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# Identification of species of *Vicia* subgenus *Vicia* (Fabaceae) using chloroplast DNA data

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**Abstract:** *Vicia* subgenus *Vicia* (Fabaceae) contains several economically important food and forage legume species. Generally, the taxonomy and identification of these species have relied heavily on a few diagnostic morphological characters (e.g., the number of flowers per inflorescence). However, there are several specimens with intermediate morphological characters that make diagnosis of these species using those characters a difficult task for nonspecialists and render results that are not always accurate. The use of molecular markers, therefore, is the most attractive alternative strategy for a more accurate identification of species of the subgenus *Vicia*. In the present study, an attempt was made to investigate polymorphism in the chloroplast DNA of 22 species of the subgenus *Vicia* using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) on 9 chloroplast regions and a set of 12 consensus chloroplast simple sequence repeat (ccSSR) primer pairs for the diagnosis of the targeted species. Based on the data generated, it was possible to identify all of the species analyzed, except for 4 species that were placed in 2 groups (1, *V. noeana* and *V. dionysiensis*; and 2, *V. serratifolia* and *V. hyaeniscyamus*), each of which had a unique chloroplast type. Species-specific markers developed here are useful for the early diagnosis of targeted *Vicia* species and can provide essential baseline data for the evolution of systematic breeding and conservation strategies, as well as guidance for germplasm collections.

**Key words:** Diagnosis, legumes, markers, taxonomy, *Vicia*

## Introduction

Legumes (Fabaceae or Leguminosae) are the third largest flowering plant family behind orchids (Orchidaceae) and asters (Asteraceae). The temperate and herbaceous genus *Vicia* L. is a member of the legume tribe Vicieae of the Papilionoideae (Frediani et al. 2004). *Vicia* comprises 166 (Allkin et al. 1986) annual or perennial species distributed mainly in Europe, Asia, and North America, also extending to the temperate regions of South America and tropical Africa (Maxted 1993; Jaaska 2005).

Taxonomic treatments of the genus have been based on the traditional morphotypological taxonomy, with subgenera and sections delimited variously by differently selected diagnostic characters (Jaaska 2005). Maxted et al. (1991) divided the genus into 2 subgenera (Kupicha 1974, 1976), *Vicilla* and *Vicia*. The 2 subgenera can be distinguished using the following characters: stipule nectary, peduncle length, style type, keel shape, legume, and canavanine (Kupicha 1976). Maxted (1993) classified the subgenus *Vicia* into 9 sections, 9 series, 38 species, 14 subspecies, and 22 varieties. The center of diversity and the possible origin for subgenus *Vicia* is the northeastern Mediterranean. This area includes Iraq, Iran, the southwestern republics of the former Soviet Union, Syria, and Turkey (Maxted 1995).

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The subgenus *Vicia* contains several economically important food and forage legume species that have a center of distribution in the eastern Mediterranean. It contains the extensively cultivated fava bean (broad bean), *Vicia faba*, and the minor forages *V. narbonensis* and *V. sativa* subsp. *sativa* (common vetch) (Maxted 1995). The subgenus also contains taxa that have a high potential for use as forages of the future: *V. hyaeniscyamus*, *V. noeana*, and *V. sativa* subsp. *amphicarpa* (Maxted et al. 1990).

Generally, diagnoses of the major subgeneric groupings within the subgenus *Vicia* have relied heavily on a few diagnostic characters that have been used commonly and consistently. These are: the presence of peduncle and, if present, the length of the peduncle in relation to either the flower or the subtending leaf length; the number of flowers per inflorescence; the presence of stipular nectarines; and the positioning of hairs around the style. Many more characters are used, however, to distinguish subgroups in the subgenus (Maxted 1995). These characters are life span, plant stature, the number of flowers per inflorescence, the number of leaflets per leaf, the size of leaflets, calyx mouth shape, flower color, vexillum shape, vexillum pubescence, legume pubescence, legume protrusion, relative legume size, relative legume shape, legume suture curvature, legume hair types, seed shape, relative hilum length, relative position of the seed hilum and lens, and seed surface type. A key to *Vicia* subgenus *Vicia* sections, series, and species was presented by Maxted (1995). Hosseinzadeh et al. (2008) reported, however, that there are several specimens with intermediate morphological characters and that there are many polymorphisms in these characters that share a common habitat. This means that species diagnosis using morphological characters is a difficult task for nonspecialists and results are not always accurate (Haider et al. 2010).

In recent years, karyological and C-banding studies have played an important role in solving taxonomic problems for the identification of some species of the subgenus *Vicia*, such as *V. sativa*, *V. grandiflora*, *V. pannonica*, *V. narbonensis* (Navratilova et al. 2003), and *V. bithynica* (İnceer and Hayırlıoğlu Ayaz 2005). A similar karyotype, however, does not always indicate affinity. For these reasons, coupled with the skill and time required to record cytological features, these techniques generally have little utility for species diagnosis.

Based on total protein, albumin, and globulin electrophoresis, Sakr et al. (2010) studied 5 wild Egyptian species of *Vicia*, 4 of which belonged to the subgenus *Vicia* (*V. sativa* subsp. *amphicarpa*, *V. ervilia*, *V. narbonensis*, and *V. villosa*). However, the limitations of seed storage protein analysis may hinder its effective use for diagnostic purposes (Haider 2011). These limitations include the low number of loci analyzed and the influence of different environmental conditions and developmental stages on band profiles.

The development of various molecular techniques that generate molecular markers, which are more stable within species, has made it possible to accurately identify plants. The use of molecular approaches for species identification (Jürgen and Weder 2002), therefore, is the most attractive alternative strategy available for a more accurate identification of species of the subgenus *Vicia*.

Due to the low rate of chloroplast DNA (cpDNA) evolution compared to the nuclear genome, the utility of polymerase chain reaction (PCR)-based markers derived from cpDNA analysis for the identification of plant species has been clearly established (Haider 2003; Haider and Nabulsi 2008; Ford et al. 2009). In the present study, PCR-restriction fragment length polymorphism (PCR-RFLP) (Haider 2012) and consensus chloroplast simple sequence repeat (ccSSR) were used to investigate polymorphism in cpDNA among 22 species of the subgenus *Vicia* for their diagnosis.

## Material and methods

### Plant material and DNA extraction

Seeds of landrace accessions belonging to 22 species of the subgenus *Vicia* were provided by the Genetic Resources Unit of the International Center for Agricultural Research in the Dry Areas (ICARDA) in Aleppo, Syria, and by the General Commission for Scientific Agriculture Research (GCSAR) in Damascus, Syria. There were generally 10 accessions for each of the subgenus *Vicia* species used, apart from

*V. sativa* (14 accessions); *V. melanops* (2 accessions); *V. lathyroides* and *V. bithynica* (9 accessions); *V. assyriaca* and *V. serratifolia* (1 accession); *V. dionysiensis*, *V. villosa*, and *V. hyaeniscyamus* (4 accessions); *V. lutea* (11 accessions); and *V. narbonensis* (12 accessions). Accessions were selected to represent as much of the geographic range of each species as possible.

A total of 5 seeds from each accession were sown in a 0.5-L plastic pot containing turf (Euroveen B.V., USA). Seeds were allowed to germinate under laboratory conditions (temperature: 21 °C) and grow for 1 month. A single randomly selected accession from each of the 22 species to be identified was used for molecular analysis (Table 1). For DNA extraction, 5-10 fresh young leaves were collected from plants 3-4 weeks after seed germination. The collected leaves were washed 3 times in sterile distilled water, immersed in liquid nitrogen, and kept at –60 °C until use. For total genomic DNA extraction, carried out according to the procedure described by Doyle and Doyle (1987), approximately 0.5 g of leaves collected from each accession was ground into a fine powder in liquid nitrogen. Recovered DNA pellets were dried under the laminar flow and then resuspended in 150 µL of double distilled and sterilized water. DNA was quantified using a GeneQuant Spectrometer (Amersham Biosciences, UK) and the concentration of all samples was set at 10 ng $\mu L^{-1}$.

Table 1. Accessions of the subgenus *Vicia* species used in this study (nomenclature according to Maxted 1993).

| No. refer to species | Genus/species | ICARDA accession used |
|---|---|---|
| 1 | *V. sativa* L. (subsp. *macrocarpa*) | IG. 64093/TUR |
| 2 | *V. lutea* L. (subsp. *lutea*) | IG. 111980/MAR |
| 3 | *V. melanops* Sibth. & Smith | IG. 60951/BGR |
| 4 | *V. lathyroides* L. | IG. 63425/SYR |
| 5 | *V. assyriaca* Boiss. | IG. 64098/TUR |
| 6 | *V. serratifolia* Jacq. | IG. 108443/SYR |
| 7 | *V. grandiflora* Scop. | IG. 64096/TUR |
| 8 | *V. noeana* (Reuter in Boiss.) Boiss. | IG. 64241/TUR |
| 9 | *V. dionysiensis* Mout. | IG. 62650/LBN |
| 10 | *V. michauxii* Sprenge | IG. 62521/JPN |
| 11 | *V. hybrida* L. | IG. 64259/TUR |
| 12 | *V. peregrina* L. | IG. 64620/TKM |
| 13 | *V. anatolica* Turrill | IG. 137455/ARM |
| 14 | *V. aintabensis* Boiss. & Hausskn. ex Boiss. | IG. 65029/TUR |
| 15 | *V. pannonica* Crant (subsp. *pannonica*) | IG. 64340/AZE |
| 16 | *V. hyaeniscyamus* Mout. | IG. 64108/SYR |
| 17 | *V. sericocarpa* Fenzl | IG. 64291/JOR |
| 18 | *V. bithynica* (L.) L. | IG. 64581/AZE |
| 19 | *V. ervilia* (L.) Willd. | IG. 63417/SYR |
| 20 | *V. villosa* Roth | IG. 683 |
| 21 | *V. faba* L. | IG. 72432/SYR |
| 22 | *V. narbonensis* L. | IG. 2388/574 |

**PCR-RFLP analysis of cpDNA**

PCR was carried out on DNA samples of targeted species to amplify a total of 9 chloroplast coding and noncoding loci using universal primer pairs. Of these loci, there were 7 loci (*rps7&rps12.2*, *psbB-F-petB-petD.3*, *23S,4.5S&5S*, *atpE&trnM*, *psaB.3*, *petB&D*, and the *psbC-trnS* intergenic spacer) that were targeted with primers developed by Haider and Wilkinson (2011). Primers that target the chloroplast *trnL* intron (Taberlet et al. 1991) were also used. The remaining locus was *matK* (FP: 5` cctatccatctggaaatcttag 3` and RP: 5` gaagccagaatggattttcc 3`).

PCR was performed in 0.2-mL microtubes (Eppendorf, Germany) using the Eppendorf Mastercycler. Each reaction contained 10× PCR buffer (Eurobio, Italy), 10× $MgCl_2$ (50 mM) (Eurobio), forward primer (15 µM) (Invitrogen, UK), reverse primer (15 µM) (Invitrogen), dNTP mix (10 mM) (Fermentas, Lithuania), and Taq polymerase (5 U $\mu L^{-1}$) (Eurobio). DNA was added to each PCR reaction at a rate of 20 ng and the total volume was adjusted with double distilled $H_2O$ to 20 µL. For 35 cycles, PCR reactions were subjected to 94 °C for 30 s for DNA denaturation, 46.5-58 °C for 1 min for the annealing of primers, 72 °C for 1 min for the extension of the target chloroplast region, and 72 °C for 5 min for the final extension. For the visualization of PCR products, 2 µL of each PCR product was loaded into 1.5% agarose gel that was run at 120 V for 30 min.

Restriction digests were performed on PCR amplicons generated from the targeted loci for the detection of single nucleotide polymorphisms (SNPs) in those loci (Haider et al. 2010). PCR products (1-5 µL) were digested according to the manufacturer (Fermentas) instructions using 23 endonucleases (Table 2). Digested fragments were separated by electrophoresis on 2% agarose gel that was run at 100 V for 2 h in 1× TAE buffer and visualized under UV light using a gel documentation system (GDS8000, UVP, UK).

**Analysis of ccSSRs**

A set of 12 consensus chloroplast primer pairs developed by Chung and Staub (2003) for amplification of ccSSRs was used for the analysis of 6 ccSSRs in the 12 species (*V. assyriaca*, *V. serratifolia*, *V. noeana*, *V. dionysiensis*, *V. hybrida*, *V. peregrina*, *V. anatolica*, *V. aintabensis*, *V. pannonica*, *V. hyaeniscyamus*, *V. sericocarpa*, and *V. narbonensis*) that could not be identified by PCR-RFLP. These were ccSSR-1 (*TrnK*), ccSSR-2 (*TrnQ-TrnS*), ccSSR-5 (*Rps2-RpoC2*), ccSSR-12 (*PsbB-PsbT*), ccSSR-15 (*Rpl2-Rpl23*), and ccSSR-16 (*TrnL*).

Table 2. The cpDNA loci and enzymes used for the restriction of their amplification products.

| Chloroplast locus | Restriction enzyme |
|---|---|
| *trnL* intron | Hae III, Taq I, Dde I, and Tru 1I |
| *rps7&rps12.2* | Rsa I |
| *psbB-F-petB-petD.3* | Hae III, Hinf I and Dpn I |
| *psbC-trnS* | Tru1 I, Hae III and Hinf I |
| *23S,4.5S&5S* | Rsa I and Alu I |
| *atpE&trnM* | Alu I and Dpn I |
| *psaB.3* | Alu I and Taq I |
| *petB&D* | Hpa II, Hae III, and Tru 1I |
| *matK* | Tru 1I, Mbo I, and Hae III |

Each 20-µL PCR reaction volume contained 3.75 mM of $MgCl_2$, 0.2 mM of each dNTP, 20 ng of DNA, 10 µM of each primer (F and R), 10× PCR buffer, and 2.5 U of Taq DNA polymerase. All amplifications were conducted using the following cycling profile: 93 °C for 1 min; 40 cycles of 94 °C for 30 s, 49-53 °C for 60 s, and 72 °C for 60 s; and 72 °C for 5 min for the final extension. For the visualization of PCR products, 5 µL of each PCR product was loaded into 2% agarose gel that was run at 100 V for 2 h.

**Detection of species specificity of generated PCR-RFLP and ccSSR markers**

Locus-enzyme combinations that were useful for generating PCR-RFLP markers that aided in the discrimination of 7 *Vicia* species [*V. bithynica*, *V. melanops*, and *V. faba* (*trnL* intron-Tru 1I); *V. sativa* (*petB&D*-Hpa II); *V. villosa* (*trnL* intron-Dde I); *V. ervilia* (*trnL* intron-Taq I); and *V. grandiflora* (*psbC-trnS*-Tru 1I)] were applied for 2-14 accessions (Table 3) of each of these species. Similarly, ccSSRs that were useful for generating markers for the identification of 4 *Vicia* species [*V. hybrida* and *V. pannonica* (ccSSR-15); *V. anatolica* (ccSSR-2); and *V. peregrina* (ccSSR-16)] were applied for 10 accessions of each of these species (Table 3).

Table 3. Accessions used for the detection of intraspecific variation of PCR-RFLP and ccSSR markers generated. The GCSAR only provided accessions of *V. lutea* subsp. *hirta* and *V. villosa* subsp. *dasycarpa*.

| Species name | Accessions analyzed | Species name | IG numbers of accessions analyzed |
|---|---|---|---|
| *V. sativa* subsp. *sativa* | | *V. pannonica* subsp. *pannonica* | 60080/DEU, 60081/HUN, and 60085/ARM |
| var. *plathyspermum* | 61973/AUS | subsp. *striata* | 60087/TUR, 116291/TUR, 62697/ HUN, 60088/TUR and 60100/GRC |
| var. *melanosperma* | 61287/TUR and 107557/MAR | *V. pannonica* | 141565/ARM |
| var. *leucosperma* | 60353/FRA and 60331/ROM | | |
| subsp. *nigra* | 63451/SYR and 63049/SYR | | |
| subsp. *amphicarpa* | 63877/SYR and 63412/SYR | | |
| subsp. *cordata* | 64215/EGY and 64187/JOR | *V. melanops* | 62523/JAP |
| *V. sativ*a | 62921/SYR and 62861/SYR | | |
| *V. lutea* | 132522/AZE | *V. peregrina* | 64169/JOR, 62544/JAP, 115825/IRN, 61156/ITA, 62550/ SYR, 60620/SYR, 60711/TUR, 61170/ TUR and 64155/DZA |
| subsp. *vestita* | 64531/DZA, 61042/AUS, 111558/TUN, and 107940/MAR | | |
| subsp. *lutea* | 107765/MAR, 116385/TUR, 64338/AZE, and 111660/TUN | | |
| subsp. hirta | 2993 | *V. anatolica* | 60971/TUR, 60969/TUR, 60967/TUR, 60965/TUR, 60960/TUR, 60992/TUR, 60959/TUR, 60980/TUR and 61024/TKM |
| *V. grandiflora* | 63664/TUR, 63285/SYR, 141522/ ARM, 126956/ARM, 64095/ TUR, 137558/ARM, 141530/ARM, 60950/ITA, 60640/USA, and 60945/SWE | | |
| subsp. *incisa* | 63641/ TUR | *V. villosa* subsp. *dasycarpa* | 3236 and 717 |
| *V. hybrida* | 59985/SYR, 60008/IRQ, 61026/ AUS, 61029/BEL, 61031/ITA, 61033/UNK, 115691/MAR, 62511/JAP, and 64286/JOR | *V. bithynica* | 64029/TUR, 60890/SWE, 60891/ MLT, 60887/ITA, 60892/DEU, 60899/GRC, 62841/SYR and 64090/TUR |
| *V. ervilia* | 63848/ SYR, 62917/ SYR 1117941/ SYR, 110589/ SYR 63562/ SYR, 63564/ SYR 63853/ SYR, 63220/ SYR and 63852/ SYR | *V. faba* | 12264/ SYR, 72492/ SYR 72426/ SYR, 12751/ SYR 72331/ SYR, 72494/ SYR 72387/ SYR, 74198/ SYR and 72449/ SYR |

## Results

### PCR-RFLP analysis

When PCR was carried out on DNA samples of the targeted species in order to amplify 9 chloroplast loci, no size variation was observed among amplicons for 8 of these loci. The exception to this rule was the amplicons generated using the *trnL* intron primers (Figure 1).

The restriction of amplicons from each locus targeted using 1-6 endonucleases was either complete or absent. The number of restriction profiles within the different locus-enzyme combinations (summarized in Table 4) that were applied ranged from 1 (positive or negative restriction in all samples) to 7 for *trnL* intron-Tru 1I. There was failure to restrict across all of the 22 samples for 10 of the 23 locus-enzyme combinations tested, while

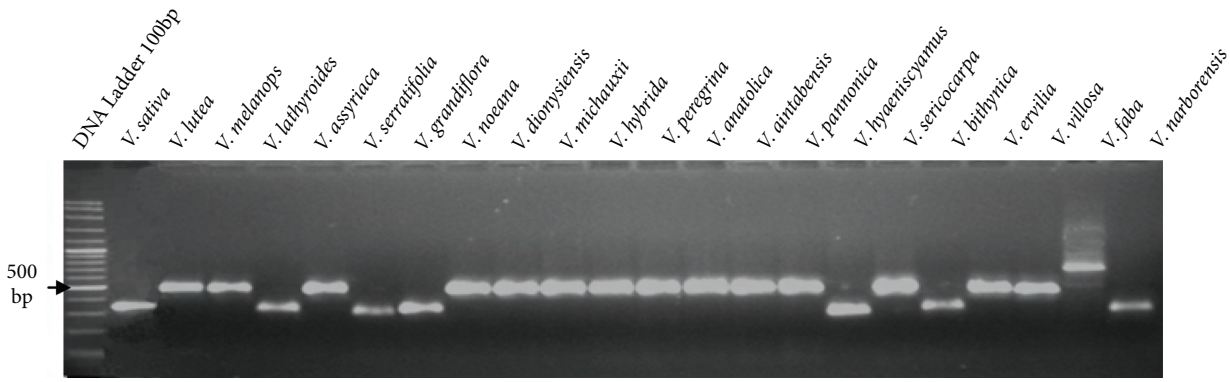


Figure 1. Amplification of *trnL* intron in the 22 species targeted.

5 of the remaining combinations had a positive and identical restriction profile in all samples. As for the 8 locus-enzyme combinations that revealed polymorphism among the species analyzed (an example is shown in Figure 2), the highest level of polymorphism was observed for *trnL* intron-Tru 1I, while the locus-enzyme combination *petB&D*-Hpa II revealed the least polymorphism (only 1 SNP unique to *V. sativa*). These combinations had diagnostic utility for 10 out of 22 species examined (*V. sativa*, *V. lutea*, *V. melanops*, *V. lathyroides*, *V. grandiflora*, *V. michauxii*, *V. bithynica*, *V. ervilia*, *V. villosa*, and *V. faba*). The remaining species were divided into 3 groups, each of which contained 3-5 species with identical restriction profiles. The first group included species *V. serratifolia*, *V. hyaeniscyamus*, and *V. narbonensis*; the second contained *V. noeana*, *V. dionysiensis*, *V. peregrina*, and *V. aintabensis*; and the third group included *V. assyriaca*, *V. hybrida*, *V. anatolica*, *V. pannonica*, and *V. sericocarpa*.

Species *V. faba* had the highest number of species-specific markers, with a total of 4. These were *trnL* intron PCR marker and *trnL* intron-Hae III, -Taq I, and -Tru 1I. This was followed by *V. villosa* (*trnL* intron-Dde I and -Tru 1I) and *V. sativa* (*petB&D*-Hpa II and *trnL* intron-Tru 1I). *PsbC-trnS*-Tru 1I was specific to *V. grandiflora*, *trnL* intron-Taq I generated a specific profile to *V. ervilia*, and *trnL* intron-Tru 1I also generated a specific profile to each of *V. lutea*, *V. melanops*, and *V. bithynica*.

**ccSSRs**

Size polymorphism among SSR products generated for the 12 species analyzed (*V. assyriaca*, *V. serratifolia*, *V. noeana*, *V. dionysiensis*, *V. hybrida*, *V. peregrina*, *V. anatolica*, *V. aintabensis*, *V. pannonica*, *V. hyaeniscyamus*, *V. sericocarpa*, and *V. narbonensis*) was observed using all primer pairs except ccSSR-1 (*TrnK*). Primer ccSSR-15 (*Rpl2-Rpl23*, Figure 3) revealed the highest polymorphism, with 4 different sizes of SSR products observed.

Specific ccSSR profiles were generated for samples of *V. narbonensis* (ccSSR-5 and ccSSR-12), *V. peregrina* and *V. pannonica* (ccSSR-16), and *V. anatolica* (ccSSR-2). The results of the ccSSR analysis are summarized in Table 5.

**Specificity of PCR-RFLP and ccSSR markers generated**

No PCR-RFLP polymorphism was observed (Figure 4) when 5 locus-enzyme combinations were applied to different representatives of 6 species (*V. bithynica*, *V. melanops*, *V. faba*, *V. villosa*, *V. ervilia*, and *V. grandiflora*) of the subgenus *Vicia* species. Polymorphism in *petB&D*-Hpa II restriction profiles was observed for the 2 samples (63877/SYR and 63412/SYR) of *V. sativa* that belong to subspecies *amphicarpa*. As for the ccSSR markers developed for 4 species (*V. narbonensis*, *V. peregrina*, *V. pannonica*, and *V. anatolica*), no ccSSR polymorphism was observed when 3 ccSSR primer pairs (ccSSR-2, ccSSR-15, and ccSSR-16) were targeted in different representatives of each of those species (an example is shown in Figure 5).

Table 4. A summary of the PCR-RFLP results generated for the 22 samples analyzed and the number of chloroplast types observed using 23 locus-enzyme combinations.

| Locus-enzyme combination | | Number of restriction profiles | Species that share the same chloroplast type |
|---|---|---|---|
| *trnL* intron | Size of PCR product | 3 | *V. sativa*, *V. lathyroides, V. serratifolia*, *V. grandiflora*, *V. hyaeniscyamus*, *V. bithynica*, and *V. narbonensis* |
| | | | *V. lutea*, *V. melanops*, *V. assyriaca*, *V. noeana*, *V. dionysiensis*, *V. michauxii*, *V. hybrida*, *V. peregrina*, *V. anatolica*, *V. aintabensis*, *V. pannonica*, *V. sericocarpa*, *V. ervilia*, and *V. villosa* |
| | | | *V. faba* |
| | Hae III | 3 | *V. sativa*, *V. lathyroides*, *V. serratifolia*, *V. grandiflora*, *V. hyaeniscyamus*, *V. bithynica*, *V. villosa*, and *V. narbonensis* |
| | | | *V. lutea*, *V. melanops*, *V. assyriaca*, *V. noeana*, *V. dionysiensis*, *V. michauxii*, *V. hybrida*, *V. peregrina*, *V. anatolica*, *V. aintabensis*, *V. pannonica*, *V. sericocarpa*, and *V. ervilia* |
| | | | *V. faba* |
| | Taq I | 4 | *V. sativa*, *V. lathyroides*, *V. serratifolia*, *V. grandiflora*, *V. hyaeniscyamus*, and *V. bithynica* |
| | | | *V. lutea*, *V. melanops*, *V. assyriaca*, *V. noeana*, *V. dionysiensis*, *V. michauxii*, *V. hybrida*, *V. peregrina*, *V. anatolica*, *V. aintabensis*, *V. pannonica*, *V. sericocarpa*, *V. villosa*, and *V. narbonensis* |
| | | | *V. ervilia* |
| | | | *V. faba* |
| | Dde I | 4 | *V. sativa*, *V. lathyroides*, and *V. grandiflora* |
| | | | *V. lutea*, *V. melanops*, *V. assyriaca*, *V. noeana*, *V. dionysiensis*, *V. michauxii*, *V. hybrida*, *V. peregrina*, *V. anatolica*, *V. aintabensis*, *V. pannonica*, *V. sericocarpa*, and *V. faba* |
| | | | *V. serratifolia*, *V. hyaeniscyamus*, *V. bithynica*, *V. ervilia*, and *V. narbonensis* |
| | | | *V. villosa* |
| | Tru 1I | 7 | *V. lathyroides*, *V. serratifolia*, *V. noeana*, *V. dionysiensis*, *V. michauxii*, *V. peregrina*, *V. aintabensis*, *V. hyaeniscyamus*, *V. ervilia*, and *V. narbonensis* |
| | | | *V. assyriaca*, *V. grandiflora*, *V. hybrida*, *V. anatolica*, *V. pannonica*, and *V. sericocarpa* |
| | | | *V. melanops* |
| | | | *V. sativa* |
| | | | *V. lutea* and *V. villosa* |
| | | | *V. bithynica* |
| | | | *V. faba* |
| *23S,4.5S&5S* | RsaI | 1 | All negative |
| | Alu I | 1 | All negative |
| *psbC-trnS* | Tru 1I | 3 | *V. sativa*, *V. lathyroides*, *V. serratifolia*, *V. hyaeniscyamus*, *V. bithynica*, *V. ervilia*, *V. villosa*, *V. faba*, and *V. narbonensis* |
| | | | *V. lutea*, *V. melanops*, *V. assyriaca*, *V. noeana*, *V. dionysiensis*, *V. michauxii*, *V. hybrida*, *V. peregrina*, *V. anatolica*, *V. aintabensis*, *V. pannonica*, and *V. sericocarpa* |
| | | | *V. grandiflora* |
| | Hae III | 1 | All negative |
| | Hinf I | 1 | All positive |
| *atpE&trnM* | Alu I | 1 | All positive |
| | Dpn I | 1 | All negative |
| *psaB.3* | Alu I | 1 | All negative |
| | Taq I | 1 | All positive |
| *psbB-F-petB-petD.3* | Hae III | 1 | All negative |
| | Hinf I | 1 | All positive |
| | Dpn I | 1 | All negative |
| *petB&D* | Hpa II | 2 | *V. sativa* |
| | | | Rest |
| | Hae III | 1 | All negative |
| | Tru 1I | 2 | *V. assyriaca*, *V. hybrida*, and *V. sericocarpa* |
| | | | Rest |
| *rps7&rps12.2* | Rsa I | 1 | All positive |
| *matK* | Tru 1I | 2 | *V. assyriaca*, *V. michauxii*, *V. hybrida*, *V. anatolica*, *V. pannonica*, and *V. sericocarpa* |
| | | | Rest |
| | Mbo I | | All negative |
| | Hae III | | All negative |

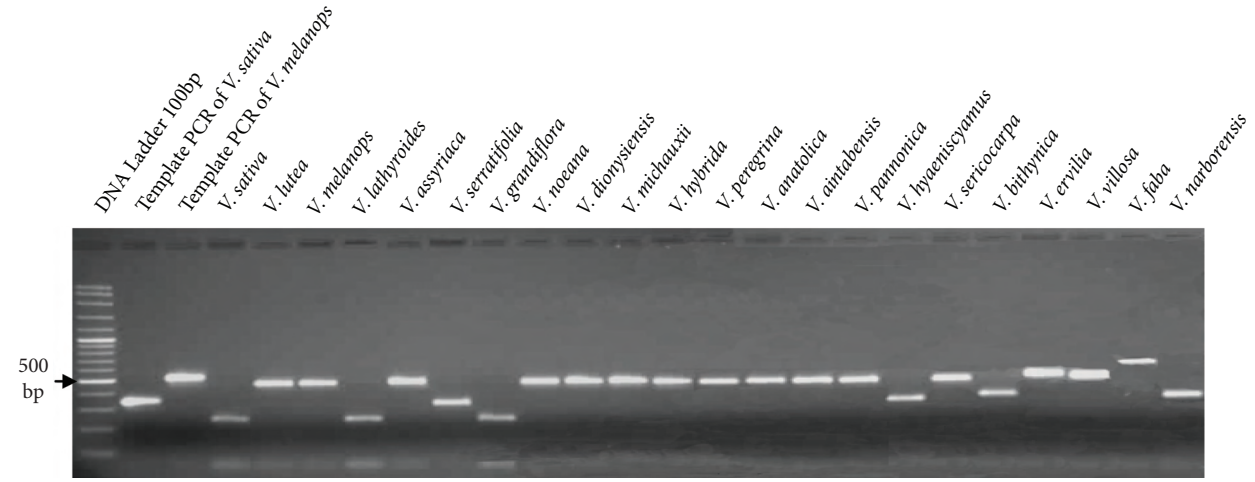


Figure 2. Restriction of *trnL* intron amplicons in the 22 species targeted with Rsa I.

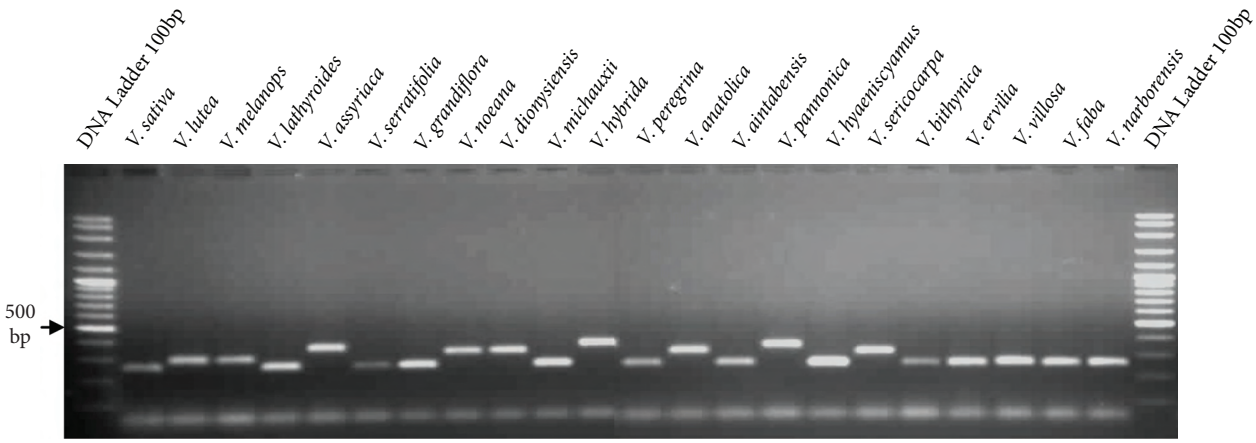


Figure 3. Amplification of ccSSR-15 (*Rpl2-Rpl23*) in the 22 species targeted.

Table 5. A summary of the ccSSR results generated for 12 samples and the number of chloroplast types observed using 6 ccSSR primer pairs (Chung and Staub 2003).

| ccSSR | Annealing T/°C | Number of ccSSR profiles | Species that share the same ccSSR profile and product size |
|---|---|---|---|
| ccSSR-1 | 53 | 1 | 195 bp |
| ccSSR-2 | 49 | 2 | *V. anatolica* (195 bp)<br>Rest (200 bp) |
| ccSSR-5 | 50 | 2 | *V. narbonensis* (280 bp)<br>Rest (265 bp) |
| ccSSR-12 | 50 | 2 | *V. sericocarpa* (240 bp)<br>Rest (230 bp) |
| ccSSR-15 | 50 | 4 | *V. assyriaca*, *V. noeana*, *V. dionysiensis*, *V. anatolica*, and *V. sericocarpa* (400 bp)<br>*V. hybrida* (450 bp)<br>*V. pannonica* (410 bp)<br>*V. serratifolia*, *V. peregrina*, *V. aintabensis*, *V. hyaeniscyamus*, and *V. narbonensis* (280 bp) |
| ccSSR-16 | 53 | 2 | *V. peregrina* and *V. pannonica* (no product)<br>Rest (360 bp) |

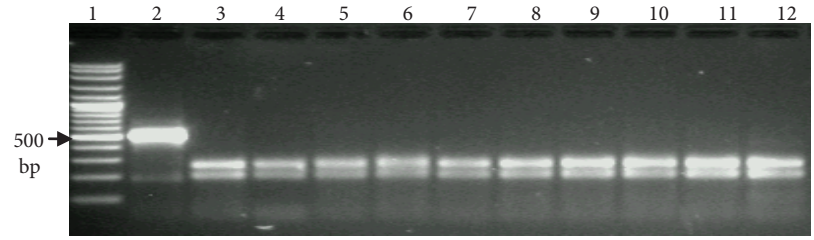


Figure 4. Restriction of amplicons of *trnL* intron in 10 representatives of *V. ervilia* with Taq I. Lane 1, 100 bp DNA ladder; lane 2, template amplicon; lanes 3-12, restriction products of *V. ervilia* samples (Table 3).

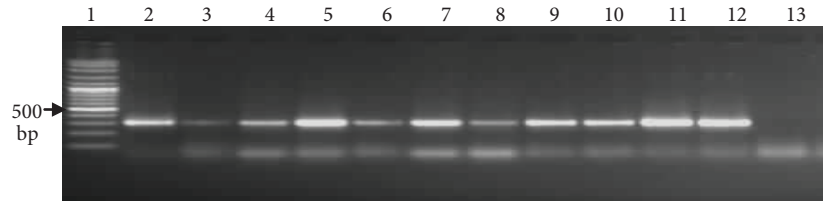


Figure 5. Amplification products using ccSSR-15 primers in 11 representatives of *V. lutea*. Lane 1, 100 bp DNA ladder; lanes 2-12, amplification products of *V. lutea* samples (Table 3); lane 13, a negative control.

**Discussion**

The high economic importance of the genus *Vicia* has led to a large body of studies on the molecular characterization and investigation of phylogenetic relationships among species that belong to this genus. These studies have been based on rDNA (Raina and Ogihara 1995), *in situ* hybridization with repetitive sequences (Navratilova et al. 2003), random amplification of polymorphic DNA (RAPD) (Haider et al. 2000; Sakowicz and Cieślikowski 2006), repetitive DNA sequences as probes (Frediani et al. 2004), capillary electrophoresis (Piergiovanni and Taranto 2005), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on seed storage proteins (MirAli et al. 2007).

The subgenus *Vicia* is one of the largest and most important in the genus *Vicia* (Haider et al. 2000). This subgenus has been most thoroughly studied with different approaches, such as morphological analysis (Maxted et al. 1993), isozymes (Jaaska 1997), RAPD and restriction of cpDNA using PCR-RFLP (Potokina et al. 1999), and a combination of morphology and isozymes (Leht and Jaaska 2002). Among these species, *V. faba*, being a major food and feed legume because of the high nutritional value of its seeds (Duc et al. 2010), has been the focus of the majority of studies based on the analysis of cpDNA (Fennell et al. 1998), mtDNA, and nDNAs (van de Ven et al. 1993). Other studies have been carried out to reveal relationships in the subgenus sections Hypechusa (e.g., Caputo et al. 2006), Peregrinae (e.g., Frediani et al. 2005), and Faba (e.g., Zimniak-Przbylska and Przybylska 1995), and between the latter and sections Bithynica and Narbonensis, which belong to the same subgenus (Sakowicz and Cieślikowski 2006).

In spite of the large body of studies that have been carried out to reveal phylogenetic relationships among species of the subgenus *Vicia*, however, very few attempts have been made to establish the diagnosis of these species using molecular markers. Haider et al. (2000) determined the genetic diversity between fava bean and the closely related species of *V. sativa*, *V. villosa*, and *V. narbonensis* based on RAPD and isozymes. Piergiovanni and Taranto (2005) applied capillary electrophoresis for the discrimination of 13 *Vicia* species belonging to 4 sections. Only 7 (*V. bithynica*, *V. ervilia*, *V. villosa*, *V. sativa*, *V. lutea*, *V. melanops*, and *V. peregrina*) of those species belong to the subgenus *Vicia*. However, these techniques have several shortcomings, such as problems in

reproducibility, reliability, species specificity, and the identification of species that are difficult to resolve. Additionally, they are time-consuming and can be influenced by environmental conditions (Haider 2011).

The efficiency of PCR-RFLP for the detection of variation in the cpDNA of *Vicia* species was established by Potokina et al. (1999), who found that PCR-RFLP of cpDNA provided more precise information concerning phylogenetic relationships among 29 subgenus *Vicia* species than RAPD analysis. Raina and Ogihara (1994) also used this technique to detect cpDNA diversity among *V. faba* and closely related species.

PCR-RFLP applied in this study to template DNAs representing 22 *Vicia* species revealed that all of the primer pairs used generated single-band amplicons of the appropriate size (Taberlet et al. 1991; Haider and Wilkinson 2011). The noncoding regions (*trnL* intron and *psbC-trnS*), which are supposed to evolve more rapidly than coding regions (Gielly and Taberlet 1994), showed a higher level of polymorphism among the species analyzed compared to the coding regions (Haider et al. 2010). Among the coding regions, *petB&D* showed the highest variation. A similar observation was previously reported by Haider and Wilkinson (2011).

PCR-RFLP detected a sufficient level of polymorphism in the cpDNA to allow for the diagnosis of the majority of the species targeted. Among all of the species analyzed, *V. faba* was the most divergent species. This was congruent with the observations of Raina and Ogihara (1994), who provided new evidence that the wild species closely related to *V. faba* did not contribute their plastomes to the evolution of *V. faba*; hence, the authors concluded that none of those species can be considered to be putative allies of the broad bean (Haider et al. 2000). The dendrogram constructed by MirAli et al. (2007) for some *Vicia* species based on the analysis of seed storage proteins showed that the 2 outcrossing species, *V. faba* and *V. villosa* (the latter revealed here to be the second most divergent species), were the most distant among all of the species they targeted. In a previous study, Haider et al. (2000) also found that *V. faba* and *V. narbonensis* have specific isozyme patterns different from those of *V. sativa* and *V. villosa*. Our data also showed that *V. bithynica* and *V. sativa* possess a unique chloroplast type. Maxted (1995) also referred to the uniqueness of these 2 species, which have peduncles longer than the subtending leaf, a characteristic that would place them in subgenus *Vicilla*; however, they are linked to subgenus *Vicia* by the presence of a nectary on the stipule and other characteristics.

The divergence observed in this study among *V. sativa*, *V. grandiflora*, *V. pannonica*, and *V. narbonensis* was also reported earlier by Navratilova et al. (2003), who observed profile variations in the positions and numbers of rDNA loci in those species.

It is worth noting that the 3 species (*V. lutea*, *V. melanops*, and *V. peregrina*) that could not be discriminated by capillary electrophoresis (Piergiovanni and Taranto 2005) were able to be successfully identified in the present study.

The high homology observed among species *V. assyriaca*, *V. melanops*, *V. hybrida*, and *V. sericocarpa* was revealed earlier by Caputo et al. (2006), since they formed a monophyletic unit based on nuclear DNA contents, rDNAs, and karyotype analysis. Two of these species (*V. sericocarpa* and *V. assyriaca*) and *V. anatolica*, which had a very similar chloroplast type to *V. sericocarpa* and *V. assyriaca*, belong to the same section, Hypechusa, as classified by Maxted (1993). A high homology was also observed among the species *V. peregrina*, *V. aintabensis*, and *V. michauxii* (sect. Peregrinae). According to Maxted (1995), these 3 species are the only ones to lack a peduncle and are obviously closely related. This led Ponert (1973) to regard the latter 2 taxa as subspecies of *V. peregrina*.

Unsurprisingly, no intraspecific variation was observed in this study for any of the *Vicia* species examined when PCR, PCR-RFLP, and ccSSR markers were applied for a geographically wide selection of accessions and representative plants of each species. Although polymorphism was observed in *V. sativa* restriction profiles of *petB&D*-Hpa II for the 2 samples of subsp. *amphicarpa*, these 2 samples were still distinct from the other 21 species samples analyzed, all of which had a negative restriction for the same locus-enzyme combination.

For the diagnosis of the 12 species that could not be diagnosed by PCR-RFLP, 6 ccSSR regions were

analyzed in these species using consensus primers, which generated single bands of a size similar to those generated by Chung and Staub (2003) for the closely related *Pisum sativum*. These primers have been previously used for the determination of genetic relationships within Cucurbitaceae (Chung et al. 2003) and among *Avena* species (Li et al. 2009). This is the first attempt to target the ccSSR regions targeted here for the identification of plant species apart from ccSSR-1, which was sequenced by Eurlings and Gravendeel (2006) to discriminate cultivated from wild gaharu (agarwood). In that study, 4 out of 5 ccSSR regions did not show any interspecific variation. The exception was the *trnR-atpA* region, which showed minor differences (a single nucleotide repeat). Therefore, the authors recommended the use of ccSSR primers for species identification. Similarly, by targeting ccSSR regions in this study, we could identify all 12 of the species examined except for 4 species that were divided in 2 groups (1, *V. noeana* and *V. dionysiensis*; and 2, *V. serratifolia* and *V. hyaeniscyamus*), each of which had a unique chloroplast type, and no intraspecific variation was found in the sizes of SSR products among species for each ccSSR. Hence, we also suggest that this approach is suitable for carrying out species discrimination since it is cheap, fast, and reliable, and it generates data that can be easily interpreted.

The species-specific markers developed here can be useful for the early diagnosis of targeted *Vicia* species and provide essential baseline data for evolving systematic breeding and conservation strategies and for guiding germplasm collections.

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