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Intraspecific Variation in *Viola suavis* in Europe: Parallel Evolution of White-flowered Morphotypes

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• *Background and Aims* *Viola* species are commonly grown for their ornamental flowers, but their evolutionary history and taxonomy are often complicated and have been poorly explored so far. This is a study of the polymorphic, typically blue-flowered species *Viola suavis*, concentrating on the white-flowered populations of uncertain taxonomic assignment that occur in Spain and central and south-eastern Europe. The aim was to resolve their origin and taxonomic status and to study the intraspecific structure and (post)glacial history of this species.
• *Methods* *Viola suavis* and five close relatives were sampled from multiple locations and subjected to molecular (AFLP, sequencing of nrDNA ITS) and morphometric analyses. Data on ploidy level and pollen fertility were also obtained, to address an assumed hybrid origin of the white-flowered populations.
• *Key Results* In *V. suavis* a strong intraspecific genetic split into two groups was observed, indicating that there has been a long-term isolation and survival in distinct glacial refugia. The white-flowered populations could be placed within the variation range of this species, and it is clear that they evolved independently in two distant areas. Their parallel evolution is supported by both morphological and genetic differentiation. The strongly reduced genetic variation and absence of unique AFLP fragments suggest their derived status and origin from the typical, blue-flowered populations.
• *Conclusions* These results suggest that intraspecific variation in *V. suavis* has been largely shaped by population isolations during the last glaciation and subsequent recolonizations, although cultivation and vegetative spread by humans have affected the present picture as well.

Key words: AFLP, central Europe, flow cytometry, ITS sequences, multivariate morphometrics, parallel evolution, Spain, Violaceae.

INTRODUCTION

Species groups with complicated phylogenetic relationships, evolutionary history and intraspecific variation are not rare among vascular plants. Due to the ambiguity of the variation patterns observed, taxa circumscription and delimitation are often surrounded by controversy, and sound taxonomic concepts are difficult to establish. Studies undertaken from the species-level perspective have actually become much more attractive and challenging since the use of molecular markers. They provide insight into the species- and population-level patterns and processes that allow us to study evolutionary radiation and speciation (Bakker *et al.*, 2005). Neverteless, species-level studies cope with a scarcity of appropriate genetic markers. Even with fast-evolving loci, there is often a limited phylogenetic signal and a lack of resolution. The nuclear ITS sequence data and non-coding cpDNA regions have proven valuable, providing enough resolution in closely related taxa (Shaw *et al.*, 2005, 2007; Mort *et al.*, 2007). Nevertheless, in some cases, insufficient variation in DNA sequences is found or the pattern is blurred due to the concerted evolution acting on ITS sequences. Potential alternatives to DNA sequence data are isozyme variation (see, for example, studies in *Viola* by Marcussen and Borgen, 2000) and fingerprinting techniques, such as AFLPs (amplified fragment length polymorphisms; Bussel *et al.*, 2005; Koopman, 2005). Although the AFLPs are not devoid of problems (see Bonin *et al.*, 2004; Archibald *et al.*, 2006), several studies have documented their strengths for investigating variation at and below the species level, mainly because of their multilocus character and assumed genome-wide distribution (e.g. Lihová *et al.*, 2003, 2007; Pelser *et al.*, 2003; Martínez-Ortega *et al.*, 2004; Cieślak *et al.*, 2007; Greimler and Jang, 2007; Pimentel and Sahuquillo, 2007).

The focus of the present study is on *Viola suavis* M. Bieb. (Violaceae), a highly polymorphic and taxonomically critical species. It has been classified in *Viola* sect. *Viola* subsect. *Viola*, which is a Eurasian group of about 25 species with both tetraploids ($2n = 20$) and octoploids ($2n = 40$) represented (see, for example, Marcussen and Borgen, 2000; Mered'a *et al.*, 2006; Hodálová *et al.*, 2008). Taxa of this subsection are well known for their taxonomic complexity, which is apparently caused by several factors: (*a*) scarcity of reliable diagnostic morphological characters; (*b*) high phenotypic plasticity; (*c*) frequent interspecific hybridizations; and (*d*) assumed past reticulate evolution within the section and subsection (Marcussen and Nordal, 1998; Marcussen and Borgen, 2000; most recently also Hodálová *et al.*, 2008).

Viola suavis ($2n = 40$) is distributed in the Mediterranean Basin from Morocco and the Iberian Peninsula to the Middle

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East. Its area extends also to central and northern Europe due to its frequent cultivation and escape to natural sites (Marcussen and Nordal, 1998). Morphologically, it can be delimited from the related species by a combination of characters: relatively short and stout stolons, long-fimbriate stipules, bracteoles inserted below the middle of the peduncle, and calycine appendages appressed to the peduncle (cf. Becker, 1910; Gams, 1925; Schmidt, 1961; Marcussen and Nordal, 1998; Hodálová *et al.*, 2008). It displays extensive morphological variation, which complicates its taxonomic treatment. Since being described by Marschall von Bieberstein (1819), a lot of new taxa have been described and attributed to this species or species complex, recently treated as separate species, subspecies or varieties or placed into the synonymy of *V. suavis*. Morphologically, they have been distinguished on the basis of slight differences in indument, shape and length of laminas and stipules, and corolla colour (varying from pale to dark blue; for further details see, for example, Becker, 1910; Gams, 1925; Schmidt, 1961; Marcussen and Nordal, 1998). The taxonomic value and reliability of these characters, however, remain questionable. Recent studies employing isozymes suggested an allo-octoploid origin of *V. suavis*. Large morphological and genetic variation indicates an old polyploidization event, followed by gene flow among the nascent octoploids (Marcussen and Nordal, 1998; Marcussen and Borgen, 2000). One of the parents appears to be *V. pyrenaica* (distributed in mountain ranges from the Atlas Mts and Pyrenees to the Caucasus). The other parent(s) could not be identified with certainty (*V. jaubertiana* Marès et Vigin, *V. odorata* L. or *V. collina* Besser are possible candidates; Marcussen and Nordal, 1998; Marcussen and Borgen, 2000). The genetic variation of *V. suavis* is surely also affected by its mixed breeding system. Seeds are formed by both outcrossing and selfing (early-spring chasmogamous flowers vs. later cleistogamous ones). The plants also propagate vegetatively by stolons (Gams, 1925; Marcussen and Nordal, 1998).

In a recent morphometric study of the Carpathian representatives of *Viola* sect. *Viola* subsect. *Viola* (*V. alba*, *V. ambigua*, *V. collina*, *V. hirta*, *V. odorata* and *V. suavis*), white-flowered populations of uncertain taxonomic status were reported. Pollen fertility and karyological and morphometric analyses revealed that they were highly fertile, octoploid ($2n = 40$) and morphologically most similar to *V. suavis* (Hodálová *et al.*, 2008). These plants, tentatively named white-flowered *V. suavis*, have not yet been reported in central Europe. Kirschner and Skalický (1990: 402) referred to similar white-flowered violets cultivated in Bohemia (Czech Republic) that sometimes escaped; they remarked that they may be of a hybrid origin, bearing morphological traits of *V. odorata*, *V. collina*, perhaps also of *V. hirta* or *V. suavis*, but in most characters allegedly resembling *V. alba* (cf. also Suda, 2002: 214).

Populations bearing white petals and violet spurs while otherwise resembling *V. suavis*, however, were frequently recorded in the Iberian Peninsula. Originally they were described as *V. catalonica* W. Becker (Becker, 1929), but in local floras they were later treated at infraspecific levels, for example, as *V. suavis* subsp. *catalonica* (W. Becker) O. Bolòs et Vigo (Bolòs and Vigo, 1974) or *V. suavis* var. *catalonica* (W. Becker) Espeut (Espeut, 1999). In *Flora Iberica* (Muñoz Garmendia *et al.*, 1993: 284), the taxon was mentioned only in a note of the *V. suavis* account as often being cultivated and distributed in the whole Iberian Peninsula. In *Flora Europaea* (Valentine *et al.*, 1968: 272), such plants are considered to be an intermediate between *V. suavis* and *V. alba*, perhaps being of a hybrid origin.

In the present study, the focus was on these peculiar, taxonomically ambiguous white-flowered populations related to *V. suavis*. The main questions were: (*a*) What is the taxonomic placement and origin of the white-flowered populations? Do they form a monophyletic group within *V. suavis* or did they arise recurrently at different locations (having multiple origins)? Do the data support their assumed hybrid origin? (*b*) Except for the anthocyan pigmentation, do white- and blue-flowered (typical) plants of *V. suavis* also exhibit other morphological differences? (*c*) Can geographic patterns in the variation of the populations sampled be found, allowing inferences of their (post)glacial histories and supporting recognition of infraspecific taxa within *V. suavis*? To answer these questions, karyological, molecular (AFLP, nuclear ITS), morphometric, and pollen fertility analyses of *V. suavis* and its closest relatives were performed.

MATERIALS AND METHODS

Plant material

The sampling strategy was based on previous morphological and genetic (ITS and isozyme) studies of *Viola suavis* and its close relatives (Marcussen and Nordal, 1998; Marcussen and Borgen, 2000; Málecot *et al.*, 2007; Hodálová *et al.*, 2008). The study focused on the geographic areas of central and south-eastern Europe (C & SE Europe hereafter) and north-eastern Spain (the area where white-flowered populations were reported and formally recognized as *V. catalonica*). There have been no published reports of similar white-flowered morphotypes from the interconnecting areas (e.g. from France, Italy; see Discussion); nor were such populations found there by the authors.

The main sampling focus was on *V. suavis*; plants were collected from typical, blue-flowered populations [plants with blue to (bluish-)violet petals and spur], as well as from populations of the white-flowered morphotype [those with white petals and a pale to deep (bluish-)violet spur] tentatively assigned to *V. suavis* (see Hodálová *et al.*, 2008). Altogether, 36 populations of *V. suavis* were sampled with as much overlap between the morphological and AFLP datasets as possible (see Appendix). In most cases the white- and blue-flowered populations were spatially well separated from each other, but intentionally material was also included from four locations where the blue- and white-flowered plants were found growing in close proximity or even partly intermingled (population nos 169, 209, 27, 206, 168, 208, 205 and 25; see Appendix). These plants were analysed in order to see if

the variation in the population is affected by hybridization or introgression between the morphotypes.

Furthermore, five close relatives of *Viola* sect. *Viola* subsect. *Viola* occurring in the area of C & SE Europe were sampled and included in molecular and karyological analyses: *V. alba* (represented by subsp. *alba*), *V. ambigua*, *V. collina*, *V. hirta* and *V. odorata*.

Details on the origin of the material used are given in Appendix and Fig. 1. All voucher specimens were deposited in the herbarium of the Institute of Botany, Slovak Academy of Sciences, Bratislava (SAV).

Karyological analyses

Plants collected in the field were cultivated in the experimental garden of the Institute of Botany, Slovak Academy of Sciences, Bratislava, Slovakia. Ploidy levels were determined by chromosome counting (two individuals per population) and by flow cytometry (1–16 individuals per population); altogether 37 populations were examined (see Appendix).

Chromosome numbers were determined from microscopic squashes prepared from root tip meristems, following the procedure specified by Hodálová *et al.* (2008). Samples for flow cytometric measurements were prepared from fresh tissues of petals and/or flower peduncles and/or young leaf petioles. Nuclear DNA amounts were estimated with propidium iodide staining, using a Becton Dickinson FACSCalibur flow cytometer with BD Cellquest Pro Software (Faculty of Science, P. J. Šafárik University, Košice, Slovakia) following the protocol described by Hodálová *et al.* (2008). *Viola reichenbachiana* Jord. ex Boreau was used as the internal or pseudo-internal standard (see Hodálová *et al.*, 2008).

Molecular analyses

Fresh leaf samples were collected and dried in silica gel. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen).

ITS sequences. Altogether 38 individuals (33 populations) from ingroup taxa (*V. alba*, *V. ambigua*, *V. collina*, *V. hirta* and *V. odorata* and both morphotypes of *V. suavis*) were analysed for ITS (ITS1-5·8S-ITS2 region of the nuclear ribosomal DNA) sequence variation. *Viola reichenbachiana* was chosen as an outgroup (based on results by Malécot *et al.*, 2007).

The ITS region was amplified with a standard PCR procedure and the universal primers P1A and P4 (Francisco-Ortega *et al.*, 1999). Amplifications were performed in a 25-μL reaction volume with 0·2 μM of each primer, 0·2 mM dNTPs, 10× PCR buffer (containing $MgSO_4$ at 2 mM in the reaction), and 0·75 U Pfu polymerase (Fermentas). Reactions were run in Mastercycler ep Gradient S (Eppendorf). The PCR cycle profile was 94 °C for 5 min, 35 cycles with 94 °C (30 s), 54 °C (30 s), 72 °C (1 min), and then a final extension at 72 °C for 10 min and incubation at 4 °C. PCR products were purified using the Spinprep PCR clean-up kit (Calbiochem). Cycle-sequencing reactions, using the original PCR primers and the BigDye® Terminator Cycle Sequencing Kit, and product separation were performed at the BITCET Consortium at the Department of Molecular Biology, Comenius University, Bratislava (ABI 3130xl Genetic Analyser). Both forward and reverse strands were sequenced with a 100 % overlap. The sequences were aligned manually using BioEdit (version 7·0·4·1; Hall, 1999). Electropherograms of ITS were inspected for the presence of overlapping peaks, indicating an occurrence of substitutions between different ITS repeats in the same individual. IUPAC ambiguity codes were used for coding such polymorphic positions.

Both maximum parsimony (PAUP* 4·0b10; Swofford, 2001) and split decomposition analyses (SplitsTree 4·8; Huson and Bryant, 2006) were conducted. The latter approach is a more effective means of representing complex patterns (e.g. low genetic divergence, persistence of ancestral sequence types and reticulate patterns), which may not be well represented by bifurcating tree models (Winkworth *et al.*, 2005). Split graphs were constructed from *p*-distances (Hamming distance), and branch lengths were optimized using the least-squares function.

AFLP fingerprinting. A total of 178 individuals originating from 45 populations were analysed (two to six plants per population). *Viola alba*, *V. ambigua*, *V. collina*, *V. hirta* and *V. odorata* were represented by 45 individuals sampled from three populations each. The main focus was on *V. suavis* with 133 individuals originating from 30 populations (two to six plants per population). Populations of *V. suavis* were *a priori* assigned to the colour morphotypes, except for one population sampled after flowering (pop. no. 25). On the basis of field research undertaken in previous years, it was known that this locality harbours both blue- and white-flowered plants growing in sympatry, but it was not possible to collect them in the flowering stage for the AFLP study.

For the AFLP, the general protocol described by Vos *et al.* (1995) and the protocol provided by Applied Biosystems (Applied Biosystems, 2005) was followed with some modifications. Double-digestion of DNA was performed using *Eco*RI and *Mse*I enzymes at 37 °C for 3 h. The reaction mix (10 μL volume) contained 5 U *Eco*RI (Fermentas), 2 U *Mse*I (New England BioLabs), 2 μL 10× Tango buffer (Fermentas) and 5 μL of the DNA extract (500–1000 ng). A ligation mix was then added in a volume of 5 μL per sample, and the reactions were incubated at 16 °C for 12 h. Aliquots of the ligation mix contained 1 U T4 DNA ligase (Fermentas), 1·5 μL T4 DNA ligase buffer (including ATP), and 1 μL of each adaptor pair (Applied Biosystems). Ligated DNA fragments were diluted 1 : 10 with TE buffer (10 mM Tris, 0·1 mM EDTA). The reaction mix of preselective amplifications (10-μL reaction volume) contained 1 μL PCR Buffer II (10 ×, Applied Biosystems), 0·6 μL $MgCl_2$ (25 mM), 0·2 μL dNTPs (10 mM each), 0·5 μL of each preselective primer (Applied Biosystems), 0·04 μL AmpliTaq DNA polymerase (5 U μL^{-1}; Applied Biosystems), and 2 μL of diluted restriction-ligation product. The PCR cycle profile was 72 °C (2 min), 30 cycles with 94 °C (30 s), 56 °C (30 s), 72 °C (2 min), followed by 72 °C for 10 min,

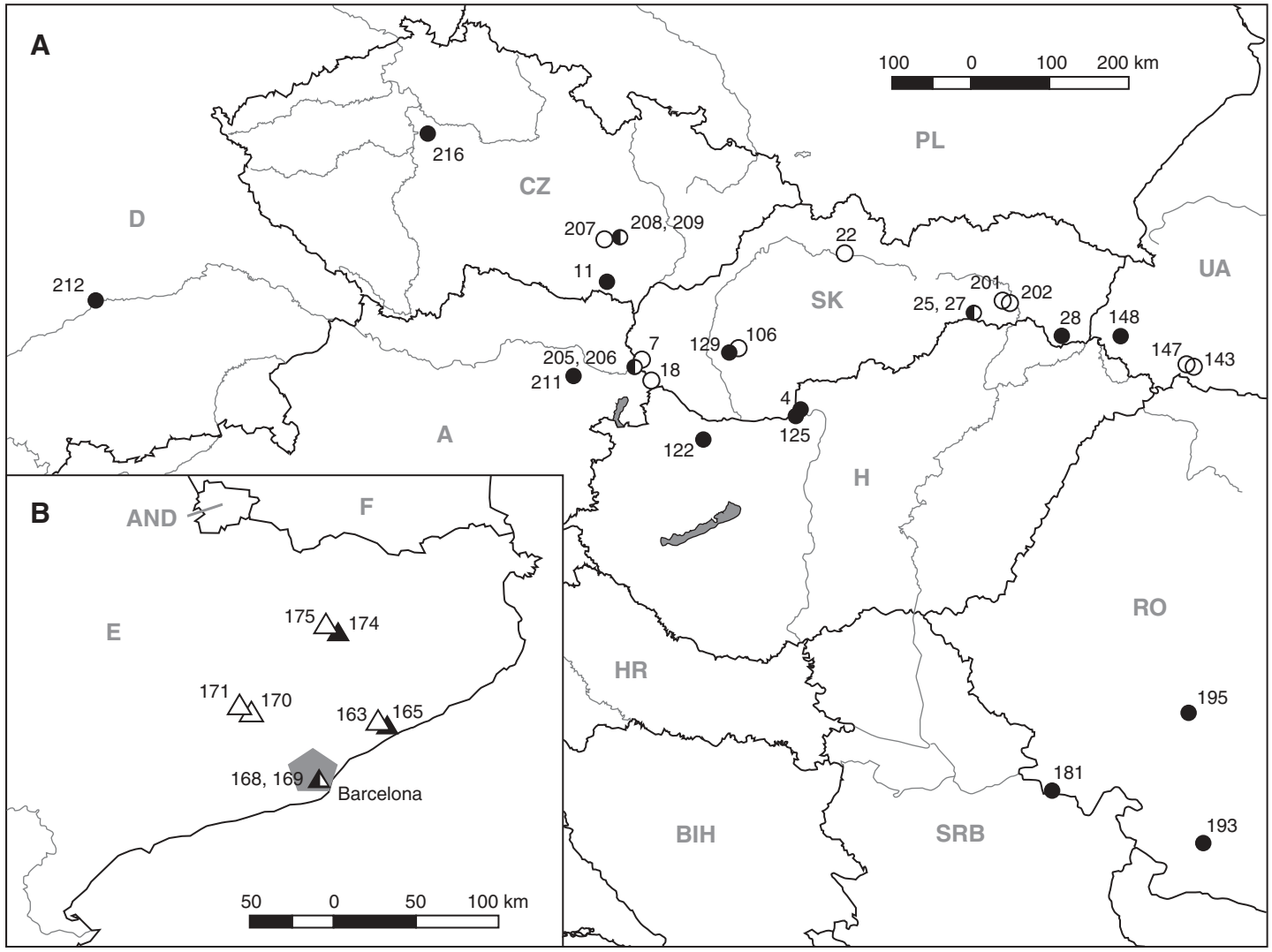

FIG. 1. Sampling sites of *Viola suavis* showing the area of (A) C & SE Europe and (B) north-eastern Spain. Full list of localities and the population numbers are given in Appendix. Blue-flowered populations are marked by black circles and triangles, white-flowered ones by white circles and triangles. Note that four localities harboured both morphotypes growing in close proximity or intermingled (half black/white symbols).

and an incubation at 4 °C (Mastercycler ep Gradient S, Eppendorf). Products of preselective amplification were diluted approx. 1 : 15 with TE buffer. A preliminary screening of 24 selective primer pair combinations was done for three samples (one *V. hirta* and two *V. suavis*). The six primer pair combinations that gave the best results with respect to polymorphism and clarity of AFLP profiles were further tested in eight samples (four *V. suavis* and four from the other species). Three pairs of selective primers were finally selected: *Eco*RI-ATC-(6-FAM)/*Mse*I-CAT, *Eco*RI-AAG-(VIC)/*Mse*I-CAC and *Eco*RI-AGC-(NED)/*Mse*I-CAA. The *Eco*RI-selective primers were 5'-fluorescent labelled. The reaction mix of selective amplifications (10 μL reaction volume) contained 1 μL PCR Gold buffer (10×; Applied Biosystems), 1 μL $MgCl_2$ (25 mM), 0·2 μL dNTPs (10 mM each), 0·5 μL of each primer (*Eco*RI at 1 μM, *Mse*I at 51 μM), 0·08 μL AmpliTaq Gold DNA polymerase (5 U μL^{-1}; Applied Biosystems), and 2 μL of diluted preselective PCR product. The PCR cycle profile was 95 °C (10 min), 13 cycles with 94 °C (30 s), 65–55·9 °C (each cycle decreasing by 0·7 °C, 1 min), 72 °C (1 min), 23 cycles with 94 °C (30 s), 56 °C (1 min), 72 °C (1 min), followed by 72 °C for 10 min, and incubation at 4 °C. The amplification products were pooled in a ratio of 1 : 1 : 1 and submitted for the fragment analysis to the BITCET Consortium, Department of Molecular Biology, Comenius University, Bratislava (ABI 3100 Avant). Size calibration was done using the internal size standard GeneScan –500 LIZ® (Applied Biosystems).

Raw AFLP data were collected and sized using the GeneScan 3·7 software (Applied Biosystems). The AFLP profiles were scored using the software Genographer 1·6·0 (available at http://hordeum.msu.montana.edu/genographer/). Only well-scorable and unambiguous fragments in the size range 75–500 bp were recorded and coded as presence (1) or absence (0). To estimate reproducibility of the AFLP data, DNA from 15 samples (8 % of the final dataset) was double-extracted, and the replicated samples were analysed independently. AFLP profiles of the replicates were scored and compared with each other to calculate the error rate (Bonin *et al.*, 2004).

For each species/group, total number of AFLP phenotypes, average number of fragments generated per individual, number of private (exclusive) fragments and number of private fixed (diagnostic) fragments were recorded. Patterns of fragment sharing were also explored.

Two data matrices were assembled: one included all species (the 'all species matrix', 178 individuals) and the other comprised only *V. suavis* individuals ('*V. suavis* matrix', 133 individuals). The genetic distance and relationships among the species and individuals were explored by neighbor-joining analysis ('all species matrix'), principal co-ordinate analyses (PCoA, Krzanowski; 1990; both matrices), and by the Bayesian model-based clustering method implemented in the software STRUCTURE 2·2 (Falush *et al.*, 2007; '*V. suavis* matrix' only). The neighbor-joining tree, based on the Nei and Li (1979) genetic distance, was constructed in PAUP* 4·0b10 (Swofford, 2001). Group support was assessed by bootstrap analyses with 5000 replications. PCoA was performed in SYN-TAX 2000 (Podani, 2001) using Jaccard's coefficient to calculate pairwise genetic similarities.

For the Bayesian inference of population structure within *V. suavis*, a model with admixture and an assumption of correlation of allele frequencies among populations were used. Settings adequate for dominant data (recessive allele model) were used as implemented in STRUCTURE 2·2. The method uses a Markov chain Monte Carlo (MCMC) algorithm to cluster genetically similar individuals on the basis of multilocus genotype data. The *K* value (a user-defined number of clusters) was set from 2 to 5. To test the stability of the results, ten replicate runs were performed for each *K*. The length of the burn-in period was set to 100 000, and the MCMC chains after burn-in were run for an additional 1000 000 replicates. R-script Structure2·1-sum (Ehrich, 2006) was used to summarize the output files, to calculate similarity coefficients between the replicate runs, and to plot the means of the estimated posterior log probability of the data over the run replicates for each *K* value, denoted as mean $L(K)$ (see also Evanno *et al.*, 2005).

The intraspecific differentiation of *V. suavis* was explored also by analyses of molecular variance (AMOVA) based on Euclidean pairwise distances using Arlequin 3·11 (Excoffier *et al.*, 2005). Different population groupings based on the results of PCoA, clustering (both neighbor-joining and Bayesian), geography and morphology were tested.

For *V. suavis* from C & SE Europe, a Mantel test (Legendre and Legendre, 1998) was calculated to test for significant correlation between pairwise genetic and geographic distances by a permutation procedure, implemented in Arlequin 3·11 (Excoffier *et al.*, 2005). Genetic distances were expressed as both pairwise population F_{ST} values (computed in Arlequin 3·11) and as individual Sørensen genetic distances (computed in SYN-TAX 2000; Podani, 2001). The geographic distances were derived from the geographic co-ordinates using the AFLPdat R-script (Ehrich, 2006). Correlation coefficients (r_m) were computed and their significance was tested with 999 permutations. In addition, 12 distance classes were defined and the correlation of the genetic distance matrix (individual pairwise Sørensen distances) was tested against a series of model matrices in which the geographical distances were coded as 0 (distance value within a predefined distance interval) or 1 (all other distances; for details, see Stehlik *et al.*, 2001). To adjust for multiple tests, the sequential Bonferroni correction was applied (García, 2004). The correlation coefficient values obtained were plotted against distance classes (Mantel correlograms) to infer the relative influences of gene flow and genetic drift on the distribution of genetic variation across different distance classes.

AFLP data format conversions and fragment presence/frequency calculations were carried out using the AFLPdat R-script (Ehrich, 2006).

Morphometric analyses

Typically, 10 (2–19) flowering plants were collected per population, depending on the population size. A total of 22 morphological characters (11 vegetative and 11 floral) were recorded, including one ratio (see Table 1). Fourteen

TABLE 1. *List of characters used in the morphometric analyses of* Viola suavis *populations, including eigenvectors expressing correlations of characters with the first and second principal components of PCA (Axis 1 and Axis 2; see Fig. 5)*

Character		Character scoring/units	Axis 1	Axis 2
Stolons				
StA*	Above-ground stolons	0 absent; 1 present	0·044	0·129
StU*	Underground stolons	0 absent; 1 present	0·230	0·090
Laminas and petioles				
LHL*	Maximum hair length (on petiole)	(mm)	−0·070	−0·127
LSL*	Lamina sinus depth	(cm)	0·293	0·262
LSA*	Lamina sinus angle	(degree)	−0·312	−0·296
LCN*	Number of crenulae along both lamina margins (= lamina dentations)	–	−0·054	0·063
LAA*	Lamina apex angle	(degree)	0·130	−0·011
LP*	Violet pigmentation of lamina	0 absent; 1 present	0·279	−0·145
Stipules (outer stipules of main rosette-leaves)				
SW*	Stipule width	(mm)	0·258	−0·001
SFN*	Number of fimbriae (= glandular fimbriae, non-glandular fimbriae and sessile glandule) along both stipule margins	–	0·234	0·204
SFL*	Maximum fimbriae length on stipule	(mm)	−0·212	0·286
Peduncules				
PL	Peduncle length	(cm)		
PL1	Peduncle length below bracteoles	(cm)		
PP*	Peduncle pigmentation	0 absence; 1 presence	0·314	−0·253
PL1/PL*	Peduncle length below bracteoles/ peduncle length	–	0·341	0·091
Calyx (sepals)				
KAL*	Anterior sepal length	(mm)	−0·075	0·146
KAW*	Anterior sepal width	(mm)	0·220	0·254
KP*	Violet pigmentation of sepals	0 absence; 1 presence	0·217	−0·201
Corolla (petals)				
CPL*	Posterior petal length	(mm)	0·109	0·368
CPW*	Posterior petal width	(mm)	0·110	0·345
CP*	Corolla colour (excluding spur)	0 white; 1 blue to violet	−0·278	0·338
CPSP*	Pigmentation of corolla in contrast to pigmentation of spur	0 spur paler than corolla; 1 spur the same colour as corolla; 2 spur darker than corolla	−0·278	−0·298

* Characters used in multivariate analyses; for characters illustrations see Hodálová *et al.* (2008).

characters were quantitative, six binary, and one semi-quantitative. Characters expressing colouration and the presence/absence of stolons (StA, StU, LP, PP, KP, CP, and CPSP; for character explanation, see Table 1) were scored immediately in the field on fresh plants. Otherwise the vegetative (leaves and stipules) and floral (peduncles, calyx and corolla) organs were attached by adhesive tape to paper, dried, and then used for measurements. Whenever possible, three measurements were made for each vegetative character, and two measurements were made for each floral character; average values were then entered into the data matrix. Twenty characters were used in morphometric analyses; two characters were used solely for calculating ratios (Table 1). Corolla colour (CP) was excluded from canonical discriminant analyses because it was constant within the groups.

Diagnostic characters in the subsection *Viola* often change after the flowering during late spring and summer (e.g. leaf shape, the length of hairs on petioles) or disappear (characters on stipules and flowers). Therefore, all morphometric analyses were performed on material collected in spring. The indument of leaves was observed on petioles of young, still-developing leaves in spring or, in some cases, on petioles of over-wintering leaves that had developed in summer or autumn of the previous year. Only open (chasmogamous) vernal flowers were considered.

Two datasets (matrices) were assembled. Matrix A included all individuals of *V. suavis* (both morphotypes; 340 individuals from 35 populations; Appendix) sampled. This matrix was used to gain insight into the overall variation pattern in *V. suavis*, especially with regard to the position of the white-flowered populations. Matrix B represented a selection of samples of *V. suavis* corresponding to the material analysed for AFLPs: from spatially separated populations all individuals sampled and measured were included; in cases where blue- and white-flowered individuals co-occurred, only the individuals analysed for AFLPs were included (245 individuals from 29 populations). Matrix B

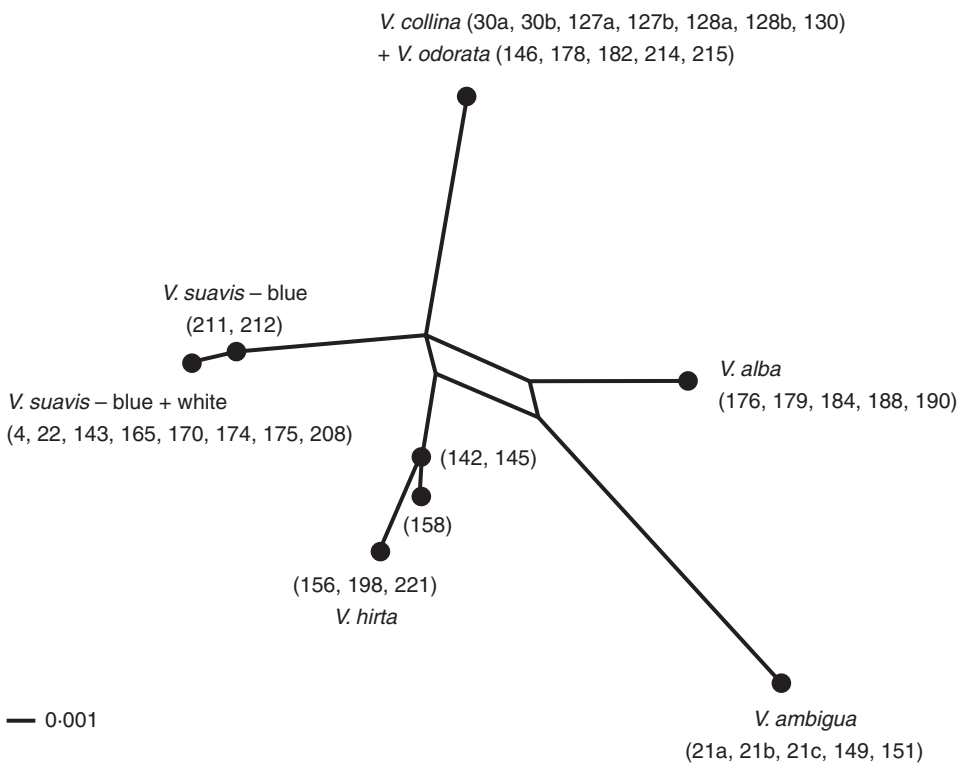

FIG. 2. Splits graph based on nrDNA ITS sequence data for the studied *Viola* species. The fit of the graph is 95·75 %. Branch lengths were optimized using the least-squares function. Accession numbers correspond to population numbers (see Appendix).

was used to test for morphological differentiation among the groups, as suggested by AFLP analyses.

Prior to multivariate analyses, a non-parametric Spearman correlation coefficient was calculated in order to eliminate pairs of highly correlated characters that might distort further analyses (cf. Legendre and Legendre, 1998). Principal component analysis (PCA) and cluster analysis were performed on matrix A, with the populations defined by the mean values of the characters measured. PCA (Sneath and Sokal, 1973), based on a correlation matrix between the characters, was used to recover the non-hierarchical structure in the dataset. Two different PCAs were run; one including all 20 characters and the other with only 15 characters, excluding the five characters that express pigmentation (i.e. LP, PP, KP, CP and CPSP; see Table 1). In the cluster analysis the UPGMA (unweighted pair-group method using arithmetic averages) and MISSQ (incremental sum of squares method – Ward's method) algorithms were employed (Everitt, 1986). The primary data were standardized by zero mean and unit standard deviation, and the Euclidean coefficient was used to compute the secondary distance matrix.

To examine the extent of morphological separation of the groups suggested by AFLP data, canonical discriminant analyses (CDA; Klecka, 1980) were computed on matrix B, with individual plants treated as objects. Discriminant analyses generally require multivariate normal distribution of the characters, but they were shown to be considerably robust against deviations in this respect (Thorpe, 1976; Klecka, 1980).

Correlations coefficients, PCA and CDA were calculated using the SAS version 9·1 statistical package (SAS Institute, 2000), while UPGMA and MISSQ were run by the SYN-TAX 2000 (Podani, 2001).

TABLE 2. *Summary information of AFLP data generated in the studied* Viola *species*

Taxon	N_{ind}	N_{phen}	N_{fragm}	N_{pr}	N_{dg}
alba	9	9	(84) 87 (89)	16	10
ambigua	9	8	(125) 128 (131)	28	23
collina	9	8	(92) 93 (95)	17	11
hirta	9	8	(94) 96 (98)	13	2
odorata	9	8	(87) 89 (91)	22	14
suavis	133	36	(112) 115 (120)	24	17
*suavis*B (C & SE)	53	23	(113) 117 (120)	9	0
*suavis*W (C & SE)	42	5	(113) 114 (117)	1	0
*suavis*B (Spain)	11	4	(112) 115 (117)	7	1
*suavis*W (Spain)	22	1	115	0	0

Viola suavis is treated as a single entity, as well as divided into four groups: *suavis*B (C & SE), *suavis*B (Spain), blue-flowered morphotype from C & SE Europe and Spain, respectively; *suavis*W (C & SE), *suavis*W (Spain), white-flowered morphotype from C & SE Europe and Spain, respectively. The columns show: number of the analysed individuals (N_{ind}), number of AFLP multilocus phenotypes (N_{phen}) resolved in a particular taxon/ group, number of AFLP fragments (N_{fragm}) per individual – (min) average (max) values, number of private (exclusive) (N_{pr}), and private fixed (diagnostic) (N_{dg}) fragments. Individuals from the population no. 25 (collected after flowering) were included in computations referring to *V. suavis* as one entity, but not assigned to the colour morphotype, and thus excluded from computations based on *V. suavis* subgroups.

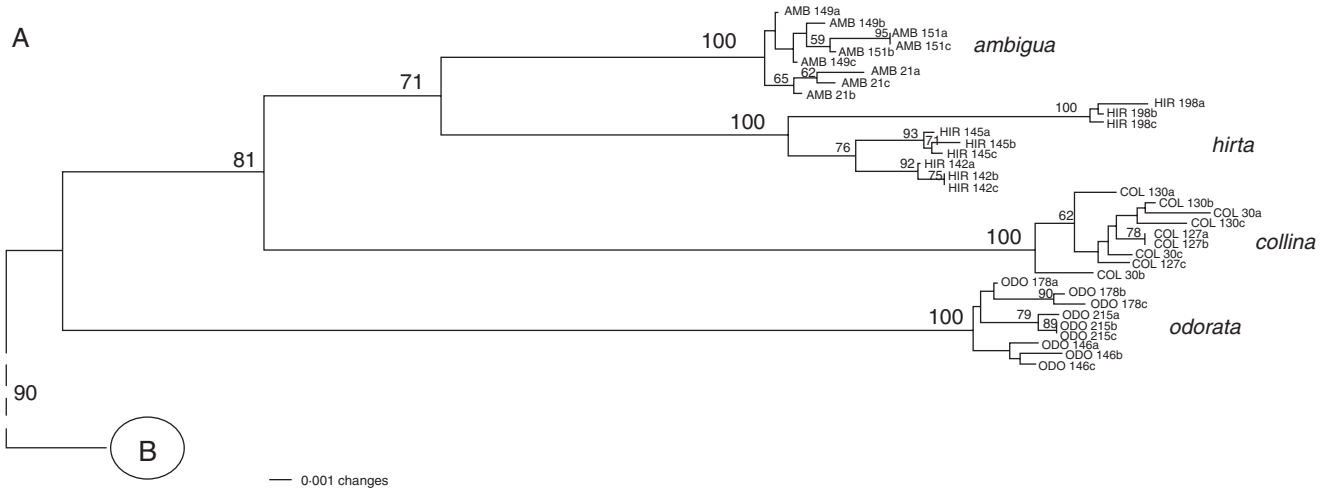

FIG. 3. (A,B) Midpoint rooted neighbor-joining tree of AFLP data (288 fragments) of 178 *Viola* individuals using Nei and Li distance. Numbers above branches indicate bootstrap support above 50 %. Accession labels include taxon abbreviation (ALB, *V. alba*; AMB, *V. ambigua*; ODO, *V. odorata*; COL, *V. collina*; HIR, *V. hirta*; SUB, *V. suavis*, blue-flowered morphotype; SUW, *V. suavis*, white-flowered morphotype) and population numbers (see Appendix).

Estimation of pollen fertility

To test the assumed hybrid origin of the white-flowered morphotype(s), all plants included in morphometric analyses were also examined for pollen viability. Anthers were removed from a single flower or a flower bud per individual and chopped in a drop of aceto-carmine jelly (Radford *et al.*, 1974) on a microscope slide to release pollen grains. One hundred pollen grains per individual were observed. The numbers of unviable (i.e. unstained and usually shrunken) and viable (well-stained and of regular shape) grains were recorded. Pollen quality was expressed as the percentage of viable grains.

RESULTS

Karyological analyses

Data on chromosome number and DNA ploidy level are presented in Appendix. All populations of *V. alba*, *V. collina*, *V. hirta* and *V. odorata* were found to be tetraploid with $2n = 20$, while all populations of *V. suavis* (both morphotypes) were octoploid with $2n = 40$. These data are consistent with previous reports (cf. Mered'a *et al.*, 2006; Hodálová *et al.*, 2008).

Molecular analyses

ITS sequences. The ITS matrix excluding the outgroup consisted of 38 sequences and 612 aligned positions; 37 sites were variable; 34 of them were parsimony informative. A few 1-bp or 2-bp indels were identified, which were not coded separately but treated as missing data. Only a few sequences contained apparent intra-individual single-site polymorphisms (overlapping peaks in electropherograms; up to two positions per sequence). The parsimony analysis (figure not shown) resulted in a strict consensus tree (2288 most parsimonious trees; $L = 50$ steps, $CI = 0{\cdot}92$, $RI = 0{\cdot}98$), which displayed a large polytomy at the base. Split decomposition analysis revealed that this is due to data conflict; the splits graph (Fig. 2) indicates that patterns of nucleotide substitutions are incompatible, and the relationships among the species are uncertain. In addition, no sequence divergence was found between *V. collina* and *V. odorata*. The monophyletic character of *V. suavis*, including both morphotypes, is evident.

AFLP fingerprinting. In the 178 individuals studied, 288 unambiguous AFLP fragments were scored; 32 fragments (11 %) were monomorphic across the dataset. Control replicates proved that the AFLP data were highly reliable (repeatability 98·6 %). Altogether, 77 different AFLP multilocus phenotypes were detected, i.e. several individuals had identical profiles. Table 2 shows the number of AFLP phenotypes and the average number of fragments per individual that were resolved in the taxa studied. In the two octoploid species, *V. ambigua* and *V. suavis*, more fragments per individual were generated than in the tetraploids, indicating a correlation with the ploidy level.

The neighbor-joining tree (Fig. 3) and PCoA (figure not shown) based on the 'all species matrix' resolved six distinct and well-differentiated groups, corresponding to the six species studied. This result was also supported by the number of species-specific fragments (Table 2). Clustering in the neighbor-joining analysis suggests closer affinities between certain species but with moderate to low bootstrap support.

With respect to variation patterns within *V. suavis* (both morphotypes), neighbor-joining clustering, PCoA and STRUCTURE analyses (the latter two based on the '*V. suavis* matrix') revealed a pronounced intraspecific genetic structure. In PCoA (Fig. 4), the accessions from Spain were clearly separated from those from C & SE Europe along the first axis, which extracted almost 32 % of the total variation. This separation was seen also in the neighbor-joining tree (Fig. 3). Consequently,

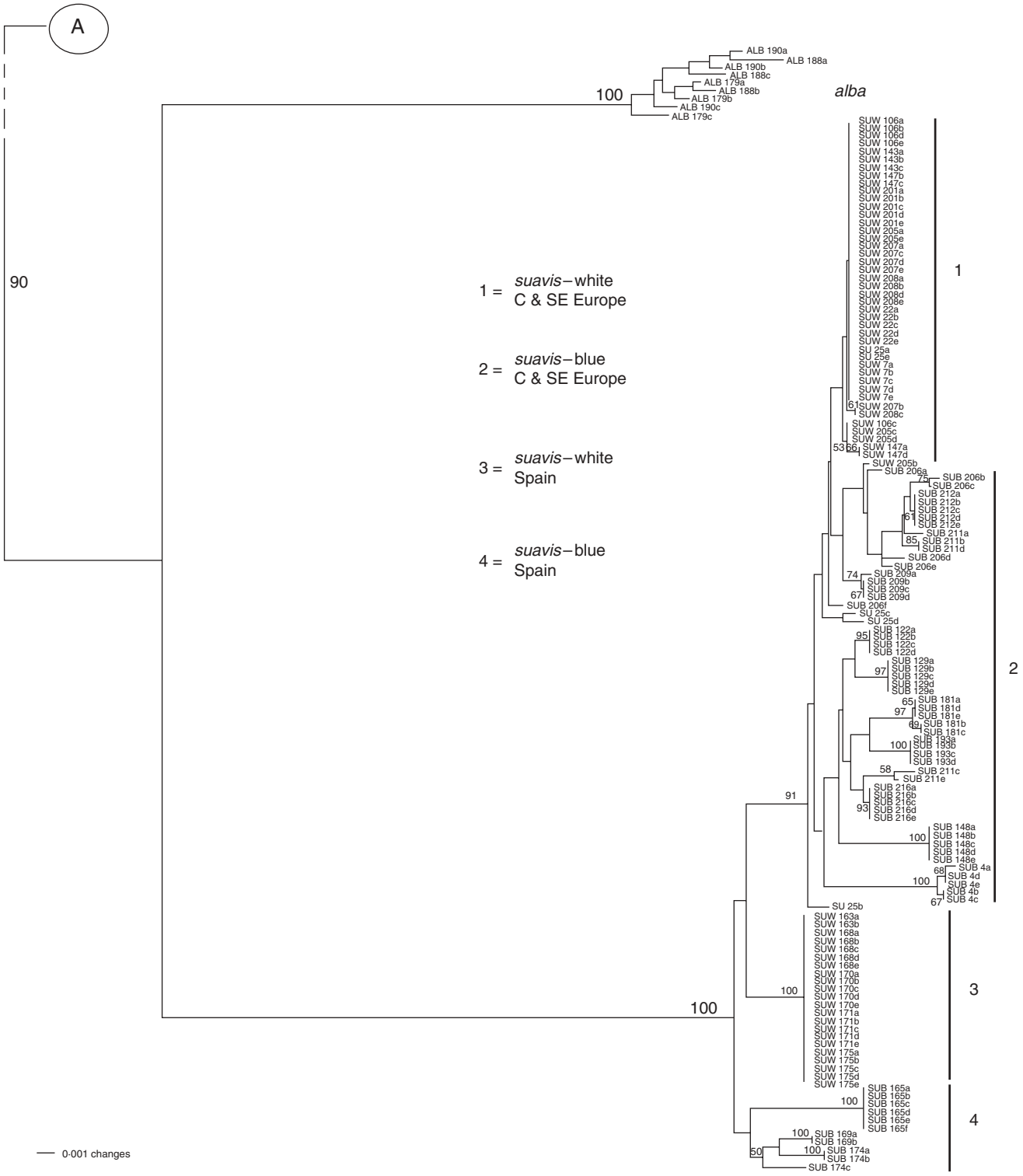

FIG. 3. *Continued*

white-flowered accessions appear clearly biphyletic. Iberian ones (five populations in total) all shared a single AFLP phenotype, which appeared close to those resolved in Iberian blue-flowered populations. The white-flowered populations from C & SE Europe formed a separate subcluster among the blue-flowered accessions from the same area (Figs 3 and 4).

Results of the Bayesian clustering (STRUCTURE) of *V. suavis* are in good agreement with the phenetic analyses. The assignment of individuals into clusters across replicate

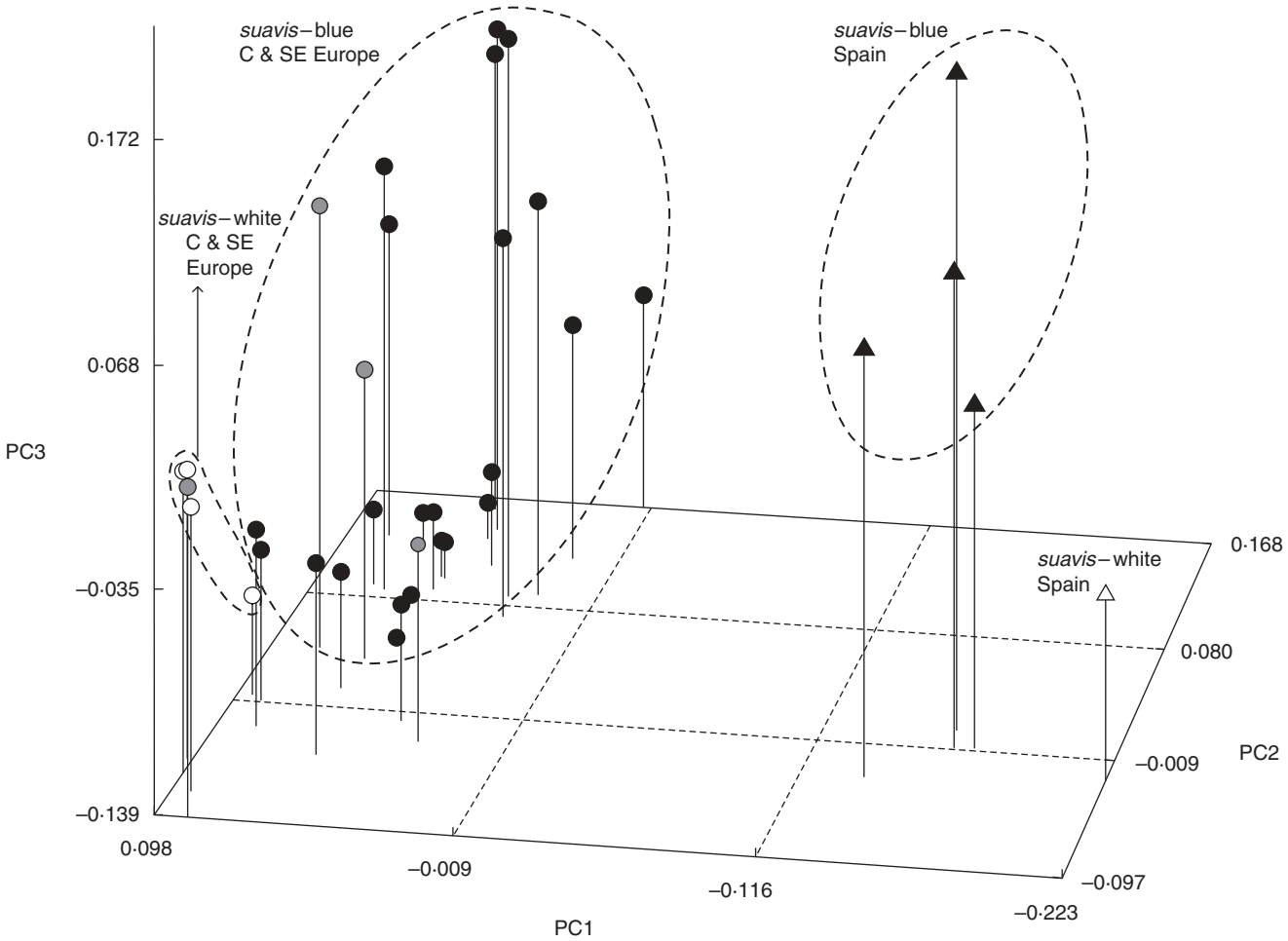

FIG. 4. Principal co-ordinate analyses of *Viola suavis* based on 133 individuals and 149 AFLP fragments using Jaccard coefficient. Geographic origin of the samples and the colour morphotypes are indicated: black circles and triangles – blue-flowered morphotype, white circles and triangles – white-flowered morphotype. Individuals from the population no. 25 (see Appendix), collected after flowering, are in grey. The first three axes explain 31·81 %, 15·25 % and 12·12 % of the total variation.

runs provided stable results only at $K = 2$. The means of the estimated posterior log probability of the data over ten run replicates for each K value [mean $L(K)$] increased with increasing values of K up to $K = 4$, but decreased at $K = 5$. At $K = 4$ and 5 some replicate runs also inferred empty clusters. At $K = 2$ the two groups resolved corresponded to the Iberian and C & SE European accessions, respectively. At $K = 3$, seven replicate runs inferred the clusters of (*a*) all Iberian populations, (*b*) the C & SE European white-flowered populations, and (*c*) the C & SE European blue-flowered populations; three replicate runs inferred the clusters of (*a*) the Iberian white-flowered populations, (*b*) the Iberian blue-flowered populations, and (*c*) all C & SE European populations.

Results of the analysis of molecular variance (AMOVA) are summarized in Table 3. The analysis revealed significant differentiation between Spain and C & SE Europe (51 % of total variance). Only 16 % of total variance was attributed to the differentiation between the white- and blue-flowered morphotypes. The highest differentiation (58 % of total variance) was achieved when considering four groups, dividing both the Iberian and C & SE European populations into two morphotypes.

Mantel tests computed for *V. suavis* from C & SE Europe revealed significant correlations between the genetic and geographic distance matrices ($r_m = 0{\cdot}283$, $P = 0{\cdot}04$ for pairwise population F_{ST} matrix, and $r_m = 0{\cdot}331$, $P = 0{\cdot}00$ for pairwise individual Sørensen distance matrix), indicating a decrease in the genetic relatedness with increasing distance between individuals and populations. Mantel tests calculated with distance classes [cf. Supplementary Information (1), available online] showed that individuals were most closely related within populations ($r_m = 0{\cdot}268$, $P = 0{\cdot}00$); this correlation remained significantly positive up to a distance of 8 km ($r_m = 0{\cdot}121$, $P = 0{\cdot}00$). For the next distance classes, the trend was equivocal (both positive and negative values obtained, a significant positive value only for the class of 161–209 km), and from 407 km on, r_m values were significantly negative. Such fluctuations may be due to the low number of AFLP genotypes and most likely also reflect genetic stochasticity acting even at relatively short distances (over 8 km).

TABLE 3. *Analysis of molecular variance (AMOVA) of AFLP data performed with different groupings within* Viola suavis *(excluding population no. 25 and 27; 128 individuals, 149 AFLP fragments)*

Grouping	Source of variation	d.f.	Sum of squares	Variance components	% of total variance
A. [Spain],[C & SE Eu]	Among groups	1	225·96	4·251	51·01 %***
	Among populations	26	448·66	3·702	44·42 %***
	Within populations	100	38·05	0·381	4·57 %***
B. [blue-fl.],[white-fl.]	Among groups	1	87·80	1·002	16·02 %***
	Among populations	26	586·83	4·871	77·89 %***
	Within populations	100	38·05	0·381	6·08 %***
C. [Spain],[C & SE Eu,	Among groups	2	317·8	3·438	50·03 %***
white-fl.],[C & SE Eu,	Among populations	25	356·82	3·054	44·44 %***
blue-fl.]	Within populations	100	38·05	0·381	5·54 %***
D. [Spain, white-fl.],	Among groups	3	387·62	4·006	57·79 %***
[Spain, blue-fl.], [C & SE	Among populations	24	287	2·545	36·72 %***
Eu, white-fl.], [C & SE	Within populations	100	38·05	0·381	5·49 %***
Eu, blue-fl.]					

blue-fl., blue-flowered populations; white-fl., white-flowered populations; C & SE Eu, central and south-eastern Europe; d.f., degrees of freedom; ***, $P < 0{\cdot}001$.

Intrapopulational variation was generally low in *V. suavis*, as seen both in low within-population component of total variance shown by AMOVA and in the neighbor-joining tree. Individuals from the same population mostly clustered together; only a few populations appeared somewhat heterogeneous (Fig. 3). Within population no. 25, sampled after flowering, the AFLP data clearly confirmed the occurrence of both the white- and blue-flowered individuals. Interestingly, much lower genetic variation was observed in the white-flowered populations than in blue-flowered ones (see the number of AFLP phenotypes summarized in Table 2). This pattern is supported also at the level of individual AFLP fragments. Private fragments were found in both blue-flowered morphotypes, but no such fragments were present in the Iberian white-flowered morphotype, and only a single one was present in the C & SE European white-flowered populations (Table 2). In addition, there were altogether 13 fragments present in the C & SE European blue-flowered populations that were absent in the white-flowered ones, but only one fragment when counting vice versa. The same number of AFLP fragments (13) was present in the Iberian blue-flowered populations but absent in the white-flowered ones, but only two fragments vice versa.

Morphometric analyses

Spearman correlation coefficients did not reveal highly correlated pairs of characters that could distort further analyses. PCA computed on all individuals of *V. suavis* (matrix A) and 20 characters showed three groupings: (1) Iberian white-flowered populations; (2) C & SE European white-flowered populations; and (3) blue-flowered populations from the whole area sampled (Fig. 5; only the two-dimensional graph is shown since the third axis did not contribute to further differentiation). Eigenvectors expressing correlations of the characters with the axes were rather low, implying that numerous characters contributed almost equally to the components and that none of them had a major impact (Table 1). In the PCA (matrix A) excluding the five characters expressing anthocyan pigmentation (LP, PP, KP, CP and CPSP) apparently less structure was seen. Nevertheless, the white-flowered populations from C & SE Europe were clearly separated from those from Spain (along axis 1), although they both overlapped with the blue-flowered samples [cf. Supplementary Information (2), available online]. The characters with the highest eigenvector values for the first axis were the shape of lamina base (LSA, −0·436; LSL, 0·399), insertion of bracteoles on peduncle (PL1/PL, 0·339), and width of anterior sepals (KAW, 0·333).

Two cluster analyses (matrix A, 20 characters) yielded results consistent with the PCA ordination (figures not shown). In the UPGMA dendrogram, the white-flowered populations formed two distinct clusters: samples from Spain grouped in a cluster formed at a higher distance level, indicating their morphological distinction, while those from C & SE Europe formed a cluster embedded in the blue-flowered populations. No geographic structuring among the blue-flowered populations was found; those from Spain were intermingled with the C & SE European ones. Clustering with the MISSQ algorithm divided the samples into two major groups: the blue-flowered populations and the white-flowered ones. Within the white-flowered group, populations from C & SE Europe and Spain constituted two separate clusters, but no geographic structuring was found within the blue-flowered group.

To test for morphological distinction of the four groups resolved by AFLP data and to see differentiation at the level of individual plants (rather than population averages as above), a series of CDA based on matrix B (figures not shown) was performed. CDA was run for the pairs of groups, both with (19 characters excluding CP, which was invariable within the groups) and without (14 characters) the pigmentation characters. The results were highly concordant with the PCA results. Clear distinction was achieved between the two white-flowered morphotypes (based on the characters PL1/PL, LSA, CPW, LSL and CPL), as well as between the two colour-morphotypes in Spain (characters CPL, CPW, CPSP, PP, KAW, LP and KAL). Some overlap, indicating less differentiation, was

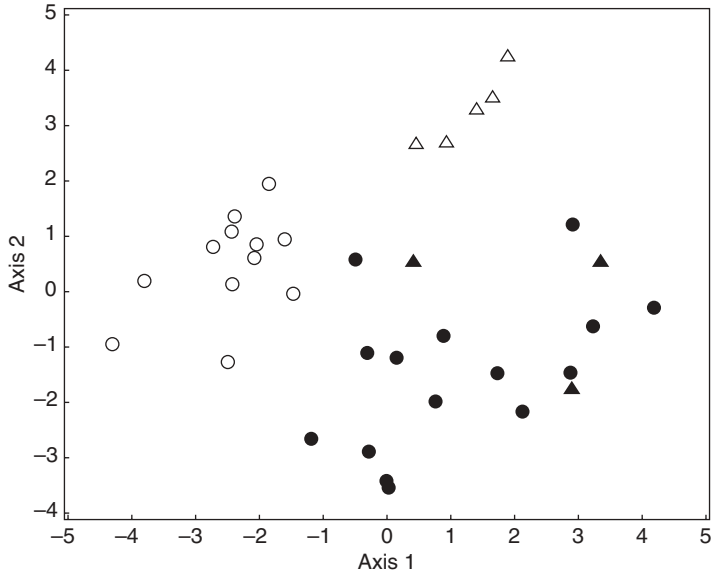

FIG. 5. Principal component analysis of 35 populations of *Viola suavis* based on 20 morphological characters. Geographic origin and colour-morphotypes are marked by different symbols: black circles, C & SE European blue-flowered populations; white circles, C & SE European white-flowered populations; black triangles, Iberian blue-flowered populations; white triangles, Iberian white-flowered populations. The first two axes explain 24·22 % and 19·14 % of the total variation.

observed between the blue-flowered populations from C & SE Europe and Spain (characters SFL, CPSP and SFN). Separation between the colour morphotypes in C & SE Europe was rather poor and based mainly on pigmentation characters (CPSP, PP and KP, but also SFL and PL1/PL). Variation of some of the best discriminating characters between the groups is shown in Supplementary Information (3) which is available online.

Estimation of pollen fertility

Male fertility, indicated by pollen stainability, was high in all populations of *V. suavis* examined, ranging from 87 % to 100 % in the blue-flowered populations, and from 88 % to 100 % in the white-flowered populations. These data agree with previously published findings in Hodálová *et al.* (2008).

DISCUSSION

Intraspecific variation in Viola suavis *and its phylogeographic interpretations*

The most pronounced intraspecific genetic split in *Viola suavis* that was inferred from AFLP markers was between populations from C & SE Europe and those from Spain. This strong geographic structuring can be considered an indication of a long-term isolation and survival in distinct glacial refugia. It can be assumed that *V. suavis* occupied a very different range during the last glaciation when the climate was much colder and drier in most parts of Europe than today (Frenzel *et al.*, 1992), and its genetic variation has been significantly shaped by these climate-induced range shifts (Hewitt, 2004). It has been shown that the distribution of deciduous oak forests, the main habitats for *V. suavis*, was largely fragmented and reduced to southern European refugia during the last glacial maximum (Brewer *et al.*, 2002; Petit *et al.*, 2002). Nevertheless, evidence that challenges the traditional paradigm of the tree-less landscape in central and eastern Europe during the last full-glacial is accumulating; it is becoming evident that populations of coniferous and some deciduous trees grew much further north and east than had been previously assumed (Willis and van Andel, 2004). It remains unclear to what extent the thermophilous oak trees were also able to survive in such regions; if so, they were most likely fragmented into small isolated patches, growing in valleys with favourable microclimatic conditions (Willis and van Andel, 2004). The distribution of *V. suavis* has presumably followed these oak range shifts; the species may have found favourable habitats during the cold periods, mainly in the southern regions, and migrated to the currently occupied area postglacially. Its current distribution is predominantly (sub-)Mediterranean (at least in its western parts; Bolòs and Vigo, 1990; Marcussen and Nordal, 1998), but is still rather poorly known, also due to misidentifications and frequent cultivation and spread by humans. It is often difficult to distinguish between anthropochorous and natural occurrences. The genetic patterns observed here suggest two major refugial areas. Since samples had not been taken in France and Italy, it was not possible to identify descendants from the putative Italian refugium, neither to investigate geographic boundaries between the genetic groups and to what extent they meet and admix at contact zones.

The genetic distinction that was observed between the Iberian and C & SE European populations might also, however, reflect a large geographic distance between the areas sampled, and thus the pattern observed may be

due to isolation by distance. Sampling conducted more equally and over the whole species range may therefore result in less geographic structuring and partly blur the deep split observed now. Mantel tests, however, showed that isolation by distance does not seem to be a major determinant of genetic structure in this species over larger geographic distances. Already at short distances, stochasticity in genetic variation is apparent, which may reflect both natural demographic processes (larger impact of genetic drift than gene flow) and human interference (cultivation and spread). It was concluded from these data that the genetic similarity and coherence of populations established in C & SE Europe is due not just to the recent gene flow and geographic proximity of the populations. A scenario is favoured in which they are descendants of relatively homogeneous source populations from glacial refugia that were distinct from those in the western part of the species range.

Many identical AFLP profiles were observed in *V. suavis*, even across distant populations, indicating that there is low genetic variation. Although the sampling of the other related species was much lower, it seems that they harboured more genetic variation than *V. suavis* (see Table 2). The low genetic variation may be due to several historical and contemporary factors and their combinations, such as the genetic impoverishment in glacial refugia, loss of variation during recolonizations (bottleneck and founder effect), limited gene flow and recombination due to the prevalence of selfing and clonal spread, and the vegetative propagation and spread by humans.

In contrast, Marcussen and Borgen (2000) found significant genetic variation in *V. suavis* from SE France while studying allozyme variation. Considering also the large morphological variation and wide ecological amplitude of the species in Europe, they concluded that there was a polytopic, maybe even polyphyletic (from different parental species) origin, and subsequent gene flow among the newly formed polyploid lineages and to related taxa. Despite the high variation, they did not reveal any geographic pattern within the area studied (France), in agreement with the present conclusions on the populations from C & SE Europe. It seems that genetic structure in this species can be seen only upon studying large areas that were putatively colonized from different source glacial populations. Thus, large-scale sampling over the whole distribution range is needed to disentangle the history of this species with respect to its polyploid origin and phylogeography.

Origin and geographic distribution of the white-flowered morphotypes

Molecular and morphological investigation of the peculiar white-flowered populations, considered as taxonomically ambiguous by several authors (see Introduction), provided a clear taxonomic assignment. Both the Iberian and C & SE European populations were unambiguously placed within the variation range of the typical (blue-flowered) *V. suavis* and thus can be considered conspecific with this species. Considering also the high pollen fertility [(88–)96–100 %] and the same ploidy level, the hypothesis of hybrid origin proposed by several authors (Valentine *et al.*, 1968: 272; Guinea Lopez and Ceballos Jimenez, 1974: 143; Kirschner and Skalický, 1990: 402; Suda, 2002: 214) can be rejected. Furthermore, AFLP and morphological analyses resulted in congruent patterns, showing that the two white-flowered morphotypes delimited here do not have a single origin but apparently evolved independently from the blue-flowered *V. suavis* in two distant areas. They are genetically distinct from each other and clearly differ in several morphological characters (mainly the position of bracteoles, shape of lamina base and petal size).

Little is known so far about the distribution of the white-flowered morphotypes of *V. suavis* in Europe, as they have been neglected (or misclassified) for a long time. In south-western Europe, the white-flowered *V. suavis* has been reported over the whole Iberian Peninsula (Muñoz Garmendia *et al.*, 1993: 284), and there are indications that is also occurs in south-eastern France (village of Taillet, SW of the town of Perpignan; Anonymous, 2008). Otherwise, they have been reported to exist only in central Europe in the Czech Republic (Kirschner and Skalický, 1990: 402; Suda, 2002: 214), Slovakia (Hodálová *et al.*, 2008; Mered'a *et al.*, 2008), and based on the present study also in the area of western Ukraine. Nevertheless, the occurrence of similar white-flowered morphotypes appears plausible also in other European countries. For instance, a unique, apparently introduced, morphotype of *V. suavis*, with pale violet to almost white corolla and a relatively dark spur, has been reported from Norway (Marcussen and Nordal, 1998). The white-flowered populations studied here are found mainly cultivated as ornamentals in gardens and parks. Escape from cultivation is quite common due to an efficient spread by rooting procumbent stolons and a high seed set per capsule. Subsequently, they often form extensive patches not only in man-made habitats, but also naturalized in semi-natural habitats close to settlements, growing in mesophilous grasslands, shrubberies and forest edges (pers. obs.).

The time, mode and exact place of origin of the white-flowered morphotypes, however, remain unclear. The highly reduced genetic diversity and absence of unique fragments in these individuals, along with the patterns of fragment sharing and population clustering, clearly demonstrate that the origin of the white-flowered populations is recent and within the typical blue-flowered *V. suavis*. Since the AFLP markers do not allow molecular dating, it can only be speculated as to whether their origin can be associated with the polyploid nature of *V. suavis* and triggered by genetic changes following the polyploidization event(s) (cf. Paun *et al.*, 2007) or whether they are much younger (maybe a few centuries old). Their establishment may have been associated with a local isolation and survival in small populations at range margins or in fragmented habitats, where rates of gene flow are reduced, genetic drift has a higher impact, and inbreeding and/or directional selection may be stronger (see, for example, Eckstein *et al.*, 2006; Eckert *et al.*, 2008). Nevertheless, the current geographic distribution and genetic variation have undoubtedly also been affected by human activity (cultivation,

vegetative propagation, and spread across relatively large distances).

Blue- (or violet)-flowered and white-flowered pigmentation morphotypes also occur within the subsection *Viola* in several other species, for example, in *V. alba*, *V. collina*, *V. odorata* and *V. thomasiana* (cf. Schmidt, 1961; Marcussen, 2003). The white-flowered morphotypes studied here, however, did not represent simple colour variants. Larger genetic divergence from the blue-flowered ones has been observed, which seems to be accompanied by (at least partial) reproductive isolation. Individuals of the different colour morphotypes sampled from the same or adjacent localities did not cluster by site, but grouped by morphotype, and no intermediate genotypes, which would indicate gene flow, were observed. No phenological differences were observed between the morphotypes, but further experiments (e.g. pollination studies) are needed to explore the potential reproductive barriers.

Taxonomic treatment of the white-flowered morphotypes and infraspecific concept of Viola suavis

The present study illustrates that a combination of detailed morphometric and AFLP analyses is a powerful approach for resolving infraspecific variation in *V. suavis*, a species reported as taxonomically critical with complicated polyploid evolution and infraspecific patterns (see Introduction). Thus, in addition to inferring the glacial history of *V. suavis*, the AFLP markers appear to be very promising for generating a sound infraspecific concept. Because sampling was limited in the present study, it would be premature to draw any taxonomic conclusions here, but once a complete genetic and morphological study of populations spanning the entire range is accomplished, long-term taxonomical controversies may be satisfactorily addressed. In the following, the two white-flowered morphotypes in Spain and C & SE Europe, which may deserve formal taxonomic recognition, are discussed. Despite growing in sympatry with their blue-flowered counterparts, they are genetically distinct, probably keep in reproductive isolation, and differ in other morphological characters besides just the pigmentation.

The Iberian white-flowered populations were assigned to the taxon previously described as *V. catalonica* (= *V. suavis* subsp. *catalonica*), although no material from the locus classicus was available and some discrepancies between the description in the protologue and the populations sampled occur. The type locality, Barcelona, El Pujolet (currently named as El Putxet) is located in an urban area of Barcelona, which is now a public park (Jardin del Turó del Putget) and there is no evidence of the presence of *V. catalonica* (P. Mered'a and I. Hodálová, pers. obs.). In the protologue of *V. catalonica*, the size of the posterior petals is reported to be 12 × 4–5 mm, while we found it to be in the range of (12·4–)14·1–16·8(–18·4) mm × (5–)6·7–8·7(–10·3) mm. It is not clear, however, whether the reported size refers to herbarium specimens or living plants and how many plants were investigated. Until now, no other detailed morphological description of *V. catalonica* has been published.

Viola alba is another species where colour variants or morphotypes have been recognized; traditionally these were assigned to different subspecies (*Viola* subsp. *alba* and subsp. *scotophylla*). Recent studies using isozyme markers, however, found no genetic distinction and disproved their formal recognition as two subspecies (Marcussen, 2003). There are examples also from other genera documenting how the taxonomic position of colour variants or morphotypes has been addressed by genetic data, either supporting or refusing their formal classification. Two bluish-flowered variants occur in the usually yellowish-flowered *Oxytropis campestris*: the Eastern Alpine assumed glacial relict subsp. *tiroliensis* and the northern Eurasian subsp. *sordida*. While AFLP data did not reveal distinction of the former, implying that the Alpine 'subspecies' does not represent a distinct entity, they did for the latter subspecies, supporting its taxonomic recognition (Schönswetter *et al.*, 2004).

Relationships within Viola *sect.* Viola *subsect.* Viola*, and phylogenetic potential of different genetic markers*

Viola sect. *Viola* subsect. *Viola*, which includes tetraploid and octoploid species, is supposed to be of an allopolyploid origin, with more recent polyploidization events giving rise to higher polyploids and resulting in complicated patterns of species relationships (Marcussen and Borgen, 2000). Reconstructing the evolutionary history of groups where polyploidization and reticulation have played major roles in evolution is not an easy task, and exploration of several marker systems appears crucial (Linder and Rieseberg, 2004; Vriesendorp and Bakker, 2005).

The ITS of nuclear ribosomal DNA has previously been used in violets and proven to be valuable in resolving relationships at subgeneric levels (cf. Ballard *et al.*, 1999; Ballard and Sytsma, 2000; Yockteng *et al.*, 2003; Malécot *et al.*, 2007). Nevertheless, the use of the highly repetitive nrDNA in taxa of allopolyploid and hybridogeneous origin may be problematic and risky due to the unpredictable intra- and interlocus sequence evolution and homogenization (Álvarez and Wendel, 2003). It still may be worth exploring the ITS sequence variation in such groups, but interpretations should be inferred carefully and in concert with other markers (see, for example, Lihová *et al.*, 2006). The present study indeed shows that the ITS region is of restricted use here, but at least it clearly supports a largely reticulated evolutionary pattern in the subsection (see Fig. 2).

The AFLP markers, on the other hand, provided resolution not only at the intraspecific level, but also seemed to allow inferences at the interspecific level. Both the AFLP data presented here and the results of allozymic studies (cf. Marcussen and Borgen, 2000; Marcussen *et al.*, 2001) showed that the studied species are genetically unambiguously differentiated (despite the excessive morphological variability and reported frequent interspecific hybridization), forming well-supported and distinct lineages. Upon further investigation into the proposed species relationships, however, we noted obvious discrepancies. The topologies of the trees that were inferred from these two markers greatly differ; in fact the only shared pattern is the close

relationship between tetraploid ($2n = 20$) *V. hirta* and octoploid ($2n = 40$) *V. ambigua*.

In conclusion, the difficulties in inferring the interspecific relationships in the subsection are apparently due to the complex reticulate history, which is difficult to disentangle even when using different molecular markers. We assume that low- or single-copy nuclear genes, which are less prone to sequence homogenization and better reflect biparental lineages (Mort and Crawford, 2004; Small *et al.*, 2004) may be particularly useful and provide more insights into the evolution of this group.

SUPPLEMENTARY INFORMATION

Supplementary information is available online at http://aob.oxfordjournals.org and contains (1) a correlogram of Mantel r_m per distance class based on AFLP data of *Viola suavis*; (2) a PCA based on populations of *V. suavis* and non-pigmentation morphological characters; and (3) variation in selected morphological characters in *V. suavis* depicted as boxplot graphs.

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LITERATURE CITED

Álvarez I, Wendel JF. 2003. Ribosomal ITS sequences and plant phylogenetic inference. *Molecular Phylogenetics and Evolution* **29**: 417–434.

Anonymous. 2008. *Fleurs du Roussillon (les plantes des Pyrénées-Orientales).* http://jeantosti.com/Fleurs.html. Accessed in March 2008.

Applied Biosystems. 2005. *AFLP® Plant mapping. Protocol*. Foster City, CA: PE Applied Biosystems.

Archibald JK, Mort ME, Crawford DJ, Santos-Guerra A. 2006. Evolutionary relationships within recently radiated taxa: comments on methodology and analysis of ISSR data and other hypervariable, dominant markers. *Taxon* **55**: 747–756.

Bakker FT, Chatrou LW, Gravendeel B, Pelser PB. 2005. *Plant species-level systematics: new perspectives on pattern and process*. Ruggell, Liechtenstein: Gantner Verlag.

Ballard Jr HE, Sytsma KJ. 2000. Evolution and biogeography of the woody Hawaiian violets (*Viola*, Violaceae): Arctic origins, herbaceous ancestry and bird dispersal. *Evolution* **54**: 1521–1532.

Ballard Jr HE, Sytsma KJ, Kowal RR. 1999. Shrinking the violets: phylogenetic relationships of infrageneric groups in *Viola* (Violaceae) based on internal transcribed spacer DNA sequences. *Systematic Botany* **23**: 439–458.

Becker W. 1910. *Violae Europaeae*. Dresden: Verlag von C. Heinrich.

Becker W. 1929. *Viola catalonica* W. Beck., sp. nova. *Cavanillesia* **2**: 43–44.

Bolòs O, Vigo J. 1974. Notes sobre taxonomía í nomenclatura de plantes, I. *Butlletí de la Institució Catalana d'Història Natural* **38**, Secció de Botànica 1: 61–89.

Bolòs O, Vigo J. 1990. Flora dels Països Catalans. *Vol*. 2. Barcelona: Editorial Barcino.

Bonin A, Bellemain E, Eidesen PB, Pompanon F, Brochmann C, Taberlet P. 2004. How to track and assess genotyping errore in population genetic studies. *Molecular Ecology* **13**: 3261–3273.

Brewer S, Cheddadi R, de Beaulieu JL, Reille M, Data contributors. 2002. The spread of deciduous *Quercus* throught Europe since the last glacial period. *Forest Ecology and Managment* **156**: 27–48.

Bussel JD, Waycott H, Chappill JA. 2005. Arbitrarily amplified DNA markers as characters for phylogenetic inference. *Perspectives in Plant Ecology, Evolution and Systematics* **7**: 3–26.

Cieślak E, Ronikier M, Koch MA. 2007. Western Ukrainian *Cochlearia* (Brassicaceae) – the identity of an isolated edge population. *Taxon* **56**: 112–118.

Eckert CG, Samis KE, Lougheed SC. 2008. Genetic variation across species' geographical ranges: the central-marginal hypothesis and beyond. *Molecular Ecology* **17**: 1170–1188.

Eckstein RL, Neill AO, Danihelka J, Köhler W. 2006. Genetic structure among and within peripheral and central populations of three endangered floodplain violets. *Molecular Ecology* **15**: 2367–2379.

Ehrich D. 2006. AFLPDAT: a collection of R functions for convenient handling of AFLP data. *Molecular Ecology Notes* **6**: 603–604.

Espeut M. 1999. Approche du genre *Viola* dans le Midi mediterranéen français. *Monde des Plantes* **464**: 15–38.

Evanno G, Regnaut S, Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**: 2611–2620.

Everitt BS. 1986. *Cluster analysis*, 2nd edn (2nd reprint). New York, NY: Gower, Halsted Press.

Excoffier L, Laval G, Schneider S. 2005. Arlequin ver. 3·0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**: 47–50.

Falush D, Stephens M, Pritchard JK. 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Molecular Ecology Notes* **7**: 574–578.

Francisco-Ortega J, Fuertes-Aguilar J, Gómez-Campo C, Santos-Guerra A, Jansen RK. 1999. Internal transcribed spacer sequence phylogeny of *Crambe* L. (Brassicaceae): molecular data reveal two Old World disjunctions. *Molecular Phylogenetics and Evolution* **11**: 361–380.

Frenzel B, Pécsi M, Velichko AA, eds. 1992. *Atlas of paleoclimates and paleoenvironments of the northern hemisphere. Late Pleistocene–Holocene*. Budapest/Stuttgart: Geographical Research Institute Hungarian Academy of Sciences/Gustav Fischer Verlag.

Gams H. 1925. Violaceae. In: Hegi G, Illustriete Flora von Mitteleuropa. Band V. München: J. F. Lehmanns Verlag, 585–657.

García LV. 2004. Escaping the Bonferroni iron claw in ecological studies. *Oikos* **105**: 657–663.

Greimler J, Jang C-G. 2007. *Gentianella stiriaca*, a case of reticulate evolution in the northeastern and eastern Central Alps. *Taxon* **56**: 857–870.

Guinea Lopez E, Ceballos Jimenez A. 1974. *Elenco de la flora vascular Española (Península y Baleares)*. Madrid: Icona.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Hewitt GM. 2004. Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London, Series B* **359**: 183–195.

Hodálová I, Mered'a Jr P, Mártonfi P, Mártonfiová L, Danihelka J. 2008. Morphological characters useful for the delimitation of taxa within *Viola* subsect. *Viola* (Violaceae): a morphometric study from the West Carpathians. *Folia Geobotanica* **43**: 83–117.

Huson DH, Bryant D. 2006. Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* **23**: 254–267.

Kirschner J, Skalický V. 1990. Violaceae Batsch. In: Hejný S, Slavík B, eds. *Květena České republiky 2*. Praha: Academia, 394–431.

Klecka WR. 1980. *Discriminant analysis*. (Sage University Papers, Series: quantitative applications in the social sciences, no. 19). Beverly Hills, CA: Sage Publications.

Koopman WJM. 2005. Phylogenetic signal in AFLP data sets. *Systematic Biology* **54**: 197–217.

Krzanowski WJ. 1990. *Principles of multivariate analysis*. Oxford: Clarendon Press.

Legendre P, Legendre L. 1998. *Numerical ecology*, 2nd edn. Amsterdam: Elsevier.

Lihová J, Tribsch A, Marhold K. 2003. The *Cardamine pratensis (*Brasssicaceae) group in the Iberian Peninsula: taxonomy, polyploidy and distribution. *Taxon* **52**: 783–802.

Lihová J, Shimizu KK, Marhold K. 2006. Allopolyploid origin of *Cardamine asarifolia* (Brassicaceae): incongruence between plastid and nuclear ribosomal DNA sequences solved by a single-copy nuclear gene. *Molecular Phylogenetics and Evolution* **39**: 759–786.

Lihová J, Kučera J, Perný M, Marhold K. 2007. Hybridization between two polyploid *Cardamine* (Brassicaceae) species in north-western Spain: discordance between morphological and genetic variation patterns. *Annals of Botany* **99**: 1083–1096.

Linder CR, Rieseberg LH. 2004. Reconstructing patterns of reticulate evolution in plants. *American Journal of Botany* **91**: 1700–1708.

Malécot V, Marcussen T, Munzinger J, Yockteng R, Henry M. 2007. On the origin of the sweet-smelling Parma violet cultivars (Violaceae): wide intraspecific hybridization, sterility, and sexual reproduction. *American Journal of Botany* **94**: 29–41.

Marcussen T. 2003. Evolution, phylogeography, and taxonomy within the *Viola alba* complex (Violaceae). *Plant Systematics and Evolution* **237**: 51–74.

Marcussen T, Borgen L. 2000. Allozymic variation and relationships within *Viola* subsection *Viola* (Violaceae). *Plant Systematics and Evolution* **223**: 29–57.

Marcussen T, Borgen L, Nordal I. 2001. *Viola hirta* (Violaceae) and its relatives in Norway. *Nordic Journal of Botany* **21**: 5–17.

Marcussen T, Nordal I. 1998. *Viola suavis*, a new species in the Nordic flora, with analyses of the relation to other species in the subsection *Viola* (Violaceae). *Nordic Journal of Botany* **18**: 221–237.

Marschall von Bieberstein FA. 1819. *Flora Taurico-Caucasica exhibens stirpes phaenogamas, in Chersoneso Taurica et regionibus Caucasicis sponte crescentes 3*. Charkouiae: Typis Academicis.

Martínez-Ortega MM, Delgado L, Albach DC, Elena-Rosselló JA, Rico E. 2004. Species boundaries and phylogeographic patterns in cryptic taxa inferred from AFLP markers: *Veronica* subgen. *Pentasepalae* (Scrophulariaceae) in the Western Mediterranean. *Systematic Botany* **29**: 965–986.

Mered'a Jr P, Hodálová I, Mártonfi P, Kolarčik V. 2006. [Report 17–22]. In: Mráz P, ed. Chromosome number and DNA ploidy level reports from Central Europe – 2. *Biologia (Bratislava)* **61**: 116–117.

Mered'a Jr P, Mártonfi P, Hodálová I, Šípošová H, Danihelka J. 2008. *Violales*. In: Goliašová K, Šípošová H, eds. *Flóra Slovenska VI/1*. Bratislava: Veda, 80–190.

Mort ME, Crawford DJ. 2004. The continuing search: low-copy nuclear sequences for lower-level plant molecular phylogenetic studies. *Taxon* **53**: 257–261.

Mort ME, Archibald JK, Randle CP, Levsen ND, O'Leary TR, Wiegang CM, *et al*. 2007. Inferring phylogeny at low taxonomic levels: utility of rapidly evolving cpDNA and nuclear ITS loci. *American Journal of Botany* **94**: 173–183.

Muñoz Garmendia F, Montserrat P, Laínz M, Aldasoro JJ. 1993. *Viola* L. In: Castroviejo S, Aedo C, Cirujano S, Laínz M, Montserrat P, Morales R, *et al*., eds. *Flora Iberica* 3. Madrid: Real Jardín Botánico, C.S.I.C., 276–317.

Nei M, Li WH. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of the USA* **76**: 5269–5273.

Paun O, Fay MF, Soltis DE, Chase MW. 2007. Genetic and epigenetic alternations after hybridization and genome doubling. *Taxon* **56**: 649–656.

Pelser PB, Gravendeel B, Van der Meijden R. 2003. Phylogeny reconstruction in the gap between too little and too much divergence: the closest relatives of *Senecio jacobaea* (Asteraceae) according to DNA sequences and AFLPs. *Molecular Phylogenetics and Evolution* **29**: 613–628.

Petit RJ, Brewer S, Bordács S, Burg K, Chelddadi R, Coart E, *et al*. 2002. Identification of refugia and post-glacial colonisation routes of European white oaks based on chloroplast DNA and fossil pollen evidence. *Forest Ecology and Managment* **156**: 49–74.

Pimentel M, Sahuquillo E. 2007. Infraspecific variation and phylogeography of the high-polyploid Iberian endemic *Anthoxanthum amarum* Brot. (Poaceae; Pooideae) assessed by random amplified polymorphic DNA markers (RAPDs) and morphology. *Botanical Journal of the Linnean Society* **155**: 179–192.

Podani J. 2001. SYN-TAX 2000. Computer programs for data analysis in ecology and systematics. User's manual. Budapest: Scientia Publishing.

Radford AE, Dickinson WC, Massey JR, Bell CR. 1974. *Vascular plant systematics*. New York, NY: Harper & Row.

SAS Institute. 2000. SAS OnlineDoc®, version 8 (available online). Cary, NC: SAS Institute.

Schmidt A. 1961. Zytotaxonomische Untersuchungen an europäischen *Viola*-Arten der Sektion *Nomimium*. *Oesterreichische Botanische Zeitschrift* **108**: 20–88.

Schönswetter P, Tribsch A, Niklfeld H. 2004. Amplified fragment length polymorphism (AFLP) reveals no genetic divergence of the Eastern Alpine endemix *Oxytropis campestris* subsp. *tiroliensis* (Fabaceae) from widespread subsp. *campestris. Plant Systematics and Evolution* **244**: 245–255.

Shaw J, Lickey EB, Beck JT, Farmer SB, Liu W, Miller J, *et al*. 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* **92**: 142–166.

Shaw J, Lickey EB, Schilling EE, Small RL. 2007. Comparison of the whole chroloplast genome sequences to choose noncoding regions for phylogenetic studies in Angiosperms: the tortoise and the hare III. *American Journal of Botany* **94**: 275–288.

Small RF, Cronn RC, Wendel JF. 2004. Use of nuclear genes for phylogeny reconstruction in plants. *Australian Systematic Botany* **17**: 145–170.

Sneath PHA, Sokal RR. 1973. *Numerical taxonomy*. San Francisco, CA: W. H. Freeman.

Stehlik I, Schneller JJ, Bachmann K. 2001. Resistance or emigration: response of the high-alpine plant *Eritrichium nanum* (L.) Gaudin to the ice age within the Central Alps. *Molecular Ecology* **10**: 357–370.

Suda J. 2002. *Viola* L. – violka. In: Hrouda L, Chrtek Jr J, Kaplan Z, Kirschner J, Kubát K, Štěpánek J, eds. *Klíč ke květene České republiky*. Praha: Academia, 207–214.

Swofford DL. 2001. *PAUP*: phylogenetic analysis using parsimony (* and other methods*). Sunderland, MA: Sinauer Associates.

Thorpe RS. 1976. Biometric analysis of geographic variation and racial varieties. *Biological Reviews* **51**: 407–452.

Valentine DH, Merxmüller H, Schmidt A. 1968. *Viola* L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM *et al*, eds. *Flora Europaea 2* (Rosaceae to Umbelliferae). Cambridge: Cambridge University Press, 270–282.

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, *et al.* 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407–4414.

Vriesendorp B, Bakker FT. 2005. Reconstructing patterns of reticulate evolution in angiosperms: what can we do? *Taxon* **54**: 593–604.

Willis KJ, van Andel TH. 2004. Trees or no trees? The environments of central and eastern Europe during the Last Glaciation. *Quaternary Science Reviews* **23**: 2369–2387.

Winkworth RC, Bryant D, Lockhart PJ, Havell D, Moulton V. 2005. Biogeographic interpretation of splits graphs: Least squares optimisation of branch lengths. *Systematic Biology* **54**: 56–65.

Yockteng R, Ballard HE, Jr., Mansion G, Dajoz I, Nadot S. 2003. Relationships among pansies (*Viola* section *Melanium*) investigated using ITS and ISSR markers. *Plant Systematics and Evolution* **241**: 153–170.

APPENDIX: PLANT MATERIAL OF *VIOLA* SPECIES USED IN THE PRESENT STUDY

The columns contain: 2*n* – chromosome number or DNA ploidy level (flow cytometric assessment); the number of individuals analysed for chromosome numbers/flow cytometry (CHN/FCM), AFLP, morphometric (Morph), and pollen viability (Pollen) analyses. In the last column Genbank accession numbers for the ITS sequences (ITS1-5·8S-ITS2) are listed. Data on chromosome numbers and DNA ploidy level marked with asterisks were taken from Hodálová *et. al.* (2008); all other data represent new records. Chromosomes were counted by L. Mártonfiová (pop. no. 27); DNA ploidy levels estimated by P. Mártonfi, P. Mered'a Jr, I. Hodálová and V. Kolarčik. Abbreviations of the collectors: JD, J. Danihelka, DD, D. Dítě, IH, I. Hodálová, DRL, D. R. Letz, PMA, P. Mártonfi, LM, L. Mártonfiová, PM_J, P. Mered'a Jr., PM_S, P. Mered'a Sen., JMM, Josep M. Montserrat, JS, J. Sádlo, HŠ, H. Šípošová. Eight populations of *V. suavis* where blue- and white-flowered plants were found in close proximity or intermigled are marked with superscripts[1–4]; they received different population numbers, but the superscript numbers indicate their co-occurrence. [#] – the five individuals sampled for AFLP from the respective locality (pop. no. 25) were collected after flowering, so their assignment to either the blue- (pop. no. 27) or the white-flowered (pop. no. 25) morphotype was not possible.

Taxon, pop. no.	Origin, collection data	2*n*	No. of plants analysed				ITS GenBank accession no.
			CHN/FCM	AFLP	Morph	Pollen	
V. alba Besser subsp. *alba* [including *alba* and *scotophylla* morphotypes]							
176	Romania, Locvei Mts, between the villages of Naidăş and Pojejena, 44°50′45″N, 21°34′14″E, 410 m, 1 April 2006; coll. IH, PM_J & DRL	~4*x* ~20	–/2	–	–	–	EU413914
179	Romania, Almăj Mts, 4·5 km SE of the village of Cozla, forest above the river Danube, 44°35′43″N, 22°01′44″E, 113 m, 3 April 2006; coll. IH & PM_J	–	–	3	–	–	EU413915
184	Romania, Almăj Mts, ca 1 km S of the village of Dubova, Cazanele Mari, above the cave Peştera Ponicova, 44°35′36″N, 22°15′17″E, 140 m, 5 April 2006; coll. IH & PM_J	~4*x* ~20	–/1	–	–	–	EU413916
188	Romania, Cerna Mts, the town of Băile Herculane, SE slopes above the spa, 44°53′18″N, 22°25′12″E, 171 m, 6 April 2006; coll. IH & PM_J	~4*x* ~20	–/1	3	–	–	EU413917
190	Romania, Mehedinţi Mts, NE of the village of Topleţ, slopes above the river Cerna, 44°49′01″N, 22°23′30″E, 130 m, 6 April 2006; coll. IH & PM_J	~4*x* ~20	–/1	3	–	–	EU413913
V. ambigua Waldst. & Kit.							
21	Slovakia, Devínska Kobyla Hills, city quarter of Bratislava-Devín, 1·2 km SW of the top of Devínska Kobyla Hill, 48°10′52″N, 16°59′12″E, 250 m, 8 April 2003; coll. IH & PM_J	8*x* = 40*	1/–	3	–	–	EU413933–35
149	Hungary, Gerecse Mts, village of Dág (SSE of town of Dorog), SE slope of Kecske-hegy Hill, 47°40′31″N, 18°42′36″E, 207 m, 14 April 2005; coll. IH & PM_J	8*x* = 40*	1/–	3	–	–	EU413936
151	Hungary, Gerecse Mts, village of Csolnok, Magos-hegy Hill (elevation point 317 m), 47°41′20″N, 18°42′10″E, 314 m, 14 April 2005; coll. IH & PM_J	8*x* = 40*	1/–	3	–	–	EU413937
V. collina Besser							
30	Austria, Lower Austria, town of Baden bei Wien, NE slope of Rauheneck Castle Hill, 48°00′34″N 16°12′25″E, 350 m, 15 April 2003; coll. IH	4*x* = 20*	2/–	3	–	–	EU413943 EU413944

Continued

APPENDIX. *Continued*

Taxon, pop. no.	Origin, collection data	2*n*	No. of plants analysed				ITS GenBank accession no.
			CHN/FCM	AFLP	Morph	Pollen	
127	Slovakia, Strážovské vrchy Mts, town of Nová Dubnica, Markovica Hill, 0·5 km SW of the summit, 48º55′30″N, 18º10′40″E, 425 m, 10 April 2004; coll. PM_J	$4x = 20$*	1/–	3	–	–	EU413941 EU413942
128	Slovakia, Strážovské vrchy Mts, village of Omšenie, W slope of Omšenská Baba Hill, 48º54′37″N, 18º14′07″E, 580 m, 11 April 2004; coll. PM_J & PM_S	$\sim 4x \sim 20$	–/1	–	–	–	EU413939 EU413940
130	Slovakia, Nízke Tatry Mts, village of Kráľova Lehota, near the settlement Hlboké, 49º02′06″N, 19º47′15″E, 605 m, 15 April 2004; coll. IH & PM_J	$4x = 20$*	1/–	3	–	–	EU413938
V. hirta L.							
221	Slovakia, Strážovské vrchy Mts, village of Omšenie, W slope of Omšenská Baba Hill, 48º54′37″N, 18º14′07″E, 500 m, 7 April 2007; coll. PM_J & PM_S	–	–	–	–	–	EU413950
145	Ukraine, village of Bytkiv (W of town of Nadvirna), 48°37′18″N, 24°29′32″E, 482 m, 10 April 2005; coll. IH & PM_J	$\sim 4x \sim 20$	–/1	3	–	–	EU413946
142	Ukraine, W of the village of Iza (N of town of Khust), NW slopes above the river Rika, 48º12′58″N, 23º18′44″E, 200 m, 9 April 2005; coll. IH & PM_J	$\sim 4x \sim 20$	–/1	3	–	–	EU413945
198	Romania, Semenic Mts, village of Caraşova, slope above the road no. 58 between the towns of Reşiţa and Anina, 45°12′01″N, 21°52′15″E, 250 m, 9 April 2006; coll. IH & PM_J	$\sim 4x \sim 20$	–/1	3	–	–	EU413949
158	Romania, Vâlcan Mts, valley of the river Jiu De Vest, above the settlement Câmpu Lui Neag, 45°17′58″N, 23°00′30″E, 837 m, 23 April 2005; coll. IH & PM_J	$\sim 4x \sim 20$	–/1	–	–	–	EU413947
156	Romania, Cozia Mts, NNE of the town of Călimăneşti, near the monastery Stânisoara, 45°18′02″N, 24°20′26″E, 743 m, 21 April 2005; coll. IH & PM_J	$\sim 4x \sim 20$	–/1	–	–	–	EU413948
V. odorata L.							
214	Czech Republic, Bohemia, 200–400 m S of the city quarter of Praha-Satalice, levee near the railway, 50°06′53″N, 14°34′40″E, 293 m, 25 April 2006; coll. IH, PM_J & JS (white-flowered morphotype)	$\sim 4x \sim 20$	–/1	–	–	–	EU413919
215	Czech Republic, Bohemia, 200–400 m S of the city quarter of Praha-Satalice, levee near the railway, 50°06′53″N, 14°34′40″E, 293 m, 25 April 2006; coll. IH, PM_J & JS (violet-flowered morphotype)	$\sim 4x \sim 20$	–/1	3	–	–	EU413921
146	Ukraine, N end of the town of Nadvirna, N of the bridge over the river Nadvirnyans'ka, 48°39′10″N, 24°34′49″E, 481 m, 10 April 2005; coll. IH & PM_J	$\sim 4x \sim 20$	–/1	3	–	–	EU413920
182	Romania, Locvei Mts, 5 km E of the village of Coronini, on the Danube river bank, 44°39′10″N, 21°43′29″E, 77 m, 4 April 2006; coll. IH & PM_J	$\sim 4x \sim 20$	–/5	–	–	–	EU413918
178	Romania, Mehedinţi Mts, Gura Văii, slopes above the tunnel Baba, 44°40′57″N, 22°31′13″E, 150–200 m, 3 April 2006; coll. IH, PM_J & DRL	$\sim 4x \sim 20$	–/1	3	–	–	EU413922
V. suavis M. Bieb. (blue-flowered populations)							
169[1]	Spain, Catalonia, Barcelona city, 'Antic Jardí Botanic de Barcelona', l'Estadi street, 41°22′02″N, 02°09′10″E, 113 m, 14 March 2006; coll. IH, PM_J, DRL & JMM	$\sim 8x \sim 40$	–/2	2	5	5	–
174	Spain, Catalonia, S of the town of Manlleu, N slope above the river Ter, 41°59′45″N, 02°16′46″E, 463 m, 17 March 2006; coll. IH, PM_J & DRL	$\sim 8x \sim 40$	–/3	3	9	9	EU413924
165	Spain, Catalonia, village of Argentona (NW of the town of Mataró), near the spring 'la font Picant', 41°32′49″N, 02°23′50″E, 98 m, 13 March 2006; coll. IH, PM_J & DRL	$\sim 8x \sim 40$	–/2	6	10	10	EU413927
212	Germany, town of Donauwörth, above the left Danube river bank, between the Michael-Imhof and Zirgesheimer streets, 48°42′53″N, 10°47′52″E, 408 m, 23 April 2006; coll. IH & PM_J	$\sim 8x \sim 40$	–/2	5	10	10	EU413925

Continued

APPENDIX. *Continued*

Taxon, pop. no.	Origin, collection data	2*n*	No. of plants analysed				ITS GenBank accession no.
			CHN/FCM	AFLP	Morph	Pollen	
211	Austria, W end of the town of Kalksburg, 48°08′02″N, 16°14′49″E, 290 m, 21 April 2006; coll. IH & PM_J	~8*x* ~40	–/10	5	10	10	EU413923
216	Czech Republic, Bohemia, ca 200 m S of the city quarter of Praha-Satalice, levee near the railway, 50°06′53″N, 14°34′40″E, 293 m, 25 April 2006; coll. IH, PM_J & JS	~8*x* ~40	–/1	5	7	7	–
11	Czech Republic, Moravia, Pavlovské vrchy Hills, village of Klentnice, Pálava Hill, 48°51′26″N, 16°38′37″E, 400 m, 4 April 2003; coll. IH & PM_J	8*x* = 40*	1/–	–	8	8	–
209[2]	Czech Republic, Moravia, town quarter of Brno-Maloměřice, Hády hill, 49°13′05″N, 16°39′58″E, 311 m, 20 April 2006; coll. IH, PM_J & JD	~8*x* ~40	–/1	4	12	12	–
4	Slovakia, Burda Hills, settlement of Kováčov, near the railway station, 47°49′25″N, 18°46′51″2E, 110 m, 2 April 2004; coll. IH & PM_J	8*x* = 40*	1/–	5	10	10	EU413926
129	Slovakia, Podunajská nížina Lowlands, town of Nitra, Šibeničný vrch Hill, Urbánkova street, 48°18′16″N, 18°04′30″E, 180 m,13 April 2004; coll. PM_J	8*x* = 40*	1/–	5	10	10	–
27[3]	Slovakia, Košická kotlina Basin, village of Turňa nad Bodvou, S foot of Turniansky hradný vrch Castle Hill, 48°36′23″N, 20°52′22″E, 205 m, 10 April 2003; coll. IH	8*x* = 40	2/–	3[#]	–	–	–
28	Slovakia, Východoslovenská nížina Lowlands, village of Hrušov, near the church, 48°26′10″N, 21°51′41″E, 105 m, 10 April 2003; coll. IH	8*x* = 40*	2/–	–	10	10	–
206[4]	Slovakia, city quarter of Bratislava-Devín, Brigádnická street 48°10′35″N, 16°59′59″E, 150 m, 11 April 2006; coll. PM_J	~8*x* ~40	–/3	6	19	19	–
125	Hungary, Pilis Mts, town of Esztergom, 0·4 km NW of the top of Vaskapu Hill, 47°47′21″N, 18°46′11″E, 340 m, 6 April 2004; IH & PM_J	ca8*x* = ca40* ~8*x* ~40*	1/1	–	10	10	–
122	Hungary, Sokoró Hills, SE of the village of Györújbarát, near the camp Ifjuságy, 47°35′12″N, 17°39′09″E, 242 m, 1 April 2004; coll. PM_J	~8*x* ~40*	–/1	4	10	10	–
148	Ukraine, 2 km E of the town of Mukacheve, near the road no. E 50, 48°28′21″N, 22°45′57″E, 164 m, 11 April 2005; coll. IH & PM_J	–	–	5	10	10	–
181	Romania, Locvei Mts, 5 km E of the village of Coronini, on the Danube river bank, 44°39′10″N, 21°43′29″E, 77 m, 4 April 2006; coll. IH & PM_J	~8*x* ~40	–/16	5	20	20	–
195	Romania, Şureanu Mts, saddle between the village of Băniţa and the town of Petroşani, 45°27′18″N, 23°18′43″E, 750 m, 8 April 2006; coll. IH & PM_J	~8*x* ~40	–/1	–	10	10	–
193	Romania, Oltenia, village of Bucovăţ (W of town of Craiova), slope above the SE end of the village, 44°17′33″N, 23°44′27″E, 130 m, 7 April 2006; coll. IH & PM_J	~8*x* ~40	–/9	4	10	10	–
Viola suavis M. Bieb. (white-flowered populations)							
171	Spain, Catalonia, between the village of Monistrol de Montserrat and the monastery of Montserrat, W of the monastery Sant Benet, near the spring Font dels Monjos, 41°36′35″N, 01°49′18″E, 457 m, 16 March 2006; coll. IH, PM_J & DRL	~8*x* ~40	–/1	5	10	10	–
170	Spain, Catalonia, village of Monistrol de Montserrat, the streets Carrer Cami del Pou, Carrer de Sant Sebastia, Carrer de Sant Jaume and Carrer de la Trinitat, 41°36′39″N, 01°50′28″E, 154 m, 15 March 2006 coll. IH, PM_J & DRL	~8*x* ~40	–/2	5	10	10	EU413930
168[1]	Spain, Catalonia, Barcelona city, 'Antic Jardí Botanic de Barcelona', l'Estadi street, 41°22′02″N, 02°09′10″E, 113 m, 14 March 2006; coll. IH, PM_J, DRL & JMM	~8*x* ~40	–/3	5	10	10	–
175	Spain, Catalonia, town of Manlleu, Carrer Torrent Magi street, 42°00′06″N, 02°17′13″E, 465 m, 17 March 2006; coll. IH, PM_J & DRL	~8*x* ~40	–/3	5	10	10	EU413931

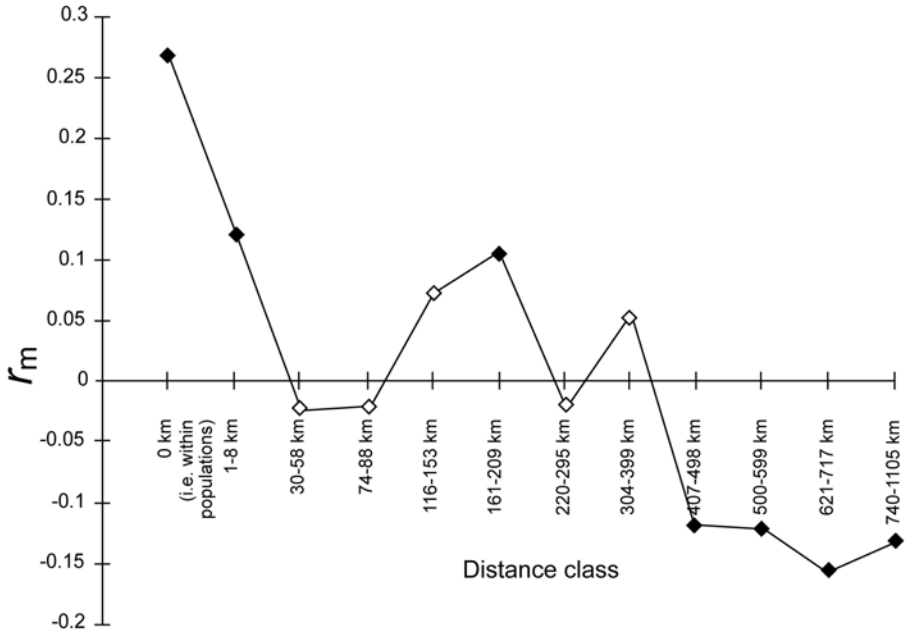
Continued

APPENDIX. *Continued*

Taxon, pop. no.	Origin, collection data	2*n*	No. of plants analysed				ITS GenBank accession no.
			CHN/FCM	AFLP	Morph	Pollen	
163	Spain, Catalonia, village of Argentona (NW of the town of Mataró), stone quarry 'pedrera de la Feu', 41°32′44″N, 02°23′13″E, 175 m, 13 March 2006; coll. IH, PM$_J$ & DRL	~8*x* ~40	–/2	2	2	2	–
207	Czech Republic, Moravia, town quarter of Brno-Rečkovice, near the Institute of Botany and Zoology, Masaryk University, Terezy Novákové street, 49°15′03″N, 16°34′25″E, 319 m, 20 April 2006; coll. IH, PM$_J$ & JD	~8*x* ~40	–/1	5	10	10	–
208[2]	Czech Republic, Moravia, town quarter of Brno-Maloměřice, Hády hill, 49°13′05″N, 16°39′58″E, 311 m, 20 April 2006; coll. IH, PM$_J$ & JD	~8*x* ~40	–/1	5	10	10	EU413929
205[4]	Slovakia, city quarter of Bratislava-Devín, Brigádnická street, 48°10′35″N, 16°59′59″E, 150 m, 11 April 2006; coll. PM$_J$	~8*x* ~40	–/2	5	6	6	–
7	Slovakia, Devínska Kobyla Hills, city quarter of Bratislava-Dúbravka, Brižite Hill, 48°11′49″N, 17°01′29″E, 240 m, 1 April 2003; coll. IH	~8*x* ~40*	–/1	5	10	10	–
18	Slovakia, Podunajská nížina Lowlands, city quarter of Bratislava-Petržalka, Dudova street, 48°06′59″N, 17°07′09″E, 135 m, 4 April 2003; coll. Hš & IH	8*x* = 40*	2/–	–	4	4	–
106	Slovakia, Podunajská nížina Lowlands, town of Nitra, Kalvária Hill, Pod Borinou street, 48°17′52″N, 18°05′22″E, 175 m, 13 April 2003; coll. PM$_J$	8*x* = 40*	2/–	5	12	12	–
25[3]	Slovakia, Košická kotlina Basin, town of Turňa nad Bodvou, S foot of Turniansky hradný vrch Castle Hill, 48°36′23″N, 20°52′22″E, 205 m, 10 April 2003; coll. IH	8*x* = 40*	2/–	2[#]	10	10	–
201	Slovakia, Košická kotlina Basin, Košice city, Botanical Garden of P. J. Šafárik University, Mánesova street, 48°44′05″N, 21°14′18″E, 227 m, 17 April 2003; coll. PMA & LM	8*x* = 40*	1/–	5	10	10	–
202	Slovakia, Košická kotlina Basin, Košice city, Humenská street, lawn in kindergarten, 48°42′23″N, 21°14′16″E, 249 m, 17 April 2003; coll. PMA & LM	8*x* = 40*	1/–	–	10	10	–
22	Slovakia, Liptovská kotlina Basin, town of Ružomberok, E of railway station, on the foot of Mních Hill, 49°05′00″N, 19°18′37″E, 490 m, 9 April 2003; coll. DD & IH	8*x* = 40*	2/–	5	6	6	EU413928
143	Ukraine, town of Khust, W foot of Castle Hill, 48°09′58″N, 23°17′46″E, 186 m, 9 April 2005; coll. IH & PM$_J$	~8*x* ~40	–/1	3	10	10	EU413932
147	Ukraine, village of Chertizh (E of the town of Khust), near the road no. P–03, 48°10′53″N, 23°15′52″E, 164 m, 11 April 2005; coll. IH & PM$_J$	~8*x* ~40	–/1	4	10	10	–
V. reichenbachiana Jord. ex Boreau							
220	Slovakia, Strážovské and Súľovské vrchy Mts, Mt Strážov, above the waterfalls, 48°57′43″N, 18°28′07″E, 900 m, 10 May 2006; coll. PM$_J$	–	–	–	–	–	EU413910–12

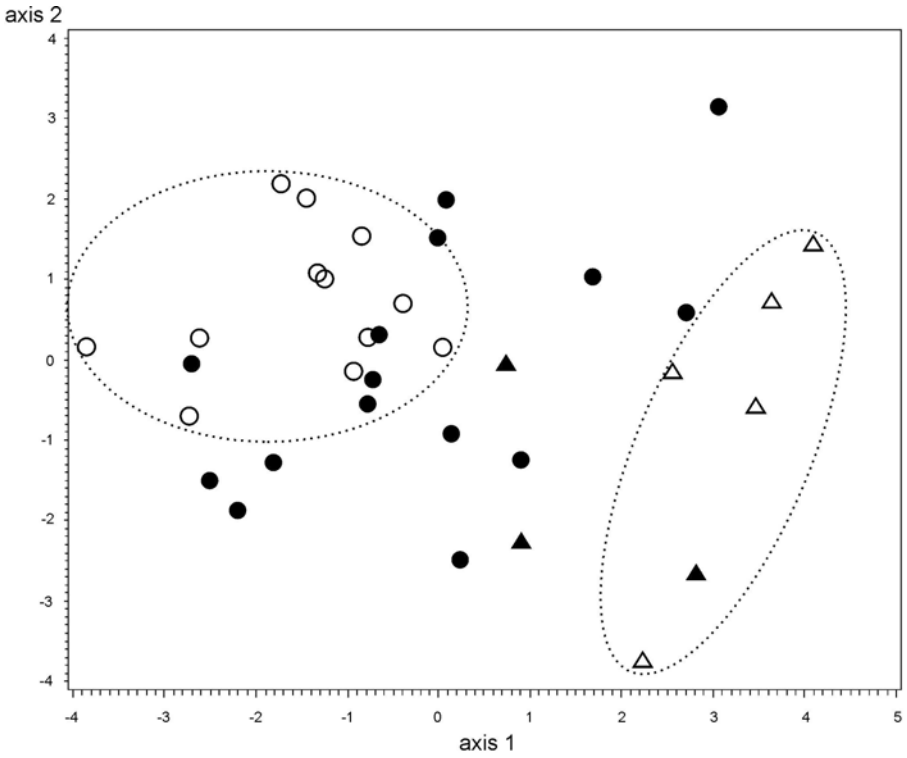
SUPPLEMENTARY INFORMATION 1

Correlogram of Mantel r_m per distance class based on AFLP data of *Viola suavis* from central and south-east Europe. Filled symbols denote r_m values significantly different from zero at $P = 0.05$ after sequential Bonferroni correction.



SUPPLEMENTARY INFORMATION 2

Principal component analysis of 35 populations of *Viola suavis* based on 15 non-pigmentation morphological characters. Geographic origin and colour morphotypes are indicated by different symbols: black circles, central and south-east European blue-flowered populations; white circles (encircled) – central and south-east European white-flowered populations; black triangles, Iberian blue-flowered populations; white triangles (encircled), Iberian white-flowered populations. The first two axes explain 28.60 % and 15.26 % of the total variation.



SUPPLEMENTARY INFORMATION 3

Variation in selected morphological characters in *Viola suavis*. SUBc, central and south-east European blue-flowered populations; SUWc, central and south-east European white-flowered populations; SUBs, Iberian blue-flowered populations; SUWs, Iberian white-flowered populations. Rectangles define the 25 and 75 percentiles; horizontal lines show the median; whiskers are from the 10 to 90 percentiles; asterisks show extreme values.