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ALLOZYMIC VARIATION AND RELATIONSHIPS WITHIN *VIOLA* SECT. *VIOLA* (VIOLACEAE) IN IRAN

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Abstract. Genetic variation and differentiation in taxa of the genus *Viola* L. sect. *Viola*, subsections *Viola* and *Rostratae* (Kupffer) W. Becker, were studied from natural populations occurring in Iran. Two isoenzyme systems, Phosphoglucoisomerase (PGI) and Aconitase (ACO), encoding four putative loci, were employed to detect interspecific and intraspecific genetic variation. Considering the patterns of isoenzyme variation in the studied taxa, it is evident that *V. caspia* (Rupr.) Freyn subsp. *caspia* and *V. capia* subsp. *sylvestroides* Marcussen are closely related. The species *V. alba* Bess. subsp. *alba* and *V. sintenisii* W. Becker are isoenzymatically well characterized as distinct genetic entities.

Key words: allozymes, genetic variation, *Viola*, PGI, ACO, Iran

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Introduction

Viola L. is widely distributed in temperate habitats of the Northern Hemisphere to higher mountainous elevations near the Equator and the Southern hemisphere (Ballard *et al*. 1999). More than 600 species have been described and currently 16 sections are recognised worldwide (Marcussen *et al*. 2015). Section *Viola* is subdivided into subsect. *Viola* and subsect. *Rostratae* (Kupffer) W. Becker. Because of extreme morphological variation, both the delimitation of the genus and its infrageneric classification are not yet universally agreed upon. Recent molecular studies have provided insights into the phylogenetic relationships, revealing some major patterns and proposing informal monophyletic groups in the genus (Wahlert *et al*. 2014; Mohammadi Shahrestani *et al*. 2015).

In spite of clear morphological differentiation, the two subsections *Viola* and *Rostratae* are shown to be phylogenetically closely related (Ballard *et al*. 1999; Marcussen *et al*. 2012; Marcussen *et al*. 2015). The taxa of subsect. *Viola* lack aerial stems and have inexplosive capsules, while those of subsect. *Rostratae* have aerial floriferous stems and explosive capsules (Becker 1925; Marcussen & Karlsson 2010). Yousefi *et al*. (2012) suggested that six species of sect. *Viola* are present in Iran: *V. alba* Bess. subsp. *alba*, *V. odorata* L., *V. sintenisii* W. Becker (subsect. *Viola*); *V. caspia* (Rupr.) Freyn, *V. reichenbachiana* Jord. *ex* Bor. and *V. rupestris* F. W. Schmidt (subsect. *Rostratae*). All treatments of *V. sintenisii* have related it to *V. alba*. *Viola sintenisii* is treated as a subspecies of *V. alba* (Becker 1918), whereas Marcussen *et al.* (2005) recorded it as a distinct species.

Becker (1924) included in *V. sieheana* W. Becker plants from the Caspian region; these Caspian plants had previously been described as *V. sylvatica* var. *caspia* Rupr and raised to species level [e.g., *V. caspia* (Rupr.) Freyn]. Morphology, ploidy levels and allozymic evidence suggest a splitting of the SE European–SW Asian *V. sieheana* into three taxa, *V. sieheana* s.str., *V. caspia* subsp. *caspia* and *V. caspia* subsp. *sylvestroides* Marcussen (Marcussen & Borgen 2011). Also, *V. sieheana* is a Eurasian species with a wide distribution in the Balkans, Turkey and Lebanon, whereas *V. caspia* has been found only

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in the Caspian-Caucasian regions westwards to Crimea. *Viola caspia* subsp. *caspia* and *V. caspia* subsp. *sylvestroides* often grow in the same habitats, but these two taxa can be distinguished on the basis of corolla color. The corolla color of *V. caspia* subsp. *caspia* is whitish, while it is lavender blue in *V. caspia* subsp. *sylvestroides* (Saeidi Mehrvarz *et al*. 2013). No evidence for gene flow between these two cytotypes has been found: that is, no records of naturally occurring hybrids, even in mixed populations, and no evidence for character introgression. *Viola caspia* is sympatric with *V. reichenbachiana,* which is normally blue-flowered but which is extremely difficult to distinguish from *V. caspia* when not in flower in N Iran. The chromosome number 2n = 20 is most common in the investigated species in sect. *Viola*, represented as tetraploid, but *V. caspia* is reported to have 2n = 40 as octoploid (Marcussen & Borgen 2011). Of the molecular techniques available, allozyme electrophoresis was deemed adequate to estimate the amount of genetic variation within and among plant populations. This technique is amenable for conservation genetic surveys because data can be obtained quickly for many individuals and it provides single-gene molecular markers that are biparentally inherited and that generally adjust to a codominant pattern of expression (Batista & Sosa 2002).

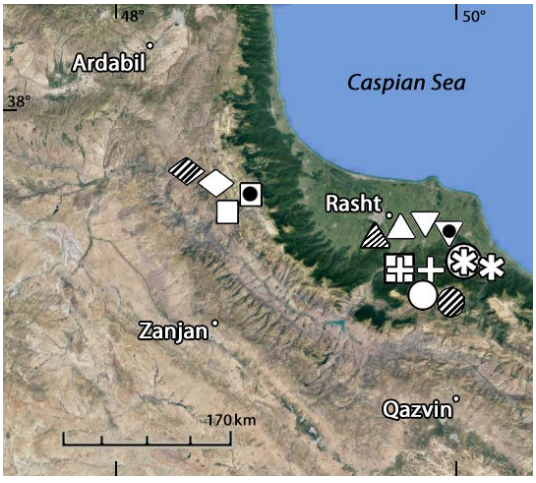

Fig. 1. Collection localities of the investigated taxa: ○ – *Viola odorata* L. in Larkhani, ◍ – *V. odorata* in Deyleman, + – *V. sintenisii* W. Becker in Lahijan, ⊞ – *V. sintenisii* in Siahkal, ✱ – *V. alba* Bess. subsp. *alba* in Lahijan, ❁ – *V. alba* subsp. *alba* in Javaher Deh, □ – *V. rupestris* F. W. Schmidt in Asalem to Khalkhal road, ■ – *V. rupestris* in Almas highlands, ◇ – *V. reichenbachiana* Jord. *ex* Bor. in Asalem to Khalkhal road, ◈ – *V. reichenbachiana* in Almas highlands, △ – *V. caspia* subsp. *sylvestroides* Marcussen in Lahijan, ◬ – *V. caspia* subsp. *sylvestroides* in Sangar, ▽ – *V. caspia* (Rupr.) Freyn subsp. *caspia* in Sheytan-kuh of Lahijan, ⍒ – *V. caspia* subsp. *caspia* in Atakuh of Lahijan.

Allozymes have been used to study intraspecific variation in a number of related species within the genus *Viola* (Marcussen & Borgen 2000; Marcussen 2003; Nordal *et al*. 2005). Recently, allozymic variation analysis on the Ponto Caucasian *V. sieheana* complex (including *V. sieheana* and *V. caspia*) was performed by Marcussen and Borgen (2011). They showed that *V. sieheana* and *V. caspia* are no more closely related to each other than to either of the other investigated European species. The aim of the present study was to assess isoenzyme variation and genetic affinities among the taxa of sect. *Viola* in Iran.

Material and methods

Sampling

Plant material is listed in Table 1 and indicated on a geographical map in Figure 1. Young and cigar-shaped leaves of 100 individuals belonging to 10 regions of N Iran were collected, representing 7 species, and analyzed for isoenzyme variation. Two populations from each species were sampled for electrophoretically detectable diversity. Collection information of the studied species is presented in Table 1. Voucher specimens are deposited in the Herbarium of Guilan University (GUH). The specimens were collected during 2012–2013, then the leaves of samples were transferred to the laboratory on ice and stored at –80°C for a few days until further use.

Extraction

Leaves (200–300 mg) were frozen with liquid nitrogen and then homogenized with 500 grinding buffer (Morden *et al*. 1987) for 20 min at 1000 rpm in 4°C. The supernatants were stored at –80°C until electrophoresis in a vertical polyacrylamide gel. Two enzymatic systems, PGI (Phosphogluco isomerase) and ACO (Aconitase), were applied in this study.

Table 1. Collection information for the studied taxa.

Taxon	Sect./Subsect.	Number of individuals	Collection data
Viola alba subsp. *alba*	*Viola*/*Viola*	20	Guilan: Lahijan, Javaher Deh, April 2012, *17321 Yeganeh*
V. sintenisii		23	Guilan: Siahkal, Lunak, August 2013, *17324 Yeganeh*
V. odorata		26	Guilan: Daylaman, Larkhani, April 2012, *17328 Yeganeh*
V. caspia subsp. *caspia*	*Viola*/*Rostratae*	22	Guilan: Sheytan-kuh of Lahijan, Atakuh of Lahijan April 2012, *17330 Yeganeh*
V. caspia subsp. *sylvestroides*		25	Guilan: Lahijan, Sangar, April 2012, *17334 Yeganeh*
V. reichenbachiana		21	Ardabil: Asalem to Khalkhal road, Almas highlands, April 2013, *17339 Yeganeh*
V. rupestris		27	Ardabil: Asalem to Khalkhal road, Almas highlands, April 2013, *17341 Yeganeh*

ELECTROPHORESIS

Allozyme electrophoresis was carried out using vertical polyacrylamide slab gel. The gel was composed of a 5% stacking gel layer and 10% separating gel layer. The buffer was 0.5 M Tris-HCl pH 6.8 for the stacking gel and 1.5M Tris-HCl pH 8.8 for the separating gel. The electrode buffer was 25 mM Tris-base, 192 mM glycine pH 8.3. Approximately 20 µl of the sample supernatant was loaded in a separate pocket of the stacking gel. The proteins contained in the supernatant were subjected to electrophoresis for 3 h at constant 100 volts. Electrophoresis was performed in acrylamide gel and cooled on ice, then stopped when the indicator dye (bromophenol blue) had migrated about 10 cm into the gel. The staining protocol followed Wendel and Weeden (1989).

DATA ANALYSIS

Zones of enzyme activity that varied independently of other such zones were considered to be coded by single gene loci. Based on the mean allelic frequencies/locus/taxon, genetic identities and distances were calculated. The band frequencies of Euclidean distances were calculated and cluster analysis with the UPGMA method using NTYSYS-PC ver. 2.02 was applied. The cluster analysis included all specimens except hybrids, while the PCO analysis, based on Dice's similarity coefficient, included hybrids (Marcussen & Borgen 2011).

RESULTS

The different band patterns of the isoenzyme systems display variation within taxa of *Viola* sect. *Viola* (Fig. 2). Band frequency values range from 0 to 0.43 within a given individual (Table 2).

Phosphoglucoisomerase (*PGI*). Nine different bands in two zones were interpreted as two isoenzyme loci of *PGI-1* and *PGI-2*. Some loci of these isoenzymes had two alleles: for example,

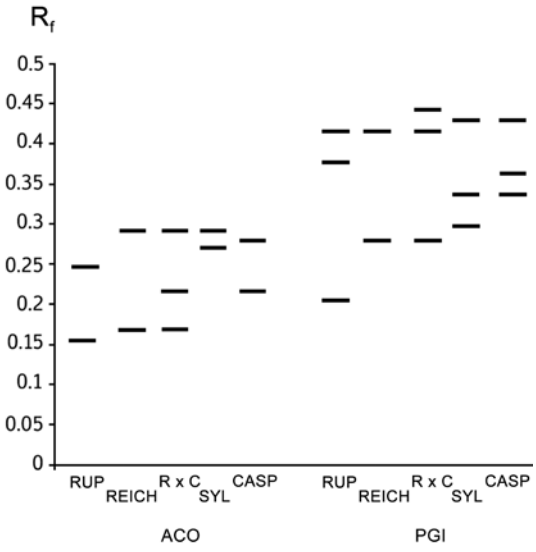

Fig. 2. Isoenzyme banding patterns of phosphogluco isomerase and aconitase of *Viola* subsect. *Rostratae* (Kupfer) W. Becker (isoenzyme bands were assigned to two loci: PGI1, PGI2; ACO1, ACO2). RUP – *V. rupestris* F. W. Schmidt, R × C – *V. reichenbachiana* × *V. caspia*, CASP – *V. caspia* (Rupr.) Freyn subsp. *caspia*, REICH – *V. reichenbachiana* Jord. *ex* Bor., SYL – *V. caspia* subsp. *sylvestroides* Marcussen.

allele D and E from *V. caspia* subsp. *sylvestroides,* and allele C and D from *V. caspia* subsp. *caspia*. *PGI-2* G and *ACO-2* G were shared as the most frequent alleles in *V. caspia* × *V. reichenbachiana, V. reichenbachiana, V. rupestris* and *V. caspia* subsp. *caspia, V. caspia* × *V. reichenbachiana, V. reichenbachiana*, respectively (Table 2).

Aconitase (*ACO*). Isoenzyme analysis of *ACO* yielded seven different bands among seven taxa. These bands included two isoenzyme loci: *ACO-1* (with slower-migrating allozymes) and *ACO-2* (with faster-migrating allozymes). *ACO-1* activity was found in three areas. Two different alleles were displayed in the anodal *ACO-b*. The allele coding for the slowest of the two bands was fixed in *V. caspia* subsp. *caspia*, and the fastest in *V. caspia* subsp. *sylvestroides* (Fig. 2). Two alleles were also displayed in the catodal *ACO-e* locus. All populations from N Iran (Fig. 2, in *V. reichenbachiana* and *V. reichenbachiana* × *V. caspia*) were fixed for the fastest-moving band; other *V. rupestris* populations displayed the slowest-moving band (Fig. 2).

The results of UPGMA analysis based on Euclidean distances are shown in Figures 2 and 3. The taxa analyzed fall into two clusters. Group A included *V. rupestris* and *V. reichenbachiana,* which was separated from other taxa. Its Euclidean distance was 0.45. This taxon (*V. rupestris*) differed in more bands, except in allele *G* of *PGI-2* which overlapped with *V. reichenbachiana.* This group segregated into two taxa. Although *V. caspia* subsp. *caspia* and *V. caspia* subsp. *sylvestroides* showed similar band patterns, they had different band patterns relative to *V. caspia* subsp. *sylvestroides* in some loci, for example, allele C of *PGI-1*, allele E of *PGI-1*, allele D of *ACO-1*, allele C of *ACO-1*, allele F of *ACO-2* and allele G of *ACO-2*.

As seen in Table 3 and Figure 6, 12 bands could be assigned to 4 isoenzyme loci: *PGI-1*, *PGI-2*, *ACO-1* and *ACO-2*. Band frequency values ranged from 0 to 0.37 (Table 3) within a given individual. Among the three taxa of *V.* subsect. *Viola*, alleles of *V. alba* subsp. *alba* were placed in a lower anodal situation versus the other two taxa (R_f values of 0.34, 0.36, 0.37, respectively). It had a locus with two alleles in allozymes A and B from isoenzyme *PGI-1*; this character was not found in other taxa.

As shown in Figure 5, the taxa analyzed fall into two clusters. Group A comprised *V. odorata* and *V. sintenisii.* These two taxa were syntenic,

Table. 2. Mean allele frequencies in the studied taxa of *Viola* subsect. *Rostrata* (Kupfer) W. Becker.

Isoenzyme	Allozyme	*V. caspia* subsp. *sylvestroides*	*V. caspia* subsp. *caspia*	*V. caspia*× *V. reichenbachiana*	*V. reichenbachiana*	*V. rupestris*
PGI-1	A	0.0	0.0	0.0	0.0	0.22
	B	0.0	0.0	0.28	0.28	0.0
	C	0.0	0.30	0.0	0.0	0.0
	D	0.34	0.34	0.0	0.0	0.0
	E	0.36	0.0	0.0	0.0	0.0
PGI-2	F	0.0	0.0	0.0	0.0	0.37
	G	0.0	0.0	0.41	0.41	0.41
	H	0.42	0.42	0.0	0.0	0.0
	I	0.0	0.0	0.43	0.0	0.0
ACO-1	A	0.0	0.0	0.0	0.0	0.16
	B	0.0	0.0	0.17	0.17	0.0
	C	0.23	0.0	0.23	0.0	0.0
	D	0.0	0.27	0.0	0.0	0.0
ACO-2	E	0.0	0.0	0.0	0.0	0.25
	F	0.28	0.0	0.0	0.0	0.0
	G	0.0	0.29	0.29	0.29	0.0

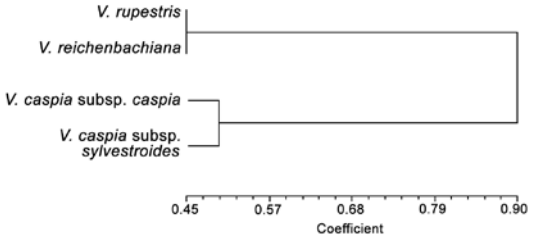

Fig. 3. Dendrogram obtained from UPGMA analysis of Euclidean distance values based on isoenzyme variation in *Viola* subsect. *Rostratae* (Kupfer) W. Becker.

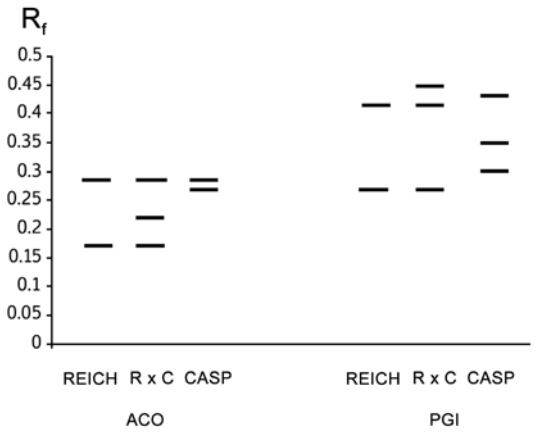

Fig. 4. Isoenzyme banding patterns of PGI and ACO revealing hybridization (R) between *V. reichenbachiana* Jord. *ex* Bohr. (REICH) and *V. caspia* (Rupr.) Freyn subsp. *caspia* (CASP).

similar to alleles found in group B except for *V. alba* subsp. *alba* which had different gene synteny.

Discussion

In population genetics, allozyme electrophoresis (Kim *et al*. 1991; Marcussen & Nordal 1998) and random amplified polymorphic DNA (RAPD) markers have been used to analyze intraspecific genetic variation and the relationships between many continental *Viola* species (Ko *et al*. 1998; Oh *et al*. 1998; Neuffer *et al*. 1999). Homoeolog number, inferred from isoenzymes and low-copy genes, suggests that 2n = 20 and 2n = 40 in sect. *Viola* correspond to the tetra- and octoploid level, respectively, based on x = 5 (Marcussen & Nordal 1998; Nordal & Jonsell 1998; Marcussen & Borgen 2011; Marcussen *et al*. 2015). Our data confirm that two subspecies of *V. caspia* fall into distinct groups defined by allelic composition in two enzyme systems (PGI and ACO). The two morphs rarely occurred within the same site. The habitat distribution of *V. caspia* subsp. *sylvestroides* was more extensive than in *V. caspia* subsp. *caspia*; the former also exhibited a longer flowering period than the latter. Surprisingly, this rather profound genetic distinction is not reflected in overall morphology except in corolla color, which is either lavender blue or nearly white. Neither do the two corolla morphs appear to differ in habitat ecology: they both have wide ecological amplitudes that

Table 3. Mean allele frequencies in the studied taxa of *Viola* L. subsect. *Viola.*

Isoenzyme	Allozyme	*V. odorata*	*V. sintenisii*	*V. alba* subsp. *alba*
PGI-1	A	0	0	0.13
	B	0	0	0.2
	C	0.24	0	0
	D	0	0.26	0
PGI-2	E	0	0	0.34
	F	0.36	0	0
	G	0	0.37	0
ACO-1	A	0	0	0.14
	B	0	0.21	0
	C	0.24	0	0
ACO-2	D	0	0.35	0.35
	E	0.37	0	0

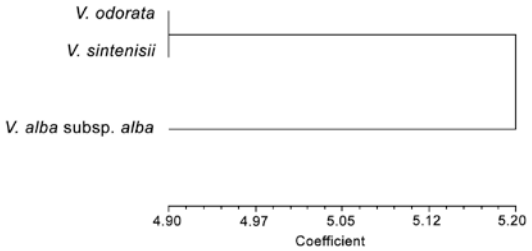

Fig. 5. Dendrogram obtained from UPGMA analysis of Euclidean distance values based on isoenzyme variation in *Viola* L. subsect. *Viola.*

range from open wooded steppe to deep soils and dense-canopy moist beech forest. The two corolla color morphs seem isolated by prezygotic barriers, as hybridization rarely seems to happen, possibly because they attract different pollinators. Based on seed size, the two subspecies of *V. caspia* differed slightly; in fact the seeds of *V. caspia* subsp. *caspia* are on average smaller than those of *V. caspia* subsp. *sylvestroides* (Marcussen & Borgen 2000). According to anatomical studies, the ratio of lateral stem cross-section diameter to pith diameter in *V. caspia* subsp. *caspia* is greater than *V. caspia* subsp. *sylvestroides.* The abaxial midrib surface of *V. caspia* subsp. *caspia* is U-shaped; it is concave in *V. caspia* subsp. *sylvestroides* (Saeidi Mehrvarz *et al*. 2013). Isoenzyme data presented in this study support the opinion of Marcussen and Borgen (2011) that *V. caspia* subsp. *caspia* and *V. caspia* subsp. *sylvestroides* are closely related but different and well-defined taxa within the genus *Viola*. According to allozymes and anatomical features, *V. rupestris* is distinct from other taxa. *Viola rupestris* with seven vascular bundles can be distinguished from *V. reichenbachiana* and *V. caspia*, which have eight and eleven vascular bundles, respectively (Yousefi *et al*. 2012)

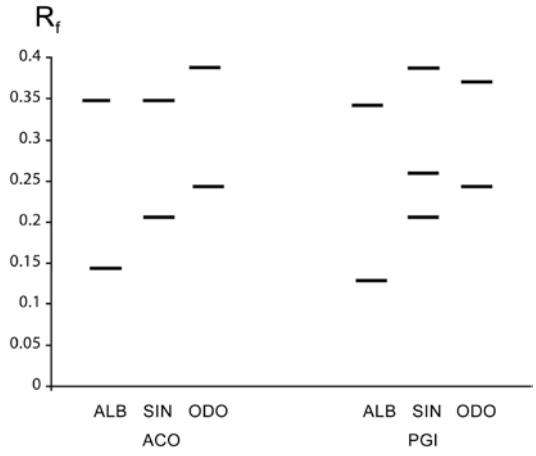

Fig. 6. Isoenzyme banding pattern Phosphoglucoisomerase and Aconitase of *Viola* L. subsect. *Viola* (isoenzyme bands were assigned to two loci: PGI1, PGI2; ACO1, ACO2). ALB – *V. alba* Bess. subsp. *alba*, SIN – *V. sintenisii* W. Becker, ODO – *V. odorata* L.

Viola caspia is sympatric with *V. reichenbachiana,* which is normally blue-flowered but extremely difficult to distinguish from *V. caspia* when they have no flowers. These two species readily hybridize to form a sterile hybrid (Marcussen & Borgen 2011). The chromosome number of *V. reichenbachiana* is 2n = 20, while that of *V. caspia* is 2n = 40 (Marcussen & Borgen 2000). According to Saeidi Mehrvarz *et al*. (2014), the pollen grains of *V. caspia* are prolate-spherodial and have a granular exine, whereas *V. reichenbachiana* and *V. rupestris* have oblate-spheroidal pollen grains with a perforate-granulate exine. Our results are completely congruent with other information, and the selected markers were suitable for distinguishing taxa of subsection *Rostratae.*

Based on the work of Marcussen *et al*. (2005), *V. sintenisii* is sympatric with *V. alba* subsp. *alba* in Azerbaijan and Iran. Sympatry clearly indicates reproductive isolation between these two taxa. *Viola sintenisii* has shorter pubescence and broader stipules than *V. alba.* Based on several morphological traits, especially leaf shape, the patterns of the indument and pigmentation, *V. alba* subsp. *alba* differs from *V. sintenisii.* The leaves of Caucasian subsp. *alba* are triangular-cordate, having straight or slightly convex margins with numerous crenules, while the leaves of *V. sintenisii* differ in being broader, triangular-reniform, with convex margins and few crenules. The leaf indument of *V. sintenisii* is much shorter than in *V. alba* subsp. *alba.* Saeidi Mehrvarz *et al*. (2014) reported that the exine of *V. alba* subsp. *alba* is perforate-subpsilate while the exine of *V. sintenisii* is perforate-granulate. The two species differ in details of root anatomy;

V. sintenisii has a pith region but this region is absent in *V. alba* subsp. *alba* (Yousefi *et al*. 2012). According to anatomical studies of leaf cross sections, *V. alba* subsp. *alba* differs from *V. sintenisii* (Saeidi Mehrvarz *et al*. 2013). It should be noted that *V. alba* subsp. *alba* and *V. sintenisii,* separated mainly on the basis of morphological and anatomical differences, are well characterized as distinct genetic entities.

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