

PHYLOGENY AND MATING SYSTEM EVOLUTION OF
TOLPIS (ASTERACEAE) IN MACARONESIA

By
© 2020

Benjamin Kerbs
B.S., Emporia State University, 2016

Submitted to the graduate degree program in the department of Ecology and Evolutionary Biology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

Mark E. Mort, Chair

Daniel J. Crawford

John K. Kelly

Robert G. Moyle

Date Defended: June 2020

The thesis committee for Benjamin Kerbs certifies that this is the approved version of the following thesis:

PHYLOGENY AND MATING SYSTEM EVOLUTION OF
TOLPIS (ASTERACEAE) IN MACARONESIA

Chair: Mark E. Mort

Date Approved: May 2020

ABSTRACT

Tolpis (Asteraceae) is a flowering plant genus with a center of distribution in the Macaronesian archipelagos. The genus has gained considerable interest, most recently, for elucidating patterns of mating system evolution, hybrid speciation, and genetic breakdown of self-incompatibility. The evolutionary history of this genus has, notwithstanding, been difficult to reveal due to recent divergence within the genus and the lack of sufficient molecular variation afforded by traditional molecular markers (e.g. inter-simple sequence repeats, chloroplast DNA restriction sites, external and internal transcribed spacers). Here we present a robust inference of the evolutionary relationships of *Tolpis* within the Macaronesian archipelagos of the Azores, Canary Islands, and Madeira. This phylogenetic study reinforces the efficacy of multiplexed shotgun genotyping (MSG) in elucidating the evolutionary history of recently-diversified plant lineages with resolution and support from the population to inter-archipelago levels. Lastly, we used the same genotyping approach to determine the mating system in two populations of *Tolpis* from Graciosa island in the Azores, where self-compatibility mutations have either arisen or been introduced. We apply genomic data and Bayesian inference (BORICE) to determine outcrossing rates, inbreeding coefficients, siring patterns for these two populations.

TABLE OF CONTENTS

Abstract	iii
List of Tables	iv
List of Figures	v
Chapter One: Introduction	1
Chapter Two: Multiplexed-shotgun-genotype (MSG) data resolve phylogenetic relationships within and among archipelagos in Macaronesian <i>Tolpis</i> (Asteraceae).	23
Chapter Three: Mating system consequences of the recent establishment of self-compatibility in two small populations on an oceanic island: <i>Tolpis succulenta</i> in the Azores	51

LIST OF TABLES:

Table 1. Table of <i>Tolpis</i> samples used in the present study, along with their population numbers, archipelago, island, locality information, and collection number	6
Table 2. Table of <i>Tolpis</i> samples used in the present study, along with their population numbers, archipelago, island, locality information, and collection number	29
Table 3. Assignment of sibship for each family across two <i>T. succulenta</i> populations	59

LIST OF FIGURES:

Figure 1. Map of Macaronesia	3
Figure 2. Maximum parsimony tree based on chloroplast DNA restriction sites (Moore et al., 2002)	9
Figure 3. Seventy-five per cent consensus tree of <i>Tolpis</i> based on nrDNA ETS data (Gruenstaeudl et al., 2013)	11
Figure 4. Maximum likelihood (ML) tree based on phylogenetic analyses of multiplexed shotgun genotyping (MSG) data (Mort et al., 2015)	12
Figure 5. Map of Macaronesia showing the four archipelagos from which samples of <i>Tolpis</i> included in the present originated. Insets show the islands of the Azores and Canaries	25
Figure 6. Maximum likelihood (ML) topology derived from analyses of multiplexed shotgun genotyping (MSG) data for <i>Tolpis</i> in Macaronesia. Clades with higher than 95% or higher ML bootstrap are indicated by asterisks. AZ, Azores; CI, Canary Islands; CV, Cape Verde; MD, Madeira; CO, continent	32
Figure 7. ML topology from MSG data for <i>Tolpis</i> in Azores and Madeira. ML bootstrap support indicated for clades with less than 95%, otherwise denoted by asterisk. Population numbers same as in Table 1. Island designations	33

for Madeira (MD) and for islands in Azores (SA, Santa Maria; SM, São Miguel; GR, Graciosa; TE, Terceira; SJ, São Jorge, FA, Faial; PI, Pico; FL, Flores)

- Figure 8.** ML topology from MSG data for *Tolpis* in the continent and Canary Islands. ML bootstrap support indicated for clades with less than 95%, otherwise denoted by asterisk. Population numbers same as in Table 1. Island designations for Cape Verde Islands: SA, Santo Antão; and for Canary Islands: T, Tenerife; EH, El Hierro; LG, La Gomera; GC, Gran Canaria; LP, La Palma. 35
- Figure 9.** Locality of two populations of *T. succulenta* (GRSC and GRBL) on Graciosa island, within the central group of the Azorean archipelago. 57
- Figure 10.** Posterior density for the overall outcrossing rate (t) across both populations 58
- Figure 11.** Individual offspring within the same rectangles are full sibs. Maternal family 1 has six sires, and families four and five have four sires. 59

CHAPTER ONE:

INTRODUCTION

Oceanic Islands and their Flora

Oceanic islands have long been regarded by biologists as natural laboratories to test, *in situ*, the patterns and processes of evolution and biogeography (Emerson, 2002). Their importance in such investigations was recognized by Darwin (1859), Wallace (1880), MacArthur and Wilson (1967) and later 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.” The notion of islands as natural laboratories is best exemplified by the fact that insular regions have discrete geographic and ecological boundaries, feature dynamic and often dateable geologic histories, and harbor a wide diversity of habitats and species (Emerson, 2002). These features may also promote especially high levels of endemism; to illustrate, among all currently described vascular plant species, 25% are endemic to oceanic islands despite only 5% of the Earth’s land surface being comprised of islands (Kreft et al., 2008; Caujape-Castells et al., 2010). The striking diversity of plant species on such islands can be explained, either singly or in concert, by differential adaptation of populations to unique island niches, by vicariance via lava flows or landslides, and/or by multiple colonization from neighboring islands or the continent (Emerson, 2002; Carson and Templeton, 1984).

A number of characteristics are associated with the flora of oceanic islands. One is a tendency toward the woody habit (Carlquist, 1974). In some species, woodiness is suggested to be the basal condition of paleoendemic island ancestors that went extinct on the continent (Bremer,

1996; Andersson and Rova, 1999); such a progression is deemed the “relict hypothesis” (Meusel, 1965; Bramwell, 1976; Cronk, 1992). More commonly, as hypothesized by Carlquist (1974), woodiness is the derived, post-colonization condition of island plants, as they adapt to a moderate climate, permitting growth throughout the year. This is evidenced by a number of molecular phylogenetic studies of island plants (Baldwin and Robichaux, 1995; Givnish et al., 1995; Kim, Crawford, and Jansen, 1996). Another feature of oceanic island flora is the so-called “island syndrome” (Carlquist, 1974). This condition describes a reduced dispersibility (Cody and Overton, 1996; Fresnillo and Ehlers, 2008; Gillespie et al., 2012), decreased competitive advantage over introduced species (Harter, 2015), and paucity of defensive mechanisms against non-native herbivores (Bowen and van Vuren, 1997; Vourc’h et al., 2001). When considered in conjunction with 1) the generally small range and overall population size of many island plants and 2) the reduced capacity of species to escape negatively changing conditions (as a result of small land size), it is evident that island plants are particularly vulnerable groups, especially in the face of climate change and human disturbance. Thus, a prompt and expanded elucidation of the evolutionary forces operant in island plants, their patterns of colonization and dispersal, and the conservation implications of such findings are all warranted.

The Macaronesian Archipelagos

One group of oceanic archipelagos, Macaronesia, has garnered much attention for its suitability as a system for testing patterns of island plant evolution. Positioned between 15 and 40° N in the Atlantic Ocean, Macaronesia represents a group of 32 volcanic islands across four archipelagos: the Azores, Madeira, the Canary Islands, and the Cape Verde Islands (Fig. 1). In contrast to

many other well-studied insular systems (e.g., Hawaii, Galápagos, Robinson Crusoe) the ages of Macaronesian islands greatly vary in age, ranging from 0.8 to 21 million years old (Carracedo et al., 2002). These islands also have great variation in terms of distance to the mainland with the Azorean island Pico as the farthest (1600 km) from the continent and

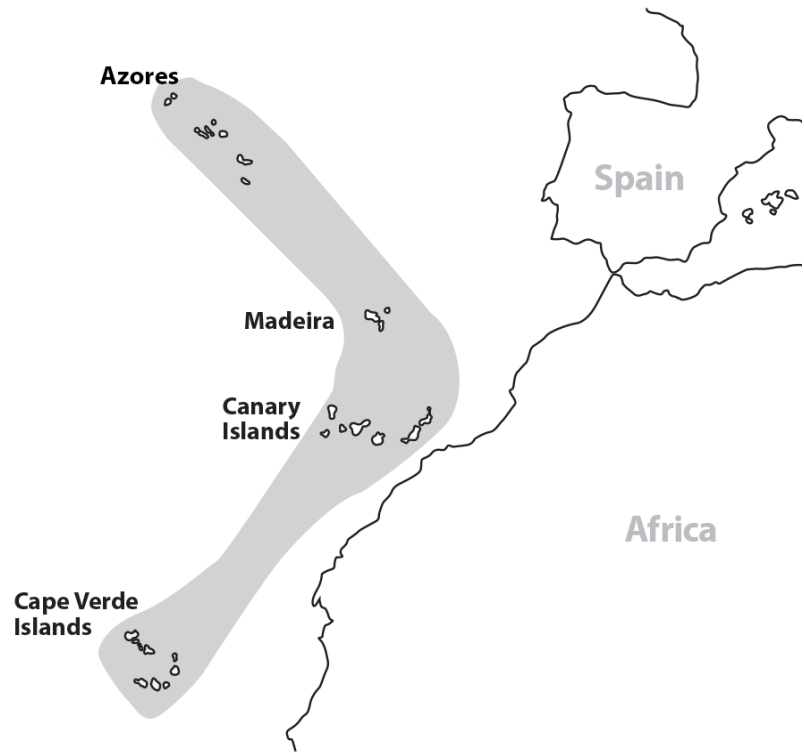


Figure 1. Map of Macaronesia

Canarian Fuerteventura as the closest (100 km) (Park et al., 2001). Lastly,

great variation also exists in the timing and magnitude of volcanism over the last 21 million years, with recent volcanic events reported in El Hierro (2012, La Restinga), La Palma (1971, Teneguía), and Tenerife (1909, Chinyero) of the Canary Islands (Juan et al., 2000; Lopez de Heredia et al., 2014). These features, in conjunction with erosional processes, make Macaronesian islands highly dynamic and heterogeneous landscapes, contributing to especially high degrees of lineage diversification (Lopez de Heredia et al., 2014); these archipelagos therefore represent a favorable system for studying the evolution of island plants.

Macaronesia is known to contain a diverse flora in which 20% of angiosperms (40% in the Canary Islands alone) are endemic (Humphries, 1979; Santos-Guerra, 1999). The endemic aspect

of Macaronesian flora is partly supported as a relict of subtropical Tertiary flora from Europe and Africa (Engler, 1879; Bramwell, 1985; Sunding 1979; Cronk, 1992; Moore et al., 2002). Instances where woodiness is found to be the ancestral pre-island condition (as in Moore et al., 2002) support the relict hypothesis, but this pattern is not always true. The vast majority of insular endemic species on Macaronesia (e.g. 72% in the Canary Islands [Aldridge, 1979]) have been shown to have continental ancestors that are herbaceous based on morphology/anatomy as well as many molecular phylogenetic studies (e.g. *Argyranthemum* Sch.Bip. [Francisco-Ortega et al., 1997]; *Echium* [Bohle, Hilger, and Martin, 1996]; *Ixanthus* Griseb. [Thiv, Struwe, and Kadereit, 1999]; *Pericallis* [Panero et al., 1999]; the *Sonchus* alliance [Kim et al., 1996]; the *Aeonium* alliance [Mort et al., 2002]). This corroborates Carlquist's (1969, 1974) hypothesis that many such plants are secondarily instead of primarily woody, as plants escaped seasonality in ocean-buffered habitats. Clearly, there is no "one size fits all" model to explain the habits of Macaronesian angiosperms.

In terms of plant colonization of Macaronesia, a number of patterns have emerged, such as: rapid radiation after a single colonization event (Francisco-Ortega et al., 1996; Bohle et al., 1996; Kim et al., 1996; Barber et al., 2007; Mort et al., 2002; Francisco-Ortega et al., 2002) multiple colonization events (Percy et al., 2002; Fuertes-Aguilar et al., 2002; Carine et al., 2004; Allan et al., 2004), and continental back-dispersal from the archipelagos (Mort et al., 2002; Carine et al., 2004; Allan et al., 2004; see Chapter 2). Kim et al. (2008) estimated the age and diversification rate of several major plant lineages endemic to Macaronesia, including the woody *Sonchus* alliance, *Echium*, *Sideritis*, *Crambe*, and the *Aeonium* alliance. These lineages were inferred to have colonized the Canary Islands during three narrow colonization windows: the middle

Miocene (15.2 Ma), Late Miocene (8.47 and 8.15 Ma), and Early Pliocene (3.73 Ma and 3.3 Ma)(Kim et al., 2008). Inter-archipelago dispersal events for these major groups were determined to have occurred recently, with four studied groups inferred to have dispersed to Madeira during the Plate Pliocene and Pleistocene, and three groups to Cape Verde during the Pleistocene (Kim et al., 2008). One notable genus not included in this study was *Tolpis*, which deviates from many other Macaronesian plant groups in the patterns of speciation and colonization, which shall be illustrated herein.

Taxon

The genus *Tolpis* Adanson (Asteraceae) comprises ca. 11-15 species ranging from herbaceous annuals to woody perennials (Jarvis, 1980). The genus is distributed in Macaronesia as well as continental Europe and Africa (Jarvis, 1980). A notable biogeographic feature of *Tolpis* is that the vast majority of species within this genus exhibit endemism in four of the five archipelagos in Macaronesia: one is endemic to Madeira, one to the Azores, one to Cape Verde Islands, and nine to the Canary Islands. Within Macaronesia, very few genera other than *Tolpis* demonstrate the same degree of endemism across archipelagos (e.g. *Carex* L. [Cyperaceae], *Euphorbia* L. [Euphorbiaceae], *Festuca* L. [Poaceae], and *Lotus* L. [Fabaceae]) (Hansen and Sunding, 1993). Though only 11 species were recognized by Jarvis (1980), it is likely that many more species are worthy of taxonomic recognition, as phylogenetic reconstructions of Macaronesian *Tolpis* utilizing molecular markers (Moore et al., 2002; Mort et al., 2015) have already lent support for four cryptic species, one of which, *T. santosii*, was recently described (Crawford et al., 2013); this highlights the necessity for greater population sampling and sequencing across taxa.

Several features combine that make *Tolpis* an ideal model system for studying patterns and processes of island plant evolution (Table 1).

These include: 1) variation in life

habit (e.g. perennial/annual), as well as the commonly associated breeding systems (self-incompatibility and self-compatibility); 2) it is a small and well-studied monophyletic group; 3) there are restricted and widespread species occurring on substrates of varying ages; 4) individuals of most species have short generation times and are easily cultivated in the greenhouse for experimental purposes; and 5) the continental sister group is well established (Kilian et al., 2009; Gruenstaeudl et al., 2013). These features permit investigation into a number of questions pertaining to phylogeny, biogeography, and reproductive strategies in *Tolpis*, the methodologies and outcomes of which may be broadly extrapolated to angiosperms as a whole. Prior work on *Tolpis* has extended the understanding of plant hybrid speciation (Kerbs et al., 2017), demonstrated the utility of DNA barcoding (Mort et al., 2010) and ISSRs (Archibald et al., 2006) in phylogenetic reconstruction, tested the validity of Baker's Law (Crawford et al., 2008), and examined the breakdown of self-incompatibility (including its genetic underpinning [Koseva et al., 2017], its impacts on mating systems [Crawford et al., 2010; Gibson et al., 2020], and influence in island colonization and dispersal [Gibson et al., 2020]). For these reasons, among others, we promote *Tolpis* as a group worthy of recognition as a model system.

Table 1. *Tolpis* as an ideal study system:

- Variation in life habit
- Variation in breeding system
- Small and well-studied monophyletic group
- Occurs on substrates of varying ages
- Short generation times
- Easily cultivated in the greenhouse
- Continental sister group is well-established

Evolutionary patterns in Macaronesian *Tolpis*

The establishment of *Tolpis* as a model system for testing the patterns of plant colonization and diversification, as well as the ascertainment of its origin in Macaronesia, begins with the inference of a robust phylogenetic reconstruction of species within this lineage. For the last two decades, a variety of markers have been utilized in the phylogenetic assessment of *Tolpis*, including DNA sequences of chloroplast markers such as *ndhF* (Park et al., 2001) and rapidly evolving cpDNA spacer regions (Mort et al., 2007), cpDNA RFLPs (Moore et al. 2002), ISSRs (Mort et al., 2003; Archibald et al., 2006), and nuclear ribosomal markers (Gruenstaeudl et al., 2013). Although the array of these traditional markers has provided considerable insight into the intergeneric placement of *Tolpis* in tribe Cichorieae of Asteraceae, well-supported inference of most low level, interspecific relationships remained a challenge until the recent advent of whole-genome sequencing.

The first phylogenetic reconstruction of *Tolpis* using molecular data was carried out by Park et al. (2001), who sequenced and aligned the chloroplast encoded gene *ndhF* and carried out phylogenetic analysis under a parsimony framework. Here, seven species of *Tolpis* from Africa, Europe, and Macaronesia were compared, as well as 29 related species from 27 Cichorieae genera and outgroups from several other tribes including Arctoteae, Cardueae, Liabeae, Mutisieae, and Vernonieae. Their results demonstrated a lack of monophyly in *Tolpis*, with two continental species, *T. capensis* (determined to be sister to *Taraxacum*) and *T. staticifolia* (within *Crepis*) as occurring outside the main *Tolpis* clade. Placement of both taxa corroborate the findings of Jarvis (1980) who excluded both species on the basis of their habit, fruit morphology, distribution, and inability to hybridize with other *Tolpis* spp. Within the core clade, *T. azorica*

was found to be sister to four species comprising two clades: clade 1) the two continental species *T. barbata* and *T. virgata* and clade 2) the Canary Islands species *T. coronopifolia* and Cape Verde species *T. farinulosa* (Park et al., 2001). Biogeographically, equally parsimonious support was found for either multiple colonization of Macaronesia from the continent or a single colonization followed by continental back dispersal.

Moore et al. (2002) increased sample size to the 13 described species and 3 putative new species of *Tolpis*. Variation between chloroplast DNA restriction sites (ultimately including 224 restriction site changes) was assessed using parsimony analysis, yielding a monophyletic tree of *Tolpis* (Fig. 1). Three major clades emerged from this analysis including one clade with all Canary Island and Cape Verde island species, another with Azorean *T. succulenta* and *T. azorica*, and a third with continental species *T. barbata* and *T. virgata* (Moore et al., 2001). Sister to the large clade comprising these three subclades are *T. macrorhiza* from Madeira, with the most basal of all species being Madeiran *T. succulenta*. The tree produced using these characters did not provide resolution of interspecific relationships of *Tolpis* in the Canary Islands. Biogeographic reconstruction based on this analysis suggested continental extinction and recolonization. Using minimum distance metrics, 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 (a biogeographic pattern strongly distinct from the aforementioned Macaronesian plant groups studied by Kim et al. [2008], or 2) a similar

dispersal to Madeira, back dispersal to the continent from Madeira, followed by colonization of and adaptive radiation in Canaries (Moore et al., 2002).

In 2003, Mort et al. assessed the applicability of inter simple sequence repeats (ISSRs) in inferring phylogenetic relationships. They scored 48 loci for 5 ISSR primers and conducted parsimony and neighbor-joining analyses which congruently revealed the relationships of six Canarian and one Madeiran species,

demonstrating a higher resolution than that previously afforded by either chloroplast RFLPs (Moore et al., 2002) or ITS (K. Jansen, pers. comm. with Mort et al., 2003). The intended outcome of the ISSR study was not to comprehensively assess relationships of *Tolpis* in Macaronesia, but rather to test the applicability and variability of the markers for phylogenetic inference. The shortfall of limited sampling was ameliorated by Archibald et al. (2006) who included nine species and three morphological variants from the Canary Islands, as well as *T. farinulosa* from the Cape Verde Islands and *T. succulenta* from Madeira. Additionally, scoring and analysis of ISSRs was

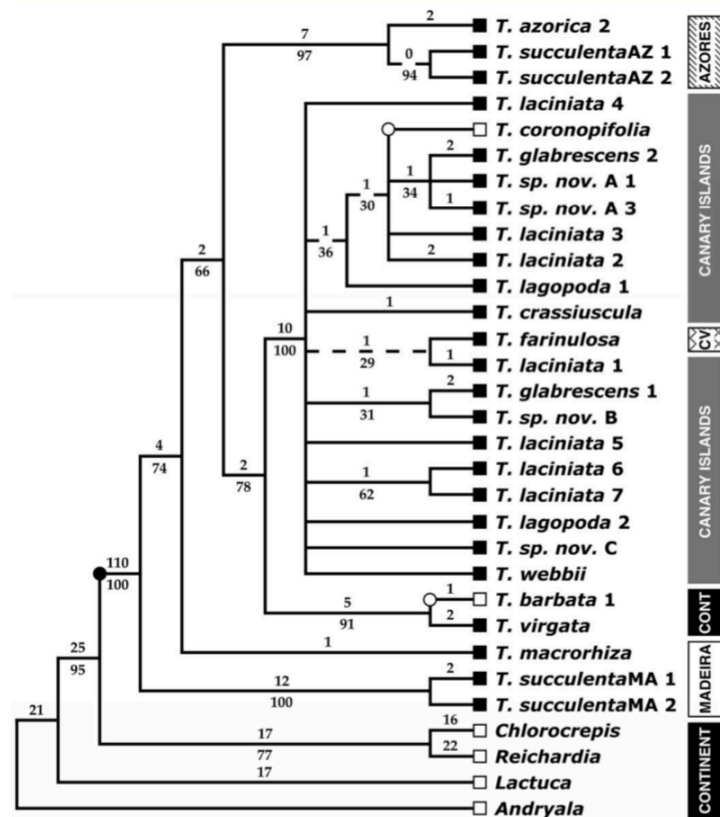


Figure 2. Maximum parsimony tree based on chloroplast DNA restriction sites (Moore et al., 2002)

automated to yield many more (1628) loci than were analyzed by Mort et al. (2003). The results of Archibald et al. (2006) demonstrated that ISSRs were most effective at grouping individuals into populations and species (i.e. confirming species circumscriptions), but the signal provided by these markers was less successful at inferring relationships at the species level, even among Canary Island species that formed distinct groups.

Partial inference of interspecific relationships was achieved through the use of plant DNA barcoding (Mort et al., 2010) and later through the use of a combination of nuclear DNA markers, including external and internal transcribed spacers and several allelic loci (Gruenstaeudl et al., 2013) (Fig. 2). Given the difficulty of determining outgroups for island genera (often because continental sister groups are highly morphologically/molecularly different), one of the most notable products of Gruenstaeudl et al. (2013) was, through investigation of 42 related genera, the identification of *Arnooseris* as the closest relative of *Tolpis*. Such findings are in line with previous morphological work done by Stebbins (1953), Jeffery (1966), and Blackmoore (1981). Although Gruenstaeudl et al. (2013) were able to reveal a number of interspecific relationships in *Tolpis*, the inferred phylogeny features a prominent basal polytomy, and still many relationships at the species and population level remained unresolved (especially among Canary Island endemics). The biogeographic reconstruction of *Tolpis* by Gruenstaeudl et al. (2013) revealed a continental origin of the genus with the most recent common ancestor (MRCA) of species from the Azores and Madeira as occurring on the continent. Similarly, they also inferred Canary Island endemic species as originating from the continent rather from other Macaronesian archipelagos.

The next-generation sequencing revolution brought forth a number of approaches which helped to resolve the issue of low resolution at shallow phylogenetic levels in recently-radiated lineages (as revealed by the previous array of molecular markers), one of those

being the multiplexed shotgun genotyping approach (MSG)

(Andolfatto et al., 2011). Mort

et al. (2015) employed this strategy to discern relationships with support previously

unachievable, including at the population, species, and deeper archipelago level (Fig. 3). Most

notably, relationships between species in the so-called “CI-CV clade” were elucidated with high support where previously (as in Gruenstaeudl et al., 2013) these relationships were obscure.

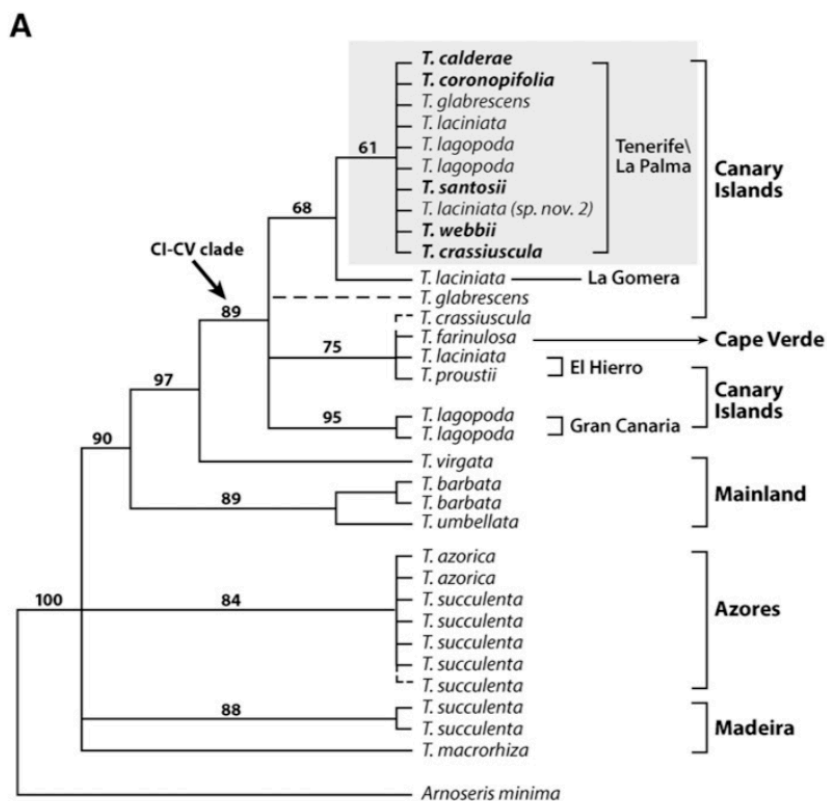


Figure 3. Seventy-five per cent consensus tree of *Tolpis* based on nrDNA ETS data (Gruenstaeudl et al., 2013)

two self-incompatible yet cross-compatible individuals sufficiently close together spatially and temporally...” Carlquist (1966, 1974) later argued that SC colonizers would have reduced potential for diversification owing to the fact that such colonizers would 1) be from inbred source populations and 2) therefore have very high genome-wide homozygosity (Charlesworth and Pannell, 2001). Mounting evidence suggests that the SI/SC condition may not be so bimodal, with numerous examples of species/populations exhibiting intermediate levels of selfing, a condition referred to as pseudo-self-compatible (PSC) (Levin 1996; Barrett 2003; Iqbal et al., 2006; Raduski et al., 2011; Pannell, 2015; Pannell et al., 2015). Even a small degree of self-compatibility might facilitate inter- and intra-island dispersal of island plants. It has been commonly accepted that *Tolpis* predominantly demonstrates the SI condition, but there are now notable exceptions, including several shifts to SC or pseudo-self compatibility (Crawford et al., 2019). Although several studies of plant breeding systems have been carried out on *Tolpis* and in island plants in general, few aim to link the breeding system to the mating system, that is who is actually mating with whom in nature (Pannell & Voillemot, 2017). Indeed, it is the relative contribution of the breeding system to the mating system that determines colonization potential, genetic structure, gene flow, and population size. The gap between breeding and mating system may be bridged through both the determination of self-seed set (a breeding system metric) and inference of outcrossing rates and inbreeding depression (two mating system metrics) of plant populations. It is an aim of Chapter 3 to demonstrate the inference of such metrics through the use of Next Generation Sequencing technology, RAD-Seq markers, and Bayesian statistics.

References

- ALLAN, G. J., J. FRANCISCO-ORTEGA, A. SANTOS-GUERRA, E. BOERNER, AND E. A. ZIMMER. 2004. Molecular phylogenetic evidence for the geographic origin and classification of Canary Island Lotus (Fabaceae: Loteae). *Molecular Phylogenetics and Evolution*. 32: 123–138.
- ANDERSSON, L., AND J. H. E. ROVA. 1999. The *rps16* intron and the phylogeny of the Rubioideae (Rubiaceae). *Plant Systematics and Evolution*. 214: 161–186.
- ANDOLFATTO, P., D. DAVISON, D. EREZYILMAZ, T. T. HU, J. MAST, T. SUNAYAMA-MORITA, AND D. L. STERN. 2011. Multiplexed shotgun genotyping for rapid and efficient genetic mapping. *Genome Research*. 21: 610–617.
- ARCHIBALD, J., D. CRAWFORD, A. SANTOS-GUERRA, AND M. MORT. 2006. The utility of automated analysis of inter-simple sequence repeat (ISSR) loci for resolving relationships in the Canary Island species of *Tolpis* (Asteraceae). *American Journal of Botany*. 93(8): 1154-1162.
- BALDWIN, B. G., AND R. H. ROBICHAUX. 1995. Historical biogeography and ecology of the Hawaiian silversword alliance (Asteraceae): new molecular phylogenetic perspectives. In W. Wagner and V. Funk [eds.], *Hawaiian biogeography: evolution on a hot spot archipelago*, 259–287. Smithsonian Institution Press, Washington D.C., USA.
- BARBER, J. C., C. C. FINCH, J. FRANCISCO-ORTEGA, A. SANTOS-GUERRA, AND R. K. JANSEN. 2007. Hybridization in Macaronesian *Sideritis* (Lamiaceae): evidence from incongruence of multiple independent nuclear and chloroplast sequence data sets. *Taxon*. 56: 74–88.

- BRAMWELL, D. 1976. VI. The endemic flora of the Canary Islands; distribution, relationships, and phytogeography. *In* G. Kunkel [ed.], *Biogeography and ecology in the Canary Islands*, 207–240. Dr. W. Junk, The Hague, The Netherlands.
- BRAMWELL, D. 1985. Contribucion a la biogeografia de las islas canaries. *Bot. Macaron.* 14: 3-34.
- BREMER, B. 1996. Phylogenetic studies within Rubiaceae and relationships to other families based on molecular data. *Opera Botanica Belgica.* 7: 33–50.
- BÓHLE, U-R, H. H. HILGER, AND W. F. MARTIN. 1996. Island colonization and evolution of the insular woody habit in *Echium* L. (Boraginaceae). *Proceedings of the National Academy of Sciences.* 93: 11740–11745.
- BOWEN, L., AND D. VAN VUREN. 1997. Insular endemic plants lack defenses against herbivores. *Conservation Biology.* 11: 1249–1254.
- CARLQUIST, S. 1969. Wood anatomy of the Goodeniaceae and the problem of insular woodiness. *Annals of the Missouri Botanical Garden.* 56: 358-390.
- CARLQUIST, S. 1974. *Island Biology*. New York: Columbia University Press.
- CARINE, M. A., S. J. RUSSELL, A. SANTOS-GUERRA, AND J. FRANCISCO-ORTEGA. 2004. Relationships of the Macaronesian and Mediterranean floras: molecular evidence for multiple colonizations into Macaronesia and back-colonization of the continent in *Colvolvulus* (Convolvulaceae). *American Journal of Botany.* 91: 1070–1085.
- CARSON, H., AND A. TEMPLETON. 1984. Genetic Revolutions in Relation to Speciation Phenomena: The Founding of New Populations. *Annual Review of Ecology and Systematics.* 15: 97-131.

- CARRACEDO, J. C., ET AL. 2002. Cenozoic volcanism II: The Canary Islands. *In*: Gibbons W, Moreno T, eds. *The Geology of Spain*. London: Geological Society Publishing House. pp 439–472.
- CAUJAPÉ-CASTELLS, J., A. TYE, D. J. CRAWFORD, A. SANTOS-GUERRA, A. SAKAI, K. BEAVER, W. LOBIN, ET AL. 2010. Conservation of oceanic island floras: present & future global challenges. *Perspectives in Plant Ecology, Evolution and Systematics*. 12: 107–129.
- CODY, M. L., AND J. M. OVERTON. 1996. Short-term evolution of reduced dispersal in island plant populations. *Journal of Ecology*. 84: 53–61.
- CRAWFORD, D. J., J. K. ARCHIBALD, D. STOERMER, M. E. MORT, J. K. KELLY, AND A. SANTOS-GUERRA. 2008. A Test of Baker’s Law: Breeding Systems and the Radiation of *Tolpis* (Asteraceae) in the Canary Islands. *International Journal of Plant Sciences*. 169: 782-791.
- CRAWFORD, D., J. ARCHIBALD, J. KELLY, M. MORT., AND A. SANTOS-GUERRA. 2010. Mixed mating in the ‘obligately outcrossing’ *Tolpis* (Asteraceae) of the Canary Islands. *Plant Species Biology*. 25(2): 114-119.
- CRONK, Q. C. B. 1992. Relict floras of Atlantic islands: patterns assessed. *Biological Journal of the Linnean Society*. 46: 91-103.
- DARWIN, C. R. 1859. *On the Origin of Species by Means of Natural Selection: Or the Preservation of Favoured Races in the Struggle for Life*. John Murray, London.
- EMERSON, B. C. 2002. Evolution on oceanic islands: molecular phylogenetic approaches to understanding pattern and process. *Molecular Ecology*. 11: 951-966.

- ENGLER, A. 1879. Versuch einer Eitwicklungsgeschichte, insbesondere der Florenggebiete seit der Tertiärperiode. I. Die extratropischen Gebiete der nördlichen Hemisphäre. Engelmann, Leipzig, Germany.
- FRANCISCO-ORTEGA, J., R. K. JANSEN, AND A. SANTOS-GUERRA. 1996. Chloroplast DNA evidence of colonization, adaptive radiation, and hybridization in the evolution of the Macaronesian flora. *Proceedings of the National Academy of Sciences*. 93: 4085–4090.
- FRANCISCO-ORTEGA, J., D. J. CRAWFORD, A. SANTOS-GUERRA, AND R. K. JANSEN. 1997. Origin and evolution of *Argyranthemum* (Asteraceae: Anthemideae) in Macaronesia. In T. J. Givnish and K. J. Sytsma [eds.], *Molecular evolution and adaptive radiation*, 407–431. Cambridge University Press, Cambridge, UK.
- FRANCISCO-ORTEGA, J., ET AL. 2002. Phylogeny of the Macaronesian endemic *Crambe* section *Dendrocrambe* (Brassicaceae) based on internal transcribed spacer sequences of nuclear ribosomal DNA. *American Journal of Botany*. 89: 1984–1990.
- FRESNILLO, B., AND B. K. EHLERS. 2008. Variation in dispersability among mainland and island populations of three wind dispersed plant species. *Plant Systematics and Evolution*. 270: 243–255.
- FUERTES-AGUILAR, J., J. M. RAY, J. FRANCISCO-ORTEGA, A. SANTOS-GUERRA, AND R. K. JANSEN. 2002. Molecular evidence from chloroplast and nuclear markers for multiple colonizations of *Lavatera* (Malvaceae) in the Canary Islands. *Systematic Botany*. 27: 74–83.
- GIBSON, M. J. S., D. J. CRAWFORD, M. T. HOLDER, M. E. MORT, B. KERBS, M. MENEZES DE SEQUEIRA, AND J. K. KELLY. 2020. Genome-wide genotyping

- estimates mating system parameters and paternity in the island species *Tolpis succulenta*. *American Journal of Botany*. In review.
- GILLESPIE, R. G., B. G. BALDWIN, J. M. WATERS, C. I. FRASER, R. NIKULA, AND G. K. RODERICK. 2012. Long-distance dispersal: a framework for hypothesis testing. *Trends in Ecology and Evolution*. 27: 52–61.
- GIVNISH, T. J., E. KNOX, T. B. PATTERSON, J. R. HAPEMAN, J. D. PALMER, AND K. J. SYTSMA. 1996. The Hawaiian lobelioids are monophyletic and underwent a rapid initial radiation roughly 15 million years ago. *American Journal of Botany*. 83: 159.
- GRUENSTAEUDL, M., A. SANTOS-GUERRA, AND R. JANSEN. 2013. Phylogenetic analyses of *Tolpis* Adans. (Asteraceae) reveal patterns of adaptive radiation, multiple colonization and interspecific hybridization. *Cladistics*. 29(4): 416-434.
- HANSEN, A., AND P. SUNDING. 1993. Flora of Macaronesia: checklist of vascular plants. 4. Revised edition. *Sommerfeltia*. 17: 1–295.
- HARTER, D., S. IRL, B. SEO, M. STEINBAUER, R. GILLESPIE, K. TRIANTIS . . . C. BEIERKUHNLEIN. 2015. Impacts of global climate change on the floras of oceanic islands – Projections, implications and current knowledge. *Perspectives in Plant Ecology, Evolution and Systematics*. 17(2): 160-183.
- HUMPHRIES, C. J. 1979. Endemism and evolution in Macaronesia. *In*: Bramwell D, ed. Plants and Islands. London: Academic Press. pp 171–200.
- JARVIS, C. E. 1980. Systematic studies in the genus *Tolpis* Adanson. Thesis, University of Reading.

- JUAN, C., B. C. EMERSON, P. OROMÍ, AND G. M. HEWITT. 2000. Colonization and diversification: towards a phylogeographic synthesis for the Canary Islands. *Trends in Ecology and Evolution*. 15: 104–109.
- KERBS, B., J. RESSLER, J. K. KELLY, M. E. MORT, A. SANTOS-GUERRA, M. J. S. GIBSON . . . D. J. CRAWFORD. 2017. The Potential Role of Hybridization in Diversification and Speciation in an Insular Plant Lineage: Insights from Synthetic Interspecific Hybrids. *AoB Plants*. 9(5): plx043 doi: 10.1093/oabpla/plx043
- KILIAN, N., B. GEMEINHOLZER, AND H. W. LACK. 2009. Cichorieae. *In*: Funk, V.A., A. Susanna, T. E. Stuessy, R. J. Bayer (Eds.). *Systematics, Evolution and Biogeography of Compositae*. IAPT, Vienna, pp. 343–383.
- KIM, S.-C., D. J. CRAWFORD, J. FRANCISCO-ORTEGA, AND A. SANTOS-GUERRA. 1996. A common origin for woody *Sonchus* and five related genera in the Macaronesian islands: molecular evidence for extensive radiation. *Proceedings of the National Academy of Sciences*. 93: 7743–7748.
- KIM, S.-C., D. J. CRAWFORD, AND R. K. JANSEN. 1996. Phylogenetic relationships among the genera of the subtribe Sonchinae (Asteraceae): evidence from ITS sequences. *Systematic Botany*. 21: 417–432.
- KIM, S., M. MCGOWEN, P. LUBINSKY, J. BARBER, M. MORT, A. SANTOS-GUERRA, AND J. STAJICH. 2008. Timing and Tempo of Early and Successive Adaptive Radiations in Macaronesia (Macaronesian Flora Age). *PLoS ONE*, 3(5): E2139.
- KOSEVA, B., D. CRAWFORD, K. BROWN, M. MORT, AND J. KELLY. 2017. The genetic breakdown of sporophytic self-incompatibility in *Tolpis coronopifolia* (Asteraceae). *New Phytologist*. 216(4): 1256-1267.

- KREFT, H., W. JETZ, J. MUTKE, G. KIER, AND W. BARTHLOTT. 2008. Global diversity of island floras from a macroecological perspective. *Ecology Letters* 11: 116–127.
- LÓPEZ DE HEREDIA, U., R. LÓPEZ,, C. COLLADA, B. EMERSON, AND L. GIL. 2014. Signatures of volcanism and aridity in the evolution of an insular pine (*Pinus canariensis*). *Heredity*. 113(3): 240-9.
- MAYR, E. 1967. The challenge of island faunas. *Australian Natural History* 15: 369–374.
- MEUSEL, H. 1965. Die Reliktvegetation der kanarischen Inseln in ihren Beziehungen zur südund mitteleuropäischen Flora. In M. Gersch [ed.], *Gesammelte Vorträge über moderne Probleme der Abstammungslehre* 1, 117–136. Friedrich-Schiller-Universität, Jena, Germany.
- MOORE, M., J. FRANCISCO-ORTEGA, A. SANTOS-GUERRA, AND R. JANSEN. 2002. Chloroplast DNA evidence for the roles of island colonization and extinction in *Tolpis* (Asteraceae: Lactuceae). *American Journal of Botany*. 89(3): 518-526.
- MACARTHUR, R. H., AND E. O. WILSON. 1967. *The Theory of Island Biogeography*. Princeton University Press, Princeton/Oxford.
- MORT, M. E., D. E. SOLTIS, P. S. SOLTIS, J. FRANCISCO-ORTEGA, AND A. SANTOS-GUERRA. 2002. Phylogenetics and evolution of the Macaronesian clade of Crassulaceae inferred from nuclear and chloroplast sequence data. *Systematic Botany*. 27: 271–288.
- MORT, M., D. CRAWFORD, A. SANTOS-GUERRA, J. FRANCISCO-ORTEGA, E. ESSELMAN, AND A. WOLFE. 2003. Relationships among the Macaronesian members of *Tolpis* (Asteraceae: Lactuceae) based upon analyses of inter simple sequence repeat (ISSR) markers. *Taxon*. 52(3): 511-518.

- MORT, M. E., J. K. ARCHIBALD, C. P. RANDLE, N. D. LEVSEN, T. R. O'LEARY, K. TOPALOY, C. M. WIEGAND AND D. J. CRAWFORD. 2007. Inferring phylogeny at low taxonomic levels: utility of rapidly evolving cpDNA and nuclear ITS loci. *American Journal of Botany*. 94(2): 173-183 doi: 10.3732/ajb.94.2.173
- MORT, M. E., D. J. CRAWFORD, J. K. KELLY, A. SANTOS-GUERRA, M. MENEZES DE SEQUEIRA, M. MOURA, AND J. CAUJAPE-CASTELLS. 2015. Multiplexed-shotgun-genotyping data resolve phylogeny within a very recently derived insular lineage. *American Journal of Botany*. 102: 634–641.
- PANERO, J. L., J. FRANCISCO-ORTEGA, R. K. JANSEN, AND A. SANTOS-GUERRA. 1999. Molecular evidence for multiple origins of woodiness and a New World biogeographic connection of the Macaronesian Island endemic *Pericallis* (Asteraceae: Senecioneae). *Proceedings of the National Academy of Sciences*. 96: 13886–13891.
- PANNELL, J. R. AND M. VOILLEMOT. 2017. Evolution and Ecology of Plant Mating Systems. In: eLS. John Wiley & Sons, Ltd: Chichester. DOI: 10.1002/9780470015902.a0021909.pub2
- PARK, S.-J., E. KOROMPAI, J. FRANCISCO-ORTEGA, A. SANTOS-GUERRA, AND R. JANSEN. 2001. Phylogenetic relationships of *Tolpis* (Asteraceae: Lactuceae) based on ndh F sequence data. *Plant Systematics and Evolution*. 226: 23-33.
- PERCY, D. M., AND Q. C. B. CRONK. 2002. Different fates of island brooms: contrasting evolution in *Adenocarpus*, *Genista*, and *Teline* (Genisteae, Fabaceae) in the Canary Islands and Madeira. *American Journal of Botany*. 89: 854–864.

- PEREIRA, M. J., M. MOURA, G. B. MACIEL, AND J. B. OLIVEIRA. 1998. Conservation of Natural Vegetation in Azores Islands. *Boletim do Museu Municipal do Funchal*. 5: 299-305.
- SANTOS-GUERRA, A. 1999. Origen y evolución de la flora canaria. *In*: Fernández-Palacios, J. M., J. J. Bacallado, J. A. Belmonte, eds. *Ecología y Cultura en Canarias*. Santa Cruz de Tenerife: Organismo Autónomo de Museos y Centros. pp 107–129.
- SJOGREN, E. 1973. Recent changes in the vascular flora of the Azores Islands. *Memórias da Sociedade broteriana*. 22: 1-453.
- SUNDING, P. 1979. Origins of the Macaronesian flora. *In*: Bramwell D. (ed.) *Plants and Islands*. Academic Press, London, pp. 13-40.
- THIV, M., L. STRUWE, AND J. W. KADEREIT. 1999. The phylogenetic relationships and evolution of the Canarian laurel forest endemic *Ixanthus viscosus* (Aiton) Griseb. (Gentianaceae): evidence from *matK* and ITS sequences, and floral morphology and anatomy. *Plant Systematics and Evolution*. 218: 299–317.
- VOURC'H, G., J.-L. MARTIN, P. DUNCAN, J. ESCARRÉ, AND T. P. CLAUSEN. 2001. Defensive adaptations of *Thuja plicata* to ungulate browsing: a comparative study between mainland and island populations. *Oecologia*. 126: 84–93.
- WALLACE, A.R. 1880. *Island Life: Or, the Phenomena and Causes of Insular Faunas and Floras, Including a Revision and Attempted Solution of the Problem of Geological Climates*. Macmillan & Co., London.

CHAPTER TWO

MULTIPLEXED-SHOTGUN-GENOTYPE (MSG) DATA RESOLVE PHYLOGENETIC RELATIONSHIPS WITHIN AND AMONG ARCHIPELAGOS IN MACARONESIAN *TOLPIS* (ASTERACEAE).

Benajmin R. Kerbs, Mark E. Mort, John K. Kelly, Lurdes Borges Silva, Mónica Moura, Miguel Menezes de Sequeira, Arnaldo Santos-Guerra, Hanno Schaefer, J. Alfredo Reyes-Bentancort, Juli Caujapé-Castells and Daniel J. Crawford

Introduction

Despite oceanic islands representing only about 5% of the land surface of the Earth, the plants endemic to islands represent about 25% of the described species of vascular plants (Kreft & al., 2008; Caujapé-Castells & al., 2010), many of the them (8-14%) in danger of extinction (Caujapé-Castells & 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 have attributes especially suitable for evolutionary studies (Mayr 1967): plant populations on islands are often more or less isolated systems of populations 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 as well as across islands of different ages. Lastly, the ages of the islands can be dated, and based on these age estimates it is now generally accepted that most insular lineages are quite young (Baldwin & al., 1998; Knope & al., 2012). The young ages and recency of insular lineages

provide better insights into the factors associated with species divergence as compared to differences that have accumulated subsequent to speciation (Templeton, 1982; Coyne & Orr 2004, p. 57).

A highly resolved and strongly supported phylogenetic hypothesis is a necessary first step in providing a framework for formulating hypotheses for the pattern and process of radiation, diversification and speciation in insular plant lineages. Initially, it was hoped that molecular markers that had been of utility for resolving relationships at the inter-generic level and lower would likewise resolve relationships in island plants. Restriction site mutations and later sequences of various regions in the nuclear and plastid genomes were used for inferring the phylogenies of island lineages (e.g., Baldwin & al., 1998, Mort & al., 2007; Knope & 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 reconstructing relationships of older clades were not sufficiently variable to provide high resolution within recently-radiated, insular lineages. Our previous study (Mort et al., 2015) demonstrated that genomic data can overcome these difficulties, resolving relationships within the Canary Island clade of *Tolpis*, a group that had confounded attempts using other markers. Here, we fully develop this approach with much greater sampling to better formulate hypotheses for the patterns and processes of radiation within this island plant lineage.

Tolpis (Asteraceae) is a small (ca. 15 species), presumably monophyletic flowering plant genus distributed largely in the Macaronesian archipelagos of the Azores, Canary Islands, Madeira and Cape Verde Islands (Fig.1), with two or three taxa in Northern Africa, Southern Europe and the

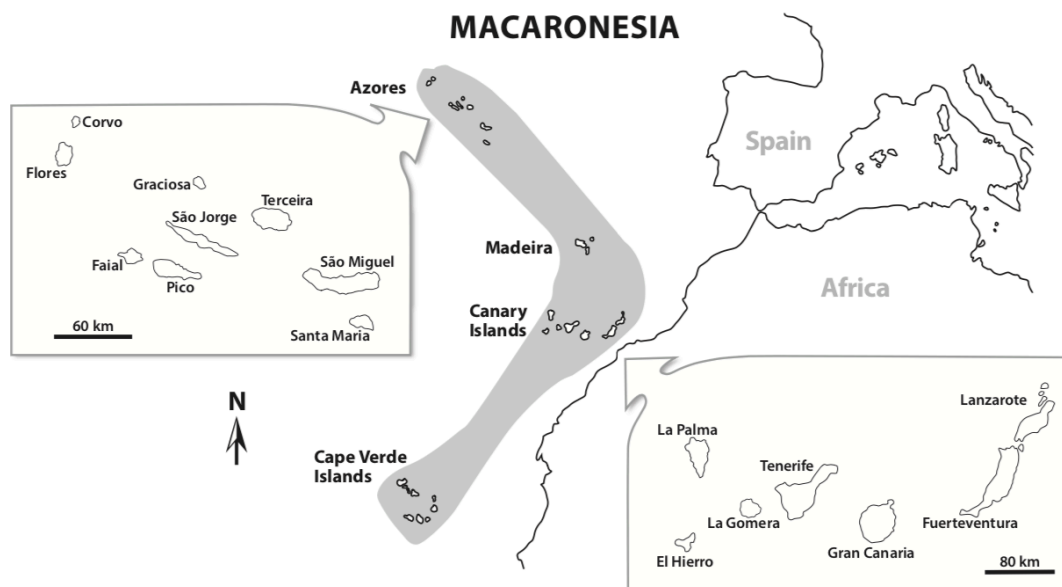


Figure 5. Map of Macaronesia showing the four archipelagos from which samples of *Tolpis* included in the present originated. Insets show the islands of the Azores and Canaries

Mediterranean (Jarvis, 1980). Despite being a relatively small lineage, there is extensive variation in *Tolpis*, including: annuals and perennials; widespread species and ones with narrow distributions and small populations; occurrence in a range of habitats (ecological zones) and a wide array of geological substrates of known age; and breeding/mating systems. Despite the extensive morphological/ecological variation in *Tolpis*, attempts to resolve phylogenetic relationships within the genus utilizing various molecular markers have been largely unsuccessful for inferring relationships within and among archipelagos (Moore & al. 2002; Mort & al. 2003, 2007, 2010; Archibald & al. 2006; Gruenstaeudl & al. 2013, 2017). Although Mort et al (2015) demonstrated the efficiency of MSG genotyping, sampling was limited for other archipelagos, with Cape Verde endemic *Tolpis farinulosa* endemic and the Madeiran endemic *T. macrorhiza* not included. The present investigation considerably expands the taxon and population sampling included in Mort & al. (2015) in order to resolve at a much finer scale the

phylogenetic relationships within and among the Macaronesian archipelagos. For example, Mort & al., (2015) focused primarily on Canarian species of *Tolpis* and thus included only three *Tolpis* populations from Madeira and the Azores. The present study expands the sampling to include 26 populations from those archipelagos. Included among these samples were three populations of the Madeiran endemic *T. macrorhiza*, which was not included by Mort & al. (2015) and whose phylogenetic placement was ambiguously resolved in previous studies (Moore & al., 2002; Gruenstaeudl & al., 2013). Furthermore, sampling was increased from two populations in Mort & al. (2015) to 10 populations of *T. succulenta* from Madeira and the Azores; and from a single population to thirteen populations of the Azorean endemic *T. azorica*. Finally, the single species of *Tolpis* currently recognized in Cape Verde, *T. farinulosa*, is included in the present study. This species was not included in Mort et al. (2015), but a previous study suggested that the colonizing ancestor(s) of *T. farinulosa* dispersed from El Hierro (Gruenstaeudl & al., 2013).

The purposes of this study are to more completely demonstrate MSG data 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) examine patterns of dispersal within and between archipelagoes and infer single island radiations versus inter-island dispersals in divergence and speciation in archipelagos; and (3) discuss the criteria for evaluating taxonomic recognition of groups of populations resolved by MSG data, and the biodiversity/conservation implications of decisions.

Materials and Methods

Taxon Sampling

A combination of fresh and dried tissue was prepared for extraction from a total of 90 *Tolpis* accessions, including 14 individuals from Madeira, 27 from the Azores, 2 from mainland Europe, 45 from the Canary Islands, and 2 individuals of the outgroup species *Arnosseris minima* (Table 1). The analyses of Tremetsberger & al. (2013) that included 42 genera of tribe Cichorieae provided strong support for *A. minima* as sister to *Tolpis*. Our sampling incorporates all currently generally-accepted species of Macaronesian *Tolpis*, as well as those potentially worthy of new taxonomic recognition. Expanded sampling for MSG analysis beyond Mort & al. (2015) includes (1) two populations of the rare species *T. macrorhiza* endemic to Madeira island, (2) two individuals from a single population of the Cape Verde endemic *T. farinulosa*, (3) four populations of *T. succulenta* from Madeira, and (4) 19 populations of Azorean species, including 6 populations of *T. succulenta* and extensive samples of the endemic *T. azorica*, including 13 populations from six of the seven islands on which it is known to occur (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 Qiagen DNEasy Plant MiniKit (Qiagen Inc., Valencia, CA, USA) and extracted samples were subsequently quantified with the Qubit dsDNA high sensitivity assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Sequencing

MSG (Andolfatto & al. 2011) libraries were created for sequencing following Mort & al. (2015). Namely, DNA was cut using the *AseI* restriction enzyme (NEB Biolabs, Ipswich, MA) 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.

Species	Population	Archipelago	Island	Collector, collection number, locality	
<i>Tolpis macrorrhiza</i>	1	Madeira	Madeira	Menezes de Sequeira, 7100, Water-channel to Caldeirão Verde, 500 m from Caldeirão Verde	
	2	Madeira	Madeira	Menezes de Sequeira, 7120, Encumeada, at the beginning of the Folhadal water-channel	
	3	Madeira	Madeira	Menezes de Sequeira, 7120, Encumeada, at the beginning of the Folhadal water-channel	
<i>Tolpis succulenta</i>	3	Madeira	Madeira	Menezes de Sequeira, 7106, Pico do Cidrão. North, exposed rocky walls	
	4	Madeira	Madeira	Menezes de Sequeira, 7106, Pico do Cidrão. North, exposed rocky walls	
	4	Madeira	Madeira	Menezes de Sequeira, 7095, Porto Moniz, near the ocean	
	5	Madeira	Madeira	Menezes de Sequeira, 7095, Porto Moniz, near the ocean	
	6	Madeira	Madeira	Menezes de Sequeira, 7094, on the road from Ribeira do Inferno to Seixal	
	7	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	Moura, MASL, São Lourenço, N 36 59' 34.1", W 25 03' 24.5"	
	9	Azores	Santa Maria	Moura, MAPA, Pico Alto, N 36 58' 56.0", W 25 05' 28.8"	
	10	Azores	Santa Maria	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"	
	11	Azores	São Miguel	Borges Silva, SMPA, Porto da Ajuda, N 37 53' 56.6", W 25 45' 01.7"	
	12	Azores	Graciosa	Moura, GRBL, Baía do Filipe, Beira Mar da Luz, N 39 01' 14.6", W 28 00' 26"	
12	Azores	Graciosa	Moura, GRBL, Baía do Filipe, Beira Mar da Luz, N 39 01' 14.6", W 28 00' 26"		
13	Azores	Graciosa	Moura, GRSC, Santa Cruz, Quitadouro, N 39 04' 37.8", W 27 59' 19.1"		
13	Azores	Graciosa	Moura, GRSC, Santa Cruz, Quitadouro, N 39 04' 37.8", W 27 59' 19.1"		
<i>Tolpis azorica</i>	14	Azores	São Miguel	Borges Silva, SMAZ, Pico da Cruz, 900 m asl	
	14	Azores	São Miguel	Borges Silva, SMAZ, Pico da Cruz, 900 m asl	
	15	Azores	São Miguel	Borges Silva, SMAZ, Pico Bartolomeu, 870 m asl	
	15	Azores	São Miguel	Borges Silva, SMAZ, Pico Bartolomeu, 870 m asl	
	16	Azores	Terceira	F. Pereira, TESB, Serra de Santa Bárbara, N 38 43' 41.1", W 27 19' 36.2"	
	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 asl	
	17	Azores	Terceira	M. Pietrzak, TRAZ, Rocha do Chambre, N 38 44' 49.6", W 27 13' 56.6", 583 m asl	
	18	Azores	São Jorge	SJPV, 1-5-68, Pico Verde, N 38 39' 14.9", W 28 05' 24.7"	
	19	Azores	São Jorge	SJPL, 4-5-6-54	
	20	Azores	São Jorge	SJPE3-67, Pico da Esperança, N 38 39' 11.2", W 28 04' 18.4"	
	21	Azores	Faial	FACA8_70, Caldeira, N 38 34' 49.1", W 28 43' 13.7"	
	22	Azores	Faial	FAAB14_71, Alto do Brejo, N 38 35' 31.3", W 28 43' 48.4"	
	23	Azores	Pico	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"	
	25	Azores	Flores	L. Serpa, Badanela, N 39 28' 10.2, W 31 12' 26.7"	
	<i>Tolpis barbata</i>	27	Continental		Crawford, s. n., commercial source
		28	Continental		Crawford, s. n., commercial source
29		Canary Isl.	Tenerife	Crawford et al., 1849, near Arafo	
<i>Tolpis farinulosa</i>	30	Cape Verde Isl.	Santo Antao	Fernandes, s.n., Santo Antao	
	30	Cape Verde Isl.	Santo Antao	Fernandes, s.n., Santo Antao	
<i>Tolpis proustii</i>	31	Canary Isl.	El Hierro	Crawford et al., 2008, Riscos de Bascos	
	31	Canary Isl.	El Hierro	Crawford et al., 2008, Riscos de Bascos	
<i>Tolpis laciniata</i>	32	Canary Isl.	El Hierro	Crawford et al., 1855, Cruz de Reyes, above Tabano, ca. 1300 asl	
	32	Canary Isl.	El Hierro	Crawford et al., 1855, Cruz de Reyes, above Tabano, ca. 1300 asl	
	33	Canary Isl.	La Gomera	Crawford et al., 2048, N 28 10' 08.4", W 17 16' 39.0"	
	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	
<i>Tolpis glabrescens</i>	36	Canary Isl.	Tenerife	Crawford et al., 1877, Anaga, Chinobre	
	37	Canary Isl.	Tenerife	R. Mesa, s.n., Anaga, Roque de Enmedio	
<i>Tolpis lagopoda</i>	38	Canary Isl.	Gran Canaria	Mort and Santos, s.n., near Artenara	
	38	Canary Isl.	Gran Canaria	Mort and Santos, s.n., near Artenara	
	38	Canary Isl.	Gran Canaria	Mort and Santos, s.n., near Artenara	
	38	Canary Isl.	Gran Canaria	Mort and Santos, s.n., near Artenara	
	39	Canary Isl.	Tenerife	Santos-Guerra, 09, near Zapatito de la Reina	
<i>Tolpis webii</i>	39	Canary Isl.	Tenerife	Santos-Guerra, 09, near Zapatito de la Reina	
	40	Canary Isl.	Tenerife	Santos-Guerra, 06, above Vilaflor	
	41	Canary Isl.	Tenerife	Crawford et al., 1941, above Esperanza, 1250 m asl	
	41	Canary Isl.	Tenerife	Crawford et al., 1941, above Esperanza, 1250 m asl	
	42	Canary Isl.	Tenerife	Santos-Guerra, 13, Masca	
	42	Canary Isl.	Tenerife	Santos-Guerra, 13, Masca	
	43	Canary Isl.	Tenerife	Crawford et al., 1949, near Mirador Ayosa	
	44	Canary Isl.	Tenerife	Crawford et al., 1949, near Mirador Ayosa	
<i>Tolpis crassiuscula</i>	45	Canary Isl.	Tenerife	Santos-Guerra, 12, Ayosa	
<i>Tolpis lagopoda</i>	46	Canary Isl.	Tenerife	Crawford et al., 1993, Teno, tunnel W of El Fraile	
	46	Canary Isl.	Tenerife	Crawford et al., 1987, Barranco del Infierno	
<i>Tolpis santosii</i>	46	Canary Isl.	Tenerife	Crawford et al., 1987, Barranco del Infierno	
	47	Canary Isl.	Tenerife	Crawford et al., 1975, Barranca Seca	
	47	Canary Isl.	Tenerife	Crawford et al., 1975, Barranca Seca	
	48	Canary Isl.	La Palma	Crawford et al., 2009, Playa de Nogales	
	48	Canary Isl.	La Palma	Crawford et al., 2009, Playa de Nogales	
<i>Tolpis calderae</i>	49	Canary Isl.	La Palma	Crawford et al., 1906, Caldera de Taburiente, near river	
	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	
<i>Tolpis laciniata</i>	50	Canary Isl.	La Palma	Crawford et al., 1887, Mirador la Cumbrecita	
	51	Canary Isl.	La Palma	Crawford et al., 1886, just S of Jedey	
	52	Canary Isl.	La Palma	Crawford et al., 1883, La Caldera	
<i>Tolpis coronopifolia</i>	52	Canary Isl.	La Palma	Crawford et al., 1883, La Caldeta	
	51	Canary Isl.	La Palma	Crawford et al., 1886, just S of Jedey	
	53	Canary Isl.	Tenerife	Crawford et al., 1834, El Guancha	
	53	Canary Isl.	Tenerife	Crawford et al., 1834, El Guancha	
	54	Canary Isl.	Tenerife	Santos-Guerra, 05, Arafo	
<i>Arnosseris minima</i>	54	Canary Isl.	Tenerife	Santos-Guerra, 05, Arafo	
	55	Spain		Montes-Moreno & Nualart, 668, Burgos: Merindad de Valdeporres, Robredo de las Puebas	
	56	Austria		Pachschwöll & Grünstäudl, 7559/3, N 48 24' 34.1", E 15 31' 49.9, 545m asl	

Table 2. Table of *Tolpis* samples used in the present study, along with their population numbers, archipelago, island, locality information, and collection number

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 & al., 2014), with parameters ILLUMINACLIP: TruSeq3-SE, LEADING: 3, TRAILING: 3, SLIDINGWINDOW 4:15, MINLEN: 36. Samples were then quality checked in FastQC (Babraham Bioinformatics, Cambridge) 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 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 v1.4.4 (<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.

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, *T. farinulosa* (Cape Verde), and the two populations of *T. barbata* from both the Canaries and the continent (Fig. 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 (Fig. 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.

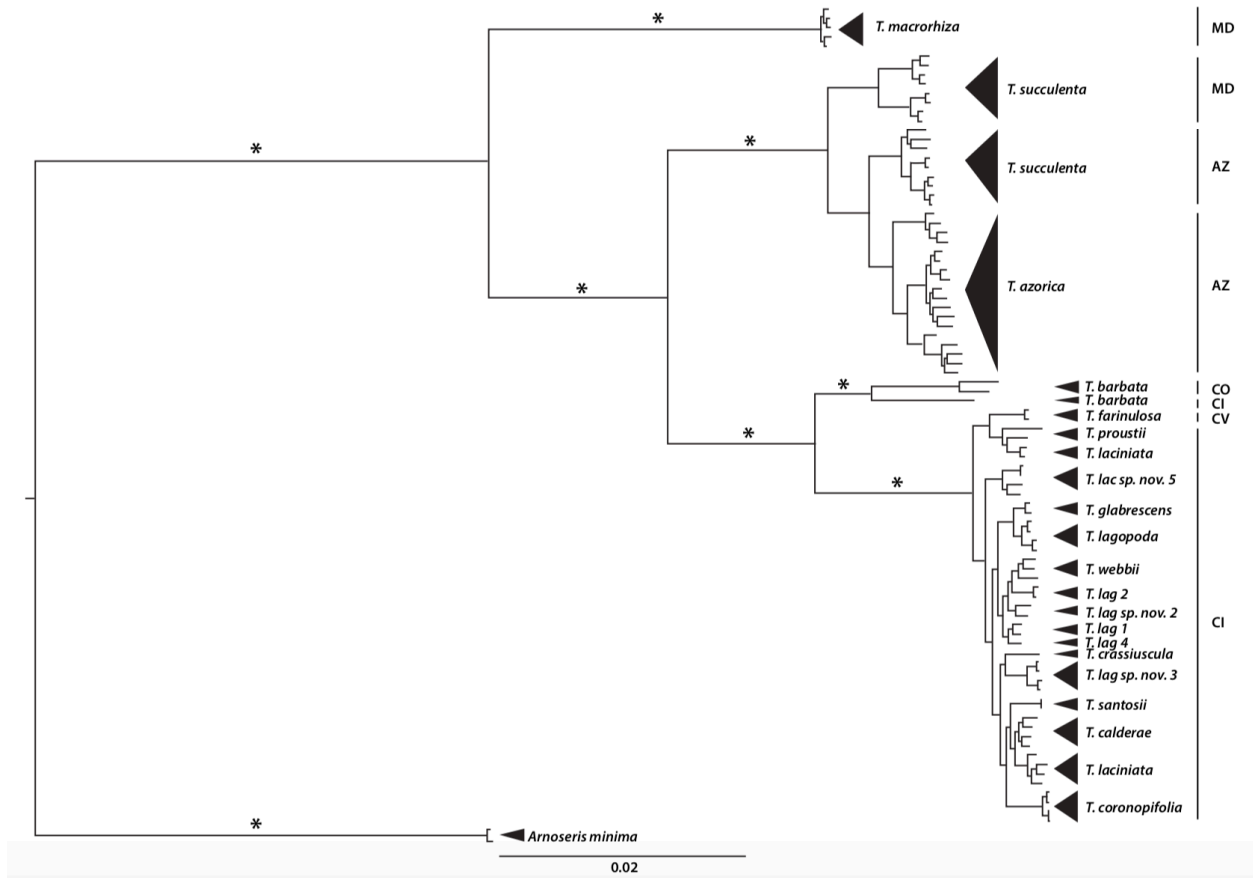


Figure 6. Maximum likelihood (ML) topology derived from analyses of multiplexed shotgun genotyping (MSG) data for *Tolpis* in Macaronesia. Clades with higher than 95% or higher ML bootstrap are indicated by asterisks. AZ, Azores; CI, Canary Islands; CV, Cape Verde; MD, Madeira; CO, continent

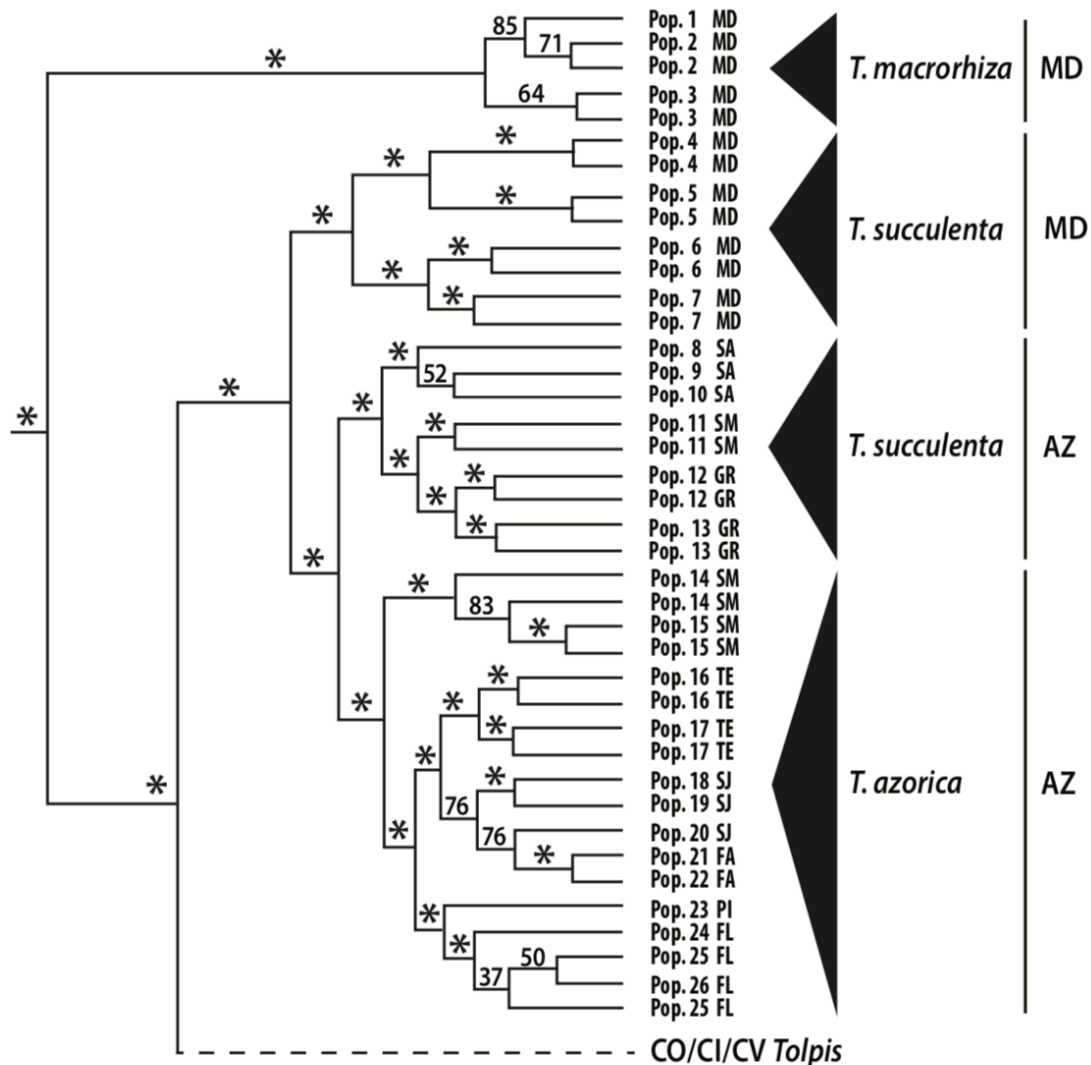


Figure 7. ML topology from MSG data for *Tolpis* in Azores and Madeira. ML bootstrap support indicated for clades with less than 95%, otherwise denoted by asterisk. Population numbers same as in Table 1. Island designations for Madeira (MD) and for islands in Azores (SA, Santa Maria; SM, São Miguel; GR, Graciosa; TE, Terceira; SJ, São Jorge; FA, Faial; PI, Pico; FL, Flores)

Even with increased taxon sampling, the patterns of relationships and levels of resolution/support within the largely Canary Island clade are consistent with Mort & al. (2015). *Tolpis barbata* (not sampled by Mort & al., 2015) is well-supported and sister to the remainder of taxa within the clade (Fig. 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 & 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 & al., 2009; Mort & al., 2015) are in turn sister to populations from the islands of La Palma, Tenerife and Gran Canaria (Fig. 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 *T. lagopoda* species complexes albeit with the recognition that it is a challenge to identify diagnostic characters for taxa within the complex (Jarvis, 1980; Crawford & 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 & 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*) considered worthy of taxonomic recognition (Archibald & al. 2006; Crawford & al. 2009; Mort & al. 2015).

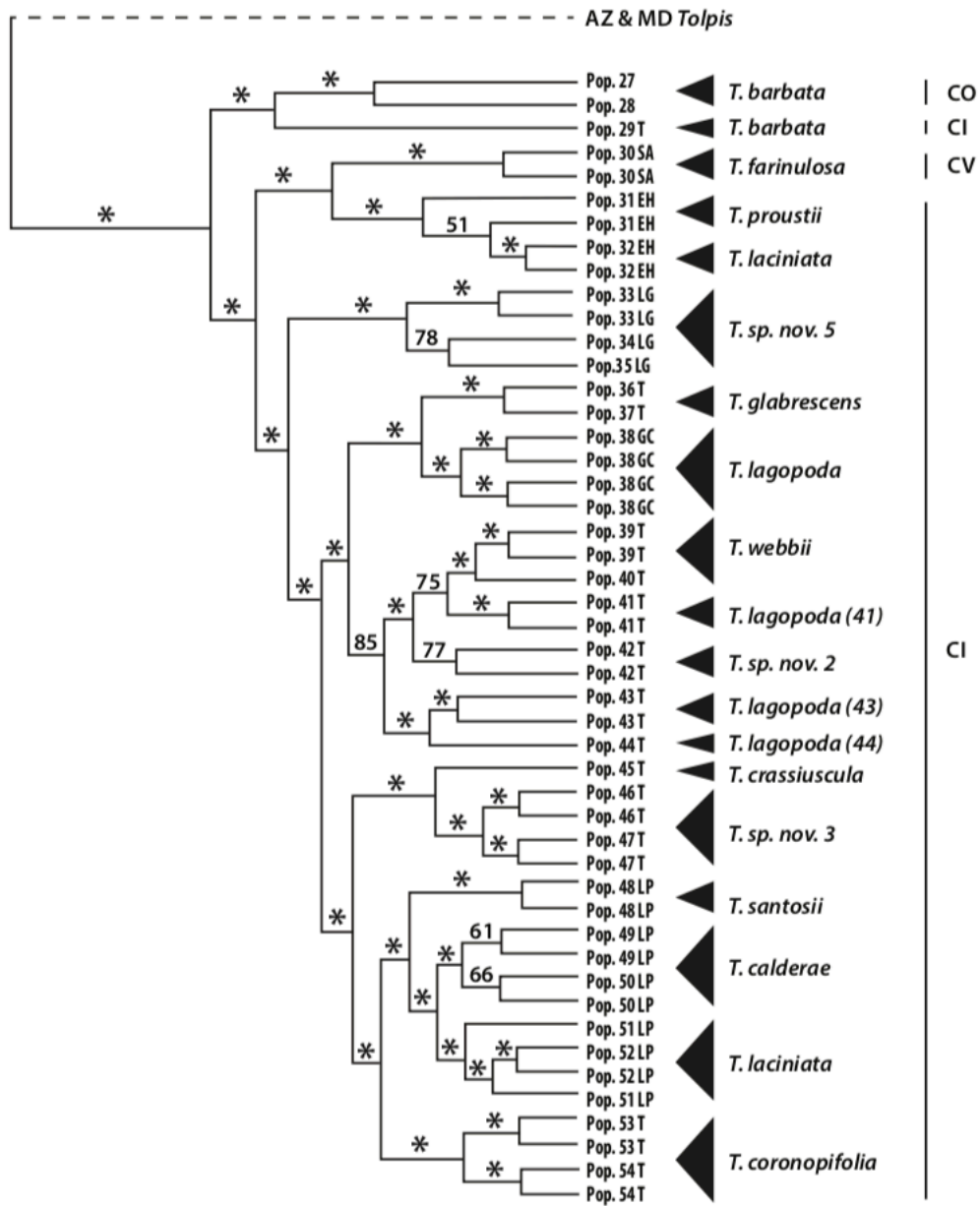


Figure 8. ML topology from MSG data for *Tolpis* in the continent and Canary Islands. ML bootstrap support indicated for clades with less than 95%, otherwise denoted by asterisk. Population numbers same as in Table 1. Island designations for Cape Verde Islands: SA, Santo Antão; and for Canary Islands: T, Tenerife; EH, El Hierro; LG, La Gomera; GC, Gran Canaria; LP, La Palma.

Discussion

Mort & al. (2015) reviewed the battery of “standard” markers that proved largely ineffective in phylogenetic studies of *Tolpis* despite having been widely successful in resolving relationships at the generic and lower levels in flowering plants. These included restriction site mutations (Moore & al., 2002), sequences of plastid DNA (Mort & al., 2007), and sequences of the internal and external transcribed spacer regions (ITS and ETS) of nuclear ribosomal DNA (Gruenstaeudl & al., 2013). This same array of markers was often equally ineffective in resolving relationships in other island lineages, with the lack of resolution generally been attributed to the young age and rapid radiations within the lineages (Baldwin & 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 & al. (2015) resolved relationships within the Canary Island clade of *Tolpis*, and provided greater resolution among the archipelagos. Likewise, Curto & 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.

One of the major insights provided by the present study is the Madeiran endemic *T. macrorhiza* resolved as sister to the remainder of *Tolpis*. Chloroplast DNA restriction site mutations placed *T. macrorhiza* in a relatively basal position; *T. succulenta* from Madeira was sister to the rest of the genus, with *T. macrorhiza* in turn sister to all other species (Moore & al., 2002). Sequences of external transcribed spacer regions of nrDNA ETS failed to resolve its position as it formed a basal polytomy with several other clades (Gruenstaeudl & al., 2013). Gruenstaeudl & al. (2017)

using ETS sequences and sequences of two low copy nuclear loci, placed *Tolpis 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 & al., 1953; Moreno Cameno, 1979; Jarvis, 1980; Delgaard, 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 & al., 2015). Synthetic F₁ hybrids between this species and *T. succulenta* on Madeira have low pollen fertility (mean 26% for 12 progeny from three different crosses (Crawford & al., 2016), suggesting strong post zygotic isolating factors between the two species.

The much greater sampling within Madeira and the Azores compared to prior studies has revealed resolution at the species and population levels. In addition to the aforementioned *Tolpis* *T. macrorhiza*, *T. succulenta* occurs on Madeira. Note that *T. succulenta* as now recognized also occurs in the Azores. Previous studies have suggested that *T. succulenta*, as currently circumscribed, was likely not monophyletic (Gruenstaeudl & al., 2013; Mort & al., 2015) and Borges Silva & 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 & 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 (Fig. 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 (Crawford & al., unpubl.). *Tolpis succulenta* occurs on all islands of the Azores, but with the exception of Santa Maria Island, it is quite rare (Schaefer, 2005; Jarvis, 1980). The Santa Maria and Graciosa populations form a strongly supported clade which is sister to the three São Miguel populations (Fig. 3). Crawford & al. (2019) showed that the Graciosa populations are highly supported as monophyletic and are highly selfing whereas other populations of *T. succulenta* are self-incompatible. The Graciosa populations differ from SI *T. succulenta* in the Azores by features of the capitula and florets (Crawford & al., 2019), and are perhaps worthy of taxonomic recognition. An extensive morphological study of Azorean *T. succulenta* is now in progress (L. Borges Silva & M. Moura, unpubl.).

The relationships among populations of *T. azorica* from six islands (no samples from Corvo island) are largely concordant with genetic structure of microsatellite loci elucidated by Borges Silva & 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 coordinates analysis (Borges Silva & al., 2016), and the two populations from this island are sister to other populations of *T. azorica* (Fig. 3). Populations from the central islands of the Azores (Faial, Pico,

São Jorge, and Terceira; Fig. 1) grouped strongly with the microsatellite data (Borges Silva & al., 2016). Similar relationships were resolved here with populations from Faial, São Jorge, and Terceira forming a strongly supported group (Fig. 3). The single sample of Pico included herein groups with the western island of Flores instead of with the central group; no samples from the other western island of Corvo were available in the present study.

Tolpis populations from the Canary Islands, Cape Verde and one accession of *T. barbata* from the Canaries and two continental samples form the last large clade (Fig. 4). Unlike the Azores and Madeira where species are few in number, generally easily recognized morphologically and populations of the same species (with the notable exception of *T. succulenta*) are monophyletic, the situation is generally more complex on the Canaries. 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* (Fig. 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 or not this species is monophyletic; however, other molecular markers (Archibald & al., 2006; Crawford & al., 2006) show this species as 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* or *T. lagopada* (Jarvis, 1980; Bramwell & Bramwell, 2001), with some 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. lagopada* 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. lagopada* 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. lagopada*. Jarvis (1980) considered most populations on La Palma to be *T. laciniata* with *T. lagopada* very rare; on Tenerife, the opposite was true with *T. lagopada* more common than *T. laciniata*. More recently, a similar geographic distribution has been suggested for the two species (A. Santos-Guerra, unpubl., cited in Crawford & al., 2009), with *T. laciniata* the only species occurring on La Gomera and El Hierro as well as at lower elevations on La Palma with *T. lagopada* much rarer and found at higher elevations on this island. The islands of Tenerife and Gran Canaria are viewed by A. Santos-Guerra (unpubl) as containing only *T. lagopada*. The present results further support the results of Gruenstaeudl & al. (2013) and Mort & al. (2015) in showing that populations of neither *Tolpis laciniata* nor *T. lagopada* as traditionally recognized are monophyletic. (Fig. 4). 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 comprises *T. laciniata*, a variant of *T. laciniata* referred to as *T. proustii*, and the Cape Verde endemic *T. farinulosa*. Mort & al. (2015) included *T. proustii*, but unfortunately, did not sample *T. laciniata* from El Hierro or *T. farinulosa* from Cape Verde. The present study, like Gruenstaeudl & al. (2013) resolves *T. laciniata* and suggests a close relationship to this taxon and the highly morphologically similar *T. proustii* (Fig. 4). *Tolpis proustii* is sometimes, but not always, recognized as a distinct species (Jarvis, 1980 versus

Bramwell & 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 current 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. Available results suggest 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) (Garcia-Maroto et al., 2009; Kim & al., 2008).

As first shown by Mort & al. (2015), populations of *T. laciniata* from La Gomera group strongly as sister to all remaining populations in the Canaries (Fig. 4). Based on field observations over several decades, A. Santos-Guerra (unpubl. cited in Gruenstaeudl & al., 2013, p. 15) recognized these plants as distinct morphologically (“a fleshy habit of up to 1.5 m, strongly pinnatifid, pubescent rosette leaves and a ring of barbed involucral bracts”) from other Canary island *Tolpis*. The present study increased the sampling of Gomeran *T. laciniata* compared to Mort & al. (2015) and again provides strong support for the three populations included. Based on the present phylogenetic results as well as the distinctive morphology, we as others have done (Crawford & al., 2009; Mort & al. (2015), designate this taxon as *T. sp nov.* 5. 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 A. Santos-Guerra (unpubl. data) assign all populations on Gran Canaria to *T. lagopada*. Gruenstaeudl & 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 & al. (2015). The four samples from one population included in this study group strongly and receive strong support in a clade with the rare tetraploid species *T. glabrescens* from Tenerife (Fig. 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 La Gomera + *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, 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, unpubl. data), 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. *Tolpis sp. nov. 2* is present only in a restricted geographic region near Barranco de Masca on the paleo-island of Teno; plants from near the village of Masca are quite distinctive morphologically in that they possess 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 unpubl. data; cited in Archibald & al., 2006; Crawford & al., 2006; Gruenstaeudl & al., 2013); 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 (Fig. 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* (Archibald & al., 2006; Crawford & al., 2009; Mort & 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 is of local occurrence 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 SC, highly-selfing species *T. coronopifolia* from Tenerife sister to the La Palma populations (Fig. 4). The recently-described *T. 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, unpubl., cited in Crawford & al., 2009) were included in this study, precluding the resolution of relationships among the populations referable to the three species on the island.

Summary

The present study further demonstrates 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 Canary and Cape Verde Islands. Resolution at the population level, when combined with morphological, geological, biosystematic and distributional data, identify lineages worthy of consideration for taxonomic recognition, and are informative for the conservation of genetic diversity in the archipelagos.

ACKNOWLEDGMENTS

We thank the following for supplying material of *Arnosseris minima*: N. Montes-Moreno and N. Nualart for collection from Spain, with A. Susanna facilitating the collection, and M. Gruenstaeudl, and C. Pachschwöll for supplying samples from Austria. M. Romeiras kindly made available material of *T. farinulosa* from the Cape Verde Islands. We also thank the staff of the Natural Island Parks of Santa Maria, São Jorge, Graciosa and Flores, the staff of Faial Botanical Garden and F. Pereira, for collecting *T. azorica* seeds.

References

- ANDOLFATTO, P., D. DAVISON, D. EREZYILMAZ, T. T. HU, J. MAST, T. SUNAYAMA-MORITA, AND D. L. STERN. 2011. Multiplexed shotgun genotyping for rapid and efficient genetic mapping. *Genome Res.* 21: 610 – 617.
- ARCHIBALD, J.K., D. J. CRAWFORD, A. SANTOS-GUERRA AND M. E. MORT. 2006. The utility of automated analysis of inter-simple sequence repeat (ISSR) loci for resolving relationships in the Canary Island species of *Tolpis* (Asteraceae). *Amer. J. Bot.* 93: 1154–1162.
- BALDWIN, B.G., D. J. CRAWFORD, J. FRANCISCO-ORTEGA, S.-C. KIM, T. SANG, AND T. F. STUESSY. 1998. Molecular phylogenetic insights on the origin and evolution of oceanic island plants. Pp. 410-444 *In*: Soltis, D., P. Soltis, and J. J. Doyle (eds.), *Molecular Systematics of Plants II. DNA Sequencing* New York: Kluwer Academic Publishers.
- BÖHLE, U.-R., H. H. HILGER, AND W. F. MARTIN. 1996. Island colonization and evolution of the insular woody habit in *Echium* L. (Boraginaceae). *Proc. Natl. Acad. Sci.* 93: 11740-11745.
- BOLGER, A.M., M. LOHSE, AND B. USADEL. 2014. Trimmomatic: A flexible trimmer for Illumina Sequence Data. *Bioinformatics*, btu170
- BORGES SILVA, L., J. SARDOS, M. MENEZES DE SEQUEIRA, L. SILVA, D. J. CRAWFORD, AND M. MOURA. 2016. Understanding intra and inter-archipelago population genetic patterns within a recently evolved insular endemic lineage. *Pl. Syst. Evol.* 302: 367–384.

- BRAMWELL, D., AND Z. BRAMWELL. 2001. Wild flowers of the Canary Islands, 2nd ed. Madrid: Editorial Rueda.
- CAUJAPÉ-CASTELLS, J. A. TYE, D. J. CRAWFORD, A. SANTOS-GUERRA, A. SAKAI, K. BEAVER, W. LOBIN, F. B. VINCENT FLORENS, M. MOURA, R. JARDIM, I. GÓMES, AND C. KUEFFER. 2010. Conservation of oceanic island floras: Present & future global challenges. *Pers. Pl. Ecol. Evol. Syst.* 12: 107–129.
- COYNE, J.A., AND H. A. ORR. 2004. *Speciation*. Sunderland, Massachusetts: Sinauer.
- CRAWFORD, D. J., J. K. ARCHIBALD, M. E. MORT, AND A. SANTOS-GUERRA. 2006. Allozyme variation within and divergence among species of *Tolpis* (Asteraceae-Lactuceae) in the Canary Islands: Systematic, evolutionary and biogeographical implications. *Amer. Jour. Bot.* 93: 656–664.
- CRAWFORD, D.J., J. K. ARCHIBALD, D. STOERMER, M. E. MORT, J. K. KELLY, AND A. SANTOS-GUERRA. 2008. A test of Baker's law: the radiation of *Tolpis* (Asteraceae) in the Canary Islands. *Inter. Jour. Plant Sci.* 169: 782–791.
- CRAWFORD, D. J., J. K. ARCHIBALD, M. E. MORT, AND A. SANTOS-GUERRA. 2009. Pollen fertility of synthetic intra- and interspecific F₁ hybrids in Canary Island *Tolpis* (Asteraceae). *Pl. Syst. Evol.* 279: 103–113.
- CRAWFORD, D. J., M. E. MORT, AND J. K. ARCHIBALD. 2013. *Tolpis santosii* (Asteraceae: Cichorieae), a new species from La Palma, The Canary Islands. *Vierea* 4: 163–169.
- CRAWFORD, D. J., G. J. ANDERSON, L. BORGES SILVA, M. MENEZES DE SEQUEIRA, M. MOURA, A. SANTOS-GUERRA, J.K. KELLY, AND M. E. MORT. 2015. Breeding

- systems in *Tolpis* (Asteraceae) in the Macaronesian islands: the Azores, Madeira and the Canaries. *Pl. Syst. Evol.* 301: 1981–1993.
- CRAWFORD, D. J., D. P. HAUBER, L. BORGES SILVA, M. MENEZES DE SEQUEIRA, M. MOURA, A. SANTOS-GUERRA, J. K. KELLY, AND M. E. MORT. 2016. Breeding relationships in Macaronesian *Tolpis* (Asteraceae-Cichorieae): F1 hybrid pollen fertility within and among populations from the Azores, Canary Islands, and Madeira. *Syst. Bot.* 41: 881–893.
- CRAWFORD, D.J., M. MOURA, L. BORGES SILVA, M. E. MORT, B. KERBS, H. SCHAEFER, AND J. K. KELLY. 2019. The transition to selfing in Azorean *Tolpis* (Asteraceae). *Pl. Syst. Evol.* 305 (4): 305–317. doi:10.1007/s00606-019-01573-7
- CURTO, M., C. SCHACHTLER, P. PUPPO, AND H. MEIMBERG. 2018. Using a new RAD-sequencing approach to study the evolution of *Micromeria* in the Canary Islands. *Mol. Phyl. Evol.* 119: 160–169.
- DALGAARD, V. 1986. Chromosome numbers in flowering plants from Madeira. *Willdenowia* 16: 221–240.
- EATON, D.A. 2014. PyRAD: assembly of de novo RADseq loci for phylogenetic analyses. *Bioinformatics* 30: 1844–1849.
- GARCÍA-MAROTO, F., A. MAÑAS-FERNÁNDEZ, J. A. GARRIDO-CÁRDENAS, D. L. ALONSO, J. L. GUIL-GUERRERO, B. GUZMÁN, AND P. VARGAS. 2009. $\Delta 6$ -Desaturase sequence evidence for explosive Pliocene radiations within the adaptive radiation of Macaronesian *Echium* (Boraginaceae), *Mol. Phyl. Evol.* 52: 563-574.

- GRUENSTAEUDL, M., A. SANTOS-GUERRA, AND R. K. JANSEN. 2013. Phylogenetic analyses of *Tolpis* Adans. (Asteraceae) reveal patterns of adaptive radiation, multiple colonization and interspecific hybridization. *Cladistics* 29: 416–434.
- GRUENSTAEUDL, M., B. C. CARSTENS, A. SANTOS-GUERRA, AND R. K. JANSEN. 2017. Statistical hybrid detection and the inference of ancestral distribution areas in *Tolpis* (Asteraceae). *Biol. J. Linn. Soc.* 121: 133–149.
- JARVIS, C. E. 1980. Systematic studies in the genus *Tolpis* Adanson. Dissertation, Reading: University of Reading.
- KIM S.-C., M. R. MCGOWEN, P. LUBINSKY, J. C. BARBER, M. E. MORT, AND A. SANTOS-GUERRA. 2008. Timing and Tempo of Early and Successive Adaptive Radiations in Macaronesia. *PLoS ONE* 3(5): e2139. doi:10.1371/journal.pone.0002139
- KNOPE, M, C. W. MORDEN, V. A. FUNK, AND T. FUKAMI. 2012. Area and the rapid radiation of Hawaiian *Bidens* (Asteraceae). *J. Biogeo.* 39: 1206–1216. doi.org:10.1111/j.1365-2699.2012.02687.
- KOSEVA, B., D. J. CRAWFORD, K. BROWN, M. E. MORT, AND J. K. KELLY. 2017. The genetic breakdown of self-incompatibility in *Tolpis coronopifolia* (Asteraceae). *New Phyt.* doi: 10.1111/nph.14759
- KREFT, H., W. JETZ, J. MUTKE, G. KIER, AND W. BARTHLOTT. 2008. Global diversity of island floras from a macroecological perspective. *Ecol. Letters* 11: 116–127.
- MAYR, E. 1967. The challenge of island faunas. *Aust. Nat. Hist.* 15: 369–374.
- MOORE, M. J., J. FRANCISCO-ORTEGA, A. SANTOS-GUERRA, AND R. K. JANSEN. 2002. Chloroplast DNA evidence for the roles of island colonization and extinction in *Tolpis* (Asteraceae: Lactuceae). *Amer. J. Bot.* 89: 51–526.

- MORENO CAMENO, M. A. 1979. Números de cromosomas de especies del genero *Tolpis* Adans. en Macaronesia. *Bot. Macaron. 5*: 61–65.
- MORT, M. E., J. K. ARCHIBALD, C. P. RANDLE, N. D. LEVSEN, T. R. O. O'LEARY, K. TOPALOV, C. M. WIEGAND, AND D. J. CRAWFORD. 2007. Utility of rapidly evolving loci for inferring phylogeny at low taxonomic levels. *Amer. J. Bot.* 94: 73–183.
- MORT, M.E., D. J. CRAWFORD, J. K. ARCHIBALD, T. R. O. O'LEARY, AND A. SANTOS-GUERRA. 2010. Plant DNA barcoding: A test using Macaronesian taxa of *Tolpis* (Asteraceae). *Taxon* 59: 581–587.
- MORT, M.E., D. J. CRAWFORD, A. SANTOS-GUERRA, J. FRANCISCO-ORTEGA, E. J. ESSELMAN, AND A. D. WOLFE. 2003. Relationships among the Macaronesian members of *Tolpis* (Asteraceae: Lactuceae) based upon the analyses of inter simple sequence repeat (ISSR) markers. *Taxon* 52: 511–518.
- MORT, M.E., D. J. CRAWFORD, J. K. KELLY, A. SANTOS-GUERRA, M. MENEZES DE SEQUEIRA, M. MOURA, AND J. CAUJAPE-CASTELLS. 2015. Multiplexed-shotgun-genotyping data resolve phylogeny within a very recently derived insular lineage. *Amer. J. Bot.* 102: 634–641.
- SCHAEFER, H. 2005. Flora of the Azores-a field guide, 2nd ed. Wilkersheim: Margraf Publishers.
- SOTO-TREJO, F., J. K. KELLY, J. K. ARCHIBALD, M. E. MORT, A. SANTOS-GUERRA, AND D. J. CRAWFORD. 2013. The genetics of self-compatibility and associated floral characters in *Tolpis* (Asteraceae) in the Canary Islands. *Int. Jour. Plant Sci.* 174: 171–178.

- STEBBINS, G. L., R. A. JENKINS, AND M. S. WALTERS. 1953. Chromosomes and phylogeny in the Compositae, tribe Cichorieae. *Univ. Calif. Publ. Bot.* 26: 401–430.
- TEMPLETON, A. R. 1982. Genetic architecture of speciation. Pp. 105-121 in: Baigozzi, C. (ed.), *Mechanisms of speciation*. New York: Alan R. Liss.
- TREMETSBERGER, K., B. GEMEINHOLZER, H. ZETZSCHE, S. BLACKMORE, N. KILIAN, AND S. TALAVERA. 2013. Divergence time estimation in Cichorieae (Asteraceae) using a fossil-calibrated relaxed molecular clock. *Org. Divers. Evol.* 13: 1–13.

CHAPTER THREE

MATING SYSTEM CONSEQUENCES OF THE RECENT ESTABLISHMENT OF SELF-COMPATIBILITY IN TWO SMALL POPULATIONS ON AN OCEANIC ISLAND: *TOLPIS SUCCULENTA* IN THE AZORES

Benjamin R. Kerbs, Daniel J. Crawford, John K. Kelly, Mark E. Mort

Introduction

The transition to selfing is one of the most common trends in flowering plants (Stebbins 1957; Iqic et al., 2008; Barrett 2013; Wright et al., 2013; Grossenbacher et al., 2017). The loss of a functional self-incompatibility locus (SI) that permits self-fertilization (SC) is a critical transition in the reproductive biology of a plant population. Self-incompatible plants are expected to be outcrossing. The outcrossing rate of SC plants may be variable depending on a variety of factors such as floral morphology, presence and behavior of biotic pollinators, the ability to self-pollinate and inbreeding depression (Pannell 2015). The loss of a functional S-locus is unidirectional (Iqic et al., 2008; Barrett, 2013; Wright et al., 2013; Grossenbacher et al., 2017) within/among populations of a species (Herman and Schoen, 2016; Layman et al., 2017) or in different congeneric species (Goodwillie, 1999; Beck et al., 2006; Urban and Bailey, 2013). The transition to selfing may be associated with the evolution of a suite of primarily floral characters (Ornduff 1969; Slotte et al., 2012; Cutter 2019). The so-called selfing syndrome in plants may include the reduction in number and size of floral parts, and the reduction of floral rewards for visitors (Kalisz et al., 2011; Fornoni et al., 2015).

When an SC mutant is introduced into an SI population, several factors may select for or against its establishment. Selfing is favored when there is lack of compatible mates or pollinators

because it provides reproductive assurance (Young and Pickup 2010; Busch and Delph 2012). The automatic transmission hypothesis favors selfing because pollen of a selfer may contribute two copies of their genome to seed production, one from selfing and one from outcrossing, whereas pollen of an SI plant contributes only to outcrossed seed (Pannell and Voilemot 2017). The automatic transmission hypothesis applies if selfing does not cause pollen discounting (reduced pollen for outcrossing) or seed discounting (reduced number of ovules available for outcrossing). Inbreeding depression is the major selective force opposing the evolution of selfing (Pannell 2015). Although inbreeding depression may initially be strong following the loss of SI, inbreeding depression may (or may not) be purged from the population with selfing (Baldwin and Schoen 2019).

Recent transitions to selfing in natural populations and the experimental introduction of SC plants into SI populations are valuable systems for assessing how mating system is affected and the selective factors involved subsequent to the loss of a functional S-locus. Empirical data on how recent transitions to SC shape mating system are limited, ostensibly due to difficulty of detecting transitions in nature. Available results vary, with SI populations of the perennial herb *Linaria cavanillesii* (Plantaginaceae) expressing high inbreeding depression, whereas inbreeding depression had been purged in SC populations (Voilemot and Pannell 2017a). However, the SC populations had a mixed mating system despite the absence of inbreeding depression and the presence of autonomous selfing (Voilemot and Pannell 2017a, b). Voilemot et al. (2019) introduced SC individuals of *Linaria cavanillesii* into SI populations of the same species and found a rapid spread of SC within the populations, driven by higher seed set and greater outcrossing. By contrast, the experimental results of Layman et al. (2017) for *Leavenworthia*

alabamica indicated that, despite the advantages of higher seed set in SC plants, seed discounting and inbreeding depression ostensibly prevent the establishment of SC mutations within populations and result in the persistence of SI populations. Studies suggest that mating system responses vary following recent transitions to SC, and additional studies are to be desired.

The major purpose of the present study was to determine the mating system (mean outcrossing rate) in two populations where SC has evolved recently. Most importantly, the application of genomic data and a Bayesian inference method (BORICE) (Colicchio et al., 2020; Gibson et al. in press) allow not only overall outcrossing rates in the populations, but also whether individual progeny of maternal plants are selfed or outcrossed. Additionally, we wished to determine the number of sires for maternal plants in each population, and whether maternal plants are selfed or outcrossed.

Tolpis succulenta (Asteraceae) consists of perennials that become woody at the base; the species as now recognized occurs in the Azores and Madeira (Jarvis 1980). In the Azorean archipelago, the species is known from very few populations in rocky areas and coastal cliffs on several islands (Jarvis 1980; Schaefer 2005). Molecular phylogenetic studies (Mort et al., 2015; Crawford et al., 2019; Mort et al. unpubl. data) have shown that Azorean *T. succulenta* and populations from Madeira occur in distinct lineages. The Azorean populations should be described as a new species, but will herein be called *T. succulenta*. The Azorean populations have evolved in the Azores from a common ancestor of the endemic *Tolpis azorica* and *T. succulenta* (Mort et al., 2015; Crawford et al., 2019). *Tolpis succulenta* is considered largely SI or pseudo-self-compatible (PSC) based on lack of or very low self-seed set from two

populations on two islands in the Azores (Crawford et al., 2015). However, a recent study (Crawford et al., 2019) of two populations from a third island of Graciosa revealed high self-seed set. Segregation for self-seed set (3 SI: 3 SC) in F₂ hybrids between Graciosa plants and SI *T. succulenta* suggest that SC is inherited mostly as a single, recessive factor (J.K. Kelly et al. unpubl.). The two populations on Graciosa are very small, with estimated census sizes of 30 to 80 plants and 10 to 20 individuals, and both occur in disturbed habitats (Crawford et al., 2019; L. Borges-Silva and M. Moura, unpubl.). The Graciosa plants are the only populations of *T. succulenta* in the Azores where SC has been documented (although one or several very small SC populations may occur on Corvo Island; H. Schaefer pers. obs.). The two Graciosa populations are sister and form a strongly supported clade in a molecular phylogeny of Azorean *T. succulenta* (Crawford et al., 2019). The floral parts in plants of these two populations are smaller than in other Azorean *T. succulenta* (Crawford et al., 2019; L. Borges-Silva and M. Moura, unpubl.) but the selfing syndrome is not nearly as highly evolved as in documented examples of the loss of SI and the transition to selfing in two other species of *Tolpis* in Macaronesean archipelagos (Crawford et al., 2008; Soto-Trejo et al., 2013; Koseva et al., 2017). The SC Graciosa populations are perennial, as in all other Azorean *T. succulenta*, and indeed in nearly all other *Tolpis* except the two aforementioned SC species. The transition to selfing was likely associated with the colonization of the disturbed habitats on Graciosa. If so, the loss of SI occurred in the last 0.7 million years, the dated age of the island (Sibrant et al., 2014) and a molecular phylogenetic study estimating divergence of the SC populations within the age of Graciosa (Kerbs et al., unpubl. data). The island age and estimated divergence time, combined with weak development of the selfing syndrome as compared to the other two SC species of *Tolpis* and the

maintenance of the ancestral perennial habit all point to a recent origin of selfing in the two populations (Crawford et al., 2019).

Methods

Sampling

This study examined two populations of *Tolpis succulenta* (herein labeled GRBL and GRSC) which occur on Graciosa island in the Azores (Fig. 1): (1). GRBL, Baía do Filipe, Beira Mar da Luz, N 39 01' 14.6", W 28 00' 26", 10 m a.s.l. *Moura GRBL*; (2) GRSC Santa Cruz, Quitadouro, N 39 04' 37.8", W 27 59' 19.1" ca 65 m a.s.l. *Moura*. GRBL, occurring on the southern side of Graciosa in costal basaltic rocks and sheltered by a rock wall, is comprised of ca. 30 (H. Schaefer, personal observation 2018) to 100 individuals (Parque Natural de Graciosa staff, personal observation 2018). GRSC, occurring on the northern side of the island on an ignimbrite wall on the slope of a volcano, has a population size between 10 (H. Schaefer, personal observation) and 20 plants (Parque Natural de Graciosa staff, personal observation). Fruits from plant capitula from four families in each population were collected and sent to the University of Kansas for cultivation. Mean and range of self-seed set in the two populations were 59.7% (31–96%) for GRBL and 41.9% (0–99%) for GRSC (Crawford et al., 2019).

Cultivation and DNA extraction

Progeny from wild maternal plants were germinated and reared in greenhouses at the University of Kansas. Tissue was collected, pressed, dried, and ground, with vouchers deposited at McGregor Herbarium (KANU) in Lawrence, Kansas. Samples were then frozen using liquid nitrogen and pulverized using chromium beads. DNA was subsequently extracted from the

ground, dried tissue using DNeasy Plant Mini Kits (Qiagen Inc., Valencia, California, USA) and DNA quantity was validated using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA).

DNA sequencing and SNP calling

RADseq libraries were constructed following the multiplexed shotgun genotyping protocol (Andolfatto et al., 2011). DNA from samples were cut using the restriction enzyme CspI, 250-300 bp fragments were selected for, and 6 bp barcodes were ligated to fragments. DNA was sequenced via Novogene, yielding 150 bp paired-end reads. Following sequencing, the demultiplexing of FastQ files carried out using STACKS (Catchen et al., 2013) and loci were *de novo* assembled using the same program with parameters $M=2$, $m=3$, $n=1$, as well as invoking the deleveraging algorithm and specifying $\alpha = 0.05$ in *ustacks*. *De novo* assembly and SNP calling yielded a total of 111,613 variant sites.

Mating system estimation and multiple paternity

The resultant VCF from STACKS were assessed and SNPs were filtered using custom python scripts (Supplemental material) in preparation for BORICE (Colicchio et al., 2020), a program which uses MCMC (Metropolis et al., 1953) to 1) estimate population outcrossing rates, 2) estimate maternal plant inbreeding coefficients, 3) ascertain whether progeny are selfed or outcrossed, and 4) assess single or multiple paternity of offspring (Gibson et al., 2020). BORICE was run with burn-in of 1000 and chain length of 4000 steps.

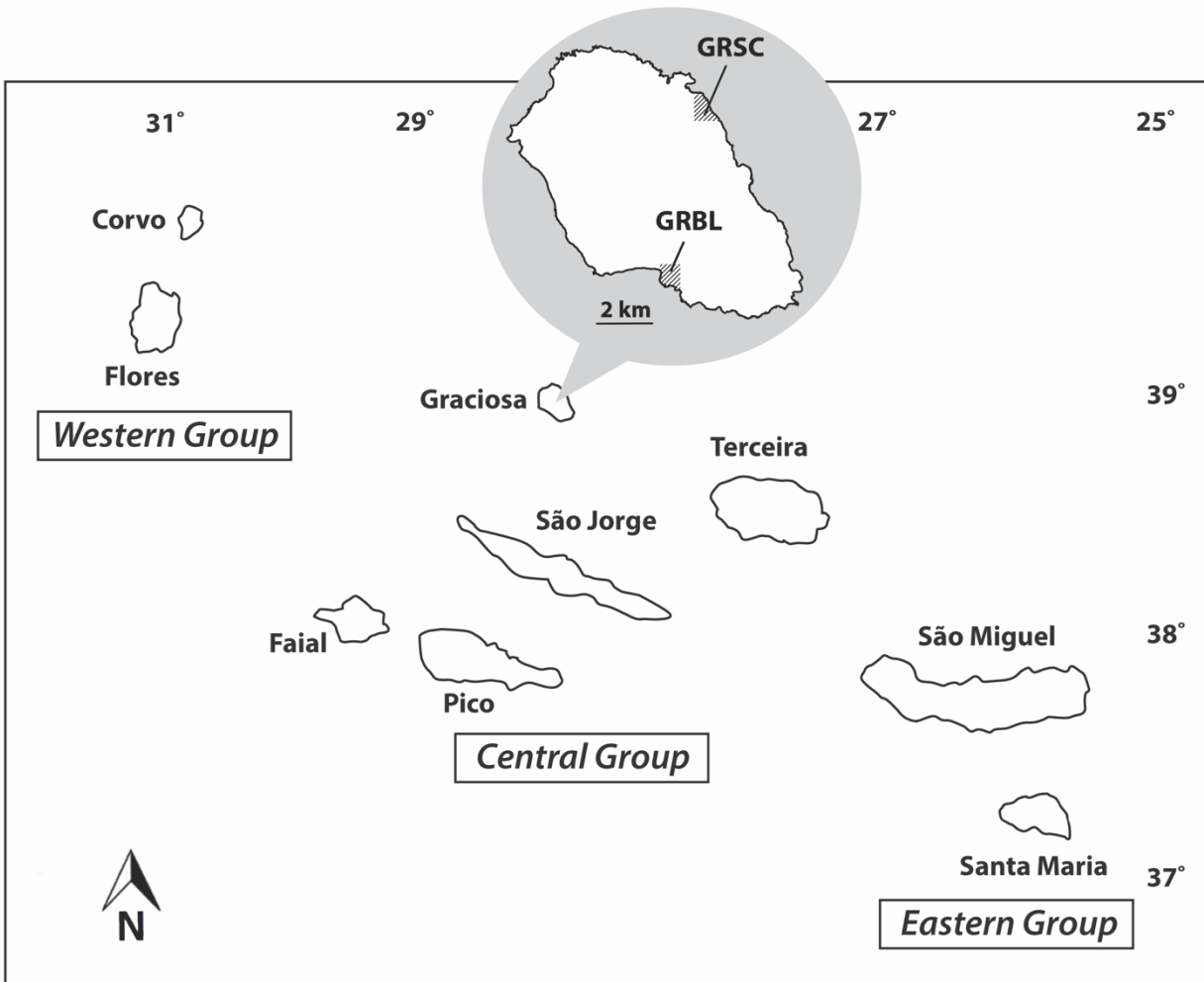


Figure 9. Locality of two populations of *T. succulenta* (GRSC and GRBL) on Graciosa island, within the central group of the Azorean archipelago.

Results

Progeny from both populations were determined to be outcrossed or selfed with strong confidence (posterior probabilities > 0.99). One offspring in family 8 from GRSC was found to be selfed, hence the posterior probability for the overall outcrossing is 0 at $t = 1$. All other offspring were found to be produced via outcrossing. Eight of eight maternal plants across the two populations were determined to be outbred with $P[\text{IH}=0] = 100\%$ for families 1, 3, 4, 5, 6,

95% for family 2, 94% and for family 7, 81% for family 8. Divergence estimates (with values comparable to F_{st} [Holsinger & Weir, 2009]) demonstrate maximum a posterior values of 0.195 in GRBL and 0.125 in GRSC, suggesting high degrees of genetic sharing between plants in each population.

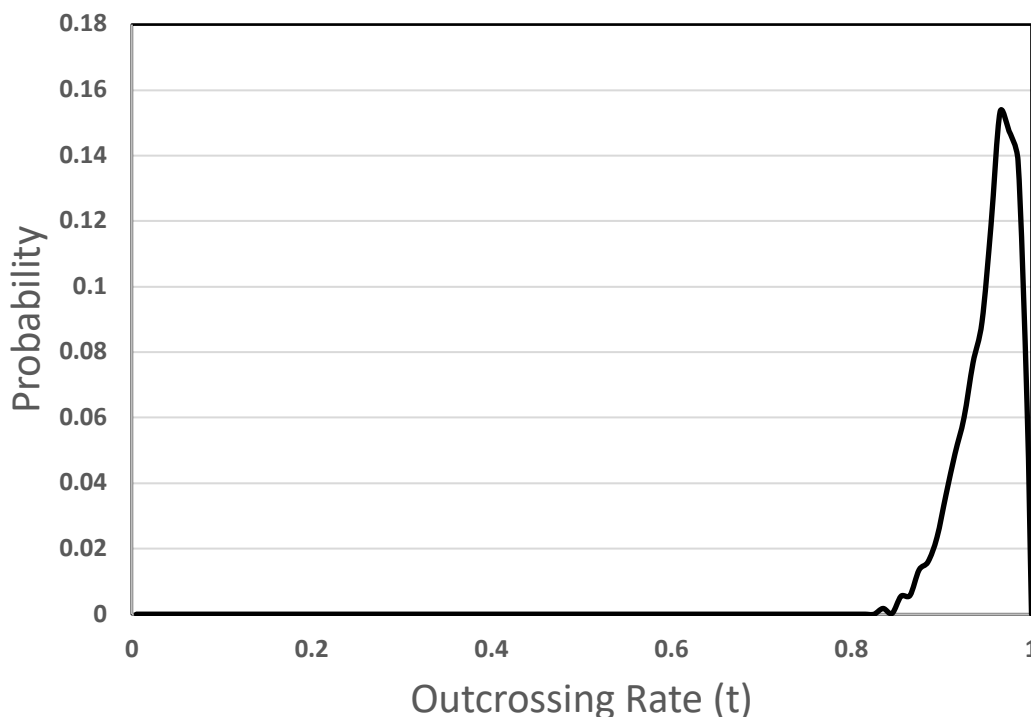


Figure 10. Posterior density for the overall outcrossing rate (t) across both populations

Across all families, the probability that progeny are full sibs is 15.3 %. No full siblings were detected in five of the eight families (Table 2). Families 1 and 4 contain one set of full sibs each, and family 5 contains two sets of full sibs (Table 2; Fig. 11). Outputs show a very high confidence (>90%) in assignment of offspring to a sire in the vast majority (78%) of contrasts. There was moderate support (~50% to 90% probability) for 23% of contrasts.

Population	Family	No. offspring	No. sires	Sibship
GRBL	Family 1	7	6	Offspring 3 & 7 full sibs
GRBL	Family 2	2	2	No full sibs
GRBL	Family 3	6	6	No full sibs
GRBL	Family 4	7	4	Offspring 3, 4, 6, & 7 full sibs
GRSC	Family 5	7	3	Offspring 1, 6, & 7 full sibs; 4 & 5 full sibs
GRSC	Family 6	6	6	No full sibs
GRSC	Family 7	2	2	No full sibs
GRSC	Family 8	5	5	No full sibs

Table 3. Assignment of sibship for each family across two *T. succulenta* populations

Maternal Family 1



Maternal Family 4



Maternal Family 5



Figure 11. Individual offspring within the same rectangles are full sibs. Maternal family 1 has six sires, and families four and five have four sires.

Discussion

Mating system

Given what was known about the two populations on Graciosa island, the rigorous documentation of a highly outcrossing mating system in both populations is rather surprising. Given the high self-seed set in progeny from the natural populations in the greenhouse and the weak expression of the selfing syndrome in the two small populations of *Tolpis succulenta* on Graciosa island (Crawford et al., 2019; Borges-Silva and M. Moura, unpubl.), we expected substantial selfing by these populations in the field. The high self-seed set occurs in the absence of potential pollinators in the greenhouse, and is likely facilitated by nyctinastic movements with capitula opening and closing daily during anthesis. These movements occur widely in Asteraceae and have been documented extensively in tribe Cichorieae, of which *Tolpis* is a member (Stirton 1983). Nyctinastic movements have been noted in all species of *Tolpis* cultivated in the greenhouse. They ostensibly bring the pollen in contact with the receptive style branches of florets in a capitulum (Crawford et al., 2019). Silva et al. (2016) suggested that the same mechanism may function in a rare species of *Sonchus* (tribe Cichorieae). Arista et al. (2017) hypothesized that in the absence of bees this mechanism, which is a kind of autonomous self-pollination, occurs after the opportunity for outcrossing has passed, resulting in delayed selfing in *Hypochoeris salzmanniana* (Cichorieae). A similar situation would seem to be feasible in natural populations of *Tolpis succulenta* on Graciosa island, yet, with the exception of the one selfed progeny, this clearly has not occurred during the reproductive period when sampling occurred. This suggests that pollinator activity was sufficient to transfer pollen among plants prior to selfing, presumably with the closing of capitula at the end of a day.

As indicated above, there are two prevalent non-mutually-exclusive hypotheses for the evolution of selfing during the colonization and establishment of small founding populations such as *Tolpis succulenta* on Graciosa. One hypothesis is reproductive assurance, where selfing is selected when the paucity of compatible mates (e.g., Reinartz and Les 1994; Young and Pickup 2010) and/or pollinators (e.g., Goodwillie 2001; Busch and Delph 2012) reduce outcrossed seed production. It is possible that there are few functioning S-alleles in the small populations of *T. succulenta* on Graciosa which could result in a paucity of compatible mates. At first glance, SSI appears to be more restrictive in terms of self- and cross-compatibility than the more common gametophytic self-incompatibility (GSI) systems. In the latter, the haploid genotype (allele at S-locus) of pollen controls compatibility and only one allele prevents fertilization. By contrast, SSI appears more restrictive because if either of the pollen alleles are the same as the stigma, then fertilization does not occur. However, factors may mitigate the seemingly more restrictive SSI. Early studies of SSI in Asteraceae (Gerstel 1950; Hughes and Babcock 1950; Crowe 1954) documented dominance relationships among S alleles. Dominance has important consequences for selfing and the number of compatible outcrossing mates because recessive alleles are not expressed, and therefore do not prevent fertilization in the presence of more dominant alleles. Extensive investigations in the genus *Senecio*, including comprehensive crossing studies, revealed a hierarchy of dominance relationships (Hiscock, 2000; Brennan et al., 2011; Brennan et al., 2013). In addition, alleles may show different dominance relationships in the stigma and pollen (Hiscock and Tabah, 2003; Brennan et al., 2006). With co-dominant alleles there is negative frequency dependent selection because as an allele becomes more frequent, it will find fewer compatible mates in a population (Byers and Meagher, 1992). By contrast, recessive alleles are not subjected to negative frequency dependent selection and will increase in a

population. Thus, species with SSI may set seed both by outcrossing and by selfing in small populations despite low S-allele diversity (Brennan et al., 2002; Silva et al., 2006). For example, Hiscock (2000) detected only four alleles in a sample of four plants from two populations of the SI colonizing species *Senecio squalidus*, but importantly a pollen/stigma recessive allele was found in all plants.

Crawford et al. (2019) discussed observations suggesting that pollinator limitation likely is not a factor favoring selfing in the two populations of *Tolpis succulenta*. The review of Weissmann et al. (2017) reported that five of 14 wild bee species on Graciosa visited Asteraceae. Also, many small halictid bees and flies were observed on plants of *T. succulenta* on Graciosa during field work in August, 2018 (H. Schaefer personal observation).

Inbreeding depression

Selfing of progeny from natural populations for two generations produced vigorous plants that flowered and had high self-self seed set (D. J. Crawford et al. unpubl. data), although more extensive experimental studies of inbreeding depression are needed.

Paternity analyses

The review of multiple paternity in plants by Pannell and Labouche (2013) discussed the variety of factors that may influence polyandry. In small populations, several factors may influence the number of sires per maternal plant. A small number of compatible mates, discussed above, could reduce the number of potential sires (Hardy et al., 2004; Young and Pickup, 2010). Pollinator

abundance and/or foraging behavior could impact sire number, with paucity of pollinators and limited foraging distance reducing siring number.

As in all Asteraceae, the inflorescence and floral morphology of *Tolpis* may affect the siring pattern. The close aggregation of small flowers in the capitulum could facilitate the pollination of many flowers by a single visit and result in correlated paternity. However, a single visit by a pollinator with pollen from prior visits to several plants (pollen carry over) could result in multiple paternity. The number of florets that can be pollinated per capitulum per visit depends on the number of open, receptive florets present in a capitulum at any given time. In some instances, individual florets may be receptive to pollen for only one or two days (Sun and Ganders 1988; (Nielsen et al., 2000; Guerrina et al., 2016). This temporal separation in receptivity should favor multiple paternity within a capitulum. The factors discussed above that could influence the number of sires per maternal plant are not known for insular *Tolpis*. It is known that individual capitula are open for at least seven days (they close nightly) (Crawford et al., 2019, unpubl data), potentially facilitating pollination within a single capitulum over a relatively prolonged period. Multiple paternity could also be promoted by the non-overlapping periods during which different individual capitula on the same plant are open. Once flowering commences on individuals of *Tolpis succulenta*, there are several open capitula during much of the flowering period (Crawford et al., unpubl.). In the present study, seed was collected in bulk from each maternal plant, thus precluding determination of intra- and inter-capitular components of multiple paternity. A study now in progress (Kerbs et al.) will provide insight into this question.

The percent of full sibs detected in the present study may be compared with other Asteraceae, including a recent study of three small populations of *Tolpis succulenta*, an SI/PSC species, on Madeira island (Gibson et al., in press). These populations had 22% full sibs, some 50% higher than detected in the two small Graciosa populations. Thus, correlated paternity is higher in the SI species in Madeira than in the SC populations on Graciosa. The reason(s) for the difference in correlated remain obscure and comments would be highly speculative. Hardy et al. (2004) found that in the rare endemic SI perennial herb *Centaurea corymbosa* (Asteraceae) 20% of sibs were full (same sire), and Sun and Ritland (1998) found 19% full sibs in the SI annual *Centaurea solstitialis*. Perhaps the important point, is that in these small insular populations the number of sires among fruits of maternal plant is comparable not only to the other few Asteraceae investigated but also to among-fruit values estimated for other plant families (Pannell and Labouche 2013). There is little evidence that particular sires are contributing disproportionately to the progeny of maternal plants, making it unlikely that biparental inbreeding is occurring in the populations.

Summary and questions for future study

There are two major findings of this study. The first is that in two small insular populations in which nonfunctional genetic SI has arisen (or been introduced) recently. These populations exhibit high self-seed set in the greenhouse, but yet appear highly outcrossing in the nature. The absence of a functional S-locus in these two small populations that have ostensibly recently colonized Graciosa would seem to favor a rapid transition to selfing (Pannell 2015). The reasons for these somewhat unexpected results remain to be determined, and in a real sense, this study

raises more questions, than it answers. One potential reason for the retention of outcrossing with the origin of SC is high inbreeding depression (Pannell 2015; Layman et al., 2017). Whether this is the situation for *Tolpis succulenta* awaits further study; two generations of selfed progeny in the greenhouse flowered and set fruit but more thorough studies are to be desired. Selection in the natural habitat may reveal reduced fitness of selfed progeny not detected in greenhouse cultivation (Armbruster and Reed 2006; Arista et al., 2017). Self-seed set is sometimes used to infer the mating system in plants from oceanic archipelagos (Anderson et al., 2001; Bernardello et al., 2001; Chamorro et al., 2012; Crawford et al., 2011) and indeed likely provide useful first estimates of mating system. The low self-seed set in the study of Gibson et al. (in press) of three populations of *Tolpis succulenta* from Madeira island was reflected in the expected highly outcrossing mating system. However, the present study provides a caveat in making such inferences. Indeed, the study of self-seed set in the two populations examined in the present study assumed, that loss of SI had resulted in a transition to selfing (Crawford et al., 2019)! The first study of mating system in Macaronesian *Tolpis* using progeny arrays and allozyme markers showed that two of six populations of an ostensibly highly outcrossing (very low self-seed set) species had mixed mating systems.

The second major result from this study is the multiple sires for all maternal plants in both populations. The markers and methods of analyses employed in the present study provide strong probabilistic assignment of progeny to different sires. The factors responsible for the low correlated paternity are not known, but possibilities include pollen carry over; individual capitula are receptive over seven days or more, and capitula of each maternal plant open progressively over the flowering season. These attributes provide the opportunity for multiple pollinator visits bearing pollen from different plants.

Field studies over several seasons would be desirable. Observations of seed set and sampling of progeny for mating system estimation over several flowering seasons from the two populations would be highly desirable to determine how “typical” the mating system estimates from one season are over several seasons. The present study indicates that both the maternal plants in the natural populations and their progeny are highly outbred, suggesting that our results apply across generations. Other observations within/over flowering seasons that could provide insights into the reproductive biology of the two populations include seed set, number of flowering plants and temporal variation in flowering, and abundance and types of floral visitors.

Gibson et al. (in press) discuss the advantages of the methods employed in the present study for studying the small populations of rare island plants, and they will be summarized briefly. A major advantage, with conservation implications, is that mating system can be inferred because individual progeny can be called as selfed or outcrossed with few maternal families and few progeny per family. This advantage can hardly be overstated given the low seed set available for progeny arrays and small population sizes of the populations. Although small populations provide challenges for mating system and paternity studies, they may offer certain advantages using genome-wide genotyping. In small populations such as the two examined in this study, it may be feasible to map all plants making it possible to detect genetic structure in the populations (Colicchio et al., 2020) and to infer not just the number of sires but the specific sires of progeny (Gibson et al., in press).

Literature Cited

- ANDERSON, G. J., G. BERNARDELLO, T. F. STUESSY, AND D. J. CRAWFORD. 2001a. Breeding system and pollination of selected plants endemic to Juan Fernández Islands. *American Journal of Botany* 88: 220-233.
- ANDOLFATTO, P., D. DAVISON, D. EREZYILMAZ, T. T. HU, J. MAST, T. SUNAYAMA-MORITA, AND D. L. STERN. 2011. Multiplexed shotgun genotyping for rapid and efficient genetic mapping. *Genome Research* 21: 610-617.
- ARISTA, M., R. BERJANO, J. VIRUEL, M. A. ORTIZ, M. TALAVERA, AND P. L. ORTIZ. 2017. Uncertain pollination environment promotes the evolution of a stable mixed reproductive system in the self-incompatible *Hypochaeris salzmanniana* (Asteraceae). *Annals of Botany* 120: 447-456.
- ARMBRUSTER, P., AND D. H. REED. 2006. Inbreeding depression in benign and stressful environments. *Heredity* 95: 235-242.
- BALDWIN S. J., AND D. J. SCHOEN. 2019. Inbreeding depression is difficult to purge in self-incompatible populations of *Leavenworthia alabamica*. *New Phytologist* 224: 1330-1338.
- BARRETT, S. C. H. 2013. The evolution of plant reproductive systems: how often are transitions irreversible? *Proceedings of the Royal Society B: Biological Sciences*. 280: 20130913. doi:10.1098/rspb.2013.0913
- BECK, J., A. I. AL-SHEBAZ, AND B. A. SCHAAL. 2006. *Leavenworthia* (Brassicaceae) revisited: testing classic systematic and mating system hypotheses. *Systematic Botany*. 31: 151-159.

- BERNARDELLO, G., G. J. ANDERSON, T. F. STUESSY, AND D. J. CRAWFORD. 2001. A survey of floral traits, breeding systems, floral visitors, and pollination systems of the angiosperms of the Juan Fernandez Islands (Chile). *Botanical Review*. (Lancaster) 67: 255-308.
- BRENNAN, A. C., S. A. HARRIS, D. A. TABA, AND S. J. HISCOCK. 2002. The population genetics of sporophytic self-incompatibility in *Senecio squalidus* L. (Asteraceae). I. S allele diversity in a natural population. *Heredity*. 89: 430-438.
- BRENNAN, A. C. S. A. HARRIS, AND S. J. HISCOCK. 2006. The population genetics of sporophytic self-incompatibility in *Senecio squalidus* L. (Asteraceae): the number, frequency, and dominance interactions of S alleles across its British range. *Evolution*. 60: 213-224.
- BRENNAN, A. C. D. A. TABAH, S. A. HARRS, AND S. J. HISCOCK. 2011. Sporophytic self-incompatibility in *Senecio squalidus* (Asteraceae): S allele dominance interactions and modifiers of cross compatibility and selfing rates. *Heredity*. 106: 113-123.
- BRENNAN, A. C. S. A. HARRIS, AND S. J. HISCOCK. 2013. The population genetics of sporophytic self-incompatibility in three hybridizing *Senecio* (Asteraceae) species with contrasting population histories. *Evolution*. 67: 1347-1367.
- BUSCH, J. W. 2005. The evolution of self-compatibility in geographically peripheral populations of *Leavenworthia alabamica* (Brassicaceae). *American Journal of Botany*.. 94(9) 1503-1512.
- BUSCH, J. W., AND L. F. DELPH. 2012. Importance of reproductive assurance and automatic selection as hypotheses for the evolution of self-fertilization. *Annals of Botany*.. 109: 553–562.

- BYERS, D. L., AND T. R. MEAGHER. 1992. Mate availability in small populations of plant species with homomorphic sporophytic self-incompatibility. *Heredity*. 68: 353-359.
- CATCHEN, J. P. HOHENLOHE, S. BASSHAM, A. AMORES, AND W. CRESKO. 2013. Stacks: an analysis tool set for population genomics. *Molecular Ecology*. doi: 10.1111/mec.12354
- CHAMORRO, S. R. HELENO, J. M. OLESEN, C. K. MCMULLEN, A. TAVASET. 2012. Pollination patterns and plant breeding systems in the Galápagos: a review. *Annals of Botany*. 110: 1489–1501.
- COLICCHIO, J., P. J. MONNAHAN, C. A. WESSINGER, K. BROWN, J. R. KERN AND J. K. KELLY. 2020. Individualized mating system estimation using genomic data. *Molecular Ecology*. doi: 10.1111/1755-0998.13094
- CRAWFORD, D. J., G.J. ANDERSON, L. BORGES SILVA, M. M. DE SEQUEIRA, M. MOURA, A. SANTOS-GUERRA A, J. K. KELLY, AND M. E. MORT. 2015. Breeding systems in *Tolpis* (Asteraceae) in the Macaronesian islands: the Azores, Madeira and the Canaries. *Plant Systematics and Evolution*. 301: 1981–1993. doi:10.1007/s00606-015-1210-5
- CRAWFORD, D. J., G. J. ANDERSON, AND G. BERNARDELLO. 2011. The reproductive biology of island plants, pp. 11-36 *In*: D. Bramwell and J. Caujape-Castells, eds, *The Biology of Island Floras*. Cambridge University Press, Cambridge.
- CRAWFORD, D. J., J. K. ARCHIBALD, D. STOERMER, M. E. MORT, J. K. KELLY, AND A. SANTOS-GUERRA. 2008. A test of Baker's law: breeding systems and the radiation of *Tolpis* (Asteraceae) in the Canary Islands. *International Journal of Plant Sciences*. 169: 782–791.

- CRAWFORD, D. J., M. MOURA, L. BORGES SILVA, M. E. MORT, B. KERBS, H. SCHAEFER AND J. K. KELLY. 2019. The transition to selfing in Azorean *Tolpis* (Asteraceae). *Plant Systematics and Evolution*. 305(4): 305–317. doi:10.1007/s00606-019-01573-7.
- CROWE, L. K. 1954. Incompatibility in *Cosmos bipinnatus*. *Heredity* 8: 1-11.
- CUTTER, A. D. 2019. Reproductive transitions in plants and animals: selfing syndrome, sexual selection and speciation. *New Phytologist*. 224: 1080–1094.
- FORNONI, J., K. BOEGE, R. PÉREZ-ISHIWARA, C. A. DOMÍNGUEZ, AND M. ORDANO. 2015. A comparison of floral integration between selfing and outcrossing species: a metaanalysis. *Annals of Botany*. 117: 299–306.
- GERSTEL, D. U. 1950. Self-incompatibility studies in *Parthenium*. 11. Inheritance. *Genetics*. 35: 482-506.
- GIBSON, J. S. M., D. J. CRAWFORD, M. T. HOLDER, M. E. MORT, B. KERBS, M. MENEZES DE SEQUEIRA AND J. K. KELLY. 2020. Genome-wide genotyping estimates mating system parameters and paternity in the island species *Tolpis succulenta*. *American Journal of Botany*. In press.
- GOODWILLIE, C. 1999. Multiple origins of self-compatibility in *Linanthus* Section *Leptosiphon* (Polemoniaceae): phylogenetic evidence from internal-transcribed-spacer sequence data. *Evolution*. 53:1387–1395.
- GROSSENBACHER, D., Y. BRANDVAIN, J. R. AULD, M. BURD, P. O. CHEPTOU, J. K. CONNER, A. G. GRANT, S. M. HOVICK, J. R. PANNELL, A. PAUW, T. PETANIDOU, A. M. RANDLE, R. R. DE CASAS, J. VAMOSI, A. WINN, B. IGIC, J.

- W. BUSCH, S. KALISZ, AND E. E. GOLDBERG. 2017. Self-compatibility is overrepresented on islands. *New Phytologist*. 215:469–478.
- GUERRINA, M., G. CASAZZA, E. CONTI, C. MACRÍ, AND L. MINUTO. 2016. Reproductive biology of an Alpic paleo-endemic in a changing climate. *Journal of Plant Research*. 129: 477-485.
- HARDY, O. J., S. C. GONZÁLEZ-MARTÍNEZ, B. COLAS, H. FRÉVILLE, A. MIGNOT, AND I. OLIVIERI. 2004. Fine-scale genetic structure and gene dispersal in *Centaurea corymbosa* (Asteraceae). II. Correlated Paternity Within and Among Sibships. *Genetics*. 168: 1601-1614.
- HERMAN, A. C., AND D. J. SCHOEN. 2016. Recent selection for self-compatibility in a population of *Leavenworthia alabamica*. *Evolution*. 70:1212–1224.
- HISCOCK, S. J. 200. Genetic control of self-incompatibility in *Senecio squalidus* L. (Asteraceae) in a successful colonizing species. *Heredity*. 85: 10-19.
- HISCOCK, S. J., AND D. A. TABAH. 2003. The different mechanisms of sporophytic self-incompatibility. *Philosophical Transactions of the Royal Society, London*. 358: 1037-1045.
- HUGHES, M. B. E. B. BABCOCK. 1950. Self-incompatibility in *Crepis foetida* L. subsp. *Rhoeadifolia* Bieb. Schinze and Keller. *Genetica*. 35: 570-588.
- IGIC, B., R. LANDE, AND J. R. KOHN. 2008. Loss of self-incompatibility and its evolutionary consequences. *International Journal of Plant Sciences*. 169: 93–104.
- KALISZ, S., A. RANDLE, D. CHAUFFETZ, M. FAIGELES, A. BUTERA, AND C. BEIGHT. 2011. Dichogamy correlates with outcrossing rate and defines the selfing syndrome in the mixed mating genus *Collinsia*. *Annals of Botany* 109: 571–582.

- KOSEVA, B, D. J. CRAWFORD, K. BROWN, M. E. MORT, AND J. K. KELLY. 2017. The genetic breakdown of self-incompatibility in *Tolpis coronopifolia* (Asteraceae). *New Phytologist*. 216:1256–1267.
- LAYMAN, N. C., M. T. R. FERNANDO, C. R. HERLIHY, AND J. W. BUSCH. 2017. Costs of selfing prevent the spread of a self-compatibility mutation that causes reproductive assurance. *Evolution*. 71:884–897. doi :10.1111/evo.13167
- METROPOLIS, N., A. W. ROSENBLUTH, M. N. ROSENBLUTH, A. H. TELLER, AND E. TELLER. 1953. Equation of state calculations by fast computing machines. *Journal of Chemical Physics*. 21: 1087-1092.
- NIELSEN, L., M. PHILIPP, H. ADERSEN, AND H. SIEGISMUND. 2000. Breeding system of *Scalesia divisa* Andersson, an endemic Asteraceae from the Galápagos Islands. Det Norske Videnskaps-Akademi I. Matematisk-Naturvidenskapelige Klasse, Shrifter, Ny Serie 39: 127–138.
- ORNDUFF, R. 1969. Reproductive biology in relation to systematics. *Taxon*. 18: 121–133.
- PANNELL, J. R. 2015. Evolution of the mating system in colonizing plants. *Molecular Ecology*. 24: 2018-2027.
- PANNELL, J. R., AND A-M. LABOUCHE. The incidence and selection of multiple mating in plants. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 368: 20120051. doi: 10.1098/rstb.2012.0051
- SIBRANT, A. L. R., F. O. MARQUES, AND A. HILDENBRAND. 2014. Construction and destruction of a volcanic island developed inside an oceanic rift: Graciosa Island, Terceira Rift, Azores. *Journal of Volcanology and Geothermal Research*. 284: 32–45.

- SILVA, J. L., A. C. BRENNAN, J. A. MEJÍAS. 2006. Population genetics of self-incompatibility in a clade of relict cliff-dwelling plant species. *AoB PLANTS* 8: plw029; 10.1093/aobpla/plw029.
- SLOTTE, T, K. M. HAZZOURI, D. STERN, P. ANDOLFATTO, AND S. I. WRIGHT. 2012. Genetic architecture and adaptive significance of the selfing syndrome in *Capsella*. *Evolution*. 66: 1360–1374.
- SOTO-TREJO, F, J. K. KELLY, J. K. ARCHIBALD, M. E. MORT, A. SANTOS-GUERRA, AND D. J. CRAWFORD. 2013. The genetics of self-compatibility and associated floral characters in *Tolpis* (Asteraceae) in the Canary Islands. *International Journal of Plant Sciences*. 174: 171–178.
- STEBBINS, G. L. 1957. Self fertilization and population variability in the higher plants. *American Naturalist*. 91: 337–354.
- STIRTON, C. H. 1983. Nocturnal petal movements in the Asteraceae. *Bothalia* 14: 1003-1006.
- SUN, M., AND F. R. GANDERS. 1988. Mixed mating systems in Hawaiian *Bidens* (Asteraceae). *Evolution*. 42: 516-527.
- URBAN, L., AND C. D. BAILEY. 2013. Phylogeny of *Leavenworthia* and *Selenia* (Brassicaceae). *Systematic Botany*. 38: 723–736.
- VOILLEMOT, M., AND J. R. PANNELL. 2017a. Inbreeding depression is high in a self-incompatible Perennial herb population but absent in a self-compatible population showing mixed mating. *Ecology and Evolution*. 7: 8535–8544.
- VOILLEMOT, M., AND J. R. PANNELL 2017b. Maintenance of mixed mating after the loss of self-incompatibility in a long-lived perennial herb. *Annals of Botany*. 119: 177–190.

- VOILLEMOT M., F. ENCINAS-VISO, AND J. R. PANNELL. 2019. Rapid loss of self-incompatibility in experimental populations of the perennial outcrossing plant *Linaria cavanillesii*. *Evolution*. 73-5: 913–926.
- WRIGHT, S. I., S. KALISZ, AND T. SLOTTE. 2013. Evolutionary consequences of self-fertilization in plants. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 280:20130133. doi:10.1098/rspb.2013.0133
- YOUNG, A. G., AND M. PICKUP. 2010. Low S-allele numbers limit mate availability, reduce seed set and skew fitness in small populations of a self-incompatible plant. *Journal of Applied Ecology*. 47: 541–54