American Journal of Botany 94(1): 29–41. 2007.

On the origin of the sweet-smelling Parma violet cultivars (Violaceae): wide intraspecific hybridization, sterility, and sexual reproduction[1]

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Parma violets are reputed for their double, fragrant flowers and have been cultivated for centuries in Europe. However, due to a rather atypical morphology their taxonomic affinity has not been clarified. Authors have proposed an origin from three possible species, *Viola alba*, *V. odorata,* or *V. suavis*, or a hybrid origin. Using both ITS sequence variation and allozyme variation in 14 putative loci, we showed that the Parma violet cultivars have their origin within *Viola alba* and that they are best included in the Mediterranean subsp. *dehnhardtii*. There is no trace of interspecific hybridization. However, the cultivars appear to have a single origin in a wide hybrid within *V. alba*, involving parental plants from the eastern and western Mediterranean region; historical literature sources seem to indicate Turkey and Italy, respectively. The Parma violet cultivars possess high levels of allozyme heterozygosity and to some extent also within-individual ITS sequence variation. Losses of heterozygosity and within-individual ITS sequence variation in some of the cultivars indicate subsequent rare events of sexual reproduction, presumably through cleistogamous seed set. We unambiguously identify the closest wild relative of this group of cultivars, allowing growers to develop new selection procedures, and show a peculiar molecular process associated with human selection.

Key words: heterozygosity; hybridization; Italy; Parma violets; polymorphism; Turkey; *Viola*; Violaceae.

Fragrant violets have long been cultivated, and the Romans were already using these flowers for cosmetic and medical purposes (Barandou, 1999). The Parma violets constitute a morphologically well-defined group of cultivars reputed for their fragrant, double flowers. They were under extensive cultivation during the late 19th and early 20th centuries, particularly in London, New York, and Toulouse in south-western France, and for that reason are frequently referred to as Toulouse Violets (Violette de Toulouse).

Different views on the geographical origin of the Parma violets have been put forward. The original plants grown in Toulouse around 1853 are assumed to have been brought from Italy (Naples or Parma) (Morard et al., 1992), but earlier literature, particularly the Italian, has suggested an origin from Byzantium, present-day Istanbul, in Turkey (Costeo in Pseudo-Mesuë, 1573; Camerarius, 1588). On the other hand, Robinson and Snocken (2002, under 'D'Udine') reported, "It is believed that the Parma violet was first introduced to Italy by the Bourbon royal family after obtaining it in Portugal during the early 17th century. Arriving first at Naples [Italy], it was then taken to Parma [Italy], from where the name for this type of violet originated."

[1] Manuscript received 19 July 2006; revision accepted 23 October 2006.

The authors thank P. Barandou for bibliographic help, J. Chassagneux and B. Cornier for translation of Latin texts, D. Charrier for illustrations, S. Vincent and members of the Conservatoire et Jardin Botaniques de Nancy for cultivating the different cultivars and botanical species of the collection, and N. Casbas who provided most of the cultivars from her private collection "*Viola*, cultivars anciens."

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Cultivars in the Parma violet group are morphologically easily characterized through several synapomorphies: their leaves are shiny and often have overlapping basal lobes, and the chasmogamous flowers are heavily fragrant and abnormal with 20 petalous floral pieces (petals and modified stamens) and no functional reproductive organs (Casbas, 1989; Barandou, 1991; Morard et al., 2000). The chasmogamous flowers, produced in winter and spring, are harvested and used commercially for floral trade, and the perfume or alimentary industry.

Due to the malformed reproductive organs of the chasmogamous flowers, members of the Parma Violet Group are normally sterile. Consequently, cultivars have been propagated by stolon cuttings, for up to 150 years in the case of 'Parme de Toulouse', and new cultivars have arisen mainly from sports (bud mutations). Nevertheless, small capsules with ripe seeds, developed from cleistogamous flowers (in which petal development is strongly suppressed), have been observed (Millet, 1898; Barandou, 1991; Casbas, 2002). Certain cultivars apparently result from such selfed seed (e.g., 'Fée Jalucine' obtained by Casbas and cited by Robinson and Snocken, 2002).

The genus *Viola* is widely distributed in the northern hemisphere and in South America with some 525–600 species and has a probable origin in the southern hemisphere (Ballard et al., 1999). With its 200 species, the blue-flowered and tetraploid section *Viola* sensu lato (Becker, 1925) is the largest in the northern hemisphere and consists of numerous more restricted groups usually treated as subsections. Much confusion has been expressed about the taxonomic origin of the Parma violets. Morphologically, they fall naturally within

subsection *Viola*, a Eurasian group of some 25 species (Chauchard et al., 2003). They show particularly strong affinity to some of the stoloniferous species of the subsection (including *V. alba* Bess., *V. odorata* L., and *V. suavis* M. Bieb). All have wide distributions in southern Europe and western Asia. Two more stoloniferous endemic taxa occur in Majorca (*V. jaubertiana* Marès & Vigin.) and in the Caspian region (*V. sintenisii* W. Becker). Also the taxonomic delimitation of these species has long been disputed (cf. Becker, 1918; Mus et al., 2000), but recent molecular studies sustain the three taxa as distinct species (Marcussen and Borgen, 2000; Marcussen et al., 2005).

Timbal-Lagrave (1854) united the Parma violets cultivated and sold on the markets in and around Toulouse under the name *Viola tolosana* Timb. In numerous later treatments, *V. tolosana* has been included with taxa today considered synonymous with *V. suavis* (i.e., *V. adriatica* Freyn, *V. beraudii* Bor., or *V. sepincola* Jord.; Timbal-Lagrave, 1862; Sudre, 1907; Becker, 1910; Gams, 1925; Barandou, 1999). However, in a recent publication, we found the chromosome number $2n = 20$ in *Viola* 'Parme de Toulouse' (Chauchard et al., 2003). If this count is correct, and excluding a polyploid series, then this rules out inclusion of the Parma violets in *V. suavis*, which invariably has $2n = 40$, and may indicate an origin in one of the widespread *V. alba* (including *V. dehnhardtii*) as proposed by some authors (e.g., Tenore, 1811; Meikle, 1963; Coombs, 1981; Marzotto-Caotorta and Lombardi, 1997; Tucker, 2000) or *V. odorata* as proposed by others (e.g., Foissy, 1884; Valatx, 1954; Chaudun and Guillaumin, 1964; Société Nationale d'Horticulture de France et al., 1997; Morard et al., 2000), which both have $2n = 20$. Alternatively, a hybrid origin could also be proposed, as hybrids within subsection *Viola* are common and often slightly fertile (Marcussen and Borgen, 2000).

Twenty years ago, the Toulouse's Region called for research on improving the growth of Parma violets for the horticultural market. Methods for in vitro culture (meristem culture and micropropagation) were successfully developed, but the botanical delineation of the Violet of Toulouse was unknown. We therefore used molecular phylogenetic techniques to elucidate the botanical origin of the Parma violet cultivars. In this study, we used data from ITS sequences and allozymes from several cultivars and all the mentioned taxa of subsection *Viola* to resolve the ambiguous taxonomic status of the Parma violets. The spacers of ribosomal genes (ITS) have been widely used to reconstruct plant phylogenies (Ainouche and Bayer, 1999; Vargas et al., 1999; Schmidt and Schilling, 2000). Within Violaceae, ITS sequence variation has been useful in resolving phylogenetic relationships among and within infrageneric groups of the genus *Viola* (Ballard et al., 1999; Ballard and Sytsma, 2000; Yockteng et al., 2003; Yoo et al., 2005) and other genera of the family (Munzinger, 2000). Allozymes have also proved powerful in the study of inter- and intraspecific variations within the genus (Nordal and Jonsell, 1998; Marcussen and Borgen, 2000; Culley and Wolfe, 2001; Batista and Sosa, 2003; Marcussen, 2003; Marcussen et al., 2005; Nordal et al., 2005).

MATERIALS AND METHODS

Phylogenetic analysis of ITS sequences—Since the cultivars apparently originated in Europe, we have included in the phylogenetic analysis, six European stoloniferous taxa that produce fragrant flowers and provide probable origins of the cultivars: *Viola alba* subsp. *alba*, subsp. *cretica* (Boiss. and Heldr.) Marcussen and subsp. *dehnhardtii* (Ten.) W. Becker, *V. odorata* and *V. suavis*, as well as *V. sintenisii* W. Becker, native of the Caucasus and western Asia. All these species have $2n = 20$, except for *V. suavis,* which has $2n = 40$. In addition, the non-stoloniferous *V. hirta* L. ($2n = 20$) of the same subsection and the more remotely related *V. mirabilis* ($2n = 20$) of subsection *Rostratae* were included. Three accessions of *Viola* 'Parme de Toulouse' (origins unknown) and four additional cultivars of Parma violet, 'Ash Vale Blue' (England, 1998). 'D'Udine' (Italy, 1903), 'Gloire de Verdun' (origins unknown), and 'Marie Louise' (Germany, 1865), were also added [location and date of description of cultivars according to Bertrand and Casbas (2001) and Robinson and Snocken (2002); spelling of cultivar epithets according to Robinson and Snocken (2002)].

The phylogenetic reconstruction was conducted using additional ITS sequences of genus *Viola* available on GenBank database (http://www.ncbi.nlm.nih.gov/). *Viola scandens* of section *Leptidium* was used as outgroup. In earlier analyses (Ballard et al., 1999; Ballard and Sytsma, 2000), this species appear sister to the remainder of the genus (except *Viola capillaris*), and its ITS sequences are less ambiguous to align than *Hybanthus* or *Noisettia* sequences. In total, 48 taxa were included in this study (Appendix).

Total DNA was extracted from either fresh or silica-gel-dried leaves (Chase and Hills, 1991), using the Dneasy Plant Mini Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. The ITS1 and ITS 2 regions were amplified simultaneously using universal primers (5′ GGA AGT AGA AGT CGT AAC AAG 3′ and 5′ TCC TCC GCT TAT TGA TAT GC 3′, respectively) (White et al., 1990). PCR reactions were performed in a total volume of 50 μl containing 2 μl DNA, 0.32 μM of each primer, 120 μM dNTPs, 1.5–2.5 mM $MgCl_2$ and 1 U *Taq* polymerase (Promega, Madison, Wisconsin, USA), in 1× buffer A (Promega). We used a Perkin Elmer 2400 Thermal Cycler (Wellesley, Massachusetts, USA), programmed for an initial step of 5 min at 94°C, followed by 35 cycles of 50 s at 94°C, 50 s at 50–55°C, 1.5 min at 72°C, and a final step of 5 min at 72°C. PCR products were visualized on a 2% agarose gel, purified using the PEG protocol standardized by Rosenthal et al. (1993), and sequenced on both strands using the same primers. Cycle sequencing products were run on the sequencer of the MWG Biotech Company (High Point, North Carolina, USA). Sequences have been deposited in the GenBank database.

The ITS sequences were aligned using ClustalW version 1.83 (Thompson et al., 1994) and edited manually in ProSeq version 2.9 beta (Filatov, 2002). The alignments of the secondary structure of ITS1 and ITS2 were computed using MFOLD (Zuker, 1989; SantaLucia, 1998). The flanking regions of 18S and 26S ribosomal genes and the sequence of the 5.8S ribosomal gene were excluded. The alignment lacked ambiguous regions that needed exclusion. The alignment is available on TreeBase (www.treebase.org, study accession no. S1578; matrix accession no. M2840). Phylogenetic reconstruction was conducted using maximum parsimony (MP) approaches using PAUP* version 4.0b10 (Swofford, 2001). Maximum parsimony analysis was conducted using the heuristic search algorithm (treating indels as missing data and without gap code), with MulTrees option on, tree-bisection-reconnection (TBR) branch swapping, and 1000 replicates of taxa random addition. Robustness of branches was tested by bootstrap (Felsenstein, 1985) (1000 replicates).

Allozyme electrophoresis—Live plants of *Viola* 'Conte di Brazza' (Italy, 1880), 'D'Udine' (Italy, 1903), 'Gloire de Verdun' (origins unknown), 'Marie Louise' (Germany, 1865), and two accessions of 'Parme de Toulouse' (origins unknown) were grown in a greenhouse at the University of Oslo (see Materials and Methods for phylogenetic analysis of ITS sequences section above for source of information on location and date of origin of cultivars). A total of 33 representative individuals for comparison were selected from within eight taxa of subsection *Viola*, the stoloniferous *V. alba* subsp. *alba*, subsp. *cretica* and subsp. *dehnhardtii*, *V. jaubertiana*, *V. odorata*, *V. sintenisii,* and *V. suavis*, as well as the nonstoloniferous *V. hirta* (Table 1), for which allozyme data were available from previous studies (Marcussen and Borgen, 2000; Marcussen, 2003, 2006; Marcussen et al., 2005).

Six isozymes, AAT (aspartate aminotransferase), AMP/LAP ([leucine] aminopeptidase), GPI (glucose-6-phosphate isomerase), IDH (isocitrate dehydrogenase), PGM (phosphoglucomutase), and SKD (shikimate dehydrogenase), were analyzed using the electrophoretic procedures of Marcussen (2003), yielding 14 putative variable loci in the $2n = 20$ taxa. Allelic and homomeric bands (heterodimers excluded) were labeled alphabetically from the most anodal position. They were scored as either present or absent (0/1), and the matrix was used to calculate a UPGMA (unpaired group method using arithmetic averages)

TABLE 1. List of *Viola* material included in allozyme analysis.

Name	Locality	Voucher (Herbarium) [herbarium sample number]
Parma violet cultivars		
V. 'Marie Louise'	Cultivated in Toulouse (N Casbas, private collection), 23 April 2002	*MH-023183* (NCY) [NCY000670]
V. 'D'Udine'	Cultivated in Toulouse (N Casbas, private collection), 23 April 2002	*MH-023175* (NCY)
V. 'Gloire de Verdun'	Cultivated in Toulouse (N Casbas, private collection), 14 September 2001	*MH-023177* (NCY) [NCY000668]
V. 'Parme de Toulouse' 8	Cultivated in Nancy (from Candiflor Society, Toulouse), 16 October 1989	*MH-953381* (NCY) [NCY000687]
V. 'Parme de Toulouse' 9	Cultivated in Nancy (from in vitro culture of *MH-953381*), 5 December 1999	*MH-993489* (NCY) [NCY000671, NCY000672]
V. 'Conte di Brazza'	Cultivated in Agen (P. Barandou, private collection), 5 December 1999	*MH-993490* (NCY) [NCY000010, NCY000011]
Viola alba		
V. alba subsp. *alba*	SWITZERLAND, Sarnen canton, Alpnachstad, below Mt. Pilatus, 450 m, 3 June 2001	*TM389-1* (O)
V. alba subsp. *alba*	GREECE, Kozánti, Euipeus Gorge at base of Mt. Olympus, 1998	*TM496-1* (O)
V. alba subsp. *alba*	CYPRUS, Lemesos, Troödos Massif, Saïttas, along Mesapotamos creek, 13 September 2001	*TM469-13* (O)
V. alba subsp. *alba*	GREECE, Evia, Mt. Dirphys, along dirt road S of the pass on Steni-Stropones road, 1040 m, 7 May 2001	*TM357-21* (O)
V. alba subsp. *alba*	FRANCE, Ain, Bourg-en-Bresse, Rue d'Ypres, April 1995	*TM039-1* (O)
V. alba subsp. *alba*	AZERBAIJAN, Şəki, Hill btw. Şəki & Baş Zəyzit, 1750 m, June 2001	*TM230-2* (O)
V. alba subsp. *alba*	TURKEY, Erzurum, Yusufeli btw. Artvin & Erzurum, 1950 m, June 1999	*TM458* (O)
V. alba subsp. *cretica*	GREECE, Crete, Rethymno, River gorge near the Arkadiuo monastery, 500 m, 8 May 2001	*TM360-10* (O)
V. alba subsp. *dehnhardtii*	SPAIN (Valencia), Castellón, Tirig N of Castellón de la Plana, c. 500 m, 1 June 1987	*TM494-1* (O)
V. alba subsp. *dehnhardtii*	FRANCE, Var, Les Lecques, St.-Cyr-sur-mer, 9 July 2001	*TM397-2* (O)
V. alba subsp. *dehnhardtii*	MOROCCO, Meknès, 25 km N of Azrou on road to Aïn Leuh, 1420 m, 1984	*TM455-1* (O)
V. alba subsp. *dehnhardtii*	FRANCE, Aveyron, 1 km N of St.-Rome-de-Cernon, 600 m, 30 May 2001	*TM384-4* (O)
V. alba subsp. *dehnhardtii*	ITALY, Sicily, near Linguaglossa, October 1995	*TM051* (O)
V. alba subsp. *dehnhardtii*	GREECE, Ioánnina, near the road Aristi-Papigo, St. Paraskeva Monastery, 11 September 2001	*TM478-2* (O)
V. alba subsp. *dehnhardtii*	TURKEY, C5 Adana, Pozantı, above Akçatehir, 1500 m, 20 June 1999	*TM484-6* (O)
V. alba subsp. *dehnhardtii*	CROATIA, Zadar, Velebit, Paklenica National Park, 500-720 m, 21 June 2001	*TM401-4* (O)
Other species		
V. hirta	NORWAY, Østfold, Fuglevik in Jeløya Island, 10-15 m, 1995	*TM004-1* (O)
V. hirta	GERMANY, Niedersachsen, Göttingen, 1996	*TM040* (O)
V. jaubertiana	SPAIN, Majorca, Sa Fosca, 1996	*TM049* (O)
V. odorata	NORWAY, Oslo, cultivated at the Biological Institute, 1997	*TM062-4* (O)
V. odorata	GREECE, Arkadía, btw. Kardaras & N. Kardaras, 990 m, 2 May 2001	*TM335-5* (O)
V. odorata	FRANCE, Alpes-Maritimes, Tourrettes-sur-Loup, cultivated, 2001	*IN4433* (O)
V. odorata	CYPRUS, Lemesos, Saïttas, along Mesapotamos creek, September 2001	*TM469-8* (O)
V. odorata	ENGLAND, Surrey, Richmond Park, 2001	*IN4431* (O)
V. odorata f. *alba*	FRANCE, Nancy, La Sapinière, 21 March 2002	*MH-93112* (NCY) [NCY000681]
V. sintenisii	AZERBAIJAN, Şamaxı, Ağsu River valley, W of Şamaxı, 700 m, June 2000	*TM235* (O)
V. sintenisii	AZERBAIJAN, İsmayilli, nr. Təzəkənd, 800 m, June 2000	*TM238* (O)
V. sintenisii	AZERBAIJAN, Dəvəxı, Çirax Castle nr. Gala-Altı, 1200 m, June 2000	*TM240* (O)
V. suavis	FRANCE, Htes-Alpes, Along road N91 W La Grave, 1400 m, 30 May 2002	*TM503-4* (O)
V. suavis	FRANCE, Alpes-Maritimes, Villeneuve-Loubet, 2001	*IN4001* (O)
V. suavis	NORWAY, Telemark, Kragerø, Valberg manor, 2 m, June 1994	*TM001-1* (O)

dendrogram (Sokal and Michener, 1958) using the Jaccard similarity index (Jaccard, 1908) and the NTSYS-pc software package (Rohlf, 1997).

RESULTS

Phylogenetic analysis of ITS sequences—ITS sequence characteristics in the studied cultivars and species of *Viola* (Table 2) were in general equivalent to values previously published for *Viola* (Ballard et al., 1999; Yoo et al., 2005). Sequence lengths were within the known range of variation in *Viola* (413–472 bases). Within section *Viola* (subsections *Viola* and *Rostratae*), sequence differences were substantial even among the subspecies of *V. alba*. Sequence divergence reached 15.6% between *Viola* 'Ash Vale Blue' and *V. calcarata* (section *Melanium*). No substantial difference was observed among the *V.* 'Parme de Toulouse' samples. The divergence between *V.* 'Parme de Toulouse' and the remaining four Parma violet cultivars ('Ash Vale Blue', 'D'Udine', 'Gloire de Verdun' and 'Marie Louise') was quite important (3.4–5.8%). These four samples presented many polymorphic sites (up to 103 in 'Gloire de Verdun'). Important variation was

TABLE 2. Sequence characteristics of the ITS region for *Viola* species and cultivars.

Sequence characteristic	ITS1	ITS2	Both
Unaligned length			
Within *Viola* *	215–268	193–209	413–472
Within *V. alba*	250–251	196–198	446–449
Within cultivars	250–252	196–197	446–449
All indels	19	12	21
Informative indels	14	5	19
GC content (%)	65.05	64.42	64.78
Transition/transversion ratio	0.73	1.56	0.91
Variable nucleotide positions	210	152	362
Informative (% of total) sites	149 (52.1)	108 (49.3)	257 (50.8)
Sequence divergence (%)			
Within *Viola* *	1.2–29.7	0.0–28.7	1.1–28.8
Within all *Viola* studied	0.8–31.3	0–28.8	0.9–28.2
Within *V. alba*	0–3.9	0–5.6	0–4.2
Within cultivars	0–6.8	0–24.6	0–13.5

* Values from Ballard et al. (1999).

observed within *V. alba* with a maximum of 3.4% sequence divergence. Three *V. alba* subsp. *dehnhardtii* samples (TM494–1, TM494–2, TM397–2) differed from the remaining *V. alba* samples (2.4–3.4%).

A heuristic search produced 3750 most parsimonious trees of 1061 steps with CI (excluding uninformative sites) = 0.5075 and RI = 0.7840. The strict consensus tree presents a good resolution (Figs. 1 and 2).

Intersection relationships in *Viola* corresponded to the previous phylogenetic analyses (Ballard et al., 1999; Yockteng et al., 2003). The three Meso- to South American sections *Leptidium*, *Rubellium,* and *Chilenium* were sisters to the rest of the genus, with low bootstrap support (BS = 62%). A first, well-supported clade (BS = 97%; clade A) included members of the paraphyletic section *Chamaemelanium* basal to the subclades of the blue-flowered section *Viola*, subsections *Stolonosae*, *Adnatae,* and *Diffusae*. The second, weakly supported clade (BS = 55%, clade B) consisted of section *Andinium* sister to a large and strongly supported clade (BS = 97%) consisting of the remnant subsections of section *Viola* (including subsection *Viola*) along with sections *Delphiniopsis*, *Melanium,* and *Nosphinium*. Within this last clade, global relationships were not supported (BS < 50% for most internal nodes), but subsection *Rostratae* appeared paraphyletic and sister to an apparently paraphyletic subsection *Viola*.

The Parma violet cultivars formed an unresolved but well-supported clade D (BS = 80%) together with *Viola sintenisii* and most specimens of *V. alba*, except for three individuals of *V. alba* subsp. *dehnhardtii*, which clustered with *V. odorata* in a strongly supported clade C (BS = 96%). The two species used as reference, *V. hirta* and *V. mirabilis*, did not cluster with the Parma violets. *Viola hirta* was sister to an unsupported clade (BS < 50%, clade E) consisting of sections *Delphiniopsis*, *Melanium* (the pansies), *Nosphinium,* and American members of section *Viola* (subsections *Mexicanae*, *Pedatae*, and *Boreali-Americanae*). *Viola mirabilis* of subsection *Rostratae* was the least related to the studied taxa and was sister to clades C, D, and E.

Within clade D (the *Viola alba*/*sintenisii* clade), a large basal polytomy with three small clades was obtained. A first clade consisted of *V. alba* from Greece and Cyprus (BS = 58%), the second of three samples of *Viola* 'Parme de Toulouse' (BS = 96%), and the third with the four remaining Parma violet cultivars (BS = 98%). In other words, the resulting trees did not confirm a supported monophyletic origin of the cultivars (i.e., Parma violet cultivars do not form a clade with bootstrap support above 70% in the consensus tree). The remaining samples of *V. alba* subsp. *alba*, subsp. *cretica*, and *V. sintenisii* were unresolved in the basal polytomy.

When branch lengths are considered (Fig. 2), the clade of the four Parma violet cultivars ('Ash Vale Blue', 'D'Udine', 'Gloire de Verdun', and 'Marie Louise') sat on a very long branch, compared to the other branch lengths in clade D. The exclusion of the four Parma cultivars drastically reduced the number of most parsimonious trees from 3750 to 12 (984 steps, CI excluding uninformative sites = 0.4982, RI = 0.7899) (Fig. 4).

Allozyme electrophoresis—The present results confirmed interpretations made elsewhere, showing that in *Viola*, $2n = 20$ refers to the tetraploid level and $2n = 40$ to the octoploid level (Marcussen and Borgen, 2000; Marcussen, 2003; Marcussen et al., 2005). Multilocus phenotypes of the analyzed specimens of taxa are presented in Table 3.

The six cultivars were rather homogenous in allozyme composition. They possessed notably higher levels of heterozygosity (15–17 markers) compared to the wild taxa on the same ploidal level, having $2n = 20$ (11–14 [15] markers), but somewhat lower than the higher-ploid *Viola suavis* with $2n = 40$ (18–20 markers). The six cultivars were generally heterozygous for up to four putative loci: *AMP* (with allozyme bands $Amp^a + Amp^f$), *GPI* ($Gpi^c + Gpi^d$), *LAP* ($Lap^d + Lap^f$), and *SKD* ($Skd^c + Skd^e$). While 'Gloire de Verdun' and 'Conte di Brazza' were heterozygous in all four putative loci, the two specimens of 'Parme de Toulouse' were homozygous in *SKD* (Skd^e), and 'D'Udine' and 'Marie Louise' were homozygous in both *AMP* (Amp^f) and *LAP* (Lap^d).

In band composition, the cultivars had strong affinities to *Viola alba*; all markers found in the cultivars were also found in this species. The cultivars shared two unique markers (Gpi^i, Skd^f) with all subspecies of *V. alba* and two (Amp^a, Gpi^d) with subsp. *dehnhardtii*. Another two markers (Amp^f, Lap^f) were shared by *V. alba, V. sintenisii* and the cultivars and one (Skd^c) by *V. alba*, *V. suavis*, and the cultivars. The remaining markers found in the cultivars were mostly widespread among taxa. In the (UPGMA) dendrogram based on Jaccard similarity (Fig. 3), the cultivars clustered together on a relatively long branch itself nested within *Viola alba* and shared a node with the Turkish sample of subsp. *dehnhardtii*.

The close relationship between the Parma violet cultivars and *Viola alba* is further elucidated in Table 4 where the cultivars are compared with regional band frequencies calculated from 281 individuals of *V. alba* (data from Marcussen, 2003). Five allozyme markers (Aat^d, Amp^b, Gpi^d, Gpi^i, Idh^a) tied the cultivars more or less tightly to *V. alba* subsp. *dehnhardtii*. One marker (Gpi^d) occurred only in subsp. *dehnhardtii* from the western Mediterranean region (in Sicily, France, and Morocco). Another three markers (Amp^f, Pgm^c, Skd^f) in the cultivars were shared with eastern Mediterranean or western Asian occurrences of either subspecies of *V. alba*.

DISCUSSION

Taxonomic affinity of the Parma violets—The results from allozymes unambiguously placed the Parma violets within the

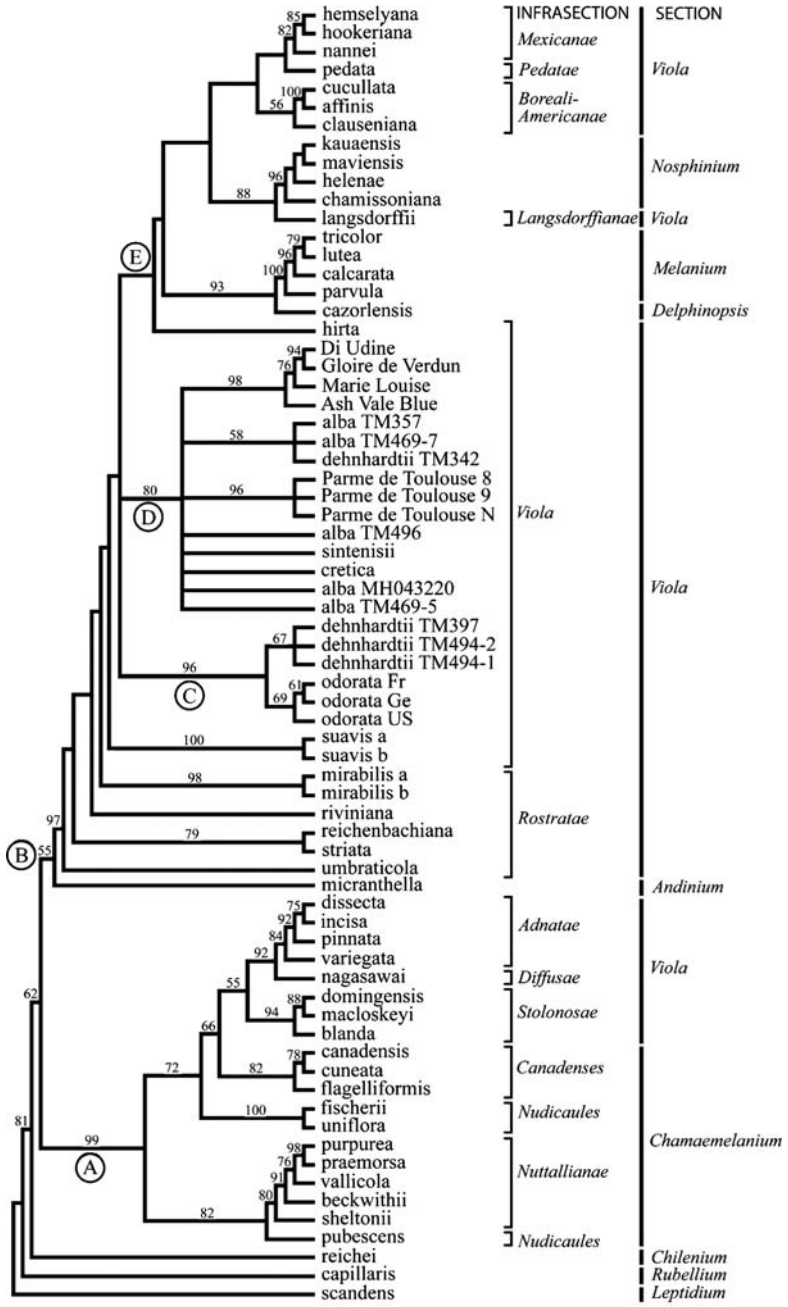

Fig. 1. Strict consensus tree based on 3750 trees. Bootstrap values above 50% are indicated along the branches. Clades A, B, C, D, and E are discussed in the Results of phylogenetic analysis of ITS sequences section. Taxa refer to the species level except within *Viola alba* s.l. where subspecies or geographical origins are indicated.

widely distributed *Viola alba* complex (Figs. 1 and 3). Presumably due to shared ancestral ITS variation and incomplete lineage sorting or possibly ancient hybridization, the results from ITS sequence data were less clear, although here the Parma violets grouped with (most) *V. alba*. These results put to rest a long-time controversy and refute the hypothesis that the cultivars originated from *V. suavis* as usually assumed, from *V. odorata,* or from an interspecific hybrid (Timbal-Lagrave, 1862; Foissy, 1884; Sudre, 1907; Becker, 1910; Gams, 1925; Valatx, 1954; Chaudun and Guillaumin, 1964; Société Nationale d'Horticulture de France et al., 1997; Barandou, 1999; Morard et al., 2000). It also follows from our results that the Parma violets should not be treated at the species level i.e., as *V. tolosana* (Timbal-Lagrave, 1854).

In spite of a paucity of interpretable floral characters, identity with *Viola alba* is not surprising from a morphological point of view. In vegetative characters, the cultivars are more similar to *V. alba* than to *V. odorata* or *V. suavis*, and in leaf blade shape and dentation, they seem to approach certain forms of *V. alba*

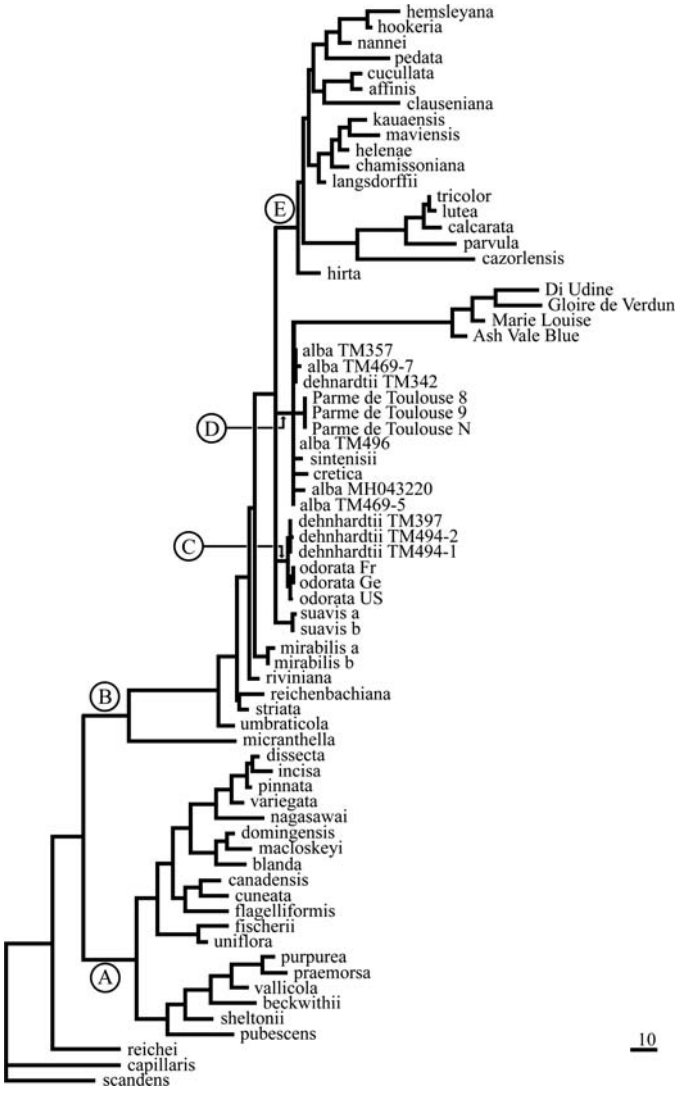

Fig. 2. Same tree as in Fig. 1 but with branch lengths, showing the long branch of some of the cultivars. Clades A, B, C, D, and E are discussed in the Results of phylogenetic analysis of ITS sequences section.

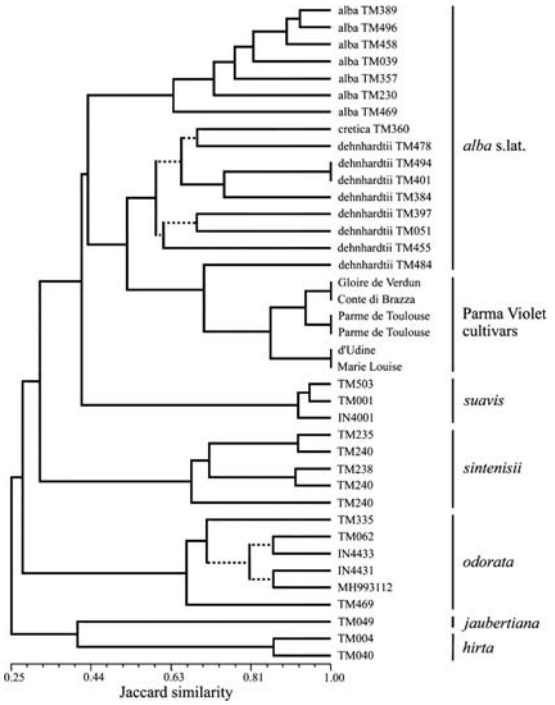

Fig. 3. One of 72 dendrograms obtained with the unpaired group method using arithmetic averages (UPGMA) based on Jaccard similarity of multilocus phenotypes. Broken lines indicate branches that collapse in the strict consensus tree. Subspecies of *Viola alba* are shown without rank.

subsp. *dehnhardtii* or subsp. *cretica*. It seems probable that not only a deviant morphology, but also the somewhat confused systematic of the subsection and the often all too wide delimitation of *V. odorata* used in horticulture may have prevented the correct determination of these cultivars.

The high similarity in allozyme composition among the cultivars seems to indicate that they have a single origin. Most likely, the Parma violet cultivars have a single origin, as reflected by their homogenous allozyme composition, although some variation in the level of heterozygosity was observed. At first glance, the ITS sequences do not support this because the cultivars fell into two well-supported clades (Figs. 1 and 2). These clades, however, coincided with the four cultivars that displayed intra-individual ITS polymorphism (i.e., heterozygosity, on the long branch of clade D) and the three cultivars that did not. It has been shown in *Amelanchier* that ITS polymorphism acquired through hybridization events and further maintained (vegetatively) through agamospermy is lost following sexual events (Campbell et al., 1997). Furthermore, ITS polymorphism implies a reduction of concerted evolution among ribosomal genes, a genetic process occurring mainly during meiosis, when unequal crossing-over allows homogenization of sequences (Liao, 1999). The situation for the Parma violets may be analogous to the one observed in *Amelanchier*: these cultivars are highly heterozygous due to a hybrid origin and are mainly vegetatively propagated, which helps to explain the maintenance of allozyme and ITS polymorphism in certain cultivars. Cultivars with lower levels of allozyme heterozygosity or lacking ITS polymorphism may be the result of rare sexual events. This is controversial because the cultivars have usually been considered as sterile. Complete sterility can indeed be assumed in the chasmogamous flowers as their generative parts are changed into petal-like structures. However like most other violets, these cultivars produce, during summer, apetallous and obligatory self-pollinating cleistogamous flowers; although their seed set obviously is not good, reports of occasional capsules containing ripe seeds have nevertheless been reported (Barandou, 1991). Our data support this finding and further suggest that these rare sexual events have played an important part in the diversification of this popular group of violet cultivars.

Applying a scenario of acquisition of ITS and allozyme heterozygosity through a hybridization event, followed by successive loss of heterozygosity through rare sexual events implies the following relationship between cultivars: the two cultivars ‘Gloire de Verdun’ and ‘Conte di Brazza’ would appear to be the most original ones. They are heterozygous for all three loci *SKD*, *AMP* and *LAP* and express ITS sequence polymorphism. They may be ancestral to two groups of

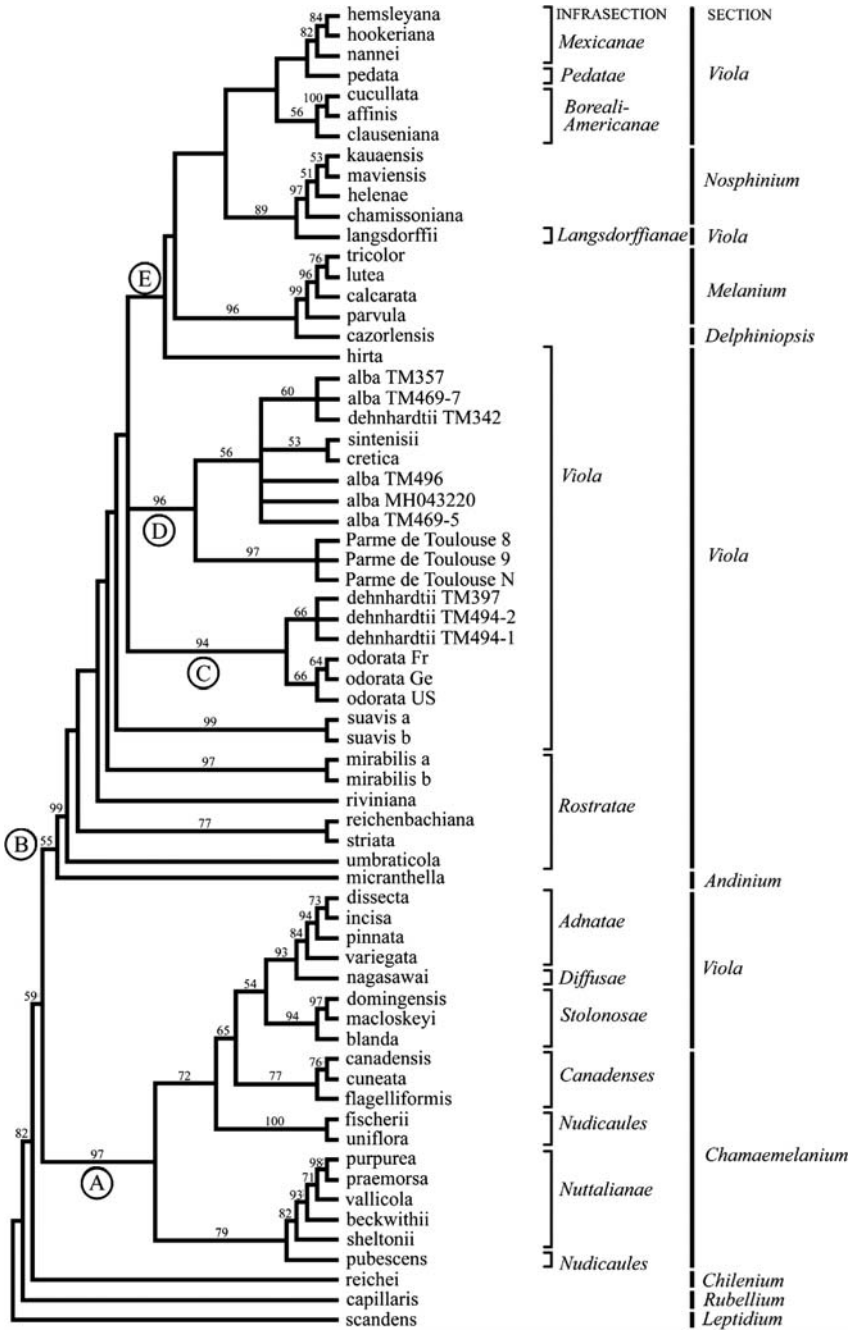

Fig. 4. Strict consensus tree when polymorphic cultivars are removed, based on 12 trees. Bootstrap values above 50% are indicated along the branches. Clades A, B, C, D, and E are discussed in the Results of phylogenetic analysis of ITS sequences section. Taxa refer to the species level except within *Viola alba* s.l. where subspecies or geographical origins are indicated.

cultivars: one group contains 'D'Udine' and 'Marie Louise' that have become homozygous for *AMP* and *LAP* but still have ITS polymorphism; and the 'Parme de Toulouse' group (MH-993489, MH-953381), which has become homozygous for *SKD* and lacks ITS polymorphism. The cultivar 'Ash Vale Blue', included in the ITS study only, may belong either to the group with the most original cultivars or to the 'D'Udine'/'Marie Louise' group.

The cultivars differed from all other investigated taxa with $2n = 20$ by having substantially higher levels of heterozygosity, 15–17 allozyme markers vs. 11–15 in the wild taxa. Similarly, there was an important number of intra-individual polymorphic ITS sites in four of the Parma violet cultivars (resulting in a polytomy within clade D in Fig. 2). Heterozygosity is, however, relatively rare in *Viola* due to predominant cleistogamous inbreeding, and high levels of heterozygosity are usually associated primarily with interspecific hybrids or with allopolyploids such as *Viola suavis* ($2n = 40$), here with 18–20 markers. Therefore, a hybrid origin of the cultivars is suggested, but as they possess no diagnostic characters of

TABLE 3. Multilocus phenotypes of the analyzed specimens. See Materials and Methods for allozyme electrophoresis for names of isozymes.

			Isozyme system and bands																											
			AAT				*AMP*							*GPI*												*IDH*				
Taxon	Origin	Accession no.	*a*	*b*	*c*	*d*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*k*	*l*	*a*	*b*	*c*	*d*	*e*
Viola 'Conte di Brazza'	—	MH-993490	—	1	—	1	1	—	—	—	—	1	—	—	—	—	1	1	—	—	—	—	1	—	—	—	1	—	—	1	—
V. 'D'Udine'	—	MH-023175	—	1	—	1	—	—	—	—	—	1	—	—	—	—	1	1	—	—	—	—	1	—	—	—	1	—	—	1	—
V. 'Gloire de Verdun'	—	MH-023177	—	1	—	1	1	—	—	—	—	1	—	—	—	—	1	1	—	—	—	—	1	—	—	—	1	—	—	1	—
V. 'Marie Louise'	—	MH-023183	—	1	—	1	—	—	—	—	—	1	—	—	—	—	1	1	—	—	—	—	1	—	—	—	1	—	—	1	—
V. 'Parme de Toulouse'	—	MH-993489	—	1	—	1	1	—	—	—	—	1	—	—	—	—	1	1	—	—	—	—	1	—	—	—	1	—	—	1	—
V. 'Parme de Toulouse'	—	MH-953381	—	1	—	1	1	—	—	—	—	1	—	—	—	—	1	1	—	—	—	—	1	—	—	—	1	—	—	1	—
V. alba subsp. *alba*	Azerbaijan	TM230-2	—	1	1	—	—	1	—	—	—	—	—	—	—	—	1	—	—	—	—	—	1	—	—	—	—	—	1	1	—
	Cyprus	TM469-13	—	1	1	—	—	1	—	—	—	—	—	—	—	—	1	—	1	—	—	—	—	—	—	—	—	—	1	1	—
	France	TM039-1	—	1	1	—	—	1	—	—	—	—	—	—	—	—	1	—	—	—	—	—	1	—	—	—	—	—	1	1	—
	Greece	TM496-1	—	1	1	—	—	1	—	—	—	—	—	—	—	—	1	—	1	—	—	—	—	—	—	—	—	—	1	1	—
	Greece	TM357-21	—	1	1	—	—	1	—	—	—	—	—	—	—	—	1	—	1	—	—	—	—	—	—	—	—	—	1	1	—
	Switzerland	TM389-1	—	1	1	—	—	1	—	—	—	—	—	—	—	—	1	—	1	—	—	—	—	—	—	—	—	—	1	1	—
	Turkey	TM458	—	1	1	—	—	1	—	—	—	—	—	—	—	—	1	—	1	—	—	—	—	—	—	—	—	—	1	1	—
V. alba subsp. *cretica*	Crete	TM360-10	—	1	—	1	—	1	—	—	—	—	—	—	—	—	1	—	—	—	—	—	1	—	—	—	1	—	—	1	—
V. alba subsp. *dehnhardtii*	Croatia	TM401-4	—	1	—	1	—	1	—	—	—	—	—	—	—	—	1	—	—	—	—	—	1	—	—	—	1	—	—	1	—
	France	TM397-2	—	1	—	1	1	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	1	—	—	—	1	—	—	1	—
	France	TM384-4	—	1	—	1	—	1	—	—	—	—	—	—	—	—	1	—	—	—	—	—	1	—	—	—	1	—	—	1	—
	Greece	TM478-2	—	1	—	1	—	1	—	—	—	—	—	—	—	—	1	—	—	—	—	1	—	—	—	—	1	—	—	1	—
	Morocco	TM455-1	—	1	—	1	—	1	—	—	—	—	—	—	—	—	—	1	—	—	—	—	1	—	—	—	1	—	—	1	—
	Sicily	TM051	—	1	—	1	1	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	1	—	—	—	1	—	—	1	—
	Spain	TM494-1	—	1	—	1	—	1	—	—	—	—	—	—	—	—	1	—	—	—	—	—	1	—	—	—	1	—	—	1	—
	Turkey	TM484-6	—	1	1	—	1	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	1	—	—	—	1	—	—	1	—
V. hirta	Germany	TM040	1	—	—	1	—	1	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	1	1	—	—	—	1
	Norway	TM004-1	1	—	—	1	—	1	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	1	1	—	—	1	—
V. jaubertiana	Majorca	TM049	1	—	—	1	—	—	1	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	1	—	1	—	—	1	—
V. odorata	Cyprus	TM469-8	—	1	—	1	—	—	—	—	1	—	—	—	—	—	1	—	—	—	—	—	—	—	1	—	1	—	—	1	—
	England	IN4431	—	1	—	1	—	—	—	—	1	—	—	—	—	—	1	—	—	—	—	—	—	—	1	—	1	—	—	1	—
	France	IN4433	—	1	—	1	—	—	—	1	—	—	—	—	1	—	—	—	—	—	—	—	—	—	1	—	1	—	—	1	—
	Greece	TM335-5	—	1	—	1	—	—	—	—	1	—	—	—	—	—	1	—	—	—	—	—	—	—	1	—	1	—	—	1	—
	Norway	TM062-4	—	1	—	1	—	—	—	1	—	—	—	—	—	—	1	—	—	—	—	—	—	—	1	—	1	—	—	1	—
	France	MH-993112	—	1	—	1	—	—	—	—	—	—	1	—	—	—	1	—	—	—	—	—	—	—	1	—	1	—	—	1	—
V. sintenisii	Azerbaijan	TM235-3	—	1	—	1	—	—	—	—	—	1	—	—	—	—	—	—	—	1	1	—	—	—	—	—	—	—	—	1	—
	Azerbaijan	TM238-1	—	1	—	1	—	—	—	—	—	1	—	—	—	—	—	—	—	1	1	—	—	—	—	—	—	—	1	—	—
	Azerbaijan	TM240-2	—	1	—	1	—	—	—	—	—	1	—	—	—	—	—	1	—	—	1	—	—	—	—	—	—	—	—	1	—
	Azerbaijan	TN240-4	—	1	—	1	—	—	—	—	—	1	—	—	—	—	—	—	1	1	—	—	—	—	—	—	—	—	1	—	—
	Azerbaijan	TM240-5	—	1	—	1	—	—	—	—	—	1	—	—	—	—	—	—	1	1	—	—	—	—	—	—	—	—	—	1	—
V. suavis	France	TM503-4	—	1	—	1	—	1	—	—	—	—	1	—	—	—	1	—	—	—	—	—	—	1	1	—	1	—	—	1	—
	France	IN4001	—	1	—	1	—	1	—	—	—	—	1	—	—	—	1	—	—	—	—	—	—	1	1	—	1	—	—	1	—
	Norway	TM001-1	—	1	—	1	—	1	—	—	—	—	1	1	—	1	—	—	—	—	—	—	1	1	—	1	—	—	1	—

TABLE 4. Cultivars compared with regional band frequencies found in *Viola alba*. See Materials and Methods for allozyme electrophoresis for names of isozymes.

		Isozyme system and bands														
		AAT			*AMP*			*GPI*					*IDH*			
Taxon	Origin	*b*	*c*	*d*	*a*	*b*	*f*	*c*	*d*	*e*	*f*	*i*	*a*	*b*	*c*	*d*
Parma Violets	—	1	—	1	0.67	—	1	1	1	—	—	1	1	—	—	1
subsp. *alba*	CW Europe	1	1	—	0.08	1	—	1	—	0.83	—	0.17	—	—	1	1
	Greece	1	0.83	—	—	1	—	1	—	0.83	—	0.23	—	—	1	1
	Cyprus	1	1	—	—	1	—	1	—	1	—	—	—	—	1	1
	N Turkey	1	1	—	—	1	—	1	—	1	—	—	—	—	1	1
	Azerbaijan	1	1	—	—	1	0.04	1	—	0.95	—	0.20	0.02	—	1	1
subsp. *cretica*	Crete	1	—	1	—	1	—	1	—	—	—	1	1	—	—	1
subsp. *dehnhardtii*	Morocco	1	—	1	—	1	—	—	1	—	—	1	1	—	—	1
	Spain	1	—	1	—	1	—	1	—	—	—	1	1	—	—	1
	S France	0.57	—	1	0.59	0.44	—	0.96	0.06	—	—	1	1	0.19	—	0.83
	Croatia	1	0.24	0.76	—	1	—	1	—	—	—	1	1	—	—	1
	Sicily	1	—	1	1	—	—	—	1	—	—	1	1	—	—	1
	Greece	1	—	1	0.21	0.79	—	0.89	—	0.24	0.13	0.63	0.79	—	0.21	1
	S Turkey	1	1	—	0.17	0.83	—	1	—	—	—	1	1	—	—	1

TABLE 3. Continued.

Taxon	Origin	Isozyme system and bands																										Bands
		LAP									*PGM*										*SKD*								
		a	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	
V. 'Conte di Brazza'	—	—	—	—	1	—	1	—	—	—	—	—	1	—	—	—	—	1	1	—	—	—	1	—	1	1	—	—	—	17
V. 'D'Udine'	—	—	—	—	1	—	—	—	—	—	—	—	1	—	—	—	—	1	1	—	—	—	1	—	1	1	—	—	—	15
V. 'Gloire de Verdun'	—	—	—	—	1	—	1	—	—	—	—	—	1	—	—	—	—	1	1	—	—	—	1	—	1	1	—	—	—	17
V. 'Marie Louise'	—	—	—	—	1	—	—	—	—	—	—	—	1	—	—	—	—	1	1	—	—	—	1	—	1	1	—	—	—	15
V. 'Parme de Toulouse'	—	—	—	—	1	—	1	—	—	—	—	—	1	—	—	—	—	1	1	—	—	—	—	—	1	1	—	—	—	16
V. 'Parme de Toulouse'	—	—	—	—	1	—	1	—	—	—	—	—	1	—	—	—	—	1	1	—	—	—	—	—	1	1	—	—	—	16
V. alba subsp. *alba*	Azerbaijan	—	—	—	1	—	—	1	—	—	—	—	—	1	1	—	—	1	—	—	—	—	1	—	1	—	—	—	—	14
	Cyprus	—	—	—	1	—	1	—	—	—	—	—	1	—	—	—	—	1	1	—	—	—	—	—	1	1	—	—	—	14
	France	—	—	—	1	—	—	—	—	—	—	—	—	1	—	—	—	1	1	—	—	—	1	—	1	—	—	—	—	13
	Greece	—	—	—	1	—	1	—	—	—	—	—	—	1	—	—	—	1	1	—	—	—	1	—	1	—	—	—	—	14
	Greece	—	—	—	1	—	1	—	—	—	—	—	—	1	1	—	—	1	—	—	—	—	1	—	1	—	—	—	—	14
	Switzerland	—	—	—	1	—	—	—	—	—	—	—	—	1	—	—	—	1	1	—	—	—	1	—	1	—	—	—	—	13
	Turkey	—	—	—	1	—	—	1	—	—	—	—	—	1	—	—	—	1	1	—	—	—	1	—	1	—	—	—	—	14
V. alba subsp. *cretica*	Crete	—	—	1	1	—	—	—	—	—	—	—	—	1	—	—	—	—	1	—	—	—	1	—	1	—	—	—	—	13
V. alba subsp. *dehnhardtii*	Croatia	—	—	—	1	—	1	—	—	—	—	—	—	1	—	—	—	1	1	—	1	—	—	—	1	—	—	—	—	14
	France	—	—	—	1	—	—	1	—	—	—	—	—	1	—	—	—	1	1	—	—	—	1	—	1	—	—	—	—	13
	France	—	—	—	1	—	—	1	—	—	—	—	—	1	1	—	—	1	—	—	1	—	—	—	1	—	—	—	—	14
	Greece	—	—	—	1	—	—	1	—	—	—	—	—	1	—	—	—	1	1	—	—	—	1	—	1	—	—	—	—	14
	Morocco	—	1	—	—	—	—	—	—	—	—	—	—	1	—	—	—	1	1	—	—	—	1	—	1	—	—	—	—	13
	Sicily	—	—	—	1	—	1	—	—	—	—	—	—	1	—	—	—	1	1	—	—	—	1	—	1	—	—	—	—	14
	Spain	—	—	—	1	—	1	—	—	—	—	—	—	1	—	—	—	1	1	—	1	—	—	—	1	—	—	—	—	14
	Turkey	—	—	—	1	—	1	—	—	—	—	—	1	—	—	—	—	1	1	—	—	—	—	—	1	1	—	—	—	14
V. hirta	Germany	—	—	—	—	1	—	—	—	1	1	—	—	—	—	—	1	1	—	—	—	1	—	—	1	—	—	—	—	14
	Norway	—	—	—	—	1	—	—	—	1	1	—	—	—	—	—	1	1	—	—	—	1	—	—	1	—	—	—	—	14
V. jaubertiana	Majorca	—	—	—	—	1	—	—	—	—	—	—	1	—	—	—	—	1	1	—	—	—	—	—	1	—	—	—	—	12
V. odorata	Cyprus	1	—	—	—	—	—	—	1	—	—	—	1	—	—	—	—	—	1	1	—	—	—	1	—	—	—	—	1	14
	England	1	—	—	—	—	—	—	1	—	—	—	—	1	—	—	—	1	1	—	—	—	—	1	—	—	—	—	1	14
	France	1	—	—	—	—	—	—	1	—	—	—	—	1	—	—	—	1	1	—	—	—	—	1	—	—	—	—	1	14
	Greece	1	—	—	—	—	—	—	1	—	—	—	1	1	—	—	—	1	1	—	—	—	—	1	—	—	1	—	—	15
	Norway	1	—	—	—	—	—	—	1	—	—	—	—	1	—	—	—	1	1	—	—	—	—	1	—	—	—	—	1	14
	France	1	—	—	—	—	—	—	1	—	—	—	—	1	—	—	—	1	1	—	—	—	—	1	—	—	—	—	1	14
V. sintenisii	Azerbaijan	—	—	—	1	—	1	—	—	—	—	—	1	—	—	—	—	1	1	—	—	—	—	—	1	—	—	—	—	12
	Azerbaijan	—	—	—	—	—	1	—	—	—	—	—	1	—	—	1	—	—	1	—	—	—	—	—	1	—	—	—	—	11
	Azerbaijan	—	—	—	—	—	1	—	—	—	—	—	1	—	—	1	—	—	1	—	—	—	—	—	1	—	—	—	—	11
	Azerbaijan	—	—	—	1	—	1	—	—	—	—	—	1	—	—	1	—	—	1	—	—	—	—	—	1	—	—	—	—	12
	Azerbaijan	—	—	—	1	—	1	—	—	—	—	—	1	—	—	1	—	1	1	—	—	—	—	—	1	—	—	—	—	13
V. suavis	France	—	—	—	1	1	—	—	—	—	—	1	—	1	1	—	—	1	1	—	—	—	1	—	1	—	—	1	—	19
	France	—	—	—	1	1	—	—	—	—	—	1	—	—	1	—	—	1	1	—	—	—	1	—	1	—	—	1	—	18
	Norway	—	—	—	1	1	—	—	—	—	—	1	—	1	1	—	—	1	1	—	—	—	1	—	1	—	—	1	—	20

TABLE 4. Extended.

Taxon	Origin	Isozyme system and bands																
		LAP					*PGM*							*SKD*				
		b	*c*	*d*	*f*	*g*	*c*	*d*	*f*	*g*	*h*	*i*	*j*	*a*	*c*	*e*	*f*	*h*
Parma Violets	—	—	—	1	0.67	—	1	—	—	—	1	1	—	—	0.67	1	1	—
subsp. *alba*	CW Europe	—	—	1	—	—	—	1	—	—	1	1	—	—	1	0.97	—	0.03
	Greece	—	—	0.90	0.50	0.03	—	1	—	0.23	0.63	0.93	—	0.03	0.97	1	—	—
	Cyprus	—	—	1	1	—	0.30	0.70	—	—	1	1	0.20	—	0.60	1	0.30	—
	N Turkey	—	—	1	1	—	—	1	—	—	1	1	—	—	1	1	—	—
	Azerbaijan	—	—	0.96	0.70	0.39	0.16	0.93	0.05	0.21	0.70	0.91	—	0.02	0.98	1	—	—
subsp. *cretica*	Crete	—	1	1	—	—	—	1	—	—	—	1	—	—	1	1	—	—
subsp. *dehnhardtii*	Morocco	1	—	—	—	—	—	1	—	—	1	1	—	—	1	1	—	—
	Spain	—	—	1	1	—	—	1	—	—	1	1	—	1	—	1	—	—
	S France	—	—	0.96	0.65	0.35	—	1	—	0.24	0.78	0.81	—	0.87	0.19	0.98	—	0.02
	Croatia	—	—	0.14	1	—	—	1	—	—	0.81	1	—	1	—	1	—	—
	Sicily	—	—	1	1	—	—	1	—	—	1	1	—	—	1	1	—	—
	Greece	—	—	1	0.84	—	—	1	—	0.71	0.89	0.29	—	0.66	0.37	0.95	—	—
	S Turkey	—	—	1	1	—	1	—	—	—	1	1	—	—	—	1	1	—

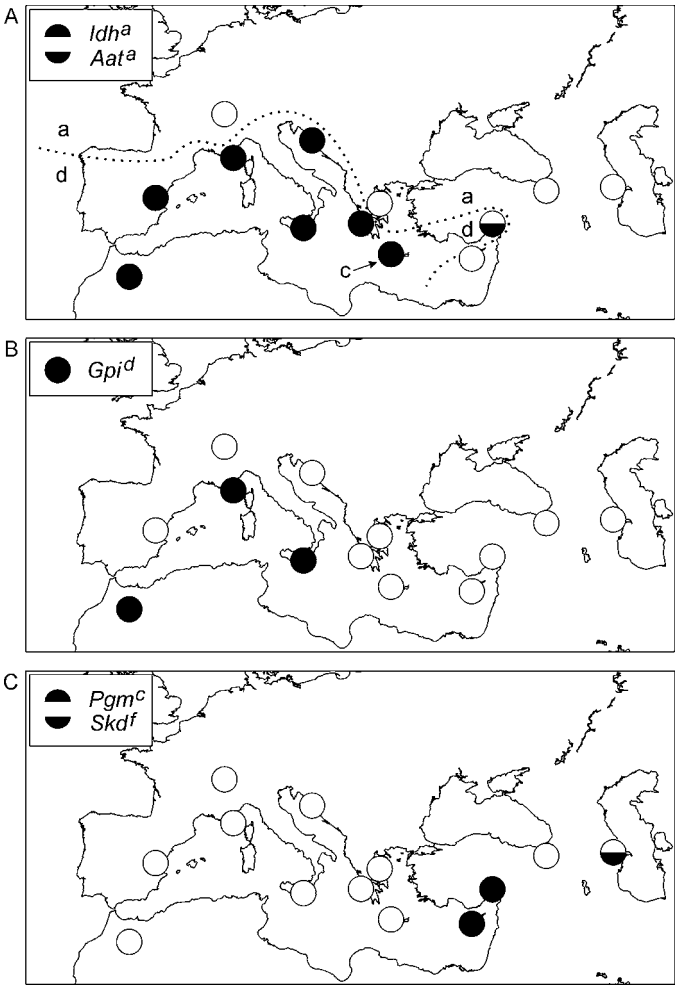

Fig. 5. Selected diagnostic allozyme markers from Parma violet cultivars and their geographic occurrence in *Viola alba*. Only markers with regional frequencies >0.02 are shown. (A) Markers connecting the Parma violets with the Mediterranean *V. alba* subsp. *dehnhardtii* (Aat^d and Idh^a). The dashed line delimits the subspecies of *V. alba*, subsp. *dehnhardtii* (d) in the Mediterranean basin, subsp. *alba* (a) to the north and east, and subsp. *cretica* (c) in Crete. (B) Markers connecting the Parma violets with western populations of *V. alba* subsp. *dehnhardtii* (Gpi^d). (C) Markers connecting the Parma violets with eastern populations of *V. alba* (Pgm^c and Skd^f).

species other than *V. alba*, we must assume they have originated from a single, wide hybridization event within *V. alba*.

Geographical origin of the Parma violets—*Viola alba* is a widely distributed species with three geographical races in Europe, currently accepted at the subspecies level (Marcussen, 2003; Marcussen et al., 2005). Subspecies *dehnhardtii* occurs in the Mediterranean region and is flanked by subsp. *alba* in the north and east (Fig. 5A), while subsp. *cretica* is endemic to Crete. These subspecies are allozymatically well-characterized, and subspp. *alba* and *dehnhardtii* have internal and additional geographically structured variation.

No diagnostic markers of subsp. *alba* were found in the cultivars. On the other hand, the sharing of five allozyme markers (Amp^b, Gpi^d, Gpi^i, and in particular Aat^d and Idh^a) with subsp. *dehnhardtii* provides ample evidence that this subspecies was involved in the origin of the cultivars (Fig. 5A). One of these markers Gpi^d is known from western Mediterranean *dehnhardtii* only (Fig. 5B). Also, the cultivars possessed three more markers (Amp^f, Pgm^c, Skd^f) characteristic of eastern Mediterranean and western Asian populations of both subsp. *alba* and *dehnhardtii* (Fig. 5C).

Historical bibliography gives some support to a trans-Mediterranean hybrid origin of the Parma violets, as suggested by allozymes. Referring to Hyeronimus, Costeo (in Pseudo-Mesuë, 1573), a professor of medicine in Turin and Bologna, mentioned a Byzantine origin (i.e., an origin in European Turkey) for violets with pleiomerous flowers and strong fragrance, implying that the cross giving rise to the modern Parma violets occurred either in Turkey before 1573 (the date of publication) or in Italy from material coming from Turkey and local *Viola alba* subsp. *dehnhardtii*. Later, Camerarius (1588, p. 177) stated that "Jeronimus, Viennese chaplain, recently sowed one of these flowers and another one taken from the first, and obtained another one of the size of a rose" (original Latin text: *Huius recentem florem unum, atque alterum prima mensa sumptum, aluum subducere instar recentis Rosae, Heyronymum Capellum Venetum rerum naturalium peritissimum obseruasse tradit*). From these historical sources, it seems possible that the original odoriferous clones came from Turkey to Italy where they hybridized with Italian plants. Between 1573 and 1730, Parma violets were only occasionally mentioned in the literature. Their horticultural development seems to be correlated with the Bourbon dynasty that ruled the kingdom of Naples from 1730 and onwards. From Naples, they may have been introduced to Parma, Toulouse, and numerous other places during the early 19th century.

Conclusions—In the present study, the phylogenetic signal obtained using ITS sequences is adequate to favor one of the three hypothetical origins of the sweet-smelling Parma violets. Allozyme analyses clarify more precisely the origin of sweet violet cultivars. Parma Violets have been variously assigned to different species in the extensive literature on these sterile and fragrant cultivated violets. Our analyses show that these cultivars belong to *Viola alba* and are best included in the Mediterranean subsp. *dehnhardtii*. They possess high allozyme heterozygosity and have probably originated only once from a hybrid involving plants from the eastern and western Mediterranean region. Segregation of heterozygosity in several cultivars indicates subsequent rare events of sexual reproduction, presumably through cleistogamy. Historical bibliography supports the results obtained by allozymes indicating that this cultivar group originated in Turkey. However, at what time and where the first cultivar exactly originated remain unclear. Thus, our results provide a decisive answer regarding the taxonomy of Parma violets, allowing growers of new cultivars to develop crosses with the wild parents whereas currently they rely on sporadic bud mutants and exceptional self-seeding. Similar methods may now allow a more precise check of the identity of cultivars and their relationships, two points important to plant property rights. Finally, this work also gives a new look at the cellular and molecular processes related to horticultural selection of an herbaceous plant that can be traced through the printed history of the Parma violets.

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APPENDIX. Voucher information and GenBank accession numbers for taxa used in this study. Voucher specimens are deposited in the following herbaria: F = Field Museum of Natural History, M = Botanische Staatssammlung München, NCY = Conservatoire et Jardins Botaniques de Nancy, NEU = Université de Neuchâtel, NTBG = National Tropical Botanical Garden, Tahuata, NY = New York Botanical Garden, O = Botanical Museum, Oslo, US = Smithsonian Institution, WIS = University of Wisconsin.

Taxon—GenBank accessions: *ITS1*, *ITS2*; Source; *Voucher specimen*; References.

Parma violet cultivars

***Viola* 'Ash Vale Blue'**—DQ358839, DQ358816; Cultivated in Toulouse (N. Casbas, private collection), 23 April 2002; *MH-023290* (NCY); This study. ***V.* 'Marie Louise'**—DQ358840, DQ358817; Cultivated in Toulouse (N. Casbas, private collection), 23 April 2002; *MH-023183* (NCY) [NCY000670]; This study. ***V.* 'D'Udine'**—DQ358841, DQ358818; Cultivated in Toulouse (N. Casbas, private collection), 23 April 2002; *MH-023289* (NCY); This study. ***V.* 'Gloire de Verdun'**—DQ358842, DQ358819; Cultivated in Toulouse (N. Casbas, private collection), 14 September 2001; *MH-023177* (NCY) [NCY000668]; This study. ***V.* 'Parme de Toulouse' 8**—DQ358843, DQ358820; Cultivated in Nancy (Originated from Candiflor Society, Toulouse), 16 October 1989; *MH-953381*, (NCY) [NCY000687]; This study. ***V.* 'Parme de Toulouse' 9**—DQ358844, DQ358821; Cultivated in Nancy (from in vitro culture of *MH-95381*), 5 December 1999; *MH-993489* (NCY) [NCY000671, NCY000672]; This study. ***V.* 'Parme de Toulouse' N**—DQ358845, DQ358822; Cultivated in Toulouse (N. Casbas, private collection, Toulouse), 23 April 2002; *MH-023288* (NCY); This study.

Viola alba

Viola alba* Bess. subsp. *alba—DQ358846, DQ358823; GREECE, Evia, Mt. Dirphys, along the dirt road S of the pass on Steni-Stropones road, 1040 m, 7 May 2001; *TM357–21* (O); This study. ***V. alba* Bess. subsp. *alba***—DQ358847, DQ358824; CYPRUS, Lemesos, Troödos Massif, Saïttas, along Mesapotamos creek, 13 September 2001; *TM469–7* (O); This study. ***V. alba* Bess. subsp. *alba***—DQ358848, DQ358825; CYPRUS, Lemesos, Troödos Massif, Saïttas, along Mesapotamos creek, 13 September 2001; *TM469–5* (O); This study. ***V. alba* Bess. subsp. *alba***—DQ358849, DQ358826; FRANCE, Nancy, Bois de l'hôpital near the aerodrome, Plateau de Malzéville, 27 March 2004; *MH-043220* (NCY) [NCY004746]; This study. ***V. alba* Bess. subsp. *alba***—DQ358850, DQ358827; GREECE, Kozánti, Euipeus Gorge at base of Mt. Olympus, 1998; *TM496* (O); This study. ***V. alba* subsp. *cretica* (Boiss. and Heldr.) Marcussen**—DQ358851, DQ358828; GREECE, Crete, Rethymno, River gorge near the Arkadiuo monastery, 500 m, 8 May 2001; *TM360* (O); This study. ***V. alba* subsp. *dehnhardtii* (Ten.) W. Becker**—DQ358852, DQ358829; GREECE, Achaía, 2 km up the road to Rogio & Kerpini, 4 May 2001; *TM 342–1* (O); This study. ***V. alba* subsp. *dehnhardii* (Ten.) W. Becker**—DQ358853, DQ358830; FRANCE, Var, Les Lecques, near St-Cyr-sur-mer, 9 July 2001; *TM397–2* (O); This study. ***V. alba* subsp. *dehnhardii* (Ten.) W. Becker**—DQ358854, DQ358831; SPAIN (Valencia), Castellón, Tirig N of Castellón de la Plana, c. 500 m, 1 June 1987; *TM494–1* (O); This study. ***V. alba* subsp. *dehnhardii* (Ten.) W. Becker**—DQ358855, DQ358832; SPAIN (Valencia), Castellón, Tirig N of Castellón de la Plana, c. 500 m, 1 June 1987; *TM494–2* (O); This study.

Other species

***Viola affinis* Leconte**—AF097251, AF097297; USA, Michigan, 17 May 1992; *Ballard 92–006* (WIS); Ballard et al. 1999. ***V. beckwithii* Torr. & Gray**—AF097227, AF097273; USA, Utah, 7 May 1995; *Ballard s.n.* (WIS); Ballard et al. 1999. ***V. blanda* Willd. var. *palustriformis* A. Gray**—AF097238, AF097284; USA, Minnesota, June 1993; *Ballard s.n.* (WIS); Ballard et al. 1999. ***V. calcarata* L.**—AF097243, AF097289; ITALY, Ligurian Alps, 5 June 1993; *Conti s.n.* (WIS); Ballard et al. 1999. ***V. canadensis* L.**—AF097231, AF097277; USA, North Carolina, 18 April 1967; *Greenlee 240* (WIS); Ballard et al. 1999. ***V. capillaris* Pers.**—AF097220, AF097266; CHILE, Prov. Concepción, 22 October 1990; *Lammers, Baeza, Peñailillo & Mazzeo 7522* (F); Ballard et al. 1999. ***V. cazorlensis* Gand.**—AY148230, AY148250; SPAIN, Cruz de Quique, August 1998; *SN-0006* (NEU); Yockteng et al. 2003. ***V. chamissoniana* Ging. subsp. *tracheliifolia* (Ging.) W. L. Wagner, Herbst & Sohmer**—AF097261, AF097307; USA, Hawai, 16 June 1993; *Nepokroeff 774* (WIS); Ballard et al. 1999. ***V. clauseniana* M. S. Baker**—AF097254, AF097300; USA, Utah, 20–30 April 1994; *Welsh & Sidles s.n.* (WIS); Ballard et al. 1999. ***V. cucullata* Aiton**—AF097252, AF097298; USA, Pennsylvania, May 1992; *Wiegman 92HB008* (Wiegman); Ballard et al. 1999. ***V. cuneata* S. Watson**—AF097234, AF097280; USA, Oregon, 18 May 1942; *Constance & Rollins 2975* (US); Ballard et al. 1999. ***V. dissecta* Ledeb.**—AY582170, AY541603; Russia, Novosibirsk, Central Siberian Botanical Garden of Siberian Branch of Russian Academy of Sciences, in the garden culture, Violets plantation of Galina P. Semenova, originally collected by M.M. Ivanova, N.I. Tupiczyna, G.P. Semenova, 8 July 1984, Krasnoyarsk Territory, Scharypovo district, Ivanovka village, Ingol' lake, south slope, heteroherbosus meadow, 19 July 2003; *Coll. Vl.V. Nikitin* (LE); Degtjareva et al. unpublished. ***V. domingensis* Urban**—AF097237, AF097283; Dominican Republic, La Nevera, 3–5 April 1971; *Liogier 17983* (F); Ballard et al. 1999. ***V. fischerii* W. Becker**—AY582168, AY541601; Altai Territory, Kur'inskyi district, in vicinity of 8-th of March village, slope of Sinyucha mountain, 18 May 1997; *A.N. Kupriyanov, O.M. Maslova, T.O. Strelnikova, I.A. Crustaleva, A.Ju. Korolyuk, Ju.A. Manakov* (LE); Degtjareva et al. unpublished. ***V. flagelliformis* Hemsley**—AF097233, AF097279; Mexico, Edo San Luis Potosi, 2 December 1983; *Fernandez & Acosta 2041* (NY); Ballard et al. 1999. ***V. helenae* C. Forbes & Lygdate**—AF097260, AF097306; USA, Hawai, 30 June 1993; *Perlman s.n.* (NTBG); Ballard et al. 1999. ***V. hemsleyana* Calderon**—AF097258, AF097304; Mexico, Edo. Veracruz, 21 June 1993; *Ballard s.n.* (WIS); Ballard et al. 1999. ***V. hirta* L.**—DQ358856, DQ358833; France, Nancy, Bois de l'hôpital près de l'aérodrome, Plateau de Malzéville, 20 April 2002; *MH-023587* (NCY) [NCY004745]; This study. ***V. hookeriana* Kunth**—AF097257,

AF097303; Mexico, Edo. Durango, 30 June 1993; *Ballard s.n.* (WIS); Ballard et al. 1999. ***V. incisa* Turcz.**—AY582172, AY541605; Russia, Novosibirsk, Central Siberian Botanical Garden of Siberian Branch of Russian Academy of Sciences, garden culture, violet plantation of Galina P. Semenova, originally collected by M.M. Ivanova, N.I. Tupiczyna, G.P. Semenova, 5 July 1982, Krasnoyarsk Territory, Scharypovo district, Ivanovka village, Ingol' lake, south steppe slope, 19 July 2003; *Coll. Vl. V. Nikitin* (LE); Degtjareva et al. unpublished. ***V. kauaensis* A. Gray**—AF097262, AF097308; USA, Hawai, no date; *Nepokroeff 786b* (WIS); Ballard et al. 1999. ***V. langsdorffii* Ging.**—AF097259, AF097305; USA, Alaska, 20 July 1975; *Craighead s.n.* (WIS); Ballard et al. 1999. ***V. lutea* Hudson**—AY148236, AY148256; France, Puy de Dôme, July 1999; *HLEB 03032* (NEU); Yockteng et al. 2003. ***V. macloskeyi* Lloyd subsp. *pallens* (Ging.) M. S. Baker**—AF097236, AF097282; USA, Michigan, 17 May 1992; *Ballard 92–005* (WIS); Ballard et al. 1999. ***V. maviensis* H. Mann**—AF097263, AF097309; USA, Hawai, 15 July 1993; *Nepokroeff 913 & Lau* (WIS); Ballard et al. 1999. ***V. micranthella* Wedd.**—AF097222, AF097268; Peru, Prov. Huaylas, 4 May 1987; *Mostacero, Leiva, Mejia, Peláez, Medina & Zelada 1959* (F); Ballard et al. 1999. ***V. mirabilis* L. (a)**—DQ358857, DQ358834; France, Nancy, La Sapinière, 21 March 2002; *MH-023589* (NCY) [NCY004743]; This study. ***V. mirabilis* L. (b)**—DQ358858, DQ358835; France, Nancy, Plateau de Malzéville, 21 March 2002; *MH-023588* (NCY) [NCY004744]; This study. ***V. nagasawai* Makino & Hayata**—AF097239, AF097285; Taiwan, Taipei Co., 12 March 1994; *Wang & Lin 9056* (WIS); Ballard et al. 1999. ***V. nannei* Polak.**—AF097255, AF097301; Costa Rica, 9°57′N 83°51′W, 10 January 1994; *Ballard 94–025 & Baker* (WIS); Ballard et al. 1999. ***V. odorata* L. (Fr)**—AY148238, AY148258; France, Orsay, Yvette, July 1999; *Yockteng SN-0002* (NEU); Yockteng et al. 2003. ***V. odorata* L. (Ge)**—AF097250, AF097296; Germany, Mainz region, 20 May 1995; *Ballard s.n.* (WIS); Ballard et al. 1999. ***V. odorata* L. (US)**—AF097249, AF097295; USA, Wisconsin, April 1993; *Ballard s.n.* (WIS); Ballard et al. 1999. ***V. parvula* Tineo**—AY148240, AY148260; Spain, Sierra Nevada, February 2000; *HLEB 03025* (NEU); Yockteng et al. 2003. ***V. pedata* L.**—AF097253, AF097299; USA, Michigan, 17 May 1992; *Ballard 92–007a* (WIS); Ballard et al. 1999. ***V. pinnata* L.**—AF097240, AF097286; USA, Ex horto Robert Faden of Smithsonian Institution, 1995; *Ballard s.n.* (WIS); Ballard et al. 1999. ***V. praemorsa* Kellogg**—AF097228, AF097274; USA, Nevada, 23 May 1987; *Tiehm 11076* (WIS); Ballard et al. 1999. ***V. pubescens* Aiton**—AF097225, AF097271; USA, Michigan, 18 May 1992; *Ballard 92–011* (WIS); Ballard et al. 1999. ***V. purpurea* Kellogg**—AF097229, AF097275; USA, Nevada, 8 May 1995; *Ballard s.n.* (WIS); Ballard et al. 1999. ***V. reichei* Skottsberg**—AF097223, AF097269; Chile, Prov. Ñuble, 11 December 1987; *Rechinger & Rechinger 64271* (M); Ballard et al. 1999. ***V. reichenbachiana* Boreau**—AF097248, AF097294; France, Alpes maritimes, 16 June 1993; *Conti s.n.* (WIS); Ballard et al. 1999. ***V. riviniana* Reichb.**—AY148242, AY148262; France, Orsay, Paris Sud University, July 1999; *SN-0009* (NEU); Yockteng et al. 2003. ***V. scandens* Roem. & Schultes**—AF097221, AF097267; Costa Rica, 10°08′N 84°09′30″W, 5 January 1994; *Ballard 94–001* (WIS); Ballard et al. 1999. ***V. sheltonii* Torr.**—AF097226, AF097272; USA, California, 2 July 1994; *Bartholomew 6787* (WIS); Ballard et al. 1999. ***V. sintenisii* W. Becker**—*DQ*358859, DQ358836; Azerbaijan, Dəvəxı, Çirax Castle nr. Gala-Altı, 1200 m, June 2000; *TM240–1* (O); This study. ***V. striata* Aiton**—AF097247, AF097293; USA, Michigan, 18 May 1992; *Ballard 92–010* (WIS); Ballard et al. 1999. ***V. suavis* Bieb. (a)**—DQ358860, DQ358837; France, Alpes-Maritimes, nr. Gourme, 2001; *IN4006* (O); This study. ***V. suavis* Bieb. (b)**—DQ358861, DQ358838; France, Alpes-Maritimes, Villeneuve-Loubet, 2001; *IN4001* (O); This study. ***V. tricolor* L.**—AY148243, AY148263; France, Saône and Loire, July 1999; *SN-0010* (NEU); Yockteng et al. 2003. ***V. umbraticola* Kunth**—AF097244, AF097290; Mexico, Edo. Durango, 30 June 1993; *Ballard s.n.* (WIS); Ballard et al. 1999. ***V. uniflora* L.**—AY582167, AY541600; Altai Territory, Krasnoschekovskyi district, 20 km to the south from the Czineta village, left bank of the river Inya, in 3 km to the north-west from the Tigirek village, ascent from Tigirek to the Razrabotnaya mountain, east slope, fir-birch forest, about 800 m, 14 August 2001; *Vl.V. Nikitin, I.D. Illarionova* (LE); Degtjareva et al. unpublished. ***V. vallicola* A. Nelson**—AF097230, AF097276; USA, Utah, 7 May 1995; *Ballard s.n.* (WIS); Ballard et al. 1999. ***V. variegata* Link.**—AY582171, AY541604; Russia, Novosibirsk, Central Siberian Botanical Garden of Siberian Branch of Russian Academy of Sciences, garden culture, violet plantation of Galina P. Semenova, originally collected by G.P. Semenova, M.M. Ivanova, 5 July 1981, Chita region, Baleiskyi district, Kunikan village, in the river valley, steppe, stone-carbonaceous slope, 19 July 2003; *Coll. Vl. V. Nikitin* (LE); Degtjareva et al. unpublished.