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ORIGINAL ARTICLE

Understanding intra and inter-archipelago population genetic patterns within a recently evolved insular endemic lineage

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Abstract The recently evolved genus *Tolpis* Adans. has its major center of diversity located in Macaronesia. Although recent advances have been made to understand the relationships of *Tolpis* species within Macaronesia, little is still known about the genetic patterns and genetic diversity of the Azorean and Madeiran *Tolpis* populations. To achieve this, a set of 8 microsatellite loci (SSR) was applied to 478 individuals of *Tolpis azorica* and *T. succulenta*. Genetic structure analysis, in addition to a spatial analysis, confirmed the existence of geographically circumscribed genetic patterns allied to a barrier effect by the sea in the Azorean *T. azorica* and *T. succulenta.* A detailed analysis of *T. azorica* revealed three different genetic groups, each group being particular to a different Azorean sub-archipelago, while the analysis conducted with *T. succulenta* confirmed the occurrence of a differential grouping between individuals from Azores and Madeira populations. The impact of catastrophic volcanic events and intense humanization of the habitats is discussed, in view of the present genetic diversity and structure of the species. In general, *T. azorica* populations showed high F_{is} values and some populations of *T. succulenta* both in Azores and in Madeira also showed signs of putative inbreeding. Conservation actions such as the eradication of invasive plant and animal species are advised but translocations of plants or diaspores between islands or between populations of a same island should not be attempted.

Keywords Azores · Endemics · Genetic structure · Madeira · Population genetics · *Tolpis*

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Introduction

The genus *Tolpis* Adans. consists of over a dozen species distributed in the Mediterranean, N. Africa, Europe, and the islands of Macaronesia, with its major center of diversity located in the Canary Islands (Hand et al. 2014). This study focuses on diversification of the genus within the Azores and Madeira archipelagos.

Tolpis azorica (Nutt.) P.Silva has been listed as the only species of the genus endemic to the Azores islands (Schaefer 2003, 2005; Silva et al. 2010) and it occurs in seven of the nine archipelago islands (being absent from Santa Maria and Graciosa; Fig. 1). *Tolpis succulenta* (Sol. in Aiton) Lowe, listed for all the Azorean islands although doubtful in Pico (Silva et al. 2010), has been considered to be a shared endemic with the Madeira archipelago, occurring in the two main islands (Madeira and Porto Santo) and the Desertas islands (Fig. 1). In Madeira Island, another endemic *Tolpis* species is present, namely, *Tolpis macrorhiza* (Lowe ex Hook.) DC.

The Azorean and Madeiran species show different ecological preferences, with *T. azorica* growing in the mountainous and very humid inlands of the Azorean islands, between 600 and 1000 m a.s.l. (Schaefer 2002), while *T. succulenta* can be found in the dryer coastal cliffs, rocks and rocky banks up to 600 m a.s.l. in Azores (Schaefer 2002) and up to 1000 m a.s.l. in Madeira, Porto Santo and Desertas (Press and Short 1994). In Madeira Island, *T. succulenta* can be seen up to 1500 m a.s.l. in open habitats, while *T. macrorhiza* prefers shady, moist rocky areas in the steeper valleys of central Madeira, above 700 m a.s.l. on rock outcrops up to 1400 m a.s.l. (this study). Despite an ecological divergence in their habitats, the presence in some islands of more than one of these endemic species, and the occurrence of at least one known inland population of *T. succulenta* in Azores raises the question of putative gene flow between their populations.

In a recent list detailing conservation priority for vascular plant species of the Azores (Silva et al. 2009), *T. succulenta* was evaluated with 4 (out of a maximum of 5 priority points) and *T. azorica* with 3 out of 5 points due to habitat degradation as a result of human activities and competition with exotic species. In addition, populations are isolated and composed of a low number of individual plants. In Madeira, *T. succulenta* is common, while *T. macrorhiza* is frequent, but rather scattered (Press and Short 1994).

A study conducted by Crawford et al. (2015) produced the first data on the breeding systems of Azorean and Madeiran *Tolpis* species and indicated that the Azorean *T. azorica* and *T. succulenta* are highly SI (self-incompatible), with low levels of PSC (pseudo-self-compatibility). In Madeira, *T. succulenta* was SI, but with capacity for occasional self-fertilization, whereas *T. macrorhiza* showed unusual characteristics such as infrequent flowering and the selfing success varying between flowering branches on a given plant.

Although phylogenetic relationships within *Tolpis* have been persistently difficult to resolve by previous studies (see Hand et al. 2014), a recent effort using rapidly

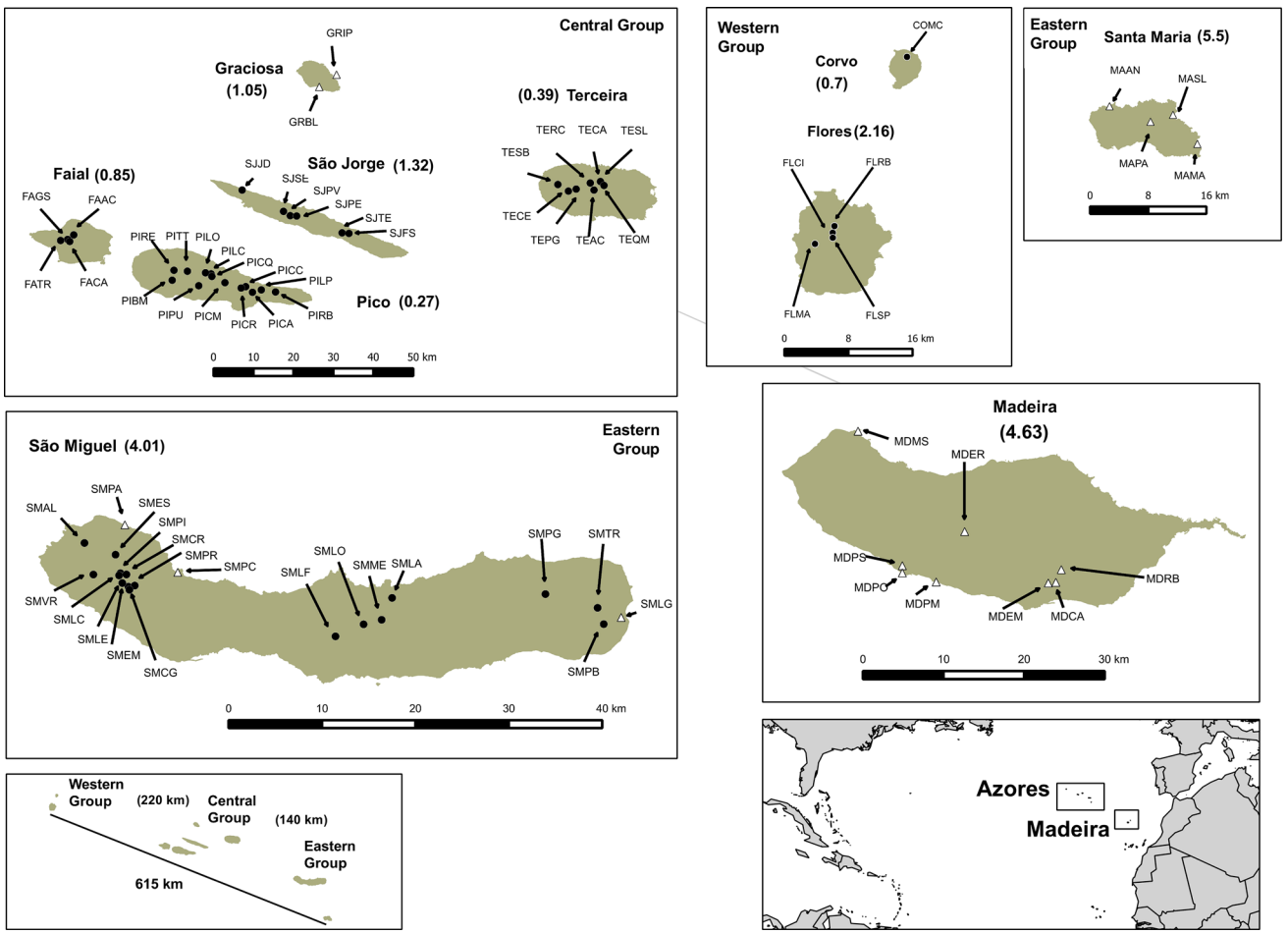

Fig. 1 Location of the *Tolpis* populations studied in the Azores archipelago and in Madeira Island. *Tolpis azorica* is represented by *dots* and *Tolpis succulenta* is represented by *triangles*. Island ages, in Myr, are shown between *brackets* (Larrea Márquez 2014; Geldmacher et al. 2000)

evolving nuclear markers (ETS) led Gruenstaeudl et al. (2012) to propose an evolutionary scenario of an early split into a Azorean-Madeiran clade and a Canarian-continental clade as well as a polyphyly in *T. succulenta*, although both of these hypothesis were not supported by all of the study markers. The polyphyly of *T. succulenta* had been suggested previously by Moore et al. (2002), from results obtained using chloroplast DNA restriction site variation. The relationships between the Azores and Madeira taxa were further resolved in a study by Mort el al. (2015), using multiplexed-shotgun-genotyping data, which clearly demonstrated that *T. succulenta* is not monophyletic, since the Madeira accession used in the study separates with strong support from the Azorean accessions of *T. succulenta* and *T. azorica*.

Furthermore, in a previous population study, ISSR markers proved to be useful in *Tolpis* for grouping individuals into populations and species although with generally low support (Mort et al. 2003), and samples from *T. succulenta* from Madeira and Porto Santo were sister to each other and clustered separately from Canarian species. However, when Archibald et al. (2006) applied an automated analysis of ISSR loci to a larger *Tolpis* dataset composed mainly of Canarian accessions and including only one sample of *T. succulenta* from Madeira, the ensuing topologies did not show support for the ancestral nodes and displayed incongruences at the deeper levels. Crawford et al. (2006), while studying the allozyme genetic variation among species of *Tolpis* in the Canary Islands, observed that it was generally similar to other insular endemics i.e. low genetic diversity within species, a relatively high proportion of the diversity among populations, and low divergence among species. In summary, although recent progress has more clearly resolved the evolutionary relationships between *Tolpis* species endemic to the Azores and Madeira, there is still a knowledge gap regarding population genetic structure and genetic diversity of those species.

Here, we focus on the population genetic structure and diversity of the Azorean-Madeiran *Tolpis* lineages using specifically developed microsatellite (SSR) primer pairs with a wide sampling spanning both archipelagos. The set of microsatellite markers developed for this study was used with a conservation purpose in mind as well as to better understand the elusive relationships between the Madeiran and Azorean lineages.

Specifically, the objectives of our study were to: (i) estimate the levels of genetic variability and diversity existing in Azores and Madeira *Tolpis* species populations; (ii) identify the populations with very low genetic diversity and thus priority for special conservation measures; (iii) determine the relationships between the Azores and Madeira *Tolpis* species and the correspondence between their currently known distributions and the genetic groups obtained; and (iv) detect the existence of other possible taxonomic entities besides those currently accepted for Azores and Madeira.

Materials and methods

Study sites and sampling

The Azores are located in the North Atlantic Ocean, within parallels 36° 55′ and 39° 43′ N and the meridians 24° 46′ and 31° 16′ W. They are geographically isolated, about 1430 km from the European continent and more than 3900 km from North America. The archipelago is composed of nine islands, distributed along a WNW-ESSE axis, and is divided into three geographic groups (Western, Central and Eastern), with several islets. The maximum spacing between the islands corresponds to the distance between Corvo and Santa Maria and exceeds 630 km (Fig. 1).

The Madeiran archipelago is located in the Atlantic Ocean southwest of the Iberian Peninsula, between latitudes 30° 01′ and 33° 07′ N and longitudes 15° 51′ and 17° 15′ W. The archipelago is formed by the island of Madeira (including three small uninhabited islets, the Desertas), the island of Porto Santo (surrounded by several small islets) situated in the extreme NE of the archipelago and the Selvagens archipelago, located between Madeira and the Canary Islands. The Desertas and the Selvagens are nature reserves.

Sampling of *T. azorica* was conducted in 53 different populations from seven islands of the Azores archipelago, resulting in 478 individuals (Fig. 1; Table 1). The sampling for *T. succulenta* was conducted in 17 different populations from three islands of the Azores and from Madeira Island, and included a total of 188 individuals. Samples of *T. macrorhiza* were collected from 24 individuals in three populations (Fig. 1; Table 1). The number of individuals sampled per population was proportional to population size and the area of occupancy of the populations was evenly sampled.

DNA extraction

Total DNA was extracted from 690 individuals of *Tolpis* using a modified Doyle and Dickson (1987) CTAB protocol. Approximately 3 cm^2 of fresh leaf tissue per sample was powdered with PolyvinylPyrrolidone and then incubated for 45 min at 65 °C in 500 µl of 2X CTAB (100 mM Tris–HCl pH 8.0; 1.4 M NaCl; 20 mM EDTA; 2 % CTAB), 50 µl of 10 % Sarkosyl buffer (100 mM Tris–HCl pH 8.8; 20 mM EDTA; 10 % Sarkosyl) and 10 µl of Proteinase K (AppliChem). The sample was thoroughly mixed with 500 µl of 24:1 chloroform: isoamyl alcohol and

Table 1 Number of samples (*N*); mean number of: alleles (*A*), polymorphic loci (*L*), expected heterozygosity (H_e), private alleles (*P*), locally common alleles (*R*; frequency ≥5 %; found in ≤25 % populations) and inbreeding coefficient (F_{is}) per population of *Tolpis azorica*, *T. succulenta* and *T. macrorhiza*

Taxon (group)	Island (N)	Population	Code	*N*	*A*	*L*	H_e	*P*	*R*	F_{is}
T. azorica (E)	São Miguel (148)	Road to Pico da Cruz	SMPI	5	3.5	7	0.603	0.0	0.8	0.12
		Lagoa do Canário	SMLC	5	3.3	6	0.559	0.0	0.8	0.27
		Caldeira do Alferes	SMAL	4	1.8	4	0.263	0.0	0.3	0.71
		Estaleiros	SMES	5	3.4	5	0.596	0.0	0.6	0.42
		Lagoa das Empadadas	SMEM	5	3.3	6	0.568	0.0	0.5	−0.47
		Pico do Carvão	SMPR	5	3.1	8	0.600	0.0	0.8	0.28
		Vista do Rei	SMVR	10	3.8	7	0.606	0.0	0.9	0.40
		Criação	SMCR	10	3.3	8	0.488	0.0	0.8	−0.67
		Lagoa do Caldeirão Grande	SMCG	3	2.8	7	0.558	0.0	0.5	−0.15
		Lagoa das Éguas	SMLE	10	3.6	4	0.567	0.0	0.9	−0.43
		Tronqueira	SMTR	16	4.3	7	0.572	0.0	1.0	0.04
		Lombadas	SMLO	10	4.0	7	0.582	0.0	0.9	0.23
		Planalto dos Graminhais	SMPG	8	2.6	7	0.331	0.0	0.1	0.61
		Monte Escuro	SMME	10	4.0	7	0.564	0.0	1.0	0.03
		Pico Bartolomeu	SMPB	13	4.0	8	0.514	0.0	1.0	0.05
		Lagoa Do Fogo	SMLF	26	4.9	7	0.569	0.0	1.3	0.11
		Lagoa do Areeiro	SMLA	3	1.9	5	0.358	0.0	0.4	0.59
T. azorica (C)	Pico (103)	Pico da Urze	PIPU	9	4.3	6	0.610	0.1	0.8	0.67
		Cabeço da Cruz	PICC	5	2.6	6	0.508	0.0	0.5	0.41
		Cabeço do Caveiro	PICA	10	3.9	7	0.554	0.0	0.6	−0.07
		Cabeço do Mistério	PICM	10	4.4	6	0.595	0.1	0.5	0.13
		Cabeço Raso	PICR	5	3.1	6	0.494	0.0	0.5	0.86
		Cabeço Redondo	PIRE	10	4.6	7	0.636	0.0	0.6	0.44
		Lagoa do Capitão	PILC	5	2.9	6	0.540	0.0	0.4	−0.51
		Lomba do Capitão	PILO	5	2.9	6	0.568	0.0	0.1	0.80
		Baldio de S. Mateus	PIBM	10	3.8	7	0.520	0.1	0.6	−0.05
		Caldeirão da Ribeirinha	PIRB	10	2.8	5	0.430	0.0	0.3	0.33
		Lagoa do Peixinho	PILP	10	3.5	6	0.541	0.0	0.3	0.31
		Transversal, near Torrinhas	PITT	10	3.3	5	0.563	0.0	0.1	0.47
		Curral Queimado	PICQ	4	3.1	7	0.603	0.0	0.1	0.53
	Terceira (105)	Algar do Carvão	TEAC	10	3.6	8	0.580	0.0	0.6	−0.39
		Caldeira de Santa Bárbara	TESB	20	5.3	6	0.618	0.1	1.5	0.25
		Moldes, Serra do Labaçal	TESL	10	3.8	7	0.596	0.0	0.8	0.64
		Rocha do Chambre	TERC	15	4.8	8	0.601	0.0	1.1	0.49
		Caldeira de Agualva	TECA	10	3.6	6	0.543	0.1	0.3	0.07
		Quinta da Madalena	TEQM	10	3.8	6	0.560	0.0	0.8	0.33
		Pico do Gaspar	TEPG	15	4.1	6	0.598	0.0	1.1	−0.01
		Cancela do Estaleiro	TECE	15	3.9	7	0.552	0.1	1.0	0.30
	S. Jorge (48)	Road to Fajã do João Dias	SJJD	1	1.4	4	0.500	0.1	0.1	0.00
		Road to Pico da Esperança	SJSE	11	4.0	6	0.580	0.0	0.5	0.83
		Pico Verde	SJPV	5	3.8	6	0.631	0.0	0.5	0.43
		Pico da Esperança	SJPE	11	5.0	7	0.663	0.0	1.3	0.51
		Terreirão	SJTE	10	3.6	7	0.538	0.0	0.1	−0.40
		Trail to Fajã do Santo Cristo	SJFS	10	4.0	6	0.568	0.0	0.3	0.34
	Faial (41)	Alto do Chão	FAAC	10	4.3	6	0.595	0.1	0.8	0.60
		Alto do Guarda-Sol	FAGS	10	4.0	5	0.637	0.0	0.5	0.18

Table 1 continued

Taxon (group)	Island (N)	Population	Code	*N*	*A*	*L*	H_e	*P*	*R*	F_{is}
		Caldeira	FACA	11	3.9	6	0.606	0.0	0.8	0.62
		Cabeço dos Trinta	FATR	10	4.1	7	0.610	0.0	0.8	0.63
T. azorica (W)	Flores (22)	Cidrão	FLCI	5	3.4	7	0.658	0.0	0.9	−0.59
		Road to Morro Alto	FLMA	2	2.5	7	0.729	0.0	0.8	0.45
		Pico dos Sete Pés	FLSP	5	2.9	7	0.478	0.1	1.0	−0.46
		Ribeira da Badanela	FLRB	10	4.3	7	0.600	0.1	1.1	0.11
	Corvo (11)	Cumeeiras do Caldeirão	COMC	11	3.8	7	0.587	0.3	1.3	−0.05
T. succulenta (E)	Sta. Maria (44)	Anjos	MAAN	10	4.8	5	0.722	0.4	1.2	−0.02
		Maia	MAMA	10	4.6	5	0.662	0.2	0.8	0.27
		Pico Alto	MAPA	14	4.6	5	0.618	0.2	1.6	−0.25
		São Lourenço	MASL	10	6.0	5	0.772	0.4	2.4	0.26
	São Miguel (52)	Praia do Lombo Gordo	SMLG	19	4.2	5	0.517	0.0	1.4	0.04
		Porto da Ajuda	SMPA	16	4.2	5	0.463	0.2	1.4	−0.14
		Porto Velho, Capelas	SMPC	17	3.6	5	0.574	0.0	1.2	−0.15
T. succulenta (C)	Graciosa (25)	Baía do Filipe, Beira-Mar da Luz	GRBL	15	2.0	4	0.219	0.0	0.2	0.92
		Ilhéu da Praia	GRIP	10	1.8	2	0.182	0.2	0.4	−0.09
T. succulenta (M)	Madeira (67)	Canhas, road to Ponta do Sol	MDPS	4	2.2	4	0.464	0.0	0.2	−0.06
		Road Encumeada-Ribeira Brava	MDER	5	2.6	2	0.528	0.0	0.2	0.11
		Road to Ribeira Brava at miradouro	MDRB	10	3.8	3	0.488	0.4	0.6	0.08
		Road to Camacha, Eira do Serrado	MDCA	8	3.2	3	0.440	0.0	0.8	−0.05
		Descent Eira do Serrado to Monte	MDEM	11	3.0	3	0.512	0.0	0.2	−0.19
		Road Monte-Santo António	MDMS	6	2.4	3	0.455	0.0	0.0	−0.23
		Porto Moniz	MDPM	12	3.0	3	0.486	0.2	0.4	−0.34
		Ponta do Sol	MDPO	11	4.4	2	0.587	0.4	0.8	0.87
T. macrorhiza (M)	Madeira (24)	Encumeada to Chão dos Louros	MDCL	6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
		Encumeada to Ribeira Brava	MDED	9	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
		Road Poiso-Ribeiro Frio	MDPR	9	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Grouped by Azorean subarchipelagos (*E* East; *C* Central; *W* Western) or by archipelago (*M* Madeira)

centrifuged 3 min at 13,000 rpm. The supernatant was mixed with 450 µl of isopropanol to allow DNA precipitation and then centrifuged 15 min at 12,000 rpm. The liquid phase was discarded and the pellet obtained was allowed to dry for 80 min at 30 °C in a dry bath before being re-suspended in 50 µl of pure water. The quality and quantity of samples were measured using a Nanodrop 2000 (Thermo Fisher Scientific) spectrophotometer. Samples were stored at −20 °C until use.

Microsatellites development, selection and genotyping

Microsatellite isolation was accomplished at the Savannah River Ecology Lab (University of Georgia) using DNA extracted from fresh leaves of *T. azorica* from São Miguel Island following the enrichment procedure described in Glenn and Schable (2005), with the exceptions described in Lance et al. (2010). Sequences were subjected to a 3′ quality trim where only one base in the last 25 bases of the sequence contains a quality score less than 20 or alternatively contains one ambiguous base. CAP3 (Huag and Madan 1999) was then used to assemble sequences at 98 % sequence identity using a minimal overlap of 75 bp. Along with singlets, contigs of two or three sequences were searched for the presence of microsatellite DNA loci using the program MSATCOMMANDER ver. 0.8.1 (Faircloth 2008) and primers designed with Primer3 (Rozen and Skaletsky 2000). The posterior testing and selection of primers were performed at the University of the Azores. One primer from each pair was extended on the 5′-end with an engineered sequence (M13R tag 5′-GGAAA-CAGCTATGACCAT-3′) to enable the use of a third primer identical to the M13R, which allows for an inexpensive fluorescent labelling of the PCR product obtained (Schuelke 2000), and a GTTT "pigtail" was added to the 5′-end of the untagged primer to facilitate accurate genotyping (Browstein et al. 1996). Out of a total of 327 sequences developed, 24 primers pairs, divided into two series by size range to allow for multiloading of PCR

products, were selected based on criteria of non-complementarities within and between primers, low secondary structures and 3′-end instability (Rychlik 1995). After an initial screening for polymorphism using a reduced subset of samples, eight primer pairs were selected for *T. azorica* and five for *T. succulenta*, while no amplification was achieved for *T. macrorhiza*. The primers and optimized protocols used in the full-scale genotyping are described in Tables 2 and 3. Five microliter of PCR product was run on a 3.5 % agarose gel, stained with SafeView™ Classic Nucleic Acid Stain (ABM Inc.) and visualized under UV to check for amplification, polymorphism and scorability of the bands. The amplification products were diluted, multiloaded and run on an ABI-3130xl Genetic Analyzer and sized with LIZ500 size standard. The genotypes were scored using the software GeneMarker® ver. 1.97 Demo version (Softgenetics®).

Data analysis

To determine the genetic structure of *Tolpis*, we used a Bayesian Markov chain Monte Carlo (MCMC) approach to estimate the number of genetic clusters. The analysis was run with the program STRUCTURE version 2.3.4 (Pritchard et al. 2000) using a batch-oriented web program package for construction of super matrices ready for phylogenomic analyses (Kumar et al. 2009). We ran 10 replicates for each K value ranging from 1 to 10 with 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 value was conducted with STRUCTURE Harvester (Earl and von Holdt 2012) following the Evanno et al. (2005) method. The optimal K repetitions were permuted in Clumpp version 1.1.2 (Jakobsson and Rosenberg 2007) using the Greedy algorithm and results were graphically represented using Distruct version 1.1 (Rosenberg 2004). As recommended by Pritchard et al. (2000), STRUCTURE analyses were also run in subsets of the complete matrix to obtain a more detailed genetic structure using the same parameters described above. To further analyse the genetic structure of *Tolpis* in Azores and Madeira, Principal Coordinate Analysis (PCoA) was performed with Genalex ver. 6.501. R_{st}-like statistics (Slatkin 1995; Excoffier and Lisher 2010) and gene flow estimates ($M = 2$ Nm; Slatkin 1991), in addition to an analysis of molecular variance (AMOVA; Excoffier et al. 1992), were calculated with Arlequin ver. 3.5.1.3. Three Mantel tests between the genetic distance matrixes and the corresponding geographic distance matrixes of populations with five or more individuals were conducted using Genalex ver. 6.501, to determine the occurrence of isolation by distance (IBD) between populations in each of the studied taxa. To estimate the order of barriers to gene flow possibly occurring within the three Azores sub-archipelagos, the same genetic distance matrixes and geographic coordinates used in the Mantel test were further analysed with the Monmonier (1973) algorithm using BARRIER version 2.2 (Manni et al. 2004). The genetic diversity of *Tolpis* in the Azores and Madeira was described using the software Arlequin ver. 3.5.1.3 (Excoffier and Lischer 2010), by calculating the number of

Table 2 Description of the eight polymorphic SSR loci that exhibited acceptable to high scorability in the genus *Tolpis*

Name	Ta	Ts	Primer	Sequences	Repeat motif	Size range (bp)	Dye
TA2A03	√	√	Forward	*CAC CTC CCT CTA CAA TGA C	$(AC)^8$	148–152	FAM
			Reverse	†AAC TCA CAA AGC GAA CAC AG			
TA3B02	√	√	Forward	†CCA ATT AAA CGG AAA GAG AC	$(AAC)^7$	240–260	FAM
			Reverse	*GCT TGC TAC TTG AGG ACT C			
TA2A01	√	√	Forward	*TAC CGT AGA ACC CAA ACT C	$(AG)^9$	175–225	NED
			Reverse	†AAA TTG TAT GAG CCC ACA AC			
TA3B05	√	√	Forward	*AAC AAC TCC ATG CCA CAC	$(AAT)^{12}$	300–350	VIC
			Reverse	†GCA ATC TTA TCG TCT GTG			
TA2A09	√	–	Forward	*GAA CGA GAA GAA AGA GAT TGT C	$(AG)^8$	125–150	VIC
			Reverse	†GAT TCC ATC CCT TTC TTT ATC			
TA2A07	√	–	Forward	*GAA GAA GAA CAA GAT CCT TTG	$(AC)^8$	175–200	VIC
			Reverse	†AAC ACG AAC GGT AAA TGT ATC			
TA4B07	√	–	Forward	*ACC TAC GAA CAT TCA TAC AAA C	$(ACAT)^6$…	180–250	PET
			Reverse	†GTA GAA GTA AAG GGC CAT TG	$(ACAT)^{14}$		
TA2A02	√	√	Forward	*GGA ATT AAT CGG AAA TTG	$(AG)^{10}$	126–140	PET
			Reverse	†CAC AAA CCC TAA CAG TTC C			

* Indicates M13R tag (5′-GGAAACAGCTATGACCAT-3′); †Indicates "pigtail" tag (5′-GTTT-3′); Ta (*T. azorica*); Ts (*T. succulenta*)

Table 3 PCR conditions for the polymorphic microsatellites obtained from the Azores and Madeira *Tolpis*

Marker	PCR mix (V_f = 25 µl–25 ng of DNA)	Cycling program
TA2A03	75 µg/ml of BSA, 1X NH4 Buffer, 3 mM MgCl2, 0.4 µM untagged primer, 0.08 µM tagged primer, 0.36 µM dye, 200 µM dNTP, 1 U of Biotaq	95 °C for 3 min; 20 cycles: 95 °C for 30 s, 65 °C (Touchdown −0.5 °C each cycle) for 30 s, 72 °C for 30 s; 20 cycles: 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; 72 °C for 10 min
TA3B02	75 µg/ml of BSA, 1X NH4 Buffer, 3 mM MgCl2, 0.4 µM untagged primer, 0.04 µM tagged primer, 0.36 µM dye, 200 µM dNTP, 1 U of Biotaq	95 °C for 3 min; 20 cycles: 95 °C for 30 s, 65 °C (Touchdown −0.5 °C each cycle) for 30 s, 72 °C for 30 s; 20 cycles: 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; 72 °C for 10 min
TA2A01	75 µg/ml of BSA, 1X ImmoBuffer, 3 mM MgCl2, 0.4 µM untagged primer, 0.08 µM tagged primer, 0.36 µM dye, 200 µM dNTP, 0.75 U of Immolase	95 °C for 4 min; 22 cycles: 95 °C for 30 s, 64 °C (Touchdown −0.5 °C each cycle) for 45 s, 72 °C for 45 s; 11 cycles: 95 °C for 30 s, 53 °C for 45 s, 72 °C for 45 s; 72 °C for 10 min
TA3B05	85 µg/ml of BSA, 1X NH4 Buffer, 3 mM MgCl2, 0.2 µM untagged primer, 0.05 µM tagged primer, 0.1 µM dye, 200 µM dNTP, 1 U of Biotaq	95 °C for 4 min; 22 cycles: 95 °C for 30 s, 64 °C (Touchdown −0.5 °C each cycle) for 45 s, 72 °C for 45 s; 11 cycles: 95 °C for 30 s, 53 °C for 45 s, 72 °C for 45 s; 72 °C for 10 min
TA2A09	75 µg/ml of BSA, 1X ImmoBuffer, 3 mM MgCl2, 0.4 µM untagged primer, 0.08 µM tagged primer, 0.36 µM dye, 200 µM dNTP, 0.5 U Immolase	95 °C for 4 min; 22 cycles: 95 °C for 30 s, 64 °C (Touchdown −0.5 °C each cycle) for 45 s, 72 °C for 45 s; 11 cycles: 95 °C for 30 s, 53 °C for 45 s, 72 °C for 45 s; 72 °C for 10 min
TA2A07	75 µg/ml of BSA, 1X NH4Buffer, 3 mM MgCl2, 0.4 µM untagged primer, 0.2 µM tagged primer, 0.2 µM dye, 200 µM dNTP, 1 U of Biotaq	95 °C for 4 min; 22 cycles: 95 °C for 30 s, 64 °C (Touchdown −0.5 °C each cycle) for 45 s, 72 °C for 45 s; 11 cycles: 95 °C for 30 s, 53 °C for 45 s, 72 °C for 45 s; 72 °C for 10 min
TA4B07	75 µg/ml of BSA, 1X ImmoBuffer, 3 mM MgCl2, 0.4 µM untagged primer, 0.08 µM tagged primer, 0.36 µM dye, 200 µM dNTP, 0.75 U Immolase	95 °C for 7 min; 96 °C for 3 min; 20 cycles: 95 °C for 30 s, 65 °C (Touchdown −0.5 °C each cycle) for 30 s, 72 °C for 30 s; 20 cycles: 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; 72 °C for 10 min
TA2A02	75 µg/ml of BSA, 1X ImmoBuffer, 3 mM MgCl2, 0.4 µM untagged primer, 0.08 µM tagged primer, 0.36 µM dye, 200 µM dNTP, 0.75 U of Immolase	95 °C for 7 min; 96 °C for 3 min; 20 cycles: 95 °C for 30 s, 63 °C (Touchdown −0.5 °C each cycle) for 30 s, 72 °C for 30 s; 20 cycles: 95 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s; 72 °C for 10 min

polymorphic loci and, across loci, the mean number of alleles, and the mean expected heterozygosity (H_e) per population, as well as the total expected heterozygosity per main genetic group or taxa using the unbiased estimate of Nei (1987). The numbers of private and locally common alleles per population (with frequency equal or above 5 % and found in 25 % or fewer populations) were calculated by Genalex ver. 6.501 (Peakall and Smouse 2012). The population matrix is available at DEMIURGE (http://www.demiurge-project.org/) with digest codes D-NMICR-102 and D-NMICR-103.

Results

Genetic structure

According to the Bayesian analysis performed with STRUCTURE and taking into consideration the variation across runs, the partitioning of individuals for each putative K and the methodology proposed by Evanno et al. (2005) (data not shown), *T. azorica* and *T. succulenta* are composed of six main genetic groups ($K = 6$; Fig. 2a). In *T. azorica,* those groups (i–vi) are predominant in: (i) individuals from São Miguel (designated in subsequent analysis as TaSM); (ii and iii) individuals from Pico, Terceira, São Jorge, and Faial (aggregated under the designation TaC); (iv) individuals from Flores and Corvo (TaW). The remaining two genetic groups mainly occur in *T. succulenta* individuals, one mostly in the Azores (v; TsAZ) and the other in Madeira (vi; TsM).

Specific analyses with STRUCTURE were subsequently conducted to detect the existence of further genetic structures in *T. azorica*. For São Miguel, two subgroups were retrieved. One subgroup prevailed in the populations of the western part of the island and another subgroup in the populations of the central and eastern part of the island (Fig. 2b). For Pico, Terceira, São Jorge and Faial (Fig. 2c), two genetic subgroups were recovered; one prevailing in individuals from Pico, São Jorge and Faial, and the other in individuals from Terceira. In Flores and Corvo, two other predominant genetic groups were found, separating both islands (TaFL and TaCO).

A STRUCTURE analysis was also conducted solely with *T. succulenta*. In the Azores, three genetic subgroups were found: (i) one subgroup was mainly found in Santa Maria and in one of São Miguel populations (Lombo Gordo) (TsLGMA), (ii) a second genetic subgroup was mainly found in two populations from São Miguel (TsSM); and (iii) a third subgroup (blue) clearly predominated in individuals from Graciosa (TsG). (Figure 2d). In Madeira, no structure was obtained (data not shown).

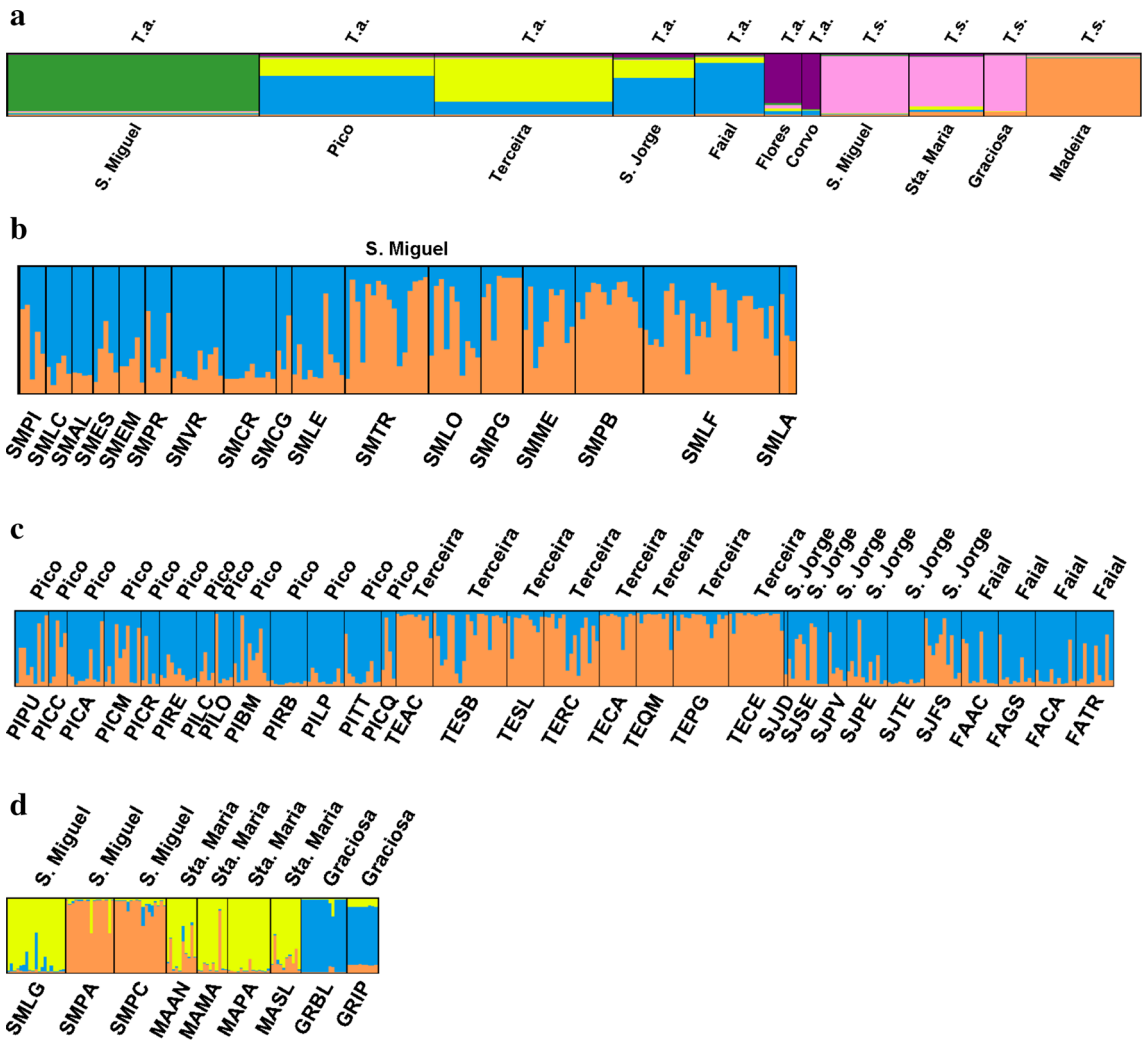

Fig. 2 Graphic display of STRUCTURE outputs. Individuals are represented as *thin vertical lines* partitioned into segments corresponding to their membership in genetic clusters indicated by the colours. **a** *Tolpis azorica* and *Tolpis succulenta* from Azores and Madeira; **b** *Tolpis azorica* from São Miguel; **c** *Tolpis azorica* from the Central Group; **c** *Tolpis succulenta* from Azores

The PCoA analysis of the *T. azorica* dataset clearly revealed three main groups corresponding to São Miguel populations, Central Group populations and Western Group populations (60.7 % of total variance explained by the first two factors; Fig. 3). Regarding *T. succulenta*, Azores populations clustered on the right side of the graph, while the individuals from Madeira clustered on the left (71.3 % of total variance explained by the first two factors; Fig. 4). Moreover, in the Azores, the two Graciosa populations were distinct and clearly separated from those of the Eastern Group (Fig. 4).

Following STRUCTURE results, three AMOVA analyses with different grouping criteria were used to detect which of these criteria resulted in the highest percentage of variation among groups (Table 4): (i) populations in two groups, according to the currently accepted taxonomic units (*T. azorica* and *T. succulenta*) accounted for the lowest percentage of variation among groups (3.4 %); (ii) populations in five groups, according to the global analysis from STRUCTURE, resulted in an increase in the percentage of variation among groups (29.9 %); and (iii) populations in eight groups, based on the subgroup analysis from STRUCTURE, lead to the highest percentage of variation among groups (39.4 %).

The Mantel tests conducted with the Azorean *T. azorica* and *T. succulenta*, as well as with the Madeiran *T. succulenta* datasets, resulted in a correlation between the genetic and geographic distances of $R = 0.789$, $P = 0.010$ for *T. azorica* and $R = 0.729$, $P = 0.020$ for *T. succulenta* in the Azores (Fig. 5a, b), while no significant correlation was retrieved for the Madeiran dataset ($R = 0.070$, $P = 0.420$).

The spatial analysis conducted with BARRIER on the Azorean populations of *T. azorica* indicated that the first-order barrier to gene flow was located between the Western

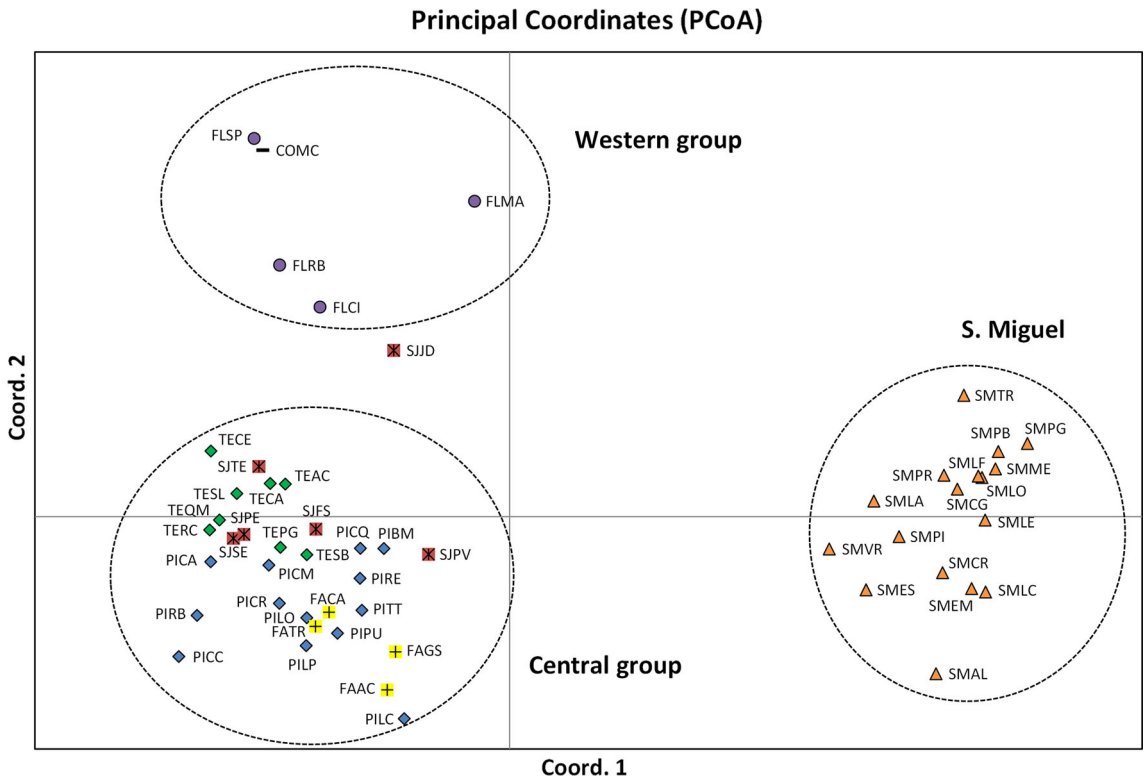

Fig. 3 PCoA obtained from the molecular data of *Tolpis azorica*

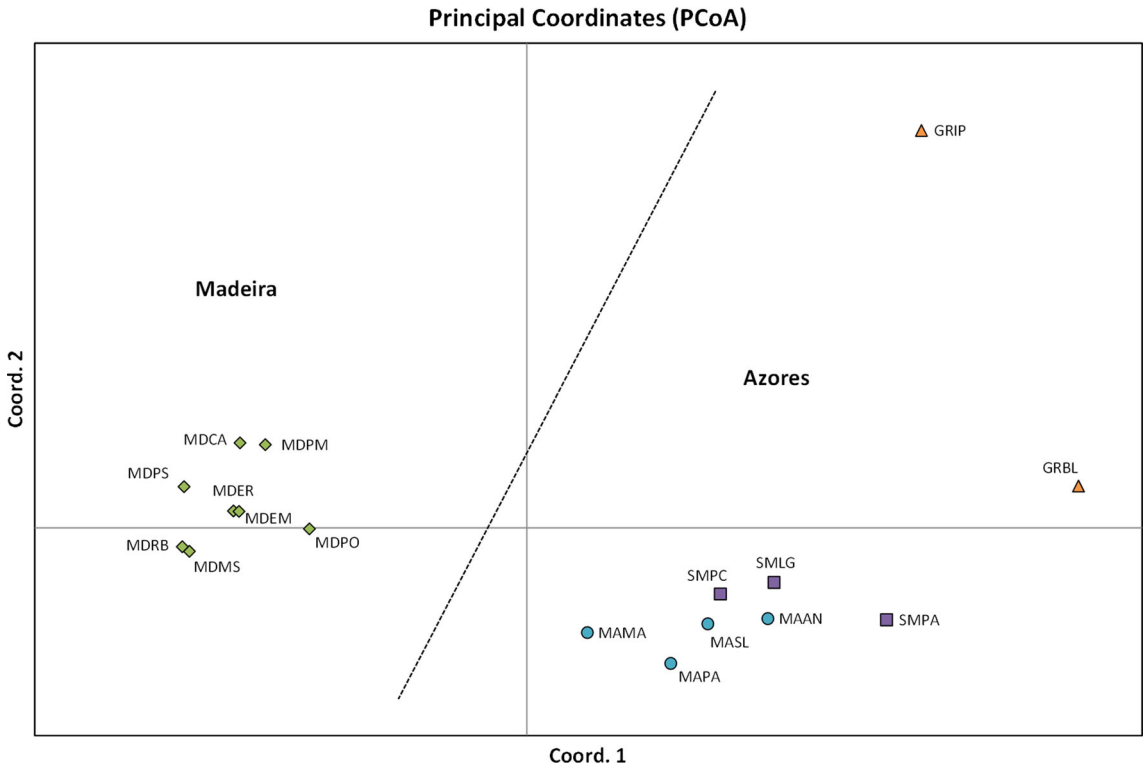

Fig. 4 PCoA obtained from the molecular data of *Tolpis succulenta*

Table 4 Analysis of molecular variance (AMOVA) applied to the populations of *T. azorica* (Azores) and *T. succulenta* (Azores and Madeira), grouped according to three different criteria: (i) the currently accepted taxonomic units; (ii) five population groups derived from STRUCTURE; and (iii) eight subgroups derived from STRUCTURE

Source of variation	*df*	S.s.	Variance	Variation (%)
TaAZ/TsAZM				
Among groups	1	6561.190	7.81623	3.4
Among populations within groups	68	126087.638	90.20220	39.5
Within populations	596	89576.548	20.01898	8.8
Within individuals	666	73432.000	110.25826	48.3
Total	1331	295657.375	228.29567	
TaSM/TaC/TaW/TsAZ/TsM				
Among groups	4	71196.158	71.12178	29.9
Among populations within groups	65	61452.669	42.31271	17.36
Within populations	596	89576.548	20.01898	8.2
Within individuals	666	73432.000	110.25826	45.2
Total	1331	295657.375	243.71173	
TaSM/TaC/TaFL/TaCO/TsLGMA/TsSM/TsG/TsM				
Among groups	7	99670.404	98.26921	39.4
Among populations within groups	63	32978.424	20.67394	8.3
Within populations	596	89576.548	20.01898	8.0
Within individuals	666	73432.000	110.25826	44.2
Total	1331	295657.375	249.22039	

Ta Tolpis azorica, *Ts T. succulenta*, Azorean sub-archipelagos: *E* East, *C* Central, *W* Western, *AZ* Azores, *FL* Flores, *CO* Corvo, *M* Madeira

and Central groups, while the second was located between the Central and Eastern groups (Fig. 6a). This is in agreement with the lack of gene flow that was generally found among the populations of the three sub-archipelagos (i.e. Western Group, Central Group, Eastern Group) using the formula $M = 2\text{Nm}$ where M is the number of migrants, Nm is the estimated gene flow and no gene flow occurs when $M < 2$ (Fig. 7a). In the *T. succulenta* dataset, the first-order barrier was located between the Graciosa island populations and the second-order barrier was located between Graciosa and São Miguel and Sta. Maria islands (Fig. 6b). This also agrees with the absence of gene flow between populations of *T. succulenta* on Graciosa, and between Graciosa populations and all other Azorean populations (Fig. 7b).

Regarding the estimation of gene flow between Madeira and Azores, very low levels (Fig. 7b) were found between the two archipelagos for *T. succulenta* except between the Madeiran populations of Canhas, Eira do Serrado-Monte and Porto Moniz and two Azorean populations of Sta. Maria (Maia and Pico Alto). In some cases, very low levels of gene flow were found among Madeiran populations (Fig. 7b).

Genetic diversity

In *T. azorica*, an average of 7.0 polymorphic loci was found, with an average allelic richness of 8.1 alleles and a total expected heterozygosity of 0.716 (Table 1). The group with the highest allelic richness and allelic size range (ASR) was the Central Group with 10.5 alleles and an ASR of 25.5, while São Miguel showed the lowest (6.9 alleles; ASR of 20).

The average allelic richness of *T. succulenta* in Azores was 9.4 alleles, obtained from 5 polymorphic loci, with a total expected heterozygosity of 0.743 (Table 1). Within Azores, the *T. succulenta* group with the highest average heterozygosity (0.724) corresponded to São Miguel and Sta. Maria, with Graciosa displaying the lowest H_e value (0.450). The number of alleles and the ASR for Graciosa populations were also the lowest (3.0 vs. 9.0 and 6.0 vs. 23.8, respectively). In Madeira, an average of 6.4 alleles was found with 3 polymorphic loci with an ASR of 23 and a total expected heterozygosity of 0.572.

For *T. azorica* (Table 1), Pico, Terceira and Corvo had the highest number of populations with private alleles, and the latter island also had the highest mean values for private alleles per population (0.3), while São Miguel did not possess any private alleles. The population of Caldeira de Sta. Bárbara in Terceira had the highest mean of locally common alleles (1.5), followed by the populations of Pico da Esperança in S. Jorge and Cumeeira do Caldeirão in Corvo, both with 1.3 locally common alleles. For *T. succulenta* (Table 1), Sta. Maria and Madeira showed the highest number of populations with private alleles (4 and 3 populations, respectively), while the population of S. Lourenço at Sta. Maria displayed the largest number of locally common alleles (2.4). The

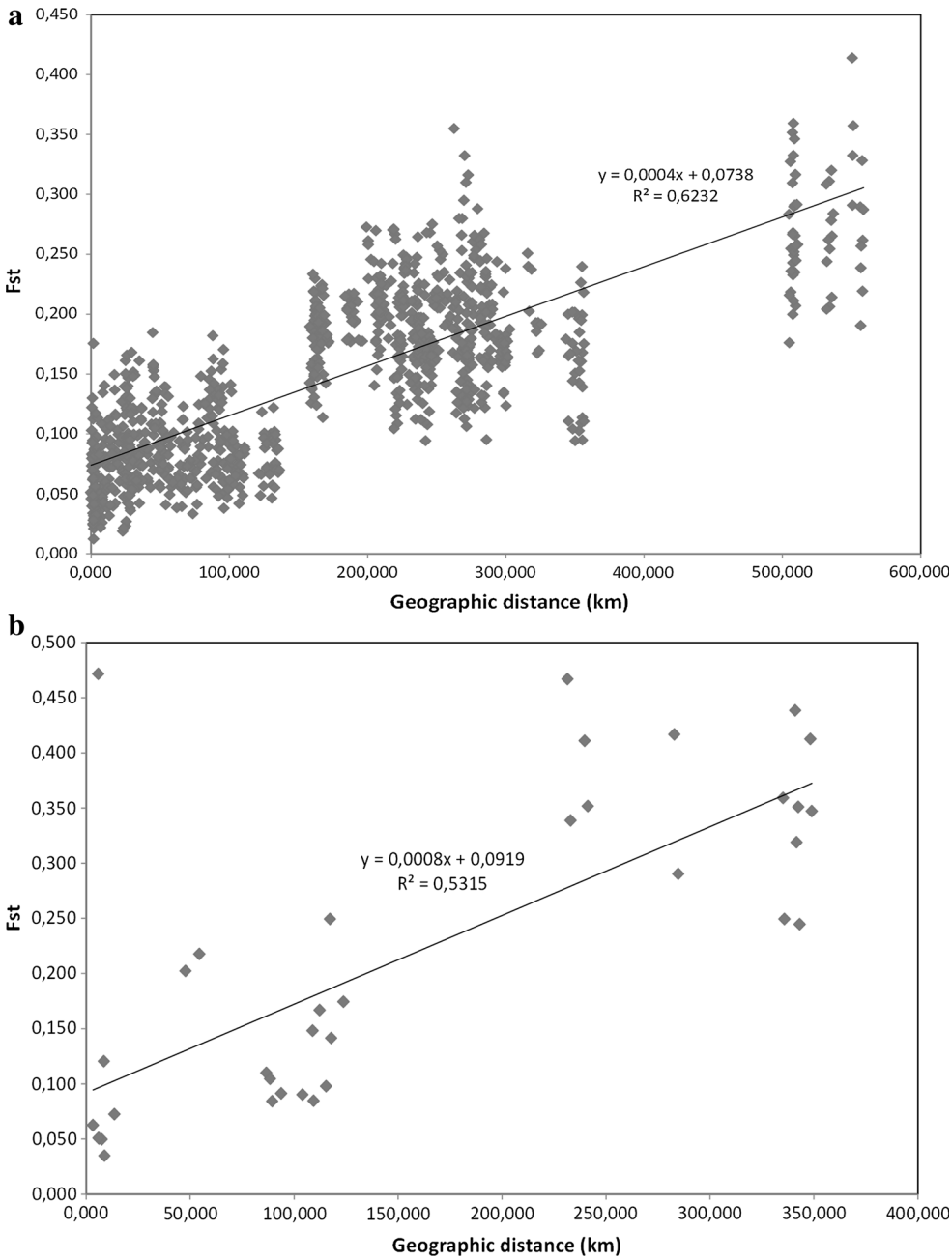

Fig. 5 Graphic display of the Mantel test calculated for *Tolpis azorica* (**a**) and *Tolpis succulenta* (**b**)

population of Monte-Santo António did not show locally common alleles.

Most populations of *T. azorica* displayed F_{is} values ranging from 0.03 (Monte Escuro) to clearly positive values such as 0.86 (Cabeço Raso), while *T. succulenta* showed an overall lower number of populations with a positive inbreeding coefficient, both in Azores (4 out of 9 populations) and in Madeira (3 out of 8 populations), with the highest values obtained for Baía do Filipe in Graciosa (0.92), and Ponta do Sol in Madeira (0.87).

Discussion

Genetic structure and putative speciation

This population genetic study of *Tolpis* species from Azores and Madeira revealed geographically circumscribed genetic patterns for *T. azorica* within the Azores archipelago and a clear separation of the Azorean and Madeiran accessions of *T. succulenta*. This complex structure was supported by AMOVA, PCoA and Bayesian model-based clustering.

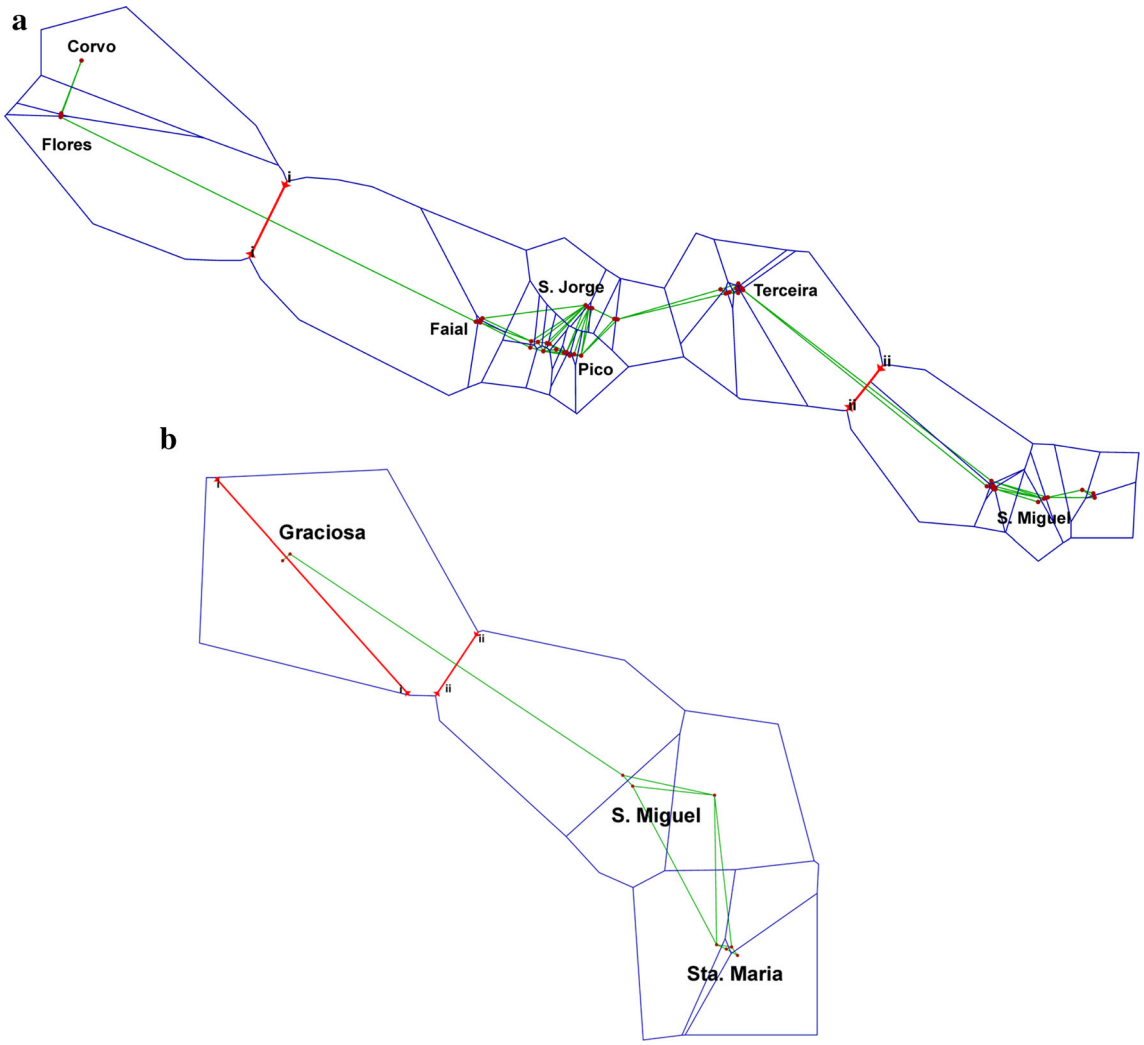

Fig. 6 Graphic display obtained with BARRIER for *Tolpis azorica* (**a**) and *Tolpis succulenta* (**b**). *Dots* populations' locations; *red lines* putative barriers to gene flow listed alphabetically according to their estimated relevancy; Delaunay triangulation shown in *green* and Voronoï tessellation in *blue*

Within the Azores, the genetic structure of *T. azorica* was composed of four main genetic groups, clearly defining three population clusters coinciding with the three sub-archipelagos (São Miguel, Central Group and Western Group). These findings could indicate the existence of overlooked cryptic species within *Tolpis* in the Azores and further studies based on morphological characters and molecular phylogenetic analysis is currently underway. The occurrence of a Linnean shortfall in the Azores, i.e. the estimation that only a fraction of the existing species has been described so far, linked to cryptic diversity in Azorean endemic taxa, was demonstrated by Schaefer et al. (2011).

The detailed STRUCTURE analysis retrieved evidence of further complexity. For example, the populations of *T. azorica* from Terceira Island are shown to be genetically distinctive compared to the remainder of the populations on the central group of Azorean islands. Other Azorean endemics with a unique pattern for Terceira include *Juniperus brevifolia* (Seub.) Antoine (Silva et al. 2011) and *Leontodon filii* (Hochst. ex Seub.) Paiva and Ormonde (Dias et al. 2014). Although it has been assumed that Terceira is the oldest island of the Central Group (3.52 Myr; see França et al. 2003 for a review), a study by Calvert et al. (2006) indicates that it is less than 0.39 Myr. Therefore, the genetic pattern obtained for Terceira may be better explained by the volcanic history of the island and its intense effect on plant populations. Massive population extirpations might have occurred due to soil sterilization by the formation of thick volcanic deposits on a large percentage of the island surface (Calvert et al. 2006). A similar explanation was also hypothesized by Silva et al. (2011) regarding the genetic pattern found in the Terceira populations of *J. brevifolia*.

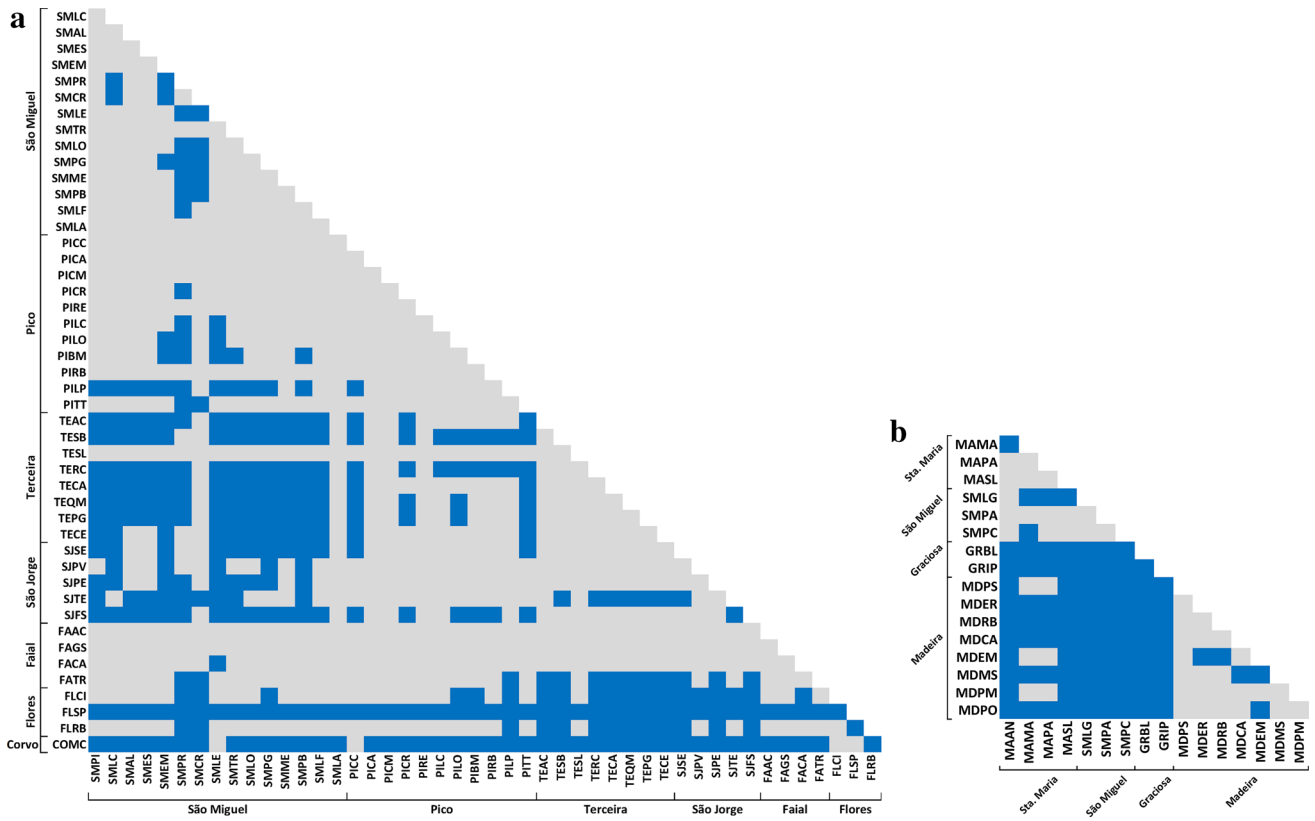

Fig. 7 Gene flow estimation according to Slatkin (1991) for *Tolpis azorica* (**a**) and *Tolpis succulenta* (**b**). *Blue squares* absence of gene flow ($M < 2$; $M = 2\text{Nm}$)

Within São Miguel Island, two genetic groups were obtained, differentiating the western populations from the central and eastern populations. Within-island genetic patterns were also reported for *Viburnum treleasei* Gand. (Moura et al. 2013). It is possible that these within-island patterns in the case of *Tolpis* could be connected to habitat fragmentation and decrease of population sizes resulting from human activities, competition with invasive alien plant species and herbivory by introduced animals such as rabbits, goats and cattle (Silva et al. 2009; Moura et al. 2013). Habitat transformation was especially relevant in the hydrographic basins of Furnas on São Miguel, where no populations of *Tolpis* were found, and in Sete Cidades (Sjögren 1973), and might explain the different genetic characteristics of the populations located in the western part of São Miguel. Populations from São Miguel showed the lowest overall values of allelic richness and of ASR, as well as mostly positive F_{is} values, suggesting the occurrence of recent genetic bottlenecks with loss of allelic diversity and heterozygosity (Nei et al. 1975; Allendorf 1986; Comps et al. 2001). Additionally, catastrophic volcanic events, such as those referred for Terceira Island, also occurred in São Miguel, with plinian-type eruptions in Lagoa do Fogo, in the central part of the island, which have occurred from prehistoric times (4.6 ky) to the most recent eruption, about one century after the arrival of Portuguese settlers (1563) (Walker and Croasdale 1971; Booth et al. 1978). These intense volcanic episodes likely led to extensive soil sterilization due to thick layers of trachytic pumice (Walker and Croasdale 1971; Booth et al. 1978), and it is possible that new populations, eventually in the same locations as those of the populations now studied, were formed by recolonization events with founders coming from surviving eastern populations. The Sete Cidades area was also affected by a series of volcanic events in the last 5000 years, the most recent and largest one occurred ca. 600 BP years (Booth et al. 1978). Thus, it is also possible that this series of partial or complete obliteration of populations due to thick pumice layering may have also contributed to the present genetic patterns observed in the westernmost populations of São Miguel studied here (Fig. 1; Table 1).

The effect of the sea as a geographical barrier to gene flow is evident in the genetic structure obtained for *T. azorica* and in the spatial analysis with the two most important barriers estimated to occur between the three subgroups of islands. In insular taxa, the occurrence of different genetic patterns is often connected to gene flow

barriers, such as the sea, which result in island isolation (Franks 2009) and speciation in an archipelago (Whittaker and Fernández-Palacios 2007). Although the values obtained for the Mantel test in *T. azorica* and *T. succulenta* were significant, they should not be interpreted as a sign of IBD since the graphical output produced for the Mantel test shows that the distribution of plots does not follow a linear model. This reasoning is further confirmed by the spatial analysis, which identified the presence of geographical barriers to gene flow between the subgroups of islands, i.e. corresponding to the sea. Eventual bias connected to IBD tests and the need to conduct additional spatial analysis to detect unaccounted overlapping effects of geographical barriers was previously discussed by Meirmans (2012). This author cautioned that by inducing allelic alterations in the sampling, the effect of geographic barriers may erroneously imply the occurrence of IBD.

Regarding the ancestry of *T. azorica*, considering the present results and the phylogeny obtained by Mort et al. (2015), one hypothetical evolution scenario would be that a widespread form of *T. succulenta*, originating from the Madeira archipelago (see further discussion below), may have undergone subsequent cladogenetic differentiation in São Miguel and in the Central and Western subarchipelagos, resulting in three putative main OTUs (i.e. TaSM, TaC and TaW) that may be presently distributed in the archipelago. However, considering that the putative OTU with higher genetic diversity occurs in the Central Group, this could be interpreted as corresponding to the most ancient form of *T. azorica* (sensu lato), which could have later dispersed to the Western Group, forming genetically more similar populations. This interpretation is further supported by the higher number of populations with private and locally common alleles in Pico, Terceira and Corvo. Private alleles have been used as a measure of population differentiation (Kalinowski 2004; Szpiech and Rosenberg 2011), while locally common alleles have been interpreted as adaptations to local habitat conditions (Marshall and Brown 1975; Brown and Marshall 1995). Considering also that the São Miguel *T. azorica* form has the lowest genetic diversity and no private alleles, notwithstanding the effect of volcanism and human activities as previously discussed, this extant diversity could also be partially connected to recent evolution, resulting from a second event of cladogenesis in the Azorean *T. succulenta*. Further analyses are needed to complement this information, and the morphological and phylogenetic revision currently underway should provide more data on the differentiating mechanisms that acted in *T. azorica*. Other Azorean Asteraceae, belonging to the genus *Leontodon* were recently confirmed to have evolved into three different species, endemic to each of the Azorean subgroups of islands (Moura et al. 2015), an evolution pattern that seems to be replicated in *Tolpis*.

The genetic structure displayed by *T. succulenta* confirms the results reported by authors such as Mort et al. (2015), Gruenstaeudl et al. (2013), and Moore et al. (2002), and corroborate that *T. succulenta* is probably polyphyletic and should be formally split into two separate taxa. The fact that three populations of *T. succulenta* from Madeira show putative gene flow with two populations from Santa Maria suggests that colonization of the Azores archipelago from the Madeira archipelago might have occurred on Santa Maria, the oldest of the Azorean islands (5-(5.5)-6 Myr; Feraud et al. 1981; Ávila et al. 2012; Fig. 1) with subsequent origin of the Azorean form of *T. succulenta* by anagenesis, followed by intra-archipelago east–west dispersal of this form. This putative dispersal route seems to be further supported by the genetic diversity values, which decrease from Santa Maria to Graciosa populations, with São Miguel populations displaying intermediate values, and by the fact that the higher number of populations with private alleles occurs in Madeira and in Sta. Maria. Low genetic diversity in insular populations has been frequently linked to the founder effect (Frankham 1997; Caujapé-Castells 2011). Successive east–west colonization events with associated loss of diversity due to the founder effect could account for the values obtained for *T. succulenta* in the Azores. An east–west dispersal pattern was also suggested for other Azorean endemics, namely, *V. treleasei* (Moura et al. 2013) and *Festuca francoi* Fern. Prieto, C. Aguiar, E. Dias and M. I. Gut, using AFLP markers (Díaz Pérez et al. 2008). An additional effect of the sea as a geographical barrier seems to have influenced the present genetic structure of the Azorean *T. succulenta* populations with a putative barrier to gene flow separating populations in the Eastern Group from those in Graciosa. It is noteworthy that the population from São Miguel (SMLG; Fig. 1) that shows putative gene flow with most of the Santa Maria populations is located on the east side of the island, nearest to Santa Maria island. A first-order barrier to gene flow was computed between the two populations of Graciosa, which are geographically apart and located on opposite sides of the island. Population GRBL is located on the SW shore of the island and GRIP is located on the Ilhéu da Praia islet facing the NE shore of Graciosa (Fig. 1). The lowest allelic number and H_e value for *T. succulenta* in Azores was obtained for the Ilhéu da Praia population, which may indicate that the islet was colonized from Graciosa. However, the genetic pattern obtained for the islet population was slightly closer to the Eastern Group populations than the pattern obtained for the population of Baía do Filipe on Graciosa, although this may have resulted from the random nature of a founder effect coupled with a geographic barrier.

Regarding the possible colonizers of the Azores archipelago, previous work (Moore et al. 2002; Gruenstaeudl et al. 2012; Mort et al. 2015) does not provide clear indication of which of the two *Tolpis* taxa that occur in the Madeira archipelago, or their ancestors, may have colonized the Azores. However, results obtained by Moore et al. (2002) suggest that *T. macrorhiza* is probably not sister to *T. succulenta* from Madeira and preliminary morphological results (Borges Silva 2012) indicate that *T. macrorhiza* is clearly distinct from both Azores and Madeira *T. succulenta*. Considering that Porto Santo (where only *T. succulenta* occurs) is the oldest island in the Madeira archipelago (Porto Santo 11.1–14.3 Myr; Madeira >4.6–0.7 Ma; Geldmacher et al. 2000) and much older than any Azorean island (Fig. 1), it is likely that Azorean founders may have come from this island instead of Madeira. The higher percentage of populations with private alleles in Madeira (47 % for Madeira, 21 % for Azores; Table 1) also seems to support this hypothesis. In addition, higher allelic richness of *T. succulenta* in Azores (9.4 alleles) when compared with *T. azorica* (8.1 alleles) also suggests that an ancient *T. succulenta* form may be at the origin of the Azorean radiation. Molecular and morphological data on Porto Santo *T. succulenta* and on *T. macrorhiza* are currently being gathered to further clarify the Azores/Madeira evolutionary paths.

Genetic diversity and conservation

The genetic diversity (H_e) obtained for the Azores species of *Tolpis* studied here was higher than the values obtained by Crawford et al. (2006) and Levsen et al. (2008) for the Canary Islands endemic *Tolpis* species, and it is also higher when compared to other Macaronesian Asteraceae such as the Azorean endemic *Leontodon* species (Dias et al. 2014), and *Sonchus gandogeri* Pitard (Kim et al. 2005), *Atractylis arbuscula* Svent. and Michaelis and *Atractylis preauxiana* Sch. Bip. (Caujapé-Castells et al. 2008a, b), all endemic to the Canary Islands. The Madeira *T. succulenta* OTU showed only slightly higher values than those obtained for *Leontodon filii* (Hochst. ex Seub.) Paiva and Ormonde (Dias et al. 2014), which occurs solely in the Azorean Central sub-archipelago (Moura et al. 2015). It is however noteworthy that, with the exception of *Leontodon*, all other total heterozygosity values were estimated using more conservative genetic markers, such as allozyme and RAPD markers.

Most populations of *T. azorica* (sensu lato) displayed F_{is} values that may indicate the occurrence of inbreeding. One of the possible causes, as previously discussed, is associated with habitat alterations due to direct (i.e. land use changes) or indirect human intervention (i.e. introductions of invasive plants and animals) that presently constitute threats to the survival of native and endemic species in the Azores (Silva et al. 2008). Although some measures have been taken to locally control invasive species, they are widespread even within Island Natural Parks (Costa et al. 2013). Among the invasive species are rabbits and goats, the latter often feral, that roam in areas where remains of native vegetation still persist, such as in Caldeirão, in Corvo Island (feral goats and cattle) and in Sete Cidades and Lagoa do Fogo, in São Miguel Island (goats and rabbits). Those threats have been recently confirmed in the case of the rare Azorean endemic, *Veronica dabneyi* Hochst. ex Seub. (Silva et al. 2015). Therefore, control and exclusion measures for herbivores should be considered as a priority in areas devoted to the preservation of the endemic flora. Likewise, plant invaders such as *Hedychium gardnerianum* (L. fil.) C. Presl and *Hydrangea macrophylla* (Thunb.) Ser., the latter regrettably often used as a tourist symbol of the Azores, should be locally removed, since they can develop into monospecific stands directly outcompeting herbaceous species such as *T. azorica* (sensu lato) (Silva et al. 2015, 2008).

Although, in general, the number of *T. succulenta* populations with positive F_{is} was fewer than *T. azorica* populations, cases like those of Baía do Filipe (Graciosa) and Ponta do Sol (Madeira) are of concern and should be addressed. Coastal endemic plants are also subject to a number of threats, which, in the Azores, include coastal landscaping, recreational use of the habitat, debris accumulation and competition with invasive plant species such as *Carpobrotus edulis* (L.) L. Bolus, *Aptenia cordifolia* (L. fil.) Schwantes, *Cyrtomium falcatum* (L. fil.) C. Presl and *Arundo donax* L. (Silva et al. 2008, 2009). An effective control of these invasive species should thus be implemented and management plans for coastal areas should give high priority to the conservation of locations where populations of endemic and native species still persist (Silva et al. 2009).

Although some of the populations displaying higher F_{is} values are likely candidates for conservation actions, translocation of individuals of *T. azorica* and *T. succulenta* should take into account the genetic structure demonstrated in this study to avoid genetic erosion from hybridization with specimens of different genetic groups. Recent studies have discussed the risks and benefits of translocations and authors, such as Weeks et al. (2011), argued that these can either have beneficial (heterosis) or deleterious (outbreeding depression) effects on reproductive fitness. According to Edmands (2007), the available data on the use of rescue techniques suggest that risks of outbreeding, particularly in the second generation, are on par with the risks of inbreeding. Edmands (2007) therefore recommends that hybridization should only be used for populations suffering from inbreeding depression, with tests of the effects of

hybridization conducted for at least two generations. Additionally, based on a meta-analysis, Frankham (2015) argues that there are no scientific impediments to the use of outcrossing to genetically rescue inbred populations of naturally outbreeding species. However, it is noteworthy that, with the exception of one species from the island of Mahé, the plants included in the study focussed on American and European continental species. Considering that the use of hybridization will maximize the genetic and adaptive similarity between populations (Edmands 2007), it will result in genetic erosion in insular plant endemics with marked genetic patterns across an archipelago, such as those observed in the Azorean *Tolpis*. Furthermore, according to Carlson et al. (2014), there is not enough evidence of the benefits or dangers of evolutionary rescue and, in cases where the effect of hybridization resulting from short-term rescue actions might lead to the fixation of adaptive alleles, there is a risk of ostensibly reducing standing genetic variation, thus compromising future rescue.

Conclusions

In agreement with previous studies, *T. succulenta* appears to be polyphyletic, since the Azorean populations have a distinctive genetic pattern when compared to *T. succulenta* from Madeira, while *T. azorica* needs further work to clarify whether other taxonomic units exist in the Azores. In the future, the current dataset should be expanded to include populations from Porto Santo, and specific SSR primers should be developed for *T. macrorhiza* because the primers used for other species in this study were not transferable to this species.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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