

Phylogeny of *Rhaponticum* (Asteraceae, Cardueae–Centaureinae) and Related Genera Inferred from Nuclear and Chloroplast DNA Sequence Data: Taxonomic and Biogeographic Implications

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• **Background and Aims** The precise generic delimitation of the *Rhaponticum* group is not totally resolved. The lack of knowledge of the relationships between the basal genera of Centaureinae could imply that genera whose position is as yet unresolved could belong to the *Rhaponticum* group. On the other hand, the affinities among the genera that are considered as members of this group are not well known. The aim of the study is to contribute to the phylogenetic and generic delineation of the *Rhaponticum* group on the basis of molecular data.

• **Methods** Parsimony and Bayesian analyses of the combined sequences of one plastid (*trnL-trnF*) and two nuclear (ITS region and ETS) molecular markers were carried out. The results of these analyses are discussed in the light of the biogeographic history.

• **Key Results** The *Rhaponticum* group appears as monophyletic, and closely related to the genus *Klasea*. The results confirm the preliminary generic delimitation of the *Rhaponticum* group, with the new incorporation of the genus *Centaurothamnus*. *Ochrocephala* is supported as a separate genus from *Rhaponticum* and, contrary to this, *Acroptilon* and *Leuzea* appear as merged into the genus *Rhaponticum*. Several nomenclatural rearrangements are made in *Klasea* and *Rhaponticum*.

• **Conclusions** The new molecular evidence is consistent with the morphological and karyological data, and suggests particularly coherent biogeographic routes of migration and speciation processes for the genus *Rhaponticum*. The biogeographic inference proposes a Near East and/or Caucasian origin for the genus. Furthermore, representatives of *Rhaponticum* could have reached Europe in two different ways: (1) expansion across central Asia to eastern Europe, and (2) expansion through the Near East, North Africa and then to the Iberian Peninsula and the Alps.

Key words: *Acroptilon*, biogeography, *Callicephalus*, *Centaurothamnus*, ETS, ITS, molecular phylogeny, *Myopordon*, *Ochrocephala*, *Oligochaeta*, *Rhaponticum*, *trnL-trnF*.

INTRODUCTION

One of the main problems which persists in the subtribe Centaureinae (Asteraceae, Cardueae) is that the phylogenetic relationships between the early branching genera are unresolved. Monographers (Dittrich, 1977; Wagenitz and Hellwig, 1996; Garcia-Jacas *et al.*, 2001; Hellwig, 2004; Susanna and Garcia-Jacas, 2006) have described informal groups of genera. One of these is the *Rhaponticum* group which comprises about seven genera and approx. 40 species. Apart from the genus *Rhaponticum* Vaill. (= *Stemmacantha* Cass.; cf. Greuter *et al.*, 2005) of approx. 25 species, it includes *Acroptilon* Cass. (two species), *Callicephalus* C. A. Mey. (one species), *Leuzea* DC. (one species), *Myopordon* Boiss. (five species), *Ochrocephala* Dittrich (one species) and *Oligochaeta* (DC.) K. Koch. (four species).

The classic morphological approach limited itself to associate *Rhaponticum* and *Acroptilon* (Dittrich, 1977), and pointed out generic delimitation problems: Holub (1973) concluded that *Leuzea* and *Rhaponticum* should be merged, and Dittrich (1983) that *Rhaponticum imatongensis* (Phillipson) Soják should be segregated to constitute a new monotypic genus, *Ochrocephala*. Later, the molecular approach allowed the addition of *Callicephalus*,

Oligochaeta (Garcia-Jacas *et al.*, 2001) and *Myopordon* (Susanna *et al.*, 2006).

These genera show the symplesiomorphic characters common to the basal genera of the Centaureinae (absence of radiant peripheral florets, lack of crystals in the phyllaries, basal hilum, no bolster cells, absence of hairs on the achene and pollen of the *Serratula* type), and some morphological traits which characterize the group: (a) a peculiar type of involucre bract with a big, soft scarious, entire or lacerate and usually silvery-white appendage (an exclusive character of the group which, unfortunately, not all the species show); (b) dimorphic achenes (the outer dorsiventrally compressed and the inner laterally compressed); and (c) the double pappus typical of all the Centaureinae, but with the peculiarity that the inner bristles are wider and longer than the outer.

The geographical distributions, environmental requirements and life cycles are very diverse in *Rhaponticum* and related genera. They are naturally distributed in North Africa (including the Canary Islands), temperate Eurasia, Siberia and the Far East, Caucasus, central and eastern Asia and eastern Australia. They grow in deserts or mountains, and are either widely distributed or narrow endemics. They can be perennial or annual, and their habit is shrubby, or hemicryptophyte from 10 cm to >1 m in height, or acaulescent. Several species are endangered to the verge

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of extinction, but one taxon, *Acroptilon*, is considered to be an invasive weed in America and Australia. The *Rhaponticum* group includes the only species of Centaureinae indigenous to Australia, *Rhaponticum australe* (Gaudich.) Soskov (Wagenitz and Hellwig, 1996). Some representatives of the group show medicinal properties which were already known in Roman culture (Plinius, 77), and various species are being marketed due to their anabolic and adaptogenic properties.

The main goals of the present study in establishing a combined molecular phylogeny were to (a) elucidate the relationships between the basal groups of Centaureinae, with the purpose of determining the taxa most closely related to the *Rhaponticum* group; (b) verify the generic delimitation of the *Rhaponticum* group and the relationships between its genera; and (c) link the findings to the group's biogeographic history.

MATERIALS AND METHODS

Plant material

The sampling includes representatives of all the genera of the *Rhaponticum* group, and all the species of the genus *Rhaponticum* (except *R. namanganicum* Iljin). The outgroups have been selected according to previous works by Garcia-Jacas *et al.* (2001) and Susanna *et al.* (2006) to represent most of the basal Centaureinae which could have phylogenetic affinities with the study group: *Centaurothamnus* Wagenitz & Dittrich, *Cheirolophus* Cass., *Klasea* Cass., *Plagiobasis* Schrenk, *Psephellus* Cass., *Rhaponticoides* Vaill., *Serratula* L. and *Stizolophus* Cass. The purpose of representing numerous outgroups is to be able, without forcing the topology, to test how good is the assignation of a taxon as an outgroup or as an ingroup, and to define the taxa which are most closely related to the *Rhaponticum* group. Both previously published and the 111 new sequences (31 ITS, 47 ETS, 33 *trnL-trnF*) were used in the analyses. The origin of the samples and GenBank sequence accession numbers are given in Table 1.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted following the mini-prep procedure of Doyle and Doyle (1987) as modified by Soltis *et al.* (1991) and Cullings (1992) from herbarium material, silica gel-dried leaves collected in the field, or fresh leaves of plants cultivated in the Botanic Institute of Barcelona. DNA of old herbarium material was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA).

nrDNA ITS region strategies. ITS1, 5-8S gene and ITS2 (the ITS region) were amplified and sequenced together with 1406F (Nickrent *et al.*, 1994), ITS1 (White *et al.*, 1990) and 17SE (Sun *et al.*, 1994) as forward primers, and ITS4 (White *et al.*, 1990) and 26SE (Sun *et al.*, 1994) as reverse primers, referring to the protocol described in Soltis and Kuzoff (1993). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc.). Direct sequencing of the amplified DNA segments was

performed using the BigDye Terminator Cycle Sequencing v3.1 (PE Biosystems, Foster City, CA, USA). Nucleotide sequencing was carried out at the Serveis Científicotècnics of the University of Barcelona on an ABI PRISM 3700 DNA analyser (PE Biosystems).

nrDNA ETS region strategies. The ETS region was amplified and sequenced with ETS1f as forward primer and 18S-2L as reverse primer (Linder *et al.*, 2000), referring to the PCR procedure described in the same publication. Purification and direct sequencing of the amplified DNA segments were performed as for the ITS region.

cpDNA trnL-trnF region strategies. The *trnL-trnF* region includes the *trnL* intron, the 3' *trnL* (UAA) exon, and the intergenic spacer between *trnL* (UAA) and *trnF* (GAA), which were amplified and sequenced together. The universal primers *trnL-c*, forward, and *trnL-f*, reverse (Taberlet *et al.*, 1991) were used to amplify and sequence the *trnL-F* region. For old material, the region was amplified and sequenced in two parts using the two precedent primers and the *trnL-e*, forward, and *trnL-d*, reverse, of the same author. The PCR procedure includes a warm start at 95 °C for 1 min 35 s, followed by 80 °C during which the polymerase (EcoTaq, Ecogen S.R.L., Barcelona, Spain) is added, and 34 cycles of 1 min denaturation at 93 °C, 1 min annealing at 58 °C, 1 min extension at 72 °C, and a final 10 min extension at 72 °C. Purification and direct sequencing of the amplified DNA segments was performed as for the ITS region.

Phylogenetic analyses

Nucleotide sequences were edited with Chromas 1.56 (Technelysium Pty, Tewantin, Australia). DNA sequences were aligned visually by sequential pairwise comparison (Swofford and Olsen, 1990).

Parsimony analysis. Parsimony analysis involved heuristic searches conducted with PAUP version 4.0b10 (Swofford, 1999) using tree bisection recognition (TBR) branch swapping with character states specified as unordered and unweighted. All most-parsimonious trees (MPT) were saved. To locate islands of most-parsimonious trees (Maddison, 1991), 100 replicates were performed with random taxon addition, also with TBR branch swapping. Trees lengths, consistency index (CI) and retention index (RI) are always given excluding uninformative characters. Bootstrap (BS; Felsenstein, 1985) was carried out to obtain support estimates of the nodes of the trees selected. Bootstrap analysis was performed using 1000 replicates of heuristic search with the default options. ACCTRAN (accelerated transformation) character-state optimization was used for all trees illustrated. To conserve the phylogenetic information of insertions–deletions and, at the same time avoiding over-estimation of lengthy indels, 'missing data' were used and the indels coded as presence–absence characters added to the end of the matrix.

Bayesian analysis. Data sets were analysed using Mr Modeltest 2.2 (Nylander, 2004) to determine the sequence evolution model that best described the present data.

TABLE 1. Origin of the materials, herbaria where the vouchers are deposited and GenBank accession numbers (new sequences are indicated by bold type)

Species	Voucher	ITS accession	ETS accession	trnL-F accession
<i>Acroptilon australe</i> Iljin	MONGOLIA: V. Grubov 301 et al. (LE)	DQ310942	DQ310990	DQ310909
<i>Acroptilon repens</i> (L.) DC.	UZBEKISTAN: Susanna 2046 et al. (BC)	AY826223	DQ310989	AY772268
<i>Callicephalus nitens</i> (M. Bieb. ex Willd.) C. A. Mey.	ARMENIA: Susanna 1578 et al. (BC)	AY826237	DQ310972	AY772281
<i>Centauranthus maximus</i> Wagenitz & Dittrich	YEMEN: Molero s. n. (BC)	AY826259	DQ310971	AY772301
<i>Cheirolophus mauritanicus</i> (Font Quer) Susanna	MOROCCO: Romo 4617 et al. (BC)	AY826261	DQ131087	AY772303
<i>Cheirolophus teydis</i> (C. Sm.) G. López	SPAIN: Susanna 1429 et al. (BC)	AY826262	DQ131092	AY772304
<i>Klasea algida</i> (Iljin) Hidalgo	TAJIKISTAN: Susanna 2558 & Romashchenko (BC)	DQ310929	DQ310968	DQ310895
<i>Klasea biebersteiniana</i> (Iljin ex Grossh.) Hidalgo	ARMENIA: Susanna 1493 et al. (BC)	DQ310928	DQ310967	DQ310894
<i>Klasea cerinthifolia</i> (Sm.) Greuter & Wagenitz	IRAN: Susanna 1700 et al. (BC)	DQ310924	DQ310963	DQ310890
<i>Klasea chartacea</i> (C. Winkl.) L. Martins	TAJIKISTAN: Susanna 2467 & Romashchenko (BC)	DQ310927	DQ310966	DQ310893
<i>Klasea coriacea</i> (Fisch. & C. A. Mey. ex DC.) Holub	ARMENIA: Susanna 1558 et al. (BC)	DQ310926	DQ310965	DQ310892
<i>Klasea grandifolia</i> (P. H. Davis) Greuter & Wagenitz	IRAN: Susanna 1709 et al. (BC)	DQ310930	DQ310969	DQ310896
<i>Klasea kuchistanica</i> (Mozaffarian) Mozaffarian	IRAN: Mozaffarian 70181 (TARI)	DQ310925	DQ310964	DQ310891
<i>Klasea serratuloides</i> (DC.) Greuter & Wagenitz	ARMENIA: Susanna 1569 et al. (BC)	AY826295	DQ310962	AY772334
<i>Leuzea berardoides</i> Batt.	MOROCCO: Hidalgo & Romo 12749 (BC)	DQ310948	DQ310998	DQ310915
<i>Leuzea conifera</i> (L.) DC.	SPAIN: Font s. n. (BC)	AY826298	DQ310996	AY772337
<i>Leuzea fontqueri</i> Sauvage	MOROCCO: Hidalgo & Romo 12621 (BC)	DQ310947	DQ310997	DQ310914
<i>Myopordon aucheri</i> Boiss.	IRAN: Carls s.n. (W)	AY826299	DQ310977	AY772338
<i>Myopordon hyrcanum</i> (Bornm.) Wagenitz	IRAN: Koelz 16395 (W)	AY826300	DQ310975	AY772339
<i>Myopordon persicum</i> Boiss.	IRAN: Remandieri s.n. (W)	AY826301	DQ310976	DQ310898
<i>Ochrocephala imatongensis</i> (Phillipson) Dittrich	ETHIOPIA: Fantahun Simon 9163 et al. (K)	DQ310931	DQ310970	DQ310897
<i>Oligochaeta divaricata</i> (Fisch. & C. A. Mey.) K. Koch	ARMENIA: Susanna 1583 et al. (BC)	AY826306	DQ310973	AY772344
<i>Oligochaeta minima</i> (Boiss.) Briq.	UZBEKISTAN: Botanical Garden of Tashrent (BC)	AY826307	DQ310974	AY772345
<i>Plagiobasis centauroides</i> Schrenk	KAZAKHSTAN: Susanna 2130 et al. (BC)	AY826312	DQ310956	DQ310887
<i>Psephellus persicus</i> (DC.) Wagenitz	IRAN: Susanna 1716 et al. (BC)	AY826316	DQ310957	AY772352
<i>Psephellus pulcherrimus</i> (Willd.) Wagenitz	ARMENIA: Susanna 1492 et al. (BC)	AY826317	DQ310958	AY772353
<i>Rhaponticoides hajastana</i> (Tzvelev) M. V. Agab. & Greuter	ARMENIA: Susanna 1587 et al. (BC)	DQ310922	DQ310959	DQ310888
<i>Rhaponticoides iconiense</i> (Hub.-Mor.) M. V. Agab. & Greuter	TURKEY: Ertugrul 1761 (BC)	DQ310923	DQ310960	DQ310889
<i>Rhaponticum acaule</i> (L.) DC.	ALGERIA: J. M. Montserrat 2331 et al. (BC)	AY826334	DQ310995	AY772369
<i>Rhaponticum aulieatense</i> Iljin	KYRGYZSTAN: Sheremetova & Lazkov (LE)	DQ310936	DQ310983	DQ310903
<i>Rhaponticum australe</i> (Gaudich.) Soskov	AUSTRALIA: Funk 12203 (BC)	AY826335	DQ310978	AY772370
<i>Rhaponticum canariense</i> DC.	SPAIN: Carqué Álamo s. n. (BC)	DQ310954	DQ311004	DQ310921
<i>Rhaponticum carthamoides</i> (Willd.) Iljin	RUSSIA: Botanical Garden of Sibiricus Centralis, Novosibirsk 2003/2004–1062 (BC)	DQ310933	DQ310980	DQ310900
<i>Rhaponticum cossonianum</i> (Ball) Greuter	MOROCCO: Gómiz 17-IV-2003 (BC)	DQ310949	DQ310999	DQ310916
<i>Rhaponticum cynaroides</i> Less.	SPAIN: Hidalgo 504 et al. (BC)	DQ310946	DQ310994	DQ310913
<i>Rhaponticum exaltatum</i> (Willk.) Greuter	SPAIN: Garcia-Jacas & Susanna 2434 (BC)	DQ310953	DQ311003	DQ310920
<i>Rhaponticum heleniifolium</i> Godr. & Gren.	Botanical Garden of Minsk, Bielorusia 1/303–2000 (BC)	DQ310945	DQ310993	DQ310912
<i>Rhaponticum insigne</i> (Boiss.) Wagenitz	IRAN: Archibald 2034 (K)	DQ310944	DQ310992	DQ310911
<i>Rhaponticum integrifolium</i> C. Winkl.	TAJIKISTAN: Makhmetov & R. Kamelin 344 (LE)	DQ310934	DQ310981	DQ310901
<i>Rhaponticum karatavicum</i> Iljin	KAZAKHSTAN: Kamelin (LE)	DQ310940	DQ310987	DQ310907
<i>Rhaponticum longifolium</i> (Hoffmanns. & Link) Dittrich	PORTUGAL: Garcia-Jacas & Susanna 2436 (BC)	DQ310950	DQ311000	DQ310917
<i>Rhaponticum lyratum</i> C. Winkl. ex Iljin	TAJIKISTAN: Konnov16-VII-1965 (LE)	DQ310935	DQ310982	DQ310902
<i>Rhaponticum nanum</i> Lipsky	TAJIKISTAN: Kochkariova 5834 (DUSH)	DQ310939	DQ310986	DQ310906
<i>Rhaponticum nanum</i> Lipsky ssp. <i>pellucidum</i> (Rech. f.) Dittrich	AFGHANISTAN: Renz 120 (W)	DQ310938	DQ310985	DQ310905
<i>Rhaponticum nitidum</i> Fisch.	RUSSIA: Kalibernova 5676 et al. (LE)	DQ310937	DQ310984	DQ310904
<i>Rhaponticum pulchrum</i> Fisch. & C. A. Meyer	RUSSIA: Popova 326 et al. (LE)	DQ310943	DQ310991	DQ310910
<i>Rhaponticum scariosum</i> Lam.	SLOVENIA: Botanical Garden of Universitatit Labacensis Slovenia 1994–180 (BC)	DQ310952	DQ311002	DQ310919
	Botanical Garden of Minsk, Bielorusia 1/304–2000 (BC)	DQ310951	DQ311001	DQ310918
<i>Rhaponticum serratuloides</i> (Georgi) Bobrov	Botanical Garden of Cluj-Napoca, Romania 636-2001 (BC)	DQ310941	DQ310988	DQ310908
<i>Rhaponticum uniflorum</i> (L.) DC.	MONGOLIA: Vallès 13–2003 (BC)	DQ310932	DQ310979	DQ310899
<i>Serratula coronata</i> L.	Botanical Garden of Vienna, Austria (BC)	AY826327	DQ310961	AY772362
<i>Stizolophus coronopifolius</i> Cass.	TURKEY: Ilarlan 4303 (ANK)	AY826337	DQ310955	AY013516

This model was used to perform a Bayesian analysis using the program Mr. Bayes 3.1.1 (<http://morphbank.ebc.uu.se/mrbayes/>; Huelsenbeck *et al.*, 2001). Four Markov chains were run simultaneously for 1000 000 generations, and these were 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 (PP) of nodes were calculated from the remaining sample.

Biogeographic distributions. The distributions were mapped on the tree using the Farris double-pass method (Farris, 1970) which provides the hypothesized distributions of the deep branches and nodes. Each taxon branch and internode was coloured as to its distribution using Adobe Illustrator.

RESULTS

Some of the ETS sequences have repeats in the 5' end of the region, as found in other groups (Baldwin and Marcos, 1998; Linder *et al.*, 2000). In most cases, these repetitions constitute autapomorphic events or characterize a group without alignment or homology problems. Conversely, the majority of *Klasea* species present a region with a different number of repetitions. Because of these repetitions, it has not been possible to obtain the entire sequence for some species; for the others, their alignment was impeded by the difficulty of establishing the homology of the repetitions. For these reasons, the unalignable sequence area, exclusive of *Klasea*, was removed from the matrix.

Parsimony analysis. Four indel characters were included in the data matrix. The results from the combined ITS, ETS and *trnL-trnF* parsimony analysis are given in Table 2.

Bayesian analysis. The GTR+G+I model was found to be the most efficient model for optimizing sequence evolution of the data set.

The two methods of phylogeny reconstruction lead to congruent results (there is no discordance for strongly

TABLE 2. Results from the combined ITS, ETS and *trnL-trnF* parsimony analysis (the consistency and homoplasy indexes are calculated excluding uninformative characters)

Data set	Combined analyses
Total characters	2950 (ITS: 669, ETS: 1354, <i>trnL-trnF</i> : 927)
Informative substitutions	549 (ITS: 149, ETS: 375, <i>trnL-trnF</i> : 25)
Number of MPTs	558
Number of steps	1582
Consistency index (CI)	0.48
Retention index (RI)	0.72
Mean pairwise distances, ingroup (%)	From 0 (<i>Acroptilon australe</i> / <i>A. repens</i> ; <i>Leuzea conifera</i> / <i>L. fontqueri</i>) to 37 (<i>Acroptilon australe</i> , <i>A. repens</i> / <i>Callicephalus nitens</i>)

supported branches) that lead to only the tree obtained with the Bayesian inference, shown in Fig. 1, being presented. This tree indicates both the bootstrap values (calculated by the parsimony analysis), the posterior probability (calculated by the Bayesian inference) and biogeography mapping.

DISCUSSION

This new molecular phylogeny makes it possible to hypothesize regarding the evolutionary history of the *Rhaponticum* group. Among all the genera selected to constitute the outgroup, the genus *Klasea* appears as the most closely related to the *Rhaponticum* group (PP = 97 %, BS = 81 %; Fig. 1). Furthermore, the results contradict the election of *Centaurothamnus* as outgroup, and suggest that it should be included in the *Rhaponticum* group. On the other hand, all the taxa previously considered as members of the *Rhaponticum* group appear in the ingroup (PP = 98 %; Fig. 1).

Klasea is sister to the Rhaponticum group

Klasea (\equiv *Serratula* section *Klasea*) comprises approx. 65 species distributed from the western Mediterranean to China and the western part of the Himalayas. The phylogenetic distance within the genus *Serratula* between *Serratula sensu stricto* (*S. coronata* L. and *S. tinctoria*) and section *Klasea* has been demonstrated by different authors, on morphological (Wagenitz and Hellwig, 1996) and on molecular bases (Martins and Hellwig, 2005). Therein, those authors were in accordance with Cassini (1825) who placed *Klasea* as a genus distinct from *Serratula*. The present results confirm these findings and clearly define *Klasea* as closely related to the *Rhaponticum* group (PP=97 %, BS=81 %; Fig. 1). This proximity was previously suggested by Lessing (1832), who considered *Klasea* Cass. as a subgenus of *Rhaponticum*. Moreover, the observation of the achene morphology has brought to light for the first time the fact that *Klasea* shows heterocarpy like the *Rhaponticum* group. The *Klasea* species constitute a monophyletic group (PP = 99 %, BS = 100 %; Fig. 1). Among them, *Serratula algida* Iljin, *S. biebersteiniana* (Iljin ex Grossh.) Takht. and *S. kuzhistanica* (Mozaffarian) Mozaffarian have not been recombined as *Klasea* until now; this has led to the establishment of new nomenclatural combinations as detailed in the Appendix.

The monophyly of the *Rhaponticum* group is supported by the Bayesian inference (PP = 98 %; Fig. 1), but the parsimony analysis shows a polytomy consisting of *Klasea*, the *Centaurothamnus* plus *Ochrocephala* clade, and the rest of the genera. Even though the parsimony analysis is equivocal in the placement of *Klasea* as sister to the *Rhaponticum* group, this hypothesis is, nonetheless, supported both by karyological and morphological data. Representatives of the genus *Klasea* have a base chromosome number of $x = 15$ (Löve and Löve, 1961; Cantó, 1982, 1984; Garcia-Jacas *et al.*, 1998a, b), while *Centaurothamnus* and *Callicephalus* have $x = 14$

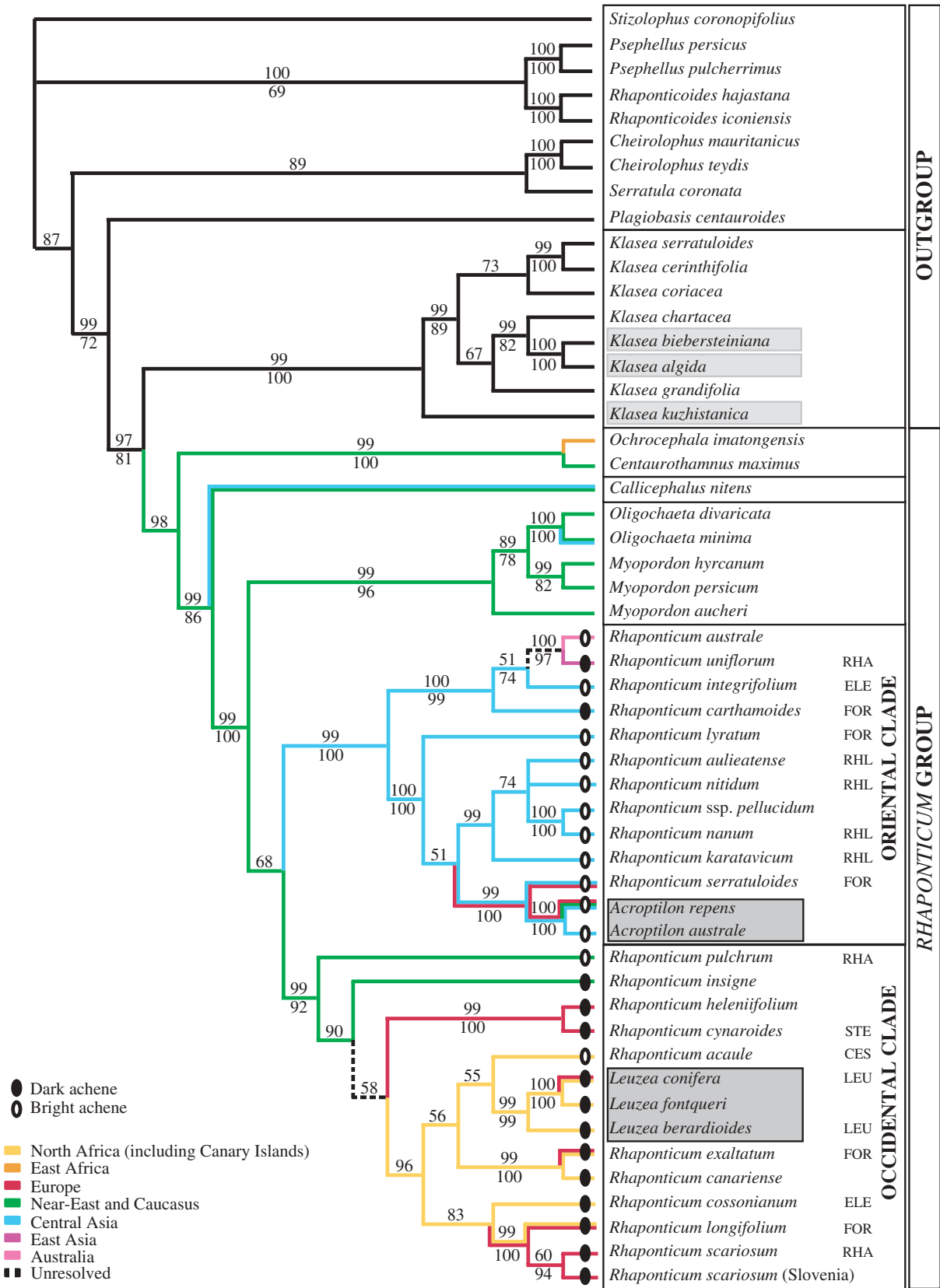


FIG. 1. Majority-rule consensus tree based on Bayesian analysis. Numbers above branches indicate Bayesian clade-credibility values (posterior probability); numbers below branches indicate parsimony bootstrap percentages. Subgenera of *Rhaponticum*: CES, *Cestrinus*; ELE, *Eleutherochaetum*; FOR, *Formicum*; LEU, *Leuzea*; RHA, *Rhaponticina*; RHL, *Rhaponticella*; STE, *Stemmacantha*.

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[Wagenitz *et al.* (1982) for the former; Chouksanova *et al.* (1968), Hellwig (1994) and Garcia-Jacas *et al.* (1998a) for the latter] and *Acroptilon*, *Leuzea* and most of the *Rhaponticum* species present $x = 13$ (Wagenitz and Hellwig, 1996). Descending dysploidy being one of the main evolutionary mechanisms in plants and in particular in the Centaureinae (Fernández Casas and Susanna, 1986; Garcia-Jacas *et al.*, 1996; Vilatersana *et al.*, 2000), this could suggest that $x = 15$ is more primitive than $x = 14$. Then, in the case of the present focus of study, this could suggest that *Klasea* should be sister to the *Rhaponticum* group. Furthermore, although *Klasea* shows heterocarpy like the *Rhaponticum* group, it does not exhibit the two other morphological apomorphies present in the other two clades, namely the typical involucre bracts (present in *Ochrocephala*) and the characteristic pappus (present in *Centaurothamnus* and *Ochrocephala*).

Centaurothamnus and Ochrocephala

The present results confirm that *Ochrocephala* is more appropriately treated as a monotypic genus (Dittrich, 1983), rather than considered as *Rhaponticum imatongensis*. Furthermore, this study allows the systematic position of the genus *Centaurothamnus* to be defined for the first time. This genus was placed with the genera of ‘uncertain position’ by Wagenitz and Hellwig (1996), Garcia-Jacas *et al.* (2001) and Hellwig (2004). With the new molecular evidence, *Centaurothamnus* appears to be closely related to the genus *Ochrocephala* (PP = 99 %, BS = 100 %; Fig. 1). This result is not surprising, because these two monotypic genera are geographically very close: *Centaurothamnus maximus* Wagenitz & Dittrich grows in south-western Arabia, in Yemen, and *Ochrocephala imatongensis* (Phillipson) Dittrich in eastern Africa (Ethiopia, Sudan and Congo). Morphologically, these two taxa share the same shrubby habit, an exclusive trait of the group. The shrubby port is uncommon within the Centaureinae, and outside *Centaurothamnus* and *Ochrocephala* it is only known from the genus *Centaurodendron* Johow, *Centaurea ptosimopappa* Hayek and the genus *Cheirolophus*. It corresponds, probably, to a secondary adaptation, this phenomenon being particularly evident for the insular taxa such as *Centaurodendron* and *Cheirolophus* (Böhle *et al.*, 1996; T. Garnatje, unpubl. res.). *Centaurothamnus* and *Ochrocephala* are genetically and morphologically distinguished: their molecular divergence for the three markers considered is 13.8 %; *Ochrocephala* shows the typical involucre bract appendages of the *Rhaponticum* group, while these are not present in *Centaurothamnus*. A new question introduced by these results is: Should *Centaurothamnus* and *Ochrocephala* be more appropriately maintained as distinct genera or should they should be merged to constitute a single genus?

Callicephalus nitens

The genus *Callicephalus* includes a single species, *Callicephalus nitens* (M. Bieb. ex Willd.) C. A. Mey., from the middle and low mountains of the Caucasus, central Asia and the Near East. It appeared within the *Rhaponticum*

group in the molecular analysis of Garcia-Jacas *et al.* (2001), but with weak statistical support. The present results strongly support the fact that *Callicephalus* belongs to the group of genera related to *Rhaponticum* (PP = 99 %, BS = 86 %; Fig. 1). Because of its annual nature, this species might show increased mutation rates, which could have induced an anomalous result in the parsimony analysis. However, the Bayesian inference method, less affected by long branch attraction, leads to an identical result. Furthermore, this hypothesis is reinforced by morphological features such as the structure of the inner pappus or the tuberculate pericarp (Garcia-Jacas *et al.*, 2001). *Callicephalus* has no closely related taxa and appears as isolated in the phylogeny. Thus, this genus may be one of the numerous ‘relict’ taxa that grow in the Caucasus. The abundance of relict and endemic plant species in this area seems largely due to the fact that it was spared glaciation during the most recent ice ages.

The rest of the ingroup belongs to a strongly supported clade (PP = 99 %, BS = 100 %, Fig. 1) which includes the genera *Acroptilon*, *Leuzea*, *Myopordon*, *Oligochaeta* and *Rhaponticum*, placed in three different groups. The relationships between these three groups are not resolved either in the parsimony or the Bayesian inference.

Oligochaeta and Myopordon

Myopordon, a small genus with five perennial species from the Near East which had been placed in the Carduinae, and *Oligochaeta*, another genus composed of four annual species from the Near East, Caucasus, Afghanistan and India, and related to *Rhaponticum*, have apparently nothing in common. However, evidence that they are closely related was provided by the molecular study of Susanna *et al.* (2006), and it is also confirmed in this analysis (PP = 99 %, BS = 96 %; Fig. 1), whose sampling of the *Rhaponticum* group species is much more complete. In spite of the morphological review of *Myopordon* by Wagenitz (1958), several questions remain open. One consists of the generic delimitation of *Myopordon* and *Oligochaeta*: the present analyses support the monophyly of *Oligochaeta* (PP = 100 %, BS = 100 %; Fig. 1) but not that of *Myopordon*. More studies are necessary to verify whether *Myopordon* and *Oligochaeta* are independent taxa or whether they should be merged. Other questions concern the morphological traits of this clade, focusing especially on their palynological characteristics, which are baffling. While all the species of the *Rhaponticum* group show a *Serratula*-type pollen, *Oligochaeta* presents a reduced form of *Serratula*-type pollen grain (Martín and Garcia-Jacas, 2000), and *Myopordon* exhibits three different pollen types: *Jacea*, *Centaurea scabiosa* and *Serratula* (Wagenitz, 1958). It is perplexing that these two specialized and divergent taxa can be so narrowly related to *Rhaponticum* up to the point that our three molecular markers are not able to segregate them.

The genus *Rhaponticum*

The genus *Rhaponticum* does not appear as monophyletic in the phylogeny established for the following two reasons.

(1) Its segregation from the clade of *Myopordon* plus *Oligochaeta* is not statistically supported (Fig. 1). Fortunately, the resolution within the genus *Rhaponticum* is better, and it shows two strongly supported clades, one 'oriental' mostly composed of central Asian species (PP = 99 %, BS = 100 %; Fig. 1) and the other 'occidental' including predominantly species from North Africa and Europe (PP = 99 %, BS = 92 %; Fig. 1). (2) The genera *Acroptilon* and *Leuzea* are firmly nested in the genus *Rhaponticum*, the first in the oriental clade, and the second in the occidental clade (Fig. 1), which leads to the paraphyly of *Rhaponticum* in its present circumscription. This implies some nomenclatural changes to reconcile the delimitation of the genus with this new evidence. Other evidence for the placement of *Acroptilon* and *Leuzea* in the genus *Rhaponticum* is that the three taxa share the same chromosome number $x = 13$, which is uncommon within the Centaureinae.

The comparison between the more comprehensive infrageneric classification of *Rhaponticum* (Holub, 1973) and the molecular phylogeny shows numerous incongruities (Fig. 1). Only two of the seven subgenera described are natural groups: the subgenus *Rhaponticella* (Soskov) Holub (PP = 99 %; Fig. 1) and the subgenus *Leuzea* DC. (PP = 99 %, BS = 99 %; Fig. 1). The present results suggest that the more appropriate division of the genus *Rhaponticum* would be into only two subgenera, these corresponding to the oriental and the occidental clades, but it has not been possible to detect any character that defines either group on morphological grounds.

The *Rhaponticum* oriental clade

The first clade (PP = 99 %, BS = 100 %; Fig. 1) consists mostly of central Asian species, but includes species from middle and eastern Asia, Australia and eastern Europe. These species have relatively restricted areas of distribution, except for two groups of taxa that have wider areas.

One group extends from western to eastern Europe, and comprises *Rhaponticum serratulooides* (Georgi) Bobrov and *Acroptilon* (PP = 99 %, BS = 100 %; Fig. 1). The incorporation of *Acroptilon* in *Rhaponticum* had never been mentioned before, and necessitates the new nomenclatural combinations detailed in the Appendix. The most recent classifications do not recognize *Acroptilon australe* Iljin as a species separate from *A. repens* (L.) DC. (Hellwig, 2004; Susanna and Garcia-Jacas, 2006), while on the contrary, Soskov (2001) considers them to be two well-defined species. It is not possible from the present results to come to a verdict, and more studies are necessary to clarify the status of *A. repens*. This is the reason why we have preferred to abstain from making a new combination for this taxon. *Acroptilon* is considered to be an invasive weed in America and Australia, where it adversely affects agronomic harvests. It is aggressively competitive and exhibits allelopathic effects. It differs from the other species of the group, most of them endemics restricted to unfavourable environments where the competition with other species is less notable, as for example the mountain screens. The structure of capitula, achenes and the type of ramification are

basically the same as *Rhaponticum*, but *Acroptilon* shows secondary adaptations due to its colonizing strategy: it is a hemicryptophyte like *Rhaponticum* but, instead of presenting few stems weakly or not ramified, this species generates numerous strongly branched stems in spring, due to its extensive root and rhizome system. Therefore, vegetative multiplication is favoured, although it also produces numerous capitula and achenes.

The other group extends from central to eastern Asia and Australia and is composed of *Rhaponticum australe* and *R. uniflorum* (L.) DC. The close relationship between these two species (PP = 100 %, BS = 97 %; Fig. 1) is a logical result, considering that *R. uniflorum* is the only species of the genus which has reached eastern Asia. From a geographic point of view, this was the best candidate to be sister to the Australian species. The fact that *R. australe* is the only species of Centaureinae indigenous to Australia is surprising because nothing explains such a long dispersal distance of the achenes of a *Rhaponticum* species. This lead Susanna and Garcia-Jacas (2006) to hypothesize that the species was doubtfully native in Australia. There is a considerable genetic divergence between *R. uniflorum* and *R. australe* for the three regions studied (8.7 %), and this means there is no possibility of a recent introduction from *R. uniflorum*. The colonizing event would have taken place during the period of lowest sea levels (between 50 000 and 84 000 years ago), from the coasts of South Asia. Was it the Aborigines that introduced the plant, and were they motivated by its medicinal properties? Had the species, on the other hand, reached Australia without human intervention, but how would this have been achieved?

The *Rhaponticum* occidental clade

This second clade within *Rhaponticum* (PP = 99 %, BS = 92 %; Fig. 1) embraces species distributed in North Africa, the Canary Islands, Europe and the Near East. *Rhaponticum pulchrum* Fisch. & C. A. Meyer, from Iran–Afganistan and the Caucasus, is situated as sister of the remainder of this group (Fig. 1), which suggests that the occidental clade originated in the Near East. A characteristic of the occidental clade is that a grouping of the North-African species is not seen in one subclade with the European species in another, but, on the contrary, various subclades combining species from North Africa, Europe and/or a mixed distribution are seen (Fig. 1). This suggests several independent passages from one continent to the other during the evolutionary history of the group.

(1) The association *Rhaponticum heleniifolium* Godr. & Gren. plus *R. cynaroides* Less. (PP = 99 %, BS = 100 %; Fig. 1) is the exception because the former one is endemic to the Alps and the latter to the Pyrenees. The two species present the particularity of exhibiting ramified inflorescences, according to the authors' observations. Few species of *Rhaponticum* show this character, and these are always <50 cm in height, while *R. heleniifolium* and *R. cynaroides* reach 1 m.

(2) The group including the *Leuzea* species (PP = 99 %, BS = 99 %; Fig. 1) shows a mixed distribution between North Africa and Europe. The present study confirms, for

the first time on a molecular basis, that *Leuzea* and *Rhaponticum* should be fused, as previously suggested by Holub (1973) for morphological reasons. *Leuzea berardioides* Batt., endemic to the High Atlas (Morocco), appears as clearly segregated from *L. conifera* (L.) DC. (the molecular divergence for the regions studied between the two species is 7%). This fact contradicts its consideration as a synonym or as a subspecies of *L. conifera* by Susanna (2002), and Greuter (2003), respectively, and implies a new nomenclatural combination of *L. berardioides* as *Rhaponticum*, as detailed in the Appendix. Another taxon, *L. fontqueri*, had been described by Sauvage (1968) as closely related to *L. berardioides*. The present results suggest that *L. fontqueri* is more closely related to *L. conifera* (PP = 100%, BS = 100%; Fig. 1), the only molecular divergences observed between these two taxa concerning polymorphic positions. *Leuzea conifera* presents a wide distribution area (western Mediterranean and Portugal) and a high morphological variability. In this sense, more studies are necessary to determine whether the differences observed with *L. fontqueri* are included in the natural variability of *L. conifera*, or if this endemic of the Chefchaouène Mountains (Morocco) merits the status of species. Meanwhile, the new nomenclatural combination for *L. fontqueri* as *Rhaponticum* is proposed in the appendix. *Leuzea conifera* had been combined previously as *Rhaponticum* by Greuter (2003). *Rhaponticum acaule* (L.) DC., is positioned at the base of the *Leuzea* group but without statistical support.

(3) *Rhaponticum canariense* DC., the only representative of *Rhaponticum* from the Canary Islands and seriously threatened with extinction, appears closely related to *R. exaltatum* (Willk.) Greuter, a species from central Spain and north-east Portugal (PP = 99%, BS = 100%; Fig. 1). Although floras do not usually indicate it, *R. exaltatum* could also be present in Morocco, in the Rif Atlas, according to a voucher from the Herbarium of Montpellier [‘montagnes de Ketama’, Sennen & Mauricio, VI-1934 (MPU); the determination of the herbarium sample was established and confirmed respectively by Maire in 1936 and Dittrich in 1976].

(4) There is another subclade which associates *R. scariosum* Lam. and *R. longifolium* (Hoffmanns. & Link) Dittrich (PP = 99%, BS = 100%; Fig. 1). *Rhaponticum cossonianum* (Ball) Greuter is positioned as sister of these two species, but with weak statistical support (Fig. 1).

An important outcome of this study is that the two representatives of *Rhaponticum* growing in the Alps, *R. scariosum* and *R. heleniifolium*, considered by several authors as subspecies of *R. scariosum* (Briquet, 1902; Rouy, 1905; Burnat, 1931; Holub, 1973; Dostál, 1976), do not appear as sisters in the phylogeny (Fig. 1). This implies *inter alia* that the colonization of the Alps took place in, at least, two independent events. In the same order of things, the biogeographic inference suggests that the two species of *Rhaponticum* indigenous to the east of Europe (*R. serratuloides* and *R. scariosum*), could have reached

this region in two different ways: one expansion across central Asia to eastern Europe generating *R. serratuloides*; the other expansion through the Near East, North Africa and then to the Iberian Peninsula, thence on to the Alps, generating *R. scariosum*.

In view of this hypothesis suggested by the present analysis, it is regrettable that some nodes of the *Rhaponticum* occidental clade are weakly supported, and it would be interesting to perform more studies to get a better understanding of its biogeographic history.

Conclusions

This study confirms the main expectations of the study. It defines the genus *Klasea* as being probably the group of taxa most closely related to the *Rhaponticum* group. The generic delimitation of the *Rhaponticum* group would include the genera *Callicephalus*, *Centaurothamnos*, *Myopordon*, *Ochrocephala*, *Oligochaeta* and *Rhaponticum* (including *Acroptilon* and *Leuzea*). The new molecular evidence is consistent with the karyological and morphological data, and suggests particularly coherent biogeographic routes of migration and speciation processes for the genus *Rhaponticum*.

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APPENDIX: NEW NOMENCLATURAL
COMBINATIONS

Klasea algida (Iljin) Hidalgo, **comb. nov.** Basionym: *Serratula algida* Iljin, *Repertorium Specierum Novarum Regni Vegetabilis* **35**: 357 (1934).

Klasea biebersteiniana (Iljin ex Grossh.) Hidalgo, **comb. nov.** Basionym: *Serratula radiata* ssp. *biebersteiniana* Iljin ex Grossh., *Flora Kavkaza* 4: 194 (1934) ≡ *Serratula biebersteiniana* (Iljin ex Grossh.) Takht. in Takhtajan & Fedorov, *Flora Erevana*: 323 (1945).

Klasea khuzistanica (Mozaffarian) Mozaffarian, **comb. nov.** Basionym: *Centaurea khuzistanica* Mozaffarian, *Iranian Journal of Botany* **5**(2): 84 (1992) ≡ *Serratula khuzistanica* (Mozaffarian)

Mozaffarian in Garcia-Jacas *et al.*, *Botanical Journal of the Linnean Society* **128**: 420 (1998).

Rhaponticum berardioides (Batt.) Hidalgo, **comb. nov.** Basionym: *Leuzea berardioides* Batt., *Contributions à la Flore Atlantique*: 55 (1919) ≡ *Rhaponticum coniferum* subsp. *berardioides* (Batt.) Greuter, *Willdenowia* **33**: 61 (2003).

Rhaponticum fontqueri (Sauvage) Hidalgo, **comb. nov.** Basionym: *Leuzea fontqueri* Sauvage, *Collectanea Botanica* (Barcelona) 59, **7**(2): 1100 (1968).

Rhaponticum repens (L.) Hidalgo, **comb. nov.** Basionym: *Centaurea repens* L., *Species Plantarum* ed. 2: 1293 (1763) ≡ *Acroptilon repens* (L.) DC., *Prodromus* **6**: 663 (1838).