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Genetic relationships among the genera *Cicer* L., *Lathyrus* L., *Lens* Mill., and *Vicia* L., together with similarity of *Lens* taxa based on morphological and AFLP markers

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Abstract: The molecular characterization of the seven taxa in the genus *Lens* Mill. with *Vicia montbretii* has not been performed, and species of the genera *Vicia* L., *Lathyrus* L., and *Cicer* L. using AFLP markers have not been elucidated so far. The objectives of the present study were the assessment of (i) genetic relationships among the genera *Lens*, *Vicia*, *Lathyrus*, and *Cicer* based on AFLP markers, (ii) relationships within the genus *Lens* including the accessions of *L. lamottei* Czefr., *L. tomentosus* Ladiz., and *L. odemensis* Ladiz., based on morphological and AFLP data, and (iii) position of the species *Vicia montbretii* (Fisch. and Mey.) Davis and Plitmann based on molecular markers. The genetic material consisted of seven *Lens* taxa comprising each three accessions of cultivated lentils (*L. culinaris* Medik.), *L. orientalis* (Boiss.) Ponert, *L. tomentosus*, *L. odemensis*, *L. ervoides* (Brign.) Grande, and *L. nigricans* (M.Bieb.) Godr., plus two *L. lamottei* accessions. Two *Vicia montbretii* accessions were also included to assess their relationship with the genus *Lens* Mill. As morphological markers the number of toothed stipules, stipule angle, aristae on peduncle, and pod pubescence were evaluated regarding lentil species. Of the 414 AFLP markers, 280 entailing the most informative and polymorphic were used for the molecular analysis. The genus *Lens* was genetically closer to the genera *Vicia* and *Lathyrus* than to the genus *Cicer*. *V. montbretti* was well separated from the genus *Lens*. The results indicated the following genetic relationship: *Lens culinaris* subsp. *culinaris*, *Lens culinaris* subsp. *orientalis*, *Lens culinaris* subsp. *tomentosus*, *Lens culinaris* subsp. *odemensis*, *L. ervoides*, *L. lamottei*, and *L. nigricans*.

Key words: *Lens*, *Cicer*, *Lathyrus*, *Vicia*, AFLP, phylogeny

1. Introduction

The cultivated lentil (*Lens culinaris* Medik.) is an annual, diploid (2n = 14), self-pollinated species. It belongs to the tribe Fabeae Rchb., which includes the genera *Pisum* L., *Lathyrus* L., *Vicia* L., *Vavilovia* A.Federov, and *Lens* Mill. (Smykal et al., 2015). The genus *Lens* is a small genus containing seven taxa: *Lens culinaris* Medik., *L. orientalis* (Boiss.) Ponert, *L. tomentosus* Ladizinsky, *L. odemensis* Ladizinsky, *L. ervoides* (Brign.) Grande, *L. nigricans* (M. Bieb.) Godron, and *L. lamottei* Czefranova (Cubero et al., 2009).

Cubero (1981) reported five lentil species: *Lens culinaris*, *L. orientalis*, *L. ervoides*, *L. nigricans*, and *L. montbretii* (Fisch. and Mey.) Davis and Plitman. *L. montbretii* was later transferred from the genus *Lens* Mill. to the genus *Vicia* L. due to its unique morphology and cytology. It is a diploid species with 2n = 12 chromosomes and it cannot be crossed with the cultivated lentil (Ladizinsky and Sakar, 1982). Morphological characteristics have been effectively and extensively utilized to distinguish lentil species (Ladizinsky, 1979; Cubero, 1981; Ladizinsky, 1997; Cubero et al., 2009). *L. lamottei* was identified amongst herbarium specimens of *L. nigricans* (Czefranova, 1971), manifesting less dentate stipules and five chromosomal rearrangements (Ladizinsky et al., 1984). *L. odemensis* was described as a new species on the basis of its unique stipules differing from those of *L. nigricans* (Ladizinsky, 1986). *L. tomentosus*, found by Ladizinsky (1997) within the wild lentil populations growing in Mardin, Turkey, morphologically differs from *L. orientalis* by its tomentose pods, a minute satellite, and one large metacentric chromosome (Ladizinsky, 1997).

The classical taxonomy of the genus *Lens* Mill. has been based on morphological characteristics (Cubero, 1981; Ferguson and Robertson, 1999; Ferguson et al., 2000; Cubero et al., 2009), which could be insufficient to denote new species. With the aid of cytological data, the genus *Lens* Mill. was taxonomically reclassified (Ladizinsky and Sakar, 1982; Ladizinsky et al., 1984; Ladizinsky, 1997; Balyan et al., 2002). Hybridizations within of the genus

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Lens have shown that the classification of the species according to their morphology is invalid. For instance, *L. orientalis* can easily be intercrossed with the cultivated lentil (Ladizinsky et al., 1984; Abbo and Ladizinsky, 1991; Ladizinsky and Abbo, 1993; van Oss et al., 1997; Fratini and Ruiz, 2006), and therefore is considered the wild progenitor species (Zohary, 1972; Ladizinsky, 1979). According to the gene pool concept, the progenitor wild species belongs to the first gene pool (Smykal et al., 2015). Hybridizations between the cultivated lentil and *L. tomentosus* failed because of hybrid embryo abortion (Ladizinsky, 1997), despite its appearance similarity with *L. orientalis*. Crossability studies between the cultivated lentil and other *Lens* species have produced fully or partly fertile hybrids (Ladizinsky et al., 1984; Abbo and Ladizinsky, 1991, 1994; Ahmad et al., 1997; van Oss et al., 1997; Fratini and Ruiz, 2006). The success in lentil crosses not only depends on the genetic similarity among species; it also depends on the interaction between the parental genomes, the embryo, and the endosperm in the hybrid zygote (Abbo and Ladizinsky, 1991). The crossability between the cultivated lentil and the wild species is prevented by pre- and postfertilization barriers (Davies et al., 2007). The morphological data have also been supported by molecular data in some studies (Ferguson et al., 2000).

Biochemical markers have potential both in the elucidation of the genetic variation within and between plant species as well as in the evaluation of the associations between cultivated and wild plants. Seed protein electrophoresis (Ladizinsky, 1979) and isozyme markers (Ferguson and Robertson, 1996) have been widely used for the classification of the genus *Lens*. The DNA-based markers, e.g., restriction fragment length polymorphisms (RFLPs) (Havey and Muehlbauer, 1989; Muench et al., 1991), random amplified polymorphic DNA (RAPD) (Abbo et al., 1992; Ahmad et al., 1996; Sharma et al., 1996; Ford et al., 1997; Eujayl et al., 1998; Ferguson et al., 2000; Sonnante and Pignone, 2001; Toklu et al., 2009; Tewari et al., 2012), simple sequence repeat (SSR) or microsatellite markers (Duran et al., 2004; Hamwieh et al., 2005; Reddy et al., 2010; Tewari et al., 2012), intersimple sequence repeat (ISSR) (Zavodna et al., 2000; Sonnante and Pignone, 2001), internal transcribed spacers (ITS) (Mayer and Bagga, 2002), nontranscribed spacer (NTS) (Fernandez et al., 2005), sequenced tagged microsatellite site (STMS) (Datta et al., 2011), single-nucleotide polymorphisms (SNPs) (Alo et al., 2011), resistance gene analogue (RGA) (Sarı et al., 2015), and amplified fragment length polymorphism (AFLP) are powerful tools to elucidate diversity, phylogeny, and taxonomy among *Lens* species. The AFLP technique developed by Vos et al. (1995) has already successfully been used for the assessment of the genetic diversity and phylogenetic relationships between the cultivated (Zavodna et al., 2000; Hamwieh et al., 2005; Tullu et al., 2008) and wild lentil relatives (Sharma et al., 1996; Eujayl et al., 1998; Duran et al., 2004). The objectives of the present study were the assessment of (i) genetic relationships between the genera *Lens* Mill., *Vicia* L., *Lathyrus* L., and *Cicer* L. based on AFLP markers, (ii) relationships among species and subspecies of the genus *Lens* Mill. including *L. lamottei*, *L. tomentosus*, and *L. odemensis* based on morphological and AFLP data, and (iii) position of the species *V. montbretii* with respect to the genus *Lens* Mill.

2. Materials and methods

2.1. Plant materials

The genera *Lens* Mill., *Vicia* L., *Lathyrus* L., and *Cicer* L. and the following seven taxa of the genus *Lens* Mill. including three cultivated lentils (*L. culinaris* Medik.), three accessions each of *L. orientalis*, *L. tomentosus*, *L. lamottei*, *L. odemensis*, *L. ervoides*, and *L. nigricans*, and two accessions of *Vicia montbretii* were grown in pots (3 L) in a plant growth room and then pots with plants were transferred from the plant growth room to the greenhouse (Table 1). The pots were filled with soil taken from the experimental area at the campus. The organic matter and total nitrogen level of the soil were low with a loamy soil texture and a pH of 7.96. During the growing period from March to June, the average temperature was adjusted to 20–30 °C during the night and throughout the day. The pots were irrigated to adjust to field capacity with well water every other day.

2.2. Morphological data

The morphological characterization of the subspecies belonging to the genus *Lens* Mill. considered: aristae presence or absence, stipule dentations and angle, peduncle/rachis length ratio, leaflet number of fully expanded leaves, and plant pubescence (Ferguson et al., 2000; Cubero et al., 2009).

2.3. DNA extraction and AFLP analysis

Genomic DNA was extracted according to the standard cetyl trimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1990) with some modifications. The youngest fully expanded leaves were sampled from each accession and put in 1.5-mL microcentrifuge tubes containing 500 µL of CTAB buffer solution. From each taxon, nine plants were grown but a single plant was used for DNA sampling. Each sample was homogenized by shredding with plastic pestles. After 3-h incubation at 65 °C, 500 µL of chloroform–isoamyl alcohol (25:1) solution was added to each tube and the samples were vortexed. Afterwards, the samples were centrifuged at 14,000 × *g* for 10 min in a table top centrifuge. Approximately, 300 µL of the supernatant was taken into new tubes and 300 µL of isoproponol was added to each sample. After gently mixing, the tubes were stored

Table 1. Plant materials and their origins.

No.	Species and subspecies	Accession no.	IG*	Origin
1	*Lens culinaris* subsp. *orientalis*	G1	72534	Egypt
2	*L. culinaris* subsp. *orientalis*	G2	72526	Turkey
3	*L. culinaris* subsp. *orientalis*	G3	72592	Uzbekistan
4	*L. lamottei*	G4	72552	Spain
5	*L. lamottei*	G5	116006	Turkey
6	*Vicia montbretii*	G6	632670	Turkey
7	*V. montbretii*	G7	515984	Turkey
8	*L. ervoides*	G8	72563	Ukraine
9	*L. ervoides*	G9	72653	Syria
10	*L. ervoides*	G10	572338	Turkey
11	*V. faba*	G11	-	Turkey
12	*V. narbonensis*	G12	-	Turkey
13	*L. culinaris* subsp. *culinaris*	G13	-	Turkey
14	*L. culinaris* subsp. *culinaris*	G14	-	Turkey
15	*L. culinaris* subsp. *culinaris*	G15	-	Turkey
16	*Lathyrus sativus*	G16	-	Turkey
17	*Lathyrus cicera*	G17	-	Turkey
18	*L. nigricans*	G18	615676	Turkey
19	*L. nigricans*	G19	572344	Spain
20	*L. nigricans*	G20	72542	Spain
21	*Cicer reticulatum*	G21	-	Turkey
22	*C. bijugum*	G22	-	Turkey
23	*L. culinaris* subsp. *tomentosus*	G23	72643	Israel
24	*L. culinaris* subsp. *tomentosus*	G24	72672	Israel
25	*L. culinaris* subsp. *tomentosus*	G25	72613	Turkey
26	*L. culinaris* subsp. *odemensis*	G26	572360	Israel
27	*L. culinaris* subsp. *odemensis*	G27	572361	Israel
28	*L. culinaris* subsp. *odemensis*	G28	72558	Israel

*IG is ICARDA germplasm number.

at –20 °C for 45 min. The samples were then centrifuged for 10 min at 14,000 × *g* and the supernatant was poured and 600 µL of 70% ethanol was added to each tube. After 5-min centrifugation at 14,000 × *g*, ethanol was removed from each sample and the tubes were left upside down to dry for 30 min. After the addition of 50 µL of sterile pure water, the tubes were stored at 4 °C for short-term and at –20 °C for long-term use.

The extracted DNA samples were loaded in wells with 1% agarose gel to check for quality along with standard molecular weight markers and run at 65 V for 30 min, after staining with ethidium bromide, and visualizing gels were photographed with a Kodak Gel Logic 200 system (Carestream Health, Rochester, NY, USA).

Amplified fragment length polymorphism amplifications (AFLPs) were performed (Vos et al., 1995), with some modifications using seven AFLP primer combinations (E_{CAA}/M_{TTT}, E_{CTT}/M_{TTT}, E_{CGG}/M_{TTT}, E_{CCC}/M_{TAA}, E_{CGG}/M_{TAA}, E_{CAA}/M_{TAA}, and E_{CTT}/M_{TAA}). In short, digestion and ligation steps were undertaken in a single tube at 37 °C. The step involved 0.55 µL of NEB4 ligase buffer, 0.0011 mg of BSA, 2.5 units of *EcoR*I enzyme, 3.125 units of *MseI* enzyme, 2.5 mM *Eco* adapter, 1.25 mM *Mse* adapter, 50 units of T4 ligase enzyme, and 100 ng of DNA in a final volume of 8.8 µL. After incubation at 37 °C for 3 h, 4 µL of each sample was checked for quality by running in 1.4% agarose gel. The remaining samples were then diluted 25-fold with pure sterile water. After preselective

amplification with *Eco*C and *Mse*T primer combinations, the samples were again checked for quality by running in 1.4% agarose gel. During the selective amplifications, the IRD700 and IRD800 labeled primers were used for the PCR reactions. The PCR products were separated using Li-Cor 4200L (Lincoln, NE, USA).

2.4. Data analysis

The AFLP bands were carefully screened and only clear-cut bands were scored, as either present (1) or absent (0). The data were analyzed using the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) version 2.1 PC program (Exeter Software, Setauket, NY, USA) (Rohlf, 1993). The genotypic similarity matrix, which reflects the relationships among accessions, was performed according to UPGMA under the SAHN module. The morphological data such as aristae presence or absence, stipule dentations and angle, leaflet number of fully expanded leaves, and pubescence were recorded as present vs. absent for each trait, while peduncle/rachis length ratio was performed using Minitab statistical software (State College, PA, USA).

3. Results

3.1. Morphological data

The size of all of the measured morphological characters was larger in the cultivated lentils than those of the wild species. According to the morphological characteristics, *L. orientalis* resembled mostly the cultivated lentil (Table 2). The peduncle of *L. orientalis* was longer than its leaf rachis; however, it was shorter or equal to that of the cultivated lentil. Although *L. tomentosus* most resembled the cultivated lentil and *L. orientalis*, it differed from *L. orientalis* by the presence of tomentose pods. *L. tomentosus* had a shorter leaf rachis than the cultivated lentil. *L. lamottei* had long aristae, while *L. ervoides* had no aristae, despite their other morphological similarities. *L. lamottei* possessed V-shaped stipules and *L. ervoides* evidenced the lowest number of leaflets per leaf among the genus *Lens* Mill., while its leaflets were larger than those of the noncultivated species (data not shown). *L. ervoides* with pubescent pods had very short aristae on the peduncle, while *L. odemensis* had slightly dentate stipules. *L. nigricans* had unique vertical and W-shaped stipules (Table 2). *V. montbretti* stood out from the genus *Lens* Mill. by its grayish color and pubescence presence (data not shown).

3.2. Molecular data

Of the 414 AFLP bands obtained, a total of 280 informative AFLP markers were scored, with more than 15 polymorphic bands per primer combination. The fragment sizes oscillated between 60 and 420 bp. The number of total bands fluctuated between 48 and 69 with an average of 59, whereas the number of polymorphic bands ranged from 34 to 47 with an average of 40 per primer combination. The highest polymorphism was obtained with the E_{CTT}/M_{TTT} primer combination, yielding 75%, while the lowest polymorphism was observed with the E_{CCC}/M_{TAA} primers, generating 63.7% (Table 3).

The relationships deduced by using the molecular markers among the genera *Cicer* L., *Lathyrus* L., *Lens* Mill., and *Vicia* L. are given in Figure 1. *Vicia* L. and *Lathyrus* L. were genetically closer to the genus *Lens* Mill. than to *Cicer* L. (Figure 1). *Cicer bijugum* K.H.Rech. and *C. reticulatum* Ladiz. were a more distinctive group than those of the tribe Fabeae (Figure 1). Genetic relationships between the genus *Lens* Mill. and *Vicia montbrettii* are depicted in Figure 2. According to the similarity tree (Figure 2), the genus *Lens* Mill. differed from *V. montbretti*. Accessions (G19 and G20) of *L. nigricans* derived from Spain may be of the same accession since they clustered closely together (Figure 2). All lentil taxa were well separated from *V. montbretti* as illustrated by the principal component analysis (PCA) (Figure 3). The mean similarity was 0.75 among accessions and the similarity tree was divided into four branches. The first group consisted of the accessions of *L. culinaris*, *L. tomentosus*, *L. orientalis*, and *L. odemensis*, while the second group included only *L. ervoides*. *L. lamottei* was the third group. The accessions of *L. nigricans* were the most distant from all of the other lentil accessions (Figure 2).

Table 2. Some specific characteristics of the genus *Lens* Mill.

Characteristic	*culinaris*	*orientalis*	*tomentosus*	*odemensis*	*ervoides*	*lamottei*	*nigricans*
Dentate stipules	No	No	No	Yes	No	V shape	W shape
Angle of stipule	Horizontal	Horizontal	Horizontal	Horizontal	Horizontal	Horizontal	Vertical
Aristae	Present	Present	Present	Present	Absent	Present	Present
Pubescence	Absent	Absent	Absent	Absent	Present	Absent	Absent
Pod tomentose	Absent	Absent	Present	Absent	Absent	Absent	Absent
Peduncle/rachis length ratio	≠ 1	>1	>1	>1	>1	>1	>1
No. of leaflets	10–16	5–8	10–12	6–8	4–6	6–10	6–8

Table 3. Primer combinations, number of total (NTB) and polymorphic bands (NPB), and polymorphism (POL).

Primer combinations	NTB	NPB	POL (%)
E_{CAA}/M_{TTT}	66	47	71
E_{CTT}/M_{TTT}	48	36	75
E_{CGG}/M_{TTT}	54	36	67
E_{CCC}/M_{TAA}	69	44	64
E_{CGG}/M_{TAA}	59	38	64
E_{CAA}/M_{TAA}	51	34	67
E_{CTT}/M_{TAA}	67	45	67
Average	59	40	68
Total	414	280	-

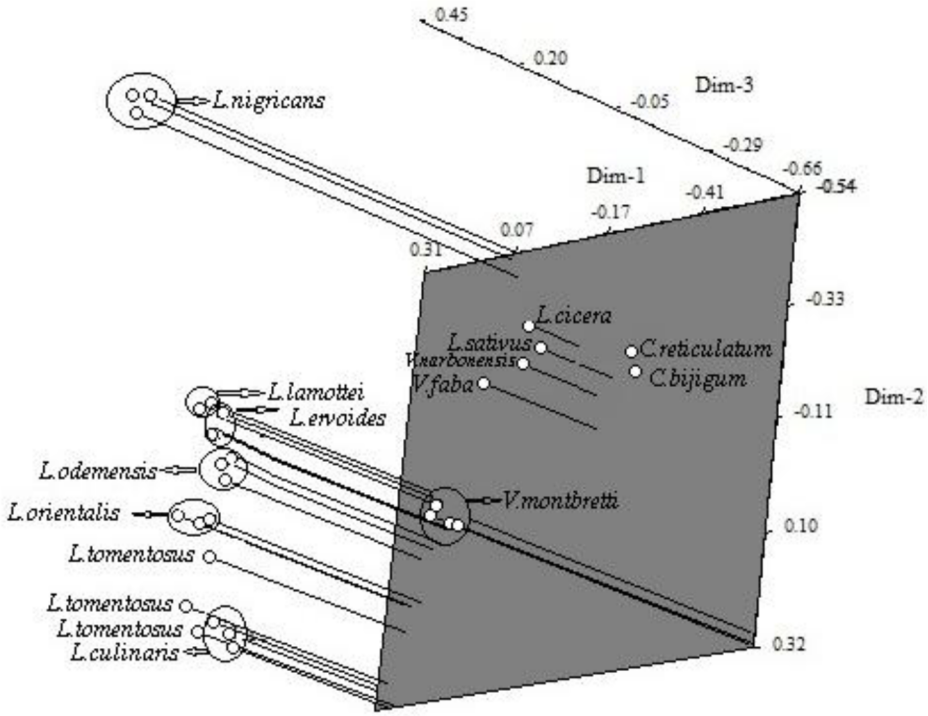

Figure 1. Genetic relationships among the genera *Lens* Mill., *Vicia* L., *Lathyrus* L., and *Cicer* L. according to principal component analysis (PCA).

Genetic similarity among the accessions of the cultivated lentil with *L. tomentosus* ranged from r = 0.70 to r = 0.73, and from r = 0.60 to r = 0.69 with *L. orientalis*. Similarly, the genetic relationships between accessions of *L. orientalis* and *L. odemensis* ranged from r = 0.50 to r = 0.63, of *L. ervoides* and *L. lamottei* from r = 0. 61 to r = 0.66, of the cultivated lentil and *L. nigricans* from r = 0.45 to r = 0.54, and of *Vicia montbretti* and the accessions of the lentil from r = 0.34 to r = 0.47 (data not shown).

4. Discussion

The genus *Lens* Mill. was found to be closer to the genera *Vicia* L. and *Lathyrus* L. than to the genus *Cicer* L. (Figure 1). Schaefer et al. (2012) studied maximum likelihood phylogeny for 470 accessions (262 species) of Fabeae using the combined chloroplast and ITS dataset, and divided into four groups. The first group consisted of *Vicia* including *Lens*, while the second group contained *Lathyrus*, *Pisum*, and *Vavilovia* Al.Fed. Two sections of *Vicia* named Ervum

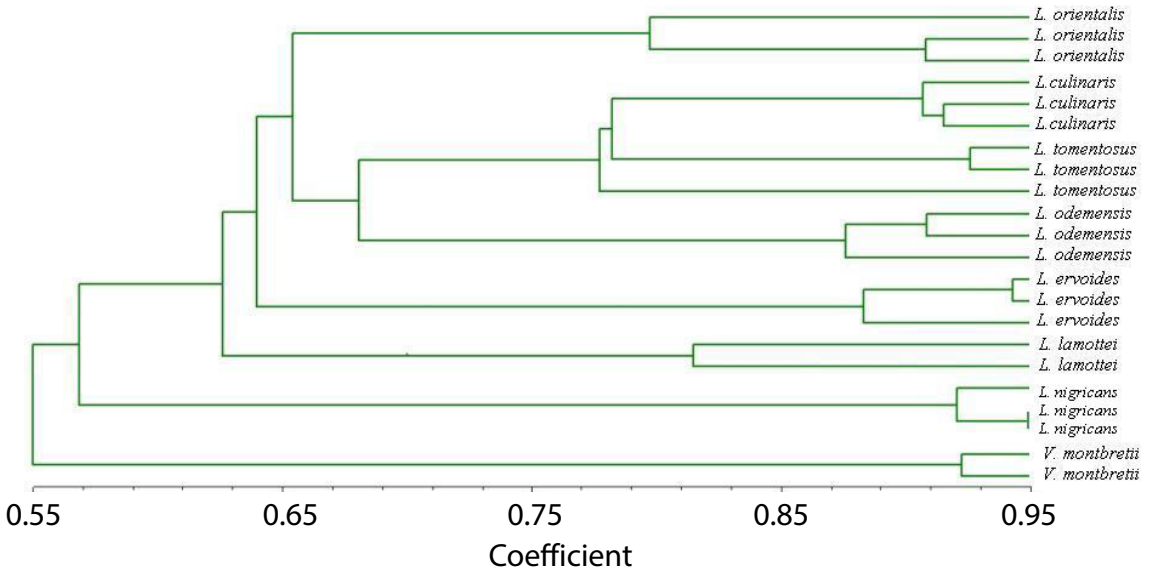

Figure 2. Genetic relationships between the genus *Lens* Mill. and *Vicia montbretii* (Fisch. & Mey.) Davis and Plitmann according to the method described by Dice (1945).

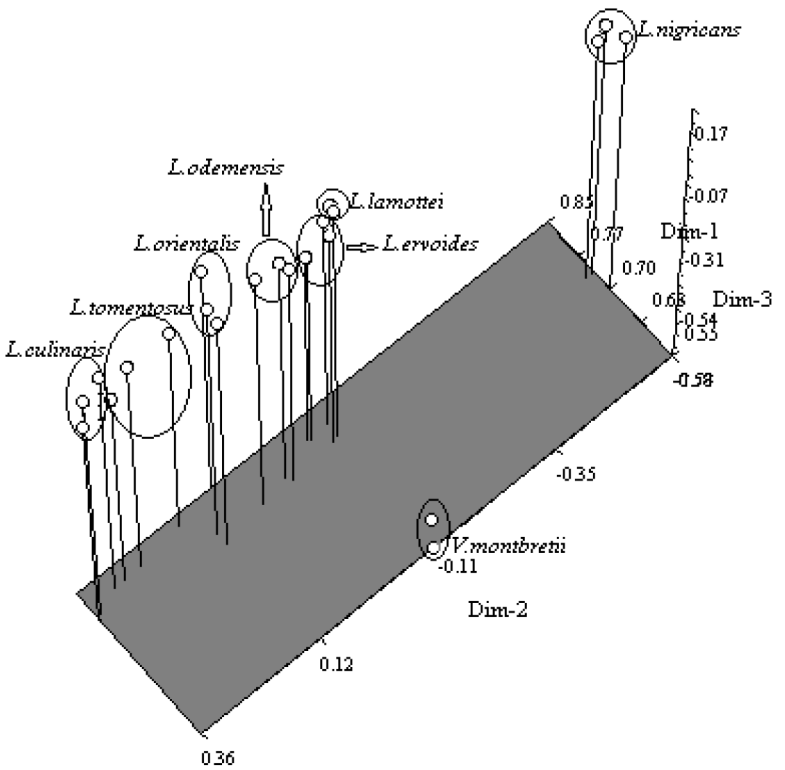

Figure 3. Genetic relationships between the genus *Lens* Mill. and *Vicia montbretii* (Fisch. & Mey.) Davis and Plitmann according to principal component analysis (PCA).

and Ervilla were the third and fourth groups (Schaefer et al., 2012). According to the most recognized phylogenetic classifications of legumes, *Lens*, *Vicia*, *Pisum*, and *Lathyrus* are included in the tribe Fabeae (Schaefer et al. 2012), while *Cicer* is included in a sister tribe.

In the present study, stipule shape and angle, presence/absence of aristae, number of leaflets per leaf, and peduncle/rachis length ratio were found to be the most important morphological characteristics in order to distinguish lentil species (Table 2). These results are consistent with earlier reports (Cubero, 1981; Ladizinsky, 1993, 1997; Ahmad et al., 1997; Ferguson and Robertson, 1999; Ferguson et al., 2000; Cubero et al., 2009). Cubero et al. (2009) also pointed out that the rachis length, the calyx teeth/corolla, and the peduncle/rachis ratios as well as the seed diameter could be used as differential characteristics to classify the lentil species. The most useful characteristics among these morphological ones were the dentation of stipules and their angle with respect to the stem, and the presence/absence of aristae (Table 2). These characteristics were already used by Ferguson et al. (2000) prior to the current study.

The cultivated lentil exhibited the largest pollen together with biggest morphological characteristics compared to the wild species (Fratini et al., 2006). Morphological similarities of the cultivated lentils with *L. orientalis* have previously been reported (Zohary, 1972; Hoffman et al., 1988). Thus, the morphological similarity and crossability allowed us to assume that the cultivated lentil derived from *L. orientalis* (Ladizinsky, 1979). As can be seen in Table 2, *L. tomentosus* was morphologically closer to *L. orientalis* than to any other species (van Oss et al., 1997; Ferguson et al., 2000) and it was different to *L. orientalis* by the pod tomentose plus evidence of a minisatellite chromosome (Ladizinsky, 1997).

According to our AFLP data (Figure 4), the findings are in agreement with the results of several other studies that have depicted similar relationship patterns between species of *Lens* and *V. montbretii* (Mayer and Soltis, 1994; Mayer and Bagga, 2002). A phylogenetic study of *Lens* taxa using restriction site-cpDNA by Mayer and Soltis (1994) evidenced *L. ervoides* and *L. nigricans* as the closest taxa (Schaefer et al., 2012). Prior to the report by Mayer and Soltis (1994), Muench et al. (1991) had obtained similar findings. In contrast, *L. ervoides* has been found to be distinct from *L. nigricans* in the current study (Figures 2 and 3). Genetic relationships among the *Lens* subspecies (Figures 2 and 3) were similar to the findings reported by Mayer and Bagga (2002) except for the position of *L. lamottei* and *L. tomentosus*.

One of the most comprehensive studies on phylogeny in the genus *Lens* using RAPD markers was carried out by Ferguson et al. (2000). However, relationships among *Lens* subspecies had still not been clarified. *L. lamottei* was depicted as the most distant species of the genus *Lens* based on RAPD analysis (Ferguson et al., 2000). However, the genetic relationships among the *Lens* subspecies in the current study are similar to the findings reported by Ferguson et al. (2000) based on isozyme polymorphisms except for the position of *L. tomentosus* and *L. odemensis*.

Prior to this study, AFLP markers have been used for lentil linkage mapping (Eujayl et al., 1998; Duran et al., 2004; Hamwieh et al., 2005), to assess genetic diversity (Sharma et al., 1996), to differentiate cultivars (Zavodna et al., 2000; Toklu et al., 2009), and to identify markers linked to specific traits (Tullu et al., 2008). From these reports using AFLP analysis, only Sharma et al. (1996) considered genetic diversity or phylogeny of the *Lens* subspecies including *L. culiuaris* subsp. *culinaris*, *L. culinaris* subsp. *orientalis*, *L. odemensis*, *L. nigricans*, and *L. ervoides*. However, the authors did not include the subspecies of *L. tomentosus*, *L. lamottei*, or *V. montbretii* (Sharma et al., 1996) compared with the present study (Table 1). Sharma et al. (1996) reported that the AFLP analysis comprised a more efficient technique compared to RAPD analysis due to a much higher level of polymorphyism disclosed. We detected about two times more markers (280 markers) than the findings reported by Sharma et al. (1996), and found different genetic relationships among the *Lens* subspecies of the study (Figures 1 and 3) in contrast to the findings published by Sharma et al. (1996). Using SSR markers, Reddy et al. (2010) found that one accession of *L. odemensis* was arbitrarily included among accessions of *L. ervoides*. Moreover, their study did not involve *L. tometosus* or *V. montbretii*. Tewari et al. (2012) studied cultivated lentils and *Lens* subspecies using 15 RAPD and 8 SSR markers detecting different genetic relationships among the *Lens* subspecies from those observed in this study (Figure 3).

Alo et al. (2011), making use of expressed sequence tags (ESTs) designed flanking one or more introns and derived from conserved exon sequences and splice sites identified through synteny between the model legume *Medicago truncatula* and *Arabidopsis thaliana*, have recently reported that *L. culinaris* subsp. *odemensi*s, *L. culinaris* subsp. *tomentosus*, and *L. lamottei* might be a single taxon. Diversity analysis of expressed sequences is much more accurate than that of random and nonexpressed fragments obtained with AFLP (Alo et al., 2011). In the present study, *L. orientalis* was found to be one of the closest taxa to *L. culinaris*, opening up the possibility that *L. tomentosus* may be a mutant derived from *L. orientalis* (van Oss et al. 1997), as *L. tomentosus* is different from *L. orientalis* by its tometose pods (Ladizinsky, 1997). van Oss et al. (1997) reported that *L. tomentosus* differed from *L. culinaris* by a mutation (*Eco* RI/ps2B), which was also shared by *L. odemensis*. One accession of *L. tomemtosus* had another mutation, which was shared by an accession of *L. orientalis* (van Oss et al., 1997). However, *L. tomentosus* is extremely difficult to cross with *L. culinaris* demanding embryo rescue (Davies et al., 2007), while Suvorova (2014) has

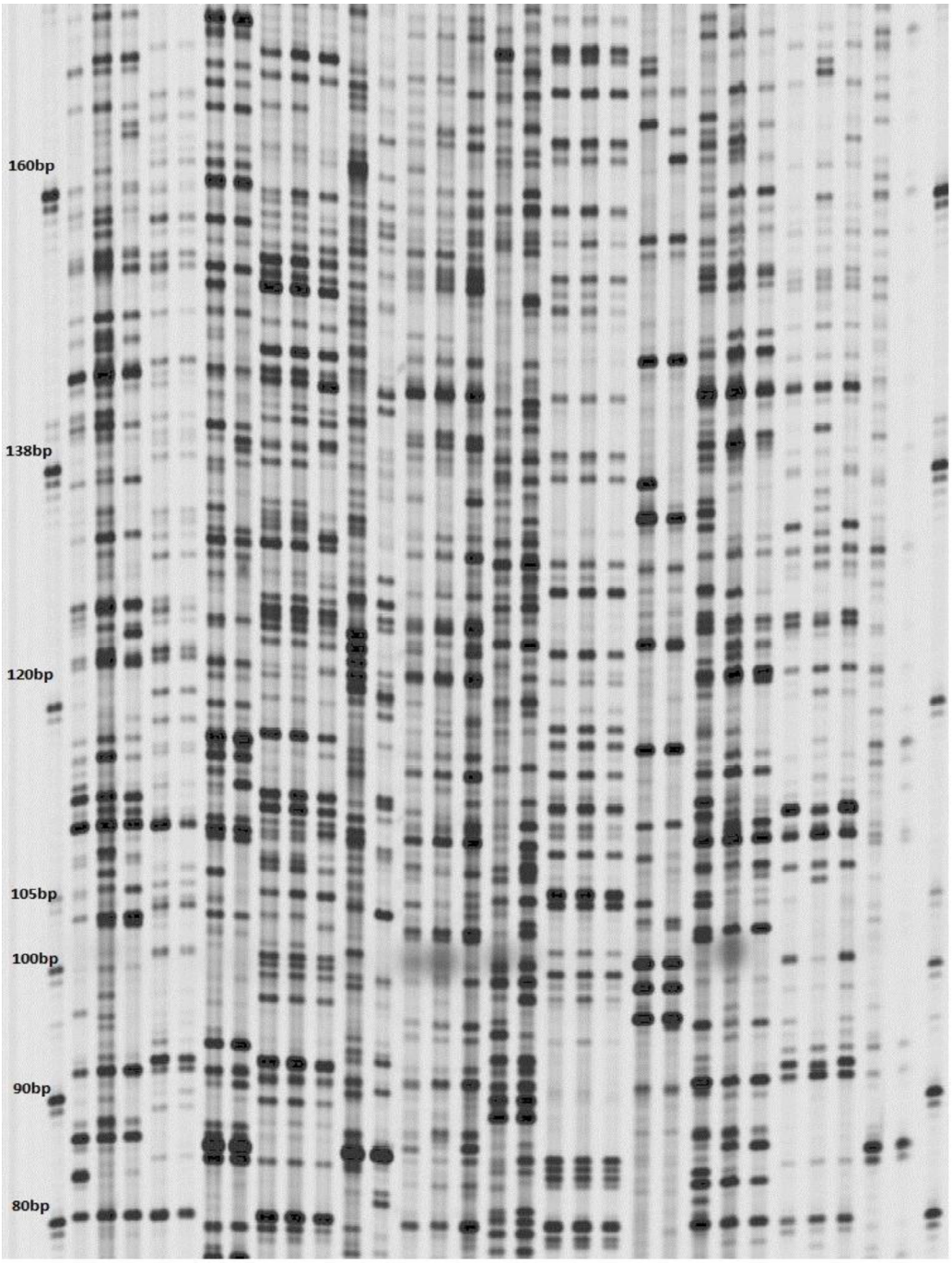

Figure 4. Amplicon profile produced by the AFLP primer combination of E_{CGG}/M_{TTT}. Lanes 1 and 32 indicate molecular weight markers. The accessions from G1 to G28 sequentially load to wells starting from lane 2. Lanes 30 and 31 are less loaded profile of G6 and G7 (*Vicia montbretii*), respectively.

recently produced hybrid seedlings between *L. culinaris* (Svetlaya) × *L. tomentosus* (ILWL 120).

Schaefer et al. (2012) used various *Lens* species (*L. culinaris* subsp. *culinaris*, *L. culinaris* subsp. *orientalis*, *L. tomentosus*, *L. odemensis*, *L. cyanea*, *L. lamottei*, *L. ervoides*, and *L. nigricans*) in their phylogeny studies. In their study, the most distinct species among *Lens* species was *L. nigricans*. Thus, our results agree with the accepted taxonomic status of *L. nigricans* in the report by Schaefer et al. (2012). *L. ervoides* was found to be different from the first and the second groups. *L. odemensis*, *L. orientalis, L. tomentosus*, *L. lamottei*, and *L. cyanea* were the first group.

In the current study (Figure 2), the position of *L. lamottei* was different from the findings reported by Schaefer et al. (2012). *L. culinaris* subsp. *culinaris* was the closer taxon to *L. culinaris* subsp. *orientalis* and shared the same group (Schaefer et al., 2012). In our study, *L. culinaris* subsp. *culinaris* and *L. culinaris* subsp. *tomentosus* were the closest taxa to each other (Figure 2). The reasons for this similarity could be the insufficiency of AFLP markers, and the statistical analysis used in the current study because *L. culinaris* subsp. *culinaris* originated from *L. culinaris* subsp. *orientalis* (Ladizinsky, 1979). In addition to these reasons, *L. culinaris* subsp. *orientalis* was the most similar taxon to the cultivated lentil according to morphological data (Table 2). In PCA, all the *Lens* accessions except *L. culinaris* subsp. *tomentosus* clustered within their taxa (Figure 1). An accession of *L. culinaris* subsp. *tomentosus* (G23) was found to be closer to accessions of *L. culinaris* subsp. *orientalis*, while two accessions of *L. culinaris* subsp. *tomentosus* (G24 and G25) were closer to accessions of *L. culinaris* subsp. *culinaris* as a hybrid.

Three different inter-subspecific crossing groups of *L.* subsp. *orientalis* have been described with cultivated lentils: (i) One completely incompatible, which requires embryo rescue, (ii) One completely compatible not requiring embryo rescue (this group is cross incompatible with the first orientalis group and vice versa), and (iii) One intermediate that can be easily crossed with the previous two orientalis groups yet gives more problems with *L. culinaris* crosses although not in need of embryo rescue F_2 descendants often display hybrid breakdown and die (Fratini et al., 2014).

As a result of the morphological and molecular data along with the cited references, the following conclusions are drawn: (i) The genus *Lens* is genetically closer to the genera *Vicia* and *Lathyrus* than to the genus *Cicer.* (ii) The *Lens* species differ from *V. montbretti*. (iii) A phylogeny of the subspecies belonging to the genus *Lens* returned *Lens culinaris* subsp. *culinaris*, *Lens culinaris* subsp. *orientalis*, *Lens culinaris* subsp. *tomentosus*, *Lens culinaris* subsp. *odemensis*, *L. ervoides*, *L. lamottei*, and *L. nigricans*.

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