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Contrasting genetic diversity and differentiation of populations of two successional stages in a Neotropical pioneer tree (*Eremanthus erythropappus*, Asteraceae)

V.L.O. Freitas[1], J.P. Lemos-Filho[2] and M.B. Lovato[3]

[1]Setor de Recursos da Terra, Fundação Centro Tecnológico de Minas Gerais, Belo Horizonte, MG, Brasil
[2]Departamento de Botânica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
[3]Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

Corresponding author: M.B. Lovato
E-mail: lovatomb@icb.ufmg.br

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ABSTRACT. *Eremanthus erythropappus*, commonly known as "candeia", is an abundant pioneer tree species, forming dense populations known as "candeial", but it is also found in forests at middle stages of succession. Trees from forests are bigger and occur in lower density than in the "candeial". The objectives of the present study were to investigate if the decrease in population density during successional process is accompanied by 1) changes in within-population genetic diversity, and 2) differentiation of populations. Eight populations, four of early successional stage ("candeial") and four of middle successional stages (forest), were analyzed with RAPD markers. The genetic diversity found was high compared to other tree species analyzed with RAPD markers. AMOVA revealed that most of the genetic variations of *E. erythropappus* were found within populations (85.7%), suggesting that this species is predominantly outcrossing. The relatively low differentiation among the populations can be attributed to small distances among the populations analyzed (0.2 to 10.8 km).

No indication that populations from middle successional habitats show lower genetic variation than populations from early successional stages was found. The percentage of polymorphic fragments (82.8 and 84.8%) and the Shannon indexes (0.442 and 0.455) were similar in "candeial" and forest, respectively. These results suggest that if an increase in selection intensity occurred during succession, it did not result in a decrease in genetic diversity or that the selection effect was balanced by other factors, such as gene flow. Higher significant differentiation among *E. erythropappus* populations from "candeial" in relation to that among populations from forest was also not detected.

Key words: Asteraceae; "Candeia"; *Eremanthus erythropappus*; Genetic diversity; Pioneer species; Succession

INTRODUCTION

Succession is characterized by a change in species composition, where pioneers are followed by early successional species, and these by species of later successional stages (Whittaker, 1993). The increase in anthropogenic pressure on natural environments results in ecosystems with various stages of recovery, and so studies of succession are important in order to improve practices for ecosystem preservation, management, and restoration. The knowledge of genetic diversity changes during succession provides a fundamental basis for these practices.

During a successional process, some pioneer long-lived species can remain in the community even after temporal changes in species composition (Pluess and Stöcklin, 2004; Goulart et al., 2005; Litrico et al., 2005). These species provide an interesting model to study ecological and genetic changes that occur during succession. Several studies have found phenotypic differences between colonizer populations and populations that persist in more advanced stages of succession, similar to differences between pioneer and late successional species. Plants from later populations are on average bigger, with a higher competitive capacity (Gray, 1987; Bazzaz, 1996) and seeds with lower dispersion capacity (Peroni, 1994). However, it is not well established if during succession genetic changes within species also occur, but alterations in allelic frequencies are presumed to happen (Gray, 1987). The first studies found differences in genotypic frequencies in isozyme loci during succession (Hancock and Wilson, 1976). It is predicted that genetic diversity will be lower in persistent populations than in founder ones (Gray, 1987) due to certain genotypes being eliminated by selection during succession (Hancock and Wilson, 1976; Gray, 1987; Hartnett et al., 1987) or due to casual mechanisms, such as founder effects (Hartnett et al., 1987).

Eremanthus erythropappus (DC) MacLeish (Asteraceae), commonly known as "candeia", is an abundant pioneer tree species forming dense populations in fields and open pastures, known as "candeial" which establishes after forest perturbation. The "candeial" has low or no vertical structuring, and "candeia" is the dominant species, along with a few other species. During succession, the population size of *E. erythropappus* decreases, and "candeia" is found in middle successional habitats in forests in lower density (CETEC, 1996; Souza et al., 2007). The trees of the "candeial" are small (up to 10 m high), with an irregular and short

trunk, whereas those from forests are bigger, with a long and cylindrical trunk (Pedralli, 1997). They are found in the States of Bahia, Minas Gerais, and Rio de Janeiro, Brazil. "Candeia" as a typical pioneer species has positive photoblastic anemochoric seeds which remain viable in the soil for a long time but have a low germination rate (CETEC, 1996). Flowering and fruiting occur from May to November, and its small purple flowers are visited by small bees (CETEC, 1996). This species has a great economic potential due to the qualities of its wood and also to its content of α-bisabolol oil which is utilized in the pharmaceutical and cosmetic industries (Clementi, 1987). Due to its economical value, "candeia" is being submitted to intensive and disordered exploitation (Souza et al., 2007).

Here, we report the patterns of genetic variation among and within populations of *E. erythropappus* from "candeial" and from forest habitats located in two reserve areas, Ecological Station of Tripuí and the State Park of Itacolomi, Ouro Preto, Minas Gerais State, Brazil. Eight populations were analyzed in four areas, constituting four successional pairs of populations, each pair containing one "candeial" population (early successional) contiguous to one from the forest (middle successional). Using random amplified polymorphic DNA (RAPD) markers, we examined how genetic variation is partitioned within and among populations of *E. erythropappus*. In general, early successional species exhibit higher genetic divergence among their populations than do late-successional species (Hamrick and Godt, 1989; Nybom and Bartish, 2000; Nybom, 2004). In a similar way, it can be hypothesized that in a comparison within the same species, early successional populations ("candeial") could be more genetically differentiated than middle successional ones (forest). In addition, considering the great phenotypic differentiation among trees from "candeial" and forest, and reduction in population density during the successional process, we also hypothesized that populations from the forest have lower genetic variation due to competition pressure and/or genetic drift.

MATERIAL AND METHODS

Sample design

The populations used in this study were located within the native range of *Eremanthus erythropappus* in Minas Gerais State, Brazil, in two neighboring reserve areas: Ecological Station of Tripuí and the State Park of Itacolomi located from 20°22'30" to 20°30'00"S and from 43°32'30" to 43°22'30"W. These areas have formations of Atlantic Forest and Cerrado (savanna vegetation) (Pedralli, 1997; Pedralli et al., 2000). The Ecological Station of Tripuí and the State Park of Itacolomi have a total area of 337 and 7543 ha, respectively.

In total, eight areas were sampled, constituting four successional pairs, each pair formed by two contiguous populations, one from "candeial" and another from forest. Three of these pairs were located in the Ecological Station of Tripuí and one pair in the State Park of Itacolomi (Table 1). The "candeial" was considered an early successional stage and the forest area, a middle successional stage. The mean density of *E. erythropappus* in the "candeial" and forest is 356.6 and 46.8 ind. ha^{-1}, respectively (CETEC, 1996).

Twenty random individuals from each of eight populations, totaling 160 individuals, were marked and had their height and circumference at breast height measured. Recently, expanded leaves were collected from each individual and stored at -70°C before DNA extraction.

DNA extraction and RAPD analysis

Genomic DNA was extracted from approximately 0.4 g of young leaves using a modification of the cetyltrimethylammonium bromide protocol described by Ribeiro (1998). After visual quantification, by comparison with standard DNA concentrations, DNAs were diluted in TE buffer to a final concentration of ≈5 ng/μL. Polymerase chain reaction was performed in 20 μL total volume containing 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM of each dNTP, 1 unit of Taq DNA polymerase, 0.25 μM of primer and approximately 20 ng of genomic DNA. Amplifications of RAPDs were carried out in a PTC-100™ (M.J. Research, Inc.) thermocycler using the following polymerase chain reaction conditions: an initial denaturation step of 95°C for 1 min, followed by 35 cycles each of 94°C for 10 s, 36°C for 1 min, and 72°C for 2 min and a final extension step of 72°C for 7 min. A negative control was included in each run, in which DNA was omitted, to verify the absence of contamination. Following amplification, the samples were subjected to electrophoresis, approximately 3 h (100 V), on a 1% agarose gel, made with 1X TBE buffer, stained with ethidium bromide, visualized by illumination with ultraviolet light and photographed for analysis. Molecular size of the fragments was estimated using a 100-bp ladder (Life Technologies).

From a pilot survey with 30 decamer primers from Operon Technologies Inc., 10 from Kit A (OPA) and 20 from Kit L (OPL), 10 were selected for the complete survey (OPA-10, OPL-01, OPL-02, OPL-04, OPL-07, OPL-08, OPL-11, OPL-13, OPL-14, OPL-19) after a detailed preliminary screening with three individuals, each one of a population that consistently revealed sharp and reproducible RAPD products (bands) over two independent runs. Furthermore, one individual was used as a standard marker, beside the 100-bp lader, for scoring bands and confirming consistent amplifications during the whole study.

Data scoring and statistical analysis

Gels were scored for the presence (1) or absence (0) of a band and a matrix of RAPD phenotypes was assembled. Only bands that showed a reproducible pattern and clearness were used for statistical analysis. One locus was considered polymorphic at the population level, if at least one individual showed or not the band (95%). The diversity within populations was quantified by two indices: 1) the Shannon index (H_O) and 2) the percentage of polymorphic bands. The Shannon index of diversity (H_O) was estimated from the frequencies of the RAPD bands within each population and also over all populations. The equation used was: $H = -\Sigma\, p_i \log_2 p_i$, where p_i was the frequency of the presence or absence of the band (Lacerda et al., 2001; Goulart et al., 2005). The among-population diversity component was calculated as $(H_{SP} - H_{POP}) / H_{SP}$, where H_{SP} is the total diversity, i.e., the diversity of all populations together and H_{POP} the mean within-population diversity value. The proportion within population was calculated as H_{POP} / H_{SP}.

We performed three different AMOVAs (Excoffier et al., 1992), based on Euclidean squared distances among individuals, with significance level evaluated after 16,000 random permutations. The first one, a one-level analysis, considered the variation among the eight populations. To test for differences in the level of genetic diversity between populations from early and middle successional stages, we used two two-level AMOVAs. The first one considered differences between the successional stages and differences among populations within the successional stages, without consideration of pair structure. The other AMOVA performed

considered the differences among the successional pairs and the differences between populations within successional pairs, i.e., between the neighbor "candeial" and forest populations.

To test the hypothesis of "isolation by distance", a genetic distance matrix (ϕ_{ST} values), obtained by AMOVA procedures, was correlated with a geographical distance matrix by the Mantel test, and its significance was tested by a nonparametric permutation procedure.

RESULTS

Morphological data revealed that *Eremanthus erythropappus* individuals from forest were on average almost two times bigger than those from "candeial" in height as well the circumference at the breast height (Table 1). A considerable amount of genetic variation within and among populations of *E. erythropappus* was detected. One hundred and sixty unique RAPD banding patterns were observed, i.e., each individual had a unique RAPD phenotype. A total of 99 fragments were scored and 89.9% of these markers were polymorphic, and the number of polymorphic fragments scored per primer varied from 5 (OPL-13) to 13 (OPL-07) (Table 2).

Table 1. Population pairs, location, successional stage, mean and standard deviation (in parentheses) of circumference at breast height (CBH) and height of populations from two successional stages of *Eremanthus erythropappus.*

Population pair	Location	Successional stage	CBH (cm)	Height (m)
1- Itacolomi-C	Itacolomi State Park	early	38.3 (8.1)	4.8 (0.9)
1- Itacolomi-F	Itacolomi State Park	middle	69.1 (26.5)	9.9 (1.8)
2- Trevo-C	Tripuí Ecological Station	early	34.4 (7.8)	6.5 (0.7)
2- Fortes-F	Tripuí Ecological Station	middle	65.8 (20.3)	9.9 (0.8)
3- Adão-C	Tripuí Ecological Station	early	30.1 (12.9)	5.0 (0.9)
3- Esperto-F	Tripuí Ecological Station	middle	57.5 (14.9)	9.1 (1.3)
4- Pomar-C	Tripuí Ecological Station	early	30.8 (16.7)	4.7 (1.6)
4- Pomar-F	Tripuí Ecological Station	middle	60.6 (21.0)	9.2 (1.0)
Early stage mean			33.4 (11.4)	5.2 (1.0)
Middle stage mean			63.2 (20.7)	9.5 (1.2)

The capital letters following the population name correspond to "candeial" (C) and forest (F).

Table 2. RAPD primer sequences, fragment size range, number of polymorphic fragments/total fragment number, and percent of polymorphic fragments in a sample of 160 individuals of *Eremanthus erythropappus*.

Primer	Sequence (5' → 3')	Fragment size range (bp)	No. of polymorphic fragments/ total No. of fragments	Percent of polymorphic fragments
OPA-10	GTGATCGCAG	500-2000	10/11	90.9%
OPL-01	GGCATGACCT	800-2000	7/7	100.0%
OPL-02	TGGGCGTCAA	500-1200	9/9	100.0%
OPL-04	GACTGCACAC	450-2000	7/9	77.8%
OPL-07	AGGCGGGAAC	500-2000	13/16	81.3%
OPL-08	AGCAGGTGGA	500-2000	9/10	90.0%
OPL-11	ACGATGAGCC	350-1800	11/11	100.0%
OPL-13	ACCGCCTGCT	450-1450	5/7	71.4%
OPL-14	GTGACAGGCT	550-1650	8/9	88.9%
OPL-19	GAGTGGTGAC	600-2000	10/10	100.0%
Total			89/99	89.9%

There was a considerable variation among populations in their diversity indexes (Table 3). The percentage of polymorphic bands within populations ranged from 54.5 (Pomar-C) to 70.7% (Trevo-C and Fortes-F), and the Shannon indexes varied from 0.382 (Pomar-C) to 0.487 (Trevo-C). However, these variations were not associated with successional stages, with populations from early ("candeial") and middle successional (forest) stages exhibiting similar levels of genetic diversity. The percent of polymorphic fragments found in total "candeial" stage was 82.8% and in forest stage was 84.8%, values not significantly different (P = 0.747) as with differences among successional pairs (P = 0.057). The successional stages also did not differ in relation to the Shannon index, with mean of populations from "candeial" equal to 0.442 and mean of populations from forest equal to 0.455 (P = 0.641), and also the values of population pairs were not significantly different (P = 0.087). In fact, it can be observed that in two successional pairs, Esperto-C/Adão-F and Pomar-C/Pomar-F, the forest populations exhibit higher Shannon index values than the respective population from "candeial", and in the two remaining pairs, Itacolomi-C/Itacolomi-F and Trevo-C/Fortes-F, the relation is inverse, with the "candeial" population having higher values than the forest one (Table 3). The test of homogeneity of molecular variance (HOMOVA) also agreed with the Shannon index, indicating that populations exhibit different levels of molecular variance (bp = 0.704; P < 0.001), but that these differences also did not have any relation to successional stages, since early and middle succession populations did not show different levels of molecular variance (P = 0.234).

Table 3. Intra-populational diversity indexes for each population of two different successional stages (early and middle) in *Eremanthus erythropappus.*

Successional stage	Population	% of polymorphic fragments	Shannon index (H_o)
Early	Itacolomi-C	67.7%	0.479
	Trevo-C	70.7%	0.487
	Adão-C	64.6%	0.421
	Pomar-C	54.5%	0.382
	Mean	64.4%	0.442
Middle	Itacolomi-F	61.6%	0.470
	Fortes-F	70.7%	0.484
	Esperto-F	65.7%	0.44
	Pomar-F	63.6%	0.424
	Mean	65.4%	0.455

To allow the partitioning of the RAPD variation within and among populations, the Shannon index and AMOVA were used. The Shannon index indicated that, on average, 17.8% of the variation was due to differences between populations (values not shown). The overall AMOVA showed a broad agreement with the Shannon index, with 14.3% of variation attributed to variation among populations (Table 4). Mantel tests were performed to examine the correlation between genetic and geographical distance. The test considering all populations showed no significance. However, another Mantel test including only comparisons between more distant populations, i.e., populations from Tripuí with populations from Itacolomi (geographical distances ranging from 7,800 to 10,300 m) revealed one positive and significant correlation (r = 0.6267, P = 0.029) between geographical and genetic distances, indicating that genetic diversity increased with increased geographical distance. Comparisons among populations geographically close (300 to 3,000 m apart), i.e., within of the same reserve, did not indicate a significant correlation.

Table 4. Summary of analysis of molecular variance for 160 individuals of eight populations of two successional stages (early and middle) of *Eremanthus erythropappus* based on RAPD markers contrasting all populations, successional pairs and successional stages.

Source of variation	d.f.	Sum of squares	Variance components	% of total variance	P
All populations					
Among populations	7	323.17	1.77	14.3%	<0.001
Within populations	152	1618.95	10.65	85.7%	
Successional pairs					
Among successional pairs	3	161.89	0.34	2.7%	0.108
Between populations/within pairs	4	161.27	1.48	11.9%	<0.001
Within populations	152	1618.95	10.65	85.4%	<0.001
Successional stages					
Among successional stages	1	38.78	-0.11	-0.9%	0.792
Among populations/within stages	6	284.39	1.84	14.8%	<0.001
Within populations	152	1618.95	10.65	86.0%	<0.001

d.f. = degrees of freedom.

To evaluate the effect of successional stages on genetic structure, we performed two different AMOVAs, with two different groupings (Table 4). The first one considered two groups, early and middle succession, and showed that populations from two stages do not diverge (-0.9% of variance due to differences between the stages, P = 0.79). This result indicated that successional stage did not explain any variation among populations. However, these analyses also indicated that within each stage, the populations are genetically divergent (14.8% of variation is due to differences among populations within stages ($P < 0.001$). Another AMOVA indicated that the successional pairs are not different, but that the population within pairs, i.e., populations located near to each other, one from "candeial" and other from forest are genetically different, with 11.9% of variation attributed to differences between populations within pairs ($P < 0.001$). Together, these AMOVAs (Table 4) and the pair-wise differences between populations (Table 5) indicate that all populations are different from each other, but the differences are not related to successional stage. The populations from "candeial" are not more divergent among themselves (ϕ_{ST} "candeial" = 0.152 ± 0.033) than are the forest populations among themselves (ϕ_{ST} forest = 0.139 ± 0.052), with no significant difference (P = 0.604, *t*-test).

Table 5. Pairwise comparisons of ϕ_{ST} values among populations of *Eremanthus erythopappus* of early and middle successional stages, based on RAPD markers.

Populations	Early				Middle			
	Itacolomi-C	Trevo-C	Adão-C	Pomar-C	Itacolomi-F	Fortes-F	Esperto-F	Pomar-F
Early								
Itacolomi-C	-							
Trevo-C	0.126*	-						
Adão-C	0.170*	0.186*	-					
Pomar-C	0.106*	0.142*	0.184*	-				
Middle								
Itacolomi-F	0.124*	0.153*	0.151*	0.107*	-			
Fortes-F	0.106*	0.113*	0.186*	0.138*	0.089*	-		
Esperto-F	0.186*	0.164*	0.143*	0.178*	0.187*	0.201*	-	
Pomar-F	0.123*	0.143*	0.096*	0.109*	0.073*	0.126*	0.157*	-

*P < 0.001.

DISCUSSION

Diversity and genetic structure of *Eremanthus erythropappus*

The diversity of populations of *Eremanthus erythropappus* can be considered high compared with other tree species from Brazil analyzed with RAPD markers. The Shannon indexes (0.382 to 0.487) were higher than in *Plathymenia reticulata* (0.301 to 0.367; Lacerda et al., 2001), *Mabea fistulifera* (0.383 and 0.405; Goulart et al., 2005) and *Hymenaea courbaril* and *H. stigonocarpa* which showed average diversity of populations of 0.301 and 0.321 (H_{POP}), respectively (Brandão, 2002). The percentage of polymorphic fragments found in populations of *E. erythropappus* (54.5 to 70.7%) was also higher than in populations of *P. reticulata* (41.7 to 55.6%; Lacerda et al., 2001), *M. fistulifera* (49.0 to 56.0%; Goulart et al., 2005), *H. courbaril* (37.7 to 47.8%), and *H. stigonocarpa* (44.1 to 48.5%) (Brandão, 2002). On the other hand, the diversity values found here are similar to that found in two other populations of *E. erythropappus* of other sites (Shannon indexes = 0.45 and 0.49; Estopa et al., 2006), which suggests that high diversity is a characteristic of this species.

Most of the genetic variation of *E. erythropappus* was found within populations (85.7%), as it is typical for outcrossing, long-lived species (Hamrick and Godt, 1989; Nybom and Bartish, 2000; Nybom, 2004). Sporophytic self incompatibility system is common in the Asteraceae family (see Ferrer et al., 2004), a mechanism that prevents selfing and biparental inbreeding (Charlesworth and Charlesworth, 1987). In fact, Moura (2005) analyzing progenies of *E. erythropappus* with isozyme markers found that it is an outcrossing species. In addition, we observed small bees visiting flowers of *E. erythropappus*. Furthermore, among-population differentiation (ϕ_{ST} = 0.143) is not high if compared with the mean values reported in studies with RAPD markers for species with the same life history traits, considering outcrossing breeding system (ϕ_{ST} = 0.27), long-lived (ϕ_{ST} = 0.25) and early successional stage (ϕ_{ST} = 0.37) (Nybom, 2004). However, maximum geographical distance between populations analyzed with RAPD markers has a strong positive effect on genetic differentiation found between populations (Nybom and Bartish, 2000; Nybom 2004). The maximum geographical distances among populations studied here (10.8 km) are much lower than the mean of maximum distances among populations (956 km) in studies reviewed by Nybom (2004). This could explain in part the comparably low value of among-population variation in *E. erythropappus*. One fact that corroborates this suggestion is that a positive correlation was found between genetic distance and geographical distance considering populations more distant, i.e., populations from Itacolomi in relation to those from Tripuí (above 7.8 km). This suggests that gene flow among populations of *E. erythropappus* is dependent on geographical distance. In fact, this also was suggested by Estopa et al. (2006) who found a value of differentiation among *E. erythropappus* populations a little higher (G_{ST} = 0.21) than that found in the present study, but also with maximum geographical distance among populations greater (37 km) than among the populations studied here. In spite of differentiation among overall populations not being high, it must be noted that even populations spatially very close, i.e, of the same reserve, exhibited considerable genetic structure (mean ϕ_{ST} = 0.151 among populations from Tripuí). This suggests that the putative pollinator of *E. erythropappus* (small bees)

exhibits small flight distance, allowing crossings to occur mainly between near trees, restricting gene flow by pollen.

Comparison of diversity and genetic structure among *Eremanthus erythropappus* populations from early and middle successional stages

We found no indication that populations from middle successional stage (forest) show lower genetic variation than populations from early successional stage ("candeial"), in opposition to our previous hypothesis. The percentage of polymorphic fragments in the two community types was high and did not differ statistically, 82.8 and 84.8% ("candeial" and forest, respectively). The populations from "candeial" also did not differ in relation to genetic variation evaluated by the Shannon index (mean = 0.442) from the populations from forest (mean = 0.455). The two indices used to estimate within population diversity in the two successional stages were concordant, and none indicated differences in degree of genetic diversity between populations from "candeial" and populations from forest. These results do not support the hypothesis that the selection of genotypes during succession leads to genetic depletion (Gray, 1987), but they are in accordance with several other studies indicating that populations maintain equal levels of intra-populational genetic diversity during succession (Aarssen and Turkington, 1985; Hartnett et al., 1987; Peroni, 1994; Pluess and Stöcklin, 2004; Solé et al., 2004; Goulart et al., 2005; Raffl et al., 2006). These results suggest that if an increase in selection intensity occurred during succession it did not result in a decrease in genetic diversity or that the selection effect was balanced by other factors, such as gene flow. Early-successional species in general exhibit a higher percentage of genetic diversity among populations than late-successional species (Hamrick and Godt, 1989; Nybom 2004). However, in evaluating within same species, we did not detect higher significant differentiation among *H. erythropappus* populations from an early successional stage in relation to that among populations from middle successional one. Differentiation among colonizing populations depends on the type of colonization (one versus several source populations) and the amount of post-colonizing recurrent gene flow, as reviewed by Raffl et al. (2006). Considering the spatial proximity among "candeial" populations studied here, gene flow among them can be considerable, and thus "candeial" populations must receive genes from different sources (populations).

Although we did not find genetic differences associated with successional status of habitats of *E. erythropappus*, the great morphological differences among trees of "candeial" and forest must be noted. RAPD markers have been generally reported to be neutral markers (Brunell and Whitkus, 1997; Heaton et al., 1999), and here we did not analyze genetic differences in relation to morphological characters among the two successional stages. The morphological differences can be due to genetic differences and/or phenotypic plasticity. To conclude about the relative influence of these two factors, it is necessary to evaluate progenies from two successional stages in a common garden experiment.

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