

Species boundaries inferred from ISSR markers in the *Myrcia laruotteana* complex (Myrtaceae)

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Abstract Myrtaceae is a family with high taxonomic complexity, especially the very rich genera such as *Myrcia*. For this genus, there is not a complete and recent taxonomic revision, and it has some species complexes that are poorly delimited. One of these complexes includes *M. laruotteana*, *M. selloi*, *M. lajeana* and *M. tomentosa*. In order to better understand the species boundaries in this complex, we analyzed the genetic variability and structure of multiple populations of these four species using inter simple sequence repeat markers, checking if there is any relationship between genetic, morphological and geographical patterns. The amount of genetic diversity found was similar ($H_E = 0.215$) compared to species with similar biological characteristics. No groupings that were previously tested (morphological, geographical and the ones used in the traditional taxonomy) showed significant genetic structure. The groups suggested by Bayesian analysis showed a higher genetic structure through analyses of molecular variance: 12 % of the variation between populations within groups and 27 % of the variation between groups. The Bayesian analysis and the neighbor-joining dendrogram showed two major groups, the first with all populations of *M. tomentosa*, and the second containing populations from the other species. According to the

unified species concept, there is enough evidence for the recognition of *M. tomentosa*, *M. laruotteana* and *M. selloi*, but not for *M. lajeana*. These data will be used as the basis of a new taxonomic classification of the species in the *M. laruotteana* complex.

Keywords *Myrcia lajeana* · *Myrcia selloi* · *Myrcia tomentosa* · Myrteae · Population genetics · Species delimitation

Introduction

Species identification is one of the aims of general biological classification and can be troublesome in some plant groups with taxa that are closely related (Rieseberg et al. 2006). Single-locus molecular markers have been successfully used as a biosystematic tool, in order to define interspecific limits within species complexes (Crawford and Mort 2004). Among these, the ISSR (inter simple sequence repeat, Zietkiewicz et al. 1994) is very useful for detecting genetic polymorphisms at species level (Gaiero et al. 2011; Amirmoradi et al. 2012; Rana et al. 2012), since it produces a large number of polymorphic fragments that can be interpreted as multiloci genetic profiles (Zietkiewicz et al. 1994).

Myrtaceae is one of these plant families where identification problems are very common (Biffin et al. 2010). Molecular studies have been conducted successfully in the family, mostly on the phylogeny and delimitation of higher taxonomic levels, such as subfamilies, tribes and subtribes (Wilson et al. 2001, 2005; Lucas et al. 2005, 2007, 2011). Nevertheless, studies at generic and specific levels are still scarce. In the family, ISSR has been used to access the relationships among species of *Eucalyptus* (Balasaravanan

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et al. 2005) and among populations of *Metrosideros polymorpha* (Wright 2007). ISSR has also been used to investigate genetic diversity in *E. grandis* (Okun et al. 2008) and *Rhodomyrtus tomentosa* (Xiaoling 2010), and the genetic structure of *Myrcia splendens* in a limited geographical area (Brandão et al. 2011).

Myrcia is one of the richest genera in tribe Myrteae, with about 400 species (Govaerts et al. 2013), all of them occurring in the Neotropics. Besides its large species number, there is not a recent taxonomic revision for the genus [the last one made by Berg (1857-59)], and the delimitation between some species is difficult. In this genus, there is a species complex, named here as the “*M. laruotteana* complex” that includes *M. laruotteana* Cambess., *M. selloi* (Spreng.) N.Silveira, *M. lajeana* D.Legrand and *M. tomentosa* (Aubl.) DC. (Lima 2013). The species in this complex occur from Panama to southern Brazil, Paraguay and Argentina: *M. laruotteana* occurs in Brazil, from the state of Maranhão to Santa Catarina, and also in Argentina and Paraguay (Legrand and Klein 1969, Sobral et al. 2013); *M. tomentosa* occurs from Panama to southern Brazil, in the state of Paraná (Kawasaki 1989, Sobral et al. 2013); *M. lajeana* occurs only in Brazil, from Paraná to Rio Grande do Sul (Sobral et al. 2013); and *M. selloi* occurs in Argentina and Brazil, from Paraná to Rio Grande do Sul (Rotman 1994, Sobral et al. 2013), but there are herbarium specimens from Minas Gerais and Bahia that seem to belong to this species. The species ranges overlap mainly in central and southern Brazil, but the four species co-occur only in the state of Paraná, in the south (Sobral et al. 2013).

All these species but *M. lajeana* were included in a phylogenetic analysis of *Myrcia* (Lucas et al. 2011), and they all belong to a single clade with a high support. This clade can be distinguished by flower buds with a constriction below the ovary; panicles that are triangular but with an abrupt apex and asymmetrical branches; free calyx lobes that are reflexed and acute; and acute bracteoles that are usually persistent after fruit abscission (Lucas et al. 2011). The distinction between *M. laruotteana*, *M. lajeana* and *M. selloi* is not very clear (Legrand and Klein 1969; *M. selloi* cited as a synonym, *M. ramulosa* DC.), but they could be recognized by leaf morphology and size, general pubescence and persistence of the hypanthium tube and calyx in the fruits: In general, *M. laruotteana* has longer and wider leaves than *M. selloi* and *M. lajeana*; *M. selloi* has caducous hypanthium and calyx, leaving the fruit with a circular apical scar. *Myrcia tomentosa* usually has denser pubescence than other three species, but is morphologically similar to some forms of *M. laruotteana* (Lucas et al. 2011). The same authors mentioned that there are specimens with intermediate character states, which they suspected to be hybrids. Overall, the characters that are traditionally used to distinguish these species are highly

variable, casting doubts about the validity of these entities as distinct taxa. The species in this complex are pollinated by bees (Gressler et al. 2006), and herbarium records show that the species flower in the same period, between September and December. Studies on reproductive biology in Myrteae are still scarce, and among the species in the complex, only *M. tomentosa* (cited as a synonym, *M. rhodosepala* Kiaersk.) has been studied (Proença and Gibbs 1994), being self-compatible.

Since the *M. laruotteana* complex shows a lot of morphological variation and overlapping character states that make it difficult to distinguish its putative taxa, we propose an investigation of the genetic variability patterns in several populations belonging to this complex. This analysis may indicate whether this complex is made up of several distinct species or of a single widespread and polymorphic species. Our goals are (1) to understand genetic variation and structure among populations of *M. laruotteana*, *M. selloi*, *M. lajeana* and *M. tomentosa* through ISSR markers; (2) evaluate genetic divergence and relationships between these populations and the taxa that have been traditionally included in this complex; and (3) evaluate the correlation between genetic and morphological patterns among the taxa.

Methods

Population sampling

We sampled 17 populations, from which five belong to *M. laruotteana*, four to *M. tomentosa*, four to *M. lajeana* and four to *M. selloi* (Table 1). The locations were chosen mainly in central and southern Brazil, where geographical ranges of the four species overlap (Fig. 1). Each population had four to fifteen individuals, totaling 200 individuals in the whole study, from which we collected leaves and one voucher per population, now in the herbaria UEC and UPCB (acronyms following Thiers 2013).

DNA extraction and amplification

The DNA was extracted from leaves stored in silica gel, following Doyle and Doyle (1987). A total of 25 ISSR primers were tested for amplification reactions, from which six were chosen due to better resolution and reproducibility (Table 2). The amplification reactions were carried out in 8.2 µL reaction volumes, using the kit *TopTaq Master Mix* (Qiagen Biotechnology) with 0.3 units of Taq polymerase, 1.5 mM of MgCl₂, 0.2 mM of dNTP, 0.8 µM of primers and 10–100 ng of genomic DNA. The amplification conditions were premelting at 94 °C for 2 min, 37 cycles with denaturing at 94 °C for 15 s, annealing at 42 °C for 30 s

Table 1 Name, geographical location and Brazilian state, number of individuals (*N*) and vouchers for populations of the *Myrcia laruotteana* complex used in the analyses with ISSR markers

Species/Population	Name	<i>N</i>	Location	Voucher
<i>Myrcia lajeana</i>				
Garuva—SC	QUI-1	6	26°01'50"S; 48°57'30"W	Lima 335
Garuva—SC	QUI-2	6	26°02'59"S; 48°57'12"W	Lima 336
Nova Petrópolis—RS	NOP	4	29°21'15"S; 51°04'19"W	Lima 347
São Francisco de Paula—RS	SFP	12	29°15'54"S; 50°18'53"W	Lima 348
<i>Myrcia laruotteana</i>				
Brasília—DF	BRA-2	7	15°43'43"S; 47°54'39"W	Faria 1983
Cambé—PR	CAM	15	23°17'59"S; 51°16'43"W	Lima 296
Campo Mourão—PR	CMOU	15	24°01'07"S; 52°21'37"W	Lima 297
Coronel Xavier Chaves—MG	CXC	12	21°03'30"S; 44°09'14"W	Lima 299
Londrina—PR	LON	12	23°27'24"S; 51°14'19"W	Lima 295
<i>Myrcia selloi</i>				
Joinville—SC	JOIN	11	26°12'25"S; 48°46'55"W	Lima 337
Niterói—RJ	NIT	15	22°58'20"S; 43°01'15"W	Lima 352
Piracicaba—SP	PIRA	15	22°42'54"S; 47°37'46"W	Lima 350
São Francisco do Sul—SC	SFS	14	26°13'44"S; 48°40'46"W	Lima 344
<i>Myrcia tomentosa</i>				
Anápolis—GO	ANA	15	16°22'58"S; 48°56'37"W	Lima 290
Brasília—DF	BRA-1	13	15°52'27"S; 47°50'50"W	Lima 294
Prados—MG	PRA	13	21°02'21"S; 44°07'06"W	Lima 300
Rio Branco do Sul—PR	RBS	15	25°05'57"S; 49°22'39"W	Lima 338

Vouchers are deposited in the herbaria UEC and UPCB. *DF* Distrito Federal, *GO* Goiás, *MG* Minas Gerais, *PR* Paraná, *RJ* Rio de Janeiro, *RS* Rio Grande do Sul, *SC* Santa Catarina, *SP* São Paulo

Fig. 1 Map with the populations of *Myrcia laruotteana*, *M. lajeana*, *M. selloi* and *M. tomentosa* sampled in this study. Open triangle *Myrcia laruotteana*; open circle *Myrcia tomentosa*; closed circle *Myrcia selloi*; open square *Myrcia lajeana*

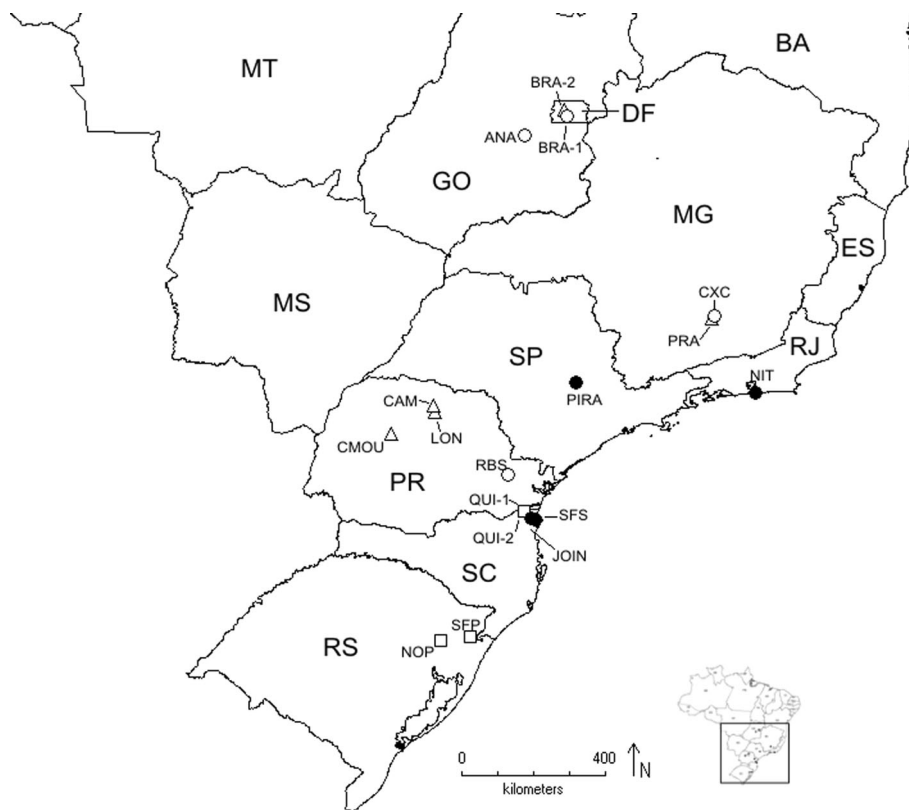


Table 2 Sequence, number and size of fragments (pb) produced by six primers used in this study

Primer	Sequence	Fragment number	Fragment size (pb)
MAO	5'-(CTC) ₄ RC-3'	11	300–1,300
UBC 813	5'-(CT) ₈ T-3'	11	400–1,200
UBC 814	5'-(CT) ₈ TG-3'	9	400–1,000
UBC 827	5'-(AC) ₈ G-3'	10	400–1,200
UBC 844	5'-(CT) ₈ RC-3'	7	500–1,000
UBC 848	5'-(CA) ₈ RG-3'	11	300–1,300

and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. PCR products were analyzed by electrophoresis on 1.5 % agarose gels with 0.5 × TAE buffer, under 100 V for approximately 3.5 h. The gels were stained with ethidium bromide at 0.5 µg/ml and photo-documented. The molecular size of the fragments was estimated using a 100-pb ladder (Ludwig Biotechnology).

Population genetic variability analysis

From the electrophoretic profiles, we built a binary matrix, where each individual was genotyped for presence (1) or absence (0) of fragments. Loci with low resolution were excluded from the analysis. All bands from the same primer with similar molecular size were considered homologous (Thormann et al. 1994). Genetic variability estimates were based on the number of polymorphic (N) and exclusive (N_{ex}) loci, percentage of polymorphic loci (P), Shannon diversity index (I) and average heterozygosity expected for the populations (H_E), assessed using the software GenAlEx 6.5 (Peakall and Smouse 2012).

Analysis of molecular variance (AMOVA) was also performed through software GenAlEx 6.5. The AMOVA provides the proportion of intraspecific genetic diversity explained by interpopulational differentiation (Φ_{ST} , analogous to F statistics from Wright 1921). The populations were tested in different grouping scenarios: (1) the four taxonomic groups (i.e., the traditional circumscription of the four species in the complex: *M. laruotteana* × *M. tomentosa* × *M. lajeana* × *M. selloi*); (2) six groups according to leaf morphology (Table 3); and (3) ten groups based on geographical distances (populations distant less than 200 km from each other and/or less than 500 m altitude from each other).

In order to infer a probability for the genotyped dataset assigned to a given number of clusters (K) and an estimation of the individual's ancestry (q), we performed a Bayesian analysis through the software STRUCTURE 2.3.4 (Pritchard et al. 2000). The values of K (genetic groups) were estimated between 1 and 17. Ten independent runs for each K were performed, with 1,000,000 MCMC (Markov Chain Monte Carlo) iterations and burn-in period of 100,000, admitting the admixture model of ancestry and correlated allele frequencies. Evanno's index (Evanno et al. 2005) was used to detect the number of clusters (K) in the dataset. The individual's ancestry (q) values of 0.9 were used to assign individuals to pure or hybrid classes (Vähä and Primmer 2006). An AMOVA was performed for the groups indicated by this Bayesian analysis through GenAlEx 6.5.

Another Bayesian analysis was performed through the package GENELAND 4.0.3 (Guillot and Santos 2010) available for the software R (<http://www.cran.r-project.org/>). In this approach, the geographical structure of populations

Table 3 Six leaf morphology patterns in the populations of the *Myrcia laruotteana* complex

	Pattern #1 (ANA, BRA-1, PRA, RBS)	Pattern #2 (CXC, BRA-2)	Pattern #3 (LON, CAM, CMOU, JOIN, SFS)	Pattern #4 (PIRA, NIT)	Pattern #5 (NOP, SFP)	Pattern #6 (QUI-1, QUI-2)
Leaf shape	Usually obovate	Elliptic-lanceolate	Usually ovate	Ovate or elliptic	Elliptic-lanceolate	Elliptic or ovate
Divergence angle of the secondary nerves	>90°	>90°	=90°	>90°	>90°	>90°
Secondary nerves	Thinning toward the margin, then joining into arches	Thinning toward the margin, then joining into arches	Same thickness toward the margin, joining the marginal nerve at a 90° angle	Same thickness toward the margin, joining the marginal nerve in a 90° angle	Same thickness toward the margin, joining the marginal nerve in a 90° angle	Thinning toward the margin, then joining into arches
Petiole length	6–13 cm	1.5–4 cm	0.8–2.5 cm	1–2.5 cm	2.5–6 cm	1–3 cm

See Table 1 for names and details of the populations

was considered. The values for K were estimated between 1 and 18, with 10 independent runs and 200,000 MCMC iterations (admitting the admixture model, with alleles correlated between populations). In this analysis, we were able to access posterior probabilities for clusters of structured populations and the probability, for each individual, to belong to the respective population or to come from an external source, and also inferring putative migration events and hybrid zones between these populations.

Another AMOVA was performed with the groups indicated by the Bayesian analyses from STRUCTURE 2.3.4 and GENELAND 4.0.3, also through GenAIEx 6.5. A matrix with the Φ_{ST} values from all group pairs indicated by this Bayesian analysis was performed through software AFLP-SURV 1.0 (Vekemans 2002). This matrix was used to build a neighbor-joining dendrogram through the “Neighbor” package, available in the software PHYLIP (Felsenstein 1993). Statistical support of the branches was calculated through the package CONSENSE, from the same software. The resulting tree was visualized with software TREEVIEW (Page 1996).

Results

Intrapopulational genetic diversity

The six primers provided 59 polymorphic loci. Each primer produced from seven to 11 different fragments, these ranging from 300 to 1,300 bp (Table 2). No exclusive fragment was found for any population. The proportion of polymorphic loci per population varied between 81.36 % (CXC) and 30.51 % (RBS).

As for the average heterozygosity expected within the populations, CXC showed the highest value (0.306), while NIT showed the lowest (0.109). The same populations CXC and NIT also showed, respectively, the highest (0.452) and lowest (0.165) values for the Shannon diversity index (Table 4).

Populational relationships and genetic structure

The highest genetic similarity was found between the populations LON and CAM (0.954), and the lowest between RBS and SFP (0.612). The value of genetic structure (Φ_{ST}) of the 17 populations was 0.377. As for the three different grouping scenarios, we found 10–17 % of variation between groups, 21–29 % of variation between the populations within the groups and 61–62 % variation inside the populations. The genetic structure of these groups (Φ_{ST}) varied from 0.385 and 0.392 (Table 5).

Table 4 Genetic variability in 17 populations in *Myrcia laruotteana* complex, based on 59 ISSR loci

Population	N	P (%)	I	H_E
ANA	51	72.88	0.364 (0.035)	0.240 (0.025)
BRA-1	53	71.19	0.337 (0.035)	0.221 (0.025)
LON	55	76.27	0.382 (0.033)	0.252 (0.024)
CAM	54	77.97	0.374 (0.034)	0.245 (0.024)
CMOU	54	76.27	0.392 (0.033)	0.259 (0.023)
CXC	52	81.36	0.452 (0.033)	0.306 (0.024)
PRA	50	71.19	0.371 (0.037)	0.250 (0.027)
QUI-1	44	47.46	0.252 (0.038)	0.169 (0.026)
QUI-2	46	52.54	0.294 (0.039)	0.200 (0.028)
JOIN	52	62.71	0.325 (0.037)	0.217 (0.026)
RBS	42	30.51	0.180 (0.037)	0.124 (0.026)
BRA-2	46	47.46	0.269 (0.039)	0.184 (0.028)
SFS	48	55.93	0.279 (0.037)	0.185 (0.026)
NOP	38	52.54	0.289 (0.038)	0.194 (0.026)
SFP	51	71.19	0.345 (0.034)	0.225 (0.024)
PIRA	51	72.88	0.403 (0.037)	0.274 (0.026)
NIT	45	32.20	0.165 (0.033)	0.109 (0.023)
Mean	48	61.91	0.322 (0.009)	0.215 (0.006)

N number of fragments in each population, P percentage of polymorphic loci, I Shannon index, H_E mean heterozygosity expected for each population; standard deviation between brackets. See Table 1 for names and details of the populations

The populations were ordered in two clusters in the Bayesian analysis through STRUCTURE 2.3.4 (Fig. 2a). One group (K #1) was made of populations of *M. tomentosa* (except by one individual of ANA) and the population NIT (except by one individual too). The second (K #2) had all other populations, mixing *M. laruotteana*, *M. lajeana* and *M. selloi* (except by one individual of CXC). The coefficient of ancestry showed that 14 samples presented q value lower than 0.9, ranging from 0.534 to 0.885, indicating that these individuals might be interpreted as hybrids. A second round of the analysis was performed only with K #2 (Fig. 2b), showing the populations clustered again in two groups, one with BRA-2 and CXC (K #3) and other with LON, CAM, CMOU, QUI-1, QUI-2, JOIN, SFS, NOP, SFP and PIRA (K #4). The genetic variation (AMOVA) found between K #1, K #3 and K #4 (14 %) is lower than between the populations in each group (27 %; Table 5).

In the other Bayesian analysis, using GENELAND 4.0.3, the populations were clustered in 11 genetic groups (Fig. 3; Table 6), with no indication that either migration or hybridization happened in these populations. No individual showed a posterior probability to belong to any other group than its original group. Populations from different species did not group together. Group #1 has the

Table 5 Molecular variance analysis (AMOVA) for the three grouping scenarios tested for the 17 populations of the *Myrcia laruotteana* complex, and for the groups based on the Bayesian analysis

Font of variation	Percentage of variation (%)				
	Morphological groups	Taxonomic groups	Geographical groups	Groups of the Bayesian analysis	
				STRUCTURE 2.3.4	GENELAND 4.0.3
Between groups	13	10	17	14	27
Between populations within groups	26	29	21	27	12
Within populations	61	61	62	59	61
Φ_{st} (pop/total)	0.392	0.392	0.385	0.412	0.387

The grouping scenarios are (1) six morphological groups; (2) four taxonomic groups; and (3) ten geographical groups. For the groups formed by the Bayesian analysis through GENELAND 4.0.3, see Table 6

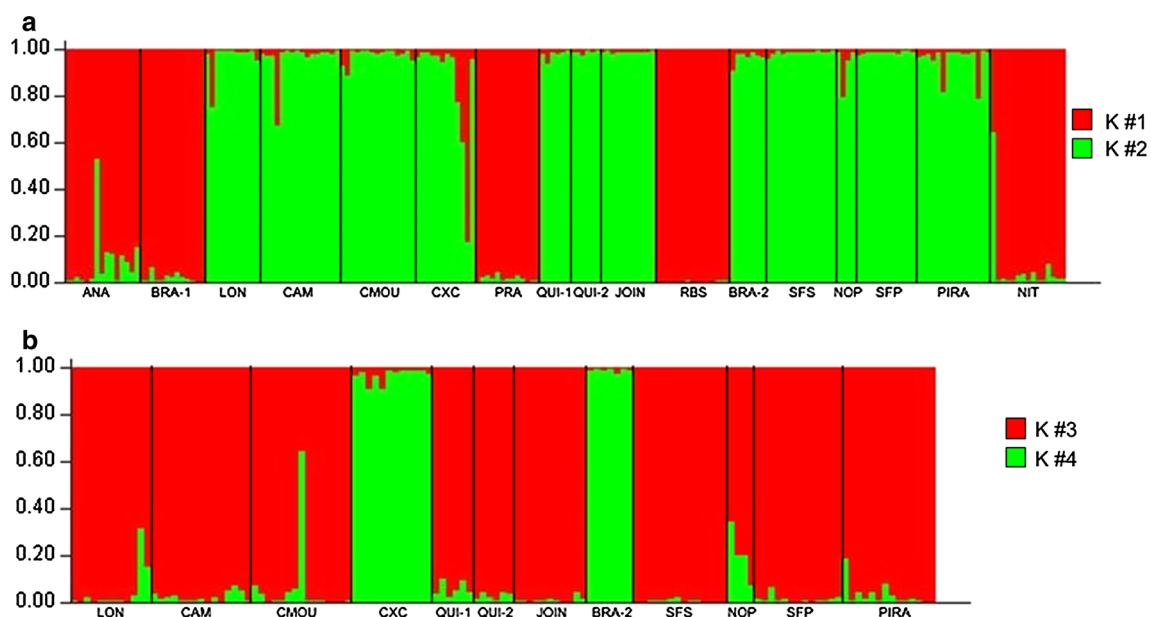


Fig. 2 Clusters obtained from the analysis through STRUCTURE 2.3.4 for individuals from the *Myrcia laruotteana* complex. **a.** 200 individuals from the whole complex; **b.** 129 individuals from a

subgroup (*K* #2, see “Results” section for its description). Each column represents an individual, and colors are used to represent their clusters

populations NOP and SFP, which are morphologically similar and are distant only about 70 km from each other. Populations QUI-1 and QUI-2 form group #2 and are also morphologically similar and share the same habitat type. Group #3 has populations SFS and JOIN, both morphologically similar, but showing a posterior probability to belong in group #2. Groups #2 and #3 are in the same geographical region, but SFS and JOIN are at sea level, while QUI-1 and QUI-2 are located at more than 1,000-m altitude. Group #4 has three populations from the same region (LON, CAM and CMOU). Group #5 has ANA and BRA-1, but these populations showed posterior probability to belong in group #6, which in turn has only BRA-2. Groups #7 and #8 have only one population each

(respectively PRA and CXC), but CXC showed posterior probability to join PRA in group #7. The last three groups also have only one population each (NIT in group #9, PIRA in group #10 and RBS in group #11). The genetic variation (AMOVA) found between these groups (27 %) is higher than between the populations in each group (12 %) (Table 5).

The consensus tree with the Φ_{ST} values showed only two branches with support higher than 50 %, and only one of these higher than 80 % (Fig. 4). The tree shows two main groups, the first one with the populations recognized as *M. tomentosa* and the second one with populations recognized as *M. laruotteana*, *M. selloi* and *M. lajeana* all mixed together.

Fig. 3 **a** Distribution of the posterior probability of a number of K genetic clusters for 17 populations of *Myrcia laruotteana* complex. **b** Map of the populations of *M. laruotteana* complex in genetic domains inferred by GENELAND 4.0.3. See Table 1 for locations of populations

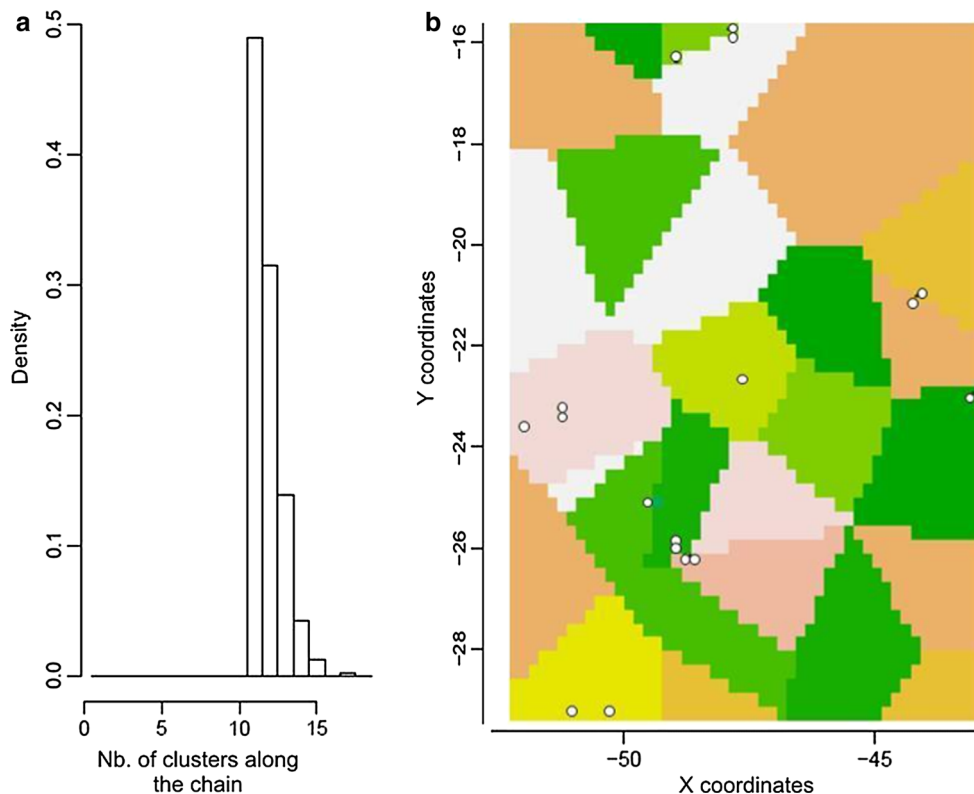


Table 6 Genetic groups and respective populations from the *Myrcia laruotteana* complex according to the Bayesian analysis performed by GENELAND 4.0.3

Group	Population
1	NOP, SFP
2	QUI-1, QUI-2
3	SFS, JOIN
4	LON, CAM, CMOU
5	ANA, BRA-1
6	BRA-2
7	PRA
8	CXC
9	NIT
10	PIRA
11	RBS

See Table 1 for names and details of the populations

Discussion

Intrapopulation genetic diversity

All primers that were tested showed a few amplified loci (ranging from seven to eleven), the same as found for other species in tribe Myrteae, either using ISSR (3–11 loci per primer in *Myrcia splendens*; Brandão et al. 2011), and RAPD (5–9 loci per primer in *Eugenia dysenterica* DC.; Trindade and Chaves 2005). On the other hand, species from other tribes in Myrtaceae seem to provide a larger number of loci per primer for ISSR, like in *Eucalyptus* sp. (44–102 loci; Balasaravanan et al. 2005) and *Metrosideros*

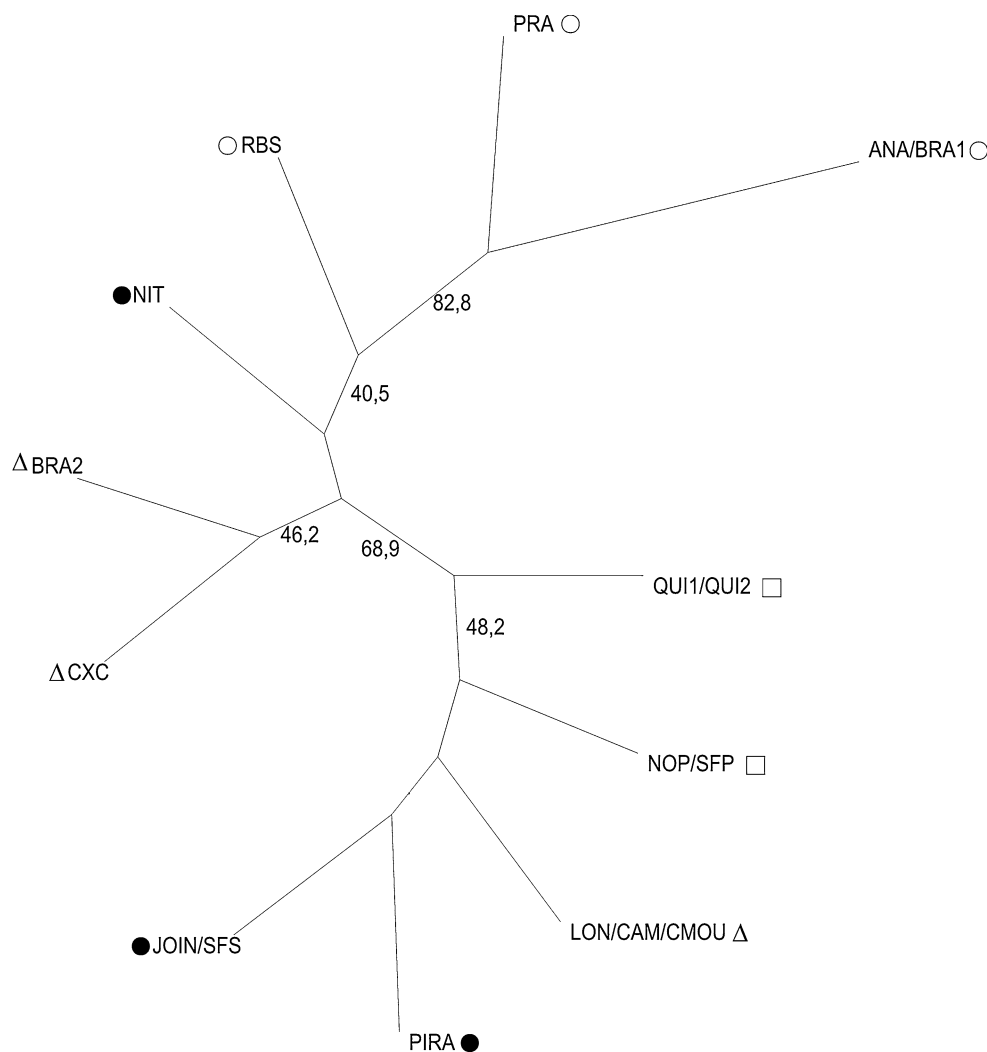
polymorpha (31–41 loci; Wright 2007). The small number of fragments amplified by ISSR may be characteristic of the species in tribe Myrteae. Despite the small amount of loci, the analyses were able to answer the initial questions proposed by these works.

As for other tropical Myrteaceae, the genetic diversity in the *M. laruotteana* complex (0.215) is similar to the one found in the polymorphic *Metrosideros polymorpha* (0.2436; Wright 2007). The species in the *M. laruotteana* complex also have a genetic diversity similar to the species with similar characters, such as perennial (0.25), cross-pollinated (0.27) or wide-distributed (0.22; Nybom 2004, using RAPD).

Populational relationships and genetic structure

The populations studied here are more structured ($\Phi_{ST} = 0.377$) than species with similar characters that are perennial (0.25) or cross-pollinated (0.27). However, this value is similar to the one found in widely distributed species (0.34; Nybom 2004). The proportion of genetic variability within populations (62 %) larger than between populations (38 %) agrees with other works on tropical tree species (Nybom and Bartish 2000; Nybom 2004). The weak genetic divergence found between populations suggests that some genetic flow may have been kept by pollen and seed transfers (Hamrick and Godt 1990). *Myrcia* is

Fig. 4 Unrooted dendrogram showing the relationships between the groups formed in the Bayesian analysis, based on the values of pairwise Φ_{ST} and neighbor-joining algorithm. Bootstrap percentage greater than 40 % are shown beside branches. *Open triangle Myrcia laruotteana*; *open circle Myrcia tomentosa*; *closed circle Myrcia selloi*; *open square Myrcia lajeana*



largely entomophyllous and zoochoric (Gressler et al. 2006), which may ensure moderate- to long-distance genetic flow (Levin 1981). The genetic structure is consequently reduced, due to allelic homogeneity (Loveless and Hamrick 1984). This also explains why populations that are close to each other are morphologically similar, since they exchange alleles more frequently than populations that are distant from each other.

The genetic variation in the grouping scenarios (morphological, geographical and taxonomic) was larger between populations inside the groups (21–35 %) than between the groups (3–17 %). This means that these groups are not genetically homogeneous, nor significantly structured. The lack of correlation between genetic structure and predetermined morphological patterns has been already found in the family (*Eucalyptus* sp.; Balasaravanan et al. 2005).

The differences between the two Bayesian analyses, through STRUCTURE and GENELAND can be explained by different approaches adopted by each method. While

STRUCTURE recovers homogeneous genetic groups, indicating lineages that can be recognized (Pritchard et al. 2000), GENELAND considers the geographical structure in addition to genetic structure, “forcing” the recognition of the lineages under a geographical context (Guillot and Santos 2010).

The Bayesian analysis performed by STRUCTURE 2.3.4 initially separated populations of *M. tomentosa* and NIT (K #1) from the others (K #2). We did not expect NIT grouping with populations of *M. tomentosa*, because this population is morphologically similar to the ones in cluster K #2, with plants regarded as *M. laruotteana*, *M. selloi* and *M. lajeana*. This analysis showed 14 possible hybrid individuals between these groups, distributed through all the sampled area, with 11 of these distributed from Goiás to São Paulo, indicating a possible hybrid zone. It suggests that populations can be exchanging alleles between them, and it also can explain the morphological diversity found in the complex. The AMOVA performed for this analysis showed lower divergence between the

clusters (14 %) than between the populations inside the clusters (27 %), showing that these groups are not significantly genetically structured. The analysis restricted to $K=2$ showed two groups, respectively, with populations from northern areas (BRA-2 and CXC) and populations from southern areas (LON, CAM, CMOU, QUI-1, QUI-2, JOIN, SFS, NOP, SFP and PIRA).

The groups determined by Bayesian analysis through GENELAND 4.0.3. showed a stronger genetic structure than the ones described above. In this case, the genetic variation between the groups (27 %) is larger than the variation between the populations inside each group (12 %). All the populations coming from a same geographical region were grouped in this analysis, except for the populations considered here as from different species. Again, populations that are located close to each other are more genetically and morphologically similar than populations that are geographically distant. Within the species in this complex, plants regarded as *M. laruotteana* are widely distributed, showing a morphological gradient that follows a geographical pattern: The populations from northern areas are morphologically more similar to plants regarded as *M. tomentosa*, while the ones from southern areas are more similar to plants regarded as *M. selloi* and/or *M. lajeana*. Clusters of *M. tomentosa* and *M. laruotteana* have a high posterior probability to cluster among themselves (ANA/BRA-1 plus PRA, with BRA-2 plus CXC, respectively), corroborating the morphological gradient.

Despite low bootstrap values found for the relationships inferred by the Bayesian analysis, patterns shown by the dendrogram are still useful to explain the morphological gradient in the *M. laruotteana* complex. Actually, the low bootstrap values found here reflect the nature of the data gathered in this study. The bootstrap method consists of resampling the characters to infer how variable the branches are in a tree (Felsenstein 1985). Therefore, whenever there are few characters in an analysis, bootstrap values are expected to be low (Soltis and Soltis 2003). Another explanation would be that these low values may reflect the absence of exclusive loci in the populations studied here.

The genetic differences between populations from northern and southern areas can also be seen in the group with the populations of *M. tomentosa*, where there is a well-supported (82.8 %) distinction between the northern populations (PRA, BRA-1 e ANA) from the one collected southwards (RBS). There is another gradient in the group formed by the populations of *M. laruotteana*, *M. selloi* and *M. lajeana*, with another well-supported (68.9 %) distinction between the populations collected to the north of the State of São Paulo (NIT, BRA-2 and CXC) and the ones to the south of it (QUI-1, QUI-2, NOP, SFP, LON, CAM, CMOU, PIRA, JOIN and SFS). In general, this structure is the same to the one provided by STRUCTURE 2.3.4.

According to the same geographic-morphological gradient, it would be expected that the populations CXC and BRA-2 (the northernmost populations of *M. laruotteana*) would be closer to the group with the populations belonging to *M. tomentosa*. Nevertheless, this did not occur, mostly because of the population NIT. This population is remarkable in the sense that it clearly belongs to *M. selloi*, and it could absolutely not be mistaken for *M. tomentosa* or *M. laruotteana*. Moreover, NIT was also the population with lower genetic diversity, which in turn could be explained by the distance between it and the other ones (Nybom 2004).

The groups shown in the tree followed the leaf morphological patterns too, as shown in Table 3. Pattern #1 was found in all populations regarded as *M. tomentosa*, which also were grouped in the dendrogram. Populations CXC and BRA-2 (the northernmost *M. laruotteana*) formed another group, and both represent pattern #2. Patterns #3, #4, #5, and #6 belong to one group, with populations from three species (*M. lajeana*, *M. laruotteana* and *M. selloi*). The population NIT did not follow the general pattern: It would be expected to be in this last big group, since it is one of the two species with leaves following pattern #5 (the other is PIRA).

The population NIT shares the caducous calyx and hypanthium with JOIN, SFS and PIRA. This character makes *M. selloi* distinct from the other species and therefore, it would be expected that all these populations should be grouped together. Again, this was not observed.

Remarks on the taxonomy of the *M. laruotteana* complex

The tests that we performed based on leaf morphological patterns and with the species that have been recognized in the traditional taxonomic circumscription did not show significant genetic structure between groups, nor a genetic discontinuity between the populations that were sampled here. This means that both morphological differences between the species and also the recognition of the four species within the complex are not supported by the genetics presented here. In general, both Bayesian analyses tend to separate populations of *M. tomentosa* from the others. Secondly, these other populations were separated according to their geographical regions. Despite the low supports, our work presents some patterns that may be useful.

Taxonomic implications based on our results may follow two lines. One interpretation would be that all populations belong to a single species, because (1) there is a morphological continuum that agrees, at least in part, with geographical distribution, and therefore, there are no clear limits between these four putative species; (2) genetic

variation within the populations is stronger than the variation among populations, and the overall diversity index is low, which means that the populations are genetically similar and moreover, there may be some genetic flow between them, irrespectively from which putative species they belong. Following this line, all species studied here should be synonymized under the older name, *M. tomentosa*. In this case, *M. tomentosa* “sensu lato” could be regarded as an ochlopecies (Cronk 1998), which means a species that is highly polymorphic but weakly polytypic. Morphological characters vary independently in an ochlopecies, and may follow complex patterns, which in turn may be related to geographical and ecological traits presented by the populations. Ochlopecies are frequently widespread, occur in a wide range of habitats, and usually have a large number of synonyms. Thus, they do not easily fit formal taxonomic treatments (White 1998).

Another interpretation for our data would be to accept that classical taxonomy may rely on molecular and statistical data, but also may deal with overall morphology of the plants. Looking at that, even accepting that there is no discontinuity between *M. laruotteana* and *M. tomentosa*, we can see that there is a clear distinction between *M. lajeana*/*M. selloi* and *M. tomentosa*. According to the unified species concept (de Queiroz 2007), species are segments of lines in a metapopulation that is constantly evolving. In this case, the traditional species concepts should be regarded as secondary expressions within this wider concept. Following this approach, there is evidence for the recognition of two separate lines, the first with *M. tomentosa* and the second with *M. laruotteana*. The limits of these two species may be explained by the two clusters shown in Fig. 4, considering that *M. lajeana* and *M. selloi* belong to *M. laruotteana*'s line. These species are subjected to evolution processes and may be because their divergence is too recent, they still share morphological and ecological characters. This character sharing occurs mainly in central and eastern Brazil, right in the places where the species co-occur. On the other hand, populations from the two species are more distinct to the north or to the south. Both facts may indicate that the divergence process between the two species may have occurred in central and eastern Brazil, followed by posterior colonization to the north and to the south, but this hypothesis can only be tested with phylogeographical studies.

Myrcia lajeana cannot be regarded as a separate line, since there is no disjunction and no genetic evidence to support it as a different species. Moreover, the characters based on which this species was described are not clearly distinct from *M. laruotteana*, as noted by Legrand and Klein (1969). In the case of *M. selloi*, despite the morphological (more precisely regarding vegetative characters) and genetic similarity toward *M. laruotteana*, there is a

reproductive character that makes it distinct. Thus, *M. selloi* could be recognized as a line distinct from *M. laruotteana* or an infraspecific entity under *M. laruotteana*, due to morphological evidence—its caducous hypanthium and calyx. The lack of genetic variation between species by itself is not necessarily a complete evidence that these represent a single species, since there may be more variable loci that were not selected for these analyses (Barrett and Freudenstein 2011), and this undetected variability could show that these species are actually distinct.

Following this scenario, taxonomic adjustments must be made for this complex. Taking into account, both our data on genetic markers and morphological and ecological aspects presented by these populations, we propose to keep the names *M. tomentosa*, *M. laruotteana* and *M. selloi*, and the synonymization of *M. lajeana* under *M. laruotteana*. As for the status of *M. selloi*, either as another species or as an infraspecific entity under *M. laruotteana*, more studies are needed to clear its taxonomic status.

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