·	·	·
C. toletana T12 6*x*	·	·	·	—	·	·	—	—	·	·	·	·	·	·	T	—	—	G	—	—	—	·	A	—	·	·	·	·	·	·	·	·	·	·	·	A
C. toletana T13 6*x*	·	·	·	·	·	·	—	—	·	·	·	·	·	·	—	—	—	·	T	·	·	·	A	—	A	·	·	·	·	·	·	·	·	·	·	·
C. toletana T14 6*x*	·	·	·	—	·	·	—	—	·	·	·	·	·	·	T	—	—	G	—	—	—	A	A	—	·	·	·	·	·	·	T	·	·	·	·	·
C. toletana T15 6*x*	·	·	·	·	·	—	—	—	·	·	·	·	·	·	—	—	—	·	—	·	·	·	A	—	·	·	·	·	A	·	·	·	·	·	·	·
C. toletana T16 6*x*	·	·	·	·	·	·	—	—	·	·	·	·	·	·	—	—	—	·	—	·	·	·	A	—	·	·	·	·	·	·	·	·	·	·	·	·
C. toletana T17 6*x*	·	·	·	·	·	—	—	—	·	·	·	·	·	·	T	—	—	·	—	·	·	·	A	—	·	·	·	·	·	·	·	·	·	·	·	A
C. podospermifolia P1 4*x*	·	·	·	·	·	·	T	—	·	T	·	·	·	·	—	—	—	·	—	·	·	·	A	—	A	·	·	·	·	·	·	·	·	·	·	·
C. podospermifolia P2 4*x*	·	·	·	·	·	·	T	—	·	T	·	·	·	·	—	—	—	·	—	·	·	·	A	—	A	·	·	·	·	·	·	·	·	·	·	·
C. granatensis G 2*x*	·	—	A	—	·	—	—	—	·	·	·	·	·	·	—	—	—	·	—	·	·	·	A	—	·	·	·	·	·	T	·	·	·	·	·	·
C. ornata O1 4*x*	·	·	·	·	T	·	T	—	C	·	·	·	·	·	—	—	—	·	—	·	·	·	A	—	·	·	·	·	·	·	·	·	·	·	·	·
C. ornata O2 4*x*	·	·	·	·	·	—	—	—	C	·	·	·	·	·	—	—	—	·	—	·	·	·	A	—	·	·	·	·	·	·	·	·	·	·	·	·
C. cephalariifolia C1 4*x*	·	·	T	·	·	·	T	T	·	·	·	·	·	·	—	—	—	·	—	·	·	·	A	—	·	·	·	·	·	·	·	·	·	·	·	·
C. cephalariifolia C2 4*x*	·	·	·	·	T	·	—	—	C	·	·	G	·	·	—	—	—	·	—	·	·	·	A	—	·	·	·	G	·	·	·	·	·	·	·	·
C. josiae J1 2*x*	·	·	·	·	·	·	—	—	·	·	·	C	T	T	—	—	—	·	—	·	·	·	A	—	·	·	·	G	·	·	·	·	·	·	G	·
C. josiae J2 2*x*	A	·	·	·	·	—	—	—	·	·	G	·	·	·	—	—	—	·	T	·	·	·	A	—	·	·	C	·	·	·	·	·	G	·	·	·
C. litardierei Li1 4*x*	·	·	·	·	·	·	—	—	·	·	·	C	T	T	—	—	—	·	—	·	·	·	A	—	·	·	·	G	·	·	·	·	·	·	G	·
C. litardierei Li2 4*x*	·	G	·	·	·	·	—	—	·	·	·	·	·	·	—	—	—	·	—	·	·	·	A	A	·	·	·	·	·	·	·	·	·	T	·	·
C. nana N1 4*x*	·	·	·	·	·	·	—	—	·	·	·	C	T	T	—	—	—	·	—	·	·	·	A	—	·	·	·	G	·	·	·	·	·	·	G	·
C. nana N2 4*x*	·	G	·	·	·	·	—	—	·	·	·	·	·	·	—	—	—	·	—	·	·	·	A	A	·	·	·	·	·	·	·	·	·	T	·	·
C. aetholica	·	·	·	·	·	·	—	—	·	·	·	·	·	·	—	T	C	·	—	·	·	·	A	—	·	·	·	·	·	·	·	·	·	·	·	·
C. raphanina	·	·	·	·	·	·	T	T	·	·	·	·	·	·	—	T	C	·	—	·	·	·	A	—	·	T	·	·	·	·	·	C	·	·	·	·
C. kandavanensis	·	·	·	·	·	·	—	—	·	·	·	·	·	·	—	T	C	·	—	·	·	·	A	—	·	T	·	·	·	·	·	C	·	·	·	·

Discussion

No one of the markers used in this study show a correlation with the systematics of the group. Species of *Acrocentron-Chamaecyanus* can not be characterized on molecular basis, which reinforces the hypothesis of extensive hybridation in the group as suggested by Font *et al.*, (2002).

Nuclear-Ribosomal DNA Evolution:

Regarding DNA evolution, the ITS spacers do not show the amount of polymorphisms that could be expected in a group with this level of hybridization: only a few positions show nucleotide additivity (Table 2), as already observed in other highly hybridized groups like *Armeria* (Gutiérrez Larena *et al.*, 2002) and *Centaurea* sect. *Willkommia* (Suárez-Santiago *et al.*, 2007). In contrast, almost every cloned individual conserves different copies of the ETS region (Fig. 1). This is a new example of the different way in which concerted evolution affects the nuclear-ribosomal spacers. In our case, concerted evolution biased towards one of the parental genomes must have been the rule for the ITS region, whilst the ETS region shows lower levels of concerted evolution.

Distribution of the ribotypes and gene flow

Diploid populations: maybe the most unexpected result of the molecular survey is the presence of three alien genomes in diploid populations of *C. toletana*. Diploid *C. toletana* accumulates four different ribotypes (Table 4): the *C. toletana* ribotype (I_T), the two ribotypes of *C. ornata* (O_I and O_A) and the ribotype of *C. podospermifolia* (I_p). Even though morphological differences between C*. toletana* and *C. podospermifolia* or *C. ornata* are obvious or even extreme, none of these crosses has left any morphological trait. However, hybrids in the *Acrocentron* group and especially those between *Acrocentron* and *Chamaecyanus* usually have intermediate characters (Fernández Casas & Susanna, 1986; Garcia-Jacas & Susanna, 1994). Repeated introgression with one of the parental species (*C. toletana* in this case) explains the disappearing of the morphology of one of the progenitors. Introgression implies homoploid hybridization, as is usually the case in *Acrocentron* (Table 6). This process –

homoploid hybridization followed by introgression- has been demonstrated in other cases (McKinnon, 2005).

The presence of a *C. podospermifoli*a ribotype in a diploid population of *C. toletana* poses a curious problem, because *C. podospermifolia* is presently tetraploid (Fernández Casas & Susanna, 1986). Certainly, the *C. podospermifolia* population implied in this hybridization must have been the diploid progenitors of the extant tetraploid.

Table 6. Homoploid hybrids within sections *Chamaecyanus* and *Acrocentron*

Species and chromosome number	Parental 1	Parental 2	Reference
Centaurea x ceballosii Fern. Casas 2n=40	*C. toletana* 2n=40	*C. cephalariifolia* 2n=40	Fernández Casas & Susanna (1986)
Centaurea x cephalariseptimae Fern. Casas & Susanna 2n=40	*C. cephalariifolia* 2n=40	*C. legionis-septimae* 2n=40	Fernández Casas & Gamarra (1986)
Centaurea x loscosii Willk. 2n=40	*C. podospermifolia* 2n=40	*C. cephalariifolia* 2n=40	Fernández Casas & Susanna (1986)
Centaurea polymorpha Lagasca 2n=40	*C. cephalariifolia* 2n=40	*C. ornata* 2n=40	Fernández Casas & Gamarra (1987)
Centaurea x tatayana Fern. Casas & Susanna 2n=40	*C. toletana* 2n=40	*C. ornata* 2n=40	Kummer (1977)

The shift of the hybrids towards a *C. toletana* morphology by introgression is indirectly supported by two cases of old hybridogenic allopolyploid species from south Iberia originated by crosses of sect.

Acrocentron x sect. *Chamaecyanus*. *Centaurea saxifraga* Coincy from South Spain is an hexaploid with 2n= 60 originated by a cross of diploid *C. mariana* Nyman from sect. *Chamaecyanus* and tetraploid *C. ornata* from sect. *Acrocentron*; *Centaurea crocata* Franco from Portugal is a tetraploid originated by the cross of diploid *C. prolongi* from sect. *Acrocentron* and an unknown species of *Chamaecyanus* also diploid (Garcia-Jacas & Susanna, 1992, 1994). In both cases, genetic isolation by polyploidy has precluded introgression with any of the progenitors, and the hybridogenic species keep some intermediate characters between *Acrocentron* and *Chamaecyanus*.

The origin of the tetraploid: Tetraploid *C. toletana* is taxonomically an autopolyploid because it cannot be distinguished morphologically from the diploid. Even though this is theoretically not enough reason for a diagnostic of autopolyploidy according to Rieseberg (1995), it is still a strong argument. However, the studied populations show five different ribotypes, four of which were already present in the diploid populations (genomes I_T, O_I, O_A and I_P, Table 4). The fifth one, *C. cephalariifolia* (C), was detected in one population. It is a good question whether *C. toletana* (4*x*), with five alien genomes, can be considered an autopolyploid. However, our results confirm that it was originated from diploid *C. toletana*, which has added to the gene pool of the tetraploid other genomes already present by previous hybridization. At least in one population, a new hybridization with *C. cephalariifolia* took place; again, followed by introgression, because morphology is pure *C. toletana*. We have represented this process in Fig. 4a. This extremely complicated example supports Wendel & Doyle (2005): maybe it is impossible to pigeonhole polyploidy just in auto- or allopolyploidy. Regarding the possibility of multiple origins of the tetraploid, we have not detected different copies of the ITS region in any population, which does not necessarily imply that they are not present. However, the ITS sequences show some hints that this was the case. There is a polymorphism in the 3' end of the ITS region that segregates diploid populations of *C. toletana* in two types: populations T1 and T2 (from the westernmost extreme of the area) have a C in the position 599, and populations

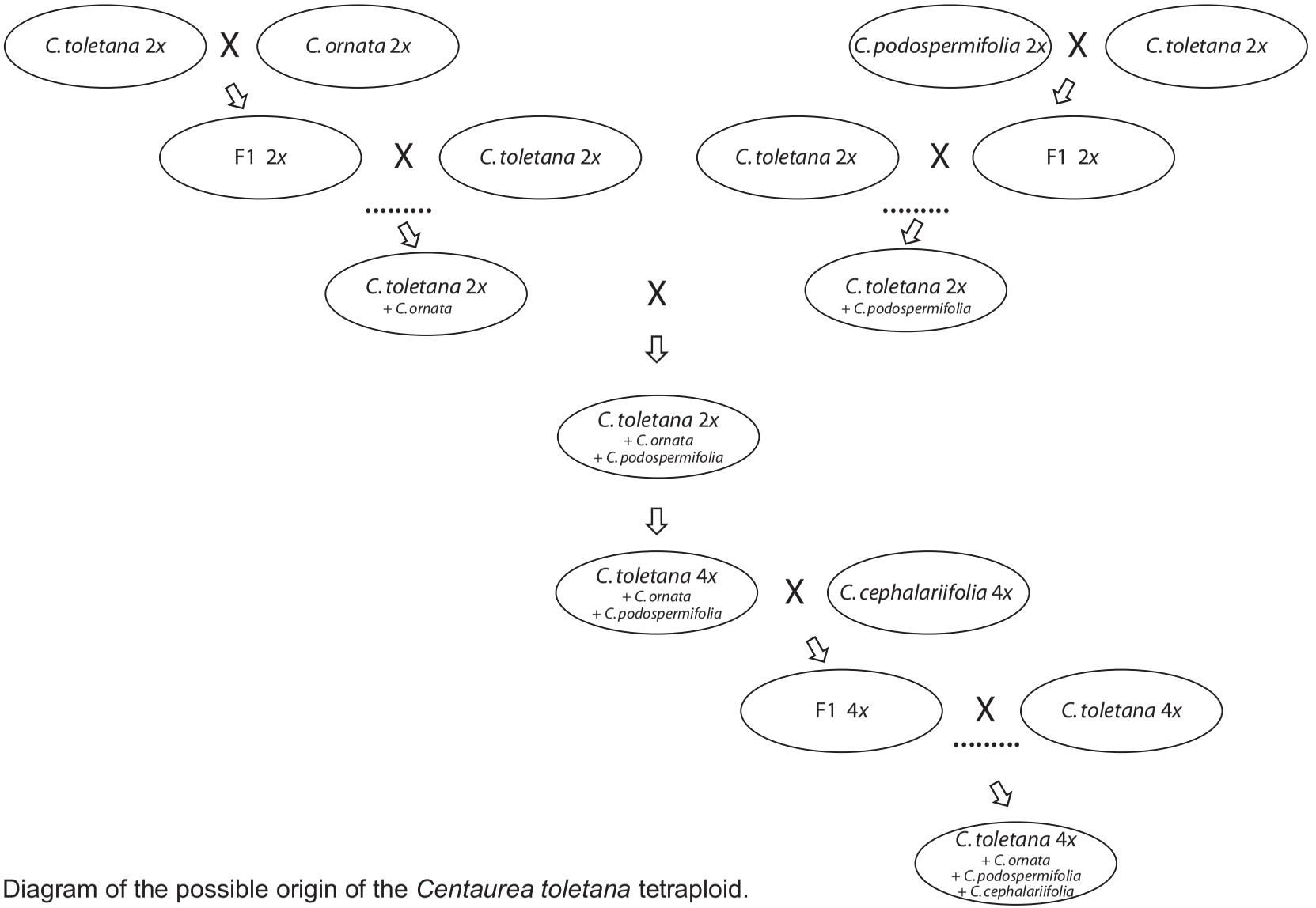

Figure 4a. Diagram of the possible origin of the *Centaurea toletana* tetraploid.

T3 and T4 (from the east part) exhibit an A (Fig. 1, Table 2). Three of the tetraploid populations, T5, T6 and T7, have a snap (M) for this position, which indicates that they are intermediate between both types. The remaining populations of the tetraploid do not have this snap, which points out that there are at least two different origins (probably more) of the tetraploid. Colonizing abilities of the tetraploid, which has broken the strict limitations of the diploid to siliceous soils, can be explained in terms of the increased genetic diversity of polyploids with multiple origins (Soltis & Soltis, 2000).

The alternate hypothesis of an allopolyploid origin is unsupported by molecular analyses. Every alien ribotype in tetraploid populations of *C. toletana* was already detected in the diploid, with the only already mentioned exception of the *C. cephalariifolia* ribotype. This cross has occurred certainly after the polyploidization event: if tetraploid *C. toletana* were a cross of *C. toletana* 2*x* and *C. cephalariifolia*, we would find its genome in all the tetraploid populations, not only in one.

The hexaploid: in the case of hexaploid *C. argecillensis*, the existence of two main ETS ribotypes in most of the studied populations strongly suggests allopolyploidy. As it was obvious on morphological grounds, *C. toletana* is certainly one of the parental species and probably the donor of the 4*x* genome to the hybrid hexaploid. The genome attributed to *C. toletana* appears in all the studied populations. The best candidate for the second parental species is *C. podospermifolia*, a species of sect. *Chamaecyanus* that grows only 100 miles away from the easternmost populations of tetraploid *C. toletana* (Fig. 1). As suggested above, the presence of *C. podospermifolia* genome in most of the studied populations of the hexaploid (Table 4) strongly supports the implication of this species in the genesis of the hexaploid, contributing the 2*x* genome. Supporting this origin, clones from the hexaploid populations T12 and T13, according to the RDP2 analysis, are recombinant of clones from population P2 of *C. podospermifolia* and from tetraploid population T7 of *C. toletana*.

Hybridization of *C. toletana* and *C. podospermifolia* would explain many of the morphological traits that separate hexaploid *C. argecillensis* and

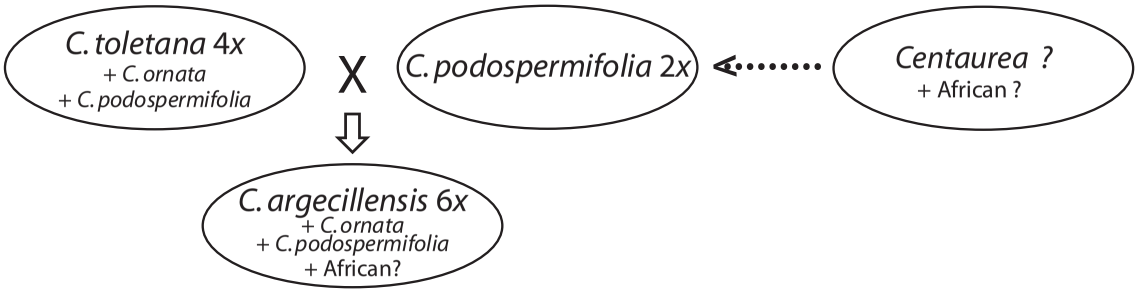

Figure 4b. Diagram of the possible origin of the *Centaurea toletana* hexaploid.

tetraploid *C. toletana*. In fact, confirming morphologic similarities, some populations of *C. argecillensis* in the northern part of its range were classified as *C. podospermifolia* (Fernández Casas & Ceballos, 1980).

The implication of tetraploid *C. toletana* and diploid *C. podospermifolia* in the genesis of *C. argecillensis* (Fig. 4b) is supported by geography: *C. argecillensis* is partly sympatric with *C. toletana* 4*x* (Fig. 1). As previously pointed out, present populations of *C. podospermifolia* are tetraploid. Nevertheless, the presence of the *C. podospermifolia*-like genome in diploid populations of *C. toletana* demonstrates that the distribution of diploid ancestors of *C. podospermifolia* overlapped with the area of *C. toletana* (Fig. 1).

Hybrid origin of hexaploid *C. argecillensis* is a fine example of sympatric polyploid speciation (Otto & Whitton, 2000).

As a result of the superposition of different genomes in hexaploid *C. argecillensis*, we can track a total of five ribotypes, most of them already present among the parental species. The first ribotype, and the only one which appears in all the studied populations, is the same found in diploid and tetraploid *C. toletana* (I_T, Table 4), confirming the identity of this species as one of the parental taxa. The second ribotype, present in most of the studied populations, is the type *C. podospermifolia* (I_P, Table 4) which appears in all but three population and is probably the second parental species. A third genome is the *C. ornata* genome O_I, already present among populations of diploid and tetraploid *C. toletana*. The fourth genome belongs to the African stock (type A, Table 4) and is found only in the population T12 (Fig. 1). This African ribotype characterizes all the included species from Morocco (*C. josiae*, *C. litardierei*, *C. nana* and *C. pubescens*), and it also appears in *C. kunkelii* from the extreme South East of the Iberian Peninsula (Font *et al*., submitted). We shall discuss below, in paleoclimatic terms, this relationship and other unexpected connections between long-separated populations.

The fifth ribotype, and indeed the most interesting one, is a combination of the Iberian *C. toletana* type and the African type (type R, Table 4). This type is present in all the studied hexaploid populations, in the purportedly pentaploid population (T11) and even in one population of *C. ornata* (Fig. 1). Even though

the RDP2 analysis does not identify this ribotype as recombinant, it is intermediate between the I_T and the African ribotypes. The first 114 base pairs of the ETS cloned region coincides with the *C. toletana* type. From positions 160 to 364, it shows a very long (205 bp) insertion that can be aligned, at least partially, with an even longer indel that characterizes the African ribotype (Fig. 3). From positions 792 to 1322, it is again very similar to the Iberian *C. toletana* type.

The role of *Centaurea ornata:* Before discussing in biogeographic terms this background of crosses among species at present not sympatric, we must discuss the role that has played *C. ornata*. This species in its widest sense (including diploid and tetraploid levels) covers the widest range of all the species of *Acrocentron* in the Iberian Peninsula (Fig. 1; see map in Garcia-Jacas & Susanna, 1992). It has been also mentioned in North Africa (Jahandiez & Maire, 1934), which is highly disputed (Susanna & Garcia-Jacas, 2002), but, indeed, some species from Morocco are morphologically very close to *C. ornata*. Besides, it also shows a high ability for hybridizing, even with species with different basic chromosome number: *Centaurea x zubiae* Sennen from North Spain is a cross between the paleohexaploid *C. lagascana* with n= 33 and tetraploid *C. ornata* with n= 20. *Centaurea ornata* shows a great variety of genomes, but maybe more significant is the fact of its two main ribotypes O_I and O_A appear in most of the studied species (Fig. 1). This combination of wide range and demonstrated crossing capability makes it the main suspect of being the vector of alien genomes through its area of distribution.

Paleoclimatic implications

The pattern of distribution of the genomes in the Iberian Peninsula and North Africa (Fig. 1) demonstrates long and frequent periods of contact among species that presently are not sympatric. How and when these species became sympatric is an obvious question that can be tackled on paleoclimatic grounds.

The Iberian Peninsula is one of the three European Peninsulas (Iberian, Italic and the Balkans) that acted as refugial area in the Ice Ages period

(Taberlet *et al.*, 1998; Hewitt, 1999). In the late Pleistocene, post-glacial climatic changes alternated cool and dry periods (Roy *et al.*, 1996; Comes & Kadereit, 1998; Hewitt, 1999). As a consequence, latitudinal and altitudinal migrations of the species were determinant in the evolutionary histories of the region. For exemple, in the genus *Armeria* in southeast Spain (Gutiérrez Larena *et al.*, 2002). In the interglacial periods, the species had zones of contacts and in these zones, intense hybridization and introgression between species occurred. Hybridization causes sometimes the loss of some species and the formation of new ones, and new climatic oscillations initiate a process of isolation and fragmentation (Hewitt, 1999). The genus *Centaurea* L., as a reflect of these processes, shows in the Iberian Peninsula and North Africa and other Mediterranean refugia (Italian Peninsula, Balkan-Greece Peninsula and the Caspian/Caucasus) many endemics usually with small populations: only a few representatives, often annual colonizers, have a wide distribution.

As is usually the case in *Acrocentron* and *Chamaecyanus*, the species involved in this genomic entanglement are mountain endemics. Most of them grow in the mountain belts above 900 m (Fig. 1). General cooling during glacial periods would make present-day conditions at 900 m to be present at 400-600, which is the mean height of the Spanish *Meseta*. This high plateau of Central Spain provides a 900 km north-south connection, from the heights of the Cantabric Mountains in the north to Sierra Nevada in the South. A similar east-west pathway would open, from the east mountain belt connecting Andalusia and the Cantabric Mountains to the western Serra da Estrela in Portugal. When all this large area became available for mountain plants, migration was a natural consequence.

As to the migration to Africa, the importance of the strait of Gibraltar as a barrier for plant migrations has been probably overestimated, as demonstrated by the frequent coincidences in modern flora between both sides (Takhtajan, 1986). This latitudinal migration was very important as demonstrated recently in *Centaurea* sect. *Willkommia* (Suárez-Santiago *et al.*, 2007). In *Centaurea* sect. *Acrocentron*, the presence of the same plastid haplotype in a population of *C. josiae* from the High Atlas in Morocco and a diploid population of *C. toletana*

from central Spain is the final proof of the latitudinal oscillations of the *Acrocentron* group.

The time span of Quaternary glacial periods has been considered too short for speciation processes by species fragmentation (Klicka & Zink, 1997). Instead, climatic rather changes triggered altitudinal and latitudinal migrations that caused extensive genetic interchange by hybridization. These views have been contested by Tzedakis *et al.* (2002), who argued that microallopatry in refuges during glaciations explains present richness of endemics in the Mediterranean flora. In *Centaurea*, we have examples that demonstrate that both points of view are not contradictory. Microallopatry is the most obvious explanation for the many local narrow endemics in *Centaurea* sect. *Willkommia* (Suárez-Santiago *et al.*, 2007), as suggested by Tzedakis *et al.* (2002). Present genomes and even local distribution of species of the *Acrocentron-Chamaecyanus* group are strongly mediated by glaciations acting on preexisting species, which migrated widely both latitudinal and altitudinally and experimented successive and massive hybridization and introgression, as suggested by Klicka & Zink (1997).

Maybe one of the best proofs of the relationships of glaciations and distribution of *Centaurea* is the narrow correlation between the distribution of some important endemics of the group and the distribution of the relictic *Abies pinsapo* Boiss. Populations of *Abies pinsapo* are the remainders of an ancestral species of *Abies* that became isolated in some small refugia in the Betic-Rif Mountains because of the glacial cooling (Scaltsoyiannes *et al.*, 1999). In every relictic population of *Abies pinsapo*, there is an accompanying narrow endemic of *Centaurea* sect. *Acrocentron* (all of them with the O_A ribotype closer to the African type), marking that present area of endemics in the group are the consequence of climatic change: *Centaurea prolongi* in Estepona (Spain), *C. clementei* Boiss. in Grazalema (Spain), and *C. carolipauana* Fern. Casas & Susanna and *C. xaveri* Garcia-Jacas & Susanna in the Rif (Morocco).

Concluding Remarks

Some important conclusions can be drawn from our results. Regarding polyploidy, the existence of different ribotypes in otherwise non-hybrid (morphologically speaking) diploid species questions the very definition of auto- and alloploidy. Even accepting that these two concepts are the extreme states of a continuum (Grant, 1981), the case of *C. toletana* makes more difficult than ever to define the differences. The other conclusion is that connections between the Iberian Peninsula and North Africa have been extensive and reached far beyond it was expected. The case of a population of *C. argecillensis* from central Iberia that shares a ribotype with *C. litardierei* from the High Atlas is a proof of this flow and witnesses the impact of glaciation-induced area shifts suffered by the Ibero-North African flora.

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BIOGEOGRAFIA I FILOGÈNIA

4.3 **Font, M.**, Garnatje, T., Garcia-Jacas, N. & Susanna, A. (2002): **Delineation and phylogeny of *Centaurea* sect. *Acrocentron* based on DNA sequences: a restoration of the genus *Crocodylium* and indirect evidence of introgression.** *Plant Systematics and Evolution* 234: 15-26

Delineation and phylogeny of *Centaurea* sect. *Acrocentron* based on DNA sequences: a restoration of the genus *Crocodylium* and indirect evidence of introgression

Resum

Les espècies del grup *Acrocentron* originàries de la mediterrània estan definides pel tipus de pol·len, pels caràcters de l'aqueni i la morfologia de les bràctees. Hem revisat la delimitació del grup seqüenciant la regió ITS del DNA ribosòmic nuclear. L'arbre de consens obtingut a partir de les anàlisis de l'esmentada regió ens permet dir que el grup *Acrocentron* és un grup natural, que inclou les seccions *Acrocentron*, *Chamaecyanus* i *Stephanochillus*, i que la secció *Chamaecyanus* s'hauria d'integrar dins de la secció *Acrocentron*, probablement com a subsecció; en canvi, les seccions *Aegialophila* i *Crocodylium* formen un grup natural, cosa que també està correlacionada amb la morfologia, sense relació amb el grup *Acrocentron*. Si s'acceptà la unió de *Centaurea crocodylium* i *Aegialophila* amb rang genèric, el nom prioritari hauria de ser *Crocodylium*.

Dels tres grups principals que es suggereixen a partir de la seqüenciació de la regió ITS, dos d'ells coincideixen amb els principals centres d'especiació de la secció *Acrocentron*. Les conclusions són, però, especulatives, ja que el baix suport intern dels arbres i l'absència de relació amb els resultats cariològics ens indiquen que la hibridació i la introgressió han jugat un paper molt important en la diversificació d'aquesta secció, cosa que era d'esperar donada l'absència de barreres reproductives, fins i tot entre les espècies amb diferents números cromosòmics bàsics.

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Delineation and phylogeny of *Centaurea* sect. *Acrocentron* based on DNA sequences: a restoration of the genus *Crocodylium* and indirect evidence of introgression

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Abstract. The Mediterranean group *Acrocentron* of the genus *Centaurea* is defined mainly on the basis of pollen type, but also by achene characters and involucral bracts morphology. We have revised the delineation of the group by cladistically comparing the sequences of the ITS spacers of the nuclear ribosomal DNA. Our results confirm that the *Acrocentron* group is a natural one, with a different delimitation from the commonly accepted. The ITS phylogeny supports that *Centaurea* sect. *Chamaecyanus* and sect. *Stephanochilus* belong to the *Acrocentron* group and suggests that sect. *Chamaecyanus* should be merged in sect. *Acrocentron* as a subsection. Contrary, sect. *Aegialophila* and sect. *Crocodylium* form a natural group that cannot be placed in the *Acrocentron* group and should be considered a different genus. The inclusion of *Centaurea crocodylium* in *Aegialophila* makes that the prioritary name for the generic level is *Crocodylium*; thus, two new nomenclatural combinations are proposed: *Crocodylium creticum* and *Crocodylium pumilum*. The groups suggested by the ITS sequences are correlated to the main geographical centers of speciation of *Acrocentron*. However, support for internal nodes of the tree is extremely poor. The low support within the tree and the absence of correlation between karyology and molecular phylogeny suggest that hybridization has played an important role in the diversification of *Acrocentron*.

Key words: Asteraceae, *Centaurea*, *Aegialophila*, *Acrocentron*, ITS sequences, hybridization.

Depending on the classification adopted, the genus *Centaurea* L. comprises between 400 and 700 species (Dittrich 1977, Bremer 1994, Wagenitz and Hellwig 1996), making it one of the largest genera in the Asteraceae. Classification of *Centaurea* and related genera has been very complicated and only recently, with the help of molecular tools, the delineation of the genera of the subtribe Centaureinae was made clear (Garcia-Jacas et al. 2001).

One of the largest sections of the genus is *Centaurea* sect. *Acrocentron* (Cass.) DC., which includes roughly one hundred species. The first description of sect. *Acrocentron* dates from Cassini (1819) who created it (as a genus) to accommodate the species of *Centaurea* with very large capitula and spiny, long decurrent appendages. Cassini (1819) differentiated his genus *Acrocentron* Cass. from the very close related genus *Lopholoma* Cass. only because *Acrocentron* had spiny bracts, whereas *Lopholoma* had unarmed appendages. Soon after, De Candolle (1838) gave both genera the rank of sections of *Centaurea*. Most of following authors have merged *Acrocentron* and

Lopholoma in a unique section, *Acrocentron* (Cass.) DC. (Hayek 1901, Gardou 1975, Wagenitz 1975, Kummer 1977, Wagenitz and Gamal-Eldin 1985, Fernández Casas and Susanna 1986). Besides morphological characters, *Acrocentron* is well defined by having one of the eight pollen types present in the genus *Centaurea*, the *Centaurea scabiosa* pollen type (Wagenitz 1955).

Though delimitation of sect. *Acrocentron* was not controversial, infrasectional classification has proven a very difficult task, as was pointed out repeatedly by Gardou (1975), Wagenitz and Gamal-Eldin (1985), Garcia-Jacas (1992) and Garcia-Jacas and Susanna (1992). A first attempt by Hayek (1901) was based on eastern representatives of the section, and the resulting classification in six subsections was not applicable to western species (Garcia-Jacas 1992). The next subdivision of *Acrocentron* was suggested by Dostál (1976), based on Hayek's subsections but with the rank of sections. This solution was also extremely confusing, because *Lopholoma* was also kept as an independent section. The case of *Centaurea x polymorpha* Lag. is a good example of this confusion. This species is the hybrid between *C. cephalariaefolia* Willk. from sect. *Lopholoma* and *C. ornata* Willd. from sect. *Acrocentron* (Garcia-Jacas and Susanna 1993), but Dostál (1976) classified it in sect. *Orientales*. The last attempt of subsectional classification was made by Routsi and Georgiadis (1994, 1999), based on the numerous group of east Mediterranean representatives of the section.

Geographic distribution of sect. *Acrocentron* is mainly Mediterranean in its widest sense. Only a few species (most of them from the *Centaurea scabiosa* complex) grow outside the Mediterranean area: they extend into the Eurosiberian region north to Finland and Ireland (Dostál 1976) and eastwards as far as to the Alatau range in Central Asia. In the Mediterranean region, two main centers of diversity are usually recognized (Garcia-Jacas and Susanna 1992): one in the Eastern Mediterranean (the Aegean Islands, Greece and Turkey, extending eastwards to the Irano-Turanian part of Turkey and reaching Iran), and another in the Western Mediterranean (the Iberian Peninsula and North Africa, with one species in Sicily). Most of species are narrow endemics; many of them are known only from a single population and even from a single gathering (Gardou 1975, Wagenitz and Gamal-Eldin 1987, Garcia-Jacas and Susanna 1992, Garcia-Jacas 1998, Routsi and Georgiadis 1999). According to Garcia-Jacas and Susanna (1992) and Garcia-Jacas et al. (1998), on the basis of karyological data, the origin of the group should be the eastern Mediterranean region.

Recently, Wagenitz and Hellwig (1996) defined several large informal groups in *Centaurea*. One of these groups was the *Acrocentron* group, formed by sect. *Acrocentron* with the addition of some other sections of *Centaurea*: *Centaurea* sect. *Aegialophila* (Boiss. & Heldr.) O. Hoffm., *C.* sect. *Chamaecyanus* Willk., *C.* sect. *Crocodylium* DC. and *C.* sect. *Stephanochilus* Coss. & Dur.

Centaurea sect. *Chamaecyanus* is a small group of species from the Iberian Peninsula that were extensively studied by Fernández Casas and Susanna (1986). This section is very closely related to *Acrocentron*: differences between both sections rely only on the combination of acaulescent habit and a reduced pappus in all the species of sect. *Chamaecyanus*. Though the boundaries are clear-cut, both sections are extremely close (Fernández Casas and Susanna 1986). The easy formation of fertile hybrids between both sections confirms this proximity.

The cases of *Centaurea* sect. *Aegialophila* (including *C.* sect. *Crocodylium* according to Garcia-Jacas et al. 2001) and *C.* sect. *Stephanochilus* are less straigthforward. The inclusion of section *Aegialophila* in the *Acrocentron* group by Wagenitz and Hellwig (1996), mainly on the basis of pollen type, was recently rejected by Garcia-Jacas et al. (2001). However, we have included all the species of the genus in our analysis to confirm this exclusion. Regarding *C.* sect. *Stephanochilus*, it is a monotypic section from North Africa. *Centau-*

rea omphalodes Coss. & Dur. which differs in some aspects from the rest of taxa of the *Acrocentron* group (Susanna and Garcia-Jacas in press). Wagenitz and Hellwig (1996) pointed out that pollen evidence favored its placement in *Acrocentron*.

The goals of this study are:

- To examine the limits of the *Acrocentron* group by verifying the position of the genus or section *Aegialophila*, and the sections *Chamaecyanus* and *Stephanochilus*, in the group.
- To explore the suggested correlation between base chromosome numbers and the phylogeny in section *Acrocentron*.
- To confirm or reject the hypothesis of an Eastern Mediterranean origin of the group.

Materials and methods

Plant material. Sampling was based on the *Acrocentron* group in its broadest sense (as defined by Wagenitz and Hellwig 1996), which includes *Centaurea* sections *Acrocentron*, *Aegialophila*, *Chamaecyanus*, *Crocodylium* and *Stephanochilus*. The studied samples have been collected in most of the area the distribution of the group *Acrocentron*, with special emphasis on the Mediterranean region.

The analysis of ITS sequences used, along with new sequence information, published sequences. Voucher data, source and GenBank sequence accession numbers are given in Table 1.

Five outgroup species, *Carduncellus dianius* Webb and *Carthamus oxyacantha* M. Bieb. from the derived clade, and *Centaurea africana* Lam., *Psephellus dealbatus* (Willd.) C. Koch and *Serratula nudicaulis* (L.) DC. from the basal grade, were chosen in the subtribe Centaureinae according to previous sequence analysis (Susanna et al. 1995, Garcia-Jacas et al. 2001).

DNA extraction, amplification and sequencing. Total genomic DNA was extracted following the CTAB method of Doyle and Doyle (1987) as modified by Soltis et al. (1991) and Cullings (1992) from silica-gel dried leaves collected in the field, or fresh leaves of plants cultivated in the Botanic Institute of Barcelona. In some cases, herbarium material was used. Double-stranded DNA of the ITS region was amplified using the 1406F primer (Nickrent et al. 1994) and ITS4 (White et al. 1990); in several taxa we used the ITS1 primer (White et al. 1990) as forward. The profile used for amplification included a warm start at 94 °C for 2 minutes, followed by 80 °C for 5 minutes, during which the polymerase (Ecotaq, Ecogen S. R. L., Barcelona, Spain) was added. 30 cycles of amplification were carried out under the following conditions: 94 °C for 1 minute 30 seconds, 55 °C for 2 minutes and 72 °C for 3 minutes, with an additional extension step of 15 minutes at 72 °C. The PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, California). Both strands were sequenced with the sequencing primers 1406F and ITS1 as forward primers, and ITS4 as reverse. Direct sequencing of the amplified DNA segments was performed using a Thermo Sequenase II Dye Terminator Cycle sequencing kit (Amersham), following the protocol recommended by the manufacturer. The nucleotide sequencing was performed at the "Serveis Científico-Tècnics" of the University of Barcelona on an ABI 377 Automated DNA Sequencer (Perkin-Elmer). Nucleotide sequences of ITS were edited using Chromas 1.56 (Technelysium Pty Ltd).

Phylogenetic analysis. DNA sequences were aligned visually by sequential pairwise comparison (Swofford and Olsen 1990). Data matrix is available on request from the first author. Parsimony analysis involved heuristic searches conducted with PAUP version 4.0b4a (Swofford 1999) using TBR branch swapping with character states specified as unordered and unweighted. The indels were coded as a fifth base as in previous studies on related groups (Garcia-Jacas et al. 2000, Vilatersana et al. 2000). All most-parsimonious trees (MPT) were saved. To locate islands of most-parsimonious trees (Maddison 1991), we performed 100 replications with random taxon addition, also with TBR branch swapping.

Bootstrap (BS) analysis (Felsenstein 1985), and decay index (DI) analysis (Bremer 1988, Donoghue et al. 1992) was performed to obtain estimates of support for the branches of the tree. Due to the practical impossibility of performing bootstrap analyses in the direct way with PAUP 4.0b4a, we used the approach by Lidén et al. (1997) using 1000 replicates, random taxon addition with 20 replicates per replicate and no branch swapping. Results obtained with this method are very similar to other approaches (Mort et al. 2000).

Table 1. Origin of the sequences, vouchers and GenBank accession numbers

Species	Voucher/Source	ITS1	ITS2
Aegialophila cretica Boiss. & Heldr.	Garcia-Jacas et al. (2001)	AY012271	AY012307
Aegialophila pumila (L.) Boiss.	Garcia-Jacas et al. (2001)	AY012272	AY012308
Carduncellus dianius Webb	Vilatersana et al. (2000)	AF140440	AF140441
Carthamus oxyacantha M. Bieb.	Vilatersana et al. (2000)	AF140452	AF140453
Centaurea aetolica Phitos & T. Georgiadis	Greece, Achaia: banks of the road Antirion-Gavrolimni, *Damboldt* 16-07-1976 (B)	AY012202	AY012236
Centaurea africana Lam.	Susanna et al. (1995)	L35863	–
Centaurea borjae Valdés-Berm. & Rivas Goday	Spain, La Coruña: near El Ferrol, cabo Prior, S side, *Garcia-Jacas* & *Susanna 2074* (BC)	AY012203	AY012237
Centaurea carduiformis DC.	Armenia, Talin: between Pokr Arthik and Bagravan, *Fajvush*, *Gabrielyan*, *Garcia-Jacas*, *Guara*, *Hovannisyan*, *Susanna 1588*, *Tamanyan & Vallès* (BC)	AY012204	AY012238
Centaurea carolipauana Fern. Casas & Susanna	Garcia-Jacas et al. (2001)	AY012278	AY012314
Centaurea cephalariaefolia Willk.	Spain, Barcelona: 1 km from Santa Coloma de Queralt on the road to La Llacuna, *Garcia-Jacas, Susanna* & *Vilatersana 12* (BC)	AY012205	AY012239
Centaurea clementei Boiss.	Susanna et al. (1995)	L35872	–
Centaurea collina L.	Spain, Alacant: between Muro de Alcoi and Beniarrés, *Garcia-Jacas, Susanna 1481* & *Vilatersana* (BC)	AY012206	AY012240
Centaurea crocata Franco	Portugal, Algarve: 5 km N of Monchique on the road to Odemira, *Roché* & *Susanna 1917* (BC)	AY012207	AY012241
Centaurea crocodylium L.	Garcia-Jacas et al. (2001)	AY012279	AY012315
Centaurea ebenoides Heldr.	Greece, Evvia island: near Limni, on the coast road, *Phitos* & *Kamari 20427* (B)	AY012209	AY012243
Centaurea euboica Rech. f.	Greece, Euboea island: Kandili mountains, between Achmet Aga and Hagios, *Rechinger 18215* (B)	AY012210	AY012244
Centaurea gabrielis-blancae Fern. Casas	Spain, Navarra: Lumbier, Foz de Lumbier, *Garcia-Jacas* & *Susanna 1592* (BC)	AY012212	AY012246
Centaurea grbavacensis (Rohlena) Stoj. & Acht.	Greece, Macedonia: Kozuf mountains, south side of mt. Tzena, 1700 m, *Greuter 14108* (B)	AY012214	AY012248
Centaurea josiae Humbert	Morocco, Ksar el Souk: Tizi'n Talrhemt, *Garnatje*, *Susanna 1792* & *Vilatersana* (BC)	AY012216	AY012250

Table 1 (continued)

Species	Voucher/Source	ITS1	ITS2
Centaurea kandavanensis Wagenitz	Iran, Mazandaran: Chalus road, 2 km from the cross to Baladeh, *Garcia-Jacas*, *Mozaffarian*, *Susanna 1621* & *Vallès* (BC)	AY012217	AY012251
Centaurea kunkelii Garcia-Jacas	Spain, Almería: road AL-411 between Roquetas and Canjáyar, *Garcia-Jacas, Susanna 1613* & *Vilatersana* (BC)	AY012218	AY012252
Centaurea lagascana Graells	Garcia-Jacas et al. (2001)	AY012284	AY012320
Centaurea lainzii Fern. Casas	Spain, Sierra Bermeja: 11 km from Estepona on the road to Igualeja, *Garcia-Jacas* & *Susanna 1330* (BC)	AY012219	AY012253
Centaurea litardierei Jahand. & Maire	Morocco, Ksar es Souk: south side of Col du Zad, *Garnatje*, *Susanna 1797* & *Vilatersana* (BC)	AY012220	AY012254
Centaurea luristanica Rech. f.	Iran, Khuzistan: ca. 15 km from Eizeh to Dehdez, 900 m, *Mozaffarian* (TARI)	AY012221	AY012255
Centaurea lydia Boiss.	Turkey, Konya: Seydisehir, Mortas, *R. Ilarsan* 4312 (ANK)	AY012222	AY012256
Centaurea ochrocephala Wagenitz	Iran, Azarbayjan-e-Gharbi: Orumiyeh, between Maranah and Haki, *Garcia-Jacas, Mozaffarian, Susanna 1693* & *Vallès* (BC)	AY012223	AY012257
Centaurea omphalodes (Coss. & Dur.) O. Hoffmann	Häffner and Hellwig (1999)	AY012301	AY012337
Centaurea ornata Willd.	Spain, Soria: near San Esteban de Gormaz, *Garcia-Jacas* & *Susanna 1823* (BC)	AY012224	AY012258
Centaurea podospermifolia Losc. & Pard.	Spain, Tarragona: Tortosa, 5 km from Monte Caro, *Garcia-Jacas*, *Martín*, *Susanna 2072* & *Vallès* (BC)	AY012226	AY012260
Centaurea prolongi Boiss.	Spain, Málaga: north side of the Sierra de Mijas, 500 m, *Garcia-Jacas* & *Susanna 1335* (BC)	AY012227	AY012261
Centaurea pubescens Willd.	Morocco, Ksar es Souk: 2 km from Oued Amesheguir, *Garnatje*, *Susanna 1795* & *Vilatersana* (BC)	AY012228	AY012262
Centaurea raphanina Sibth. & Sm.	Greece, Chanion: Kydonias, mounts Levka, NW part, near Omalos, along track to Eos Katafygion, *Strid 15093* & *Papanicolau* (B)	AY012229	AY012263
Centaurea rupestris L.	Greece, Macedonia: road E-90 between Véria and Kozáni, *Roché* & *Susanna 1967* (BC)	AY012211	AY012245
Centaurea saharae Pomel	Morocco, Oujda: 28 Km N of Tendrara, *Garnatje*, *Susanna 1778* & *Vilatersana* (BC)	AY012230	AY012264

Table 1 (continued)

Species	Voucher/Source	ITS1	ITS2
Centaurea salonitana De Vis.	Greece, Macedonia: 600 m W from Efkarpia, *Roché & Susanna 2005* (BC)	AY012231	AY012265
Centaurea saxicola Lag.	Spain, Murcia: La Azohía, near the watchtower, *Garcia-Jacas, Susanna 1616 & Vilatersana* (BC)	AY012232	AY012266
Centaurea scabiosa L.	France, Lozère: between Le Sec and l'Aumède, near Chanac, *Vilatersana 52* (BC)	AY012233	AY012267
Centaurea tauromenitana Guss.	Botanical Garden of Catania (BC)	AY012234	AY012268
Centaurea toletana Boiss. & Reut.	Susanna et al. (1995)	L35866	–
Psephellus dealbatus (Willd.) C. Koch	Susanna et al. (1995, as *Centaurea dealbata* Willd.)	L35886	–
Serratula nudicaulis (L.) DC.	Susanna et al. (1995)	L35862	–

Table 2. Numeric results of the phylogenetic analysis

Data set	ITS1	ITS2
Total characters	249–257	210–217
Informative characters	44	43
Range of divergence, ingroup (%)	0–14.59	0–13.77
Number of MPTs	1524	
Number of steps	211	
Consistency index (CI)	0.5681	
Retention index (RI)	0.7540	
Homoplasy index (HI)	0.4319	

Regarding decay analysis, the search of trees longer than the shortest trees was impossible in all the cases. Therefore, the decay analyses were conducted using the clade-constraint approach as discussed in Morgan (1997).

Results

Numeric results of the analysis are summarized in Table 2. The ITS1 and ITS2 alignment of 42 taxa consisted of 476 positions and contained 80 phylogenetically informative substitutions and 7 phylogenetically informative indels 1-2 bp long. ITS1 mean pairwise distances (as calculated by PAUP) within ingroup varied from 0% (between seven pairs of species of *Centaurea*) to 14.59% (between *Aegialophila pumila* (L.) Boiss. and *Centaurea saxicola* Lag.). Pairwise distance between ingroup and outgroup varied from 5.15% (between *Carthamus oxyacantha* and *Centaurea gabrielis-blancae* Fern. Casas) to 14.17% (between *Serratula nudicaulis* and *Centaurea saxicola*).

ITS2 mean pairwise distances (as calculated by PAUP) within ingroup varied from 0% (between nine pairs of species) to 13.77% (between *Aegialophila pumila* and *Centaurea crocata* Franco). Pairwise distance between ingroup and outgroup varied from 5.73% (between *Carduncellus dianius* and *Centaurea omphalodes*) to 13.08% (between *Centaurea africana* and *Centaurea lydia* Boiss.).

In both ITS regions, distances within the ingroup are always higher that distances between the outgroup and the ingroup. In both cases, the involved species was *Aegialophila pumila*.

The parsimony analysis yielded 1524 MPTs of 211 steps in one island. The strict consensus of all the trees is shown in Fig. 1.

The strict consensus of the 1524 MPTs produced from the ITS analysis (Fig. 1) supports monophyly of *Centaurea* sect. *Acrocentron* including *Centaurea* sect. *Stephanochilus* (*C. omphalodes*) as sister, but excluding sections *Aegialophila* and *Crocodylium*, with

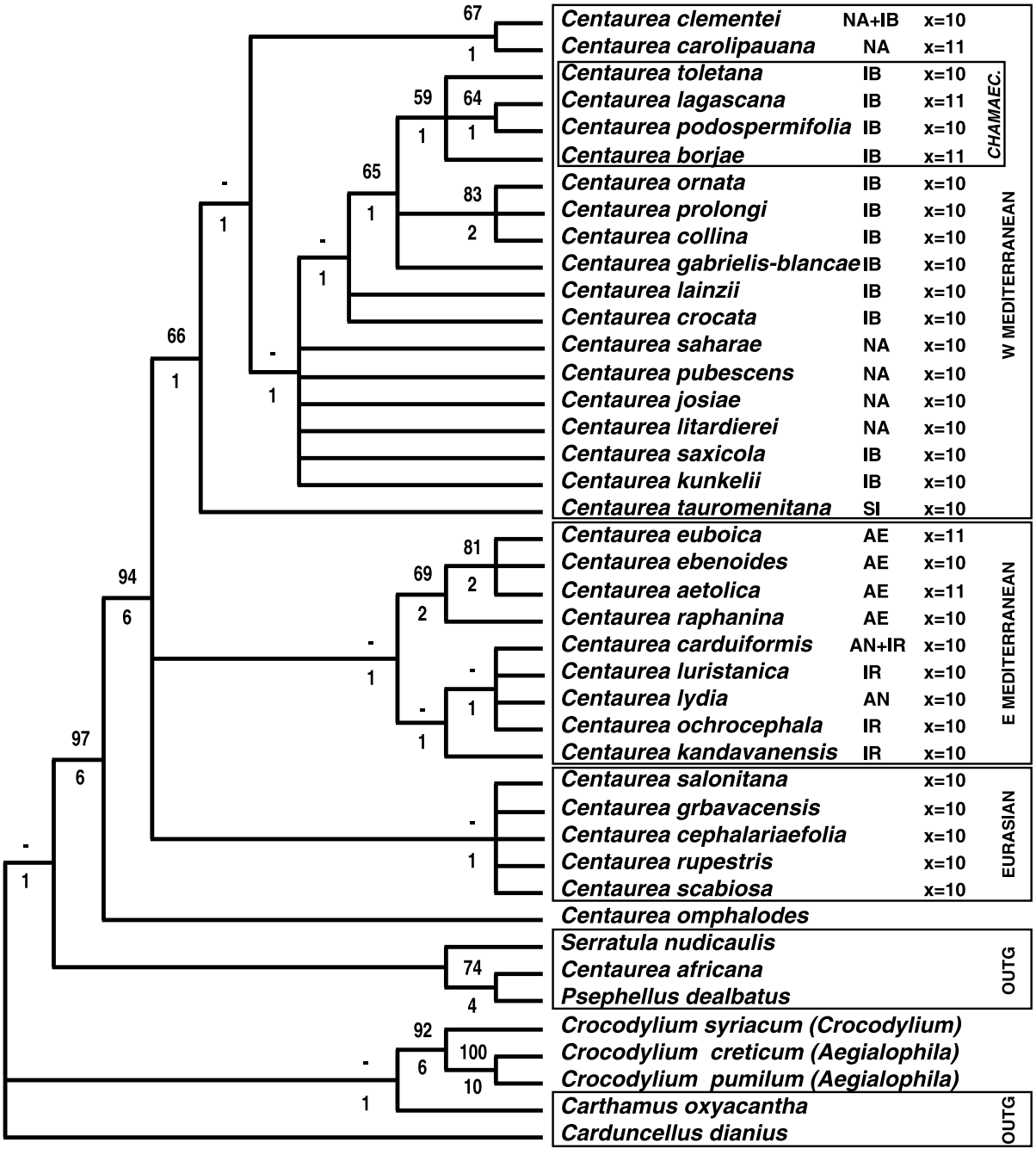

Fig. 1. Strict consensus tree of the 1524 equally most-parsimonius trees obtained in the heuristic search (211 steps, CI excluding uninformative characters = 0.5681; RI = 0.7540; HI = 0.4319). Numbers above the branches are bootstrap values; numbers below the branches are decay indices. *Chamaec* = *Centaurea* sect. *Chamaecyanus*. Regional acronyms: **AE** Aegean; **AN** Anatolian; **IB** Iberian; **IR** Iranian; **NA** North African; **SI** Sicilian. **OUTG** Outgroup

BS = 97% and DI = 6. The next clade was formed by all the species of the genus *Centaurea* that have been included in sections *Acrocentron* and *Chamaecyanus*, with a strong support (BS = 94%, DI = 6).

The internal branches of the consensus tree had no support from the bootstrap or the decay index. However, three large groups could be defined, that we have marked with boxes (Fig. 1):

- A clade formed by species with Eurasian distribution (represented mainly by the *Centaurea scabiosa* complex), with the addition of some species of east Mediterranean distribution as *Centaurea salonitana*.
- A clade which includes the species with an eastern Mediterranean distribution (Greece and Anatolia). This clade includes the representatives from the Irano-Turanian region (Iran).
- A third clade with the western Mediterranean representatives, from Sicily (*Centaurea tauromenitana*) to Spain and North Africa.

Discussion

The delimitation of the *Acrocentron* group. The delimitation of the *Acrocentron* group coincides with the proposal of Wagenitz and Hellwig (1996) regarding *Centaurea* sects. *Acrocentron, Chamaecyanus* and *Stephanochilus*. *Centaurea omphalodes* (sect. *Stephanochilus*) is sister to the rest of *Acrocentron* with very high support (BS = 94%, DI = 6). This is a very intriguing position, because geographic distribution of *Centaurea omphalodes* is marginal to the distribution of *Acrocentron*: it is a strictly Saharan plant, extending northwards to central Algeria (Jahandiez and Maire 1934). Obviously, it is an extremely isolated representative of the group, as is patent in two characters: it is the only annual plant in the entire group, and the achene is very different from the *Acrocentron* model (Susanna and Garcia-Jacas, in press). Regarding *Centaurea* sect. *Chamaecyanus,* its position in the tree (Fig. 1), strongly suggest that it should be merged into sect. *Acrocentron*.

Contrary, *Centaurea* sections *Aegialophila* and *Crocodylium* do not belong to the group, as was recently pointed out by Garcia-Jacas et al. (2001) on the basis of ITS and *mat*K sequence analysis and Vilatersana et al. (2001) on the basis of pollen type, and must be kept as a segregate genus (Fig. 1). Vilatersana et al. (2001) revised extensely the morphological traits that purportedly related *Aegialophila* (and *Crocodylium*) to the *Acrocentron* group, and concluded that they were misinterpreted.

According to our results, the only species of *Centaurea* sect. *Crocodylium*, *Centaurea crocodylium* L., must be moved to *Aegialophila*. However, at the genus level, *Crocodylium* Hill (1762) is prioritary over *Aegialophila* Cass. (1819). As the genus status looks most appropriate for this group (Garcia-Jacas et al. 2001; Susanna and Garcia-Jacas, in press), some nomenclatural changes are needed (see Appendix).

Not only the ITS supports the merging of sections *Aegialophila* and *Crocodylium*, but also the morphology. By example, the very big, patent, showy peripheral florets of *C. crocodylium* are very similar to those of *Aegialophila*, as is the case of the sterile achenes of the peripheral florets. Sterile achenes are also present in sect. *Acrocentron*, but they are quite different (Susanna and Garcia-Jacas, in press).

Biogeography and evolution in *Centaurea* sect. *Acrocentron*. As previously marked, support for the internal nodes of the tree is extremely low, and our conclusions are only speculative. However, three main groups are defined, and two of them coincide with the main centers of speciation of the group.

The Eurasian clade is formed by two representatives of the *Centaurea scabiosa* group (*C. cephalariaefolia* and *C. scabiosa* L.), with the addition of two species of wide distribution in the Balkans, extending eastwards to north Italy: *Centaurea rupestris* L. and *C. salonitana* De Vis.

In the eastern Mediterranean group, there are two clades. One of them includes narrow endemics from the Aegean (*Centaurea euboica*

Rech. f., *C. ebenoides* Heldr., *C. aetolica* Phitos & Georgiadis and *C. raphanina* Sibth. & Sm.), with the three insular isolated species (*C. euboica*, *C. ebenoides* and *C. aetolica*) forming one of the rare branches with good bootstrap support (Fig. 1). The other clade includes the Iranian and Irano-Anatolian representatives, and one species of wider distribution in Anatolia (*C. lydia*). Based on the absence of the archaic base chromosome number x = 11 among Iranian species, Garcia-Jacas et al. (1998) suggested that the Iranian taxa conform a center of speciation secondary to the Aegean-Turkish center. Our analysis does not contradict this view.

The western Mediterranean group is very suggestive from a biogeographic standpoint. *Centaurea tauromenitana* Guss. from Sicily stands as sister to the rest of the clade, reinforcing previous suggestions of an east-to-west route of expansion for the group by Garcia-Jacas and Susanna (1992). On the other hand, the Iberian species are grouped in a clade with the north African representatives. Two species, *Centaurea clementei* Boiss. and *C. carolipauan*a Fern. Casas & Susanna, are sister to the rest of the Ibero-North African clade. *Centaurea clementei* grows both in South Spain and Morocco (Garcia-Jacas and Susanna 1989), and *C. carolipauana* is restricted to a single locality in Morocco (Garcia-Jacas 1992). Morphological characters of leafs and bract appendages support this relationship between the two species, but they have different chromosome numbers: x = 11 for *C. carolipauana* and x = 10 for *C. clementei*. If we conclude that *C. clementei* derives from *C. carolipauana*, descending dysploidy in *Acrocentron* has appeared more than one time, as they form a very isolated group. This view is confirmed by the general lack of correlation between molecular phylogeny and chromosome numbers (Fig. 1).

Two species from south east Spain, *Centaurea saxicola* and *C. kunkelii* Garcia-Jacas, are placed out of the Iberian clade and ranged in the basal grade of African representatives. Dostál (1976) subordinated *C. saxicola* to *C. ornata* as subspecies, but our analysis supports its status as a different species. Regarding *C. kunkelii*, it was described very recently (Garcia-Jacas 1998), on the basis of a plant from south Spain that had been much confused, again, with *C. ornata*. Curiously, Garcia-Jacas (1998) suspected an hybridogenic origin for this species, and one of the hypothesized parental species was *C. saxicola*.

The placement of two Iberian species among the polytomy of African taxa reinforces the hypothesis of a narrow floristic connection between the floras of north Africa and south-east Spain. In the same line, two more species from south Iberia, *Centaurea lainzii* Fern. Casas and *C. crocata* Franco, are placed in an intermediate position between the north African grade and the Iberian clade (Fig. 1). These connections suggest that a North-African migration originated the Iberian group of species of sect. *Acrocentron*, with the exception of *Centaurea cephalariaefolia*, which is connected to the Eurasian group of *Centaurea scabiosa*.

Finally, within the strictly Iberian clade, the only branch that has moderate support is formed by three species that do not share their distribution: *Centaurea collina* L. from east Spain and south France; *Centaurea ornata* from central Spain; and *C. prolongi* Boiss. from south Spain (Fig. 1). Probably the fact of being allopatric- and thus not intrograding- is the reason for this support, as we will discuss later.

Regarding *Centaurea* sect. *Chamaecyanus*, the ITS analysis suggests that it is a monophyletic group, but- as the rest of the internal nodes of the tree- without support. Surprisingly, *Centaurea borjae* Valdés-Berm. & Rivas Goday is grouped with *Chamaecyanus*. This species lacks the pappus that would made clear its position in *Chamaecyanus* or *Acrocentron*. Valdés Bermejo and Rivas Goday (1980) described a new section for this species, sect. *Borjae*. Fernández Casas and Susanna (1986), on the basis of achene surface characters, moved it to sect. *Acrocentron*. According to the ITS analysis, it is connected to *Chamaecyanus* (Fig. 1).

Hybridization in *Acrocentron*. The low levels of support for the internal nodes of the tree strongly suggest that hybridization and introgression are very extended in the group, as it could be expected because of the lack of barriers between species (Kummer 1977, Fernández Casas and Susanna 1986, Garcia-Jacas 1992, Garcia-Jacas and Susanna 1994, Garcia-Jacas 1998). In a recent study of the *Carthamus* complex, the removal of the species of suspected hybrid origin resulted in a dramatic increase of the bootstrap and decay values for the branches within the genus *Carthamus* (Vilatersana et al. 2000). However, in the case of *Acrocentron*, removal of the suggested hybridogenic *Centaurea crocata* and *C. kunkelii* (Garcia-Jacas and Susanna 1994, Garcia-Jacas 1998) did not improve the support for the branches.

A significant peculiarity of the analysis supports our view: the only clades within the tree that have support from the bootstrap, the Aegean group and the group of three Iberian species *Centaurea collina*, *C. ornata* and *C. prolongi* (Fig. 1), are formed by species that do not share their geographic distribution. The low number of synapomorphies that define internal clades (actually, one change in the clade of *Centaurea collina*, *C. ornata* and *C. prolongi*) would made easy that homoplasy caused by introgression blurr the result of the analysis. In fact, hybrids of all the three species are known from old, but always with species from outside this clade: e. g., *Centaurea ornata* has been found to hybridize with *C. lagascana* Graells, *C. cephalariaefolia* and *C. toletana* Boiss. & Reut. (Kummer 1977, Fernández Casas and Susanna 1986).

Actually, hybridization is one of the most conflicting features of the *Acrocentron* group. Fertile hybrids are frequent not only in sect. *Acrocentron* (Kummer 1977, Fernández Casas and Susanna 1986), but also between sects. *Acrocentron* and *Chamaecyanus*. The problem relies in the difficulties in detecting hybridization within sect. *Acrocentron*, only evident if the parental species have florets of different color. Alternately, when one of the parental species belong to sect. *Chamaecyanus*, the hybrid origin is obvious from the achene characters (Garcia-Jacas 1992, Garcia-Jacas and Susanna 1994, Garcia-Jacas 1998). Three of these putative hybrids, *C. crocata*, *C. kunkelii* and *C. saxifraga* Coincy, are now established as new species and grow unconnected to their presumed progenitors (Garcia-Jacas 1998).

Human activity has been crucial in the generalized introgression in the group. Species of *Acrocentron* have intense vegetative reproduction; by example, a sterile triploid, *Centaurea lainzii*, has managed to colonize the south face of the Sierra Bermeja range in south Spain (Fernández Casas and Fernández Morales 1979). They colonize very quickly open habitats, like the margins of the roads. The opening of new ways made many allopatric species to come in contact and intrograde. Actually, the best place for observing hybrid populations of *Acrocentron* are the old roads, where banks and ditches are not cleaned very often, and all the new hybrid species between *Acrocentron* and *Chamaecyanus* were first spotted and collected along roadsides (Fernández Casas and Susanna 1986).

The group of taxa of wider distribution must lie at the basis of this intense gene flow that emerges as the main reason of the low support for the internal nodes of the tree. *Centaurea* sect. *Acrocentron* can be added to the long list of groups posing very complex problems of introgression, specially in molecular systematics, as was extensively revised in a recent paper by Wendel and Doyle (1998).

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Appendix

Crocodylium Hill, Veg. Syst. 4: 22 (1762).

= *Aegialophila* Boiss. & Heldr. ex Boiss., Diagn. pl. orient. ser. 1.2, 10: 105 (1849).

Crocodylium syriacum Cass., Dict. Sci. Nat. 12: 19 (1819).

= *Crocodylium crocodylium* Hill, nom. inv.

= *Centaurea crocodylium* L., Sp. Pl. 919 (1753).

Crocodylium pumilum (L.) Garcia-Jacas & Susanna, **comb. nov.**

Basionym: *Centaurea pumilio* L., Amoen. Acad. 4: 292 (1759).

= *Aegialophila pumila* Boiss. & Heldr. ex Boiss., Diagn. Pl. Orient. ser. 1.2 (10): 105 (1849).

Crocodylium creticum (Boiss. & Heldr.) Garcia-Jacas & Susanna, **comb. nov.**

Basionym: *Aegialophila cretica* Boiss. & Heldr. ex Boiss., Diagn. Pl. Orient. ser. 1.2 (10): 106 (1849).

= *Centaurea aegialophila* Wagenitz, Notes R. Bot. Gard. Edinburgh 33: 230 (1974).

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4.4 **Font, M.**, Garcia-Jacas, N., Vilatersana, R. & Susanna, A. En revisió. **Biogeography and introgression in *Centaurea* section *Acrocentron* inferred from nuclear and plastid DNA sequence analyses.** *Taxon*.

Biogeography and introgression in *Centaurea* section *Acrocentron* inferred from nuclear and plastid DNA sequence analyses

Resum

La majoria de les espècies de la secció *Acrocentron* del gènere *Centaurea* estan localitzades a l'àrea mediterrània, tot i que trobem unes poques espècies que creixen fora d'aquesta àrea. Dins la mediterrània s'hi reconeixen dos centres de diversificació, un centrat a l'est d'aquesta àrea i l'altre a l'oest. Si tenim en compte les dades cariològiques l'origen del grup s'hauria d'atribuir a la zona est de la mediterrània. Hem analitzat les seqüències del DNA ribosòmic nuclear i del DNA cloroplàstic amb l'objectiu de confirmar els límits i d'intentar reconstruir la història de l'expansió de la secció *Acrocentron* dins la mediterrània.

Els resultats de les anàlisis corroboren que la secció *Chamaecyanus* és en realitat una subsecció dins d'*Acrocentron*; de tota manera, per poder establir una classificació subseccional del grup, les dades moleculars no ens són de gran ajuda, perquè l'intens flux genètic que es dóna en aquest grup amaga la reconstrucció de les afinitats filogenètiques. A més, l'extrema homogeneïtat dels caràcters morfològics també dificulta aquesta classificació.

La combinació de les dades provinents de les anàlisis de DNA ens mostren que hi ha quatre centres d'especiació i que la radiació ha seguit dues vies separades en el temps; des d'Anatòlia, les espècies d'*Acrocentron* van radiar cap a les illes gregues i l'Egeu que constituirien la via més a l'est de la penetració i que estaria datada al final del miocè on el pas entre Anatòlia i l'Egeu estava obert. De l'Egeu va passar als Balcans per un cantó, i per l'altre, la baixada del nivell del mar (període messinià) va permetre el pas cap a l'extrem sud d'Itàlia i Sicília i d'aquí a la zona nord-africana i a la Península Ibèrica. La segona radiació, més tardana, s'hauria donat durant els períodes interglacials de l'holocè a partir de les espècies provinents dels Balcans seguint

una línia de distribució contínua eurasiàtica des del centre de la Península Ibèrica fins al Kazakhstan.

La filogènia dels tàxons ibèrics i nord-africans no queda clara. La successió de glaciacions va forçar les espècies a migrar a més baixes latituds (a la inversa en períodes interglacials), i espècies que estaven aïllades van esdevenir simpàtriques, fet que va donar lloc a una intensa introgressió.

Biogeography and introgression in *Centaurea* section *Acrocentron* inferred from nuclear and plastid DNA sequence analyses

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Section *Acrocentron* of the genus *Centaurea* is analyzed using plastid and nuclear DNA sequence analysis. The resulting phylogeny has implications for the sectional classification of *Acrocentron*, and the merging of sect. *Chamaecyanus* into *Acrocentron* as a subsection is mandatory. Previous suggestions of an Eastern Mediterranean origin of the group are confirmed. Radiation followed two waves, widely separated in the time scale. The oldest migration, from Turkey to Greece and the northern Balkans and then to North Africa and Iberia, should be dated at the end of the Miocene in the Messinian period. It reached the Iberian Peninsula by the south, following a route that is landmarked by several relictic taxa in Sicily and North Africa. A later radiation during the Holocene interglacial periods followed involving species from the north of the Balkan Peninsula, this time along an Eurasian pathway reaching from north and Central Iberia to the steppes of Kazakhstan. A generalized pattern of reticulation is also evident from our results, indicating past contacts between presently separated species. The implications of gene flow and introgression between African and Iberian populations for the biogeographic history of the Iberian Peninsula are shortly discussed.

KEYWORDS: Cardueae, ETS, ITS, Mediterranean, *psb*A-*trn*H, phylogeny, reticulation

INTRODUCTION

The tribe Cardueae (Compositae) has been deeply reviewed and widely modified in the past ten years. Most of the many systematic problems within the tribe, from the subtribal classification to the genus delineation, are now more clear (Susanna & al., 1995; Garcia-Jacas & al., 2001; Susanna & al., 2006), a complicated task because the tribe includes some of the largest genera of the whole family (*Centaurea* L., *Cirsium* Mill., *Cousinia* Cass. and *Saussurea* DC.). Our present research centres in one of these large genera, *Centaurea*.

In its present delimitation as adopted in the latest revision of the Cardueae (Susanna & Garcia-Jacas, 2007), *Centaurea* comprises ca. 250 species (from 400 in earlier classifications; e. g. Dittrich, 1977). *Centaurea* encompasses only the large *Jacea* group (revised by Garcia-Jacas & al., 2006), the section *Cyanus* and the *Acrocentron* group.

The *Acrocentron* group is formed by *Centaurea* sect. *Acrocentron* (Cass.) DC. with the addition of two much smaller sections: *Centaurea* sect. *Chamaecyanus* Willk. and *C.* sect. *Stephanochilus* Coss. & Dur. A previous proposal by Wagenitz & Hellwig (1996) also included *Centaurea* sect. *Crocodylium* DC. and *Centaurea* sect. *Aegialophila* (Boiss. & Heldr.) O. Hoffm., but these two sections should be excluded and merged in a different genus, *Crocodylium* Cass. (Garcia-Jacas & al., 2001; Vilatersana & al., 2001; Font & al., 2002). Among other characters, the *Acrocentron* group is well defined by having one of the four pollen types described in the genus *Centaurea*, the *Centaurea scabiosa* pollen type (Wagenitz, 1955).

The largest part of the *Acrocentron* group is *Centaurea* sect. *Acrocentron*, a huge section with ca. 100 species mainly in the Mediterranean region. The first mention of *Acrocentron* dates from Cassini (1819) who created it (as a genus) to accommodate the species of *Centaurea* with very large capitula and spiny, long decurrent appendages. Cassini (1819) differentiated genus *Acrocentron* Cass. from the very closely related genus *Lopholoma* Cass. only because *Acrocentron* had spiny bracts, whereas *Lopholoma* had unarmed appendages. Soon after, De Candolle (1838) gave both genera the rank of sections of *Centaurea*. Most of following authors have merged *Acrocentron* and *Lopholoma* in a unique section *Acrocentron* (Hayek, 1901;

Gardou, 1975; Wagenitz, 1975; Kummer, 1977; Wagenitz & Gamal-Eldin, 1985; Fernández Casas & Susanna, 1986).

Though delimitation of sect. *Acrocentron* is not controversial, infrasectional classification has proven a very difficult task, as was pointed out repeatedly by Gardou (1975), Wagenitz & Gamal-Eldin (1985), Garcia-Jacas (1992), Garcia-Jacas & Susanna (1992) and Font & al. (2002). A first attempt by Hayek (1901) was based on eastern representatives of the section, and the resulting classification in six subsections was not applicable to western species (Garcia-Jacas, 1992). The next subdivision of *Acrocentron* was suggested by Dostál (1976), based on Hayek's subsections but with the rank of sections. This solution was also extremely confusing, because Dostál (1976) also kept *Lopholoma* as an independent section. The case of *Centaurea* x *polymorpha* Lag. is a good example of this confusion. This species is a hybrid between *C. cephalariifolia* Willk. from sect. *Lopholoma* and *C. ornata* Willd. from sect. *Acrocentron* (Garcia-Jacas & Susanna, 1993), but Dostál (1976) classified it in sect. *Orientales*.

The latest attempt of subsectional classification was made by Routsi and Georgiadis (1994, 1999) and, just like Hayek (1901) one century before, they based their sectional division on the numerous group of east Mediterranean representatives of the section. In consequence, this proposal is difficult to evaluate until applied to the whole of the section.

The second section of the *Acrocentron* group is *Centaurea* sect. *Chamaecyanus* Willk. This section is a small group of eight species from the Iberian Peninsula that were extensively studied by Fernández Casas & Susanna (1986). This section is very closely related to sect. *Acrocentron*: Differences between both sections rely only on the combination of acaulescent habit and a reduced pappus in all the species of sect. *Chamaecyanus*. Though the boundaries are clear-cut, both sections are extremely close (Fernández Casas & Susanna, 1986). The easy formation of fertile hybrids between both sections confirms this proximity and it has been suggested that *Chamaecyanus* should be considered a subsection of *Acrocentron* (Font & al., 2002).

The third section and the smallest of the *Acrocentron* group is monotypic, *Centaurea* sect. *Stephanochilus* from North Africa. Our previous molecular survey

confirmed that it was part of the *Acrocentron* group and it was sister to the rest of the group (Font & al., 2002).

Besides taxonomy, the most important point of interest of the *Acrocentron* group is biogeography. *Acrocentron* is centred in the Mediterranean Floristic Region in its widest sense. Only few species (most of them from the *Centaurea scabiosa* complex) grow outside the Mediterranean area: They extend from the north Balkans to north Italy, and then into the Eurosiberian region reaching Finland and Ireland to the north (Dostál, 1976) and eastwards as far as to the Alatau range in Central Asia (Susanna, pers. obs). In the Mediterranean region, two main centers of diversity are usually recognized (Garcia-Jacas & Susanna, 1992): One in the Eastern Mediterranean (the Aegean Islands, Greece and Turkey, extending eastwards to the Irano-Turanian part of Turkey and reaching Iran), and another in the Western Mediterranean (the Iberian Peninsula and North Africa, with one species in Sicily). Most of species are narrow endemics; many of them are known only from a single population and even from a single gathering (Gardou, 1975; Wagenitz & Gamal-Eldin, 1985; Garcia-Jacas & Susanna, 1992; Garcia-Jacas, 1998; Routsi & Georgiadis, 1999). According to Garcia-Jacas & Susanna (1992) and Garcia-Jacas & al. (1998), on the basis of karyological data, the origin of the group should be the eastern Mediterranean region. Reconstruction of paleogeography of plants is a relatively novel focus in botany, and present distribution of *Acrocentron* in the Mediterranean region makes the group an excellent object of study. A recent paper dealing with the related *Acrolophus-Phalolepis* complex of *Centaurea* has contributed to our knowledge of the epoch of expansion of *Centaurea* in the Iberian Peninsula and North Africa (Suárez-Santiago & al., 2007). It is tempting to verify whether *Acrocentron* follows a similar pattern of colonization of the Mediterranean.

In our previous molecular survey of the *Acrocentron* group, we used sequences of the ITS region. The delineation of *Acrocentron* with the unambiguous inclusion of sect. *Stephanochilus*; the exclusion of the restored genus *Crocodylium*; the difficulties for keeping *Chamaecyanus* as a different section; and the existence of three main centres of speciation (Anatolian-Aegean, North Balkanic-Eurasian, and Iberian-North African) were the most important results. However, the phylogeny suggested by the ITS sequences was flawed by an extremely low support for most of

the clades that was considered a reflect of intense gene flow (Font & al., 2002). Hybridization in the *Acrocentron* group is well documented (Kummer, 1977; Fernández Casas & Susanna, 1986) and recent surveys involving extensive cloning (Font & al., in prep.) suggest that homoploid hybridization, and subsequent introgression with both parentals, is a generalized phenomenon that obscures phylogenetic reconstructions.

Thereafter, we decided to carry out a new study with the addition of the ETS region of the nuclear-ribosomal DNA and the non-coding intergenic spacer *trn*H-*psb*A of chloroplast DNA. The ETS region has proven a good tool for the phylogenetic reconstruction of closely related species in many genera from the Compositae (*Calycadenia*, Baldwin & Markos, 1998; *Helianthus*, Linder & al., 2000; *Lessingia*, Markos & Baldwin, 2001; *Xylothamia* and *Gundlachia*, Urbatsch & al., 2003; *Montanoa*, Plovanich & Panero, 2004) and has already been very useful in the Cardueae-Carduinae (Kelch & Baldwin, 2003) and Cardueae-Centaureinae (Hidalgo & al., 2006; Suárez-Santiago & al., 2007). Our goals with this combined approach are:

-To examine whether a phylogenetic subsectional classification of sect. *Acrocentron* is supported by molecular data;

-To confirm on molecular grounds the inclusion of sect. *Chamaecyanus* in sect. *Acrocentron* with subsectional rank;

-To verify the existence of the suggested centres of speciation of the group;

-To explore and reconstruct the history of the expansion of *Acrocentron* through the Mediterranean and establishing the centre of origin of the group.

MATERIAL AND METHODS

Plant material. – The ETS (external transcribed spacer) and ITS (internal transcribed spacer) regions of the nrDNA of 45 taxa of the sect. *Acrocentron* and sect. *Chamaecyanus* were sequenced. In some cases, more that one population was sequenced. Our ITS1 and ITS2 sequences from previous works (Font & al., 2002) have been completed with the sequence of the 5.8 S gene. Sampling included all the geographical areas of distribution of the section: Eastern Mediterranean (Balkan Peninsula and Anatolia), Irano-Turanian, Western Mediterranean (Iberian and Italian Peninsulas, and North Africa) and some species of wide distribution like *Centaurea scabiosa* L.

A chloroplast non-coding region, the intergenic spacer *trn*H-*psb*A, was also sequenced. In view of the very low number of informative characters found in this region, only a representative subset of taxa (30 taxa) was sequenced.

Outgroup was chosen among the genera of the "derived clade" of the subtribe Centaureinae (Garcia-Jacas & al. 2001), where the *Acrocentron* group belongs, and included one species each from the *Cyanus* and *Jacea* groups of *Centaurea* and the genera *Carduncellus* Adans. and *Phonus* Hill.

Voucher data, source and GenBank sequence accession numbers are given in Appendix 1.

DNA Extraction, Amplification and Sequencing. – Total genomic DNA was extracted following the CTAB method of Doyle & Doyle (1987) and Cullings (1992) from silica gel dried leaves collected in the field. In some cases, herbarium material was used.

nrDNA ITS and ETS region and cpDNA amplification strategies: Double-stranded DNA of the ITS region was amplified using ITS1 (White & al., 1990) and 1406F (Nickrent & al., 1994) as the forward primer and ITS4 as the reverse primer (White & al., 1990). The profile used for PCR amplification follows the protocol described in Susanna & al. (2006). The ETS region was amplified with ETS1F as the forward primer and as reverse primers 18S-2L (Linder & al., 2000) or 18SETS (Baldwin & Markos, 1998). The PCR was executed with the following conditions: 5

min denaturing at 95 ºC, followed by 30 cycles of 94 ºC denaturing for 45 s, 48 ºC annealing for 45 s and 72 ºC extension for 40 s, with an additional 7 min at 72 ºC. PCR products of the species with more than one band were cloned using TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, except that only half reactions were used. When possible, from eight to fifteen positive colonies from each reaction were screened with direct PCR using T7 and M13R universal primers following the protocol of Vilatersana & al. (2007). Five to eight PCR products were selected for sequencing in both directions using the same primers.

The intergenic spacer *trn*H-*psb*A was amplified using the primers trnH (GUG)F and psbAR (Hamilton, 1999). The profile used for PCR amplification follows the protocol of Vilatersana & al. (2007).

nrDNA and cpDNA sequencing strategies: Chloroplast and nuclear PCR products were purified with the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Direct sequencing of the amplified DNA segments was performed using a BigDye Terminator Cycle Sequencing v3.1 (Applied Biosystems, Foster City, CA), following the manufacturer's protocol. Nucleotide sequencing was performed on an ABI PRISM 3100 DNA Analyzer (Applied Biosystems, Foster City, CA). Unique substitutions in clones from one accession were excluded. Consensus sequences were generated for each accession and region, condensing the single base-pair differences among clones. This reduced the size of the matrices as well as the impact of potential PCR artifacts (chimerical sequences and Taq errors; Cline & al., 1996; Popp & Oxelman, 2004).

Phylogenetic analyses. **–** Sequences were aligned visually by sequential pairwise comparison (Swofford & Olsen, 1990). The data matrices are available on request from the corresponding author. In view of the practical impossibility of unambiguously aligning the most variable 5' end of the ETS region of the ingroup and the outgroup, two analyses were carried out. First, we analyzed the more conserved 3'ETS region plus the ITS region including the outgroups, with the aim of identifying the basal clade of *Acrocentron*. Second, we carried out the analysis of the complete region ETS plus ITS, including only the species of the *Acrocentron* group,

using as outgroup the clade identified as basal in the first analysis. For the analysis of the *trn*H-*psb*A plastid region, we used as outgroup some representatives from the clade identified as basal in the first analysis.

Phylogenetic analyses were conducted using both parsimony and Bayesian inference optimality criteria. The plastid dataset was independently analyzed. Both nrDNA regions (ETS plus ITS) were combined and analyzed in one matrix because both datasets resulted in fully compatible topologies. A partition homogenity test was impossible to complete because heuristic search collapsed after reaching the tree limits of PAUP in the first replicate.

Parsimony analysis involved heuristic searches conducted with PAUP ver. 4.0b10 (Swofford, 2002) using tree-bisection-reconnection (TBR) and branch swapping with character states specified as unordered and unweighted. The indels were treated as missing data in all the analyses. All most-parsimonious trees were saved. To locate other potential islands of most-parsimonious trees (Maddison, 1991) 100 replications were performed with random taxon addition, also with TBR branch swapping. Consistency index (CI) and retention index (RI) are always given excluding uninformative characters. Bootstrap analyses (BS) was performed for assesing the confidence of the branches (Felsenstein, 1985). For the combined matrices (ETS plus ITS), bootstraping followed the approach by Lidén & al. (1997) using 1000 replicates, random taxon addition with 10 replicates, and no branch swapping.

For the Bayesian analysis, the data sets were analyzed using MrModeltest ver. 2.2 (Nylander, 2004) to determine the sequence evolution model that best described our data. This model was used to perform a Bayesian analysis using the program MrBayes ver. 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Four Markov chains were run simultaneously for 1,000,000 generations and sampled every 100 generations. Data from the first 1000 generations were discarded as the “burn-in” period, after confirming that likelihood values had stabilized prior to the 1000^{th} generation. The 50% majority rule consensus phylogeny and posterior probability of nodes (PP) were calculated from the remaining sample.

RESULTS

Numerical results of the analyses are shown in Table 1.

Table 1. Numeric results of the phylogenetic analyses.

Data set	ETS+ITS	3'ETS+ITS	*trn*H-*psb*A
Taxa	45	49	30
Number of sequences	61	65	33
Total characters	1859	1142	443
Informative characters	225	173	8
Number MPTs	815072	34446	16
Number of steps	494	399	13
Consistency index (CI)	0.5423	0.5396	0.3913
Retention index (RI)	0.8582	0.8090	0.6410
Range of divergence, ingroup (%)	0-0.1193	0.0008-0.091	0-0.875
Model	GTR+I+G	GTR+I+G	GTR+I+G

The analysis of the 3'ETS plus ITS regions is shown in Fig. 1. The *Acrocentron* group is monophyletic with high support (BS= 100%, PP= 1.0). The basal group of *Acrocentron* is the clade of species of Eastern origin (BS= 58%, PP= 1.0); firstly the Anatolian and Irano-Turanian taxa (clade ANT) and afterwards the Aegean taxa (clade AEG). Some species of wide distribution (the *Centaurea scabiosa* group) ranging from the Iberian Peninsula to Central Asia, together with a group of taxa from the North Balkans and North Italy (clade BAL), are sister to the rest (BS= 65%, PP= 1.0). Finally, there is a dichotomy comprising two clades. The first subclade is a well-supported group (BS= 80%, PP= 1.0) with all the species from North Africa and Sicily and some others from the Iberian Peninsula (clade NA). The second subclade (BS= 96%, PP= 1.0) includes only species from the Iberian Peninsula (clade IB).

The analysis of the complete ETS plus ITS region (Fig. 2) shows an important difference with the partial analysis: The Iberian and North African clades, which formed a well-supported clade in the first analysis (clades IB and NA, Fig. 1), are not grouped together in the complete analysis. In this second analysis, the Balkan clade

is sister to the Iberian clade, and this joint clade is sister to the North African clade (clades BAL, IB and NA; Fig. 2). Besides this diverging result, there are some other minor but striking differences. The clones of *C. josiae* Humbert form a monophyletic clade in the partial analysis (Fig. 1), but one of the clones is associated to *C. amourensis* Pomel in the complete analysis (Fig. 2). One of the clones of *C. argecillensis* Gredilla (species from Central Iberia), which in the partial analyses occupies an unresolved position at the base of the North African clade (Fig. 1), is ranged together with *C. litardierei* Jahand. & Maire in the complete analysis (Fig. 2). There is also a difference in the group formed by the clones of Portuguese *C. prolongi* Boiss., which appear on different clades in the partial analysis (clade IB, Fig. 1) and unsupportedly united in the complete analysis (clade IB, Fig. 2). Finally, *C. lainzii* Fern. Casas, which appears as an isolated taxon in the partial analysis (clade NA, Fig. 1), is united to *C. prolongi* and *C. haenseleri* Boiss. in the complete analysis (clade NA, Fig. 2).

The complete analysis suggests that the species from the Aegean (clade AEG, Fig. 2) are closer to the outgroup formed by the Anatolian and Irano-Turanian species (clade ANT, Fig. 2). Next, there come two clades, one with species from the Balkans and some others of wide distribution (clade BAL, Fig. 2), and the taxa from the Iberian Peninsula (clade IB, Fig. 2); and a clade with species of North Africa, Sicily and the rest of the Iberian taxa (clade NA, Fig. 2) with high support both from BS and PP.

The results of the chloroplast sequencing are summarized in Table 1. The low number of informative characters explains the lack of resolution in the phylogenetic analysis (tree not shown). Table 2 shows the changes of the sequences in the chloroplast dataset.

Table 2. Variable characters in the cloroplast trnH-psb A sequences. Variable characters in respec to to the first sequence are boldfaced. Number above each character in the sequence indicates in each data seat (- =gap; OR= Origin; AR= Armenia;ANT = Anatolia, BAL= Balcans; I= Iberian; IR= Iran; NA= North Africa; WD= wide distribution).

Species and population	OR						1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
		6	7	7	7	8	2	2	3	3	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	5	6	6	7	7	7	7	7	2	2	2	2	2	2	2	2	2
		5	5	7	8	0	2	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	1	8	9	0	1	2	5	9	0	1	2	3	4	5	6	7	8	9
C. glehnii	AR	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. lydia	ANT	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	G	G	–	–	–	–	–	–	–	–	–
C.salonitana	BAL	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	T	T	T	T	A	T	C	T	T	T
C. amblensis	I	C	T	T	T	T	–	T	T	T	A	G	T	A	G	T	A	T	T	T	A	C	C	T	T	A	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. argecillensis	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. borjae	I	C	A	A	A	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. cephalariifolia	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. clementei	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. crocata	I	C	A	A	A	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. collina	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. gabrielis-blancae	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. galianoi	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. granatensis	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. haenseleri	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. kunkelii	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. lasgacana	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. lainzii	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. legionis-septimae	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. mariana	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	A	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. ornata	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. podospermifolia	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. prolongi 1335	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. prolongi 1353	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. saxicola	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. toletana 1817	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. toletana 1818	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. toletana 1819	I	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. kandevanensis	IR	C	A	A	A	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. amourensis	NA	C	A	A	A	A	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	A	–	–	–	A	A	–	–	–	–	–	–	–	–	–
C. carolipauana	NA	C	A	A	A	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	A	A	A	A	A	G	–	–	–	–	–	–	–	–	–
C. litardierei	NA	C	A	A	A	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–
C. pubescens	NA	C	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	A	–	–	–	A	A	–	–	–	–	–	–	–	–	–
C. scabiosa	WD	T	T	T	T	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–

Species and population	OR	2	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
		3	3	3	1	1	1	1	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3	3	6	6	6	6	6	8	8	8	8	8	8	9
		0	1	2	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	9	2	5	6	7	8	1	2	3	4	5	8	8
C. glehnii	AR	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	A	–	–	–	–	–	–	–	–	–	A	A
C. lydia	ANT	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	A	–	–	–	–	–	–	–	–	–	A	A
C.salonitana	BAL	G	T	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	A	–	–	–	–	–	–	–	–	–	C	A
C. amblensis	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. argecillensis	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. borjae	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	A	T	A	T	T	–	–	–	–	–	A	A
C. cephalariifolia	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. clementei	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. crocata	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. collina	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	A	–	–	–	–	–	–	–	–	–	A	A
C. gabrielis-blancae	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. galianoi	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	A	–	–	–	–	–	–	–	–	–	A	A
C. granatensis	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. haenseleri	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. kunkelii	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	C
C. lasgacana	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	G	–	–	–	–	–	–	–	–	–	A	A
C. lainzii	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	A	–	–	–	–	–	–	–	–	–	A	A
C. legionis-septimae	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	A	–	–	–	–	–	–	–	–	–	A	A
C. mariana	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	T	–	–	–	–	–	–	–	–	–	A	A
C. ornata	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. podospermifolia	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. prolongi 1335	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	A	–	–	–	–	–	–	–	–	–	A	A
C. prolongi 1353	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. saxicola	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. toletana 1817	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. toletana 1818	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. toletana 1819	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. kandevanensis	IR	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	T	T	T	T	A	A	A
C. amourensis	NA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A
C. carolipauana	NA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	G	–	–	–	–	–	–	–	–	–	A	A
C. litardierei	NA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	C	G	–	–	–	–	–	–	–	–	–	A	A
C. pubescens	NA	–	–	–	T	C	A	A	A	A	T	T	A	T	A	A	C	T	A	A	A	A	T	A	G	A	C	T	–	–	–	–	–	–	–	–	–	A	A
C. scosa	WD	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	A	–	–	–	–	–	–	–	–	–	A	A

DISCUSSION

Taxonomic implications: Sectional classification. – The classic taxonomic classification in two sections, *Acrocentron* and *Chamaecyanus*, is untenable after our results. The segregation of sect. *Chamaecyanus* (species of this section are marked CH in the Figs. 1 and 2) would leave *Acrocentron* paraphyletic. In the Iberian clade (Figs. 1 and 2), species of both sections are part of a polytomy. Both sections are morphologically well defined, but intermediates between them are very frequent (Fernández Casas & Susanna, 1986), a fact that is probably the reason of this entanglement and the low support for a clade that comprises many of the species of *Chamaecyanus* (Figs. 1 and 2). The position of *Centaurea crocata* Franco as sister to the Iberian clade is in this sense very significant: On the basis of morphology, *C. crocata* is suspected a hybrid between taxa of both sections (Garcia-Jacas & Susanna, 1994), which would explain this basal position. The most appropriate status for *Chamaecyanus* is the subsectional within *Acrocentron*, confirming previous suggestions (Font & al., 2002).

Regarding the possibility of establishing a subsectional classification within *Acrocentron* as suggested by previous workers (Hayek, 1901; Routsi & Georgiadis, 1994), molecular data are of little help because intense gene flow hinders reconstruction of phylogenetic affinities, and the extreme homogeneity of morphologic characters makes any suggestion of infrasectional classification a very difficult task. As is evident from Figs. 1 and 2, molecular data group the taxa on a geographic basis.

Hybridization and reticulation. – In our previous study based on the ITS region (Font & al., 2002), we suggested that the very low definition within *Acrocentron* was probably caused by the high levels of hybridization and reticulation within the group. Our new results support this view, as exemplified in the presence of an ETS clone of *C. argecillensis* from Central and North East Iberia within the North African clade, whilst the rest of the clones of this species are placed in the Iberian Peninsula clade (Figs. 1 and 2). There are some other examples, maybe not so extreme but also indicating intense gene flow: *C.*

toletana Boiss. & Reut. (4*n*) has a clone united to *C. podospermifolia* Loscos & J. Pardo with high PP support, and another one ranged within the Iberian polytomy (Figs. 1 and 2). Another case is a population of *C. prolongi* Boiss. from Portugal, firstly cited as *C. collina* L., later on identified as *C. prolongi* (Garcia-Jacas & Susanna, 1994) and even considered a new species, *C. occasus* Fern. Casas (Fernández Casas, 1997). The two clones of this population of *C. prolongi* appear in the same clade, but in different position (*C. prolongi* Port, clade IB; Fig. 1).

Centaurea prolongi poses another problem. Populations of this species appear in two different clades: The Portuguese population is associated to the taxa from Central and North Iberia, whilst populations from the extreme south of Spain are placed in the African clade (*C. prolongi* Pop. 1 and Pop. 2, clade NA; Figs. 1 and 2). This segregation shows that they have different ribotypes, a fact that could reinforce the hypothesis that they are two different species. However, morphological differences alleged by Fernández Casas (1997) are only quantitative (namely, the size of the leaves and florets) and could be easily explained by the different climatic conditions of both localities (milder and wetter in Portugal). The alternate explanation, which we favour until more evidence is available, is gene flow: In the populations of *C. prolongi* and other species of *Acrocentron* growing nearby in South Spain (*C. haenseleri* and *C. lainzii*), repeated onsets of African genomes have been detected (Font & al., in prep.). To the contrary, the studied Portuguese locality was far apart from the waves of African taxa and the affinities of the ribotypes found in *C. prolongi* from Portugal point towards Central Iberia. Inconsistencies regarding clones of *C. prolongi* and other differences discussed above between complete and partial analyses can be explained by reticulation, more obviously reflected in the phylogeny when the more variable 5' end of the ETS is included.

Other related examples also support reticulation. Extensive and repeated hybridization and introgression has been detected in the *C. toletana* complex, the species of which usually exhibit more that one ribotype (Font & al., in prep.). In our analyses, *C. legionis-septimae* Fern. Casas & Susanna, a narrow endemic from the mountains of northern Spain, is united in our analyses to *C.*

cephalariifolia Willk., a species from the *C. scabiosa* widespread group. This position contradicts morphologic and biogeographic evidence and must be considered another evidence of hybridization, as confirmed by the existence of hybrid populations between both taxa described as *C. x cephalariiseptimae* Fern. Casas & Susanna. Gene flow is extremely favoured by the fact that most of the hybridization in *Acrocentron* is homoploid (Font & al., in prep.).

There are many polyploid species in *Acrocentron*, most of them hexaploids and tetraploids. Hexaploid taxa are obviously allopolyploids by addition of di- and tetraploid genomes. As to the frequent tetraploid species in *Acrocentron*, Font & al. (in prep.) strongly suggest that tetraploid *C. toletana* is an autotetraploid, the successful establishment of which depended on the availability of an appropriate ecological niche for the new chromosomal race. These findings could suggest that polyploidy is not necessarily linked to hybridization in many other tetraploids from the *Acrocentron* group.

The position of the Balkan clade, which is united to the Iberian clade in the phylogenetic analysis (Fig. 2), can also be explained in terms of hybridization and introgression. This relationship is caused by the gene flow of the widely distributed taxa from the clade BAL and the Iberian species. The culprit is certainly *C. cephalariifolia*, a tetraploid race of the widespread diploid *C. scabiosa*: To date, up to six naturally occurring hybrids have been reported between *C. cephalariifolia* and other Iberian taxa (we have discussed above one of them, *C. x cephalariiseptimae*). In four cases, the crosses involve two tetraploid parental species and the hybrids are also tetraploid (Kummer, 1977; Fernández-Casas & Susanna, 1986) and thereafter they are not karyologically isolated from their progenitors.

The final reason for considering high levels of introgression is the result of the chloroplast sequencing (Table 2): The few changes observed in the sequences of the intergenic spacer *trn*H-*psb*A do not agree with any known relationship, either taxonomic or biogeographic. Cytoplasmic gene flow is one of the main causes of low resolution in phylogenies (Rieseberg & Soltis, 1991) and in *Acrocentron* chloroplastic gene flow has been extensive from the beginning of its diversification and expansion.

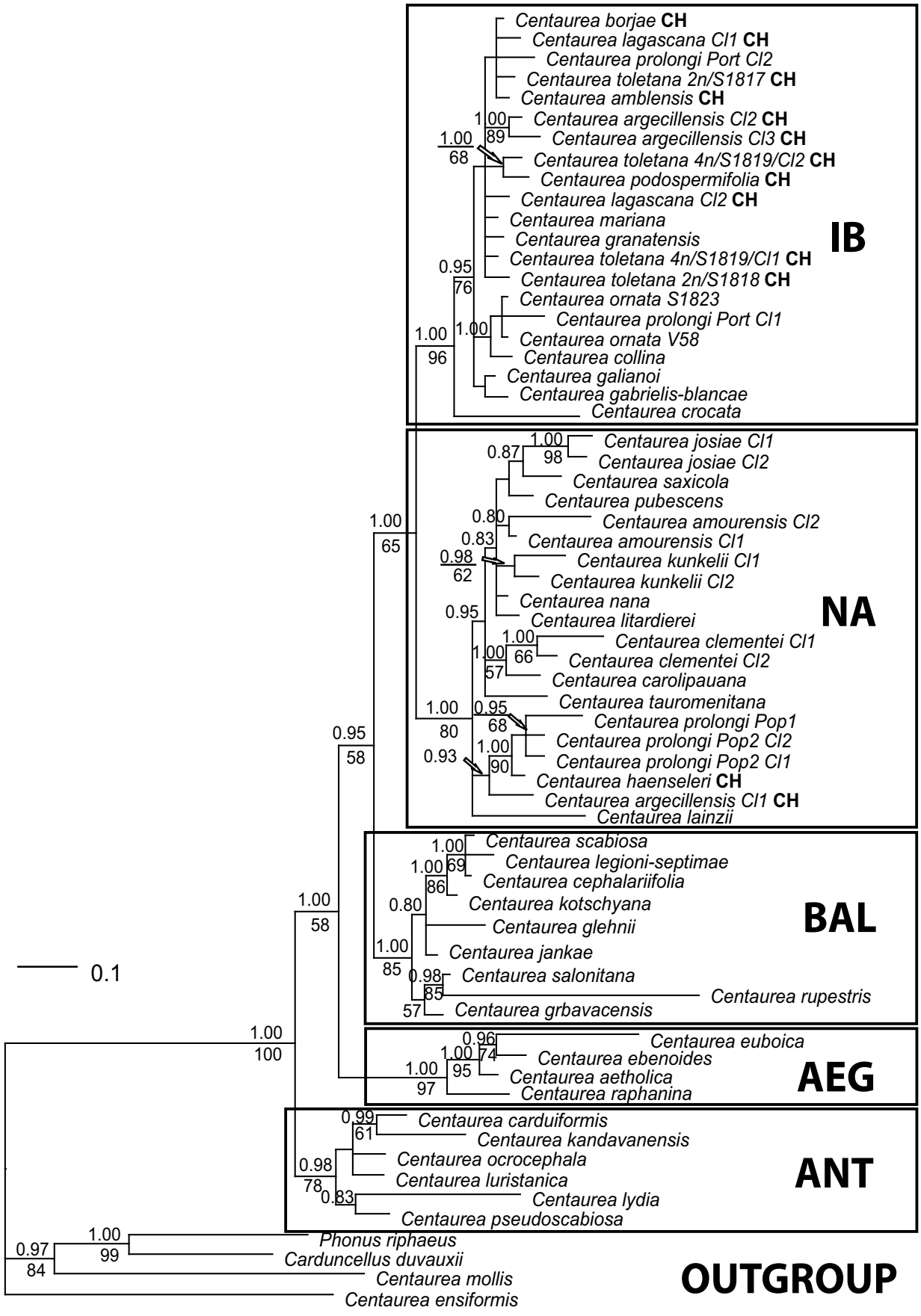

Fig. 1. Consensus tree obtained from 9,000 Bayesian trees with higher posterior probability (PP) from the combined 3'ETS and ITS data with an extended outgroup. Numbers above branches indicate Bayesian-credibility values (PP); numbers below branches indicate parsimony BS. AEG= Aegean clade; ANT= Anatolian-Iranian clade; BAL= Balkan clade; IB= Iberian clade; NA= North African clade. CH= section *Chamaecyanus*; Cl= clone; Pop= population.

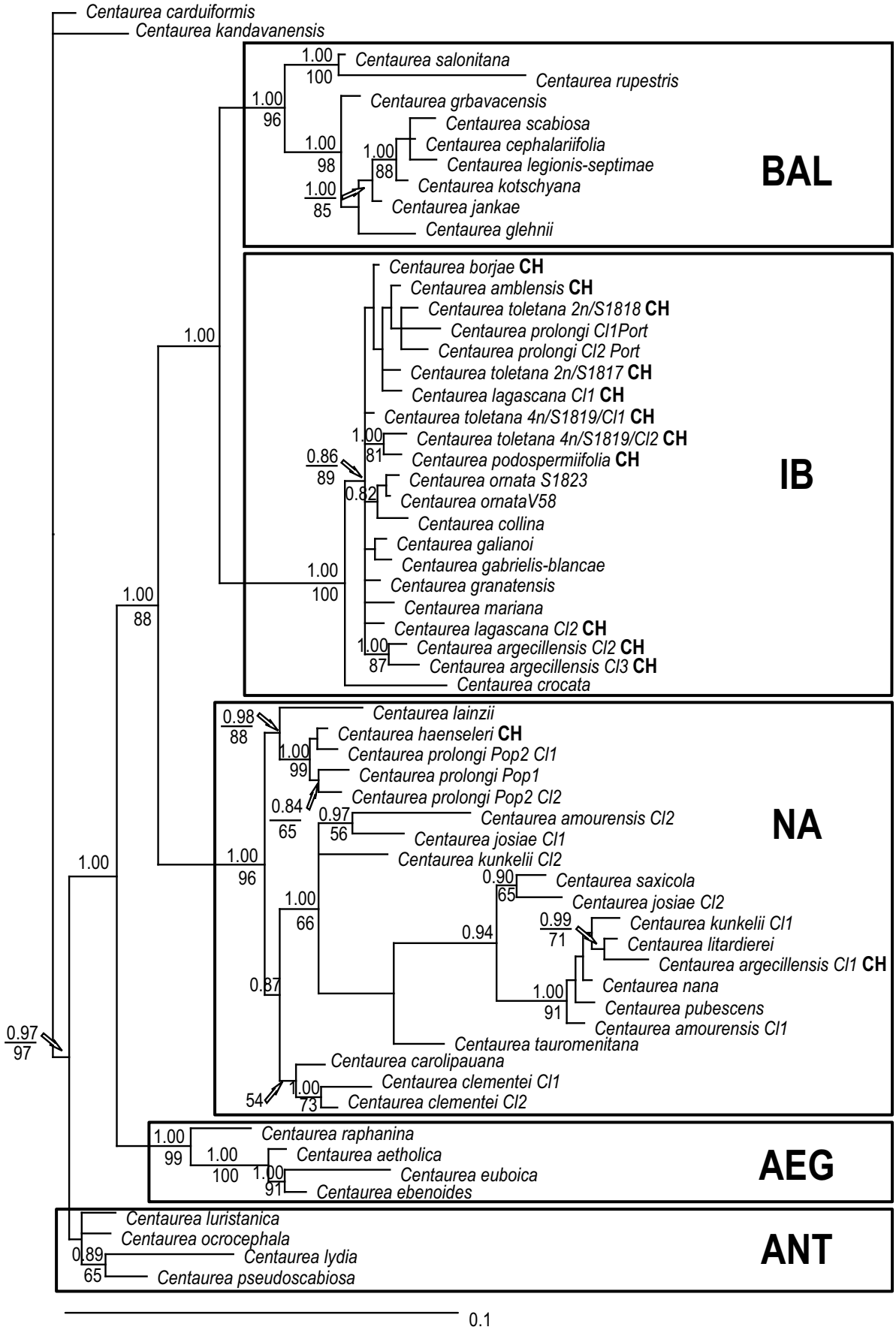

Fig. 2. Consensus tree obtained from 9,000 Bayesian trees with higher posterior probability (PP) from the combined 3'ETS and ITS data with the Anatolian-Iranian clade as outgroup. Numbers above branches indicate Bayesian-credibility values (PP); numbers below branches indicate parsimony BS. AEG= Aegean clade; ANT= Anatolian-Iranian clade; BAL= Balkan clade; IB= Iberian clade; NA= North African clade. CH= section *Chamaecyanus*; Cl= clone; Pop= population.

Biogeography. – The combined ITS plus ETS phylogeny (Figs. 1 and 2) shows that they are four speciation centers: Anatolia-Iran, the Aegean (these two were united in one by Font & al., 2002), the Balkans (including some species of wide Eurasian distribution), the Iberian Peninsula and North Africa. Geographic denominations for the clades are general and do not imply that all the taxa within this clade grow in this precise geographic area, but they are accurate enough for the purpose of briefly discussing each centre and the history of the geographic distribution of *Acrocentron* (Fig. 3).

Distribution of *Acrocentron* has been presented as an East to West migration (Garcia-Jacas & Susanna, 1992). In confirmation of this hypothesis, the Anatolian-Iranian centre is basal to the rest in the first of our analyses, 3'ETS plus ITS (Fig. 1). The origin of *Acrocentron* should be placed in the Eastern Mediterranean, much like the rest of the subtribe according to the higher richness of genera of the Centaureinae in this area (Susanna & Garcia-Jacas, 2007). We must recall, however, that the hypothesis of the Irano-Turanian region as the place of origin of the group was rejected on karyologic grounds and instead it was considered a later expansion from Anatolian populations (Garcia-Jacas & al., 1998).

From Anatolia, species of *Acrocentron* radiated to the Aegean islands, the species of which (*C. aetholica* Phitos & T. Georgiadis, *C. ebenoides* Heldr., *C. euboica* Rech. f. and *C. raphanina* Sibth. & Sm.) form a well-supported clade (AEG in Figs. 1 and 2) that is sister to the next branches. The Aegean islands and south Greece constitute the easiest way of penetration from Anatolia to the Western Mediterranean, which is consistent with the intermediate position of the Aegean clade in the phylogeny (Figs. 1 and 2).

The Aegean clade is at the basis of the two main radiations of *Acrocentron*, as reflected in our phylogeny (Fig. 1). In the north of its range, it originated the Balkan clade in, and in the south it migrated to extreme south of the Italian Peninsula (Fig. 3).

The Messinian salinity crisis (between 6 and 5.2 MYA) offers a good opportunity for dating the migration of the *Acrocentron* group. In the East, a pass between Anatolia and the Aegean was already open by middle Miocene

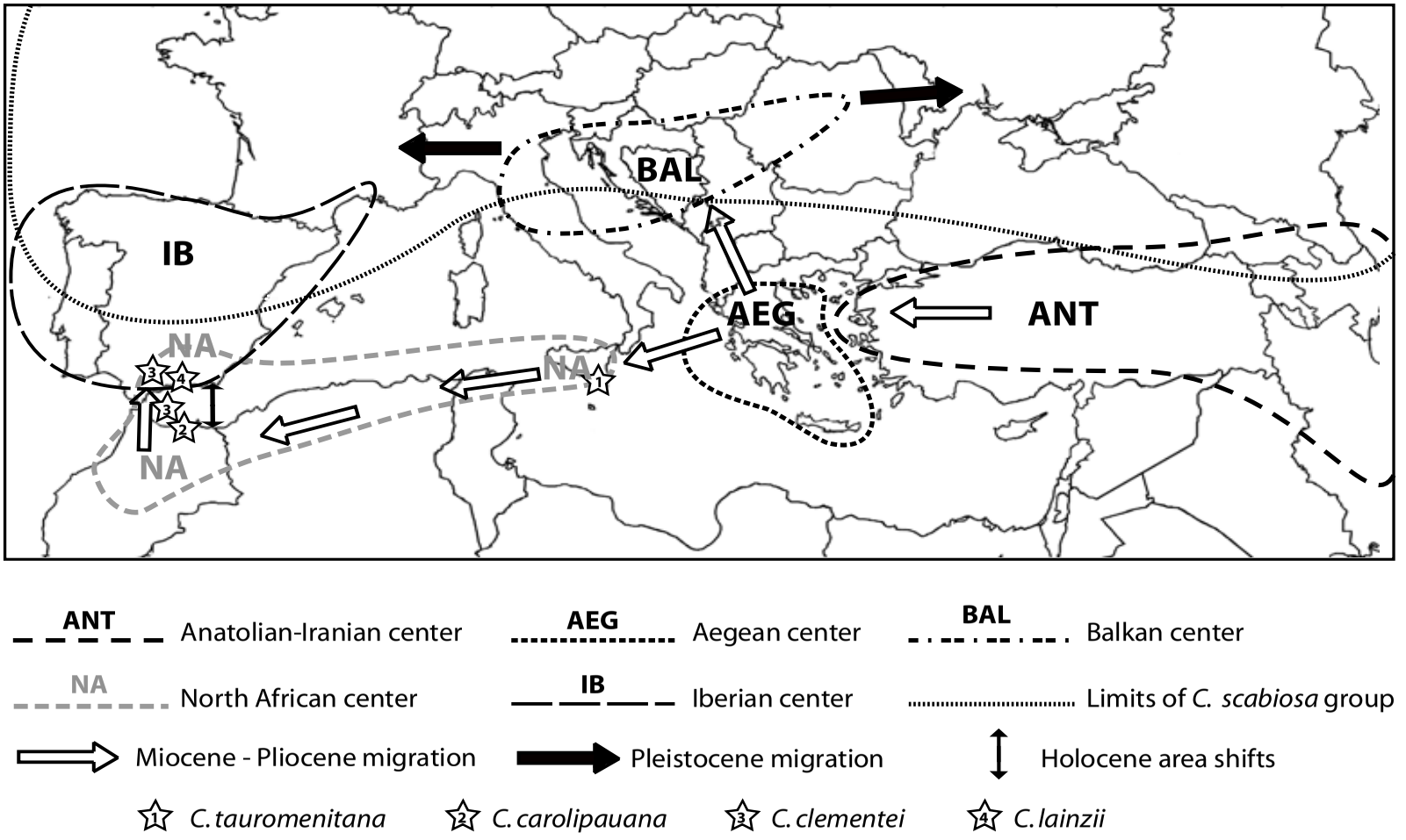

Fig. 3. Geographic representation of the two migration pathways of section *Acrocentron* in the Mediterranean.

(Çağatay & al., 2006). The lowering of the sea level opened the way from the Aegean and Greece to the extreme South of Italy and Sicily, and then to North Africa and the Iberian Peninsula (Fig. 3). The main migration of *Acrocentron* through this southern pathway must have terminated in the Pliocene because species of *Acrocentron* are absent in all the western Mediterranean archipelagos, which that during the Messinian were connected to the mainland. This datation is consistent with the suggested date for the expansion of the related complex *Acrolophus-Phalolepis* of the genus *Centaurea* (Suárez-Santiago & al., 2007), a group highly speciated in Iberia but also absent from the Balearic Islands.

Besides paleogeographic arguments, a series of landmarks illustrate this path. Four species closely related on morphological grounds mark the southern way of the migration: *C. tauromenitana* Guss. from Sicily, *C. carolipauana* Fern. Casas & Susanna from Northern Morocco, *C. clementei* Boiss. from Northern Morocco and South Iberia and *C. lainzii* from South Iberia (Fig. 3). All of them are relictic mountain plants and, with the exception of *C. clementei*, are known from a single locality. They are chasmophytes sharing some unusual characters in *Acrocentron* like the very wide involucral bracts ending in an unarmed black appendage (Fernández Casas & Fernández Morales, 1979). Figure 3 summarizes the hypothesized way of expansion of the group, together with the distribution of these landmark species. The expansion of the group to the rest of the Iberian Peninsula and North Africa was the natural consequence, favoured by the developing of a Mediterranean climate. The poverty in species of *Acrocentron* in the North African lowlands stretching between Algeria and Libya reflect the deep changes in the vegetation of this area in recent times. Only one species of the group survived in the desert, but at the price of a dramatic adaptation: *Centaurea omphalodes* from the oasis of Algeria is the only annual species of *Acrocentron*.

As a final proof of the predominance of the southern way of migration, in northern and middle Italy some species of *Acrocentron* can be found, but they are related to the Balkan clade and did not cross the huge barrier of the Alps into France (Fig. 3). The only species of *Acrocentron* present in France belong

to the widespread *C. scabiosa* group, with the only exception of one species shared with East Iberia (*C. collina*).

Migration through the southern path would also explain the pattern of distribution of *Acrocentron* in Iberia. Figure 4 shows the richness in number of species in Iberian Peninsula and Morocco. As we go north, the number of taxa diminishes and in northern Iberia species of *Acrocentron* are scarce and most of them (three out of four) are relictic paleopolyploids (Garcia-Jacas & Susanna, 1992).

A later entry of *Acrocentron* following a northern pathway can be tracked in the Iberian Peninsula, originated among the species of the Balkan clade. The continuous distribution of species of this group in Europe and Middle Asia, from the Iberian Peninsula and Great Britain to Kazakhstan, is a recent radiation based in a single species, *C. scabiosa*. Many local variants of this species have been described as new taxa on insufficient morphological basis, which confirms that they are of very recent age. Even those taxa from the *C. scabiosa* complex that are usually accepted by synantherologists, *C. cephalariifolia* from Spain (a tetraploid), *C. glehnii* Trautv. from Armenia and *C. kotschyana* Heuff. from Poland, have been diversely considered at subspecific rank within *C. scabiosa*. This extreme geographic radiation (Fig. 3) was favoured by the occurrence of a cold steppe continuum from Iberia to Asia along the repeated interglacial periods in the Pleistocene-Holocene (Prentice & al., 2000).

In our molecular reconstruction based on ITS plus ETS region, phylogeny of the Iberian and African taxa is obscure and unresolved because present distribution of ribotypes in Iberia is deeply mediated by the troublesome climatic history of the Iberian Peninsula and North Africa during the Holocene (reviewed in Font & al. in prep.). The succession of glaciations forced mountain species of *Acrocentron* to migrate to lower latitudes, and species that were isolated became sympatric. Iberian species could migrate south to Africa during glacial maximums, and African taxa moved to the north during the warmer interglacial periods (Fig. 3). The resulting pattern of generalized introgression is reflected in the nrDNA phylogeny, with even North Iberian clones deeply nested in the North African clade. Maybe the most striking result, which confirms the

impact of glaciation-mediated migrations and hybridization in *Acrocentron*, is the otherwise inplausible group formed by one clone of *C. argecillensis* from Central Spain and a clone of *C. litardierei* from the High Atlas (Figs. 1 and 2).

CONCLUDING REMARK

Our study demonstrates the usefulness of the analyses of the ETS region for phylogenetic and biogeographic investigation of highly hybridized groups. The unravelling of the pathways followed by the *Acrocentron* group offers a possible model for many other groups with a suggested Eastern Mediterranean origin and secondary centres of speciation in the Iberian Peninsula and North Africa.

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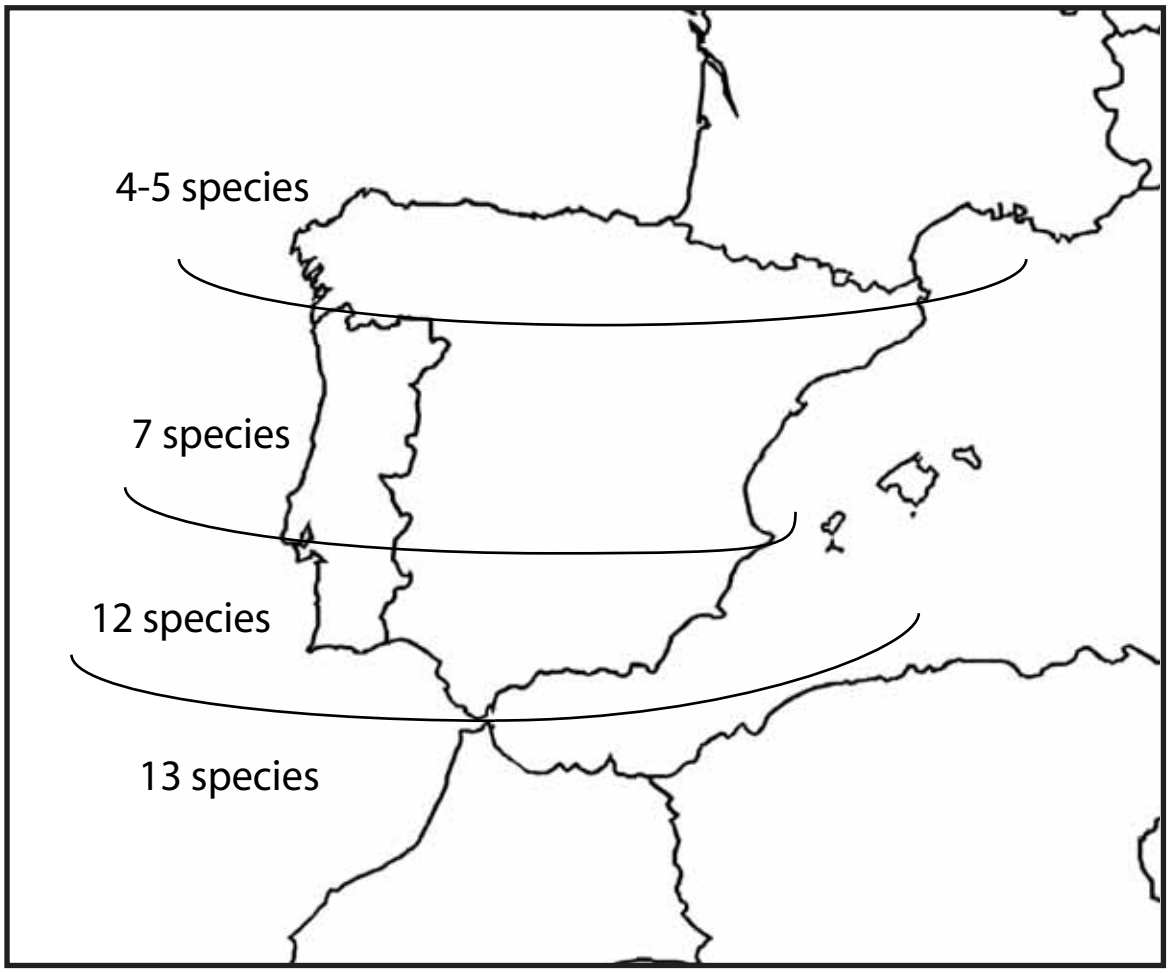

Fig. 4. Density of species gradient of the *Acrocentron* group in the Iberian Peninsula and North Africa

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Appendix 1. Origin of the material, herbaria where the vouchers are deposited and GenBank accesions numbers. *Taxa that have been cloned. Species, origin, herbaria, and GenBank accession number (ITS, ETS, *trn*H-*psb*A).

Ingroup: *Centaurea aetholica* Phytos & Georgadis, Greece, Achaia: Antirion-Gavrolimni, *Damboldt s. n.* (B); *C. amblensis* Graells, Spain, Ávila: El Barco de Ávila, sine col. (BC); **C. amourensis* Pomel, Morocco, Zagora: North east side of the Atlas, *M. Standinger & M. Finckh* (BC); **C. argecillensis* Gredilla, Spain, Guadalajara: Argecilla, mountain slopes on the road to Ledanca, *Font & Susanna 1811* (BC); *C. borjae* Valdés-Berm. & Rivas Goday, Spain, La Coruña: near El Ferrol, cabo Prior, S side, *Garcia-Jacas & Susanna 2074* (BC); *C. carduiformis* DC., Armenia, Talin: between Pokr Arthik and Bagravan, *Susanna 1527 & al.* (BC); *C. carolipauana* Fern. Casas & Susanna, Morocco, Tetuan: Djebel Tasaot, 14.5 km from the cross road to Talembote, *Garcia-Jacas & Susanna 1437* (BC); *C. cephalariaefolia* Willk., Spain, Huesca: between Capdesaso and San Lorenzo del Flumen, *Vilatersana 20* (BC); **C. clementei* Boiss., Spain, Cádiz: Grazalema, overhanging limestone, *Garcia-Jacas & Susanna 1350* (BC); *C. collina* L., Spain, Alacant: between Muro de Alcoi and Beniarrés, *Garcia-Jacas, Susanna 1481 & Vilatersana* (BC); *C. crocata* Franco, Portugal, Algarve: 5 km N of Monchique on the road to Odemira, *Roché & Susanna 1917* (BC); *C. ebenoides* Heldr., Greece, Euboea island: near Limni, on the coast road, *Phytos & Kamari 20427* (B); *C. euboica* Rech. f., Greece, Euboea island: Kandili mountains, between Achmet Aga and Hagios, *Rechinger 18215* (B); *C. gabrielis-blancae* Fern. Casas, Spain, Navarra: Lumbier, Foz de Lumbier, *Garcia-Jacas & Susanna 1592* (BC); *C. galianoi* Fern. Casas & Susanna, Spain, Huelva: Sierra de Aracena, between Linares de la Sierra and Alaján, *Garnatje 55 & Vilatersana* (BC); **C. glehnii* Trautv., Armenia, Peninsula of Artanish, *Susanna 1527 & al.* (BC); *C. granatensis* Boiss., Spain, Granada: Sierra Guillimona, Gorgas de Torilla, *Valdés & al.* (SEV); *C. grbavacensis* (Rohlena) Stoj. & Acht., Greece, Macedonia: Kozuf mountains, south side of mt. Tzena, *Greuter 14108* (B); **C. haenseleri* Boiss., Spain, Málaga: 10 km to Jubrique, *Garcia-Jacas & Susanna 1888* (BC); *C. jankae* Brandza, Dobrogea,

Badarau s. n. (CLCB); **C. josiae* Humbert, Morocco, Ksar el Souk: Tizi'n Talrhemt, *Garnatje, Susanna 1792 & Vilatersana* (BC); *C. kandavanensis* Wagenitz, Iran, Mazandaran: Chalus road, 2 km from the cross to Baladeh, *Susanna 1621 & al*. (BC); *C. kotschyana* Heuff., Botanical Garden "Al. Borza", Cluj-Napoca, Rumania, (BC); **C. kunkelii* N. Garcia, Spain, Almería: road AL-411 between Roquetas and Canjáyar, *Garcia-Jacas, Susanna 1612 & Vilatersana* (BC); **C. lagascana* Graells, Spain, Palencia: 500 m from Alba de los Cardaños, *Font & Susanna* 1822 (BC); *C. lainzii* Fern. Casas, Spain, Sierra Bermeja: 11 Km from Estepona on the road to Igualeja, *Garcia-Jacas & Susanna* 1330 (BC); *C. legionis-septimae* Fern. Casas, Spain, León: pr. vicum Crémenes, *Fernández Casas & Susanna* (SEV); *C. litardierei* Jahand. & Maire, Morocco, Ksar es Souk: south side of Col du Zad, *Garnatje, Susanna 1797 & Vilatersana* (BC); *C. lydia* Boiss., Turkey, Konya: Seydisehir, Mortas, *R. Ilarsan 4312* (ANK); *C. luristanica* Rech. f., Iran, Khuzistan: ca. 15 km from Eizeh on the way to Dehdez, Mozaffarian (TARI); *C. mariana* Nyman, Spain, Almería: north side of the Sierra María, *Garcia-Jacas & Susanna 1342* (BC); *C. nana* Desf., Morocco, Ksar es Souk: north side of Col du Zad, 1 km from Aïn Leuh on the road to Azrou, *Garnatje, Susanna 1799 & Vilatersana* (BC); *C. ocrocephala* Wagenitz, Iran, Azarbayjan-e-Gharbi: Orumiyeh, between Maranah and Haki, *Susanna 1693 & al.* (BC); *C. ornata* Willd., Spain, Soria: near San Esteban de Gormaz, *Garcia-Jacas & Susanna 1823* (BC); Spain, Huesca: road A-132 between Salinas and reservoir de la Peña, *Vilatersana 58* (BC); *C. podospermifolia* Losc. & Pard., Spain, Tarragona: Tortosa, 5 km from Monte Caro, *Susanna 2072 & al*. (BC); **C. prolongi* Boiss., Spain, Málaga: north side of the Sierra de Mijas, *Garcia-Jacas & Susanna 1335* (BC), population 1; Spain, Málaga: Sierra of Almijara, Frigiliana, *Galbany & Arrabal*, s. n., population 2; Portugal, Algarve: near Loulé, Cerro Botello over Fonte da Murta, *Garcia-Jacas & Susanna 1353* (BC); *C. pseudo-scabiosa* Boiss. & Buhse, Turkey, Erzurum: between Erzurum and Varto, 13 km from Varto, *Uysal 893* (KON); **C. pubescens* Wiilld., Morocco, Ksar es Souk: 2 km from Oued Amesheguir, *Garnatje, Susanna 1795 & Vilatersana* (BC); *C. raphanina* Sibth. & Sm., Greece, Chanion: Kydonias, mounts Levka*, Strid 15093 & Papanicolau* (B); *C.*

rupestris L., Greece, Macedonia: road E-90 between Véria and Kozáni, *Roché & Susanna 1967* (BC); *C. salonitana* De Vis., Greece, Macedonia: W from Efkarpia, *Roché & Susanna 2005* (BC); *C. saxicola* Lag., Spain, Murcia: La Azohía, near the watchtower, *Garcia-Jacas, Susanna 1616 & Vilatersana* (BC); *C. scabiosa* L., France, Lozère: between Le Sec and L'Aumède, near Chanac, *Vilatersana 52* (BC); *C. tauromenitana* Guss., Botanical Garden of Catania (BC); **C. toletana* Boiss. & Reut. (4x), Spain, Madrid: Redueña, road N-320, 4 km to Torrelaguna, *Font & Susanna 1819* (BC); *C. toletana* Boiss. & Reut. (2x), Spain, Toledo: Risco de las Paradas, *Font & Susanna 1817* (BC); Spain, Toledo: mountains above San Pablo on the track to Baños del Robledillo, *Font & Susanna 1818* (BC). **Outgroup:** *Carduncellus duvauxii* Batt. & Trab., Morocco, Al-Hoceima: 8 km S to Tafraoute, *Gómiz s. n.* (BC); *Centaurea ensiformis* P. H. Davis, Turkey, Mugla: Köycegiz district, Sandras Dağ range 13 km from Ağla, *Susanna 2251 & al.* (BC); *C. mollis* Waldst. & Kit., Ukraine, Podolia: Lysa Hora, 2 km E of Vilshanitsa near Zolochiv, *Boratyñski & Romo 0506D* (BC); *Phonus riphaeus* (Font Quer & Pau) G. López, Morocco, Al-Hoceima: Tleta oued Laou between Tarerha and Azenti, *J. M. Montserrat 4360, Pallàs & Veny* (BC).

5- CONCLUSIONS

CONCLUSIONS

L'ORIGEN DE LA POLIPLOÏDIA EN *ACROCENTRON*

- Les poblacions diploides de *Centaurea toletana* presenten més d'un ribotip, la qual cosa comporta que hi hagi hagut d'haver diverses hibridacions homoploides.

- L'origen de la forma tetraploide de *Centaurea toletana* ha estat degut a múltiples hibridacions intraespecífiques, fet que queda demostrat per la superposició de diferents ribotips ja presents a les poblacions diploides.

- En una poblacion tetraploide de *Centaurea toletana* s'hi ha trobat un ribotip diferent dels detectats a les poblacions diploides, el que demostra que hi ha hagut una hibridació homoploide després de la gènesi del poliploide.

- Podem concloure que la forma tetraploide de *Centaurea toletana* s'ha originat per autopoliploïdia a partir de *C. toletana,* diploide, confirmant el què ja es suposava pels caràcters morfològics. Els nostres resultats, però, posen en qüestió l'us tradicional dels termes autopoliploide i al·lopoliploide.

- *Centaurea argecillensis* és una espècie al·lopoliploide originada a partir de *C. toletana* tetraploide i una espècie diploide, probablement la predecessora de l'actual *C. podospermifolia*.

- Els processos de poliploïdia en el grup estan condicionats pel intens flux genètic entre les espècie Ibèriques i les Nord africanes. Aquest flux ha portat que moltes espècies del grup *Acrocentron* presentin més d'un ribotip.

- Els patrons de bandes dels cromosomes no ens han permès establir la relació existent en la sèrie poliploide de *Centaurea ornata*, ni tampoc hem pogut establir quins han estat els possibles parentals de *C. kunkelii.*

FILOGÈNIA I BIOGEOGRAFIA DE LA SECCIÓ *ACROCENTRON*

- El grup *Acrocentron* queda reduït a les seccions *Acrocentron* i *Stephanochilus*; la secció *Aegialophila* hauria de ser considerada un gènere apart, *Crocodylium*.

- La separació clàssica d'*Acrocentron* i *Chamaecyanus* en dues seccions no és sostenible. Segons les dades moleculars, l'estatus més apropiat per *Chamaecyanus* és el de subsecció dins la secció *Acrocentron.*

- Les dades moleculars no permeten establir una classificació subseccional dins d*'Acrocentron*.

- Les espècies de la sect. *Acrocentron* presenten uns alts nivells d'hibridació i introgressió. Aquests fets queden patents en la baixa definició filogenètica del grup.

- L'origen de la sect. *Acrocentron* queda establert a l'est de l'àrea mediterrània, en concret a la zona d'Anatòlia, i a partir d'aquí s'ha anat distribuint a la resta de centres d'especiació.

- Des d'Anatòlia, les espècies de la sect. *Acrocentron* van radiar cap a les illes de l'Egeu (pas obert en el miocè mitjà), i aquí es formà la base de les dues radiacions principals del grup. L'expansió més antiga, que finalitzà al pliocè, va portar a la sect. *Acrocentron* cap al sud d'Itàlia, Sicília, Àfrica del Nord i la Península Ibèrica. L'altra radiació, molt posterior (durant les glaciacions del neogen), va estendre *Acrocentron* des del nord dels Balcans cap al nord de la Península Ibèrica i fins a Àsia Central, en una distribució contínua eurasiàtica.