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Mountains and refuges: Genetic structure and evolutionary history in closely related, endemic *Centaurea* in continental Greece ☆

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ABSTRACT

Mountains of continental Greece are one of the main Mediterranean biodiversity hotspots, very rich in endemic species. The speciation in this area might have resulted from two main factors: a complex orography and its role as a refugium during past glaciations. We have investigated genetic diversity and population structure for a group of narrow endemics of *Centaurea* subsect. *Phalolepis*, with three main goals: to investigate population structure of these narrow endemics, to check whether patterns of genetic variation are in agreement with recognized species boundaries, and to get insights into the process of diversification within this group. Fifteen populations belonging to seven species were genotyped using cpDNA (*rpl32-trnL* region) sequences and nuclear microsatellites (eight loci). SSR were used to assess genetic variability, to analyse molecular variance, to identify genetic barriers, to estimate recent and historical gene flow, and to carry out a model-based Bayesian clustering. Analysis of cpDNA was used to construct a haplotype network. Despite being narrow endemics, all the studied species show moderate to high SSR genetic diversity. Genetic isolation of populations is very high, with no current gene flow among them. Patterns of genetic structure indicate that there are more genetic clusters than there are currently recognized taxa. Genetic data suggest that isolation in mountain ranges and subsequent allopatric speciation would be the main driver of diversification in the group; the refugial nature of the mountains of continental Greece has allowed the maintenance of high within-population genetic diversity.

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1. Introduction

The Mediterranean Basin with its 25,000 species, including 13,000 endemic species, is the third richest global biodiversity hotspot containing some 4.3% of the world's plants (Myers et al., 2000); of these, 60% are specifically narrow endemics (Thompson et al., 2005). One of the subregions of the Mediterranean that shows high levels of endemism is Greece, with 1278 endemic species, which corresponds to 22.2% of the country's total native plants (Dimopoulos et al., 2013).

The mainland of Greece is a mountainous region and specifically central and western Greece is divided by many irregular and interrupted mountain ranges (Strid, 1986). In this area, two separate Middle Pleistocene glaciations have been identified, one dating to before the last interglacial period, i.e. prior to ca. 127,000 cal. years BP, and a more extensive one that occurred over 350,000 years ago (Hughes et al., 2006). Climatic fluctuations resulted in extensive changes in the vegetation (Tzedakis et al., 2006). Conceivably, the combination of climatic–ecological fluctuations during the Quaternary and rugged terrain provided intense selection, whereby the expansion of some species and their subsequent isolation are believed to have resulted in the evolution of many narrow endemics. As a result, endemics in continental Greece mostly occur at moderate to high altitudes (above 600 m; Georghiou and Delipetrou, 2010), a natural consequence of the role of mountains as centres of speciation (Tzedakis et al., 2002).

One of the genera that shows a high degree of species richness and endemism in Greece is *Centaurea* with 76 endemic species out of a total of 141 native taxa. Many of the endemic taxa are range-restricted (Dimopoulos et al., 2013). As currently delimited (e.g. Susanna and Garcia-Jacas, 2007), *Centaurea* has a circum-Mediterranean distribution, especially the section *Centaurea* (Hilpold et al., 2014a,b). The large section *Centaurea* is usually divided into subsects. *Centaurea*, *Phalolepis* and *Willkommia* (Hilpold et al., 2014a). To date, molecular analyses of sect. *Centaurea* have not succeeded in offering a well-resolved

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phylogeny: sect. *Centaurea* is monophyletic, but subsectional classification, which is clear on morphological grounds, is completely blurred (*Centaurea* group in Hilpold et al., 2014b) and subsects. *Centaurea* and *Phalolepis* were clustered together. However, morphological differences between both groups are evident and Hilpold et al. (2014a) proposed to treat them as subsections.

Our study focuses on subsect. *Phalolepis*, which is mostly composed of species with restricted distribution area, with the only exception of two taxa with large distributional ranges such as *Centaurea alba* L. from the Iberian Peninsula and *Centaurea deusta* Ten. from the Italian and the Balkan Peninsulas (Matthäs, 1981). The latter region, and in particular Greece, constitutes one of the centers of diversity for subsect. *Phalolepis* along with the Anatolian, Crimean, Italian and Iberian Peninsulas (based on number of species). In Greece there are 22 species of *Centaurea* subsect. *Phalolepis* of which 16 are endemic (Gamal-Eldin and Wagenitz, 1991; Georgiadis et al., 1996; Dimopoulos et al., 2013). Many narrow Greek or Balkan endemics [e.g. *Centaurea albanica* Halácsy, *C. brunnea* (Halácsy) Halácsy, *C. formanekii* Halácsy, *C. heldreichii* Halácsy, *C. princeps* Boiss. & Heldr. and *C. subciliaris* Boiss. & Heldr.] have, in the past, been subordinated to species having a wider distribution, namely, either *C. alba* or *C. deusta* (Dostál, 1976), whereas other authors (e.g. Kalpoutzakis and Constantinidis, 2004) consider them to be independent taxa. Half of the species endemic to Greece are regarded as rare and/or threatened, and they are included either in the *Red Data Book of Rare and Threatened Plants of Greece* (Phitos et al., 2009) or in the *IUCN Red List of Threatened Species* (IUCN, 2013).

The large number of threatened narrow endemics of subsect. *Phalolepis* found in the mountain ranges of contintental Greece makes the group a good subject for studies focused on population genetics and conservation. In addition, by analyzing their genetic structure we can get insights on the diversification process and evolutionary history on the mountain flora of the eastern Mediterranean Basin. Allopatric speciation has been proposed as one of the main (if not the main) modes of speciation for plants in the Mediterranean Basin, especially on its eastern side (Verlaque et al., 1997). The role of allopatry in the diversification of *Centaurea* has already been emphasized, e.g. within sect. *Centaurea* (Hilpold et al., 2011). The narrow range of most of the Greek endemic taxa of subsect. *Phalolepis* suggests that they may have arisen as a consequence of allopatry. Another factor, however, that has emerged as one of the causes of speciation in *Centaurea* is introgression (Suárez-Santiago et al., 2007; Garcia-Jacas et al., 2009). Molecular markers show in many cases the consequences of introgression in the form of multiple copies in the nuclear markers, even in diploid species, because hybridization is very often homoploid (Garcia-Jacas et al., 2009; Mameli et al., 2014) and difficults phylogenetic reconstructions.

We selected a highly variable type of marker, microsatellites (simple sequence repeats or SSRs) for our study. These markers allow straightforward analyses of genetic structure given their codominant inheritance, reproducibility, and very high level of variability. Previous studies in *Centaurea* using microsatellites have cleared out the population structure of narrow endemics (Fréville et al., 2001; Mameli et al., 2008; Marrs et al., 2008a,b; Guarino et al., 2013).

As the subject of our study we chose three narrow Greek endemics, namely *C. chrysocephala* Phitos & T. Georgiadis, *C. messenicolasiana* T. Georgiadis, Dimitrellos & Routsi, and *C. princeps* from Central Greece, all of them diploid, which constitute a homogeneous group on morphological grounds (Georgiadis et al., 1996). We added some other narrow diploid endemics of subsect. *Phalolepis* that are closely related to the former group: *Centaurea brunnea*, *C. heldreichii*, *C. litochorea* T. Georgiadis & Phitos, and one widespread species (*C. deusta*) to which some of the aforementioned species were once assigned (Fig. 1). Some of the microspecies were, in fact, subordinated to *C. alba*, but this species grows only in the western Mediterranean (Iberian Peninsula and southern France), and the Italian and Balkan populations referred to *C. alba* are, in fact, *C. deusta* (López and Devesa, 2011). Since one of the goals of the study would be verifying the independence of the narrow endemics in front of the widespread species, we included only the populations of *C. deusta* that are geographically closer to central Greece.

Our specific goals are: (i) to investigate population structure by estimating genetic diversity within and between narrow endemics; (ii) to check whether there is any correlation between morphological differentiation and genetic diversity of the closely related *C. chrysocephala*, *C. messenicolasiana* and *C. princeps*; (iii) to check whether patterns of genetic variation correspond to recognized species boundaries; and (iv) to get insights into the process of diversification and speciation. Given that most of the selected species are threatened (see below), genetic data obtained from this study may be helpful when designing plans for their management and recovery.

2. Material and methods

2.1. Plant material

The studied material comprises three closely related Greek endemic *Centaurea* species (*C. chrysocephala*, *C. messenicolasiana* and *C. princeps*) plus three other narrow endemic species (*C. brunnea*, *C. heldreichii* and *C. litochorea*), totalling 13 populations, and two populations of the more widespread *C. deusta*, all of which are assigned to subsect. *Phalolepis*.

Centaurea chrysocephala, a biennial or perennial plant, was known from three sites in central Greece, i.e. around the monasteries of Meteora, close to Klinovo village, and close to Pertouli village (Georgiadis et al., 1996). Another site was discovered during this study near the village of Kastania. The populations of the species are all small, probably comprising not more than 150–300 mature plants per site. It prefers rocky places and cliffs but can also be found in stony places, road margins and walls, on limestone, sandstone, serpentine and conglomerate substrates. It has been classified as R (Rare) by Phitos et al. (1995).

Centaurea messenicolasiana is a recently described and a very narrow perennial species, found in a small area of the foothills of Mount Agrapha, north-east of Tavropos artificial lake (central Greece), at an altitude of 500–800 m. It forms small groups of 10–60 individuals in an area measuring ca. 4 km^2, mostly in openings and margins of deciduous forests, on flysch. The species has been listed as VU (Vulnerable) by Phitos et al. (2009).

Centaurea princeps is a local endemic known only from Mount Timfristos in central Greece, with two populations comprising 200–300 individuals. Its extent of occurrence is 9 km^2. It is a biennial taxon growing on limestone cliffs and screes (at altitudes of 1100–1850 m) and is taxonomically related to *C. chrysocephala* and *C. messenicolasiana* (Georgiadis et al., 1996). Listed as VU (Vulnerable) by Phitos et al. (2009), it too has recently been upgraded to EN (Endangered; IUCN, 2013).

This group of three species is remarkable by their whitish florets and the involucre appendages lacking a hyaline margin. *Centaurea chrysocephala* differs from *C. princeps* by its narrower involucre (10–15 mm vs. 17–22 mm), smaller, distinctly convex appendages which are uniformly straw-colored (with a brownish central part in *C. princeps*) and slightly longer mucro (2–3 mm vs. 1–2 mm). *Centaurea messenicolasiana* has even narrower involucre (8–11 mm), pearl-white instead of straw-colored appendages on shorter involucral bracts, and lacks auricles at the base of the appendages.

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