Botanical Journal of the Linnean Society, 2019, **191**, 365–380. With 5 figures.

Colonization routes, microevolutionary genetic structure and conservation concerns in a remote widespread insular endemic grass: the case of the Azorean tussock grass *Deschampsia foliosa*

MÓNICA MOURA[1*], PILAR CATÁLAN[2,3,4], ANTÓNIO BREHM[5] and
MIGUEL MENEZES DE SEQUEIRA[1,6]

[1]*CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Pólo dos Açores, Universidade dos Açores, Rua da Mãe de Deus 13A, 9501-801 Ponta Delgada, Açores, Portugal*
[2]*Universidad de Zaragoza-Escuela Politécnica Superior de Huesca, 22071 Huesca, Spain*
[3]*Grupo de Bioquímica, Biofísica y Biología Computacional (BIFI, UNIZAR), Unidad Asociada al CSIC, Zaragoza E-50059, Spain*
[4]*Institute of Biology, Tomsk State University, Tomsk 634050, Russia*
[5]*Life Science Faculty, University of Madeira, Campus da Penteada, 9020-105 Funchal, Madeira*
[6]*Madeira Botanical Group, Life Science Faculty, University of Madeira, Campus da Penteada, 9000-390 Funchal, Madeira*

Received 7 December 2018; revised 25 May 2019; accepted for publication 30 July 2019

Population genetic structure and diversity and phylogeographical dispersal routes were assessed for the Azorean endemic grass *Deschampsia foliosa* using AFLP markers. This species occurs on seven islands in the archipelago and a sampling of populations from the three main geographical groups of islands was used, covering its known distribution. Principal coordinates analyses (PCoAs), Bayesian analyses and phylogenetic networks revealed different degrees of admixture for the central group (C) populations and a clear differentiation for the western group (W) and São Miguel island (in the eastern group, E) populations. The best *K* values corresponded to nine and 11 genetic groups, which were also confirmed by analysis of molecular variance. A low but significant correlation between genetic data and geography was observed, with most relevant barriers to gene flow generally placed between sub-archipelagos. We suggest a west-to-east isolation by distance dispersal model across an island age continuum with Flores–Corvo (W) and Pico (C) at the extremes of the dispersal path. An alternative scenario, also supported by the genetic data, implies an initial colonization of São Jorge (C), dispersal within C and following bidirectional dispersal to the W and E. The phylogeographical framework detected might be related to island age and to highly destructive volcanic events, and it supports the occurrence of cryptic diversity within *D. foliosa*. Genetic diversity estimators were highest for Pico island populations (C), lowest for São Miguel (E) and Flores (W) populations, and more divergent for the Corvo population (W). Conservation measures should be taken to preserve the genetic structure found across sub-archipelagos and islands.

ADDITIONAL KEYWORDS: Azores – conservation genetics – microevolution – phylogeography.

INTRODUCTION

The Macaronesian region is part of the Mediterranean Basin Biodiversity Hotspot, but the number of extant endemic lineages present in each archipelago varies. In recent years, studies have addressed the issue of why fewer vascular endemic plants apparently evolved in the Azores and Cabo Verde, compared to other Macaronesian archipelagos, such as the Canary Islands (Silva *et al.*, 2010; Menezes de Sequeira *et al.*, 2012). Jardim & Menezes de Sequeira (2008) proposed

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*Corresponding author. E-mail: monica.mt.moura@uac.pt

a link between the distinct levels of diversity found among Macaronesian archipelagos and the patterns of ecosystem use and destruction after human colonization, suggesting possible pre-Linnean extinctions of taxa as potential drivers of the current diversity. Carine & Schaefer (2010) suggested that palaeoclimatic variation during the Quaternary was largely absent in the Azores, which limited allopatric inter-island diversification. Triantis *et al.* (2012), however, argued in favour of geological, geographical and ecological specificities of the archipelago, suggesting that the Azores are too young, too small and too environmentally homogeneous in comparison with other Macaronesian archipelagos. The possible occurrence of a Linnean shortfall (Brown & Lomolino, 1998) was proposed by Schaefer *et al.* (2011), who suggested that current taxonomic knowledge of endemic diversity on oceanic islands, such as the Azores, may be incomplete, biasing studies of diversity patterns. Connor *et al.* (2013), working with preliminary palynological data from the Azorean islands of Flores and Pico, concluded that the impact of palaeoclimate on vegetation was minor, and that vegetation was primarily influenced by volcanism, soil formation and human impact. Connor *et al.* (2013) also questioned whether high levels of cryptic endemism in the Azorean flora, as suggested by Schaefer *et al.* (2011), might be related to the broad elevational range of endemic plants in the archipelago. Meanwhile, growing evidence for a Linnean shortfall in the Azorean flora has been amassed in several taxonomic groups, including Asteraceae (Moura *et al.*, 2015a; Schafer *et al.*, 2015), Orchidaceae (Bateman, Rudall & Moura, 2013; Bateman *et al.*, 2014) and Cupressaceae (Elias & Dias, 2014). In these groups, new endemic taxa have recently been described or native taxa have been re-circumscribed as endemics (Moura, Carine & Menezes de Sequeira, 2015b; Mort *et al.*, 2015). Furthermore, studies using population genetic markers have also suggested that geological and geographical conditions might have had a major influence on the present genetic patterns and the microevolution of Azorean endemic lineages (Silva *et al.*, 2011; Borges Silva *et al.*, 2016; Dias *et al.*, 2016; Vieira, Dias & Moura, 2018).

Interpretation of the geology of the archipelago, specifically estimation of the age of some Azorean islands, has undergone critical changes in recent studies, suggesting that the archipelago is much younger than previously considered [see Ávila *et al.* (2016) for ages of Macaronesian islands]. Santa Maria is still the oldest island in the archipelago (*c.* 6 Myr; Ramalho *et al.*, 2017), although in the context of colonization by terrestrial plant life, it should be considered younger, because the island was totally submerged and only resurfaced *c.* 3.5 (or 2.8) Mya (Ramalho *et al.*, 2017). Volcanism has equally been considered a crucial factor to explain the population genetic structure of several endemic plants. On some Azorean islands, such as São Miguel and Terceira, volcanic events were particularly destructive and thus possibly responsible for extensive soil sterilization, leading to subsequent recolonization events and an alteration of the genetic structure of the populations on these islands (Díaz-Perez *et al.*, 2008; Silva *et al.*, 2011; Borges Silva *et al.*, 2016; Dias *et al.*, 2016).

Geographical barriers to gene flow have also been detected, often overlapping with the expanses of sea between the islands (Moura, Silva & Caujapé-Castells, 2013; Borges Silva *et al.*, 2016; Dias *et al.*, 2016; Vieira *et al.*, 2018), although others (e.g. mountains; Dias *et al.*, 2016) also occur. Additional factors affecting population genetic structure are ecological heterogeneity linked to different elevations and exposure conditions (Vieira *et al.*, 2018) and human- or animal-mediated dispersal, as with fleshy fruit tree taxa historically used as timber (Martins *et al.*, 2013; Moreira *et al.*, 2013).

Caujapé-Castells *et al.* (2017) presented a view on the origins and evolution of genetic diversity in the Canary Islands. They hypothesized the importance of geographical and ecological complexity as driving forces of natural fragmentation and gene flow cessation, thus leading to low genetic diversity. In contrast, if the geographical and ecological complexity of an island decreases, an increase in population genetic diversity is expected due to secondary contact between some populations previously isolated and the flux of colonizers coming to the island from other areas.

Deschampsia P.Beauv. *s.l.* is a genus of *c.* 30 species, several of which remain taxonomically doubtful, distributed in cold temperate regions of both hemispheres (Chiapella & Zuloaga, 2010). Recent evolutionary systematic studies supported its split into three divergent clades, representing the currently recognized genera *Deschampsia s.s.*, *Avenella* (Bluff & Fingerh.) Drejer and *Vahlodea* Fr. (Chiapella, 2007; Soreng *et al.*, 2015). Traditionally, *Deschampsia* was considered to include three Macaronesian endemics: *D. foliosa* Hack. in the Azores (Silva *et al.*, 2010), and *D. argentea* Lowe and *D. maderensis* (Hack. & Bornm.) Buschm. in the Madeira archipelago (Buschmann, 1950). However, a recent phylogenetic study by Quintanar, Castroviejo & Catalán (2007) suggested that *D. maderensis* may belong to *Avenella*.

Deschampsia foliosa, occurring between *c.* 200 and 900 m altitude, is a hemicryptophyte with hydrochorous dispersal and an estimated total abundance of >100 000 individuals (Schaefer, 2003, 2005). It is distributed on seven islands of the archipelago but is absent from Graciosa and Santa Maria. The species grows in moist, steep locations such as craters, mountains, ravines, around lakes, near creeks and in natural meadows associated with *Holcus rigidus* Hochst. ex Seub. and *Festuca francoi* Fern.Prieto, C.Aguiar, E.Dias & M.I.Gut. It also occurs in high-elevation permanent and semi-permanent pastures and other human-disturbed vegetation, in mountain natural woodlands dominated by *Juniperus brevifolia* (Seub.) Antoine, and in scrublands dominated by *Calluna vulgaris* (L.) Hull and *Juniperus brevifolia* (Silva *et al.*, 2009, 2010; Elias *et al.*, 2016; this study; for species syntaxonomy see also Fernández Prieto, Aguiar & Dias, 2012). The species is included on the list of 90 priority species for conservation in the Azores, ranking 57, due to isolation of populations, low population density, disturbance of sensitive areas, habitat degradation, changes in land use, invasion by alien species, herbivory, trampling and natural disturbances such as landslides (Silva *et al.*, 2009).

AFLP markers have been successfully applied to population genetics and phylogeographical studies of other endemic Azorean grasses, namely *Festuca francoi* Fern.Prieto, C.Aguiar, E.Dias & M.I.Gut. and *F. petraea* Guthn. ex Seub. (Díaz-Pérez *et al.*, 2008). These authors suggested that the Azorean species probably diverged *in situ*, from a common mainland ancestor, following ecological adaptation, according to an east-to-west volcanism-associated dispersion for *F. francoi* and a west-to-east colonization direction for *F. petraea*.

In this study, we aim to provide further insight into the colonization and dispersal mechanisms occurring in the Azores and provide the first data on the genetic diversity of *D. foliosa* populations. The fact that *D. foliosa* is widespread in the Azores makes it an interesting subject for examining the potential occurrence of cryptic evolutionary patterns at the population level and for testing which of the previously proposed dispersal hypotheses and factors affecting genetic structure might be operating in the archipelago. In addition, we provide baseline data on the genetic diversity of *D. foliosa*, as a fundamental step in developing an adequate conservation or action plan.

MATERIAL AND METHODS

Study site and sampling

The Azores archipelago consists of nine volcanic islands located in the North Atlantic Ocean, spanning *c*. 600 km on a WNW–ESE axis between 36°55′–39°43′N and 25°00′–31°15′W. The archipelago is situated *c*. 840 km north-west of Madeira, 1370 km west of Cabo da Roca on the Portuguese coast and 3510 km east of Nova Scotia, and it is divided into three island groups or sub-archipelagos. The eastern group (E) includes two islands (São Miguel and Santa Maria), the central group (C) five (Terceira, Graciosa, São Jorge, Pico and Faial) and the western group (W) two (Corvo and Flores) (Fig. 1C). A wide sampling was conducted in 23 natural populations of *D. foliosa* (Fig. 1A, B) located on six islands across the three island groups (Fig. 1C). In populations with <20 individuals, all were sampled. The maximum number of samples obtained per population was 20, with a minimum distance of 1 m between individuals (see Table 1 for samples sizes and other details).

DNA extraction

Fresh plant material previously dried and stored in silica gel was used to extract total DNA using 100 mg of dry leaf material, employing a CTAB protocol (Doyle & Doyle, 1987), with minor modifications. DNA quantity and quality were checked using electrophoresis on a 1% TBE-agarose gel, and its concentration was estimated by comparison with 200 ng of tomato DNA. Finally, DNA was diluted to a final concentration of 100 ng μL^{-1} and stored at −20 °C.

AFLP marker selection and full-scale amplification

AFLP analysis followed Vos *et al.* (1995) using the AFLP Analysis System I (Invitrogen Life Technologies), with minor modifications. Genomic DNA (200 ng) was digested by *Mse*I and *Eco*RI enzymes in a 12.5-μL volume at 37 °C for 2 h. The enzymes were inactivated after 15 min at 70 °C. Digested DNA was ligated to the *Eco*RI and *Mse*I adapters (12.5 μL) with T4 DNA ligase (0.5 U) at 20 °C for 2 h. This was followed by a pre-selective amplification step, which consisted of 30 cycles (94 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s) using *Eco*RI and *Mse*I primers, each with one selective nucleotide. PCR products were diluted ten- to 50-fold and used as templates for selective amplification with *Eco*RI and *Mse*I primers, each with three selective nucleotides. The amplification profile was one cycle consisting of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 60 s, followed by reduction of the annealing temperature at each cycle by 0.7 °C for 12 cycles, and finally 23 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s. Three of the nine primer pair combinations tested in a pilot study (E-AAG + M-CTC, E-AGC + M-CAG, E-AGC + M-CTA) were chosen based on the number of amplified fragments, clarity and number of bands. The reaction products were separated by electrophoresis on 6% denaturing polyacrylamide gels and the AFLP fragments were visualized by silver staining.

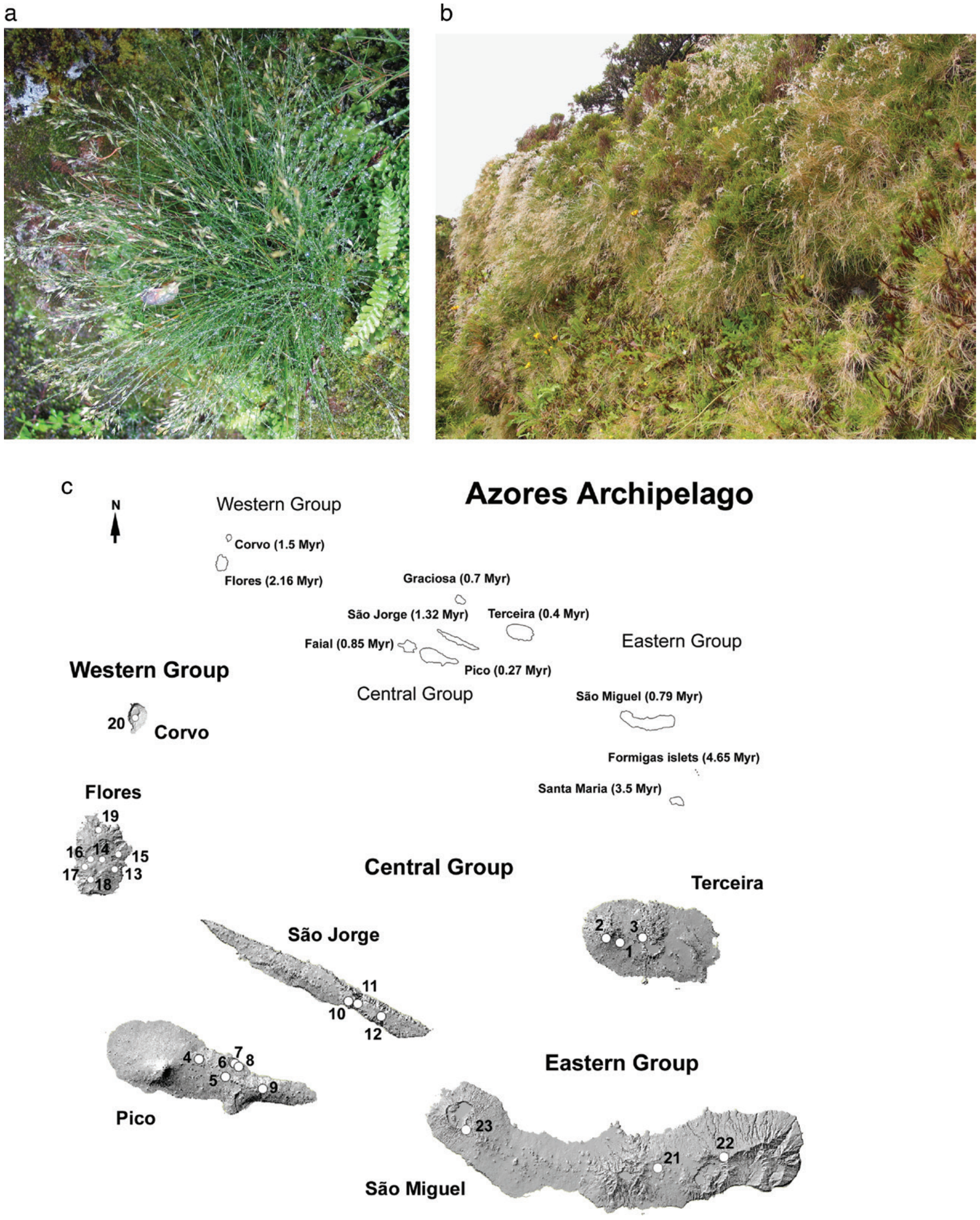

Figure 1. *Deschampsia foliosa*. A, flowering plant (Terceira: Lagoa das Patas); B, species ecology (Terceira: Serra de Santa Bárbara); C, the Azores archipelago and location of populations sampled in this study. Population codes are as listed in Table 1. Island ages according to Sibrandt et al. (2015), Ávila *et al.* (2016) and Ramalho *et al.* (2017).

AFLP DATA ANALYSES

AFLP profiles were scored for each individual as discrete characters (presence or absence of amplified products) across all individuals from all populations and for each primer used. Only products that gave clear, unambiguous, intense bands in the range 70–330 bp were used in the genetic analysis. The preliminary matrix, consisting of 292 loci, was subsequently revised and downsized to 280, with <2% and >98% frequency loci removed.

Table 1. Populations of *Deschampsia foliosa* sampled per island, with island code (I), population code (P), number of specimens amplified (*N*), locality, exposure, elevation and collector number (C)

Island (I)	P	*N*	Locality	Exposure	Elevation (m)	Collector number
Terceira (TE)	1	8	At base of Serra de Santa Bárbara caldera, on a creek downstream of Lagoa das Patas		542	4399(2)
	2	11	Serra de Santa Bárbara caldera, at the exposed crests and steep slopes 1 km before the summit	W	870	4401(8)
	3	4	Furna do Enxofre, on a slope under *Juniperus brevifolia*	W 279		4410A
Pico (PI)	4	11	Lagoa do Capitão, on lake margin slopes, under partial cover of *Erica azorica* and *Juniperus brevifolia*, with native ferns and mosses	SW 223	791	4438(15)
	5	12	Lagoa do Caiado	NE 28	834	4443(1)
	6	3	Creek at Prainha de Cima, human altered with construction debris and urban waste, shaded by *Pittosporum undulatum*, with high abundance of *Hedychium gardenerianum*	NE	213	4447A
	7	3	Creek, east of Prainha de Cima, on creek bed where water flows	NE 26	213	4450C
	8	5	Creek, east of Prainha de Cima, on creek bed where water flows	NE 23	188	4451E
	9	1	Lagoa da Laje	SSE	844	4452
São Jorge (SJ)	10	2	Road to Topo, on a steep slope at the rim of a *Pittosporum* woodland		465	4464
	11	18	Road to Topo, on a slope at Ribeira do Capadinho	SSE 153	690	4465(1)
	12	9	Road to Topo, near Ribeira do Capadinho, on a slope		738	4468(1)
Flores (FL)	13	10	Road to Lagoa da Lomba, on a slope between pastures, with introduced Poaceae and *Hydrangea macrophylla*		446	4472A
	14	4	On Santa Cruz–Fajãzinha road, on narrow slopes between *Cryptomeria japonica*, *Hydrangea* and pastures		609	4473E
	15	5	Descent to Santa Cruz	N 100	572	4474(A)
	16	6	Lagoa Negra margin	W 265	586	4476(1)
	17	2	Miradouro Craveiro Lopes, on a slope	SE	483	4477B
	18	5	Lagoa Rasa	S 177	519	4478(1)
	19	1	Road to Ponta Delgada, on a slope			4483
Corvo (CO)	20	20	Caldeirão south border, on soil heavily trampled by cattle	WSW 251	595	4486(1)
S. Miguel (SM)	21	7	Near Lagoa do Fogo, on an SSE slope with *Festuca jubata*	SSE 155	639	4494(1)
	22	5	Near Salto do Cavalo, slope on the rim of a pasture heavily invaded by *Gunnera tinctoria*	W	776	4495(15)
	23	5	Near Pico do Carvão, on a slope	N	670	4498(1)

Material was collected by M. Menezes de Sequeira. Vouchers are deposited at the University of Madeira.

A principal coordinates analysis (PCoA) with a superimposed minimum spanning tree (MST) was calculated with NTSYS-pc v.2.2 (Rohlf, 2008), based on pairwise genetic distances between individuals from all 23 populations using Nei & Li's (1979) genetic distances. A second PCoA, calculated with Genalex v.6.501 (Peakall & Smouse, 2012), was obtained from the matrix of average population genetic distances. For the latter analysis and all subsequent ones, populations with fewer than five individuals were excluded and two populations that were <1 km apart (Pico populations 6 and 7; Fig. 1C) were merged.

The NeighborNet algorithm (Bryant & Mouton, 2004) was applied in SplitsTree v.4.14.5 (Huson and Bryant, 2006), using default settings, albeit excluding constant sites, to identify possible reticulate evolution. Additionally, a statistical parsimony TCS network (Templeton, Crandall & Sing, 1992) was constructed with PopART v.1.7 (Leigh & Bryant, 2015).

An estimation of the existence and number of genetic clusters (*K*) was made through a Bayesian Markov chain Monte Carlo (MCMC) approach, as implemented in TESS v.2.3.1 (Chen *et al.*, 2007) and STRUCTURE v.2.3.4 (Pritchard, Stephens & Donnelly, 2000). Several TESS runs were calculated using the BYM model with *K* ranging from 2 to the maximum number of putatively different populations with ≥5 individuals (16 populations), ten replicates for each *K*, 50 000 sweeps and a burn-in length of 10 000. The lowest deviance information criterion (DIC) values obtained were used to filter the top 10% best runs. A similar analysis was conducted with ParallelStructure in the CIPRES Science Gateway v.3.3 (Miller, Pfeiffer & Schwartz, 2010), for the same range of *K* values, using ten replicates, a burn-in length of 50 000 and 500 000 iterations of each chain with the admixture model and the assumption of correlated allele frequencies between groups (Falush, Stephens & Pritchard, 2003). The best *K* values were determined by the method of Evanno, Regnault & Goudet (2005) in STRUCTURE Harvester (Earl & von Holdt, 2012). The optimal *K* repetitions were permuted using the Greedy algorithm (Jakobsson & Rosenberg, 2007) and graphically represented following Rosenberg (2004), in Clumpak (Kopelman *et al.*, 2015).

To confirm the genetic structure estimations obtained, six analyses of molecular variance (AMOVAs) were calculated with Arlequin v.3.5.2.2 (Excoffier and Lischer, 2010), based on geographical grouping criteria, i.e. by sub-archipelago and by island, and on the clustering obtained in TESS and STRUCTURE. In the latter, groups were constructed in accordance with the main genetic group or admixture pattern. Additionally, Bayesian genetic structure ($G_{st\beta}$) was also estimated in Hickory (Holsinger, Lewis & Dey, 2002; Holsinger & Lewis, 2003) with the f free model.

To determine the occurrence of isolation by distance (IBD) between populations with five or more individuals, a Mantel test was calculated between a genetic distance matrix, obtained with Genalex v.6.501 using Nei's formula (Nei, 1972, 1978), and the corresponding geographical distance matrix. To estimate the order of putative barriers to gene flow occurring between populations, the same matrixes used in the Mantel test were further analysed with the Monmonier (1973) algorithm using BARRIER v.2.2 (Manni, Guérard & Heyer, 2004). The first ten barriers calculated by the program were depicted in a Voronoï tessellation representing a polygonal neighbourhood for each population, and the Delaunay method of triangulation connected the sampled localities by a set of triangles (Manni *et al.*, 2004).

To evaluate possible models of dispersal within the archipelago, a series of Mantel tests between the matrix of Nei's pairwise genetic distances between populations and several geographical distance matrices were calculated in Genalex v.6.501, assuming the occurrence of IBD. A geographical distance matrix was obtained for each putative dispersal model (Table 3), by calculating the pairwise cumulative sum of distances between every two populations along a connective path defined in each model (Table 3).

Genetic diversity estimates were obtained with AFLPsurv (Vekemans *et al.*, 2002) following a Bayesian analysis with non-uniform prior distribution, and the method of Lynch & Milligan (1994) to estimate several parameters, namely: (1) the proportion of polymorphic loci (PLP) at the 5% level; (2) expected heterozygosity or Nei's genetic diversity (H_j) and its variance components, such as (3) the proportion of variance due to sampling of individuals (VarI%); and (4) the proportion of variance due to sampling of loci (VarL%). Species-average Bayesian genetic diversity (H_s) was obtained with Hickory for all populations, and as an average total. The frequency-down-weighted index (DW; Schönswetter & Tribsch, 2005) was estimated with AFLPdat (Ehrich, 2006). Overall values for PLP at the 5% level and band richness (Br) were obtained with AFLPdiv (Coart *et al.*, 2005), with a rarefaction of 5 to account for different population sizes (Petit, El Mousadik & Pons, 1998; Coart *et al.*, 2005). The population matrix is available at DEMIURGE (http://www.demiurge-project.org/).

RESULTS

AFLP MARKER POLYMORPHISM

Three of the nine primer pair combinations tested were chosen based on the number of amplified products, clarity of the bands and number of variable presence bands. The first combination (B; E-AAG + M-CTC) resulted in 175 operational taxonomic units (OTUs) and was inferior to the other two combinations tested (F; E-AGC + M-CAG) and (G; E-AGC + M-CTA) with, respectively, 190 and 198 OTUs. Using all combinations simultaneously further reduced the matrix to 157 OTUs.

Genetic structure and phylogeography

PCoAs computed with average pairwise population genetic distances roughly clustered populations per sub-group of islands (Fig. 2). The 3D plot with a superimposed MST (84.6% of the total variance explained by the first three coordinates) suggested a connection between central group Terceira and Pico populations, whereas São Jorge populations appeared to be connected to the latter group, to western group Flores populations and to eastern group São Miguel populations, which in turn were also connected to Flores populations (Fig. 2A). The 2D plot (48.4% of the total variance explained by the first two coordinates, Fig. 2B) showed the heterogeneity of the central group populations, with a distinct separation of the Serra de Santa Bárbara (2) population in Terceira and of Lagoa do Capitão (4) and Lagoa do Caiado (5) populations in Pico, from the remaining central and western sub-archipelago populations across coordinate 1, which explained 29.9% of the variance obtained, whereas eastern group São Miguel populations (21–23) were separated from all other populations across coordinate 2 (18.5%).

The NeighborNet network (Fig. 3) confirmed the connections implied by the PCoA MST, with significant loops estimated between Flores, Corvo and São Jorge, between São Jorge and Pico, and between Pico and Terceira. São Miguel individuals of *D. foliosa* showed less reticulation compared with the other populations and islands. A similar result was observed in the TCS network (Supporting Information, Figures S1 and S2).

The best results obtained in STRUCTURE and TESS are summarized in Figure 4 and correspond to $K = 3$ and $K = 5$ (ΔK = 544.314 and 498.174, respectively; all others between 19.073 and 0.075) in STRUCTURE, and to $K = 9$ (DIC = 25 510.3, run 77) and $K = 11$ (DIC = 25 584.7, run 91) in TESS. The genetic structure depicted by the two higher K values mostly correspond to the results obtained by the other analyses. This structure indicates a distinctiveness of São Miguel individuals (populations 21–23), and of Corvo and most of Flores individuals from the rest (populations 15–20), in addition to different degrees of admixture between Terceira and Pico, São Jorge, Pico and Flores, and Flores and Corvo.

The highest percentages of variance among groups and lowest percentages among populations within groups obtained in the AMOVAs (*c*. 25% and 11%, respectively; Table 2) corresponded to the criteria based on the main genetic group or admixture pattern retrieved for the best K values in TESS. These criteria significantly discriminated populations of: São Miguel (21–23); Terceira (1); Terceira (2); São Jorge (11) and Pico (6 & 7, 8); São Jorge (12, second best model) or São Jorge (12, first best model) and Flores (13); Pico (4, 5); Flores (15, 16, 18); and Corvo (20). The lowest percentage of variance among groups and highest percentage among populations within groups was obtained when grouping by sub-archipelagos (13.2% and 25.6%, respectively). Additionally, $G_{st\beta}$ calculated with the f free model was 0.32.

A Mantel test (Supporting Information, Fig. S2) resulted in a significant ($P = 0.02$) albeit low correlation between Nei's genetic distance and geographical distance matrices ($R^2 = 0.1145$). The most important barriers to gene flow, computed between the populations of *D. foliosa* (Fig. 5A), were generally placed between sub-archipelagos (b, c), with the notable exception of the first-order barrier (a) that separated the two eastern and coastal Pico populations (6 & 7 and 8) from those inland (4 and 5), dividing the archipelago islands into two large population groups along a west–east axis: (1) eastern island of São Miguel plus central Terceira, São Jorge and the eastern coastal Pico populations; and (2) western Flores and Corvo plus inland Pico populations. Lower order barriers separated within-island populations on São Miguel, Terceira and Flores.

Of the ten models of dispersal tested only two were statistically significant at the 5% level, with R^2 values of 0.57 and 0.28 (Table 3). The model with higher correlation (Fig. 5B) connects sampled islands along an island continuum with Flores and Corvo at one end of the connective path that further goes from Flores to São Jorge, from São Jorge to São Miguel and from São Miguel to Terceira and Pico, although the model does not imply the direction of dispersal, i.e. younger to older or vice versa. The second-best model (Fig. 5B) indicates a path between Flores and Corvo to Terceira and from Terceira to the remaining islands sampled in the study.

Genetic diversity

The highest percentage of polymorphic loci per population, highest expected heterozygosity (H_j) and highest Bayesian genetic diversity (H_s) were always in Lagoa do Capitão (population 4) on Pico (PLP = 78.9, $H_j = 0.280$, $H_s = 0.263$). Lowest diversity values were estimated for Salto do Cavalo (population 22), São

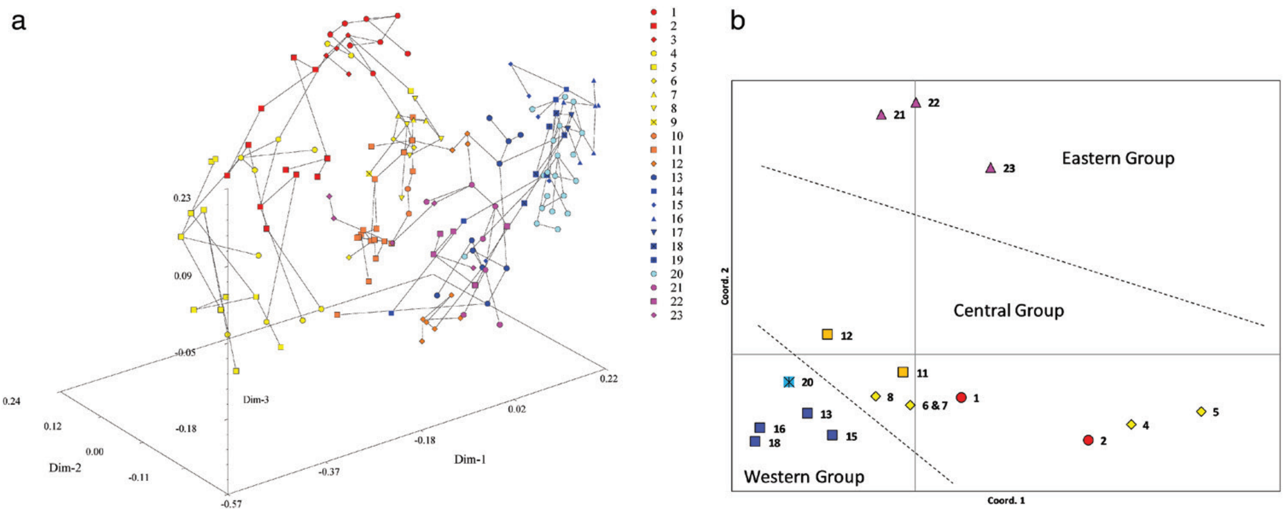

Figure 2. Principal coordinates analysis (PCoA) of 23 populations of *Deschampsia foliosa*: A, 3D plot with a superimposed minimum spanning tree (MST), based on the genetic distance matrix of Nei & Li (1979); B, PCoA using average population genetic distances [populations with fewer than individuals were excluded from the analysis, and populations <1 km apart were merged (Pico 6 and 7)]. Terceira (red); Pico (yellow); São Jorge (orange); Flores (blue); Corvo (light blue); and São Miguel (magenta). Population codes are as in Table 1.

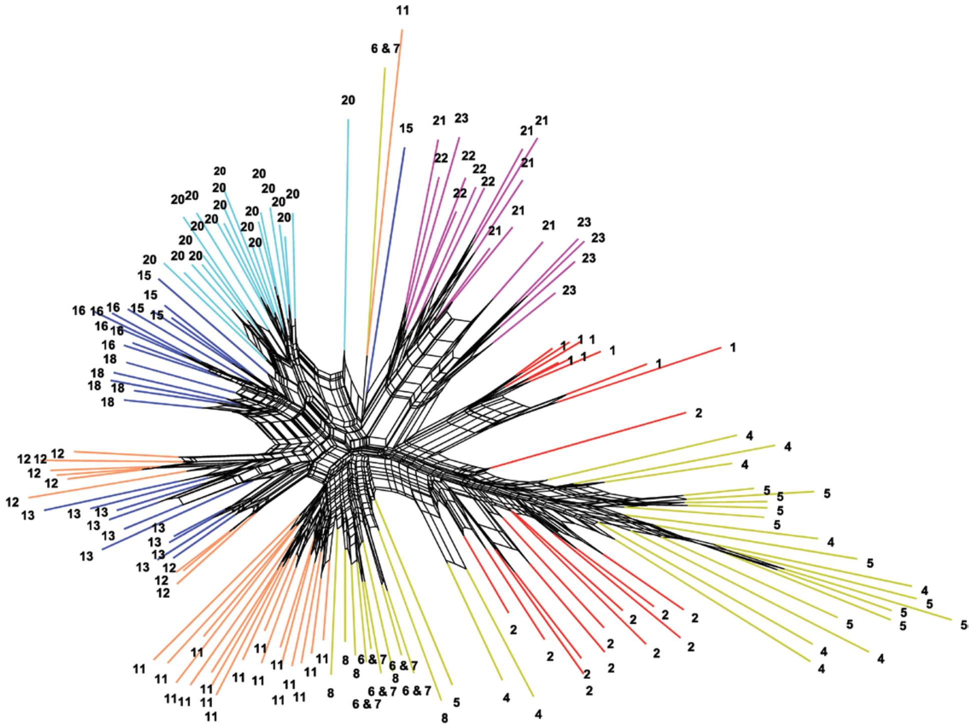

Figure 3. NeighborNet network of *Deschampsia foliosa* populations calculated with SplitsTree. Terceira (red); Pico (yellow); São Jorge (orange); Flores (blue); Corvo (light blue); and São Miguel (magenta). Population codes are as in Table 1.

Miguel (PLP = 62.1) and Lagoa Negra (population 16) on Flores island (H_j = 0.187, H_s = 0.175). At the species level, H_j was 0.24, with the greatest proportion of variance (VarI%) attributed to sampling of individuals, and H_s was 0.20. Using a rarefaction of 5, average PLP for *D. foliosa* was 46% at the 5% level, and average band richness (Br) was 1.37. The highest DW value, estimated as a measure of divergence, was

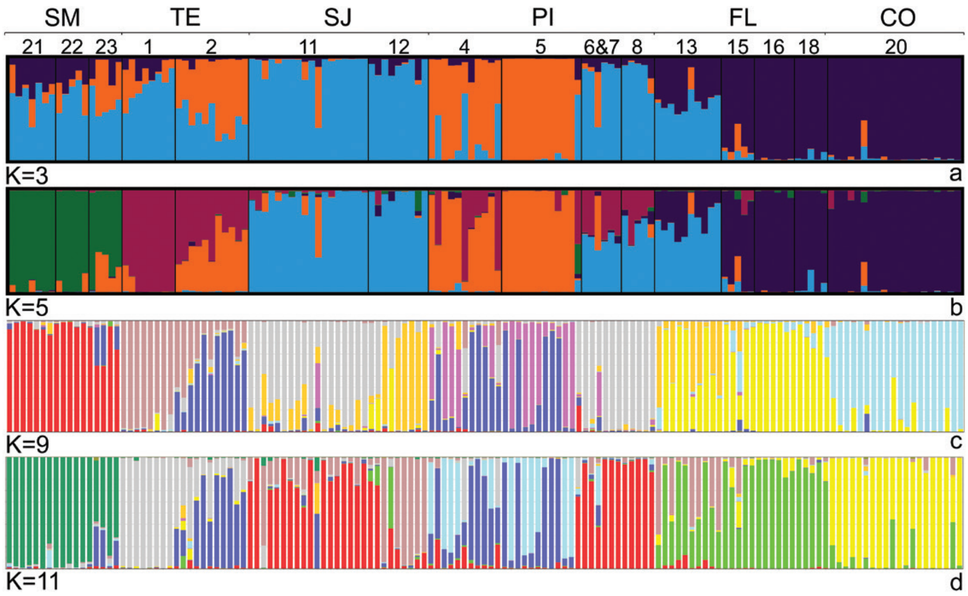

Figure 4. Graphical outputs for the best *K* and DIC values obtained through the Bayesian structure analyses implemented in STRUCTURE (A, B) and TESS (C, D) to infer the genetic structure of the *Deschampsia foliosa* populations. Population and island codes are as in Table 1.

obtained for Caldeirão (population 20) on Corvo island (89.4) and the lowest for Pico do Carvão (population 23), a population of São Miguel island (9.2).

DISCUSSION

COLONIZATION AND DISPERSAL ROUTES IN *D. FOLIOSA*

In recent years, estimates of the geological ages of several Azorean islands have been reviewed and significantly lowered. These changes provided a completely different spatio-temporal scenario regarding the reconstruction of ancient plant colonization pathways and within-archipelago dispersal routes. Most relevant changes affected earlier estimations for Santa Maria (6.3 Mya but resurfacing again at 3.5 Mya; Ramalho *et al.*, 2017), São Miguel (0.79 Mya; Sibrandt *et al.*, 2015) and Terceira (0.4 Mya; Hildenbrand *et al.*, 2014). Even in the absence of a close outgroup that could root the evolutionary tree for *D. foliosa*, our best supported IBD dispersal model suggests an initial colonization landing point and source of subsequent dispersals located in the westernmost islands of the archipelago (Table 3), currently also known to be the earliest to arise after the second emersion of Santa Maria (Ramalho *et al.*, 2017). Given that Flores is the oldest island in the western sub-archipelago, it is thus probable that it was the first to be colonized by an ancestral form of *D. foliosa* with subsequent dispersal to the nearby Corvo. Dispersal from Flores to the oldest island in the central group (São Jorge) and from there to the closest eastern group island (São Miguel) is a possible scenario. A dispersal end point at Terceira and Pico is in accordance with the current estimated ages for both islands, i.e. the most recently constructed in the archipelago (Sibrandt *et al.*, 2015; Ávila *et al.*, 2016; Ramalho *et al.*, 2017).

In contrast, the PCoA with superimposed MST (Fig. 2), the NeighborNet network (Fig. 3) and the haplotype network (Supporting Information, Fig. S1) indicate connections between São Jorge and islands of the central and western groups, whereas a connection between São Jorge and São Miguel is also clear in the MST and haplotype network. On average, our genetic diversity data (Table 4) show higher parameter values in the central sub-archipelago islands (São Jorge and Pico populations), suggesting potential ancestry and accumulated variation over time (as proposed for *Lactuca watsoniana* Trel.; Dias *et al.*, 2016), with less diverse populations in the western or eastern sub-archipelago islands (e.g. Flores), which could have resulted from more recent founder events and genetic drift (Borges Silva *et al.*, 2016). These results suggest an alternative scenario to the IBD model, with an initial colonization taking place on São Jorge, followed by dispersal within the central group and bidirectional dispersal to the western and eastern groups (Fig. 5B). Our data support previous findings on a similar dispersal pattern for the Azorean endemic temperate grass *Festuca francoi*, which showed a west-to-east direction of colonization, although an alternative origin in the Central Group was also

Table 2. AMOVA performed for *Deschampsia foliosa* populations according to geographical and genetic structure criteria

Source of variation	d.f.	s.s.	Var.	% V
Sub-archipelagos; groups 21,22,23 / 1,2,11,12,4,5,6&7,8 / 13,15,16,18,20				
Among groups	2	756.716	5.950	13.2
Among populations within groups	13	1661.904	11.540	25.6
Within populations	127	3497.758	27.541	61.2
Total	142	5916.378	45.031	
Islands; groups 21,22,23 / 1,2 / 11,12 / 4,5,6&7,8 / 13,15,16,18 / 20				
Among groups	5	1447.171	6.740	15.5
Among populations within groups	10	971.448	9.195	21.2
Within populations	127	3497.758	27.541	63.4
Total	142	5916.378	43.479	
Based on K = 3; groups 21,22,13 / 23,1 / 2 / 11,12,6&7,8 / 4 / 5 / 15,16,18,20				
Among groups	6	1541.155	6.809	15.6
Among populations within groups	9	877.464	9.194	21.1
Within populations	127	3497.758	27.541	63.3
Total	142	5916.378	43.544	
Based on K = 5; groups 21,22,23 / 1 / 2,4 / 11,12 / 5 / 6&7,8 / 13 / 15,15,18,20				
Among groups	7	1848.427	10.490	24.0
Among populations within groups	8	570.192	5.631	12.9
Within populations	127	3497.758	27.541	63.1
Total	142	5916.378	43.662	
Based on K = 9 and 11; groups 21,22,23 / 1 / 2 / 11,6&7,8 / 12 / 4,5 / 13 / 15,16,18 / 20				
Among groups	8	1989.006	11.014	25.4
Among populations within groups	7	429.613	4.833	11.1
Within populations	127	3497.758	27.541	63.5
Total	142	5916.378	43.388	
Based on K = 9 and 11; groups 21,22,23 / 1 / 2 / 11,6&7,8 / 12,13 / 4,5 / 15,16,18 / 20				
Among groups	7	1916.558	11.143	25.6
Among populations within groups	8	502.061	4.8178	11.1
Within populations	127	3497.758	27.541	63.3
Total	142	5916.378	43.502	

Groups are represented in italics, commas separate populations within the same group and slashes separate groups. Population codes are as in Table 1. All variance (% V) values are significant at the $P < 0.001$ level.

suggested (Díaz-Pérez *et al.*, 2008). Other studies also point to São Jorge as the original place of dispersal for *Azorina vidalii* (H.C.Watson) Feer (Menezes *et al.*, 2018). It is also possible that recurrent colonization events originating from different sources, some of them after widespread extirpations due to volcanism (Borges Silva *et al.*, 2016), could have contributed to shape the current genetic patterns of *D. foliosa*, obscuring the genetic footprint associated with early dispersal events.

DRIVERS OF GENETIC DIVERSITY AND STRUCTURE IN *D. FOLIOSA*: A BASELINE FOR CONSERVATION GENETICS

Several genetic molecular studies of Azorean endemic plants have detected the presence of previously unknown genetic structure at the population level, revealing cryptic diversity in island ecosystems (Crawford & Stuessy, 2016). In *D. foliosa*, although the occurrence of admixture is significant in the populations of the central group, it is also clear that populations from São Miguel and from both islands of the western group are distinct, with individuals pertaining mostly to island-specific genetic groups (Fig. 4; Table 2). Similarly, geographically connected distinction (although other factors might be acting as well) has been observed in other Azorean endemic plants such as *Euphorbia stygiana* H.C.Watson (Schaefer *et al.*, 2011), species of *Tolpis* Adans. (Borges Silva *et al.*, 2016), *Lactuca watsoniana* (Dias *et al.*, 2016, 2018) and *Festuca francoi* (Díaz-Pérez *et al.*, 2008). Given the currently estimated young age of the Azores archipelago (6.3/3.5–0.27 Myr; Ávila *et al.*, 2016), most Azorean lineages are therefore quite recent and the presence of these cryptic evolutionarily significant units (ESUs) is of upmost evolutionary importance because they probably reflect ongoing radiation and speciation processes.

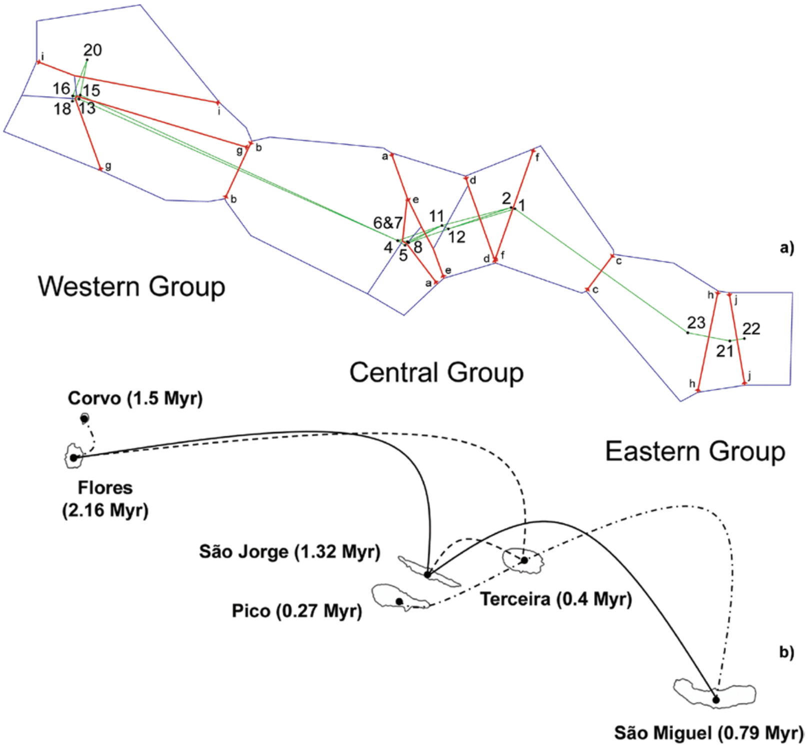

Figure 5. A, graphical representation of barriers to gene flow obtained with BARRIER for *Deschampsia foliosa*. Dots represent geographical locations of populations; red lines are the putative barriers to gene flow listed alphabetically according to their estimated relevancy; Delaunay triangulation shown in green and Voronoï tessellation in blue. B, summary of best IBD dispersal model and alternative dispersal model. Solid lines correspond to best model dispersal routes, dashed lines to alternative model routes and dot-dashed lines to dispersal routes common to both models. Population codes are as in Table 1.

Table 3. Models of dispersal of *Deschampsia foliosa* tested under the assumption of IBD

Model	Connective paths	R^2	
1	CO-FL//FL-SJ//SJ-SM//SM-TE-PI	0.57	*
2	CO-FL//FL-TE-SJ/TE-PI//TE-SM	0.28	*
3	CO-FL//FL-SM//SM-TE-SJ/TE-PI	0.27	
4	CO-FL//FL-SM//SM-SJ/SJ-PI-TE	0.27	
5	CO-FL//FL-SJ/SJ-PI-TE//SJ-SM	0.25	
6	CO-FL//FL-SM//SM-TE-SJ-PI	0.25	
7	CO-FL//FL-TE//TE-SJ-PI//TE-SM	0.24	
8	CO-FL//FL-SJ/SJ-PI/SJ-TE//SJ-SM	0.23	
9	CO-FL//FL-SJ-PI/SJ-TE//TE-SM	0.21	
10	CO-FL//FL-SJ-PI-TE//TE-SM	0.18	
11	CO-FL//FL-PI-SJ-TE//TE-SM	0.14	
12	CO-FL//FL-SJ-PI//SJ-SM//SM-TE	0.05	

Double-slashes indicate dispersal events to a different sub-group of islands of the Azores archipelago and single slashes indicate dispersal within the same sub-group. R^2 indicates the fraction of the variance on the matrix of genetic distances explained by the cumulative sum of geographical distances calculated according to each model. Asterisks indicate a significant correlation at the 5% level. Island codes are as in Table 1.

Widespread insular endemics are particularly interesting study cases to understand the effect of evolutionary forces in remote and isolated islands. Geography has been frequently suggested to explain extant genetic structure within the Azores, namely the existence of large sea barriers that separate the

Table 4. Estimation of genetic diversity indexes obtained for the populations of *Deschampsia foliosa*

Island	Code	PLP (%)	H_j	VarI%	VarL%	H_s	DW
TE	1	62.9	0.195 ± 0.011	42.2	57.8	0.176 ± 0.007	25.0
	2	70.0	0.248 ± 0.011	25.8	74.2	0.212 ± 0.006	21.8
PI	4	78.9	0.280 ± 0.011	18.4	81.6	0.263 ± 0.008	44.8
	5	75.4	0.217 ± 0.010	18.8	81.2	0.229 ± 0.011	26.6
	6&7	72.1	0.272 ± 0.011	54.4	45.6	0.207 ± 0.007	13.8
	8	67.5	0.236 ± 0.010	73.9	26.1	0.190 ± 0.007	10.4
SJ	11	76.1	0.253 ± 0.010	25.5	74.5	0.211 ± 0.007	62.0
	12	75.0	0.256 ± 0.010	47.4	52.6	0.206 ± 0.006	28.8
FL	13	70.4	0.213 ± 0.010	41.9	58.1	0.181 ± 0.006	23.2
	15	67.1	0.255 ± 0.011	64.4	35.6	0.197 ± 0.007	9.5
	16	63.6	0.187 ± 0.010	62.9	37.1	0.175 ± 0.007	13.5
	18	67.9	0.205 ± 0.010	90.5	9.5	0.184 ± 0.008	14.6
CO	20	72.9	0.217 ± 0.010	25.2	74.8	0.184 ± 0.007	89.4
SM	21	70.7	0.263 ± 0.011	44.0	56.0	0.211 ± 0.007	17.8
	22	62.1	0.245 ± 0.011	51.0	49.0	0.199 ± 0.007	9.5
	23	64.3	0.265 ± 0.011	48.2	51.8	0.208 ± 0.007	9.2

PLP (%): proportion of polymorphic loci at the 5% level; H_j: Nei's genetic diversity; VarI%: proportion of variance due to sampling of individuals; VarL%: proportion of variance due to sampling of loci; H_s: speciesaverage Bayesian genetic diversity; DW: frequency-down-weighted index. Island and population codes are as in Table 1.

three island groups (Moura *et al.*, 2013; Borges Silva *et al.*, 2016; Dias *et al.*, 2016; Viera *et al.*, 2018). In *D. foliosa*, and providing evidence against a first-order barrier between Pico island populations, the second- to fourth-order barriers were all placed between the three sub-archipelagos (Fig. 5A). The first-order barrier computed for Pico could be associated with ecologically distinct conditions as it separated within-island populations from coastal populations. It thus appears that barriers to gene flow have contributed to shape the observed genetic structure of *D. foliosa* in the Azores. Similar findings were obtained for other Azorean endemic plants with marked genetic structure (e.g. *Lactuca watsoniana*; Dias *et al.*, 2018). Although of lesser importance (except for Pico), putative barriers to gene flow were estimated within single islands for all islands except São Jorge and Corvo (which had only one sampled population) (Fig. 5A).

Furthermore, cryptic diversity can be connected to island ages in archipelagos, but also to different substrates of varying ages (such as those connected to recent volcanism) and to areas of natural and human-mediated disturbances on single islands, creating 'islands within islands' (Crawford & Stuessy, 2016). In the Azores, several factors have been proposed to explain the genetic structure detected in various endemic plant taxa. Geology was one of the proposed factors, such as for *Viburnum treleasei* Gand., with Santa Maria island (the oldest island of the archipelago) showing a distinctive, albeit mainland-connected, genetic pattern (Moura *et al.*, 2013). In the case of *D. foliosa*, our best IBD model suggests a geologically driven dispersal scenario connected to the age of emergence of the Azorean islands (Fig. 5b). Structure may relate to ancient volcanism and resulting patterns of island formation (*Festuca francoi* and *F. petraea*; Díaz-Pérez *et al.*, 2008). In fact, large-scale volcanic events that took place in Terceira (Gertisser *et al.*, 2010) may have led to a recolonization process accounting for the observed within-island structure (Figs 3, 4).

Although reticulation was seen in *D. foliosa*, particularly in the central group (Figs 3, 4), possibly indicating mating through facilitated gene flow resulting from historical and recent human intervention but also from wind-mediated pollen dispersal and/or zoochory, it is also unquestionable that the populations of the western and eastern sub-archipelagos, and even some of the central sub-archipelago, mostly pertain to specific genetic groups and are distinct (Fig. 4; Table 2). As such, it is not advisable to translocate diaspores to these islands originating from populations of other groups of islands. Although artificially facilitating gene flow between isolated and low-density populations (two of the problems for *D. foliosa* pinpointed by Silva *et al.*, 2009) is often seen as a positive conservation effort to rescue genetically depauperate populations, it is also known that induced hybridization can sometimes increase the risk of outbreeding depression and compromise the evolutionary processes taking place in geographically isolated populations, therefore hampering the natural development of future biodiversity (Edmands, 2007; Weeks *et al.*, 2011; Crawford & Stuessy, 2016; Moura, Dias & Maciel, 2018).

Other threats to *D. foliosa* indicated by Silva *et al.* (2009), namely disturbance of sensitive areas, habitat degradation, changes in land use, invasion by alien species, herbivory and trampling, were evident in several of the sampled locations and require regulatory action by regional and local governments. In fact, *D. foliosa* was, in some cases, sampled in locations corresponding to ecosystems greatly affected by invasive species such as *Hedychium gardnerianum* Sheppard ex Ker Gawl., *Hydrangea macrophyla* (Thunb.) Ser. and *Gunnera tinctoria* (Molina) Mirb. Furthermore, cattle trampling was evident in the Caldeirão border on Corvo island (a UNESCO Biosphere Reserve, which harbours several conservation priority endemics) and in many other locations.

CONCLUSIONS

Population genetic diversity and structure data suggest that *D. foliosa* followed a similar dispersal pattern to one of the scenarios proposed by Díaz-Pérez *et al.* (2008) for *Festuca petraea*, i.e. colonization from the central group and posterior bidirectional dispersion to the western and eastern islands. However, it is also possible that the ages of the islands might have also shaped dispersal with an old-to-new, west-to-east, dispersal route. The observed genetic structure seems to reflect the concept of 'islands within islands' and possibly signals the occurrence of several ESUs. Conservation actions to reinforce *D. foliosa* populations should consider the population structure detected to avoid translocations that might counteract ongoing speciation processes. Augmentation of the populations will also benefit from eradication or control of invasive species and fencing to avoid herbivory and trampling by cattle.

ACKNOWLEDGMENTS

P.C. was funded by a Bioflora research team grant co-founded by the Spanish Aragón Government and the European Social Fund. This work was supported by a Portuguese Ministry of Science and Technology (POCTI/BME/39640/2001) research grant project.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Figure S1. Parsimony haplotype network for 23 populations of *Deschampsia foliosa*, obtained with PopART using the TCS Network algorithm. Black dots represent haplotypes not present in the sample. The number of steps (mutations) between haplotypes is given in parentheses. Terceira (red); Pico (yellow); São Jorge (orange); Flores (blue); Corvo (light blue) and São Miguel (magenta). Island codes are as in Table 1.

Figure S2. Scatter plot of a correlation test conducted between Nei's pairwise genetic distances matrix and a geographical distances matrix (metres) obtained for the *Deschampsia foliosa* populations studied. Equations are the linear functions of the Mantel test for isolation by distance (IBD). The permutation test showed a significant correlation ($P < 0.05$).