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Relationships among pansies (*Viola* section *Melanium*) investigated using ITS and ISSR markers

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Abstract. Sequences of the nuclear region ITS and the variable molecular markers ISSR were used to estimate the phylogeny of the section *Melanium* of the genus *Viola*. We confirm that the so-called pansies form a derived and monophyletic group. Two floral characters, the upturned side petals and the large size of pollen grains appear to be synapomorphies in *Melanium*. The *Melanium* species are very closely related, as shown by the reduced genetic variation compared to the other sections of *Viola*. Our analyses suggest $x = 5$ or $x = 7$ as the possible base chromosome number of the section *Melanium*. Polyploidy and hybridization would have played an important role in the evolutionary history of this clade resulting in a wide range of chromosome number. The low genetic differentiation and the complex cytological evolution suggest that diversification in *Melanium* is the result of a reticulate evolution and rapid radiation in Europe and Northern Africa.

Key words: *Viola*, phylogeny, ISSR, ITS, chromosome numbers, rapid radiation.

Introduction

Viola L. is the largest genus of the Violaceae family, with 525–600 species (Clausen 1964, Ballard 1996), distributed throughout most frost-free regions of the world. The genus probably arose in South America but most centers of morphological and taxonomic diversity occur in the Northern Hemisphere. Numerous taxonomic studies on particular species complexes have been published over the 20th century based largely on morphology (Becker 1925) and chromosome numbers (Clausen 1927, 1929). A recent molecular phylogenetic analysis (Ballard 1996, Ballard et al. 1999) has clarified the composition and relationships of the main groups and reevaluated the placement of controversial assemblages.

Section *Melanium* Ging., which includes the so-called pansies, is derived and probably monophyletic. It is a morphologically well-defined group of about 80–100 species (Ballard 1996). Most species are herbaceous, caulescent, with frontally flattened flowers, a yellow throat, big divided leaf-like stipules as well as entire stipules and a well-developed spur of variable length among species. The side petals are upturned (downturned in the other sections) and the bottom petal is

enlarged, serving probably as a landing platform for insect pollinators. The style has a characteristic capitate or globose shape with a stigmatic orifice on a ventral rostellum. Cleistogamy has not been reported for members of this section (Knuth 1908, Herrera 1993) although it occurs in most species in the rest of the genus. The geographical distribution spans Europe and westernmost Asia. A few species are found in Northern Africa and one species is disjunct and probably native in North America (Clausen et al. 1964). The composition of section *Melanium* varies according to authors. Ballard (1996) hypothesizes that the woody sections *Xylinosium* and *Delphiniopsis* may have arisen from within *Melanium*. The woody stems, linear leaves and long spurred flowers, adapted to hawk moth pollination in the three *Delphiniopsis* species, have been traditionally interpreted as primitive characters by some taxonomists (Beattie 1974) However, these are readily argued to represent instead evolutionary specializations, as are the woody stems of the four *Xylinosium* species in the Fynbos and Mediterranean regions. Both sections have the frontally flattened flowers with a yellow corolla throat, which characterize the section *Melanium*, and share cytological features with different groups of this section (Ballard 1996). If this most recent viewpoint is maintained, section *Melanium* is composed of three groups: *Elongatae*, *Delphiniopsis* and *Xylinosium*.

Cytological diversity is a striking feature of this section. Whereas the base chromosome number is relatively stable in the rest of the genus ($x = 12$, $x = 10$ or $x = 6$), it is extremely diverse in the section *Melanium* where it ranges from $x = 5$ to $x = 17$, with polyploid series based on most of them (Ballard 1996, Erben 1996). Erben (1996) proposes $x = 11$ as the original base number, while Ballard has suggested either $x = 5$, $x = 6$ or $x = 10$ based on the molecular phylogenetic relationships of the whole clade containing *Melanium* and its nearest sister groups (Ballard et al. 1999) Determination of which hypothesis is correct would depend on a robust phylogeny for *Melanium* species themselves and placement of certain ploidy levels within that phylogeny. Intra-specific variation of chromosome number has been reported (Küpfer 1971).

Hybridization is common throughout the genus and often leads to fertile derivatives (Stebbins et al. 1963, Ballard 1996, Erben 1996, Ko et al. 1998, Neuffer et al. 1999) in spite of ploidy differences. Many species from the section *Melanium* are believed to originate from hybridization events (Clausen 1927, Küpfer 1971, Erben 1996), which suggests potentially close relationships among species. One can wonder to what extent hybridization has driven the expansion of the section. Could hybridization combined with allopolyploidy and aneuploidy account for the high diversity in chromosome numbers? Reconstructing the cytological history of such a group, with a putatively reticulate evolution, is a challenge. A few attempts, based on chromosome numbers, have been made in the past (Clausen 1927, Küpfer 1971), but there is no comprehensive study of the section. The increasing sophistication of molecular markers offers the possibility to investigate the phylogenetic relationships of closely related species (Small et al. 1998, Wolfe and Randle 2001), to detect the hybrid origin of species (Mes et al. 1997, Morrell and Rieseberg 1998, Sang and Zhang 1999) and reticulate evolution (Rieseberg 1991, Sang et al. 1995) and to ultimately place cytogenetic evolution within a broader, independently derived context.

In this paper, we present a molecular phylogeny of the section *Melanium*. Two kinds of markers have been used for this purpose: Internal Transcribed Spacer (ITS) nuclear DNA sequences, which are routinely used for resolving phylogenetic relationships within genera (Yuan 1996, Compton et al. 1998, Torrell et al. 1999, Blattner et al. 2001), and Inter Simple Sequence Repeat (ISSR) markers, which have been used for assessing intraspecific as well as interspecific genetic diversity and potential hybridization (Salimath et al. 1995, Fang et al. 1998, Ge and Sun 1999, Joshi

et al. 2000, Martin and Sánchez-Yélamo 2000, Wolfe and Randle 2001). The phylogenetic position of species belonging to the groups *Xylinosium* and *Delphiniopsis* is examined, and we propose scenarios for cytological evolution in the section *Melanium*.

Materials and methods

Plant material and DNA isolation. Table 1 lists the 46 species studied and their taxonomic position according to Ballard et al. (1999). Section *Melanium* Ging. is represented by 25 species of 80–100 and sections *Delphiniopsis* and *Xylinosium* by 4 species; the rest of the species are used as outgroups to root the trees. The 25 *Melanium* species used in the study were selected to represent the geographic range of pansies. However, the number species included was limited by the accessibility to the plant material. A handful of species could not be amplified successfully for both approaches, but most taxa yielded data for both ITS sequences and ISSR markers. Specimens were obtained either in the field, from botanical gardens or from the herbarium of the University of Neuchâtel. Voucher specimens are deposited at University of Neuchâtel. Total DNA was extracted from either fresh or dried leaves, using the Dneasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions.

ITS sequencing. 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). PCR reactions were performed in a total volume of 50 µl containing 2 µl DNA, 0.32 µM of each primer, 140 µM dNTPs, 2.5 mM $MgCl_2$ and 1U *Taq* Polymerase (Promega, Madison, WI), in 1X buffer A (Promega, Madison, WI). We used a PTC-100 Thermal cycler (MJ-Research, Inc.) or, for some ITS sequences, a Perkin Elmer 2400 model, programmed for an initial step of 3 min at 94 °C, followed by 35 cycles of 40 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 QIAquick PCR purification kit (Qiagen) or the Promega Wizard PCR Preps kit and sequenced on both strands with the DNA sequencing Big Dye terminator kit (ABI Prism). Cycle sequencing products were run on an ABI Prism 3700 DNA Analyzer automated sequencer or a 310 capillary sequencer (PE Biosystems, Foster City, California, USA). Sequences were deposited in GenBank.

ISSR. The seven primers employed were based on SSR motifs ($(CA)_6$-GC, $(CT)_8$-AC, $(CT)_8$-TG, $(CA)_6$-AC, $(CT)_8$-GC, $(CA)_6$-AG and $(CA)_6$-GT) reported for flowering plants (Wolfe et al. 1998, Esselman et al. 1999). Reactions consisted of 1X *Taq* DNA polymerase buffer, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 1.3 µM primer, 2% Formamide, 1 µl DNA and 1U *Taq* DNA polymerase (Promega Corp.) in a total volume of 15 µl. Amplifications were performed in a PTC-100 thermal cycler (MJ Research, Inc.) using the following temperature profile: 90 s at 94 °C, 35 cycles [40 s at 94 °C, 45 s at 44 °C and 90 s at 72 °C] followed by 45 s at 94 °C, 45 s at 44 °C and 5 min at 72 °C. Amplification products were visualized on 1.5% agarose gels stained with ethidium bromide and photographed under UV light. Band sizes were estimated with the 200–10,000 bp Ladder and the 100–1000 bp Ladder (Smart Ladder, Eurogentec, Belgium).

Data analyses. ITS Sequences were aligned using Clustal X (Thompson et al. 1997) with final corrections made manually. The 5.8S coding region was excluded from the alignment. Phylogenetic analyses were performed with PAUP 4.0b8a* (Swofford 2001). Indel regions were scored as additional characters using Gapcoder (Young and Healy 2002). Maximum Parsimony analysis (MP) was performed using the heuristic search algorithm, with the MULTREES option on, TBR branch swapping, and 1000 replicates with random addition saving 100 trees per replicate. Bootstrapping (100 replicates) was done using the previous parameters. Jukes-Cantor pairwise distances were used for distance analysis with the Neighbor Joining (NJ) method. Maximum Likelihood analysis was performed using the heuristic search algorithm with TBR branch swapping, 10 replicates with random addition option and the following model of evolution: TIM + G, alpha shape parameter equal to 1.3044, estimated using Modeltest version 3.06 (Posada 2001).

The DNA fragments obtained using the ISSR primers were scored as present (1) or absent (0). Only fragments with a strong and clear signal were scored. Bands of identical size were assumed homologous across species sampled in this study. The 0/1 matrix was used to calculate a similarity

Table 1. List of species included in this study. Taxonomic classification and haploid chromosome number are given. Voucher data include primary collector or herbarium voucher are deposited. GenBank numbers are included. Extractions from herbarium material are indicated by an asterisk

Sections	Species	Species Distribution	n	Ref.	Voucher	GenBank Number
Melanium						
	Viola aetolica Boiss. & Heldr.	Greece, Albany, Yougoslavia	8	1	HR1	AY148225,
	Viola alpina Jacq.	NE Alps, Carpaths (Carinthia)	11	2	SN-0007	AY148245
	Viola arvensis Murr.	Europe	17	2	SN-0003	AY148226, AY148246
	Viola bertolonii De Salis	Corsica	20	2	HR3	AY148227, AY148247
	Viola calcarata L.	Alps, Balkans (Europe)	20	2, 4	SN-0005	AY148229, AY148249
	Viola cheiranthifolia Humb. & Bonpl.	Canary Isl. Tenerife	circa 32[c]	3	RBGE: 19841491	AY148231, AY148251
	Viola comollia Messara*	S Alps (Italy)	11	3	HIB 25831	AY148232, AY148252
	Viola cornuta L.*	Pyrénées (S Europe)	11	2, 4	HIB 03064	
	Viola corsica Nyman	Corsica, Sardinia	60, 26	2, 3	HR5	
	Viola dyris Maire*	Morocco	10, 11	2, 6	HIB 03336	
	Viola eugeniae Parl.	Appenines (Italy)	17[b]	2	HR7	AY148233, AY148253
	Viola gracilis Sibth. & Sm.	Greece	10[q]	17	SN-0004	AY148234, AY148254
	Viola kitaibeliana Roem. & Schultes	Europe, Asia, USA	7, 8, 12, 18, 24	2	SN-0008	AY148235, AY148255
	Viola lutea Hudson*	CE Europe, S-Pyrenees	24	2, 4	HIB 03032	AY148236, AY148256
	Viola magellensis Porta & Rigo ex Strobl.	Appenines, Greece, Albany	11	2	HIB?	AY148237, AY148257
	Viola minuta Bieb.*	Caucase	?		HIB 03456	
	Viola montcaunica Pau*	Spain	11	7	HIB 03024	
	Viola argenteria B. Moraldo & G.Forneris*	Alps, Corsica (Europe)	7[c]	3	HIB 03038	

Table 1 (continued)

	Viola oreades Bieb.*	Anatolia, Caucas, Crimea	?		HIB 03026	
	Viola palmensis Webb & Berth.	Canary Isl. La Palma	?		HR8	AY148239, AY148259
	Viola parvula Tineo*	S Europe, Syria	5	2.8	HIB 03025	AY148240, AY148260 AY148241, AY148261
	Viola perinensis W. Becker	Greece, Bulgaria (Macedonia)	10, 11	2, 1	HR9	
	Viola stojanowii W. Becker	Greece, Bulgaria (Macedonia)	13	1	SN-0001	
	Viola tricolor L.	Europe, North America	13	2, 4	SN-0010	AY148243, AY148263
	Viola valderia All.	Alps	10	2, 4	HIB 03056	AY148244, AY148264
Xylinosium						
	Viola arborescens L.	Mediterranean region	26	3	KEW 316-74.02529 - 10220	
	Viola saxifraga Maire	Marocco	26	4	HR10	
	Viola decumbens L.f.	South Africa				
Delphiniopsis						
	Viola cazorlensis Gand.	Spain	10	4, 5	SN-0006	AY148230, AY148250
Viola I – Outgroup						
	Viola rotundifolia Michx.	N America	6		Ballard et al. 1999	AF0972241, AF097287
Viola II – Outgroup						
	Viola blanda Brainerd	N America	22	14.15	Ballard et al. 1999	AF097238, AF097284
	Viola jalapaensis W. Becker	Mexico			Ballard et al. 1999	AF097235, AF097281
	Viola pinnata L.	Eurasia, N America	24	3	Ballard et al. 1999	AF097240, AF097286

Table 1 (continued)

Sections	Species	Species Distribution	n	Ref.	Voucher	GenBank Number
Viola III – Outgroup						
	Viola odorata L.	C Europe, S and N America, Asia	10	2	SN-0002	AY148238, AY148258
	Viola reichenbachiana Jordan ex Boreau	Europe W-Asia, Middle East, North Africa	10			AF097248, AF097294
	Viola riviniana Reichb.	Europe, Mediterranean region, Middle East	20		SN-0009	AY148242, AY148262
Dischidium – Outgroup						
	Viola biflora L.*	Boreal and Temperate Regions	6	2	HIB 03068	AY148228, AY148248
Chamaemelanium I – Outgroup						
	Viola barroetana Hemsley	Mexico			Ballard et al. 1999	AF097224, AF097270
	Viola pubescens Aiton	N America	6	13	Ballard et al. 1999	AF097225, AF097271
	Viola purpurea Kellog	N America, Baja California	6	11	Ballard et al. 1999	AF097229, F097275
	Viola sheltonii Torr.	N America	6	11	Ballard et al. 1999	AF097226, AF097272
	Viola vallicola A. Nelson	N America, Baja California	6	16	Ballard et al. 1999	AF097230, AF097276
Chamaemelanium II – Outgroup						
	Viola cuneata S. Watson	N America, N Mexico			Ballard et al. 1999	AF097234, AF097280
	Viola flagelliformis Hemsley	Mexico	6	13	Ballard et al. 1999	AF097233, AF097279

Table 1 (continued)

Leptidium – Outgroup						
	Viola scandens Willd. Ex Roem. & Schult.	Mesoamerica, West Indies, S America	27	12	Ballard et al. 1999	AF097221, AF097267
Chilenium – Outgroup		Greece, Albany, Yougoslavia				
	Viola reichei Skottsberg	Chili, Argentina			Ballard et al. 1999	AF097222, AF097268
Outgroup						
	Hybanthus concolor (T. F. Forst.) Spreng.	N America			Ballard et al. 1999	AF097218, AF097264

1: (Erben 1985); 2: (Bolkhovskikh et al. 1969); 3: (Merxmüller and Lippert 1977); 4: (Clausen 1927); 5: (Küpfer 1972); 6: (Favarger et al. 1979); 7: (Merxmüller 1974); 8: (Schmidt 1962); 9: (Galland 1985); 10: (Strid 1996); 11: (Clausen 1964); 12: (Damboldt and Phitos 1971); 13: (Clausen 1929); 14: (Gershoy 1932); 15: (Canne 1987); 16: (Davidse 1976); 17: (Tutin et al. 1968)
HR: Collection Heinz Rehfeld, D.-Erxleben Str.9 06484 Quedlinburg, Germany; RBGE: Royal Botanic Garden, Edinburgh, UK ; KEW: Royal Botanic Gardens, Kew, UK; HIB: Herbarium of Institute of Botany, University of Neuchâtel, Switzerland; SN: New samples deposited in the HIB; Ballard et al. 1999: Data used from Ballard et al. (1999)

matrix using the Dice coefficient (Salimath et al. 1995). A phenogram was constructed with UPGMA using NTSYS 1.8 (Rohlf 1999). An analysis of maximum parsimony was also done with a heuristic search using PAUP 4.0b8a (Swofford 2001). Robustness of the UPGMA and Maximum Parsimony trees was evaluated by a bootstrap analysis. Principal coordinates analysis (PCoA) was used to find relationships between ISSR markers and specimens without a priori division of the samples into discrete groups (Wiley 1981). PCoA was conducted under MatLab (version 5, The Math Works Inc.) using a Dice distance matrix (1 – Dice similarity coefficient).

We traced the distribution of haploid chromosome number on the Neighbor Joining ITS tree and the consensus ISSR cladogram (MP) using Mac Clade (Maddison and Maddison 1992).

Results

ITS. The alignment of ITS1 and ITS2 sequences consisted of 510 positions (ITS1: 293 positions and ITS2: 217 positions). 127 indels were coded as additional characters. This alignment included the sequences from 20 individuals from the section *Melanium* (representing 17 species), from 19 species representing the other sections of the genus and from *Hybanthus concolor* (Violaceae), used as an outgroup (Table 1). 227 sites were parsimony informative (35.5%). The parsimony informative positions were reduced to 14 when only species from section *Melanium* were included, revealing highly reduced genetic divergence within the pansies.

The heuristic search generated 8300 most parsimonious trees of 875 steps with a consistency index of 0.644 and a retention index of 0.752, indicating a moderate level of homoplasy. The strict consensus tree resulted in a largely unresolved polytomy for the pansies, but their monophyletic origin is supported with a 100% bootstrap value (Fig. 1). The species representing the sections *Delphiniopsis* and *Xylinosium* are located apart from the *Melanium* species. *Viola cazorlensis* (section *Delphiniopsis*) appears as sister group to *Melanium* in all analyses, although this relation-

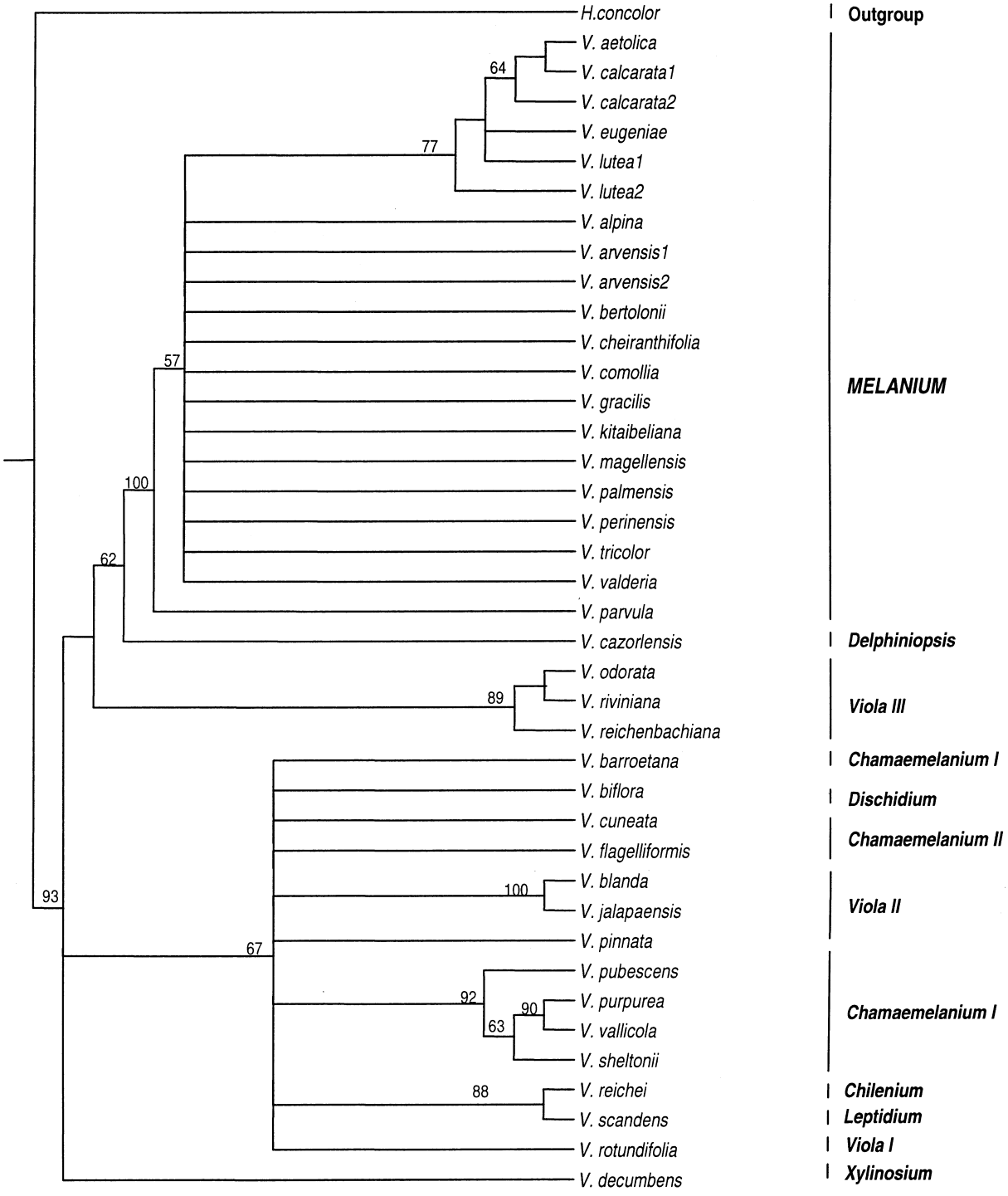

Fig. 1. Strict consensus tree of 8300 most parsimonious trees resulting from a heuristic search with TBR, random addition sequence (nreps = 1000) and MulTrees ON, based on a data set of ITS sequences. Bootstrap values higher than 50% are indicated above branches. Sections are according to (Ballard et al. 1999)

ship is only moderately supported in the Maximum Parsimony tree (bootstrap value of 62%). In both distance (NJ) and Maximum Likelihood analyses (not shown), *Xylinosium* is sister group to a clade comprising *Melanium*, *Delphiniopsis* and *Viola*.

The level of ITS sequence divergence within section *Melanium* was estimated using uncorrected pairwise distance and compared to other sections of *Viola*. Significantly lower values were found for the pansy group (mean value = 0.023) compared to related sections (0.032 to 0.092) and subsections (0.051 to 0.068) (Kolmogorov-Smirnov test, $p < 0.01$ in SAS System).

ISSR. ISSR data from 7 primers were obtained for 32 species (3 from section *Viola*, 1 from sections *Dischidium* and *Delphiniopsis*, 2 from section *Xylinosium* and 25 from section *Melanium*). Species from sections other than *Melanium* were used as outgroups. We selected primers that displayed interspecific variation but no or little intraspecific variation.

A total of 177 polymorphic fragments ranging from 230 to 1556 bp were scored. We scored 27 bands for the primer $(CA)_6$-GC, 15 bands for $(CT)_8$-AC, 20 bands for $(CT)_8$-TG, 33 bands for $(CA)_6$-AC, 24 bands for $(CT)_8$-GC, 33 bands for $(CA)_6$-AG and 25 bands for $(CA)_6$-GT. 84% of the 177 characters consisted of bands shared by at least two species, and were therefore parsimony informative. sixty-six bands were found exclusively in species belonging to Melanium and were therefore autapomorphies of this section, although none of the bands were shared by all 25 pansies. The smallest pairwise similarities were found among outgroups, ranging between 0 and 0.355. Values ranged between 0.127 and 0.759 among pansies, the highest similarity being between *V. cheiranthifolia* and *V. palmensis*, from the Canary Islands. Whatever method was used for phylogenetic inference, MP (Fig. 2) or distance (not shown), all species belonging to *Melanium* were clustered together and all the species chosen as outgroups were basal to *Melanium*. *Viola arborescens* and *V. saxifraga* were grouped together, according to their placement in section *Xylinosium* (Galland 1998) Within *Melanium*, the tree topology shows a basal group of 5 species including *V. parvula*, confirming the result obtained with the ITS sequence data for this species (Fig. 1). The rest of the species are distributed in three main cluster. One regroups 4 species widely distributed in Europe (*V. arvensis, V. lutea, V. cornuta* and *V. tricolor*). Another cluster regroups 7 more narrowly endemic species (*V. stojanowii*, *V. bertolonii*, *V. corsica*, *V. calcarata*, *V. perinensis*, *V. cheiranthifolia* and *V. palmensis*) found in Southern Europe or the Canary Islands. The position of *V. aetolica*, *V. eugeniae*, *V. gracilis* and *V. alpina* varies between distance and MP analyses. The principal coordinates analysis (Fig. 3) supports the inclusion of these 4 species in the two cluster above mentioned, as found in the MP analysis. *Viola kitaibeliana* has an intermediate position between this group and the more basal species.

Chromosome numbers. Chromosome number evolution was reconstructed with MacClade using the MP tree based on ISSR data (Fig. 4). It gives $x = 7$ as a hypothetical base chromosome numbers for the section *Melanium*. Reconstruction using the NJ tree based on ITS data gives $x = 5$. The number $x = 11$ appears to be the ancestral state for the most derived groups of *Melanium*, which display the highest diversity of chromosome numbers.

Discussion

For sorting out phylogenetic relationships in a group of closely related plants, the most widely used markers are ITS and non-coding chloroplast regions such as *trn*L-F (Gielly and Taberlet 1994, Gielly et al. 1996, Johansson 1998, Fukuda et al. 2001). As a database of ITS sequences of *Viola* species was already available, we chose to start our investigation of phylogenetic relationships in section *Melanium* using ITS sequences, in the hope of finding phylogenetic structure in the section and identifying clusters of species. Based on our results, section *Melanium*, which consists of all

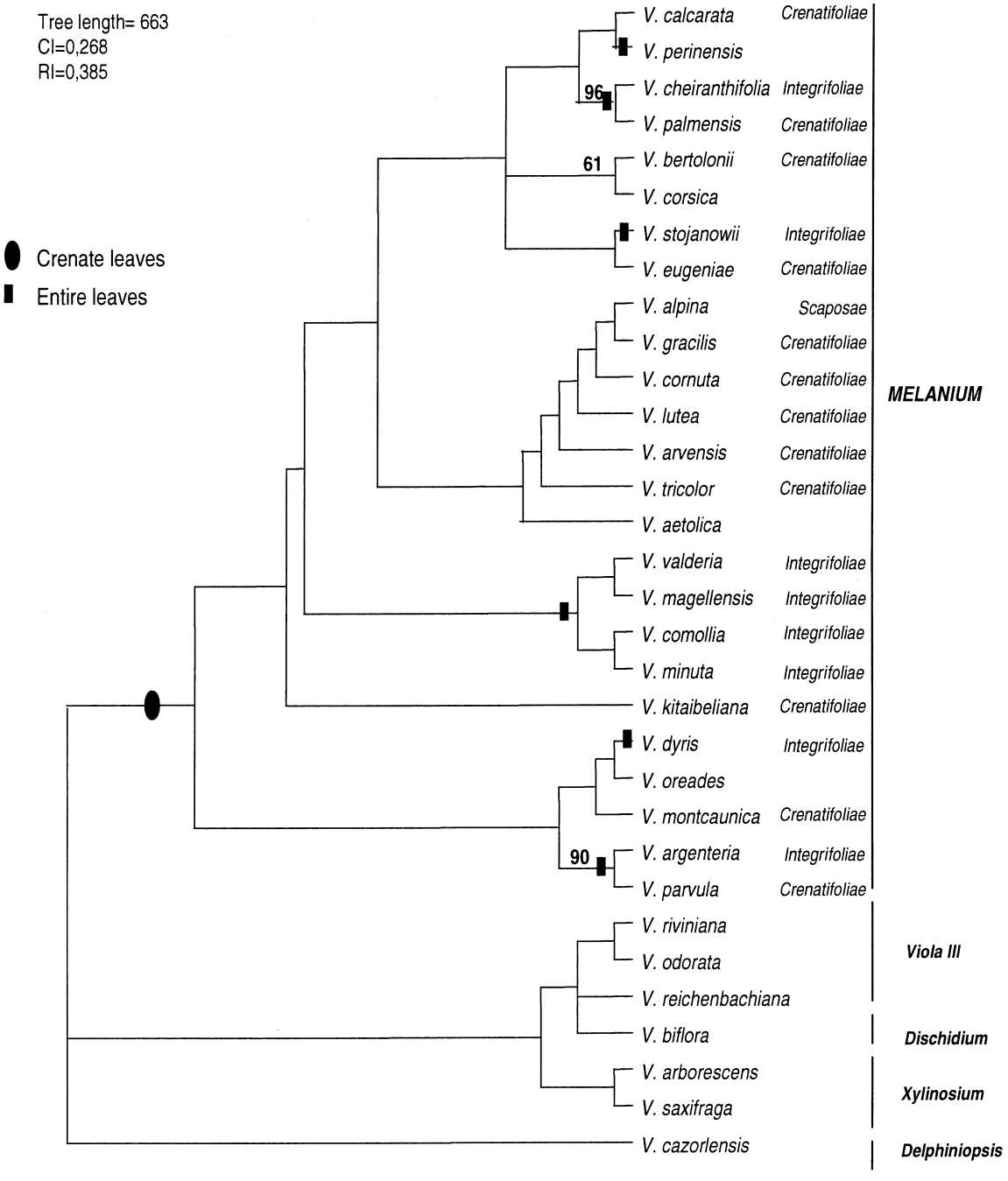

Fig. 2. Strict consensus of 4 trees resulting from a heuristic search based on ISSR data from 25 *Melanium* species and 7 outgroup species. Bootstrap values higher than 50% are indicated above branches. Sections in bold are according to Ballard et al. (1999), divisions following species names are according to Melchior (1960)

the species considered as "pansies", is clearly monophyletic, as shown by the strong bootstrap support. It confirms that the orientation of lateral petals, used as a criterion for distinguishing between pansies and violets, is a synapomorphy of pansies. This particular shape of flower is encountered in only one other complex species of violets, the circumboreal *V. biflora* species complex. ITS sequence data confirm morphological classifi-

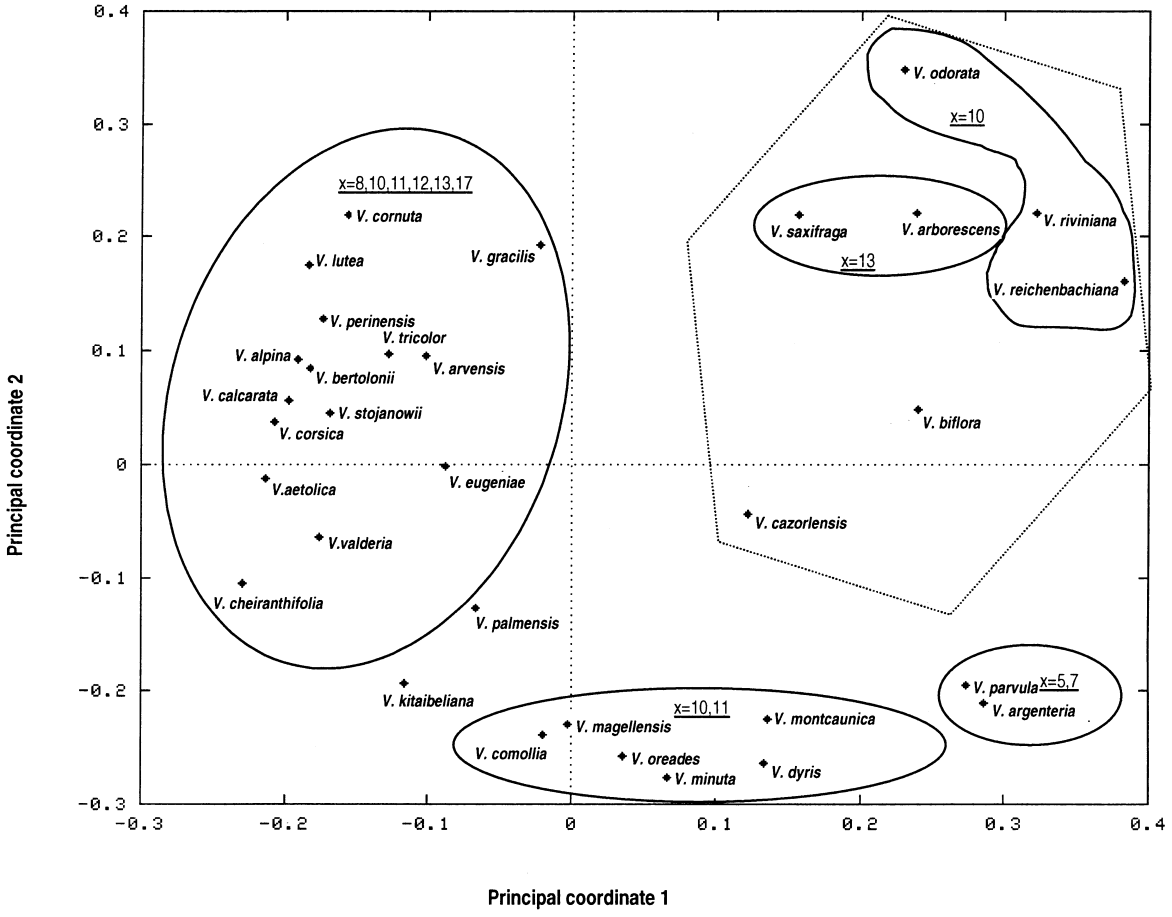

Fig. 3. Principal Coordinates Analysis (PCoA) plot based on ISSR data. The two principal coordinates account for an accumulate variation of 21.2%. Chromosome numbers (n) are indicated

cations, which consider this feature as a convergence: this species is placed in section *Dischidium*, not directly related to section *Melanium* (Fig. 1). Another morphological synapomorphy of pansies is the size of pollen grains, which was measured for a sample of the species included in this study. Pollen grains are significantly larger in section *Melanium* than in outgroups (mean length 56 μm within *Melanium*, 33 μm for outgroups; T test $p < 0.01$). It can be noted that the size of pollen in sections *Xylinosium* and *Delphiniopsis* is not significantly different from the size of pollen in the other outgroups which confirms the distinction between section *Melanium* and sections *Xylinosium* and *Delphiniopsis*.

However, the alignment of ITS1 + ITS2 sequences revealed very little variation among pansy species. The number of nucleotide differences between species ranges from 0 to 16 (0 to 4.3%). In fact, intraspecific variation evaluated by the sequences of two individuals for *V. lutea* (3 differences), *V. arvensis* (2 differences) and *V. calcarata* (6 differences), was comparable to interspecific variation. The large polytomy observed in the tree presented in Fig. 2 shows a complete lack of resolution. Consequently, whereas ITS sequence data proved useful to clarify relationships among sections (Compton et al. 1998, Esselman et al. 1999, Torrell et al. 1999, Bell and Patterson 2000), it clearly offers no help within section *Melanium*. Chloroplast regions are now used routinely for infrageneric phylogenetic studies (Gielly and Taberlet 1994, Gielly et al. 1996, Johansson 1998, Wang et al. 1999). The

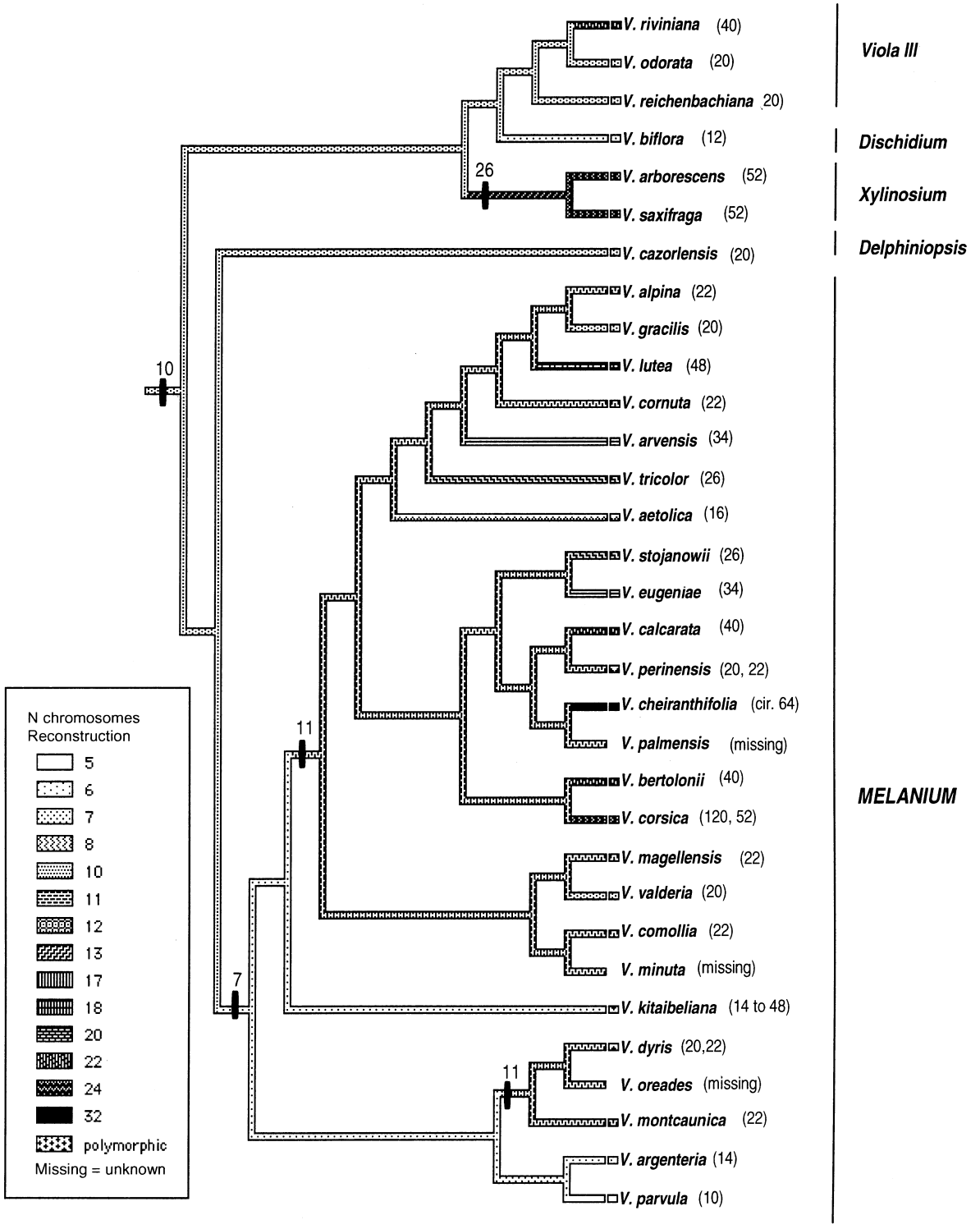

Fig. 4. Reconstruction of the evolution of chromosomal number using Equivocal Cycling option of MacClade 3.08 (Maddison and Maddison 1992), using the ISSR MP tree. First of 684 most parsimonious reconstructions. Branch numbers are haploid (meiotic) chromosome numbers, whereas those in parentheses following species names are diploid (sporophytic) numbers

potential variability of the chloroplast genome within *Melanium* was evaluated by using the PCR-RFLP technique. Two regions, *rpo*C1 and *trn*H-*trn*K, were amplified and digested with an array of 4-base and 6-base cutting enzymes (data not shown). No variation at all was detected among pansies, indicating a high degree of sequence identity. Instead of sequencing very long regions, with limited chances to find variation, our choice was to use ISSR markers. These markers have a level of variability similar to or greater than that of RAPDs, but do not generally suffer as much from the problem of reproducibility. They have been used mostly for assessing genetic diversity among populations (Esselman et al. 1999, Gilbert et al. 1999, Camacho and Liston 2001) but they have also been used successfully for studying phylogenetic relationships and potential hybridization and introgression among closely related species (Fang et al. 1998, Joshi et al. 2000, Wolfe and Randle 2001).

Although most nodes on the distance or the MP trees (Fig. 2) are not supported by high bootstrap values, it can be noted that the general clustering pattern of species is similar in both trees and in the PCoA (Fig. 3). None of the classifications of the section proposed previously are in agreement with our results (Drabble 1909, Shishkin 1949, Melchior 1960), suggesting a high level of homoplasy in morphological distinctions of subsections. *Melanium* was divided in two groups by Melchior (1960): *Scaposae* (absence of above-ground stem) and *Elongatae* (presence of above-ground stem), the latter being subdivided again in *Integrifoliae* (entire leaves) and *Crenatifoliae* (crenate leaves). Our tree (Fig. 2) does not support this classification. Most pansies have crenate leaves and the few species with entire leaves have evolved independently. The only species representing the group *Scaposae*, *V. alpina*, appears derived from *Elongatae*. The occurrence of hybridization events could affect the morphological characters as the leaf form leaving the phylogenetic value of these type of character very questionable. Additionally, the ISSR results do not follow a geographic pattern: no clear correlation appears in the trees between species relationship and geographic distribution. Although the ISSR markers bring valuable information about the relationships among pansies, the interpretation could be complex if *Melanium* species have undergone a reticulated evolution. In this case, introgression events could affect the pattern of molecular markers in species originated by hybridization. To confirm the occurrence of reticulation processes during the evolutionary history of *Melanium*, it will be necessary to conduct a thorough molecular analysis in order to find specific markers to identify the hypothetical parental species and verify the co-occurrence of these markers in species formed by hybridization.

Some remarks can be made about chromosomal evolutionary pattern. In a paper discussing the role of hybridization in forming species in *Melanium*, Erben (1996) suggested that this section would have evolved from an ancestor with $x = 11$, with decreasing and increasing dysploidy taking place through structural changes. Our results do not contradict this hypothesis, since a paraphyletic group of species with $2n = 22$ is located near the base of the section. The number $2n = 20$ would be derived from $2n = 22$ by the fusion of 2 chromosomes, forming a large metacentric chromosome (Erben 1996). Under such a scenario of evolution, the low chromosome numbers of *V. parvula* ($2n = 10$) and *V. argenteria* ($2n = 14$) would be interpreted as derived, resulting from decreasing dysploidy. However, our reconstruction of the chromosome number evolution suggest that the base number of the section is either $x = 5$ using the ITS tree or $x = 7$ using the ISSR tree. The derived chromosome number $x = 11$ would be then the result of hybridization process between species with $x = 7$ and $x = 5$ and further fusion of chromosomes. Under this scenario, *V. parvula* (and possibly also *V. argenteria*) is wrongly positioned in some of the ISSR trees, and is in fact sister group to a clade including all other *Melanium* species, as confirmed by the ITS

data set. Pollen morphology also supports this scenario. Both *V. parvula* and *V. argenteria* have smaller pollen grains than the remaining species of the section, and pollen grains are predominantly 3-aperturate, like violets, whereas all other pansies examined produce mostly 4-aperturate pollen grains. These two species could then be representative of a sister group of section *Melanium* in which decreasing dysploidy would have happened. Further addition of species and use of other types of DNA data, would be interesting in order to examine more closely the phylogenetic position of *Melanium* species with low base numbers.

In the ISSR tree, the most derived group of pansies shows extremely variable chromosome numbers, ranging from $2n = 16$ to 120, and no clear pattern of evolution appears from the tree (Fig. 4). It has been suggested that pansies have undergone a reticulate evolution (Küpfer 1971, Erben 1996): indeed, crosses between species are easy in pansies, even with different chromosome numbers, and often give fertile hybrids. This phenomenon would explain the weakly supported relationships among pansies. It would also explain why most species of pansies display pollen heteromorphism: this phenomenon has been shown to arise as a consequence of polyploidization (Bronckers 1963, Mignot et al. 1994, Nadot et al. 2000) which is often associated with hybridization. Polyploidization and/or hybridization could also account for the larger size of pollen grains in pansies, as it has been shown that hybridization can affect pollen morphology (Chaturvedi et al. 1999) The low level of genetic differentiation revealed by the ITS analysis could be interpreted as resulting from explosive and quite recent radiation (Hodges and Arnold 1994, Givnish and Sytsma 1997, Yuan and Küpfer 1997, Ainouche and Bayer 1999, Hahn and Sytsma 1999, Blattner et al. 2001). When examining different sections and subsections of *Viola*, pansies appear to have the lowest level of genetic differentiation of ITS (0.023%), even compared to the most derived group in the genus, namely the Hawaiian violets of section *Nosphinium* (0.032%) (Ballard et al. 1999, Ballard and Sytsma 2000). Their sequence identity is therefore likely to reflect an explosive radiation, and not simply a recent origin. The apparition of a key innovation, such as the different orientation of lateral petals perhaps coupled with lateral petal trichomes, corolla color differentiation and modification of the shape, could have triggered the diversification of section *Melanium*, the changes in flower morphology allowing them to access a broader array of pollinators compared to the "ancestral" types (Hodges and Arnold 1994).

The molecular study presented here is only preliminary and further work is needed in order to understand the evolution of pansies. To clarify conclusively the relationships among pansies, it would be helpful to increase the ISSR data set by adding extra species or using markers such as Amplified Fragment Length Polymorphisms (AFLP's). In addition, such a complex reticulate evolution will be better understood by using cytogenetic methods such as Fluorescent or Genomic In Situ Hybridization, by conducting artificial hybridizations and observing meiotic behavior of both synthetic and putative hybrids, and by comparison of data from biparentally and uniparentally inherited genomes (e.g. nuclear and chloroplast simple sequence repeats).

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References

Ainouche A. K., Bayer R. J. (1999) Phylogenetic relationships in *Lupinus* (Fabaceae: Papilionoideae) based on internal transcribed spacer (ITS)

of nuclear ribosomal DNA. Amer. J. Bot. 86: 590–607.

Ballard H. E. (1996) Phylogenetic relationships and infrageneric groups in *Viola* (Violaceae) based on morphology, chromosome numbers, natural hybridization and Internal Transcribed Spacer (ITS) sequences. PhD Thesis, University of Wisconsin, Maddison.

Ballard H. E., Sytsma K. J. (2000) Evolution and biogeography of the woody hawaiian violets (*Viola*, Violaceae): arctic origins, herbaceous ancestry and bird dispersal. Evolution 54(5): 1521–1532.

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 DNA sequences. Syst. Bot. 23(4): 439–458.

Beattie A. J. (1974) Floral evolution in *Viola*. Ann. Missouri Bot. Gard. 61: 781–793.

Becker W. (1925) *Viola*. In: Engler A., Prantl K. (eds.) Die natürlichen Pflanzenfamilien. Verlag von Wilhelm Engelmann, Leipzig, Germany, pp. 363–376.

Bell C. D., Patterson, R. W. (2000) Molecular phylogeny and biogeography of *Linanthus* (Polemoniaceae). Amer. J. Bot. 87(12): 1857–1870.

Blattner F. R., Weising K., Bänfer G., Maschwitz U., Fiala B. (2001) Molecular analysis of phylogenetic relationships among myrmecophytic *Macaranga* species (Euphorbiaceae). Mol. Phylogent. Evol. 19(3): 331–344.

Bolkhovskikh Z., Grif V., Matvejeva, T., Zakharyena O. (1969) Chromosome numbers of flowering plants. Leningrad.

Bronckers F. (1963) Variations polliniques dans une série d'autopolyploïdes artificiels d'*Arabidopsis thaliana* (L.) Heyhn. Pollen Spores 5: 233–238.

Camacho F. J., Liston A. (2001) Population structure and genetic diversity of *Botrychium pumicola* (Ophioglossaceae) based on inter-simple sequences repeats (ISSR). Amer. J. Bot. 88(6): 1065–1070.

Canne J. M. (1987) Determinations of chromosome numbers in *Viola* (Violaceae). Canad. J. Bot. 65: 653–655.

Chaturvedi M., Datta K., Pal M. (1999) Pollen anomaly – a clue to natural hybridity in *Argemone* (Papaveraceae). Grana 38(6): 339–342.

Clausen J. (1927) Chromosome number and the relationship of species in the genus *Viola*. Ann. Bot. 41(156): 677–714.

Clausen J. (1929) Chromosome number and the relationship of some North American species of *Viola*. Ann. Bot. 63: 741–764.

Clausen J. (1964) Cytotaxonomy and distributional ecology of western North American violets. Madroño 17: 173–204.

Clausen J., Channell R. B., Nur U. (1964) *Viola rafinesquii*, the only *Melanium* violet native to North America. Rhodora 66: 32–46.

Compton J. A., Culham A., Gibbings J. G., Jury S. L. (1998) Phylogeny of *Actaea* including *Cimicifuga* (Ranunculaceae) inferred from nrDNA ITS sequence variation. Biochem. Syst. Ecol 26: 185–197.

Damboldt J., Phitos D. (1971) IOPB Chromosome number reports 34. Taxon 20: 785–797.

Davidse G. (1976) A study of some intermountain violets (*Viola*, sect. *Chamaemelanium*). Madroño 23: 274–283.

Drabble E. (1909) The British pansies. J. Bot. 47: 1–32.

Erben M. (1985) Cytotaxonomische Untersuchungen an südosteuropäischen *Viola* – Arten der Sektion *Melanium*. Mitt. Bot. Staatssamm. München 21: 339–740.

Erben M. (1996) The significance of hybridization on the forming of species in the genus *Viola*. Bocconea 5: 113–118.

Esselman E. J., Jianqiang L., Crawford D. J., Winduss J. L., Wolfe A. D. (1999) Clonal diversity in the rare *Calamagostris porteri* ssp. *insperata* (Poaceae): comparative results for allozymes and random amplified polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR) markers. Molec. Ecol. 8: 443–451.

Fang D., Krueger R. R., Roose M. L. (1998) Phylogenetic relationships among selected *Citrus* germplasm accessions revealed by Inter-Simple Sequence Repeat (ISSR) markers. J. Amer. Soc. Hort. Sci. 123(4): 612–617.

Favarger C., Galland N., Küpfer P. (1979) Recherches cytotaxonomiques sur la flore orophile du Maroc. Nat. Monspel., Ser. Botanique 29: 1–64.

Fukuda T., Yokoyama J., Ohashi H. (2001) Phylogeny and biogeography of the genus *Lycium* (Solanaceae): inferences from chloroplast DNA sequences. Mol. Phylogenet. Evol. 19(2): 246–258.

Galland N. (1985) IOPB Chromosome Number Reports 87. Taxon 34: 346–351.

Galland N. (1998) Recherche sur l'origine de la flore orophile du Maroc: étude caryologique et cytogéographique. Travaux de l'Institut Sci., Univ. Mohammed V.; Séries Botanique 35: 1–168.

Ge X. J., Sun M. (1999) Reproductive biology and genetic diversity of a cryptoviviparous mangrove *Aegiceras corniculatum* (Myrsinaceae) using allozyme and intersimple sequence repeat (ISSR) analysis. Mol. Ecol. 8: 2061–2069.

Gershoy A. (1932) Descriptive notes for *Viola* exhibits. I. General considerations, Vermont Agricultural Experiment Station Publication.

Gielly L., Taberlet P. (1994) Chloroplast DNA polymorphism at the intrageneric level and plant phylogenies. Compt. Rend. Acad. Paris 317: 685–692.

Gielly L., Yuan Y., Küpfer P., Taberlet P. (1996) Phylogenetic use of noncoding regions in the genus *Gentiana* L.: chloroplast *trn*L (UAA) intron versus nuclear ribosomal internal transcribed spacer sequences. Mol. Phylogent. Evol. 5(3): 460–466.

Gilbert J. E., Lewis R. V., Wilkinson M. J., Caligari P. D. S. (1999) Developing an appropriate strategy to assess genetic variability in plant germplasm collections. Theor. Appl. Genet. 98: 1123–1131.

Givnish T. J., Sytsma K. J. (1997) Molecular evolution and adaptive radiation. New York, NY, Cambridge University Press.

Hahn W. J., Sytsma K. J. (1999) Molecular systematics and biogeography of the Southeast Asian genus *Caryota* (Palmae). Syst. Bot. 24(4): 558–580.

Herrera C. M. (1993) Selection on floral morphology and environmental determinants of fecundity in a hawkmoth-pollinated violet. Ecol. Monogr. 63: 251–275.

Hodges S. A., Arnold M. L. (1994) Columbines: A geographically widespread species flock. Proc. Natl. Acad. Sci. USA 91: 5129–5132.

Johansson J. T. (1998) Chloroplast DNA restriction site mapping and the phylogeny of *Ranunculus*. Plant Syst. Evol. 213: 1–19.

Joshi S. P., Gupta V. S., Aggarwal R. K., Ranjekar P. K., Brar D. S. (2000) Genetic diversity and phylogenetic relationships as revealed by inter simple sequence repeat (ISSR) polymorphism in the genus *Oryza*. Theor. Appl. Genet. 100: 1311–1320.

Knuth P. (1908) Handbook of flower pollination Vol II. London, Clarendon Press.

Ko M. K., Yang J., Jin Y. H., Lee C. H., Oh B. J. (1998) Genetic relationships of *Viola* species evaluated by random amplified polymorphic DNA analysis. J. Horticult. Sci. Biotech 73(5): 601–605.

Küpfer P. (1971) Contribution à l'étude cytologique et phylogénétique de la section *Melanium* Ging. du genre *Viola* L. Compt. Rend. Acad. Sci.; Série D 272: 1085–1088.

Küpfer P. (1972) Cytotaxonomie et cytogéographie de quelques groupes d'orophytes du bassin occidental de la Méditerranée et des Alpes. Compt. Rend. Hebdo. Séances Acad. Sci.; Séries D 275: 1753–1756.

Maddison W. P., Maddison D. R. (1992) Mac Clade: analysis of phylogeny and character evolution. Sinauer, Sunderland, Mass.

Martin J. P., Sánchez-Yélamo M. D. (2000) Genetic relationships among species of the genus *Diplotaxis* (Brassicaceae) using inter-simple sequence repeat markers. Theor. Appl. Genet. 101: 1234–1241.

Melchior H. (1960) Violaceae. In: Engler A., Prantl K. (eds.) Die natürlichen Pflanzenfamilien. Duncker and Humblot, Berlin.

Merxmüller H. (1974) Veilchenstudien I-I *Viola*. Phyton 16: 137–158.

Merxmüller H., Lippert W. (1977) Veilchenstudien V-VII. Mitt. Bot. Staatssamml. München 13: 503–534.

Mes T. H. M., Wijers G. J., Hart H. (1997) Phylogenetic relationships in *Monanthes* (Crassulaceae) based on morphological, chloroplast and nuclear DNA variation. J. Evol. Biol. 10(2): 193–216.

Mignot A., Hoss C., Dajoz I., Leuret C., Henry J. P., Dreuillaux J. M., Heberle-Bors E., Till-Bottraud I. (1994) Pollen aperture polymorphism in the angiosperms: importance, possible causes and consequences. Acta Bot. Gallica 141: 109–122.

Morrell P. L., Rieseberg L. H. (1998) Molecular tests of the proposed diploid hybrid origin of *Gilia achilleifolia* (Polemoniaceae). Amer. J. Bot. 85(10): 1439–1453.

Nadot S., Ballard H. E., Creach J. B., Dajoz I. (2000) The evolution of pollen heteromorphism

in *Viola*: a phylogenetic approach. Plant Syst. Evol. 223: 155–171.

Neuffer B., Auge H., Mesch H., Amarell U., Brandl R. (1999) Spread violets in polluted pine forest: morphological and molecular evidence for the ecological importance of interspecific hybridization. Molec. Ecol. 8: 365–377.

Posada D. (2001) ModelTest. Department of Zoology Brigham Young University, Provo USA.

Rieseberg L. H. (1991) Homoploid reticulate evolution in *Helianthus* (Asteraceae): Evidence from ribosomal genes. Amer. J. Bot. 78(9): 1218–1237.

Rohlf J. (1999) NTSYS-pc version 1.80. Applied Biostatistics.

Salimath S. S., deOliveira A. C., Godwin I. D., Bennetzen J. L. (1995) Assessment of genome origins and genetic diversity in the genus *Eleusine* with DNA markers. Genome 38(4): 757–763.

Sang T., Crawford D. J., Stuessy T. F. (1995) Documentation of reticulate evolution in paeonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA – Implications for biogeography and concerted evolution. Proc. Natl. Acad. Sci. USA 92(15): 6813–6817.

Sang T., Zhang D. (1999) Reconstructing hybrid speciation using sequences of low copy nuclear genes: hybrid origins of five *Paeonia* species based on *Adh* gene phylogenies. Syst. Bot. 24(2): 148–163.

Schmidt A. (1962) Eine neue Grundzahl in der Gattung *Viola* (Zytotaxonomische Untersuchungen an *Viola parvula* Tin. und *Viola occulta* Lehm.). Ber. Deutsch. Bot. Ges. 75: 78–84.

Shishkin B. K. (1949) Flora of the USSR. Vol. XV. Malvales, Parietales, Myrtiflorae, Academy of Science of the USSR.

Small R. L., Ryburn J. A., Cronn R. C., Seelanan T., Wendel J. F. (1998) The tortoise and the hare: choosing between noncoding plastome and nuclear *Adh* sequences for phylogeny reconstruction in a recently diverged plant group. Amer. J. Bot. 85(9): 1301–1315.

Stebbins G. L., Harvey B. L., Cox E. L., Rutger J. N., Jelencovic G., Yagil E. (1963) Identification of the ancestry of an amphidiploid *Viola* with the aid of paper chromatography. Amer. J. Bot. 50(8): 830–839.

Strid A. (1996) Mountain flora of Greece vol 1. Cambridge, Cambridge UP.

Swofford D. L. (2001) PAUP*. Phylogenetic Analysis Using Parsimony and other methods. Sinauer Assoc., Sunderland, MA.

Thompson J. D., Gibson T. J., Plewnoak F., Jeanmougin F., Higgins D. G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24: 4876–4882.

Torrell M., Garcia-Jacas N., Alfonso S., Vallès J. (1999) Phylogeny of *Artemisia* (Asteraceae, Anthemideae) inferred from nuclear ribosomal DNA (ITS) sequences. Taxon 48: 721–736.

Valentine D. H., Merxmüller H., Schmidt A. (1968) *Viola* L. In: Tutin T. G., Heywood V. H., Burges N. A., Moore D. M., Valentine D. H., Walters S. M., Webb D. A. (eds.) Flora Europaea, Vol 2. (Rosaceae to Umbelliferae). Cambridge University Press, Cambridge.

Wang X., Tsumura Y., Yoshimaru H., Nagasaka K., Szmidt A. E. (1999) Phylogenetic relationships of Eurasian pines (*Pinus*, Pinaceae) based on chloroplast *rbc*L, *mat*K, *rpl*20-*rps*18 spacer, and *trn*V intron sequences. Amer. J. Bot. 86(12): 1742–1753.

Wiley E. O. (1981) Phylogenetics: The theory and practice of phylogenetic systematics. New York, Wiley.

Wolfe A., Randle C. P. (2001) Relationships within and among species of the holoparasitic genus *Hyobanche* (Orobanchaceae) inferred from ISSR banding patterns and nucleotide sequences. Syst. Bot. 26(1): 120–130.

Wolfe A. D., Xiang Q., Kephart S. R. (1998) Assessing hybridization in natural populations of *Penstemon* (Scrophulariaceae) using hypervariable intersimple sequence repeat (ISSR) bands. Mol. Ecol. 7: 1107–1125.

Young N. D., Healy J. (2002) Gap Coder: A computer program for including indels in phylogenetic analysis. http://www.trinity.edu/nyoung/GapCoder, Trinity University Biology Dept.

Yuan Y. (1996) Infrageneric phylogeny of the genus *Gentiana* (Gentianaceae) inferred from nucleotide sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA. Amer. J. Bot. 83(5): 641–652.

Yuan Y., Küpfer P. (1997) The monophyly and rapid evolution of *Gentiana* sect. *Chondrophyllae* Bunge s.l. (Gentianaceae) Evidence from the nucleotide sequences of the internal transcribed

spacers of nuclear ribosomal DNA. Bot. J. Linn. Soc. 123: 25–43.

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