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Genetic diversity assessed by microsatellite markers in the amphicarpic species *Trifolium polymorphum* Poir.

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ABSTRACT

Trifolium polymorphum Poir. is an amphicarpic forage legume from southern Brazil, Uruguay, Argentina, Paraguay and Chile. Information on the genetic diversity of natural populations in natural grasslands in southern Brazil is limited. In order to increase the knowledge about this species, an analysis of the genetic diversity was carried out in 10 natural populations of *T. polymorphum* with the use of 20 microsatellite markers. The expected heterozygosity in *T. polymorphum* populations ranged from 0.40 to 0.43, with a mean of 0.42. A total of 193 alleles were detected with a mean of 9.3 alleles per locus and polymorphic information content (PIC) for these markers of 0.62 to 0.89 with a mean of 0.84.The grouping based on the Jaccard's coefficient of similarity classified populations, regardless of their regions of origin, into two groups with a mean similarity coefficient of 0.32, reflecting the high genetic variability of the populations, especially those located in the Campanha phytogeographic region. This information on diversity can be used to plan future germplasm collection strategies for conservation purposes and also for the breeding of the species.

Key words: genetic variability, microsatellites, natural grasslands, Pampa biome.

INTRODUCTION

Trifolium polymorphum Poir., is a stoloniferous perennial legume endemic to southern Brazil, Uruguay, Argentina, Paraguay and Chile (Burkart 1987, Zohary and Heller 1984) and one of the two amphicarpic species of the genus *Trifolium*, i.e., capable of producing flowers and aerial and subterranean fruits in the same individual (Lev-Yadun 2000). Amphicarpy can be considered a response to different selection pressures, including herbivory, draught, fire, desiccation, seed predation (Cheplick 1987, Kaul et al. 2000, Kumar et al. 2012). *T. polymorphum* also has a vegetative

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reproduction through stolons and regrowth of the reserve roots separate from the mother plant (Speroni et al. 2014). The subterranean flowers are obligatory cleistogamous with four to five anthers, while the aerial flowers with 10 anthers (Conterato and Schifino-Wittmann 2014) have been reported as being allogamous, self-compatible and that benefit from pollinators for seed production (Real et al. 2007). The other amphicarpic species of the genus, *T. argentinense* Speg, has been extensively studied in relation to seed dimorphism (Conterato et al. 2010), seed production, morphology, reproductive biology and life strategy (Conterato et al. 2013).

During winter *T. polymorphum* is an important forage component of natural grasslands of Rio Grande do Sul (between 30ºS and 34ºS) due to its high palatability and good forage quality (Speroni and Izaguirre 2003). However, the loss of large areas of these grasslands in Rio Grande do Sul due to conversion to annual agricultural crops, especially soybean and corn and silviculture (mainly of eucalyptus and pine), and the degradation associated with the invasion of exotic species such as *Eragrostis plana* Ness (called capim anoni in Brazil) is a major threat to the genetic diversity of this highly biodiverse natural ecosystem that provides soil carbon storage, erosion control, soil water infiltration, pollinator availability and forage production (Pillar et al. 2012). Therefore, conservation strategies to understand patterns of genetic variation within and between populations are crucial to assess the current status of populations (Béjaoui et al. 2011, Corlett 2016) and to establish a collection strategy for the conservation and use of germplasm (Rao and Hodgkin 2002, Whitlock et al. 2016).

Microsatellites, also known as SSR (Simple Sequence Repeats) markers, have gained considerable importance for their many desirable genetic attributes, including hypervariability, broad genomic distribution, co-dominant inheritance, reproducibility, multi-allelic nature and specific chromosomal location (Verma et al. 2015), and for their wide use in analyzing genetic diversity (Dalla Rizza et al. 2007, Conterato et al. 2013, Dugar and Popov 2013, Njung'e et al. 2016, Liang et al. 2007, Sinha et al. 2015, Swift et al. 2016), plant breeding (Islam et al. 2012, Tan et al. 2014), phylogenetic studies (Kang et al. 2015), among others.

With the exception of the studies by Dalla Rizza et al. (2007) and Real et al. (2007), which evaluated the genetic diversity in Uruguayan populations of *T. polymorphum* with ISSR and SSR molecular markers, respectively, no other study was conducted with this purpose for this species. The objective of this study was to evaluate the genetic diversity in populations of *T. polymorphum* occurring in Rio Grande do Sul, from the germination of aerial seeds.

MATERIALS AND METHODS

PLANT MATERIAL

Seeds from ten accessions (naturally occurring populations) of *T. polymorphum* were collected in November or December of 2002, 2005, 2006, 2007, 2008 and 2010 along the species area of distribution in physiographic regions of Rio Grande do Sul in the Pampa and Mata Atlântica (Atlantic Forest) biomes (Table I).The taxonomic vouchers are kept at Herbarium HBEI (Universidade Federal do Pampa, Campus São Gabriel, Brazil) (Table I). In each locality, between 30 and 60 aerial inflorescences with mature legumes were collected in paper bags from at least 15 plants around a ca. 300m^2 area. Afterwards, the legumes of each accessions were opened and the seeds bulked and stored in a refrigerator at the Departamento de Plantas Forrageiras e Agrometeorologia/Faculdade de Agronomia da Universidade Federal do Rio Grande do Sul and later used for molecular analysis.

TABLE I
Acessions of *T. polymorphum* analyzed, collector's number, physiographic region and expected heterozigosity.

Acession/ Place of Collection	Collector's number	Physiographic region	[b]HZ
01-Caçapava do Sul (30º 30' S, 53º 29' W)	HBEI 1446	Serra do Sudeste	0.426
[a]04-São Jerônimo (29º 57' S, 51º 43' W)	HBEI 1437	Depressão Central	0.410
[a]06-Rio Pardo (29º 59' S, 52º 22' W)	HBEI 1440	Campanha	0.424
07-Bagé (31º 19' S, 54º 06' W)	HBEI 1448	Campanha	0.432
10-Bagé (31º 19' S, 54º 06' W)	HBEI 1439	Campanha	0.411
12-Pelotas (31º 46' S, 52º 20' W)	HBEI 1445	Encosta do Sudeste	0.434
17-Eldorado do Sul (30º 05' S, 51º 36' W)	HBEI 1442	Depressão Central	0.413
18-Santana do Livramento (30º 53' S, 55º 31' W)	HBEI 1441	Campanha	0.434
20-Pinheiro Machado (31º 34' S, 53º 22' W)	HBEI 1447	Campanha	0.429
21-São Gabriel (30º 20' S, 54º 19' W)	HBEI 1449	Campanha	0.405
Average			0.422

[a]04-São Jerônimo and 06-Rio Pardo are accessions from Mata Atlântica (Atlantic Forest) biome and other accessions are from Pampa biome. The values cited of latitude (S) and longitude (W) refers to the municipality where the collection was carried and not to the collection site itself.
[b]Expected heterozigosity.

MOLECULAR ANALYSIS

In April 2010, 15 aerial seeds of each accession were scarified with sandpaper and germinated in petri dishes with moistened filter paper. The germinated seedlings were transferred to 200 ml capacity plastic cups filled with commercial substrate and in August 2010, 10 plants were transferred to plastic boxes (34 cm x 14 cm x 11.5 cm) filled with commercial substrate, one plant per box in an open area. Two plants of each population did not persist and the experiment was conducted with eight plants of each population. The total genomic DNA of each sample (100-150 ng) was extracted from a bulk of young leaves of eight plants per accession, following Doyle and Doyle (1990) protocol with slight modifications. DNA concentration and quality were evaluated in 1% agarose gel, using 100 and 500 ng fago λ DNA as standard.

Five microsatellite (SSR) primers developed for *T. repens* L. and 19 primers developed for *Lotus japonicus* L. (Regel) Larsen were tested and, from these, 20 were selected to be used plants grown from aerial seeds. PCR reactions were prepared in a final volume of 6 µL per reaction, containing 2 µL of the work DNA solution (25 ng/µL), 1 µL of PCR 10X buffer, 0.6 µL of $MgCl_2$ (50 nM), 0.8 µL of DNTP mix containing 2.5 mM of each of the four nucleotides, 0.6 µL of the forward primer (10 µM), 0.6 µL of the reverse primer (10 µM), 0.2 µL of *Taq* polymerase Quiagen (5U/µL) and Miliq sterilized water to complete the volume. PCR conditions followed Dias et al. (2008) with the following modifications: initial denaturation at 94ºC for 5 min, followed by seven cycles of 1 min at 94ºC, 1 min at 61ºC, 1 min at 72ºC, and annealing temperature reduction of 1ºC per cycle, followed by 25 cycles of 1 min at 94ºC, 1 min at 55ºC and 1 min at 72ºC and six cycles of 45 seconds at 94ºC, 45 seconds at 54ºC and 45 seconds at 72ºC and a final extension for 8 minutes at 72ºC and storage at 7ºC. The amplification products were separated in a 3% high resolution agarose gel (Agarose 1000) stained with 0.08 µL/mL ethidium bromide (10 mg/mL) and immersed in TBE 1X buffer under an electrophoretic current of 96 V for approximately 2

hours. After electrophoresis, the gel was visualized in an ultraviolet light (wavelength of 260 nm) and photographed in order to compare the samples fragments with a 100 base pair (bp) ladder, using the Kodak EDAS 290 (Electrophoresis Documentation and Analysis System) program.

The dendrogram of genetic similarity among accessions was obtained from the similarity coefficients using the UPGMA (Unweighted Pair-Group Method Using an Arithmetic Average). Cluster analysis was performed using the Numerical Taxonomy and Multivariate Analysis System NTSYSpc program version 2.1 (Rohlf 2001) and the similarity matrix constructed with Jaccard's coefficient. The total number of alleles per locus (A), the allelic frequencies and the Polymorphism Information Content (PIC) for each locus (PICi = 1 - ∑ Pi, where Pi is the frequency of allele I band) were calculated. The expected heterozygosity (HZ) of each identified band was calculated using the following formula: $HZi = 1 - (+)$, where P_{ip} and P_{ia} are the frequency of presence and absence of the *i*th band (Isobe et al. 2013). The individuals of each population were assessed in bulk; therefore, the mean *HZ* value of identified bands generated by a marker in a population was substituted for the *HZ* of each population.

RESULTS AND DISCUSSION

The level of expected heterozygosity is a measure of diversity that provides information on the proportion of different alleles in populations (Pagnotta et al. 2011). The values of expected heterozygosity among the accessions (HZ) ranged from 0.40 (21-São Gabriel accession) to 0.43 (12-Pelotas and 18-Santana do Livramento accessions), with a mean of 0.42 (Table I), showing a moderate differentiation between the populations of *T. polymorphum* for the analyzed loci. Based on the literature, the expected heterozygosity for species of the *Trifolium* does not contemplate *T. polymorphum*, which makes the present results important, since it complements information on the species. This data differs from that obtained in cultivars of *T. pratense* by Dugar and Popov (2013) where the expected heterozygosity ranged from 0.22 to 0.80 (mean 0.70) with the use of SSR, and by Pagnotta et al. (2011) who found expected heterozygosity between 0.18 and 0.43 (mean 0.31) with the use of amplified fragment length polymorphism (AFLP) markers in Italian red clover genotypes, including local varieties, natural populations and ancient cultivars of the species.

Amplification of the 20 SSR primers in the 10 accessions of *T. polymorphum* resulted in a total of 193 alleles, ranging in size from 50 to 420 base pairs. The number of alleles per locus ranged from a minimum of seven (SSR TM0080) to a maximum of 12 (SSR TM0070), with a mean of 9.3 alleles per locus (Table II), which are comparable to previous studies in the amphicarpic *T. argentinense* (8.90, Conterato et al. 2013), and in the allogamous *Trifolium pratense* L. (11.1, Dias et al. 2008 and 8.70, Dugar and Popov 2013). In Uruguayan populations of *T. polymorphum*, Real et al. (2007), using only five SSRs, found from three to nine alleles per locus. According to Shiferaw et al. (2012), the number of alleles detected per marker and the level of genetic diversity depend on the number and origin of the genotypes analyzed and it is not easy to compare the level of diversity between different studies. However, both studies confirm the importance of SSR to access diversity in *T. polymorphum*.

The PIC values, which reflect allelic diversity and frequency, were high, ranging from 0.62 (SSR TM0118) to 0.89 (SSR prs461, SSR TM0180, SSR TM0065 and SSR TM0406), with a mean of 0.84 (Table II), similar to the PIC variation between 0.52 to 0.86 in Uruguayan populations and as expected for a highly heterogeneous cross-pollinated species such as *T. polymorphum* (Real et al. 2007). Similar results were found for the *T. argentinense*, with

TABLE II
Characteristics of the 20 microsatellite marker used for *T. polymorphum*.

Primer	Primer sequence	Alelle size (pb)	Alelle number	PIC
[a]TRSSRAXX31	F: TCTGTTTTGTTGGCCATGC R: TTGCAAAGTGTTTGGAAGGA	150-400	11	0.80
[a]prs461	F: ACCTTCCGATATCCCAAACC R: ATGGTGCGTTTGGAGATAGC	80-380	10	0.89
[a]prs 582	F: CCGGTTCGATTCAACAAGTT R: CTGCAGATCCAGTAATGATTTCC	100-390	08	0.84
[a]prs 612	F: TTGAACTAGTCGTTGGATGGG R: GAGAGGGTTTCAGGAACATACG	100-380	10	0.85
[b]TM0095	F: CTTGTGGTTTTCTTCCCGAC R: GGGACAAAAGAAAATAACGC	90-420	09	0.88
[b]TM0180	F: CAGTTATTTAGGAACGGAGG R: AAACAAACAGTAAGTGTCTCATC	70-400	11	0.89
[b]TM0817	F: TTGCTCATGTGAGAAAGAAC R: GCTTTAAAATGACGTCCTAATC	70-420	08	0.86
[b]TM0118	F: TGCAATTTACTCTTTATATTTCG R: TCCAATTCAAATTATTTTATAGAG	70-400	11	0.62
[b]TM0314	F: TGTGATTAGTGATTAGAAAGTGAG R: TTTGACCAAACTTCCTTCAC	110-420	08	0.85
[b]TM0056	F: CATTAGAATATTGAATGCACC R: TCTCTTCTCTCTGTTATTTATAGC	70-400	10	0.85
[b]TM0436	F: ATGTTGTCTGTGTGTCTGTG R: AAATTGATTGAAAAGGGGTG	160-400	09	0,86
[b]TM0771	F: CACTTCCTTTGAGAGCAGTC R: GTTCCCTTGAAAATTGAATG	50-400	10	0.88
[b]TM0070	F: CTTAACAAAAGTCTGGGGTG R: GAATGTGTGGTAGCTTGTTG	80-400	12	0.88
[b]TM0080	F: AACAAAATACTAAACTATAGCAAAG R: CGTCCCACAACTCTCTTTAC	100-400	07	0.72
[b]TM0654	F: TCAGTTGAGACCTCAAAATC R: TCCTGATAGAAGTGATTTGG	80-400	10	0.87
[b]TM0065	F: AATGCTAGTTAAGCGCTCTC R: CCCAAAGGTCTATAATTATAAGG	100-400	11	0.89
[b]TM0076	F: TCAAATGTGATGAGTGACATAC R: AAAACATGTAGCTAAGAAACTAAAA	150-400	08	0.88
[b]TM0059	F: TCCTTCATTCATTCATAACC R: TGAGAAGAGAATGAAAAGCG	100-400	09	0.82
[b]TM0406	F: CAGACAGAGAAAAGGGTCAG R: TGCATCTTCAGTTGCTCTTG	80-400	11	0.89
[b]TM 0711	F: CCTAAGATCATTTAGACAAAACTC R: AAAGATGTTCTCTTGGGTTG	100-400	10	0.84
Total average			193(9.3)	0.84

[a]Primer developed for *Trifolium repens* L.
[b]Primer developed for *Lotus japonicus* (Regel) Larsen.

PIC values between 0.66 and 0.93 (Conterato et al. 2013), the *Trifolium riograndense* Speg, between 0.48 and 0.80 (Conterato et al. 2012) and *T. pretense,* between 0.70 and 0.86 (Dias et al. 2008). In *T. polymorphum* plants grown from aerial seeds, Real et al. (2007) identified a greater 'inter-patch' than 'intra-patch' variability with the use of SSR, explaining the high polymorphism between populations by cross-pollination aerial seed dispersal, and the low intra-patch variability by vegetative propagation and underground seeds. However, in this study only plants grown from aerial seeds were analyzed.

The similarity between the accessions ranged from 0.16 to 0.56 with a mean of 0.32 (Fig. 1), reflecting the high genetic variability present in the species, even with the substitution by crops of almost 54% of the original area of the Pampa biome (MMA 2012), and remaining only 12.5% of the original area of the Atlantic Forest biome (SOS Mata Atlântica 2014). Real et al. (2008) also found a high morphological variation for the leaf mark, leaf margin, central leaflet length, hairiness, number of stolons and plant size for *T. polymorphum*. The results of genetic diversity of the present work are similar to those found by Conterato et al. (2012) in *T. riograndense*, but distinct from the coefficient of similarity between 0.50 and 0.90 observed in *T. pratense* populations by Ahsyee et al. (2014). The co-expressed correlation coefficient of the dendrogram (Fig. 1) was 0.94, indicating a good fit between the original distance matrix and the graphical distance. The UPGMA grouping of genetic distance resulted in two groups using the mean similarity of 0.32 as a cut point: group 1 (04-São Jerônimo, 17-Eldorado do Sul, 12-Pelotas,

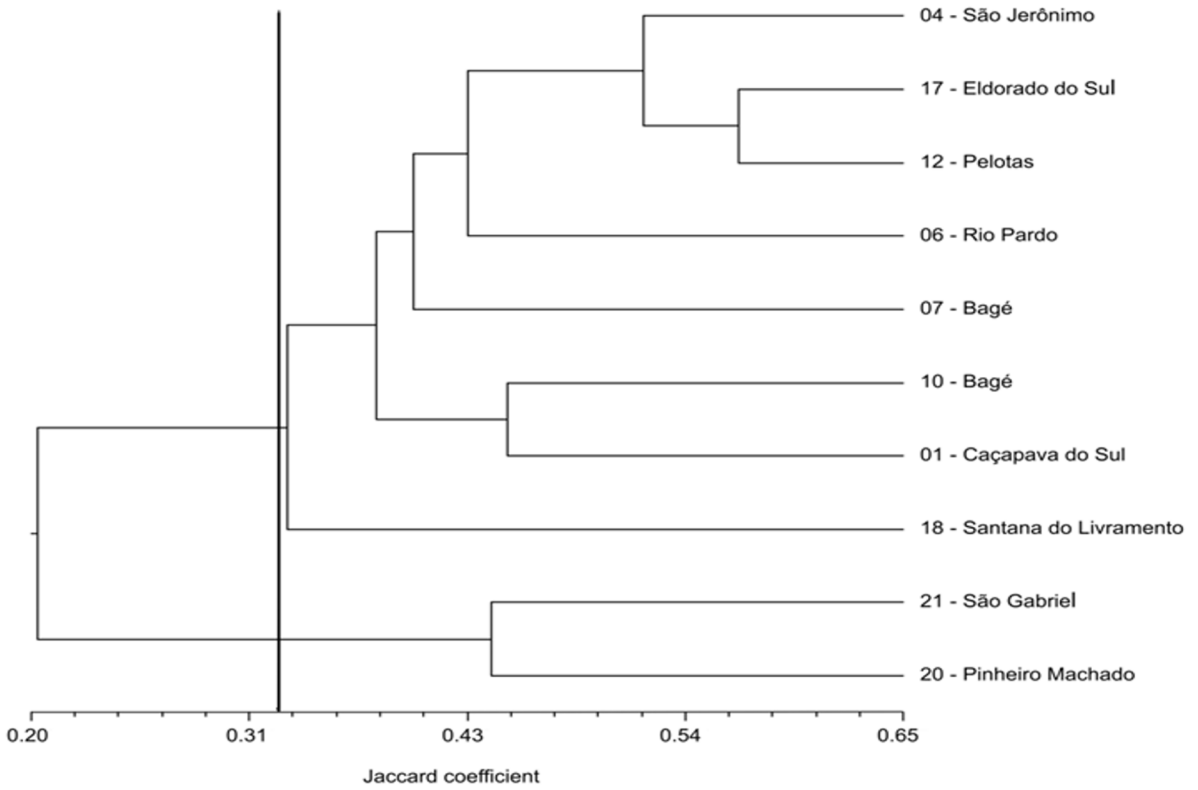

Figure 1 - Dendrogram of 10 *T. polymorphum* accessions revealed by UPGMA cluster analysis based on SSR markers data using Jaccard's similarity coefficient.

06-Rio Pardo, 07-Bagé, 10-Bagé, 01-Caçapava do Sul and 18-Santana do Livramento accessions) and group 2 (21-São Gabriel and 20-Pinheiro Machado accessions).

In group 1, the subgroup among the 04-São Jerônimo, 17-Eldorado do Sul and 12-Pelotas accessions does not reflect its geographical origin. Acessions 04-São Jerônimo and 17-Eldorado do Sul, are both municipalities in the physiographic region of the Central Depression showed an average similarity of about 50% (Fig. 1), while the 17-Eldorado do Sul and 12-Pelotas accessions, distant about 270 km from each other and belonging to the Central Depression and Sudeste Encosta regions, respectively, were the most similar (about 57% similarity), a result similar to that of the 10-Bagé and 01-Caçapava do Sul subgroup. The 10-Bagé and 07-Bagé accessions, however, did not remain in the same subgroup (Fig. 1), suggesting that the diversity can be high even in geographically close places. A similar result in group 1 was also observed among the 07-Bagé, 18-Santana do Livramento and 06-Rio Pardo accessions, reflecting the high diversity of *T. polymorphum*in the physiographic region of the Campanha. In group 2 the 21-São Gabriel and 20-Pinheiro Machado accessions were grouped according to the region of origin.

No well-defined relation between genetic diversity assessed by SSR and geographic origin can be established in the present study (Fig. 1), the same being found by Conterato et al. (2012) for *T. riograndense* and by Ahsyee et al. (2014) for red clover (*T. pratense*). In sorghum, Gerrano et al. (2014) found some relation between genetic diversity and geographic origin, and in common beans the relation between the majority of genotypes and the origin of cultivation were strongly correlated (Zargar et al. 2016). The low genetic similarity observed between the accessions of *T. polymorphum* can be a reflection of the contrasting ecogeographic location, absence of gene flow due to the distance between the populations, plus the absence of seed dispersion, low fruit yield, with values ranging from 0.6 to 12 (Speroni and Izaguirre 2003), as well as the high morphological variation naturally present in the species (Zohary and Heller 1984, Real et al. 2007).

The high genetic diversity observed in *T. polymorphum* populations in all phytogeographic regions of Rio Grande do Sul increases the genetic knowledge on this important amphicarpic species of the natural grasslands of Rio Grande do Sul. This information can be used as a tool to plan future collection strategies of this important forage germplasm for conservation purposes and also for the purpose of the species breeding.

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