

RESEARCH NOTE

Molecular characterization of *Lotus corniculatus* cultivars using transferable microsatellite markers

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Abstract

D. Alem, R. Narancio, P. Díaz Dellavalle, M. Rebuffo, R. Zarza, and M. Dalla Rizza. 2011. Molecular characterization of cultivars of *Lotus corniculatus* using transferable microsatellite markers. Cien. Inv. Agr. 38(3): 453-461. *Lotus corniculatus* L. is the most important agricultural species in the genus *Lotus* and is the most widely distributed *Lotus* species worldwide. *L. corniculatus* genotypes form complex groups that are difficult to recognize both morphologically and biochemically. Given the extensive and expensive process of isolating Simple Sequence Repeats (SSR, also called microsatellites), the possibility of using microsatellites already identified in related species is highly attractive. The aim of this work was the identification and validation of transferable microsatellite markers in *L. corniculatus*, and using those markers to study the genetic variability among four cultivars. Each cultivar of *L. corniculatus* was represented by 15 genotypes. Ten microsatellite markers were evaluated, and from those, four were selected based on their discriminative values observed among cultivars. We detected 29 alleles for the four markers, and there was an average of 7.25 alleles per locus. The marker TM0197 had the fewest number of alleles (5) and TM0083 had the highest number of alleles (10). The polymorphic information content (PIC) for the selected markers varied from 0.19 to 0.35, and the markers were therefore classified as highly informative. Based on the markers, we found high variability between individuals of the same cultivar. The use of transferable microsatellite markers could be useful to differentiate individuals at a relatively low cost, showing a great potential for use in breeding programs.

Key words: Genetic variability, *Lotus japonicus*, SSR.

Introduction

Lotus corniculatus L. is the most important and widely distributed crop from the *Lotus* genus (Díaz *et al.*, 2005). *L. corniculatus* is a warm-season cross-pollinating legume forage species that is

perennial and tetraploid ($2n=4x=24$) (though there are some reports of diploid plants) (Grant and Small, 1996). Although the species originated in temperate areas of Europe and north Africa, it has spread throughout Europe, Asia, Africa, North and South America, Australia and New Zealand as a result of cultivation for use in pastures, ensilage and hay production (Duke, 1981; Beuselinck and Grant, 1995; Ayala and Carámbula, 2009).

L. corniculatus has several important traits that make it attractive as an agricultural species. For example, it is adaptable to relatively extreme environments, such as acidic and poorly drained soils (Duke, 1981; Beuselinck and Grant, 1995; Ayala and Carámbula, 2009). Additionally, it has a high nutritive value for grazing, and does not produce bloat in cattle (Carámbula, 1977; Ramírez-Restrepo and Barry, 2005; Ayala and Carámbula, 2009).

In Uruguay, the most frequently used cultivars of *L. corniculatus* are as follows: (1) San Gabriel, which was originated from a population from Rio Grande do Sul (Brazil); (2) Estanzuela Ganador, which is a selection of FAO lines originally introduced from Argentina; (3) INIA Draco, which was derived from crosses of Estanzuela Ganador and a population from eastern Uruguay (Colonia department, Uruguay) after two rounds of recurrent selection; and (4) Rigel, which was obtained after two more rounds of selection from INIA Draco.

Microsatellites are considered to be a useful genetic marker system because they are abundant sequences, highly polymorphic, locus specific, multiallelic, codominant and robust (Weber, 1990; Morgante and Olivier, 1993). The major disadvantage of the use of this marker is its high development cost (Zane *et al.*, 2002; Squirrel *et al.*, 2003; Saha *et al.*, 2006). To overcome this problem, it is sometimes possible to transfer knowledge of microsatellite markers already developed in well-known species to less studied species. Given the vast information on microsatellites available in the literature and in genetic databases, markers developed in species related to those of interest can be searched and tested. This makes it possible to circumvent marker development, which makes the use of microsatellite easier and cheaper (Peakall *et al.*, 1998; Dalla Rizza *et al.*, 2005; Real *et al.*, 2007).

The cross amplification of microsatellite markers among species can improve as the

phylogenetic distance decreases (Peakall *et al.*, 1998). For example, Peakall *et al.* (1998) reported the transferable use of SSR markers from soy (*Glycine max*) for other members of the Fabaceae family. The amplification observed among different genera of Fabaceae was between 3 and 13%, while there was 65% amplification among species from the genus *Glycine* (Peakall *et al.*, 1998). Similar results were obtained using transferable microsatellite markers in tall fescue (*Festuca arundinacea* Schreb.), wheat (*Triticum aestivum* L.), ryegrass (*Lolium multiflorum* Lam.) and rice (*Oriza sativa* L.) (Saha *et al.*, 2006).

Generally, *L. corniculatus* genotypes tend to form highly related complexes which are difficult to define, both biochemically and morphologically (Steiner and Poklemba, 1994). Although there are few examples of the use of molecular markers in *L. corniculatus*, those that are available report high genetic variability. *L. corniculatus* populations have been evaluated using isoenzyme (*e.g.*, cyanogen) reactions (Blaise *et al.*, 1991), globulin electrophoretic profiles (Steiner and Poklemba, 1994), RAPD markers to assess relatedness among *Lotus* species (Campos *et al.*, 1994; Steiner and García de los Santos, 2001; García de los Santos and Steiner, 2003), inheritance studies with RAPD and RFLP markers (Fjellstrom *et al.*, 2001) and AFLP and microsatellite markers in natural populations (Savo Sardaro *et al.*, 2008). High intra-population variability and even higher inter-population variability was observed within and among natural populations in Italy using potentially transferable microsatellite markers that were useful for accession discrimination (Savo Sardaro *et al.*, 2008).

The purpose of this study was to develop and modify molecular tools for the characterization of *L. corniculatus* cultivars using transferable microsatellite markers from *L. japonicus*, and to assess the intra- and inter-cultivar variability among four cultivars.

Materials and methods

Plant material

Fifteen individuals from each cultivar (San Gabriel, Draco, Ganador and Rigel) were used in this study. *Lotus japonicus* individuals were used as positive reference controls (three genotypes each of Miyakojima MG-20 and Gifu B-129). Genomic DNA was extracted according to the protocol of Doyle and Doyle (1987).

Ten primers previously developed in *L. japonicus* (Sato *et al.*, 2001) were used for this assay. Annealing temperatures were adjusted with gradient PCR in a Palm cycler CG1-96 (Corbett Research, Australia). Four primers were selected from the 10 evaluated based on their repeatability, polymorphic content and sharpness of the bands obtained in polyacrilamide gels (Table 1). PCR reactions were performed under the following conditions: 20 µL of total volumen reaction, 40-80 ng of genomic DNA, reaction buffer -75 mM Tris-HCl, 50 mM of KCl, (NH₄)₂SO₄ 20 mM-, 1.0 mM of MgCl₂, 100 µM of dNTPs, 1.0 µM of each primer, and 1.0 U Taq polymerase (Biotools). PCR was programmed for 4 min of initial melting at 94 °C, followed by 30 cycles each at 94 °C for 30 s, annealing temperatures between 53 °C and 60 °C (depending on the primer) (Table 1) for 20 s, 72 °C for 20 s, and a final extension step at 72 °C for 7 min. Amplification products were run on denaturing 8% polyacrilamide gels (Urea 7.0 M, TBE buffer 1X, Bio-rad Sequi-Gen GT Nucleic Acid Electrophoresis Cell, USA) for approximately 90 min. The bands were visualized using the silver stain method (Tixier *et al.*, 1997).

Analysis

Sets of primers where there was no amplification, or when amplification was weak or illegible, were discarded. The staining method used was not quantitative, and therefore the allelic dose inferred by the banding intensity was not considered. The

presence of null alleles is another source of error when trying to determine allelic dosage. A null allele results from a mutation in the region of DNA where the primers hybridize, leading to a consequent lack of the corresponding band (Luo *et al.*, 2000); null alleles are more common in plants with a polysomal inheritance than in diploids (Luo *et al.*, 2000). There are several precedents in polyploid species where microsatellites are considered as dominant markers to overcome these limitations (Mengoni *et al.*, 2001; George *et al.*, 2006). This approach was adopted for the analysis considering the microsatellite-transferable elements obtained as dominant markers, and it was assumed that each band corresponds to a locus with two alleles. Band presence (1) or absence (0) was determined visually. Band sizes were determined by comparisons with the reference microsatellite alleles known from *L. japonicus* (Miyakojima MG-20 and Gifu B-129) and 100 bp (molecular weight) markers (Invitrogen, Table 1).

To determine the genetic distances between plants, a binary matrix was constructed and a Jaccard similarity coefficient calculated.

$$J = \frac{b + c}{a + b + c}$$

where:

a = number of bands present in xi and xj

b = number of bands present in xi and absent in xj

c = number of bands present in xj and absent in xi,

where xi and xj are the i-th and j-th individuals.

Cluster analysis was performed using the Neighbor-Joining (NJ) method, which is based on finding neighbors sequentially to minimize the total tree length (Saitou and Nei, 1987). In this study we used the Gascuel (1997) version, which uses the weighted average to calculate the Unweighted Neighbor-Joining (UNJ) (Gascuel, 1997). The genetic similarity matrix was performed using the software DARWIN 5.0 (Darwin software <http://darwin.cirad.fr/darwin>). The dendrogram is a branching description of the similarity or genetic distance between individuals. We also carried out

Table 1. Microsatellites markers evaluated in this work.

Marker name	SSR motif	Forward primer (5' to 3')	Band size in <i>Lotus japonicus</i>		Annealing temperatures
	Number of repeats	Reverse primer (5' to 3')	MG-20	B-129	(°C)
TM0197 ¹	(AAC) ₈	CATTCACATGTCATTGGACC TTAGATCGAGTTCGAGGGAG	121	119	56
TM0304	(CT) ₁₈	CGTTCTCTGGGAACCTTATGAC ATTTGTTGTGTGCACTCAGG	127	115	NA
TM0212 ¹	(CT) ₁₃	CTTCCTTCTCACCACCTTAG TAAACGAAAATGAAGCAGAG	153	159	57
TM0203 ¹	(AT) ₁₂	CTGTTTTGACGAATCGATAG TACGAAGGTTTCTTCATTCC	151	179	56
TM0023 ²	(AT) ₁₂	CATAAGCACACAATTTCATAG GTTGTGGTTCAAAGTTAGGG	185	179	53
BM1397 ³	(AT) ₈₊₁₁	ATTCAGTAACAGAAATCCCG AAAGACTCGAGACCAATACC	180	200	53
TM0083 ¹	(CT) ₁₉	CTCTCTCTCAGGGCCAACAC TGATTCTTGAAGATTTAAAAGATG	161	148	59
TM1335	(CT) ₉	ATTAGACCACCACGGGCTGC CCAAAGTTACTCAATGTAGCG	153	155	NR
TM1150	(CT) ₉	ATCCAGAACTCCATCCAAG TCGTTTTCAAAGTGCATCG	156	162	NR
TM0360	(AT) ₁₀₊₁₇	CATGCTTGGAATGTTACTGC GTGTATTGGATGTTATGGGG	113	95	NA

¹primers used in this work.

²Monomorphic.

³Band patterns were not legible.

NR, not repeatable. NA, no amplification detected. MG-20 and B-129, *Lotus japonicus* lines.

Adapted from <http://www.kazusa.or.jp/lotus/>

a principal coordinates analysis (PCA) using the similarity matrix. PCA is a multivariate technique used to find the dominant patterns in a multivariate dataset.

The genetic similarity matrix was used in an analysis of molecular variance (AMOVA) to estimate the variability within and between cultivars. AMOVA partitions, and therefore estimates, the total variance components (Excoffier *et al.*, 1992). This analysis was performed using GenAlEx

6.2 (Australian National University, Canberra, Australia).

The polymorphism information content (PIC) was calculated as: $PIC = 1 - \sum P_i$, where P_i is the band frequency of the i -th allele. For dominant marker values, $PIC = 1 - p^2 - q^2$, where p = band frequency and q = no-band frequency (Ghislain *et al.*, 1999). The PIC value represents the possible combinations between the different alleles and is an index of the informative capacity of the microsatellite marker.

Results and discussion

Eight of the 10 tested primers amplified, demonstrating the transferability of markers to *L. corniculatus* (Table 1). Four markers, TM0083, TM0212, TM0197 and TM0203, were selected according to the above-mentioned criteria. We obtained amplification products with the TM0083, TM0197 and TM0212 primers for all of the individual plants tested. The TM0203 primer did express alleles for plants 10 and 3 from Estanzuela Ganador and INIA Draco, respectively. The other six primers showed weak patterns, which made the analysis difficult. This was not surprising, since primers from different species do not always show strong patterns. Accounting for the genetic background of the studied cultivars, the high variability of the microsatellite sequences made it possible to find differences between plants with a low genetic diversity (see also Peakall *et al.*, 1998). Mutations could cause the emergence of null alleles or the fading of microsatellite sequences, which leads to bands that do not amplify.

Twenty-nine alleles were found from the visual analysis of the polyacrilamide gels (Figure 1). The maximum number of alleles per plant considered was 4. The average number of allele per locus was 7.25. TM0197 had the fewest alleles (5) while TM0083 had the highest number of alleles (10) (Table 2). Savo Sardaro *et al.* (2008) analyzed 88 plants from 11 *L. corniculatus* in Italy with microsatellite markers (the TM0083, TM0212, and TM0203 markers were also used here), and reported a higher

number of alleles (68) and also a higher average of alleles per locus (13.6) than found here. These different results could have been because Savo Sardaro *et al.* studied natural populations from different locations, while cultivars with a lower expected variability were studied here. In addition, the number of plants analyzed in the current study was lower than in the study by Savo Sardaro *et al.* (60 vs. 88), and therefore the probability of finding more alleles should be also lower.

Table 2. Number of alleles and PICs value obtained for the markers used. Sixty plants per primer were analyzed (15 plants per cultivar).

Marker	Number of alleles	PIC
TM0212	6	0.19
TM0083	10	0.28
TM0203	8	0.35
TM0197	5	0.23
Average	7.25	0.26

The polymorphic information content (PIC) results are listed in Table 2. The PIC, which is equivalent to Nei's genetic diversity index, is frequently used to evaluate the discriminatory power of a microsatellite locus (Roussel *et al.*, 2004; George *et al.*, 2006). This is based on the expected heterozygosity and the allele frequencies (Botstein and White, 1980). There is more than one equation that defines the PIC, and the value ranges between 0 and 1. Because the microsatellite markers were scored as dominant, the maximum PIC value should be between 0.01 and 0.5. Higher PIC values indicate

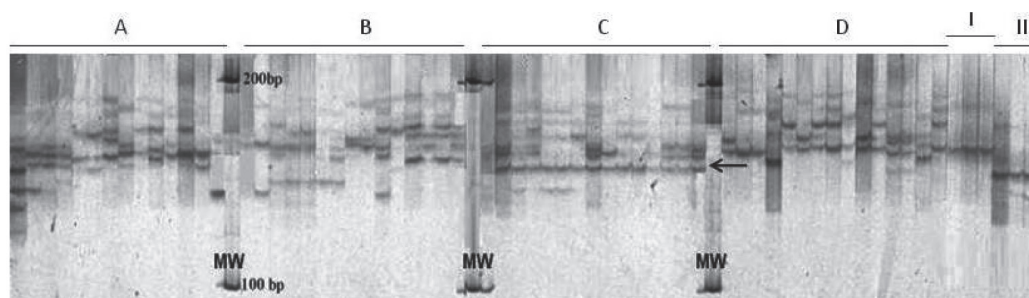


Figure 1. Polyacrilamide denaturing gel (8%) obtained with the TM0083 primer. The arrow points to a band primarily present in the San Gabriel cultivar. A: INIA Draco; B: Estanzuela Ganador; C: San Gabriel; D: Rigel. I: Miyakojima MG-20 and II: Gifu B-12. MW: Molecular weight marker.

more informative content (Ghislain *et al.*, 1999). The average PIC value for all the evaluated loci was 0.26. The maximum PIC value was 0.35 (TM0203) and the lowest was 0.19 (TM0212).

The AMOVA evaluated the distribution of genetic variance both within and among cultivars. Eighty-two percent of the variance was explained by the within-cultivar genetic differences, while 18 % was explained by the among-cultivar differences. The high within-cultivar variability found in this study is consistent with others studies of allogamous plants (Rajasekar S. *et al.*, 2005). This could make the cultivar differentiation difficult with the primers used. Thus, to find informative markers, it is necessary to test a higher number of primers in order to differentiate cultivars.

Line grouping

A genetic distance matrix was calculated using the Jaccard's coefficient and a grouping analysis was performed. There was a tendency of associa-

tion between individuals from the San Gabriel cultivar, but there was no clear association pattern for individuals of the other cultivars (Figure 2). In fact, as mentioned above, San Gabriel has a genetic background from Rio Grande do Sul (Brasil), which is geographically far from the other cultivars analyzed. The explanation for the lack of differentiation observed among the Estanzuela Ganador, INIA Draco and Rigel cultivars could be that the INIA Draco and Rigel lines were both obtained from the Estanzuela Ganador cultivar. Similar results were obtained from the PCA, in which there was no discernable pattern among individuals (results not shown). There is generally a high rate of gene flow and germplasm exchange among allogamous plants, which could explain these results. The close origin of the cultivars supports this tendency. Although none of the cultivars could be differentiated, we found some band association in the San Gabriel cultivar.

To conclude, the molecular markers used in this study confirmed the high allelic variability within and among the *L. corniculatus* cultivars

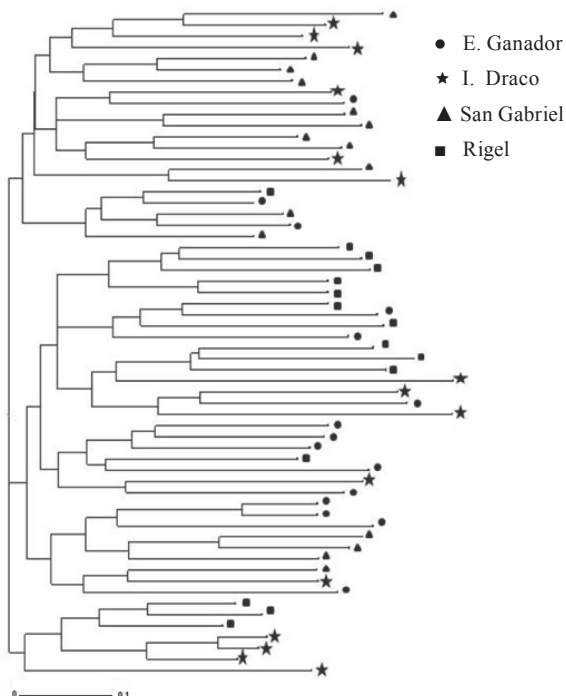


Figure 2. Dendrogram for the 60 *Lotus corniculatus* genotypes obtained from the Unweighted Neighbour joining (UNJ) analysis.

evaluated. Further, a higher number of markers would likely permit the discrimination among the four cultivars. Regardless, the markers used in this study could be useful for differentiating less closely related populations of *L. corniculatus*, or for distinguishing *L. corniculatus* from other related *Lotus* species.

The use of transferable microsatellite markers has been shown to be a promising tool for biodiversity evaluation and for breeding *L. corniculatus* germplasm. Transferable microsatellites were reproducible and fast, with a relatively low cost.

In addition, this study shows that the transfer of microsatellite markers from molecularly well-known species to agronomically important species with little sequence information is feasible.

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Resumen

D. Alem, R. Narancio, P. Díaz Dellavalle, M. Rebuffo, R. Zarza y M. Dalla Rizza. 2011. Caracterización molecular de cultivares de *Lotus corniculatus* mediante el empleo de marcadores microsatélites transferibles. Cien. Inv. Agr. 38(3): 453-461. *Lotus corniculatus* L. es la especie de mayor importancia agronómica en el género *Lotus* y la de mayor distribución a nivel mundial. Los genotipos de *L. corniculatus* forman grupos genéticos complejos y difíciles de reconocer tanto morfológica como bioquímicamente. Dado el proceso largo y costoso de aislar secuencias simples repetidas (SSR, microsatélites), resulta altamente atractiva la posibilidad de utilizar microsatélites ya identificados en una especie relacionada. El objetivo del trabajo fue la identificación y validación de marcadores microsatélites transferibles y la caracterización molecular de cuatro cultivares. Cada cultivar de *L. corniculatus* fue representado por 15 genotipos. Se evaluaron 10 marcadores microsatélites provenientes de *L. japonicus* L. Se comprobó la transferibilidad de marcadores de *L. japonicus* en *L. corniculatus*. Se detectaron un total de 29 alelos para los 4 microsatélites transferibles seleccionados, mientras que el promedio de alelos por locus fue de 7,25. El marcador TM0197 fue el que menos alelos presentó (5 alelos) y el TM0083 el que más presentó (10 alelos). El contenido de información polimórfica (PIC) para los marcadores seleccionados varió entre 0,19 y 0,35 calificando en el rango de altamente informativos. Se comprobó una alta variabilidad entre individuos del mismo cultivar. El empleo de marcadores microsatélites puede ser de gran utilidad para diferenciar individuos a un bajo costo, presentando un gran potencial para su uso en mejoramiento.

Palabras clave: *Lotus corniculatus*, SSR, variabilidad genética.

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