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Is the extremely rare Iberian endemic plant species *Castrilanthemum debeauxii* (Compositae, Anthemideae) a 'living fossil'? Evidence from a multi-locus species tree reconstruction

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ABSTRACT

The present study provides results of multi-species coalescent species tree analyses of DNA sequences sampled from multiple nuclear and plastid regions to infer the phylogenetic relationships among the members of the subtribe *Leucanthemopsidinae* (Compositae, Anthemideae), to which besides the annual *Castrilanthemum debeauxii* (Degen, Hervier & É.Rev.) Vogt & Oberp., one of the rarest flowering plant species of the Iberian Peninsula, two other unispecific genera (*Hymenostemma, Prolongoa*), and the polyploidy complex of the genus *Leucanthemopsis* belong. Based on sequence information from two single- to low-copy nuclear regions (*C16, D35*, characterised by Chapman et al. (2007)), the multi-copy region of the nrDNA internal transcribed spacer regions ITS1 and ITS2, and two intergenic spacer regions of the cpDNA gene trees were reconstructed using Bayesian inference methods. For the reconstruction of a multi-locus species tree we applied three different methods: (a) analysis of concatenated sequences using Bayesian inference (MrBayes), (b) a tree reconciliation approach by minimizing the number of deep coalescences (PhyloNet), and (c) a coalescent-based species-tree method in a Bayesian framework (*BEAST). All three species tree reconstruction methods unequivocally support the close relationship of the subtribe with the hitherto unclassified genus *Phalacrocarpum*, the sister-group relationship of *Castrilanthemum* with the three remaining genera of the subtribe, and the further sister-group relationship of the clade of *Hymenostemma* + *Prolongoa* with a monophyletic genus *Leucanthemopsis*. Dating of the *BEAST phylogeny supports the long-lasting (Early Miocene, 15–22 Ma) taxonomical independence and the switch from the plesiomorphic perennial to the apomorphic annual life-form assumed for the *Castrilanthemum* lineage that may have occurred not earlier than in the Pliocene (3 Ma) when the establishment of a Mediterranean climate with summer droughts triggered evolution towards annuality.

1. Introduction

The annual species *Castrilanthemum debeauxii* (Degen, Hervier & É.Rev.) Vogt & Oberp. (Compositae, Anthemideae) is one of the rarest flowering plant species of the Iberian Peninsula (Vargas, 2010; Jiménez-Mejías et al., 2012). It is the sole member of the genus *Castrilanthemum* Vogt & Oberpr., which has been described based on the type species *Pyrethrum debeauxii* Degen, Hervier & É.Rev. in 1996 (Vogt and Oberprieler, 1996) and for which, besides the type specimen collections of Élisée Reverchon dating to the year 1903, only a single further collection made by J. Leal Pérez-Chao in 1978 was available until most recently. Presently, only one restricted population in Sierra de Guillimona is known and has appeared with continuity during the last decade. Its remote and very local potential distribution in some Sierras (Sierra de Castril, Sierra de Cuarto, Sierra de la Cabrilla, Sierra de Guillimona) in the provinces of Jaen and Granada (SE Spain) and its ephemeral appearance as an annual plant led to the inclusion of the species in the Red List of the Spanish Vascular Flora as 'critically endangered' (Moreno, 2011).

The phylogenetic position of *Castrilanthemum* has been studied by Vogt and Oberprieler (1996) based on morphological characters and by Oberprieler and Vogt (2000), Oberprieler (2005), and Oberprieler et al. (2007) using molecular phylogenetic methods based on nrDNA ITS and cpDNA trnL/trnF intergenic spacer (IGS) sequences. While cladistic analyses of morphological data (Vogt and Oberprieler, 1996) turned out to be equivocal in respects to the phylogenetic position of *Castrilanthemum* in the subtribe

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Leucantheminae sensu Bremer and Humphries (1993), the subsequent molecular studies focussing on the Mediterranean representatives of the tribe *Anthemideae* (Oberprieler and Vogt, 2000; Oberprieler, 2005) and on the whole tribe (Oberprieler et al., 2007, 2009) elaborated the consistent placement of the genus in a small and well-supported monophyletic group of genera with a western Mediterranean core distribution. This generic group was raised to subtribal rank as *Leucanthemopsidinae* Oberpr. & Vogt by Oberprieler et al. (2007) and, besides *Castrilanthemum*, comprises the larger (6 species) perennial genus *Leucanthemopsis* (Giroux) Heywood and the two annual unispecific genera *Hymenostemma* Willk. and *Prolongoa* Boiss. Within that group, *Castrilanthemum* was found to be the sister-group to the other three genera with a 6–7 Ma long period of independent evolutionary history (Oberprieler, 2005). This phylogenetic isolation, together with its geographical restrictedness and its rarity, makes *C. debeauxii* a potential candidate for its designation as 'living fossil', a term with some potential for grabbing attention but with an equally divergent history of semantic connotations in evolutionary biology (Darwin, 1859; Stanley, 1979; Eldredge and Stanley, 1984; Fisher, 1990; Vrba, 1984; Gould, 2002).

The molecular phylogenetic reconstructions mentioned suffer from two main shortcomings that hamper a more substantiated discussion of the 'living fossil' topic for *Castrilanthemum*: (1) all previous studies were based on a restricted sampling of the members of subtribe *Leucanthemopsidinae,* with the name-giving genus *Leucanthemopsis* only represented by the single species *L. alpina* (L.) Heywood and all other taxa included only sampled from single accessions; (2) the previous studies were based on either the nrDNA ITS region alone or on a combined analysis of this standard region with the cpDNA trnL/trnF IGS region. Since especially the multi-copy nuclear region nrDNA ITS is quite problematic due to phenomena like concerted evolution and high levels of homoplasy (Álvarez and Wendel, 2003), the usage of low- and single-copy nuclear regions have gained further attraction for phylogenetic studies. Candidate single-copy regions for application in the sunflower family (Compositae) were proposed by Chapman et al. (2007) and have been successfully applied since then in a number of studies (Smissen et al., 2011; Brennan et al., 2012; Guo et al., 2012; Gruenstaeudl et al., 2013). With this new array of phylogenetic regions available, however, problems come into focus that are connected to the fact that stochastic mechanisms may produce discordance among the individual gene trees and that those gene trees may not correspond to the underlying species tree (e.g., Brower et al., 1996; Maddison, 1997; Avise and Wollenberg, 1997; Kingman, 1982, 2000; Degnan and Rosenberg, 2009; Edwards, 2009).

The challenge for the systematists who want to undertake a phylogenetic study based on data from multiple loci is that usually widespread incongruence among gene trees is found as the number of regions taken into account increases. In the past, the standard and universally accepted way to deal with multi-locus data was the concatenation of the sequences from the different regions and the analysis of the obtained 'supergene' with the traditional methods used in molecular phylogeny, despite the awareness of the processes leading to different evolution between unlinked genes. Weisrock et al. (2012) have shown that, when processing regions with high levels of discordance, concatenated analyses may produce robust, well-supported, but inaccurate phylogenetic reconstructions. As a consequence, an increasing number of methods have been proposed to estimate the correct species tree without concatenation of sequence data, especially for those cases when incomplete lineage sorting (ILS) is the reason for incongruence among gene trees (Mossel and Roch, 2007; Liu, 2008; Than and Nakhleh, 2009; Liu et al., 2009; Heled and Drummond, 2010; Knowles and Kubatko, 2010; Fan and Kubatko, 2011).

With the present study we aim therefore to (i) reconstruct a well resolved phylogeny of the *Leucanthemopsidinae,* (ii) to verify the monophyly of the subtribe as well as the monophyly of the genera included in it, shedding light also on the relationships among the different taxa of the subtribe, and (iii) to apply a molecular clock approach to find out the absolute time of the divergence of *Castrilanthemum debeauxii* from the lineage of its closest relatives. In order to achieve these goals we used two plastid regions (cpDNA), the ribosomal internal transcribed spacer (nrDNA ITS), and two single-copy nuclear regions, for a representative number of accessions for each taxon of the subtribe. We used three different approaches to reconcile the results from the different regions, including (i) an analysis based on concatenated sequences, (ii) a tree reconciliation approach by minimizing the number of deep coalescences (Maddison, 1997), and (iii) a coalescent-based species-tree method in a Bayesian framework (Heled and Drummond, 2010).

2. Material and methods

2.1. Plant material

During 2010 and 2011, individuals belonging to all the taxa of the subtribe *Leucanthemopsidinae* plus the outgroup taxon *Phalacrocarpum oppositifolium* were collected in the Iberian Peninsula, Corsica, and the Alps. With regards to the *Leucanthemopsidinae*, three specimens were used for *Castrilanthemum debeauxii*, two for *Hymenostemma pseudoanthemis,* and *Prolongoa hispanica,* and 12 for the different *Leucanthemopsis* species with at least one accession per taxon. Since the infrageneric phylogeny of *Leucanthemopsis* was beyond the scope of the present analysis and inclusion of polyploid taxa from that genus reaching tetra- and hexaploid levels would have complicated sequencing and analysis, mainly diploid representatives of this genus were included.

In order to test for the monophyly of the subtribe, further 14 accessions for the analysis came from species belonging to several subtribes of *Anthemideae* besides the *Leucanthemopsidinae*. Among those accessions, two individuals belonging to *Phalacrocarpum oppositifolium*, a species which is still unassigned to any subtribe of the *Anthemideae* (Oberprieler et al., 2009) but considered to be presumably related to the *Leucanthemopsidinae,* were analysed. A total amount of 31 accessions were included in the present study.

Almost all of the specimens of *Leucanthemopsidinae* used in the study were collected in the field and instantly dried in silica gel. *Leucanthemopsis pallida* subsp. *virescens* (sample number LPS185) and *L. pallida* (LPS186) were sampled from specimens kept at MA herbarium. The accessions for *Leucanthemopsis alpina* subsp. *tatrae* (LPS037) and *Phalacrocarpum oppositifolium* subsp. *oppositifolium* (LPS147) were sampled from specimens kept at M herbarium and from the private herbarium of the first author (S.T.), respectively. A complete list of the accessions used in the present study is given in Table 1.

2.2. DNA extraction, amplification and sequencing

For the outgroup samples included in the present analysis, we employed DNA extracts stored at the Institute of Plant Sciences of Regensburg University and used in former studies (Oberprieler and Vogt, 2000; Oberprieler, 2004a,b; Himmelreich et al., 2008). All silica-gel samples belonging to subtribe *Leucanthemopsidinae* and collected in the Iberian Peninsula during 2011 were extracted using the DNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands). *Leucanthemopsis pallida* (LPS186), *L. pallida* subsp. *virescens* (LPS185), *L. pallida*, *L. alpina* subsp. *alpina* (LPS074-1), *L. alpina* subsp. *tatrae* (LPS037), *L. alpina* subsp. *tomentosa* (LPS181-3), and *L. pallida* var. *alpina* (LPS157-3) were extracted using a modified

Table 1
Comprehensive list of the samples used in the present study including voucher information and GenBank accession numbers. Asterisks (*) beside accession numbers indicate samples cloned for some of the marker used. Number in brackets behind GenBank accession numbers refer to sequences from former studies: (1) Himmelreich et al. (2008), (2) Oberprieler (2004a), (3) Oberprieler (2004b), (4) Oberprieler & Vogt (2000), (5) Lo Presti et al. (2010), and (6) Sonboli et al. (2012).

Taxon	Sample no.	Voucher information	ITS1	ITS2	psbA-trnH	trnC-petN	*C16*	*D35*
Achillea tenuifolia Lam.	A205	Armenia, 18.06.2002, Oberprieler 10094 (Herb. Oberprieler).	KM589804	KM589830	FR689911(5)	FR690061(5)	KM589665	KM589719
Artemisia vulgaris L.	A838*	Germany, Regensburg, 16.09.2010, Konowalik s.n. (Herb. Konowalik).	KM589806-KM589809	KM589836	KM589761	KM589799	KM589674	KM589718
Heliocauta atlantica (Litard. & Maire) Humphries	A176	Marocco, Toubkal, 3850 m, 23.08.1992, Kreisch 920589 (Herb. Kreisch).	AJ748782(2)	AJ748782(2)	FR689913(5)	FR690063(5)	KM589666	KM589720
Ismelia carinata (Schousb.) Sch. Bip.	A007*	Morocco, Agadir, 26.04.1994, Kilian 3384 (B).	KM589810	KM589832	KM589759	KM589797	KM589675 KM589676	KM589730
Leucanthemum vulgare (Vaill.) Lam. subsp. *pujulae* Sennen	A045	France, Pyrenees orientales, Prats de Mollo la Preste, 850 m, 24.08.1986, Vogt 5053 & C. Prem (Herb. Vogt).	AJ3296398(4)	AJ3296433(4)	FR689914(5)	FR690064(5)	KM589667	KM589721
Matricaria discoidea DC.	A069	Germany, Jena, Botanischer Garten, Oberprieler 9762 (Herb. Oberprieler, B).	AJ3296412(4)	AJ3296447(4)	FR689917(5)	FR690067(5)	KM589668	KM589722 KM589723
Nananthea perpusilla DC.	A170*	Italy, Sardinia, Sulcis, bay NW Portoscuso, 0–20 m, 9.-20.4.1966, Merxmüller 21023 & Oberwinkler (M).	AJ864579(3)	AJ864579(3)	AB683361 (6)	XXX	KM589672 KM589673	KM589748-KM589750
Plagius flosculosus (L.) Alavi & Heyw	A793	Italy, Sardinia, Sassari, Ittiri, near Bacino Cuga, 19.8.1996, leg. L. Zedda s.n. (Herb. Vogt).	AJ3296403(4)	AJ3296403(4)	FR689918(5)	FR690068(5)	KM589669	KM589724
Santolina rosmarinifolia L.	A077*	Morocco, Er-Rachidia, Tounfite - Boumia, 1810–1850 m, 01.07.1989, Oberprieler 1950 (cult. in HB Berol. 071-52-91-10).	AJ3296387(4)	AJ3296422(4)	KM589760	KM589798	KM589677 KM589678	KM589728 KM589729
Tanacetum coccineum (Willd.) Grierson	A197	Armenia, 12.06.2002, Oberprieler 10045 (Herb. Oberpieler).	KM589805	KM589831	FR689920(5)	FR690070(5)	KM589670	KM589725
Tripleurospermum caucasicum (Willd.) Hayek	A192	Armenia, 30.06.2002, Oberprieler 10192 (Herb. Oberpieler).	AJ864590(3)	AJ864590(3)	FR689921(5)	FR690071(5)	KM589717	KM589726 KM589727
Ursinia anthemoides (L.) Poir. subsp. *vesicolor* (DC.) Prassler	A436*	South Africa, Cape Province, Kamiesbergpas, ENE Kamieskroon, 800–1000 m, 12.09.1993, Strid & Strid 37382 (S).	AM774473(1)	AM774473(1)	HE818814(5)	HE818929(5)	KM589671	KM589751-KM589755
Phalacrocarpum oppositifolium (Brot.) Willk. subsp. *oppositifolium*	LPS147	Portugal, Serra de Estrela, Manteigas - La Torre, 1000 m, 10.05.2011, Tomasello 281 (MA).	KM589820	KM589843	KM589770	KM589790	KM589710	KM589744
Phalacrocarpum oppositifolium subsp. *anomalum* (Lag.) Vogt & Greuter	LPS162–1	Spain, Venta Pepin, Puerto de las Piedraluengas, 1200 m, 16.06.2011, Tomasello 360 (MA).	KM589822	KM589845	KM589771	KM589803	KM589712	KM589745
Hymenostemma pseudoanthemis (Kunze) Willk.	LPS130-7	Spain, Pinar del Hierro, Chiclana de la Frontera, 30 m, 16.04.2011, Tomasello 195 (MA).	KM589815	KM589838	KM589765	KM589784	KM589700	KM589740
Hymenostemma pseudoanthemis (Kunze) Willk.	LPS131-9	Spain, Arcos de la Frontera, 260 m, 17.04.2011, Tomasello 197 (MA).	KM589828	KM589839	KM589766	KM589785	KM589701	KM589741
Prolongoa hispanica G. López & C. E. Jarvis	LPS133-6*	Spain, Las Nieves (Nambrocas), 650 m, Tomasello 212 (MA).	KM589816	KM589840	KM589767	KM589786	KM589702-KM589706	KM589742
Prolongoa hispanica G. López & C. E. Jarvis	LPS135-10	Spain, Puente Duero, 695 m, 23.04.2011, Tomasello 221 (MA).	KM589818	KM589842	KM589768	KM589788	KM589708	KM589743
Castrilanthemum debeauxii (Degen & al.) Vogt & Oberpr.	IA2169-20*	Spain, Sierra Guillimona, 10.06.2011, Alvarez 2169 & Tomasello (MA).	KM589811	KM589833	KM589762	KM589781	KM589682-KM589686	KM589738
Castrilanthemum debeauxii (Degen & al.) Vogt & Oberpr.	IA2170-4*	Spain, Sierra Guillimona, 10.06.2011, Alvarez 2170 & Tomasello (Herb. Tomasello, MA).	KM589812	KM589834	KM589763	KM589782	KM589687-KM589693	KM589739
Castrilanthemum debeauxii (Degen & al.) Vogt & Oberpr.	IA2171–28*	Spain, Sierra Guillimona, 10.06.2011, Alvarez 2171 & Tomasello (MA).	KM589813	KM589835	KM589764	KM589783	KM589694-KM589697	KM589746
Leucanthemopsis pulverulenta (Lag.) Heywood	LPS134-1	Spain, Puente Duero, 695 m, 23.04.2011, Tomasello 217 (MA).	KM589817	KM589841	KM589769	KM589787	KM589707	KM589734
Leucanthemopsis pallida var. *alpina* (Boiss. & Reuter) Heywood	LPS157-3	Spain, La Mira (Sierra de Gredos), 2300 m, 12.06.2011, Tomasello 332 (MA).	KM589821	KM589844	KM589774	KM589801	KM589711	KM589755 KM589756
Leucanthemopsis pallida (Mill.) Heywood	LPS186	Spain, Pico Revolcadores (Murcia), 1970 m, 01.05.2005, Aedo 11398 (MA).	KM589827	KM589850	KM589777	KM589794	KM589716	KM589737
Leucanthemopsis pallida subsp. *virescens* var. *bilbilitanum* (Pau) Heywood	LPS138-1*	Spain, Puerto de Aguaron (Sierra del Vicort), 1000 m, 1.05.2011, Tomasello 247 & Hilpold (MA).	KM589819	KM589851 KM589852	KM589772	KM589789	KM589709	KM589733

Leucanthemopsis pallida subsp. *virescens* (Pau) Heywood var. *virescens*	LPS163-2	Spain, Sierra del Brezo (Montaña Palentina), 1670 m, 17.06.2011, Tomasello 361 (MA).	KM589823	KM589846	KM589773	KM589791	KM589713	KM589732	
Leucanthemopsis pallida subsp. *virescens* (Pau) Heywood var. *virescens*	LPS185*	Spain, Valle de Losa, Monte Peñalta, 1000–1095 m, 20.04.2006. Uribe-Echebarria 77690 (MA).	KM589826	KM589849	KM589780	KM589802	KM589679-KM589681	KM589757 KM589758	
Leucanthemopsis pectinata (L.) G. López & C. E. Jarvis	LPS166-11	Spain, Veleta (Sierra Nevada), 13.07.2011, Vargas, Cano & Tomasello 372 (MA).	KM589824	KM589847	KM589775	KM589792	KM589714	KM589735	
Leucanthemopsis alpina subsp. *tomentosa* (Loisel.) Heywood	LPS181-3	France, Corsica, Monte Renoso, 2300 m, 08.08.2011, Tomasello 409 (Herb. Tomasello).	KM589825	KM589848	KM589776	KM589793	KM589715	KM589736	
Leucanthemopsis alpina (L.) Heywood subsp. *alpina*	LPS074-1	France, Vallouise, Glacier Blanc, 2380 m, 18.07.2010, Tomasello 40 (Herb. Tomasello).	KM589814	KM589837	KM589778	KM589795	KM589698	KM589747	
Leucanthemopsis alpina subsp. *tatrae* (Vierh.) Holub	LPS037	Romania, Gilort, Papusa - Lespezi, c. 2100 m, 29.06.1963, Buia, Paum, Casanova, Fulga & Olaru s.n. (M).	KM589829	KM589853	KM589779	KM589800	KM589699	KM589731	

protocol based on the CTAB method by Doyle and Doyle (1987). The quality of the extracted DNA was checked on 1.5% TBE-agarose gels.

For the phylogenetic analyses, we used two intergenic spacer regions on the plastid genome (*psbA-trnH* and *trnC-petN*), the nuclear ribosomal internal transcribed spacer region (nrDNA ITS), and two single-copy nuclear regions *(C16, D35)* characterised by Chapman et al. (2007). The plastid spacer *psbA-trnH* was amplified using the primers psbAf and trnHr (Sang et al., 1997), whereas for *trnC-petN* we used the primers trnC (Demesure et al., 1995) and petN1r (Lee and Wen, 2004). PCR amplification was performed using the Taq DNA Polymerase Master-mix Red (Ampliqon, Odense, Denmark) in a final volume of 12.5 µl, using the protocol suggested of the company. The following temperature profile was employed: 2 min at 95 °C, then 36 cycles of 30 s at 95 °C, 60 s at 62 °C, 60 s at 72 °C, with a final extension of 5 min at 72 °C.

Concerning the nrDNA ITS region, ITS1 and ITS2 were amplified separately using the primers 18SF (Rydin et al., 2004) and P2B (White et al., 1990) for ITS1 and P3 (White et al., 1990) and SR (Blattner et al., 2001) for ITS2. The temperature profile used for nrDNA ITS was the same as for the plastid regions, with the only difference that the annealing temperatures of 50 °C and 60 °C were used for ITS1 and ITS2, respectively. The two single-copy nuclear regions *(C16, D35)* were amplified using either a touch-down PCR program as recommended by Chapman et al. (2007) or the same program used for the ITS and plastid regions with an annealing temperature of 58 °C.

The PCR products were purified using Agencourt AMPure magnetic beads (Agencourt Bioscience Corporation, Beverly, Massachusetts, USA). Cycle sequencing was performed using the DTCS Sequencing Kit (Beckman Coulter, Fullerton, California, USA), following the protocol suggested of the manufacturer. Sequences were analysed on a CEQ 8000 sequencer (Beckman Coulter, Fullerton, California, USA) and the obtained electropherograms were carefully checked for ambiguities using Chromas Lite 2.10 (Technelysium Pty Ltd., Tewantin, Australia; http://technelysium.com.au/chromas.html). We used the IUPAC ambiguity code to indicate single nucleotide polymorphisms. In the electropherograms, a site was considered polymorphic when more than one peak was present and the weakest one reached at least the 25% of intensity of the strongest one (Fuertes Aguilar et al., 1999; Mansion et al., 2005). We considered reliable those sequences where the percentage of polymorphisms was not higher than approximately 2% of the total sequence (Lo Presti et al., 2010). Eleven accessions needed to be cloned either for nrDNA ITS or for one of the low-copies nuclear regions (see Table 1 for details). Cloning was done using the CloneJET PCR cloning kit (Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's recommendations. Eight colonies were picked for each accession cloned in order to sample the two possible alleles of a heterozygous individual with a probability of 0.99 (formula from Joly et al., 2006).

2.3. Data processing and phylogenetic analyses

Alignments were done using the Clustal W progressive method for multiple sequences alignment (Thompson et al., 1994) as implemented in BioEdit, version 7.0.5.3 (Hall, 1999; http://www.mbio.ncsu.edu/bioedit/bioedit.html) and improved in MAFFT (Katoh et al., 2002; http://mafft.cbrc.jp/alignment/software/), which uses a two-cycle progressive method called FFT-NS-2 (Katoh and Toh, 2008). Alignments were finally checked and edited manually. In the *trnC-petN1* alignment, the region between alignment positions 392 and 422 was deleted due to a poly-A microsatellite motive that produced non-informative, presumably highly homoplastic differences among sequences. Gaps were coded as binary characters using the simple gap coding method of Simmons and Ochoterena (2000)

as implemented in the software programme GapCoder (Young and Healy, 2003).

A maximum parsimony analysis (MP) was done for the plastid regions (*psbA-trnH* and *trnC-petN* concatenated into a single alignment), nrDNA ITS, *C16,* and *D35* separately using PAUP* 4.0 version beta 10 (Swofford, 2002). For the plastid alignment, nrDNA ITS, and *D35* the heuristic search was performed with TBR branch swapping in action, for 1000 random addition replicates. Support for clades was evaluated using bootstrap analyses (Felsenstein, 1985). These were performed using 1000 bootstrap replicates, 100 random addition sequence replicates per bootstrap replicate, with a time limit of 10 s per random addition sequence replicate, and ACCTRAN, TBR, and MULPARS in action. For *C16*, the same settings as above were used with the only difference that a time limit of 60 s per replicate was used in the heuristic search.

As in the MP analyses, Bayesian inference (BI) phylogenetic analyses were performed with MrBayes, version 3.2.1 (Ronquist et al., 2012) for the plastid regions, nrDNA ITS, *C16*, and *D35* separately. BI is dependent on assumptions about the process of DNA substitution (a model of DNA evolution). Therefore, the models that best fit the sequence information for each of the different regions were selected based on the Akaike Information Criterion (AIC) in MODELTEST, version 3.7 (Posada and Crandall, 1998). Information concerning the evolution model and the parameter values accepted for each region is provided in Appendix A.

The BI analyses were conducted using seven heated chains and one cold one, with a chain heating parameter of 0.2 in the individual runs. The Metropolis-coupled Markov chain Monte Carlo (MC^3) chains were run for 10^8 generations, with trees sampled every 1000th generation. Reaching of convergence among searches was checked by examining the average standard deviation of split frequencies and by comparing likelihood values and parameter estimates in Tracer, version 1.5 (Rambaut and Drummond, 2007). A burn-in equal to 25% of the run-length was applied as by default (Ronquist et al., 2011). The remaining trees were used to estimate topology and posterior probability (PP) using the 'halfcompat' setting for the consensus tree.

2.4. Total-evidence tree inference and dating

The first approach to infer a total-evidence tree from the four gene trees was done producing a supermatrix dataset from the five different regions and running a concatenated analysis. One major problem for the implementation of concatenated analyses is the selection of alleles when the accessions (OTUs) are heterozygous at multiple loci. Weisrock et al. (2012) showed that phylogenetic results are influenced by the selection of alleles in the concatenation process and that it is preferable to produce multiple analyses pruning randomly different allele copies across regions each time than choosing arbitrarily only one of the alleles or producing accession-wise consensus sequences of alleles. In contrast to these suggestions, however, we decided to produce allelic consensus sequences for those accessions which had more than one allele per region. This was done because of the observation that in all heterozygous cases in our dataset, the different allelic forms of an accession (OTU) formed monophyletic groups in the gene trees. A MP analysis was performed for the concatenated dataset for 1000 random addition replicates, with a time limit of 60 s for each replicate. A bootstrap analysis was run using 100 bootstrap replicates, 1000 random addition sequence replicates per bootstrap replicate and a time limit of 60 s per random addition sequence replicate. For the Bayesian analysis, we used a partitioned approach with the model parameters for each locus as in the single region analyses (see above). Two runs, each of eight MC^3 chains (seven heated and one cold one, chain heating parameter of 0.2) were run for 10^7 generations, with trees sampled every 1000th generation. A 'halfcompat' consensus tree was estimated after applying a burn-in equal to the 25% of the total number of sampled trees.

For the coalescent-based, multi-locus tree inference using the minimizing deep coalescences (MDC) criterion, we followed three different procedures: (i) we used the four gene trees obtained from the Bayesian analyses to produce a MDC species tree using the computer package PhyloNet (Than et al., 2008). (ii) We employed the method in an exploratory way, so that we obtained not only the optimal clique (i.e., the MDC tree(s) with the minimum number of extra lineages), but also the sub-optimal cliques with higher numbers of assumed extra lineages (Than and Nakhleh, 2009). In analogy to maximum-parsimony (MP) analyses in gene tree studies, we summarised those sub-optimal clique species-trees by computing a strict consensus tree. In order to express the robustness of clades in the optimal species tree, we calculated equivalents to 'Bremer support' or decay index (Bremer, 1988) values known from MP analyses by successively computing strict consensus trees with one, two, or more steps (i.e., number of extra lineages) longer than the most parsimonious species trees and inferring whether a certain clade was still present in those suboptimal solutions. This was done for a total of 40 suboptimal trees with the number of extra lineages up to six steps longer than the number in the optimal reconstruction. (iii) Since the described procedures assume that the gene trees are correct and that their incongruence is a consequence of incomplete lineage sorting alone, we proceeded to infer the species tree under the MDC method to account also for topological uncertainty in the gene tree reconstructions as follows: we used Phylm (Guindon et al., 2010) to produce 100 bootstrap replicate ML gene trees for each of the four independent region sets (cpDNA, nrDNA ITS, *C16, D35*) using the same evolutionary models as for the Bayesian analyses (see above). We then estimated 100 MDC trees in PhyloNet based on the replicate ML gene trees obtained from the Phyml analyses and finally computed a 50%-majority-rule consensus tree from these MDC trees using PAUP* 4.0.

In a third approach to infer a total-evidence tree based on all regions and accessions, we submitted the complete dataset to the species tree reconstruction and divergence time estimation procedure in the program *BEAST (Heled & Drummond, 2010). The BEAST.xml input files were produced using BEAUti, version 1.8 (Drummond et al., 2012) and comprised 10 different partitions (the sequence information plus the binary coded gap sequences for each of the 5 regions). During the tree search, monophyly was enforced for the Eurasian taxa (all except *Ursinia anthemoides* subsp. *vesicolor* from S Africa). Nucleotide substitution models were chosen as in the Bayesian analyses (see above), but allowed to vary in parameter space around a mean value corresponding to the one given by ModelTest in a normal distribution manner, whereas for the five indel partitions the stochastic Dollo model was employed following Alekseyenko et al. (2008), who argued that this model does not allow back-mutation, being therefore more appropriate to treat indel mutations. A Yule speciation process was chosen as species tree prior, along as the 'piece-wise linear and constant root' model for population size. In order to test whether sequences evolved in a clock-like manner, we ran two independent analyses with BEAST version 1.8 (Drummond et al., 2012) for 5×10^8 generations, sampling every 50,000th generations, and applying in the first analysis a strict-clock model and an uncorrelated log normal relaxed-clock model (Drummond et al., 2006) in the second one. We performed marginal likelihood estimation (MLE) using stepping-stone sampling (SS; Xie et al., 2011; chain length for the MLE = 10^6, number of steps = 100 and alpha = 0.3), for allowing comparison between the two models. Since the uncorrelated log normal relaxed-clock performed better than the strict-clock model (log marginal likelihood: −11721.13

and −11896.88, respectively) an additional analysis was run using the relaxed-clock and the rest of settings as described above. After checking convergence and determining burn-in values in Tracer v1.6, the two independent *BEAST runs were merged using LogCombiner v1.8 (Drummond et al., 2012) and applying a burn-in period of 10% of the total amount of trees sampled. Finally, the remaining 18,000 trees were used to construct a maximum-clade-credibility tree with a posterior probability limit set to 0.5 using TreeAnnotator v1.8 (Drummond et al., 2012).

Two calibration points were used in order to obtain absolute divergence times: The first one was the crown age of the tribe *Anthemideae* (i.e., the age of the node at the split between *Ursinia* and the Euro-Mediterranean clade of the tribe). It was estimated following Oberprieler (2005), using *ndhF* data for the whole family of *Compositae* (Kim and Jansen, 1995) but adding to the dataset the *ndhF* sequence of *Artemisia absintium* L. Re-calibration of these analyses was also necessary because of the fact that a new 'oldest' fossil of the family *Compositae* from North-Western Patagonia suggests an origin of the family in the Early Eocene (50 Ma; Barreda et al., 2010). As a consequence, the age of the tribe *Anthemideae* was estimated to range between 28 Ma and 38 Ma (for more details about the analysis see Appendix B). Owing to these age estimates for the tribe, a normally distributed prior for the time to the most recent common ancestor (*tmrca*) was used for the root age (mean: 33.8 Ma, SD: 3 Ma). The second calibration point was the age of *Artemisia:* The earliest records of *Artemisia* type pollen fossils are from the Lower and Upper Oligocene, in the provinces of Xinjiang and Qinghai, in North-Eastern China (Wang, 2004). This allowed us to set a *tmrca* prior for a subset of taxa including the whole Eurasian grade and Euro-Mediterranean clade of *Anthemideae*, to calibrate the split between the *Artemisiinae* (the subtribe of *Anthemideae* exhibiting the *Artemisia* pollen type) and the accessions belonging to the rest of the subtribes of the Euro-Mediterranean clade included in our analysis. Therefore, we applied a log normal prior for this calibration point with an offset of 23.05 Ma (mean: 2.7, SD: 0.5).

3. Results

Detailed information on the different regions used in the present study is given in Table 2. The nuclear regions are fairly more variable than the two plastid intergenic spacer regions. The most variable region is the low-copy nuclear gene *D35*, which exhibits 159 variable sites (134 of which being parsimony-informative) along its total length of 318 bp. Although being the shorter of the plastid regions, *psbA-trnH* provides a higher number of variable sites and indels.

3.1. Gene trees

The four gene trees with support values obtained both from the BI and the MP analyses are shown in Fig. 1. They are characterised by different degrees of resolution and a considerable amount of topological incongruence among each other. In general, the results of the MP analyses were consistent with those obtained from the BI analyses in all four region sets.

The accessions belonging to subtribe *Leucanthemopsidinae* form a monophyletic group with strong support (PP and BS values) in both the cpDNA and the nrDNA ITS analyses (Fig. 1a and b) where *C. debeauxii* is always found holding the basal position. Conversely, the monophyly of the subtribe is not supported in the trees obtained from the two low copies nuclear regions: In region *D35* (Fig. 1d), the genus *Leucanthemopsis* forms a monophyletic and well-supported group, while the other three annual genera of the subtribe are found as a further monophyletic group with no supported sister-group relationship. In region *C16* (Fig. 1c), *Leucanthemopsidinae* are split into two clades, the first being formed by all cloned sequences from *Castrilanthemum,* while the second comprises *Leucanthemopsis*, *Hymenostemma* and *Prolongoa* accessions. The gene tree based on *C16* is highly unresolved when further relationships among genera are considered, presumably a consequence of the high degree of variation exhibited by this region causing higher levels of homoplasy in the data set (CI = 0.7993, RI = 0.9085).

While in the cpDNA data set, the Iberian genus *Phalacrocarpum* is strongly supported as the sister-group of *Leucanthemopsidinae,* unresolved relationships in the remaining gene trees based on nrDNA ITS, *C16,* and *D35* render this association equivocal. Besides the above mentioned remoteness of *Castrilanthemum* from the other three genera of the subtribe seen in the cpDNA, nrDNA ITS, and *C16* trees, relationships within the subtribe consistently point towards a bipartition within the genus *Leucanthemopsis* with accessions LPS037 and LPS074 of the Central European *L. alpina* on the one and the Iberian species (*L. pallida, L .pectinata, L. pulverulenta*) on the other hand, while the position of *L. alpina* subsp. *tomentosa* (LPS181) from Corsica remains equivocal.

3.2. Total-evidence tree based on concatenated sequences

The analyses based on concatenated sequences resulted in well-resolved trees with strong support from posterior probability and bootstrap values (Fig. 2). The MP analysis yielded six equally-parsimonious trees with a length of 1482 steps and a topology corresponding to the tree found in the BI analysis. The monophyly of the subtribe *Leucanthemopsidinae* is strongly supported, along with *Castrilanthemum* being the basal taxon of the clade. The sister-group relationship of *Phalacrocarpum* with the subtribe is also found with considerable support (PP: 1.0, BS: 89%). Within subtribe *Leucanthemopsidinae,* the monophyly of each of the four genera is well-supported. As in the individual gene trees, the taxa of *Leucanthemopsis* are again grouped into two distinct, well-supported clades, with only *L. alpina* subsp. *tomentosa* (LPS181) remaining unassigned as a consequence of its ambiguous position in the gene trees (cpDNA, nrDNA ITS, and *D35* vs. *C16*).

3.3. Coalescent-based multi-locus tree inference with MDC

The MDC analysis based on four gene trees from three nuclear and two plastid regions produced five equally parsimonious trees. All of them required 54 extra lineages to reconcile the four gene trees used. The strict consensus tree based on the equally parsimonious trees obtained from the MDC analysis is shown in Fig. 3.

Table 2
Characteristics, substitution models, and number of parsimony-informative characters (PI) for each of the molecular markers used.

Marker	Length	Model	Variable sites	PI variable sites	Indels	PI indels
psbA-trnH	493	TVM + G	138	77	49	19
trnC-petN	617	TVM + G	134	69	26	10
nr DNA ITS	495	SYM + G	226	152	40	18
C16	349	HKY + G	139	75	29	11
D35	318	K81uf + I	156	134	31	21

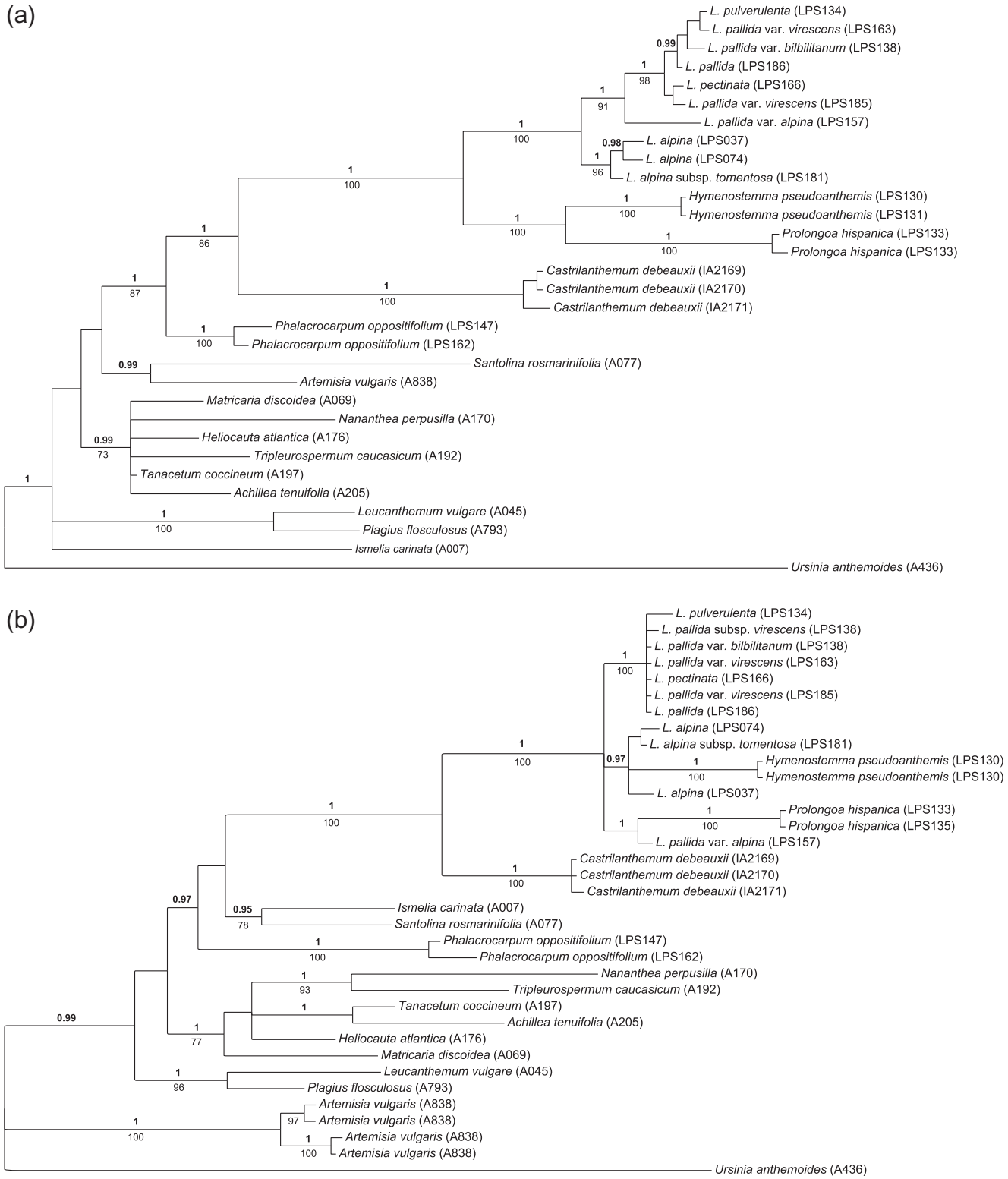

Fig. 1. Half-compat consensus gene trees obtained from the Bayesian analyses (BI) of (a) the concatenated data set of the two plastid intergenic spacer regions *psbA-trnH* and *trnC-petN*, (b) the nrDNA ITS region, (c) the single/low-copy region *C16* (Chapman et al., 2007), and (d) the single/low-copy region *D35* (Chapman et al., 2007). Numbers above branches indicate Bayesian posterior probabilities, while those below the branches refer to the bootstrap support values from the Maximum Parsimony (MP) analyses. Accession codes (see Table 1) are given in brackets in the leaf labels.

Incongruence among these equally parsimonious species/accession trees were only found concerning the relative position among the outgroup taxa from subtribes *Matricariinae* and *Anthemidinae* but not in the ingroup of subtribe *Leucanthemopsidinae*. The bootstrapped analysis produced as expected less resolved results. This is especially pronounced within the genus *Leucanthemopsis*, where the topology of the clade of Iberian representatives is completely unsupported. On the other hand, the monophyly of subtribe

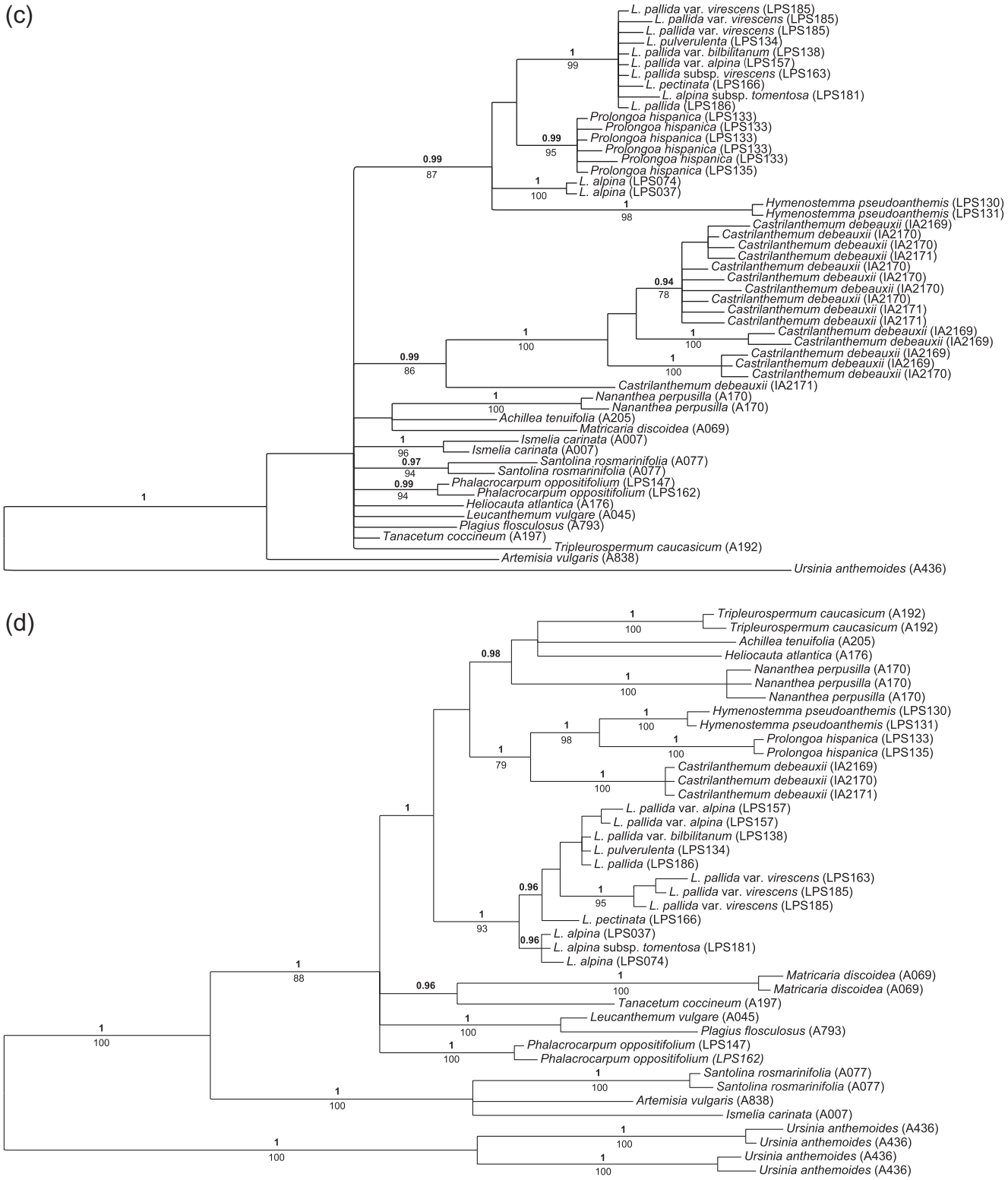

Fig. 1 (*continued*)

Leucanthemopsidinae, the basal position of *Castrilanthemum* within the subtribe, the bipartition of *Leucanthemopsis* species into accessions of *L. alpina* on the one side and the Iberian species on the other receive support from bootstrap and decay index values. The sister-group relationship of *Phalacrocarpum* and *Leucanthemopsidinae* is supported by the bootstrap although it shows a low decay index value.

3.4. Coalescent-based multi-locus tree and chronogram inference with *BEAST

The maximum clade credibility (MCC) tree obtained from the *BEAST analysis is shown in Fig. 4. Besides its topology, which is corresponding in all major aspects to the species tree reconstruction via the MDC method (see above), it provides time estimates

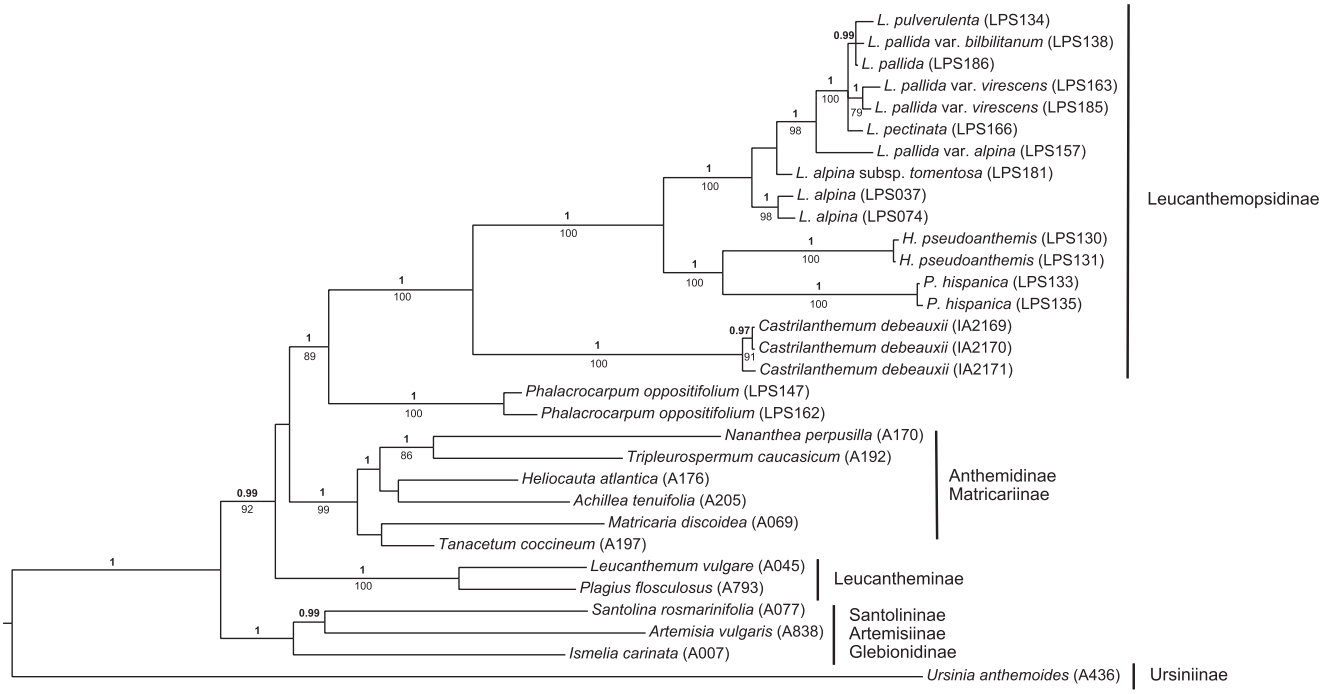

Fig. 2. Total-evidence tree obtained from the Bayesian analysis of the data set with concatenated sequences from all five regions (*psbA-trnH, trnC-petN,* nrDNA ITS, *C16, D35*). Numbers above branches indicate Bayesian posterior probabilities, while those below the branches refer to the bootstrap support values from the Maximum Parsimony (MP) analysis.

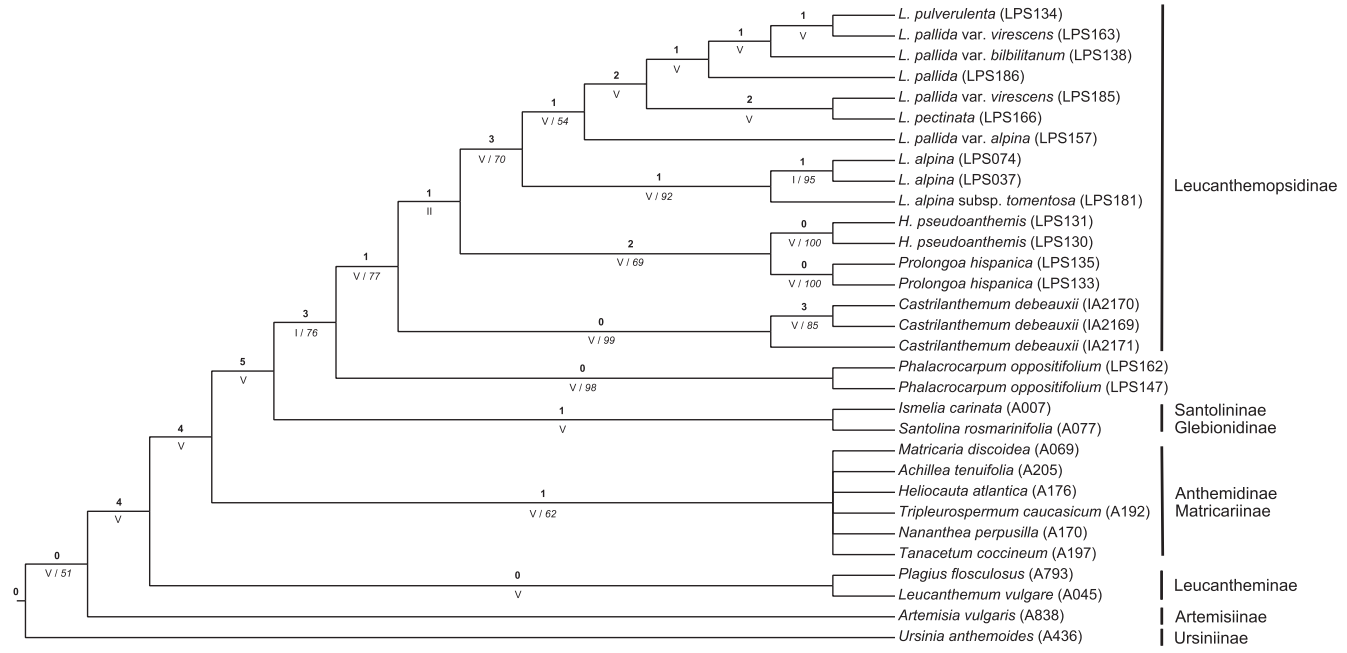

Fig. 3. Strict consensus tree summarising the five MDC species tree inferred using the four gene trees (cpDNA, nrDNA ITS, *C16,* and *D35*). The number of extra lineages is given in bold above each branch. Below the branches the support values obtained from the bootstrap analysis (in italics) and those from the decay index analysis (in roman numbers) are shown. The lowest value for the decay index found (I) is given to clades which are found only in one of the five sub-optimal cliques obtained when running the analyses in an exploratory manner, while the highest values for this index (V) indicates that a clade is found in all the five sub-optimal cliques considered in the exploratory analysis.

for many important nodes in the evolution of the subtribe *Leucanthemopsidinae* (Table 3). Following these reconstructions, *Phalacrocarpum* forms the sister-group of the subtribe and diverged from the common ancestor of *Leucanthemopsidinae* around 20 Ma (16.6–24.1 Ma) ago. The divergence of *Castrilanthemum* is dated to the Early Miocene (13.2–20.8 Ma) while the split between the annuals *Hymenostemma* and *Prolongoa* and the perennial genus *Leucanthemopsis* is dated to around 10 Ma ago. Finally, the speciation processes within *Leucanthemopsis* seem to be all influenced by the glaciation cycles during the Pleistocene, with the crown age of the genus (split between *L. alpina* and the Iberian taxa *L. pallida, L. pectinata,* and *L. pulverulenta*) dated to 4.4 Ma (2.6–6.4 Ma) and the

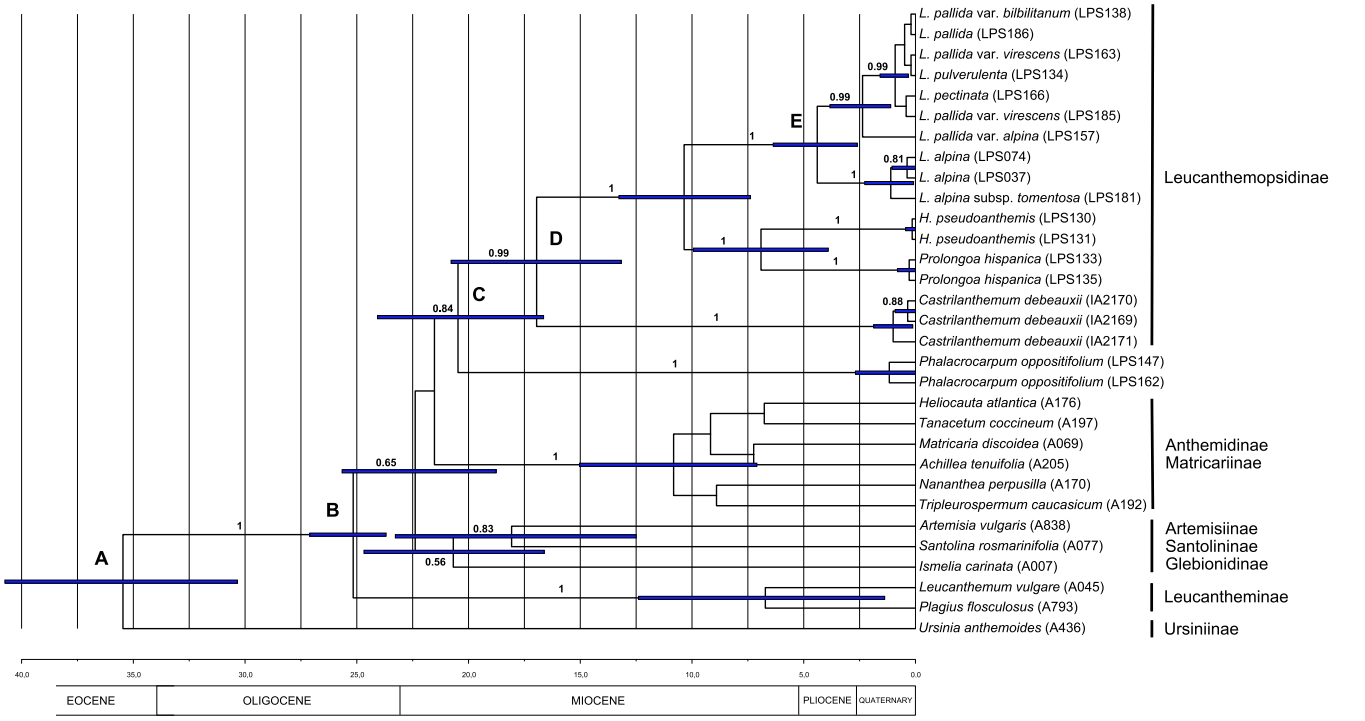

Fig. 4. Dated multi-locus species tree for the subtribe *Leucanthemopsidinae* estimated using ∗BEAST. The chronogram was inferred using sequence data from the five regions (*psbA-trnH, trnC-petN,* nrDNA ITS, *C16, D35*). The error bars indicate the 95% highest posterior density (HPD) intervals for the divergence times estimates. Numbers above branches indicate Bayesian posterior probabilities. Age estimates for the nodes used for calibration (A and B) as well as age estimates for other important branching events in the subtribe *Leucanthemopsidinae* (C–E) are detailed in Table 3.

Table 3
Prior and posterior distributions of age estimates for the calibration points (A and B) and for important nodes (C–E) of the *BEAST chronogram (see Fig. 4).

Node	Description	Prior distribution		Posterior distribution	
		Median age	95% HPD interval	Mean age	95% HPD interval
A	Root age	33.78	28.85–38.72	35.46	30.34–40.45
B	Euro-Asian grade crown age	25.43 (offset = 23.05)	24.1–28.47	25.16	23.69–27.10
C	Phalacrocarpum stem age			20.47	16.64–24.07
D	Leucanthemopsidinae crown age			16.95	13.17–20.78
E	Leucanthemopsis crown age			4.39	2.61–6.37

further speciation processes within these two sub-groups to more recent times.

4. Discussion

The phylogenetic relationships among members of the subtribe *Leucanthemopsidinae* of Compositae–Anthemideae presented in this study are based on DNA sequence information from three nuclear and two plastid regions analysed in both a traditional manner after concatenation of sequences and using multi-species coalescent species tree methods. The latter interpret incongruence among gene trees as the result of incomplete lineage sorting (ILS) which is known to negatively influence the soundness of phylogenetic inference especially in the most recent branches of an organism group (Knowles and Kubatko, 2010). Despite some incongruence among the four underlying gene trees (three for the nuclear regions, one for the jointly analysed plastid regions), however, we observe a (nearly) complete correspondence between the phylogenetic reconstruction based on a sequence concatenation on the one hand and the two methods of coalescent-based species tree reconstruction (minimising deep coalescences, MDC; Bayesian species tree reconstruction, *BEAST) on the other hand. We think that this result indicates that the often-disturbing effects of incomplete lineage sorting observed in comparable studies (e.g., Sanchez-Garcia and Castresana, 2012) are minimal in the present study group and/or region set. Possible explanations for this lack of dramatic consequences of incomplete lineage sorting in the study group may be due to the small effective population sizes of the mostly narrowly distributed species of *Leucanthemopsidinae* in conjunction with long branches of species reaching back from between 15 and 25 Ma in the cases of *Castrilanthemum* and *Phalacrocarpum,* and the prevailing of short generation times in the subtribe, with only *Leucanthemopsis* exhibiting perennial life-forms. As a consequence, the most distinctive differences between the concatenated and the coalescent-based analyses are found concerning the relationships among the taxa of *Leucanthemopsis* (e.g., the position of *L. alpina* subsp. *tomentosa*), where species are more widespread, the generation times of all species are longer, and their radiation into the present diploid taxa was presumably caused by allopatric differentiation processes not earlier than during the Pleistocene.

In this respect, the concatenated analysis exhibits the higher degree of resolution and shows highly supported groups even in the clade of the Iberian representatives of *Leucanthemopsis,* while in the results obtained from the two species-tree reconstruction approaches this is not the case. We consider this observation being

a further example for the general trend described by Weisrock et al. (2012) that in the presence of incomplete lineage sorting concatenated analyses could produce well-resolved and highly supported, but untrustworthy clades. Concerning the relationships among taxa within *Leucanthemopsis*, it seems clear that incomplete lineage sorting has played a major role and that more comprehensive studies are needed to shed light on the reticulate evolution of the genus, surely influenced further by polyploidy and (possibly) homoploid hybridisation.

Irrespective of the two reconstruction strategies (concatenated sequences vs. species tree reconstruction methods) or sub-strategies [species tree reconstruction based on a fast maximum parsimony method (MDC) vs. a more time-consuming model-based Bayesian inference method (*BEAST)] the subtribe *Leucanthemopsidinae* is found to form a monophyletic group with high statistical support. While this is in accordance with previous studies solely based on nrDNA ITS and cpDNA trnL/trnF intergenic spacer sequences (Oberprieler and Vogt, 2000; Oberprieler, 2005; Oberprieler et al., 2007), the phylogenetic relationships among the four genera of the subtribe found in the present, more comprehensive analysis are deviating from these older reconstructions: the two unispecific genera *Hymenostemma* and *Prolongoa* form a well-supported monophyletic group being itself sister to *Leucanthemopsis* in the present reconstructions while the previous ones pointed towards a sister-group relationship between *Prolongoa* and *Leucanthemopsis.* However, since our present analyses are based on more regions, a more representative sampling of taxa (all diploid species of *Leucanthemopsis,* more accessions of the three monotypic genera), and more sophisticated reconstruction methods, we consider the relationships among the four genera of *Leucanthemopsidinae* as depicted in Figs. 2–4 more trustworthy. Despite its strongly supported monophyly in the molecular phylogenetic analyses, the subtribe is less well-defined in morphological and anatomical respects: while the three core-genera *Leucanthemopsis, Hymenostemma,* and *Prolongoa* according to a cladistic analysis by Bremer and Humphries (1993) share the presumed synapomorphies of a reduced number of achene ribs and the joint possession of a scarious adaxial achene corona, the fruits of *Castrilanthemum* with its ten ribs and its lack of an apical corona (Vogt and Oberprieler, 1996) changed the circumscription of the subtribe considerably. Because the closely related genera *Hymenostemma* and *Prolongoa* are also quite different in fruit morphological and anatomical respect (with 5–7 equally sized ribs in *Hymenostemma* and two large and three small ribs in *Prolongoa,* Oberprieler et al., 2006), it is only the annual life-form that is shared between these two genera, which contrasts with the perennial life-form realised in *Leucanthemopsis,* and that may be considered as a synapomorphy of the two (but see discussion of life-form evolution below).

In contrast to previous nrDNA-based phylogenetic studies of the Compositae–Anthemideae (Oberprieler, 2005; Oberprieler et al., 2007) our present results point towards a sister-group relationship between subtribe *Leucanthemopsidinae* and the hitherto unclassified genus *Phalacrocarpum,* represented here by one of its two species endemic to the Iberian Peninsula. While some morphological features support this interpretation (*Phalacrocarpum* achenes are 7–9-ribbed as in *Hymenostemma* and *Leucanthemopsis* and apically rounded as in *Castrilanthemum*), others like the opposite leaf arrangement and the lack of myxogenic cells on the fruit walls (Oberprieler et al., 2006) set *Phalacrocarpum* aside from the *Leucanthemopsidinae*. However, the strong support from the plastid sequence data (Fig. 1a) together with the lack of hard incongruence between the cpDNA topology and each of the gene trees based on the three nuclear regions and along with the observation that the basal-most leaves of *Castrilanthemum* are also arranged in opposite pairs (Oberprieler et al., 2006) corroborate this formerly discussed (Vogt and Oberprieler, 1996) but never re-evaluated hypothesis of a closer phylogenetic relationship between *Phalacrocarpum* and the Leucanthemopsidinae.

A further discrepancy between our present analyses and those previous ones based on nrDNA ITS sequences (Oberprieler, 2005; Oberprieler et al., 2007) concerns the temporal diversification of the *Leucanthemopsidinae.* While the split between *Castrilanthemum* and the other three genera was dated to the Late Miocene (6–7 Ma) in Oberprieler (2005), this split was shifted towards the Early Miocene (13.2–20.8 Ma) in our present multi-locus *BEAST reconstruction (Fig. 4) and renders *Castrilanthemum* to be an extremely old unispecific lineage. This temporal discrepancy is considerable, but we think that our present reconstructions are more trustworthy because the former estimate has been based on a single region (nrDNA ITS), which is considered quite problematic for phylogenetic reconstructions due to its nature of being a multi-copy region, potentially comprising multiple paralogous copies that show signs of concerted evolution (Álvarez and Wendel, 2003). Additionally, the former reconstruction (Oberprieler, 2005) used a less sophisticated molecular dating method (non-parametric rate smoothing, NPRS, Sanderson, 1997) with only a single calibration point at the base of the tree (the crown age of the tribe Anthemideae as being 21 Ma) and no internal ones. Finally, our present dating was now based on a newly determined and higher crown age of Anthemideae (27–42 Ma; see Appendix B) as a consequence of a recently discovered fossil of Compositae from NW Patagonia, which suggests the origin of the family to date back to the Early Eocene (50 Ma; Barreda et al., 2010) as compared to the hitherto alleged maximum age of 35–42 Ma of the family (Graham, 1996) used in Oberprieler (2005).

While the Mediterranean region has experienced a trend towards aridification in the Late Miocene between 12 and 7 Ma (Ivanov et al., 2002; Fortelius et al., 2006; Van Dam, 2006), the stabilization of a truly Mediterranean climate with summer droughts that may have triggered the switch towards annuality as an efficient adaptation was observed not earlier than in the Pliocene at 5–3 Ma (Suc, 1984; Bertoldi et al., 1989; Thompson, 2005). It is therefore evident that the divergence of *Castrilanthemum* from the closest lineages predates significantly the establishment of the Mediterranean climate in Europe as well as the salinity crisis occurred during the Messinian (5.96–5.33 Ma; Fauquette et al., 2006; Krijgsman et al., 1999). Instead it coincides nicely with the uplift of the Prebaetic System, comprising today's Sierra de Guillimona and Sierra de Castril, occurred during the Middle Miocene, approximately 16 Ma (Sanz de Galeano, 1990; Braga et al., 2003). Since the Prebaetic chain emerged as a island system between the water bodies formed by the Guadalquivir depression on the one side and the 'Infra Mountains basins' on the other (Vera, 2000), the split between the *Castrilanthemum* lineage and its sister-lineage giving rise to the three other genera of the Leucanthemopsidinae could have been the consequence of an allopatric or peripatric speciation process.

When *Phalacrocarpum* is considered to be the sister-group to *Leucanthemopsidinae,* two equally parsimonious scenarios emerge concerning the evolution of life form in the study group: either we have to assume that a primarily perennial life form evolved into annuality in the most recent common ancestor of the tribe (17–20.5 Ma) and reversed to perennial in the stem species leading to *Leucanthemopsis* (4.5–10.5 Ma), or that an annual life form evolved independently in *Castrilanthemum* (1–17 Ma) and in the ancestor of *Hymenostemma* and *Prolongoa* (7–10.5 Ma). While it has been demonstrated in other plant groups (e.g., Orobanchaceae subtribe Castillejinae; Tank and Olmstead, 2008) that a perennial life-form may evolve form an annual one, the palaeoclimatological evidences on the settlement of a Mediterranean climate in Southern Europe discussed above, are considered to add more weight to the latter scenario (parallel gain of annuality during the Late

Miocene and/or Pliocene) than to the former one (evolution of an annual life form in the Early Miocene) and support an interpretation of *Leucanthemopsidinae* evolution with a permanently perennial stock of mountain-dwelling *(Leucanthemopsis*-like*)* species as a backbone that shifted towards annuality in at least two independent lineages *(Castrilanthemum, Hymenostemma/Prolongoa*).

Following Gould (2002), living fossils are species 'belonging to ancient lineages from which most species are now extinct, and which have undergone relatively little evolutionary change' (Wright et al., 2012). While our present analyses demonstrate that the criterion of taxonomical independence or low taxonomic diversity along with a great antiquity of the lineage is certainly true for *Castrilanthemum debeauxii,* the proof of a long-lasting morphological and eco-physiological conservatism is hard to show when fossil evidences are missing, as it is the case in this small and herbaceous representative of Compositae–Anthemideae. As reasoned above, however, it appears reproducible to assume that the shift from an originally perennial to the annual life form of *Castrilanthemum* might have been happened along the long branch leading to its present-day representative *C. debeauxii,* presumably not longer than 3 Ma ago. As a consequence, in respect of this important life-history trait, the modern representative of this lineage might be deviating from the stem species of this branch and, therefore, may not be in accordance with morphological conservatism required for its perception as being a 'living fossil'. Nevertheless, the evolutionary distinctiveness and the scarcity of this rare species definitively prioritise it and its habitat for conservation efforts (Rosauer et al., 2009; Cadotte and Davies, 2010).

Acknowledgments

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Appendix A. Supplementary material

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