



Allopolyploidisation in a geological collision zone: on the origin of the tetraploid *Anthemis cupaniana* Nyman (Compositae, Anthemideae) in Sicily

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Abstract

The genus *Anthemis* has a circum-Mediterranean distribution and comprises c. 175 annual, biennial, and perennial species with polyploid species and species complexes found in its section *A. sect. Hiorthia*. In Sicily, the genus is represented by 13 species, one of these being the tetraploid *A. cupaniana*, which is endemic to the island and is distributed throughout the limestone mountains at elevations between 500 and 1800 m a.s.l. Discordant positions in phylogenetic trees based on two plastid regions (*psbA-trnH* and *trnC-petN*) and on one nuclear marker (nrDNA ITS1 + 5.8S + ITS2) reveal that the species is of allopolyploid origin, with the maternal parent from the species group around *A. cretica* and the paternal one from a monophyletic group comprising mostly N African representatives around *A. pedunculata*. Owing to the fact that the latter group also comprises the Sicilian perennial and diploid *A. ismelia* and that *A. cretica* has been found with diploid populations in the C Sicilian Madonie Mts, these two species are considered being the most probable parental diploids of *A. cupaniana*. The close relationship of *A. ismelia* with the N African *A. pedunculata* group supports the biogeographical interpretation of *A. cupaniana* being the result of a ‘collision’ between diploids from the southern (African) and northern (Eurasian) platforms after dispersal along a Pleistocene corridor in the Sicilian channel.

Keywords Asteraceae · Biogeography · Miocene · Pleistocene · Polyploidy · Sicilian channel · Speciation

Introduction

Despite some controversy concerning the evolutionary significance of polyploidisation for the longer-term diversity of higher plants (Fawcett and Van der Peer 2010; Arrigo and Barker 2012; Mayrose et al. 2011; but see Soltis et al. 2014 for a completely opposite view), polyploid speciation plays a paramount role in phytodiversity respects on smaller time-scales. The study by Wood et al. (2009) showed that about 35% of vascular plant species are recent polyploids

(“neopolyploids”, having formed since their genus arose). As a consequence, polyploid species and species-rich polyploidy complexes are undeniable building blocks of the actual plant biodiversity and, therefore, highly important drivers of ecological processes at the community and global levels. For example, evidence is amassing in the last years for the fact that polyploidisation has a crucial influence on the invasiveness of plant species by changing the morphology, physiology, ecological tolerance, phenotypic plasticity, and competition capabilities of newly formed polyploids (e.g. teBeest et al. 2012, Hahn et al. 2013, Linder and Barker 2013).

On the other hand, however, polyploid species formation creates considerable challenges for the taxonomical assessment and documentation of biodiversity. Autopolyploidy may lead to reproductively isolated evolutionary units (biological species) that go unnoticed when a morphology-based taxonomic (or phenetic) species concept is applied. This may cause the presence of ‘cryptic species’ and may lead to a gross underestimation of the number of species (Soltis et al. 2007). Allopolyploid populations could be

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formed repeatedly based on the hybridisation of the same two diploid ancestral species and may evolve into evolutionary independent and morphological distinct lineages, while in other cases multiple formation of polyploids (often under reciprocal parentage) may result in a single polyploid evolutionary entity with unrestricted gene-flow among its populations (Soltis and Soltis 1999).

Molecular techniques of the last decades not only helped to corroborate hypotheses of allopolyploid species formation in textbook examples like *Tragopogon* L. (Ownbey 1950; Soltis et al. 2004) and *Galeopsis* L. (Bendiksby et al. 2011), but also in many non-model angiosperm and fern groups (e.g. *Cerastium* L., Brysting et al. 2007; *Melampodium* L., Weiss-Schneeweiss et al. 2011; *Viola* L., Marcussen et al. 2012; *Fumaria* L., Bertrand et al. 2015; *Silene* L., Frajman et al. 2018; *Cystopteris* Bernh., *Gymnocarpium* Newman, Rothfels et al. 2017). Despite considerable progress in polyploidy phylogenetics (Oxelman et al. 2017; Rothfels 2021), discordance between gene trees based on nuclear ribosomal genes and spacer regions (nrDNA ITS and/or ETS) and those based on spacer/intron sequence information from the chloroplast genome (cpDNA), along with the additivity of ribotype polymorphisms inferred by amplicon cloning or scrutinising electropherograms of the ribosomal multi-copy marker, is still used in an overwhelming majority of contributions aiming at the inference of the ancestry of polyploid lineages and species.

The genus *Anthemis* L. (Compositae, Anthemideae) comprises around 175 species in the Mediterranean region and adjacent areas (Oberprieler et al. 2009, 2022) and harbours polyploid species and complexes in its section *A. sect. Hiorthia* (DC.) R.Fern. that comprises the perennial representatives of the genus (Oberprieler 1998). In Italy, the genus is represented by 16 species, of which 13 are also found in Sicily (Lo Presti et al. 2018). When studying the phylogeny of this extremely species-rich genus based on nuclear and plastidic markers sampled for nearly all of its species, Lo Presti et al. (2010) noticed the obvious discordant position of the Sicilian endemic tetraploid *A. cupaniana* Nyman in the two gene trees reconstructed, with a close relationship to a clade of perennial and often polyploid species around the S European-SW Asian *A. cretica* L. in terms of cpDNA sequence variation and a contradicting position of the species among annuals and perennials from N Africa in nrDNA respects. Owing to the fact that *A. cupaniana* was included in the mentioned study with only two accessions and sequence information for other taxa of the genus endemic to Sicily [i.e. *A. aetnensis* Spreng., *A. cretica* subsp. *messanensis* (Brullo) Giardina and Raimondo, *A. ismelia* Lojac., and *A. pignatiorum* Guarino, Raimondo and Domina] were not or only incompletely and geographically underrepresentatively sampled, the present contribution aims at a more extensive analysis on the origin of this tetraploid.

Materials and methods

Plant material, DNA extraction, PCR, and sequencing

The present study comprised 70 herbarium accessions of 26 *Anthemis* species from the central and western Mediterranean region (Online Resource 1). DNA sequences have been either gained in previous studies [Oberprieler and Vogt (2000), Oberprieler (2001), Lo Presti and Oberprieler (2009), Lo Presti et al. (2010)] or were newly produced in the present study. In the latter case, genomic DNA was extracted according to the CTAB DNA extraction protocol of Doyle and Dickson (1987) and Doyle and Doyle (1987). PCR amplification of the markers followed protocols given by Oberprieler et al. (2007) and Lo Presti and Oberprieler (2009) for nrDNA ITS1 and ITS2 and Lo Presti et al. (2010) for the two intergenic spacer regions *psbA-trnH* and *trnC-petN* on the chloroplast genome. After purification of PCR amplicons with AmpliClean (Nimagen, Nijmegen, The Netherlands) magnetic beads, Sanger sequencing was carried out by a contract sequencing company (MacroGen Europe, Amsterdam, The Netherlands).

For some ITS amplicons that were not readable due to ambiguous electropherograms caused by polymorphisms, we were able to pinpoint indels for sequence shifts and split reads into the two overlaying sequences (a and b in labels of Online Resource 3 and Fig. 2), which were subsequently used in the phylogenetic analysis. In cases of ITS amplicons that were not editable at all (i.e. those of accessions A1206 of *A. aetnensis* and A0689, A0728, A1189, and A1208 of *A. cupaniana*), purified PCR products were cloned into NEB Turbo *E. coli* (New England Biolabs, Frankfurt am Main, Germany) with the CloneJET PCR Cloning Kit (Fermentas, St. Leon-Rot, Germany). All reactions were conducted following the manufacturer's protocols. After 12 h of incubation at 37 °C, 11–17 clones of each transformation were picked, dissolved in water, and used as templates in a second PCR reaction. The primers and temperature profile were identical to the initial PCR. Following another purification step, the cloned amplicons were sequenced.

Phylogenetic analyses

Electropherograms were edited with CHROMAS v2.6.6 (Technelysium Pty Ltd 1998–2018) with usage of the IUPAC code for polymorphisms, and marker-wise alignments were done with BioEDIT v7.2.5 (Hall 1999). As outgroups for the subsequent phylogenetic analyses, sequences from *A. scariosa* Banks and Sol. and from *Cota*

melonoloma (Trautv.) Holub were included following previous results of more comprehensive phylogenetic analyses of *Anthemis* s.l. by Lo Presti and Oberprieler (2009) and Lo Presti et al. (2010).

For gene-tree reconstructions in MRBAYES v3.2.7 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003; Ronquist et al. 2012), substitution models were selected using the ‘automated model selection’ option in PAUP* v.4.0a169 (Swofford 2021). Gene-tree reconstructions were performed with a coding of indels (done with SEQSTATE v.1.4.1; Müller 2008) based on the simple indel-coding scheme of Simmons and Ochoterena (2000) and the splitting of data into a DNA and a ‘coded gaps’ partition. For the nrDNA ITS dataset, the symmetrical model with a gamma model of rate heterogeneity (SYM+G) was chosen; the two concatenated cpDNA intergenic spacer regions were allowed to evolve independently, each following the F81 model. The indel partitions were modelled with an equal-rates parameter. The gene-tree analyses were run for 2 million generations in two parallel runs with a number of four chains each and a temperature of 0.1 until convergence of runs was attained (average standard deviation of split frequencies < 0.01). Sampled trees were summarized with 25% of trees discarded as burn-in.

Results

Chloroplast haplotype phylogeny

The alignments of the two intergenic spacer regions of the chloroplast genome *trnC-petN* and *psbA-trnH* were 566 bp and 418 bp long and comprised 18 and 22 coded indels, respectively (Online Resource 2). The phylogenetic tree resulting from the Bayesian analysis is depicted in Fig. 1. It provides a well-supported bipartition of taxa, with representatives of *A. aetnensis*, *A. alpestris*, *A. cretica*, *A. cupaniana*, and *A. hydruntina* H.Groves on the one hand (the *A. cretica* clade) and all other species on the other (the *A. pedunculata* clade). While all members of the former have been assigned to *A. sect. Hiorthia* (DC.) R.Fernandes in former taxonomic treatments of the genus (e.g. Fernandes 1976; Oberprieler 1998), the latter clade comprises species from the annual series *A. sect. Anthemis* ser. *Bourgaeiniana* (*A. mauritiana* Maire and Sennen, *A. stiparum* Pomel, *A. zaiatica* Oberpr.), ser. *Chrysanthae* (*A. gharbensis* Oberpr., *A. maroccana* Batt. & Pitard, *A. tenuisecta* Ball), ser. *Secundirameae* [*A. confusa* Pomel, *A. cyrenaica* Coss., *A. glareosa* E.A.Durand & Barratte, *A. muricata* (DC.) Guss., *A. secundiramea* Biv., *A. ubensis* Pomel] together with perennials of *A. sect. Hiorthia* [*A. abylaea* (Font Quer & Maire) Oberpr., *A. ismelia*,

A. maritima L., *A. pedunculata*, *A. pignattiorum*, *A. punctata* Vahl]. In both clades, accessions and/or infraspecific taxa of the species surveyed do not form well-supported monophyletic groups.

Nuclear ribosomal DNA ITS phylogeny

The nrDNA ITS alignment comprised 158 accessions or cloned sequences and was 743 bp long, with 34 coded indels (Online Resource 3). The phylogenetic tree resulting from the Bayesian analysis is depicted in Fig. 2. Again, with the exception of clone C7 from accession A0689 (*A. cupaniana*) and accession A0286 (*A. hydruntina*), two well-supported monophyletic groups can be distinguished that correspond largely to the aforementioned *A. cretica* and *A. pedunculata* clades. However, this comes with a very important difference: all accessions and cloned sequences of *A. cupaniana* (with the exception of clone C7 from accession A0689 and clone C7 from accession A1189) are now found being members of the *A. pedunculata* clade, while all other taxa of the cpDNA-based *A. cretica* clade remained in the monophyletic group around *A. cretica*. As in the phylogenetic tree based on cpDNA sequence variation, monophyletic groups comprising all accessions of a species are not found; with the exception of all accessions and nearly all clones representing *A. cupaniana* (supported by a posterior probability of 0.97 within the *A. pedunculata* clade).

Discussion

Despite the lack of sufficient resolution in both phylogenetic trees resulting in the non-monophyly of the majority of the species included, the unique position of the focal species of the present study, the Sicilian endemic *A. cupaniana*, is unequivocal. While all other taxa are found being member of either the *A. cretica* or the *A. pedunculata* clade in the cpDNA and the nrDNA phylogenies, *A. cupaniana* combines its position in the former clade in respects to its chloroplast genome with membership in the latter one in terms of its sequences from the internal transcribed spacer regions of the ribosomal repeat. This pattern is interpreted best by assuming that this tetraploid species is of allopolyploid origin, with the maternal, plastid-inheriting parent being a diploid from the *A. cretica* group and the paternal one being a diploid from the *A. pedunculata* group. An alternative explanation to this could be a chloroplast-capture event via hybridisation between a diploid or tetraploid member of the *A. cretica* group (as maternal parent) and an autotetraploid of the *A. pedunculata* group.

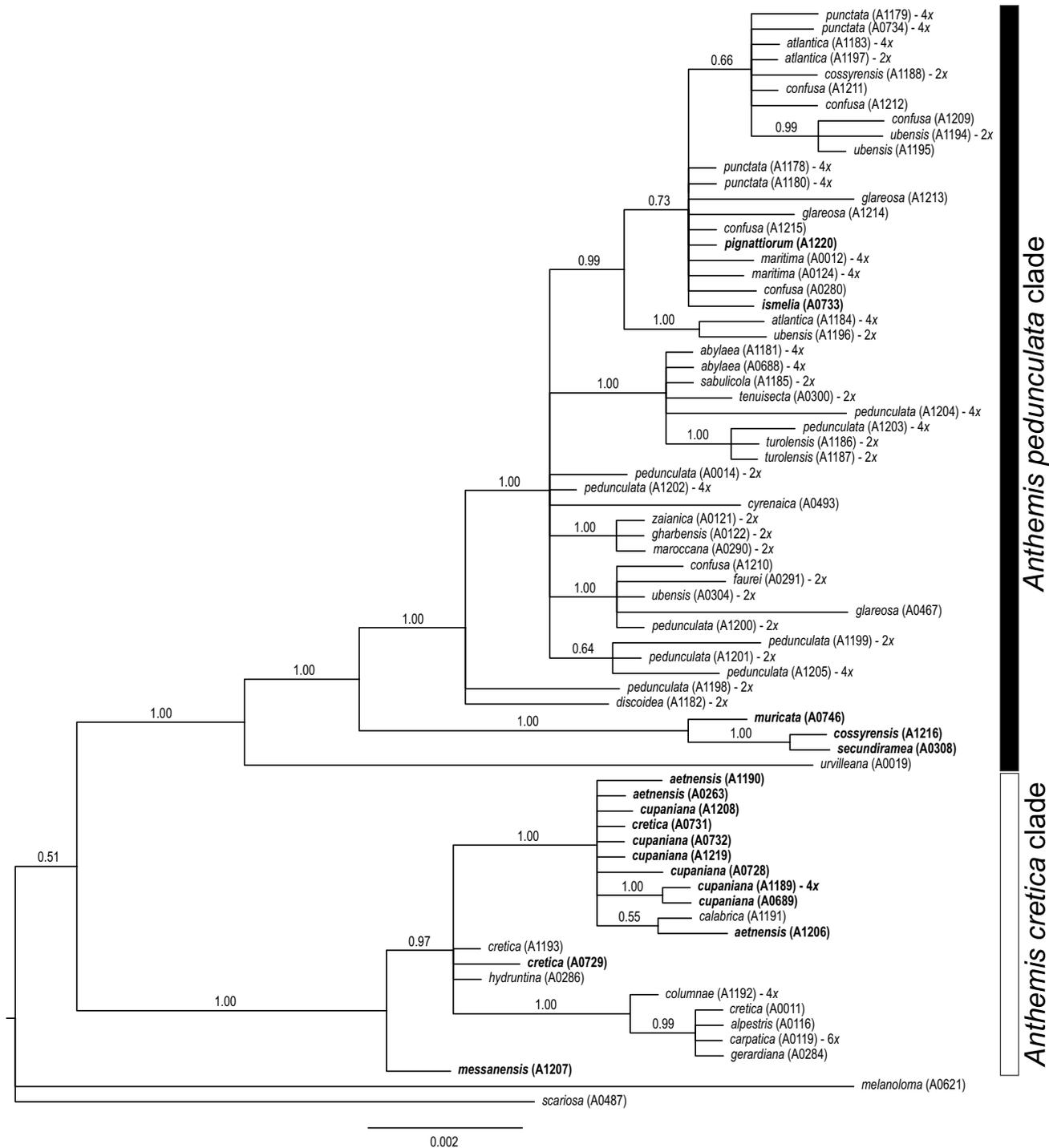


Fig. 1 Bayesian inference (majority-rule consensus) tree of western and central Mediterranean *Anthemis* representatives based on sequence variation for the two intergenic spacer regions *psbA-trnH* and *trnC-petN* on the plastid genome, with information on branch

support (posterior probabilities), accession numbers, ploidy level (2x, 4x, 6x, when inferred for accessions), and accessions from Sicily in bold

Diploid candidate species for this assumed allopolyploidisation leading to *A. cupaniana* are present in Sicily: from the *A. cretica* group, diploid chromosome numbers

were reported by Brullo and Pavone (1978) for a collection of *A. cretica* (subsp. *cretica*) from Piano Zucchi (Madonie Mts), which is close to the sampling localities of the two

A. cretica accessions A0729 and A0731 of the present study, while *A. ismelia* is a diploid (Bartolo et al. 1981) endemic to Monte Gallo and Monte Pecoraro close to Palermo (Cusimano et al. 2017) and belongs to the *A. pedunculata* group. The tetraploid *A. cupaniana* shares its ribbed, but smooth achenes with *A. cretica* (tuberculate in *A. ismelia*), while large capitula and a trend towards branched capitulescences is shared with *A. ismelia* (smaller capitula and unbranched stems in *A. cretica*). Additionally, the elevational distribution of *A. cupaniana* (500–1800 m a.s.l.) is more similar to *A. cretica* than to *A. ismelia* (10–650 m a.s.l.). The nearly identical chloroplast sequences of *A. cupaniana* accessions with accession A0731 of *A. cretica* from Mte Quacella (Madonie Mts) and the strongly supported clade of the majority of nrDNA clones from *A. cupaniana* formed with accession A0733 of *A. ismelia* from Mte Gallo (Palermo) and north African annuals and perennials (i.e. *A. confusa*, *A. glareosa*, *A. pedunculata* subsp. *atlantica*, *A. pedunculata* subsp. *pedunculata*, *A. secundiramea* var. *cosyrensis* Guss., *A. secundiramea* subsp. *secundiramea*, and *A. ubensis*) support this interpretation.

Participation of *A. cretica* in the polyploid formation of *A. cupaniana* is not only supported by the possession of *A. cretica*-type chloroplasts alone; the finding of nrDNA ITS clones that completely (accession A1189, clone C7) or partly (accessions A0689, clones C7 and C14; accession A0728, clone C2) are identical with ITS sequences from *A. cretica* (Table 1) support this conclusion impressively. Especially convincing are the two clones of accessions A0728 (clone C2) and A0689 (clone C7), where in the former, ITS1 typical for *A. ismelia* is combined with ITS2 typical for *A. cretica*, while in the latter, ITS2 contains a mixture of nucleotide positions typical for both parental species. These deviating ITS clone sequences are best explained by assuming recombination between the two parental nrDNA ITS arrays (cassettes) in the tetraploid. This could be expected either (a) when a tetraploid exhibits a tetrasomic inheritance and recombination between homeologous chromosomes of the two parental genomes is realised, or (b) when illegitimate recombination events between parental genomes happen as exception to a regular disomic behaviour of parental chromosome sets, or (c) when hybridisation of the tetraploid with one or both parental diploids leads to the formation of early recombinants of parental ribotypes. Owing to the fact that the two parental lineages (i.e. the *A. cretica*- and the *A. pedunculata* group) diverge from each other already around 9 Ma ago (Lo Presti and Oberprieler 2009), tetrasomic inheritance appears less reasonable than the two other hypotheses. The explanation of the observed pattern caused by recent hybridisation between the tetraploid *A. cupaniana* and the diploid *A. cretica*, however, receives plausibility due

to the sympatric and even syntopic (Rivas 1964) distribution of the two taxa in the Madonie Mts. of central Sicily. Recent hybridisation may also explain why after homogenisation of ITS repeats towards the *A. ismelia*-side in the allopolyploid *A. cupaniana* (as seen in accessions A0732, A1208, and A1219) as a consequence of concerted evolutionary mechanisms (unequal crossing-over, gene conversion; Álvarez and Wendel 2003) patterns of incomplete homogenisation are observed in some individuals (A0689, A0728, and A1189). Detailed cytological and molecular studies in the mixed stands of *A. cretica* and *A. cupaniana* in the Madonie Mts. may reveal signatures of inter-ploidy hybridisation (presence of triploids, additional individuals with recombinant nrDNA ITS arrays) that would support the latter scenario.

In biogeographical respects, the allopolyploid formation of *A. cupaniana* in Sicily is an impressive example for the important role of the Sicilian Channel as a barrier and corridor for the floristic exchange between the African and the Eurasian plate during the Quaternary as reviewed by Nieto (2014). A dated phylogenetic and biogeographical reconstruction for *Anthemis* s.l. provided by Lo Presti and Oberprieler (2009) shows that the most recent common ancestor of the *A. cretica* and *A. pedunculata* groups lived in the Miocene (Tortonian-Messinian, 8.8 ± 1.9 Ma ago), with the Balkan Peninsula and Asia Minor being the most probable ancestral area of the former and north-west Africa being the ancestral area of the latter species group. While the *A. cretica* group subsequently diversified on the northern platform into a species group of perennials distributed throughout the southern European and southwest Asian mountain ranges from the Iberian Peninsula in the west to the Caucasus and northern Iran in the east, the common ancestor of the *A. pedunculata* group evolved into an array of annuals and perennials in northern Africa (Lo Presti and Oberprieler 2009; Fig. 3). The onset of the glaciation cycles of the Pleistocene c. 2.5 Ma ago triggered numerous speciation events in the former group and led to dispersal events of the latter group onto the Eurasian platform (e.g. *A. muricata* or *A. secundiramea* to Sicily, *A. pedunculata* to the Iberian Peninsula). The role of the Sicilian Channel as a corridor for floristic exchange caused by eustatic sea-level shifts during the Pleistocene has been demonstrated for a number of plant (e.g. *Silene*, Naciri et al. 2010; *Anacamptis*, Zitari et al. 2011; *Anthemis secundiramea*, Lo Presti and Oberprieler 2011; *Linaria*, Fernández-Mazuecos and Vargas 2011) and animal groups (e.g. *Bufo*, Stöck et al. 2008). The presence of a member of the *A. cretica* group in North Africa [i.e. *A. cretica* subsp. *columnae* (Ten.) R.Franzén in the Kabylie Mts of Algeria; Oberprieler 1998] and of species of the *A. pedunculata*

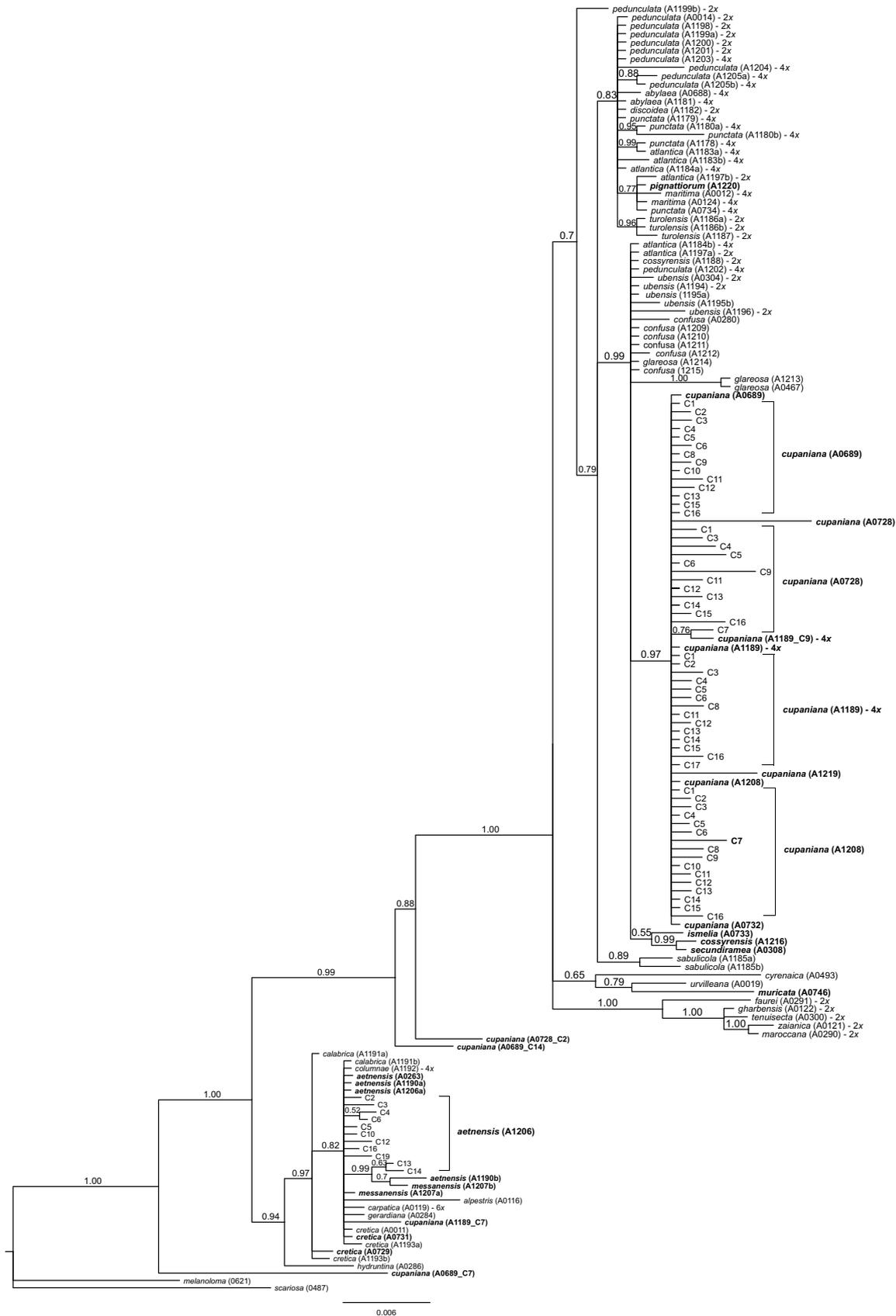


Fig. 2 Bayesian inference (majority-rule consensus) tree of western and central Mediterranean *Anthemis* representatives based on sequence variation for the nuclear ribosomal repeat comprising ITS1, 5.8S, and ITS2, with information on branch support (posterior probabilities), accession numbers (with “C” denoting sequences from cloned PCR products), ploidy level (2x, 4x, 6x, when inferred for accessions), and accessions from Sicily in bold

group in Sicily (i.e. *A. ismelia*, *A. muricata*, *A. pignattiorum*, *A. secundiramea*; present study) exemplifies this exchange nicely and provides biogeographical evidence to the formation of the allotetraploid *A. cupaniana* under participation of members of both groups as hypothesised above.

Table 1 Comparison of polymorphic sequence positions in the intergenic spacer regions ITS1 and ITS2 of the nuclear ribosomal repeat (nrDNA) of accessions of *Anthemis cretica*, *A. ismelia*, and *A. cupa-*

niana. Positions typical for *A. cretica* in bold, those of *A. ismelia* in italics. Accession numbers with “C” denote sequences from cloned PCR products

Taxon	Accession	ITS 1	ITS 2																				
			0	0	1	1	1	1	1	2	2	4	4	5	5	5	5	5	5	5	6	6	
<i>A. cretica</i> L	A0729	T	C	C	G	T	T	T	A	T	C	T	A	T	C	C	C	A	G	T	C	A	C
	A0731	T	C	C	G	T	T	T	A	T	C	T	A	T	C	C	C	A	G	T	C	A	C
<i>A. cupaniana</i> Nyman	A1189-C7	T	C	C	G	T	T	T	A	T	C	T	A	T	C	C	C	A	G	T	C	A	C
	A0689-C7	T	C	T	G	T	T	T	A	T	C	A	T	A	T	C	T	A	A	T	G	T	C
	A0689-C14	C	T	T	T	A	C	A	A	T	C	T	A	T	C	C	T	A	G	T	C	A	C
	A0728-C2	C	T	T	T	A	C	A	G	C	C	T	A	T	C	C	C	A	G	T	C	A	C
	A0732	C	T	T	T	A	C	A	G	C	T	A	T	A	T	T	T	T	A	C	G	C	T
	A1208	C	T	T	T	A	C	A	G	C	T	A	T	A	T	T	T	T	A	C	G	T	T
<i>A. ismelia</i> Lojac	A1219	C	T	T	T	A	C	A	G	C	T	A	T	A	T	T	T	A	C	G	C	T	T
	A0733	C	T	T	T	A	C	A	G	C	T	A	T	A	T	T	T	A	C	G	T	T	T

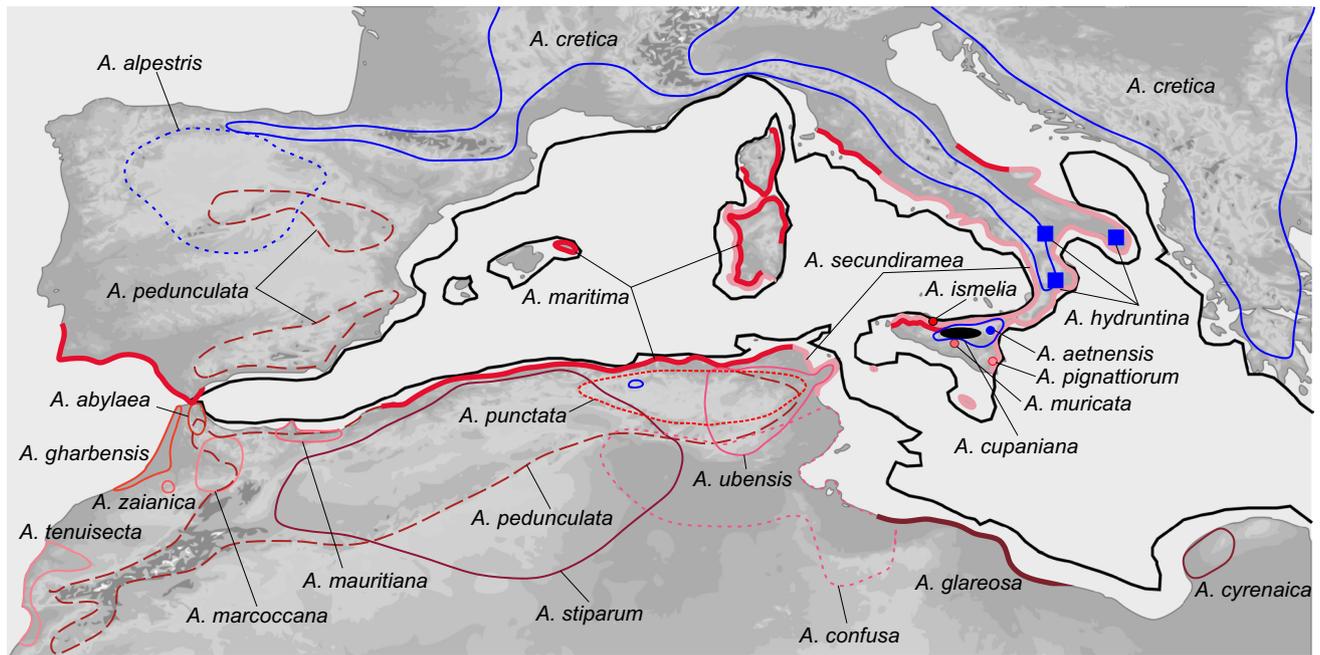


Fig. 3 Distribution map of the species involved in the present study, with members of the *Anthemis cretica* clade in blue, those of the *A. pedunculata* clade in red, and the focal species *A. cupaniana* in

black. Black lines illustrate the approximated paleo-coastline during the Last Glacial Maximum (after Thiede 1978)

Information on Electronic Supplementary Material

Online Resource 1. List of taxa and sources of plant material, and voucher specimens used for the present molecular study. The two accession numbers for nrDNA ITS of some taxa represent ITS1 and ITS2 regions, respectively. Bold type denotes sequences new to science; other sequences were published in (a) Oberprieler and Vogt (2000), (b) Oberprieler (2001), (c) Lo Presti and Oberprieler (2009), (d) Lo Presti et al. (2010). Information on ploidy is based on the very same individuals published in Oberprieler (1998) or (in brackets) came from literature sources based on other plants/populations. Ploidies in bold (and with an asterisk) are reported here for the first time for the surveyed populations.

Online Resource 2. Alignment in Nexus format for the concatenated plastidic intergenic spacer regions *trnC-petN* (positions 1-566) and *psbA-trmH* (positions 567-984), with information on 40 indels coded in addition to the sequence information.

Online Resource 3 Alignment in Nexus format for the nrDNA ITS1+5.8S+ITS2 marker, with information on 34 indels coded in addition to the sequence information.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00606-022-01823-1>.

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Author contributions CO planned and conveyed the study. Gianniantonio Domina provided plant material from Sicily. EV did the laboratory work and the phylogenetic analyses in the course of her Bachelor thesis in the Evolutionary and Systematic Botany Group of CO at Regensburg University. The latter wrote a first draft of the manuscript, to which GD contributed further improvements.

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Declarations

Conflict of interest The authors declare no conflict of interests.

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