Theor Appl Genet (1994) 89:206–210

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Inheritance of mitochondrial DNA in lentil (*Lens culinaris* Medik.)

Received: 7 February 1994 / Accepted: 17 February 1994

Abstract Restriction fragment analysis was used to examine the inheritance of lentil mitochondrial DNA (mtDNA) in F_1 and F_5 progeny from intrasubspecific (*Lens culinaris* ssp. *culinaris*) crosses and in F_1 progeny from intersubspecific (*Lens culinaris* ssp. *orientalis* × *L. culinaris* ssp. *culinaris*) crosses. Southern blots of digested parental and progeny DNA were hybridized to heterologous maize mtDNA probes specific to *coxI* and *atp6* genes. Two restriction fragment polymorphisms separated *L.c.* ssp. *culinaris* 'Laird' and 'Eston' from *L.c.* ssp. *culinaris* 'ILL5588', and one restriction fragment polymorphism distinguished *L.c.* ssp. *culinaris* 'Laird' and 'Eston' from *L.c.* ssp. *orientalis* 'LO4'. Twelve of 13 F_1 progeny and all F_5 progeny from the intrasubspecific crosses, and all F_1 progeny from intersubspecific crosses had only maternal mtDNA restriction fragments. One F_1 plant from an 'Eston' × 'ILL5588' cross inherited mtDNA fragments from both parents. Nuclear DNA inheritance was biparental in all F_1 progeny.

Key words *Lens culinaris* · Mitochondrial DNA · Maternal inheritance · Biparental mitochondrial inheritance · Restriction fragment length polymorphism

Introduction

Uniparental-maternal transmission of mitochondrial DNA (mtDNA) is generally assumed in angiosperms, but precise information on mitochondrial inheritance in plants has been very limited in the past due to a lack of phenotypic markers. The analysis of restriction fragment length polymorphism (RFLP) now provides precise molecular genetic markers for examining mitochondrial inheritance, and a great majority of the studies on angiosperms that have used RFLP markers indicate a uniparental-maternal inheritance of mtDNA (review in Smith 1989; see also Schmitz 1988; Schumann and Hancock 1989; Horlow et al. 1990; Monroy et al. 1990; Radetzky 1990; Rajora et al. 1992). However, in some angiosperms, biparental inheritance of mtDNA has been observed to varying degrees (Soliman et al. 1987; Erickson and Kemble 1990).

Lentil (*Lens culinaris* Medik.), a legume of the Fabaceae, is an important food crop of the Indian subcontinent, the Middle East, and North Africa because of its high nutritional quality and drought resistance (Simpson and Conner-Ogorzaly 1986). *Lens culinaris* is subdivided into three cross-compatible subspecies: ssp. *culinaris* (cultivated), ssp. *orientalis* (wild), and ssp. *odemensis* (wild) (Ladizinsky et al. 1984). Recent genetic studies have examined variation and the inheritance of nuclear genes in *Lens* using morphological, isozyme, and RFLP markers (listings in Vaillancourt and Slinkard 1992; see also Havey and Muehlbauer 1989a, b). Also, restriction fragment variation of chloroplast (cp)DNA has been reported for *Lens* (Muench et al. 1991), but as yet there is no information on the inheritance and variation of its mtDNA. Indeed, with the exception of *Medicago sativa*, for which uniparental-maternal inheritance of mtDNA has been reported (Schumann and Hancock 1989), to our knowledge there is no genetic information on the mode of mtDNA inheritance in Fabaceae.

The objective of the investigation reported here was to examine the inheritance of *Lens culinaris* mtDNA by means of RFLP analysis of F_1 progeny and F_2-derived F_5 lines from intrasubspecific crosses and F_1 progeny from intersubspecific crosses. The results presented demonstrate the existence of biparental inheritance in addition to the more usual uniparental-maternal mode of mtDNA inheritance.

Materials and methods

Controlled crosses

Three accessions of *Lens culinaris* ssp. *culinaris* ('Laird', 'Eston', and 'ILL5588') and one accession of *L. culinaris* ssp. *orientalis* ('LO4')

Communicated by H. F. Linskens

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were used as parents of the controlled crosses. 'Laird' and 'Eston' are commercial varieties from Saskatchewan, and 'ILL5588' is a Syrian accession from the International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria, that has improved resistance to *Ascochyta* blight.

Single-seed descent lines of the accessions 'Laird', 'Eston', and 'ILL5588', and F_2-derived F_5 lines from a 'Laird' × 'ILL5588' cross were procured from Dr. A. E. Slinkard of the Crop Development Centre, Saskatoon, Saskatchewan, Canada. Bulked seeds of accession 'LO4' of *L.c.* ssp. *orientalis* were obtained from Dr. F. J. Muehlbauer of the USDA/ARS Grain Legume Program, Washington State University, Pullman. Plants were grown in pots of Turface (Applied Industrial Materials Corp, Deerfield, Ill.) in a controlled growth chamber with a 16-h photoperiod (PAR 400 µmol quanta $m^{-2} s^{-1}$) and day/night temperatures of 20°/15 °C.

Reciprocal intrasubspecific crosses between 'Laird' or 'Eston' and 'ILL5588' and unidirectional intersubspecific crosses between *L.c.* ssp. *orientalis* and *L.c.* ssp. *culinaris* ('LO4' × 'Laird', and 'LO4' × 'Eston') were made in the summer of 1990 by emasculation and hand pollination in a growth chamber. The reciprocal *L.c.* ssp. *culinaris* × *L.c.* ssp. *orientalis* controlled cross could not be made because of insufficient pollen from 'LO4'. F_1 hybrids were germinated and then grown as described above. The following F_1 plants were obtained and analyzed: 1 from a cross between 'Laird' parent1 × 'ILL5588' parent2, 2 from 'Laird' parent2 × 'ILL5588' parent1, 1 from 'ILL5588' parent1 × 'Laird' parent2, 4 from 'ILL5588' parent1 × 'Laird' parent1, 3 from 'Eston' × 'ILL5588' parent1, 2 from 'ILL5588' parent1 × 'Eston', 7 from 'LO4' × 'Laird' parent1, 1 from 'LO4' × 'Laird' parent 2, and 2 from a 'LO4' × 'Eston' cross.

DNA extraction, restriction, electrophoresis, and Southern blotting

Total cellular DNA was isolated from 0.5–2.0 g fresh weight of leaf tissue from each individual by a modification (to be presented elsewhere by Rajora and Dancik) of the CTAB method of Doyle and Doyle (1987). DNA, purified by ultracentrifugation in a CsCl gradient produced the same results as unpurified DNA. DNA (5–10 µg) from individual plants was digested with 15–20 units of the restriction enzymes *Ava*I, *Bam*HI, *Bcl*I, *Bgl*II, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pst*I, *Pvu*II, *Sal*I, *Xba*I, and *Xho*I for 5 h according to the manufacturer's recommendations (Boehringer Mannheim Canada, Laval, Quebec or BRL, Bethesda, USA).

Restriction fragments were separated on 20 × 20 cm 0.8% agarose gels (in TBE buffer, Maniatis et al. 1982) containing ethidium bromide by electrophoresing at 1.5–1.75 V/cm for about 18 h in TBE buffer. The DNA fragments were transferred to nylon membranes (Gene Screen Plus, Du Pont Canada, Mississauga, Ontario) or Hybond-N + (Amersham Canada, Oakville, Ontario) using the alkaline transfer method of Chomczynski and Qasba (1984).

mtDNA probes

A 10-kb mtDNA fragment containing the cytochrome c oxidase subunit I (*coxI*) gene (probe CoxI) from maize (Issac et al. 1985) and a 2.7-kb mtDNA fragment containing the ATPase subunit 6 (*atp6*) gene (probe Atp6) from maize (Dewey et al. 1985) were used as probes for mtDNA. Restriction fragment variation of mtDNA among the parents of the controlled crosses was screened by employing these two probes and all 15 restriction enzymes. Two informative enzyme-probe combinations (*Bgl*II-CoxI, and *Xba*I-Atp6) were used to examine the mode of mtDNA inheritance in intrasubspecific *L.c.* ssp. *culinaris* crosses, and one combination (*Hin*dIII-CoxI) was used to determine mtDNA inheritance in intersubspecific *L.c.* ssp. *orientalis* × *L.c.* ssp. *culinaris* crosses.

Nuclear DNA probe

A 0.64-kb cDNA clone CMH52 from lentil (Havey and Muehlbauer 1989b) was used as the probe for nuclear DNA. CMH52 was hybridized with *Bgl*II, *Xba*I (intrasubspecific crosses), and/or *Hin*dIII (intersubspecific crosses) digests of the parents and progeny DNAs on the same blots after stripping off maize mtDNA probes and/or on fresh blots.

Probe preparation, hybridization, washing, and autoradiography

Plasmid DNA containing maize mtDNA or lentil cDNA inserts was isolated and purified by following the protocol described for Qiagen columns (Qiagen, Chatsworth, Calif.). The probes were prepared by random prime labeling with $\alpha - [^{32}P]$dCTP according to the specifications of the kit manufacturer (Boehringer Mannheim Canada). Unincorporated nucleotides were removed from the labeled recombinant probes by using Elutip D Columns (Schleicher and Schuell, Keene, N.H.). Prehybridizations for 6–8 h and hybridizations for a minimum of 16 h were conducted at 60 °C (Rajora and Dancik 1992). Hybridized blots were washed (Rajora and Dancik 1992) and then exposed to X-ray films with intensifying screens for 3–48 h at −70 °C. For quantitative estimates of radioactivity, developed X-ray films were scanned with a Molecular Dynamics (Sunnyvale, Calif.) Computing Densitometer, and relative band densities were determined by the linear integration of each lane.

Results and discussion

mtDNA inheritance in intrasubspecific crosses of *L. culinaris* ssp. *culinaris*

Restriction fragment patterns produced by the *Bgl*II-CoxI and *Xba*I-Atp6 combinations differentiated the parents of the intrasubspecific crosses (Table 1; Figs. 1 and 2). The hybridization of probes to any of the parent plants within an accession revealed the same mtDNA fragments. *Bgl*II digests hybridized with CoxI showed 'ILL5588' to have 6.0-, 3.6-, 2.5- and 1.1.-kb fragments, the first two of which were also seen in 'Laird' and 'Eston' samples (Fig. 1). However, the autoradiographic intensity of the 3.6-kb band was relatively greater in 'Laird' and 'Eston' samples than in 'ILL5588'. In fact, densitometry of the autoradiogram estimated an average of 90% of the total radioactivity of 'Eston' and 'Laird' samples to be in the 3.6-kb band, as compared to 38% for the 'ILL5588' sample, suggesting that there were two 3.6-kb *Bgl*II-CoxI fragments in 'Laird' and 'Eston', whereas an additional restriction site within one of these fragments in 'ILL5588' produced the 2.5- and

Table 1 Restriction fragment polymorphisms of mtDNA revealed by maize mtDNA probes CoxI and Atp6 among the four parents of the controlled crosses of lentil and subsequently used for determining the mode of mtDNA inheritance. (+ present, − absent)

Enzyme	Probe	DNA fragment(s) (size in kb)	*L.c.* ssp. *culinaris*			*L.c.* ssp. *orientalis*
			Laird	Eston	ILL-5588	LO4
*Bgl*II	CoxI	3.6[a] = 3.6, 3.6	+	+	−	+
		3.6, 2.5, 1.1	−	−	+	−
*Hin*dIII	CoxI	2.4	−	−	−	+
		0.9	+	+	+	−
*Xba*I	Atp6	2.9	+	−	+	−

[a] Double intensity as compared to the 3.6-kb fragment in 'ILL5588'

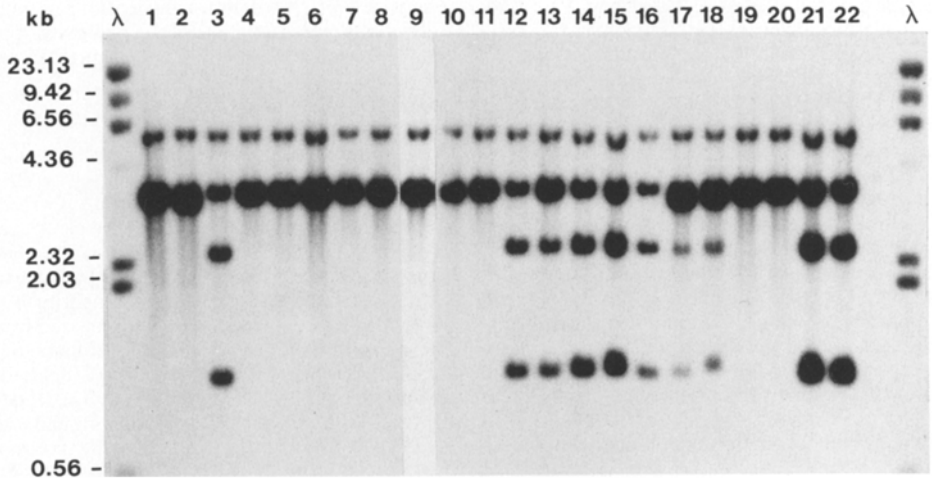
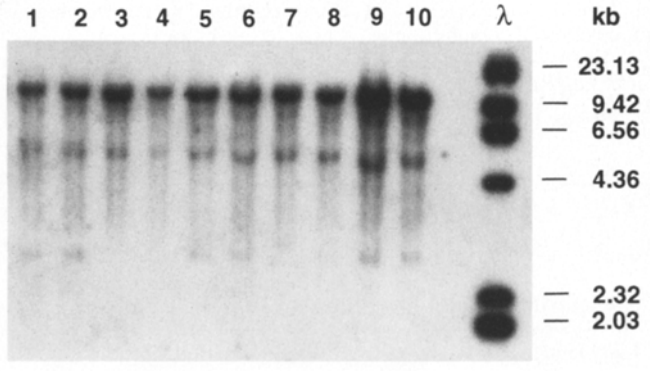


Fig. 1 Autoradiograph illustrating the mode of mtDNA inheritance in intrasubspecific controlled crosses of *L. c.* ssp. *culinaris*. Parents and progeny DNAs were restricted with *Bgl*II and hybridized with the 10-kb maize mtDNA probe CoxI. All crosses are as female × male. *Lane1* 'Laird', *2* 'Eston', *3* 'ILL5588', *4–6* F_1 offspring of two 'Laird' × 'ILL5588' crosses, *7–11* F_2-derived F_5 offspring of a 'Laird' × 'ILL5588' cross, *12–16* F_1 hybrid progeny of two 'ILL5588' × 'Laird' controlled crosses, *17–20* F_1 hybrids of an 'Eston' × 'ILL5588' cross – *17* and *18* are of the same F_1 individual from two different DNA extractions: *17* DNA extracted on November 28, 1990, and *18* DNA extracted on January 14, 1991, *21* and *22* F_1 progeny of an 'ILL5588' × 'Eston' cross

1.1-kb fragments (Table 1). To verify that the double-density 3.6-kb fragment in 'Laird' and 'Eston' was not a result of incomplete *Bgl*II digestion, the same amount (5–10 µg) of 'Laird', 'Eston', and 'ILL 5588' DNA was restricted with two-, three-, four-, and fivefold *Bgl*II concentrations (20–50 units/digest) and hybridized to CoxI. In each case, the same results were obtained. To verify that the observed *Bgl*II-CoxI polymorphism was not related to cpDNA, we probed *Bgl*II digests of 'Laird', 'Eston', and 'ILL5588' DNA with each of the seven *Pst*I fragments of *Petunia hybrida* cpDNA (23, 21, 19, 15.3, 9.2, 9.0, and 2.6 kb; Palmer et al. 1983), totaling 99.1 kb in size (lentil's cp genome size is approximately 125 kb). In no case did we observe the same or similar restriction fragment polymorphisms. Therefore, 'Laird', and 'Eston' were distinct from 'ILL5588' by the loss or gain of a *Bgl*II restriction site in and/or around the region of the mitochondrial gene *coxI*. *Xba*I digests hybridized with Atp6 indicated that 'Laird' and 'ILL5588' were distinct from 'Eston' by the loss or gain of a restriction site in and/or around the region of the *atp6* gene (Table 1; Fig. 2). Thus, the *Xba*I-Atp6 combination was informative only for the crosses between 'Eston' and 'ILL5588'.

Fig. 2 Autoradiograph illustrating mtDNA inheritance in *L.c.* ssp. *culinaris* intrasubspecific 'Eston' × 'Ill5588' and its reciprocal 'ILL5588' × 'Eston' crosses. *Xba*I restriction digests of the parents and progeny DNAs were hybridized to the 2.7-kb maize mtDNA fragment Atp6. Crosses are indicated as female × male. *Lanes 1* and *2* 'ILL5588', *3* and *4* 'Eston', *5–8* F_1 hybrids of an 'Eston' × 'ILL5588' cross — *5* and *6* are of the same F_1 individual from two separate DNA extractions as *17* and *18* in Fig. 1, *9* and *10* F_1 progeny of an 'ILL5588' × 'Eston' cross



The *Bgl*II-CoxI restriction fragment patterns of all F_1 progeny and all 5 F_2-derived F_5 lines from the reciprocal intrasubspecific *L.c.* ssp. *culinaris* crosses between 'Laird' and 'ILL5588' were consistent with the maternal inheritance of mtDNA (Table 2; Fig. 1). As well, 4 of the 5 F_1 offspring from the reciprocal crosses between 'Eston' and 'ILL5588' inherited *Bgl*II-CoxI and *Xba*I-Atp6 mtDNA restriction fragments from their maternal parents (Table 2; Figs. 1 and 2), confirming the general maternal inheritance of mtDNA. However, 1 F_1 plant from the 'Eston' × 'ILL5588' cross had *Bgl*II-CoxI (Fig. 1, lanes 17 and 18) as well as *Xba*I-Atp6 (Fig. 2, lanes 5 and 6) mtDNA restriction fragments from both parents, indicating the transmission of maternal as well as paternal mtDNA. The detection of these paternal fragments in independent restriction digests of DNA extracted at different times during growth of the same plant (Figs. 1 and 2) shows that the observed mtDNA restriction fragment patterns were characteristic of the genotype, and not the result of contamination. Nevertheless, the estimated radioactivity of these 2.5- and 1.1-kb paternal fragments, expressed as a percentage of the total autoradiographic density of the lanes (Fig. 1), averaged only 11% in the 2 samples from this plant as compared to a mean ± SD of 56 ± 4% for 'ILL5588' and 6 other samples from crosses with 'ILL5588' as the maternal parent; this result suggests a lower ratio of paternal to maternal mtDNA in this F_1 hybrid. In

Table 2 Mitochondrial DNA inheritance in lentil controlled crosses

Cross	Number of crosses	Generation	Number of progeny		
			Total	Maternal	Biparental
L. culinaris ssp. *culinaris* × *L. culinaris* ssp. *culinaris*					
Laird × ILL5588	2	F_1	3	3	0
	1	F_5	5	5	0
ILL5588 × Laird	2	F_1	5	5[a]	0
Eston × ILL5588	1	F_1	3	2	1
ILL5588 × Eston	1	F_1	2	2	0
L. culinaris ssp. *orientalis* × *L. culinaris* ssp. *culinaris*					
LO4 × Laird	2	F_1	8	8	0
LO4 × Eston	1	F_1	2	2	0

[a] One of these F_1 progeny may have biparental mtDNA inheritance

Brassica napus, the concentration of the paternal mtDNA in progeny with biparental mtDNA inheritance ranged from 28% to 84% (Erickson and Kemble 1990). Densitometry also identified another sample (Fig. 1, lane 13) with unusual ratios of radioactivity in the different bands. In this sample, the 3.6-kb band comprised 63% of the total as compared to a mean ± SD of 36 ± 4% in 7 samples with the 'ILL5588' pattern and 90 ± 2% in 12 samples with the 'Eston'/'Laird' pattern. This intermediate pattern may also indicate transmission of some mtDNA from the paternal 'Laird' parent; however, even if the possibility of incomplete digestion were eliminated, such quantitative evidence is clearly less compelling than the detection of restriction fragments unique to the paternal parent.

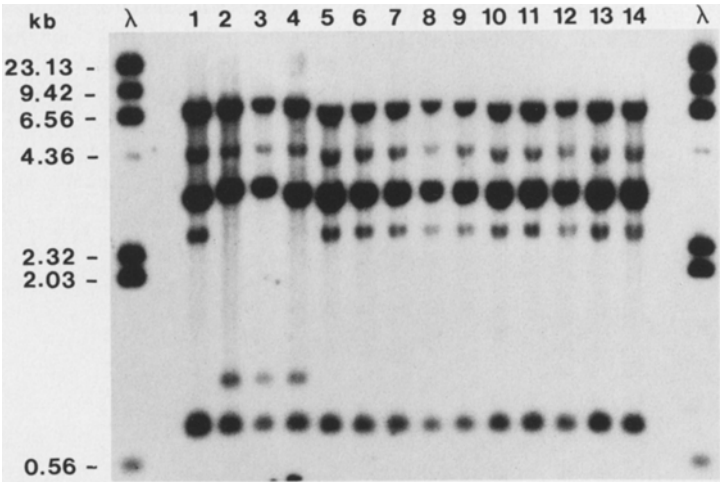
mtDNA inheritance in intersubspecific crosses of *L. culinaris* ssp. *orientalis* × *L. culinaris* ssp. *culinaris*

The *L.c.* ssp. *orientalis* parent 'LO4' differed from the *L.c.* ssp. *culinaris* parents 'Laird' and 'Eston' by one subspecies-specific mtDNA RFLP in and/or around the region of *coxI* gene as defined by hybridization of *Hin*dIII digests with maize mtDNA probe CoxI (Table 2; Fig. 3). Whether this mtDNA restriction fragment polymorphism is due to a deletion/insertion event or genome rearrangement is not clear, but all 10 F_1 progeny of the 3 intersubspecific lentil crosses (Table 2) had mtDNA restriction fragments only from their maternal parent 'LO4' (Fig. 3), supporting a general uniparental-maternal inheritance of mtDNA in *L. culinaris*.

Nuclear DNA inheritance

Nuclear DNA inheritance was biparental in all 13 F_1 progeny of the six intrasubspecific crosses of *L. culinaris* ssp. *culinaris* (Table 1) and all 10 F_1 hybrid progeny of the three *L.c.* ssp. *orientalis* × *L.c.* ssp. *culinaris* controlled crosses (Table 1). This was shown by hybridization of

Fig. 3 Autoradiograph demonstrating maternal inheritance of mtDNA in intersubspecific lentil controlled crosses. mtDNA restriction fragment patterns of the parents and F_1 progeny of the *L.c.* ssp. *orientalis* × *L.c.* ssp. *culinaris* ('LO4' × 'Laird', and 'LO4' × 'Eston') controlled crosses were obtained by hybridization of the 10-kb maize mtDNA probe CoxI to the *Hin*dIII-restricted total DNAs of the parents and offspring. All crosses are indicated as female × male. *Lane 1* 'LO4', *2* 'Laird' parent1, *3* 'Laird' parent2, *4* 'Eston', *5–11* F_1 progeny of 'LO4' × 'Laird' parent1, *12* F_1 progeny of 'LO4' × Laird' parent2, *13* and *14* F_1 progeny of 'LO4' × 'Eston'



the lentil cDNA clone CMH52 to *Bgl*II, *Xba*I, or *Hin*dIII digests of the parents and progeny DNA on the same membranes after removal of mtDNA probes and also on fresh blots. 'Laird', 'Eston', and 'ILL5588' and 'LO4', respectively, had 22-, 21.2-, and 19-kb *Bgl*II, 2.4-, 2.32-, and 2.1-kb *Hin*dIII, and 2.9-, 2.82-, and 2.6-kb *Xba*I fragments at a CMH52 locus. All of the F_1 progeny were heterozygous for their parental alleles. F_2-derived F_5 lines from the 'Laird' × 'ILL5588' cross segregated for these two alleles and were either homozygous or heterozygous.

The genetic variability in mtDNA restriction fragment patterns between the mitochondrial genomes of different accessions within *L.c.* ssp. *culinaris*, and between *L.c.* ssp. *culinaris* and *L.c.* ssp. *orientalis* allowed the examination of mtDNA inheritance in this species. The results clearly demonstrate the predominant uniparental-maternal mode of mtDNA inheritance in lentil as 11 of the 13 F_1 progeny from intrasubspecific crosses, all 5 F_2-derived F_5 lines from an intrasubspecific cross, and all 10 F_1 progeny from three intersubspecific crosses inherited mtDNA restriction fragments from only their maternal parents. That this resulted from maternal inheritance rather than self-fertilization of the maternal parent was shown by the heterozygosity of all of the F_1 progeny at the nuclear gene locus homologous to cDNA clone CMH52. Our results also demonstrate clear evidence of biparental inheritance of mtDNA in 1 F_1 offspring from an intrasubspecific cross between *L.c.* ssp. *culinaris* 'Eston' and 'ILL5588' and the possibility of biparental inheritance by a plant from an 'ILL5588' × 'Laird' cross. Because of the small size of the populations studied, it is not possible to determine with any accuracy the frequency of paternal transmission of mtDNA. However, this is the first evidence of the existence of biparental inheritance of mtDNA in the genus *Lens*, and to our knowledge, in any of the Fabaceae. Uniparental-maternal inheritance seems to be the most common mode of mtDNA inheritance in angiosperms (review in Smith 1989; and see Schmitz 1988; Schumann and Hancock 1989; Horlow et al. 1990; Monroy et al. 1990; Radetzky 1990; Rajora et al. 1992), but biparental inheritance of mtDNA has also been reported for F_1 progenies of *Brassica napus* (Erickson and Kemble 1990) and intergeneric *Hordeum* × *Secale* hybrids (Soliman et al. 1987).

Acknowledgements We thank Colin Kindrachuk for technical assistance, Dr. C. S. Levings III for providing maize mitochondrial DNA fragments, Dr. J. D. Palmer for providing *Petunia* chloroplast DNA fragments, and Dr. F. J. Muehlbauer for a gift of lentil cDNA clones. This research was supported by the Saskatchewan Agriculture Development Fund (Project No. R-89-05-04580).

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