Naturally fragmented but not genetically isolated populations of *Podocarpus sellowii* Klotzsch (Podocarpaceae) in southeast Brazil

F.R. Gonçalves[1], F.A. Vieira[2] and D. Carvalho[3]

[1]RGA Engenharia & Meio Ambiente, Passos, MG, Brasil
[2]Universidade Federal do Rio Grande do Norte, Macaíba, RN, Brasil
[3]Universidade Federal de Lavras, Lavras, MG, Brasil

Corresponding author: F.A. Vieira
E-mail: vieirafa@gmail.com

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ABSTRACT. In southeastern Brazil, the majority of the riparian ecosystems are fragmented and degraded mainly due to human activities. The perennial gymnosperm *Podocarpus sellowii* Klotzch is a typical tree species from a gallery forest in the rupestrian area. Ten alloenzymatic loci were used to estimate the allelic frequency of 232 individuals distributed in eight subpopulations naturally divided by rock outcrops. The results indicated high genetic variability for the species in all subpopulations, with H_O varying from 0.593 to 0.658, and H_E, from 0.484 to 0.502. No endogamy was observed within (f = -0.292) and for the population set (f = -0.264). Genetic divergence of the species between subpopulations was 2.1%. Historic gene flow was low between subpopulations located in different water streams, corroborating the positive and significant correlation between genetic and geographical distance (r_m = 0.496, P = 0.022). Co-ancestry

revealed that only population A presents continuous distribution of the genotypes up to 94 m. *Sp* statistics did not indicate significant spatial genetic structure in the populations. In all the subpopulations, values of effective sizes were higher than the numbers of sampled individuals. The balance between mutation and drift was not verified, indicating the occurrence of a recent population bottleneck. These data can be used to determine the most effective strategies for the genetic conservation of this species.

Key words: Allozymes; Conifers; Genetic diversity; Rock outcrops; Natural fragmentation

INTRODUCTION

The presence of *Podocarpus sellowii* Klotzsch (Podocarpaceae), a perennial gymnosperm of discontinuous geographical distribution, is common in the Brazilian gallery forests, with a large number of individuals evenly distributed over the water streams. The *Podocarpus* genus is represented by more than 100 species, many of which are of forest interest, and is classified as a cosmopolitan group since it has a wide geographical distribution. There have been few reports on the genetic diversity of the *Podocarpus* genus (Quiroga and Premoli, 2007, 2010) and of *P. sellowii* species (Dantas et al., 2015). Studies conducted in South America revealed low genetic diversity and a positive correlation between the genetic and geographical distances of *Podocarpus parlatorei* (Quiroga and Premoli, 2007). Compared with *Podocarpus nubigena* (Quiroga and Premoli, 2010), there was high genetic diversity and genetic differentiation between populations. These studies show contrasting results for genetic diversity; therefore, further studies on other species of the *Podocarpus* genus are required for a better understanding of the genetic structure of natural populations.

Although the number of studies on anthropogenic habitat fragmentation in plants is increasing (Silva et al., 2015; Álvares-Carvalho et al., 2016), to date, there have been no reports on the genetic structure of populations that are naturally fragmented. The present study represents the first case study addressing this characteristic, which is common in some Brazilian ecosystems. The perception of species distribution in naturally fragmented environments is important to evaluate the genetic behavior of these populations and to determine how they can perpetuate, since there are natural barriers to gene flow. Therefore, it is important that levels of variability and genetic structure of populations occurring in naturally fragmented environments are understood.

In this context, determination of the neutral genetic divergence between populations and subpopulations using allozymes has been useful in the quantification of genetic diversity to assist *in situ* conservation practices (Vieira et al., 2012). The following issues were addressed in the present study: i) the levels of genetic variability between populations and subpopulations located in different water streams, ii) the sufficiency of gene flow to allow populations and subpopulations to remain cohesive, iii) isolation by distance in naturally fragmented populations, iv) the levels of genetic structure between populations over the landscape and within populations, and the possible implications for *in situ* genetic conservation.

To address these issues, this study characterized the genetic diversity of *P. sellowii* populations using samples from areas under natural conditions with a high degree of

conservation. The genetic structure of *P. sellowii* in eight subpopulations was analyzed in order to quantify the genetic variability, to estimate the gene flow between subpopulations, to study the spatial distribution of genotypes, and to estimate the effective population size.

MATERIAL AND METHODS

Study species

P. sellowii Klotzsch is a Gymnospermae, of the Coniferales order and Podocarpaceae family (Joly, 1991), commonly known as "pinho-bravo" and "louro-pinho". Despite its wide geographical distribution in Brazil, the *Podocarpus* genus has restricted ecological distribution, which leads to discontinuous geographical occupation of available space. It is therefore found in areas with altitude of 400 to 1800 m, with special soil and climate characteristics, such as light, siliceous, humus, airy soils, under conditions of low temperature and high humidity. The pollination and dispersal systems of this species are not well known. It produces diaspores, and has a stem with a succulent dark purple terminal, which has a sweet flavor when ripe. These characteristics suggest that it is dispersed by animals (especially birds), water, and gravity. Although *P. sellowii* wood does not have high economic value, it is used widely as plywood, toys, matchsticks, crates, ceilings, woodworks, and pencils. Nevertheless, its characteristics and application are similar to the Brazilian pine, *Araucaria angustifolia*. As an ornamental species, it can also be used in landscaping. Another possible use is in seedling production for ecological restoration, especially in permanent preservation areas.

Study area and sampling

The studied area is characterized by gallery forests, which have well-defined boundaries with a non-forest formation (rock outcrop) (21°21'42.8"S, 044°46'05.2"W, at 1000 m in average altitude; Figure 1). Eight subpopulations of *P. sellowii* Klotzsch were sampled in three different water streams. Subpopulations were characterized due to the disruption of natural forest formations along the water streams by rock outcrops, which is characteristic of the area. Subpopulations of the same water stream form a population, and thus the following subpopulations were defined: A1, A2, A3, and A4, belonging to Batatal Stream, population A; subpopulations B1 and B2, belonging to Farias Stream, population B; and subpopulations C1 and C2, belonging to the Morro da Janela Stream, population C (Figure 1). Distance between subpopulations varied between 206 and 2228 m (Table 1). It is important to note that the streams belong to adjacent micro basins, which are delimited by water disruptions. These disruptions are accentuated along the micro basins, which form natural barriers. The slope of streams decreases as the altitude rises, and reaches zero at the top. In the lower parts, the slope is so great that it forms natural barriers, such as waterfalls.

Adult trees were randomly sampled across the subpopulations. Both grouped and isolated individuals were collected. Sampled individuals were identified and georeferenced using the global positioning system. The genetic structure of *P. sellowii* was investigated using samples from 232 individuals, with the aim of collecting at least 30 trees per subpopulation. Subpopulations B1 and B2 contained fewer samples due to their low local occurrence. Leaf samples from each individual were collected, identified, and placed in a cooler with ice for transportation. The plant material was stored in a freezer at -80°C until alloenzyme extraction.

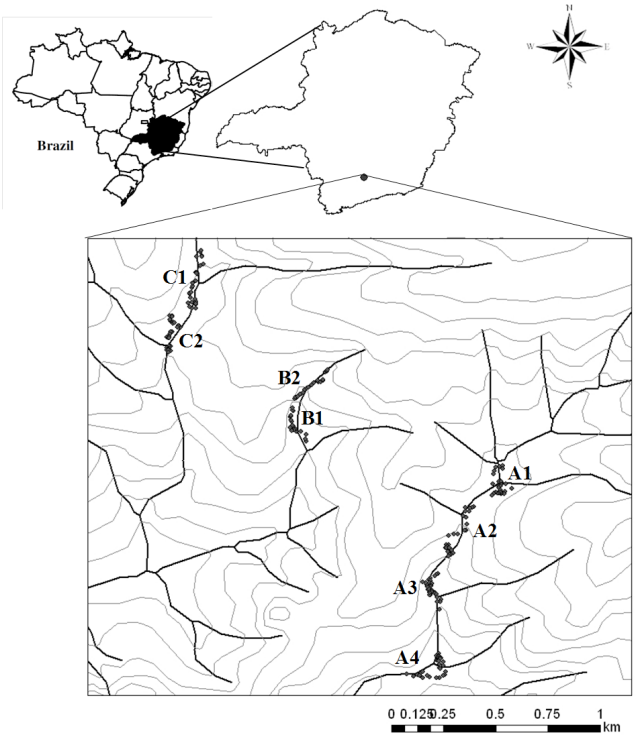

Figure 1. Location and characterization of subpopulations of *Podocarpus sellowii* Klotzsch in the experimental area.

Table 1. Sample size (N), altitude, and geographical distance (m) among the eight populations of *Podocarpus sellowii* Klotzsch.

Subpopulations	N	Altitude (m)	A1	A2	A3	A4	B1	B2	C1	C2
A1	30	1030	-	327	634	989	1046	1035	1754	1741
A2	33	1010		-	311	695	954	1008	1750	1638
A3	32	990			-	409	1037	1144	1870	1759
A4	32	935				-	1391	1522	2228	2088
B1	21	1015					-	206	837	731
B2	24	1045						-	742	708
C1	30	1055							-	285
C2	30	1040								-

Enzymatic extraction and electrophoresis

For alloenzyme extraction, 300 mg leaf tissue was used with polyvinylpyrrolidone and 1.5 mL buffer solution number 1 as described by Brune et al. (1998). Electrophoresis was performed using polyacrylamide gels (10 and 4% gels for separation and concentration, respectively). For electrophoresis, 10 mA was applied to each gel at 300 V. Each electrophoretic run lasted approximately 3 h 30 min at 4°C. Gel preparation, sample application, and electrophoresis were carried out according to the methods described by Brune et al. (1998). Fifteen enzyme systems were tested.

Gel staining was based on the methodology described by Brune et al. (1998). Details of each enzyme, as well as the solutions and staining methodology, are described in Brune et al. (1998). Following zymogram interpretation (**Table S1**), allele frequencies at each locus were obtained.

Intrapopulational genetic diversity

The following diversity indices were obtained: percentage of polymorphic loci (P), mean number of alleles per locus (N_A), mean observed heterozygosity (H_O), and mean expected heterozygosity (H_E) according to the Hardy-Weinberg equilibrium (HWE). Estimates were obtained using the BIOSYS 2 software (Swofford and Selander, 1997). The F coefficient of Wright (1951) was obtained by locus and by the loci mean, from the following ratios: $f = 1 - H_O / H_E$ (locus) and $f = 1 - SH_O / SH_E$ (loci mean), where: f = Wright fixation index estimate. Confidence intervals at 95% probability were obtained by the bootstrap procedure based on 10,000 replications. Analyses of mean fixation indices were carried out with the aid of the GDA software (Lewis and Zaykin, 2000).

Genetic structure

The genetic structure between and within populations was analyzed using co-ancestry coefficients (Cockerham, 1969). Confidence intervals were estimated at 95% probability by the bootstrap resampling method, based on 10,000 replications. Analyses of variance and bootstrapping were carried out with the aid of the GDA software. Genetic divergence (F_{ST}) between pairs of subpopulations was obtained by the FSTAT 2.9.3.2 software (Goudet, 2002).

Nei (1978)'s genetic identity was used as a measure of genetic identity between pairs of populations. Dendrograms were constructed by the estimates of genetic identity, using the unweighted pair group method with arithmetic mean (UPGMA) method. The consistency of the nodes in each cluster was evaluated by the tools for population genetic analysis 1.3 software (Miller, 1997). Multivariate genetic identity analysis (UPGMA) was carried out using the numerical taxonomy and multivariate analysis system (NTSYS) 1.5 package. Deviations in genotype frequencies deviations obtained for expected frequencies by the proportions of the HWE were estimated and tested with the BIOSYS 2 software (Swofford and Selander, 1997).

Gene flow

Estimates of gene flow between populations were based on the methodology proposed by Wright (1951), which considers the number of migrants (N_m) and the genetic divergence between populations (F_{ST}), and has been commonly used in population genetics. These estimates were obtained according to the equation: $N_m = 1/4a\ (1 / F_{ST} - 1)$, being $a = (n / [n - 1])^2$, where n = number of populations.

The genetic distance matrix (F_{ST}) was compared with the geographical distance matrix by the Mantel test (Manly, 1997). The Mantel test was performed in the PCOrd 4 program (McCune and Mefford, 1999) using 1000 random permutations to test the significance of the correlation matrix.

Spatial genetic structure

Sp statistics (Vekemans and Hardy, 2004) were used to analyze the spatial structure

of *P. sellowii*, and the co-ancestry coefficient [$F_{(ij)}$] was estimated between plants for each distance class using the SPAGeDi 1.2 software (Hardy and Vekemans, 2002). Mean standard errors of the estimates between loci were obtained by jack knife resampling. Confidence intervals were obtained at 95% probability of the mean coancestry coefficient estimated for each distance class. The absence of spatial genetic structure was tested for each distance class, using 1000 permutations. *Sp* statistics were calculated as $Sp = b_F / (F_{(1)} - 1)$, in which: $F_{(1)}$ = mean coancestry coefficient between individuals in the first distance class and b_F = inclination of the linear regression curve of the $F_{(r)}$ coefficient against the distance logarithm. When $b_F = 0$, the null hypothesis of random spatial genetic structure is accepted.

Effective population size

Estimates of effective population size were obtained using the method described by Vencovsky (1997). For single populations without genetic structure, the effective population size was calculated by: $N_E = n / (1 + f)$, in which, n = number of adult plants and f = intrapopulation inbreeding coefficient. For individuals of several populations, in a model of infinite populations (without correction for finite population size), the following formula was used: $N_E = 0.5 / \theta p\ (1 + C_p / p - 1 / n) + 1 + F / 2n$, in which, θp = coancestry coefficient related to the population, p = number of evaluated populations, n = total number of individuals evaluated in the populations, C_p = square of the coefficient of variation of the number of individuals (n_i) between populations, and F = fixation index for the population set.

Genetic bottleneck

The BOTTLENECK 1.2.02 software (Cornuet and Luikart, 1996) was used to test if the populations are in equilibrium between mutation and genetic drift, following the methodology described by Cornuet and Luikart (1996). According to Luikart et al. (1998), populations, which have undergone a recent bottleneck, present a temporary excess in heterozygosity. This makes mean H_E higher than the H_E in equilibrium between mutation and drift (H_{eq}) since it is calculated from the number of alleles (Cornuet and Luikart, 1996; Piry et al., 1999). Significance was evaluated using the Wilcoxon signed rank test, since it is the most adequate test when used for <20 loci (Piry et al., 1999), based on 5000 replications.

RESULTS

Isoenzymatic systems

Nine of the 15 isozyme systems tested in *P. sellowii* were selected: α-esterase (α-EST), phosphoglucomutase (PGM), glucose dehydrogenase (GDH), β-galactose dehydrogenase (GLDH), glutamate dehydrogenase (GTDH), malate dehydrogenase (MDH), peroxidase (PO), sorbitol dehydrogenase (SDH), and shikimate dehydrogenase (SKDH). The nine isozyme systems used revealed 27 active loci, of which 10 were used for interpretation. α-EST, GDH, GLDH, GTDH, MDH, PO, SDH, and SKDH presented three activity zones, and two loci were discarded due to difficult interpretation.

Allele frequencies

Analysis of allele frequencies at the 10 loci revealed no unique alleles in the populations and subpopulations of *P. sellowii* (Tables 2 and 3). For populations, the highest allele frequencies were observed for SKDH (allele 2 = 0.650) population A, PO (allele 2 = 0.585) population B, and MDH (allele 1 = 0.664) population C. For subpopulations, the highest allele frequencies were: SKDH (allele 2 = 0.717), GTDH (allele 2 = 0.742), MDH (allele 2 = 0.650), SDH (allele 2 = 0.672), PO (allele 2 = 0.625), SDH (allele 1 = 0.667), MDH (allele 1 = 0.648), and MDH (allele 1 = 0.679), for A1, A2, A3, A4, B1, B2, C1, and C2, respectively.

Table 2. Allele frequencies and sample size (N) for 10 isozyme loci analyzed in *Podocarpus sellowii* (Klotz.) for the population set and for the three populations.

Locus	Alleles	Population set	Population		
			A	B	C
α-EST	1	0.507	0.492	0.522	0.526
	2	0.493	0.508	0.478	0.474
	N	220	118	45	57
GDH	1	0.421	0.399	0.477	0.424
	2	0.579	0.601	0.523	0.576
	N	221	119	43	59
GLDH	1	0.444	0.423	0.537	0.422
	2	0.556	0.577	0.463	0.578
	N	216	117	41	58
GTDH	1	0.421	0.372	0.500	0.474
	2	0.579	0.628	0.500	0.526
	N	222	125	40	57
MDH	1	0.465	0.364	0.448	0.664
	2	0.535	0.636	0.512	0.336
	N	213	118	40	55
PGM-1	1	0.465	0.468	0.478	0.450
	2	0.535	0.532	0.522	0.550
	N	231	126	45	60
PGM-2	1	0.474	0.508	0.533	0.358
	2	0.526	0.492	0.467	0.642
	N	229	124	45	60
PO	1	0.500	0.529	0.415	0.500
	2	0.500	0.471	0.585	0.500
	N	216	119	41	56
SDH	1	0.451	0.390	0.537	0.518
	2	0.549	0.610	0.463	0.482
	N	216	118	41	57
SKDH	1	0.424	0.350	0.549	0.491
	2	0.576	0.650	0.451	0.509
	N	218	120	41	57

Genetic variability indices

Loci analyzed in *P. sellowii* presented 100% polymorphism (*P*) (Table 3). The N_A value was 2.0 for the population set and for the subpopulations. H_O and H_E showed high values both for the population set and for subpopulations. The H_O/H_E ratio provided negative fixation indices (*f*) in all of the analyzed subpopulations, revealing a higher proportion of heterozygotes (Table 4).

Table 3. Allele frequencies and sample size (N) for 10 isozyme loci analyzed in *Podocarpus sellowii* (Klotz.) for the eight sub-populations.

Locus	Alleles	Subpopulations							
		A1	A2	A3	A4	B1	B2	C1	C2
α-EST	1	0.571	0.450	0.550	0.400	0.524	0.521	0.481	0.567
	2	0.429	0.550	0.450	0.600	0.476	0.479	0.519	0.433
	N	28	30	30	30	21	24	27	30
GDH	1	0.393	0.300	0.516	0.383	0.429	0.523	0.517	0.328
	2	0.607	0.700	0.484	0.617	0.571	0.477	0.483	0.672
	N	28	30	31	30	21	22	30	29
GLDH	1	0.482	0.355	0.433	0.429	0.444	0.609	0.448	0.397
	2	0.518	0.645	0.567	0.571	0.556	0.391	0.552	0.603
	N	28	31	30	28	18	23	29	29
GTDH	1	0.450	0.258	0.383	0.406	0.472	0.523	0.517	0.429
	2	0.550	0.742	0.617	0.594	0.528	0.477	0.483	0.571
	N	30	33	30	32	18	22	29	28
MDH	1	0.308	0.391	0.350	0.400	0.421	0.548	0.648	0.679
	2	0.692	0.609	0.650	0.600	0.579	0.542	0.352	0.321
	N	26	32	30	30	19	21	27	28
PGM-1	1	0.483	0.439	0.453	0.500	0.429	0.521	0.433	0.467
	2	0.517	0.561	0.547	0.500	0.571	0.479	0.567	0.533
	N	29	33	32	32	21	24	30	30
PGM-2	1	0.483	0.530	0.533	0.484	0.571	0.500	0.367	0.350
	2	0.517	0.470	0.467	0.516	0.429	0.500	0.633	0.650
	N	30	33	30	31	21	24	30	30
PO	1	0.643	0.469	0.483	0.533	0.375	0.452	0.603	0.389
	2	0.357	0.531	0.517	0.467	0.625	0.548	0.397	0.611
	N	28	32	29	30	20	21	29	27
SDH	1	0.483	0.333	0.417	0.328	0.400	0.667	0.552	0.482
	2	0.517	0.667	0.583	0.672	0.600	0.333	0.448	0.518
	N	29	30	30	29	20	21	29	28
SKDH	1	0.283	0.333	0.450	0.333	0.475	0.619	0.500	0.481
	2	0.717	0.667	0.550	0.667	0.525	0.381	0.500	0.519
	N	30	30	30	30	20	21	30	27

Table 4. Genetic diversity of *Podocarpus sellowii* (Klotz.) in the three populations and eight subpopulations studied.

	N_A	*P*	Mean H_O	Mean H_E	*f*
A	2.0	100.0	0.620 (0.012)	0.485 (0.005)	-0.282* [-0.323 to -0.236]
B	2.0	100.0	0.629 (0.023)	0.503 (0.001)	-0.255* [-0.342 to -0.170]
C	2.0	100.0	0.643 (0.034)	0.492 (0.006)	-0.311* [-0.429 to -0.180]
A1	2.0	100.0	0.658 (0.033)	0.484 (0.011)	-0.370* [-0.461 to -0.261]
A2	2.0	100.0	0.608 (0.016)	0.468 (0.012)	-0.305* [-0.357 to -0.266]
A3	2.0	100.0	0.610 (0.023)	0.497 (0.005)	-0.232* [-0.323 to -0.137]
A4	2.0	100.0	0.608 (0.024)	0.487 (0.007)	-0.255* [-0.335 to -0.174]
B1	2.0	100.0	0.670 (0.036)	0.502 (0.003)	-0.347* [-0.477 to -0.218]
B2	2.0	100.0	0.593 (0.020)	0.499 (0.006)	-0.192* [-0.283 to -0.113]
C1	2.0	100.0	0.679 (0.024)	0.496 (0.005)	-0.377* [-0.457 to -0.289]
C2	2.0	100.0	0.607 (0.047)	0.485 (0.008)	-0.257* [-0.437 to -0.076]

Number in parentheses = Standard deviation; numbers in brackets = confidence interval; *significant at 5% probability. N_A = mean number of alleles per locus; *P* = percentage of polymorphic loci; mean H_O = mean observed heterozygosity; mean H_E = mean expected heterozygosity; *f* = fixation index.

Most subpopulations showed similarities in the indices of observed heterozygosity according to the standard deviation, especially the subpopulation C2, which was the only one that did not differ from the other subpopulations, also according to the standard deviation. H_E was 0.485 for population A, 0.503, for population B, and 0.492, for population C.

Fixation index (*f*) was significantly different from zero in all the subpopulations at 95% probability, indicating an excess of heterozygotes in relation to the HWE assumptions. The highest fixation values occurred in subpopulations C1 (-0.377) and A1 (-0.370), and the lowest fixation value was observed in subpopulation B2 (-0.192) (Table 3).

Genetic structure

Mean estimates were negative, indicating the absence of inbreeding within (f = -0.292) and also in the population set (F = -0.264). Mean inbreeding coefficients were significantly different from zero, showing heterozygote excess in relation to that expected by the HWE.

Genetic divergence between the eight *P. sellowii* subpopulations obtained by the F_{ST} estimate was low (F_{ST} = 0.021). This indicates that 2.1% variability was found between subpopulations, and that 97.9% of this variability occurred within subpopulations. Adherence to HWE was verified in the eight subpopulations (**Table S2**). Fisher's exact test showed that for *P. sellowii,* the proportion of loci adherent to HWE was 70, 90, 90, 80, 80, 90, 50, and 50% in subpopulations A1, A2, A3, A4, B1, B2, C1, and C2, respectively. Only α-EST, PGM-1, and PGM-2 were in HWE in all the subpopulations.

The observed genetic divergences were significant after Bonferroni correction on eight occasions (Table 5). The highest difference was between A2-B2 subpopulations (6.8%), followed by subpopulations A2-C1 (5.1%), A4-B2 (4.5%), A1-C2 (4.3%), A1-B2 (4.2%), A1-C1 (3.2%), A2-C2 (3.2%), and A4-C2 (2.7%).

Table 5. Geographical distance (in meters, above the diagonal) and genetic divergence ($F_{ST,}$ below the diagonal) of *Podocarpus sellowii* (Klotz.) subpopulations.

Subpopulations	A1	A2	A3	A4	B1	B2	C1	C2
A1	-	327	634	989	1046	1035	1754	1741
A2	0.019	-	311	695	954	1008	1750	1638
A3	0.006	0.009	-	409	1037	1144	1870	1759
A4	0.005	0.002	0.002	-	1391	1522	2228	2088
B1	0.016	0.010	0.008	0.003	-	206	837	731
B2	0.042*	0.068*	0.022	0.045*	0.016	-	742	708
C1	0.032*	0.051*	0.023	0.029	0.023	0.009	-	285
C2	0.043*	0.032*	0.026	0.027*	0.014	0.024	0.010	-

*Significant after Bonferroni correction (α = 0.05).

The dendrogram constructed by the UPGMA method based on the genetic identity matrix (Nei, 1978) showed consistency with significant genetic differences observed in this study (Figure 2). The existence of a hierarchical pattern of genetic similarity suggests that two clusters exist [(A1, A4, A3, B1), A2], [(B2, C1), C2]. Those two clusters have consistency values of 63 and 55%, respectively. They show genetic divergence between populations A and C, and similarity between subpopulations of population B (geographically intermediate) with the populations A and C. Thus, it is suggested that population B could have a role in genetic exchange between A and C, and its extinction may, in the future, increase the divergence between these populations.

Table 6 shows the genetic flow for each pair of subpopulations. When the estimated values for gene flow exceed 1.0, this will be sufficient to prevent differentiation due to genetic drift. In this case, only A2 x B2 (0.9) is below 1.0 and effects of drift can be anticipated. Therefore, these results show that for subpopulations A1 x A3, A1 x A4, A2 x A3, A2 x A4,

A2 x B1, A3 x A4, A3 x B1, A4 x B1, B2 x C1, and C1 x C2, more than four migrants per generation is sufficient to counteract the effects of genetic drift (Wright, 1951). The Mantel test showed that there is significant correlation between the geographical distance and the estimated genetic distances (r_m = 0.496; P = 0.022).

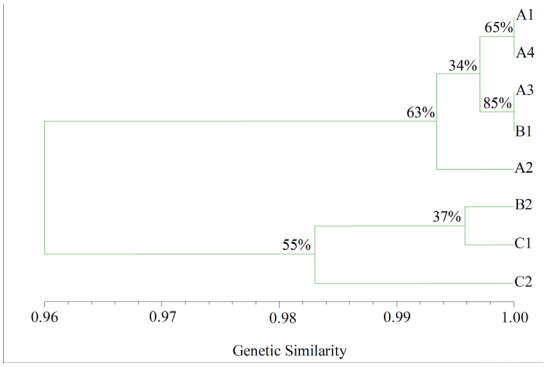

Figure 2. Cluster analysis of Nei (1978)'s genetic identity between the eight subpopulations of *Podocarpus sellowii* (Klotz.).

Table 6. Gene flow (N_m) obtained from estimates of genetic divergence (F_{ST}) between the eight subpopulations of *Podocarpus sellowii* (Klotz.).

Subpopulations	N	nf	F_{ST}	N_m
A1-A2	63	2	0.019	3.3
A1-A3	62	2	0.006	11.2
A1-A4	62	2	0.005	12.0
A1-B1	51	2	0.016	3.9
A1-B2	54	2	0.042*	1.4
A1-C1	60	2	0.032*	1.9
A1-C2	60	2	0.043*	1.4
A2-A3	65	2	0.009	6.8
A2-A4	65	2	0.002	34.4
A2-B1	54	2	0.009	6.5
A2-B2	57	2	0.067*	0.9
A2-C1	63	2	0.051*	1.2
A2-C2	63	2	0.032*	1.9
A3-A4	64	2	0.002	29.1
A3-B1	53	2	0.008	7.9
A3-B2	56	2	0.022	2.8
A3-C1	62	2	0.023	2.6
A3-C2	62	2	0.026	2.3
A4-B1	53	2	0.003	23.2
A4-B2	56	2	0.045*	1.3
A4-C1	62	2	0.028	2.1
A4-C2	62	2	0.027*	2.2
B1-B2	45	2	0.016	3.9
B1-C1	51	2	0.023	2.7
B1-C2	51	2	0.014	4.5
B2-C1	54	2	0.009	7.0
B2-C2	54	2	0.024	2.6
C1-C2	60	2	0.010	6.2
All populations	232	8	0.021	8.8

*Significant after Bonferroni's correction (α = 0.05). N = number of individuals analyzed; nf = number of subpopulations analyzed.

Spatial genetic structure

With the exception of population A, all populations were randomly distributed (Figure 3). In this population, significant and positive co-ancestry values were observed in the first distance class, that is, individuals located up to 94 m apart have some kinship. For the other populations, the results indicate low structuring of genotypes within populations.

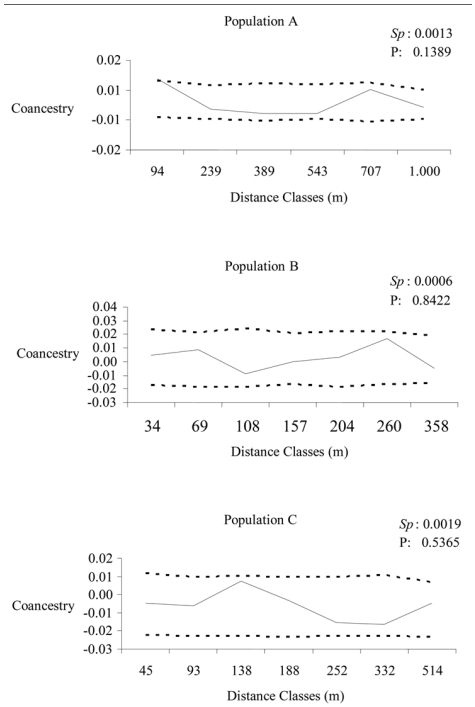

Figure 3. *Sp* statistics, probability (P), and spatial genetic structure with co-ancestry coefficient [$F_{(ij)}$]. Full rows represent the regression coefficient $F_{(ij)}$ against the logarithm; dotted lines represent the 95% confidence interval.

Effective population size

In all subpopulations, the values for effective sizes were superior to the number of sampled individuals, ranging from 30 to 48 (Table 7). This was due to the large number of heterozygote individuals indicated by the negative fixation indices observed in subpopulations. This was expected, since the equation used proposed by Vencovsky (1997) relates to the sample size and population fixation index, which results in more representativeness for heterozygosity. Thus, the values observed for effective population size (N_E) confirm the existence of low inbreeding in the studied subpopulations of *P. sellowii*

Significant deviations from the mutation equilibrium and drift (such as bottleneck effects) were tested by adapting the infinite allele mutation model (Cornuet and Luikart, 1996; Luikart and Cornuet, 1998; Piry et al., 1999). No equilibrium was found in any of the populations, which indicates that recent population bottlenecks occurred (within 12 generations, according to Van Rossum and Prentice, 2004) (Table 8).

Table 7. Effective size (N_E) and number of sampled individuals (n) of *Podocarpus sellowii* (Klotz.) subpopulations.

Subpopulations	N_E	n	N_E/n
A1	48	30	1.59
A2	47	33	1.44
A3	42	32	1.30
A4	43	32	1.34
B1	32	21	1.53
B2	30	24	1.24
C1	48	30	1.61
C2	40	30	1.35
Set	41	-	-

Table 8. Equilibrium tests between mutation and genetic drift for eight *Podocarpus sellowii* (Klotz.) subpopulations under the infinite allele mutation model.

Subpopulations	N*	Deficit[1]	Excess[2]	P	Wilcoxon's test
A1	4.56	0	10	0.00038	0.00098
A2	4.28	0	10	0.00020	0.00098
A3	4.38	0	10	0.00025	0.00098
A4	4.37	0	10	0.00025	0.00098
B1	4.58	0	10	0.00039	0.00098
B2	4.34	0	10	0.00024	0.00098
C1	4.40	0	10	0.00027	0.00098
C2	4.44	0	10	0.00029	0.00098
Set	3.80	0	10	0.00006	0.00098

*Expected number of loci with heterozygosity excess in the model, followed by: [1]loci with heterozygosity deficit and [2]loci with heterozygosity excess. P = probability.

DISCUSSION

This is the first report describing the genetic structure of populations that are naturally fragmented, as well as the first case study addressing this characteristic, which is common in some Brazilian ecosystems. Although codominant molecular markers such as microsatellites have been used, in the absence of specific primers, isozyme markers are a useful and valid tool to assess the genetic diversity of populations, especially in developing countries, where there is shortage of financial resources for nature conservation. Thus, recent studies on isozyme markers with different goals have been published (Ballian et al., 2013; Chung et al., 2013).

In studies on different tree species, such as *Protium spruceanum* Benth., Vieira and Carvalho (2008) used 10 polymorphic loci to characterize the genetic diversity and structure of fragmented populations. However, Bacles et al. (2004) studied allele frequencies through allozyme frequencies, and sampled between 2 and 27 individuals in eight remnants. These authors noted that besides reflecting the fragmented status of the landscape, the sampling strategy varied according to the size and isolation of populations, of altitude, and of accessibility to individuals. Therefore, the present study on *P. sellowii*, which occurs in naturally fragmented environments, was well sampled, including 10 polymorphic loci and the 232 individuals in subpopulations (Table 2).

Genetic diversity and structure

Compared to other studies on gymnosperms, the results obtained in the present study for *P. sellowii* revealed higher genetic diversity than reported for species of the genera *Abies* (0.130), *Picea* (0.218), *Pinus* (0.136), and *Pseudotsuga* (0.163) (Hamrick et al., 1992). Mantovani et al. (2006) reported a 0.389 index for *A. angustifolia*. These differences may be due to the natural history of each population or to the exploitation of each area. In studies with *Pinus*, the genetic diversity was 0.179 for *Pinus halepensis* (Loukas et al., 1983) and 0.146 for *Pinus rigida* (Guries and Ledig, 1982). These data show that the genetic diversity values differ for various tree species, and that there is no pattern for species with similar ecological characteristics. High levels of genetic variability enable the occurrence of a large number of new genotypic combinations, thus increasing the evolutionary potential of the species due to their increased capacity to adapt to environmental changes.

Subpopulations of *P. sellowii* did not present significant genetic diversity values, except between subpopulations of different locations. This indicates that there may be factors limiting gene flow between populations, such as water disruptions between populations that form a natural barrier. However, this was not observed in subpopulations of the same populations, as no significant values of genetic divergence were found. High genetic divergence between subpopulations A2 x B2, A2 x C1, A4 x B2, A1 x C2, A1 x B2, A1 x C1, A2 x C2, and A4 x C2, reinforced by the low similarity observed from the identity matrix of Nei (1978), can be influenced by low or absent gene flow. Most of the genetic diversity of tree species is comprised of the genetic variability within a population (Hamrick et al., 1992), which is also observed for the genetic structure data of *P. sellowii*. In addition, there was a correlation between the geographical position and the genetic distance of populations, characterizing isolation by distance. Therefore, it is necessary to investigate how the genetic variability of populations is spatially organized. The correlation coefficient between genetic and geographic matrices of *P. sellowii* subpopulations suggests that this genetic structure probably originated from a stochastic process, such as high gene flow between close subpopulations, and low gene flow between distant subpopulations, which characterize isolation by distance.

HWE deviations at supposedly neutral loci, such as allozymes, implies that the population is reproductively subdivided into groups with a degree of kinship, and that random crossing (selfing and biparental crosses) or genetic drift do not occur, and the subdivision may be associated with the existence of family structure within the population. To analyze whether heterozygote maintenance exists in *P. sellowii*, subsequent generations should be studied.

Loveless and Hamrick (1984) reported that typically allogamous plants present high genetic variability within populations. The higher the gene flow between populations, the lower the divergence between them. The estimated genetic divergence found for *P. sellowii* (2.1%) is consistent with that observed for other tropical wood species, i.e., most of the genetic variability is within populations. HWE takes into account random crosses, mutation absence, migration, genetic drift, selection, and the infinite size of populations (Futuyma, 1992). Thus, due to the assumptions of random mating, it is expected that a panmitic population retains the frequency of its alleles in every generation, and only redistribution of alleles within the genotypes of the new generation can change the genetic composition of the population (Futuyma, 1992).

Spatial distribution of genotypes

Seed dispersal is the main cause of genetic structure in tree species. However, other factors, such as the crossing system and pollen flow may favor the spatial structure genetic (Epperson and Alvarez-Buylla, 1997). All *P. sellowii* populations presented random distribution of genotypes, with the exception of population A. For the other populations, the results indicated low structuring of genotypes within populations. In population A, genotypes presented familiar structure up to a distance of 94 m. This information is important for seed collection for genetic improvement programs and for the recovery of degraded areas, maximizing sampling for greater genetic variability.

In the present study, *Sp* statistical analysis revealed no significant spatial genetic structure in any of the populations (Figure 3). The methods employed in *Sp* statistics provide good resolution, and it is possible to identify structured genetic families within a few meters (Vekemans and Hardy, 2004). In areas with a high density of individuals, seeds from different trees overlap, resulting in a mixture of different progenies and, in such cases, weak spatial genetic structure is expected.

N_E

N_E is a key parameter when *ex situ* and *in situ* conservation are taken into account, since it considers the genetic representativeness of plant, seed, or propagule samples. Since estimates of effective sizes indicate genetic representativeness of the samples from the allele frequencies, some dynamic factors that affect the distribution of allele frequencies should be considered. Frankel et al. (1996) observed fluctuations in population size between generations, variation in fertility between individuals, and overlapping generations.

Vencovsky (1997) reported that information regarding the genetic representation of the population matrices (N_E) is important to maximize seed collection activities, as the number of matrices to be sampled can be calculated. That author considers that progenitor plants should be randomly selected, rather than their seeds. In vegetation-enrichment strategies and in recovery areas, using this principle to collect seeds will result in numerous new genotype recombinations in the population, increasing its evolutionary potential. Ratios between the effective population size and the number of sampled individuals (N_E/n) have been applied to conservation (Frankham, 1995). Therefore, according to the ratio (N_E/n) observed for subpopulations, it is recommended that more seeds are collected in populations with low ratios in order to ensure the maintenance of seed genetic variability.

Equilibrium between mutation and genetic drift

Luikart et al. (1998) reported that populations subjected to recent bottlenecks present a temporary heterozygosity excess ($H_E > H_{eq}$). This was observed in all subpopulations in the present study, since they all had a significant number of loci with heterozygosity excess, this is, H_E by the HWE assumptions in polymorphic loci is greater than the H_{eq} between mutation and drift. Because the history of the studied area is not known, and represents a preserved and non-anthropic region, the cause of the population bottlenecks is unknown.

Van Rossum and Prentice (2004) evaluated significant deviations in mutation and drift equilibrium in populations of *Silene nutans* (Caryophyllaceae) in Sweden and northern

Finland, and found evidence for recent genetic bottlenecks in various populations. These were interpreted to have been a consequence of population fragmentation as a result of human disturbance. In contrast, Bacles et al. (2004) did not observe recent genetic bottlenecks for *Sorbus aucuparia* (Rosaceae) in southern Scotland. Those authors reported that habitat destruction in the study area is approximately 6000 years old. Luikart et al. (1998) suggested that, after detecting a bottleneck, the probability that the deleterious effects of this event can be avoided is higher, since mitigating management procedures, or the introduction of immigrants can be carried out. In addition, such practices can be effective if associated with knowledge on ecological and demographic factors of the species. This further confirms the necessity of investigating ecological factors associated with *P. sellowii*, such as reproductive biology. The detection of populations subjected to recent bottlenecks is important, since it allows local extinction risks to be analyzed as a result of the reduced population size.

Implications for genetic conservation

The high genetic diversity observed in *P. sellowii* populations indicates their potential use in genetic conservation programs. Mitigation processes, such as the introduction of immigrants or vegetation corridors (Brandão et al., 2015), may be carried out in areas that have suffered genetic bottlenecks, or when gene flow is not sufficient to counteract the effects of drift. The implementation of *in situ* conservation strategies by introducing divergent genotypes may be utilized to increase N_E and by minimizing outbreeding depression (Vieira et al., 2010). For seedling production, the number of trees for seed collection in each subpopulation should be close to N_E. For population A, a limit of 94 m between the matrices should be respected for the collection, since a clustered distribution of genotypes was observed at this distance. No genetic structure was detected for the other populations. Especially for the studied area, management and compliance with legislation that protects these areas are required so that the high genetic diversity can be maintained. The extinction of population B may increase the genetic divergence between populations A and C. The presence of rock masses, which act as barriers to gene flow, may influence the genetic structure of *P. sellowii.* Thus, variation in the estimates of genetic variability may be influenced by the particular characteristics of each habitat.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Table S1. Allozyme loci (genotype data) for *Podocarpus sellowii*.

Table S2. Probabilities of Fisher's exact test for analysis of Hardy-Weinberg Equibrium of *P. sellowii* (Klotz.) individuals in the studied subpopulations.