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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.

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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 & Oberp., 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/trnT 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 (Smitsen et al., 2011; Brennan et al., 2012; Guo et al., 2012; Gruenstaedl 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 pseudoanthesis*, 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

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