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Phylogeny of *Asperula* L. sect. *Cynanchicae* (DC.) Boiss. (Rubiaceae)

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Abstract

Aim of the study

The present study is aimed at a phylogenetic reconstruction of *Asperula* L. sect. *Cynanchicae* (DC.) Boiss., the richest section for number of taxa belonging to the genus *Asperula* (tribe *Rubieae*, family Rubiaceae). Systematic boundaries in the tribe *Rubieae* are somehow artificial, as several genera resulted to be paraphyletic or even polyphyletic (i.e. *Galium*, *Asperula*). However, it is not conceivable to re-arrange generic assemblages without previously understanding and establishing intra-specific circumscriptions.

In addition to strictly phylogenetic issues, this work also includes a phylogeographic effort on a single species, the endemic *Asperula crassifolia* L., which was chosen as a model species to undertake a study focused on population diversity and conservation issues.

Methods

Different phylogenetic approaches (maximum parsimony, maximum likelihood and Bayesian inference) were employed for reconstructing the evolutionary history of approximately 70 entities of *Asperula* sect. *Cynanchicae*, using nuclear ribosomal Internal Transcribed Spacers sequences and two chloroplast spacers (*trn*C-*pet*N, *pet*N-*psb*M). Moreover, divergence time within the section were estimated using a Bayesian approach and biological processes (e.g., reticulation, incomplete lineage sorting) subtending phylogenetic relationships were evaluated.

Asperula crassifolia was sampled for its entire distribution range (the island of Capri, Sorrentine Peninsula and Sirenusae archipelago) and nuclear simple sequence repeats (SSR) were isolated for the species. Furthermore, *rps*16 intron variability was investigated in order to formulate phylogeographic hypothesis about the divergence of different populations.

Results

Molecular homogeneity of *Asperula* sect. *Cynanchicae* is extremely evident; divergence time estimation indicates that the section originated during Miocene and terminal nodes are the outcome of geological and climatic vicissitudes occurred during the Tertiary and Quaternary. Probably, reproductive barriers between some taxa of the section are totally lacking, contributing to obscure phylogenetic signal. Hybridization is likely also involved, and this partly prevented the homogenization of the ITS sequences as a consequence of concerted evolution.

As far as biogeographical history of *A. crassifolia* is concerned, the maximum number of haplotypes is recorded for Capri; therefore the origin of the species appears more plausibly to have occurred in this island. Nevertheless, the sympatric presence of *A. aristata* subsp. *aristata* does not allow to exclude crossing between the two entities. The rarity of *A. crassifolia* gives strong indications for safeguard by law.

Riassunto

Scopo della ricerca

Il presente studio è incentrato sulla ricostruzione filogenetica di *Asperula* L. sect. *Cynanchicae* (DC.) Boiss., la sezione più ricca per numero di taxa appartenente al genere *Asperula* (tribù *Rubieae*, famiglia Rubiaceae). Le delimitazioni sistematiche nella tribù delle *Rubieae* sono spesso fittizie, in quanto numerosi generi risultano parafiletici o finanche polifiletici (ad esempio *Galium*, *Asperula*). Ad ogni modo, la riorganizzazione intergenerica non può prescindere da una preliminare comprensione dei limiti infraspecifici.

In aggiunta a un discorso prettamente filogenetico, col presente studio è stata avviata un'indagine filogeografica sulla specie endemica *Asperula crassifolia* L., scelta come modello per intraprendere uno studio focalizzato sulla diversità popolazionistica e le dinamiche di conservazione.

Metodi

Differenti approcci filogenetici (massima parsimonia, massima verosimiglianza e inferenza Bayesiana) sono stati impiegati per la ricostruzione della storia evolutiva di circa 70 specie appartenenti ad *Asperula* sect. *Cynanchicae*, usando gli spaziatori interni del DNA ribosomale (ITS) e due spaziatori intergenici plastidiali (*trn*C-*pet*N, *pet*N-*psb*M). Inoltre, è stata condotta una stima dei tempi di divergenza all'interno della sezione usando un approccio Bayesiano, e sono stati valutati i processi biologici (fra cui evoluzione reticolata, *lineage sorting*) alla base delle relazioni filogenetiche.

Asperula crassifolia è stata campionata per l'intero areale di distribuzione (l'Isola di Capri, la Penisola Sorrentina e l'arcipelago di Li Galli) e per essa sono state isolate sequenze microsatellitari (SSR) nucleari. Inoltre è stata analizzata la variabilità dell'introne *rps*16, la quale è servita a formulare un'ipotesi filogeografica sulla divergenza delle differenti popolazioni.

Risultati

Dallo studio è emersa l'estrema similarità tra i membri della *Cynanchicae*; la stima dei tempi di divergenza indica che la sezione si è originata durante il Miocene e i taxa attualmente noti sono il risultato di vicissitudini geologiche e climatiche avvenute durante il Terziario e il Quaternario. È inoltre molto probabile l'assenza totale di barriere riproduttive tra alcuni taxa della sezione, e ciò contribuisce a confondere il segnale filogenetico. Verosimilmente, un altro fenomeno coinvolto è l'ibridazione, e ciò ha parzialmente impedito l'omogeneizzazione delle sequenze ITS per opera dell'evoluzione concertata.

Per quanto riguarda la storia filogeografica di *A. crassifolia,* il numero massimo di aplotipi *rps*16 è stato rinvenuto nella popolazione di Capri. Di conseguenza, appare molto più plausibile che Capri sia il centro di origine di questa specie. Ciononostante, la presenza simpatrica di *A. aristata* subsp. *aristata* non consente di escludere la presenza di ibridi tra le due specie. Infine, la rarità di *A. crassifolia* rende necessario attuare misure di protezione.

Chapter 1 – General introduction

The family Rubiaceae and its subdivisions

Rubiaceae Juss. is the fourth-largest angiosperm family, with 13,000 species classified into approximately 600 genera (Govaerts *et al.*, 2006; Davis *et al.*, 2009). This family, differentiated about 90 mya (Bremer & Eriksson, 2009), has a cosmopolitan distribution with maximum diffusion at the tropical latitudes, where it represents up to 20% of the biodiversity and has important ecological roles, supplying food for the faunal community by virtue of the abundant production of fruit, nectar and leaves. At these latitudes, Rubiaceae are mostly woody, whereas in the temperate regions they are represented by herbaceous, perennial plants. Besides the ecological relevance of the Rubiaceae, some species have remarkable economic importance. The coffee plant (*Coffea arabica* L. and allied species) belongs to this family; *Cinchona* spp. contain the alkaloid historically used for the treatment of malaria (Humphrey, 2000); *Rubia tinctorum* L. (the dyer's madder) is a tinctorial plant whose root is traditionally used as a natural dye (Cannon & Cannon, 2003).

In the last 20 years Rubiaceae have been the subject of at least 70 studies at different taxonomic levels (e.g., Bremer & Jansen 1991; Andreasen & Bremer, 1996; Bremer & Thulin, 1998; Andersson & Rova, 1999; Bremer *et al.*, 1999; Nepokroeff *et al.*, 1999; Bremer & Manen, 2000; Persson, 2000; Malcomber, 2002; Delprete & Cortes-B, 2004; Motley *et al.*, 2005; Backlund *et al.*, 2007; Bremer & Eriksson, 2009) aimed at the systematic revision of the family. Besides the clarification of the phylogenetic relationships at infra- and intergeneric levels, molecular analyses led to the assignation of Rubiales to order Gentianales, and to the suppression of the family Theligonaceae, whose members are now included in Rubiaceae (APGII, 2003). A correct understanding of the phylogenetic relationships in the family is crucial, especially in connection to the substantial presence of monotypic genera, which represent about 35% of the total number of genera (Davis *et al.*, 2009). In spite of this, albeit a great part of them does not probably have reason to exist, several monotypic genera represent uniqueness from an evolutionary point of view: extinction of these taxa might represent the demise of entire lineages. Hence, it is essential to create

safeguards for the protection of these species.

Current knowledge allows to assert that Rubiaceae are monophyletic, divided into 3 subfamilies (*Cinchonoideae*, *Ixoroideae*, *Rubioideae*) and 43 tribes, several of which monogeneric and many which turned out to be paraphyletic or polyphyletic (e.g., *Gardenieae*, *Morindeae*). Only one tribe (*Coptosapeltae*) cannot be ascribed to any subfamily; it occupies, anyway, a rather basal position in the Rubiaceae phylogenetic tree, as sister group of the Asiatic genus *Luculia* Sweet, which is not attributable to any superior taxonomic rank as well (Bremer, 2009).

This study focuses on the genus *Asperula* L., which belongs to tribe *Rubieae*, subfamily Rubioideae. The latter includes 11 of the 20 largest genera of Rubiaceae. Many molecular studies corroborated the hypothesis of monophyly for the subfamily (Natali *et al.*, 1995; Bremer, 1996; Andersson & Rova, 1999; Bremer & Manen, 2000). In the work of Natali *et al.* (1995), phylogenetic support was obtained using chloroplast intergenic spacer *atp*B-*rbc*L, which showed a 204-nucleotides deletion for the sampled taxa. For the same spacer, members of tribe *Rubieae* showed an additional 50-bp deletion, which suggested the monophyletic nature of the group. Eventually several studies attested the monophyly of the tribe (Natali *et al.*, 1996; Bremer, 1996; Andersson & Rova, 1999; Bremer & Manen, 2000; Nie *et al.*, 2005; Backlund *et al.*, 2007; Bremer & Eriksson, 2009; Rydin *et al.*, 2009; Soza & Olmstead, 2010). Concerning morphological peculiarities, *Rubieae* show a bilocular ovary with one ovule per locule, a rudimentary calyx, several pollen traits (Huysmans *et al.*, 2003) and leaf-like whorls that differentiate *Rubieae* from the remaining Rubiaceae. Moreover, prevailing herbaceous *habitus* and temperate distribution are distinctive characters of the 13 genera of *Rubieae*.

Rubieae have mostly hermaphroditic or andromoecious flowers with entomophilous pollination. They are mainly self-incompatible outbreeders; autogamy has been documented for several annuals, (e.g., *Galium aparine* L. and *Sherardia arvensis* L.; Tao & Ehrendorfer, 2011) and, to date, only for the perennial *A. daphneola* O.Schwarz (Gücel & Seçmen, 2009).

Rubieae evolutionary radiation appears to be relatively recent, as suggested by fossil pollen dating back to the Miocene (Van Campo, 1976; Menke, 1976; Graham, 2009); although *Asperula*, *Galium* L., *Rubia* L. and *Cruciata* Mill. exhibit

indistinguishable pollen morphology (Huysmans *et al.*, 2003), fossil pollen from Alaska was indicated and accepted as *Galium* pollen (Graham, 2009). The tribe is thought to have originated in Eurasia; subsequently it probably underwent at least 8 independent dispersion events in Northern America, followed by diversification in Southern America (Soza & Olmstead, 2010); the estimated divergence time of the tribe is 28.6 Ma with a crown age of 18.1 Ma (Bremer & Eriksson, 2009)

Molecular studies place *Rubia* as the basal-most genus, as however suggested from the *habitus*, the fruits (berries), the 5-lobed corolla and the whorls of 4-6 elements (Natali *et. al*, 1995; Soza & Olmstead, 2010). The most recent classification indicates the presence of the following genera: *Asperula*, *Callipeltis* Steven, *Crucianella* L., *Cruciata*, *Didymaea* Hook.f., *Galium*, Kelloggia Torr. ex Benth. & Hook.f., *Mericarpaea* Boiss., *Microphysa* Schrenk, *Phuopsis* Benth. & Hook.f., *Rubia*, *Sherardia* L., *Valantia* L. (Govaerts *et al.*, 2006; Soza & Olmstead, 2010; Tao & Ehrendorfer, 2011).

The genus *Asperula* L.

Asperula includes approximately 130 species, generally perennial and suffruticose, rarely annual and herbaceous, divided into ten sections (Table 1; Ehrendorfer *et al.*, 2005). Leaves are associated to stipulae in a variable number (up to 14 elements); inflorescence is thyrsoid, with a corymbose, subcapitate or subspicate cyme. Flowers are hermaphrodite and bracteate with a calyx which is either null or consisting of short teeth; corolla, pentamerous or tetramerous (rarely trimerous), is hypocrateriform, infundibuliform or campanulate. Styles are two, with a globose stigma; fruit is a schizocarp (Ehrendorfer & Krendl, 1976; Ehrendorfer *et al.*, 2005).

The genus appears really heterogeneous, in terms of morphological and karyological traits. In fact, its systematic boundaries have always been a challenging issue, especially in connection to other historical members of Rubiaceae (e.g., *Galium*, *Sherardia*). Among them, monotypic genus *Sherardia* is progressively losing reason to exist from a molecular point of view; in fact, it appears to be closer to *Asperula* sect. *Hexaphylla* Ehrend., rather than being external to the genus (Soza & Olmstead, 2010). Moreover, present separation between *Asperula* and *Galium*, based upon two

morphological traits i.e., loss of bracteoles in *Galium* and longer corolla-tube in *Asperula* (e.g., Ehrendorfer & Krendl, 1976), turned out to be related to homoplastic characters, and the non-monophyly of the two genera is confirmed (Manen *et al.*, 1994; Natali *et al.*, 1996; Soza & Olmstead, 2010). Although several members of *Asperula* have been moved to *Galium* (e.g., *A. odorata*, *A. aparine*, etc.), the effort is far from its conclusion. Some *Galium* members appear to be phylogenetically closer to *Asperula* than to other co-generic species as, for instance, the representatives of the sect. *Aparinoides* (Jordan) Gren. and sect. *Depauperata* Pobed. (Natali *et al.*, 1995; Ehrendorfer *et al.*, 2005; Soza & Olmstead, 2010). Such a condition is probably due to the fact that *Asperula* is an assemblage of paraphyletic group, which share plesiomorphic traits. Loss of floral bracteoles (and often of the bracts), reduction of the corolla-tube and, finally, depigmentation of flowers gave rise to *Galium* (Ehrendorfer *et al.*, 2005).

The origin of *Asperula* is traceable to Eurasia, and the center of its diversity is South-Western Asia, with a distribution ranging from Mediterranean and Western Europe to the Eastern Asia; some taxa belong to Australian and New Zealand floras (Sect. *Dioicae* Airy Shaw & Turril). From a phylogeographic and conservational perspective, it is interesting that several species are narrowly endemic.

Table 1 – Sections of the genus *Asperula* (Ehrendorfer & Krendl, 1976; Ehrendorfer *et al.*, 2005; Govaerts *et al.*, 2006)

Sections	# species (approx.)
Asperula L.	3
Cruciana Griseb.	8
Crucianelloides Boiss.	1
Cynanchicae DC. ex Boiss.	71
Dioicae Shaw & Turrill	22
Glabella Griseb.	9
Hexaphylla Ehrend.	10
Oppositifoliae Schischk. ex E. Schönb.-Tem.	13
Thliphthisa (Griseb.) Ehrend.	14
Trichodes Boiss.	2
Tricostella Schönb.-Tem. & Ehrend.	1

Chapter 2 – Phylogenetic reconstruction of *Asperula* L. sect. *Cynanchicae* (DC.) Boiss.

Introduction

The section *Cynanchicae* encloses about 100 entities, including species and subspecies (Appendix A). These plants are dwarf shrubby perennials, mainly calciphylous and growing on dry and rocky grounds. Leaves and stipules per node are at maximum in number of 4, the cauline being linear-lanceolate with a hyaline apex. Corolla is always tetramerous, hypocrateriform or infundibuliform, purplish, pink, greenish or yellowish. Anthers and stigma are generally not protruding; fruit is obovate and never entirely smooth (Ehrendorfer & Krendl, 1976; Ehrendorfer *et al*., 2005).

The section is predominantly distributed in the Mediterranean and Aegean areas, but the range covers Great Britain northerly and Caucasus easterly. In Anatolia it is represented by approximately 20 species, mostly endemic (Ehrendorfer *et al*., 2005; Minareci & Yildiz, 2010; Öztürk, 2013). The type species of the section is *Asperula cynanchica* L., whose geographical range covers the distribution of the whole section. In the past, the forenamed binomial included almost all the entities of the section (Parlatore, 1857; Fiori, 1925) and this reflects the extreme morphological similarity within the group; in fact, interspecific distinction is often hard, as it is based upon quantitative and poorly discriminating traits. Moreover, several authors pointed out the existence of intermediates between some different species (see Table 2; Del Guacchio & Caputo, 2013). Such a situation typically occurs when dealing with groups in which hybridization and polyploidization is widespread (Rieseberg *et al*., 1993; Popp *et al*., 2005; Guo *et al*., 2008; Schmidt-Lebuhn *et al*., 2012). Hybrid nature of many entities is presumed (*A.* x *jordanii* Perrier & Songeon, 1855; *A.* x *portae* Peruzzi; Bernardo *et al*., 2010; Ehrendorfer, 1976); furthermore, various taxa exhibit tetraploidy or incostant ploidy (see Appendix A); karyotypes, however, are not available for the majority of the sect. *Cynanchicae*. Regardless, it is possible to note that inconstant ploidy marks the most widespread entities of the section (e.g., *A. cynanchica* and *A. aristata*), whereas diploid species tend to be narrowly endemic. Such a circumstance might be not a coincidence; in fact, the wider the distribution, the

larger the number of sympatric situations which could represents the basis for hybridization, and subsequent introgression, if reproductive barriers are weak.

Reticulation due to hybridization phenomena obscures to a certain extent phylogenetic reconstruction, as repeated crossing between lineages may determine the impossibility to outline a tree-like phylogeny (Erixon & Oxelmann, 2008; Willyard *et al*., 2009). Clues to reticulate evolution may appear as a conflict between different sources of data (molecular, biogeographic and morphologic) or, referring to molecular data, as conflicting topologies deriving from datasets of different markers (McDade, 1992). At a topology level, in particular, signals of the occurrence of hybrid taxa are the increase of the homoplasy in the dataset, a decrease in resolution and, lastly, the evidence of non-monophyly of conspecific entities (Fuertes Aguilar & Feliner, 2003), although, other phenomena such as the retention of ancestral polymorphism (i.e. incomplete lineage sorting), may also reasonably explain some of the above mentioned difficulties (Avise 1994, 2000; Comes & Abbott, 2001; Schimdt-Lebuhn *et al*., 2012).

A good starting point to unravel relationships when hybridization is suspected is to follow the phylogenetic positions of the endemic and isolated taxa.

As previously said, some diploid entities are narrowly endemic taxa; Gutermann & Ehrendorfer (2000) illustrated an interesting biogeographic hypothesis about a group of endemic members of *Asperula* sect. *Cynanchicae*, whose geographic location is probably the relic distribution of a diploid common ancestor dating back to Pliocene.

The above mentioned hypothesis arises from the observation that several Mediterranean chasmophytes exhibit similar ecological behaviors: small, isolated and specialized populations confined in stable habitats, residual of a wider geographic range fragmented by geological rearrangements occurred during the late Tertiary. The group of species in question is informally known as "*Palaeomediterraneae*", and comprehends: *A. crassifolia* L., *A. deficiens* Viv., *A. paui* Font Quer, *A. garganica* Huter, Porta & Rigo ex Ehrend. & Krendl, *A. calabra* (Fiori) Gavioli, *A. borbasiana* (Korica) Korica, *A. naufraga* Ehrend. & Gutermann, *A. visianii* Korica, *A. staliana* Vis. (and relative subspecies), *A. woloszczakii* Korica (Gutermann & Ehrendorfer, 2000; see Appendix A for ploidy data).

The authors conjecture that some of these are probably involved in the origin

of tetraploid taxa which, in turn, played a role in the speciation of polyploid entities; it is known, indeed, that diploid genome represents the raw material on which speciation via polyploidization operates (Rešetnik *et al.*, 2014).

Table 2 – Literature reports of intermediate and hybrid forms (Ehrendorfer & Krendl, 1976; Ehrendorfer & Schönbeck-Temesy, 1982; Schönbeck-Temesy & Ehrendorfer, 1991; Bernardo *et al.*, 2010; Greuter, 2012)

Taxon	Intermediates indication
A. aristata	Extremely polymorphic and polyploid, often transitional towards *A. cynanchica*
A. beckiana	Sometimes difficult to distinguish from *A. wettsteinii*
A. cynanchica	Very variable species with local races, transitional towards *A. aristata*, *A. rupicola* and *A. rumelica*
A. diminuta *A. graveolens* *A. littoralis*	Indicated as geographical vicariants connected by transitional forms
A. lutea *A. mungieri* *A. rigidula*	Previously indicated as subspecies of *A. lutea*, they are prone to form intermediate in the areas of contact; sometimes indicated as vicariant at different altitudes
A. nitida	Highly polymorphic species
A. pulvinaris	Intermediates with *A. lutea* and *A. rigidula* occur
A. rumelica	Very polymorphic species
A. stricta	Highly polymorphic species
A. tenella	Intermediates with *A. rumelica* have been observed
A. x jordanii	Hybrid between *A. aristata* subsp. *oreophila* and *A. cynanchica*. Difficult to distinguish from *A. rupicola* and *A. cynanchica* subsp. *pyrenaica*
A. x portae	Hybrid between *A. calabra* and *A. aristata* subsp. *oreophila*

Materials and methods

Sampling

50 species of the total 65 belonging to *Asperula* sect. *Cynanchicae* were employed in the analyses (Appendix B). Several entities were collected in the field, but most frequently DNAs were extracted from herbarium material, which was routinely re-identified. Some *Rubieae* were included as outgroup, by virtue of the evidence shown in the work by Soza & Olmstead (2010): *Didymaea alsinoides* (Cham. & Schltdl.) Standl, as genus *Didymaea* together with *Rubia* was reported as the basal-most of the tribe *Rubieae*; *Asperula purpurea* belonging to sect. *Thliphthisa* and *Sherardia arvensis*, which appeared to be closer to the section *Cynanchicae* than other *Asperula* sections.

Molecular methods

DNA extractions were carried out with the CTAB method (Doyle e Doyle, 1987) For herbarium material extraction, the DNA precipitation steps were modified; some precipitation steps were carried out in isopropanol at -20°C for three days, some others in ethanol 90% overnight, followed by purification using GENECLEAN® II Kit (MP Biomedicals). These procedures allowed the extraction and the subsequent amplification of material more than a hundred century old.

Several primers were tested in order to select variable markers. The chloroplast spacers *ndh*F-*rpl*32, *psb*A-*trn*H and the gene *mat*K were discarded as essentially invariable; primers for the spacers *trn*S-*trn*G, *trn*Q-*rps*16, *atp*B-*rbc*L failed to amplify a specific product, as well as the primers for the LEAFY gene. The intron *rps*16 gave interesting and unusual results: in the first part of the sequence it was very variable, in the remaining part of the sequence it was almost invariable. The intron turned out to be useful for intraspecific identification of haplotypes (see Chapter 3). Nuclear markers 5S-NTS and ETS failed to be sequenced efficiently, as a result of non-specific signals. At last, two chloroplast spacers (*trn*C-*pet*N, *pet*N-*psb*M) provided good results in terms of variability and were chosen for the investigation; in the nuclear DNA, I chose Internal Transcribed Spacers of the ribosomal DNA (ITS), traditionally employed for phylogenetic analyses (Baldwin, 1992; Baldwin *et al.*, 1995; Kårehed, 2008). Primers

used are listed in Table 3.

PCR amplifications were carried out in a final volume of di 25 µL, with a variable DNA concentration (2-10 ng), *DreamTaq DNA Polymerase* 1 U (Fermentas, Thermo Scientific), *DreamTaq Buffer* 1X, MgCl$_2$ 0.5-2.5 mM, dNTPs 0.2 mM, primers 0.5 µM, water to the final volume and the following PCR program: initial denaturation at 94° C for 3 minutes, 30 cycles of 94° C for 30", annealing for 30" at a variable temperature (Table 3), 45"-1 minute of extension at 72° C, followed by 7 minutes of final extension. Thermo Scientific Phire Plant Kit was used for recalcitrant DNAs, with the following concentration: Phire Hot Start II DNA Polymerase 1U, 1X Phire Plant PCR Buffer, primers 0.5 µM and water to the final volume of 20 µL. PCR program was modified as follows: initial denaturation at 98°C for 1 minute, 35 cycles at 98°C for 1", variable annealing temperature for 30" and extension at 72°C for 45"-1 minute; final extension at 72°C for 1 minute.

PCR products were purified with PolyEthylene Glycol 8000 protocol (http://www.mcdb.lsa.umich.edu/labs/olsen/files/PCR.pdf). When necessary, ITS amplification products were cloned using the CloneJet PCR Cloning Kit (Fermentas, Thermo Scientific).

Sequencing was carried out using BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Life Technologies). Capillary electrophoresis was conducted on an ABI 3130 Genetic Analyzer (Applied Biosystems, Life Technologies).

Electropherograms were inspected in search of incorrect assignments using the software BioEdit v7.2.5 (Hall, 1999). When chromatograms showed an overlapping peak of two nucleotides, a standard IUPAC ambiguity code was assigned (Y for C-T; W for A-T; R for A-G; S for C-G; M for A-C; K for G-T) if the lower peak was at least one third of the highest one. Sequences were aligned with Mesquite v2.75 (Maddison & Maddison, 2011) using the Muscle option (Edgar, 2004). Alignments were inspected and manually edited. Gaps were generally treated as missing data except an indel found in the chloroplast matrix, which was coded as binary characters.

Table 3 – Primers used for amplifications

Marker	Primer pairs	T_m
ITS (Aceto *et al.*, 1999)	**F:** 5' – GGA GAA GTC GTA ACA AGG TTT CCG – 3'	65° C
	R: 5' – CCA AAC AAC CCG ACT CGT AGA CAG C – 3'	63° C
	Internal pair:	
	F: 5' – TTG CAG AAT CCC GTG AAC CAT CG – 3'	65° C
	R: 5' – ATC CTG CAA TTC ACA CCA AGT ATC G – 3'	64° C
trnC-petN (Shaw *et al.*, 2005; Soza & Olmstead, 2010; modified)	**F:** 5' – CCA GTT CRA ATC YGG GTG – 3'	56° C
	R: 5' – CTC GTT CTA CAA TCA CGA TGT C – 3'	60° C
	Internal pair:	
	F: 5' – ATG GAT ATA GTA AGT CTY GCT TGG GC – 3'	65° C
	R: 5' – GCC CAA GCR AGA CTT ACT ATA TCC AT – 3'	65° C
petN-psbM (Shaw *et al.*, 2005; Soza & Olmstead, 2010; modified)	**F:** 5' – GACATCGTGATTGTAGAACGAG – 3'	60° C
	R: 5' – ATG GAA GTA AAT ATT CTY GCA TTT ATT GCT – 3'	63° C

Phylogenetic methods

Phylogenetic reconstruction was carried out using different analytical approaches: maximum parsimony (Kluge & Farris, 1969; Farris, 1970; Fitch, 1971), maximum likelihood (Felsenstein, 1981) and Bayesian inference (Yang & Rannala, 1997).

For inferring the model of evolution that best fit the data, chloroplast and nuclear matrices were analyzed with jModelTest v2.1.3 (Posada, 2008) using the Aikaike Information Criterion (AIC; Akaike, 1974).

Firstly, Bayesian analyses were carried out on each single dataset, employing the software MrBayes v3.2.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003), using the default prior options for the analyses of each marker. MCMC sampling was performed with 2 runs and a *Metropolis coupling* of 4 chains (1 cold and 3 heated); sampling frequency was 1000 generations and a relative burnin of 25%. After a number of generations equal to $5x10^{6}$, the average standard deviation of split frequencies was less than 0.005 for the nuclear matrix and slightly less than 0.01 for chloroplast dataset. Consequently, one million additional generations were carried out for the latter matrix. Convergence diagnostics was obtained with Tracer v1.5 (Rambaut & Drummond, 2009).

Subsequently MrBayes analysis was carried out specifying two distinct partitions for nuclear and chloroplast datasets, for $40x10^{6}$ generations, with a number of runs equal to 4 and 4 chains per run. In this case the analyses were run using a parallel version of MrBayes on the SCOPE supercomputing GRID facility of the University of Naples Federico II, gratefully acknowledged here. Convergence was assessed with Tracer v1.5 (Rambaut & Drummond, 2009) and AWTY online (Wilgenbusch *et al.*, 2004; Nylander *et al.*, 2008). The latter, in particular allows to examine different runs with the functions: "compare", "cumulative" and "var". They compare, respectively, split frequencies between the runs, split cumulative frequencies for each run and tree divergence between and among the runs (*symmetric tree-difference score*; Penny & Hendy, 1985).

PAUP* v4.0 (Swofford, 2003) was employed for the maximum parsimony analysis. Analysis was executed using a stepwise addition algorithm, with 500 bootstrap repeats and a TBR branch swapping.

A maximum likelihood hypothesis was obtained with the software RAxML v7.2.6 (Stamatakis, 2006), with the rapid bootstrap algorithm which allows to draw supports on the topology that maximizes the likelihood (Stamatakis, 2008). The optimal number of replicates was automatically determined with the bootstopping criteria, which allows to establish when stable supports values are obtained (Pattengale *et al.*, 2009), under the autoMRE option and the GTR model.

Divergence time estimation

Bremer & Eriksson (2009) conducted a thorough divergence time estimation for the whole family of Rubiaceae, in which all tribe ages were estimated. Consequently, a secondary calibration was possible for the group of the present study; in particular the crown age of *Rubieae* provided the age of the tree.

Unfortunately, no fossil record assigned with certainty to *Asperula* is available to date; Graham (2009) reported a fossil pollen from Miocene attributed to *Galium*, but employing it for fossil calibration s not without peril, as *Galium* is clearly non-monophyletic (Ehrendorfer *et al.*, 2005; Soza & Olmstead, 2010).

The software Beast v1.8.0 (Drummond & Rambaut, 2007; Drummond *et al.*, 2012) was employed to estimate divergence times. The .xml input file was created using the Beauti v1.8.0 software, assuming the model of sequence evolution specified for the previous analyses (GTR and GTR+G), a lognormal relaxed clock (Lepage *et al.*, 2007; Kishino *et al.*, 2001) and a Yule process of speciation (Yule, 1925). The crown age of *Rubieae* (18.1 Ma) was used as the prior for the height of the root, which was approximated to a normal distribution with a standard deviation equal to one; the analyses were run for 30 million generations. Convergence was checked examining the log file with Tracer v1.5 (Rambaut & Drummond, 2009); in particular, the ESS (Estimate Effective Sample Size) was considered satisfactory if it reached at least the value of 200 for each parameter. The maximum clade credibility tree was prepared using TreeAnnotator v1.8.0, setting a "burnin" depending on the likelihood examination in Tracer.

An additional analysis was set up to assess how the presence of ambiguities in the ITS matrix may affect divergence time estimation. In general, Bayesian Inference software do not take into account the ambiguities, i.e. the latter do not contribute to the likelihood estimation; Beast, however, allows attributing different priors. In particular, the .xml file was edited in the command <treeLikelihood id="treeLikelihood" useAmbiguities="false">, substituting the "false" with "true".

The Beast software only depicts fully resolved topologies, even for the clades with inconsistent posterior probabilities; the latter are obviously not considered when speculating about divergence time estimations.

Inference of the species tree

A further Bayesian approach was used to estimate a species tree of *Asperula* sect. *Cynanchicae*. The option *Beast (Heled & Drummond, 2010), implemented in the Beast v1.8.0 software allows to deal with situation of incomplete lineage sorting, which is one of the source of incongruence between gene tree and species tree. This option, in fact, combines the multispecies coalescent model (Rannala & Yang, 2003) and a Markov chain Monte Carlo sampling to generate a species tree drawn from the analysis of the single gene trees, in this case referring to nrDNA and cpDNA.

Several exploratory analyses were conducted for parameter tuning, in order to detect possible reductions in the ESS values due to over-parameterization. Consequently, the RootHeight priors used in the previous Beast analyses were removed and GTR model was switched to HKY to avoid the artificial extreme decreases of the ESS values of posteriors, priors and likelihood. Furthermore, setting a mean value for the lognormal relaxed molecular clock allowed avoiding negative branch lengths in the gene trees. In particular, ucld.mean was set to uniform with a range of 5×10^{-4} to 5×10^{-2} substitutions per site per My for the ITS matrix and 1×10^{-4} to 1.0×10^{-2} s/s/My for the chloroplast dataset. These rates largely contain the estimates for plants ITS rates (1.7-8.3×10^{-3} s/s/My; Kay *et al.*, 2006) and chloroplast rates (1.0-3.0×10^{-3} s/s/My; Wolfe *et al*, 1987).

Six independent runs of 50 million generations each were run, with a sampling frequency of 1000 generations; resulting trees were combined with LogCombiner v1.8.0, decreasing the frequency of sampling to 2000, and then summarized in a maximum credibility tree with TreeAnnotator v1.8.0.

Ancestral state reconstruction

As previously stated, it is difficult to identify discrete morphological characters that clearly discriminate among members of *Asperula* sect. *Cynanchicae*; in fact, most of the taxonomic keys are based upon quantitative characters (e.g., Ehrendorfer & Krendl, 1976) which are hardly adequate to sharply separate the taxa of the section. There is only one character that, albeit not discrete, behaves in a broad sense as a categorical trait: the length of corolla-tube with respect to the lobes. The state of this character for each member of *Asperula* sect. *Cynanchicae* is indicated in the Appendix C. Therefore, this distinction was used to operate an ancestral state reconstruction, as implemented in Mesquite v2.75 (Maddison & Maddison, 2011). The reconstruction was carried out under parsimony and maximum likelihood, using the Mk1 model (Lewis, 2001) for the latter framework, which attributes equally probable changes. The software needs a categorical file which specifies characters attribution and a topology with a good resolution (virtually complete); consequently, the maximum clade credibility tree deriving from the Beast analysis was used.

Results

Alignment length and characters information are summarized in Table 4. ITS sequences resulted rich in ambiguous sites, so they required a relevant time of editing. Some regions of the alignments were excluded: 5,8S gene, sites difficult to align and regions corresponding to the internal primers annealing. Homoplasy level of the ITS dataset (CI= 0.38) notably affected the concatenated analysis (Table 4).

The most adequate model for nuclear matrix resulted the General Time Reversible model (GTR; Lanave *et al.*, 1984; Tavaré, 1986), whereas for the chloroplast data, the model emerged was GTR with gamma distributed rate variation among sites (GTR+G).

In MrBayes analysis, convergence diagnostics on the outputs allowed to discard 25% of the samples.

Table 4 – Alignments and parsimony information

	# total analyzed characters	# constant characters	# parsimony informative characters	CI (parsimony analysis)	Tree length (parsimony analysis)
ITS1-ITS2	620	451	69	0.38	335
*trn*C-*psb*M	1234	1021	49	0.82	276
Total matrix (indels included)	1854	1490	118	0.40	625

Phylogenetic reconstruction

Bayesian inference revealed a poorly resolved topology for nuclear dataset, whereas chloroplast data gave better resolution; however topology was globally similar for both datasets (data not shown). When using the merged matrix, Bayesian approach produced the most resolved topology; maximum parsimony and maximum likelihood analyses provided an identical topology for the resolved clades but did not accomplish a satisfactory hypothesis of relationships in terms of global resolution. Bootstrap supports deriving from the aforesaid analyses are shown on the Bayesian phylogram Figure 1; for the geographical origin of the samples, see Appendix B.

Basal structure of the tree appears partly unresolved. *Asperula bornmuelleri* from Turkey seems to share only plesiomorphic states with the remaining part of sect. *Cynanchicae*, with a low posterior probability (0.55). A large basal politomy (Figure 1) encompasses predominantly Greek taxa, several of which are associated from a morphological point of view and are known to engender intermediate forms (Table 2). Interestingly, this politomy includes three of the four accessions of *A. cynanchica* analyzed in the study (sampled in Great Britain, Romania and Austria), and other non-Greek species: *A. rumelica*, an extremely variable entity distributed in the Balkan countries, Turkey and Russia, *A. pestalozzae* from Turkey and, lastly, *A. neilreichii* and *A. staliana* subsp. *arenaria*, endemic of different locations of the Balkans.

In this collapse some sister group relationships and few partially resolved clades emerge. Sister group relationships involve: *A. icarica* and *A lilaciflora* subsp. *runemarkii* (PP 1), which populate west Aegean isles; Turkish elements *A. affinis*, *A. woronowii*, *A. stricta* s.l., *A. nitida* and *A. tenuifolia* constitute an unresolved clade, supported by a posterior probability of 0.9; ultimately, the clade composed by Russian taxa *A. graveolens* subsp. *danilewskiana* and *A. diminuta* (PP 1), which are allied to the sister species *A. littoralis* and *A. tephrocarpa* (respectively from Turkey and Russia; PP 0.65,) form the unstable sister group of Clade A (Figure 1).

Clade A is the most resolved group in the phylogram; *A. supina* and *A. suffruticosa* play an outgroup role (respectively with a PP of 0.9 and 1). The cluster groups members of *A. aristata* s.l. in different phylogenetic relationships: *A. aristata* subsp. *aristata* 8H is closer to the southern Italian endemic *A. garganica*, to *A. calabra* (PP 1) and to the putative hybrid *A. x portae*. *Asperula aristata* subsp. *oreophila* 131,

from central Apennines, is related to *A. wettsteinii* and *A. rupicola*, two Alpine species (PP 0.7). The subspecies *A. aristata* subsp. *thessala* and subsp. *nestia*, sampled on Mount Olympus (Greece), form a sister group (PP 0.8), giving evidence of being closer than the others intraspecific taxa of *A. aristata*. Other accessions of this species remain in uncertain position (Figure 1).

Clade A also includes several taxa of the informal "*Palaeomediterraneae* group", in disparate and often collapsed positions (e.g., *A. naufraga*, *A. crassifolia*). Subclade 1 (PP 0.9) points out interesting connections concerning palaeo-endemic species *A. deficiens,* growing on the isle of Tavolara (Sardinia), *A. paui*, from Ibiza (Balearic Archipelago), and the Balkan assembly of *A. staliana* (except *A. staliana* subsp. *arenaria*), *A. borbasiana*, *A. visianii*, *A. woloszczakii* (PP 0.94). In this subclade, non-palaeo-endemic taxa are: the hypothetic hybrid *A. x jordanii*, *A. cynanchica* 128, from central Apennines, and *A. neglecta*, an endemic of the Gran Sasso massif (Apennines), which shares synapomorphies with *A. staliana* subspecies, *A. visianii* and *A. borbasiana* (PP 0.95).
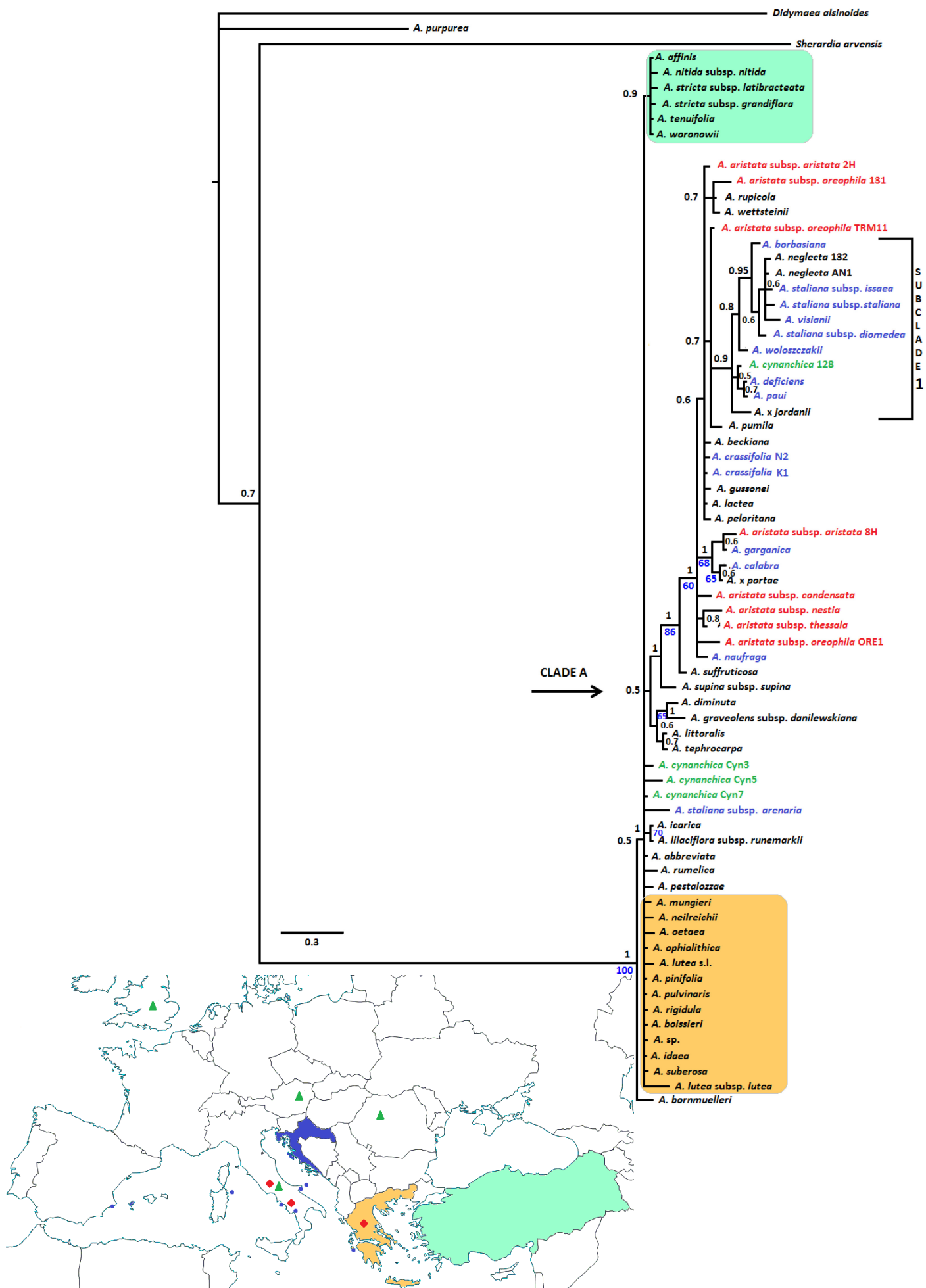
Figure 1 – Total evidence phylogram; node values in black represent posterior probabilities, values in blue represent bootstrap supports (maximum likelihood analysis). Terminals in blue indicate the *Palaeomediterraneae* assemblage (sensu Gutermann & Ehrendorfer, 2000); red terminals are *A. aristata* accessions, whereas green terminals are *A. cynanchica* accessions. Colors in the map refer to the terminals.

Divergence time estimation

The outputs of Beast analyses were checked for convergence in Tracer v1.5 (Rambaut & Drummond, 2009); all parameters reached an ESS value greater than 200. Maximum clade credibility trees were summarized using a "burnin" value of 10%.

The divergence time estimation yielded by Beast was reliable exclusively for some nodes of the chronogram, as posterior probabilities were generally low (Figure 2, Figure 3).

The time of divergence of *Asperula* sect. *Cynanchicae* turned out to be approximately 8.5 mya, hence relatively recent with respect to the crown age of *Rubieae* (18.1 myr). Divergence ages are dating back to Pleistocene for almost all the supported terminal nodes.

A comparison with the chronogram obtained after the treatment the ambiguities as indicated in Materials and Methods (Figure 3), reveals that the two approaches are essentially congruous: the time of divergence of the section *Cynanchicae* resulted the same, all the clusters which are simultaneously supported in both chronograms have approximately the same time of divergence and terminal nodes have a last common ancestor never more older than 2.5 My. Some clades are exclusively supported by one of the treatments and are accordingly absent in the other topology, e.g., the node separating *A. bornmuelleri* from the remaining taxa of the section (Figure 2, clades indicated by blue arrow), the node supporting the two main clades (Figure 3, clades indicated by blue arrow); however, the alternative clades that they constitute are not supported, so overlapping between the two topologies cannot be excluded.

Another minor difference concerns the number of well supported nodes: ambiguities treatment yielded 18 supported nodes versus the 22 provided by the simple treatment.

In terms of divergence times, it appears that the Turkish *A. bornmuelleri* diverged earlier than the remaining taxa of the section (8.5 mya, Figure 2). In Figure 3, the origin of sect. *Cynanchicae* begot the differentiation of two main clades, one predominantly composed by Turkish and Greek taxa and the other grouping taxa distributed in the western Mediterranean area and the Balkans.

Supported sister relationships are, in general, concordant with the framework outlined in Figure 1, except for the sister group relationship between *A. suffruticosa* and *A. supina* subsp. *supina* highlighted after the Beast analysis; they seem to have diverged about 2.5 mya.

Sister species *A. diminuta* and *A. graveolens* subsp. *danilewskiana* are linked to the sister group made up by *A. littoralis* and *A. tephrocarpa*, from which they probably diverged more than 2.5 mya (Figure 2, Figure 3). *Asperula icarica* and *A. lilaciflora* subsp. *runemarkii* are, once again, close species diverged in the late Pleistocene.

Politomy of Turkish taxa previously emerged from the Bayesian reconstruction (Figure 1) and composed by *A. affinis*, *A. stricta* s. l., *A. tenuifolia*, *A. nitida* subsp. *nitida*, *A. woronowii* diverged in the middle Pleistocene (Figure 2, Figure 3).

The robust clade (PP 0.9 in both the chronograms) mainly containing the presumed Palaeo-endemism and Italian taxa (clade A, Figure 2, Figure 3), shows that the separation between the putative palaeo-endemic taxa *A. calabra* and *A. garganica* with respect to the remaining ones, had already occurred at the end of the Miocene (Figure 2, Figure 3), with a PP > 0.9 in both the chronograms. Latest divergence among palaeo-endemic taxa occurred from 2.5 mya: the Balkan group (except *A. woloszczakii*) diverged from a cluster composed by *A. deficiens* and *A. paui* and, indeed, *A. woloszczakii*. *Asperula deficiens*, *A. paui* and *A. cynanchica* (not belonging to the *Palaeomediterraneae*), in particular, seem the most recently diverged. *Asperula borbasiana*, *A. visianii*, *A. neglecta* (another non-palaeo-endemic) and *A. staliana* assemblage (except *A. staliana* subsp. *arenaria*), on the contrary, had their last common ancestor about 2 mya.

Regarding infra-specific divergence, there are essentially three cases of deep divergence: 1) *A. cynanchica* taxa have their last (supported) common ancestor dating back to the Miocene, coinciding with the divergence time of the section; 2) the same circumstance is evident for *A. staliana* s.l., attributable especially to the disjoint position of *A. staliana* subsp. *arenaria*; 3) the *A. aristata* assemblage diverged about 5.5 mya.
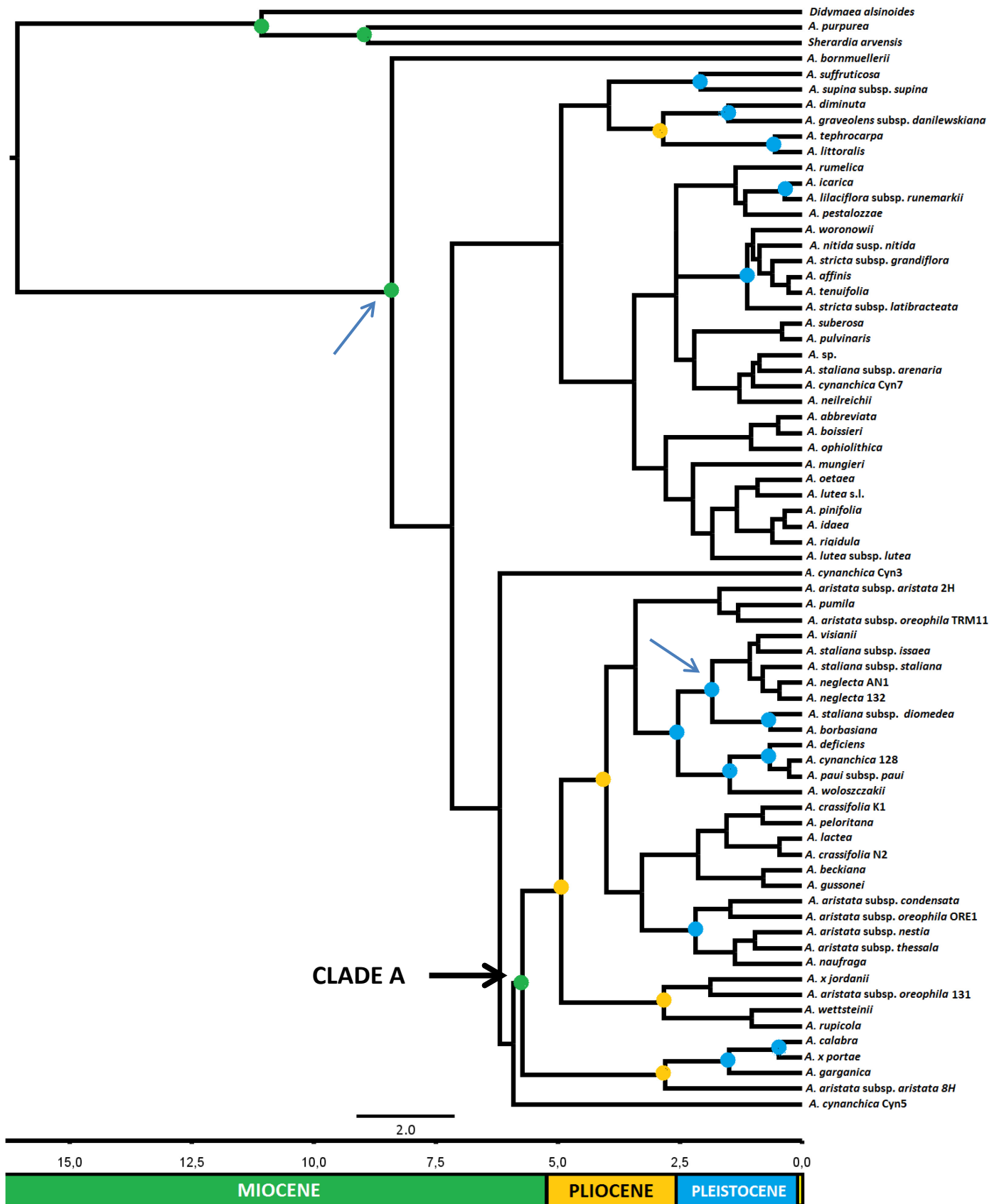
Figure 2 – Chronogram showing divergence time estimation for *Asperula* sect. *Cynanchicae*, based upon the ITS and *trn*C-*psb*M spacers merged dataset. The ambiguities are here treated as missing data. Nodes in evidence are supported by posterior probability values greater than 0.7.
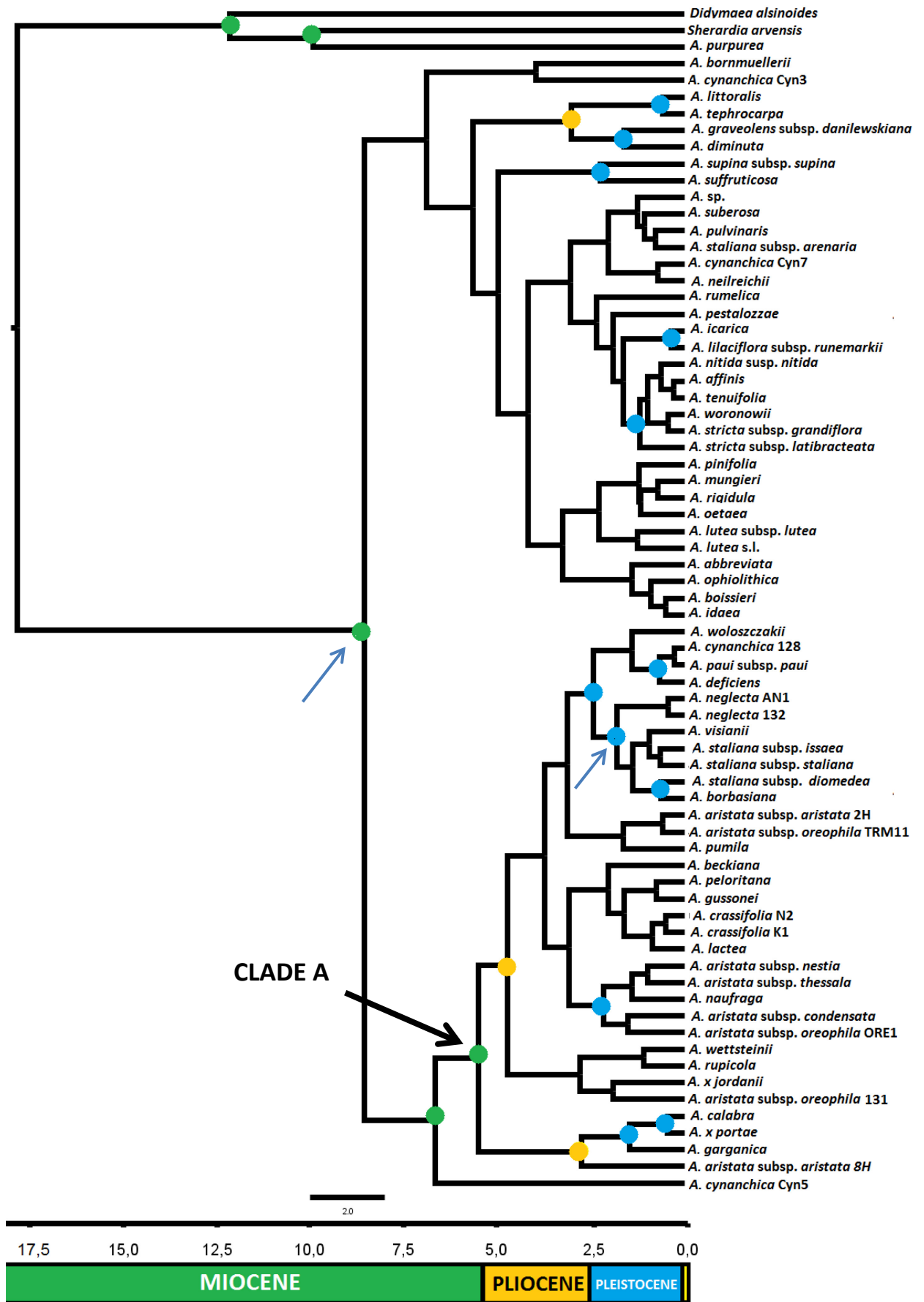
Figure 3 – Chronogram as in fig. 2, but with the treatment of ambiguities described in the text. Nodes in evidence are supported by posterior probability values greater than 0.7.

Inference of the species tree

Resulting log files from the *Beast analyses were inspected in order to assess that ESS> 200 was reached for all parameters. ITS and cpDNA trees are shown in Figure 4. Positions of *A. staliana* subsp. *arenaria* and *A. x jordanii* are incongruent; *A. staliana* subsp. *arenaria* appears related to the other conspecific members exclusively in the ITS tree (PP 0.84), whereas it seems close to other taxa in the cpDNA tree. *Asperula x jordanii* is related to *A. paui*, *A. deficiens* and *A. cynanchica* 128 according to the ITS tree; chloroplast dataset reveals its connection with *A. wettsteinii*, *A. rupicola* and *A. aristata* subsp. *oreophila* 131. Anyway, ITS tree resolution is lacking and this obscures further contingent incongruences.

*Beast analysis failed to generate a reliable species tree; the merger of trees obtained after the 6 separate analyses, in fact, did not provide posterior probabilities greater than 0.5.
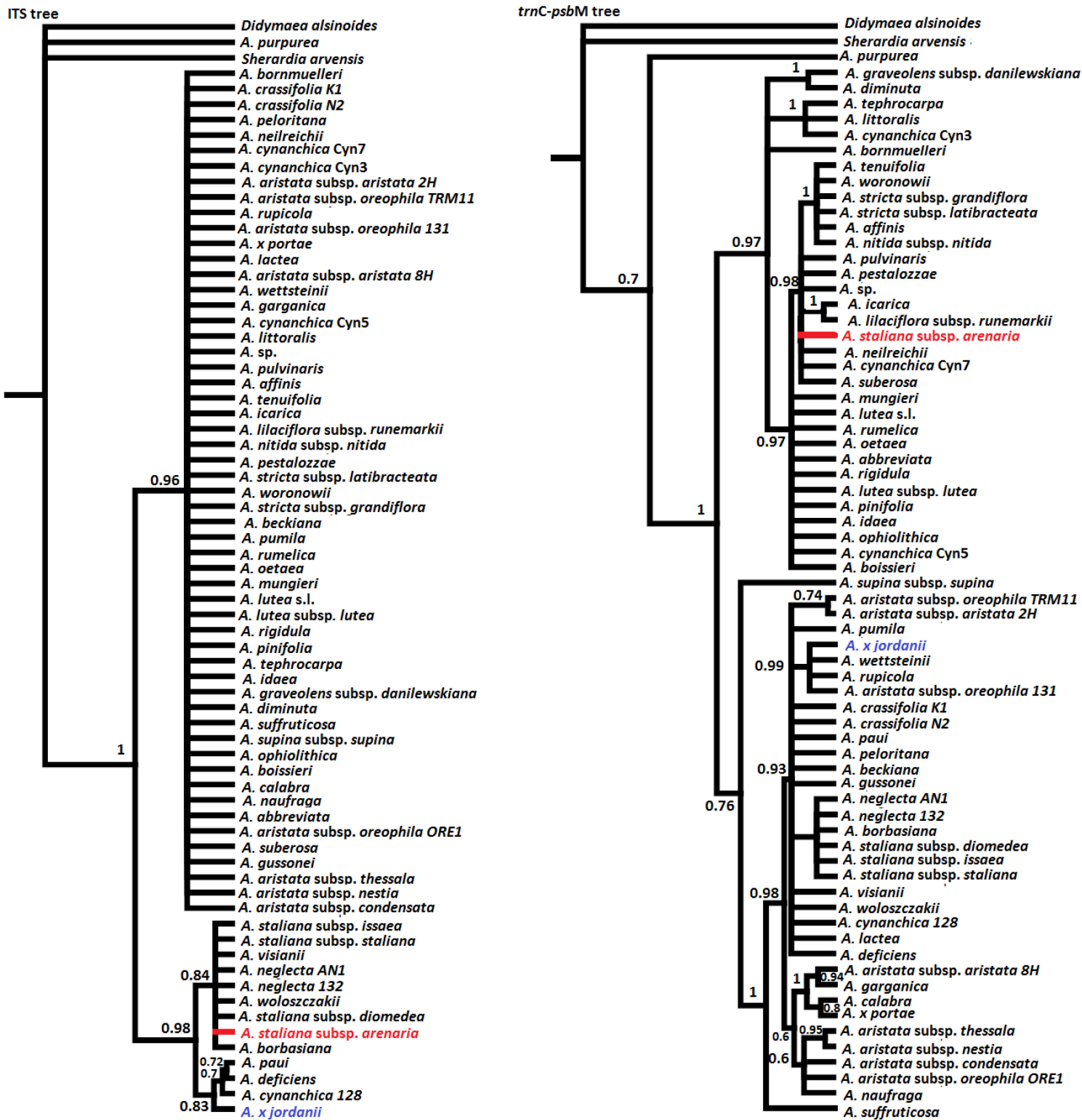
Figure 4 – Cladograms showing "gene trees" obtained after the *Beast analysis. Terminals in colour indicate incongruent positions. Node numbers are posterior probability values.

Ancestral state reconstruction

Corolla tube/lobe ratio shows a distribution which does not correspond to monophyletic units within the section, having homoplasiously varied several times within the inclusive group in study. Parsimony treatment as an unordered character (Figure 5) and likelihood estimation with Mk1 (Figure 6) gave similar pattern.

Figure 5 – Maximum credibility tree deriving from Bayesian estimation, displaying the parsimony ancestral reconstruction of the corolla tube/lobe ratio (see Appendix C), obtained using the Mesquite package.
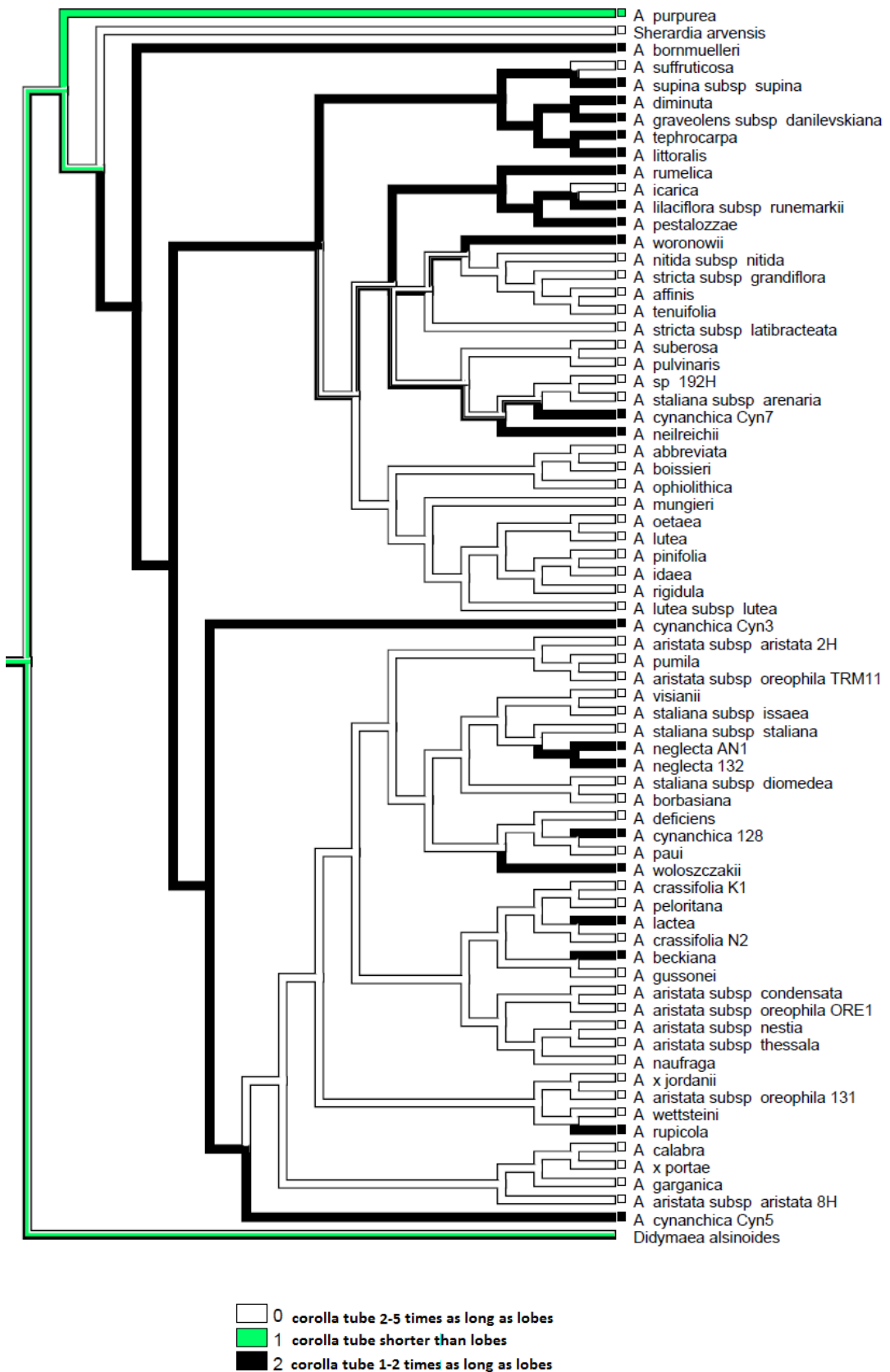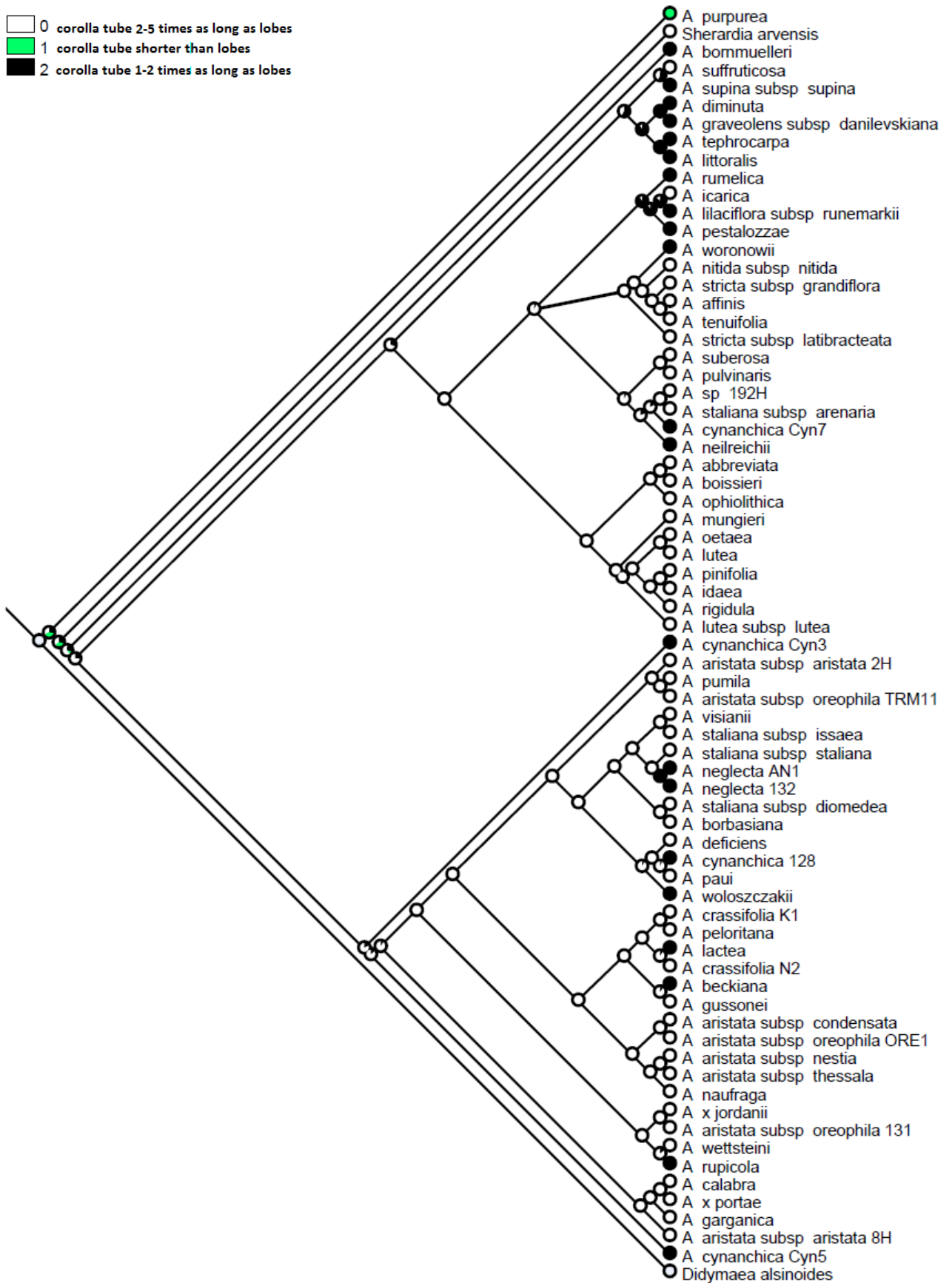
Figure 6 – Maximum credibility tree deriving from Bayesian estimation, displaying the likelihood ancestral reconstruction of the corolla tube/lobe ratio (see Appendix C), obtained using the Mesquite package.

Discussion

Approach to data analysis

In the last decade, parsimony methods have been relegated to an exploratory role, due to the relative speed of this approach as compared to the time consuming Bayesian and maximum likelihood methods. The latter two represent, to date, the most complete method to retrace phylogenetic histories. In the present work, two different Bayesian software were employed and their outputs were compared.

In the Beast software, the enormous flexibility in defining priors is a double-edged sword, as doubtlessly it allows to obtain a very robust phylogenetic estimation, but only provided that biological process underlying the data are exhaustively known.

Some authors suggest that the implementation of a relaxed molecular clock should be a normal routine in phylogenetics, not exclusively when the purpose is estimation of divergence (Drummond *et al.*, 2006; Pybus, 2006; Wertheim *et al.* 2010). Moreover, applying the coalescent model (Rannala & Yang, 2003) offers the opportunity to detect biological processes, such as incomplete lineage sorting (obviously, unless other phenomena influencing coalescence – e.g., hybridization - are also at work) which cannot be detected with a common matrix concatenation method. (Kubatko & Degnan, 2007; Heled & Drummond, 2010; Degnan *et al*., 2012; Heled *et al*., 2013).

Last but not least, the Beast software allowed exploring different treatments of ambiguities. The presence of ambiguities was restricted to the nuclear matrix, implying biological phenomena which contrast the ordinary concerted evolution of ITS sequences. Treatment of ambiguities in the Beast software did not affect divergence time estimation to a large extent. At any rate this may depend on the biological process that originated these ambiguities, and cannot be extended to other situations of ambiguous nuclear matrices.

Phylogenetic hypothesis

The first evidence shown by the phylogenetic exploration is the incomplete global resolution. Basically, shared apomorphies define some widely collapsed clades but, within them, it is rarely possible to detect further sinapomorphies diagnostic of dichotomous relationships. Conversely, several terminals exhibit apomorphies, often consisting in the indels of the cpDNA dataset. The ITS dataset has a quite low phylogenetic signal and this is probably the main reason of the failure of species tree reconstruction.

For various entities, however, probably real separation has never occurred (for instance in the case of collapsed Greek and Turkish entities). Morphological diversity may be attributable to ecological adaptations (i.e. altitudinal) and conclusive divergence will possibly occur in the future.

The second evidence is the presence of non-monophyletic specific assemblages, exemplified by the disjoint positions of various *A. staliana* subsp. *arenaria* and *A. cynanchica* accessions and particularly by the *A. aristata* assemblage. Subspecific members of the latter, in particular, have disparate phylogenetic positions in the trees and some of them have a very high divergence time as compared to the other species of *Asperula* sect. *Cynanchicae*.

Non-monophyly of a species is a well-known phenomenon documented in recent works of molecular phylogeny (Fuertes Aguilar & Feliner, 2003; Syring *et al.*, 2007; Schmidt-Lebuhn *et al.*, 2012) occurring when not all the entities assembled in a single species share molecular apomorphies. The reason of this intra-specific heterogeneity may be attributed to taxonomic inaccuracy (i.e. wrong attributions), incomplete lineage sorting (consisting into the differential persistence of ancestral polymorphic alleles) or hybridization and introgression (reticulate evolution; Wendel & Doyle, 1998). Unfortunately understanding which – or the combination of which – of the said processes is involved in the problem at hands is arduous (e.g., Doyle *et al.*, 1999; Sang & Zhong, 2000; Maureira-Butler *et al.*, 2008); it is possible, however, to formulate a hypothesis on which may be the most probable among the alternative explanations.

Hybridization hypothesis

It may be argued that non-monophyly of the species is due to the absence of reproductive isolation among members of *Asperula* sect. *Cynanchicae*. Evidences for this hypothesis are: 1) a geographical structure rather than a taxonomic structure in the phylogenetic tree, in particular for the most widespread entities (e.g., *A. aristata*, *A. cynanchica*), which are often in simpatry with other members of the section; 2) the well documented presence of intermediate phenotypes; 3) the variable and often polyploid chromosome numbers; 4) the large amount of ambiguities in the nuclear matrix, suggesting incomplete concerted evolution.

A conceivable scenario might be the following: hybridization could account for the relatively little divergence, virtually absent in the taxa without molecular apomorphies; reduced divergence in this case depends upon the continuous gene flow between species and, consequently, upon the homogeneity from both a morphological and a molecular standpoint (collapsed clade with short branches). High levels of homoplasy, especially in the nuclear matrix, is probably related to partial concerted evolution: ribotypes are unambiguous in some species (perhaps plesiomorphic or apomorphic and already subject to concerted evolution) whereas are ambiguous in other taxa. Indeed these ambiguities do not allow reliable phylogenetic reconstruction, because of reticulation.

Specific examples in which hybridization is highly probable include *A. cynanchica* and *A. aristata*. The propensity of some plant species to "capture" genomic portions of other species via hybridization and introgression has been object of several studies (Harlan & De Wet, 1963; Fuertes Aguilar et al., 1999; Fuertes Aguilar & Feliner, 2003), and entities with such behaviour were defined "compilospecies" (from the Latin compilo). *Asperula cynanchica* and *A. aristata,* whose intermediate forms are often observed (e.g., Ehrendorfer & Krendl, 1976; Schönbeck-Temesy & Ehrendorfer, 1991), may very well be compilospecies. If this is the case, they might be responsible of some biodiversity decline among *Asperula* sect. *Cynanchicae*. After the climatic vicissitudes of the Quaternary, range expansion may have caused contact of these "genetically aggressive" species with schizo-endemics. In spite of their differentiation because of fragmentation (Thompson, 2005), the absence of reproductive barriers may have made the involved endemics prone to be robbed out of their genomic unicity.

A case in which introgression was presumed concerns *Asperula x portae,* whose chromosome number ($2n$=35) was attributed to a hybridization/introgression. Phylogenetic reconstruction locates it as sister group of one of the putative parental species, *A. calabra* (its position is confirmed in the cpDNA tree), but with no special connection to the other putative parent, *A. aristata* subsp. *oreophila* of the same region (ORE1). ITS tree does not contribute to cast light on the issue but, however, the cpDNA identity between *A. calabra* and *A. x portae* is strongly suggestive of hybridization*.*

Asperula x jordanii* was described as an intermediate between *A. cynanchica* and *A. aristata* subsp. *oreophila* of the Alps. This hypothesis cannot be either confirmed or excluded; in fact, cpDNA tree shows its affinity to *A. aristata* subsp. *oreophila* 131, *A. wettsteinii* and *A. rupicola*, whereas it appears close to *A. paui, A. cynanchica* 128 and *A. deficiens* in the ITS tree.

In the Balkan assemblage of *A. staliana*, non-monophyly is attributable to the cpDNA of subspecies *arenaria* (Figure 4), which diverges from those of the other intraspecific taxa*.* ITS DNA of *A. staliana* subsp. *arenaria*, on the contrary, does not depart to a great extent from those of other conspecific individuals, regardless of the subspecies. As morphological evidence attests the cohesiveness of the *A. staliana* complex (and indeed, of all the Balkans palaeo-endemics), inaccurate taxonomy can be excluded.

Asperula staliana* subsp. *arenaria* may have arisen from a crossing of a male belonging to *A. staliana* and an unknown tetraploid female individual (all subspecies of *A. staliana* are tetraploid), followed by repeated backcrossing with a male member of *A. staliana.* This would explain both the presence of cpDNA extraneous to *A. staliana* s.l. and, at the same time, nuclear and morphological homogeneity of subsp. *arenaria* with the other subspecies of *A. staliana*. The identity of the hypothetical contributor to the maternal lineage cannot be ascertained, as *A. staliana* subsp. *arenaria* haplotype is present also in various other members of sect. *Cynanchicae*.

Lineage sorting hypothesis

A second possible explanation for the non-monophyly of some species in sect. *Cynanchicae* may be the existence of incomplete lineage sorting (Pamilo & Nei, 1988; Maddison, 1997).

According to the nuclear dataset, *A. staliana* subsp. *arenaria* may be assimilated to the *A. staliana* complex; consequently, the incomplete lineage sorting may more probably have involved cpDNA. A hypothesis may be the following: cpDNA was heterogeneous in the ancestral population of *A. staliana*, but only subspecies *arenaria* retained a different haplotype. The observation of the chloroplast dataset reveals that the incongruence of *A. staliana* subsp. *arenaria* is based on the whole cpDNA haplogroup, not only on a single haplotype. This haplogroup is almost identical to the one of the Eastern members of sect. *Cynanchicae* (Turkish, Russian and several Greek taxa), so an incomplete lineage sorting hypothesis would imply the existence of an ancestral, nearly panmictic population which included also the ancestor(s) of the Eastern members of sect. *Cynanchicae*. Moreover this would entail that, after speciation, this haplogroup remained almost unvaried in the two lineages, in spite of the fact that both ITS and morphological characters in the *A. staliana* group moved toward cohesiveness.

Referring now to *A. cynanchica* and *A. aristata*, doubtlessly the existence of wide-distributed large population makes the incomplete lineage sorting hypothesis more probable (Nei & Kumar, 2000). In spite of this, the clear geographical structure in the non-monophyletic assemblage that they constitute conveys the impression that incomplete lineage sorting may be only a concomitant factor, but not the prevailing one: hybridization, in fact, seems more likely to be at the foundation of the lack of monophyly at species level in *A. cynanchica* and *A. aristata*.

Chapter 3 – Analysis of the endemic *Asperula crassifolia* L.: conservation and biogeographical implications

Introduction

Asperula crassifolia L. is a narrowly endemic species of southern Italy. To date, its distribution is circumscribed to three locations: the island of Capri, the Sirenusae archipelago (also known as Li Galli) and a locality in the southern part of the Sorrentine Peninsula (Nerano; Figure 7). The largest population of *A. crassifolia* is located in Capri, where it is distributed in different areas of the island, ranging from Mount Solaro (589 m) to the sea level (Marina Piccola). In the first decades of '900 the botanist Guadagno discovered the population located in the Sirenusae archipelago (Guadagno, 1913); finally, in the eighties of the same century, Caputo *et al*. found the population located in the Sorrentine Peninsula (1989-90).

The entity was firstly described in pre-Linnaean era by Boccone (1697) as *Gallium totum villosum, supinum, folio retuso*; later, it was formally described by Linnaeus (1767). Afterwards, *A. crassifolia* was indicated by Tenore as *Asperula tomentosa* Ten. (1811) but, subsequently, the identity of descriptions associated to the two names was repeatedly noted (e.g., De Candolle, 1830; Nyman, 1879); the latter name is now accepted as a synonym (Ehrendorfer & Krendl, 1976; Govaerts *et al*., 2006).

Asperula crassifolia is a dwarf shrub, woody at the base (suffrutescent chamaephyte) growing on calcareous rocks (Figure 8). It is prevailing on well-exposed slopes, but is also found on shady rocks. Every element of the plant is generally hairy. Cauline leaves are lanceolate or linear, coupled from the middle part of the stem upwards; the basalmost leaves are obovate and crowded; leaf margins are revolute. Inflorescence is pyramidal and partial inflorescences are capitate. Corolla is hypocrateriform and yellowish, with a tube 2-3 times longer than lobes (Ehrendorfer & Krendl, 1976; Ehrendorfer, 1982).

This species is reported as a Tyrrhenian palaeo-endemism (Ehrendorfer, 1982; Gutermann & Ehrendorfer, 2000), belonging to the informal "*Palaeomediterraneae* group"; in this perspective, *A. crassifolia* modern distribution may represent the relict distribution of a previous larger geographical range. Unfortunately no reliable divergence time estimation was obtained for this taxon (Figure 2, Figure 3), although it is likely that the most common ancestor lived during the late Tertiary, as asserted by Gutermann & Ehrendorfer (2000). Current distribution of *A. crassifolia* populations may derive from a vicariance event rather than a dispersion event, although the latter hypothesis cannot be excluded in principle. If vicariance is accepted, then the origin of *A. crassifolia* predates the definitive separation of Capri and Sirenusae from the Sorrentine Peninsula, dating back respectively 18,000 to 15,000 ya for the former and approximately 12,000 ya for the latter (Barattolo *et al*., 1992; Lambeck *et al*., 2004; Lambeck *et al*., 2011).

Asperula crassifolia embodies a good model for evolutionary study within sect. *Cynanchicae*; in fact, vicariant populations might be assimilated to the disjunct endemisms typical of the section and, moreover, the species may epitomize an ancestor, at least for a fraction of the *Cynanchicae* group, as suggested by Gutermann & Ehrendorfer (2000) on account of its diploid status. Indeed, its palaeomediterranean distribution and its diploid chromosome number may suggest similarity to the patroendemic responsible for the origin of (some) tetraploid and polyploid taxa (Thompson, 2005).

Apart from the evolutionary relevance, focus on *A. crassifolia* may have important conservational implications. The first step to adopt any conservation strategy, indeed, is the detailed assessment of the consistency and ecology of the populations of the involved species (Giam *et al*., 2011). The definition of endemism is not necessarily linked to rarity and vulnerability (Kruckeberg & Rabinowitz, 1985); abundance and habitat specificity are also important clues for considering a species as rare (Prober & Austin, 1990), whereas reproductive strategies, resilience and anthropic perturbations may also affect survival (Schemske *et al*., 1994).

Some members of *Asperula* sect. *Cynanchicae* are already included in the Red List of Threatened plants (IUCN, 2001), whereas no status is currently provided for *A. crassifolia*. In particular, one member of the section, indicated as Vulnerable (VU), is *A.*

daphneola O.Schwarz. This entity is distributed in 5 localities of southern Greece, covering a total area of 2.5 km^2 and a total number of individuals estimated in approximately 8000 (Gücel & Seçmen, 2009). An exhaustive study focusing on the pollination biology of this species demonstrated that autogamy is partially involved into the decline of the abundance for *A. daphneola*, to such an extent that the authors propose to change the IUCN category in Critically Endangered (CR; IUCN, 2001; Gücel & Seçmen, 2009). This proves that danger of extinction is not always strictly related to rarity.

In order to elucidate the phylogeography of *A. crassifolia* and to outline the genetic structures of this entity, a population analysis has been undertaken, using both nuclear microsatellite fragments and cpDNA *rps*16 intron sequences. In addition, the abundance and the state of the habitat of *A. crassifolia* were assessed during the sampling procedure; this is mandatory to evaluate the level of risk (IUCN, 2001; Rodrigues *et al.*, 2006) and to establish a possible status of protection (Rodrigues *et al.*, 2004).
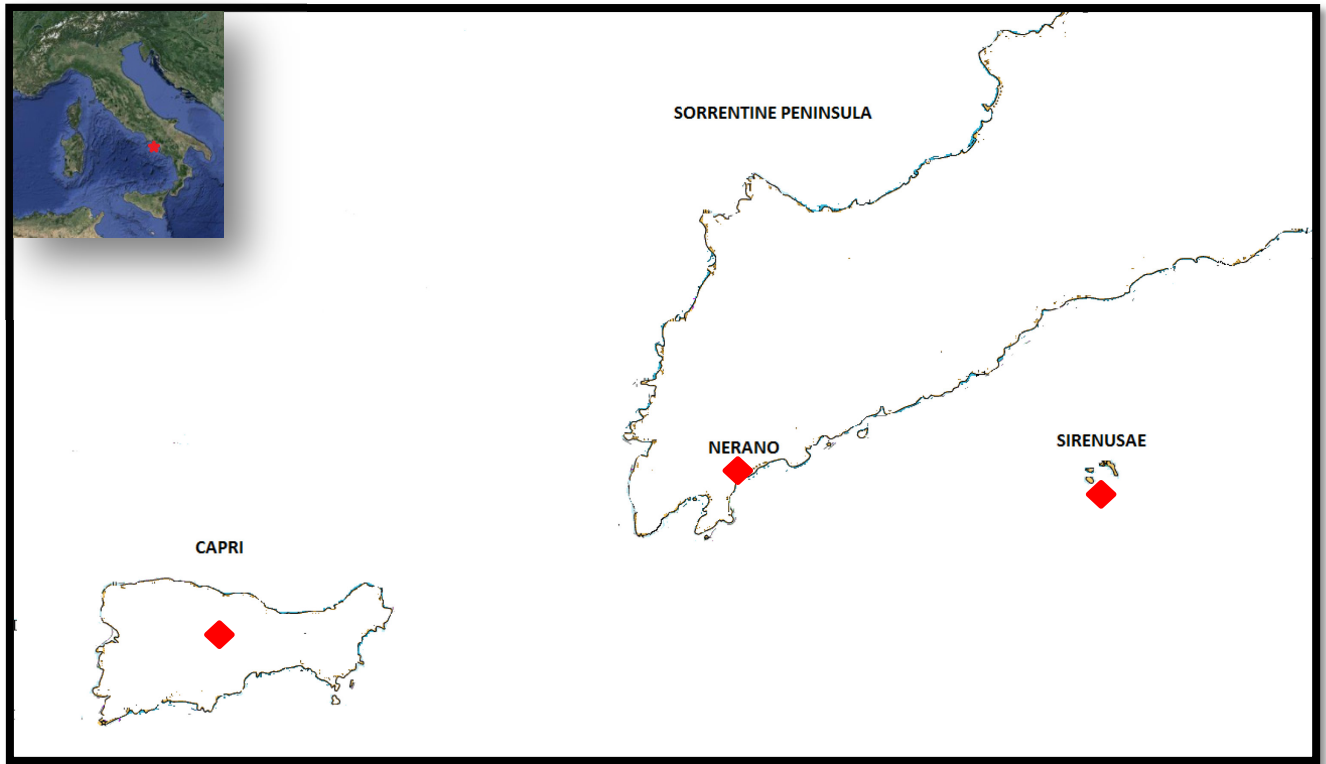
Figure 7 – *Asperula crassifolia* **distribution**

Figure 8 – *Asperula crassifolia* **(right: detail of a partial inflorescence)**

Material and methods

Sampling

Sampling was conducted during the flowering period of *A. crassifolia*; plants were searched in all the locations indicated in literature (e.g., Tenore, 1811; Guadagno 1913; Ricciardi, 1996, Caputo *et al.*, 1989-1990). In addition to field-collected plants, various historical herbarium specimens were also employed. In all cases, sampling was non-destructive. Collection and sample data are listed in Table 5 and Table 6.

DNA extraction and SSR protocol

Specific primers for *Asperula* are not available to date so the first effort was the isolation of repeated motifs suitable for primers construction.

Isolation of microsatellites was conducted following the SSR-patchwork protocol (Di Maio & De Castro, 2013a). DNA was extracted from the sample K1 (Table 5), with lysis in liquid nitrogen followed by CTAB method (Doyle & Doyle, 1990) including an RNase step. Two µg of genomic DNA were cut with restriction enzymes (MseI and EcoRI); the fragments of interest (ranging from 250 to 500 bp) were selected on 1% agarose gel, then purified using GENECLEAN® II Kit (MP Biomedicals). Adapters for the cut produced by the two restriction enzymes were prepared and ligated to the fragments; subsequently a first enrichment was performed, in which ligated fragments were amplified.

The preparation of the biotinylated probe was carried out employing two motifs, GA_{12} e CAA_{10}; probes were then hybridized to the products of the first enrichment. The isolation of the probe-product complex was operated using the Vetrex Avidin D (Vector Laboratories); afterwards, PCRs were performed for a second enrichment (for the PCR set-up, see Di Maio & De Castro, 2013a).

PCR products were purified using PEG precipitation protocol (http://www.mcdb.lsa.umich.edu/labs/olsen/files/PCR.pdf) and cloned using the Thermo Scientific CloneJet PCR Cloning Kit. Colony PCR was performed in a final volume of 20 µL, using FIREPol DNA Polymerase 1 U (Solis BioDyne), *Reaction Buffer B* 1X, $MgCl_2$ 2 mM, dNTPs 0.2 mM, primers 0.25 µM, and water to final volume.

Sequencing was carried out using BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Life Technologies), with the following thermocycler program: 25 cycles of 96° C for 10", 50° C for 5", 60° C for 4 minutes. Capillary electrophoresis was conducted on an ABI 3130 Genetic Analyzer (Applied Biosystems, Life Technologies), after an EDTA-Sodium Acetate purification step of the sequencing products.

Table 5 – Sampling details

Sample	Location	Sample	Location
S1	Capri - Monte Solaro	G6	Gallo Lungo
S2	Capri - Monte Solaro	G7	Gallo Lungo
S3	Capri - Monte Solaro	G8	Gallo Lungo
S4	Capri - Monte Solaro	G9	Gallo Lungo
S5	Capri - Monte Solaro	G10	Gallo Lungo
T1	Capri - Via Tragara	N1	Nerano
T2	Capri - Via Tragara	N2	Nerano
T3	Capri - Via Tragara	N3	Nerano
T4	Capri - Via Tragara	N4	Nerano
K1	Capri - Via Krupp	N5	Nerano
K2	Capri - Via Krupp	N6	Nerano
K3	Capri - Via Krupp	N7	Nerano
K4	Capri - Via Krupp	N8	Nerano
K5	Capri - Via Krupp	N9	Nerano
K6	Capri - Via Krupp	N10	Nerano
K7	Capri - Via Krupp	N11	Nerano
K8	Capri - Via Krupp	N12	Nerano
K9	Capri - Via Krupp	N13	Nerano
K10	Capri - Via Krupp	N14	Nerano
G1	Gallo Lungo	N15	Nerano
G2	Gallo Lungo	N16	Nerano
G3	Gallo Lungo	N17	Nerano
G4	Gallo Lungo	N18	Nerano
G5	Gallo Lungo	N19	Nerano

Table 6 – *A. crassifolia* herbarium specimens

Herbarium specimens	Label indications
PAS1 (herbarium Pasquale NAP)	"Scala di Anacapri" – 1868
PAS2 (herbarium Pasquale NAP)	"Scala di Anacapri" – 1868
GUS1 (herbarium Gussone NAP)	"Rupi di Capri" – 1808
GUS3 (herbarium Gussone NAP)	"Rupi di Capri" – 1808
GUS5 (herbarium Gussone NAP)	"Capri" – 1808
BOR1 (herbarium Bornmueller B)	"In rupibus insulae Caprearum" – 1901
BOR2 (herbarium Bornmueller B)	"Capri: Anacapri, ad rupes verticales" – 1933
BOR3 (herbarium Bornmueller B)	"Capri: ad Marina Piccola, in rupibus umbrosis" – 1933
BAS (herbarium Baschant B)	"Capri" – 1930
GUA (herbarium Guadagno B)	"Rupi. Isola di Capri" 1900 (approx.)

The *rps*16 intron

The *rps*16 intron was amplified for each sample, using the primers *rps*F (5'-GTG GTA GAA AGC AAC GTG CGA CTT-3') and *rps*R2 (5'-TCG GGA TCG AAC ATC AAT TGC AAC-3') by Oxelman (1997), and an internal reverse primer designed *ad hoc* for *Asperula* (Asp-rpsRI: 5'-CCG GCA ATT AGT GAG ACG GTG-3').

Alignment of *rps*16 intron sequences were conducted with Muscle (Edgar, 2004) as implemented in Mesquite v2.75 (Maddison & Maddison, 2011). The *rps*16 haplotypes were inferred by the observation of the aligned sequences; indels were treated as point mutations (i.e., single-step events). A. parsimony-based phylogenetic network was constructed with TCS v1.21 (Clement *et al.*, 2000). The outgroup was chosen on the basis of the observation of the haplotypes in related species: various members of the section *Cynanchicae* from Southern Italy (i.e. *A. lactea*, *A. calabra*, etc.) and especially the geographically close *A. aristata* subsp. *aristata* 8H from Sorrentine Peninsula (Mount Faito).

Results

Habitat observation

The habitat at Nerano is extremely deteriorated, surrounded by cliffs protected by containment nets and by dry stone walls. The populations in Capri are located mostly along tourists' paths. The most undisturbed population is the one at Sirenusae: the Gallo Lungo Island, on which sampling was conducted, is in fact privately owned.

Primer design

Electropherograms were inspected and edited using BioEdit v7.2.5 (Hall, 1999). This step is crucial in order to design primers correctly, as it is essential to remove the part of the sequence which belongs to the cloning vector and the part belonging to the restriction enzyme adaptors (which occasionally can be found interspersed in the sequence and not necessarily at the ends).

Approximately 150 clones were screened; the majority of them showed the same sequence, others gave sequences not suitable for primer constructions and others again provided no repeated motif. Isolated microsatellites are shown in Table 7.

Table 7 – Isolated repeats for *A. crassifolia*

	SSR repeats
Clone 1	$(AG)_5$
Clone 2	$(AG)_{18}$
Clone 3	$(GA)_4(AG)_5(AG)_4$
Clone 4	$(GA)_{15}(AG)_6$
Clone 5	$(GA)_9$
Clone 6	$(GA)_{26}$
Clone 7	$(GA)_3(GAGG)_3$
Clone 8	$(GA)_{24}GT(GA)_{10}$
Clone 9	$(AG)_{10}$
Clone 10	$(AG)_4(GA)_8$
Clone 11	$(GAAA)_2(GA)_4(GAAA)_4$
Clone 12	$(AG)_{15}C(GA)_5A(GA)_{10}$
Clone 13	$(CT)_{28}$
Clone 14	$(TCC)_5$
Clone 15	$(AG)_3(GT)_4(CT)_5$
Clone 16	$(CT)_{20}$
Clone 17	$(CT)_6C(CT)_9$
Clone 18	$(GA)_{10}$
Clone 19	$(TC)_{13}$
Clone 20	$(TC)_{63}AAATATT(CT)_4A(TC)_7C(CT)_{11}(AT)_8$
Clone 21	$(AG)_7$
Clone 22	$(AG)_4(GT)_4(CT)_{20}$
Clone 23	$(AG)_3TG(GA)_4(GT)_4A(GT)_3GA(GT)_3$
Clone 24	$(AG)_6$
Clone 25	$(AG)_8AA(AG)_{12}$
Clone 26	$(CT)_9(TC)_4$
Clone 27	$(TC)_{19}$
Clone 28	$(CT)_6$

(continued)

	SSR repeat
Clone 29	$(GTT)_5(GGTTT)_3G(GTT)_3GTA(GGTTT)_3$
Clone 30	$(GTT)_4$
Clone 31	$(TGG)_5$
Clone 32	$(GT)_5(TG)_4(GT)_3$
Clone 33	$(GTT)_4$
Clone 34	$(AG)_4(GT)_9$
Clone 35	$(TGG)_5$
Clone 36	$(GTT)_3(AG)_3$

The *rps*16 intron

The observation of the *rps*16 sequences in closely related species allowed to determine the putative ancestral haplotype, that was invariant in the chosen outgroup.

The *rps*16 intron is highly variable in the first part of the sequence, which includes indels; four haplotypes were globally detected (Appendix D). Populations of Nerano and Sirenusae share the same haplotype, whereas populations of Capri show all the detected haplotypes. Network is shown in Figure 9: the most represented haplotype is that indicated with A, found in 52% of the entities sampled; then haplotype C, which coincides with the ancestral one and is present in the 27% of the individuals. Haplotype B has a percentage of 19%; haplotype D is the least represented (2%), being present in one single accession.

The hot spot of *A. crassifolia* diversity turned out to be the island of Capri. In particular, 3 of the 4 haplotypes (B, C, D) were observed in recently sampled accessions; haplotype A belongs to an historical herbarium specimen (PAS2; Table 6). The label of this specimen indicates "Scala di Anacapri", a location in which, unfortunately, no accession was found recently. The A haplotype is the only haplotype found in Nerano and Sirenusae as well.
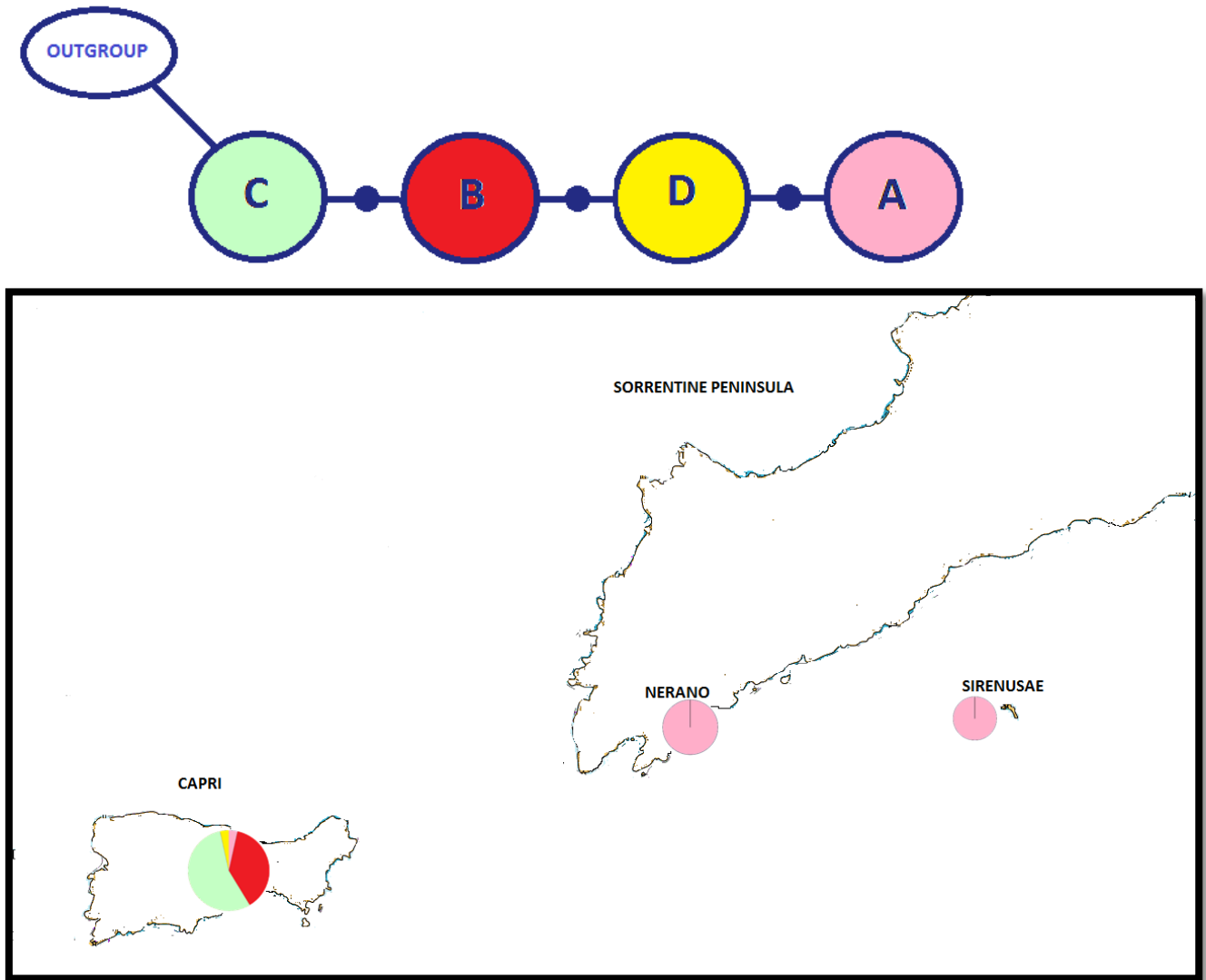
Figure 9 – The network displaying the haplotypes detected in the populations of *A. crassifolia*. Below: geographical distribution of haplotypes. Coloured areas are proportional to the number of individuals.

Discussion

Biogeographic hypothesis

Asperula crassifolia represents a rare and endemic species whose peak of variability was recorded for the Isle of Capri. Hypothesis on its origin may be reconstructed in two different ways.

It may be argued that *A. crassifolia* originated on the mainland during the late Tertiary, as generally indicated in Chapter 2; the original population split on the nearby islands (Capri and Sirenusae) as a consequence of the eustatic oscillations occurred during the Quaternary. This scenario need further assumptions to justify the reason of the greater variability recorded for Capri population. In this context, Nerano population may really be the residual of a previous larger distribution on the mainland, and a bottleneck led to the persistence of a unique haplotype in Nerano; in this perspective, the existence of the same haplotype in Sirenusae may be ascribed to the connection of these little islands to the mainland or to a dispersion event occurred after separation, approximately 12,000 ya (later than the estimated separation of Capri; Barattolo *et al*., 1992; Lambeck *et al*., 2004; Lambeck *et al*., 2011). The variability for the Island of Capri might be depending on ancient polymorphism, but may also be the result of outcrossing with the sympatric *A. aristata* subsp. *aristata* (Ricciardi, 1996). Unfortunately, individuals belonging to the foresaid species were not found (albeit repeatedly searched) during the sampling.

A second possibility to justify the results shown here is that *A. crassifolia* originated in Capri, where the genetic variability is highest, and subsequent events of vicariance or dispersion towards Nerano and Sirenusae. However, not even in this case possible outcrossing with the sympatric *A. aristata* may be ruled out.

Proposed IUCN conservation category

The habitat inspection revealed that, albeit *A. crassifolia* is not in a forthcoming danger, there are several reasons for which the species should be considered vulnerable. Unfortunately, no precise estimation of the relative abundance of each population was possible, as the specie grows on rocky slopes not easy to monitor. Consequently data concerning the risk of decline are lacking as well.

Populations of Nerano and Capri, the latter in particular, are exposed to anthropogenic impact (tourism); moreover, Nerano belongs to a regional park, but safeguards are not guaranteed. The habitat of *A. crassifolia*, in fact, is surrounded by cultivations and dry stone walls covered with containing nets. Population of Sirenusae is probably the least threatened, as two of the three isles are inaccessible and the largest one is a private estate.

On the basis of observations, *A. crassifolia* should be considered as Vulnerable (VU), and in particular it should be attributed to the VU-D2 category (IUCN, 2001). In fact, a taxon qualifies for this category if the area of occupancy is very restricted (typically less than 20 km^2, although the numerical threshold is not intended to be interpreted strictly), or the taxon exists at typically five or fewer locations. Moreover, a necessary requisite to be included in this category is the plausibility of a stochastic event (anthropogenic or natural) which leads to make the taxon Critically Endangered or even Extinct within one or two generations after the event (IUCN, 2013). Tourism in Capri and downfall of boulders in Nerano (where, furthermore, nearby cultivations are present) constitute serious threats to *A. crassifolia* individuals.

General conclusions

Phylogeny of *Asperula* sect. *Cynanchicae* appears as a mixture of different evolutionary histories, in terms of distributions, ecological needs and interactions with allied species; on account of this, it is impossible to seek an univocal evolutionary explanation without examining each species at population level.

The failure of species tree reconstruction and, to a genetic level, the extensive failure of complete concerted evolution leads to the conclusion that recent hybridization is the predominant force affecting the evolution of *Asperula* sect. *Cynanchicae*. Probably hybridization has periodically occurred during the whole Quaternary as a consequence of repeated range expansions and contractions, contributing to entangle relationships to such an extent as to compromise a reliable tree-like phylogeny. In addition, possible further subtle events of reticulation may be concealed, due to the lack of molecular resolution.

Besides hybridization, probably many species of the section are not sharply differentiated, as corroborated by the absence of clear morphological synapomorphies (Schmidt-Lebuhn *et al.*, 2012)

Another important evidence arisen from the study is that using single accessions of widely distributed taxa may be extremely misleading for phylogenetic reconstruction, as shown for other organisms (Comes & Abbott, 2001; Spinks *et al.*, 2013). This is due to the possible occurrence of different selective pressures or other biological phenomena which may have differentially affected individuals of the same species.

Asperula crassifolia embodies an emblematic example for section *Cynanchicae*; population-level analysis disclosed an unexpected variability. If we extend the peculiarity of *rps*16 intron to the whole genome, the populations of Nerano and Sirenusae represent an evolutionary *unicum* and, as such, they deserve safeguards.

Literature cited

Aceto S., Caputo P., Cozzolino S., Gaudio L., Moretti A., 1999 – Phylogeny and evolution of *Orchis* and allied genera based on ITS DNA variation: morphological gaps and molecular continuity. *Molecular Phylogenetics and Evolution*. **13:** 67-76.

Akaike H., 1974 – A new look at the statistical model identification. *IEEE Transactions on Automatic Control.* **19.6**: 716-723.

Aldrich J., 1997 – R. A. Fisher and the making of maximum likelihood 1912–1922. *Statistical Science.* **12 (3):** 162–176.

Andersson L., Rova J. H. E., 1999 – The *rps*16 intron and the phylogeny of Rubioideae (Rubiaceae). *Plant Systematics and Evolution*. **214:** 161-186.

Andreasen K., Bremer B., 1996 – Phylogeny of the subfamily Ixoroideae (Rubiaceae). *Opera Botanica Belgica*. **7:** 119-138.

APG – The Angiosperm Phylogeny Group, 2003 – An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. *Botanical Journal of the Linnean Society*. **141:** 399-436.

Avise J.C., 1994 – Molecular markers, natural history and evolution. *Chapman and Hall, New York.*

Avise J.C., 2000 – Phylogeography: The history and formation of species. *Harvard University press, Cambridge, MA.*

Backlund M., Bremer B., Thulin M., 2007 – Paraphily of Paederieae, recognition of Putorieae and expansion of *Plocama* (Rubiaceae). *Taxon*. **56:** 315-328.

Baldwin B.G., 1992 – Phylogenetic utility of the Internal Transcribed Spacers of Nuclear Ribosomal DNA in Plants: an example from the Compositae. *MoleculaPhylogenetics and Evolution*. **1(1):** 3-16.

Baldwin B.G., Sanderson M. J., Porter J. M., Wojciechowski M. F., Campbell C. S., Donoghue M. J., 1995 – The ITS region of Nuclear Ribosomal DNA: A valuable

source of evidence on Angiosperm Phylogeny. *Annals of Missouri Botanical Garden*. **82(2):** 247-277.

Barattolo F., Cinque A., D'Alessandro E., Guida M., Romano P., Russo Ermolli E., 1992 – Geomorfologia ed evoluzione tettonica quaternaria dell'Isola di Capri. *Studi geologici camerti, volume speciale,* pp. 221-229.

Bayes T., Price R., 1763 – An Essay towards solving a problem in the doctrine of chance. By the late Rev. Mr. Bayes, communicated by Mr. Price, in a letter to John Canton, M. A. and F. R. S. *Philosophical Transactions of the Royal Society of London.* **53:** 370–418.

Bernardo l., passalacqua n. G., peruzzi l., 2010 – Notula: 1741. *A. x portae* (Rubiaceae). In: Nepi C., Peccenini S., Peruzzi L. (eds.), Notulae alla Checklist della flora italiana: 10. *Informatore Botanico Italiano: Bollettino della Società Botanica Italiana*. **42(2):** 530.

Boccone P.S., 1697 – Museo di Piante rare della Sicilia, Malta, Corsica, Italia, Piemonte e Germania. *B. Zuccaro, Venezia*, p.172.

Bremer B., Jansen R.K., 1991 – Comparative restriction site mapping of chloroplast DNA implies new phylogenetic relationships within Rubiaceae. *American Journal of Botany*. **78:** 198-213.

Bremer B., 1996 – Phylogenetic studies within Rubiaceae and relationship to other families based on molecular data. *Opera Botanica Belgica*. **7:** 33-50.

Bremer B., Thulin M., 1998 – Collapse of Isertieae, re-establishment of Mussaendeae, and a new genus of Sabiceeae (Rubiaceae): phylogenetic relationships based on *rbc*L data. *Plant Systematics and Evolution*. **211:**71-92.

Bremer B., Jansen R.K., Oxelman B., Backlund M., Lantz H., Kim K.J., 1999 – More characters or more taxa for a robust phylogeny in a case study from the coffee family (Rubiaceae). *Systematic Biology*. **48:** 413-435.

Bremer B., Manen J.F., 2000 – Phylogeny and classification of the subfamily Rubioideae (Rubiaceae). *Plant Systematics and Evolution*. **225:** 71-92.

Bremer B., Eriksson T., 2009 – The time tree of Rubiaceae: Phylogeny and dating the family, subfamilies, and tribes. *International Journal of Plant Sciences*. **170 (6): 766-793.**

Bremer B., 2009 – A review of molecular phylogenetic studies of Rubiaceae. *Annals of Missouri Botanical Garden*. **96:** 4-26.

Brullo C., Brullo S., Giusso del Galdo G.P., Scuderi L., 2009 – Taxonomical notes on the Sicilian populations of *Asperula gussonei* (Rubiaceae): *Asperula peloritana* sp.nov. *Anales del Jardín Botánico de Madrid*. **66(1):** 85-92.

Cannon J., Cannon M., 2003 – Dye Plants and Dyeing (2nd ed.). *Timber Press in Association with The Royal Botanic Gardens: Kew,* pp. 76, 80.

Caputo G., La Valva V., Nazzaro R., Ricciardi M., 1989-90 – La flora della penisola sorrentina (Campania). Delpinoa *N.S.,* **31-32:** 57.

Clement M., Posada D., Crandall K.A., 2000 – TCS: a computer program to estimate gene genealogies. *Molecular Ecology*. **9:** 1657-1659. http://darwin.uvigo.es/software/tcs.html

Comes H.P., Abbott R.J., 2001 – Molecular phylogeography, reticulation and lineage sorting in mediterranean *Senecio* sect. *Senecio* (Asteraceae). *Evolution*. **55(10):** 1943–1962.

Cronquist A., 1968 – The evolution and classification of flowering plants. *Boston: Houghton Mifflin*.

Davis A. P., Govaerts R., Bridson D.M., Ruhsam M., Moat J., Brummit N.A., 2009 - A global assessment of distribution, diversity, endemism, and taxonomic effort in the Rubiaceae. *Annals of the Missouri Botanical Garden.* **96(1)**:68-78.

De Candolle A.P., 1830 – Prodromus systematis naturalis regni vegetabilis, sive, enumeratio contracta ordinum generum specierumque plantarum huc usque cognitarium, juxta methodi naturalis, normas digesta. (**4**): 583-584.

Degnan J., Rosenberg N., Stadler T., 2012 – The probability distribution of ranked gene trees on a species tree. *Mathematical Bioscience.* **235:**45-55.

DEL GUACCHIO E., CAPUTO P., 2013 – Taxonomic notes on *Asperula* sect. *Cynanchicae* (Rubiaceae). *Taxon*. **62(2):** 394-400.

DELPRETE P.G., CORTES-B R., 2004 – A phylogenetic study of the tribe Sipaneeae (Rubiaceae, Ixoroideae), using *trn*L-F and ITS sequence data. *Taxon*. **53:** 347-356.

DI MAIO A., DE CASTRO O., 2013a – SSR-Patchwork: an optimized protocol to obtain a rapid and inexpensive SSR library using first-generation sequencing technology. *Application in Plant Sciences*. **1(1):** 1200158.

DI MAIO A., DE CASTRO O., 2013b – Development and characterization of 21 microsatellite markers for *Pancratium maritimum* L. (Amaryllidaceae). *Conservation Genetics Resources*. **5:** 911-914.

DI PIETRO R., WAGENSOMMER R. P., 2008 – Analisi fitosociologica su alcune specie rare e/o minacciate del Parco Nazionale del Gargano (Italia centro-meridionale) e considerazioni sintassonomiche sulle communità casmofitiche della Puglia. *Fitosociologia*. **45:** 177-200.

DOYLE J.J., DOYLE J.L., BROWN A.H.D., 1999 – Origins, colonization, and lineage recombination in a widespread perennial soybean polyploid complex. *PNAS, Proceedings of the National Academy of Sciences.* **96:** 10741-10745.

DRUMMOND A.J., HO S.Y.W., PHILLIPS M.J., RAMBAUT A., 2006 – Relaxed phylogenetics and dating with confidence. *PLoS Biology.* **4(5):** e88.

DRUMMOND A.J., RAMBAUT A., 2007 – BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology.***7**:214. http://www.beast2.org/wiki/index.php/Main_Page

DRUMMOND A.J., SUCHARD M. A., XIE D., RAMBAUT A., 2012 – BEAST: Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution.* **29 (8):** 1969-1973.

EHRENDORFER F., KRENDL F., 1976 – *Asperula* L., IN: TUTIN T. G., BURGES N. A., CHATER A. O., EDMONDSON J. R., HEYWOOD V. H., MOORE D. M., VALENTINE D. H., WALTERS S. M., WEBB D. A. (Eds.), Flora Europaea, *Cambridge.***3:** 4-14.

EHRENDORFER F., 1982 – *Asperula* L., IN: PIGNATTI S., Flora d'Italia. *Edagricole, Bologna.***2:** 355-359.

EHRENDORFER F., SCHÖNBECK-TEMESY E., 1982 – *Asperula* L., IN: P. H. DAVIS (ed.), Flora of Turkey and the East Aegean Islands, vol. 7. *Edinburgh University Press, Edinburgh, Scotland, U.K.,* pp. 742-757.

EHRENDORFER F., 1982 – *Asperula* L., IN: MOORE D.M., Flora Europaea. Check-list and Chromosome Index. *Cambridge University Press,* pp. 168-173; 221-222.

EHRENDORFER F., SCHÖNBECK-TEMESY E., PUFF C., RECHINGER W. , 2005 – Rubiaceae, IN: Flora Iranica. *Verlag des Naturhistorischen Museums Wien, Vienna, Austria.***176**.

EDGAR R. C., 2004 – MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*. **32 (5):** 1792-1797. http://www.drive5.com/muscle/

ERIXON P., OXELMAN B., 2008 – Reticulate or tree-like chloroplast DNA evolution in *Sileneae* (Caryophyllaceae)? *Molecular Phylogenetics and Evolution*. **48:** 313-325.

FARRIS J.S., 1970 – Estimating phylogenetic trees from distance matrices. *American Nature.* **106:** 645-668**.**

FARRIS J. S., KÄLLERSJÖ M., KLUGE A. G., BULT C., 1995a – Constructing a significance test for incongruence. *Systematic Biology*. **44:** 570-572.

FARRIS J. S., KÄLLERSJÖ M., KLUGE A. G., BULT C., 1995b – Testing significance of incongruence. *Cladistics*. **10:** 315–319.

FELSENSTEIN J., 1981 – Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution.* **17:** 368–376.

FIORI A., 1925 – Nuova Flora Analitica d'Italia. *Firenze*. **2:** 500-503.

FITCH W. M., 1971 – Toward defining the course of evolution: minimum change for a specific tree topology. *Systematic Zoology.* **20:** 406-416**.**

FUERTES AGUILAR J., ROSSELLÓ J., FELINER J. N., 1999 – Molecular evidence for the Compilospecies Model of reticulate evolution in *Armeria* (Plumbaginaceae). *Systematic Biology.* **48(4):** 735-754.

FUERTES AGUILAR J., FELINER J. N., 2003 – Additive polymorphisms and reticulation in an ITS phylogeny of thrifts (*Armeria*, Plumbaginaceae). *Molecular Phylogenetics and Evolution.* **28:** 430–447.

GEYER C. G., 1991 – Markov chain Monte Carlo maximum likelihood. IN: KERAMIDAS E. M., KAUFMAN S. M. (Eds.), Computing Science and Statistics: Proceedings of the 23rd Symposium on the Interface. *Interface Foundation, Fairfax Station,* p. 156-163.

GIAM X., SODHI N.S., BROOK B.W., TAN H.T.W., BRADSHAW C.J.A., 2011 – Relative need for conservation assessments of vascular plant species among ecoregions. *Journal of Biogeography.* **38:** 55-68.

GOLDSTEIN D. B., LINARES A. R., CAVALLI-SFORZA L. L., FELDMAN M. W., 1995 –An Evaluation of Genetic distances for use with microsatellite loci. *Genetics* **139:** 463-471.

GOLDSTEIN D. B., POLLOCK D. D., 1997 – Launching Microsatellites: a review of mutation processes and methods of phylogenetic inference. *Journal of Heredity.* **88:** 335-342.

GOVAERTS R., RUHSAM M., ANDERSSON L., ROBBRECHT E., BRIDSON D. M., DAVIS A. P., SCHANZER I., SONKE B., 2006 - World checklist of Rubiaceae. *The Board of Trustees of the Royal Botanic Gardens, Kew*. http://www.kew.org/wcsp/rubiaceae/

GRAHAM A., 2009 – Fossil record of the Rubiaceae. *Annals of the Missouri Botanical Garden*. **96(1):** 90-108.

GREUTER W., 2012 – Results of the Seventh "Iter Mediterraneum" in the Peloponnese, Greece, May to June 1995. *Bocconea* **25:** 107-108.

GUADAGNO M., 1913 – Prime notizie sulla vegetazione delle Isole Sirenuse. *Bullettino dell'Orto Botanico della R. Università di Napoli.* **3:** 75-91.

GÜCEL S., SEÇMEN O., 2009 – Conservation biology of *Asperula daphneola* (Rubiaceae) in Western Turkey. *Turkish Journal of Botany*. **33:** 257-262.

GUO, Y.-P., SAUKEL, J., EHRENDORFER, F., 2008. – AFLP trees versus scatter plots: Evolution and phylogeography of the polyploid complex *Achillea millefolium* agg. (Asteraceae). *Taxon*. **57:** 153-169.

GUTERMANN W., EHRENDORFER F., 2000 – *Asperula naufraga* (Rubiaceae), a new species from Zakinthos (Ionian Islands, Greece), with notes on its ecology, karyology and relationship (Material towards a Flora Ionica, 1). *Botanika Chronika*. **13:** 61-70.

HALL T.A., 1999 – BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series.* **41:** 95-98. http://www.mbio.ncsu.edu/bioedit/bioedit.html

HARLAN J. R., DE WET J. M. J., 1963 – The Compilospecies Concept. *Evolution.* **17(4):** 497-501.

HELED J., DRUMMOND A. J., 2010 – Bayesian inference of species trees from multilocus data. *Molecular Biology and Evolution.* **27:** 570-580.

HELED J., BRYANT D., DRUMMOND A. J., 2013 – Simulating gene trees under the multispecies coalescent and time-dependent migration. *BMC Evolutionary Biology*. **13**:44.

HUELSENBECK J. P., RONQUIST F., 2001 – MrBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics.* **17 (8):** 754–755. http://mrbayes.sourceforge.net/download.php

IUCN, 2001 - IUCN Red List categories and criteria: version 3.1. IUCN Species Survival Commission. *Gland, Switzerland and Cambridge, UK.*

IUCN STANDARDS AND PETITIONS SUBCOMMITTEE, 2013 – Guidelines for using the IUCN Red List categories and criteria. Version 10.1. *Prepared by the Standards and Petitions Subcommittee*. http://www.iucnredlist.org/documents/RedListGuidelines.pdf

LOREN, HUMPHREY, 2000 – Quinine and Quarantine: Missouri Medicine Through the Years. *University of Missouri Press*.

HUYSMANS S., DESSEIN S., SMETS E., ROBBRECHT E., 2003 – Pollen morphology of NW European representatives confirms monophyly of Rubieae (Rubiaceae). *Review of Palaeobotany and Palynology*. **127:** 219-240.

KÅREHED J., GROENINCKX I., DESSEIN S., MOTLEY T. J., BREMER B., 2008 – The phylogenetic utility of chloroplast and nuclear DNA markers and the phylogeny of the Rubiaceae tribe Spermacoceae. *Molecular Phylogenetics and Evolution*. **49:** 843-866.

Kay K.M., Whittall J.B., Hodges S.A., 2006 – A survey of nuclear ribosomal internal transcribed spacer substitution rates across angiosperms: an approximate molecular clock with life history effects. *BMC Evolutionary Biology*. **6:** 36.

Kishino H., Thorne J. L., Bruno W.J., 2001 – Performance of a divergence time estimation method under a probabilistic model of rate evolution. *Molecular Biology and Evolution.* **18(3):** 352-361.

Kluge A. G., Farris J.S., 1969 – Quantitative phyletics and the evolution of anurans. *Systematic Zoology.* **18:** 1-32**.**

Korica S., Lausi D.,Ehrendorfer F., 1992 – A new subspecies of the trans-Adriatic *Asperula staliana* from the IsoleTremiti: subsp. *diomedea*, and its ecology. *Flora mediterranea*. **2:** 65-76.

Klokov M. V., Pobedimova E. G., Schischkin B. K., 1958 – *Asperula* L.In: Schischkin B. K. (Ed.) Flora of the U.R.S.S. *Smithsonian Institution Libraries. Washington DC (2000)*. **28:** 184-271.

Kruckeberg A.R., Rabinowitz D., 1985 – Biological aspects of endemism in higher plants. *Annual Review of Ecology and Systematics*. **16:** 447-479.

Kubatko L., Degnan J., 2007 – Inconsistency of phylogenetic estimates from concatenated data under coalescence. *Systematic Biology.* **56:** 17-24*.*

Lambeck K., Antonioli F., Purcell A., Silenzi S., 2004 – Sea level change along the Italian coast for the past 10,000 yr. *Quaternary Science Reviews.* **23:** 1567-1598.

Lambeck K., Antonioli F., Anzidei M., Ferranti L., Leoni G., Scicchitano G., Silenzi S., 2011 – Sea level change along the Italian coast during the Holocene and projections for the future. *Quaternary International.* **232:** 250-257.

Lanave C., Preparata G., Saccone C., Serio G., 1984 – A new method for calculating evolutionary substitution rates. *Journal of Molecular Evolution.* **20 (1):** 86-93.

Lepage T. Bryant D., Philippe H., Lartillot N., 2007 – A General Comparison of Relaxed Molecular Clock Models. *MolecularBiology and Evolution.* **24 (12):** 2669-2680.

LEWIS P.O., 2001 – A likelihood approach to estimating phylogeny from discrete morphological character data. *Systematic Biology*. **50(6):** 913-925.

LINNEUS C., 1767 – *Mantissa Plantarum.* 37.

LIÒ P., GOLDMAN N., 1998 – Models of molecular evolution and phylogeny. *Genome Research.* **8 (12):** 1233-1244.

LIU Z.W., ZHOU J., LIU E.D., PENG H., 2010 – A molecular phylogeny and a new classification of *Pyrola* (Pyroleae, Ericaceae). *Taxon.* **59:** 1690-1700.

MADDISON W. P., 1997 – Gene trees in species trees. *Systematic Biology*. **46 (3):** 523-536.

MADDISON W.P., MADDISON D.R., 2011 – MESQUITE: a modular system for evolutionary analysis, version 2.75. HTTP://MESQUITEPROJECT.ORG

MALCOMBER S.T., 2002 – Phylogeny of *Gaertnera* Lam. (Rubiaceae) based on multiple DNA markers: evidence of a rapid radiation in a widespread, morphologically diverse genus. *Evolution*. **56:** 42-57.

MANEN J. F., NATALI A., EHRENDOFER F., 1994 – Phylogeny of Rubiaceae-Rubieae inferred from the sequence of cpDNA intergene region. *Plant Systematics and Evolution*. **190:** 195-211.

MARTÌNEZ-AZORÌN M., CRESPO M.B., JUAN A., FAY M.F. , 2011 – Molecular phylogenetics of subfamily Ornithogaloideae (Hyacinthaceae) based on nuclear and plastid DNA regions, including a new taxonomic arrangement. *Annals of Botany*. **107:** 1-37.

MAUREIRA-BUTLER I.J., PFEIL B.E., MUANGPROM A., OSBORN T.C., DOYLE J.J., 2008 – The Reticulate History of Medicago (Fabaceae). *Systematic Biology.* **57(3):** 466-482.

MCDADE L.A., 1992 – Hybrids and phylogenetic systematics. II. The impact of hybrids on cladistics analysis. *Evolution*. **46:** 1329-1346.

MENKE B., 1976 – Pliozäne und latest quartäre Sporen und Pollenflora von Schleswig-Holstein. *Geologisches Jahrbuch*. **A32:**3-197.

MINARECI E., YILDIZ K. 2010 – *Asperula pseudochlorantha* var. *antalyensis* comb. et stat. nov.(Rubiaceae). *Annales Botanici Fennici*. **47(2)**.

MOTLEY T. J., WURDACK K. J., DELPRETE P. G., 2005 – Molecular systematics of the Catesbaeeae-Chiococceae complex (Rubiaceae): flower and fruit evolution and biogeographic implications. *American Journal of Botany*. **92:** 316-329.

NATALI A., MANEN J.C., EHRENDORFER F., 1995 –Phylogeny of the Rubiaceae-Rubioideae, in particular the tribe Rubieae: evidence from a non-coding chloroplast DNA sequence. *Annals of Missouri Botanical Garden*. **82:** 428-439.

NATALI A., MANEN J.C., KIEHN J.-F., EHRENDORFER F., 1996 –Tribal, generic and specific relationship in the Rubioideae-Rubieae (Rubiaceae) based on sequence data of a cpDNA intergene region. *Opera Botanica Belgica*. **7:** 193-203.

NEI M., KUMAR S., 2000 – Molecular evolution and phylogenetics. *Oxford University Press.*

NEPOKROEFF M., SYTSMA K. J., BREMER B., 1999 –Reorganization of the genus *Psychotria* and tribe Psychotrieae (Rubiaceae) inferred from ITS and rbcL sequence data. *Systematic Botany*. **24:** 5-27.

NIE Z. L., WEN J., SUN H., BARTHOLOMEW B., 2005 – Monophyly of *Kelloggia* Torrey ex Benth. (Rubiaceae) and evolution of its intercontinental disjunction between western North America and eastern Asia. *American Journal of Botany*. **92:** 642-652.

NYLANDER J. A. A., WILGENBUSCH J. C., WARREN D. L., SWOFFORD D. L., 2008 – AWTY (are we there yet?): a system for graphical exploration of MCMC convergence in Bayesian phylogenetics. *Bioinformatics.* **24 (4):** 581–583.

NYMAN C.F., 1878-1882. Conspectus florae europaeae. *Örebro: typis officinae Bohlinianae,* p. 334.

OXELMAN B., LIDÉN M., BERGLUND D., 1997 – Chloroplast *rps*16 intron phylogeny of the tribe *Sileneae* (Caryophyllaceae). *Plant Systematics and Evolution*. **206:** 393-410

ÖZTÜRK M., 2003 – *Asperula anatolica* (Rubiaceae), a new species from south-east Anatolia, Turkey. *Turkish Journal of Botany.* **37(1)**.

PAMILO P., NEI M., 1988 – Relationships between gene trees and species trees. *Molecular Biology and Evolution.* **5(5):** 568-583.

Parlatore F., 1857 –Flora italiana. *Firenze*. **7:** 73-95.

Pattengale N.D., Alipour M., Bininda-Emonds O.R.P., Moret B.M.E., Stamatakis A., 2009 – How Many Bootstrap Replicates Are Necessary? *Research in Computational Molecular Biology* **5541:** 184-200.

Penny D., Hendy M.D., 1985 – The use of tree comparison metrics. *Systematic Zoology*. **34:** 75-82.

Perrier E., Songeon A., 1855 – Indication de quelques plantes nouvelles, rares ou critiques, observées en Savoie, suivie d'une revue de la section *Thylacites* du genre *Gentiana*. *Bulletin de la Societe de Histoire Naturelle Savoie de 1854*, p.44.

Persson C., 2000 –Phylogeny of the Neotropical *Alibertia* group (Rubiaceae), with emphasis on the genus *Alibertia,* inferred from ITS and 5S ribosomal DNA sequences. *American Journal of Botany*. **87:** 1018-1028.

Peruzzi L., Gargano D., Passalacqua N.G., 2004 – Considerazioni tassonomiche su *Asperula* L. sect. *Cynanchicae* (Rubiaceae) nell'Italia meridionale. *Informatore Botanico Italiano.* **36:** 154–157*.*

Popp M., Erixon P., Eggens F., Oxelman B., 2005 – Origin and evolution of a circumpolar polyploid species complex in Silene (Caryophyllaceae) inferred from low copy nuclear RNA polymerase introns, rDNA, and chloroplast DNA. *Systematic Botany*. **30(2):** 302-313.

Posada D, 2008 – JModelTest: phylogenetic model averaging. *Molecular Biology and Evolution.* **25:** 1253-1256. http://code.google.com/p/jmodeltest2/

Prober S.M., Austin M.P., 1991 – Habitat peculiarity as a cause of rarity in *Eucalyptus paliformis*. *Australian journal of ecology*. **16(2):** 189-205.

Pybus O.G., 2006 – Model selection and the molecular clock. *PLoS Biology*. **4(5):** e151.

Rambaut A., Drummond A. J., 2009 – Tracer v1.5. MCMC trace analysis package. http://tree.bio.ed.ac.uk/software/

RANNALA B, YANG Z, 2003 – Bayes estimation of species divergence times and ancestral population sizes using DNA sequences from multiple loci. *Genetics*. **164(4):** 1645-1656.

RAZAFIMANDIMBISON S.G., KAINULAINEN K., WONG K.M., BEAVER K., BREMER B., 2011 – Molecular support for a basal grade of morphologically distinct, monotypic genera in the species-rich Vanguerieae alliance (Rubiaceae, Ixoroideae): Its systematic and conservation implications. *Taxon.***60 (4):** 941-952.

REŠETNIK I., FRAJMAN B., BOGDANOVIĆ S., EHRENDORFER F., SCHÖNSWETTER P., 2014 – Disentangling relationships among the diploid members of the intricate genus *Knautia* (Caprifoliaceae, Dipsacoideae). *Molecular Phylogenetics and Evolution*. **74:** 97–110.

RICCIARDI M., 1996 – Flora di Capri (Golfo di Napoli). *Annali di Botanica*. **54:** 7-169.

RIESEBERG L.H., ELLSTRAND N.C., 1993 – What can molecular and morphological markers tell us about plant hybridization? *Critical Reviews in Plant Sciences.* **12(3):** 213-241.

ROBBRECHT E., 1988 –Tropical woody Rubiaceae. *Opera Botanica Belgica.***1:**1-171.

RODRIGUES A.S.L., ANDELMAN S.J., BAKARR M.I., BOITANI L., BROOKS T.M., COWLING R.M., FISHPOOL L.D.C., DA FONSECA G.A.B., GASTON K.J., HOFFMANN M., LONG J.S., MARQUET P.A., PILGRIM J.D., PRESSEY R.L. , SCHIPPER J., SECHREST W., STUART S.N., UNDERHILL L.G., WALLER R.W., WATTS M.E.J., YAN X., 2004 – Effectiveness of the global protected area network in representing species diversity. *Nature.* 428: 640-643.

RODRIGUES A.S.L., PILGRIM J.D., LAMOREUX J.F., HOFFMANN M., BROOKS T. M., 2006 – The value of the IUCN Red List for conservation. *Trends in Ecology & Evolution.* **21(2):** 71-76.

RODRIGUEZ F., OLIVER J. L., MARIN A., MEDINA J. R., 1990 – The general stochastic model of nucleotide substitution. *Journal of theoretical biology*. **142:** 485-501.

RONQUIST F., HUELSENBECK J. P., 2003 – MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*. **19:** 1572-1574.

RUTISHAUSER R., 1999 – Polymerous leaf whorls in vascular plants: Developmental morphology and fuzziness of organ identities. *International Journal of Plant Sciences.* **160 (6 Suppl.):** S81-S103.

RYDIN C., RAZAFIMANDIMBISON S. G., KHODABANDEH A., BREMER B., 2009 – Evolutionary relationships in the Spermacoceae alliance (Rubiaceae) using information from six molecular loci: insights into systematic affinities of *Neohymenopogon* and *Mouretia*. *Taxon*. **58(3):** 793-810.

SANG T., ZHONG Y., 2000 – Testing Hybridization Hypotheses Based on Incongruent Gene Trees. *Systematic Biology.* **49(3):** 422-434.

SANGER F., NICKLEN S., COULSON R., 1977 – DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the USA.* **74:** 5463-5467.

SCHEMSKE D.W., HUSBAND B.C., RUCKELSHAUS M.H., GOODWILLIE C., PARKER I.M., BISHOP J.G., 1994 – Evaluating Approaches to the Conservation of Rare and Endangered. *Ecology*. **75(3):** 584-606.

SCHMIDT-LEBUHN A.N., DE VOS J.M., KELLER B., CONTI E., 2012 – Phylogenetic analysis of *Primula* section *Primula* reveals rampant non-monophyly among morphologically distinct species. *Molecular Phylogenetics and Evolution.* **65 (1):** 23–34.

SCHÖNBECK-TEMESY E., EHRENDORFER F., 1991 – *Asperula* L. IN: STRID A., TAN K., Mountain Flora of Greece. *Edinburgh University Press.* **2:**281-300.

SCHUELKE M., 2000 – An economic method for the fluorescent labelling of PCR fragments. *Nature Biotechnology.* **18:**233–234.

SHAW J., LICKEY E. B., SCHILLING E. E., SMALL R. L., 2007 – Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III.*American Journal of Botany*. **94 (3):** 275-288.

SOZA V.L., OLMSTEAD R.C., 2010a –Molecular systematics of tribe Rubieae (Rubiaceae): Evolution of major clades, development of leaf-like whorls and biogeography. *Taxon*. **59 (3):** 755-771.

Spinks P.Q., Thomson R.C., Pauly G.B., Newman C.E., Mount G., Bradley Sheffer H., 2013 – Misleading phylogenetic inferences based on single-exemplar sampling in the turtle genus *Pseudemys*. *Molecular Phylogenetics and Evolution.* **68:** 269-281.

Stamatakis A., 2006 – RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics.* **22 (21):** 2688-2690. http://www.exelixis-lab.org/.

Stamatakis A., 2008 – A Rapid Bootstrap Algorithm for the RAxML Web Servers. *Systematic Biology.* **57 (5):** 758-771.

Strid A., Franzen R., 1981 – Chromosome number reports LXXIII. *Taxon* **30:** 829-842**.**

Sullivan J., Joyce P., 2005 – Model selection in phylogenetics. *Annual Review of Ecology, Evolution and Systematics*. **36:** 445-466.

Swofford D.L., 2003 – PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods) version 4. *Sinauer Associates, Sunderland, MA.*

Syring J., Farrell K., Businský R., Cronn R., Liston A., 2007 – Widespread genealogical nonmonophyly in species of *Pinus* subgenus *Strobus*. *Systematic Biology.* **56(2):** *163-181.*

Takeda H., 1916 –Some points in the morphology of the stipules in the *Stellatae*, with special reference to *Galium*. *Annals of Botany (Oxford)*. **30:** 197-214.

Tao C., Ehrendorfer F., 2011 – 28. *Galium* Linnaeus, Sp. Pl. 1: 105. In: Flora of China. Vol. 19 (Cucurbitaceae through Valerianaceae, with Annonaceae and Berberidaceae). *Missouri Botanical Gardens Press, St. Louis.* **(19):** 104–141.

Tavare S., 1986 – Some probabilistic and statisical problems on the analysis of DNA sequences. *Lectures on mathematics in the life sciences*. **17:** 57-86.

Tenore M., 1811 – Flora Napolitana. *Naples*. **1:**34-36.

Thompson J.D., 2005 – Plant evolution in the Mediterranean. *Oxford: Oxford University Press.*

VAN CAMPO E., 1976 – Le flore sporopollénique du gisement Miocène terminal de venta de Moro (Espagne). *Thesis of the University of Montpellier, Montpellier*.

WENDEL J. F., DOYLE, J. J., 1998. – Phylogenetic incongruence: window into genome history and molecular evolution. IN: Soltis P.S., Soltis D.E., Doyle J.J. (Eds.), Molecular Systematics of Plants II: DNA Sequencing. *Kluwer Academic Publishers, Dordrecht, Netherlands*. 265–296.

WERTHEIM J.O., SANDERSON M.J., WOROBEY M., BJORK A., 2010 – Relaxed molecular clocks, the bias-variance trade-offs and the quality of phylogenetic inference. *Systematic Biology.* **59(1):** 1-8.

WILGENBUSCH J. C., WARREN D. L., SWOFFORD D. L., 2004 – AWTY: A system for graphical exploration of MCMC convergence in Bayesian phylogenetic inference. http://ceb.csit.fsu.edu/awty

WILLYARD A., CRONN R., LISTON A., 2009 – Reticulate evolution and incomplete lineage sorting among the ponderosa pines. *Molecular Phylogenetics and Evolution*. **52:** 498-511.

WOLFE K.H., LI W.H., SHARP P.M., 1987 – Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proceedings of the National Academy of Sciences.* **84:** 9054–9058.

YANG Z., RANNALA B., 1997 – Bayesian phylogenetic inference using DNA sequences: a Markov chain Monte Carlo method. *Molecular Biology and Evolution.* **14:** 717-724.

YAO X., YE Q., FRITSCH P. W., CRUZ B. C., HUANG H., 2008 – Phylogeny of *Sinojackia* (Styracaceae) based on DNA sequence and Microsatellite data: implications for taxonomy and conservation. *Annals of Botany.* **101(5):** 651-659.

YULE G.U., 1925 – A mathematical theory of evolution, based on the conclusions of Dr. J.C. Willis, F.R.S. *Philosophical Transactions of the Royal Society B Biological Sciences*. **213:** 21-87.

Appendix A – Members of *Asperula* sect. *Cynanchicae* (DC.) Boiss.

From Govaerts, 2006; Ehrendorfer & Krendl, 1976; Ehrendorfer, 1982; Schönbeck-Temesy & Ehrendorfer, 1991; Peruzzi *et al.* 2004; Bernardo *et al.*, 2010; Gutermann & Ehrendorfer, 2000; Ehrendorfer, 2005; Brullo *et al.*, 2009; Gücel & Seçmen, 2009; Caputo & Del Guacchio, 2013.

Species	Author	Distribution	Ploidy
Asperula abchasica	V.I. Krecz.	Caucasus	
Asperula abbreviata	(Halàcsy) Rech. f.	Greece (Cyclades: Naxos, Amorgos)	
Asperula affinis	Boiss. & A.Huet	Turkey	
Asperula aristata	L.f.		
subsp. *aristata*		S Europe, France and N-W Balkans	Variable: x= 10-11
subsp. *condensata*	(Heldr. ex Boiss.) Ehrend. & Krendl	W-C Balkan Peninsula	Diploid: 2*n*=20
subsp. *nestia*	(Rech. f.) Ehrend. & Krendl	N Greece; S Bulgaria	Tetraploid: 2*n*=40
subsp. *oreophila*	(Briq.) Hayek	S Alps, E. Pyrenees, Apennines	Variable: x= 10-11
subsp. *thessala*	(Boiss. & Heldr.) Hayek	E Greece	Tetraploid: 2*n*=44
Asperula beckiana	Degen	Croatia, Slovenia, Bosnia (Dynaric Alps)	
Asperula boissieri	Heldr. ex Boiss.	Greece (Mount Kyllini)	Diploid: 2*n*=22
Asperula borbasiana	(Korica) Korica	Croatia (Krk)	Tetraploid: 2*n*=40
Asperula bornmuelleri	Velen. ex Bornm.	C Turkey	
Asperula brachyphylla	Trigas & Iatroù	Greece (Euboea)	
Asperula bryoides	Stapf	Turkey	
Asperula calabra	(Fiori) Gavioli	Italy (southern Apennines)	Tetraploid: 2*n*=40
Asperula capitellata	Hausskn. & Bornm.	Turkey	
Asperula crassifolia	L.	Italy (Isle of Capri, Sorrentine Peninsula, Sirenusae isles)	Diploid: 2*n*=20
Asperula cretacea	Willd. ex Roem. & Schult.	Krimea	
Asperula cynanchica	L.		
subsp. *cynanchica*		Europe up to GB e EIRE (except Portugal, Corsica, Czech Republic and Slovakia)	Variable: x=10-11
subsp. *occidentalis*	(Rouy) Stace	N Spain; S-W France; S-W Great Britain; Ireland	
subsp. *pyrenaica*	(L.) Nyman	France, Spain (Pyrenees)	
Asperula daphneola	O. Schwarz	Turkey (Nif Mountain - Kemalpaşa)	
Asperula deficiens	Viv.	Sardinia (Tavolara)	Diploid: 2*n*=20
Asperula diminuta	Klokov	S Russia to Caucasus	
Asperula garganica	Huter, Porta & Rigo ex Ehrend & Krendl	Italy (Mount Gargano)	Diploid: 2*n*=20
Asperula graveolens	M.Bieb. ex Schult. & Schult.f.		
subsp. *danilewskiana*	(Basiner) Pjatunina	SE Russia to C Asia	
subsp. *graveolens*		E Bulgaria, E Romania, Russia	
subsp. *leiograveolens*	(Popov & Chrshan.) Pjatunina	C Ukraine	
Asperula glareosa	Ehrend.	Lebanon, S Turkey	
Asperula gussonei	Boiss.	N Sicily (Madonie)	
Asperula icarica	Ehrend. & Schönb.-Tem.	Greece (Icaria)	

(continued)

Species	Author	Distribution	Ploidy
Asperula idaea	Halàcsy	Greece (Crete)	Tetraploid: 2*n*=44
Asperula inopinata	Schönb.-Tem.	Iraq	
Asperula x jordanii	E.P. Perrier & Songeon	Alps, Pyrenees	
Asperula lactea	(Porta) Brullo, Gargano, N.G.Passal. & Peruzzi	SW Italy (Calabria)	Tetraploid: 2*n*=40
Asperula lilaciflora	Boiss.		
subsp. *coa*	(Rech. f.) Ehrend.	Greece (Kos isle)	
subsp. *lilaciflora*		Turkey	
subsp. *mutensis*	Schönb.-Tem.	S Turkey (Içel)	
subsp. *phrygia*	(Bornm.) Schönb.-Tem.	W Turkey	
subsp. *runemarkii*	Ehrend. & Schönb.-Tem.	Turkey (Aegean isles)	
Asperula littoralis	Sm.	NW-SW Turkey	
Asperula lutea	Sm.		
subsp. *euboea*	Ehrend.	Greece (Euboea)	
subsp. *griseola*	Greuter	Greece	
subsp. *lutea*		SC Greece	
Asperula lycia	Stapf	SW Turkey	
Asperula malevonensis	Ehrend. & Schönb.-Tem.	Greece	
Asperula mungieri	Boiss. & Heldr.	S Greece	
Asperula naufraga	Ehrend. & Gutermann	Greece (Zakynthos)	Diploid: 2*n*=20
Asperula neglecta	Guss.	Central Apennines(Abruzzo, Italy)	
Asperula neilreichii	Beck	NE Alps	Diploid: 2*n*=20
Asperula nitida	Sm.		
subsp. *hirtella*	(Boiss.) Ehrend.	W & C Turkey	
subsp. *mytilinica*	Ehrend.	Greece (Lesbos)	
subsp. *nitida*		NW Turkey (Olympus Bithynicus)	
subsp. *subcapitellata*	Ehrend.	N Turkey	
Asperula oetaea	(Boiss.) Heldr. ex Halácsy	S Greece	
Asperula ophiolithica	Ehrend.	Greece (NW Euboea)	
Asperula paui			
subsp. *paui*	Font Quer	Balearic islands	Diploid: 2*n*=22
subsp.*dianensis*	(Font Quer) De la Torre, Alcaraz & M.B. Crespo	Spain (Alicante)	
Asperula peloritana	C.Brullo, Brullo, Giusso & Scuderi	NE Sicily	
Asperula pestalozzae	Boiss.	N & C Turkey	
Asperula pinifolia	(Boiss.) Heldr. ex Ehrend. & Schonb.-Tem.	Greece	
Asperula pontica	Boiss.	N Turkey to W Transcaucasus	
Asperula x portae	Peruzzi	Southern Apennines (Calabria, Italy)	2*n*=35
Asperula pulvinaris	Heldr. ex Boiss.	S Greece	Tetraploid: 2*n*=44
Asperula pumila	Moris	Sardinia (Oliena)	
Asperula rigidula	(Halácsy) Halácsy	SE Greece (including Euboea)	Tetraploid: 2*n*=44
Asperula rumelica	Boiss.	SE & E Europe to NW Turkey	
Asperula rupicola	Jord.	SW Alps	
Asperula samia	D.H.Christ & Goeriadis	Aegean islands (Samos)	

Species	Author	Distribution	Ploidy
Asperula sintenisii	Asch. ex Bornm.	Turkey	
Asperula staliana	Vis.		Tetraploid: 2*n*=40
subsp. *arenaria*	Korica	Biševo (Croatia)	
subsp. *diomedea*	Korica, Lausi & Ehrend.	Tremiti isles (Adriatic sea, Italy)	
subsp. *issaea*	Korica	Vis (Croatia)	
subsp. *staliana*		Biševo (Croatia)	
Asperula stricta	Boiss.		
subsp. *elmaniensis*	Schönb.-Tem.	SW Turkey	
subsp. *grandiflora*	Schönb.-Tem.	S Turkey	
subsp. *latibracteata*	(Boiss.) Ehrend.	C & SE Turkey	
subsp. *monticola*	Ehrend.	SW Turkey	
subsp. *stricta*		S Turkey to Lebanon	
Asperula suberosa	Sm.	N Greece, SW Bulgaria	Tetraploid: 2*n*=44
Asperula suffruticosa	Boiss. & Heldr.	Greece (C Euboea)	
Asperula supina	M. Bieb.		
subsp. *supina*		Russia to Caucasus	Diploid: 2*n*=20
subsp. *caespitans*	(Juz.) Pjatunina	Krimea	
Asperula tenella	Heuff. ex Degen	Ungary, Bulgaria, Greece, Russia, Turkey	
Asperula tenuifolia	Boiss.	Eastern Aegean Islands to SW Turkey	
Asperula tephrocarpa	Czern. ex Popov & Chrshan.	E Ukraine to Russia	
Asperula visianii	Korica	Svetac (Croatia)	Tetraploid: 2*n*=40
Asperula wettsteinii	Adamovic	Bosnia, Montenegro (Dynaric Alps)	
Asperula woloszczakii	Korica	Croatia (Kvarner isles)	Diploid: 2*n*=40
Asperula woronowii	V.I.Krecz.	NE Turkey	

Appendix B: accessions analyzed

Accession	Specimen origin	Geographical information
Didymaea alsinoides	259233557; FJ695442.1 288190803; GU357441.1	Central America
Sherardia arvensis	Sampled in this study	Southern Italy
Asperula purpurea	Herbarium of E. Del Guacchio	Southern Italy
Asperula abbreviata	UPA	Naxos (Cyclades, Greece)
Asperula affinis	G	Gümüşhane (Northern Turkey)
Asperula aristata subsp. *aristata* 2H	Herbarium of E. Del Guacchio	Potenza (Southern Italy)
Asperula aristata subsp. *aristata* 8H	Herbarium of E. Del Guacchio	Mount Faito (Southern Italy)
Asperula aristata subsp. *condensata*	CP	Mount Kerkini (or Belasica; Greece)
Asperula aristata subsp. *nestia*	CP	Mount Olympus (Greece)
Asperula aristata subsp. *oreophila* 131	Sampled in this study	Scanno (Central Apennines, Italy)
Asperula aristata subsp. *oreophila* ORE1	Herbarium of E. Del Guacchio	Serra Dolcedorme (Southern Apennines, Italy)
Asperula aristata subsp. *oreophila* TRM11	Herbarium of E. Del Guacchio	Mount Terminio (Southern Apennines, Italy)
Asperula aristata subsp. *thessala*	CP	Mount Olympus (Greece)
Asperula beckiana	CP	Velebit (Croatia)
Asperula boissieri	B	Giona Massif (Greece)
Asperula borbasiana	NAP	Krk (Croatia)
Asperula bornmuelleri	G	Kastamonu (Northern Turkey)
Asperula calabra	Herbarium of E. Del Guacchio	Serra Dolcedorme (Southern Apennines, Italy)
Asperula crassifolia K1	Sampled in this study	Capri (Southern Italy)
Asperula crassifolia N2	Sampled in this study	Nerano (Southern Italy)
Asperula cynanchica 128	Sampled in this study	Gran Sasso-La Madonnina (Central Apennines, Italy)
Asperula cynanchica Cyn3	CP	Transylvania
Asperula cynanchica Cyn5	CP	England
Asperula cynanchica Cyn7	CP	Austria
Asperula deficiens	FI	Tavolara isle (Sardinia)
Asperula diminuta	CP	Dagestan
Asperula garganica	Herbarium of R.P. Wagensommer	Mount S. Angelo (Puglia, Southern Italy)
Asperula graveolens subsp. *danilewskiana*	CP	Kazakhstan
Asperula gussonei	CAT	(Madonie massif) Sicily
Asperula icarica	G	Icaria (Greece)
Asperula idaea	CP	Crete
Asperula lactea	CLU	Mount Consolino (Southern Italy)
Asperula lilaciflora subsp. *runemarkii*	UPA	Chios (Greece)
Asperula littoralis	B	Istanbul (Turkey)
Asperula lutea s.l.	CP	Mount Parnassos (Greece)

(continued)

Accession	Specimen origin	Geographical information
Asperula lutea subsp. *lutea*	CP	Peloponnese (Achaea, Greece)
Asperula mungieri	L	Mount Taygetus (Greece)
Asperula naufraga	NAP	Zakynthos (Greece)
Asperula neglecta 132	Cult. Hort. CRFA acc. n°488/11 (legit F. Bartolucci, N. Ranalli)	Vado di Corno (Central Apennines, Italy)
Asperula neglecta AN1	Sampled in this study	Campo Imperatore (Central Apennines, Italy)
Asperula neilreichii	L	Raxalpe (Austria)
Asperula nitida subsp. *nitida*	L	Mount Ulu Dagh (Turkey)
Asperula oetaea	CP	Peloponnese (Achaea, Greece)
Asperula ophiolithica	ACA	Euboea (Greece)
Asperula paui subsp. *paui*	B	Ibiza (Balearic Islands)
Asperula peloritana	CAT	Mount Scuderi (Sicily)
Asperula pestalozzae	E (http://data.rbge.org.uk/herb/E00639182)	Turkey
Asperula pinifolia	UPA	Mount Tymfristos (central Greece)
Asperula pulvinaris	UPA	Mount Pateras (Greece)
Asperula pumila	FI	Sopramonte d'Oliena (Sardinia)
Asperula rigidula	CP	Peloponnese (Greece)
Asperula rumelica	Mustafa Kemal University	Hatay (Southern Turkey)
Asperula rupicola	L	Alps (France)
*Asperula sp.**	ACA	Mount Ochi (Euboea Greece)
Asperula staliana subsp. *arenaria*	B	Biševo (Croatia)
Asperula staliana subsp. *diomedea*	Herbarium of E. Del Guacchio	San Nicola, Isole Tremiti (Italy)
Asperula staliana subsp. *issaea*	B	Vis (Croatia)
Asperula staliana subsp. *staliana*	B	Biševo (Croatia)
Asperula stricta subsp. *grandiflora*	E (http://data.rbge.org.uk/herb/E00639181)	Turkey
Asperula stricta subsp. *latibracteata*	E (http://data.rbge.org.uk/herb/E00270784)	Turkey
Asperula suberosa	CP	Mount Athos (Greece)
Asperula suffruticosa	ACA	Mount Dirphis (Euboea, Greece)
Asperula supina subsp. *supina*	MWG	Krasnodar(Russia)
Asperula tenuifolia	E (http://data.rbge.org.uk/herb/E00639174)	Turkey
Asperula tephrocarpa	B	Saratov (Russia)
Asperula visianii	NAP	Svetac (Croatia)
Asperula wettsteinii	G	Dinaric Alps (Serbia-Montenegro)
Asperula woloszczakii	NAP	Krk (Croatia)
Asperula woronowii	E (http://data.rbge.org.uk/herb/E00639173)	Turkey
Asperula x jordanii	CP	Alps (France)
Asperula x portae	Herbarium of E. Del Guacchio	Serra Dolcedorme (Southern Apennines, Italy)

*this specimen has been left unidentified after multiple attempts by various botanists;
it is probably related to *A. rigidula* (Dr. Panayiotis Trigas, Pers. Comm.)*.

Appendix C: corolla tube/lobes ratio

Character states of corolla tube/lobes ratio in *Asperula* sect. *Cynanchicae*; scoring is as follows:

- 0: corolla tube 2-5 times as long as lobes;
- 1: corolla tube shorter than lobes (only for the outgroup *A. purpurea*);
- 2: corolla tube 1-2 times as long as lobes;

Didymaea alsinoides has a corolla which is not comparable with those of *Asperula*, so it was scored as not applicable; (Ehrendorfer & Krendl, 1976; Ehrendorfer, 1982; Schönbeck-Temesy & Ehrendorfer, 1991; Bernardo *et al*., 2010; Gutermann & Ehrendorfer, 2000; Ehrendorfer, 2005; Brullo *et al*., 2009).

Taxa	Corolla tube
Didymaea alsinoides	Not applicable
Sherardia arvensis	long tube
Asperula purpurea	long lobes
Asperula abbreviata	long tube
Asperula affinis	long tube
Asperula aristata subsp. *aristata*	long tube
Asperula aristata subsp. *aristata*	long tube
Asperula aristata subsp. *condensata*	long tube
Asperula aristata subsp. *nestia*	long tube
Asperula aristata subsp. *oreophila*	long tube
Asperula aristata subsp. *oreophila*	long tube
Asperula aristata subsp. *oreophila*	long tube
Asperula aristata subsp. *thessala*	long tube
Asperula beckiana	short tube
Asperula boissieri	long tube
Asperula borbasiana	long tube
Asperula bornmuelleri	short tube
Asperula calabra	long tube
Asperula crassifolia	long tube
Asperula crassifolia	long tube
Asperula cynanchica	short tube
Asperula cynanchica	short tube
Asperula cynanchica	short tube
Asperula cynanchica	short tube
Asperula deficiens	long tube
Asperula diminuta	short tube
Asperula garganica	long tube
Asperula graveolens subsp. *danilevskiana*	short tube
Asperula gussonei	long tube
Asperula icarica	long tube
Asperula idaea	long tube
Asperula lactea	short tube

(continued)

Taxa	Corolla tube
Asperula lilaciflora subsp. *phrygia*	short tube
Asperula lilaciflora subsp. *runemarkii*	short tube
Asperula littoralis	short tube
Asperula lutea s.l.	long tube
Asperula lutea subsp. *lutea*	long tube
Asperula mungieri	long tube
Asperula naufraga	long tube
Asperula neglecta	short tube
Asperula neglecta	short tube
Asperula neilreichii	short tube
Asperula nitida subsp. *nitida*	long tube
Asperula oetaea	long tube
Asperula ophiolithica	long tube
Asperula paui subsp. *paui*	long tube
Asperula peloritana	long tube
Asperula pestalozzae	short tube
Asperula pinifolia	long tube
Asperula pulvinaris	long tube
Asperula pumila	long tube
Asperula rigidula	long tube
Asperula rumelica	short tube
Asperula rupicola	short tube
Asperula sp.	long tube
Asperula staliana subsp. *arenaria*	long tube
Asperula staliana subsp. *diomedea*	long tube
Asperula staliana subsp. *issaea*	long tube
Asperula staliana subsp. *staliana*	long tube
Asperula stricta subsp. *grandiflora*	long tube
Asperula stricta subsp. *latibracteata*	long tube
Asperula suberosa	long tube
Asperula suffruticosa	long tube
Asperula supina subsp. *supina*	short tube
Asperula tenuifolia	long tube
Asperula tephrocarpa	short tube
Asperula visianii	long tube
Asperula wettsteinii	long tube
Asperula woloszczakii	short tube
Asperula woronowii	short tube
Asperula x jordanii	long tube
Asperula x portae	long tube

Appendix D: *rps*16 haplotypes found in *Asperula crassifolia*

Specimen	Haplotype	Specimen	Haplotype
S1	C	N1	A
S2	C	N2	A
S3	C	N3	A
S4	C	N4	A
S5	C	N5	A
T1	C	N6	A
T2	C	N7	A
T3	B	N8	A
T4	C	N9	A
K1	B	N10	A
K2	C	N11	A
K3	C	N12	A
K4	C	N13	A
K5	D	N14	A
K6	C	N15	A
K7	C	N16	A
K8	C	N17	A
K9	C	N18	A
K10	B	N19	A
G1	A	PAS1	B
G2	A	PAS2	A
G3	A	GUS1	B
G4	A	GUS3	B
G5	A	GUS5	B
G6	A	BOR1	B
G7	A	BOR2	B
G8	A	BOR3	B
G9	A	BAS	B
G10	A	GUA	C

Ackowledgments