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Identity of the *Calcarata* species complex in *Viola* sect. *Melanium* (*Violaceae*)

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Abstract: The *Calcarata* species complex in *Viola* sect. *Melanium* (*Violaceae*) is a group of species from Italy and neighbouring islands. The complex is of considerable evolutionary interest because several hypotheses about hybrid speciation within the group have been previously proposed. Because the *Calcarata* complex is not well characterized morphologically, we used 142 samples representing 92 (of c. 120) species of *V.* sect. *Melanium* plus three outgroup species. Nuclear ITS and ETS and plastid *trnS–trnG* intergenic spacer sequences were analysed to test the monophyly of the *Calcarata* complex and to infer relationships among the constituent species. Both nuclear and plastid sequences resulted in very limited phylogenetic resolution. Based on the nuclear dataset, most species of the *Calcarata* complex were recovered in four clades that also contained species not previously associated with the complex. Results from the plastid dataset recovered most species of the complex in a large polytomy. However, one larger clade containing only *Calcarata* complex species could be recovered, and species of all four nuclear clades were part of this larger plastid clade. The *Calcarata* complex clearly could not be resolved as monophyletic. We hypothesize that the lack of phylogenetic resolution may result mainly from frequent hybridization and hybrid speciation, processes that are well documented for *Viola* and *V.* sect. *Melanium*.

Key words: *Calcarata* species complex, ETS, hybrid speciation, hybridization, ITS, *trnS–trnG*, *Viola*, *Viola* sect. *Melanium*, *Violaceae*

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Introduction

Interspecific hybridization has enormous evolutionary potential and can result in homoploid or polyploid hybrid speciation in addition to introgressive hybridization (Arnold 1997). Homoploid hybrid speciation appears to be rare and has been documented more or less convincingly in fewer than 30 cases (Yakimowski & Rieseberg 2014; Kadereit 2015; Schumer & al. 2014; Nieto Feliner & al. 2017). In contrast, polyploid hybrid speciation may account for 2% to 15% of speciation events in flowering plants (Otto & Whitton 2000; Wood & al. 2009).

Viola L. (*Violaceae*) is a genus of approximately 525 (Ballard & al. 2014) or 580 to 620 species (Wahlert & al. 2014) distributed in temperate regions and montane areas in the tropics worldwide (Ballard & al. 2014). Several species have long been the subject of intensive "biosystematic" studies (Briggs & Walters 2016), including studies of hybrid speciation based mainly on chromosome numbers, morphology, ecology and geographical distribution (Clausen 1926, 1927, 1931; Valentine 1950, 1958; Moore & Harvey 1961; Schmidt 1961, 1962, 1963, 1964; Harvey 1966; Küpfer 1971; Merxmüller 1974; Merxmüller & Lippert 1977; Erben 1985).

Several of these studies examined the Italian species of the informally named *Calcarata* complex (Merxmüller 1974; Merxmüller & Lippert 1977; Pignatti 1994; Erben & Raimondo 1995; Fenaroli & Moraldo 2003). The unranked taxon *Calcaratae* was first introduced by Becker (1910). In his treatment of *Viola* in the 2nd edition of *Die natürlichen Pflanzenfamilien* (Becker 1925), which is the last comprehensive treatment of the entire genus, Becker characterized his *V.* sect. *Melanium* Ging. [unranked] *Elongatae* W. Becker [unranked] *Crenatifoliae*

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W. Becker [unranked] *Calcaratae* W. Becker as violets with large flowers with long or short spurs, often basally dentate sepals and pinnate to dentate stipules. Gams (1925) characterized *V.* [unranked] *Calcaratae* as having long and creeping primary axes with leaves and flowers on lateral axes. Becker (1925) divided *V.* [unranked] *Calcaratae* into *V.* [unranked] *Eucalcaratae* W. Becker with long and acute spurs and *V.* [unranked] *Altaicae* W. Becker with long or short but obtuse spurs. These two groups were claimed to be distributed mostly in the Alps and southern European mountains, the Mediterranean islands (*V.* [unranked] *Eucalcaratae*) and from North Africa to East Asia (*V.* [unranked] *Altaicae*).

Following Becker (1925), *Viola* [unranked] *Eucalcaratae* contains *V. calcarata* L. from the Alps, *V. bertolonii* Pio emend. Merxm. & W. Lippert from Italy and *V. corsica* Nyman from Corsica, while *V.* [unranked] *Altaicae* contains *V. altaica* Ker Gawl. from southeastern Europe through the Caucasus to China, *V. dichroa* Boiss. from Turkey, *V. arsenica* Beck from southeastern Europe and *V. eugeniae* Parl., *V. nebrodensis* Presl and *V. pseudogracilis* Strobl from Italy as well as *V. munbyana* Boiss. & Reut. from North Africa.

In the course of time, the use of the name *Viola* [unranked] *Calcaratae*, as the *Calcarata* complex (*V. calcarata*-Gruppe; Schmidt 1964), was limited to mostly Italian species, and the complex was considered by Schmidt (1964) to contain taxa with large flowers. Many of the species considered to belong to the *Calcarata* complex or found to be associated with it by us, have flowers > 2 cm, and sometimes up to 4 cm wide (Valentine & al. 1968; Pignatti 2017). Pignatti (2017) characterized the complex as often having dimorphic leaves where the lower and upper cauline leaves differ in shape, complex stipules (pinnately or palmately divided), and thin and rather long (20–30 cm) ascending axes. According to Pignatti (2017) the complex occurs at higher elevations in Italy and on Sicily, Sardinia, Elba and Corsica. Following Schmidt (1964) and Pignatti (1994, 2017), the Italian *Calcarata* complex contains the following 15 species: *V. aethnensis* Parl., *V. bertolonii*, *V. calcarata*, *V. corsica*, *V. culminis* F. Fen. & Moraldo, *V. dubyana* Burnat ex Gremli, *V. etrusca* Erben, *V. eugeniae*, *V. ferrarinii* Moraldo & Ricceri, *V. merxmuelleri* Erben, *V. nebrodensis*, *V. pseudogracilis*, *V. tineorum* Erben & Raimondo, *V. ucriana* Erben & Raimondo and *V. valderia* All. Several of these species were treated by Fiori (1924) as varieties of *V. calcarata*. These species were grouped by Pignatti (2017) with *V. argenteria* Moraldo & Forneris, *V. cenisia* L., *V. comollia* Massara and *V. magellensis* Porta & Rigo ex Strobl, which all have more or less undivided stipules, in *V.* sect. *Melanium* group I [unranked] *Heterophyllae* Pignatti. It is conceivable that some of the species newly described by Ricceri & al. (2018) also belong to this complex. Schmidt (1964) suspected that the Italian *Calcarata* complex species might be closely related to species from the Balkans and Greece.

The *Calcarata* complex clearly belongs to *Viola* sect. *Melanium*, a group of more than 120 species (Valentine & al. 1968; Merxmüller & Lippert 1977; Erben 1984, 1985, 1986, 1989; Erben & Raimondo 1995; Ballard & al. 1999; Erben 2000; Fenaroli & Moraldo 2003; Blaxland 2004; Moraldo & al. 2011; Marcussen & al. 2015; Margini & Scoppola 2015; Ricceri & al. 2018; Perrino & al. 2018). *Viola* sect. *Melanium* is widely distributed in western Asia and Europe, with its centre of diversity in the hills and mountains of southern Europe, especially the Balkan Peninsula and the Apennines (Erben 1996). The only species of *V.* sect. *Melanium* to occur in the New World is *V. bicolor* Pursh from eastern North America (Clausen & al. 1964). Previous studies have recovered *V.* sect. *Melanium* as monophyletic (Ballard & al. 1999: two species; Yockteng & al. 2003: 20 species; Marcussen & al. 2015: three species; Slomka & al. 2015: 25 species), but very little phylogenetic resolution within the section was obtained using ITS sequences (Yockteng & al. 2003; Slomka & al. 2015).

In preparation of a detailed study of hybrid speciation in the *Calcarata* complex using restriction-site associated DNA sequencing (RADseq), we set out to investigate whether this *Calcarata* complex, which is not well-characterized morphologically, can be resolved as monophyletic using Sanger sequencing data, and whether species from outside Italy and the listed Mediterranean islands also belong to this *Calcarata* complex. For this purpose, we inferred a phylogeny of *Viola* sect. *Melanium* using 92 species and DNA sequence data from the nuclear ribosomal internal transcribed spacer region (ITS), the nuclear ribosomal external transcribed spacer region (ETS) and the plastid *trnS–trnG* intergenic spacer region.

Material and methods

Taxon sampling

A total of 142 samples of *Viola* sect. *Melanium*, representing 92 species (including seven species with more than one subspecies or variety), were included in our analysis. Leaf material was taken either from herbarium specimens or was available as silica-dried material collected during fieldwork in 2018. Based on the results by Marcussen & al. (2015), *V. hirta* L. (*V.* sect. *Viola*), *V. cazorlensis* Gand. (*V.* sect. *Delphiniopsis* W. Becker) and *V. scorpiuroides* Coss. (*V.* sect. *Xylinosium* W. Becker) were included as outgroups. These three sections had been identified as close relatives of *V.* sect. *Melanium*. Specimen voucher information is listed in Supplementary Table 1 (see Supplemental content online).

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) fol-

lowing the manufacturer's protocol but with lysis for 30 minutes. We sequenced the nuclear ITS and ETS regions and the plastid *trnS–trnG* intergenic spacer, which included part of the *trnS* gene (71 bp). PCR amplification of the ITS region was performed using the primer pair ITS1/ITS4 (White & al. 1990). For the ETS region we used the primer pair jKETS-9/ETS-18S (Mitchell & al. 2009; Wright & al. 2001), and for *trnS–trnG*, the primer pair *trnS*/*trnG* (Cennamo & al. 2011). PCR products were purified with ExoSap-IT PCR Clean-Up (Affymetrix, Santa Clara, California, U.S.A.). Cycle sequencing was carried out with BigDye Terminator 3.1 (Applied Biosystems, Foster City, California, U.S.A.), using the same primers as for the PCR amplifications. The fluorescence labelled samples were run on an ABI 3130xl Genetic Analyzer at Johannes Gutenberg-Universität Mainz (Germany) for sequencing.

DNA sequence alignment and phylogenetic analysis

Contigs of forward and reverse sequences were assembled and manually edited using Sequencher v.4.1.4 (Gene Codes, Ann Arbor, Michigan, U.S.A.). Sequences were aligned automatically with Mafft7 (Rozewicki & al. 2019) and manually adjusted using MEGA V7.0.21 (Kumar & al. 2016). Phylogenetic analyses were carried out using Maximum Likelihood (ML) and Bayesian inference (BI). The resulting topologies were compared and inspected for supported conflicts as described by Pirie & al. (2008). Since there were no conflicts between the nuclear datasets, we combined ITS and ETS sequences. All sequences obtained in this study were submitted to GenBank (https://www.ncbi.nlm.nih.gov/genbank/), and accession numbers for the sequences are given in Supplementary Table 1 (see Supplemental content online). The ML analyses for each individual marker were run on the CIPRES Science Gateway (Miller & al. 2010), using RAxML v.8.2.8 (RAxML-HPC2 on XSEDE) with default settings (Stamatakis 2014). Bayesian inference was performed using BEAST v.1.8.3 (Drummond & Rambaut 2007; Drummond & al. 2012). For each aligned locus, the best-fit substitution model was detected using PartitionFinder2 on XSEDE (Lanfear & al. 2016). HKY + G + I was suggested as the most appropriate nucleotide substitution model for the *trnS–trnG* spacer region, ITS1, ITS2 and ETS, and JC for *trnS* and 5.8S. The Birth-Death Process was chosen as the tree prior. The individual output log files were examined using Tracer v.1.5 (Rambaut & Drummond 2009) to assess convergence. The first 1000 trees (10 %) were discarded as burn-in, and a maximum clade credibility tree was computed using TreeAnnotator v.1.8.3 (Drummond & al. 2012).

ITS substitution rate

In order to compute an ITS substitution rate based on a crown group age of *Viola* sect. *Melanium* of between 12.76 and 15.26 (means) million years (ma) as estimated by Marcussen & al. (2015), we used r8s (Sanderson 2003) following the protocol by Lanfear & al. (2013) and using 12.76 ma as minimum and 15.26 ma as maximum crown group ages.

Results

The sequence alignments were 491 bp (ITS), 471 bp (ETS) and 628 bp (*trnS–trnG*, *trnS* gene) long. The combined nuclear dataset and the plastid dataset contained 132 and 134 accessions, respectively. The ML and BI phylogenies from the combined nuclear dataset and from the plastid dataset showed supported topological differences. In general, the ML/BI plastid phylogenies were far less resolved than the nuclear phylogenies. Apart from the absence of a few clades in the ML phylogeny, the ML and BI topologies did not differ. Accordingly, the phylogenetic trees shown (Fig. 1, 2) represent the BI topology with ML bootstrap support (BS) added. Branches with BS < 50 or posterior probabilities (PP) < 0.95 were collapsed.

Viola sect. *Melanium* was recovered as monophyletic in all analyses. In the following, we will describe our results with reference to the *Calcarata* complex as described in the introduction. Most species of the *Calcarata* complex group in four larger clades in the BI analysis of the nuclear dataset (Fig. 1). *Viola aethnensis* subsp. *aethnensis*, *V. corsica* subsp. *limbarae* Merxm. & W. Lippert, *V. dubyana*, *V. merxmuelleri* and *V. valderia* were recovered outside these clades in larger polytomies. In the following description, clades with species of the *Calcarata* complex are given capital letters in the nuclear dataset (Fig. 1) and numbers in the chloroplast dataset (Fig. 2). These clades are also listed in Table 1.

Clade **A** (PP 1, BS 86) contains *Viola nebrodensis*, *V. ucriana*, *V. tineorum*, *V. bertolonii* and *V. ferrarinii* of the *Calcarata* complex together with *V. cornuta* L. from the Pyrenees, *V. grisebachiana* Vis. and *V. orphanidis* Boiss. both from the Balkan peninsula and *V. paradoxa* Lowe from Madeira. In this clade, three species of the *Calcarata* complex from Sicily, *V. nebrodensis*, *V. ucriana* and *V. tineorum*, form a supported subclade (PP 1, BS 76). Of the *Calcarata* complex species of clade **A**, *V. ferrarinii* and *V. bertolonii* fall into clade **2** (PP 1, BS 64) in the BI analysis of the plastid data (Fig. 2; Table 1), and *V. nebrodensis* and *V. ucriana* form a supported clade (**1**) (PP 1, BS 70; Table 1). Clade **B** (PP 0.98, BS 62) contains *V. eugeniae* subsp. *levieri* (Parl.) A. F. W. Schmidt, *V. eugeniae* subsp. *eugeniae*, *V. pseudogracilis* subsp. *cassinensis* (Strobl) Merxm. & A. F. W. Schmidt, *V. aethnensis* subsp. *splendida* (W. Becker) Merxm. & W. Lippert, *V. corsica* subsp. *ilvensis* (W. Becker) Merxm., *V. aethnensis* subsp. *calabra* (A. Terracc.) Peruzzi and *V. pseudogracilis* subsp. *pseudogracilis* of the *Calcarata* complex plus the Italian *V. magellensis* and the Greek *V. rausii* Erben, *V. graeca* (W. Becker) Halacsy and *V. sfikasiana* Erben. Of the *Calcarata* complex species of clade **B**, *V. corsica*

subsp. *ilvensis* is part of clade **2** in the BI analysis of the plastid data (Fig. 2; Table 1). All other *Calcarata* complex taxa are part of large polytomies in this analysis. Clade **C** (PP 1, BS 72) contains *V. corsica* subsp. *corsica* and *V. etrusca* of the *Calcarata* complex together with *V. montcaunica* Pau from Spain. Of the *Calcarata* complex species of clade **C**, *V. corsica* subsp. *corsica* is part of clade **2** in the BI analysis of the plastid data (Fig. 2a; Table 1) and *V. etrusca* and *V. montcaunica* are supported sister to each other (clade **3**; PP 0.99, BS 68; Fig. 2; Table 1). Clade **D** (PP 1, BS 88) contains *V. culminis*, *V. calcarata* subsp. *calcarata*, *V. calcarata* subsp. *cavillieri* (W. Becker) Merxm. & W. Lippert, *V. calcarata* subsp. *zoysii* (Wulf.) Merxm. and *V. calcarata* subsp. *villarsiana* (Roem. & Schult.) Merxm. of the *Calcarata* complex. They group together with the widespread *V. lutea* Huds. subsp. *lutea* and *V. beckiana* Fiala from the southern Balkan peninsula. Of the *Calcarata* complex taxa of clade **D**, *V. calcarata* subsp. *villarsiana* and *V. calcarata* subsp. *cavillieri* are part of clade **2** in the BI analysis of the plastid data (Fig. 2; Table 1). *Viola culminis* is part of a supported clade (**4**) together with *V. comollia* in the plastid phylogeny (PP 1, BS 60; Fig. 2; Table 1), and the remaining *Calcarata* complex taxa of clade **D** are part of large polytomies in this analysis.

In summary, species of the *Calcarata* complex fall into four larger clades in the BI analysis of the nuclear dataset. Interestingly, some species from these four clades are part of the only larger supported clade (**2**) in the plastid phylogeny, which also contains *Viola corsica* subsp. *limbarae*, which is not part of any larger clade in the nuclear phylogeny. All species recovered in clade **2** of the plastid phylogeny are part of the *Calcarata* complex.

The ITS substitution rate calculated using the crown group age of *Viola* sect. *Melanium* estimated by Marcussen & al. (2015) is 0.57×10^{-9} substitutions/site/year.

Table 1. Clades recovered in the analyses of the ITS/ETS and *trnS–trnG* datasets. Species of the *Calcarata* complex are marked in bold script. Placement of species of ITS/ETS clades in *trnS–trnG* clades and of species of *trnS–trnG* clades in ITS/ETS clades is indicated in parentheses.

ITS/ETS	*trnS–trnG*
Clade A	Clade 1
Viola nebrodensis (Clade 1)	***Viola nebrodensis*** (Clade A)
V. ucriana (Clade 1)	***V. ucriana*** (Clade A)
V. tineorum	Clade 2
V. cornuta	***V. corsica*** subsp. ***ilvensis*** (Clade B)
V. bertolonii (Clade 2)	***V. corsica*** subsp. ***limbarae***
V. grisebachiana	***V. ferrarinii*** (Clade A)
V. paradoxa	***V. corsica*** subsp. ***corsica*** (Clade C)
V. orphanidis	***V. calcarata*** subsp. ***villarsiana*** (Clade D)
V. ferrarinii (Clade 2)	***V. calcarata*** subsp. ***cavillieri*** (Clade D)
Clade B	***V. bertolonii*** (Clade A)
V. eugeniae subsp. ***levieri***	Clade 3
V. eugeniae subsp. ***eugeniae***	***V. etrusca*** (Clade C)
V. pseudogracilis subsp. ***cassinensis***	*V. montcaunica*
V. magellensis	Clade 4
V. rausii	*V. comollia*
V. aethnensis subsp. ***splendida***	***V. culminis*** (Clade D)
V. corsica subsp. ***ilvensis*** (Clade 2)	
V. aethnensis subsp. ***calabra***	
V. graeca	
V. sfikasiana	
V. pseudogracilis subsp. ***pseudogracilis***	
Clade C	
V. corsica subsp. ***corsica*** (Clade 2)	
V. montcaunica	
V. etrusca (Clade 3)	
Clade D	
V. lutea subsp. *lutea*	
V. culminis (Clade 4)	
V. calcarata subsp. ***calcarata***	
V. calcarata subsp. ***cavillieri*** (Clade 2)	
V. calcarata subsp. ***zoysii***	
V. calcarata subsp. ***villarsiana*** (Clade 2)	
V. beckiana	

Discussion

Considering the phylogenetic topologies obtained by us from the analysis of nuclear (ITS/ETS) and plastid (*trnS–trnG*) sequences (Fig. 1, 2), it is obvious that neither the Italian *Calcarata* complex as understood by Schmidt (1964) and Pignatti (1994, 2017) nor Becker's (1925) *Viola* [unranked] *Calcaratae* was recovered as a monophyletic group. In the nuclear dataset, most taxa of the Italian *Calcarata* complex – except *V. aethnensis* subsp. *aethnensis*, *V. corsica* subsp. *limbarae*, *V. dubyana*, *V. merxmuelleri* and *V. valderia* – fall into four larger clades which, however, all also contain additional species that had not previously been considered part of the *Calcarata* complex. In the plastid dataset, some species of all four nuclear clades fall into clade **2**, which contains only *Calcarata* complex species. However, the majority of *Calcarata* complex species are found in unresolved positions in a large polytomy.

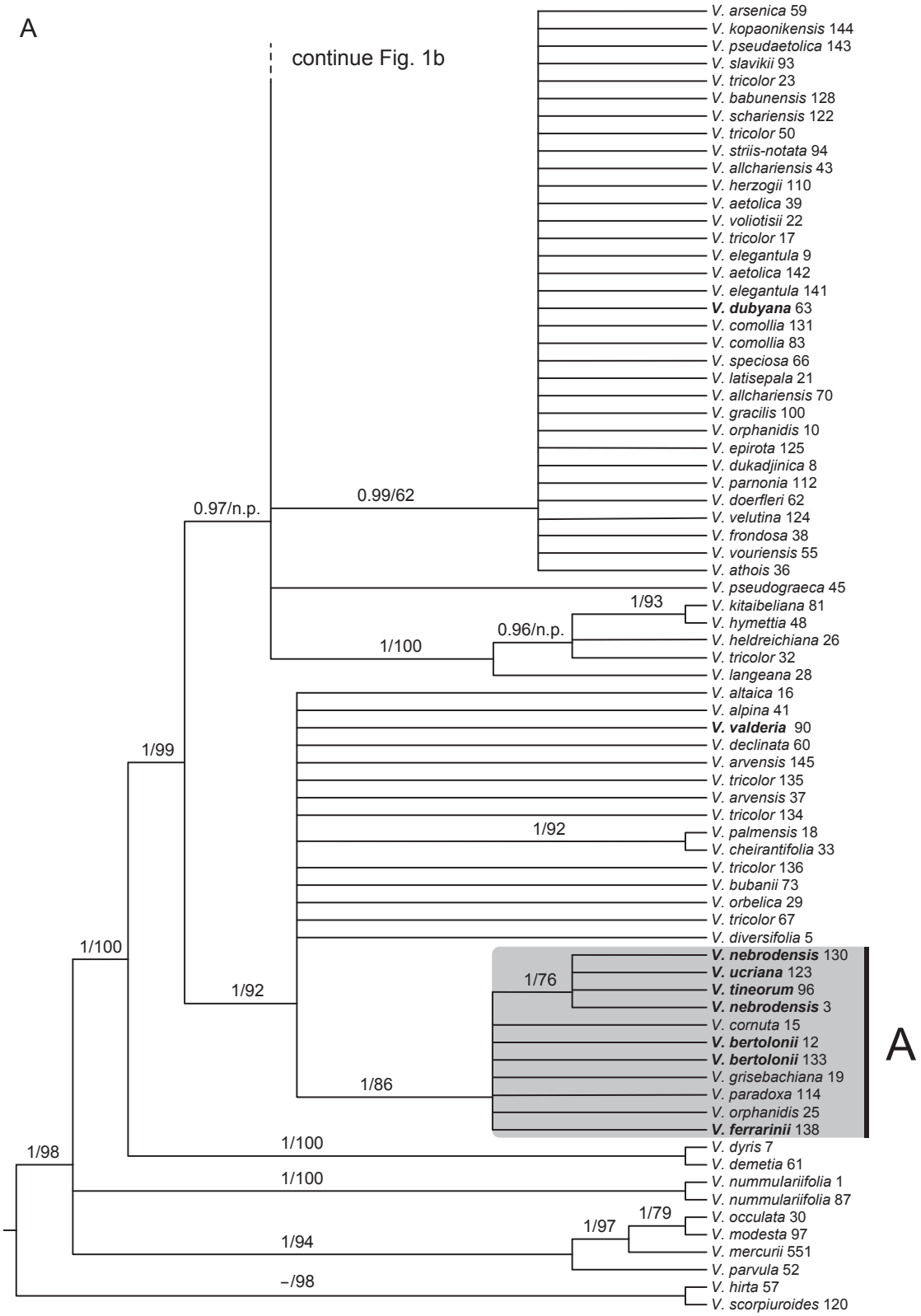

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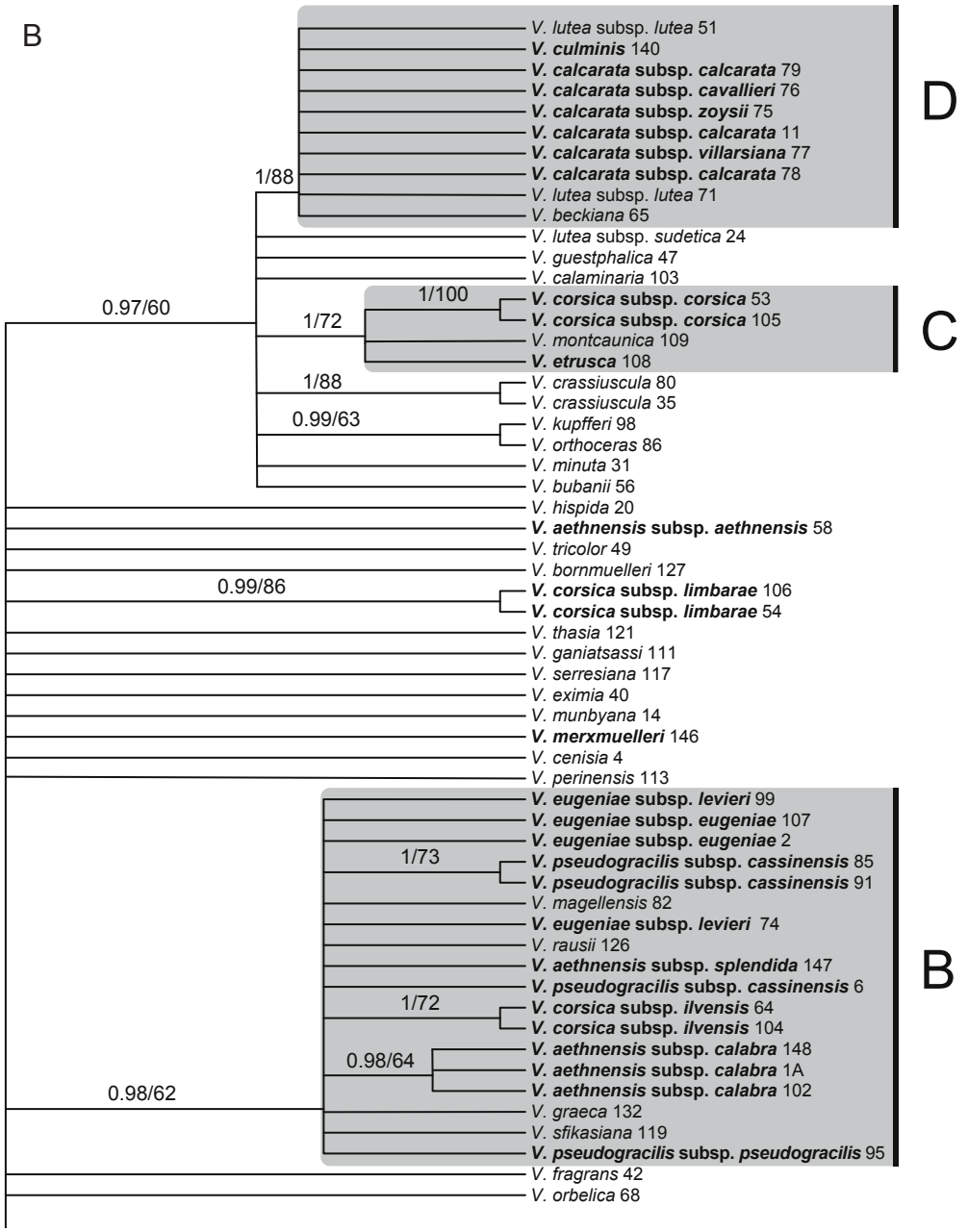
Fig. 1. Bayesian inference (BI) phylogeny of *Viola* sect. *Melanium* based on the combined nuclear dataset (ITS/ETS). Values above branches are Bayesian posterior probability and maximum likelihood bootstrap values (PP/BS). Only posterior probabilities ≥ 0.95 and bootstrap values ≥ 50 are shown. A hyphen (-) represents posterior probabilities < 0.95 or bootstrap values < 50. Absence of clades is indicated by n.p. (not present). Capital letters refer to clades discussed in the text. Species in bold script have been considered part of the *Calcarata* complex in the past.



The question arises as to why the *Calcarata* complex/ *Viola* [unranked] *Calcarateae* is not resolved as monophyletic and why, more generally, phylogenetic resolution is rather poor in both our nuclear and plastid phylogenies, as also found by others (e.g. Ballard & al. 1999; Yockteng & al. 2003; Wahlert & al. 2014; Slomka & al. 2015). The lack of resolution could be caused by insufficient sequence divergence (Maddison 1989), incomplete lineage sorting, or hybridization (Doyle 1992; Maddison 1997). In this case, we postulate that hybridization is the major problem in reconstructing phylogenetic relationships in *Viola* in general and in *V.* sect. *Melanium* in particular. First, there exist a large number of studies which have either documented interspecific hybridization in the genus and in *V.* sect. *Melanium* (Clausen 1927; Fothergill 1938; Erben 1985, 1996; Pettet 1964; Kakes 1979; Krahulcová 1996; Marcussen & al. 2001; Conesa & al. 2008) or hybrid speciation (Erben 1996; Küpfer 1971; Merxmüller & Lippert 1977; Pignatti 1994; Erben & Raimondo 1995; Erben 1996; Fenaroli & Moraldo 2003; Siuta & al. 2005; Marcussen & al. 2012), and the role of hybridization in the origin of supraspecific lineages in *Viola*, suspected previously by Ballard & al. (1999), has been convincingly demonstrated by Marcussen & al. (2015). An example of hybrid speciation in *V.* sect. *Melanium* inferred by nuclear-plastid incongruence may be highlighted by *V. corsica* subsp. *ilvensis.* This taxon has been hypothesized by Pignatti (1994) to have originated from hybridization between *V. bertolonii*, with which it groups in clade **2** of the plastid phylogeny (Fig. 2), and *V. eugeniae* or *V. pseudogracilis*, with which it groups in clade **B** in the nuclear phylogeny (Fig. 1). Second, evidence for hybridization may also be provided by the lim-

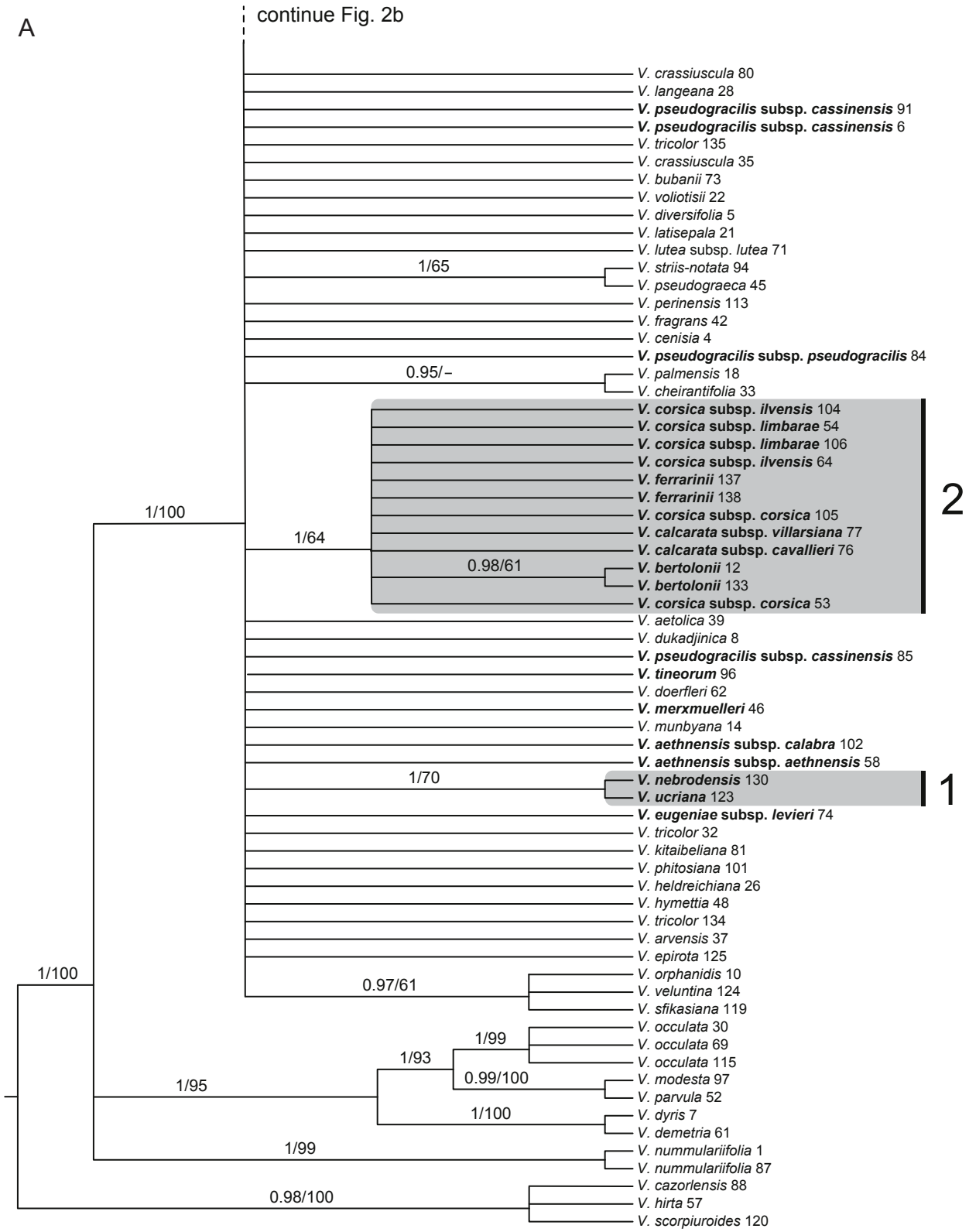


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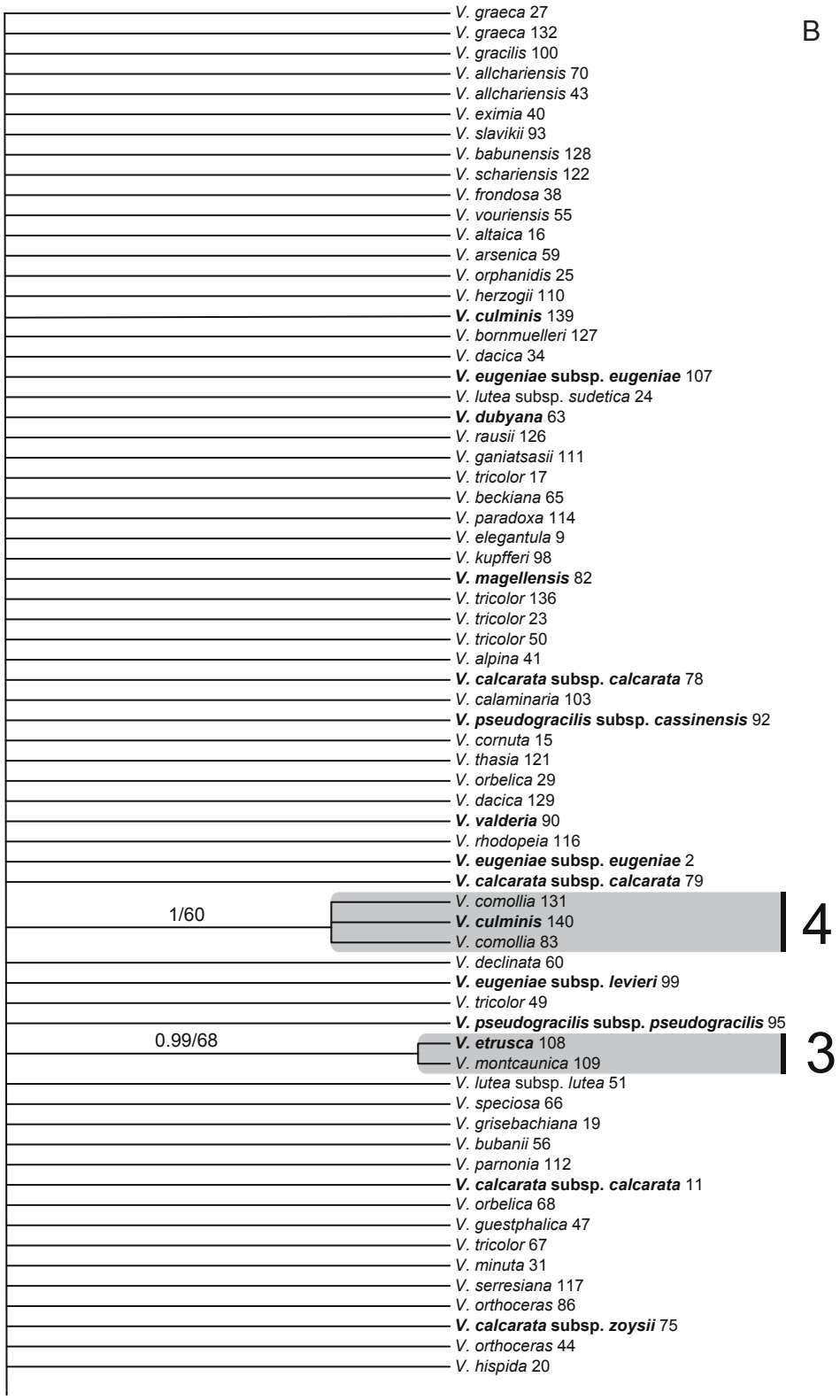
Fig. 2. Bayesian inference (BI) phylogeny of *Viola* sect. *Melanium* based on the plastid dataset (*trnS–trnG*). Values above branches are Bayesian posterior probability and maximum likelihood bootstrap values (PP/BS). Only posterior probabilities ≥ 0.95 and bootstrap values ≥ 50 are shown. A hyphen (-) represents bootstrap values < 50. Numbers refer to clades discussed in the text. Species in bold script have been considered part of the *Calcarata* complex in the past.



ited amount of ITS sequence divergence in the section. If indeed, as estimated by Marcussen & al. (2015), *Viola* is of Oligocene origin and the crown group age of *V.* sect. *Melanium* is between 12.76 and 15.26 (means) ma, the ITS substitution rate calculated by us is 0.57×10^{-9} substitutions/site/year. Such a rate clearly falls outside the known range of ITS substitution rates for herbaceous annual or perennial plant species of 1.72×10^{-9} to 8.34×10^{-9} substitutions/site/year and is similar to the lowest rates otherwise found only in woody plants (Kay & al. 2006). The low substitution rate found by us is then perhaps best explained by hybridization across the entire section, resulting in homogenization of younger ribotypes leading to the exclusion of older ribotypes.

Based on our findings, we hypothesize that hybridization has been so frequent in the evolution of *Viola* sect. *Melanium* that tree building methods such as ML and BI are not suitable for the reconstruction of phylogenetic relationships (Posada & Crandall 2001).

Irrespective of the likely great importance of hybridization in the evolution of *Viola* sect. *Melanium*, we will briefly examine (1) those species of the Italian *Calcarata* complex that did not fall into the four major nuclear clades, (2) those species that fell into these nuclear clades but had not been explicitly associated with the *Calcarata* complex or *V.* [unranked] *Calcaratae* before, and (3) those species that fell into the plastid clade but had not been explicitly associated with the *Calcarata* complex or *V.* [unranked] *Calcaratae* before. The four major nuclear clades found by us do not correspond to the major morphological groups among Italian *Calcarata* complex species identified by Pignatti (2017).

As for the five taxa not falling into the four larger clades of *Calcarata* complex species in our nuclear phylogeny, i.e. *Viola aethnensis* subsp. *aethnensis*, *V. corsica* subsp. *limbarae*, *V. dubyana*, *V. merxmuelleri* and *V. valderia*, their relationship to the *Calcarata* complex has never been doubted from a morphological point of view (Pignatti 2017). In the case of *V. aethnensis* subsp. *aethnensis* and *V. corsica* subsp. *limbarae*, other subspecies of these two species group in the four major nuclear clades found by us, and *V. corsica* subsp. *limbarae* falls into clade **2** of our plastid phylogeny.

Species not strictly associated with the Italian *Calcarata* complex or *Viola* [unranked] *Calcaratae* are *V. cornuta*, *V. grisebachiana*, *V. orphanidis* and *V. paradoxa* in clade **A**, *V. graeca*, *V. magellensis*, *V. rausii* and *V. sfikasiana* in clade **B**, *V. montcaunica* in clade **C** and *V. beckiana* and *V. lutea* subsp. *lutea* in clade **D**. Of these 11 species, the widespread *V. lutea* subsp. *lutea* (clade **D**) has been considered closely related to *V. calcarata* by Reiche & Taubert (1895), and Becker (1925) included *V. dubyana*, a species of the *Calcarata* complex, in his *V.* [unranked] *Luteae* W. Becker, which also included *V. lutea*. *Viola lutea* was found in a clade containing *Calcarata* complex species also by Hildebrandt & al. (2006). *Viola beckiana* (clade **D**), a species from serpentine or calcareous rocks in the southern Balkan peninsula, is large-flowered and has palmately or pinnately divided stipules (Valentine & al. 1968). The Spanish *V. montcaunica* (clade **C**) and *V. cornuta* (clade **A**; both with 2n =22), of which the former was described as essentially a smaller form of the latter by Valentine & al. (1968), have palmately divided stipules like *V. valderia* (see above) and share relatively large flowers and long spurs with species of the *Calcarata* complex. *Viola magellensis* was included in *V.* sect. *Melanium* group I [unranked] *Heterophyllae*, which also includes the *Calcarata* complex, by Pignatti (2017). Of the Greek species (all clade **B**), *V. graeca* has large flowers and long spurs, *V. rausii* has long spurs and dimorphic leaves and *V. sfikasiana* has dimorphic leaves, all characters found in at least part of the *Calcarata* complex. *Viola orphanidis* and *V. cornuta*, grouping with species of the *Calcarata* complex in clade **A**, are similar to each other in leaf and stipule shape. Following Gams (1925), Becker included *V. orphanidis*, a species from the Balkans, in *V. lutea*, and *V. lutea* had previously been linked to the *Calcarata* complex (see above). *Viola grisebachiana* (clade **A**) from the Balkans is acaulescent according to Valentine & al. (1968) and has no obvious similarities to the *Calcarata* complex. Finally, *V. paradoxa* (clade **A**) from Madeira has been postulated to be a close relative of *V. calcarata* by Lowe (1868); the species has long axes, dimorphic leaves and large flowers (Short 1994). In the plastid clades, only *V. montcaunica* (clade **3**) and *V. comollia* (clade **4**) fell into supported clades with *Calcarata* complex species (Fig. 2). Of these, *V. montcaunica* has been discussed above, and *V. comollia* was included in *V.* sect. *Melanium* group I [unranked] *Heterophyllae* by Pignatti (2017).

With the exception of *Viola grisebachiana*, a relationship of all those species that have not been associated with the *Calcarata* complex but falling into nuclear *Calcarata* complex clades seems possible on the basis of their morphology or on the basis of their classification by earlier authors. However, in the absence of clear morphological characters defining the *Calcarata* complex – all characters used by Pignatti (2017) to characterize the *Calcarata* complex can also be found in other species of *V.* sect. *Melanium* (Valentine & al. 1968) – association of non-*Calcarata* species with the *Calcarata* complex based on morphological characters is not fully convincing.

In summary, tree-building methods such as ML and BI do not appear to be suitable for the reconstruction of phylogenetic relationships in *Viola* and *V.* sect. *Melanium* because of rampant interspecific hybridization. Reconstruction of relationships instead requires both larger DNA sequence datasets and tree-building methods that take hybridization into account (e.g. Wen & al. 2018).

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