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Original Article

Conservation of Nature

Genetic Diversity and Structure of *Calophyllum brasiliense* Along the Santa Catarina Coast

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ABSTRACT

Population genetics studies can provide knowledge in support of conservation efforts for plant populations. We studied the genetic diversity and structure in nine populations of *Calophyllum brasiliense* from Santa Catarina state, Brazil, aiming to establish conservation strategies. Allozymic markers were used to genotype about 50 individuals from each population. Moderate genetic diversity (mean = 0.135) and high fixation indexes (mean = 0.259) were estimated. Genetic divergence was significant, equal to 0.140, and a Bayesian analysis found two different genetic groupings. The results show clear signs of risk of diversity loss, basically related to restrictions of effective size. As conservation efforts, we suggested the use of *C. brasiliense* in restoration programs and for wood production. We also suggested the protection of its associated fauna and the development of more protected areas. Finally, populations 430, 640, 642 and 913 were indicated as priorities for conservation, based on their genetic indexes.

Keywords: conservation, effective size, *olandi*, quaternary coastal plains.

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1. INTRODUCTION

Population genetics studies allow us to understand the origin, amount, and distribution of genetic variation present in populations of organisms, as well as its spatiotemporal variation (Templeton, 2011). This, in turn, forms a foundation for understanding the consequences of anthropic actions on populations (e.g., Hall et al., 1996; Bittencourt & Sebbenn, 2009; Silva & Reis, 2010) and proposing conservation strategies for populations at risk (e.g., Hmeljevski et al., 2011; Ferreira et al., 2012; Rogalski et al., 2017). Studies proposing conservation efforts for plant populations commonly use molecular markers to investigate genetic diversity and structure (e.g., Botrel et al., 2006; Reis et al., 2009; Nazareno & Reis, 2014).

Calophyllum brasiliense Cambess. (Clusiaceae), popularly known as *olandi* or *guanandi*, is a tree species with wide natural occurrence area, from latitude 18° N in Costa Rica, to 28°10' S in Brazil (Carvalho, 2003). It occurs in distinct biomes along Brazil (Cerrado, Amazon and Atlantic Forests and Restinga), and in Santa Catarina state (SC), its occurrence is restricted to the quaternary coastal plains where it is one of the dominant species (Klein, 1978; Lingner et al, 2013). *Calophyllum brasiliense* occurs in soils with poor drainage and in an area further characterized by humidity and periodic flooding (Reitz et al., 1978; Carvalho, 2003). Forests occurring on quaternary coastal plains are subject to flooding and thus commonly termed as *caixetais* or *olandizais*, owing to the dominance of *Tabebuia cassinoides* (*caixeta*) and *C. brasiliense* (*olandi*) (Reitz et al., 1978; Galvão et al., 2002).

Forests of the quaternary coastal plains, collectively called *restinga*, exhibit distinct vegetation forms, ranging from grasslands with predominance of herbaceous plants to scrubs and forest physiognomies (Marques et al., 2015). In SC, these forests are threatened by real estate speculation and silvopastoral activities (Reis et al., 2012; Schultz, 2012; Lingner et al., 2013); moreover, *C. brasiliense* is considered endangered in SC, having been categorized as critically endangered (Santa Catarina, 2014). This calls for studies to support conservation strategies for this species and its habitat. Other studies have also pointed to the need for conservation actions for ecosystems in quaternary coastal plains (Scarano, 2006; Reis et al., 2012; Marques et al., 2015).

Thus, the present study explored the genetic diversity and structure of *C. brasiliense* populations, aiming to establish conservation strategies for the species and its habitat in SC. This study is part of the project entitled "Floristic and Forest Inventory of Santa Catarina", from which the objective is to evaluate forest remnants of SC in order to form a basis for proposing public conservation policies directed toward the forestry sector (Vibrans et al., 2012).

2. MATERIAL AND METHODS

2.1. Sampling and genotyping

Fresh leaves of approximately 50 adult individuals, spaced at least 50 m apart, were collected in nine populations along the area of occurrence of *C. brasiliense* in SC (Figure 1). The samples were kept refrigerated until the time of genotyping.

To assess the genetic diversity levels of the populations, we used allozymic markers, resolved and stained in maize starch gel (13%), according to the recommendations of Kephart (1990) and Alfenas (1998). Tris-Citrate buffer pH 7.5 (Tris 27 g/l and citric acid 16.52 g/l) was used to solve the following allozymic systems: 6-phosphogluconate dehydrogenase (6PGDH, Enzyme Commission 1.1.1.44), diaphorase (DIA, EC 1.8.1.4), glutamate oxalacetate transaminase (GOT, EC 2.6.1.1), isocitrate dehydrogenase (HDI, EC 1.1.1.42), malic enzyme ME, EC 1.1.1.40), malate dehydrogenase (MDH, EC 1.1.1.37), phosphoglucoisomerase (PGI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 5.4.2.2), peroxidase (PO, EC 1.11.1.7), and shikimate dehydrogenase (SKDH, EC 1.1.1.25), totaling 10 systems.

2.2. Data analysis

Based on genotypes observed in gel, we estimated the allelic frequencies, percentage of polymorphic loci ($\hat{P}$), number of alleles ($\hat{k}$), average number of alleles per locus ($\hat{A}$), number of rare ($\hat{A}_r$ – frequency < 5%) and private alleles ($\hat{A}_{ex}$ – occuring in only one population), observed heterozygosity ($\hat{H}_O$), expected heterozygosity under Hardy Weinberg Equilibrium ($\hat{H}_E$) (Nei, 1973), and the fixation index ($\hat{f}$) for each studied population. The genetic divergence between populations ($\hat{\theta}$) was estimated as proposed by Weir & Cockerham (1984). Statistical significance ($p < 0.05$)

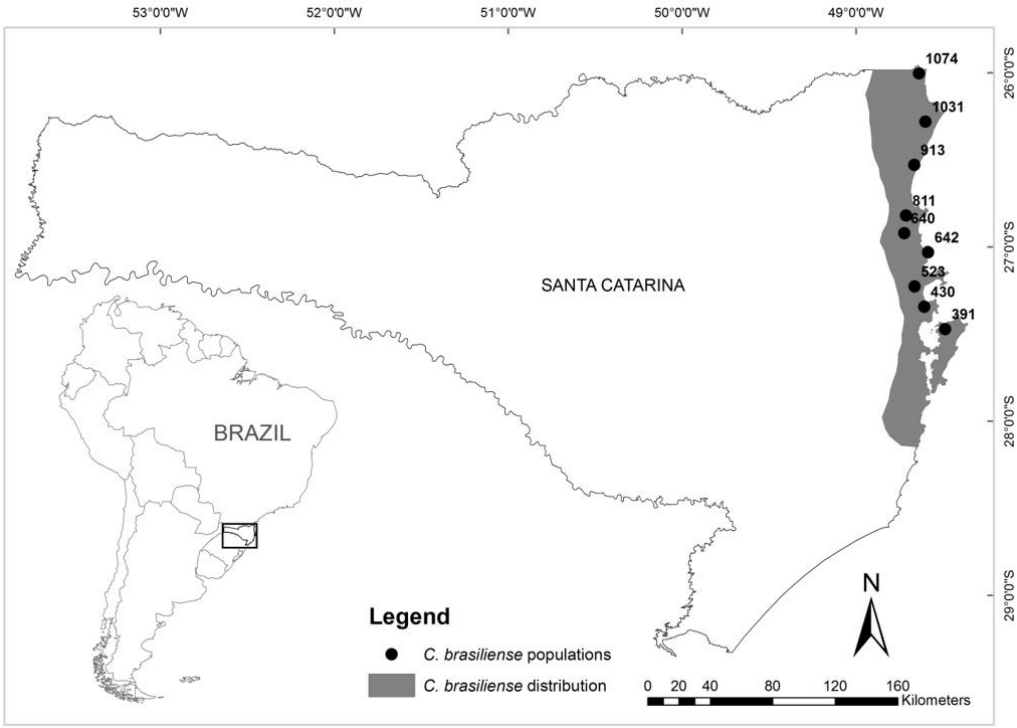

Figure 1. Map of nine sampled populations (dots) of *Calophyllum brasiliense* in Santa Catarina state, Brazil. Coloring represents the occurrence area of *Calophyllum brasilense* Cambess. (Reitz et al., 1978).

for $\hat{f}$ values was obtained by permutations of alleles between individuals and within populations, and for $\hat{\theta}$ values, by 1,000 *bootstraps* between loci. Effective size ($\hat{N}_e$) was estimated using Equation 1, as proposed by Li (1976). Except for $\hat{N}_e$, all other estimates were calculated using FSTAT, version 2.9.3.2 (Goudet, 2002).

$$\hat{N}_e = n / \left(1 + \hat{f}\right) \quad (1)$$

where: n = sample size and $\hat{f}$ = fixation index.

Based on the $\hat{N}_e$ of each population, we estimated the minimum numbers of seed trees to be sampled in the event of seed collection to establish new populations with effective sizes of 25, 50 and 500, as proposed by Sebbenn (2002). Therefore, the ratio between the sample size (n) and the $\hat{N}_e$ of each population was multiplied by 25, 50 and 500.

To assist in the identification of priority conservation areas, a Bayesian analysis of the genetic structure was conducted, aiming to estimate an optimal number of populations to conserve, depending on the number of genetic groups (K), using STRUCTURE, version 2.3.4 (Pritchard et al., 2000). The analysis was performed under the admixture model with correlated allele frequencies, with 50,000 burn-in length periods and 100,000 Markov chain Monte Carlo replications. Each run was iterated 10 times, with K ranging from 1 to 9. The most probable value of K was selected using the highest value of ΔK (Evanno et al., 2005) in the STRUCTURE HARVESTER program (Earl & vonHoldt, 2012).

A Mantel test (Mantel, 1967) was conducted in order to identify if geographical distances (km) influence the levels of genetic divergence ($\hat{\theta}$). Thus, a Pearson correlation was estimated between the pairwise geographic distances and genetic divergences between populations with both distances transformed $[log(km)] \sim [\hat{\theta} / (1 - \hat{\theta})]$, according to Rousset (1997). Statistical significance ($p < 0.05$) for the correlation was obtained using a t test (Equation 2).

$$t = r\sqrt{\left(n - 2 / 1 - r^2\right)} \quad (2)$$

where: r = Pearson correlation coefficient and n = number of populations pairs.

3. RESULTS

3.1. Genetic diversity

The 10 allozymic systems allowed the interpretation of 14 loci, of which nine were polymorphic. The allelic frequencies (Table 1) within each polymorphic locus were mostly concentrated in only one of the alleles, a fact that reduced the estimates of $\hat{H}_E$. Thirty distinct alleles were identified among the nine populations, out of which three were private: alleles 6PGDH-2-3 and PO-2-3 in population 391, and allele DIA-3-1 in population 640 (Table 1).

The estimated genetic diversity indexes for the studied populations are shown in Table 2. All populations presented at least one rare allele ($\hat{A}_r$). Estimates of $\hat{H}_O$ were lower than $\hat{H}_E$ for all populations, resulting in positive and significant fixation indexes. Consequently, the estimated effective sizes ($\hat{N}_e$) were always smaller than the sample sizes, reflecting the estimated $\hat{f}$ for each population (Table 2).

Table 1. Allelic frequencies for nine populations of *Calophyllum brasilense* Cambess. using 14 allozymic loci.

Locus	Allele	391	430	523	642	640	811	913	1031	1074	All
6PGDH-1	1	0.971	0.981	0.962	NE	1	0.981	0.419	0.99	0.962	0.934
	2	0.029	0.019	0.038	NE	0	0.019	0.581	0.01	0.038	0.066
6PGDH-2	1	0.048	0.115	0.009	NE	0.333	0.096	0.984	0.188	0.03	0.185
	2	0.952	0.865	0.991	NE	0.667	0.904	0.016	0.813	0.97	0.812
	3	0	0.019	0	NE	0	0	0	0	0	0.003
DIA-1	1	1	1	1	1	1	1	1	1	1	1
DIA-2	1	1	1	1	1	1	1	1	1	1	1
DIA-3	1	0	0	0	0	0.058	0	0	0	0	0.006
	2	0.865	0.824	0.925	1	0.913	1	1	1	1	0.948
	3	0.135	0.176	0.075	0	0.029	0	0	0	0	0.046
GOT	1	1	1	1	1	1	1	1	1	1	1
IDH	1	0	0	0.047	0	0	0	0	0	0.019	0.007
	2	1	1	0.953	1	1	1	1	1	0.981	0.993
ME	1	1	1	1	1	1	1	1	1	1	1
MDH-1	1	0.077	0.147	0.269	0.167	0.102	0.329	0.135	0.208	0.16	0.173
	2	0.038	0.118	0.038	0.069	0.046	0	0.115	0.047	0.06	0.06
	3	0.885	0.735	0.692	0.765	0.852	0.671	0.75	0.745	0.78	0.766
MDH-2	1	1	1	1	1	1	1	1	1	1	1
PGI-2	1	0.788	0.683	0.698	0.88	0.676	0.873	0.935	0.877	0.863	0.805
	2	0.192	0.317	0.302	0.12	0.287	0.127	0.043	0.123	0.137	0.187
	3	0.019	0	0	0	0.037	0	0.022	0	0	0.009
PGM	1	0	0	0.023	0.133	0.113	0.365	0.302	0.031	0.054	0.112
	2	0.442	0.547	0.398	0.337	0.226	0	0.417	0.5	0.489	0.373
	3	0.558	0.453	0.58	0.531	0.66	0.635	0.281	0.469	0.457	0.515
PO-2	1	0.01	0.01	0	0	0	0	0	0	0	0.002
	2	0.99	0.95	1	1	1	1	1	1	1	0.994
	3	0	0.04	0	0	0	0	0	0	0	0.004
SKDH	1	0.721	0.721	0.761	0.806	1	0.814	0.73	0.802	1	0.82
	2	0.279	0.269	0.239	0.153	0	0.186	0.24	0.151	0	0.167
	3	0	0.01	0	0.041	0	0	0.03	0.047	0	0.013

NE = not evaluated.

Table 2. Population genetics estimates for nine *Calophyllum brasilense* Cambess. populations sampled in Santa Catarina state, Brazil.

Population	n	$\hat{N}_e$	$\hat{P}$	$\hat{k}$	$\hat{A}$	$\hat{A}_r$	$\hat{A}_{ex}$	$\hat{H}_E$	$\hat{H}_O$	$\hat{f}$
391	52	42	0.57	24	1.71	5	0	0.133	0.102	0.238*
430	52	42	0.57	26	1.86	5	2	0.175	0.136	0.226*
523	52	42	0.57	24	1.71	5	0	0.149	0.112	0.246*
642	51	35	0.43	21	1.58	1	0	0.127	0.072	0.437*
640	54	38	0.36	23	1.64	3	1	0.132	0.080	0.392*
811	51	41	0.43	20	1.43	1	0	0.119	0.091	0.234*
913	48	40	0.43	24	1.71	4	0	0.153	0.123	0.197*
1031	51	43	0.43	23	1.64	4	0	0.130	0.105	0.193*
1074	51	44	0.43	22	1.57	3	0	0.095	0.079	0.168*
Average	51	41	0.47	23	1.65	-	-	0.135	0.100	0.259
All	454	335	0.64	30	2.14	-	-	0.153	0.099	0.354

n = sample size; $\hat{N}_e$ = effective size; $\hat{P}$ = percentage of polymorphic loci; $\hat{k}$ = number of alleles; $\hat{A}$ = average number of alleles per locus; $\hat{A}_r$ = number of rare alleles; $\hat{A}_{ex}$ = number of private alleles; $\hat{H}_E$ = genetic diversity; $\hat{H}_O$ = observed heterozygosity; $\hat{f}$ = fixation index; *p < 0.05.

3.2. Genetic structure

Populations exhibited significant genetic divergence ($\hat{\theta}$), equal to 0.140. The Mantel test presented a non-significant correlation of 0.08 between genetic divergence and geographic distances. Bayesian analysis returned an ideal number of genetic groupings (K) equal to 2 (Figures 2 and 3).

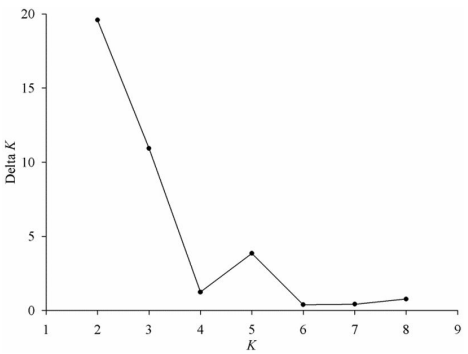

Figure 2. Ideal number of genetic clusters (K) according to ΔK (Evanno et al., 2005) for nine *Calophyllum brasilense* Cambess. populations.

4. DISCUSSION

4.1. Genetic diversity

The presence of fixed loci (Table 1) suggests loss of alleles from genetic drift. The diversity indexes found by the present study were lower than the averages compiled by Hamrick & Godt (1989) for long-lived tree species (Table 3). The same trend was observed when our results were compared to indexes estimated by studies with other populations of *C. brasilense* (Table 3). Also noteworthy is the fact that most studies with populations of *C. brasilense* estimated lower $\hat{f}$ per population than those estimated by the present study (Table 3).

The fact that all studied populations presented rare alleles, more susceptible to the effects of genetic drift, and high $\hat{f}$, owing to significant deviations between $\hat{H}_O$ and $\hat{H}_E$, shows that the populations of *C. brasilense* risk the loss of diversity in the next generations. These findings may result from aspects related to the mating system of the species or to historical population reduction caused by exploitation and fragmentation.

It is not yet known if *C. brasiliense* presents an outcrossed, autogamous or mixed mating system. The species is pollinated by bees of the Halictidae family (Fischer & Santos, 2001), which indicates at least a tendency for cross-fertilization. However, *C. brasiliense* presents hermaphrodite and male flowers in separate individuals, or in different proportions in the same individual (Fischer & Santos, 2001; Botrel et al., 2006), and that can be a sign of some level of autogamy. Tree species with an outcrossed system have higher diversity indexes within species and populations than species with mixed or autogamous systems (Hamrick et al., 1992). Therefore, the mating system of a species can help to explain the genetic indexes.

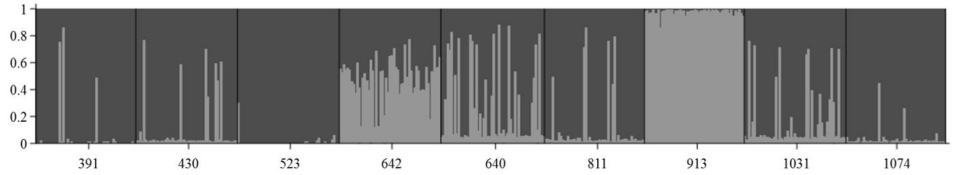

Figure 3. Assignation of individuals to genetic clusters when $K = 2$ for nine *Calophyllum brasilense* Cambess. populations. The color in each barplot represents the probability of each individual belonging to a genetic cluster.

Table 3. Comparison of the mean indexes of genetic diversity obtained in the present study with other studies.

Study	Pop.	$\hat{A}$	$\hat{H}_E$	$\hat{H}_O$	$\hat{f}$
Hamrick & Godt (1989)[†]	115	1.79↑	0.149↑	NE	NE
Kawaguici & Kageyama (2001)[††]	1	1.64↓	0.223↑	0.141↑	0.372↑
Botrel et al. (2006)[††]	2	1.75↑	0.131↓	0.119↑	0.091↓
		1.5↓	0.112↓	0.111↑	0.008↓
Souza et al. (2007)[††]	2	2.00↑	0.430↑	0.444↑	-0.031↓
		2.00↑	0.438↑	0.492↑	-0.125↓
Reis et al. (2009)[††]	1	2.00↑	0.419↑	0.488↑	-0.166↓
Present study	9	1.65	0.135	0.100	0.259

Higher (↑) or lower (↓) index when compared to the same index estimated in the present study. Pop. = number of sampled populations; $\hat{A}$ = average number of alleles per locus; $\hat{H}_E$ = genetic diversity; $\hat{H}_O$ = observed heterozygosity; $\hat{f}$ = fixation index; [†] compilation of 115 populations of tropical species; [††] studies with populations of *Calophyllum brasilense* Cambess; NE = not evaluated.

Accordingly, this question needs to be evaluated in more depth for *C. brasiliense* in order to make more accurate inferences about its conservation status and also to better design strategies to conserve the genetic diversity of the species in remnant populations.

On the other hand, even in the absence of specific estimates, forests of the Brazilian coastal plains are highly fragmentated (Marques et al., 2015). In addition, populations of *C. brasiliense* went through the logging process at different levels of intensity. Reitz et al. (1978) have already pointed out the rarity of the species, even in its occurrence area in SC, and later, Marques & Joly (2000) reported that populations of *C. brasiliense* were under heavy pressure from illegal logging and/or the advance of agricultural areas.

Fragmentation and exploitation events act to isolate and diminish populations, respectively. As a consequence, populations are more susceptible to the effects of genetic drift and, in the long term, they may present an increase in fixation indexes owing to the increased frequency of crosses between related individuals (Ellstrand & Elam, 1993; Kageyama et al., 1998) and the absence of gene flow. Thus, the estimated fixation indexes for the studied populations may be related to the history of environmental fragmentation and exploration, as reported for the quaternary coastal plains and for *C. brasiliense*, respectively. Effects of fixation indexes are already perceived in $\hat{N}_e$ estimates, which were, on average, 20% smaller than the sample sizes.

4.2. Genetic structure

The estimation of genetic divergence ($\hat{\theta}$) indicates that 14% of the genetic diversity is not shared by the populations, and although the most distant populations are approximately 160 km apart, geographic distance did not significantly influence genetic divergence, as evidenced by the Mantel test. This result indicates that events of fragmentation and exploitation, or even founder effects, are more important for the generation

of genetic structure than geographic distance between populations.

Bayesian analysis of the genetic structure demonstrated the existence of two distinct genetic groups, one formed by populations 642 and 913, and the other formed by the other populations (Figure 3). This clustering is useful for determining priority populations for conservation based on the genetic structure because it demonstrates which populations present significant distinct genetic compositions. Therefore, aiming to conserve the greatest amount of genetic diversity with the lowest number of populations, we should choose at least one population from each group.

4.3. Conservation of genetic diversity

The results show that the populations of *C. brasiliense* risk the loss of diversity related to restrictions of effective size, as imposed by the habitats' exploitation and fragmentation history. Therefore, the suggestion of conservation measures should take this into account. However, questions linked to the mating system cannot be discounted (e.g., possible levels of autogamy).

Increasing gene flow among populations can mitigate the effects of genetic drift and high fixation indexes. This could be accomplished by including this species in reforestation programs, taking advantage of its tolerance to poor drainage, humidity and periodic flooding (Reitz et al., 1978; Carvalho, 2003; Oliveira & Joly, 2010), as well as its interaction with fauna (Fischer & Santos, 2001; Carvalho, 2003). Other authors have also pointed out this possibility (Oliveira & Joly, 2010; Schultz, 2012).

We know that the mating system in relation to fauna plays an important role in gene flow between and within populations of *C. brasiliense*, especially since flowers are pollinated by bees (Fischer & Santos, 2001), and fruits are dispersed by bats (Phyllostomidae) and birds, such as *Cyanocorax caeruleus* (Fischer & Santos, 2001; Carvalho, 2003). Hence, the preservation of fauna is important to the maintenance of *C. brasiliense* gene flow, especially in fragmented areas of quaternary coastal plains (Marques et al., 2015).

Calophyllum brasiliense presents high-quality wood in addition to its moderate growth (Carvalho, 2003). Consequently, the establishment of plantations for wood production could be a viable alternative to increase *C. brasiliense* populations and the connectivity between them, even if they are temporary. Thus, with the adoption of seed collection criteria, forest fragments could serve as a source of seeds for local plantations.

Sebbenn (2002) recommends the collection of seeds in 25 seed trees, aiming to establish reforestation of less than 100 ha, in 40 to 50 seed trees for reforestation with an area between 100 and 500 ha, and in 400 to 500 seed trees for establishing reforestation in areas larger than 500 ha. However, since all populations presented high $\hat{f}$ (Table 2), the number of seed trees collected should be, correspondingly, higher, as shown in Table 4.

Based on Bayesian analysis of genetic structure and on the genetic diversity estimates, populations 430, 640, 642 and 913 are indicated as priorities for conservation. The first two have private alleles (Table 2) and are part of a distinct genetic grouping from the last two, which, in turn, are the most genetically distinct populations (Figure 3).

Table 4. Number of seed trees required for seed collection aiming at an effective size of 25 ($\hat{N}_e$ = 25), 50 ($\hat{N}_e$ = 50) and 500 ($\hat{N}_e$ = 500).

Population	n	$\hat{N}_e$	$n/\hat{N}_e$	$\hat{N}_e$ = 25	$\hat{N}_e$ = 50	$\hat{N}_e$ = 500
391	52	42	1.24	31	62	619
430	52	42	1.23	31	61	613
523	52	42	1.25	31	62	623
642	51	35	1.44	36	72	719
640	54	38	1.39	35	70	696
811	51	41	1.23	31	62	617
913	48	40	1.20	30	60	599
1031	51	43	1.19	30	60	597
1074	51	44	1.17	29	58	584

n = sample size; $\hat{N}_e$ = effective size.

5. CONCLUSION

The *Calophyllum brasiliense* populations studied had, in general, lower genetic diversity than expected for a long-lived tree species, along with high fixation indexes, implying constraints in effective size in the past. This condition plus the contemporary habitat fragmentation endangers the maintenance of genetic diversity of the species, putting specially the rare alleles, which were sampled in all populations, at risk of loss.

Conservation efforts should involve expanding populations and increasing connectivity between them. Therefore, we suggest the following conservation strategies: 1) the use of *C. brasiliense* in restoration programs of degraded areas; 2) the establishment of plantations with *C. brasiliense* for wood production; 3) the protection of the fauna associated with *C. brasiliense*; and 4) the inclusion of more fragments of quaternary coastal plains in protected areas.

Populations 430, 640, 642 and 913 are indicated as priorities for conservation. The first two represent the presence of private alleles (Table 2), and the last two represent the most genetically distinct (Figure 3). For a better understanding of the factors influencing the levels of genetic diversity between and within populations, studies to elucidate the mating system of the species are crucial.

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