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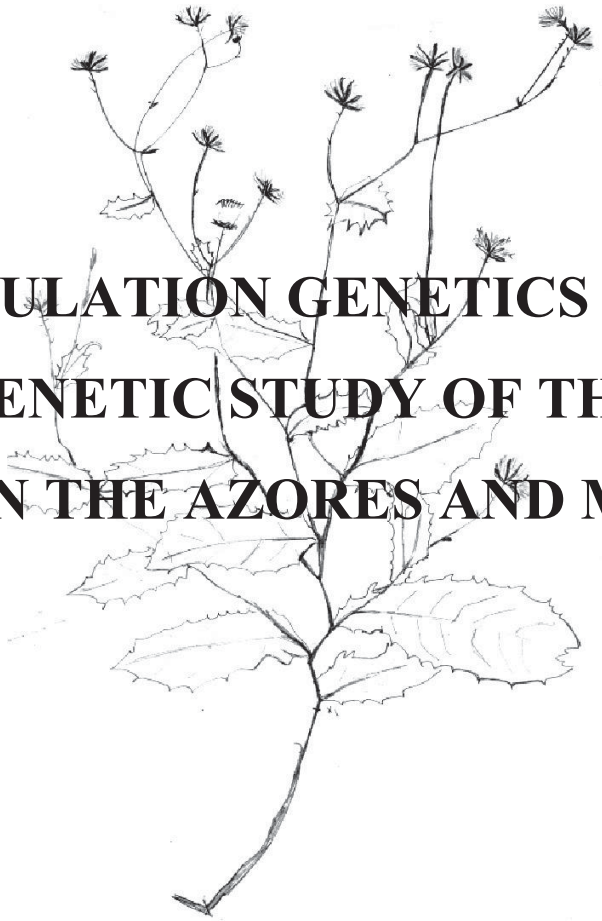

POPULATION GENETICS AND PHYLOGENETIC STUDY OF THE GENUS *TOLPIS* IN THE AZORES AND MADEIRA

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Resumo

O género *Tolpis* é constituído por aproximadamente 12 espécies que se encontram distribuídas por África, Europa e pelas ilhas da Macaronésia. O género *Tolpis* está incluído na tribo cosmopolita Lactuceae Cass. pertencente à família Asteraceae. No arquipélago dos Açores, *T. azorica* (Nutt.) P. Silva, é considerada como endêmica e está atualmente indicada como presente em todas as ilhas do arquipélago com excepção de Santa Maria e Graciosa. *T. succulenta* (Dryander in Aiton) Lowe é indicada para todas as ilhas do arquipélago dos Açores, ocorrendo também no arquipélago da Madeira.

Para estimar a variabilidade intra e intergenética nas várias populações de *Tolpis* spp. nos Açores e na Madeira, foi utilizado um conjunto de 5 microsatélites (SSR) em 478 indivíduos de *Tolpis* spp. Para estes marcadores, *T. azorica* apresenta 59 alelos no total (média de 11,8), oscilando entre 5, para o marcador TA3B02, e 22 para o marcador TA3B05, apresentando um excesso global de homozigotia (Multilocus Fis=0,298, variando desde -0,05 para TA3B05 a 0,49 para TA2A01). Quanto a *T. succulenta,* foram obtios 56 alelos no total (média de 11,2), variando entre 7 para o marcador TA2A02 e 18 para TA3B05, com um excesso global de homozigotia (Multilocus Fis=0.383, desde 0.06 para TA3B05 a 0.582 para TA2A01). As duas espécies têm um valor equivalente de alelos raros, nomeadamente 55.9% para *T. azorica* e 57.1% para *T. succulenta.* Após a realização de uma Análise de Componentes Principais (ACP) e análises Bayesianas confirmou-se a existência de uma estrutura populacional composta por três grupos bem definidos. Nesta estrutura *T. azorica* surge dividida em dois grupos, sendo o terceiro composto por *T. succulenta*. Uma análise mais detalhada a *T. succulenta* confirmou um agrupamento diferencial entre indivíduos pertencentes a populações dos Açores e da Madeira.

Análises morfológicas e dados moleculares obtidos a partir de sequencias da região nuclear ITS foram utilizadas para esclarecer as relações filogenéticas entre as espécies de *Tolpis* endémicas presentes nos arquipélagos dos Açores e da Madeira. Os resultados obtidos são congruentes com os obtidos através da análise da estrutura genética populacional e têm como resultado final a nomeação de duas novas espécies, que são aqui descritas pela primeira vez. Uma actualização da circunscrição geográfica das espécies de *Tolpis* endémicas dos Açores é aqui também indicada.

Acknowledgments

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I also want to dedicate this accomplishement to my deceased grandfather António Borges, I wouldn't be who I am and be able to persue my dreams without his life example and his personal love of botany. For always supporting and be there a very special thank you to my boyfriend Lisardo, to my sister Paula that always has been my safe harbour, also to my mother Luísa an my grandmother Lurdes for all their support and worries. Finally to my nephews and the rest of the family.

Abstract

Tolpis consists of approximately 12 species distributed in Africa, Europe, and the islands of Macaronesia. The genus *Tolpis* is included in the cosmopolitan tribe Lactuceae Cass., within the Asteraceae family. In the Azores Islands, *T. azorica* (Nutt.) P. Silva, is an endemic specie to the archipelago and is currently listed as present in all the islands except Santa Maria and Graciosa. *T. succulenta* (Dryander in Aiton) Lowe is said to occur in all the Azores Islands and is shared with the Madeira archipelago.

To evaluate intra and intergenetic variability in several populations of Azorean and Madeiran *Tolpis* spp., a set of 5 microsatellite loci (SSR) were applied to 478 individuals of *Tolpis* spp. As main results, *T. azorica* exhibits 59 alleles in total (average of 11.8), ranging from 5 for marker TA3B02 to 22 for TA3B05, and an overall excess of homozygotes (Multi*locus* Fis=0.298, ranging from -0.05 for TA3B05 to 0.49 for TA2A01), while *T. succulenta,* exhibits 56 alleles in total (average of 11.2), ranging from 7 for marker TA2A02 to 18 for TA3B05, and an overall excess of homozygotes (Multi*locus* Fis=0.383, ranging from 0.06 for TA3B05 to 0.582 for TA2A01). The two species have an equivalent value of rare alleles, 55. 9% for *T. azorica* and 57.1% for *T.succulenta.* A Principal Component Analysis (PCoA) and a Bayesian approach confirmed the existence of a population genetic structure with three well-confirmed groups, where *T. azorica* is divided in two groups, with the third one aggregating the *T. succulenta* individuals. A further detaled analysis to *T. succulenta* confirmed the occurrence of a diferencial grouping between individuals from Azores and Madeira populations.

Morphological analysis and molecular sequence data from the ITS nuclear region was used for elucidating the phylogenetic relationships of the endemic *Tolpis* spp. in the archipelagos of Azores and Madeira. The results are congruente with the population genetic structure analyses obtained, and as main results two new species are named and described here for the first time. An update of the geographic circunscription of the Azorean endemic *Tolpis* spp. is also indicated here.

Introduction

Island endemics represent some of the most unusual and rare plants in the world (Carlquist, 1974) and have long fascinated biologists because of their different morphologies and interest as systems for the study of plant evolution (Stuessy and Ono, 1998; Emerson, 2002).

The endemic floras of oceanic islands provide some of the clearest and most well known examples of adaptive radiation (Givnish & Systsma, 1997). High rates of endemism are due to biocontainment of such islands, which are usually colonized by only a very small number of continental species that evolve, leading frequently to speciation. This is common in herbaceous settlers which often radiate after colonizing new types of habitats (Carlquist 1974, Givnish 1998).

In this study we focus on the genetic variability of several *Tolpis* species which are part of the endemic flora of the Azores and Madeira archipelagos.

The Azores and Madeira archipelagos belong to the Macaronesian Region, composed by a set of North Atlantic islands with biological affinities, due to the colonization process. In addition to the Azores and Madeira, it includes the archipelagos of Cape Verde, Canary Islands and the Salvage Islands. There are many endemic species in the Macaronesian archipelagos, with a greater number of endemics in the Canary Islands (Moore et al., 2002).

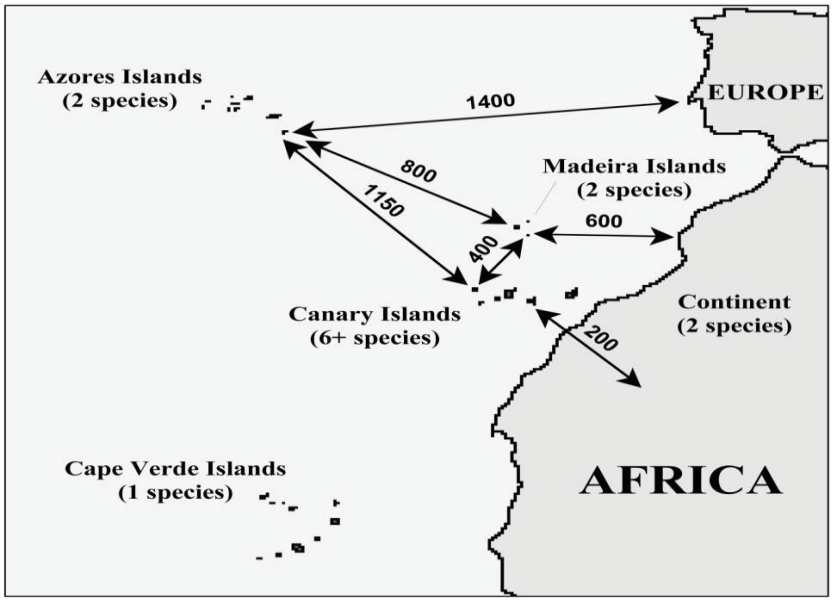


Fig. 1. Distribution of *Tolpis* across Macaronesia and the adjacent continents of Africa and Europe, along with distances used in computing minimum distance (*D)* scores. Adapted from Morre et al, 2002

The Azores are located in the North Atlantic Ocean, within a range bounded by the parallel 36 º 55 '43" and 39 º 43' 02" N and the meridians 24 º 46 '15" and 31 º 16' 02" W. The position it occupies manifests itself in a strong geographic isolation, since it is roughly about 1430 km from the European continent and more than 3900 km from North America. The archipelago, consisting of nine islands, is divided into three distinct groups (Western, Central and Eastern) with several islets and has a direction WNW-ESSE, due to regional tectonic phenomena. The maximum spacing between the islands exceeds 340 nautical miles (630 km), and corresponds to the distance between Corvo and Santa Maria (Azevedo, 1996).

The surface of the islands (2334 km²) is about 2.6% of the country (88,797 km²). However, the islands show very unequal size: the largest, São Miguel (745.8 km²), Pico (448.4 km²) and Terceira (403.4 km²), representing 70% of the total area, São Jorge (245, 9 km²), Faial (173.8 km²) and Flores (141.6 km²) have an intermediate size, Santa Maria (97.1 km²), Graciosa (61.2 km²) and Corvo (17.2 km²) are the smallest. Given the criteria of UNESCO, which defines "small islands" with an area less than 1000 km2, all Azores islands are included in this classification (Azevedo, 1996).

The maximum elevation of the islands is highly variable, ranging from 402 m in Graciosa and 2351 m at Pico Mountain, the highest point of Portugal. Pico Island is the most eccentric in terms altimetric with 16% of the area above 800m.

The Madeira archipelago is located in the Atlantic Ocean southwest of the Iberian Peninsula, roughly between latitudes 30 °01'N and 33 °31'N and longitudes15º17º30'W 51'W of Greenwich. The archipelago has a total area of approximately 796.8 km², and is formed by the island of Madeira with 736 km², a length of about 58 km in the direction E – W and a width of 23 km in direction N - S (Ribeiro, 1949), and the island of Porto Santo with 42.26 km², situated in the extreme NE of the archipelago, and, therefore, closer to the European and African continents. Near Madeira two groups of uninhabited islands occur, the Desertas and the Salvage Islands, which are nature reserves in the archipelago. The first covers an area of 14.2 km² and include the islands of the Deserta Grande, Bugio and Chão. The latter with an area of 3.6 km², comprising the islands of Selvagem Grande, Selvagem Pequena and the islet of Fora. Together, these constitute an archipelago individualized, reaching 250 km SSE of the eastern extremity of Madeira.

Tolpis is characterized by a suite of morphological characters, including usually annual or perennial herbaceous habit, mostly rosulate leaves, capitula arranged in

corymbs or panicles yellow florets, styles short, cypselas ribbed and glabrous, and a pappus of few to many long scabrid bristles interspersed with minute scales (Park et al. 2001). The genus belongs to the Asteraceae, which is one of the largest botanical families, consisting of approximately 110 genera and 2500 species. There have been several systematic approaches on the Asteraceae family (Cronquist, 1955; Poljakov, 1967; Carlquist, 976; Wagenitz, 1976; Jeffrey, 1978; Robinson, 1981, 1983; Thorne, 1983; Bremer, 1987) and most of these studies acknowledged that there are two subfamilies, Lactucoideae and Asteroideae, with *Tolpis* nested in the sub-family Lactucoideae.

The genus *Tolpis* is included in the cosmopolitan tribe Lactuceae Cass. It is currently considered a tribe within the Asteraceae family comprising 98 genuses and over 1550 species (Bremer, 1994). A phylogenetic study conducted in the Asteraceae based on morphological (Karis et al., 1992) and molecular (Jansen et al., 1991, Him et al., 1992) data indicated that Lactuceae is monophyletic, and their closest relatives Liabeae Rydb Cass. and Vernoniae Cass.

The classification of infratribal Lactuceae has been the focus of several morphological and molecular studies to find the right place of *Tolpis* (Stebbins 1953; Jeffrey, 1966; Tomb, 1975, 1977; Baagoe, 1980; Blackmore, 1981; Whitton, 1981; Bremer, 1994). Stebbins (1953) placed *Tolpis* in subtribe Cichoriinae with seven other genera and suggested that *Tolpis* was most closely allied to *Arnoseris* and *Hispidella* (Park et al., 2001). Jeffrey (1966) placed *Tolpis* in an informal *Tolpis* subgroup and allied it to *Arnoseris* based on several morphological characters. Pollen investigations of Blackmore (1981) suggested that the small pollen grains with double rows of spines on the equatorial ridges support a close relationship between *Arnoseris*, *Hispidella* and *Tolpis*.

Bremer's (1994) subtribal treatment, which was based on a morphological cladistic analysis of 22 genera representing the major monophyletic groups of Lactuaceae, classified *Tolpis* in the Hieraciinae with six other genera. Jarvis (1980) suggested that morphological similarities of the pappus and cypselas and chromosome number suggest a close relationship between *Tolpis* and *Hieracium.*

Based on studies of Park *et al.* (2001), their analysis of the Lactuceae using the chloroplast gene *ndhF* found *Tolpis* to be monophyletic and nested within the Lactuceae as a sister group to a large clade containing such genuses as *Lactuca* L., *Crepis* L., *Reichardia* Roth, *Dendroseris* and *Hypochoeridinae*. However the morphological

cladogram did not resolve satisfactorily the intergeneric relationships in the Lactuceae (Park et al. (2001).

Tolpis consists of approximately 12 species distributed in Africa, Europe, and the islands of Macaronesia (Jarvis, 1980; Park et al., 2001; Moore et al., 2002). Eleven species are Macaronesian endemics, with at least one species native to each of the four major island group (Jarvis, 1980).

In the Azores Islands, *T. azorica* (Nutt.) P. Silva, has been listed as the only species of the genus endemic to the archipelago (Silva et al., 2011), and considered present in all the islands except Santa Maria and Graciosa. *T. succulenta* (Dryander in Aiton) Lowe, said to ocurr in all the Azorean islands, is shared with the Madeira archipelago. Another *Tolpis* endemic occurring in the Madeira archipelago is *T. macrorhiza* Lowe.

T. azorica grows in the mountainous, very humid, inlands of the islands while *T. succulenta* in the Azores and Madeira archipelagos is found on the dryer coastal cliffs, rocks and rocky banks up to 1000 m, in Madeira also occasionally up to 1500 mm in open habitats. Despite this ecological cleavage in their habitats, the presence in some islands of both of these endemics raises the question of putative gene flows between the two species.

An extensive synonymy revision of *T. azorica* and *T. succulenta* was conducted by Lack in 1981 (see "Taxonomic treatment" in Discussion). The holotype of *T. azorica* is a specimen from Pico collected by the Hochstetter (father and son) in 1838, and held at TUB herbarium. All other types of different synomys are from the central or western groups of islands. The holotype of *T. succulenta* can be found at BM and was cultivated at Kew from material obtained at the Madeira Island. All synonym types are equally originated from Madeira.

Lack (1981), observed that unusual plants of *T. azorica* have been collected in São Miguel and Santa Maria islands with long hairs at the base of the achenes and somehow odd distribution of the leaves along the stem, indicating that the systematic position of these conspicuous variants needed further analyses. Furthermore, recent molecular studies showed that there is some variability between individuals of *T. succulenta* from Madeira and the Azores (Moore et al., 2002). Other molecular studies conducted recently in the Azores with different species have been unravelling the occurrence of intra-archipelago patterns of diversity in the endemic flora that were

previously unaccounted for, producing evidence that the current taxonomic groups may not be accurately defined (Carine & Schafer, 2010, Moura *et al.*, 2010a).

Morphological and molecular data analysis has been helpful to understand the phylogenetic relationships among plants. Nuclear and chloroplastidial genomic DNA sequences have been widely used to achieve this goal in plants. The selection of the marker depends of the purpose and objectives of the study and the relationship level among species (Mayer & Soltis, 1999).

In this investigation, we used the information contained in the nuclear region of the internal transcribed spacer ITS (ITS1-5.8S-ITS2), to propose a molecular phylogenetic hypothesis for the position of *Tolpis* spp. in the archipelagos of Azores and Madeira. The ITS region has often been used as a tool to study genetic diversity (Schmichl *et al.*, 2010) and to develop phylogenetic studies for comparisons by genus and other higher taxonomical categories (Baldwin et al., 1995; Schmichl et al., 2010). This application has been extended with success to Macaronesian (Vargas *et al.*, 1999; Valcárcel *et al.*, 2003) and Azorean endemic taxa (Moura, 2005; Carine & Schaefer, 2010; Moura *et al.*, 2010a,b). ITS sequences have excellent advantages for phylogenetic studies, namely their universality, biparental inheritance, simplicity, intragenomic uniformity, intergenomic variability and low functional constraint (Mort et. al, 2007).

Although the use of ITS sequence data is very common in phylogenetic studies, characteristics of nrDNA evolution may result in comparison of paralogs if sufficient caution is not exercised (Alvarez and Wendel, 2003). Bailey et al. (2003) investigated the phylogenetic implications of paralogy resulting from the tandem existence of both nrDNA pseudogenes and functional copies within an individual. While the presence of pseudogenes is not necessarily confounding to phylogenetic analysis if all copies have been sampled from each individual, rates of evolution among functional and nonfunctional paralogs may be sufficiently different to result in long-branch artifacts (Bailey et al., 2003).

Alvarez and Wendel (2003) discussed other problems: the effect of secondary structure on base substitution resulting in nonindependence of characters, difficulties with alignment, problems with contamination resulting from universal primers, generally high levels of homoplasy due to rapid evolution, and difficulties with amplification that arise from secondary structure and the existence of multiple rDNA arrays. Although several of these concerns are common to most rapidly evolving regions, they did emphasize the need for caution in molecular phylogenetic studies.

In order to deal with the above mentioned problems related to ITS, and further support our phylogenetic analysis of *Tolpis*, morphological data analysis was added to the study. Morphological characters were gathered from literature and personal observations, subject to a preliminary screening for adequacy and then measured in several herbarium specimens of *T. azorica*, *T. succulenta* (from Azores and Madeira) and *T. macrorhiza*.

The second part of our study includes the evaluation of intra and intergenetic variability in several populations of Azorean and Madeiran *Tolpis* using microsatelites loci (SSR). In a recent review, Bussell et al. (2005) stated that SSR markers might be useful for phylogenetic analyses involving species that are closely related and represent recent radiations. The set of microsatellites markers obtained in this study were used to elucidate the relationship between the populations within and between species at the scale of the archipelago and to compare the species micro-evolution within the genus.

In previous population studies, ISSR markers proved to be useful in *Tolpis* for grouping individuals into populations and species (Archibald, 2006). According to Jarvis (1980), the lack of clustering showed by members of *Tolpis* may be the result of the presence of a suite of ancestral polymorphic markers segregating within populations referable to more than one species in the current taxonomy of the genus. Gene flow between populations could also serve to slow or prevent the sorting of particular loci or combinations of loci within populations. These processes would result in more ISSR loci segregating within populations than there are markers or arrays of markers that define populations or groups of populations (Archibald, 2006).

In a study by Crawford et al. (2006), the allozyme variation among species of *Tolpis* in the Canary Islands was generally similar to other insular endemics in having low genetic diversity within species, a relatively high proportion of the diversity among populations, and low divergence among species. *Tolpis* differs from some other endemics in the lower genetic diversity within and higher proportion of diversity among populations of self-compatible species compared to self-incompatible species.

As the genus *Tolpis* comprises recent colonization/speciation events within the genus , the transferability of the SSR developed from the Azorean species was tested on *T. macrorhiza* and the homonymous but distinct *T. succulenta* (Moore et al. 2002) that are both endemic to Madeira Islands.

Compared with other classes of markers, microsatellites or Simple Sequence Repeats (SSRs), are highly polymorphic due to its repetitive nature and have been

widely used to study the effect of genetic drift and gene which determine population genetic diversity (Wright and Bentzen, 1994; Collevatti et al., 1999; Daynandan et al., 1997).

These markers have a number of advantages, since they are abundant and extensively cover the genome, have multiallelic nature, require small amounts of DNA for analysis, are easy to detect by PCR (polymerase chain reaction), have Mendelian inheritance type and are expressed as co-dominant alleles (Schlotterer& Pemberton, 1994; Ellegren, 2004).

The most efficient measure to assess population structure is based on Wright's *F*-statistics (1951), Wright's inbreeding coefficient (*FST*, also called θ) being particularly useful for analysing microsatellite markers because it is able to discriminate between alleles, especially that rare ones, although *FST* produced using such markers can sometimes be overestimates of the true value.

Microsatellite markers include loci with a large number of alleles, but one question that should be asked is whether a large number of loci or a large number of alleles is more important in genetic assessment. Working on the relationship between the allele number and the coefficient of variation of four genetic distances, Kalinowski (2002) used simulated data to show that highly polymorphic loci provided better estimates of genetic distance than less polymorphic loci and that increased allele number was associated with a decrease in the coefficient of variation of each of the four genetic distances studied. These results show that there is no requirement to examine either highly polymorphic loci or large numbers of loci, the only requirement being that a sufficient number of alleles are examined.

However, the high mutation rate of microsatellites can also invalidate many assumptions used in some conventional population structure analysis because different populations may share homoplasic alleles at frequencies that depend on both the rate and the details of the mutation process (Estoup *et al*., 2002). When such effects are ignored the rate of gene flow or genetic introgression can be overestimated (Balloux *et al*. 2000). Slatkin (1995) developed the *RST* statistic (analogous to *FST*) to take into account the effects of mutation, but although *RST* performs better than *FST* in some circumstances it can also be sensitive to details of the mutation process (Balloux and Goudet, 2002). Since mutation rate varies widely between loci within species (Di Rienzo *et al*. 1998) one advantage of loci with a high mutation rate is that genetic

differentiation reaches equilibrium faster, offering the possibility of obtaining estimates from larger and more widely spaced populations.

The objectives of our study were: (i) increase the sampling of *Tolpis* spp. populations in order to complete the already existing collection at AZB; (ii) to understand the intra and interpopulational genetic variability existing in *Tolpis* spp. populations and their relationships, with an emphasis in S. Miguel Island; (iii) identify cases of populations with very low genetic variability; (iv) determine the correspondence between the currently accepted distribution of *Tolpis* spp. in the Azores and the main groups obtained; (v) confirm the occurrence of other taxonomic groups besides those currently listed.

This research was part of the Demiurge Project, financed by FEDER, the initiative MAC/1/C020-Transnational Cooperation Programme Madeira-Açores-Canarias (MAC) 2007-2013, which has as one of this aims to collect data from genetic populations of several species of the endemic flora of Macaronesia.

Material and Methods

1. Phylogenetic study

1.1. Taxon sampling

The sample included 26 populations of *Tolpis*. Fifteen of these populations correspond to *T. azorica* from to the Azores. Seven populations of *T. succulenta* from the Azores islands where they occur, as well as two individuals of *T. succulenta* and two specimens of *T. macrorhiza* of Madeira Island.

Our total sample was of 26 different populations from 9 islands of the Azores, and Madeira, in a total of 26 individuals (Table 1).

Table 1. DNA Bank collection codes and localities for the populations included in this study.

Species	GenBank	Locality
Tolpis azorica	TA-SMLC-11	Azores, São Miguel, Lagoa do Canário
	TA-SMTR-58	Azores, São Miguel, Tronqueira
	TA-SMLF-33	Azores, São Miguel, Lagoa do Fogo
	TA-PIBM-03	Azores, Pico, Montanha Baldio de São Mateus
	TA-PIRB-07	Azores, Pico, near Caldeirão da Ribeirinha
	TA-TESB-07	Azores, Terceira, Caldeira de Santa Bárbara
	TA-TEQM-01	Azores, Terceira, Curral Queimado
	TA-SJFS-07	Azores, São Jorge, Caminho da Fajã para Caldeira Santo Cristo
	TA-SJPE-09	Azores, São Jorge, Pico da Esperança
	TA-FACA-09	Azores, Faial , Caldeira
	TA-FAAC-09	Azores, Faial, Alto do Chão
	TA-FLRB-04	Azores, Flores, Ribeira da Badanela
	TA-FLMA-02	Azores, Flores, Morro Alto
	TA-COMC-01	Azores, Corvo, Caminho do Marco do Caldeirão
	TA-COMC-10	Azores, Corvo, Caminho do Marco do Caldeirão
Tolpis succulenta	TS-SMPA-02	Azores, São Miguel, Porto da Ajuda
	TS-SMLG-02	Azores, São Miguel, Praia do Lombo Gordo
	TS-MAPA-09	Azores, Santa Maria, Pico Alto
	TS-MAMA-04	Azores, Santa Maria, Maia
	TS-MASL-02	Azores, Santa Maria, Baia de São Lourenço
	TS-GRBL-02	Azores, Graciosa, Baia do Filipe, Beira-mar da Luz
	TS-GRIP-03	Azores, Graciosa, Ilhéu da Praia
	TS-6729-H	Madeira, Eira do Serrado
	TS-6727-D	Madeira, entre Encumeada e Ribeira Brava
Tolpis macrorhiza	TM-6728-C	Madeira, entre Poiso e Ribeiro Frio
	TM-6725-E	Madeira, entre Encumeada e Chão dos Louros

1.2. DNA extraction and amplification

Leaves of 26 individuals of *Tolpis* collected were kept dry in silica gel. DNA was extracted from dry leafs and all the material was processed using a modified Doyle & Dickson CTAB protocol for DNA extraction. Due to the difficulties encountered to obtain high quality DNA, these modifications, ensued from Borges *et al.* (2009),

consisted in using 700 µl of 3 X CTAB, 50 µl of Sarcosyl and 10 µl of Proteinase K for the initial lyses step, in performing a first washing step with 500µl of chloroform-isoamylalchol (24:1) (SEVAG) and then carrying out an additional wash with 500µl of SEVAG and 200µl of 3 X CTAB. DNA was then precipitated by adding 450µl of isopropanol. Finally, the pellet obtained was suspended in 50µl of pure water. Each individual was subsequently purified following the modified protocol of QIAquick PCR purification kit from Qiagen. These modifications were the substitution 50 ul of buffer EB by 25µl of water pre-heated to 60ºC in the final step of purification. Quality and quantity of DNA was determined using the Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific). The primers AB101 and AB102 (Douzery et al., 1999) were used to amplify the entire ITS region. The PCR products from the internal transcribed spacer region were obtained using the amplification protocol of Carine et al. (2004). The amplification reaction was performed on a T-gradient amplifier (Whatman Biometra). All products with the exception of the primers, DNA and pure water, were provided by the PCR kit from Bioline©. The DNA fragments resulting from amplification products were separated on an agarose gel 4% and in TBE buffer, stained with DNA SafeView Stain and visualised with a Visidoc–IT (UVP) system. A molecular marker for 50-2000 base pairs (Sigma-Aldrich) was used as reference. After evaluation of the amplification, the PCR products were purified using the same QIAquick PCR protocol. Quality and quantity of DNA was checked and the amplification products were sequenced at STABVida.

1.3. Alignment of sequences and analysis

Preliminary alignments were obtained in Bioedit 7.0.4.1 (Hall 1999) using ClustalW (Thompson et al. 1994). The alignments were then manually inspected and verified. Phylogenetic analysis were conducted in PAUP* 4.0b10 (Swofford 2003), using the maximum parsimony (MP) criterion. The analysis used 100 heuristic searches with random stepwise addition, tree-bisection-reconnection branch swapping (TBR), zero length branches collapsed, and all characters equally weighted. A 50% majority-rule consensus tree was calculated and clade robustness was assessed by bootstrap analysis, using 1000 bootstrap replicates each comprising 1000 heuristic searches, random stepwise addition and TBR branch swapping. jModeltest (Posada 2008) was used to determine the best-fitting model of sequence evolution selected among 88 possible

models pertaining to the JC, HKY and GTR families, according to the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). A Maximum Likelihood search (ML) was conducted in RAxML BlackBox for Web-servers version 7.2.3 (Stamatakis et al. 2008) and a Bayesian estimation of phylogeny used MrBayes 3.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) in the Cipres portal (Miller et al., 2010). The Bayesian analysis was performed with the following settings: The maximum likelihood model employed 6 substitution types ("nst=6"), with rate variation across sites modelled using a gamma distribution (rates="gamma"). The Markov chain Monte Carlo search was run with 4 chains for 5000000 generations, with trees sampled every 100 generations (the first 500000 trees were discarded as "burnin"). MrBayes output files were analysed in the Tracer software (Rambaut, 2009) to confirm the parameters and the burn values. The most distal outgroup in all analyses was *Crepis biennis*.

2. Morphological study

2.1. Taxon sampling and characters measured

Our total sample was of 71 different specimens from 9 islands of the Azores, and Madeira (Table 2), stored at the AZB herbarium`s Azorean Flora DNA Bank. We included specimens pertaining to all the groups obtained in the molecular analyses. A total of 61 characters were gathered from literature and personal observations and evaluated according to Hickey et al. (2000).

The morphological characters measured were of two types: quantitative and qualitative. The quantitative characters were: stem ramification; leaf stem length and width; leaf basilar length and width; petiole length; peduncle length; number of bracts per peduncle; peduncle bracts length and width; capitula number; capitula diameter including corolla and excluding corolla; for each series the involucral number of bracts; bracts length and width; corolla tube length; ligule length and width; achenes length and width; achenes surface; achens pappus number; pappus length for each series. The qualitative characters were: life form; stem surface; stem pubescence; stem consistence; leaf pubescence; leaf insertion; lamina shape; lamina apex; lamina base; leaf margin; peduncle position; peduncle form; peduncle indument; capitula colour; involucral indument; involucral bracts shape; bracts pubescence per series and achenes color.

Table 2 . Specimens included in the morphological analysis

Taxa	Voucher Data		Locality
	TA-SMPI-01	(AZB)	São Miguel, Caminho Pico da Cruz
	TA-SMCR-01	(AZB)	São Miguel, Criação
	TA-SMLC-24	(AZB)	São Miguel, Lagoa do Canário
	TA-SMLC-76	(AZB)	São Miguel, Lagoa do Canário
	TA-SMVR-01	(AZB)	São Miguel, Vista do Rei (Sete Cidades)
	TA-SMLF-100	(AZB)	São Miguel, Lagoa do Fogo
	TA-SMME-01	(AZB)	São Miguel, Monte Escuro
	TA-SMTR-63	(AZB)	São Miguel, Tronqueira antes do Miradouro (Nordeste)
	TA-SMTR-68	(AZB)	São Miguel, Tronqueira antes do Miradouro (Nordeste)
	TA-SMTR-96	(AZB)	São Miguel, Tronqueira antes do Miradouro (Nordeste)
	TA-SMPG-05	(AZB)	São Miguel, Planalto dos Graminhais (Nordeste)
	TA-PIBM-01	(AZB)	Pico, Montanha (Baldio de S.Mateus)
	TA-PIRB-11	(AZB)	Pico, Perto Caldeirão da Ribeirinha
	TA-PIPU-01	(AZB)	Pico, Pico da Urze
	TA-PIPU-02	(AZB)	Pico, Pico da Urze
	TA-PITT-01	(AZB)	Pico, Transversal perto Torrinhas
Tolpis azorica	TA-TESB-34	(AZB)	Terceira, Caldeira de santa Bárbara
	TA-TESB-41	(AZB)	Terceira, Caldeira de santa Bárbara
	TA-TEQM-09	(AZB)	Terceira, Quinta da Madalena
	TA-TERC-03	(AZB)	Terceira, Rocha do Chambre
	TA-SJPE-01	(AZB)	São Jorge, Pico da Esperança
	TA-SJPV-01	(AZB)	São Jorge, Pico Verde
	TA-SJFS-10	(AZB)	São Jorge, Caminho para Fajã Caldeira Santo Cristo
	TA-SJTE-02	(AZB)	São Jorge, Terreirão
	TA-SJTE-03	(AZB)	São Jorge, Terreirão
	TA-FACA-01	(AZB)	Faial, Caldeira
	TA-FAAC-01	(AZB)	Faial, Alto do Chão
	TA-FAGS-01	(AZB)	Faial, Alto do Guarda Sol
	TA-FLMA-01	(AZB)	Flores,Estrada do Morro Alto
	TA-FLRB-01	(AZB)	Flores, Ribeira da Badanela
	TA-FLSP-01 (a/b)	(AZB)	Flores, Pico dos Sete Pés
	TA-FLCI-02	(AZB)	Flores, Cidrão
	TA-COMC-11	(AZB)	Corvo, Caminho Ponta do Marco do Caldeirão
	TS-SMPA-21	(AZB)	São Miguel, Planalto dos Graminhais (Nordeste)
	TS-SMPA-24	(AZB)	São Miguel, Porto da Ajuda Bretanha
	TS-SMLG-01	(AZB)	São Miguel, Praia do Lombo Gordo
Tolpis succulenta	TS-SMPC-01	(AZB)	São Miguel, Porto Velho das Capelas
	TS-SMPC-18	(AZB)	São Miguel, Porto Velho das Capelas
	TS-GRBL-01	(AZB)	Graciosa, Baía do Filipe- Beira Mar da Luz
	TS-GRIP-01	(AZB)	Graciosa, Ilhéu da Praia

Table 2. Specimens included in the morphological analysis (continued)

Taxa	Voucher Data		Locality
	TS-MAMA-09	(AZB)	Santa Maria, Maia
	TS-MAMA-10	(AZB)	Santa Maria, Maia
	TS-MASL-04	(AZB)	Santa Maria,São Lourenço
	TS-MASL-05	(AZB)	Santa Maria, São Lourenço
	TS-MAAN-02	(AZB)	Santa Maria, Anjos
	TS-MAPA-02	(AZB)	Santa Maria, Pico alto
	TS-MAPA-11	(AZB)	Santa Maria, Pico Alto
	TS-MAPA-14	(AZB)	Santa Maria, Pico Alto
	MS -6727-F	(AZB)	Madeira, Estrada encumeada-Ribeira Brava
	MS-6729 A	(AZB)	Madeira, Estrada do Serrado, estrada para a Camacha
	MS-6714	(AZB)	Madeira, Canhas, Estrada para a Ponta do Sol
	MS-6736 D	(AZB)	Madeira, Ribeira Brava, no miradouro
Tolpis succulenta	MS -6736-E	(AZB)	Madeira, Ribeira Brava, no miradouro
	MS-7085	(AZB)	Madeira, Subida para encumeada, Pousada dos Vinháticos
	MS-7059 A	(AZB)	Madeira, Porto Novo
	MS-7061 C	(AZB)	Madeira, Maroços antes do túnel para o Porto da Cruz
	MS-7062 B	(AZB)	Madeira, Porto da Cruz
	MS-7084 B	(AZB)	Madeira, Funchal (Santo António)
	MS-6731 B	(AZB)	Madeira, Funchal-Estrada Monte para St. António
	MS-7094 C	(AZB)	Madeira, Ribeira do Inferno
	MS-7094 A	(AZB)	Madeira, Ribeira do Inferno Seixal
	MS 6743 K	(AZB)	Madeira, Ponta do Sol
	MS 6743 D	(AZB)	Madeira, Ponta do Sol
	P3 SF3	(AZB)	Madeira, Porto Santo
	Planta C	(AZB)	Madeira, Porto Santo-Castelo Branco
	MS 6725 C	(AZB)	Madeira, Encumeada, descida para o chão dos Louros
	MS 6728 E	(AZB)	Madeira, Estrada Poiso para o ribeiro Frio
Tolpis macrorhiza	MS 6728 C	(AZB)	Madeira, Estrada Poiso para o ribeiro Frio
	MS 6726 I	(AZB)	Madeira, Encumeada, descida para a Ribeira Brava
	MS 6726 E	(AZB)	Madeira, Encumeada, descida para a Ribeira Brava

2.2. Statistical analysis

The data were subjected to statistical analysis using the software SPSS v. 18.0 in order to access the variation between the different taxa studied.

The means and standard errors were calculated for each variable. The occurrence of significant differences between groups was analyzed using the chi-square test. An Analysis of Variance (ANOVA) was used to compare the different features between groups. A Principal Components Analysis (PCoA) was used to describe the greatest variance between some of the quantitative variables studied.

3. Population genetics study

3.1 Taxon sampling

The collection of *Tolpis* spp. samples obtained during previous field work in all the islands of the Azores archipelago, stored at the AZB herbarium´s Azorean Flora DNA Bank, was completed with 148 samples collected in 2010 at the island of São Miguel and 67 samples from Madeira.

Our sampling of *T. azorica* was conducted in 53 different populations from 7 islands of the Azores archipelago, resulting in 478 individuals (Table 3). The sampling for *T. succulenta* was conducted in 17 different populations from 3 islands of the Azores and from Madeira Island, in a total of 188 individuals (Fig. 2, Fig. 3 and Table 4).

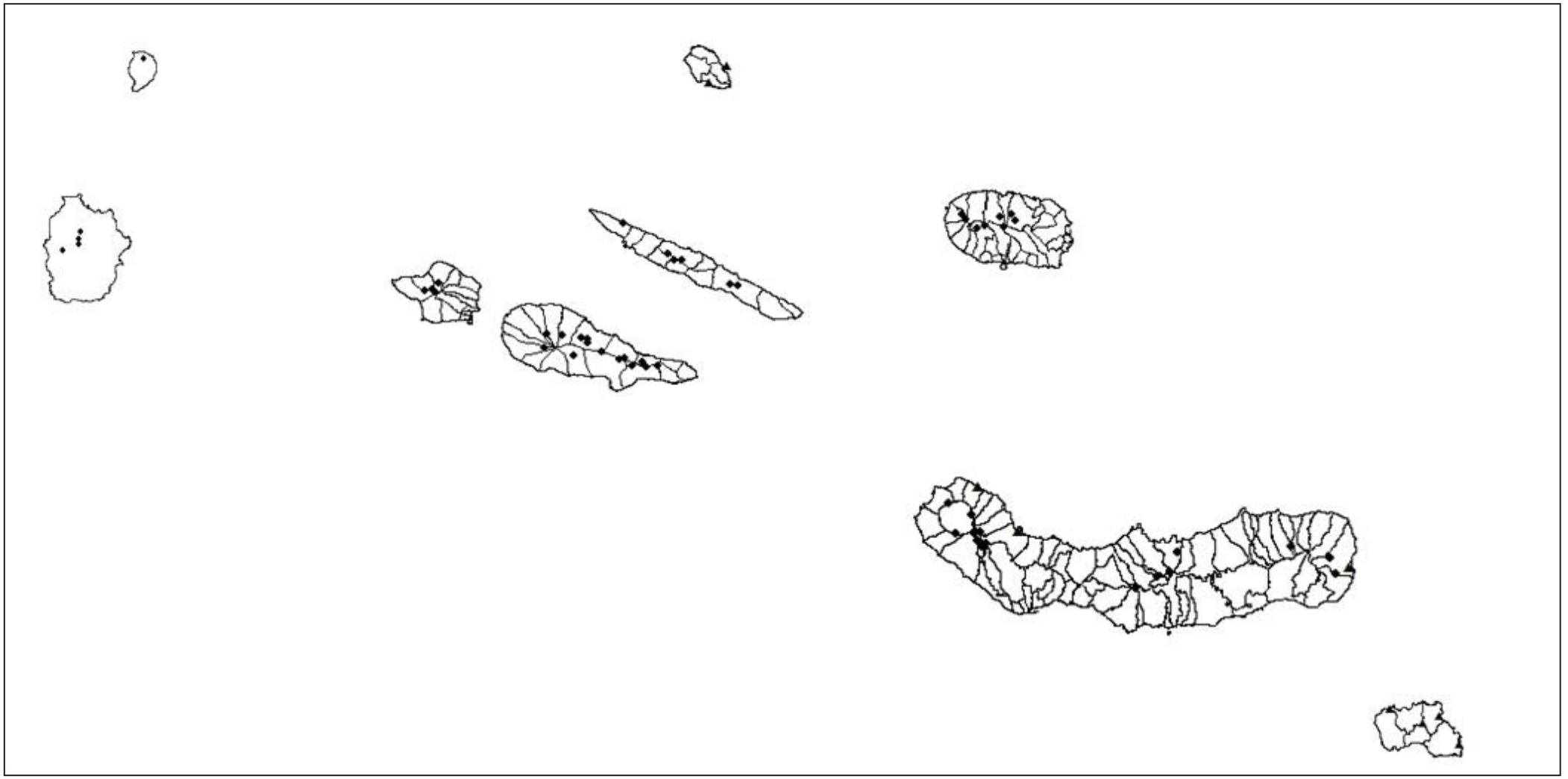


Fig.2. Location of the *Tolpis* samples collected in the three groups of the Azores archipelago. *T. azorica* is represented with dots and *T. succulenta* is represented by triangles.

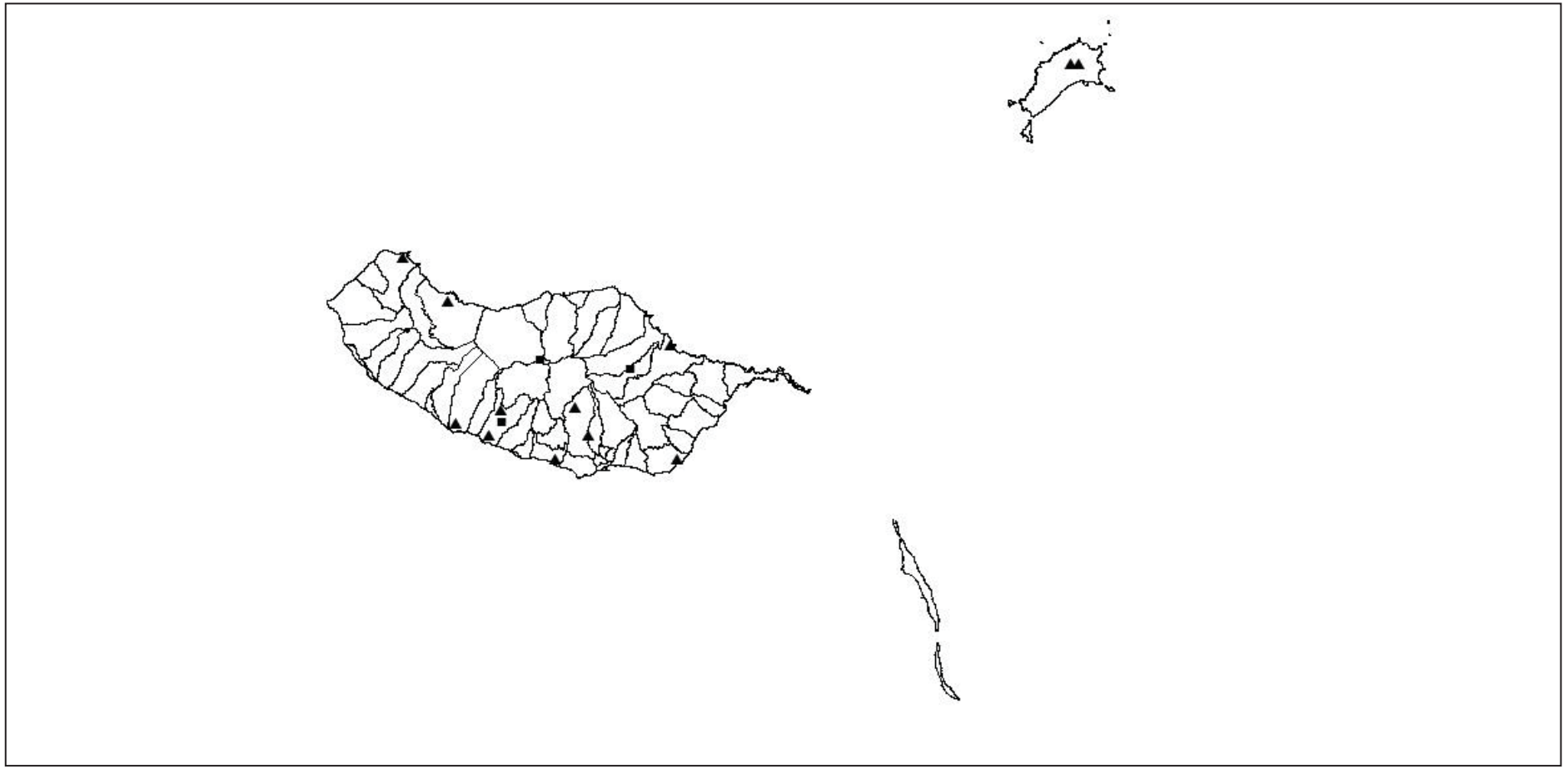


Fig. 3. Location of the Tolpis sample collected in the Madeira archipelago. *T. succulenta* is represented with triangles and *T. macrorziha* is represented by squares.

Table 3. Description of the geographical distribution of the samples of *T. Azorica*. Total of 478 individuals were collected.

Island Group	Island (N)	Populations	Codes	N	Total
Oriental	Santa Maria (0)	-	-	-	148
	São Miguel (148)	Caminho Pico da Cruz	SMPI	5	
		Lagoa do Canário	SMLC	5	
		Caldeira do Alferes (Sete Cidades)	SMAL	4	
		Estaleiros (Sete Cidades)	SMES	5	
		Lagoa das Empadadas	SMEM	5	
		Pico do Carvão	SMPR	5	
		Vista do Rei (Sete Cidades)	SMVR	10	
		Criação	SMCR	10	
		Lagoa Do Caldeirão Grande	SMCG	3	
		Lagoa Das Éguas	SMLE	10	
		Tronqueira (Nordeste)	SMTR	16	
		Lombadas	SMLO	10	
		Planalto Dos Graminhais (Nordeste)	SMPG	8	
		Monte Escuro (Interior)	SMME	10	
		Pico Bartolomeu	SMPB	13	
		Lagoa Do Fogo	SMLF	26	
		Lagoa do Areeiro (Vila Franca do Campo)	SMLA	3	
Central	Pico (103)	Pico da Urze	PIPU	9	287
		Cabeço da Cruz	PICC	5	
		Cabeço do Caveiro	PICA	10	
		Cabeço do Mistério	PICM	10	
		Cabeço do Raso	PICR	5	
		Cabeço do Redondo	PIRE	10	
		Lagoa do Capitão	PILC	5	
		Lomba do Capitão	PILO	5	
		Montanha Baldio de S. Mateus	PIBM	10	
		Perto do Caldeirão da Ribeirinha	PIRB	10	
		Lagoa do Peixinho	PILP	10	
		Transversal, perto de Torrinhas	PITT	10	
		Curral Queimado	PICQ	4	
	Terceira (105)	Algar do Carvão	TEAC	10	
		Caldeira de Santa Bárbara	TESB	20	
		Serra do Labaçal, Moldes	TESL	10	
		Rocha do Chambre	TERC	15	
		Caldeira de Agualva	TECA	10	
		Quinta da Madalena	TEQM	10	
		Pico do Gaspar	TEPG	15	
		Cancela do Estaleiro	TECE	15	
	São Jorge (48)	Caminho da Fajã do João de Dias	SJPV	1	
		Subida para o Pico da Esperança	SJPE	11	
		Pico Verde	SJPV	5	
		Pico da Esperança	SJPE	11	
		Terreirão	SJTE	10	
		Caminho da Fajã da Caldeira Santo Cristo	SJFS	10	
	Faial (41)	Alto do Chão	FAAC	10	
		Alto do Guarda-Sol	FAGS	10	
		Caldeira	FACA	11	
		Cabeço dos Trinta	FATR	10	
	Graciosa (0)	-	-	-	
Ocidental	Flores (22)	Cidrão	FLCI	5	43
		Estrada do Morro Alto	FLMA	2	
		Pico dos Sete Pés	FLSP	5	
		Ribeira da Badanela	FLRB	10	
	Corvo (11)	Caminho da Ponta do Marco do Caldeirão	COMC	11	

Table 4. Description of the geographical distribution of the collected individuals (N) of *T.succulenta.* A total of 188 samples were collected.

Island Group	Island (N)	Populations	Codes	N	Total
Oriental	Santa Maria (44)	Anjos	MAAN	10	96
		Maia	MAMA	10	
		Pico Alto	MAPA	14	
		São Lourenço	MASL	10	
	São Miguel (52)	Praia do Lombo Gordo	SMLG	19	
		Porto da Ajuda, Bretanha	SMPA	16	
		Porto Velho das Capelas	SMPC	17	
Central	Pico (0)	-	-	-	25
	Terceira (0)	-	-	-	
	São Jorge (0)	-	-	-	
	Faial (0)	-	-	-	
	Graciosa (25)	Baía do Filipe, Beira-mar da Luz	GRBL	15	
		Ilhéu da Praia	GRIP	10	
Ocidental	Flores (0)	-	-	-	-
	Corvo (0)	-	-	-	
Madeira	Madeira (67)	Canhas	6714	4	67
		Estrada entre Ribeira Brava e Encumeada	6727	5	
			6736	10	
		Estrada entre Ribeira Brava e Miradouro	6729	8	
			6730	11	
		Eira do Serrado	6731	6	
		Descida da Eira do Serrado,Monte	6735	12	
		Santo António, Funchal	6743	11	
		Porto Moniz			
		Ponta do Sol			

3.2. DNA Extraction

We extracted total DNA from 666 individuals for the population study of *Tolpis spp.* using a modified Doyle & Dickson (1987) CTAB protocol for DNA extraction. Briefly, 3 cm² of fresh leaves were powdered with PolyvinylPyrrolidone. They were then incubated during 45 minutes at 65ºC 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% Sarcosyl buffer (100 mM Tris-HCl pH 8.8; 20 mM EDTA; 10% Sarcosyl) and 10 µl of Proteinase K (AppliChem). The sample was thoroughly mixed with 500 µl of 24:1 chloroform: isoamylacohol and centrifuged 3 min at 13000 rpm. The supernatant was slowly mixed with 450 µl of isopropanol to allow DNA precipitation. The tube was then centrifuged 15 minutes at 12000 rpm, the liquid phase was discarded and the pellet obtained was

allowed to dry 80m at 30ºC in a dry bath before being re-suspended in 50 µl of pure water.

DNA samples' qualities and quantities were then measured using a Nanodrop 2000 (Thermo Fisher Scientific) spectrophotometer. Samples were conserved at -20ºC until use.

3.3. Characterization of the microsatellites

The molecular markers used were developed by Savannah River Ecology Lab (University of Georgia) and the posterior testing and selection were performed at he University of the Azores (Sardos et al., in prep).Out of 24 only putative microsatellites, eight were used for *T. azorica* and five for *T. succulenta* (Table 4).

Table 5. Description of the eight polymorphic SSR loci that exhibited acceptable to high scorability in the genus *Tolpis.* * Indicates M13R tag (5'- GGAAACAGCTATGACCA – 3') ; † Indicates "pigtail" tag (5' – GTTT – 3') ; T.a (*T. azorica*); T.s (*T. succulenta*).

Name	T.a	T.s	Primer	Sequences	Repeat motif	Size Range	Dye
TA2A03	✓	✓	Forward	*ATCACCTCCCTCTACAATGAC	(AC)^8	148-152 bp	FAM
			Reverse	†AACTCACAAAGCGAACACAG			
TA3B02	✓	✓	Forward	†CCAATTAAACGGAAAGAGAC	(AAC)^7	240-260 bp	FAM
			Reverse	*TGCTTGCTACTTGAGGACTC			
TA2A01	✓	✓	Forward	* TACCGTAGAACCCAAACTC	(AG)^9	175-225 bp	NED
			Reverse	†AAATTGTATGAGCCCACAAC			
TA3B05	✓	✓	Forward	*ATAACAACTCCATGCCACAC	(AAT)^12	300-350 bp	VIC
			Reverse	†TTGCAATCTTATCGTCTGTG			
TA2A09	✓	-	Forward	*GAACGAGAAGAAAGAGATTGTC	(AG)^8	125-150 bp	VIC
			Reverse	†GATTCCATCCCTTTCTTTATC			
TA2A07	✓	-	Forward	*GAAGAAGAACAAGATCCTTTG	(AC)^8	175-200 bp	VIC
			Reverse	†AACACGAACGGTAAATGTATC			
TA4B07	✓	-	Forward	*ACCTACGAACATTCATACAAAC	(ACAT)^6...(ACAT)^14	180-250 bp	PET
			Reverse	†GTAGAAGTAAAGGGCCATTG			
TA2A02	✓	✓	Forward	*AT GGA ATT AAT CGG AAA TTG	(AG)^10	126-140bp	PET
			Reverse	†TCA CAA ACC CTA ACA GTT CC			

3.4. Full scale genotyping

The following PCR programs were optimized and described in Sardos et al. (in prep.) for the eight polymorphic primer pairs. The primers are described in Table 5.

After optimization, the amplifications for the whole sample were performed using the protocols presented in table 6 which describe the PCR conditions for the eight primers. The M13R was labelled either with PET, FAM, NED or VIC. The Taq polymerase chosen for 4 SSR (TA2A03, TA3B02, TA3B05, TA2A07) was Biotaq (Bioline) for the others 4 primers (TA2A01, TA2A09, TA4B07, TA2A02) Immolase (Bioline) was used. The amplification products were then diluted, multiloaded and were

run on an ABI-3130xl Genetic Analyzer and sized with LIZ500 size standard. The genotypes were scored using the software GeneMarker® V.1.97 Demo version (Softgenetics®).

Table 6. PCR conditions for the polymorphic microsatellites obtained from the Açores and Madeira Tolpis.

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

3.5. Data analysis

The diversity of the overall sample of *Tolpis* in the Azores and Madeira was described using the software GENETIX 4.02 (Belkhir *et al.* 2002) by calculating the mean number of alleles, the observed and expected mean heterozygosities (Hobs and Hexp, respectively) and Wright's fixation index (Fis) at each *locus* under the null hypothesis of Hardy-Weinberg (H-W) equilibrium. The expected mean heterozygosity

was determined using the unbiased estimate method of Nei (1978); Fis values were calculated following Weir and Cockerham (1984).

For each SSR primer and population, we determined the Fst of Wright, the effective population size (Ne) and the value of gene flow (Nm). Distance was calculated based on the proportion of alleles shared, from which will produce a dendrogram. The distance matrix is further analyzed using AMOVA. This was done using the software Arlequin v.3.11 (Excoffier et al., 2005).

The molecular data obtained from the *Tolpis* samples from the Azores were then analysed using the computer software DARwin version 5.0 (Perrier and Jacquemmoud-Collet 2006; Perrier *et al.* 2003) using a Principal Coordinate Analysis (PCoA) based on the dissimilarity matrix between genotypes. Dissimilarities were calculated using the simple matching indices for allelic data.

To further determine the genetic structure of *Tolpis* in Azores and Madeira, we used a Bayesian Markov Chain Monte Carlo (MCMC) approach to estimate the number of genetic clusters. This model-based analysis was run with the program STRUCTURE version 2.3.3 (Pritchard *et al.* 2000) using a batch-oriented web program package for construction of supermatrices ready for phylogenomic analyses (Kumar *et al.* 2009). For a pre-assigned number of genetic clusters K in the dataset, each of which characterized by a set of allele frequencies at each *locus* under the assumption of H-W and linkage equilibrium. STRUCTURE calculates the posterior probabilities of the data for each K called Ln P(D). We ran 10 replicates for each K value ranging from 1 to 10 with a burn-in length of 50,000 followed by 500,000 iterations of each chain using the admixture model along with the assumption of correlated allele frequencies between groups (Falush *et al.* 2003). STRUCTURE then partitioned individuals of the sample according to the membership coefficient Q, that ranges from 0 (lowest affinity to the group) to 1 (highest affinity to a group), across the K groups. The optimal value of K was then determined by examining both Ln P(D) and ΔK, an ad hoc quantity related to the second order rate of change of the log probability of data with respect to the number of clusters (Evanno *et al.* 2005). Graphics of STRUCTURE results were produced by using DISTRUCT 1.1 (Rosenberg 2004).

Results

1.1-*Tolpis* spp. (5 primers)

According to the Bayesian analysis performed with STRUCTURE, *Tolpis* spp. is composed of three genetic clusters (Fig. 4). The posterior probabilities of the data for each K, called Ln P(D) in STRUCTURE output, along with their variance across runs are presented in Figure 4.A. The maximum value of Ln P(D) is reached for K = 10 but the plateau seems to start as soon as K = 2. Additionally, variance across runs is very low for K = 3 and the results obtained following the methodology of Evanno *et al.* (2005), compiled in Figure 4, resulted in two peaks of ΔK. The first at K = 3 is the highest, the second at K = 7 is the smallest. Taking into consideration the variation across runs, the partitioning of individuals for each putative K (data not shown) and the methodology proposed by Evanno *et al.* (2005), we assume K=3 as the real value of K with a Fst value of 0.149 that supports the differentiation between the clusters

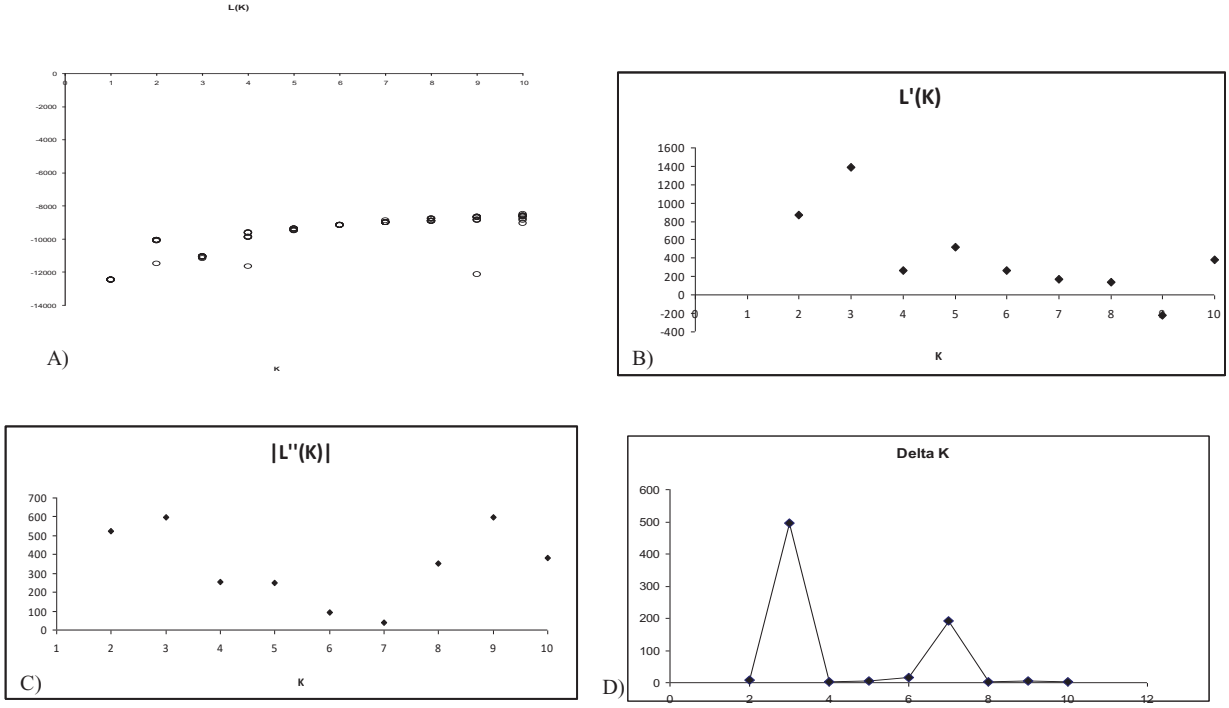


Fig. 4. Methodology from Evanno *et al.* (2005) for the interpretation of STRUCTURE results. A) Median Ln(K), B) Ln'(K), C) Ln''(K) and D) Median Delta K are presented for each value of K. The two peaks of Median Δ K at K = 3 and K = 7 indicate two putative right values for K

The graphical display of STRUCTURE output for K=3 is represented in Figure 4. Colours represent the proportion of each individual that belongs to each cluster. The first group is composed of individuals of *T. azorica* from São Miguel, the second one is composed of all the other individuals of *T. azorica* from Pico, Terceira, São Jorge, Faial, Flores and Corvo and the third group is composed of all the individuals of *T. succulenta* from the Azores and Madeira.

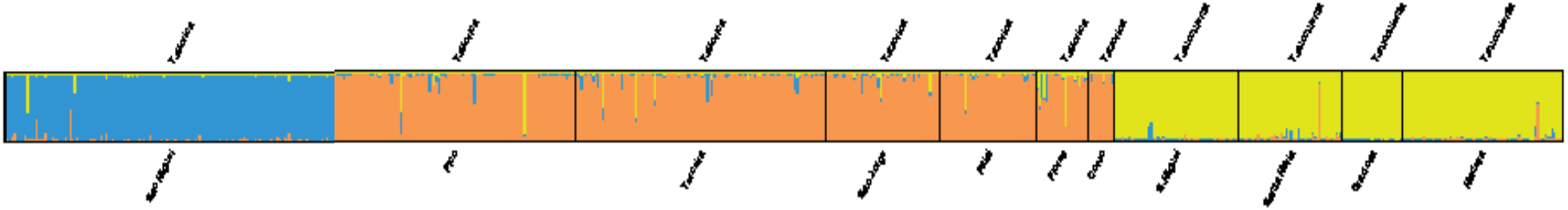


Fig. 5. Graphic display of STRUCTURE output *Tolpis* spp. Individuals are represented as thin vertical lines partitioned into segments corresponding to their membership in genetic clusters indicated by the colours.

For *Tolpis* spp. the number of alleles, observed and expected heterozigosity, Wright's fixation index and percentage of rare alleles were calculated for each *locus* and for all loci (Table 7).

Regarding *T. azorica*, the matrix exhibits 59 alleles in total (average of 11.8), ranging from 5 for marker TA3B02 to 22 for TA3B05, and an overall excess of homozygotes (Multi*locus* Fis=0.298, ranging from -0.05 for TA3B05 to 0.49 for TA2A01), and displays 55.9 % of rare alleles. *Tolpis azorica* in São Miguel, Pico, Terceira and Flores exhibits positive overall Fis values.

In São Miguel, *T. azorica* exhibits 36 alleles in total and the multi*locus* Fis value, 0.279, suggests an excess of homozygotes. Pico and Terceira display the higher amount of alleles (39 and 38 alleles, respectively), with an elevated excess of homozygotes (Fis=0.257 and Fis=0.17 respectively) and Flores exhibit Fis value of 0.149 (Table 6).

In Faial, *T. azorica* exhibits a multilocus Fis value 0.03 suggesting that the species in this island is under Hardy-Weinberg equilibrium. However, locus specific Fis values at locus TA2A01 (0.59) suggests an heterozygosity deficit while Fis value at locus TA3B02 (-0.86) suggest an excess of heterozygotes, the Fis values at remaining loci seem to be close to zero.

In Corvo and Flores, *T. azorica* exhibit low proportion of rare alleles, with 10.5%, and 25% respectively. In Corvo, *T. azorica* (30 alleles in total, ranging from 2 on locus TA3B05 and TA2A09 to 5 in locus TA2A03 and TA4B07) exhibits negative Fis value in 4 out of the 5 loci, TA2A03, TA3B02. TA2A01 and TA2A02 displaying a Fis value strongly negative (-0.25, -1.00, -0.59 and -0.30, respectively) which suggests excess of heterozygotes. The multilocus Fis value is equal to -0.34, which demonstrate the presence of an excess of heterozygotes (Table 7).

As for *T. succulenta,* the matrix exhibits 56 alleles in total (average of 11.2), ranging from 7 for marker TA2A02 to 18 for TA3B05, and an overall excess of homozygotes (Multi*locus* Fis=0.383, ranging from 0.06 for TA3B05 to 0.582 for TA2A01), and displays 57.1% of rare alleles.

All of the *T. succulenta* exhibits positive multilocus Fis values, Graciosa exhibit the higher value of Fis (0.773) suggests an excess of homozygotes.

Regarding the rares alleles, *T. succulenta* exhibits high value in all the populations except Graciosa with only 20% (Table 7).

Table 7. Number of genotyped individuals, number of alleles, Hobs, Hexp, Fis, percentage of rare alleles (frequency q<0.05), for each *locus* of the *T. azorica* and *T. succulenta* populations distributed in the seven islands of the Azores and Madeira Archipelagos.

Species	Island	N	TA2A03					TA3B02					TA3B05					TA2A01					TA2A02					Multi*locus*				
			Alleles	Hexp	Hobs	Fis	Rare alleles (%)	Alleles	Hexp	Hobs	Fis	Rare alleles (%)	Alleles	Hexp	Hobs	Fis	Rare alleles (%)	Alleles	Hexp	Hobs	Fis	Rare alleles (%)	Alleles	Hexp	Hobs	Fis	Rare alleles (%)	Alleles	Hexp	Hobs	Fis	Rare alleles (%)
T.azorica	São Miguel	148	4	0.36	0.18	0.50	25.00	5	0.70	0.66	0.06	20.00	11	0.75	0.57	0.23	54.50	12	0.81	0.53	0.34	41.70	4	0.44	0.26	0.41	50.00	36	0.61	0.44	0.28	41.67
	Pico	103	7	0.77	0.65	0.16	28.57	5	0.32	0.27	0.16	60.00	15	0.85	0.64	0.25	60.00	11	0.82	0.50	0.39	45.50	1	0.00	0.00	-	0.00	39	0.55	0.41	0.26	48.70
	Terceira	105	7	0.73	0.67	0.08	42.90	4	0.56	0.78	-0.41	50.00	11	0.79	0.58	0.27	54.55	13	0.84	0.37	0.57	46.15	3	0.08	0.09	-0.03	66.70	38	0.60	0.50	0.17	50.00
	São Jorge	48	10	0.77	0.81	-0.05	50.00	3	0.57	0.92	-0.63	0.00	10	0.77	0.69	0.11	40.00	9	0.77	0.30	0.61	44.40	2	0.02	0.02	0.00	50.00	34	0.58	0.55	0.06	41.20
	Faial	41	5	0.61	0.61	-0.01	40.00	3	0.53	0.98	-0.86	33.30	10	0.86	0.80	0.07	40.00	8	0.83	0.35	0.59	25.00	2	0.02	0.02	0.00	50.00	28	0.57	0.55	0.03	35.71
	Flores	22	6	0.75	0.73	0.03	33.33	3	0.63	0.86	-0.39	0.00	13	0.88	0.86	0.02	46.20	5	0.68	0.00	1.00	0.00	5	0.78	0.73	0.07	0.00	32	0.74	0.64	0.15	25.00
	Corvo	11	5	0.74	0.91	-0.25	20.00	2	0.52	1.00	-1.00	0.00	4	0.69	0.55	0.22	0.00	4	0.65	1.00	-0.59	0.00	4	0.64	0.82	-0.30	25.00	19	0.65	0.85	-0.34	10.52
	Overwal	478	11	0.77	0.53	0.30	54.50	5	0.64	0.67	-0.05	20.00	22	0.91	0.63	0.30	63.63	15	0.87	0.45	0.49	53.33	6	0.29	0.16	0.47	66.70	59	0.70	0.49	0.30	55.90
T.succulenta	São Miguel - SMLG	33	5	0.37	0.39	-0.08	40.00	4	0.56	0.42	0.25	25.00	5	0.61	0.64	-0.04	40.00	8	0.77	0.30	0.61	50.00	6	0.62	0.70	-0.13	66.67	28	0.59	0.49	0.17	54.20
	Stª Maria + SMLG	63	7	0.62	0.48	0.23	42.86	5	0.68	0.71	-0.05	20.00	13	0.83	0.67	0.20	61.50	10	0.74	0.51	0.32	50.00	7	0.62	0.62	0.00	42.86	42	0.70	0.60	0.15	47.62
	Graciosa	25	3	0.63	0.00	1.00	0.00	5	0.44	0.44	-0.01	40.00	2	0.47	0.08	0.83	0.00	3	0.63	0.00	1.00	0.00	2	0.08	0.00	1.00	50.00	15	0.45	0.10	0.77	20.00
	Madeira	67	5	0.57	0.37	0.35	40.00	6	0.66	0.91	-0.38	33.33	10	0.78	0.70	0.11	50.00	10	0.85	0.41	0.52	20.00	1	0.00	0.00	-	0.00	32	0.57	0.48	0.17	57.14
	Overwall	188	10	0.77	0.36	0.53	60.00	8	0.74	0.70	0.06	37.50	18	0.89	0.59	0.34	61.11	13	0.87	0.36	0.58	53.84	7	0.52	0.33	0.37	71.43	56	0.76	0.47	0.38	57.14

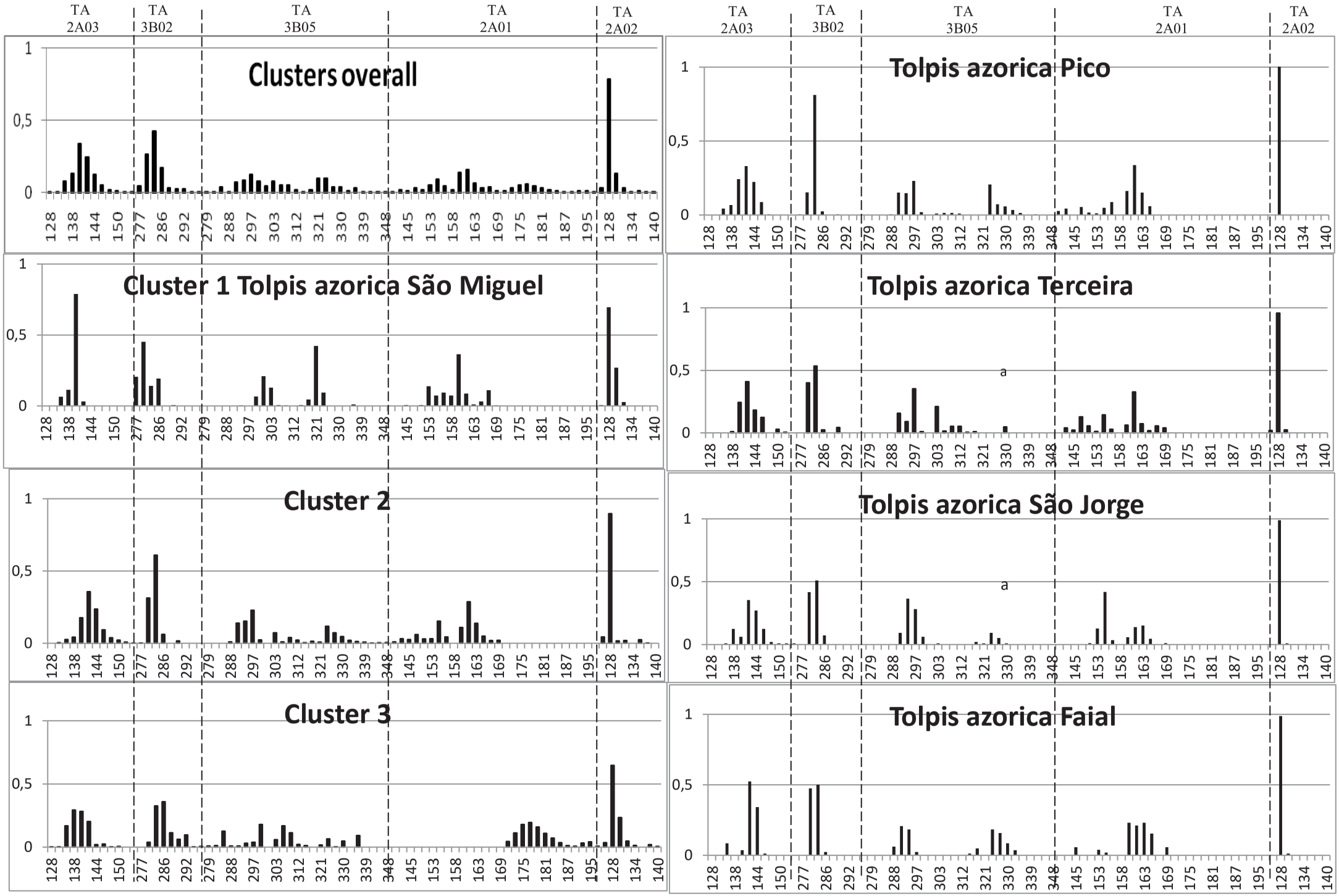
The allelic richness for all islands is given as the average number of alleles per loci. Private alleles are the estimated number of alleles per island that do not appear elsewhere in the sample, for our set of five markers the allelic richness varies from an average of 3 alleles Graciosa (*T. succulenta*) to 8 alleles at Pico, Terceira (*T. azorica*) and Santa Maria + Praia do Lombo Gordo (*T. succulenta*). The island with a higher percentage of private alleles is Madeira (28.1%) and the island with a lower percentage of private alleles is Terceira and Faial with a lack of private alleles (Table 8).

For the allelic frequencies at the five *locus*, the general pattern indicates a distribution of genetic diversity higher in cluster 2 (*T. azorica* from central and western groups), with the presence of more alleles than in cluster 1 (*T. azorica* from São Miguel) and cluster 3 (*T. succulenta*) (Figure 6).

For cluster 1 the dominant alleles for the five markers are similar to cluster 2. In cluster 2, the diversity of dominant alleles between the five *locus* is higher. In the case of *T. azorica* from Flores, *T. succulenta* from São Miguel and Santa Maria a large range of alleles present at locus TA2A02(Figure 6).

Table 8. Allelic richness and private alleles based on 5 microsatallites (SSR) loci.

Species	Islands	Alleles	Average alleles	Private alleles %
T. azorica	São Miguel	36	7	5.6
	Pico	39	8	5.1
	Terceira	38	8	0.0
	São Jorge	34	7	2.9
	Faial	28	6	0.0
	Flores	32	6	9.4
	Corvo	19	4	10.5
T. succulenta	São Miguel -Praia Lombo Gordo	28	6	3.6
	Santa Maria +Praia do Lombo Gordo	42	8	23.8
	Graciosa	15	3	6.7
	Madeira	32	6	28.1


TA 2A03
TA 3B02
TA 3B05
TA 2A01
TA 2A02
Clusters overall
Cluster 1 Tolpis azorica São Miguel
Cluster 2
Cluster 3
Tolpis azorica Pico
Tolpis azorica Terceira
Tolpis azorica São Jorge
Tolpis azorica Faial
a
a
1
0,5
0
128 138 144 150 277 286 292 279 288 297 303 312 321 330 339 348 145 153 158 163 169 175 181 187 195 128 134 140

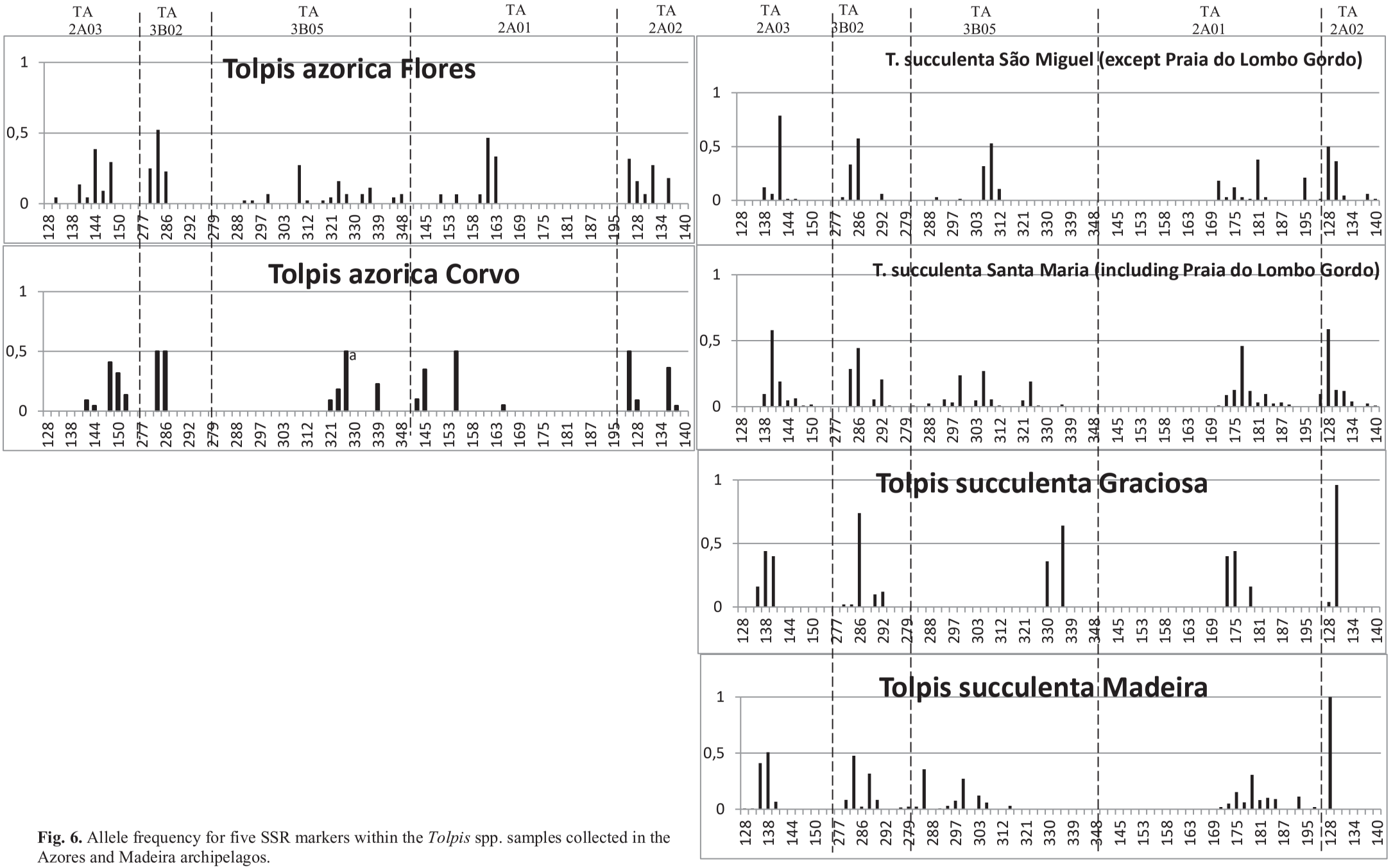


Fig. 6. Allele frequency for five SSR markers within the *Tolpis* spp. samples collected in the Azores and Madeira archipelagos.

The AMOVA analyses based on the genetic structure defined by STRUCTURE, for *T. azorica* in the central and western groups confirmed that the highest percentage of variation occurs within populations (84.1%), while the lowest occurs among populations within groups (6.5%; Table 9). Equally, in the São Miguel populations, the highest percentage of variation occurs within populations (84.5%) and the lowest among populations within groups (7%; Table 10).

Table 9. Molecular variance analyses (AMOVA) applied to 36 populations of *T. azorica* from central and western groups, calculated for five locus.

Source of variation	d.f.	Sum of squares	Variance of componentes	Percentage of variation
Among Groups	5	95.950	0.15582 Va	9.36
Among populations within groups	30	100.482	0.10855 Vb	6.52
Within populations	624	874.312	1.40114 Vc	84.13
Total	659	1070.744	1.66551	

Table 10. Molecular variance analyses (AMOVA) applied to 17 populations of *T. azorica* from São Miguel, calculated for five locus.

Source of variation	d.f.	Sum of squares	Variance of componentes	Percentage of variation
Among Groups	1	23.924	0.13910 Va	8.51
Among populations within groups	15	49.286	0.11450 Vb	7.00
Within populations	279	385.300	1.38100 Vc	84.49
Total	295	458.510	1.63460	

Relatively to *T. azorica*, we observed the presence of gene flow between all the islands of the Azores, however the higher gene flow values occur between the islands of the western group. Between Pico and Terceira we obtained a value of M=11, between Terceira and São Jorge the value of M=10 and between São Jorge and Faial, M=8 (Table 11).

Table 11: Matrix of M values (gene flow) obtained for *T. azorica* in the Azores archipelago.

	São Miguel	Pico	São Jorge	Terceira	Corvo	Faial	Flores
São Miguel							
Pico	2						
São Jorge	2	7					
Terceira	2	11	10				
Corvo	1	1	1	1			
Faial	2	7	8	7	1		
Flores	2	2	2	2	3	2	0

In São Miguel, and for *T. azorica*, the highest values of gene flow occur between the population of Lagoa do Fogo (SMLF) and two populations of the central part of the island (SMLO and SMME) as well with three populations of the eastern part of the island (SMTR, SMPG and SMPB). Relatively to the western part of the island, the population of Pico do Carvão (SMPR) presents high values of M with the neighbor populations of SMCG, SMES, SMLC along with three populations of the central part SMLF, SMLO and SMME. Furthermore, the populations with the lowest gene flow values with all other populations are SMAL, SMLA.

Table 12. Matrix of M values (gene flow) obtained for *T. azorica* from São Miguel.

	SMPI	SMLC	SMAL	SMES	SMEM	SMPR	SMVR	SMCR	SMCG	SMLE	SMTR	SMLO	SMPG	SMME	SMPB	SMLF	SMLA
SMPI																	
SMLC	24																
SMAL	2	3															
SMES	16	8	1														
SMEM	inf	inf	2	inf													
SMPR	12	13	1	12	11												
SMVR	6	8	2	5	37	4											
SMCR	3	5	1	3	8	2	32										
SMCG	4	57	1	3	8	17	3	2									
SMLE	7	19	1	7	9	inf	4	3	29								
SMTR	4	2	1	3	3	7	3	2	2	4							
SMLO	7	3	1	5	4	16	4	3	4	6	inf						
SMPG	3	1	1	3	2	2	2	1	1	2	4	4					
SMME	8	3	1	5	5	19	4	3	4	6	inf	inf	4				
SMPB	6	2	1	3	3	8	3	2	3	3	inf	inf	5	inf			
SMLF	6	3	2	4	4	11	4	2	5	6	20	28	28	53	15		
SMLA	2	1	0	2	2	1	1	1	0	1	1	1	1	1	1	1	0

Regarding *T. succulenta*, the AMOVA analyses again resulted in a high percentage of intrapopulational genetic variability (58,79%; Table 13).

Table 13. Molecular variance analyses (AMOVA) applied to 17 populations of *T. succulenta* from Azores and Madeira calculated for five locus.

Source of variation	d.f.	Sum of squares	Variance of componentes	Percentage of variation
Among Groups	3	178.735	0.55706 Va	26.09
Among populations within groups	13	103.812	0.32280 Vb	15.12
Within populations	359	450.589	1.25512 Vc	58.79
Total	375	733.136	2.13499	

For *T. succulenta* from Azores and Madeira, the M values are generally very low. The GRIP population of Graciosa Island has a lack of gene flow. The only populations that have a high value of gene flow are: three populations of Santa Maria (between MASL and MAAN and MAMA) and in Madeira island between 6727 and 6714, 6736, 6743 and between 6736 and 6731.

Table 14. Matrix of M values (gene flow) obtained for *T. succulenta* in Azores and Madeira archipelagos.

	SMLG	SMPA	SMPC	MAAN	MAMA	MAPA	MASL	GRBL	GRIP	6714	6727	6729	6730	6731	6735	6736	6743
SMLG	0																
SMPA	1	0															
SMPC	1	2	0														
MAAN	2	1	2	0													
MAMA	3	1	3	5	0												
MAPA	4	1	1	6	5	0											
MASL	2	1	3	31	10	4	0										
GRBL	1	0	1	1	1	1	1	0									
GRIP	0	0	0	0	0	0	0	0	0								
6714	1	0	1	1	2	1	2	0	0	0							
6727	1	1	1	1	2	1	2	0	0	11	0						
6729	1	0	1	1	1	1	1	0	0	6	5	0					
6730	1	1	1	1	2	1	2	0	0	6	6	3	0				
6731	1	0	1	1	1	1	1	0	0	4	9	1	3	0			
6735	1	0	1	1	1	1	1	0	0	2	2	3	4	2	0		
6736	1	1	1	1	1	1	1	0	0	4	10	2	3	17	2	0	
6743	1	1	1	1	1	1	2	0	0	3	17	2	5	5	2	3	0

1.2- *Tolpis succulenta* (5 Primers)

The factorial analysis performed on the distance matrix between genotypes of the entire sample of *T. succulenta* is presented in Figure 7. Factors 1 and 2 represented 26.73 % of the total variance observed. Individuals from *T. succulenta* of the Azores are mainly in right side of the graphic and the individuals from Madeira clustered exclusively in the left side of the graphic.

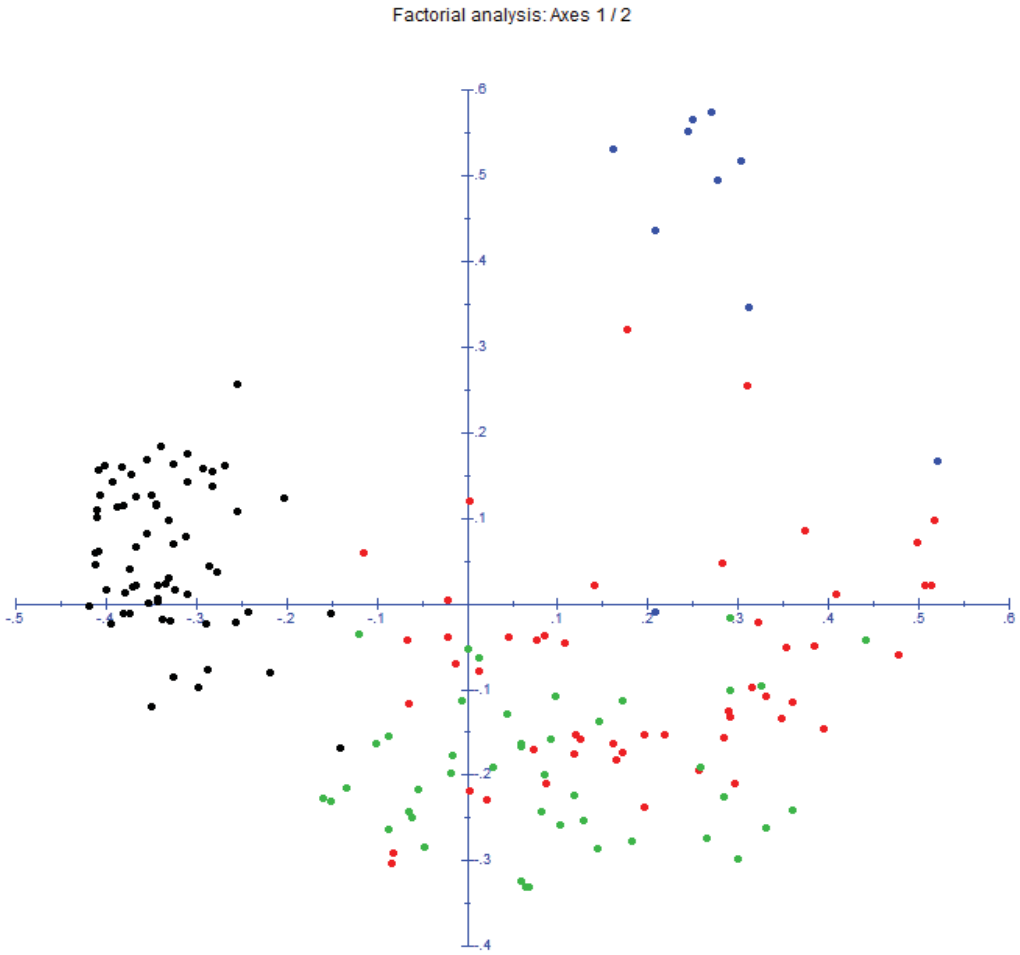


Fig 7. PCoA performed on simple matching dissimilarities matrix obtained from the molecular data of *T. succulenta*. Acessions from São Miguel are represented with red dots, accessions from Santa Madeira with green dots, accessions with Graciosa with blue dots and Madeira are represented with black dots.

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Due to this structure, obtained with the software DARwin, we ran an analysis with STRUTURE to detect the existence of subgroups.

The results obtained following the methodology of Evanno *et al.* (2005) are compiled in Figure 8. We obtained two peaks of ΔK. The first at K=4 is the highest, the second at K=7 is the smallest and we run DISTRUCT for the results of K=4 and K=7 (data not shown) to compare the two graphs. Considering the partitioning of individuals and the methodology proposed by Evanno *et al.* (2005), we assumed K=4 as the real value of K with a Fst value of 0.277.

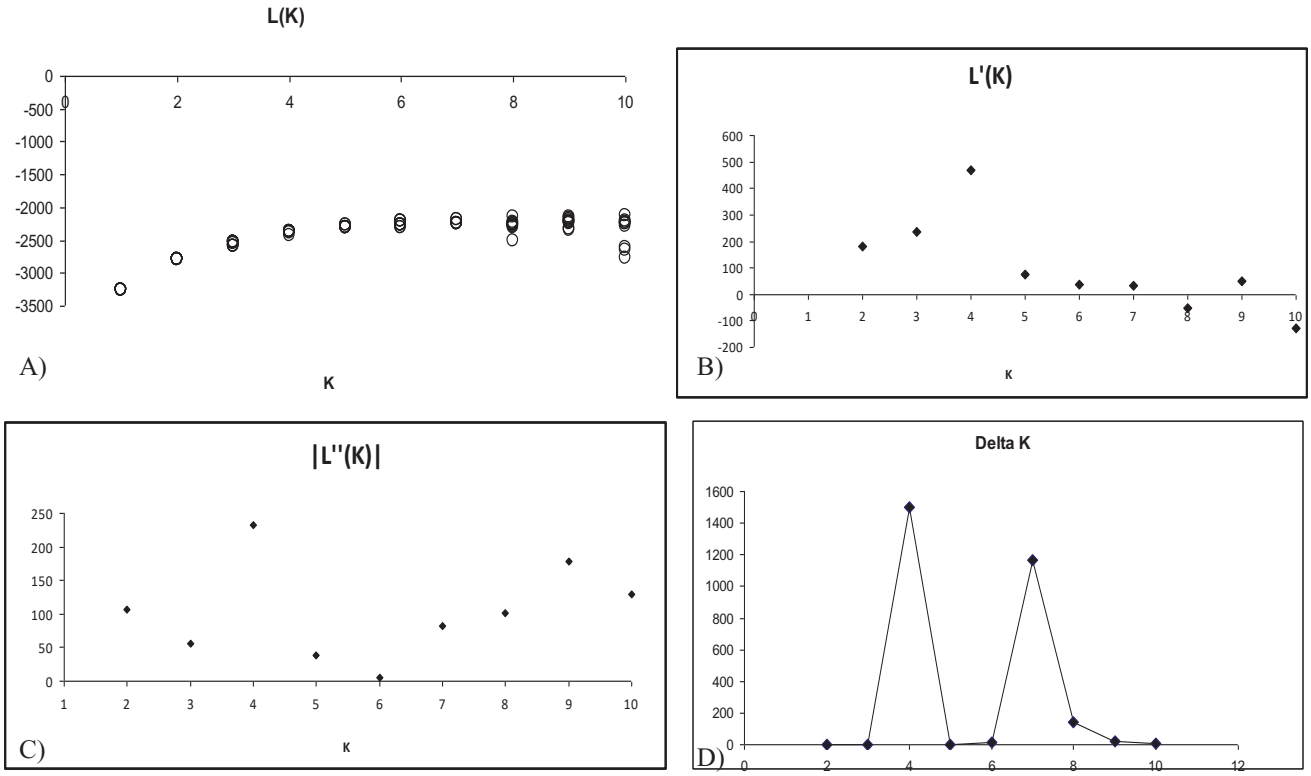

Fig. 8. Methodology from Evanno *et al.* (2005) for the interpretation of STRUCTURE results. A) Median Ln(K), B) Ln'(K), C) Ln''(K) and D) Median Delta K are presented for each value of K. The two peaks of Median Δ K at K=4 and K=7 indicate two putative right values for K

The graphical display of STRUCTURE output for K=4 is represented in Figure 9. Colours represent the proportion of each individual that belongs to each cluster.

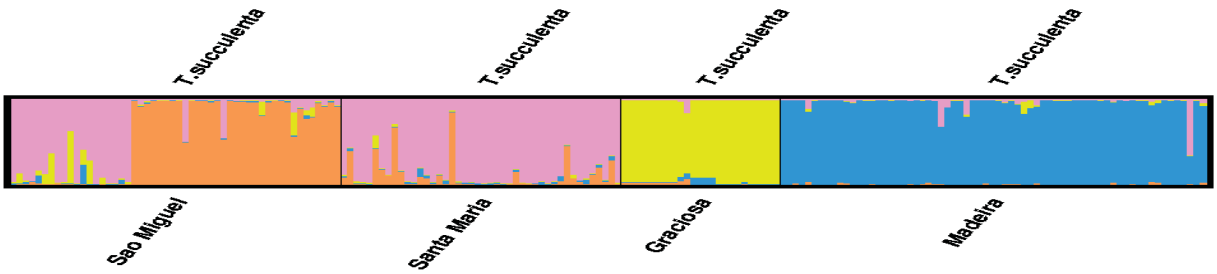

Fig. 9. Graphic display of STRUCTURE output for *T. succulenta*. Individuals are represented as thin vertical lines partitioned into segments corresponding to their membership in genetic clusters indicated by the colours.

The first of the subgroups identified by STRUCTURE corresponds to the island of São Miguel where some individuals (Praia do Lombo Gordo population, SMLG) are strongly admixed with individuals of the second group. The second subgroup is represented by individuals of Santa Maria. The third one is composed of the entire samples of Graciosa while the individuals from Madeira composed the fourth group.

We then rerun DARwin for the São Miguel and Santa Maria groups identified by STRUCTURE, and a Neighbor-Joining Tree (NJ) was performed for each cluster for a more perceptible graphical display of the clustering, both analyses confirmed the data from STRUCTURE. For the cluster composed of *T. succulenta* from São Miguel along with the samples of *T. succulenta* from Santa Maria, (Figure 10). According to the results obtained in STRUCTURE with this set of markers, the two of the tree populations collected in the island of São Miguel appears to belong to the first cluster and the population of SMLG belongs to the second cluster along with all the populations of Santa Maria (Figure 11).

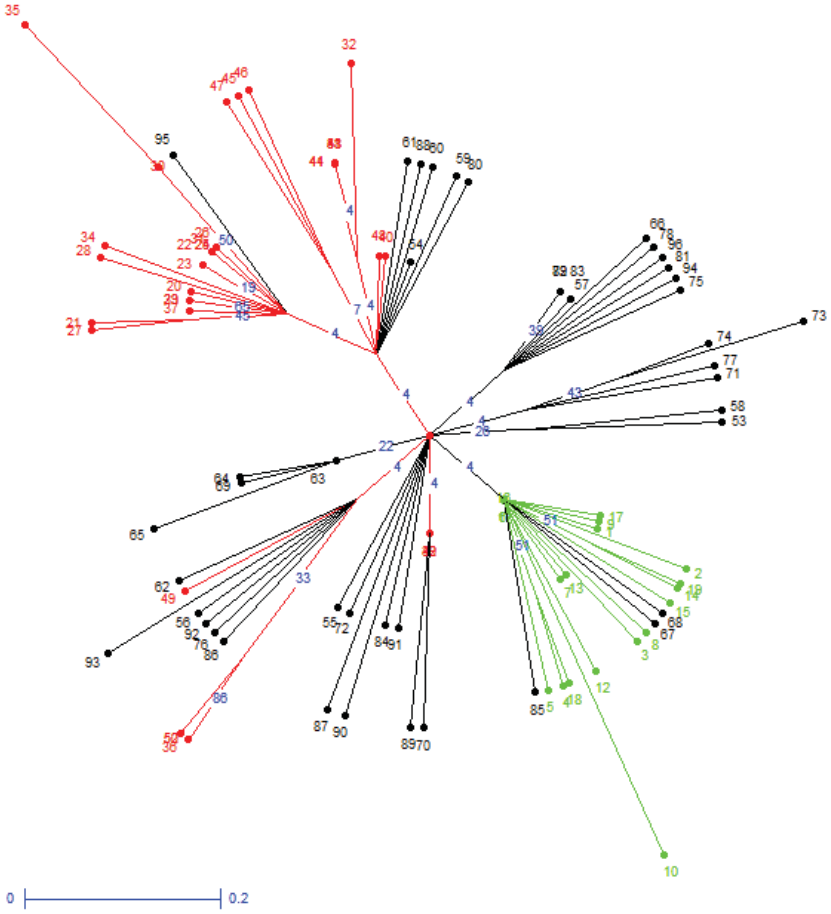

Fig. 10. Neighbor-Joining Tree (NJ) obtained from the molecular data of *T. succulenta* from São Miguel and Santa Maria. Accessions from Porto Velho das Capelas and Porto da Ajuda Bretanha are represented in red, accessions from Praia do Lombo Gordo are represented in green and all the populations of Santa Maria are represent in Black.

The graphical display of STRUCTURE output for K= 2 is represented in Figure 11. Colours represent the proportion of each individual that belongs to each cluster.

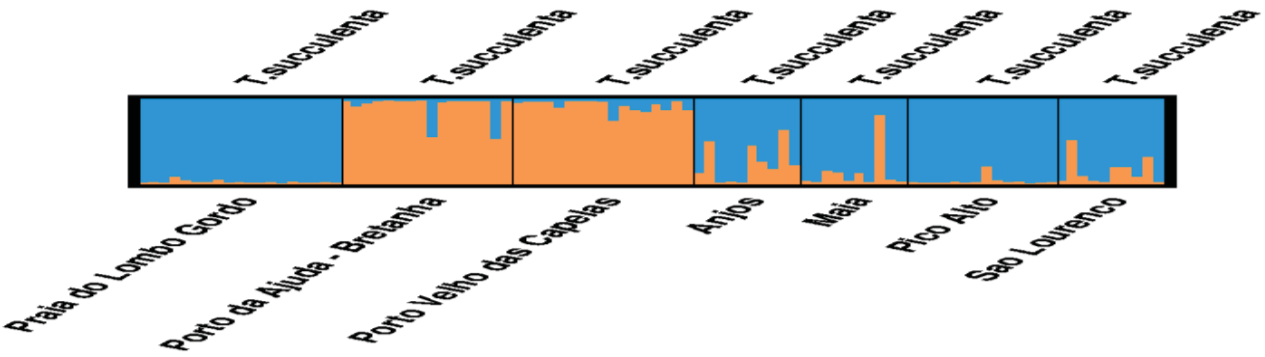

Fig.11. Graphic display of STRUCTURE output for *T. succulenta* of São Miguel and Santa Maria. Individuals are represented as thin vertical lines partitioned into segments corresponding to their membership in genetic clusters indicated by the colours.

For *T. succulenta* of São Miguel and Santa Maria, STRUCTURE identified 2 subgroups (data not shown). The first one is composed of two populations of São Miguel, Porto Velho das Capelas (SMPC) and Porto da Ajuda da Bretanha (SMPA). The second subgroup is represented by all individuals of Santa Maria and the individuals of SMLG of São Miguel. We also noticed some levels of admixture between the two clusters. We then reran DARwin only for São Miguel and factors 1 and 2 represented 42.25% of the total variance observed. The PcoA clearly displayed the separation of the two subgroups (SMPC and SMPA) on the left side of the graphic and SMLG in the right part of the graphic such as been identified by STRUCTURE (data not shown).

For the cluster composed by *T. succulenta* collected in Madeira, factors 1 and 2 represent 26.07% of the total variance observed (Figure 12). This PCoA displayed interesting results and shows a consistency between the sampling locations and the clustering. We run STRUCTURE software for the populations of *T. succulenta* of Madeira but no structure was detected (data not shown).

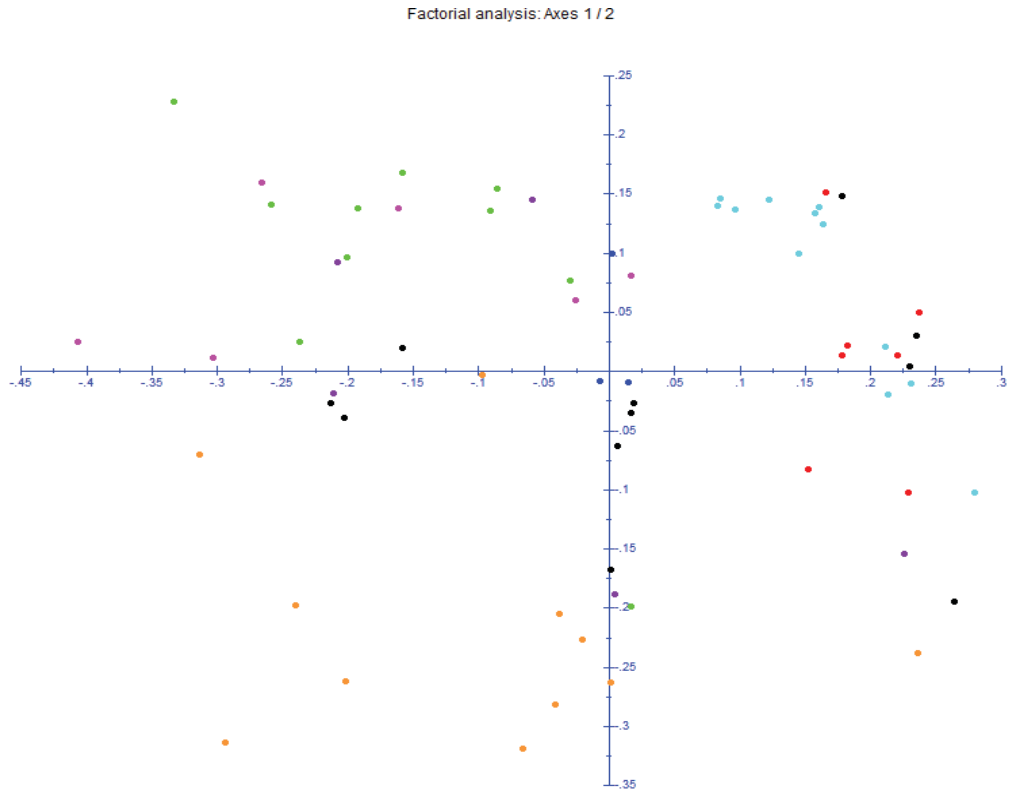

Fig. 12. PCoA performed on simple matching dissimilarities matrix obtained from the molecular data of *T. succulenta* from Madeira. Acessions from Canhas are represented dark blue dots, accessions from Descida da Eira do Serrado para o Monte with black dots, accessions from Eira do Serrado with red dots, accessions from Ribeira Brava-Estrada da Encumeada with purple dots, accessions from Estrada do Monte para Santo António with pink dots, accessions from Ponta do Sol with orange dots, accessions from Porto Moniz with light blue dots and Ribeira Brava are represented with green dots.

1.3-Tolpis azorica (8 primers)

The factorial analysis performed on the distance matrix between genotypes of the entire sample of *T. azorica* is presented in Figure 13. Factors 1 and 2 represented 17.92% of the total variance observed. The *T. azorica* accessions from Pico, Terceira, São Jorge, Faial, Flores and Corvo clustered together in the left side of the graphic. All of the individuals from São Miguel clustered in the right side of the graphic.

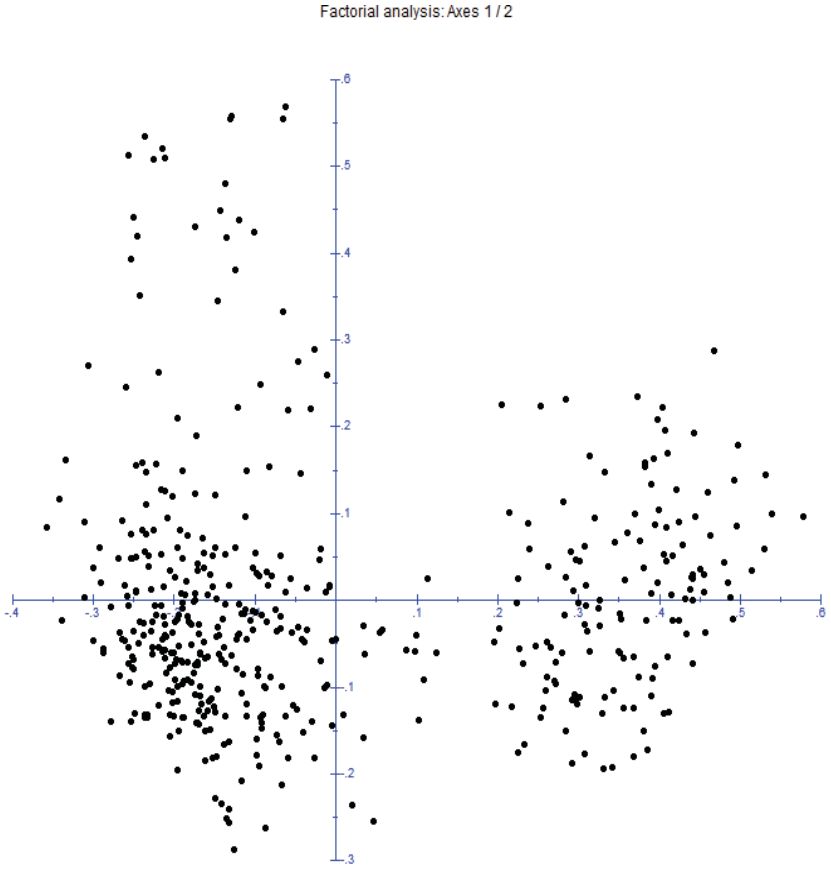

Fig. 13. PCoA performed on simple matching dissimilarities matrix obtained from the molecular data. Accessions from *T. azorica* are represented with black dots.

Due to the clustering obtained with the software DARwin, we tried a Bayesian approach by performing an analysis with STRUCTURE.

The posterior probabilities of the data for each K, called Ln P(D) in STRUCTURE output, we observed one peak of ΔK (K=2) are represented in Figure 13. Taking into consideration the variation across runs, the partitioning of individuals and the methodology proposed by Evanno *et al.* (2005) (Fig.14), we assume K=2 as the real value of K with the Fst value of 0.204.

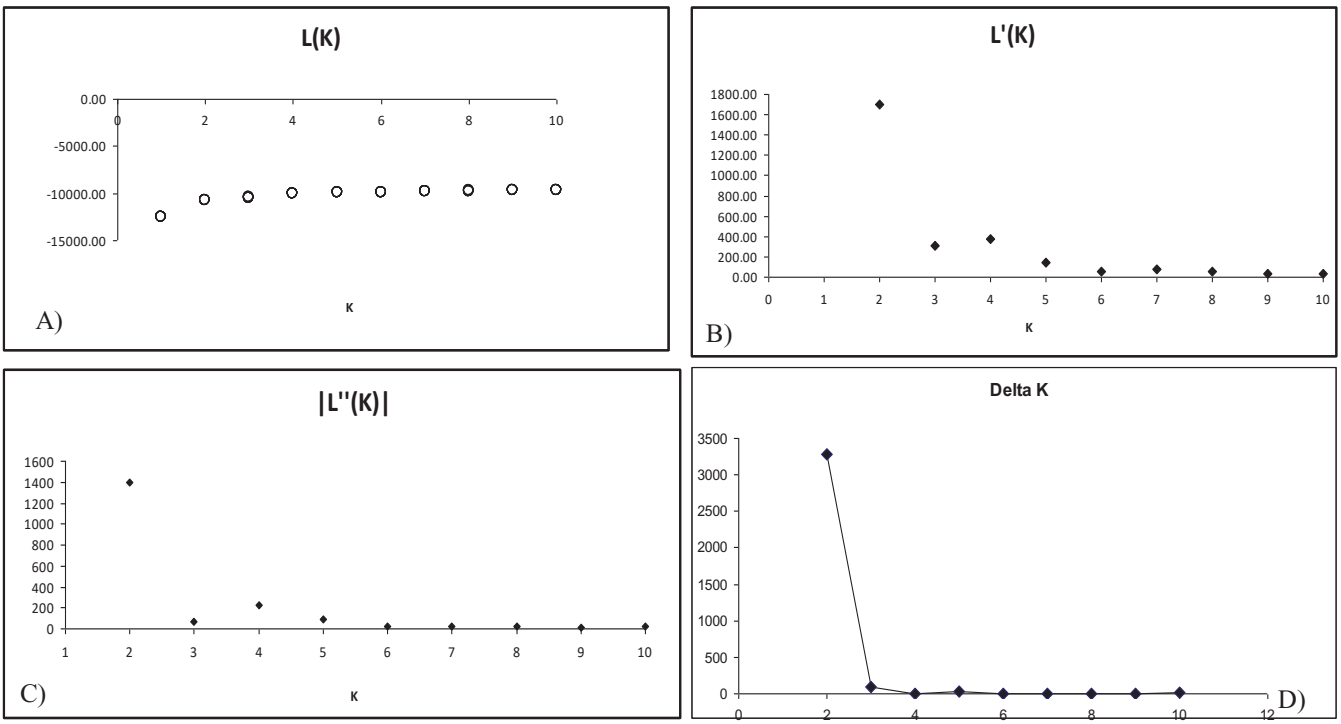

Fig. 14. Methodology from Evanno *et al.* (2005) for the interpretation of STRUCTURE results. A) Median Ln(K), B) Ln'(K), C) Ln''(K) and D) Median Delta K are presented for each value of K. One peaks of Median Δ K at K = 2 indicate one putative right value for K

The graphical display of STRUCTURE output for K=2 is represented in Figure 15. Two genetic groups were detected for *T. azorica*. Cluster 1 is composed of individuals from São Miguel, the populations of Pico, Terceira, São Jorge, Flores while Corvo belongs to cluster 2.

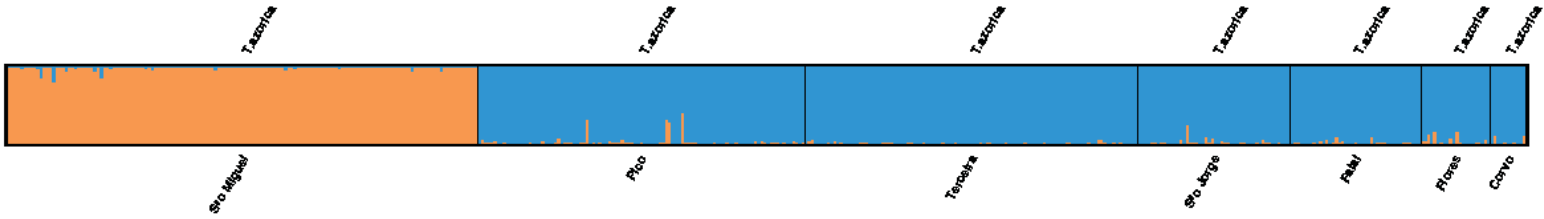

Fig 15. Graphic display of STRUCTURE output for *T. azorica*. Individuals are represented as thin vertical lines partitioned into segments corresponding to their membership in genetic clusters indicated by the colours.

As recomended by the user manual of STRUCTURE version 2.3.3 (Pritchard *et al.* 2000), we ran a new analysis separately for São Miguel (cluster 1) and Pico, Terceira, São Jorge, Faial, Flores and Corvo (cluster 2) to detect the existence of sub-structures inside the two groups.

For São Miguel, STRUCTURE identified two subgroups. The first subgroup is composed only by the populations of the western part of the island (SMPI, SMAL, SMES, SMEM, SMPC, SMVR, SMCR, SMCG and SMLE), the second subgroup is composed by the populations of the central and eastern part of the island (SMLO, SMME, SMLF, SMLA, SMTR, SMPB and SMPG; Figure 16).

The PCoA obtained didn't show a clear separation of the individuals of São Miguel. However, the individuals from the western seem to derive mainly from cluster one while those from individuals from center and east seem to derive mainly from cluster 2 (Figure 17).

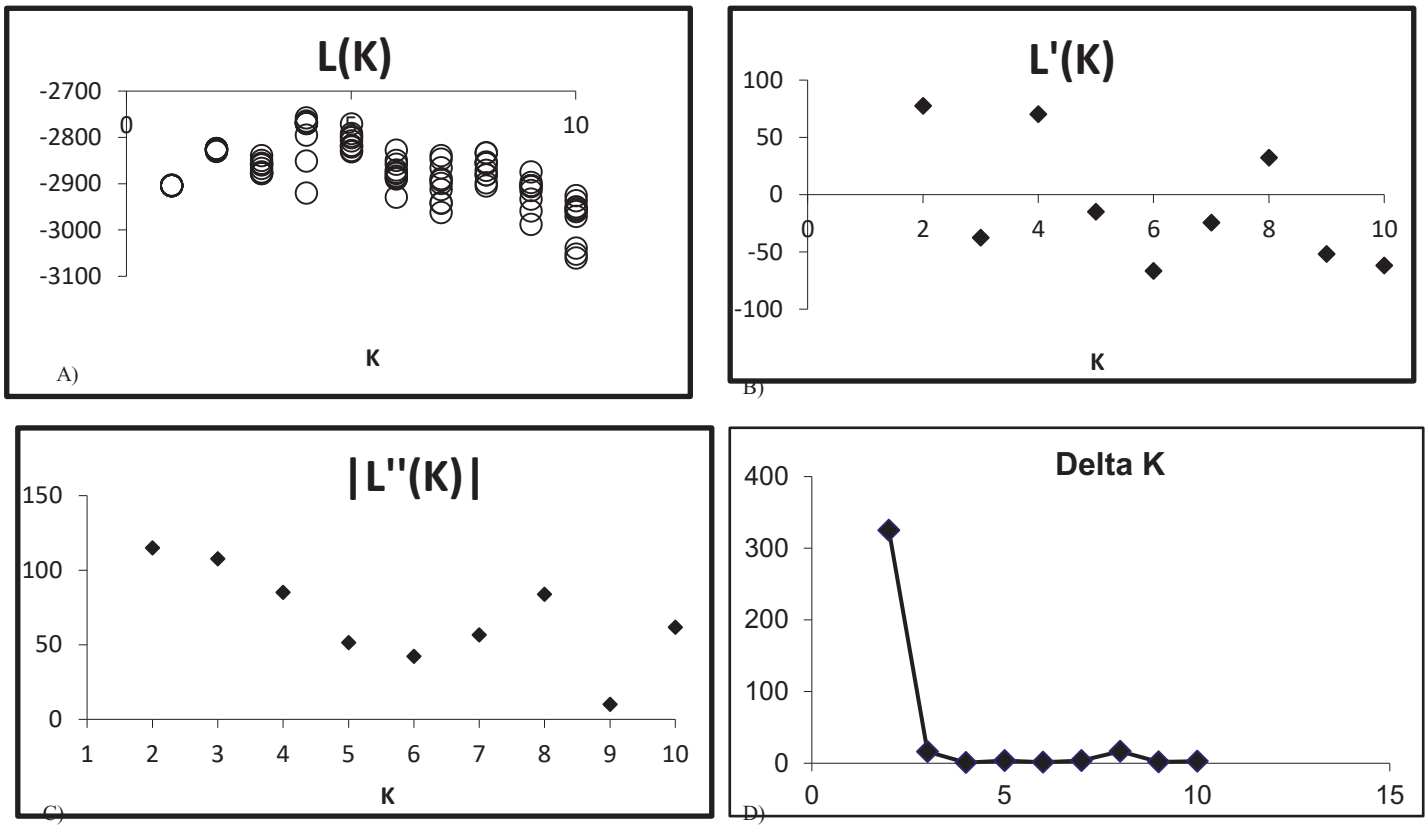

Fig. 16. Methodology from Evanno *et al.* (2005) for the interpretation of STRUCTURE results. A) Median Ln(K), B) Ln'(K), C) Ln''(K) and D) Median Delta K are presented for each value of K. One peaks of Median Δ K at K = 2 indicate one putative right value for K

The graphical display of STRUCTURE output for K=2 is represented in Figure 17. *T. azorica* from São Miguel is thus composed of two genetic groups.

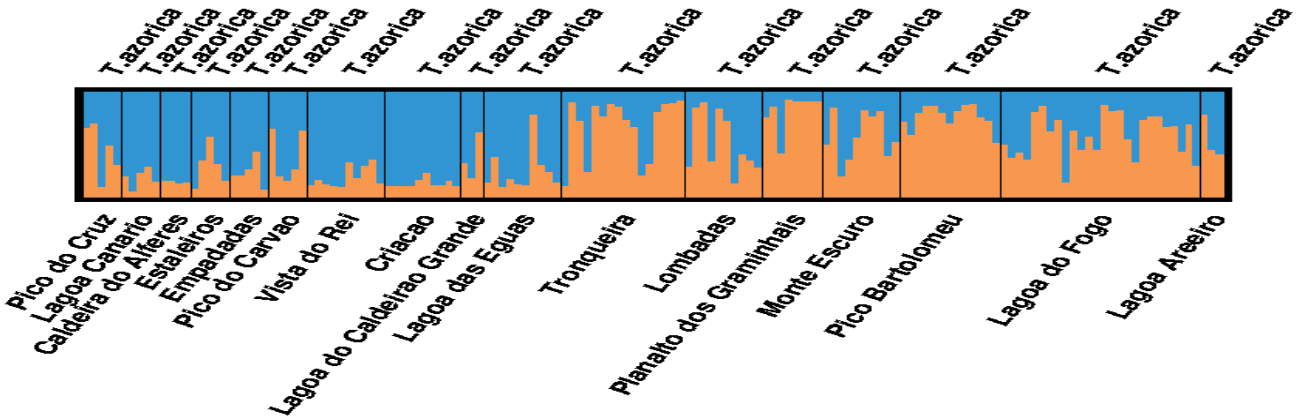

Fig.17. Graphic display of STRUCTURE output for *T. azorica* from São Miguel. Individuals are represented as thin vertical lines partitioned into segments corresponding to their membership in genetic clusters indicated by the colours.

For Pico, Terceira, São Jorge, Faial, Flores and Corvo, the results obtained following the methodology of Evanno *et al.* (2005) are compiled in Figure 18. We observed one peak of ΔK, and assumed K=3 as the real value of K with a Fst value of 0.155.

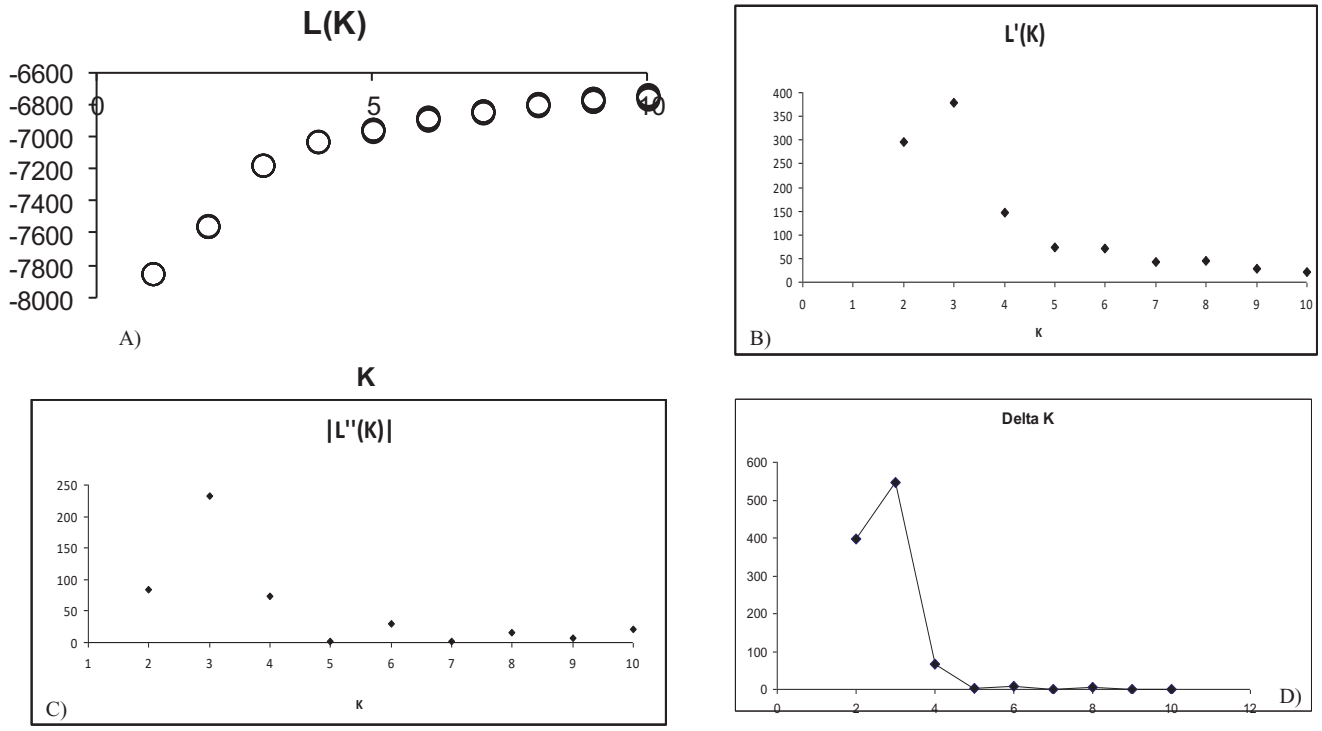

Fig.18. Methodology from Evanno *et al.* (2005) for the interpretation of STRUCTURE results. A) Median Ln(K), B) Ln'(K), C) Ln''(K) and D) Median Delta K are presented for each value of K. One peak of Median Δ K at K=3 one putative right value for K.

The graphical display of STRUCTURE output for K=3 is represented in Figure 19. Colours represent the proportion of each individual that belongs to each cluster.

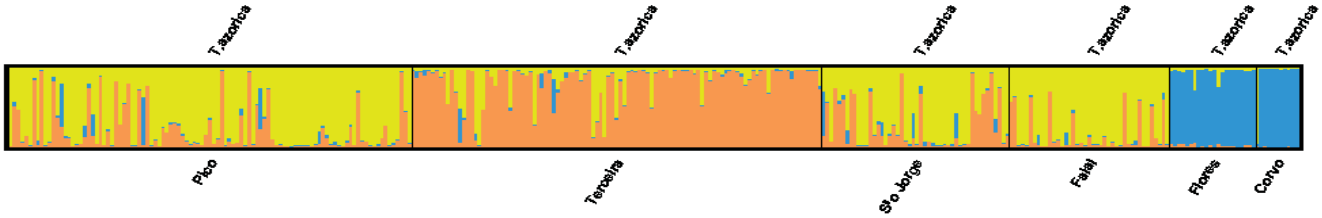

Fig.19. Graphic display of STRUCTURE output for *T. azorica* from the central and western groups. Individuals are represented as thin vertical lines partitioned into segments corresponding to their membership in genetic clusters indicated by the colours.

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According to STRUCTURE, for Pico, Terceira, São Jorge, Faial, Flores and Corvo is composed by tree genetic subgroups. The first one is represented by individuals from Pico, São Jorge and Faial. The second group from individuals from Terceira and the thirty group is composed by individuals from Flores and Corvo. We noted some admixture between group 1 and group 2.

Finally we ran Darwin and the PCoA showed that the populations from Flores and Corvo are separated from the rest of the sample (data no shown).

For *T. azorica* the number of alleles, observed and expected heterozigosity, Wright's fixation index and percentage of rare alleles were calculated for each *locus* and for all loci (Table 8). In total *T. azorica* exhibits 93 alleles (average of 11.62), ranging from 4 for marker TA2A09 to 22 for TA3B05, and an overall excess of homozygotes (Multi*locus* Fis=0.25, ranging from 0.01 for TA2A09 to 0.49 for TA2A01), and displays 55.6% of rare alleles

In Pico and Terceira *T. azorica* displays the higher amount of alleles (61 and 59 alleles, respectively), Pico with an elevated excess of homozygotes (Fis=0.15) and Terceira in Hardy-Weinberg equilibrium (Fis=0.05) along with São Jorge and Faial (Fis=0.08 and Fis=0.04 respectivley).

In São Miguel, *T. azorica* exhibits 55 alleles in total and the multi*locus* Fis value, 0.22, suggests an excess of homozygotes.

In *Corvo T.azorica* (30 alleles in total, ranging from 2 on locus TA3B05 and TA2A09 to 5 in locus TA2A03 and TA4B07) exhibits negative Fis value in 4 out of the 8 loci, TA2A03, TA3B05 and TA2A07 displaying a Fis value strongly positive (0.25, 0.22 and 0.36, respectively). The multilocus Fis value is equal to -0.23, which demonstrate the presence of an excess of heterozygotes (Table 15).

Table 15. Number of genotyped individuals, number of alleles, Hobs, Hexp, Fis, percentage of rare alleles (frequency q<0.05), for each *locus* of the . Populations of *T. azorica* distributed in the seven islands of the Azores archipelago.

Species	Sub-Group	Island	N	TA2A03					TA3B02					TA3B05					TA2A07					TA2A09				
				Alleles	Hexp	Hobs	Fis	Rare alleles (%)	Alleles	Hexp	Hobs	Fis	Rare alleles (%)	Alleles	Hexp	Hobs	Fis	Rare alleles (%)	Alleles	Hexp	Hobs	Fis	Rare alleles (%)	Alleles	Hexp	Hobs	Fis	Rare alleles (%)
Tolpis azorica	Cluster 1	São Miguel	148	4	0.36	0.18	0.5	25	5	0.7	0.66	0.06	20	11	0.75	0.57	0.23	54.5	6	0.75	0.58	0.22	33.3	3	0.39	0.43	-0.1	0
	Cluster 2	Pico	103	7	0.77	0.65	0.16	28.57	5	0.32	0.27	0.16	60	15	0.85	0.64	0.25	60	5	0.66	0.75	-0.13	20	4	0.58	0.8	-0.38	25
		Terceira	105	7	0.73	0.67	0.08	42.9	4	0.56	0.78	-0.41	50	11	0.79	0.58	0.27	54.6	8	0.7	0.95	-0.36	50	3	0.57	0.85	-0.48	0
		São Jorge	48	10	0.77	0.81	-0.05	50	3	0.57	0.92	-0.63	0	10	0.77	0.69	0.11	40	8	0.77	0.94	-0.22	37.5	3	0.44	0.38	0.16	33.3
		Faial	41	5	0.61	0.61	-0.01	40	3	0.53	0.98	-0.86	33.3	10	0.86	0.8	0.07	40	6	0.65	0.93	-0.43	50	3	0.64	0.73	-0.15	0
		Flores	22	6	0.75	0.73	0.03	33.33	3	0.63	0.86	-0.39	0	13	0.88	0.86	0.02	46.2	6	0.76	0.5	0.35	16.7	3	0.22	0.24	-0.09	33.3
		Corvo	11	5	0.74	0.91	-0.25	20	2	0.52	1	-1	0	4	0.69	0.55	0.22	0	4	0.7	0.45	0.36	0	2	0.09	0.09	0	50
	Overall		478	11	0.77	0.53	0.3	54.5	5	0.64	0.67	-0.05	20	22	0.91	0.63	0.3	63.6	12	0.79	0.76	0.04	58.3	4	0.61	0.61	0.01	25

(**Table 15**. Continued)

TA2A01					TA4B07					TA2A02					Multi*locus*				
Alleles	Hexp	Hobs	Fis	Rare alleles (%)	Alleles	Hexp	Hobs	Fis	Rare alleles (%)	Alleles	Hexp	Hobs	Fis	Rare alleles (%)	Alleles	Hexp	Hobs	Fis	Rare alleles (%)
12	0.81	0.53	0.34	41.7	10	0.6	0.54	0.1	60	4	0.44	0.26	0.41	50	55	0.6	0.47	0.22	41.8
11	0.82	0.5	0.39	45.5	13	0.83	0.52	0.37	53.8	1	0	0	-	0	61	0.61	0.52	0.15	45.9
13	0.84	0.37	0.57	46.2	10	0.61	0.38	0.38	70	3	0.08	0.09	-0.03	66.7	59	0.61	0.58	0.05	50.9
9	0.77	0.3	0.61	44.4	9	0.83	0.52	0.37	33.3	2	0.02	0.02	0	50	54	0.62	0.57	0.08	38.9
8	0.83	0.35	0.59	25	9	0.84	0.37	0.57	33.3	2	0.02	0.02	0	50	46	0.62	0.6	0.04	34.8
5	0.68	0	1	0	6	0.74	0.68	0.08	33.3	5	0.78	0.73	0.07	0	47	0.68	0.58	0.16	25.5
4	0.65	1	-0.59	0	5	0.68	0.91	-0.36	40	4	0.64	0.82	-0.3	25	30	0.59	0.72	-0.23	16.7
15	0.87	0.45	0.49	53.3	15	0.85	0.5	0.41	60	6	0.29	0.16	0.47	66.7	90	0.72	0.54	0.25	55.6

A high value of Multilocus rare alleles (55.6%) are displayed by *T. azorica* for São Miguel, Pico, Terceira, São Jorge and Faial (41.8%, 45.9%, 50.9%, 38.9% and 34.8%, respectively), while Corvo and Flores exhibit a low proportion (16.7% and 25.5%, respectively; Table 15).

For our set of eight markers the allelic richness varies from an average of 4 alleles for Corvo to 8 alleles for Pico. The island with a higher percentage of private alleles is Corvo (10 %), and the island with a lower percentage of private alleles is Faial with a lack of private alleles (Table 16).

Regarding, for the allelic frequencies for the eight *locus*, the general pattern indicates a distribution of genetic diversity higher in cluster 1 São Miguel, with the presence of more alleles than in cluster 2 from central and western group (Figure 20).

For cluster 1 the dominant alleles for the eight markers are similar to cluster 2. Particular differences appear in Flores that exhibits unique patterns of alleles in *locus* TA2A02 (Figure 20).

Table 16. Allelic richness and private alleles based on 8 microsatellites (SSR) loci.

Species	Islands	Alleles	Average alleles	Private alleles %
Tolpis azorica	São Miguel	55	7	3.6
	Pico	61	8	4.9
	Terceira	59	7	3.4
	São Jorge	54	7	1.9
	Faial	46	6	0.0
	Flores	47	6	6.4
	Corvo	30	4	10.0

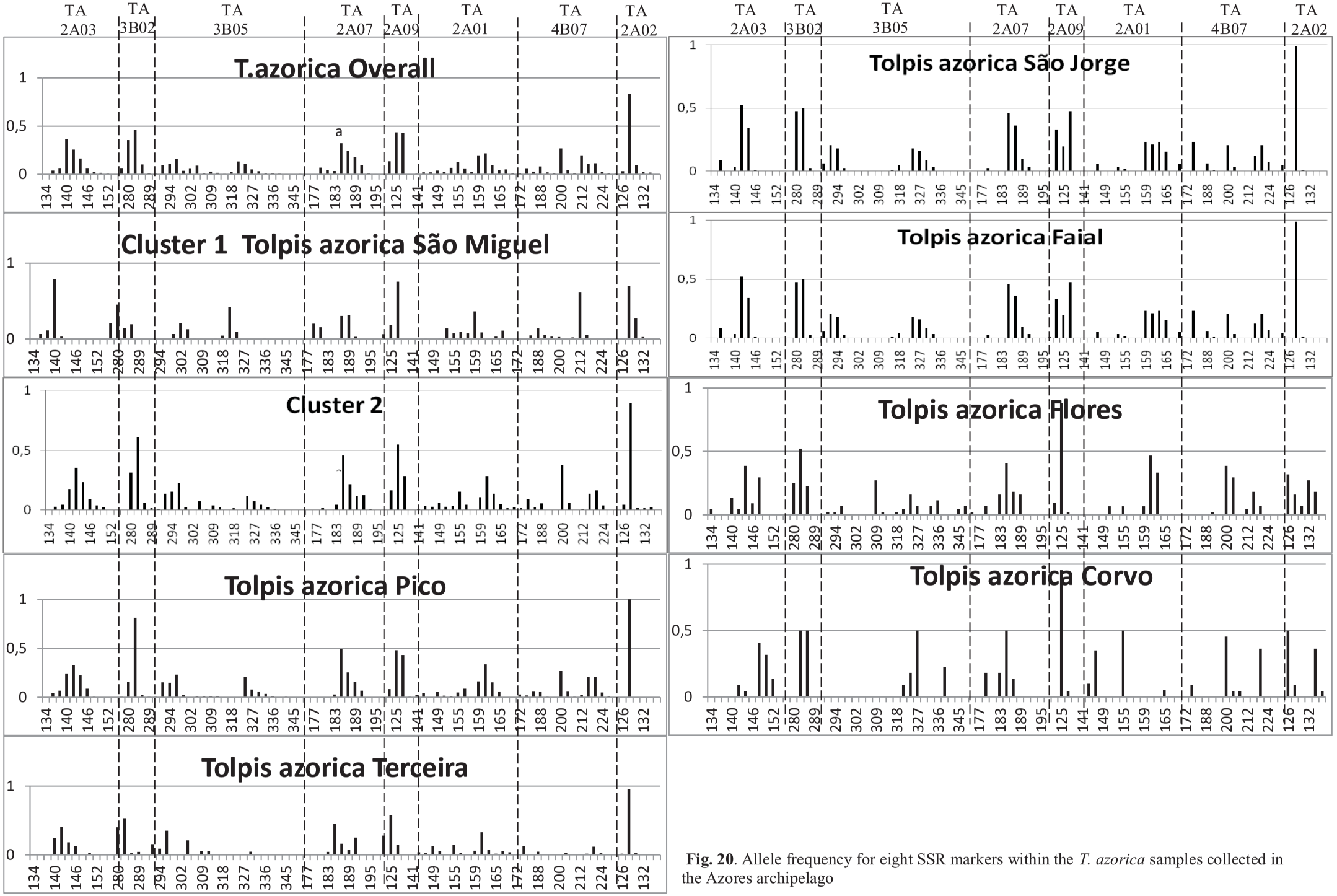

Fig. 20. Allele frequency for eight SSR markers within the *T. azorica* samples collected in the Azores archipelago

The AMOVA analyses based on the genetic structure defined by STRUCTURE, for *T. azorica* in central and western groups confirmed that the highest percentage of variation occurs within populations (85.56%) while the lowest occurs among populations within groups (6,21 %; Table 17). Equally, in São Miguel populations, the highest percentage of variation occurs within populations (86.94%) and the lowest among populations within groups (7,20%; Table 18).

Table 17. Molecular variance analysis (AMOVA) applied to 36 populations of *T. azorica* from central and western groups, calculated for eight locus.

Source of variation	d.f.	Sum of squares	Variance of componentes	Percentage of variation
Among Groups	5	141.704	0.22546 Va	8.23
Among populations within groups	30	161.816	0.16999 Vb	6.21
Within populations	624	1461.883	2.34276 Vc	85.56
Total	659	1765.403	2.7381	

Table 18. Molecular variance analyses (AMOVA) applied to 17 populations of *T. azorica* from São Miguel, calculated for eight locus

Source of variation	d.f.	Sum of squares	Variance of componentes	Percentage of variation
Among Groups	1	27.662	0.14897 Va	5.87
Among populations within groups	15	78.703	0.18272 Vb	7.20
Within populations	279	615.841	2.20732 Vc	86.94
Total	295	722.206	2.53900	

1.4- Molecular Phylogeny

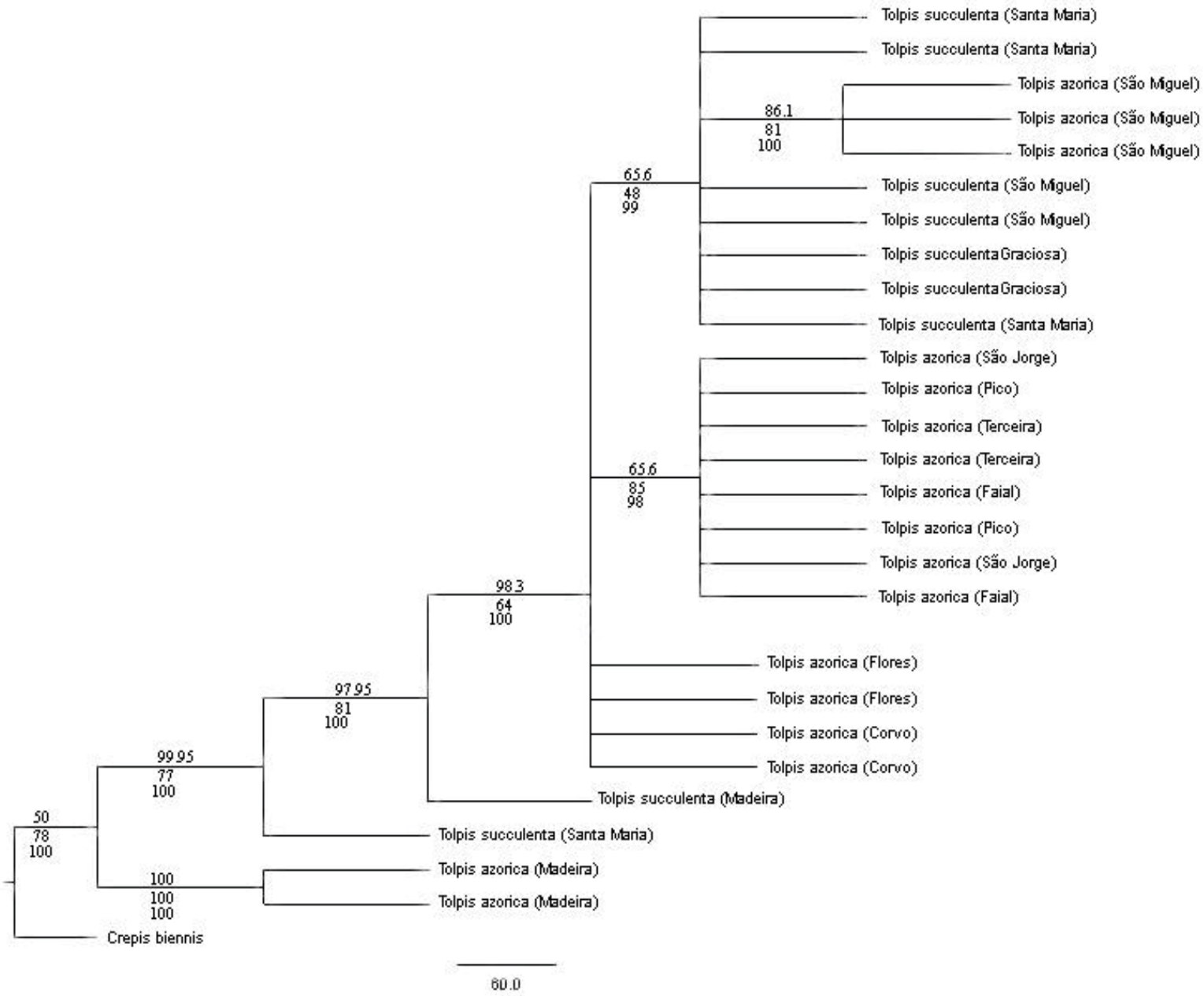

Fig.21. The 50% majority-rule consensus tree of endemic *Tolpis* spp. in Azores and Madeira archipelagos based on ITS sequences. The bootstrap value obtained for the maximum parsimony analysis is found above the branches, while the bootstrap value of RAxML and MrBayes posterior probabilities are found below branches, in respective order.

Parsimony analysis of the combined data matrix generated the upper tree (Figure 21) that was rooted with *Crepis biennis* as outgroup. The most parsimonious tree was recovered with 45 informative characters, the length value of 151 steps, a consistency index value (CI) of 0.98 and a retention index value (RI) of 0.97. The best fitting model selected by AIC for the ITS data matrix had an nst=6 value, hence the RaxML web-servers model settings were deemed appropriate for conducting a ML analysis. The BIC criterion selected an nst=1 model, which was henceforth applied in the Bayesian analysis. The bootstrap algorithm of the RAxML web-servers

resulted in a single tree with a final ML optimization likelihood of -lnL=-1857.682215. The Bayesian output, analyzed with Tracer, converged on similar lnL scores, and became stationary no later than 500000 generations.

All phylogenetic analyses used in this study suggested that ITS is a potentially informative marker for reconstructing the phylogenetic relationships between the Azores and Madeira endemic *Tolpis* spp. The most basal of all *Tolpis* species studied is *T. macrorhiza*. The two *T. succulenta* accessions group in different, while nested clades and one of the accessions is sister to all the Azorean accessions. The Azorean *Tolpis* spp. are grouped in a separate clade with a high support value. Within the Azorean clade, *T. succulenta* separates from the central group accessions of *T. azorica* with medium support values. A third clade composed by all the western group accessions of *T. azorica* is suggested in the tree, although with low support.

1.5- Morphological Variation

From a total of 61 characters evaluated according to Hickey et al. (2000), only the results obtained for the characters either qualitative (Tables 19 and 20) or quantitative, deemed as significant by the chi-square test, are presented**.**

For the qualitative characters, the lamina shape for *T. azorica* in São Miguel exhibited 10 lanceolate individuals and 1 individual with ovate shape; *T. azorica* from central and western groups exhibited 14 and 5 individuals with ovate shape, respectively; *T. succulenta* from Azores displayed 13 lanceolate individuals and 1 with spatulate shape; *T. succullenta* from Madeira exhibited 12 filiforme individuals and 2 individuals with spatulate shape; and *T. macrorhiza* exhibited 5 lanceolate individuals and 2 individuals with ligulate shape.

Additionally, the average leaf ratio (length:width) was calculated for each group (data not shown) as follows: *T. azorica* from São Miguel with a ratio of 2.13; *T. azorica* from central group, 1.05; *T. azorica* from western group, 1.03; *T. succulenta* from Azores, 2.91; *T. succulenta* from Madeira, 8.33 and *T. macrorhiza* 5.47. These ratios are complementary and support the results of Table 19.

Table 19. Significant qualitative vegetative characters observed in *Tolpis* spp.

Specie/Groups	*Tolpis azorica* from São Miguel		*Tolpis azorica* from Central Group of Azores		*Tolpis azorica* from Western Group of Azores		*Tolpis succulenta* from Azores		*Tolpis succulenta* from Madeira		*Tolpis macrorhiza* from Madeira		
Variables	**N**	**Description**	**N**	**Description**	**N**	**Description**	**N**	**Description**	**N**	**Description**	**N**	**Description**	**P**
Growth form	11	Chamaephyte	17	Chamaephyte	5	Chamaephyte	15	hemicryptophyte	15	hemicryptophyte	7	Perenial	<0,001
Stem surface	11	striate	17	striate	5	striate	15	rugose	15	rugose	7	striate	<0,001
Caulinar leaf Insertion	11	alternate	17	alternate	4	alternate	6	alternate	5	alternate	6	alternate	<0,001
					1	rosette	2	rosette	9	rosette	1	rosette	
Lamina shape	10	lanceolate	14	ovate	5	ovate	13	lanceolate	12	filiformes	5	lanceolate	<0,001
	1	ovate	1	oblong			1	spatulate	2	spatulate	2	ligulate	
			2	elliptic					1	ligulate			
Lamina apex	9	acute	10	acute	3	acute	2	acute	15	acute	2	acute	<0,001
	2	acuminate	4	acuminate	2	rounded	12	acuminate			5	acuminate	
			3	rounded			1	rounded					
Lamina bases	6	cuneada	5	cuneada	1	cuneada	15	cuneada	15	acuminate	3	cuneada	<0,001
	5	obtuse	12	obtuse	4	obtuse					2	obtuse	
											2	acuminate	
Leaf margins	8	dentate	16	dentate	1	dentate	8	dentate	1	dentate	5	dentate	<0,001
	1	dentaticulate	1	serrate	4	serrate	2	acentuate dentate	8	entire	2	entire	
	2	acentuate dentate					4	entire	6	incised			
							1	incised					

In addition, we also present the results obtained for the qualitative characters pertaining to reproductive traits for each *Tolpis* group studied, which showed significance according to the chi-square test. These characteristics are peduncles form, and involucral bracts shape from the first, second and third series (Table 20).

Table 20. Significant qualitative reproductive characters observed in *Tolpis* spp.

Specie/Groups		*Tolpis azorica* from São Miguel		*Tolpis azorica* from Central Group of Azores		*Tolpis azorica* from Western Group of Azores		*Tolpis succulenta* from Azores		*Tolpis succulenta* from Madeira		*Tolpis macrorhiza* from Madeira	
Variables	**N**	**Description**	**N**	**Description**	**N**	**Description**	**N**	**Description**	**N**	**Description**	**N**	**Description**	**P**
Peduncles form	6	narrow	3	narrow	2	narrow	9	aclavate	7	narrow	5	narrow	0,001
	3	aclavate	4	aclavate			3	narrow to aclavate	7	aclavate			
	1	narrow to aclavate	8	narrow to aclavate					1	narrowt o aclavate			
Involucral bracts shape, 1st series	8	triangulate	14	triangulate	2	oblong	10	linear	15	linear	2	linear	< 0,001
Involucral bracts Shape, 2nd series	3	triangulate	5	triangulate	2	oblong	2	linear	7	linear	2	linear	< 0,001
	5	oblong	9	oblong			8	lanceolate	8	lanceolate			
Involucral bracts shape, 3rd series	8	oblong	12	oblong	1	oblong	4	lanceolate	7	lanceolate	2	linear	< 0,001

Regarding the involucral bracts shape in the first series, observed in *T. azorica* from São Miguel and central group, the character exhibits a triangular shape, while in *T. azorica* from the western group, it is oblong. In *T. azorica* from São Miguel and central group, the involucral bracts from the second series are oblong and triangulate, while in *T.azorica* from the western group we observed an oblong shape. Regarding the third series of bracts, all the *T. azorica* specimens observed exhibited oblong involucral bracts.

Regarding *T. succulenta*, the first series of involucral bracts display a linear shape. The involucral bracts of the third series of *T. suculenta* from Azores are lanceolate in 8 individuals and linear in 2, *T. succulenta* from Madeira displays lanceolate and linear shapes. The third series of bracts exhibits a lanceolate shape. Finally, *T. macrorhiza* displays a linear shape in all involucral bracts series.

We used ANOVA to compare quantitative, vegetative and reproductive traits between all the groups that resulted from the molecular analysis, namely *T. azorica* from São Miguel, *T. azorica* from Central group, *T. azorica* from the western group, *T. succulenta* from Azores and Madeira and *T. macrorhiza.*

There were significant differences between those groups for vegetative traits, namely: leaf length (df=5; F=3.148; P=0.014), leaf width (df=5; F=16.188; P=0.000), and petiole length (df=5; F=11.207; P=0.000).

Regarding leaf length, the only significant difference was found between *T. azorica* from the western group, which presented the largest value and *T. succulenta* from Madeira with the smallest. For the leaf width, significant differences were found between *T. azorica* from the western group (largest leaf width), and *T. succulenta* from Azores and Madeira and *T. macrorhiza* with the smallest. Regarding petiole length, significant differences were found between *T. azorica* from the western and central groups (largest petiole), *T. succulenta* from Azores (medium petiole), and *T. macrorhiza* (smallest).

Furthermore, there were significant differences between the studied groups regarding the reproductive traits, namely: peduncle length (df=5; F= 3.605; P=0.008); peduncle bracts length (df=5; F=4.632; P=0.002); peduncle bracts width (df= 5; F=27,441; P=0,000); capitula number (df= 5; F=24,676; P=0,000); bracts length 1st series (df=5; F=8.904; P=0.000), bracts width 1st series (df=5; F=15.870; P=0.000), bracts width 2nd series (df=5; F=3.031; P=0.020).

Regarding peduncle length, a significant difference was found between groups, with the largest length occurring in *T.azorica* from São Miguel and *T. macrorhiza*, and the smallest in *T. azorica* from the western group. As for peduncle bracts length, a significant difference was found with the largest length observed in *T. azorica* from the western group, and the smallest in *T. azorica* from São Miguel and *T. succulenta* from Azores and Madeira. Regarding the peduncle bracts width, a significant difference was found with the largest width occurring in *T. macrorhiza*, medium in *T. azorica* from the western group, and smallest in *T. azorica* from São Miguel and *T. succulenta* from Azores. As for the capitula number the only significant difference was found in *T. azorica* from the western group with the smallest number. For the bracts width, a significant difference was found with the largest width observed in *T. azorica* from the western group, and the smallest in *T. macrorhiza*.

Below are the results of a discriminant analysis representing the sampled individuals according to the first and second canonical functions, which explained 83% of the variance (Figure 22)

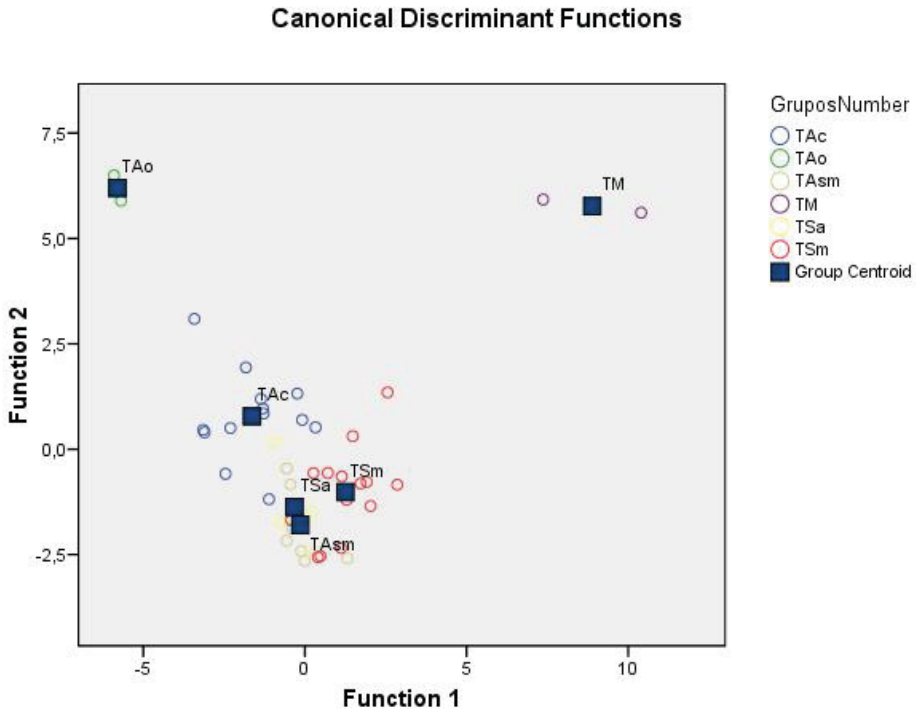

Fig 22. Comparison of the groups that resulted from the molecular analysis, namely *T. azorica* from São Miguel (TAsm), *T. azorica* from Central group (TAc), *T. azorica* from western group (TAo), *T. succulent*a from Azores (TSa), *T. succulenta* from Madeira (TSm), and *T. macrorhiza* (TM), based on quantitative vegetative and reproductive traits.
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Discussion

In this research, a population molecular genetics study of *Tolpis* spp., performed with a set of 5 microsatellite markers, identified three well-defined groups (*T. azorica* of São Miguel, *T. azorica* of the central and western groups and *T. succulenta* of Azores and Madeira). This clustering is confirmed by a Bayesian analysis (Figures 4 and 5), even though the PCoA performed was not very informative. However, this pattern with the PCoA can be explained easily by the very high number of samples used in the study and the high level of genetic heterogeneity within the different species.

The division of *T. azorica* obtained in our study differs from the currently accepted geographical circumscription of the genus *Tolpis spp*. in Azores and is confirmed by the 8 SSR

study (Figure 13 and 15), the phylogenetic study (Figure 21) and the morphological study (Figure 22).

The difference between *T. azorica* from São Miguel and *T. azorica* from other islands seems to be better well-defined (Figure 13) than the difference between *T. succulenta* from Azores and *T. succulenta* from Madeira (Figure 7).

The cladograms from the phylogenetic tree analyses (MP, ML, MB) are concordant with each other and display a well-supported topography for two major clades (Figure 21): one including all *T. azorica* from the central group, and a second clade including accessions of *T. succulenta* from the Azores and of *T. azorica* from São Miguel. A third clade with *T. azorica* from the Azores western group of islands is suggested but does not have sufficient support. The results of the morphological analysis are in congruence with the phylogenetic results as they show that *T. azorica* specimens from the central and western groups have significant differences when compared with specimens from S. Miguel (Figure 22).

The analyses further suggest that *T. azorica* from São Miguel is sister to *T. succulenta* from the Azores, although the *T. succulenta* accessions did not resolve well. However, considering also the populations structure obtained and the morphological results, we postulate here that *T. azorica* in São Miguel is a new taxonomic unit and that its classification should be revised. In addition and considering the phylogenetic relationships obtained (Figure 21) for *T. succulenta* from Azores and *T. succulenta* from Madeira, which cluster in different and well-supported clades, we consider that the taxonomic position of the Azores *T. succulenta* should be revised.

The high consistency index (0.98) obtained in the MP analysis apparently indicates that the spacer regions were not subjected to homoplasy across the lineages during the evolution and the diversification of *Tolpis.* Monophyly of the Azorean lineages is evident and suggest that all species derived from a single ancestral species. Relationships among these clades and the two species of *Tolpis* endemic to Madeira, *T. succulenta* and *T. macrorhiza,* are also resolved in our phylogeny, with strong bootstrap and posterior probability support. The most basal of all *Tolpis* species is *T. macrorhiza*.

These results are in consonance to the ones of Moore et al. (2002) in their analyses of chloroplast DNA restriction site variation for all species of *Tolpis* occurring in Azores, Madeira, Canary and Cape Verde Islands..

Relatively to the Fis values for *T. azorica* a reduction in observed heterozygosity was obtained (increased homozygosity), probably because of pooling discrete subpopulations with different allele frequencies that do not interbreed, as a single randomly mating unit.

Corvo displayed a multilocus Fis value strongly negative which demonstrate the presence of an excess of heterozygotes in the set of loci. Corvo is also the island with a higher percentage

of private alleles for *T. azorica.* Since it is a single population, protection measures should be undertaken.

T. succulenta exhibits 56 alleles on 5 loci in total and all the groups exhibited overall excesses of homozygotes. However, Fis locus by locus display a heterogeneous pattern similar to those observed in *T. azorica* in São Miguel, Pico and Flores. Madeira is the island with the higher percentage of private alleles in *T. succulenta* which can be explained by the fact of being a separate taxonomical unit unique to the Madeira archipelago.

For *T. azorica* STRUCTURE identified two clusters in São Miguel but all the genotypes in our sample displayed admixed profiles. However, the genetic backgrounds of individuals from the western part of the island derived mainly from cluster 1 while those from individuals collected in the central and eastern part of the island derived mainly from cluster 2 (Figure 17). The morphologically results show that leaves from São Miguel specimens displayed differences when compared with the ones from the central and western groups of islands. In São Miguel the lamina shape is lanceolate while in the others islands are widely ovate. The holotype of *T. azorica* is a specimen illustrated by Seubert (1844) originated from the island of Pico.

The phylogenetic study and the genotyping with 8 SSR analyses also revealed a secondary level of clustering within *T. azorica* as the samples from Flores and Corvo differ from the central group and compose a homogeneous sub-group (Figure 16).

Both Flores-Corvo subgroup and Graciosa subgroup in *T. succulenta* display a lack of rare alleles (Tables 6 and 8). Allelic loss occurs more rapidly than loss of genic heterozygosity. Rare alleles are lost especially rapidly (Maruyama et al. 1985). A genetic bottleneck causes loss of allelic diversity and heterozygosity (Nei et al. 1975; Allendorf 1986), and rare alleles are most likely to be lost (Nei et al. 1975; Houlden et al. 1996; Luikart et al. 1998; England et al. 2003), which can be visualized as a departure from an L-shaped distribution of allele frequencies (Luikart et al. 1998). Moreover, loss of rare alleles causes a temporary excess in heterozygosity and this can be a basis for detection of bottlenecks (Cornuet and Luikart 1996). Therefore, it seems that *T. azorica* in Flores and Corvo and *T. succulenta* in Graciosa suffered a recent bottleneck. We recommend implementation of protection and conservation measures in order to safeguard these populations.

In this study we observed that, with our set of markers, the intrapopulational genetic variability (within populations) is high for *Tolpis* spp. in the Azores and Madeira archipelagos (Table 10, 11 and 14) and this factor is the main responsible for the genetic variability found (84.13, 84.49 and 58.79 for *T. azorica* from central and western groups, São Miguel and *T. succulenta*, respectively). However the interpopulational genetic variability (among populations within groups) is very low (6.52, 7 and 15.2 for *T. azorica* from central and western groups, São Miguel and *T. succulenta*, respectively).

Taxonomic Treatment

Here we propose a taxonomic and nomenclatural revision of the Azorean *Tolpis* species, including two new species never before described for the archipelago. Synonymy of *T. azorica* and *T. succulenta*, according to Lack (1981), is also transcribed for clarity.

Tolpis micaelensis Silva L. B. & M. Moura. sp. nova. ined. HOLOTYPE : AZORES. São Miguel: 1845, T. C. Hunt, s.n (BM 33318).

Description – *Growth form* chamaephyte. *Stem* with striated surface, erect, woody at base, glabrous. *Leaf* 4.3-9.6 x 2.8-4.5 cm, alternate, lanceolate to ovate, base cuneate to obtuse, apex acute, often deeply dentate and glabrous. *Petiole* short or absent. *Peduncles* 4.10-14.3 cm, with bracts, erect to curved ascendant and descendent, thin to clavate. *Inflorescence* 2.2-2.6 cm wide simple cyme or bostryx with capitula in number from 3 to 29. *Involucre* diameter 1.3-1.5 cm, bracts series from 3 to 4. *Bracts* glabrous or pubescent. *Ligule* 5.44 x 5.8 mm, yellow. *Achenes* 1.17 x 0.36 mm, surface with 4 to 12 transverse striae, light brown to dark brown. *Pappus setae* biseriate, first serie 4.8 mm, second serie 1.95 mm.

Distribution and Habitat – Only in São Miguel Island. Found in natural forests (*Ilex, Laurus, Juniperus, Juniperus* with peat bogs), margins of permanent and semi-natural pastures, natural meadows (*Holcus, Festuca, Deschampsia*), wet meadows, lake shores and watercourse margins, young lava flows with pioneer vegetation, roadside slopes, strongly exposed areas, steep slopes, ravines and craters. Altitudinal limits can reach below 437 m (Caldeira do Alferes) and above 898 m (Lagoa do Fogo, Pico da Barrosa).

Representative Specimens Examined – *Tolpis azorica*. AZORES. SÃO MIGUEL: Caminho do Pico da Cruz, Silva, L.B. and Dias, E.F. TA-SMPI-03 (AZB); Lagoa do Canário, Silva. L.B. and Dias, E.F. TA-SMLC-024,TA-SMLC-076 (AZB); Criação, Silva, L.B. and Dias, E.F TA-SMCR-01 (AZB); Vista do Rei, Silva, L.B. and Dias, E.F TA-SMVR-01 (AZB); Lagoa do Fogo, Sardos, J. TA-SMLF-100 (AZB); Monte Escuro, Silva, L.B. and Dias, E.F TA-SMME-01 (AZB); Tronqueira, Silva, L.B. and Dias, E.F TA-SMTR-063,TA-SMTR-068, TA-SMTR-096 (AZB); Planalto dos Graminhais, Silva.L.B., Dias, E.F and Moura M. TA-SMPG-05 (AZB).

- *Tolpis maritima* Silva L. B. & M. Moura sp. nova. ined. HOLOTYPE: AZORES. São Miguel: 6 July 2010, M. Moura, TS-SMPC-018 (AZB 1327).

Description – *Growth form* hemicryptophyte herb. *Stem* surface rugose, procubent to ascending, woody at base, glabrous. *Leaf* 4.80-8.38 cm, alternate and rosulate, elliptic to lanceolate, base acute or acuminate, apex acuminate or rounded, entire or coarsely dentate. *Leaf indument* with generally simple trichomes in upper leaf surface, lower leaf surface glabrous. *Petiole* 0.5-2 mm. *Peduncles* 3.84- 8.93 cm, with bracts, erect to curved ascendant and descendent. *Inflorescence* 2-2.8 cm wide simple cyme, capitula number from 2 to 40. *Involucre* diameter 1.2-1.8 cm, bracts series varie from 2 to 4. *Bracts* glabrous, pubescent, pilose and tomentose. *Ligule* 4.94 x 8.89 mm yellow. *Achenes* 1.62 x 0.60 mm, surface with 5-8 transverse striae, light brown to marron. *Pappus setae* biseriate, first serie 4.05 mm, second serie 2.11 mm.

Distribution and Habitat – Present in the islands of São Miguel, Santa Maria and Graciosa. Found near the coast, dry and strongly exposed areas, coastal rocks and cliffs, coastal lava flows, coastal scrubland (*Erica, Morela*, mixed), *Erica* scrubland, steep roadside slopes and rocks, especially in sea wind exposed places. Altitudinal limits can reach sea level (São Miguel) and above 600 m (Santa Maria).

Representative Specimens Examined – *Tolpis succulenta*. AZORES. SÃO MIGUEL: Porto da Ajuda Bretanha, Silva L.B. TS-SMPA-21,TS-SMPA-24 (AZB); Praia do Lombo Gordo, Moura M. and Silva L. TS-SMLG-01 (AZB); Porto Velho das Capelas, Moura M. TS-SMPC-01,TS-SMPC-18 (AZB); SANTA MARIA: Maia, Moura M. and Silva L. TS-MAMA-09,TS-MAMA-10 (AZB); São Lourenço, Moura M. and Silva L. TS-MASL-04,TS-MASL-05 (AZB); Pico Alto, Moura M. and Silva L. TS-MAPA-11, TS-MAPA-14, TS-MAPA-02 (AZB); Anjos, Moura M. and Silva L. TS-MAAN-02 (AZB); GRACIOSA: Baía do Filipe, Beira-mar da Luz, Moura M. and Dias E.F. TS-GRBL-01 (AZB); Ilhéu da Praia, Moura M and Dias E.F. TS-GRIP-01 (AZB).

Tolpis azorica (Nutt.) Pinto da Silva in Palh., Cat. Pl. Vasc. Açores 129 (1966) ≡ *Caladonta azorica* Nutt. in Trans. Amer. Phil. Soc., N. Ser., 7: 448 (1841). – Typus: Açores, Fayal, Caldera, *Nuttall* (Holotypus: BM! Isotypus: K!).

= *Tolpis nobilis* Hochst. ex Seub., Fl. Azorica 33 (1844). – Typus: Açores, Pico, Pic de Pico, alt circ. 3000', 18.7.1838, *C. hochstetter* (Holotypus: TUB!).

= *Tolpis nobilis* Hochst. ex Seub. var. *petiolaris* Trel. in Annual Rep. Missouri Bot. Gard. 8: 125 (1897) ≡ *Tolpis azorica* (Nutt.) Pinto da Silva in Palh. var *petiolaris* (Trel.) Pinto da Silva in Palh., Cat. Pt. Vasc. Açores 129 (1966). – Syntypi: Açores, Flores, *Trelease 470* (MO!), *471* (MO!), *475* (MO!).

Tolpis succulenta (Dryander in Aiton) Lowe, Man. Fl. Madeira 1:525 (1868) ≡ *Crepis succulenta* Dryander in Aiton, Hort. Kew. 3:128 (1789). – Typus: Im Hortus Kewensis Kultivierter Beleg mit der Beschriftung, „Hort.Kew 1779“ auf der Rückseite und *„Crepis succulenta* Mss.” In Dryanders Schrift auf der Vorderseite (Holotypus: BM!).

= *Crepis filiformis* Dryander in Aiton, Hort. Kew. 3:128 (1789) ≡ *Tolpis filifornes* (Dryander in Aiton) DC., Prodr. 7:87 (1838) ≡ *Schmidtia filiformis* (Dryander in Aiton) Schultz Bip. in Webb & Berth., Phyt. Canar. 3 (2) 399 (1847-48) ≡ *Tolpis succulenta* (Dryander in Aiton) Lowe subsp. *filiformis* (Dryander in Aiton) Menezes, Fl. Arch. Madeira 99 (1914) ≡ *Tolpis succulenta* (Dryander in Aiton) Lowe subsp. v*ulgaris* Menezes var. *filiformes* (Dryander in Aiton) Menezes in Brotéria, sér. Bot. 22:185 (1926). –Typus: Im Hortus Kewensis kultivierter Beleg mit der Beschriftung „Hort.Kew.” auf der Rückseite und „Crepis filiformis mss.” in Dryanders Schrift auf der Vorderseite (Holotypus: BM!).

= *Schmidtia fruticosa* Moench, Meth. Suppl. 217 (1802). – Typus: Im Marburger Botanischen Garten Kultivierte Pflanze.

= *Hieracium fruticosum* Willd., Sp.Pl.3:1591 (1803) ≡ *Tolpis fruticosa* (Willd.) Schrank, Pl. rar.5:46 (1819) ≡ *Tolpis succulenta* (Dryander in Aiton) Lowe subsp. *fruticosa* (Willd.) Menezes, Fl.Arch. Madeira 99 (1914) ≡ *Tolpis succulenta* (Dryander in Aiton) Lowe subsp. *succulenta* var. *fruticosa* (Willd.) Menezes in Brotéria, sér. Bot.22:135 (1926). – Typus: Hb*, Willd. 14724* (Holotypus: B!).

= *Crepis pectinata* Lowe in Trans. Cambridge Philos. Soc. 4:24 (1831) ≡ *Tolpis pectinata* (Lowe) DC., Prodr. 7:87 (1838). – *Tolpis succulenta* (Dryander in Aiton) Lowe subsp. *pectinata* (Lowe) Menezes, Fl.Arch. Madeira 99 (1914) ≡ *Tolpis succulenta* (Dryander in Aiton) Lowe subsp. *vulgaris* Menezes var. *pectinata* (Lowe) Menezes in Brotéria, sér. Bot. 22:134 (1926),- Syntypi: Sloane Herbarium, Hortus siccus 5:4 (planta dextra) (BM!); auf der ersten Cookschen Weltumseglung gesammelter,, *Crepis tenuifolia* Ms. Madera “ beschrifteter Beleg /BM!)

=Tolpis succulenta (Dryander in Aiton) Lowe var. *multifida* Lowe, Man. Fl. Madeira 527 (1868). = Tolpis succulenta (Dryander in Aiton) Lowe var. *ligulata* Lowe, Man. Fl. Madeira 527 (1868). = *Tolpis succulenta* (Dryander in Aiton) Lowe var. *linearifolia* Lowe, Man. Fl. Madeira 528 (1868). = *Tolpis succulenta* (Dryander in Aiton) Lowe var. *oblongifolia* Lowe, Man. Fl. Madeira 528

(1868). = *Tolpis succulenta* (Dryander in Aiton) Lowe subsp. *succulenta* var. *propinqua* Menezes in Brótéria, sér. Bot.22:135 (1926). – Typus: Madeira, Magdalena, *J.G. da Costa* (COI).

Conclusion

Tolpis specimens from the highlands of São Miguel Island showed a completely distinct genetic and morphological variability from the *T. azorica* which occurs in the other islands of the Azores archipelago. therefore we propose here to consider it as a new endemic species, under the name of *T. micaelensis* Silva L. B. & M. Moura. Furthermore, *T. azorica* (sensu Lack) is only circumscribed to the central and western groups of the Azores archipelago, although the populations of the western group already show a slight genetic differentiation when compared to the rest of the *T. azorica* from the central group. T. micaelensis is a single island endemic and thus a special conservation measures should be taken, in order to avoid genetic contamination from other islands.

In Azores, *T. succulenta* also needs to be redefined as a new taxonomic unit, since it presents distinct genetic differentiation and is morphologically different, when compared to *T. succulenta* from Madeira, therefore we propose the name *T. maritima* Silva L. B. & M. Moura.

The populations of *T. azorica* from Flores and Corvo along with *T. maritima* from Graciosa, are cases of very low genetic variability, and these populations should be considered as endangered, and conservation measures should be undertaken in order to avoid their disappearance..

All the different approaches (molecular phylogenetics, population genetics and morphological analyses) in this study are congruent among themselves, but it's necessary in future researches to increase the sampling of the *Tolpis* spp. from the Madeira archipelago in order to have a clearer insight of the intra and inter populational dynamics and evolution.

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