Tribulus cistoides: An Evolutionary Model System to Understand Unique Species Interactions on Islands

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Montreal, Québec

August 2023

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

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ABSTRACT

Islands have long served as a model for understanding the relationships that shape organismal form and function. The appeal of islands comes from their often-unique environmental conditions, their independent "replicates" (i.e., different islands), and their simplified set of species interactions. As a result, islands often generate evolutionary relationships that can be novel and unique even for species that are widespread across the globe, such as *Tribulus cistoides*.

Tribulus cistoides is a perennial plant distributed across arid zones throughout the world. The species' ubiquitous nature makes it an excellent model system for comparisons between locations and for understanding how a widespread, and sometimes invasive, species might be adapting to new conditions. Indeed, insularity and specialized endemic species interactions appear to be driving phenotypic divergence of *Tribulus* populations on islands. Specifically, on *Tribulus* fruit traits, also called mericarps, which are a hard and thorny fruit that contains seeds. I used three approaches to understand this divergence in Galápagos, especially to the specialist seed predators: Darwin's finches.

This thesis starts with a literature review of the Galápagos – *Tribulus* -Finch system. Then, in Chapter 2, I use a comparative analysis of variation in *T. cistoides* between island and continental populations, with a particular emphasis on traits involved in antagonistic (seed defense) and mutualistic (floral traits) interactions. I find that island populations have larger mericarps with fewer "lower" spines compared to continental populations, with environmental variation among islands explaining variation in the spines. Flower petal length was consistently smaller on islands, especially on Galápagos. This work highlights how bioclimatic variables play a role in shaping the evolution of traits associated with species interactions in this globally distributed species.

In my next chapter, I conduct surveys over 5 years to estimate mericarp survival in relation to natural variation in mericarp traits, and I conduct a

manipulative mark-recapture field experiment to enhance variation and better estimate natural selection. In both cases, I focus on selection imposed by endemic seed predators, Darwin's finches. The surveys reveal that larger and spinier mericarps in island populations are the result of adaptation to predation and environmental conditions. The mark-recapture experiments reveal that manipulated mericarp traits experienced enhanced selection. Together, these studies reveal that mericarps are generally well-adapted to finch predation, although gene flow within islands and trade-offs with dispersal cause some maladaptation, I suggest that recent increases in human activity have imposed new forms of selection on some traits. This chapter emphasizes the importance of monitoring natural populations and conducting field experiments to gain insights into the role of natural selection in shaping adaptation.

In my final chapter, I use chloroplast and nuclear markers and phylogeographic methods to reconstruct the spatial and temporal history of *T. cistoides* populations on Galápagos, and their potential mainland sources. I find that *T. cistoides* most likely arrived in the Galápagos through a single colonization event around 0.92 million years ago, indicating its native status rather than being introduced. I also found that *T. cistoides* and the finches have been interacting since their arrival on the islands, suggesting that *T. cistoides* might have played a significant role in the speciation of finches.

In combination, this research demonstrates that *T. cistoides*, despite not being an endemic species, serves as a valuable model for studying island biogeography, natural selection, and coevolution. It highlights the significance of species interactions in sustaining endemic communities on islands, emphasizing the importance of multiple methods (herbarium samples, field experiments and molecular methods) to understand the evolutionary aspects of these interactions. Moreover, the study illustrates that even non-endemic species exhibit unique adaptations on islands, reaffirming the importance of studying such species to gain insights into island ecology and evolution.

ABRÉGÉ

Les îles servent depuis longtemps de modèle pour comprendre les relations qui façonnent la forme et la fonction des organismes. L'attrait des îles provient de leurs conditions environnementales souvent uniques, de leurs "répliques" indépendantes (c'est-à-dire des îles différentes) et de leur ensemble simplifié d'interactions entre les espèces. Par conséquent, les îles génèrent souvent des relations évolutives qui peuvent être nouvelles et uniques, même pour des espèces répandues dans le monde entier, comme *Tribulus cistoides*.

Tribulus cistoides est une plante vivace répartie dans les zones arides du monde entier. L'omniprésence de l'espèce en fait un excellent système modèle pour les comparaisons entre lieux et pour comprendre comment une espèce répandue, et parfois envahissante, peut s'adapter à de nouvelles conditions. En effet, l'insularité et les interactions entre espèces endémiques spécialisées semblent être à l'origine de la divergence phénotypique des populations de *Tribulus* sur les îles. Plus précisément, les traits des fruits du *Tribulus*, également appelés méricarpes, sont des fruits durs et épineux qui contiennent des graines. J'ai utilisé trois approches pour comprendre cette divergence sur les Galápagos, en particulier en ce qui concerne les prédateurs spécialisés dans les graines: les pinsons de Darwin.

Cette thèse commence par une revue de la littérature sur le système Galápagos - *Tribulus* - Pinson. Ensuite, dans le chapitre 2, j'utilise une analyse comparative de la variation chez *T. cistoides* entre les populations insulaires et continentales, avec un accent particulier sur les traits impliqués dans les interactions antagonistes (défense des graines) et mutualistes (traits floraux). Je constate que les populations insulaires ont des méricarpes plus grands avec moins d'épines "inférieures" par rapport aux populations continentales, la variation environnementale entre les îles expliquant la variation des épines. La longueur des pétales des fleurs était systématiquement plus petite sur les îles, en particulier sur les Galápagos. Ces travaux mettent en évidence le rôle des variables bioclimatiques

dans l'évolution des traits associés aux interactions entre espèces chez cette espèce répartie dans le monde entier.

Dans le chapitre suivant, je mène des enquêtes sur 5 ans pour estimer la survie des méricarpes en relation avec la variation naturelle des caractéristiques des méricarpes, et je mène une expérience manipulative de marquage-recapture sur le terrain pour augmenter la variation et mieux estimer la sélection naturelle. Dans les deux cas, je me concentre sur la sélection imposée par les prédateurs endémiques des graines, les pinsons de Darwin. Les études révèlent que les méricarpes plus grands et plus épineux dans les populations insulaires sont le résultat d'une adaptation à la prédation et aux conditions environnementales. Les expériences de marquage-recapture révèlent que les caractéristiques manipulées des méricarpes ont fait l'objet d'une sélection accrue. L'ensemble de ces études révèle que les méricarpes sont généralement bien adaptés à la prédation par les pinsons, bien que les flux de gènes au sein des îles et les compromis avec la dispersion soient à l'origine d'une certaine mal-adaptation. En outre, je suggère que l'augmentation récente de l'activité humaine a imposé de nouvelles formes de sélection sur certains traits. Ce chapitre souligne l'importance du suivi des populations naturelles et de la réalisation d'expériences sur le terrain pour mieux comprendre le rôle de la sélection naturelle dans la formation de l'adaptation.

Dans mon dernier chapitre, j'utilise des marqueurs chloroplastiques et nucléaires ainsi que des méthodes phylogéographiques pour reconstruire l'histoire spatiale et temporelle des populations de *T. cistoides* aux Galápagos et leurs sources continentales potentielles. Je constate que *T. cistoides* est très probablement arrivé aux Galápagos par le biais d'un seul événement de colonisation il y a environ 0,92 million d'années, ce qui indique qu'il s'agit d'une espèce indigène et non d'une espèce introduite. J'ai également constaté que *T. cistoides* et les pinsons interagissent depuis leur arrivée sur les îles, ce qui suggère que *T. cistoides* pourrait avoir joué un rôle important dans la spéciation des pinsons.

L'ensemble de cette recherche démontre que *T. cistoides*, bien que n'étant pas une espèce endémique, sert de modèle précieux pour l'étude de la biogéographie insulaire, de la sélection naturelle et de la coévolution. Elle met en évidence l'importance des interactions entre espèces pour le maintien des communautés endémiques sur les îles, en soulignant l'importance de méthodes multiples (échantillons d'herbiers, expériences sur le terrain et méthodes moléculaires) pour comprendre les aspects évolutifs de ces interactions. En outre, l'étude montre que même les espèces non endémiques présentent des adaptations uniques sur les îles, ce qui réaffirme l'importance d'étudier ces espèces pour mieux comprendre l'écologie et l'évolution des îles.

ACKNOWLEDGEMENTS

During the development of my thesis, I received the support of many people and institutions to whom I will always be grateful.

I would like to start by thanking my supervisor, Andrew Hendry, for giving me the opportunity to be part of his lab team. I would always remember the first time we met, changing my interest from fish to plants but at the core keep my enthusiasm for evolutionary biology. I am grateful to Andrew for being an extraordinary guide and support in every step of the way. I am also grateful to all members of DRYBAR lab. Especially to Lotte, Paola, Felipe, Betzi, Jonathas, Tim, Grant, David, Marco, Alan, Åsa, Allegra, Lea, Chelsea, Janay, Victoria, Ananda, and MK for providing a friendly environment to learn and develop new skills. I would always remember our discussions over coffee or beers at Thompson House. I would like to specifically, thank Sofía Carvajal who guided me and was always willing to help me from the start of my PhD, always with enthusiasm and openness to share your knowledge with me. To BESS, and my BESS cohort especially to Angelly, Dirley, Camilo, Ilse, Andrea, Daniel, Francis, Heather, Chris, José, Carmen, and Ximena. To my Redpath family, for being great friends and figuring out together our new lives in Montreal and the PhD program.

I am grateful to Dan Schoen, Jaime Chaves, and March Johnson for being part of my Supervisory Committee, for providing feedback on my projects, for sharing their time and space to successfully accomplish the goals needed for this thesis. I would like to express my gratitude to Marc, who closely guided me during many steps of my PhD, the development of Chapter 2, my first publication, for always sharing his wisdom to improve my research and support my ideas. To Jaime who give me the unique chance to travel like never before and be part of a research expedition across Galápagos and to being my support while in Ecuador. Finally, to Dan, my constant support at McGill, who provided me with the space needed, despite the pandemic, and allowed me to continue making progress in the lab. I hope we can keep working together.

I would like to thank the institutions that have provided funding and local collaborators. NSERC CREATE BESS program, QCBS, the Redpath Museum, the Department of Biology at McGill University, and the McGill-STRI NEO Program. To the Charles Darwin Station in Galápagos, to Patricia Jaramillo, and the Galápagos National Park and the Galápagos Science Center.

I would like to thank Ancil Gittens and Caroline LeBlond for their logistical support.

During these past six years, in addition to academic and logistical support, I have received constant support from friends and family without whom I would not have been able to pursue this goal. My dearest friend Juan, who taught me so much over the years. Thank you for sharing your wisdom with me. I deeply treasured our conversations and your teachings. Thanks for helping me becoming a better person. Mon cher grand ami, Marc Perron, pour tous les moments agréables passés avec lui à écouter de la musique, à apprendre de bonnes recettes et à me faire aimer la culture québécoise. Our friends Stani, Carole and Nicolas who with always shared the funniest conversations. My Ecuadorian family in Montreal, Iván, Melissa, Ricardo, Javier, Laura, and Victor, who made me feel welcomed and who with I share fond memories of your concerts and music. My friends from the Club Eco-Evo Latinoamérica, Luis, Diego, Ana e Ixchel, are the best team I have the pleasure to work with! My dear friend, Paola Elizalde, who started at the same time the PhD journey, on our separate ways, but always supporting each other. Finally, to my *papus*, Carlitos, Ricardo, Christian, Andrés and Irwing for being always there despite the distance, thank you so much for your love and friendship.

Le quiero dar las gracias a mis padres, Karen Corral y Winer Reyes, a mi familia, mi hermana Daniela, Titi, Letty, mi mami Lala y mi abuelita Aida, a todos mis tíos, tías y primos por apoyarme a la distancia, por su pacencia, por su ayuda, sus consejos y por siempre darme ánimos para ser mejor persona cada día. Los amo muchísimo.

Quiero agradecer a mis amigos en Manabí. A la Reserva Bosque-Seco Lalo Loor; a Mariela, José, Irma y Camote por apoyarme dándome un espacio donde trabajar, por nuestros almuerzos juntos y por compartir con ustedes mientras escribí esta tesis. Y a mis queridos amigos de Don Juan y Manta: Adri, Erika, Bryan, papu Edu y Majita por su apoyo y su alegría. A la Biblioteca A Mano Manaba, Ruth, Esteban y Miriam, estoy eternamente agradecido por recibirme, por apoyarme y por permitir compartir mi pasión por la biología con los niños y niñas de la Biblioteca. Un abrazo muy fuerte para todos los quiero mucho.

Finalmente, quiero agradecer a mi reina, María José, mi serendipia, la persona que se convirtió en mi compañera de vida. Agradezco tu paciencia, tu amor y apoyo. Me encanta compartir mis *miércoles de tarde* contigo y espero poder compartir muchos más. Gracias por siempre enseñarme nuevas cosas, nuevas perspectivas, darme fuerzas y ser mi mejor amiga. Te amo mucho. Muchas gracias a Mamá María, a José y a todos los tíos por recibirme y hacerme sentir parte de una sola familia.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

All manuscript chapters of this thesis (Chapters 2 -4) constitute original contributions to scientific knowledge.

In Chapter 2, I examine the morphological variation of flowers and fruits of *Tribulus cistoides* across islands and continent populations to understand the observed phenotypic divergence related to antagonistic and mutualistic interactions. I conclude that *T. cistoides* exhibits phenotypic differences in fruit and floral traits between island and continental habitats, consistent with antagonistic and mutualistic interactions driving divergent evolution between continental and insular populations, while in other cases climatic variation appears to be the main driver, or at least modulates biotic selection.

In Chapter 3, I examine the factors influencing selection between Darwin's ground finches and *T. cistoides* in Galápagos, using two methods to estimate natural selection: First, annual monitoring of natural populations and using field experiments. I find that mericarps are generally well adapted to finch predation, specifically that larger and spinier mericarps are the result of adaptation to finch predation. I also found that there are still some populations under selection and environmental variables influence selection due to predation. Suggesting that other mechanisms such as gene flow or environmental trade-offs influence selection. Finally, the selection experiment indicates that predation across islands varies in intensity, but in general, has the same trend and *Tribulus cistoides* across islands are locally adapted to differences in finch communities.

In Chapter 4, I add historical and evolutionary context to these interactions and dynamics, by estimating the number of colonization events in Galápagos, the time of divergence, and some genetic structure of *T. cistoides* populations. I used chloroplast and nuclear markers to infer this information to build phylogeny and haplotype networks. I find that *T. cistoides* from Galápagos form a monophyletic group, indicating most likely a single colonization event. The time of divergence was

around a million years ago, indicating that *Tribulus* populations are native to the islands (not introduced by humans). Also, indicates that *Tribulus* already coexisted with the common ancestor of Darwin's finches, suggesting that their interactions likely influenced the evolution and speciation of the endemic species. Finally, the haplotype network shows unique haplotypes corresponding to *Tribulus cistoides* from Galápagos, indicating differentiation but also some populations share haplotypes with populations from Central America and México, the Caribbean and Oceania. Suggesting that these are most likely their sources.

CONTRIBUTION OF AUTHORS

Chapter 1: Winer Daniel Reyes-Corral wrote the chapter with input from Lota Skovmand.

Chapter 2: Peter R. Grant, B. Rosemary Grant and Marc Johnson conceptualized the study. Winer Daniel Reyes-Corral, Sofia Carvajal-Endara, and Marc T. J. Johnson collected, curated, and analyzed data. Molly Hetherington-Rauth collected data. Jaime A. Chaves, Andrew P. Hendry, and Marc T. J. Johnson acquired funding. Winer Daniel Reyes-Corral wrote the original manuscript. Marc T. J. Johnson wrote and supervised the original manuscript. All co-authors reviewed the manuscript.

Chapter 3: Winer Daniel Reyes-Corral, Marc T. J. Johnson and Andrew P. Hendry conceptualized the study. Marc T. J. Johnson, Sofia Carvajal-Endara, and Winer Daniel Reyes-Corral collected data and designed the selection experiment. Patricia Jaramillo provided volunteers to monitor the selection experiment and provided and collect data. Andrew P. Hendry and Jaime A. Chaves provided funding. Jaime A. Chaves and Patricia Jaramillo provided permits for field samples and herbarium samples. Winer Daniel Reyes-Corral and Andrew P. Hendry wrote the first draft of the paper.

Chapter 4: Winer Daniel Reyes-Corral collected field samples, curated, processed, extracted, and analyzed sequences. Jaime A. Chaves provided permits and logistics for sample collection. Molly Hetherington-Rauth collected herbarium samples. Marc T. J. Johnson, Andrew P. Hendry, and Daniel Schoen conceptualized, provided funding, and supported the lab study. Winer Daniel Reyes-Corral and Jaime A. Chaves wrote the first draft of the paper.

Chapter 5: Winer Daniel Reyes-Corral wrote the chapter with input from Lota Skovmand.

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INTRODUCTION

The main objective of this thesis is to evaluate the ecological and evolutionary factors that drive the adaptation of populations of *Tribulus cistoides* on islands, showing the potential of *T. cistoides* as a model for evolutionary studies. Specifically, to understand how *T. cistoides* flower (petal length) and mericarp traits (size and defense) respond to selection by unique environmental factors on the Galápagos, and how mutualists and endemic seed predators present on the islands, such as Darwin's finches (*Geospiza fortis, Geospiza magnirostris*), drive these changes. The particular interest in studying *T. cistoides* is the ecological and evolutionary context that has been shown on the Galápagos. Darwin's finches have shaped *T. cistoides* evolution, selecting for specific *T. cistoides* traits such as mericarp size and spines with selection varying between years and partially being explained by precipitation (Boag & Grant, 1981; Carvajal-Endara et al., 2020; P. R. Grant, 1981; P. R. Grant & Grant, 2014). Likewise, *T. cistoides* influences the selection of beak size for ground finches that specialize in seeds. This selection is stronger during the dry years when other seeds are not available and only finches with large enough beaks survive (Boag & Grant, 1981; P. R. Grant, 1981).

Besides this dynamic, *T. cistoides'* unique ecological context allows for more specific studies. For example, *T. cistoides* is not endemic to the Galápagos, but rather a widespread species across arid and tropical areas around the world (E. Johnson, 1932; Kearney et al., 2020; Scott & Morrison, 1996; Squires, 1979; Wiggins & Porter, 1971). These attributes make *T. cistoides* a great model for comparison studies of phenotypic divergence between island and continent populations and testing the effects of specific island conditions (Chapter 2). The abundance of *T. cistoides* also allows field experiments directly test selection in natural or semi-natural populations (Chapter 3). In addition, *T. cistoides* is closely related to human activities, which allows one to test the potential anthropogenic effects on the

evolution dynamics of *T. cistoides* in an island context compared to continental habitats (Rivkin et al., 2021).

Each chapter of this thesis evaluates specific aspects of *T. cistoides* adaptation to island ecosystems with a focus on unique island species interactions. Chapter 1 is a literature review that gives theoretical context to the thesis looking at the concepts of natural selection, island biogeography and species interactions on islands. The chapter explains the context of the study system, *T. cistoides* and its interactions with Darwin's finches in the Galápagos. Chapter 2 addresses that *T. cistoides* mericarps show great phenotypic variation between populations of islands and continents that may be explained by unique species interactions or unique environmental conditions on islands. Chapter 3 focuses on the factors that drive selection of *T. cistoides* traits, focusing on endemic predators, Darwin's finches. Finally, Chapter 4, using molecular methods, adds the historical and evolutionary context of *T. cistoides* populations in the Galápagos and its interactions.

The main objective of Chapter 2 is to understand how isolated ecosystems, like islands, influence the divergence and speciation of species, and how ecological interactions, particularly between plants and other organisms, impact the phenotypic traits of *T. cistoides*. I found that flower and mericarp traits differ between island and continental populations. *T. cistoides* mericarps on islands are larger and their spine number is variable while on the continent spine number is consistent. *T. cistoides* flowers on the other hand do not differ much between the islands and continents. However, *T. cistoides* flowers on the Galápagos specifically are smaller than anywhere else, adding to the specific island syndrome of flower size for the Galápagos (Hetherington-Rauth & Johnson, 2020). The results imply that phenotypes of *T. cistoides* are driven by island environments and that different species interactions shape the phenotypic divergence observed. These findings also show the importance of measuring herbarium samples to quantify phenotypes from multiple locations and show that even widespread species such as *T. cistoides* exhibit divergence.

The main objective of Chapter 3 is to investigate the influence of natural selection and trait variation on the adaptation of *T. cistoides* to specific ecological conditions, particularly in response to the endemic seed predators, Darwin's finches. The chapter aims to determine whether the observed phenotypic traits of the mericarps represent well-adapted states influenced by selection pressures, either biotic or abiotic factors in the unique island environment. I found that larger and spinier mericarps are the result of adaptation due to predation and environmental conditions on islands. Mericarps are generally well-adapted, but still, some populations have not reached their optima. I argue that gene flow or a defense/dispersal trade-off might be the explanation for these specific populations. I also found that phenotypic variation exists among island populations. Finally, using a field selection experiment, I inflated phenotypic variation to show that mericarp traits, such as size and defense, are selected across the islands. These findings suggest that variations seen in mericarp phenotypes are indeed the result of natural selection by endemic predators. The chapter shows the importance of monitoring natural populations along with field experiments to understand natural selection processes.

Finally, for Chapter 4, the main objective is to provide historical context to *T. cistoides* populations on the Galápagos Islands. I used phylogeographic methods to reconstruct the spatial and temporal history of *T. cistoides* populations on the Galápagos Islands and potential mainland sources. In this chapter, I found that *T. cistoides* populations arrived in Galápagos most likely from a single colonization event and that there has been some migration between the islands. The estimated time of divergence was 2.4 million years ago, suggesting that *T. cistoides* is native and not introduced, which has been previously implied (Hooker, 1847b; Porter, 1971; Traveset et al., 2013). In this context, our findings also suggest that *T. cistoides* and the finch common ancestor have been interacting since their arrival to the islands. Given the role of *T. cistoides* in shaping finch evolution, this suggests that *T. cistoides* may be a key factor in the speciation process of finches.

Overall, these chapters show evidence that *T. cistoides*, despite being a non-endemic species, is a great model to study island biogeography, natural selection, and coevolution. This thesis shows the importance of species interactions in the maintenance of endemic communities on islands (de la Torre et al., 2018; Strauss & Irwin, 2004). It shows the importance of molecular methods to add evolutionary context to species interactions. Finally, it shows that even non-endemic species show unique adaptations on islands (Schlaepfer et al., 2011).

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CHAPTER 1: Literature Review

Evolution is defined as any directional or cumulative change in the characteristics of organisms or populations over many generations (Endler, 1986). Evolution, as the origin of change, is directed by natural selection but doesn't fully explain it (Endler, 1986; MacColl, 2011). There are other processes, for example, genetic drift, that can also generate change between generations, though through a random sampling process of alleles (Lande, 1976). Thus, natural selection is a predictable process resulting from biological differences among individuals, potentially leading to genetic change or evolution. Although natural selection cannot explain the origin of new variants or adaptations; it can explain the increased frequency of relatively better adaptations (Endler, 1986; Wade & Kalisz, 1990).

Evolutionary changes within populations often occur over short-time scales, and it is often possible to quantify natural selection on contemporary time scales (Hendry, 2017; Reznick & Ghalambor, 2005). To define if a particular trait is under selection, we need to define three conditions: 1) variation in a trait should exist among individuals, 2) a consistent relationship should exist between that trait and fitness differences (mating ability, fertility, etc.), and 3) a consistent relationship should exist between parents and their offspring for that trait, which is partially independent of common environmental effects (Endler, 1986). When these conditions are met, it results in predictable differences in trait frequency distribution between the trait distribution of parents and offspring when the population is not at equilibrium.

Several methods exist to estimate natural selection either directly or indirectly (Endler, 1986; Schluter, 1988). Often, multiple methods need to be used together to confirm the presence of natural selection. For example, one can show a correlation with environmental variables as selective factors in geographically varying selection, as opposed to traits varying independently of environmental factors (For example, see: (Endler, 1978, 1980)). Another method is long-term

studies of trait frequency distributions. This method assesses whether traits show long-term stability or directional changes over time, which could suggest whether the population is at a selective equilibrium or not (For examples, see: (Dunham et al., 1979; P. R. Grant, 1999; Schluter, 1984)). Finally, another method is genetic demographic or cohort analysis. This method involves gathering data on individuals, including survivorship, and examining whether specific demographic patterns (e.g., survival rates) are associated with particular trait values (fitness differences) (For examples, see: (Arnold, 1983; Arnold & Wade, 1984; Hoffmann & Watt, 1974)). These methods have been applied to different systems, and among those, island ecosystems are the most used to test these concepts.

Island ecosystems, especially oceanic islands, are considered valuable environments for seeking answers to complex questions about natural selection mechanisms and patterns (Bramwell, 2011; Whittaker & Fernandez-Palacios, 2007). Islands have contributed important ideas, theories, models, and tests to mainstream biology. Such as species diversification, trait evolution (Darwin, 1875, p. 18; P. R. Grant, 1999, p. 199; Losos et al., 1998; Schluter, 2000), island biogeography (Burns, 2019; Cody, 2006; Losos & Ricklefs, 2009; Whittaker & Fernandez-Palacios, 2007), and species interactions (Case & Bolger, 1991; J. M. Diamond, 1975; Holt, 1996). Islands are important to natural selection, because they provide relatively fewer complex environments and interactions, and can be used as relative replicating units where field experiments can be done (Losos & Ricklefs, 2009; Reznick & Ghalambor, 2005). Among the different island models, plant organisms are of particular importance. Plants on islands can serve as model systems for investigating the genetic basis of adaptation, natural selection, and speciation. For example, there are a series of island syndromes exclusive to plants (Carlquist, 1974). Including the loss of dispersal (Bowen & Vuren, 1997), loss of flower attractiveness (Bramwell & Caujapé-Castells, 2011; Carlquist, 1974), and development of woodiness (Whittaker & Fernandez-Palacios, 2007). These examples demonstrate that the study of plants on islands can show some general patterns. However, other studies have shown that many island generalizations have their

exceptions that depend again, on the plant taxa, island system, and species interactions found there, which means that studying specific species is still valuable to test island theories and hypotheses (Burns, 2019; Moreira et al., 2021).

These species-specific studies are valuable because they can show the importance of microevolutionary mechanisms (phenotypic divergence, rapid adaptation, and genetic differentiation) in explaining more complex patterns (species interactions, speciation, and adaptive radiation) (Whittaker & Fernandez-Palacios, 2007). These microevolutionary processes, such as founder effects, genetic drift, bottlenecks, and directional selection lead to phenotypic and genetic divergence but do not necessarily involve speciation. Also, these processes can be driven by island-specific species interactions, which may be important in studying island-specific divergence on non-endemic species (Arbogast et al., 2006; Clegg et al., 2002; Emerson, 2002).

On already established and flourishing island ecosystems, studying non-endemic plants can serve as useful models to better understand how these species interact with island-specific mechanisms and how these initial processes of divergence due to island conditions drive subsequent adaptations on these species. In addition, using non-endemic plants can be useful for comparative studies and field experiments to directly calculate natural selection as explained above, and the role of unique island interactions in the process of natural selection of non-endemic species. In this thesis, I focus on the plant *Tribulus cistoides*, a non-endemic plant species, and their interaction with the endemic predator, Darwin's finches (*Geospiza*) on the Galápagos Islands to understand the microevolutionary mechanisms that drive phenotypic divergence in the *Tribulus*-Finch interaction.

Study system

Galápagos Islands

The Galápagos islands are located approximately 1000 km west of the Ecuadorian coast. It comprises 14 main islands, which range between 10 km^2 to 4700 km^2, and over 40 islets (Tye & Francisco-Ortega, 2011). The Galápagos

archipelago likely started to emerge approximately 80-90 million years ago when the Nazca plate moved over a tectonic hotspot (Christie et al., 1992; D. Geist, 1996; D. J. Geist et al., 2014). During the Pleistocene, the Galápagos had a larger area, and land bridges existed between major and minor islands. Also, the presence of drowned seamounts to the east of the oldest islands suggests the emergence and sinking of previous islands to the present. This suggests that older islands could have been colonized as early as 14 million years ago (Heads & Grehan, 2021; Werner et al., 1999). However, the current islands range in age, with the oldest situated in the southeast and dating back around 3.5 million years, while the youngest is in the northwest and approximately 0.4 million years old (D. J. Geist et al., 2014).

In contrast to other islands in the region, the Galápagos climate is notably dry, with a distinct intra-annual seasonality (Hamann, 1979; Itow, 2003). The rainy season is around January to May and the cool and dry season starts from June to December. Within each island, the topography also plays a role in modifying the climate, with higher elevations displaying different climatic zones such as dry highlands, humid highlands, and dry lowlands, while lower elevation islands are predominantly dry (Trueman, 2010). Moreover, major climate patterns such as El Niño Southern Oscillation (ENSO) significantly influence the archipelago's climate, featuring two irregular cyclic events, every three to six years. The warm phase, El Niño, leads to intense rains and an extended warm season, and the cold phase, La Niña, results in colder weather and severe droughts (Riedinger et al., 2002; Snell & Rea, 1999). These climatic factors have considerable effects on vegetation patterns and animal population dynamics on the islands (Parent et al., 2008; Trueman, 2010).

The volcanic origin of the Galápagos Islands implies that all the flora of the Galápagos came from somewhere else (Tye & Francisco-Ortega, 2011). The Islands have a significant affinity with the Andean region of South America, with a smaller portion originating from Central America, the Caribbean, or North America

(Hamann, 2011). The colonization of the islands occurred over considerable time scales, allowing some species to become endemic (Porter, 1978). Native plants from Galápagos are approximately 488 species with approximately 50% of endemic species. Over time vegetation zones started to form based on the island topology (Tye & Francisco-Ortega, 2011; Wiggins & Porter, 1971). It has been suggested that seeds arrived in the archipelago by marine birds, oceanic currents, and wind (Porter, 1978). This thesis, however, does not focus on the endemic plants of the archipelago, rather, it will focus on *Tribulus cistoides*, a widespread plant that is also established on the Islands, and which has a unique role in the island community.

Tribulus cistoides

Tribulus cistoides is a perennial plant species belonging to the Zygophyllaceae family, native to Africa and spread across the world in tropical and subtropical regions, mainly by human activities (Bowman et al., 1983; Porter, 1972; Schweickerdt, 1948). *T. cistoides* typically grow on loose, sandy, or gravel soil and are mainly found by field margins, roads, or paths in arid lowlands (Goeden & Ricker, 1973; Squires, 1979). *T. cistoides* spread on the ground from a central node and have pubescent leaves that are opposite and pinnate, with unequal leaflets (Kearney et al., 2020). *T. cistoides* produces perfect, symmetrical, yellow flowers with 5 petals, measuring 20 – 40 mm in diameter (Wiggins & Porter, 1971, 1971). Flowers and floral glands are a key taxonomic trait that serves to distinguish between *Tribulus* species (Porter, 1971; Schweickerdt, 1948). *T. cistoides* produces a thorny fruit, called a schizocarp, composed of 5 segments, called mericarps. The mericarps are the units of dispersal, they vary morphologically in size and spine number (Scott & Morrison, 1996). Mericarps are dorsally armed with two strong spines and contain two additional seeds separated by transverse partitions. Mericarp size is correlated with seed number and a mericarp can hold between 2 – 6 seeds (Boydston, 1990). Mericarps are produced in great quantity, with a single plant producing hundreds of mericarps per year (Boydston, 1990). The main mechanism of dispersal is by attaching to larger animals using their large thorns

(Boydston, 1990; Kearney et al., 2020). This mechanism also makes humans and human activities excellent vectors of dispersal for *Tribulus* mericarps (E. Johnson, 1932; M. K. A. Johnson et al., 2020). In some places, *T. cistoides* is considered a plague, because it affects cattle when ingested and damages agricultural equipment (E. Johnson, 1932; Schweickerdt, 1948; Squires, 1979). Biological controls are used to limit its growth, such as weevils (*Microlarinus lereynii*, Curculionidae) that eat the seeds (Huffaker et al., 1983; Maddox, 1976; Stegmaier, 1973). Only the Galápagos and Hawaii have reports of bird predation on *T. cistoides* mericarps (Conant, 1988; P. R. Grant, 1981; Pimm, 1988).

Tribulus cistoides most likely arrived at the Galápagos Islands by attaching to large animals. Some suggest that marine birds were most likely the dispersal vector since some species are large enough to carry *Tribulus* mericarps, others suggest that *Tribulus* arrived in Galápagos when humans started activities on the islands (Hooker, 1847b, 1847a; Porter, 1971; Wiggins & Porter, 1971). Species of the genus *Tribulus* were first described on the Galápagos by Hooker (1847a, 1847b), who found at least two genera of Zygophyllaceae in the Galápagos: *Tribulus* and *Kallstroemia*. These genera are closely related phylogenetically (Wu et al., 2015) and they can be identified morphologically, mainly by their flower size and differences in floral gland structures (Wiggins & Porter, 1971). In Galápagos, there is a single species of *Kallstroemia, K. ascendens.* For *Tribulus*, at least two species have been identified: *T. cistoides* and *T. terrestris*.

The coexistence of these two *Tribulus* species in the Galápagos is almost unique to the islands, besides the Caribbean (Pope, 1929). *T. cistoides* and *T. terrestris* are native to two different regions. The first is native to Africa, the latter is native to the Mediterranean (Scott & Morrison, 1996). Although, there are reports of variants of *Tribulus* based on intermediate floral structures, such as nectarine glands, and mericarp size the variation found on Galápagos is not warrantied to be considered subspecies (Hooker, 1847a; Pope, 1929; Porter, 1971; Wiggins & Porter, 1971). In addition, morphological differences exist between island and continental

populations, mainly in flower size, with flowers in Galápagos being smaller than continental samples, with an unequal number of leaflets (Robinson, 1902). This evidence suggests that even if *Tribulus* is not an endemic species to the islands, the unique environmental conditions and origins of the archipelago may provide the mechanisms for divergence in this group. In addition, unique island interactions such as the endemic Darwin's finches or endemic pollinators provide new selection pressures, that could further explain the morphological divergence found on the islands.

Darwin's finches

Darwin's finches are the most well-studied example of rapid adaptive radiation (P. R. Grant & Grant, 2014). There are 15 recognized species of finches, although others mention at least 18 (Rubin et al., 2022), that diverged from a common ancestor approximately 1.5 million years ago (Lamichhaney et al., 2015; Sato et al., 2001). Darwin's finches evolved different beak shapes and sizes to adapt to various food resources (P. R. Grant, 1999; P. R. Grant & Grant, 2014). Several factors contributed to the rapid species radiation of finches such as isolation, unique ecological niches, natural selection, and gene flow (De León et al., 2012; P. R. Grant & Grant, 2006; Lamichhaney et al., 2015; T. D. Price et al., 1984; Rubin et al., 2022). In addition, changes in climate, mainly seasonality and ENSO altered available food resources and influenced the evolution of finch groups. Climate conditions lead to later divergence between the two main groups of finches: the tree finches (specialized insect feeders on trees) and the ground finches (specialized seed feeders on the ground) are linked to climate (Lamichhaney et al., 2015). These different niches lead to changes in beak shape, with tree finches having pointier and narrower beaks to feed on insects, and ground finches having blunt and deeper beaks, which enable them to crack open seeds. Genetic studies later revealed further evidence of the genes involved in beak divergence, linking some genes related to craniofacial and beak development (Lamichhaney et al., 2015, 2016).

Among Darwin's finches, the seed predators or ground finches (*Geospiza*) show the most specialization. The adaptive evolution of finch beaks has been extensively studied within the ground finch group, with their diets closely linked to beak morphology and food availability (De León et al., 2011, 2012; P. R. Grant & Boag, 1980; T. D. Price et al., 1984; Schluter, 1982b; Schluter & Grant, 1984). For example, although finch diets can widely overlap when resources are abundant, finches switch to specialized food sources when resources start to diminish (De León et al., 2014; P. R. Grant & Grant, 2014). In these conditions, the large ground finch (*Geospiza magnirostris*), which has the largest beak, leans towards larger and harder seeds; the medium ground finch (*G. fortis*), eats medium-sized seeds; the small ground finch (*G. fuliginosa*), which has the smallest beak, eats smaller seeds; and the cactus finch (*G. scandens*), with a longer beak specialized in flowers and fruits of *Opuntia* cacti (De León et al., 2014; Smith et al., 1978, p. 1). It is also during this time that ground finches are under the most selective pressure for beak morphology. In a 40-year study on Daphne Major Island, Grant, and Grant (2014), found that during an intense drought, the scarcity of food led to a decline in the *G. fortis* population. The finches adapted by selecting larger and harder seeds, causing directional selection for larger beaks. However, the study also showed that strong directional selection over short timescales does not always predict long-term evolutionary dynamics, as beak morphology selection varies with unpredictable climate changes.

Finches are selective feeders, preferring seeds and fruit that they can break and eat, based on size and hardness, leading to strong selection on beak morphology. This close interaction between diet and beak preferences may result in co-evolutionary interactions between finches and plants, where seed traits selected by finches trigger evolutionary changes in both species. One example, and the focus of this thesis, is the interactions of ground finches and *Tribulus cistoides* mericarps. The Finch-*Tribulus* interaction becomes more prevalent during dry years/seasons. Also, *T. cistoides* mericarps are available all year round, being during the dry times one of the few food sources for finches, imposing selection on finch beak size during

these conditions. Finally, and most importantly, only *G. fortis* and *G. magnirostris* can eat *Tribulus* seeds, selecting for potentially specific fruit traits (P. R. Grant, 1981).

Observations of finch feeding behavior on *Tribulus* seeds have indicated differences in foraging efficiency based on bill size. Larger-billed finches were more effective at extracting seeds from the mericarps, leading to variations in feeding preferences among species (P. R. Grant, 1981, 1999), this is also true for the Hawaiian species, the Laysan finch (Conant, 1988; Pimm, 1988). *Tribulus* appears to be rarely consumed by other birds in the Galápagos. This thesis explores whether finches could be influencing the morphology of *Tribulus* through their feeding behavior and if this potential interspecific selection could explain the differences observed in mericarp traits among individuals and populations within the islands.

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Linking statement 1

As mentioned in the Literature Review, natural selection is a predictable process that results in evolution or change. Natural selection involves changes in a phenotype or groups of phenotypes frequencies on average among populations of a species. These changes are related to interactions with the environment or with other species. It is possible to quantify selection in short-time scales and is common to observe that species interactions can also influence the selection of certain traits. There are specific conditions that must be met to conclude that a trait is under the process of natural selection: 1) if there is phenotypic variation, 2) if the trait is related to the fitness of the individual and 3) if it has a genetic component that can be inherited.

In the following chapter, we will describe the phenotypic variation of the fruits and flowers of *Tribulus cistoides* in the context of island and mainland populations. As mentioned above, *Tribulus* is a plant that because of its unique context in the Galápagos Islands has the potential to be a model for understanding microevolutionary processes. Specifically, how endemic predators drive phenotypic divergence of plant traits.

The next chapter focuses directly on the fruits and flowers of *Tribulus cistoides*. Using samples collected in the field and herbarium collections, the chapter describes the phenotypic variability of these traits and demonstrates that phenotypic variation and divergence exist among populations.

CHAPTER 2: Phenotypic divergence of traits that mediate antagonistic and mutualistic interactions between island and continental populations of the tropical plant, *Tribulus cistoides* (Zygophyllaceae)

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This chapter is published in *Ecology and Evolution* (2023) 13, e9766.

Abstract

Island systems have long served as a model for evolutionary processes due to their unique species interactions. Many studies of the evolution of species interactions on islands have focused on endemic taxa. Fewer studies have focused on how antagonistic and mutualistic interactions shape the phenotypic divergence of widespread nonendemic species living on islands. We used the widespread plant *Tribulus cistoides* (Zygophyllaceae) to study phenotypic divergence in traits that mediate antagonistic interactions with vertebrate granivores (birds) and mutualistic interactions with pollinators, including how this is explained by bioclimatic variables. We used both herbarium specimens and field-collected samples to compare phenotypic divergence between continental and island populations. Fruits from island populations were larger than on continents, but the presence of lower spines on mericarps was less frequent on islands. The presence of spines was largely explained by environmental variation among islands. Petal length was on average 9% smaller on island than continental populations, an effect that was especially accentuated on the Galápagos Islands. Our results show that *Tribulus cistoides* exhibits phenotypic divergence between island and continental habitats for antagonistic traits (seed defense) and mutualistic traits (floral traits). Furthermore, the evolution of phenotypic traits that mediate antagonistic and mutualistic interactions partially depended on the abiotic characteristics of specific islands. This study shows the potential of using a combination of herbarium and field samples for comparative studies on a globally distributed species to study phenotypic divergence on island habitats.

Introduction

Islands have long served as models for understanding the processes that shape the evolution of life. Species living on islands provide powerful systems for testing evolutionary hypotheses and theories (Bramwell & Caujapé-Castells, 2011; Losos & Ricklefs, 2009; Whittaker & Fernandez-Palacios, 2007). The appeal of island systems comes from their unique species diversity and simplified species interactions, making it easier to identify the drivers of adaptive evolution (Barrett, 1996; P. R. Grant, 1998; Traveset & Navarro, 2018). Moreover, large differences in the biotic and abiotic environments between island and continental habitats can lead to divergent selection between conspecific populations, potentially leading to phenotypic differentiation of island populations and speciation (Whittaker & Fernandez-Palacios, 2007). Here we compared conspecific populations of a globally distributed tropical plant, *Tribulus cistoides* L. (Zygophyllaceae), to understand whether divergent antagonistic and mutualistic communities between islands and continental habitats drive divergent phenotypic plant traits that mediate species interactions.

Island and continental habitats frequently differ in their biotic communities. Islands typically have fewer native mammalian herbivores, favouring birds and reptiles with higher dispersal capacity over water (Burns, 2019). This discrepancy can lead to the evolutionary loss of antiherbivore defences in plants (Baier & Hoekstra, 2019; Cummins et al., 2020). For example, spines largely evolve as protection against vertebrate herbivores, and in the case of the Island Bush Poppy (*Dendromecon rigida harfordii*) on the Island of Santa Cruz, California, where these plants evolved reduced spines due to a historical lack of herbivores (Bowen & Vuren, 1997). However, the loss of antiherbivore defences on islands is not universal for all species (Meredith et al., 2019; Monroy & García-Verdugo, 2019; Moreira et al., 2021). The Hawaiian Prickly Poppy (*Argemone glauca*) evolved greater spine density than their continental sister species (*A. mexicana*), putatively because of selection by an extinct herbivorous duck that was common on Hawaii (Hoan et al.,

2014). Additionally, a recent meta-analysis of plant defences found no significant difference in either plant physical or chemical defences between insular and continental plant populations, and in fact there was a trend for physical defences to be higher on islands (Moreira et al., 2021). This range of results show how variation in antagonistic interactions between island and continental communities can influence evolutionary processes of defence traits. However, there is still the need for more studies of insular plant-animal interactions to understand the conditions that lead to the evolution for increased versus decreased defences on islands compared to continental populations.

Mutualistic interactions also frequently differ between island and continental communities, with the diversity of mutualists (e.g., pollinators and dispersers) typically being lower on islands. It is often predicted that the absence of mutualistic species could lead to the loss of traits that mediate mutualistic species interactions on islands (Janzen, 1973). Specifically, in the case of pollination, pollinators tend to be less diverse and less specialized on islands than on the continent (Barrett, 1996; Burns, 2019; Traveset & Navarro, 2018). Less specialized pollinators can give an advantage to more generalized flowers, and lead to the evolution of selfing and wind-pollination and thus smaller attractive structures (Bramwell & Caujapé-Castells, 2011; Burns, 2019; Carlquist, 1965). Various studies support these observations (Inoue & Amano, 1986; Martén-Rodríguez et al., 2015; Yamada et al., 2010). However, as with antiherbivore defences, there is large variation in results, calling into question whether general predictions can be made. A recent comparative analysis between continental insect-pollinated taxa and their island endemic sister taxa showed that on average there was no overall reduction in flower size on islands, although specific lineages (e.g. Asteraceae, Solanaceae) and island groups (e.g. Galápagos, Revillagigedo Islands) did fit that expectation (Hetherington-Rauth & Johnson, 2020). These results show that the evolution of reproductive traits such as flower size on islands is species-specific and context dependent, making it difficult to generalize. Thus, warranting further research that

investigates divergent evolution of reproductive traits between island a continental population (Burns, 2019), which our study seeks to address.

Tribulus cistoides (L., Zygophyllaceae) is an excellent system to study the phenotypic variation of reproductive traits on islands in response to species interactions. *T. cistoides* is found on many tropical islands and continents throughout the world. The nature of *T. cistoides* fruits makes them ideal to be carried by larger animal and/or seabirds, which facilitate their arrival to islands, with the possibility of many native populations (Hooker, 1847a; Porter, 1971). In the same way, humans are also effective dispersers and have helped the plant distribute throughout the world (M. K. A. Johnson et al., 2020). Classic expectations for the evolution of *Tribulus* antiherbivore defences of their fruits are complex owing to the evolution of endemic granivores on some island archipelagos. With respect to mutualistic interactions, *T. cistoides* in continental populations are typically pollinated by a diversity of insects, including bees and butterflies (Huffaker et al., 1983). On islands, *T. cistoides* is mainly pollinated by an endemic community of pollinators. These attributes make *T. cistoides* well suited to study how the unique communities and environment of islands affect the phenotypic divergence of traits associated with antagonistic and mutualistic interactions (Carvajal-Endara et al., 2020; Morrison & Scott, 1996; Rivkin et al., 2021; Scott & Morrison, 1996).

Here we investigate whether *T. cistoides* exhibits phenotypic divergence in traits associated with antagonistic and mutualistic interactions across continental and island habitats. Our main question was: How does insularity affect phenotypic divergence in plant reproductive traits that mediate species interactions with antagonist vertebrate granivores (mericarp size and number of spines) and mutualistic pollinators (flower size)? We expect that plant traits that mediate species interactions will diverge between island and continental populations due to differences in community interactions and/or divergent environmental conditions found on islands. Specifically, for antagonistic interactions, we expect that *T. cistoides* fruits to be larger and have more spines (better defended) on islands where

vertebrate granivores are present whereas on the continent there are mainly insect predators. For flowers that mediate mutualistic interactions with pollinators, we expect that island *T. cistoides* populations will evolve smaller flowers because islands generally have depauperate and generalised pollinator communities compared to the continent (Burns, 2019). Our study uses a combination of field collected samples and multiple herbaria samples to account for both fruit defensive traits and floral mutualistic traits. Fruit samples were collected from the field and from herbarium collections and floral traits were collected exclusively from herbarium samples (Appendix 2.1). The inclusion of herbarium samples allowed us to test our expectations more broadly and to compare multiple continental and island populations throughout the world.

Materials and methods

Study system

Tribulus cistoides is a perennial plant that is widely distributed in tropical and subtropical regions across the world (Porter, 1971, Appendix 2.2). Plants spread on the ground via long prostrate stems that radiate out from a central root stock (Kearney et al., 2020). *T. cistoides* has perfect flowers with five petals arranged in a radially symmetric pattern, measuring 20 - 40 mm in diameter (Porter, 1971; Wiggins & Porter, 1971). Petals have nectaries at their base, and although they can self-pollinate, they are usually outcrossed by insect pollinators (Porter, 1971). Plants typically grow in well-drained sandy or gravel soil on beaches, loose soil by field margins, roadsides or paths, and arid lowlands (Goeden & Ricker, 1973; Squires, 1979). *Tribulus cistoides* produce hard fibrous fruits called schizocarps, which have five individual segments called mericarps, each containing 1-7 seeds (Figure 2.1). As mericarps mature, they dry and fall adjacent to the plant. Mature mericarps can hold viable seeds for many years (Goeden & Ricker, 1973; E. Johnson, 1932). Mericarp changes are minimal once they fall from the plant, although spines tend to wear and break over time due to dispersal (Ernst & Tolsma, 1988; Scott & Morrison, 1996). Mericarps vary substantially in overall size, as well as the number

and length of spines. Spine size and number can change due to selection for both dispersal (M. K. A. Johnson et al., 2020) and protection against avian granivores (Carvajal-Endara et al., 2020) (Figure 2.1).

Antagonistic interactions such as seed predation differ between continental and island populations of *Tribulus*. Insect predation is prominent on continental populations, where weevils (e.g. *Microlarinus lareynii, M. lypriformis* (Coleoptera: Curculonidae)) are used as a control agent to prevent *Tribulus terrestris* from spreading on cropland (Huffaker et al., 1983), and the weevil also attacks *T. cistoides* (Maddox, 1976; Stegmaier, 1973). Other studies report predation by cattle, although this is not intentional and potentially harms the animal (E. Johnson, 1932; Squires, 1979). Bird predation of *T. cistoides* seeds has been observed on Laysan Island in Hawaii (Conant, 1988), but is best known from the Galápagos islands (Carvajal-Endara et al., 2020; P. R. Grant, 1981). Several species of ground finch (*Geospiza* spp.) feed on the seeds of *T. cistoides*, and their feeding behaviour differs among species depending on their beak size. The largest beaked species, *Geospiza magnirostris* and *Geospiza conirostris*, crack mericarps more quickly than the medium ground finch *Geospiza fortis* (P. R. Grant, 1981). Being able to crack *T. cistoides* mericarps increases the survival of *G. fortis*, especially during dry years when preferred seeds of other species are depleted (P. R. Grant, 1981; P. R. Grant & Boag, 1980). Correspondingly, *T. cistoides* imposes selection on *G. fortis* beak size (Boag & Grant, 1981), which drives rapid adaptive evolution (Boag & Grant, 1981). Finch predation, in turn, imposes selection on *T. cistoides* mericarp morphology (Carvajal-Endara et al., 2020). Mericarp size and spine number affect the probability of seed predation by finches. Specifically, the presence of lower spines (Figure 2.1) decreases predation in populations where *G. fortis* are present, but it does not affect predation by *G. magnirostris* (Carvajal-Endara et al., 2020).

Mutualistic interactions, such as plant-pollinator interactions, also differ for *Tribulus* between island and continental communities. In continental communities, *T. cistoides* interacts with a more diverse array of generalist and specialized insects,

such as Hymenoptera (mainly various species of Apidae but also Scolidae), Diptera, Coloeptera, Lepidoptera, and Thysanoptera, to name a few groups (Austin, 1972; Reddi, 1981). On the Galápagos Islands, *T. cistoides* is considered a network hub for endemic and introduced pollinators alike (Traveset et al., 2013). Its most generalized pollinator is the endemic carpenter bee *Xylocopa darwinii* (Hymenoptera). Apart from another endemic, *Leptotes parrhasioides* (Lepidoptera), its other pollinators include introduced insects: a lycaenid, a wasp (Hymenoptera), and a hoverfly (Diptera) (Traveset et al., 2013).

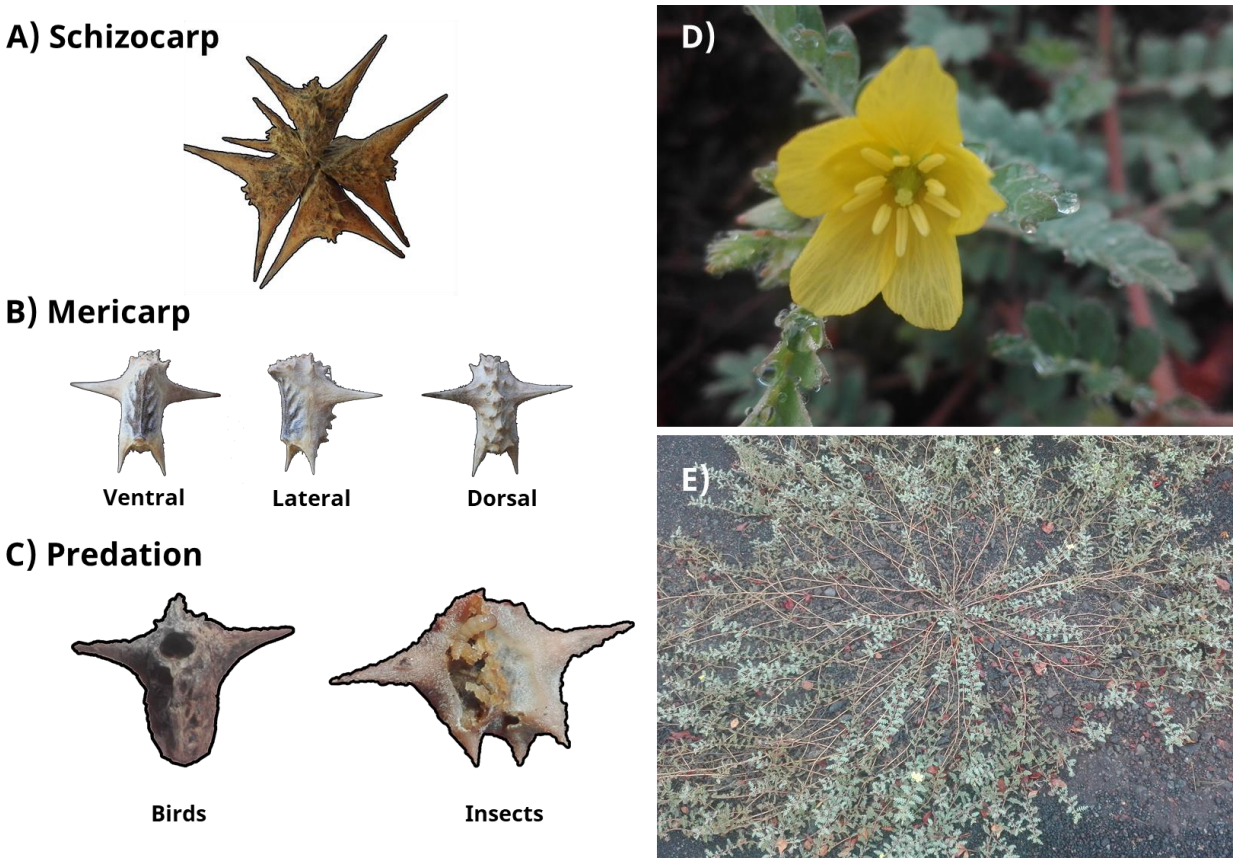

Figure 2. 1 Morphology of Tribulus cistoides fruits and flowers. (A) A mature *T. cistoides* schizocarp, containing four developed mericarps plus one underdeveloped mericarp. (B) *T. cistoides* mericarps, showing their upper and lower spines. C) Mericarp predation. The left mericarp was depredated by birds, showing the open gap that remains after seed removal. At right is a mericarp being fed on by insect larvae. (D) Flower showing both male (anthers) and female (pistil) parts. (E) An individual *T. cistoides*, showing prostrate growth habit.

Data collection

Mericarps – Mericarps (n = 5084) were collected from field and herbarium samples. Field samples were collected from Galápagos and Florida. Herbarium samples were collected from the California Academy of Science (CAS), the Missouri Botanical Garden (MOBot), Harvard University Herbarium (HUH), and the Charles Darwin Research Station Herbarium (CDRS). Mericarp samples were collected between 1873 and 2018, and across 12 countries on three continents (Figure 2.2, see Appendix 2.1 for details on sample size). Linear mixed models were used to account for the unbalanced design as described below (see Statistical Analyses).

The morphology of mericarps was characterised by measuring five traits. These traits included mericarp length (mm), width (mm), depth (mm), spine tip distance (mm) (hereafter "spine size"), and the presence/absence of lower spines (See Results section). These traits were included because they vary among mericarp populations (Appendix 2.3) and they have been shown to be subject to selection by Darwin's finches in past studies (Carvajal-Endara et al., 2020; P. R. Grant, 1981; Rivkin et al., 2021). For herbarium mericarps, we only measured mericarps that had complete spines, and we did not measure mericarps that showed damage.

Flowers – We characterised floral morphology from herbarium specimens. We obtained high-resolution images of specimens (n = 772) from the Smithsonian Institute Herbarium, the Harvard University Herbarium (HUH), and the Charles Darwin Research Station Herbarium (CDRS). Collection dates ranged from 1800 - 2014 and included samples from 42 countries across five continents (Fig 2.2, see Appendix 2.1 for details on sample size). We focused on flower size quantified as the length of petals because flower size is a key trait influencing pollinator attraction, and this trait could be reliably measured from most flowering herbarium samples. Petal length (mm) was measured from the base to the tip of the petal, from up to three separate flowers per plant (See Results section). All measurements were performed using ImageJ (Schneider et al., 2012).

Bioclimatic data – We downloaded the data from the WorldClim database at a 30s resolution (~1 km^2) (Fick & Hijmans, 2017). We used variables Bio1 (Annual Mean Temperature), Bio4 (Temperature seasonality), Bio12 (Annual precipitation) and Bio15 (Precipitation seasonality). The locations coordinates and climate data were matched in QGIS (version 3.18.2-Zürich) (QGIS Development Team, 2022). We used the tool Fill No Data by a maximum distance of 10 pixels to project the climate information and reduce NAs from locations that may be too small to have estimated data. Then, we extracted the bioclimate information using the Sampled Raster Values tool and included the estimated data into our mericarp and flower datasets. In addition, we used the projected bioclimate estimates of Weigelt et al. (2013) for specific locations that we were unable to extract using the projected maps (Shungu-Mbili island, Tanzania; Heron Island, Australia, the Kure, Pearl and Hermes Atolls, Hawaii; and the Lucayan Islands, Bahamas). However, for these locations, Weigelt did not estimate Bio4.

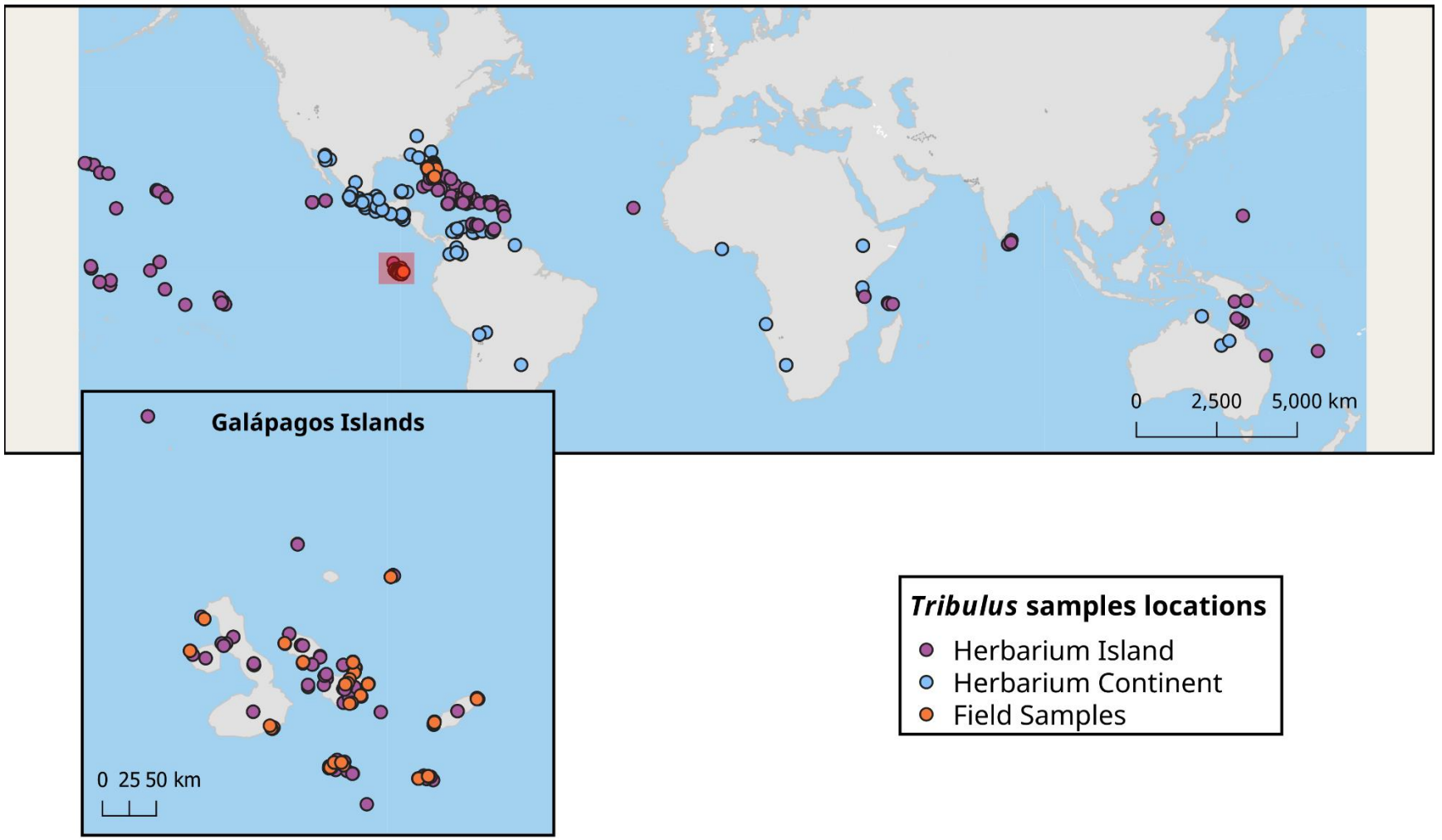

Figure 2. 2 Distribution of samples of *Tribulus cistoides* collected for this study. Most samples around the world were collected from herbarium collections. Field samples were collected by the authors are marked as orange circles including samples from Galápagos and Florida. In the large map, the Galápagos archipelago is outlined in red, with a blow-up of the archipelago shown as an inset. The mericarp dataset was collected mainly from a combination of field collected samples and herbarium vouchers. The flower dataset was exclusively collected from herbarium samples. See Appendix 2.1 for details on sample numbers for each location.

Statistical Analyses

We used linear mixed-effects models implemented in R version 4.0.3 (R Core Team, 2022). Our analytical approach involved the use of two models. Model 1 compared differences between populations located on continental versus island habitats. We used the definition of true oceanic islands mentioned by Whittaker and Fernández-Palacios, as land surrounded by water (2007). For this distinction, we used the definition mentioned by Model 2 focused on islands only and compared populations on the Galápagos versus other island systems. We used the *lmer* package (Bates et al., 2022) for the analysis of most traits, except for the presence of lower spines, which were fitted to binomial and negative binomial type II distributions, respectively, with a log link function implemented in the *glmmTBT* package (Brooks et al., 2017). Trait values typically varied among years, and so year of collection was included as a quantitative covariate. We also included whether samples came from herbarium or field samples, to test any potential effect of shrinkage due to age, and sample ID was treated as a random effect to reflect the non-independence of multiple measurements made per sample. Our full statistical model (Model 1) for testing the effects of islands versus continents on traits was: $trait \sim continental/island + year + herbarium + (1 | ID)$. For flower size, we also contrasted the Galápagos islands versus other islands using the following model (Model 2): $petal\ length \sim Galápagos/other\ island + year + (1 | ID)$. Model 2 did not include the herbarium covariate because all flower samples came from herbaria. We omitted model 2 in our mericarp dataset because we did not have enough samples from other islands to perform a robust analysis (Appendix 2.1). Sample ID allowed us to take multiple measurements from a single location, allowing us to accurately estimate the effects of each factor in the model without pseudoreplication, while accommodating the unbalanced sampling design inherit to using a mixture of field and herbarium samples. Sample ID referred to a single herbarium specimen or single field location for field samples. Year of collection was significant for some traits, and it allowed us to partition temporal trends in plant traits that may be associated with phenotypic change or collector bias. There was no significant

difference between herbarium and field samples. For lower spines, the model differed, and we removed the effect of the year: lower spines ~ continental/island + herbarium + (1 | ID) because the model would not converge otherwise.

We used the *Anova* function from the car package (Fox et al., 2012) and fit the models to Type II sums-of-squares to test for the significance of fixed effects in the model, with marginal means estimated using the package *emmeans* (Lenth et al., 2022). We used the *Dharma* package (Hartig & Lohse, 2022) to assess whether residuals met assumptions of homogeneity of variance and normality in *lmer* models. Based on these diagnostics, we assessed whether the raw data or transformed data better fit model assumptions. For mericarp traits in model 1, untransformed values of mericarp length and depth met model assumptions, whereas width was square root transformed. For spine size, 3.1% of mericarps (n = 158) lacked upper spines (Appendix 2.3), but only on the Galápagos. Even so, no difference was evident between continental and island populations for the presence/absence of upper spines ($\chi^2 = 0.7423$, p = 0.3889), and so we removed all mericarps lacking upper spines from subsequent analyses of this trait. We further removed two large outliers (residuals>| 9 |) for spine size. For flower traits in model 1, we filtered outliers (residuals >| 5 |). For model 2, we square root transformed petal length to meet assumptions of ANOVA.

We re-ran the models described above to include all bioclimatic variables as covariates in Model 1 and Model 2 to understand whether abiotic environmental variables helped to explain phenotypic divergence: *trait ~ continental/island + year + herbarium + Bio1 + Bio4 + Bio12 + Bio15 + (1 | ID)* (Model 1); *petal length ~ Galápagos/other island + year + Bio1 + Bio4 + Bio12 + Bio15 + (1 | ID)* (Model 2); and *lower spines ~ continental/island + herbarium + Bio1 + Bio4 + Bio12 + Bio15 + (1 | ID)* for the presence of lower spines. We expected that the first set of analyses without bioclimatic variables would show whether there is an overall effect of island on phenotypic evolution. The second set of models that included bioclimate variables, tested if the climate of the island drove the results instead of insularity

per se (i.e., bioclimate variables were significant and continent/island became non-significant after being initially significant), or if there was an effect of island independent of climate, which would indicate that insularity of plant-animal interactions itself influences evolution (island/continent is significant after including bioclimate variables).

Given our unequal replication between sampling location, we considered three different approaches to further asses the robustness of our results for mericarps. First, we took the mean trait value from each sampling location and reran the analyses to test for divergence between island and continental populations. Second, we removed some individual herbarium vouchers that account for whole island systems to further reduce potential individual bias. We removed samples from two island systems, Cape Verde (n = 3) and Shungo-Mbili Island (n=5) and reran the analysis between island and continental populations. Finally, to assess the unbiased sampling effort from Galápagos, which accounts for most of our field collected samples (n=3245). We removed Galápagos from the analysis and reran the models with only samples from other island systems. Then, we reran the analysis using only the Galápagos samples to compare results. All these analyses showed similar effects and results to the original analyses and are presented in the supplements (Appendix 2.4, 2.5, 2.6, and 2.7, respectively).

Finally, we used multivariate analysis to further explore how mericarp morphology differed between continental and island populations because mericarp length, width, depth, and spine size strongly covary (Appendix 2.8). First, we normally standardised each variable using the scale function in R and performed principal component analysis (PCA) using the *prcomp* function. We visualised the PCA using the *FactoExtra* package in R (Kassambara, 2017). Then, we extracted the scores from PC 1 and used the values to fit model 1 used for the univariate analysis above. We used the *Anova* function to test for the significance of the effect of habitat and bioclimate variables. We performed multivariate analysis for the additional analyses mentioned above when applicable (Appendix 2.4, 2.6 and 2.7).

Results

Phenotypic divergence between island and continental habitats

Mericarp morphology

Mericarps phenotypically diverged between island and continental populations. Mericarps were on average 7% longer, 6% wider, and 12% deeper on islands compared to continental populations. Spine size was also 6% longer on islands (Table 2.1). At the same time, lower spines were 59% more common in continental populations than on islands (Figure 2.3). When we included bioclimatic variables in analyses, the effect of island/continent were qualitatively similar in the direction of effect but became non-significant for length ($P = 0.388$), width ($P = 0.132$), spine size ($P = 0.393$), and lower spines ($P = 0.215$), while it remained significant ($P= 0.01$) for mericarp depth. Bioclimatic variables significantly explained variation in multiple traits: BIO 4 (Temperature Seasonality) predicted variation in mericarp length and BIO 15 (Annual precipitation) predicted mericarp depth (Table 2.2). All bioclimatic variables (Annual Mean Temperature, Temperature Seasonality, Annual precipitation, and Precipitation Seasonality) predicted variation in the presence/absence of lower spines (Table 2.2). These changes in the significance of the effect of islands implies that some of the divergence in mericarp traits is explained by variation in bioclimatic differences between islands and continents instead of the insularity of plant-animal interactions itself (Table 2.2).

Table 2. 1 Model estimates of the effects of population and year of collection on mericarp and flower traits. A-B) Model 1 estimates from continental and island populations. A) Individual mericarp traits and mericarp size (PC1). B) Petal length. C) Model 2 estimates from the effect of Galápagos and other non-Galápagos islands populations on petal length.

A) Mericarp – continental vs island						
Trait	**Continental/Island**		**Year**		**Field/Herbarium**	
	χ^2	**P**	**χ^2**	**P**	**χ^2**	**P**
Length	**14.139**	**<0.001**	**6.176**	**0.012**	0.489	0.484
Width	**12.047**	**<0.001**	3.228	0.072	0.012	0.91
Depth	**51.506**	**<0.001**	**11.107**	**<0.001**	0.309	0.578
Spine size	**5.85**	**0.015**	0.731	0.392	1.077	0.299
Lower spines	**77.921**	**<0.001**	-	-	3.254	0.071
Mericarp Size (PC1)	**24.992**	**<0.001**	7.298	0.006	0.198	0.655
B) Flowers – continental vs island						
Petal length	1.386	0.239	15.623	<0.001	-	-
C) Flowers – Galápagos vs other islands						
	Galápagos/Other islands		**Year**		**Field/Herbarium**	
	χ^2	**P**	**χ^2**	**P**	**χ^2**	**P**
Petal length	157.147	<0.001	9.453	0.002	-	-

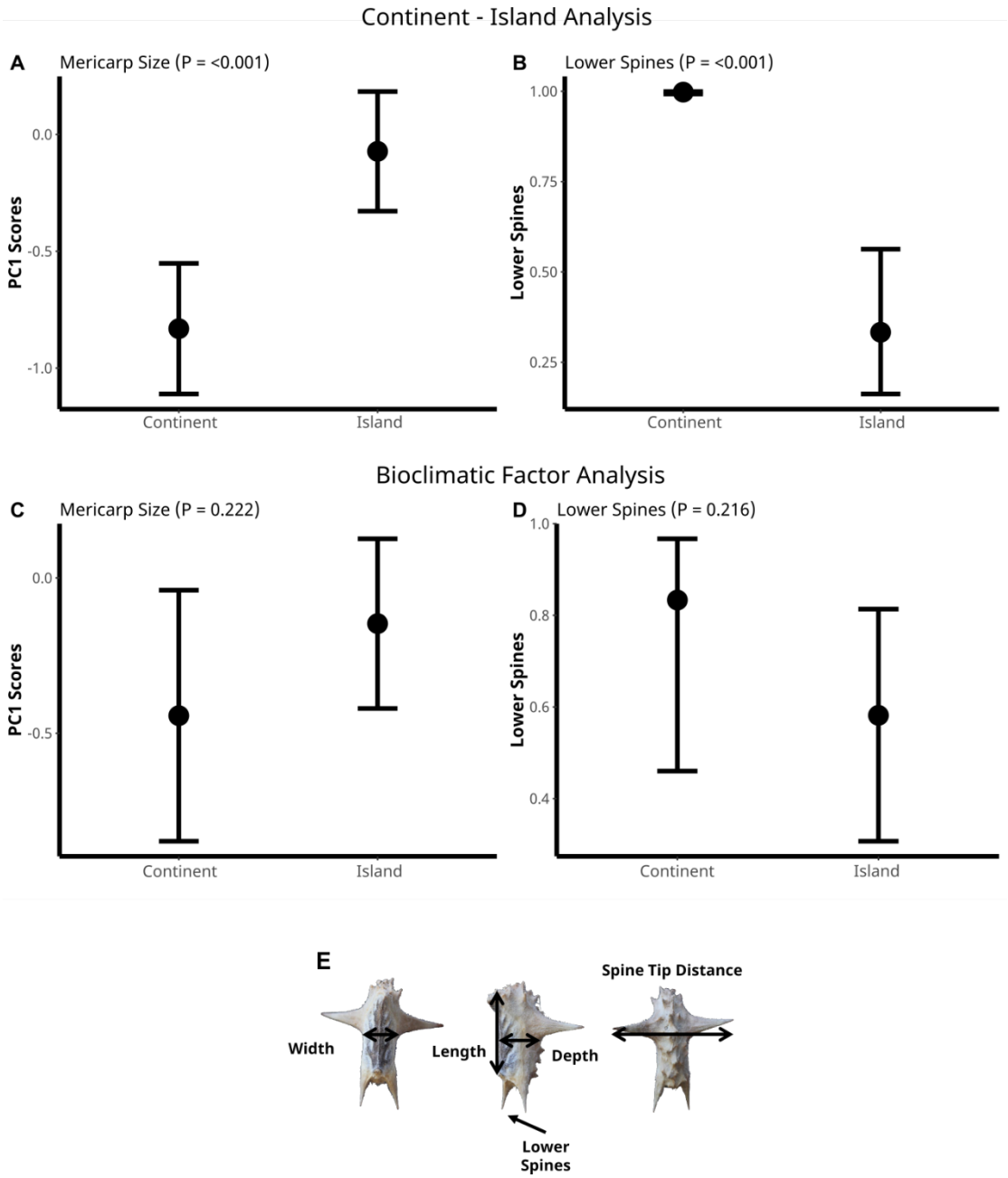

Figure 2. 3 Mericarp traits compared between island and continental locations. Plots show the least-squares mean estimates (± 1 SE) using PC1 as a summary of mericarp size (length, width, depth, and spine size) and the presence or absence of lower spines. P-values correspond to the difference between island and continental plants. (A-B) Estimates of continental and island populations only. (C-D) Estimates of the island effect from the model after accounting for bioclimatic variation. (E) Diagram of mericarp measurements: Length was measured along the ventral border of the mericarp where the seeds are contained within. Width was measured as the distance across the base of the upper spines. Depth was measured as the distance from the ventral and dorsal border in the middle of the mericarp. Spine size was the distance between the upper spine tips. Lower spines were considered present if they were longer than 1 mm and located at the base of the mericarp.

Table 2. 2 Model estimates of the effects of population, year of collection, and bioclimatic variables on mericarp and flower traits. Nomenclature on bioclimate variables was taken from the WorldClim dataset (https://worldclim.org/). We used variables Bio1 (Annual Mean Temperature), Bio4 (Temperature Seasonality), Bio12 (Annual precipitation), Bio15 (Precipitation Seasonality). A-B) Model 1 estimates from continental and island populations. A) Individual mericarp traits and mericarp size (PC1). B) Petal length. C) Model 2 estimates from the effect of Galápagos and other island populations on petal length.

A) Mericarp – *continental vs island*														
Trait	**Continental/Island**		**Year**		**Field/ Herbarium**		**Bio1**		**Bio4**		**Bio12**		**Bio15**	
	X^2	**P**	**X^2**	**P**	**X^2**	**P**	**X^2**	**P**	**X^2**	**P**	**X^2**	**P**	**X^2**	**P**
Length	0.664	0.414	**5.646**	**0.017**	0.006	0.937	0.067	0.794	**4.373**	**0.036**	2.252	0.133	3.097	0.078
Width	2.202	0.137	2.829	0.092	0.028	0.865	0.214	0.643	0.845	0.357	0.077	0.78	2.547	0.11
Depth	**5.669**	**0.017**	**11.402**	**<0.001**	0.586	0.443	0.132	0.716	0.119	0.729	0.466	0.494	**6.879**	**0.008**
Spine size	0.597	0.439	0.597	0.408	0.495	0.481	0.563	0.453	0.553	0.456	1.562	0.211	0.049	0.824
Lower spines	1.53	0.216	-	-	0.001	0.97	**8.605**	**0.003**	**10.701**	**0.001**	**19.497**	**<0.001**	**18.157**	**<0.001**
Mericarp Size (PC1)	1.487	0.222	**7.384**	**0.006**	0.067	0.795	0.042	0.836	1.941	0.163	2.345	0.125	2.934	0.086
B) Flowers – *continental vs island*														
Petal length	**10.043**	**0.001**	**15.95**	**<0.001**	-	-	**13.657**	**<0.001**	**15.299**	**<0.001**	2.167	0.14	**18.533**	**<0.001**
C) Flowers – *Galápagos vs other islands*														
Trait	**Galápagos/Other islands**		**Year**		**Field/ Herbarium**		**Bio1**		**Bio4**		**Bio12**		**Bio15**	
	X^2	**P**	**X^2**	**P**	**X^2**	**P**	**X^2**	**P**	**X^2**	**P**	**X^2**	**P**	**X^2**	**P**
Petal length	**84.867**	**<0.001**	**5.188**	**0.022**	-	-	1.909	0.167	0.471	0.492	2.622	0.105	0.522	0.47

Our additional analysis showed the same trend. There was a general effect of increased mericarp size that was later lost after accounting for environmental factors which explained the observed variation (Appendices 2.6 and 2.7). However, we found that lower spines were not significant when we removed the Galápagos from analysis (P = 0.246) (Appendix 2.6, Table S2.7).

Multivariate analysis explained 86% of the variation in mericarp morphology and further supported the univariate analyses, showing that mericarps differed between continental and island populations, but also becoming non-significant when bioclimatic variables were added (Figure 2.3) (Table 2). PC_1 explained 71% of the variance in mericarp morphology and was mostly associated with mericarp size (length, depth, width), and PC_2 explained 15% of the variance and was mainly associated with spine size (Figure 2.4).

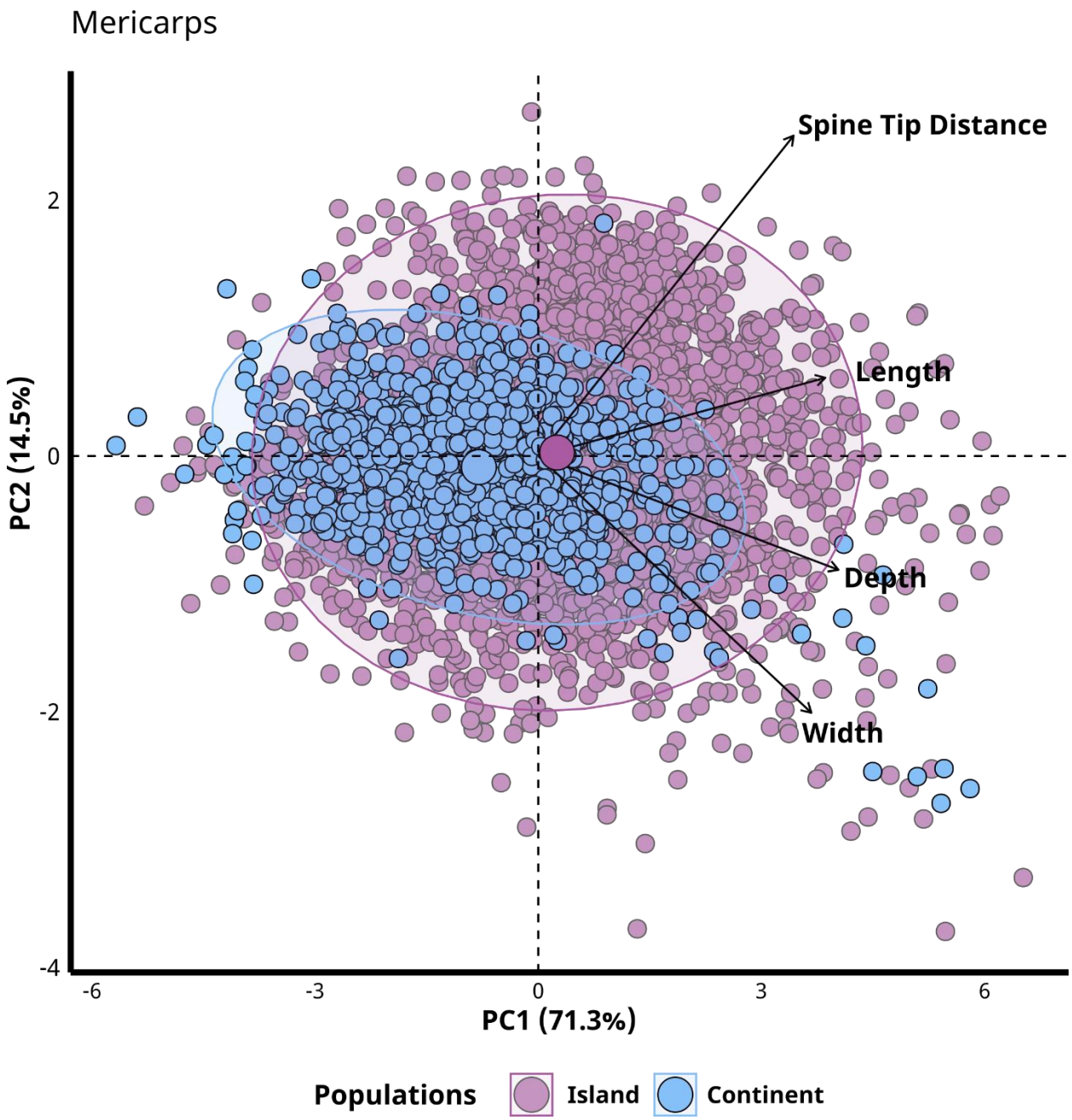

Figure 2. 4 Principal component analysis of mericarp traits. Points represent all individual mericarps sampled. Vectors are proportional to the contribution and direction associated with each trait. Groups are separated into island and continental populations. Larger circles represent the centroid of the ellipses with a 95% confidence interval. Although individual mericarps are shown here, statistical tests between island/continental sites were based on scores along PC1 fit to a GLMM using Model 1, which accounted for non-independence of mericarps from the same sampling location.

Flower size

Flower size differed between island and continental habitats, but these effects were only apparent after accounting for bioclimatic variation among sample sites (Figure 2.5) (Table 2.2). When we fit Model 1 there was no clear effect of island/continent (P = 0.239, Table 2.1), but when we included bioclimatic variables the effect of island/continent became highly significant (P = 0.001, Table 2.2), with petals on the continent being on average 9% longer than petals on islands. BIO1 (Annual Mean Temperature), BIO4 (Temperature Seasonality), and BIO15 (Precipitation Seasonality) all predicted variation in petal size (Table 2.2). This result shows that abiotic factors have a large impact on divergence of flower size among sampling locations, and island/continent divergence in flower size is only apparent after accounting for this effect.

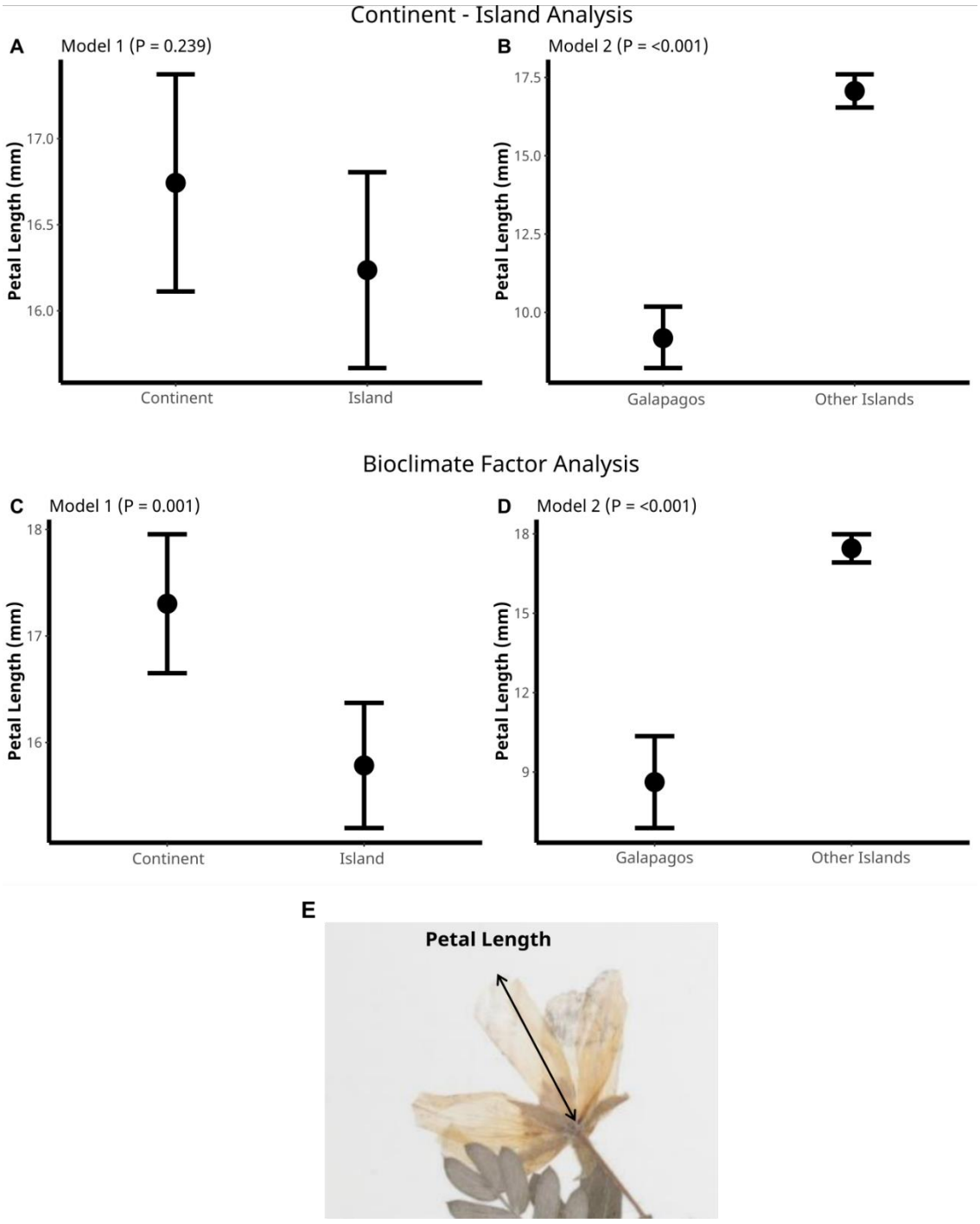

Figure 2. 5 Petal length estimates from island and continental plants. The plots show the least-squares mean estimates (± 1 SE) using petal length. P-values correspond to the difference between island and continental plants (Model 1), and the difference between the Galápagos Islands and Other (non-Galápagos) islands (Model 2). (A) Estimates of continental and island populations only. (B) Estimates of Galápagos and other (non-Galápagos) islands only. (C-D) Estimates of the island effect from the models after accounting for bioclimatic variation. (E) Diagram of how petal length was measured: from the base to the tip of the petal.

Our additional analysis showed that the insularity effect becomes non-significant when we remove the Galápagos samples and only use Other Islands (P = 0.118) (Appendix 2.6, Table S2.7). But we found a similar trend effect when we include bioclimate variables, with the same variables becoming significant to predict variation of insularity (Appendix 2.6, Table S2.8).

Phenotypic divergence between the Galápagos islands and other island groups

Flower size

We found that *T. cistoides* flowers on the Galápagos were smaller than on other islands. Specifically, the petal length of *T. cistoides* was 46% shorter on the Galápagos than on other islands (Figure 2.5). This effect was apparent whether bioclimatic variables were included or not, with no bioclimatic variables significantly predicting variation in flower size when only island sites were included in analyses (Table 2.2).

Discussion

We found that fruit and floral traits that mediate antagonistic and mutualistic species interactions with *T. cistoides* frequently diverged between island and continental populations. Mericarps were larger and deeper on islands, but more frequently lacked lower spines in comparison to continental populations. After accounting for climatic variation, the divergence in all mericarp traits except depth became non-significant, while climatic variables frequently predicted variation in mericarp morphology. By contrast, flower size consistently diverged to be smaller on island than continental populations, particularly after accounting for bioclimatic variation among sampling sites. Plants on the Galápagos islands had substantially smaller flowers than plants from other islands. We discuss the importance of these results for understanding how insularity influence the evolution of traits associated with species interactions.

Divergence of antagonistic traits between islands and continent

The morphological divergence observed between island and continental populations is partially consistent with our expectations of evolution in response to changes in herbivore communities. We expected that mericarps would be larger and better defended on islands if vertebrate seed predation was an important agent of selection (Boag & Grant, 1981; Carvajal-Endara et al., 2020). We found that mericarp depth was still significantly different between island and continental plants after the inclusion of bioclimate variables. Increased mericarp depth may increase survival of mericarps when vertebrate predators are present. In the case of ground finches on the Galápagos, the birds crack the mericarps transversely, twisting the lower surface of the mericarp, a deeper mericarp may increase handling time for finches (P. R. Grant, 1999). Mericarp size is especially important for finches with medium sized beaks because it takes them more time to handle large mericarps when extracting seeds. By contrast, the less numerous large beaked finches open larger mericarps more easily to extract seeds (P. R. Grant, 1999). Previous field experiments showed that on average the ground finches on the

Galápagos imposed phenotypic selection in favour of larger *Tribulus* mericarps, and longer upper spines (Carvajal-Endara et al., 2020; Rivkin et al., 2021).

We expected that if mericarps were better defended on islands, then lower spines would be more frequent than in continental plants. For example, the presence of lower spines on the Galápagos increases survival from vertebrate predators (Carvajal-Endara et al., 2020). However, we found that there is no effect of lower spines when compared with other island systems and that there is an insularity effect mainly on Galápagos (Appendix 2.6 and 2.7). In general, we found that lower spines were less common and bioclimatic variation was the best explanation for variation in the presence/absence of lower spines. This may have occurred because precipitation and seasonality drive increased seed production of a diversity of plant species on islands. The abundance of alternative seed sources alleviates predation and antagonistic selection on defence traits of *T. cistoides*, which is a non-preferred food source when other more easily acquired seeds are available (Carvajal-Endara et al., 2020; P. R. Grant, 1999; P. R. Grant & Boag, 1980). The effects of seasonal climatic variation in predation could facilitate the maintenance of variation in traits like lower spines.

Another explanation for the decreased frequency of lower spines on islands could be differences in dispersal between islands and continents (Cody, 2006; Cody & Overton, 1996). Upper and lower spines of *T. cistoides* are involved in dispersal as well as defence, in that the fruits become attached to animals (M. K. A. Johnson et al., 2020; Porter, 1971; Wiggins & Porter, 1971). These spines might be an especially important mechanism for dispersal in continental populations, but could be disadvantageous on islands, especially if dispersal disproportionately leads to seeds being deposited in unfavourable habitats. As suggested by Porter (1971), seabirds may carry *T. cistoides* mericarps, potentially depositing them in the ocean. Alternatively, Larger seeds may simply help seedling establishment while islands may lack dispersal agents, leading to higher costs of maintaining lower spines without substantial benefits (Burns, 2019; Kavanagh & Burns, 2014). If the cost-

benefit ratio of maintaining spines is high on islands, then larger seeds could explain both why fruits tend to be larger and lower spines are less frequent on islands.

Conflicting selection due to antagonistic and mutualistic interactions on fruits may frequently lead to phenotypic divergence among populations. For example, Siepielski and Benkman (2010) found that seed predation by squirrels led to selection for pine cones to be more defended and contain fewer seeds. In the absence of squirrels, seed dispersal by nutcrackers selected for pinecones to have lower defences and larger seeds. When both agents of selection were present, it led to contrasting selection and greater phenotypic variation within populations. Notably, we also observed greater variation in morphological traits on islands than continents (Figure 4). This type of opposing selection by antagonistic and mutualistic interactions may similarly explain why fleshy fruits that rely on seed dispersers often have spines (e.g., *Ribes* spp., *Durio* spp.) and why many types of fruits are chemically defended (e.g., *Solanum* spp., *Hippomane mancinella*). These contrasting traits may allow plants to attract beneficial dispersers and deter costly predators. It seems likely that the evolution of many plants' fruit and seed traits reflect a balance of conflicting selection between antagonistic and mutualistic interactions (Blake et al., 2012; Jordano, 1995; O'Farrill et al., 2013; Stiles, 1980).

Divergence of mutualistic traits between islands and continents

We expected that flowers on islands would be consistently smaller because islands commonly have a lower diversity and more generalized pollinators. This expectation is founded on the Island Floral Syndrome Hypothesis (Hetherington-Rauth & Johnson, 2020), which stems from observations by naturalists during the past two centuries (Bramwell & Caujapé-Castells, 2011; Carlquist, 1974; Darwin, 1845; Wallace, 2013). These naturalists claimed that islands typically have small inconspicuous flowers. Our results differ from this expectation considering other island systems, where there was no effect for petal length between island and continent populations (Appendix 2.6). However, they are consistent with this

expectation after accounting for climatic variation among sampling sites on the Galápagos archipelago, where flowers were about half the size of flowers on other islands or continental populations.

Our results for other island systems are supported by a recent large comparative analysis across the Pacific Islands. Hetherington-Rauth and Johnson (2020) found that, across many taxa, flowers were not on average smaller on islands than on the American continents. Interestingly, Galápagos was a notable exception in their study, where endemic species' flowers were consistently smaller on the archipelago compared to their continental sister taxa. *Tribulus cistoides* was not used in that study, and so it is striking that our results align with their previous macroevolutionary results for other species on the same archipelago. This correspondence raises the question: why is the Galápagos an exception and why do we observe the evolution of smaller flowers both within and between species?

In the case of *T. cistoides* in Galápagos, changes in flower size could be explained by the evolution of increased selfing, divergence in pollinator communities, or climatic differences. *Tribulus cistoides* is self-compatible (Chamorro et al., 2012), but seed production is thought to rely mostly on outcrossing mediated by pollinators (Reddi, 1981). It is conceivable that island populations of *T. cistoides*, are evolving increased selfing rates and consequently smaller flower sizes. This possibility deserves further investigation. Divergent pollinator communities on islands may also contribute to the evolution of smaller flowers. For example, there is only a single native bee species found on the Galápagos, the large endemic carpenter bee *Xylocopa darwinii*. The paucity of bee species in the Galápagos could lead to high competition among plant species for attracting pollinators, which might drive evolution of smaller flower sizes in *T. cistoides* to attract different pollinators, such as smaller Hymenoptera, day flying butterflies, or even nocturnal moths, all of which are introduced. These introduced species comprise ~25% of all Galápagos insect species (Traveset et al., 2013). If introduced pollinators are sustained by non-endemic or generalist plants, they may impose selection for smaller flowers. Finally,

it was clear from our results that environmental variation among populations explained differences in flower size. Specifically, there was a significant effect of temperature and precipitation seasonality for island and continental populations. It is possible that environmental factors are the main source of selection for flowers, or that they modulate the strength and direction of biotic selection, as suggested by the differences in flower size between islands and continents that were only apparent after accounting for climate.

An alternative explanation to the possibilities above, is that the smaller flowers of *T. cistoides* are not an example of adaptive evolution to a depauperate pollinator community, but instead reflect recent hybridization. The Galápagos is just one of two locations in the world where the larger flowered *T. cistoides* and the smaller flowered *T. terrestris* coexist. If these species have hybridised on the Galápagos, then the observation of their smaller flowers could reflect segregating hybrid variation. Porter (1971) reported that diagnostic floral characters of these two species were intermediate and variable among *T. cistoides* plants on the Galápagos. Our own data shows that mericarps on islands exhibit greater variation in morphology than on the continent (Figure 5), as expected in the case of hybrid segregating variation on Galápagos but not elsewhere. These observations are not conclusive evidence of hybridization, and genomic analyses would be required to further test whether hybridization is occurring on the islands and accounting for increased phenotypic variance.

Limitations

Our study has two main limitations that need to be considered when interpreting our results. First, our mericarp dataset was unbalanced, in that we had an abundance of mericarp data from the Galápagos islands, yet relatively few mericarp samples from other island systems. Few herbarium specimens containing mericarps were available from other islands and visiting many additional islands across *T. cistoides*' global distribution was logistically infeasible. As such, most of our mericarp samples came from the Galápagos, field samples from continental

Florida, and other continental field samples distributed around the world. Thus, our results that compare continental and Galápagos populations are robust, but our ability to compare morphological patterns of mericarps on the Galápagos versus other islands is limited. Second, our results reflect phenotypic differences among field samples, yet we cannot partition the effects of genetic versus environmental differences. Given our large replication and the magnitude of the differences we observed, plus previous results showing evidence consistent with heritable variation in some of these traits (Carvajal-Endara et al., 2020), it reasonable to conclude that much of this variation is genetically based.

Conclusions and future directions

Our results show that *T. cistoides* exhibits phenotypic differences in fruit and floral traits between island and continental habitats. Many of these differences are consistent with antagonistic and mutualistic interactions driving divergent evolution between continental and insular populations, while in other cases climatic variation appears to be the main driver, or at least modulates biotic selection. This study shows the potential of using a species that is globally distributed and shows unique interactions in the context of island populations. These characteristics makes *Tribulus* a potential species for multiple venues of research answering questions on island evolution and ecology. The global distribution of *T. cistoides* and the inclusion of herbarium samples is an asset for pursuing large scale comparative studies in the tropics. For island systems specifically, there is potential for controlled experiments to address specific dynamics of vertebrate and invertebrate predation on islands. Observed phenotypic divergence on mericarps or flowers could be further explored with common gardens that would allow for the partitioning of genetic and plastic differences in phenotypic traits (i.e., presence of lower spines) observed within and among populations. Molecular analyses would help establish whether hybridization on islands is contributing to the observed phenotypic variation in plant traits of *T. cistoides*. Furthermore, field experiments of pollinators and dispersers could help to establish how selection by mutualists is

shaping the evolution of mericarp and floral traits. Overall, our study shows the potential for using *Tribulus* as a study system to understand co-evolutionary dynamics driven by mutualistic and antagonistic interactions.

Acknowledgements

The authors thank the support of the Connaught Fellowship to MH-R, NSERC Discovery Grants and Canada Research Chairs to APH and MTJJ, a Steacie Fellowship to MTJJ, and NSERC CREATE in Biodiversity, Ecosystem Services and Sustainability (BESS) to WDRC. Field work was supported by a Research Travel Award and a Graduate Research Enhancement Travel Award (GREAT) to WDRC. Samples were collected under the "Contrato Marco Acceso de Recursos Genéticos: Investigación Genética en la provincia de Galápagos" (MAE-DPNG/DGA-2016-0198), from the Environment Ministry and USFQ; the Scientific Research Permits, PC-14-17 and PC-28-19 from the Galápagos National Park, under the supervision of JACH and the Galápagos Science Centre (GSC). We thank Tiffani Harrison and Wanzhang Wang for collecting the herbarium flower data. Missouri Botanical Garden (MOBot), Harvard University Herbarium (HUH), California Academy Sciences (CAS) and the Charles Darwin Research Station (CDRS) made herbarium samples available to us for floral and fruit measurements. Patricia Jaramillo and Sarita Mahtani-Williams from Charles Darwin Research Station facilitated data collection from Galápagos samples. We thank the Galápagos Science Centre (GSC) and Anita Carrion, Sylvia Sotamba, Karla Vasco, and Diego Páez-Rosas for including us in their annual sampling expedition across the Galápagos. We thank Pedro Moncayo, Andy Torres, Rene Freire, Agustin Masaquiza, Mario Pilla, Secundino Masaquiza, Alberto Alarcon from the Galápagos National Park (GNP) for facilitating our research on the *Sierra Negra* ship. Finally, Andrea Loyola (GNP), Jorge Torres (GNP), Eduardo Espinoza (GNP), Juan Pablo Muñoz (GSC), José Luis Viera (Tropical Herping), and David Salazar (Universidad Indoamérica) who were part of the scientific crew in the expedition were WDRC collected the field samples.

Data availability statement

Data is available at DRYAD: https://doi.org/10.5061/dryad.h70rxwdnz

Scripts are available at GitHub at: https://github.com/Winer-DanielR/Tribulus-mericarp-morphology

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Linking statement 2

In the previous chapter, we showed that *Tribulus cistoides* mericarps differ phenotypically between island and continental populations. These differences are likely influenced by antagonistic and mutualistic interactions, as well as climatic variation. Mericarps on islands were larger and deeper but lacked lower spines compared to continental populations. When accounting for climatic variation, most mericarp traits showed non-significant divergence, with climatic factors playing a role in mericarp morphology variation. Flower size consistently diverged to be smaller on islands, especially after considering bioclimatic differences among sampling sites. Galápagos islands showed particularly smaller flowers than other islands. In short, the previous chapter showed that *T. cistoides* mericarps and flowers have great phenotypic variation and island populations of *Tribulus* diverge phenotypically. These results also suggest that the first condition for natural selection is met in the *Tribulus* system.

Further, the chapter suggests that potential mutualistic and antagonistic interaction may explain better why specific traits differ between populations. In the next chapter, we explore this further, using annual monitoring of *Tribulus* populations and field experiments to estimate the natural selection of mericarps in the context of endemic seed predators, Darwin's finches. I use the presence of open or closed mericarps as a measurement of survival to finch predation. The first method is a yearly monitoring of populations across four islands. The second method is a mark-recapture field experiment that inflates phenotypic variation to better understand patterns of mericarp selection by finches.

We found that mericarps on the Galápagos are generally well adapted to finch predation, resulting in larger and spinier mericarps. Although we found variation among island populations. The next chapter shows that mericarp traits are closely related to survival, checking the second condition of natural selection.

CHAPTER 3: Factors influencing selection on a non-endemic plant species (*Tribulus cistoides*; Zygophyllaceae) by an endemic seed predator (Darwin's finches).

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This chapter is being prepared for submission.

Introduction

To understand the diversity of organismal form and function, we need to establish relationships among interacting species, such as predators and prey, hosts and parasites, competitors, and mutualists (Endler, 1986; P. R. Grant, 1999). A critical part of this understanding is how a given species imposes natural selection on the phenotypic traits of another species. Comparisons of the strength and form of this selection to the observed phenotypic distribution can answer important questions such as: How important is the species interaction to patterns of trait variation? How does selection imposed by one species influence the other species' traits? and How spatially consistent is the outcome of selection between interacting species? (Schluter, 2000; Vellend, 2016; Vermeij, 1982, 2002). These and other questions can be answered by measuring contemporary selection in natural populations (Endler, 1986; Kingsolver et al., 2001; Siepielski et al., 2013), comparing that selection among times or places characterized by different species interactions (Alcántara & Rey, 2003; Siepielski & Benkman, 2007), comparing patterns of selection to patterns of trait variation (Benkman, 1999; Hoekstra et al., 2001), and manipulating phenotypes to enhance phenotypic variation and sharpen inferences about the traits selection has wrought (Møller et al., 1998; Reznick & Ghalambor, 2005; Rivkin et al., 2021). We will leverage all these approaches to understand a particular predator-prey interaction in Galápagos.

Island systems offer excellent opportunities to explore how natural selection and adaptation are driven by species interactions. Island communities often have fewer species and fewer interactions that often produce relationships that can be novel and unique (Burns, 2019; Whittaker & Fernandez-Palacios, 2007). For instance, many studies have focused on how species radiation and endemism on islands are shaped by species interactions (Bramwell & Caujapé-Castells, 2011; Carlquist, 1965, 1974; Whittaker & Fernandez-Palacios, 2007). These studies often show that, as long-established lineages become endemic and more specialized on islands, they might then strongly select for specific traits in newly arrived or

generalized species. Putative examples include dwarfism in mammals on Mediterranean islands (Reyment, 1983), flightlessness in island birds (J. Diamond, 1991), and various unique feeding specializations (e.g., blood-feeding finches and mockingbirds in Galápagos (Curry & Anderson, 1987; Koster & Koster, 1983), honeycreepers in Hawaii (Freed et al., 1987; Pratt, 2005), and spiders, beetles and snails in The Canarias (Báez et al., 2001). These new relationships that generate new selective pressures are expected to shape unique aspects of species that colonize islands, generating novel phenotypic variation.

Among the many types of new species associations studied on islands, plant–animal interactions have proven to have a particular fascination. In most cases, these interactions are often driven by super-generalist endemic or native plants or pollinators (Olesen et al., 2002; Traveset et al., 2015). But in other cases, plants on islands have evolved specialized endemic forms from originally generalized colonist species. For example, the multiple species of Hawaiian lobeliad endemics that radiated and adapted to multiple habitats might have originated from a single wind-dispersed, bird-pollinated, shrub ancestor (Givnish et al., 2009). Second, animals on the islands often specialize in those specialized plants. For example, Hawaiian honeycreepers adapted to different food resources endemic to the islands (Freed et al., 1987). Finally, more recently colonizing generalist plants can then be subject to intense selection from the existing specialist predators on islands. For example, seed predation by native crabs and insects on Enewetak Atoll reduced the seed survival of the Indian almond (*Terminalia catappa*), affecting plant distribution on the island (Louda & Zedler, 1985). Our study will explore this last step in the above sequence by studying how a plant (*Tribulus cistoides*) that appears to be a relatively recent colonist on the Galápagos (Wiggins & Porter, 1971) is responding to specialist Darwin's finch seed predators.

Darwin's finches are a classic example of island adaptation radiation and specialization. These finches radiated due to the diversity of food resources that, in turn, also help them avoid competition that varies in space and time (Boag & Grant,

1981; T. D. Price et al., 1984; Schluter & Grant, 1984). In particular, the beak shapes and sizes of different species are specialized to consume different food sources, such as flowers, seeds, plants, insects, or blood (De León et al., 2012; P. R. Grant, 1999). That is, finch species are (at least partly) adapted to different niches afforded by the various plants that colonized the island. Perhaps the strongest example here is the specialization of the cactus finch (*Geospiza scandens*) on the *Opuntia* cactus (B. R. Grant & Grant, 1981; Millington & Grant, 1983). Most other interactions between finches and plants appear more diffuse, with a given finch species feeding on multiple plants and a given plant species being fed on by more than one finch species. However, when conditions are difficult – such as during dry seasons, droughts, and La Niña events – each finch species increasingly specializes in particular plant species for which they are particularly suited (De León et al., 2014; P. R. Grant, 1981). *Tribulus cistoides* is one of those plants.

Tribulus cistoides is a perennial plant found around the world in tropical and arid zones (Porter, 1971). Mature plants produce hard fibrous fruits called schizocarps that have segments called mericarps, each containing 1-7 seeds (Kearney et al., 2020). Mericarp size is related to the number of seeds they can hold (Carvajal-Endara et al. 2020). These mericarps provide a defense against seed predators (Carvajal-Endara et al., 2020; P. R. Grant, 1981; E. Johnson, 1932) and have spines that influence predation and dispersal (M. K. A. Johnson et al., 2020; Porter, 1971). Interestingly, *T. cistoides* mericarps on the Galápagos differ from those in the rest of the species range. First, island mericarps are larger: on average, 7% longer, 6% wider, and 12% deeper than those found elsewhere (Reyes-Corral et al., 2023) (Chapter 2). Second, although mericarps in the rest of the species range have both "upper" and "lower" pairs of spines, some Galápagos mericarps completely lack lower spines, upper spines, or both (Robinson, 1902). In the present study, we will explore whether these Galápagos-specific trait variations are shaped by selection from specialist vertebrate seed predators – the Darwin's finches.

On islands, vertebrate predation on *T. cistoides* mericarps occurs mainly by birds, with evidence coming from Galápagos (P. R. Grant, 1981) and Hawaii (Conant, 1988; Fleischer et al., 1991; Freed et al., 1987). In both locations, *T. cistoides* appears to influence seed predators by selecting birds that have a beak strong enough to crack the mericarps and get the seeds. Of these two cases, the interactions with Darwin's finches are particularly well documented. For starters, the *Geospiza* ground finches specialize in the consumption of seeds (T. Price, 1987) and then two species within that group feed on *T. cistoides* seeds: *Geospiza fortis* (medium ground finch) and *Geospiza magnirostris* (large ground finch). For *G. fortis*, only the largest individuals can break the mericarps to extract the seeds – and, even then, with difficulty and many failures (P. R. Grant, 1999). *G. magnirostris* have a much easier time obtaining the seeds, obtaining twice the amount of seeds during the same time it takes *G. fortis* to manipulate a mericarp (P. R. Grant, 1999). Both species – and especially the *G. fortis* – tend to feed on *T.* cistoides only during dry periods when other preferred foods become rare (Carvajal-Endara et al., 2020; De León et al., 2014; P. R. Grant, 1981, 1999). *T. cistoides* imposes natural selection on finch beaks during these drought periods – specifically favoring beak width and depth in *G. fortis* (P. R. Grant, 1999). We suspect that this predation reciprocally causes selection on *T. cistoides* mericarp traits (Carvajal-Endara et al., 2020) – perhaps shaping the unique traits this species exhibits in Galápagos, but due to species distributions not all locations are well adapted to specialist local predators.

To guide efforts to understand the relationships between specialized endemic predators (*G. fortis* and *G. magnirostris*) on mericarp traits of *T. cistoides*, we use two sets of data. The first data set is a "point-in-time" record of predation and survival over multiple populations of *T. cistoides* on multiple islands over 5 years. The second data set is a mark-recapture experiment that manipulated specific mericarp traits to inflate variation and thus further explore selection.

Hypothesis and questions:

1. Are Galápagos mericarps under a general form of selection?

Relative to continental *T. cistoides* mericarps, those on the Galápagos tend to be larger and more often lack lower spines (Reyes-Corral et al., 2023) (Chapter 2). If the current trait values reflect a well-adapted state, we might not expect any further contemporary selection acting on them; see Haller and Hendry (2014). If, however, trait values are not currently at (or near) their optima – which could occur due to insufficient time for adaptation or various other constraints; we might expect to still see positive contemporary selection acting on mericarps in Galápagos. These mericarps might still experience a general (across populations and islands) pattern of selection for larger size and an absence of lower spines. To answer this question, we used the point-in-time dataset (see explanation below) of estimates of selection imposed by finches based on 10,814 mericarps sampled across 32 populations on 6 islands over 5 years (Figure 3.1; Appendix 3.1, Table S3.1).

2. Are among-island mericarp differences correlated with selection?

It seems unlikely that specific local populations of *T. cistoides* are perfectly adapted to the local selection pressures – because a variety of forces (most obviously gene flow) should constrain adaptive divergence (Garant et al., 2007). Under such constraints, we might expect a correlation between directional selection and mean trait values (Bolnick & Nosil, 2007). That is, *T. cistoides* populations with the largest mericarps still might be under the strongest contemporary selection for larger mericarps. Similarly, *T. cistoides* populations with lower frequencies of lower spines might be under stronger selection to eliminate those spines. We answer this question with the same dataset as the first question – but we here focus on variation among sites in the Galápagos – as opposed to a shared Galápagos-wide signal considered above.

3. Are the among-island differences in selection and traits associated with environmental variables?

Question 2 considers the relationship between contemporary selection and mean trait values – without reference to the potential biological drivers of that selection. The present question goes a step further to ask whether the among-site differences in selection or mean trait values are associated with candidate biotic or abiotic factors. For biotic factors, the presence of finches that can open mericarps (e.g., *G. magnirostris* and *G. conirostris*) is expected to influence the strength of selection (Carvajal-Endara et al., 2020)). For abiotic drivers, trait variation could conceivably be shaped by temperature, precipitation, or seasonality – whether directly or indirectly via their effect on other variables. Here we again leverage the above point-in-time dataset from Questions 1 and 2, but we now add various environmental drivers to the statistical models.

4. Does inflated trait variation uncover additional selection?

As hinted above, when populations are well-adapted to local environments, selection on them should be rather weak – that is "selection erases its traces" (Haller & Hendry, 2014). Thus, if mericarp trait differences among Galápagos populations reflect strong local adaptation (i.e., constraints are weak), contemporary selection on the current trait variation might be weak. If so, it will be hard to infer how selection drove the among-population differences. In such cases, manipulations that inflate trait variation can provide more power to infer how selection might have favored the current trait values (Hendry, 2017). To this end, we used a mark-recapture seed predation experiment where we increased mericarp trait variation and then estimated selection based on 2400 mericarps across 3 sites (one per island) over up to 1.5 years (Figure 3.1; Appendix 3.1).

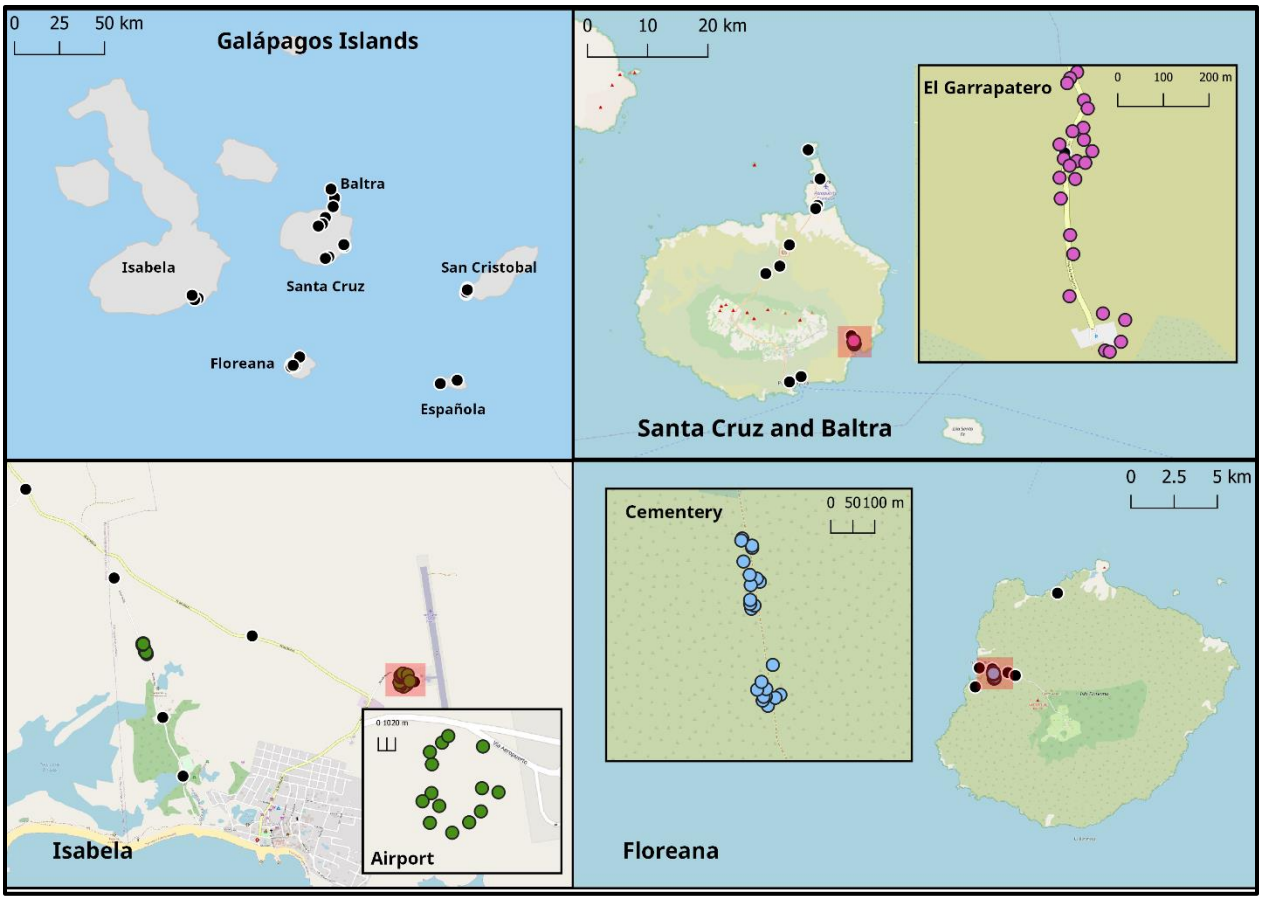

Figure 3. 1 Distribution of monitored populations of *Tribulus cistoides* in the Galápagos Islands. Top left. Overview of all *T. cistoides* point-in-time populations across islands. Top right. Overview of Santa Cruz and Baltra islands showing point-in-time populations. The area highlighted in red shows the location of the mark-recapture experiment in El Garrapatero. Pink dots show the plate locations that contained the marked mericarps. Bottom left. Overview of Puerto Villamil in Isabela Island showing the location of point-in-time populations. The area highlighted in red shows the location of the mark-recapture experiment close to the Airport. Green dots show the plate locations. Bottom right. Overview of Floreana island, showing the distribution of point-in-time populations. The area highlighted in red shows the location of the mark-recapture experiment close to the Cemetery. Blue dots show the plate locations.

Methods

Tribulus cistoides measurements

Each collected mericarp was measured for a set of phenotypic traits and examined for signs of predation and germination. We took linear measurements with digital calipers (Electronix, EAGems, IP54; up to 0.01 mm resolution) of mericarp size (length, width, and depth) and spine traits (spine length, spine tip distance, and spine position), and we recorded the presence/absence of lower spines

(Figure 3.2). Length was the distance along the ventral border of the mericarp where seeds are contained. Width was measured as the distance across the base of the upper spines. Depth was measured as the distance from the ventral and dorsal edge in the middle of the mericarp. Spine length was measured as the distance of the base to the tip of the upper spine. Spine tip distance was between the upper spine tips. Spine position was defined as the position of the upper spine axis in relation to the body axis, with the body axis as the base and the upper spine axis starting from the top (0%) up to the bottom (100%) of the mericarp making a semi-circle shape. Spine position was observed and recorded by placing the mericarp on its ventral end and estimating the position of the upper spine. Mericarps fell between 7 angles (10-70%) with 40% being the most common (22% of total mericarps). For example, if the spine was perpendicular to the body axis, it was scored as 50% (Figure 3.2). For all spine traits, if a mericarp lacked spines at all they were scored as an *NA*.

We scored whether a mericarp was "open" (1) if at least one seed was removed from the mericarp, if there was no evidence of predation it was scored as "closed" (0), meaning the seeds were uneaten and still surviving (Figure 3.2). On Galápagos, bird predation is much more common than insect predation – indeed, we found only 54 insect-eaten mericarps, representing 0.5% of the total 10,814 mericarps examined and 1.4% of the 3810 predation events, and we therefore henceforth exclude insect predation from analyses. The predation behavior of finches allowed us to score the seed position that was predated. A mericarp was also considered "germinated" (1) when they showed signs of germination and recorded germinated seed position, germinated mericarps were excluded from analysis (Figure 3.2).

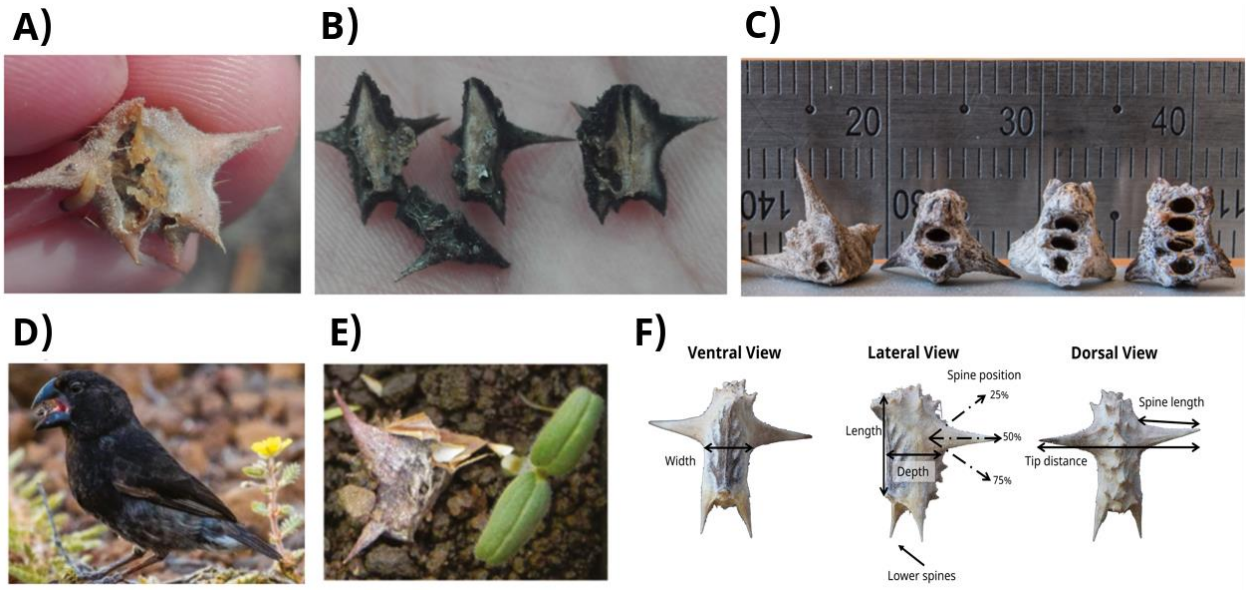

Figure 3. 2 *Tribulus cistoides* mericarps with evidence of different types of predations. Predation by insects differs from birds in that insect larvae drills holes on the sides of mericarps and usually attacks the whole schizocarp. (A) Predation by insects on an immature mericarp. (B) Predation by insects on a mature mericarp. (C) Predation by birds. Bird predation cracks the ventral side of mericarp and leaves open the seed gap. (D) A ground finch manipulating a mericarp with its beak. (E) A germinated seed next to a mericarp. Germination leaves a different opening on the mericarps, leaving one side intact. This way, we can differentiate germination from insect or bird predation. Open mericarps were scored as (0) "eaten", whole mericarps as (1) "uneaten". (F) Diagram showing mericarp position (0% - 100%) and the seven traits measured. Pictures (A) and (B) were taken by WDRC. Picture (C) was taken by APH. Dr. Kiyoko Gotanda took picture (D). Picture (E) was taken by SCE. Picture (F) was taken by MTJJ, diagram was made by WDRC.

Experimental designs

Point-in-time sampling

Tribulus mericarps persist in the soil for years after they are produced by the plants (Goeden & Ricker, 1973). As a result, we can visit a location, collect mericarps from the ground, and have a time-integrated signature of trait variation and selection. We previously collected and analyzed these point-in-time samples from 30 populations on 7 islands from 2015 to 2017 (Carvajal-Endara et al., 2020). The number of populations per island varied from six to eight due to spatial variation in the abundance of plants. For the present study, we re-analyze these existing data combined with two more years (2018-2019) of data from the populations on Santa Cruz, San Cristóbal, Isabela, and Floreana (Appendix 3.1). From each population, 100 mericarps were collected (when possible) haphazardly across the population area.

Mark-recapture experiment

The mark-recapture experiment was done on the largest monitored population of each island: Santa Cruz, Isabela, and Floreana (not enough plants were present on San Cristóbal). We started this experiment in May 2018 and replicated it (with modifications) in May 2019. From each population, we collected 400 mature mericarps that were still attached from the plants, thus controlling for generation and development stage. Mericarps were divided into four categorical groups representing each combination of mericarp size (small and large) and spines (present or absent), spines treatments were contained within the size groups. We measured these mericarps (as described above) and then marked them on their dorsal side using nail polish. Each mericarp was marked based on a system of mark position and colour that indicated their assignment to the above four groups (Appendix 3.2). Marked mericarps were distributed across the population area in Petri dishes (n=25) that contained an even number of mericarps for each treatment (16 mericarps per dish) (Appendix 3.1, Table S3.3). Individual identification of mericarps was possible by accounting the color, mark position and plate they were

grouped in. The specific colors and positions per treatment changed between islands (Appendix 3.2). For the 2018 experiment, within each size group, we divided mericarps into four spine treatments: All spines, Lower spines, Upper spines, and No spines. These spine treatments were manipulations, spines were removed with calipers. We had 50 mericarps per spine treatment. For each treatment we kept spines intact, removed upper spines, removed lower spines, and removed all spines respectively. (Appendix 3.1, Table S3.3). For the 2019 experiment, we only used two spine groups within each size group, (All spines and No spines) as the proportions of size groups changed between years and we could not find enough mericarps to match 2018's treatments.

The plates were located randomly, but based on finch forage observations, the total area of the *Tribulus* population and accessibility for monitoring. We prioritized places that would be marked easily (close by a tree or bush) but that were open and closer to feeding areas. We recorded the plates' GPS coordinates and used marking tape as a visual reference to easily locate the plates (Appendix 3.2, Figure S3.1). The mericarps on these plates were then checked every three months for 12 months to determine which were still present and to record survival as seeds that had been removed and the number of seeds removed by finches or insects. After 12 months, the remaining mericarps were recovered, identified, and measured again to test the effect of time on trait variation. For the 2019 experiment, the marked mericarps were monitored only once after being placed, in August 2019. Due to COVID travel restrictions in early 2020, we could not monitor the plates further.

Additional to the survival scores explained above, we also recorded the presence/absence of mericarps. We made this distinction for our analysis to test the assumption that missing mericarps were "eaten". Also, we recorded the seed position of eaten and germinated mericarps, with the first seed defined as the one closer to the upper spines and if multiple seeds were eaten for a particular mericarp.

Data preparation, models, and analysis

Principal component analyses

We used a principal component analysis using the Point-in-time dataset to answer questions 1, 2 and 3. Mericarp traits are highly correlated, so we generated composite variables for mericarp size, defense, and spine position. This PCA used individual mericarp values. We also generated a PCA for bioclimate variables for Question 3, using population values. We standardized each variable and used the *prcomp* function to perform the PCA in R version 4.0.3 (R Core Team, 2022). Then, extracted the PCA loadings and contribution scores for each trait to better associate PC axis with specific traits. The PC1 from the point-in-time dataset showed negative loadings, that were multiplied by -1 and transformed to positive values to better interpret our results (Appendix 3.3). We visualized each PCA using *FactoExtra* package (Kassambara, 2017) and extracted the PC scores into the datasets for model analysis.

Point-in-time PCA

The Point-in-time PCA generated 6 PC axis. The first three PCs explained 79.6% of the total variation and were used in subsequent analysis. PC1 (43.7%) will be considered as "mericarp size" as it positively correlates with three size traits (length, width, and depth). PC2 (18.8%) will be considered "mericarp defense" as it was positively correlated (increasing "spininess") with the presence of lower spines and with increasing upper spine length. Finally, PC3 (17.1%) was associated with spine position. Hereafter, we are going to refer to these PC axes as *mericarp trait classes* associated with traits groups (*Size*, *Defense* and *Spine position*). These were our main tested variables.

Bioclimatic variables PCA

Bioclimatic data was collected following Reyes-Corral *et. al* (2023) (Chapter 2). Bioclimate data was downloaded from the WorldClim database at 30s resolution (~ 1 Km^2). These bioclimatic variables represent the average for the years 1970 - 2020 (Fick & Hijmans, 2017). We used the variables Bio1 (Annual Mean

Temperature), Bio4 (Temperature Seasonality), Bio12 (Annual Precipitation) and Bio15 (Precipitation Seasonality). Location coordinates and climate data were matched in QGIS (version 3.18.2-Zürich) (QGIS Development Team, 2022). We used the tool *Fill No Data* by a maximum distance of 10 pixels to project the climate information and reduce NAs from locations that may be too small to have estimated data. Then, we extracted the bioclimate information using the *Sampled Raster Values* tool. The information was incorporated into the Point-in-time dataset to answer Question 3, at the population level.

The Bioclimatic PCA generated 4 PC axis. The first two PCs explained 78.3% of the total variation. PC1 (54.5%) was associated with Precipitation and Temperature Seasonality. PC2 (23.8%) was associated with Annual Temperature and Precipitation. We used all PC axis for model analysis because the PC loadings of PC3 and PC4 were associated with Temperature Seasonality and Precipitation Seasonality respectively (Appendix 3.3. Fig S3.6).

Mark-recapture data preparation

We used mark-recapture data to answer Question 4. We prepared the data in two ways, one by summarizing the total number and percentages of eaten, present (uneaten) and missing mericarps per monitoring time, across treatments of size and spines on each island. In this way, we were able to compare the years (2018 – 2019). However, this approach would not allow individual survival estimates. The second way to summarize data would allow us to calculate individual estimates of survival. Survival days were the number of days passed from the start of the experiment (day 0) up to the date of the following monitoring time until the mericarp was no longer found or was marked as eaten. In this way, mericarps that survived longer have a larger number of days survived. This dataset was also used to discard potential effects due to biases of the experimental design (marking, color, and plate).

The mark-recapture PCA performed using only the mericarp size related variables (Length, Width and Depth), due to the nature of the experiment that required spine manipulation, it was difficult to incorporate this information without bias. Mericarp

size was the main criterion for the categories and the PCA shows a clear difference between groups (Appendix 3. Fig S9). The first two PCs explained 93.9% of total variation. PC1 (79.6%) was associated with mericarp size. PC2 (14.3%) was associated mostly with mericarp length and width. PC3 (6.1%) was associated with mericarp depth. We used PC1 for model testing and results.

Selection estimates

Selection estimates (S) were calculated as the difference between the mean trait value of the closed ("uneaten") and open ("eaten") mericarps. The means of closed and open mericarps were estimated by population within each island using the PC axis corresponding to Size, Defense, and Spine Position (Appendix 3.1). The selection estimates were used in Questions 2 and 3 and were incorporated for model testing and results. Note, these estimates are not the same as formal selection estimates used to predict evolutionary change – as those estimates would be the difference in mean trait value of the closed ("uneaten") and all the mericarps (including both closed and open mericarps) (Lande & Arnold, 1983). However, the estimates we use are sensitive and have better properties (e.g., the same data points are not in both categories) for statistical models.

Models and analysis

All models and analysis were performed in R version 4.0.3. (R Core Team, 2022). We used generalized linear mixed models for all our questions as implemented in the package glmmTBT (Brooks et al., 2017). We also used the Anova function from the car package (Fox et al., 2012) and fit the models to Type II sums-of-squares. We used the package DHARMa (Hartig & Lohse, 2022) to check whether residuals met assumption of homogeneity of variance and normality (when appropriate). The model structure for each question was as follows.

Question 1: We used individual trait values (PCA scores) in GLMMs as the predictor of individual mericarp *survival* (open versus closed mericarps). These models were univariate – that is, they used PCA scores separately for each *mericarp trait class* (Size, Defense and Spine Position). The models also included *island* and

population (nested within island) as random factors and used a binomial logit distribution.

Question 2: These GLMMs used *mean trait values* for each population as a predictor of *mericarp selection* (S) on the population. These models were again univariate – considering mean PCA scores separately for each *mericarp trait class* (Size, Defense and Spine Position). The models included *island* as random factor and used a Gaussian distribution.

Question 3: Here we used mean values (as in Question 2) in two GLMM models for each trait class. The first model used the *bioclimate variables* (PC axis of bioclimate PCA) and *finch beak* composition (presence versus absence of *Geospiza magnirostris* and *G. conirostris* on islands) to predict *mericarp selection* (S). The second model used the same predictors and tested their effects on *mean trait values*.

For Questions 2 and 3, R^2 values were computed for mixed-effect models using the function *r.squaredGLMM* from the R package *MuMIn* v. 1.47.5 (Bartoń, 2023); we estimated R^2 values associated with fixed effects (R^2 marginal), and R^2 values associated with fixed and random effects (R^2 conditional).

Question 4: Here we used two GLMMs for each trait class. The first model type tested the fixed effects of *group treatments* (Size and Spine groups) and *islands* as predictors of *days survived* for individual mericarps and used a Gaussian distribution. This model was also used to account for potential bias introduced by variables related to the *experimental design*, which were incorporated as random factors (mericarp color, marked position nested within color, and plates nested within islands). This first model used only the 2018 dataset because the 2019 experiment only had one monitoring time – and so that second experiment required a different model. This second model type thus tested the effects of *group treatments, monitoring time* (time 1 – 3) and *islands* as predictors of the *number of eaten mericarps*, with a Poisson distribution. This model is directly testing mericarps that were recovered and eaten in the experiment and uses both 2018 and 2019 datasets. We used the package *emmeans* (Lenth et al., 2022) to estimate the

marginal means of days survived and number of eaten mericarps per each category within the treatment group and the function *emmip* to plot predicted survival and number of eaten mericarps for each model (Appendix 3.5), to better interpret their results.

Results

Question 1. Are Galápagos mericarps under a general form of selection?

Tribulus mericarps on Galápagos are currently under some common patterns of directional selection; that is, some shared trends were evident across populations and islands (Table 3.1). These general patterns, and deviations from them, can be visualized by plotting the population mean trait value of closed "uneaten" mericarps (y-axis) against the population mean trait value of open "eaten" mericarps (x-axis). In such plots for mericarp size (PC1), fifteen populations were above the 1:1 line, suggesting that larger mericarps have better chances of survival in those locations. Nine other populations were below the 1:1 line, suggesting the opposite. Whether positive or negative, however, estimates for many of these populations were near the 1:1 line, suggesting that – in fact – selection was usually weak or absent. The largest deviations – and therefore cases where selection was likely strongest – were all above the 1:1 line. Thus, cases where finch-imposed selection on mericarp size is strong, it seems to favor larger mericarp size. For mericarp defense (PC2), almost all populations were very close to the 1:1 line, again suggesting weak or no selection. However, four populations showed large deviations below the 1:1 line, suggesting occasionally strong selection against spininess. For spine position (PC3), all populations, except for one, were below the 1:1 line, suggesting, common and consistent selection for lower spine angles (Figure 3.3). In summary, although some shared patterns of selection (especially on spine angles) could be inferred, considerable heterogeneity was evident among populations. This variation among populations will be leveraged to test our next two questions.

Table 3. 1 Model estimates for Questions 1 – 3 using the Point in time dataset and question 4 using the Mark Recapture dataset. The model structure for each question is shown in the subheading. Bioclimate PCs are associated with Temperature and Precipitation. PC1 is associated with the interaction between these variables (Annual Mean and Seasonality). PC2 is associated with Mean Annual Temperature and Precipitation. PC3 is associated with Temperature Seasonality. PC4 is associated with Precipitation Seasonality. Finch beak is the presence or absence of large ground finches per location.

A) Question 1: ***eaten ~ mericarp trait + (1|island/population)***				
Traits		X^2		***P***
Mean Size		15.591		**<0.001**
Mean Defense		3.8715		**0.049**
Mean Spine Position		186.8		**<0.001**
B) Question 2: ***Selection ~ mean trait + (1|island/population)***				
Traits		X^2		***P***
S Size		8.1257		**0.004**
S Defense		3.1963		0.073
S Spine Position		0.7001		0.402
C) Question 3a: ***Mean trait ~ PC1 - PC4 bioclimate + finch beak + (1|island/population)***				
Traits		X^2		***P***
Mean Size	*Bioclimate PC1*	0.004		0.95
	Bioclimate PC2	8.157		**0.004**
	Bioclimate PC3	0.28		0.596
	Bioclimate PC4	1.966		0.161
	Finch Beak	4.221		**0.04**
Mean defense	*Bioclimate PC1*	1.543		0.214
	Bioclimate PC2	4.084		**0.043**
	Bioclimate PC3	2.723		0.099
	Bioclimate PC4	4.709		**0.03**
	Finch Beak	0.022		0.881
Mean Spine Position	*Bioclimate PC1*	8.425		**0.004**
	Bioclimate PC2	8.433		**0.004**
	Bioclimate PC3	0		0.995
	Bioclimate PC4	5.706		**0.017**
	Finch Beak	13.601		**<0.001**
C) Question 3b: ***Selection ~ PC1 - PC4 bioclimate + finch beak + (1|island/population)***				
Traits		X^2		***P***
S Size	*Bioclimate PC1*	2.6377		**0.003**
	Bioclimate PC2	3.901		0.104
	Bioclimate PC3	0.4783		**0.048**
	Bioclimate PC4	3.4286		0.489
	Finch Beak	4.6648		0.064
S Defense	*Bioclimate PC1*	2.098		0.148
	Bioclimate PC2	0.791		0.374
	Bioclimate PC3	0.000		0.997
	Bioclimate PC4	0.040		0.841
	Finch Beak	1.206		0.272
S Spine Position	*Bioclimate PC1*	0.067		0.796
	Bioclimate PC2	0.403		0.526
	Bioclimate PC3	0.212		0.645
	Bioclimate PC4	11.169		**0.001**
	Finch Beak	0.958		0.328
E) Question 4a: ***Days survived ~ Categories + island + (1|plate/island) + (1|color) + (1|mark position/color)***				
Traits		X^2	***Df***	***P***
Days survived	*Treatments*	47.473	3	**<0.001**
	Island	41.719	2	**<0.001**
F) Question 4b: ***number eaten ~ Categories + time + island***				
Number of eaten mericarps	*Treatments*	16.619	3	**<0.001**
	Time	70.52	2	**<0.001**
	Island	43.433	2	**<0.001**

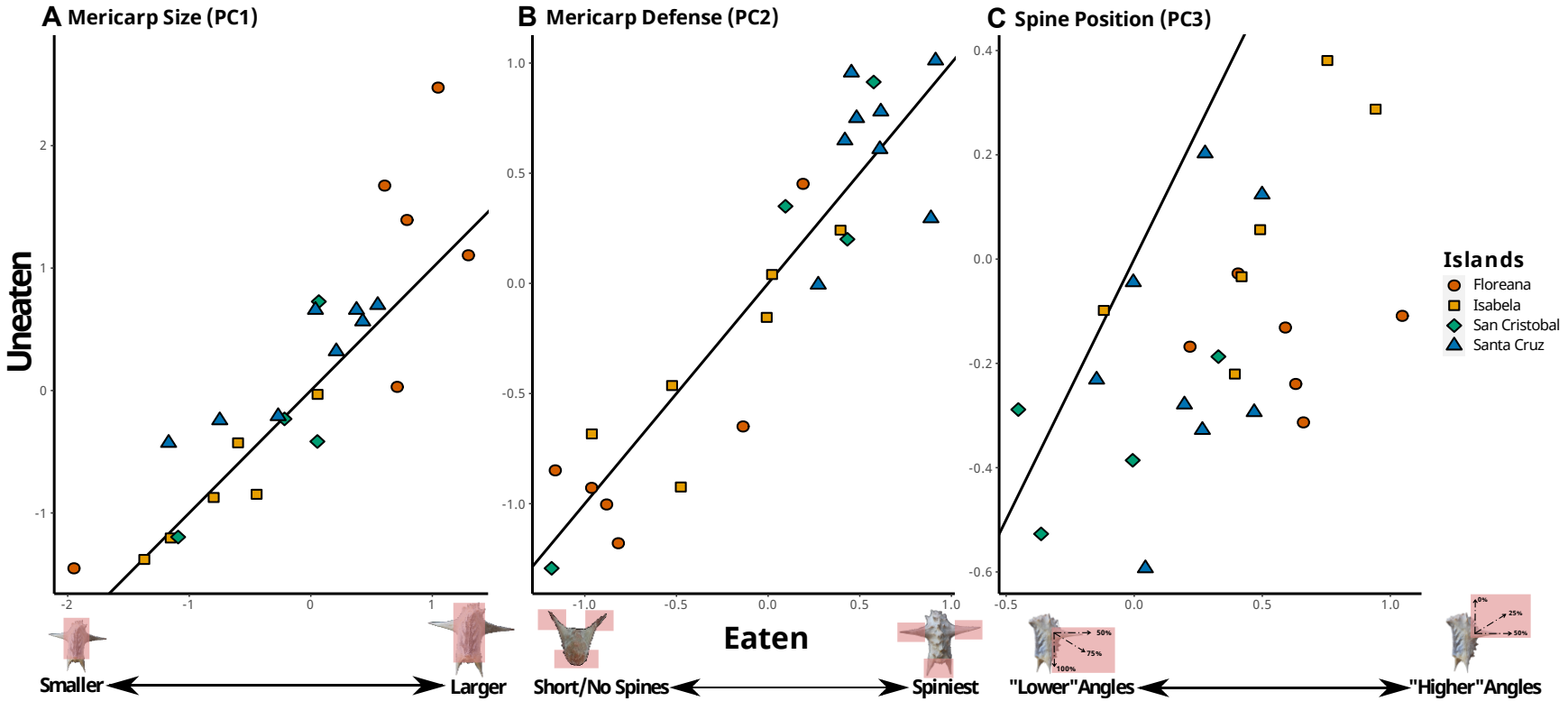

Figure 3. 3 PC plots of mean Eaten (open) and Uneaten (closed) mericarps by population per island. Below each plot, there is a mericarp drawing that highlights in red the associated traits with each axis and an arrow describing the direction of those traits. A) Shows scores of PC1, related to mericarp size. Negative values correspond to smaller mericarps. Positive values correspond to larger, wider, and deeper mericarps. B) Shows scores of PC2 related to mericarp defense. Negative values correspond to mericarps with short upper spines and no lower spines. Positive values correspond to mericarps with larger upper spine length and the presence of lower spines. C) Shows scores of PC3 related to spine position. Negative values correspond to "lower" angles, closer to the lower end of the mericarp body (50% - 100%). Positive values correspond to "higher" angles closer to the upper end of the mericarp (0% – 50%). For a more detailed description of spine position, please see the Measurements Section in the Methods. Plot symbols and colors represent a unique Island: Floreana (orange circles), Isabela (yellow squares), San Cristóbal (green diamond), and Santa Cruz (blue triangle) respectively.

Question 2. Are among-island mericarp differences correlated with selection?

For two of the trait classes, our analyses revealed among-population correlations between mean trait values and the intensity of selection on those traits. First, populations with larger mericarps were under stronger selection for larger mericarps ($R^2_{marginal}$ = 0.25; $R^2_{conditional}$ = 0.27; P < 0.001; Figure 4A). Second, populations with more spiny mericarps (PC2 combining larger spines and the presence of lower spines) were under marginally stronger selection for those very characteristics ($R^2_{marginal}$ = 0.12; $R^2_{conditional}$ = 0.13; P = 0.073). Some of this among-population variation was associated with island. For instance, mericarps on Floreana were typically (but not always) larger than those on other islands and Floreana populations were typically (but not always) under stronger selection for larger size. Mericarps on Isabela were often on the opposite end of this correlation. As another example, mericarps on Santa Cruz often (but not always) were the spiniest, while often (but not always) showing the strongest selection for increased spininess. In contrast to these two trait classes, no noteworthy or significant effects were evident for spine position ($R^2_{marginal}$ = 0.04; $R^2_{conditional}$ = 0.32) (Table 3.1; Fig 3.4C). Comparable analyses of individual traits (rather than trait classes based on PC scores) generally showed similar results, except for spine position that showed significant results (Appendix 3.4). The next question was whether any of the among-island variation in selection or trait sizes could be explained by bioclimatic variables.

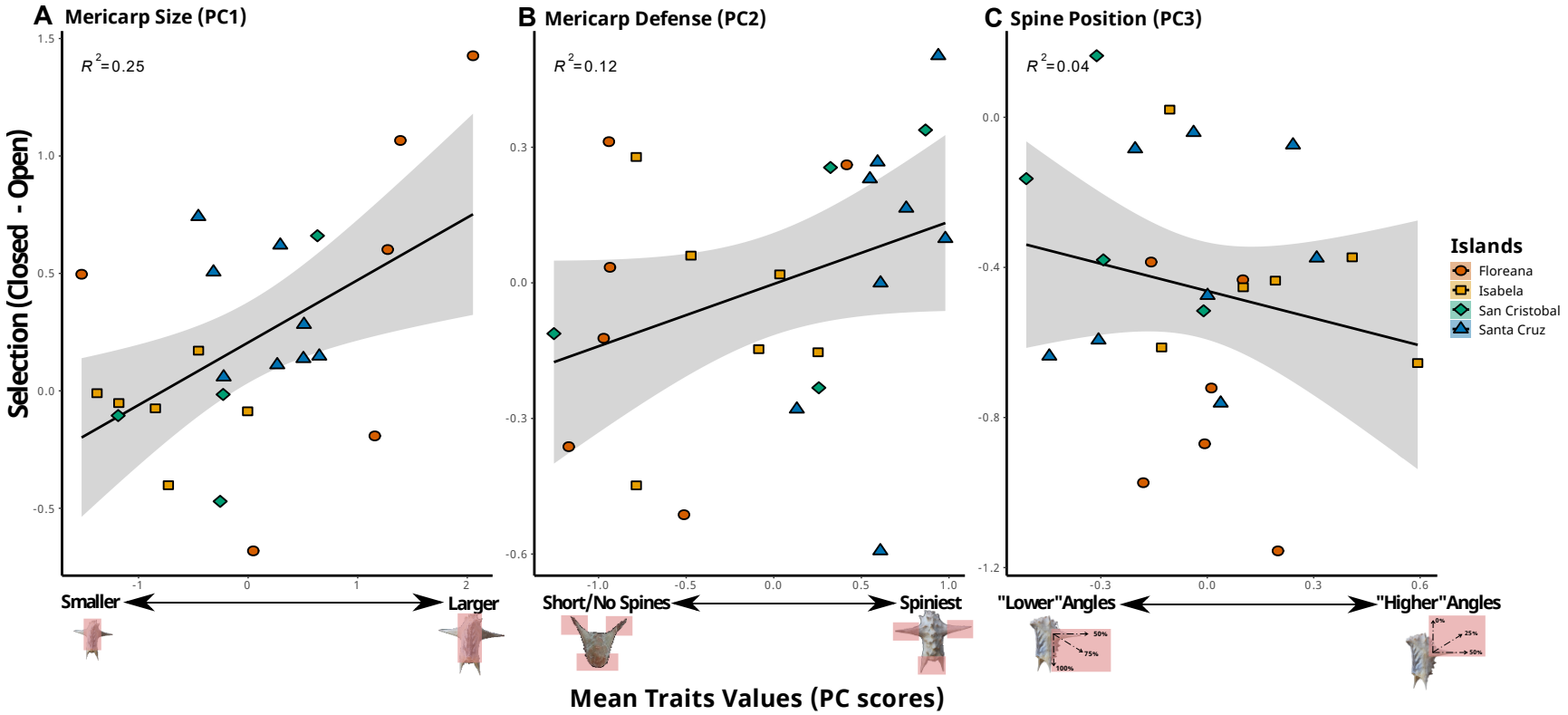

Figure 3. 4 Plots showing the mean PC scores per population by island and the selection estimate, calculated as the difference between the means of closed and open mericarps. Below each plot, there is a mericarp drawing that highlights in red the associated traits with each axis and arrows describe the direction of those traits. A) Shows scores of PC1 related to mericarp size (length, width, and depth) and selection for size. B) Shows scores of PC2 related to mericarp defense (spine length and presence of lower spines) and selection for defense. C) Shows scores of PC3 related to spine position and selection for spine position. The line on each plot is the regression line estimated from the model and the gray area shows the 95% confidence interval of the line. On the top left of each plot is the fixed factor's marginal R^2 estimated from the model. Each color and symbol represent a unique island: Floreana (orange circles), Isabela (yellow squares), San Cristóbal (green diamonds), and Santa Cruz (blue triangles) respectively.

Question 3. Are the among-island differences in selection and traits associated with environmental variables?

Some population mean trait values could be predicted by abiotic variables. First, increasing annual temperature and precipitation (PC2 of bioclimate variables) increased mean mericarp size ($R^2_{marginal}$ = 0.43; $R^2_{conditional}$ = 0.51; based on model; P < 0.004) and mean mericarp spininess ($R^2_{marginal}$ = 0.47; $R^2_{conditional}$ = 0.55; based on model; P = 0.043) (Figure 3.5A). Second, mean mericarp spininess also is higher on islands with strong seasonal variation (PC4 of bioclimate variables), such as Santa Cruz and San Cristóbal ($R^2_{marginal}$ = 0.47; $R^2_{conditional}$ = 0.55; based on model; P = 0.03). (Figures 3.5B and 3.5D). On other islands like Floreana Island, which have no lower spines, this effect may be associated with higher mean spine length. Finally, changes in mean spine positions were associated with changes in biotic variables (PC1). Specifically, mean higher angles are associated with annual temperature changes (PC2), whereas lower angles are more associated with precipitation seasonality (PC4) ($R^2_{marginal}$ = 0.52; $R^2_{conditional}$ = 0.94; based on model; P = 0.004, P = 0.004 and P = 0.017, respectively) (Figure 3.5C and 3.5E). Such is the case of Isabela, that has a higher angle mean and San Cristóbal, that has a lower angle mean.

Some selection estimates also could be predicted by abiotic variables. First, increasing changes in biotic variables (PC1), specifically changes in annual and seasonal temperature (PC3) impose stronger selection of mean mericarp size ($R^2_{marginal}$ = 0.24; $R^2_{conditional}$ = 0.51; based on model; P = 0.003 and P = 0.048, respectively). We also found that increasing changes in seasonal precipitation (PC4) impose stronger selection for spine position, favoring populations with lower angles ($R^2_{marginal}$ = 0.39; $R^2_{conditional}$ = 0.73; based on model; P = 0.001). In contrast with these two trait classes, we did not find significant effects of changes in the environment explaining selection for defense traits ($R^2_{marginal}$ = 0.09; $R^2_{conditional}$ = 0.52) (Figure 3.6).

Finally, we considered if mean trait values or selection estimates could be predicted by a biotic variable, specifically the presence versus absence of large beaked finches capable of opening mericarps. We found that islands where large beaked finches were absent tended to have larger mericarps ($R^2_{marginal}$ = 0.43; $R^2_{conditional}$ = 0.51; P = 0.04) and lower spine angles ($R^2_{marginal}$ = 0.52; $R^2_{conditional}$ = 0.94; based on model; P < 0.001) (Figure 3.7A and 3.7B). We did not find effects of finches on mean mericarp defense, nor on selection estimates for defense or spine position. However, on islands where large beak finches are present, selection estimates for mericarp size were more constrained and closer to zero, whereas on islands where large beak finches were absent selection for mericarp size was more variable ($R^2_{marginal}$ = 0.24; $R^2_{conditional}$ = 0.51; based on model; P = 0.064) (Figure 3.7C). Suggesting that mericarps on islands where large beak finches are present are well-adapted to that selective pressure, and selection estimates are rather weak. The next question will use phenotypic manipulations that inflate trait variation and provide more power to infer how selection favors the current trait values.

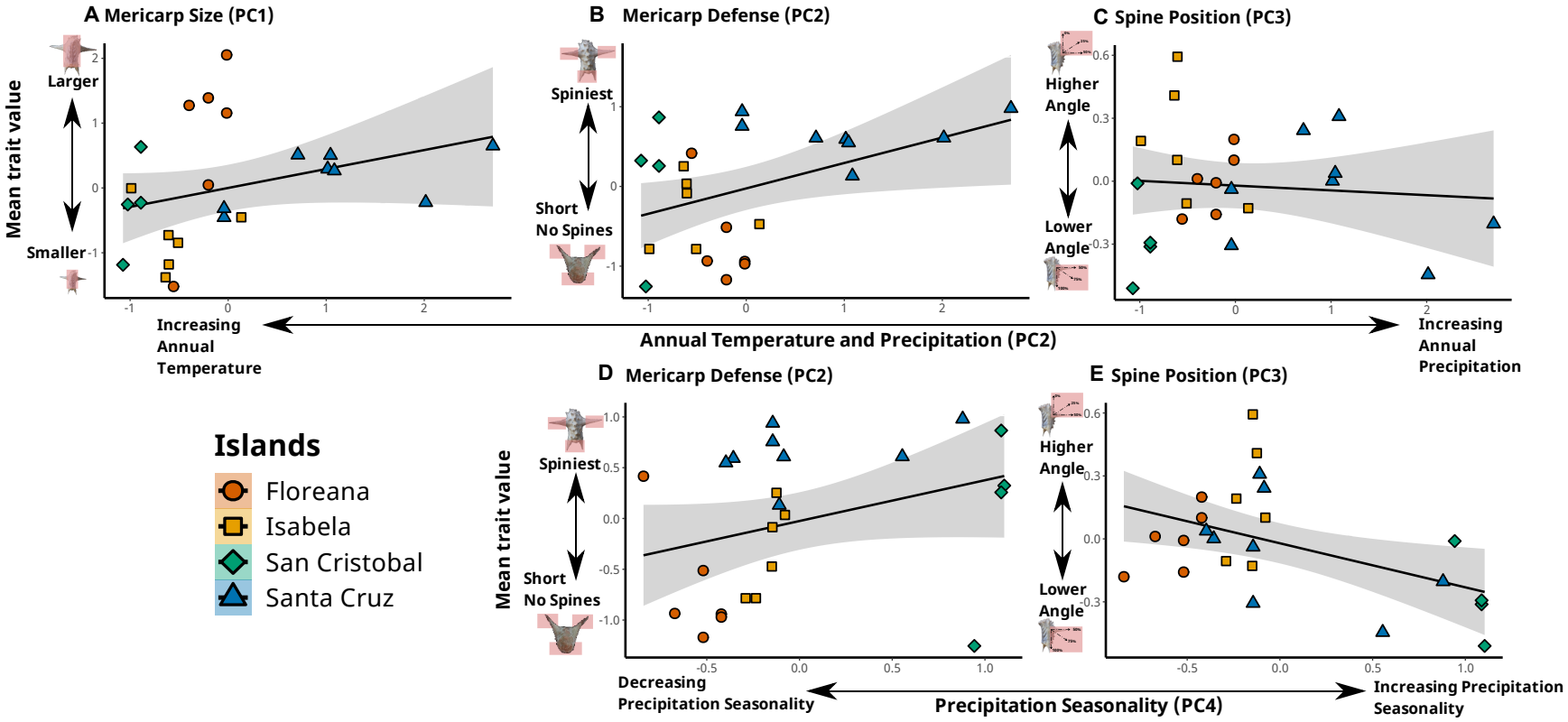

Figure 3. 5 Plots showing the significant effects of bioclimate variables, PC2 (top) and PC4 (bottom) scores and the mericarp mean trait values. Next to each plot there is a mericarp drawing that highlights in red the associated traits of each axis. Arrows next and below plots axis indicate the direction of associated traits. The Y axis is associated with mericarp traits, the X axis is associated with bioclimate variables. Negative values of Bioclimate PC2 are associated with Increasing Annual Temperature. Positive values are associated with Increasing Annual Precipitation. Bioclimate PC4 is associated with Precipitation Seasonality. Negative values are associated with decreasing precipitation and positive values with increasing precipitation seasonality. A) Shows scores of PC2 related to mericarp size (mericarp length, width, and depth). B) Shows scores of PC2 related to mericarp defense (spine length and presence of lower spines). C) Shows scores of PC2 related to spine position (0% - 100%). D) Shows scores of PC4 related to mericarp defense. E) Shows scores of PC4 related to spine position. The line on each plot is the regression line estimated from the model and the gray area shows the 95% confidence interval of the line. Each color and symbol represent a unique island: Floreana (orange circles), Isabela (yellow squares), San Cristóbal (green diamond), and Santa Cruz (blue triangles) respectively.

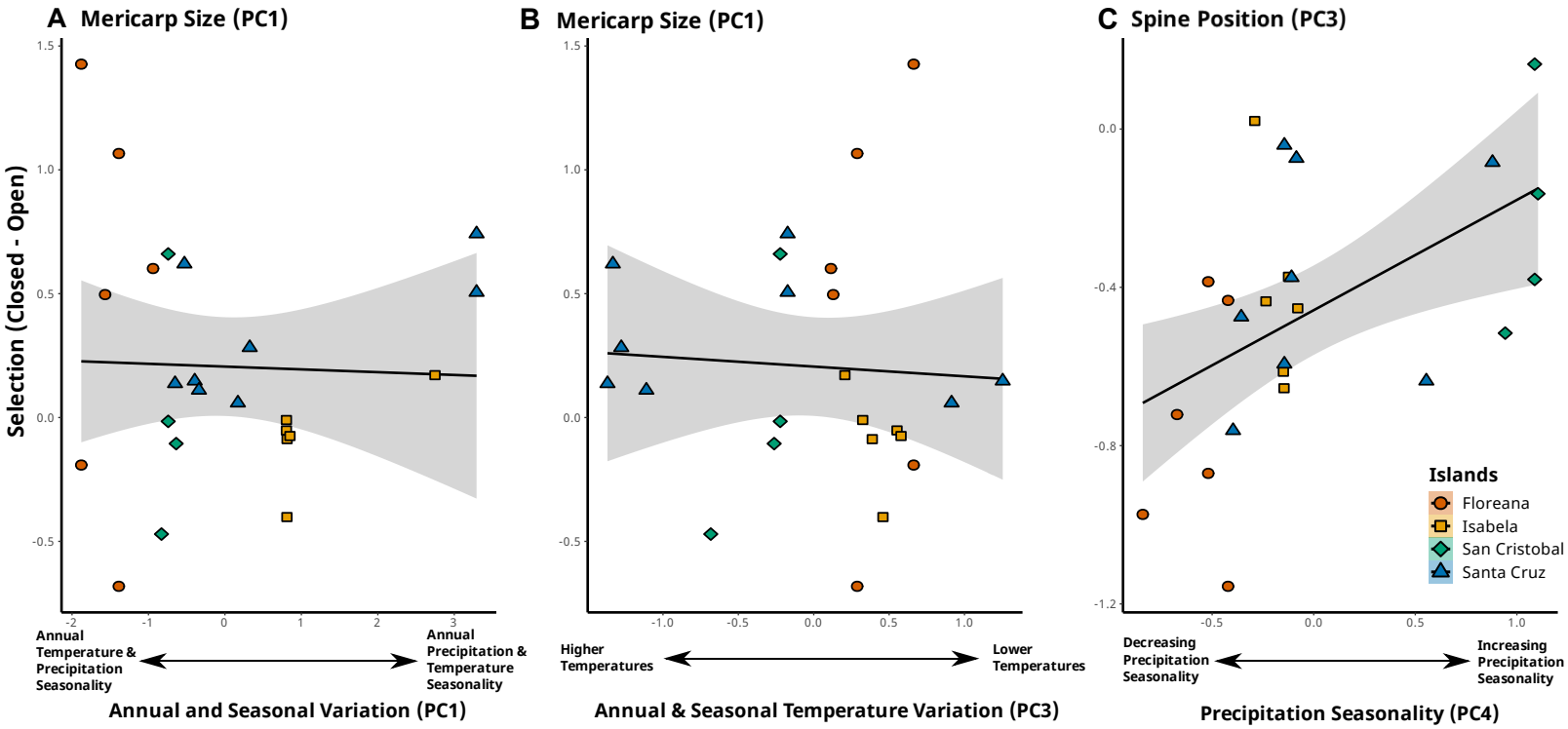

Figure 3. 6 Plots showing the significant effects of bioclimate variables scores and the selection estimates. Arrows below show the direction and association of each bioclimate PC. PC1 was associated with overall annual and seasonal variation. Negative values were associated with annual temperature and precipitation seasonality. Positive values were associated with annual precipitation and temperature seasonality. PC3 was associated with annual and seasonal temperature variation. Negative values were associated with higher temperatures and positive values with lower temperatures. Finally, PC4 was associated with precipitation seasonality. Negative values were associated with decreasing precipitation and positive values win increasing precipitation seasonality. The selection estimate was calculated as the difference between mean closed and mean open mericarps. A) Shows scores of PC1 related to mericarp size (length, width, and depth). B) Shows scores of PC3 related to mericarp defense (spine length and presence of lower spines). C) Shows scores of PC4 related to spine position. The line on each plot is the regression line estimated from the model and the gray area shows the 95% confidence interval of the line. Each color and symbol represent a unique island: Floreana (orange circles), Isabela (yellow squares), San Cristóbal (green diamonds), and Santa Cruz (blue triangles) respectively.

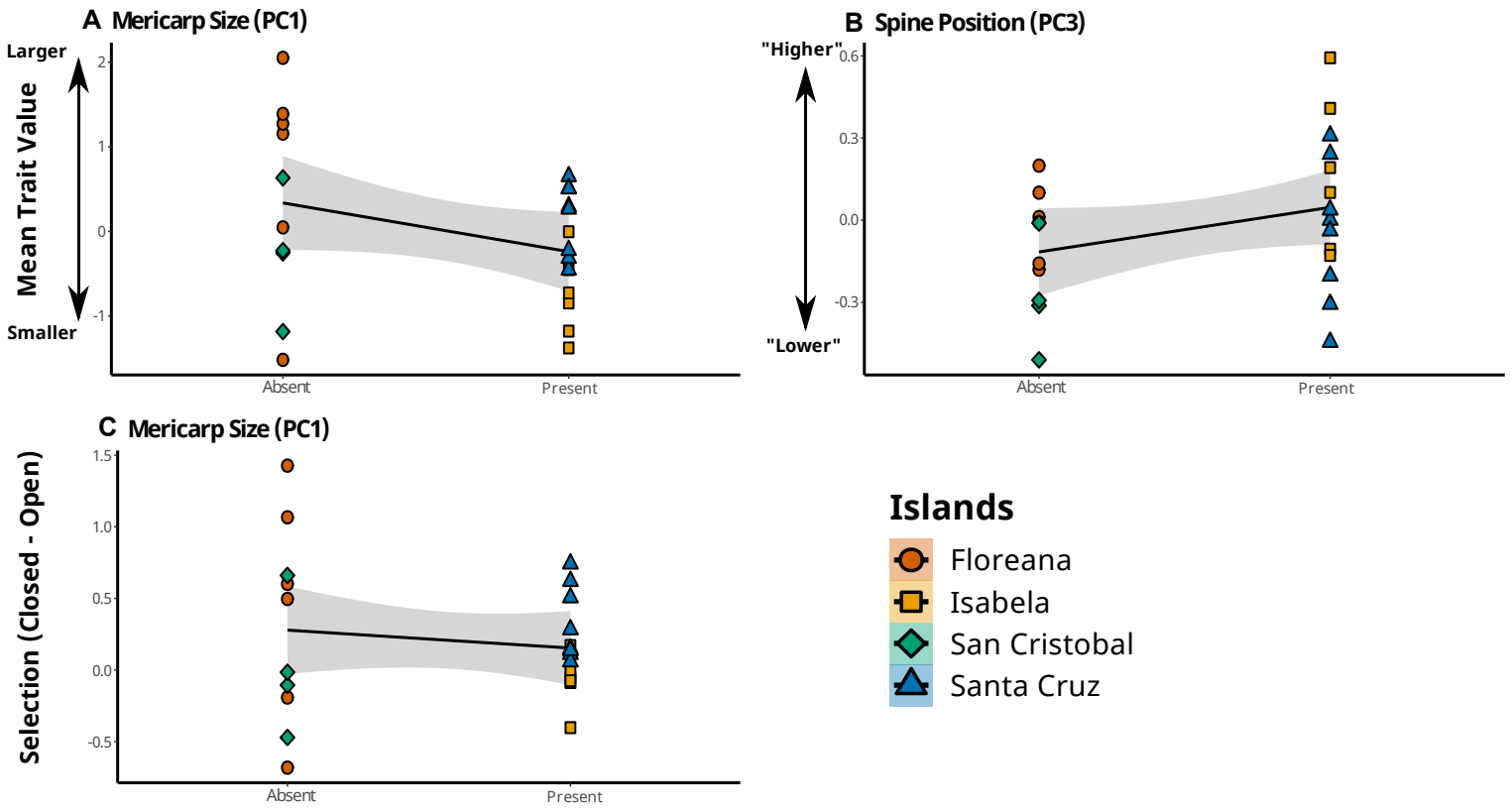

Figure 3. 7 Plots showing the presence or absence of large beak finches (*Geospiza. magnirostris* and *G. conirostris*), in the absence of these species, the medium ground finch, *Geospiza fortis* is the main predator. Plots A and B show the mean trait variation of mericarp size and spine position and the C plot shows the selection estimate, calculated as the difference between mean uneaten and mean eaten mericarps for mericarp size. A) Mean mericarp size. B) Mean spine position. C) Mean size selection. The line on each plot is the regression line estimated from the model and the gray area shows the 95% confidence interval of the line. Each color and symbol represent a unique island: Floreana, Isabela, San Cristóbal, and Santa Cruz respectively.

Question 4. Does inflated trait variation uncover additional selection?

When we experimentally inflated trait variation in factorial mark-recapture seed predation experiments, we found that *Tribulus* intensity of predation differed across islands. For example, when we checked the estimated survival of mericarps (mericarps that remained unopened the longest), overall, mericarps on Floreana island survived 48.3% more compared to the other islands (*Marginal means* = 221 days, CI (95%) = 197.8 – 244), followed by Isabela that survived 43% (*Marginal means* = 193 days, CI (95%) = 168.7 – 218) and Santa Cruz mericarps survived only 27% compared to other islands (*Marginal means* = 120 days, CI (95%) = 97.7 – 142). In addition, when we estimated the rate of mericarp consumption, Santa Cruz Island showed the largest rate compared to other islands (*Marginal means* = 6% CI (95%) = 4.6 – 7.55), followed by Floreana (*Marginal means* = 3% CI (95%) = 2.19 – 4.2) and finally, Isabela (*Marginal means* = 2% CI (95%) = 1.14 – 2.56) (Appendix 5). Suggesting that mericarp consumption differed across islands and may be influenced by other factors.

However, *Tribulus* mericarps experienced similar (across islands) patterns of contemporary selection for the experimental treatments after controlling for variables related to the experimental design. Mericarps that survived the longest tended to be the largest mericarps with all spines intact. These mericarps survived in Floreana 60% of the time of the experiment (Estimated *marginal mean* of 274 days of 458 total days survived; CI (95%) = 243 - 304) more compared to the other categories in that island. In Isabela they survived 50% more (*Marginal mean* of 225 days, 447 of total days survived; CI (95%) = 187 - 262), and in Santa Cruz 33% more compared to the other categories (*Marginal mean* of 145 days, 437 of maximum of days survived; CI (95%) = 113 - 177). The mericarps that survived the least were small mericarps with removed spines. These mericarps survived only 37% of the time of the experiment in Floreana and 37% in Isabela. However, in Santa Cruz, small mericarps with all spines survived the least with only 21% compared to the other categories (Figure 3.8). Suggesting that mericarp size and spines are crucial for mericarp survival.

When we estimated the rate of mericarp consumption per category, we found that the pattern was similar across islands, but the experimental categories differed between these estimates and survival estimates. We found that mericarps with their spines present were eaten the most than mericarps without spines, with smaller mericarps getting eaten more than larger mericarps within these categories. For example, small mericarps with spines were considered predated the most. Small spined mericarps were predated at different rates across islands. In Floreana these mericarps were eaten 5% more compared to other experimental categories (CI (95%) = 3.41 – 8.31). In Isabela they were eaten 2% more (CI (95%) = 1 – 4.1) and on Santa Cruz 10% more compared to the other categories (CI (95%) = 7.26 – 14.16). The least predated mericarps were large mericarps with all spines intact. In Floreana these mericarps were eaten only 2% of the time, and on Isabela only 1%. However, on Santa Cruz, the least predated mericarps were large with spines all removed at 3% (CI (95%) = 0.67 – 3.42; CI (95%) = 0.37 – 2.72; CI (95%) = 1.88 – 5.76, respectively). This suggests that survival estimates explained above, and mericarp predation shown here coincide on size treatments but not on spine treatments (Appendix 3.5).

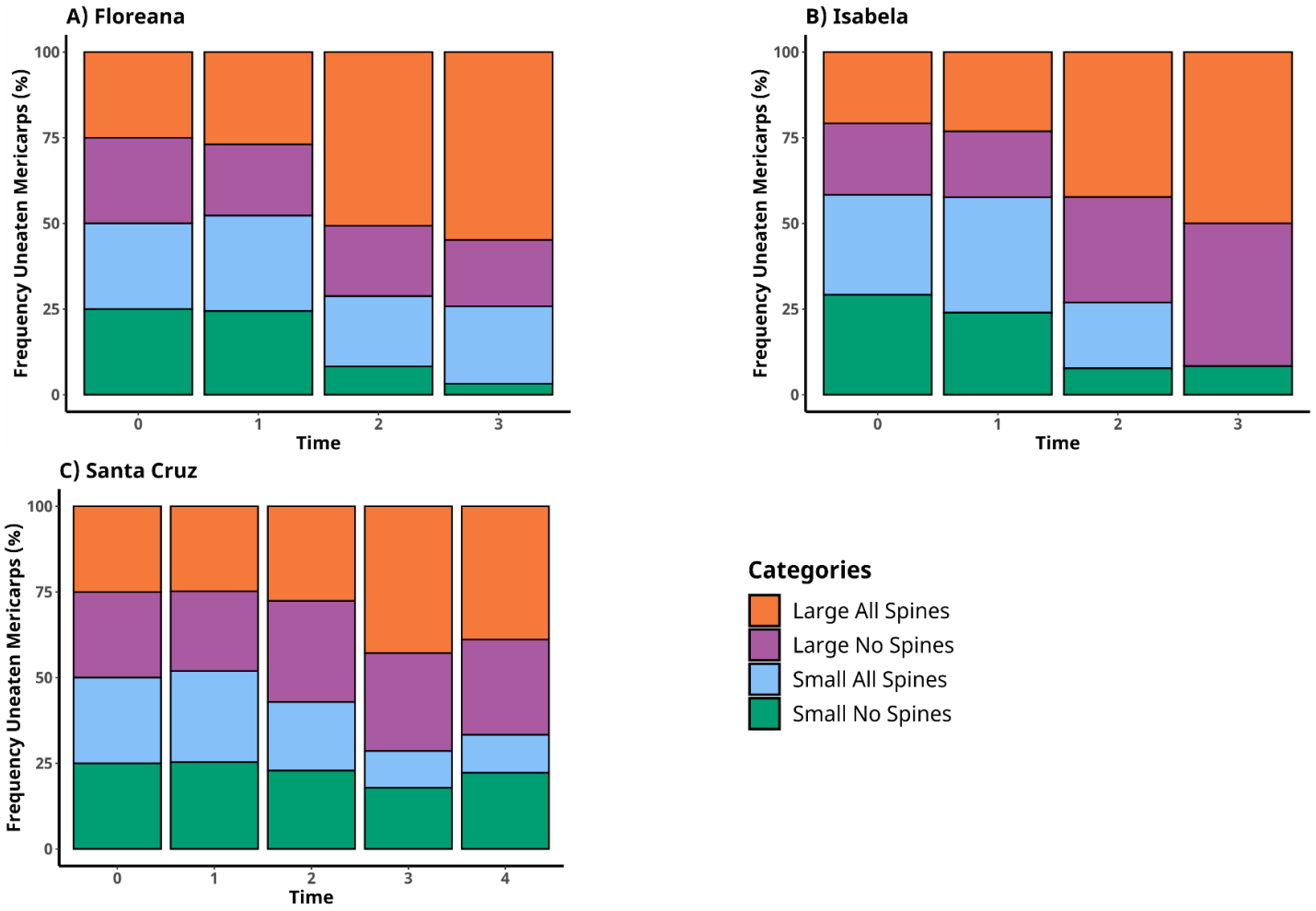

Figure 3. 8 Frequency plots for each group category in the Mark-recapture experiment. The frequencies are from survived – uneaten mericarps. Each plot shows a different island. At the start of the experiment all categories have the same proportion of mericarps. Over time the proportion of some categories was reduced. At the end of the experiment mostly large mericarps survived.

Discussion

We used multiple approaches to explore the evolutionary processes shaping plant traits on islands: specifically defensive structures (spines) and the overall size of *Tribulus* mericarps on Galápagos. Our specific focus was on explaining trait variation as a function of selection imposed by specialist seed-predators: Darwin's finches in the genus *Geospiza*. Our first analysis revealed only weak evidence for any consistent patterns of selection across Galápagos populations; instead, the primary pattern was for large among-population heterogeneity in both trait means and selection. Our second analysis showed how some of the among-population variation in selection was positively associated with among-population variation in mean traits, particularly for mericarp size (PC1) and marginally so for mericarp defense (PC2). That is, populations with larger mericarps (relative to populations with smaller mericarps) were under somewhat stronger contemporary selection favoring larger mericarps. Our third analysis revealed how some of the among-population variation in mean trait values and selection was associated with biotic or abiotic factors. For biotic factors, the presence of finches with the largest beaks (*Geospiza magnirostris* or *Geospiza conirostris*) was associated with smaller mericarps and "higher spine angles" (that is, the upper spines lean more towards the upper end of the mericarp body). For abiotic factors, greater annual temperatures were associated with mericarps that were smaller and less spiny, and with their spines at "low angles" (that is, the upper spines lean more towards the lower end of the mericarp body). Further, greater annual precipitation was associated with mericarps that were larger and spinier, with their spines at higher angles. In our final analysis, we inflated trait variation in a factorial mark-recapture experiment that revealed how the mericarps least likely to be opened (i.e., no seeds eaten by finches) were largest and had the full complement of spines. In the following sections, we unfold explanations for, and implications of, these results in the context of the evolution of species interactions.

Question 1. Are Galápagos mericarps under a general form of selection?

Tribulus cistoides mericarps are larger on Galápagos than elsewhere in their global distribution (Reyes-Corral et al., 2023) (Chapter 2), which suggest some archipelago-wide factor favoring larger mericarps. We started by asking if specialized seed predators (the granivorous *Geospiza* finches) are still imposing selection on this trait – or on other traits, such as overall spininess or spine position. Such consistent contemporary selection acting on already-divergent traits would be expected if (1) insufficient time has passed for *Tribulus* to fully adapt to seed predation by finches, or (2) trait values that resist predation are subject to a trade-off with other selective factors (e.g., dispersal) or fitness component (e.g., germination success). In Chapter 4, we used phylogenetic analyses to estimate that *Tribulus* has been present on Galápagos for approximately 0.92 million years, and so the first of these hypotheses seems unlikely (but see below). Trade-offs, however, do seem likely; yet, as we will now explain, they do not appear to maintain archipelago-wide selection.

For mericarp size and spininess, most populations were not under much (if any) directional selection, implying that the generally large and spiny mericarps of Galápagos *Tribulus* are at least reasonably well adapted for finch predation. This interpretation makes sense given how finches eat *Tribulus*: they manipulate the body of the mericarp and avoid spines by rotating it towards the frontal end, where the seeds are located, before then cracking the fruit (Carvajal-Endara et al., 2020; P. R. Grant, 1981; P. R. Grant & Grant, 2014; T. Price, 1987). As such, larger and spinier mericarps should be harder to manipulate and open for most finches (Carvajal-Endara et al., 2020; P. R. Grant, 1981). Some specific populations, however, were still experiencing strong selection (Figure 3), suggesting that their trait values remain short of the fitness peak for resisting local finch predation. Such population-specific (as opposed to archipelago-wide) trait mal-adaptation might be explained by several factors (Brady, Bolnick, Angert, et al., 2019; Brady, Bolnick, Barrett, et al., 2019). First, high gene flow from populations with smaller trait values could prevent strong adaptation where larger mericarps are favored, thus

maintaining local selection at those places. Second, environmental conditions might recently have changed in some populations, thus imposing increased contemporary selection. Third, trade-offs with other selective factors or fitness components (as noted earlier) could be stronger in some populations, thus constraining local adaptation to finch predation in specifically locations. Attempting to dissect these various potential causes requires consideration of population-specific factors and is therefore reserved for the subsequent questions.

In contrast to mericarp size and spininess, almost all populations experienced a similar direction of selection for spine position (Figure 3.3). This consistent selection across populations cannot be explained by gene flow because that force would not generate a system-wide direction of selection. Trade-offs, however, could provide an explanation – especially with respect to dispersal. In *Tribulus*, the upper spines are oriented in a way that, when a mericarp is on the ground, the spines are mostly pointing upward (M. K. A. Johnson et al., 2020). As such, it is possible that "high" spine angles are good for dispersal whereas "low" spine angles are good for avoiding predation. Interestingly, this balance between opposing selective forces might recently have changed given that most dispersal is now driven by human influences, such as car tires, bike tires, shoe soles, and the hooves of feral goats or donkeys (Goeden & Ricker, 1973; E. Johnson, 1932; M. K. A. Johnson et al., 2020; Squires, 1979). Hence, it is quite possible that a change recent selection by these new forces has dragged spine angle off its finch-adapted peak and thus (again) enhanced selection via the trade-off between dispersal and predation defense. Also, note that this explanation resurrects the above "insufficient time" argument: that is, even though *Tribulus* has been present on Galápagos for more than a million years, these new dispersal-related selection pressures have only become intense in the last 100 years or so (Benítez et al., 2018). The system might not yet have reached a new equilibrium.

Question 2. Are among-island mericarp differences correlated with selection?

Question 1 started by asking whether any patterns were shared across *Tribulus* populations in Galápagos. As explained above, however, the primary result – at least for mericarp size and spininess – was instead substantial variation among populations. For instance, the five populations with the largest mericarps (and the population with the smallest mericarps) are found on Floreana, seven of the eight populations with the spiniest mericarps are found on Santa Cruz, and spine angles are generally the lowest on San Cristóbal and highest on Isabela. This variation among populations – and especially that among islands – suggests an explanation based on local selective conditions.

Our starting point for understanding this among-population variation was to ask if mean trait values were correlated with contemporary selection (Figure 3.4). For spine position, no correlation was evident, which is consistent with the above finding of a shared archipelago-wide direction of selection – perhaps due to a trade-off with other selective agents, which might recently have changed. For mericarps size and spininess, however, a positive correlation was evident: that is, populations with larger mericarps were more likely to be under selection for even larger mericarps, and populations with spinier mericarps were more likely to be under selection for even spinier mericarps. These associations are perhaps most consistent with the gene flow explanation that we entertained earlier: that is, populations where the most extreme phenotypes are favored are most likely to be held short of their optima (and therefore remain under selection) as a result of gene flow among populations (Bolnick & Nosil, 2007).

This gene flow scenario is perhaps best illustrated by considering variation on Floreana. First, populations on Floreana were often under the strongest selection (Figure 3.3). Second, Floreana showed the most among-population variation in mericarp size, including five populations with the largest mericarps and the population with the smallest mericarps. Third, the populations with the largest

mericarps were located closer to the main town (Puerto Velasco Ibarra) and the only road up to the highlands. The population with the smallest mericarps was located in Post Office Bay, a tourist site on the northern end of the island. Boats with tourist connect these two points directly and may act as dispersal vectors for mericarps between populations. It is therefore quite likely that mericarps are moved back and forth between these populations, perhaps constraining adaptation in both general areas. In addition, the above-described dispersal/predation trade-off might play out differently on Floreana. In particular, populations on Floreana lack lower spines, which are mainly associated with mericarp defense (Carvajal-Endara et al., 2020; M. K. A. Johnson et al., 2020). Hence, upper spines might be selected more strongly for defense rather than dispersal and perhaps size is indirectly being selected for more effective dispersal. Of course, additional work will be required to formally test these mechanistic hypotheses.

Question 3. Are the among-island differences in selection and traits associated with environmental variables?

Even if gene flow – perhaps recently accentuated by humans – is an important contributor to among-population differences in contemporary selection (as suggested above), we still need to seek an explanation for why some populations have dramatically different trait values from other populations. Founder effects or genetic drift seem unlikely given that substantial trait variation is present at all levels: among islands, among populations within islands, and within populations (see Chapter 2 and Carvajal-Endara *et. al* (2020)). More profitable would be analysis of various biotic or abiotic correlates of trait variation or selection (Hendry, 2017; MacColl, 2011; Wade & Kalisz, 1990). For biotic factors, we considered presence/absence of the large-beaked finch species (e.g., *G. magnirostris* and *G. conirostris*) that can most easily open mericarps (De León et al., 2014; P. R. Grant & Grant, 2014; T. Price, 1987). For abiotic factors, we considered various estimates of temperature, precipitation, or seasonality.

For biotic factors, our study showed some similarities to, and also some differences from, previous work relating trait variation and selection in *Tribulus* to distributions of the large-beaked finch species (Carvajal-Endara et al., 2020). Rather than detailing the specific similarities and differences between these studies, we here emphasize that statistical significance and effect sizes were weak in both cases. In short, the shared interpretation of both studies is that factors other than the presence/absence of the large-beaked finch species must be the most important determinants of selection and trait variation. This result is not surprising because other finch species can also (albeit with greater difficulty) open mericarps (Conant, 1988; P. R. Grant, 1981; Pimm, 1988), and the large-beaked species are generally quite rare at any given location (Dvorak et al., 2012; P. R. Grant, 1999; Schluter, 1982a). Further, finch predation varies through time at any given location. Hence, much of the variation in selection imposed by finches is likely driven by other factors, some of which might interact with the presence of large finches. For instance, Carvajal-Endara *et al.* (2020) found that finch-imposed selection on *Tribulus* traits varied dramatically among years with different rainfall levels. In our study, we extended these temporal considerations to the spatial dimension by asking if variation among populations in climate is correlated with mean trait values and selection.

Abiotic factors were indeed associated with some of the among-population variation in mean trait values. For instance, a trend was present for populations experiencing the highest rainfall (most of these were on Santa Cruz) to have larger and spinier mericarps than other populations. Another modest trend was for spinier mericarps at locations with greater precipitation seasonality, especially on San Cristóbal (Figure 3.5D). Spine position (angle) again showed perhaps the most striking pattern, with sites having greater precipitation seasonality also having mericarps with lower angles (spines leaning towards the lower end of the mericarp body) and precipitation seasonality also associated with strong selection for lower angles (Figure 3.6C). Determining potential causal effects underlying these associations will require further work. For instance, wetter conditions might cause

plastic changes that generate larger and spinier mericarps. These conditions may favour growth of reproductive organs such as seeds, fruit, and flowers. Plants may allocate more resources to their reproduction increasing investment in those traits, or wet conditions might alter predation rates by finches, which may prefer other seed resources aside from hard fruits such as *Tribulus* (which might then alter selection). Overall, however, the observed patterns point to the value of further exploring interactive effects of biotic (finches) and abiotic (especially precipitation) influences on spatial variation in traits and contemporary selection.

Question 4. Does inflated trait variation uncover additional selection?

The results discussed above suggest that *Tribulus* traits in Galápagos, at least mericarp size and spininess, are reasonably well adapted to finch predation (Question 1). In such cases, past selection could "erase its traces" to the point that estimates of contemporary selection are not very informative about the process of adaptation (Haller & Hendry, 2014). One solution is to conduct experiments that inflate trait variation to reveal again the selection that generated the current well-adapted state. We inflated mericarp trait variation by selecting from extremes of the trait distribution (large or small) and by manipulating traits (clipping spines). We then marked the individual mericarps, placed them into natural settings, and monitored them for more than a year to detect finch predation. This approach worked extremely well in enhancing and expanding the above inferences about adaptation in this system.

For starters, our experimental manipulation revealed strong and consistent (across years and islands) finch-imposed selection that favored larger and spinier mericarps (i.e., such mericarps were less likely to be opened by finches). This result clearly supports our assertions that the exceptionally large and spiny mericarps of *Tribulus* from Galápagos, relative to elsewhere, are the result of past selection imposed by finch predation.

We also found differences between selection and predation rate across islands. As mentioned above on all island's selection favored larger and spinier

mericarps. However, selection varied across islands. For example, in Floreana, mericarps survived on average the longest number of days, with a difference between mericarps with and without spines of 47 days for large mericarps and 41 days for smaller mericarps. Whereas on Santa Cruz, mericarps survived the least, with a difference between mericarps with and without spines of 8 days for large mericarps and 13 days for smaller mericarps (Appendix 3.5). This suggests that selection for mericarps is strongest in Floreana than Santa Cruz. These differences in selection could be explained by the total predation rate, which was higher on Santa Cruz compared to any other island, whereas mericarps in Isabela were eaten the least.

Also, our experiment helped to disentangle the effects of upper and lower spines on finch predation risk. Previous studies suggested that upper spines are related to defense and dispersal, whereas lower spines are exclusively related to defense (M. K. A. Johnson et al., 2020). Our experiment supported the first part of this argument because mericarps with upper spines survived the longest; yet it also showed that the survival benefits of lower spines depended on mericarp size (Appendix 5). Small mericarps with all spines present survived the least time. The study by Rivkin *et. Al* (2021) also found increased predation of smaller mericarps with all spines present. In combination, these results suggest that the presence of upper and lower spines is not an additive effect and further experiments will be required to disentangle the reasons for this interaction.

Limitations and Future Work

Our study had several limitations that help suggest useful future work on *Tribulus* in Galápagos. For example, focused work should investigate the genetic basis for mericarp traits and for trait differences between populations, such as through common-garden rearing, reciprocal transplants, or QTL mapping of trait differences. Another avenue to explore would be population structure and gene flow within and between *Tribulus* populations in Galápagos – as those parameters would help inform some of the inferences we have attempted, such as gene flow imposing a

constraint on the adaptation of extreme phenotypes. In Chapter 4, I found unique haplotype variation within some *Tribulus* populations that stemmed from a monophyletic group, suggesting that any potential genetic differences occurred when *Tribulus* colonized and then expanded across islands. However, that study did not attempt to infer gene flow among populations on islands, nor in relation to recent human influences on dispersal. It would be interesting to explore these trade-offs on islands where human activities are less present, given that our populations are on islands with higher human activity, our results may reflect this factor. Additionally, inferences would benefit from better information on the relative abundance of large beaked ground finches, as such information would be much more useful than simply presence/absence at the island level.

Conclusions and Implications

We conclude that phenotypic divergence between *Tribulus* mericarps on Galápagos versus the rest of their global distribution mainly reflects adaptation to their specialist speed predators – the Darwin's finches. Further, trait differences among populations within Galápagos reflect a combination of biotic and biotic factors, likely interacting with each other. We further conclude that instances of contemporary selection could reflect recent human influences on the dispersal/defense trade-off and gene flow. In addition, our study is yet another example of the importance of using experimental manipulations and natural population trait variation to uncover past selection (Reznick & Ghalambor, 2005). Similar to other studies of natural selection in the field (Endler, 1980; Losos et al., 1998), our experiment revealed the importance of population structure in shaping adaptation, and also showed evidence of consistent patterns of selection and potential trade-offs that better described the factors driving selection in the mericarp population of Galápagos. Thus, our study provided a better understanding of how phenotypes respond to selection on a non-endemic species that in part also shapes the evolution of its endemic predator and highlights how environments found on oceanic islands can hide complex and dynamic interactions between biotic and abiotic factors that are both historical and contemporary.

We know that *Tribulus* traits have influenced the adaptive radiation of finches and their contemporary (rapid) evolution (P. R. Grant, 1999; P. R. Grant & Grant, 2014). Our recent work has highlighted the reciprocal effect: how the finches and other factors are altering *Tribulus* evolution, (see also: Carvajal-Endara et al., 2020; Rivkin et al., 2021). These interactions between finches and *Tribulus* surely interact to shape the evolution and success (distribution and abundance) of both and, given that finches and *Tribulus* both influence other species in the environment, this reciprocal evolution surely alters those environments (i.e., eco-evolutionary dynamics and feedbacks). As such, the recent human influences that we are suggesting could well disrupt these dynamics and generate highly modified evolutionary trajectories of both *Tribulus* and finches, potentially altering their distribution and population dynamics, and hence their effects on other species. Indeed, we have already shown how specific human influences are shaping finch evolution (De León et al., 2011, 2014, 2018; Hendry et al., 2006), and Tribulus traits (M. K. A. Johnson et al., 2020; Rivkin et al., 2021) – so these interactive effects seem almost inevitable. Here then is perhaps the next inferential realm for research on Galápagos finches: how human influences alter evolutionary trajectories of species interactions and thereby alter ecological dynamics.

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Linking statement 3

In the previous chapter, we showed that *Tribulus* mericarps are well adapted to predation by finches. Larger and spinier mericarps are the results of *Tribulus* response to predation, suggesting that *Tribulus* and finches have coevolved mericarp size and beak shape traits in response to their interactions.

However, in these cases we need a historical context to determine how long this interaction has been going on, and with that evidence to debate whether it is possible that this interaction and these changes are the product of natural selection, but at the same time evidence of adaptation and differentiation. To provide historical context we need molecular methods that allow us to quantify differences in certain regions of the species' DNA. This information also allows us to determine more specific questions such as divergence times, population structure, and colonization events.

Phylogeography is a field of historical biogeography focused on investigating the spatial and temporal history of taxa by integrating patterns of genetics and geography (Avise, 2009; Avise et al., 1987). These studies are conducted at the level of species or intraspecific populations to uncover how current distribution patterns have been influenced by past events such as geological changes and colonization patterns. Phylogenetic methods assist in determining the number of colonization and the origins of geographical sources by testing if groups of interest are monophyletic or not. If groups are monophyletic, then is assumed that a single colonization event occurred, whereas if there are multiple groups, then is concluded that there were multiple colonization events, either from the same source or from multiple geographical locations. We can estimate the time of divergence of these phylogenetic relationships using molecular clock models and tree calibrations either with fossils or secondary calibration points (Baele et al., 2013; Drummond et al., 2006). Then, haplotype reconstructions help identify unique genetic fingerprints in specific areas, such as between islands which can provide evidence of populations' native genetic divergence if enough time has passed. Together these methods

provide evidence of evolutionary historical context of the species of interest in the islands.

For the next chapter, using phylogeographic tools we will estimate the historical context of *Tribulus cistoides* in the Galápagos Islands. This will help us further understand the microevolutionary mechanisms and the phenotypic variation treated in the previous chapters.

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CHAPTER 4: Phylogeography of *Tribulus cistoides* in the Galápagos Islands: insights into evolutionary history

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This chapter is being prepared for submission.

Introduction

Oceanic islands offer a natural setting to study organisms' abilities to colonize and establish (I. Thornton, 2007; P. Vargas et al., 2014). Over time, the interplay between these two characteristics is thought to influence species composition, range expansion, genetic structure, and phenotypic characteristics of species island communities (Burns, 2019; Heleno & Vargas, 2015; I. Thornton, 2007). Ultimately, the outcomes of colonization and establishment could also determine novel interactions between species impacting the evolutionary trajectory of one or multiple of these interacting species. In some cases, some colonists could facilitate the establishment of other species, or outcompete others affecting their persistence through time (Heleno & Vargas, 2015; Hulme & Barrett, 2013).

In this sense, a plant colonist, once established, is the baseline of any volcanic island community (I. Thornton, 2007). Plants on islands give the starting point for sustaining many species interactions, either serving as a food source or sustaining mutualist networks of dispersers and pollinators. Over time, some of these interactions lead to divergence and in some cases to specialized, often unique organisms. On the other hand, other plant species may not necessarily become endemic, but they can still become key species to sustain the island community by supporting or serving as resources for other (even endemic) species. For example, species of *Ficus* on Anak Krakatau have been correlated with the colonization of fruit bats, frugivorous birds, and fig wasps. These species may not be established otherwise, once *Ficus* was already on the island, making *Ficus* a key species in the island ecosystem (I. Thornton, 2007; I. W. Thornton et al., 1996; I. W. B. Thornton et al., 1993; Zann & Darjono, 1992).

The value of these key plant species has been underappreciated (Schlaepfer et al., 2011), but it has been shown that they can play an important role in the evolution of endemic species (Boag & Grant, 1981; Carvajal-Endara et al., 2020; P. R. Grant, 1981; P. R. Grant & Grant, 2014, 2014). This is the case of *Tribulus cistoides* on the Galápagos, a potentially non-native plant, even considered

introduced that has been demonstrated to play a role in beak selection for Darwin's finches. We previously demonstrated that *T. cistoides* diverged phenotypically relative to continental populations, having larger and spinier mericarps (Reyes-Corral et al., 2023). Also, we have demonstrated that *T. cistoides* larger mericarps are the result of adaptation to finch predation (Chapter 3, but also (Carvajal-Endara et al., 2020)). However, we still have not determined the historical context of *Tribulus* in the Galápagos and its implications in the evolution of this plant and its interactions with endemic species.

There are two species present of the genus *Tribulus* in Galápagos. *T. cistoides* is a widespread plant, hypothesized to be originally from Africa, and *T. terrestris*, originated from the Mediterranean (Wiggins & Porter, 1971). These species are commonly found in tropical and subtropical regions around the world including islands (Kearney et al., 2020). Both species produce hard fibrous fruits called mericarps. *T. cistoides* mericarps are larger than *T. terrestris*. Mericarps, are great units of dispersal because they can hold viable seeds for many years (Goeden & Ricker, 1973; E. Johnson, 1932), and have spines that allow them to be dispersed by larger animals, among those, seabirds, and humans. These vectors facilitate their arrival to islands (Hooker, 1847a; M. K. A. Johnson et al., 2020; Porter, 1971). There has not been definitive evidence on how long the *Tribulus*-finch interaction has been going on, especially since there has been speculation about whether *Tribulus* is old or young on the islands (i.e. millions/thousand vs. hundreds of years) (Porter, 1971; Traveset et al., 2013; Wiggins & Porter, 1971). On one hand, *Tribulus* may have been introduced naturally, by birds, and on the other hand it could have been introduced by humans in recent times. The importance of addressing this problem stems from the impact of these interactions in determining the adaptive radiation and evolution of Darwin's finches on the Galápagos. If the origin of *Tribulus* is old, it implies these interactions could have naturally been ongoing for millennia. Alternatively, if *Tribulus* is young and potentially introduced by humans, then finch-plant interactions could be a recent factor affecting these evolutionary outcomes and thus changing our understanding of this iconic avian radiation as an

outcome of anthropogenic disturbance. Thus, understanding how *Tribulus* in the Galápagos has helped shape the current communities, divergence, and interactions is an important aspect of the study. Specifically putting *Tribulus* in a historical context to determine times of divergence and colonization patterns, either via phylogeography studies or haplotype reconstructions.

In this study, we used field collected and herbarium samples of closely related *Tribulus* species to reconstruct the phylogeny and haplotype networks of this plant in the context of the Galápagos Islands. Our objectives are 1) to determine the number of colonization events (either single or multiple), 2) to estimate divergence times of each of these events, 3) to describe genetic differentiation on islands using haplotype networks, 4) to provide historical context of *Tribulus* and Darwin's finches interactions, based on the time estimates from the phylogeny. We used chloroplast and nuclear markers to infer these estimates between Galápagos and continental populations and used previous phylogenetic studies on Zygophyllaceae as calibration for our estimates (Böhnert et al., 2020; Godoy-Bürki et al., 2018; Wu et al., 2018).

Methods

Sample collection

To generate the first molecular phylogenetic hypothesis in this group, we collected *Tribulus* leaf tissue samples from the field and herbarium vouchers. Field samples were collected in the Galápagos Islands based on a Collaborative Agreement in 2017 between the Universidad San Francisco de Quito (USFQ), the Galápagos Science Center (GSC), and the Galápagos National Park (PNG), in their annual population monitoring of emblematic species across the archipelago (NPC-14-17) and under the Contrato Marco de Acceso a Recursos Genéticos, from the Environment Ministry of Ecuador (MAE-DNB-CM-2016-0041). Tissue samples were collected with disinfected tweezers, deposited in individually labelled coin envelopes per population. Samples were stored in resealable bags containing dry silica gel

beads. We collected a total of 81 tissue samples across 11 islands: Champion (5), Española (11), Fernandina (4), Floreana (10), Genovesa (3), Isabela (4), Rábida (9), Santiago (10), San Cristóbal (7), Santa Cruz (12), Seymour Norte (6). Española and Floreana samples were collected in two different locations per island: Punta Suárez (3), and Bahía Gardner (8) for Española; Post Office (8) and Las Cuevas (2) for Floreana. The rest of the samples were collected from one location per island (Figure 4.1) (See also, Appendix 4.1; Table S4.1). We also collected vouchers (n = 71) and mericarps (n = 1613) that were used for morphological data (Reyes-Corral et al., 2023). The vouchers and mericarps were deposited in the Charles Darwin Research Station Herbarium (CDRS), in Puerto Ayora, Galápagos in 2019.

Herbarium samples were collected from the Missouri Botanical Garden (n = 100) based on a Material Transfer Agreement in 2016. Collection dates ranged from 1970 - 2011 and included samples from 29 countries across 4 continents (Figure 4.1). Herbarium tissue samples were preserved in tubes with silica gel beads. Samples were collected for 13 *Tribulus* species, that based on herbarium labels were identified as: *T. aff. occidentalis* (1); *T. alatus (1); T. bimucronatus* (2); *T. cistoides* (58); *T. cristatus* (4); *T. eichlerianus* (2); *T. forrestii* (1); *T. hystrix* (1); *T. longipetalus* (2); T. *macropterus* (1); *T. petandrus* (2); *T. pterophorus* (2); *T. terrestris* (15); and *T. zehyeri* (6). We corroborated the herbarium labels using taxonomic guides and published floras (Appendix 2). Based on these guides we made the following corrections: We have 1 species complex; *T. pentandrus* complex (6) and the following species: *T. bimucronatus* (2); *T. cistoides* (58); *T.* cristatus (4); *T. eichlerianus* (2); *T. forrestii* (1); *T. hystrix* (1); *T. micrococcus* (2*)*; *T. minutus* (1); *T. pterophorus* (5); *T. terrestris* (12); *T. zeyheri* (6). The discrepancy between herbarium IDs was settled when we built the phylogeny. Thus, we show the corrected species names in our results (Appendix 4.1; Table S4.2). Field collected and herbarium tissue samples were stored at -20°C until we proceeded with DNA extractions.

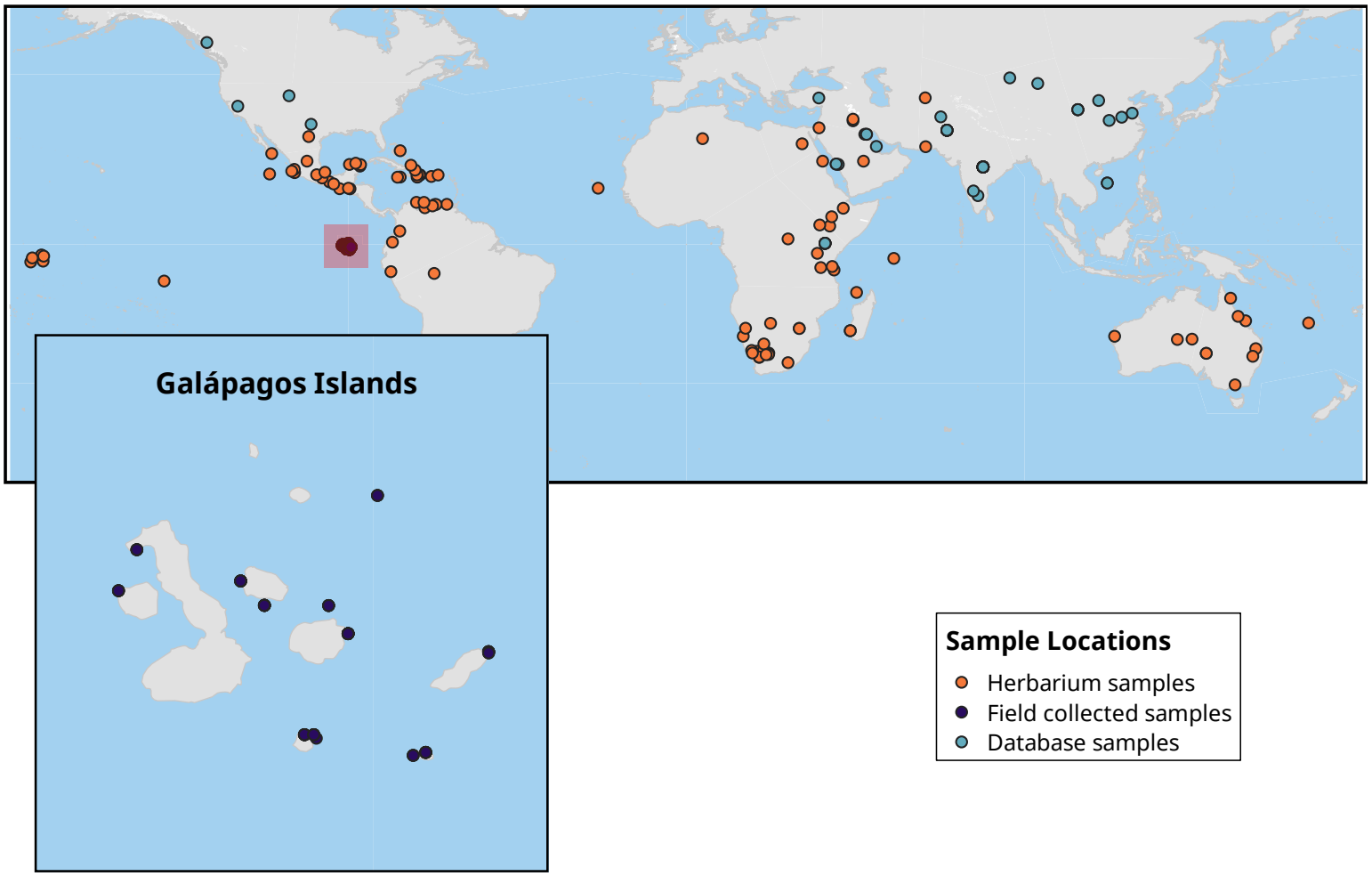

Figure 4. 1 Map showing the locations of *Tribulus* samples and their origin, from herbarium samples (orange), field collected samples (purple) or database (NCBI) samples (light blue). The area highlighted in red represents the Galápagos Islands, zoomed in below.

DNA extractions

We prepared the DNA extractions by weighing 20 mg of leaf tissue when possible. We grounded the tissue using a pair of 2mm metal beads and a TissueLyser® (QIAGEN) for 30 seconds to 1 minute, until it was converted into a fine powder and no tissue clumps were visible. DNA was extracted using the DNeasy® plant kit (No. 69104; QIAGEN) and followed kit instructions. After DNA extractions, we measured the DNA concentration of all samples using a NanoDrop1000. DNA concentrations were highly variable (2 - 129 ng/µl) with herbarium samples showing the lowest yield (Appendix 4.3). We suspended herbarium samples at a reduced volume (100µl, buffer AE) compared to field collected samples (200µl, buffer AE, following kit instructions). In total we extracted DNA from 172 samples (Appendix 4.3). DNA samples were stored at -20°C until PCR reactions were done.

Primer selection

After a general literature review, we used 4 markers sets for our analysis (Appendix 4.4). Three chloroplast markers (*rpl32-trnL*; *ndhF*; and *psbD* - $trnT^{(GGU)}$; hereafter *rpl32, ndhF* and *psbD*) and a nuclear marker (*ITS* regions 1 and 2; hereafter *ITS*). We used the markers proposed in Shaw *et. al* (2007) for phylogeography studies, based on their diversity and variability across chloroplast regions. This is part of a review series (Shaw et al., 2005, 2007, 2014) that provided useful information for marker selection at low level resolution. The selected markers showed great potential for differentiation on single to closely related species, such as our study. We used the marker sets from Simpson *et. al* (2004) which were used in *Krameria* (Krameriaceae). Simpson's work offered a set of *ITS* markers that allowed us to amplify *ITS1* and *ITS2* regions together but also used a set that allowed us to amplify the *ITS* regions separately for cases with samples that were more difficult to amplify (Table 4.1). Hereafter, the markers from these previous works are going to be referred to as the "original markers" since we also developed nested primers in this study.

Nested markers.

The nested markers were modified from the original markers to increase the yield of herbarium samples and any other difficult sample (Table 4.1). We specified which primers sets were used for all the samples (Appendix 4.3). We used Geneious®V.2022.2.1 to align the original markers to NCBI references and previously sequenced samples in this study to create the nested primers. We used a complete genome sequence of *Tribulus terrestris* chloroplast (MN164624) and an *ITS* sequence from *T. terrestris* (AY260972) as references. The PCR protocols were modified accordingly to meet the nested primer conditions.

PCR protocols

The PCR protocols of the original markers were based on their publications. For the chloroplast markers, we used the protocol based on Shaw *et. al* (2005): 5 min at 80°C; 35 cycles of 1 min at 94°C; 1 min at 50°C with a ramp of 0.3°C/s; 5 min at 65°C; and 4 min at 65°C. For *ITS* region, we used the protocol of Simpson *et. al* (2004): 3min at 94°C; 1 min at 50°C; 1 min at 72°C; 35 cycles of 1 min at 94°C, 1 min at 50°C, and 45 sec at 72°C with an additional 3 sec per cycle; 7 min at 72°C and a final cooling to 15°C.

We modified the original PCR protocols for our nested primers. For chloroplasts markers we modified the protocol as: 5min denaturation at 80°C; 30 cycles of 1 min at 95°C; a ramp up from 50°C to 65°C at a rate of 0.3C/s; an extension of 4 min at 72°C; and a final extension for 5 min at 65°C. The nested *ITS* protocol was modified as follows: 3 min initial denature at 94°C; 35 cycles of 1 min at 94°C; 15 sec at 50°C annealing; 45 sec at 72°C extension; and a final extension for 5 min at 72°C.

1 Table 4. 1 List of "original primers" and "nested primers" information and sequences.

Primer pairs	Region	Primers	Direction	Sequence	Size (bp)	Average amplified product (bp)	Tm (°C)	GC%	Reference
rpl32 - trnL	Chloroplast	Original	Forward	CAGTTCCAAAAAAACGTACTTC	22	1034	54.7	36.4	(Shaw et al., 2007)
	Chloroplast	Original	Reverse	CTGCTTCCTAAGAGCAGCGT	20		59.3	55	(Shaw et al., 2007)
psbD - trnT$^{(GGU)}$	Chloroplast	Original	Forward	CTCCGTARCCAGTCATCCATA	21	1486	57.9	47.62	(Shaw et al., 2007)
	Chloroplast	Original	Reverse	CCCTTTTAACTCAGTGGTAG	20		55.3	45	(Shaw et al., 2007)
ndhF - rpl32	Chloroplast	Original	Forward	GAAAGGTATKATCCAYGMATATT	23	527	51.7	30	(Shaw et al., 2007)
	Chloroplast	Original	Reverse	CCAATATCCCTTYYTTTTCCAA	22		52.8	35	(Shaw et al., 2007)
ITS (P1a)	Nuclear	Original	Forward	GGAAGGAGAAGTCGTAACAAGG	22	754	60.3	50	(Simpson et al., 2004), based on (Downie & Katz-Downie, 1996)
ITS (P4)	Nuclear	Original	Reverse	TCCTCCGCTCATTGATATGC	20		57.3	50	(Simpson et al., 2004), based on (T. J. White et al., 1990)
ITS 1 (P1a)	Nuclear	ITS 1	Forward	GGAAGGAGAAGTCGTAACAAGG	22	436	60.3	50	(Simpson et al., 2004), based on (Downie & Katz-Downie, 1996)
ITS 1 (P2B)	Nuclear	ITS 1	Reverse	CTCGATGGAACACGGGATTCTGC	35		74.2	57.1	(Simpson et al., 2004), based on (Helfgott, 2000)
ITS 2 (P3)	Nuclear	ITS 2	Forward	GCATCGATGAAGAACGCAGC	20	397	59.3	55	(Simpson et al., 2004), based on (T. J. White et al., 1990)
ITS 2 (P4)	Nuclear	ITS 2	Reverse	TCCTCCGCTCATTGATATGC	20		57.3	50	(Simpson et al., 2004), based on (T. J. White et al., 1990)
psbD - trnT$^{(GGU)}$ Nested	Chloroplast	Nested	Forward	TTTTAACTCAGCGGTAGAGTAAC	23	1434	57.1	39	**This study,** based on (Shaw et al., 2007)
	Chloroplast	Nested	Reverse	CGTAACCAGTCATCCATAATATC	23		57.1	39	**This study,** based on (Shaw et al., 2007)
rpl32 - trnL Nested	Chloroplast	Nested	Forward	TTCCAAARAARCGTACTTCTCTATC	25	971	56.4	34.8	**This study,** based on (Shaw et al., 2007)
	Chloroplast	Nested	Reverse	CTTCCTAAGAGCAGCGTGTCTACCG	20		59.3	55	**This study,** based on (Shaw et al., 2007)
rpl32 - trnL Nested 2	Chloroplast	Nested	Forward	CGTACTTCTCTATCAAAAAAGC	22		54.7	36.4	**This study,** based on (Shaw et al., 2007)
ndhF - rpl32 Nested	Chloroplast	Nested	Forward	AGGTATGATCCATGAATATTGATATG	26	473	54.9	30.8	**This study,** based on (Shaw et al., 2007)
	Chloroplast	Nested	Reverse	ATATCCCTTTTTTTTTCCAAATATTC	25		53.1	24	**This study,** based on (Shaw et al., 2007)
ITS Nested	Nuclear	Nested	Forward	GGAGAAGTTGTAACAAGGTTTC	22	746	56.5	40.9	**This study,** based on (Simpson et al., 2004)
	Nuclear	Nested	Reverse	CCGCTCATTGATATGCTTAAG	21		55.9	42.9	**This study,** based on (Simpson et al., 2004)

We used a standard 20 μL PCR reaction mix. The mix consisted of a final concentration of 0.02 U/μL DreamTaq Green DNA polymerase (5 U/μL) with 1X of its respective PCR buffer (10X; 20mM Mg^{2+}) (No. EP0712; Thermo Fisher Scientific); a final concentration of 0.2 mM of dNTPs Mix (10mM each nucleotide) (No. R0192; Thermo Fisher Scientific); 0.4 μM of each primer; and 1 μL of template DNA. The amplified PCR product was verified by checking its size using a 1KB DNA Ladder (No. M103R-1, Bio Basic, Inc) in a standard 1% agarose gel (No. IB70041; IBI Scientific) that ran at a constant 400 mA, 120 V for 45 mins. Once verified with the average amplified product size (Table 4.1), the PCR products were divided into aliquots and sent for sequencing.

Sequencing and editing

We sent the forward and reverse PCR products separately for sequencing, to avoid potential sequencing errors due to the length of our markers (900 to 1.2k bp). Sequencing products separately is the recommended approach because Sanger Sequencing is more reliable for markers between 700 – 800 bp (Genome Quebec, personal communication 2021). The sequencing was done by Genome Québec (Montreal, QC, Canada) using their Sanger sequencing service. We checked the quality of the chromatograms and edited any ambiguous nucleotide curves using Geneious® v.2022.2.1.

We aligned the edited forward and reverse chromatograms to generate a consensus sequence for each sample. We used Geneious default parameters: Global Alignment with a cost matrix of 65% similarity, a gap open penalty of 15, a gap extension penalty of three, and two refinement iterations as conditions for all the alignments. Once aligned, we extracted the consensus of the chromatograms to make individual sequences. We obtained a total of 575 sequences: 144 for *rpl32*; 147 for *ndhF*; 130 for *pdbD*; and 154 for *ITS*. Single nucleotide polymorphisms that showed chromatogram curves of similar height were settled using the IUPAC nucleotide ambiguity code. Once any potential errors were corrected, we aligned the sequences and proceeded with the phylogeny analysis and haplotype networks.

Galápagos sequences selection

We subset the Galápagos samples in the subsequent analyses because most individual sequences were identical. For the haplotype networks we used all island sequences to keep haplotype frequency (n = 71). However, for the phylogeny trees we only used individuals that reflected unique haplotypes samples per island (Appendix 5). This allowed us to reduce redundancy when estimating the phylogeny relationships and define better any within Galápagos groups in the context of the global samples.

Additional sequences and outgroup selection

We found only one sequence for *rpl32* (KC593444). We did not find any individual sequences for *ndhF* and *psbD* for any *Tribulus* species. We expected these results given that our markers are not necessarily the most commonly used (Shaw et al., 2007). We found complete chloroplast genomes and extracted our markers (*rpl32, ndhF* and *psbD*) using Geneious® alignment tools. We found two complete chloroplast genomes for *Tribulus terrestris* (MN164624 and KC593444) from China and whole chloroplast sequencing of three closely related species: *Larrea tridentata* (NC028023), *Guaiacum angustifolium* (NC043796), and *Balanites aegyptiaca* (OL703321) that we used as outgroups (Appendix 4.1, Table S4.3). After these additional samples we had a total of 107 sequences for *rpl32*, 113 for *ndhF*, and 111 for *psbD*.

We selected the best outgroup combination after a series of tests for the chloroplast sequences. We found that when we included all three outgroups for the chloroplast markers the resolution of the trees was affected. Thus, we generated two phylogenies, one using only *Balanites* as an outgroup, the other using *Larrea* and *Guaiacum*. Using the outgroups separately was informative for better resolution of individual chloroplast trees. *Balanites* is the closest relative of *Tribulus* which allowed us to infer the most recent branches and *Larrea* and *Guaiacum* allowed us to infer trees for more conserved markers, like *ndhF* (Appendix 4.6).

The nuclear marker had more available sequences that we incorporated in our study. We found a total of 56 additional sequences for *Tribulus ITS* on NCBI (Appendix 4.1, Table S4.3) that included geographical information. We also found *ITS* sequences for *Kallstroemia californica* (MF963813.1), *Kallstroemia sp.* (MH699457), *Kallstroemia parviflora* (AY260973) which are the closest outgroup of *Tribulus* (Böhnert et al., 2020; Wu et al., 2018). In addition, we used samples from *Balanites aegyptiaca* (MH699439) and *Balanites maughamii* (MH990661) as additional outgroups. (Appendix 1; Table S3). In total we used 173 sequences for *ITS* phylogeny.

Phylogenetic analysis

Selection of best substitution models

Once the samples were selected and aligned, we used Jmodeltest v2.1.7 (Darriba et al., 2012) to test the best model fit given our aligned sequences. We used 11 substitution schemes, for a total of 88 evaluated models. We included inversed and gamma rate variations. The base tree for likelihood calculations was optimized for maximum likelihood (ML) and we use an NNI base tree search. The best model outputs per marker were for *NdhF*, F81+I+G; for *rpl32*, TIM1+I+G; for *psbD*; and for *ITS*, TPM2+G (Appendix 4.7).

Phylogeny reconstruction

Individual markers

We used MrBayes v3.2.7 (Ronquist et al., 2012) to reconstruct the phylogenetic tree of *Tribulus*, we replaced some of the substitution models to fit MrBayes. For *rpl32* and *psbD* TIM+I+G was replaced by GTR+G+I. For *ITS* *TPM2*+G was replaced to its equivalent GTR+G, finally, *NdhF* was kept the same (Lecocq et al., 2013). All reconstructions used four Markov chain Monte Carlo (MCMC) chains (three heated and one cold chain, temperature of 0.1) and sampling one tree every 1000 generations, starting from random trees. The convergence was defined when the phylogenies reached an average standard deviation of 0.001 or less (Ronquist et al., 2012). For *rpl32*, the phylogeny converged at 400 million

generations using *Balanites* as an outgroup, and 200 million using *Larrea* and *Guaiacum* as outgroups. For *ndhF*, it converged at 200 million for using *Balanites* as outgroup and 650 million using *Larrea* and *Guaiacum*. For *psbD*, it converged at 400 million generations using *Balanites* as outgroup and 600 million using *Larrea* and *Guaiacum* as outgroups. Finally, for the *ITS* the phylogeny converged at 200 million generations. We used Tracer v1.7 (Rambaut et al., 2018) to assess the effective sample sizes (ESS) values of each run and make sure all of them are above 200.

Concatenated analysis

We also generated a concatenated analysis using all markers combined. The concatenated tree was used for the time divergence dating analysis, described below. The concatenated analysis used samples that had all four markers sequenced (n = 61, Appendix 4.8). We selected these samples to prevent any further errors in the phylogeny inference. In this way, we avoided the use of dummy sequences between markers and prevented the variance observed in our individual trees when we used different chloroplast outgroups (Appendix 4.6). This concatenated tree specified the same substitutions models as the individual trees. We used the *Balanites* and *Kallstroemia* outgroups (Appendix 4.1, Table S4.3). The tree converged at 100 million generations.

Time of divergence dating

Divergence times were estimated in BEAST2 version 2.7.4 (Bouckaert et al., 2014, p. 2), which uses a Bayesian MCMC approach to estimate the topology, rates and node ages of trees. We used the concatenated tree from MrBayes as template for the topology. The four gene partitions were linked with respect to clock and tree models, but we specified the substitution models according to the outputs by Jmodeltest mentioned above. We used a relaxed lognormal clock with an estimated clock rate and a birth-death model as tree prior (Drummond et al., 2006; Gernhard, 2008). The fossil record within the Zygophyllaceae is sparse (Bellstedt et al., 2012), and the few documented fossils cannot be confidently assigned to any member of

extant taxa. Thus, we used a secondary calibration approach, using already inferred trees (Böhnert et al., 2020; Wu et al., 2015, 2018), we used a normal distribution for the secondary tree priors and used their mean and upper and lower 95% Higher Posterior Density (HPD) intervals. The concatenated analysis used the root age for the *Balanites* and *Kallstroemia* estimates, the root age of *Kallstroemia* and *Tribulus* clade and the divergence time estimates between the *Balanites* clade (Table 4.2). We ran two independent analyses of 15 million MCMC that later were combined using LogCombiner, from the BEAST2 package with a burn-in of 10%. The log file was checked using Tracer version 1.71 (Rambaut et al., 2018). The Maximum Clade Credibility Tree was produced using TreeAnnotator, summarizing the mean heights, and a posterior probability of 0.95 for the HPD intervals for node estimates. The annotated tree was edited in FigTree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Table 4. 2 Age constrains used for normal prior distribution in BEAST2. This includes the 95% HPD intervals and the mean estimates. The nodes column shows the corresponding nodes for the outgroups we used. Bold rows are the estimates used for the concatenated analysis.

	Min	Max	Mean	Nodes	Reference	Markers used
1	**9.897**	**29.639**	**18.640**	***Balanites - Kallstroemia***	(Wu et al., 2018)	***rbcL, trnL, trnL-F, ITS***
2	**4.485**	**15.205**	**9.390**	***Kallstroemia - Tribulus***	(Wu et al., 2018)	***rbcL, trnL, trnL-F, ITS***
3	17.121	43.001	30.080	*Larrea - Guaiacum*	(Wu et al., 2018)	*rbcL, trnL, trnL-F, ITS*
4	38.061	80.712	59.890	*Larrea - Tribulus*	(Wu et al., 2018)	*rbcL, trnL, trnL-F, ITS*
5	1.931	7.253	4.200	*T. cistoides - T. terrestris*	(Wu et al., 2018)	*rbcL, trnL, trnL-F, ITS*
6	9.043	36.261	21.620	*Larrea - Guaiacum*	(Wu et al., 2015)	*trnL, trnL-F, ITS*
7	34.696	80.826	56.950	*Larrea - Tribulus*	(Wu et al., 2015)	*trnL, trnL-F, ITS*
8	3.388	11.173	7.101	*Kallstroemia-Tribulus*	(Böhnert et al., 2020)	
9	19.023	39.153	28.664	*Larrea - Guaiacum*	(Böhnert et al., 2020)	
10	51.205	77.199	64.267	*Larrea - Tribulus*	(Böhnert et al., 2020)	

Haplotype networks and genetic diversity

We generated haplotype networks for each marker using the open source software PopArt v1.7 (Leigh & Bryant, 2015). We trimmed the ends of each sequence to have a uniform size (*rpl32, ndhF, psbD,* and *ITS*) used the individual Galápagos samples (n = 81) and the samples from their sister clade (including the samples of *T. cistoides*) to generate the networks for each marker (Table 4.3 shows the samples used for the *ITS* marker). We used the Integer Neighbor Joining (intNJ) method with an $\alpha = 0.5$ to generate the networks (Bandelt et al., 1999). The program removed samples that had significantly more undefined states and

sequences that were partially sequenced (Appendix 4.9). After comparing between markers, we selected *ITS* as the most informative network because it showed more variation than the others (Appendix 4.9).

Genetic diversity estimates were obtained using DNAsp v6.12.03 (Rozas et al., 2017). We estimated Haplotype diversity (*Hd),* which is the measure of the uniqueness of haplotypes in a population, nucleotide diversity (π), defined as the average number of differences per site between any two sequences chosen randomly from the sampled population, number of segregating sites, number of parsimony-informative sites, and Tajima's *D* for all markers. Tajima's *D* neutrality test calculates the difference between *Theta* estimation from the number of polymorphic sites and the *Theta* estimation from the average number of pairwise differences. Tajima's *D* value departs from neutrality to negative values in cases of demographic expansion or purifying selection (Tajima, 1989).

Genetic subdivision was estimated by analysis of molecular variance (AMOVA) and by calculating pairwise population's F_{ST} using PopArt v1.7. To estimate the amount of variation among populations and within populations, analyses were categorized at the region level (Continent vs Galápagos). The AMOVA analyses the covariance components using the information on haplotype frequencies and nucleotide distance between haplotypes (Excoffier et al., 1992).

Table 4. 3 Number of samples for the *ITS* haplotype network from the *T. cistoides* clade. Samples were separated into locations for non-Galápagos samples and into sites for Galápagos samples. Genetic divergence analysis was done at the region level.

Region	Location/Island	Site	Species	Samples
Galápagos	Champion	Champion	*Tribulus spp.*	5
	Española	Punta Suárez	*Tribulus spp.*	3
		Bahía Gardner	*Tribulus spp.*	8
	Fernandina	Cabo Douglas	*Tribulus spp.*	4
	Floreana	Post Office	*Tribulus spp.*	8
		Las Cuevas	*Tribulus spp.*	2
	Genovesa	Salvaje de corazón	*Tribulus spp.*	3
	Isabela	Pta. V. Roca	*Tribulus spp.*	4
	Rábida	Playa Roja	*Tribulus spp.*	9
	San Cristóbal	Punta Pitt	*Tribulus spp.*	7
	Santa Cruz	Plazas	*Tribulus spp.*	12
	Santiago	Pto. Egas	*Tribulus spp.*	10
	Seymour Norte	Bahía Seymour	*Tribulus spp.*	6
Non-Galápagos	Africa	Tanzania	*T. cistoides*	2
	Caribbean	Dominican Republic	*T. cistoides*	2
		Haiti	*T. cistoides*	1
		Puerto Rico	*T. cistoides*	1
		Bahamas	*T. cistoides*	1
		Jamaica	*T. cistoides*	2
	Central America - Mexico	Mexico	*T. cistoides*	10
		Guatemala	*T. cistoides*	1
	Oceania	Republic of Kiribati	*T. cistoides*	2
		French Polynesia	*T. cistoides*	1
	South America	Colombia	*T. cistoides*	1
		Venezuela	*T. cistoides*	4
	Southern Africa	South Africa	*T. cistoides*	1

Results

Phylogeny reconstruction and time estimates

Individual trees

Individual trees from chloroplast markers (*ndhF, psbD,* and *rpl32*) showed high polyphyly across locations, suggesting that these markers have not been much differentiated across *Tribulus* species. Even when we used different outgroups, the results for chloroplast trees were similar with a single clade showing a high posterior probability (PP = 100%) polyphyly (Appendix 4.6). However, when we used the most basal outgroup, *Larrea* and *Guaiacum*, we did observe some clade differentiation based on specific geographical regions. Most of the polyphyly corresponds to samples from Galápagos, Mexico, Oceania, and South America. Populations from Africa, Madagascar, Southern Africa, and the Middle East showed some differentiation and are the most basal clades. The marker *ndhF*, for example, shows a paraphyletic group of *Tribulus* from those regions but is not well supported (PP = 57%) (Appendix 4.6).

However, the *ITS* marker showed the most complete and well supported structure across species and regions (Figure 4.2). This tree showed two main paraphyletic clades. The first clade grouped all the samples from *Tribulus cistoides*, and includes all the samples from Galápagos, the Caribbean, Central America – Mexico, Oceania, South America, Africa, and Southern Africa for that species (PP = 87%), suggesting that our samples are *T. cistoides*. A single *T. cistoides* sample from Madagascar (PP = 100%) is shown at the base of the clade. Samples from Galápagos specifically, showed a monophyletic clade with high support (PP = 96%). However, within the Galápagos clade, we found some polyphyly that included samples from South America and the Caribbean. At the base of the Galápagos clade, we found a single sample from South America (PP = 82%) (Figure 4.2).

The second main clade showed two well differentiated paraphyletic groups within (PP = 100%). The first group show mostly samples from *T. terrestris* (PP = 81%), across North American, Central American – Mexico, Middle Eastern, African,

Southern African, and Asian samples. At the base of this group, we found a highly supported monophyletic clade (PP = 93%) composed of samples from *T. cistoides* and one *T. zeyheri* sample from Oceania, Africa, and Southern Africa, respectively. The second group shows samples from multiple *Tribulus* species but mostly from two locations Asia and Middle East, these samples show polyphyly and a monophyletic group at the base from *T. zeyheri* from Southern Africa (PP = 100%) (Figure 2). This second main clade also showed one not-so-well differentiated paraphyletic group (PP = 57%) This group contained species from the *T. pentandrus complex* and some *T. cistoides*. However, within this group there was some structure. For example, there is a monophyletic group of *T. micrococcus* from Oceania (PP = 85%) and the *T. pentandrus* samples were mostly from the Middle East (PP =100%) (Figure 4.2). Finally, at the base of the phylogeny, we found three monophyletic groups. The first one, at the base of these well-differentiated clades shows *T. cistoides* and *T. terrestris* samples from South America. The second one, groups *T. pterophorus* and *T. zeyheri* samples from Southern Africa. The third one, groups *T. cristatus,* is also from Southern Africa. At the base of the whole phylogeny, we found a monophyletic group with two samples of *T. cistoides* and *T. forrestii* (PP = 100%) from Central America – Mexico and Oceania (Figure 4.2).

As shown above, the *ITS* tree holds the most information and differentiation of all markers, despite, some of the fine detail structures still show many samples that are para/polyphyletic, suggesting that the taxonomy of this group has some pending revision. Note that such detailed phylogenetic and taxonomic revision is beyond the scope of the present study.

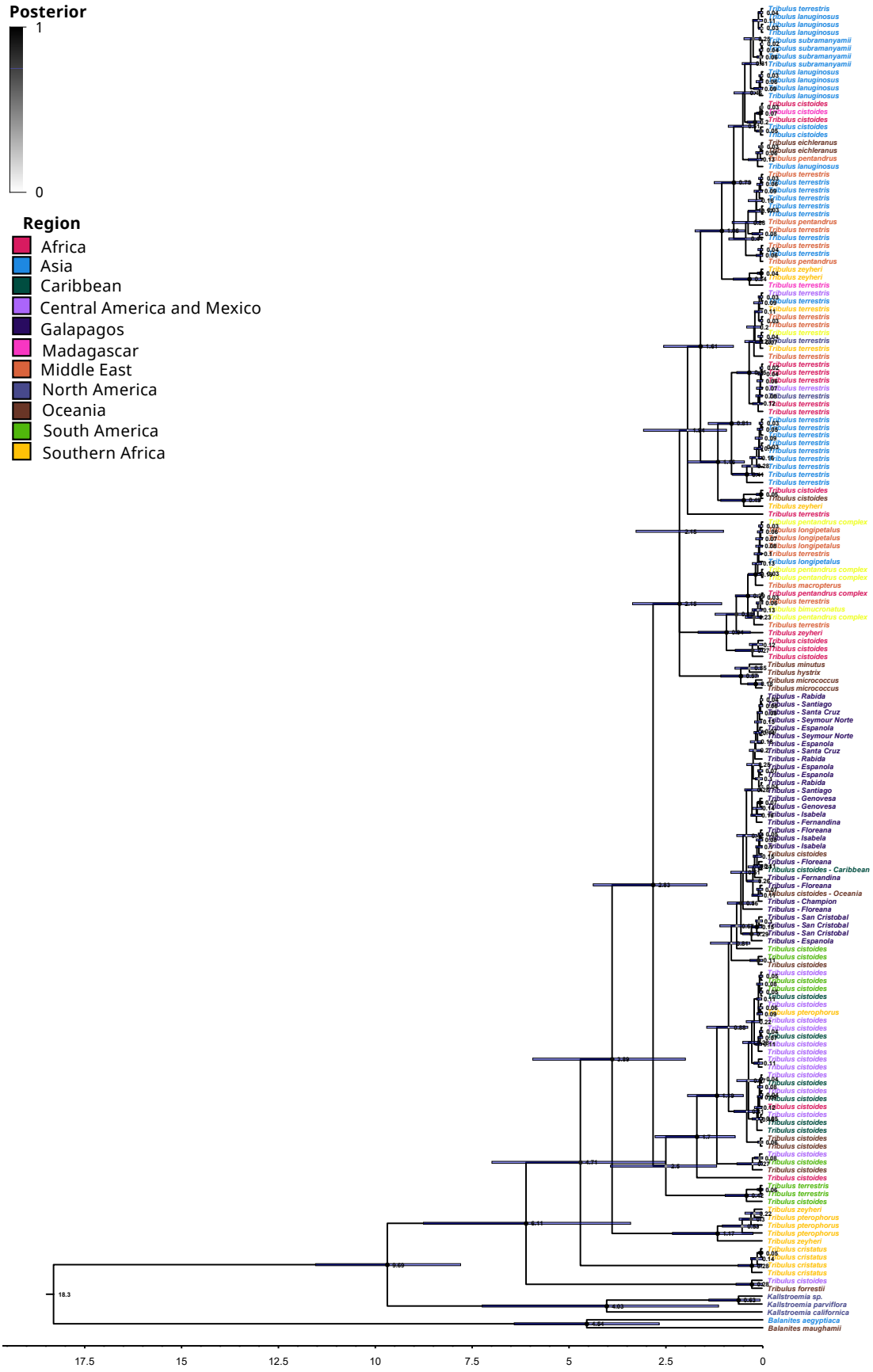
Posterior
1
0
Region
Africa
Asia
Caribbean
Central America and Mexico
Galapagos
Madagascar
Middle East
North America
Oceania
South America
Southern Africa
17.5
15
12.5
10
7.5
5
2.5
0

Figure 4. 2 Phylogenetic tree based on the *ITS* markers. The tree was plotted using BEAST2 with multiple outgroups (*Balanites* and *Kallstroemia*). The tree is arranged in increasing order of the nodes. Node numbers show age estimates based on secondary calibrations. Bars show 95% Highest Posterior Density (HPD) confidence intervals of age estimates. Circles are color coded based on the gradient on the left and represent the node's posterior support. The bottom scale shows an estimate timeline of the tree in millions of years from the present. Colors from the tips represent the regions from which the samples were collected. Tip names are the species taxa based on the reclassified species descriptions (see Methods). The Galápagos clade (in purple) also shows the names of the islands where the samples were collected.

Time divergence estimates

We selected only the samples that were amplified for all four markers samples to assure the best time estimates and overcome some of the polyphyletic groups observed in the individual trees (Figure 4.3). The concatenated alignment included 60 samples corresponding to 16 species from 23 countries and had a concatenated length of 7209 bp. The topology of the concatenated tree was congruent between the MrBayes and the two BEAST runs, with a high ESS support and high posterior probabilities (Appendix, 4.8, Figure 4.3).

The estimated divergence times ranged between 6.19 to 0.08 Mya for the *Tribulus* tree. Samples from Galápagos formed a single monophyletic clade and is the basal group of a well-supported (PP: 100%) *Tribulus cistoides* clade that groups samples from Central America and Mexico, the Caribbean, and the South American continent. The age of the node dividing Galápagos and the continental samples was estimated at 2.4 Mya (95% HPD: 1.01 – 4.03) (Figure 4.3). In addition, the basal group from the *T. cistoides* clade is a medium-supported (PP: 53%) *Tribulus* clade from Oceania, containing *T. minutus, T. eichleranus, T. hystrix,* and *T. micrococcus*. The parental node of the Oceania clade and the *T. cistoides* clade as was estimated at 2.93 Mya (95% HPD: 1.2 – 4.8). Finally, as an outgroup of all the above, there was a single *T. cistoides* sample from Africa, with an estimated time of divergence of 3.14 Mya (95% HPD: 1.27 – 5.08).

We found three groups within the Galápagos clade. The estimated divergence time of the Galápagos clade was 0.92 Ma (95% HPD: 0.21 – 1.86). Suggesting that *Tribulus* populations within Galápagos started diverging between less than a million years ago. From the common ancestor node, the most basal group contained

samples from Fernandina and Floreana that diverged between them 0.11 Mya (HPD: 0 – 0.44). Then, we found two groups that diverged from a common node estimated at 0.7 Mya (95% HPD: 0.17 – 1.39). The first group includes samples from Champion, Española, and Isabela (as an outgroup). The second group contained samples from Genovesa, Rabida, Santa Cruz, and Isabela (as an outgroup) (Figure 4.3).

The rest of the tree shows evidence for geographical structure with most clades being grouped by region. The next clade that diverged from the common ancestral node (3.45 Mya (95% HPD: 1.58 – 5.55)) of the previous groups, was composed of samples of various *Tribulus* species mainly from Africa and Southern African samples, these samples diverged within at 2.6 Mya (95% HPD: 1.01 – 4.36). At the base of all diverging around 4.69 Mya (95% HPD: 2.2 – 7.36), there was a well-supported group from Southern Africa, composed mainly by samples of *T. zeyheri* and *T. pterophorus*. Finally, a basal group composed of Southern African samples of *T. cristatus* and one sample of *T. forrestii* with an estimated time of 4.89 Mya (95% HPD: 1.71 – 8.09) (Figure 4.3).

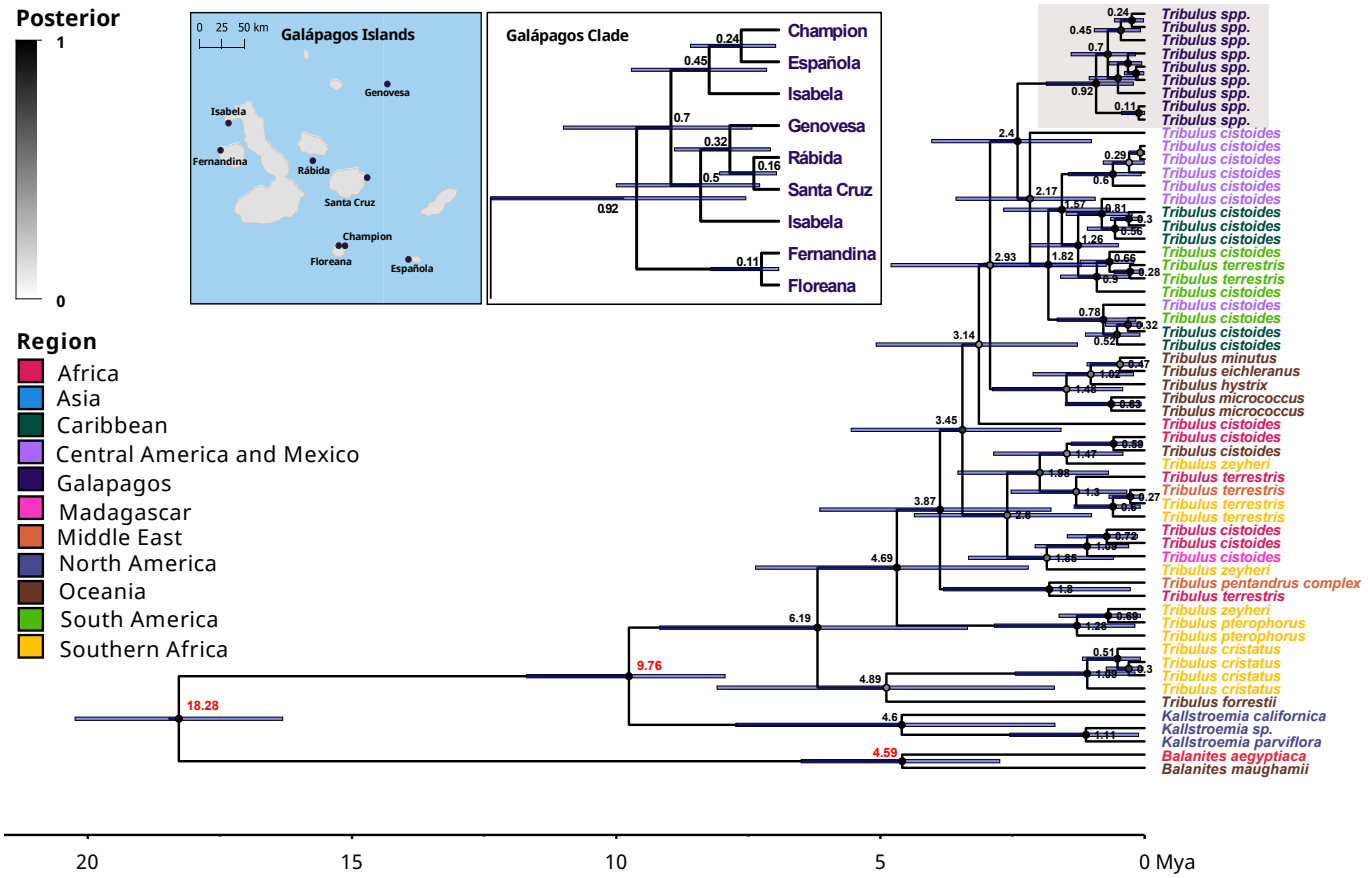

Figure 4. 3 Concatenated global reconstruction of *Tribulus* phylogeny using nuclear marker *ITS* and chloroplast markers *ndhF, psbD* and *rpl32* plotted using BEAST2 with multiple outgroups (*Balanites* and *Kallstroemia*). The tree is arranged in increasing order of the nodes. Nodes numbers show age estimates based on secondary calibrations. Bars show 95% Highest Posterior Density (HPD) confidence intervals of age estimates. Circles are color coded based on the gradient on the left and represent the node's posterior support. The bottom scale shows an estimate timeline of the tree in millions of years from the present. Age estimates highlighted in red are the calibration points used based on previous tree analyses. Colors from the tips represent the regions from which the samples were collected. Tip names are the species taxa based on the reclassified species descriptions (see Methods). Highlighted in gray is the Galápagos clade. On the left there is a closer view of the clade, with tip names representing the islands where samples were collected and their age estimates and confidence intervals. Next to it, a map of the Galápagos Islands, showing the sampling location.

ITS Haplotype network and genetic diversity

We used 92 sequences for the haplotype analysis. The *ITS* region revealed 23 variable sites among 15 haplotypes of those, 12 are unique haplotypes are from Galápagos (Table 4.4). The overall haplotype diversity and nucleotide diversity of Galápagos and Non-Galápagos samples was 0.724 and 0.31% respectively (Table 4.4). When we estimated the genetic diversity per islands, we found that the highest haplotype diversity was observed on Floreana and San Cristóbal islands with similar values (0.833), followed by Española Island (0.711). The highest nucleotide diversity was found on Floreana (0.48%) and Española (0.42%) respectively. Floreana and Española were islands that were sampled in two sites per island. The lowest haplotype diversity values were observed at Champion, Fernandina, and Isabela where all sequences were similar with no unique haplotypes, and estimates were not possible to calculate. The lowest haplotype and nucleotide diversity values that were estimated were found on Rábida Island with 0.222 and 0.04% respectively. The test for neutrality did report significant Fs values and deviations from neutrality for all samples and non-Galápagos samples. However, tests for neutrality and deviations from neutrality between Galápagos islands showed non-significant values (Table 4.4).

Table 4. 4 Genetic diversity (*Hd* = haplotype diversity, π = nucleotide diversity and demographic parameters (Tajima's D and Fu's *Fs*) estimated from the *ITS* region (639 bp) of A) Galápagos and Non-Galápagos and B) Galápagos Island specific *Tribulus cistoides* populations. N = number of samples; S = variables sites; H = total number of haplotypes; *Hd* = Haplotype diversity (± sd = standard deviation); and π = nucleotide diversity (%). Tajima's D and Fu's *Fs* were significant for overall samples and non-Galápagos samples ($p < 0.005$) showed in bold. Some values were not determined (n.d) or not applicable, due to lack of differences in the sequences (n.a.).

Region analysis							
Population	**N**	**S**	**H**	**Hd ± sd**	**π (%)**	**Tajima's *D***	**Fu's *Fs***
All samples	92	23	15	0.724 ± 0.035	0.31	**-1.8647**	**-5.764**
Non-Galápagos	29	7	3	0.2 ± 0.098	0.10	**-1.958**	-0.784
Galápagos	63	20	12	0.781 ± 0.035	0.36	-1.5463	-3.374
Island analysis							
Population	**N**	**S**	**H**	**Hd ± sd**	**π (%)**	**Tajima's *D***	**Fu's *Fs***
Champion	4	0	1	0	0	n.d.	n.a.
Española	10	6	4	0.711 ± 0.117	0.0042	0.9778	1.217
Fernandina	4	0	1	0	0	n.d.	n.a.
Floreana	4	6	3	0.833 ± 0.222	0.0048	-0.8086	0.731
Genovesa	3	1	2	0.667 ± 0.314	0.00106	n.d.	0.201
Isabela	4	0	1	0	0	n.d.	n.a.
Rábida	9	1	2	0.222 ± 0.166	0.00035	-1.0882	-0.263
San Cristóbal	4	3	3	0.833 ± 0.222	0.00264	0.1677	-0.133
Santa Cruz	6	2	2	0.333 ± 0.215	0.00106	-1.132	0.952
Santiago	10	1	2	0.356 ± 0.159	0.00057	0.015	0.417
Seymour Norte	5	1	2	0.4 ± 0.237	0.00063	-0.8165	0.09

Overall AMOVA analysis revealed that most of the variance was found among locations (81.57%). The F_{ST} value (0.818) indicates that 81.18% of variance in haplotype frequencies is caused by the combined effects of groups and island populations (Table 4.5).

Table 4. 5 Analysis of molecular variance (AMOVA) using the *ITS* region of *Tribulus cistoides*. Statistics: F_{CT} = 0.002, F_{SC} = 0.817, and F_{ST} = 0.818. After 1000 permutations, values for F_{CT} were non-significant ($p = 0.238$) and values for F_{SC} and F_{ST} were significant at $p < 0.001$; df = degrees of freedom.

Genetic differentiation	**Df**	**Sum of squares**	**σ^2**	**% variation**
Among groups (Galápagos/Non-Galápagos samples)	1	18.43	0.0006	0.20
Among populations (Locations/Islands)	17	212.074	2.57	81.57
Within populations	73	41.92	0.574	18.23
Total	91	272.424	3.151	

The total number of haplotypes (H) was higher on Española (4), followed by Floreana and San Cristóbal (3). The network showed three main nodes that grouped samples from multiple locations and another 12 nodes grouping samples from the same regions. The main node was Node III grouped most of the haplotypes from non-Galápagos, mostly from Central America – Mexico, the Caribbean, and South

America and samples along with the islands that showed no unique haplotypes, such as: Champion, Fernandina, and Isabela. Suggesting that these regions are the most likely sources of origin from the Galápagos haplotypes and from there they stemmed into more unique Galápagos haplotypes, found on the other main nodes (I and II). Node I grouped samples from Rábida, Española (Bahía Gardner site), Seymour Norte and Santa Cruz, and a unique Santa Cruz haplotype stemmed from this node. Node II grouped mostly samples from Santiago Island, Seymour Norte, Española (Bahía Gardner site) and Rábida, and a unique Bahía Gardner haplotype stemmed from this node. Interestingly, San Cristóbal island and Punta Suárez on Española Island showed the most separation with San Cristóbal specifically showing 3 unique haplotypes (Figure 4.4).

Table 4. 6 Haplotype variations of all the sequences evaluated. Variations were classified using haplotype 1 as a base. Locations in the sequences are described and classified accordingly for the 15 haplotypes.

	Base location relative to haplotype 1																						
Haplotype	21	38	47	79	114	189	221	223	231	273	280	308	352	401	405	418	420	424	435	489	582	596	614
Hap 1	C	G	C	C	C	C	A	G	T	C	C	G	A	C	T	C	C	G	C	A	C	G	C
Hap 2	.	.	T	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
Hap 3	.	A	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 4	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 5	.	.	.	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
Hap 6	.	.	.	.	.	.	.	.	.	.	.	A	T	.	.	T	T	.	.	.	.	.	.
Hap 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T
Hap 8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	T	T
Hap 9	T	.	.	.	.	T	T	A	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.
Hap 10	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
Hap 11	T	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
Hap 12	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	.	.	G	.	.
Hap 13	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	G	.	.
Hap 14	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	.	.
Hap 15	.	A	.	T	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.

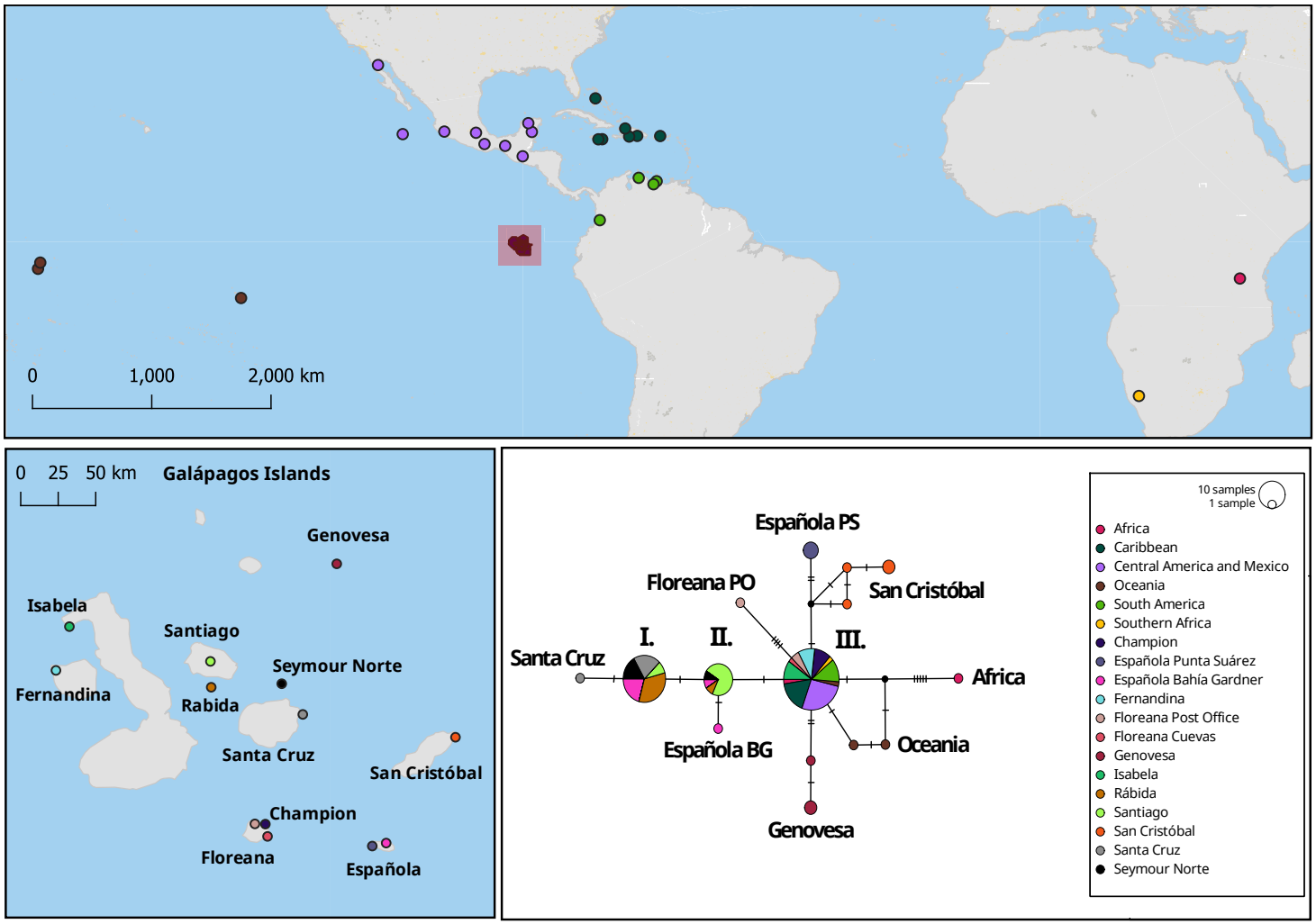

Figure 4. 4 Haplotype network map using the nuclear *ITS* marker. Circles on the map represent unique haplotypes; the size of the circles is proportional to the number of individuals sharing that haplotype; lines across each branch represent the mutations between haplotypes, with each line representing a mutation. Colors represent each sampling region, and the Galápagos islands including sites within the same island, as the case of Floreana and Española.

Discussion

Based on our analysis of both fresh and herbarium samples using chloroplast and nuclear markers, we have found that the populations of *Tribulus* on the Galápagos Islands can be traced back to a single colonization event. The split from Galápagos and continental samples is estimated at 2.4 Mya (95% HPD: 1.01 – 4.03), and a divergence time within islands at 0.92 (95% HPD: 0.21 – 1.86) million years ago. We found unique haplotypes for some islands suggesting local differentiation. These findings indicate that our Galápagos samples are *T. cistoides* populations, and they are native, and not introduced by humans. It also suggests that the *Tribulus*-finch interactions have been occurring for millennia and differences observed across populations are the result of adaptation and coevolution.

Number of colonization events and divergence times

Our results, based on the concatenated tree indicate that there is a monophyletic clade for *Tribulus* populations in Galápagos. This suggests that the Galápagos populations come from a single colonization event and then most likely dispersed throughout the islands. This form of colonization has also been found in other Galápagos species. For example, Darwin's finches most likely followed a similar pattern (Sato et al., 2001) and Galápagos mockingbirds (Arbogast et al., 2006). We also found that the *Tribulus* group from Galápagos is a paraphyletic group from the *Tribulus cistoides* clade. This suggests that our samples are most likely *T. cistoides* rather than, *T. terrestris*, the second species that coexists on the islands (Wiggins & Porter, 1971). This also suggests that the distribution of *T. cistoides* is more abundant than *T. terrestris* on the islands. We consider that our samples, although including multiple islands, come from one location per island, so it is likely that *T. terrestris* populations were not sampled in our efforts but could be located on those islands in other locations (Wiggins & Porter, 1971).

The age of the common node between the Galápagos and other *T. cistoides* samples was estimated at 2.4 Mya (95% HPD: 1.01 – 4.03). Within the Galápagos clade, we have divergence times inferred at 0.92 Mya (95% HPD: 0.21 – 1.86). Indicating that

T. cistoides colonized the islands at least 2.4 Mya but started diverging within the archipelago less than a million years ago. To determine this timeframe, we relied on secondary calibrations using previous phylogenetic reconstructions of the Zygophyllaceae family (Böhnert et al., 2020; Wu et al., 2015, 2018). Our results fit into the expected timing frames of those previous reconstructions, but most importantly they align with geological evidence regarding the formation time of the Galápagos Islands. Previous studies estimated that the approximate age of the Galápagos Islands in their current state is around 3 – 3.3 million and not much older than 4 million years ago, based on the geology of the islands (Bailey, 1976; D. Geist, 1996; Hall, 1983; Heads & Grehan, 2021; Hickman & Lipps, 1985; Simkin, 1984; W. M. White et al., 1993). In addition, due to the nature of the Galápagos hotspot, the age of specific islands varies, with islands on the east being older than islands from the west, closer to the hotspot (Merlen, 2014). For example, Fernandina has the youngest emergence age estimates between 0.032 – 0.06 Ma. Whereas Española is one of the oldest, with an estimated age between 3 – 3.5 Ma (D. J. Geist et al., 2014). These ages fit into our time divergence estimations, based on our concatenated tree. Indicating that *T. cistoides* arrived in the Galápagos in the "modern" state of the islands rather than older, presently submerged islands (D. J. Geist et al., 2014).

If a species colonization pattern matches the pattern of island formation it is said that the species follow the Progression Rule (Funk & Wagner, 1995). In the case of *T. cistoides*, our phylogenies show some discrepancies. On one hand, the concatenated tree shows a basal group with samples from Floreana and Fernandina, the youngest island (Figure 4.3). If Fernandina is grouped in the basal clade, it suggests that the island was colonized much earlier but started diverging at 0.11 Mya (HPD: 0 – 0.44) (Figure 3). On the other hand, if we consider the individual *ITS* tree that contains more samples, we find a different result. The *ITS* tree includes samples from Española, and San Cristobal islands, which are not included in the concatenated tree (Figure 4.2). These islands are the oldest ones, and they form a well-supported basal group on the *ITS* tree suggesting that these

islands may have been colonized earlier and that *Tribulus* may follow the east-west patterns (Figure 2). Certain lineages within the Galápagos Islands, such as the Galápagos giant tortoise (Poulakakis et al., 2020) and the Galápagos lava lizard (Kizirian et al., 2004), show a clear progression from older to younger islands. However, not all faunal groups in the Galápagos follow this progression rule, such as Darwin's Daisies, (*Scalesia*) (Fernández-Mzuecos et al. 2020), Darwin's finches (*Geospiza, Camarhynchus, Cactospiza, Platyspiza, Certhidea*) (Sato et al., 2001), endemic moths (*Galagete*) (Schmitz et al., 2007), and weevils (*Galapaganus*) (Sequeira et al., 2008). In conclusion, samples from the Galápagos show a monophyletic group that indicates a single colonization event and upon establishment, individuals were spread between islands. However, the mechanisms of dispersal between islands and the timing of these dispersals need further evidence (see below).

Genetic differentiation on islands

The haplotype network shows the current state of the genetic diversity of *T. cistoides* populations in the Galápagos. The haplotype networks show unique genetic differentiation on some Galápagos islands whereas other islands are more homogeneous. For *ITS*, we found that unique haplotypes correspond to Floreana, San Cristóbal, Española, and Genovesa Islands (Figure 4.4), whereas shared haplotypes are mostly located between western/central islands, with the lowest haplotype diversity found on Champion, Fernandina, and Isabela. For two chloroplast markers, *ndhF* and *psbD* we also found unique haplotypes on San Cristóbal, Española, and Floreana. However, they differ from Champion, where chloroplast markers only show one haplotype. For *rpl32*, however, we did not find unique haplotypes it shows a single haplotype for all Galápagos samples. This suggests that, even if we found differences between chloroplast and nuclear haplotype networks, the rise of unique haplotypes on eastern islands is consistent between some markers. The rise of unique haplotypes suggests that in some populations, enough time has passed for genetic differentiations to accumulate, and they are evidence of genetic divergence. Interestingly, these diverging populations

are mainly found on the eastern islands, perhaps supporting the idea of eastern island populations being older.

In addition, most of the other islands show a single haplotype that is shared with continental samples suggesting that other factors, such as gene flow, may be homogenizing any potential genetic differentiation. This could be related to humans and human activity. Humans are excellent dispersers of *Tribulus* (Goeden & Ricker, 1973; E. Johnson, 1932; M. K. A. Johnson et al., 2020), and gene flow due to human activities may have homogenizing effects on the populations. We suggest more studies focusing on population genetics and gene flow, to determine if the haplotypes found are being homogenized on islands where the flow of people is greater and how stable these populations are beginning to differentiate genetically.

Historical context of *Tribulus* in Galápagos

Based on our results, the origins of *T. cistoides* in the Galápagos are most likely from a single colonization event around 2.4 million years ago but started diverging within the islands around 0.92 million years ago. The *Tribulus* clade from Galápagos is a sister clade of *Tribulus cistoides* from Central America, Mexico, South America, and the Caribbean. Indicating the potential origin of the *T. cistoides* populations is from those regions. Finally, we found unique haplotypes for the Galápagos on the eastern islands, which suggests further differentiation on older islands than younger ones. Thus, *T. cistoides* on the Galápagos may follow the island progression rule.

The most likely origin of the Galápagos is linked to Central America, Mexico, South America, and the Caribbean common ancestor. These regions are part of three well-known and previously established biogeographic patterns for the origin of many Galápagos species: 1) Galápagos-south western Mexico; 2) Galápagos, western Americas, and the Caribbean, and 3) Galápagos – Caribbean, which are defined by geological and phylogeny events (Heads & Grehan, 2021). These biogeographic patterns are the source of many endemic Galápagos plant and animal species. Such as the *Scalesia* family (Fernández-Mazuecos et al., 2020; Schilling & Panero, 2002),

Castela galapageia (Simaroubaceae) (Thomas, 1990), and the plant *Erigeron* (Asteraceae) (Andrus, 2002), to name a few plant examples. The Caribbean region, for example, has the highest number of identified origins (45), according to Heads and Grehan (2021). Also, Tye and Francisco-Ortega (2011), suggested that there is a strong floristic connection between Galápagos and the Caribbean, which can be explained if the Galápagos hotspot produced the Caribbean plateau (Heads & Grehan, 2021; Nerlich et al., 2014). Tribulus likely followed one of these routes. However, more specific studies are needed to ensure which of these three regions is the exact origin of *Tribulus*.

Tribulus cistoides colonized the islands around 2.4 million years ago, which has interesting implications in the context of the *Tribulus*-finch interaction. Time estimates based on finch phylogeny suggest that the common ancestor of Darwin's finches arrived on the islands around 1.2 ± 0.8 Ma (Sato et al., 2001). This suggests that *T. cistoides* was already well established on the islands compared to the common ancestor of Darwin's finches. Implying that *T. cistoides* may have helped the common ancestor of finches to establish as well. According to Sato et al. (2001), the closest living relative of the finches' common ancestor is *Tiaris obscura,* a bird of finches fed most likely on seeds. It is possible that the common ancestor of finches also fed on seeds making the already established *T. cistoides* populations a potential food source. It is likely that this may have occurred, given that *T. cistoides* seeds are available all year round. However, it is more likely that the shift of the common ancestor to eat *T. cistoides* may occurred later, because the hard mericarps of *T. cistoides* may imposed strong selective pressures on the not-yet-well-adapted ancestral populations of finches making them select other available seeds (De León et al., 2014; P. R. Grant, 1981; T. Price, 1987).

Interestingly, the age that the Galápagos *T. cistoides* clade started diverging within was estimated at 0.92 million years ago, not so long after the arrival of the common ancestor of finches. Up to the present day, we have two specialized species that feed on *Tribulus*, the medium ground finches (*Geospiza fortis*) and large ground

finches (*G. magnirostris*) (Boag & Grant, 1984; P. R. Grant, 1981; T. Price, 1987). These specialized seed predators diverged around 0.258 ± 0.07 Ma (Lamichhaney et al., 2015), implying that *T. cistoides* may be a factor in finch evolution since arrival because *T. cistoides* mericarps does select for beak size (Boag & Grant, 1981; P. R. Grant, 1981; P. R. Grant & Grant, 2014). Over time, it would be likely that *T. cistoides* and other plant species further accentuated beak morphology differences between medium and large ground finches over generations and vice versa, Darwin's finches also played are role in the diversification of *T. cistoides* across islands, as the abundance of specialized predators started to distribute and establish. We provided strong evidence that suggests that island selection pressures could lead to *T. cistoides* phenotypic divergence. However, we could not discard that *T. cistoides* phenotypic divergence could also have started by genetic (founder effects, gene flow, dispersal) or environmental factors (precipitation). As a result, traits associated with survival against predation, such as upper and lower spines, likely remained relevant since *Tribulus's* arrival and were not entirely lost following colonization (Reyes-Corral *et al.*, 2023; Chapter 2 and Chapter 3).

Limitations and future directions

Concatenated analysis

The use of concatenated phylogenies often offers higher accuracy estimates and solves discrepancies from the individual markers (Gadagkar et al., 2005; Gontcharov et al., 2004). In our study, we relied on a concatenated analysis to solve any potential discrepancies compared with individual markers. First, we used the concatenated tree to compare the topology of the Galápagos clade. We found that on both the individual trees (mainly, *ITS*) and the concatenated tree, the Galápagos forms a monophyletic clade. In addition, the concatenated tree partially solved some polytomies within the clade with higher support on the nodes than the individual trees. However, we believe this is due to the decreased number of Galápagos samples used on the concatenated tree (9 compared to 29 for individual trees) and that any potential group structure may be lost in the concatenated analysis. Second,

we used the concatenated analysis to increase the accuracy of time of divergence estimates. The concatenated analysis did show more accurate estimates of time divergence, compared with individual trees. Individual trees estimated earlier times of divergence especially for inner, not-well-supported nodes within the more established clades (Figure 3 and Appendix 6). Finally, we used the concatenated analysis as additional support to identify potential sources of colonization of the Galápagos populations. However, the concatenated analysis is not the definitive approach in this regard and it must be used with caution (O. M. Vargas et al., 2017), as we discuss later (See Historical context below), the individual trees and the haplotype networks together may form a better idea of the origins of the Galápagos clade.

Selection of DNA markers

The *ITS* region has been used in previous studies of *Tribulus*, and is in general the most informative DNA marker for phylogeny (Böhnert et al., 2020; Wu et al., 2015) and species discrimination (Balasubramani et al., 2010). In our dataset, *ITS* was the marker with the most samples. In contrast, our chloroplast markers, *ndhF*, *rpl32,* and *psbD* were less common in data repositories. We selected our chloroplast markers based on the review series of Shaw (Shaw et al., 2005, 2007, 2014), which tested multiple chloroplast regions and identified potential markers for studies at multiple taxonomic levels. Our study is at the genus level, which requires markers with high genetic variability to elucidate differences (Dong et al., 2012). However, our results based on the phylogeny reconstruction of individual chloroplast markers show high levels of polytomy for *Tribulus*, making difficult any sort of differentiation (Appendix 4.6).

Another approach is to analyze the whole chloroplast genome in search for specific and highly variable chloroplast markers (Y. Wang et al., 2021). In Zygophyllaceae, there have been studies that compared and described whole chloroplast genomes for other species, including *Tribulus Terrestris* (Al-Juhani et al., 2022; X. Wang et al., 2022; Yan et al., 2019). Based on those studies, there is an indication of the

potential genomic variation of our chloroplast markers. For example, the study by Al-Juhami (2022) on *Balanites* suggests that markers such as *rpl32* have faster evolutionary rates and are under positive selection, indicating potential higher genetic variation. However, the studies cited above have focused on the loss of chloroplast genes, and further studies are needed to properly identify good chloroplast marker genes to solve genus-level phylogenies. That was not the focus of our study, but our results suggest that the use of proper chloroplast markers is crucial for a more confident inference of a particular species phylogeny. Identifying these markers would be an immediate next step for *Tribulus cistoides* phylogenetics, or at least the use of more informative techniques, such as RADseq would be better to elucidate the phylogenetic differences observed in our study. Nevertheless, our findings do provide the information necessary to answer our questions, especially when we concatenated our phylogenies.

Our study is perhaps another case of discrepancies between the phylogenetic inferences of nuclear and organelle markers (Klicka et al., 2023; O. M. Vargas et al., 2017). Both cpDNA and nuclear DNA play important roles in phylogeographic assessments. CpDNA, with its maternal transmission and non-recombining nature, provides insights into the evolutionary history and biogeography of plant populations. Meanwhile, nDNA, despite its technical and biological challenges, offers more information and the potential to extract explicit genealogical information, contributing to our understanding of population dynamics and genetic diversity (Avise, 2009). In some cases, the nuclear marker shows evidence of admixture, which is not detectable in organelle markers (Klicka et al., 2023). In others, discrepancies include the detection of hybridization and introgression, which confound the phylogenies of organellar markers due to their mode of inheritance (O. M. Vargas et al., 2017). In the case of our study, the cpDNA showed different tree topologies compared to the nuclear marker, so it is worth exploring in depth if these mechanisms (admixture, hybridization, and introgression) are common in *Tribulus*. Perhaps more in-depth studies using other methods such as RADseq could provide better evidence of these mechanisms. Finally, information about *Tribulus* gene flow

between islands would be crucial to understanding the haplotype patterns shown in this study.

Tribulus taxonomy

Another limitation was that the taxonomy of *Tribulus,* our phylogenetic analysis grouped taxon that were classified as another species based on herbaria classification. We revised the taxonomic classification and tried to characterize the species correctly (Appendix 4.2), however, that is not the focus of our study. It is necessary to review the taxonomy of this group in detail and update it based on this new evidence. This is an important point concerning the herbarium specimens we used, and it is possible to use our phylogenetic evidence to update this information. Although we did not use morphological characters to classify some of our Galápagos samples, we did observe that some individuals have intermediate morphological characters in some of our vouchers. Specifically, in the floral glands and nectarines, crucial for *Tribulus* species classification (Porter, 1971; Wiggins & Porter, 1971). Interestingly, our phylogeny shows that our Galápagos samples correspond to a single species, *T. cistoides*. Nevertheless, it is worth following up on the presence of these intermediate morphological characters that historically may suggest more complex species interactions such as hybridization. This is an aspect that we did not explore, and we did not find evidence for but is likely to occur in the islands. The Galápagos and the Caribbean are the only two places where the species *T. terrestris* and *T. cistoides* coexist, suggesting the potential for hybridization between these plants (Porter, 1971). We recommend exploring this further by sampling these individuals and potentially identifying pure individuals on the islands.

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CHAPTER 5: General Discussion

In this chapter, I examine *Tribulus*-finch interactions in the Galápagos and the adaptation of *Tribulus* to the island environment. This thesis shows the potential of *Tribulus cistoides* as a model for evolutionary studies. I will start discussing the main limitation found for this thesis. Then, I will discuss unresolved questions and propose potential avenues for future research that can be supported by the findings presented in this study.

First, we found that *T. cistoides* phenotypic traits, mainly those related to mutualistic and antagonistic interactions, differ between islands and continental populations. We found that *T. cistoides* mericarps on islands are larger and their number of spines is more variable than those found on the continent. In addition, flowers on the Galápagos islands are smaller than elsewhere, even on other island systems, suggesting that phenotypes of *T. cistoides* are driven by island conditions (either environmental or biotic factors) (Chapter 2). We also found that the trait variation observed in mericarps is the result of interactions between *Tribulus* and their predators, the Darwin's finches. Larger and spinier mericarps are the result of adaptation to predation. Populations of *T. cistoides* are generally well-adapted to predation, but still, some populations have not reached their optima. In general, a trade-off may exist between predation defense and dispersal. However, potential gene flow between the islands may also explain differences found in maladapted populations (Chapter 3). Finally, we found that *T. cistoides* populations from the Galápagos arrived at the islands millions of years ago, meaning that this species is native to the islands and the phenotypic divergence observed is most likely the result of adaptation. *T. cistoides* was already established on the islands when the finch ancestor arrived, suggesting that *Tribulus*-finch interactions might have helped the diversification/specialization of Darwin's finches (Chapter 4).

Thesis limitations

In Chapter 2 and Chapter 4, we mention some form of data limitations. The main critique of Chapter 2 was the unbalanced dataset with a lack of samples from certain island systems. In Chapter 4 we mentioned the limited informativeness of chloroplast markers, resulting in restricted conclusions.

The unbalanced dataset in Chapter 2 was the result of the limited but very important use of herbarium specimens. Herbarium collections offer valuable information about the history of species and provide useful phenotypic and genetic information for comparison studies (Besnard et al., 2018). Relying on only natural populations for a large comparative study is logistically unfeasible, much so if one needs to compare multiple, even uninhabited, island systems. Ideally, a combination of both herbarium and natural population samples solves any potential unbalanced sampling. However, there may still be limited samples, especially for island systems, so any number of island samples were highly valuable, and they were the only feasible way to increase the size and spatial extent of sampling. In Chapter 2, all floral samples were from herbarium collections, whereas we had herbarium and natural population data for both island and continental mericarp samples. We then performed extensive additional analysis to show that there was no major bias in our results (Appendix 2.4 to 2.7).

Proper marker selection is crucial for the analysis and objectives of any phylogeographic project. Genetic markers should have significant genetic variation at the appropriate taxonomic level (Schaal et al., 1998). The interspecific level of taxonomy is the most challenging (Schaal et al., 1998). In our study, we selected markers that should have enough variation at the genus level. However, for Chapter 4, the chloroplast markers we used show little to no variation for *Tribulus*, despite some evidence of being highly variable in other taxa (Shaw et al., 2005, 2007, 2014). Other studies were successful at finding differences at the interspecific level. For example, Aguirre-Liguori et. al (2014) used *ndhF*, *rpl32*, and *psbJ* to identify nine haplotypes within *Fouquieria shrevei*. One explanation for our results

is the inheritance of organelle genes and nuclear genes (Petit & Vendramin, 2007). While organelle genes exhibit high geographic structure due to their single locus inheritance through seeds, they cannot generate multiple replicates of colonization history. In contrast, nuclear genes segregate independently, allowing better evaluation of genetic stochasticity but combining colonization and gene flow history (Petit & Vendramin, 2007). Another more likely explanation is that simply these markers are not that variable in *Tribulus* or the Zygophyllaceae, than in other species. For example, Shaw (2014) indicated that species such as *Acorus, Nicotiana, Oryza,* and *Phyllostachys* show little to no variation in these chloroplast regions. In regard to our study, Chapter 4 was rather exploratory, thus we selected specific chloroplast and nuclear markers. However, our study may be improved greatly from an initial screening of the chloroplast region, specifically for *Tribulus*, where potential markers can be determined and used more confidently.

Unexplored and future research themes

Heritability of *Tribulus cistoides* mericarps traits

In Chapter 1, I explained that evolution is the accumulation of change, and that many mechanisms can generate that change, but that it is mainly directed by natural selection (Endler, 1986). The main results of our chapters suggest that *T. cistoides* mericarps are under the process of natural selection. In our study, I found direct evidence of two of the three conditions of natural selection (Chapter 1). First, we observed the phenotypic variation between the islands and continents. Second, we found a consistent relationship between mericarp size, spininess, and survival due to finch predation. Finally, *T. cistoides* populations were established and have been interacting with the island ecosystems and predators for many generations.

However, the fact that *T. cistoides* has been established in the islands for many generations is not a direct measurement of the third condition of natural selection (Chapter 1). This condition was explained as a consistent relationship between parents and their offspring for the trait of interest (Endler, 1986). In our case, we would need to measure the heritability of mericarp size and defense. To

estimate the heritability of a trait is necessary to know the phenotypic variation of parental individuals and track their offspring in an experiment commonly known as common garden (de Villemereuil et al., 2016; Mousseau & Roff, 1987). A common garden experiment is mainly used to separate the environmental effect from the genetic effect on trait variation and estimate the overall heritability of a trait (Roff, 1997). In our case, it would involve measuring the mericarps of parental plants, raising that offspring until they produce mericarps themselves and then measuring those mericarps. After enough replicates, we can have a reliable estimate of the parent-offspring relationship (Roff, 1997).

Early in my research, I attempted a common garden experiment to elucidate the effects of the environment and estimate the heritability of mericarp traits. The idea of this common garden experiment was to estimate the heritability of mericarps traits on Santa Cruz Island. We collected at least 10 mericarps from at least 10 specific plants across the yearly monitored populations of Santa Cruz (total 8 populations, see Appendix 3.1) and raised them in lowland and highland conditions to estimate the effect of environmental conditions (plasticity) and calculate heritability of mericarp trait variation. However, the seeds did not germinate, and only a handful of mericarps germinated but did not survive. The experiment started in early June and finished in October 2019. After some consideration, I concluded that *Tribulus* seeds must only germinate during specific environmental conditions, most likely during the wet season. Previous attempts at *Tribulus* germination experiments also reported somewhat lower germination rates and mentioned that breaking seed dormancy was the main concern in their experiments (Ernst & Tolsma, 1988; Yankova-Tsvetkova et al., 2011). In one case, less than 50% of germination was achieved (Ernst & Tolsma, 1988). However, in another study, a high germination rate occurred, likely also due to soil and air temperatures with high and dry conditions between 20-37°C (Petkov, 2010).

Despite our unsuccessful attempt, we believe that mericarp traits do have a heritable component. During our yearly surveys, we observed individuals producing

particular phenotypes. These unique phenotypes were mostly related to spine number, with plants producing mericarps without lower spines, without upper spines, and even without spines at all (Appendix 2.3). Over the years, we found that *Tribulus* individuals from Santa Cruz Island that produced 2-spined mericarps often produced 2 spines and that plants that produced 4 spines often produced 4 spines. Thus, our observations suggest spine number to be genetically determined and a component of this trait could be independent of environmental conditions. We can test this with an experimental plot, where "unique" and "common" mericarps are collected and placed on a clean area and then followed over time. If spine number is heritable, one would expect that plants growing on these plots would produce the same number of spines over generations. A study like that would provide more direct evidence of the third condition of natural selection on *T. cistoides* mericarps.

Effects of the Environment on seed germination of *T. cistoides*

In chapters 2 and 3, we showed the effect of environmental factors on trait variation and selection. Chapter 2 described that mericarp size, spines and flower size are mainly explained by environmental factors. In Chapter 3, we found that environmental factors affect mericarp selection as well, specifically mericarp size and spine angle. However, other life-history traits are strongly related to environmental conditions that we did not fully explore.

For instance, as we mentioned above, seed germination is strongly related to environmental conditions. I believe that the variability in germination rates and the relationship between seed dormancy in *T. cistoides* is a venue for research worth exploring. For instance, Boydston (1990) germinated *Tribulus* mericarps that were on the soil for years, showing the potential for "resurrection" experiments (Franks et al., 2007, 2018; Krishna et al., 2022). Also, Verdú and Mas (2004), conducted a cohort-dependent study and demonstrated that earlier cohorts of T. *terrestris* had higher survival probability than well-established populations. Finally, Kigel (1995) reported that seedlings' survival is dependent on earlier seed germination, and their

ability to tolerate desiccation. Opening potential studies on population dynamics and the effect of seasonality on plant establishment and mortality. For instance, one can link the genetic structure of seasonal populations over yearly populations, in the case of *T. cistoides*.

These potential research topics can be applied to the context of the Galápagos. Due to the seasonal variation on the islands, it might be that *T. cistoides* seed dormancy and germination is linked with seasonality. In addition, a relationship may exist between seed predation and germination. Finches eat mericarp seeds in a specific order, with seeds closer to the upper spines always being predated first (Grant, 1981). Predated mericarps may trigger the germination of other seeds within the mericarp ensuring survival. In Chapter 3, I collected data on seed germination for the yearly populations. Unfortunately, we did not have enough information for a proper analysis, but it is an idea that can be followed immediately after resuming population monitoring.

Potential research topics on the genetics of *T. cistoides*

Advances in technology and the reduction of costs of genetic techniques makes it more feasible to use larger amounts of genetic information for answering specific questions on non-model species. One technique that can be applied to these proposed studies is the Restriction-site Associated DNA Sequencing (RADseq). RADseq is an umbrella term for similar techniques that involve using restriction enzymes to digest genomic DNA, followed by the ligation of customized adaptors and barcodes for PCR amplification and sequencing. The number of sampled genomic regions depends on factors like genome size, choice of enzymes, and genetic diversity (Andrews et al., 2016; Parchman et al., 2018). The low level of sequence divergence in many island lineages makes RADseq suitable for application in island studies (Crawford & Archibald, 2017). In plant studies, RADseq has been used, mainly for non-model species without previous genetic information (Egan et al., 2012; Peterson et al., 2012). It can be used to estimate gene flow (Salmona et al., 2023), linkage mapping (Zhou et al., 2014), population structure and history

(Stojanova et al., 2020), molecular quantitative genetics (Zhigunov et al., 2017), and phylogenetic analysis (Paetzold et al., 2019). Here I discuss some potential research topics using this technique that would expand based on the findings of this thesis.

Gene flow of T. cistoides on the Galápagos Islands

In Chapters 3 and 4, I delve into the genetic differentiation among populations on different islands and the colonization history of *T. cistoides*. I mentioned the role of gene flow, either as an explanation of patterns of selection in certain populations (Chapter 3) or to explain the potential homogeneity of haplotypes (Chapter 4). Here, I will discuss further the implications of gene flow in the context of this study, and suggest future studies based on our results.

Gene flow is defined as the movement of individuals, or genes from one population to the other (Slatkin, 1985). Gene flow can be influenced by factors such as population size, density, breeding systems, surrounding vegetation, and dispersal mechanisms (Levin & Kerster, 1974). Factors that can vary spatially and temporally. In plants, gene flow is driven by the movement of gametes, either pollen (male-inherited) or seeds (often motherly inherited) (Petit & Vendramin, 2007; Slatkin, 1985). The effects of gene flow are multiple. On one hand, it can increase genetic variability in populations, as new genes arrive in a small population, closer to extinction (Lacy 1987, Tallmon 2004). But on the other hand, if these small populations are under a process of differentiation (either by new environmental conditions or new species interactions) it can homogenize populations and erase any rising differences (Slatkin, 1987). In the case of island populations, often gene flow is seen under that second perspective.

In this thesis, I found that gene flow could explain why some populations within islands have not reached complete adaptation (Chapter 3) and that gene flow may affect genetic differentiation across islands (Chapter 4). In the future, potential studies can focus on aspects of gene flow within and between islands. For example, a study of gene flow of *Tribulus* within island populations could help explain our findings in Chapter 3. Specifically, it would be worth exploring gene flow on

Floreana Island, where most of the populations are still under selection for mericarp traits. Using specific genetic markers, we can differentiate populations and test for gene flow. In Chapter 4, we found that haplotype structure in Floreana, at least for *ITS,* is well differentiated for two populations from opposing ends of the island (Figure 4.3). However, we did not sample the populations in town. So, a study estimating gene flow between town populations and other locations on the island could provide further evidence of the mechanisms that keep populations on Floreana still under selection for mericarp size. A second study can look at overall gene flow across the archipelago. The sampling scheme in Chapter 4 focused on a few individuals but from multiple locations. The same samples can be used for RADseq and provide more information about the populations across islands and overall estimates of gene flow and population structure for *T. cistoides* in Galápagos. Chapter 4 was limited in our methodological approach. Although it was enough to provide evidence for our objectives, some intricacies of *T. cistoides* populations in Galápagos will be resolved in future studies.

Potential hybridization or introgression of Tribulus cistoides in the Galápagos.

Plants are propending to hybridize and introgress (Stebbins, 1950). Previous work, based on morphological traits and taxonomy, suggested that *Tribulus* in Galápagos exhibits intermediate traits (Porter, 1971). Specifically, between extrastaminal glands, that help differentiate *T. terrestris* from *T. cistoides*. In the Galápagos, intrastaminal gland morphology of *T.* cistoides is usually connate and from *T. terrestris,* usually free. However, some individuals of *T. terrestris* show connate glands (Porter, 1971). Suggesting potential hybridization between these species in Galápagos. In our study, our samples were only from *T. cistoides* as suggested by our phylogeny results (Chapter 4), but it is possible to find specimens of both species and potentially provide genetic evidence of hybridization. Previous research indicated that hybrid compatibility can persist over long evolutionary periods, potentially contributing to genetic variation. The impact of gene flow on genetic variation among closely related organisms is influenced by factors like natural history, geographic distribution, selection, and mutation (Slatkin, 1985,

1987). The genomic era has revealed frequent instances of introgressive hybridization across evolutionary tiers and between species. In plants, hybridization is recognized as a source of genetic variation, enhancing the potential for adaptive responses to environmental changes (Stebbins, 1950). Interspecific gene flow through hybridization could introduce new alleles that enhance short-term adaptive potential.

Genetic basis of adaptation of Tribulus cistoides mericarps

This thesis focused mainly on phenotypic variation and phenotypic selection. Above, I mentioned the importance of the genetic component to understand the mechanisms of selection on *Tribulus* mericarps. If mericarp traits are genetically based, then it would be interesting to explore the genetic basis of mericarp traits. We already know that specific traits are under selection and environmental effects also affect trait variation. However, traits such as spine number, seem to be heritable, explained by environmental factors and important for survival. In addition, some individuals produce rare mericarp phenotypes, that lack some set of spines. We could use these rare phenotypes, to understand the genetic basis and mechanisms that originate these phenotypes. Focusing on linking candidate genes or going further by looking at mechanisms of fruit development. One approach would be conducting genome-wide studies and identifying candidate genes associated with specific traits. We can gain a deeper understanding of the underlying genetic mechanisms driving adaptation to island conditions.

Potential anthropogenic effects on *Tribulus cistoides* evolution and genetics

As mentioned throughout this thesis, humans are great dispersers of *T. cistoides* (M. K. A. Johnson et al., 2020), however, this thesis did not directly explore any potential effects of humans on *Tribulus* - finch interactions or *Tribulus* evolution. This is often observed in the large frequency of *Tribulus* on roads and paths, and how activities like hiking, biking and cars can help the dispersal of *Tribulus* mericarps (E. Johnson, 1932; Schweickerdt, 1948). Likely, human

activities influence gene flow of *Tribulus* populations, and human activities may affect haplotype distributions in the Galápagos.

Other studies on islands have found that humans influence population structure. For example, Crispo *et al.* (2011) found that humans can increase genetic exchange among groups of individuals. Either by bringing previously isolated groups into contact, or by disrupting reproductive barriers. In the context of the Galápagos, tourists flow daily across the main inhabited islands, and often to some uninhabited ones (de Groot, 1983). Even if there are controls present on each dock that focuses on limiting the exchange of seeds on shoes and luggage, some mericarps are likely carried around. For example, I have personally found *T. cistoides* mericarps on the daily boats across Santa Cruz and Floreana, and even on boats offering tours within the same island. If we consider the dormancy aspects of *T. cistoides* seeds discussed earlier, then it is possible that mericarps move across islands and can be established between populations. However, we still do not know how much humans affect gene flow on *Tribulus*. If humans have a large effect as has been suggested in this thesis, then *T. cistoides* would be another example of how human activities are indirectly conflicting with the evolution of this species.

Conservation and management of species interactions

Often conservation efforts are species focused, however, these efforts may weaken naturally occurring species interactions and restored populations may not become self-sustainable, which should be the ultimate goal in conservation (Heinen et al., 2020). Another approach that aims to self-sustainability is focusing on species interactions, an idea that is often unexplored in conservation and management (Schlaepfer et al., 2011). Identifying, tracking and understanding, species interactions are crucial to reach this goal. By knowing how different species depend on each other, we can identify key species or interactions crucial for maintaining ecosystem stability (Heinen et al., 2020). For *Tribulus*, conservation efforts on the mainland have focused mainly on eradication of the plant using natural insect predators (Huffaker et al., 1983; Kirkland & Goeden, 1978). In the context of

islands, *Tribulus* is crucial for maintaining endemic predator and pollinator populations (Carvajal-Endara et al., 2020; Traveset et al., 2013).

In addition, many human activities (overharvesting, habitat fragmentation and degradation, exotic species, and chains of extinction; (Diamond, 1989) are also strong selective pressures that could change the evolutionary trajectories of species and species interactions (Stockwell et al., 2003). Thus, gathering information such as this thesis, where we quantify how *Tribulus* responds to selection within a few years, is the basis for conservation and management decisions towards holistic, self-sustained conservation goals. The *Tribulus*-finch interaction can be used as evidence of the importance of species interactions and contemporary evolution in the conservation and management of endemic species.

Future studies focused on conservation efforts in the Galápagos should note the findings of this thesis as an example of the importance of species interactions. There are many efforts for the conservation of endemic species in the Galápagos, some successfully eradicated invasive species from the islands (Carrion et al., 2011). However, the eradication of these species has problematic effects on other species. For example, the reduction of Galápagos hawks after goat eradication (Rivera-Parra et al., 2012). More recently, there are planning large efforts of restoring a whole island ecosystem ('Restoring Floreana', 2023). We strongly suggest considering species interactions in their efforts, to self-sustain populations and to provide enough resources and not limit species' ecological role. For example, the pink pigeon on Mauritius (*Nesoenas mayeri*), after a successful breeding program became ecologically limited by not serving as a disperser of other plant species and depending mostly on feeders (Florens, 2013). Preserving these interactions can be essential for protecting biodiversity and ensuring the resilience of ecosystems, particularly in the face of environmental disturbances and climate change.

Conclusion and Summary

In summary, *Tribulus cistoides* is a widespread plant that offers unique opportunities to understand island processes and species interactions further. *T.*

cistoides shows potential as a model to study microevolutionary processes, phenotypic divergence, plant establishment, germination, plasticity, and in general, adaptation to island environments. In addition, the abundance of *Tribulus cistoides*, on the islands means it can be used in a wide range of experimental settings and field experiments.

Finally, we now further understand how plants respond and adapt to predation by Darwin's finches. This study shows the importance of species interactions driving adaptation and is another example of native, non-endemic species playing a crucial role in the specialization and endemism of other species as our findings suggest. The thesis emphasizes the importance of focusing on species interactions rather than a single species for conservation purposes, given that interactions are the key processes that maintain species and populations over time.

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APPENDIX

Chapter 2 Appendix

Appendix 2.1

Table S2. 1 Sample distribution of mericarp and flower datasets. The table shows the distribution of samples from island and continents and their source, from herbarium or field samples.

Sets	Continents	Population	Source	n	Total
Mericarps	Africa	Island	Herbarium	8	47
			Field samples	-	
		Continent	Herbarium	39	
			Field samples	-	
	North and Central America	Island	Herbarium	86	1291
			Field samples	200	
		Continent	Herbarium	81	
			Field samples	924	
	South America	Island	Herbarium	505	3829
			Field samples	3245	
		Continent	Herbarium	79	
			Field samples	-	
Flowers	Africa	Island	Herbarium	6	26
			Field samples	-	
		Continent	Herbarium	20	
			Field samples	-	
	Asia	Island	Herbarium	22	22
			Field samples	-	
		Continent	Herbarium	-	
			Field samples	-	
	North America and Caribbean	Island	Herbarium	260	512
			Field samples	-	
		Continent	Herbarium	252	
			Field samples	-	
	Oceania	Island	Herbarium	52	58
			Field samples	-	
		Continent	Herbarium	6	
			Field samples	-	
	South America	Island	Herbarium	69	154
			Field samples	-	
		Continent	Herbarium	85	
			Field samples	-	

Table S2. 2 Distribution of mericarp and flower samples from Galápagos and Other Islands.

Sets	Islands	Continent	Island Location	n	Total
Mericarps	**Galápagos**	South America	Baltra	193	3745
			Champion	51	
			Daphne Major	88	
			Daphne Minor	42	
			Darwin	2	
			Enderby	6	
			Espanola	264	
			Fernandina	73	
			Floreana	605	
			Genovesa	29	
			Guy Fawkes West	5	
			Isabela	671	
			Pinta	12	
			Plaza Norte	2	
			Rabida	124	
			San Cristobal	405	
			Santa Cruz	1005	
			Santiago	55	
			Seymour Norte	113	
	Oher Islands	Africa	Cape Verde Islands	3	8
			Shungu-Mbili Island	5	
		North and Central America	Boca Grande	100	286
			Clarion Island	7	
			India Key	15	
			Key Biscayne, FL	4	
			Key West, FL	20	
			Marathon	100	
			Big Coppett Key	5	
			Socorro Island	35	
		South America	Isla de Salamanca	5	5
Flowers	**Galápagos**	South America	Baltra	5	48
			Champion	2	
			Daphne Major	4	
			Darwin	2	
			Eden	1	
			Fernandina	1	
			Floreana	1	
			Gardner	2	
			Isabela	15	
			Plaza Norte	1	
			Plaza Sur	3	
			Santa Cruz	7	
			Santiago	4	
	Other Islands	Africa	Seychelles Islands	3	6
			Cape Verde Islands	1	
			Zanzibar	2	
		Asia	Philippines	4	22
			Sri Lanka	18	
		North America and Caribbean	Antigua and Barbuda	5	252
			Bahamas	18	
			British Virgin Islands	2	
			Clarion Island	6	
			Cuba	17	
			Dominican Republic	22	
			Guadeloupe	6	
			Haiti	30	
			Jamaica	49	
			Martinique	1	
			The Revillagigedo Islands	8	
			Socorro Island	9	

			Puerto Rico	6	
			Turks and Caicos Islands (Lucayan Archipelago)	13	
			U.S. Virgin Islands	8	
			Florida Keys	23	
			Hawaiian Islands	29	

Appendix 2.2

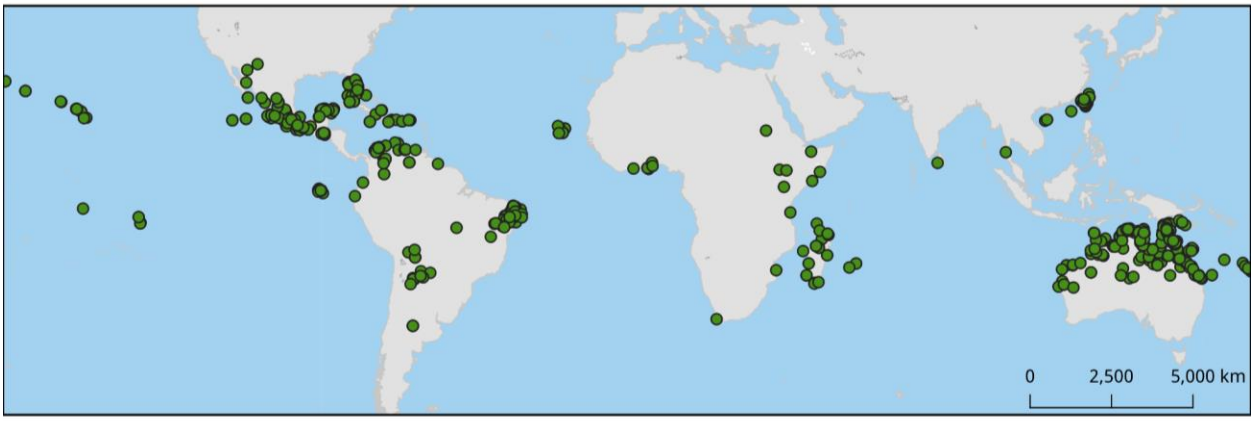


Figure S2. 1 Global distribution of *Tribulus cistoides* based on specimens from the Global Biodiversity Information Facility (GBIF) (n = 1213). The collection ranges from 1770 - 2021.

Appendix 2.3

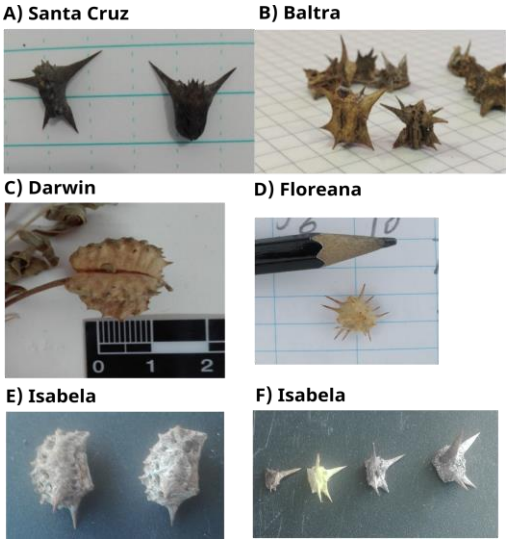


Figure S2. 2 Mericarp phenotypic variation found on the Galápagos Islands. Mericarps shown are taken from A) Santa Cruz, B) Baltra, C) Darwin, D) Floreana, and E-F) Isabela islands. Mericarps on the Galápagos differ in size, shape, and spine number. Some phenotypes lack lower or upper spines. These phenotypes coexist and individual plants produce the same phenotype. Although the frequency of mericarps with no spines is less common, they can be found in populations that are close to towns and roads. Photos by WDRC.

Appendix 2.4

Table S2. 3 Model estimates of the effect of population and year of collection on mean mericarp traits. The table shows the model estimates per trait and the mean PC1 estimates. The means were from Galápagos and Florida populations to help account for unbalanced sampling. Other locations were kept as individual observations. Estimating the means of these two locations gave a total of 561 observations. Means were calculated using ID, that identifies the populations or locations of the samples.

Mericarp Means – *continental vs island*							
	Trait	**Continental/Island**		**Year**		**Field/Herbarium**	
		X^2	**P**	X^2	**P**	X^2	**P**
	Length	**15.2892**	**<0.001**	**13.6705**	**<0.001**	1.6815	0.19472
	Width	**9.0171**	**0.00268**	2.2999	0.12938	0.0061	0.938
	Depth	**33.5116**	**<0.001**	**9.9451**	**0.00161**	0.1914	0.66178
	Spine size	0.578	0.44724	3.6738	0.05528	1.3526	0.24483
	Mericarp Size (PC1)	**12.453**	**<0.001**	10.6016	0.001	0.6904	0.4060

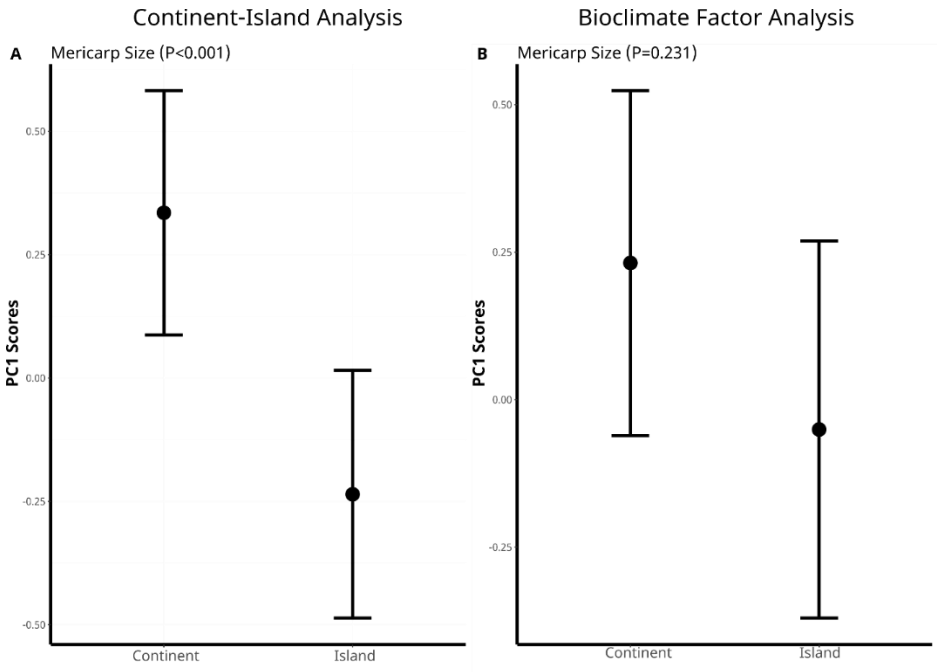


Figure S2. 3 Mean mericarp traits compared between island and continental locations. Means were estimated to account for unbalanced sampling from Galápagos and Florida (n = 3829, n = 1291). Locations were grouped by ID. Plots are the least-squares means ± one standard error. On top of each plot, it shows the p-values from the ANOVA. A) Mean mericarp trait plots of continent and island populations only. B) Mean mericarp trait plots including bioclimate variables.

Table S2. 4 Model estimates of the effect of population and year of collection on mean mericarp traits including bioclimate variables. The table shows the model estimates per trait and the mean PC1 estimates. The means were from Galápagos and Florida populations to account for unbalanced sampling. Nomenclature on bioclimate variables was taken from the WorldClim dataset (https://worldclim.org/). We used variables Bio1 (Annual Mean Temperature), Bio4 (Temperature Seasonality), Bio12 (Annual precipitation), Bio15 (Precipitation Seasonality).

Mericarp Means – *continental vs island*														
	Trait	**Continental/Island**		**Year**		**Field/Herbarium**		**Bio1**		**Bio4**		**Bio12**		**Bio15**	
		X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**
	Length	3.533	0.0602	**10.601**	**0.0011**	0.028	0.8668	0.023	0.8793	**6.485**	**0.0108**	1.016	0.3134	2.245	0.1340
	Width	2.766	0.0963	1.242	0.2651	0.528	0.4673	0.287	0.5916	2.684	0.1013	0.017	0.894	**5.078**	**0.024**
	Depth	**5.962**	**0.0146**	**8.796**	**<0.001**	0.031	0.8601	0.374	0.5406	1.411	0.2347	0.395	0.5296	**6.592**	**0.0102**
	Spine size	0.167	0.6824	2.731	0.0984	0.200	0.6544	0.807	0.3689	1.536	0.2151	0.023	0.8781	1.064	0.3023
	Mericarp Size (PC1)	1.432	0.2314	**8.574**	**0.0034**	0	0.9972	0.077	0.7812	**4.735**	**0.0295**	1.162	0.2809	**3.892**	**0.0485**

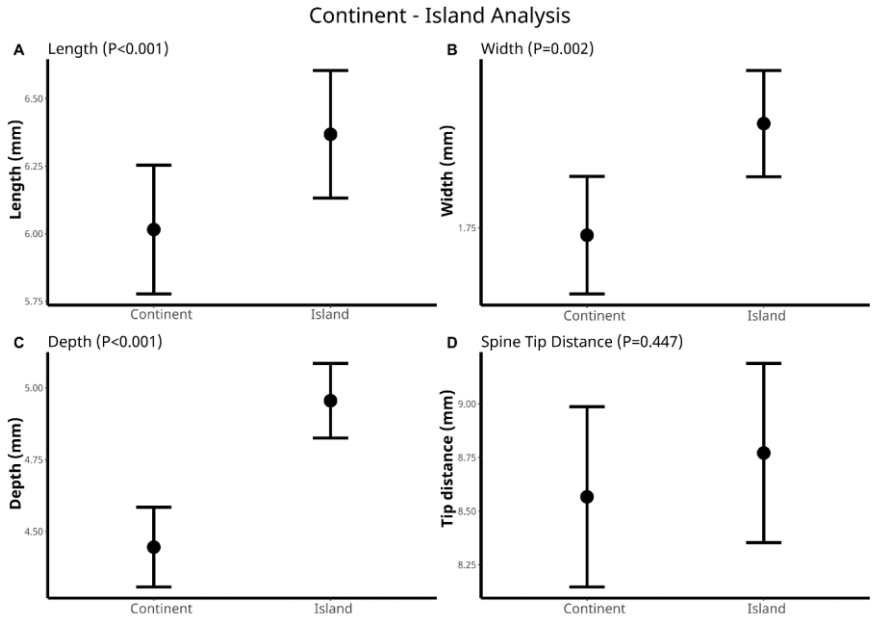


Figure S2. 4 Mean individual mericarp traits compared between island and continental locations. Means were estimated to account for unbalanced sampling from Galápagos and Florida (n = 3829, n = 1291). Locations were grouped by ID. Plots are the least-squares means ± one standard error. On top of each plot, it shows the p-values from the ANOVA. A-D) Mericarp trait plots without bioclimatic variables included.

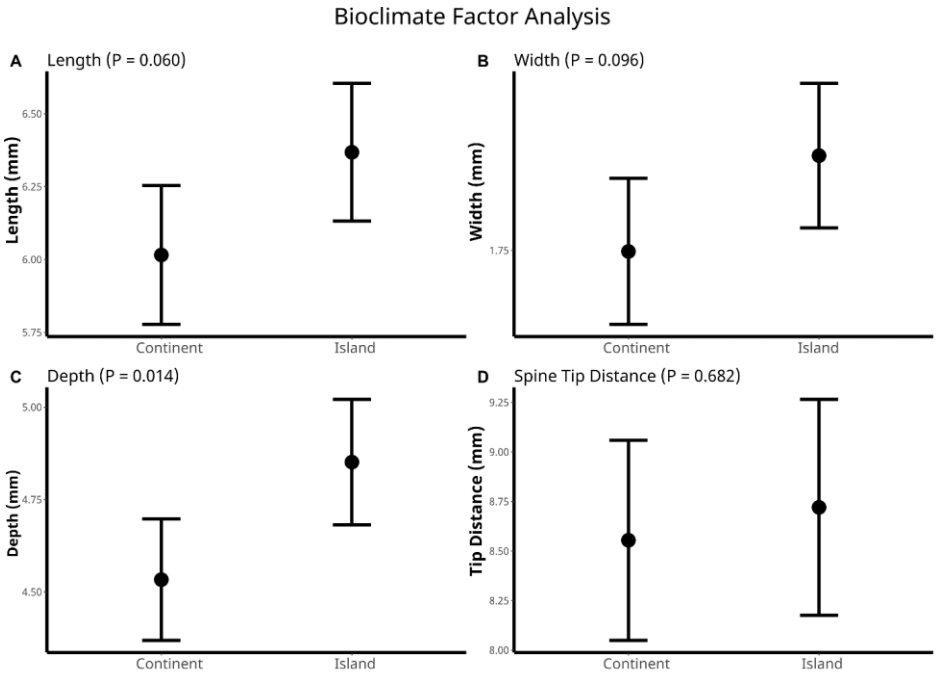


Figure S2. 5 Mean individual mericarp traits compared between island and continental locations. Means were estimated to account for unbalanced sampling from Galápagos and Florida (n = 3829, n = 1291). Locations were grouped by ID. Plots are the least-squares means ± one standard error. On top of each plot, it shows the p-values from the ANOVA. A-D) Mericarp trait plots include bioclimatic variables.

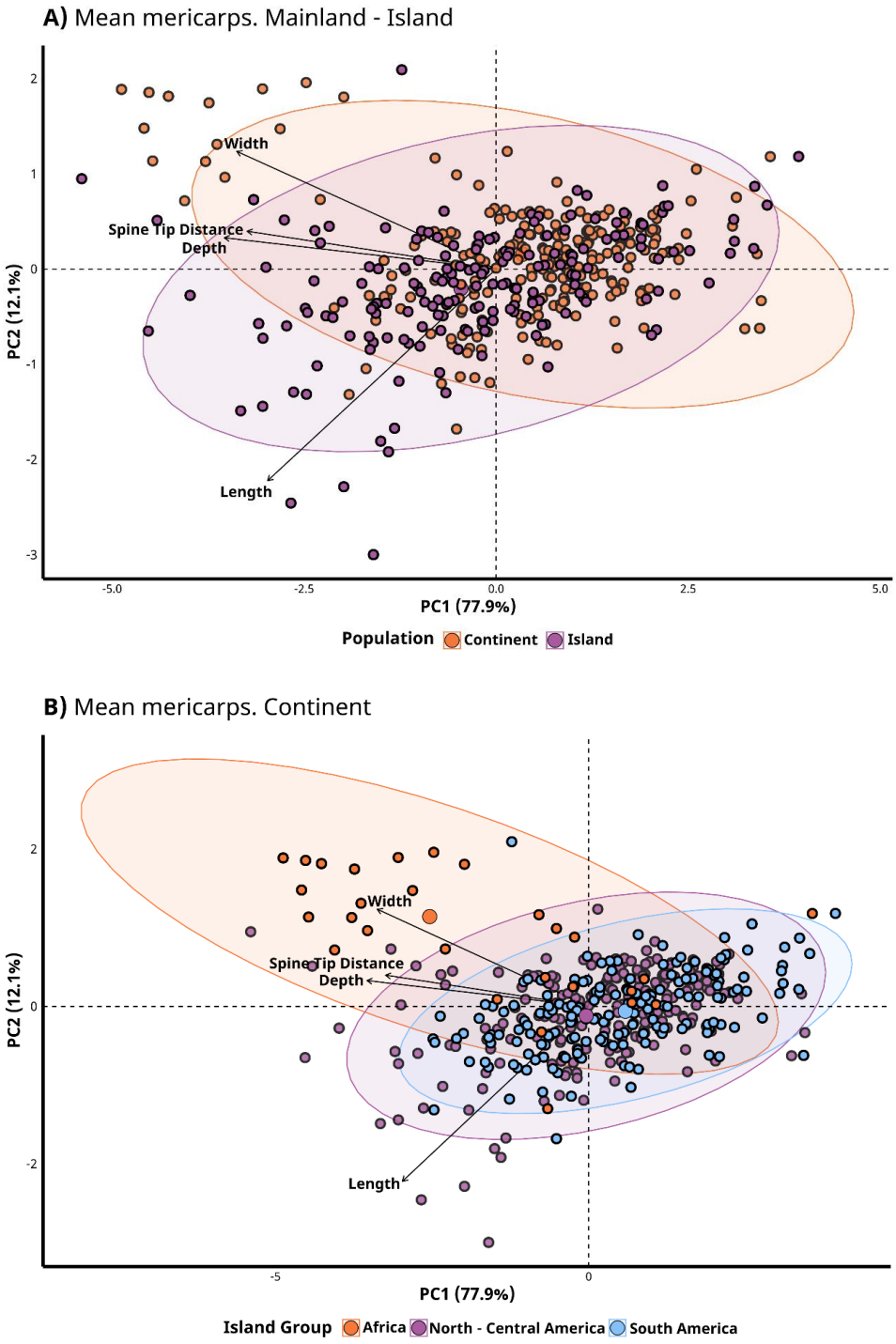


Figure S2. 6 Principal component analysis of mean mericarp traits, length, depth, width, and spine size. Points represent individual mericarps and estimated means of mericarps for Galápagos and Florida locations. Locations were grouped by ID. Other locations kept their individual values. Means were estimated to account for unbalanced sampling from these locations (n = 3829, n = 1291). Trait vectors are proportional to the contribution and direction associated with each trait. Larger circles represent the centroid of the ellipses with a 95% confidence interval. Top represents PCA with ellipses representing continental and mainland populations. Bottom, PCA representing main continental groups used in the analysis.

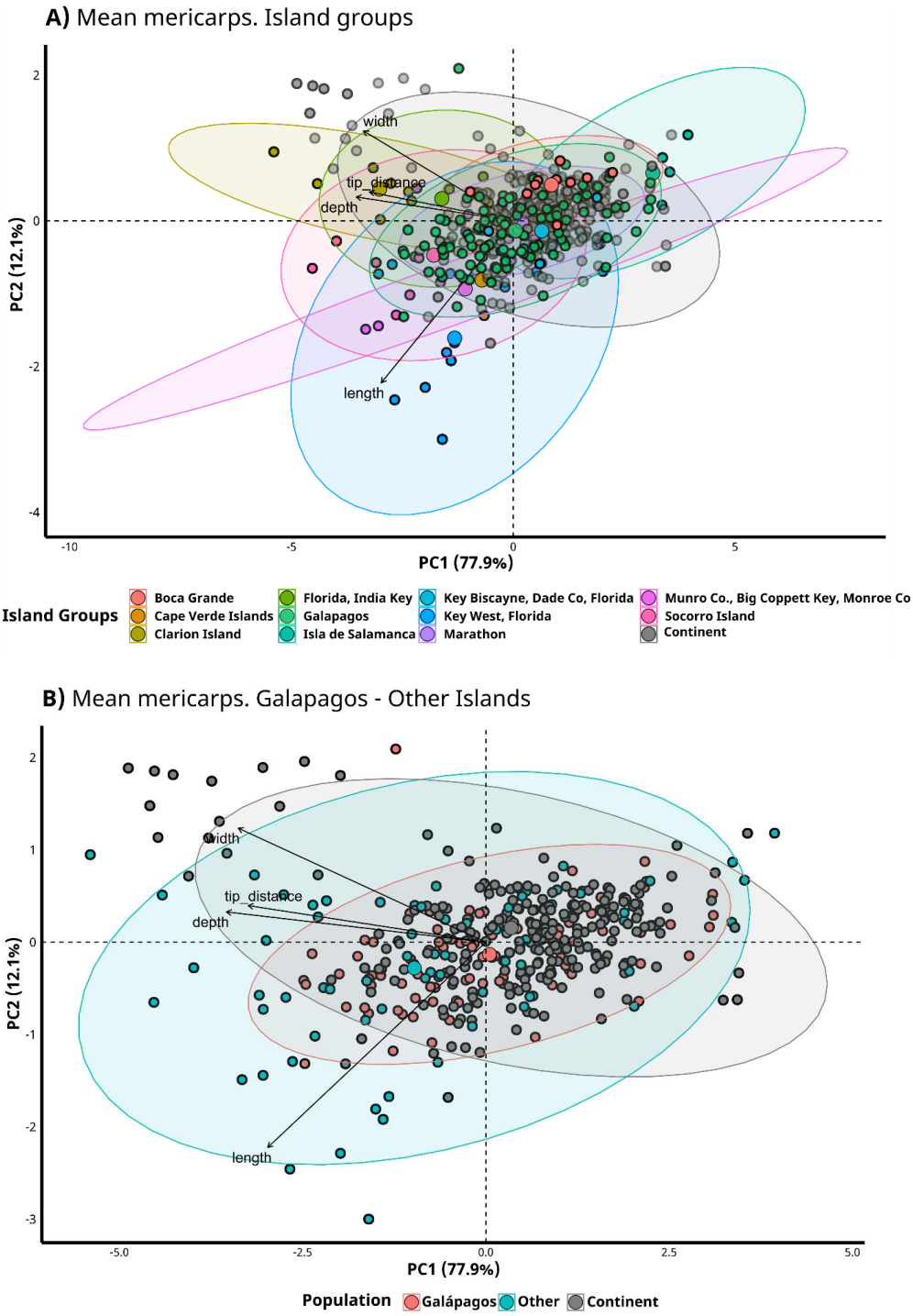


Figure S2. 7 Principal component analysis of mean mericarp traits, length, depth, width, and spine size. Points represent individual mericarps and estimated means of mericarps for Galápagos and Florida locations. Locations were grouped by ID. Other locations kept their individual values. Means were estimated to account for unbalanced sampling from these locations (n = 3829, n = 1291). Trait vectors are proportional to the contribution and direction associated with each trait. Larger circles represent the centroid of the ellipses with a 95% confidence interval. Top represents PCA with ellipsis representing island groups. Bottom, PCA representing Galápagos and Other Island groups used in the analysis.

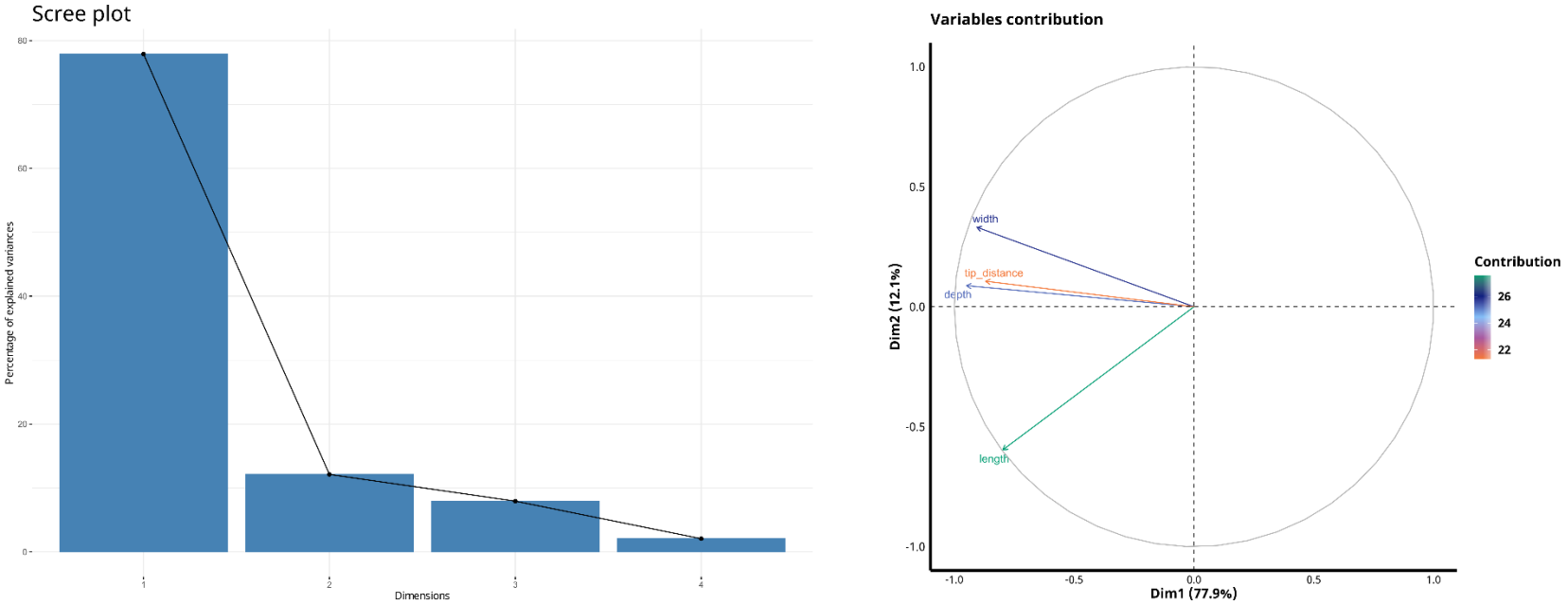


Figure S2. 8 Eigenvector contribution plot and percentage of explained variation for each PC axis associated with mericarp morphology for mean mericarps of Galápagos and Florida. Locations were grouped by ID. Other locations kept their individual values. Means were estimated to account for unbalanced sampling from these locations (n = 3829, n = 1291).

Appendix 2.5

Table S2. 5 Model estimates of the effect of population and year of collection on individual mericarp traits including bioclimate variables with African Islands removed. African Islands were samples from a single herbarium voucher and represents only one individual per location. The table shows the model estimates per trait and the PC1 estimates.

A) Mericarp – *continental vs island* - African Islands removed							
	Trait	**Continental/Island**		**Year**		**Herbarium**	
		X^2	**P**	X^2	**P**	X^2	**P**
	Length	**18.275**	**<0.001**	**10.179**	**0.001**	0.7783	0.377
	Width	**12.930**	**<0.001**	1.019	0.312	0.164	0.684
	Depth	**55.408**	**<0.001**	**7.989**	**0.004**	0.203	0.651
	Spine tip distance	**7.086**	**0.007**	0.163	0.686	0.519	0.470
	Lower spines	**73.388**	**<0.001**	-	-	3.077	0.0793
	Mericarp Size (PC1)	**26.374**	**<0.001**	**4.298**	**0.038**	0.063	0.800

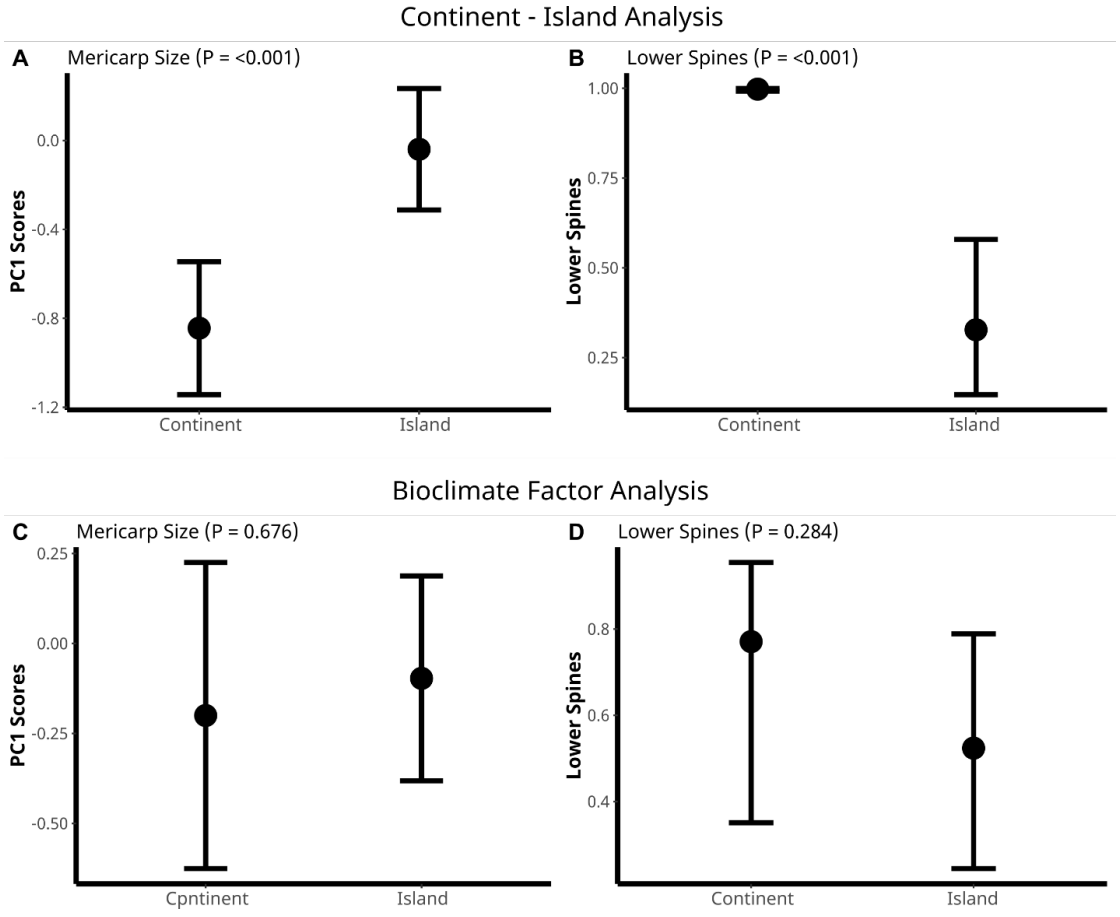


Figure S2. 9 Mericarp traits compared between island and continental locations with African Island (n=8) samples removed. Plots show the least-squares mean estimates (± 1 SE) using PC1 as a summary of mericarp size (length, width, depth, and spine size) and the presence or absence of lower spines. P-values correspond to the difference between island and continental plants. (A-B) Estimates of continental and island populations only. (C-D) Estimates of the island effect from the model after accounting for bioclimatic variation.

Table S2. 6 Model estimates of the effect of population and year of collection on individual mericarp traits including bioclimate variables with African Islands removed. African Islands were samples from a single herbarium voucher and represents only one individual per location. The table shows the model estimates per trait and the PC1 estimates. Nomenclature on bioclimate variables was taken from the WorldClim dataset (https://worldclim.org/). We used variables Bio1 (Annual Mean Temperature), Bio4 (Temperature Seasonality), Bio12 (Annual precipitation), Bio15 (Precipitation Seasonality).

A) Mericarp – *continental vs island* - African Islands removed														
	Trait	**Continental/Island**		**Year**		**Herbarium**		**Bio1**		**Bio4**		**Bio12**		**Bio15**	
		X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**
	Length	0.463	0.495	**9.824**	**0.001**	0.014	0.905	0.068	0.068	**5.699**	**0.016**	**4.096**	**0.042**	2.150	0.142
	Width	0.647	0.420	0.755	0.384	0.652	0.419	0.028	0.866	3.726	0.053	2.903	0.088	0.459	0.498
	Depth	2.645	0.103	**8.212**	**0.004**	0.068	0.794	0.031	0.860	2.494	0.114	**4.445**	**0.034**	**4.276**	**0.038**
	Spine tip distance	0.010	0.918	0.272	0.601	0.012	0.910	0.456	0.499	**4.089**	**0.043**	**6.971**	**0.008**	0.039	0.843
	Lower spines	1.147	0.284	1.477	0.224	0.384	0.535	**5.835**	**0.015**	**7.912**	**0.004**	**20.423**	**<0.001**	**15.961**	**<0.001**
	Mericarp Size (PC1)	0.173	0.676	**4.242**	**0.039**	0.184	0.667	0.0004	0.984	**7.119**	**0.007**	**8.852**	**0.002**	1.345	0.246

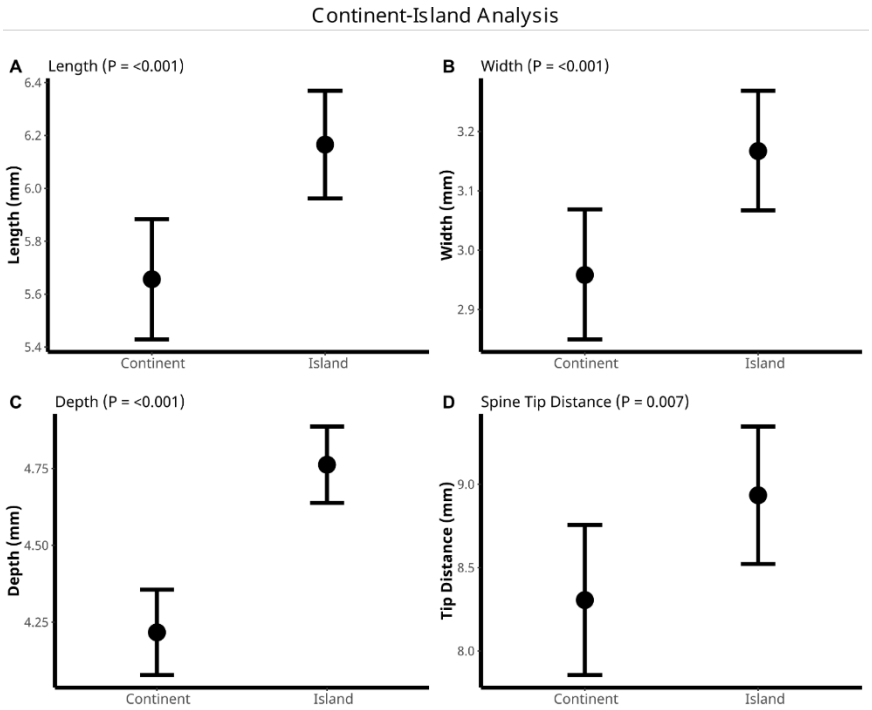


Figure S2. 10 Individual mericarp traits compared between island and continental locations with African Islands (n =8) samples removed. Plots are the least-squares means ± one standard error. On top of each plot, it shows the p-values from the ANOVA. A-D) Mericarp trait plots without bioclimatic variables included.

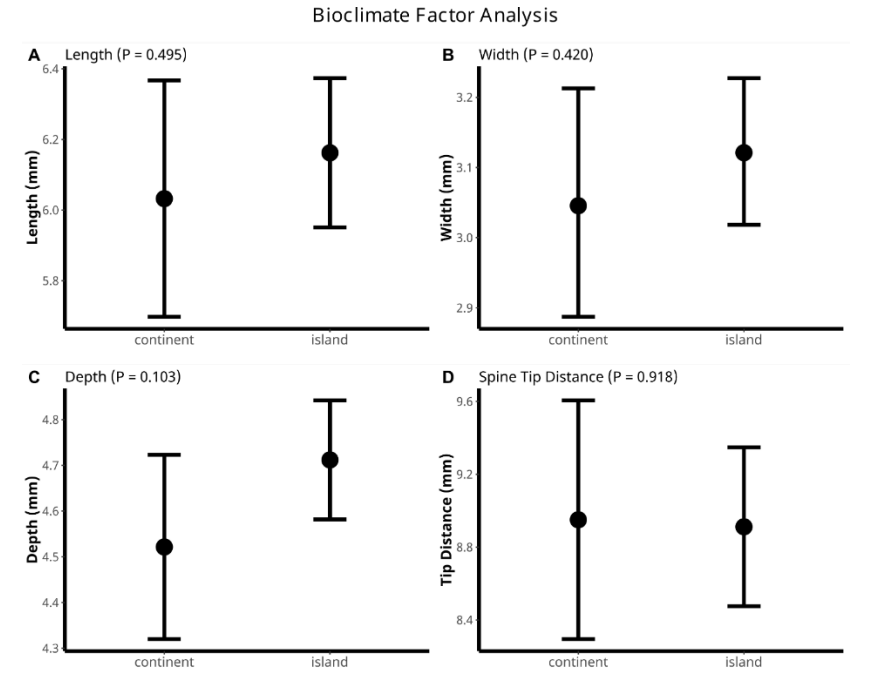


Figure S2. 11 Individual mericarp traits compared between island and continental locations with African Islands (n =8) samples removed. Plots are the least-squares means ± one standard error. On top of each plot, it shows the p-values from the ANOVA. A-D) Mericarp trait plots include bioclimate variables.

Appendix 2.6

Table S2. 7 Model estimates of the effect of population and year of collection on individual mericarp and flower traits from Other Islands (removing Galápagos samples) and Continental populations. A) Model estimates per mericarp traits and the PC1 estimates. B) Model estimates for petal length.

A) Mericarp – *continental vs island - Galápagos removed*							
	Trait	**Continental/Island**		**Year**		**Herbarium**	
		χ^2	**P**	χ^2	**P**	χ^2	**P**
	Length	0.072	0.787	**31.511**	**<0.001**	**10.341**	**0.001**
	Width	2.795	0.094	0.991	0.319	0.018	0.893
	Depth	**5.843**	**0.015**	**5.662**	**0.017**	0.033	0.855
	Spine tip distance	**4.591**	**0.032**	0.039	0.842	0.002	0.957
	Lower spines	1.342	0.246	-	-	**5.364**	**0.020**
	Mericarp Size (PC1)	3.252	0.071	**6.054**	**0.013**	0.526	0.467
B) Flowers – *continental vs island - Galápagos removed*							
	Petal length	2.440	0.118	**9.040**	**0.002**	-	-

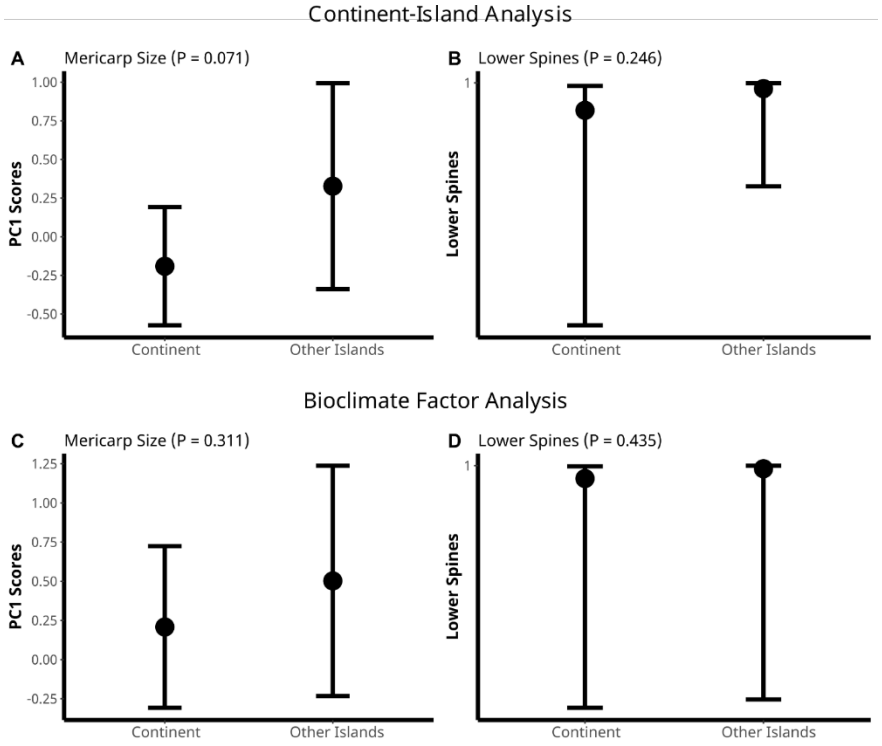


Figure S2. 12 Mericarp traits compared between the Other Islands and continental populations. Plots show the least-squares mean estimates (± 1 SE) using PC1 as a summary of mericarp size (length, width, depth, and spine size) and the presence or absence of lower spines. P-values correspond to the difference between island and continental plants. (A-B) Estimates of continental and island populations only. (C-D) Estimates of the island effect from the model after accounting for bioclimatic variation.

Table S2. 8 Model estimates of the effect of population and year of collection on individual mericarp and flower traits including bioclimate variables from Other Islands (removing Galápagos samples) and Continental populations. A) Model estimates per mericarp traits and the PC1 estimates. B) Model estimates for petal length. Nomenclature on bioclimate variables was taken from the WorldClim dataset (https://worldclim.org/). We used variables Bio1 (Annual Mean Temperature), Bio4 (Temperature Seasonality), Bio12 (Annual precipitation), Bio15 (Precipitation Seasonality).

A) Mericarp – *continental vs island - Galápagos removed*														
Trait	**Continental/Island**		**Year**		**Herbarium**		**Bio1**		**Bio4**		**Bio12**		**Bio15**	
	X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**
Length	0.016	0.898	**17.491**	**<0.001**	0.871	0.350	0.059	0.807	3.453	0.063	0.337	0.561	0.100	0.751
Width	1.063	0.302	0.010	0.918	1.462	1.462	0.046	0.829	**4.199**	**0.040**	**4.316**	**0.037**	0.072	0.787
Depth	1.713	0.190	1.446	0.229	0.313	0.575	0.083	0.772	**3.891**	**0.048**	3.027	0.081	**6.717**	**0.009**
Spine tip distance	2.993	0.083	1.429	0.231	2.501	0.113	0.008	0.925	**4.767**	**0.029**	**18.585**	**<0.001**	**8.598**	**0.003**
Lower spines	0.608	0.435	0	0.999	1.033	0.309	1.115	0.290	0.027	0.868	1.991	0.158	**6.906**	**0.008**
Mericarp Size (PC1)	1.022	0.311	0.932	0.334	0.758	0.383	0.232	0.629	**6.009**	**0.014**	**9.032**	**0.002**	0.128	0.72
B) Flowers – *continental vs island - Galápagos removed*														
Trait	**Continental/Island**		**Year**		**Herbarium**		**Bio1**		**Bio4**		**Bio12**		**Bio15**	
	X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**	X^2	**P**
Petal length	0.004	0.947	**7.880**	**0.004**	-	-	**4.781**	**0.028**	**8.149**	**0.004**	1.353	0.244	**7.630**	**0.005**

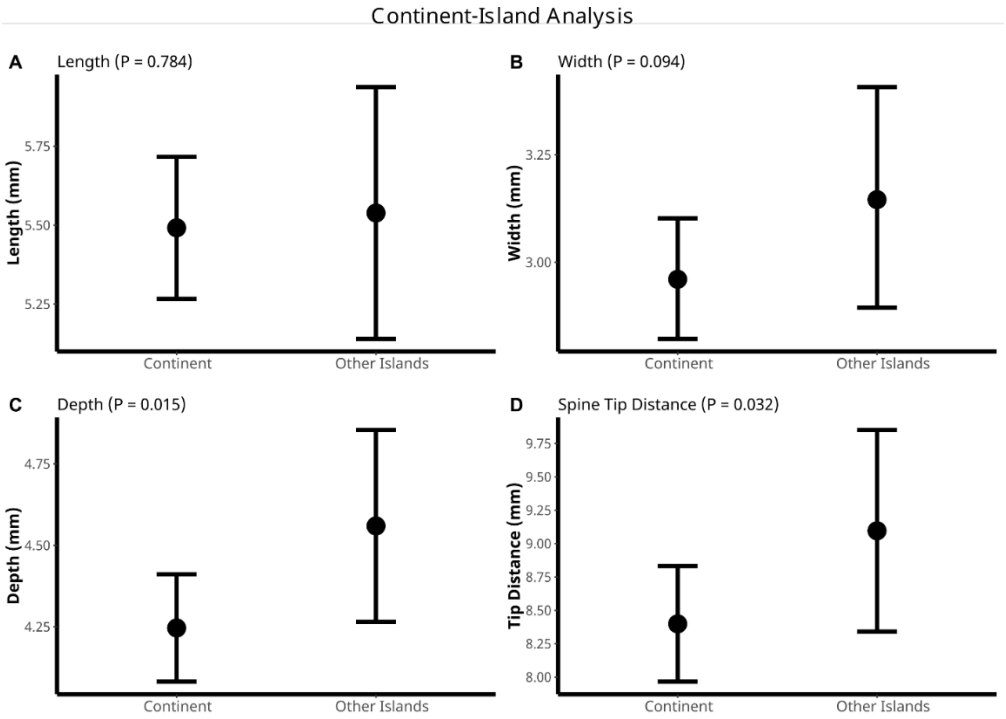


Figure S2. 13 Individual mericarp traits compared between the Other Islands and continental populations. Plots are the least-squares means ± one standard error. On top of each plot, it shows the p-values from the ANOVA. A-D) Mericarp trait plots without bioclimate variables.

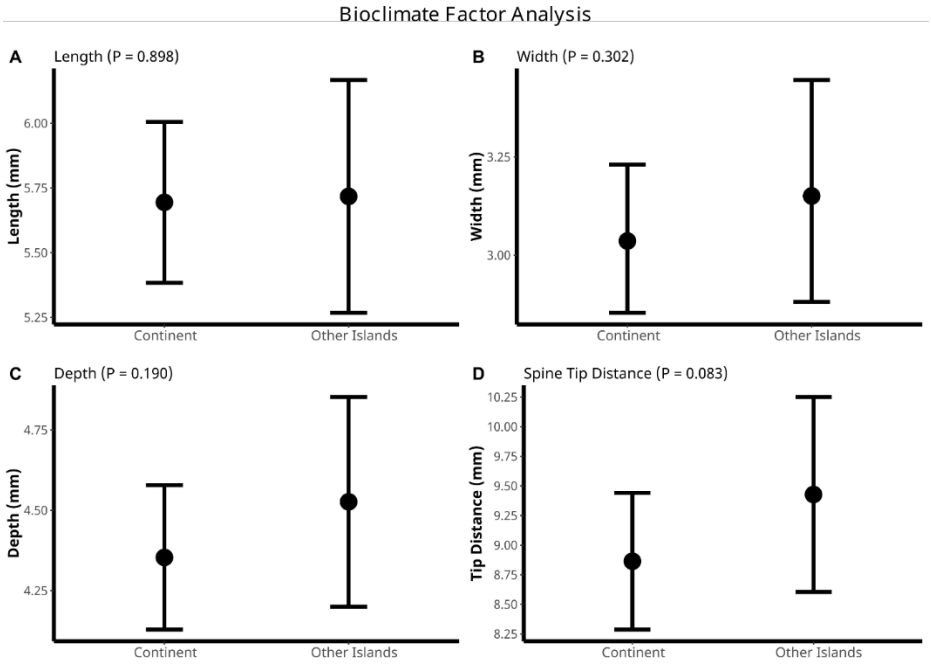


Figure S2. 14 Individual mericarp traits compared between the Other Islands and continental populations. Plots are the least-squares means ± one standard error. On top of each plot, it shows the p-values from the ANOVA. A-D) Mericarp trait plots include bioclimate variables.

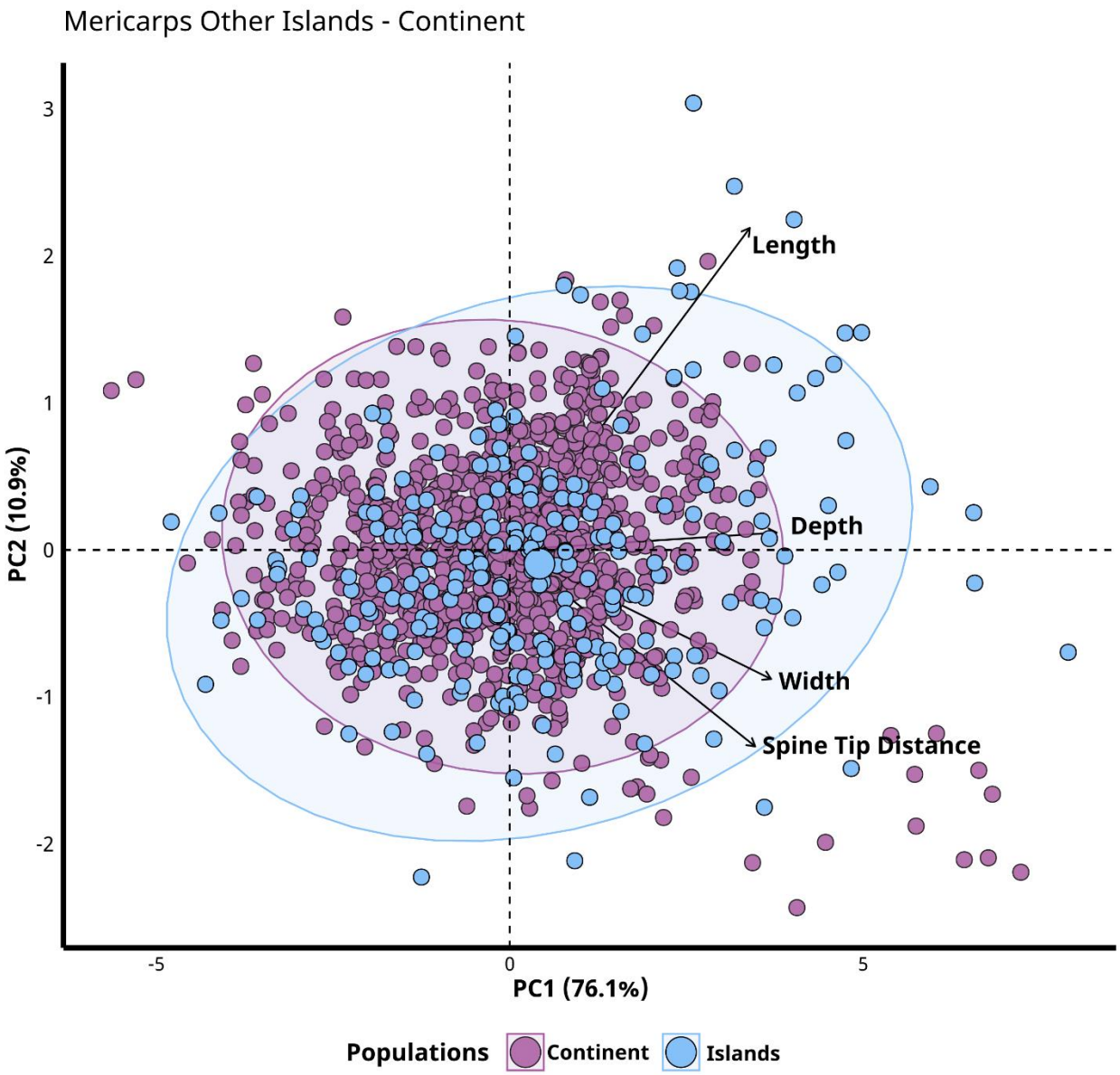


Figure S2. 15 Principal component analysis of mean mericarp traits, length, depth, width, and spine size. Points represent individual mericarps from other islands and continental populations. Trait vectors are proportional to the contribution and direction associated with each trait. Larger circles represent the centroid of the ellipses with a 95% confidence interval.

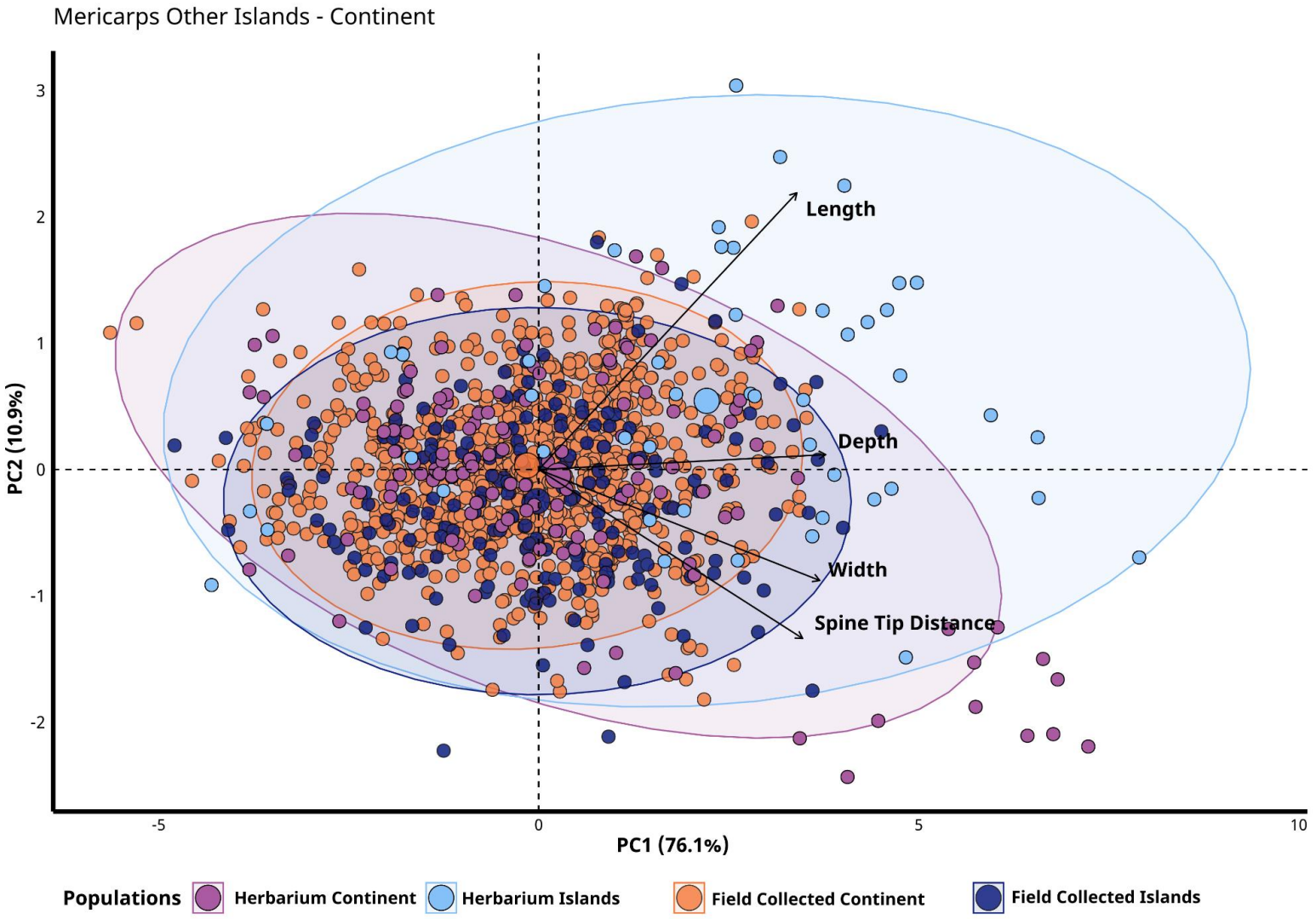


Figure S2. 16 Principal component analysis of mean mericarp traits, length, depth, width, and spine size. Points represent individual mericarps divided into groups from herbarium and field collected samples from other islands and continent populations. Trait vectors are proportional to the contribution and direction associated with each trait. Larger circles represent the centroid of the ellipses with a 95% confidence interval.

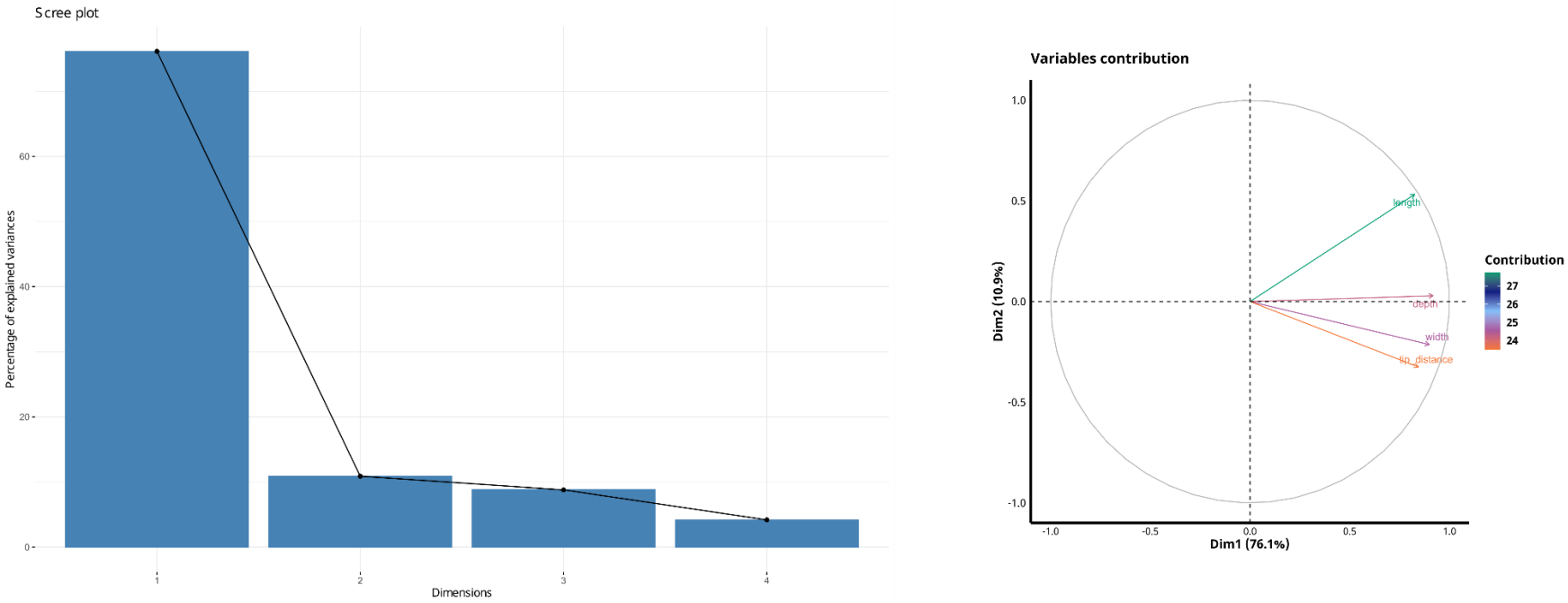


Figure S2. 17 Eigenvector contribution plot and percentage of explained variation for each PC axis associated with mericarp morphology for other islands and continental populations.

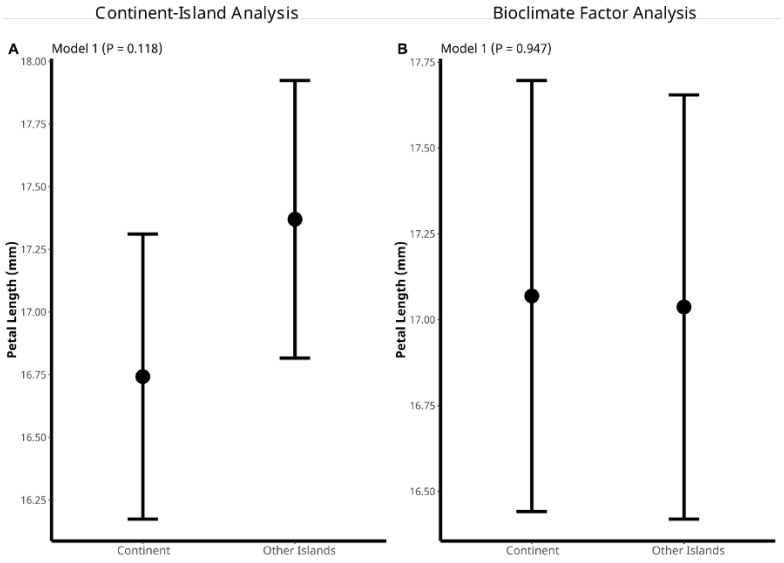


Figure S2. 18 Petal length estimates from other islands and continental plants. The plots show the least-squares mean estimates (± 1 SE) using petal length. P-values are shown on top of each plot. (A) Estimates of continental and island populations only. (B) Estimates including bioclimate variables.

Appendix 2.7

Table S2. 9 Model estimates of the effect of population and year of collection on individual mericarp and flower traits from Galápagos and Continental populations only. A) Model estimates per mericarp traits and the PC1 estimates. B) Model estimates for petal length.

A) Mericarp – *Galápagos - Continent*							
	Trait	**Continental/Island**		**Year**		**Herbarium**	
		X^2	**P**	**X^2**	**P**	**X^2**	**P**
	Length	**15.495**	**<0.001**	0.021	0.883	0.002	0.957
	Width	**12.327**	**<0.001**	0.463	0.496	0.039	0.843
	Depth	**59.523**	**<0.001**	3.361	0.066	0.163	0.685
	Spine tip distance	**5.083**	**0.024**	0.251	0.615	0.948	0.33
	Lower spines	**78.411**	**<0.001**	-	-	3.603	0.057
	Mericarp Size (PC1)	**26.227**	**<0.001**	1.490	0.222	0.059	0.807
B) Flowers – *Galápagos vs Continent*							
		Continental/Island		**Year**		**Herbarium**	
		X^2	**P**	**X^2**	**P**	**X^2**	**P**
	Petal length	**97.100**	**<0.001**	**5.842**	**0.015**	-	-

Table S2. 10 Model estimates of the effect of population and year of collection on individual mericarp and flower traits including bioclimate variables from Galápagos and Continental only. A) Model estimates per mericarp traits and the PC1 estimates. B) Model estimates for petal length. Nomenclature on bioclimate variables was taken from the WorldClim dataset (https://worldclim.org/). We used variables Bio1 (Annual Mean Temperature), Bio4 (Temperature Seasonality), Bio12 (Annual precipitation), Bio15 (Precipitation Seasonality).

A) Mericarp – *Galápagos - Continent*														
	Trait	**Continental/Island**		**Year**		**Herbarium**		**Bio1**		**Bio4**		**Bio12**		**Bio15**	
		χ^2	**P**	χ^2	**P**	χ^2	**P**	χ^2	**P**	χ^2	**P**	χ^2	**P**	χ^2	**P**
	Length	2.203	0.137	0.046	0.829	0.249	0.617	0	0.995	2.854	0.091	0.004	0.949	**5.276**	**0.021**
	Width	3.090	0.078	0.467	0.494	0.004	0.945	0.001	0.967	0.07	0.790	0.640	0.423	0.423	0.504
	Depth	**10.159**	**0.001**	3.293	0.069	0.716	0.397	0.191	0.661	0.788	0.374	2.575	0.108	**5.974**	**0.014**
	Spine tip distance	0.066	0.796	0.249	0.617	1.064	0.302	0.904	0.341	0.067	0.795	1.212	0.270	0.010	0.919
	Lower spines	**17.470**	**<0.001**	0.158	0.691	0.225	0.635	2.039	0.153	**0.153**	**0.014**	0.573	0.449	**16.087**	**<0.001**
	Mericarp Size (PC1)	1.807	0.178	1.507	0.219	0.091	0.762	0.199	0.655	0.025	0.872	0.005	0.941	2.775	0.095
B) Flowers – *Galápagos vs Continent*															
	Trait	**Continental/Island**		**Year**		**Herbarium**		**Bio1**		**Bio4**		**Bio12**		**Bio15**	
		χ^2	**P**	χ^2	**P**	χ^2	**P**	χ^2	**P**	χ^2	**P**	χ^2	**P**	χ^2	**P**
	Petal length	**64.747**	**<0.001**	**5.489**	**0.019**	-	-	**11.319**	**<0.001**	**6.614**	**0.010**	0.434	0.509	**11.044**	**<0.001**

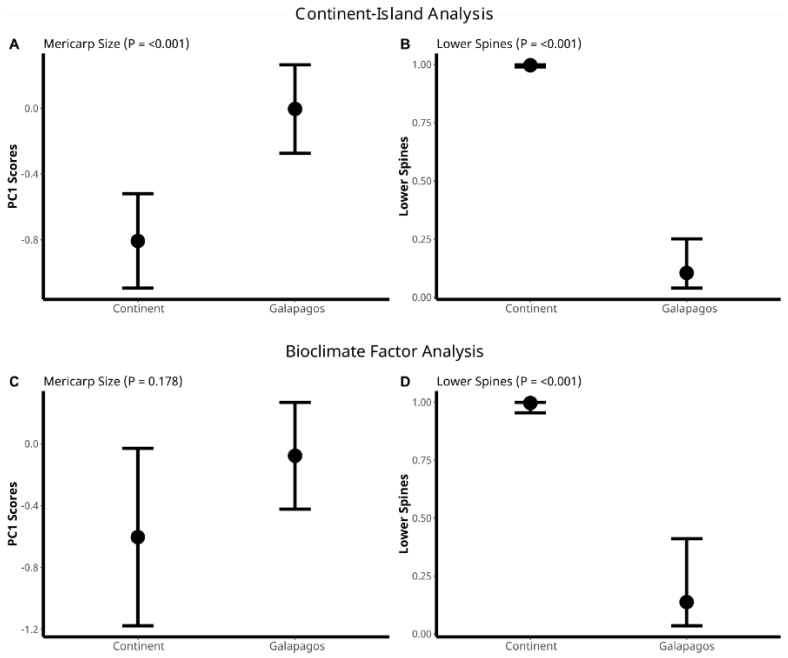


Figure S2. 19 Mericarp traits compared between the Galápagos Islands and continental populations. Plots show the least-squares mean estimates (± 1 SE) using PC1 as a summary of mericarp size (length, width, depth, and spine size) and the presence or absence of lower spines. P-values correspond to the difference between island and continental plants. (A-B) Estimates of continental and island populations only. (C-D) Estimates of the island effect from the model after accounting for bioclimatic variation.

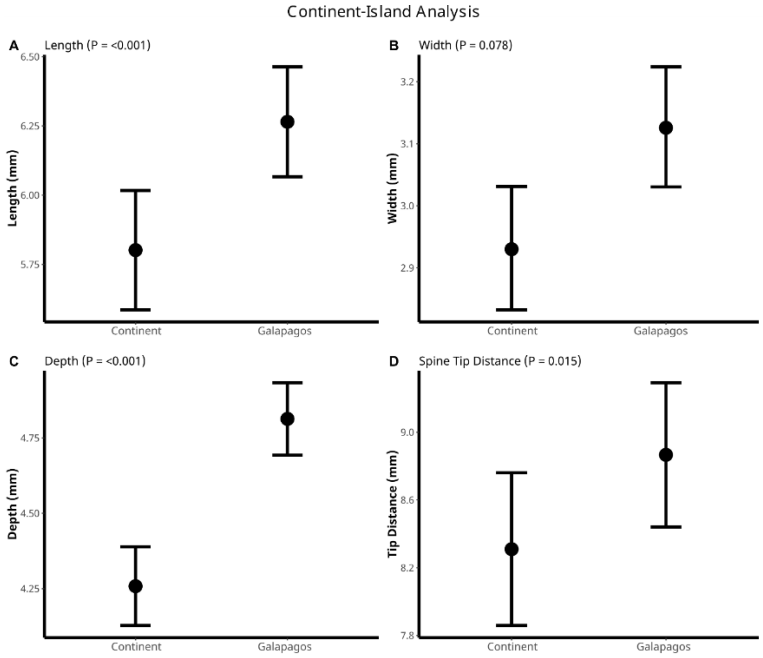


Figure S2. 20 Individual mericarp traits compared between the Galápagos Islands and continental populations. Plots are the least-squares means ± one standard error. On top of each plot, it shows the p-values from the ANOVA. A-D) Mericarp trait plots without bioclimate variables.

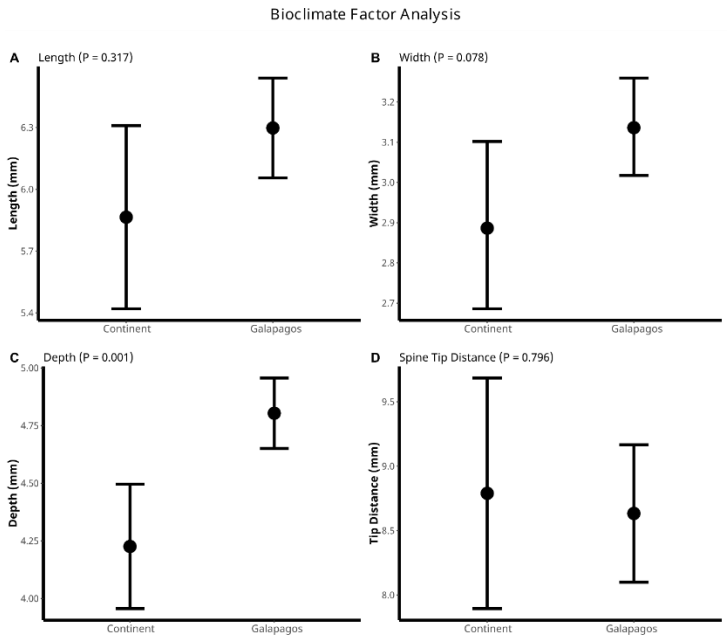


Figure S2. 21 Individual mericarp traits compared between the Galápagos Islands and continental populations. Plots are the least-squares means ± one standard error. On top of each plot, it shows the p-values from the ANOVA. A-D) Mericarp trait plots include bioclimate variables.

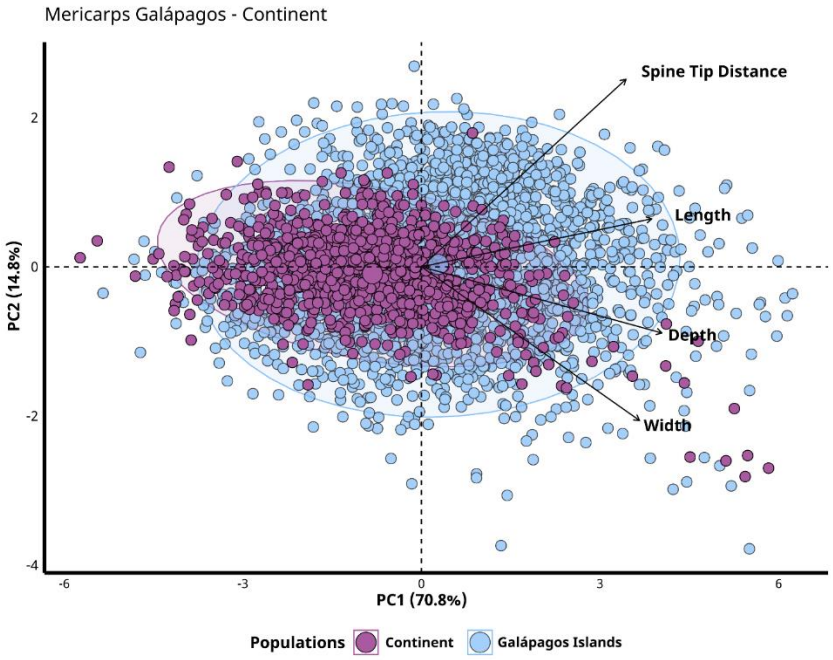


Figure S2. 22 Principal component analysis of mean mericarp traits, length, depth, width, and spine size. Points represent individual mericarps from Galápagos and continental populations. Trait vectors are proportional to the contribution and direction associated with each trait. Larger circles represent the centroid of the ellipses with a 95% confidence interval.

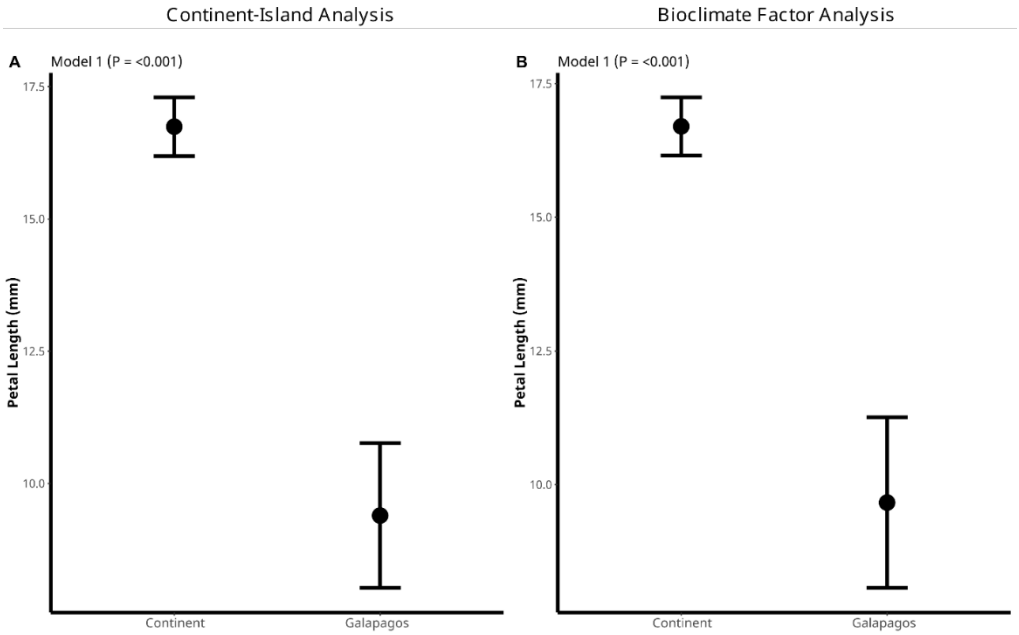
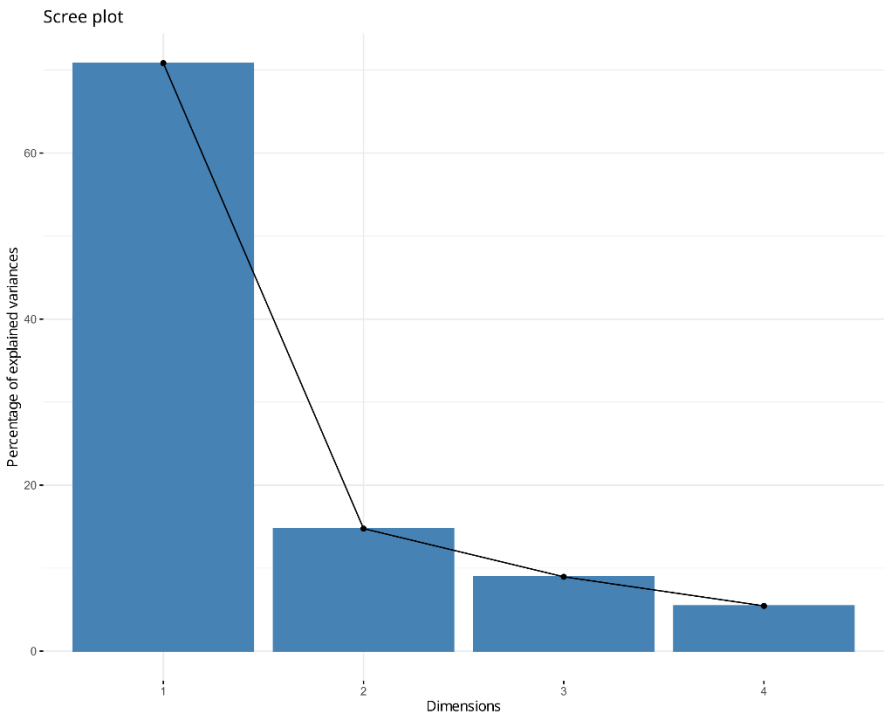


Figure S2. 23 Petal length estimates from the Galápagos Islands and continental plants. The plots show the least-squares mean estimates (± 1 SE) using petal length. P-values are shown on top of each plot. (A) Estimates of continental and island populations only. (B) Estimates including bioclimate variables.



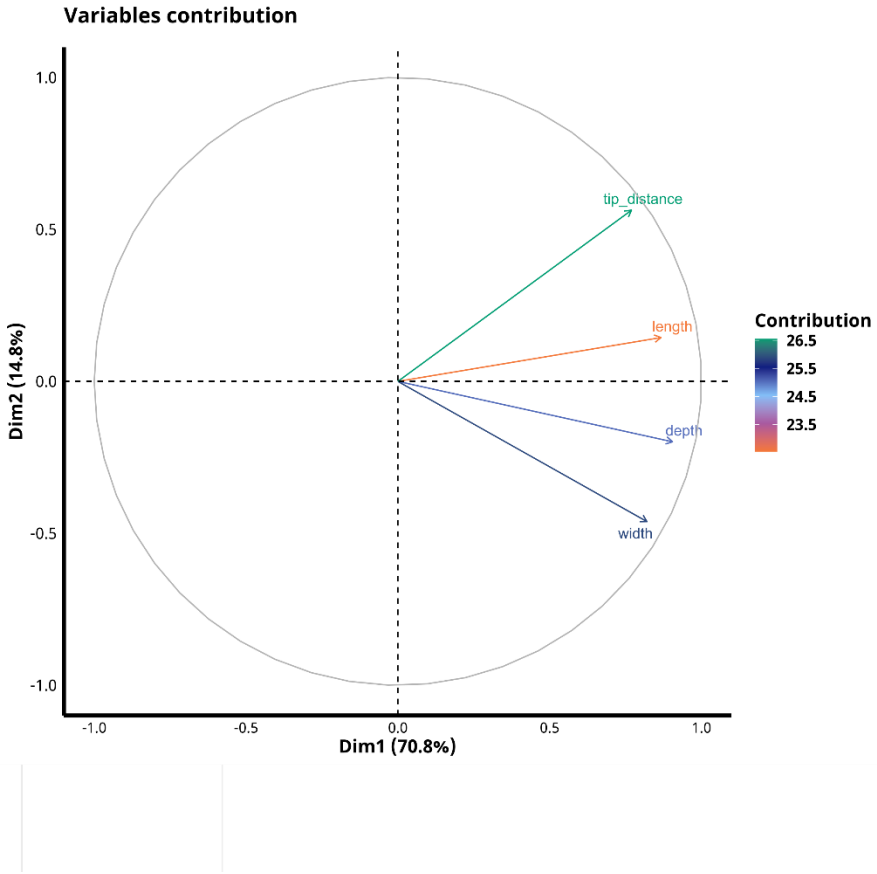


Figure S2. 24 Eigenvector contribution plot and percentage of explained variation for each PC axis associated with mericarp morphology for Galápagos and continental populations.

Appendix 2.8

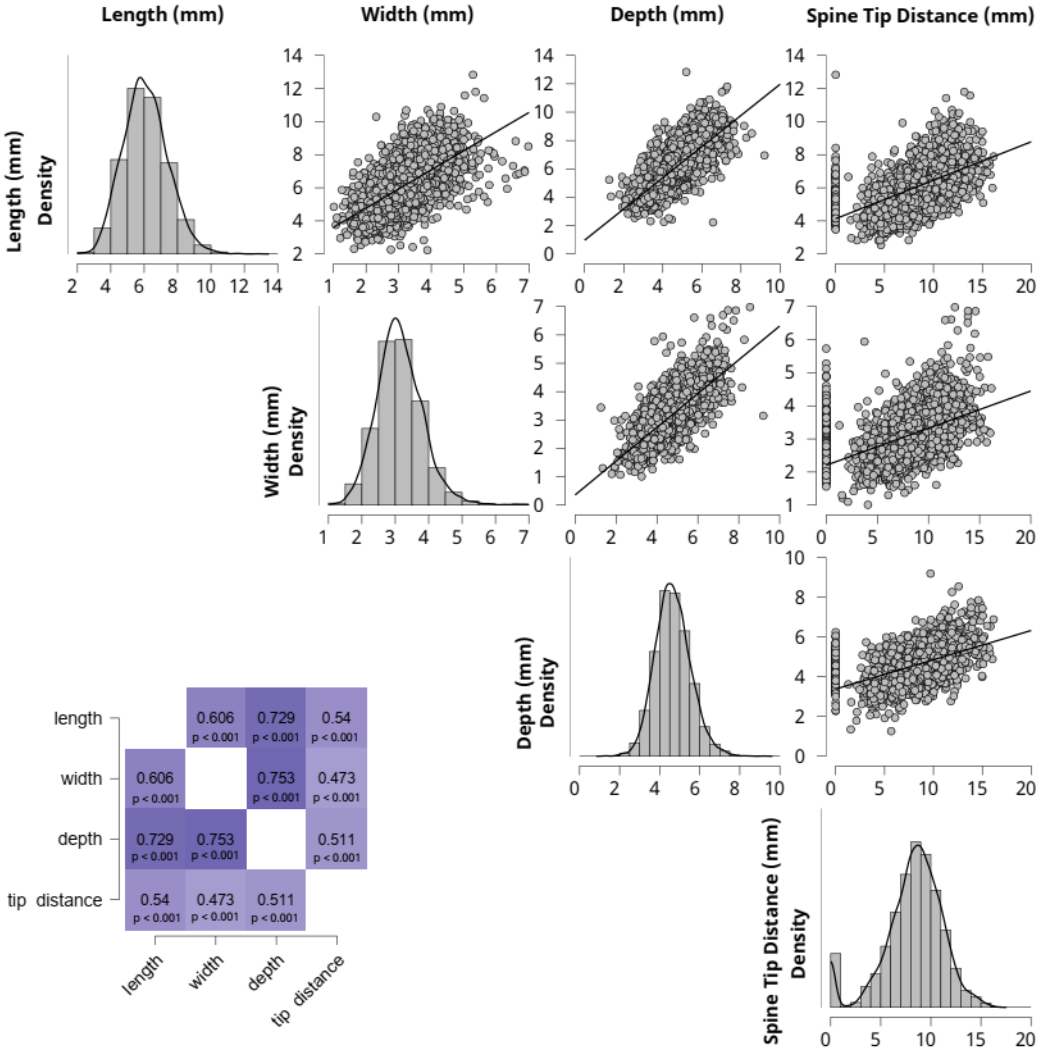


Figure S2. 25 Distributions of mericarp traits and the correlations between traits. Upper right: The frequency distribution of each trait and the bivariate scatterplot between traits with the line of best fit show. Lower left: Symmetrical matrix of Pearson correlation coefficients between traits with heat darkness of colour corresponding to the strength of the correlation. All R-values are significant at P<0.001.

Appendix 2.9

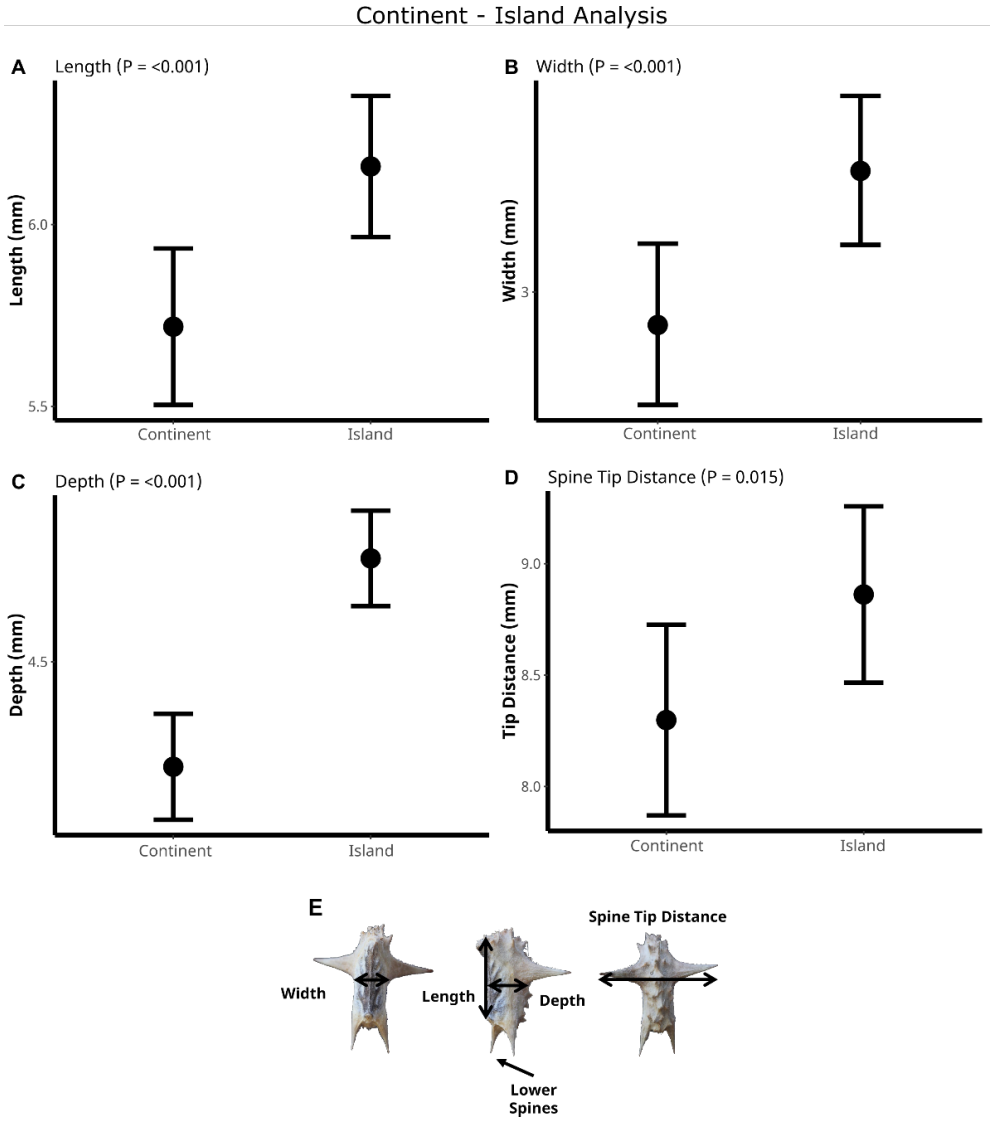


Figure S2. 26 Mericarp traits compared between island and continental locations. Plots are the least-squares means ± one standard error. On top of each plot, it shows the p-values from the ANOVA. A-D) Individual mericarp trait plots. E) Diagram of how these traits were measured as described in Figure 3.

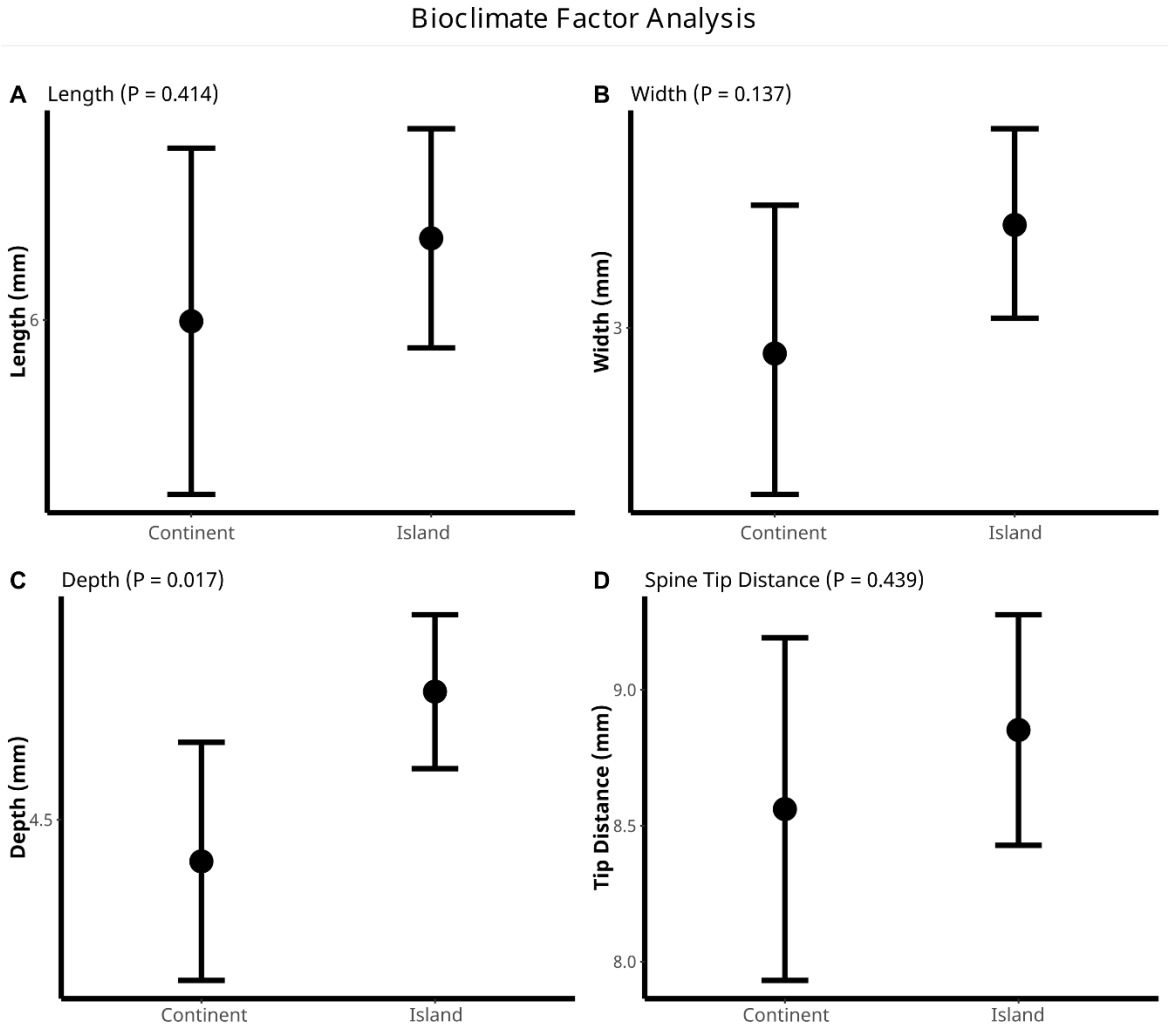


Figure S2. 27 Mericarp traits compared between island and continental locations. Plots are the least-squares means ± one standard error. On top of each plot, it shows the p-values from the ANOVA. A-D) Individual mericarp trait plots including bioclimate variables.

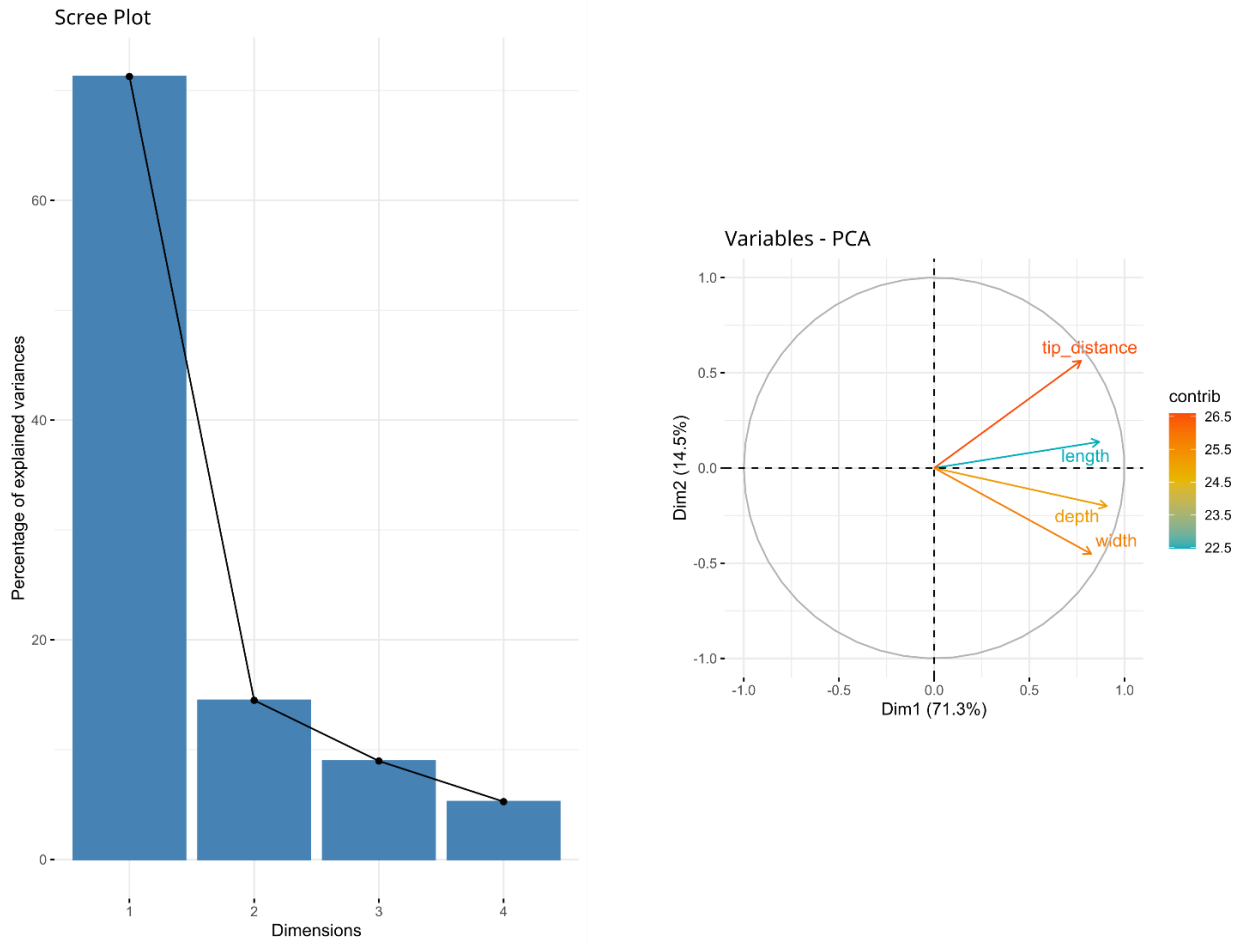


Figure S2. 28 Eigenvector contribution plot and percentage of explained variation for each PC axis associated with mericarp morphology.

Appendix 2.10

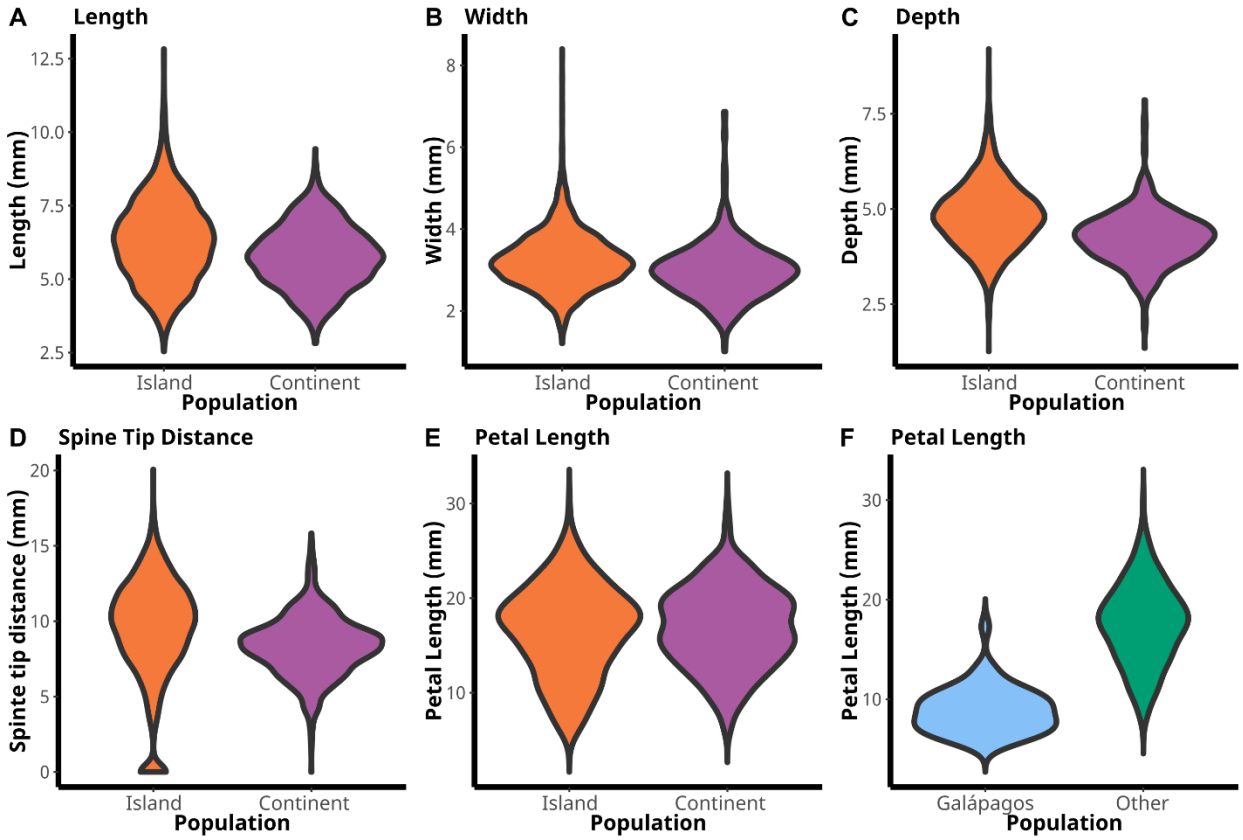


Figure S2. 29 Trait distributions within measured traits groups shown as violin plots. Continental and island populations for mericarps and flowers (A-E) and for flower size between Galápagos and other islands (F).

Chapter 3 Appendix

Appendix 3.1

Data summary of Tribulus experiments

Point in time populations.

Table S3. 1 *Tribulus cistoides* populations collected for the point-in-time dataset. The table shows the number of mericarps collected per year for each population.

Island	Population	Populations per island	Latitude	Longitude	Elevation(m)	2015	2016	2017	2018	2019	Site notes
Baltra	CH	2	-0.48328	-90.2772		-	100	-	-	-	Chanel bus stop
	AP2		-0.44381	-90.2733	53	-	100	-	-	-	On the road to the airport. Close to the airport and in front of the airport entrance
Española	BG	2	-1.35404	-89.6601		-	100	-	-	-	Bahía Gardner
	PS		-1.37093	-89.7447		-	200	-	-	-	Punta Suárez. On the tourists' trail
Floreana	PN	8	-1.27689	-90.4881	9	-	101	100	50	100	Close to the beach PN
	CC		-1.27733	-90.4813	42	-	100	-	50	-	Close to cemetery (heliport)
	CD		-1.27927	-90.4732	93	100	100	100	50	100	app. 1km north of cemetery (close to dump)
	CM		-1.28085	-90.4694	161	100	100	100	50	100	app. 1.5km north of cemetery (close to mine)
	WC		-1.28238	-90.4803	86	100	-	100	50	100	app. 1km west of cemetery
	LB		-1.28675	-90.4903		100	-	-	-	-	La lobería app. 1km east of town
	POB		-1.23804	-90.4475		-	-	100	-	-	Post Office Bay
	WC2		-1.27988	-90.48		-	-	-	100	100	app. 600m north of cemetery (close to dump)
Isabela	TP	6	-0.9478	-90.9744		100	100	100	100	100	Outside of tortoise breeding center "Centro de crianza de tortugas Arnaldo Tupiza" (Tortuga pens)
	ECR		-0.93697	-90.9782	17	100	91	100	100	100	East to crossroad
	RSA		-0.94147	-90.9674	26	100	100	100	100	100	On the road to Volcán Sierra Negra (Roadside South of the Airport)
	AP		-0.94503	-90.9548		100	100	101	100	100	Airport
	RTP		-0.95237	-90.9728		100	-	100	100	100	On the road to tortoise breeding center

Island	Population	Populations per island	Latitude	Longitude	Elevation(m)	2015	2016	2017	2018	2019	Site notes
											"Arnaldo Tupiza"
	RVS		-0.93007	-90.9851		100	-	100	107	100	On the road to Volcán Sierra Negra app 4 km NW from the airport
San Cristóbal	USFQ	5	-0.8959	-89.6089		100	100	100	100	100	Close to USFQ GAIA
	LB2		-0.92228	-89.6153	5	100	100	100	50	71	La Lobería (entrance)
	RLB		-0.91453	-89.6151	42	100	-	-	-	-	On the road towards La Lobería (close to mine entrance)
	ORO		-0.89987	-89.6093		-	-	-	50	100	Playa Oro
	PK		-0.90295	-89.6095	18	-	-	-	100	100	Park in center of town
Saymour Norte	TT	1	-0.40064	-90.2912	11	-	100	-	-	-	On tourists' trail
Sta. Cruz	EG	8	-0.68631	-90.2231	35	100	100	100	100	100	Close to El Garrapatero beach (old parking lot)
	EG2		-0.67742	-90.2268	58	100	100	100	100	100	On the road to El Garrapatero (~500 m before to arrive to the old parking lot)
	AB		-0.7382	-90.3017		100	100	100	100	100	Charles Darwin Research Station (close to the cliff)
	TB		-0.74614	-90.3191	22	100	100	100	96	100	Entrance to Tortuga Bay
	ITC		-0.48803	-90.2801	13	100	100	-	100	100	Close to Itabaca channel
	ITC2		-0.54211	-90.3192	100	100	100	100	100	100	6.5 km from Itabaca
	MGN		-0.57375	-90.3335	267	100	100	100	100	97	Close to mina de granillo negro
	DP		-0.58483	-90.3544	328	100	100	100	100	100	Close to Dump

Point in time germinated mericarps.

Table S3. 2 Number of *Tribulus cistoides* germinated mericarps from the point-in-time dataset.

Island	Population Name	Populations per island	Latitude	Longitude	Elevation(m)	2017	2018	2019	Site notes
Floreana	PN	4	-1.27689	-90.4881	9	-	2	4	Close to the beach PN
	CD		-1.27927	-90.4732	93	-	-	10	app. 1km north of cemetery (close to dump)
	WC		-1.28238	-90.4803	86	-	1	4	app. 1km west of cemetery
	WC2		-1.27988	-90.48	0	-	2	-	app. 600m north of cemetery (close to dump)
Isabela	TP	6	-0.9478	-90.9744	0	34	3	10	Outside of tortoise breeding center "Centro de crianza de tortugas Arnaldo Tupiza" (Tortuga pens)
	ECR		-0.93697	-90.9782	17	33	3	12	East to crossroad
	RSA		-0.94147	-90.96746	26	32	3	3	On the road to Volcán Sierra Negra (Roadside South of the Airport)
	AP		-0.94503	-90.9548	0	-	1	5	Airport
	RTP		-0.95237	-90.9728	0	24	5	1	On the road to tortoise breeding center "Centro de crianza de tortugas Arnaldo Tupiza"
	RVS		-0.93007	-90.9851	0	28	-	-	On the road to Volcán Sierra Negra app 4 km NW from the airport
San Cristóbal	USFQ	4	-0.8959	-89.6089	0	-	2	1	Close to USFQ GAIA
	LB2		-0.92228	-89.6153	5	-	4	2	La Lobería (entrance)
	ORO		-0.89987	-89.6093	0	-	-	100	Playa Oro
	PK		-0.90295	-89.6095	18	-	3	4	Park in center of town
Sta. Cruz	EG	7	-0.68631	-90.2231	35	28	-	-	Close to El Garrapatero beach (old parking lot)
	EG2		-0.67742	-90.2268	58	21	-	-	On the road to El Garrapatero (~500 m before to arrive to the old parking lot)
	AB		-0.7382	-90.3017	0	10	-	-	Charles Darwin Research Station (close to the cliff)
	TB		-0.74614	-90.3191	22	24	1	2	Entrance to Tortuga Bay
	ITC2		-0.54211	-90.3192	100	22	-	-	6.5 km from Itabaca
	MGN		-0.57375	-90.3335	267	10	-	-	Close to mina de granillo negro
	DP		-0.58483	-90.3544	328	22	-	-	Close to Dump

Mark-recapture experiment

Table S3. 3 *Tribulus cistoides* populations collected for the mark-recapture dataset. The table shows the number of mericarps collected per group.

Year	Island	Source	Groups								Mericarps per plate		Total plates	Times sampled
			Large				Small			Total				
			All spines	*Upper spines*	*Lower spines*	*No spines*	*All spines*	*Upper spines*	*Lower spines*	*No spines*				
2018	Santa Cruz	Garrapatero	50(1)	50	50	50	50	50(1)	50	50	400	16 (2 per group)	25	3
	Isabela	Airport	50	50	50	50	50	50	50	50	400	16 (2 per group)	25	3
	Floreana*	Dump	100			100	100			100	400	16 (4 per group)	25	3
2019*	Santa Cruz	Garrapatero	100			100	100			100	400	16 (4 per group)	25	1
	Isabela	Airport	75			75	125			125	400	16 (10 small, 6 large)	25	1
	Floreana	Dump	100			100	100			100	400	16 (4 per group)	25	1

*Floreana's mericarps do not have lower spines. On 2019 I decided to keep the groups the same and did only two groups with and without spines.

*For 2019 we changed the treatment groups to only two groups

Mark-recapture germinated mericarps

Table S3. 4 Number of *Tribulus cistoides* germinated mericarps from the mark-recapture experiment.

Year	Island	Source	Groups								
			Large				Small				Total
			All spines	*Upper spines*	*Lower spines*	*No spines*	*All spines*	*Upper spines*	*Lower spines*	*No spines*	
2018	Santa Cruz	Garrapatero	1	-	-	-	-	1	-	-	2
	Isabela	Airport	1	1	4	-	-	1	-	-	7
	Floreana*	Dump	4			2	2			-	8
2019*	Santa Cruz	Garrapatero	1			3	4			5	13
	Isabela	Airport	3			1	1			1	6
	Floreana	Dump	-			-	-			1	1

Appendix 3.2

Mark-recapture experimental setup.

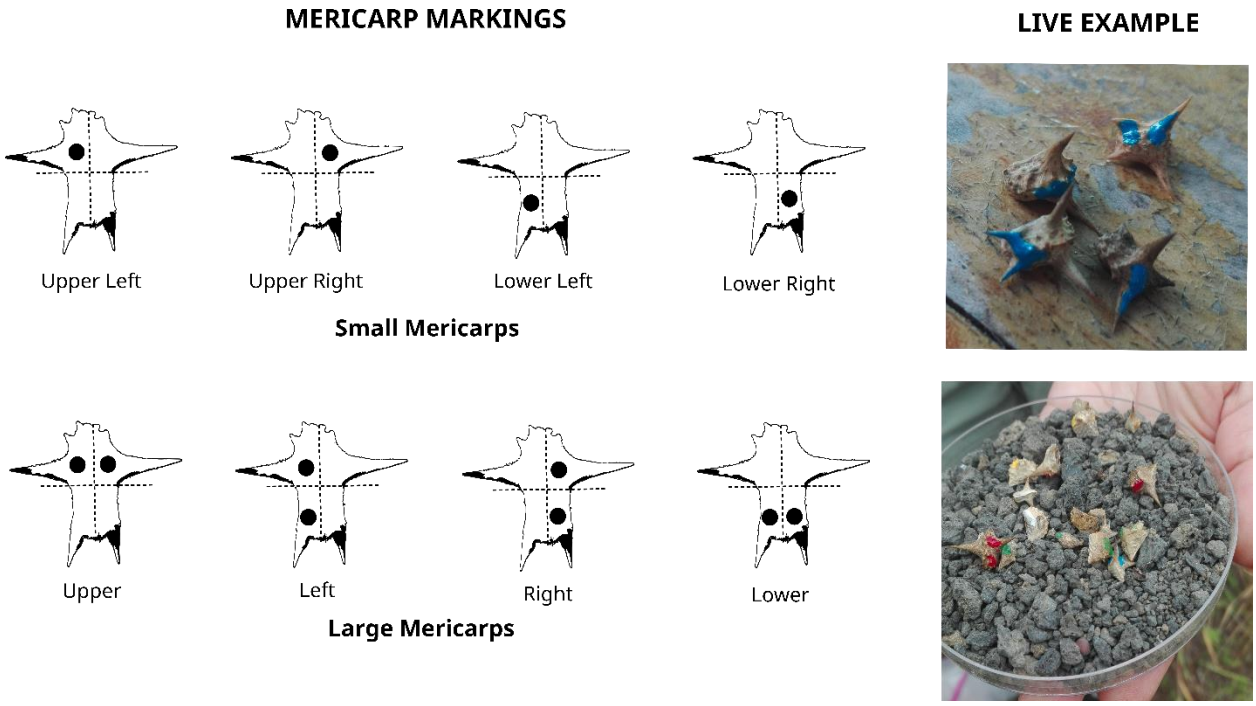


Figure S3. 1 Diagram of mericarp marks for the mark-recapture treatments. Marks were located in the dorsal end of the mericarp and labelled using nail polish. Vertebrate predators handle the mericarps by their ventral end where seeds are located, usually spines protect the dorsal end of the mericarp. Small and Large mericarps were marked following a quadrant. We used only one mark for small mericarps and two marks for large mericarps. On the right, the pictures shows live examples of marked mericarps showing the respecitve upper, left, right and lower marks. Below, there is a close-up of a plate, showing marked mericarps for the different size and spine treatments differenciated by their respective colours.

Figure S3. 2 An example of a mark-recapture plate located close to a marked tree in El Garrapatero site, Santa Cruz Island. The plate contained the same substrate as the area, with mericarps for each treatment group.

Color distribution

Table S3. 5 Colour distributions among the mark-recapture treatments.

2018			
Santa Cruz*			
Plates 1 – 12			
Small		**Large**	
All spines (Green)	Lower spines (Red)	All spines (Green)	Lower spines (Red)
Upper spines (Red)	No spines (Green)	Upper spines (Green)	No spines (Green)
Plates 14 - 25			
Small		**Large**	
All spines (Red)	Lower spines (Green)	All spines (Red)	Lower spines (Green)
Upper spines (Green)	No spines (Red)	Upper spines (Red)	No spines (Red)
*Santa Cruz used a two-color system.			
Isabela*			
Small		**Large**	
All spines (Green)	Lower spines (Blue)	All spines (Green)	Lower spines (Blue)
Upper spines (Red)	No spines (Yellow)	Upper spines (Red)	No spines (Yellow)
*Isabela used a four-color system			
Floreana*			
Small		**Large**	
All spines (Blue)	No spines (Green)	All spines (Red)	No spines (Yellow)
*All Floreana's populations lacked mericarps with lower spines.			
2019			
Santa Cruz*			
Small		**Large**	
All spines (Blue)	No spines (Green)	All spines (Red)	No spines (Yellow)
* Treatments changed in 2019 because not enough mericarps were collected to match 2018's treatments			
Isabela*			
Small		**Large**	
All spines (Blue)	No spines (Green)	All spines (Red)	No spines (Yellow)
* Treatments changed in 2019 because not enough mericarps were collected to match 2018's treatments			
Floreana*			
Small		**Large**	
All spines (Blue)	No spines (Green)	All spines (Red)	No spines (Yellow)
*All Floreana's populations lacked mericarps with lower spines.			

Variables definitions for Mark recapture experiment

Date: Date of the start of the mark recapture experiment per site.

Time: Factor. Time 0 marks the start of the experiment up to time 3 when it was last checked.

Days passed: Number of days passed from the start of the experiment up to the next monitoring date.

Island: Factor. Name of the island.

Treatment: Factor. Names the types of treatment. All spines, No spines, Upper Spines and Lower spines are categories for the 2018 experiment. In 2019, we only used No spines and All spines.

Size: Factor. Large or Small

Color: Color used for each treatment. Check each site for the colors used. For example, Santa Cruz, 2018 only used red and green.

Mark position: Factor. Position on the back of the mericarps where colors were added. It will depend on the type of treatment group. For example, mericarps marked upper left were small mericarps. Mericarps marked on the right side were large mericarps. This is specific for each site and year.

Mericarp: Individual mericarp number per population 1- 400

Mericarp traits: Length, width, depth, longest spine, spine tip distance, lower spine (binomial), spine position.

Plate: Factor. Plate ID 1 - 25

Present: Binomial. Whether the mericarp was recovered (present, 1) or not (0). A present mericarp could be eaten or not. A missing mericarp could be eaten if we assume that missing mericarps were picked up and eaten elsewhere.

Eaten birds: Binomial. Whether the mericarp shows at least one seed predation (1) or not (0).

Eaten insects: Binomial. Whether the mericarp shows insect predation (1) or not (0).

Number of seeds eaten: Count. The total of seeds eaten per mericarp. 1 - 4.

Germinated: Binomial. Whether the mericarp shows evidence of germination (1) or not (0).

Seed germinated: Count. The number of seeds germinated.

Appendix 3.3

Principal component analysis for each dataset

Point in time PCA.

Table S3. 6 PCA loadings from the point in time dataset. Notice that PC1 scores were mostly negative values, thus they were transformed in subsequent analyses.

Trait	PC1	PC2	PC3	PC4	PC5	PC6
Length	-0.51898	0.15459	-0.13921	-0.05076	-0.74396	0.362388
Width	-0.48219	-0.2053	0.210771	0.539637	0.435382	0.447387
Depth	-0.56536	-0.10172	0.041806	0.136468	-0.03609	-0.8052
Longest spine	-0.38785	0.486741	0.112513	-0.63282	0.437571	0.089808
Spine position	-0.10056	-0.01418	-0.9601	0.058056	0.253149	0.021044
Lower spines	0.13407	0.828546	8.05E-04	0.53269	-0.01047	-0.10802

Table S3. 7 Trait contributions per PC axis from the point in time dataset. PC1 was mostly associated with mericarp size, PC2 with mericarp defense and PC3 with spine position.

Trait	PC1	PC2	PC3	PC4	PC5	PC6
Length	26.93437282	2.389805064	1.937935907	0.257617534	55.34774	13.13253
Width	23.25060881	4.214786872	4.4424592	29.12086131	18.95577	20.01551
Depth	31.96345616	1.0347672	0.174770201	1.862346317	0.130277	64.83438
Longest spine	15.0428177	23.69166429	1.265922505	40.04622859	19.14681	0.806556
Spine position	1.011279509	0.020102622	92.1788474	0.337044868	6.40844	0.044285
Lower spines	1.797465006	68.64887395	6.48E-05	28.37590138	0.010958	1.166737

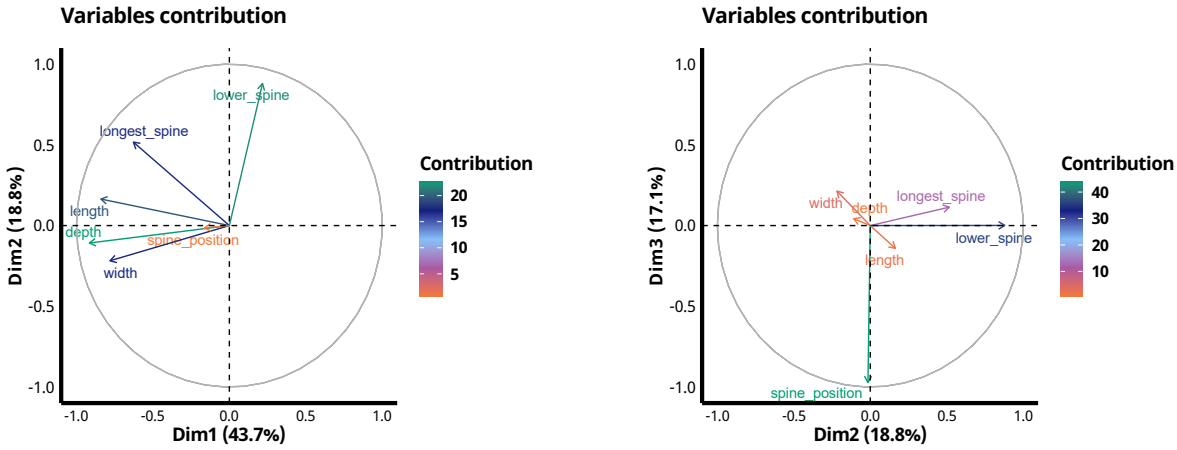


Figure S3. 3 Plots showing the variable contributions of mericarp traits for the Point in time populations. Left, PC1 and PC2 contributions. Right PC2 and PC3 contributions.

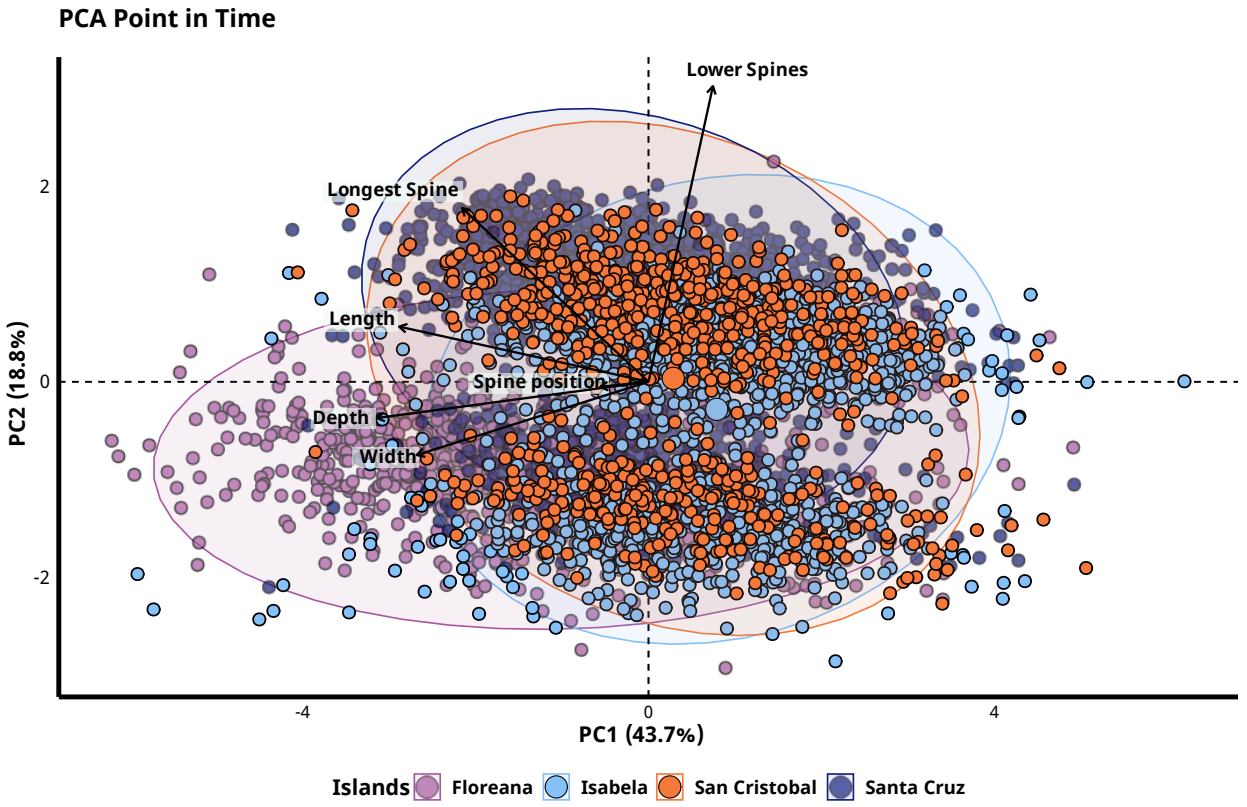


Figure S3. 4 PCA biplot of the Point in time mericarps. Each point corresponds to a single mericarp. Each color represents an island. Percentages represent the relative explained variation by each PC. PC1 was mostly associated with mericarp size. PC2 was associated with the presence of lower spines and longest spine.

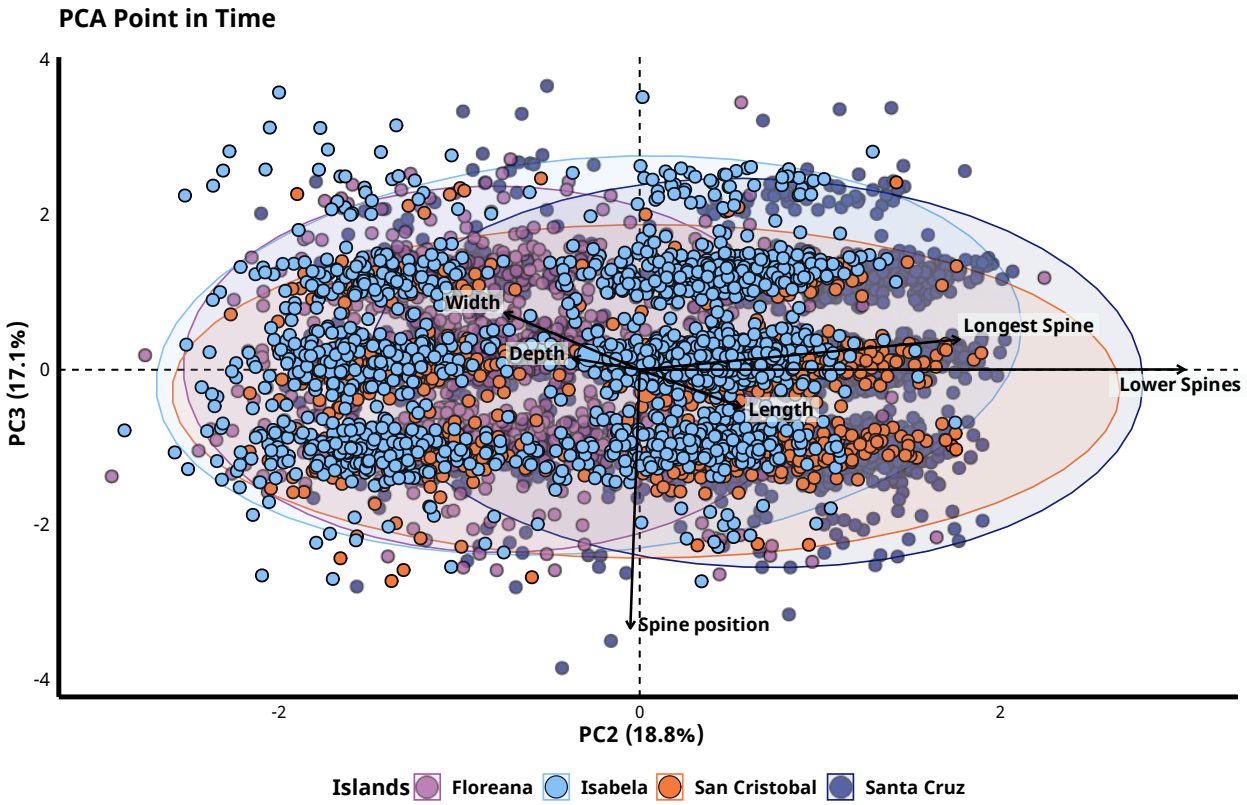


Figure S3. 5 PCA biplot of the Point in time mericarps. Each point corresponds to a single mericarp. Each color represents an island. Percentages represent the relative explained variation by each PC. PC2 was mostly associated with mericarp size. PC3 was associated with the presence of lower spines and longest spine.

Bioclimate variables PCA

Table S3. 8 PCA loadings from the bioclimatic variables. Nomenclature on bioclimate variables was taken from the WorldClim dataset (https://worldclim.org/). We used variables Bio1 (Annual Mean Temperature), Bio4 (Temperature Seasonality), Bio12 (Annual precipitation), Bio15 (Precipitation Seasonality).

Bioclimate	PC1	PC2	PC3	PC4
Bio_1: Mean Annual Temperature	0.439174	-0.63447	-0.57657	-0.2686
Bio_4: Temperature Seasonality	-0.5059	0.369165	-0.77914	-0.02671
Bio_12: Mean Annual Precipitation	-0.48316	-0.60623	0.004821	0.631679
Bio_15: Precipitation Seasonality	0.56369	0.306018	-0.24593	0.726722

Table S3. 9 Trait contributions from the bioclimatic variables PC scores. Nomenclature on bioclimate variables was taken from the WorldClim dataset (https://worldclim.org/). We used variables Bio1 (Annual Mean Temperature), Bio4 (Temperature Seasonality), Bio12 (Annual precipitation), Bio15 (Precipitation Seasonality).

Bioclimate	PC1	PC2	PC3	PC4
Bio_1: Mean Annual Temperature	19.28736	40.25519	33.24303	7.214427
Bio_4: Temperature Seasonality	25.59392	13.6283	60.70642	0.071364
Bio_12: Mean Annual Precipitation	23.34406	36.75183	0.002324	39.90178
Bio_15: Precipitation Seasonality	31.77466	9.364681	6.048232	52.81243

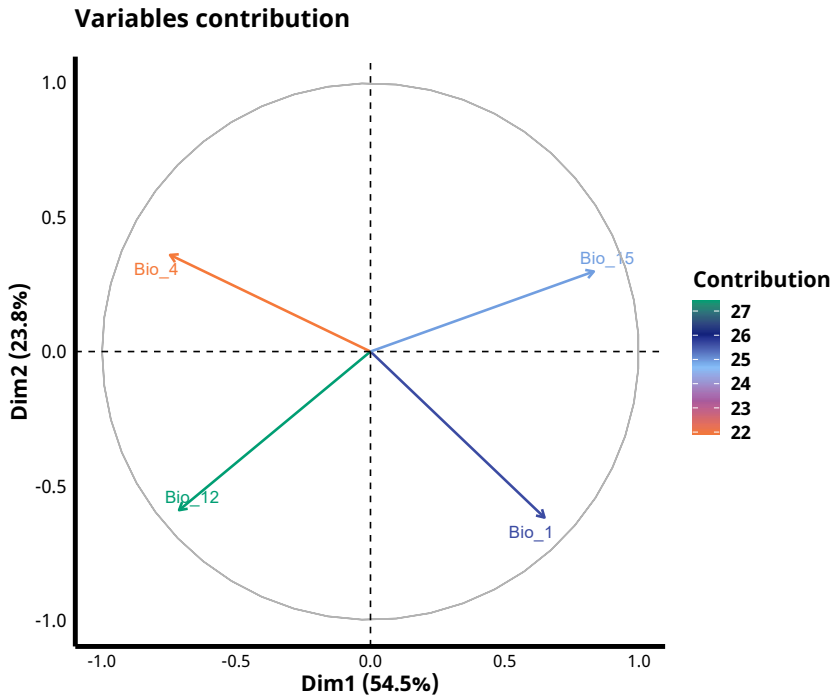


Figure S3. 6 Plot showing the variable contributions of mericarp traits for the Bioclimatic variables. PC1 was associated with Temperature and Precipitation. PC2 was associated with seasonality and annual measurements. Bio1 (Annual Mean Temperature), Bio4 (Temperature Seasonality), Bio12 (Annual precipitation), Bio15 (Precipitation Seasonality).

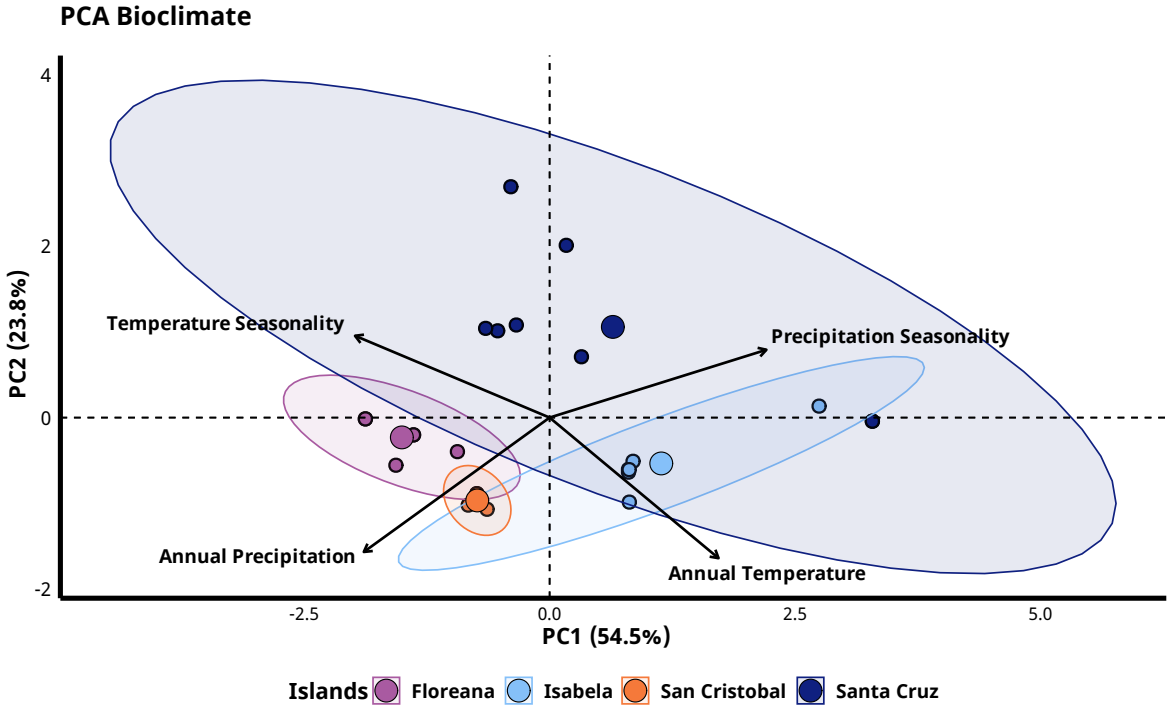


Figure S3. 7 PCA of bioclimate variables and grouped per island. Nomenclature on bioclimate variables was taken from the WorldClim dataset (https://worldclim.org/). We used variables Bio1 (Annual Mean Temperature), Bio4 (Temperature Seasonality), Bio12 (Annual precipitation), Bio15 (Precipitation Seasonality).

Mark Recapture PCA

Table S3. 10 PCA loadings from the Mark Recapture experiment. Notice that PC1 scores were mostly negative values, thus they were transformed in subsequent analyses.

Trait	PC1	PC2	PC3
Length	-0.56284	-0.69527	0.447003
Width	-0.55961	0.71853	0.412979
Depth	-0.60832	-0.01771	-0.7935

Table S3. 11 Trait contributions per PC axis from the Mark Recapture experiment. PC1 was mostly associated with mericarp size. PC2 was associated with mericarp length and width. PC3 was associated with mericarp depth.

Trait	Dim.1	Dim.2	Dim.3
Length	31.67881	48.34006	19.98114
Width	31.31624	51.62859	17.05517
Depth	37.00496	0.03135	62.96369

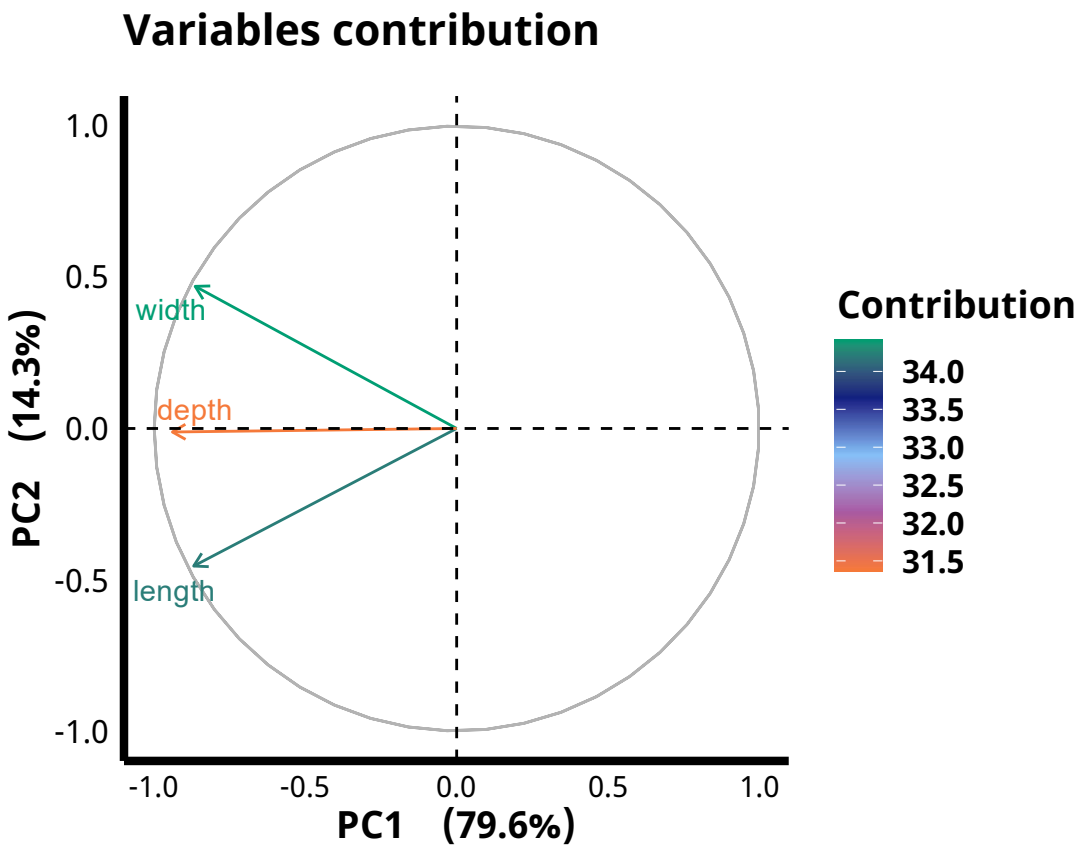


Figure S3. 8 Plot showing the variable contributions of mericarp traits for the Mark Recapture Experiment. The PCA only used mericarp size to estimate PC scores. In this way, we included the spine manipulation treatments without biasing the PC estimates.

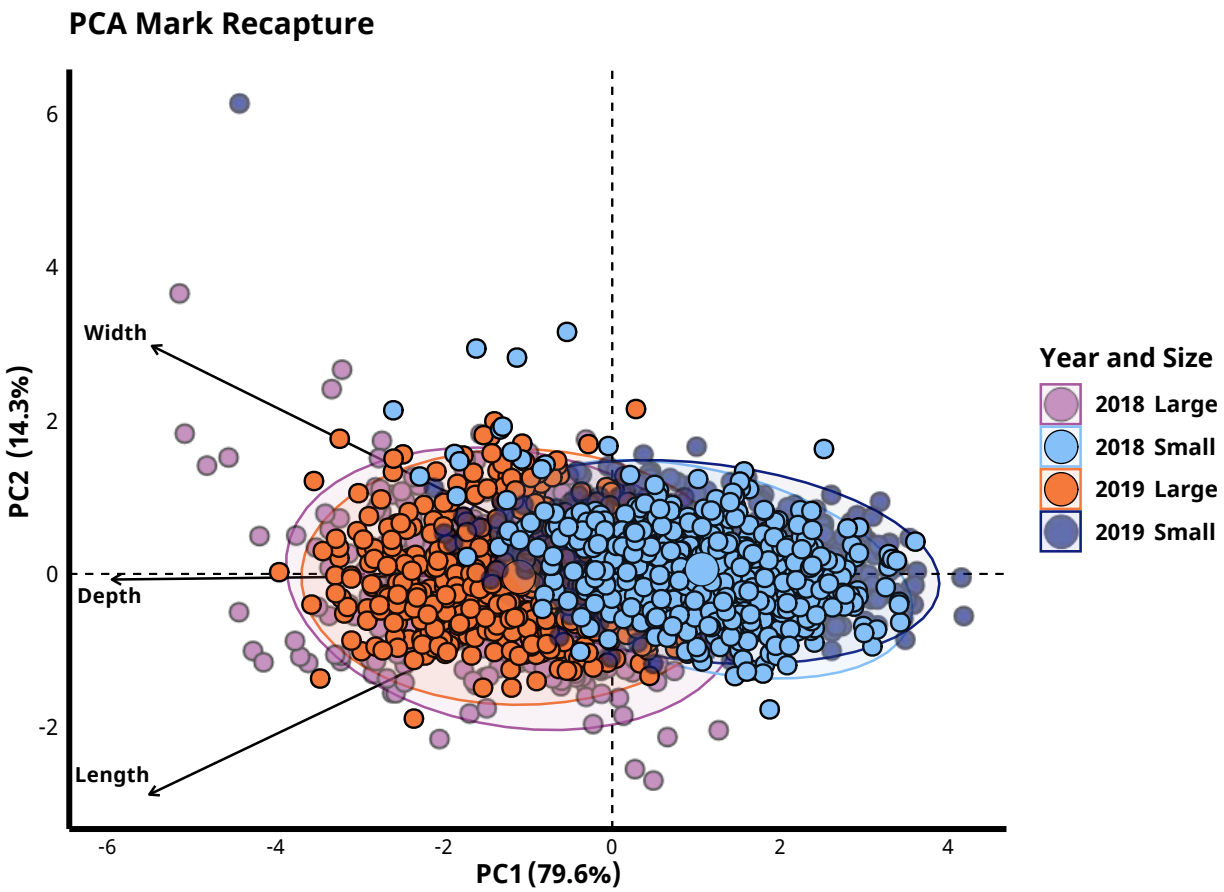


Figure S3. 9 PCA biplot per Categories for the Mark Recapture Experiment. The PCA only used mericarp size to estimate PC scores. In this way, we included the spine manipulation treatments without biasing the PC estimates. Notice the difference between size treatments for both years shows a clear separation between groups.

Appendix 3.4

Individual traits model outputs

Question 1. Are Galápagos mericarps under a general form of selection?

Table S3. 12 Model estimates of the effects of survival on individual mericarps traits. Bold values are statistically significant.

ANOVA (II)		
Traits	***Chisq***	***Pr(>Chisq)***
Depth	79.381	**<0.001**
Length	11.564	**<0.001**
Longest Spine	*92.826*	**<0.001**
Lower Spine	2.873	0.09008
Spine position	336.831	**<0.001**
Tip Spine Distance	33.152	**<0.001**
Width	4.3202	**0.0376**

Question 2. Are among-island mericarp differences correlated with selection?

Table S3. 13 Model estimates of the effect of selection on mean mericarp traits. Bold values are statistically significant. Underlined values are barely significant.

ANOVA (II)		
Traits	***Chisq***	***Pr(>Chisq)***
Lower Spine Frequency	90.4881	**<0.001**
Mean Depth	0.9727	0.324
Mean Length	3.0812	0.0792
Mean Longest Spine	0.3895	0.5325
Mean Spine position	4.3499	**0.037011**
Mean Tip distance	1.8887	0.1693
Mean Width	0.1674	0.6825

Question 3. Are the among-island differences in selection and traits associated with environmental variables?

Table S3. 14 Model estimates of the effects of bioclimate and finch community on the mericarp selection. Bold values are statistically significant. Underlined values are barely significant.

ANOVA (II)			
Traits		***Chisq***	***Pr(>Chisq)***
S Depth	*PC1_bioclimate*	1.8676	0.17175
	PC2_bioclimate	2.2571	0.133
	PC3_bioclimate	0.6068	0.436
	PC4_bioclimate	2.869	**0.0903**
	Finch Beak	3.2336	**0.07214**
S Length	*PC1_bioclimate*	0.790	0.374
	PC2_bioclimate	0.973	0.324
	PC3_bioclimate	0.001	0.982
	PC4_bioclimate	1.530	0.216
	Finch Beak	3.499	0.061
S Longest Spine	*PC1_bioclimate*	6.1372	0.01324
	PC2_bioclimate	0.3453	0.5568
	PC3_bioclimate	0.8332	0.36135
	PC4_bioclimate	3.0573	0.08037
	Finch Beak	0.1622	0.68717
*S Lower Spines**	*PC2_bioclimate*	1.8101	0.17849
	PC3_bioclimate	4.7169	**0.02987**
	PC4_bioclimate	0.042	0.83768
	Finch Beak	0.239	0.62495
S Spine Position	*PC1_bioclimate*	0.5767	0.447628
	PC2_bioclimate	1.0008	0.317112
	PC3_bioclimate	0.3615	0.547685
	PC4_bioclimate	13.2925	**0.000267**
	Finch Beak	0.7576	0.384081
S Tip Distance	*PC1_bioclimate*	0.1345	0.713792
	PC2_bioclimate	3.6405	0.056391
	PC3_bioclimate	3.6203	0.057078
	PC4_bioclimate	16.8119	4.13E-05
	Finch Beak	8.4497	0.003651
S Width	*PC1_bioclimate*	1.7526	0.18555
	PC2_bioclimate	0.4157	0.5191
	PC3_bioclimate	0.4031	0.52549
	PC4_bioclimate	2.9947	0.08354
	Finch Beak	0	0.99861

Table S3. 15 Model estimates of the effects of bioclimate and finch community on the mean mericarp trait value. Bold values are statistically significant. Underlined values are barely significant.

ANOVA (II)			
Traits		***Chisq***	***Pr(>Chisq)***
Mean Depth	*PC1_bioclimate*	0.0001	0.99079
	PC2_bioclimate	4.4627	**0.03464**
	PC3_bioclimate	0.2828	0.59485
	PC4_bioclimate	6.5836	**0.01029**
	Finch Beak	4.69	**0.03034**
Mean Length	*PC1_bioclimate*	3.4236	0.06427
	PC2_bioclimate	37.449	**9.38E-10**
	PC3_bioclimate	4.1895	**0.04067**
	PC4_bioclimate	0.0163	0.8985
	Finch Beak	15.4278	**8.57E-05**
Mean Longest Spine	*PC1_bioclimate*	0.3934	0.53052
	PC2_bioclimate	7.4972	**0.006179**
	PC3_bioclimate	1.9436	0.163279
	PC4_bioclimate	0.1257	0.722882
	Finch Beak	0.372	0.541918
Lower Spine Frequency	*PC1_bioclimate*	0.0082	0.928
	PC2_bioclimate	0.0068	0.9341
	PC3_bioclimate	0.0438	0.8342
	PC4_bioclimate	0.0417	0.8383
	Finch Beak	0.0164	0.8982
Mean Spine Position	*PC1_bioclimate*	8.3722	**0.00381**
	PC2_bioclimate	7.1496	**0.007498**
	PC3_bioclimate	0.2792	0.597222
	PC4_bioclimate	8.7795	**0.003046**
	Finch Beak	15.8424	**6.88E-05**
Mean Tip Distance	*PC1_bioclimate*	0	0.9976
	PC2_bioclimate	0.0097	0.9217
	PC3_bioclimate	0.033	0.8559
	PC4_bioclimate	0.0701	0.7912
	Finch Beak	0.0075	0.9309
Mean Width	*PC1_bioclimate*	1.3671	0.2423
	PC2_bioclimate	0.0846	0.7711
	PC3_bioclimate	0.1911	0.662
	PC4_bioclimate	1.3857	0.2391
	Finch Beak	0.284	0.5941

Appendix 3.5

Mark recapture experiment. Predicted model plots per island and experimental treatments.

Individual survival days model

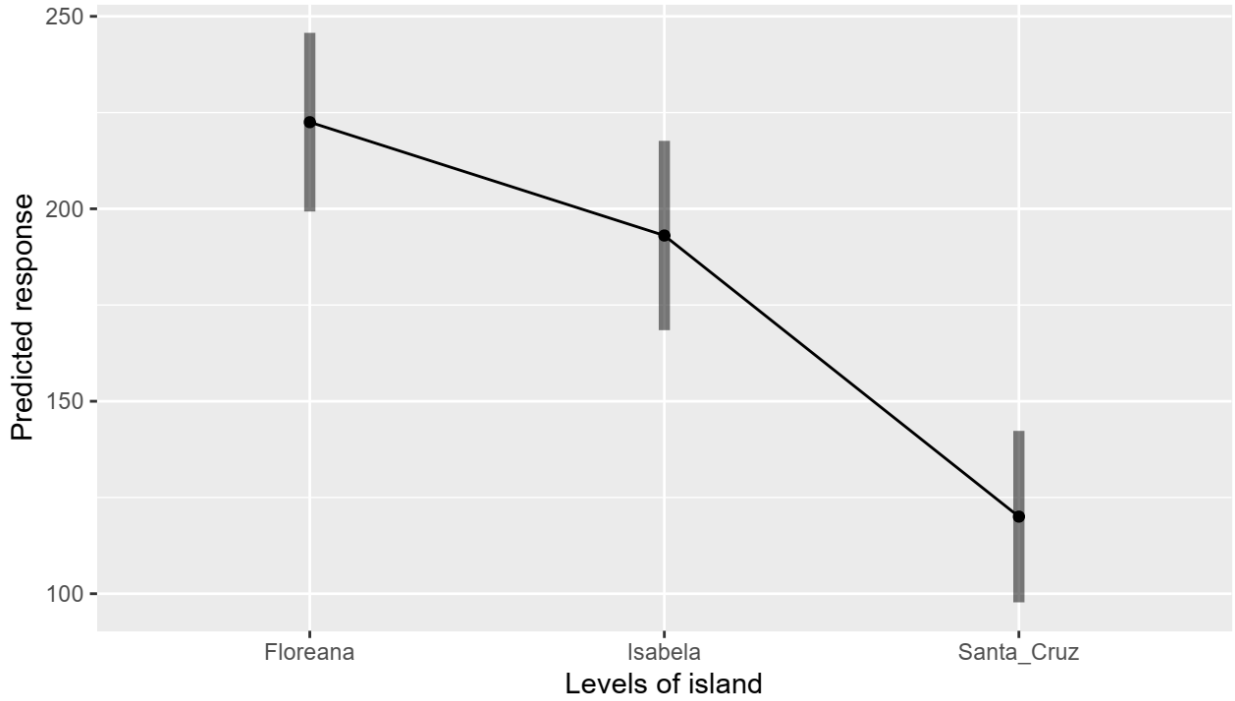


Figure S3. 10 Predicted model responses based on the mark recapture experiment. The predicted values use the marginal estimated means and represent mericarp survival. The plot shows mericarp survival for each island. From left to right: Floreana, Isabela and Santa Cruz.

Table S3. 16 Estimated mean survival days of uneaten mericarps per island. The percentage of survival was estimated based on the total number of days of each experiment per island.

Island	emmean	%	SE	df	lower.CL	upper.CL
Floreana	221	48.3	11.9	543	197.8	244
Isabela	193	43.2	12.5	543	168.7	218
Santa Cruz	120	27.5	11.3	543	97.7	142

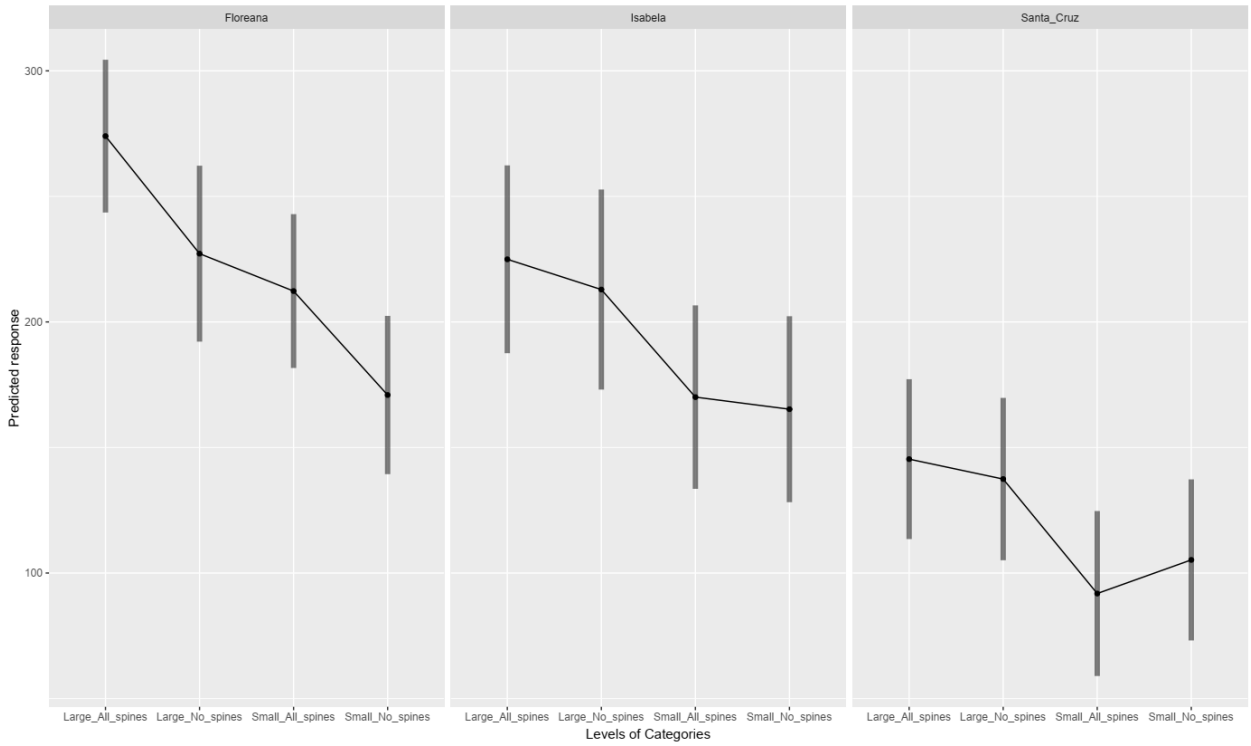


Figure S3. 11 Predicted model responses based on the mark recapture experiment. The predicted values use the marginal estimated means and represent mericarp survival. The plot shows mericarp survival for each experimental treatment. From left to right: Large all spines, large no spines, small all spines and small no spines.

Table S3. 17 Estimated mean survival days of uneaten mericarps per category per island. The percentage of survival was estimated based on the total number of days of each experiment per island. The categories are sorted by survival percentage.

Floreana						
Categories	**emmean**	**%**	**SE**	**df**	**lower.CL**	**upper.CL**
Large All Spines	274	59.8	15.5	543	243.6	304
Large No Spines	227	49.6	17.8	543	192.2	262
Small All Spines	212	46.4	15.6	543	181.7	243
Small No Spines	171	37.3	16	543	139.4	202
Isabela						
Categories	**emmean**	**%**	**SE**	**df**	**lower.CL**	**upper.CL**
Large All Spines	225	50.3	19	543	187.6	262
Large No Spines	213	47.6	20.3	543	173.1	253
Small All Spines	170	38.1	18.6	543	133.5	207
Small No Spines	165.2	37.0	18.8	543	128.2	202
Santa Cruz						
Categories	**emmean**	**%**	**SE**	**df**	**lower.CL**	**upper.CL**
Large All Spines	145	33.3	16.2	543	113.5	177
Large No Spines	137	31.4	16.5	543	105.1	170
Small No Spines	105.2	24.1	16.3	543	73.2	137
Small All Spines	91.8	21.0	16.7	543	59	125

Eaten mericarps model.

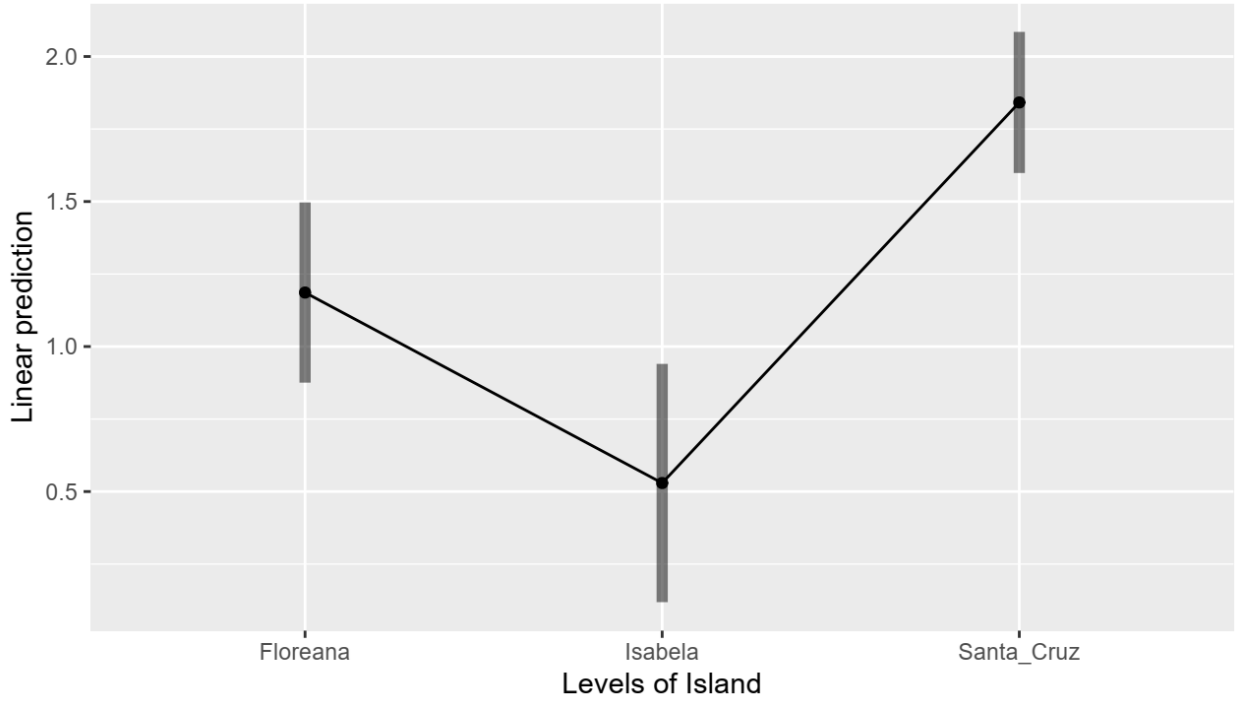


Figure S3. 12 Predicted values based on the marginal mean estimates of the number of eaten mericarps per island.

Table S3. 18 Estimated emmeans of eaten mericarps for each island. Results are averaged over the levels of: Categories and Time. Confidence levels used 0.95. Intervals are back transformed from the log scale.

Island	rate	SE	asymp.LCL	asymp.UCL
Santa Cruz	5.89	0.747	4.6	7.55
Floreana	3.03	0.505	2.19	4.2
Isabela	1.71	0.354	1.14	2.56

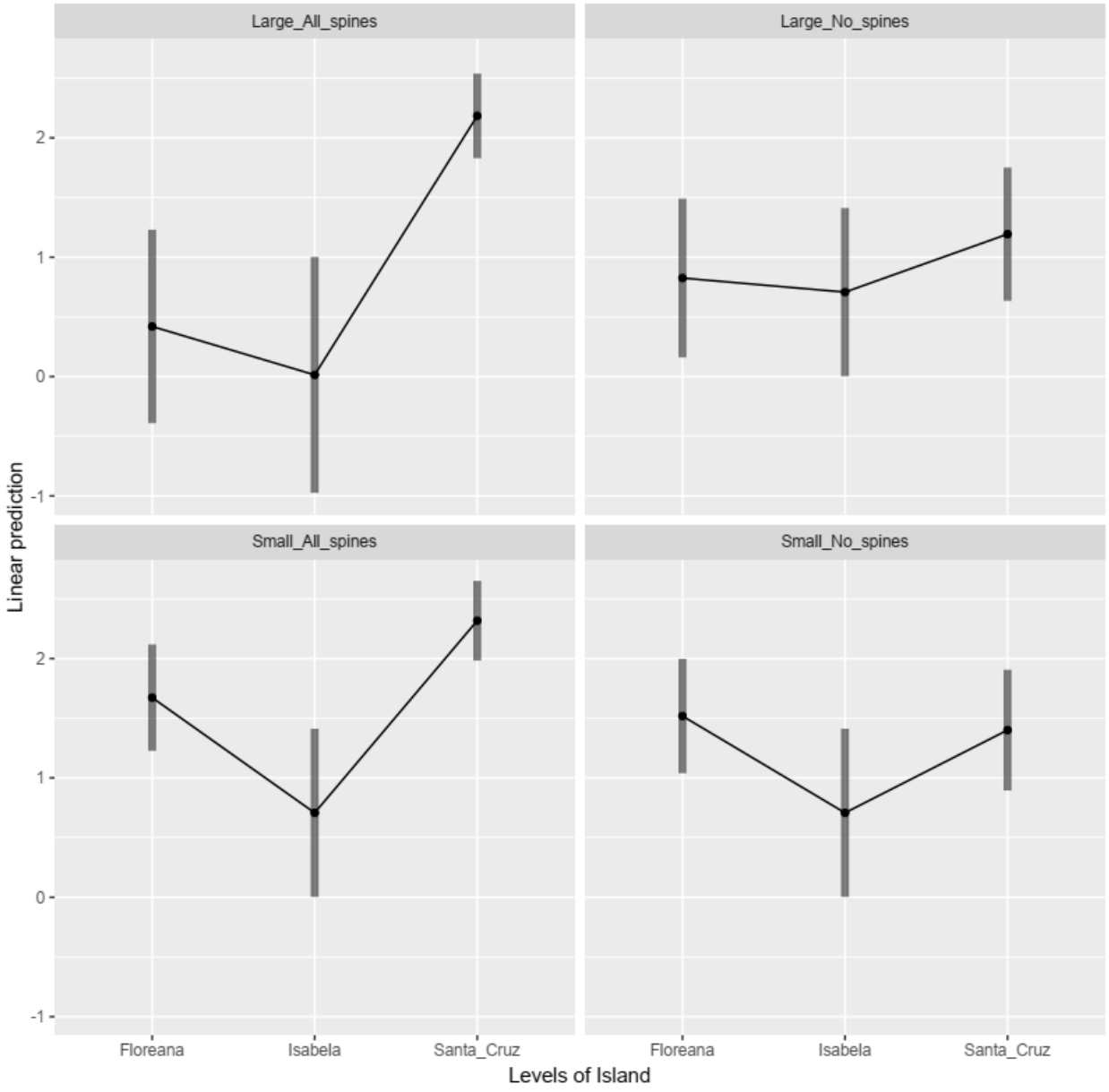


Figure S3. 13 Predicted values based on the marginal mean estimates of the number of eaten mericarps per experimental treatment.

Table S3. 19 Estimated emmeans of eaten mericarps for each category per island. Confidence levels used 0.95. Intervals are back transformed from the log scale.

Floreana				
Categories	**rate**	**SE**	**asymp.LCL**	**asymp.UCL**
Small All Spines	5.33	1.21	3.412	8.31
Small No Spines	4.56	1.114	2.829	7.36
Large No Spines	2.28	0.774	1.174	4.44
Large All Spines	1.52	0.629	0.677	3.42
Isabela				
Categories	**rate**	**SE**	**asymp.LCL**	**asymp.UCL**
Large No Spines	2.03	0.729	1.004	4.1
Small All Spines	2.03	0.729	1.004	4.1
Small No Spines	2.03	0.729	1.004	4.1
Large All Spines	1.01	0.511	0.378	2.72
Santa Cruz				
Categories	**rate**	**SE**	**asymp.LCL**	**asymp.UCL**
Small All Spines	10.14	1.727	7.266	14.16
Large All Spines	8.88	1.602	6.232	12.64
Small All Spines	4.06	1.046	2.448	6.73
Large No Spines	3.3	0.938	1.888	5.76

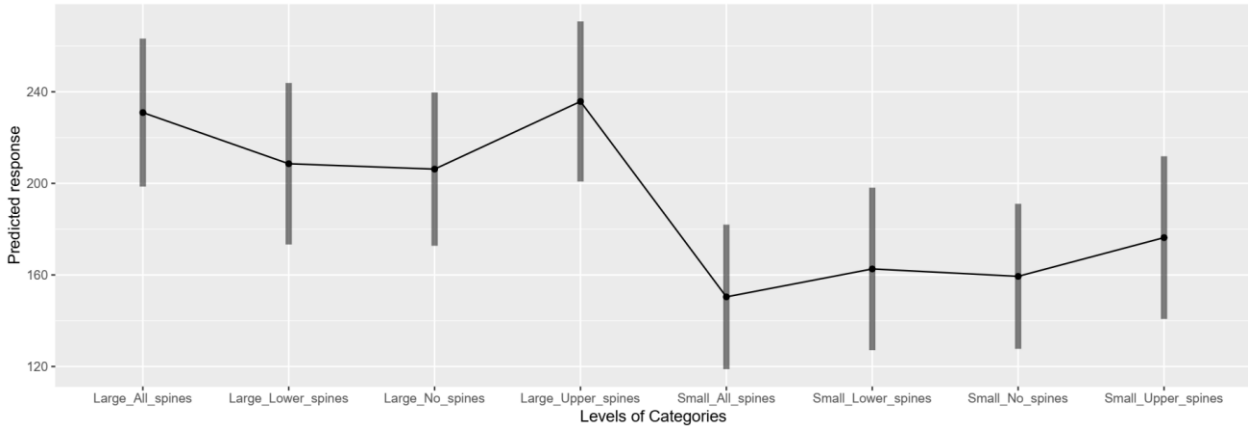


Figure S3. 14 Predicted means of survival days for the additional categories of the mark-recapture experiment used in 2018.

Table S3. 20 Estimated emmeans of eaten mericarps for 2018's additional categories per island. Confidence used 0.95. Intervals are back transformed from the log scale.

Floreana						
Categories	**emmean**	**%**	**SE**	**df**	**lower.CL**	**upper.CL**
Large Upper Spines	270.8	59	21.3	883	229.1	313
Large All Spines	266	58	18.7	883	229.2	303
Large Lower Spines	243.6	53	22.1	883	200.3	287
Large No Spines	241.3	53	19.6	883	202.8	280
Small Upper Spines	211.3	46	21.5	883	169.2	254
Small Lower Spines	197.7	43	22.1	883	154.3	241
Small No Spines	194.4	42	18.5	883	158.1	231
Small All Spines	185.5	41	19.2	883	147.8	223
Isabela						
Categories	**emmean**	**%**	**SE**	**df**	**lower.CL**	**upper.CL**
Large Upper Spines	260.8	58	19.2	883	223	299
Large All Spines	255.9	57	18.7	883	219.3	293
Large Lower Spines	233.6	52	19.7	883	194.8	272
Large No Spines	231.2	52	19.9	883	192.1	270
Small Upper Spines	201.3	45	19.7	883	162.7	240
Small Lower Spines	187.6	42	19.9	883	148.5	227
Small No Spines	184.4	41	18.7	883	147.7	221
Small All Spines	175.4	39	18.6	883	138.8	212
Santa Cruz						
Categories	**emmean**	**%**	**SE**	**df**	**lower.CL**	**upper.CL**
Large Upper Spines	175.7	40	20.4	883	135.7	216
Large All Spines	170.9	39	20	883	131.7	210
Large Lower Spines	148.5	34	19.4	883	110.4	187
Large No spines	146.2	33	19.4	883	108.1	184
Small Upper Spines	116.2	27	20.5	883	76	156
Small Lower Spines	102.6	23	19.5	883	64.3	141
Small No Spines	99.3	23	19.3	883	61.4	137
Small All Spines	90.4	21	18.6	883	53.9	127

Chapter 4 Appendix

Appendix 4.1 Sample information and summaries

Table S4. 1 Summary of field collected samples from the Galápagos Islands. The table shows the location of each sample, the number of vouchers, mericarps, tissue and individuals sampled.

Island	ID	Site	Vouchers	Mericarps	Individuals	Leaves	Latitude	Longitude
Champion	CHA_001	Trail	5	50	5	15	-1.237364961	-90.38716396
	CHA_002						-1.237396980	-90.38720797
	CHA_003						-1.237374013	-90.38719397
	CHA_004						-1.237423969	-90.38722498
	CHA_005						-1.237421036	-90.38721601
Española	ESP_001	Punta Suárez	NA	167	3	9	-1.371727958	-89.743469
	ESP_002						-1.371989977	-89.74312199
	ESP_003						-1.372240009	-89.74269602
	ESP_004	Bahía Gardner	8		8		-1.352375988	-89.66170697
	ESP_006						-1.352448994	-89.66159902
	ESP_007						-1.352451006	-89.66156398
	ESP_008						-1.352479002	-89.66155099
	ESP_009						-1.352494005	-89.66150103
	ESP_010						-1.352531975	-89.66147698
	ESP_011						-1.352531975	-89.66147698
Fernandina	FER_001	Cabo Douglas	4	63	4	20	-0.304568019	-91.653088
	FER_002						-0.304638008	-91.653103
	FER_003						-0.304742027	-91.653118
	FER_004						-0.304930033	-91.65356602
Floreana	FLO_001	Post Office	9	250	8	16	-1.238039033	-90.44726497
	FLO_002						-1.238074992	-90.44730102
	FLO_003						-1.238064012	-90.44740202
	FLO_004						-1.238037022	-90.44746597
	FLO_005						-1.237700991	-90.44785498
	FLO_006						-1.237154994	-90.44858697
	FLO_007						-1.260962030	-90.37057299
	FLO_008						-1.238037022	-90.44746597
	FLO_009	Cuevas			2		-1.237154994	-90.44858697
	FLO_010						-1.26096203	-90.37057299
Genovesa	GEN_001	Salvaje Corazón	3	203	3	15	0.312611042	-89.97429203
	GEN_002						0.312611964	-89.97425599
	GEN_003						0.312636020	-89.97426203
Isabela	ISA_001	Punta Vicente Roca	3	27	4	12	-0.039147008	-91.53424602
	ISA_002						-0.038794968	-91.53406296
	ISA_003						-0.038822964	-91.53403002
	ISA_004						-0.038781976	-91.53390597

Island	ID	Site	Vouchers	Mericarps	Individuals	Leaves	Latitude	Longitude
Rabida	RAB_001	Playa Roja	9	233	9	27	-0.399709996	-90.70682896
	RAB_002						-0.399703039	-90.70684003
	RAB_003						-0.399699016	-90.70692996
	RAB_004						-0.399690969	-90.70699199
	RAB_005						-0.399687029	-90.707022
	RAB_006						-0.399683006	-90.707123
	RAB_007						-0.399631960	-90.70727899
	RAB_008						-0.399617041	-90.70793001
	RAB_009						-0.399596002	-90.70826001
Santiago	SAN_001	Puerto Egas	7	103	10	30	-0.242061988	-90.86111801
	SAN_002						-0.241987975	-90.86110904
	SAN_003						-0.242023012	-90.86106
	SAN_004						-0.242106998	-90.86094299
	SAN_005						-0.242171036	-90.86120602
	SAN_006						-0.241392022	-90.861663
	SAN_007						-0.241190018	-90.86182603
	SAN_008						-0.241150958	-90.861836
	SAN_009						-0.241150958	-90.861836
	SAN_010						-0.241150958	-90.861836
San Cristóbal	SCR_001	Punta Pitt	7	101	7	21	-0.699737035	-89.25405001
	SCR_002						-0.699757989	-89.25407398
	SCR_003						-0.699725971	-89.25400802
	SCR_004						-0.699653970	-89.25390098
	SCR_005						-0.699821021	-89.254292
	SCR_006						-0.707835965	-89.25378498
	SCR_007						-0.708027994	-89.25410097
Santa Cruz	SCZ_001	Plazas	10	191	12	24	-0.582552999	-90.16483499
	SCZ_002						-0.582561968	-90.16486399
	SCZ_003						-0.582625000	-90.16474304
	SCZ_004						-0.582773024	-90.16477204
	SCZ_005						-0.583296977	-90.16504001
	SCZ_006						-0.583177032	-90.16516004
	SCZ_007						-0.583122969	-90.16519499
	SCZ_008						-0.583672989	-90.16540504
	SCZ_009						-0.583710037	-90.16534704
	SCZ_010						-0.583457993	-90.16517697
	SCZ_011						-0.583522031	-90.16520396
	SCZ_012						-0.583357997	-90.16517898
Seymour	SYN_001	Bahía Seymour	6	225	6	18	-0.400695037	-90.29118799
	SYN_002						-0.400685985	-90.29117198
	SYN_003						-0.400684979	-90.29120701
	SYN_004						-0.400652960	-90.29119796
	SYN_005						-0.400684979	-90.29120701
	SYN_006						-0.400684979	-90.29120701
TOTAL			71	1613	81	207		

Table S4. 2 *Tribulus* herbarium samples summary. The table shows the reclassified taxa based on our guide. Also includes the location information of each sample and voucher accession number from the Missouri Botanical Garden. Bold IDs show corrected taxa.

ID	Herbarium name label	Re-classified taxon as	Region	Country	Province	Additional Locality Notes	Latitude	Longitude	Collection Year	MoBot Accession #
MBG-026	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Oaxaca	Plantas del Istmo de Tehuantepec. Planicie inundable a 1.4 km en linea recta al SO (192d) de Nizanda, Mpio de Asuncion Ixtaltepec, Dto. de Juchitan, Oaxaca	16.64527778	-95.01472222	1998	5804631
MBG-027	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Oaxaca	Valle de Tehuaan-cuicatlan. Mun. San Juan Bautista Cuicatlan. Barranca de las guacamayas, San Jose El Chilar	17.67758333	-96.96530556	2002	4756224
MBG-028	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Chiapas	La Cebadilla, municipio de Tapachula	14.823232	-92.313908	1984	5566961
MBG-029	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Chiapas	10 miles S of Arriaga. Municipio of Arriaga	16.090391	-93.895031	1972	2366945
MBG-030	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Colima	Along highway 200, ca. 3 miles south of Manzanillo between Manzanillo and Colima	19.098289	-104.292039	1979	5549600
MBG-031	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Jalisco	22km N or Autlan de Navarro on Highway 80.	19.964198	-104.375588	1985	5549832
MBG-032	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Isla Socorro	Naval Base on Isla Socorro	18.727634	-110.950342	1987	5074076
MBG-033	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Puebla	5 miles SE of Izucar de Matamoros in arroyo	18.535538	-98.424231	1972	4321862
MBG-034	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Quintana Roo	En la entrada de la Brecha a Vallarta, a	20.833422	-86.88946	1980	5654986

ID	Herbarium name label	Re-classified taxon as	Region	Country	Province	Additional Locality Notes	Latitude	Longitude	Collection Year	MoBot Accession #
						2 km al Sur de Puerto Morelos				
MBG-035	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Yucatan	3 km al O de Puerto Progreso, sobre el ca mino a Yucalpeten	21.27653	-89.693299	1986	5654983
MBG-036	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Quintana Roo	6 km al S de la zona urbana de Isla de Mujeres, sobre la carretera perimetral	21.230607	-86.733873	1987	5549606
MBG-037	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Vera Cruz	Rte 150 2 km W of bridge over Rio Atoyac	19.199681	-96.221507	1970	2027365
MBG-038	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Yucatan	4 km West of las Coloradas	21.607845	-88.026498	1989	4246334
MBG-039	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Guatemala	Chiquimula	Chiquimula. entrada principal de Chiquimula via Zacapa	14.791854	-89.545215	2003	5861329
MBG-040	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Chamela	Mpio. La Huerta, Jalisco.	19.5	-105.05	1985	5549822
MBG-041	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Mexico	Baja California	Bahia de La Paz about 5 miles NW of La Paz, Mexico	24.168553	-110.38577	1974	2244042
MBG-042	*Tribulus cistoides*	*cistoides*	Central America-Mexico	Guatemala	El Progresso	San Agustin Acasaguastlan	14.947645	-89.96968	2003	5861072
MBG-044	*Tribulus cistoides*	*cistoides*	South America	Colombia	Valle del Cauca	Mun. Palmira	3.537972	-76.297166	1993	5035926
MBG-045	*Tribulus cistoides*	*cistoides*	South America	Peru	Cajamarca	Procedencia: Chilete-Magdalena. Dpto. Cajamarca	-7.2507	-78.658312	1981	2918132
MBG-046	*Tribulus cistoides*	*cistoides*	South America	Venezuela	Penninsula de Araya	26 km NW of Cariaco by air, immeditely west of turnoff to new road	10.65833333	-63.76666667	1981	2926391
MBG-047	*Tribulus cistoides?*	*cistoides*	South America	Venezuela	Zulia	Dtto. Mara: cerca de la playa Santa Fe	11.189188	-71.868397	1979	2920135
MBG-048	*Tribulus cistoides*	*cistoides*	South America	Venezuela	Lara	2 km S of Quibor	9.75	-69.61666667	1982	3335114
MBG-049	*Tribulus cistoides*	*cistoides*	South America	Venezuela	Federal	municipio Vargas. Parroquia Catia la Mar	10.6	-67.03333333	1989	3761078
MBG-050	*Tribulus cistoides*	*cistoides*	South America	Venezuela	Vargas	between Naiguatá and Los Caracas	10.622016	-66.641065	1975	3335112
MBG-051	*Tribulus*	*cistoides*	South America	Venezuela	Aragua	University central de	10.245441	-67.585458	1973	2916321

ID	Herbarium name label	Re-classified taxon as	Region	Country	Province	Additional Locality Notes	Latitude	Longitude	Collection Year	MoBot Accession #
	cistoides?					Venezuela Maracay				
MBG-052	*Tribulus cistoides*	*cistoides*	South America	Venezuela	Falcon	Falcon	11.181067	-69.859741	1980	3903343
MBG-053	*Tribulus cistoides*	*cistoides*	Caribbean	Dominican Republic	Guano, 30 Km	Loma el guano, 30 km SW of Pedernales	17.95	-71.58333333	1985	321758
MBG-054	*Tribulus cistoides*	*cistoides*	Caribbean	Dominican Republic	Azua	en la zona comprendida entre el puente sobre el rio Tabara y approx. 10 km en el trayecto Tabara Abajo-Barahona	18.48333333	-70.86666667	1984	4317988
MBG-055	*Tribulus cistoides*	*cistoides*	Caribbean	Dominican Republic	Km. East	Prov. Barahona, 8km. east of Quita Coraza.	18.468646	-71.069019	1979	6022767
MBG-056	*Tribulus cistoides*	*cistoides*	Caribbean	Domican Republic	Pedernales	Al sur del Puerto de Cabo Rojo	17.90833333	-71.66666667	1981	6022759
MBG-057	*Tribulus cistoides*	*cistoides*	Caribbean	Dominica Republic	Independencia	Isla Cabritos en el Lago Enriquillo, 2 km al Oeste de la casea del Parque nacional	18.5	-71.71666667	1981	6022762
MBG-058	*Tribulus cistoides*	*cistoides*	Caribbean	Haiti	Ducroix	Dept. Du Nord: Ducroix, near Cap-Hatien	19.78333333	-72.21666667	1988	6112272
MBG-059	*Tribulus cistoides*	*cistoides*	Caribbean	Puerto Rico	Isla de Mona	Isla de Mona. Playa Sardinera	18.082077	-67.892338	1991	3801855
MBG-060	*Tribulus cistoides*	*cistoides*	Caribbean	Puerto Rico	San Juan	San Juan: Puerta de Tierra	18.46638	-66.096912	1995	5936594
MBG-061	*Tribulus cistoides*	*cistoides*	Caribbean	Bahamas	The Bahama	Plants of the Bahama Islands: Great Inagua: Northwest point in coconut grove	21.065607	-73.323708	1974	2433188
MBG-062	*Tribulus cistoides*	*cistoides*	Caribbean	Bahamas	The Bahama	Plants of the Bahama Islands: Eleuthera: in weedy open slpe avoe town of Governor's Harbour	24.931365	-76.189906	1977	2636472
MBG-063	*Tribulus cistoides*	*cistoides*	Caribbean	Jamaica	Morant Point	Along road to lighthouse, Morant Point	17.918415	-76.184614	1990	5711025
MBG-064	*Tribulus*	*cistoides*	Caribbean	Jamaica	Hellshire	Jamaica. St.	17.88333333	-76.9	1993	4578294

ID	Herbarium name label	Re-classified taxon as	Region	Country	Province	Additional Locality Notes	Latitude	Longitude	Collection Year	MoBot Accession #
	cistoides					Catherine: Hellshire hills, E portion, vicinity of Fort Clarence				
MBG-065	*Tribulus cistoides*	*cistoides*	Africa	Tanzania	Dar es Salaam	Dar es Salaam, Ras Chokir	-6.816828	39.30025	1974	2574392
MBG-066	*Tribulus cistoides?*	*cistoides*	Africa	Ethiopia	Borena	Sidamo Region 3 km N of Yavello along main road to Agere Maryam	4.883333333	38.15	1997	4822553
MBG-067	*Tribulus cistoides?*	*cistoides*	Africa	Ethiopia	Sidamo	46 miles SE of Neghelle	7.359798	38.669094	1974	2651126
MBG-068	*Tribulus cistoides?*	*cistoides*	Africa	Cape Verde Islands	Praia	Desert area NE of the international airport	14.958081	-23.495511	1993	5709279
MBG-069	*Tribulus cistoides?*	*cistoides*	Africa	Tanzania	Pangani District	Mbigiri (Zanzibari) south of Mto Kama, 12 km south of Mkwaja Tanga Region	-5.879839	38.807242	1974	2574435
MBG-070	*Tribulus cistoides*	*cistoides*	Africa	Togo		cultivated	1.45701	27.047297	1987	4916970
MBG-071	*Tribulus cistoides*	*cistoides*	Africa	Sudan	Ecuatoria Oriental	lower omo valley expedition. SE extreme Ilemi Triangle. 7 mi WSW Kibish Police.	5.16439	35.493757	1970	2265147
MBG-072	*Tribulus cistoides*	*cistoides*	Africa	Tanzania	Dodoma	Dodoma Distriot	-6.172932	35.764048	1973	2296824
MBG-073	*Tribulus cistoides*	*cistoides*	Oceania	New Caledonia			-20.904303	165.618041	1980	2924336
MBG-074	*Tribulus cistoides*	*cistoides*	Oceania	Austraila	Queensland	Pelican Island. Great Barrier Reef Islands	-20.335915	148.853166	1973	2355459
MBG-075	*Tribulus cistoides*	*cistoides*	Oceania	Austrailia	Queensland	Flora of Queensland. Stapleton Island. Grat Barrier Reef	-14.319097	144.85009	1973	2355472
MBG-076	*Tribulus cistoides*	*cistoides*	Oceania	Australia	Magnetic Island	Nelly Bay, Magnetic Island	-19.156807	146.84968	1981	4329742
MBG-077	*Tribulus cistoides*	*cistoides*	Oceania	Republic of Kiribati	Sidney Island	Phoneix islands. Sydney Island, north side of atoll near lagoon	-4.45572	-171.238081	1973	2363769
MBG-078	*Tribulus cistoides*	*cistoides*	Oceania	Republic of Kiribati	Gardner Atoll	Phoenix Islands: Gardner Atoll.	-4.674522	-174.522874	1975	2304987

ID	Herbarium name label	Re-classified taxon as	Region	Country	Province	Additional Locality Notes	Latitude	Longitude	Collection Year	MoBot Accession #
						northeast rim east of main channel (Taraia)				
MBG-079	*Tribulus cistoides*	*cistoides*	Oceania	Republic of Kiribati	Canton Island	Phoenix Islands: Canton Island 2 kms S of NW point of island	-2.814525	-171.670602	1973	2363819
MBG-080	*Tribulus cistoides*	*cistoides*	Oceania	Republic of Kiribati	Enderbury Island	Phoenix Islands: Enderbury island. outhwest corner of island, near old colonist camp and red and white monument	-3.123547	-171.08701	1975	2310026
MBG-081	*Tribulus cistoides*	*cistoides*	Oceania	Republic of Kiribati	McKean Island	Phoenix Islands: McKean Island: Abundant over much of island, dominant on flat coral sand	-3.594938	-174.122795	1975	2310012
MBG-082	*Tribulus cistoides*	*cistoides*	Oceania	French Polynesia	Hanamenu, Site	Hivaoa Island. Hanamenu, site of old village deerted ca. 10 years ago	-9.754673	-139.021122	1975	4327972
MBG-083	*Tribulus cistoides*	*cistoides*	Madagascar	Madagascar	Seychelles	Plants of seychelles Island. Bird Island	-3.723912	55.205543	2002	5694571
MBG-084	*Tribulus cistoides?*	*cistoides*	Madagascar	Madagascar	Petite-Terre	Petite-Terre. Labattori. Plage De Moya	-12.785259	45.295648	1976	2578472
MBG-096	*Tribulus pterophorus*	*pterophorus*	Southern Africa	Namibia	Karas	Haib River, west of Warmbad	-28.449038	18.733313	1976	3884399
MBG-097	*Tribulus zehyeri*	*zeyheri*	Southern Africa	South Africa	North Cape	Northern Cape. just past Die Poort on road to Groblershoop	-28.926995	21.930462	1996	5298059
MBG-098	*Tribulus zehyeri*	*zeyheri*	Southern Africa	Namibia	Desert	Ganab in Namib Desert Park. sandy open plains below hill	-24.448066	15.126926	1976	2477136
MBG-099	*Tribulus zehyeri*	*zeyheri*	Southern Africa	South Africa	North Cape	Cape province. Namaqualand and flower reserve. springbok.	-30.0593	19.383769	1973	2254110
MBG-100	*Tribulus zehyeri*	*zeyheri*	Southern Africa	South Africa	Limpopo	Messina c. 56 myl N.W. op plaas Greefswald. Herbarium Pretoria.	-22.33889	30.038973	1974	2342647

ID	Herbarium name label	Re-classified taxon as	Region	Country	Province	Additional Locality Notes	Latitude	Longitude	Collection Year	MoBot Accession #
						waterpoort. Regio: Tvl.				
MBG-101	***Tribulus zehyeri***	***pterophorus***	Southern Africa	Namibia	Erongo	Swakop River Valley at Husab. sandy floodplain. open ground. regio: SWA	-22.310258	15.742037	1976	2576690
MBG-103	***Tribulus terrestris***	***zeyheri***	Southern Africa	South Africa	Limpopo	Transvaal. dist. messina. Farm Erfrus. at roadsides, also in veld near roads	-22.381251	30.031855	1982	5969905
MBG-104	***Tribulus zehyeri***	***pterophorus***	Southern Africa	south Africa	south Africa	south africa. Northern cape, Upington. Swartmodder. Along road R360 to Twee Rivieren, approx. 60 km norht of Lutzputz. alt. 800m	-28.03996667	20.55995	2002	4797369
MBG-105	***Tribulus cristatus***	***pterophorus***	Southern Africa	Namibia	Karas	55-60km NW of Noordoewer, gravel dunes at junction of Gamkab and Orange River	-28.232084	17.363478	1984	3202050
MBG-106	***Tribulus zehyeri***	***pterophorus***	Southern Africa	South Africa	North Cape	Cape province. Kalahari-Gemsbok National Park. road in Audo River	-26.489326	20.615278	1989	3909512
MBG-107	*Tribulus pterophorus*	*cristatus*	Southern Africa	South Africa	North Cape	North Cape. Sendeling scent. very stoney hill ca. 5 km from octha Llts road-covered in yellow flowers	-29.297445	21.85686	1982	3394500
MBG-108	*Tribulus cristatus*	*cristatus*	Southern Africa	South Africa	North Cape	NW Cape. Namaqualand. just S of Vyfmylspoort (Vioolsdrift)	-29.981697	19.378253	1986	4389017
MBG-109	*Tribulus cristatus*	*cristatus*	Southern Africa	South Africa	North Cape	Northern Cape. Kenhardt District. Farm: Brypaal. ca. 65 km SW of Kakamas. orange river Nama Karoo	-29.354065	21.212958	2006	6173957
MBG-110	*Tribulus*	*cristatus*	Southern Africa	South	North Cape	Southwest Africa. 20	-28.902977	17.574946	1974	2228813

ID	Herbarium name label	Re-classified taxon as	Region	Country	Province	Additional Locality Notes	Latitude	Longitude	Collection Year	MoBot Accession #
	cristatus			Africa		km north of Vioolsdrif.				
MBG-111	***Tribulus aff. occidentalis***	***minutus***	Oceania	Australia	Northern Territory	Northern Territory. Uluru (Ayers Rock-Mt Olga) National Park. Kata Tjuta (the Olgas), on the Docker River road, 45 km WNW of the Ranger Station	-25.3	130.6833333	1988	4335756
MBG-112	***Tribulus terrestris***	***micrococcus***	Oceania	Australia	Queensland	8 km along Leyburn road from Pittsworth-Milmerran road, Queensland	-27.74899	151.558121	1981	2980501
MBG-113	***Tribulus terrestris***	***micrococcus***	Oceania	Australia	New South Wales	Myall creek, 18 km SW of Delungra on raod to Bingara	-29.78333333	150.7666667	1987	4335763
MBG-114	*Tribulus hystrix*	*hystrix*	Oceania	Australia	South Australia	South Australia. Region 2: Lake Eyre. Dulkaninna Station. Large sanddunes approximately 12 km W of Dulkaninna Field Station by track. Dulkaninna Field Station is c. 80 km N or Marree on the Birdsville Track	-28.98805556	138.3775	1997	5026472
MBG-115	*Tribulus forrestii*	*forrestii*	Oceania	Australia	Western Australia	Western Australia. 68.8 km S of Minilya	-24.45	113.9833333	1985	4620007
MBG-116	*Tribulus eichlerianus*	*eichlerianus*	Oceania	Australia	South Australia	South Australia, Region 2: Lake Eyre. Dulkaninna Station. Large sandunes, approimtely 12 km W of Dulkaninna Field Stion by track. Dulkaninna Field Statio is c. 80 km N or Marree on the Birdsville Track	-28.98805556	138.3775	1997	5026470
MBG-117	*Tribulus eichlerianus*	*eichlerianus*	Oceania	Australia	Northern Territory	9km N of Colsons Pinnacle, Horeshore Bend Sations.	-25.21666667	134.5833333	1993	5044240

ID	Herbarium name label	Re-classified taxon as	Region	Country	Province	Additional Locality Notes	Latitude	Longitude	Collection Year	MoBot Accession #
						State/District: NT/CS. spreading annual, yellow flowers. Rare, woodland, loamy soil				
MBG-118	***Tribulus pentandrus***	***pentandrus complex***	Africa	Egypt	Egypt	W. Zeidun. sandy soil	26.77422	30.799403	1980	5969873
MBG-119	***Tribulus pentandrus***	***pentandrus complex***	Africa	Egypt	Egypt	Sinai Penisula: ca. 25 km north of N Sharm el Sheikh near the road to Dahab, ca. 50 m. semidesert	28.140182	4.299471	1991	6050336
MBG-120	*Tribulus zehyeri*	*zeyheri*	Africa	Ethiopia	Ethiopia	Harerge, along road to Erer Gota, 12km out of Kire Dawa	9.616666667	41.75	1975	6488093
MBG-122	***Tribulus alatus***	***pentandrus complex***	Middle East	Iraq	Babylon	Large depression in Iskandariya desert. Babylon province, Iraq	32.889585	44.347304	1971	2228727
MBG-123	*Tribulus bimucronatus*	*bimucronatus*	Middle East	Saudi Arabia	Saudi Arabia	km 106.5, Makkah Bypass. soft damp sand	21.362897	39.853193	1982	3189025
MBG-124	***Tribulus macropterus***	***pentandrus complex***	Middle East	Turkmenistan	Turkmenistan	Chardzhoutoray region	39.004131	63.568808	1979	5652119
MBG-125	***Tribulus longipetalus***	***pentandrus complex***	Middle East	Saudi Arabia	Riad	Aflja Wells.	22.149423	47.136213	1976	3273509
MBG-126	***Tribulus longipetalus***	***pentandrus complex***	Middle East	Pakistan	Balochistan	c. 40 miles form Turbat on way to Hushab. sandy soil. Karachi University herbarium	25.995846	63.683009	1972	2230811
MBG-127	*Tribulus bimucronatus*	*bimucronatus*	Middle East	Israel	Israel	Dead Sea Area: Nahal Pere, Junction with Sodom-Dimona Rd.	31.014164	35.290694	1986	4609343
MBG-128	*Tribulus terrestris*	*terrestris*	Africa	Egypt	Gebel Elba	Sudan Government administration area, Gebel Elba, Wadi Aideib	22.15	36.26	1985	3339292
MBG-129	*Tribulus terrestris*	*terrestris*	Africa	Tanzania	Mara	Acacia-Albizia woodland	-2.378333333	34.85194444	2004	5902804
MBG-130	*Tribulus terrestris*	*terrestris*	Madagascar	Madagascar	Toliara		-22.97222222	43.61527778	2006	6034067
MBG-131	*Tribulus*	*terrestris*	Madagascar	Madagasca	Toliara		-	43.61555556	2006	6128855

ID	Herbarium name label	Re-classified taxon as	Region	Country	Province	Additional Locality Notes	Latitude	Longitude	Collection Year	MoBot Accession #
	terrestris			r			22.97222222			
MBG-132	*Tribulus terrestris*	*terrestris*	Middle East	Iraq	Baghdad	Poaghdad-Gaderia	33.318028	44.359143	1988	4339235
MBG-133	*Tribulus terrestris*	*terrestris*	Central America and Mexico	Mexico	San Luis Potosi	Eastern outskirts of city of San luis Potosi.	22.15647	-100.985541	1982	5573055
MBG-134	*Tribulus terrestris*	*terrestris*	Central America and Mexico	Mexico	Coahuila	Camino a Piedras Negras. Coahuila	28.691618	-100.540862	NA	5667507
MBG-135	*Tribulus terrestris*	*terrestris*	South America	Ecuador	Imbabura	0d.35m N| 78d.15m W	0.583336111	-78.25	1990	3854045
MBG-136	*Tribulus terrestris*	*terrestris*	South America	Argentina	San Luis	Depto. La Captial: cerca del límite con Mendoza, alrededores de Ruta Nacional	-7.727222222	-67.14305556	2011	6700615
MBG-137	*Tribulus terrestris*	*terrestris*	Southern Africa	Botswana			-21	22.4	1997	5782637
MBG-138	*Tribulus terrestris*	*terrestris*	Southern Africa	South Africa	East Cape		-31.47083333	27.04277778	1997	5782638
MBG-139	*Tribulus terrestris*	*terrestris*	Oceania	Australia	Simarloo, Property	South Aust. Simarloo, property.	-37.379672	146.048595	1976	2659968

Table S4. 3 Summary of complementary sequences and their geographic locations found on the NCBI database for *Tribulus sp*. The table shows the datasets and sample ID. It indicates which samples were used on which dataset, either *ITS*, or chloroplast (*ndhF, rpl32,* and *psbD*).

Accession number	Sample ID	Function	Species	Location	Country	ITS	Chloroplast markers
NC028023	OUT_001	Outgroup	*Larrea tridentata*	North America	USA	-	X
MF963813.1	OUT_002	Outgroup	*Kallstroemia californica*	North America	USA	X	-
MH699457	OUT_003	Outgroup	*Kallstroemia sp.*	North America	USA	X	-
AY260973	OUT_004	Outgroup	*Kallstroemia parviflora*	North America	USA	X	-
MH699439	OUT_005	Outgroup	*Balanites aegyptiaca*	Oceania	Australia	X	-
MH990661	OUT_006	Outgroup	*Balanites maughamii*	Oceania	Australia	X	-
NC043796	OUT_007	Outgroup	*Guaiacum angustifolium*	North America	USA	-	X
OL703321	OUT_008	Outgroup	*Balanites aegyptiaca*	Middle East	Saudi Arabia	-	X
MN164624.1	NCBI-001	Complement	*Tribulus terrestris*	Asia	China	-	X
KC593444	NCBI-002	Complement	*Tribulus terrestris*	Asia	China	-	X
MH699504	NCBI-003	Complement	*Tribulus longipetalus*	Asia	India	X	-
MH699485	NCBI-004	Complement	*Tribulus macropterus*	Middle East	Afghanistan	X	-
MH768348	NCBI-005	Complement	*Tribulus cistoides*	Asia	China	X	-
MH768349	NCBI-006	Complement	*Tribulus cistoides*	Asia	China	X	-
AY260972	NCBI-007	Complement	*Tribulus terrestris*	North America	USA	X	-
KP087777	NCBI-008	Complement	*Tribulus terrestris*	Asia	China	X	-
KX282460	NCBI-009	Complement	*Tribulus terrestris*	Middle East	Kuwait	X	-
KX282461	NCBI-010	Complement	*Tribulus terrestris*	Middle East	Kuwait	X	-
KX282462	NCBI-011	Complement	*Tribulus terrestris*	Middle East	Kuwait	X	-
MF440358	NCBI-012	Complement	*Tribulus terrestris*	Western Asia	Turkey	X	-
MH547545	NCBI-013	Complement	*Tribulus terrestris*	Middle East	Saudi Arabia	X	-
MH547526	NCBI-014	Complement	*Tribulus terrestris*	Middle East	Saudi Arabia	X	-
MG256344	NCBI-015	Complement	*Tribulus terrestris*	Middle East	Pakistan	X	-
MG256343	NCBI-016	Complement	*Tribulus terrestris*	Middle East	Pakistan	X	-
MG256342	NCBI-017	Complement	*Tribulus terrestris*	Middle East	Pakistan	X	-
MG256341	NCBI-018	Complement	*Tribulus terrestris*	Middle East	Pakistan	X	-
MG256340	NCBI-019	Complement	*Tribulus pentandrus*	Middle East	Pakistan	X	-
MG256339	NCBI-020	Complement	*Tribulus pentandrus*	Middle East	Pakistan	X	-
MG256338	NCBI-021	Complement	*Tribulus pentandrus*	Middle East	Pakistan	X	-
MG256337	NCBI-022	Complement	*Tribulus longipetalus*	Middle East	Pakistan	X	-
MG256336	NCBI-023	Complement	*Tribulus longipetalus*	Middle East	Pakistan	X	-
MG256335	NCBI-024	Complement	*Tribulus longipetalus*	Middle East	Pakistan	X	-
MK261309	NCBI-025	Complement	*Tribulus cistoides*	Africa	Kenya	X	-
MK261139	NCBI-026	Complement	*Tribulus cistoides*	Africa	Kenya	X	-
KR734183	NCBI-027	Complement	*Tribulus terrestris*	Africa	Kenya	X	-
KR734173	NCBI-028	Complement	*Tribulus terrestris*	Africa	Kenya	X	-
KR733998	NCBI-029	Complement	*Tribulus terrestris*	Africa	Kenya	X	-
KR733795	NCBI-030	Complement	*Tribulus terrestris*	Africa	Kenya	X	-
MK216501	NCBI-031	Complement	*Tribulus terrestris*	Asia	India	X	-

Accession number	Sample ID	Function	Species	Location	Country	ITS	Chloroplast markers
MH203153	NCBI-032	Complement	*Tribulus terrestris*	Western Asia	Bahrain	X	-
MG236002	NCBI-033	Complement	*Tribulus terrestris*	North America	Canada	X	-
MH809166	NCBI-034	Complement	*Tribulus terrestris*	Asia	China	X	-
MH809167	NCBI-035	Complement	*Tribulus terrestris*	Asia	China	X	-
MH809168	NCBI-036	Complement	*Tribulus terrestris*	Asia	China	X	-
DQ233661	NCBI-037	Complement	*Tribulus terrestris*	Asia	China	X	-
MK792324	NCBI-038	Complement	*Tribulus lanuginosus*	Asia	India	X	-
MK792325	NCBI-039	Complement	*Tribulus subramanyamii*	Asia	India	X	-
MK792326	NCBI-040	Complement	*Tribulus lanuginosus*	Asia	India	X	-
MK792327	NCBI-041	Complement	*Tribulus subramanyamii*	Asia	India	X	-
MK792328	NCBI-042	Complement	*Tribulus lanuginosus*	Asia	India	X	-
MK792329	NCBI-043	Complement	*Tribulus lanuginosus*	Asia	India	X	-
MK792330	NCBI-044	Complement	*Tribulus terrestris*	Asia	India	X	-
MK792331	NCBI-045	Complement	*Tribulus terrestris*	Asia	India	X	-
MK792332	NCBI-046	Complement	*Tribulus terrestris*	Asia	India	X	-
MK792333	NCBI-047	Complement	*Tribulus lanuginosus*	Asia	India	X	-
MK792334	NCBI-048	Complement	*Tribulus subramanyamii*	Asia	India	X	-
MK792335	NCBI-049	Complement	*Tribulus lanuginosus*	Asia	India	X	-
MK792336	NCBI-050	Complement	*Tribulus lanuginosus*	Asia	India	X	-
MK792337	NCBI-051	Complement	*Tribulus terrestris*	Asia	India	X	-
MK792338	NCBI-052	Complement	*Tribulus lanuginosus*	Asia	India	X	-
MK792339	NCBI-053	Complement	*Tribulus subramanyamii*	Asia	India	X	-
MK792340	NCBI-054	Complement	*Tribulus terrestris*	Asia	India	X	-
MK792341	NCBI-055	Complement	*Tribulus terrestris*	Asia	India	X	-
MW591544	NCBI-056	Complement	*Tribulus terrestris*	Asia	India	X	-

Appendix 4.2

Tribulus taxonomy criteria to re-classify herbarium samples. Excel file: **Tribulus taxonomy.**

Appendix 4.3

List of samples with DNA concentrations and the primers used to amplify each sample. Excel file: **Summary of DNA extractions.**

Appendix 4.4

Notes on the chloroplast markers ranked based on variability.

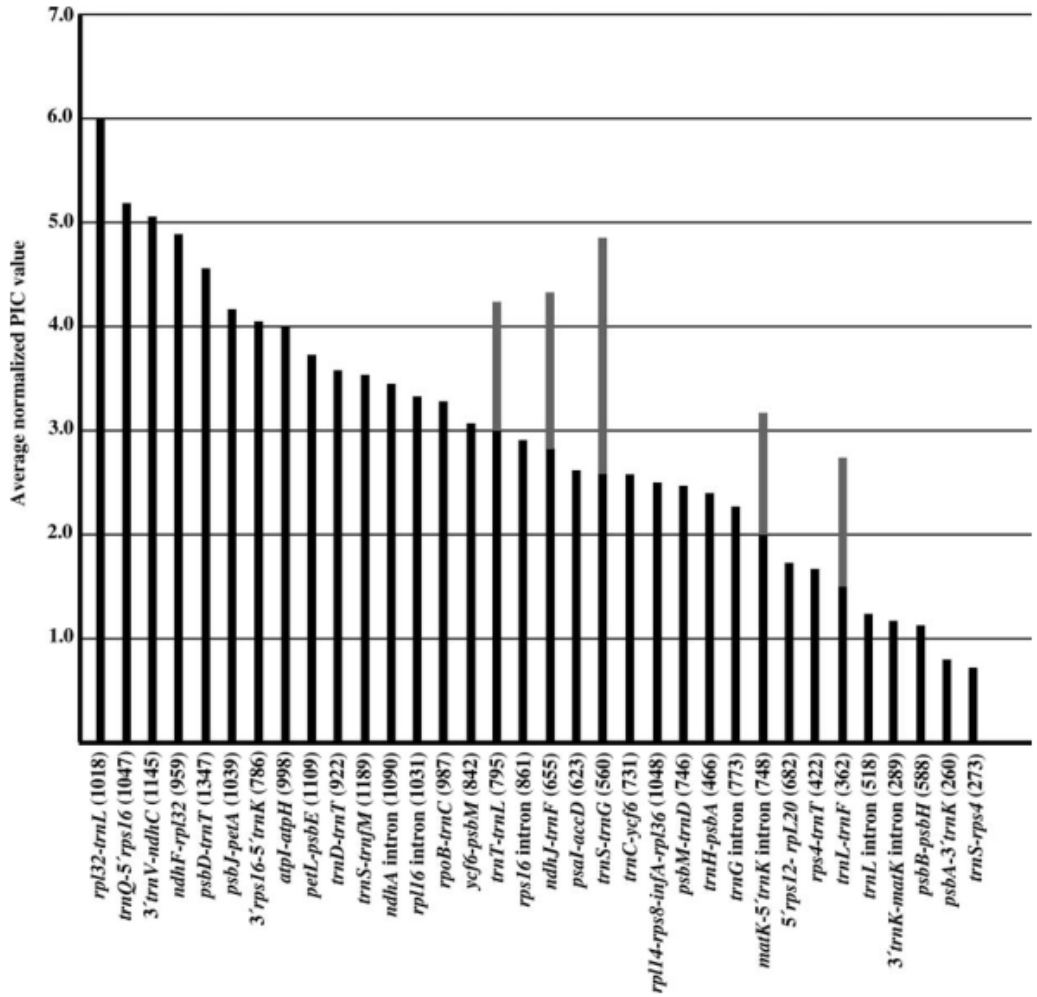


Fig. 4. The normalized PIC (potentially informative character) value of each of the 34 regions (21 are from Shaw et al., 2005, and 13 are from this study). The 34 regions are oriented from most to least number of PICs (left to right). Gray bars stacked on top of black bars indicate the normalized PIC value of often-combined regions. From left to right these are *trnT-trnL-trnL*, *ndhJ-trnF-trnL*, *trnS-trnG-trnG*, both halves of the *trnK* intron, and *trnL-trnL-trnF*. Numbers in parentheses indicate average length of the region.

1. ndhF-rpl32 (Shaw et al., 2007, p. 20)

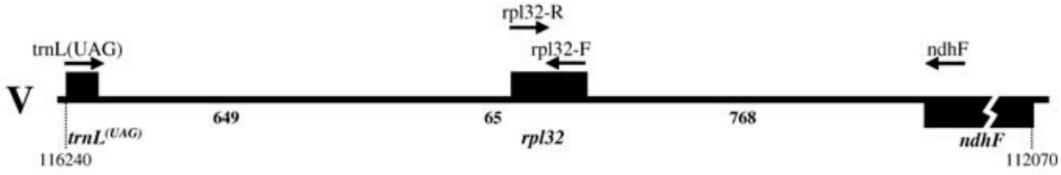
The ndhF-rpl32 intergenic spacer is in the SSC region of the chloroplast genome (Fig. 2V) that is adjacent to rpl32-trnL(UAG).

2. rpl32-trnL(UAG) (Shaw et al., 2007)

Unexplored regions up to the publication of the paper. This region is likely to offer more potential informative characters (PICs) than the best regions of Shaw et al. (2005). PICs are the number of nucleotide substitutions, indels and inversions. Having this marker to survey prior to beginning an all-out molecular sequencing study should ultimately lead to better resolved sequence-based studies and a more accurate dependent hypothesis. Potential to concatenate with ndhF-rpl32-trnL but this region is relatively long at 2kb. Located in the SSC region of the chloroplast genome. This is the best region of the 34 surveyed for low-level studies. Amplified with the ndhF-rpl32 intergenic spacer. Most informative according to the 2014 review (Shaw et al., 2014).
PCR conditions:

- 80°C for 5 min
- 30x (95°C for 1min, 50°C for 1min, ramp 0.3°C/s to 65°C, 65°C for 4 min)
- 65°C, 5min

Genome Location:



3. trnQ-5' - rps16 (Shaw et al., 2007)

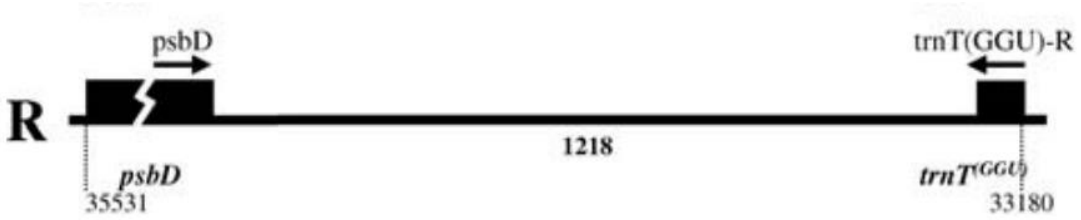
Offers a level of variability previously unseen in the chloroplast genome compared to Shaw (2005). It is the intergenic spacer located in the LSC region and was noted as highly variable by both Daniell et al (2006) and Timme et al. (2007) (As cited in Shaw (2007)). Multiple small tandem repeats (AT) or single nucleotide repeats (A/T). Useful for cpSSR studies.
PCR conditions:

- 80°C for 5 min
- 30x (95°C for 1min, 50°C for 1min, ramp 0.3°C/s to 65°C, 65°C for 4 min)
- 65°C, 5min

Genome Location:

4. psbD - trnT(GGU) (Shaw et al., 2007)- LSC

This region is likely to offer more potential informative characters (PICs) than the best regions of Shaw et al. (2005). PICs are the number of nucleotide substitutions, indels and inversions. Intergenic spaces are found in the LSC. Status: Amplified
Genome Location:



5. petL – psbE (Shaw et al., 2007) - LSC

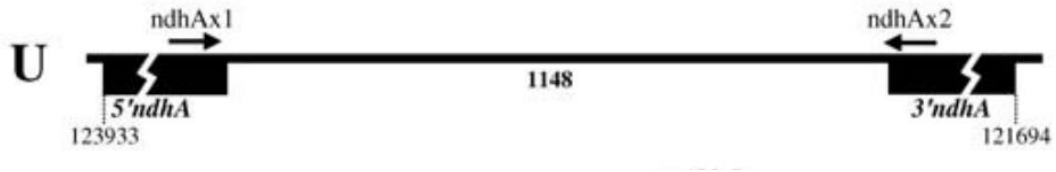
This region is likely to offer more potential informative characters (PICs) than the best regions of (Shaw et al., 2005). PICs are the number of nucleotide substitutions, indels and inversions. Is an intergenic spacer being a region of the LSC.
Genome Location:

6. ndhA intron (Shaw et al., 2007) - SSC

Amplified well in the gymnosperm taxa used in the study. The only gene of the SSC region with an intron. Fewer potential characters than *rpl16, trnT - trnL, trnL-trnF.* Ranked slightly above the median in number of PICs compared to other regions surveyed. Not statistically better than the *rpl16 intron.*
PCR conditions:

- 35x, denaturation 95C for 30s, primer annealing at 55C for 30s and primer extension at 72C for 2 minutes.

Genome Location:



7. rpL16 (Shaw et al., 2005)

This marker is considered a Tier 2. Other references mentioned here say this region has high sequence divergence in flowering plants (Wolfe et al, 1987). Primarily used for phylogenetic analysis at the infrageneric and familial levels. There have been some reports of variability within species, but only a few studies used it for intraspecific variation (Xu et al., 2000; Kimura et al., 2003) as cited in Shaw *et al.* (2005).
PCR specific conditions:

- 80°C for 5 min
- 30x (95°C for 1min, 50°C for 1min, ramp 0.3°C/s to 65°C, 65°C for 4 min)
- 65°C, 5min

8. trnL - F spacer (Wu et al., 2015)

Named in Taberlet *et al.* (1991) as the trnL (UAA)3' exon and trnF (GAA) spacer. Potentially very useful for evolutionary studies of related species and probably of populations of the same species (Taberlet et al., 1991). At least closely related species. Frequently used in other studies

PCR conditions

- 25uL reactions. 2 mM MgCl, 200uM dNTPs, 1pM primer, 0.025 U/uL Taq, 1 - 2uL DNA.
- 2 min 95C denaturation
- 35x of 30s at 95C, 20 - 50s at 52 - 56C for annealing, 1 min 30s at 72C for extension
- 72C for 10min

9. trnL intron (Wu et al., 2015)

The trnL (UAA) intron is probably less variable due to the fact it has catalytic properties and forms secondary structures (Taberlet et al., 1991). It could be more useful for evolutionary studies at higher taxonomic levels (Taberlet et al., 1991).

10. rcbL (Savolainen et al., 2000)

Used a reference for Zygophyllaceae. Zygophyllaceae is not well supported.

11. ITS (Simpson et al., 2004; Wu et al., 2015)

Primers from Wu used in their study of Zygophyllum. Useful for calibration points with this study. Variable nuclear marker.
Primers from Simpson *et al.* (2004) are more flexible, it allows for amplification of individual ITS regions.

Appendix 4.5

Phylogeny trees using all Galápagos individuals.

Below is the series of phylogenies produced in MrBayes, for all four markers (*ITS, ndhF, psbD,* and *rpl32*), using all Galápagos individuals. Due to sequence redundancy from some individuals, we filtered out and selected unique samples that represented all the haplotypes per island., see the table.

For the chloroplast markers we generated two sets of trees, one using only *Balanites* as an outgroup and the other using *Larrea* and *Guaiacum* (see Methods section for further details).

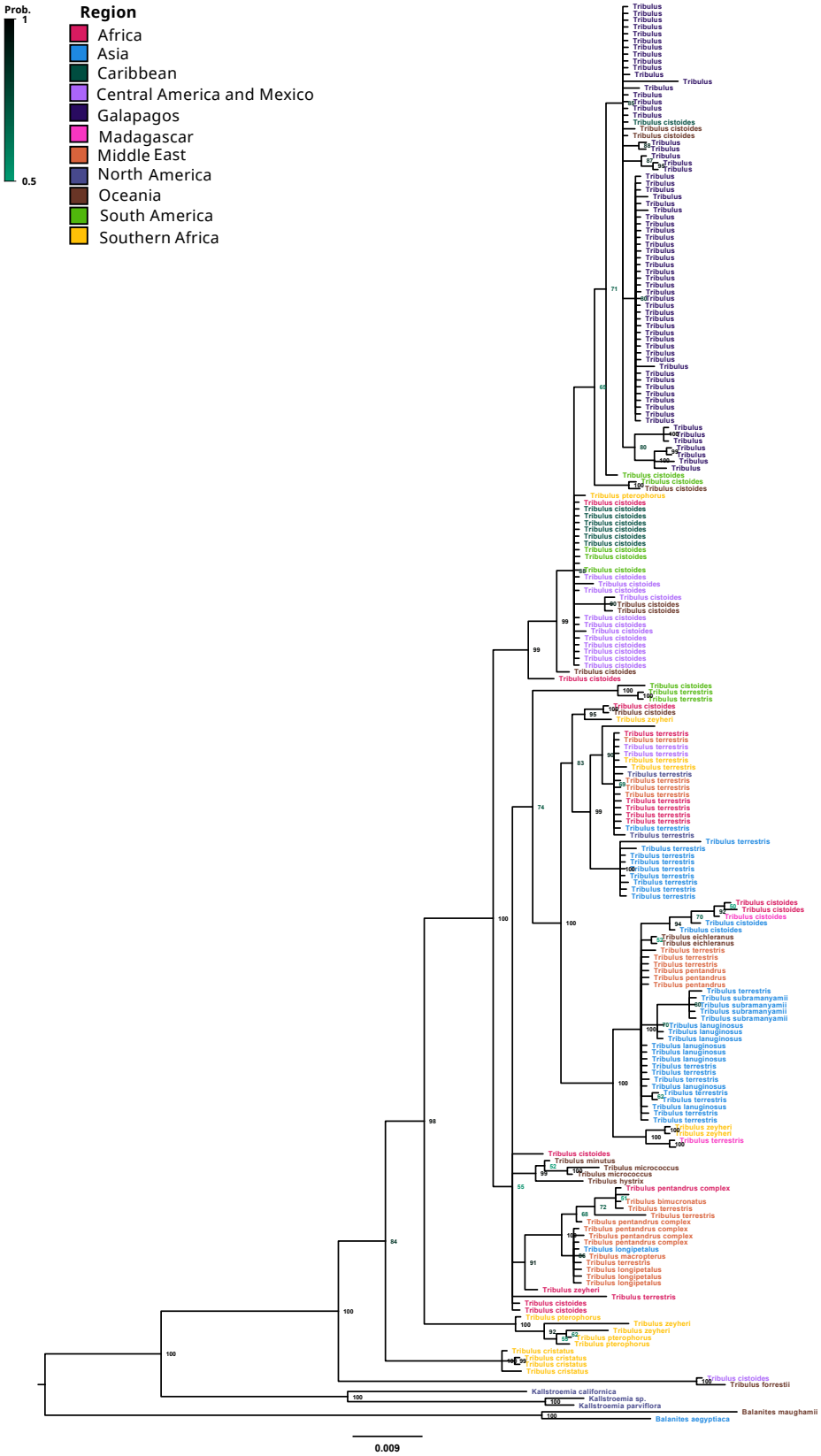


Figure S4. 1 ITS phylogeny tree including all Galápagos samples. Tree was produced in MrBayes.

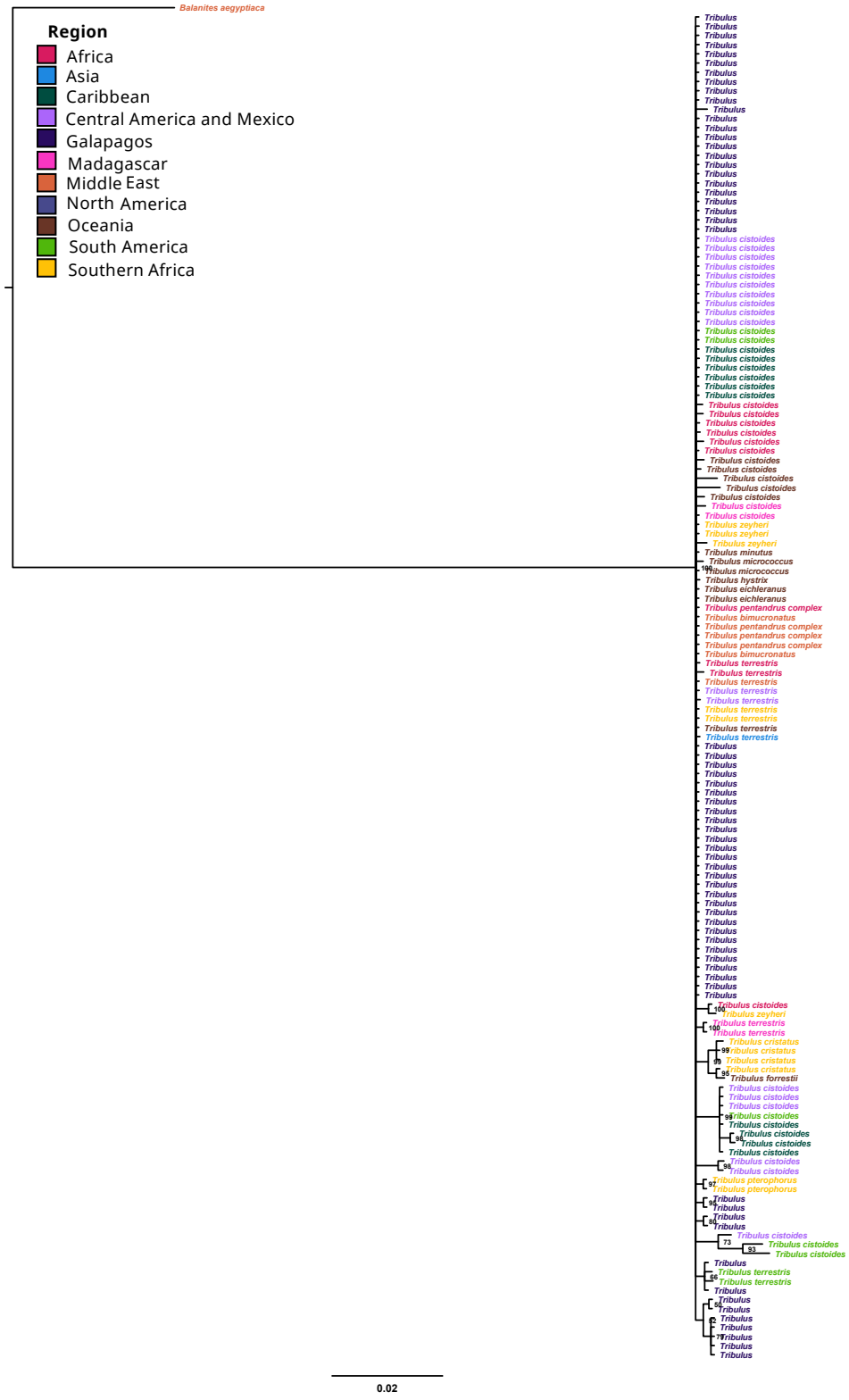


Figure S4. 2 *ndhF* tree including all Galápagos samples, using only Balanites as an outgroup.

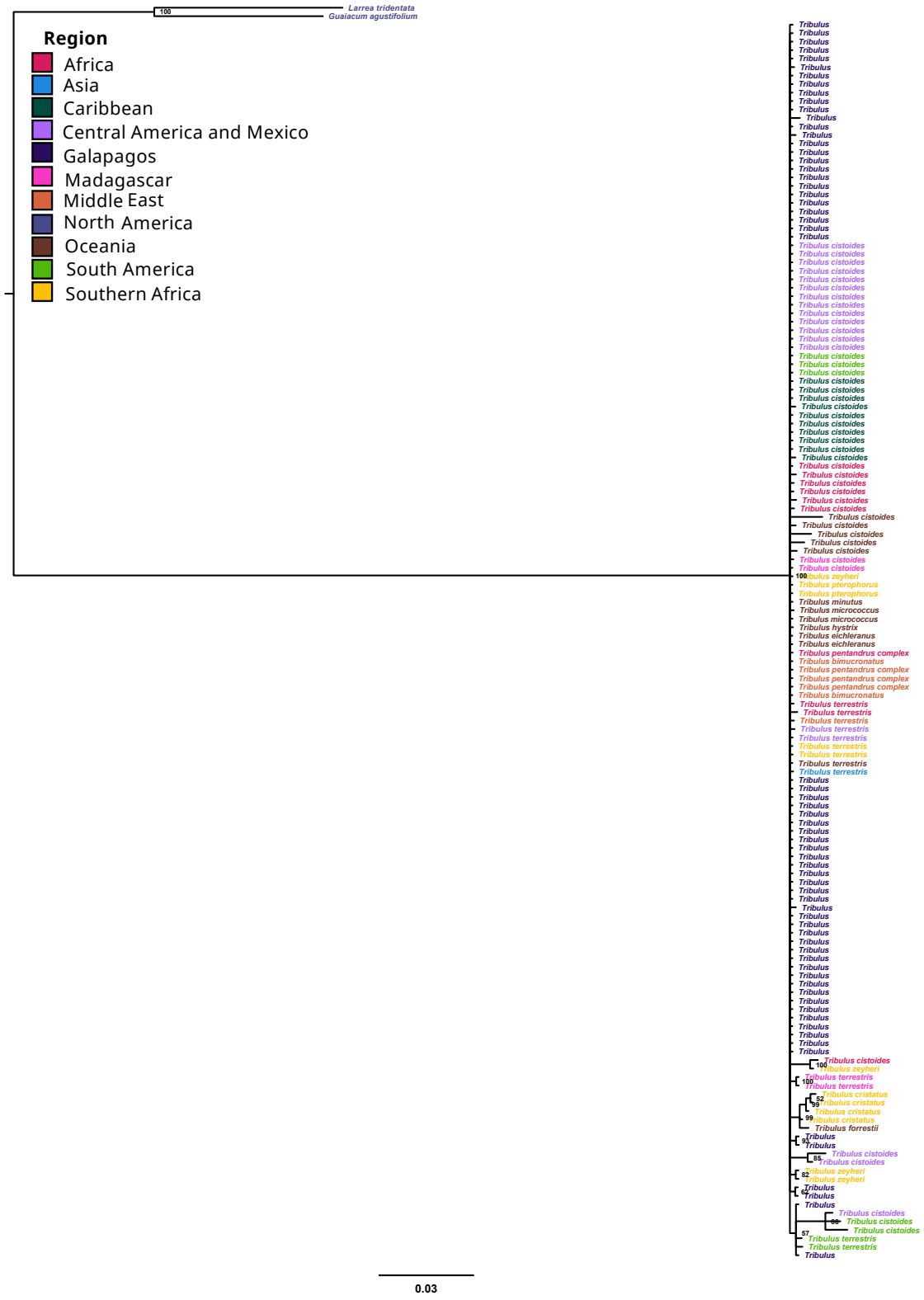


Figure S4. 3 *ndhF* tree including all Galápagos samples, using *Larrea* and *Guaiacum* as outgroups.

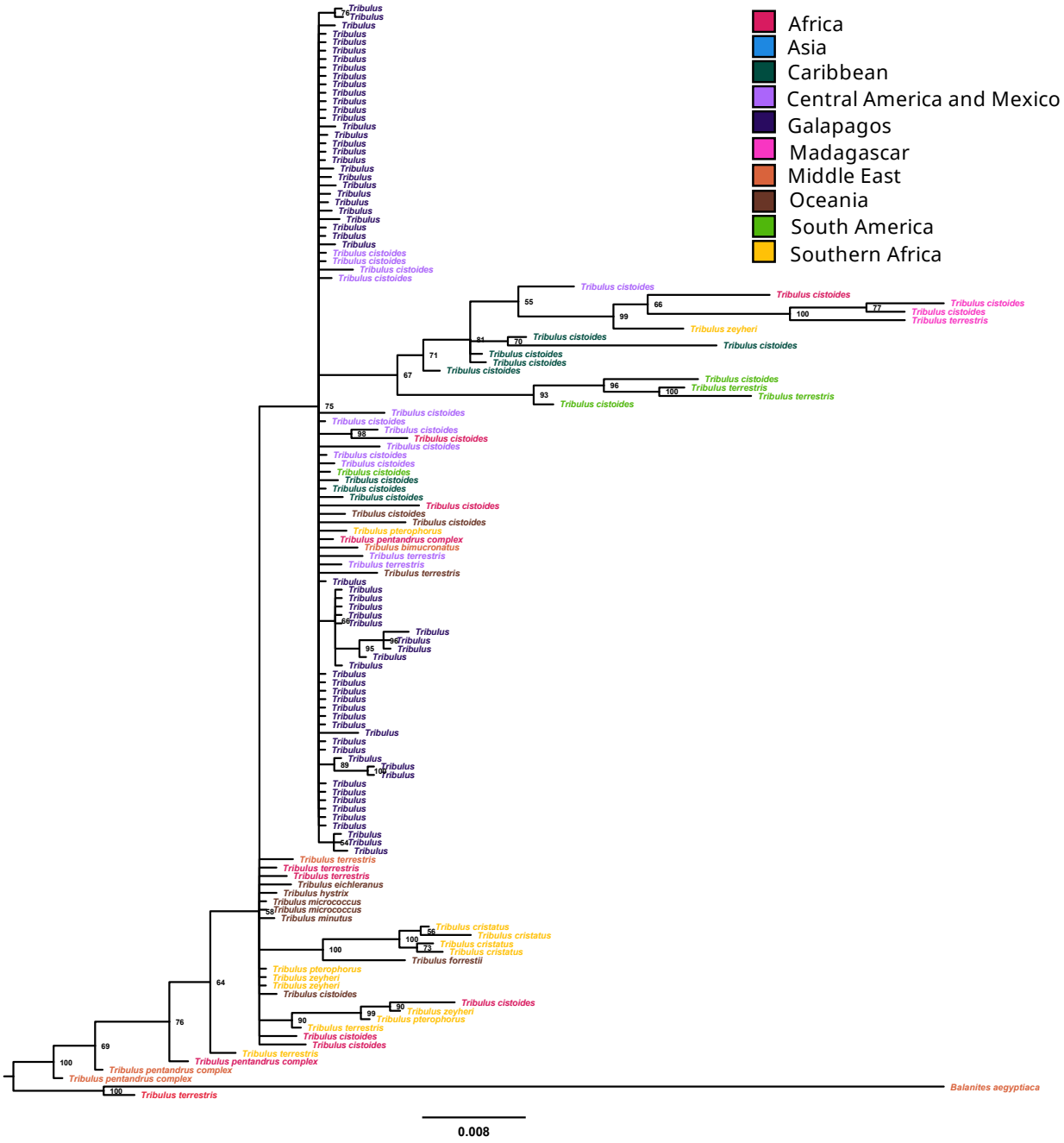


Figure S4. 4 *psbD* tree including all Galápagos samples, using only *Balanites* as an outgroup.

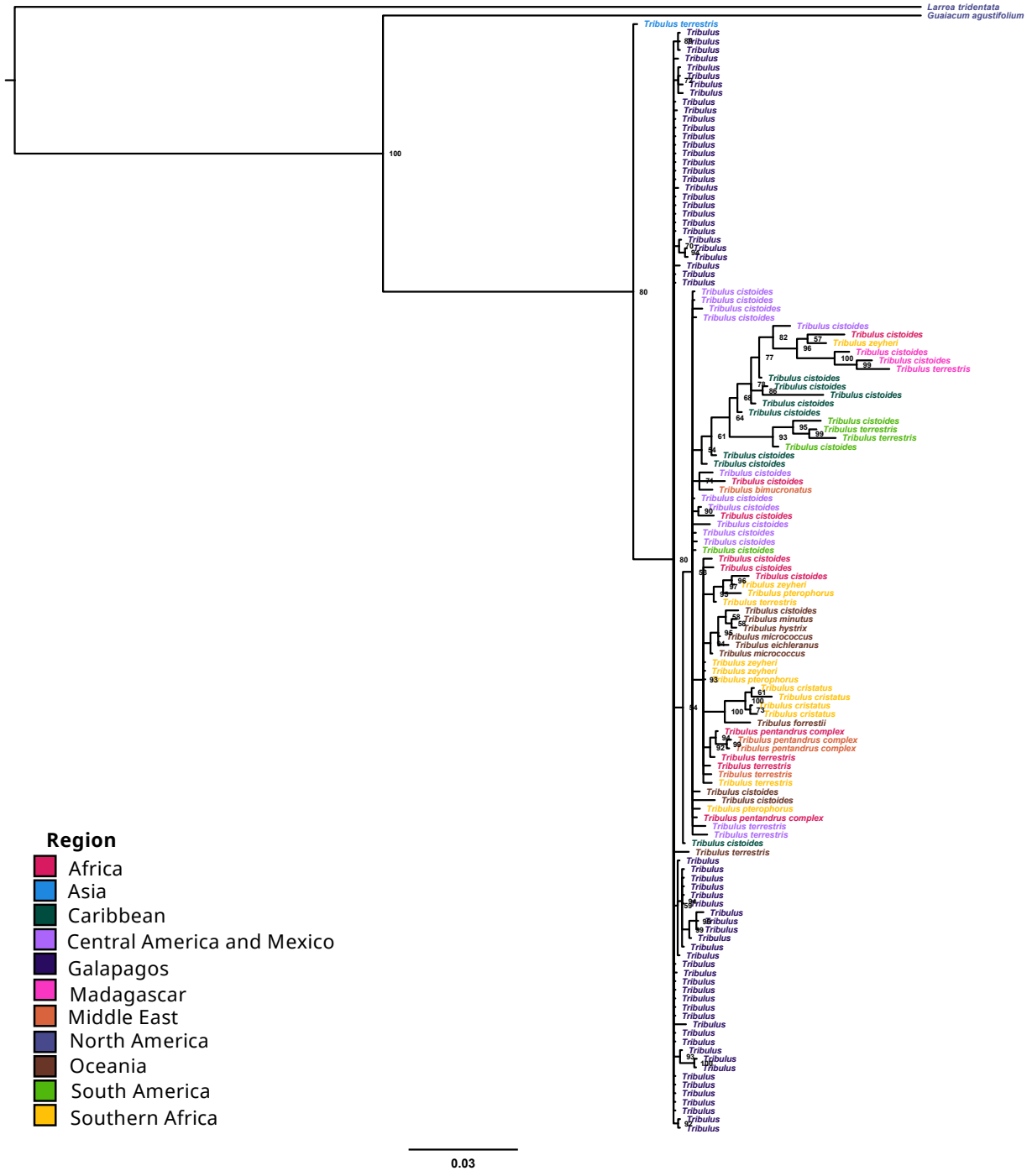


Figure S4. 5 *psbD* tree including all Galápagos samples, using *Larrea* and *Guaiacum* as outgroups.

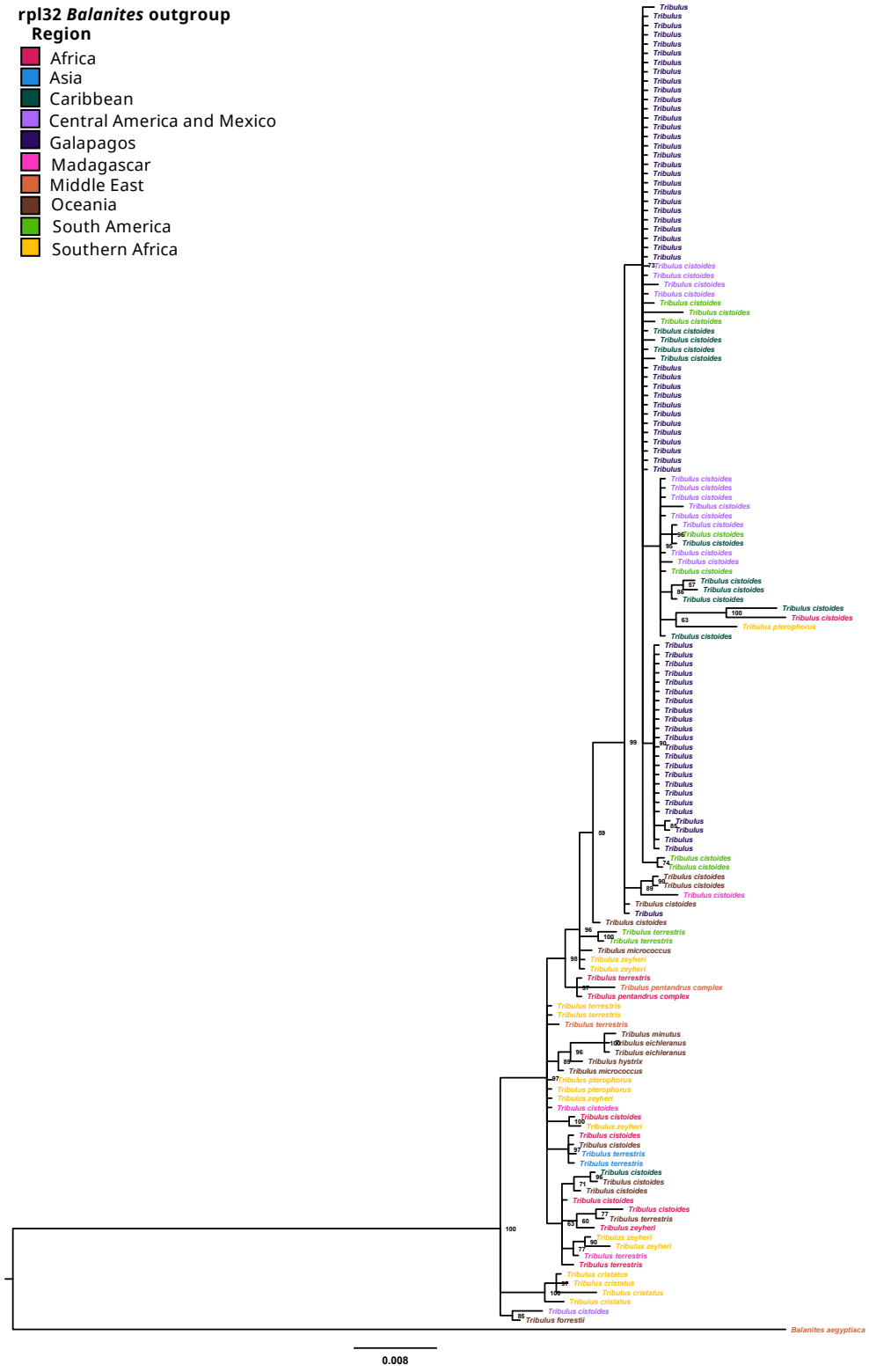


Figure S4. 6 *rpl32* tree including all Galápagos samples, using only *Balanites* as an outgroup.

Figure S4. 7 *rpl32* tree including all Galápagos samples, using Larrea and Guaiacum as outgroups.

Galápagos unique haplotype sequences

Table S4. 4 The table below shows the list of unique samples used for the individual phylogenies per marker. The sequences differences can be observed in the alignment files.

Markers				**Island**
ITS	***ndhF***	***psbD***	***rpl32***	
CHA_001	CHA_001	CHA_001	CHA_001	Champion
	CHA_003	CHA_002	CHA_004	
		CHA_003		
		CHA_004		
		CHA_005		
ESP_001	ESP_001	ESP_001	ESP_001	Espanola
ESP_004	ESP_002	ESP_002	ESP_003	
ESP_005	ESP_004	ESP_004		
ESP_007	ESP_011	ESP_005		
ESP_009		ESP_007		
FER_001	FER_001	FER_001	FER_001	Fernandina
FER_002	FER_002	FER_002	FER_003	
	FER_003	FER_003		
		FER_004		
FLO_001	FLO_001	FLO_001	FLO_001	Floreana
FLO_004	FLO_009	FLO_003		
FLO_005		FLO_005		
FLO_010		FLO_009		
		FLO_010		
GEN_001	GEN_001	GEN_001	GEN_001	Genovesa
GEN_002		GEN_002		
ISA_001	ISA_001	ISA_001	ISA_001	Isabela
ISA_003	ISA_002	ISA_002	ISA_003	
ISA_004	ISA_004	ISA_004	ISA_004	
RAB_001	RAB_001	RAB_001	RAB_001	Rabida
RAB_005		RAB_003	RAB_004	
RAB_009		RAB_004		
SAN_002	SAN_001	SAN_001	SAN_001	Santiago
SAN_004	SAN_007	SAN_003	SAN_002	
SCR_001	SCR_001	SCR_002	SCR_002	San Cristobal
SCR_003	SCR_004	SCR_003	SCR_003	
SCR_004	SCR_007			
SCZ_001	SCZ_001	SCZ_001	SCZ_001	Santa Cruz
SCZ_003	SCZ_002	SCZ_002	SCZ_002	
	SCZ_003	SCZ_006		
SYN_003	SYN_001	SYN_002	SYN_001	Seymour Norte
SYN_004		SYN_003	SYN_003	
		SYN_004		

Appendix 4.6

This appendix includes the phylogenies per marker (*ITS, ndhF, psbD, rpl32*) generated in MrBayes, using only samples that represent the unique haplotypes from Galápagos.

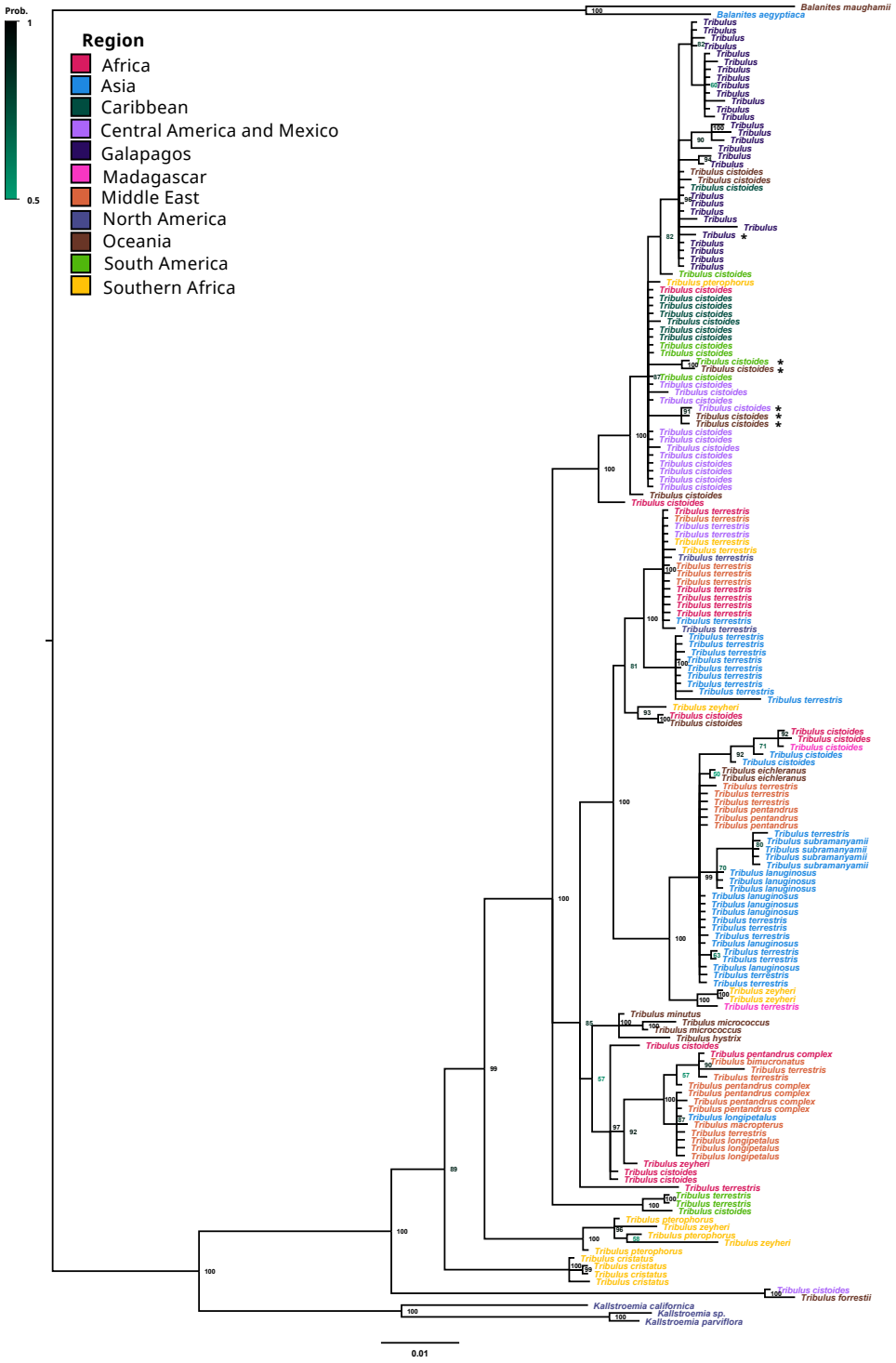


Figure S4. 8 MrBayes tree for *ITS* regions. The tree includes only the haplotype representatives for Galápagos. Star marked samples were the ones removed from the haplotype network.

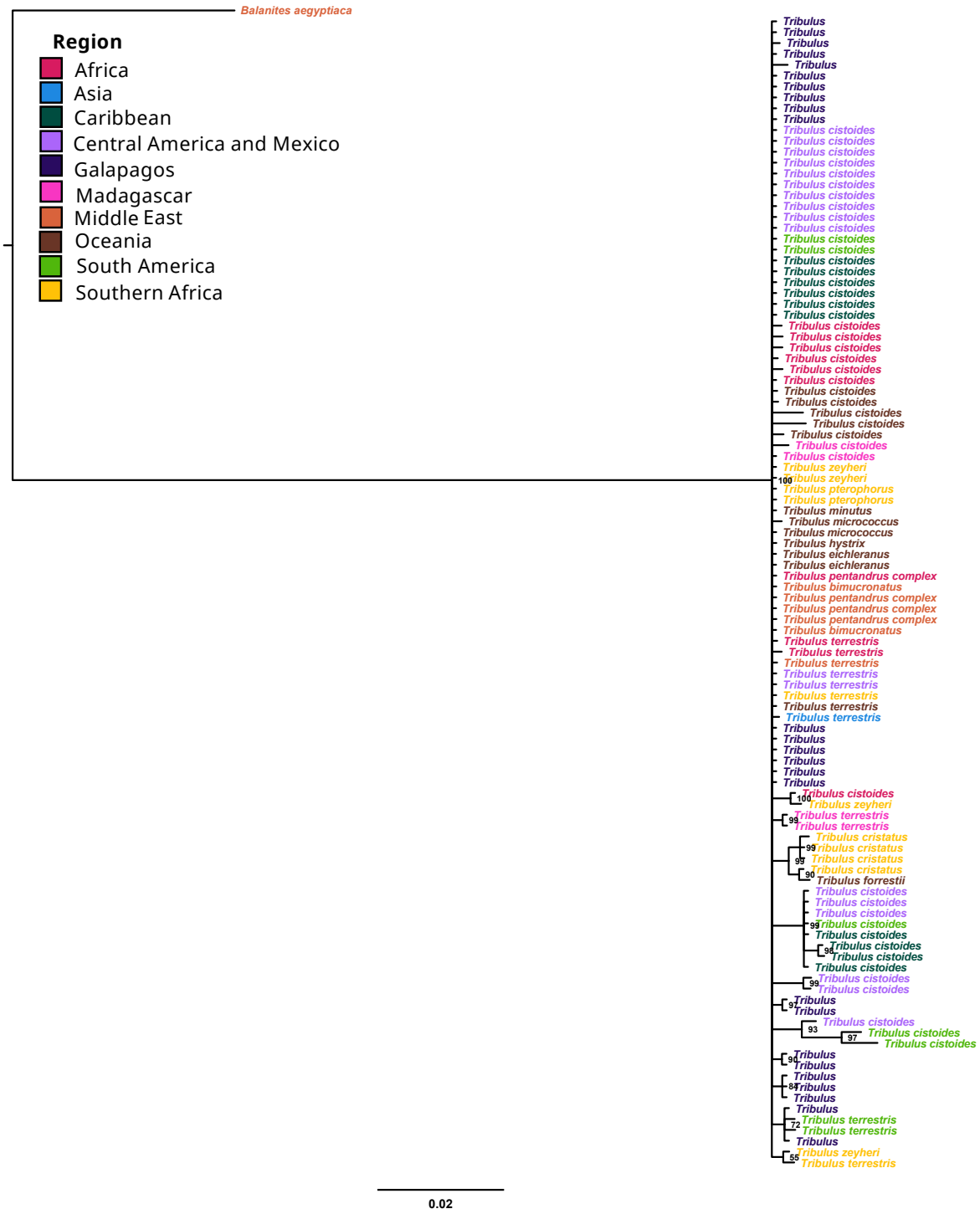


Figure S4. 9 MrBayes tree for *ndhF*. The tree includes only the haplotype representatives for Galápagos. The tree uses only the *Balanites* outgroup.

Figure S4. 10 MrBayes tree for *ndhF*. The tree includes only the haplotype representatives for Galápagos. The tree uses *Larrea* and *Guaiacum* as outgroups.

Figure S4. 11 MrBayes tree for *psbD*. The tree includes only the haplotype representatives for Galápagos. The tree uses only the *Balanites* outgroup.

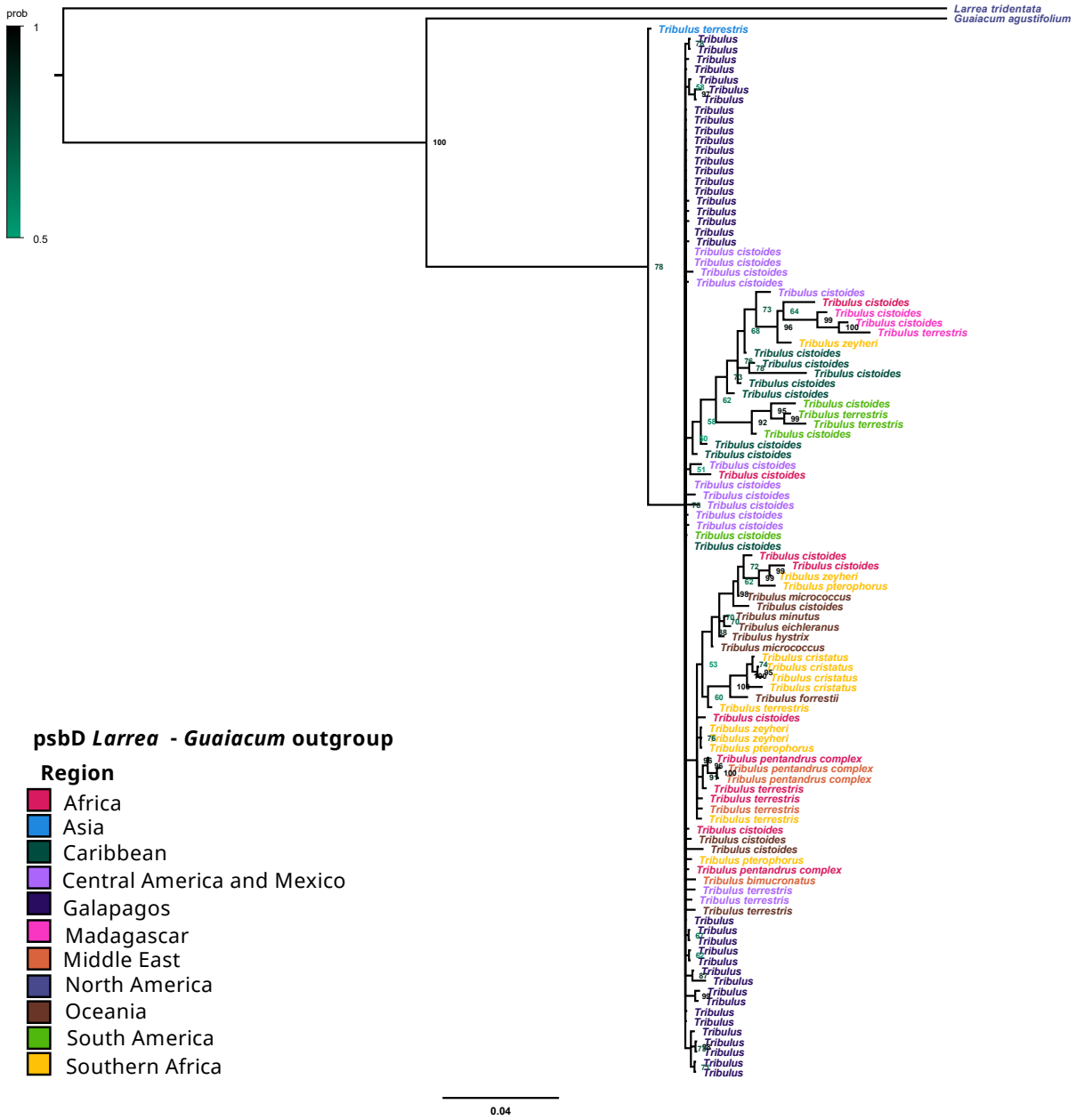


Figure S4. 12 MrBayes tree for *psbD*. The tree includes only the haplotype representatives for Galápagos. The tree uses *Larrea* and *Guaiacum* as outgroups.

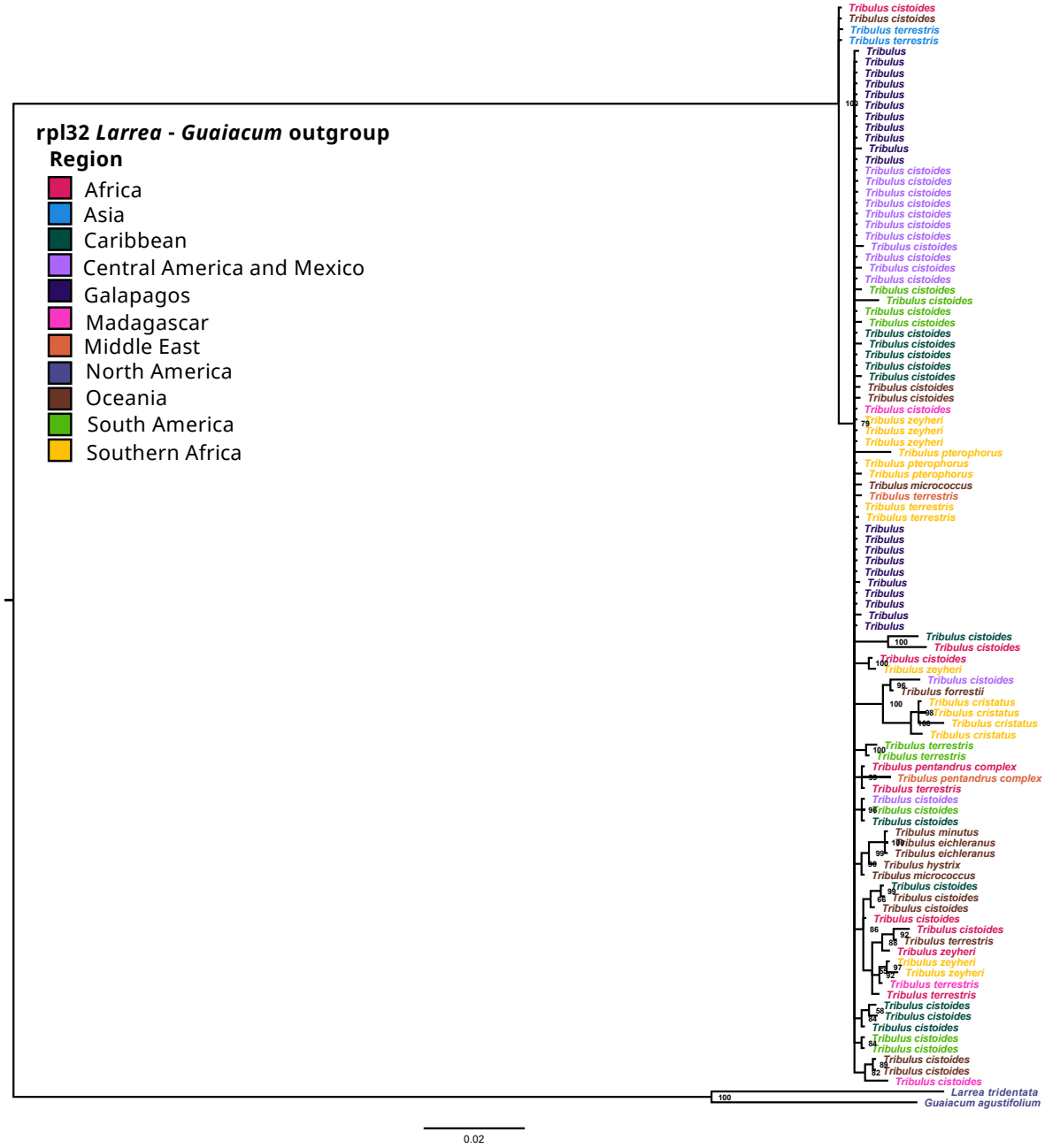


Figure S4. 13 MrBayes tree for *rpl32*. The tree includes only the haplotype representatives for Galápagos. The tree uses *Larrea* and *Guaiacum* as outgroups.

Figure S4. 14 MrBayes tree for *rpl32*. The tree includes only the haplotype representatives for Galápagos. The tree uses only the *Balanites* outgroup.

Appendix 4.7

Substitution model selection results from jmodeltest. The table shows the top 3 results based on model selection criteria for each marker alignment. Excel file: **jmodeltest results.**

Appendix 4.8

This Appendix includes the samples and results for concatenated analysis. We used samples that were amplified for all 4 markers. This includes a summary list on an Excel file that contains the samples used First sheet, shared samples across markers. Second sheet, samples missing from each marker Excel file: **Concatenated sample list.**

Appendix 4.9

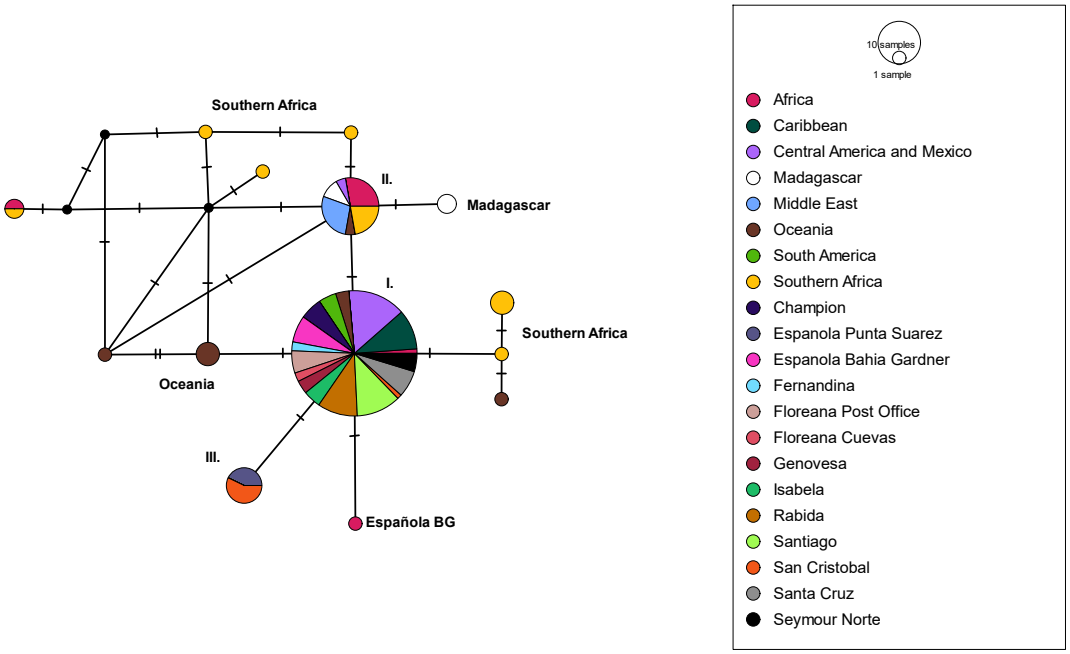


Figure S4. 15 Haplotype networks using the *ndhF* gene and all Galápagos samples from the *Tribulus cistoides* clade. The network was made using the Integer Neighbor Joining method with an alpha of 0.5. This method shows intermediate haplotypes (black nodes) that better connect the samples used. Node size is relative to the number of samples on that node. Branches connecting the nodes show the number of mutations as crossed lines. The *ndhF* network shows three main nodes. Node I. Groups samples from Africa and the Middle East. Node II. Groups samples from Galápagos, Central America, Caribbean, and Oceania. Node III. Groups samples from Galápagos, specifically, Española Island (Punta Suárez) and San Cristóbal Island. A map of the Galápagos Archipelago is shown in Figure 3.

Table S4. 5 Nucleotide diversity summary for the *ndhF* haplotype network

Region analysis							
Population	*N*	*S*	*H*	*Hd*	*π (%)*	*Tajima's D*	*Fu's Fs*
All Samples	131	19	21	0.607 ± 0.047	0.31%	-1.7301	-15.946
Non-Galápagos	67	17	18	0.768 ± 0.04	0.44%	-1.3885	-10.228
Galápagos	64	6	8	0.477 ± 0.075	0.14%	-1.1662	-4.46
Island specific analysis							
Population	*N*	*S*	*H*	*Hd*	*π (%)*	*Tajima's D*	*Fu's Fs*
Champion	5	1	2	0.6 ± 0.175	0.12%	1.2247	0.626
Española	9	4	4	0.694 ± 0.147	0.24%	-0.8426	-0.722
Fernandina	2	2	2	1 ± 0.5	0.41%	n.d.	0.693
Floreana	7	1	2	0.286 ± 0.196	0.06%	-1.0062	-0.095
Genovesa	3	0	1	0	0.00%	n.d.	n.a.
Isabela	4	1	2	0.5 ± 0.265	0.10%	-0.6124	0.172
Rabida	9	0	1	0	0.00%	n.d.	n.a.
San Cristóbal	4	0	1	0	0.00%	n.d.	n.a.
Santa Cruz	6	1	2	0.533 ± 0.172	0.11%	0.8506	0.625
Santiago	10	1	2	0.2 ± 0.154	0.04%	-1.1117	-0.339
Seymour Norte	4	0	1	0	0.00%	n.d.	n.a.

Table S4. 6 Analysis of molecular variance (AMOVA) using the *ndhF* region of *Tribulus cistoides*. Statistics: F_{CT} = 0.148, F_{SC} = 0.081, and F_{ST} = 0.218. After 1000 permutations, values for F_{CT}, F_{SC} and F_{ST} were significant (p = 0.004, 0.021 and <0.001 respectively); df = degrees of freedom.

Genetic differentiation	**Df**	**Sum of squares**	**σ^2**	**% variation**
Among groups (Galápagos/Non-Galápagos samples)	1	10.412	0.142	14.82
Among populations (Locations/Islands)	19	21.752	0.066	6.94
Within populations	108	80.867	0.749	78.25
Total	128	113.031	0.957	

Table S4. 7 Haplotype variations of all the sequences evaluated. Variations were classified using haplotype 1 as a base. Locations in the sequences are described and classified accordingly for the 21 haplotypes of *ndhF*.

	Base location relative to haplotype 1																		
Haplotype	29	46	76	81	88	91	136	141	212	249	293	295	310	344	377	389	395	503	515
Hap_1	T	A	A	T	T	T	A	C	G	C	A	T	A	A	T	G	A	T	T
Hap_2	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
Hap_4	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_5	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_6	.	.	T	.	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_7	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	.
Hap_8	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
Hap_9	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_10	A	.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	.
Hap_11	.	.	.	.	A	.	.	.	.	.	.	.	T	C	G	.	C	.	.
Hap_12	.	.	.	.	A	.	.	.	.	.	.	.	C	.	G	.	.	.	.
Hap_13	.	.	.	.	A	.	.	.	.	.	.	A	T	.	G	.	.	.	.
Hap_14	.	.	.	.	A	.	.	.	.	.	T	.	T	.	G	.	.	.	.
Hap_15	.	.	.	.	A	.	.	.	.	.	T	.	.	.	G	.	.	.	.
Hap_16	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.
Hap_17	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
Hap_18	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
Hap_19	.	.	.	.	.	.	G	.	.	T	.	.	.	.	.	.	.	.	.
Hap_20	.	.	.	.	A	.	.	G	.	.	.	.	.	.	G	.	.	.	.
Hap_21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A

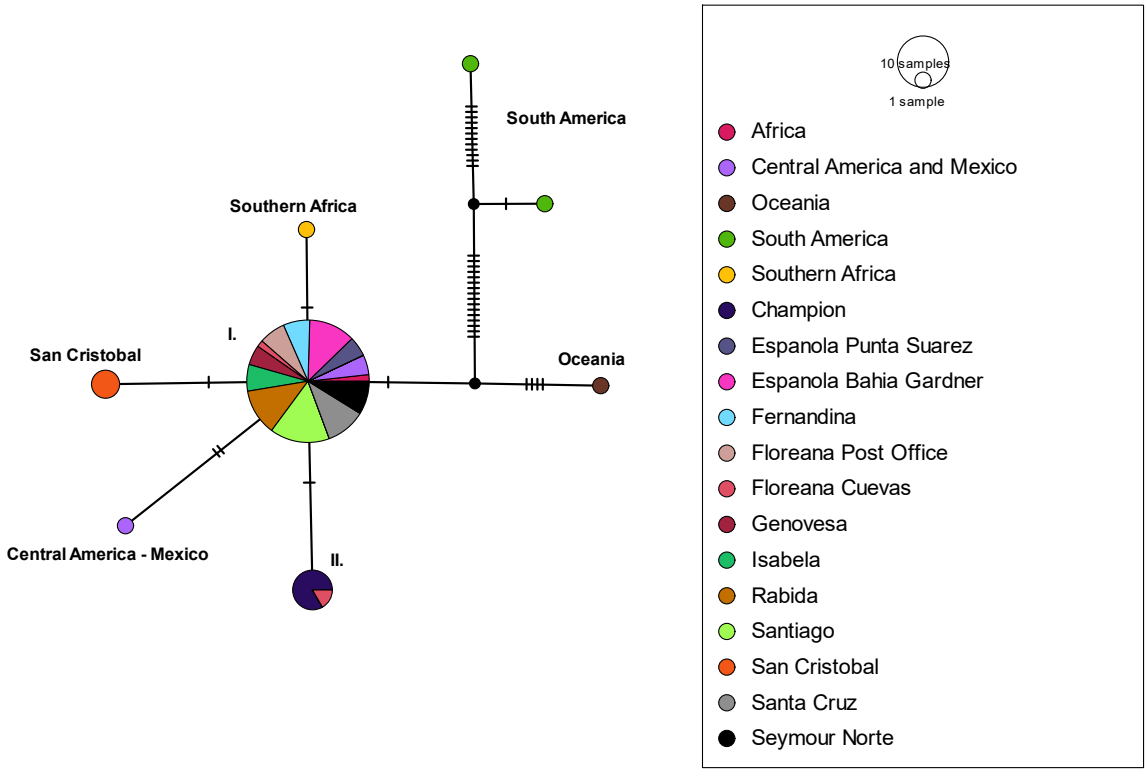


Figure S4. 16 Haplotype networks using the *psbD* gene and all Galápagos samples from the *Tribulus cistoides* clade. The network was made using the Integer Neighbor Joining method with an alpha of 0.5. This method shows intermediate haplotypes (black nodes) that better connect the samples used. Node size is relative to the number of samples on that node. Branches connecting the nodes show the number of mutations as crossed lines. The *psbD* network has one main node that groups all samples from Galápagos and Central America – Mexico and Oceania. There is another node for San Cristóbal Island. This network also differentiates samples from the Caribbean, the South American continent, Madagascar, and Southern Africa.

Table S4. 8 Nucleotide diversity summary for the *psbD* haplotype network.

Region Analysis							
Population	*N*	*S*	*H*	*Hd ± sd*	*π (%)*	*Tajima's D*	*Fu's Fs*
All Sequences	71	36	8	0.351 ± 0.071	0.14%	-2.4575	-0.362
Non-Galápagos	9	38	6	0.833 ± 0.127	0.94%	-0.6265	2.061
Galápagos	62	2	3	0.262 ± 0.069	0.02%	-0.6159	-0.712
Island Analysis							
Population	*N*	*S*	*H*	*Hd ± sd*	*π (%)*	*Tajima's D*	*Fu's Fs*
Champion	5	1	2	0.6 ± 0.175	0.04%	1.2247	0.626
Española	10	1	2	0.467 ± 0.132	0.04%	0.8198	0.818
Fernandina	4	1	2	0.5 ± 0.265	0.04%	-0.6124	0.172
Floreana	6	4	5	0.933 ± 0.122	0.13%	-0.0572	-2.429
Genovesa	3	1	2	0.667 ± 0.314	0.05%	n.d.	0.201
Isabela	4	2	3	0.833 ± 0.222	0.07%	-0.7099	-0.887
Rabida	7	1	2	0.476 ± 0.171	0.04%	0.559	0.589
San Cristóbal	3	3	2	0.667 ± 0.165	0.15%	n.d.	1.609
Santa Cruz	6	4	3	0.6 ± 0.314	0.10%	-1.295	0.297
Santiago	9	2	3	0.556 ± 0.215	0.05%	-0.0638	-0.239
Seymour	5	5	4	0.9 ± 0.161	0.21%	1.124	-0.445

Table S4. 9 Analysis of molecular variance (AMOVA) using the *psbD* region of *Tribulus cistoides*. Statistics: F_{CT} = 0.392, F_{SC} = 0.893, and F_{ST} = 0.935. After 1000 permutations, values for F_{CT} were significant (p = 0.001). Values for F_{SC} were non-significant (p = 0.076) and values for F_{ST} were significant (p = < 0.001); df = degrees of freedom.

Genetic differentiation	**Df**	**Sum of squares**	**σ^2**	**% variation**
Among groups (Galápagos/Non-Galápagos samples)	1	200.253	10.034	39.16
Among populations (Locations/Islands)	16	902.522	13.927	54.36
Within populations	53	88	1.666	6.48
Total	70	1190.775	25.621	

Table S4. 10 Haplotype variations of *psbD*. Variations were classified using haplotype 1 as a base. Locations in the sequences are described and classified accordingly.

	Base location relative to haplotype 1																																		
Haplotypes	170	187	215	240	272	282	298	316	323	395	408	423	431	460	468	475	483	497	501	546	548	587	606	612	615	625	667	669	672	694	696	711	727	735	803	1087
Hap_1	T	T	T	A	A	A	T	A	T	A	A	T	T	T	T	T	A	A	A	A	A	T	G	T	A	A	T	T	T	A	T	T	T	A	T	G
Hap_2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_4	G	C	C	.	.	G	G	C	G	G	T	C	C	G	G	G	.	G	C	C	C	A	T	G	G	G	C	C	C	.	.	G	C	T	G	.
Hap_5	G	C	C	T	.	G	G	C	G	G	.	C	.	G	G	G	.	G	.	.	.	.	T	G	.	.	C	C	.	.	.	.	.	T	.	.
Hap_6	.	.	.	.	G	.	.	.	.	G	.	.	.	.	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	C	A
Hap_7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.
Hap_8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.	.	.	.	.

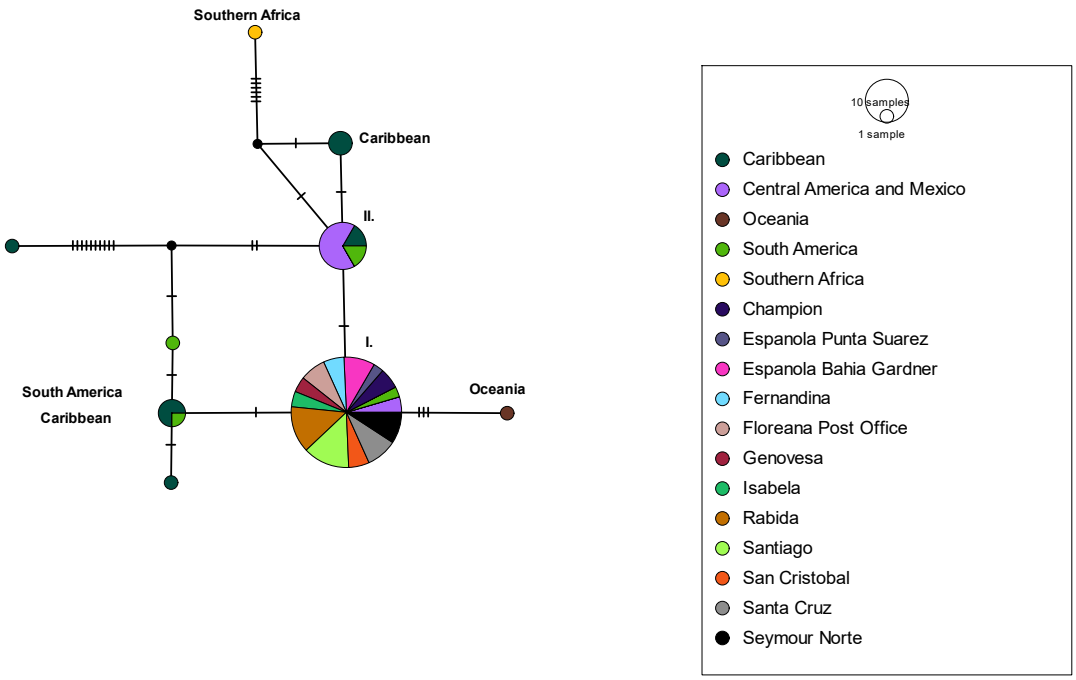


Figure S4. 17 Haplotype networks using the *rpl32* gene and all Galápagos samples from the *Tribulus cistoides* clade. The network was made using the Integer Neighbor Joining method with an alpha of 0.5. This method shows intermediate haplotypes (black nodes) that better connect the samples used. Node size is relative to the number of samples on that node. Branches connecting the nodes show the number of mutations as crossed lines. The *rpl32* network shows two main nodes. Node I. groups all Galápagos samples with some Central America – Mexico and South American continent samples. Node II: Groups most of Central America and Mexico samples with samples of the Caribbean and South America.

Table S4. 11 Nucleotide diversity summary for the *rpl32* haplotype network.

Region Analysis							
Population	*N*	*S*	*H*	*Hd ± sd*	*π (%)*	*Tajima's D*	*Fu's Fs*
All Sequences	90	30	13	0.483 ± 0.063	0.18%	-2.2832	-4.811
Non-Galápagos	29	31	15	0.909 ± 0.043	0.46%	-1.759	-4.77
Galápagos	61	1	2	0.064 ± 0.039	0.01%	-0.8926	-1.056
Island Analysis							
Population	*N*	*S*	*H*	*Hd ± sd*	*π (%)*	*Tajima's D*	*Fu's Fs*
Champion	4	0	1	0	0.00%	n.d.	n.a.
Española	8	0	1	0	0.00%	n.d.	n.a.
Fernandina	4	1	2	0.5 ± 0.265	0.06%	-0.6124	0.172
Floreana	5	0	1	0	0.00%	n.d.	n.a.
Genovesa	3	0	1	0	0.00%	n.d.	n.a.
Isabela	3	0	1	0	0.00%	n.d.	n.a.
Rabida	9	0	1	0	0.00%	n.d.	n.a.
San Cristóbal	4	0	1	0	0.00%	n.d.	n.a.
Santa Cruz	7	1	2	0.038 ± 0.196	0.03%	-1.006	-0.095
Santiago	9	0	1	0	0.00%	n.d.	n.a.
Seymour	6	1	2	0.533 ± 0.172	0.06%	0.8506	0.625

Table S4. 12 Analysis of molecular variance (AMOVA) using the *rpl32* region of *Tribulus cistoides*. Statistics: F_{CT} = 0.051, F_{SC} = 0.245, and F_{ST} = 0.284. After 1000 permutations, values for F_{CT} were significant (p = 0.017). Values for F_{SC} were non-significant (p = 0.079) and values for F_{ST} were significant (p = 0.020); df = degrees of freedom.

Genetic differentiation	**Df**	**Sum of squares**	**σ^2**	**% variation**
Among groups (Galápagos/Non-Galápagos samples)	1	14.631	0.164	5.14
Among populations (Locations/Islands)	15	89.799	0.74	23.22
Within populations	73	166.615	2.282	71.63
Total	89	271.044	3.186	

Table S4. 13 Haplotype variations of *rpl32*. Variations were classified using haplotype 1 as a base. Locations in the sequences are described and classified accordingly.

	Base location relative to haplotype 1																					
Haplotype	10	11	14	18	52	120	182	250	280	323	338	342	359	402	549	566	588	599	600	693	715	758
Hap_1	G	G	A	T	C	T	C	T	T	A	T	C	G	G	C	A	A	T	T	A	A	A
Hap_2	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
Hap_3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
Hap_4	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.
Hap_5	.	.	.	.	.	.	A	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
Hap_6	.	.	G	A	G	C	.	C	.	C	C	T	.	A	T	C	G	A	.	.	.	.
Hap_7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	.	.
Hap_8	A	A	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
Hap_9	.	.	.	.	.	.	T	.	C	C	.	T	A	.	.	.	.	.	.	T	T	C

Table S4. 14 List of samples from the *Tribulus cistoides* clade for each maker that were removed by the haplotype network analysis. The table shows the country and locations of the removed samples.

Haplotype Network Removed Samples			
ITS			
Label	**Region**	**Country**	**Location**
FLO_003	Galápagos	Ecuador	Floreana
FLO_004	Galápagos	Ecuador	Floreana
FLO_009	Galápagos	Ecuador	Floreana
MBG_036	Central America and Mexico	Mexico	Quintana Roo
MBG_049	South America	Venezuela	Federal
MBG_074	Oceania	Australia	Queensland
MBG_075	Oceania	Australia	Queensland
MBG_077	Oceania	Republic of Kiribati	Sidney Island
ndhF			
Label	**Region**	**Country**	**Location**
FER_001	Galápagos	Ecuador	Fernandina
MBG_028	Central America and Mexico	Mexico	Chiapas
MBG_031	Central America and Mexico	Mexico	Jalisco
MBG_036	Central America and Mexico	Mexico	Quintana Roo
MBG_037	Central America and Mexico	Mexico	Vera Cruz
MBG_039	Central America and Mexico	Guatemala	Chiquimula
MBG_050	South America	Venezuela	Vargas
MBG_052	South America	Venezuela	Falcon
MBG_056	Caribbean	Dominican Republic	Pedernales
MBG_069	Africa	Tanzania	Pangani District
MBG_075	Oceania	Australia	Queensland
MBG_076	Oceania	Australia	Magnetic Island
MBG_077	Oceania	Republic of Kiribati	Sidney Island
MBG_080	Oceania	Republic of Kiribati	Enderbury Island
MBG_114	Oceania	Australia	South Australia
MBG_118	Africa	Egypt	Egypt
MBG_126	Middle East	Pakistan	Balochistan
MBG_129	Africa	Tanzania	Mara
MBG_138	Southern Africa	South Africa	East Cape
psbD			
Label	**Region**	**Country**	**Location**
MBG_029	Central America and Mexico	Mexico	Chiapas
MBG_030	Central America and Mexico	Mexico	Colima
MBG_053	Caribbean	Dominican Republic	Guano, 30 Km
MBG_062	Caribbean	Bahamas	The Bahama
MBG-067	Africa	Ethiopia	Sidamo
MBG_071	Africa	Sudan	Ecuatoria Oriental
MBG_130	Madagascar	Madagascar	Toliara
rpl32			
Label	**Region**	**Country**	**Location**
CHA_003	Galápagos	Ecuador	Champion
ESP_006	Galápagos	Ecuador	Española
ESP_009	Galápagos	Ecuador	Española
ESP_011	Galápagos	Ecuador	Española
FLO_001	Galápagos	Ecuador	Floreana
GEN_003	Galápagos	Ecuador	Genovesa
MBG_032	Central America and Mexico	Mexico	Isla Socorro
MBG_035	Central America and Mexico	Mexico	Yucatan
MBG_038	Central America and Mexico	Mexico	Yucatan
MBG_042	Central America and Mexico	Guatemala	El Progresso
MBG_044	South America	Colombia	Valle del Cauca
MBG_047	South America	Venezuela	Zulia
MBG_049	South America	Venezuela	Federal
MBG_050	South America	Venezuela	Vargas
MBG_056	Caribbean	Dominican Republic	Pedernales
MBG_063	Caribbean	Jamaica	Morant Point
MBG_075	Oceania	Australia	Queensland

MBG_078	Oceania	Republic of Kiribati	Gardner Atoll
MBG_079	Oceania	Republic of Kiribati	Canton Island
MBG_080	Oceania	Republic of Kiribati	Enderbury Island
MBG_084	Madagascar	Madagascar	Petite-Terre
SCZ_006	Galápagos	Ecuador	Santa Cruz
SCZ_007	Galápagos	Ecuador	Santa Cruz