

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

Authors: Krause, Sandra, and Kadereit, Joachim W.

Source: Willdenowia, 50(2) : 195-206

Published By: Botanic Garden and Botanical Museum Berlin (BGBM)

URL: <https://doi.org/10.3372/wi.50.50205>

---

BioOne Complete ([complete.BioOne.org](https://complete.BioOne.org)) is a full-text database of 200 subscribed and open-access titles in the biological, ecological, and environmental sciences published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Complete website, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at [www.bioone.org/terms-of-use](https://www.bioone.org/terms-of-use).

Usage of BioOne Complete content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

---

BioOne sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

SANDRA KRAUSE<sup>1\*</sup> & JOACHIM W. KADEREIT<sup>1</sup>

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

Version of record first published online on 12 May 2020 ahead of inclusion in August 2020 issue.

**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

**Article history:** Received 21 January 2020; peer-review completed 10 March 2020; received in revised form 2 and 6 April 2020; accepted for publication 6 April 2020.

**Citation:** Krause S. & Kadereit J. W. 2020: Identity of the *Calcarata* species complex in *Viola* sect. *Melanium* (Violaceae). – Willdenowia 50: 195–206. doi: <https://doi.org/10.3372/wi.50.50205>

## 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 “bio-

systematic” 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*

<sup>1</sup> Institut für Organismische und Molekulare Evolutionsbiologie, Johannes Gutenberg-Universität Mainz, 55099 Mainz, Germany; \*e-mail: [sankraus@uni-mainz.de](mailto:sankraus@uni-mainz.de) (author for correspondence); [kadereit@uni-mainz.de](mailto:kadereit@uni-mainz.de)

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 Greml, *V. etrusca* Erben, *V. eugeniae*, *V. ferrarinii* Moraldo & Ricceri, *V. merxmulleri* Erben, *V. nebrodensis*, *V. pseudogracilis*, *V. tineorum* Erben & Raimondo, *V. uciana* 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* Mas-sara 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 Tree-Annotator 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 estimat-

ed 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. merxmulleri* 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 Marcusen & al. (2015) is  $0.57 \times 10^{-9}$  substitutions/site/year.

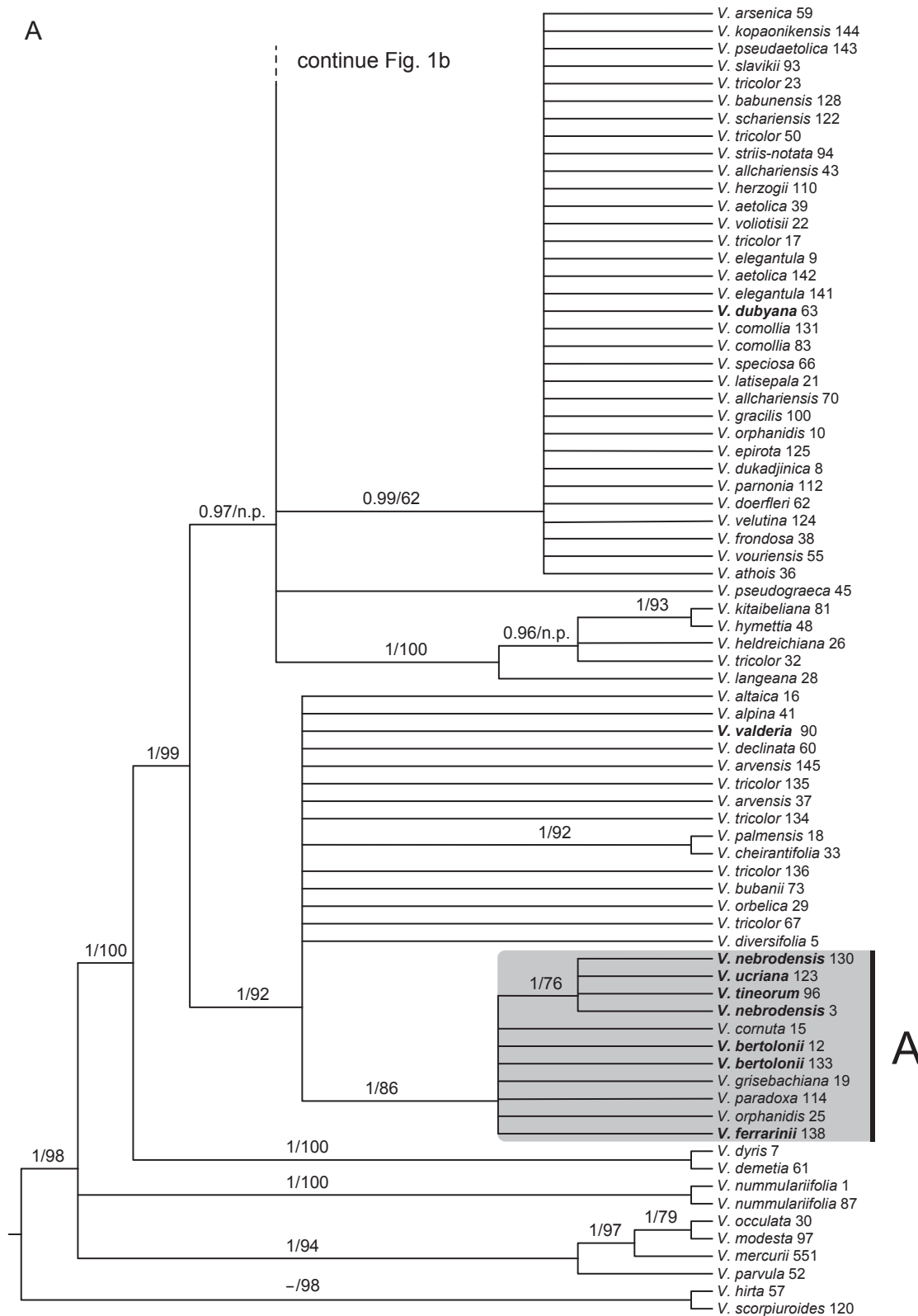
## Discussion

Considering the phylogenetic topologies obtained by us from the analysis of nuclear (ITS/ETS) and plastid

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.

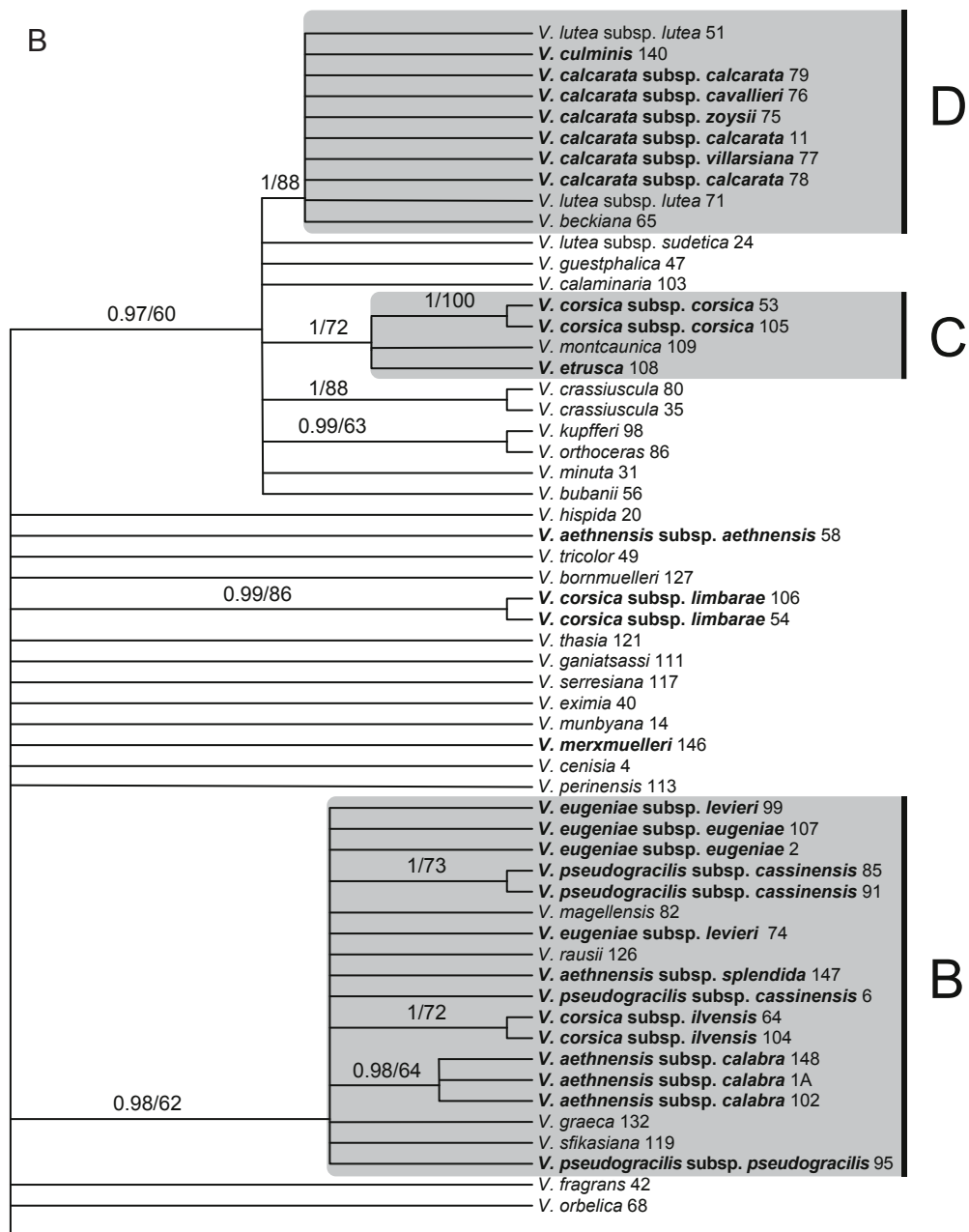
ITS/ETS	<i>trnS-trnG</i>
Clade A	Clade 1
<b><i>Viola nebrodensis</i></b> (Clade 1)	<b><i>Viola nebrodensis</i></b> (Clade A)
<b><i>V. ucriana</i></b> (Clade 1)	<b><i>V. ucriana</i></b> (Clade A)
<b><i>V. tineorum</i></b>	Clade 2
<i>V. cornuta</i>	<b><i>V. corsica</i></b> subsp. <b><i>ilvensis</i></b>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

(*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. merxmulleri* 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.



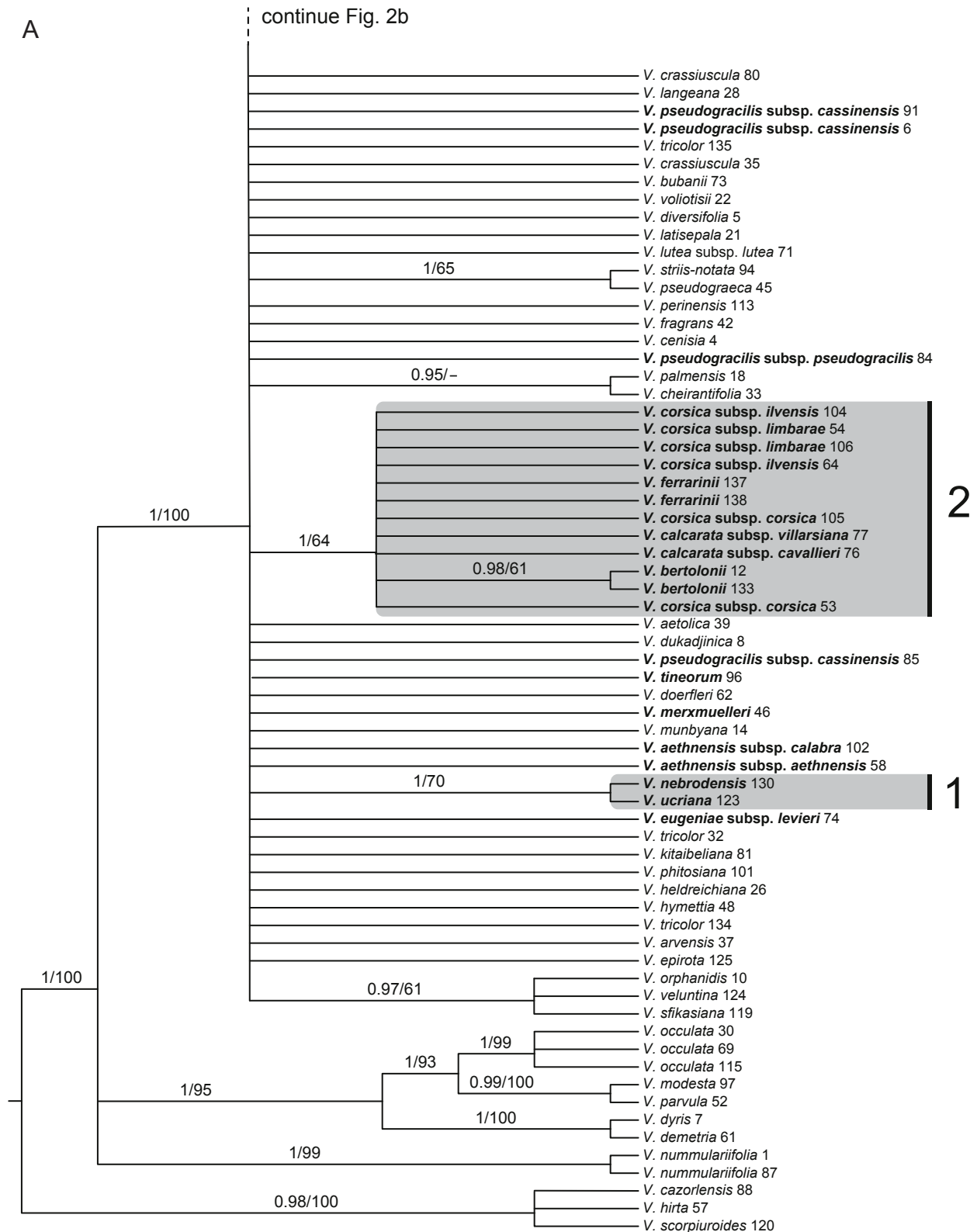
continued on next page

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  $\geq 0.95$  and bootstrap values  $\geq 50$  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] *Calcaratae* 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 &

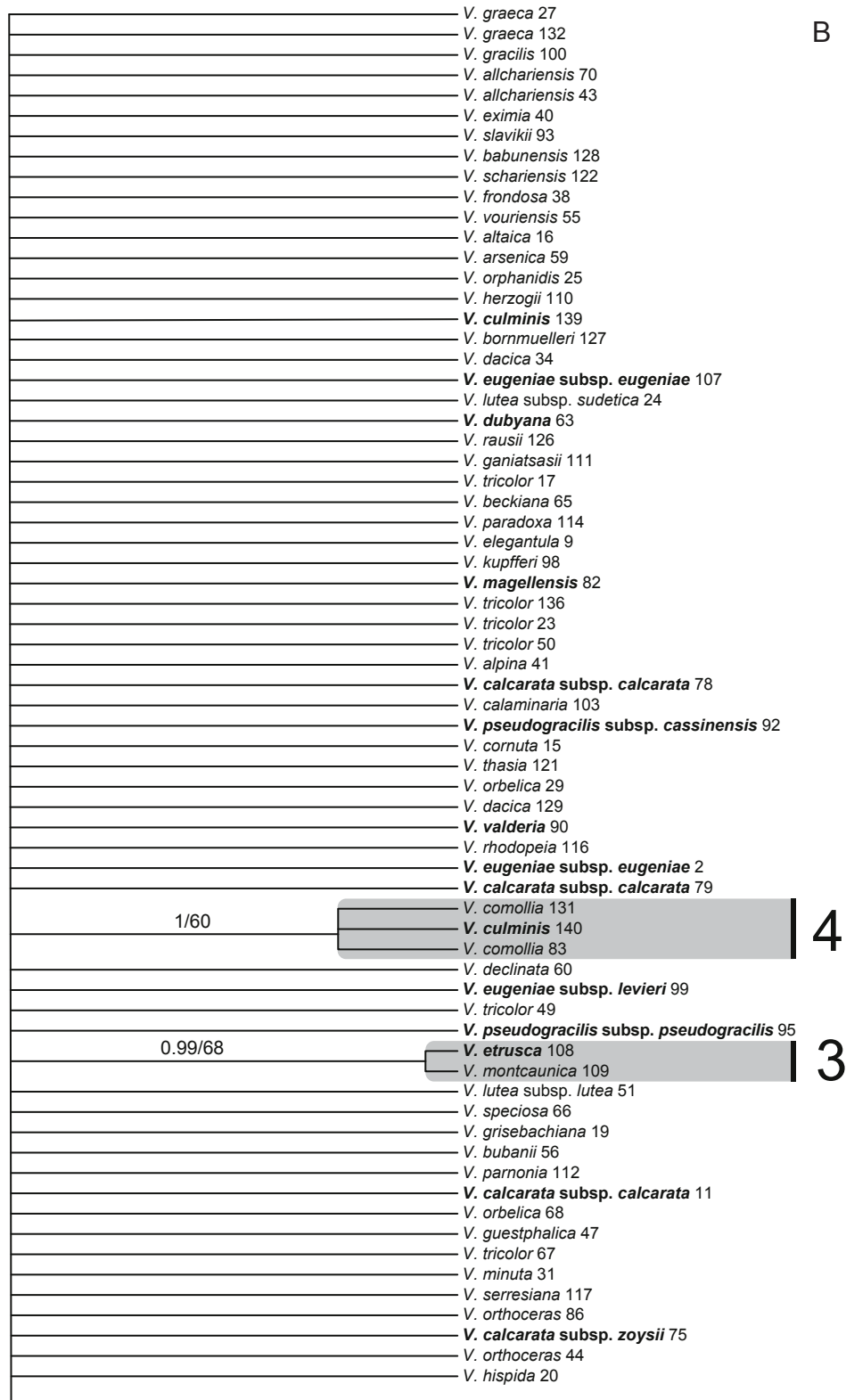
al. 2001; Conesa & al. 2008) or hybrid speciation (Erben 1996; Küpfer 1971; Merxmüller & Lippert 1977; Pignatti 1994; Erben & Raimondo 1995; Erben 1996; Fenaroli & Moraldo 2003; Siuta & al. 2005; Marcussen & al. 2012), and the role of hybridization in the origin of supraspecific lineages in *Viola*, 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-



continued on next page

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  $\geq 0.95$  and bootstrap values  $\geq 50$  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  $0.57 \times 10^{-9}$  substitutions/site/year. Such a rate clearly falls outside the known range of ITS substitution rates for herbaceous an-

nual or perennial plant species of  $1.72 \times 10^{-9}$  to  $8.34 \times 10^{-9}$  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. merxmulleri* 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).

## Acknowledgements

We would like to thank the directors and curators of B, FI, FIAF, HBBS, M and MSB for permission to use their material for DNA extraction. We are grateful to Dr. Lorenzo Cecchi (Firenze, Italy), Prof. Giannantonio Domina (Palermo, Italy), Prof. Dmtar Lakušić (Beograd, Serbia) and Dr. Filippo Scafidi (Palermo, Italy) for sending us leaf material collected in the field or taken from herbarium specimens. We are grateful to Dr. Andreas Fleischmann and Dr. Matthias Erben (both München, Germany) for assistance during visits to their institution by one of us (SK). We thank Doris Franke (Mainz, Germany) for help in preparing the figures. Dr. Markus S. Dillenberger (Mainz, Germany) is gratefully acknowledged for help in data analysis. We are also grateful to

Dr. Kevin Thiele (Perth, Australia) and an anonymous reviewer for their very helpful comments on an earlier version of this article.

## References

- Arnold M. L. 1997: Natural hybridization and evolution. – Oxford: Oxford University Press.
- Ballard H. E., de Paula-Souza J. & Wahlert G. A. 2014: *Violaceae*. – Pp. 303–322 in: Kubitzki K. (ed.), The families and genera of vascular plants **XI**. Flowering plants. Eudicots. *Malpighiales*. – Berlin: Springer-Verlag.
- Ballard H. E., Sytsma K. J. & Kowal R. R. 1999: Shrinking the violets: phylogenetic relationships of infrageneric groups in *Viola* (*Violaceae*) based on internal transcribed spacer DNA sequences. – *Syst. Bot.* **23**: 439–458.
- Becker W. 1910: *Violenstudien II*. – Pp. 289–390 in: Uhlworm U. & Schinz H. (ed.), *Beih. Bot. Centralbl.*, Abt. 2, **XXVI**. – Dresden: Verlag von C. Heinrichs.
- Becker W. 1925: *Viola*. – Pp. 363–376 in: Engler A. & Prantl K. (ed.), *Die natürlichen Pflanzenfamilien*, ed. 2, **21**. – Leipzig: Verlag von Wilhelm Engelmann.
- Blaxland K. 2004: A new species of *Viola* (*Violaceae*) from south-west Turkey. – *Bot. J. Linn. Soc.* **145**: 505–509.
- Briggs D. & Walters S. M. 2016: *Plant variation and evolution*, ed. 4. – Cambridge: Cambridge University Press.
- Cennamo P., Guacchio E., Jury S. L. & Caputo P. 2011: Molecular markers in *Viola* L. subsect. *Viola*: Application and taxonomic implications for the identification of dubious herbarium specimens. – *Pl. Biosyst.* **145**: 306–323.
- Clausen J. 1926: Genetical and cytological investigations on *Viola tricolor* L. and *Viola arvensis* Murr. – *Hereditas* **8**: 1–156.
- Clausen J. 1927: Chromosome number and relationship of species in the genus *Viola*. – *Ann. Bot.* **41**: 677–714.
- Clausen J. 1931: Cyto-genetic and taxonomic investigations on *Melanium* violets. – *Hereditas* **15**: 219–308.
- Clausen J., Channel R. B. & Nur U. 1964: *Viola rafinesquii*, the only *Melanium* violet native to North America. – *Rhodora* **66**: 32–46.
- Conesa M. A., Mus M. & Rosselló J. A. 2008: Hybridization between insular endemic and widespread species of *Viola* in non-disturbed environments assessed by nuclear ribosomal and cpDNA sequences. – *Pl. Syst. Evol.* **273**: 169–177.
- Doyle J. J. 1992: Gene trees and species trees: Molecular systematics as one-character taxonomy. – *Syst. Bot.* **17**: 144–163.
- Drummond A. J. & Rambaut A. 2007: BEAST: Bayesian evolutionary analysis by sampling trees. – *BMC Evol. Biol.* **7**: 214.
- Drummond A. J., Suchard M. A., Xie D. & Rambaut A. 2012: Bayesian phylogenetics with BEAUti and the BEAST 1.7. – *Molec. Biol. Evol.* **29**: 1969–1973.
- Erben M. 1984: *Viola merxmulleri* – eine neue Art der Gattung *Viola* der Sektion *Melanium* aus Mittel-Italien (Gargano). – *Mitt. Bot. Staatssamml. München* **20**: 29–38.
- Erben M. 1985: Cytotaxonomische Untersuchungen an südosteuropäischen *Viola*-Arten der Sektion *Melanium*. – *Mitt. Bot. Staatssamml. München* **21**: 339–740.
- Erben M. 1986: *Viola acrocerauniensis* und *Viola etrusca* – zwei neue *Viola*-Arten aus der Sektion *Melanium*. – *Mitt. Bot. Staatssamml. München* **22**: 493–506.
- Erben M. 1989: *Viola ganiatsasii* – eine neue Art der Sektion *Melanium* aus den südlichen Rhodopen. – *Bios (Thessaloniki)* **1**: 67–73.
- Erben M. 1996: The significance of hybridization on the forming of species in the genus *Viola*. – *Bocconea* **5**: 113–118.
- Erben M. 2000: *Viola serresiana*, eine neue Art von *Viola* sect. *Melanium* aus Nordost-Griechenland. – *Bot. Chron. (Patras)* **13**: 51–59.
- Erben M. & Raimondo F. M. 1995: *Viola tineoreum* e *Viola ucriana* nuove specie dei Monti del Palermitano (Sicilia). – *Giorn. Bot. Ital.* **129**: 79–92.
- Fenaroli F. & Moraldo B. 2003: *Viola culminis*, una nuova specie delle prealpi bresciane (Lombardia, N-Italia). – *Nat. Bresciana* **33**: 21–29.
- Fiori A. 1924: *Nuova flora analitica d'Italia* **1**. – Firenze: Tipografia di M. Ricci.
- Fothergill P. G. 1938: Studies in *Viola*, 1: the cytology of a naturally-occurring population of hybrids between *Viola tricolor* L. and *Viola lutea* Huds. – *Genetica* **20**: 159–186.
- Gams H. 1925: *Viola*. – Pp. 586–656 in: Hegi G., *Illustrierte Flora von Mittel-Europa Bd. V, 1*. – München: Carl Hanser Verlag.
- Harvey M. J. 1966: Cytotaxonomic relationships between the European and North American rostrate violets. – *New Phytol.* **65**: 469–476.
- Hildebrandt U., Hoef-Emden K., Backhausen S., Bothe H., Bozek M., Siuta A. & Kuta E. 2006: The rare, endemic zinc violets of Central Europe originate from *Viola lutea* Huds. – *Pl. Syst. Evol.* **257**: 205–222.
- Kadereit J. W. 2015: The geography of hybrid speciation in plants. – *Taxon* **64**: 673–687.
- Kakes P. 1979: Genecological investigations on zinc plants III. Cytology of hybrids between *Viola calaminaria* (Lej.) Ernst and *Viola arvensis* Murr. – *Genetica* **51**: 135–142.
- 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 Evol. Biol.* **6**: 36.
- Krahulcová A., Krahulec F. & Kirschner J. 1996: Introgressive hybridization between a native and an intro-

- duced species: *Viola lutea* subsp. *sudetica* versus *V. tricolor*. – *Folia Geobot. Phytotax.* **31**: 219–244.
- Kumar S., Stecher G. & Tamura K. 2016: MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. – *Molec. Biol. Evol.* **33**: 1870–1874.
- Küpfer P. 1971: Contribution à l'étude cytologique et phylogénétique de la section *Melanium* Ging. du genre *Viola* L. – *Compt. Rend. Hebd. Séances Acad. Sci., Sér. D*, **272**: 1085–1088.
- Lanfear R., Frandsen P. B., Wright A. M., Senfeld T. & Calcott B. 2016: PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. – *Molec. Biol. Evol.* **34**: 772–773.
- Lanfear R., Ho S. Y. W., Davies J. T., Moles A. T., Aarssen L., Swenson N. G., Warman L., Zanne A. E. & Allen A. P. 2013: Taller plants have lower rates of molecular evolution. – *Nature Commun.* **4**: 1879.
- Lowe T. 1868: A manual flora of Madeira and the adjacent islands of Porto Santo and the Desertas. – London: John Van Voorst.
- Maddison W. P. 1989: Reconstructing character evolution on polytomous cladograms. – *Cladistics* **5**: 365–377.
- Maddison W. P. 1997: Gene trees in species trees. – *Syst. Biol.* **46**: 523–536.
- Marcussen T., Borgen L. & Nordal I. 2001: *Viola hirta* (*Violaceae*) and its relatives in Norway. – *Nordic J. Bot.* **21**: 5–17.
- Marcussen T., Heier L., Brysting A. K., Oxelman B. & Jakobsen K. S. 2015: From gene trees to a dated allopolyploid network: insights from the angiosperm genus *Viola* (*Violaceae*). – *Syst. Biol.* **64**: 84–101.
- Marcussen T., Jakobsen K. S., Danihelka J., Ballard H. E., Blaxland K., Brysting A. K. & Oxelman B. 2012: Inferring species networks from gene trees in high-polyploid North American and Hawaiian violets (*Viola*, *Violaceae*). – *Syst. Biol.* **61**: 107–126.
- Margini S. & Scoppola A. 2015: Further studies in *Viola* sect. *Melanium* (*Violaceae*). Identity and typification of *Viola nana* and *V. henriquesii*, two neglected European Atlantic taxa. – *Phytotaxa* **230**: 259–266.
- Merxmüller H. 1974: Veilchenstudien I–IV. – *Phyton* (Horn) **16**: 137–158.
- Merxmüller H. & Lippert W. 1977: Veilchenstudien V–VII. – *Mitt. Bot. Staatssamml. München* **13**: 503–534.
- Miller M. A., Pfeiffer W. & Schwartz T. 2010: Creating the CIPRES Science Gateway for inference of large phylogenetic trees. – Pp. 45–52 in: *Proceedings of the Gateway Computing Environments Workshop (GCE) New Orleans, Louisiana, 14 Nov. 2010.* – Piscataway: IEEE.
- Mitchell A. D., Heenan P. B. & Paterson A. M. 2009: Phylogenetic relationships of *Geranium* species indigenous to New Zealand. – *New Zealand J. Bot.* **47**: 21–31.
- Moore D. M. & Harvey M. J. 1961: Cytogenetic relationships of *Viola lactea* Sm. and other West European arosulate violets. – *New Phytol.* **60**: 85–95.
- Moraldo B., Ricceri C., Fiorini G. & Demaria G. 2011: *Viola ferrarinii* (*Violaceae*), a new species from the northern Apennines (Italy). – *Webbia* **66**: 45–55.
- Nieto Feliner G., Álvarez I., Fuertes-Aguilar J., Heuertz M., Marques I., Moharrek F., Piñeiro R., Riina R., Rosselló J. A., Soltis P. S. & Villa-Machío I. 2017: Is homoploid hybrid speciation that rare? An empiricist's view. – *Heredity* **118**: 513–516.
- Otto S. P. & Whitton J. 2000: Polyploid incidence and evolution. – *Annual Rev. Genet.* **34**: 401–437.
- Perrino E. V., Silletti G. N., Erben M. & Wagensommer R. P. 2018: *Viola cassinensis* subsp. *lucana* (*Violaceae*), a new subspecies from the Lucanian Apennine, southern Italy. – *Phyton* (Horn) **58**: 109–115.
- Pettet A. 1964: Studies on British pansies. II. The status of some intermediates between *Viola tricolor* L. and *V. arvensis* Murr. – *Watsonia* **6**: 51–69.
- Pignatti S. 1994: *Ecologia del paesaggio.* – Torino: Utet.
- Pignatti S. 2017: *Violaceae.* – Pp. 378–388 in: Pignatti S., Guarino R. & La Rosa M., *Flora d'Italia*, ed. 2, **2**. – Milano: Edagricole – New Business Media.
- Pirie M. D., Humphreys A. M., Galley C., Barker N. P., Verboom G. A., Orlovich D., Draffin S. J., Lloyd K., Baeza C. M., Negritto M., Ruiz E., Cota Sanchez J. H., Reimer E. & Linder H. P. 2008: A novel supermatrix approach improves resolution of phylogenetic relationships in a comprehensive sample of danthonioid grasses. – *Molec. Phylogen. Evol.* **48**: 1106–1119.
- Posada D. & Crandall K. A. 2001: The effect of recombination on the accuracy of phylogeny estimation. – *J. Molec. Evol.* **54**: 396–402.
- Rambaut A. & Drummond A. J. 2009: Tracer, MCMC trace analysis tool. Computer program and documentation distributed by the authors. – Published at <http://tree.bio.ed.ac.uk/software/tracer/>
- Reiche K. & Taubert P. 1895: *Violaceae.* – Pp. 322–336 in: Engler A. & Prantl K. (ed.), *Die natürlichen Pflanzenfamilien III(6)*. – Leipzig: Verlag von Wilhelm Engelmann.
- Ricceri C., Moraldo B. & Pisani G. 2018: Contributo alla conoscenza di *Viola* L. sect. *Melanium* Ging. (*Violaceae*) dell'Appennino centro-meridionale e della Sicilia (Italia). – *Bollag [Boll. Accad. Gioenia Sci. Nat. Catania]* **51**: 181–234.
- Rozewicki J., Li S., Amada K. M., Standley D. M. & Katoh K. 2019: MAFFT-DASH: integrated protein sequence and structural alignment. – *Nucl. Acids Res.* **47**: W5–W10.
- Sanderson M. J. 2003: r8s: inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock. – *Bioinformatics* **19**: 301–302.



- Schmidt A. 1961: Zytotaxonomische Untersuchungen an *Viola*-Arten der Sekt. *Melanium*. – Ber. Bayer. Bot. Ges. **34**: 93–95.
- Schmidt A. 1962: Eine neue Grundzahl in der Gattung *Viola*. – Ber. Deutsch. Bot. Ges. **75**: 78–84.
- Schmidt A. 1963: Zytotaxonomische Untersuchungen an griechischen *Viola*-Arten der Sektion *Melanium*. – Oesterr. Bot. Z. **110**: 285–293.
- Schmidt A. 1964: Zytotaxonomische Beiträge zu einer Neugliederung der Sektion *Melanium* der Gattung *Viola*. – Ber. Deutsch. Bot. Ges. **77**: 95–99.
- Schumer M., Rosenthal G. G. & Andolfatto P. 2014: How common is homoploid hybrid speciation? – *Evolution* **68**: 1553–1560.
- Short M. J. 1994: *Violaceae* – Pp. 223–225 in: Press J. R. & Short M. J. (ed.), *Flora of Madeira*. – London: HMSO.
- Siuta A., Bożek M., Jedrzejczyk M., Rostański A. & Kuta E. 2005: Is the blue zinc violet (*Viola guestphalica* Nauenb.) a taxon of hybrid origin? Evidence from embryology. – *Acta Biol. Cracov., Ser. Bot.* **47**: 237–245.
- Slomka A., Godzik B., Szarek-Łukaszewska G., Shukac L., Hoef-Emden K. & Bothe H. 2015: Albanian violets of the section *Melanium*, their morphological variability, genetic similarity and their adaptations to serpentine or chalk soils. – *J. Pl. Physiol.* **174**: 110–123.
- Stamatakis A. 2014: RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. – *Bioinformatics* **30**: 1312–1313.
- Valentine D. H. 1950: The experimental taxonomy of two species of *Viola*. – *New Phytol.* **49**: 193–212.
- Valentine D. H. 1958: Cytotaxonomy of the rostrate violets. – *Proc. Linn. Soc. London* **169**: 132–134.
- Valentine D. H., Merxmüller H. & Schmidt A. 1968: *Viola* L. – Pp. 270–282 in: Tutin T. G., Heywood V. H., Burges N. A., Moore D. M., Valentine D. H., Walter S. M. & Webb D. A. (ed.), *Flora europaea* **2**. – Cambridge: Cambridge University Press.
- Wahlert G. A., Marcussen T., de Paula-Souza J., Feng M. & Ballard H. E. Jr. 2014: A phylogeny of the *Violaceae* (*Malpighiales*) inferred from plastid DNA sequences: implications for generic diversity and intra-familial classification. – *Syst. Bot.* **39**: 239–252.
- Wen D.-Q., Yu Y., Zhu J.-F. & Nakhleh L. 2018: Inferring phylogenetic networks using PhyloNet. – *Syst. Biol.* **67**: 735–740.
- White T. J., Bruns T., Lee S. & Taylor J. W. 1990: Amplification and direct sequencing of fungal ribosomal RNA for phylogenetics. – Pp. 315–321 in: Innis M. A., Gelfand D. H., Sninsky J. J. & White T. J. (ed.), *PCR protocols: a guide to methods and applications*. – San Diego: Academic Press.
- Wood T. E., Takebayashi N., Barker M. S., Mayrose I., Greenspoon P. B. & Rieseberg L. H. 2009: The frequency of polyploid speciation in vascular plants. – *Proc. Natl. Acad. Sci. U.S.A.* **106**: 13875–13879.
- Wright S. D., Young C. G., Keeling D. J., Dawson J. W. & Gardner R. C. 2001: Stepping stones to Hawaii: a transequatorial dispersal pathway for *Metrosideros* (*Myrtaceae*) inferred from nrDNA (ITS + ETS). – *J. Biogeogr.* **28**: 769–774.
- Yakimowski S. B. & Rieseberg L. H. 2014: The role of homoploid hybridization in evolution: a century of studies synthesizing genetics and ecology. – *Amer. J. Bot.* **101**: 1247–1258.
- Yockteng R., Ballard H. E. Jr., Mansion G., Dajoz I. & Nadot S. 2003: Relationships among pansies (*Viola* section *Melanium*) investigated using ITS and ISSR markers. – *Pl. Syst. Evol.* **241**: 153–170.

## Willdenowia

Open-access online edition [bioone.org/journals/willdenowia](https://bioone.org/journals/willdenowia)



Online ISSN 1868-6397 · Print ISSN 0511-9618 · 2018 Journal Impact Factor 1.156

Published by the Botanic Garden and Botanical Museum Berlin, Freie Universität Berlin

© 2020 The Authors · This open-access article is distributed under the CC BY 4.0 licence