



Conservation genetics of two rare and endemic *Myrceugenia* species (Myrtaceae) from the Brazilian Atlantic Forest

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Abstract

Genetic diversity is essential to the survival and resilience of all species, yet very few studies characterizing the intraspecific genetic diversity of rare species have been carried out. In this study, we assessed and described the genetic diversity and structure of two rare and endemic *Myrceugenia* species (Myrtaceae) from the Brazilian Atlantic Forest using microsatellite markers developed specifically for each species. We also followed the IUCN guidelines to assess their conservation status and discussed conservation strategies for these species. Our results suggested a moderate level of genetic diversity for both species. *Myrceugenia basicordata*, distributed at lower altitudes, is the species with the lowest genetic diversity, although with low differentiation among populations. *Myrceugenia joinvillensis* presented higher genetic diversity and a more distinct genetic structure, possibly because of its exclusive occurrence at higher altitudes. Both species require conservation efforts to avoid future declines in population size and distribution range. The main threat to these species is habitat loss or fragmentation due to urban expansion. Considering this, our practical recommendations for conserving these species include immediate actions, such as including the species in official red lists, and long-term actions, such as *ex situ* cultivation for future reintroduction. This study represents the first conservation effort focused on *Myrceugenia* species from the Atlantic Forest, marking an important initial step towards understanding these and other rare species from this biome.

Keywords Genetic diversity and structure · Microsatellites · Rare species · SSRs

Introduction

Conservation genetics provides tools for the conservation of biodiversity (Frankham 2010; Willi et al. 2021). The Group on Earth Observations - Biodiversity Observation Network (GEO BON), who is committed to improving and disseminating biodiversity information at different levels (GEO BON 2024), introduced the concept of Essential Biodiversity Variables (EBVs), a set of fundamental metrics designed to standardize and interpret complex biodiversity data (Jetz et al. 2019; Pereira et al. 2013). EBVs include genetic composition, species populations, species traits, community composition, ecosystem structure, and ecosystem function (Pereira et al. 2013). For the genetic composition category (genetic EBVs), four metrics are considered: (i) genetic diversity; (ii) genetic differentiation; (iii) inbreeding; and (iv) effective population size (N_e) (Hoban et al. 2022).

The estimation of EBVs for threatened species remains a challenge due to lack of information. Rare species,

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including those with a narrow distribution, restricted habitat tolerance, in some cases with small populations, or any combination of these factors (Drury 1974; Harnik et al. 2012; Leitão et al. 2016), are usually considered threatened because they are more susceptible to habitat fragmentation and, consequently, to extinction, as they are unable to adapt to environmental changes (Brown et al. 2023; Davies et al. 2004). Notably, there is a high estimated occurrence of rare and threatened species in nature, suggesting that three out of every four undescribed species are already threatened to some degree (Brown et al. 2023). For this reason, improving our knowledge of rare species is essential to understanding their ecological niche and indirectly promoting the preservation of other species in the process (Dee et al. 2019).

The IUCN (International Union for Conservation of Nature) is one of the most important organizations for monitoring threatened biodiversity through the IUCN Red List assessments (IUCN Red List 2024). Based on systematic criteria, such as geographic range, demographic factors (e.g., number of mature individuals), and quantitative analyses, the IUCN Red List classifies threatened species into three main categories: critically endangered (CR), endangered (EN), and vulnerable (VU) (IUCN 2024). Additionally, the IUCN Red List recognizes genetic metrics as an important data source for assessing the conservation status of species. However, these metrics are not frequently used in species assessments, primarily due to practical challenges, including difficulties in data collection and interpretation, as well as the absence of clear guidelines, such as specific criteria for incorporating genetic data (Garner et al. 2020; IUCN 2024).

In the Brazilian flora, Myrtaceae is one of the families with the largest number of species, mainly distributed in the Atlantic Forest and Cerrado domains, with great ecological importance. Myrtaceae species are frequently used in ecological corridors and in the restoration of degraded areas (Zamith and Scarano 2006), provide food for animals through the production of fruits throughout the year (Staggemeier et al. 2017), and are the main pollen source for native bees (Martins et al. 2023). *Myrceugenia* (Myrtaceae) includes 46 species (POWO 2024), most of which (34) are found in Brazil, especially in the Atlantic Forest (Vieira and Meirelles 2024). Recently, *Myrceugenia basicordata* and *M. joinvillensis* were described, both endemic to the Atlantic Forest and with a distribution restricted to the northeastern portion of the state of Santa Catarina, in southern Brazil. The aims of this study were to characterize the genetic diversity and structure in the two mentioned *Myrceugenia* species, considering their rarity and potential threats, and to discuss conservation strategies for these species.

Materials and methods

Study species

The two *Myrceugenia* species (Myrtaceae) included in our study are species with restricted distributions, endemic to Santa Catarina state, Brazil, and with few records of occurrence. They are both considered rare and threatened in the Brazilian Atlantic Forest. *Myrceugenia basicordata* F.C.S.Vieira, Molz & Sobral is a small tree, up to 2 m tall. It is currently restricted to a few forested areas, close to the urban area of Joinville, in northeastern Santa Catarina. It generally occurs on slopes at ca. 200 m above sea level (masl) of the submontane dense ombrophilous forest (Sobral et al. 2019), in the Serra do Mar Mountain range. The species' habitat is at the foothills/sedimentary area of the elevations, not found on slopes with a gradient of 45° or more (FCSV, field observations). *Myrceugenia joinvillensis* F.S.C.Vieira exhibits habits that range from shrub to arboreal, up to 3 m tall. The species is found at the edge of the Atlantic Forest mountains in northeastern Santa Catarina, as well as in the Serra do Mar, but primarily at higher altitudes between 400 and 700 masl (Vieira 2020), usually in mature forests. However, the species occupies relatively distinct areas of the forest characterized by the absence of a closed canopy and clearly defined strata (FCSV, field observations). As recently described species, there is no specific information about the ecology of *M. basicordata* and *M. joinvillensis*.

Data collection and field work

We used online platforms to search for basic information about the species, such as distribution area and number of populations. The consulted sources were Specieslink (<http://www.splink.org.br/>), GBIF – Global Biodiversity Information Facility (<https://www.gbif.org/>) and REFLORA Virtual Herbarium (<http://reflora.jbrj.gov.br/>). We also searched the main herbarium collections from South and South-East Brazil for neglected specimens and no new records were found.

We conducted field work in 2022 and 2023 and found all eight populations, four for *M. basicordata* and four for *M. joinvillensis* (Table 1). A total of 50 individuals of *M. basicordata* and 52 of *M. joinvillensis* were sampled. We collected young leaves from all the individuals (tree height: two to three meters) found at a minimum distance of 10 m apart. Leaf tissue was dried in silica gel. One individual from each population was herborized and deposited at the herbarium FLOR (acronym following Thiers 2024).

Table 1 Information about the populations collected for *Myrceugenia basicordata* and *M. joinvillensis* all localities are inserted in Santa Catarina State, Brazil

Species	N of populations	Population ID	N of individuals sampled	Voucher	Locality and municipality
<i>M. basicordata</i>	4	FIN	8	Augustin 36	Morro do Finder, Joinville
		QUI	13	Augustin 38	Picadão do Quiriri, Garuva
		CG	15	Augustin 47	Morro Santa Galo, São Francisco do Sul
		CUB	14	Vieira 3465	Morro Cubatão, Joinville
<i>M. joinvillensis</i>	4	BAR	18	Lima 565	Barragem do 8º Salto, Schroeder
		JUR	14	Augustin 41	Morro Jurapê, Joinville
		MC	10	Vieira 3473	Monte Crista, Garuva
		MUT	10	Vieira 3466	Morro Mutucas, Joinville

Population identification: FIN=Finder, QUI=Quiriri, CG=Canta Galo, CUB=Cubatão, BAR=Barragem, JUR=Jurapê, MC=Monte Crista and MUT=Mutucas

DNA extraction, high-throughput sequencing and quality control

The extraction of genomic DNA was carried out using the CTAB protocol (Doyle and Doyle 1987). After extraction, DNA integrity was evaluated using gel electrophoresis; quantification was carried out using Nanodrop and Qubit. One sample from each species (*Augustin 47 and Lima 565*) was selected for high-throughput sequencing (HTS). Genomic libraries from each sample were prepared separately using Illumina DNA preparation kit and sequenced on Illumina NextSeq platform (2×100 bp; with an average coverage of 10 million clusters per sample). The construction of genomic libraries and all the sequencing procedures were carried out by NGS Genomic Solutions (Piracicaba, Brazil).

The quality of the raw HTS data (reads) was evaluated using FastQC v. 0.12.1 (Andrews 2010). All the reads had high quality and trimming process was performed by NGS Genomic Solutions. The assembly of contigs (*de novo* assembly) was conducted using the online platform Galaxy (<https://usegalaxy.org/>), through the SPAdes v. 3.15.3 tool, suitable for short reads (Bankevich et al. 2012). Reads and contigs were visualized in Geneious v. 9.0.5 (Kearse et al. 2012). Due to the high number of contigs generated, and to optimize the computational processing, we used a custom Python script to filter and select only those with more than 10,000 bp for the subsequent steps.

Development of SSRs markers and amplification

Microsatellite markers were developed using GMATA software v. 2.3 (Wang and Wang 2016). We searched and selected sequences with di-, tri- and tetranucleotides motifs with at least five repetitions, and sequences with penta- and hexa-nucleotides with at least three repetitions. We only selected markers from different contigs and of nuclear origin (as inferred after a BLAST search against the NCBI (National Center for Biotechnology Information, [\[ast.ncbi.nlm.nih.gov/\]\(https://ast.ncbi.nlm.nih.gov/\), database\). Markers whose sequences were in organellar regions or had inconclusive results in BLAST were excluded \(Hagl et al. 2021\).](https://bl</p>
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The selected primers and markers were tested in two steps: first, we virtually validated the markers in GMATA (E-mapping function) and SPCR v. 3.0 (Cao et al. 2005). Twenty markers for each species showing good patterns of virtual amplification (reliable single fragment amplification) were selected for the second step, which consisted of laboratory tests and temperature optimizations. Twelve markers for each species showed good results in the second step and were selected for the genotyping process. The transferability of the final SSR markers (from *M. basicordata* to *M. joinvillensis* and vice-versa) was also virtually tested using SPCR software.

The primers were constructed using the M13 method, which consists in adding an oligonucleotide tail to the forward primer. This allows the annealing of a fluorochrome during the PCR reactions (Schuelke 2000), which facilitates fragment analysis after capillary electrophoresis. Amplifications were carried out in a final volume of 10 µL, with 6 µL of DreamTaq PCR Master Mix (2x) (Thermo Scientific), 0.5 µL of reverse primer (10µM), 0.5 µL of M13 tailed forward primer (10µM), 0.5 µL of labelled-M13 fluorescent dye (FAM or HEX), 0.5 µL of Bovine Serum Albumin (BSA) and 1 µL of pure water. The PCR were performed as follows: 94°C for 5', followed by 30 cycles of 94°C for 30'', specific annealing temperature for 45'' (see Table 2), 72°C for 45'', 8 cycles of 94°C for 30'', 53 °C for 45'', 72°C for 45'', and a final extension of 72°C for 10' (Schuelke 2000).

Fragment analysis and SSRs scoring

Fragment analysis was conducted using ABI 3500xL Genetic Analyzer (Applied Biosystems). We used one µL of non-diluted PCR product in a solution containing 8.75 µL of formamide and 0.25 µL of GeneScan™ 500 LIZ™ Size Standard (Applied Biosystems), in a final volume of 10 µL. Fragment sizing and scoring were performed in GeneMapper

Table 2 Information on the SSR markers used to assess genetic composition in *Myrceugenia basicordata* (MB) and *M. joinvillensis* (MJ)

Locus	Label	Primer's sequence (5'-3')	Allele size range (bp)	Motif	Ta (°C)
MB22	FAM	F: TGGTTCATGGGCAATACTCC R: ACGCTGCATAACGGTCTCTT	390–396	(CT) ₁₁	55
MB45*	HEX	F: GGTGGCAAACCTCGTGATCT R: AGCTTCCTCAAGCACAAGG	217–234	(TTTA) ₅	60
MB64	HEX	F: CGTAGCCTTCCGAGAAGATG R: GAGCGAACTTTGGTTGGAG	393–402	(AGG) ₅	60
MB92	FAM	F: AAAATCCCATGGTTCCACAA R: CGTCCAACCTCTTCATGTTC	387–394	(TC) ₅	56.8
MB116	FAM	F: AGTCGCCCAATGAAGAGAGA R: CATCAAAAGCACAGGCAGAA	352–354	(CT) ₅	56.8
MB118	FAM	F: TTGTTTTCCCTGGTGTAGC R: TCGAGGCGAAGAAGAAATGT	341–344	(TGA) ₅	55
MB153	FAM	F: CGAGATCAATCCCGATCACT R: GCTTCATCCCAAGTCCTGAA	405–407	(CT) ₅	56.8
MJ18	FAM	F: ATGTGTGGCGACCAACTGTA R: TTCATGACGGTGAACCTCAA	323–338	(ATT) ₅	60.6
MJ31	HEX	F: AATGGACAGTGGCAATGGTT R: TTGGTCGGAATTTTGAATC	352–364	(AGG) ₆	60.6
MJ38	FAM	F: TGGGTTAATTGGGTTTCGAC R: ATGGGCATTGGACGATTTA	269–274	(ACCCA) ₃	63.2
MJ56	HEX	F: TTGAACCCACCCAAATCTA R: GCTGAATCCCGTACTTGGAA	206–220	(TC) ₅	56.8
MJ61*	FAM	F: TTGGTTCAGCAAATCGAGTG R: CCTACATCACTCCCGGAAA	381–383	(AG) ₆	56.8
MJ71	FAM	F: TCCTTGTTGAGCTCCCATCT R: TCCCACGACAATCTTCTTCC	415–421	(CT) ₁₁	63.2
MJ103**	HEX	F: TGCATTGATCTCTTGCTTG R: CTTTCAAACGCATCGGTCTT	358–368	(GA) ₈	60.6

* For MB45 and MJ61, many individuals displayed doubtful heterozygous peaks and were considered as homozygous. ** MJ103 worked for two populations (MUT and MC), partially worked for one population (BAR) and did not work for the JUR population. Ta indicates the annealing temperature used during PCR

Software 6 (Applied Biosystems). We excluded five of the markers for each species during the genotyping and scoring processes because they either appeared as monomorphic, failed for most of the individuals, or their associated electropherograms were difficult to interpret and score. We used manual bin sets for SSR scoring. Finally, fourteen different polymorphic SSR markers were chosen to evaluate genetic diversity and differentiation in *M. basicordata* and *M. joinvillensis* (Table 2), seven for each species.

All the electropherograms were checked by two co-authors, to agree on the allele calling performed by the GeneMapper software. In some cases, allele sizes did not reflect the expectation based on the microsatellite motif. We handled this using two conservative approaches to avoid overestimating genetic diversity: when we observed such mutant allele sizes in more than one individual, we decided to keep it as a true allele; however, when we observed mutant allele sizes inconsistently, we attributed them to stuttering and therefore we approximated the size to the closest allele size.

In addition, we used the Micro-Checker software v. 2.2.3 (Van Oosterhout et al. 2004) to search for scoring errors and

null alleles and performed the analysis on the final set of markers for all populations.

Population genetic analyses

We estimated allele frequencies (N_a), observed and expected heterozygosity (H_o and uH_E), and private alleles using the GenAlEx V6.51b2 plugin (Peakall and Smouse 2006). We evaluated whether populations were deviating from the Hardy-Weinberg proportions using the “HW exact tests”, checking specifically for heterozygote deficit and excess in the GenePop (v. 4.8.3 Rousset 2008) software. Additionally, we carried out tests for genotypic linkage disequilibrium (all default parameters for each locus pair across all populations), to test the null hypothesis of independence between loci. A significance level of $\alpha=0.05$ was considered and adjusted using the Holm-Bonferroni correction for multiple comparisons (Holm 1979). The inbreeding coefficient (F_{IS}) was calculated using the Rho(is) approach, which takes into account allele sizes rather than allele identity (Weir and Cockerham 1984) in the GenePop software. Allelic richness

(A_r) was calculated in FSTAT software v. 2.9.3.2, considering a minimum sample size of four diploid individuals (Goudet 2001).

Genetic structure was investigated in the Structure software v. 2.3.4 (Pritchard et al. 2000) which implements a Bayesian algorithm, using the Admixture model without sampling sites as a prior assumption. We tested K values (number of genetic clusters) ranging from 1 to 7, with 500,000 burn-in replicates, 500,000 MCMC replicates, and 25 iterations. To detect the optimal K value that best represented our dataset, we used the online tool Structure Selector (Li and Liu 2018) and summarized the results through CLUMPAK (Kopelman et al. 2015). For optimal K value detection in Structure Selector, we tested two different methods: Puechmaile method (Puechmaile 2016) with a threshold of 0.5, and Evanno method, including ΔK and MeanLnP(K) (Evanno et al. 2005). Additionally, we performed a Discriminant Analysis of Principal Components–DAPC (Jombart et al. 2010) in R environment v. 4.4.1 (R Core Team 2024), using the *adeigenet* package v. 2.1.10 (Jombart et al. 2008). As the Structure approach assumes panmictic populations (Pritchard et al. 2000), the DAPC analysis is an alternative method to assess the clustering of all individuals without the assumption of panmixia.

In Arlequin v. 3.5.2 (Excoffier and Lischer 2010), we performed a hierarchical Analyses of Molecular Variance (AMOVA) to evaluate genetic structure within and among populations (Excoffier et al. 1992). All the populations were combined into one group. Additionally, we performed population comparisons by computing pairwise F_{ST} for short genetic distances. For both analyses, we computed the distance matrix and set the sum of squared size difference (R_{ST}) as the distance method, with 1,000 permutations each analysis (Gargiulo et al. 2021). To construct the pairwise F_{ST} matrix, we used Arlequin xml outputs in R environment v. 4.4.1 (R Core Team 2024).

We estimated the effective population size (N_e) using NeEstimator, v. 2.1 (Do et al. 2014) with the linkage disequilibrium method (LD), assuming random mating (Hill 1981; Waples and Do 2008) and isolated populations. Runs were performed without frequency restriction, excluding singletons. Jackknife confidence intervals were estimated (Jones et al. 2016). Although our small sample sizes might limit the accuracy of our N_e estimation, we proceeded with the analysis, as even approximate estimates can provide valuable insights for conservation decision-making (England et al. 2006; Garner et al. 2020).

To detect evidence of historical reductions in population size, we used Bottleneck v. 1.2.02 (Piry et al. 1999). We selected all three mutation models implemented in the software: Infinite Allele Model (IAM), Stepwise Mutation Model (SMM), and Two-Phase Model (TPM). The last

two, especially TPM, are considered more appropriate for microsatellite data (Piry et al. 1999). For TPM, we used the default parameters defined by the software, which consists of a variance of 30, a proportion of SMM in TPM of 70%, and 1,000 iterations. We selected all tests available in the software, which assumes mutation-drift equilibrium (Piry et al. 1999): sign test, standardized differences test, and Wilcoxon sign-rank test. We also verified the occurrence of a shift in the allele frequencies with the qualitative test of mode-shift (Gargiulo et al. 2021).

IUCN red list assessments

We conducted an assessment for both species using the IUCN Red List guidelines (IUCN 2024), based on: (1) geographical information, through Extent of Occurrence (EOO) and Area of Occupancy (AOO) evaluations, performed using the online tool Geospatial Conservation Assessment Tool–GeoCAT (Bachman and Moat 2012); (2) the population analyses presented here; and (3) all current known information about the species, their ecology and projections in future scenarios (Augustin et al. in prep.). For EOO and AOO calculations, we included all points available on online platforms as described above, excluding points located in the centers of cities, and duplicates from the same collector number.

Results

Sequencing resulted in 11,026,738 reads for *M. basicordata* and 15,862,298 for *M. joinvillensis*, in a length average of 100 base pairs. The guanine and cytosine (GC) percentages were 42% and 43%, and the number of contigs generated was 234,722 and 249,015, respectively. To reduce computer processing, we only selected contigs with at least 10,000 base pairs, resulting in 80 and 87 contigs for each species, respectively.

In silico tests for marker transferability suggested that MJ61 and MJ71 (*M. joinvillensis* markers) could be further investigated for *M. basicordata*, as they showed amplification with the expected fragment size. Other markers also showed amplification signals in the virtual tests (MB45 and MB118 for *M. joinvillensis*, and MJ31, MJ38, and MJ56 for *M. basicordata*), although with unexpected fragment sizes.

Genetic diversity and differentiation

We found 24 alleles across all populations of *M. basicordata*. In the Micro-Checker analyses, we did not find evidence of scoring errors due to stuttering, large allele dropout, and null alleles. The number of different alleles per locus

ranged from two to six. After Holm-Bonferroni corrections, the null hypothesis for genotypic linkage disequilibrium test was accepted, showing that all loci could be considered as independent. Three out of the seven loci showed significant values for heterozygote excess: MB118 (pop C: $p=0.0004$, Q: $p=0.0057$ and CG: $p=0.0033$), MB116 (CG $p=0.0013$) and MB92 (CG $p=0.0096$). Canta Galo (CG) exhibited a heterozygote excess at all three loci (MB118, MB116, and MB91). Only for the FIN population there was no evidence of heterozygote excess. For the heterozygote deficit test, no deviations from Hardy-Weinberg proportions were found.

Observed heterozygosity (H_o) ranged from 0.512 (QUI) to 0.661 (CG), while unbiased expected heterozygosity (uH_e) ranged from 0.283 (FIN) to 0.498 (CUB). Allelic richness (A_r) values followed the same pattern of uH_e and N_a , where FIN had the lowest value (1.62) and CUB had the highest value (2.33). The inbreeding coefficient (F_{IS}) was negative in three populations (FIN, CG, and QUI), and only CUB showed a positive value (0.10). In some cases, F_{IS} values were based on a subset of loci, as the software reported “no information” for loci that were completely homozygous: F_{IS} values in FIN were based on four loci, while in CUB, QUI, and CG values were based on six loci. Private alleles were detected in all populations, except in FIN, with CUB being the population with the highest number of private alleles ($n=3$) (Table 3).

The most likely K value for *M. basicordata* changed depending on the method used. The Puechmaille method gave $K=3$ as the most likely value. However, results from the Evanno method were more ambiguous (Supplementary Information 1). ΔK was associated with $K=2$, while pre-plateau value in MeanLnP(K) was $K=3$. In this case, we decided to keep $K=3$ as the final K value (Fig. 1a), based on Puechmaille results, and the MeanLnP(K) results. The three clusters correspond to (1) FIN, (2) CG and (3) QUI+CUB (Fig. 1b), but some individuals showed some level of admixture.

The DAPC analysis showed low differentiation among the populations of *M. basicordata* (Fig. 2a). For the final

results, 15 PCs were retained, and three clusters were considered after performing cross-validation procedures (Supplementary Information 2). Individuals from FIN and CG appeared as genetically very close. Individuals from CUB formed a different cluster and appeared in a sparse cloud. Likewise, individuals from QUI appeared as the most genetically differentiated when compared with those from other populations.

The AMOVA analysis suggested that most of the variation ($\sim 80\%$) is distributed within populations, and only 20% among populations ($F_{ST} = 0.206$, $p=0.000$). For this analysis, five loci were used by Arlequin and two were discarded because of missing data. The pairwise F_{ST} comparisons (Fig. 3a) indicated moderate differentiation, with the highest differentiation occurring between FIN and QUI (0.24), followed by CG and QUI (0.23).

The estimated effective population size (N_e) in *Myrceugenia basicordata* ranged from 6.9 individuals in CUB (95% CI: 1.1 - infinite) to 11.6 (95% CI: 0.6 - infinite) in CG. The point estimate for the FIN population and the confidence intervals for all the populations had infinite upper value suggesting that the small sample sizes do not allow enough resolution to estimate N_e reliably.

The Bottleneck results suggested that, among all four populations of *Myrceugenia basicordata*, CUB showed evidence of historical reduction in population size. For all the statistical tests and mutation models performed, CUB showed p -value < 0.05 (except for SMM model in sign test). CG and FIN also presented some evidence for a recent bottleneck, with p -values < 0.05 in at least two of the three statistical tests, under at least two of the three mutation models tested. QUI presented p -values > 0.05 for all statistical tests performed. All populations presented evidence of a shift in allele frequencies (Supplementary Information 3).

In *M. joinvillensis*, we found 31 alleles in total. As for *M. basicordata*, the Micro-Checker analysis did not reveal evidence of scoring errors due to stuttering, large allele dropout, and null alleles. The number of alleles per locus ranged from three to eight in all 52 samples. After Holm-Bonferroni

Table 3 Genetic diversity indices for populations of *M. basicordata* and *M. joinvillensis*

Indices	Myrceugenia basicordata				Myrceugenia joinvillensis			
	FIN	CG	CUB	QUI	BAR	JUR	MC	MUT
A_r	1.62	1.84	2.33	2.32	1.93	2.23	2.43	2.73
N_a (SE)	1.714 (0.286)	2.000 (0.218)	2.571 (0.369)	2.140 (0.286)	2.266 (0.360)	2.143 (0.404)	2.714 (0.184)	3.143 (0.340)
H_o (SE)	0.625 (0.183)	0.661 (0.158)	0.646 (0.135)	0.518 (0.120)	0.656 (0.161)	0.335 (0.088)	0.439 (0.046)	0.457 (0.091)
uH_e (SE)	0.283 (0.105)	0.395 (0.087)	0.498 (0.090)	0.465 (0.088)	0.399 (0.089)	0.370 (0.080)	0.424 (0.044)	0.478 (0.048)
F_{IS}	-0.929	-0.631	0.102	-0.152	-0.812	0.186	-0.204	-0.432
NPA	-	1	3	1	2	-	4	6
N_e (CI)	Infinite (0.1 - Infinite)	11.6 (0.6 - Infinite)	6.9 (1.1 - Infinite)	8.2 (0.9 - Infinite)	Infinite (0.4 - Infinite)	21.8 (2.2 - Infinite)	0.8 (0.2–87.1)	2 (0. - Infinite)

SE=Standard errors; A_r = Allelic richness (minimum sample size 4 diploid individuals); N_a =Average number of different alleles per locus; H_o = Observed heterozygosity; uH_e = Unbiased expected heterozygosity; F_{IS} = Inbreeding coefficient; NPA=Private alleles; N_e = Effective population size. CI=Confidence intervals

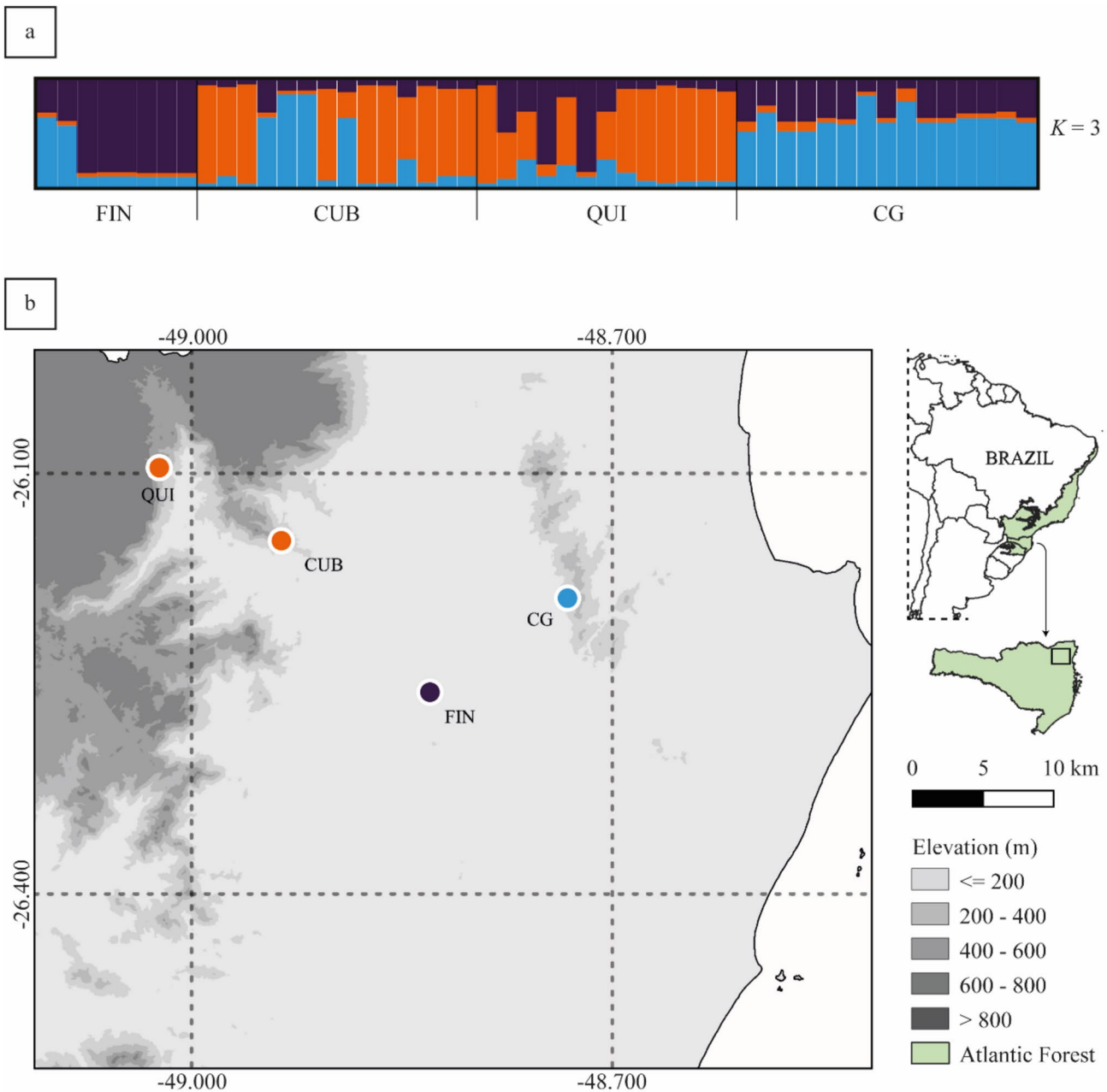


Fig. 1 (a) Bar plots from Structure, showing the best K value for *Myrceugenia basicordata* ($K=3$). (b) Geographic distribution of all populations of *M. basicordata*, Santa Catarina state, South Brazil. Colors refer to the main genetic clusters in the bar plots

corrections, the tests for genotypic linkage disequilibrium were not significant, confirming the independence between all pairs of loci. Hardy-Weinberg exact tests for heterozygote deficit suggested significant values for one locus in JUR, MJ56 ($p=0.0010$). MC population had $p=1$ for all loci, except for MJ56 ($p=0.0195$). Heterozygote excess test suggested significant values for MJ38 ($p=0.0000$) and MJ71 ($p=0.0001$) in the BAR population.

Observed heterozygosity (H_O) ranged from 0.335 (JUR) to 0.656 (BAR) and uH_E ranged from 0.370 (JUR) to 0.478 (MUT). Allele richness ranged from 1.93 (BAR) to 2.73 (MUT). The estimated values of F_{IS} were negative in all populations, except in JUR. In BAR and JUR, F_{IS} values were based on six loci, as the software reported “no information” for completely homozygous loci. Additionally, private alleles were detected in all populations, except in JUR.

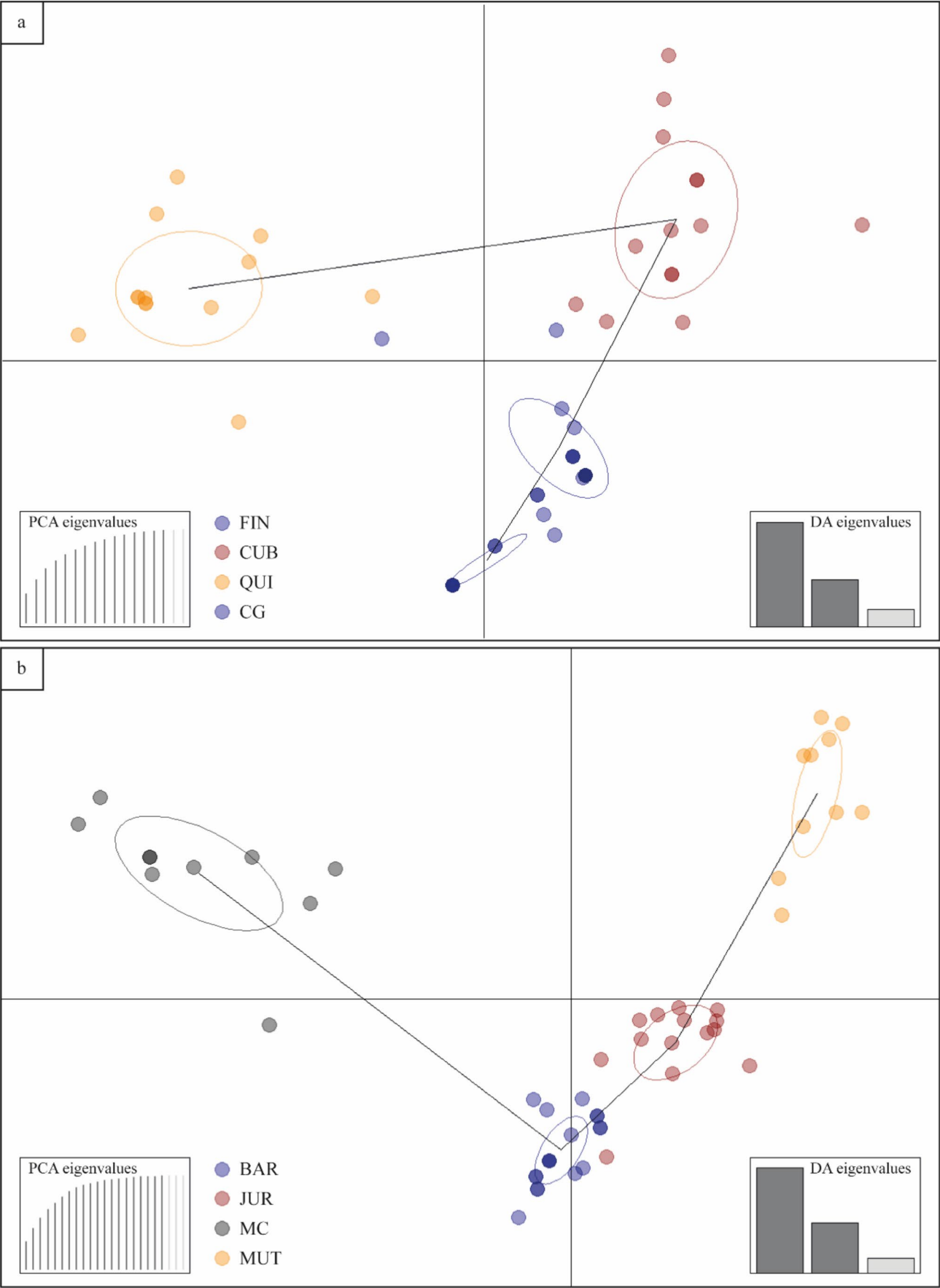


Fig. 2 Discriminant Analysis of Principal Components (DAPC). **(a)** *Myrceugenia basicordata* populations, considering three genetic clusters. Black line linking populations indicates a minimum spanning tree based on their squared distance within the entire space. **(b)** *Myrceugenia joinvillensis* populations, considering four genetic clusters. Black line linking the populations indicates a minimum spanning tree based on their squared distance within the entire space

MUT presented the highest number of private alleles ($n=6$), followed by MC (4) and BAR (2) (Table 3).

After the Structure analysis, the two best K -value in *M. joinvillensis* according to the Puechmaille and Evanno method were $K=4$ and $K=2$, respectively (for both ΔK and MeanLnP(K), see Supplementary Information 4). When considering $K=2$, the groups were composed of (1) BAR+MC and (2) JUR+MUT. However, when considering $K=4$, the groups corresponded to the four populations: (1) BAR, (2) JUR, (3) MC and (4) MUT (Fig. 4a and b). Based on the Puechmaille method, we considered $K=4$ as the most likely number of genetic clusters.

Retaining 20 PCs and four clusters (Supplementary Information 5), DAPC analysis suggested a clear differentiation among *M. joinvillensis* populations (Fig. 2b). Individuals from BAR, JUR and MUT were more closely related compared with MC, which formed an isolated cluster. Additionally, although BAR is the population in which we observed and sampled more individuals (18), it is also the population with the highest number of genetically similar individuals (Fig. 2b).

The hierarchical AMOVA for *M. joinvillensis* suggested that around 81% of the variation is within populations, and approximately 19% is among all four populations ($F_{ST} = 0.193$, $p=0$). For this analysis, Arlequin used two loci over the seven, after excluding those with missing data. The pairwise F_{ST} comparisons (Fig. 3B) indicated that the highest differentiation occurs among BAR and MUT individuals (0.399), followed by MUT and JUR (0.212), corroborating the DAPC results.

The estimated effective population size (N_e) in *M. joinvillensis* ranged from 0.8 (CI 95% 0.2–87.1) in MC to 21.8 in JUR (CI 95% 2.2 - Infinite) and was infinite in BAR. All populations, except MC, presented an infinite upper confidence interval, indicating that our small sample sizes do not allow a reliable N_e estimation.

The Bottleneck tests, in general, did not indicate a clear recent reduction in populations of *M. joinvillensis* (Supplementary Information 6). Significant values were found in: BAR when considering the IAM model in both standardized differences test and Wilcoxon test; JUR, when considering the IAM mutation model for Wilcoxon test, and MUT, when considering the SMM in the Standardized differences test. MC did not exhibit significant p-values in any of the performed tests. For the mode-shift analysis, BAR and MC

showed shifts in allele frequencies, while JUR and MUT were associated with a normal L-shaped distribution.

IUCN red list assessments

We compared the data on *M. basicordata* and *M. joinvillensis* with all categories and criteria from the IUCN Red List guidelines. For geographic range calculations, *M. basicordata* presented an EOO of 166.96 km² and an AOO of 20 km². Similarly, *M. joinvillensis* presented an EOO of 72.95 km² and an AOO of 20 km². We incorporated some of the genetic results presented here in the C criterion. As a result, both species fit in the threatened category of “endangered”, for criteria B (geographic range), C (small population size and decline—only for *M. basicordata*; based on the bottleneck analyses) and E (quantitative analysis, such as modeling approaches). The complete assessment report is in preparation to be submitted to the IUCN Red List (<https://www.iucnredlist.org/>).

Discussion

Genetic diversity and differentiation

We aimed to assess the Essential Biodiversity Variables (EBVs) for genetic composition for two rare and narrow endemic species of *Myrceugenia* (Myrtaceae) and to discuss these parameters in the context of conservation strategies. Our findings showed moderate levels of genetic diversity and differentiation for both species, especially considering the context of rare, threatened, and endemic species (Gargiulo et al. 2019; López-Pujol et al. 2013; Martínez-León et al. 2022; Turchetto et al. 2016).

Myrceugenia basicordata showed the lowest genetic diversity between the two species. Among the four populations of *M. basicordata*, FIN showed the lowest values for allelic richness (A_r), number of different alleles per locus (N_a), and expected heterozygosity (uH_E). Additionally, FIN is the population with the smallest observed number of individuals, where only eight samples were collected from an urban park in Joinville (the largest city in the Santa Catarina state, according to IBGE 2024). Despite being a relatively protected green area, this urban park shows clear signs of human activity, such as ecotourism and leisure activities that might have led to the decline of the population.

Finder (FIN) also presented a strongly negative inbreeding coefficient (F_{IS}), suggesting heterozygote excess (-0.93). We interpreted this result as artefactual, likely due to the reduced number of loci used in the analysis (4 out of 7 loci) and the extremely small sample size at this site (8 individuals). However, nearly all the populations of *M. basicordata*

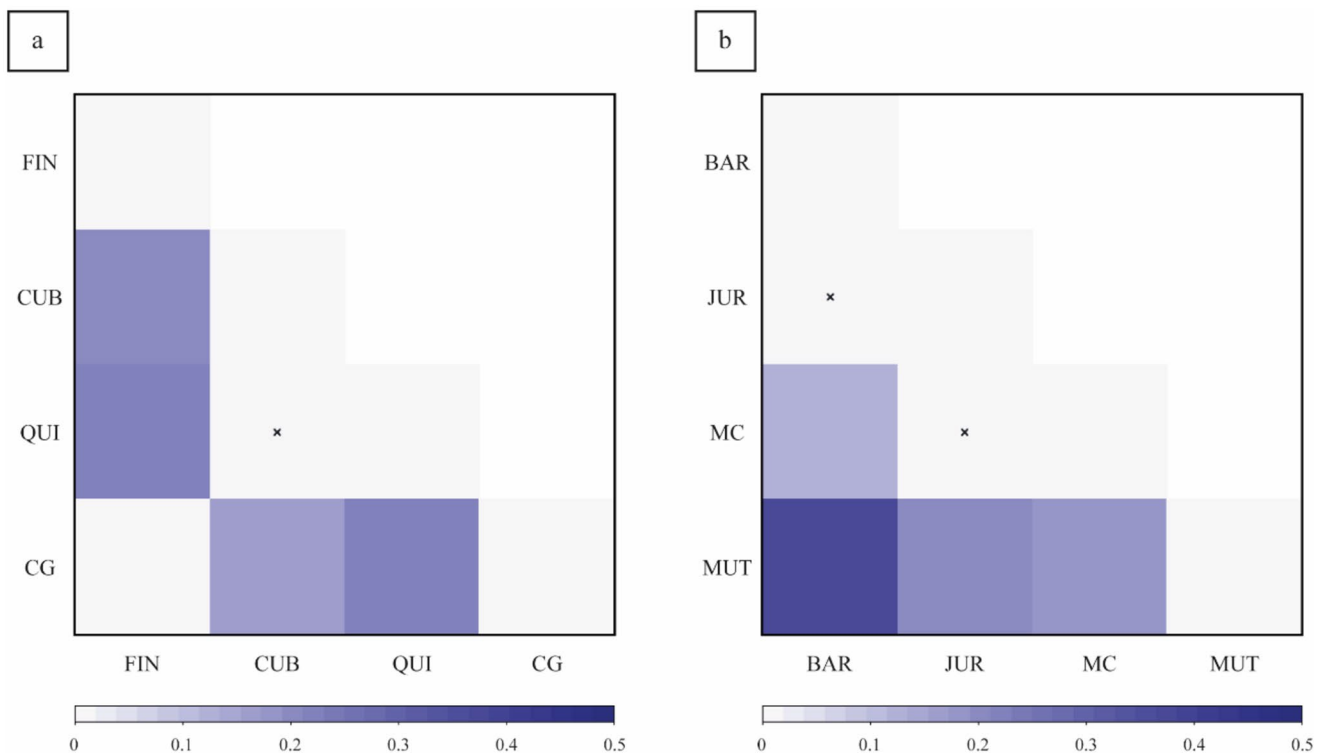


Fig. 3 Pairwise F_{ST} values in (a) *Myrceugenia basicordata* results, and (b) *Myrceugenia joinvillensis*. Population pairs marked with “x” indicate non-significant p values ($p > 0.05$)

showed slightly negative F_{IS} values, which we considered normal given the species’ outcrossing system, although the opposite is typically expected for small population sizes (Allendorf et al. 2013; Ellstrand and Elam 1993). CUB was the only population to show a positive F_{IS} value (0.10), and had the highest number of private alleles (NPA=3), providing evidence of isolation and possibly high relatedness among individuals (Allendorf et al. 2013).

Our genetic diversity results for *Myrceugenia basicordata* can be more closely compared to another rare *Myrceugenia* species (*M. correifolia*) in Chile, where the genus is also diversified (Pérez et al. 2017). Using eight SSR markers and 124 sampled individuals from 16 sites, the authors found a higher mean number of alleles/locus (N_a) and similar levels of uH_E for *M. correifolia*. *Myrceugenia basicordata*, in contrast, presented higher H_O and negative F_{IS} values, suggesting a stronger tendency toward outbreeding than *M. correifolia*. Contrary to *M. basicordata*, whose distribution is more fragmented in the Atlantic Forest, *M. correifolia* occupies deep canyons in Mediterranean regions, forming large populations, which likely contributes to its higher allelic richness.

In terms of genetic structure, *M. basicordata* populations did not show clear differentiation according to the Structure and DAPC results. Both analyses indicated three clusters, with a slight difference in the groups: DAPC indicated

one group formed by the FIN and CG populations, another group only including CUB, and the last group only including QUI. The Structure analysis clustered FIN and CG in a separate group, but both analyses suggested admixture within clusters, which is also supported by the pairwise F_{ST} results. FIN and CG appeared to be more closely related to each other compared to the other populations. Additionally, QUI and CUB also appeared closely related, particularly according to the Structure analysis. It is important to note that QUI population is the most geographically distant, and its samples were collected over a wider area.

We observed that genetic diversity increases as *M. basicordata* populations move further away from the central urban region. FIN and CG (lower genetic diversity) are located near Joinville, whereas CUB and QUI (higher genetic diversity) are located in a wider area. We attribute this pattern to two main dependent factors: first, a possible reduction in pollinators (mainly bees in the case of Myrtaceae, as noted by Fidalgo and Kleinert 2009) within large urban centers, potentially caused by pollution, pesticides, and/or urban intensification, which has already been demonstrated in empirical studies (e.g., Baldock 2020). Second, populations with higher genetic diversity are located in protected habitats, in green areas at a certain distance from cities with lower human interference.

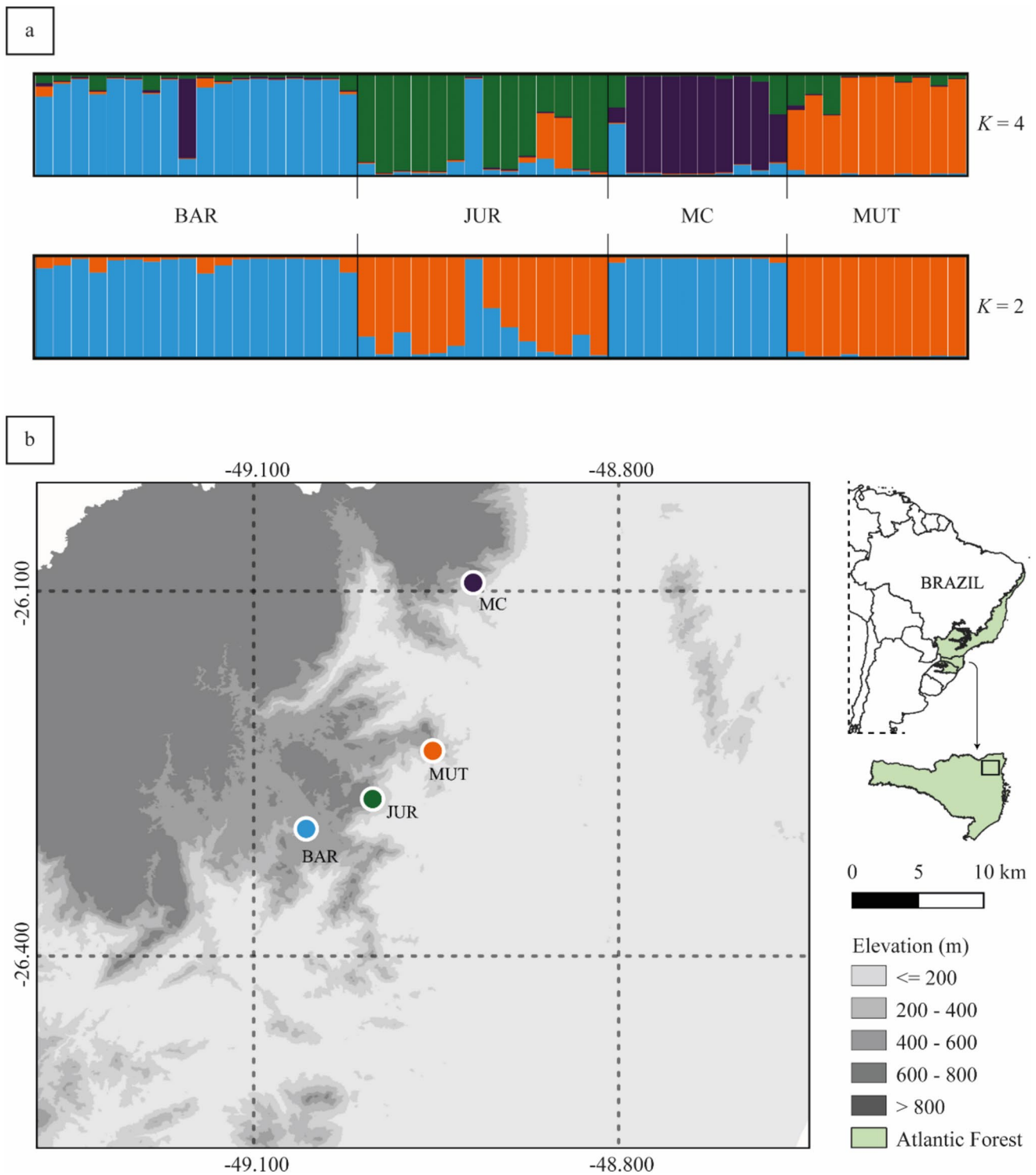


Fig. 4 (a) Bar plots from the Structure, showing the best K values for *M. joinvillensis* with $K=4$ and $K=2$, for comparison. (b) Geographic distribution of all populations of *M. joinvillensis*, Santa Catarina state,

South Brazil. Colors refer to the main genetic clusters in the bar plots, considering $K=4$

Although population declines have not been documented in previous studies, our bottleneck simulations suggested a historical reduction in population size in nearly all *M. basicordata* populations (except QUI), and particularly in CUB. Therefore, we strongly recommend continuous monitoring of these populations to detect any disturbance factors and prevent further declines. For *M. joinvillensis*, we did not find strong evidence of a bottleneck effect, which suggests that populations might be naturally small.

Myrceugenia joinvillensis presented higher values of genetic diversity, except for the BAR and JUR populations, which showed lower values for allelic richness (A_r), number of different alleles per locus (N_a), and expected heterozygosity (uH_E), despite larger observed population size. Jurapê is located within a protected area, while BAR is situated in a dam area, on a private property owned by an electricity company, likely subjected to frequent disturbance. In BAR, we observed that individuals were relatively closer to each other compared to other sites, all of them seemed to be mature as presented reproductive structures, and were found next to a flooded area.

Jurapê was the only population with a positive F_{IS} value (0.186), indicating some level of inbreeding among its individuals. All other *M. joinvillensis* populations showed slightly negative F_{IS} values, consistent with the outcrossing reproductive strategy (Allendorf et al. 2013). However, BAR presented a relatively strong negative value (-0.812), which could be explained by reproductive dynamics, such as mating between different genotypes due to recent gene flow from neighboring populations (Allendorf et al. 2013). However, an artefactual excess of heterozygotes cannot be excluded, as only two of the seven loci (MJ38 and MJ71) showed significant heterozygote excess after the Hardy-Weinberg exact tests.

The analysis of genetic differentiation in *M. joinvillensis* indicated four discrete and distinguishable clusters, according to both the Structure and DAPC results, with minor levels of admixture. BAR and JUR are more closely related, as further supported by the pairwise F_{ST} results. Although MC is the most geographically distant population, MUT is the most genetically different according to the F_{ST} and DAPC results, and also showed some degree of isolation, with the highest number of private alleles ($NPA=6$).

Considering the geographical distribution of the populations and the results obtained, genetic diversity in *M. joinvillensis* increases alongside the linear distribution range of the species (BAR to MC populations, especially in MUT), with populations at higher altitudes (around 600 m) with higher genetic diversity. The observed genetic differentiation could be explained by the lower abundance of biotic pollinators at higher altitudes (Brito et al. 2016). In contrast, populations of *M. basicordata* located far from urban

centers present higher genetic diversity values (FIN and CG to CUB and QUI, Fig. 1b), although genetic differentiation is less evident.

During the development of this study, we identified several caveats. First, we recognize the relatively small number of loci used as SSR markers, which may have led to limited resolution in our results. Second, some loci exhibited a significant percentage of missing data, preventing their inclusion in all the proposed analyses. Despite the challenges, our results have important implications for the conservation of *M. basicordata* and *M. joinvillensis* and allow us to discuss potential conservation strategies for the two species and for species with similar life-history traits and distribution.

Our results point out the need for clarifying genetic diversity in other rare Myrtaceae species occurring in the Brazilian Atlantic Forest with narrow distributions. Numerous studies focused on common Myrtaceae species, often linked to economic interests, such as *Eugenia* and *Campanesia* spp. (e.g., Moreira et al. 2022; Telles et al. 2013; Turchetto-Zolet et al. 2016), or on important widespread species for habitat management, such as *Myrcia* and *Psidium* spp. (e.g., De Oliveira Bernardes et al. 2022; Brandão et al. 2015; Franceschinelli et al. 2007). However, we found only one study concerning narrow endemic or threatened Myrtaceae species in the Atlantic Forest, focusing on *Curitiba prismatica* (D.Legrand) Salywon & Landrum, a vulnerable species from South Brazil (Flora e Funga do Brasil 2024; Silva et al. 2022). There are no studies on *Myrceugenia* for comparison, and this highlights a significant gap in the knowledge regarding threatened biodiversity, especially in contrast to other biodiversity centers of Myrtaceae distribution (see examples in Simmons et al. 2023; Rutherford et al. 2019; Rutherford et al. 2022 for Australia and Pérez et al. 2017 for Chile).

Implications for the conservation of endangered species

This study represents the first conservation genetic effort focused on species of *Myrceugenia* from the Atlantic Forest, marking an important initial step towards understanding these and other rare species from this hotspot of biodiversity. Both *M. basicordata* and *M. joinvillensis* require conservation efforts to avoid future declines in population size and species ranges. These species are threatened by habitat loss or fragmentation caused by urban expansion, as they are not currently protected for their economic interest, or cultivated as ornamental plants. The state of Santa Catarina experienced an estimated population growth of 5.89% in 2023, making it the eighth state with the highest population increase in Brazil (IBGE 2024). Additionally, climate change may also negatively impact the species' survival, as

it has been shown that rare and/or endemic species are more vulnerable than widespread species when their habitats are altered (Manes et al. 2021; Vincent et al. 2020). Furthermore, species distribution modeling approaches suggest a significant reduction in suitable habitats for these *Myrceugenia* species by 2100 (Augustin et al. in prep).

The species studied here are certainly not isolated cases within the Atlantic Forest domain. In fact, comprehensive conservation assessments for Atlantic Forest endemic trees revealed that 82% of them are already threatened to some degree (De Lima et al. 2024). Considering the gaps in knowledge about threatened Brazilian Myrtaceae, we strongly recommend future studies focused on the conservation of *Myrceugenia*. Based on the reduced number of records in online databases (such as Specieslink), we suggest that species such as *M. decussata* Mattos, *M. foveolata* (O.Berg) Sobral, *M. hatschbachii* Landrum and *M. mesomischia* (Burret) D.Legrand & Kausel should also be investigated for genetic composition.

Our findings also considered other biological and environmental aspects to evaluate the conservation status of *M. basicordata* and *M. joinvillensis*. The IUCN Red List assessments we carried out qualified both species as endangered (EN) according to the IUCN Red List criteria B, C and E. For *M. joinvillensis*, we also observed an extensive mining area nearby MUT, even if this population is located in a protected area (FCSV observation; Google 2024). The IUCN Red List is extremely important for raising awareness of threatened species worldwide, due to its accessibility (e.g., Omar and Elgamar 2021; Harfoot et al. 2021). For *Myrceugenia*, 22 species (47%) have already been evaluated and are currently included on IUCN Red List, most of them are classified as least concern (9), but some of them are classified as endangered (2), vulnerable (8) or as near threatened (3).

Local lists of threatened species with federal and regional scope are equally important and essential for immediate conservation policies. Ideally, global and regional red lists should be interconnected and constantly updated which, unfortunately, is not the case for many Brazilian species (Moraes et al. 2014; Morais et al. 2012). For the state of Santa Catarina, for example, an official list of threatened species was made available in 2014 (CONSEMA 2014), but it has not been updated since then. For Myrtaceae, for which four species are mentioned, there are also many inconsistencies, such as taxonomic problems and an evident underestimation of the number of threatened species (Caddah and Lima 2022; Drischel and Caddah, in prep.).

We used the above considerations to recommend some practical conservation actions for *Myrceugenia basicordata* and *M. joinvillensis*, to guide future conservation plans

for these species and possibly other species in the Atlantic Forest:

Practical recommendations for *M. basicordata* and *M. joinvillensis*

Our practical recommendations are divided into immediate actions, which should be considered for the next 1–2 years, and future actions, which should be considered for the next 3–10 years, and dependent on future studies clarifying the biology and the ecology of the two species.

Immediate actions

Continue monitoring populations and conduct further studies on the biology and ecology of the species

Monitoring populations of *M. basicordata* and *M. joinvillensis* is extremely important to evaluate whether or not a reduction in the number of individuals in situ is occurring. Although our analyses suggested a recent decrease in population sizes in *M. basicordata*, there are no available studies or historical documentation that can confirm population decline. Additionally, *Myrceugenia joinvillensis* populations are particularly difficult to access, at high altitudes on mountain peaks (Fig. 4b), often covered by dense montane rainforest vegetation. Therefore, future monitoring efforts could leverage modern technologies based on drones, sensors IoT (Internet of Things) and Radio Frequency Identification tags (RFID) for example (Kocer et al. 2021; Sujanani et al. 2016; Tavilla et al. 2024).

Involving local communities might also be a powerful strategy for monitoring populations. Some citizen science projects have already been conducted for other endangered species in Brazil (e.g., “Faveiro-de-Wilson case, Fernandes et al. 2022). The involvement of local communities might help increase the number of discovered individuals and, consequently, provide better estimates of population sizes to incorporate into effective conservation action plans for the species (Fernandes et al. 2022). This approach could also be a cheaper option for future monitoring after in situ conservation actions have been implemented.

Finally, we strongly recommend further investigations into basic biological processes in *Myrceugenia*, including seed germination (e.g., viability, storage behavior, and overall development), reproductive systems, types of pollinators, and phenology (e.g., Nadarajan et al. 2021; Van der Walt et al. 2021). Although the ecology of Myrtaceae species is well-studied, their physiology exhibits significant variability, with germination periods ranging from 10 days to over a year (Lughadha and Proença 1996). Experiments focusing on the genus *Myrceugenia* are extremely important.

Understanding their reproductive systems might enable the *ex-situ* growth of seedlings for future restoration strategies.

Include the species in official red lists

We recommend the inclusion of *M. basicordata* and *M. joinvillensis* in official red lists for the Atlantic Forest, such as the regional list (CONSEMA 2014) for the state of Santa Catarina, and the Brazilian national list (*Lista Oficial de Espécies da Flora Brasileira Ameaçadas de Extinção*, MMA 2022). In addition to disseminating knowledge about threatened flora and standardizing monitoring protocols, this will allow companies specialized in environmental restoration to encourage and prioritize the planting of seedlings of these species for restoration projects in suitable areas.

Future actions

Introduce new individuals in areas of high-suitability

The introduction of new individuals into suitable habitats has already been tested as a successful conservation strategy (e.g., Pavlik et al. 1993; Soorae 2021). Therefore, we recommend the introduction of new populations of *M. basicordata* and *M. joinvillensis* in highly suitable areas (for example, those suggested in Augustin et al. in prep), preferably protected areas of the Atlantic Forest. Seedlings should originate from *ex situ* efforts (e.g., after germination experiments) targeted at generating genetically diverse and resilient material for reintroduction (Di Sacco et al. 2021), possibly excluding germplasm from populations that have reduced genetic diversity and small populations (FIN and JUR, for example). Analogous strategies should be used to reinforce populations at known sites, by facilitating cross-pollination or introducing seedlings that have been generated using local genetic diversity.

Deposit in seed banks

Most species within Myrtaceae from the Atlantic Forest have recalcitrant seeds, which are not tolerant to desiccation (Mayrink et al. 2019), posing challenges to the standard seed preservation; however, we are not aware of studies focused on *Myrceugenia*. If further studies clarified the best strategies to preserve seed *ex-situ*, depositing viable seeds from *M. basicordata* and *M. joinvillensis* might also serve as an important strategy to safeguard the genetic material necessary for species recovery, habitat restoration, and biodiversity conservation in the face of climate change and habitat loss.

We recommend depositing viable seeds from all known populations but prioritizing those with higher genetic

diversity and, when possible, larger populations. For example, for *M. basicordata*, QUI and CUB populations should be considered as priority targets since they likely occupy a larger area and they display higher genetic diversity. For similar reasons, the populations MUT and MC of *M. joinvillensis* should be considered for seed collections. If new individuals/populations were to be discovered, further studies clarifying their population genetics would be advisable.

Ex situ cultivation

When feasible, cultivation in botanical gardens, public plant nurseries, degraded areas that are targets of restoration projects, and private orchards (which also may involve local communities) might serve as a supplementary strategy for *ex situ* conservation efforts, but also as a tool to observe the ecology of the species more closely.

Conclusion

Despite its rarity, *M. basicordata* and *M. joinvillensis* presented moderate levels of genetic diversity. *Myrceugenia basicordata* showed the lowest indices of genetic diversity, suggesting a historical reduction in population size, despite more evidence of gene flow among populations. *Myrceugenia joinvillensis*, by contrast, presented higher indices of genetic diversity, but more discrete genetic groups, suggesting more limited gene flow among populations. Habitat loss and fragmentation are the most pressing threats to these species, which need to be safeguarded through increased knowledge and monitoring and long-term conservation measures. Our study represents the first conservation effort focused on *Myrceugenia* species from the Atlantic Forest, marking an important initial step toward understanding these and other rare species from this biome.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10592-025-01703-4>.

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Author contributions MKC, DFL and RG conceived the idea; AFA, DLF, MKC and FCSV conducted fieldworks; AFA conducted labwork; AFA, RG and VMS performed analyses; AFA and RG wrote the first draft of the manuscript; all authors reviewed the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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