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Genetic structure of *Dicksonia sellowiana* Hook. (Dicksoniaceae) reveals clinal distribution along the latitudinal gradient of the Atlantic Forest

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ABSTRACT

Dicksonia sellowiana is the only species of the genus occurring in Brazil. Its distribution is restricted to humid areas of the Atlantic Forest biome. The distribution pattern of biodiversity in this biome is known to have been influenced by historical and environmental factors, although the pattern for ferns remains unknown. This study is first to describe the genetic structure of *D. sellowiana* along the latitudinal gradient of the Atlantic Forest biome. We use microsatellite markers to estimate genetic diversity and structure for 267 individuals representing 14 populations of *D. sellowiana* from the Atlantic Forest. The results (Ho, He, Fst, Fis, distance genetic) support the hypothesis of a pattern of biodiversity discontinuity. We found greater genetic variability in populations located in regions of higher humidity and milder temperatures. Our data suggest that there is a clinal distribution pattern of genetic variation along the north to south latitudinal gradient of the Atlantic Forest. This clinal variation has a genetic basis in the frequencies of the two genetic groups. This structure does not evidence long-standing historical barriers to gene flow and favors the influence of landscape characteristics on the establishment of populations.

Keywords: conservation, neotropics, gene flow, genetic diversity, microsatellites, tree fern

Introduction

Biodiversity studies of hotspots aim to increase knowledge about the richness and dynamics of native species in order to safeguard biomes (Myers *et al.* 2000). The Neotropics covers biomes with high diversity and endemism of species (Antonelli & Sanmartín 2011). Native species of the Atlantic Forest exhibit different patterns of biological diversity as a result of its extensive latitudinal gradient, which, in turn, is correlated with environmental variation along the area of occurrence of the biome in the east coast region of the Neotropics (Caley & Schluter 1997; Gaston 2000; Ribeiro *et al.* 2009). Furthermore, environmental characteristics associated with habitat reduction through fragmentation may restrict the distribution of native species, making them more vulnerable to environmental changes and increasing their risk for extinction (Laurance 1991; Myers *et al.* 2000; SOS Mata Atlântica/INPE 2008). Latitudinal variation of the Atlantic Forest has resulted in a mosaic of heterogenous phytophysiognomies, leading to a considerable number of narrowly endemic species throughout its distribution — about 526 species of vertebrates and 8000 species of plants are endemic to the Atlantic Forest (Myers *et al.* 2000).

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The patterns of species diversity observed in the Atlantic Forest can also be explained by historical factors associated with aspects of geography and climate (Wiens 2007). Oscillations in temperature and humidity during the Quaternary resulted in the distribution of species in refuges along the entire latitudinal gradient of the Atlantic Forest (Behling 1997; 2002; Carnaval & Bates 2007; Carnaval *et al.* 2014). Studies on the reconstruction of flora and climate show evidence of forest contraction in regions of high humidity and low elevation during the last glacial maximum (Behling, 1997; 2002; Pinheiro *et al.* 2011). Historical factors related to the distribution of flora can be understood via studies of the patterns of genetic diversity of native species of the Atlantic Forest (Behling 1997; 2002; CBD 2016).

Dicksonia sellowiana (Dicksoniaceae.) is an endemic tree fern species of the Neotropics. The species is popularly known as xaxim or xaxim-bugio, and is considered endangered (CITES 2009). Dicksoniaceae emerged at the end of the Jurassic period, about 157 million of years ago (Myr) (Noben *et al.* 2017). There is high endemism among species of pteridophytes in the Neotropical Region (Noben *et al.* 2018). The processes involved in the emergence and speciation of species of *Dicksonia* were related to Gondwanan biogeographic elements, the distribution of which are likely associated with tectonic events (McLoughlin 2001; Heads 2005; Noben *et al.* 2017; 2018). *Dicksonia* contains 30 species that are distributed in humid environments with temperate temperatures in the northern regions of Australia, New Zealand, Malaysia, southern North America (Mexico), and Central and South America (Kramer & Green 1990; Noben *et al.* 2018). *Dicksonia sellowiana, D. gigantea, D. karsteniana* and *D. stuebelli*. are endemic to the Neotropics (Tryon & Tryon 1982; Perez-Garcia 1995), while *D. sellowiana* is the only species of the genus registered for Brazil and occurs in fragments of Atlantic Forest (Condack 2015). The species occurs in altitudinal humid forests from the states of Espirito Santo and Minas Gerais in the north to northern Rio Grande do Sul State in the Serra Geral region to the south (Condack 2015). Dispersion events for the genus show that South American species derived from Central American regions with distributions along the Pacific Coast to the South of the continent and later into areas of the east coast of Brazil (Noben *et al.* 2017). Therefore, it is possible that *D. sellowiana* is no older than 2 Myr (Noben *et al.* 2017).

The history of the formation of the Atlantic Forest resulted in a highly heterogeneous biome with different distribution patterns at different levels of biodiversity. Thus, our main goal was to understand the genetic diversity of natural populations of *D. sellowiana* in Brazil, considering its area of occurrence along the east coast of the Atlantic Forest biome. This is the first population genetics study of *D. sellowiana* of the Atlantic Forest. The species is highly endangered and the acquisition of molecular data will help to better understand the historical-evolutionary context of ferns in the Neotropical region.

Materials and methods

Sampling and DNA extraction

Our sampling included a total of 267 individuals representing 14 populations of *Dicksonia sellowiana* from the Southeast and South regions of Brazil (Tab. 1). The sampled populations are in areas of native forest and covered the distribution of the species along the latitudinal gradient of the Atlantic Forest (Fig. 1). Samples were collected from fronds and preserved on silica gel. Extraction of DNA was based on the protocol of Roy *et al.* (1992), with subsequent storage at -20 °C. Vouchers were deposited at the herbarium of Universidade Federal do Paraná (UPCB).

PCR-Microsatellites

Molecular analysis was conducted using eight microsatellite loci (*Simple Sequence Repeats* SSR) developed by Nazareno *et al.* (2013) for *D. sellowiana*. Polymerase chain reaction (PCR) was conducted with a final volume of 15μL containing 1X PCR *buffer*, 1 mM of $MgCl_2$, 0.25 mM of each dNTP, 0.5 μM of each *primer,* 1 U of *Taq* DNA polymerase and 15 ng of genomic DNA. Conditions for PCR included initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of 94 °C for 30 seconds, annealing temperature of the *primer* for 45 seconds and 72 °C for 1 min, and finally 72 °C for 15 min for final extension of the fragments. The annealing temperatures for the SSR *loci* are provided in Table 2. The results of the reactions were evaluated in 2 % agarose gel stained with ethidium bromide (0.5 μg mL^{-1}). Alleles of the SSR *loci* were subsequently analyzed by automatic DNA genotyping (*3500xL Genetic Analyzer*) using LIZ-600 (*GeneScan – Applied Biosystems*) as the standard for fragment size. Individuals from each population were genotyped according to the alleles present at each locus using Genemaker *software* (SoftGenetics LLC).

Analysis of SSR data

Average observed heterozygosity (Ho), average expected heterozygosity (He), Nei's genetic distance, Wright's F statistics and gene flow were calculated using GenAIEx 6.5016.501 software (Peakall & Smouse 2012). Analysis of molecular variance (AMOVA) and estimated overall and pairwise Fst's were performed to assess the partitioning of genetic variation among populations using Arlequin software version 3.5 (Excoffier & Lischer 2010) with 1,000 permutations. Pairwise genetic distances among populations were estimated using Populations 1.2.32 software (Langella 2002). The relationships were viewed in a neighbor-joining tree derived from chord genetic distances (Cavalli-Sforza & Edwards 1967). Each node in the tree was evaluated with

Table 1. Location (municipality, latitude, longitude and altitude), voucher and number of individuals sampled (N) for each of the studied populations of *Dicksonia sellowiana* in Brazil.

Population	Municipality	Latitude (S)	Longitude (W)	Altitude (m)	Voucher	N
ES1	Castelo	20°36'19.1"	41°12'10.1"	370	UPCB 68341	16
ES2	Dores do Rio Preto	20°33'13.6"	41°49'03.4203.42"	942	UPCB 68340	20
MG1	Alto Caparaó	20°27'28.2"	41°57'02.5"	1620	UPCB 68339	20
MG2	Alto Caparaó	20°27'26.6"	41°57'02.8"	1540	UPCB 68338	20
RJ1	Teresópolis	22°25'01.3"	43°03'27.1"	1562	UPCB 67236	20
RJ2	Resende	22°28'09.8"	44°37'29.4"	1010	UPCB 68342	20
SP1	São José do Barreiro	22°38'44.9"	44°35'05.1"	1220	UPCB 68336	19
SP2	São José do Barreiro	22°38'46.4"	44°35'04.7"	1188	UPCB 68337	20
PR1	Balsa Nova	25°34'39.1"	49°38'10.9"	863	UPCB 67355	12
PR2	Quatro Barras	25°22'49.8"	49°04'36.7"	891	UPCB 67351	20
SC1	Irani	27°01'31.5"	51°53'57.7"	650	UPCB 67350	20
SC2	Vargem Bonita	27°00'21.3"	51°44'58.2"	740	UPCB 67354	20
RS1	São Francisco de Paula	29°25'53.7"	50°32'15.7"	854	UPCB 67352	20
RS2	São Francisco de Paula	29°25'54.4"	50°32'16.3"	855	UPCB 67352	20
Total						**267**

ES: Espírito Santo, MG: Minas Gerais, RJ: Rio de Janeiro, SP: São Paulo, PR: Paraná, SC: Santa Catarina, RS: Rio Grande do Sul; UPCB: Herbarium of Universidade Federal do Paraná.

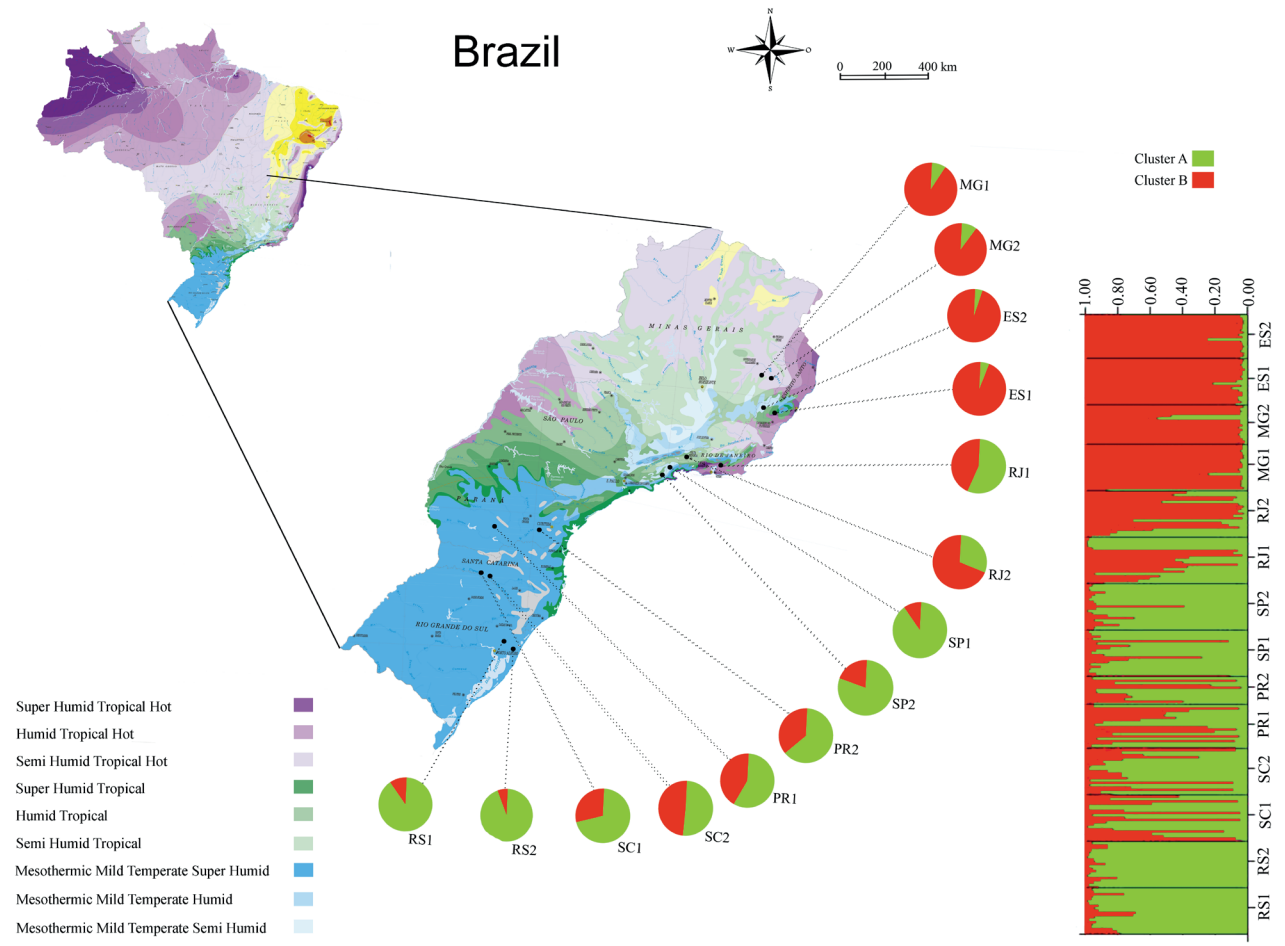

Figure 1. Genetic structure of *Dicksonia sellowiana* Hook. according to the genetic (K) clusters established by Bayesian analysis using eight SSR loci (Table 2). The populations sampled are identified in the Brazilian Climate Map (IBGE 2002). The colors correspond to the climatic categories according to the IBGE (2002) and in the legend are shown only the categories where the populations of the species studied were sampled.

1,000 repetitions of bootstraps over loci. The dendrogram of the neighbor-joining tree was visualized in TreeView 1.6.6 (Page 1996). Correlation between estimated values of Nei's distance among populations and geographical distance was tested by the Mantel test with 1,000 permutations using NTSYS 2.01 software (Mantel 1967; Podani 2000).

Linkage disequilibrium (LD) was estimated according to the D statistic of Kimura & Ohta (1969) using PopGene 1.32 (Yeh *et al.* 1999). The percentage of null alleles was estimated to evaluate the information content of the loci using Cervus 3.0.3 software (Marshall *et al.* 1998; Kalinowski *et al.* 2007). The program STRUCTURE version 2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003; Pritchard *et al.* 2010) was used to determine the distribution of *clusters* among populations through grouping based on the Bayesian model. In order to determine the ideal number of genetic groups (number of *clusters* = K) simulations were performed assuming that it was possible to obtain any number of *clusters* between one and 15. The *admixture* ancestry model was used for this analysis, with the allele frequencies correlated for 250,000 *burn-in* and, subsequently 1,000,000 Markov Chain Monte Carlo (MCMC) repetitions. The most probable K among those proposed by the analysis was defined using the criterion of Evanno *et al.* (2005) by means of the program Structure Harvester version 6.93 (Earl & Vonhold 2012).

Pearson correlation ($\alpha = 0.05$) calculated with R *software* (R Development Core Team 2017) was used to assess correlations between abiotic factors and the genetic diversity of *D. sellowiana*. Mean temperature (°C) and precipitation (mm) for the last 25 years and elevation (m) and latitude (°) of each population were used as predictor variables (Tab. 1). Values for temperature and precipitation were obtained from the most recent climate classification of Köppen (1936) published by Alvares *et al.* (2014). The response variables (genetic diversity) used were Ho, He and the inbreeding coefficient (F_{IS}).

Results

The eight SSR *loci* used in this study were all polymorphic and amplified 32 alleles for an average of 3.8 alleles per *locus*. The greatest number of amplified alleles (seven) was for *locus* DIC03 (Tab. 2). Three exclusive alleles were identified, one each for *loci* DIC06, DIC10 and DIC12 for populations in the South Region of Brazil (Paraná [PR], Santa Catarina [SC] and Rio Grande do Sul [RS]). Mean values for the genetic diversity indices for the species in Brazil were: He = 0.50, Ho = 0.45 and F_{IS} = 0.08 (Tab. 3). Populations of the states of Espírito Santo (ES), PR, SC and RS had the highest heterozygosity indices, with observed heterozygosity in these regions being greater than 0.50, with emphasis on the populations of Quatro Barras (PR2) and Irani (SC1). Pairwise F-statistics revealed a significant F_{ST} value among populations (F_{ST} = 0.15) with average gene flow (Nm) between all populations being 1.89 individuals per generation.

The AMOVA showed that most of the variation was within populations (63 %), with a significant F_{ST} (F_{ST} = 0.15, $p < 0.001$; Tab. 4). The dendrogram based on genetic distances grouped the 14 populations into three groups with high bootstrap values (>96 %; Fig. 2). In general, the most distinctive and isolated populations were from South Region. The populations of RS and SC formed a group isolated from the other populations. Interestingly, populations in the Southeast Region were grouped with populations of PR, where this represents the most external group in this clade. The Mantel test indicated a non-significant relationship between genetic and geographic distances (r = 0.42657; p = 0.03). The percentage of null alleles was less than 1 % for all *loci* (Tab. 2). The D statistic proposed by Ohta (1982) varied among the evaluated *loci* as: $D_{IS}^2 < D_{ST}^2$ and $D'_{IS}^2 > D'_{ST}^2$. In other words, according to mean values, *loci* exhibited non-significant linkage disequilibrium ($p > 0.001$) in the studied populations.

The number of *clusters* defined by STRUCTURE was K = 2 (Fig. 1). Genetic group A was predominant in the populations of São Paulo (SP), PR, SC and RS, and genetic group B was most represented in the populations of ES, Minas Gerais (MG) and Rio de Janeiro (RJ).

According to Pearson correlation analysis, latitude was significantly positively correlated with He (r = 0.80, p = 0.0005). In addition, regional precipitation was found to be directly correlated with population He (r = 0.61; p =

Table 2. Genetic parameters for each microsatellite locus for the 14 studied populations of *Dicksonia sellowiana*.

Locus	AT	Size Range (pb)	A	Ho	He	FIS	FST	FIT	Nul
DIC01	50	262 - 286	5	0.48	0.24	0.13	0.28	0.17	0.34
DIC02	52	207 - 214	4	0.61	0.46	0.12	0.16	0.27	0.09
DIC03	55	240 - 270	7	0.65	0.23	0.48	0.11	0.54	0.56
DIC06	56	280 - 298	3	0.50	0.56	-0.03	0.07	0.04	0.12
DIC08	50	215 - 223	3	0.49	0.52	-0.27	0.13	-0.09	0.08
DIC10	58	272 - 274	3	0.43	0.37	-0.04	0.12	0.07	0.10
DIC11	58	162 - 172	4	0.56	0.34	-0.01	0.12	0.33	0.36
DIC12	56	268 - 282	3	0.69	0.52	0.26	0.11	0.34	0.19
Mean			**3.8**	**0.49**	**0.59**	**0.10**	**0.15**	**0.24**	**-**

A_T: *Primer* annealing temperature (°C); A: Number of alleles; Ho: observed heterozygosity; He: expected heterozygosity; I: Shannon's index, F_{IS}: inbreeding coefficient, Nul: null alleles (%).

0.0204). Elevation was significantly negatively correlated with Ho (r = -0.67, p = 0.007) and He (r = -0.62, p = 0.01). The Ho of populations of *D. sellowiana* was not significantly (p > 0.05) influenced by precipitation or latitude, and F_{IS} was not influenced by any of the abiotic variables evaluated (p > 0.05). The response variables analyzed — Ho, He and F_{IS} — were not significantly (p > 0.05) correlated with temperature.

Discussion

We studied the genetic diversity and population structure of *Dicksonia sellowiana*, an endemic species of the Neotropics. Our main goal was to understand how populations are genetically structured along the latitudinal gradient of the distribution of the species in Brazil and to understand

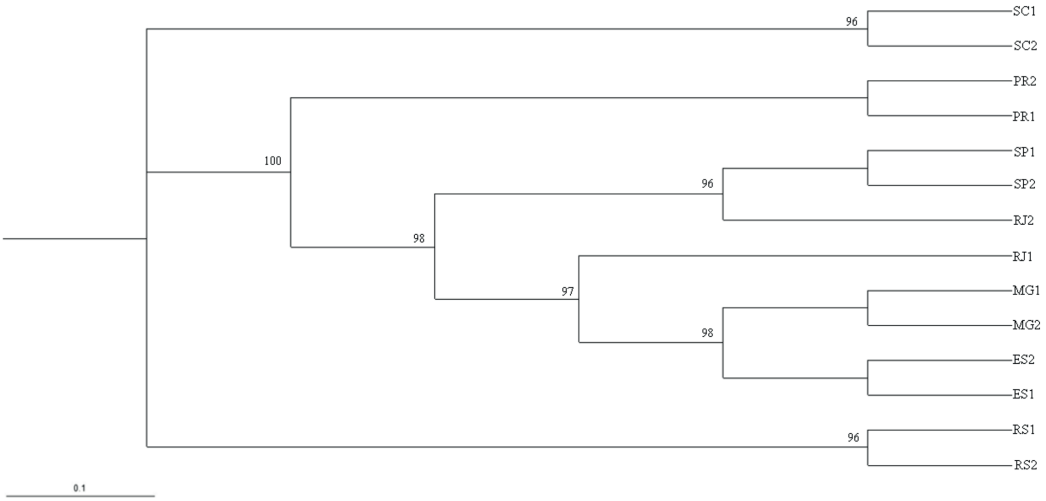

Figure 2. Neighbor-joining (NJ) tree for the 14 *Dicksonia sellowiana* Hook. populations based on DC genetic distance (Cavalli-Sforza and Edwards, 1967). Numbers at nodes represent percentages 1000 bootstrap replicates.

Table 3. Genetic parameters based on eight microsatellite loci for the 14 studied populations of *Dicksonia sellowiana*.

Population	Ho	He	F_{IS}
ES1	0.60	0.45	-0.32
ES2	0.55	0.46	-0.21
MG1	0.18	0.38	-0.53
MG2	0.45	0.43	0.03
RJ1	0.25	0.38	0.28
RJ2	0.13	0.39	0.57
SP1	0.19	0.34	0.57
SP2	0.34	0.47	0.20
PR1	0.58	0.61	0.03
PR2	0.73	0.64	0.15
SC1	0.78	0.65	-0.21
SC2	0.51	0.64	0.18
RS1	0.50	0.58	0.12
RS2	0.51	0.60	0.13
Mean	**0.45**	**0.50**	**0.08**

Ho: observed heterozygosity; He: expected heterozygosity; F_{IS}: inbreeding coefficient.

Table 4. Analysis of Molecular Variance (AMOVA) of the 14 studied populations of *Dicksonia sellowiana* of the Atlantic Forest.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	p*
Among populations	13	189.6	0.305	12	< 0.001
Among individuals within groups	253	751.9	0.659	25	< 0.001
Among individuals within populations	267	441.5	1.654	63	< 0.001
Total	**553**	**1383.08**	**2.618**	**100**	
Fixation index = F_{ST}: 0.15					

d.f.: degrees of freedom; *p = significance tests (1023 permutations)

the processes involved. Using SSR markers and AMOVA analysis, we found that most of the genetic diversity of *D. sellowiana* is within populations (Tab. 4).

The distribution of *Dicksonia sellowiana* in Brazil is restricted, which implies a tendency for inbreeding within populations as suggested by high and significant F_{IS} values (Tab. 3; Fiori *et al.* 2009). The species is homosporous and reproduction by gametophytes may be the key to explaining why its distribution is limited to the Atlantic Forest, which is fundamental to understanding its population structure (Fiori *et al.* 2009; Noben *et al.* 2017). Spores of *D. sellowiana* are small and light, similar to dust, and can be easily dispersed by rain and wind, which facilitates the dispersal of individuals (Tryon & Tryon 1982; Tryon & Lugardon 1991; Fernandes 2000; Fiori *et al.* 2009). However, the species is dependent on high humidity for reproductive success since spore germination and fertilization of gametes in the gametophytic phase occur in the presence of water (Fernandes 2000; Fiori *et al.* 2009).

We found through Mantel´s test that the genetic distance between populations was not related to the geographical distance. This was also shown by the dendrogram with populations from RS and SC each forming its own group isolated from the other populations (Fig. 2). Gene flow is conditioned by different factors, such as dispersal ability, geographical distance between populations, characteristics of the environment and ecological factors (Cushman *et al.* 2016; Mäder *et al.* 2019). Values for the indexes of genetic diversity (He and Ho) and their respective correlations with characteristics of the environment show that the dynamics of gene movement among populations of *D. sellowiana* can clearly be explained by differences in characteristics of the landscape (*i.e.,* precipitation and altitude) throughout the distribution of the Atlantic Forest (Schwartz & Gasper 2020). The F_{ST} value supports intermediate differentiation between populations, according to Wright (1965). This pattern of discontinuity of genetic diversity associated with the value of F_{ST} was also observed for *Araucaria angustifolia* (Bittencourt & Sebbenn 2009; Souza *et al.* 2009), a tree species that co-occurs with *D. sellowiana* in Brazil (Biondi *et al.* 2009; Mallmann *et al.* 2018).

Perhaps, the most prominent finding of our results is the decrease in genetic variability from north to south, which was strongly correlated with latitude. These data suggest a clinal distribution pattern for gene frequencies of *D. sellowiana* along the latitudinal gradient of the Atlantic Forest (Endler 1973; Noben 2017). Latitudinal clinal variation has a genetic basis in the frequencies of the two structured groups from north to south on the east coast (Cushman *et al.* 2016). Furthermore, there is no evidence of longstanding historical barriers to gene flow, which supports the influence of landscape characteristics on the establishment of populations, such as anthropic fragmentation of the biome, for example (Tryon & Tryon 1982; SOS Mata Atlântica/INPE 2008; Fiori *et al.* 2009; Noben *et al.* 2017).

The latitudinal gradient of the Atlantic Forest presents variation in humidity from north to south according to the classification of phytophysiognomies that compose the biome (Fig. 1; Morellato & Haddad 2000; Pellegrino *et al.* 2005; Thomé *et al.* 2014). In the states of PR, SC and northern RS, the vegetation is characterized by the Araucaria Moist Forest (IBGE 2012). The climate in these regions lacks a biologically dry period and a long winter period (Leite 2002; Alvares *et al.* 2014). The sampled populations with lower values for genetic variability correspond to regions of Dense Evergreen Forest (IBGE 2012). Periods of low humidity can be restrictive for *D. sellowiana* because the species requires water for gametophyte reproduction and spore germination and does not possess adaptations for dry periods (Fiori 2009; Schwartz & Gasper 2020). The characteristics of the landscapes where the populations were sampled may be reflected in the observed pattern of genetic distance between them (Fig. 2), in the Ho and He indices (Tab. 3) and in the frequencies of the genetic groups (Fig. 1). The populations of SP, RJ, MG and ES were sampled in relict wetland areas at high altitudes (IBGE 2012). Forest areas in PR, SC and RS are more extensive, which allows the establishment of more individuals and increases the chances of reproductive success due to favorable climatic conditions (Fiori *et al.* 2009; IBGE 2012; Alvares *et al.* 2014; Carnaval *et al.* 2014; Schwartz & Gasper 2020).

This is the first genetic study to assesses whether the genetic diversity of a native fern is distributed according to already-known patterns of biodiversity structuring for the Atlantic Forest (Behling 2002; Carnaval & Moritz 2008; Pinheiro *et al.* 2011; Carnaval *et al.* 2014). The microsatellite flanking regions are conserved among related species, especially those with more recent diversifications, such as *D. sellowiana* (Nazareno *et al.* 2013; Moodley *et al.* 2015; Fagundes *et al.* 2016; Noben *et al.* 2017; Mäder *et al.* 2019). Our genetic diversity data (Ho, He, Fst, Fis, genetic distance) support the hypothesis that there is a pattern of biodiversity discontinuity in the Atlantic Forest biome (Behling 1997; 2002; Carnaval & Moritz 2008; Ribeiro *et al.* 2011; Pinheiro *et al.* 2011; Turchetto-Zolet *et al.* 2013; Thode *et al.* 2014; Thome *et al.* 2014; Carnaval *et al.* 2014). However, for *D. sellowiana*, this structure was more recent than the barriers evidenced in studies with other species, and showed a pattern of clinal variation from north to south (Behling 1997; 2002; Carnaval & Moritz 2008; Ribeiro *et al.* 2011; Pinheiro *et al.* 2011; Turchetto-Zolet *et al.* 2013; Thode *et al.* 2014; Carnaval *et al.* 2014; Leite *et al.* 2016; Rosa *et al.* 2017; Stefenon *et al.* 2019). This different pattern for *D. sellowiana* is related to its more recent origin than other native and endemic ferns of the Neotropics (Noben *et al.* 2017). The set of microsatellites used here proved to be an effective tool for testing the hypotheses of biodiversity discontinuity due to the latitudinal gradient of the Atlantic

Forest. In this way, we showed that the genetic structure of *D. sellowiana* may be under the influence of anthropogenic fragmentation of the biome, which is one of the main factors responsible for the loss of biodiversity in global hotspots.

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