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Lessons from *Plectocephalus* (Compositae, Cardueae-Centaureinae): ITS disorientation in annuals and Beringian dispersal as revealed by molecular analyses

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- *Background and Aims* The geographic distribution of the genus *Plectocephalus* comprises a single species in Ethiopia, two in North America and possibly four more in South America, in a striking disjunction that is exceptional for genera of the tribe Cardueae. The enormity of this disjunction cast doubts on the precise taxonomic delineation of the genus, which is not unanimously recognized as a natural entity. The aims of this study were to define the generic boundaries of *Plectocephalus* and to formulate a hypothesis that would explain its natural range.
- *Methods* A combined molecular approach, using nuclear internal transcribed spacers (ITS) and external transcribed spacers (ETS), and plastid *trnL-trnL-F*, *rpl32-trnL*^{UAG} and *ndhF* markers, was chosen for phylogenetic reconstruction by maximum parsimony and Bayesian inference.
- *Key Results* Phylogenetic analysis shows that *Plectocephalus* is a natural genus that includes the African species *P. varians*, together with all the native South American species, currently classified as *Centaurea*, *C. cachinalensis*, *C. floccosa* and *C. tweediei*. The recognition of *Centaurodendron* as an independent genus, which we consider appropriate, would make *Plectocephalus* paraphyletic. Affinities of *Plectocephalus* should lie with eastern representatives of Centaureinae. Geographic disjunction is explained as a consequence of dispersal via the Bering Land Bridge during the Miocene–Pliocene. The phylogeny of the basal grade of Centaureinae differs from previous phylogenies, and artefacts resulting from differences in mutation rates of annual and perennial taxa are confirmed. Sensitivity of ITS to these differences was the highest observed for all DNA regions used in this study.
- *Conclusions* The natural status of the genus *Plectocephalus* is confirmed and several nomenclatural combinations are proposed. New evidence contributes to the debate concerning problems posed by the use of ITS in the phylogenetic reconstruction of groups that differ in terms of their life cycles. Dispersal from Caucasus and Anatolia along the Siberian route and then across the Bering Land Bridge follows a route previously proposed for other taxonomic groups.

Key words: *Plectocephalus*, Compositae, Cardueae, Centaureinae, *Centaurea*, Bering Land Bridge, ETS, ITS, migration, *ndhF*, phylogeny, *rpl32-trnL*^{UAG}, *trnL-trnL-F*.

INTRODUCTION

It is not possible to discuss *Plectocephalus* D. Don without first discussing the generic concept of *Centaurea* L. (Compositae, Cardueae-Centaureinae). From the first description of *Centaurea* by Linnaeus (1753), it was clear that this genus was an artificial assemblage. Cassini (1817) declared: '*Linné a reuni, sous ce nom, en un seul et même genre, une multitude d'espèces, qui ont en effet beaucoup de caractères communs, mais qui cependant peuvent et doivent être distribuées dans plusieurs genres, ne fût-il que pour rendre leur étude plus facile et plus commode.* [...] *Il est vrai que Linnaeus a divisé ses centaurées en plusieurs sections; mais cet expédient ne suffit pas pour prevenir la confusion qui résulte surtout du même nom générique appliqué à un trop grand nombre d'espèces*'. [Linnaeus has united, under this name, in a single genus, a multitude of species that indeed have many characters in common, but which should of necessity be distributed amongst several genera, if only to make it easier and more convenient to study. [...] Linnaeus certainly assigned his centaureas to many sections, but this was not sufficient to prevent the confusion that resulted, especially as he used the same generic name for too many species]. Evidence for the artificial nature of *Centaurea* was accumulating, but it was a pollen study by Wagenitz (1955), in which he described eight different pollen types in a single genus, that sounded the death knell for the broad Linnean concept. Molecular tools were to provide the final evidence (Susanna *et al.*, 1995; Garcia-Jacas *et al.*, 2001, 2002, 2006) and, as late as 2001, a new type species was chosen for the genus, putting an end to the old problem of the delineation of *Centaurea* (Greuter *et al.*, 2001). The latest revisions of subtribe Centaureinae recognized the new circumscription of *Centaurea* as a natural group comprising only 250 species (Susanna and Garcia-Jacas, 2007, 2009), an important reduction when compared with the broad concept of the genus in Dittrich (1977) who recognized 400 species. Currently accepted genera are *Cheirolophus* Cass., *Crupina* (Pers.) DC., *Mantisalca* Cass., *Phalacrachena* Iljin, *Plectocephalus*, *Psephellus* Cass., *Rhaponticoides* Vaill., *Rhaponticum* Vaill. and *Stizolophus* Cass., all of them formerly included in the broad Linnean concept of *Centaurea*.

Taxonomy and phylogenetic relationships

Plectocephalus was one of the earliest segregates of *Centaurea* and contained a single species, *Centaurea americana* Nutt. from Texas that was eventually renamed *Plectocephalus americanus* (Nutt.) D. Don (Sweet, 1830). It was distinguished from the rest of the heterogeneous *Centaurea* assemblage by its very large and showy peripheral florets, the scariose bracts with unarmed silvery appendages, and by its arcuate and faintly ribbed achenes. Don (cited in Sweet, 1830) described the pappus as triseriate, a misinterpretation of the obscurely multiseriate, easily deciduous pappus that is typical of *Plectocephalus*. This is in contrast to the neatly double and persistent pappus of *Centaurea sensu stricto* (Susanna and Garcia-Jacas, 2007). Another botanist with the same name, G. Don (cited in Loudon, 1855), described a second species for the genus, namely *Plectocephalus chilensis* G. Don ex Loudon from central Chile.

Boissier (1856) was the first to accept the new genus and he added a further two new species, *Plectocephalus abyssinicus* Boiss. and *Plectocephalus cyanoides* Boiss., though these are currently considered to be conspecific and assigned the name *Plectocephalus varians* (A. Rich.) C. Jeffrey ex Cufod. from Ethiopia. Nevertheless, despite obvious differences between *Centaurea* and *Plectocephalus*, the latter genus was soon forgotten and, consequently, the next closest relative of *Plectocephalus americanus* to be discovered was named *Centaurea rothrockii* Greenm. (from New Mexico). In fact, most works dealing with the flora of North America still tend to use the older names *Centaurea americana* Nutt. and *C. rothrockii* (e.g. Correl and Johnston, 1970; Martin and Hutchins, 1981), whereas only the latest flora of the USA recognizes *Plectocephalus* (Keil and Ochsmann, 2006). Indeed, major works generally consider *Plectocephalus* to be merely a section of a widely circumscribed *Centaurea* (Bentham, 1853; Hoffmann, 1894; Dittrich, 1977; Bremer, 1994). Besides *C. americana*, *C. chilensis* and *C. rothrockii*, sect. *Plectocephalus* included another three *Centaurea* species from South America, namely *Centaurea cachinalensis* Phil., *C. floccosa* Hook. & Arn. from Chile, and *C. tweediei* Hook. & Arn. from Argentina, Brazil and Uruguay.

Jeffrey (1968) was the next to recognize *Plectocephalus* as a genus distinct from *Centaurea*, soon to be followed by Nordenstam and El-Ghazaly (1977). However, the exact limits of *Plectocephalus* were not unanimously accepted. Even though Hind (1996) recognized the genus, he excluded the African and South American species and suggested that *Plectocephalus* should include only the two North American species *P. americanus* and *P. rothrockii* (Greenm.) D.J.N. Hind. Susanna and Garcia-Jacas (2007, 2009) considered *Plectocephalus* in its broader sense (including the species from Africa and the Americas), but this delineation was only tentative and they did not propose any formal nomenclatural combinations owing to lack of molecular evidence. None of the major molecular surveys of Cardueae to date has included many representatives of *Plectocephalus*.

The only other genus of Centaureinae native to America is *Centaurodendron* Johow (including *Yunquea* Skottsb.), with three species occurring on the Juan Fernández Islands. On both morphological and biogeographical grounds, *Centaurodendron* is considered a close relative of *Plectocephalus*, as suggested by Carlquist (1958) and Nordenstam and El-Ghazaly (1977). Indeed, despite anatomical (Carlquist, 1958) and palynological (Parra, 1969–70) differences, Hellwig (2004) even proposed incorporating *Centaurodendron* in *Plectocephalus*. As with *Plectocephalus*, *Centaurodendron* has not been included in any previous molecular survey. As well as problems in delineating the genus, the positions of *Plectocephalus* and *Centaurodendron* within Centaureinae remain obscure. Both genera have *Serratula* type pollen (Wagenitz, 1955; Parra, 1969–70). Species having this kind of pollen form an unresolved polytomy at the base of the subtribe (Garcia-Jacas *et al.*, 2001), and all the molecular surveys have failed to elucidate the phylogeny of the basal branches of Centaureinae (Hidalgo *et al.*, 2006).

Biogeography

The geographical distribution of *Plectocephalus* and *Centaurodendron* is one of the most interesting features of the group, as it exhibits a striking disjunction. Were the placing of *P. varians* within *Plectocephalus* confirmed, the genus would comprise one species in East Africa, two in North America and four in South America, together with the diversification of *Centaurodendron* (three species) on the Juan Fernández Islands. As well as displaying a remarkable Afro-American disjunction, *Plectocephalus* species grow at the margins of regions considered to be the natural range of Centaureinae. This subtribe originated at the edges of the Mediterranean and Irano-Turanian regions, close to the Caucasus (Wagenitz and Hellwig, 1996; Susanna and Garcia-Jacas, 2009). Its present distribution is mainly Mediterranean and, to a lesser extent, Eurasian, with some rare species extending as far as East Africa whilst *Rhaponticum australe* (Gaudich.) Soskov occurs in Australia. A few genera (*Plagiobasis* Schrenk, *Russowia* C. Winkl. and *Schischkinia* Iljin) reach as far as Middle Asia, but only the genera *Goniocaulon* Cass. and *Tricholepis* DC. extend beyond the natural geographical barrier formed by the high peaks of Central Asia (Tien San, Himalaya and Hindu Kush), reaching India and even Burma. Thus, *Plectocephalus varians* has the distinction of being one of the southernmost species of the whole subtribe in East Africa, with *Plectocephalus* and *Centaurodendron* being the only native representatives of the Centaureinae on the American continent (Susanna and Garcia-Jacas, 2009).

Disjunctions between African and South American taxa are known to occur among the basal Compositae. This is contrary to what is found here, in that South American genera from the basal groups of the family have some African-derived representatives (Ortiz *et al.*, 2009). However, none of these Afro-South American distributions includes North America, where the basal branches of the Compositae are represented by a single, monotypic genus, *Hecastocleis* A. Gray (Funk and Hind, 2009). Other disjunctions that parallel the Afro-North American disjunction of *Plectocephalus* are the genera *Datisca* L. and *Plantago* L. (Stebbins and Day, 1967), *Styrax* L. (Fritsch, 1996, 2001), and the tribe Betoideae of the Chenopodiaceae (Hohmann *et al.*, 2006). Should the relationships of *Plectocephalus varians* and the American species be confirmed, there are two possible ways of explaining this disjunction: (1) migration by continuous range expansion through East Asia and the Bering Land Bridge (BLB) as postulated for *Datisca* and *Plantago* (Stebbins and Day, 1967); or (2) long-distance dispersal, as proposed by Raven (1972) for some Mediterranean–Californian disjunctions and by Fritsch (1996, 2001) for *Styrax*. If one takes into account the relatively young age of the genus, estimated to be approx. 12 million years (cf. Barres *et al.*, Botanic Institute of Barcelona, in prep.), the hypothesis that a North-Atlantic migration took place, as suggested for older floristic elements in the Paleogene, can be disregarded (see Hohmann *et al.*, 2006).

Defining the limits of the genus and identifying the ancestors of *Plectocephalus* is a critical and necessary step in determining the route by which this group migrated. In view of the low resolution of basal branches of the subtribe achieved in previous analyses (Garcia-Jacas *et al.*, 2001; Hidalgo *et al.*, 2006), the only possible approach is that of combined molecular analysis, including plastid and nuclear markers. Consequently, information was collected from five different regions [internal transcribed spacers (ITS) and external transcribed spacers (ETS) as nuclear markers, and *trnL-trnL-F*, *rpl32-trnL*^{UAG} and *ndhF* as plastid markers) with the following aims: (*a*) reaching a precise delineation of *Plectocephalus* and determining the systematic position of the African *P. varians*, and the South American *P. chilensis*, *Centaurea cachinalensis*, *C. floccosa* and *C. tweediei*; (*b*) verifying the relationships of *Centaurodendron* and *Plectocephalus*; (*c*) exploring the affinities of *Plectocephalus* within the basal genera of the Centaureinae; (*d*) using this new molecular evidence to improve our knowledge regarding the relationships of the 'Basal Grade' of the subtribe Centaureinae; and (*e*) determining the putative routes of geographic expansion and the largest area occupied by any native Cardueae.

MATERIALS AND METHODS

Plant material

The data set consisted of 178 accessions, including all the basal genera in subtribe Centaureinae, with the only exceptions of *Karvandarina* Rech. f. and *Ochrocephala* Dittrich (Susanna and Garcia-Jacas, 2007). The sampling was primarily aimed so as to represent the taxonomic and geographical diversity of the *Plectocephalus* group, for which the majority of the genomic regions were sequenced for the first time. We were able to sample only one of the three species of *Centaurodendron*. However, the general consensus is that all three species attributed to this genus constitute a natural group that includes monospecific *Yunquea* Skottsb., with the species *Y. tenzii* Skottsb. now considered a synonym of *Centaurodendron tenzii* (Skottsb.) Skottsb (Dittrich, 1977; Susanna and Garcia-Jacas, 2007).

For the plastid data set, we targeted three regions: *ndhF*, *rpl32-trnL*^{UAG} and *trnL-trnL-F*, which were sequenced for 40 species of Centaureinae. Some of the sequences of the *trnL-trnL-F* region have previously been published, but the protein-encoding *ndhF* region and *rpl32-trnL*^{UAG} (including a portion of the *rpl32* gene and the complete *rpl32-trnL* intergenic spacer) data are new. Nuclear regions were newly sequenced for 12 (29 %, ITS) and 23 (65 %, ETS) of the species studied.

The outgroup was chosen from representatives of the sister clade of subtribe Centaureinae (Garcia-Jacas *et al.*, 2002; Susanna *et al.*, 2006). It included one species from the genus *Cousinia* and one from *Saussurea.* Voucher data, source of material and GenBank sequence accession numbers are given in Table 1.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted following the cetyltrimethylammonium bromide (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.

Nuclear ribosomal DNA (nrDNA) ETS and ITS 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 followed the protocol described by Susanna *et al.* (2006). The ETS region was amplified with ETS1F as the forward primer (Linder *et al.*, 2000) and 18SETS as the reverse primer (Baldwin and Markos, 1998). In some cases, AST-1 and AST-2 were also used as internal primers (Markos and Baldwin, 2001). The profile used for PCR amplification was as described by Galbany-Casals *et al.* (2009). For both regions, reactions were performed in 25 µL volumes with 10 % of 10× AmpliTaq buffer, 10 % of 25 mM $MgCl_2$, 10 % of 2 mM dNTP mix, 4 % of each primer at 5 µM, 0·5 µL of DMSO (dimethylsulfoxide; Sigma-Aldrich, St Louis, MO, USA), 1 U of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) and 2 µL of template DNA of an unknown concentration. This was made up to 25 µL using sterile, distilled water.

Chloroplast DNA (cpDNA) amplification strategies. The *trnL-F* region was amplified using the trnL-c, forward, and trnL-f, reverse, primers (Taberlet *et al.*, 1991). In some cases, trnL-d, reverse, and trnL-e, forward, were also used (Taberlet *et al.*, 1991). The profile used for amplification of this region was as described by Susanna *et al.* (2006). The

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

Taxon	Voucher	Country	*trnL-trnF*	*rpl32-trnL*	*ndhF*	ITS	ETS
Acroptilon repens (L.) DC.	*Susanna* 2046 (BC)	UZB	AY772268	JF754869	JF754831	AY826223	DQ310989
Amberboa turanica Iljin	*Susanna* 1643 (BC)	IRN	JF754753	JF754870	JF754832	AY012311, AY012275	JF754783
Callicephalus nitens (M. Bieb. ex Willd.) C. A. Mey.	*Susanna* 1578 (BC)	ARM	AY772281	JF754871	JF754833	AY826237	DQ310972
Centaurea cachinalensis Phil.	*Belov s.n.* (BC)	CHL	JF754755	JF754872	JF754834	JF754804	JF754784
Centaurea floccosa Hook. & Arn.	*Belov s.n.* (BC)	CHL	JF754756	JF754873	JF754835	JF754805	JF754785
Centaurea tweediei Hook. & Arn.	*Dematteis 43 & Gutiérrez* (BC)	ARG	JF775384	JF775400	JF775396	JF775392	JF775388
Centaurodendron palmiforme Skottsb.	*Tobar s.n. & Arredondo* (BC)	CHL	JF754757	JF754874	JF754836	JF754806	JF754786
Centaurothamnus maximus (Forssk.) Wagenitz & Dittrich	*Molero s.n.* (BC)	YEM	AY772301	JF754875	JF754837	AY826259	DQ310971
Cheirolophus mauritanicus (Font Quer) Susanna	*Romo 4617* (BC)	MAR	AY772303	JF754876	JF754838	AY826261	DQ131087
Cheirolophus teydis (C.Sm.) G.López	*Susanna 1429* (BC)	ESP	AY772304	JF754877	JF754839	AY826262	DQ131092
Cousinia microcarpa Boiss.	*Susanna 2160 et al.* (BC)	KAZ	AY772312	JF754878	JF754840	AY826270	JF754787
Crupina crupinastrum Vis.	*Vilatersana s.n.* (BC)	ITA	JF754762	JF754879	JF754841	JF754829, JF754830	JF754788
Crupina vulgaris Cass.	*Susanna 1813* (BC)	ESP	AY772320	JF754880	JF754842	AY826280	JF754789
Goniocaulon indicum C.B.Clarke	*Fris s.n. et al.* (K)	ETH	JF775385	JF775401	JF775397	JF775393	JF775389
Klasea coriacea (DC.) J.Holub	*Susanna 1558* (BC)	ARM	DQ310892	JF754881	JF754843	DQ310926	DQ310965
Klasea serratuloides (DC.) Greuter & Wagenitz	*Susanna 1569* (BC)	ARM	AY772334	JF754882	JF754844	AY826295	DQ310962
Leuzea conifera (L.) DC.	*Font s.n.* (BC)	ESP	AY772337	JF754883	JF754845	AY826298	JF754790
Mantisalca salmantica (L.) Briq. & Cavill.	*Susanna 1457* (BC)	ESP	JF754765	JF754884	JF754846	AY012328, AY012292	JF754791
Myopordon aucheri Boiss.	*Carls s.n.* (BC)	IRN	AY772338	JF754885	JF754847	AY826299	DQ310977
Myopordon persicum Boiss.	*Remandieri s.n.* (BC)	IRN	DQ310898	JF754886	JF754848	AY826301	DQ310976
Oligochaeta divaricata K. Koch	*Susanna 1583* (BC)	ARM	AY772344	JF754887	JF754849	AY826306	DQ310973
Phalacrachena calva (Ledeb.) Iljin	*Tipsgko s.n.* (LE)	KAZ	JF754767	JF754888	JF754850	JF754815	JF754792
Phalacrachena inuloides (Fisch.) Iljin	*Romaschenko 402 & Didukh* (BC)	UKR	JF754768	JF754889	JF754851	JF754816	JF754793
Plagiobasis centauroides Schrenk	*Susanna 2130* (BC)	KAZ	DQ310887	JF754890	JF754852	AY826312	DQ310956
Plectocephalus americanus D.Don	*Quayle 765* (TEX)	USA	JF754769	JF754891	JF754853	JF754817	JF754794
Plectocephalus chilensis G.Don ex Loudon	Jardí Botànic de Barcelona s.n. (BC)	CHL	JF775386	JF775402	JF775398	JF775394	JF775390
Plectocephalus rothrockii (Greenm.) D.J.N.Hind	*Hiett s.n.* (TEX)	USA	JF754770	JF754892	JF754854	JF754818	JF754795
Plectocephalus varians (A.Rich.) C.Jeffrey in Cufod.	*Ortiz s.n. & Vivero* (BC)	ETH	JF775387	JF775403	JF775399	JF775395	JF775391
Psephellus persicus (DC.) Wagenitz	*Susanna 1716 et al.* (BC)	IRN	AY772352	JF754893	JF754855	AY826316	DQ310957
Psephellus pulcherrimus (Willd.) Wagenitz	*Susanna 1492 et al.* (BC)	ARM	AY772353	JF754894	JF754856	AY826317	DQ310958
Rhaponticoides hajastana (Tzvelev) M.V.Agab. & Greuter	*Susanna 1587 et al.* (BC)	ARM	AY772279	JF754895	JF754857	AY826235	DQ310959
Rhaponticoides iconiensis (Hub.-Mor.) M.V.Agab. & Greuter	*Ertuğrul 1761* (BC)	TUR	DQ310889	JF754896	JF754858	DQ310923	DQ310960
Rhaponticum acaule DC.	*Montserrat 2331 et al.* (BC)	DZA	AY772369	JF754897	JF754859	AY826334	DQ310995
Russowia sogdiana B.Fedtsch	*Kamelin s.n.* (LE)	TJK	JF754775	JF754898	JF754860	AY826320	JF754796
Saussurea maximowiczii Herder	Kanagawa Prefect. Ofuna Bot. Gard. s.n. (BC)	JPN	AY772359	JF754899	JF754861	AY826324	JF754797
Schischkinia albispina (Bunge) Iljin	*Botschantzev 827* (LE)	TKM	AY772360	JF754900	JF754862	AY826325	JF754798
Serratula coronata L.	Vienna Univ. Bot. Gard. s.n. (BC)	AUT	AY772362	JF754901	JF754863	AY826327	DQ310961
Stizolophus balsamita (Lam.) Cass. ex Takht.	*Susanna 1547 et al.* (BC)	ARM	AY772371	JF754902	JF754864	AY826336	JF754799
Stizolophus coronopifolius Cass.	*Ilarslan 4303* (ANK)	TUR	AY772372	JF754903	JF754865	AY826337	DQ310955
Tricholepis tibetica Hook.f. & Thomson ex C.B.Clarke	*Nüsser 1055* (B)	PAK	JF754780	JF754904	JF754866	AY826341	JF754800
Volutaria crupinoides (Desf.) Maire	*Vogt 11075 & Oberprieler* (B)	MAR	AY772379	JF754905	JF754867	AY826344	–
Zoegea leptaurea L.	*Susanna 1704 et al.* (BC)	IRN	AY772383	JF754906	JF754868	AY826349	JF754801

Country codes follow ISO A3 standard. An dash indicates a region that was not sequenced.

rpl32-trnL^{UAG} intergenic spacer was amplified with the primers rpL32F as forward and trnL-UAG as reverse (Shaw *et al.*, 2007) with the following termocycler settings: 4 min denaturing at 94 °C, followed by 35 cycles of 60 s denaturing at 95 °C, 90 s annealing at 54 °C and 2 min extension at 72 °C, with an additional final step of 10 min at 72 °C. The major part of the *ndhF* encoding region was amplified using a set of four primers. The 5′ end portion of the gene was not used in analysis because of its low substitution level (Kim and Jansen, 1995). Overlapping sequence fragments were obtained by amplifying the 3′ end portion of the gene in two pieces. For the 5′ quarter, we used 3′F as forward primer (Eldenas *et al.*, 1999) and 1783R (R. Vilatersana, Botanic Institute of Barcelona, pers. comm.) as reverse primer. For the 3′

quarter, we used 1626F (R. Vilatersana, Botanic Institute of Barcelona, pers. comm.) as forward primer and +607 (Kim and Jansen, 1995) as reverse primer. The profile for amplifications was used as in Kim *et al.* 2002. The PCRs were performed following the protocol used for the nuclear regions, but with the addition of 2·5 μL of 400 ng μL^{-1} BSA (bovine serum albumin; New England Biolabs, NE, USA).

nrDNA and cpDNA sequencing strategies. Plastid and nuclear PCR products were purified using a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA). Direct sequencing of the amplified DNA segments was performed using a BigDye Terminator Cycle Sequencing v3·1 kit (Applied Biosystems) in accordance with the manufacturer's recommended protocol. Nucleotide sequencing was performed at the 'Serveis Científico-Tècnics' of the University of Barcelona on an ABI PRISM 3100 DNA Analyzer (Applied Biosystems).

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. A graphical representation of the changes in sequence divergence for all the data sets was constructed using the Jukes–Cantor value, as implemented in PAUP. Eight data sets were prepared and analysed: ITS, ETS, combined ITS–ETS, *ndhF*, *rpl32-trnL*^{UAG}, *trnL-F*, combined plastid and combined ETS–plastid regions.

Maximum parsimony and Bayesian analysis were used to infer phylogeny. The maximum parsimony analysis was conducted in PAUP* 4·0b10 (Swofford, 2002). A heuristic search was performed employing the tree-bisection and reconnection (TBR) branch-swapping algorithm. The bootstrap statistical support was calculated using 1000 replicates and ten random addition sequences per replicate. The majority rule consensus tree of the 1000 resulting best trees found for each bootstrap reweighed data set was constructed. Bootstrap support (BS) values of 90 − 100 % were interpreted as strong support.

Bayesian posterior probabilities (PPs) were estimated using MrBayes v3·1·2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The evolutionary models for separate regions and combined data sets were selected using jModeltest (Posada, 2008). The maximum likelihood parameters were specified according to the Akaike Information Criterion (AIC). The plastid data set and combined ETS–plastid data sets were partitioned according to the number of regions in use, and the relevant substitution models were applied for each partition (Table 2). The extent of rate variation across sites for individual data partitions and for the combined data set was estimated by the shape parameter of the gamma distribution (σ). The results of partitioned runs were tested against the Bayesian analyses of the combined sets according to the single model of substitution. The ITS data set was analysed separately.

Each Bayesian analysis was initiated with random starting trees and was run for 1 million generations with sampling frequency of trees set at the 100th iteration. For all analyses, the variance of split sequences was <0·01, which indicated convergence of chains (Huelsenbeck and Ronquist, 2001). The fraction of sampled values discarded as burn-in was set at 0·25. Posterior probabilities of 0·95 − 1·00 were considered statistically significant.

A partition homogeneity test [incongruence length difference (ILD); Farris *et al.* (1995*a*, *b*)] implemented in PAUP v. 4·0b10 (Swofford, 2002) was carried out to verify the congruence of plastid and nuclear data sets. The *P*-value (adjusted at <0·01) was scored after 1000 replications run for two established partitions excluding uninformative characters and using heuristic search and random addition of sequences. In addition to the ILD test, the topologies obtained from the separate analyses were examined in order to detect any contradictory placement of taxa.

RESULTS

The topology of the trees obtained for separate regions by maximum parsimony and Bayesian approaches, respectively, was consistent. Therefore, only Bayesian majority-rule consensus trees are shown, with Bayesian PPs and parsimony BS added to the branches. With the exception of the ITS data, the phylogenetic analysis of the remaining regions revealed similar relationships for major groups. The ITS strongly supported a different placement for several annual species of interest belonging to the genera *Schischkinia*, *Stizolophus* and *Zoegea* L. compared with that shown by plastid and ETS data. The ILD test also indicated significant incongruence between the data sets of ITS and the remaining regions. Therefore, only plastid and ETS regions were used in combination, while the ITS data were processed separately. The value of the gamma shape parameter varied from 0·17 to 0·68 for the separate regions, which indicated substantial rate variation among sites in our data. The partitioned runs of the combined data sets under specified substitution models for each DNA region strengthened support for several crown nodes, as compared with searches made using the single model of substitution. The summary of the phylogenetic analyses of ITS, ETS, *rpl32-trnL*^{UAG}, *trnL-F*, *ndhF* and combined data sets is given in Table 2. Two trees are shown: ITS alone (Fig. 1) and combined ETS–plastid regions (Fig. 2). Sequence divergence rates among the species studied are detailed in Fig. 3.

In the combined ETS–plastid analysis (Fig. 2), the *Volutaria* Cass. group includes *Schischkinia* with strong parsimony BS and the highest PP value (BS = 98 %; PP = 1·00). This clade is sister to the remaining taxa representing basal Centaureinae. Within the *Volutaria* group, there are two supported clades. The first includes *Goniocaulon* and *Tricholepis* (BS = 100 %; PP = 1·00), whereas the second comprises *Amberboa* Vaill., *Plagiobasis*, *Russowia*, *Schischkinia* and *Volutaria* (BS = 60 %; PP = 0·97).

The crown node for the clade of basal Centaureinae received good support (BS = 95 %; PP = 1·00). However, relationships among the main groups in this clade remain poorly resolved, forming a general trichotomy. It includes a strongly supported clade of two species of *Cheirolophus* (BS = 100 %; PP = 1·00), another equally supported clade (BS = 100 %; PP =

TABLE 2. *Phylogenetic characteristics of the four plastid regions, ITS, ETS and combined regions used in the study*

	ETS	ITS	*ndhF*	*rpl32-trnL*	*trnL-F*	Plastid	Plastid + ETS
Total characters	522	650	1277	1086	816	3026	3531
Number of parsimony-informative characters	202	181	65	78	32	172	361
Tree length	806	759	213	273	102	578	1349
Consistency index (CI)	0·5298	0·5059	0·8216	0·8278	0·9314	0·8408	0·6597
Homoplasy index (HI)	0·4702	0·4941	0·1784	0·1722	0·0686	0·1592	0·3403
CI excluding uninformative characters	0·4515	0·415	0·6696	0·6781	0·8444	0·6954	0·5219
HI excluding uninformative characters	0·5485	0·585	0·3304	0·3219	0·1556	0·3046	0·4781
Retention index (RI)	0·6199	0·5318	0·819	0·8105	0·9255	0·821	0·6781
Rescaled consistency index (RC)	0·3284	0·2691	0·6729	0·671	0·862	0·6903	0·4474
Akaike Information Criterion (AIC)	TIM2 + G	SYM + G	TVM + G	TVM + G	TPM1	TVM + G	TPM1uf + G
Gamma shape parameter (σ)	0·684	0·438	0·176	0·176	–	0·27	8·169

1·00) which encompasses *Rhaponticum* (including former *Acroptilon* Cass. and *Leuzea* DC.), *Myopordon* Boiss. and *Oligochaeta* (DC.) K. Koch., and a third clade containing the remaining taxa which has a high PP value (1·00), but no statistically significant BS. Consequently, the latter clade encompasses four subsumed clades which separate in the following order. (1) The clade with a high PP value (1·00) but no BS support which includes *Callicephalus*, *Centaurothamnus* Wagenitz & Dittrich, *Klasea* Cass., *Mantisalca* and *Serratula* L. as sister to the joint clade (BS = 87 %; PP = 1·00) of the remaining Centaureinae. (2) The well-supported clade (BS = 100 %; PP = 1·00) of several species of the genera *Centaurodendron*, *Plectocephalus* and three South American species hitherto included in the genus *Centaurea* (*C. cachinalensis*, *C. floccosa* and *C. tweediei*). In this clade, the representatives of the *Plectocephalus* group are placed in two equally strongly supported clades (BS = 100 %; PP = 1·00). In the first, *P. varians* is sister to a moderately supported clade (BS = 81 %; PP = 0·54) that contains *P. americanus*, *P. rothrockii* and *C. tweediei*, while the second clade includes *Centaurodendron palmiforme* Skottsb. as sister to a strongly supported clade (BS = 100 %; PP = 1·00) comprising *P. chilensis*, *C. cachinalensis* and *C. floccosa*. (3) The unsupported joint clade (BS = 61 %; PP = 0·69) includes a strongly supported clade (BS = 100 %; PP = 1·00) of two species of *Stizolophus*, as well as a moderately to strongly supported clade (BS = 71 %; PP = 1·00) that contains the five remaining genera: *Crupina*, *Phalacrachena*, *Psephellus*, *Rhaponticoides* and *Zoegea*. Each of the four genera, *Crupina*, *Phalacrachena*, *Psephellus* and *Rhaponticoides*, is represented by two species placed in separate well-supported clades (BS = 100 %; PP = 1·00). On the whole, the taxa are arranged in three cognate, phylogenetic groups: the *Zoegea–Psephellus* group with strong support for the joint clade (BS = 96 %; PP = 1·00), the *Rhaponticoides* group and the *Crupina–Phalacrachena* group (BS = 96 %; PP = 1·00). Of these groups, *Rhaponticoides* is rendered sister to the *Crupina–Phalacrachena* group, with virtually no statistical support (BS = 62 %; PP = 0·92).

The topology resulting from ITS analysis (Fig. 1) mainly differs from that obtained from combined plastid and combined ETS–plastid analyses in the placement of the annuals *Schischkinia*, *Stizolophus* and *Zoegea*, which in the ITS tree are, in turn, sisters to the entire clade of the basal Centaureinae.

DISCUSSION

Taxonomic implications

Delineation of Plectocephalus. Monophyly of *Plectocephalus*, including African *P. varians*, North American *P. americanus* and *P. rothrockii*, together with South American *Centaurea cachinalensis*, *C. chilensis*, *C. floccosa* and *C. tweediei*, could be confirmed, were it not for the position of *Centaurodendron*, deeply nested in the clade formed by the perennial species of the group (Figs 1 and 2). If *Centaurodendron* is recognized as a separate genus on molecular grounds, then *Plectocephalus* remains paraphyletic. Nevertheless, there are some arguments against incorporating *Centaurodendron* in *Plectocephalus*. First, the position of

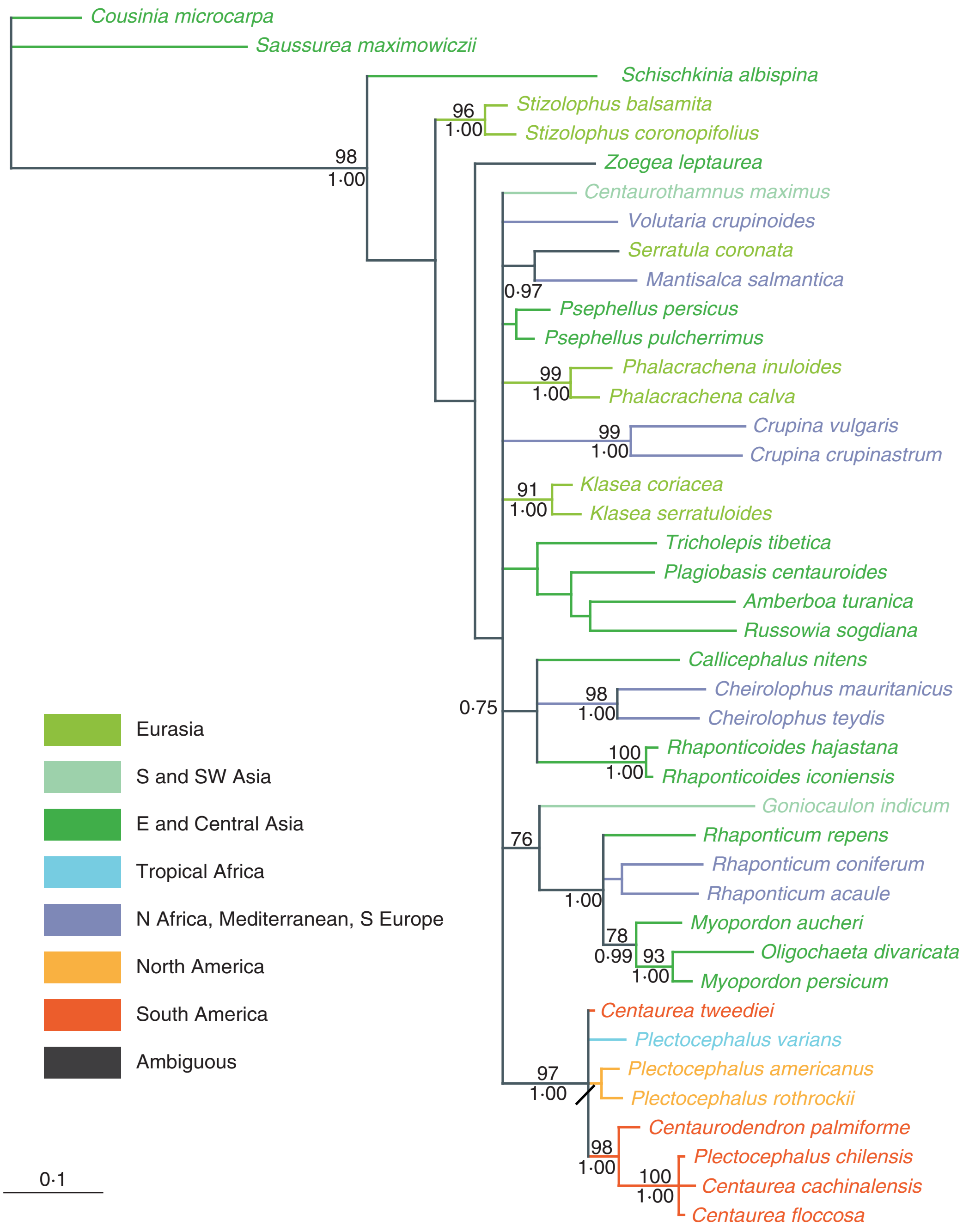

FIG. 1. Majority-rule consensus tree based on Bayesian MCMC analysis of the ITS region. Numbers above branches are parsimony bootstrap percentages (BS); numbers below branches are Bayesian posterior probability values (PP). Colour codes of geographical distribution according to Funk *et al.* (2009).

Centaurodendron within the clade of perennial species from Chile might reflect the peculiarities of genome evolution that are associated with perennial vs. annual life cycles, as will be discussed below. Secondly, the morphological differences that exist between *Centaurodendron* and *Plectocephalus* are substantial and are not limited solely to the habit (Parra, 1969–70). There are also differences in geographic distribution between Chilean *Plectocephalus* and *Centaurodendron*, in that the latter is a small tree characteristic of the Fernandezian Floristic Region, while species of *Plectocephalus* from Chile belong to the Northern and Middle Chilean Provinces (Takhtajan, 1986). Finally, the case of *Centaurodendron* is a timely example of the unwanted consequences of a strict cladistic interpretation of paraphyly in endemics from oceanic archipelagos as discussed by Hörandl and Stuessy (2010). When dealing with genera that are downgraded to species because of paraphyly, they pointed out: ‘The loss of endemic generic [. . .] status, however, greatly lowers their conservation importance. When one multiplies this result in oceanic islands worldwide, the decrease in island endemism at the generic level declines substantially. One has to question the advisability of this approach, particularly

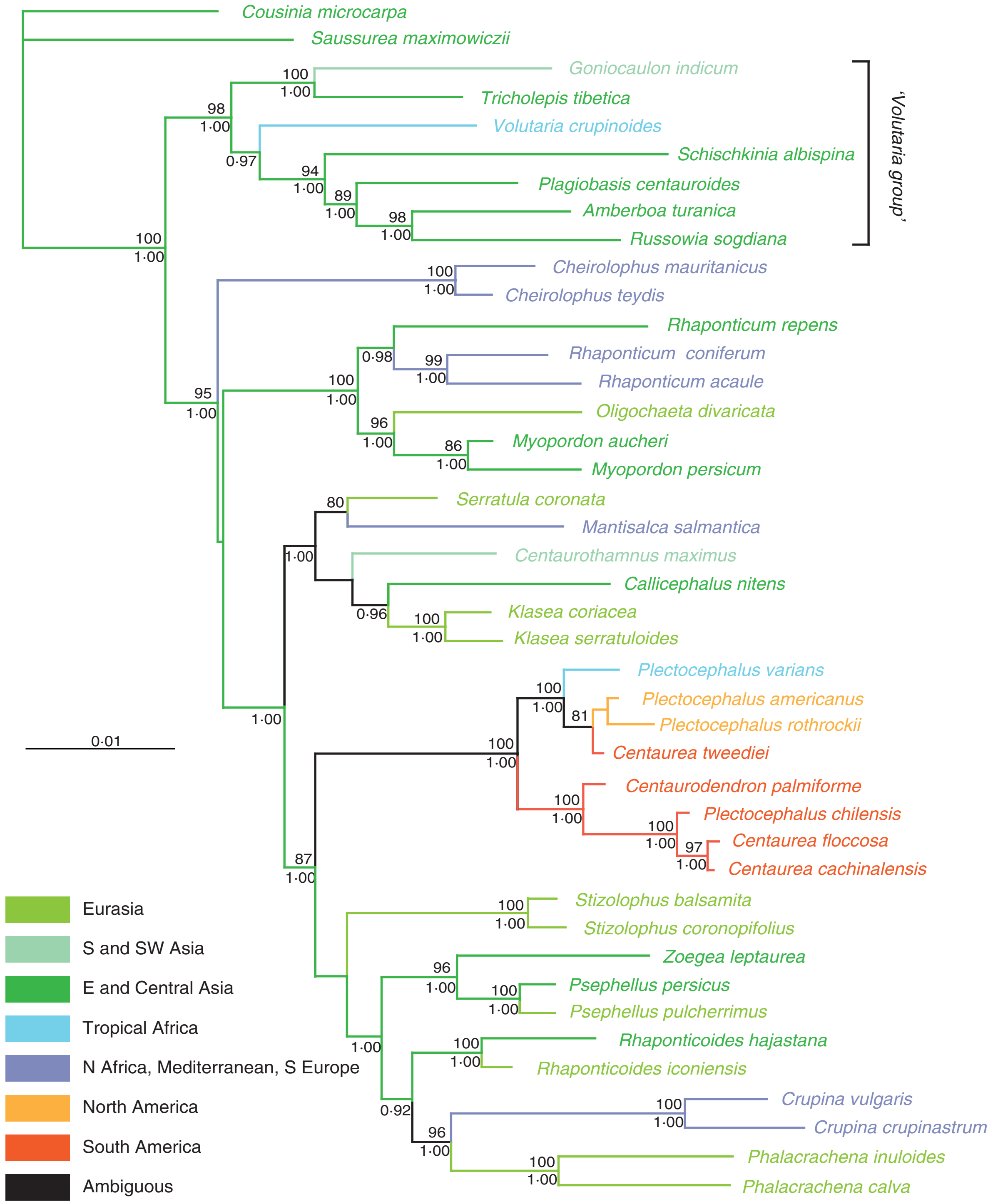

FIG. 2. Majority-rule consensus tree based on Bayesian MCMC analysis of the combined 3′ ETS and plastid regions. Numbers above branches are parsimony bootstrap percentages (BS); numbers below branches are Bayesian posterior probability values (PP). Colour codes are according to Funk *et al.* (2009).

because the reason the taxa have been treated as good genera in the first place is because they are highly morphologically divergent from their continental relatives.' We must conclude that it is preferable to keep *Centaurodendron* as an independent genus.

All analyses confirm that *Plectocephalus* should also include African and South American species formerly assigned to *Centaurea*. Consequently, adequate nomenclatural combinations are proposed (see Appendix 1). However, relationships within the genus are difficult to explain. The combined molecular phylogeny defines two clades: the first, containing the annual species *P. americanus*, *P. rothrockii*, *P. tweediei* and *P. varians* (Fig. 2); and the second, the shrubby perennials *Centaurea cachinalensis*, *C. floccosa*, and

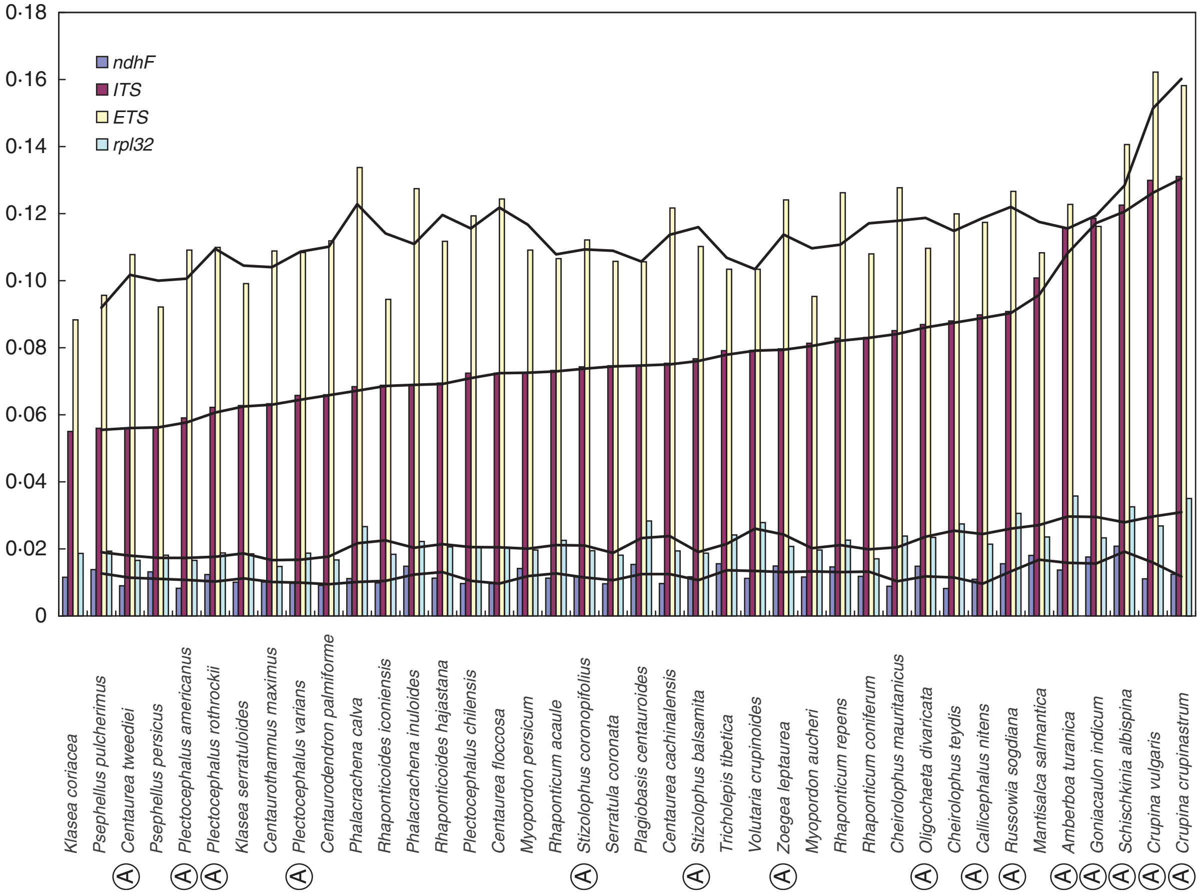

FIG. 3. Graphic of the sequence divergence (Jukes–Cantor coefficient) calculated for all the sequenced regions. 'A' indicates an annual species.

Plectocephalus chilensis, as well as the arborescent *Centaurodendron*. If one accepts this result as reflecting true phylogenetic relationships, then one must also accept that two different dispersal events from North to South America must have occurred, the first giving rise to *P. tweediei*, and the second to the remainder of the species found in Chile and Juan Fernández. However, the most-parsimonious explanation is the occurrence of a single colonization event from North America. Based on our results, it is difficult to decide between these two hypotheses, but the character of the groups defined within the clade *Plectocephalus–Centaurodendron* (see above) makes us suspect its artefactual nature. In this case, a single introduction would be the most plausible explanation.

The affinities of Plectocephalus

Our results show *Plectocephalus* to be part of an unsupported polytomy, together with two other clades (Fig. 2): the first contains the genus *Stizolophus*, whereas the other is a moderately supported clade, containing the genera *Crupina*, *Phalacrachena*, *Psephellus*, *Rhaponticoides* and *Zoegea*. Based on strict morphological grounds, the only genera to share some similarities with *Plectocephalus* are *Phalacrachena* (especially the achenes) and *Psephellus* (in particular the sterile florets). Further studies are necessary to confirm which genus is the most closely related, but our results indicate that *Plectocephalus* belongs to an assemblage of genera that share an Eastern–Mediterranean and Irano-Turanian distribution (Fig. 2). The only exceptions are the circum-Mediterranean colonizing species of *Crupina*. However, *Crupina* is sister to the Siberian *Phalacrachena* with high statistical support, suggesting that it too could have had an eastern origin, as previously proposed by Garnatje *et al.* (2002).

The sister group of the Centaureinae: do annuals frustrate ITS analyses?

The results presented here show topological modification of the previous phylogeny with regard to the basal clade of Centaureinae, and this deserves further discussion.

A previous survey of Centaureinae (Garcia-Jacas *et al.*, 2001) identified the monotypic genus *Schischkinia* as sister to the rest of the subtribe (Fig. 4). Other successive sisters included two small genera, namely *Zoegea* (four species) and *Stizolophus* (two species). ITS analysis presented here yielded a similar phylogeny (Fig. 1). The only character shared by these three genera is their annual habit, and, therefore, it is possible that their presumed position is, in fact, incorrect (Garcia-Jacas *et al.*, 2001; Susanna *et al.*, 2006; Susanna and Garcia-Jacas, 2007, 2009). On morphological

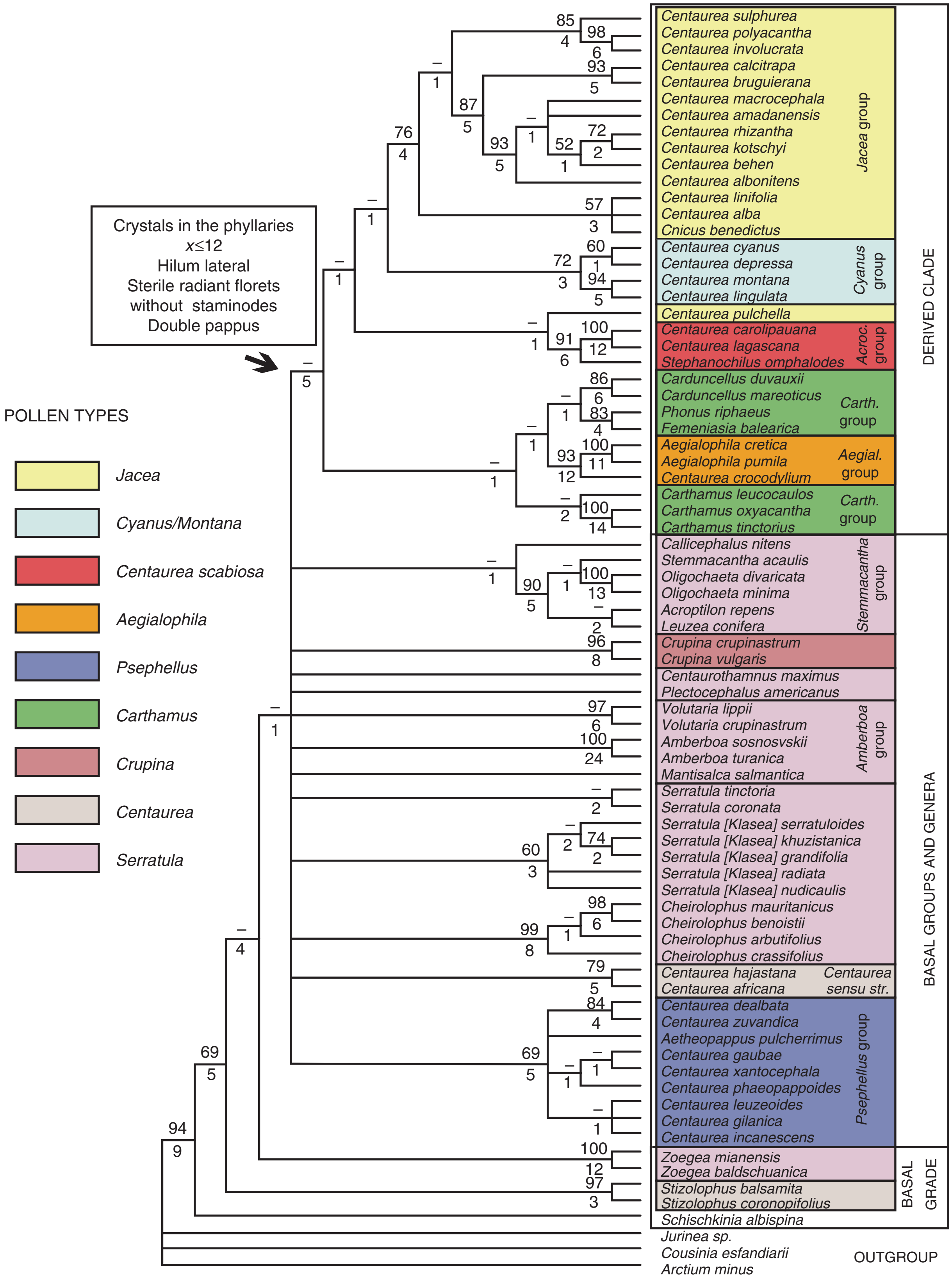

FIG. 4. Strict consensus tree resulting from the ITS analysis of the subtribe Centaureinae by Garcia-Jacas *et al.* (2001).

grounds, there are no plesiomorphic characters to support this position. Other cases of annual species being placed in a basal position in the tribe Cardueae are discussed in López-Vinyallonga *et al.* (2009).

The addition of plastid regions (*ndhF*, *rpl32-trnL*^{UAG} and *trnL-F*) strongly suggests that a difference in life cycle is the cause of the basal position of *Schischkinia*, *Zoegea* and *Stizolophus. Schischkinia* is nested within the *Volutaria* group, a complex of seven genera (Susanna and Garcia-Jacas, 2007, 2009) now defined as the basalmost group in the Centaureinae. *Zoegea* is currently considered sister to *Psephellus* Cass., a genus found in Anatolia, the Caucasus and northern Iran. *Stizolophus* appears to be unrelated to any genus, but is well nested within the Centaureinae, and distant from the basal branches (Fig. 2). These relationships and other unexpected associations, such as the link that exists between *Crupina* and *Phalacrachena* (Fig. 2), need to be reviewed on morphological and palinological grounds. However, this lies beyond the scope of the present paper. Even so, the present results confirm the aberrant position of some annuals in phylogenetic reconstructions, and this phenomenon has been much discussed in recent years. Aïnouche and Bayer (1999), Andreasen and Baldwin (2001), Smith and Donoghue (2008) and Smith and Beaulieu (2009), as well as many others, speculated that plant lineages with longer generation times, as a general rule, have lower molecular evolution rates than lineages with shorter generation times. This is surely true for the three genera whose position has been so difficult to explain in previous studies, no doubt because of the high sensitivity of the ITS region to differences in the life cycle (Aïnouche and Bayer, 1999; Andreasen and Baldwin, 2001).

The most sensitive region is the ITS, with a divergence rate ranging from 0·055 to 0·13 (Fig. 3). The ETS is also sensitive to the annual habit and shows a steep divergence peak for some annuals, the difference between maximum and minimum divergence being less marked. However, sensitivity to the different life cycles is not limited to raw divergence: Two of the misplaced genera, *Stizolophus* and *Zoegea*, are placed in the middle range of divergence (Fig. 3), and annual species of *Plectocephalus* that form a group, here suspected to be artefactual (see above), occur interspersed among perennial species. This suggests that difficulties relating to the placement of these annuals on the basis of ITS sequences cannot be attributed to raw divergence, but that other processes must be involved in the molecular evolution of annuals. Long-branch attraction, a frequent problem in parsimony analysis and less evident in Bayesian approaches (Swofford *et al.*, 2001), could also be responsible for some of the unwanted results of ITS analyses. It is significant that the retention index for the ITS analysis is the lowest of all data sets, while the homoplasy index is the highest (Table 2).

This deficiency of ITS lends support to other criticisms relating to the widespread and automatic use of this marker (sensitivity to reticulation and proclivity to lineage sorting following biased concerted evolution; see Nieto Feliner and Rosselló, 2007). As suggested by Álvarez and Wendel (2003), ITS should always be used in combination with other markers, and workers should be fully aware of the limitations and deficiencies of this particular region. Based on our results, we recommend that the use of ITS should be carefully weighted for phylogenetic reconstruction when working with plants having different types of life cycle (annuals vs. perennials).

Evolution of life cycles within the group is also of interest. Species of the *Plectocephalus* group are either annual (*P. americanus*, *P. rothrockii*, *C. tweediei* and *P. varians*) or perennial (*Centaurea cachinalensis*, *C. floccosa*, *Centaurodendron* sp. pl. and *Plectocephalus chilensis*). Ancestral taxa for the group are likely to be perennial, as this is the ancestral state for all genera of Cardueae, a tribe derived from shrubby African ancestors of subfamily Carduoideae (Ortiz *et al.*, 2009). Significantly enough, the most likely ancestors for subtribe Centaureinae (*Cousinia*, *Jurinea* and *Saussurea*) include mainly perennial species: there are only four annuals in total for these three genera that together sum >1000 species (Susanna *et al.*, 2006; Susanna and Garcia-Jacas, 2007). Evidence that annual species developed only recently in the *Plectocephalus* group is based on low divergence rates (Fig. 3). While most annual species of other genera are grouped together at the right end of the graphic and show high levels of divergence, in contrast all the annual species of the *Plectocephalus* group are found at the other end of the range. It is thus interesting to note the affirmation made by Hind (1996) that the two North American species of *Plectocephalus* can be facultatively annual, biennial or perennial, a striking case of phenotypic plasticity that reinforces the hypothesis that the annual life cycle was recently adopted by species of this genus. The development of the arborescent habit in *Centaurodendron* is yet another example of a very rapid response to island habitats (Crawford *et al.*, 1992). In fact, the Juan Fernández Islands are very young, the oldest being only 4 million years old (Stuessy *et al.*, 1984).

In brief, perennial ancestors of *Plectocephalus* developed an annual life cycle, probably in response to climate change; but, in more stable conditions, they reverted to the perennial life cycle.

The migration of Plectocephalus

Susanna and Garcia-Jacas (2007, 2009) have suggested that Centaureinae originated at the boundaries of the Mediterranean and Irano-Turanian regions (Caucasus and Northern Iran). Since the affinities of *Plectocephalus* lie with Eastern–Mediterranean and Irano-Turanian groups, the occurrence of *Plectocephalus varians* in Ethiopia is likely to be due to the migration of more boreal ancestors. This is also known to occur in another rare species of the subtribe that grows in East Africa, namely *Ochrocephala imatongensis* (Philipson) Dittrich, which belongs to the mostly Eurosiberian *Rhaponticum* group (Hidalgo *et al.*, 2006). Taking into account the Caucasian or north-Irano-Turanian origin of *Plectocephalus*, its disjunction parallels that of two of the best studied examples to date, namely subtribe Betoideae of the Chenopodiaceae (Hohmann *et al.*, 2006) and the genus *Styrax* (Fritsch, 1996). A discussion of the two possible hypotheses for the origin of American *Plectocephalus*, namely long-distance dispersal or migration (in the sense of continuous range expansion), now follows.

Long-distance dispersal was the preferred hypothesis for *Styrax* (Fritsch, 1996), not just based partly on Axelrod's (1975) strong opposition to migration, but also because only a frost- and drought-tolerant ancestor could travel through the cold latitudes between Caucasus and Beringia. If the migration of this ancestor was as hypothesized, then it is also possible to speculate about its extinction. Despite the objections of Fritsch (1996), extinction of intermediate populations is supported by other disjunctions between North America and the Mediterranean. The *Filago* group (Compositae, Gnaphalieae) is a case in point. According to Ward *et al.* (2009) and Galbany-Casals *et al.* (2010), species of this group are mostly annuals that are adapted to xeric or high-mountain habitats. The *Filago* group has a Mediterranean distribution extending eastwards to Central Asia, with another centre of speciation in the south of the USA (California, Texas and Arkansas) and north of Mexico (Stebbins and Day, 1967). Thus, the most plausible explanation for this distribution is a continuous range expansion from Central Asia to North America via the BLB, followed by the extinction of landmark species in Far East Asia. This could also be the case for *Datisca* (Liston *et al.*, 1992) distributed throughout the East Mediterranean, Central Asia and North America, as well as the tribe Betoideae (Hohmann *et al.*, 2006).

Ever since Stebbins and Day (1967) suggested the existence of such a corridor, the possibility that species from xeric habitats were able to cross Central and East Asia on their way to the BLB has been much debated. The presence of two species of the genus *Phalacrachena* (assigned to subtribe Centaureinae) in Siberia indicates that this expansion was, at least to some extent, possible. Distribution of *Phalacrachena* cannot be considered direct evidence for migration by way of Siberia to North America because, despite some morphological similarities (especially the achenes), a close relationship between *Phalacrachena* and *Plectocephalus* is not supported by molecular analyses. Nevertheless, *Phalacrachena* does still provide indirect evidence. Close examination of the leaves of *Phalacrachena* and *Plectocephalus* reveals the presence of many sessile glands. These are frequent in many North American desert plants, and occur as critical adaptations to xeric habitats, since they greatly increase the reflectance capacity of leaves (Ehleringer, 1984). This same role has also been suggested for sessile glands in Mediterranean taxa such as *Origanum* L. (Kokkini *et al.*, 1994). The presence of glands in *Phalacrachena*, together with its somewhat incrassate leaves, demonstrates that this genus belongs to a stratum of xeromorphic plants that has had the opportunity to migrate in a north-easterly direction (Fig. 5). Other examples of xeric taxa that migrated in the same way are cited by Yurtsev (2001) and, as previously stated, the case for the *Filago* group is very convincing (Galbany-Casals *et al.*, 2010). The hypothesis for a corridor that allowed the migration of xerophytes via East Asia is increasingly supported, despite being on a very different time scale from the Palaeogene suggested by Stebbins and Day (1967).

The hypothesis that migration occurred via the BLB is also supported by the time framework. As for Betoideae (Hohman *et al.*, 2006), separation of *Plectocephalus* from the basal Centaureinae occurred no later than the middle Miocene [approx. 12 million years ago (Mya), cf. Barres *et al.*, Botanic Institute of Barcelona, in prep.], and this agrees with the time scale proposed for the period when the BLB was most used as a migration corridor (Marincovich and Gladenkov, 1999).

Within this time scale, the event that triggered the processes of extinction, transformation and migration of *Plectocephalus*

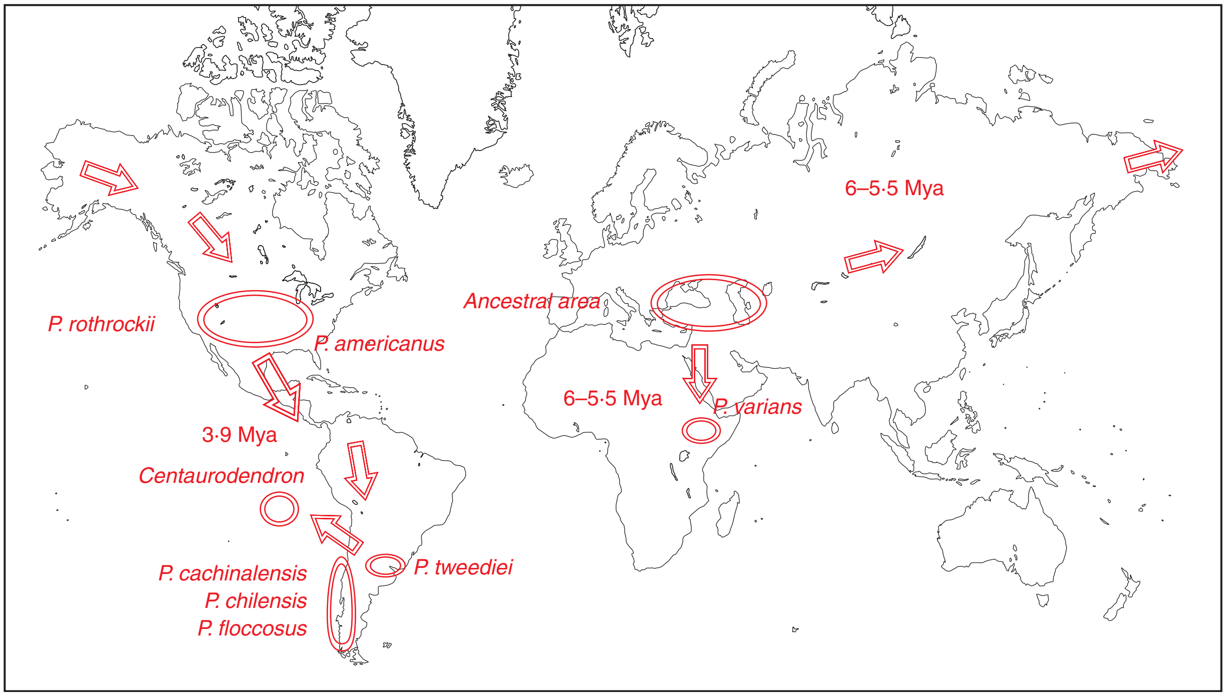

Fig. 5. Geographic distribution of the genera *Centaurodendron* and *Plectocephalus*. Arrows indicate the hypothesized migration route with possible dates. Mya = million years ago.

may have been the Messinian Salinity Crisis (MSC), which reached its zenith at about 5·5 Mya (Rögl, 1998, 1999; Suc and Popescu, 2005). The date of 5·5 Mya is also generally regarded as the period when the BLB closed, and it is possible that the migration of *Plectocephalus* also took place at about this time. The BLB then reopened approx. 2·4 Mya (Ogasawara, 1998) and, thus, it is equally possible that colonization could have happened then. However, there is an argument that favours the date of approx. 6–5 Mya. Environmental transformations triggered by the MSC (and emanating outwards from the Mediterranean region) may have resulted in the front-line migration of some plant species. These followed the edges of the drastically changing and expanding climatic zones, and it is assumed that the latter provided conditions that favour annual forms of *Plectocephalus*. Had a later migration taken place during the intermittent reopening of the BLB, then it would have been necessary for representatives of this genus to survive in some of the East Asian Tertiary refuges for a period of approx. 3 million years. If this is so, then one would expect some species of the group still to grow in Far East Asia, much like three other genera of tribe Cardueae: *Atractylodes* DC. and *Synurus* Iljin in Korea and Japan, and *Tricholepis* in Burma (Susanna and Garcia-Jacas, 2009).

The emergence of the Panamanian Land Bridge (Isthmus of Panama) 3–3·5 Mya (Webb, 1991; Graham, 1992; Coates and Obando, 1996) dramatically changed the climatic condition of both Americas. The Pliocene climate was much cooler than that of the Late Miocene, with more conspicuous seasonal changes and ecological zonality (Pascual *et al.*, 1996). Additionally, the final phase of the Andean orogenesis produced a rain-shadow effect that resulted in the establishment of extremely xeric conditions (Pascual *et al.*, 1996). At the time when the two Americas were connected, the savannah–grassland environments were predominant on both sides of the Isthmus of Panama, and this facilitated the first phase of the Great American Biotic Interchange (Pascual *et al.*, 1996; Webb and Rancy, 1996; Koepfli *et al.*, 2007). It is assumed that this was the time when the annual ancestors of the modern branch of the South American *Plectocephalus* and *Centaurodendron* migrated via the Isthmus of Panama. The alternative explanation (an earlier migration involving long-distance dispersal) is less plausible for the following reasons: (1) the probability that species became established in a totally new environment through long-distance dispersal is very low (Nathan, 2006); and (2) in the late Miocene, most of South America was covered by flooded plains, while elevated territories were occupied mostly by tropical forests. The first appearance of C_4 grasses in the diet of grazing animals as indicators of xerophytization in response to the climate occurred no earlier than 6·5 Mya and became widespread by 3·9 Mya, i.e. by the time the Isthmus of Panama closed (McFadden *et al.*, 1996). The xerothermic habit of *Plectocephalus* would be inconsistent with the supposed new environment, even if seed were successfully transported by birds.

Therefore, the expansion of *Plectocephalus* to South America can be dated to the time when the latter was still connected to North America and the subsequent establishment of an arid environment throughout the territory of migration. However, the increasing zonality and complexity of the developing Pliocene climates presented new challenges to the adaptive capacity of *Plectocephalus*. This time, it resulted in new transformations in response to, and as adaptations to new environments. These included the perennial habit and finally, the arborescent habit.

An outline of the speculated pathway and dates of migration of *Plectocephalus*, from its place of origin to East Africa, East Asia, North America and South America, is presented in Fig. 5.

Concluding remarks

The study of *Plectocephalus* confirms that it is a natural genus comprising African (*P. varians*), North American (*P. americanus* and *P. rothrockii*) and South American (*P. cachinalensis*, *P. chilensis*, *P. floccosus* and *P. tweediei*) species. We have confirmed that the genus *Centaurodendron* from Juan Fernández Islands derived from the genus *Plectocephalus* from continental Chile in a fine example of budding. The group made an astonishing journey, from the Caucasus to Africa and to North America, South America and finally to the Juan Fernández Islands. The results presented here support the existence of a migration route for Mediterranean xerophilous taxa via Beringia. This is supported by other cases that together indicate the late Miocene as the most plausible time for the opening of this pathway. Finally, this study stresses the importance of using plastid and nuclear DNA regions in combination for inferring the phylogenies of groups with different life cycles. In the case of the basal Centaureinae, a study using the nuclear marker ITS in combination with two plastid markers resulted in an incorrect phylogeny. This is most likely because molecular evolution in annuals differs from that of perennials and because ITS are extremely sensitive to such differences.

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APPENDIX

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Plectocephalus tweediei (Hook. & Arn.) N. Garcia & Susanna, **comb. nov.;** basionym: *Centaurea tweedieii* Hook. & Arn., Companion to the Botanical Magazine 1: 110. 1835.