ANNALS OF BOTANY
Founded 1887

Evolution and biogeography of *Centaurea* section *Acrocentron* inferred from nuclear and plastid DNA sequence analyses

Mònica Font[1], Núria Garcia-Jacas[1,*], Roser Vilatersana[1], Cristina Roquet[2] and Alfonso Susanna[1]

[1]*Institut Botànic de Barcelona (CSIC – ICUB), Pg. del Migdia, s/n, E08038 Barcelona, Spain and* [2]*Unitat de Botànica, Facultat de Ciències, Universitat Autònoma de Barcelona, E08193 Bellaterra, Spain*

• *Background and Aims* Section *Acrocentron* of the genus *Centaurea* is one of the largest sections of *Centaurea* with approx. 100 species. The geographic distribution, centred in the Mediterranean, makes it an excellent example for studies of the biogeographic history of this biodiversity-rich region.

• *Methods* Plastid (*trn*H-*psb*A) and nuclear (ITS and ETS) DNA sequence analysis was used for phylogenetic reconstruction. Ancestral biogeographic patterns were inferred by dispersal-vicariance analysis (DIVA).

• *Key Results* The resulting phylogeny has implications for the sectional classification of *Acrocentron* and confirms merging sect. *Chamaecyanus* into *Acrocentron* as a subsection. Previous suggestions of an eastern Mediterranean origin of the group are confirmed. The main centres of diversification established in previous studies are now strongly supported. Expansion of the group in two different radiations that followed patently diverse paths is inferred.

• *Conclusions* Radiation followed two waves, widely separated in time scale. The oldest one, 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 from 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, along a Eurasian pathway running from Central Iberia to the steppes of Kazakhstan. A generalized pattern of reticulation is also evident from the results, indicating past contacts between presently separated species. Molecular data also confirmed the extent of hybridization within *Acrocentron* and were successful in reconstructing the paleogeography of the section.

Key words: *Centaurea* sect. *Acrocentron*, Cardueae, dispersal-vicariance, ETS, ITS, Mediterranean, phylogeny, *psb*A-*trn*H, radiation, reticulation.

INTRODUCTION

The tribe Cardueae (Compositae) has been deeply reviewed and widely modified in the past 10 years. Most of the many systematic problems within the tribe, from the subtribal classification to the delineation of genera, are now more clear (Susanna *et al.*, 1995, 2006; Garcia-Jacas *et al.*, 2001). This has been 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 and Garcia-Jacas, 2007), *Centaurea* comprises approx. 250 species (from 400 in earlier classifications; e.g. Dittrich, 1977). *Centaurea* encompasses only the large *Jacea* group (revised by Garcia-Jacas *et 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 and 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 *et al.*, 2001; Vilatersana *et al.*, 2001; Font *et 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). A previous molecular survey of the group using sequences of the ITS region (Font *et al.*, 2002) only confirmed the inclusion of sect. *Stephanochilus* and the exclusion of the restored genus *Crocodylium*, and suggested merging *C.* sect. *Chamaecyanus* in *C.* sect. *Acrocentron*. However, for the rest, the resulting trees consisted largely of unsupported polytomies. The largest part of the *Acrocentron* group is *Centaurea* sect. *Acrocentron*, a huge section with approx. 100 species mainly in the Mediterranean region.

An aspect of special interest to the present contribution is the history of expansion of *Centaurea* sect. *Acrocentron* through the Mediterranean. Reconstruction of the paleogeographic history of plants is a relatively novel focus in botany, and the present distribution of *C.* sect. *Acrocentron* in the Mediterranean region makes the group an excellent object of study. In our previous molecular survey, the

* For correspondence. E-mail ngarciajacas@ibb.csic.es

existence of three main centres of speciation (Anatolian–Aegean, North Balkanic–Eurasian, and Iberian–North African) was suggested. However, as previously noted, the resulting phylogeny was flawed by an extremely low support for most of the clades. A detailed phylogeny of the related *Centaurea* group *Jacea* (Garcia-Jacas *et al.*, 2006) confirmed the existence of the three centres of speciation hinted at in Font *et al.* (2002). A recent paper dealing with the related *Acrolophus–Phalolepis* complex of *Centaurea* has contributed to our knowledge of the expansion of *Centaurea* in the Iberian Peninsula and North Africa (Suárez-Santiago *et al.*, 2007). It is tempting to verify whether *Acrocentron* follows similar patterns of colonization of the Mediterranean.

The general lack of support in our previous report was considered a result of intense historical gene flow (Font *et al.*, 2002). Hybridization in the *Acrocentron* group is well documented (Kummer, 1977; Fernández Casas and Susanna, 1986; Garcia-Jacas and Susanna, 1993, 1994; Garcia-Jacas, 1998; Hellwig *et al.*, 1994). Hybrids are often homoploid (Kummer, 1977; Fernández Casas and Susanna, 1986), which favours gene flow because homoploid hybrids act as a bridge between the parental species (McKinnon, 2005).

Based on these previous results 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 plastid DNA. Incongruence between nuclear and plastid phylogenies is an important indicator of histori-cal interspecific gene flow (Wendel and Doyle, 1998). The ETS region has proved to be a good tool for the phylogenetic reconstruction of closely related species in many genera from the Compositae (*Calycadenia*, Baldwin and Markos, 1998; *Helianthus*, Linder *et al.*, 2000; *Lessingia*, Markos and Baldwin, 2001; *Xylothamia* and *Gundlachia*, Urbatsch *et al.*, 2003; *Montanoa*, Plovanich and Panero, 2004) and has already been very useful in the Cardueae–Carduinae (Kelch and Baldwin, 2003) and Cardueae–Centaureinae (Hidalgo *et al.*, 2006). Moreover, the ETS region has demon-strated great potential for unraveling the extent of gene flow in *Centaurea* sect. *Willkommia* (Suárez-Santiago *et al.*, 2007). Our goals with this combined approach are as follows: (1) to examine whether a phylogenetic subsectional classification of *Centaurea* sect. *Acrocentron* is supported by molecular data and to confirm the inclusion of *C.* sect. *Chamaecyanus* in sect. *Acrocentron* with subsectional rank; (2) to seek for molecular evidence of hybridization and gene flow among *C.* sect. *Acrocentron*; (3) to verify the existence of the suggested centres of speciation of the group; and (4) to explore and reconstruct the history of the expansion of *C.* sect. *Acrocentron* through the Mediterranean and establish the centre of origin of the group.

MATERIAL AND METHODS

Plant material

The ETS (external transcribed spacer) and ITS (internal tran-scribed spacer) regions of the nrDNA of 45 taxa of *Centaurea* sect. *Acrocentron* and *C.* sect. *Chamaecyanus* were sequenced. In some cases, more than one population was sequenced. Our ITS1 and ITS2 sequences from previous work (Font *et al.*, 2002) were complemented by sequencing the 5·8S region. 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, such as *Centaurea scabiosa* L.

A plastid 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 31 taxa was sequenced (Table 1).

Outgroups were chosen among the genera of the 'derived clade' of the subtribe Centaureinae (Garcia-Jacas *et 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 Table 1.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted following the CTAB method of Doyle and 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 *et al.*, 1990) and 1406F (Nickrent *et al.*, 1994) 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 ETS region was amplified with ETS1F as the forward primer and as reverse primer either 18S-2L (Linder *et al.*, 2000) or 18SETS (Baldwin and Markos, 1998). The PCR was executed with the following conditions: 5 min denaturing at 95 °C, fol-lowed by 30 cycles of 94 °C denaturing for 45 s, 48 °C anneal-ing for 45 s and 72 °C extension for 40 s, with an additional 7 min at 72 °C. PCR products of the species with more than one band were cloned using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, except that only half of the reaction volume was 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 *et 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 followed the protocol of Vilatersana *et al.* (2007).

nrDNA and cpDNA sequencing strategies. Plastid and nuclear PCR products were purified with the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Direct sequen-cing of the amplified DNA segments was performed using a

TABLE 1. *Species, origin of the material, herbaria and GenBank accession numbers*

Species	Voucher	ITS accession	ETS accession	*trn*H-*psb*A accession
Centaurea aetholica Phytos & Georgadis	Greece, Achaia: Antirion-Gavrolimni, *Damboldt s. n.* (B).	FJ459646	FJ459583	–
Centaurea amblensis Graells	Spain, Ávila: El Barco de Ávila, sine col. (BC).	FJ459647	FJ459584	FJ459697
Centaurea amourensis Pomel	Morocco, Zagora: north-east side of the Atlas, *M. Staudinger & M. Finckh* (BC).*	FJ459648	FJ459585 FJ459586	FJ459698
Centaurea argecillensis Gredilla	Spain, Guadalajara: Argecilla, mountain slopes on the road to Ledanca, *M. Font & Susanna 1811* (BC).*	FJ459649	FJ459587 FJ459588 FJ459589	FJ459699
Centaurea borjae Valdés-Berm. & Rivas Goday	Spain, La Coruña: near El Ferrol, cabo Prior, S side, *Garcia-Jacas & Susanna 2074* (BC).	FJ459650	FJ459590	FJ459700
Centaurea carduiformis DC	Armenia, Talin: between Pokr Arthik and Bagravan, *Susanna 1527 & al.* (BC).	FJ459651	FJ459591	–
Centaurea carolipauana Fern. Casas & Susanna	Morocco, Tetuan: Djebel Tasaot, 14·5 km from the cross to Talembote, *Garcia-Jacas & Susanna 1437* (BC).	AY826253	FJ459592	FJ459701
Centaurea cephalariifolia Willk.	Spain, Huesca: between Capdesaso and San Lorenzo del Flumen, *Vilatersana 20* (BC).	FJ459652	FJ459593	FJ459702
Centaurea clementei Boiss.	Spain, Botanical Garden of Barcelona (BC).*	FJ459653	FJ459594 FJ459595	FJ459703
Centaurea collina L.	Spain, Alacant: between Muro de Alcoi and Beniarrés, *Susanna 1481 & al.* (BC).	FJ459654	FJ459596	FJ459704
Centaurea crocata Franco	Portugal, Algarve: 5 km N of Monchique on the road to Odemira, *Roché & Susanna 1917* (BC).	FJ459655	FJ459597	FJ459705
Centaurea ebenoides Heldr.	Greece, Euboea island: near Limni, on the coast road, *Phytos & Kamari 20427* (B).	FJ459656	FJ459598	–
Centaurea euboica Rech. f.	Greece, Euboea island: Kandili mountains, between Achmet Aga and Hagios, *Rechinger 18215* (B).	FJ459657	FJ459599	–
Centaurea gabrielis-blancae Fern. Casas	Spain, Navarra: Lumbier, Foz de Lumbier, *Garcia-Jacas & Susanna 1592* (BC).	FJ459658	FJ459600	FJ459706
Centaurea galianoi Fern. Casas & Susanna	Spain, Huelva: Sierra de Aracena, between Linares de la Sierra and Alaján, *Garnatje 55 & Vilatersana* (BC).	FJ459659	FJ459601	FJ459707
Centaurea glehnii Trautv.	Armenia, Peninsula of Artanish, *Susanna 1527 & al.* (BC).*	FJ459660 FJ459661	FJ459602	FJ459708
Centaurea granatensis Boiss.	Spain, Granada: Sierra Guillimona, Gorgas de Torilla, *Valdés s. n. & al.* (SEV).	FJ459662	FJ459603	FJ459709
Centaurea grbavacensis (Rohlena) Stoj. & Acht.	Greece, Macedonia: Kozuf mountains, south side of mt. Tzena, *Greuter 14108* (B).	FJ459663	FJ459604	–
Centaurea haenseleri Boiss.	Spain, Málaga: 10 km to Jubrique, *Garcia-Jacas & Susanna 1888* (BC).	FJ459664	FJ459605	FJ459710
Centaurea jankae Brandza	Romania: Dobrogea, *Badarau s. n.* (CLCB).	FJ459665	FJ459606	–
Centaurea josiae Humbert	Morocco, Ksar el Souk: Tizi'n Talrhemt, *Susanna 1792 & al.* (BC).*	FJ459666	FJ459607 FJ459608	–
Centaurea kandavanensis Wagenitz	Iran, Mazandaran: Chalus road, 2 km from the cross to Baladeh, *Susanna 1621 & al.* (BC).	FJ459667	FJ459609	FJ459711
Centaurea kotschyana Heuff.	Romania, Botanical Garden 'Al. Borza', Cluj-Napoca (BC).	FJ459668	FJ459610	–
Centaurea kunkelii N. Garcia	Spain, Almería: road AL-411 between Roquetas and Canjáyar, *Susanna 1612 & al.* (BC).*	FJ459669	FJ459611 FJ459612	FJ459712
Centaurea lagascana Graells	Spain, Palencia: 500 m from Alba de los Cardaños, *M. Font & Susanna 1822* (BC).*	FJ459670	FJ459613 FJ459614	FJ459713
Centaurea lainzii Fern. Casas	Spain, Sierra Bermeja: 11 Km from Estepona on the road to Igualeja, *Garcia-Jacas & Susanna 1330* (BC).	FJ459671	FJ459615	FJ459714
Centaurea legionis-septimae Fern. Casas & Susanna	Spain, León: pr. vicum Crémenes, *Fernández Casas 5600 & Susanna* (SEV).	FJ459672	FJ459616	FJ459715
Centaurea litardierei Jahand. & Maire	Morocco, Ksar es Souk: south side of Col du Zad, *Susanna 1797 & al.* (BC).	FJ459673	FJ459617	FJ459716
Centaurea luristanica Rech. f.	Iran, Khuzistan: ca. 15 km from Eizeh on the way to Dehdez, *Mozaffarian s. n.* (TARI).	FJ459674	FJ459618	–
Centaurea lydia Boiss.	Turkey, Konya: Seydisehir, Mortas, *R. Ilarsan 4312* (ANK).	FJ459675	FJ459619	FJ459717
Centaurea mariana Nyman	Spain, Almería: north side of the Sierra María, *Garcia-Jacas & Susanna 1342* (BC).	FJ459676	FJ459620	FJ459718
Centaurea nana Desf.	Morocco, Ksar es Souk: north side of Col du Zad, 1 km from Aïn Leuh on the road to Azrou, *Susanna 1799 & al.* (BC).	FJ459677	FJ459621	–
Centaurea ochrocephala Wagenitz	Iran, Azarbayjan-e-Gharbi: Orumiyeh, between Maranah and Haki, *Susanna 1693 & al.* (BC).	FJ459678	FJ459622	–
Centaurea ornata Willd.	Spain, Soria: near San Esteban de Gormaz, *Garcia-Jacas & Susanna 1823 (*BC), population 1.	FJ459679	FJ459623	FJ459719

Continued

TABLE 1. *Continued*

Species	Voucher	ITS accession	ETS accession	*trn*H-*psb*A accession
	Spain, Huesca: road A-132 between Salinas and reservoir la Peña, *Vilatersana 58* (BC), population 2.	FJ459680		
Centaurea podospermifolia Losc. & Pard.	Spain, Tarragona: Tortosa, 5 km from Monte Caro, *Susanna 2072 & al.* (BC).	FJ459681	FJ459624	FJ459720
Centaurea prolongi Boiss.	Spain, Málaga: north side of the Sierra de Mijas, *Garcia-Jacas & Susanna 1335* (BC), population 1.	FJ459682	FJ459625	FJ459721
	Spain, Málaga: Sierra of Almijara, Frigiliana, *Galbany & Arrabal, s. n.* (BC), population 2.*	FJ459683	FJ459627 FJ459628	–
	Portugal, Algarve: near Loulé, Cerro Botello over Fonte da Murta, *Garcia-Jacas & Susanna 1353* (BC), population 3.*	FJ459684 FJ459685	FJ459629	FJ459722
Centaurea pseudoscabiosa Boiss. & Buhse	Turkey, Erzurum: between Erzurum and Varto, 13 km from Varto, *Uysal 893 (*KON).	FJ459686	FJ459630	–
Centaurea pubescens Wiilld.	Morocco, Ksar es Souk: 2 km from Oued Amesheguir, *Garnatje, Susanna 1795 & Vilatersana* (BC).*	FJ459687	FJ459631	FJ459723
Centaurea raphanina Sibth. & Sm.	Greece, Chanion: Kydonias, mounts Levka, *Strid 15093 & Papanicolau* (B).	FJ459688	FJ459632	–
Centaurea rupestris L.	Greece, Macedonia: road E-90 between Véria and Kozáni, *Roché & Susanna 1967* (BC).	FJ459689	FJ459633	–
Centaurea salonitana De Vis.	Greece, Macedonia: W from Efkarpia, *Roché & Susanna 2005* (BC).	FJ459690	FJ459634	FJ459724
Centaurea saxicola Lag.	Spain, Murcia: La Azohía, near the watchtower, *Garcia-Jacas, Susanna 1616 & Vilatersana* (BC).	FJ459691	FJ459635	FJ459725
Centaurea scabiosa L.	France, Lozère: between Le Sec and L'Aumède, near Chanac, *Vilatersana 52* (BC).	FJ459692	FJ459636	FJ459726
Centaurea tauromenitana Guss.	Italy, Sicily: Botanical Garden of Catania (BC).	FJ459693	FJ459637	–
Centaurea toletana Boiss. & Reut. (2*x*)	Spain, Toledo: mountains above San Pablo on the track to Baños del Robledillo, *M. Font & Susanna 1818 (*BC), population 1.	FJ459694	FJ459638	FJ459727
	Spain, Toledo: Risco de las Paradas, *M. Font & Susanna 1817* (BC), population 2.	FJ459695	FJ459639	FJ459728
Centaurea toletana Boiss. & Reut. (4*x*)Outgroup	Spain, Madrid: Redueña, road N-320, 4 km to Torrelaguna, *M. Font & Susanna 1819* (BC).*	FJ459696	FJ459640 FJ459641	FJ459729
Carduncellus duvauxii Batt. & Trab.	Morocco, Al-Hoceima: 8 km S to Tafraoute, *Gómiz, s. n.* (BC).	AY826239	FJ459642	–
Centaurea ensiformis P. H. Davis	Turkey, Muğla: Köycegiz district, Sandras Dağ range 13 km from Agla, *Susanna 2251 & al.* (BC).	DQ319112	FJ459643	–
Centaurea mollis Waldst. & Kit.	Ukraine, Podolia: Lysa Hora, 2 km E of Vilshanitsa near Zolochiv, *Boratyñski & Romo 0506D* (BC).	DQ319133	FJ459644	–
Phonus riphaeus (Font Quer & Pau) G. López	Morocco, Al-Hoceima: Tleta oued Laou between Tarerha and Azenti, *J. M. Montserrat 4360 & al.* (BC).	AY826310	FJ459645	–

* Samples that have been cloned (for these taxa, each different accession number correspond to one cloned sequence).

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). When cloned sequences from one accession presented only a single substitution with regard to the others, they were excluded. Consensus sequences were generated for each accession and region, condensing the single base-pair differences among cloned sequences into a degenerated base. This strategy reduced the size of the matrices as well as the impact of potential PCR artifacts (chimerical sequences and Taq errors; Cline *et al.*, 1996; Popp and Oxelman, 2001).

Phylogenetic analyses

Sequences were aligned visually by sequential pairwise comparison (Swofford and 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 analysed the more conserved 3′ETS region plus the ITS region including the outgroups, with the aim of identifying the earliest-branching clade of *Acrocentron*. Second, we carried out an analysis of the complete-region ETS plus ITS, including only the species of the *Acrocentron* group, using as outgroup the clade identified as earliest-branching 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 earliest-branching in the first analysis.

Phylogenetic analyses were conducted using both parsimony and Bayesian inference optimality criteria. The plastid dataset was analysed independently. Both nrDNA regions (ETS plus ITS) were combined and analysed in one matrix because both datasets resulted in fully compatible topologies. A partition homogeneity test was impossible to complete because the 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 analysis (BS) was performed in order to assess the confidence of the branches (Felsenstein, 1985). For the combined matrices (ETS plus ITS), bootstraping followed the approach of Lidén *et al.* (1997) using 1000 replicates, random taxon addition with ten replicates, and no branch swapping.

For the Bayesian analysis, the data sets were analysed using MrModeltest ver. 2.2 (Nylander, 2004) to determine the sequence evolution model that best described the data. This model was used to perform a Bayesian analysis using the program MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and 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 1000th generation. The 50 % majority rule consensus phylogeny and posterior probability of nodes (PP) were calculated from the remaining sample.

Dispersal-vicariance analysis (DIVA)

Ancestral biogeographic reconstruction was inferred with the dispersal-vicariance analysis implemented in DIVA (Ronquist, 1996, 1997). Eight areas of endemism were defined by the presence of one or more endemic taxons: A = Iberian; B = North African; C = Balkanic; D = Aegean; E = Anatolian + Iranian; F = Eurasian; G = Caucasian; and H = Sicilian. Because DIVA needs a dichotomic tree, we used as input data the tree with the highest posterior probability, obtained with the first analysis (3'ETS plus ITS data; see Fig. 1). Outgroup taxa were omitted when running the DIVA because these taxa were not biogeographically informative. The analysis was carried out constraining the number of ancestral areas to a maximum of two, which is the maximum geographic range of the most widespread species.

RESULTS

Numerical results of the analyses are shown in Table 2. The analysis of the 3'ETS plus ITS regions is shown in Fig. 1. *Centaurea* sect. *Acrocentron* group is monophyletic with high support (BS = 100 %, PP = 1·0) only if we include *C.* sect. *Chamaecyanus*, which is not confirmed as a natural group. The earliest-branching group of *Acrocentron* is the clade of the Anatolian and Irano-Turanian taxa (clade ANAT), without parsimony support but with very high Bayesian suport (BS = 58 %, PP = 1·0). Next, there comes the Aegean taxa (clade AEG) almost without support (BS = 58 %, PP = 0·95). 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 Balkans), 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 North Africa). The second subclade (BS = 96 %, PP = 1·0) includes only species from the Iberian Peninsula.

The analysis of the complete ETS plus ITS region (Fig. 2) shows an important difference with the partial analysis: the Iberian Peninsula and North African clades, which formed a well-supported clade in the first analysis (Fig. 1), are not grouped together in the complete analysis. In this second analysis, the Balkans–Eurasia clade is sister to the Iberian Peninsula clade, and this joint clade is sister to the North African clade (Fig. 2). Besides this divergent result, a few additional differences become apparent. The cloned sequences of *C. josiae* Humbert form a monophyletic clade in the partial analysis (Fig. 1), but one of the cloned sequences is associated with *C. amourensis* Pomel in the complete analysis (Fig. 2). One of the cloned sequence 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 grouped together with *C. litardierei* Jahand. & Maire in the complete analysis (Fig. 2). There is also a difference in the group formed by the cloned sequences of Portuguese *C. prolongi* Boiss. (Pop. 3), which appear on different clades in the partial analysis (clade Iberian Peninsula, Fig. 1) and unsupportedly united in the complete analysis (clade Iberian Peninsula, Fig. 2). Finally, *C. lainzii* Fern. Casas, which appears as an isolated taxon in the partial analysis (clade North Africa, Fig. 1), is united with *C. prolongi* (Pop. 1 and 2) and *C. haenseleri* Boiss. in the complete analysis (clade North Africa, Fig. 2). Differences in the topology resulting from the two analyses (ITS + ETS 3', Fig. 1; ITS + ETS 5' and 3', Fig. 2) are caused by the number of informative characters yielded by the markers used in each case – much higher for the ETS 5' end.

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

The low number of informative characters in the plastid dataset (Table 2) explains the lack of resolution in the phylogenetic analysis (tree not shown). Table 3 shows the changes of the sequences in the plastid dataset.

The output of the DIVA is the most-parsimonious ancestral area for each node, and the results are reflected in Fig. 1. Three equally parsimonious solutions for the ancestral area are suggested for the deepest node (Iberian/Anatolian, Balkanic/Anatolian and Aegean/Anatolian). This ancestor would have then dispersed to the Iberian/Aegean or Balkanic/Aegean areas. The ancestral area suggested for the third deepest node is Iberian/Balkanic. Beyond this node, the DIVA results suggest predominance of vicariance.
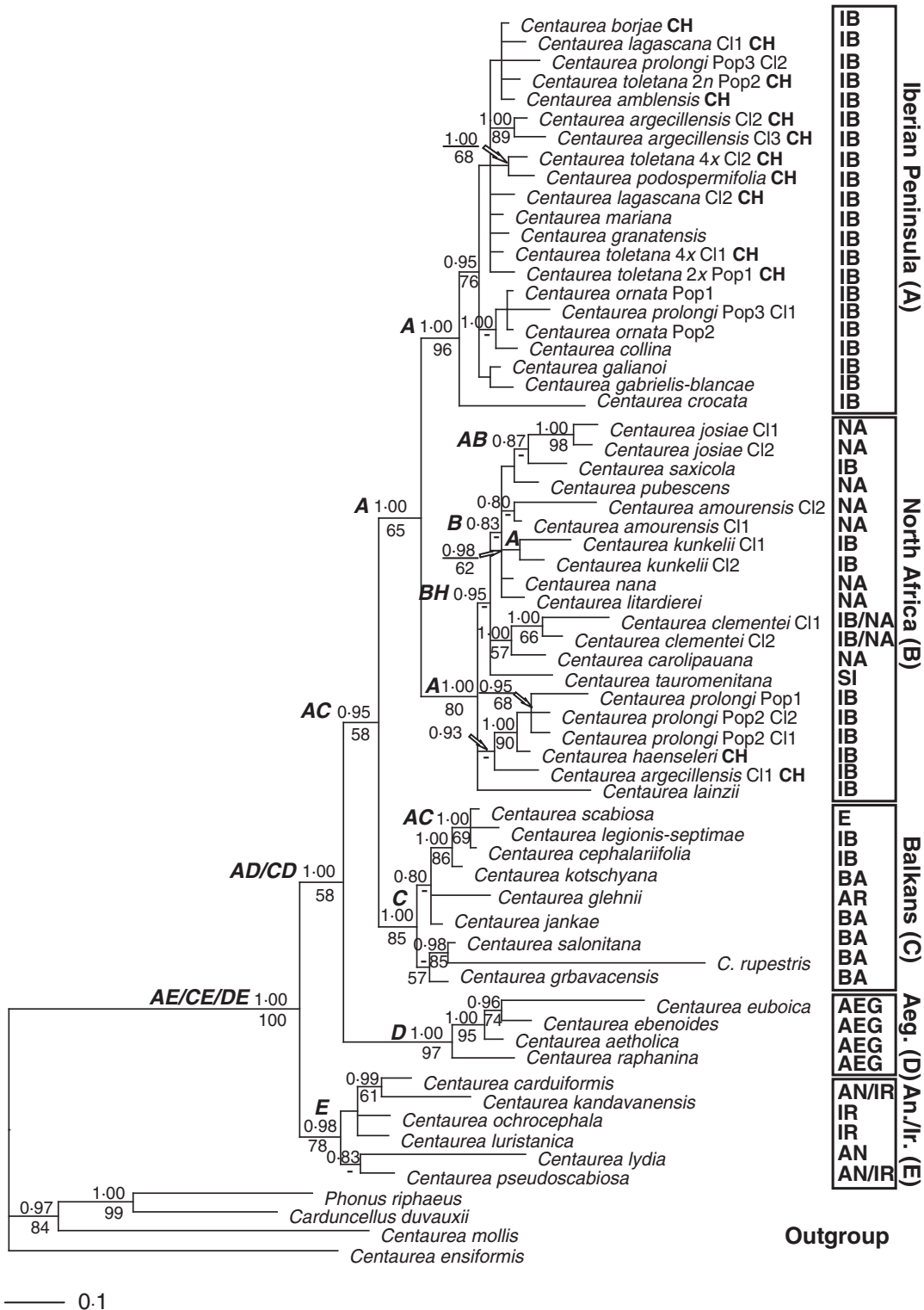
Font et al. — *Evolution and biogeography of* Centaurea *sect.* Acrocentron

FIG. 1. Consensus phylogram obtained from 9000 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 bootstrap values. The lettering of the nodes indicates the areas defined for the DIVA: A = Iberian Peninsula; B = North Africa; C = Balkans; D = Aegean; E = Anatolia + Iran; H = Sicily. Biogeographic regions on the right of the figure are: AEG = Aegean; AN = Anatolia; AR = Armenia; BA = Balkans; E = Eurasia; IB = Iberian Peninsula; IR = Iran; NA = North Africa; SI = Sicily. Other abbreviations: CH = section *Chamaecyanus*; Cl = clone; Pop = population.

TABLE 2. *Numeric results of the phylogenetic analyses*

Data set	3′ETS + ITS	5′ and 3′ ETS + ITS	*trn*H-*psb*A
Taxa	49	45	31
Number of sequences	64	60	33
Total characters	1859	1142	443
Informative characters	225	173	8
Number MPTs	815 072	34 446	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
Bayesian model of evolution	GTR + I + G	GTR + I + G	GTR + I + G

DISCUSSION

Concerted evolution in the ETS–ITS regions

The presence of different rybotypes in both regions (ETS and ITS) confirms that in some species of the group concerted evolution has not occurred or has been incomplete. This is an acute contrast with the related *Centaurea* sect. *Willkommia* in which biased concerted evolution was extensive (Suárez-Santiago *et al.*, 2007).

Taxonomic implications: sectional classification

The classic taxonomic classification in two sections, *Acrocentron* and *Chamaecyanus*, is untenable after our results. The segregation of *Centaurea* sect. *Chamaecyanus* (species of this section are marked CH in Figs 1 and 2) would leave *C.* sect. *Acrocentron* paraphyletic. The most appropriate status for *C.* sect. *Chamaecyanus* is subsectional within *C.* sect. *Acrocentron*, confirming previous suggestions (Font *et al.*, 2002). In the Iberian clade (Figs 1 and 2), species of both sections are part of a polytomy, and *C.* sect. *Chamaecyanus* is not resolved as a natural group. However, both sections are morphologically very well defined (Fernández Casas and Susanna, 1986), and we suspect that the lack of confirmation of the monophyly of *C.* sect. *Chamaecyanus* is caused by introgression. Intermediates between them are very frequent (Fernández Casas and Susanna, 1986), a fact that is probably the cause of this entanglement and the reason for the low support for the clade that comprises many of the species of *C.* sect. *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 as a hybrid between taxa of both sections (Garcia-Jacas and Susanna, 1994), which would explain this basal position.

Regarding the possibility of establishing a subsectional classification within *C.* sect. *Acrocentron* as suggested by previous workers (Hayek, 1901; Routsi and Georgiadis, 1994), molecular data are of little help because intense gene flow hinders molecular reconstruction of phylogenetic affinities, and the extreme homogeneity of morphological 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 geographical basis.

Hybridization and reticulation

In our previous study based on the ITS region (Font *et al.*, 2002), we suggested that the very low definition within *Centaurea* sect. *Acrocentron* was probably caused by the high levels of hybridization and reticulation within the group. Our new results could support this view, for example the presence of an ETS cloned sequence of *C. argecillensis* from central and north-east Iberia within the North African clade, whilst the rest of the cloned sequences of this species are placed in the Iberian clade (Figs 1 and 2). There are some other examples, perhaps not so extreme but also indicating intense gene flow: *Centaurea toletana* Boiss. & Reut. ($2n = 4x$) has a cloned sequence 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* from Portugal, firstly cited as *C. collina* L., later on identified as *C. prolongi* (Garcia-Jacas and Susanna, 1994) and even considered a new species, *C. occasus* Fern. Casas (Fernández Casas, 1997). The two cloned sequences of this population of *C. prolongi* (Pop. 3) appear in the same clade, but in different positions (*C. prolongi* 3, clade Iberian Peninsula; Fig. 1).

Gene flow could also explain the differences within *C. prolongi*: the Portuguese population is associated with 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 North Africa; Figs 1 and 2). This segregation shows that they have different ribotypes, a fact that could reinforce the hypothesis that they are different species. However, morphological differences alleged by Fernández Casas (1997) are only quantitative (namely, the size of the leaves and florets), which could easily be explained by the different climatic conditions of both localities (milder and wetter in Portugal). A preferable explanation for the positions of populations of *C. prolongi* in opposite clades is gene flow: populations of *C. prolongi* in south Spain are very close to the African coast (Fig. 3), and other species of *C.* sect. *Acrocentron* growing nearby (*C. haenseleri* and *C. lainzii*) also appear in the African group (Figs 1 and 2). In contrast, the studied Portuguese locality was far apart from the waves of African taxa.

In our analyses, *C. legionis-septimae* Fern. Casas & Susanna, a narrow endemic from the mountains of northern Spain, is united with *C. cephalariifolia* Willk., a species from the *C. scabiosa* widespread group. This position contradicts morphologic evidence, which relates *C. legionis-septimae* to *C. ornata* Willd. (Garcia-Jacas and Susanna, 1992), and could be considered another indication of hybridization, as suggested by the existence of homoploid hybrid populations between both taxa described as *C.* ×*cephalariiseptimae* Fern. Casas & Susanna (Fernández Casas and Susanna, 1985).

The position of the Balkan clade, which is united to the Iberian clade in the phylogenetic analysis (Fig. 2), could be explained in terms of hybridization and introgression. This relationship could be caused by gene flow of the widely distributed taxa from the clade Balkans–Eurasia and the Iberian species. The culprit is
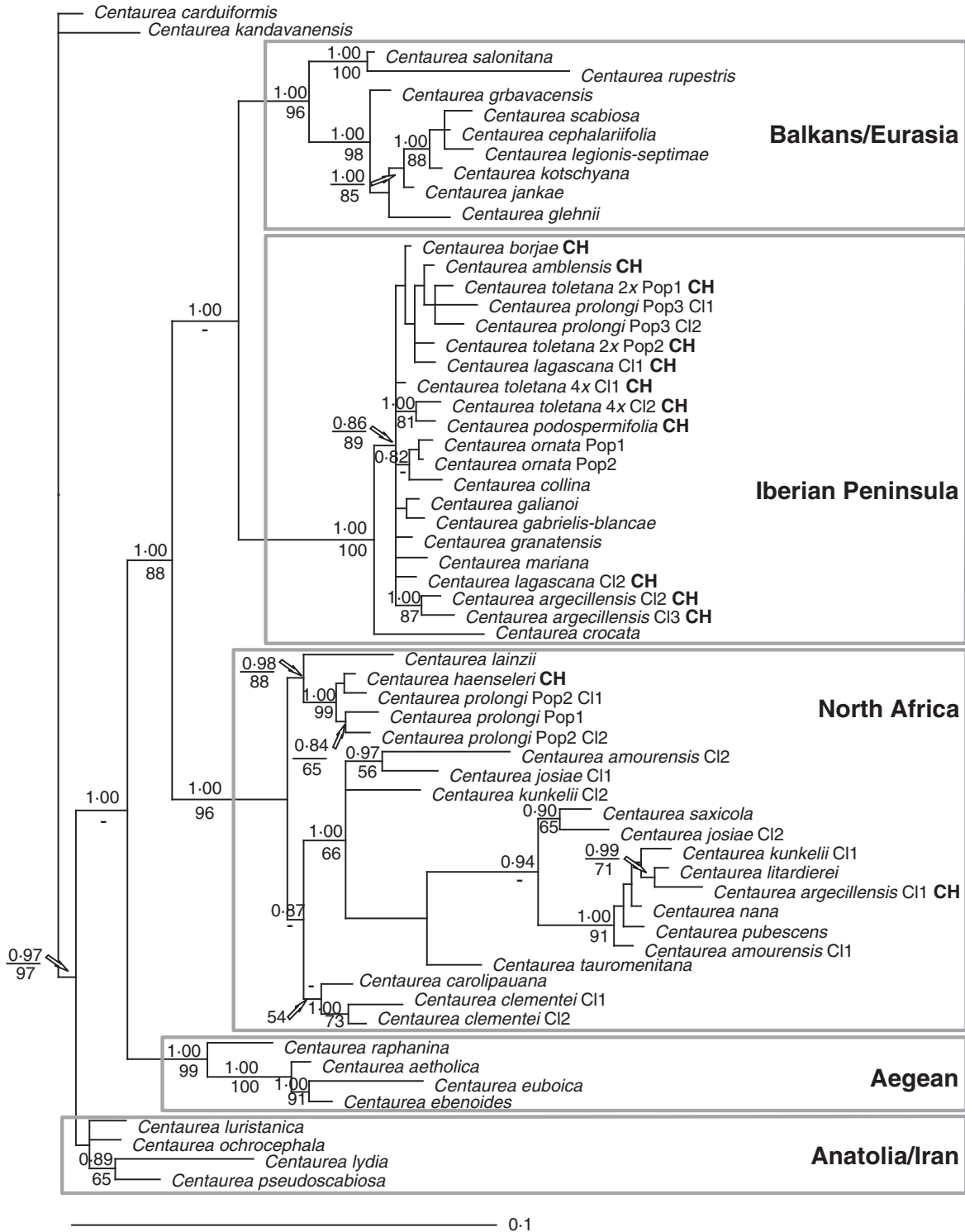
FIG. 2. Consensus phylogram obtained from 9000 Bayesian trees with higher posterior probability (PP) from the combined 5′-3′ ETS and ITS data with the Anatolian–Iranian clade as outgroup. Numbers above branches indicate Bayesian-credibility values (PP); numbers below branches indicate parsimony bootstrap values. CH = section *Chamaecyanus*; Cl = clone; Pop = population.

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 one of them above, *C. ×cephalariiseptimae*). In four cases, the crosses involve tetraploid parentals and the hybrids are also tetraploid (Kummer, 1977; Fernández Casas and Susanna, 1986) – thereafter they are not genetically isolated from their progenitors.

TABLE 3. *Informative characters in the chloroplast* trn*H*-psb*A sequences. Variable characters with respect to the first sequence are in bold type*

Species and population	Origin*						1 5 1	1 6 8	1 6 9	1 7 5	1 7 9	3 3 9	3 6 2	3 8 8	3 9 8
		6 5	7 5	7 7	7 8	8 0									
C. glehnii	AR	C	T	T	T	T	A	–	–	A	G	C	A	A	A
C. lydia	ANT	C	T	T	T	T	A	–	–	**G**	G	C	A	A	A
C. salonitana	BAL	C	T	T	T	T	A	–	–	A	G	C	A	**C**	A
C. amblensis	IB	C	T	T	T	T	A	–	–	A	G	**A**	A	A	A
C. argecillensis	IB	C	T	T	T	T	A	–	–	A	G	**A**	A	A	A
C. borjae	IB	C	**A**	**A**	**A**	**A**	A	–	–	A	G	C	A	A	A
C. cephalariifolia	IB	C	T	T	T	T	A	–	–	A	G	**A**	A	A	A
C. clementei	IB	C	T	T	T	T	A	–	–	A	G	**A**	A	A	A
C. crocata	IB	C	**A**	**A**	**A**	**A**	A	–	–	A	G	**A**	A	A	A
C. collina	IB	C	T	T	T	T	A	–	–	A	G	C	A	A	A
C. gabrielis-blancae	IB	C	T	T	T	T	**C**	–	–	A	G	**A**	A	A	A
C. galianoi	IB	C	T	T	T	T	A	–	–	A	G	C	A	A	A
C. granatensis	IB	C	T	T	T	T	A	–	–	A	G	**A**	A	A	A
C. haenseleri	IB	C	T	T	T	T	A	–	–	A	G	**A**	A	A	A
C. kunkelii	IB	C	T	T	T	T	A	–	–	A	G	**A**	A	A	**C**
C. lagascana	IB	C	T	T	T	T	A	–	–	A	G	**A**	**G**	A	A
C. lainzii	IB	C	T	T	T	T	**C**	–	–	A	G	C	A	A	A
C. legionis-septimae	IB	C	T	T	T	T	A	–	–	A	G	C	A	A	A
C. mariana	IB	C	T	T	T	T	A	**A**	**A**	A	G	C	**T**	A	A
C. ornata Pop 1	IB	C	T	T	T	T	A	–	–	A	G	**A**	A	A	A
C. podospermifolia	IB	C	T	T	T	T	A	–	–	A	G	**A**	A	A	A
C. prolongi Pop 1	IB	C	T	T	T	T	A	–	–	A	G	C	A	A	A
C. prolongi Pop 3	IB	C	T	T	T	T	A	–	–	A	G	**A**	A	A	A
C. saxicola	IB	C	T	T	T	T	A	–	–	A	G	**A**	A	A	A
C. toletana 2x Pop 1	IB	C	T	T	T	T	A	–	–	A	G	**A**	A	A	A
C. toletana 2x Pop 2	IB	C	T	T	T	T	A	–	–	A	G	**A**	A	A	A
C. toletana 4x	IB	C	T	T	T	T	A	–	–	A	G	**A**	A	A	A
C. kandavanensis	IR	C	**A**	**A**	**A**	**A**	A	–	–	A	G	**A**	A	A	A
C. amourensis	NA	C	**A**	**A**	**A**	**A**	A	**A**	**A**	A	**A**	**A**	A	A	A
C. carolipauana	NA	C	**A**	**A**	**A**	**A**	A	**A**	**A**	A	G	C	**G**	A	A
C. litardierei	NA	C	**A**	**A**	**A**	**A**	A	–	–	A	G	C	**G**	A	A
C. pubescens	NA	C	T	T	T	T	A	**A**	**A**	A	**A**	C	**T**	A	A
C. scabiosa	E	**T**	T	T	T	T	A	–	–	A	G	**A**	A	A	A

Positions in data set

* AR = Armenia; AN = Anatolia, BA = Balkans; E = Euro-Siberian; IB = Iberian; IR = Iran; NA = North Africa; – indicates gap.

A final reason for considering high levels of introgression is the result of the plastid sequencing (Table 3). 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 could be one of the main causes of low resolution in phylogenies (Rieseberg and Soltis, 1991) or, alternately, *C*. sect. *Acrocentron* shows high levels of homoplasy in the *trn*H-*psb*A (Hamilton *et al.*, 2003).

Biogeography

The combined ITS plus ETS phylogeny (Figs 1 and 2) shows that there are five speciation centres: Anatolia–Iran, the Aegean (these two were united into one by Font *et al.*, 2002), the Balkans (including some species of wide Eurasian distribution), the Iberian Peninsula, and North Africa. These geographic denominations for the clades are general and do not imply that all the taxa within the 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 *C*. sect. *Acrocentron* (Fig. 3).

Distribution of *Acrocentron* has been presented as an east-to-west migration (Garcia-Jacas and Susanna, 1992). As a confirmation of this hypothesis, the Anatolian–Iranian centre is the earliest-branching clade in the first of our analyses, 3'ETS plus ITS (Fig. 1). This is one of the three hypotheses suggested by the DIVA, and indeed the most likely because the two areas are contiguous. The alternate solutions suggest either an unlikely Iberian–Anatolian origin for the group or a Balkanic–Anatolian origin that would obviate the Aegean milestones. The origin of *Acrocentron* should be placed in the eastern Mediterranean, much like the rest of the subtribe according to the greater richness of genera of the Centaureinae in this area (Susanna and Garcia-Jacas, 2007). The hypothesis of the south of the Irano-Turanian region (Iranian plateau) as the place of origin of the group was rejected on karyologic grounds, and Iranian species of *C*. sect. *Acrocentron* were considered a later expansion from Anatolian populations (Garcia-Jacas *et al.*, 1998).

From Anatolia, species of *C*. sect. *Acrocentron* radiated to the Aegean islands, the species of which (*C. aetholica* Phitos & T. Georgiadis, *C. ebenoides* Heldr., *C. euboica*
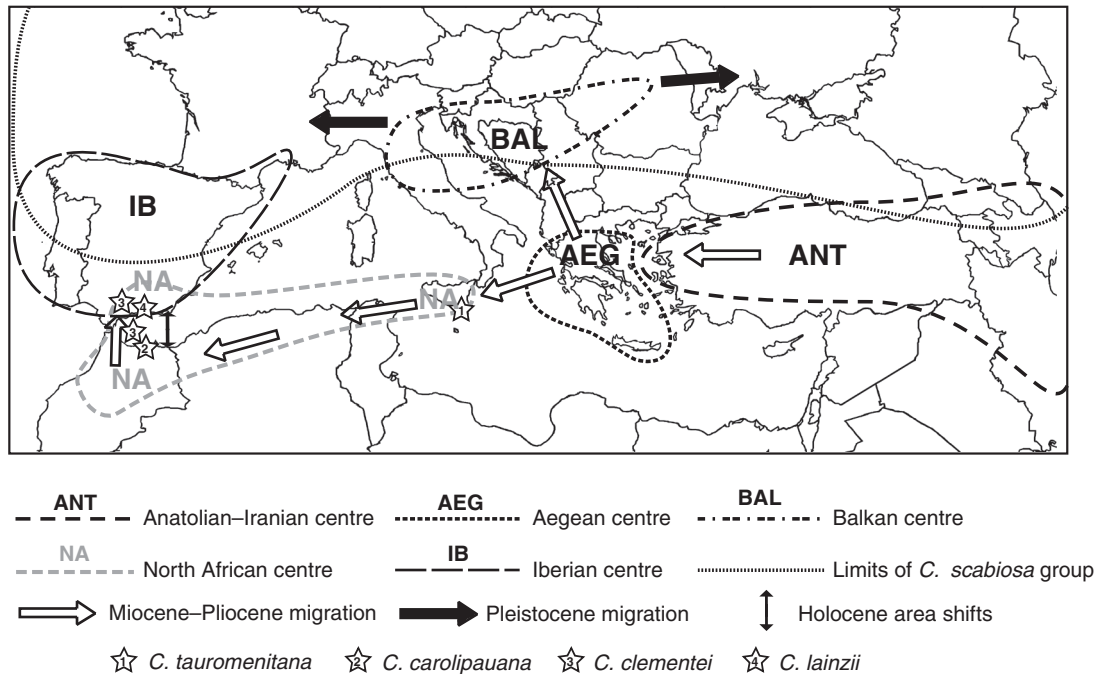
FIG. 3. Geographic representation of the two migration pathways of sect. *Acrocentron* in the Mediterranean, and the landmark species in Sicily and North Africa.

Rech. f. and *C. raphanina* Sibth. & Sm.) form a well-supported clade (AEG in Fig. 1 and Aegean in Fig. 2) that is sister to the next branches. This radiation is supported by the DIVA results, which suggests two equally possible solutions for this dispersal: an unlikely (not contiguous) Iberian–Aegean ancestral area, or the Balkanic–Aegean hypothesis (Fig. 1). 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 *C.* sect. *Acrocentron*, as reflected in our phylogeny (Fig. 1). In the north of its range, it originated the Balkanic clade, and in the south it migrated to the extreme south of the Italian Peninsula (Fig. 3). A dual radiation is supported by the DIVA results (Fig. 1), which suggest an Iberian–Balkanic ancestral area for this node. Predominance of Iberian species in the upper part of the tree could be the reason for DIVA assigning an Iberian origin to this branch, which should be better considered as North African–Balkanic.

The Messinian salinity crisis (between 6 and 5·2 MYA) offers a good opportunity for hypothesizing a date for the migration of the *Acrocentron* group. In the east, a pass between Anatolia and the Aegean was already open by the middle Miocene (Çağatay *et 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 *C.* sect. *Acrocentron* through this southern pathway probably would have terminated in the Pliocene because species of *C.* sect. *Acrocentron* are absent in all the western Mediterranean archipelagos, which during the Messinian were connected to the mainland. This dating has also been suggested for the

expansion of the related complex *Acrolophus–Phalolepis* of the genus *Centaurea* (Suárez-Santiago *et al.*, 2007), a group highly speciated in Iberia but absent from the Balearic Islands.

Besides paleogeographic arguments, a series of landmarks illustrate this hypothetical path. Four species closely related on morphological grounds mark the southern path 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 *C.* sect. *Acrocentron*, such as very wide involucral bracts ending in an unarmed black appendage (Fernández Casas and Fernández Morales, 1979). Figure 3 summarizes the hypothesized pathes 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 a natural consequence, favoured by the development of a Mediterranean climate. The poverty in species of *C.* sect. *Acrocentron* in the North African lowlands stretching between Algeria and Libya reflects 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* (Coss. & Dur.) O. Hoffm. from the oasis of Algeria is the only annual species of the *Acrocentron* group.

As a final argument for the predominance of the southern path of migration, in northern and middle Italy some species of *C.* sect. *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 *C.* sect. *Acrocentron*
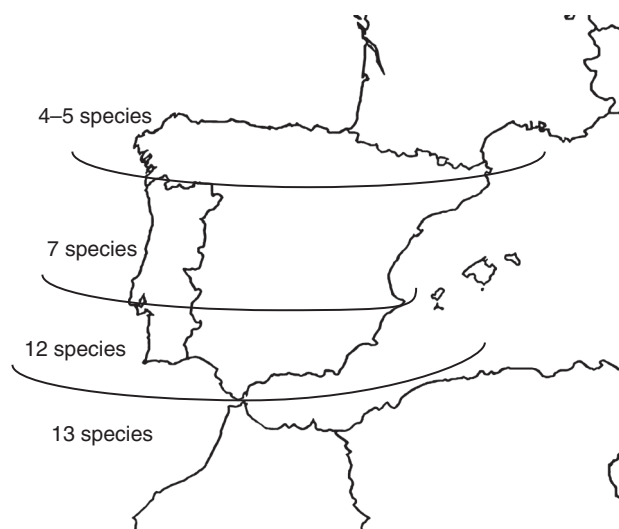
FIG. 4. Species density gradient for the *Acrocentron* group in the Iberian Peninsula and North Africa.

present in France belongs to the widespread *C. scabiosa* group, with the exception of only one species shared with east Iberia (*C. collina*).

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

In contrast, DIVA reconstruction suggests the Iberian Peninsula as the ancestral area for the clade comprising Iberian, North African and Sicilian species (Fig. 1). This solution seems unlikely because it contradicts biogeographic evidence: if taxa of *Acrocentron* migrated from Anatolia to the Aegean and the south Balkans, the easiest way to the Iberian Peninsula is the southern route via Sicily and North Africa, where landmark species exist (Fig. 3). When interpreting the DIVA results, we have to be aware that the DIVA method does not take in account geographic connections between the areas defined for the analysis (Ronquist, 1996, 1997).

A later entry of *C.* sect. *Acrocentron* following a northern pathway can be tracked in the Iberian Peninsula, originating 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, should be considered a recent radiation based on 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 during the

repeated interglacial periods in the Pleistocene–Holocene (Prentice *et al.*, 2000).

In our molecular reconstruction based on the ITS plus ETS regions, the phylogeny of the Iberian and African taxa is obscure and unresolved because the present distribution of ribotypes in Iberia is significantly mediated by the troublesome climatic history of the Iberian Peninsula and North Africa during the Holocene. The succession of glaciations forced mountain species of *Acrocentron* to migrate to lower latitudes (Peñalba *et al.*, 1997), and species that were isolated became sympatric. Iberian species could migrate south to Africa during glacial maxima, 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 cloned sequences deeply nested in the North African clade. Perhaps the most striking result, which confirms the impact of glaciation-mediated migrations and hybridization in *C.* sect. *Acrocentron*, is the otherwise implausible group formed by one cloned sequence of *C. argecillensis* from central Spain and another of *C. litardierei* from the High Atlas mountain range (Figs 1 and 2).

CONCLUSIONS

Our study demonstrates the usefulness of the analysis 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.

ACKNOWLEDGEMENTS

LITERATURE CITED

Baldwin BG, Markos S. 1998. Phylogenetic utility of the external transcribed spacer (ETS) of 18S-26S rDNA: congruence of ETS and ITS trees of *Calycadenia* (Compositae). *Molecular Phylogenetics and Evolution* **10**: 449–463.
Cline J, Braman JC, Hogrefe HH. 1996. PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Research* **24**: 3546–3551.
Cullings KW. 1992. Design and testing of a plant-specific PCR primer for ecological and evolutionary studies. *Molecular Ecology* **1**: 233–240.
Çağatay MN, Görür N, Flecker R, *et al*. 2006. Paratethyan–Mediterranean connectivity in the Sea of Marmara region (NW Turkey) during the Messinian. *Sedimentary Geology* **188–189**: 171–187.
Dittrich M. 1977. Cynareae – systematic review. In: Heywood VH, Harborne JB, Turner BLeds. *The biology and chemistry of the Compositae*. London, New York, San Francisco: Academic Press, 999–1015.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin, Botanical Society of America* **19**: 11–15.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

Fernández Casas FJ. 1997. *De Centaureis occidentalibus notulae sparsae* X. *Fontqueria* **48**: 203–222.

Fernández Casas FJ, Fernández Morales MJ. 1979. *Centaurea lainzii*, un triploide natural. *Mémoires de la Société Botanique de Genève* **1**: 115–122.

Fernández Casas FJ, Susanna A. 1985. *De Centaureis occidentalibus notulae sparsae*, VII. *Fontqueria* **9**: 13–16.

Fernández Casas FJ, Susanna A. 1986. Monografía de la sección *Chamaecyanus* Willkomm del género *Centaurea* L. *Treballs de l'Institut Botànic de Barcelona* **10**: 5–174.

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.

Garcia-Jacas N. 1998. *Centaurea kunkelii*, a new hybridogenic endecaploid species of sect. *Acrocentron* from Spain. *Annales Botanici Fennici* **35**: 159–167.

Garcia-Jacas N, Susanna A. 1992. Karyological notes on *Centaurea* sect. *Acrocentron*. *Plant Systematics and Evolution* **179**: 1–18.

Garcia-Jacas N, Susanna A. 1993. *Centaurea ×polymorpha* Lagasca: los problemas de un híbrido. *Fontqueria* **36**: 65–66.

Garcia-Jacas N, Susanna A. 1994. *Centaurea prolongi* and *Centaurea crocata* in Portugal: an old confusion. *Nordic Journal of Botany* **14**: 31–38.

Garcia-Jacas N, Susanna A, Mozaffarian V. 1998. New chromosome counts in the subtribe Centaureinae (Asteraceae, Cardueae) from West Asia, III. *Botanical Journal of the Linnean Society* **128**: 413–422.

Garcia-Jacas N, Susanna A, Garnatje T, Vilatersana R. 2001. Generic delimitation of phylogeny of the subtribe Centaureinae (Asteraceae): a combined nuclear and chloroplastic DNA analysis. *Annals of Botany* **87**: 503–515.

Garcia-Jacas N, Uysal T, Romashchenko K, Suárez-Santiago VN, Ertuğrul K, Susanna A. 2006. *Centaurea* revisited: A molecular survey of the *Jacea* group. *Annals of Botany* **98**: 741–753.

Hamilton MB. 1999. Four primers pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology* **8**: 521–523.

Hamilton MB, Braverman JM, Soria-Hernanz DV. 2003. Patterns and relative rates of nucleotide and insertion/deletion evolution at six chloroplast intergenic regions in New World species of the Lecythidaceae. *Molecular Biology and Evolution* **20**: 1710–1721.

Hayek AV. 1901. Die *Centaurea* Arten Oesterreich-Ungarns. *Denkschriften der Kaiserlichen Akademie der Wissenschaften, Mathematisch-Naturwissenschaftliche Klasse* **70**: 585–773.

Hellwig FH, Oberprieler C, Vogt R, Wagenitz G. 1994. Chromosome numbers of North African phanerogams. III. Some counts in *Centaurea* (Compositae). *Willdenowia* **24**: 249–254.

Hidalgo O, Garcia-Jacas N, Garnatje T, Susanna A. 2006. Phylogeny of *Rhaponticum* (Asteraceae, Cardueae–Centaureinae) and related genera inferred from nuclear and chloroplast DNA sequence data: taxonomic and biogeographic implications. *Annals of Botany* **97**: 704–714.

Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–755.

Kelch DG, Baldwin BG. 2003. Phylogeny and ecological radiation of New World thistles (*Cirsium*, Cardueae–Compositae) based on ITS and ETS rDNA sequence data. *Molecular Ecology* **12**: 141–151.

Kummer C. 1977. Untersuchungen von natürlichen Bastarden zwischen *Centaurea cephalariifolia* Willkomm und *Centaurea ornata* Willdenow. *Mitteilungen der Botanischen Staatssammlung München* **13**: 129–202.

Lidén M, Fukuhara T, Rylander J, Oxelman B. 1997. Phylogeny and classification of Fumariaceae, with emphasis on *Dicentra s. l.* based on the plastid gene *rps*16 intron. *Plant Systematics and Evolution* **206**: 411–420.

Linder CR, Goertzen LR, Vanden Heuvel B, Francisco-Ortega J, Jansen RK. 2000. The external transcribed spacer of the rDNA repeat: a new nuclear region for low-level taxonomic analysis of the Asteraceae and closely allied families. *Molecular Phylogenetics and Evolution* **14**: 285–303.

Maddison DR. 1991. The discovery and importance of multiple islands of most-parsimonious trees. *Systematic Zoology* **40**: 315–328.

Markos S, Baldwin BG. 2001. Higher-level relationships and major lineages of *Lessingia* (Compositae, Astereae) based on nuclear rDNA internal and external transcribed spacers (ITS and ETS) sequences. *Systematic Botany* **26**: 168–183.

McKinnon GE. 2005. Reticulate evolution in higher plants. In: Henry RJ, ed. *Plant diversity and evolution: genotypic and phenotypic variation in higher plants*. Wallingford, UK: CABI Publishing, 81–96.

Nickrent DL, Schuette KP, Starr EM. 1994. A molecular phylogeny of *Arceuthobium* (Viscaceae) based on nuclear ribosomal DNA internal transcribed spacer sequences. *American Journal of Botany* **81**: 1149–1160.

Nylander JAA. 2004. *MrModeltest ver. 2*. Program distributed by the author. Uppsala, Sweden: Evolutionary Biology Centre, Uppsala University.

Peñalba MC, Arnold M, Guiot J, Duplessy JC, Beaulieu JL. 1997. Termination of the last glaciation in the Iberian Peninsula inferred from the pollen sequence of Quintanar de la Sierra. *Quaternary Research* **48**: 205–214.

Plovanich AE, Panero JL. 2004. A phylogeny of the ITS and ETS for *Montanoa* (Asteraceae: Heliantheae). *Molecular Phylogenetics and Evolution* **31**: 815–821.

Popp M, Oxelman B. 2001. Inferring the history of the polyploid *Silene aegaea* (Caryophyllaceae) using plastic and homoeologous nuclear DNA sequences. *Molecular Phylogenetics and Evolution* **20**: 474–481.

Prentice IC, Jolly D & BIOME 6000 participants. 2000. Mid-Holocene and glacial-maximum vegetation geography of the northern continents and Africa. *Journal of Biogeography* **27**: 507–519.

Rieseberg LH, Soltis DE. 1991. Phylogenetic consequences of cytoplasmic gene flow in plants. *Evolutionary Trends in Plants* **5**: 65–84.

Ronquist F. 1996. *DIVA ver. 1.1. Computer manual*. Available from http://www.ebc.uu.se/systzoo/research/diva/diva.html. Uppsala, Sweden: Evolutionary Biology Centre, Uppsala University.

Ronquist F. 1997. Dispersal-vicariance analysis: a new approach to quantification of historical biogeography. *Systematic Biology* **46**: 195–203.

Ronquist F, Huelsenbeck JP. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Routsi E, Georgiadis T. 1994. Contribution to the systematics of the genus *Centaurea* section *Acrocentron* (Asteraceae) in Greece. *Nordic Journal of Botany* **14**: 369–378.

Suárez-Santiago VN, Salinas MJ, Garcia-Jacas N, Soltis PS, Soltis DE, Blanca G. 2007. Evolution by reticulation of the *Acrolophus* subgroup (*Centaurea* L., Compositae) in the occidental Mediterranean: origin and diversification of the section *Willkommia* Blanca. *Molecular Phylogenetics and Evolution* **43**: 156–172.

Susanna A, Garcia-Jacas N. 2007. Tribe Cardueae. In: Kadereit JW, Jeffrey C, eds. *Flowering plants. Eudicots. Asterales* (vol. 8 in series *The families and genera of vascular plants*, Kadereit JW, ed.). Berlin, Heidelberg: Springer Verlag, 123–146.

Susanna A, Garcia-Jacas N, Soltis DE, Soltis PS. 1995. Phylogenetic relationships in tribe Cardueae (Asteraceae) based on ITS sequences. *American Journal of Botany* **82**: 1056–1068.

Susanna A, Garcia-Jacas N, Hidalgo O, Vilatersana R, Garnatje T. 2006. The Cardueae (Compositae) revisited: insights from ITS, *trn*L-*trn*F, and *mat*K nuclear and chloroplast DNA analysis. *Annals of the Missouri Botanical Garden* **93**: 150–171.

Swofford DL. 2002. PAUP*. Phylogenetic analysis using parsimony *(*and other methods)*, ver. 4.0b10. Sunderland: Sinauer Associates.

Swofford DL, Olsen GJ. 1990. Phylogeny reconstruction. In: Hillis D, Moritz C, eds. *Molecular systematics*. Sunderland: Sinauer Associates, 411–501.

Urbatsch LE, Roberts RP, Karaman V. 2003. Phylogenetic evaluation of *Xylothamia*, *Gundlachia*, and related genera (Asteraceae, Astereae) based on ETS and ITS nrDNA sequence data. *American Journal of Botany* **90**: 634–649.

Vilatersana R, Brysting AK, Brochmann C. 2007. Molecular evidence for hybrid origins of the invasive polyploids *Carthamus creticus* and *C. turkestanicus* (Cardueae, Asteraceae). *Molecular Phylogenetics and Evolution* **44**: 610–621.

Vilatersana R, Martín J, Susanna A, Garcia-Jacas N, Garnatje T. 2001. Pollen studies in subtribe Centaureinae (Asteraceae): the *Carthamus*

complex and the genus *Aegialophila* analyzed with electron microscopy. *Plant Biology* **3**: 607–615.

Wagenitz G. 1955. Pollenmorphologie und Systematik in der Gattung *Centaurea* L. *s. l. Flora* **142**: 213–279.

Wagenitz G, Hellwig FH. 1996. Evolution of characters and phylogeny of the Centaureinae. In: Hind DJN, Beentje HG, eds. *Compositae: systematics. Proceedings of the International Compositae Conference, Kew, 1994.* Kew, London: Royal Botanic Gardens, 491–510.

Wendel JF, Doyle JJ. 1998. Phylogenetic incongruence: window into genome history and molecular evolution. In: Soltis PS, Soltis DE, Doyle JJ, eds. *Molecular systematics of plants II.* Dordrecht: Kluwer, 265–296.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR protocols: a guide to methods and applications.* San Diego: Academic Press, 315–322.