

BIOGEOGRAPHY AND PHYLOGENETICS OF THE HAWAIIAN ENDEMIC

*HIBISCADELPHUS, HAU KUAHIWI* (MALVACEAE)

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This work is one such that is built upon much of the previous efforts of others, both conceptually and academically. I am specifically referring to the Progression Rule which, while initially described by Willi Hennig in his book "Phylogenetic Systematics" in 1966, was later reexamined and thoughtfully applied to many lineages of the Hawaiian flora and fauna in the book "Hawaiian Biogeography: Evolution on a Hot Spot Archipelago", an excellent publication from the Smithsonian edited by Vicki A. Funk and Warren L. Wagner. I was lucky enough to have met Vicki briefly while working on my thesis. She was a visiting researcher wrapping up a project with a collaborator in the Botany Department. It was great speaking to her about such specifics of the Progression Rule and my far-flung suspicions of the origin of the *Hibiscadelphus* lineage. Vicki encouraged me to follow up on my preliminary results that suggested some of the phylogenetically closest related species to *Hibiscadelphus* were indeed from Madagascar. She suggested I contact the Missouri Botanical Gardens about their field house in Madagascar and told me of her time there doing research. It was fascinating and certainly helped to motivate me to this day, I still wish to go to Madagascar, Socotra, and the Mascarenes in hopes of tracing the ancient roots of *Hibiscus*. Vicki Funk passed away October 22, 2019 at age 71, but I am certain her contributions to botany, biogeography, and phylogenetics will continue to inspire researchers far into the future.

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## Abstract

The endemic Hawaiian flowering plant genus *Hibiscadelphus* was described by Joseph F. Rock based on two species found on Hawaii island and one species from Maui in 1911. In the years since, five additional species have been described, one from Hawaii island, two from Kauai, and one each from Lanai and Maui. In April 2012, A new species was discovered with a small remote population of ca. 99 mature individuals, the genus as whole is critically endangered. It had been proposed that the island colonization history of species in the genus followed the “Progression Rule”, a well-documented pattern in many Hawaiian taxa of progressive colonization from the older to younger islands, as new islands emerged from the sea. However, no molecular phylogeny has been published that tests this hypothesis for *Hibiscadelphus*. It is the purpose of this work to generate a phylogenetic hypothesis of *Hibiscadelphus* and its relationship to other genera of the Malvaceae based on chloroplast and nuclear DNA sequenced from a combination of samples from living plants, herbarium specimens, and accessions from the Hawaiian Plant DNA Library. DNA sequences were examined by both Bayesian and Maximum Likelihood approaches for phylogenetic reconstruction. Further, a fossil and island age calibrated BEAST analysis was performed with a Relaxed Log Normal molecular clock to estimate lineage divergence timing within the Hawaiian archipelago. The molecular clock estimate corroborates generally accepted dates for island ages and supports the hypothesis of the *Hibiscadelphus* radiation following the Progression Rule. The resulting phylogenies also indicate *Hibiscadelphus* as a monophyletic clade that is nested within the African *Hibiscus* section Calyphylli, from which they appear to have diverged approximately 10 mya. These results indicate the relationship of *Hibiscus* and *Hibiscadelphus* is paraphyletic and *Hibiscadelphus* should be reclassified within *Hibiscus*. This insight further suggests that there were five independent colonization events of *Hibiscus* to the Hawaiian Islands.

Keywords: Biological Radiation, Long Distance Dispersal, Conservation, Botany, *Hibiscus*, Island Biogeography, *Hibiscadelphus*, Molecular Clock, Phylogenetic Dating, Progression Rule.

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## INTRODUCTION

Commonly known as the mallows, the Malvaceae are a diverse and economically important plant family. Agriculturally, this family encompasses species important for textile manufacturing (e.g., cotton, kenaf), food production (e.g., okra, marshmallows, roselle, cacao, and durian), and many species are also horticulturally important (e.g., hibiscus and hollyhocks). The Malvaceae family, *sensu lato*, is currently comprised of 4,225 species, 244 genera, and nine subfamilies: the Byttnerioideae, Grewioideae, Sterculioideae, Tilioideae, Dombeyoideae, Brownlowioideae, Helicteroideae, Bombacoideae, and the Malvoideae (Stevens, 2001).

Within the subfamily Malvoideae there are four tribes: Malveae, Gossypieae, Kydieae and the Hibisceae (Bayer and Kubitzki, 2003). The Malveae include *Althea officinalis* L., the original marshmallow, and *Abutilon* Mill., or Indian Mallow. The latter has a number of species endemic to the Hawaiian archipelago. The tribe Gossypieae includes the species *Gossypium hirsutum* L., the species from which most commercial cotton is bred. *Gossypium tomentosum* Nutt. ex Seem is a species of cotton endemic to Hawaii. Furthermore, *Kokia* Lewton is a Hawaiian endemic genus within the Gossypieae (Morden and Yorkston, 2018). The Kydieae are a tribe with the four genera, *Dicellostyles* Benth., *Julostylis* Thwaites, *Kydia* Roxb., and *Nayariophyton* T.K. Paul (Bates, 1968).

The Hibisceae tribe, as evident by the tribe's nomenclature, includes the genus *Hibiscus* L., as well as *Abelmoschus* Medik. (commonly known as Okra). In the Hawaiian Islands, there are a great variety of *Hibiscus* including single island endemics, and others that are widely dispersed across the archipelago. Notably, some Hawaiian *Hibiscus* flowers are scented, unlike many of the cultivated hybrids and *Hibiscus rosa-sinensis* L., the type species of the genus. The genus *Hibiscadelphus* Rock has likewise been prescribed to the tribe Hibisceae, based on morphological characters and a marked resemblance to *Hibiscus*. The Hibisceae is comprised of five major sections. A paraphyletic Madagascan clade (Megistohibiscus), Phylloglandula, Trionum, Euhibiscus, and section Calyphylli (Koopman and Baum, 2008).

The latest species estimates identify the number of *Hibiscus* between 300-350 species (Pfeil and Crisp, 2005). *Hibiscus* species are known to hybridize interspecifically and there are patterns of recent polyploidy throughout the group based on differences in chromosomal number (Pfeil and Crisp, 2005). Historically, Hawaiian *Hibiscus* were important to early efforts in creating cultivated hybrids. Referencing this broadly, Darwin states that “the fertility of first crosses between species, and of the hybrids produced from them, is largely governed by their systematic affinity” (Darwin, 1859). Hybridization is also a prominent feature of *Hibiscadelphus*, and species have been shown to hybridize readily in the wild and exhibit hybrid vigor in F<sub>1</sub> and further generations; this tendency has raised concern among natural resource managers that hybrid swarms could supplant parental types (Baker, 1980).

In Hawaii, there are seven generally accepted species of *Hibiscus* from four separate colonization events (Wagner *et al.*, 1990). These seven species include *Hibiscus tiliaceus* L., *Hibiscus brackenridgei* A.Gray, *Hibiscus furcellatus* Desr., *Hibiscus arnottianus* A.Gray, *Hibiscus waimeae* A.Heller, *Hibiscus kokio* Hillebr., and *Hibiscus clayi* O.Deg. & I.Deg. (Wagner *et al.*, 1990). Three species are believed to have arisen from three separate colonization events. *Hibiscus tiliaceus* is the “sea hibiscus” or *hau* in

Hawaiian. The seeds of the *hau* tree are known to have small internal air spaces that allow them to float on saltwater and remain viable at sea for greater than three months (Nakanishi, 1988; Takayama *et al.*, 2006). *Hibiscus furcellatus* or *akiohala* is indigenous to Hawaii and it can also be found in Florida, the Caribbean, Central and South America in marshes and low wetland areas. This disjunct distribution suggests a long-distance dispersal event potentially from the Caribbean Sea to Hawaii. *Hibiscus brackenridgei* is the state flower of Hawaii. The remaining species form a distinct clade of red and white flowered *Hibiscus* (Huppman, 2013), the white flowered species sometimes being scented.

### *Hibiscadelphus*

There are eight species of the Hawaiian endemic genus *Hibiscadelphus* that have been described. In 1911, Joseph F. Rock established the genus *Hibiscadelphus* to include three concurrently described species, *Hibiscadelphus wilderianus* Rock, *H. hualalaiensis* Rock, and *H. giffardianus* Rock (Rock, 1913). They are distinguished from the genus *Hibiscus* by a zygomorphic corolla, the separable endocarp, and deciduous calyx (Bishop and Herbst, 1973). The close relationship of *Hibiscus* is evident in the Latin name, referencing *Hibiscadelphus* as the “brother of *Hibiscus*”. *Hibiscadelphus wilderianus* was described from material collected in Auwahi, Maui on the slopes of Haleakala by Rock (1910). *Hibiscadelphus giffardianus*, was also collected by Rock in March 1911 on the slopes of Kilauea, at Kipuka Puauulu and described later that year. The oldest collected sample of *Hibiscadelphus hualalaiensis*, housed by the Bishop Museum, is a “cotype” (= syntype) collected by Rock in 1909 from Hualalai. The original label of the specimen is dated “June 18th 09” and is annotated as “*Hibiscus* sp.” (personal observation, 2019).

*Hibiscadelphus bombycinus* Forbes, was described by C.N. Forbes (1920) from the material collected by Hillebrand and Lydgate in Kawaihae during the mid 1800's (no specific date available) (personal observation, 2019). There are no further collections of *H. bombycinus*, although it is worth noting that the original label for the holotype is annotated *Hibiscus bombycinus*.

*Hibiscadelphus distans* Bishop & Herbst, was first collected in June 1972 and described the next year with the specific epithet *distans* to reflect “the fact that this species stands well apart from other members of the genus, both in distribution and in fruit morphology” (Bishop and Herbst, 1973). *Hibiscadelphus distans* has been found almost exclusively in Waimea Canyon, Kauai, particularly on steep cliffs above Koaie stream. Based on fruit morphology and distribution, it was presumed to be the basal lineage in Hawaii (Bishop and Herbst, 1973).

*Hibiscadelphus crucibracteatus* Hobdy, was described from a single tree discovered on Puhielelu Ridge, Lanai by Peter Connally in 1981 (Hobdy, 1984). The only known tree died soon after its discovery from depredation by axis deer (*Axis axis*); seeds were collected, but none were successfully germinated (Oppenheimer *et al.*, 2014) (Hobdy, 1984).

*Hibiscadelphus woodii* Lorence & Wagner, was described from material collected in March 1991 by Kenneth Wood of the National Tropical Botanical Garden (NTBG) (Lorence and Wagner, 1995). This species was known from only four individuals on the lowland mesic cliffs of Kalalau Rim (990–1020 m), North of Kahuamaa Flat on Kauai.



The last individual was observed to have died in August of 2011; all attempts of propagating *H. woodii* had failed, and the species was thought to be extinct (Wood, 2012). However, in 2019, the National Tropical Botanical Garden discovered a population of *H. woodii* by use of unmanned aerial vehicle (UAV) technology thus offering a new hope to secure this species from extinction (Lee, 2019).

*Hibiscadelphus stellatus* was discovered by Hank Oppenheimer, Keahi Bustamente, and Steve Perlman in 2012 during fieldwork in Kauaula Valley, West Maui (Oppenheimer *et al.*, 2014). The authors reported 99 plants found, making *H. stellatus* the largest known wild population of the genus. Additionally, it is the only species known to have any natural seedling recruitment (Oppenheimer *et al.*, 2014).

To summarize, of the eight species of *Hibiscadelphus*, three species are extinct (*H. bombycinus*, *H. wilderianus*, and *H. crucibracteatus*), and two species are extinct in the wild (*H. giffardianus* and *H. hualalaiensis*), and the other three species have fragmented at-risk populations. Only three of the eight species have any naturally occurring representatives. Each of the eight species are single island endemics (Oppenheimer *et al.*, 2014).

## Colonization of the Hawaiian Islands

Long distance dispersal to the Hawaiian Islands prior to human settlement was an exceedingly rare event, with an estimated 259 colonization events giving rise to approximately 1000 species of native angiosperm (Price and Wagner, 2018). Natural mechanisms of dispersal to the Hawaiian Islands are often characterized by Wind, Wing or Wave (the three W's). This suggests, seeds or spores small enough to get airborne could have floated to the islands on air currents, while seeds that are sticky, barbed or small enough may have hitchhiked with the avian visitors to Hawaii. Almost certainly the seeds of *hau kuahiwi*, like other Hawaiian Malvaceae, would have made the long journey by “wave”, floating across thousands of miles of open ocean (Zimmerman, 1948). These seeds, perhaps borne on oceanic currents that have since been disrupted by land masses likely dispersed across the Pacific no later than three million of years ago, prior to the formation of the isthmus of Panama (Slater *et al.*, 2014). This was accomplished either by rafting or an ancestral adaptation to dispersal across saltwater. The namesake of *hau kuahiwi* is *hau* (*Hibiscus tiliaceus*), the seeds of which are known to have small internal air spaces that allow them to float on saltwater and remain viable for three months or more (Nakanishi, 1988; Takayama *et al.*, 2006). Seemingly, conservation of the traits that make sea-drifted seeds possible in this group appear to be under stabilizing selection.

Eight species of *Hibiscadelphus* have been described across the Hawaiian archipelago. The range of the genus appears to be restricted to the main Hawaiian high islands, with the conspicuous exception of the island of Oahu. This chainwide distribution suggests a pattern of colonization dependent upon the formation of new volcanic islands with subsequent dispersal and diversification. The oldest island on which the genus is found is Kauai, which is estimated to be approximately 5 million years old (Price and Clague 2002; Clague *et al.*, 2010; Obbard *et al.* 2012). The two species found on Kauai (*H. distans*, and *H. woodii*) are cliff-dwelling shrubs. They are both, generally speaking, smaller than the other representatives of the genus. The remainder of the genus

is made up of small trees with floral morphology that is much larger than the Kauai representatives (Wagner *et al.* 1990).

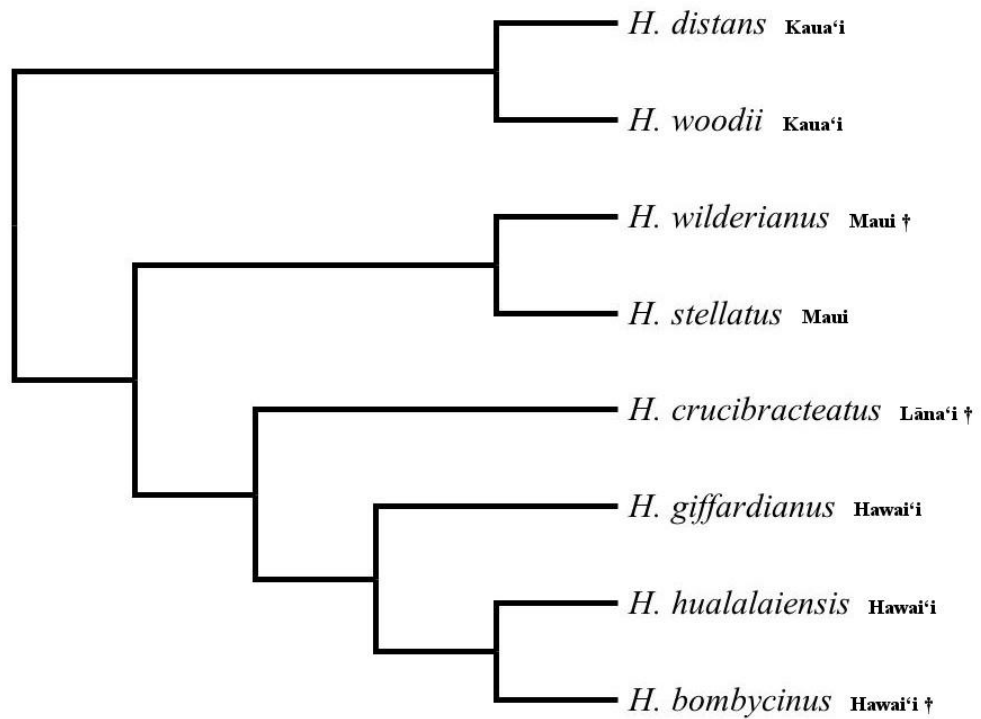
Willi Hennig was a phylogenetic systematist widely credited as the founder of cladistics (Brower, 2013) and is responsible for the biogeographical theory known as the “progression rule” (Hennig, 1966). Hennig's progression rule states, “the species with the most primitive characters are found within the earliest part of the area, which will be the center of origin of that group” (Hennig, 1966). When applied to volcanic islands, such as the Hawaiian Islands, the progression rule dictates that the center of origin should be the island on which the ancestor first established. It is possible the center of origin for the *Hibiscadelphus* radiation may very well be on islands now submerged, however there is a geological upper limit to Hawaiian terrestrial lineages of approximately 33 mya when no islands existed in the Hawaiian Archipelago (Clague *et al.*, 2010). While Oahu has never had a species of *Hibiscadelphus* described, although an early extinction is a possibility, the pattern of well-defined clades on younger islands skipping Oahu is not uncommon as is the case with the Silversword alliance (Funk and Wagner, 1995).

If the small shrub-like habit of the Kauai endemics is indeed an ancestral character and the larger trees with enlarged flowers of the younger islands are derived, then we may speculate that the group is potentially  $\geq 5$  million years old (the approximate age of Kauai Island). The last high before Kauai was what is known as the Koko Seamount, from its current distribution it appears *Hibiscadelphus* only occurs on high islands as none are known from the Northwestern Hawaiian Islands (Clague *et al.*, 2010; Wagner *et al.* 1990). The Koko Seamount was a prominent high island of the archipelago before ca. 33 million years ago (Clague *et al.*, 2010). This ca. 28-million-year gap in suitable habitat suggests a few possibilities. The simplest possibility places Kauai Island as the center of origin for the group, being the oldest island with *hau kuahiwi* represented. The other possibility is a dispersal from the Northwestern Hawaiian Islands, which has never been known to harbor any species of the genus.

## OBJECTIVES

The four aims of this study are as follows: First, to establish the relationships among the different species of *Hibiscadelphus*. Second, identify the closest relative of *Hibiscadelphus* within the Hibisceae and determine when the ancestor of *Hibiscadelphus* is likely to have first established in Hawaii. Third, determine if subsequent colonization of other islands follows the progression rule (Figure 1). Fourth, to resolve the number of colonization events of *Hibiscus* species within the Hawaiian Islands.

Plastid markers are particularly useful for phylogenetic comparison of maternal lineages due to their conserved mutational rates (Shaw *et al.*, 2005). Additionally, chloroplast capture is the process by which the seedling is endowed with the chloroplast of the maternal parent, preserving the continuity of maternal chloroplast lineages from mother to offspring. The phylogenetic analysis provided here is the result of two compared plastid markers: *matK*, and *ndhF*. *matK* encodes the enzyme Maturase K that is essential for the splicing of group II introns in the chloroplast genome (Mohr & Lambowitz, 2003). *ndhF* encodes NADH dehydrogenase subunit F protein that is a conserved single copy region gene of the chloroplast genome that has been widely used to resolve taxonomic classifications in plants (Schnabel and Wendel 1998). Chloroplast



**Figure 1.** Modified cladogram from Funk and Wagner (1995). The above cladogram is a hypothetical phylogeny based on a morphological analysis utilizing a number of different character states to infer phylogenetic relatedness within the clade *Hibiscadelphus*. This cladogram incorporates two new species and placement on the cladogram for those species is based solely on island distribution (*Hibiscadelphus woodii*, and *Hibiscadelphus stellatus*).

markers are useful in discerning the phylogeny of sea drifted seeds (Takayama *et al.*, 2006). However, chloroplast markers are not useful for elucidating the paternal lineage of a plant. This is somewhat analogous to the case of mitochondrial DNA in animals. However, phylogenetic signal is often very strong for nuclear regions as compared to cpDNA regions within island systems (Baldwin *et al.* 1995). For this reason, the nuclear ribosomal internal transcribed spacer is also examined here to confirm phylogeny generated by the chloroplast markers with a marker known to evolve more rapidly and account for the paternal admixture (ITS; Baldwin *et al.* 1995).

## MATERIALS and METHODS

Voucher specimens of the genus *Hibiscus* were collected from Koko Head Crater Botanical Garden, and Waimea Botanical Garden (Table 1). True species of non-hybrid origin from diverse geographic localities were specifically targeted. Voucher specimens were then deposited in the Joseph F. Rock Herbarium (HAW) at the University of Hawaii at Manoa. *Hibiscadelphus stellatus* material was supplied by Hank Oppenheimer from the Olinda Rare Plant Nursery on Maui. Leaves from six plants representative of the wild population were placed in gel silica packets for shipment, their DNA extracted and accessioned into the Hawaiian Plant DNA Library (HPDL; Morden *et al.*, 1996; Randell and Morden 1999) as were all specimens collected for this research. Remaining species of *Hibiscadelphus* were prior accessions of the Hawaiian Plant DNA Library. *Kokia drynarioides* (HPDL accession 1196) of the related tribe Gossypieae was used as an outgroup species.

CTAB and silica extractions were performed to extract cpDNA and nuDNA. Once genomic DNA was accessioned, Polymerase Chain Reaction (PCR) was employed to amplify the plastid regions *matK* and *ndhF* (Pfeil and Crisp, 2005). Additionally, the nuclear encoded ITS region was also amplified (Baldwin *et al.*, 1995). Amplified PCR products were run on an electrophoresis gel to assess the quality of amplification. Quality PCR product was prepped and supplied to the ASGPB lab (Advanced Studies in Genomics, Proteomics and Bioinformatics) sequencing facility at the University of Hawaii Manoa Campus. Sequences were edited, assembled and made into contiguous regions with Sequencher DNA Sequence Analysis Software, both forward and reverse primers were sequenced.

PCR amplifications were carried out in 25 µl reaction volumes under the following conditions: ca. 25 ng of DNA, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 1X Taq Polymerase buffer, 1.5 mM MgCl<sub>2</sub>, 0.50 mg bovine serum albumin (BSA), 0.2 µM forward and reverse primers, and ca. 1 unit of Taq DNA Polymerase (Promega, Madison, Wisconsin). PCR was performed in a DNA thermocycler (MJ Research). PCR amplified products were examined on 1.5% agarose gels, stained with EtBr, and visualized with an ultraviolet (UV) light source. Amplified DNA fragment size was confirmed using either the 100 bp ladder (Promega) or a pBS plasmid (Stratagene, La Jolla, California) digested with restriction enzymes to produce fragments in a size range from 0.448 to 2.96 kb. Final gel products were viewed with the Bio-Rad Gel Doc XR System and recorded with the Quantity One Software. The PCR products were cleaned using Exo-Sap-It (Affymetrix, Santa Clara, California) according to the manufacturer's instructions. Double stranded PCR products were sequenced in forward and reverse directions, and

**Table 1. Voucher Table** Abbreviations in collections: PI = USDA Plant Inventory number, PCMB = Pacific Center for Molecular Biodiversity, SCBGP = Specialty Crop Block Grant Program

Species	Authority	Collection	Geography	GenBank <i>matK</i>	GenBank <i>ndhF</i>	GenBank ITS	HPDL
<i>Abelmoschus manihot</i>	(L.) Medik.	PI639674	India, Central China, Malaysia			KP222436	
<i>Abutilon eremitopetalum</i>	Caum	PCMB2765	Lanai			EF219364	
<i>Anotea flavida</i>	Ulbr.	<i>Fryxell 785630</i> (ISC)	Mexico			U56775	
<i>Helicteropsis microsiphon</i>	(Baill.) Hochr.	<i>P. B. Phillipson 1955</i> (TAN)	Madagascar	EF207264	EF207296		
<i>Hibiscadelphus crucibracteatus</i>	Hobdy	BISH442299	Lanai			MT250676	10895
<i>Hibiscadelphus distans</i>	L.E. Bishop & D.R. Herbst	<i>S. Perlman s.n.</i>	Kauai	MT250701	MT250705	MT250675	4465
<i>Hibiscadelphus giffardianus</i>	Rock	<i>L. Pratt</i>	Big Island	MT250700	MT250704	MT250674	1127
<i>Hibiscadelphus hualalaiensis</i>	Rock	Waimea Botanical Gardens	Big Island	MT250699	MT250703	MT250673	1125
<i>Hibiscadelphus stellatus</i>	H. Oppenh., Bustamente, & Perlman	<i>H. Oppenheimer</i>	Maui	MT250698	MT261418	MT250672	10146
<i>Hibiscadelphus woodii</i>	Lorence & W.L. Wagner	<i>S. Montgomery</i>	Kauai	MT250697	MT250702	MT250671	1131
<i>Hibiscus acetosella</i>	Welw. ex Hiern	<i>4293 Tanmoy AM et al 2015</i>	Africa x Brazil			FJ621494	
<i>Hibiscus arnottianus</i>	A. Gray	<i>Huppmann 189</i>	Hawaii	MT250696			6392
<i>Hibiscus bojerianus</i>	Baill.	<i>D. Baum 383</i> (MO)	Madagascar	EF207275	EF207306		
<i>Hibiscus boryanus</i>	DC.	<i>S. Champion 008</i> (HAW)	Mauritius, Réunion	MT250695		MT250670	10157
<i>Hibiscus brackenridgei</i>	A. Gray	<i>Huppmann 156</i>	Hawaii	MT250694			6520
<i>Hibiscus caeruleus</i>	Baill.	<i>M. Koopman 233</i> (MO)	Madagascar	EF207265	EF207297		
<i>Hibiscus calyphyllus</i>	Cav.	<i>E. Huppmann 47</i>	Africa, W. Indian Ocean	MT250693			6563
<i>Hibiscus cannabinus</i>	L.	<i>R. Small s.n.</i> (TENN)	Tropical & S. Africa, SW. Arabian Peninsula	EF207259			
<i>Hibiscus clayi</i>	O. Deg. & I. Deg.	BISH 709201	Kauai	MT250692		AY962408	6497
<i>Hibiscus clypeatus</i>	L.	<i>Areces et al. 588</i> (UPRRP)	Puerto Rico, Jamaica, Mexico	KX984264		KX984255	
<i>Hibiscus columnaris</i>	Cav.	<i>S. Champion 005</i> (HAW)	Mauritius, Réunion	MT250691		MT250669	10154
<i>Hibiscus costatus</i>	A.Rich.	<i>Baum et al. 2004</i>	Americas	AY589057			
<i>Hibiscus diplocrater</i>	Hochr.	<i>S. Champion 002</i> (HAW)	Madagascar	MT261421		MT250668	10141
<i>Hibiscus diriffan</i>	A.G.Mill.	<i>S. Champion 004</i> (HAW)	Socotra	MT250690		MT250667	10143
<i>Hibiscus diversifolius</i>	Jacq.	<i>E. Huppmann</i>	Africa, Australia, New Zealand	MT250689			7109
<i>Hibiscus dongolensis</i>	Caill. ex Delile	<i>R. Small s.n.</i> (TENN)	Africa	EF207271			
<i>Hibiscus elatus</i>	Sw.	<i>Takayama et al. 2006</i>	Jamaica, Cuba	AB233276			
<i>Hibiscus ferrugineus</i>	Cav.	<i>M. Koopman 235</i> (MO)	Africa, Madagascar	EF207268	EF207300		

<i>Hibiscus fragilis</i>	DC.	<i>S. Champion 006</i> (HAW)	Mauritius, Réunion	MT250688	MT250666	10155
<i>Hibiscus furcellatus</i>	Desr.	PI585122	Hawaii, Tropical & Subtropical America	MT250687	EU188880	6554
<i>Hibiscus genevii</i>	Bojer ex Hook.	<i>S. Champion 007</i> (HAW)	Mauritius	MT250686	MT250665	10156
<i>Hibiscus glaber</i>	Matsum. ex Nakai	<i>Takayama 298</i>	Ogasawara	AB181077		
<i>Hibiscus grandidieri</i>	Baill.	<i>R. Ramanjananhary 182</i> (TAN)	Madagascar	EF207263		
<i>Hibiscus hamabo</i>	Siebold & Zucc.	<i>4541</i> (UPRRP)	China, Korea, Japan	KR259988	KR259988	KX984261
<i>Hibiscus humbertianus</i>	Hochr.	<i>DuPuy, DuPuy R. &amp; Rovonjiansoa</i> s.n. (P)	Madagascar	EF207266	EF207298	
<i>Hibiscus insularis</i>	Endl.	<i>S. Champion 011</i> (HAW)	Philip Island	MT250685	MT250664	10159
<i>Hibiscus kokio</i>	Hillebr. Ex Wawra	<i>E. Huppmann 58</i>	Hawaii	MT250684	AY962407	6485
<i>Hibiscus ludwigii</i>	Eckl. & Zeyh.	<i>E. Huppmann 41</i>	Africa	MT250683		6562
<i>Hibiscus macrogonus</i>	Baill.	<i>M. Koopman 289</i> (MO)	Madagascar	EF207273	EF562456	
<i>Hibiscus macrophyllus</i>	Roxb. ex Hornem.	<i>Ohl HTT009</i> (TI)	China, Tropical Asia	AB181100		
<i>Hibiscus mandrarensis</i>	Humbert ex Hochr.	<i>P. B. Philipson et al. 3978</i> (TAN)	Madagascar	EF207274	EF207305	
<i>Hibiscus mutabilis</i>	L.	SCBGP199_1	China, Taiwan		KP092993	
<i>Hibiscus ovalifolius</i>	(Forssk.) Vahl	<i>S. Champion 001</i> (HAW)	Africa, Yemen, India	MT250682	MT250663	10140
<i>Hibiscus pacificus</i>	Nakai ex Jôtani & H.Ohba	<i>S. Champion 015</i> (HAW)	Ogasawara	MT250681	MT250662	10163
<i>Hibiscus palmatifidus</i>	Baker	<i>S. Champion 003</i> (HAW)	Madagascar	MT261420	MT250661	10142
<i>Hibiscus pernambucensis</i>	Arruda	<i>Takayama et al. 2005</i> (Takayama 591 (TI))	Tropics & Subtropics	AB233275		
<i>Hibiscus rosa-sinensis</i>	L.	<i>Huppmann 45</i>	Unknown	MT250680	JX856461	6548
<i>Hibiscus schizopetalus</i>	(Dyer) Hook.f.	<i>Huppmann 43</i>	Africa, Madagascar	MT250679	KX452505	6547
<i>Hibiscus splendens</i>	Fraser ex Graham	<i>S. Champion 014</i> (HAW)	Australia	MT261419	MT250660	10162
<i>Hibiscus striatus</i>	Cav.	<i>S. Champion 013</i> (HAW)	S. America	MT250678	MT250659	10161
<i>Hibiscus surattensis</i>	L.	<i>R. Small s.n.</i> (TENN)	Tropical & Subtropical Old World	EF207258	EF207289	
<i>Hibiscus syriacus</i>	L.	<i>R. Small s.n.</i> cultivated (TENN)	China, Taiwan	EF207270	EF207302	
<i>Hibiscus taiwanensis</i>	S.Y. Hu	<i>S. Champion 012</i> (HAW)	Taiwan	MT250677	MT250658	10160
<i>Hibiscus tiliaceus</i>	L.	<i>Ohl HTT012</i> (TI)	Tropical & Subtropical Old World	AB181086		
<i>Hibiscus tozerensis</i>	Craven & B.E.Pfeil	<i>B. Pfeil</i> Garraway Ck <i>M. Koopman s.n.</i> WIS Greenhouse collected #880903	Australia	EF207269	EF207301	
<i>Hibiscus waimeae</i>	A. Heller		Kauai	EF207262	EF207294	
<i>Humbertiella decaryi</i>	(Hochr.) Dorr	<i>D. Baum 385</i> (MO)	Madagascar	EF207279	EF207310	

<i>Humbertiella henrici</i>	Hochr.	<i>M. Koopman 236</i> (MO)	Madagascar	EF207281	EF207312	
<i>Humbertiella quararibeoides</i>	Hochr.	<i>D. Baum 389</i> (WIS)	Madagascar	EF207280	EF207311	
<i>Jumelleanthus perrieri</i>	Hochr.	<i>Wolhauser and Andriamalaza 432</i> (TAN)	Madagascar	EF207272	EF207304	
<i>Kokia drynarioides</i>	Lewton	<i>C. Morden 1196</i> (HAW)	Big Island	KY569594	U55330	U56784
<i>Kosteletzkya diplocrater</i>	(Hochr.) Hochr.	<i>D. Baum 387</i> (MO)	Madagascar	EF207276	EF207307	
<i>Kosteletzkya reflexiflora</i>	Hochr.	<i>M. Koopman 258</i> (MO)	Madagascar	EF207283	EF207314	
<i>Kosteletzkya velutina</i>	Garcke	<i>M. Koopman 215</i> (MO)	Madagascar	EF207282	EF207313	
<i>Kosteletzkya virginica</i>	(L.) C. Presl ex A. Gray	<i>R. Small 230</i> (TENN)	USA, Bermuda, Cuba, S. Europe to N. Iran	EF207257	EF207288	
<i>Kydia calycina</i>	Roxb.	<i>R. Neupaney s.n.</i> (KWNNU)	India, China	EF207261	EF207293	
<i>Macrostelia laurina</i>	(Baill.) Hochr. & Humbert	<i>S. Malcomber 2806</i> (MO)	Madagascar	EF207267	EF207299	
<i>Malachra radiata</i>	(L.) L.	<i>Moran 8724 and Areces</i> (INB)	Tropical & Subtropical America, Africa	KT966993	KT967031	
<i>Malvaviscus arboreus</i>	Cav.	<i>Alverson 2181</i> (WIS)	Mexico, Trinidad, Peru	AY589061		
<i>Malvaviscus penduliflorus</i>	Moc. & Sessé ex DC.	<i>Areces and Vega 925</i> (UPRRP)	Texas, Venezuela, Peru	KT966994		KT966956
<i>Megistostegium microphyllum</i>	Hochr.	<i>D. Baum 382</i> (MO)	Madagascar	EF207278	EF207309	
<i>Megistostegium perrieri</i>	Hochr.	<i>R. Ranairojaona 534</i> (TAN)	Madagascar	EF207277	EF207308	
<i>Modiola caroliniana</i>	(L.) G.Don	<i>J. Beck 6298</i> (TENN)	S. America	EF207256	EF207287	
<i>Pavonia cauliflora</i>	(Nees & Mart.) Fryxell	<i>Baum et al.</i> 2004	Brazil	AY589056		
<i>Pavonia fruticosa</i>	(Mill.) Fawc. & Rendle	<i>605</i> (UPRRP)	Tropical America	KT966991		
<i>Pavonia spinifex</i>	(L.) Cav.	<i>602</i> (UPRRP)	Florida, Caribbean	KT966992		
<i>Perrierophytum humbertii</i>	Hochr.	<i>P. B. Phillipson et al. 3480</i> (US)	Madagascar	EF207284	EF207315	
<i>Perrierophytum rubrum</i>	Hochr.	<i>P. B. Phillipson 2543</i> (MO)	Madagascar	EF207285	EF207316	
<i>Sphaeralcea angustifolia</i>	(Cav.) G.Don	<i>R. Small 306</i> (TENN)	California, Nebraska, Mexico	EF207255	EF207286	
<i>Trochetiopsis erythroxylon</i>	(G.Forst.) Marais	<i>Chase 18170</i>	Saint Helena			JF939205
<i>Urena lobata</i>	L.	<i>J. Beck 5143</i> (TENN)	Tropics & Subtropics	EF207260	EF207291	
<i>Wercklea tulipiflora</i>	(Hook.) Fryxell	<i>Ickes 194</i> (UPRRP)	Lesser Antilles			KX984258

with amplification and internal primers as needed at the University of Hawaii Advanced Studies in Genomics, Proteomics and Bioinformatics lab.

Sequence alignments were constructed using Multiple Sequence Comparison by Log-Expectation (MUSCLE; Edgar, 2004) and truncated in Geneious Bioinformatics Software for Sequence Data Analysis (Geneious Prime, 2019). Additional sequences were collated from National Center for Biotechnology Information (NCBI). Preliminary Neighbor-Joining consensus trees were aligned with MUSCLE in Geneious and R (phangorn package) for exploratory data analysis with Tamura-Nei genetic distance model (Schliep, 2011). Final analysis was accomplished with Bayesian Evolutionary Analysis Sampling Trees (BEAST) (Bouckaert *et al.*, 2019) and Mr. Bayes (Huelsenbeck and Ronquist, 2001) modules installed on the University of Hawaii High Performance Computing Cluster, with an HKY evolutionary model (Hasegawa *et al.*, 1985). Phylogenetic relationships and clade divergence times were estimated separately for the nuclear, plastome, and concatenated datasets using BI implemented in BEAST2 v. 2.6.0 (Ronquist *et al.* 2012) and Mr. Bayes (Huelsenbeck and Ronquist, 2001), with an HKY+I +G evolutionary model (Hasegawa *et al.*, 1985) on the University of Hawaii High Performance Computing cluster. Separate Bayesian analyses were conducted for each gene region, the two plastome and one nuclear region to assess congruence.

The BEAST tree was modeled under a Yule process using a random starting tree, the HKY+I+G substitution model, and a relaxed clock with a lognormal distribution. A calibration points was placed at the crown of the Hibisceae based on the previous dating analysis by Koopman and Baum 2008 which estimated the crown age of Hibisceae to be 15-19 Mya. A normal distribution was used and set the mean to 17.4 Mya, with the standard deviation set to include the 95% HPD. For a second calibration point, the age of section Euhibiscus was used to constrain the crown of the clade. Estimates for the age of Euhibiscus were based on previous dating analysis by Koopman and Baum, 2008 which estimated the crown age of Euhibiscus 9-11.6 Mya. For this second calibration point, a normal distribution was used and set the mean to 10.4 Mya, with the standard deviation set to include the 95% HPD. For a third calibration point, the age of the Malagasy section of Euhibiscus was used to constrain the crown of the clade. Estimates for the age of the clade were based on a previous dating analysis by Koopman and Baum, 2008 which estimated the crown age as 3.7-4.9 Mya. For this third calibration point, a normal distribution was used and set the mean to 4.3 Mya, with the standard deviation set to include the 95% HPD. For a fourth calibration point, the age of the Megistohibiscus was used to constrain the crown of the clade. Estimates for the age of the clade were based on previous dating analysis by Koopman and Baum, 2008 which estimated the age as 11.5-14.5 Mya. For this fourth calibration point, a normal distribution was used and set the mean to 13.03 Mya, with the standard deviation set to include the 95% HPD.

Age of the oldest surface rocks have been used for island age calibrations in other dating analyses of Hawaiian genera (Obbard *et al.* 2012). Here, the age of oldest surface rock is used for island age calibration within the monophyletic clade *Hibiscadelphus* (but see discussion of this issue in Knope *et al.* 2020). Most species of *Hibiscadelphus* are single volcano endemics (with the exception of the Kauai island species, *H. distans* and *H. woodii*). This allows for interior calibration points within the clade based on island age. For the first of these island age calibration points, the age of Kauai Island was used



to constrain the crown of the entire *Hibiscadelphus* clade (*H. distans*, *H. woodii*, *H. stellatus*, *H. hualalaiensis*, *H. giffardianus*). Estimates for the age of Kauai Island were based on a previous dating analysis by Obbard *et al.* 2012, which estimated the age of oldest surface rock as 5.13 Mya. For this calibration point, a normal distribution was used and the mean set to 5.1 Mya, with the standard deviation set to include the 95% HPD. For the second of these island age calibration points, the age of Maui Island was used to constrain the crown of *H. stellatus*, *H. hualalaiensis*, and *H. giffardianus*. Estimates for the age of Maui Island were based on previous dating analysis by Obbard *et al.* 2012 which estimated the age of shield completion as 1.37 Mya. For this calibration point shield completion was used instead of oldest surface rock to account for vicariance between species of *Hibiscadelphus* on the prehistoric island Maui Nui (species which were likely united populations prior to the break-up of the island and separation of *H. crucibracteatus* on Lanai). A normal distribution was used and the mean set to 1.3 Mya, with the standard deviation set to include the 95% HPD. For the third of these island age calibration points, the age of Hawaii Island was used to constrain the crown of *H. hualalaiensis*, and *H. giffardianus*. Estimates for the age of Hawaii Island were based on previous dating analysis by Obbard *et al.* 2012 which estimated the age of oldest surface rock as 0.46 Mya. For this calibration point, a normal distribution was used and the mean set to 0.5 Mya, with the standard deviation set to include the 95% HPD. A Markov Chain Monte Carlo (MCMC) analyses was run for 100 million generations, sampling every 1,000 generations. Independent runs were merged in LogCombiner v. 2.5.1 (Bouckaert *et al.* 2014), and chain convergence was checked in Tracer v. 1.7.1 (Rambaut *et al.* 2014) by examining log-likelihood plots and ensuring that Effective Sample Size (ESS) values were well above 200. After discarding 20% of the trees as burn-in, a maximum clade credibility tree with mean node heights was constructed using TreeAnnotator v. 2.5.1 (Bouckaert *et al.* 2014). Tree topology and node support were examined in Figtree v. 1.4.4. The R package GGtree was employed to finalize the phylogenies (Yu *et al.*, 2017).

The fossil calibrated *matK/ndhF* phylogeny dataset used by Koopman and Baum (2008) is adapted here. The authors used a concatenated cpDNA gene tree (*matK* and *ndhF*) with penalized likelihood in combination with pollen fossil priors of *Echiperiporites estalae* and *Malvacearumpollis bakonyensis* to create a time calibrated phylogeny (Koopman and Baum, 2008). This study expands on the Hibisceae branch with updated Bayesian methods and additional taxa from the Hawaiian Plant DNA Library. Prior assumptions (priors) were parameterized from ranges in the literature for specific clades within the Hibisceae (Koopman and Baum, 2008). Within the *Hibiscadelphus*, generally accepted ages for the islands were included as priors in the phylogeny. These island age priors were necessary to achieve a normal distribution of the Effective Sample Size (ESS) and an acceptable trace of posterior probabilities.

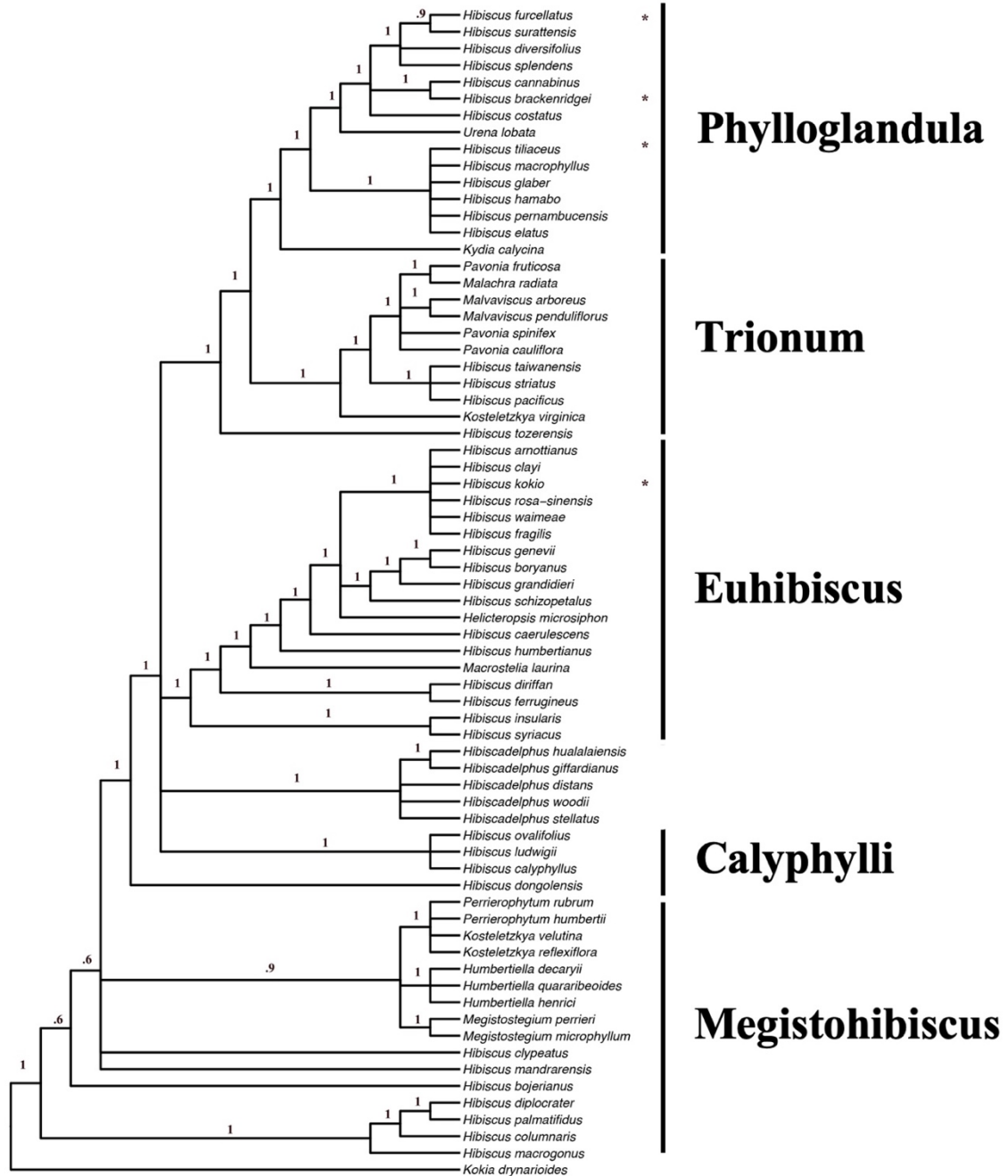
## RESULTS

For the paternal lineage to be unobscured by chloroplast capture, the following nuclear DNA (nDNA) phylogeny were not included in the total evidence chloroplast DNA (cpDNA) tree. Upon combination of nDNA and cpDNA, the phylogenies become paraphyletic in the tribe (Hibisceae) due likely to a high degree of polyploidization and/or hybridization that obfuscates the relationships. Thus, we interpret cpDNA and nDNA

separately in turn to best understand the evolution of the genes in the chloroplast and nucleus, respectively. The chloroplast gene *rpl16* was examined for over 20 species of tribe Hibisceae with very limited phylogenetic signal and was not used further for this study.

### cpDNA Phylogeny: *matK*

Two trees were generated, the first tree includes 70 taxa with representation across the entire tribe based upon an aligned sequence of 1519 bp (Figure 2). The second tree included only those species for which species representation was mirrored in *ndhF* and is based upon an alignment of 1931 bp (see next section and Figure 3). As shown in the entire tribe phylogeny (Figure 2), the Hibisceae segregated into six distinct clades including each of the five sections of *Hibiscus* plus *Hibiscadelphus*. Phylloglandula is a monophyletic clade that contains three Hawaiian *Hibiscus* species that are not closely related to each other, *H. furcellatus*, *H. brackenridgei* and *H. tiliaceus*. Sister to Phylloglandula is section Trionum which is paraphyletic and contains no Hawaiian *Hibiscus* representatives. *Hibiscus tozerensis* (included within section Trionum) is sister to a clade that includes both section Phylloglandula and the remaining taxa of section Trionum. Together sections Phylloglandula and Trionum form a monophyletic clade, which is recovered as part of a polytomy with sections Euhibiscus, Calyphylli, and *Hibiscadelphus*. Euhibiscus is a monophyletic section with the greatest Hawaiian *Hibiscus* representation. Four closely related Hawaiian species, *H. arnottianus*, *H. waimeae*, *H. kokio*, and *H. clayi* form a well-supported polytomy (pp = 1) with *Hibiscus rosa-sinensis* and *Hibiscus fragilis*. These four Hawaiian species are closely related and form a monophyletic clade in other analyses of the endemic Hawaiian *Hibiscus* (Huppman, 2013). Section Calyphylli is a paraphyletic section containing *Hibiscus calyphyllus* Cav., the closely related *Hibiscus ovalifolius* (Forssk.) Vahl, and *Hibiscus ludwigii* Eckl. & Zeyh. *Hibiscus dongolensis* Caill. ex Delile is included in this clade (Koopman and Baum 2008) but is shown here as sister to all sections discussed above. Section Megistohibiscus is a paraphyletic clade of almost exclusively Madagascan origin with one exception recovered in this phylogeny (the Neotropical *Hibiscus clypeatus* L.). The Megistohibiscus are a basally branching clade relative to all other sections within the Hibisceae and may exhibit some characteristics of what a Gondwanan *Hibiscus* lineage could have looked like. The monophyletic clade comprised of *Hibiscadelphus* forms a polytomy with sections Calyphylli, Trionum and Euhibiscus in this phylogeny. However, resolution within the clade show that *H. hualalaiensis* and *H. giffardianus* form a well-supported (pp = 1) clade separate from the older islands', Kauai and Maui species of *Hibiscadelphus*.



**Figure 2.** Bayesian phylogenetic analysis of Hibisceae representatives based on 1519 bp (aligned) of the *matK* sequence. Section names are adjacent to clades in bold. Posterior probabilities are given on each branch. Asterisks denote colonization events for a given clade of *Hibiscus* to the Hawaiian Islands. This phylogeny is comprised of 70 taxa across the entire tribe Hibisceae.



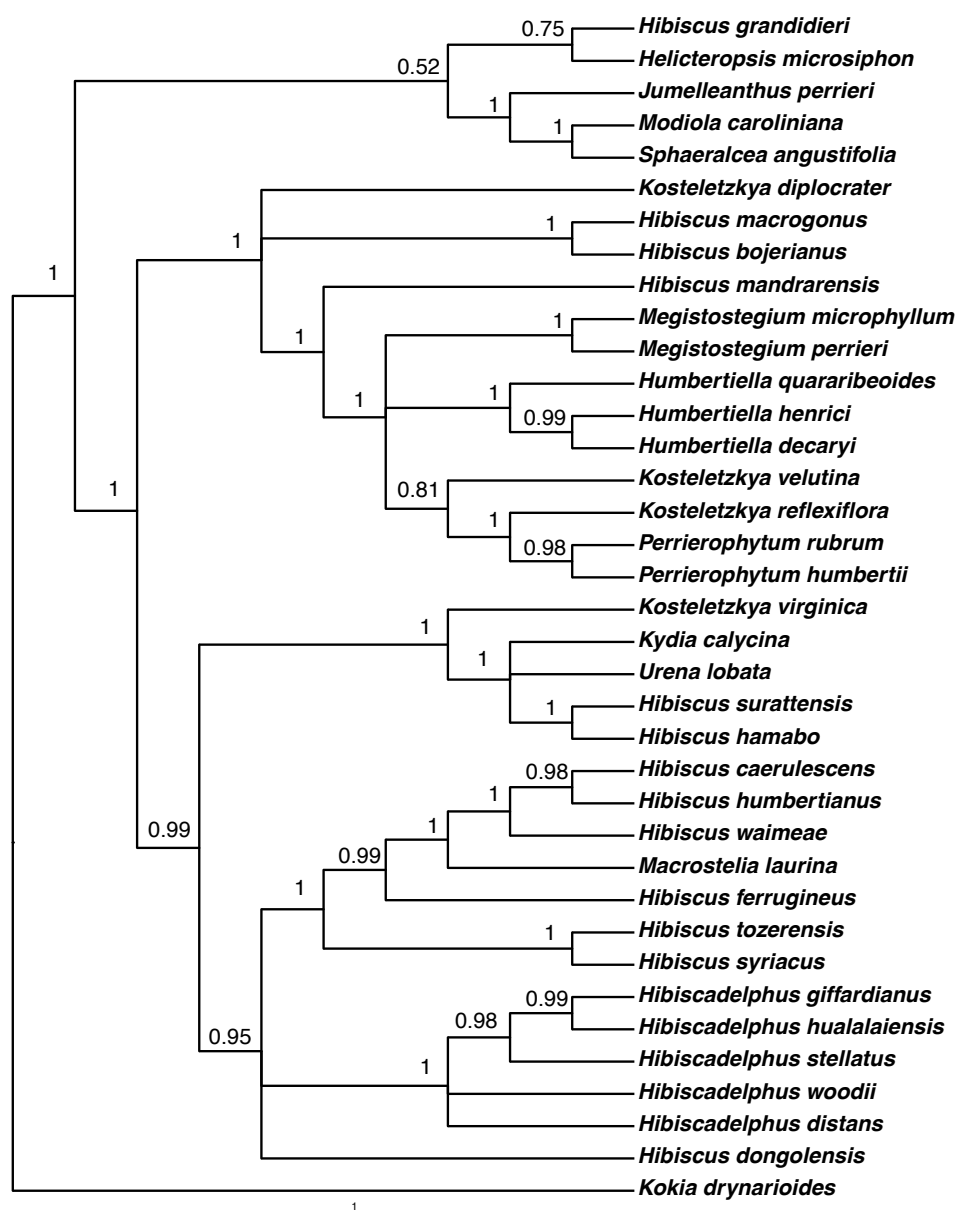
### cpDNA Phylogeny: *ndhF*

The *ndhF* (2080 bp) phylogeny recovered four major clades of Hibisceae (Figure 4). Most basal are the species mostly of the tribe Malveae (*Modiola caroliniana*, *Sphaeralcea angustifolia* and *Jumelleanthus perrieri*) but also included two species of section Euhibiscus in the Hibisceae (*Hibiscus grandidieri* and *Helicteropsis microsiphon*). The remaining three clades include species of sections Megistohibiscus (pp = 1.0) and Phylloglandula (pp = 0.99), and a well-supported trichotomy (pp = 0.95) that includes *Hibiscus dongolensis* (section Calyphylli), species of section Euhibiscus and species of *Hibiscadelphus*. Section Euhibiscus also includes *H. tozerensis* which was sister to the Trionum-Phylloglandula clade in the *matK* analysis. The clades of Euhibiscus species and *Hibiscadelphus* species are both well-supported (pp = 1.0). Section Trionum is not represented as sequences were not available to be mirrored in the *matK* phylogeny.

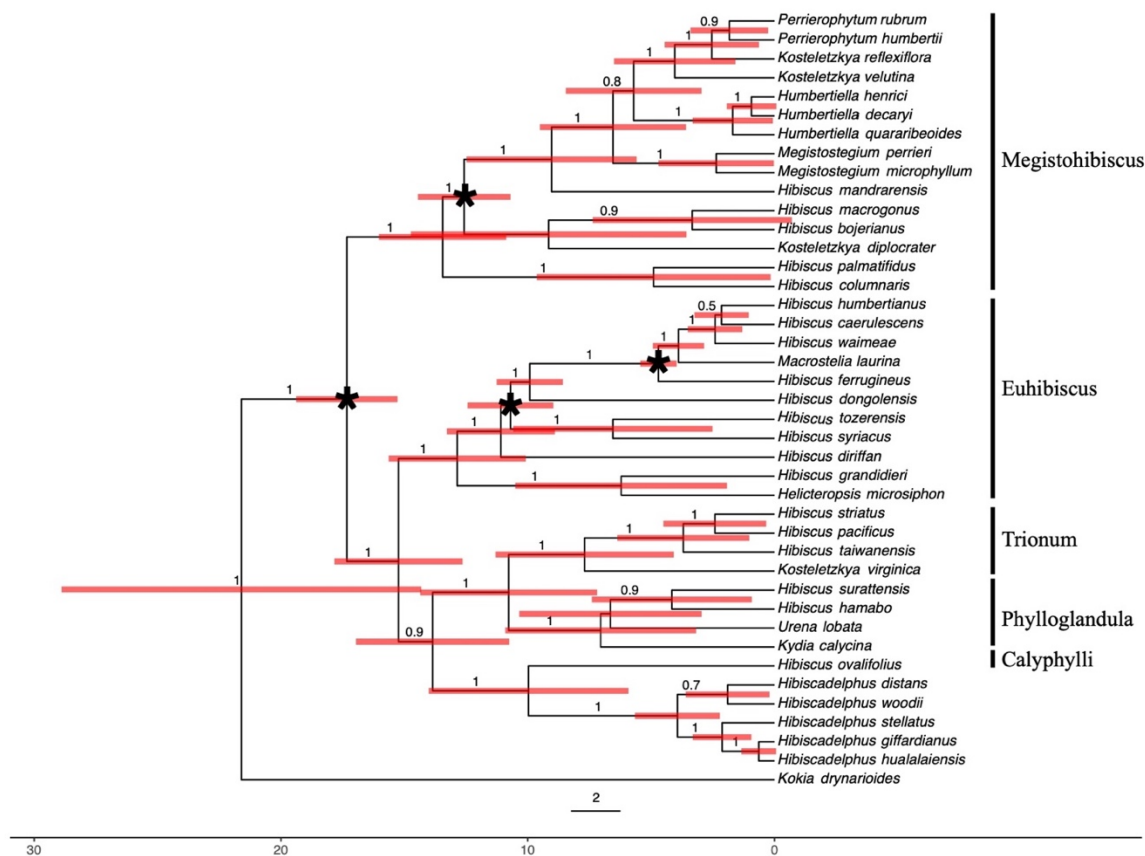
### Concatenated *matK/ndhF* Bayesian Estimation of Species Divergence Times

The concatenated *matK/ndhF* analysis was examined using BEAST to estimate species divergence times and confirm topology of single gene phylogenies. The combined *matK/ndhF* gene phylogeny (3879 bp) recovered greater resolution among species and clades throughout the Hibisceae (Figure 5). Five clades are evident in the analysis. Section Megistohibiscus is a basally branching clade (pp = 1) sister to a clade comprised of the remainder of the Hibisceae. Section Euhibiscus and a clade consisting of sections Phylloglandula-Trionum-Calyphylli are well-supported sister clades (pp = 1). Section Trionum is a well-supported clade (pp = 1) sister to the well-supported clade of section Phylloglandula (pp = 1). A clade with sect. Calyphylli (represented by *only H. ovalifolius*) and a monophyletic *Hibiscadelphus* is sister to the clade of sections Trionum and Phylloglandula. Greater resolution within the *Hibiscadelphus* clade resolve the Kauai Island species are sister to the Maui and Hawaii Island species (pp = 0.7) and the Maui Island taxon (*H. stellatus*) is sister to the species from Hawaii Island (*H. giffardianus* and *H. hualalaiensis*) (pp = 1.0). Placement of two other species are of note. *Hibiscus dongolensis* which falls clearly within sect. Euhibiscus here where in the *matK* phylogeny it was sister to all sections except Megistohibiscus, but in the *ndhF* phylogeny was a part of a trichotomy with sections Euhibiscus and *Hibiscadelphus*. *Hibiscus tozerensis* also is placed here within sect. Euhibiscus whereas in the *matK* phylogeny it is basally branching sister to the Trionum-Phylloglandula clade.

The Bayesian analysis was calibrated with a Relaxed Log Normal molecular clock (Figure 5). The asterisk at the nodes indicate the calibrated fossil priors from Koopman and Baum (2008); island ages were used to calibrate within the *Hibiscadelphus* clade (Price and Clague, 2002). This allows for a divergence time estimation at the nodes of the tree with 95% highest posterior densities (red bars). This model estimates that divergence of sect. Megistohibiscus from the remainder of the Hibisceae occurred ca. 17.3 million years ago (15.3 to 19.4 mya), Euhibiscus diverged from the Trionum-Phylloglandula-Calyphylli-*Hibiscadelphus* clade ca. 15.5 mya (12.5 to 18.5 mya), and *Hibiscadelphus* diverged from its most recent common ancestor (MRCA) with *H.*



**Figure 4.** Bayesian phylogenetic analysis of Hibisceae representatives based on 2080 bp (aligned) of the *ndhF* sequence. Posterior probabilities are given on the branch for each node. Only taxa replicated in *matK* dataset shown here.



**Figure 5.** Bayesian phylogenetic analysis with time divergent estimation based on combined *matK* and *ndhF* (aligned 3879 bp). The stratigraphic calibration points based on fossilized pollen evidence of *Echiperiporites estalae* and *Malvacearumpollis bakonyensis* are denoted by asterisks at the nodes of the phylogeny. Section names are adjacent to clades in bold. Posterior probabilities are given on each branch from BEAST. Scale bar along bottom is measure of divergence times in million years ago (mya).

*ovalifolius* (section Calyphylli) about 10 mya (6 to 14 mya). Divergence among the *Hibiscadelphus* species began ca. 3.95 mya (2.2 to 5.7 mya) with the separation of the Kauai species from those of Maui and Hawaii Island; divergence among Maui and Hawaii Island species occurred ca. 2.1 mya (0.9 to 3.3 mya). Divergence among the two Hawaii Island species occurred ca. 0.6 mya (0.04 to 1.5 mya).

### **nDNA Phylogeny: Internally Transcribed Spacer Region**

Analysis of the ITS gene tree (814 bp) resulted in similar results to those of the cpDNA gene trees although with some apparent differences (Figure 6). The five sections are represented as monophyletic groups with the exception of sect. Trionum. The placement of *H. clypeatus* (section Megistohibiscus) is basal to the entire Hibisceae clade. *Hibiscadelphus* is sister to *Hibiscus ovalifolius* (sect Calyphylli). The monophyly of *Hibiscadelphus* is supported here (including the extinct *H. crucibracteatus*), but there is no resolution among the species with them all forming a polytomy.

## **DISCUSSION**

### **Phylogenetic Relations of *Hibiscadelphus***

The only species not contained within either Rock's original description or these phylogenies is *Hibiscadelphus bombycinus* from Kawaihae, Hawaii Island, which was circumscribed by C.N. Forbes in 1920. The holotype specimen for *Hibiscadelphus bombycinus* was originally collected by Hillebrand in the mid-1800's with the name "*Hibiscus bombycinus*" on the specimen label (Hillebrand s.n., BISH accession 572446). Similarly, the first *Hibiscadelphus* collection by Joseph Rock near Puuwaawaa, Hawaii Island, was tentatively identified as "*Hibiscus* sp?" (Rock 53, BISH accession 511386). This collection was later designated as the type of *Hibiscadelphus hualalaiensis* and the type species of the genus (Rock, 1911; personal observation, 2019). The strong support for the genus being in a well-supported clade sister to *H. ovalifolius* (*Hibiscus* section Calyphylli) indicates the genus *Hibiscadelphus* should be relegated to the *Hibiscus* as it was originally considered by both Hillebrand and Rock, respectively. The close relationship of *Hibiscadelphus* species to the species of *Hibiscus* section Calyphylli (*H. ovalifolius*, *H. ludwigii*, and *H. calyphyllus*), is evident although not strongly supported in a phylogeny with all species represented.

This study supports monophyly of the genus *Hibiscadelphus* based on molecular evidence for all genes and gene regions examined. This was presumed based upon a number of character states as defined by Funk and Wagner (1995). The postulated close relatedness of the younger islands' species compared to the older island representatives is also well supported. The Kauai Island species *H. woodii* and *H. distans* form a distinct clade sister to the species endemic to the younger islands. *Hibiscadelphus crucibracteatus* is not included in the cpDNA analyses as it is extinct, however the internal transcribed spacer from a nearly 40-year-old voucher specimen (*Peter Connally* 44229, BISH) did amplify and that data is included in the ITS analysis (DNA was not amplifiable for cpDNA regions).





The genus *Hibiscadelphus* has established itself on Kauai, Maui, Lanai, and Hawaii Island. The radiation of this lineage has resulted in three species present on Hawaii Island, the most species on any one island. Maui Nui (the prehistoric large island consisting of Maui, Molokai, Lanai and Kahoolawe) is home to three species. These islands have become separated since the last glacial maximum (ca. 20,000 years ago) due to island subsidence and ocean level rise (Price & Elliot-Fisk, 2004).

Diploid counts in the genus *Hibiscus* range from  $2n = 20$  to  $2n = 180$ . Additionally, Carr and Baker's (1977) cytogenetic examination of *Hibiscadelphus* unite the genus based upon chromosomal counts for the Kauai species *H. distans* and the two extant Hawaii Island species *H. giffardianus* and *H. hualalaiensis* with the common counts of  $2n = 40$  for all three species. A diploid count of  $2n = 40$  is relatively low for Hawaiian *Hibiscus*, with most having diploid counts between  $2n = 80$  to 144 (Hinsley, 2012). This indicates that *Hibiscadelphus* is likely a distinct lineage from another Hawaiian *Hibiscus*, even prior to considering the molecular DNA evidence. While there is a dearth of chromosome counts for the Malvaceae as a whole, there are many taxa with no chromosomal counts among the early divergent sections of *Hibiscus* such as Section Megistohibiscus. It is tempting to try to explain some of the evolutionary history of the Malvaceae by an analysis of the polyploid series within the Hibisceae. However, no chromosomal counts are known for some of the basal lineages from Madagascar, Mauritius and Reunion Islands in the Indian Ocean. One implication of these phylogenies is that *Hibiscadelphus* is nested in groups native to South Africa, Madagascar and the Indian Ocean region. Specifically, section Calyphylli and other species of Section Megistohibiscus originated from around this region of the Indian Ocean. This is not surprising considering that several of the early branching lineages have circum-Indian Ocean distribution (Koopman and Baum, 2008)

### **Sectional Affiliation of *Hibiscadelphus***

The phylogenies recovered from the various chloroplast (*matK*, *ndhF*) and nuclear (ITS) loci indicate the monophyletic clade *Hibiscadelphus* is nested within *Hibiscus* section Calyphylli. The section is based on a species that has been referred to by many different names. First named *Hibiscus ovalifolius* (Forssk.) Vahl in 1790 (originally named *Urena ovalifolia* Forssk. in 1775), it became widely traded and renamed by others. The type species for section Calyphylli, *Hibiscus calyphyllus* Cav. (named in 1788), was pantropically dispersed from Southeastern Africa and Madagascar. It was traded widely in the late 1800's under the monikers *Hibiscus chrysanthus* W. Bull and *Hibiscus calycinus* Willd. In Hawaii, this species has been referred to as both *Hibiscus rockii* Degener & I. Degener and *Hibiscus brackenridgei* A. Gray var. *kauaiana* Caum. The status of *Hibiscus rockii* has been the subject of some debate as to whether this species is a naturalized plant on Kauai Island or an endemic variety, but there is speculation it established in Hawaii early after Western contact via ballast water discharge (personal communication, Waimea Botanical Garden).

## ***Hibiscadelphus* Coevolution with Hawaiian Avifauna**

The floral morphology of *hau kuahiwi* on the younger islands, combined with the Hawaiian Honeycreeper colonization timeline would appear to suggest a diffuse coevolutionary relationship between the avifauna and the native flora. *Hibiscadelphus* has evolved an ornithophilous pollination syndrome with a salverform floral morphology for pollination by endemic Hawaiian avifauna such as the Honeycreepers (Drepanidinae). Observations of *amakihi* (*Hemignathus virens*) and *iiwi* (*Drepanis coccinea*) floral visits are thoroughly documented (Wood, 2012) (Baker & Allen, 1976). The flowers have been shown to produce copious amounts of nectar and it appears there is also “remarkable similarity between bill curvature and corolla curvature” for the extinct Hawaii Island *mamo* (*Drepanis pacifica*) and *akialoa* (*Akialoa obscura*) (Baker & Allen, 1976). The nectarivorous drepanidines began to colonize Hawaii approximately 5.7 million years ago (Lerner *et al*, 2011). It is therefore likely incompatible with the diversification of ornithophilous lobelioids which is estimated to have occurred 8-17 mya (Givnish, 1998; Givnish *et al*. 1995). The results of this work (Figure 5) suggest that *Hibiscadelphus* diversity began ca. five million years ago as well, possibly offering an attractive alternative hypothesis in terms of a coevolutionary relationship between an endemic Hawaiian plant lineage and the Drepanidinae.

## **Fossil Calibration of *Hibiscus* sections and *Hibiscadelphus***

The fossil calibrated Bayesian phylogeny indicates the lineage that would eventually give rise to *Hibiscadelphus* diverged from other members of the section Calyphylli about 10 million years ago, this phylogeny also indicates that *Hibiscadelphus* began to diversify into the species we recognize today around 5 million years ago (Kauai Island is 5.1 million years old approximately), this is also the crown age for another endemic Hawaiian radiation; the Silversword alliance (Baldwin and Sanderson, 1998). A long-distance dispersal event of an ancestral *Hibiscus* species from section Calyphylli about 10 million years ago would have likely been from an African lineage. All other species in section Calyphylli are from Southern or Eastern Africa and Madagascar. Sister section Phylloglandula harbors plants with seed airspace (e.g.; *Hibiscus tiliaceus*, *Talipariti* sp.) and salt tolerance for over three months (Nakanishi, 1988; Takayama *et al.*, 2006). This timeline suggests that perhaps the MRCA between *Hibiscadelphus* and the rest of section Calyphylli dispersed into the Pacific some other way in the last 10 million years. It is clear that the species of Sect. Megistohibiscus are sister to all other sections in the tribe and that section Calyphylli is an early diverging branch (Koopman and Baum, 2008; phylogeny produced herein). One likely dispersal route for these tropical early divergent *Hibiscus* might have been via the Neotropics. The Panamanian Seaway present prior to the close of the Isthmus of Panama was most likely the only warm water route for sea drifted seeds from Africa to disperse into the Pacific (Slater *et al.*, 2014).

## Possible Origins of *Hibiscadelphus*

The Isthmus of Panama was closing approximately three million years ago (Slater *et al.*, 2014). The consequence of this event initiated the Great American Biotic Interchange of large-scale migrations of many animals from North to South America and vice versa. However, this likely ended another biotic interchange, one facilitated by oceanic currents flowing from the Atlantic into the Pacific Ocean that may have been taking place for 100 million years prior. Sea-drifted plants, fish, and marine mammals that once had the Panamanian Seaway as a dispersal route between the Americas then became isolated populations. Some of the best evidence of this, besides sea-drifted seed plants, is the close relatedness of Hawaiian Monk Seals and the now extinct Caribbean Monk Seal (Slater *et al.*, 2014).

In the 5 to 10-million-year period in which the *Hibiscadelphus* lineage diverged from other members of the section, there were other related taxa that may have established in the Americas or the Caribbean. One such species is *Hibiscus clypeatus* that shares several morphological characters with *Hibiscadelphus* including an erubescens and zygomorphic corolla marking a parallel evolution among these lineages (Macfadyen, 1837). The larger *matK* phylogeny reveals *H. clypeatus*, a native to the West Indies, is nested within the exclusively Madagascan Section Megistohibiscus, making it a basally branching lineage compared to all other sections of *Hibiscus*. *Hibiscus clypeatus*, native to the Caribbean and Yucatan Peninsula, is the only closely related taxa from the Americas in an otherwise exclusively Madagascan clade.

The ITS phylogeny also resolves the relationship of *Hibiscadelphus* as nested in section Calyphylli. *Hibiscus ovalifolius* ( $2n = 40$ ; Hinsley, 2012) branches basally in this clade and is sister to *Hibiscadelphus*. This phylogeny, in addition to morphological and cytological data, suggest the possibility of a hybridization and polyploidization event where a presumed diploid ( $2n = 20$ ) ancestor may have doubled its ploidy level to become tetraploid. The only known chromosome count for any species of Megistohibiscus is *H. clypeatus* ( $2n = 20$ ). A neotropical relation in the Americas would account for the approximately 5-million-year divergence in the lineage prior to the formation of the current Hawaiian Islands. It is tempting to invoke successive hybridization and polyploidization between these closely related sections of *Hibiscus* (Megistohibiscus and Calyphylli) to explain the evolutionary history of *Hibiscadelphus*. However, the work produced here cannot definitively confirm this. Further taxon sampling, cytogenetics, and genomics may help to elucidate such a potentially fascinating evolutionary history.

## Stepping-Stone Model of *Hibiscadelphus* Island Colonization

After Kauai formed about 5 million years ago (Price and Clague 2002; Clague *et al.*, 2010; Obbard *et al.* 2012), the genus *Hibiscadelphus* began to diversify about 4 million years ago according to the fossil calibrated phylogeny. This is in agreement with generally accepted island ages with upper limits of the interval just outside of the 5-million-year estimate for the age of Kauai. The lower limit of the interval also encompasses the island of Oahu which is generally considered 2.2 – 3.4 million years old. However, Oahu has never had a member of the genus described or collected, an

apparent trend for some Hawaiian lineages (Funk and Wagner, 1995). The island ages are in agreement with the remainder of the radiation across the archipelago with the mean for the Maui clade being 2.1 million years old and the mean for the Hawaii Island clade being 0.6 million years old. These values support the hypothesis that the distribution of *Hibiscadelphus* follows the progression rule.

The phylogenies also support the hypothesis that *Hibiscadelphus* is deeply nested within *Hibiscus sensu lato*. The other Hawaiian lineages of *Hibiscus* account for four separate colonization events to these islands, a red and white clade comprised of the Hawaiian *Hibiscus* of section *Euhibiscus*, two yellow flowered clades from section *Phylloglandula* and a fourth taxa *Hibiscus furcellatus* also from section *Phylloglandula*. Including *Hibiscadelphus* among the *Hibiscus* lineages, there have been five total colonization events of *Hibiscus* to the Hawaiian Islands, three resulting in completely endemic lines (*Hibiscadelphus* species, red/white flowered clade of *Hibiscus*, and *H. brackenridgei*) and two indigenous lines (*H. tiliaceus* and *H. furcellatus*). By comparison, *Ipomoea* (Convolvulaceae) has five species resulting from five separate colonization events, but only one species that is endemic (Wagner *et al.* 1990). Hawaiian species of *Peperomia* (Piperaceae) are descended from four colonization events resulting in two indigenous species and two radiations of endemic species (Lim *et al.* 2019).

A clearer understanding of the evolutionary history and phylogenetic relationship of these plants is informative from a natural resource management and conservation perspective. The survivorship of the genus is being impacted by inbreeding depression, invasive species, habitat loss, and depredation by pests (Baker, 1980). Conservation agencies, National Parks, and horticulturalist are keenly interested in how to implement restoration efforts for the genus, but vigorous hybrids have previously removed from natural areas in fear of introgression with parental species (Baker, 1980). The results here suggest that species of *Hibiscadelphus* be reclassified as species of *Hibiscus*. By doing so, we begin to gain an understanding of the importance of the genus *Hibiscus* to the Hawaiian Islands. It is not just the state flower, but a genus of flowering plants that was able to disperse repeatedly across thousands of miles of open ocean most readily and successfully establish, adapt, and proliferate across the entire archipelago. With five separate colonization events and two distinct radiations of species, *Hibiscus* represents an important example of evolutionary divergence and adaptation, and an important icon of the Hawaiian Islands.

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