RESEARCH ARTICLE

Botany
American Journal of

Multiplexed shotgun genotyping (MSG) data resolve phylogenetic relationships within and among archipelagos in Macaronesian *Tolpis*

Mark E. Mort[1] | Benjamin R. Kerbs[1] | John K. Kelly[1] | Lurdes Borges Silva[2] |
Mónica Moura[2] | Miguel Menezes de Sequeira[3,4] | Arnoldo Santos-Guerra[5] |
Hanno Schaefer[6] | J. Alfredo Reyes-Betancort[7] | Juli Caujapé-Castells[8] |
Daniel J. Crawford[1,9]

[1]Department of Ecology and Evolutionary Biology, University of Kansas, 1200 Sunnyside Avenue, Lawrence, Kansas 66045, USA

[2]CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Pólo dos Açores, Universidade dos Açores, Rua da Mãe de Deus 13 A, 9501-801, Ponta Delgada, Açores, Portugal

[3]Madeira Botanical Group, Faculty of Life Sciences, Campus Universitário da Penteada, University of Madeira, 9020-105, Funchal, Portugal

[4]CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Pólo dos Açores, 9501-81, Ponta Delgada, Portugal

[5]Calle Guaidil 16, Urbanización Tamarco, Tegueste, Tenerife, Canary Islands 38280, Spain

[6]Department of Life Science Systems, Plant Biodiversity Research, Technical University of Munich, Emil-Ramann-Str. 2, 85354, Freising, Germany

[7]Unidad de Botánica Aplicada, Instituto Canario de Investigaciones Agrarias, Jardín de Aclimatación de La Orotava, Puerto de La Cruz, Santa Cruz de Tenerife, Spain

[8]Departamento de Biodiversidad Molecular y Banco de ADN, Jardín Botánico Canario "Viera y Clavijo"-Unidad Asociada CSIC, Cabildo de Gran Canaria, Camino al Palmeral 15, Tafira Alta, 35017, Las Palmas de Gran Canaria, Spain

[9]Department of Ecology & Evolutionary Biology, and the Biodiversity Institute, University of Kansas, Lawrence, Kansas 66045-7534, USA

Correspondence
Mark E. Mort, Department of Ecology and Evolutionary Biology, University of Kansas, 1200 Sunnyside Avenue, Lawrence, Kansas 66045, USA.
Email: memort@ku.edu

Abstract

Premise: Plants endemic to oceanic archipelagos are suitable for studying evolution, being isolated on substrates of different ages. Evolution has been recent, rendering traditionally employed sequences insufficiently variable for resolving relationships. This study includes sampling in the genus *Tolpis* (Asteraceae) from the Azores, Madeira, and Cape Verde, and expands upon an earlier study demonstrating the efficacy of multiplexed shotgun genotyping (MSG) for resolving relationships in Canarian *Tolpis*.

Methods: Genomic libraries for 90 accessions of *Tolpis* and two from the outgroup were generated for genotyping individuals using MSG. Loci were de novo assembled with iPyrad, which clusters MSG loci within and between samples. A maximum likelihood phylogeny was generated with RAxML. Ancestral area reconstruction was inferred using R package BioGeoBEARS.

Results: MSG data recovered a highly resolved phylogeny from population to inter-archipelago levels. Ancestral area reconstruction provided biogeographic hypotheses for the radiation of Macaronesian *Tolpis*.

Conclusions: Four major clades were resolved. The Madeiran endemic *T. macrorhiza* is sister to other *Tolpis*. Species from the Canaries, Cape Verdes, and the continent are sister to *T. succulenta* from Madeira, which has a sister subclade of Azorean populations composed of *T. succulenta* and *T. azorica*. Population-level resolution suggests unrecognized taxa on several archipelagos. Ancestral reconstruction suggests initial dispersal from the continent to Madeira, with dispersal to the Azores, then

dispersal from Madeira to the Canary Islands, with both subsequent dispersal to the Cape Verdes and back-dispersal to the continent. Single-island radiations and inter-island dispersal are implicated in divergence in Macaronesian *Tolpis*.

In a general context, *islands* may refer to situations ranging from mountain tops to caves or forest fragments and may not involve water (Watson, 2009). For the commonly recognized concept of islands as areas of land surrounded by water, two general types have been distinguished. Continental islands surrounded by water result from fragmentation of an adjacent continent and rifting or by isolation from the continental source area by rising sea levels. Continental islands may be ancient and quite large; well-known examples include New Caledonia and Madagascar (Watson, 2009). By contrast, oceanic islands such as Hawaii or the Galápagos are formed de novo by volcanic activity and have never been physically connected to a continent (Nunn, 2009); they are not as old or as large as the most well-known continental islands. An important biological distinction between the two types is that continental islands take with them descendants of the biota from their landmass source areas. By contrast, oceanic islands are initially devoid of living organisms, making dispersal a critical process in the origin and evolution of their biota. The Macaronesian archipelagos that are the focus of the present study consist of oceanic islands, and thus their biota originated from dispersal from continental source areas.

Despite oceanic islands representing only ~5% of the land surface of the Earth, the plants endemic to islands represent ~25% of the described species of vascular plants (Kreft et al., 2008; Caujapé-Castells et al., 2010), many of them (8%–14%) in danger of extinction (Caujapé-Castells et al., 2010). Plant species of oceanic islands have long been of interest because they are often rare and very morphologically distinct from continental relatives, and occur in small, remote landmasses (typically of volcanic origin) in the oceans. Island floras and faunas have attributes especially suitable for evolutionary studies (Mayr, 1967): plant populations on islands are often more or less isolated systems found in a diversity of habitats occurring over small spatial scales. In addition, natural processes such as volcanoes and landslides create new open habitats, resulting in a mosaic of substrates of different ages, both on single islands and across islands of different ages. Lastly, the ages of the islands can be dated, and in this regard, the hypothesis of Carlquist (1974, p. 18) is that most insular lineages are relatively young—with few exceptions, such as *Lactoris* on Robinson Crusoe Island (Stuessy et al., 2018, pp. 229, 230)—and are not relicts. Molecular phylogenetic studies of insular lineages have provided broad support for the Carlquist hypothesis (Baldwin et al., 1998; Knope et al., 2012). The distinction between stem age (time of initial colonization) and crown age (from initiation of

diversification in the island setting) is important (García-Verdugo et al., 2019). The review by García-Verdugo et al. (2019), in which various molecular markers were used to estimate crown ages for almost a third of the endemic plant lineages in the Hawaiian Islands and the Canary Islands, indicated a mean of 3.5 ± 2.9 myr for the former archipelago and 2.1 ± 2.4 myr for the latter. Keeping in mind the large confidence intervals in some estimates, the range in mean crown ages for lineages in the Canaries ranged from <1 myr to >8 myr, and for Hawaii the range was from <1 myr to >13 myr. The recent diversification of many insular lineages provides better insights into the factors associated with species divergence as compared to differences that have accumulated subsequent to speciation (Templeton, 1982; Coyne and Orr, 2004, p. 57).

A highly resolved and strongly supported phylogenetic hypothesis is a necessary first step in providing a framework for formulating hypotheses in regard to the pattern and process of radiation, diversification, and speciation in insular plant lineages. Initially, it was hoped that molecular markers that had been of use in resolving relationships at the inter-generic and intra-generic levels would likewise resolve relationships in island plants. Restriction site mutations and later DNA sequences of various regions in the nuclear and plastid genomes were used to infer the phylogenies of island lineages (e.g., Baldwin et al., 1998; Mort et al., 2007; Knope et al., 2012). While molecular data proved somewhat useful for inferring relationships in island plants, it became clear that some of the commonly employed markers for inferring relationships of older clades were not sufficiently variable to provide high resolution within recently radiated, insular lineages. Mort et al. (2015) demonstrated that genomic data can overcome these difficulties, resolving relationships within the Canary Island clade of *Tolpis* (Asteraceae), a group that had confounded attempts using other markers. Other recent studies have likewise provided elegant evidence of the utility of genomic data for phylogenetic studies of insular lineages (Fernández-Mazuecos et al., 2020; White et al., 2020). Here, we more fully develop this approach with much higher sampling than in Mort et al. (2015) to formulate better hypotheses for the patterns and processes of radiation within *Tolpis*.

Tolpis Adans. is a small (~15 species; Jarvis, 1980), presumably monophyletic flowering plant genus distributed largely in the Macaronesian archipelagos of the Azores, Canary Islands, Madeira, and Cape Verde Islands (Figure 1), with two or three taxa in Northern Africa, Southern Europe, and the Mediterranean (Jarvis, 1980). Despite its being a relatively small lineage, there is extensive

FIGURE 1 Map of Macaronesia showing the four archipelagos from which samples of *Tolpis* included in this study originated. Insets show the islands of the Azores and Canaries.

morphological variation in *Tolpis*, including habit (annuals and perennials), distribution (widespread species and ones with narrow distributions and small populations), ecology (occurrence in a range of habitats, or ecological zones, and a wide array of geological substrates of known age), a diverse breeding system (e.g., varying from highly self-compatible to strongly self-incompatible, with notable occurrences of pseudo-self-compatibility), and a diverse mating system (e.g., highly outcrossing to highly selfing). Despite the extensive morphological/ecological variation in *Tolpis*, attempts to resolve phylogenetic relationships within the genus by utilizing various molecular markers have been largely unsuccessful at inferring relationships within and among archipelagos (Moore et al., 2002; Mort et al., 2003, 2007, 2010; Archibald et al., 2006; Grünstäudl et al., 2013, 2017). Although Mort et al. (2015) demonstrated the efficiency of multiplexed shotgun genotyping (MSG) for estimating phylogeny in insular lineages, their sampling was very limited for archipelagos other than the Canary Islands, and did not include the Cape Verde endemic *T. farinulosa* or the Madeiran endemic *T. macrorhiza*. The present investigation considerably expands upon the taxon and population sampling in Mort et al. (2015), which included only three *Tolpis* populations from Madeira and the Azores; we include a total of 26 populations from those two archipelagos, including three populations of the Madeiran endemic *T. macrorhiza*, which was not included by Mort et al. (2015) and whose phylogenetic placement was ambiguously resolved in previous studies (Moore et al.,

2002; Gruenstaeudl et al., 2013). Furthermore, sampling was increased from two populations in Mort et al. (2015) to 10 populations of *T. succulenta* from Madeira and the Azores; and from a single population to 13 populations of the Azorean endemic *T. azorica*. Finally, the present study includes the single species of *Tolpis* currently recognized in Cape Verde, *T. farinulosa*. The results of Grünstäudl et al. (2013) suggested a Canary Island origin of the colonizing ancestor(s) of *T. farinulosa*, probably from El Hierro.

Biogeographic inference of *Tolpis* in Macaronesia has been a contentious topic over the past two decades. Early work by Park et al. (2001) demonstrated equally parsimonious support for either multiple colonization of Macaronesia from the continent or a single colonization followed by continental back-dispersal. The biogeographic reconstruction of *Tolpis* carried out by Moore et al. (2002) suggested the latter—that is, continental extinction and subsequent recolonization. Using minimum distance metrics, they showed that the two most likely scenarios for dispersal of *Tolpis* to Macaronesia were either (1) dispersal to Madeira, which served as "a center of dispersal for the genus" to other archipelagos, including the Azores and Canaries, followed by back-dispersal to the continent from the Canary Islands; or (2) a similar dispersal to Madeira, back-dispersal to the continent from Madeira, followed by dispersal to the Canaries (Moore et al., 2002).

The purposes of this study are to demonstrate more completely that MSG data have great utility for resolving relationships at various taxonomic levels in *Tolpis* and to use the resulting phylogeny to (1) test the monophyly of recognized

species and variants that may be worthy of recognition; (2) discuss the criteria for evaluating taxonomic recognition of groups of populations resolved by MSG data, and the biodiversity/conservation implications of decisions; and (3) conduct statistical inference(s) of historical biogeographic patterns of dispersal within and between archipelagos.

MATERIALS AND METHODS

Taxon sampling

The analyses of Tremetsberger et al. (2013) that included 42 genera of tribe Cichorieae suggest that *Arnoseris minima* is sister to *Tolpis*. Our sampling incorporates all currently generally accepted species of Macaronesian *Tolpis*, as well as populations showing significant morphological variation, in some cases corresponding to taxa not currently accepted (i.e., species, subspecies, or varieties). A combination of fresh and dried tissue was prepared for extraction from a total of 89 *Tolpis* accessions from 56 populations, including 13 individuals (7 populations) from Madeira, 27 individuals (19 populations) from the Azores, two plants from one population in Cape Verde, 45 individuals (25 populations) from the Canary Islands, two plants (two populations) from mainland Europe, and two individuals from two populations of the outgroup species *A. minima* (Table 1, Figures 2–4). This sampling was expanded for MSG analysis beyond Mort et al. (2015) to include populations from the Azores, Madeira, and Cape Verde archipelagos (Table 1). All sampled species are diploid with the exception of *T. glabrescens*, which is a tetraploid species from the Canary Islands (Jarvis, 1980). DNA was extracted using the DNEasy Plant MiniKit (Qiagen, Valencia, California, USA), and extracted samples were subsequently quantified with the Qubit dsDNA high sensitivity assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Sequencing

MSG (Andolfatto et al., 2011) libraries were created for sequencing following Mort et al. (2015). Namely, DNA was cut using the Ase I restriction enzyme (NEB Biolabs, Ipswich, Massachusetts, USA) and was size selected for fragments between lengths of 250 and 300 bp. Sample-specific 6 bp barcodes were ligated to the digested fragments and barcoded DNA was then pooled and sequenced on four lanes using Illumina HiSeq. 2500, located at the University of Kansas Genomics Core, Lawrence, Kansas. Voucher specimens are deposited at the McGregor Herbarium (KANU), Lawrence, Kansas (Table 1).

Genotyping and tree construction

The resultant, demultiplexed reads were each 70 bp in length and trimmed to 66 bp to remove the restriction overhang TAAT. Reads were further filtered using Trimmomatic (Bolger et al., 2014), with parameters ILLUMINA-CLIP: TruSeq. 3-SE, LEADING: 3, TRAILING: 3, SLIDINGWINDOW 4:15, MINLEN: 36. Samples were then quality checked in FastQC (Babraham Bioinformatics, Cambridge, UK), and loci were de novo assembled in iPyrad (Eaton, 2014) with the following notable parameters: a clustering threshold of 85%, a minimum depth of coverage of 6, and a minimum number of samples for a locus to be output of 23. Following the clustering and alignment of consensus sequences, 26,478 loci were retained and concatenated into a supermatrix; the output phylip file was used for maximum likelihood (ML) phylogeny construction in RAxML, wherein we specified the GTRGAMMA model of evolution and assigned the computation of 500 bootstrap replicates to assess support. Using FigTree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/), a graphical representation of the tree was produced and the tree was re-rooted by specifying *A. minima* as the outgroup.

Biogeography

The same loci used in ML analyses were concatenated into a supermatrix in nexus format and were used to produce a phylogeny under a Bayesian framework via MrBayes, parallel version (Huelsenbeck and Ronquist, 2001). We ran the program using a clock model with uncorrelated lognormal distributed rate variation, a birth-death tree prior, and a fixed tree age prior of 5.8 myr, based on divergence time established by Tremetsberger et al. (2013). We allowed the MCMC chain to run for 1 million generations with a sample frequency of 20,000, and these were run in parallel across 32 computer cores at the KU Center for Research Computing (Appendix S1). The output tree was pruned to the species level using the R package phytools (Revell, 2012) (Appendix S2). Each species was assigned to one or more Macaronesian archipelagos (i.e., Azores, Canaries, Cape Verdes, Madeira) and/or the continent, based on occurrence (Appendix S3). The aforementioned pruned tree and geography files were then imported to R. Ancestral area reconstruction was carried out using the R package BioGeoBEARS (Matzke, 2014) under the dispersal-extinction-cladogenesis (DEC) model (Appendix S4).

RESULTS

Analyses of MSG data resolve three major, well-supported lineages within *Tolpis*, including *T. macrorhiza*, which is sister to two large clades: one comprising the remaining taxa from Madeira and the Azorean taxa sampled, and a second large clade consisting of Canary Island populations, Cape Verde endemic *T. farinulosa*, and the two populations of *T. barbata* from both the Canaries and the continent

TABLE 1 Samples used in this study, with population numbers, archipelago, island, locality information, and collection number.

Species	Population	Archipelago	Island	Collector, collection number, locality
Tolpis macrorhiza	1	Madeira	Madeira	Menezes de Sequeira, 7120, Encumeada, at the beginning of the Folhadal water-channel
	2	Madeira	Madeira	Menezes de Sequeira, 7100, Water-channel to Caldeirão Verde, 500 m from Caldeirão Verde
	3	Madeira	Madeira	Menezes de Sequeira, 7106, Pico do Cidrão. North, exposed rocky walls
T. succulenta	4	Madeira	Madeira	Menezes de Sequeira, 7095, Porto Moniz, near the ocean
	5	Madeira	Madeira	Menezes de Sequeira, 7094, on the road from Ribeira do Inferno to Seixal
	6	Madeira	Madeira	Menezes de Sequeira, 7058, Formosa beach, on promenade that leads to Câmara de Lobos
	7	Madeira	Madeira	Menezes de Sequeira, 7104, Road to Pico do Arieiro, circa Poço da Neve
	8	Azores	Santa Maria	N. Moura, MASL, São Lourenço, N 36 59′ 34.1″, W 25 03′ 24.5″
	9	Azores	Santa Maria	N. Moura, MAPA, Pico Alto, N 36 58′ 56.0″, W 25 05′ 28.8″
	10	Azores	Santa Maria	N. Moura, MAMA, Maia, N 36 56′ 53.0″, W 25 01′ 09.8″
	11	Azores	São Miguel	Borges Silva, SMPA, Porto da Ajuda, N 37 53′ 56.6″, W 25 45′ 01.7″
	12	Azores	Graciosa	M. Moura, GRBL, Baía do Filipe, Beira Mar da Luz, N 39 01′ 14.6″, W 28 00′ 26″
	13	Azores	Graciosa	Parque Natural da Graciosa, GRSC, Santa Cruz, Quitadouro, N 39 04′ 37.8″, W 27 59′ 19.1″
T. azorica	14	Azores	São Miguel	Borges Silva, SMAZ, Pico da Cruz, 900 m a.s.l.
	15	Azores	São Miguel	Borges Silva, SMAZ, Pico Bartolomeu, 870 m a.s.l.
	16	Azores	Terceira	F. Pereira, TESB, Serra de Santa Bárbara, N 38 43′ 41.1″, W 27 19′ 36.2″
	17	Azores	Terceira	M. Pietrzak, TRAZ, Rocha do Chambre, N 38 44′ 49.6″, W 27 13′ 56.6″, 583 m a.s.l.
	18	Azores	São Jorge	D. Braga & F. Mendes, SJPV, 1-5-68, Pico Verde, N 38 39′ 14.9″, W 28 05′ 24.7″
	19	Azores	São Jorge	D. Braga & F. Mendes, SJPL, 4-5-6-54
	20	Azores	São Jorge	D. Braga & F. Mendes, SJPE3-67, Pico da Esperança, N 38 39′ 11.2″, W 28 04′ 18.4″
	21	Azores	Faial	C. Freita & P. Casimiro, FACA8_70, Caldeira, N 38 34′ 49.1″, W 28 43′ 13.7″
	22	Azores	Faial	C. Freita & P. Casimiro, FAAB14_71, Alto do Brejo, N 38 35′ 31.3″, W 28 43′ 48.4″
	23	Azores	Pico	E. Dias, PIAP, Prainha, N 38 26′ 26.5″, W 28 11′ 10.0″
	24	Azores	Flores	FLRB1_72, Ribeira da Badanela, N 39 28′ 10.1″, W 31 12′ 26.9″
	25	Azores	Flores	L. Serpa, Badanela, N 39 28′ 10.2″, W 31 12′ 26.7″
	26	Azores	Flores	L. Serpa, Cidrão, N 39 27′ 40.2″, W 31 12′ 36.7″
T. barbata	27	Continental		Crawford, s.n., commercial source
	28	Continental		Crawford, s.n., commercial source
	29	Canary Isl.	Tenerife	Crawford et al., 1849, near Arafo

(Continues)

TABLE 1   (Continued)

Species	Population	Archipelago	Island	Collector, collection number, locality
T. farinulosa	30	Cape Verde Isl.	Santo Antao	Fernandes, s.n., Santo Antao
T. proustii	31	Canary Isl.	El Hierro	Crawford et al., 2008, Riscos de Bascos
T. laciniata	32	Canary Isl.	El Hierro	Crawford et al., 1855, Cruz de Reyes, above Tabano, ca. 1300 m a.s.l.
	33	Canary Isl.	La Gomera	Crawford et al., 2048, N 28 10′ 08.4″, W 17 16′ 39.0″
	34	Canary Isl.	La Gomera	Crawford et al., 2049, N 28 07′ 46.4″, W 17 19′ 06.2″
	35	Canary Isl.	La Gomera	Crawford et al., 1918, along roadside toward Epina
T. glabrescens	36	Canary Isl.	Tenerife	Crawford et al., 1877, Anaga, Chinobre
	37	Canary Isl.	Tenerife	R. Mesa, s.n., Anaga, Roque de Enmedio
T. lagopoda	38	Canary Isl.	Gran Canaria	Mort and Santos, s.n., near Artenara
T. webbii	39	Canary Isl.	Tenerife	Santos-Guerra, 09, near Zapatito de la Reina
	40	Canary Isl.	Tenerife	Santos-Guerra, 06, above Vilaflor
T. lagopoda	41	Canary Isl.	Tenerife	Crawford et al., 1941, above Esperanza, 1250 m a.s.l.
	42	Canary Isl.	Tenerife	Santos-Guerra, 13, Masca
	43	Canary Isl.	Tenerife	Crawford et al., 1949, near Mirador Ayosa
	44	Canary Isl.	Tenerife	Santos-Guerra, 12, Ayosa
T. crassiuscula	45	Canary Isl.	Tenerife	Crawford et al., 1993, Teno, tunnel W of El Fraile
T. lagopoda	46	Canary Isl.	Tenerife	Crawford et al., 1987, Barranco del Infierno
	47	Canary Isl.	Tenerife	Crawford et al., 1975, Barranca Seca
T. santosii	48	Canary Isl.	La Palma	Crawford et al., 2009, Playa de Nogales
T. calderae	49	Canary Isl.	La Palma	Crawford et al., 1906, Caldera de Taburiente, near river
	50	Canary Isl.	La Palma	Crawford et al., 1887, Mirador la Cumbrecita
T. laciniata	51	Canary Isl.	La Palma	Crawford et al., 1886, just S of Jedey
	52	Canary Isl.	La Palma	Crawford et al., 1883, La Caldera
T. coronopifolia	53	Canary Isl.	Tenerife	Crawford et al., 1834, El Guancha
	54	Canary Isl.	Tenerife	Santos-Guerra, 05, Arafo
Arnoseris minima	55	Spain		Montes-Moreno & Nualart, 668, Burgos: Merindad de Valdeporres, Robredo de las Pueblas
	56	Austria		Pachschwöll & Grünstäudl, 7559/3, N 48 24′ 34.1″, E 15 31′ 49.9″, 545 m a.s.l.

(Figure 2). This is the first analysis showing *T. macrorhiza* to be sister to the remainder of *Tolpis*. Within the Madeira + Azores clade, the four sampled populations of *T. succulenta* from Madeira form a strongly supported subclade. Within this subclade the two individuals sampled from each population are resolved with strong support (Figure 3). A second subclade resolved with strong support comprises *Tolpis* from the Azores. In this subclade there is strong support for a lineage that includes the six populations currently recognized as *T. succulenta* that were sampled; the three populations for which two individuals were sampled group with strong support. The 13 populations of *T. azorica* sampled likewise form a highly supported clade.

Even with increased taxon sampling, the patterns of relationships and levels of resolution/support within the largely Canary Island clade are consistent with Mort et al. (2015). *Tolpis barbata* (not sampled by Mort et al., 2015) is well supported and sister to the remainder of the taxa within the clade (Figure 4). The two populations sampled from El Hierro form a well-supported clade, within which *T. proustii* is not resolved as monophyletic, but without support. *Tolpis farinulosa* (not sampled by Mort et al., 2015), which is endemic to Cape Verde, is sister to the El Hierro clade with strong support. Populations from La Gomera, which previously had been suggested as possibly worthy of taxonomic recognition (Crawford et al., 2009;
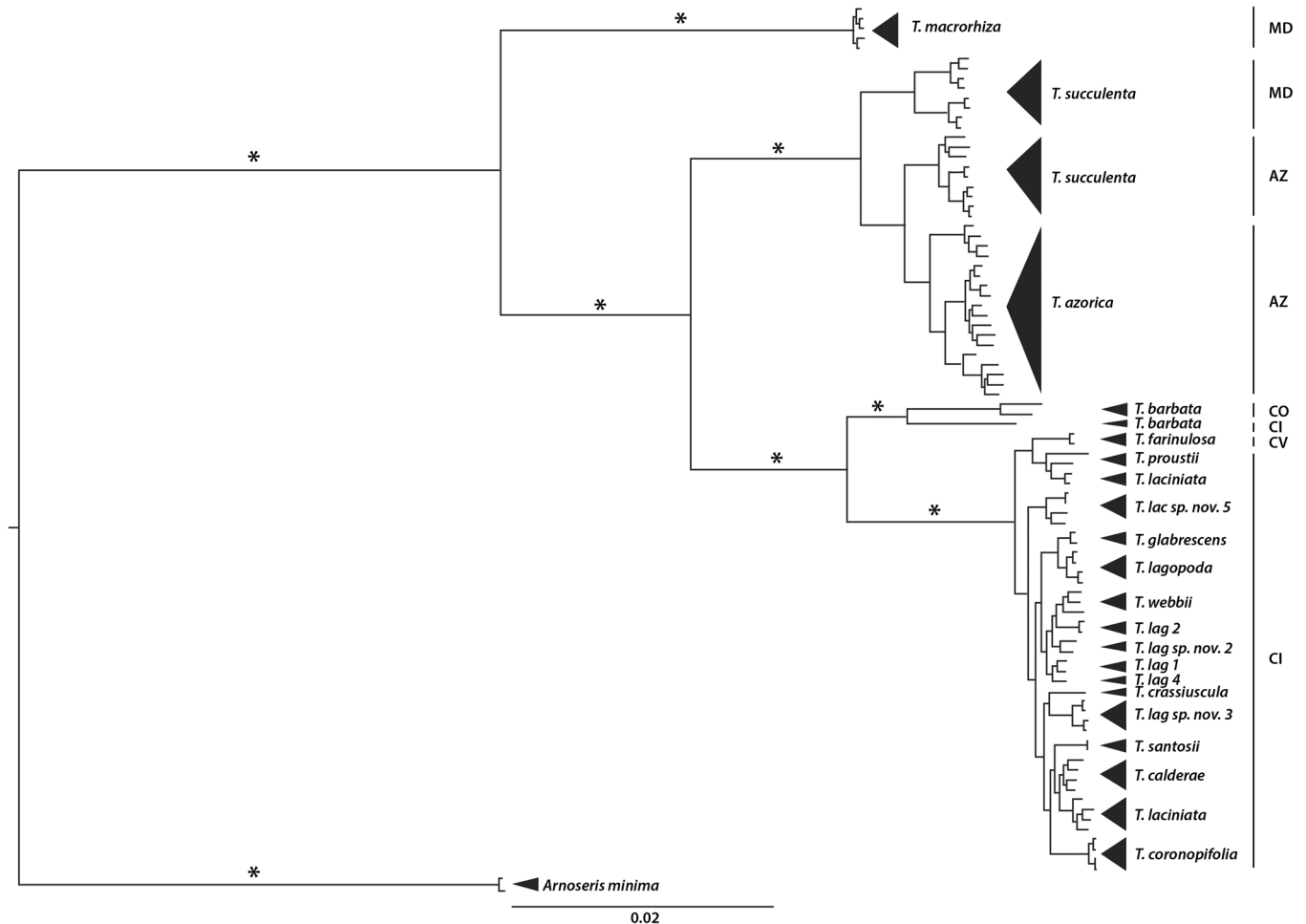
FIGURE 2 Maximum likelihood (ML) topology derived from analyses of multiplexed shotgun genotyping data for *Tolpis* in Macaronesia. Clades with 95% or higher ML bootstrap are indicated by asterisks. Abbreviations: AZ = Azores, CI = Canary Islands, CV = Cape Verde, MD = Madeira, CO = continent.

Mort et al., 2015) are in turn sister to populations from the islands of La Palma, Tenerife, and Gran Canaria (Figure 4). Within the latter lineage, the four individuals from a single population on Gran Canaria group with strong support; however, populations from Tenerife and La Palma do not form geographically defined clades, suggesting that dispersal is common between these islands. Within the La Palma + Tenerife lineage, there is strong support for the monophyly of the annual, selfing *T. coronopifolia* as well as the tetraploid *T. glabrescens*; the recognition of these species has rarely been questioned. The remaining taxa in this clade have a complex, confused taxonomic history, with species historically assigned to either the *T. laciniata* or the *T. lagopoda* species complex, albeit with the recognition that it is a challenge to identify diagnostic characters for taxa within the complex (Jarvis, 1980; Crawford et al., 2009). However, analyses of MSG provide strong support for several morphologically/ecologically distinct taxa such as *T. webbii*, *T. crassiuscula*, *T. calderae*, and the recently described *T. santosii* (Crawford et al., 2013). Furthermore, there is strong support for three *laciniata/ lagopoda* variants (i.e., *T. sp. nov. 2*, *T. sp. nov. 3*, *T. sp. nov. 5*) deemed worthy of serious consideration for taxonomic

recognition (Archibald et al., 2006; Crawford et al., 2009; Mort et al., 2015).

Ancestral area reconstruction of *Tolpis* infers a primary dispersal event from the continent to Madeira (Figure 5). From there, *Tolpis* likely colonized the Azores to the northwest, followed by the Canary Islands to the south (Figure 5). For the most recent common ancestor of Azorean *Tolpis* and Madeiran *T. succulenta*, equal probabilities exist for Madeira or the Azores as ancestral areas; nevertheless, there appears to have been recolonization of Madeira from the Azores, represented by the extant *T. succulenta* (Mad) (Figure 5). From the Canary Islands, there was an apparent back-dispersal to the continent as previously posited by Moore et al. (2002) as well as dispersal to the Cape Verde Islands (Figure 5).

DISCUSSION

Mort et al. (2015) reviewed the battery of "standard" markers that proved largely ineffective in phylogenetic studies of *Tolpis* despite having been widely successful in
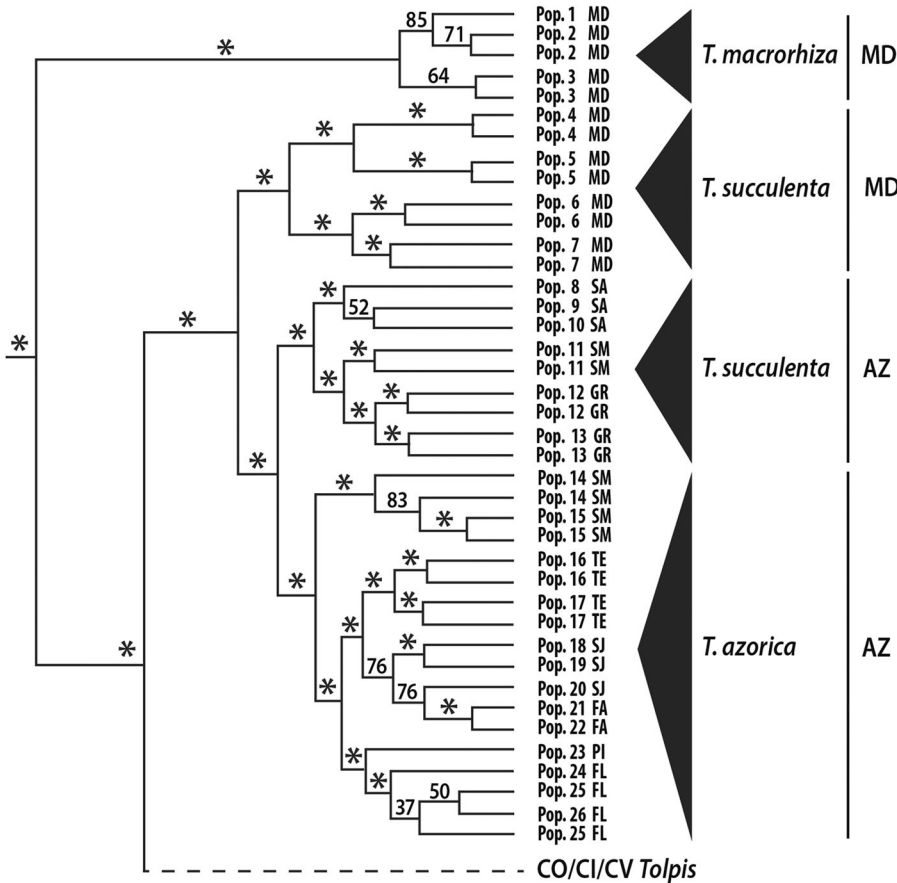
FIGURE 3 Maximum likelihood (ML) topology from multiplexed shotgun genotyping data for *Tolpis* in Azores and Madeira. ML bootstrap support is indicated for clades with <95% support (otherwise denoted by asterisk). Population numbers are the same as in Table 1. Abbreviations: MD = Madeira; Azores (AZ): SM = Santa Maria, SA = São Miguel, GR = Graciosa, TE = Terceira, SJ = São Jorge, FA = Faial, PI = Pico, FL = Flores.

resolving relationships at the generic and lower levels in flowering plants. These include restriction site mutations (Moore et al., 2002), sequences of plastid DNA (Mort et al., 2007), and sequences of the internal and external transcribed spacer regions (ITS and ETS) of nuclear ribosomal DNA (Grünstäudl et al., 2013). This same array of markers was often equally ineffective in resolving relationships in other island lineages, with the lack of resolution generally attributed to the young age and rapid radiations within the lineages (Baldwin et al., 1998). That is, there has been a disconnect between morphological/ecological divergence and the molecular markers that were routinely being employed. In contrast to most prior insular studies, Mort et al. (2015) resolved relationships within the Canary Island clade of *Tolpis* as well as greater resolution among the archipelagos. Likewise, Curto et al. (2018) employed genomic data to provide much higher resolution of relationships in the genus *Micromeria* in the Canary Islands than had been achieved with other molecular data. Other examples of the utility of genomic data for inferring evolutionary/biogeographic insights into insular lineages previously unattainable with sequences from various nuclear and/or plastid regions include White et al. (2020) for *Argyranthemum*, the largest endemic genus in the Macaronesian archipelagos, and Fernández-Mazuecos et al. (2020) for *Scalesia*, the largest genus endemic to the Galápagos Islands.

One of the major insights provided by the present study is the resolution of the Madeiran endemic *T. macrorhiza* as

sister to the remainder of *Tolpis*. Chloroplast DNA restriction site mutations placed *T. macrorhiza* as one of the first branching lineages in *Tolpis*; *T. succulenta* from Madeira was sister to the rest of the genus, with *T. macrorhiza* in turn sister to all other species (Moore et al., 2002). Sequences of external transcribed spacer regions of nrDNA (ETS) failed to resolve its position as it was placed in a basal polytomy with several other clades (Grünstäudl et al., 2013). Grünstäudl et al. (2017), using ETS sequences and sequences of two low copy nuclear loci, placed *T. macrorhiza* in a basal clade with Madeiran *T. succulenta*. As with all insular *Tolpis* endemics, except the tetraploid *T. glabresens*, *T. macrorhiza* is a diploid ($n = 9$) species (Stebbins et al., 1953; Moreno Cameno, 1979; Jarvis, 1980; Dalgaard, 1986). It is a perennial with large rhizomes (rare in *Tolpis*) and annual stems. In addition, the capitula are small compared to most other *Tolpis* (Jarvis, 1980; Crawford et al., 2015). Synthetic F_1 hybrids between this species and *T. succulenta* from Madeira have low pollen fertility (mean = 26% for 12 progeny from three different crosses; Crawford et al., 2016), suggesting postzygotic isolating factors between the two species.

The much greater sampling within Madeira and the Azores than in prior studies has revealed resolution at the species and population levels. In addition to the aforementioned *T. macrorhiza*, *T. succulenta* also occurs on Madeira. Note that *T. succulenta* as now recognized also occurs in the Azores, and previous studies have suggested that

FIGURE 4 Maximum likelihood (ML) topology from multiplexed shotgun genotyping data for *Tolpis* in the continent (CO), Canary Islands (CI), and Cape Verde (CV). ML bootstrap support is indicated for clades with <95% support (otherwise denoted by asterisk). Population numbers are the same as in Table 1. Abbreviations, Cape Verde Islands: SA = Santo Antão; Canary Islands: T = Tenerife, EH = El Hierro, LG = La Gomera, GC = Gran Canaria, LP = La Palma.
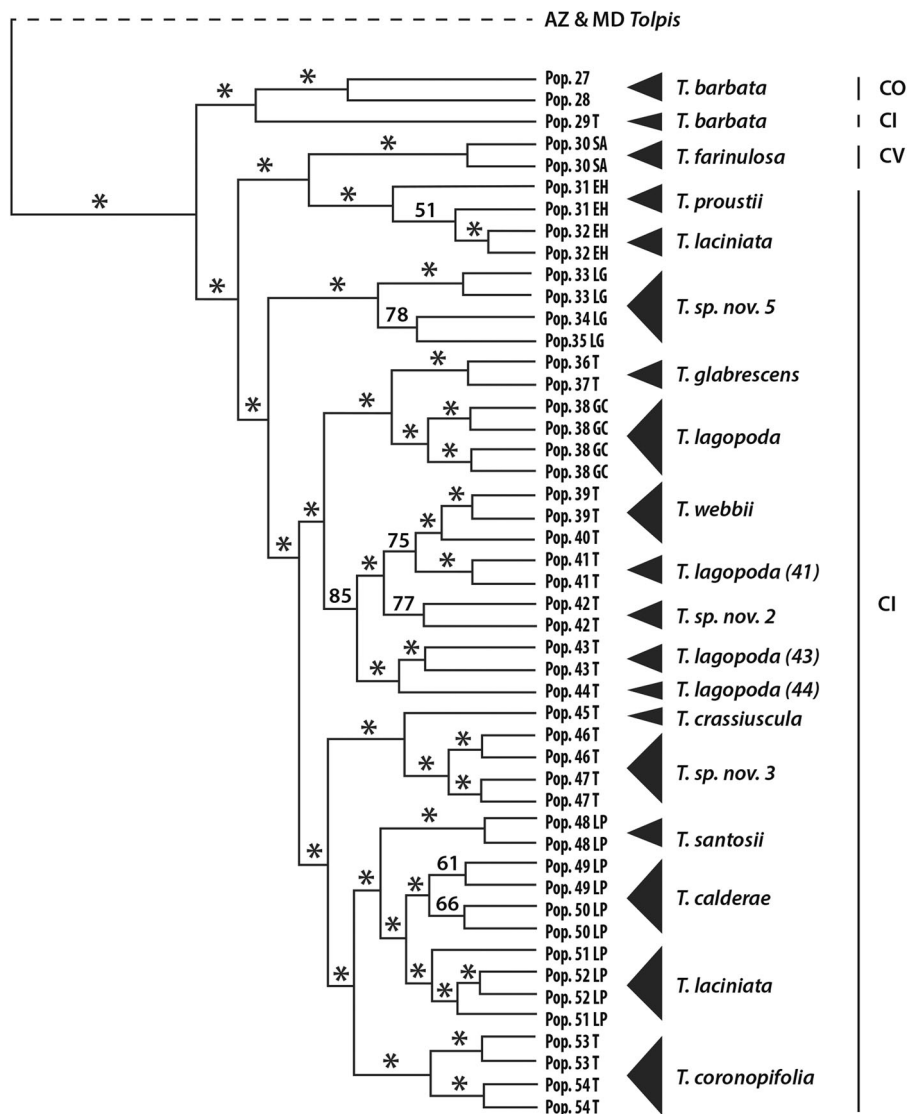
T. succulenta, as currently circumscribed, is not monophyletic (Grünstäudl et al., 2013; Mort et al., 2015). Borges Silva et al. (2016) showed that populations from the two archipelagos form distinct groups with microsatellite loci. The present study represents the most robust sampling of this species for phylogenetic analyses from both archipelagos and provides strong support for the recognition of populations in the two archipelagos as distinct species. In support of species recognition, mean pollen fertility of hybrids between plants of *T. succulenta* from the two archipelagos is 32% (four crosses, 21 plants), as compared to 64% for hybrids between different populations on Madeira (Crawford et al., 2016).

Consider next the resolution among populations of *T. succulenta* in each of the archipelagos. The four populations from Madeira are resolved as two strongly supported subclades, each of which includes two populations that also form well-supported lineages (Figure 3). It has long been recognized that Madeiran *T. succulenta* is variable in leaf characters, with several of the forms having been recognized taxonomically (Jarvis, 1980). In

addition to strong support from MSG data, the populations resolved in the two subclades are also distinguished morphologically, differing in both the degree of leaf dissection and capitulum diameter (D. J. Crawford et al., unpublished data).

However, genomic and morphological studies of additional populations are needed before taxonomic decisions are made. *Tolpis succulenta* occurs on all islands of the Azores, but with the exception of Santa Maria Island, it is quite rare (Jarvis, 1980; Schaefer, 2005). The Santa Maria and Graciosa populations form a strongly supported clade, which is sister to the three São Miguel populations (Figure 3). Crawford et al. (2019) showed that the Graciosa populations are highly selfing whereas populations of *T. succulenta* on Santa Maria are self-incompatible. The Graciosa populations differ from self-incompatible *T. succulenta* on Santa Maria by features of the capitula and florets (Crawford et al., 2019) and are perhaps worthy of taxonomic recognition. An extensive morphological study of Azorean *T. succulenta* is now in progress (L. Borges Silva and M. Moura, unpublished data).
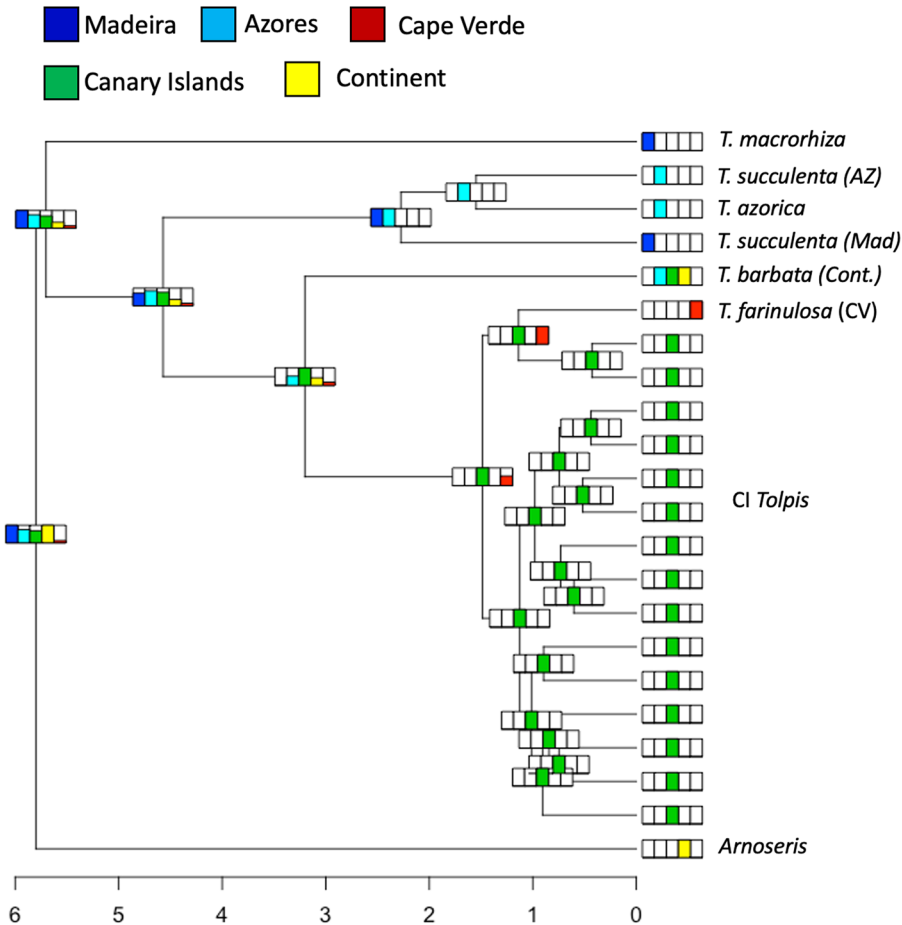
The relationships among populations of *T. azorica* from six islands (no samples from Corvo Island) are largely concordant with the genetic structure of microsatellite loci elucidated by Borges Silva et al. (2016), which included 53 populations from all seven islands where the species is known. Populations from São Miguel form a distinct group in principal coordinate analysis (Borges Silva et al., 2016), and the two populations from this island are sister to other populations of *T. azorica* (Figure 3). Populations from the central islands of the Azores (Faial, Pico, São Jorge, and Terceira; Figure 1) group strongly with the microsatellite data (Borges Silva et al., 2016). Similar relationships were resolved here, with populations from Faial, São Jorge, and Terceira forming a strongly supported group (Figure 3). By contrast, our analyses place the single sample of Pico included herein with the western island of Flores instead of with the central group. This is a surprising result, which is not supported by any of the previous genetic and morphological studies, so more material from Pico should be tested to exclude the possibility of some kind of labeling error. No samples from the other western island of Corvo were available in the present study.

Tolpis populations from the Canary Islands and the Cape Verdes and the samples of *T. barbata* (one accession from the Canaries and two continental samples) form the last large clade (Figure 4). Unlike in the Azores and Madeira

—where accepted species are few in number and generally easily recognized morphologically, and where populations of the same species (with the notable exception of *T. succulenta*) are monophyletic—the situation is generally more complex in the Canaries.

Whether or not the fact of fewer recognized species in the Azores and Madeira is a result of less intensive study or of lower diversity in the two archipelagos than in the Canaries remains an open question. Several Canary Island endemic species that are generally recognized are resolved with strong support, including the highly selfing *T. coronopifolia*, the rare tetraploid *T. glabrescens*, and *T. webbii* (Figure 4). Only one sample of the very rare, morphologically distinct species *T. crassiuscula*, restricted to the paleo-island of Teno on Tenerife, was examined; thus, we were not able to determine whether this species is monophyletic. However, other molecular markers (Archibald et al., 2006; Crawford et al., 2006) have indicated that this species is divergent from other Canary Island *Tolpis*.

The remaining populations from the Canaries have been variously assigned to one or the other of two morphologically variable species complexes, *Tolpis laciniata* and *T. lagopoda* (Jarvis, 1980; Bramwell and Bramwell, 2001), with variants sometimes recognized taxonomically (see below). Jarvis (1980) provided an extensive discussion of

morphological variation in *T. laciniata* and, to a lesser extent, in *T. lagopoda*, which he viewed as a much more morphologically uniform species. In his diagnostic key, he used the characters of "flowering axes markedly leafy, pendent to ascending" to distinguish *T. lagopoda* from *T. laciniata* and several other species that were described as "flowering axes not markedly leafy, erect." Jarvis (1980) considered La Gomera and El Hierro to harbor only *T. laciniata*, whereas all plants on Gran Canaria were viewed as *T. lagopoda*. Jarvis (1980) considered most populations on La Palma to be *T. laciniata*, with *T. lagopoda* very rare; on Tenerife, the opposite was true, with *T. lagopoda* more common than *T. laciniata*. More recently, a similar geographic distribution has been suggested for the two species (A. Santos-Guerra, unpublished data, cited in Crawford et al., 2009), with *T. laciniata* the only species occurring on La Gomera and El Hierro as well as at lower elevations on La Palma; *T. lagopoda* is considered much rarer and found only at higher elevations on La Palma. The islands of Tenerife and Gran Canaria are viewed by one of us (A.S.-G.) as containing only *T. lagopoda*. The present results further support the results of Grünstäudl et al. (2013) and Mort et al. (2015) in showing that populations of neither *T. laciniata* nor *T. lagopoda* as traditionally recognized are monophyletic (Figure 4). The non-monophyly of these taxa is perhaps to be expected given the recency of their divergence. Freudenstein et al. (2017) make the important point that there has been too much emphasis on recognizing lineages resolved as monophyletic with molecular data as species, when in reality, with progenitor-derivative species (and in other situations), paraphyletic groups are to be expected with recent divergence, yet these groups may be phenotypically and ecology distinct ("fill roles") and should be named. In the present study, it seems that the populations are mostly monophyletic, and we point out that some are morphologically distinct and others are not. It is of interest, however, to consider the resolution of populations assigned to these two species.

Within the Canary Island clade, populations from the youngest island of El Hierro are sister to all other samples from the archipelago. This clade includes *T. laciniata*, a presumed variant of *T. laciniata* referred to as *T. proustii*, and the Cape Verde endemic *T. farinulosa* (Figure 4). Mort et al. (2015) included *T. proustii* but did not sample *T. laciniata* from El Hierro or *T. farinulosa* from Cape Verde. The present study, like Grünstäudl et al. (2013), resolves *T. laciniata* and suggests a close relationship with the highly morphologically similar *T. proustii* (Figure 4). *Tolpis proustii* is sometimes, but not always, recognized as a distinct species (Jarvis, 1980, vs. Bramwell and Bramwell, 2001). The two samples of *T. proustii*, both from the same population at the type locality, do not form a clade, and thus the present study shows no support per se for recognition of the species. Additional samples from other populations in the same geographic area of the island are to be desired. The placement of the Cape Verde endemic *T. farinulosa* in the El

Hierro clade suggests long-distance dispersal from El Hierro to Cape Verde; relatively recent long-distance dispersals from other Macaronesian archipelagos to Cape Verde have been detected in other taxa, such as *Echium* (Boraginaceae), *Sonchus* (Asteraceae), and *Aeonium* (Crassulaceae) (Kim et al., 2008).

As first shown by Mort et al. (2015), populations of *T. laciniata* from La Gomera group strongly as sister to all remaining populations in the Canaries (Figure 4). Based on field observations over several decades, one of us (A.S.-G.) recognized these plants as morphologically distinct from other Canary Island *Tolpis*, based on their fleshy habit of ≤1.5 m, strongly pinnatifid, pubescent rosette leaves and a ring of barbed involucral bracts. The present study increased the sampling of Gomeran *T. laciniata* compared to Mort et al. (2015) and again provides strong support for the three populations included. Based on the present phylogenetic results as well as the distinctive morphology, and as others have done (Crawford et al., 2009; Mort et al., 2015), we designate this taxon as *T.* sp. nov. 5 (Figure 4). Future studies will robustly assess the recognition of the Gomeran populations as a new single-island endemic species.

The remaining Canary Island populations are distributed on the other three high islands of Gran Canaria, La Palma, and Tenerife. Both Jarvis (1980) and one of us (A.S.-G.) assign all populations on Gran Canaria to *T. lagopoda*. Grünstäudl et al. (2013) included two samples from Gran Canaria, one assigned to *T. lagopoda* and the other a morphological variant termed ecotype "Faneque." They form a strongly supported group, but are one element in a basal polytomy for Canarian *Tolpis*, and thus their relationships are unresolved. Representatives from Gran Canaria were not included in Mort et al. (2015). The four samples from one population included in the present study group strongly and receive strong support in a clade with the rare tetraploid species *T. glabrescens* from Tenerife (Figure 4). Additional populations should be sampled from Gran Canaria, both to include morphological/geographical variation on the island and to further test the placement of Gran Canaria plants in the present study as sister to a rare species from another island.

The Gran Canaria + *T. glabrescens* clade is sister to a clade comprising taxa sampled from Tenerife. The latter clade includes *T. webbii*, a species endemic to the Cañadas region of Tenerife, which forms a clade with population 41 (Table 1) of *T. lagopoda* from the high central mountains near Esperanza. *Tolpis* populations in the Esperanza area typically have narrow leaves compared to populations of *T. lagopoda* in other parts of Tenerife (A. Santos-Guerra, personal observation), but it is unclear at this time if these taxa are worthy of species-level recognition. A variant of *T. lagopoda* that does seem to warrant recognition (*T.* sp. nov. 2) is resolved, but with only moderate support (77%) and sister to the *T. webbii* + *T. lagopoda* (41) subclade (Figure 4). *Tolpis* sp. nov. 2 is known only from a restricted geographic region near Barranco de Masca on
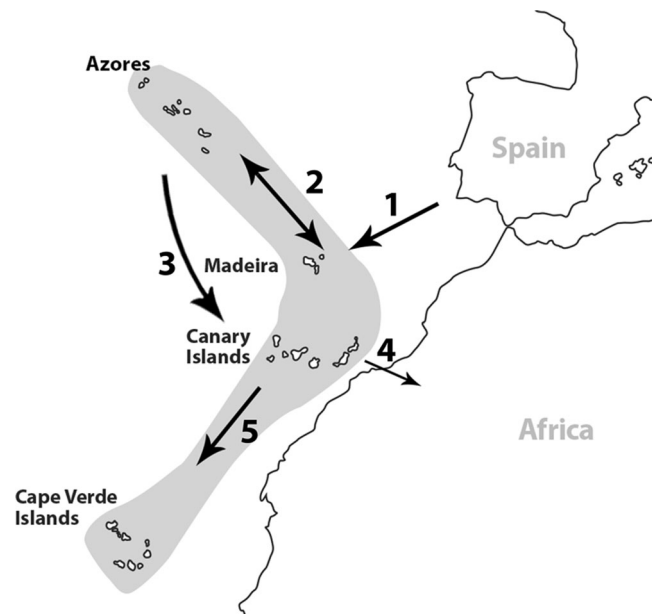
FIGURE 6 Inferred chronological pattern of inter-archipelago *Tolpis* dispersal within the past ~6 myr, based on dispersal-extinction-cladogenesis (DEC) model results.

the paleo-island of Teno; plants from near the village of Masca are quite distinctive morphologically in possessing leaves that are nearly entire and distributed along the stem (as opposed to a basal rosette) and have very woody bases. This taxon should be considered for taxonomic designation (A. Santos-Guerra, personal observation); however, additional samples are required for further study.

The last strongly supported clade in the Canaries includes all populations from La Palma and plants from Tenerife (Figure 4). One subclade consists of two populations of the morphologically distinct (large plants, highly branched stems; inflorescences highly branched with large capitula) *T.* sp. nov. 3 (Figure 4; Archibald et al., 2006; Crawford et al., 2009; Mort et al., 2015). Each population occurs in a different isolated canyon in the paleo-island Adeje region of southern Tenerife. The one sample of the aforementioned *T. crassiuscula*, which occurs locally on the paleo-island of Teno, groups strongly with the populations from the other paleo-island. The second subclade resolves all populations from La Palma in one group and the two populations of the morphologically distinct, self-compatible, highly selfing species *T. coronopifolia* from Tenerife as sister to the La Palma populations (Figure 4). *Tolpis santosii* (Crawford et al., 2013) is sister to other populations, suggesting an early divergence within the island. The one sample of the sometimes-recognized species *T. calderae* (a morphological form of *T. laciniata*) included by Mort et al. (2015) was sister, with weak support, to two samples from one population of *T. laciniata*. In the present study, two individuals from an additional population of each of the taxa were included, and they were resolved with high support as two groups. However, no samples from higher-elevation plants on La Palma referable to *T. lagopoda* (A. Santos-Guerra, unpublished data, cited in Crawford

et al., 2009) were included in this study, precluding the resolution of relationships among the populations referable to the three species on the island.

A number of geological and environmental features corroborate the hypothesized dispersal pattern of *Tolpis* in Macaronesia (Figure 5). The first is that, across Macaronesia, variation exists in the patterns of wind that may carry the pappus-bearing achenes of *Tolpis*. Though all islands in Macaronesia are affected by southeasterly trade winds, monsoonal winter winds tend toward the southwest in the inter-tropical convergence zone below 20°N, a region that includes the Canaries and Cape Verde (Cropper, 2013). Thus, trade winds may explain dispersal events 1 and 5, while the shifting monsoon winds support continental back-dispersal (event 4) at lower latitudes (Figure 6). The inference of Madeira as the first archipelago to be colonized is supported by reconstructions of ancient shorelines showing that, during glacial periods, several now-sunken islands existed between Iberia and Madeira (García-Talavera, 1999). During these periods, the longest distance required for a fruit to reach Madeira from the continent was 200 km, as opposed to the present-day distance of 800 km (Moore et al., 2002). Our results corroborate those of Moore et al. (2002), who posited that these sunken islands may have been instrumental in the dispersal of *Tolpis* from the continent to Macaronesia.

CONCLUSIONS

The results of the present study further demonstrate the utility of MSG data for resolving phylogenetic relationships from the inter-population to inter-archipelago levels in *Tolpis* in the Macaronesian archipelagos. Clades at all levels

received strong support, with most bootstrap values above 95% even at the population level. The highly resolved phylogeny provides insight into single-island radiations and inter-island dispersal in the radiation of *Tolpis* in the Canaries and Cape Verdes. Resolution at the population level, when combined with morphological, geological, biosystematic, and distributional data, identifies lineages worthy of consideration for taxonomic recognition, and this is informative for the conservation of genetic diversity in the archipelagos. Ancestral area reconstructions provide biogeographical hypotheses and possible dispersal events for Macaronesian *Tolpis*.

AUTHOR CONTRIBUTIONS

ACKNOWLEDGMENTS

DATA AVAILABILITY STATEMENT

The MSG data presented in this study are available for download at NCBI SRA under project no. PRJNA827639.

ORCID

Mark E. Mort http://orcid.org/0000-0003-3338-1009

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. MrBayes parameters.

Appendix S2. Phytools scripts.

Appendix S3. Geography input file for BioGeoBears.

Appendix S4. BioGeoBears script.