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Testing hypotheses for morphological differences among populations of *Miconia sellowiana* (Melastomataceae) in southern Brazil

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ABSTRACT. Previous studies have uncovered considerable variability in foliar morphology and anatomy for *Miconia sellowiana* in different types of vegetation (Grassland, Montane Atlantic forest, Upper Montane Atlantic forest and Araucaria Pine forest). Although such variability could be due to phenotypic plasticity, an alternative explanation for this phenomenon is the existence of genetic differentiation among populations resulting from genetic drift or adaptation to different environments. The goal of the present study was to investigate the extent of genetic structures among populations of *Miconia sellowiana* using a neutral dominant genetic marker (RAPD - Random Amplification of Polymorphic DNA). There was considerable variability in the studied samples, resulting in 96.5% polymorphic loci and a Gst = 0.13. The analysis of molecular variance showed the populations are genetically structured ($p < 0.001$). The subpopulations of *M. sellowiana* were grouped similarly together using genetic (based on a neutral marker) or morphological dendrograms, suggesting that the morphological differences observed are the result of local genetic differentiation by genetic drift and not the alleged phenotypic plasticity of the species.

Keywords: gene flow, genetic adaptation, local selection, phenotypic plasticity.

Testando hipóteses para as diferenças morfológicas entre populações de *Miconia sellowiana* (Melastomataceae) do sul do Brasil

RESUMO. Estudos prévios relatam a variabilidade na morfologia e anatomia de *Miconia sellowiana* em diferentes formações vegetacionais (Estepe Gramínio-Lenhosa, Floresta Ombrófila Densa Montana, Floresta Ombrófila Densa Alto-Montana e Floresta Ombrófila Mista). Apesar dessa variabilidade poder ser devido à plasticidade fenotípica, uma explicação alternativa para o mesmo fenômeno é a existência de diferenciação genética entre as populações, resultado de deriva genética ou adaptação aos diferentes ambientes. O objetivo do presente estudo foi investigar a existência de estruturação genética entre as populações de *M. sellowiana*, utilizando um marcador genético dominante e neutro (RAPD - "Random Amplification of Polymorphic DNA"). Foi encontrado um grau considerável de variabilidade nas amostras estudadas, sendo que 96,5% dos locos foram polimórficos e o valor de Gst foi de 0,13. O número estimado de migrantes por geração foi de 3,19, o que consiste com a existência de um fluxo gênico reduzido entre os locais estudados. Esse resultado foi confirmado pela análise de variância molecular ($p < 0,001$). As subpopulações de *M. sellowiana* ficaram igualmente agrupadas nos dendrogramas dos dados genéticos (baseado no marcador molecular neutro) e morfológicos, sugerindo que as diferenças morfológicas encontradas são resultado da diferenciação genética local e não por plasticidade fenotípica da espécie.

Palavras-chave: fluxo gênico, adaptação genética, seleção local, plasticidade fenotípica.

Introduction

Miconia sellowiana Naudin (Melastomataceae) has a wide distribution, occurring in different vegetation types that apparently influence its great morphological variation (BOEGER et al., 2008). Previous studies of morphological and anatomical differences for leaves of this species in different vegetation types (Grassland (GL), Montane Atlantic Forest (MAF), Upper Montane Atlantic Forest (UMAF), and Araucaria Forest (AF)) in the State of Paraná, Brazil suggested that there is considerable phenotypic variability (BOEGER et al., 2008). The results of an analysis of morpho-anatomical variables, such as foliar area, dry weight, stomatal density and total mesophyll thickness, indicated significant differences between the leaves of individuals from those vegetation types. The variation among

these populations is also reflected in marked differences in plant heights. For the populations in AF and MAF, the individuals reach up to 6 m, whereas for the population in UMAF, the individuals are not taller than 2 m and in the GL they are about 1 m tall. These differences have been considered to represent differential responses to environmental conditions to which they were exposed (BOEGER et al., 2008). Although the authors suggested that the differences reflect phenotypic plasticity, local adaptations and genetic drift cannot be completely ruled out with the available data.

This study analyzed the genetic structure for those four populations of *M. sellowiana* in the State of Paraná, Brazil based on RAPD - PCR data to test hypotheses concerning the underlying basis for the observed morpho-anatomical variability.

Material and methods

Collection sites throughout the State of Paraná, Brazil are indicated in Figure 1. The Grassland area (GL) is located at Buraco do Padre near the city of Ponta Grossa (25°30' S, 48°59' W). The Montane Atlantic Forest (MAF) area is located at the Parque Mananciais da Serra near the city of Piraquara (25°29' S, 49°59' W). The Upper Montane Atlantic Forest (UMAF) is located at Morro do Canal near the city of Piraquara (25°30' S, 49°50' W). The Araucaria Forest (AF) is located at the Canguiri Experimental Farm near the city of Pinhais (25°0' S, 49°58' W). Environmental characteristics for each site are described in Table 1.

Leaves from ten different individuals of *M. sellowiana* were collected in each vegetation type, except for MAF from which only seven plants were sampled. All populations considered in this study are small with sparsely distributed individuals, which hindered collection of greater numbers of individuals. Leaves were fixed in absolute ethanol and stored at -20°C. DNA was extracted with the DNeasy Plant Mini® kit (Qiagen).

Random amplification of polymorphic DNA (RAPD) was used to characterize each sampled individual plant. The polymerase chain reaction (PCR) was performed in a 30 μL reaction volume composed of 0.83 mM dNTPs (Biotools®), 1X reaction buffer, 3.33 mM $MgCl_2$, 0.08 u μL^{-1} Taq Platinum DNA Polymerase (Invitrogen®), 0.33 μM primers, 5 $\mu g\ \mu L^{-1}$ BSA (Bovine Serum Albumin) and 10 $\mu g\ \mu L^{-1}$ plant DNA.

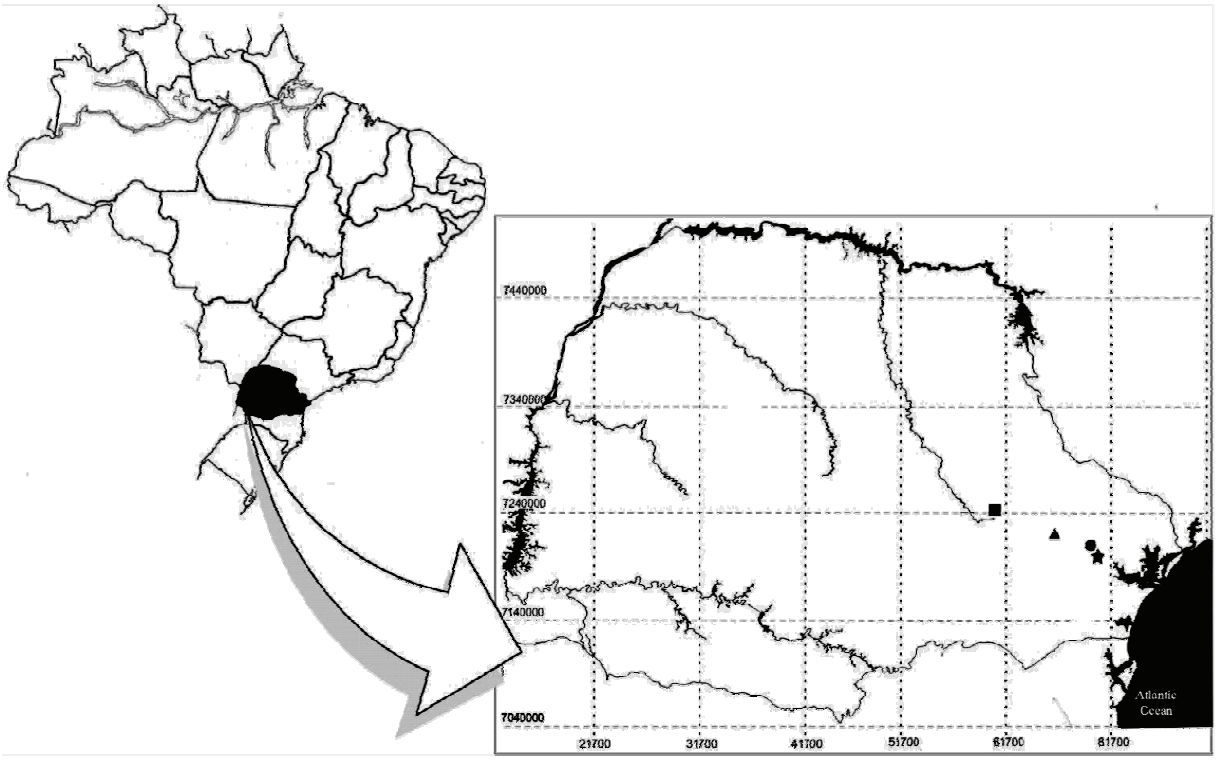

Figure 1. Geographic locations of the sampled population areas. ■Grassland (Buraco do Padre); ●Montane Atlantic Forest (Mananciais da Serra Park); ▲Araucaria Forest (Experimental Farm of Canguiri); ★Upper Montane Atlantic Forest (Morro do Canal).

Table 1. Environmental characteristics for the four forest types: GL (Grassland), AF (Araucaria Forest), MAF (Montane Atlantic Forest) and UMAF (Upper Montane Atlantic Forest). Subtitles = PAR (Photosynthetically Active Radiation). *According to the Brazilian classification. Modified from Boeger et al. (2009).

Environmental characteristics	GL	AF	MAF	UMAF
Latitude	25°10' S	25°0' S	25°29' S	25°30' S
Longitude	48°59' W	49°58' W	49°59' W	49°50' W
Elevation				
Mean annual temperature (°C) (min. - max.)	17.8 (14 - 20)	18 (13 - 24)	16.6 (13 - 20)	18 (12 - 24)
Mean annual precipitation (mm)	1.497	1.451	2.008	1.384
Type of weather	CfB	CfB	CfB	CfB
Soil*	"Neossolo Litólico Distrófico Típico"	"Cambissolo Háplico Alumínico Típico"	"Neossolo Litólico Húmico Típico"	"Neossolo Litólico Húmico Típico"
Altitude (m)	975	775	1,100	1,370
Mean relative humidity (%)	77	91	88	80
Luminous intensity in PAR (μmol s^{-1} m^2)	1550.25	78.09	60.51	1666.52

The amplification was performed in a Personal Cycler PCR System (Eppendorf) with initial denaturation at 94°C for 3 min. followed by 35 cycles of denaturation (94°C for 15 sec.), annealing (35°C for 30 sec.) and extension (72°C for 1 min.) with a final extension for 4 min. at 72°C. 20 primers of the Operon® series 1 were tested on a reduced group of plants from each location and 10 primers (Table 2) were chosen for the analysis. PCR products of each individual plant were electrophoresed in a 1.5% agarose gel such that individuals from the same location were never loaded in neighboring wells. This strategy was designed to avoid any scoring error caused by the proximity of bands from individuals of the same population in the gel. RAPD bands were digitalized with photo-documentation equipment (Vilber Loumart®) and aligned and scored using the software Gel Pro Analyzer®. The identity of each band was determined by comparison with the standard banding pattern of a 1-kb ladder. A negative control was included in all PCR to check for contamination.

Table 2. Primers used in PCR amplifications.

Primers	Sequences
OPA-1	CAGGCCCTTC
OPA-7	GAAACGGGTG
OPA-8	GTGACGTAGG
OPA-9	GGGTAACGCC
OPA-10	GTGATCGCAG
OPA-11	CAATCGCCGT
OPA-12	TCGGCGATAG
OPA-13	CAGCACCCAC
OPA-15	TTCCGAACCC
OPA-18	AGGTGACCGT

The computer program POPGENE® 1.30 (YEH et al., 1999) was used to estimate gene diversity (NEI, 1973), the Shannon index (SHANNON; WEAVER, 1949), population differentiation through Gst and Nm (SLATKIN; BARTON, 1989) and genetic distance (NEI, 1978). Neutrality of the RAPD bands was tested with the Ewens-Watterson test for neutrality (MANLY, 1985). Genetic structuring between populations was tested by Analysis of Molecular Variance (AMOVA) using the application available at the website "The Dyer Laboratory of Populations Genetics" (http://www.dyer2.bio.vcu.edu). Using the calculated Nei's genetic distances, a Mantel's test was performed using the Arlequin software v.3.1 (EXCOFFIER et al., 2005) to examine a correlation between genetic and geographic distances for the sampled populations.

A genetic dendrogram of the analyzed populations was constructed based on standard genetic distances using the neighbor joining method (SAITOU; NEI, 1987) with 1000 bootstrap support for both NJ and Upgma, using the software Dispan (OTA, 1993) and Tfpga® (MILLER, 2007). Morphological relationships between populations were inferred through the neighbor joining method (with 1000 bootstrap support for both NJ and Upgma) based on Euclidean distances using the program Past v. 1.88 (HAMMER et al., 2001). The morphological variables used are those presented by Boeger et al. (2008). Only those showing phenotypic plasticity among the four studied populations of *M. sellowiana* were used (Table 3).

The results of the genetic population analysis were used as additional data to test the alleged hypothesis of phenotypic plasticity (BOEGER et al., 2008). If no population structuring is detected, phenotypic plasticity may indeed occur but selection may not be ruled out. If the populations of *M. sellowiana* are genetically structured, the observed morphological variations are likely the result of exclusive microevolutionary events within each population (e.g., drift or local selection).

Table 3. Mean values (± SE) for morphological characteristics of leaves of *Miconia sellowiana* from different vegetation types (n = 96). Stomatal frequency (n = 192). Grassland (GL); Montane Atlantic Forest (MAF); Upper Montane Atlantic Forest (UMAF); and Araucaria Forest (AF).

Characteristics	UMAF	GL	MAF	AF
Leaf area (cm^2)	3.51 (0.09) d	4.33 (0.14) c	7.55 (0.24) b	10.77 (0.46) a
Leaf dry weight (g)	0.05 (0) d	0.06 (0) c	0.07 (0) b	0.08 (0) a
Stomatal frequency (no. mm^{-2})	1383.64 (26.92) a	1133.2 (11.64) b	769.54 (11.71) c	556.34 (9.87) d
Leaf density (mg mm^{-3})	0.92 (0.01) b	0.89 (0.02) b	0.80 (0.01) b	1.52 (0.16) a
Palisade tissue thickness (μm)	45.62 (3.33) b	64.09 (4.01) a	48.42 (3.62) b	23.69 (1.49) c
Spongy tissue thickness (μm)	108.89 (7.72) a	77.5 (2.95) b	47.18 (2.51) c	35.7 (1.49) d
Leaf thickness (μm)	163.92 (12.23) a	161.63 (4.25) a	116.87 (6.25) b	76.63 (2.06) c

Distinct letters in the same column represent statistically different mean values (Fisher's test, $p < 0.05$).

Further testing was performed by comparing dendrograms built with independent data using a neutral genetic marker (RAPD) and morphologic features of the leaves. Congruence between dendrograms is considered to provide further resolution favoring local adaptation (incongruent dendrograms) or genetic drift (congruent dendrograms).

Results

RAPD - PCR amplifications from 37 individuals using the 10 selected RAPD primers produced a total of 203 diagnosable bands, of which 196 are polymorphic (96.55%). The Ewens-Watterson test for neutrality indicated that six bands were only marginally neutral and they were removed from subsequent analyses. The band patterns obtained showed differences between populations and between individuals of the same population. A comparison of the banding patterns among populations indicated that 22 bands are unique to a single population, with 3 belonging to the population from AF, 4 to MAF, 7 to UMAF and 8 to GL.

The analysis of population genetic parameters showed evidence of population differentiation (Gst = 0.13, AMOVA with a $p < 0.001$; Table 4). In addition, pairwise AMOVAs between populations indicated that all populations are genetically distinct from each other (Table 5). Mantel's test showed no correlation between genetic and geographical distances ($p > 0.05$).

The dendrograms for genetic and morphological similarities are strictly congruent (Figure 2). Both indicate that the population in GL is most proximally related to that in UMAF, whereas the population in AF is more proximally related to that in MAF. Considering that there are five possible rooted dendrograms to describe the relationship between four populations, the probability that the dendrograms are strictly congruent as in this case is $p = 0.04$ ($p = 1/5 \star 1/5$). Thus, congruence between dendrograms is unlikely to be the result of chance alone.

Table 4. Analysis of Molecular Variance for all studied populations of *M. sellowiana* ($p < 0.001$).

	df	SS	MS
Between populations	3	151.93	50.64
Within populations	33	843.71	25.56
Total	36	995.64	

df = degrees of freedom; SS = sum of square; MS = mean square.

Table 5. Paired AMOVA between the studied populations and respective values of Nm.

	AF	MAF	UMAF
Grassland (GL)	$\sqrt{st} = 0.13$ Nm = 3.94 $p < 0.000001$	$\sqrt{st} = 0.13$ Nm = 3.8 $p < 0.000001$	$\sqrt{st} = 0.07$ Nm = 5.77 $p < 0.000001$
Upper Montane Atlantic Forest (UMAF)	$\sqrt{st} = 0.09$ Nm = 4.55 $p < 0.000001$	$\sqrt{st} = 0.06$ Nm = 4.73 $p < 0.000001$	
Montane Atlantic Forest (MAF)	$\sqrt{st} = 0.09$ Nm = 4.26 $P < 0.000001$		

AF = Araucaria Forest; MAF = Montane Atlantic Forest; UMAF = Upper Montane Atlantic Forest.

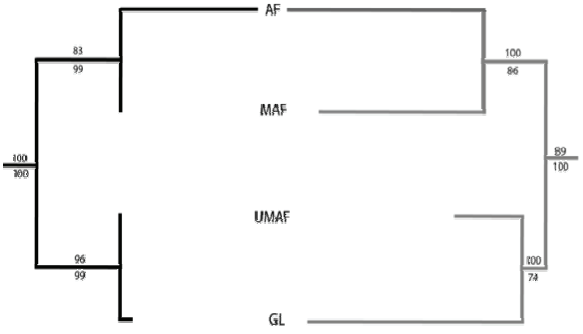

Figure 2. Dendrograms of genetic (gray) and morphological (black) data for four populations of *M. sellowiana*. Bootstrap values for the neighbor joining method are shown above lines and for the Upgma method below lines.

Discussion

The AMOVA and other measurements of population differentiation we obtained (Gst, Φst) indicate that the populations of *M. sellowiana* analyzed in this study are moderately structured ($p < 0.001$). In addition, a comparison of dendrograms for morphological data and genetic distances (Figure 2) resulted in congruent topologies. The population relationships depicted by the genetic dendrogram are also supported by

statistical analysis of the morpho-anatomic data provided by Boeger et al. (2008). Quantitative leaf characteristics vary considerably among vegetation types and many of these differences are statistically significant (Table 3). According to these authors, leaves from GL and UMAF share many characteristics, such as smaller area, larger stomatal frequency, thicker palisade and spongy parenchyma and thicker leaves, as compared to MAF and AF. These differences in leaf morphology were suggested to be plastic, allowing the studied species to grow in different environments and develop several strategies to overcome local conditions when unfavorable (BOEGER et al., 2008).

The statistically significant congruence between dendrograms (Figure 2) strongly suggests that the events associated with the observed divergence between populations are the same for genetic structure, as inferred with a neutral marker, and for foliar structure of individuals. This coincidence supports the hypothesis that the morpho-anatomical differences observed between populations are a consequence of local differentiation by genetic drift rather than an ability of plants in this species to express different phenotypes with the same genotype (phenotypic plasticity) or local selection. Indeed, the observed small size of the sampled populations is consistent with the genetic drift hypothesis. *Miconia sellowiana* is also an apomictic species (SARAIVA et al., 1996), and this reproductive mode may contribute to the postulated genetic differentiation of the populations.

A study on *Atriplex halimus* L. (ORTÍZ-DORDA et al., 2005) also showed genetic structuring which agrees with the morphological differences found in the considered populations. Furthermore, Knight et al. (2006) found specific genes for two populations of *Boechera holboellii* (Hornem.) using AFLP. These populations had different water availabilities, one being well irrigated and the other more xeric. Crossed transplantation of individuals in the latter study resulted in low or no survival, suggesting local adaptation based on genetic differentiation.

Inclusion of additional populations of *M. sellowiana* in the analysis may result in a more robust test of the observed congruence between dendrograms based on putatively independent datasets. Moreover, morpho-anatomical analysis of individuals transplanted from one population to another (crossed transplantation) would provide an additional test for the hypothesis that the observed morphological differences are indeed associated with local genetic differentiation resulting from drift rather than with phenotypic plasticity of the species.

Conclusion

The genetic structure detected between the populations of *M. sellowiana* together with the congruence of the morphological and genetic dendrograms support the hypothesis that the morpho-anatomical differences are probably the result of genetic drift rather the effect of phenotypic plasticity.

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