

Length polymorphism in the ribosomal DNA intergenic spacers of *Lens* species

M. Fernández¹, M. L. Ruiz² and M. Pérez de la Vega^{1*}

¹ Área de Genética. Facultad de Ciencia Biológicas y Ambientales. Universidad de León. 24171 León. Spain

² Instituto de Recursos Naturales. Universidad de León. 24171 León. Spain

Abstract

Analysis of the ribosomal DNA (rDNA) intergenic spacers (IGS) (i.e., the IGS of 18S, 5.8S and 24S rDNA, and the NTS or non-transcribed spacer of 5S rDNA) of lentil cultivars and landraces (*L. culinaris* spp. *culinaris*) showed to have very low intraspecific length variability. PCR products of approximately 3200 bp were obtained from all cultivated materials; no significant electrophoretic differences were found between them. However, observable intraspecific polymorphism increased with the digestion of PCR-amplified IGSs by the restriction endonucleases *Bsp*HI, *Stu*I and *Bst*BI. This was also the case when specific PCR primers designed to amplify a particularly variable segment of the IGS were used. This segment includes two families of short, tandemly arranged, repeated sequences. Both methods revealed differences between some of the cultivated materials, yielding genetic markers that might be useful in further genetic mapping and breeding studies. Several NTS amplification products were observed in each plant, ranging from approximately 300 to 900 bp in the cultivated lentil. Differences between *L. culinaris* spp. *culinaris* and its related wild species (including the wild ancestor of the cultigen, *L. culinaris* spp. *orientalis*) were observed in the total length of their IGSs, the restriction patterns of the latter, and in their NTS variants.

Additional key words: genetic markers, IGS, legumes, lentil, NTS.

Resumen

Polimorfismos de tamaño de los espaciadores intergénicos del DNA ribosomal de *Lens*

Los espaciadores intergénicos del DNA ribosomal (el IGS o espaciador intergénico del rDNA 18S, 5.8S, y 24S, y el NTS o espaciador no transcrito del rDNA 5S) de lenteja (*L. culinaris* spp. *culinaris*) mostraron muy poca variabilidad intraespecífica, tanto en cultivares como en variedades locales. De todos los materiales de esta especie se obtuvieron productos de amplificación por PCR con una longitud de unas 3200 pb, no observándose diferencias significativas mediante separación por electroforesis. Sin embargo, el polimorfismo intraespecífico aumentó mediante la digestión con endonucleasas de restricción (*Bsp*HI, *Stu*I, y *Bst*BI) de los IGS amplificados por PCR o por el uso de cebadores específicos que amplificaban segmentos particularmente variables del IGS. Estos segmentos incluyen dos familias de secuencias cortas agrupadas. Con ambos métodos se observaron diferencias entre materiales de lenteja, utilizables como posibles marcadores genéticos en posteriores trabajos de mejora o genética, como la confección de mapas genéticos. Se observaron varios productos de amplificación NTS en cada planta, cuyo tamaño osciló entre unas 300 y 900 pb en la lenteja cultivada, no observándose diferencias entre estos materiales. Sí se observaron diferencias entre *L. culinaris* spp. *culinaris* y las especies silvestres del género *Lens* (incluido el ancestro silvestre del cultigen, *L. culinaris* spp. *orientalis*), tanto en la longitud del IGS, como en su patrón de restricción, y en los conjuntos de NTS.

Palabras clave adicionales: IGS, legumbres, lenteja, marcadores genéticos, NTS.

* Corresponding author: degmpv@unileon.es

Received: 18-07-05; Accepted: 24-10-05.

Introduction

The lentil (*L. culinaris* spp. *culinaris*) is an important pulse grown in many semi-arid areas with temperate climates. As in many other crop species, genetic maps are becoming a useful tool in its breeding and in the study of its genetics (Rubeena *et al.*, 2003; Durán *et al.*, 2004; Hamwieh *et al.*, 2005). The development of genetic markers that can identify lentil chromosomes helps in assigning the latter to linkage groups. Ribosomal DNA (rDNA) *loci*, i.e., nucleolar organiser (*Nor*) and 5S *loci*, are examples of such markers. The *Nor* is a complex genetic *locus* (found at one or more chromosomal locations) consisting of numerous tandemly arranged copies of ribosomal RNA (rRNA) genes (Rogers and Bendich, 1987). The basic organisation of rDNA has been maintained in most eukaryotic systems. One repeating unit consists of the 18S, 5.8S and 25S rDNA-coding regions, the corresponding internal transcribed spacers (ITS), and an intergenic spacer (IGS). In contrast to the conserved coding regions, internal spacers and IGSs are variable portions of rDNA cistrons. Indeed, IGSs frequently show sufficient variation to allow the analysis of the genetic relationships between closely related species, populations or cultivated varieties (Polanco and Pérez de la Vega, 1995, 1997; Penteado *et al.*, 1996; Nickrent and Patrick, 1998). They can vary widely in length between plant species groups (ranging from approximately 1 kb to over 12 kb; see Rogers and Bendich, 1987) and within species (Polanco and Pérez de la Vega, 1995; 1997; Penteado *et al.*, 1996), mainly due to the presence of one or more tandem or dispersed subrepeat sequences. As in most eukaryotic species, the 5S rDNA of plant species is organised in tandem repetitive units located at one or more *loci*. Each repetitive unit includes 120 bp coding for the 5S rRNA, and a non-transcribed spacer (NTS) which typically ranges between 100 and 900 bp in length (Sastri *et al.*, 1992).

The IGS of *Lens culinaris* Medik. cv. Verdina is 2939 bp long (EMBL GenBank accession number AJ245998) and is composed of non-repeated sequences and four tandems of repeated sequences named A to D (Fernández *et al.*, 2000). Each subrepeat of the A family is 85-86 bp long, the length of B subrepeats is 170-173 bp, C subrepeats are 66-68 bp long (each formed by a set of 11 bp short repeats; consensus motif TGGGCATTTCG), and finally D subrepeats are

formed by the repetition of short consensus sequences 21 bp long. In addition to complete subrepeats there are also some that are truncated.

IGS sequence similarities exist between lentil and other legume species, in particular those of the *Vicieae* tribe (Fernández *et al.*, 2000). In the genus *Lens*, a single *Nor locus* for 18S-5.8S-25S rRNA on a pair of homologous chromosomes has been described for each species (Balyan *et al.*, 2002). Having a single *Nor locus* means that any genetic marker linked to an IGS polymorphism is associated with this chromosome. Polymorphism of the ITSs in *Lens culinaris* is very low or nil according to Sonnante *et al.* (2003). The polymorphism of *Lens* 5S NTS has previously been studied by PCR amplification (Ford *et al.*, 1997) and hybridisation with heterologous probes (Patil *et al.*, 1995). Two gene *loci* for 5S rRNA on different pairs of homologous chromosomes have been mapped in *Lens* species (Balyan *et al.*, 2002), although it is possible to distinguish between them on the basis of their different NTS size variants (Fernández *et al.*, 2005).

The aims of the present work were to analyse the polymorphism of the IGS and NTS rDNA spacers as potential markers for localizing other genetic markers on chromosomes 3, 2 and 6 of *L. culinaris*, according to the nomenclature of Balyan *et al.* (2002). These markers may be useful for constructing genetic maps of *Lens culinaris*, and for studying spacer variability among *Lens* species.

Material and Methods

The materials used in this study included 12 varieties of the cultivated species *Lens culinaris* ssp. *culinaris* (Medik.), two accessions of *L. culinaris* ssp. *orientalis* (Boiss.) Ponert, four accessions of *L. odemensis* Godr., and one accession each of *L. ervoides* (Bring.) Grande, *L. nigricans* (Bieb.) Godr., *L. lamottei* Czefranova, and *L. tomentosus* Ladizinsky (the two last samples kindly provided by Dr. Ladizinsky). Of the cultivated materials, eight varieties came from mainland Spain (five of the microsperma and three of the macrosperma type), two from the Canary Islands (microsperma) and two from Lithuania (microsperma) (Table 1). Genomic DNA was isolated from individual dry seeds of *Lens* species by the method of Dellaporta *et al.* (1983) with minor modifications. IGSs were amplified using the «touchdown» PCR method,

Table 1. *Lens* materials used

Species	Variety/Accession	Status	Origin
<i>L. c. ssp. culinaris</i>	'Alpo' (m)	Cultivar	León (Spain)
	'Armuña' (M)	Land race	Salamanca (Spain)
	'Castellana' (M)	Land race	León (Spain)
	'Diskiai' (m)	Cultivar	Lithuania
	'Lupa' (m)	Cultivar	León (Spain)
	'Mala' (m)	Land race	Lanzarote (Canary Islands, Spain)
	'Mosa' (M)	Cultivar	León (Spain)
	'Pardina' (m)	Land race	León (Spain)
	'Paula' (m)	Cultivar	León (Spain)
	'Tetir' (m) ¹	Land race	Fuerteventura (Canary Islands, Spain)
	'Smelinuliai' (m)	Cultivar	Lithuania
<i>L. c. ssp. orientalis</i>	'Verdina' (m)	Land race	León (Spain)
	ILWL-7 ²	Wild	ICARDA (Syria)
<i>L. odemensis</i>	BG-16880 ³	Wild	Israel
	ILWL-39 ²	Wild	ICARDA (Syria)
	ILWL-235 ²	Wild	ICARDA (Syria)
	ILWL-238 ²	Wild	ICARDA (Syria)
<i>L. nigricans</i>	ILWL-252 ²	Wild	ICARDA (Syria)
	BG 16873 ³	Wild	Yugoslavia
<i>L. ervoides</i>	BG 16877 ³	Wild	Israel
<i>L. lamottei</i>	244 ⁴	Wild	Morocco
<i>L. tomentosus</i>	133 ⁴	Wild	Turkey

M: Macroserma. m: microsperma. Samples: ¹ The Tetir land-race is also known as Rosario. ² From the Germplasm Bank of the International Centre for Agricultural Research in Dry Areas (ICARDA), Aleppo, Syria. ³ From the Spanish Germplasm Bank, Alcalá de Henares, Madrid, Spain. ⁴ Kindly provided by Prof. G. Ladizinsky.

employing the primers SPA-F and SPA-R described by Polanco and Pérez de la Vega (1994) and Fernández *et al.* (2000). For the amplification of the fragment including the C and D subrepeats, the primers IGS-CD-F (TTCCTAGGCCTTTATGCT) and IGS-CD-R (TTTTAGCCCAATTTTATAGG) were used. The NTSs of the 5S rDNA were PCR amplified using the 20-mer RRN5-A and RRN5-B primers described by Ko *et al.* (1994). PCR products were cloned into the pGEM-T plasmid (Promega) following the manufacturer's instructions. Five nanograms of DNA were amplified in a final volume of 50 µl with 0.5 µM of each primer in a buffer containing 10 mM TrisHCl, pH 8, 50 mM KCl, 1.5 mM MgCl₂ and 2 U of *Taq* DNA polymerase (Promega Madison, USA). The PCR conditions were as follows: denaturation at 94°C for 1 min and 30 cycles at 94°C for 10 s, 50°C for 20 s, and 72°C for 60 s. DNA sequencing was performed using the dideoxynucleotide chain termination method, employing universal primers, the Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham Pharmacia Biotech, NJ), and automatic sequencing (with ALFTM Manager v.2.6 software; Amersham Pharmacia

Biotech, NJ). DNA restriction reactions were performed following the manufacturer's instructions. After PCR, the IGS amplification product from the 12 lentil accessions was separately digested with each of the following ten enzymes: *AccI*, *DdeI*, *EcoRV*, *HaeIII*, *HindIII*, *PstI*, *Sau3A*, *SphI*, *TaqI*, *KpnI*. IGS PCR and restriction products were electrophoresed in 1.5 or 2.0 w v⁻¹ agarose (Seakem GTG, BioProducts FMC) gels for 24 h. For variability analysis, DNA was extracted from at least five seeds per sample; for sequencing, DNA was extracted from a single seed per species.

Results

A first analysis of the PCR amplification products obtained with the SPA-F and SPA-R primers (electrophoresis in 1.5 wv⁻¹ agarose gels for 24 h) showed no appreciable IGS length variability (approximately 3200 bp) among the cultivated lentil accessions (Table 1). However, some differences were seen between these and the wild *Lens* materials (Fig. 1). No differences among accession digestion patterns

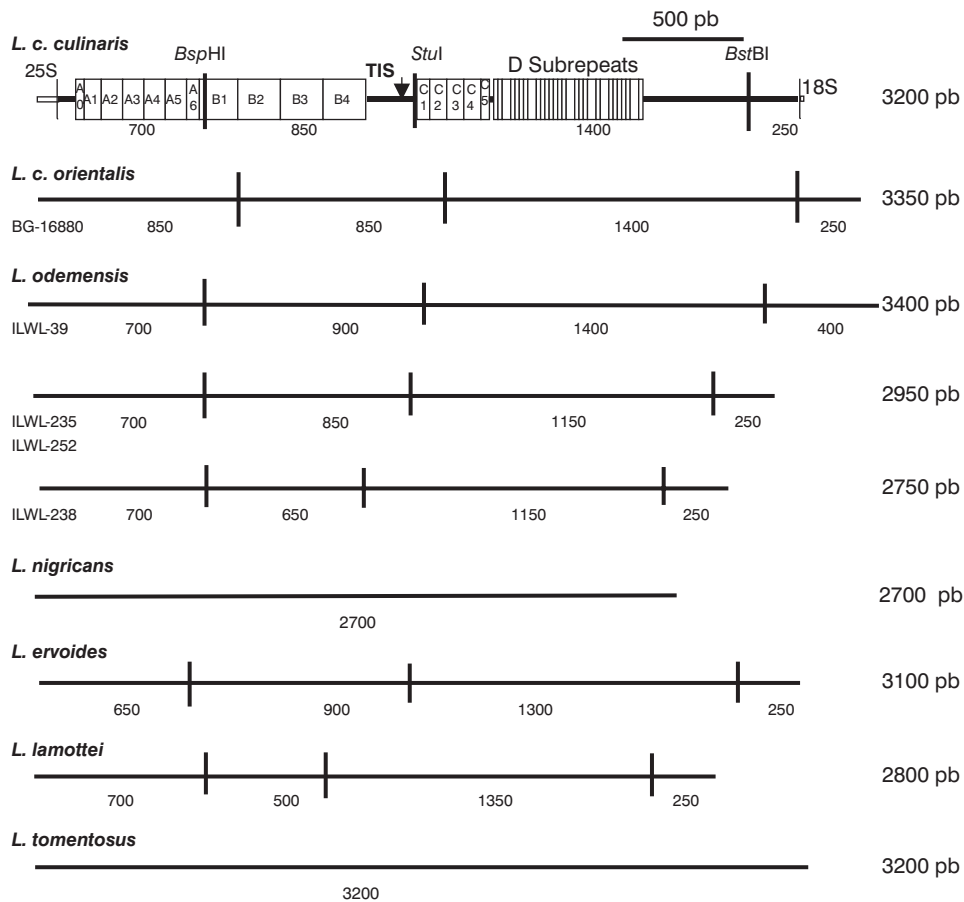


Figure 1. Size and restriction pattern of the PCR products from different *Lens* species amplified with the SPA-F and SPA-R primers. Figures below the different fragments indicate the approximate size in bp. For *L. culinaris* ssp. *culinaris* cv. Verdina, the different subrepeats and the transcription initiation site are indicated. The accessions showing each pattern are indicated below each sequence on the left. *Lens culinaris* ssp. *orientalis* accession ILWL-7 showed the same pattern as cultivated lentil.

were observed with any of the restriction enzymes for the cultivated materials listed in Table 1.

Since most IGS length polymorphisms have been attributed to different numbers of subrepeats in other plant species, three additional restriction enzymes were selected with a single target motif located between the different subrepeat families within the IGS sequence. This was to allow the detection of any differences in the number of short subrepeats within a subrepeat family (such as C and D) that were difficult to resolve in the complete IGS sequence. The *Bsp*HI restriction site was located within the first B subrepeat at 583 bp from the 5' IGS end. The *Stu*I site was located just before the C subrepeats and 43 bp downstream from the

transcription initiation site (TIS) in a central position in the PCR amplified sequence. The *Bst*BI site was located after the D subrepeats, 217 bp before the IGS 3' end. Figure 1 shows the PCR amplification products of *Lens culinaris* ssp. *culinaris* and its wild relatives after digestion with these three enzymes. The IGSs of *L. nigricans* and *L. tomentosus* were not digested by any of these three enzymes. The four expected restriction products were observed in all other species. No appreciable differences were seen between the cultivated lentil accessions. In all the accessions assayed, the fragment lengths were of approximately 250, 700, 850 and 1400 bp. Figure 1 shows the 250 bp fragment at the 3' end of the IGS and the 5' end of the

downstream 18S rDNA. The 700 bp fragment included some nucleotides of the 3' end of the 25S rDNA and the IGS at the end of the A subrepeats. The 850 bp fragment included the IGS B subrepeats and a non-repeated TIS sequence. Finally, the 1400 bp fragment comprised the IGS C and D subrepeats and part of the IGS 3' non-repeated sequence.

When the *L. c. ssp. culinaris* restriction products were electrophoresed in higher resolution 2.0% agarose gels, differences between the accessions were seen, but were limited to slight positional changes. These were evident for the 1400 bp fragment, and in the resolution of the 700 bp fragment into two or three different sized fragments in some accessions. In particular «Smelinuliai» showed two bands of approximately 650 and 725 bp, and «Mala» three bands of approximately 650, 700 and 725 bp. The most likely cause for the presence of multiple fragments is that in these two accessions there are IGSs with different numbers of A subrepeats.

After digestion of the PCR products with *Bsp*HI, *Stu*I and *Bst*BI, two accessions of *L. c. ssp. orientalis* (the wild ancestor of the cultivated lentil) showed different restriction patterns. While ILWL-7 showed the same pattern as the cultivated lentil, accession BG-16880 showed only three fragments of 250, 850 and 1400 bp (Fig. 1). In the latter accession, the A family of subrepeats probably has more copies, and instead of the 700 bp fragment there is a sequence of approximately 850 bp. The four accessions of *L. odemensis* showed three different patterns (Fig. 1). *Lens ervoides* and *L. lamottei* also showed a pattern of four fragments with distributions and sizes as shown in

Fig. 1. On the contrary, *L. nigricans* and *L. tomentosus* were not digested by any of the three enzymes, indicating the lack of restriction sites specific for them.

Agarose gel (2% wv⁻¹) electrophoresis of the amplified NTS of the 5S rDNA revealed several bands ranging from approximately 275 bp to 1000 bp (Table 2). All samples of the cultivated lentil (*L. c. culinaris*) showed a similar pattern of seven bands, although those between 400 and 475 bp showed differences in intensity depending upon the cultivar. Table 2 shows the results for this and other *Lens* species. The 300 bp and 900 bp bands seemed to be common to most species. The exceptions were *L. ervoides*, which lacked the 300 bp band, and *L. lamottei*, which lacked the 900 bp band. However, *L. ervoides* showed the 275 bp and 325 bp bands, which were not observed in other species (Table 2). The size classes of the *L. c. culinaris* NTS were differentially distributed between the two *loci*, and are therefore useful for identifying chromosome pairs 2 and 6.

Primers IGS-CD-F and IGS-CD-R were designed to amplify the IGS region corresponding to the 1400 bp segment which showed slight differences in mobility among the different cultivars (as mentioned above). IGS-CD-F annealed from 39 bp downstream of the TIS to the beginning of the C subrepeats and included the *Stu*I site, while IGS-CD-R annealed 24 bp downstream of the end of the D subrepeats. Thus, the sequence expected to be amplified in the cultivar «Verdina» was of 1004 bp. After amplification and electrophoresis in 1.5% agarose gel for 24 h, two bands were observed for all the cultivated accessions, except for «Tetir». The first band had approximately 700 bp with no observable

Table 2. Comparison of the 5S NTS of *Lens* species obtained in this study and by Ford *et al.* (1997). The fragments of similar size in these studies are indicated in bold. Lightly stained fragments are underlined

Species	Present work ¹		Ford <i>et al.</i> (1997)	
	Number of samples	Band size (bp)	Number of samples	Band size (bp)
<i>L. c. culinaris</i>	12	300 , 350, 400, 425 , 475, <u>675</u> , 900	2	300 , 420 , 670 , 910
<i>L. c. orientalis</i>	BG-16880	300 , 350, 425 , 900	2	300 , 420 , 670, 910
<i>L. c. orientalis</i>	ILWL-7	500, 550, 1000		
<i>L. odemensis</i>	4	300 , 350, 425, 675 , <u>900</u>	3	300 , 670 , 910
<i>L. nigricans</i>	1	300 , 350, 400, 675 , 900	3	300 , 670 , 910
<i>L. ervoides</i>	1	275 , 325 , 350, 400 675 , <u>900</u>	2	290 , 310 , 650, 690
<i>L. lamottei</i>	1	300, 350, 425, 675		
<i>L. tomentosus</i>	1	<u>300</u> , 900		

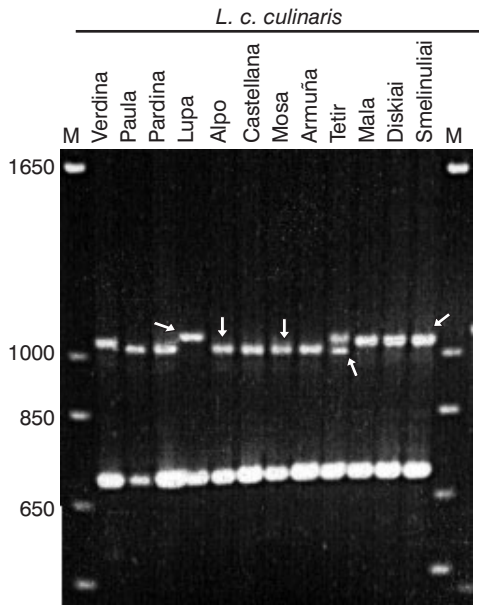


Figure 2. Amplification products obtained with the IGS-CD-F and IGS-CD-R primers. M, size marker standard. Arrows indicate the sequenced bands.

variation between cultivars; the second was the expected band of approximately 1004 bp, although some size differences among cultivars were observed (Fig. 2). The landrace «Tetir» showed three bands: the 700 bp band plus two others of 1002 bp and approximately 1016 bp (Fig. 2). The bands of approximately 1000 bp from the accessions «Alpo», «Mosa», «Smelinuliai», «Tetir» and «Lupa» were sequenced since they represented all the size variants observed. From «Tetir», only the 1002 bp band was sequenced. The exact sizes of these bands were 980 bp for «Mosa», 982 bp for «Alpo», 1002 bp for «Tetir», 1004 bp for «Smelinuliai» (the same size as the corresponding segment in «Verdina»), and 1016 bp for «Lupa». The corresponding EMBL GenBank accession numbers are AM040285 to 40289. The sequences from

«Alpo» and «Mosa» differed with respect to the reference sequence from «Verdina» by a deletion of 21 bp in the C3 subrepeat. This deletion approximately corresponded to two of the 11 bp short repeats which form the C subrepeat family. «Lupa» showed a 12 bp insertion (GGGCATTTTCGT) - a variant of a short repeat - in the C3 subrepeat (Fig. 3). Other differences between the sequences were due to single nucleotide indels or base substitutions. The approximately 700 bp band amplified in «Verdina» was also sequenced, yielding a 704 bp sequence (including both primers) with a 60% A+T content. Dot-Plot analysis revealed no repetitions in this sequence, nor was any significant similarity found with any sequence in the databases or with the lentil IGS, nor did it show any significant open reading frame. The similarity with the C and D set of repeats was 39.5%; it was therefore most likely unrelated to rDNA.

Discussion

The lengths of the PCR-amplified IGS in *Lens* species agree with those reported in RFLP analyses of the whole rDNA repeat (Patil *et al.*, 1995). Further digestions by restriction enzymes of the PCR products showed low intraspecific IGS polymorphism in *L. culinaris* ssp. *culinaris*. This indicates strong conservation of the IGS sequence in this species. Low intraspecific levels of variability have also been described in *Vicia pisiformis*, *Glycine soja*, *G. max* and *Capsicum* spp. (Doyle and Beachy, 1985; Gustafsson and Gustafsson, 1994; Youn-Kyu *et al.*, 1999), and previous RFLP studies on lentil rDNA reported no polymorphism in six cultivars (although polymorphism was detected by RAPD in these cultivars) (Kraic *et al.*, 1995). Greater genetic variation was observed in the wild lentil ancestor than in the cultivated form. The same has been reported for other characters and

C3 subrepeats	
Verdina	TTCGTGGGCATTTTCGTGGCCGCGGTGGGCATTTTCGT-----TGGCTCGGTGGGCATTTTCGTGGGCATT
Smelinuliai	TTCGTGGGCATTTTCGTGGCCGCGGTGGGCATTTTCGT-----TGGCTCGGTGGGCATTTTCGTGGGCATT
Tetir	TTCGTGGGCATTTTCGTGGCCGCGGTGGGCATTTTCGT-----TGGCTCGGTGGGCATTTTCGTGGGCATT
Lupa	TTCGTGGGCATTTTCGTGGCCGCGGTGGGCATTTTCGTGGGCATTTTCGTTGGCTCGGTGGGCATTTTCGTGGGCATT
Alpo	TTCGTGGGCATTTTCGTGGCCGCGGTGGGCATTTTCGT-----GGGCATT
Mosa	TTCGTGGGCATTTTCGTGGCCGCGGTGGGCATTTTCGT-----GGGCATT

Figure 3. Sequence differences between C3 subrepeats of several lentil cultivars responsible for the length polymorphism observed in the larger PCR products obtained with the IGS-CD-F and IGS-CD-R primers.

markers (Ahmad *et al.*, 1996; Ferguson and Robertson, 1996; Ladizinsky, 1999). The selective amplification of the more variable internal IGS segments, which include two families of short repeats sequences, increased the observable polymorphism in *L. c. ssp. culinaris*, improving the usefulness of rDNA as a genetic marker.

The amplification and digestion results obtained with *L. nigricans* and *L. tomentosus* suggest that the IGSs of these two species are different in sequence and number of subrepeats compared to the cultivated species. This agrees with the fact that *L. nigricans* is more distantly related to *L. culinaris* (Van Oss *et al.*, 1997; Ferguson, 2000; Mayer and Bagga, 2002; Sonnante *et al.*, 2003; Durán and Pérez de la Vega, 2004). *Lens tomentosus*, however, seems to be phylogenetically close to *L. culinaris* (Van Oss *et al.*, 1997; Ferguson *et al.*, 2000; Durán and Pérez de la Vega, 2004).

The NTS sizes detected agree, with few exceptions, with those reported by Ford *et al.* (1997), who also used PCR for the amplification of *Lens* species NTSs (Table 2). The 670 bp band of *L. c. orientalis* was not observed in the present *L. orientalis* samples but was seen in *L. c. culinaris*. Thus, the only band not observed in the present study was the 650 bp band of *L. ervoides*. Some of the differences in NTS size observed by Ford *et al.* (1997) and in the present study can be attributed to the use of different accessions since intra-specific NTS variation was observed in both analyses (at least for the wild species). Other discrepancies are most probably due to the lower PCR annealing temperature used in the present work, which allowed for the amplification of additional sequences. The hybridisation of a 5S probe from rye with lentil genomic DNA digested with *Bam*H1, produces a similar band pattern (Patil *et al.*, 1995). Discounting the 120 bp of the 5S rDNA yielded NTSs ranging from 230 to 900 bp in length; the 230 bp band was specific to *L. ervoides*. The data of Patil *et al.* (1995), Ford *et al.* (1997) and the present results indicate that *L. ervoides* has NTS lengths that are not seen in any other *Lens* species. A further source of NTS size polymorphism is the variation in microsatellite and subrepeat numbers (Fernández *et al.*, 2005). This can cause slight changes in size and therefore in the position of the bands in the gel, or generate wide and more diffuse bands by accumulation of several fragments of similar length (Fernández *et al.*, 2005).

In summary, despite the relatively low polymorphism of lentil IGSs, the use of PCR

amplification and endonuclease restriction, and the design of specific primers which amplify particularly variable IGS segments, generated several genetic markers that can be used to locate genes in the NOR-carrying chromosome of this species. NTS size variants might also be of use for genetic mapping when analysing families derived from *L. c. culinaris* x *L. c. orientalis* crosses.

Acknowledgements

This research was supported by the grants AGF99-0192, BMC2001-1297-CO2-02 and AGL2002-0023 from the Spanish Research Directorate General, and a PhD grant to M. Fernández from the University of León.

References

- AHMAD M., MCNEIL D.L., FAUTRIER A.G., ARMSTRONG K.F., PATERSON A.M., 1996. Genetic relationships in *Lens* species and parentage determination of their interspecific hybrids using RAPD markers. *Theor Appl Genet* 92, 1091-1098.
- BALYAN H.S., HOUBEN A., AHNE, R., 2002. Karyotype analysis and physical mapping of 18S-5.8S-25S and 5S ribosomal RNA loci in species of genus *Lens* Miller (*Fabaceae*). *Caryologia* 55, 121-128.
- DELLAPORTA S.L., WOOD J., HICKS J.B., 1983. A plant DNA miniprep: Version II. *Plant Mol Biol Rep* 1, 19-21.
- DOYLE J.J., BEACHY R.N., 1985. Ribosomal gene variation in soybean (*Glycine*) and its relatives. *Theor Appl Genet* 70, 369-376.
- DURÁN Y., PÉREZ DE LA VEGA M., 2004. Assessment of genetic variation and species relationships in a collection of *Lens* using RAPD and ISSR. *Span J Agric Res* 2, 538-544.
- DURÁN Y., FRATINI R., GARCÍA P., PÉREZ DE LA VEGA M., 2004. An intersubspecific genetic map of *Lens*. *Theor Appl Genet* 108, 1265-1273.
- FERGUSON M., 2000. *Lens* spp: Conserved resources, priorities and future prospects. In: Linking research and marketing opportunities for pulses in the 21st century (R. Knight, ed). Kluwer Academic Publishers. The Netherlands, pp. 613-620.
- FERGUSON M.E., ROBERTSON L.D., 1996. Genetic diversity and taxonomic relationships within the genus *Lens* as revealed by allozyme polymorphism. *Euphytica* 91, 163-172.
- FERGUSON M.E., MAXTED N., VAN SLAGEREN M., ROBERTSON L.D., 2000. A re-assessment of the

- taxonomy of *Lens* Mill. (*Leguminosae*, *Papilionoideae*, *Vicieae*). Bot J Linn Soc 133, 41-59.
- FERNÁNDEZ M., POLANCO C., RUIZ M.L., PÉREZ DE LA VEGA M., 2000. A comparative study of the structure of the rDNA intergenic spacer of *Lens culinaris* Medik., and other legume species. *Genome* 43, 597-603.
- FERNÁNDEZ M., RUIZ M.L., LINARES C., FOMINAYA A., PÉREZ DE LA VEGA M., 2005. The 5S rDNA genome regions of *Lens* species. *Genome* (in press).
- FORD R., PANG E.C.K., TAYLOR P.W.J., 1997. Diversity analysis and species identification in *Lens* using PCR generated markers. *Euphytica* 96, 247-255.
- GUSTAFSSON L., GUSTAFSSON P., 1994. Low genetic variation in Swedish populations of the rare species *Vicia pisiformis* (*Fabaceae*) revealed with RFLP (rDNA) and RAPD. *Plant Syst Evol* 189, 133-148.
- HAMWIEH A., UDUPA S.M., CHOUMANE W., SARKER A., DREYER F., JUNG C., BAUM M., 2005. A genetic linkage map of *Lens* sp. based on microsatellite and AFLP markers and the localization of fusarium vascular wilt resistance. *Theor Appl Genet* 110, 669-677.
- KO H.L., HENRY R.J., GRAHAM G.C., FOX G.P., CHADBONE D.A., HAAK, I.C., 1994. Identification of cereals using the polymerase chain reaction. *J Cereal Sci* 19, 101-106.
- KRAIC J., GREGOVÁ E., BENKOVÁ M., ZÁK I., 1995. Evaluation of protein and DNA polymorphism in lentil (*Lens culinaris* L.) for genotypes and cultivars distinguishing. *Rostlinna Výroba* 41, 181-184.
- LADIZINSKY G., 1999. Identification of the lentil's wild genetic stock. *Genet Resour Crop Evol* 46, 115-118.
- MAYER M.S., BAGGA S.K., 2002. The phylogeny of *Lens* (*Leguminosae*): new insight from ITS sequence analysis. *Plant Syst Evol* 232, 145-154.
- NICKRENT D.L., PATRICK J.A., 1998. The nuclear ribosomal DNA intergenic spacers of wild and cultivated soybean have low variation and cryptic subrepeats. *Genome* 41, 183-191.
- PATIL P.B., VRINTEN P.L., SCOLES G. J., SLINKARD A.E., 1995. Variation in the ribosomal RNA units of the genera *Lens* and *Cicer*. *Euphytica* 83, 33-42.
- PENTEADO M.I. DE O., GARCÍA P., PÉREZ DE LA VEGA M., 1996. Genetic variability and mating system in three species of the genus *Centrosema*. *J Hered* 87, 124-130.
- POLANCO C., PÉREZ DE LA VEGA M., 1994. The structure of the rDNA intergenic spacer of *Avena sativa* L.: A comparative study. *Plant Mol Biol* 25, 751-756.
- POLANCO C., PÉREZ DE LA VEGA M., 1995. Length polymorphism in the ribosomal DNA intergenic spacer of rye and slender wild oats. *J Hered* 86, 402-407.
- POLANCO C., PÉREZ DE LA VEGA M., 1997. Intergenic ribosomal spacer variability in hexaploid oat cultivars and landraces. *Heredity* 78, 115-123.
- ROGERS S.O., BENDICH A.J., 1987. Ribosomal RNA genes in plants: Variability in copy number and the intergenic spacer. *Plant Mol Biol* 9, 509-520.
- RUBEENA, FORD R., TAYLOR P.W.J., 2003. Construction of an intraspecific linkage map of lentil (*Lens culinaris* ssp. *culinaris*). *Theor Appl Genet* 107, 910-916.
- SASTRI D.C., HILU K., APPELS R., LADUDAH E.S., PLAYFORD J., BAUM B.R., 1992. An overview of evolution in plant 5S DNA. *Plant Syst Evol* 183, 169-181.
- SONANTE G., GALAZO I., PIGNONE D., 2003. ITS sequence analysis and phylogenetic inference in the genus *Lens* Mill. *Ann Bot* 91 49-54.
- VAN OSS H., ARON Y., LADIZINSKY G., 1997. Chloroplast DNA variation and evolution in the genus *Lens* Mill. *Theor Appl Genet* 94, 452-457.
- YOUN KYU P., KIM B.D., KIM B.S., ARMSTRONG K.C., KIM N.S., 1999. Karyotyping of the chromosomes and physical mapping of the 5S rRNA and 18S-26S gene families in five different species in *Capsicum*. *Genes Genet Syst* 74, 1149-157.