



# Microsatellites reveal high levels of genetic admixture in the natural populations of *Laurus azorica*, Lauraceae

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## Abstract

*Laurus* (Lauraceae) species are currently restricted to isolated refugia in the southern Black Sea, Mediterranean Basin, southern Morocco, and Macaronesian archipelagos. One to three species of *Laurus* has been recognized: the Azorean endemic *Laurus azorica*, *L. nobilis* from the Mediterranean, and *L. novocanariensis* from Madeira and the Canary Islands. This study aims to determine the population structure, genetic diversity, and associated patterns of gene flow within and between Azorean populations, using eight existing SSR markers. We also included plant material from *L. nobilis* populations found in the Azores, for comparison. Amplification was performed in 212 samples of *L. azorica* and in 30 samples of *L. nobilis*. For *L. azorica*, 78 alleles were amplified (average 9.75 alleles per loci). Bayesian analysis with STRUCTURE unveiled five genetic groups for the Azorean accessions, with high level of genetic admixture. Genetic diversity was generally high, with moderate levels of genetic differentiation among *L. azorica* populations. Moreover, high gene flow levels, likely mediated by past human translocations and naturally, by birds, might have contributed to the high level of genetic admixture observed possibly reflecting hybridization events between *L. nobilis* and *L. azorica*. Conservation measures should be applied to some populations of São Miguel, Santa Maria, and Flores Islands, based on the number of private alleles, and further, ad hoc translocation events should be avoided. Conservation in situ and the preservation of laurel forest remains are recommended. The study of this species' taxonomy, genetics, and population dynamics in the Macaronesian region should be continued.

**Keywords** Azores · Conservation strategies · Genetic diversity · *Laurus azorica* · Population structure · Private alleles

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## Introduction

The genus *Laurus* L. (Lauraceae) is native to Europe (Arroyo-García et al. 2001). In the mid-Tertiary, its distribution ranged from southern Europe to northern Africa, but, presently, it is restricted to isolated refugia in the southern Black Sea area, Mediterranean Basin, south of Morocco and in the Macaronesian archipelagos of the Azores, Madeira, and the Canary Islands (Rodríguez-Sánchez et al. 2009). *Laurus* was initially described by Linnaeus (von Linné 1800) and included *Laurus nobilis* L. Seubert (Seubert and Hochstetter 1844), initially described the Azorean specimens as *Persea azorica*, this taxon was later transferred by Franco (1960) to *Laurus azorica* (Seub.) Franco, and POWO (2023) currently lists six synonyms for the Azorean species, but at least one variety is not generally accepted. The Azores also host other Lauraceae genera, such as *Persea* and *Ocotea*, which were likely introduced (Góis-Marques et al. 2020).

In recent years, the delimitation of species within the Macaronesian *Laurus* has been the object of controversy. Rivas-Martínez et al. (2002) added a third species to this group, *Laurus novocanariensis* Rivas Mart., Lousã, Fern. Prieto, E.Dias, J.C.Costa & C.Aguiar, which is geographically restricted to the Canary Islands, Madeira and south of Morocco, circumscribing *Laurus azorica* as endemic to the Azores (Rivas-Martínez et al. 2002). Other authors continue to acknowledge the existence of only two species, *L. azorica* and *L. nobilis*, although this distinction is not supported by a recent molecular study (Rodríguez-Sánchez et al. 2009). Kondraskov et al. (2015) showed that island divergence during the Pleistocene resulted in low support distinctions between the three taxa, with *L. nobilis* from the Mediterranean being sister to Spanish *L. nobilis*, *L. azorica*, and *L. novocanariensis* (the latter two species being mostly separated, with a few individuals nested inside the other). Morphological traits have also proven to be questionable (Ferguson 1974; Rodríguez-Sánchez et al. 2009) and inconclusive (Arroyo-García et al. 2001), since they are mostly based on leaf shape and tomentum (Moura et al. 2015). In *L. nobilis*, leaves are mostly glabrous (Schaefer 2005; Arroyo-García et al. 2001), while in *L. azorica*, leaves are smoothly pubescent, wider, and densely brown tomentose in younger shoots (Arroyo-García et al. 2001), with an elliptic to obovate shape (Schaefer 2005).

Another differing character in *Laurus* s.l. is chromosomal numbers (*Laurus nobilis*:  $2n=48, 42$ ; *L. canariensis* Webb:  $2n=36$ ). Plants with  $2n=36$  and  $42$  may show irregular meiosis and may result in hybrids, between  $2x$  and  $4x$ , and between  $3x$  and  $4x$ , as reported by Battaglia (1947). The most frequently mentioned karyotype for the genus is tetraploid ( $n=4x=48$ ; Ehrendorfer et al. 1968; Arroyo et al. 2010). A potential threat to *L. azorica* is the possible occurrence of hybridization events with *L. nobilis*, as intermediate forms are sometimes found, although such events have never been confirmed (Moura et al. 2015; Matos et al. 2019).

Palaeobotanical studies suggest that *L. azorica* and *L. nobilis* both derive from the same common ancestor, which was widely distributed in Europe from the Miocene until the Pleistocene (Barbero et al. 1981; Arroyo-García et al. 2001). Moura et al. (2015), including samples of both *L. azorica* and *L. nobilis*, detected the presence of three different haplotypes for the Azores, using *trnK-matK* and *trnD-trnT*, also suggesting the existence of genetic diversity within and between Azorean islands, as well as some degree of genetic differentiation toward clear *L. nobilis* morphotypes, mainly distinguished by lanceolate leaves with undulated margins, as opposed to *L. azorica* specimens with oblong leaves, without a clear undulation.

Human activity has had profound effects on the local natural vegetation, especially in laurel forest (Elias et al.

2016). Although Rull et al. (2017) found evidence of human presence in São Miguel Island in the last quarter of the thirteenth century, the first records related to the archipelago's native vegetation were written by Gaspar Frutuoso (1522–1591), after the Portuguese colonization of the Azores, in the fifteenth century. Frutuoso (1589) refers that the landscape was dominated by forests, and the transformation of native ecosystems began with the discovery of the islands and the consequent human settlement (Elias et al. 2016). Frutuoso refers that not only *Laurus azorica*, but other endemic, such as the cedar (*Juniperus brevifolia*), or “sanguinho” (*Frangula azorica*), and native trees were present in the Azores Islands, which were extremely common and abundant (Frutuoso 1589).

Silva et al. (2009) estimated that the natural populations of *Laurus azorica* include more than 100,000 individuals, scattered across the archipelago. Submontane *Laurus* forests probably dominated the Azorean landscape when the first Portuguese settlers arrived on the islands (Elias et al. 2016). Presently, these forests occur quite sparsely, usually between 300 and 600 m a. s. l., and are most often invaded by *Pittosporum undulatum* Vent (Dias 1996; Elias et al. 2016; Pavão et al. 2019). In the Azores, *L. azorica* also occurs in several other habitats, including coastal scrublands, low-altitude forests, such as *Erica-Morella* forests and *Picconia-Morella* forests. Although not the dominant species, *L. azorica* is frequent at higher altitudes (600–900 m) in juniper forests (Silva et al. 2009; Elias et al. 2016; Pavão et al. 2019). This species can also be found scattered in ravines and on steep slopes, in lava flows, margins of cultivated land and on forested peat bogs (Schaefer 2005; Silva et al. 2009). Despite being found in all the Azores's islands, several populations of *L. azorica* have clearly declined. In fact, it is very rare in Graciosa and rare in Corvo and Santa Maria islands. These populational bottlenecks are most likely connected to past human alterations (Silva et al. 2011), due to the intensive land clearance and replacement of laurel forests by production forests and pastures. Presently, it is threatened due to the expansion of invasive alien trees such as *Pittosporum undulatum* (Hortal et al. 2010; Lourenço et al. 2011; Borges Silva et al. 2018; Dutra Silva et al. 2019; Liang et al. 2019).

*Laurus azorica* is dioecious and Forfang & Olesen (1998) found that the sex/ratio is male-biased. It has obligate outbreeding as breeding system, and the adult individuals can develop numerous secondary and tertiary stems from the primary trunk base (Forfang & Olesen 1998). This species vigorously regenerates through seed, and pollinating insects belong to *Hymenoptera*, *Diptera*, *Lepidoptera*, *Coleoptera*, and *Hemiptera* (Forfang & Olesen 1998). Flowering starts in November or December, lasting until April, with a fruiting period that extends through the summer and autumn (Schaefer 2005). *Laurus azorica* is also a micro- or

mesophanerophyte with endozoochorous dispersal. Among the native bird species, only the blackbird, *Turdus merula azorensis* Hartert, 1905 and the common wood pigeon, *Columba palumbus azorica* Hartert, 1905 are reported to eat large drupes and seed cones and perform long-distance dispersal (Guppy 1917; Dias et al. 2007; Rumeu et al. 2011), assisting in the dispersal of endemic plants such as *Laurus* (Guppy 1917).

The Mediterranean *L. nobilis* is known worldwide for its uses in culinary and health (Alejo-Armijo et al. 2017). The endemic *L. azorica* was valued by humans, since the oil from its berries was used in traditional medicine, as a disinfectant, and for illumination (Viveiros et al. 2022). However, the use of *L. azorica* as a condiment is not recommended since its leaves are toxic (Vinha et al. 2015).

So far, few studies addressed the population structure and genetic diversity of the Azorean *Laurus*. Among those is included a detailed phylogenetic study to attempt the detection of hybrids (Moura et al. 2015), and phylogenetic (Arroyo-García et al. 2001) and phylogeographical studies (Rodríguez-Sánchez et al. 2009), which included *L. azorica* and *L. nobilis* samples, but with very low coverage of the Azores archipelago, although useful SSR markers were developed by Arroyo et al. (2010). The use of such markers has been referred by Arroyo et al. (2010) as fully adequate for the characterization of local and regional-scale levels of genetic variation and studying patterns of pollen and self-mediated gene flow, representing, thus, a helpful tool to inform effective protection and management strategies for this emblematic and potentially threatened relict genus.

Here we aim to determine the genetic diversity of *L. azorica* and the genetic structure of its populations, using previously developed nuclear microsatellite markers (Arroyo et al. 2010). This information is fundamental for future conservation plans that may involve *Laurus*, which was considered as a species with conservation priority (Cardoso et al. 2008; Silva et al. 2009, 2015). Furthermore, the inclusion of *L. nobilis* samples may also contribute to further clarify eventual hybridization events between the two taxa by comparing the alleles and their frequencies in individuals of the two species that occur sympatrically in São Miguel Island and that probably occur in other islands of the archipelago. Finally, we hope to contribute to a better understanding of the global patterns of diversity and genetic structure of endemic plants in the Azores, complementing those already found for other endemic woody species such as *Juniperus brevifolia* (Silva et al. 2011), *Viburnum treleasei* Gand. (Adoxaceae) (Moura et al. 2013), *Prunus lusitanica* subsp. *azorica* (Mouill.) Franco (Moreira et al. 2013), and *Picconia azorica* (Tutin) Knobl. (Oleaceae) (Martins et al. 2013).

Our starting hypothesis is that there might be a high level of genetic diversity in *L. azorica* populations, although with

a relatively low level of genetic differentiation between populations, assuming that, for example, birds will be able to assist in seed dispersal, thus ensuring gene flow (Martins et al. 2013). However, due to the fragmentation of the populations, fluctuations in allele frequencies might have occurred due to genetic drift (Slatkin 1985).

## Methods

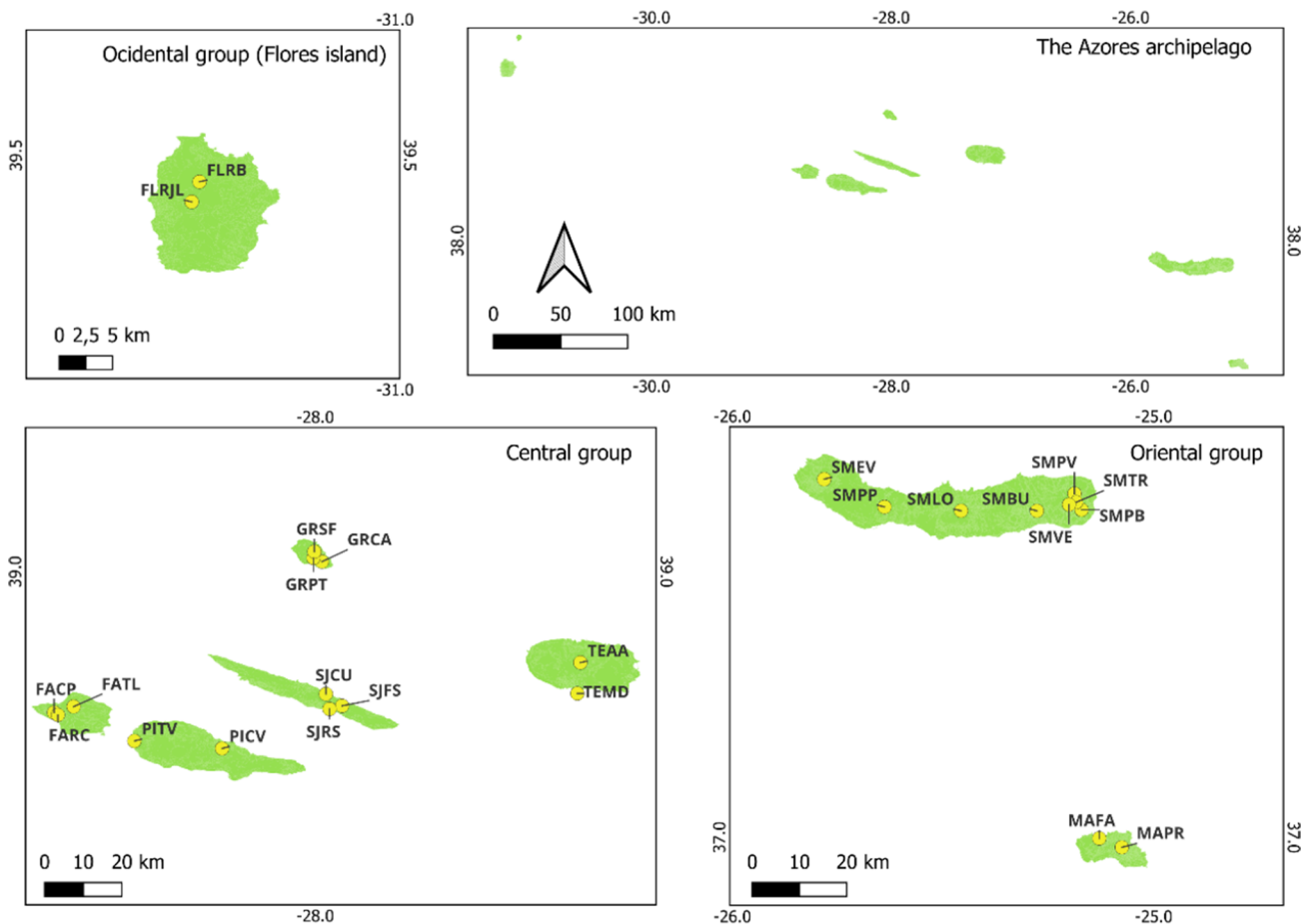
### Study site

The Azorean archipelago (36° 55'–39° 43' N, 25° 00'–31° 15' W) is situated in the north Atlantic Ocean and is composed of nine volcanic Islands divided into three groups: Western (Flores and Corvo), Central (Terceira, Graciosa, São Jorge, Pico, and Faial), and Eastern (São Miguel and Santa Maria). The islands are scattered over a distance of 615 km on a WNW–ESE alignment and cover a total of 2532 km<sup>2</sup>. The islands' topography is characterized by large hydrographic basins, ravines, and seasonal water streams, with maximum elevations ranging from 450 m in Graciosa to 2351 m in Pico with several islands peaking near 1000 m (Moreira et al. 2013). The geological age of the islands ranges from 0.27 to 4.1 Mya (Fig. 1; Ávila et al. 2016).

The Azorean vascular plant flora comprises about 1000 species (Silva et al. 2011). Of these, nearly 300 are considered native and approximately 80 of these endemic species (Silva et al. 2010).

### Plant material

Field trips were undertaken to all the archipelago islands, with the objective of collecting samples of *Laurus* from additional populations, complementing and enhancing the existing collections already databased and maintained at the AZB herbarium and at the DNA Bank Collection of the Azores University. Therefore, a total of 212 samples of *L. azorica* were collected from 24 populations of eight of the nine islands, with exception of Corvo Island. We also sampled 30 individuals of *L. nobilis* from four populations in three islands (São Miguel, Terceira, and Graciosa; Table 1). The morphological distinction between the two laurel species was made based on leaf shape, margin shape, and indumentum. Putative *L. azorica* specimens were considered as those with simple, oblong to elliptic shaped leaves, with entire margin and presence of brown indumentum, while putative *L. nobilis* were distinguished by elliptic to lanceolate leaves, margin undulated, and absence or indumentum. In some cases, samples had mixed characteristics, and the specimens were noted as possible hybrids ( $\pm$  indumentum, obovate to lanceolate leaves, and undulated leaf margin). At least one representative voucher specimen per population



**Fig. 1** The islands of the Azores archipelago and locations of the *Laurus* populations studied

was also collected and stored at the AZB herbarium (Fig. 2). Leaves were kept in silica gel vacuum-sealed bags until dry. The locations of the sampled populations were georeferenced in the field and mapped using Quantum GIS 3.28.2 (QGIS Development Team 2022).

### Extraction of DNA

DNA was extracted using dry leaf material and the CTAB extraction method (Doyle and Dickson 1987), which used 700  $\mu\text{l}$  of 3 X CTAB, 50  $\mu\text{l}$  of Sarcosyl, and 10  $\mu\text{l}$  of Proteinase K, for the initial step of lysis. A first washing step was performed using 500  $\mu\text{l}$  of SEVAG (chloroform-isoamyl alcohol, 24:1), followed by an additional wash with 500  $\mu\text{l}$  of SEVAG and 200  $\mu\text{l}$  of 3 X CTAB. Finally, 450  $\mu\text{l}$  of isopropanol was added to achieve DNA precipitation. The pellet obtained was then suspended in 50  $\mu\text{l}$  of pure water. The quality and quantity of the DNA extracted was measured using a Nanodrop 2000 (Thermo Fisher Scientific) spectrophotometer. The samples were kept at  $-20\text{ }^{\circ}\text{C}$  until use.

### Microsatellite selection and screening

From the 20 microsatellites developed by Arroyo et al. (2010), a set of 15 microsatellite pairs was selected and purchased from Invitrogen™ (Thermo Fischer Scientific Corporation, USA), to perform preliminary screening (Table 2). The forward primer from each pair was tagged on the 5'-end with an M13R universal tail (GGAAACAGCTATGACCAT) to enable fluorescent labeling (Schuelke 2000), while the tagged primers were added with the 5'GTTT "pigtail," to ensure consistency in amplicon size (Brownstein et al. 1996). All 15 primer pairs were tested on seven samples of *L. azorica* and on one sample of *L. nobilis* using a Biometra TGradient thermocycler, and an amplification mix with a final volume of 25  $\mu\text{l}$ , consisting of 25 ng of DNA, 75  $\mu\text{g}/\text{ml}$  BSA 0.1%,  $1\times\text{NH}_4$  Buffer, 3 mM  $\text{MgCl}_2$ , 0.4  $\mu\text{M}$  of untagged primer, 0.08  $\mu\text{M}$  of tagged primer, 0.36  $\mu\text{M}$  of M13R labeled with fluorescent dye (NED, VIC, FAM or PET), 200  $\mu\text{M}$  of dNTPs, 0.75 U of Immolase (Bioline, UK). The initial amplification program consisted of the following steps, as described by Arroyo et al. (2010): 2 min of denaturation at  $94\text{ }^{\circ}\text{C}$ , followed by 17 cycles at  $92\text{ }^{\circ}\text{C}$  for

**Table 1** List of populations sampled, with codes and the number of collected individuals per population (*N*)

Island	COL. ID	Population	Code	<i>N</i>	Total
Sta. Maria	<i>L.azorica</i>	1—Barreiro da Faneca	MAFA	6	
	<i>L.azorica</i>	2—Miradouro da Pedra Rija	MAPR	11	17
São Miguel	<i>L.azorica</i>	3—Pico do Buraco	SMBU	14	
	<i>L.azorica</i>	4—Road to the Village (Sete Cidades)	SMEV	5	
	<i>L.azorica</i>	5—Lombadas	SMLO	6	
	<i>L.azorica</i>	6—Pico Bartolomeu	SMPB	4	
	<i>L.azorica</i>	7—Pinhal da Paz	SMPP	12	
	<i>L.azorica</i>	8—Pico da Vara	SMPV	7	
	<i>L.azorica</i>	9—Tronqueira (Nordeste)	SMTR	10	
	<i>L.azorica</i>	10—Pico Verde	SMVE	17	75
Terceira	<i>L.nobilis</i>	25—Pinhal da Paz	SMPP	13	13
	<i>L.azorica</i>	11—Trail Algar do Carvão—Caldeira de Aqualva	TEAA	13	13
Graciosa	<i>L.nobilis</i>	26—Monte Brazil	TEMD	9	9
	<i>L.azorica</i>	12—Caldeira	GRCF	4	
São Jorge	<i>L.azorica</i>	13—Pico Timão	GRPT	5	
	<i>L.azorica</i>	14—Serra das Fontes	GRSF	5	14
	<i>L.nobilis</i>	27—Caldeira	GRCF	5	
	<i>L.nobilis</i>	28—Pico Timão	GRPT	3	8
Pico	<i>L.azorica</i>	15—Fajã dos Cubres	SJCU	5	
	<i>L.azorica</i>	16—Trail Topo—Fajã Caldeira Santo Cristo	SJFS	9	
	<i>L.azorica</i>	17—Fajã da Ribeira Seca	SJRS	3	17
Faial	<i>L.azorica</i>	18—Chão Verde	PICV	13	
	<i>L.azorica</i>	19—Criação Velha Vineyard trail	PITV	10	23
Flores	<i>L.azorica</i>	20—Road to Capelo	FACP	16	
	<i>L.azorica</i>	21—Rocha do Cabo forest trail	FARC	9	
	<i>L.azorica</i>	22—Levada trail	FATL	8	33
Flores	<i>L.azorica</i>	23—Ribeira da Badanela	FLRB	10	
	<i>L.azorica</i>	24—Rochão do Junco forest trail	FLRJL	10	20

Column named COL. ID (collectors' identification) indicates if the samples collected were initially identified as *L. azorica* or *L. nobilis*, based in situ on samples' morphological traits and scent and later on observation of all herbarium specimens from the sampled populations

30 s, annealing at 60–44 °C for 30 s (1 °C decrease in each cycle), and extension at 72 °C for 30 s; 25 cycles at 92 °C for 30 s, 44 °C for 30 s and 72 °C for 30 s; and a final extension of 5 min at 72 °C. Two microliters of amplification products was run on a 3.5% agarose gel, stained with Safe-View™ Classic (abm®), and visualized under UV to determine amplification success, polymorphism, and approximate length of amplification products. The preliminary screening allowed to select eight polymorphic SSR markers (Table 2) that exhibited readable amplified products to conduct the complete analyses.

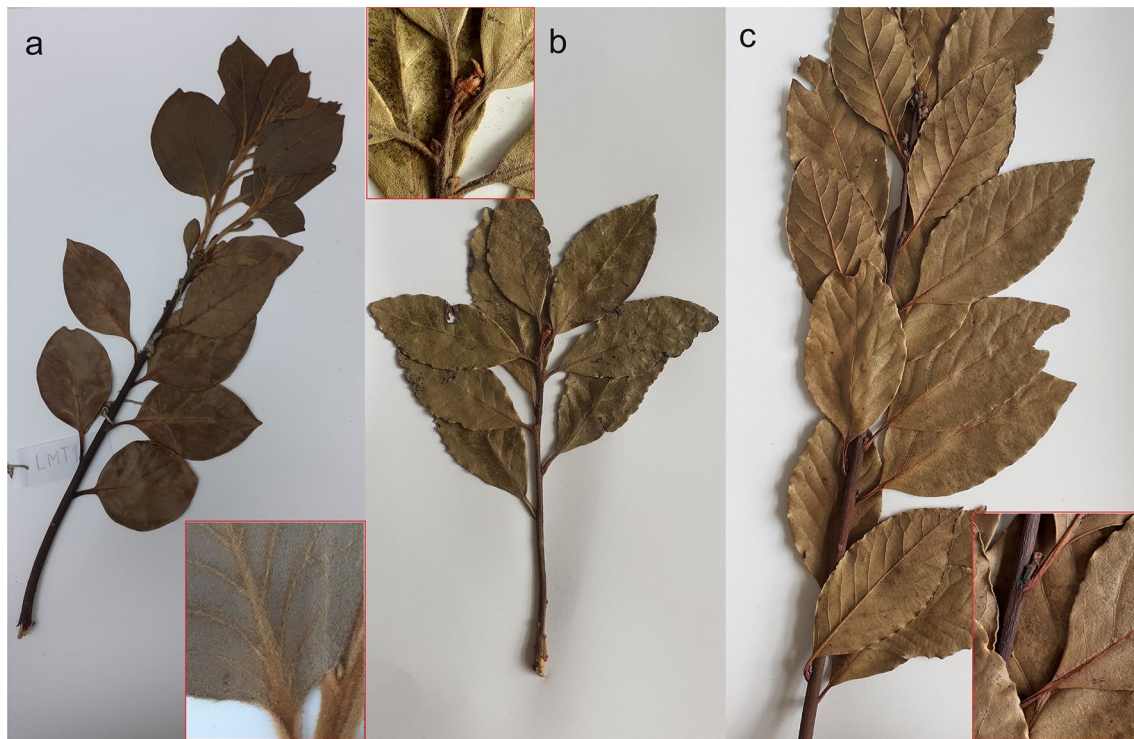
### Full-scale genotyping

After primer selection, amplifications were conducted for the whole sample set. The amplification products were diluted, multiplexed, and run on an ABI-3130xl Genetic Analyzer with LIZ500 size standard (Thermo Fischer Scientific).

Then, the obtained genotypes were scored using the micro-satellite plugin in Geneious Pro 5.6.7 software (Biomatters Ltd., <http://www.geneious.com/>).

### Data analysis

To determine whether any clusters were formed among individuals of the Azorean *Laurus*, we used a principal coordinate analysis (PCoA), calculated with GenAlEx version 6.501 (Peakall and Smouse 2012) using the Codom-genotypic distance matrix obtained with the same program. Additionally,  $F_{st}$  and gene flow estimates (Wright 1949), and an analysis of molecular variance (AMOVA; Excoffier et al. 1992), were calculated, again with GenAlEx. Bayesian Markov chain Monte Carlo (MCMC) approaches, as implemented in STRUCTURE version 2.3.4 (Pritchard et al. 2000), were used to estimate the existence and number of genetic clusters. We used STRUCTURE with *K* ranging



**Fig. 2** Voucher specimens of *Laurus* used for morphological determination of species. **a** Voucher specimen of *L. azorica* from Tronqueira, with detail of dense brown tomentum in leaves and twigs. **b** Specimen of *L. azorica* or intermediate form from Pico Timão (Graciosa)

and detail of brown tomentum on twigs and petiole. **c** Voucher specimen of *L. nobilis* from Pinhal da Paz and detail of leaves, and twig, with absence of tomentum

from 2 to the maximum number of putatively different populations sites (28), using 10 replicates and a burn-in length of 50,000 and 500,000 iterations of each chain, using the admixture model along with the assumption of correlated allele frequencies between groups (Falush et al. 2003). Estimation of the best  $K$  values obtained by STRUCTURE was conducted with STRUCTURE Harvester (Earl and von Holdt 2012) following the Evanno et al. (2005) method. The  $K=5$  repetitions were permuted in Clumpak v.1.1 (Kopelman et al. 2015) using the Greedy algorithm (Jakobsson and Rosenberg 2007), which was also used to graphically represent the results following Rosenberg (2004).

We used the genetic structure suggested by the best model obtained with STRUCTURE and that provided the contribution of each genetic cluster for each individual sample. This matrix was analyzed in R (R Development Core Team 2023) according to the following steps: i) calculation of Bray–Curtis, chord, and Euclidean distances between individuals, ii) application of two agglomeration schedules (Ward's and UPGMA); iii) calculation of cophenetic correlation and selection of the best method; iv) calculation of the number of groups of individuals based on two algorithms (); and v) representation of the individuals and of the respective

groups in a two-dimensional plot using non-metric multidimensional scaling (Pavão et al. 2019).

Several AMOVA's were performed with Arlequin (Excoffier and Lischer 2010), using different population grouping criteria, namely: i) island; ii) sub-archipelago; iii) morphological identification (putative *L. azorica*, putative *L. nobilis*, possible intermediate forms). Additional criteria were tested, namely: iv) STRUCTURE's output for  $K=5$  (Supplementary material, Fig. S1), which was generated creating artificial populations according to this output.

A Mantel test between Nei's genetic distances matrix and the corresponding geographic distance matrix of sites with at least two individuals was also conducted using GenAlEx version 6.501, to determine the occurrence of isolation by distance (IBD) between sites. To estimate the order of barriers to gene flow possibly occurring within the Azores, the same matrices were further analyzed with the Monmonier (1973) algorithm using BARRIER version 2.2 (Manni et al. 2004). To avoid calculation bias, only populations with three or more individuals were included in the full-scale genotyping.

Population genetic diversity was analyzed using GenAlEx version 6.501 (Peakall and Smouse 2012), by calculating the number of polymorphic loci per population and the mean values, over loci and populations, of alleles ( $N_a$ ), effective

**Table 2** List of the selected SSR loci for *Laurus azorica* and *L. nobilis* (Arroyo et al. 2010), with forward and reverse sequences, repeat motif and allele size range

Name	Primer	Sequences (5' - 3')	Repeat motif	Dye	Size range (bp)		Total number of alleles
					<i>L. azorica</i>	<i>L. nobilis</i>	
LnA106	Forward	<b>GGAAACAGCTATGACCATCAAATGATTTCAAGGACC</b> AC	(AC) 12	VIC	157–167	157–165	11
GU344697	Reverse	<i>GTTTAGGGGTCTTACTTCTATGAAGG</i>					
LnB2	Forward	<b>GGAAACAGCTATGACCATTATTTGAAGGTTTCTCT</b> CAGA	(GA) 24	PET	242–293	244–279	9
GU344693	Reverse	<i>GTTTATAAAGCGTGTCATTGTGAAC</i>					
LnB116	Forward	<b>GGAAACAGCTATGACCATGCTTTCTCTCCTCCCTG</b> TC	(TC) 17	PET	175–221	184–218	6
GU344695	Reverse	<i>GTTTACCCTCTCAATAATGGTTTGG</i>					
LnB119	Forward	<b>GGAAACAGCTATGACCATGGTAAGCAACAGAGC</b> ACATC	(TC) 27	FAM	185–235	185–241	24
GU344686	Reverse	<i>GTTTAGGAAAACAGTCAATAACTCC</i>					
LnB124	Forward	<b>GGAAACAGCTATGACCATTGGAATGTATGGCTCTGA</b> ACTC	(CT) 16	FAM	223–285	225–273	19
GU344698	Reverse	<i>GTTTCCAATCACAACCAGAAAGACAG</i>					
LnD101	Forward	<b>GGAAACAGCTATGACCATTTTCTTACTCCATAGA</b> CACG	(TCA) 8	PET	241–263	251–263	4
GU344696	Reverse	<i>GTTTGGCTCAAGGTAGACTAGAATG</i>					
LnD106	Forward	<b>GGAAACAGCTATGACCATTGCTCTACGTTTTGTGAA</b> GATC	(ATC) 8	NED	152–167	152–161	11
GU344691	Reverse	<i>GTTTCATTGGAGGGAACCTCTTTTAC</i>					
LnD109	Forward	<b>GGAAACAGCTATGACCATGCTGCTTATTGACACAAC</b> CAC	(ATG) 7	FAM	275–287	281–287	12
GU344690	Reverse	<i>GTTTGAAGGGAAACTGTAGGGCATA</i>					

The M13R tail is in bold (5'-GGA AAC AGC TAT GAC CAT-3'); "pigtail" in italics (5'-GTTT-3')

alleles ( $N_e$ ), Shannon diversity index ( $I$ ), observed ( $H_o$ ) and expected ( $H_e$ ), heterozygosity, and the fixation index ( $F$ ). The same software was used to estimate the numbers of private alleles ( $P$ ) and the number of locally common alleles ( $L50$  and  $L25$ , freq.  $\geq 5\%$ ), found in 50% or fewer and in 25% or fewer populations. Estimations were conducted in separate analyses for each putative species and for the overall dataset.

## Results

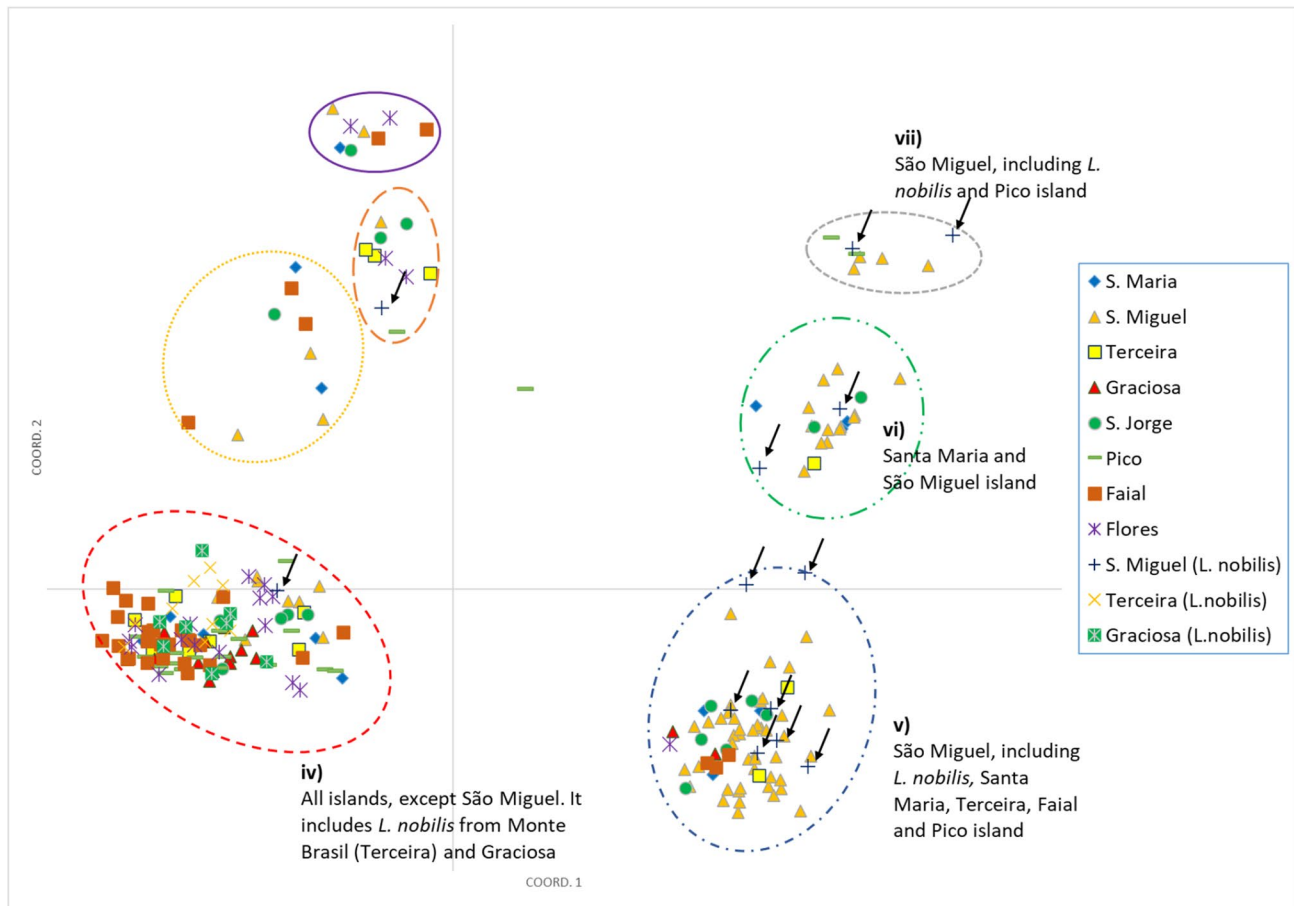
### Transferability and profile of the SSRs markers

From the fifteen primer pair sequences used, eight pairs presented a polymorphism and were selected to conduct the study for the whole dataset. Allelic patterns were manually inspected on Geneious Pro 5.6.7 and whenever peaks were located outside binning areas and had abnormal shapes, they were considered as artifacts or stutter. No differences were observed among the two species, *L. azorica* and *L. nobilis*, in terms of ploidy levels, for the same marker.

Regarding the genetic diversity of the eight polymorphic SSR loci, a total of 81 alleles were amplified, 78 alleles for *L. azorica* (mean of 9.75 alleles per locus) across 21 populations, and 38 alleles were amplified for *L. nobilis* (mean of 4.75 per loci) from four populations. The number of alleles per marker ranged from 3 (LnD101) to 17 (LnB119). The percentage of polymorphic loci across populations ranged from 75 to 100.00%, with the mean percentage established in  $86.61\% \pm 1.43\%$  SE.

### Genetic structure

A PCoA of the complete *Laurus* dataset (Fig. 3), based on covariance standardized approach of pairwise Nei's genetic distances, exhibited seven well-defined groups of individuals that compose the genetic structure of this species. However, there was not a clear correlation with the PCoA genetic structure and the in situ populations, since for the most part, individuals from a same population appeared scattered through several groups. Most of the smaller groups were composed by few samples from populations located in two, three, or four islands (Groups i, ii, iii, vi, and vii). Group iv



**Fig. 3** Principal coordinate analysis (PCoA) of the complete *Laurus* dataset calculated with GenAlEx. Arrows are pointing to *L. nobilis* samples from Pinhal da Paz (S. Miguel Island). Coordinate 1 was a percentage of explained variability of 20.01%, while Coordinate 2 was 28.79%

encompassed individuals from most of the populations and islands, with exception of São Miguel, while group v was mostly composed by São Miguel populations, albeit also including a few samples from Santa Maria, Terceira, Faial, and Pico.

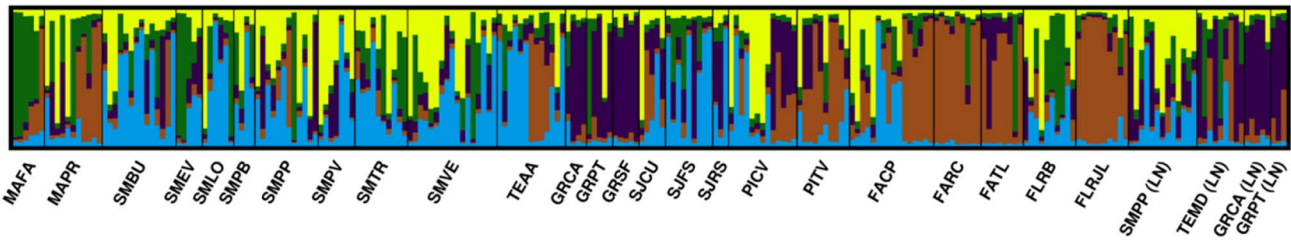
Bayesian analyses performed with STRUCTURE determined that the best  $K$  value is 5, therefore existing five main genetic groups. The analysis retrieved high levels of genetic admixture among and within populations and, in some cases, among individuals within populations. This is apparent in the populations of Miradouro da Pedra Rija (Santa Maria), Trail Algar do Carvão-Caldeira de Aqualva and Monte Brasil (Terceira), Chão Verde (Pico), Road to Capelo (Faial), and Ribeira da Badanela (Flores). The graphical representation given by Clumpak for the major cluster (Fig. 4), indicates that: (i) the populations of Santa Maria show similarities with one population from São Miguel (Road to Sete Cidades) and another from Flores (Ribeira da Badanela); (ii) a large group is formed with the remaining populations of São Miguel, including the *L. nobilis* population from Pinhal da Paz; (iii) a clearly distinct genetic pattern is observed in

the populations of Graciosa Island; (iv) populations of *L. azorica* from Terceira and São Jorge islands exhibit similar genetic patterns, although some individuals from Terceira's population are genetically similar to those from Pico, Faial, and Flores; (v) Pico, Faial, and Flores present a complex structure, since some populations of these islands show similar genetic patterns, while other populations from the same islands are genetically similar toward the islands of São Miguel and Santa Maria; (vi) the population of *L. nobilis* from Monte Brazil shows different genetic patterns among individuals.

Bray–Curtis coupled with non-metric dimensional scaling (Fig. S1, Supplementary material) provided the contribution of each genetic cluster for each individual, showing that the groups of individuals located into the extremities of the star-shaped graph likely represent pure allelic lineages of *Laurus azorica* and *L. nobilis*, differing from those that converge in the middle of the graphic, which share genetic characteristics from various genetic groups, as a result of gene flow or hybridization events between populations and species, contributing for the increased genetic admixture.



K=5



**Fig. 4** Population codes as in Table 1. Graphic representation of the genetic structure analysis conducted with STRUCTURE, for K=5. Each vertical line represents an individual, and the genetic groups

in the bar chart are represented by different colors. The proportions of colors in an individual represent the contribution of each genetic group

The AMOVA analyses, performed according to different grouping criteria (Table 3), indicated that the highest percentage between groups (6.58%) occurred following criteria iv), in which individuals were grouped into artificial populations, in accordance with results shown in Online Resource 1a. An additional AMOVA, which subjectively, i.e., visually, grouped populations with similar

genetic profiles (patterns of colors obtained in Clumpak), resulted in 5.05% between groups (data not shown).

A Mantel test conducted with the entire *Laurus* dataset (Fig. 5) resulted in a non-significant correlation between the genetic differentiation and geographic distance ( $R=0.152$ ;  $P=0.110$ ).

The spatial analysis conducted with BARRIER (Fig. 6) indicated a first-order barrier (a) to gene flow within the

**Table 3** Analysis of molecular variance (AMOVA) applied to the Azorean populations of *Laurus azorica* and *L. nobilis*, according to STRUCTURE K=5

Source of variation: K=5	df	S.s	Variance	%
Criteria: Island grouping: 1,2 // 3–10,25 // 11,26 // 12–14,27,28 // 15–17 // 18,19 // 20–22 // 23,24				
Among groups	7	40.178	0.074 Va	4.52
Among populations within groups	20	30.927	0.021 Vb	1.33
Among individuals within populations	214	254.123	– 0.355 Vc	– 21.70
Within individuals	242	459.5	1.898	115.85
Total	483	784.729	1.639	
Criteria: Sub-archipelago grouping: 1–10,25 // 11–22,26–28 // 23,24				
Among groups	2	14.913	0.036 Va	2.19
Among populations within groups	25	56.193	0.062 Vb	3.82
Among individuals within populations	214	254.123	– 0.355 Vc	– 21.66
Within individuals	242	459.5	1.898 Vd	115.64
Total	483	784.729	1.641	
Criteria: Morphological distinction: 1,2,11,15–24 // 3–10,25 // 12–14 // 26 // 27,28				
Among groups	4	25.993	0.063 Va	3.86
Among populations within groups	23	45.113	0.044 Vb	2.67
Among individuals within populations	214	254.123	– 0.355 Vc	– 21.54
Within individuals	242	459.5	1.898 Vd	115.01
Total	483	784.729	1.651	
Criteria: STRUCTURE's output for K=5: Individuals were clustered into artificial populations, according to the genetic group they belong (from 1 to 5)				
Among groups	4	50.391	0.108 Va	6.58
Among populations within groups	5	8.316	0.016 Vb	1.00
Among individuals within populations	232	266.522	– 0.375 Vc	– 22.74
Within individuals	242	459.5	1.898 Vd	115.17
Total	483	784.729	1.649	

Six of the eight loci were used to compute the AMOVAS (LnD101 and LnD109 were excluded), due to the tolerated level of missing data of 0.1. The hypothesis tested is represented by slanted bars separating groups of populations (numbers separated by comas)

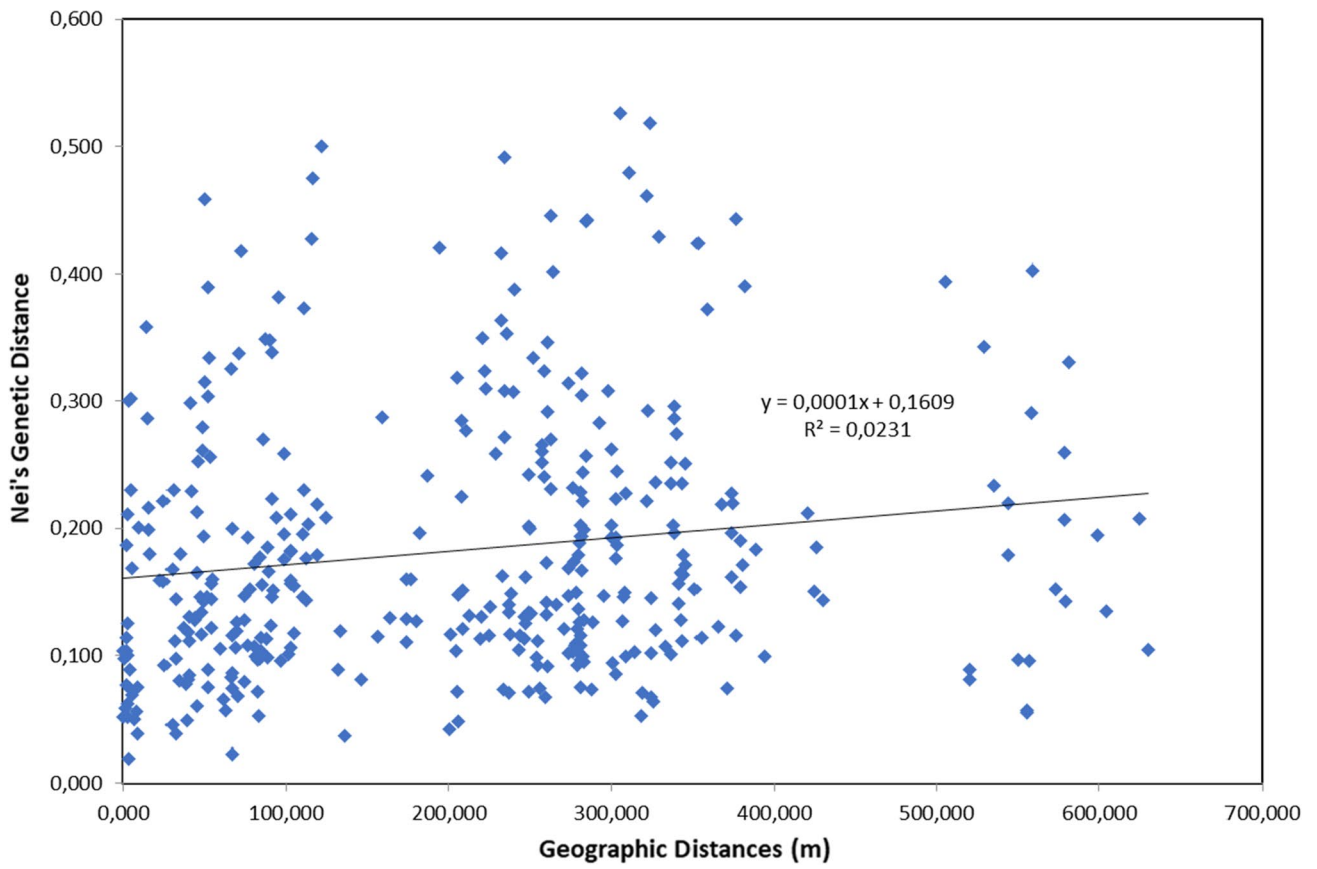


Fig. 5 Graphic representation of the Mantel test for the entire *Laurus* dataset, relating geographic distance and Nei's genetic distance

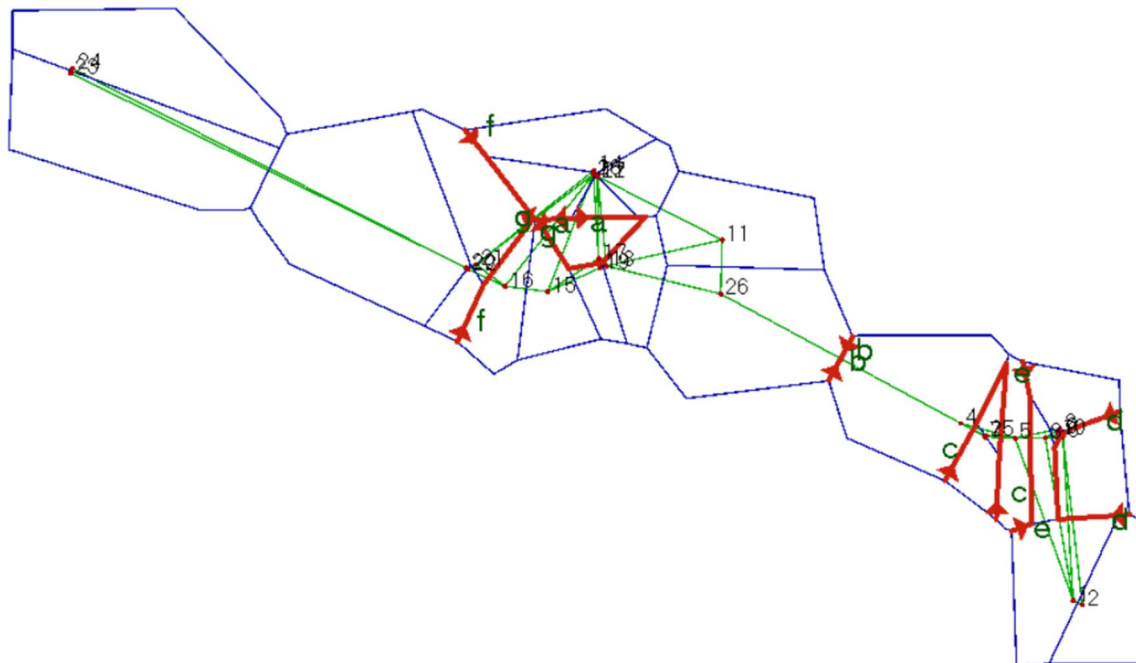


Fig. 6 Barriers to gene flow according to the Monmonier (1973) algorithm and after the spatial analysis obtained with BARRIER. Barriers are represented by letters a–g, while populations are represented by numbers from 1 to 28

Island of São Jorge, isolating the population of Fajã dos Cubres from the remaining islands of the central group. The second-order barrier (b) separated the eastern group from the other sub-archipelagos, while the next three barriers (c–e) were placed inside the Island of São Miguel, isolating the populations of Pinhal da Paz and Pico Bartolomeu, respectively, and splitting the island in half, separating the populations from the eastern and western parts of São Miguel. Finally, the remaining barriers to gene flow restricted the islands of the central sub-archipelago, separating Faial, and Flores Island, from the Western group, from the remaining islands.

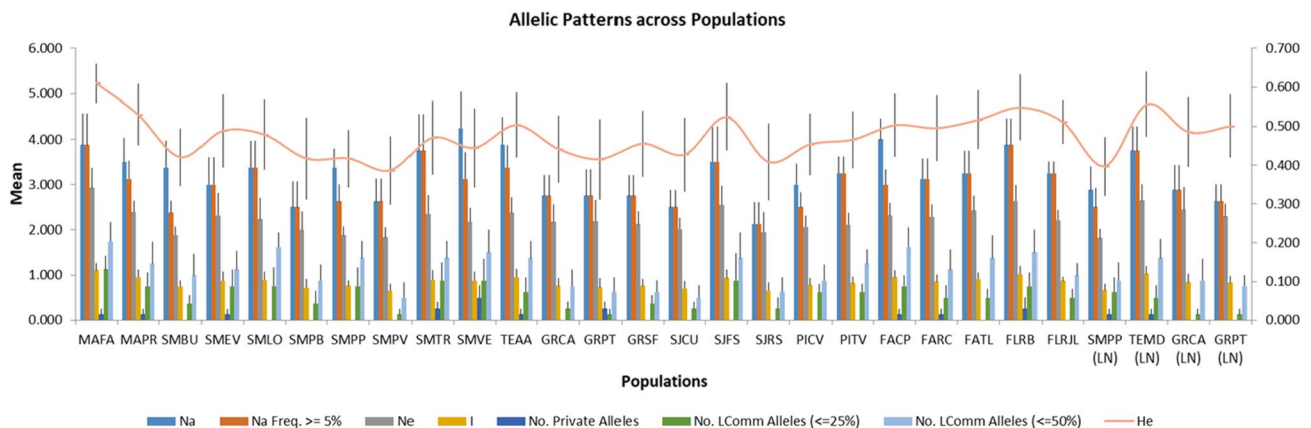
The estimated  $F_{ST}$  values for *L. azorica* fell between 0.01 and 0.24, (median 0.077), while the global  $F_{ST}$  value was around 0.098. In terms of gene flow ( $NM$ ), the results were very similar in both between populations and regarding the groups of populations established, reflecting the genetic structure obtained. Between populations, gene flow values varied from 0.795 to 18.784 (Online Resource 1b), and highest values were retrieved between the populations of São Miguel, Terceira, Pico, and Faial Islands (Pico da Vara – Pico Verde:  $NM = 18.784$ ; Caminho Algar do Carvão-Caldeira de Agualva and Road to Capelo:  $NM = 14.858$ ; Chão Verde and Criação Velha vineyard trail:  $NM = 13.731$ ). Gene flow was mostly absent between certain populations of the Islands of São Miguel and Santa Maria and those from Graciosa and São Jorge Islands. Between groups, the gene flow was, in general, higher and occurred between all the genetic groups that were detected in this study. The highest values were predominantly found between populations of *L. azorica* from São Miguel, Terceira, Faial, Pico, and Flores Islands. In addition, the populations of the endemic laurel from the central group islands Pico and Faial and the Western group island Flores exhibited high affinity with *L. nobilis* populations, suggesting that hybridization can be occurring between laurel species from these populations.

## Genetic diversity

The number of different alleles ( $N_a$ ) was highest in Pico Verde (4.25), followed by Road to Capelo (4.00) and Barreiro da Faneca and Ribeira da Badanela (3.875), while Shannon's information index ( $I$ ) and expected heterozygosity ( $H_e$ ) were higher at Santa Maria's population of Barreiro da Faneca and lowest in Pico da Vara. Observed heterozygosity ( $H_o$ ) was lowest in the population of Pinhal da Paz and highest at Rochão do Junco forest trail. The fixation index ( $F$ ) was not always negative and, at the population level, was highest at Serra das Fontes, a population of *L. nobilis*. The highest  $F$  value retrieved for a putative *L. azorica* population was observed at Fajã da Ribeira Seca and the lowest, again in Pinhal da Paz (Fig. 7). On average, for putative *L. azorica* populations,  $N_a = 3.234$ ,  $I = 0.842$ ,  $H_o = 0.582$ ,  $H_e = 0.472$ , and  $F = -0.240$ .

Regarding the number of effective ( $N_e$ ), private ( $P$ ), and locally common alleles obtained for putative *L. azorica* (Fig. 7), the population of Barreiro da Faneca obtained the highest  $N_e$  (2.929), as well as the highest percentages of locally common alleles ( $L_{25} = 1.125$  and  $L_{50} = 1.750$ , respectively). In a large majority of populations, private alleles were not detected (São Miguel, with exception of Road to Sete Cidades, Tronqueira, and Pico Verde (São Miguel); Pico Timão (Graciosa); São Jorge, Pico, Faial (Levada trail), and Flores (Rochão do Junco). Among these populations, the highest values of private alleles ( $P$ ) were retrieved in Pico Verde (0.50), following Tronqueira, Pico Timão, and Ribeira da Badanela (0.25) and the populations of Santa Maria, Terceira, and Faial Islands (0.125; Online Resource 1c).

The fixation index ( $F$ ) calculated for the global dataset was negative ( $-0.237$ ), which indicates an absence of an inbreeding in laurels; however, as seen above, positive values were obtained in few populations of São Miguel Island.



**Fig. 7** Allelic patterns across populations of putative *Laurus azorica* and *L. nobilis* included in this study

## Discussion

### Marker transferability and ploidy profiling

The SSR markers developed by Arroyo et al. (2010) effectively produced new and relevant data regarding the genetic structure and diversity of *L. azorica*. Their suitability for estimating local and regional levels of genetic variation and gene flow in *Laurus* accessions from the Azorean archipelago is thus in accordance with Arroyo et al. (2010) and is an important tool toward the conservation of this relict genus (Rodríguez-Sánchez et al. 2009).

Regarding ploidy levels, the results from microsatellite fingerprinting revealed the occurrence of diploid individuals in both *L. azorica* and *L. nobilis*. These results were not expected, considering previous suggestions of tetraploidy, namely, from Ehrendorfer et al. (1968) and Arroyo et al. (2010).

### Genetic structure at taxa level and occurrence of putative interspecific hybrids

Although preceding molecular studies did not find strong evidence supporting the distinction between *L. azorica* and *L. nobilis* (Rodríguez-Sánchez et al. 2009; Kondraskov et al. 2015), and our results for the Azores exhibit great levels of genetic admixture and possible hybridization within *Laurus* s.l., we consider that previously described morphological characteristics (Franco 1960; Schaefer 2005), biochemical properties (Vinha et al. 2015; Viveiros et al. 2022), and this study's findings, particularly the genetic structure output obtained in STRUCTURE (Fig. 4) and Bray–Curtis coupled with NMDS analysis (Fig. S1), show that it is still genetically possible to distinguish between pure lineages of *L. azorica* s.l. and *L. nobilis*. This is also supported by the specific allelic combinations obtained for *L. nobilis* (Fig. 4, color code: dark purple) for the population of Monte Brazil in Terceira (e.g., LnB2, 246), while in other islands (e.g., Santa Maria and Pico), individuals present specific allelic combinations that appear in typical *L. azorica* populations phenotype (e.g., LnD109, 272; LnB116, 180), scattered across the archipelago.

Putative hybrid individuals of *L. nobilis* from Graciosa Island show intermediate morphological forms, such as the presence of brown tomentum on twigs, petiole and in young leaves, obovate to elliptic leaves and undulated leaf margins, which are also found in individuals from both species at Pinhal da Paz population, in São Miguel.

Given the considerable amounts of genetic admixture found, supported by the fact that gene flow can occur

between populations of both laurels and within locations where these species are sympatric, constitute strong evidence that hybridization might have occurred in the past, among populations and individuals of *L. azorica*, and between *L. nobilis* and *L. azorica*. This can be attributed to the human colonization in large part, mainly due to the arrival of great amounts of wood materials, from other Islands to São Miguel, during the Portuguese colonization (Frutuoso 1589). These were mainly used in construction, due to timber depletion resulting from the replacement of native forests with introduced species and farmlands (Guppy 1917; Dias 2007). Furthermore, it is possible that birds, as natural dispersers (Heleno et al. 2011), might also have played a key role as sources of some of the hybridization evidence detected. Ultimately, the geological construction of São Miguel Island in different phases (Sibrant et al. 2015) could also have resulted in the genetic structure presented for the laurel accessions in this island.

### Genetic population structure within the archipelago

The populations of putative *L. azorica* were split in five genetic groups and high levels of genetic admixture were found between individuals and populations, especially in São Miguel Island, but also across the islands. This is the likely result of gene flow between populations, as found in past studies for the Azorean endemics *Juniperus brevifolia* (Silva et al. 2011) and *Picconia azorica* (Martins et al. 2013). Additionally, most genetic variation was retrieved within individuals, among the clusters detected in the best output obtained with STRUCTURE, which is in accordance with the previous results obtained by Rodríguez-Sánchez et al. (2009).

We find that the most fitting criteria used in the AMOVA were grouping by “Island,” which provided the best explanation for the genetic patterns exhibited in populations within and between some islands.

A relevant genetic pattern that was found in this study is shared between populations from Santa Maria Island, with populations from São Miguel and Flores, and has been observed in previous molecular studies with other Azorean endemics (Martins et al. 2013; Dias et al. 2014). This could be the result of an early dispersal event that occurred from east to west, since according to several authors (França et al. 2003; Ávila et al. 2016), these three islands are among the oldest islands of the archipelago. Additionally, the island-specific genetic profiles, and levels of private alleles found here for Santa Maria, similarly to other studies (e.g., Ferreira et al. 2011; Moura et al. 2013) can contribute for a better understanding of Santa Maria's evolutionary mechanisms and reinforce the distinctiveness of this island's flora, due to ecological speciation (Ferreira et al. 2011; Schaefer et al. 2011).

In previous studies with other endemic tree species, populations from São Miguel showed some affinity with those from the islands of the central sub-archipelago of Pico, Faial, and Flores (Silva et al. 2011; Martins et al. 2013; Moreira et al. 2013), but in this case, this relationship is not clear, since some populations of Pico, Faial, and Flores share a genetic pattern, while other populations, or groups of individuals within a population, seem to share a similar genetic pattern with one found in São Miguel Island.

São Jorge and Terceira showed a relatively homogeneous genetic profile, as found in *Picconia azorica* (Martins et al. 2013), and the populations of Graciosa Island presented a distinct and uniform genetic pattern, regardless of the initial morphological identification as *L. nobilis* or *L. azorica*. In addition, the PCoA revealed that the genetic structure of this island is closely related to the putative population of *L. nobilis* from Monte Brazil, suggesting that the populations from Graciosa are, most likely, from *L. nobilis*, or some hybrid form between the two laurel species. This indicates that *L. azorica* may be extinct in the wild, in Graciosa Island, excluding translocated plant material from São Miguel.

### Occurrence of barriers to gene flow within *Laurus azorica* populations

The spatial analysis with BARRIER retrieved putative barriers between populations which agree with most of the genetic patterns observed. Although for Azorean laurels, isolation by distance (IBD) does not appear to occur, it seems that geographical barriers, like the sea, can hinder gene flow, with the example of the islands of the oriental sub-archipelago, São Miguel, and Santa Maria, being isolated from the remaining islands of the archipelago. In addition, geological barriers promoted by intense volcanism appear to correspond to barriers to gene flow within São Miguel Island (Calvert et al. 2006), promoting the isolation of some populations located near volcanic complexes, e.g.: Road to Sete Cidades which is located on Sete Cidades Volcanic Complex (França et al. 2003). However, in this case, the effect of ecological/geological barriers is not as relevant as observed for other Azorean endemics (Moura et al. 2013; Vieira et al. 2018). The major barriers retrieved are also in concordance with the results of gene flow between sub-archipelagos. For instance, there were no barriers between the populations of some islands of the central sub-archipelago, namely between Pico and the remaining islands or between Faial and Flores, where gene flow was generally high.

The  $F_{ST}$  values obtained for *L. azorica* ranged between 0.01 and 0.24, which suggests the occurrence of genetic admixture among populations and low genetic differentiation, in general, probably due to fluctuations in the expected heterozygosity (Meirmans and Hedrick 2011),

or maybe because of the genetic flow that contributed for the admixture found between populations of this species. It seems that populations that share stronger links [e.g., (i) Pico Buraco and Tronqueira; (ii) Pico Vara and Pico Verde; (iii) Caminho Algar do Carvão-Caldeira de Agualva and Road to Capelo] and higher rates of gene flow are prone to have a lower  $F_{ST}$  value. This was observed between some populations of São Miguel, Terceira, Faial, and Flores. To summarize, these results show that *L. azorica* still maintains moderate levels of genetic differentiation, although among populations, the level of genetic divergence is becoming dangerously low. Because birds are vectors of zoochoric seed dispersal of endemic trees, the low level of genetic differentiation among populations may be likely attributed to this type of colonization, as seen elsewhere (Vargas 2007; García-Verdugo et al. 2009; Martins et al. 2013). Martins et al. (2015) found high genetic diversity and similar values of  $F_{ST}$  among three species of *Ocotea*, ranging from 0.086 in *O. odorifera* to 0.148 in *O. catharinensis*. Previous genetic studies including *L. azorica* and *L. nobilis* samples (Arroyo-García et al. 2001; Rodríguez-Sánchez et al. 2009) did not allow to make comparisons or to take conclusions regarding the evolutionary processes influencing the structure of genetic variation within and among populations. Only Arroyo-García et al. (2001) reported the possible existence of geographic sub-differentiation in laurel samples from Madeira Island, among other samples from the Iberian Peninsula, North of Spain, and the Canaries, but not from the Azores.

The global  $F_{ST}$  value (0.098) for *L. azorica* is low when compared with the one obtained for *Juniperus brevifolia* (Silva et al. 2011) and somewhat in accordance with the value obtained for the endemic tree *Picconia azorica* (Martins et al. 2013). When comparing *L. azorica* with the Tropical Lauraceae *Cryptocarya aschersoniana* Mez, Moraes and Derbyshire (2003) found that the  $F_{ST}$  values ranged between 0.279 and 0.395, higher than those found for the Azorean tree.

### Genetic diversity

Regarding genetic diversity, we find that the average number of alleles per loci of *L. azorica* (9.75) is slightly lower than those observed for other endemic trees, such as *Prunus lusitanica* subsp. *azorica* (Moreira et al. 2013) and *Picconia azorica* (Martins et al. 2013). Regarding the allelic frequencies of *L. azorica* and *L. nobilis* from the São Miguel population of Pinhal da Paz, where the two species coexist and where the possibility of hybridization was considered (Moura et al. 2015; Tavares 2015), a comparison shows that the values of the allelic frequencies of the two species in this area are very similar, but the fixation index ( $F$ ) is positive for *L. azorica* (0.168) and negative for *L. nobilis* (− 0.002),

suggesting that some level of consanguinity is occurring in *L. azorica*. Looking at the allelic richness, both the specific patterns of the populations from São Miguel Island, and the shared patterns of São Miguel with the remaining islands of the archipelago, were always very similar.

Our results show that most of the genetic variation resides within individuals (87%), which is similar to what was found for other woody Azorean endemics (Moreira et al. 2013; Martins et al. 2013; Moura et al. 2013).

## Conservation concerns

In the last decades, *Laurus azorica* has achieved a stable trend in numbers and its current conservation status is least concern (LC), according to the International Union for Conservation of Nature (Silva and Beech 2017). However, past alterations on the natural vegetation of the islands (Frutuoso 1589) led to massive declines in numbers, largely reducing the area of occupancy of *L. azorica* in some islands of the Azores (Elias et al. 2016). Currently, this species still faces ongoing threats, connected with human activities and competition with various invasive alien species.

The possible hybridization between *L. azorica* and *L. nobilis* found in this study can jeopardize the conservation and survival of the endemic *L. azorica*. Genetic contamination (Whelan et al. 2006) and counter speciation (Arnold 1992) in the Azorean laurel may be related to the deliberate introduction of the edible taxon *L. nobilis*. Continuing this practice should be avoided, as it can lead to the total loss of distinct genetic features still present in the extant allelic lineages of *L. azorica*.

Another important criterion for classifying population diversity in a genetic conservation context is allelic richness (Marshall and Brown 1975; Schoen and Brown 1993; Szpiech and Rosenberg 2011; Vieira et al. 2018), due to giving a measure of their distinct biodiversity. Based on low allelic richness, the populations of Pico Bartolomeu (São Miguel) and the populations of São Jorge, Fajã dos Cubres, and Fajã da Ribeira Seca need to be protected. Furthermore, some *L. azorica* populations should also be considered a priority for conservation, due to the occurrence of private alleles, namely Barreiro da Faneca from Santa Maria, Pico Verde and Tronqueira (São Miguel), and Ribeira da Badanela (Flores).

Considering these results, we reinforce the importance of conserving *L. azorica*, as mentioned by other authors (Cardoso et al. 2008; Silva et al. 2009; Liang et al. 2019; Matos et al. 2019), and similarly to what was recommended in previous molecular studies for other Azorean relict species (Silva et al. 2011; Moura et al. 2013; Vieira et al. 2018).

In situ conservation of *Laurus azorica* is a priority, and management measures are fundamental to achieve that goal,

including the preservation of the last patches of laurel forests by establishing a legal framework, as recommended, for example, in the case of the Moroccan *Laurus* populations, by Ettaqy et al. (2023). Habitat restoration, control, and eradication of alien species accompanied by information campaigns and management of traditional land uses, such as agriculture (Silva et al. 2009), will likely improve the conservation outcomes for *L. azorica*.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00606-023-01888-6>.

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**Author contributions** RMCR, ÂFV, LS, and MM helped in study conception and design. All authors have contributed for sampling. Material preparation was performed by RMCR, ÂFV and RR. Analysis was performed by RMCR, LS, and MM. The first draft of the manuscript was written by RMCR, ÂFV, and MM, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. LS and MM acquired the funding. The study was supervised by LS and MM.

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## Declarations

**Conflict of interest** All the authors have no conflict of interest regarding this research.

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