Euphytica **56**: 213–218, 1991.

Determination of genetic variation and taxonomy in lentil (*Lens* Miller) species by chloroplast DNA polymorphism

D.G. Muench[1], A.E. Slinkard & G.J. Scoles
Dept. of Crop Science and Plant Ecology, Univ. of Saskatchewan, Saskatoon, SK, Canada S7N OWO;
[1] Dept. of Genetics, Univ. of Alberta Edmonton, AB, Canada T6G 2E9

Received 2 April 1991; accepted 3 September 1991

Key words: chloroplast DNA, *Lens*, polymorphism, restriction fragments, taxonomy

Summary

Chloroplast DNA restriction fragment length polymorphisms (RFLP) were used to examine the taxonomic relationships of cultivated and wild lentil (*Lens* Miller) species and identify the extent of genetic variation in this genus. Twelve accessions representing all *Lens* subspecies were digested with four hexanucleotide-recognizing restriction endonucleases. These digests randomly surveyed 540 base pairs, or 0.4% of the approximately 125 kilobase lentil chloroplast genome. A high degree of fragment length conservation was seen among members of crossability group I, i.e., *L. c.* ssp. *culinaris*, *L. c.* ssp. *orientalis* and *L. c.* ssp. *odemensis*. Accessions of the two subspecies comprising crossability group II, i.e., *L. n.* ssp. *nigricans* and *L. n.* ssp. *ervoides*, showed the greatest amount of variation when compared to the cultivated lentil, *L. c.* ssp. *culinaris*. Limited variation was observed within subspecies except for *L. n.* ssp. *nigricans*, where accessions of the normal cytotype were highly polymorphic to those of the differentiated cytotype. Chloroplast DNA RFLPs reaffirm hypotheses that propose *L. c.* ssp. *orientalis* as the progenitor to the cultivated lentil. The implications of this study on taxonomy and genetic resources is also discussed.

Introduction

A requisite to any plant breeding program is a comprehensive understanding of the genetic variation available to the breeder as well as the taxonomic relationships of a crop and its wild relatives. Many different methods of fullfilling these requirements are available. The chloroplast genome is well suited for comparative molecular analysis and determination of species relationships because of its small size (120 to 150 kilobases in angiosperms) and sequence conservation (Palmer et al., 1987). Chloroplast DNA restriction fragment length polymorphism (RFLP) has been used to infer genetic variation in many genera, including *Lycopersicon* (Palmer & Zamir, 1987), *Pisum* (Palmer et al., 1985), *Hordeum* (Holwerda et al., 1986), *Coffea* (Berthau et al., 1983), *Nicotiana* (Kung et al., 1982) and *Clarkia* (Syksma & Gottlieb, 1986), and has become a useful tool in augmenting existing criteria for assessment of taxonomic relationships in plant taxa.

Lentil (*Lens culinaris* ssp. *culinaris* Medik.) is one of the oldest cultivated species known and today production is widespread, traditionally being grown on the Asian subcontinent and northern Africa, but recently it has become an important alternative crop in Canada and the United States. Cubero (1984) considered lentil a crop with a poor research history in terms of genetic resources and taxonomy.

The genus *Lens* contains two species based on

crossability and cytological studies: *L. culinaris* and *L. nigricans*. The first crossability group (*L. culinaris*) contains three subspecies; ssp. *culinaris* (the cultivated lentil), ssp. *orientalis*, and ssp. *odemensis*. Two subspecies were defined within *L. nigricans* (crossability group 2): ssp. *nigricans* and ssp. *ervoides* (Ladizinsky et al., 1984). *L. c.* ssp. *orientalis* has been hypothesized as the progenitor to the cultivated lentil, based on affinities in morphology and cytology, as well as in isozyme, seed protein and nuclear RFLP patternings (Ladizinsky, 1979; Ladizinsky et al., 1984; Hoffman et al., 1986; 1988; Havey & Muehlbauer, 1989). Two accessions currently exist within *L. n.* ssp. *nigricans* that differ from other accessions of this subspecies by four reciprocal translocations and a paracentric inversion, and from *L. n.* ssp. *ervoides* by only two translocations (Ladizinsky et al., 1983; 1984), and are referred to as the differentiated cytotype of *L. n.* ssp. *nigricans*. This cytotype of *L. n.* ssp. *nigricans* currently does not fit well into any of the existing *Lens* taxa.

Recent studies involving morphology, isozyme and nuclear RFLP patterns (Hoffman et al., 1986; Hoffman et al., 1988; Havey & Muehlbauer, 1989) have shown contradictions to the existing taxonomy, thus indicating that further research into the genetic relationships in the genus *Lens* is required. In this study we report the use of chloroplast DNA RFLPs in an effort to better understand these relationships, and gain more information on the sources of genetic variation for lentil crop improvement.

Materials and methods

Two accessions of each of the five *Lens* subspecies plus two accessions of the differentiated cytotype of *L. n.* ssp. *nigricans* were chosen for chloroplast DNA RFLP analysis (Table 1). Chloroplast DNA was isolated from 200 to 300, two- to four-week-old seedlings, which had been subjected to complete darkness for two days. The chloroplast DNA extraction procedure was similar to that of Holwerda et al. (1986), and restriction endonuclease digestion and end-labelling of restriction fragments was as per Maniatis et al. (1982). Four hexanucleotide-recognizing restriction endonucleases, viz., Bam HI, Eco RI, Bgl II and Hind III, were used in approximately ten times excess activity. Klenow-mediated end-labelling was performed to fill in the 3′ recessed ends with deoxyadenosine 5′-([^{35}S] thio)-triphosphate and electrophoresis of the labelled DNA was either through 0.8 or 1.2% agarose gels at 40 volts/cm. The gels were then vacuum dried, and used to expose Kodak XAR-50 film from 4 to 25 days.

Estimates of variability were calculated by the 'fragment method' of Upholt (1977), by determining the fraction (F) of conserved fragments (Nei & Li, 1979). A dendogram was then constructed based on the fraction of conserved fragments using the unweighted pair-group method of analysis (UPGMA) computer program (Gentzbittel and Nicolas, 1989).

Results

Approximately 90 fragments were scored for each lentil accession, the exact number depending on

Table 1. Accession number, source and origin of the lentil accessions used for chloroplast DNA RFLP analysis

Species and subspecies	Accession or cultivar	Source	Origin
L. culinaris			
ssp. *culinaris*	Eston	U of S[a]	Canada (Turkey)
	PI 297797	USDA[b]	Yugoslavia
ssp. *orientalis*	49	U of S	Israel
	4	U of S	Uzbekistan
ssp. *odemensis*	20	U of S	Israel
	94	USDA	Turkey
L. nigricans			
ssp. *ervoides*	38	U of S	Yugoslavia
	54	U of S	Israel
ssp. *nigricans*	23	U of S	Yugoslavia
	93	USDA	Turkey
	14[c]	USDA	France
	29[c]	USDA	Spain

[a] U of S – University of Saskatchewan, Saskatoon, Saskatchewan, Canada.
b USDA – United States Department of Agriculture, Pullman, Washington, USA.
[c] Differentiated cytotype of *L. n.* ssp. *nigricans*.

the gain or loss of restriction sites, and occasional poor resolution of the fragments. Summation of restriction fragments gave an estimated average chloroplast genome size of 126 kilobases, a value similar to that of other members of the Vicieae (Palmer et al., 1987). The use of hexanucleotide-recognizing restriction endonucleases resulted in a pseudo-random sampling of approximately 540 base pairs, or 0.4% of the chloroplast genome. Although each accession had a unique fragment pattern, variation within subspecies was limited (F = 0.99 to 0.97), except for *L. n.* ssp. *nigricans* where both accessions of the normal cytotype differed substantially from those of the differentiated cytotype. Variation among subspecies was much higher, with the normal cytotype of *L. n.* ssp. *nigricans* being the farthest removed from the other four subspecies (Table 2).

The fraction of conserved fragments (F, Table 2) among subspecies indicated that the three subspecies of *L. culinaris* were genetically similar to one another (F = 0.94 to 0.98), with *L. n.* ssp. *orientalis* closest to the cultivated lentil, *L. c.* ssp. *culinaris*. *L. n.* ssp. *ervoides* shared more fragments in common with the three subspecies of *L. culinaris* (F = 0.88 to 0.91) than with its conspecific, *L. n.* ssp. *nigricans* (F = 0.78). The differentiated cytotype of *L. n.* ssp. *nigricans* was more similar to *L. n.* ssp. *ervoides* (F = 0.85) and to the subspecies of *L. culinaris* (F = 0.80 to 0.81) than to the normal cytotype of *L. n.* ssp. *nigricans* (F = 0.78), as the normal cytotype of *L. n.* ssp. *nigricans* was the farthest removed from the other *Lens* subspecies (F = 0.76 to 0.78). A dendogram was constructed which reflects these conclusions (Fig. 1).

Table 2. Matrix of the fraction (F) of conserved chloroplast DNA fragments, based on the observed fragment patterns among subspecies

Accession	cul[a]	ori	ode	erv	nin
ori	0.98				
ode	0.96	0.94			
erv	0.89	0.88	0.91		
nin	0.77	0.76	0.77	0.78	
nid	0.80	0.81	0.81	0.85	0.78

[a] cul = *L. c.* ssp. *culinaris*; ori = *L. c.* ssp. *orientalis*; ode = *L. c.* ssp. *odemensis*; erv = *L. n.* ssp. *ervoides*; nin = *L. n.* ssp. *nigricans*, normal cytotype; and nid = *L. n.* ssp. *nigricans*, differentiated cytotype.

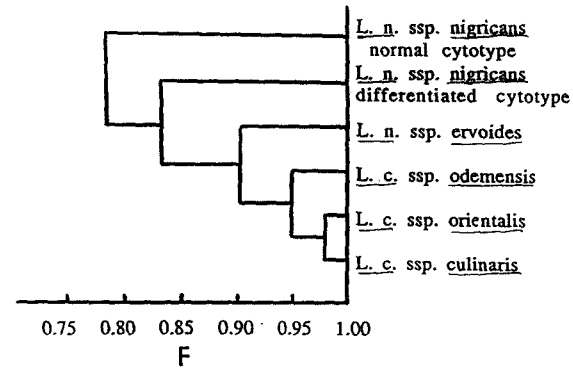

Fig. 1. Dendogram indicating chloroplast DNA affinity within the genus *Lens*, based on the fraction (F) of conserved chloroplast DNA fragments among subspecies. F values are represented on the horizontal axis.

Most of the detectable variation in fragment length was due apparently to insertions and deletions of 50 to 500 base pairs in length, which were recognized in the moderate to low molecular weight fragments as slight changes in migration distance (Fig. 2). The presence of restriction site mutations was also indicated by gains or losses in the number of fragments, but were often difficult to ascribe to particular fragments due to the large amount of variation present among subspecies.

Discussion

Most previous taxonomic studies on the genus *Lens* show that *L. c.* ssp. *orientalis* is most likely the progenitor to the cultivated lentil, *L. n.* ssp. *culinaris*. Results of this study on chloroplast DNA RFLP further confirm this since *L. c.* ssp. *orientalis* shared more chloroplast DNA fragments with *L. c.* ssp. *culinaris* (F = 0.98) than does any other subspecies. Ladizinsky et al. (1984) classified *L. c.* ssp. *odemensis* in the same crossability group as *L. c.* ssp. *culinaris* and *L. c.* ssp. *orientalis*, and this study indicated that *L. c.* ssp. *odemensis* has a high frequency of fragment conservation with these two subspecies (F = 0.96 and 0.94, respectively). This

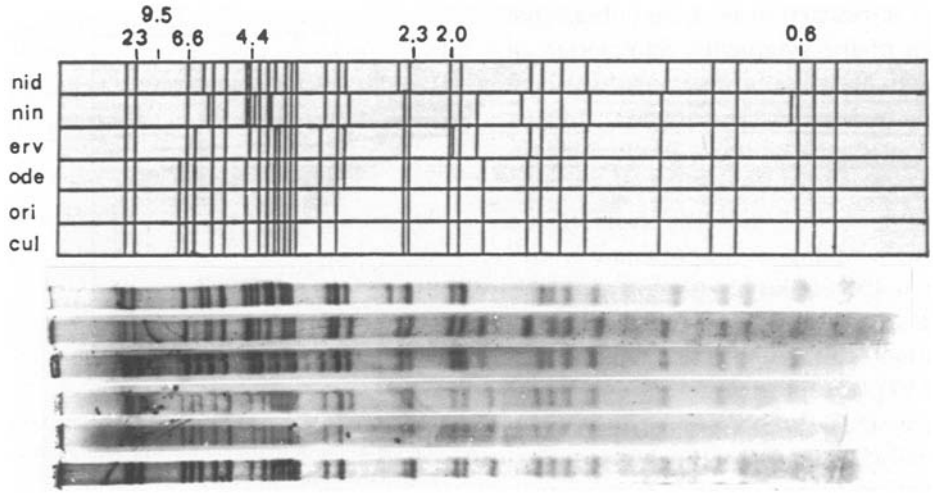

Fig. 2. Chloroplast DNA Bgl II restriction digest of representatives of the five subspecies of *Lens* including the differentiated cytotype of *L. n.* ssp. *nigricans*. Lane designations are as in Table 2. Lambda DNA Hind III molecular weight size standards are given in kilobases.

observation is also consistent with isozyme and nuclear RFLP studies (Hoffman et al., 1986; Havey & Muehlbauer, 1989), and as a result the taxonomic placement of these three subspecies within crossability group 1 in the classification of Ladizinsky et al. (1984) now appears conclusive.

The taxonomic placement of the subspecies within crossability group 2 does not correspond as well with the results obtained in this and other studies. The similarity between *L. n.* ssp. *nigricans* (normal cytotype) and *L. n.* ssp. *ervoides* in terms of chloroplast DNA RFLP is relatively low (F = 0.78), supporting previous studies using morphology, isozyme polymorphism and nuclear DNA RFLP, indicating that these two subspecies of *L. nigricans* are not closely related (Hoffman et al., 1986; 1988; Havey & Muehlbauer, 1989). This study also shows that *L. n.* ssp. *ervoides* is genetically closer to the subspecies of *L. culinaris* than to its conspecific *L. n.* ssp. *nigricans*, a result consistent with isozyme and nuclear RFLP data, and in terms of chromosomal rearrangements (Ladizinsky et al., 1984; Hoffman et al., 1986; Havey & Muehlbauer, 1989). Conversely, morphological studies have shown that *L. n.* ssp. *nigricans* (normal cytotype) is closer to members of the first crossability group than to *L. n.* ssp. *ervoides*, as *L. n.* ssp. *ervoides* was the most differentiated subspecies (Hoffman et al., 1988). This observation supports the suggestion by Hoffman et al. (1986) that *L. n.* ssp. *nigricans* (normal cytotype) and *L. n.* ssp. *ervoides* might best be returned to the specific level.

The taxonomic status of the differentiated cytotype of *L. n.* ssp. *nigricans* also remains unclear as it showed a higher affinity with *L. n.* ssp. *ervoides*, *L. c.* ssp. *culinaris*, *L. c.* ssp. *orientalis* and *L. c.* ssp. *odemensis* in this study than with the normal cytotype of *L. n.* ssp. *nigricans*, although hybridization between the two cytotypes of *L. n.* ssp. *nigricans* has been demonstrated (Ladizinsky et al., 1984). Isozyme conservation studies show a similar result to the data (Hoffman et al., 1986). Chloroplast DNA RFLP also shows that the differentiated cytotype of *L. n.* ssp. *nigricans* is actually closer to *L. n.* ssp. *ervoides* than to any other subspecies. Since *L. n.* ssp. *ervoides* is crossable with the differentiated cytotype of *L. n.* ssp. *nigricans* and the resulting hybrids are partially fertile, the differentiated cytotype may be more closely related to *L. n.* ssp. *ervoides* than to any other subspecies and might best be placed within *L. n.* ssp. *ervoides* or elevated to the subspecies status itself.

Ladizinsky et al. (1983) speculated that both the normal and differentiated cytotypes of *L. n.* ssp.

nigricans might be a remnant of early cultivation, each being derived from different stocks. Hoffman et al. (1988) found that plants of the differentiated cytotype of *L. n.* ssp. *nigricans* and *L. c.* ssp. *odemensis* were morphologically similar and they suggested that the normal cytotype was derived from Southern European stocks, and the differentiated cytotype from Near Eastern stocks. Alternatively, the differentiated cytotype may have arisen from a rare hybridization event between *L. n.* ssp. *ervoides* and the normal cytotype of *L. n.* ssp. *nigricans*, subsequently retaining some traits from each. In any event, the status of the differentiated cytotype in *Lens* taxonomy remains debatable.

This study indicates that the subspecies of crossability group 2 show the greatest divergence from the cultivated lentil. Many economically important genes are likely present within this group, and it would be advantageous if these genes could be transferred to the cultivated varieties. Unfortunately, the genepool of the cultivated lentil is apparently restricted to crossability group 1, as introgression of genes from *L. n.* ssp. *nigricans* and *L. n.* ssp. *ervoides* is blocked as a result of hybrid embryo breakdown (Ladizinsky et al., 1984; Muench, 1989). Cohen et al. (1985) and Ladizinsky et al. (1985) reported rare hybrid formation via embryo rescue in crosses between cultivated lines and *L. n.* ssp. *ervoides*, but these results have not been repeatable. This emphasizes the importance of further germplasm collection to increase the possibility of obtaining lines possessing improved crossability, in addition to increasing the genetic resources of lentil. This is critical, in light of the fact that the habitat of certain wild lentil populations is continually being destroyed (Sohl & Erskine, 1981). If partially fertile *culinaris/ervoides* hybrids could be produced consistently, a bridge linking crossability group 1 and 2 would be possible and could have a tremendous impact on lentil breeding.

Acknowledgements

We would like to thank Allen Good and Greg Penner for their help in DNA purification and labelling, and to the University of Saskatchewan and the Canadian Wheat Board for their financial support throughout this project.

References

Berthau, F., C. Mathieu & F. Vedel, 1983. Chloroplast and mitochondrial DNA variation as an indicator of phylogenetic relationships in the genus *Coffea* L. Theor. Appl. Genet. 65: 77–84.

Cohen, D., G. Ladizinsky, M. Ziv & F.J. Muehlbauer, 1984. Rescue of interspecific *Lens* hybrids by means of embryo culture. Plant Cell Tissue Org. Cult. 3: 343–348.

Cubero, J.I., 1984. Taxonomy, distribution and evolution of the lentil and its wild relatives. In: J.R. Witcomb & W. Erskine (Eds). Genetic resources and their exploitation-chickpeas, fababeans, lentils. Martinus Nijhoff/Dr. W. Junk Publishers, the Hague, the Netherlands.

Gentzbittel, L. & P. Nicolas, 1989. A BASIC program to construct evolutionary trees from restriction endonuclease data. J. Hered. 80: 254.

Havey, M.J. & F.J. Muehlbauer, 1989. Variability for restriction fragment length and phylogenies in lentil. Theor. Appl. Genet. 77: 839–843.

Hoffman, D.L., F.J. Muehlbauer & G. Ladizinsky, 1988. Morphological variation in *Lens* (Leguminosae). Syst. Bot. 13: 87–96.

Hoffman, D.L., D.E. Soltis, F.J. Muehlbauer & G. Ladizinsky, 1986. Isozyme polymorphism in *Lens*. Syst. Bot. 11: 392–402.

Holwerda, B.C., S. Jana & W.L. Crosby, 1986. Chloroplast and mitochondrial DNA variation in *Hordeum vulgare* and *Hordeum spontaneum*. Genetics 114: 1271–1291.

Kung, S.D., Y.S. Zhu & G.F. Shen, 1982. *Nicotiana* chloroplast genome. III. Chloroplast DNA evolution. Theor. Appl. Genet. 61: 73–79.

Ladizinsky, G., 1979. Species relationships in the genus *Lens* as indicated by seed protein electrophoresis. Bot. Gaz. 140: 449–451.

Ladizinsky, G., D. Braun & F.J. Muehlbauer, 1983. Evidence for the domestication of *Lens nigricans* (M. Bieb.) Godron in S. Europe. Bot. J. Linn. Soc. 87: 169–176.

Ladizinsky, G., D. Cohen & F.J. Muehlbauer, 1985. Hybridization of the genus *Lens* by means of embryo culture. Theor. Appl. Genet. 70: 97–101.

Ladizinsky, G., D. Braun, D. Goshen & F.J. Muehlbauer, 1984. The biological species of the genus *Lens* L. Bot. Gaz. 154: 253–261.

Maniatis, T., E.F. Fritsch & J. Sambrook, 1982. Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.

Muench, D.G., 1989. Crossability, cytology and chloroplast DNA variation in *Lens* Miller. Masters thesis. University of Saskatchewan.

Nei, M. & W. Li, 1979. Mathematical model for studying genetic

variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76: 5269–5273.

Palmer, J.D., 1987. Chloroplast DNA evolution and biosystematical uses of chloroplast DNA variation. Amer. Nat. (suppl.) 130: S6–S29.

Palmer, J.D. & D. Zamir, 1982. Chloroplast DNA evolution and phylogenetic relationships in *Lycopersicon*. Proc. Natl. Acad. Sci. USA 79: 5006–5010.

Palmer, J.D., R.A. Jorgensen & W.F. Thompson, 1985. Chloroplast DNA variation and evolution in *Pisum*: Patterns of change and phylogenetic analysis. Genetics 109: 195–213.

Palmer, J.D., B. Osorio, J. Aldrich & W.F. Thompson, 1987. Chloroplast DNA evolution among legumes: loss of a large inverted repeat occurred prior to other sequence rearrangements. Curr. Genet. 11: 275–286.

Sohl, M. & W. Erskine, 1981. Genetic resources. In: C. Webb & G. Hawton (Eds). Lentils. Commonwealth Agric. Bureax. London, pp. 53–68.

Syksma, K.J. & L.D. Gottlieb, 1986. Chloroplast DNA evolution and phylogenetic relationships in *Clarkia* sect. Peripetasma (Onagraceae). Evolution 40: 1248–1261.

Upholt, W.B., 1977. Estimation of DNA sequence divergence from comparison of restriction endonuclease digestion. Nucleic Acids Res. 4: 1257–1265.