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Dear Dr Hidalgo

Re : Manuscript reference number 05/438 Phylogeny of *Rhaponticum* (Asteraceae, Cardueae-Centaureinae) and related genera inferred from nuclear and chloroplast DNA sequence data: taxonomic and biogeographic implications

Your paper has now been assessed by two referees (comments enclosed) and the decision editor, Dr Christian Lexer, who writes as follows:

Based on the referees' reports I recommend that your paper be accepted for publication in *Annals of Botany* after minor revision. It will be important for you to explain how you have dealt with each of the referees' criticisms.

In the light of these remarks I am happy to accept the paper subject to our receiving a suitably revised manuscript. Please send the revised version of the typescript in electronic form as a Word or RTF document (including any tables) to the Assistant Editor Dr David Frost, as an e-mail attachment (e-mail: [annals-botany@bristol.ac.uk](mailto:annals-botany@bristol.ac.uk)), or by post on a disk if the total file size is too large. Figures are usually best placed in separate files. Before sending, do check the technical requirements in the section of our Instructions to Authors entitled 'Preparing an Accepted Paper for Production' ([www.aob.oupjournals.org](http://www.aob.oupjournals.org)). Please quote the paper's reference number in the correspondence. Dr Frost will pass the revised version to Dr Lexer for further evaluation. In a covering letter, you should outline the changes you have made in response to the referees' comments and justify your position if you have disagreed with the referees. Also, please prepare a draft for the Content Snapshots following the enclosed instructions.

You are asked to revise your paper within 4 weeks or risk a considerable further delay in publication. If this return time is not practicable, please let me know the reason as soon possible. We look forward to receiving your revised paper in due course.

Yours sincerely

MB Jackson  
Chief Editor

1 **Phylogeny of *Rhaponticum* (Asteraceae, Cardueae-Centaureinae) and related**  
2 **genera inferred from nuclear and chloroplast DNA sequence data: taxonomic and**  
3 **biogeographic implications.**

4

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9

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1 **Phylogeny of *Rhaponticum* (Asteraceae, Cardueae-Centaureinae) and related**  
2 **genera inferred from nuclear and chloroplast DNA sequence data: taxonomic and**  
3 **biogeographic implications.**

4

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10

11 Running title: Combined DNA phylogeny of *Rhaponticum* and related genera.

12

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14

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

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

11 ● Key Results. The *Rhaponticum* group appears as monophyletic, and closely related to  
12 the genus *Klasea*. Our results confirm the preliminary generic delimitation of the  
13 *Rhaponticum* group, with the new incorporation of the genus *Centaurothamnus*.  
14 *Ochrocephala* is supported as a separate genus from *Rhaponticum* and, contrary to this,  
15 *Acroptilon* and *Leuzea* appear as merged into the genus *Rhaponticum*.

16 ● Conclusions. The new molecular evidence is consistent with the morphological and  
17 karyological data, and suggests particularly coherent biogeographic routes of migration  
18 and speciation processes for the genus *Rhaponticum*.

19

20 **Keywords:** *Acroptilon*, *Callicephalus*, *Centaurothamnus*, Compositae, ETS, ITS,  
21 molecular phylogeny, *Myopordon*, *Ochrocephala*, *Oligochaeta*, *Rhaponticum*, *trnL-*  
22 *trnF*

## INTRODUCTION

1

2  
3 One of the main problems which persists in the subtribe Centaureinae (Asteraceae,  
4 Cardueae) is that the phylogenetic relationships between the basal genera are  
5 unresolved. Monographers (Dittrich, 1977; Wagenitz and Hellwig, 1996; Garcia-Jacas  
6 *et al.*, 2001; Hellwig, 2004; Susanna and Garcia-Jacas, in press) have described  
7 informal groups of genera. One of these is the *Rhaponticum* group which comprises  
8 about 7 genera and approximately 40 species. Apart from the genus *Rhaponticum* Vaill.  
9 (~25 sp.), it includes *Acroptilon* Cass. (2 sp.), *Callicephalus* C. A. Mey. (1 sp.), *Leuzea*  
10 DC. (1 sp.), *Myopordon* Boiss. (5 sp.), *Ochrocephala* Dittrich (1 sp.) and *Oligochaeta*  
11 (DC.) K. Koch. (4 sp.).

12 The classic morphological approach limited itself to associate *Rhaponticum* and  
13 *Acroptilon* (Dittrich, 1977), and pointed out generic delimitation problems: Holub  
14 (1973) concluded that *Leuzea* and *Rhaponticum* should be merged, and Dittrich (1983)  
15 that *Rhaponticum imatongensis* (Phillipson) Soják should be segregated to constitute a  
16 new monotypic genus: *Ochrocephala*. Later, the molecular approach allowed the  
17 addition of *Callicephalus*, *Oligochaeta* (Garcia-Jacas *et al.*, 2001) and *Myopordon*  
18 (Susanna *et al.*, submitted).

19 These genera show the symplesiomorphic characters common to the basal genera of the  
20 Centaureinae (homogamous capitula, lack of crystals in the phyllaries, basal hilum, no  
21 bolster cells, absence of hairs on the achene and pollen of the *Serratula* type), and some  
22 morphological traits which characterize the group:

- 1       - a peculiar type of involucre bract with a big, soft scarious, entire or lacerate and
- 2       usually silvery-white appendage (an exclusive character of the group which,
- 3       unfortunately, not all the species show);
- 4       - dimorphic achenes (the outer dorsiventrally compressed and the inner laterally
- 5       compressed);
- 6       - and finally the double pappus typical of all the Centaureinae, but with the
- 7       peculiarity that the inner bristles are wider and longer than the outer.

8   The geographical distributions, environmental conditions and life cycles are very

9   diverse in *Rhaponticum* and related genera. They are naturally distributed in North

10   Africa (including Canary Islands), temperate Eurasia, Siberia and the Far East,

11   Caucasus, Central and Eastern Asia, and Eastern Australia. They grow in deserts or

12   mountains, and are either widely distributed or narrow endemics. They can be perennial

13   or annual, and their habit is shrubby, or hemicryptophyte from 10 cm to more than 1 m

14   in height, or acaulescent. Several species are endangered to the verge of extinction, but

15   one taxon, *Acroptilon*, is considered to be an invasive weed in America and Australia.

16   The *Rhaponticum* group includes the only species of Centaureinae indigenous to

17   Australia: *Rhaponticum australe* (Gaudich.) Soskov (Wagenitz and Hellwig, 1996).

18   Some representatives of the group show medicinal properties which were already

19   known in Roman culture (Plinius, 77), and various species are being marketed due to

20   their anabolic and adaptogenic properties.

21   The main goals of our study in establishing a combined molecular phylogeny were to:

22   (1) elucidate the relationships between the basal groups of Centaureinae, with the

23   purpose of determining the taxa most closely related to the *Rhaponticum* group; (2)

1 verify the generic delimitation of the *Rhaponticum* group and the relationships between  
2 its genera; (3) link our findings to the group's biogeographic history.

3

4

## MATERIAL AND METHODS

5

### 6 *Plant Material*

7 The sampling includes representatives of all the genera of the *Rhaponticum* group, and  
8 all the species of the genus *Rhaponticum* (except *R. namanganicum* Iljin). The  
9 outgroups have been selected according to previous works by Garcia-Jacas *et al.* (2001)  
10 and Susanna *et al.* (submitted) to represent most of the basal Centaureinae which could  
11 have phylogenetic affinities with our focus of study: *Centaurothamnus* Wagenitz &  
12 Dittrich, *Cheirolophus* Cass., *Klasea* Cass., *Plagiobasis* Schrenk, *Psephellus* Cass.,  
13 *Rhaponticoides* Vaill., *Serratula* L. and *Stizolophus* Cass. The purpose in representing  
14 numerous outgroups is to permit us, without forcing the topology, to test the good  
15 assignation of a taxon as outgroup or as ingroup, and to define the more closely related  
16 taxa to the *Rhaponticum* group. Both previously published and the 111 new sequences  
17 (31 ITS, 47 ETS, 33 *trnL-trnF*) were used in the analyses. The origin of the samples and  
18 GenBank sequence accession numbers are given in Table 1.

19

### 20 *DNA Extraction, Amplification, and Sequencing*

21 Total genomic DNA was extracted following the miniprep procedure of Doyle and  
22 Doyle (1987) as modified by Soltis *et al.* (1991) and Cullings (1992) from herbarium  
23 material, silica-gel-dried leaves collected in the field, or fresh leaves of plants cultivated

1 in the Botanic Institute of Barcelona. DNA of old herbarium material was extracted  
2 using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA).

3 *nrDNA ITS region strategies.* ITS1, 5.8S gene and ITS2 (the ITS region) were amplified  
4 and sequenced together with 1406F (Nickrent *et al.*, 1994), ITS1 (White *et al.*, 1990)  
5 and 17SE (Sun *et al.*, 1994) as forward primers, and ITS4 (White *et al.*, 1990), 26SE  
6 (Sun *et al.*, 1994) as reverse primers, referring to the protocol described in Soltis and  
7 Kuzoff (1993). PCR products were purified using the QIAquick PCR Purification Kit  
8 (Qiagen Inc., Valencia, CA). Direct sequencing of the amplified DNA segments was  
9 performed using the BigDye Terminator Cycle Sequencing v3.1 (PE Biosystems, Foster  
10 City, CA). Nucleotide sequencing was carried out at the Serveis Científicotècnics of the  
11 University of Barcelona on an ABI PRISM 3700 DNA analyzer (PE Biosystems, Foster  
12 City, CA).

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

17 *cpDNA trnL-trnF region strategies.* The *trnL-trnF* region includes the *trnL* intron, the 3'  
18 *trnL* (UAA) exon, and the intergenic spacer between *trnL* (UAA) and *trnF* (GAA),  
19 which were amplified and sequenced together. The universal primers *trnL-c*, forward,  
20 and *trnL-f*, reverse (Taberlet *et al.*, 1991) were used to amplify and sequence the *trnL-F*  
21 region. For old material we have amplified and sequenced the region in two parts using  
22 the two precedent primers and the *trnL-e*, forward, and *trnL-d*, reverse, of the same  
23 author. The PCR procedure includes a warm start at 95°C for 1 min 35 s, followed by  
24 80°C during which the polymerase (Ecotaq, Ecogen S.R.L., Barcelona, Spain) is added,



1 and 34 cycles of 1 min denaturation at 93°C, 1 min annealing at 58°C, 1 min extension  
2 at 72°C, and a final 10 min extension at 72°C. Purification and direct sequencing of the  
3 amplified DNA segments was performed as for the ITS region.

4

#### 5 *Phylogenetic Analyses*

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

9 *Parsimony analysis* involved heuristic searches conducted with PAUP version 4.0b4a  
10 (Swofford, 1999) using Tree Bisection Recognition (TBR) branch swapping with  
11 character states specified as unordered and unweighted. All most-parsimonious trees  
12 (MPT) were saved. To locate islands of most-parsimonious trees (Maddison, 1991), we  
13 performed 100 replicates with random taxon addition, also with TBR branch swapping.  
14 Trees lengths, consistency index (CI) and retention index (RI) are always given  
15 excluding uninformative characters. Bootstrap (BS; Felsenstein, 1985) was carried out  
16 to obtain support estimates of the nodes of the trees selected. Bootstrap analysis was  
17 performed using 1000 replicates of heuristic search with the default options.  
18 ACCTRAN (accelerated transformation) character-state optimization was used for all  
19 illustrated trees. In order to conserve the phylogenetic information of insertions-  
20 deletions and at the same time avoiding over-estimation of lengthy indels, we have used  
21 “missing data” and coded the indels as presence-absence characters added to the end of  
22 the matrix.

23 *Bayesian analysis*: data sets were analysed using Modeltest 3.04 (Posada and Crandall,  
24 1998) to determine the sequence evolution model that best described our data. This

1 model was used to perform a Bayesian analysis using the program Mr. Bayes 3.0  
2 (<http://morphbank.ebc.uu.se/mrbayes/>; Huelsenbeck *et. al.*, 2001). Four Markov chains  
3 were run simultaneously for 1.000.000 generations, and these were sampled every 100  
4 generations. Data from the first 1000 generations were discarded as the “burn-in”  
5 period, after confirming that likelihood values had stabilized prior to the 1000<sup>th</sup>  
6 generation. The 50% majority rules consensus phylogeny and posterior probability of  
7 nodes (PP) were calculated from the remaining sample.

8 Biogeographic distributions: the distributions were mapped on the tree using the Farris  
9 double pass method (Farris, 1970) which provides the hypothesized distributions of the  
10 deep branches and nodes. Each taxon branch and internode was colored as to its  
11 distribution using Adobe illustrator.

## 12 13 RESULTS

14  
15 Some of our ETS sequences have repeats in the 5' end of the region, as found in other  
16 groups (Baldwin and Marcos, 1998; Linder *et al.*, 2000). In most cases, these repetitions  
17 constitute autapomorphic events or characterize a group without alignment or homology  
18 problems. Conversely, the majority of *Klasea* species present a region with a different  
19 number of repetitions. Because of these repetitions, we have not obtained the entire  
20 sequence for some species; and for the others their alignment was impeded by the  
21 difficulty of establishing the homology of the repetitions. For these reasons, this  
22 repeated zone of *Klasea* was removed from the matrix.

23 Parsimony analysis: the results from the combined ITS, ETS and *trnL-trnF* parsimony  
24 analysis are given in Table 2.

1 Bayesian analysis: the GTR+G+I model was found to be the most efficient model for  
2 optimizing sequence evolution of our data set.  
3 The two methods of phylogeny reconstruction lead to congruent results (there is no  
4 discordance for strong supported branches) that lead us to present only the tree obtained  
5 with the Bayesian inference, shown in Fig. 1. This tree indicates both the bootstrap  
6 values (calculated by the parsimony analysis), the posterior probability (calculated by  
7 the Bayesian inference), and the biogeography mapping.

8

9

## DISCUSSION

10

11 This new molecular phylogeny allows us to draw some hypotheses regarding the  
12 evolutive history of the *Rhaponticum* group. Among all the genera selected to constitute  
13 the outgroup, the genus *Klasea* appears as the most closely related to the *Rhaponticum*  
14 group (PP=97 %, BS=81 %, Fig. 1). Furthermore, our results contradict the election of  
15 *Centaurothamnus* as outgroup, and suggest that it should be included in the  
16 *Rhaponticum* group. On the other hand, all the taxa previously considered as members  
17 of the *Rhaponticum* group appear in the ingroup (PP=98 %, Fig. 1).

18

19 *Klasea*, sister of *Rhaponticum* group?

20 *Klasea* ( $\equiv$  *Serratula* section *Klasea*) comprises ca. 65 species distributed from the  
21 Western Mediterranean to China and the Western part of the Himalayas. The  
22 phylogenetic distance within the genus *Serratula* between the *Serratula* sensus stricto  
23 (*S. coronata* L. and *S. tinctoria*) and the *Klasea* section, has been demonstrated by  
24 different authors, on morphological (Wagenitz and Hellwig, 1996) and on molecular

1 bases (Martins and Hellwig, 2003). Therein, those authors were in accordance with  
2 Cassini (1825) who placed *Klasea* as a distinct genus from *Serratula*. Our results  
3 confirm these findings and, furthermore, clearly define *Klasea* as closely related to the  
4 *Rhaponticum* group (PP=97 %, BS=81 %, Fig. 1). This proximity was previously  
5 suggested by Lessing (1832), who considered *Klasea* Cass. as a subgenus of  
6 *Rhaponticum*. Moreover, the observation of the achene morphology has brought to light  
7 for the first time the fact that *Klasea* shows the same achene polymorphism as the  
8 *Rhaponticum* group. The *Klasea* species constitute a monophyletic group (PP=99 %,  
9 BS=100 %, Fig. 1). Among them, *Serratula algida* Iljin, *S. chartacea* C. Winkl., *S.*  
10 *biebersteiniana* (Iljin ex Grossh.) Takht. and *S. kuzhistanica* (Mozaffarian) Mozaffarian  
11 have not been recombined as *Klasea* until now; this has lead us to establish new  
12 nomenclatural combinations detailed in the appendix.

13

14 The monophyly of the *Rhaponticum* group is supported by the Bayesian inference  
15 (PP=98 %, Fig. 1), but the parsimony analysis shows an irresolution between *Klasea*,  
16 the *Centaurothamnus* plus *Ochrocephala* clade, and the rest of the genera. Even though  
17 the parsimony advices us to hesitate before placing *Klasea* as sister to the *Rhaponticum*  
18 group, this hypothesis is, nonetheless, supported both by karyological and  
19 morphological data. Representatives of the genus *Klasea* have a base chromosome  
20 number of  $x=15$  (Löve and Löve, 1961; Cantó, 1982 and 1984; Garcia-Jacas *et al.*,  
21 1998a and 1998b), while *Centaurothamnus* and *Callicephalus* have  $x=14$  (Wagenitz *et*  
22 *al.*, 1982 for the former; Chouksanova *et al.*, 1968; Hellwig, 1994; Garcia-Jacas *et al.*,  
23 1998a, for the second one), and *Acroptilon*, *Leuzea* and most of the *Rhaponticum*  
24 species present  $x=13$  (Hidalgo, unpubl. res.). Descending dysploidy being one of the

1 main evolutionary mechanisms in plants, and in particular in the Centaureinae  
2 (Fernández Casas and Susanna, 1986; Garcia-Jacas *et al.*, 1996; Vilatersana *et al.*,  
3 2000), this could suggest that  $x=15$  is more primitive than  $x=14$ . Then, in the case of our  
4 focus of study, this could suggest that *Klasea* should be sister to the *Rhaponticum*  
5 group. Furthermore, although *Klasea* shows achene polymorphism as the *Rhaponticum*  
6 group, it does not exhibit the two other morphological apomorphies which define it,  
7 namely the typical involucre bracts (present in *Ochrocephala*) and the characteristic  
8 pappus (present in *Centaurothamnus* and *Ochrocephala*).

9

#### 10 *Centaurothamnus* and *Ochrocephala*:

11 Our results confirm that *Ochrocephala* is more appropriately treated as a monotypic  
12 genus (Dittrich 1983), rather than considered as *Rhaponticum imatongensis*.  
13 Furthermore, this study allows us to define for the first time the systematic position of  
14 the genus *Centaurothamnus*. This genus was placed with the genera of «uncertain  
15 position» by Wagenitz & Hellwig (1996), Garcia-Jacas *et al.* (2001) and Hellwig  
16 (2004). With the new molecular evidence, *Centaurothamnus* appears as closely related  
17 to the genus *Ochrocephala* (PP=99 %, BS=100 %, Fig. 1). This result is unexpected but  
18 not surprising, because these two monotypic genera are geographically very close:  
19 *Centaurothamnus maximus* Wagenitz & Dittrich grows in Southwestern Arabia, in  
20 Yemen, and *Ochrocephala imatongensis* (Phillipson) Dittrich in Eastern Africa  
21 (Ethiopia, Sudan and Congo). Morphologically, these two taxa share the same shrubby  
22 habit, an exclusive trait of the group. The shrubby port is uncommon within the  
23 Centaureinae, and outside *Centaurothamnus* and *Ochrocephala* it is only known from  
24 *Centaurodendron* Johow, *Centaurea ptosimopappa* Hayek and *Cheirolophus*. It

1 corresponds, probably, to a secondary adaptation, this phenomenon being particularly  
2 evident for the insular taxa such as *Centaurodendron* and *Cheirolophus* (Böhle *et al.*,  
3 1996; Garnatje *et al.* unpubl. res.). In the case of *Centaurothamnus* and *Ochrocephala* it  
4 could be a secondary adaptation of hemicryptophytes colonizing extremely dry habitats,  
5 where they suffer from physiologic constraints and lack of pollinators.  
6 *Centaurothamnus* and *Ochrocephala* are genetically and morphologically distinguished:  
7 their molecular divergence for the three markers considered is 13.8 %; *Ochrocephala*  
8 shows the typical involucral bract appendages of the *Rhaponticum* group, while these  
9 are not present in *Centaurothamnus*. A new question induced by these results could be  
10 to determine whether *Centaurothamnus* and *Ochrocephala* should be more  
11 appropriately maintained as distinct genera or whether they should be merged to  
12 constitute a single genus.

13

14 *Callicephalus nitens*:

15 The genus *Callicephalus* includes a single species, *Callicephalus nitens* (M. Bieb. ex  
16 Willd.) C. A. Mey., from the middle and low mountains of the Caucasus, Central Asia,  
17 and the Near East. It appeared within the *Rhaponticum* group in the molecular analysis  
18 of Garcia-Jacas *et al.* (2001), but with a weak statistical support. Our results strongly  
19 support the fact that *Callicephalus* belongs to the *Rhaponticum* related genera (PP=99  
20 %, BS=86 %, Fig. 1). Because of its annual nature, this species shows a high rate of  
21 mutation, which could have induced distortions in the parsimony analysis. However, the  
22 Bayesian inference method, less affected by the long branch attraction, leads to an  
23 identical result. Furthermore, this hypothesis is reinforced by morphological features  
24 such as the structure of the inner pappus or the tuberculate pericarp (Garcia-Jacas *et al.*

1 2001). *Callicephalus* has no closely related taxa and appears as isolated in the  
2 phylogeny. Thus, this genus should be one of the numerous «relict» taxa which grow in  
3 the Caucasus. The abundance of relict and endemic plant species in this area seems  
4 largely due to the fact that it was spared glaciation during the most recent Iceages.

5

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

10

11 *Oligochaeta* and *Myopordon*:

12 *Myopordon*, a small genus with 5 perennial species from the Near East which had been  
13 placed in the Carduinae, and *Oligochaeta*, another genus composed of 4 annual species  
14 from the Near East, Caucasus, Afghanistan and India, and related to *Rhaponticum*, have  
15 apparently nothing in common. However their close relationship was put in evidence in  
16 the molecular study of Susanna *et al.* (submitted), and is also confirmed in this analysis  
17 (PP=99 %, BS=96 %, Fig. 1), whose sampling of the *Rhaponticum* group species is  
18 much more complete. In spite of the morphological review of *Myopordon* by Wagenitz  
19 (1958), several questions remain open. One consists of the generic delimitation of  
20 *Myopordon* and *Oligochaeta*: our analyses support the monophyly of *Oligochaeta*  
21 (PP=100 %, BS=100 %, Fig.1) but not that of *Myopordon*. More studies are necessary  
22 to verify whether *Myopordon* and *Oligochaeta* are independent taxa or whether they  
23 should be merged. Another question could be the morphological traits of this clade,  
24 focusing especially on their palynological characteristics, which are baffling. While all

1 the species of the *Rhaponticum* group show a *Serratula*-type pollen, *Oligochaeta*  
2 presents a reduced form of *Serratula*-type pollen grain (Villodre and Garcia-Jacas,  
3 2000), and *Myopordon* exhibits 3 different pollen types: *Jacea*, *Centaurea scabiosa* and  
4 *Serratula* (Wagenitz, 1958). An additional question would be to understand how these  
5 two specialized and divergent taxa can be so narrowly related to *Rhaponticum* up to the  
6 point that our three molecular markers are not able to segregate them.

7

8 The genus *Rhaponticum*:

9 The genus *Rhaponticum* does not appear as monophyletic in the phylogeny established  
10 for the following two reasons. Firstly, its segregation from the clade of *Myopordon* plus  
11 *Oligochaeta* is not statistically supported (Fig. 1). Fortunately, the resolution within the  
12 genus *Rhaponticum* is better, and shows two strongly supported clades, one oriental  
13 (PP=99 %, BS=100 %, Fig. 1) and the other occidental (PP=99 %, BS=92 %, Fig. 1).  
14 The second reason lead us to the paraphyly of *Rhaponticum* in its present  
15 circumscription: this is, that the genera *Acroptilon* and *Leuzea* are firmly nested in the  
16 genus *Rhaponticum*, the first in the oriental clade, and the second in the occidental clade  
17 (Fig. 1). This implies some nomenclatural changes to reconcile the delimitation of the  
18 genus with this new evidence. Another evidence for the placement of *Acroptilon* and  
19 *Leuzea* in the genus *Rhaponticum* is that the three taxa share the same chromosome  
20 number  $x=13$  (Hidalgo, unpubl. res.), which is uncommon within the Centaureinae.  
21 The comparison between the more comprehensive infrageneric classification of  
22 *Rhaponticum* (Holub, 1973) and the molecular phylogeny shows numerous  
23 incongruences (Fig. 1). Only two of the seven subgenera described are natural groups:  
24 the subgenus *Rhaponticella* (Soskov) Holub (PP=99 %, Fig. 1) and the subgenus *Leuzea*



1 DC. (PP=99 %, BS=99 %, Fig. 1). Our results suggest that the genus *Rhaponticum*  
2 should be more appropriately divided into only two subgenera, these corresponding to  
3 the oriental and the occidental clades. Nevertheless, we have been unable to detect any  
4 character for defining either group on morphological grounds.

5

6 The *Rhaponticum* oriental clade:

7 The first clade (PP=99 %, BS=100 %, Fig. 1) consists mostly of Central Asian species,  
8 but includes species from Middle and Eastern Asia, Australia and the Eastern Europe.  
9 These species have relatively restricted areas of distribution, except two groups of taxa  
10 which have wider areas.

11 (1) One group extends from the Western to Eastern Europe, and is constituted by  
12 *Rhaponticum serratuloides* (Georgi) Bobrov and *Acroptilon* (PP=99 %, BS=100 %, Fig.  
13 1). The incorporation of *Acroptilon* in *Rhaponticum* had never been mentioned before,  
14 and this implies new nomenclatural combinations detailed in the appendix. The most  
15 recent classifications do not recognize *Acroptilon australe* Iljin as a separate species  
16 from *A. repens* (L.) DC (Hellwig, 2004; Susanna and Garcia-Jacas, in press), while on  
17 the contrary Soskov (2001) considers them as two well-defined species. Our results do  
18 not allow us to pronounce a verdict, and more studies are necessary to clarify the status  
19 of *A. repens*. This is the reason why we have preferred to abstain from making a new  
20 combination for this taxon. *Acroptilon* is considered as an invasive weed in America  
21 and Australia, where it adversely affects agronomic harvests. It is aggressively  
22 competitive and exhibits allelopathic effects. It differs from the other species of the  
23 group, most of them endemics restricted to unfavorable environments where the  
24 competition with other species is less notable, as for example the mountain screes. The

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

7 (2) The other group expands from Central to Eastern Asia and Australia and is  
8 composed of *Rhaponticum australe* and *R. uniflorum* (L.) DC. The close relationship  
9 between these two species (PP=100 %, BS=97 %, Fig. 1) is a logical result, considering  
10 that *R. uniflorum* is the only species of the genus which has reached Eastern Asia. From  
11 a geographic point of view, this was the better candidate as sister to the Australian  
12 species. The fact that *R. australe* is the only species of Centaureinae indigenous to  
13 Australia is surprising because nothing explains such a long dissemination distance of  
14 the achenes of a *Rhaponticum* species. This lead Susanna and Garcia-Jacas (in press) to  
15 hypothesize that the species was doubtfully native in Australia. There is a considerable  
16 genetic divergence between *R. uniflorum* and *R. australe* for the three studied regions  
17 (8.7 %), and this allows us to rule out the possibility of a recent introduction from *R.*  
18 *uniflorum*. The colonizing event would have taken place during the period of lowermost  
19 sea level (between 50,000 and 84,000 years ago), from the coasts of South Asia. Was it  
20 the Aborigines that introduced the plant, and were they motivated by its medicinal  
21 properties ? Had the species, on the other hand, reached Australia without human  
22 intervention, we would need to ask ourselves how ?

23

24 The *Rhaponticum* occidental clade:

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

10 (1) the association *Rhaponticum heleniifolium* Godr. & Gren. plus *R. cynaroides* Less.  
11 (PP=99 %, BS=100 %, Fig. 1) is the exception because the former one is endemic of the  
12 Alps and the latter of the Pyrenees. The two species present the particularity of  
13 exhibiting ramified inflorescential stems. Few species of *Rhaponticum* show this  
14 character, and those are always less than 50 cm in height, while *R. heleniifolium* and *R.*  
15 *cynaroides* reach 1 m.

16 (2) one of these passages could be the case for the group including the *Leuzea* species  
17 (PP=99 %, BS=99 %, Fig. 1). Our study confirms, for the first time on a molecular  
18 basis, that *Leuzea* and *Rhaponticum* should be fused, as previously suggested by  
19 Holub (1973) on morphological bases. *Leuzea berardioides* Batt., endemic to the High  
20 Atlas (Morocco), appears as clearly segregated from *L. conifera* (L.) DC. (the molecular  
21 divergence for the studied regions between the two species is 7 %). This fact contradicts  
22 its consideration as a synonym or as a subspecies of *L. conifera* by Susanna (2002), and  
23 Greuter (2003), respectively, and implies a new nomenclatural combination of *L.*  
24 *berardioides* as *Rhaponticum*, as detailed in the appendix. Another taxon, *L. fontqueri*,

1 had been described by Sauvage (1968) as closely related to *L. berardioides*. Our results  
2 suggest that *L. fontqueri* is more closely related to *L. conifera* (PP=100 %, BS=100 %,   
3 Fig. 1), the only molecular divergences observed between these two taxa concerning  
4 polymorphic positions. *Leuzea conifera* presents a wide distribution area (W  
5 Mediterranean and Portugal) and a high morphological variability. In this sense, more  
6 studies are necessary to determine whether the observed differences with *L. fontqueri*  
7 are included in the natural variability of *L. conifera*, or if this endemic of the  
8 Chefchaouène Mountains (Morocco) merits the status of species. Meanwhile, the new  
9 nomenclatural combination for *L. fontqueri* as *Rhaponticum* is proposed in the  
10 appendix. *Leuzea conifera* had been previously combined as *Rhaponticum* by Greuter  
11 (2003). *Rhaponticum acaule* (L.) DC., is positioned at the base of the *Leuzea* group, but  
12 without statistical support.

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

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

1 An important outcome of this study is that the two representatives of *Rhaponticum*  
2 growing in the Alps, *R. scariosum* and *R. heleniifolium*, considered by several authors  
3 as subspecies of *R. scariosum* (Briquet 1902; Rouy 1905; Burnat 1931; Holub 1973;  
4 Dostál 1976), do not appear as sisters in the phylogeny (Fig. 1). This implies *inter alia*  
5 that the colonization of the Alps took place in, at least, two independent events. In the  
6 same order of things, the biogeographic inference suggests that the two species of  
7 *Rhaponticum* indigenous to the East of Europe (*R. serratuloides* and *R. scariosum*),  
8 could have reached this region in two different ways: one expansion across Central Asia  
9 to Eastern Europe generating *R. serratuloides*; the other expansion through the Near  
10 East, North Africa and then to the Iberian Peninsula, thence on to the Alps, generating  
11 *R. scariosum*.

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

16  
17 To conclude, this study fulfills our main expectations. It defines the genus *Klasea* as  
18 being probably the most closely related group of taxa to the *Rhaponticum* group. The  
19 generic delimitation of the *Rhaponticum* group would include the genera *Callicephalus*,  
20 *Centaurothamnus*, *Myopordon*, *Ochrocephala*, *Oligochaeta*, and *Rhaponticum*  
21 (including *Acroptilon* and *Leuzea*). The new molecular evidence is consistent with the  
22 karyological and morphological data, and suggests particularly coherent biogeographic  
23 routes of migration and speciation processes for the genus *Rhaponticum*.

24

1

2 APPENDIX

3

4 *Klasea* Cass., in Cuvier, Dict. Sci. Nat. 35: 173 (1825).

5 *K. algida* (Iljin) Hidalgo comb. nov. ≡ *Serratula algida* Iljin, *Repertorium Specierum*  
6 *Novarum Regni Vegetabilis* 35: 928-935 (1934).

7 *K. chartacea* (C. Winkl.) Hidalgo comb. nov. ≡ *Serratula chartacea* C. Winkl., in Tr.  
8 Peterb. Bot. Sada 9,2: 524 (1886).

9 *K. biebersteiniana* (Iljin ex Grossh.) Hidalgo comb. nov. ≡ *Serratula radiata* ssp.  
10 *biebersteiniana* Iljin ex Grossh., Fl. Kavk. 4 (1934) ≡ *Serratula biebersteiniana* (Iljin ex  
11 Grossh.) Takht., in Takht. et Fed., Fl. Erevana : 323 (1945).

12 *K. kuzhistanica* (Mozaffarian) Mozaffarian comb. nov. ≡ *Centaurea khuzistanica*  
13 Mozaffarian, *Iranian Journal of Botany* 5(2): 84 (1992) ≡ *Serratula khuzistanica*  
14 (Mozaffarian) Mozaffarian in Garcia-Jacas *et al.*, *Botanical Journal of the Linnean*  
15 *Society* 128: 420 (1998).

16

17 *Rhaponticum* Adans.

18 *R. berardioides* (Battand.) Hidalgo, comb. nov. ≡ *Leuzea berardioides* Batt., Contr. Fl.  
19 Alt., 55 (1919) ≡ *Rhaponticum coniferum* (L.) Greuter subsp. *berardioides* (Battand.)  
20 Greuter, *Willdenowia* 33:61 (2003).

21 *R. fontqueri* (Sauvage) Hidalgo, comb. nov. ≡ *Leuzea Fontqueri* Sauvage, *Collectanea*  
22 *Botanica* 59, 7(2) : 1100-1104 (1968).

23 *R. repens* (L.) Hidalgo, comb. nov. ≡ *Centaurea repens* L., Species Plantarum ed. 2,  
24 1293 (1763) ≡ *Acroptilon repens* (L.) DC., Prodrumus 6: 663 (1838).

1

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1 TABLE 1. Origin of the materials, herbaria where the vouchers are deposited and  
 2 GenBank accession numbers.

SPECIES	VOUCHER	ITS ACCESSION	ETS ACCESSION	trnL-F ACCESSION
<i>Acroptilon australe</i> Iljin	MONGOLIA: <i>V. Grubov 301 et al.</i> (LE)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Acroptilon repens</i> (L.) DC.	UZBEKISTAN: <i>Susanna 2046 et al.</i> (BC)	AY826223	xxxxxxx	AY772268
<i>Callicephalus nitens</i> (M. Bieb. ex Willd.) C. A. Mey.	ARMENIA: <i>Susanna 1578 et al.</i> (BC)	AY826237	xxxxxxx	AY772281
<i>Centauranthus maximus</i> Wagenitz & Dittrich	YEMEN: <i>Molero s. n.</i> (BC)	AY826259	xxxxxxx	AY772301
<i>Cheirolophus mauritanicus</i> (Font Quer) Susanna	MOROCCO: <i>Romo 4617 et al.</i> (BC)	AY826261	xxxxxxx	AY772303
<i>Cheirolophus teydis</i> (C. Sm.) G. López	SPAIN: <i>Susanna 1429 et al.</i> (BC)	AY826262	xxxxxxx	AY772304
<i>Klasea algida</i> (Iljin) Hidalgo	TAJIKISTAN: <i>Susanna 2558 &amp; Romaschenko</i> (BC)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Klasea biebersteiniana</i> (Iljin ex Grossh.) Hidalgo	ARMENIA: <i>Susanna 1493 et al.</i> (BC)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Klasea cernitifolia</i> (Sm.) Greuter & Wagenitz	IRAN: <i>Susanna 1700 et al.</i> (BC)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Klasea chartacea</i> (C. Winkl.) Hidalgo	TAJIKISTAN: <i>Susanna 2467 &amp; Romaschenko</i> (BC)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Klasea coriacea</i> (Fisch. & Mey. ex DC.) Holub	ARMENIA: <i>Susanna 1558 et al.</i> (BC)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Klasea grandifolia</i> (P. H. Davis) Greuter & Wagenitz	IRAN: <i>Susanna 1709 et al.</i> (BC)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Klasea kuzhistanica</i> (Mozaffarian) Mozaffarian	IRAN: <i>Mozaffarian 70181</i> (TARI)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Klasea serratuloides</i> (DC.) Greuter & Wagenitz	ARMENIA: <i>Susanna 1569 et al.</i> (BC)	AY826295	xxxxxxx	AY772334
<i>Leuzea berardioides</i> Batt.	MOROCCO: <i>Hidalgo &amp; Romo 12749</i> (BC)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Leuzea conifera</i> (L.) DC.	SPAIN: <i>Font s. n.</i> (BC)	AY826298	xxxxxxx	AY772337
<i>Leuzea Font-Quer</i> Sauvage	MOROCCO: <i>Hidalgo &amp; Romo 12621</i> (BC)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Myopordon aucheri</i> Boiss.	IRAN: <i>Carls s. n.</i> (W)	AY826299	xxxxxxx	AY772338
<i>Myopordon hyrcanum</i> (Bornm.) Wagenitz	IRAN: <i>Koelz 16395</i> (W)	AY826300	xxxxxxx	AY772339
<i>Myopordon persicum</i> Boiss.	IRAN: <i>Remandieri s. n.</i> (W)	AY826301	xxxxxxx	xxxxxxx
<i>Ochrocephala imatongensis</i> (Phillipson) Dittrich	ETHIOPIA: <i>Fantahun Simon 9163 et al.</i> (K)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Oligochaeta divaricata</i> (Fisch. & C. A. Mey.) K. Koch	ARMENIA: <i>Susanna 1583 et al.</i> (BC)	AY826306	xxxxxxx	AY772344
<i>Oligochaeta minima</i> (Boiss.) Briq.	UZBEKISTAN: <i>Botanical Garden of Tashrent</i> (BC)	AY826307	xxxxxxx	AY772345
<i>Plagiobasis centauroides</i> Schrenk	KAZAKHSTAN: <i>Susanna 2130 et al.</i> (BC)	AY826312	xxxxxxx	xxxxxxx
<i>Psephellus persicus</i> (DC.) Wagenitz	IRAN: <i>Susanna 1716 et al.</i> (BC)	AY826316	xxxxxxx	AY772352
<i>Psephellus pulcherrimus</i> (Willd.) Wagenitz	ARMENIA: <i>Susanna 1492 et al.</i> (BC)	AY826317	xxxxxxx	AY772353
<i>Rhaponticoides hajastana</i> (Tzvelev) M. V. Agab. & Greuter	ARMENIA: <i>Susanna 1587 et al.</i> (BC)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Rhaponticoides iconiensis</i> (Hub.-Mor.) M. V. Agab. & Greuter	TURKEY: <i>Ertugrul 1761</i> (BC)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Rhaponticum acaule</i> (L.) DC.	ALGERIA: <i>Montserrat 2331 et al.</i> (BC)	AY826334	xxxxxxx	AY772369
<i>Rhaponticum aulicetense</i> Iljin	KYRGYZSTAN: <i>Sheremetova &amp; Lazkov</i> (LE)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Rhaponticum australe</i> (Gaudich.) Soskov	AUSTRALIA: <i>Funck 12203</i> (BC)	AY826335	xxxxxxx	AY772370
<i>Rhaponticum canariense</i> DC.	SPAIN: <i>Carqué Alamo s. n.</i> (BC)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Rhaponticum carthamoides</i> (Willd.) Iljin	RUSSIA: <i>Botanical Garden of Sibiricus Centralis, Novosibirsk 2003/2004-1062</i> (BC)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Rhaponticum cossonianum</i> (Ball) Greuter	MOROCCO: <i>Gómiz 17-IV-2003</i> (BC)	xxxxxxx	xxxxxxx	xxxxxxx
<i>Rhaponticum cynaroides</i> Less.	SPAIN: <i>Hidalgo 504, et al.</i> (BC)	xxxxxxx	xxxxxxx	xxxxxxx

<i>Rhaponticum exaltatum</i> (Willk.) Greuter	SPAIN: <i>Garcia-Jacas &amp; Susanna 2434</i> (BC)	XXXXXXXX	XXXXXXXX	XXXXXXXX
<i>Rhaponticum helenifolium</i> Godr. & Gren.	<i>Botanical Garden of Minsk, Bielorusia 1/303-2000</i> (BC)	XXXXXXXX	XXXXXXXX	XXXXXXXX
<i>Rhaponticum insigne</i> (Boiss.) Wagenitz	IRAN: <i>Archibald 2034</i> (K)	XXXXXXXX	XXXXXXXX	XXXXXXXX
<i>Rhaponticum integrifolium</i> C. Winkl.	TAJIKISTAN: <i>Makhmetov &amp; R. Kamelin 344</i> (LE)	XXXXXXXX	XXXXXXXX	XXXXXXXX
<i>Rhaponticum karatavicum</i> Iljin	KAZAKHSTAN: <i>Kamelin</i> (LE)	XXXXXXXX	XXXXXXXX	XXXXXXXX
<i>Rhaponticum longifolium</i> (Hoffmanns. & Link) Dittrich	PORTUGAL: <i>Garcia-Jacas &amp; Susanna 2436</i> (BC)	XXXXXXXX	XXXXXXXX	XXXXXXXX
<i>Rhaponticum lyratum</i> C. Winkl. ex Iljin	TAJIKISTAN: <i>Kossov</i> (LE)	XXXXXXXX	XXXXXXXX	XXXXXXXX
<i>Rhaponticum nanum</i> Lipsky	TAJIKISTAN: <i>Kochkariova 5834</i> (DUSH)	XXXXXXXX	XXXXXXXX	XXXXXXXX
<i>Rhaponticum nanum</i> Lipsky ssp. <i>pellucidum</i> (Rech. f.) Dittrich	AFGHANISTAN: <i>Renz 120</i> (W)	XXXXXXXX	XXXXXXXX	XXXXXXXX
<i>Rhaponticum nitidum</i> Fisch.	RUSSIA: <i>Kalibernova 5676, et al.</i> (LE)	XXXXXXXX	XXXXXXXX	XXXXXXXX
<i>Rhaponticum pulchrum</i> Fisch. & C. A. Meyer	RUSSIA: <i>326 et al.</i> (LE)	XXXXXXXX	XXXXXXXX	XXXXXXXX
<i>Rhaponticum scariosum</i> Lam.	SLOVENIA: <i>Botanical Garden of Universitatis Labacensis Slovenia 1994-180</i> (BC)	XXXXXXXX	XXXXXXXX	XXXXXXXX
	<i>Botanical Garden of Minsk, Bielorusia 1/304-2000</i> (BC)	XXXXXXXX	XXXXXXXX	XXXXXXXX
<i>Rhaponticum serratuloides</i> (Georgi) Bobrov	<i>Botanical Garden of Cluj-Napoca, Romania 636-2001</i> (BC)	XXXXXXXX	XXXXXXXX	XXXXXXXX
<i>Rhaponticum uniflorum</i> (L.) DC.	MONGOLIA: <i>Vallès 13-2003</i> (BC)	XXXXXXXX	XXXXXXXX	XXXXXXXX
<i>Serratula coronata</i> L.	<i>Botanical Garden of Vienna, Austria</i> (BC)	AY826327	XXXXXXXX	AY772362
<i>Stizolophus coronopifolius</i> Cass.	TURKEY: <i>Ilarslan 4303</i> (ANK)	AY826337	XXXXXXXX	AY013516

- 1 TABLE 2. Results from the combined ITS, ETS and *trnL-trnF* parsimony analysis. The  
 2 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 MTPs	558
Number of steps	1582
Consistency index (CI)	0.48
Retention index (RI)	0.72
Mean pairwise distances, ingroup (%)	from 0 [ <i>Acroptilon australe/A. repens; Leuzea conifera/L. fontqueri</i> ] to 37 [ <i>Acroptilon australe, A. repens/Callicephalus nitens</i> ]



1 FIG1. Majority-rule consensus tree based on Bayesian analyse. Numbers above branches  
2 indicate Bayesian clade-credibility values (Posterior probability); Numbers below  
3 branches indicate parsimony bootstrap percentages. Subgenus of *Rhaponticum*:  
4 CES=*Cestrinus*; ELE=*Eleutherochaetum*; FOR=*Fornicium*; LEU=*Leuzea*;  
5 RHA=*Rhaponticina*; RHL=*Rhaponticella*; STE=*Stemmacantha*.  
6

