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Research Article

Genetic diversity and DNA content of three South American and three Eurasiatic *Trifolium* species

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Abstract

Six species of *Trifolium* (*T. polymorphum* Poir., *T. riograndense* Burkart, *T. argentinense* Speg., *T. medium* L., *T. pratense* L. and *T. repens* L.) were analyzed using inter-simple sequence repeats (ISSR) markers. Six selected primers generated 186 polymerase chain reaction (PCR) products exploring 112 loci in 34 genotypes analyzed with molecular sizes ranging from 200 to 1300 bp. These primers were able to discriminate among and within species, with the PCR products being on average 41.6% species-specific and 59.9% polymorphic at the within species level. Nuclear DNA content was determined by flow cytometry and revealed variation among species. The 1Cx genome size values were calculated and were found to range from 0.46 pg (*T. pratense*) to 0.96 pg (*T. polymorphum*). Genome size values of South American species were higher than those of Eurasiatic origin. The analyses of the molecular data grouped the six species in agreement with their geographical origin and clearly differentiate *T. polymorphum* from *T. argentinense*. The Eurasiatic group showed the highest average of species-specific bands (45.3%) and the South American group exhibited the highest amount of total bands (59.7). The highest level of intra-species polymorphisms was detected in *T. argentinense* (92.9%), followed by *T. medium* (89.5%).

Key words: DNA content, genetic variability, inter-SSR, *Trifolium* spp.

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Introduction

The genus *Trifolium* consists of about 230 species (Gillet *et al.*, 2001) with a wide distribution and adaptation to different agro-ecological regions (Izaguirre, 1995; Ellison *et al.*, 2006; Vižintin *et al.*, 2006), although, only a few of them are commercially used as forage crops.

There are three *Trifolium* species native to the Campos region of southern Brazil, western Argentina and Uruguay which are *T. argentinense, T. polymorphum,* and *T. riograndense*. These native *Trifolium* species contribute to the high quality production of beef, dairy products, leather and wool with low levels of inputs to the system. The difficulty of producing interspecific hybrids even among closely related *Trifolium* taxa has spurred interest in the evaluation of the agronomic potential of locally utilized and currently uncultivated species (Ellison *et al.*, 2006). In Uruguay, germplasm collections of native *Trifolium* species were made in 1997, 2003 and 2005. These native species are long-lived perennials in the Campos region environment, with *T. argentinense* and *T. polymorphum* being unique species in the genus *Trifolium* because they bear both typical aerial flowers in clusters and also small subter-

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ranean basal flowers arising from the axis of the stolons and leaves (Izaguirre, 1995; Real *et al.*, 2007). These two species with this particular characteristic belong to the Campos region produce a very unusual patch-like growth form in the open field. Although *T. argentinense* is very similar to *T. polymorphum* the former is mostly glabrous, has yellow to cream flowers and is usually found in waterlogged areas (Izaguirre and Beyhaut, 1998). Zohary and Heller (1984) considered *T. argentinense* and *T. polymorphum* as synonyms. The other species, *T. riograndense* is native to southern Brazil (Schifino-Wittmann, 1985; Schifino-Wittmann, 2000) and northern Uruguay (Izaguirre and Beyhaut, 1998). All these Campos region's native populations have evolved into adapted ecotypes through long exposure to environmental stresses, grazing and by competing with the native C_3 and C_4 grasses which have dominated the Campos vegetation over a long period of time.

Eurasiatic *Trifolium* species include *T. medium*, *T. pratense* and *T. repens*, with the latter two being the main perennial *Trifolium* species in the world. However, these two species lack persistence in the temperate to subtropical environment found in the Campos region and have to rely on their reseeding capacity to perenniate. A long-lived perennial is the rhizomatous *T. medium*, and this species is currently being studied for its ability to adapt to conditions in Uruguay.

The majority of *Trifolium* species are diploid; a few are tetraploid while higher ploidy levels are rare. The most common basic chromosome number (80% of species) is x = 8 (Vižintin *et al.*, 2006). Recently, Ellison *et al.* (2006) and Vižintin *et al.* (2006) reported detailed and comprehensive studies of the molecular phylogenetic framework for the genus *Trifolium*. There are different molecular methods for germplasm evaluation using simple and effective markers based on the polymerase chain reaction (PCR) (Barcaccia *et al.*, 1999; Fernández *et al.*, 2002; Barreto Dias *et al.*, 2004; Real *et al.*, 2004). For germplasm variability evaluation, identification of cultivars and genetic distance estimation, dominant markers such as amplified fragment length polymorphisms (AFLPs), random amplified polymorphic DNAs (RAPDs) and inter-simple sequence repeats (ISSR) have been widely utilized during the past 15 years (for reviews, see Antonius-Klemola, 1999 and Ulloa *et al.*, 2003). The AFLP technique is a powerful and reliable tool capable of probing a large number of genomic loci per experiment and discriminating genetic differences, but is a laborious and somewhat expensive methodology based on the selective amplification of restriction fragments and requires the purchase of a kit and polyacrylamide gels for separating the products. In *Trifolium*, primarily RAPDs (dominant markers) have been utilized for the evaluation of genetic diversity in *T. pratense* (Kongkiatngam *et al.*, 1996; Campos and Ortega, 2001; Gustine *et al.*, 2002; Greene *et al.*, 2004; Sica *et al.*, 2005). However, the ISSR-PCR method (Wolfe and Liston, 1998) using primers based on di-tri-tetra-penta-nucleotide repeats without the requirement for prior knowledge of the genome sequence seems particularly suitable for germplasm comparison. The advantage of the use of ISSR as a dominant marker compared to RAPD has been the repeatability of ISSR methodology reported for several species (Jain *et al.*, 1999; Fernández *et al.*, 2002; Sica *et al.*, 2005).

In the study described in this paper were used 14 ISSR markers to study the *inter*- and *intra*-species variability in six *Trifolium* species (three of Eurasiatic origin and three of South American origin). We also estimated the nuclear DNA content of these species using flow cytometry. Using these simple and reliable markers it was possible to differentiate between the two previously ambiguous closely related species *T. argentinense* and *T. polymorphum.*

Materials and Methods

Plant material

Two accessions of *Trifolium polymorphum* Poir. were collected at the North Regional of the National Institute of Agricultural Research (Instituto Nacional de Investigación Agropecuaria – INIA) located in Tacuarembó, Uruguay (31°44'20" S, 55°8'46" W) while *Trifolium argentinense* Speg was collected in fields near the Uruguayan towns of Tacuarembó (31°46'35" S, 55°40'19" W) and Florida (33°30'13" S, 55°49'26" W). We grew *Trifolium medium* L. (donated by the genebanks of Gatersleben, Germany and Kew Botanical Garden, United Kingdom) and *Trifolium riograndense* Burkart (INIA 'La Estanzuela' genebank) from seeds, while *Trifolium pratense* L. and *Trifolium repens* L. are naturalized in Uruguay and the accessions utilized in this study were collected at INIA Las Brujas, Uruguay (34°40'19" S, 56°20'24" W). All the species studied were mainly cross-pollinated (Schifino-Wittmann, 1985; Gillet *et al.*, 2001; Real *et al.*, 2007), except *T. argentinense* because its breeding system is not reported in the literature.

For *T. medium, T. polymorphum* and *T. riograndense*, two accessions represented by four genotypes each were assayed. For *T. argentinense* two accessions were studied, one with four genotypes and the other with two genotypes. The *T. pratense* and *T. repens* were represented by one accession with two genotypes each. In total, 34 genotypes were studied from the six species.

Determination of nuclear DNA content by flow cytometry

The DNA measurements were performed according to Doležel and Göhde (1995). Small pieces of plant leaves (60 mg to 100 mg) from the sample and the appropriate standard were mixed in a 96 mm diameter glass petri dish with 0.5 mL ice-cold Otto buffer I (0.1 M citric acid + 0.5% (v/v) Tween 20) and cross-chopped with a sharp razor blade, the buffer containing the chopped plant being fil-

tered through a 45 µm mesh nylon membrane into a 5 mL cytometry tube and kept at room temperature for 10-60 min. Finally, after addition of 0.5 mL Otto II buffer (0.4 M $Na_2HPO_4.12H_2O$), RNAse (50 µg/mL) and propidium iodide (50 µg/mL), plant cell nuclei were immediately analyzed by flow cytometry. The DNA content analysis was carried out in a FACS Vantage flow cytometer (Becton Dickinson, San José, California) using an Innova 300 laser at 488 nm and CELLQuest software (Becton Dickinson). Chicken red blood cells (CRBC) were used to calibrate the flow cytometer to optimize forward and side scatter and fluorescence parameters. Pulse processing for cytometric DNA content estimation was adjusted using DNA QC Particles (Becton Dickinson) to check instrument linearity and doublet discrimination performance following the instructions of the manufacturer, with *Zea mays* cv CE-777 (2C = 5.43 pg) being used as the internal reference standard. Two plants per species were analyzed and three DNA estimations were carried out for each plant (5000 to 10000 nuclei per analysis). Nuclear DNA content was calculated as: (Sample peak mean/Standard peak mean) x 2C DNA content of the standard (in pg). The Cx-values were obtained by dividing the nuclear DNA content by the corresponding ploidy level. For Cx-value calculations, chromosome numbers of *Trifolium* species were taken from Schifino-Wittmann (2000), Vižintin *et al.* (2006) and Mowforth (1986).

Genomic DNA extraction and ISSR amplification

DNA was extracted from freshly harvested young leaflets from actively growing plants in the field or in the greenhouse, using a small-scale sodium dodecyl sulfate (SDS) procedure (Dalla Rizza *et al.*, 2004). The DNA analyses were performed on individual genotypes as well as on *a posteriori* bulked DNA using 20 ng of DNA of each genotype to produce the bulk (Kongkiatngam *et al.*, 1996). Since the bulk was performed using the accession genotypes it was used as an indicator to easily find and confirm individual PCR products. The DNA concentration was assessed both spectrophotometrically at 260 nm and by 0.9% (w/v) agarose gel electrophoresis, quantified with λ DNA and stained with ethidium bromide. Each polymerase chain reaction (PCR) was done in a 20 µL volume containing 80 ng of genomic DNA, 1.25 mM of MgCl2, 75 mM Tris-HCl (pH 8.8), 20 mM (NH4)2SO4, 0.01% (v/v) Tween 20, 200 µM each of dCTP, dGTP, dATP and dTTP, 0.2 mM of primer and 1 unit of T*aq* DNA Polymerase (Fermentas Life Science, CA, USA). The DNA amplification was performed with 14 synthetic ISSR primers (Tsumura *et al.*, 1996; Jain *et al.*, 1999). Based on previously results reported in plant species surveyed using ISSR markers, different types of nucleotide motifs were assayed. The simple-sequence primers used in this study were $(AG)_8T$, $(AG)_8TA$, $(AG)_8CA$, $(AC)_8CT$, $(GT)_8CG$, $(CT)_8GG$, $(GACA)_4$, $(GACAC)_3$, $(TGTC)_4$, $(CAG)_5$, $(GGAGA)_3$, $(TGC)_6$, $(AGT)_6$ and $(CTTCA)_3$ and were obtained from Operon (Cologne, Germany). The PCR conditions were as follows: pre-incubation for 7 min at 94 °C, followed by 45 cycles of 30 s at 94 °C, 45 s at 52 °C, 1.5 min at 72 °C and a final 7 min extension at 72 °C. The annealing temperature was assayed 48 °C for $(GACA)_4$, $(GACAC)_3$ and $(TGTC)_4$ and 60 °C for $(CAG)_5$. The PCR was performed in a Genius thermal cycler (Techne Ltd., Cambridge, UK) and the PCR products were separated on 3% (w/v) agarose gels using 1 x TAE buffer (Maniatis *et al.*, 1982), 100 bp standard markers (GeneRuler, Fermentas) and stained with ethidium bromide.

ISSR marker scoring and data analyses

Band patterns were digitized using the gel image analysis system Gel-Pro imager (MD, USA). Bands were scored directly into a spreadsheet and ISSR markers were scored as presence (1) and absence (0) of a band. Only reproducible well-marked amplified bands were scored. Faint amplified products and irreproducible fragments were not considered for the analysis. Bands with the same mobility were considered as identical fragments, irrespective of band intensity. According to Tsumura *et al.* (1996), ISSR markers are considered to be dominant markers and consequently effectively act as bi-allelic loci (band presence *vs.* absence). To determine genetic distances, the uncentered correlation coefficient was used. Hierarchical cluster analysis was performed using the average method (Everitt *et al.*, 2001). To asses the uncertainty in hierarchical cluster analysis, multiscale bootstrap resampling was done (10000 samples) and an approximately unbiased (AU) p-value was calculated for each cluster (Shimodaira, 2004). The P-value of a cluster indicates how strong the cluster is supported by the data, and lies between 0 and 1 (strongest). The matrix of genetic similarities was represented in a dendrogram together with AU values. Clusters with high AU p-values (0.95) were highlighted. All the analyses were performed using the pvclust package in the statistical software R version 2.5.0 (http://www.r-project.org/, Suzuki, 2005). The polymorphism information content (PIC) was calculated as: $PIC = 1 - \sum Pi$, where P*i* is the band frequency of the i-th allele; for ISSR values $PIC = 1 - p^2 - q^2$, where p = band frequency and q = no-band frequency. The PIC values for each ISSR marker generated by the same primer were summed and denominated the ISSR primer index (IPI) according to Ghislain *et al.* (1999).

Results

In the genus *Trifolium* basic chromosome numbers form an aneuploid series of x = 5, 6, 7 and 8. The most common number is x = 8, with the great majority of species being diploid, a few being tetraploid and higher ploidy levels being very rare (Vižintin *et al.*, 2006). Since there are both

inter- and intra species differences in ploidy level, the assessment of nuclear DNA content is essential for breeding programs. The ploidy level of *T. argentinense* is unknown. The nuclear DNA content of *Trifolium* species estimated by flow cytometry varied between 0.91 pg for *T. pratense* and 8.58 pg for *T. medium*. No *intra*-specific variations were found between the different accessions of *T. argentinense*, *T. polymorphum*, *T. pratense*, *T. repens* and *T. riograndense*. However, different nuclear DNA values were observed in the two accessions of *T. medium* (6.86 pg and 8.58 pg), which might be explained by different ploidy levels between the two accessions (Table 1).

Calculated genome sizes (1Cx-value in pg) of the six *Trifolium* species of known ploidy level (Table 1) ranged from 0.46 pg for *T. pratense* to 0.96 pg for *T. polymorphum*, with similar values for the South American species having a higher genome size in comparison with those of Eurasiatic origin (Table 1).

From the 14 ISSR primers screened in the six *Trifolium* species, only six were selected as the most informative ones, which generated 186 total bands. The other eight ISSR primers gave either smeared, poor or irreproducible bands in all species and were therefore rejected. Thus, the ISSR primer useful for analyses of the genus *Trifolium* were: $(AG)_8T$, $(AG)_8TA$, $(AG)_8CA$, $(AC)_8CT$, $(GACA)_4$ and $(GACAC)_3$ (Figure 1).

The PCR products obtained ranged from 200 bp to 1300 bp. All six selected ISSR primers revealed polymorphism among the *Trifolium* species with an average of 31 total scorable bands by primer, ranging, on average, for each species from 2.8 for $(AG)_8CA$ to 9.3 for $(GACA)_4$ (Table 2). The PIC index was used to characterize the capacity of each primer to reveal or detect polymorphic loci, the ISSR primer showing the highest average PIC value being $(AG)_8T$ and the primers with the highest IPI were $(GACA)_4$, $(AG)_8TA$ and $(GACAC)_3$ (Table 2).

Considering the average percentage of *intra*-species polymorphism per primer, *T. argentinense* and *T. medium* showed the highest values, as opposed to *T. repens* for which no polymorphisms were observed (Tables 3 and 4).

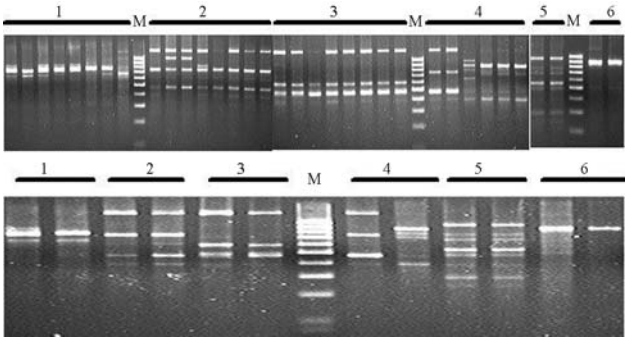

Figure 1 - Inter-simple sequence repeats (ISSR) profiles of six *Trifolium* species based on the $(GACAC)_3$ primer. A: individual profiles of accessions. B: profiles bulked by accession, of 1) *T. medium*, 2) *T. polymorphum*, 3) *T. riograndense*, 4) *T. argentinense*, 5) *T. pratense*, and 6) *T. repens*. M: 100 bp molecular size markers (Fermentas).

The $(GACAC)_3$ primer inter-SSR profiles for individual genotypes and bulked DNA are shown in Figure 1. The South American species showed a higher number of bands than the Eurasiatic species and *T. argentinense* showed the highest number of bands with 42 out of a total of 186 bands (Table 4). The Eurasiatic species showed a higher percentage of species-specific bands (45.3%) than the South American species (26.1%). When bands shared among *Trifolium* species were considered (at least one band between three species), *T. medium* and *T. repens* showed the lowest values (Table 4).

A distance matrix was produced for the 34 genotypes using the uncentered correlation coefficient (Figure 2). Cluster analysis based on a pairwise genetic distance matrix, using the average method, grouped the 34 genotypes into clusters with distance ranging from 0 to 0.9. The accessions within the species were grouped into four clusters (AU = 0.95). The species clusters are shown in Figure 2, from which it can be seen that the South American species were grouped together as were the Eurasiatic species.

Table 1 - Somatic chromosome number (2n) and nuclear DNA content of six *Trifolium* species.

Species	Somatic chromosome number (2*n*)	Ploidy level (x)	2C-value + SD (pg)	1Cx-value (Mbp)
Eurasiatic origin				
T. medium[1]	80 (Mowforth, 1986)	10	6.86 (+0.04)	675
T. medium[1]	96 (Vižintin *et al.*, 2006)	12*	8.58 (+0.03)	704
T. pratense[1]	14 (Vižintin *et al.*, 2006)	2	0.91 (+0.02)	450
T. repens[1]	32 (Vižintin *et al.*, 2006)	4	2.22 (+0.04)	548
South American origin				
T. argentinense[2]	nd	nd	1.92 (+0.02)	
T. polymorphum[2]	16 (Schifino-Wittmann, 2000)	2	1.93 (+0.03)	939
T. riograndense[2]	16 (Schifino-Wittmann, 2000)	2	1.79 (+0.03)	880

Cx value = (2C-value/ploidy level) x 1 pg DNA; 1 pg DNA = 978 Mbp (Doležel *et al.*, 2003); nd = non determined SD = standard deviation.
*Tentative ploidy level.

Table 2 - Characteristics of selected inter-simple sequence repeats (ISSR) primers for four *Trifolium* species.

ISSR primers	Total bands generated	Species average number of bands per primer	Species exhibiting most polymorphisms	Average PIC*	ISSR marker index (IPI)†
(AG)$_8$TA	29	4.8	*T. medium*, *T. riograndense*	0.27	4.94
(AG)$_8$CA	17	2.8	*T. argentinense*	0.29	2.89
(AG)$_8$T	24	4	*T. argentinense*	0.30	4.19
(AC)$_8$CT	29	4.8	*T. argentinense*, *T. medium*	0.21	3.55
(GACA)$_4$	56	9.3	*T. argentinense*, *T. medium* *T. riograndense*	0.21	6.86
(GACAC)$_3$	31	5.2	*T. argentinense*, *T. medium*	0.22	4.38

*PIC, Polymorphism information content.
†IPI, ISSR primer index.

Table 3 - Inter-simple sequence repeats (ISSR) primer analysis of six *Trifolium* species. Total bands (T) and percentage of intra-species polymorphisms (%P) generated with the (AG)$_8$TA, (AG)$_8$CA, (AG)$_8$T, (AC)$_8$CT, (GACA)$_4$ and (GACAC)$_3$ primers.

	Primers											
Trifolium	(AG)$_8$TA		(AG)$_8$CA		(AG)$_8$T		(AC)$_8$CT		(GACA)$_4$		(GACAC)$_3$	
species	T	%P	T	%P	T	%P	T	%P	T	%P	T	%P
T. medium	6	100.0	1	0.0	7	71.4	10	100.0	10	100.0	4	75.0
T. pratense	3	0.0	2	0.0	1	0.0	1	0.0	3	0.0	6	0.0
T. repens	2	0.0	3	0.0	2	50.0	3	66.7	9	88.9	2	0.0
T. argentinense	8	87.5	4	100.0	3	100.0	7	85.7	11	100.0	9	88.9
T. polymorphum	6	83.3	4	25.0	4	50.0	1	0.0	7	28.6	6	50.0
T. riograndense	4	100.0	3	66.7	7	71.4	7	85.7	16	93.8	4	0.0

Table 4 - Total bands, percentage of species specific bands, percentage of *intra*-species polymorphic bands and proportion of shared band among six *Trifolium* species generated with six Inter-simple sequence repeats (ISSR) primers.

Trifolium species	Total bands	Percentage of species-specific bands	Percentage of *intra*-species polymorphism	Proportion of shared bands*
T. medium	38	39.5	89.5	18.4
T. pratense	16	50.0	0.0	31.3
T. repens	21	52.4	52.4	23.8
T. argentinense	42	33.3	92.9	42.9
T. polymorphum	28	21.4	46.4	39.3
T. riograndense	41	19.5	78.0	29.3

*with at least 3 species.

Discussion

Flow cytometry studies showed a higher Cx-value for the South American species and an increased intra-specific variation in *T. medium* (Table 1). Similar Cx-values for *T. pratense* (0.427 pg), *T. repens* (0.559 pg), *T. medium* (0.645 pg) and *T. polymorphum* (1.025 pg) have been reported by Vižintin *et al.* (2006). There were different ploidy levels among the six *Trifolium* species studied (Table 1) and this might have affected band intensity due to the presence of different copies of the same locus in the genome, because of which differences in band intensity for the molecular analysis were not considered in our study. The ISSR markers applied to the six *Trifolium* species proved to be suitable for the analysis of genetic diversity. In all the species, the analysis of common bands shared by most of the individual genotypes inside the accession group were congruent when compared with the bulked DNA sample and confirmed the methodology employed for band evaluation.

Ecogeographical and species discrimination based on individual fingerprinting or bulked DNA of accessions was achieved. Moreover, all the species generated profiles with intra-polymorphic bands and species-specific bands. Since

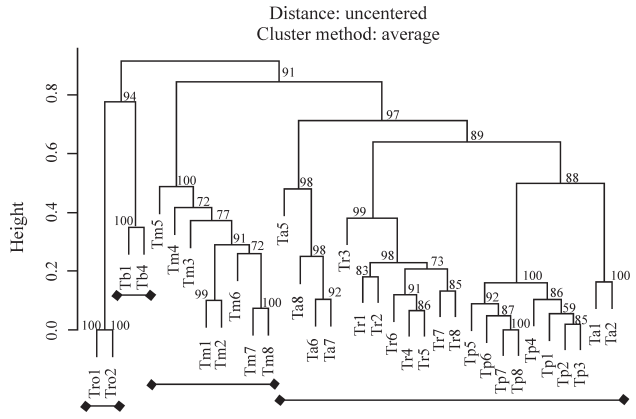

Figure 2 - Dendrogram for the 34 *Trifolium* genotypes using the average method of genetic distance estimates (uncentered correlation coefficient) with an approximately unbiased (AU) p-value computed by multiscale bootstrap resampling, Clusters with AU value greater than 95% are highlighted by horizontal bars.

ISSR markers cover all the genome, the six primers analyzed 112 loci, and the selection of consistent bands led to reliable estimates of genetic relationships. Considering the repeatability and the simplicity of the analysis, it might be considered as supplementary evidence for determining rights of inventor when summed to the descriptor data of a variety (Jain *et al.*, 1996; Lanham and Brennan, 1999; Barreto Dias, 2004).

The analysis of *Trifolium* species using ISSR markers can be complemented with agronomic data and may thus present a readily accessible means for studying ISSR-associated polymorphism and as marker assisted selection Tsumura *et al.*, 1996; Sica *et al.*, 2005).

For the *Trifolium* species studied, this is, to our knowledge, the first report of molecular variability evaluation using ISSR markers. In this study, *T. medium* and *T. argentinense* showed the highest level of intra-species polymorphism (Table 4), supporting the work of Zohary and Heller (1984) who reported that *T. medium* was an extremely polymorphic species. The South American species generally yielded the highest total number of primer bands, showing 59.7% of all recorded bands. This characteristic might be due to differences in genome size, because, in effect, the South American species showed a higher genome size than the Eurasiatic species (Table 1), as has also been reported by Vižintin *et al.* (2006). The larger the genome the more putative loci can be explored by dominant markers. In our study, the differences in genome size were correlated with the geographical origins, also previously reported by agreement with Vižintin *et al.* (2006). Furthermore, our results show that *T. argentinense* was the most polymorphic of the accessions, revealing the potential of this type of marker in this species. A low similarity was observed among accessions for *T. argentinense,* probably due to the contrasting ecogeographical location. Nevertheless, *T. argentinense* was grouped with *T. polymorphum* and *T. riograndense*. All the ISSR markers clearly discriminated between *T. argentinense* and *T. polymorphum* and suggests that they are in fact different species, supporting the view of several workers (Burkart, 1987; Izaguirre, 1995; Ellison *et al.*, 2006) but contrasting with the opinion of Zohary and Heller (1984) that they are different species.

Isobe *et al.* (2002) reported hybridization between the Eurasiatic *Trifolium* species *T. medium* and *T. pratense*, and these species were clearly separated in the present study by the six ISSR assayed, while the group of South American species analyzed showed similarities that encourage the possibilities of combining characteristics through interspecific crosses. The South American species were clustered together, clearly separating them from the Eurasiatic species but also showing intra-species variability (Figure 2). The clade formed agreed with the classification of Ellison *et al.* (2006), who placed *T. argentinense*, *T. polymorphum* and *T. riograndense* in the Section *Involucrarium* and belonging to the clade O of their study, while *T. medium* and *T. pratense* were placed in the Section *Trifolium* and *T. repens* in the Section *Trifoliastrum*. Furthermore, Vižintin *et al.* (2006) found similar relationships between the Eurasiatic and American species based on Internal Transcribed Spacer (ITS) polymorphisms. Our genetic-distance estimates within each cluster showed the extensive genetic variation within the species, with *T. polymorphum* exhibiting lower levels of genetic variability as compared with *T. argentinense*. We found no variability in the allogamous species *T. repens*, probably due to the low number of genotypes analyzed.

The information generated in this study established an easy, fast and reliable marker system which could be transferred to other laboratories. All the ISSR markers could clearly discriminate between *T. argentinense* and *T. polymorphum*, indicating that they are in fact different species. These results indicate that the ISSR markers used in our study could be used in future research to evaluate the variability present in each of the six *Trifolium* species studied.

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