Molecular polymorphism in *Lonchocarpus cultratus* (Fabaceae) from riparian areas of natural reforesting in Upper Paraná River, Brazil

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ABSTRACT. *Lonchocarpus cultratus* (Vell.) A.M.G. Azevedo and H.C. Lima is an important plant species in areas of natural reforesting in South and Southeastern Brazil, including riparian areas. This arboreous species seems to reproduce by seeds in undisturbed forests. In regenerating forests, however, *L. cultratus* has been observed mostly as patches of aggregates, consisting of highly similar plants, resembling clones. Sprouting from root buds had been previously observed in *L. cultratus* in a forest affected by fire. In the present study, RAPD polymorphism revealed high genetic diversity among plants from *L. cultratus* aggregates in a natural restoring riparian forest affected by fires, in the Upper Paraná River, Brazil. These data allow the suggestion that sexual reproduction has been the *L. cultratus* usual reproductive strategy to colonize the reforesting riparian area.

Keywords: *Lonchocarpus*, Paraná River, RAPD, reforesting.

RESUMO. Polimorfismo molecular em *Lonchocarpus cultratus* (Fabaceae) de áreas ripárias de reflorestamento natural no alto rio Paraná, Brasil. No Sul e Sudeste do Brasil, *Lonchocarpus cultratus* (Vell.) A.M.G. Azevedo e H.C. Lima é uma espécie arbórea importante para o reflorestamento natural, incluindo áreas ripárias. Em florestas regeneradas, diferentemente de florestas não perturbadas, esta espécie tem sido observada como agregados de plantas semelhantes entre si, ao ponto de ter sido formulada a hipótese de serem clones. Em um estudo prévio, brotamento de raízes foi observado em *L. cultratus*, em uma floresta afetada pelo fogo. No presente estudo, análises de polimorfismo de RAPD revelaram alta diversidade genética entre as plantas de agregados de *L. cultratus*, em uma floresta ripária afetada pelo fogo, no alto rio Paraná. Os dados permitem sugerir que reprodução sexual tem sido a usual estratégia reprodutiva de colonização de *L. cultratus*, nesta área de reflorestamento natural.

Palavras-chave: *Lonchocarpus*, rio Paraná, RAPD, reflorestamento.

Introduction

In many tropical and subtropical regions, forest destruction has been a major concern regarding to environmental damage. Basic knowledge on plant populations structure and their reproductive strategy is required both for understanding forest dynamics and for designing appropriate reforesting guidelines. *Lonchocarpus cultratus* (Vell.) A.M.G. Azevedo and H.C. Lima is one of the first and most abundant arboreous species in several areas of South and Southeastern Brazil. It plays a fundamental role in the natural regeneration of devastated forest areas, including riparian areas. This species, popularly known as 'feijão-cru' or 'imbira', can reach heights of about 24m, and it is capable to grow in diverse soil conditions, including poor and dry soils. In undisturbed forests, *L. cultratus* plants are scarce, irregularly distributed, and apparently established through seed germination (Souza, 1998). However, in regenerating forests affected by fire, *L. cultratus* has been observed mostly as plant aggregates, resembling clones (Souza, 1998). Sprouting from root buds was recently described for this species in a forest affected by fires (Rodrigues *et al*., 2004). The plant aggregates resembling clones are the main pattern of *L. cultratus* in reforesting riparian areas

from the Upper Paraná River basin, located at the border of Northern Paraná and Southeastern Mato Grosso do Sul States, Brazil.

In the past few decades, the environment of a major portion of the Upper Paraná River basin has been severely impacted by a significant demographic increase, by the expansion of agriculture and pastures, and by the building of many hydroelectric dams. In this hydrographic basin, hydroelectric reservoirs have changed the main rivers into almost a succession of lakes. The recent and rapid environmental changes have caused serious modifications in the aquatic life and have rapidly destroyed large extensions of riparian forests both along tributaries and main rivers of this basin (Vazzoler *et al*., 1997). The floodplain of Upper Paraná River is an important and rare stretch which is not confined in a reservoir. It represents the only remaining running water stretch of this river in Brazilian territory. Its right margin is usually flooded during annual raining season, and this unique ecosystem comprises an Environmental Protection Area (Vazzoler *et al*., 1997). The riparian vegetation of this ecosystem has been studied regarding phytosociology (Campos and Souza, 2002), chemical composition (Sator *et al*., 1999; Coelho *et al*., 1998), and seed dispersion (Souza-Stevaux *et al*., 1994). Fires have naturally affected the vegetation at both margins of the river, which also have been affected by human activities. Previous studies had demonstrated that *L. cultratus* plays an important role in the natural reforesting of the left margin of the Upper Paraná River floodplain, such as in Porto Rico County (Souza, 1998, 1999). In this riparian area, previously affected by fires, L. cultratus distribution pattern has been observed mostly as aggregates of plants that resemble clones, because they are established as clusters of plants with similar age, height, and stem diameter (Souza, 1998). This characteristic distribution has suggested that this species could be preferentially established through vegetative propagation during natural reforesting. Successful plants would form groups of adult clones in recovered forests. Molecular markers could be highly useful to evaluate the colonization strategy of *L. cultratus* during the natural regeneration of these riparian forests.

The molecular markers RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter-Simple Sequence Repeat), also referred as SPAR (Single Primers Amplification Reactions), have been suitable for polymorphism detection in studies of genetic relatedness among organisms. Both techniques consist of relatively uncomplicated procedures for the analysis of DNA fragments amplified by Polymerase Chain Reaction (PCR), and produce results at a low statistic error rate (Williams *et al.*, 1990; Gupta *et al.*, 1994). Single primers of randomized sequences are used in the RAPD technique. Because these primers do not discriminate between coding and non-coding regions, it has been assumed that the RAPD technique samples the genome in a highly randomized way, and the number of loci that can be analyzed is immensely high (Williams *et al*., 1990). In a wide range of organisms, RAPD markers have been successfully used in studies of plant and animal natural populations, including genetic relatedness among individuals and clone identification, (Bastianel *et al.*, 1998; Mohana *et al*., 2001; Oliveira *et al*., 2002; Jover *et al*., 2003; Zhou *et al*., 2003).

Units of DNA sequences repeated in *tandem*, smaller than 200 base pairs, are dispersed in the genome of eucariotic organisms. They are called microsatellites, and they can be highly polymorphic (Britten and Kohne, 1968; Tautz and Renz, 1984). The ISSR technique consists of PCR amplification of genomic DNA sequences occurring between two microsatellites sequences of the same repeat, and located within a distance that enables PCR amplification. Only one primer is used, consisting of a few repeat units, characteristics of the microsatellite of interest. Primers composed of tetranucleotide microsatellite repeats have been described as an efficient tool to identify diagnostic loci, useful to distinguish both intraspecific and interspecific polymorphic patterns in plants and animals (Gupta *et al.*, 1994; Godwin *et al*., 1997; Albert *et al*., 1999; Camacho and Liston, 2001).

In the present work, RAPD and ISSR markers were used to evaluate molecular polymorphism among *L. cultratus* plants from aggregates from a riparian forest in regeneration in the floodplain of Upper Paraná River basin, attempting to contribute to a better understanding of this species' colonization strategy during natural reforesting.

Material and methods

Plant Sampling and DNA Extraction. Young leaves were sampled from *L. cultratus* adult plants from a regenerating riparian forest at the left margin of the Paraná River floodplain, in Porto Rico, State of Paraná, Brazil (22° 47' 37" S and 53° 19' 03" W). Samples were taken from five plant aggregates consisting of trees of apparently similar age, located about 50m from the river margin. Trees of similar height, about five meters, and similar stem diameter, were randomly selected in an approximate row

distribution, within lines from five to 10 meters. Leaves were sampled from 32 plants, ranging from three to 10 plants of each aggregate. Leaf samples were frozen in liquid nitrogen and total DNA samples were extracted according to Murray and Thompson (1980), and then quantified on agarose gel electrophoresis by comparison with λ DNA of known concentrations.

RAPD. PCR amplifications were tested with RAPD primers from kits OPA, OPX, and OPW (Operon Technologies, CA, USA). The selected primers OPA02, OPA04, OPA07, OPA17, OPX01, OPX03, 0PX04, OPW03, OPW11, and OPW13 were used in RAPD analysis. PCR reaction mixture consisted of buffer Tris-KCl (20 mM Tris-HCl pH 8.4 and 50 mM KCl), 2 mM $MgCl_2$, 0.46 μM primer, 0.2 mM dNTP, 1 U/Reaction of *Taq* DNA polymerase (Gibco BRL), 10 ng DNA, and sterile deionized water to a total volume of 13 μl. The reaction mixture was heated at 92°C for 4 min, followed by 40 cycles of 1 min at 92°C, 1min 30s at 40°C, and 2min at 72°C. Immediately after the last amplification cycle, reaction mixture was kept at 72°C for 2min and then cooled at 4°C for 20min. PCR amplified DNA fragments were separated by electrophoresis in agarose gel (1.4%), stained with ethydium bromide (20 μg/100 ml), at 3 $V.cm^{-1}$ for 5–6h. A sample without template DNA was included as a negative control in each experiment. In addition, one to three samples of PCR products, containing *L. cultratus* DNA fragments previously amplified and analyzed with the same primer, were included in each agarose gel. Electrophoretic profile was visualized under UV radiation and photographed with Kodak EDAS-290. Sizes of DNA fragments were estimate by comparison with standard Ladder 100 pb (Gibco BRL). Electrophoretic profiles were analyzed for polymorphism based on the presence and absence of DNA bands on agarose gel.

ISSR. ISSR primers composed of tetranucleotide repeats $(GGAC)_4$, $(GGAC)_3T$, $(AAGC)_4$, and $(GACA)_4$ were selected for analyses. PCR reaction mixture consisted of the same components above described for RAPD analysis, but replacing the primer by one of the selected ISSR primers. Amplifications were done according to Albert *et al*. (1999), in 5 cycles of 45s at 94°C, 1 min at 51°C, and 1min at 72°C, immediately followed by 30 cycles of 45s at 94°C, 1min at 48°C, and 1min at 72°C. The reaction mixture was kept at 72°C for 2min and then cooled at 4°C. A reaction mixture without template DNA was included in each experiment, as a negative control. PCR amplified DNA fragments were separated by electrophoresis, visualized and photographed using the above described procedure used for RAPD.

Analysis. It was assumed that the occurrence of diversity among plants for presence or absence of DNA fragments was a demonstration of sexual reproduction. Frequencies of RAPD polymorphic loci were estimated within each plant aggregate and overall plant aggregates. Shannon diversity indexes (Zar, 1974) and Jaccard similarity coefficients were estimated among plants, combined in pairs within each aggregate, and also among the five aggregates. Shannon diversity indexes were estimated with POPGENE 1.31 software. Arithmetic complements of Jaccard similarity coefficients were estimated through GENES software (Cruz, 2001). Clustering was performed by the neighbor-joining method with MEGA 2.1 (Kumar *et al*., 2001).

Results

Selected RAPD primers amplified 82 loci, and 54 of those loci (65.85%) were identified as polymorphic. Genetic variability was detected among specimens from all five aggregates. As represented in Figure 1 and Table 1, RAPD markers revealed molecular polymorphism among plants within the aggregates. The percentage of polymorphic loci ranged from 12.2% to 52.44% within plant aggregates. Each primer amplified a number of polymorphic loci, which were enough to discriminate several, but not all, specimens within each plant aggregate. However, when analyzed together, these loci amplified by the 11 selected RAPD primers clearly revealed genetic differences among individuals from all five aggregates. As a contrast, PCR amplifications using four different ISSR primers, composed of tetranucleotide microsatellite repeats, resulted in extremely low polymorphism within and among plant aggregates. Therefore, the ISSR markers were excluded from analyses.

Table 1. Number and percentage of polymorphic loci (PL) within each plant aggregate of *L. cultratus* from a riparian forest in the floodplain of Upper Paraná River.

Aggregates	Number of polymorphic loci	% PL	Shannon Index
A	25	30.49	0.16
B	28	34.15	0.19
C	10	12.20	0.07
D	29	35.47	0.19
E	43	52.44	0.28
Total	54	65.85	

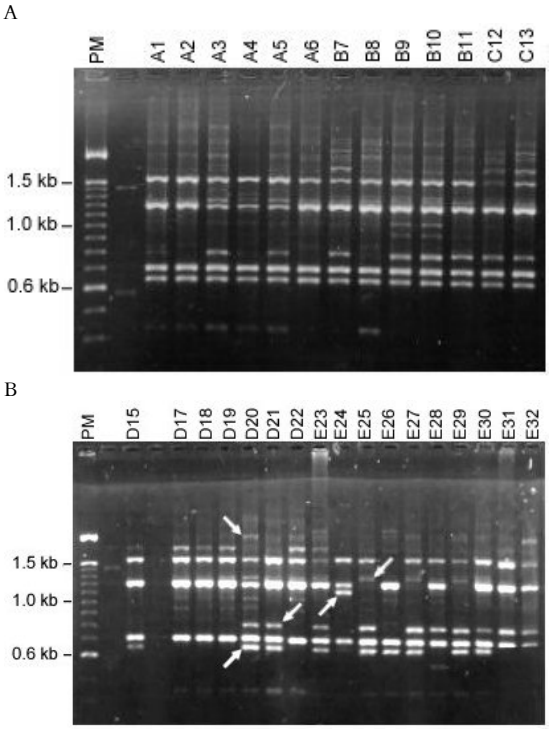

Figure 1. RAPD profiles for five plant aggregates (A-E) of *Lonchocarpus cultratus* from the floodplain of Upper Paraná River, using primer OPA-07. (**A**) Trees from aggregates A (**Lanes 3-8**), B (**Lanes 9-13**), and C (**Lanes 14-15**). (**B**) Trees from aggregates D (**Lanes 3**, **5-10**) and E (**Lanes 11-20**). In both eletrophoretic profiles, **Lane 1 (PM)** contains DNA molecular weight markers (Ladder 100 bp, Gibco BRL), and **Lane 2** contains a PCR negative control. Arrows indicate several polymorphic loci.

As shown in Table 2, the coefficients of Jaccard similarity varied from 0.63 to 0.97, and the Shannon diversity indexes ranged from 0.01 to 0.21, demonstrating genetic differences among all plants, except for plants A3 and A5. These latter plants did not show polymorphism for the analyzed RAPD loci. Moreover, as shown in Table 3, the estimates of Jaccard similarity coefficients among aggregates varied from 0.79 to 0.90. Therefore, the range of genetic similarities among aggregates was clearly comparable to the range of genetic similarities within the aggregates.

The Shannon diversity indexes among aggregates ranged from 0.19 to 0.30. Within aggregates, Shannon diversity indexes varied from 0.07 to 0.28 (Table 1). Therefore, the degree of genetic variability among aggregates was similar to the genetic variability degree obtained within aggregates (Tables 1 and 3). Moreover, in the neighbor-joining tree, the genetic variability did not group the sampled plants according to their respective aggregates. Therefore, aggregates were not distinguished upon the clustering in the neighbor-joining dendrogram, and indicated that the genetic variation within each aggregate was also present among aggregates (Figure 2).

Table 2. Jaccard similarity coefficients (below diagonal) and Shannon diversity indexes (above diagonal), based on RAPD markers, estimated among plants of five *L. cultratus* aggregates from a riparian forest of the floodplain of Upper Paraná River.

	A1	A2	A3	A4	A5	A6				
A1	-	0.14	0.15	0.17	0.15	0.13				
A2	0.75	-	0.08	0.13	0.08	0.06				
A3	0.73	0.83	-	0.06	0.00	0.07				
A4	0.68	0.74	0.86	-	0.06	0.10				
A5	0.73	0.83	1.00	0.86	-	0.07				
A6	0.76	0.87	0.85	0.79	0.85	-				
	B1	B2	B3	B4	B5					
B1	-	0.16	0.14	0.16	0.12					
B2	0.70	-	0.10	0.12	0.12					
B3	0.73	0.81	-	0.02	0.10					
B4	0.70	0.78	0.96	-	0.08					
B5	0.77	0.78	0.81	0.82	-					
	C1	C2	C3							
C1	-	0.06	0.10							
C2	0.89	-	0.07							
C3	0.81	0.87	-							
	D1	D2	D3	D4	D5	D6	D7			
D1	-	0.14	0.15	0.16	0.17	0.11	0.14			
D2	0.74	-	0.01	0.05	0.16	0.12	0.05			
D3	0.73	0.97	-	0.04	0.15	0.14	0.06			
D4	0.72	0.90	0.93	-	0.11	0.15	0.03			
D5	0.71	0.72	0.75	0.81	-	0.09	0.14			
D6	0.79	0.76	0.74	0.73	0.84	-	0.12			
D7	0.75	0.90	0.88	0.95	0.77	0.77	-			
	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
E1	-	0.11	0.16	0.14	0.11	0.09	0.11	0.13	0.16	0.17
E2	0.80	-	0.13	0.11	0.14	0.08	0.12	0.10	0.15	0.16
E3	0.70	0.75	-	0.16	0.15	0.11	0.13	0.11	0.20	0.21
E4	0.75	0.80	0.70	-	0.17	0.11	0.15	0.13	0.16	0.17
E5	0.78	0.74	0.70	0.69	-	0.08	0.04	0.10	0.17	0.18
E6	0.82	0.84	0.78	0.79	0.83	-	0.08	0.08	0.13	0.14
E7	0.79	0.77	0.74	0.72	0.91	0.84	-	0.08	0.15	0.16
E8	0.76	0.81	0.78	0.76	0.80	0.84	0.84	-	0.13	0.14
E9	0.71	0.73	0.64	0.71	0.69	0.75	0.72	0.76	-	0.01
E10	0.70	0.72	0.63	0.70	0.67	0.74	0.71	0.75	0.98	-

Table 3. Jaccard similarity coefficients (below diagonal) and Shannon diversity indexes (above diagonal), based on RAPD markers, estimated among five *L. cultratus* plant aggregates (A-E) from a riparian forest of the floodplain of Upper Paraná River.

Aggregate	A	B	C	D	E
A	—	0.22	0.19	0.22	0.28
B	0.89	—	0.22	0.26	0.30
C	0.84	0.79	—	0.22	0.28
D	0.88	0.90	0.83	—	0.29
E	0.82	0.82	0.80	0.88	—

Discussion

RAPD polymorphism revealed genetic diversity among all studied *L. cultratus* plants in the sampled aggregates from the left margin of the Upper Paraná River floodplain. RAPD markers clearly demonstrated genetic polymorphism within and among plant aggregates. The genetic diversity revealed by RAPD rejected the hypothesis of

preferential clonal propagation for the establishing of *L. cultratus* population during the reforesting of the studied area affected by fire.

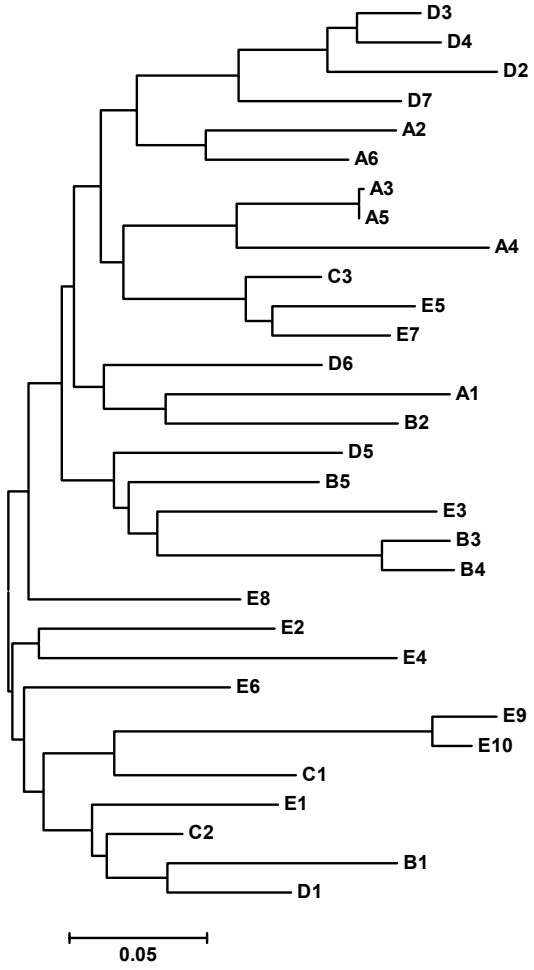

Figure 2. Neighbor-joining dendrogram based on arithmetic complements of Jaccard similarity coefficients among five *L. cultratus* plant aggregates (A-E), obtained from RAPD markers.

In the analyzed *L. cultratus* plants, the polymorphism detected by ISSR primers was low, much lower than the RAPD polymorphism. Primers composed of tetranucleotide microsatellite repeats have been reported as useful to identify diagnostic loci capable to distinguish both intraspecific and interspecific polymorphic patterns in several plants and animals (Gupta *et al.*, 1994; Godwin *et al*., 1997; Albert *et al*., 1999; Camacho and Liston, 2001; Reddy *et al*., 2002). Data in the present work indicated that these primers are not suitable to analyze the studied *L. cultratus* plant aggregates.

RAPD technique has been extensively reported as suitable to analyze intraspecific genetic similarity among organisms, including clone identification in plants (Williams *et al.*, 1990; Triest *et al*., 2000; Mohana *et al*., 2001; Jover *et al*., 2003; Torimaru *et al*., 2003; Zhou *et al*., 2003). RAPD demonstrated that *L. cultratus* plants within and among aggregates were genetically different from each other, with only one exception. Therefore, excluding plants A3 and A5, the genetic variability among studied plants discarded the possibility of clones, which would be generated by vegetative propagation after forest destruction by fires (Rodrigues *et al*., 2004). Although the studied *L. cultratus* individuals were distributed in patches of plant aggregates that resemble clones, the molecular markers demonstrated that just about all of them were not generated by vegetative propagation. The genetic variability detected among these *L. cultratus* plants could only be attributed to sexual reproduction. Therefore, the studied *L. cultratus* population must have established, at least preferentially, through seedlings in this area under natural regeneration.

The destruction of riparian vegetation in the Upper Paraná River basin has been especially threatening to forest structure and aquatic life (Vazzoler *et al.*, 1997). In this hydrographic basin, the riparian communities are represented mainly by Semideciduous Seasonal forests. These communities have unique characteristics, such as lower humidity levels and denser low vegetation, as compared to other large forests from Brazil, such as the Amazonian and Atlantic forests (Leitão-Filho, 1987). The phanerogam flora of riparian areas from Upper Paraná River has been studied and it comprises trees of large, medium and small sizes, as well as bushes, herbs, lianas, and a few epiphytes (Romagnolo *et al.*, 1994; Souza *et al.*, 1997; Souza e Souza, 1998; Souza, 1999; Prioli *et al*., 2004). In the remnant forest, near Porto Rico, *L. cultratus* occupies the first position in the total importance value (IV) of this community, estimated as one quarter, including relative frequency, density and dominance. Therefore, this species has been of high importance in the natural regeneration of these riparian forests, where it occurs mostly as plant aggregates resembling clones.

In a previous work, it was reported that root sprouting in *L. cultratus* represents an effective process of spacial reoccupation of Semideciduous Seasonal forests previously affected by several fires (Rodrigues *et al*., 2004). In undisturbed forests, however, this species seems to propagate through dispersed seeds (Souza, 1998). Probably, the *L. cultratus* preferential mode of propagation can be highly affected by the level of devastation or disturbance caused by fires or other factors. RAPD

polymorphism revealed that in the studied riparian Semideciduous Seasonal forest in regeneration, at Porto Rico, in Upper Paraná River basin, the *L. cultratus* pioneer population was preferentially established through sexual reproduction, by seedlings. Contribution of sexual reproduction in the initial establishment of a plant population on devastated areas has been recently reported in *Polygonum cuspidatum,* as inferred from molecular markers (Zhou *et al*., 2003). This species can propagate both by seeds and by rhizome extension, and it is distributed as patches scattered in a volcanic scoria on Mount Fuji. The most recent eruption of Mount Fuji, in 1707, destroyed all vegetation on its Southeast slope and covered the ground with volcanic ash and scoria. It was reported that in this area, *P. cuspidatum* populations have been initially established from dispersed seeds, before starting vegetative propagation by rhizome extension (Zhou *et al*., 2003). Based on data from the present study, it could be hypothesized that fires might have destroyed the *L. cultratus* mother plants, but their seeds possibly could have survived the harsh environmental in heterogeneous microenvironmental conditions. During the initial reestablishment of this riparian vegetation, the germination of *L. cultratus* seeds might have occurred concurrently, and the population was recovered as aggregates composed of plants with similar age, as it can be observed nowadays.

In Brazil, the recent rapid destruction of riparian forests have made urgent the need of official regulations requiring reforesting of riparian areas along the main rivers and their tributaries, such as the Upper Paraná River basin. It should be expected that reforesting must be formulated within procedures as close as possible to natural models of forest dynamics and reforesting. The results obtained in the present work represent a basic knowledge on the genetic variability of a *L. cultratus* population and on its reproductive strategy in natural forest regeneration. This basic knowledge stands for a fundamental awareness required both for a better understanding of forest dynamics and for designing ecological consistent reforesting guidelines.

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