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DIFFERENTIATING THE NECHES RIVER ROSE MALLOW (HIBISCUS DASYCALYX) FROM ITS CONGENERS BY MEANS OF PHYLOGENETICS AND POPULATION GENETICS

Julia Norrell

DIFFERENTIATING THE NECHES RIVER ROSE MALLOW (*HIBISCUS DASYCALYX*) FROM ITS CONGENERS BY MEANS OF PHYLOGENETICS AND POPULATION GENETICS

by

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Abstract

DIFFERENTIATING THE NECHES RIVER ROSE MALLOW (*HIBISCUS DASYCALYX*) FROM ITS CONGENERS BY MEANS OF PHYLOGENETICS AND POPULATION GENETICS

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The University of Texas at Tyler
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This study used molecular phylogenetic methods to attempt to resolve the taxonomic status of the federally threatened East Texas-endemic wildflower, the Neches River Rose Mallow (*Hibiscus dasycalyx). Hibiscus dasycalyx* co-occurs with two other closely related congeners that are currently not of conservation concern: the halberdleaf rose mallow (*H. laevis)*; and the crimson-eyed rose mallow *(H. moscheutos).* This study assessed the phylogeny of these three *Hibiscus* species, and attempted to determine if there is possible hybridization occurring between them. To this end, Restriction Site Associated DNA Sequencing (RAD-Seq), a Next Generation Sequencing method, was used to generate genome-wide polymorphic genetic data.

Two phylogenies were constructed utilizing Maximum Likelihood and Bayesian coalescence approaches. The Maximum likelihood phylogeny identified *H. dasyclayx*, *H. laevis,* and *H. moscheutos* as distinct monophyletic taxa. The Bayesian coalescence approach suggested *H. moscheutos* is a monophyletic sister clade to *Hibiscus laevis,* but suggested that *H. dasycalyx* and *H. laevis* are one monophyletic group and that *H. dasycalyx* is paraphyletic. AMOVAs did not show significant levels of admixture occurring between *H. laevis, H. moscheutos,* and *H.*

dasycalyx. Bayesian clustering implemented in STRUCTURE was used determine the species relationships and gene flow between species, and revealed that *H. dasycalyx* clusters separately from *H. laevis*, and that the two species were differentiated from each other in this analysis with no evidence of admixture.

The results overall do not have enough support to suggest the need, nor at the same time discredit a reclassification of *H. dasycalyx*. Further analysis of *H. dasycalyx* and *H. laevis* are needed to help better understand the taxonomic relationship between them.

Introduction and Background Information

Introduction

As of 2016, the United States Endangered Species Act listed 944 threatened or endangered species of plants (USFWS, 2017). This is a substantial number of species, and given the limited amount of conservation funding available, it is imperative that resources to protect these species are allocated appropriately. Additional research on taxonomic and population level relationships is therefore necessary to ensure that the target species are properly identified and classified (Schemske et al., 1994). Species targeted for conservation should be able to satisfy all of the standard criteria for categorization as distinct species, or else their taxonomic statuses should be reconsidered (Mace et al., 2008).

Modern genomic methods can help with determining whether these taxonomic classifications are accurate, or if modifications need to be made to include the appropriate groups for priority protection. Taking a genome-wide molecular genetic approach to develop a more accurate taxonomy of the federally threatened wildflower, *Hibiscus dasycalyx*, and its congeners *H moscheutos* and *H. laevis*, will hopefully yield such a clarification.

Malvaceae

The genus *Hibiscus* belongs to the family Malvaceae, commonly referred to as the mallow family, the members of which are found in tropical, sub-tropical and temperate regions. Members of this family also include okra, cotton, and cacao (Ploetz, 2007). Malvaceae contains over 4,000 species with *Hibiscus* being the largest genus with over

300 species (Akpan, 2007). The genus contains annuals, herbaceous perennials, shrubs and small trees; and some *Hibiscus* species are known to be valuable as sources of food and medicine (Wilson and Menzel, 1964).

Section Muenchhusia

A section is a taxonomic rank listed below the genus, but above the species level. *Hibiscus* section *Muenchhusia* was separated from the large *Hibiscus* section *Trionum* by Blanchard (Fryxell, 1988). Section *Muenchhusia* is comprised of a group of five closely related and recently evolved *Hibiscus* species (*H. moscheutos. H. laevis, H. grandiflonts. H. coccineus* and *H. dasycalyx*) uniquely designated "rose mallows," whose range is confined mainly to marshy habitats in the eastern half of the United States (Blanchard, 1976; Small, 2004). The plants in this section exhibit a shared ecological wetland niche, similar morphological characteristics, a shared growth habit, and common geographic distribution throughout eastern and central North America (Blanchard, 1976). *Hibiscus dasycalyx* co-occurs with two other closely related congeners, the halberd leaf rose mallow, *H. laevis*, and the crimson-eyed rose mallow, *H. moscheutos*, that have similar ecological ranges but that are not considered imperiled (Blanchard, 1976; Sain, 2015).

Hibiscus dasycalyx

Hibiscus dasycalyx is a perennial that can only be found in the wetlands of East Texas, including Cherokee, Houston, and Trinity counties (TPWD, 2011). *Hibiscus dasycalyx* is distinguished from its congeners by a combination of long, thin leaves that are lobed at the end as well as hairy calyces (Figure 1). *Hibiscus dasycalyx* possesses vegetative parts that are glabrous (hairless), and leaves that are deeply and narrowly

three-lobed. The petals moderately spread beyond the calyx tube, and are of white color with a red base. *Hibiscus dasycalyx* is very similar to *H. laevis*, except for its highly pubescent (covered with erect hairs) calyx and fruit and extremely narrowly and deeply lobed leaves. *Hibiscus dasycalyx* is threatened by interspecific hybridization with *H. laevis* and *H. moscheutos*, as well as loss of preferred wetland habitat along the Neches River and its tributaries (Klips, 1995).

Hibiscus laevis

Hibiscus laevis has glabrous vegetative parts, including the calyx and capsule. The leaves are triangularly three-lobed in general outline (Klips, 1995). The middle leaf lobe is two to six times as long as the width of the body of the leaf. The petals are pink or white with a red base, moderately spreading beyond the calyx tube (a common trait in the *Hibiscus* genus), and is bee-pollinated (Klips, 1995). The entirely glabrous parts and reddish- pubescent seeds help to distinguish *H. laevis* from the similar *H. moscheutos* (Blanchard, 1976).

Hibiscus moscheutos

Hibiscus moscheutos is characterized by vegetative structures that are pubescent to a certain degree (Klips, 1995). The leaf is unlobed to broadly triangular-ovate. The calyx has star shaped hairs and is densely pubescent with matted, soft white woolly hairs. The capsule is variously pubescent, with hair ranging from simple, or stellate, to glandular. The petals are usually white or pink, with a red base in the center, near the calyx, and like the previous *Hibiscus* species they are also bee-pollinated (Klips, 1995).

Taxonomic statuses

Since these species are very similar in terms of habitat use and morphology, the concern has been raised whether *H. dasycalyx* is a distinct species or a misidentified subgroup of *H. laevis* or *H. moscheutos*. This possibility is presented in recent work by Sain (2015), indicating that *H. dasycalyx* is genetically similar to *H. laevis* at the *GRANULE-BOUND STARCH SYNTHASE I* (*GBSSI*) gene. Klips (1995) also raised the question whether *H. dasycalyx* was a distinct species and conducted laboratory breeding experiments to test for possible hybridization between *H. laevis* and *H. moscheutos*. He found cross pollination was able to occur in the lab, and hybrid offspring are robust and fertile between *H. dayscalyx-H. laevis* and *H. dasycalyx-H. moscheutos* (Klips, 1995). Klips found *H. dasycalyx* and *H. laevis* both had electrophoretically detectable enzyme alleles that distinguish them both from *H. moscheutos*. This research suggested the possibility that *H. laevis* is so genetically similar to *H. dasycalyx* that it might better be regarded as an ecotype or variety of *H. laevis* rather than a separate species. On the other hand, this it is possible that *H. dasycalyx* repeatedly back-crossed with *H. laevis* following one or more hybridization events between the two wide-ranging species. His research was not able to tell apart these scenarios using the available data.

On the other hand, some of the research into these species suggests they are all genetically distinct from one another. Small (2004) created a phylogeny of the *Muenchhusia* section and his results showed *H. dasycalyx, H. laevis*, and *H. moscheutos* grouping as separate monophyletic taxa.

Furthermore a Bayesian clustering analysis implemented using the program STRUCTURE (Pritchard et al., 2000) with *GBSSI* data suggested that *H. dasycalyx*

individuals have a genetic affinity for one another that distinguishes them from *H. laevis* and *H. moscheutos* (Banta, unpublished data).

Given the conflicting evidence available to date, the taxonomic status of *H. daycalyx* requires further attention. This study utilized Restriction Site Associated DNA Markers (RAD-Seq) to yield genome-wide polymorphic genetic data, as opposed to data from just one or a few genes. This is important because gene phylogenies can be misleading regarding evolutionary relationships (Spinks et al., 2013). The objectives were to address the following: (1) Is *H. dasycalyx* a distinct taxon from *H. laevis* and *H. moscheutos*? (2) If so, to what degree is hybridization (or advanced-generation hybridization, also known as introgression and admixture) between *H. laevis*, *H. dasycalyx,* and *H. moscheutos* occurring?

Phylogenetics

Phylogenetics is the study the evolutionary relationships of organisms (Hedges, 2002). With this type of research the understanding of how individuals or species should be grouped to reflect their relatedness. In previous research, phylogenetic analysis of the gene *GBSSI* was used to find differentiation between *H. laevis*, *H. moscheutos*, and *H. dasycalyx* (Sain, 2015). The results showed that that while *H. dasycalyx* was not distinguishable from *H. laevis* phylogenetically, both species were distinguishable from *H. moscheutos*. The phylogenetic tree did not resolve *H. dasycalyx* to be distinct from *H. laevis*, but the results were left ambiguous because of low support values for the nodes (Sain, 2015).

Phylogenetic relationships among recently diverged species are often difficult to resolve due to insufficient markers and confliction among gene trees (Eaton and Ree,

2013; O'Meara, 2010). Additional genome-wide data is needed to fully understand the relatedness between *H. laevis* and *H. dasycalyx*, as single-gene analyses often lack resolution to confidently infer phylogenetic relationships among species. (Gontcharov, 2004; Cariou, 2013; Maddison and Knowles, 2006). Applying similar methods to RAD-Seq will provide more robust context on the evolutionary history of these species, and hence their proper taxonomic groupings.

RAD-Seq

RAD-Seq is a fractional genome sequencing strategy designed to interrogate the selected genome (Baird et al., 2008; Floragenex, 2015). Genomic DNA from the study specimens is digested with a restriction nuclease, then a series of adapters are attached to the resulting DNA fragments, allowing for amplification and tagging for Illumina sequencing. Following high-throughput sequencing, thousands of genetic variations such as SNPs (single nucleotide polymorphisms) are obtained, permitting robust phylogenetic analysis across the study specimens. RAD-Seq-derived SNPs can also be used for assessing population structure, linkage, and quantitative trait locus mapping (Narum et al., 2013). Phylogenetic methods such as RAxML (Stamatakis, 2014) or BEAST2 (Drummond and Rambaut, 2007) have been popular to determine evolutionary relationships among individuals (Ogilvie et al., 2016).

Population Genetics

Many evolutionary processes, such as natural selection, local adaptation, and genetic drift strongly depend on a species' past and present population structure (Meirmans, 2012). Assessment of population structure also has practical importance in

conservation biology and the study of invasive species (Meirmans, 2012), because species of conservation and invasive concern often have closely related congeners with whom they co-occur in the wild and with whom they can exchange alleles. Hybridization is one of the chief threats to conservation species (Rhymer and Simberloff, 1996).

Phylogenetics is not designed to study population structure, so additional analyses are required to infer history of hybridization and advance-generation backcrossing (also known as introgression/admixture). Population genetic analyses like Bayesian clustering with STRUCTURE (Prichard et al., 2000) as well as Analysis of Molecular Variance (AMOVAs) (Excoffier et al., 1992) provide the information necessary to understand the patterns of allele sharing that are occurring between *H. dasycalyx* and the congeners that are co-occurring in the same habitat range. The amount of inbreeding and outbreeding occurring within and among these populations will help illustrate whether gene flow is occurring among species, suggesting certain patterns of hybridization, introgression, and gene flow.

Gene flow with *H. laevis* or *H. moscheutos* is a possible threat to future persistence of *H. dasycalyx* populations. As seen in Klips (1995), hybridization was possible when in a lab breeding setting between *H. dasycalyx*, *H. laevis*, and *H. moscheutos*. Evidence has also been suggested by Bayesian clustering analysis of the gene *GBSSI* that admixture is possibly occurring between these species (M. Sain unpublished data). The five species of the *Muenchhusia* section have been recorded to have a chromosome number of n = 19, and they are known to form hybrids relatively easy (Winters, 1970; Small, 2004).

To assess how diversity is partitioned across the different groups, Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992) was performed to assess the relative divergence of the three species from one another as compared to the divergences within each of the species. From the AMOVA, F_{st} was calculated, measuring the degree of inbreeding of each species relative to a single panmictic (random mating) population. Furthermore, Bayesian clustering analysis was performed to graphically assess the degree of allele sharing and haplotypic differentiation within and among the three species. The same RAD-Seq derived data was used here as described above.

Figure 1: From left to right: *Hibiscus dasycalyx* with narrow leaves and hairy calyx; *Hibiscus laevis* with wider leaves and no hairs on the calyx; *Hibiscus mosechuetos* (photos by J Norrell and JK Marlov).

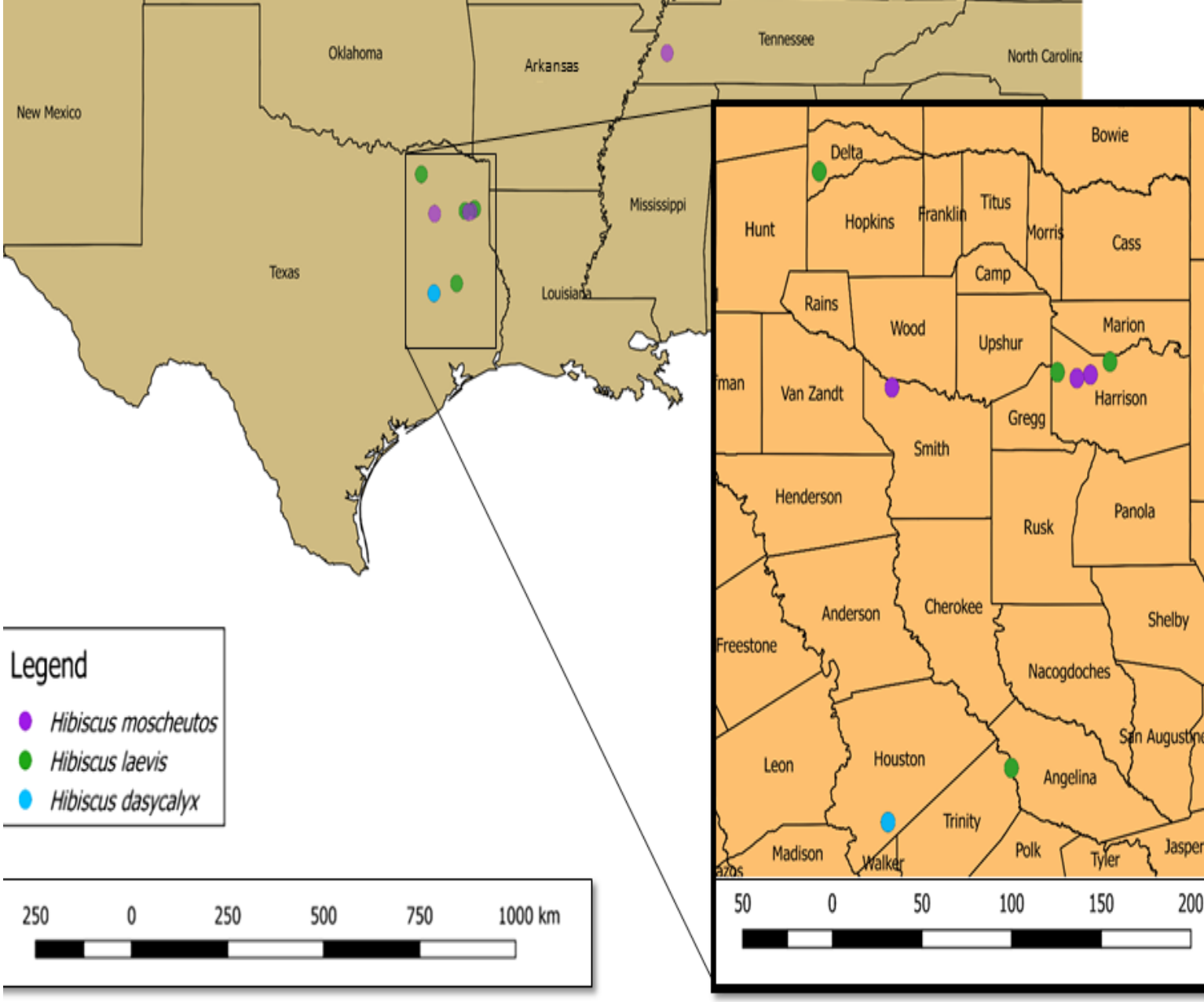

Figure 2: Map showing the populations of *Hibiscus* specimens sampled in Texas and Tennessee with surrounding states outlined. Species represented by the following colored dots: *Hibiscus dasycalyx* (blue), *Hibiscus laevis* (green), and *Hibiscus moscheutos* (purple). Map inset shows counties where *Hibiscus* species were collected in Texas.

Methods

Phylogenetic Methods

Plant Collection

The *H. dasycalyx*, *H. laevis,* and *H. moscheutos* sampled for this study came from wild-collected populations, and *H. trionum* was initially obtained from a commercial source and provided by Dr. Edwige Moyroud at the University of Cambridge. Locations of plant sampling are recorded in Table 1 and exemplar herbarium specimens will be submitted to the Botanical Research Institute of Texas (BRIT). The plant samples used were collected from the field from June-October 2014, and in April 2016. The distribution of the populations and the areas where the specimens were collected in both Texas and Tennessee were recorded (Figure 2). As shown, all samples for *H. dasycalyx* came from the *Hibiscus* preserve for the *H .dasycalyx* species in Lovelady, TX (http://www.texaslandconservancy.org/lands/properties-list/east-texas/97-hibiscus-preserve-houston-county).

DNA Extraction and RAD-Seq

Tissue samples were stored at -80 Celsius following collection. The leaves of each plant collected were used for DNA extractions performed with the Qiagen DNeasy Plant Mini Kit. DNA extraction samples of each species *H. dasycalyx* (6 samples), *H. laevis* (4 samples), and *H. moscheutos* (5 samples), and 1 outgroup species *Hibiscus trionum* were sent to the Floragenex lab for RAD-Seq analysis.

Once the extracted DNA quality was confirmed via gel electrophoresis and Nanodrop as per the standards set by Floragenex (see Appendix), samples were sent off to the Floragenex lab for RAD- sequencing and SNP identification. The Florgenex

protocols were as follows. The genome was first digested with a restriction endonuclease *PstI*, and then a series of sequencing adapters were ligated to the resulting DNA fragments. The DNA fragments were subjected to 1x100bp Seq on Illumina Hi Seq 2000 15-30x (Bentley et al., 2008).

Following Floragenex's standard bioinformatics pipeline, sample M7 was assembled *de novo* and used as the pseudoreference to call SNPs for the rest of the samples. Filters were applied at three levels of stringency: relaxed, standard, and stringent. Subsequent analyses used the SNPs called by the standard criteria, specifically a cluster depth of 10 – 1000 and 2 – 4 variants per cluster. The resulting genome-wide SNP data were used to: (a) determine the heterozygosity at specific loci, (b) quantify the gene flow among the three species, and (c) construct a single phylogeny based on all of the samples and the SNPs within all of the genetic fragments. This helped create a picture of the evolutionary and genetic relationships among these species, which takes into account multiple genomic fragments and multiple individuals (Davey et al., 2011; Heled and Drummond, 2010). Sequence data has been archived under NCBI BioProject PRJNA382435.

Maximum Likelihood Phylogeny

Randomized Axelerated Maximum Likelihood (RAxML) is a program for phylogenetic analysis of large datasets, which implements a tree search algorithm that returns trees with reliable likelihood scores (Stamatakis, 2014). JModeltest 2.16 v20140903 identified a General Time Reversible (GTR) model as the best model of sequence evolution for the concatenated SNP alignment under the Akaike information criterion (AIC). A phylogeny was constructed in RAxML 3.1 using the rapid

bootstrapping with subsequent ML search option under a GTR model of evolution with an ascertainment bias correction (ASC), given that only variant SNP sites were included in the alignment (as discussed in the RAxML manual). RAxML assessed support for the phylogeny using non-parametric bootstrap resampling of 100 replicates (Felsenstein, 1981). The output was then visualized in a phylogenetic tree using used FigTree v1.4.3. *Hibiscus trionum* was selected as the outgroup because the section in which it is placed (*Trionum*) is closely related to section *Muenchhusia*, and this outgroup has been used in previous studies with *H. dasycalyx* (Small, 2004).

Bayesian Coalescence Phylogeny

An additional phylogeny was constructed using the program Bayesian Evolutionary Analysis by Sampling Trees (BEAST) (Bryant et al., 2012), with the add-on package SNP and AFLP Package for Phylogenetic analysis (SNAPP) (Bryant et al., 2012). This package is designed for inferring species trees and species demographics from independent (unlinked) biallelic markers such as well spaced SNPs (Bryant et al., 2012). This program implements a full coalescent model, but uses a novel algorithm to integrate over all possible gene trees, rather than sampling them explicitly. Following Yoder et al. (2013), we analyzed our SNP data using a multispecies coalescent approach in SNAPP version 1.3.0 within BEAST2 v2.3.2. The analysis utilized the same GTR model of evolution and proceeded for 10,000,000 generations with 1,000,000 (10%) discarded as burnin. The full SNP data were converted to a 0, 1, 2 format for analysis, with 1 representing a heterozygous genotype. Once the program completed, the results were analyzed in Tracer (Drummond and Rambaut et al., 2007) for performance and accuracy. As a primary analysis, we used all individuals of our focal in-group species and

a single individual of *H. trionum* as an outgroup to facilitate rooting as with the maximum likelihood phylogeny. The output was then visualized in a phylogenetic tree using used FigTree v1.4.3.

Bayesian clustering analysis

The potential number of genetic clusters and the membership of each individual were estimated using STRUCTURE Ver. 2.3.4 (Pritchard et al., 2000). The software uses Markov chain Monte Carlo (MCMC) simulations to estimate those parameters, with the number of clusters to be tested (K) specified by the user (Blanco-Bercial and Bucklin, 2016). The MCMC simulation was run for 300,000 iterations, after a burn-in period of 100,000 iterations. The traces were examined graphically to confirm chain convergence. The most likely *K* present in the data was inferred following Evanno et al. (2005). For each value of K (number of potential ancestral populations, which ranged from 1 to the number of presumed populations + 1), the genetic ancestry of each individual was estimated based on the admixture model without any prior population assignment. For the entire population set, K ranged from 1 to 10. The optimal K between the species in the 10 subsets was visualized and then chosen using the lowest log-likelihood (Rohlf and Sokal, 1995).

AMOVA

Analysis of Molecular Variance (AMOVA) was used to quantify differentiation among species (Excoffier et al., 1992). It was conducted in *Arlequin* 3.5 to examine the

variation within and among groups of genetically similar species. AMOVA uses the amount of variance explained among groups via F-statistics (see below) to assess whether there is well-defined population structure. AMOVA also assigns populations into a *priori* groups. AMOVA was conducted with 1,000 simulated annealing permutations. Separate AMOVAs were conducted for (i) the entire data set and (ii) *H. dasycalyx* and *H laevis* samples only.

F-statistics (Wright, 1951) are used to quantify genetic differentiation between different groups. In this study the *F*-statistic was used to measure differentiation among the species rather than among subpopulations. The fixation index we call F_{st} measures genetic differentiation of species relative to the total genetic diversity of all samples. This statistic calculates how genetically similar two species are to one another; for the AMOVA with all three species, the F_{st} reported is the average F_{st} of all pairwise comparisons. The values range from 0 to 1. Zero indicates the species have open gene flow among them, and therefore have higher amounts of genetic diversity shared among them. A higher F_{st} indicates there is possible inbreeding occurring within the species and low amounts of gene flow are happening among the species, which results in lower amounts of genetic diversity between the populations. These data are used to help understand the degree of gene flow among species. F_{st} was calculated by *Arlequin* as part of the AMOVA described above.

Results

RAD-Seq Results

The RAD-Seq analysis yielded large amounts of genome-wide data for the three *Hibiscus* species. The number of quality filtered RAD tags via the standard output of reads passing FASTQ quality filters were 14,354,883, and the number of failing reads was 480,151. The total number of contigs extracted from the provisional clusters were 44,054, and the total number of contigs in the final assembly were 71,194 with an average base pair length of 92. The total cluster length was 6,549,848 bp.

Out of the 16 samples screened, the total number of candidate variants detected was 117,026, and the number of candidate variants filtered (due to missing or low quality data) was 102,622. The number of candidate variants passing all filters was 14,062. The average number of polymorphisms within 200 bp of each variant was 3.1. The number of homozygous genotypes found was 197,488, and the number of heterozygous genotypes found was 16,379.

Maximum likelihood phylogeny

The rooted maximum likelihood tree shows *H. dasycalyx* and *H. laevis* to be more closely related to each other than either are to *H. moscheutos*. Furthermore, it shows *H. laevis* and *H. dasycalyx* to each be a separate monophyletic group, albeit closely related. The analysis separated the three species into two major clades (Figure 3): one clade contained only *H. moscheutos*, and the other clade contained both *H. dasycalyx* and *H. laevis*. Within the H. *dasycalyx-H.laevis* clade, the two species were monophyletic sister taxa. Bootstrap support for all nodes was high, except for some internal nodes within the *H. dasycalyx* clade.

Bayesian Coalescence Phylogeny

The rooted Bayesian coalescent tree also showed two major clades (Figure 4): one containing only *H. moscheutos* and one containing both *H. dasycalyx* and *H. laevis*. The difference in this analysis was that *H. dasycalyx* was a paraphyletic taxon within *H. laevis*. The major clades described here, as well as the paraphyly of *H. dasycalyx*, had high posterior support.

Bayesian clustering analysis

For the Bayesian cluster analysis of all three species, the most parsimonious number of inferred ancestral groups was two. It shows that *H. moscheutos* clusters separately from *H. dasycalyx* and *H. laevis*, but that *H. laevis* and *H. dasycalyx* do not cluster separately from one another. It also shows no evidence of admixture among *H. moscheutos* and the *H. dasycalyx*/*H. laevis* group (Figure 5). For the Bayesian cluster analysis of just *H. dasycalyx* and *H. laevis* the most parsimonious number of inferred ancestral groups was six. In this case, the analysis was able to detect more fine-scale differentiation between the two species, revealing that *H. dasycalyx* clusters separately from *H. laevis* (Figure 6). While *H. laevis* shows evidence of genetic diversity in the form of multiple inferred ancestral contributions to its genome, these inferred ancestral contributions are not shared by *H. dasycalyx*. Furthermore, the two inferred ancestral groups comprising *H. dasycalyx* were not shared by *H. laevis*. Thus, the two species were reciprocally differentiated from each other in this analysis with no evidence of admixture.

AMOVAs

The AMOVA provided the percentage of molecular variation that is explained by variation 1) among species, 2) among individuals within species, and 3) within species. F_{st} in this case represents the proportion of molecular variation explained by variation among species and ranges from zero to one. For the AMOVA examining all three *Hibiscus* species (Table 1), F_{st} is 0.58 and the *P*-value is < 0.01, rejecting the null hypothesis of no genetic differentiation among the three species. For the AMOVA examining only *H. dasycalyx* and *H. laevis* (Table 3), the percentage of molecular variation explained by variation within species is much larger than the percentage of variation explained by variation among species. However, the F_{st} value is still significantly different from zero (F_{st} = 0.2249; P < 0.01), rejecting the null hypothesis that there is no genetic differentiation between *H. dasycalyx* and *H. laevis*.

Appendix A

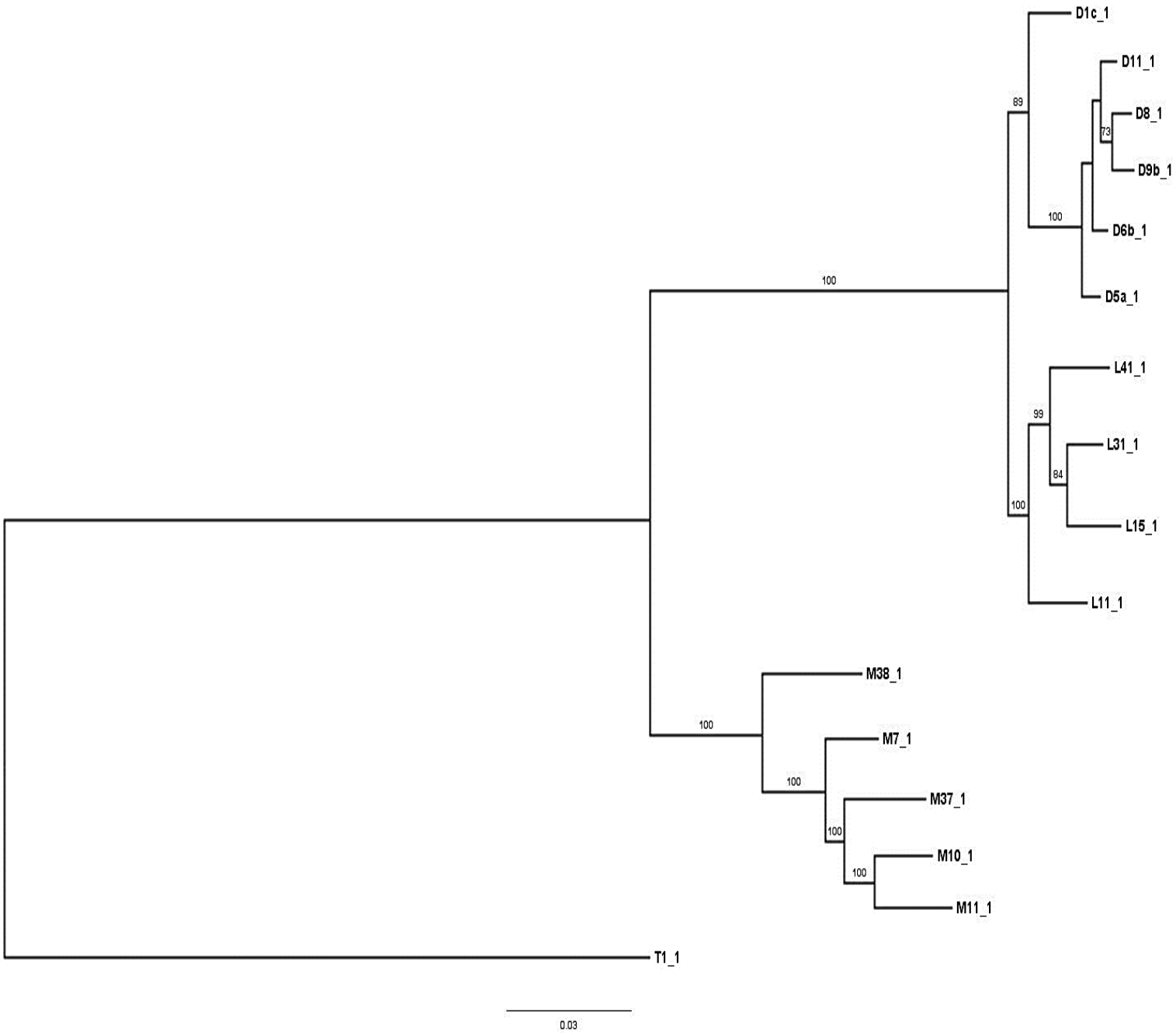

Figure 3 Rooted maximum likelihood tree showing phylogenetic relationships of *H. dasycalyx*, *H. laevis*, and *H. moscheutos* inferred from RAD-seq. Bootstrap values greater than 60% are shown on each branch. Each accession is labeled by species D represent *H. dasycalyx*, L represents *H. laevis*, M represents *H. moscheutos*, and T1 represents the outgroup *H. trionum*.

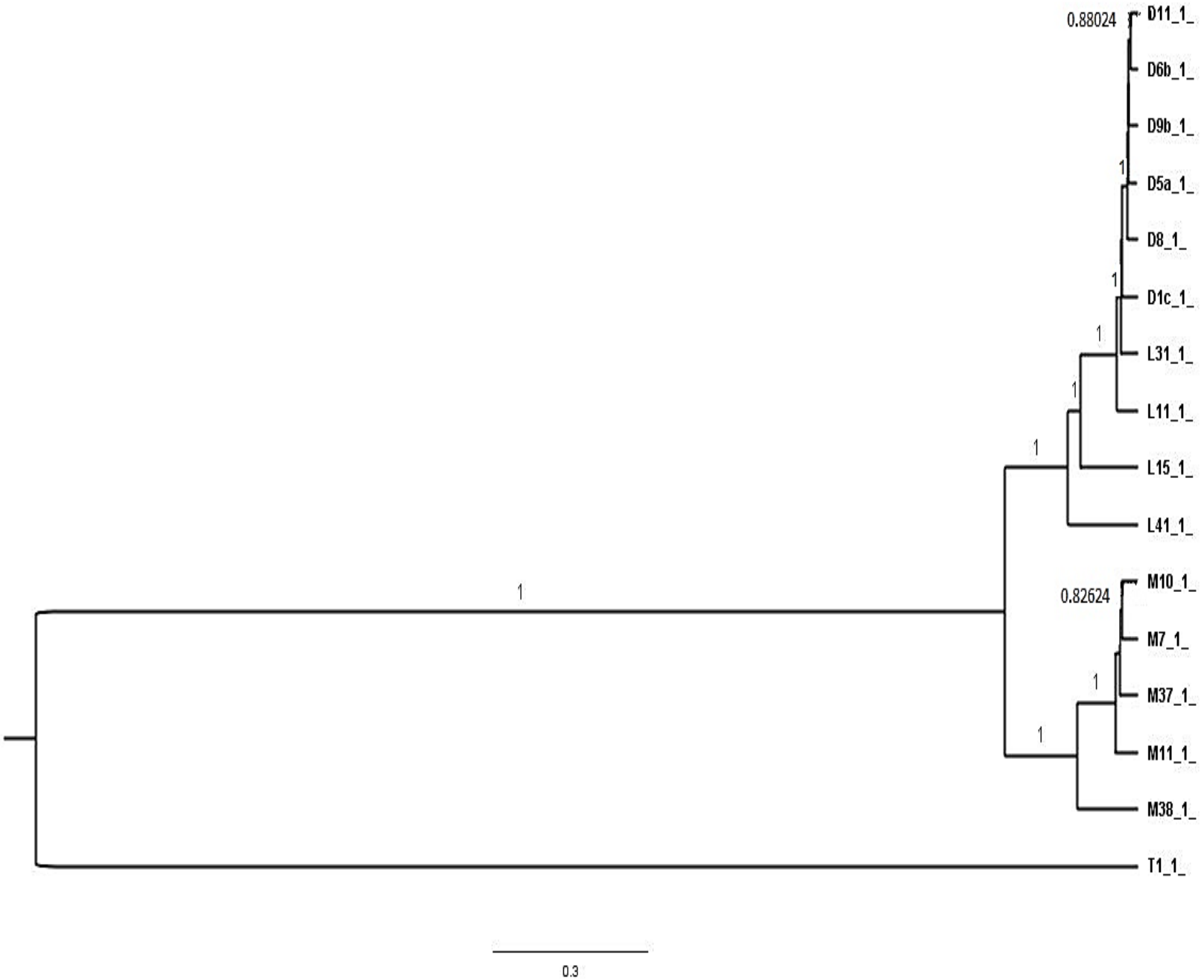

Figure 4 Rooted SNAPP tree showing phylogenetic relationships of *H. dasycalyx*, *H. laevis*, and *H. moscheutos* inferred from RAD-seq. Posterior support values greater than 0.7 are shown above each branch. Each accession is labeled by species D represent *H. dasycalyx*, L represents *H. laevis*, M represents *H. moscheutos*, and T1 represents the outgroup *H. trionum*.

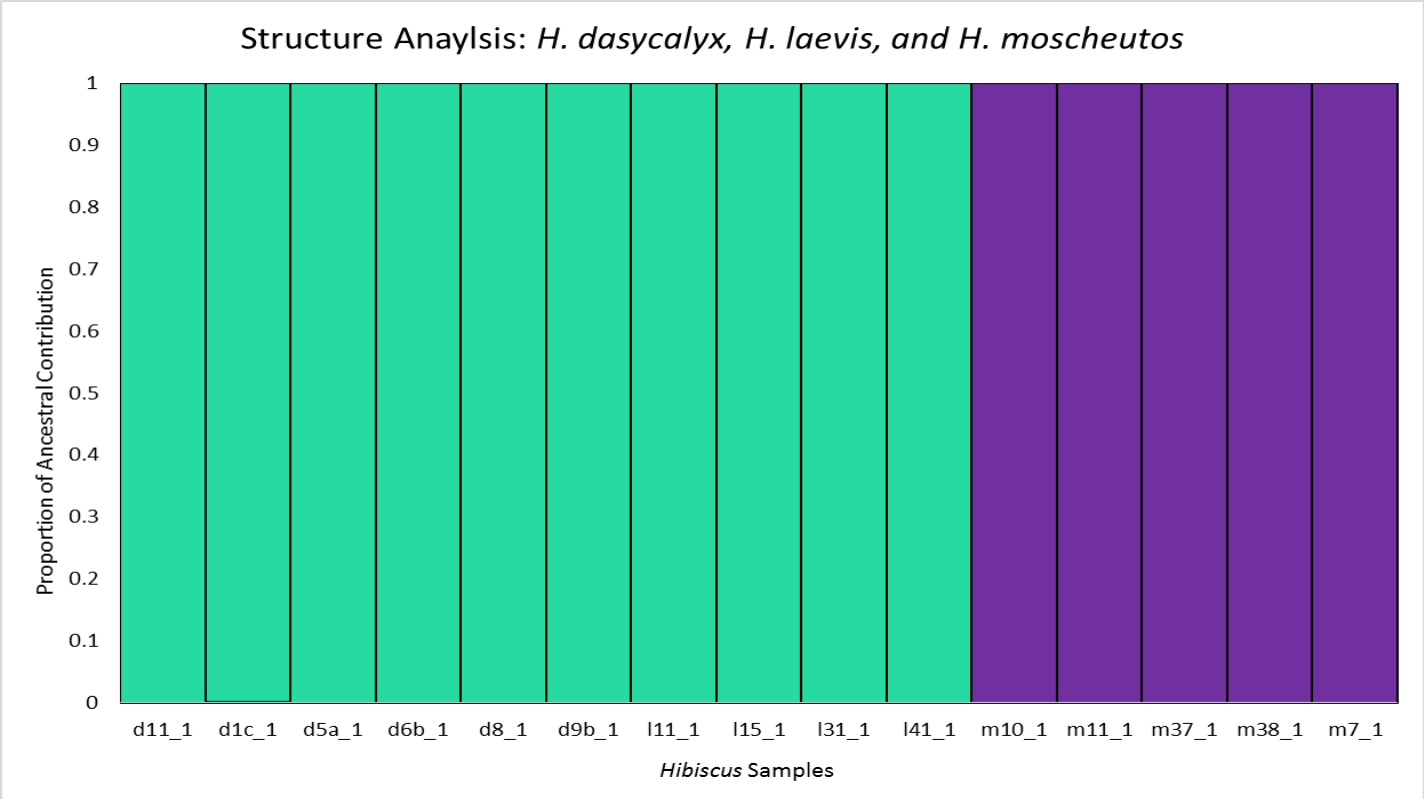

Figure 5. STRUCTURE analysis of all three *Hibiscus* species *Hibiscus dasycalyx* labeled as d, *Hibiscus laevis* labeld as l, and *Hibiscus moscheutos* labeled as m. This analysis shows *moscheutos* clusters separately from *H. dasycalyx* and *H. laevis*, but that *H. laevis* and *H. dasycalyx* do not cluster separately from one another.

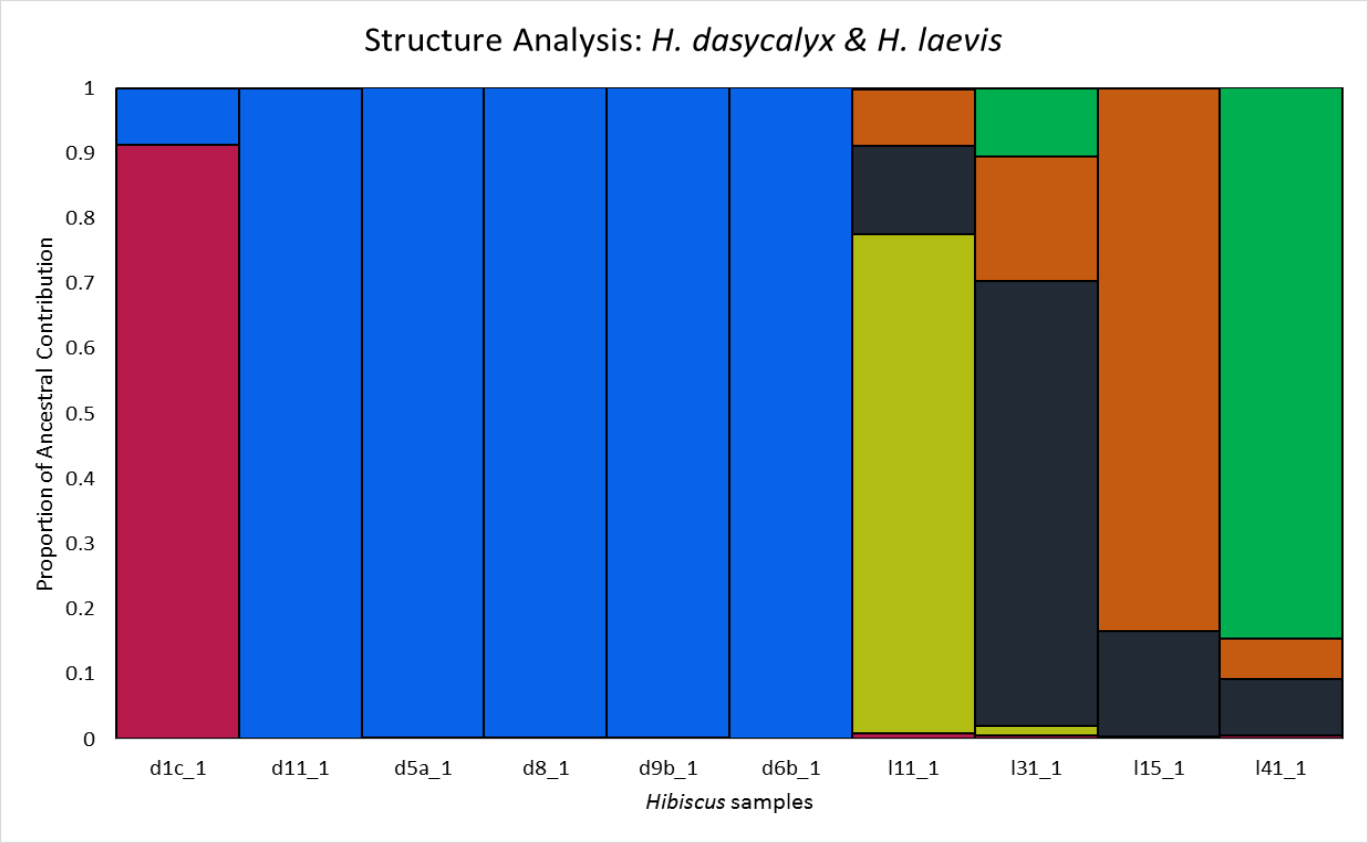

Figure 6. STRUCTURE analysis of *Hibiscus dasycalyx* labeled as d, and *Hibiscus laevis* labeled as l. This analysis shows *H. dasycalyx* and *H. laevis* clustering differently from one another, and this suggests there is differentiation between *H. dasycalyx* and *H. laevis.*

Table 1. AMOVA design and results (average over 9602 loci): *H. dasycalyx*, *H. laevis*, and H. *moscheutos*

Source of variation	**Sum of squares**	**Variance components**	**Percentage variation**	**Fst**	**P- value**
Among species	21074.915	1026.48345	57.86718	0.57867	0.00
Among individual within species	10684.625	195.30710	11.01028		
Within species	7940.500	552.07070	31.12254		
Total	39700.040	1773.86125	100		

Table 2. AMOVA design and results (average over 3962 loci): *H. dasycalyx*, *H. laevis*

Source of variation	**Sum of squares**	**Variance components**	**Percentage variation**	**Fst**	**P-value**
Among species	2239.715	173.32480	22.48949	0.22489	0.00
Among individual within species	5185.367	88.59673	11.49573		
Within species	4883.000	508.77083	66.01478		
Total	12308.082	770.69236	100		

Discussion

Phylogenetics

Next generation sequencing (NGS) represents an opportunity to better clarify the taxonomic status of rare species and thereby make more rational conservation decisions (Andrews et al., 2016). To ascertain the certainty that resources and funding are being appropriated to the correct individuals, these advanced NGS techniques can be used to determine whether a taxon is evolutionarily and phylogenetically distinct from another, and therefore whether the current taxonomy is justified. In this study, we used phylogenetic methods on genome-wide data to ascertain whether *H. dasycalyx* is rightfully considered to be a separate taxon from *H. laevis* and *H. moscheutos*. Previous work by Small (2004) suggested that *H. dasycalyx* was monophyletic and excluded *H. laevis* and *H. moscheutos*, but subsequent work by Sain (2015) using more samples suggested that *H. laevis* and *H. dasycalyx* are not easily distinguishable using a single-gene phylogeny. The previous studies (Small, 2004; Sain, 2015), however, used only one phylogenetically informative gene to draw this conclusion (*GBSSI*), and both called for more research using additional loci to accurately resolve the phylogeny of this group. Such a study is provided here.

Our maximum likelihood phylogeny suggests that *H. dasycalyx* and *H. laevis* are, in fact, monophyletic sister taxa, while *H. moscheutos* is more distantly related, with high bootstrap support (100%). In the Bayesian coalescent phylogeny, however, *H. dasycalyx* and *H. laevis* were not resolved as sister monophyletic taxa. Instead this analysis suggests that *H. dasycalyx* + *H. laevis* are monophyletic, but that *H. dasycalyx* is paraphyletic.

The findings by both phylogenetic tree construction methods support previous research suggesting that *H. moscheutos* is more distantly related to the clade containing *H. dasycalyx* and *H. laevis* (Small, 2004; Sain, 2015, Klips, 1995). Furthermore, while the two methods differ as to whether *H. dasycalyx* and *H. laevis* are reciprocally monophyletic, they both suggest that these two species are very closely related. One possible explanation as to why the two methods differ regarding reciprocal monophyly between *H. dasycalyx* and *H. laevis* is that the Bayesian coalescent approach implemented here performs better as the number of taxa increases (Leache et al., 2014); it could be that we did not have enough individuals represented to obtain an accurate picture of the relationships. The maximum likelihood method, however, could have had biases regarding which taxa are heterozygous at each location, which is why this tree postulates different taxonomic relationships than the Bayesian coalescent tree, which applies a completely different model of evolution (Brumfield et al. 2003). Whatever the reason, this illustrates the sensitivity of the results to different software packages that implement phylogenetic methods in different ways, even when the methods themselves are supposed to be similar.

Samples of *H. laevis* and *H. moscheutos* that were used in this study were mostly collected from Texas populations, and fewer samples of each species were used in this study than in Sain (2015). The advantage of this study over previous research on this group is the inclusion of genome-wide data comprising thousands of loci, as opposed to single or multiple gene data. While RAD-Seq provides a vast increase in the amount of data that can be readily generated over what has been possible with previous-generation genotyping methods (Hipp et al., 2014), this does not compensate for having as

geographically broad a representation of samples as possible. In the future, more samples of all three species should be studied to achieve broader representation of the genotypes of all three species. This may be more enlightening about patterns of phylogenetic differentiation within and among the three species. A caution in interpreting these findings is that this study only included *H. dayscalyx* specimens from a single population, while the *H. laevis* and *H. moscheutos* samples came from multiple counties across East Texas and Tennessee. This could influence the fact that *H. dasycalyx* appeared to be less genetically diverse than *H. laevis*, but it should not influence the conclusion that there is a core group of *H. dasycalyx* specimens that are differentiated from *H. laevis*. This would appear to add more weight to the conclusion that *H. dasycalyx* is a separate taxon from *H. laevis*, as the current taxonomy implies. Taken together with the ambiguous phylogenetic results (one analysis showing reciprocal monophyly between *H. laevis* and *H. dasycalyx*, the other one not), there is no clear signal emerging from our data that would recommend either affirming the current taxonomic rank of *H. dasycalyx* as a species or reclassifying it as a variety of *H. laevis.* Therefore, the most prudent approach at this point is to continue treating *H. dasycalyx* as a distinct taxon until the weight of evidence tips the scales to some other conclusion.

This study used clearly morphologically delineated specimens, which could have had an impact on the results. All *H. dasycalyx* samples came from a single population where the plants are clearly identified as *H. dasycalyx* morphologically (J. Norrell, personal observation and photo documentation; USFWS 2016), and the samples of *H. laevis* and *H. moscheutos* were likewise morphologically unambiguous representatives of their respective taxa (J. Banta, personal communication). Samples of *H. dasycalyx* from

other populations were not considered for this study, even though individuals in some of the other documented populations of this species are more intermediate and ambiguous in their phenotypes, and co-occur with *H. laevis* and *H. moscheutos* at those sites (TPWD, 2011). Including these other sites may make the phylogenetic results more nuanced and complex. Even in this simplified study, using only a single isolated population of morphologically unambiguous *H. dasycalyx*, the Bayesian coalescence approach could not recover *H. dasycalyx* as a monophyletic group (although the maximum likelihood approach did); and uncertainty regarding the taxonomic status of *H. dasycalyx* still remains.

The AMOVA and the Bayesian clustering analysis (STRUCTURE) both support the conclusions derived from our maximum likelihood tree in that *H. moscheutos* is a sister taxon to *H. dasycalyx* and *H. laevis*. The AMOVA and the Bayesian clustering analysis both showed that most of the genetic variation among species in this group is due to variation among *H. moscheutos* and the *H. laevis + H. dasycalyx* clade; an AMOVA including only *H. laevis* and *H. dasycalyx* dropped the value of F_{st} by more than half, as compared to the one including all three species; and in a Bayesian clustering analysis containing all three species, the genetic differentiation of *H. moscheutos* so eclipsed the genetic differences between *H. laevis* and *H. dasycalyx* that they could not be detected. Interestingly, a Bayesian clustering analysis of only *H. laevis* and *H. dasycalyx* did reveal genetic diversity in the inferred ancestral contributions to the individuals, with most of this diversity within *H. laevis.* Furthermore, *H. laevis* and *H. dasycalyx* did not share overlapping inferred ancestral contributions, suggesting they are genetically distinct

from one another (Giolo et al., 2012). Thus the Bayesian clustering analysis finds all three species to be genetically distinct from one another, with no evidence of admixture.

Interestingly, the population genetic analyses did not find any clear evidence of gene flow among these three species. This would have manifested itself in the Bayesian cluster analysis results, where the same inferred ancestral groups would be evident in multiple different species, but this was not the case. This could be in part an artifact of the paucity of sampling for *H. dasycalyx*, discussed above, so this negative result is perhaps not surprising. Furthermore, morphologically pure representatives of each of three species were used, which would further bias the results against detecting admixture that may be occurring in nature. More sampling of all three species from a larger number of populations, including morphologically ambiguous specimens, is warranted to strenuously test for hybridization/introgression in this group.

Conclusion

Even using advanced NGS RAD-Seq methods, the phylogenetic genetic results presented here failed to recover *H. dasycalyx* as a separate taxon from *H. laevis* when a Bayesian coalesce phylogenetic approach was used. The maximum likelihood phylogeny did show *H. dasycalyx* as a separate taxon from *H. laevis*, so it is possible that *H. dasycalyx* and *H. laevis* are reciprocally monophyletic. Both phylogenies indicate that *H. laevis* and *H. dasycalyx* are very closely related to each other; *H. moscheutos* however, was clearly shown in both phylogenies to be a distinct taxa from *H. laevis* and *H. dasycalyx.*

AMOVA and Bayesian clustering methods did not show a significant level of admixture occurring between *H. laevis, H. moscheutos*, and *H. dasycalyx*. This is possibly due to the sampling of the populations: each specimen was chosen based on distinct morphology and collected from areas where the species did not overlap. It is possible that samples taken from sites where all three species occur could show signs of hybridization.

Ultimately, the results were inconclusive and did not produce a strong argument for amending the current taxonomic status of *H. dasycalyx*. The current conservation status of *H. dasycalyx* is therefore warranted. It is important to note that, while these results do not call for a change in the current taxonomic status of the *H. dasycalyx*, there is also not enough evidence in these findings to firmly state *H. dasycalyx* and *H. laevis* should not be reclassified into one taxa. Further analysis of *H. dasycalyx* and *H. laevis* are needed to help better understand the taxonomic relationship between them.

Appendix B

Table 3: Showing all the specimens there sample name, GPS coordinates from the collection site, and the county and state they were collected from.

Species	Sample Name	GPS Coordinates (latitude, longitude)	Location
Hibiscus trionum	T1	N/A	Donated by Dr. Edwige Moyroud at the University of Cambridge; previously obtained from a commercial source
Hibiscus moscheutos	M7	-95.458304, 32.58368287	Smith County, TX
Hibiscus moscheutos	M10	-94.51598, 32.62743	Harrrison County, TX
Hibiscus moscheutos	M11	-94.51598, 32.62743	Harrrison County, TX
Hibiscus moscheutos	M37	-94.580391, 32.615227	Harrrison County, TX
Hibiscus moscheutos	M38	-89.42619323, 35.58216476	Haywood County, TN
Hibiscus laevis	L11	-9580344937, 33.32034211	Delta County, TX
Hibiscus laevis	L15	-94.67286, 32.63586	Harrrison County, TX
Hibiscus laevis	L31	-94.42331, 32.67161	Harrrison County, TX
Hibiscus laevis	L41	-94.8912, 31.286128	Trinity County, TX
Hibiscus dasycalyx	D1c	-95.476861, 31.101333	Houston County, TX
Hibiscus dasycalyx	D5a	-95.476861, 31.101333	Houston County, TX
Hibiscus dasycalyx	D6b	-95.476861, 31.101333	Houston County, TX
Hibiscus dasycalyx	D8	-95.476861, 31.101333	Houston County, TX
Hibiscus dasycalyx	D9b	-95.476861, 31.101333	Houston County, TX
Hibiscus dasycalyx	D11	-95.476861, 31.101333	Houston County, TX

Supplemental Links

Supplemental data and file report from Floragenex is available on:

https://www.dropbox.com/s/5eqp8zq3bwb6bv0/JNorrell_UTexasTyler_Hibiscus_20160413-01409_Project_Report%20%281%29.pdf?dl=0

Standards for DNA to be processed by RAD-Seq is available on:

https://www.dropbox.com/s/3ly897ta85um8dh/Microsoft%20Word%20-%20STARTING%20YOUR%20FLORAGENEX%20PROJECT%20PREMIUM_130D.pdf?dl=0

References

Adenyo, C., Hayano, A., Kayang, B. B., Owusu, E. H., & Inoue-Murayama, M. 2013. Mitochondrial D-loop diversity of grasscutter (Thryonomys swinderianus Rodentia:Hystricomorpha) in Ghana. *Open Journal of Animal Biology*

Akpan, G. A. 2007. Hibiscus. In *Flower Breeding and Genetics* (pp. 479-489). Springer Netherlands.

Andrews, K. R., J. M. Good, M. R. Miller, G. Luikart, & P. A. Hohenlohe. 2016. Harnessing the power of RADseq for ecological and evolutionary genomics. Nature Review Genetics 17:81-92.

Baird, Nathan A., Paul D. Etter, Tressa S. Atwood, Mark C. Currey, Anthony L. Shiver, Zachary A. Lewis, Eric U. Selker, William A. Cresko, & Eric A. Johnson. 2008."Rapid SNP discovery and genetic mapping using sequenced RAD markers." *PloS one* 3, no. 10 e3376.

Bentley, D. R., Balasubramanian, S., Swerdlow, H. P., Smith, G. P., Milton, J., Brown, C. G, & Boutell, J. M. 2008. Accurate whole human genome sequencing using reversible terminator chemistry. *Nature*, *456*(7218), 53-59.

Blanchard, O. J. 1976. A revision of species segregated from *Hibiscus* sect. *Trionum* (Medicus) de Candolle sensu lato (Malvaceae). Ph.D. dissertation, Cornell University, Ithaca, NY.

Blanco-Bercial, L., & Bucklin, A. 2016. New view of population genetics of zooplankton: RAD-seq analysis reveals population structure of the North Atlantic planktonic copepod Centropages typicus. *Molecular ecology*.

Brumfield, R. T., Beerli, P., Nickerson, D. A., & Edwards, S. V. 2003. The utility of single nucleotide polymorphisms in inferences of population history. *Trends in Ecology & Evolution*, *18*(5), 249-256.

Bryant, David, Remco Bouckaert, Joseph Felsenstein, Noah A. Rosenberg, & Arindam Roy Choudhury. 2012. Inferring species trees directly from biallelic genetic markers: by passing gene trees in a full coalescent analysis. *Molecular biology and evolut*ion 29, no. 8 :1917-1932.

Cariou, M., Duret, L., & Charlat, S. 2013. Is RAD-seq suitable for phylogenetic inference? An in silico assessment and optimization. *Ecology and evolution*, *3*(4), 846-852.

Eaton, D. A., & Ree, R. H. 2013. Inferring phylogeny and introgression using RADseq data: an example from flowering plants (Pedicularis: Orobanchaceae). *Systematic Biology*, *62*(5), 689-706.

Darriba, D., G. L. Taboada, R. Doallo, & D. Posada. 2012. "jModelTest 2: more models, new heuristics and parallel computing". *Nature Methods* 9:772.

Davey, J. W., Hohenlohe, P. A., Etter, P. D., Boone, J. Q., Catchen, J. M., & Blaxter, M. L. 2011. Genome-wide genetic marker discovery and genotyping using next-generation sequencing. *Nature Reviews Genetics*, *12*(7), 499-510.

dos Santos, C. H. D. A. 2014. Genetic relationships between captive and wild subpopulations of Arapaima gigas (Schinz, in Cuvier, 1822). *International Journal of Fisheries and Aquaculture*, *6*(10), 108-123.

Drummond AJ, Suchard MA, Xie D & Rambaut A .2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7 *Molecular Biology And Evolution* **29**: 1969-1973

Ellstrand, N. C., & Elam, D. R. 1993. Population genetic consequences of small population size: implications for plant conservation. *Annual review of Ecology and Systematics*, *24*(1), 217-242.

Evanno, G., Regnaut, S., & Goudet, J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular ecology*, *14*(8), 2611-2620.

Excoffier, L. G. Laval, & S. Schneider .2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online **1**:47-50.

Felsenstein, J. 1981. Evolutionary trees from DNA-sequences-a maximum likelihood approach. *Journal of Molecular Evolution.* 17: 368-376.

Floragenex, inc. 2015: 4640 SW Macadam Ave, Portland, OR, 97239, United States.

Fryxell, P. A. 1988. Malvaceae of Mexico. Systematic Botany Monographs 25.

Gadagkar, S. R., Rosenberg, M. S., & Kumar, S. 2005. Inferring species phylogenies from multiple genes: concatenated sequence tree versus consensus gene tree. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *304*(1), 64-74.

Giolo, Suely R., Julia MP Soler, Steven C. Greenway, Marcio AA Almeida, Mariza De Andrade, J. G. Seidman, Christine E. Seidman, José E. Krieger, & Alexandre C. Pereira. 2012. Brazilian urban population genetic structure reveals a high degree of admixture. *European Journal of Human Genetics* 20, no. 1:111-116.

Gontcharov, A. A., Marin, B., & Melkonian, M. 2004. Are combined analyses better than single gene phylogenies? A case study using SSU rDNA and rbcL sequence comparisons in the Zygnematophyceae (Streptophyta). *Molecular Biology and Evolution, 21*(3), 612-624.

Hedges, S. B. (2002). The origin and evolution of model organisms. *Nature Reviews Genetics, 3*(11), 838-849.

Heled, J., & Drummond, A. J. (2010). Bayesian inference of species trees from multilocus data. *Molecular biology and evolution, 27*(3), 570-580.

Hipp, A. L., Eaton, D. A., Cavender-Bares, J., Fitzek, E., Nipper, R., & Manos, P. S. 2014. A framework phylogeny of the American oak clade based on sequenced RAD data. *PLoS One, 9*(4), e93975.

Klips. R. A. 1995. Genetic affinity of the rare eastern Texas endemic. *Hibiscus dasycalyx*. (Malvaceae). *American Journal of Botany*. 82: 1463-1472.

Leaché, A. D., M. K. Fujita, V. N. Minin, & R. R. Bouckaert. 2014. Species delimitation using genome-wide SNP data. Syst. Biol. 63:534-542.

Mace, Georgina M., Nigel J. Collar, Kevin J. Gaston, C. R. A. I. G. Hilton-Taylor, H. Resit Akçakaya, N. I. G. E. L. Leader-Williams, Eleanor Jane Milner- Gulland, & Simon N. Stuart. 2008. Quantification of extinction risk: IUCN's system for classifying threatened species. *Conservation Biology* 22, no. 6: 14241442.

Maddison W.P., Knowles L.L. 2006. Inferring phylogeny despite incomplete lineage sorting, *Syst. Biol.*, vol. 55:21-30.

Meirmans, P. G. 2012. AMOVA-based clustering of population genetic data. *Journal of Heredity, 103*(5), 744-750.

Narum, S. R., Buerkle, C. A., Davey, J. W., Miller, M. R., & Hohenlohe, P. A. 2013. Genotyping-by-sequencing in ecological and conservation genomics. Molecular ecology, 22(11), 2841-2847.

Ogilvie, H. A., Heled, J., Xie, D., & Drummond, A. J. (2016). Computational performance and statistical accuracy of* BEAST and comparisons with other methods. *Systematic biolo*syv118.

O'Meara B.C. 2010. New heuristic methods for joint species delimitation and species tree inference, *Syst. Biol*., vol. 59: 59-73.

Padial, J. M., Miralles, A., De la Riva, I., & Vences, M. (2010). The integrative future of taxonomy. *Frontiers in zoology*, *7*(1), 16.

Pfeil, B. E., C. L. Brubaker, L. A. Craven, & M. d. Crisp. 2002. Phylogeny of *Hibiscus* and the tribe Hibisceae (Malvaceae) using chloroplast DNA sequences of *ndh*F and the *rpl*16 intron. *Systematic Botany*. 27: 333-350.

Ploetz, R. C. 2007. Cacao diseases: important threats to chocolate production worldwide. *Phytopathology*, *97*(12), 1634-1639.

Pritchard, J. K., Stephens, M., & Donnelly, P. 2000. Inference of population structure using multilocus genotype data. *Genetics*, *155*(2), 945-959.

R Development Core Team. 2008. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0.

Rhymer, J. M. & D. Simberloff. 1996. Extinction by hybridization and introgression. Annual Review of Ecology and Systematics 27:83-109.

Rodríguez-Ezpeleta, N., Bradbury, I. R., Mendibil, I., Álvarez, P., Cotano, U., & Irigoien, X. 2016. Population structure of Atlantic mackerel inferred from RAD-seq-derived SNP markers: effects of sequence clustering parameters and hierarchical SNP selection. *Molecular ecology resources*, *16*(4), 991-1001.

Rohlf, F. J., & Sokal, R. R. (1995). *Statistical tables*. Macmillan.

Sain, Melody P., "Distinguishing the Neches River Rose Mallow, Hibiscus Dasycalyx, from its Congeners Using DNA Sequence Data and Niche Modeling Methods" (2015). *Biology Theses.* Paper 26.

Schemske, D. W., Husband, B. C., Ruckelshaus, M. H., Goodwillie, C., Parker, I. M., & Bishop, J. G. (1994). Evaluating approaches to the conservation of rare and endangered plants. *Ecology*, *75*(3), 584-606.

Small, R. L. 2004. Phylogeny of Hibiscus sect. Muenchhusia (Malvaceae) based on chloroplast rpL16 and ndhF, and nuclear ITS and GBSSI sequences. Systematic Botany, 29(2), 385-392.

Spinks, P. Q., Thomson, R. C., Pauly, G. B., Newman, C. E., Mount, G., & Shaffer, H. B. (2013). Misleading phylogenetic inferences based on single-exemplar sampling in the turtle genus Pseudemys. *Molecular Phylogenetics and Evolution*, *68*(2), 269-281.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, *30*(9), 1312-1313.

Stevison, L. 2008. Hybridization and gene flow. *Nature Education* 1(1):111

US Fish & Wildlife Service. 2017. Endangered and threatened wildlife: Flowering planta

US Fish & Wildlife Service. 2013. 50 CFR Part 17: Endangered and threatened wildlife and plants; designation of critical habitat for Texas Golden Gladecress and Neches River Rose-Mallow

U.S. Senate Committee on Environment & Public Works. "Endangered species act of 1973" December 2012.

Van Wyngaarden, Mallory, Paul VR Snelgrove, Claudio DiBacco, Lorraine C. Hamilton, Naiara Rodríguez-Ezpeleta, Nicholas W. Jeffery, Ryan RE Stanley, & Ian R. Bradbury. 2017."Identifying patterns of dispersal, connectivity and selection in the sea scallop, Placopecten magellanicus, using RADseq-derived SNPs." *Evolutionary Applications* 10, no. 1 102-117.

Wise, D. A., & M. Y. Menzel. 1971. Genetic affinities of the North American species of *Hibiscus* sect. *Trionum*. *Brittonia*. 23: 425-437.

Wilson, F. D., & Menzel, M. Y. 1964. Kenaf (Hibiscus cannabinus), roselle (Hibiscus sabdariffa). *Economic Botany*, *18*(1), 80-91.

Wright, S. 1965. The interpretation of population structure by F-statistics with special regard to systems of mating. *Evolution*, 395-420.

Yoder, J. B., Roman B., Joann M., Andrew F., Timothy P., Kelly S., George D. W. 2013. "Phylogenetic signal variation in the genomes of Medicago (Fabaceae)." *Systematic biology* 62, no. 3: 424-438.