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Exploring the intricate evolutionary history of the diploid–polyploid complex *Veronica* subsection *Pentasepalae* (Plantaginaceae)

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Veronica subsection *Pentasepalae* is a diploid–polyploid complex of *c*. 20 species distributed in Eurasia and North Africa, in which species boundaries are difficult to determine. Here, we present the first comprehensive phylogenetic analysis of *V*. subsection *Pentasepalae* based on nucleotide sequences [internal transcribed spacer (ITS) and the plastid *trnH-psbA* and *ycf6-psbM* spacers] combined with ploidy estimations. Our results support the monophyly of the subsection. Five well-supported clades are recovered in the ITS sequence analyses, corresponding to broad geographical areas. The causes of the extensive incongruence found between the ITS and plastid DNA datasets, namely incomplete lineage sorting and/or hybridization and polyploidization, are discussed. Most of the diploids traditionally recognized based on morphological characters and one tetraploid are each recovered as monophyletic by the ITS sequence analyses. The Balkan species *V. kindlii* is resurrected. DNA ploidy level for *V. teucrioides* is reported here for the first time (2*x*). Diploid populations have been found for *V. orbiculata*, which was previously thought to be only tetraploid. Past contact in the amphi-Adriatic area between *V. orsiniana* and *V. orbiculata* is suggested. Finally, molecular analyses show that diploid *V. jacquinii* and diploid *V. orbiculata* are unrelated. This study contributes to our understanding of the evolutionary history of polyploid complexes, especially those in southern Europe, and highlights the importance of using multiple lines of evidence to investigate species boundaries in such actively diversifying groups. *Botanical Journal of the Linnean Society*, 2015, **179**, 670–692.

ADDITIONAL KEYWORDS: hybridization – incomplete lineage sorting – ITS – phylogenetic analysis – plastid DNA – polyploidy – reticulate evolution – taxonomy.

INTRODUCTION

Veronica subgenus *Pentasepalae* (Benth.) M.M.Mart. Ort., Albach & M.A.Fisch. is the second largest subgenus of *Veronica* L. (Plantaginaceae *sensu* APG III, 2009), comprising *c*. 70–75 species (Albach *et al*., 2008) of perennial herbs distributed throughout Eurasia and northern Africa. Based on the phylogenetic analysis of internal transcribed spacer (ITS) and plastid DNA sequence data this subgenus is monophyletic (Albach *et al*., 2004a,b). In the most recent classification of *Veronica* L. (Albach *et al*., 2008) four subsections were recognized in this subgenus, in accordance with the molecular, morphological and karyological data available at that time [*V*. subsections *Armeno-Persicae* Stroh, *Orientales* (Wulff) Stroh, *Petraea* Benth. and *Pentasepalae* Benth.], but in this treatment many species (13%) were left unclassified. The first three subsections mentioned are distributed in southwestern Asia, with some species reaching southeastern Europe (e.g. *V. thymifolia* Sibth. & Sm. distributed in southern Greece). The last, *V*. subsection

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Pentasepalae, is the main focus of this study. It includes *c*. 20–25 taxa, depending on authors, that grow in meadows and stony places from lowlands to high alpine zones. Most of them are distributed in Europe, with the Balkan Peninsula being home to the highest number of species, although there are also representatives in North Africa and Asia (Turkey, Caucasus, and Siberia). The species included in *V.* subsection *Pentasepalae* are characterized by a pentapartite calyx (rarely tetrapartite) with the fifth sepal significantly smaller. Plants are usually woody at the base, with prostrate to erect stems, sometimes caespitose and bearing a vegetative apical shoot. The leaves are sessile, sometimes short-petiolate, entire to pinnatisect. The inflorescences are axillary and racemose. The four-lobed corolla is slightly zygomorphic, violet, pale violet or dark blue, rarely whitish or pink. The capsules are laterally compressed, widely obovate to widely ovate, suborbicular or elliptical in outline and usually emarginate. The seeds are flattened with a reticulate–verrucate coat (Muñoz-Centeno *et al*., 2006). The base chromosome number is $x = 8$ (Albach *et al*., 2008).

The selection of morphological characters supporting classifications has always been controversial in the subgenus and therefore different subsections or groups have been described within it (e.g. Riek, 1935; Elenevsky, 1977). Additionally, species boundaries in *V.* subsection *Pentasepalae* are difficult to establish in some cases, due to overlapping character states and the existence of intraspecific variation in ploidy. As a result, several taxonomic treatments have been proposed in partial monographs and floras (e.g. Watzl, 1910; Stroh, 1942; Borissova, 1955; Hartl, 1966; Walters & Webb, 1972; Elenevsky, 1977; Fischer, 1982; Martínez-Ortega, Sánchez-Agudo & Rico, 2009), which differ substantially regarding taxonomic concepts. Moreover, taxon sampling for previous molecular phylogenetic studies has been limited (Albach, Martínez-Ortega & Chase, 2004c; Albach *et al*., 2004b; Martínez-Ortega *et al*., 2004) and the uncertainty regarding the phylogenetic relationships among species remains high.

According to previous authors (Lehmann, 1937; Scheerer, 1949) hybridization is possible between certain taxa of the group and there might be complex relationships among polyploids and their diploid relatives, but this has never been experimentally tested. In contrast to the polyploids, which are often widespread in Eurasia, the diploids belonging to *V*. subsection *Pentasepalae* are mainly distributed in southern Europe and North Africa, coinciding with areas that represent putative glacial refugia in the Mediterranean basin (Hewitt, 2000; Médail & Diadema, 2009): *Veronica rosea* Desf. in North Africa, the '*V. tenuifolia* complex' (*V. fontqueri* Pau; *V. javalambrensis* Pau, Fig. 1J; *V. tenuifolia* Asso) in the Iberian Peninsula, *V. orsiniana* Ten. (Fig. 1E) in the Iberian Peninsula, south-eastern France, Italy and the Balkans, and *V. crinita* Kit. (Fig. 1A, B), *V. rhodopea* (Velen.) Degen. ex Stoj. & Stef. (Fig. 1C), *V. teucrioides* Boiss & Heldr. and *V. turrilliana* Stoj. & Stef. (Fig. 1D) in the Balkan Peninsula. The exceptions are *V. prostrata* L. (Fig. 1F, G) reaching northern Europe and Siberia (Borissova, 1955; Kosachev, 2003) and *V. krylovii* Schischk., a Siberian endemic. Many of these species are endemic to geographically restricted areas. Importantly, Mediterranean orophytes such as *V. rhodopea* or *V. teucrioides* (also the tetraploid *V. aragonensis* Stroh) are rare with narrow distributions that face a high risk of extinction with climate warming (Thuiller *et al*., 2005). However, before these species can be protected effectively, their species boundaries need to be clarified.

Among the polyploids, the tetraploid *V. satureiifolia* Poit. & Turp. is widespread throughout north-western Europe, from the Alps to the Pyrenees, whereas the tetraploid *V. aragonensis* (Fig. 1K) is an orophyte endemic to the Iberian Peninsula that grows on scree slopes. It is well represented in the Pyrenees and there is also a disjunct population in the southern Iberian mountains (Sierra de la Sagra). The tetraploid *V. orbiculata* A.Kern is endemic to the western part of the Balkan Peninsula (southern Croatia and Bosnia and Herzegovina). Individuals of *V. jacquinii* Baumg. (Fig. 1H) are highly variable regarding morphology and ploidy ($2x$, $4x$, $6x$, $8x$, $10x$; Albach *et al*., 2008). They are widespread in eastern Europe, from the Balkans to the Caucasus and from Slovakia to Greece. Several infraspecific taxa have been described under *V. jacquinii* based on morphological characters (Watzl, 1910; Ghisa, 1960; Peev, 1995) and on cytotypes (Peev, 1972). In spite of their variability, they are characterized by pinnatifid to pinnatisect leaves and they are predominantly hexaploid (see supplementary data associated with Albach *et al*., 2008; available at http://www.researchgate.net/publication/258769259_cariologia2013). *Veronica dentata* F.W.Schmidt and *V. austriaca* L. *s.s.* are both hexaploid and are distributed in central and eastern Europe [some authors have included *V. dentata* or *V. jacquinii* within the variation of *V. austriaca* (e.g. Walters & Webb, 1972; Fischer, 2011)]. Morphologically intermediate populations between *V. dentata*, *V. jacquinii* and *V. orbiculata* are found in nature and botanists have interpreted them either as the result of hybridization between species (Lehmann, 1937; Scheerer, 1949) or as transitional forms caused by phenotypic plasticity in one large, variable species (Watzl, 1910). *Veronica teucrium* L. (Fig. 1I) is octoploid and is widely distributed throughout Europe and Asia, north of the Pyrenees to Siberia (Borissova, 1955). In the Iberian Peninsula the octoploid level is represented by *V. sennenii* (Pau)

Figure 1. Morphological diversity in *Veronica* subsection *Pentasepalae*. Species are denoted by the names finally accepted by us. Herbarium number or locality is indicated in parentheses. A, *V. crinita* (*SALA 149289*; detail of the inflorescence in fruit); B, *V. crinita* (*SALA 149290*; detail of the leaf indument – abaxial face); C, *V. rhodopea* (*SALA 149321*); D, *V. turrilliana* (*SALA 149334*); E, *V. orsiniana* (*SALA 155872*); F, *V. prostrata* (*SALA 149311*); G, *V. prostrata* (*SALA 149319*; detail of the leaf indument – adaxial face); H, *V. austriaca* subsp. *jacquinii* (*SALA 149366*); I, *V. teucrium* (*SALA 149414*); J, *V. tenuifolia* subsp. *javalambrensis* (Mt. Torozos, Valladolid, Spain); K, *V. aragonensis* (*SALA 121540*). Photographs: A, F, G, H and I, B. M. Rojas-Andrés; B, C, D, E and K, M. M. Martínez-Ortega; J, S. Andrés-Sánchez.

M.M.Mart.Ort. & E.Rico, which is endemic to a small area in the northern Iberian Peninsula.

Despite molecular phylogenetic studies on *Veronica* (e.g. Albach *et al*., 2004b, c; Meudt *et al*., 2015), a comprehensive molecular phylogenetic study of *V*. subsection *Pentasepalae* is still lacking. Two recent studies of the subsection used amplified fragment length polymorphism (AFLP) and morphometric data to delimit species boundaries (Martínez-Ortega *et al*., 2004; Andrés-Sánchez *et al*., 2009), but included only a small percentage of the species from *V*. subsection *Pentasepalae* and therefore have not fully tested its monophyly.

Here, we have investigated the phylogenetic relationships among species of the diploid–polyploid complex *V*. subsection *Pentasepalae*. We sequenced the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) and the *trnH-psbA* and *ycf6-psbM* spacers of maternally inherited plastid DNA. To test the monophyly of the subsection and embed our results in a wider phylogenetic framework, we complemented the newly generated sequences with previously published ITS sequences of the species belonging to the other three subsections of *V.* subgenus *Pentasepalae*. The aims of this study are to: (1) investigate the phylogenetic position of *V*. subsection *Pentasepalae* in *V*. subgenus *Pentasepalae* and test the monophyly of the subsection; and (2) reconstruct the evolutionary patterns of *V*. subsection *Pentasepalae* and elucidate the phylogenetic relationships among all species currently included in the subsection to provide a taxonomic framework for further investigations.

MATERIAL AND METHODS

Plant material

Sampling was taxonomically comprehensive and included all widely accepted species, subspecies, varieties and forms, collected from the type localities whenever possible. Sampling was also geographically comprehensive, covering the whole geographical distribution of the subsection (Fig. 2) based on a revision of specimen locality data from 63 European herbaria. Among the morphologically intermediate populations, two accessions identified as '*V. jacquinii-orbiculata*' were included. For samples of *V*. subsection *Pentasepalae*, leaf material was collected in the field and immediately stored in silica gel for flow cytometric analyses and DNA sequencing. DNA extractions for *V. krylovii* were made from herbarium specimens (Table A1). Plants were identified following the taxonomy of Borissova (1955), Walters & Webb (1972), Fischer (1991, 2011), Peev (1995) and Martínez-Ortega *et al*. (2009). As a unique taxonomic treatment was not followed, basionyms were used to name the taxa (Supporting Information Table S1). Exceptions were *V. rhodopea* and *V. sennenii* because in those cases the corresponding basionyms (*V. surculosa* var. *rhodopea* Velen. and *V. prostrata* var. *sennenii* Pau) might be misleading. The names finally accepted in the nomenclatural revision by Rojas-Andrés, Rico & Martínez-Ortega (in press) are indicated in parentheses (when they are different from the basionyms) in Figure 3 and they are preferentially used along the discussion section.

The ITS region (55 individuals) and two plastid DNA regions (49 individuals) were analysed for 18 (100%) diploid and polyploid species and subspecies of *V*. subsection *Pentasepalae*. To assess the monophyly of the subsection and the placement of the members in *V.* subgenus *Pentasepalae*, 19 species (*c*. 36%) (23 individuals) from the remaining three subsections recognized by Albach *et al*. (2008) were included.

Species (one individual of each) belonging to *V.* subgenus *Pocilla* (Dummort) M.M.Mart.Ort., Albach & M.A.Fisch. (*V. polita* Fr.) and *V.* subgenus *Chamaedrys* W.D.J.Koch (*V. arvensis* L. and *V. chamaedrys* L.) were used as outgroups for the phylogenetic analysis of dataset 1 (see below), based on previous analyses (e.g. Albach *et al*., 2004b, c; Muñoz-Centeno *et al*., 2006). One individual of *V. armena* Boiss. & A.Huet from *V*. subsection *Armeno-Persicae* was additionally used as an outgroup with *V. polita* and *V. arvensis* for the analyses performed with dataset 2 (see below).

Eighty-one ITS sequences (of which 50 were newly generated here), 52 *trnH-psbA* sequences and 52 *ycf6-psbM* sequences (all newly generated here) were included in the analyses. Voucher information, the source of material and GenBank accession numbers are given in Table A1.

Flow cytometry

Ploidies were estimated by flow cytometry for 36 of the sampled populations of *V*. subsection *Pentasepalae* (Table A1). Three individuals from each sampled population were measured separately, although only one of them was used for the phylogenetic analyses (except for the population of *V. orbiculata* var. *hercegovinica* K. Malý, for which two individuals of different ploidies were used for sequencing; Table A1). For nine populations (Table A1), ploidies based on direct chromosome counts were available from previous studies (Martínez-Ortega *et al*., 2004; Albach *et al*., 2008). In these cases chromosome counts were made on individuals from the same populations used in the phylogenetic analyses and were therefore directly used here.

For the flow cytometry measurements, a nuclear suspension was prepared following the protocol of Galbraith *et al*. (1983). Given the difficulty of using fresh material of individuals sampled in remote locations we used silica-gel-dried leaves for our flow cyto-

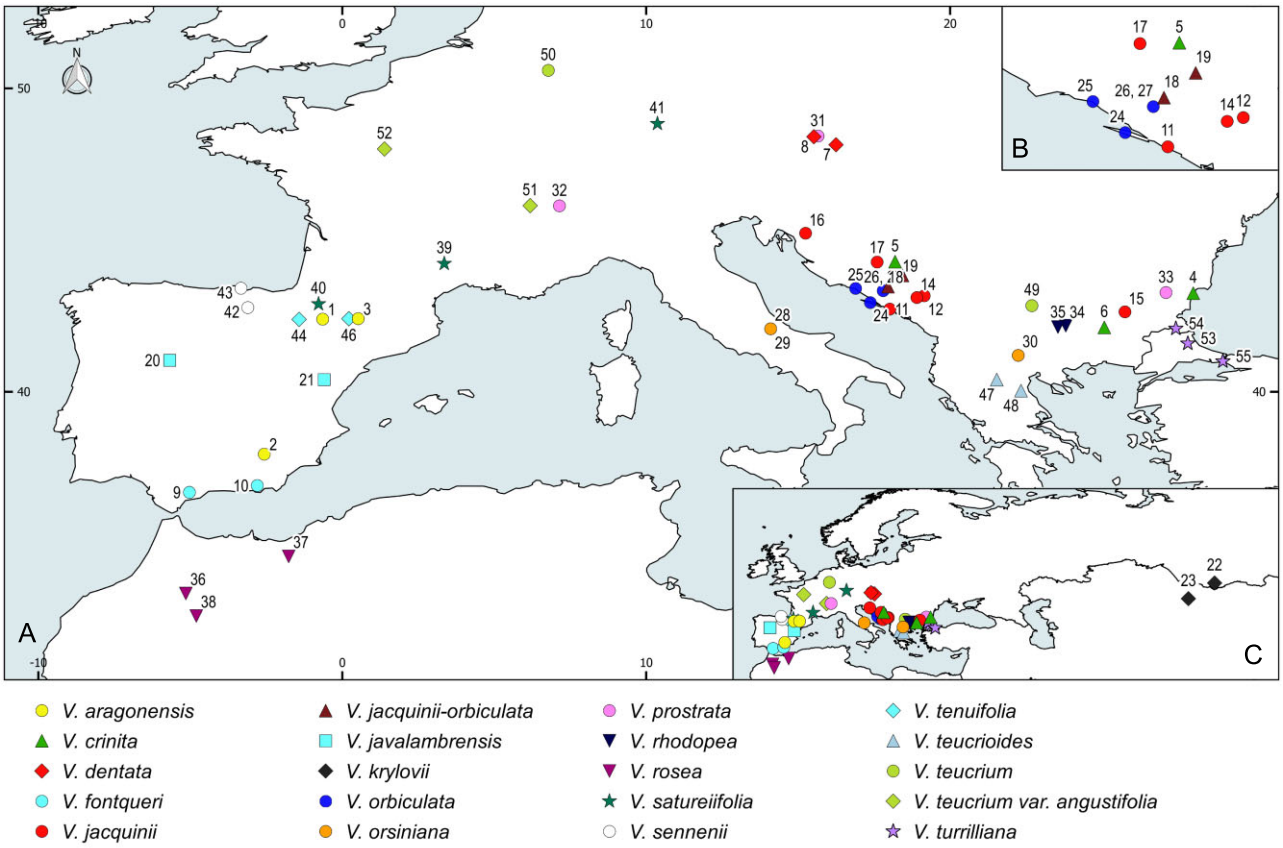

Figure 2. Map of the *Veronica* subsection *Pentasepalae* samples analysed in this study numbered according to Table A1. Basionyms are used to name the species, subspecies and varieties (except for *V. sennenii* and *V. rhodopea*). No. 5 corresponds to *V. crinita* forma *bosniaca*, No. 6 to *V. thracica*, No. 25 to *V. austriaca* var. *emarginata*, and No. 26 and 27 to *V. orbiculata* var. *hercegovinica*. A, main study area; B, detail of the studied area corresponding to Bosnia and Herzegovina, Montenegro and southern Croatia; C, overview of the study area showing the provenance of the samples of *V. krylovii*.

metric measurements as has been done previously for *Veronica* by Meudt *et al*. (2015). Woody plant buffer (WPB; Loureiro *et al*., 2007) was used with slight modifications. For propidium iodide (PI) staining, 1 mL of the nuclear suspension, prepared as above, was filtered through a 48-µm nylon gauze, mixed with RNase (Sigma) to a final concentration of 0.15 mg mL^{-1} and digested at 37 °C for 30 min. A total of 450 µL of the nuclear suspension was then mixed with 2 mL of the PI staining solution (60 µg mL^{-1} PI in doubled distilled water) and measured after at least 10 min. *Solanum pseudocapsicum* L. (2C = 2.589 pg; Temsch, Greilhuber & Krisai, 2010), *Zea mays* L. 'CE-777' (2C = 5.43 pg; Lysak & Doležel, 1998), *Pisum sativum* L. 'Ctirad' (2C = 9.09 pg; Doležel *et al*., 1998) and *Pisum sativum* L. 'Kleine Rheinländerin' (2C = 8.84 pg; Greilhuber & Ebert, 1994) were used as internal standards depending on the C-value and standard availability. For each individual, one run of 5000 counts was made on a CyFlow SL system (Partec GmbH) equipped with a solid state laser featuring blue excitation at 488 nm. For four samples (Table A1), however, it was impossible to obtain flow cytometry measurements suitable for ploidy estimation (CV ≤ 10%; Suda & Trávnícek, 2006). This was probably either because the material has been stored in silica gel for a long time (more than 4 years) or because the available material was from herbarium vouchers. Thus, we assume the diploid level for the populations of *V. krylovii* used here based on previous chromosome counts (Rostovtseva, 1977) and on a flow cytometry measurement obtained for a sample not included in the phylogenetic analyses (Russia, Republic of Altai, Chike-Taman-Pass, *Albach 1275*, OLD). The ploidy of *V. crinia* forma *bosniaca* Fiala remains undetermined.

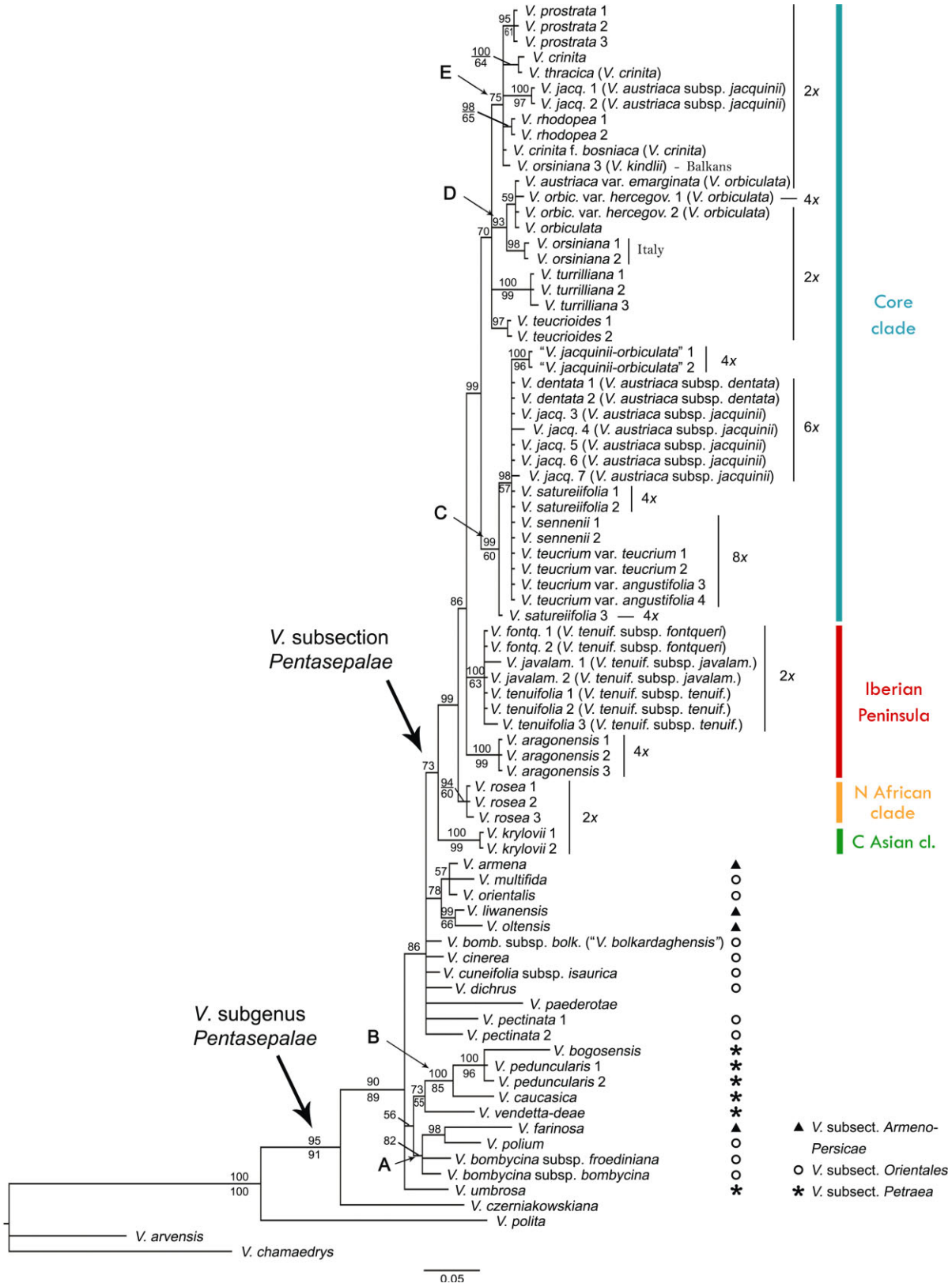

Figure 3. See caption on next page.

Figure 3. Majority rule consensus tree obtained from the Bayesian analyses of the ITS region for *Veronica* subgenus *Pentasepalae* (dataset 1) and ploidies found for each sample. Numbers above the branches indicate Bayesian posterior probability (PP) values. Bootstrap support values (BS) > 50% from the parsimony analyses are indicated below the branches. Accessions are denoted by the basionym (except for *V. sennenii* and *V. rhodopea*) and a number (when there is more than one accession per taxon) according to Table A1. The names finally accepted by us are indicated in parentheses when they differ from the basionym. The scale refers to the expected substitutions per site.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from *c*. 20–30 mg silica-gel-dried material following the CTAB protocol (Doyle & Doyle, 1987) with slight modifications. The quality of the extracted DNA was checked on 1% TAE-agarose gels and the amount of DNA was estimated using a spectrophotometer (T60 UV/VIS, PG Instruments) at 260 nm. DNA extractions are deposited at the Biobanco de ADN Vegetal (University of Salamanca, Spain).

The ITS region was amplified using the LEU1 forward (Vargas, Baldwin & Constance, 1998) and ITS4 reverse (White *et al*., 1990) primers and includes ITS1, 5.8S rDNA and ITS2. For this study 12 plastid regions were tested (*matK*, *matK-psbA*, *ndhF-rpl32*, *rpl16*, *rpoB-trnC*, *rps16*, 3′*rps16*-5′*trnK*^{UUU}, *trnC-ycf6*, *trnH-psbA*, *trnQ-rps16*, *trnT-trnL*, *ycf6-psbM*) using subsets of individuals of different species. Of these regions, only *trnH-psbA* and *ycf6-psbM* showed appropriate levels of variability (4.89 and 2.32% variability, respectively) and were used further. The *ycf6-psbM* spacer was amplified using the *ycf6*F forward and *psbM*R reverse primers (Shaw *et al*., 2005). The *trnH-psbA* spacer was amplified with the forward primer *psbA* (Sang, Crawford & Stuessy, 1997) and the reverse primer *trnH*2 (Tate & Simpson, 2003). PCR conditions for ITS amplification were 2 min 30 s at 94 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 53 °C and 1 min 15 s at 72 °C, followed by 10 min at 72 °C. PCR conditions for *ycf6-psbM* were 2 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C and 2 min 30 s at 72 °C, followed by 10 min at 72 °C. PCR conditions for *trnH-psbA* were 2 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min 20 s at 72 °C, followed by 5 min at 72 °C. Reaction volumes of 25 μL included 36 ng genomic DNA, 5 μL 5× Green GoTaq Reaction Buffer (Promega), 0.2 mM dNTPs, 0.3 μM each primer and 0.85 units GoTaq DNA Polymerase (Promega). PCRs for the ITS region included 1.25 μL dimethylsulphoxide (DMSO) and 0.25 μL bovine serum albumin (BSA; 1 mg mL^{-1}). All amplified fragments were visualized on 1% TBE-agarose gels and purified with ExoSap-IT (USB Corporation) following the manufacturer's instructions. PCR products were sequenced by Macrogen Inc. (Seoul, Korea) using an ABI Prism 3730XL DNA analyser (Applied Biosystems).

Editing and alignment

Sequence identities were confirmed using the NCBI public BLAST service as implemented in Geneious Pro version 5.5.8 (Biomatters). Sequences were edited in Geneious and aligned with SATé-II version 2.2.5 (Liu *et al*., 2011). Nucleotide polymorphisms were coded using the NC-IUPAC ambiguity codes. Mononucleotide repeats and inversions were excluded from further analyses, as they are prone to homoplasy (Kelchner, 2000). Indels were coded according to the 'simple indel coding' method of Simmons & Ochoterena (2000) as implemented in SeqState (Müller, 2005) and datasets were analysed (using Bayesian inference) with the indels either coded or not coded. Prior to the phylogenetic analysis of dataset 1 (see below) the ends of each sequence were trimmed to match the shortest sequence. Sequences from the plastid regions were concatenated based on the assumption that the plastid forms a single linkage group.

Phylogenetic analyses

The available and newly generated sequence data were analysed at two different taxonomic levels. In the first approach (dataset 1; Table 1) the ITS sequences corresponding to 81 individuals (three of which were outgroups, 55 from *V*. subsection *Pentasepalae*, four from *V*. subsection *Armeno-Persicae*, 11 from *V*. subsection *Orientales*, six from *V*. subsection *Petraea* and two species that still have not been assigned to any subsection; see Table A1 for further details) were included. Our aim was to test the monophyly of *V.* subsection *Pentasepalae* and to investigate its phylogenetic position within the subgenus.

In the second approach, our objective was to revise the relationships among the species of *V*. subsection *Pentasepalae.* In this case two subsets were considered. The first (dataset 2; Table 1) included sequences of the nuclear ITS region from 58 individuals plus plastid DNA sequences (corresponding to two plastid regions) from 52 individuals from 20 species (diploids and polyploids) of *V*. subsection *Pentasepalae* and three outgroup species. The second (dataset 2-diploids; Table 1) is exactly the same as dataset 2, but includes 33 ITS and 29 plastid DNA sequences from diploid individuals only, plus three outgroup sequences.

Table 1. Information regarding the DNA markers and datasets used in this study

	Dataset 1	Dataset 2				Dataset 2-diploids			
	ITS	ITS	*trnH-psbA*	*ycf6-psbM*	Plastid DNA	ITS	*trnH-psbA*	*ycf6-psbM*	Plastid DNA
No. of individuals	81	58	52	52	52	36	32	32	32
Sequence length (bp)	626–759	626–739	285–338	649–680	–	626–738	285–338	649–679	–
Aligned length (bp, after trimming)	598	637	348	688	1036	620	332	682	1014
No. of coded indels (length in bp)	20 (1–12)	11 (1–2)	13 (1–29)	19 (1–13)	32 (1–29)	10 (1–2)	10 (1–29)	18 (1–12)	28 (1–29)
No. of variable sites	219 (36.6%)	168 (27.7%)	150 (43.1%)	153 (22.2%)	303 (29.2%)	150 (24.2%)	121 (36.7%)	132 (19.4%)	253 (25%)
No. of potentially parsimony-informative characters	112	62	24	18	42	49	22	19	41
No. of most parsimonious trees	11880	720	12	966	334	378	48	396	64
Tree length of most parsimonious trees	380	188	85	75	165	168	75	70	149
Consistency index (CI)*	0.50	0.65	0.67	0.50	0.61	0.69	0.74	0.59	0.67
Retention index (RI)*	0.77	0.86	0.83	0.83	0.84	0.85	0.86	0.81	0.83
Model of molecular evolution	000121 + I + G + F	000120 + G + F	001100 + G + F	001000 + F	Multiple	000120 + G + F	001100 + G + F	001000 + F	Multiple

*CI and RI were calculated excluding uninformative characters.

Test of recombination

Recombination is the genetic exchange that occurs between nucleotide sequences (either homologous or non-homologous) (Lemey & Posada, 2009). It is thought to be common after hybridization, resulting in chimaeric sequences (Álvarez & Wendel, 2003). In addition, artificial recombination can occur during PCR amplification. It is important to take recombination into account given the potential misleading effect in evolutionary inferences (Lemey & Posada, 2009). Recombination in ITS has been observed in other *Veronica* hybrids (D. Albach, unpubl. data). Evidence for recombination for the ITS sequences (dataset 1) was tested using the pairwise homoplasy index (PHI) test (Bruen, Philippe & Bryant, 2006) as implemented in SplitsTree4 (Huson & Bryant, 2006). In addition, an exploratory analysis was performed in RDP4 software (Martin *et al*., 2010) using *P*-value set to 0.05 and the multiple comparison correction.

Analysis of secondary structure

Secondary structures for all ITS1 and ITS2 sequences were generated using RNAstructure, version 5.7 (Reuter & Mathews, 2010), and examined to determine whether pseudogenes were present in the dataset.

Test of incongruence

Congruence among nuclear and plastid matrices of dataset 2 and 2-diploids was first evaluated observing topological congruence (Figs 3, 4A, 5), and then performing the incongruence length difference (ILD) test (Farris *et al*., 1994). ILD significance values were calculated on informative characters in TNT v.1.1 (Goloboff, Farris & Nixon, 2003) with the INCTST script with 1000 replicates.

Parsimony and Bayesian analyses

All datasets were analysed using parsimony and Bayesian inference. These analyses were always performed for each marker independently (ITS, *trnH-psbA* and *ycf6-psbM*) and for the combined plastid DNA data sets. A combined analysis for the three regions was not performed in any case (i.e. dataset 2 and 2-diploids), due to the extensive incongruence found between ITS and plastid DNA phylogenetic trees (see below). Concatenation of sequences from multiple genes is highly inadvisable when gene trees of different loci are discordant because it may lead to highly supported misleading phylogenetic trees when incongruence is due to incomplete lineage sorting (ILS) (Kubatko & Degnan, 2007).

Parsimony analyses were conducted using TNT v.1.1 (Goloboff *et al*., 2003) applying the traditional search option with equal character weights. In a first run, 10 000 replicates of random addition sequence

and tree-bisection-reconnection (TBR) branch-swapping, saving nine trees per replicate, were performed. As some replicates reached the maximum number of trees that can be saved, the trees from the first run were used as starting trees in a second heuristic search. Bootstrap support (BS) was calculated with 2000 replicates, each consisting of 1000 replicates of random addition sequence and TBR branch-swapping (saving ten trees per replicate); BS ≥ 70% was considered to be an indication of good support for that node. Consistency index (CI), homoplasy index (HI), retention index (RI) and rescaled consistency index (RC) were calculated using PAUP* v.4.0b10 (Swofford, 2002).

Bayesian analyses were performed using MrBayes v.3.2 (Ronquist *et al*., 2012). The best model of DNA substitution for each marker was determined with Jmodeltest v.2.1.2 (Darriba *et al*., 2012) under the Bayesian information criterion (BIC), which was shown to be the most accurate criterion of model selection (Darriba *et al*., 2012). The best-fitting model for each region was used in each case for all the analyses, and partitions were defined when necessary in combined analyses. Two simultaneous and independent runs of 5000 000 generations were conducted, each one with four chains using Markov chain Monte Carlo searches. One out of every 500 generations was sampled, which resulted in a total of 10 000 sampled trees in each run. To check for convergence the standard deviation of split frequencies (< 0.01), the potential scales reduction factor (PSRF) and the 'sump'-plot were examined. The first 10% of the trees of each analysis were discarded as burn-in before computing the majority rule consensus tree. Posterior probability support (PP) was estimated to be significant for nodes with PP ≥ 95%.

Additionally, for dataset 2-diploids (excluding outgroups *V. polita* and *V. arvensis*), a supernetwork analysis (Huson *et al*., 2004) was performed using SplitsTree4 (Huson & Bryant, 2006) to investigate competing topologies of the ITS and plastid DNA trees obtained from the Bayesian analyses. Supernetworks are useful tools for summarizing and visualizing complex information in cases of gene tree incongruence (Huson & Bryant, 2006; McBreen & Lockhart, 2006). The reliability of the individual gene-trees used as input is crucial to reconstruct with confidence the reticulate evolutionary history of a particular group (McBreen & Lockhart, 2006). The strategy of excluding polyploids at this step was adopted because a highly supported polytomy was displayed for most of the polyploids in the ITS phylogenetic tree, and it could represent a misleading topology due to the limitation of tree-building methods when reticulate evolution is involved (Linder & Rieseberg, 2004; Hörandl, 2006).

RESULTS

Ploidies of 45 populations from *V.* subsection *Pentasepalae* ranged from 2*x* to 8*x* (Table A1). For most of the individuals sampled, ploidies estimated by flow cytometry were in accord with previous chromosome counts for each particular species. For *V. teucrioides* no direct chromosome count exists and DNA ploidy level (terminology following Suda *et al*., 2006) is given here for the first time. The genome size obtained for *V. teucrioides* was similar to that of other diploids from the subsection (which are uniformly so), which is why we infer the diploid level for *V. teucrioides* here based on flow cytometry. *Veronica orbiculata* was considered to be a tetraploid species based on chromosome counts, but we found DNA contents suggesting a diploid level. Individuals from three different populations of *V. orbiculata* were included in this study. Two populations contained only diploids (*V. orbiculata* and *V. austriaca* var. *emarginata* K. Malý ex Watzl, Table A1, Fig. 3) whereas the other was composed of diploid and tetraploid individuals (*V. orbiculata* var. *hercegovinica* 2 and 1, respectively, Table A1, Fig. 3). The individuals measured from the populations identified as '*V. jacquinii-orbiculata*' 1 and 2 were found to be tetraploid.

Sequence and data set statistics are given in Table 1. All ITS sequences contained the three conserved 5.8S motifs described for Viridiplantae (Harpke & Peterson, 2008). In addition, all sequences conserved the typical secondary structure of ITS (results not shown). The highly conserved motif of Liu & Schardl (1994), GGCRY-(4- to 7*n*)-GYGYCAAGGAA, was present in ITS1 forming a hairpin structure. The GC content varied from 56.5 to 59%. Some degree of intraspecific variation was detected in ITS sequences, which ranged from one substitution in *V. javalambrensis*, *V. rosea* and *V. turrilliana* to 14 substitutions in *V. jacquinii*, in which variation was mainly found between diploid and polyploid individuals. For the plastid regions, one to six substitutions or indels were found in certain species (data not shown). Of the three DNA regions in dataset 2, the plastid *trnH-psbA* was the shortest (348 bp, aligned) but also the most variable (Table 1).

The PHI test did not reveal statistically significant evidence for recombinant ITS copies ($P = 0.265$). Likewise, no potential recombinant sequences were detected during the exploratory analysis performed in RDP4. The results of the ILD test reported significant incongruence between the nrDNA and the plastid (*trnH-psbA* + *ycf6-psbM*) DNA regions ($P = 0.001$). Bayesian analyses of datasets with coded indels provided similar topologies with higher PP values than for those with uncoded gaps. Because gap characters thus have the same phylogenetic signal as nucleotide substitution data, only results from the datasets with coded indels are shown, as they are more robust

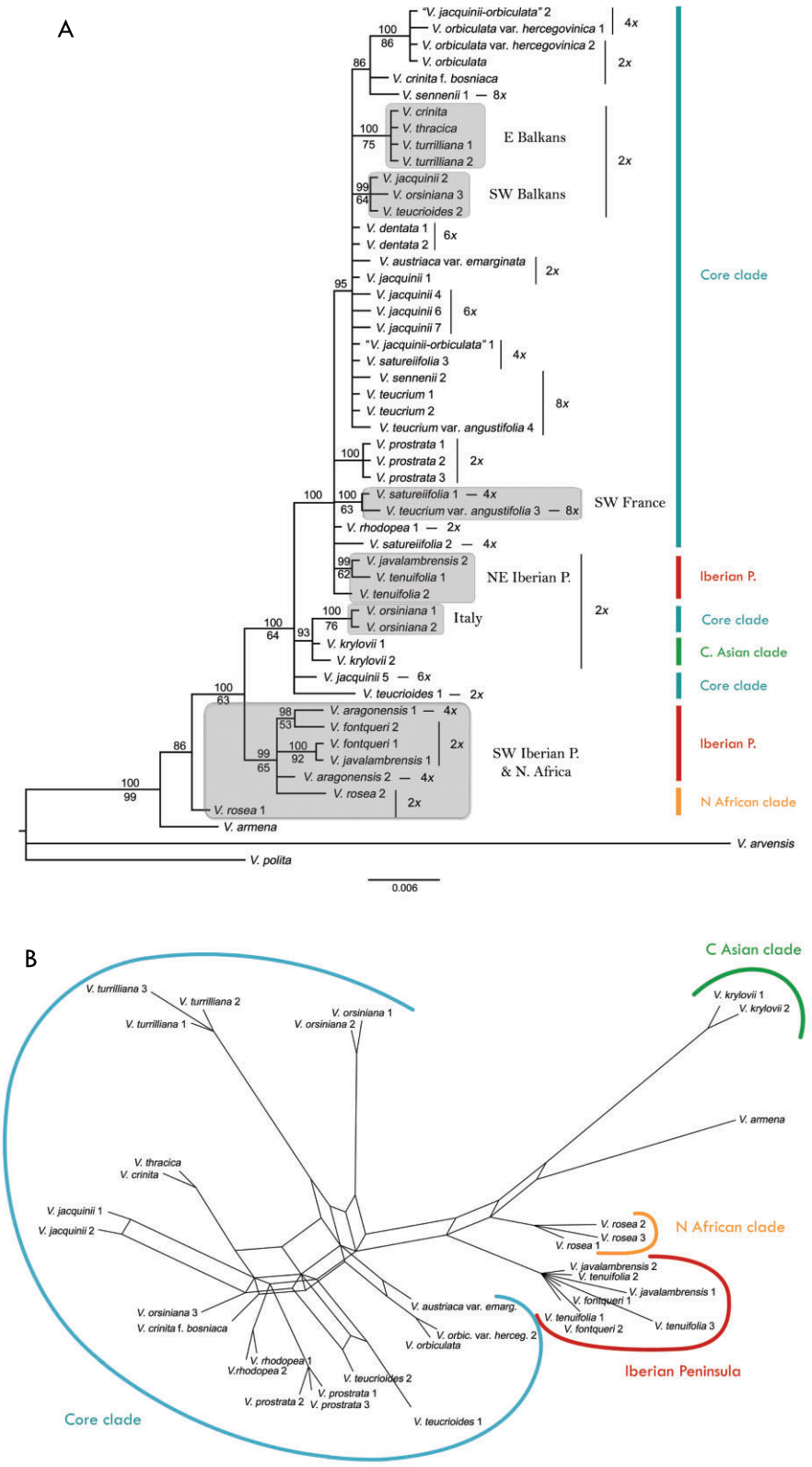

Figure 4. See caption on next page.

Figure 4. A, majority rule consensus tree obtained from the Bayesian analyses of the two plastid regions (*trnH-psbA* + *ycf6-psbM*) for *Veronica* subsection *Pentasepalae* (dataset 2). Numbers above the branches indicate Bayesian posterior probability (PP) values. Bootstrap support values (BS) > 50% from the parsimony analyses are indicated below the branches. Ploidy of each sample is indicated. The scale refers to the expected substitutions per site. B, supernetwork constructed with the ITS and plastid DNA majority rule consensus trees from the Bayesian analyses of dataset 2-diploids. In both A and B, individuals are denoted by the basionym (except for *V. sennenii* and *V. rhodopea*) and a number (when there is more than one individual per species) according to Table A1.

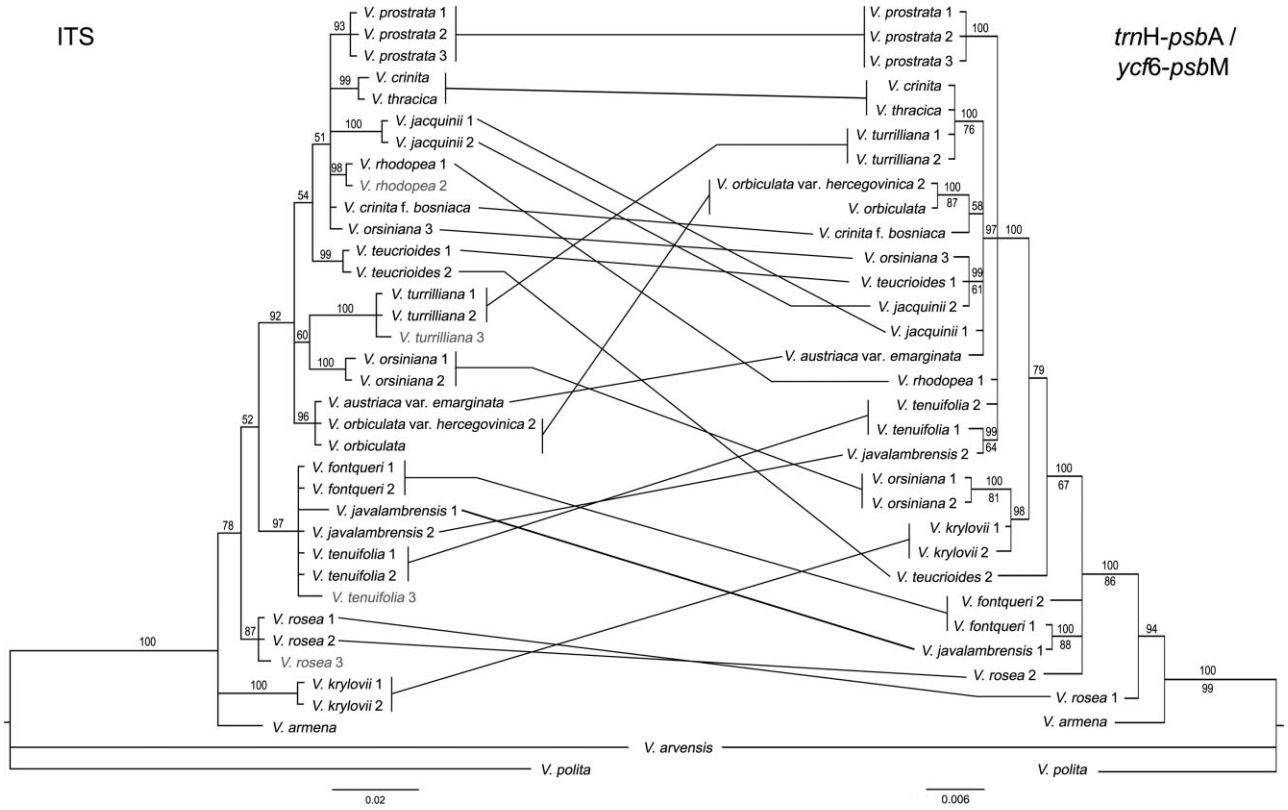

Figure 5. Majority rule consensus trees obtained from the Bayesian analyses of the ITS region (left) and the two plastid regions (right) for *Veronica* subsection *Pentasepalae* (dataset 2-diploids). Numbers above the branches indicate Bayesian posterior probability (PP) values. Bootstrap support values (BS) > 50% from the parsimony analyses are indicated below the branches. Accessions are denoted by the basionym (except for *V. rhodopea*) and a number (when there is more than one accession per taxon) according to Table A1. The scale refers to the expected substitutions per site.

taking into account both nucleotide substitutions and indel information. As the topology of the parsimony 50% majority rule consensus trees was almost identical to that of the Bayesian trees, but less resolved and less supported, only the Bayesian results are displayed. Similarly, only the ITS trees of dataset 1 (Fig. 3) and 2-diploids (Fig. 5) are shown as the ITS analyses of datasets 1 and 2 did not differ in topology and had only slight differences in PP values.

The Bayesian analysis of dataset 1 supported the monophyly of *V.* subgenus *Pentasepalae* (95% PP), *V.* subsection *Pentasepalae* (99% PP excluding *V. krylovii*) and *V.* subsection *Petraea* (73% PP excluding *V. umbrosa* M.Bieb) (Fig. 3). Conversely, the species ascribed to *V.* subsections *Orientales* and *Armeno-Persicae* were scattered across early-branching nodes.

In *V.* subsection *Pentasepalae*, five main clades were revealed (Fig. 3): a central Asian clade (*V. krylovii*, 100% PP), a North African clade (*V. rosea*, 94% PP), two clades corresponding to species endemic to the Iberian Peninsula (the '*V. tenuifolia* complex' and *V. aragonensis*, both 100% PP) and a core clade (99% PP) which comprises the remaining species, mostly from northern and central Europe, Italy and the Balkans. Although the phylogenetic affinities among the diploids and polyploids remain unresolved, most of the diploid species traditionally recognized based on morphological traits are recovered as monophyletic,

excluding *V. orsiniana* and *V. crinita* (Fig. 3). In the polyploid representatives of the complex, ITS sequences showed nearly no variation and most of them were displayed in a polytomy, with the exception of the putatively autotetraploid *V. orbiculata* var. *hercegovinica* 1 and *V. aragonensis* (Fig. 3).

The phylogenetic analysis of plastid DNA data corresponding to 52 individuals (Fig. 4A) additionally supported the monophyly of *V.* subsection *Pentasepalae* (100% PP; 86% PP including the individual *V. rosea* 1). Whereas the ITS tree showed taxonomic structuring (Fig. 3), the plastid DNA tree exhibited geographical structure to a certain extent (Fig. 4A). However, in some cases a taxonomic structure is observed (i.e. *V. prostrata* and the Italian populations of *V. orsiniana*).

The supernetwork obtained from the ITS and plastid DNA trees of dataset 2-diploids (Figs 4B, 5) revealed that reticulate evolution has played an important role in the phylogenetic history of *V.* subsection *Pentasepalae* and especially among the members of the core clade. The supernetwork retained the taxonomic structure and the four main geographical groups identified by the ITS sequences (i.e. core clade, Iberian Peninsula, central Asian clade and North African clade).

DISCUSSION

The present work is the first extensive molecular phylogenetic analysis that covers the whole geographical range and the full spectrum of morphological variation of the taxonomically complicated group *V.* subsection *Pentasepalae* and is a test of monophyly of the subsection as recognized by Albach *et al*. (2008). To delimit species in the subsection, we use multiple lines of evidence (i.e. integrative taxonomy; Dayrat, 2005). Specifically, we rely on our phylogenetic analyses of ITS data coupled with evidence from morphology (N. López-González, B. M. Rojas-Andrés & M. M. Martínez-Ortega, unpubl. data), cytology (B. M. Rojas-Andrés, N. López-González, M. de Pedro, D. Albach & M. M. Martínez-Ortega, unpubl. data) and geographical distribution. Plastid DNA regions evolve at a slower mutation rate than nrDNA regions (Small, Cronn & Wendel, 2004) and are usually more introgressed in plants (Petit & Excoffier, 2009). Therefore, they are not always the best markers to define boundaries among closely related species, as seems to be the case here. Even with these shortcomings, these results represent a significant contribution toward understanding the patterns of diversification and evolutionary history of the group, allow circumscription mainly of diploid taxa and provide a taxonomic framework on which future studies will be based.

Circumscription, phylogenetic affinities and position of *V.* subsection *Pentasepalae* in *V.* subgenus *Pentasepalae*

The results presented here confirm the general east-to-west migration pattern of *V.* subgenus *Pentasepalae* along the Mediterranean (Albach *et al*., 2004c; Martínez-Ortega *et al*., 2004) and demonstrate that *V.* subsection *Pentasepalae* constitutes a monophyletic lineage that probably evolved from the Near Eastern Asiatic species (or their ancestors). However, a west-to-east migration route was followed by some representatives of the subsection that reached Siberia (*V. krylovii* and the Russian populations of *V. prostrata* and *V. teucrium*). Support for a monophyletic *V.* subsection *Pentasepalae* is lower when *V. krylovii* is included (73 vs. 99% without it). Pending additional analyses, we advocate maintaining this species in the subsection based on morphological characters (e.g. Borissova, 1955) and plastid DNA analyses (Fig. 4A). Relationships and classification of the other subsections in the subgenus will need to be reconsidered with adequate taxon sampling and other molecular markers.

Evolutionary history and sources of incongruence in *V.* subsection *Pentasepalae*

Trying to reconstruct the evolutionary history and phylogenetic relationships in *V.* subsection *Pentasepalae* is challenging due to extensive incongruence found between the phylogenetic analyses obtained from different data sets. Incongruence between gene trees can be attributed to phylogenetic and sampling error, recombination, sampling of pseudogenes, unrecognized gene duplication and loss (paralogy), ILS and/or hybridization (e.g. Wendel & Doyle, 1998). In our case, phylogenetic error due to poor taxon sampling can be excluded, because the sampling is thorough with regard to number of individuals per species and distribution range. Although some clades are well supported in the nuclear and plastid DNA trees, resolution is low regarding some relationships, especially in the plastid DNA phylogenetic analysis (Fig. 4A). Therefore, sampling error (i.e. few informative characters) may be causing part of the incongruence observed between gene-trees. The possibility of recombinant ITS sequences (artificial or from a recent event) confounding phylogenetic analysis (Álvarez & Wendel, 2003) was excluded according to the results from the recombination analyses. Given the results obtained from the analyses of secondary structure the presence of pseudogenes in the ITS data set is also improbable. In addition, none of the ITS sequences showed a lower GC content than expected and no longer branches were obtained in the ITS tree. Given that we are working at low taxonomic levels (i.e. at the species level), unrec-

ognized gene duplication and random loss are also unlikely (Maddison, 1997). However, this possibility cannot be completely ruled out.

Veronica subsection *Pentasepalae* is a diploid–polyploid complex composed of closely related taxa, as demonstrated by the low resolution and short branches found in the ITS and especially the plastid DNA tree. This low genetic variation and the fact that several diploid representatives are still extant also suggests that the species have recently diverged [mean crown age of 2.87 Mya (1.37–4.53 Mya) for *V.* subsection *Pentasepalae*; Meudt *et al.*, 2015]. Recent divergences usually correspond to high levels of ILS, especially if effective population size (N_e) is large compared with the time between divergences (Maddison & Knowles, 2006). Population sizes are usually quite small for species of *V.* subsection *Pentasepalae* (often fewer than 10–20 individuals; M. M. Martínez-Ortega & B. M. Rojas-Andrés, pers. observ.) and therefore retention of ancestral polymorphism is not likely to occur. Additional analyses are required to elucidate the role that ILS has played in the evolution of the group.

Hybridization and polyploidization are known to be important speciation mechanisms in plants. Considering the incidence of polyploidy in *V.* subsection *Pentasepalae*, the discovery of mixed-ploidy populations and the variability of monoploid genome size (B. M. Rojas-Andrés *et al.*, unpubl. data), the reticulate patterns observed are best explained by hybridization and introgression. The presence of some geographical structure coupled with the lack of taxonomic structure shown in the plastid DNA tree (Fig. 4A) could also reflect hybridization, introgression and low seed dispersal capacity. Maternally inherited genomes are less appropriate for species delimitation because of their higher rates of introgression (Petit & Excoffier, 2009) and their generally lower mutation rates, which are less likely to reflect speciation events. The lack of resolution of the plastid DNA tree (Fig. 4A) may reflect a scenario in which the present lineages are in the initial stages of divergence. Thus, nuclear markers will be the best option for this purpose in future studies of *V.* subsection *Pentasepalae* and other groups of closely related taxa in which hybridization and introgression are thought to occur.

Coalescent-based approaches distinguishing between hybridization and ILS require either several independent DNA markers or reliable information on mutation rates (Holland *et al.*, 2008; Degnan & Rosenberg, 2009; Joly, McLenachan & Lockhart, 2009), but this kind of information is still lacking for *V.* subsection *Pentasepalae*, and our data set consists only of two independent DNA markers. Highly variable molecular markers (e.g. AFLPs or simple sequence repeats) or new approaches such as RAD-Sequencing (Dufresne *et al.*, 2014) may provide the variation needed to resolve relationships among the polyploids of *V.* subsection *Pentasepalae*.

An additional challenging question for the reconstruction of the phylogenetic relationships in *V.* subsection *Pentasepalae* are the polyploids. Although the ITS phylogenetic analyses allowed delimitation of most of the traditionally recognized diploid taxa as monophyletic, almost no variation was found for the polyploids belonging to the core clade, which were recovered in a highly supported clade, thus apparently suggesting a common origin for all of them (clade C, Fig. 3). A scenario in which the tetraploid *V. satureiifolia* (or one of its ancestors) gave rise to the rest of the polyploids cannot be rejected. However, the morphological variation shown by the polyploid individuals included in this study, which almost equates to that of the diploid individuals, does not seem to favour the explanation of a common origin. In the particular case of *V. jacquinii*, the polyploid specimens are morphologically indistinguishable from the diploid specimens, suggesting that the diploids may have been involved in the origin of hexaploid *V. jacquinii* and in the formation of those polyploids that show an intermediate morphology between *V. jacquinii* and *V. orbiculata* (see below). The recurrent formation of polyploids has been shown to be the rule rather than the exception in angiosperms (Soltis & Soltis, 1999; Doyle *et al.*, 2003), with examples also in *Veronica* (*V. cymbalaria* Bodard, Albach, 2007; *V. chamaedrys* group, Bardy *et al.*, 2010). A common origin of all these polyploids is therefore improbable. One possible explanation for the apparent monophyly of the polyploids would be that concerted evolution acted in the same direction in all polyploids (i.e. all ITS copies would have evolved towards the same DNA sequence type), and few subsequent mutational changes have occurred. Despite the fact that we cannot reject such a scenario, it is also improbable. The apparent monophyly shown by the ITS data could alternatively be attributed to methodological artefacts (i.e. tree building methods that fail to depict evolutionary relationships when reticulate or anagenetic evolution is involved; Hörandl, 2006; Naciri & Linder, 2015). In contrast to the assumptions of cladistics, some speciation processes do not imply the extinction of the parental species. In cases of speciation such as budding of a part of a species (Mayr & Bock, 2002) and subsequent rapid divergence, the progenitor-derivative relationship will be depicted in the cladogram as a sister relationship. Interspecific hybridization can produce misleading cladograms whereby the hybrid appears either in a polytomy with both parental taxa due to conflicting signals, as sister to one of the parental taxa or as sister to both (Hörandl, 2006). Given the importance of polyploidy in *V.* subsection *Pentasepalae*, it is likely that the common origin shown by the polyploids and their sister rela-

tionship to the diploid taxa of the core clade are incorrect, actually representing a progenitor-derivative relationship, although perhaps not involving any extant diploid genotype. Likewise, the lack of phylogenetic resolution encountered among the polyploids may be attributed to conflicting signals in the data set due to hybrid speciation and polyploidization or to a lack of differentiation of the ITS copies because of a recent origin of these polyploids.

In summary, reticulate evolutionary patterns in *V.* subsection *Pentasepalae* are probably due to ILS, polyploid hybridization or a combination of both. In addition, the diploid–polyploid relationship is probably a progenitor-derivative one. Thus, the widely distributed polyploid members of the core clade have probably arisen from Balkan diploids (or their ancestors) through hybridization and polyploidization. Recurrent polyploidization and interbreeding of individuals with different genotypes might be obscuring phylogenetic relationships in the diploid–polyploid complex of *V.* subsection *Pentasepalae*, as seen in several other polyploid complexes (e.g. Brochmann *et al*., 1998; Španiel *et al*., 2011; Himmelreich, Breitwieser & Oberprieler, 2014). Moreover, retention of ancestral polymorphisms may also contribute to the intricate evolutionary history of this species group. Future studies with other nuclear markers are needed to address these issues.

Establishing taxonomic concepts in *V.* subsection *Pentasepalae*

The phylogenetic tree based on ITS sequences presented here allows the recognition of ten monophyletic diploid species in *V.* subsection *Pentasepalae*, each discussed in turn below. However, the relationships among them remain unresolved and low resolution is found in the trees resulting from both plastid (Fig. 4A) and nuclear DNA sequence data (Fig. 3). All diploids have previously been identified based on morphological characters, although they have not always been accepted in traditional taxonomic treatments. In contrast to other Mediterranean plant groups (e.g. the "*Cardamine maritima* group" refers to several species: *C. maritima* D.C and *C. monteluccii* Br.-Cat. & Gubell., among others; please see Kucera, Marhold & Lihova, 2010 for details), our study demonstrates the feasibility of disentangling speciation at the diploid level in polyploid complexes in which reproductive barriers are permeable.

Individuals of *V. krylovii* and *V. rosea* occupy the earliest branching clades in the ITS phylogenetic tree (Fig. 3) and are rather separated from the other individuals in the supernetwork (Fig. 4B). Endemic to Siberia (*V. krylovii*) or North Africa (*V. rosea*), both occur at the margin of the distribution range of *V.* subsection *Pentasepalae*. *Veronica krylovii* was initially described as a subspecies of *V. teucrium* (*V. teucrium* subsp. *altaica* Watzl) due to the strong morphological similarity of both species, but its distinctiveness is clear according to genetic data. *Veronica rosea* is retrieved as monophyletic (94% PP, Fig. 3), a surprising result given its great morphological variation (Martínez-Ortega, 1999), but in agreement with the results obtained using AFLP by Martínez-Ortega *et al*. (2004).

The diploid Iberian endemic '*V. tenuifolia* complex' is monophyletic (100% PP, Fig. 3). The variability of the ITS sequences is not sufficient to differentiate among the three subspecies currently recognized (*V. tenuifolia* subspp. *tenuifolia*, *fontqueri* and *javalambrensis*), which can be distinguished otherwise based on morphology and AFLP data (Martínez-Ortega *et al*., 2004, 2009; Andrés-Sánchez *et al*., 2009). In the plastid DNA tree (Fig. 4A) the south-western and north-eastern Iberian populations appear separated, the latter together with individuals from the core clade. Indeed, AFLP data showed that populations with maximum genetic diversity are concentrated in the north-eastern Iberian Peninsula (Martínez-Ortega *et al*., 2004). This suggests that gene flow among the north-eastern populations of the '*V. tenuifolia* complex' and those from the core clade seems to have occurred at least historically.

Veronica rhodopea and *V. turrilliana* are narrow endemic diploids from the Balkan Peninsula. They are registered in the Red List of Bulgarian vascular plants (Petrova & Vladimirov, 2009) as vulnerable and endangered, respectively, and grow in Important Plant Areas [IPAs; IPA online database (http://www.plantlifeipa.org/reports.asp)] that display exceptionally rich floras of biogeographical interest. The first, from the mountains in southern Bulgaria, was described as *V. surculosa* var. *rhodopea* Velen. *Veronica surculosa* Boiss. & Balansa is a member of *V.* subsection *Orientales* described from Anatolia and not sampled in our analyses, but our ITS data support that *V. rhodopea* is a monophyletic diploid (98% PP, Fig. 3) that should be included in *V.* subsection *Pentasepalae*. Degen had already proposed (Stojanov & Stefanoff, 1925) the separation and recognition of *V. rhodopea* at the species level based on morphological characters. *Veronica turrilliana* (Stojanov & Stefanoff, 1923), which occurs in south-eastern Bulgaria and European Turkey (Strandzha Planina) and has always been recognized at the species level, is monophyletic (100% PP, Fig. 3). The long branch length (Figs 3, 4B) suggests that the species is quite isolated in *V.* subsection *Pentasepalae*. Nevertheless, according to our field observations it is able to hybridize with sympatric *V. crinita* in natural conditions (see specimens *SALA 149335* and *SALA 149283* lodged at the herbarium of the University of Salamanca), providing additional

evidence for the relative permeability of reproductive barriers among species in this subsection.

Veronica teucrioides from north-western Greece and Macedonia is monophyletic (97% PP, Fig. 3). It has been treated as a subspecies or variety by some authors [*V. austriaca* var. *teucrioides* (Boiss. & Heldr.) Halácsy; *V. orsiniana* subsp. *teucrioides* (Boiss. & Heldr.) M.A.Fischer], but neither a close relationship with the representatives from the '*V. austriaca* complex' included in this study nor a close relationship with *V. orsiniana* is supported by genetic data. Species rank for *V. teucrioides* is warranted given the results obtained here and that some morphological characters can be found to identify it (mainly glabrous leaves, calyx and capsules; N. López-Gónzalez, B. M. Rojas-Andrés & M. M. Martínez-Ortega, unpubl. data).

The data presented here suggest that *V. orsiniana* as circumscribed in relatively modern revisions (e.g. Fischer, 1982, 1991; Martínez-Ortega *et al*., 2009) is polyphyletic, as the Italian (*V. orsiniana* 1 and 2) and Balkan (*V. orsiniana* 3) populations are neither monophyletic nor closely related (Figs 3, 4A, B). Slight morphological differences in capsule and calyx morphology can be found between populations from the Italian and Balkan peninsulas (B. M. Rojas-Andrés, E. Rico & M. M. Martínez-Ortega, unpubl. data). In addition, they have different and non-continuous distribution areas and the Italian *V. orsiniana* (type locality in the Abruzzi Mountains) is recovered with moderate support in clade D (93% PP, Fig. 3) as a sister group of *V. orbiculata*, whereas the Balkan population appears in clade E. For these reasons we propose to recognize the Balkan populations as a separate species and use the next available name *V. kindlii* Adamović for them. Further examples of taxa with an amphi-Adriatic distribution that are now considered different species can be found in *Campanula* L. (Park *et al*., 2006) and *Androsace* L. (Schönswetter & Schneeweiss, 2009). *Veronica kindlii* was published by Adamović (1904) for plants from the present border between Greece and Former Yugoslav Republic of Macedonia, and was later included by Hayek (1929) in the Prodromus Florae Peninsulae Balcanicae. It has been used in identification labels and botanical works (e.g. Watzl, 1910; Dimopoulos & Georgiadis, 1995) for those plants morphologically close to *V. orsiniana* from the Balkan Peninsula. A lack of information on its phylogenetic affinities (Walters & Webb, 1972) has hampered a wider use of this name until now, but many recent internet resources are using the name *V. kindlii* for these plants [e.g. EUNIS (http://eunis.eea.europa.eu/index.jsp); Euro + Med Plantbase (Marhold, 2011); The Plant List (http://www.theplantlist.org/); Tropicos (http://www.tropicos.org/); USDA-ARS (www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?432713; PESI (www.eu-nomen.eu/portal)]. We reserve the name *V. orsiniana* for the plants distributed across a continuous area from north-eastern Spain and southern France to Italy, whereas for the populations from the Balkans the name *V. kindlii* would apply.

Veronica crinita is also recovered as non-monophyletic (Figs 3, 4B). Whereas *V. crinita* forma *bosniaca* appears in clade E in an unresolved position, *V. crinita* and *V. thracica* appear together with high support (100% PP; Fig. 3) also in clade E. However, with the data available so far it is difficult to know whether the non-monophyly is due to low levels of sequence variation, ILS and/or hybridization. Alternatively, these taxonomic entities might represent independent evolutionary linages (i.e. cryptic speciation). Cryptic taxa have been shown to occur in *V.* subsection *Pentasepalae* in the Iberian Peninsula (Martínez-Ortega *et al*., 2004). A more in-depth analysis using highly variable molecular markers is currently under development by our research group to answer these questions regarding the evolution of *V. crinita*. Pending further molecular, cytotypic and morphological analyses, *V. crinita* forma *bosniaca* might be elevated at the specific or subspecific rank. Given the continuous distribution area of the *V. crinita* populations, the uncertainty concerning the ploidy of *V. crinita* forma *bosniaca* and the few morphological differences found between this form and typical *V. crinita* (basically the smaller size of the former), we include *V. crinita* forma *bosniaca* within the variation of *V. crinita* as a synonym.

A perfect congruence between the ITS and plastid DNA data sets is observed in showing *V. prostrata* as monophyletic. This suggests strongly a lack of gene flow with other species of the subsection and reinforces the taxonomic recognition at the species level, thus confirming the view of most previous taxonomic treatments (e.g. Borissova, 1955; Fischer, 1982, 2011; Peev, 1995).

Among the diploid–polyploid species, *V. jacquinii* (mostly 2*x* and 6*x*, according to the data obtained here) and *V. orbiculata* (2*x* and 4*x*, according to data obtained here) have been traditionally considered to be phylogenetically related as members of the '*V. austriaca* complex' (Beck, 1887; Watzl, 1910; Walters & Webb, 1972). Nevertheless, the diploid individuals analysed here that correspond to *V. jacquinii* and *V. orbiculata* are recovered as monophyletic unrelated taxonomic entities (Figs 3, 4B). The sister-group relationship found between *V. orbiculata* and the Italian *V. orsiniana* may suggest contacts in the amphi-Adriatic area (probably occurring during the glacial periods of the Pleistocene), as found in other species (e.g. *Cardamine maritima* group; Kučera *et al*., 2010). The tetraploid accession identified as *V. orbiculata* var. *hercegovinica* 1 (Fig. 3; Table A1) groups together with the conspecific diploids and was found in a

mixed-ploidy population. This suggests an autopolyploid origin of this tetraploid, which would need further testing.

Veronica jacquinii is polyphyletic according to the phylogenetic analysis of ITS sequence data and, therefore, diploid and hexaploid populations should be treated as different species under the criterion of monophyly. However, there are several reasons against splitting *V. jacquinii* into different species, namely: (1) most polyploid species are polyphyletic (Soltis & Soltis, 1999) and therefore most would have to be split into several species; and (2) the lack of morphological characters to identify individuals of different ploidies (N. López-González, B. M. Rojas-Andrés & M. M. Martínez-Ortega, unpubl. data) might hamper their unequivocal determination. For these reasons, we prefer to consider *V. jacquinii* as a cytologically variable taxonomic entity, at least until further detailed analyses are conducted.

Diploid populations of *V. jacquinii* (and of *V. orbiculata*) are restricted to a narrow range along the Dalmatian coast (only in southern Croatia and Montenegro) and the southern part of the Dinaric Alps (B. M. Rojas-Andrés *et al.*, unpubl. data), coinciding with two putative refugial areas (Médail & Diadema, 2009). In contrast, polyploid populations of *V. jacquinii* are more widespread extending towards the eastern and northern parts of the Balkan Peninsula. Diploid populations of *V. jacquinii* and *V. orbiculata* may have survived in those places during glacial periods. After the retreat of the ice sheet they probably gave rise to polyploid populations, which in the case of *V. jacquinii* would have extended along the Balkan Peninsula, whereas tetraploid *V. orbiculata* would have remained in the western part of the peninsula.

The morphologically intermediate populations '*V. jacquinii-orbiculata*' 1 and 2 grouped together in a well-supported clade (100% PP, Fig. 3) with higher levels of genetic differentiation (slightly longer branch length) than the remaining polyploids from the core clade. This suggests an older origin of these tetraploids, which were sampled from populations in which only tetraploid individuals have been detected. These results, together with the fact that the geographically restricted diploid populations of *V. jacquinii* and *V. orbiculata* are recovered as unrelated taxonomic entities, suggest that the morphologically intermediate populations (relatively frequent in the field) of variable ploidy (B. M. Rojas-Andrés *et al.*, unpubl. data) between *V. jacquinii* and *V. orbiculata* are probably a result of interspecific hybridization and introgression rather than transitional forms distributed along a gradient of ecological conditions.

Veronica aragonensis is the only polyploid species that is recovered as monophyletic in the nuclear DNA phylogenetic analysis. It appears in a strongly supported clade (100% PP, Fig. 3) distant from the remaining polyploids outside the core clade, but clearly as a part of the subsection. In addition, the branch length defining *V. aragonensis* in the ITS tree is relatively long compared with other branch lengths. Martínez-Ortega *et al*. (2004) have suggested that this species is a palaeopolyploid, which is supported by the results obtained here. However, in contrast to the lack of interspecific gene flow found by those authors using AFLP (mostly nuclear markers), the plastid DNA tree shows probable past interspecific gene flow among populations of *V. aragonensis*, *V. tenuifolia* and *V. rosea* (i.e. the '*Hispano-Africanae* group' *sensu* Riek, 1935) or their ancestors. Alternatively, the pattern observed in the plastid DNA tree might reflect ILS. *Veronica aragonensis* seems to be reproductively isolated and interspecific gene flow between this species and other taxa occurring in the Iberian Peninsula probably does not occur at present. Morphologically intermediate populations have never been observed, even in those rare cases in which *V. aragonensis* grows in close vicinity (< 1 km) to *V. orsiniana* (M. M. Martínez-Ortega, pers. observ.).

With regard to the other polyploids from *V.* subsection *Pentasepalae*, the phylogenetic relationships among them remain largely unresolved and taxonomic conclusions are hampered by the lack of resolution of the ITS and plastid DNA trees. In this situation, we can only base further taxonomic decisions on morphological (N. López-González, B. M. Rojas-Andrés & M. M. Martínez-Ortega, unpubl. data), cytological (B. M. Rojas-Andrés *et al.*, unpubl. data) and chorological considerations. Thus, we recognize the following polyploid species and subspecies: *V. austriaca* subsp. *austriaca*, *V. austriaca* subsp. *dentata* and *V. austriaca* subsp. *jacquinii* in a large '*V. austriaca* complex' (mostly 6*x*), *V. satureiifolia* (4*x*), *V. sennenii* (8*x*) and *V. teucrium* (8*x*).

This study reiterates (Suárez-Santiago *et al*., 2007; Pessoa *et al*., 2012) the importance of using multiple and complementary sources of evidence (i.e. integrative taxonomy; Dayrat, 2005) for delimiting species in polyploid complexes. Using information from DNA-based phylogenetic analyses, morphological characters, ploidy and biogeography, we have been able to establish a reliable taxonomic framework for the diploid members of the group. On this basis, we will be able to disentangle the patterns of reticulation among the polyploids using more informative molecular markers.

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APPENDIX

Table A1. Details of specimens of *Veronica* included in this study, including locality, geographical coordinates, herbarium information, collectors, ploidy and GenBank accession numbers. Sequences newly generated for this study are in bold. Dashes indicate missing data

No.	Taxon name	Collector and individual number	Ploidy*	Locality†	Latitude	Longitude	Herbarium (voucher)	Collectors‡	ITS GenBank	*trnH-psbA* GenBank	*ycf6-psbM* GenBank
Subsection *Pentasepalae*											
1	*V. aragonensis* 1	AA14-6	–	E: Huesca, collado de Ceresa, Peña Montañesa	42.41182N	0.52454E	*SALA 121537*	AA, ATR, MMO, XG	**KT361667**	**KT361717**	**KT361769**
2	*V. aragonensis* 2	MO885-1	4*x* (C)	E: Granada, Huéscar, Sierra de la Sagra	37.94617N	2.56752W	*SALA 93528*	JSA, MMO	**KT361668**	**KT361718**	**KT361770**
3	*V. aragonensis* 3	MO1045	–	E: Huesca, Turbón massif, between the Turbonet and Aligas peak	–	–	*SALA 121536*	MMO	AY741517		
4	*V. crinita*	MS1244-1	2*x* (FCM)	BG: Varna, between Vinitsa and Aldza monastery	43.25097N	28.00242E	*SALA 149037*	BR, MMO, MS, XG	KJ630594	**KT361719**	**KT361771**
5	*V. crinita* forma *bosniaca*	s.n.	§	BiH:, Ravan Planina, Mt. Tajan	44.28861N	18.19028E	*SALA 149244*	BFR	**KT361669**	**KT361720**	**KT361772**
6	*V. thracica*	MS1227-4	2*x* (FCM)	BG: Plodiv, near Popovitsa	42.12294N	25.07567E	*SALA 149038*	BR, MMO, MS, XG	**KT361670**	**KT361721**	**KT361773**
7	*V. dentata* 1	BR178-2	6*x* (FCM)	A: Krems, between Weissenkirchen and Dürnstein	48.40502N	15.51789E	*SALA 149043*	BR, MMO, XG	KJ630593	**KT361722**	**KT361774**
8	*V. dentata* 2	MO6041-7	6*x* (FCM)	A: Wien, Kalksburg	48.14111N	16.24728E	*SALA 149383*	BR, MMO, XG	**KT361671**	**KT361723**	**KT361775**
9	*V. fontqueri* 1	MO858-2	2*x* (C)	E: Málaga, Sierra de las Nieves, Puerto de los Pilones	–	–	*MGC 46659*	FJH, MMO, JSA	**KT361672**	**KT361724**	**KT361776**
10	*V. fontqueri* 2	MO890-1	2*x* (C)	E: Almería, Sierra de Gádor, Llanos de Boliches	36.91023N	2.79794W	*SALA 95041*	FJH, MMO, JSA	**KT361673**	**KT361725**	**KT361777**
11	*V. jacquinii* 1	BR112-1	2*x* (FCM)	HR: Dubrovnik, Gromača	42.72444N	18.01778E	*SALA 149039*	BR, MMO, SA, XG	KJ630595	**KT361726**	**KT361778**
12	*V. jacquinii* 2	SA392-2	2*x* (FCM)	MNE: Žabljak	43.16378N	19.15008E	*SALA 149287*	BR, MMO, SA, XG	**KT361674**	**KT361727**	**KT361779**
13	*V. jacquinii* 3	Albach 70	–	Botanischer Garten Bonn, cultivated	–	–	*BONN*	DA	AF313000		
14	*V. jacquinii* 4	BR121-1	6*x* (FCM)	MNE: Treskavac Mts. between Borkovici and Boricje	43.10678N	18.90789E	*SALA 149369*	BR, MMO, SA, XG	**KT361675**	**KT361728**	**KT361780**
15	*V. jacquinii* 5	MO4595-9	6*x* (FCM)	BG: Stara Zagora, Nova Mahala, near Nikolaev	42.63394N	25.75508E	*SALA 149377*	BR, MMO, MS, XG	**KT361676**	**KT361729**	**KT361781**
16	*V. jacquinii* 6	MO5528-1	6*x* (FCM)	HR: Josipdol, between Oštarije and Ribarići	45.22533N	15.24603E	*SALA 149042*	BR, MMO, SA, XG	KJ630596	**KT361730**	**KT361782**
17	*V. jacquinii* 7	SA382-8	6*x* (FCM)	BiH:, Travnik, Vlašić	44.27483N	17.59997E	*SALA 149389*	BR, MMO, SA, XG	**KT361677**	**KT361731**	**KT361783**
18	*V. jacquinii-orbiculata* 1	BR102-2	4*x* (FCM)	BiH:, Potoci, Porim planina, Rujiste	43.46342N	17.95917E	*SALA 149041*	BR, MMO, SA, XG	KJ630600	**KT361732**	**KT361784**
19	*V. jacquinii-orbiculata* 2	SA377-7	4*x* (FCM)	BiH:, Sarajevo, Trebević	43.83508N	18.43531E	*SALA 149355*	BR, MMO, SA, XG	**KT361678**	**KT361733**	**KT361785**

Appendix *Continued*

No.	Taxon name	Collector and individual number	Ploidy*	Locality†	Latitude	Longitude	Herbarium (voucher)	Collectors‡	ITS GenBank	*trnH-psbA* GenBank	*ycf6-psbM* GenBank
20	*V. javalambrensis* 1	BR222-3	2*x* (FCM)	E: Salamanca, La Mata de la Armuña	41.03783N	5.67681W	*SALA 149328*	BR, NLG	**KT361679**	**KT361734**	**KT361786**
21	*V. javalambrensis* 2	ER7085-1	2*x* (C)	E: Valdelinares, El Hornillo	40.39994N	0.59625W	*SALA 110650*	ER, XG	**KT361680**	**KT361735**	**KT361787**
22	*V. krylovii* 1	s.n.	§	RUS: Rep. Altai, Ulaganskii distr., vicin. of lake Choibekkel	50.43672N	87.59361E	*ALTB*	AIS & al.	**KT361681**	**KT361736**	**KT361788**
23	*V. krylovii* 2	s.n.	§	KZ: Ridge Tarbagataj	47.15000N	82.10000E	*ALTB*	SS, DG, EA	**KT361682**	**KT361737**	**KT361789**
24	*V. orbiculata*	BR110-12	2*x* (FCM)	HR: Peljesak peninsula, between Trstenik and Pijavičino	42.93728N	17.37764E	*SALA 149294*	BR, MMO, SA, XG	**KT361683**	**KT361738**	**KT361790**
25	*V. austriaca* var. *emarginata*	MO5537-12	2*x* (FCM)	HR: between Omis and Makarska, Brela	43.40383N	16.89364E	*SALA 149337*	BR, MMO, SA, XG	**KT361684**	**KT361739**	**KT361791**
26	*V. orbiculata* var. *hercegovinica 1*	BR100-12	4*x* (FCM)	BiH:, Mostar, Mt. Hum	43.32728N	17.79939E	*SALA 149336*	BR, MMO, SA, XG	**KT361685**	**KT361740**	**KT361792**
27	*V. orbiculata* var. *hercegovinica 2*	BR100-2	2*x* (FCM)	BiH:, Mostar, Mt. Hum	43.32728N	17.79939E	*SALA 149336*	BR, MMO, SA, XG	**KT361686**	**KT361741**	**KT361793**
28	*V. orsiniana* 1	A3248-3	2*x* (FCM)	I: Abruzzo, La Majella	–	–	*SALA 149297*	ER & al.	**KT361687**	**KT361742**	**KT361794**
29	*V. orsiniana* 2	A3267-1	2*x* (FCM)	I: Abruzzo, La Majella	–	–	*SALA 149298*	ER & al.	**KT361688**	**KT361743**	**KT361795**
30	*V. orsiniana* 3	MO5569-16	2*x* (FCM)	MK: Gevgelija, Mt. Kozuf	41.20006N	22.24369E	*SALA 149278*	BR, MMO, SA, XG	**KT361689**	**KT361744**	**KT361796**
31	*V. prostrata* 1	BR182-9	2*x* (FCM)	A: Rohrendorf bei Krems, Saubühel	48.42783N	15.65614E	*SALA 149040*	BR, MMO, XG	KJ630602	**KT361745**	**KT361797**
32	*V. prostrata* 2	BR215-1	2*x* (FCM)	CH: Valais, Charrat	46.12739N	7.14564E	*SALA 149312*	BR, MMO, XG	**KT361690**	**KT361746**	**KT361798**
33	*V. prostrata* 3	MS1239-3	2*x* (FCM)	BG: Shumen, Madara	43.27253N	27.10661E	*SALA 149317*	BR, MMO, MS, XG	**KT361691**	**KT361747**	**KT361799**
34	*V. rhodopea* 1	BR12-3	2*x* (FCM)	BG: Pazardzhik, Belmeken, near the lake	42.17653N	23.80769E	*SALA 149321*	BR, MMO, MS, XG	**KT361692**	**KT361748**	**KT361800**
35	*V. rhodopea* 2	s.n.	–	BG: Mt. Rila	–	–	*SOM*	Bondev	AF144459		
36	*V. rosea* 1	DP783-2	2*x* (FCM)	MA: Ifrane, Azrou, Djebel Hebri	33.35294N	5.14817W	*SALA 149323*	DP, ER, TR, VL	**KT361693**	**KT361749**	**KT361801**
37	*V. rosea* 2	MO5502-5	2*x* (FCM)	DZ: Tlemcen, Col de Krorchef	34.57506N	1.76428W	*SALA 149324*	AJ, JPG, MMO, SB	**KT361694**	**KT361750**	**KT361802**
38	*V. rosea* 3	MO1501	2*x* (FCM)	MA: Midelt, Great Atlas, Cirque de Jaffar	32.61139N	4.80333W	*SALA 121638*	LD, MMO, XG	AY741519		
39	*V. satureiifolia* 1	BR204-3	4*x* (FCM)	F: Dep. Lozère, Aven Armand	44.22481N	3.35617E	*SALA 149356*	BR, MMO, XG	**KT361695**	**KT361751**	**KT361803**
40	*V. satureiifolia* 2	MO768-1	4*x* (C)	E: Huesca, Ansó, Linza, Paso del'Onso	–	–	*SALA 124593*	LD, MMO	**KT361696**	**KT361752**	**KT361804**
41	*V. satureiifolia* 3	MO1093	4*x* (C)	D: Baden Württemberg, Bopfingen, Härtsfeldhausen, Rohrbachmühle	48.83556N	10.37028E	*SALA 124594*	MMO	**KT361697**	**KT361753**	**KT361805**
42	*V. sennenii* 1	BR223-1	8*x* (FCM)	E: Álava, Salinas de Añana, way up to the collado de la Rastrilla	42.77514N	3.10808W	*SALA 149394*	BR, MMO, NLG	**KT361698**	**KT361754**	**KT361806**
43	*V. sennenii* 2	BR224-1	8*x* (FCM)	E: Cantabria, Sonabia	43.41250N	3.33350W	*SALA 149395*	BR, MMO, NLG	**KT361699**	**KT361755**	**KT361807**
44	*V. tenuifolia* 1	MO652-1	2*x* (C)	E: Navarra, Cáseda, at the intersection of road NA534 and Canal de Bárdenas	42.38666N	1.42078W	*SALA 95040*	LD, MMO	**KT361700**	**KT361756**	**KT361808**
45	*V. tenuifolia* 2	MO670-1	2*x* (C)	E: Huesca, Arro, near the road to Los Molinos	42.40878N	0.21723E	*SALA 93496*	LD, MMO	**KT361701**	**KT361757**	**KT361809**
46	*V. tenuifolia* 3	MO1043	2*x* (C)	E: Huesca, Arro.	–	–	*SALA 93496*	MMO	AY741516		

Appendix *Continued*

No.	Taxon name	Collector and individual number	Ploidy*	Locality†	Latitude	Longitude	Herbarium (voucher)	Collectors‡	ITS GenBank	*trnH-psbA* GenBank	*ycf6-psbM* GenBank
47	*V. teucrioides* 1	AH3633	§	GR: Kozani, Mt. Siniátsikon, over Námata	40.40333N	21.53722E	*SALA 149270*	AH & al.	**KT361702**	**KT361758**	**KT361810**
48	*V. teucrioides* 2	BR48-1	2*x* (FCM)	GR: Mt. Olympus	40.03872N	22.33369E	*SALA 149330*	BR, MMO, MS, XG	**KT361703**	**KT361759**	**KT361811**
49	*V. teucrium* var. *teucrium* 1	MO4574-2	8*x* (FCM)	BG: 3 km E from Tran, road to Pernik.	42.83794N	22.69272E	*SALA 149044*	BR, MMO, MS, XG	KJ630603	**KT361760**	**KT361812**
50	*V. teucrium* var. *teucrium* 2	MO6025-3	8*x* (FCM)	D: Nordrhein-Westfalen, Euskirchen, between Iversheim and Arloff	50.59158N	6.77928E	*SALA 149414*	AA, BR, MMO, NLG	**KT361704**	**KT361761**	**KT361813**
51	*V. teucrium* var. *angustifolia* 3	BR168-10	8*x* (FCM)	F: Dep. Haute-Savoie, Mt. Salève	46.13512N	6.18264E	*SALA 149399*	BR, MMO, XG	**KT361705**	**KT361762**	**KT361814**
52	*V. teucrium* var. *angustifolia* 4	MO6022-7	8*x* (FCM)	F: Dep. Eure et Loir, Châteaudun, Thiville	48.00259N	1.38930E	*SALA 149413*	AA, BR, MMO, NLG	**KT361706**	**KT361763**	**KT361815**
53	*V. turrilliana* 1	BR45-3	2*x* (FCM)	TR: Vize, towards Kömürköy-Alkpinar	41.59472N	27.82417E	*SALA 149333*	BR, MMO, MS, XG	**KT361707**	**KT361764**	**KT361816**
54	*V. turrilliana* 2	MS1247-1	2*x* (FCM)	BG: 15 km N of Malko Turnovo, near the bridge on the river Veleka	42.08506N	27.42903E	*SALA 149334*	BR, MMO, MS, XG	**KT361708**	**KT361765**	**KT361817**
55	*V. turrilliana* 3	Albach 278	–	TR: Istanbul			*WU*	DA	AF486360		
V. subsection *Armeno-Persicae*											
	V. armena	AH1747-1	–	TR: Erzurum, Mt. Palandöken	39.51 N	41.17 E	*MA 687629*	AH & al.	**KT361709**	**KT361766**	**KT361818**
	V. farinosa	Alava 13656	–	–	–	–	*TUR*	Alava	AY741518		
	V. liwanensis	Struwe 1411	–	NYBG, cultivated	–	–	*WU*	Struwe	AF312997		
	V. oltensis	Sfruwe 1405	–	NYBG, cultivated	–	–	*WU*	Struwe	AF312995		
V. subsection *Orientalis*											
	V. bombycina subsp. bolkardaghensis	Struwe 1406	–	NYBG, cultivated	–	–	*WU*	Struwe	AF486358		
	V. bombycina subsp. bombycina	Struwe 1403	–	NYBG, cultivated	–	–	*WU*	Struwe	AF486353		
	V. bomb subsp. froediniana	Albach 709	–	TR: Pelli Dagi	–	–	*WU*	DA	**KT361710**		
	V. cinerea	Albach & Chase, 113		RBG Kew, cultivated	–	–	*K*	DA & MC	AY144458		
	V. cuneifolia subsp. isaurica	Struwe 1409	–	NYBG, cultivated	–	–	*WU*	Struwe	AF486354		
	V. dichrus	Struwe 1407	–	NYBG, cultivated	–	–	*WU*	Struwe	AF312998		
	V. multifida	Albach 1143	–	TR: prov. Antalya, Taurus	–	–	*OLD*	DA	**KT361711**		
	V. orientalis	Albach 701	–	TR: Van, Karabel pass	–	–	*WU*	DA	AY741515		
	V. pectinata 1	CA6210-1	–	TR: Bolu, S of Abant Golu	40.35 N	31.17 E	*MA 688478*	CA & al.	**KT361712**		
	V. pectinata 2	Struwe 1410	–	–	–	–	*WU*	Struwe	AY144460		
	V. polium	Albach 712	–	TR: Pelli Dagi	–	–	*WU*	DA	**KT361713**		
V. subsection *Petraea*											
	V. bogosensis	s.n.	–	–	–	–	*MO*	Prima	AF486359		
	V. caucasica	Albach 326	–	GE: Kazbegi	–	–	*WU*	DA	AF486357		
	V. peduncularis 1	MO1554-3	–	GE: Mtskheta, monastery Sedaseni	41.88028N	44.77778E	*SALA 110319*	MMO, XG, LMC	**KT361714**		
	V. peduncularis 2	Albach 325	–	GE: Kazbegi	–	–	*WU*	DA	AF486356		
	V. umbrosa	s. n.	–	RBG Kew, cultivated	–	–	*K*	Lancaster	AF486355		
	V. vendetta-deae	Albach 327	–	GE: Kazbegi	–	–	*WU*	DA	AF486361		

Appendix *Continued*

No.	Taxon name	Collector and individual number	Ploidy*	Locality†	Latitude	Longitude	Herbarium (voucher)	Collectors‡	ITS GenBank	*trnH-psbA* GenBank	*ycf6-psbM* GenBank
Not assigned to any subsection											
	V. czerniakowskyana	Terme 39177E	–	IR: Khorassan: Kopet Dagh	–	–	*EVIN*	Terme	AF486362		
	V. paederotae	Klein 7901	–	IR: Prov. Mazandaran	–	–	*WU*	Klein	AF509783		
Outgroup											
	V. arvensis	BR229-1	–	E: Salamanca	40.96561N	5.67911W	*SALA 149232*	BR	**KT361715**	**KT361767**	**KT361819**
	V. polita	s.n.	–	E: Salamanca, Ciudad Rodrigo	40.59611N	6.55194W	*SALA 149255*	JSA	**KT361716**	**KT361768**	**KT361820**
	V. chamaedrys	1970-1438	–	RBG Kew	–	–	*K*	MC	AF313003		

*Ploidy determined by direct chromosome count (C) or flow cytometry (FCM) is indicated in parentheses.†A, Austria; BG, Bulgaria; BiH, Bosnia and Herzegovina; D, Germany; DZ, Algeria; E, Spain; GE, Georgia; GR, Greece; HR, Croatia; I, Italy; IR, Iran; KZ, Kazakhstan; MA, Morocco; MK, FYR of Macedonia; MNE, Montenegro; NYBG: New York Botanical Garden; RBG Kew, Royal Botanic Gardens, Kew; RUS, Russia; SRB, Serbia; TR, Turkey.‡AA, A. Abad de Blas; AH, A. Herrero; AIS, A. I. Schmakov; AJ, A. Juan-Gallardo; AT, A. Tribsch; BFR, B. Frajman; BR, B. M. Rojas-Andrés; CA, C. Aedo; DA, D. Albach; DG, D. German; DP, D. Pinto-Carrasco; EA, E. Antoyuk; ER, E. Rico; FJH, F. J. Hernández-García; JPG, J. Peñas-de Giles; JSA, J. A. Sánchez-Agudo; LD, L. Delgado-Sánchez; LMC, L. M. Muñoz-Centeno; MC, M. Chase; MMO, M. M. Martínez-Ortega; MS, M. Santos-Vicente; NLG, N. López-González; SA, S. Andrés-Sánchez; SB, S. Barrios-de León; SS, S. Smirnov; TR, T. Romero; VL, V. Lucía-García; XG, X. Giráldez.§Individuals for which flow cytometric measurements failed.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Final accepted names for the taxa included in this study and their corresponding basionyms. In the cases indicated with an asterisk (*) the corresponding basionyms have not been used (see Materials and methods).