Multiplexed-shotgun-genotyping data resolve phylogeny within a very recently derived insular lineage[1]

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- *Premise of the study:* Endemic plants on oceanic islands have long served as model systems for studying patterns and processes of evolution. However, phylogenetic studies of island plants frequently illustrate a decoupling of molecular divergence and ecological/morphological diversity, resulting in phylogenies lacking the resolution required to interpret patterns of evolution in a phylogenetic context. The current study uses the primarily Macaronesian flowering plant genus *Tolpis* to illustrate the utility of multiplexed shotgun genotyping (MSG) for resolving relationships at relatively deep (among archipelagos) and very shallow (within archipelagos) nodes in this small, yet diverse insular plant lineage that had not been resolved with other molecular markers.
- *Methods:* Genomic libraries for 27 accessions of Macaronesian *Tolpis* were generated for genotyping individuals using MSG, a form of reduced-representation sequencing, similar to restriction-site-associated DNA markers (RADseq). The resulting data files were processed using the program pyRAD, which clusters MSG loci within and between samples. Phylogenetic analyses of the aligned data matrix were conducted using RAxML.
- *Key results:* Analysis of MSG data recovered a highly resolved phylogeny with generally strong support, including the first robust inference of relationships within the highly diverse Canary Island clade of *Tolpis*.
- *Conclusions:* The current study illustrates the utility of MSG data for resolving relationships in lineages that have undergone recent, rapid diversification resulting in extensive ecological and morphological diversity. We suggest that a similar approach may prove generally useful for other rapid plant radiations where resolving phylogeny has been difficult.

  Key words: Asteraceae; Canary Islands; genome sequencing; Macaronesia; multiplexed shotgun genotyping; phylogenomics; phylogeny; radiation; *Tolpis*.

The biota of oceanic islands have long fascinated biologists and laypeople alike because of the image of unique species occurring on isolated specks of lava in the vast oceans. Darwin (1859) and Wallace (1870), the two major figures in the formulation of the modern theory of evolution, recognized the significance of oceanic islands as systems for studying and understanding the evolutionary process. The value of islands for evolutionary studies was further emphasized by the distinguished 20th century biologist Ernst Mayr (1967, p. 369) who stated, "It is as if nature has made a whole series of experiments and it was merely our task to analyze the results." Several factors make oceanic archipelagos ideal settings for evolutionary studies. They are closed, isolated systems that harbor a diversity of habitats and climates over small spatial scales. Most insular systems comprise two or more islands with known maximum ages. Furthermore, ongoing natural disturbances (e.g., volcanism or landslides) create a mosaic of habitats of broad geological age and composition that are conducive to lineage diversification. Nearly 25% of described vascular plant species are endemic to islands, which represent a mere 5% of the Earth's land surface (Kreft et al., 2008; Caujapé-Castells

et al., 2010). Some 60% of the world's 34 biodiversity hotspots include islands (Myers et al., 2000), and somewhere between 8 and 14% of insular plant endemics are in danger of extinction (Caujapé-Castells et al., 2010).

For the past three decades, oceanic island plants have been prominent subjects of studies utilizing various molecular markers, with one of the early and continuing results being a "decoupling" of variation between ecological/morphological characters and molecular variation, with minimal divergence in the latter despite extensive variation in the former (Lowrey et al., 2001; Givnish et al., 2009; Knope et al., 2012). A pronounced case of such decoupling occurs in the small genus *Tolpis* (Asteraceae; Fig. 1). *Tolpis* is primarily distributed in Macaronesia (i.e., the Azores, the Canary Islands, and Madeira; Fig. 2), with continental occurrences in the Mediterranean regions and North Africa (Jarvis, 1980). Of the 12 *Tolpis* species recognized by Jarvis (1980), 10 are insular, and two are continental. Six of the strictly insular species recognized by Jarvis (1980) are endemic to the Canaries, with the Azores, Cape Verde, and Madeira each having one endemic species, and another species shared by the Azores and Madeira. Despite the application of a battery of genetic markers (see below), previous studies have resulted in few well-supported nodes for the phylogeny of Macaronesian *Tolpis*; the most recent estimate of phylogeny is shown in Fig. 3A.

In contrast to the low molecular variation, Macaronesian *Tolpis* has diversified in a variety of characters associated with speciation in flowering plants. Self-compatibility and the suite of floral features typically associated with the transition to selfing (i.e., the "selfing syndrome", see Ornduff, 1969) have originated at least once and possibly multiple times (Crawford et al., 2008); a shift from the perennial to annual habit parallels the transition to selfing. *Tolpis* occurs in substrates of different ages, ranging from decades via recent natural disturbance to several million years (Hoernle and Carracedo, 2009). *Tolpis* has radiated into all the commonly recognized vegetation zones in the Canaries (Bramwell and Bramwell, 2001), with several putative "cryptic" species restricted to localized substrates. The lineage in the Canaries occurs in all five of the high western islands, but the roles of intra-island radiation and inter-island dispersal in diversification of the genus remain obscure because of the poor resolution in molecular phylogenetic studies (e.g., Fig. 3A). As a result of the poor resolution in Macaronesian *Tolpis*, the patterns and processes of diversification and speciation cannot be studied within a phylogenetic framework.

If the potential of insular archipelagos as model systems for studying evolution is to be fully achieved, more highly resolved and robust phylogenies are needed. Recent advances in genomic DNA sequencing have made it possible to generate large volumes of molecular data for a variety of evolutionary studies, including resolving relationships at various phylogenetic scales (Rubin et al., 2012; Cariou et al., 2013; Eaton and Ree, 2013; Cruaud et al., 2014; Hipp et al., 2014) in taxonomically difficult groups. In particular, the resolution at both relatively deep (among archipelagos) and very shallow (within archipelagos) levels within a single lineage still represents a major challenge to understanding patterns and processes of plant evolution in oceanic archipelagos. Here, we report on the utility of one genome-scale sequencing method (MSG; Andolfatto et al., 2011) for resolving phylogeny in Macaronesian *Tolpis*, both among archipelagos and within a lineage of morphologically diverse yet molecularly similar species in the process of diverging.

MATERIALS AND METHODS

Taxon sampling—Much of the known diversity of *Tolpis* in the Macaronesian archipelagos was sampled, including the generally recognized species, putative segregate taxa, a recently described species, and populations that have been suggested as worthy of taxonomic recognition based on morphology, molecular markers, and occurrence on particular substrates (Table 1). All the taxa sampled for the current study are diploids. The only widely accepted Canary Island species not included is the very rare tetraploid species *T. glabrescens*. The equally rare species *T. farinulosa* from Cape Verde and *T. macrorhiza* from Madeira were also not included. No populations of *Tolpis* from Gran Canaria, where the genus is also quite rare, were included, although there are no known endemic species from this island.

DNA sequencing—We generated genomic libraries for genotyping individuals using the multiplexed-shotgun-genotyping (MSG) method (Andolfatto et al., 2011). We digested genomic DNA from each plant using the restriction enzyme AseI (NEB Biolabs, Ipswich, Massachusetts, USA). For increasing the amount of sequence data obtained, each DNA sample was ligated to two distinct bar-coded adaptors, each containing a unique 6-bp code. After PCR, we size-selected our library for fragments between 250 and 300 bp using a Pippin Prep (Sage Science, Beverly, Massachusetts, USA). Subsequent to size-selection, we performed PCR reactions (14 cycles) using Phusion High-Fidelity PCR Master Mix (NEB Biolabs, Ipswich, Massachusetts, USA) and primers that bind to common regions in the adaptors. Sequences were generated in two separate Illumina (San Diego, California, USA) HiSEq. 2500 lanes in combination with samples for a separate study at the Genome Sequencing Core, University of Kansas, Lawrence, Kansas, USA. The first lane included eight accessions that were sequenced as part of an SR100 lane, whereas the other 20 accessions were included within a PE100 lane. We included a 10% phiX spike-in for all lanes to provide additional sequence complexity. Regardless of the type of run, we demultiplexed the FastQ files into sample-specific sequence files and ignored read-pairing for subsequent analyses.

Processing data and phylogenetic analyses—The demultiplexed FastQ files were processed using the program pyRAD vers. 2.12 (Eaton, 2014), employing USEARCH vers. 7.0 (Edgar, 2010) with a user specified similarity threshold of 90% and a minimum depth of coverage of 12. Similarly, consensus sequences were generated for all sampled individuals in pyRAD by clustering and filtering putative MSG loci for all 27 sampled individuals using the same similarity threshold and randomized sequence input order. Loci shared by 50% or more of the sampled taxa were aligned in the program MUSCLE vers. 3.8 using default settings (Edgar, 2004, 2010), and following Eaton and Ree (2013), any locus with shared heterozygous positions in three or more individuals was discarded as likely paralogs. The resulting data set was used to infer a ML topology using the program RAxML vers. 7.4.2 employing the GTRGAMMA model (Stamatakis, 2014); support for phylogenetic relationships was assessed via 1000 replicates of ML bootstrap analysis.

RESULTS

Processing of the MSG data resulted in a data set of 686 663 aligned nucleotide positions with about 50% missing data (Appendix S1, see Supplemental Data with the online version of this article). A range of similarity thresholds and minimum coverage settings were explored in pyRAD (Eaton, 2014), which resulted in data sets of different sizes and levels of missing data; however, analyses of these data sets recovered the same topology presented here (Fig. 3B), but with some minor differences in clade support. ML analysis resulted in a topology (Fig. 3B) with resolution and support far greater than previous attempts to infer relationships in Macaronesian *Tolpis* (Moore et al., 2002; Mort et al., 2003, 2007, 2010; Archibald et al., 2006; Gruenstaeudl et al., 2013). There is strong support for a clade comprising four species from the Azores, Madeira, and the mainland (99% bootstrap). Although the level of taxon sampling from these geographic locations is admittedly low, the current study is the first to resolve taxa from these areas as

Fig. 1. Diversity of *Tolpis* in the Canary Islands. (A) Habitat of *T. proustii* from the island of El Hierro. (B) Capitulum of the outcrossing *T. lagopoda* (*lag-3*) from La Palma. (C) Habitat and typical growth form of *T. santosii* from La Palma. (D) Captitulum of the selfing *T. coronopifolia* from Tenerife.
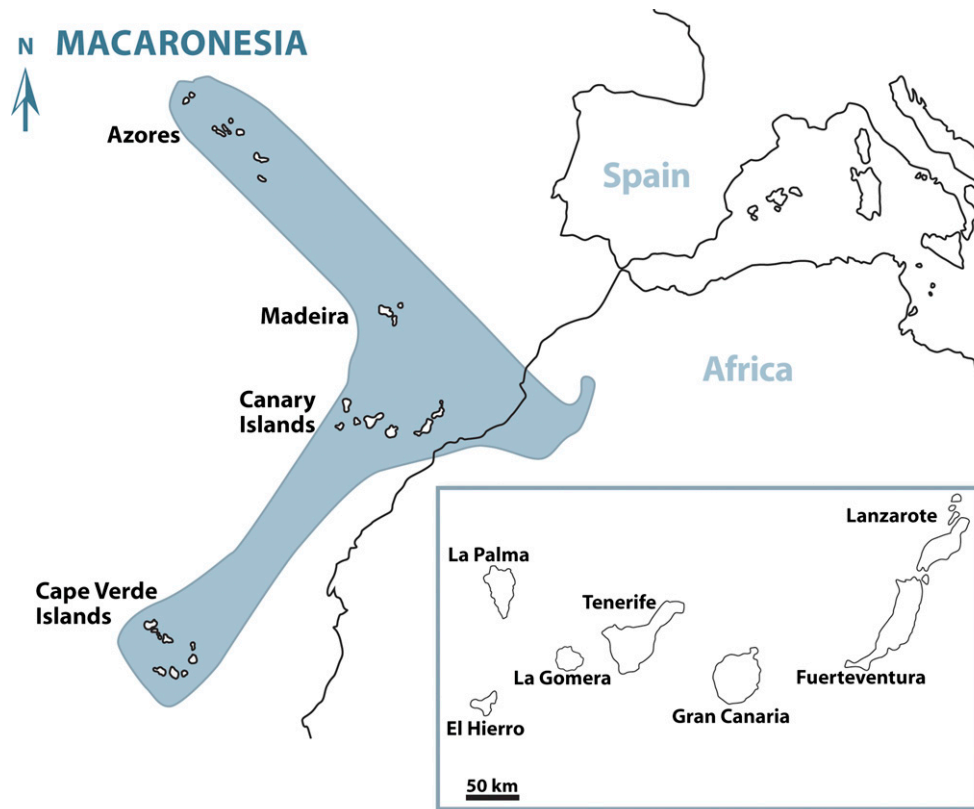
Fig. 2.   Map of Macaronesia (shaded) with a detailed map showing the seven major islands in the Canarian archipelago.

monophyletic. There is 99% bootstrap support for a clade of species from the Canary Islands (CI). Although *T. farinulosa* from Cape Verde (CV) has yet to be sequenced for MSG loci, we will refer to the clade from these geographic regions as the CI-CV clade for consistency with previously published phylogenies for Macaronesian *Tolpis* (e.g., Fig. 3A). Furthermore, phylogenetic relationships within the CI-CV clade are highly resolved, and many receive moderate to strong bootstrap support. This phylogeny does not conflict with the most recent estimate of relationships for *Tolpis* based on analyses of nrDNA ETS (Fig. 3A). Particularly noteworthy is that MSG data provide resolution within a clade of populations sampled from the islands of Tenerife and La Palma, greatly exceeding that achieved in previous studies (see shaded boxes in Fig. 3B). There is weak support for the grouping of an accession from Tenerife with one from El Hierro, but this is likely an artifact of sampling, rather than inter-island dispersal. A prior analysis resolved plants from El Hierro as a moderately supported clade that does not include elements from Tenerife (Fig. 3A).

DISCUSSION

***Utility of MSG for inferring phylogeny in radiating taxa*—** Resolving phylogenetic relationships within Macaronesian *Tolpis* has been very difficult due to limited molecular variation in a wide variety of commonly employed molecular markers (Moore et al., 2002; Mort et al., 2003, 2007, 2010; Archibald et al., 2006; Gruenstaeudl et al., 2013). Regardless of the markers

used, the general outcome has been phylogenies that resolve a clade of *Tolpis* populations from the Azores and a clade of *Tolpis* taxa from the Canary Islands, including *T. farinulosa* from Cape Verde (i.e., CI-CV clade). However, there has been minimal well-supported resolution within these clades, and the two species present in Madeira have not formed a clade. A brief summary of the major findings of previous phylogenetic studies follows.

An extensive cpDNA restriction site study (Moore et al., 2002) of 16 species of *Tolpis* provided strong support for the monophyly of *Tolpis* and resolved a CI-CV clade, and an Azorean subclade; however, three populations sampled from Madeira were not resolved as monophyletic. Relationships within the CI-CV clade, which encompasses the most diversity in Macaronesian *Tolpis*, were largely unresolved by cpDNA RFLPs. Two studies employed analyses of intersimple sequence repeat (ISSR) data to estimate relationships in *Tolpis* (Mort et al., 2003; Archibald et al., 2006). The more comprehensive study (Archibald et al., 2006) included 1628 loci from 264 individuals from 36 populations. These analyses recovered some population clusters, but the only well-supported node linked two populations of the morphologically and ecologically distinctive, selfing *T. coronopifolia*. A DNA sequence survey of six rapidly evolving cpDNA regions from 20 populations of *Tolpis* generated a data set of 4400 bp of which only seven (0.16%) were parsimony informative (Mort et al., 2007). A second cpDNA-based study employed sequences of two sets of markers proposed as DNA barcodes (Chase et al., 2007) in 19 *Tolpis* populations (Mort et al., 2010). Each data set distinguished
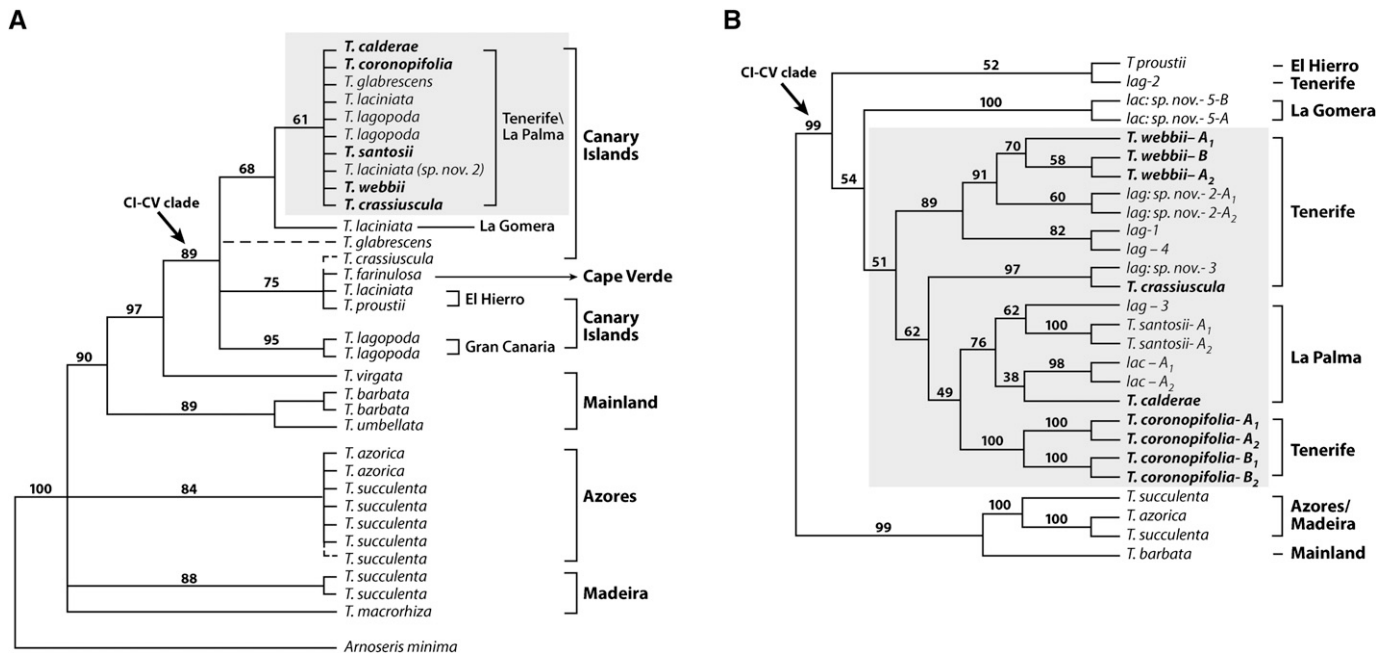
Fig. 3.   (A) Seventy-five percent consensus tree of *Tolpis* based on analyses of nrDNA ETS with maximum likelihood (ML) bootstrap support greater than 50% indicated (modified from Gruenstaeudl et al., 2013). Dashed lines indicate putative hybrid ETS orthologs. The accession referred to as *T. laciniata* "Nogales" in Gruenstaeudl et al. (2013) represents the newly named *T. santosii* and is labeled as such in both topologies. (B) Maximum likelihood (ML) topology derived from analyses of multiplexed shotgun genotyping (MSG) data with ML bootstrap support indicated. Note that some species sampled by Gruenstaeudl et al. (2013) have yet to be included in the MSG data set. Within the CI-CV clade (indicated by arrow), taxa widely recognized as distinct from *T. laciniata* or *T. lagopoda* are in bold; taxa of uncertain status are referred to as *lac* or *lag* (Table 1). Shaded boxes highlight the increased resolution and bootstrap support in one clade provided by MSG data.

some taxa; however, the neighbor joining dendrogram lacked support for most internal branches (Mort et al., 2010).

The most recent effort to estimate phylogeny for Macaronesian *Tolpis* (Gruenstaeudl et al., 2013) used external transcribed spacer sequences (nrDNA ETS) for 29 *Tolpis* accessions (Fig. 3A) and, once again, resolved clades from the Azores and the CI-CV clade, but failed to resolve the populations from Madeira. Similarly, there is a lack of resolution at the base of the tree and within the CI-CV clade (Fig. 3A).

The generally low level of sequence divergence within *Tolpis*, while confounding previous attempts at phylogeny reconstruction, actually facilitates using MSG data for estimating phylogeny. A principal shortcoming of MSG data for phylogenetic inference is nonrandom dropout of loci due to increasing numbers of mutations in restriction sites with increasing divergence among lineages. Fortunately, the problem posed by recent radiations, namely minimal molecular divergence, is least problematic when using MSG loci. Furthermore, recent advances in de novo assembly and SNP calling of MSG data, such as STACKS (Catchen et al., 2013) and pyRAD (Eaton, 2014) greatly enhance the accuracy of selecting homologous loci for phylogeny reconstruction.

In contrast to previous studies of *Tolpis*, the MSG data presented here show great promise for resolving phylogenetic relationships within recently radiated lineages, such as insular endemics. Furthermore, MSG data provide robust resolution at relatively deeper as well as shallow nodes (Fig. 3B). For example, there is strong support (100% bootstrap) for a clade comprising the Madeiran and Azorean *Tolpis* species, whereas populations from those two regions are placed in a polytomy by ETS (Fig. 3A). In agreement with Gruenstaeudl et al. (2013),

the accessions of *T. succulenta* from Madeira and Azores are not monophyletic.

Particularly noteworthy is the resolution provided by MSG data among the morphologically and ecologically diverse members of a La Palma/Tenerife subclade of CI-CV *Tolpis* (shaded in Fig. 3B). The analyses of Gruenstaeudl et al. (2013) resolved this lineage, but phylogenetic relationships lacked resolution among the populations sampled from these islands (Fig. 3A). ML analysis of MSG loci for an expanded sampling of this lineage (19 vs. 10 accessions) recovered generally well-supported resolution among accessions (Fig. 3B). Within this lineage, there is strong support (100% bootstrap) for the relationship between two populations of *T. coronopifolia*, a species that has evolved a selfing reproductive strategy and the associated selfing morphological syndrome (i.e., small capitula, lower pollen–ovule ratios, smaller stigmatic surfaces, etc.; Ornduff, 1969). Sister to this is a clade (76% bootstrap) comprised of La Palma endemic taxa. This clade includes two populations currently referred to as *T. laciniata* (*lac*-A) or *T. lagopoda* (*lag*-3), as well as the newly recognized *T. santosii* (Crawford et al., 2013), and a morphological variant recognized by some taxonomists as *T. calderae*. The latter species was considered a variant of *T. laciniata* by Jarvis (1980), who noted that its morphology falls within the overall range of variation seen in *T. laciniata*; however, based primarily on leaf characters Bramwell and Bramwell (2001) recognized *T. calderae* as a species distinct from *T. laciniata*. Similarly, MSG data resolve a clade (89% bootstrap) of Tenerife-endemic taxa including *T. webbii* and three populations currently ascribed to *T. lagopoda*. However, the latter "species" is not a true evolutionary lineage, being dispersed across the phylogeny and likely representing multiple cryptic

TABLE 1. Accessions sampled for the present study including designation used on the phylogenetic tree, archipelago where collected, island of origin for Canary Island endemic taxa, and collector and collection number. Taxa sampled from the Canary Islands are grouped as those that are well-defined morphologically, which are single-island endemics, and those that are part of the geographically widespread *Tolpis laciniata-lagopoda* complex. Voucher specimens are deposited in the McGregor Herbarium (KANU) at the University of Kansas.

Species	Designation on phylogeny	Archipelago and island of origin	Collector and collection number
T. azorica	*T. azorica- Az*	Azores- São Miguel	*Borges Silva TA* 4.1
T. succulenta	*T. succulenta- Az*	Azores- São Miguel	*Borges Silva TS* 3.2
T. succulenta	*T. succulenta- Mad*	Madeira- Madeira	*Menezes de Sequeira 7095*
T. barbata	*T. barbata- Cont*	Mainland	*Crawford et al., 1849*
Well-defined Canary Island species			
T. calderae	*T. calderae*	Canary Islands- La Palma	*Crawford et al., 1906*
T. coronopifolia	*T. coronopifolia- A_1*	Canary Islands- Tenerife	*Santos-Guerra 5*
T. coronopifolia	*T. coronopifolia- A_2*	Canary Islands- Tenerife	*Santos-Guerra 5*
T. coronopifolia	*T. coronopifolia- B_1*	Canary Islands- Tenerife	*Crawford et al., 1834*
T. coronopifolia	*T. coronopifolia- B_2*	Canary Islands- Tenerife	*Crawford et al., 1834*
T. crassiuscula	*T. crassiuscula*	Canary Islands- Tenerife	*Crawford et al., 1993*
T proustii	*T proustii*	Canary Islands- El Hierro	*Santos-Guerra 10*
T. santosii	*T. santosii- A_1*	Canary Islands- La Palma	*Crawford et al., 2009*
T. santosii	*T. santosii- A_2*	Canary Islands- La Palma	*Crawford et al., 2009*
T. webbii	*T. webbii- A_1*	Canary Islands- Tenerife	*Santos-Guerra 09*
T. webbii	*T. webbii- A_2*	Canary Islands- Tenerife	*Santos-Guerra 09*
T. webbii	*T. webbii- B*	Canary Islands- Tenerife	*Santos-Guerra 06*
Members of the *T. laciniata-lagopoda* species complex			
T. laciniata	*lac: sp. nov.- 5-A*	Canary Islands- La Gomera	*Crawford et al., 1911*
T. laciniata	*lac: sp. nov.- 5-B*	Canary Islands- La Gomera	*Crawford et al., 2048*
T. laciniata	*lac − A_1*	Canary Islands- La Palma	*Crawford et al., 1883*
T. laciniata	*lac − A_2*	Canary Islands- La Palma	*Crawford et al., 1883*
T. lagopoda	*lag: sp. nov.- 2-A_1*	Canary Islands- Tenerife	*Santos-Guerra 13*
T. lagopoda	*lag: sp. nov.- 2-A_2*	Canary Islands- Tenerife	*Santos-Guerra 13*
T. lagopoda	*lag-1*	Canary Islands- Tenerife	*Crawford et al., 1949*
T. lagopoda	*lag-2*	Canary Islands- Tenerife	*Crawford et al., 1941*
T. lagopoda	*lag: sp. nov.- 3*	Canary Islands- Tenerife	*Crawford et al., 2030*
T. lagopoda	*lag- 3*	Canary Islands- La Palma	*Santos-Guerra 14*
T. lagopoda	*lag- 4*	Canary Islands Tenerife	*Santos-Guerra 12*

taxa. This illustrates the great potential of analyses of MSG data for species delimitation and discovery within lineages that contain cryptic morphological variants, such as *Tolpis* in Macaronesia.

The well-supported resolution across all phylogenetic levels in Macaronesian *Tolpis* is consistent with empirical studies such as Hipp et al. (2014), that found MSG data useful for phylogeny inference in a group up to 33 million years old (Myo), and simulation studies that suggest MSG to be useful in lineages 50 Myo (Rubin et al., 2012) to even 63 Myo (Cariou et al., 2013). The current study illustrates the potential of MSG data for resolving phylogeny in *Tolpis* across archipelagos, as well as within the very recently diverging Tenerife/La Palma lineage, thereby establishing the necessary framework within which evolutionary hypotheses might be tested in this group. Previous studies (Kim et al., 2008) date the most recent common ancestor of five major radiations of flowering plants in Macaronesia (*Tolpis* was not included) at between 3.3 and 15.2 Myo. Thus, the results presented here suggest that analyses of MSG data can provide the much-needed phylogenies for comprehensive evolutionary studies of Macaronesian endemic plants. Given the estimated ages of radiations on other archipelagos—e.g., *Bidens* (3.1–1.3 Mya, Knope et al., 2012), silverswords (5.2 Mya, Baldwin and Sanderson, 1998), lobeliads (13.6 ± 1.5 Mya, Givnish et al., 2009), and honeycreepers (5.7 Mya, Lerner et al., 2011) in Hawaii, and *Geospiza* (2.3 Mya, Sato et al., 2001) from the Galapagos—it is very likely that MSG data will prove similarly useful in a variety of groups in different oceanic archipelagos.

***Patterns of evolution in* Tolpis—**There is little doubt that both inter-island dispersal (island hopping) and "adaptive" radiation on a single island play important roles in the diversification of insular flowering plants (e.g., Francisco-Ortega et al., 1997; Mort et al., 2002; Goodson et al., 2006; Givnish et al., 2013; Morden and Harbin, 2013; Talavera et al., 2013; Jones et al., 2014). On the Canary Islands, the emerging pattern of evolution for most endemic lineages is colonization via a single founder event, followed by diversification, mostly through inter-island dispersal, but with infrequent within-island radiations (Mort et al., 2002; Talavera et al., 2013; Jones et al., 2014). However, to-date, most studies have lacked the comprehensive population sampling and robust framework of relationships necessary to document the role of each process.

The unprecedented resolution attained with MSG data allow us to illustrate that not only inter-island dispersal, but also within-island diversification (including possible shifts in ecological zones) have played a role in the evolution of *Tolpis* on the Canary Islands. For example, a clade (76% bootstrap) of six accessions from four populations on La Palma, including *T. santosii* and *T. calderae* (Fig. 3B), is nested within a lineage comprising taxa endemic to Tenerife. Similarly, analyses of MSG data recover a robust clade of seven accessions from five Tenerife populations (89%), where there is complete resolution

of relationships even among populations of "*T. lagopoda*," including a putative segregate species. The overall pattern illustrates a high degree of within-island diversification coupled with periodic dispersal between islands. We are currently deepening our taxon sampling to infer more robustly patterns of diversification across these islands.

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