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Chloroplast DNA variation and evolution in the genus *Lens* Mill

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Abstract Chloroplast DNA (cpDNA) restriction site diversity was assessed by 21 enzyme/probe combinations in 30 accessions of six *Lens* species, including the recently recognized *L. lamottei* and *L. tomentosus*. A total of 118 fragments were scored and 26 restriction site mutations were identified. The cpDNA restriction pattern supports circumscribing *L. lamottei* and *L. tomentosus* as independent species. The value of the data for reconstructing phylogeny in the genus is discussed. The cpDNA of all 13 accessions of the lentil's wild progenitor, *L. culinaris* subsp. *orientalis*, differed from that of the single lentil cultivars used in this study. This diversity indicates that other populations of this subspecies from Turkey and Syria examined by Mayer and Soltis (1994) are potentially the founder members of lentil. Examination of *L. lamottei* × *L. nigricans* hybrids between accessions having different restriction patterns showed paternal plastid inheritance in *L. nigricans*.

Key words Lentil · Evolution · Domestication · cpDNA · Plastid inheritance

Introduction

Since the last review of taxonomy and species relationships in the genus *Lens* (Ladizinsky 1993) two more species, *L. lamottei* Czefranova (Czefranova 1971) and *L. tomentosus* Ladizinsky (Ladizinsky 1996), have been brought to light. *Lens lamottei* was identified among herbarium material of *L. nigricans* (M. Bieb.) Grand. on the basis of its horizontal, less dentate stipules. This morphological type was also recognized as a unique cytogenetical stock within *L. nigricans* following breeding experiments. Two populations of this type, from France and Spain, differs from other populations of *L. nigricans* by five chromosomal rearrangements (Ladizinsky et al. 1984). An additional population of *L. lamottei* has recently been discovered by one of us (G.L.) in Morocco. One population of *L. tomentosus* in the Mardin area of southeastern Turkey, has been known about for sometime, but it was regarded as a variant of *L. culinaris* Medik. subsp. *orientalis* (Boiss.) Hand.-Maz. Morphologically, it is distinguished by tomentose pods, and karyotypically by a minute satellite and one large metacentric chromosome. It is also cross-incompatible with most *L. culinaris* lines as well as other *Lens* species (Ladizinsky and Abbo 1993). *Lens tomentosus* was delimitade as a new species following the discovery of an additional two populations in the Mardin area (Ladizinsky 1996) and partly because of data presented in this paper.

Breeding experiments have revealed three crossability groups among the six *Lens* species: (1) *L. culinaris* Medik., *L. odemensis* Ladiz. (2) *L. ervoides* (Bring.) Grand., *L. nigricans*, *L. lamottei*; (3) *L. tomentosus*. Interspecific crosses within groups produce hybrids which are sterile or semisterile, but crosses between members of different groups result in early abortion of the hybrid embryos (Ladizinsky et al. 1984). *Lens culinaris* is exceptional because it contains a few wild populations which show various degrees of cross-incompatibility (Ladizinsky and Abbo 1993). One of these accessions has since been assigned to *L. tomentosus* (Ladizinsky 1996). Further insight into species relationships in *Lens* has been achieved by enzyme electrophoresis (Pinkas et al. 1985; Hoffman et al. 1986), restriction fragment length polymorphism (RFLP) (Havey and Muehlbauer 1989), chloroplast DNA (cpDNA) (Muench et al. 1991; Mayer and Soltis 1994) and random amplified polymorphic DNA (RAPD) analyses (Abo-elwafa et al. 1995; Sharma et al.

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1995). In this paper we describe variations in the cpDNA of selected accessions from the six *Lens* species. They represent the extreme ranges of distribution of their species, unique chromosome types and crossability peculiarities. Since the domesticated lentil was thoroughly examined by Mayer and Soltis (1994), and appeared to be highly uniform, only a single accession of this group was studied. This particular accession has been used as a tester line in seed-protein elecrophoresis (Ladizinsky 1979), allozyme studies (Pinkas et al. 1985) and breeding experiments (Ladizinsky et al. 1984).

Materials and methods

Plant material

Thirty accessions representing all the known *Lens* species were employed in this study. The origins of the various accessions are given in Table 1.

Chloroplast DNA clones

Fourteen tobacco cpDNA fragments digested by *Pst*I covering the entire genome (Fluhr et al. 1983) were used as probes. Three of them, ps5, ps6 and ps12, hybridized poorly in the preliminary survey and were dropped from the analysis.

RFLP protocol

Total DNA was isolated using a modification of the Bernatsky and Tanksley (1986) method. Each DNA isolate was digested with three restriction enzymes, *Eco*RI, *Hin*dIII, and *Dra*I, which in a preliminary study were found to be the most informative. Digestion was performed overnight according to the supplier's instructions (Boehringer Mannheim). The resulting fragments of the digested DNA were separated by electrophoresis through agarose gels, denaturated and transferred to Gene Screen Plus nylon membranes (Gamidor NEF-976) alkaline transfer (Southern 1975). The filters were prehybridized for at least 6 h with hybridization buffer containing 1% boiled ST DNA (salmon testes DNA type II).They were then hybridized with random hexamer-labeled plasmid (Feinberg and Vogelstein 1983). Hybridization was carried out overnight at 65°C in hybridization buffer (containing, per litre) 44 g NaCl, 36.8 g trisodium dihydrate citric acid, 30 ml 20% SDS, 50 ml 1 *M* phosphate buffer, 50 ml Denhardt's solution × 100, 10 ml 0.25 *M* EDTA, 100 ml 50% dextran sulfate. The filters were then washed at increasing stringency in 2 × SSC, 0.1% SDS at 65°C for 20 min, then in 1 × SSC, 0.1% SDS for 20 min and in 0.5 × SSC, 0.1% SDS. The filters were wrapped in Saran Wrap and exposed to X-ray films (X-OMAT, Kodak) with an amplifier screen for 1-5 days, depending on the strength of the signal. Autoradiography was performed by developing the films with Kodak Developer D-19 Acid Fixer.

Data analysis

The absence or addition of individual DNA fragments was interpreted as a restriction site mutation, as was the lack of a specific band accompanied by the occurrence of two bands with the same combined molecular weight. Restriction site divergence and distance between accessions were estimated according to Nei and Lei (1979) and were computed with the PHYLIP statistical package (http://evolution genetics. Washington edu/phylip/softwar html).

Table 1 Origin of the various accessions used in this study

Accession	Species/line	Origin
1	*L. culinaris* ssp. *culinaris* LC2	Israel
2	ssp. *orientalis* S74	N Syria
3	ssp. *orientalis* S76	N Syria
4	ssp. *orientalis* 142	N Syria
5	ssp. *orientalis* 24	Jerusalem, Israel
6	ssp. *orientalis* 42	Mt Elburz, Iran
7	ssp. *orientalis* 117	Lengam forest, SW Turkey
8	*L. tomentosus* 133	19 km SW of Mardin, Turkey
9	*L. tomentosus* 137	Near Midyat, SE Turkey
10	ssp. *orientalis* 138	Near Midyat, SE Turkey
11	ssp. *orientalis* 141	Tokat, Turkey
12	ssp. *orientalis* 229	Karakala, Turkemenistan
13	ssp. *orientalis* 233	Konaka, Dushanbe, Tadjikistan
14	ssp. *orientalis* 234	Varzob, Tadjikistan
15	ssp. *orientalis* 238	Komsomolabad, Tadjikistan
16	ssp. *orientalis* 242	50 km E Angren, Uzbekistan
17	*L. odemensis* 36	Golan Heights, Israel
18	*L. odemensis* S101	S Syria
19	*L. odemensis* SII2	S Syria
20	*L. odemensis* S122	S Syria
21	*L. ervoides* 32	Yechiam, Israel
22	*L. ervoides* 46	Djurjura, Algeria
23	*L. ervoides* 228	Aleltu, Ethiopia
24	*L. nigricans* 59	Massafra, Italy
25	*L. nigricans* 65	Makarska, former Yugoslavia
26	*L. nigricans* 68a	Unknown
27	*L. lamottei* 73	Jativa, Spain
28	*L. nigricans* 172	Yeshilova, Turkey
29	*L. nigricans* 186	La Palma, Canary Islands
30	*L. lamottei* 244	Volubilis, Morocco

Results

Chloroplast DNA analysis of 30 *Lens* accessions by 21 enzyme/probe combinations yielded 118 clearly visible fragments. The polymorphic fragments were interpreted as consequences of at least 26 restriction site mutations. In most enzyme/probe combinations only a single mutation was detected, but 3 different mutations were resolved by *Hin*dIII/ps10, and the same number by *Eco*RI/ps3A. Some mutations were found only in one species, but these were then usually confined to a small number of accessions, and several others were found in more than one species. The variation observed in each of the *Lens* species was as follows.

Lens culinaris The 14 accessions of this species included a single domesticated form, subsp. *culinaris*, and the rest were of the wild form, subsp. *orientalis*. They represented populations growing throughout this taxon's main natural geographical area, including the Middle East, Iran and central Asia. Accession nos. 24 and 42 differed from one another by two reciprocal

translocations, and each differed by a single translocation from the rest of the accessions. The examined accessions also varied in their crossability potential, presenting three crossability groups. Accession no. S76 was cross-incompatible with most other accessions because of hybrid-embryo abortion. S74 and 138 were cross-compatible with no. S76 and the rest of the accessions.

Diversity was relatively low, and only 7 of the 26 detected mutations were found in this species. The restriction pattern of the single cultivated line differed from those of all of the examined subsp. *orientalis* accessions. Each of the 2 mutations in the domesticated line revealed by *Hin*dIII/ps10 were observed in some subsp. *orientalis* accessions, either individually or in combination with other mutations. Three accessions, no. 42 from Iran, no. 117 from southwestern Turkey and no. 242 from Uzbekistan, did not reveal any restriction site mutations with any of the enzyme/probe combinations. Accession nos. 233 and 234, both from Tadjikistan, had the same restriction site mutations. The 2 accessions of the intermediate crossability group (S74 and 138) also shared the same restriction sites, but these were also found in another 7 accessions.

Lens tomentosus Morphologically, *L. tomentosus* is closer to *L. culinaris* subsp. *orientalis* than to any other taxon, but it is distinguished by a hairy pod and a modified karyotype, and is isolated from the latter by hybrid-embryo breakdown, complete sterility and five chromosomal rearrangements. The 2 *L. tomentosus* accessions differed from all *L. culinaris* accessions by a mutation revealed by *Eco*RI/ps2B, which was also shared by all *L. odemensis* accessions. Accession no. 137 had another mutation that was shared by no. 24 of subsp. *orientalis*.

Lens odemensis Three mutations were observed in the *L. odemensis* accessions, of which 2 were shared by the 3 accessions. The first was revealed by *Eco*RI/ps2B and was also shared by *L. tomentosus*; another was revealed by *Eco*RI/3B and was shared by all *L. ervoides* and *L. nigricans* accessions. The third mutation revealed by *Hin*dIII/ps2A was found only in accession no. 36 as well as in accession nos. 233, 234 and S76 of subsp. *orientalis*.

Lens ervoides The 3 accessions from Israel, Algeria and Ethiopia that were examined represent the extreme geographical ranges of this species and adaptation to different ecological conditions. They all shared mutations revealed by *Eco*RI/ps2A, *Eco*RI/ps3A and *Eco*RI/ps3B. The first and third mutations were also shared by all the *L. nigricans* accessions. Another mutation was revealed by *Eco*RI/ps4 in the Ethiopian accession and was also observed in all the *L. nigricans* accessions. Another 2 mutations of *Hin*dIII/ps2A and *Hin*dIII/ps3B in no. 32 were shared by 2 subsp. *orientalis* accessions, nos. 233 and 234 from central Asia, and the latter mutation was also found in no. 141 from Turkey.

Lens nigricans This species showed the largest number of restriction site mutations and the greatest interspecific diversity (Table 2). Of its 21 mutations, 13 were unique to this taxon. Ten mutations, revealed by combinations of two enzymes and seven probes, were shared by all the *L. nigricans* accessions. Of these 10, 1 (*Eco*RI/ps3B) was also shared by all *L. odemensis* and *L. ervoides* accessions, and 1 (*Eco*RI/ps2A) by all *L. ervoides* accessions. The mutation revealed by *Eco*RI/ps10 was also found in the Ethiopian accession of *L. ervoides*; another common mutation (*Eco*RI/ps4) was found in accession no. 141 of subsp. *orientalis* from Turkey. Another 3 mutations which were detected in only 1 or 2 *L. nigricans* accessions were found in several accessions of subsp. *orientalis*. A mutation revealed by *Hin*dIII/ps10, for example, was unique to accession no. 65 of *L. nigricans* from Yugoslavia and was also found in the domesticated line of *L. culinaris* and in 3 accessions of subsp. *orientalis* from Turkey and 2 from central Asia.

Lens lamottei Seven mutations were found in the 2 accessions of this species, of which 4 were common to both. These 7 mutations were also found in *L. nigricans*, while 5 of them were present in *L. ervoides*. Of the latter, 1 was also shared by 2 accessions of subsp. *orientalis* from central Asia and with *L. ervoides* from Israel.

Clustering according to the cpDNA divergence of the 30 accessions representing the six *Lens* species resulted in three groups: (1) *L. culinaris*, *L. tomentosus* and *L. odemensis*, with relatively small differences

Table 2 Chloroplast DNA genetic distances within and between *Lens* species

	culinaris (C)	*tomentosus* (T)	*odemensis* (O)	*ervoides* (E)	*lamottei* (L)	*nigricans* (N)
C	0.115					
T	0.132	0.055				
O	0.217	0.081	0.092			
E	0.292	0.280	0.276	0.124		
L	0.351	0.346	0.264	0.135	0.134	
N	0.864	0.871	0.802	0.613	0.610	0.201

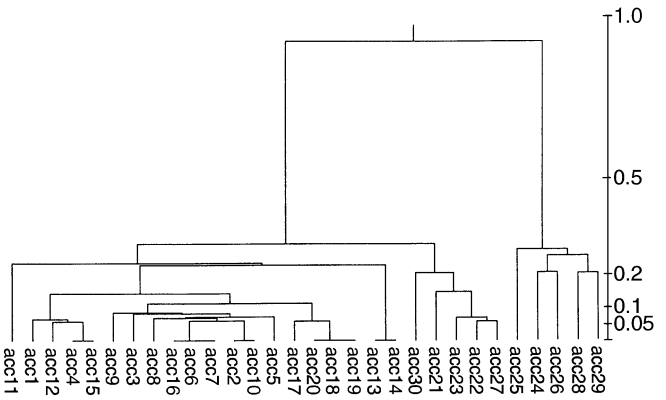

Fig. 1 Genetic distances (Nei and Lei 1979) between 30 *Lens* accessions belonging to six species (see Table 1)

among them; (2) *L. ervoides* and *L. lamottei*; (3) *L. nigricans*, which is distantly related to all of the any other species (Fig. 1)

Mode of cpDNA transmission

The different restriction patterns were used to determine the mode of plastid transmission in lentil. Hybrids were produced between *L. nigricans* accessions nos. 59 and 68a, and *L .lamottei* nos. 73, and 244. Four mature F_1♀73 × ♂68a plants were analyzed with *Eco*RI/ps4, *Eco*RI/ps3A and *Dra*III/ps2B, and another four F_1♀244 × ♂59 plants by the *Eco*RI/ps4, *Eco*RI/ps2B. Some of the F_1 plants had the female phenotypes, others the male phenotype, indicating biparental plastid transmission. When the same plants were analyzed with different enzyme/probe combinations, the plastid populations in one plant of each of the two F_1 hybrid groups appeared to be of mixed origin (Table 3).

Discussion

The extent of cpDNA polymorphism revealed in this study is greater than that previously reported (Muench et al. 1991; Mayer and Soltis 1994). In fact, none of the six species was monomorphic, although the degree of diversity varied among species (Table 2). In part, this could be a reflection of the number and origin of the accessions examined in each species, but it also indicates genuine differences. Of the 26 restriction site mutations over the 30 *Lens* accessions, only 5 were species-specific and shared by all members of that species and therefore of possible taxonomic value. They all were found in *L. nigricans*, but in view of the wide range of diversity in this species, it is not unlikely that an examination of additional accessions would reveal variation in some of these site mutations as well.

Table 3 Distribution patterns of specific cpDNA fragments in parental lines and four F_1 plants

Fragment origin	Parental lines		F_1			
	73	68a	1	2	3	4
*Eco*RI/ps4	−	+	−	+	+	−
*Eco*RI/ps3A	−	+	−	+	+	−
*Dra*III/ps2B	+	−	+	+	−	+
	244	59	1	2	3	4
*Eco*RI/ps4	−	+	+	−	−	+
	+	−	−	+	+	+
*Eco*RI/ps2B	−	+	+	−	−	+

The distribution of the variable restriction sites among the different species constitutes more supportive evidence for circumscribing *L. tomentosus* and *L. lamottei* as independent species. *Lens tomentosus* is presently known from a restricted geographical area in southeastern Turkey where *L. culinaris* subsp. *orientalis* is particularly common and no other *Lens* species grows. The two are morphologically similar, except for the hairy pod of *L. tomentosus*. However, they are widely differentiated karyotypically and genetically. Although the cpDNA of *L. tomentosus* contains only 2 of the 26 variable restriction sites, the one which was common to both accessions was absent in *L. culinaris*. This mutation was also shared by all 4 *L. odemensis* accessions, and these two species contained the most similar chloroplast genomes in the genus (Table 2). The chromosomal relationship between *L. odemensis* and *L. tomentosus* has not been studied because they are isolated by hybrid-embryo abortion, but their karyotypes are indicative of several chromosomal rearrangements.

The relationships between *L. nigricans* and *L. lamottei* are also complicated. The morphological differences between them are minute and confined to stipule shape. The two are cross-compatible but differ by four reciprocal translocations and one paracentric inversion, resulting in complete sterility of their hybrids (Ladizinsky et al. 1984). They also differ in their allozyme profiles (Hoffman et al. 1986) and their cpDNA restriction pattern. However, all 7 restriction sites of *L. lamottei* were also present in *L. nigricans*, although the latter was much more variable and contained 14 additional mutations.

Of special interest are the relationships between *L. lamottei* and *L. tomentosus*. Morphologically they are indistinguishable from one another, but they are separated geographically, the former being confined to the western and the latter to the eastern part of the Mediterranean. Their cytogenetic relationship has not been studied because they are isolated by hybrid-embryo abortion. They are, however, distinguished by 3 cpDNA mutations and the overall divergence between them is twice that between *L. lamottei* and *L. ervoides* (Table 2), with which it is cross-compatible.

To date, intraspecific diversity in *Lens* has been assessed for chromosomal arrangement, allozymes,

Table 4 Mean intraspecific diversity of four molecular and DNA class characters in *Lens*

	L. culinaris	*L. odemensis*	*L. ervoides*	*L. nigricans*
Allozymes[a]	0.11	0.15	0.11	0.07
RAPD[b]	0.21	0.45	0.16	0.35
RFLP[c]	0.27	0.27	0.06	0.09
cpDNA	0.11	0.09	0.12	0.20

[a] Calculated from Hoffman et al. (1986)
[b] Abo-elwafa et al. (1995)
[c] Havey and Muehlbauer (1989)

RAPD, RFLP, and cpDNA restriction sites. Chromosomal variation is relatively high in *L. culinaris* subsp. *orientalis* in which populations may vary from each other by one or two chromosomal rearrangements. However, no such variation has been found in its domesticated derivative subsp. *culinaris*. Chromosomal rearrangements are rare in both *L. ervoides* and *L. nigricans* (Ladizinsky et al. 1984) and are absent in *L. odemensis* (Ladizinsky and Abbo 1993). The extent of chromosomal variation in *L. lamottei* and *L. tomentosus* is insufficiently known because only a few populations have been available for examination. Comparisons of the diversity of allozymes, RAPD, RFLP and cpDNA restriction sites reveal considerable differences between the level of diversity in each of the class characters (Table 4). There is no single character which is consistently the most variable across species, nor a species which has the highest level of diversity in the four class characters. The mean diversity across the four class characters is 0.175, 0.240, 0.112 and 0.117 in *L. culinaris*, *L. odemensis*, *L. ervoides* and *L. nigricans*, respectively, suggesting that *L. odemensis* is the richest and *L. ervoides* the poorest in genetic diversity.

Using cladistic analysis of the cpDNA, Mayer and Soltis (1994) proposed two phylogenetic lineages in *Lens*, *L. culinaris*, *(senso lato)*, and *L. odemensis*, *L. ervoides* and *L. nigricans*. The latter shared 3 restriction-site mutations that were absent in *L. culinaris*. In the present study, only a single mutation of that kind was detected, and it was observed in *L. lamottei* as well. This so-called conserved mutation constituted 0.047, 0.142, 0.142 and 0.333 of the mutations observed in *L. nigricans*, *L. lamottei*, *L. ervoides* and *L. odemensis*, respectively. In *L. nigricans*, it is 1 of the 5 which seem, at the moment, to be conserved mutations. However, it is possible that the examination of additional accessions may reveal diversity in these mutations as well. Furthermore, the recognition of two phylogenetic lineages in *Lens* implies that the 4 mutations shared by some accessions of *L. culinaris* and members of the other lineage occurred independently. If conversion has indeed been quite common in *Lens* cpDNA, it probably also occurred in the "ancestral" mutations. The phylogeny proposed by Mayer and Soltis (1994) is also incompatible with their and our data on the cpDNA divergence and the wealth of evidence from other studies attempting to assess divergence by allozyme, RFLP and RAPD polymorphism.

The tracing of *Lens* phylogeny is becoming more and more complex. Initially, crossability was suggested as a basic criterion and, accordingly, two groups were defined: *culinaris-odemensis* and *ervoides-nigricans* (Ladizinsky et al. 1984). Now it is obvious that *L. tomentosus* forms an additional group and, furthermore, intraspecific cross-incompatibility has been found in *L. culinaris* (Ladizinsky and Abbo 1993) and in *L. tomentosus* (Ladizinsky 1996). Diversity in molecular and DNA markers is even more inconclusive, with different groupings having been proposed by different authors. Prior to the recognition of *L. tometosus* as an independent species, *L. odemensis* was regarded as the most akin to *L. culinaris*, though Pinkas et al. (1985) placed *L. ervoides* in that position. Similarly, *L. nigricans* has been the most distantly related, except with the respect to RAPD markers (Abo-elwafa et al. 1995). Moreover, the mean ranges of diversity vary considerably among the different studies, with occasional overlapping of intra- and interspecific ranges.

Mayer and Soltis (1994) reported that 112 of the 114 lentil cultivars examined by them were cpDNA monomorphic with the same restriction pattern in 3 of the 4 accessions they examined of subsp. *orientalis*, the lentil wild progenitor. In this study 7 mutations were found, suggesting that the narrow diversity observed by Mayer and Soltis (1994) is not because subsp. *orientalis* is depauperate in cpDNA diversity, but is a result of founder effect (Ladizinsky 1985). The role of the founder effect in lentil domestication is also evidenced by the pattern of divergence in allozymes (Pinkas et al. 1985; Hoffman et al. 1986), RFLP (Havey and Muehlbauer 1989) and RAPD (Abo-elwafa et al. 1995; Sharma et al. 1995). On cytogenetical grounds populations of subsp. *orientalis* from Israel and Iran, and some from Syria and Turkey have been excluded from the ancestry of the cultivated lentil because they differ by one or more chromosomal rearrangements (Ladizinsky et al. 1984). Other populations from Turkey and Syria have been excluded because they are cross-incompatible with the cultivated lentil (Ladizinsky and Abbo 1993). Now the populations of central Asia can be excluded because they have different cpDNA restriction patterns. It would be interesting to see if the 3 subsp. *orientalis* accessions with matching cpDNA restriction pattern studied by Mayer and Soltis (1994) also possess the necessary chromosomal and crossability attributes to be considered the founder populations of lentil.

The pattern of plastid transmission observed in this study supports Rajora and Mahon's (1995) report on biparental plastid inheritance in *L. culinaris*. The paternal plastid inheritance observed in *L. lamottei* × *L. nigricans* hybrids indicates that the same biparental inheritance occurs in *L. nigricans*, and probably in the

rest of the genus. The evolutionary significance of biparental plastid inheritance seems negligible in a highly autogamous plant such as lentil. However, it could be a mechanism for the utmost unitization of rare outcrossing by creating new genome-plasmone interactions.

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