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Assessment of genetic differentiation among relict populations of *Calophyllum brasiliense* Camb. (Calophyllaceae) from Northeast Argentina

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Abstract

Calophyllum brasiliense is a tropical tree that grows exclusively in riparian forest and in almost permanently flooded areas. In Argentina two small populations located within riparian forest from Misiones and Corrientes Provinces have been recently identified. These riparian communities have been extensively fragmented and are at risk of local extinction due to flooding caused by a nearby dam and other anthropogenic changes that threaten this habitat. Genetic characterization using information from 56 RAPD loci revealed low expected heterozygosity in both populations (*He* = 0.273). Most genetic variability was distributed within populations, and a significant ϕ_{ST} statistic value (0.283, $p<0.05$) showed the existence of a large genetic differentiation between them. Furthermore, the SGS analysis revealed a nonrandom distribution of genotypes in Misiones' population. Although these populations could have belonged to a large and continuous forest in the past, the process of habitat fragmentation may have favoured the divergence between them; sufficient time has passed to cause their genetic differentiation. As these populations represent the new southernmost species distribution, the genetic information obtained in this study should be analyzed in conjunction with ecological evaluations in order to develop management strategies that can ensure its conservation.

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Introduction

The biogeographic Paranaense Forest Province includes Misiones and the Northeast of Corrientes Argentinean Provinces (Morrone, 2001) (Fig. 1). Within the Paranaense Forest Province, a peculiar plant formation stands out for growing in almost permanently flooded soils. This formation type shows a differentiated structure and floristic composition which is known as "floresta higrófila", "mata de brejo" (Leitão-Filho, 1982), or "seasonal semideciduous forest" with permanent fluvial influence (Rodrigues, 2000). Lobo and Joly (2000) pointed out that there are typical or exclusive species at hygrophile forests, such as *Calophyllum brasiliense*, which are absent in other forest classes.

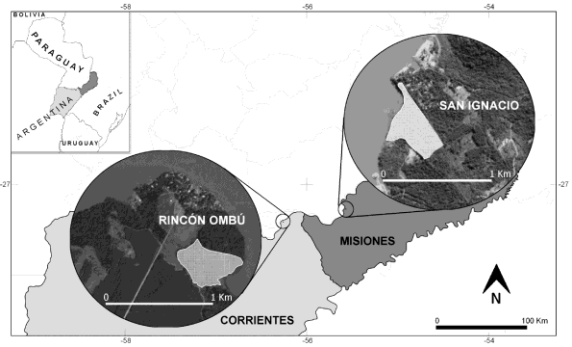

Fig. 1. Location of Corrientes and Misiones Provinces in the Northeast of Argentina (inset). Circles: Remnants of riparian forest with populations of *C. brasiliense*. A: Rincón Ombú (Corrientes) and B) San Ignacio (Misiones).

C. brasiliense, arary, is a tree species specialist in habitat, abundant in riparian environments (Reis *et al.,* 2009). It has been classified as a flood-tolerant tree due to its capability of maintaining carbon assimilation and growing in flooded conditions (De Oliveira and Joly, 2010). The species possesses several flooding-induced characteristics that allow it to survive and be successful in natural seasonally flooded areas; therefore, *C. brasiliense* populations exhibit high density, dominancy, and frequency indexes within riparian communities (Marques *et al.*, 2003). Sanjurjo (1994) described it as a "rare tree species" for standing in small and almost pure patches, far away from its common habitat. Due to all its properties and adaptations, De Oliveira and Joly (2010) proposed arary as a suitable species for native flora rehabilitation in riparian areas.

The species geographical distribution extends from southern Mexico to northeastern Argentina, its new southern boundary delimited by the populations that have been found in Corrientes and Misiones Provinces (Rodríguez *et al.*, 2009). Several human activities have caused reduction in both populations, with ecological and genetic consequences such as a loss of variability that has compromised the local viability of the species (Reis *et al.*, 2009). Recently, the water level of the Paraná River increased due to the nearby downstream Yacyretá Dam. Ecological and genetic studies of at-risk species and populations have demonstrated the effects of human modifications in natural environments, and have helped define the best genetic parameters to use in the conduct and monitoring of conservation activities in damaged ecosystems (Kageyama *et al.*, 1998).

There are several matters to take into account when planning conservation actions: (a) understanding the local distributions of genotypes, which could be useful for delimiting conservation areas; (b) collecting seeds or pollen for a germplasm bank and other natural population conservation strategies; and (c) determining the existence of Spatial Genetic Structure (SGS), or nonrandom distribution of genotypes, which is common in natural plant populations (Doligez and Joly, 1997; Hardy *et al.*, 2006). Molecular markers have contributed significantly to these topics and have been widely used in plant science. Random amplified polymorphic DNA (RAPD) analysis is the simplest and most cost-effective of the many DNA markers available, and does not require a sophisticated laboratory for most of its application (Babu *et al.*, 2014).

RAPD markers have been used for prompt genetic characterization of a great number of plant species (Fernandes de Souza *et al.*, 2012; Liu *et al.*, 2012;

Chhipi Shrestha *et al.*, 2013; Inoue *et al.*, 2013). Although the method is limited by the dominant nature of RAPD markers and by the difficulties in technique reproducibility, RAPD markers are usually the most practical alternative for the analysis of the numerous poorly studied tropical tree species, for which codominant markers are not yet available (Fernandes de Souza *et al.*, 2012). In addition, this technique can simultaneously examine multiple genome loci. Therefore, the RAPD technique has the advantage of being able to quickly screen a wide proportion of the DNA without prior sequence knowledge of the genome under study (Allendorf *et al.*, 2013; Babu *et al.*, 2014).

We hypothesized that these Argentinean *C. brasiliense* populations belonged in the past to a continuum of large forest which underwent a profound fragmentation process that resulted in genetic differentiation of the Argentinean *C. brasiliense* populations. Our overall goals were to contribute to understanding of this process and achieve a rapid genetic characterization of the local at-risk and fragmented population. Specific objectives were 1) to characterize the distribution of genetic variation within and between the two *C. brasiliense* populations from Northeast Argentina, and 2) to evaluate the existence of spatial genetic structure autocorrelation.

Materials and Methods

Study Area

Misiones and Corrientes are Argentinean provinces bordered by the Paraná and Uruguay rivers in the Northeast of the country. Misiones contains the largest remaining tract of Paraná Atlantic Forest ecoregion and it was historically 90–95% covered with Upper Paraná Atlantic Forest. Both provinces are characterized by humid, subtropical climate with no distinct dry season. Argentinean populations of *C. brasiliense* analyzed are both in remnants of riparian forest on the Argentinean side of the Paraná River. One of them is located at the Private Reserve Luis Jorge Velázquez in San Ignacio (SI-Misiones-ARG; 27°16'34.4"S, 55°34'11.9"W) and the other one is downstream, at Rincón Ombú Refuge in Ituzaingó (RO-Corrientes-ARG; 27°24'54.42"S, 56°29'43"W). The geographical distance between these populations is approximately 100 km (Fig. 1).

Plant Material

Grids of plots (10 m × 10 m) were outlined for SI and RO populations that covered 0.21 ha and 0.14 ha, respectively. Each individual was identified with a number written on a piece of sheet metal and the label was attached to the tree bark with a nail, or a seal in the case of young trees. For each individual, spatial coordinates were registered using a GPS (GARMIN 60CSX).

Leaves of 30 individuals separated by a distance of at least 10 m were collected, cleaned, and placed in small ziplock plastic bags with silica gel until they were completely dry for the DNA extraction.

Genetic Analysis

Total genomic DNA extraction was carried out based on the protocol described by Stange (1998), modified to include the incorporation of 2% polyvinylpyrrolidone (PVP), 5 mM ascorbic acid, 4 mM sodium diethyldithiocarbamate trihydrate (DIECA), and 1.2% β-mercaptoethanol into the digestion buffer in a 2 ml plastic tube to improve the homogenization (Percuoco, 2007). Extractions were carried out in a room separated from the one in which polymerase chain reaction (PCR) amplifications were conducted to prevent cross contamination of samples.

The PCR amplifications were conducted in a final volume of 40 µl containing 10 ng of genomic DNA; 200 µM each of dATP, dCTP, dGTP, and dTTP; 1X buffer; 2.5 mM $MgCl_2$; 1.25 units of FlexiTaq (Promega-Biodynamics); and 0.2 µM of each primer. Samples were amplified in a DNA thermal cycler TechnePHC-3 programmed to perform 40 cycles of 94 °C for 1 min, 36 °C for 1 min, and 72 °C for 2 min. A negative control (no DNA added) was included in each PCR run to test for contamination. The

amplifications were carried out in triplicate to assess profile reproducibility. Two series of 10 primers each were screened (Table 1) in order to select those that generated reproducible and clear profiles for the analysis. Nonspecific bands that appeared in negative controls and in each sample, due to the low stringency of the reaction, were excluded from the sample profiles in subsequent analysis (Thormann *et al.*, 1994; Casas *et al.*, 1999). The amplicons were resolved in 1.4 % agarose gels with BrEt (Promega) and visualized in a UVP-TM-20 transilluminator. Images were captured for the next analysis step using a Kodak Easy Share B102-1 Camera.

Table 1. Details of the RAPDs primers assayed (Biodynamics Series A and B).

Primer ID	Sequence 5'- 3'	Primer ID	Sequence 5'- 3'
A01	CCCAAGGTC	**B01**	TCGAAGTCCT
A02	GGTGCGGGAA	**B02**	CGATGTCAGA
A03	AAGACCCCTC	**B03**	ACTTCGACAA
A04	CTTCACCCGA	**B04**	TGCCATCAGT
A05	CACCAGGTGA	**B05**	GCGCTCACGC
A06	GAGTCTCAGG	**B06**	GTGACATGCC
A07	CCCGATTCGG	**B07**	AGATCGAGCC
A08	ACGCACAACC	**B08**	TCACCACGGT
A09	CTAATGCCGT	**B09**	ATGGCTCAGC
A10	ACGGCGTATG	**B10**	CAGGCACTAG

Genetic Data Analysis

RAPD markers were named using the primer identification code plus the band molecular weight. The molecular sizes in bp were estimated using Gqmol software (http://www.ufv.br/dbg/gqmol/gqmol. htm). Bands for each selected primer were scored as present (1) or absent (0) for each individual resulting in a binary data matrix to estimate allelic frequencies, polymorphic loci (%P), number of private bands (PB), and expected heterozygosity. In order to describe the genetic diversity distribution, Shannon's Information Index (*I*) (Shannon, 1948), and Nei's genetic distance (Nei, 1972; 1978) were calculated and a Principal Coordinates Analysis (PCoA) was conducted to identify existence of clusters within and between populations. A genetic distance matrix was obtained to test for genetic structure (differentiation) via Analysis of Molecular Variance (AMOVA) computing Phi-statistics (ϕ_{ST}) based on 9999 permutations.

A geographic distance matrix was obtained and compared with the genetic distance matrix through a Mantel test to assess for correlation, comparing the observed Rxy versus random Rxy obtained from 9999 permutations. Finally, a multilocus spatial autocorrelation analysis was performed for each population, with class size of 10 m, 9999 permutations, and 10 000 bootstraps. All parameters and spatial genetic analysis mentioned above were done using GeneAlEx versión 6.5 (Peakall and Smouse, 2006; 2012).

Results

All samples (N= 60) were successfully amplified, allowing us to obtain reproducible profiles in all samples derived from SI (N=30) and RO (N=30) populations. Only three of the primers tested (A03, B06, and B10) were selected for the genetic analysis. These primers generated distinct band patterns from which it was possible to identify 56 bands that ranged in size from 198 to 1780 bp (Table 2). Of the 56 loci analyzed, 82.14% were polymorphic (Table 2), with a total *He* = 0.273 and a mean Shannon's Index of 0.406. In addition, the San Ignacio population exhibited 9 private bands (17.64%), whereas Rincón Ombú shown only 5 among all loci analyzed (10.63%) (Table 3). Mean parameter values obtained for each population are detailed in Table 3. Finally, the Nei's genetic distance estimated between populations was 0.185.

Table 2. Summary of the data obtained from primer band patterns selected. TNB: Total Number of Bands, NPB: Number of Polymorphic Bands. %P: Percentage of Polymorphic Loci.

Primer ID	TNB	NPB	%P	Amplicon Size Range (bp)
A03	19	18	94.7	245 – 1630
B06	13	12	92.3	325 – 1780
B10	24	24	100	198 – 1750

Table 3. Population parameter estimated based on 56 RAPD loci in SI and RO. %P: Polymorphic Loci, PB: Private Bands, *He*: Expected Heterozygosity, and *I*: Shannon's Information Index. Standard deviation is between brackets.

Parameter	Population		Mean
	San Ignacio	Rincón Ombú	
% P	80.36	83.93	82.14 (1.79)
PB	5	9	-
He	0.250 (0.023)	0.296 (0.025)	0.273 (0.017)
I	0.379 (0.032)	0.434 (0.034)	0.406 (0.023)

The results obtained from the PCoA analysis carried out explained the 38.55% of the genetic variability in the three first axes, segregating the two populations (Fig. 2). Through the AMOVA it was determined that 72% of the variation found was within populations and only 28% of it was between them (Table 4). The ϕ_{ST} calculated was 0.283, and was significant at $p<0.001$. The Mantel test carried out to detect correlation between geographic and genetic distance showed significance for San Ignacio (Rxy = 0.183, $p \leq 0.007$). However, the Rxy obtained in Rincón Ombú was not significant (Rxy = 0.084, $p \leq 0.07$) (Fig. 3a,c). The spatial autocorrelation analysis indicated that the genetic variability is spatially structured in San Ignacio with significant positive r values in the 20-m class and significant negative r values in 80-, 100-, 120-, and 130-m classes. On the other hand, lack of significant values indicated that genetic variation is randomly distributed in Rincón Ombú (Fig. 3b,d).

Table 4. Analysis of Molecular Variance (AMOVA) in SI and RO populations. df: degree of freedom, SS: Squares Sum, MS: Mean Squares.

	df	SS	MS	Variance	% Variance
Among POP	1	106,531	106,531	3,226	28
Within POP	59	481,486	8,161	8,161	72
Total	60	588,017		11,387	100

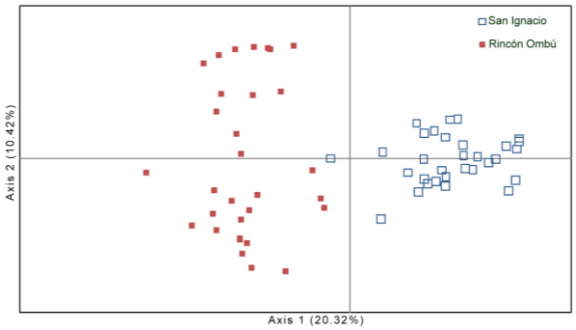

Fig. 2. Principal Coordinates Analysis (PCoA) of the genetic variability in San Ignacio and Rincón Ombú. The explained variability is between brackets in each axis.

Discussion

The data obtained in the present work allowed us to estimate population parameters useful for describing the genetic variability in these southernmost *C. brasiliense* populations. The estimated *He* seems to be lower than values obtained in other arary populations from countries that represent the middle of the species' range. AFLPs analysis carried out in four populations from the Amazonian region, southeastern Brazil, and Costa Rica detected heterozygosity in the range of 0.190 to 0.320 (Carnavale-Bottino, 2006). Even higher *He* measurements were obtained through isoenzyme electrophoresis analysis; 0.430 and 0.438 were observed in two other populations of *C. brasiliense* located in the gallery forest from Minas Gerais State, Brazil (de Souza *et al.*, 2007).

The second index commonly used to express the genetic diversity through dominant markers is Shannon's Index *(I)*. Although this parameter was not developed for genetic studies, it is extensively employed just for its independence of Hardy-Weinberg equilibrium assumption (Lewontin, 1972; Lynch and Milligan, 1994; Arias *et al.*, 2012). The Shannon's *I* estimates for both Argentinean populations in this study were lower than those reported by Galvão Mendoça (2006) for Brazilian populations from Minas Gerais State. The author analyzed two arary populations through RAPD markers and obtained Shannon's *I* values of 0.503

and 0.530. The fact that the two diversity indicators (*He* and Shannon's *I*) were lower in the southernmost populations of the species could well be a consequence of the edge effect and the minor genetic variability that is usually observed in species distribution range boundaries (Geber, 2008).

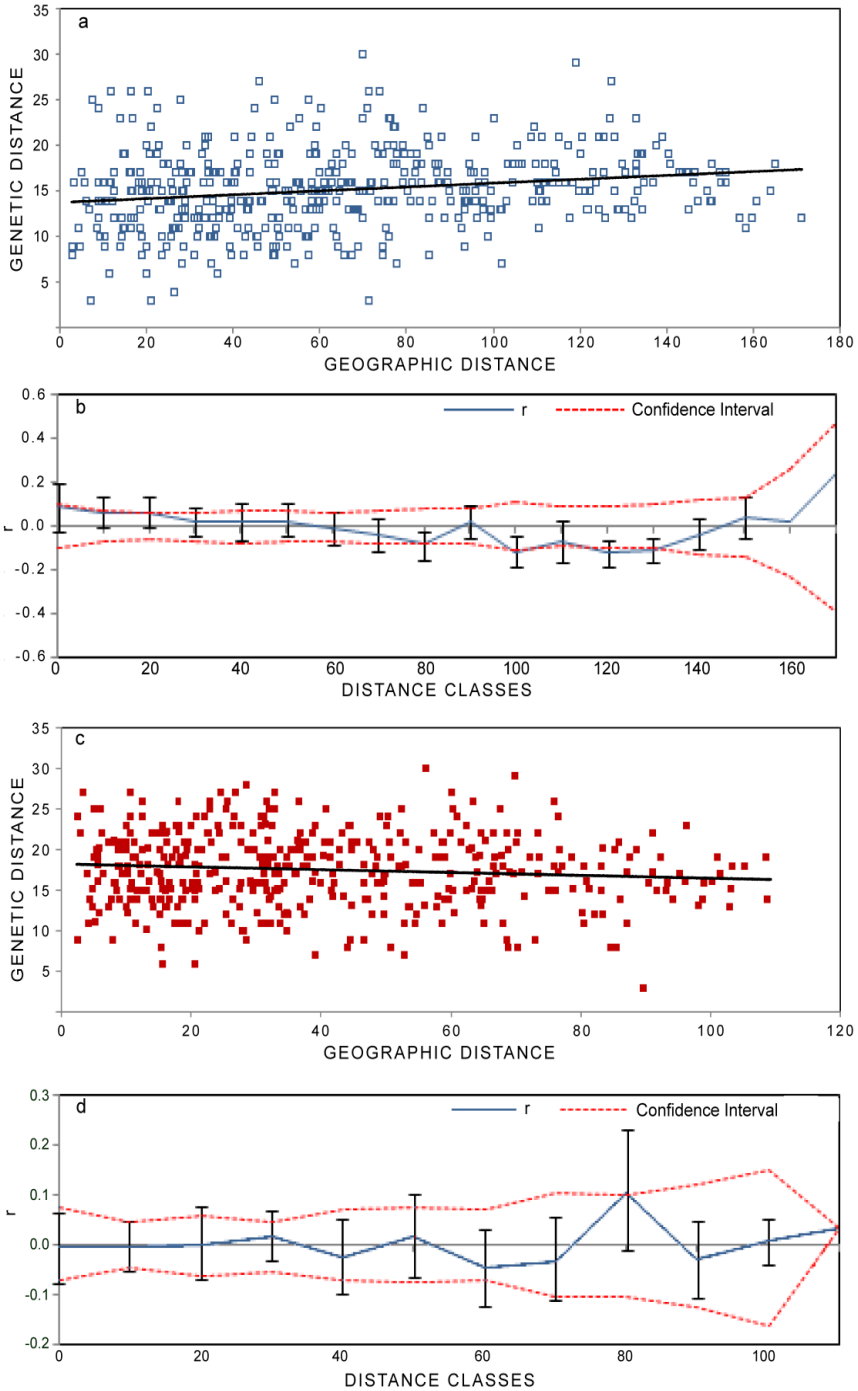

Fig. 3. Correlation tests between Genetic and Geographic Distances based on 9999 permutations**. a and c**: Mantel Test **a**: Rincón Ombú. **c:** San Ignacio. **b and d**: Spatial Autocorrelation Analysis. Error bars are shown around each *r* class estimated with 10 000 bootstraps at p< 0.05 **b:** Rincón Ombú. **d:** San Ignacio.

Looking above population level, we obtained a Nei genetic distance of 0.185 between the two analyzed populations, which is the highest found in *C. brasiliense* populations. The maximum distance previously reported for the species was 0.150 between two populations located 5400 km apart (Carnavale-Bottino, 2006). Our study's value is also higher than those reported for other tropical tree populations (Dayanandan *et al.*, 1999; Figueroa-Esquivel *et al.*, 2010). The maximum genetic distance between populations of *Calycophyllum spruceanum*, a tree species that lives in riverine floodplains in the Amazonian basin, ranged from 0.020 to 0.103 (Russell *et al.*, 1999). The high genetic distance estimated between SI and RO populations could be a consequence of the geographic distance between them together with the time since both diverged. In addition, the size and weight of *C. brasiliense* fruits limits their long-distance transport and dispersion by water courses, and the absence of other populations nearby that could enable genetic exchange could be issues that also contribute to the high genetic distance estimated.

According to the high Nei's genetic distance estimated, we observed that most genetic variability in these two Argentinean *C. brasiliense* populations is distributed within populations (72%) in contrast with a minor proportion observed between populations (28%). These results are similar to those reported for Brazilian *C. brasiliense* populations (71% within vs. 29% between; Galvão Mendoça, 2006). Conversely, Carnavale-Bottino (2006) found only 63% variability within four *C. brasiliense* populations 300 to 5400 km apart analyzed through 514 AFLP loci. However, there are tree species that exhibit more population structure; for example, 91% variability was found among individuals of *C. spruceanum* populations (Russell *et al.*, 1999). With the exception of variations that can be related to the genetic marker employed and the number of loci evaluated, our results are in accordance with a common pattern observed in tropical tree species: a tendency to have the major variance within populations and high differentiation among them (Degen *et al.*, 2001a).

The ϕ_{ST} value (0.283) estimated for SI and RO indicated that both populations displayed a high genetic structure based on Wright´s classification (1978); this is best visualized in the PCoA graphic (Fig. 2), where the two population clusters are evident. In addition, the existence of exclusive alleles in each population supports the level of divergence found between them. The same situation was reported for the two *C. brasiliense* populations analyzed by Galvão Mendoça (2006) (ϕ_{ST} = 0.295). The estimated ϕ_{ST} value suggested to us that the Argentinean populations are differentiated enough to be treated as independent management units (MUs). However, the estimated ϕ_{ST} value is based exclusively on the allele frequency differentiation and therefore should not be used by itself to determine MUs. Indeed many kinds of information should be integrated for identification of conservation units (Allendorf *et al.*, 2013). Inferring demographic independence requires taking into account other parameters such as the effective population size and the number and proportion of migrants. Outside of these considerations, the differentiation found between the Argentinean populations is strong enough to indicate the urgent need for joint conservation efforts to preserve and restore the riparian forest in the Northeast of Argentina.

In concordance with the Mantel test results, which revealed significant correlation in the SI population, spatial autocorrelation was found in SI, but neither correlation nor spatial structure was found in RO. Similarly, only one of the two Brazilian populations from Minas Gerais analyzed by Galvão Mendoça (2006) shown spatial genetic structure; however, those populations were bigger than SI and RO, so the class size employed to test for spatial autocorrelation was 224 m. In contrast, due to the small Argentinean population size, class size used in the present work was 10 m and five of the r values observed in SI were significant (see 95% confidence intervals in Fig. 3d).

For *ex situ* preservation, and based on the distance class that shown significant values for genetic structure, Galvão Mendoça (2006) recommended collecting seeds from parents at least 443 m apart and advised taking this distance into account when planning *in situ* conservation areas. Minimal distance to prevent inbreeding seems to be an intrinsic property of each population. For example, in the SGS analysis that involved the four *C. brasiliense* populations analyzed by Carnavale-Bottino in 2006, a nonrandom genotype distribution was observed in individuals located within 75 meters only in the population derived from Rio de Janeiro. Applying the same concept to the SI Argentinean population distance classes of 20, 80, 100, 120, and 130 m showed significant r values ($p<0.05$), suggesting that a pair of individuals should be 20 m apart to be randomly distributed.

Differences shown by the SGS analysis between the Argentinean studied populations could be due to several factors. First, local spatial structure could be generated after a few generations as a consequence of fine-scale genetic processes, such as limited seed and pollen flow and local selection pressures (Degen *et al.*, 2001a), which would result in a genotype spatial rearrangement dependent on the presence of pollen and seed dispersal agents. In a study conducted to test for spatial genetic structure in tropical tree species, Degen *et al.* (2001b) reported that four species exhibited spatial structure in the first classes (150 – 300 m), which corresponded to those pollinated by insects and hummingbirds or those whose seeds are dispersed by gravity and small rodents. They observed a weak or absent structure in species whose pollinators and seed dispersal agents included other animals, like bats and monkeys. *C. brasiliense* is known to be pollinated by bees (Fischer and Dos Santos, 2001), whereas seed dispersal is mediated by water, gravity, and bats (Fischer and Dos Santos, 2001; Passos and Graciolli, 2004; Marques and Fischer, 2009); thus, the absence of SGS in RO could be related to the absence of pollinators and dispersal species. Another important factor in SGS generation is intra- and interspecific competition. Reis *et al.* (2009) evaluated spatial autocorrelation in four life history stages of *C. brasiliense* populations, concluding that only seedlings generated a family structure within 10-m patches. Older classes did not show genetic structure and the authors attributed the results to intra- and interspecific competition in that case. However, in higher-density populations, many seed shadows will overlap, and the probability of adjacent seedlings being related is lower (Allendorf *et al.*, 2013). In this respect, the fact that the population density estimated in RO is higher than in SI could mask a weak SGS. In addition, soil composition and drainage conditions are important limiting factors in the viability of *C. brasiliense* seedlings. We are currently analyzing ecological data about soil composition and humidity, population structure, specific pollinators, and dispersal agents in SI, RO, and other recently identified arary populations. These analyzes will be useful in achieving a more complete understanding of the genetics observations made in SI and RO in this study.

Our hypothesis for this investigation was that the Argentinean arary populations have a common origin but that a large fragmentation process caused their genetic divergence. The large differentiation found between the studied populations does not allow us to reject this hypothesis. In any case, this information must be compared and contrasted with results from other species and community investigations that are being conducted by our research group. This study represents the first contribution to the analysis of genetic diversity in this habitat with the focus on conservation of this vulnerable species, and is one of four projects that are studying *C. brasiliense* and the riparian community in Argentina. Future efforts will be aimed at integrating ecological and genetic data in order to make recommendations for the most appropriate management strategies to protect these habitats.

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References

Allendorf FW, Gordon L, Aitken SN. 2013. Conservation and the genetics of populations. UK: Wiley-Blackwell.

Arias DM, Albarrán-Lara AL, González-Rodríguez A, Peñaloza-Ramírez J, Dorado O, Leyva E. 2012. Genetic diversity and structure of wild populations of the tropical dry forest tree *Jacaratia mexicana* (Brassicales: Caricaceae) at a local scale in Mexico. Revista de Biología Tropical **60(1),** 01-10.

Babu KN, Rajesh MK, Samsudeen K, Minoo D, Suraby EJ, Anupama K, Ritto P. 2014. Randomly amplified polymorphic DNA (RAPD) and derived technique. Molecular Plant Taxonomy: Methods in Molecular Biology **1115**, 191–209.

Carnavale-Bottino M. 2006. Análise da diversidade genética de populações de *Calophyllum brasiliense* Camb. (Clusiaceae) utilizando marcadores AFLP. Graduate thesis, Universidade Federal do Rio de Janeiro, Brazil, 101.

Casas AM, Igartua E, Balaguer G, Moreno MA. 1999. Genetic diversity of *Prunus* rootstocks analyzed by RAPD markers. Euphytica **110(2),** 139–149.

Chhipi Shrestha JK, Bhattarai T, Sijapati J, Rana N, Maharjan J, Rawal DS, Raskoti BB, Shrestha S. 2013. Assessment of Genetic Diversity in Nepalese Populations of *Swertia chirayita* (Roxb. Ex Fleming) H. Karst Using RAPD-PCR Technique. American Journal of Plant Science **4,** 1617–1628.

Dayanayan S, Dole J, Bawa K, Kesseli R. 1999. Population structure delineated with microsatellite markers in fragmented populations of a tropical tree, *Carapa guianensis* (Meliaceae). Molecular Ecology **8**, 1585–1592.

de Oliveira VC, Joly CA. 2010. Fooding tolerance of *Calophyllum brasiliense* Camb. (Clusiaceae): morphological, physiological and growth responses. Trees **24(1),** 185–193.

de Souza AM, de Carvalho D, de Almeida Vieira F, do Nascimento LH, de Lima DC. 2007. Estrutura genética e espacial de populações naturais de *Calophyllum brasiliense* Camb. em mata de galería. Cerne **13(3),** 239–247.

Degen B, Petit R, Kremer A. 2001a. SGS—Spatial Genetic Software: A Computer Program for Analysis of Spatial Genetic and Phenotypic Structures of Individuals and Populations. Heredity **92(5),** 447–449.

Degen B, Caron H, Bandou E, Maggia L, Chevallier MH, Leveau A, Kremer A. 2001b. Fine-scale spatial genetic structure of eight tropical tree species as analyzed by RAPDs. Heredity **87**, 497–507.

Doligez A, Joly HI. 1997. Genetic diversity and spatial structure within a natural stand of a tropical

forest tree species, *Carapa procera* (Meliaceae) in French Guiana. Heredity **79**, 72–82.

Fernandes de Souza R, Duarte Ziroldo B, Rossetto EF, Cavalheiro AL, Domingues Torezan JM, Laforga Vanzela AL. 2012. The use of genetic structure as a guide for seed gathering for forest restoration. Revista Brasileira de Biociências **10(3),** 309–313.

Figueroa-Esquivel EM, Olivares FP, Eguiarte LE, Núñez-Farfán J. 2010. Estructura genética de un árbol tropical dispersado por aves (*Dendropanax arboreus*) en un paisaje fragmentado en México. Revista Mexicana de Biodiversidad **81**, 789-800.

Fischer E, Dos Santos FAM. 2001. Demography, phenology and sex of *Calophyllum brasiliense* (Clusiaceae) trees in the Atlantic forest. Journal of Tropical Ecology **17**, 903–909.

Galvão Mendoça E. 2006. Análise da diversidade genética de *Calophyllum brasiliense* Camb. por marcadores RAPDs em populações de mata ciliar. Graduate thesis, Universidade Federal de Lavras, Brazil, 67.

Geber MA. 2008. To the edge: studies of species' range limits. New Phytologist **178**, 228–230.

Hardy OJ, Maggia L, Bandou E, Breyne P, Caron H, Chevalier MH, Doligez A, Dutech C, Kremer A, Latouche-Hallé C, Troispoux V, Veron V, Degen B. 2006. Fine-scale genetic structure and gene dispersal in 10 neotropical tree species. Molecular Ecology **15**, 559–571.

Inoue M, Kelley KJ, Frary A, Craker LE. 2013. A measure of genetic diversity of goldenseal (*Hydrastis canadensis* L.) by RAPD analysis. Genetic Resources and Crop Evolution **60**, 1201–1207.

Kageyama PY, Gandara FB, Souza LMI. 1998. Conseqüências genéticas da fragmentação sobre populações de espécies arbóreas. Série Técnica Instituto de Pesquisas e Estudos Florestais **12(32),** 65–70.

Leitão-Filho HF. 1982. Aspectos taxonômicos das florestas do Estado de São Paulo. Silvicultura em São Paulo **16A,** 197–206.

Lewontin RC. 1972. The apportionment of human diversity. In: Dobzhansky T, Hecht MK, Steere WC, eds. Evolutionary Biology 6. New York, USA: Appleton-Century-Crofts, 381–398.

Liu Y, Xing M, Zhao W, Jun Fan R, Luo S, Chen X. 2012. Genetic diversity analysis of *Rhododendron aureum* Georgi (Ericaceae) located on Changbai Mountain using ISSR and RAPD markers. Plant Systematics and Evolution **298**, 921–930.

Lobo PC, Joly CA. 2000. Aspectos ecofisiológicos da vegetação de mata ciliar do sudeste do Brasil. In Rodrigues RR, Leitão Filho HF, eds. Matas Ciliares: conservação e recuperação. São Paulo, Brazil: Universidade de São Paulo, 143–157.

Lynch M, Milligan BG. 1994. Analysis of population genetic structure with RAPD markers. Molecular Ecology **3**, 91-99.

Marques MCM, Fischer E. 2009. Effect of bats on seed distribution and germination of *Calophyllum brasiliense* (Clusiaceae). Ecotropica **15(1/2)**, 1–6.

Marques MC, Silva SM, Salino A. 2003. Florística e estrutura do componente arbustivo-

arbóreo de uma floresta higrófila da bacia do rio Jacaré-Pepira, SP, Brasil. Acta Botanica Brasilica **17(4),** 495–506.

Morrone JJ. 2001. Biogeografía de América Latina y el Caribe. M&T. Manuales & Tesis Vol. 3. Zaragoza, España: SEA, p. 148.

Nei M. 1972. Genetic distance between populations. American Naturalist **106**, 283–392.

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics **89**, 583–590.

Passos FC, Graciolli G. 2004. Observações da dieta de *Artibeus lituratus* (Olfers) (Chiroptera, Phyllostomidae) em duas áreas do su do Brasil. Revista Brasileira de Zoologia **21(3),** 487–489.

Peakall R, Smouse PE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes **6**, 288–295.

Peakall R, Smouse PE. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. Bioinformatics **28**, 2537–2539.

Percuoco C. 2007. Obtención de marcadores RAPDs en una población de *Calophyllum brasiliense* Cambess. (Clusiaceae) de San Ignacio – Mnes. Graduate thesis, Universidad Nacional de Misiones, Argentina, 58.

Reis CAF, de Souza AM, Galvão E, Rodrigues GF, Guimarães RM, de Carvalho D. 2009. Diversidade e estrutura genética espacial de *Calophyllum brasiliense* Camb. (Clusiaceae) em uma floresta paludosa. Árvore **33(2),** 265–275.

Rodrigues RR. 2000. Florestas ciliares? Uma discussão nomenclatural das formações ciliares. In Rodrigues RR, Leitão Filho HF, eds. Matas Ciliares: conservação e recuperação. São Paulo, Brazil: Universidade de São Paulo, 91–99.

Rodríguez ME, Cardozo AE, Krauczuk ER, Fontana JL, Iriart D. 2009. *Calophyllum brasiliense* (Clusiaceae) nuevo registro para la flora de la Argentina. Boletín de la Sociedad Argentina de Botánica **44**, 361–366.

Russell J, Weber JC, Booth A, Powell W, Sotelo Montes C, Dawson K. 1999. Genetic variation of *Calycophyllum spruceanum* in the Peruvian Amazon Basin, revealed by amplified fragment length polymorphism (AFLP) analysis. Molecular Ecology **8**, 199–204.

Sanjurjo MD. 1994. El arary, un árbol en extinción en el Paraguay. Revista Crítica **9**, 51–55.

Shannon CE. 1948. A Mathematical Theory of Communication. The Bell System Technical Journal **7**, 379–423, 623–656.

Stange C, Prehn D, Arce-Johnson P. 1998. Isolation of *Pinus radiate* genomic DNA suitable for RAPD analysis. Plant Molecular Biology Reporter **16**, 1–8.

Thormann CE, Ferreira ME, Camargo LEA, Tivang JG, Osborn TC. 1994. Comparison of RFLP and RAPD markers to estimating genetic relationships within and among cruciferous species. Theoretical and Applied Genetics **88(8),** 973-980.

Wright S. 1978. Evolution and the Genetics of Populations. Variability within and among natural populations. Vol 4. Chicago, USA: University of Chicago Press.