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Research Article

Genetic and morphological variation in *Viola suavis* s.l. (Violaceae) in the western Balkan Peninsula: two endemic subspecies revealed

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The Balkan Peninsula, with many endemic species, is known as one of the most important speciation and diversification centres in Europe. Here, we present a study of the western Balkan populations of the polymorphic European species, *V. suavis* s.l., which have been reported under the name *V. adriatica*, but their taxonomic status and position within the genus have remained uncertain. *Viola suavis* s.l. and nine close relatives sampled across Europe were subjected to molecular (sequencing of nuclear ribosomal internal transcribed spacers and amplified fragment length polymorphism), karyological and morphometric analyses. Our results revealed the presence of four allopatric, genetically and morphologically differentiated lineages within *V. suavis* s.l. in Europe, which are suggested here to be recognized at the subspecific rank. Populations from the western Balkans were segregated into two distinct entities: (1) those from north-western Croatia correspond to the previously recognized taxon, *V. suavis* subsp. *adriatica* and (2) those from southern Dalmatia (southern Croatia, southern Bosnia and Herzegovina, and south-western Montenegro) are described here as *V. suavis* subsp. *austrodalmatica* subsp. nov. The other two lineages of *V. suavis* s.l., which both harbour blue- and white-flowered morphotypes, occur in central and eastern Europe (*V. suavis* subsp. *suavis*) and in north-eastern Spain (plants provisionally treated as *V. suavis* 'Spain'). The AFLP and morphological data indicate gene flow between the nominate subspecies and *V. suavis* subsp. *adriatica* in a few localities. The distribution of the two western Balkan subspecies is discussed and an identification key to the *V. suavis* subspecies in Europe is presented.

Key words: AFLP, flow cytometry, ITS sequences, multivariate morphometrics, *Viola* subsect. *Viola*, western Balkans

Introduction

One of the most important centres of European and Mediterranean biodiversity is the Balkan Peninsula (Turrill, 1929; Polunin, 1997; Kryštufek & Reed, 2004), which harbours extremely rich and diversified fauna and flora (for examples, see Griffiths *et al*., 2004). Approximately 6530 plant species are represented in the area, of which about one-third are endemic (Horvat *et al*., 1974; Polunin, 1997). Such high species diversity can be explained by several specific features of this area (Polunin, 1997; Reed *et al*., 2004; Stefanović, *et al*., 2008): (1) a geographic position at the transition of different floral and faunal provinces; (2) a relatively large and topographically very diverse terrain, with high climatic, geological and edaphic complexity; and (3) the relatively high environmental stability throughout the geologic history – during the Pleistocene glaciations, the Balkan Peninsula served as one of the most important European refugial areas (e.g. Petit *et al*., 2003; Hewitt, 2004; Tzedakis, 2004).

Although the importance of the floristic and faunal diversity in the Balkan Peninsula has been long recognized, its patterns and processes are still only weakly understood. Until the 1980s, the biodiversity had been studied mainly through morphology and karyology. Since the 1990s, there have been an increasing number of studies that also assess the Balkan biota from the perspective of its genetic variation (e.g. Krystufek *et al*., 2007; Stefanović *et al*., 2008). However, there is still a lack of literature focusing on Balkan endemics that use a combined approach of morphometrics, karyology and molecular markers. In recent years, such studies have been performed only in a few herbaceous species groups – for example, *Cardamine* (Kučera *et al*., 2008, 2010), *Onosma* (Kolarčik *et al*., 2010; Kolarčik *et al*., in prep.), *Pilosella* (Šingliarová *et al*., 2011), and *Veronica* (Bardy *et al*., 2010, 2011).

Our study focused on another representative of the herbaceous endemics of the Balkan Peninsula, *Viola adriatica*

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Freyn from the *V. suavis* complex, which is among the less-known species of the European taxa of *Viola* subsect. *Viola*. This subsection includes approximately 25 species distributed in the Mediterranean area and the temperate and subtropical regions of Eurasia. Eleven species occur in Europe (including the Caucasus); the remainder grow in Asia (from Turkey to Japan) (Marcussen & Borgen, 2000; Dinç *et al*., 2003; Malécot *et al*., 2007; Hodálová *et al.*, 2008). Two chromosome numbers, 2n = 20 and 2n = 40, are known in the species of *Viola* subsect. *Viola*; individuals with 2n = 20 are hereafter referred to as diploids and those with 2n = 40 as tetraploids (but see Marcussen & Borgen, 2000; Van den Hof *et al*., 2008; Marcussen *et al.*, 2011).

Most authors (see below) have treated *V. adriatica* as a member of the widespread and polymorphic species, *V. suavis* s.l. *Viola suavis* s.l. is distributed in the Mediterranean and sub-Mediterranean region from Morocco, eastwards to the Caucasus and Ural regions (Fig. 1A); however, due to cultivation, its distribution area has expanded also to some parts of western, central and northern Europe (Marcussen & Nordal, 1998). In the Caucasus and the Middle East, *V. suavis* Marschall von Bieberstein (1819) is often confused with *V. sintenisii* W. Becker and *V. odorata* Linnaeus, and, therefore, a re-examination of their distribution in this area is needed (Marcussen *et al*., 2005; Marcussen, 2011 in litt.). The main habitats of *V. suavis* s.l. are dry grasslands, shrubs and open, deciduous woods; it frequently occurs also in man-made or man-influenced habitats, such as gardens, parks and cemeteries. *Viola suavis* s.l. is morphologically an extraordinarily variable species, and its intraspecific classification is still in dispute. It is assumed that *V. suavis* s.l. has an allopolyploid origin from *V. pyrenaica* Ramond ex de Candolle, a diploid (2n = 20) distributed in mountain ranges from the Atlas and Cantabria to the Caucasus (Dakskobler & Peljhan, 2007), and another, so far unidentified, diploid (Marcussen & Borgen, 2000; Hepenstrick, 2009).

More than 40 taxa have been attributed to *V. suavis* s.l. Currently, however, none of them is widely accepted (e.g. Marcussen & Nordal, 1998). In a previous study (Mereďa *et al*., 2008) we revealed that two allopatric and genetically differentiated major lineages of *V. suavis* s.l. occur in Europe: one in north-eastern Spain (NE Spain, hereafter) and the other in central and south-eastern Europe (C & SE Europe, hereafter). We also showed that both lineages are represented by two colour morphotypes (sublineages), the blue- and the white-flowered ones, and that the white-flowered morphotypes occurring in the two distant areas evolved independently from the typical, blue-flowered populations.

Viola adriatica has been described by J. F. Freyn from the Croatian seaside, near the town of Buccari [Bakar] (Freyn, 1884). This taxon has been variously interpreted on morphological grounds either as (1) a well-established species (e.g. Becker, 1929; Merxmüller, 1982), (2) an infraspecific taxon of *V. suavis* s.l. (e.g. Gams, 1925; Haesler, 1975), (3) identical to *V. suavis* s.str. (Schmidt, 1961), (4) intermediate between *V. suavis* s.l. and *V. alba* Besser, perhaps being of a hybrid origin (Valentine *et al*., 1968) or (5) as a glabrous derivative of *V. alba* (Becker, 1909: 20). Most recently, authors dealing with the Balkan flora have continued to use the original species-level treatment and have considered *V. adriatica* as an endemic to the Illyrian–Adriatic region (e.g. Domac, 1994; Šegulja, 1997; Milović, 2002; Nikolić, 2009). All the above-mentioned opinions were based on the traditional taxonomy, and thus far, *V. adriatica* has not been evaluated using a biosystematic approach. Consequently, this taxon remains one of the least-known European violets of the subsection. Characters that have been considered diagnostic for *V. adriatica* are an absence of indument, narrow stipules and triangular-cordate laminas. This species has been reported along the Adriatic seaside from the provinces of Gorizia and Trieste in north-eastern Italy (NE Italy, hereafter), Slovenia, Croatia (from islands and the coastal mainland), Bosnia and Herzegovina, to Montenegro (Fig. 1B; Pospichal, 1897; Becker, 1929; Merxmüller, 1982; Rakar, 2008; Nikolić, 2009). It can be found only in the Mediterranean climate area from the sea level up to the colline (montane) belt (however, Degen, 1937, gave its occurrence up to 1400 m a.s.l.). It inhabits rocky karst places, dry pastures, shrubberies, open deciduous or mixed forests, and man-influenced habitats, such as parks, cemeteries, gardens and lawns.

The aim of the present study was to examine the genetic, karyological and morphological variation of *V. adriatica* and its closest relatives from Europe. The main questions addressed by this study were as follows: (1) What are the phylogenetic positions and relationships of the western Balkan populations to the other European taxa from *Viola* subsect. *Viola*? (2) Are the western Balkan populations of *V. suavis* s.l. (recognized as the separate species, *V. adriatica*) genetically, morphologically and/or karyologically differentiated from the other populations of *V. suavis* s.l.? Are they homogeneous, or do they possess some variation? (3) Is there any support for the recognition of *V. adriatica* as a separate taxonomic entity?

To address these questions, we used a combination of molecular, karyological and morphometric approaches, which have proven to be powerful tools in similar studies of other plant genera (e.g. Kučera *et al*., 2008; Španiel *et al*., 2011) and has also been successfully applied in our previous study of *V. suavis* s.l. (Mereďa *et al*., 2008). As molecular markers, we used sequencing of nuclear ribosomal internal transcribed spacers (ITS of nrDNA) and amplified fragment length polymorphism (AFLP), which have been used in violets by many authors resolving relationships at both subgeneric (ITS, e.g. Malécot *et al*., 2007; Conesa *et al*., 2008) and subspecific levels (AFLP markers, e.g. Cieślak *et al*., 2006; Eckstein *et al*., 2006).

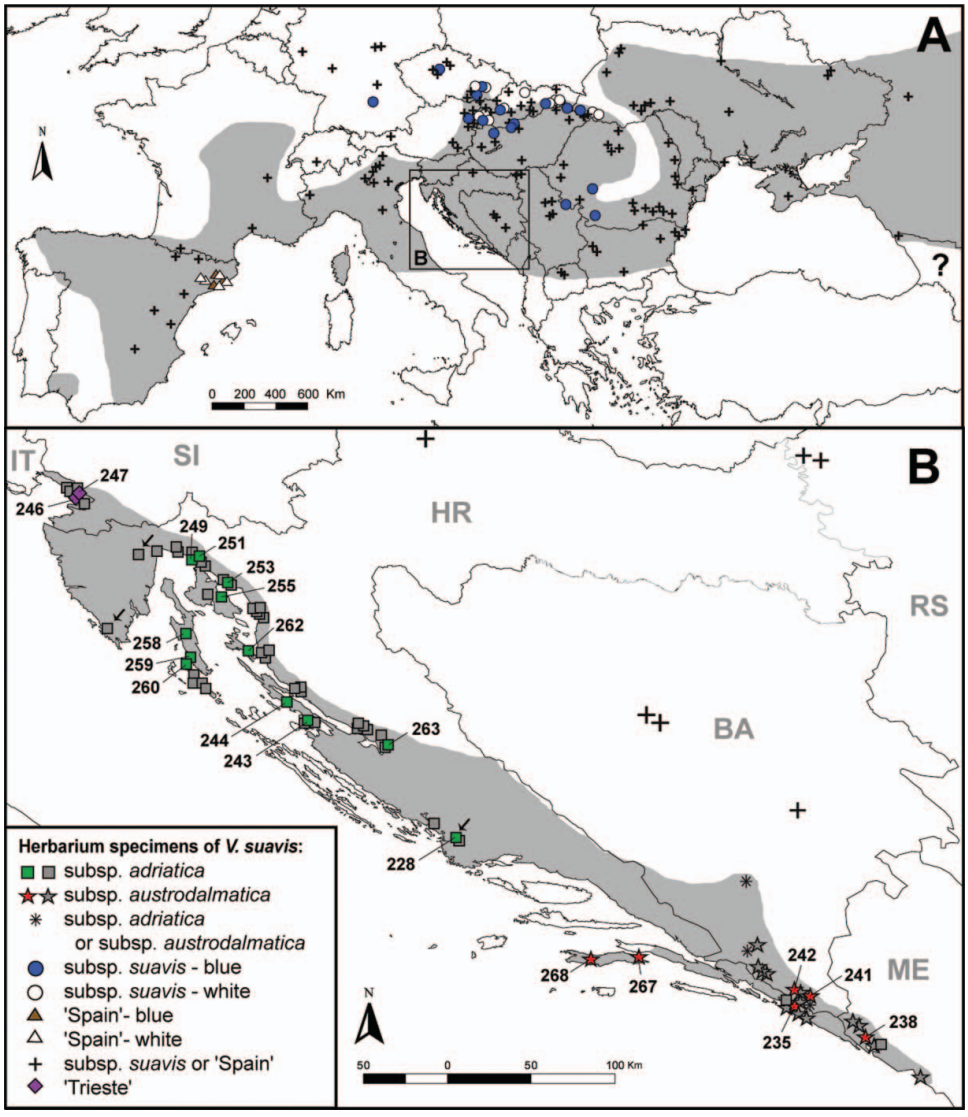

Fig. 1. Map of the sampling sites and geographic distribution of the studied taxa of *Viola suavis* s.l. based on field observations and herbarium records. Herbarium specimens investigated with uncertain identity (i.e. those with characters not well maintained for reliable determination) are given as symbols '+' (= blue- and white-flowered populations of *V*. *suavis* subsp. *suavis* or *V*. *suavis* 'Spain') or '*' (= populations of *V*. *suavis* subsp. *adriatica* or *V*. *suavis* subsp. *austrodalmatica*). **A**, overview of the study area; the area of the assumed native European distribution range of *V*. *suavis* s.l., as based on herbarium records and data in the literature (cf. Marcussen & Nordal, 1998), is shaded; doubtful records are labelled '?'; **B**, detailed view of the western Balkans; the distribution range of *V*. *suavis* subsp. *adriatica* and *V*. *suavis* subsp. *austrodalmatica*, as based on herbarium records (cf. Appendix 10, see supplementary material, which is available via the Supplementary Content table of the article's online page at http://dx.doi.org/10.1080/14772000.2011.603903) and literature data (cf. Pospichal, 1897; Becker, 1929; Merxmüller, 1982; Rakar, 2008; Nikolić, 2009), is shaded; the populations of presumably hybrid origin between *V*. *suavis* subsp. *adriatica* and the blue-flowered morphotype of *V*. *suavis* subsp. *suavis* are marked with arrows; populations with numbers represent those included in our analyses (see Appendix 1, supplementary material, which is available via the Supplementary Content table of the article's online page at http://dx.doi.org/10.1080/14772000.2011.603903).

Materials and methods

Plant material

The sampling focused on the area of the western Balkan Peninsula where *Viola adriatica* populations have been reported. Information on the distribution of *V*. *suavis* s.l. (incl. *V*. *adriatica*) in the western Balkans was collected from the following sources: (a) the BP, BRA, BRNM, BRNU, CL, KRAM, LI, LW, PR, PRC, SAV, SLO, W, WU, ZA and ZAHO herbaria (acronyms according to Holmgren *et al*., 1990); (b) published literature sources, including all of the regional Floras of the Balkan countries and the floristic database, Flora Croatica Database (Nikolić, 2009); and (c) field observations. The morphotype corresponding to

V. adriatica has not been reported from other areas of Europe (e.g. from the Apennine Peninsula or central Balkans). Altogether, 20 populations covering the entire range of *V. adriatica* (Fig. 1B), were sampled in 2009, including its type locality. Primarily, we aimed to sample indigenous populations; only in one case (pop. nr. 267), did we also collect individuals that were apparently in cultivation.

The populations sampled from the western Balkans were compared with those of *V. suavis* s.l. from NE Spain and C & SE Europe that were analysed in our previous study (Mereďa *et al*., 2008). Finally, all of the studied populations of *V. suavis* s.l. were compared with the herbarium material of *V. suavis* s.str. from the area of its type locality deposited in LE (the village of Mereffi, near Kharkov in Ukraine). The lectotype selected by Nikitin (1998) (the village of Mereffi [Merefa], near Kharkov, coll. in 1816 by M. Bieberstein) was not available, as it could not be found. The morphometric analyses included only populations of *V. suavis* s.l., whereas the karyological and molecular analyses were based on a broader taxon sampling; along with *V. suavis* s.l., representative samples of its nine closest relatives from *Viola* subsect. *Viola* that occur in Europe were analysed (Appendix 1, see supplementary material, which is available online).

Details on the origin of the material used are given in Appendix 1 (see supplementary material) and Fig. 1. All of the voucher specimens were deposited at the SAV herbarium.

Karyological analyses

Plants collected in the field were cultivated in the greenhouse of the Institute of Botany, Slovak Academy of Sciences, Bratislava, Slovakia, and their DNA ploidy levels were estimated using flow cytometry (FCM). We focused on the populations that were used in our molecular and/or morphometric analyses and were not karyologically analysed in our previous study (Mereďa *et al*., 2008) (Appendix 1, see supplementary material, which is available via the Supplementary Content table of the article's online page at http://dx.doi.org/10.1080/14772000.2011.603903). First, samples of reference plants with known chromosome numbers (*V. suavis*, pop. 4: Slovakia, settlement of Kováčov; 2n = 40, cf. Appendix 1) were analysed simultaneously with an internal DNA reference standard (*Bellis perennis* Linnaeus, 2C = 3.38 pg; Schönswetter *et al*., 2007), and the ratio of their G_0/G_1 peak positions was recorded. The DNA ploidy levels of the analysed plants (of unknown chromosome number) were then assessed by their peak position relative to the DNA reference standard peak. *Bellis perennis* was selected as an internal reference standard because its genome size does not differ markedly from those of both diploid and tetraploid *Viola* samples, and the histogram peaks of the standard and samples did not overlap.

Sample preparation followed the simplified two-step nuclei isolation procedure using Otto buffers (Doležel *et al*., 2007). In order to avoid the problems associated with sliming and the interference of secondary metabolites encountered in *Viola*, the use of the petioles of very young and still cigar-shaped leaves proved to be the best tissue for a successful analysis. Fresh tissues of the *Viola* sample (~ 0.5 cm^2), together with an appropriate amount of fresh leaf tissue of the internal reference standard, were chopped using a sharp razor blade in a plastic Petri dish containing 0.9 ml of ice-cold Otto I buffer (0.1 M citric acid monohydrate, 0.5% Tween 20). The resulting suspension of nuclei was filtered through a 42-μm nylon mesh and stored for 10–15 minutes (min) at room temperature. The flow-through fraction was stained with 0.9 ml of Otto II buffer (0.4 M $Na_2HPO_4 \times 12H_2O$) supplemented with ß-mercaptoethanol and the AT-selective fluorochrome, 4′,6-diamidino-2-phenylindole (DAPI), at final concentrations of 2 μg/ml and 4 μl/ml, respectively. After incubation for 5–15 min at room temperature with occasional shaking, the fluorescence intensities were analysed using a Partec CyFlow ML flow cytometer (Partec GmbH, Germany) equipped with an HBO 100W mercury arc lamp. The resulting histograms were evaluated using Partec FloMax software (Partec GmbH, Germany). For each measurement, the coefficients of variation (CVs) of the standard and the analysed sample were calculated. If the CV of the G_0/G_1 peak of the standard or (pooled) sample(s) exceeded the 4.5% threshold or the quality of the histograms was otherwise low (i.e. high background), the analysis was discarded and the sample was re-analysed.

Molecular analyses

ITS sequencing and data analyses. Genomic DNA was extracted from silica gel-dried leaf samples using the DNeasy Plant Mini Kit (Qiagen). Newly generated ITS sequence data (ITS1-5.8S-ITS2 region; 24 sequences) of nuclear ribosomal DNA (nrDNA) were combined with already published sequences of taxa from *Viola* subsection *Viola* (except for the cultivars). Among the published sequences we included only those which did not contain numerous intra-individual single-site polymorphisms and could be unambiguously assigned to the particular taxon (one sequence from Ballard *et al*., 1999; one from Yockteng *et al*., 2003; 10 from Malécot *et al*., 2007; five from Conesa *et al*., 2008; 41 from Mereďa *et al*., 2008; for details see Appendix 1).

Altogether 79 individuals of the following ten ingroup taxa (i.e. from *Viola* subsection *Viola*) were analysed: *V. alba* subsp. *alba*, *V. alba* subsp. *dehnhardtii* (M. Tenore) W. Becker, *V. ambigua* Waldstein & Kitaibel, *V. collina* Besser, *V. hirta* Linnaeus, *V. jaubertiana* Marès & Vigineix, *V. odorata*, *V. pyrenaica*, *V. suavis* s.l., and *V. thomasiana* Songeon & E.P. Perrier. Thus, all of the taxa

from the subsection *Viola* occurring in Europe (including the Caucasus), except three – *V. alba* subsp. *cretica* (Boissier & Heldreich) Marcussen (endemic to Crete, 2n = 20; Marcussen, 2003), *V. chelmea* Boissier & Heldreich (incl. *V. dinarica* Trinajstić; a montane to alpine species from the *V. libanotica* group native to former Yugoslavia and Greece, 2n = 20; Schmidt, 1961), and *V. sintenisii* (the west Asian species, 2n = 20; Marcussen *et al*., 2005) – were included. *Viola reichenbachiana* Jordan ex Boreau, which belongs to the phylogenetically closest subsection, *Viola* subsect. *Rostratae* (Kupffer) W. Becker (cf. Malécot *et al*., 2007; Van den Hof *et al*., 2008; Yoo & Jang, 2010; Marcussen *et al*. 2011) was chosen as an outgroup.

The amplification protocol and PCR cycling conditions followed those described in Mereďa *et al*. (2008); for some problematic samples we used the following touchdown PCR cycle described by Chapman *et al*. (2007): 95°C for 3 min; 10 cycles of 30 seconds (s) at 94°C, 30 s at 60°C (each cycle decreasing by 1°C), and 45 s at 72°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C, 45 s at 72°C and then a final extension at 72°C for 20 min, and an incubation at 4°C. Purified PCR products (NucleoSpin PCR clean-up kit, Macherey-Nagel) were submitted to the BITCET Consortium at the Department of Molecular Biology, Comenius University, Bratislava for cycle-sequencing reactions and analyses using an ABI 3130xl Genetic Analyser.

ITS sequence data were edited and assembled manually using BioEdit (version 7.0.5.3; Hall, 1999). The electropherograms were inspected for the presence of overlapping peaks, indicating intra-individual single-site polymorphisms. IUPAC ambiguity codes were used to code such polymorphic positions.

The ITS alignment, excluding the outgroup, comprised 79 sequences and 609 nucleotide positions, out of which 56 sites were variable and 51 were parsimony informative. Intra-individual single-site polymorphisms were recorded in eight sequences (one to three positions per sequence; altogether 18 positions). Only one of the detected polymorphic positions has an additive pattern among the analysed taxa or clades. Thus, all those eight sequences were included into the phylogenetic analysis without a risk of collapsing a hierarchical structure of the resulting tree. The alignment contained only a few short indels; eight 1-bp and one 2-bp indels were recorded. Five of them were coded as additional binary characters (following the simple gap coding according to Simmons & Ochoterena, 2000), the others were treated as missing data because of their ambiguity.

Both maximum parsimony (MP; PAUP* version 4.0b10; Swofford, 2001) and Neighbor-Net (NN; SplitsTree version 4.11.3; Huson & Bryant, 2006) analyses were conducted to reconstruct the phylogenetic relationships among the analysed taxa. Heuristic searches used in the MP analysis were performed with the following settings: gaps treated as missing data, single-site polymorphisms as uncertainties, tree construction with stepwise addition, 100 replicates with random taxon addition, tree bisection-reconnection (TBR) branch swapping, keeping multiple trees found during branch swapping (MULTREES option in effect) and saving no more than 500 trees of ≤69 steps (based on initial heuristic searches) in each replicate. For character state optimization, the accelerated character transformation (ACCTRAN) option was used. Bootstrap analyses were performed using 10 000 resamplings with the fast-heuristic search as implemented in PAUP*. Neighbor-Net was constructed using uncorrected *P* distances.

AFLP fingerprinting and data analyses. AFLP data were generated from 192 individuals sampled from 31 populations (four to seven individuals per population) of *V. pyrenaica* and *V. suavis* s.l. (Appendix 1). *Viola suavis* s.l. was represented by 20 populations from the western Balkans and eight populations that originated from NE Spain and C & SE Europe. The latter were chosen from our previous study (Mereďa *et al*., 2008), representing four main (genetic and morphological) groups detected in this species – the blue- and white-flowered morphotypes from NE Spain and the blue- and white-flowered morphotypes from C & SE Europe.

The AFLP procedure (Vos *et al*., 1995) followed the protocol described in detail in Mereďa *et al*. (2008), including the same three selective primer combinations that gave the best profiles with respect to their clarity and reproducibility. To estimate the reproducibility of the AFLP data, we included 15 replicates (distributed across different populations; 8% of the final dataset) and calculated the error rate (Bonin *et al*., 2004) expressed as the ratio of mismatches (scoring 1 vs. 0) over matches (1 vs. 1) in these replicated samples. The AFLP products were submitted for fragment analysis to the BITCET Consortium, Department of Molecular Biology, Comenius University, Bratislava (ABI 3130xl). The size calibration was done using the internal size standard GeneScan-500 LIZ® (Applied Biosystems).

AFLP trace files were read and analysed using the DAx software (Van Mierlo Software Consultancy, the Netherlands). We recorded markers ranging between 50 and 500 bp that could be scored unambiguously and coded them as present (1) or absent (0). A binary data matrix was generated.

Three data matrices were assembled: one included both *V. pyrenaica* and *V. suavis* s.l. samples (the '*V. pyrenaica*+*suavis* s.l. matrix'; 192 individuals), the second matrix comprised *V. suavis* s.l. individuals ('*V. suavis* s.l. matrix'; 172 individuals), and the third comprised only *V. suavis* s.l. individuals from C & SE Europe, NE Italy (province of Trieste) and north-western Croatia (NW Croatia, hereafter) ('*V. *suavis*+Trieste+*adriatica* matrix'; 124 individuals). The overall genetic structure and relationships among the studied populations and accessions were first explored by Neighbor-Joining clustering (NJ; Saitou & Nei, 1987; only the '*V. pyrenaica*+*suavis* s.l. matrix') based on

Sørensen's similarities transformed into a distance matrix (d = 1−s), and by principal coordinate analysis (PCoA; Krzanowski, 1990; all three matrices), using Jaccard's coefficient for calculating pairwise genetic similarities. Both analyses were performed using the FAMD 1.108 beta software (Schlüter & Harris, 2006). Support for each node in the NJ tree was assessed by bootstrap analyses with 5000 replicates. Furthermore, the network-generating Neighbor-Net analysis (NN; all three matrices) based on the Nei & Li (1979) genetic distances was conducted using SplitsTree 4.11.3 (Huson & Bryant, 2006), which can shed light on some possible conflicts in the data.

Additionally, Bayesian non-hierarchical clustering based on a Markov chain Monte Carlo (MCMC) method (software STRUCTURE 2.2.3; Falush *et al*., 2007; '*V. suavis* s.l. matrix' and '*V. *suavis*+Trieste+*adriatica* matrix') was employed to assign AFLP multilocus data into an optimal number of genetic clusters and to detect genetic admixture at both the population and individual levels. We used a recessive allele model, assuming admixture and independence of allele frequencies among the populations. The number of user-defined clusters (*K*) was used as a prior value; ten replicates for each *K* value from 1 to 10 were run with a burn-in period of 10^5 iterations, followed by an additional 10^6 MCMC iterations. The STRUCTURE computations were carried out on the freely accessible Bioportal (www.bioportal.uio.no). The R-script Structure-sum-2009 (part of AFLPdat; Ehrich, 2006) was used to summarize the resulting assignments and calculate the similarity coefficients between the replicate runs, means of the estimated posterior log probability of the data over the run replicates for each *K* value (denoted as the mean L[*K*]) and a quantity based on the second-order rate of change with respect to *K* of the likelihood function – Δ*K* (see Evanno *et al*., 2005). These statistics were used to identify the optimal number of clusters (*K*). Final graphical visualization was generated using the CLUMPP ver. 1.1.1 (Jakobsson & Rosenberg, 2007) and DISTRUCT (Rosenberg, 2004) software.

Differentiation patterns in *V. suavis* s.l. were also explored by the analysis of molecular variance (AMOVA) using Euclidean pairwise distances and a significance test with 10 000 permutations, carried out in Arlequin 3.11 (Excoffier *et al*., 2005). AMOVA was employed to study the variance partitioning within and among populations and among the population clusters suggested by the above-mentioned clustering or ordination analyses.

For each population, we recorded the total number of AFLP genotypes, the average number of AFLP markers scored per individual (± S.D.), the percentage of polymorphic markers (P%), and the average proportion of pairwise differences between individuals (Nei's gene diversity, D_{Nei}; Nei & Li, 1979) using R-script AFLPdat (Ehrich, 2006). The genetic divergence of the analysed populations was assessed by calculating the number of rare markers (those present at a frequency <10%), private markers (those confined to a certain population, not necessarily present in all of its individuals), and private fixed markers (i.e. diagnostic, those confined to a certain population and present in all of its individuals), using FAMD (Schlüter & Harris, 2006). We also calculated a rarity index, the frequency-down-weighted marker value (Schönswetter & Tribsch, 2005), as implemented in AFLPdat (DW1; Ehrich, 2006).

Morphometric analyses

The morphometric analyses included only taxa of *V. suavis* s.l. The analyses were based on 193 individuals (20 populations) from the western Balkans and 340 individuals (35 populations) from NE Spain and C & SE Europe analysed in our previous study (Mereďa *et al*., 2008). Typically, 10 (2–19) flowering plants were collected per population, depending on the population size. A total of 21 morphological characters (12 vegetative, eight reproductive and one ratio derived; 14 quantitative, six binary and one semi-quantitative) were examined on each individual (see Appendix 2, supplementary material, which is available via the Supplementary Content table of the article's online page at http://dx.doi.org/10.1080/14772000.2011.603903). Character scoring and measurements were performed in the same way as in our previous study (Mereďa *et al*., 2008). The characters used included those reported as diagnostic for the studied taxa in literature (e.g. Marcussen & Nordal, 1998), or found useful based on our previous study of the group (Mereďa *et al*., 2008) or during our field sampling.

The morphological data were examined using both univariate (box-plots and the Tukey–Kramer multiple comparison analysis) and multivariate methods. Prior to multivariate analyses, non-parametric Spearman correlation coefficients (Legendre & Legendre, 1998) based on the matrix including all of the studied plants were computed in order to eliminate pairs of highly correlated characters from further analyses. Both discriminant analyses (canonical discriminant analyses [CDA] and parametric classificatory discriminant analyses [PCDA]; Klecka, 1980) and principal component analysis (PCA; Sneath & Sokal, 1973; Krzanowski, 1990) were employed. All of the individuals were assigned to four groups, as resolved by the AFLPs, corresponding to the populations from: (1) NE Spain; (2) C & SE Europe, including populations nr. 246 and 247 from the province of Trieste; (3) NW Croatia; and (4) southern Dalmatia, including populations from southern Croatia, southern Bosnia and Hercegovina, and south-western Montenegro (S Dalmatia, hereafter) (see Results). Populations nr. 228, 258, 259 and 260, which exhibited genetic admixture as detected by the Bayesian clustering analyses, were omitted from the CDA and PCAs, and their affiliation to the morphological groups was tested separately by PCDA (see below). Three measurements were made for each leaf character (lamina, petiole and stipule); floral traits (peduncle, calyx and corolla) were scored from two fully expanded (chasmogamous) flowers.

The average values of those (three or two) measurements for each plant were entered into the data matrix for discriminant and principal component analyses, whereas each value (three or two measurements per individual) was entered into the Tukey–Kramer multiple comparison analysis. Morphometric data analyses were performed using SAS v.9.1.3 software (SAS Institute, 2007) and are available from the first author upon request.

To reveal the degree of morphological separation of the above-mentioned four genetic groups, two CDA based on 20 morphological characters (one character, corolla colour, was excluded because of its uniformity within some of the groups) were computed. CDA 1 was performed on the populations of the whole dataset (51 populations as operational taxonomic units [OTUs] characterized by average values of 20 morphological characters, '*V. suavis* s.l. populations matrix'), while CDA 2 was computed on the individuals of the whole dataset (494 individuals as OTUs, 20 morphological characters, '*V. suavis* s.l. individuals matrix'). Discriminant analyses generally require a multivariate, normal distribution of the characters, but they have been shown to be considerably robust against deviations in this respect (Klecka, 1980).

PCA 1–4 (based on individuals as OTUs, 21 morphological characters and correlation matrices between the characters) were used to test the morphological homogeneity of the genetic groups revealed by the AFLPs (excluding the genetically admixed populations, nr. 228, 258, 259 and 260). Four different subsets were assembled and subjected to PCA: (1) '*V.* *Spain matrix' (66 individuals from NE Spain, PCA 1), (2) '*V.* **suavis*+Trieste matrix' (294 individuals from C & SE Europe, including two morphologically unclear populations, nr. 246 and 247, from the province of Trieste, PCA 2), (3) '*V.* **adriatica* matrix' (81 individuals from NW Croatia, PCA 3) and (4) '*V.* **austrodalmatica* matrix' (53 individuals from S Dalmatia, PCA 4).

The Tukey–Kramer multiple comparison analysis at the probability level $P \leq 0.05$ (Tukey test for unequal sample sizes; Zar, 1999), was calculated to determine which characters show significant differences among the groups. Boxplots of quantitative morphological characters were generated from all of the plants, except those from population nr. 228.

Finally, two PCDA, based on probability models, were performed to assess the morphological position of (1) plants of an assumed hybrid origin between the C & SE European (excluding populations nr. 246 and 247 from the province of Trieste) group and the NW Croatian group, as inferred from AFLP data and (2) a herbarium specimen of *V. suavis* s.str. from the type locality near Kharkov (the village of Mereffi) in Ukraine (not included in the molecular analyses). In PCDA 1, a classificatory criterion was derived from the genetically homogeneous individuals of both 'parental' groups (training data set 1) and, consequently, applied to a partial dataset with genetically admixed individuals from populations nr. 228, 258, 259 and 260. In PCDA 2, a classificatory criterion was derived from the whole dataset (training data set 2) and, consequently, applied to a specimen from the type locality.

Results

Karyological analyses

Data on the DNA ploidy levels are presented in Appendix 1 (see supplementary material). All of the individuals of *V. alba* subsp. *dehnhardtii*, *V. pyrenaica* and *V. thomasiana* were found to be diploid with $2x \sim 20$, while all of the populations of *V. suavis* s.l. (incl. *V. adriatica*) were tetraploid with $4x \sim 40$. These data are consistent with previous reports (e.g. Schmidt, 1961; Mereďa *et al*., 2008).

Molecular analyses

ITS sequences. Maximum parsimony (MP) analysis resulted in 3871 trees of 69 steps with a consistency index (CI) of 0.887 and a retention index (RI) of 0.984. The strict consensus tree showed five main clades in basal polytomy (figure not shown), while the majority rule consensus tree indicated a somewhat more hierarchical structure with three basal clades (Fig. 2A). One of the well-supported clades in the strict consensus tree (clade A; 93% bootstrap support, BS) included the accessions of *V. suavis* s.l. and *V. pyrenaica*; the latter placed in a basal polytomy to the subclade of *V. suavis* s.l. (clade B; 64% BS). Thus, all of the accessions of *V. suavis* s.l. (i.e. Spanish, French, central European and W Balkan) formed a monophyletic group. In addition, individuals from the southern Balkan range (from Korčula Island to Montenegro; i.e. populations nr. 235, 238, 241, 242, 267, 268) formed a distinct subclade (clade C; 86% BS) within *V. suavis* s.l. that was supported by two unique substitutions. The ITS sequences of the population from Montenegro (nr. 238) were, in addition, characterized by three adjacent intra-individual single-site polymorphisms displaying no additivity to any of the sequences in the alignment. The second main clade (clade D; 96% BS) comprised the individuals of *V. alba* subsp. *dehnhardtii*, *V. collina*, *V. jaubertiana* and *V. odorata*. Only *V. alba* subsp. *dehnhardtii* and *V. jaubertiana* formed their own specific subclades with a moderate bootstrap support (64% BS and 81% BS, respectively). Finally, the third main clade (clade E, < 50% BS, resolved as three separate clades in the strict consensus tree) included (1) *V. alba* subsp. *alba*, together with a single accession of *V. alba* subsp. *dehnhardtii* from Greece (99% BS), (2) *V. ambigua* (100% BS) and (3) *V. hirta*, together with *V. thomasiana* (79% BS). Thus, the accessions of *V. alba* were unexpectedly split into two divergent clades, denoted here as the 'alba I' (99% BS) and 'alba II' clade/ribotype (64% BS).

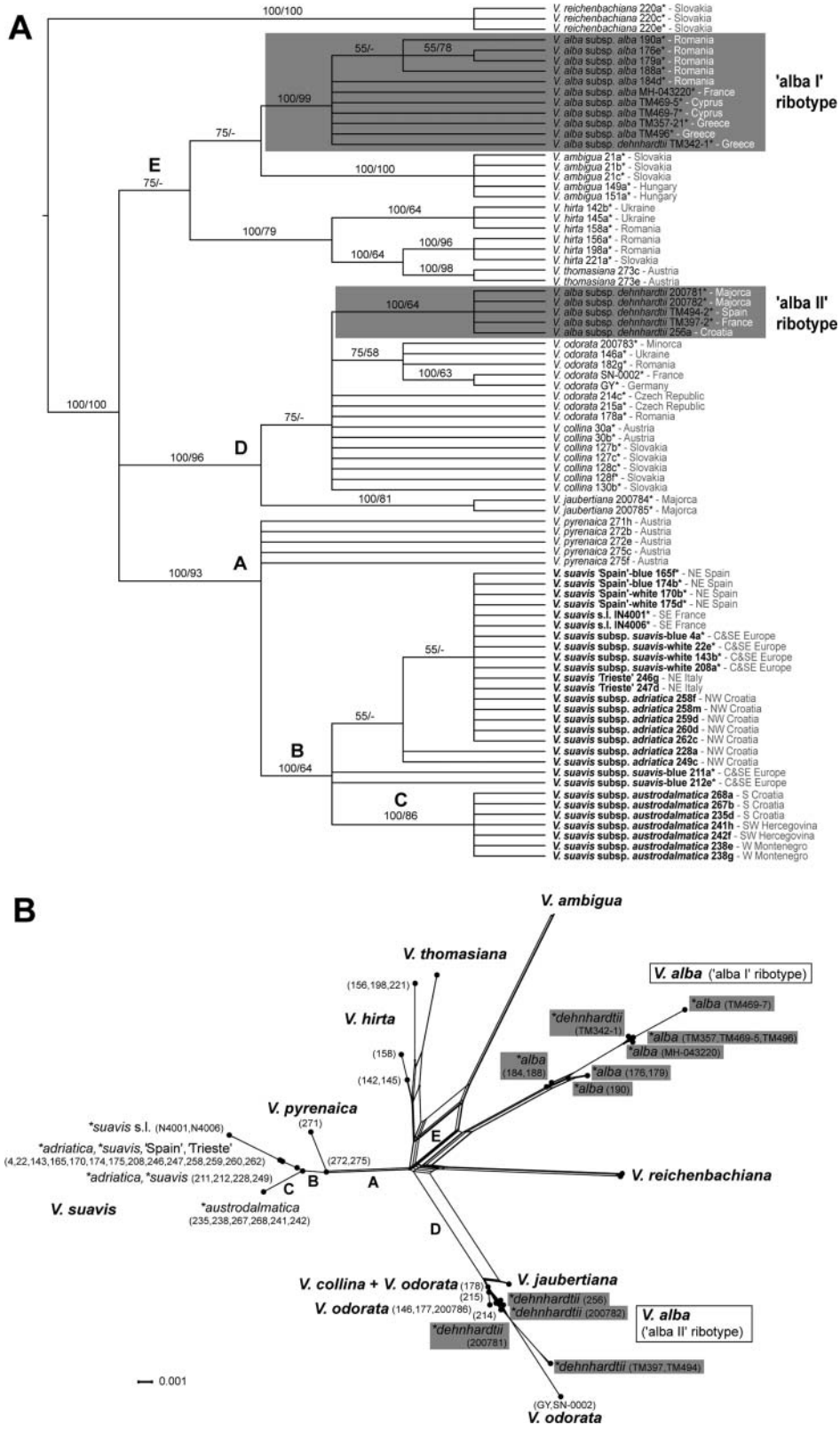

Fig. 2. **A**. Majority rule consensus tree of 3871 most parsimonious trees (MPTs) based on nrDNA ITS sequence data. The numbers along branches refer to the percentage of MPTs displaying the given clade/bootstrap support values of ≥50%. Major clades denoted as A, B, C, D, E, 'alba I' ribotype, and 'alba II' ribotype (the latter two highlighted in grey) are discussed in the text. The accession labels include the taxon name, population number and individual letter; for *Viola suavis* s.l., also the geographic origin and colour morphotypes are given. Asterisks in the accession labels indicate the sequences retrieved from GenBank (see Appendix 1, supplementary material, which is available via the Supplementary Content table of the article's online page at http://dx.doi.org/10.1080/14772000.2011.603903, for details). **B.** Neighbor-Net diagram based on uncorrected *P* distances of nrDNA ITS sequence data. Splits corresponding to the clades of the MP consensus tree (A–E) are indicated; accessions bearing 'alba I' and 'alba II' ribotypes are highlighted in grey.

The NN analysis (Fig. 2B) resulted in a split network displaying five main splits within the ingroup, corresponding to the major clades detected in the strict consensus tree of the MP analysis, and supported also the substructure within the clade A (*V. suavis* s.l. and *V. pyrenaica*). Incompatible phylogenetic signal, however, was revealed in the centre of the network, corresponding to the backbone of the MP consensus trees and the base of clade E, indicating uncertain evolutionary relationships among the main lineages of the subsection.

AFLPs. Replicate samples indicated high reproducibility of the AFLP data, with the error rate being 0.2%. Altogether, 182 AFLP markers (52−486 bp in length) were scored in the 192 analysed individuals; 126 (69%) of these markers were polymorphic. A total of 138 multilocus AFLP genotypes were found; thus, several individuals harboured the same AFLP profiles. A few populations were detected to be genetically uniform: populations nr. 170 (Monistrol de Montserrat, Spain), 207 (Brno-Řečkovice, Czech Republic) and 267 and 268 (both from Korčula Island, Croatia). The number of AFLP markers scored per individual ranged between 76 and 77 in *V. pyrenaica* and 110 and 118 (mean ± S.D. = 113 ± 2) in *V. suavis* s.l. The dataset of *V. suavis* s.l. comprised 178 AFLP markers scored in 172 individuals; 122 (67%) markers were polymorphic, and 127 different multilocus AFLP genotypes were inferred. Four AFLP markers occurred exclusively in *V. pyrenaica* (two of them present in all of its individuals) and 99 were restricted to *V. suavis* s.l. (15 of them fixed across all of the individuals). Altogether, 31% of all of the scored markers were shared between *V. pyrenaica* and *V. suavis* s.l.

The NJ tree (Appendix 3, see supplementary material, which is available online), PCoA (figure not shown), and the NN diagram (figure not shown), based on the '*V. pyrenaica*+*suavis* s.l. matrix', revealed a clear separation of *V. pyrenaica* (100% BS in NJ) from *V. suavis* s.l.

PCoA ordination based on the '*V. suavis* s.l. matrix' (figure not shown) supported the general structure seen in the NJ tree (Appendix 3), resolving four clusters within *V. suavis* s.l. that corresponded to the populations from (1) NE Spain (53% BS in the NJ tree, with the white- and blue-flowered individuals placed in two separate subclusters), (2) C & SE Europe (< 50% BS in the NJ tree, with white- and blue-flowered individuals forming separate subclusters), (3) NW Croatia (from the Primorsko-Goranska county to the Šibenik-Knin county, < 50% BS), and (4) S Dalmatia (from Korčula Island to Montenegro, 88% BS). The S Dalmatian populations and those from NE Spain were resolved as the two most divergent groupings in the PCoA ordination space, whereas the NW Croatian and C & SE European populations were placed into two, somewhat closer, groupings. Two geographically very close populations from the province of Trieste showed somewhat striking positions. While one of them (pop. nr. 246) was found in the ordination space within the C & SE European grouping, the other (pop. nr. 247) was placed in an intermediate position between the C & SE European and NW Croatian groupings. In the NJ tree, both populations clustered with the C & SE European populations.

In congruence with the NJ and PCoA, the NN diagram based on the '*V. suavis* s.l. matrix' also showed the division of *V. suavis* s.l. into four genetically distinct groupings (Fig. 3). Within the S Dalmatian grouping, the southernmost population (nr. 238, Kameno, Montenegro) appeared as more distinct from the others, in accordance with the ITS sequence variation. The two populations from the province of Trieste (nr. 246 and 247) were placed within the C & SE European cluster, but shifted (especially the population nr. 247) towards the cluster of the NW Croatian populations. The NN also confirmed the position of two white-flowered morphotypes of *V. suavis* from NE Spain and C & SE Europe, respectively, which formed separate subclusters among the blue-flowered accessions from the same area, as resolved in our previous study (Mereďa *et al*., 2008) with more extensive sampling.

In the Bayesian clustering based on the '*V. suavis* s.l. matrix', the mean L(K) increased with the increasing K; however, ΔK showed the highest value at $K = 2$, and it was also slightly increased at $K = 4$. The replicate runs only produced stable results with high values of the similarity coefficient for $K = 2$ (similarity coefficient = 1.0) and $K = 4$ (similarity coefficient = 0.89). The two genetic clusters ($K = 2$) that were resolved corresponded to the populations from S Dalmatia as one cluster and all of the other populations of *V. suavis* s.l. as the other cluster (Appendix 4A, see supplementary material, which is available online). Both clusters were highly homogeneous, with no uncertain assignments at the population or individual level. Nine out of the ten runs at $K = 4$ (Appendix 4B, see supplementary material, which is available online) resulted in the same four clusters as those identified in the above analyses (NJ, PCoA and NN). Populations from the province of Trieste (nr. 246 and 247) were unequivocally assigned to the cluster of the C & SE European populations; in one run (exhibiting, however, a much lower posterior log probability), however, population nr. 247 was assigned to the cluster of the NW Croatian populations. The four clusters were largely homogeneous; only slight genetic admixture was observed in a few individuals from NW Croatia (pop. nr. 228, 258, 259 and 260) towards the C & SE European cluster.

To get more insights into the distinction and possible genetic admixture between the C & SE European and the NW Croatian populations, we extracted these populations into a separate partial dataset (the '*V.* **suavis*+Trieste+*adriatica* matrix'), which was again subjected to the Bayesian clustering and PCoA. The same two Bayesian clusters ($K = 2$, figure not shown; but see Appendix 5A, supplementary material, which is available online), with the similarly low level of genetic admixture, were inferred as for the

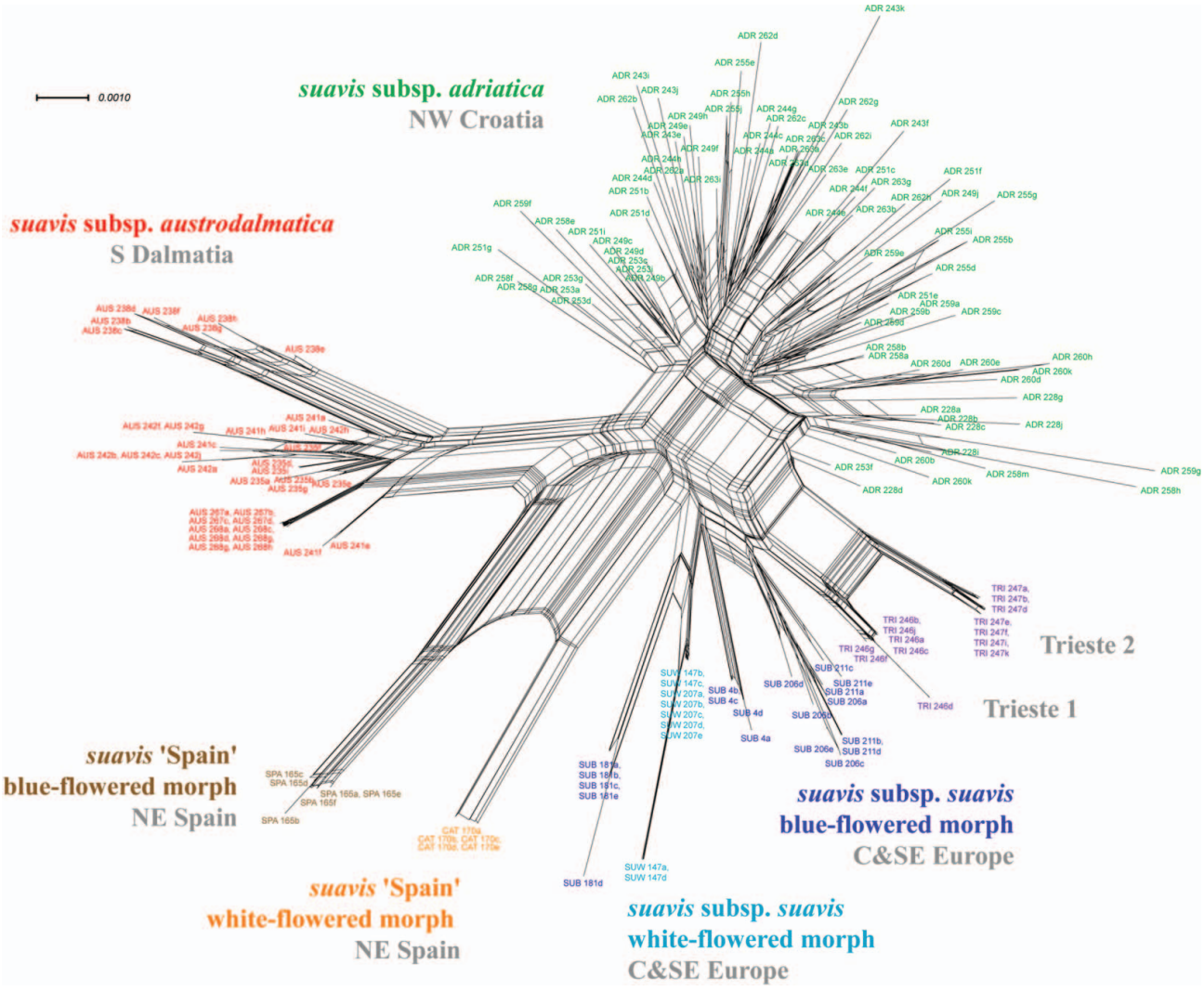

Fig. 3. Neighbor-Net diagram based on the AFLP data of 172 individuals of *Viola suavis* s.l. The accession labels include taxon abbreviation (ADR, *V. suavis* subsp. *adriatica*; AUS, *V. suavis* subsp. *austrodalmatica*; CAT, *V. suavis* 'Spain', white-flowered morphotype; SPA, *V. suavis* 'Spain', blue-flowered morphotype; SUB, *V. suavis* subsp. *suavis*, blue-flowered morphotype; SUW, *V. suavis* subsp. *suavis*, white-flowered morphotype; and TRI, *V. suavis* 'Trieste'), population number, and individual letter (see Appendix 1, supplementary material, which is available via the Supplementary Content table of the article's online page at http://dx.doi.org/10.1080/14772000.2011.603903, for details).

complete dataset of *V. suavis* s.l. (Appendix 4B). In PCoA, the C & SE European populations and those from NW Croatia were clearly separated along the first axis; population nr. 247 from the province of Trieste was, in addition, somewhat separated along the third axis (figure not shown).

The non-hierarchical AMOVA revealed strong differentiation among the populations, as 67.32% ($F_{ST} = 0.67$, df = 27, $P < 0.001$) of the total variation accounted for the among-population variation, yet a significant portion, 32.68% of variation, still occurred within the populations. The four population groupings within *V. suavis* s.l., as defined above, explained 40.65% ($F_{CT} = 0.41$, df = 3, $P <$ 0.001) of the total variation; 30.81% accounted for the variation among populations and 28.54% for that within populations. The other AMOVA computations (e.g. omitting the four NW Croatian populations showing a slight genetic admixture or separating the populations from the province of Trieste into a distinct grouping) yielded very similar percentages of the among-group variance component (approximately 40%).

The AFLP data summary is provided in Appendix 6 (see supplementary material, which is available online). Genetic diversity values (expressed as the percentage of polymorphic loci and Nei's gene diversity) indicated that the populations from NW Croatia were generally more diverse

than those from the other geographic regions. Conversely, two populations from Korčula Island in S Dalmatia (pop. nr. 267, represented by only three cultivated plants, and pop. nr. 268), and two populations of the white-flowered morphotypes from NE Spain (pop. nr. 170) and C & SE Europe (pop. nr. 207) were found to be genetically uniform. Genetic divergence indicators (the presence of private, diagnostic and rare markers and DW values), however, did not show any obvious geographic correlations. However, the three highest values (DW of 18.89−21.34) were recorded in the southern regions (NE Spain, Montenegro and SE Romania).

Morphometric analyses

Spearman correlation coefficients did not reveal the presence of any highly correlated pairs of characters (exceeding the value 0.90); the highest value obtained was 0.803 (between characters CP and CPSP; for character explanations, see Appendix 2, thus all characters could be used in further analyses.

Canonical discriminant analysis (CDA 1, the '*V. suavis* s.l. populations matrix'), classifying populations into a priori defined groups on the basis of AFLP data, showed that the genetic groups were morphologically highly differentiated and formed four distinct clusters (Appendix 7, available online) corresponding to the following regions: (1) NE Spain, (2) C & SE Europe, including populations nr. 246 and 247 from the province of Trieste, (3) NW Croatia and (4) S Dalmatia. Characters showing the highest correlations with the first three canonical axes (Appendix 2), and, thus, best separating the groups, were predominantly those on leaves (LCN, LSA, LHD, LAA and LHM), including stipules (SW, SFN and SFL). CDA 2, based on individuals as objects (the '*V. suavis* s.l. individuals matrix'), showed an extensive overlap among the plants from all of the four genetic groups, however, a tendency toward their separate grouping was still evident (Fig. 4). Similarly, as in CDA 1, the groups differed, especially in the characters on leaves (LHL, LCN, LSA, LHD and LHM), including those on stipules (SW).

To test the morphological homogeneity of the genetic groups revealed by the AFLPs, four PCAs (1–4) based on different data matrices were computed. PCA 1, based on the '*V.* *Spain matrix', showed two slightly differentiated groups corresponding to the blue- and white-flowered morphotypes of *V. suavis* (graph not shown; the results are in concordance with those of Mereďa *et al*., 2008). PCA 2, based on the '*V.* **suavis*+Trieste matrix', resulted in two groups clearly separated along the second and third axes: plants from C & SE Europe were placed in the upper back part, while those from the province of Trieste (populations nr. 246 and 247) were placed in the lower front part of the diagram (Appendix 8, see supplementary material, which is available online). Moreover, there was a tendency towards a further separation of the plants from C & SE Europe along the first axis, which corresponded to colour morphotypes: the blue-flowered plants were shifted towards the right and the white-flowered ones towards the left. The two remaining data sets, based on the '*V.* **adriatica* matrix' (PCA 3) and '*V.* **austrodalmatica* matrix' (PCA 4), were morphologically homogeneous, with no structure observed in the PCA scatterplots (graphs not shown).

According to PCA 1–4, seven morphological groups/taxa were revealed within the whole dataset of *V. suavis* s.l. Despite the overlaps indicated among these groups (Appendix 9, see supplementary material, which is available online), all of the groups/taxa differed significantly in a majority of traits (notably, the characters on the lamina, stipule, petiole and corolla) according to the Tukey–Kramer multiple comparison analysis ($P \leq 0.05$). Individuals from NW Croatia significantly differed from the rest (except of the Trieste populations) in the number of hairs along the lamina margin, hair density, maximum hair length on the petiole, number of crenulae along the lamina margin and stipule width. Significant differences between the populations from S Dalmatia and the remaining groups (except of the Trieste populations) were found for the number of crenulae along the lamina margin, stipule width and pigmentation of the corolla, in contrast to pigmentation of the spur. The unique morphological characters for the populations from NE Spain and C & SE Europe were as follows: peduncle pigmentation and the posterior petal width for the white-flowered morphotype from NE Spain; the maximum hair length on the petiole, peduncle pigmentation, and the position of the bracteole on the peduncle for the white-flowered morphotype from C & SE Europe; the maximum fimbriae length on the stipule and pigmentation of corolla, in contrast to pigmentation of the spur, for the blue-flowered morphotype from C & SE Europe; and the blue-flowered morphotype from NE Spain was characterized by the number of hairs along the lamina margin and the hair density (but not in contrast to the populations from S Dalmatia). Finally, the populations from the Trieste province were significantly differentiated from the remaining six groups by the maximum fimbriae length on the stipule. In other characters, they possessed an intermediate position between the blue-flowered morphotype from C & SE Europe and the populations from NW Croatia, or they were identical with one of these groups/taxa.

The parametric classificatory discriminant analysis (PCDA 1) showed that the individuals from three genetically slightly admixed populations, nr. 258, 259 and 260 (Appendix 4B and 5A), morphologically corresponded to the other populations from NW Croatia (their assignments to this group varied from 80.61% to 100%), whereas the plants from population nr. 228 were clearly assigned to the C & SE European populations (their assignments varied from 83.86% to 100%) (Appendix 5B). PCDA 2 revealed that the plant from the type locality (the village of Mereffi

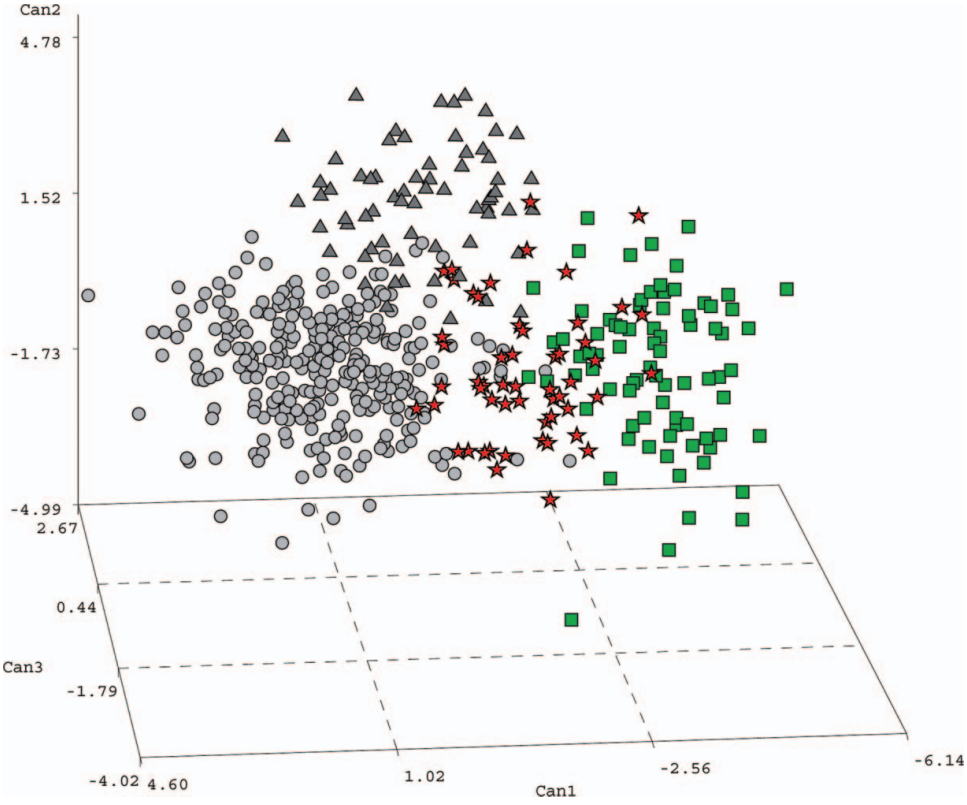

Fig. 4. Canonical discriminant analysis of 494 individuals of *Viola suavis* s.l. based on 20 morphological characters. The four groups defined on the basis of AFLP data represent: *V. suavis* 'Spain' from NE Spain (triangles), *V. suavis* subsp. *suavis* from C & SE Europe, including populations nr. 246 and 247 from the province of Trieste (circles), *V. suavis* subsp. *adriatica* from NW Croatia, excluding populations nr. 228, 258, 259, 260 (squares), *V. suavis* subsp. *austrodalmatica* from S Dalmatia (stars). The first three axes explain 66.3%, 20.94% and 12.75% of the variation.

near Kharkov, Ukraine) was clearly assigned to the C & SE European populations (with an assignment of 97.60%).

Discussion

Species relationships in *Viola* subsect. *Viola*

Our ITS sequencing results, in accordance with previously published data (Mereďa *et al*., 2008), lend strong support to the idea that all of the studied populations of *V. suavis* s.l. form a monophyletic group, clearly separated from the other taxa of *Viola* subsect. *Viola*. Within the *V. suavis* s.l. dataset, the populations from S Dalmatia were determined to be the most divergent and monophyletic evolutionary unit.

Viola pyrenaica (2n = 20) is shown here as the most closely related species to the tetraploid, *V. suavis* s.l. (2n = 40). Our ITS sequence data (the first for *V. pyrenaica*), thus, confirmed the close relationship of these taxa, as has also been ascertained in the allozyme study by Marcussen & Borgen (2000) and microsatellite analyses by Hepenstrick (2009).

Viola thomasiana, another diploid mountain species reported as endemic to the Alpine coniferous forests of France, Switzerland, Italy and Austria (Becker, 1909; Gams, 1925; Schmidt, 1961) (although Nikitin [1998] gave its occurrence also from the Caucasus), clustered with *V. hirta* with a moderate bootstrap support (79% BS). A close relationship between these taxa is supported also by the relatively high homology of their genomes and the resulting high fertility of their hybrid referred to by Schmidt (1961). On the other hand, the sequence data presented here (the first for *V. thomasiana*) do not support the previous assumptions about its subspecific status within tetraploid *V. ambigua* (inferred on the basis of morphology; Gams, 1925) or its close position to diploid *V. collina* (estimated on the basis of morphology and ecology; Gerstlauer, 1943).

Regarding the systematic position of *V. jaubertiana* (an endemic to Mallorca Island), the ITS sequence data presented here are in congruence with those of Conesa *et al*. (2008), placing this species in a well-supported clade with *V. collina*, *V. odorata* and *V. alba* subsp. *dehnhardtii*. Thus,

ITS analyses do not support a relict and isolated position of *V. jaubertiana* among the members of *Viola* subsect. *Viola*, which has been postulated on morphological (Schmidt, 1961) and isozyme bases (Marcussen & Borgen, 2000).

The presence of two distinct ITS ribotypes among the *V. alba* samples ('alba I', forming a separate clade within the subsection and 'alba II', placed in a clade with *V. odorata* and *V. collina*; see Fig. 2), is another intriguing result of our ITS analyses of the members of *Viola* subsect. *Viola*. Although the same results were obtained in the previous studies by Malécot *et al*. (2007) and Conesa *et al*. (2008), this pattern has not been discussed yet. Diploid ($2n = 20$) *V. alba* s.l. is well known for its complex infraspecific variation. Much attention has been paid to this group by several authors, who have suggested different taxonomic solutions (for a review, see Marcussen, 2003). Currently, the *V. alba* complex is recognized as consisting of two species, *V. sintenisii* (distributed on the Caspian coast from Azerbaijan to Turkmenistan, and probably only cultivated in Uzbekistan and Afghanistan) and *V. alba*. The latter is divided into three subspecies: *V. alba* subsp. *alba*, occurring from the Caucasus, westwards to Central Europe and northern Spain; *V. alba* subsp. *dehnhardtii*, growing in the Mediterranean region from Turkey, westwards to the Iberian Peninsula and Morocco; and *V. alba* subsp. *cretica*, endemic to Crete (Marcussen, 2003; Marcussen *et al*., 2005).

The two ribotypes resolved in *V. alba* s.l. seem to be associated with geography and taxonomy. The 'alba I' ribotype has been reported from populations occurring in the eastern and northern parts of the species distribution area (Azerbaijan, Cyprus, Crete, Greece, Romania and western France) and has been identified in *V. sintenisii*, *V. alba* subsp. *alba*, *V. alba* subsp. *cretica* and in cultivars known as 'Parme de Toulouse' (Malécot *et al*., 2007; Conesa *et al*., 2008; the present study). This ribotype has also been found in one population attributed to *V. alba* subsp. *dehnhardtii* (Greece, pop. TM 342); however, the allozyme study (Marcussen, 2003: 62) indicated that this population is transitional between *V. alba* subsp. *alba* and *V. alba* subsp. *dehnhardtii* and located in the contact zone of the two subspecies. In contrast, the 'alba II' ribotype has been to date found only in the western part of the area of *V. alba* s.l. (Majorca, Spain, southern France and Croatia) and it is associated exclusively with *V. alba* subsp. *dehnhardtii* (Malécot *et al*., 2007; Conesa *et al*., 2008; the present study).

The presence of two different ribotypes in *V. alba* subsp. *dehnhardtii* may be due to incomplete lineage sorting of ancestral ITS variation. As a result the taxon may harbour ITS variants that are older than the taxon itself. Another explanation might be ancient hybridization, and the presence of two divergent ribotypes could reflect the polymorphisms brought by the ancestral species – *V. alba* s.l. (with an 'alba I' ribotype) on one side and *V. odorata* (with an 'alba II' ribotype) on the other. This explanation would also correspond to the morphology of *V. alba* subsp. *dehnhardtii*, since among the members of the *V. alba* group, this subspecies shows the most morphological similarities (leaves ± obtuse with convex margins and a shorter indument) with *V. odorata*. In any case the present-day absence of intra-individual polymorphisms in *V. alba* subsp. *dehnhardtii* and the 'western-eastern' geographical pattern in the distribution of the 'alba I' and 'alba II' ribotypes suggest bidirectional homogenization of ITS sequences in this taxon and their geographic sorting. Nevertheless, these scenarios represent hypotheses that need to be confirmed by additional data and other molecular markers (such as single- or low-copy genes, including chloroplast DNA sequences).

Taxonomy of *Viola suavis* s.l.

Results of the AFLP analyses, in conjunction with those of the multivariate morphometrics, brought strong support for the recognition of four major lineages within *V. suavis* s.l. in the studied area, which are associated with the following regions: (1) NE Spain, (2) C & SE Europe, (3) NW Croatia and (4) S Dalmatia. The populations from the province of Trieste had a somewhat uncertain position and could not be unequivocally assigned (see below for a more detailed discussion). Considering the extent of the genetic, morphological and chorological differentiation among these lineages, we think that it is most appropriate to use the subspecific rank for these entities. We are able to attribute two of the above-mentioned genetic lineages to previously described taxa. The herbarium specimen of *V. suavis* from the type locality (the village of Mereffi near Kharkov, Ukraine) clearly fell within the morphological variation of the blue-flowered morphotype from C & SE Europe, thus, the name *V. suavis* subsp. *suavis* should be attributed to the C & SE European populations. Accordingly, the name *V. suavis* subsp. *adriatica* (Freyn) Haesler is applicable to the populations from NW Croatia. There is no name available for the lineage from S Dalmatia, which is described here as a new subspecies, *V. suavis* subsp. *austrodalmatica* Mereďa & Hodálová (see Taxonomic treatment below). The taxonomic solution for the populations from NE Spain is more complicated. The AFLP results presented here (Fig. 3 and Appendix 3 and 4) and those of Mereďa *et al*. (2008) indicate that there is clear differentiation between the populations from NE Spain and those from C & SE Europe. However, this molecular pattern was only partly confirmed by morphometric data (cf. Appendix 9, see supplementary material, which is available online, and Mereďa *et al*., 2008), and, in addition, we lack material from the intervening areas. This lineage is provisionally referred to as *V. suavis* 'Spain'. Before any taxonomic decision is drawn, comparative analyses based on a more detailed sampling from the whole European species range is necessary.

According to our study, the leaf indument is the most reliable character that distinguishes the subspecies of *V. suavis* s.l. (see Fig. 5, Appendix 9, presented as supplementary

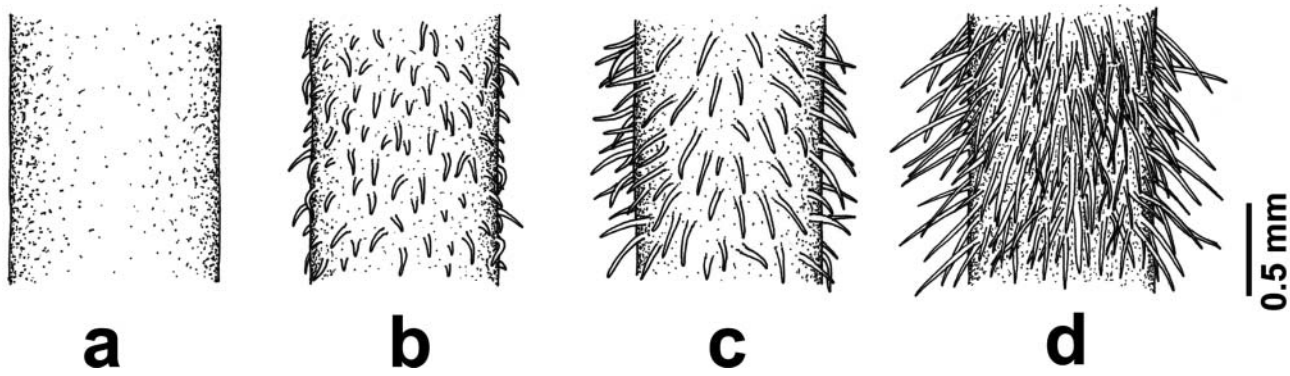

Fig. 5. Indument of petiole of cigar-shaped leaf: (a, b) *V. suavis* subsp. *adriatica* (glabrous and considerably hairy individuals), (c) *V. suavis* subsp. *austrodalmatica*, (d) *V. suavis* subsp. *suavis*. Drawings by P. Mereďa Jr.

material and available online, and the Identification Key below). The leaves of the nominate subspecies and *V. suavis* from NE Spain are relatively densely- and long-hairy. On the contrary, the leaf indument in *V. suavis* subsp. *adriatica* is significantly reduced and usually entirely absent. The indument of the leaf lamina of *V. suavis* subsp. *austrodalmatica* is almost identical with those of *V. suavis* from C & SE Europe and NE Spain, while the indument of the leaf petiole is intermediate between the extremes observed in the nominate subspecies and *V. suavis* subsp. *adriatica*: the hairs are relatively short, and in the flowering period often obscure. The determination of *V. suavis* subsp. *austrodalmatica* will probably cause the greatest difficulties. During early phenological stages, it resembles *V. suavis* subsp. *adriatica*, while after anthesis, it is very similar to *V. suavis* subsp. *suavis*. This was most likely the reason why *V. suavis* subsp. *austrodalmatica* has remained overlooked. However, it can be reliably determined during both phenological stages by a combination of characters, such as the number of hairs along the lamina margin and the length of the hairs on the petioles (see the Identification Key). It should be noted that despite the transitional status of most of the morphological characters observed in *V. suavis* subsp. *austrodalmatica*, there is no evidence in the genetic data (neither in the ITS, nor in the AFLP markers) that would indicate its hybridogenous origin from *V. suavis* subsp. *suavis* and *V. suavis* subsp. *adriatica*.

In contrast to the information given in most of the literature (including the protologue; e.g. Freyn, 1884; Pospichal, 1897; Gams, 1925; Haesler, 1975; Merxmüller, 1982), *V. suavis* subsp. *adriatica* is not always entirely glabrous. In some plants, hairs can be observed (Fig. 5b). Hairy plants, but otherwise morphologically identical with the glabrous ones, can be found in populations throughout the whole distribution range of *V. suavis* subsp. *adriatica*. An indument is frequently present on leaf petioles, less often on the lamina surface, lamina margin or stipules, and only occasionally on the capsule surface. Hairs are present either on all of the mentioned plant parts or only on some of them (e.g. the leaf petiole is hairy, but the lamina surface and/or lamina margin remain glabrous). Such hairy plants have been often confused with *V. suavis* subsp. *suavis* (see the Discussion below). It could be argued that they may represent hybrids between subsp. *adriatica* and subsp. *suavis*, but the AFLP analyses do not support this; both the hairy and glabrous individuals from NW Croatia constituted a single group, and no genetic admixture was shown by the Bayesian clustering (except of populations nr. 228, 258, 259 and 260, see below). Therefore, there is no evidence that the presence of hairs in *V. suavis* subsp. *adriatica* would indicate recent hybridization events.

Patterns of genetic diversity of the Balkan endemics, *Viola suavis* subsp. *adriatica* and *V. suavis* subsp. *austrodalmatica*

Populations of *V. suavis* subsp. *adriatica* showed a higher level of genetic diversity in comparison with those of *V. suavis* from NE Spain and C & SE Europe (cf. Mereďa *et al*., 2008). The distribution of genetic variation among the populations of *V. suavis* subsp. *adriatica* showed little geographical structure (Appendix 6, available online). Interestingly, we did not find any diversity gradient that correlated with the latitude, which is typically observed as a consequence of gradual colonization processes accompanied by founder and bottleneck effects (Petit *et al*., 2003; Hewitt, 2004). However, we did observe the highest diversity and divergence values in several island populations (Appendix 6). The clustering patterns (cf. Appendix 3) did not show obvious geographic correlation, as the mainland and island populations were rather intermingled. These results suggest some phylogeographical

implications regarding the origin of populations and the level of genetic exchange between them.

The species of *Viola* subsect. *Viola* are characterized by restricted pollen and seed dispersal. Flowers are insect- or self-pollinated. Cross-pollination results from the visits of a variety of insects, including bumblebees, honeybees, solitary bees, hoverflies and beeflies (Beattie, 1971). The plants propagate via seeds, or in some species (e.g. *V. alba*, *V. odorata* and *V. suavis* s.l.), also vegetatively by rooting stolons (Gams, 1925; Marcussen & Nordal, 1998). The seeds of the species of *Viola* subsect. *Viola* belong to the largest among the violets; they are passively released from the capsule that prostrates on the ground during dehiscence and are dispersed via myrmecochory because they have an elaiosome that attracts ants (Beattie & Lyons, 1975). Occasional endozoochory, for example, by birds, cannot be excluded (cf. Gams, 1925: 593). Thus, the levels of over-sea dispersals should be considerably limited in this group. It can be expected that the populations with such restricted gene flow would be characterized by higher rates of genetic differentiation, endemicity and possibly also decreased genetic variation on islands, which contrasts with our observations.

Although Adriatic islands seem to have a lower level of endemism than the rest of the Mediterranean, they harbour 35 narrow endemic taxa (Nikolić *et al*., 2008). In particular, south Adriatic islets (such as Jabuka, Kamik, Palagruža, Svetac, Vis, Biševo and Sušac) have the appropriate conditions for genetic differentiation and the development of endemism (Nikolić *et al*., 2008). An example of an island differentiation observed in the Croatian islands has been recently reported in the *Cardamine maritima* group, where populations from the islands in the Kvarner Bay in the north to Šipan Island in the south represent *C. maritima* Portenschlag-Ledermayer ex de Candolle (s.str.), whereas those from the Croatian mainland should be treated as a separate species, *C. adriatica* Jar. Kučera, Lihová & Marhold (Kučera *et al*., 2010). Although the continental island systems (i.e. islands that have recently become disconnected from each other and the mainland by rising sea levels such as those of the Adriatic Archipelago) provide excellent laboratories to study microevolutionary processes, there remains a lack of reports dealing with the genetic variation of plant populations occurring in such regions (cf. Bittkau & Comes, 2005), and, to the best of our knowledge, similar studies based on the Adriatic Archipelago are still largely lacking (but see Kučera *et al*., 2010; Surina *et al.*, 2011).

To get a broader picture of the genetic variation of *V. suavis* s.l. in the western Balkans and to test the predictions of genetic diversification of island populations of this group, we gathered data from several Croatian islands (Krk, Cres, Lošinj, Rab, Pag and Korčula) where *V. suavis* subsp. *adriatica* has been reported (*V. suavis* s.l. has not been reported from more southern Adriatic islets, except Vis; however, data on its occurrence on this island are most likely erroneous – the only herbarium specimen from Vis deposited in ZA is in fact *V. alba*, and *V. suavis* s.l. was not found in this island during our field research; see Appendix 10). Two scenarios would be expected in plants with restricted pollen and seed dispersal, such as the violets from the subsection *Viola*. First, if the island populations of *V. suavis* subsp. *adriatica* were old (autochthonous) and sufficiently isolated, the level of their genetic divergence should be high, and the extent of genetic diversity may depend on such factors as the demographic history, population size and life-history traits. Second, if the island populations were only recently introduced, both the genetic divergence and diversity would be low (due to founder events). The genetic patterns observed in *V. suavis* subsp. *adriatica* (no differentiation between the island and mainland populations, and a high diversity and divergence harboured by the island populations) indicate that the island populations are autochthonous, and there is significant gene flow between the islands and mainland. The over-sea gene flow could be maintained by the following three processes: (a) the transfer of whole plants by humans (see the two populations from Korčula Island), (b) pollen exchange by insects, and (c) the transfer of seeds via endozoochory by birds.

The two highly divergent allopatric intraspecific lineages of *V. suavis* s.l., corresponding to subsp. *adriatica* and subsp. *austrodalmatica*, in the western Balkans suggest their independent glacial differentiation centres. We assume that the topographical complexity of the Balkans promoted allopatry and isolation of populations of *V. suavis* s.l. into two areas during the Pleistocene glaciations. Ecological factors were probably not the major speciation force, as there is no apparent ecological differentiation between the subspecies. They both can be found in sunny or shady sites, karst regions, dry pastures, shrubs, open deciduous or mixed forests, and they are also well adapted to man-made habitats, such as parks, cemeteries, gardens and lawns.

The genetic patterns in *V. suavis* subsp. *adriatica* indicate that they may have survived the last glaciation in an area situated close to their current distribution. The altitudinal, rather than latitudinal, movements during the glacial cycles have been suggested also in other species that are distributed in the western Balkan Peninsula, for example, those from the *Cardamine maritima* group (Kučera *et al*., 2008). The high level of genetic divergence in the island populations of *V. suavis* subsp. *adriatica* suggests their refugial character, rather than a postglacial recolonization from the Croatian mainland. During the glacial periods, the level of the Mediterranean Sea was 100–200 m lower than at present (Dawson, 1992; Voges, 1995). All of the Adriatic islands were interconnected to the mainland during the last glacial maximum, and south-facing slopes in this area may have served as refugia for *V. suavis* subsp. *adriatica*.

Regarding *V. suavis* subsp. *austrodalmatica*, we can only speculate about its glacial survival because only a few

populations were analysed. Southern Montenegro is one of the candidate refuge regions. The same AFLP genotype shared in all of the individuals from the two populations of Korčula Island – one cultivated in a garden (nr. 267) and the other growing in a park in the centre of the town of Blato (nr. 268) – is clear evidence for a recent introduction and a significant contribution of clonal propagation in their subsequent dispersal. More extensive geographic sampling in Montenegro and surrounding countries could help address the phylogeographic history of *V. suavis* subsp. *austrodalmatica*.

Taxonomically uncertain and presumable hybrid populations

Our molecular and morphometric analyses revealed several populations (individuals) in the western Balkans that showed uncertain or intermediate status. They could be divided into the following two groups: (1) the genetically and morphologically homogeneous populations nr. 246 and 247 from the province of Trieste (NE Italy) and (2) the genetically and morphologically heterogeneous populations nr. 228, 258, 259 and 260 from NW Croatia.

From a morphological perspective, the populations (nr. 246 and 247) from the province of Trieste look, at first sight, identical with those of *V. suavis* subsp. *adriatica*. They possess glabrous leaves, which are the most important diagnostic feature of *V. suavis* subsp. *adriatica*. This morphological feature is reflected also in their assignment to *V. adriatica* by earlier authors (e.g. Becker, 1909, 1929; Schmidt, 1961; Merxmüller, 1982). For other morphological traits (e.g. the number of crenulae along the lamina margin, the width of the stipule, the insertion of bracteoles on the flower peduncle, the length of the sepals and the length of the posterior petals), however, they possess an intermediate position between *V. suavis* subsp. *adriatica* and the blue-flowered morphotype of *V. suavis* subsp. *suavis* from C & SE Europe. They also have a unique trait, the extreme length of the fimbriae, that is significantly different from the other lineages of *V. suavis* s.l. (Appendix 9). This character, observed in the Italian populations, has been mentioned previously by several authors (e.g. Becker, 1909; Schmidt, 1961). However, whether this character has a greater evolutionary significance should be tested. The position of these two Italian populations was ambiguous also with respect to the AFLP analyses. Whereas population nr. 246 mostly clustered with the accessions of the blue-flowered morphotype of *V. suavis* subsp. *suavis* from C & SE Europe, population nr. 247 possessed a rather intermediate position between the accessions of *V. suavis* subsp. *adriatica* and the blue-flowered morphotype of *V. suavis* subsp. *suavis* from C & SE Europe. It remains unclear whether these two Italian populations are of a hybrid origin between *V. suavis* subsp. *adriatica* and (probably) the blue-flowered morphotype of *V. suavis* subsp. *suavis* from C & SE Europe or whether they represent a separate evolutionary lineage. Broader taxon sampling in the territory of Slovenia and northern Italy with a more detailed study using additional molecular markers (such as single- or low-copy genes, including chloroplast DNA sequences) may shed more light on this interesting problem.

The other individuals with uncertain positions were found in the NW Croatian populations (nr. 258, 259 and 260) from the Cres-Lošinj archipelago and in population nr. 228 from northern Dalmatia. Although these individuals were unequivocally placed among the *V. suavis* subsp. *adriatica* accessions (Fig. 3 and Appendix 3) by the several analyses (NJ, PCoA and NN) of the AFLP data, genetic admixture was revealed in a few individuals by the Bayesian clustering analysis (Appendix 4B and 5A). An interesting pattern was observed for these individuals in the morphometric analyses. The parametric classificatory discriminant analysis (PCDA 1, Appendix 5B) showed that the individuals from Croatian populations nr. 258, 259 and 260 morphologically corresponded to the other populations from NW Croatia, whereas the plants from population 228 were clearly assigned to the C & SE European populations. Individuals from populations 258, 259 and 260 possessed almost glabrous leaves, and they also fell within the morphological range of *V. suavis* subsp. *adriatica* in other characters. In contrast, individuals from population 228 differed from the rest of the studied material of *V. suavis* subsp. *adriatica* by a higher density of hairs on the leaf surface and lamina margin, having longer hairs on the petiole and a higher number of crenulae along the lamina margin. All of these characters shifted this population towards *V. suavis* subsp. *suavis*.

We assume that the genetic admixture observed in populations 258, 259 and 260 indicates either incomplete lineage sorting (i.e. insufficient genetic differentiation between the two subspecies) or introgression (a low level of gene flow between the subspecies). On the contrary, a conflict between the genetic and morphological data observed in population nr. 228 favours a hybrid origin of this population. Similar cases of genetic admixture revealed by the Bayesian clustering of AFLP multilocus genotypes have been recently reported in several plant taxa occurring in the Balkan Peninsula, for example, *Onosma malkarmayorum* Teppner (Kolarčik *et al*., 2010), *Veronica chamaedrys* Linnaeus (Bardy *et al*., 2010) and *Alyssum montanum* subsp. *pluscanescens* (Jos. Baumgartner) Trpin (Španiel *et al*., 2011). However, it needs to be pointed out that the NN analysis, which is a method used for uncovering reticulate relationships (Huson & Bryant, 2006), did not identify any individuals of these four Croatian populations as being intermediate between the NE Croatian and C & SE European groups.

Distribution of *Viola suavis* s.l. in the western Balkans

Viola suavis subsp. *suavis*, subsp. *adriatica* and subsp. *austrodalmatica* seem to be allopatric based on the available chorological data (Fig. 1). In the Balkan Peninsula, *Viola suavis* subsp. *suavis* has the main distribution east of the Dinaric Mountains, in contrast to the coastal subsp. *adriatica* and subsp. *austrodalmatica*. It is, however, uncertain how far the nominate subspecies extends westwards and whether its range overlaps with those of the other two subspecies in some regions. Although *V. suavis* subsp. *adriatica* shows a shift to more extreme (xerothermous and rocky) habitats, the ecological requirements of all of the *V. suavis* subspecies are very similar. The lack of apparent ecological barriers among them suggests that they may occur in sympatry at least in some regions.

The occurrence of *V. suavis* subsp. *suavis* west of the Dinaric Mountains is questionable. Although some herbarium specimens (as well as pop. nr. 228 in our study) seem to be of a hybrid origin between *V. suavis* subsp. *adriatica* and subsp. *suavis*, we have not seen any typical herbarium specimen of the nominate subspecies from this territory. The data in older literature from Istria given as *V. austriaca* or *V. suavis* (e.g. Schlosser von Klekowski & Farkaš-Vukotinović, 1869; Freyn, 1884; Pospichal, 1897) will probably refer to hairy individuals of *V. suavis* subsp. *adriatica*, or to those of the presumably hybrid origin between subsp. *adriatica* and subsp. *suavis*. *V. suavis* subsp. *suavis* is currently reported in Croatia only from the surroundings of Vransko zero Lake (Nikolić, 2009 as a 'field observation'). However, the identity of these plants needs to be verified.

Viola suavis subsp. *adriatica*, as currently defined, occupies the area from Istria to the Šibensko-Kninska županija County in Croatia (see Fig. 1B and Appendix 10). Most recently, it has been confirmed also from SW Slovenia (Rakar, 2008). Its occurrence in NE Italy (the provinces of Gorizia and Trieste; Pospichal, 1897; Becker, 1929; Merxmüller, 1982) should be verified (see the Discussion above). The same applies to its occurrence in western Bosnia and Herzegovina (where the subspecies could extend from Croatia), and in southern Croatia and NW Montenegro. Although there are old herbarium specimens morphologically identical with *V. suavis* subsp. *adriatica* collected in the town of Dubrovnik and in the Bay of Kotor (Appendix 10), the identity of these plants is unclear, as we were not able to find such plants in these areas during our field research. Questionable remains also the occurrence of *V. suavis* subsp. *adriatica* in southern Bosnia and Hercegovina, from where several herbarium specimens with uncertain identity (due to characters not well maintained for reliable determination) exist. These specimens could represent either *V. suavis* subsp. *adriatica* or *V. suavis* subsp. *austrodalmatica* (see Appendix 10).

The distribution range of *V. suavis* subsp. *austrodalmatica* is, according to our knowledge, restricted to a relatively narrow area of southern Croatia, southern Bosnia and Herzegovina and south-western Montenegro (Fig. 1B). There are no records of this taxon from Albania or Serbia. However, more information is needed regarding its northern-, eastern- and southernmost occurrences. In any case, our analyses underline the importance for the conservation of populations of *V. suavis* s.l. from southern Dalmatia.

Taxonomic treatment of the taxa of *Viola suavis* s.l. occurring in the western Balkans

Based on this study, we recognize two subspecies in the western Balkan Peninsula: *V. suavis* subsp. *adriatica* and *V. suavis* subsp. *austrodalmatica*. For reliable identification of both subspecies, we intentionally included in the key the following two subspecies/lineages of *V. suavis* s.l. revealed in our study, although they were not confirmed to the western Balkans: *V. suavis* subsp. *suavis* (i.e. the blue- and white-flowered morphotypes from C & SE Europe) and *V. suavis* 'Spain' (i.e. the blue- and white-flowered morphotypes from NE Spain). We also include *V. pyrenaica*, which is genetically and morphologically the closest relative of *V. suavis* s.l. occurring in the western Balkans. The key values were derived from all of the plants, except those from population 228. In both the key and morphological descriptions, value ranges of quantitative characters correspond to the 10th and 90th percentiles, with the minima and maxima in parentheses. The main diagnostic characters (i.e. those sufficient for the safe identification of particular taxa) are separated from the supplementary ones (which are less reliable and have no complementary statements consistently given in the other branch of the key) by the symbol •.

A list of the studied herbarium specimens of *V. suavis* s.l. from the western Balkans is presented in Appendix 10. The geographic distribution of *V. suavis* s.l. in this territory is depicted in Fig. 1B.

Key to *Viola suavis* and closely related *V. pyrenaica*

1a. Plants without stolons (but sometimes with a thick, many-headed rhizome), or with short under- or above-ground stolons up to 3.5 cm long; lamina truncate to shallowly cordate at base, with sinus angle (0–)60–140(–170)°; petioles with the longest hairs 0.2–0.4 mm long • Leaves yellowish-green; 2n = 20; (submontane−) montane to subalpine (−alpine) belt . . . . . ***V. pyrenaica***

1b. Plants with at least one under- or above-ground stolon, 3.5–30 cm long, sometimes without stolons (especially in young plants); lamina shallowly to deeply cordate at base, with sinus angle

([−60]–)0–100(−150)°; petioles glabrous or with hairs max. 1.1 mm long • Leaves pale to dark green, rarely yellowish-green; 2n = 40; lowlands to submontane belt . . . . . ***V. suavis***

Ia. Lamina margin of all leaves glabrous or rarely sparsely hairy (number of hairs along 3 mm of lamina margin 0–2[–38]); petioles usually glabrous, rarely hairy and the longest hairs 0.05–0.15(–0.30) mm long; upper leaf surface of all leaves glabrous or sparsely hairy (number of hairs on 12 mm^2 0–30[–110]) • Number of crenulae along lamina margin on leaves in the flowering period (14–)21–34(–42); stipules (except of fimbriae) (0.8–)1.5–3.1(–4.2) mm wide . . . . . ***V. suavis* subsp. *adriatica***

Ib. Lamina margin of the majority of leaves hairy (number of hairs along 3 mm of lamina margin [4–]10–40[–58]); petioles usually hairy, the longest hairs 0.10–1.10 mm long, rarely glabrous; upper leaf surface usually sparsely to densely hairy, rarely glabrous (number of hairs on 12 mm^2 [0–]10–180[–250]) . . . II

IIa. Petioles glabrous or hairy, on the youngest leaves (usually cigar shaped) the longest hairs 0.10–0.25(–0.35) mm long, after anthesis, petioles with the longest hairs up to 0.40(–0.50) mm long • Number of crenulae along lamina margin in the flowering period (19–)24–46(–56); stipules (except of fimbriae) (1.7–)2.2–4.0(–5.0) mm wide . . . ***V. suavis* subsp. *austrodalmatica***

IIb. Petioles hairy, on the youngest leaves (usually cigar shaped) the longest hairs (0.10–)0.20–0.60(–0.85) mm long, after anthesis, petioles with the longest hairs up to (0.10–)0.30–0.85 (–1.10) mm long • Number of crenulae along lamina margin in the flowering period (20–)31–49(–68); stipules (except of fimbriae) (1.4–)2.6–4.7(–6.2) wide . . . ***V. suavis* subsp. *suavis* (**C & SE Europe, incl. blue- and white-flowered morphotypes) and ***V. suavis* 'Spain'** (NE Spain, incl. blue- and white-flowered morphotypes)

Viola suavis subsp**. *adriatica*** (Freyn) Haesler, Mitteilungen der Botanischen Staatssammlung München 12: 111, 1975.

≡ *Viola adriatica* Freyn, Flora 67: 679, 1884. ≡ *Viola suavis* var. *adriatica* (Freyn) Pospichal, Flora des Oesterreichische Küstenlandes. Vol. 1: 550, 1897. ≡ *Viola sepincola* subsp. *adriatica* (Freyn) Gams, in Hegi, Illustrierte Flora von Mittel-Europa 5: 648, 1925. ≡ *Viola beraudii* subsp. *adriatica* (Freyn) E. Mayer, Seznam praprotnic in cvetnic slovenskega ozemlja: 99, 1952. – Ind. loc.: 'Croatia, Buccari [Bakar]. In vinetis in colle Turcinae silvuli marginibus, leg. Hirc (sub *V. austriaca*)'. – Type: Bei Buccari [Bakar] in Weingärten u. [und] einem Wäldchem a. [an dem] westlichen Abhange d. [des] Gipfels Turčina [a hill situated east of the town], May, leg. and det. D. Hirc as *Viola austriaca*, s.n. (lectotype designated here: BRNU 21181/33!).

= *V. cyanea* var. *perfimbriata* f. *istrica* W. Becker, Beihefte zum Botanischen Centralblatt 26(2): 17, 1909. – Ind. loc.: 'die Pflanzen Istriens, Kroatiens und Dalmatiens. . .'. – Type: Kroat. in Weinbergen bei Buccari [Bakar], häufig, 13. V. 1894, leg. B. [G. Beck], det. W. Becker 1909, s.n. (lectotype designated here: PRC!)

Viola suavis* subsp. *austrodalmatica Mereďa & Hodálová, subsp. nov.

Type: Croatia, Dubrovnik-Nertva County, north of the village of Bosanka (east of the town of Dubrovnik), 270 m, 42°38′45″N, 18°07′46″E, 9 March 2009, leg. and det. I. Hodálová & P. Mereďa jun., s.n. (holotype: SAV).

Planta acaulis, rhizomata subterranea vel supraterranea, (1–)4–15(–30) cm longa, interdum absentes. Laminae plerique foliorum sparse ad dense pilosae, raro glabrae, planitiae 12 mm^2 cum (0–)10–110(–190) pilis, margine piloso-dentato cum (19–)24–46(–56) dentibus, densunus longitudine 3 mm (13–)15–37(–54) pilorum continent. Laminae tempore florendi profunde cordatae [angulus ([−65]–)10–80(–145)°]. Cursu florescentiae maior pars petiolorum glabra sed plerumque saltem petiolus unus breve pilosus 0.10–0.25(–0.35) mm longus, post florescentiam pili longitudine 0.40(–0.50) mm. Stipulae externae rosellae foliorum principalis (1.7–)2.2–4.0(–5.0) mm latae, margine dentorum glabrae aut sparse pilosae, pilus (0.3–)0.7−1.5(–2.2) mm longus. Bracteolae in parte inferiore pedunculi locatae (2–)20–55(–80) longitudinis. Flores modice ad gravi odorati. Sepala pedunculi astricta (4–)5–7(–8) mm longa. Petala (9–)11–15(–18) mm longa, caerulea aut caeruleo-violacea, ad basin plerumque cum insigna macula alba, calcar caeruleum aut caeruleo-violaceum, raro fere album. Capsulae sparse pilosae, tantum raro glabrae.

DNA ploidy level: 2n ∼ 4x ∼ 40.

Habitats: rocky karst places, dry pastures, shrubs, open deciduous or mixed forests, as well as man-influenced habitats such as parks, cemeteries, gardens and lawns; mainly on basic substrata; from sea level to colline (submontane) belt.

Distribution area: southern Croatia, southern Bosnia and Herzegovina and south-western Montenegro (Fig. 1B, Appendix 10).

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