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Biology-Genetics

Genetic diversity of *Trifolium polymorphum* Poir. (Fabaceae) in natural populations of Rio Grande do Sul, Brazil

Diversidade *Trifolium polymorphum* Poir. (Fabaceae) em populações naturais no Rio Grande do Sul, Brasil

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ABSTRACT

The genus *Trifolium* (Fabaceae) is composed of herbaceous plants of forage interest that contribute to the maintenance of the productive capacity of soils, enabling the fixation of atmospheric nitrogen. Genetic diversity is an important factor for the response of populations to environmental changes. The present work aimed at analyzing the genetic diversity and population structure of *Trifolium polymorphum* Poir., a native species of the Pampa biome, using ISSR (Inter Simple Sequence Repeat) molecular markers. Leaves and stolons from five populations were collected in São Borja, São Gabriel, Tupanciretã, Eldorado do Sul, and São Pedro do Sul and stored in silica gel until DNA extraction. The DNA was extracted and quantified in 1% agarose gel. Regions were amplified via ISSR-PCR with four primers: (GA)8 YC, (AG)8 T, (GACAC)3, and (AG)8 TA. The data were analyzed using statistical software GenAlEx 6.5, Structure, and Structure Harvester. The results showed greater genetic variation within populations (56%) than between populations (44%). Cluster analysis and the scatter plot from the main coordinate analysis separated the accessions according to geographical origin. However, the mean genetic diversity (H=0.113) for all primers was low when compared to other studies with the same genus and marker. This diversity information can be used to adopt measures that favor the best forms of conservation and management for the sustainable use of the biodiversity of native species.

Keywords: Conservation; ISSR; Molecular markers; Pampa

RESUMO

O gênero *Trifolium* (Fabaceae) é composto por plantas herbáceas de interesse forrageiro. As leguminosas de interesse forrageiro contribuem para a manutenção da capacidade produtiva dos solos, possibilitando a fixação do nitrogênio atmosférico. A diversidade genética é um fator importante para a resposta das

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populações às mudanças ambientais. O presente trabalho teve como objetivo analisar a diversidade genética e a estrutura populacional de *Trifolium polymorphum* Poir., uma espécie nativa do bioma Pampa, utilizando marcadores moleculares ISSR (Inter Simple Sequence Repeat). Folhas e estolhos de cinco populações foram coletados em São Borja, São Gabriel, Tupanciretã, Eldorado do Sul e São Pedro do Sul e armazenados em sílica gel até a extração do DNA. O DNA foi extraído e quantificado em gel de agarose a 1%. As regiões foram amplificadas via ISSR-PCR com quatro primers: (GA)8 YC, (AG)8 T, (GACAC)3 e (AG)8 TA. Os dados da matriz binária foram analisados utilizando os softwares estatístico GenAlEx 6.5, Structure e Structure Harvester. Os resultados mostraram maior variância genética dentro das populações (56%) do que entre populações (44%). A análise de agrupamento e o gráfico de dispersão da análise de coordenadas principais separaram os acessos de acordo com a origem geográfica. Porém, a diversidade genética média (H=0,113) para todos os primers foi baixa quando comparada a outros estudos com o mesmo gênero e marcador. Essas informações sobre diversidade podem ser utilizadas para adotar medidas que favoreçam as melhores formas de conservação e manejo para o uso sustentável da biodiversidade de espécies nativas.

Palavras-chave: Conservação; ISSR; Marcadores moleculares; Pampa

1 INTRODUCTION

Fabaceae consists of the third largest family in species diversity of angiosperms, surpassed only by Orchidaceae and Asteraceae (Lewis et al., 2005), and comprises 770 genera and about 19,500 species (LPWG, 2017). In Brazil, Fabaceae is the richest family, represented by 234 genera and 2.941 species, of which 15 genera and 1,568 species are endemic.

The state of Rio Grande do Sul (RS), located in the extreme south of Brazil, between coordinates 27º and 33º S and 49º and 57º W, covers an area of approximately 280.000 km^2, bordering Uruguay and Argentina, and has large extensions of grasslands. Part of the state is included in the Atlantic Forest biome and part in the Pampa biome (Pillar et al., 2012). The Pampa biome occupies an area of 193.836 thousand km^2 (about 2 % of the national territory) and is composed mainly of grassland vegetation (herbaceous plants, shrubs, and some trees). In Brazil, the Pampa is restricted to the state of Rio Grande do Sul, occupying 63 % of the gaucho territory and portions of the territories of Argentina and Uruguay (IBGE, 2019).

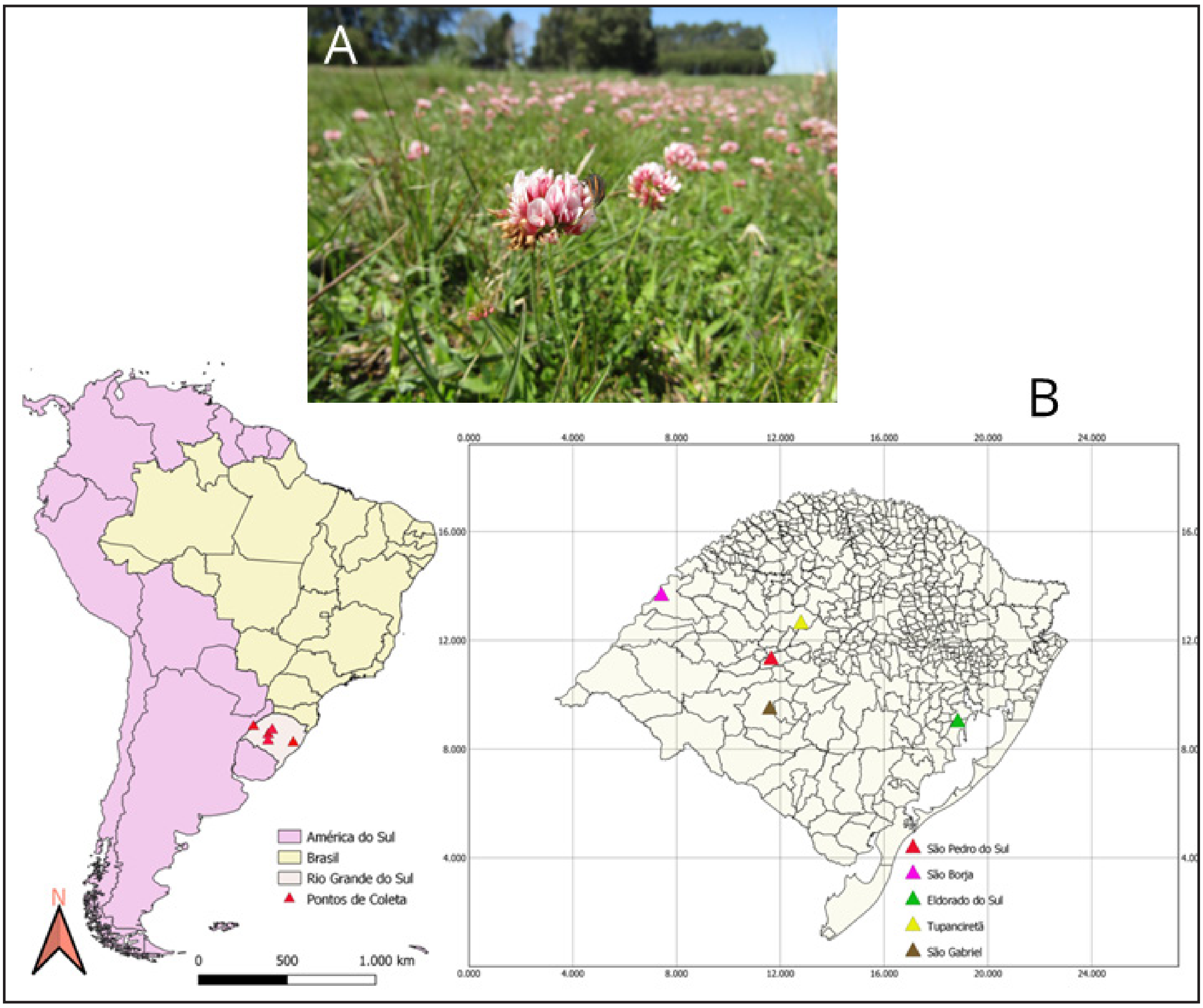
For the grassland formations of the state of Rio Grande do Sul, about 670 legume species are estimated (The Brazil Flora Group, 2021), demonstrating the relevance of this family in the composition of the Atlantic Forest and Pampa biomes. The genus *Trifolium*, which includes species known as true clovers, is a group of legumes that play an essential role in ecosystems and cropping systems due to their biological capacity to fix atmospheric nitrogen and for their resilience to the effects of climate change (Liew et al., 2014).

In addition, legume species have an essential function for the food security of cattle and especially sheep during the winter period in the regions of the gaucho pampa. Food security is linked to the development of cultivars to support and optimize the production of meat and milk on pastures (Soares and Rosinha, 2019). Furthermore, they are important for their high protein concentration, which can reach values of up to 22 % (Speroni; Izaguirre 2003).

Ecologically, the species of the genus *Trifolium* also stand out due to their ecological adaptations to predators and to the environmental conditions of the biomes in which they are found. One of the most interesting adaptations of these species is the phenomenon called amphicarpy, in which a species has the ability to generate fruits in both its aerial and underground parts (Barker, 2005). Amphicarpy enables the species to have a higher survival rate for the herbivores that inhabit their regions of occurrence (Speroni et al. 2014). However, it is an uncommon characteristic and an extremely interesting object of study since it occurs only in approximately 50 other angiosperms (Lev-Yadun, 2000). Other factors such as their high adaptability and plasticity to variations in soil pH and humidity (Izaguirre, 2001) ensure the success of these plants in natural conditions and make their cultivation as a forage species even more advantageous (Speroni; Izaguirre, 2003).

Trifolium polymorphum Poir, popularly known as “pink clover” (*trevo-rosa*), is a perennial herb common in grasslands in the southern half of the state of Rio Grande do Sul (Figure 1b). It is a stoloniferous plant with leaves composed of three green leaflets and can belong to one of three leaf mark categories: no leaf mark, white central leaf mark, or red leaf mark (Real et al., 2007). The flowers, gathered in delicate globose inflorescences, are pinkish or reddish in hue. Flowering occurs between September and November. The rusticity, capacity to cover the soil and the aspect of the leaves and flowers give this plant the ornamental characteristics for cultivation as bed lining and as a way to diversify vegetation cover. At flowering, it adds color (Figure 1a) since the inflorescences stand out among the vegetation (Stumpf, 2009).

Figure 1 – A. *Trifolium polymorphum* in natural environment, in the town of Tupanciretã, Rio Grande do Sul, Brazil. B. Map with location of *T. polymorphum* sampling sites, built in Qgis software



Source: Letícia Kraetzig, 2020

The interest related to studies with the species is manifold, Speroni et al. (2009), developed studies on the intrafloral phenology of the aerial flowers of *Trifolium polymorphum*; Conterato et al. (2018) evaluated genetic diversity with microsatellite markers in *Trifolium polymorphum* in samples collected from 2002 to 2010; Conterato et al. (2019) analyzed amphicarpy as a reproductive strategy with respect to the number, size, and weight of aerial and underground seeds of two populations of *T. polymorphum* grown from reserve root regrowth and in the absence of herbivory; and Conterato and Schifino-Wittmann (2014), investigated the reproductive strategies of the amphicarpic species *Trifolium polymorphum*.

Molecular biology has provided valuable tools for analysis of genetic diversity and population structure in plant species. Molecular markers are one of these tools, detecting differences in DNA levels between two or more individuals and assisting in the molecular characterization of the analyzed material (Giustina et al., 2014). Among the molecular markers, ISSRs (Inter-simple sequence repeats) have been widely used since they prove to be a fast and efficient technique, with high reproducibility and high polymorphism rates, providing a large number of data at low cost, while being transferrable to any type of plant (Rivas et al., 2013, Giustina et al., 2014, Rossi et al., 2014).

Although many species of the genus have publications involving genetic diversity with ISSR molecular markers, research with this marker is still scarce in *T. polymorphum* in southern Brazil. Considering that the species is a native of the Pampa Biome, it is important to evaluate the possible changes in genetic diversity of natural populations, considering that the Brazilian Pampa Biome has been reduced in almost 30 % of its area from 1985 to 2021 (MapBiomas, 2023). As a native species of forage value, genetic studies of natural populations are necessary for conservation purposes and even as a starting point for genetic improvement. Taking these factors into account, the present study aimed at evaluating the diversity and genetic structure in natural populations of

Trifolium polymorphum occurring in the Pampa biome, in the state of Rio Grande do Sul, Brazil, using ISSR markers and more recently collected samples, contributing to the monitoring of the genetic diversity of the species.

2 MATERIAL AND METHODS

Five collection sites were selected where the species naturally occur in the state of Rio Grande do Sul, Brazil, in the municipalities of São Borja, São Gabriel, Tupanciretã, Eldorado do Sul and São Pedro do Sul (Figure 1). Each site is formed by a study population, totaling five natural populations. The collections occurred during the flowering period of the species, from October to November 2020. According to Santos (2018), the classification of the soils of the collection cities are: Tupanciretã (Typical dystrophic red-yellow Argisol), São Gabriel (Typical eutrophic haplic Planosol), São Borja (Typical dystrophic red Nitosol), Eldorado do Sul (Typical dystrophic red Argisol), and São Pedro do Sul (Arenic dystrophic red Argisol).

Leaf and stolon material were collected from each individual, observing a distance of 3 meters between each individual sampled to avoid collecting clones, since the species also reproduces vegetatively through stolons (Speroni et al., 2014). All material was identified in the field, stored in bags containing silica gel and transported to the Laboratory of Plant Genetics and Evolution (GENEV) at the Universidade Federal de Santa Maria, in Santa Maria, Rio Grande do Sul, Brazil, where it was stored at room temperature until DNA extraction. A total of 103 individuals were sampled. A sample from each population was herborized and deposited as a voucher in the SMDB Herbarium at the Federal University of Santa Maria (Table 1).

Table 1 - Collection site, voucher and number of individuals (samples) collected per population of *T. polymorphum*

Municipality	**Latitude (S)**	**Longitude (W)**	**Voucher**	**N° samples**
Eldorado do Sul	30°53’15.1”	051°41’12.16”	SMDB20489	17
São Borja*	28°66’08.3”	056°00’38.89”	SMDB20488	17
São Gabriel	30°20’42.6”	054°16’08.4”	SMDB20492	34
São Pedro do Sul	29°29’20.3”	054°11’16.7”	SMDB20483	17
Tupanciretã	29°60’73.86”	054°30’25.83”	SMDB20486	18

* Location of headquarters - the latitude (S) and longitude (W) values refer to the municipality where the collection was carried out and not to the collection site itself. Source: Authors (2025)

Total DNA extraction was performed using the CTAB (cetyltrimethylammonium bromide) method described by Doyle and Doyle (1987), adapted to microcentrifuge tubes, using 50 mg of dried leaf material macerated in liquid nitrogen. DNA integrity and quantification was analyzed by electrophoresis in 0.8% agarose gel, stained with ultrasensitive fluorescent nucleic acid dye (GelRed) in a 0.5X TBE buffer (Tris, boric acid, EDTA). Quantification of genomic DNA extracted from *T. polymorphum* leaves was performed by visual comparison of band intensity with phage λ DNA on 0.8% agarose gel. Samples were compared with standard DNA (λ). Standardized aliquots (25 nanograms of DNA) were prepared in sterile ultrapure water for the PCR reactions. A total of 20 primers were tested, out of which four were selected to be used $(GA)_8YC$, $(AG)_8T$, $(GACAC)_3$ and $(AG)_8TA$).

The PCR reactions were performed in a final volume of 25 µL, containing: ultrapure H_2O (15.9 µL), 10X Buffer with 20 Mm $MgCl_2$ (2.5 µL), 25 mM $MgCl_2$ (2.4 µL), primer 25 µM (1 µL), 200 µM dNTP (1µL), DMSO (1µL), Taq DNA polymerase (0.2 µL) and sample DNA (1 µL). The amplifications were developed using a thermal cycler (MJ Research Minicycler). The amplification conditions were established as an initial cycle of 5 min at 94 ºC; followed by 40 cycles at 94 ºC for 1 min (denaturation); 45 sec at the specific temperature of each primer (annealing); 2 min at 72 ºC (extension); and a final extension of 5 min at 72 ºC. Amplified products were separated by electrophoresis on

1.2% agarose gel in a 0.5X TBE buffer under constant voltage of 100 volts and compared with two ladders (50 bp and 1Kb). The gels were stained with GelRed and the bands visualized under ultraviolet light in a UV transilluminator (HOEFER-MacroVue/UV-20).

2.1 Statistical analysis

After performing the PCR reactions, a binary matrix was prepared considering presence (1) and absence (0) of the amplified fragments (online resource 1). Descriptive analyses of the data including total number of bands (NTB), number of polymorphic bands (NBP) and percentage of polymorphic bands (PBP) were also performed. Subsequently, the presence/absence matrix data were subjected to analyses using the statistical software GenAlEx 6.5 (Peakall and Smouse, 2012) and the programs Structure (Pritchard et al., 2000), and Structure Harvester (Earl and vonHoldt, 2012).

3 RESULTS

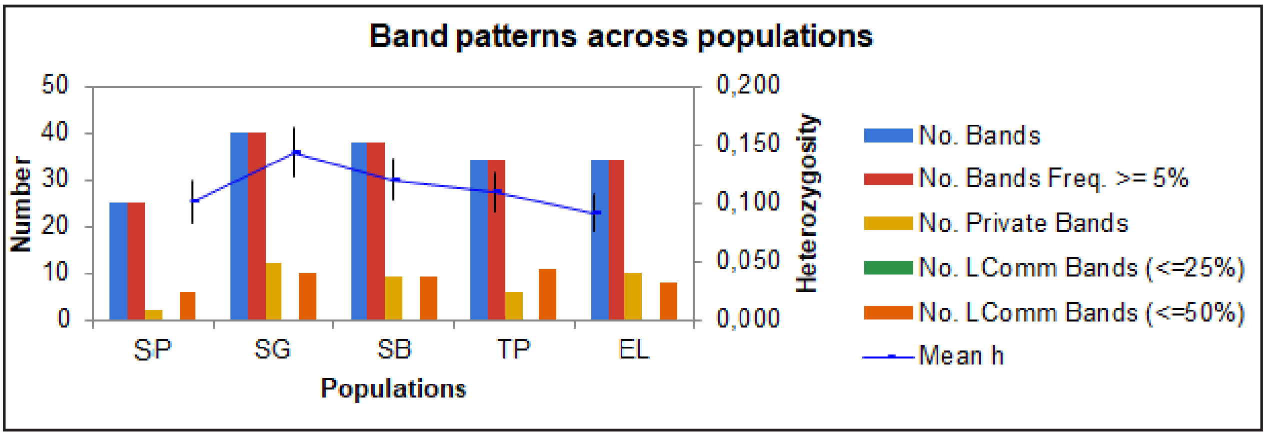
The genetic diversity of a total of 64 individuals from five populations of *T. polymorphum* was then analyzed using ISSR markers. The products amplified with the different primers ranged from 100 bp to 2900 bp between populations and between individuals (Table 2).

Table 2 – Minimum, average, and maximum size of the bands in base pairs, for each *primer* analyzed

Primer	Band size (bp) Minimum	Average	Maximum
F4	100	760	1600
I1	100	970	2500
I4	100	950	2900
I5	100	820	2000

Source: Authors (2025)

Figure 2 – Banding patterns of the five populations of *Trifolium polymorphum*



Populations: SP - São Pedro do Sul; SB- São Borja; SG - São Gabriel; EL-Eldorado do Sul; TP-Tupanciretã. - Diversity. The orange line indicates the He mean

This number of data allowed us to identify and analyze many *loci* (172), as can be seen in Figure 2, which shows the banding patterns for the different populations, including total number of bands, common bands, unique bands, and diversity.

The percentage of polymorphic *loci* showed variation according to the collection site (Table 3), with the lowest values (29.07%) in the São Pedro do Sul population, and the highest values for the São Borja (44.19%) and São Gabriel (43.02%) populations.

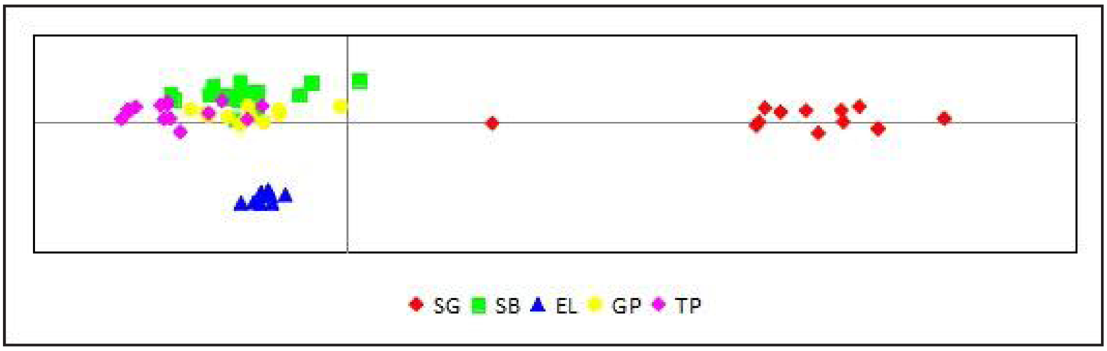
Table 3 – Mean genetic diversity indices in the analyzed populations of *T. polymorphum*

Population	**P (%)**	**Na**	**Ne**	**I**	**H**
São Gabriel	43.02%	0.895	1.244	0.217	0.143
São Borja	44.19%	0.884	1.187	0.190	0.120
Eldorado do Sul	31.40%	0.721	1.142	0.147	0.094
São Pedro do Sul	29.07%	0.581	1.169	0.151	0.100
Tupanciretã	39.53%	0.791	1.163	0.175	0.110

P - Percentage of polymorphic *loci*; Na - Number of different alleles; Ne - Number of effective alleles; *I* - *Shannon* Index and h - Diversity. Source: Authors (2025)

Of the five populations evaluated, the São Gabriel population had the highest genetic diversity index (H= 0.143) and the highest Shannon index (I= 0.217), while the Eldorado do Sul population had the lowest diversity index (= 0.094) and lowest Shannon index (I =0.147) (Table 3). Principal Coordinates Analysis (PCoA) was able to clearly separate the populations into three genetic groups (Figure 3).

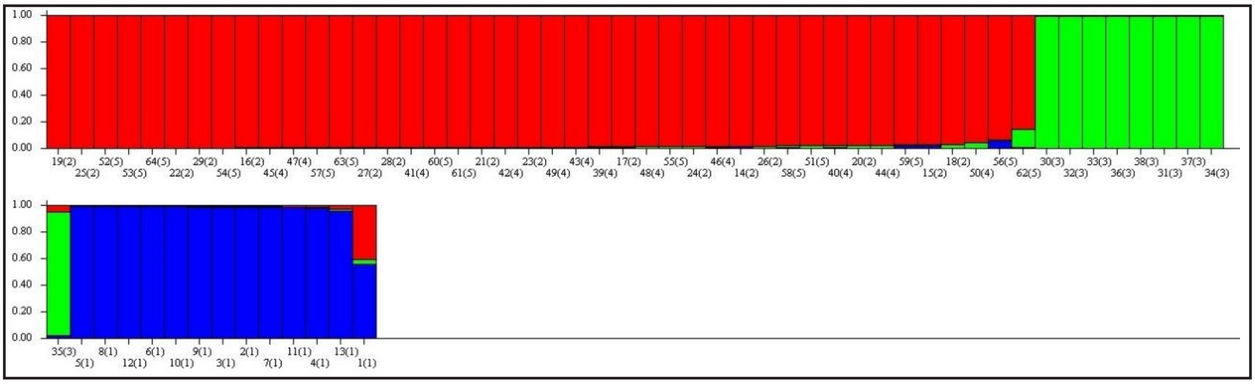
Figure 3 – Principal Coordinates Analysis (PCoA) in *Trifolium polymorphum* populations in Rio Grande do Sul, Brazil. SP - São Pedro do Sul; SB- São Borja; SG - São Gabriel; EL- Eldorado do Sul; TP- Tupanciretã



Source: Authors (2025)

Thus, what was indicated is that samples collected in two localities have genetically very distinct individuals (São Gabriel and Eldorado do Sul), while three other populations are genetically very close (São Borja, Tupanciretã, and São Pedro do Sul). Bayesian inference analysis separated the five populations into three clusters. Whole populations were grouped into a single cluster with little mixing between individuals from different populations (Figure 4).

Figure 4 – Genetic structure of the five *Trifolium polymorphum* populations with the formation of three *clusters*. Blue (1) = Eldorado do Sul. Red (2) = Tupanciretã; São Pedro do Sul, São Borja. Green (3) = São Gabriel

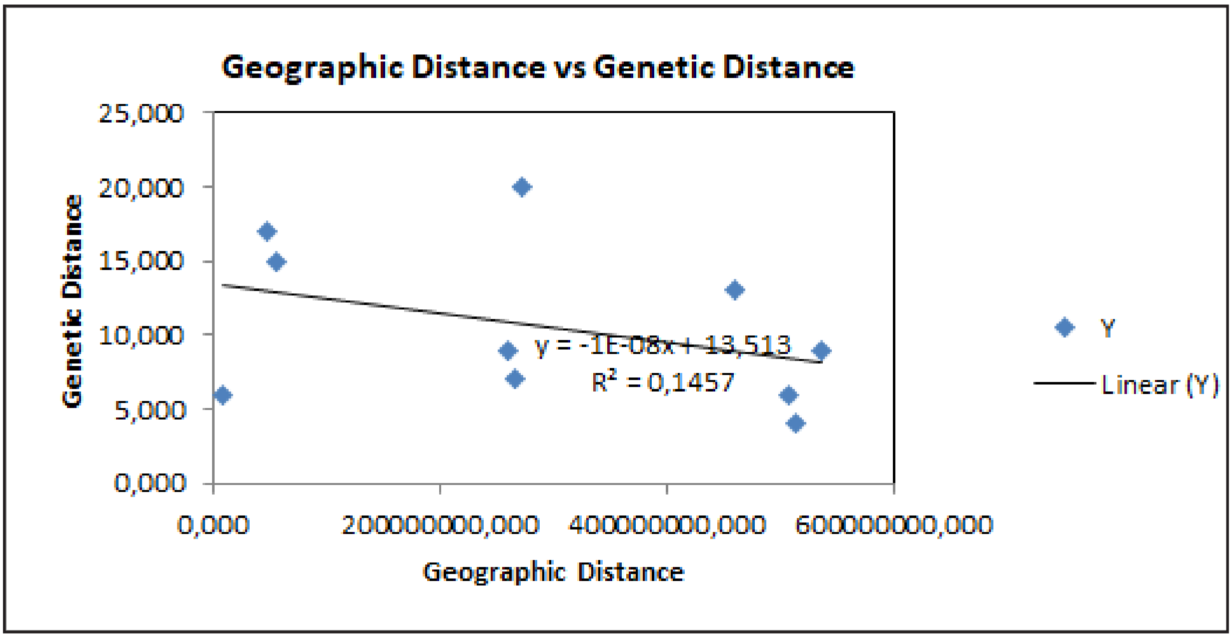


Source: Software *Structure*

The PCoA analysis (Figure 3) shows that the populations of Eldorado do Sul and São Gabriel are isolated from the others. Although there is mixing between populations, a single gene pool still prevails, suggesting that our sampling strategy likely archieved the correspondence to natural populations.

With the result of the Mantel Test (Figure 5), which analyzes the relationship between geographic distance and genetic distance, it is possible to see that geographic distance is not the main factor of this genetic structuring (r^2 = 0.145), with grouping of populations occurring at a distance of up to 215 km (São Pedro do Sul - São Borja). Although there is no correlation between genetic and geographical distance, it is interesting to note that populations from municipalities in the northern plateau of Rio Grande do Sul tended to group together, while the populations of São Gabriel (*Campanha* Region) and Eldorado do Sul, (Central Depression), were clearly separated.

Figure 5 – Result of the Mantel Test, performed on GenAlEx, testing the correlation of geographic distance and genetic distance. Result for 99 permutations. r2=0.1457



Source: Authors (2025)

4 DISCUSSION

ISSR molecular markers are used in different species of the family Fabaceae, including the genus *Trifolium*, confirmed by the works of Dalla Rizza et al. (2007); Aryanegad et al. (2013); Nosrati et al. (2015); Hwang and Man (2016); Abate (2017); Oppmann and Morris (2021).

According to Xia et al. (2016), the percentage of polymorphic *loci* has been used to evaluate genetic diversity in studies with natural populations and dominant markers. Of the five populations evaluated, the São Gabriel population had the highest genetic diversity index (H= 0.143) and the highest Shannon index (I= 0.217). These data corroborate studies using SSRs by Conterato et al. (2018), evidencing a higher diversity of the species in the phytogeographic region of the *Campanha*. The Eldorado do Sul population, on the other hand, obtained the lowest diversity index (H= 0.094) and Shannon index (I=0.147).

When performing an AMOVA for the partitioning of genetic variation at two levels, within populations and between populations, it was observed that for the patterns of all primers, the highest variation occurred most frequently within populations (56 %) than between populations (44 %). This information, that the highest variation occurred within populations (56 %), agrees with studies by Dalla Rizza et al. (2007) who used ISSR molecular markers for species of the same genus, *T. medium* and *T. argentinense*, which also showed the highest level of polymorphism within populations, 89 % and 92 %, respectively. This also corroborates studies by Zohary and Heller (1984), who identified the high degree of intraspecies polymorphism in other studies with plants of the genus. Hwang and Huh (2016), in a genetic diversity study of five species of the genus *Trifolium*, also using ISSRs, identified a higher variation of polymorphism (62%) among populations.

The proposed classification for the breeding system of the *T. polymorphum* is that it is an allogamous, self-compatible species that benefits from pollinators to set seed (Real et al., 2007). However, *T. polymorphum* is an amphicarpic species (produces aerial and subterranean seeds on the same plant) and propagates vegetatively by regrowth from storage roots as well (Speroni et al., 2014; Conterato et al., 2019).

A possible explanation for the greater variation within populations is that the original population could be composed mainly of plants derived from aerial seeds (individuals were collected respecting 3 m of distance) and, therefore, the collected plants would be derived from different genotypes.

Dalla Rizza et al. (2007) studied the genetic diversity of 34 genotypes of six South American (*T. polymorphum* Poir., *T. riograndense* Burkart, *T. argentinense* Speg.) and Eurasian (*T. medium* L., *T. pratense* L. and *T. repens* L.) *Trifolium* species using ISSR markers. The South American species showed a higher number of bands than the Eurasian species. The percentage of intraspecies polymorphism was 46.4 % for *T. polymorphum*, 78 % for *T. riograndense* Burkart and 92.9 % for *T. argentinense* Speg.. It is important to emphasize that none of these three species are from natural grasslands in Brazil, but from Uruguay. However, this information of intraspecies polymorphism of South American species corroborates the results found in this study.

Aryanegad et al. (2013) characterized the genetic variation of 14 accessions of three *Trifolium* species from Iran and obtained 75 bands in total, all polymorphic, with 60 % polymorphism in *T. fragiferum*, 58.67 % in *T. hybridum* and 77.32 % in *T. pratense*, with within-species variation accounting for 71% of the total molecular variation. "

Information on the genetic diversity of natural populations of *T. polymorphum* from southern Brazil was scarce. Conterato et al. (2018) performed an analysis of genetic diversity in 10 natural populations of *T. polymorphum* collected between 2002 and 2010, using 20 microsatellite markers. From the sampling of these ten populations, our work sampled from two common municipalities (Eldorado do Sul and São Gabriel), however, in different geographic coordinates. In comparison with our study, the genetic diversity observed in *T. polymorphum* in the work of Conterato et al. (2018) was high in all the populations from the different phytogeographic regions of Rio Grande do Sul sampled.

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, especially between different molecular markers. However, several studies (Dalla Rizza et al., 2007; Dabkevičienė et al. 2011; Dabkevičienė and Paplauskienė, 2012; Hwang and Huh, 2016) confirm the importance of using molecular markers, specifically ISSRs, to assess diversity in *T. polymorphum*.

In the Principal Coordinates Analysis (PCoA), the São Gabriel and Eldorado do Sul populations were isolated, corroborating a greater number of unique bands exhibited by these populations. The other populations (São Pedro do Sul, São Borja and Tupanciretã) appear grouped together.

Another way to measure molecular genetic diversity is to use the Shannon Diversity Index, which can be employed both to compare diversity between species and between populations of the same species. The mean value for the Shannon Diversity Index for *T. polymorphum* was 0.17 and the highest range was 0.21, with no great variation between the values. According to the literature, the Shannon index can vary between 0 and 1, with 1 being the maximum genetic diversity of a population (Lewontin ,1972). Thus, the values found in this study show that the populations have low genetic diversity.

In comparison with the Shannon index found in other studies, these values are low. Haerinasab et al. (2020), in a genetic diversity study using microsatellites of *Trifolium fragiferum* L. in Iran, obtained an average of diversity index (H) and Shannon index (I) of H= 0.23 and I= 0.38. Wesen (2017), in studies with the species of *Trifolium steudneri*, native to the highlands of Ethiopia, using ISSR markers, obtained an average diversity H = 0.26 and Shannon I= 0.39. In *Trifolium quartinianum* (Abate and Tesfaye, 2017) in Ethiopia, the authors obtained the following indices, H=0.29 and I=0.44.

In general, the ISSR molecular markers selected were efficient to reveal and quantify the genetic diversity of *T. polymorphum* in natural populations from the state of Rio Grande do Sul, Brazil. The populations analyzed showed low genetic diversity and, yet, they have a definite genetic structuring among the populations analyzed.

The use of a dominant molecular marker in this study has expanded the genetic diversity information of the species, contributing with important data and still emphasizing that there is more molecular variation within populations than between them.

Since environmental change is a continuous process, the loss of genetic diversity decreases the adaptive value of the species, making it less apt to such changes. The low genetic diversity found in *T. polymorphum*, when compared to other studies (Abate and Tesfaye, 2017; Conterato et al., 2018), cannot be ignored since its loss can lead to inbreeding and deficit in reproductive success.

The results found in this work on diversity have several implications for effective strategies that can be used to plan future germplasm collection for the management conservation purposes of natural grasslands in the state of Rio Grande do Sul, in particular, the need to monitor the size of natural populations and the genetic diversity in the coming years to ensure that genetic erosion is avoided.

One suggested action would be to monitor grasslands fragmentation, since fragmentation of the ecosystems have a huge impact on genetic diversity of the species. Another action could be to create or to expand germplasm collections of the species, with the purpose to be a source of different genetic types for grasslands restoration.

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