

SPECIATION

Evolution of the central Mediterranean *Centaurea cineraria* group (Asteraceae): Evidence for relatively recent, allopatric diversification following transoceanic seed dispersal

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Abstract We explored the spatiotemporal diversification of the *Centaurea cineraria* group based on AFLP fingerprints and plastid DNA sequences applied to a broad sampling of Central Mediterranean taxa of the Acrolophus subgroup. Despite its morphological distinctness, monophyly of the *C. cineraria* group was not supported by our data. A distinct lineage mostly restricted to Sicily (the Sicily group) comprised some members of the *C. cineraria* group but also included *C. parlatoris*, assumed to be a member of the *C. dissecta* group. The relationships of the Sicily group with other members of the Acrolophus subgroup could not be clarified. Molecular dating supported recent, presumably allopatric diversification whose onset dates back to less than 250,000 years within the Sicily group. Tunisia, the Aeolian Islands and the Island Ventotene in the central Tyrrhenian Sea were likely colonised from Sicily. The young age of diversification within the Sicily group rejects land bridges between Africa and Sicily, which were only available during significantly older periods, in favour of transoceanic seed dispersal. Dispersal might have been favoured by low sea levels during cold stages of the Pleistocene. The molecular data indicate that taxonomy of the *C. cineraria* group needs to be revised.

Keywords AFLP; biogeography; Mediterranean; phylogeography; Sicily; transoceanic dispersal

■ INTRODUCTION

The Mediterranean is one of the global centres of biodiversity (Myers & al., 2000) owing to a complex geographic and climatic history (Thompson, 2005). Moreover, the Mediterranean Basin is situated at the crossroads of three continents and has served as meeting ground and eventually melting pot for a variety of lineages (Blondel & Aronson, 1999). A plethora of diversification processes resulted in a highly “reticulate” biogeographical history (Sanmartín, 2003). The overall richness of about 25,000 plant species (Quézel, 1985) is to a large extent due to the presence of a high number of locally or regionally endemic taxa (Greuter, 1991; Médail & Diadema, 2009). Islands and mountain ranges are known for their richness (Médail & Quézel, 1997), with mountainous islands exhibiting the highest rates of species diversity and endemism. One such example is Sicily: with 2,700 plant species it is the island with the greatest species richness in the Mediterranean (Raimondo & al., 1994; Médail & Quézel, 1997; Giardina & al., 2007) and is considered one of ten “hot spots” of biodiversity within the Mediterranean (Médail & Quézel, 1997). The percentage of endemics among vascular plants ranges from 10% to 20% in the mountainous interior part of the island (Médail & Quézel, 1997; Conti & al., 2005). In addition to complex history (see below), a precondition for biotic diversity is a highly diverse landscape. Coastal regions of the island harbour evergreen, thermo-mediterranean vegetation whereas the top of Mt. Etna, Europe’s highest active volcano, is

snow-covered for most of the year. In between, a wide range of natural and anthropogenic habitats on both volcanic and calcareous substrate can be observed. This natural diversity offered an outstanding playground for evolutionary processes during an eventful geographical and geological history.

Sicily existed as an island through most of the Miocene (23.0–5.3 Ma [million years]; Meulenkeamp & Sissingh, 2003; Goes & al., 2004), and was in tight contact with Calabria (S. Italy), Corsica, and Sardinia especially during late stages of this epoch (Speranza & al., 2002; Goes & al., 2004). Consequently, Sicily has probably had an important function as migration route and stepping stone between Africa and Europe and between the eastern and the western Mediterranean (shown for *Centaurea* subg. *Acrocentron*; Font & al., 2009). The connection with Corsica and Sardinia is evidenced, for example, in Araceae (Mansion & al., 2008). A direct connection with the African continent certainly existed during the Messinian Salinity Crisis (MSC, 5.96–5.33 Ma), when the Mediterranean desiccated due to the closure of the Strait of Gibraltar (Hsü & al., 1972; Butler & al., 1999; Krijgsman & al., 1999). With the end of the MSC, Sicily became an island again (Blanc, 2002; Garcia-Castellanos & al., 2009). The Strait of Sicily functioned as barrier between Sicily and Africa, whereas the Strait of Messina and the Isthmus of Catanzaro were barriers between Sicily and the Italian mainland (Bonfiglio & al., 2002). This fact, in combination with the subsequent onset of the Mediterranean climate around 3.2–2.8 Ma (Suc, 1984;

Thompson, 2005), may have been crucial for the development of a specific Sicilian flora. The Pleistocene climatic oscillations led to strong sea level fluctuations (Rohling & al., 1998). During warm periods, the island was much more isolated than during cold periods, when the sea level was 120–150 m below the recent level (Rohling & al., 1998; Lambeck & Bard, 2000; Lambeck & al., 2004). The distance to Africa was only 50 km during the Last Glacial Maximum (Thiede, 1978; Stöck & al., 2008), i.e., less than half of today's distance. The Strait of Messina was almost or entirely closed and various small islands around Sicily were connected to the main island (Lambeck & al., 2004). However, there is no evidence for a land bridge to Africa after the MSC.

The islands to the north of Sicily originated from volcanic eruptions during the last one million years. The larger Aeolian Islands in the Southern Tyrrhenian Sea were formed between 430,000 and 120,000 years BP (Barca & Ventura, 1991; Crisci & al., 1991; Astis & al., 1997), and the rise of Ventotene and Ponza situated in the northern Tyrrhenian Sea happened more or less at the same time (Bellucci & al., 1999). The distance of the Aeolian Islands to Sicily is small suggesting that their colonisation may have taken place mostly from Sicily. The islands in the northern Tyrrhenian Sea, however, are close to the Italian mainland rendering colonisation from there more probable.

Despite its biotic richness and the high number of endemics, Sicily has been largely neglected in phylogeographic studies. In contrast, disjunctions across the Strait of Gibraltar have been examined in detail (e.g., Martínez-Solano & al., 2004; Veith & al., 2004; Busack & Lawson, 2008; Cano-Maqueda & al., 2008; Ortiz & al., 2009). Most phylogeographic studies concerning Sicily and the adjacent areas affect a broader region, such as the entire central or western Mediterranean, and are primarily phylogenetic (e.g., Mansion & al., 2008). Whereas the paramount role of the MSC for plant migrations was emphasised for Holm Oak (*Quercus ilex* L., Lumaret & al., 2002), other studies demonstrated post-Messinian diversifications (*Matthiola* W.T. Aiton: Sánchez & al., 2005; *Jacobaea* Mill.: Passalacqua & al., 2008; *Anthemis* L.: Lo Presti & Oberprieler, 2009).

A good model to explore biogeographic relationships among Sicily, Northern Africa, the Apennine Peninsula and the islands of the Tyrrhenian Sea is provided by the relatives of *Centaurea cineraria*, perennial herbs with tomentose leaves and purple florets. They belong to the morphologically defined *C.* sect. *Acrolophus* (Candolle, 1838), which was combined with *C.* sect. *Phalolepis* and sect. *Willkommia* to create the molecularly defined, monophyletic “Acrolophus subgroup” of the “*Jacea* group” (García-Jacas & al., 2006; Suárez-Santiago & al., 2007b; Hilpold & al., 2009). The *Jacea* group was suggested to have originated in the late Miocene in the eastern Mediterranean (Suárez-Santiago & al., 2007b). The classification of the Acrolophus subgroup is not sufficiently resolved as extensive hybridisation has likely played a key role in its evolution (Suárez-Santiago & al., 2007b). The *C. cineraria* group (Pignatti, 1982; Cela-Renzoni & Viegi, 1982; Raimondo & Bancheva, 2004; Raimondo & al., 2004) comprises ten species, three of which are further divided into subspecies

(*C. cineraria*, *C. aeolica*, *C. panormitana*). Four species occur in Sicily (*C. busambarensis*, *C. erycina*, *C. panormitana*, *C. saccensis*), three grow in adjacent areas (*C. aeolica* on islands of the southern and central Tyrrhenian Sea, *C. cineraria* on the southwestern coast of the Apennine Peninsula and *C. papposa* in Tunisia), two more around the northern Tyrrhenian Sea (*C. gymnocarpa* and *C. veneris*) and *C. leucadea* at the southeastern tip of Italy (Fig. 1). To date, it is unclear if the latter three species belong to the *C. cineraria* group. It is noteworthy that *C. aeolica* is a rare example of a species which inhabits the two different volcanic island archipelagos of the Tyrrhenian Sea; subsp. *aeolica* occurs on the Aeolian Islands while subsp. *pandataria* occurs on Ventotene offshore Naples. All members of the *C. cineraria* group inhabit rocky places, predominantly coastal cliffs. Sicily is the only area where species occur in mountain areas (*C. busambarensis* and *C. erycina*) and *C. saccensis* is restricted to an inland gorge. The preferred bedrock is mostly calcareous; an exception is *C. aeolica* from volcanic islands. All species except *C. gymnocarpa* possess achenes with a pappus, but the achene is glabrous, fairly heavy and the pappus is short. Consequently, gravity appears to be the only mechanism of seed dispersal (Bancheva, unpub.). In *C. corymbosa* Pourr., a close relative of the *C. cineraria* group (Hilpold & al., 2009), the average seed dispersal distance was only 32 cm (Colas & al., 1997; Hardy & al., 2004). Pollination is mainly carried out by insects (Cela-Renzoni & Viegi, 1982) and reproduction is mostly sexual, but facultative apospory was reported in *C. cineraria*, *C. gymnocarpa* and *C. panormitana* (Cela-Renzoni & Viegi, 1982). The chromosome number is $2n = 18$ (Cela-Renzoni & Viegi, 1982; Raimondo & Bancheva, 2004), only a single tetraploid individual was ever counted (Damboldt & Matthäs, 1975).

Although several molecular studies were conducted in the Acrolophus subgroup (Ochsmann, 2000; García-Jacas & al., 2006; Suárez-Santiago & al., 2007a,b), the *C. cineraria* group has been fairly neglected with only a single isoenzyme study available (Bancheva & al., 2006). Here, we apply complementary molecular markers, i.e., maternally inherited (Zhang & al., 2003), slowly evolving plastid DNA sequences and biparentally inherited, rapidly evolving (Bussell & al., 2005) AFLPs to a dense population sampling throughout the distribution area of all taxa of the *C. cineraria* group plus an exhaustive sample of potential outgroups. Our aim is to answer the following questions. (1) Does the *C. cineraria* group constitute a natural, monophyletic entity and which species of the Acrolophus subgroup in the Central Mediterranean are the next relatives? (2) If the *C. cineraria* group is monophyletic, at what time did it diversify? (3) Furthermore, we test the two hypotheses for the origin of the disjunction across the strait of Sicily, i.e., vicariance due to the fragmentation of a land-bridge established during the Messinian Salinity Crisis or transoceanic seed dispersal. (4) In addition, we aim to elucidate the phylogenetic relationships of the two subspecies of *C. aeolica* from geographically distant volcanic islands in the Tyrrhenian Sea in order to trace their colonisation history. Finally (5), we point out shortcomings of the presently used taxonomic framework.

■ MATERIALS AND METHODS

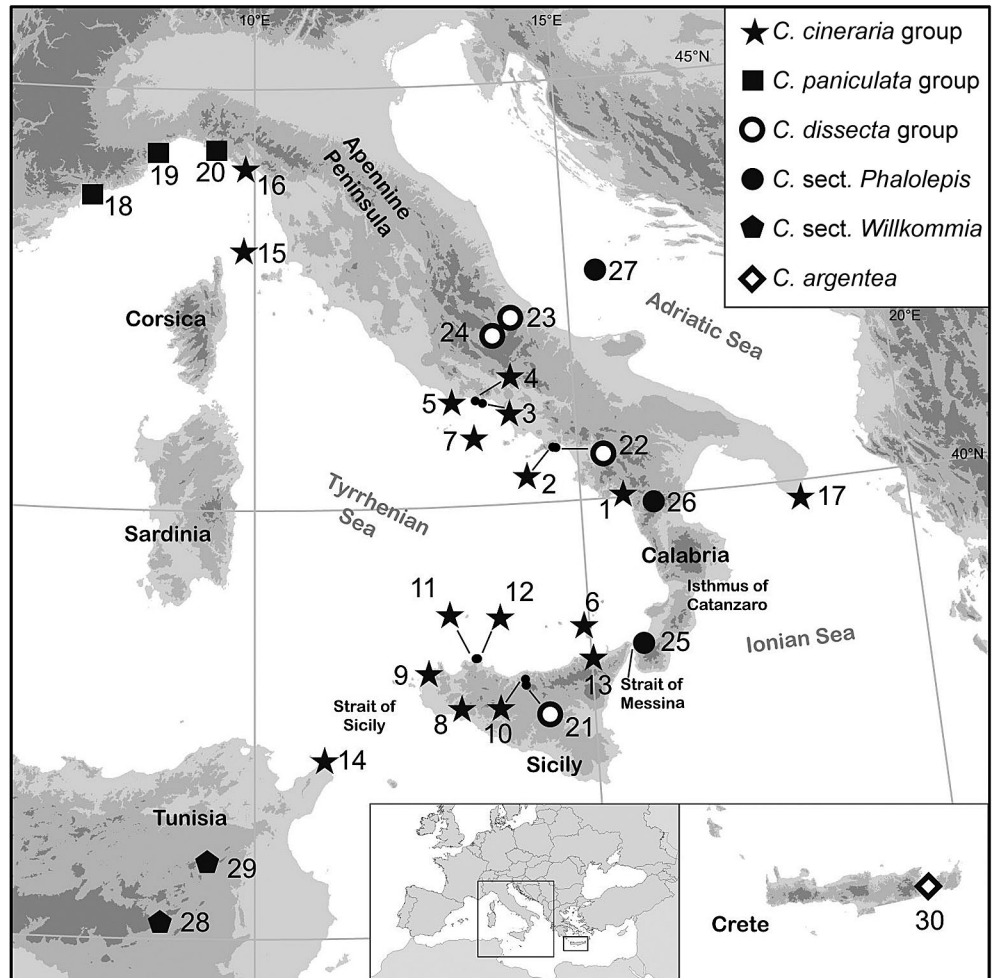
In the summer of 2008, 30 sample sites (subsequently referred to as “populations”) of *Centaurea* sect. *Acrolophus*, sect. *Phalolepis* and sect. *Willkommia* were visited in the field (Fig. 1; Table 1). In addition to all taxa of the *C. cineraria* group, we also included southern Italian members of the two other groups constituting sect. *Acrolophus*, i.e., the *C. paniculata* and *C. dissecta* groups (Pignatti, 1982) as well as *C. argentea*, a member of sect. *Acrolophus* from Crete. *Centaurea subtilis* Bertol. was not included, as it belongs to the *Jacea-Lepteranthus* clade (Hilpold & al., 2009) rather than to the *C. cineraria* group. Voucher specimens were determined based on Pottier-Alapetite (1981), Pignatti (1982), Breitwieser & Podlech (1986), Turland & Chilton (2000), Arrigoni (2003), Raimondo & Bancheva (2004) and Raimondo & al. (2004). Nomenclature follows Greuter (2008) and Peruzzi (2008). Leaf material was collected and immediately stored in silica gel. Voucher specimens are deposited at the herbarium of the Institut Botànic de Barcelona (BC).

Total genomic DNA was extracted from silica gel-dried tissue (ca. 10 mg) of eight to ten plants from each population (only two plants in population 13). Extraction followed the

CTAB-protocol of Doyle & Doyle (1987) with the modifications of Tel-Zur & al. (1999), including three washing steps with sorbitol buffer and a few further modifications: after precipitation with isopropanol and subsequent centrifugation, the DNA pellet was washed in 70% ethanol, dried at 37°C and re-suspended in TE-buffer. The quality of the extracted DNA was checked on 0.7% TBE agarose gels.

The AFLP procedure followed Vos & al. (1995) with the modifications described in Schönswetter & al. (2009). Initially, selective primers were screened using 24 selective primer combinations. The two final primer combinations for the selective PCR (fluorescent dye in brackets) were *EcoRI* (6-Fam)-ACA/*MseI*-CAC and *EcoRI* (NED)-AGC/*MseI*-CTG. Five microlitres of each selective PCR product were combined and purified using Sephadex G-50 Superfine (GE Healthcare Bio-Sciences, Uppsala, Sweden) applied to a Multi Screen-HV plate (Millipore, Molsheim, France). One microlitre of the elution product was mixed with 10 µl formamide (Applied Biosystems, Foster City, California, U.S.A.) and 0.1 µl GeneScan 500 ROX (Applied Biosystems) and run on an ABI 3130x automated capillary sequencer. Nineteen individuals were used as replicates to calculate the error rate and to exclude non-reproducible fragments from the analysis. Raw AFLP data were aligned with

Fig. 1. Sampled populations of *Centaurea* in the central Mediterranean and in Crete. Details of the collected populations are given in Table 1. Symbols reflect the taxonomic framework of Pignatti (1982) and Breitwieser & Podlech (1986).



the internal size standard using ABI Prism GeneScan v.3.7.1 (Applied Biosystems), and imported into Genographer v.1.6.0 (available at <http://hordeum.oscs.montana.edu/genographer>) for scoring. The error rate (Bonin & al., 2004) was calculated as the ratio of mismatches (scoring of 0 vs. 1) over matches (1 vs. 1) in the AFLP profiles of replicated individuals.

A Neighbour-joining (NJ) analysis based on a Nei-Li distance matrix (Nei & Li, 1979) was conducted and bootstrapped (2000 pseudo-replicates) with TreeCon 1.3b (Van de Peer & De Wachter, 1997). Population mixture analysis implemented in BAPS v.5.2 (Corander & al., 2004) was used to infer the genetic structure of the Sicily group (see Results for a definition). This software can handle dominant markers like AFLPs under the module “clustering with linked loci” (Corander & Tang, 2007). Mixture analysis was conducted with the maximal number of groups (K) set to 2 to 10. Each run was replicated ten times and the results were averaged according to the resultant likelihood scores. Admixture coefficients were estimated using 500 iterations, and the significance of these coefficients was estimated by employing the simulation strategy described by Corander & Marttinen (2006) using 50 reference individuals and 10 iterations each. An Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree among clusters was inferred based on Nei distances.

Out of several plastid DNA markers screened, only the *rpl32-trnL* intergenic spacer (primers *rpl32-F*, *trnL* (UAG); Shaw & al., 2007) showed a sufficient degree of variation, while other markers were either entirely invariable or exhibited only very few variable sites. This result is in accordance with the survey of Shaw & al. (2007), who identified the *rpl32-trnL* intergenic spacer as the most variable plastid DNA region out several tested. It was sequenced from five to six individuals per population from the Sicily group and the geographically distant *C. argentea*. Reactions were performed in 25 µl volumes with 2.5 µl 10× AmpliTaq buffer, 2.5 µl 25 mM MgCl₂, 2.5 µl 2 mM dNTPs mix, 1 µl of each primer at 5 µM, 2.5 µl of 400 ng/µl BSA (bovine serum albumine; New England Biolabs, Ipswich, Massachusetts, U.S.A.), 1 U AmpliTaq DNA polymerase (Applied Biosystems) and 2 µl of template DNA of unknown concentration. PCR was conducted using the following conditions: 3 min at 95°C, followed by 35 cycles of 95°C for 40 s, 54°C for 40 s and 72°C for 1 min 40 s, followed by 10 min at 72°C. The PCR product was purified with ExoSAP-IT (USB Corp., Cleveland, Ohio, U.S.A.). Direct sequencing of the amplified DNA segments was performed using BigDye Terminator Cycle Sequencing v.3.1 (Applied Biosystems), following the manufacturer’s protocol at the University of Florida ICBR Core Facility on an ABI 3730xl capillary sequencer (Applied Biosystems). Nucleotide sequences were edited using BioEdit v.7.0.5.3 (Hall, 1999) and were aligned manually.

A statistical parsimony haplotype network was constructed using TCS v.1.21 (Clement & al., 2000). For the latter analysis, insertions/deletions longer than one base pair were re-coded as single base pair mutations, and sequence gaps were treated as a fifth character state.

Molecular dating of the onset of plastid DNA diversification within the Sicily group was conducted using BEAST v.1.5.3

(Drummond & Rambaut, 2007). The best substitution model had an Akaike weight (determined with Modeltest v.3.6; Posada & Crandall, 1998) of 0.31 and 17 models (out of 56 tested) were included until the cumulative Akaike weight exceeded 0.95. The included models ranged from having only three (F81) up to eight free parameters, and for the final analysis we used HKY + Gamma which is a medium complex model with five free parameters, using six rate categories instead of the default four. Given the low level of sequence variation within the Sicily group (see Results), we used a strict clock model with a prior on the substitution rate modelled as a lognormal distribution and a model of constant population size as demographic model. Setting a rate of 4.5×10^{-3} substitutions per site and per million years and a deliberately wide standard deviation of 3×10^{-2} resulted in the mode of the prior distribution at 4.0×10^{-3} substitutions per site and per million years in accordance with published substitution rates for plastid markers (Yamane & al., 2003; Smith & al., 2008). After initial analyses, the root of the tree was constrained to be maximally 5 million years old. The Markov chain was run three times for 5×10^7 generations each, sampling every 1000th generation. After removing the first 10% of sample points as burn-in, parameter estimates and their 95% highest posterior density intervals (HPD) were obtained from 135,000 generations.

■ RESULTS

We scored 476 AFLP fragments ranging from 100 to 550 base pairs. The error rate amounted to 4.7% and was thus within the range deemed acceptable by Bonin & al. (2004). Neighbour-joining analysis (Fig. 2) linked all individuals of each population to separate clusters with bootstrap support (BS) between 94% and 100%. Exceptions were the two geographically close populations of *C. panormitana* (populations 11 and 12) as well as *C. delicatula* (population 28). One individual of *C. busambarensis* (population 10) grouped with low support (BS 51%) with *C. parlatoris* (population 21). *Centaurea argentea* (population 30) was separated along the longest branch in an unrooted NJ analysis and was therefore used to root the tree. Only a few deeper nodes had BS > 50%. A branch formed by *C. leucadea*, *C. deusta* and *C. scillae* (populations 17, 26, 25, respectively) was sister to all other populations with BS 60%. Within this latter group three members of the *C. paniculata* group s.str. and *C. veneris* constituted a branch with BS 81%. The best supported larger group within the tree consisted of nine populations of the *C. cineraria* group from Sicily, the Tyrrhenian Islands and Tunisia and *C. parlatoris* from Sicily. The group received BS 93% and is referred to as the Sicily group. The Sicily group was split into two smaller groups with low support, the first consisting of the two subspecies of *C. aeolica* from the Tyrrhenian Islands and the Tunisian *C. papposa*, the second consisting of all populations from Sicily.

Mixture analysis of the Sicily group identified eight clusters that consisted mainly of single populations or several populations of the same taxon, with the exception of a cluster joining *C. parlatoris* (population 21), *C. panormitana* subsp.

seguenzae (population 13) and one individual of *C. busambarensis* (population 10). The UPGMA tree among the clusters identified two main groups (Fig. 3A). The first was distributed from Tunisia over Sicily to the islands of the central and northern Tyrrhenian and comprised two subgroups, constituted by *C. saccensis* (population 8) plus the Tunisian *C. papposa* (population 14), and by *C. aeolica* subsp. *aeolica* (population 6) plus subsp. *pandataria* (population 7). The second group was restricted to Sicily and fell into a northwestern (populations 9, 11, 12) and an eastern subgroup (populations 10, 21, and 13).

The subsequent admixture analysis revealed admixture in a single individual from population 13 and is presented on a geographical basis in Fig. 3C.

The *rpl32-trnL* sequences of members of the Sicily group were 843–867 bp long and yielded six haplotypes altogether (Tables 1–2; Fig. 3B). Haplotype IV occurred in six populations (Fig. 3C): *C. busambarensis* (population 10), *C. parlatoris* (population 21), both subspecies of *C. aeolica* (populations 6 and 7), *C. erycina* (population 9) and *C. papposa* (population 14). The haplotypes of the remaining populations differed only in a

Table 1. Population number (Pop.), taxon, collecting locality and collection number, coordinates, number of individuals investigated with AFLPs, cpDNA haplotype for members of the Sicily group (for a definition, see text) and GenBank accession numbers of 30 sampled populations of ...

	Pop.	Taxon
<i>C. cineraria</i> group	1	<i>Centaurea cineraria</i> L. subsp. <i>cineraria</i>
	2	<i>Centaurea cineraria</i> L. subsp. <i>cineraria</i>
	3	<i>Centaurea cineraria</i> L. subsp. <i>cineraria</i>
	4	<i>Centaurea cineraria</i> L. subsp. <i>cineraria</i>
	5	<i>Centaurea cineraria</i> L. subsp. <i>circae</i> (Sommier) Cela Renz. & Viegi
	6	<i>Centaurea aeolica</i> Lojac. subsp. <i>aeolica</i>
	7	<i>Centaurea aeolica</i> Lojac. subsp. <i>pandataria</i> (Fiori & Bég.) Anzal.
	8	<i>Centaurea saccensis</i> Raimondo & al.
	9	<i>Centaurea erycina</i> Raimondo & Bancheva
	10	<i>Centaurea busambarensis</i> Guss.
	11	<i>Centaurea panormitana</i> Lojac. s.l.
	12	<i>Centaurea panormitana</i> Lojac. s.l.
	13	<i>Centaurea panormitana</i> Lojac. subsp. <i>seguenzae</i> (Lacaita) Greuter
	14	<i>Centaurea papposa</i> (Coss.) Greuter
	15	<i>Centaurea gymnocarpa</i> Moris & De Not.
	16	<i>Centaurea veneris</i> (Sommier) Bég.
	17	<i>Centaurea leucadea</i> Lacaita
<i>C. paniculata</i> group	18	<i>Centaurea leucophaea</i> Jord. subsp. <i>controversa</i> (Briq. & Cavill.) Kerguélen
	19	<i>Centaurea aplolepa</i> Moretti subsp. <i>aplolepa</i>
	20	<i>Centaurea aplolepa</i> Moretti subsp. <i>levantina</i> (Arrigoni) Greuter
<i>C. dissecta</i> group	21	<i>Centaurea parlatoris</i> Heldr.
	22	<i>Centaurea lacaitae</i> Peruzzi
	23	<i>Centaurea ambigua</i> Guss.
	24	<i>Centaurea scannensis</i> Anzal. & al.
<i>Centaurea</i> sect. <i>Phalolepis</i>	25	<i>Centaurea scillae</i> Brullo
	26	<i>Centaurea deusta</i> Ten.
	27	<i>Centaurea diomedeae</i> Gasp.
<i>Centaurea</i> sect. <i>Willkommia</i>	28	<i>Centaurea delicatula</i> Breitw. & Podlech
	29	<i>Centaurea delicatula</i> Breitw. & Podlech
<i>Centaurea</i> sect. <i>Acrolophus</i> (Incertae sedis)	30	<i>Centaurea argentea</i> L. subsp. <i>argentea</i>

few mutational steps from haplotype IV. Haplotypes from populations 2 and 5 of the outgroup *C. cineraria* were also linked directly to this haplotype. *Centaurea argentea* (population 30) is separated from haplotype IV by five mutational steps.

Using a model of constant population size, the onset of diversification within the Sicily group was inferred to be (given as mean/median) 0.248/0.207 Ma (95% highest posterior density interval 0.576–0.018 Ma). The use of a more complex demographic model (extended Bayesian Skyline Plot) resulted in slightly younger age estimates (not shown).

DISCUSSION

Despite the clear morphological diagnostic features of the *Centaurea cineraria* group (Pignatti, 1982; Cela-Renzoni & Viegi, 1982; Raimondo & Bancheva, 2004; Raimondo & al., 2004), our AFLP data suggest that even though most populations are strongly reciprocally divergent, little higher-level structure is evident. Monophyly of the *C. cineraria* group in the traditional circumscription is not supported. Only a mostly geographically defined group centred on Sicily, but also including

... *Centaurea* from the central Mediterranean and Crete. All vouchers are deposited in the herbarium BC. Collections numbers are those of Vilatersana (V) or Galbany-Casals (G).

Locality and collection number	Coordinates (E, N)	N AFLP	Haplotype	GenBank accession
Italy, Potenza: Acquafredda, <i>V-1120</i>	15°39'53", 40°02'21"	8	–	
Italy, Salerno: Minori, <i>V-1118</i>	14°37'48", 40°38'55"	10	–	
Italy, Latina: Gaeta, <i>V-1112</i>	13°34'00", 41°12'30"	10	–	
Italy, Latina: Torre Capoverde, <i>V-1107</i>	13°27'45", 41°14'30"	10	–	
Italy, Latina: S. Felice Circeo, <i>V-1104</i>	13°05'22", 41°13'38"	10	–	
Italy, Messina: Lipari, <i>V-1181</i>	14°57'44", 38°30'17"	10	IV (6)	HQ441794–HQ441799
Italy, Latina: Isola di Ventotene, <i>V-1113</i>	13°24'55", 40°47'13"	9	IV (6)	HQ441800–HQ441805
Italy, Agrigento: Lago Arancio, <i>V-1159</i>	13°03'39", 37°37'35"	10	I (6)	HQ441776–HQ441781
Italy, Trapani: Erice, <i>V-1160bis</i>	12°35'17", 38°02'10"	10	IV (6)	HQ441782–HQ441787
Italy, Palermo: Isnello, <i>V-1144</i>	14°00'08", 37°56'45"	10	IV (4), V (1), VI (1)	HQ441765–HQ441770
Italy, Palermo: Sferracavallo, <i>V-1166</i>	13°17'24", 38°12'42"	10	II (6)	HQ441752–HQ441757
Italy, Palermo: Mondello, <i>V-1167</i>	13°18'44", 38°12'55"	9	II (5)	HQ441758–HQ441762
Italy, Messina: Tindari, <i>V-1173</i>	15°02'27", 38°08'40"	2	III (2)	HQ441763, HQ441764
Tunisia, Nabeul: Cap Bon, <i>V-1384</i>	11°01'41.9", 37°04'22.3"	9	IV (6)	HQ441788–HQ441793
Italy, Livorno: Isola di Capraia, <i>V-1248</i>	09°50'35", 43°03'00"	10	–	
Italy, La Spezia: Palmaria, <i>V-1255</i>	09°51'04", 44°02'33"	10	–	
Italy, Lecce: S. Maria di Leuca, <i>V-1205</i>	18°22'22", 39°47'57"	10	–	
France, Alpes-Maritimes: Eze, <i>V-1274</i>	07°21'02", 43°42'59"	10	–	
Italy, Savona: Spotorno, <i>V-1265</i>	08°24'02", 44°13'46"	10	–	
Italy, Genova: Sestri Levante, <i>V-1262</i>	09°23'19", 44°16'11"	10	–	
Italy, Palermo: Piano Battaglia, <i>V-1150</i>	14°00'45", 37°52'24"	8	IV (4), V (1)	HQ441771–HQ441775
Italy, Salerno: Capo d'Orso, <i>V-1116</i>	14°40'42", 40°38'10"	9	–	
Italy, Pescara: Eremo S. Spirito, <i>V-1223</i>	14°04'20", 42°10'30"	8	–	
Italy, L'Aquila: Gole del Sagittario, <i>V-1225</i>	13°47'59", 41°59'30"	9	–	
Italy, Reggio Calabria: Bagnara, <i>V-1140</i>	15°47'07", 38°16'28"	10	–	
Italy, Potenza: Prastio, <i>V-1131</i>	16°08'13", 39°56'15"	10	–	
Italy, Foggia: San Domino, <i>V-1211</i>	15°29'42", 42°42'13"	8	–	
Tunisia, Siliana: Jebel Seri, <i>V-1346</i>	09°23'27.7", 35°51'24.4"	9	–	
Tunisia, Kasserine: Jebel ech Chambi, <i>V-1355</i>	08°39'53.9", 35°12'09.2"	9	–	
Greece, Crete: Kavousi, <i>G-2008</i>	25°49'12.2", 35°06'17.4"	8	–	HQ441806

C. papposa from North Africa, *C. aeolica* from the Tyrrhenian Islands and the morphologically clearly distinct *C. parlatoris* from Sicily form a supported monophyletic entity termed here the “Sicily group”. The next relatives of the Sicily group cannot be determined due to insufficient resolution (Fig. 2). Our AFLP data provide no direct evidence that hybridisation, previously suggested to be a driving force in the diversification of *Centaurea*, is the main cause for the lack of coherence of the *C. cineraria* group. If at all, hybridisation had already occurred in early stages of diversification.

Highly fragmented distribution patterns as exemplified by the *C. cineraria* group were considered indicative for old, “relic” groups that failed to re-gain a wider distribution after restriction to the warmest available habitats such as coastal cliffs during cold stages of the Pleistocene (Ferrarini, 1971; Cela-Renzoni & Viegi, 1982). In contrast, plastid DNA data generated for the Sicily group strongly discourage an old age but instead suggest a fairly recent diversification taking place less than 250,000 years ago. As suggested by the AFLP data (Fig. 2) albeit without bootstrap support, the closest extant relatives of the Sicily group may be sought for in northern populations of *C. cineraria* subsp. *cineraria*. In any event, the ancestors of the Sicily group have most probably evolved on the Italian mainland and subsequently dispersed to Sicily. As southernmost Italy is predominated by granitic bedrock, an unsuitable substrate for members of the *C. cineraria* group, long-distance seed dispersal across the Tyrrhenian Sea appears more probable than the significantly shorter crossing of the Strait of Messina.

Diversification within Sicily and further dispersal into circumjacent areas (North Africa, Tyrrhenian Islands) was accompanied by genetic divergence in probably small and isolated populations as well as by rapid morphological diversification. Differentiation of populations may have been fostered by natural habitat fragmentation. Sandy beaches and river estuaries have probably acted as barriers for members of the Sicily group, which are restricted to rock faces close to the sea. Despite the fact that their achenes lack adaptations for long-distance dispersal (Bancheva, unpub.), members of the Sicily group were obviously able to expand their distribution areas. We found the same plastid DNA haplotype in populations from

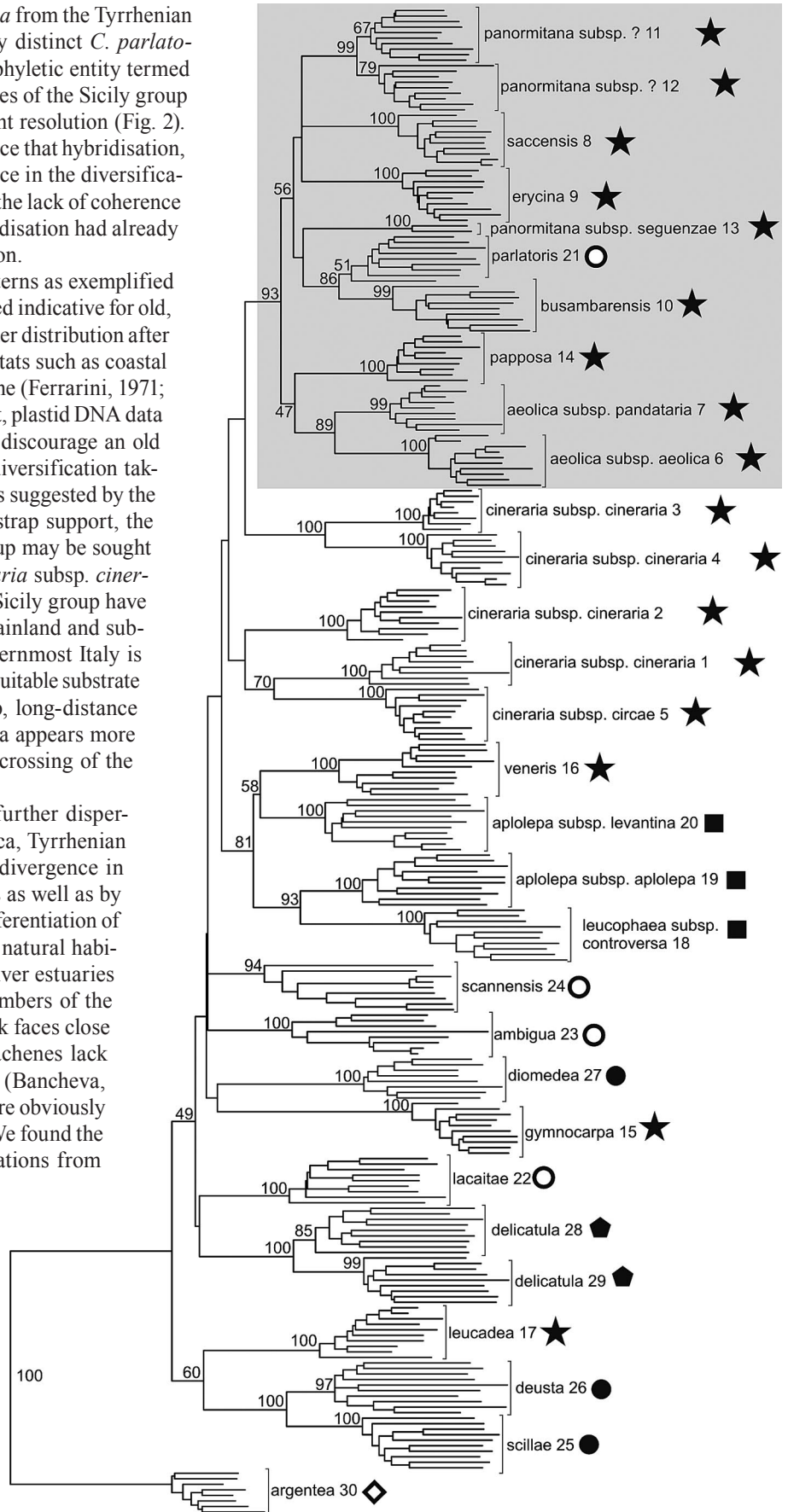


Fig. 2. Neighbour-joining tree of AFLP data of 30 populations of *Centaurea* in the central Mediterranean and in Crete. Numbers along branches are bootstrap support values above 45%. As in Fig. 1, symbols reflect the taxonomic framework of Pignatti (1982) and Breitwieser & Podlech (1986). The Sicily group (for circumscription see text) is shaded in grey.

North Africa on the one hand and Sicily and the Tyrrhenian Islands on the other hand (Fig. 3C). As the earliest diversification within the Sicily group clearly post-dates the MSC when a land bridge connected Sicily and Africa, vicariance can be outright rejected as cause for the disjunction in favour of transoceanic seed dispersal. The dispersal from Sicily to northern Africa likely occurred during cold periods of the Pleistocene when the distance between Sicily and Tunisia was much smaller (Thiede, 1978; Stöck & al., 2008), and may have been easier leaped. Similarly, the disjunction seen in *C. aeolica* between Lipari and Ventotene over a distance of about 250 km is best explained by long-distance seed dispersal. The relatively recent emergence of these islands during the last one million years fits well with our dating of the group's diversification. Colonisation likely occurred directly from the Aeolian Islands to Ventotene and Ischia, where the species was reported in the 19th century (Gussone cit. in Pignatti, 1982), but appears to be extinct now. *Centaurea aeolica* grows on volcanic soil, but the rocks along the coast south of Naples are almost exclusively calcareous or granitic, rendering dispersal across the mainland difficult. Seed dispersal probably occurred during cold periods with lower sea level and may have involved a stepping stone, the volcanic Palinuro seamount that nowadays lies below sea level, but was a large, flat island during cold periods (Kenyon & al., 2003; Siani & al., 2004). Similar disjunction patterns between

the volcanic area of Naples and the nearby Tyrrhenian Island Ventotene on the one hand and the Aeolian Islands on the other hand are rarely observed; further examples are provided by *Bassia saxicola* (Guss.) A.J. Scott and *Heliotropium suaveolens* M. Bieb. subsp. *bocconeii* (Guss.) Brummitt (Pignatti, 1982).

Fig. 3. AFLP and plastid DNA variation in populations of the Sicily group of *Centaurea* from Sicily, Tunisia and the Tyrrhenian (for definition, see text). **A**, Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree based on Nei distances among gene pools derived by Bayesian clustering with BAPS. **B**, Statistical parsimony network of plastid DNA haplotypes. A mutational step not sampled is given as small black dot. The asterisk shows the connection with haplotypes sampled in the outgroups *Centaurea cineraria* and *C. argentea*. **C**, Geographical distribution of plastid DNA haplotypes derived from *rpl32-trnL* sequences (indicated with roman numerals) and of eight gene pools as defined by Bayesian clustering (indicated in colour).

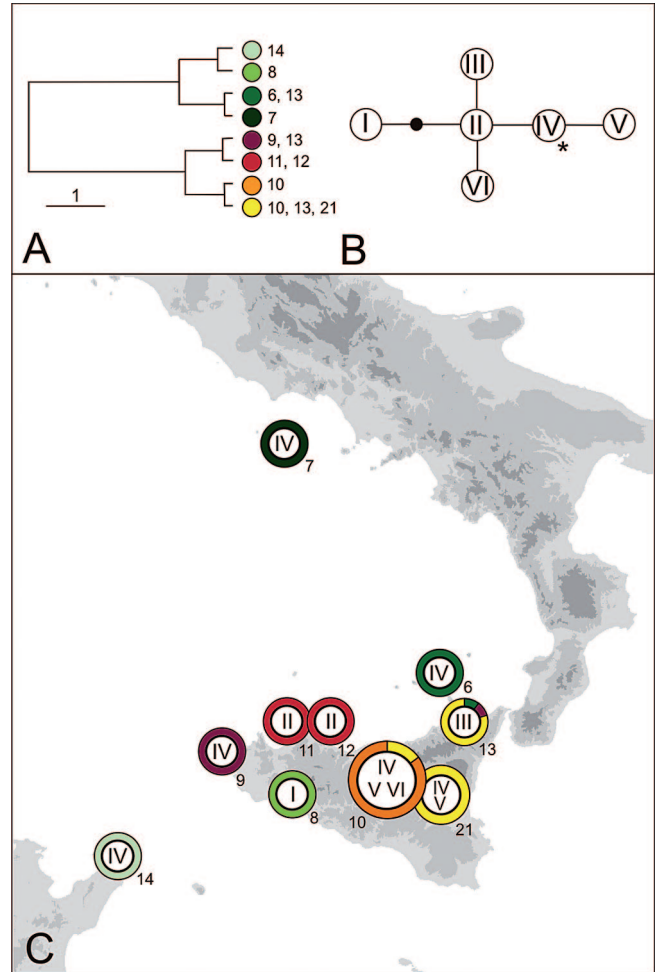


Table 2. List of haplotypes in the studied populations of the Sicily group and in *Centaurea argentea* with indication of nucleotide site variation within the chloroplast region *rpl32-trnL*.

Haplotype	2	3	4	523–534	6	770–773	Taxon (population number)
Haplotype I	A	T	G	CTATAAGGATTT	G	T A GAAA	<i>Centaurea saccensis</i> (8)
Haplotype II	A	T	G	CTATAAGGATTT	G	G A GAAA	<i>Centaurea panormitana</i> (11, 12)
Haplotype III	A	G	G	CTATAAGGATTT	G	G A GAAA	<i>Centaurea panormitana</i> subsp. <i>sequenzae</i> (13)
Haplotype IV	A	T	G	CTATAAGGATTT	G	G A GAAA	<i>Centaurea aeolica</i> subsp. <i>aeolica</i> (6), <i>C. aeolica</i> subsp. <i>pandataria</i> (7), <i>C. busambarensis</i> (10), <i>C. papposa</i> (14), <i>C. parlatoris</i> (21)
Haplotype V	A	T	G	CTATAAGGATTT	G	G A –	<i>Centaurea parlatoris</i> (21)
Haplotype VI	A	T	G	CTATAAGGATTT	G	G A GAAA	<i>Centaurea busambarensis</i> (10)
Haplotype <i>C. argentea</i>	G	T	A	CTTCTTAATTATC	T	G C GAAA	<i>Centaurea argentea</i> (30)

It is known that AFLPs are no marker of choice for resolving relationships at deep nodes as the frequency of non-homologous fragments being scored as one AFLP band increases in parallel with the phylogenetic distance. The very limited plastid DNA variation, however, suggests that diversification within the group is recent and we therefore do not expect that our AFLP dataset contains much homoplasy. The molecular data suggest that the current taxonomic framework needs to be significantly revised. As taxonomic ranks should be applied in a comparable way throughout the entire genus once phylogenetic relationships are better understood and because a detailed morphometric analysis of the *C. cineraria* group is underway (Guarino, in prep.), we here refrain from taking taxonomic decisions but rather point out shortcomings of the presently used classification. (1) First of all, the lack of supported hierarchical structure in the AFLP dataset does neither support the division of the *Acrolophus* subgroup into sects. *Acrolophus*, *Phalolepis* and *Willkommia* (Candolle, 1838; Blanca, 1981) nor the division into informal groups as proposed by Pignatti (1982). It has been suggested that hybridisation and introgression within this group confound reconstruction of sectional boundaries (Suárez-Santiago & al., 2007b) but our data provide no evidence for this hypothesis. (2) It was previously shown (Schönswetter & al., 2009) that in groups that exhibit geographically isolated, fairly small populations and were suggested to have undergone relatively recent, synchronous, allopatric differentiation, geographically close populations tend to be related independent of their taxonomic assignment, the latter often being based on a single or very few characters only. As our data strongly suggest recent diversification in the *C. cineraria* group and because populations are often small, allowing for rapid fixation of probably parallelly evolving characters, we expect that entirely morphology-based classifications such as those by Dostál (1976) and Pignatti (1982) are suited for practical purposes but do not reflect a natural system. This is well illustrated by the morphologically divergent *C. parlatoris* that was previously assigned to the *C. dissecta* group. It is not only deeply nested in the Sicily group but is also genetically only weakly differentiated from its next relative, *C. busambarensis* (Fig. 2), growing in geographic proximity. (3) Taxonomic ranks used for members of the Sicily group and for *C. cineraria* do not reflect observed patterns of genetic divergence. For example, the four investigated populations of *C. cineraria* subsp. *cineraria* are strongly reciprocally divergent and do not even form a monophyletic group; their genetic differentiation is at least as strong as that among the constituents of the Sicily group classified at specific level (Fig. 2). It is unclear if this pattern is exclusively caused by genetic drift in small, geographically isolated populations or if facultative apospory previously reported in *C. cineraria* (Cela-Renzoni & Viegi, 1982) also plays a role. (4) Of the remaining three species previously included in the *C. cineraria* group, *C. veneris* from the northern Tyrrhenian is more closely related to the *C. paniculata* group (Fig. 2). *Centaurea gymnocarpa* and *C. leucadea*, in contrast, clearly fall outside both groups. *Centaurea leucadea* groups with weak support with two members of sect. *Phalolepis* (*C. deusta*, *C. scillae*). In consequence, similarities of *C. gymnocarpa*, *C. leucadea* and *C. veneris* to

C. cineraria might be caused by convergence or by gene flow between these groups. The later hypothesis is supported by the presence of morphologically intermediate individuals in some populations not included in this study (A. Hilpold, pers. obs.). (5) As a minor point, the subspecies of *C. panormitana* are highly doubtful. On Mt. Pellegrino, where all three subspecies should occur, neither morphological differentiation was possible (A. Hilpold, pers. obs.) nor did the AFLP data suggest any differentiation. In contrast, subsp. *sequenzae* is morphologically separated by prolonged bract appendages and is clearly not the next relative of the other subspecies (Fig. 2).

In conclusion, our study shows that diversification in groups that – based on their highly disjunct distribution – were assumed to be old relics may be surprisingly recent. A further example for a recent, homoploid diversification in the Mediterranean is provided by the genus *Nigella* L. in the Aegean (Bittkau & Comes, 2008). Moreover, it becomes more and more apparent that disregarding the “flat tail of the dispersal curve” might lead to erroneous conclusions regarding the evolutionary significance of long-distance seed dispersal. As illustrated by the *C. cineraria* group, this concerns not only species with diaspores that are morphologically adapted to dispersal, but also those which are lacking such adaptations. As a consequence, we could reconstruct a large number of biogeographic processes, i.e., various allopatric-vicariant divergences in Sicily as well as transoceanic dispersal across the Strait of Sicily and between the geographically distant but edaphically similar volcanic islands Lipari and Ventotene in the Tyrrhenian Sea.

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