MORPHOLOGICAL AND MOLECULAR APPROACHES TO DISENTANGLING THE TAXONOMY OF *PLUMERIA* SPECIES

(APOCYNACEAE)

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

TROPICAL PLANT AND SOIL SCIENCES

AUGUST 2019

By

B. Kauahi Perez

Dissertation Committee:

Richard M. Manshardt, Chairperson
Richard A. Criley
Michael B. Kantar
Teresita D. Amore
Kenneth W. Leonhardt
Sterling C. Keeley

Keywords: *Plumeria*, morphology, DNA barcoding, taxonomy

DEDICATION

This dissertation is dedicated to scientists and plant enthusiasts who love every and anything about *Plumeria.*

ACKNOWLEDGEMENTS

First and foremost, I'd like to thank my dissertation committee for all their time, effort, contributions, and support throughout my research endeavors. Everyone has been extremely helpful and patient, and I am forever grateful for every one of your contributions to making my dissertation writing so much stronger.

Many thanks go to the Southern California Plumeria Society for repeated funding and helping me get my research off the ground, and to the Plumeria Society of America for the extramural funding that has helped maintain my research and keep me going. Here, too, I'd like to thank the UH Manoa Graduate Student Organization for travel funding so that I could disseminate my research findings to the greater scientific community at the American Society for Horticultural Sciences conferences.

Much appreciation goes to Tex Norwood and Carol Vannoorbeeck (Florida Colors Nursery), Hetty Ford and Sandra Rigotti-Santos (Naples Botanical Garden), Dr. Chad Husby (Fairchild Tropical Botanic Garden), Dr. Alan Meerow (Miami USDA), Rae Matthews (National Tropical Botanical Garden), Donald Hodel (University of California), Carlo Morici (The Palmetum of Santa Cruz de Tenerife), and Michael Ferrero for helping me gain access to a whole bunch of *Plumeria* samples. And a big *mahalo* to Barbara Kennedy and Clyde Imada (Bishop Museum Herbarium) for helping me with the vouchering process of some of my *Plumeria* accessions from Waimanalo. And, not to forget Dr. Richard Criley, who has been the best chauffer ever! Thank you for being my driver to and from Waimanalo and trekking me around Florida to collect *Plumeria*. One of these days I look forward to being your chauffer. And thank you for helping me translate a lot of the Latin descriptions of *Plumeria* into English.

I also would like to extend my gratitude to Elizabeth Winnicki, Mona Al-Tamimi, Preksha Bapna, and Sunisa Kogar for all of their time spent working with me in the lab, helping to process my many samples of *Plumeria* leaf material from DNA extraction and PCR to DNA sequencing, and helping me to hone my teaching and mentoring skills.

A big *mahalo nui* to my dear friend, Dr. Martha Jane Gauthier, who kindly provided me with a suite of DNA primers to jump start my molecular endeavors. I'd also like to thank many of the graduate students of the Tropical Plant and Soil Sciences

Department, especially Emily Teng and Peter Toves, who have helped me to persevere in my doctoral journey through their moral support. Many other friends outside of the department have also been extremely helpful in talking with me about my research to help me better communicate my findings.

Most of all, I'd like to thank my family, especially my mom and dad, who has been extremely supportive throughout my entire college career. Thank you all so much for your words of encouragement to keep me going. To my nieces and nephews, thank you for cheering me up when the research bogged me down. You were all the mental break that I've needed from my research. I've learned so much more about juggling family and work life because of my family. Without their endless support, I would not have had the opportunity to pursue a PhD.

ABSTRACT

This dissertation investigated the taxonomy of *Plumeria*, a popular ornamental plant in the Apocynaceae. A brief introduction to the plant and its current taxonomy is provided in Chapter 1 along with a proposal for research to identify *Plumeria* spp. by morphological and molecular approaches. The overall goal of this research was to evaluate morphological and molecular characters that are useful for identifying *Plumeria* spp. so that we can delineate species boundaries, verify our taxonomic understanding of *Plumeria*, and begin to understand their evolutionary history. The use of qualitative and quantitative morphology to diagnose *Plumeria* spp. from the literature is difficult because of the multitude of descriptions given by various authors, even for the same species. Furthermore, the criteria for delineating currently recognized *Plumeria* spp. is unclear. Hence, in Chapter 2 the use of descriptive morphology is evaluated to determine its effectiveness at identifying *Plumeria* spp. Using iterative principal component analyses, it was found that a combination of descriptive vegetative characters was useful for identifying most *Plumeria* spp. However, other species could not be identified based solely on descriptive morphology, due to morphological variation of descriptors used. Instead, it would require the use of quantitative measurements and the use of other morphological characters, such as fruits and flowers, to properly diagnose these species. Chapter 3 explores molecular approach to delineating species and investigates the phylogenetic utility of five candidate loci. Some regions were able to identify operational taxonomic units as true species, but no single region could be used to identify all the putative species in our sampling. In fact, not all species could be recovered as distinct clusters even with a data set that combined four molecular regions. On the other hand, it did result in a well resolved phylogeny that agrees with

prior findings, notably that most *Plumeria* are distinguishable by molecular means and that some *Plumeria* form a species complex comprised of morphologically variable members that share very similar molecular characters. Chapter 4 concludes with a synthesis of morphological and molecular findings and future directions in the realm of disentangling the taxonomy of *Plumeria* spp.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS ..... **iv**

ABSTRACT ..... **vi**

LIST OF TABLES ..... **xi**

LIST OF FIGURES ..... **xii**

CHAPTER 1 INTRODUCTION TO *PLUMERIA* **1**

Horticultural Importance of Plumeria 1

The Taxonomic Issue with Plumeria 2

Current Taxonomy of Plumeria 3

The Need for (Macro) Morphological Investigations 4

The Need for Molecular Investigations 5

The Research Plan 9

Objectives 9

Research Questions 9

CHAPTER 2 USING DESCRIPTIVE MORPHOLOGY TO IDENTIFY *PLUMERIA* SPECIES IN LIVING COLLECTIONS **13**

ABSTRACT 13

INTRODUCTION 14

Plumeria Distribution and Taxonomy 15

The Search for Persistent Morphological Characters 17

MATERIALS AND METHODS 18

Taxon sampling 18

Principal Component Analyses 19

Limitations 21

RESULTS 21

Iterative PCA: Step one 21

Iterative PCA: Step two 26

Iterative PCA: Step three 29

Iterative PCA: Step four 33

DISCUSSION 35

P. clusioides Griseb. 35

P. stenophylla Urb. 36

P. subsessilis A. DC. 37
P. pudica Jacq. 39
P. caracasana J.R. Jhonst. 39
P. alba L. 40
P. rubra L. 42
P. stenopetala Urb. 42
Remaining Taxa of the *P. obtusa* Complex 43
Utility and Limitations of Descriptive Foliar Characters 47
Prospects 49

CONCLUSION 50

ACKNOWLEDGEMENTS 50

CHAPTER 3 EVALUATING NUCLEAR AND PLASTID REGIONS TO DELIMIT *PLUMERIA* SPECIES 51

ABSTRACT 51

INTRODUCTION 52
The Search for Informative Molecular Characters 53

MATERIALS AND METHODS 60
Sample Collection, DNA Extraction, and Purification 60
DNA Amplification, Sequencing, and Alignment 60
Maximum Likelihood and Bayesian Analyses 63
DNA Barcoding Gap Analysis 64
Operational Taxonomic Units as Species Clusters 64
Limitations 64

RESULTS 65
Sequence Characteristics 65
DNA Barcode Gap Analysis 65
Analysis of *ITS2* 70
Analysis of *matK* 72
Analysis of *trnH-psbA* 74
Analysis of *psbJ-petA* 76
Analysis of *rpl32-trnL* 78
Analysis of Combined Data 80

DISCUSSION 84
Phylogenetic utility of regions 84
DNA Barcodes for *Plumeria* 84
P. rubra L. 86
P. alba L. 86
P. subsessilis A. DC. and *P. stenopetala* Urb. 86
The *P. obtusa* Complex and the Sericifolia Group 87

P. pudica Jacq., *P. caracasana* J.R. Jhonst., and *P.* sp. 'Isabella' 89
Future Directions 89
CONCLUSION 90
ACKNOWLEDGEMENTS 90
CHAPTER 4 SYNTHESIS, PROSPECTS, AND CONCLUSION 91
INTRODUCTION 91
SYNOPSES OF CHAPTERS 91
Chapter 1 91
Chapter 2 92
Chapter 3 92
SYNTHESIS 93
PROSPECTS 94
CONCLUSION 95
APPENDICES 96
Appendix A. Accession data for morphological analyses 96
Appendix B. Morphological matrix of taxa and characters 101
Appendix C. Morphological characters, character states, and definitions 110
Appendix D. Accession data for molecular analyses 113
Appendix E. Diagnostic molecular characters of *Plumeria* taxa 119
Appendix F. Taxonomic status and natural geographic distribution of *Plumeria* 122
REFERENCES 125

LIST OF TABLES

Table 1.1. Preliminary intergenic spacer (IGS) region results 6
Table 2.1. Summary of the first three principal components (PCs) and loadings 22
Table 3.1. Taxonomic status and natural geographic distribution of *Plumeria* 54
Table 3.2. Publicly available *Plumeria* sequences 59
Table 3.3. PCR primer sequences of nuclear and chloroplast regions tested 62
Table 3.4. Summary statistics of molecular regions of *Plumeria* taxa 67
Table 3.5. Intraspecific and interspecific sequence divergences 68

LIST OF FIGURES

Figure 1.1. Potentially useful characters to incorporate into new *Plumeria* hybrids 2
Figure 1.2. Preliminary testing of intergenic spacer regions 7
Figure 1.3. A potentially informative molecular marker 8
Figure 2.1. Top 20 variables that contributed to the construction of PC1 24
Figure 2.2. Top 20 variables that contributed to the construction of PC2 25
Figure 2.3. Second iteration PCA. 28
Figure 2.4. Third iteration PCA 32
Figure 2.5. Fourth iteration PCA 34
Figure 2.6. Morphological characters that distinguish *P. clusioides*, *P. stenophylla*, and *P. subsessilis* 38
Figure 2.7. Morphological characters that distinguish *P. pudica*, *P. caracasana*, and *P. alba*. 41
Figure 2.8. Morphological characters that distinguish *P. rubra* and *P. stenopetala* 45
Figure 2.9. Morphological diversity of taxa within the *P. obtusa* complex 46
Figure 3.1. Leaf morphologies of *Plumeria* accessions from the University of Hawai'i Waimanalo Research Station 58
Figure 3.2. Average sequence divergences among molecular regions tested 69
Figure 3.3. ML and BI consensus cladograms of the *ITS2* region 71
Figure 3.4. ML and BI consensus cladograms of the *matK* region 73
Figure 3.5. ML and BI consensus cladograms of the *trnH-psbA* region 75
Figure 3.6. ML and BI consensus cladograms of the *psbJ-petA* region 77
Figure 3.7. ML and BI consensus cladograms of the *rpl32-trnL* region 79
Figure 3.8. ML consensus cladogram of the 4-region concatenated data set 81
Figure 3.9. BI consensus cladogram of the 4-region concatenated data 83
Figure 3.10. Potentially diagnostic nucleotides of the *trnH-psbA* region 85

CHAPTER 1

INTRODUCTION TO *PLUMERIA*

Horticultural Importance of Plumeria

The genus *Plumeria* L., otherwise known as frangipani or temple tree (Woodson, 1938a), is a plant that holds great significance in many parts of the world. In tropical Asia plumerias are grown as ornamentals for their color and fragrance, and also for offerings and religious purposes (Staples and Herbst, 2005). In other parts of the world, *Plumeria* has ethnobotanical uses to which various medicinal qualities have been ascribed (Eggenberger and Eggenberger, 2005; Choudhary et al., 2014). In the United States, plumerias are valued as ornamentals for landscaping and hobby collectors (Little, 2006), and are also utilized in perfumery (Tohar et al., 2006; Joulain, 2008). Specifically in Hawaii, plumerias are commonly used in landscaping and commercially grown for their flowers which are used in lei making (Criley, 2009) and hair adornments. In short, *Plumeria* is widely used in many parts of the world.

The vast majority of cultivated plumeria varieties belong to two species, *P. rubra* and *P. obtusa*. Varieties of *P. rubra* predominate over *P. obtusa* varieties in most cases. However, many of the former varieties are susceptible to Plumeria rust (*Coleosporium plumeriae*) and the Plumeria stem borer (*Lagocheirus undatus*) (Criley, 2005). Other *Plumeria* species are gaining popularity and show great potential for use in breeding programs to generate cultivars with pest and disease resistance (Little, 2006). In addition, other traits such as non-deciduous leaves (evergreen), dwarfing, variable leaf shapes, and different flower forms can be produced using these new species (Eggenberger and Eggenberger, 2005) (Figure 1.1 A).

The plants under study in this research include those collected by horticulturists from local and national botanical gardens (see Appendices 2.1 and 3.1). Prior to their placement in botanical gardens, a majority of the species were collected from the wild in Central America, Costa Rica, Cuba, and the Caribbean (R. Criley, personal communication). The chief collection criterion has been ornamental value, and consequently, most accessions are currently identified as *P. rubra*, which appears to have the best source of genetic variation in petal number and purple, red, or pink petal

colors on white or yellow backgrounds (Fig. 1.1 A). However, these colorful variants are susceptible to rust and stem borer damage, limiting their overall value. On the other hand, other *Plumeria* spp. appear to exhibit notable differences in disease resistance to Plumeria rust (*Coleosporium plumeriae*) and the Plumeria stem borer (*Lagocheirus undatus*) (Criley, 2009; Nelson, 2009) (Fig. 1.1 B).

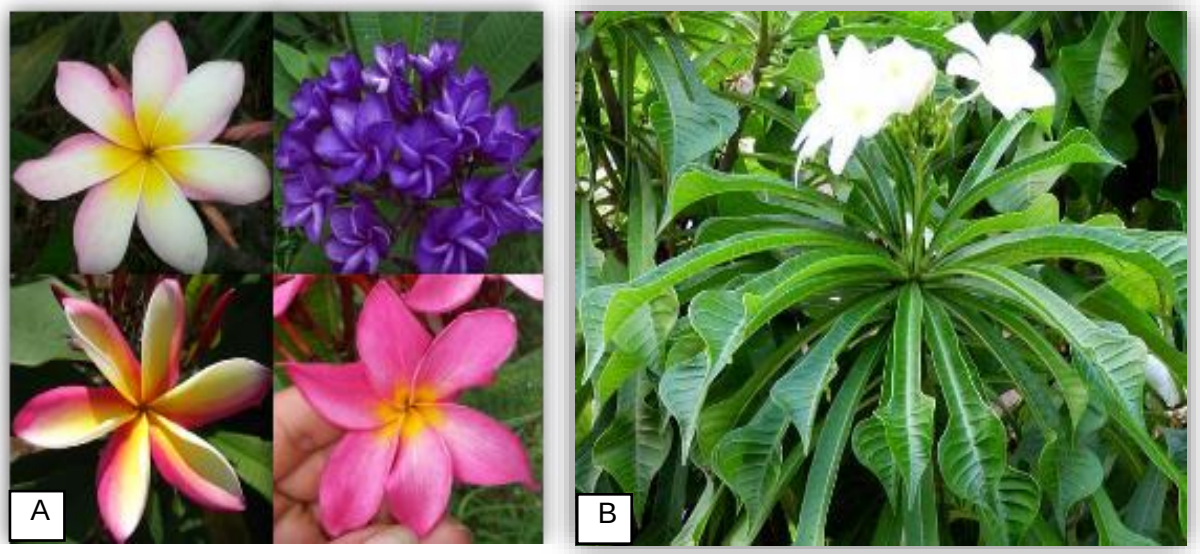


Figure 1.1. Potentially useful characters to incorporate into new *Plumeria* hybrids. A: Color forms and petal number variants of *P. rubra* cultivars. B: The evergreen trait and unusual leaf shape of *P. pudica*, which is also resistant to Plumeria leaf rust disease, can be harnessed to generate novel plumeria cultivars that have interesting leaf shape, are rust-resistant, and do not shed leaves as most current *Plumeria* cultivars do.

The Taxonomic Issue with Plumeria

The taxonomy of *Plumeria* is problematic because many invalid or unverified species names are used among collectors (Criley, 2009), and unconfirmed or mislabeled specimens exist in botanical gardens, including those in Hawaii. This has led to the accumulation and perpetuation of misnomers (i.e. botanical gardens using incorrect or synonymous names for different specimens) in *Plumeria* collections. Morphological and molecular investigations are two powerful tools that can aid in verifying species names in these collections. Thus, evidence to support or refute the

taxonomic status of existing species names will be beneficial to botanical gardens, horticulturists and growers, and the greater scientific community. A combination of morphological and molecular data analyses will provide a comprehensive verification of *Plumeria* accessions.

A collection that is referenced by accurate morphological descriptions and molecular determinations of ancestry will also be more accessible and likely generate more interest for research, landscape, breeding, and educational purposes. The short-term benefit of the proposed research will be to allow for better documentation of *Plumeria* accessions in public gardens. In turn this will produce long term benefits in greater utilization of the genetic resources for public gardens, development of new ornamental cultivars, and training of horticulturists and geneticists. Furthermore, the proposed research will provide a phylogenetic foundation for more in-depth investigations and contribute to evolutionary understanding within the Apocynaceae.

Current Taxonomy of Plumeria

The family Apocynaceae is comprised of over 5,500 species within 410 genera (The Plant List, 2013). Genera are grouped within one of five subfamilies, 25 tribes, and 49 subtribes (Endress et al., 2014). The family includes taxa that are mostly distributed throughout the tropics, but with a few from temperate regions (Endress and Bruyns, 2000; Sennblad and Bremer, 2002; Endress et al., 2007a). Nearly all taxa are poisonous, due to the presence of indole alkaloids and cardenolides, and many have ethnobotanical or ornamental uses (Judd et al., 2008). Taxa within the family are characterized by the presence of milky latex, a highly-modified gynoecium with separate ovaries, a differentiated stigmatic head, and unique combinations of molecular sequences (Leeuwenberg, 1994; Soltis et al., 2005; Judd et al., 2008; Nazar et al., 2013; Selvaraj et al., 2015). Rauvolfioideae is the most basal subfamily of the Apocynaceae and is comprised of genera with simple flowers, anthers detached from the style head, and seeds that lack hairs (Endress and Bruyns, 2000; Simões et al., 2007; Nazar et al., 2013).

The genus *Plumeria* L. is a member of subfamily Rauvolfioideae (Simões et al., 2007) and is characterized by thick, succulent branches, corky bark, showy flowers with

a waxy corolla and a narrow base, and linear fruits (follicles) with dehiscent pods containing winged seeds (Staples and Herbst, 2005). Woodson (1938a) who did the last taxonomic study of the genus, recognized seven species and several subspecies or varieties that are native to the New World tropics.

The Need for (Macro) Morphological Investigations

Woodson's proposed species descriptions were based on morphological characters including leaf shape, surface, venation, perianth features, and provenance. Although Woodson's study was thorough, it was limited by availability of material as his studies were conducted solely on herbarium specimens from European and American sources. As such, some morphological features of leaves and flowers, which are readily visible in live specimens, may have been lost during pressing and drying of herbarium material. He also admittedly ignored subtle morphological features, including slight variations in leaf outline, which may have led him to mistakenly subsume some *Plumeria* taxa into one of the seven species he recognized, including taxa that comprised the *P. obtusa* complex (i.e. *P. bahamensis*, *P. cubensis*, etc.). Aguoru et al. (2015) have recently proposed the use of epidermal cell shape, leaf size, and stomata index to distinguish among the three *Plumeria* species *P. rubra*, *P. obtusa*, and *P. lutea* that occur in Nigeria. However, their study was limited to only these three *Plumeria* spp. in this region so the utility of these three characters to distinguish other *Plumeria* spp. remains unknown. Furthermore, the other morphological characters that Aguoru et al. (2015) used were either present in all three species or applied to only one of the three species and may not be sufficient for distinguishing among other extant *Plumeria* taxa. Quick and accurate identification of species in the field based on morphological characters that are easy to score is critical to many areas of biology besides systematics (i.e. ecology, physiology, etc.) (Wiens, 2004). Hence, a taxonomic update using novel morphological characters to distinguish species in this genus is warranted. Therefore, as one component of this dissertation, descriptive foliar morphological characters that can accurately discriminate among *Plumeria* spp. will be explored.

The Need for Molecular Investigations

Using morphology to identify species can be difficult due to variation in the environment as well as the innate natural variation within any given taxon. In addition, under cultivation random somatic mutations do arise during clonal propagation altering the morphology of a plant and sometimes limiting the use of morphology to diagnose a species. Developmental stage also affects morphology, for example seedlings of *Plumeria* spp. can be so variable as to make floral and foliar morphology useless due to character state overlap which may lead to misidentification. Since morphology alone may not be dependable for species distinctions, using molecular markers to verify *Plumeria* (species) in collections will be an extremely valuable tool.

Molecular data can support the monophyly of taxa that were originally recognized on the basis of morphology and place taxa whose relationships were once problematic or unknown (Judd et al., 2008). Molecular markers have been used in the identification of plant material in germplasm as well as in native plants (Ford-Lloyd, 2001). This can increase targeting of plant material for collection and aid in the exchange of germplasm material at local, national, and international botanical gardens. It can also help in identifying duplicates in plant collections, especially in instances where duplicated specimens are not morphologically identical. Furthermore, by identifying molecular markers that can distinguish species, genetic relationships among taxa can be determined (Ford-Lloyd, 2001), which can then be used to answer evolutionary and ecological questions.

Studies have shown that the utility of different coding and noncoding chloroplast DNA (cpDNA) and nuclear DNA (nrDNA) regions within a taxonomic group can be tremendously variable (Kress et al., 2005; Qiu et al., 2013; Tripathi et al., 2013; Cantley et al., 2014; Hochbach et al., 2015). Thus, Shaw et al. (2005; 2007; 2014) argue that choosing the appropriate molecular marker region to carry out investigations at a given taxonomic level is dependent on the taxa under study. Indeed, many studies in the Apocynaceae aimed at characterizing genetic relationships have reported success using different molecular regions (chloroplast or nuclear) and in combination with morphology (Potgieter and Albert, 2001; Simões et al., 2004; Simões et al., 2007;

Simões et al., 2010; Fishbein et al., 2011; Selvaraj et al., 2015). The basic criteria for determining the suitability of molecular markers for genetic analyses include:

1) a bifurcating tree—with minimal polytomies—is produced;
2) outgroup taxa are distinctly separate from ingroup taxa;
3) multiple accessions of a species cluster together;
4) clades are well supported by bootstrap values of 70% or more;
5) individual regions surveyed are characterized by high **inter**specific (between species) DNA sequence variation and relatively low **intr**aspecific (within species) DNA sequence variation (Table 1.1).

Table 1.1. Preliminary intergenic spacer (IGS) region results demonstrating that intraspecific (within species) DNA sequence variation is lower than interspecific (between species) sequence variation. Such regions are candidate markers for detecting species.

	DNA Region			
	***psbJ-petA* IGS**		***rpl32-trnL* IGS**	
Taxon	Within	Between	Within	Between
P. ekmanii	0.000	0.002	0.005	0.014
P. pudica	0.000	0.001	0.001	0.006
P. caracasana	0.000	0.001	0.001	0.006
P. obtusa var. *sericifolia*	0.000	0.004	0.006	0.047
P. alba	0.001	0.004	0.005	0.009

In preliminary work using morphologically distinct *Plumeria* spp., only two out of seven non-coding chloroplast markers were able to accurately distinguish these species using the aforementioned criteria (Fig. 1.2). Moreover, genetically distinct taxa were also morphologically distinct. In addition, some regions revealed insertion/deletions (indels) that in some cases indicated mislabeled accession (Fig. 1.3). This confirms the argument by Shaw et al. (2005) that molecular marker regions must first be tested before a full-blown taxonomic study is undertaken. Additionally, Shaw et al. (2014) recommend using at least three genetic regions, from the chloroplast and/or nucleus, to achieve better discriminatory power from regions of the plant genome.

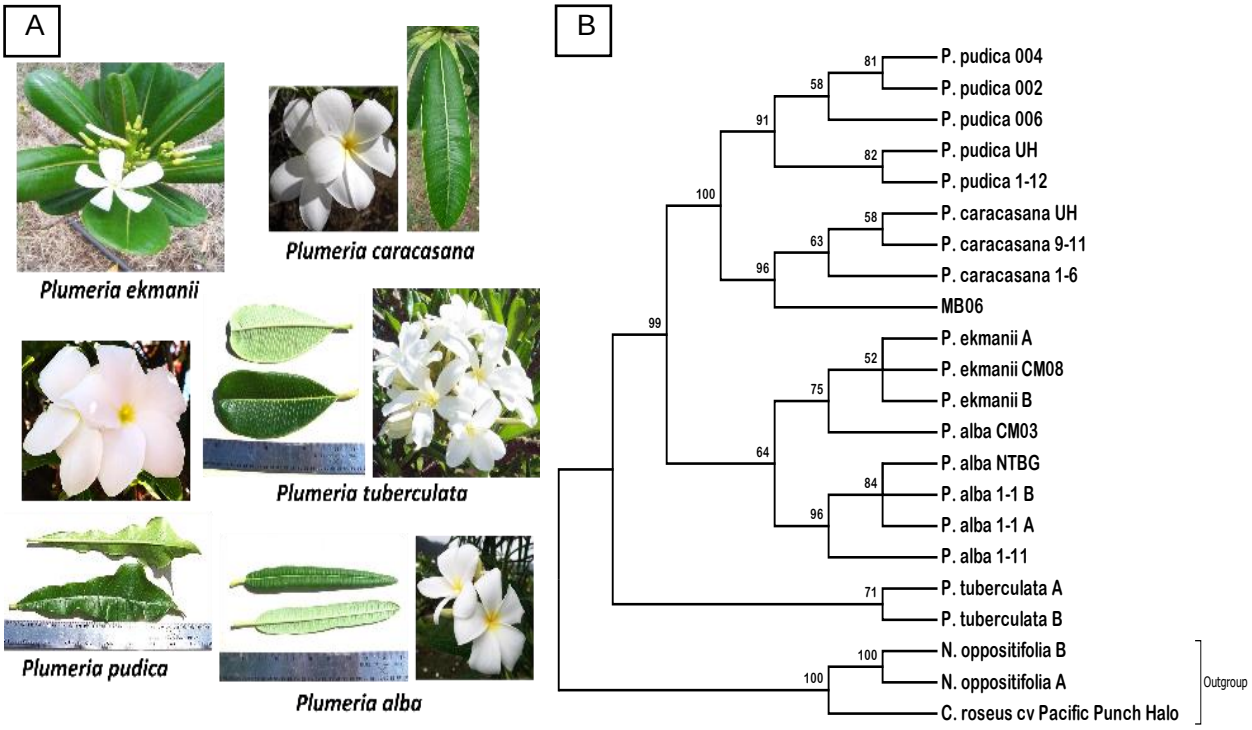


Figure 1.2. Preliminary testing of intergenic spacer regions with morphologically distinct *Plumeria*. A: Morphologically distinct *Plumeria* spp. used to evaluate molecular regions *psbJ-petA* and *rpl32-trnL*. B: A combined neighbor-joining tree of the psbJ-petA and rpl32-trnL intergenic spacer regions, verifying their potential use as molecular markers using criteria for evaluating genetic regions to determine genetic relationships.

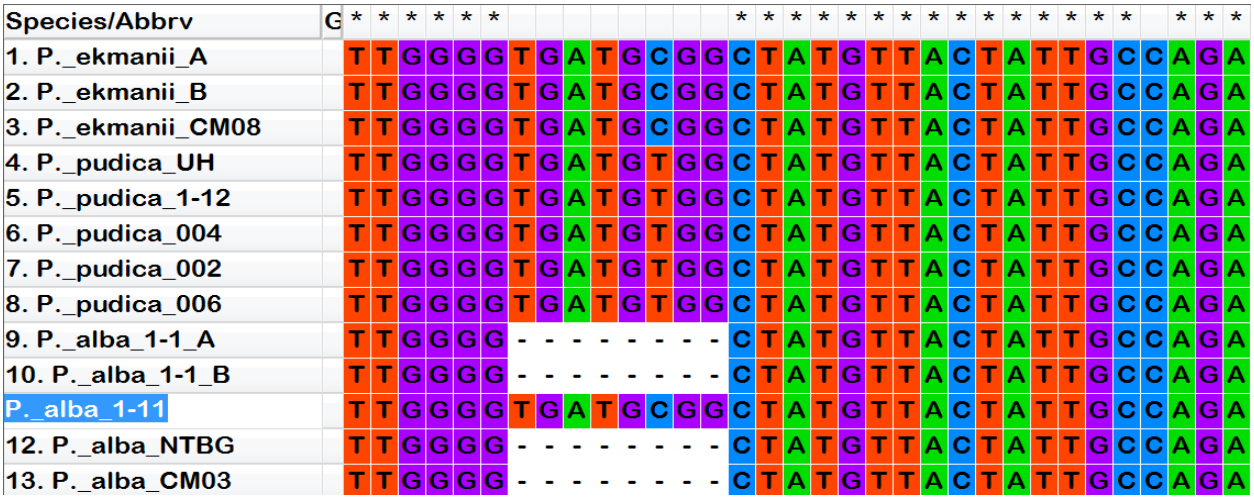


Figure 1.3. A potentially informative molecular marker. An intergenic spacer region is informative if it contains genetic regions that are unique to distinct *Plumeria* spp. Such an informative region will be able to authenticate *Plumeria* spp. in a collection. In this example, *P. alba* 1-11 contains an insertion, whereas the other accessions of *P. alba* do not, indicating that *P. alba* 1-11 is not a *P. alba*. However, it could be closely related to *P. ekmanii*.

Many molecular markers have been tested for their taxonomic utility. However, certain markers are gaining popularity because of their applicability across a wide range of plant taxa. Within the chloroplast genome, Simões et al. (2007) have shown that the *matK* gene was useful in resolving genetic relationships within the subfamily Rauvolfioideae. Shaw et al. (2005) have also demonstrated that cpDNA regions *psbJ-petA* and *rpl32-trnL* possess many potentially informative characters across a wide range of plant taxa. The plastid intergenic spacer region *trnH-psbA* and the nuclear ribosomal internal transcribed spacers (*ITS*) are two loci that are also commonly used as supplemental genetic regions for delineating species (Fazekas et al., 2012; Fišer Pečnikar and Buzan, 2014). However, Selvaraj et al. (2015) have shown that using a subset of the ribosomal cassette (*ITS2*) can also discriminate species within the Apocynaceae. Therefore, these five regions will be used as molecular markers to assess their utility in identifying species and genetic relationships within the genus *Plumeria*.

The overarching goal of this dissertation is to provide a means to accurately identify *Plumeria* spp. As a necessary first step, morphological and molecular data must be evaluated for their ability to species. Once species are identified, it will then be possible to understand the genetic relationships among the species. Identifying genetic relationships among the taxa included in this proposed research will then provide a framework for taxonomic separation. Furthermore, the linkage between genetic and morphological characters can then be compared as to whether they are good indicators of relationships as reflected in results obtained from these data sources.

The Research Plan

Research Goal: To evaluate the utility of descriptive morphology and molecular regions to delineate species of *Plumeria*.

Overall Hypothesis: Morphological and molecular analyses of *Plumeria* accessions will allow clear recognition and verification of species, establish genetic relationships and provide criteria for the delimitation of currently unrecognized ones.

Morphological and molecular analyses of *Plumeria* accessions will characterize genetic relationships and allow for the verification of extant species, and delimitation of unrecognized ones.

Objectives

1) To identify *Plumeria* spp. using descriptive morphological characters that are easy to score.
2) To identify DNA loci that can delineate species and resolve genetic relationships in *Plumeria* by examining separate and combined molecular regions.

Research Questions

1) What qualitative foliar characters can be used to distinguish *Plumeria* spp.?
2) What individual molecular markers (chloroplast and/or nuclear) can be used to distinguish *Plumeria* spp.?

3) Does some combination of chloroplast and/or nuclear DNA markers better distinguish among taxa?
4) Are these regions phylogenetically informative?
5) How well does the molecular data reflect the morphological data regarding the identification of *Plumeria* spp.?

Objective 1: To identify Plumeria spp. using descriptive morphological characters that are easy to score.

Research Question 1: What qualitative foliar characters can be used to distinguish Plumeria spp.?

Hypothesis: No one character will be sufficient to distinguish species, but rather it will require a combination of characters.

Methodology:

1. Leaf samples of *Plumeria* accessions will be collected from local botanical gardens on Oahu, Kauai and Waimanalo Research Station, and morphological features of specimens from more distantly located *Plumeria* collections (i.e. Florida Colors Nursery, Naples Botanical Garden, Miami USDA, Fairchild Tropical Botanic Garden) will be photographed.

2. Fresh and photographed specimens will be assessed for potentially useful foliar characters.

3. A morphological data matrix of presence/absence characters and binary character states will be developed and analyzed using principal component analysis (PCA) to assess how the different taxa under study cluster with one another given the morphological characters used.

4. Results will be discussed and conclusions on the efficacy of these descriptive morphological characters to identify species will be discussed.

Objective 2: To identify DNA loci that can delineate species and resolve genetic relationships in Plumeria by examining separate and combined molecular regions.

Research Question 2: What individual molecular markers (chloroplast and/or nuclear) can be used to distinguish Plumeria spp.?

Research Question 3: Does some combination of chloroplast and/or nuclear DNA markers better distinguish among taxa?

Research Question 4: Are these regions phylogenetically informative?

Hypothesis: No single region will be sufficient to distinguish species, but rather it will require a combination of molecular regions.

Methodology:

1. DNA extraction kits will be used to extract genomic DNA (gDNA) from leaf samples of *Plumeria* accessions that were collected from the UH Waimanalo Research Station, National Tropical Botanical Garden—Allerton Garden, Florida Colors Nursery, Naples Botanical Garden, Miami USDA, and Fairchild Botanical Garden.

2. For each accession (sample), nuclear ITS2, partial chloroplast matK gene, and intergenic spacer regions trnH-psbA, rpl32-trnL, and psbJ-petA will be amplified via polymerase chain reaction (PCR) with primers that have been optimized for each genetic region, and using established primers and thermocycler conditions from Shaw et al. (2005), Simões et al. (2007), and Selvaraj et al. (2015) to amplify these regions. PCR products will be verified via gel electrophoresis prior to preparation for sequencing.

3. Unincorporated dNTPs and primers will be removed from PCR products (amplicons) using ExoSap-IT (USB), and purified products will be used as

template for sequencing at The Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) lab on UH Manoa campus. Both forward and reverse DNA strands of amplicons will be sequenced.

4. Sequence traces will be checked for base call quality, edited manually as the need arises, and contigs assembled using Geneious Prime, v.1.1 (Kearse et al., 2012). Multiple alignments will also be done using Geneious. Maximum Likelihood [ML] and Bayesian Inference [BI] Analyses will be carried out using RaxML and MrBayes, respectively, as implemented on the CIPRES Science Gateway (Miller et al., 2010; Ronquist et al., 2012; Stamatakis, 2014).

5. Summary statistics for each region (total length in final alignments, nucleotide ranges and average PCR product lengths will be calculated as implemented in Geneious for each region. Interspecific and intraspecific sequence divergences for each region will be calculated for each molecular region using MEGA X (Knyaz et al., 2018).

6. Molecular regions will be analyzed separately and in combination (concatenated) with one another, if individual regions yield similar tree topologies. Regions that do not yield similar tree topologies will not be concatenated.

7. Results will be discussed on the efficacy of my proposed molecular regions to characterize relationships and identify species.

Synthesis Question: How well does the molecular data reflect the morphological data regarding the identification of *Plumeria* spp.?

CHAPTER 2

USING DESCRIPTIVE MORPHOLOGY TO IDENTIFY *PLUMERIA* SPECIES IN LIVING COLLECTIONS

ABSTRACT

Species of the genus *Plumeria* in the plant family Apocynaceae are used for ornamental, cosmetic, and ethnomedicinal purposes. However, only a few of the species, specifically *P. rubra*, *P. obtusa*, and their cultivated varieties, find their way into commercial purposes, while other potentially useful species remain untapped sources of horticultural, medicinal, and cosmetic value. One of the major reasons for this is that there is much disagreement among collectors and taxonomists as to the number of recognized *Plumeria* species. Thus, the number of species in the genus *Plumeria* remains unknown. Moreover, numerous synonyms apply to taxa within this genus, many of them misused or unconfirmed. Here, we evaluate whether a combination of descriptive morphological characters is good enough to identify groups of taxa as species within this genus. Fifty-seven *Plumeria* specimens from a variety of available germplasm collections were scored for 43 qualitative morphological characters, including foliar, stem, and reproductive characters. Principal component analysis (PCA) was used to determine informative characters that characterized a *Plumeria* species. PCA revealed that some specimens formed distinct clusters, suggesting that certain taxa should be recognized as legitimate species, and verified that descriptive morphological characters will suffice for eight out of the 11 *Plumeria* species and putative species examined. However, not all taxa were identified by distinct clusters, especially those belonging to the *P. obtusa* complex, thus indicating that other characters are needed to sort these taxa. Instead, the use of quantitative, reproductive, and anatomical characters is likely required for proper diagnosis. In conclusion, the use of qualitative morphological characters has merit for identifying Plumeria species in most cases.

INTRODUCTION

The family Apocynaceae is comprised of over 5,500 species within 410 genera (The Plant List, 2013). Genera are grouped within one of five subfamilies, 25 tribes, and 49 subtribes (Endress et al., 2014). The family includes taxa that are mostly distributed throughout the tropics, but with a few from temperate regions (Endress and Bruyns, 2000; Sennblad and Bremer, 2002; Endress et al., 2007a). All members from this family are potentially poisonous due to the presence of cardiac glycosides and various alkaloids (Judd et al., 2008). However, many have medicinal uses, such as treating leukemia, hypertension, heart stimulation, and tranquilizers (Staples and Herbst, 2005; Judd et al., 2008). Moreover, many members of this family are economic ornamentals, including *Adenium* (desert rose), *Allamanda* (trumpet vine), *Asclepias* (milkweed), *Carrisa* (Natal plum), *Catharanthus* (Madagascar periwinkle), *Nerium* (oleander), *Vinca* (periwinkle), and *Plumeria* (frangipani) (Judd et al., 2008).

Named by Tourneforte in tribute to the botanist Charles Plumier (Woodson, 1938a), the genus *Plumeria* Tourn. ex L. is comprised of culturally important taxa in many parts of the world (Seth, 2003). It has become the city flower in Palermo (Italy) since its arrival in the early 1800s (Criley, 2009). In tropical Asia and West Indies, plumerias find use as ornamentals for their color and fragrance, and also for offerings and religious purposes (Brussell, 2004; Staples and Herbst, 2005). In Sri Lanka and India, plumerias are considered sacred and are planted near temples. In other parts of the world, *Plumeria* has ethnobotanical uses to which various pharmacological and medicinal qualities have been ascribed (Rahman et al., 2014; Shinde et al., 2014). Decoctions of leaves, bark, and flowers are used to treat skin ailments, venereal disease, diarrhea, and are also used as a purgative (Brundu and Camarda, 2004; Brussell, 2004; Eggenberger and Eggenberger, 2005; Staples and Herbst, 2005; Gupta et al., 2006; Wu-Yang et al., 2007; Choudhary et al., 2014). In the United States, plumerias are valued as ornamentals for landscaping and hobby collectors (Little, 2006; Criley, 2009), but are also utilized in perfumery (Knudsen et al., 2006; Tohar et al., 2006; Joulain, 2008). In Hawaii and Polynesia, plumerias commonly find use in landscaping, especially in xeriscapes, and are cultivated for lei making and hair adornments (Whistler, 1988; Criley, 1992; Wong, 2008).

As popular as this group of plants may be, only a few of the species find their way into commercial production. The vast majority of plumeria cultivated varieties (cultivars) are hybrids that belong to two species, *P. rubra* and *P. obtusa*. *Plumeria rubra* cultivars predominate over those of *P. obtusa*, since *P. rubra* appears to have the best source of genetic variation in petal number and purple, red, or pink petal colors on white or yellow backgrounds (Watson et al., 1965; Chinn and Criley, 1982). However, many of these varieties are susceptible to scale insects, whiteflies, mealybugs, leafhoppers, Plumeria rust (*Coleosporium plumeriae*), and Plumeria stem borers (*Lagocheirus undatus*) (Criley, 2005; Hodel et al., 2017). On the other hand, other *Plumeria* species (spp.) are gaining popularity and show great potential for use in breeding programs to generate cultivars with improved horticultural traits, including pest and disease resistance, evergreeness, dwarfing, interesting leaf shapes, early and late blooming, and different flower forms, fragrances, and colors (Woodson, 1938a; Watson et al., 1965; Chinn and Criley, 1982; Eggenberger and Eggenberger, 2005; Little, 2006). Furthermore, these species remain an untapped and potentially useful source of medicinal and cosmetic qualities. Yet, one of the biggest questions about this genus that remains unanswered is: How many species of *Plumeria* are there?

Plumeria Distribution and Taxonomy

Plumeria are naturally distributed in southern Mexico, Central America, northern South America from Panama to southern Brazil, and the Greater and Lesser Antilles (West Indies), which includes The Bahamas, Jamaica, Cuba, Puerto Rico and Virgin Islands (Woodson and Seibert, 1938; Duke, 1965; Woodson et al., 1970; Haber, 1984; Sloan et al., 2007). Taxa of this genus can be found growing in diverse habitats including topographically rugged terrain, dry coastal areas, rocky cliffs, dry hillsides, and moist coastal and limestone forests (Harshberger, 1903; Britton, 1915; Gleason and Killip, 1939; Seifriz, 1943; Duke, 1965; Sánchez-Sánchez and Islebe, 2002; Brussell, 2004; Boal et al., 2006).

However, *Plumeria* have been anthropogenically distributed to other tropical regions of the world, such as Polynesia, Australia, Malaysia, India, and Africa (Merrill, 1937; Watson et al., 1965; Whistler, 1988; Seth, 2003; Brundu and Camarda, 2004). The fact that it is easily propagated by cuttings or seeds (Watson et al., 1965; Little,

2006) and produces fragrant and showy flowers (Woodson et al., 1970) nicely explain the wide dispersal and cultural adoption of these taxa in areas where they were introduced and cultivated.

The genus *Plumeria* (syn. *Plumiera*, *Plumieria*) (The Missouri Botanical Garden, 1938) belongs to the subfamily Rauvolfioideae of Apocynaceae (Britton, 1915; Simões et al., 2007), and are characterized by thick, succulent branchlets with pronounced leaf scars, spiral to alternate phyllotaxis, waxy and salverform or infundibuliform corollas with narrow bases and sinistrorse aestivation, stamens deeply included and adnate to the corolla tube, subinferior ovaries that are bicarpellate and apocarpous, bifollicular dehiscent fruit that are basally united, and basally winged seeds with a thin endosperm (Woodson, 1938a; Woodson et al., 1970; Leeuwenberg, 1994).

According to The Plant List (2013), the genus is currently comprised of 12 species. However, various *Plumeria* spp. Have also been described from expeditions throughout the Antilles (West Indies) and Central America (Britton, 1910; Johnston, 1912; Britton, 1915; Britton and Millspaugh, 1920; Hollick, 1922; Britton, 1923; Woodson, 1938a; Woodson et al., 1970; Williams, 1996; Acevedo-Rodríguez and Strong, 2012). In addition, there are disparities among authors regarding legitimacy of described species. For instance, Goaverts et al. (2003) do not recognize *Plumeria clusioides* Griseb. as a legitimate species, whereas Acevedo-Rodríguez and Strong (2012) do. In fact, one can find many examples of such disparities by simply comparing the species recognized within these two sources. Yet, many more discordances exist within the *Plumeria* literature, especially in the treatment of synonymous names for *Plumeria* spp. Furthermore, numerous appellations have been applied to taxa within this genus, many of which are misused or unconfirmed among collectors (Criley, 2009), thereby exacerbating the problem of species nomenclature within this genus. Clarifying such disparities have become the motivating force for this present study.

The only formal taxonomic treatment of the genus comes from Robert Woodson, Jr. (1938a), according to his survey of *exsiccatae* (herbarium specimens) from American and European herbaria. Woodson recognized seven species and several botanical varieties, primarily sorting species on floral shape, and secondarily on leaf characters. Prior to Woodson's work, various species had been described (Britton,

1910; Johnston, 1912; Britton, 1915; Britton and Millspaugh, 1920; Hollick, 1922; Britton, 1923), mostly based on leaf characters. Woodson (1938a), however, attempted to synthesize these previously described taxa, recognizing seven *Plumeria* spp. As a result of this "lumping," many previously described species (i.e. *P. bahamensis*, *P. clusioides*, *P. cubensis*) fell under one of two varieties of *P. obtusa*—var. typica or var. sericifolia. Woodson also acknowledged the difficulty of separating taxa of the *P. obtusa* L. complex (Obtusa complex), stating that the variability of *P. obtusa* specimens from numerous collections renders the use of leaf characters imperfect. Additionally, he included other morphologically distinct taxa like *P. caracasana*, *P. stenophylla*, and *P. stenopetala* within *P. pudica*, *P. filifolia*, and *P. rubra* x *P. subsessilis*, respectively. The limitation to Woodson's work was that he was not able to view live specimens, which can have a dramatically different appearance as compared to their pressed and dried counterparts in herbaria.

The Search for Persistent Morphological Characters

Currently, the use of DNA polymorphism is the preferred tool of choice to delimit species (Kress et al., 2005; Shaw et al., 2005; Shaw et al., 2007; Steele and Pires, 2011; Shaw et al., 2014). However, finding useful DNA sequences can be expensive and time-consuming as different molecular regions are only effective at delimiting species in certain genera and plant families (Shaw et al., 2005; Shaw et al., 2007; Judd et al., 2008; Calonje et al., 2009; Pang et al., 2012; Shaw et al., 2014). Instead, the use of morphological characters to identify species can provide a more affordable option, especially in the era of digitized herbarium collections, and still has proven its utility in delimiting species relationships in a number of taxonomically distinct genera and families (Allred and Gould, 1983; García-Lara et al., 2015; da Silva, 2017).

Flowering of *Plumeria* is seasonal (Murashige, 1966; Lawton and Akpan, 1968; Sloan et al., 2007), and in my observations many of the live specimens examined produced leaves prior to flowering. This inhibits the use of floral characters for diagnosing species when specimens are collected out of season. Furthermore, floral descriptions in the literature (Britton, 1910; Johnston, 1912; Britton, 1915; Britton and Millspaugh, 1920; Hollick, 1922; Britton, 1923; Woodson, 1938a; Woodson et al., 1970), when they can be found, can be variable even for one species, and flowers may look

quite different as compared to their descriptions when examining live specimens. Clearly, there is a need for finding other characters that persist when flowers are not present, even in *exsiccatae*. Moreover, some morphological characters, such as midvein pubescence and leaf margin color, may have been overlooked or ignored by earlier taxonomists studying this genus. Hence, we examined the utility of leaf characters as an aid to identifying taxa in this genus.

Therefore, the objective of this study is to evaluate vegetative morphological characters for their effectiveness in identifying *Plumeria* taxa. The question we aim to answer is: What vegetative morphological characters can be used to distinguish *Plumeria* species? The expectation is that no single character will be enough to distinguish species, but rather it will require a combination of characters. The over-arching goal of this study is to find descriptive morphological characters that are easy to score, which can then be incorporated into an existing dichotomous key (Woodson, 1938a) to expedite identification of *Plumeria* species. To my knowledge, this is the most recent investigation since Woodson that evaluates descriptive morphology specific to the genus *Plumeria*.

MATERIALS AND METHODS

Taxon sampling

Fifty-seven accessions, representing species and botanical varieties considered in Woodson's (1938) manuscript, were collected from *Plumeria* collections at Florida Colors Nursery, Fairchild Tropical Botanic Garden (Florida), Naples Botanical Garden (Florida), McBryde Garden (Kaua'i), and University of Hawaii's Waimanalo Research Station (O'ahu). Accession data can be found in Appendix A. Provenance data indicate that most of the specimens were originally collected from the wild in Central America and the Antilles. Since many of the specimens in these collections were small, either because of recent plantings or because conditions were not fit for collecting whole branches, a minimally destructive sampling procedure was used in which only leaves were harvested for subsequent morphological analyses. In addition, close-up pictures were taken of leaves, branches, and trunks to analyze the presence or absence of

certain characters, such as tubercles on branches and trunks, which are difficult to score when taken at a distance.

Each taxon was scored for 43 qualitative morphological characters having binary character states based on presence or absence of the character. These characters are defined in Appendix B, and are based in part on characters that have been previously used to differentiate or describe species of *Plumeria* (Britton, 1910, 1915; Britton and Millspaugh, 1920; Hollick, 1922; Britton, 1923; Woodson, 1938a). To define characters, the morphological character descriptions of Harris and Harris (2011) and Staples and Herbst (2005) were used. The morphological matrix included leaf characters (shape, apex, base, margin, surface, and venation) in addition to trunk, growth, and reproductive characters. Binary characters and presence/absence character states were used for easier interpretation of results. In some instances, certain leaf character states were difficult to score immediately (i.e. venation, leaf bases, etc.), due to variability in such character states on a leaf or plant. In these cases, the more prevalent character state was selected after close examination among many leaves of a given plant from images. After constructing the morphological matrix, the entire matrix was scanned by eye to eliminate any invariant morphological characters. That is, any character which all taxa had in common or lacked were deleted from the data set as these characters were deemed uninformative.

Principal Component Analyses

Principal component analysis (PCA) on morphological data sets, used here, has been employed in other studies to characterize the taxonomic status of species in such plant families as Arecaceae, Fabaceae, Oleaceae, Passifloraceae, and Poaceae (Henderson, 2006; Jin-Yong et al., 2009; Marr et al., 2011; Nagahama et al., 2014; Robbiati et al., 2014; García-Lara et al., 2015; Espinoza et al., 2018). PCA is a multivariate statistical technique that aids in data reduction, allowing for the use of only a few principal components (PCs) to explain variation in a data set (Mead et al., 2003). However, PCA can also be used to identify variables that contribute little information to underlying relationships (Iezzoni and Pritts, 1991). Such characters can be omitted, yielding a more refined data set and more meaningful results. The data matrix consisting of 57 taxa and 43 morphological characters was subjected to multiple

iterations of PCA using R Software Version 1.1.456 (R Core Team, 2013) with the 'FactoMineR' package (Le et al., 2008). Data were also visualized using a combination of 'factoextra' and 'ggplot2' packages to aid in interpretation of results (Wickham, 2016; Kassambara and Mundt, 2017).

Following the methods of Marr et al. (2011) and Viera Barreto et al. (2018), multiple rounds of PCA were employed, and the data set was refined after each iteration. An initial round of PCA was conducted to determine the most informative morphological characters, resulting in a refined data set. Thereafter, successive rounds of PCA were employed to identify taxa clusters, each of which were representative of a species.

An initial round of PCA was done to identify morphological characters that were informative and those that could be dropped from further analyses (Iezzoni and Pritts, 1991). This was done by examining the loadings of characters on components and contributions to the construction of these components. Furthermore, to interpret the value to each PC loading (PCL), contributions to PC1 and PC2 were visualized using the *fviz_pca_var* function as implemented in the 'factoextra' package.

Since the first several principal components (PCs) usually explain the most variation in the data set (Iezzoni and Pritts, 1991; Mead et al., 2003), only the first three PCs were inspected after the initial PCA. However, to do an initial reduction in the data set, only the top 20 contributions of characters to the construction of PC1 and PC2 were examined, all other characters were dropped from further analyses, resulting in a data set containing a reduced number of characters.

Following the methods of Marr et al. (2011) and Viera Barreto et al. (2018), subsequent iterations of PCA was conducted on refined data sets. After each iteration, individuals were plotted on the axes of the first two PCs according to their coordinates to identify clusters that were clearly separated from the remaining taxa. Other PC dimensions up to PC4 were also explored to look for alternative clustering of taxa. Taxa that grouped together and were distinct from other taxa in the plot were removed from subsequent PCA iterations. By doing this, we hoped to enhance resolution of other species on the PCA plots. In addition, if a single specimen's position on the PCA plot differed greatly from others of the same species cluster, it was re-examined for errors in

scoring of morphological characters. Prior to running subsequent iterations of PCA, the data set was sequentially refined to exclude taxa that formed distinct clusters, as mentioned above. Morphological characters that were found only among those clusters of taxa were also removed, as were characters that were present or absent among all the remaining taxa in the data set. A total of four rounds of PCA were carried out.

Limitations

All samples were collected from specimens within botanical gardens, the Plumeria collection at the University of Hawaii Waimanalo Experiment Station, or from private collections. So, there may be some concern that none of my specimens were collected from the wild. However, at some point in time these specimens were still collected from the wild, including accessions at the Honolulu Botanical Garden and Waimea Arboretum, and their provenance is documented. Moreover, particular accessions may no longer exist in the wild or are currently difficult to collect. Another limitation with doing research on specimens collected from live collections in gardens/nurseries is that growth habit of such specimens may be different as compared to their native habitat, as cultivated specimens may have more optimum conditions in which to develop.

RESULTS

Iterative PCA: Step one

Using PCA, informative morphological characters were identified and this confirmed the discrimination of 8 out of 11 *Plumeria* spp. featured in this study. In comparison with the initial PCA, the inclusion of only the top 20 characters that were important to the construction of PCs 1 and 2 in successive rounds of PCA resulted in a refined data set of morphological characters and enhanced resolution of certain clusters of taxa. Resolution of other species was further enhanced by successive removal of clusters of the more distinct species in successive rounds of PCA.

After the initial round of PCA, approximately 14.5% of the variation in the data was explained by PC1, whereas 14.3% and 9.5% of the variation was explained by PC2 and PC3, respectively. These components also had relatively high eigenvalues of 6.24, 6.14, and 4.10 for PC1, PC2, and PC3 (Table 2.1). Since there was a marked decrease

in the amount of variation explained by PC3 in comparison to PC1 and PC2, only the first two components were reviewed for the characters that contributed to the construction of the respective PCs (Figs. 2.1 & 2.2). A review of the top 20 contributing characters to the first two PCs showed that lanceolate, oblong, and elliptical leaf shapes, acute and mucronate leaf apices, equilateral leaf bases, and decurrent or direct secondary venation characters did not contribute significantly to the construction of these components (Table 2.1, Figs. 2.1 & 2.2) and were dropped from subsequent analyses.

Table 2.1. Summary of the first three principal components (PCs) and loadings (p<0.05) of characters that contributed to the construction of these three PCs in the preliminary PCA. The primary variables on which taxa are separated on each component are in boldface. "--" denotes that a variable did not contribute to the construction of the corresponding PC.

	PC 1	**PC 2**	**PC 3**
Eigenvalue:	6.24	6.14	4.10
Variation explained (%):	14.50	14.27	9.53
Characters			
Glabrous_or_CoriaceousAB	**0.69**	0.48	--
Sunken_or_RaisedABv	**0.69**	**-0.65**	0.31
Connivent	**0.65**	-0.43	--
Tomentose_Midvein	**0.64**	-0.52	0.49
Puckering	0.49	0.32	--
Mucronulate	0.45	0.39	--
Oblique	0.41	--	--
Recurved_Lf	0.39	-0.29	0.39
Revolute_Margin	0.34	0.34	--
Glabrous_or_ScabrousAD	0.32	--	--
Follicle_Occurrence	0.31	--	**-0.69**
Retuse	0.30	0.29	--
Prominent_MargVein	0.29	0.27	--
Emarginate	0.27	--	--
Tubercles_Trunk	0.26	0.28	--
Opposite_or_AlternateSV	-0.33	-0.35	--
Cuneate	-0.35	0.47	0.40
Spatulate	-0.36	--	--
Obtuse	-0.46	0.40	--
Flat_Orientation	**-0.60**	0.47	--

Table 2.1. (Continued) Summary of the first three principal components (PCs)

	PC 1	PC 2	PC 3
Characters			
Sunken_or_RaisedAD	**-0.65**	0.52	--
InconspicuousABv	**-0.66**	0.43	--
Pink_Midvein	**-0.67**	0.48	0.45
Pink_Margin	**-0.67**	0.48	0.45
Angular_or_PerpendicularSV	--	**0.66**	--
Leaf_Attachment	--	0.54	**-0.69**
Leaf_Margin	--	0.45	-0.35
Tubercles_Branch	--	0.44	--
Obelliptic	--	0.43	--
Recurved_Margin	--	0.29	-0.33
Growth_Habit	--	-0.53	0.54
Cordate_Acuminate	--	-0.53	0.54
Acuminate	--	-0.53	--
Oblanceolate	--	-0.54	-0.47
Attenuate	--	**-0.61**	-0.50
Conduplicate_Ptyxis	--	**-0.83**	--
Lanceolate	--	--	0.42
Mucronate	--	--	-0.44

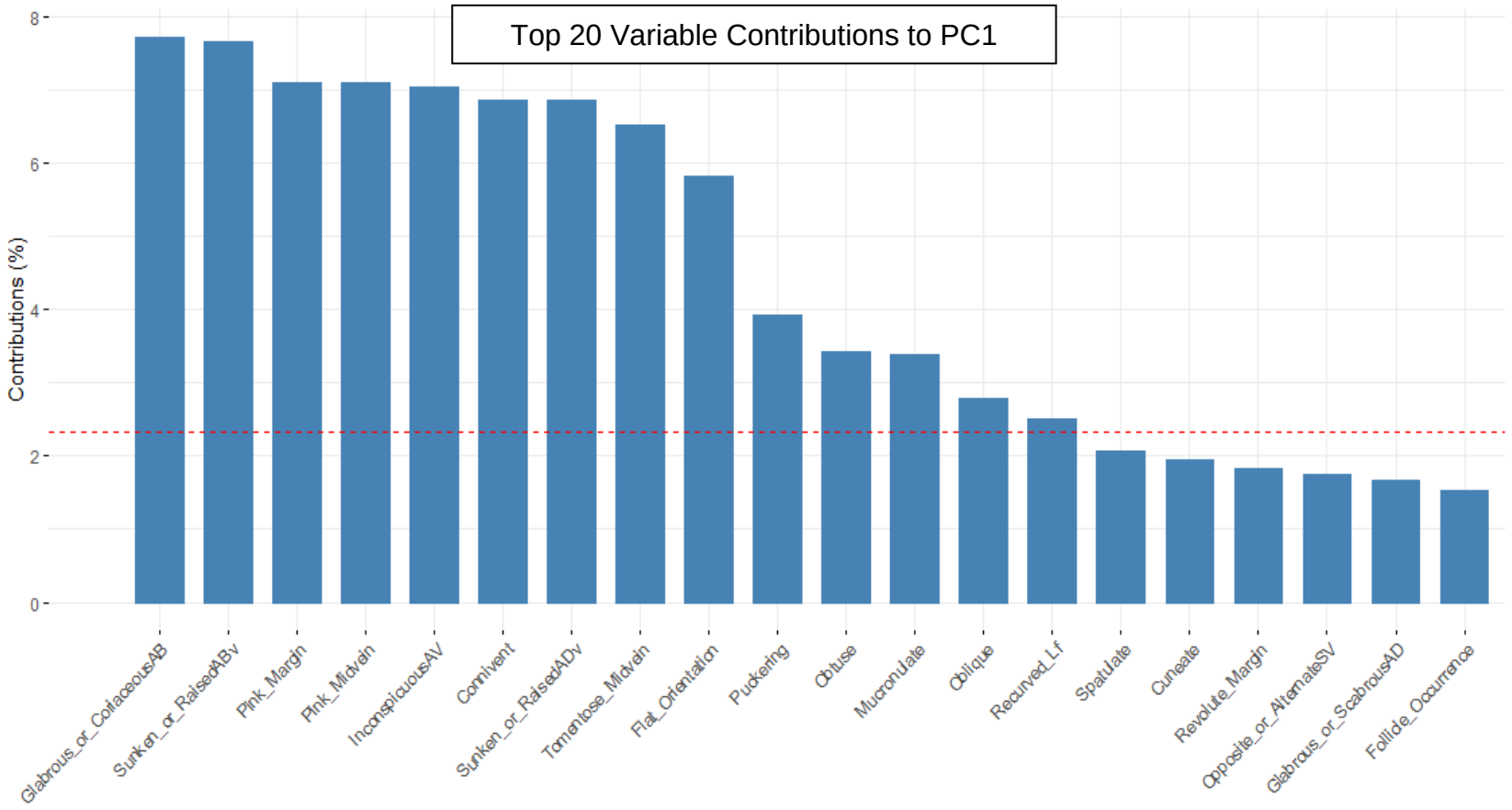


Figure 2.1. First iteration PCA: Top 20 variables that contributed to the construction of PC1. The red dashed line on the graphs above indicate the expected average contribution of variables to the construction of the components. Variables with a contribution larger than this cutoff are considered as important in contributing to the components.

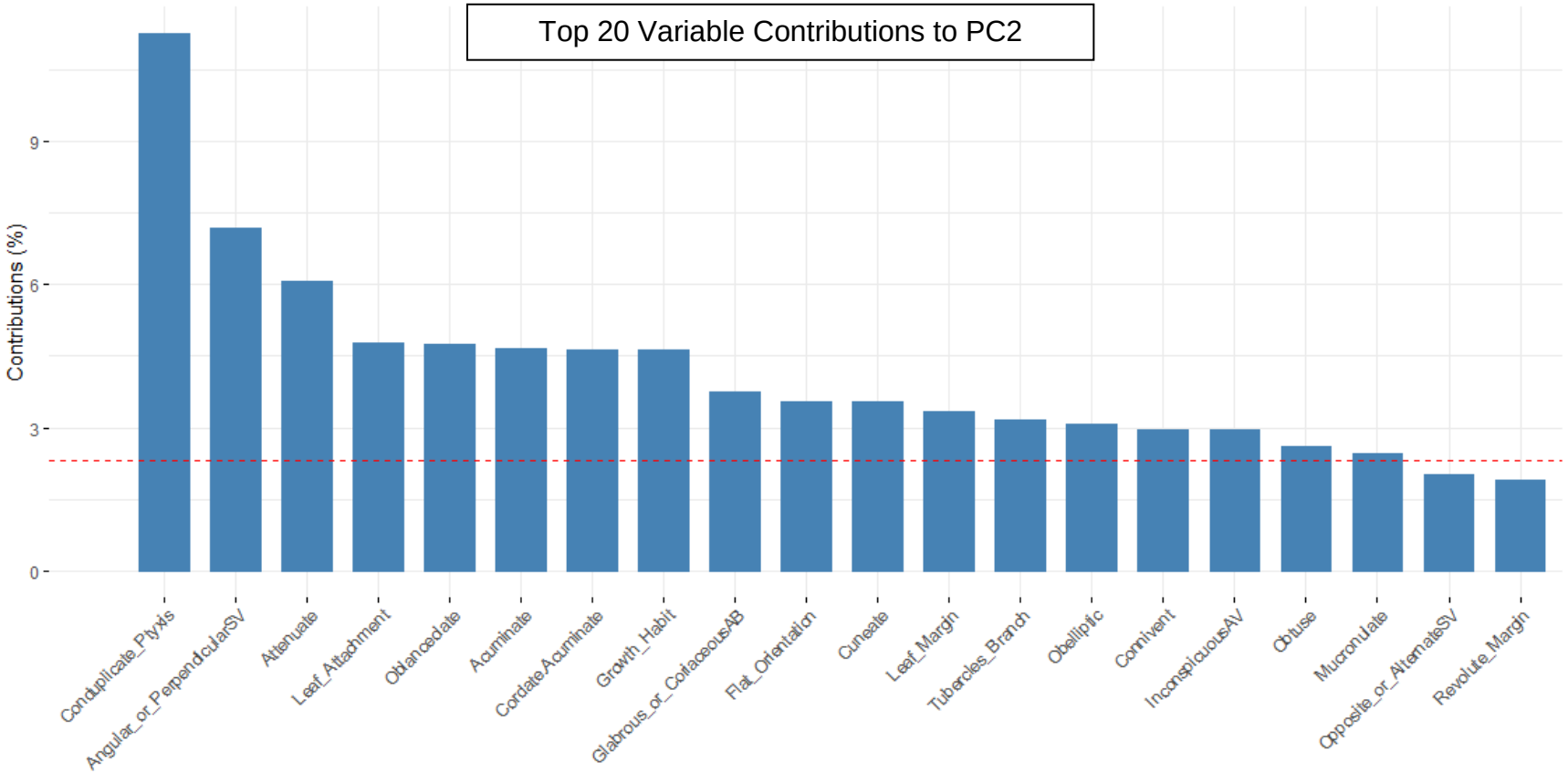


Figure 2.2. First iteration PCA: Top 20 variables that contributed to the construction of PC2. The red dashed line on the graphs above indicate the expected average contribution of variables to the construction of the components. Variables with a contribution larger than this cutoff are considered as important in contributing to the components.

Iterative PCA: Step two

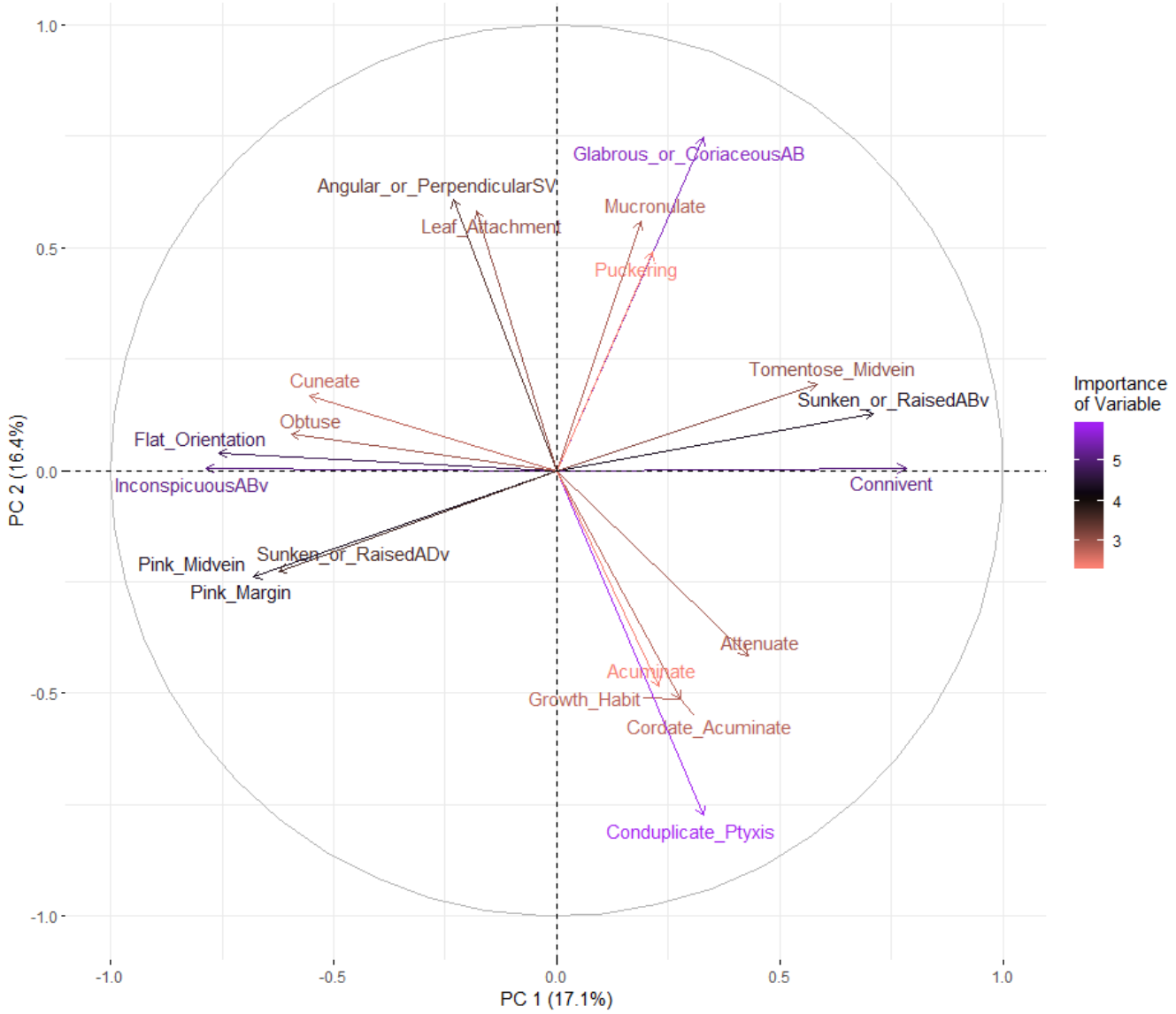
A second round of PCA was carried out on the refined data set containing 36 morphological characters to identify taxa that formed separate and distinct clusters from other samples. The first component accounted for 17.1% of the variation (see above for list of component characters), whereas 16.4% and 10.3% of the variation was accounted for by components two and three, respectively. Taxa were predominantly separated on PC1 according to characters for connivent leaf bases, inconspicuous abaxial venation, flat leaf orientation, sunken or raised abaxial venation, pink leaf margins and midveins, and sunken or raised adaxial venation, whereas conduplicate leaf ptyxis, glabrous or coriaceous abaxial leaf surfaces, angular or perpendicular secondary venation, and leaf attachment characterized PC2 (Fig. 2.3A).

Biplots containing individuals and morphological characters were constructed using the first two PCs according to their coordinates, and distinct clusters of *P. clusioides* and *P. pudica* were identified (Fig. 2.3B). Clu_FCN, Clu_WES, and Clu_NBG are representative of *P. clusioides*. These specimens formed the most distinctive cluster and shared the same data points, indicating homogeneity of morphological characters for the species. Specifically, these taxa possessed inconspicuous abaxial venation, flat leaf orientation, pink midveins and margins, raised adaxial secondary venation, obtuse leaf tips, and cuneate leaf bases.

Representatives of *P. pudica* (Pud_NBG, Pud_CG NBG, and Pud_WESa & b) also formed a cluster of individuals (Fig. 2.3B). Like *P. clusioides*, these taxa also shared a common point, indicating the stability of the morphological characters that unite them. These were clustered on the combination of characters most closely associated with PC2, namely conduplicate leaf ptyxis, angular secondary venation, cordate-acuminate leaf apices, subsessile leaf attachment, and columnar growth habit.

Since these taxa formed distinct clusters from others, these were removed from the data set prior to running a third iteration of PCA. Columnar growth habit and cordate-acuminate leaf apices were only unique to *P. pudica*. Therefore, these characters were also removed prior to a third iteration of PCA.

A


Glabrous_or_CoriaceousAB
Angular_or_PerpendicularSV
Mucronulate
Leaf_Attachment
Puckering
Tomentose_Midvein
Sunken_or_RaisedABv
Cuneate
Obtuse
Flat_Orientation
InconspicuousABv
Connivent
Pink_Midvein
Sunken_or_RaisedADv
Pink_Margin
Attenuate
Acuminate
Growth_Habit
Cordate_Acuminate
Conduplicate_Ptyxis
PC 2 (16.4%)
PC 1 (17.1%)
1.0
0.5
0.0
-0.5
-1.0
Importance of Variable
5
4
3

B

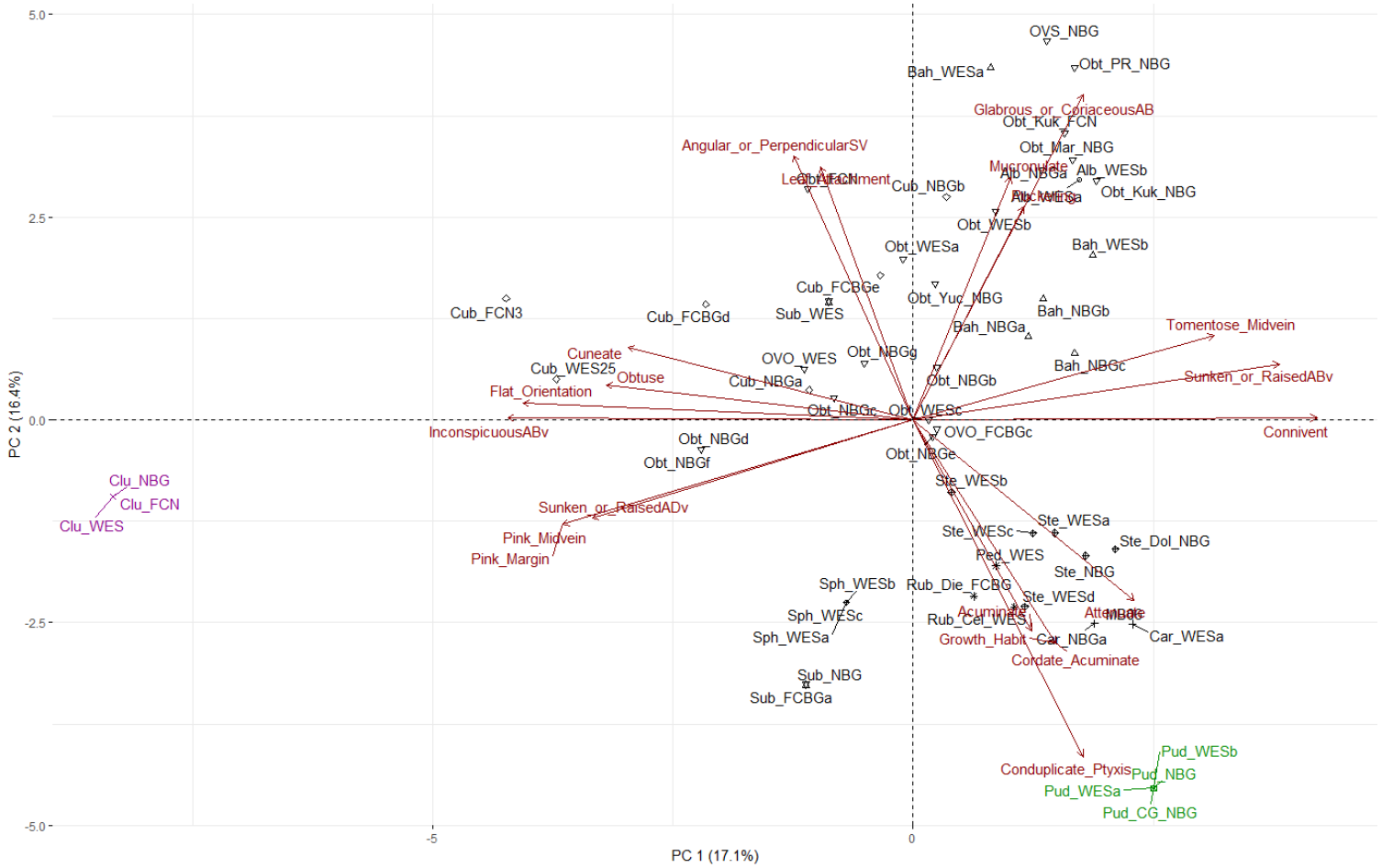


Figure 2.3. Second iteration PCA. A: Factor map showing the top 20 characters that contributed to the construction of PC1 and PC2. Proximity of arrow points to the perimeter of the circle indicate the strength of correlation and colors represent the importance of each character to the construction of PC1 and PC2. B: Biplot of individuals and associated variables of PC1 and PC2. Taxa represent 11 putative *Plumeria* species, with only *P. clusioides* (Clu) and *P. pudica* (Pud) colorized to highlight placement of these taxa into distinct clusters.

Iterative PCA: Step three

The third iteration of PCA contained 34 characters and 50 taxa. In the third iteration of PCA, the first two PCs explained approximately 31% of the total variation (PC1, 17.1%; PC2 13.6%). However, examination into PC3 and PC4, which respectively accounted for 10.8% and 8.2% of variation, allowed for the identification of another distinct cluster of taxa. Conduplicate ptyxis, glabrous or coriaceous abaxial surfaces, oblanceolate leaf shape, and angular or perpendicular secondary venation characters were more strongly associated with PC1. Tomentose midvein, pink margin and midvein, sunken or raised abaxial venation, leaf attachment and follicle occurrence were more strongly associated with PC2.

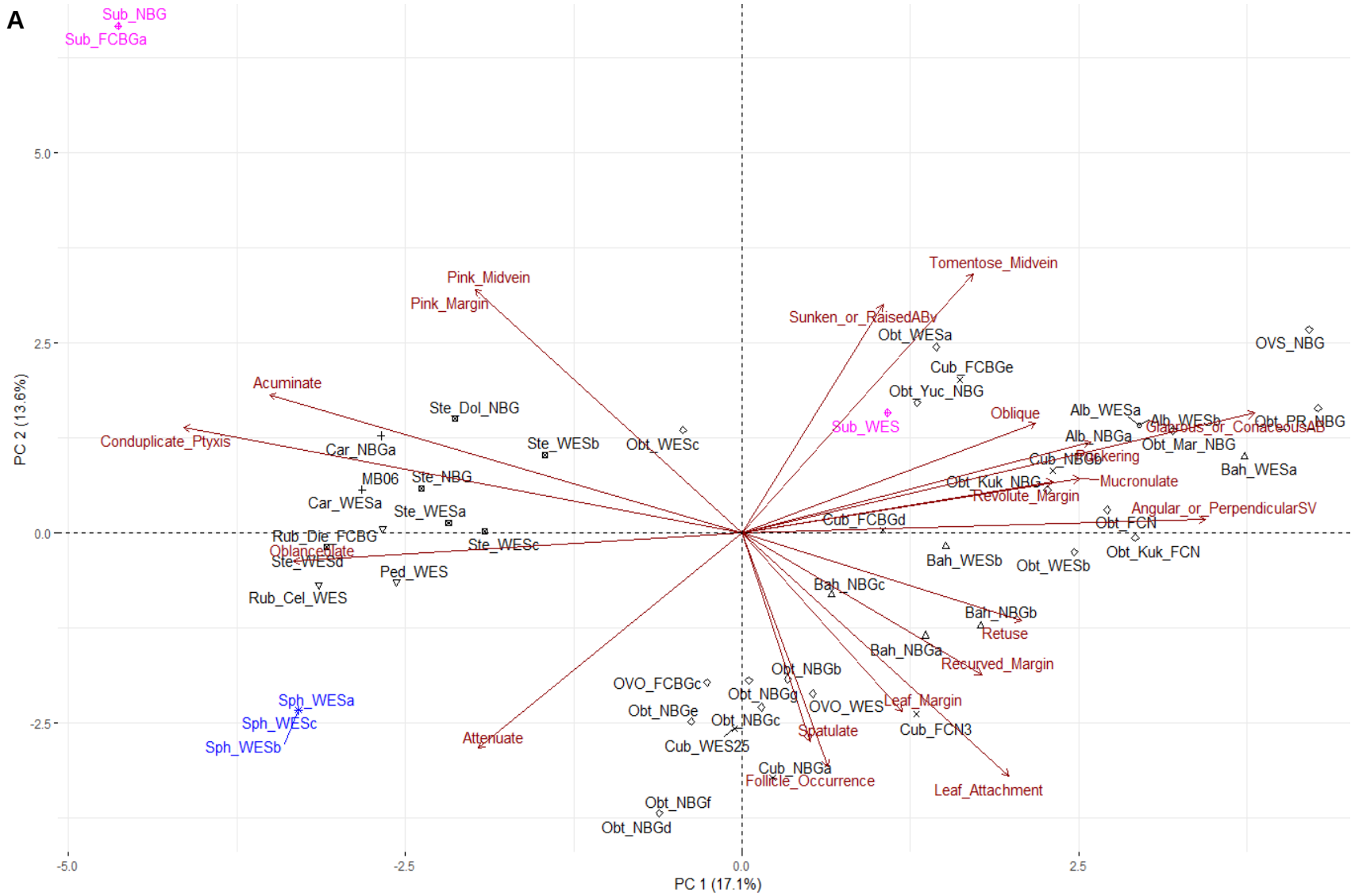
Taxa were plotted according to their coordinates for the first two PCs (Fig. 2.4A). The taxa Sub_FCBGa, Sub_NBG, and Sub_WES represent *P. subsessilis*. Apart from Sub_WES, these taxa shared a similar morphology as indicated by a single shared point. These taxa possessed pink margin and midvein, subsessile leaf attachment, conduplicate leaf ptyxis, and acuminate leaf apex characters. Sub_WES, on the other hand, lacked these four characters. Moreover, it clustered with other *P. obtusa* taxa, which suggests that this specimen is not a *P. subsessilis* but could be a member of *P. obtusa*. Prior to its placement in the Waimanalo Experiment Station, this specimen was accessioned in Nong Nooch Tropical Botanical Garden as *P. subsessilis,* but further collection data is missing. Therefore, this specimen should be re-examined for potential reclassification as a *P. obtusa* or at least a member of the *P. obtusa* complex.

Taxa of *P. stenophylla* (Sph_WESa,b,c) also formed a distinct cluster (Fig. 2.4A). These taxa were united by such characters as oblanceolate leaf shape, attenuate leaf bases, sunken abaxial venation, lack of tomentose midveins, and glabrous abaxial surfaces. Although the character for mucronate leaf apex does not appear in the PCA, these accessions are the only taxa in the data set that possessed this character.

Observations into PC3 and PC4 showed a cluster of *P. alba* (Fig. 2.4 B). *P. alba* taxa (Alb_NBGa, Alb_WESa & b) were united by characters for recurved leaves, puckering of the laminal surface, oblique leaf bases, and revolute leaf margins. These taxa, too, share common coordinates thus indicating homogeneity in characters of which they share. One accession of *P. obtusa* (Obt_PR_NBG) clustered closely with *P.*

alba taxa due to the characters that they share (i.e., puckering, oblique leaf bases, recurved leaves, and revolute leaf margins). However, this clustering only occurred when analyzing PC3 and PC4.

Prior to a fourth iteration of PCA, these taxa were removed from the data set. The characters for pink midvein, pink margin, leaf attachment, and sunken or raised adaxial secondary venation were also removed since these were not found in the remaining taxa. The final data set for the fourth iteration of PCA contained 41 taxa and 30 morphological variables.


A
Sub_NBG
Sub_FCBGa
Pink_Midvein
Pink_Margin
Tomentose_Midvein
Sunken_or_RaisedABv
Obt_WESa
OVS_NBG
Cub_FCBGe
Obt_Yuc_NBG
Acuminate
Ste_Dol_NBG
Oblique
Alb_WESa
Alb_WESb
Obt_PR_NBG
Glaberous_or_CoriaceousAB
Conduplicate_Ptyxis
Car_NBGa
Ste_WESb
Obt_WESc
Sub_WES
Alb_NBGa
Obt_Mar_NBG
Puckering
Cub_NBGb
Bah_WESa
MB06
Ste_NBG
Obt_Kuk_NBG
Mucronulate
Car_WESa
Revolute_Margin
Ste_WESa
Angular_or_PerpendicularSV
Cub_FCBGd
Obt_FCN
Rub_Die_FCBG
Oblanceolate
Ste_WESc
Ste_WESd
Bah_WESb
Obt_WESb
Obt_Kuk_FCN
Ped_WES
Bah_NBGc
Rub_Cel_WES
Bah_NBGb
Retuse
Bah_NBGa
Recurved_Margin
Obt_NBGb
OVO_FCBGc
Obt_NBGg
Leaf_Margin
Sph_WESa
Obt_NBGe
OVO_WES
Sph_WESc
Obt_NBGc
Spatulate
Cub_FCN3
Attenuate
Sph_WESb
Cub_WES25
Cub_NBGa
Follicle_Occurrence
Leaf_Attachment
Obt_NBGf
Obt_NBGd
5.0
2.5
0.0
-2.5
-5.0
-2.5
0.0
2.5
PC 2 (13.6%)
PC 1 (17.1%)

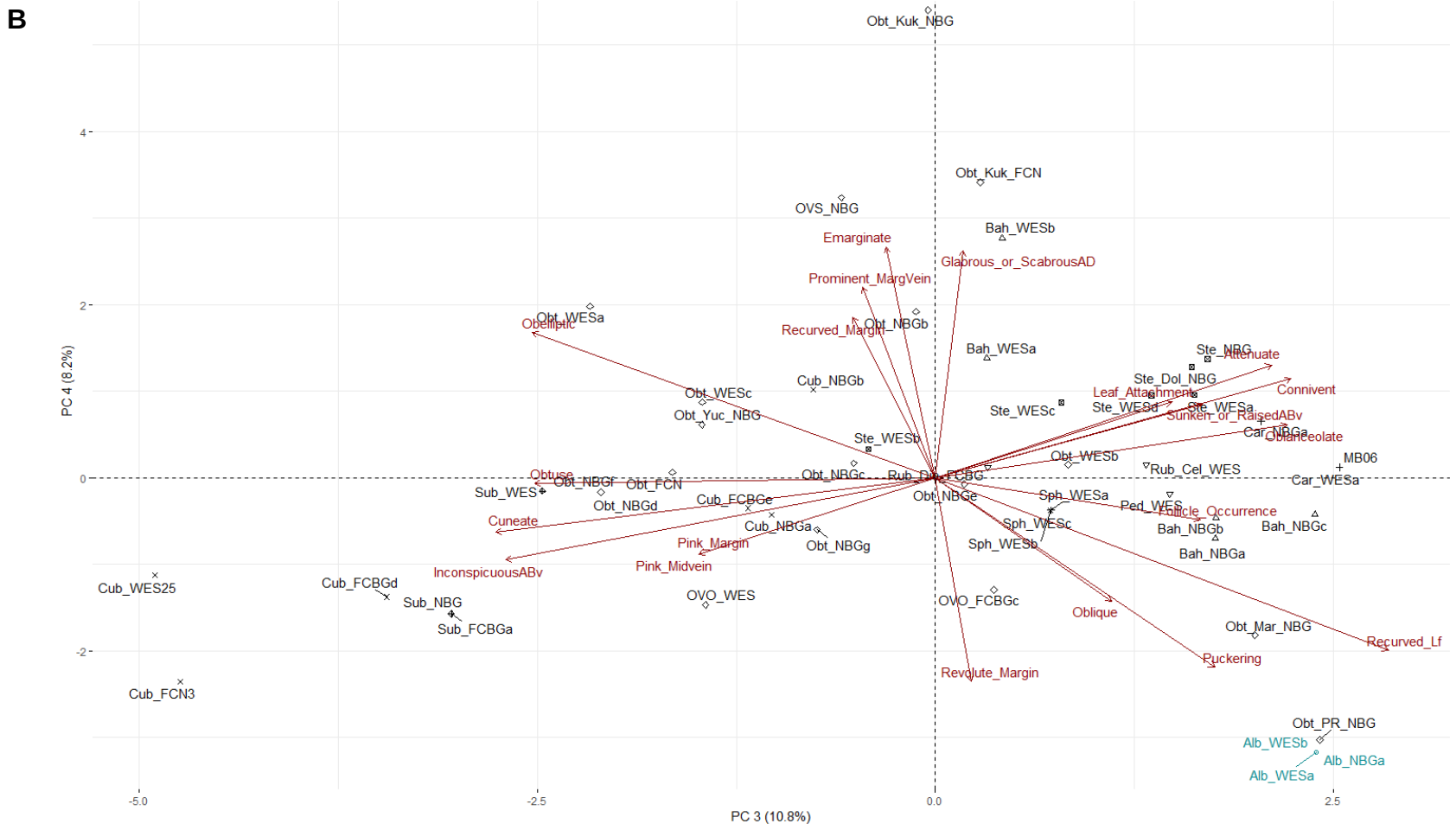


Figure 2.4. Third iteration PCA. A: Biplot of individuals and characters associated with PC1 and PC2. Taxa of *P. stenophylla* (Sph) and *P. subsessilis* (Sub) are colorized to highlight placement of these taxa into clusters. B: Biplot of individuals and characters associated with PC3 and PC4. Taxa of *P. alba* (Alb) colorized to show their placement.

Iterative PCA: Step four

PC1 explained 17.9% of the variation, whereas roughly 14.6% of the variation was explained by PC2. Taxa were primarily sorted on PC1 based on glabrous or coriaceous abaxial surface, acuminate leaf tips, conduplicate ptyxis, and oblanceolate leaf shape. On PC2, taxa were sorted primarily on presence or absence of inconspicuous abaxial venation, sunken or raised abaxial venation, and presence or absence of tomentose midveins.

Accessions of *P. caracasana*, *P. stenopetala*, and *P. rubra* formed a complex cluster based on the shared presence of conduplicate ptyxis, oblanceolate leaf shape, and acuminate leaf apices (Fig. 2.5). However, this complex cluster could be further subdivided into smaller clusters consisting of *P. caracasana*, *P. rubra*, and *P. stenopetala*.

Aside from the presence of oblanceolate leaf shape, acuminate leaf apex, attenuate leaf bases, and conduplicate leaf ptyxis, specimens of *P. caracasana* (Car_NBGa, Car_WESa, and MB06) could be further distinguished by the presence of recurved leaves and undulate leaf margins (Fig. 2.5, see also Appendix A). MB06 and Car_WESa share the same point, indicating that both taxa share the same morphological features. Car_NBGa differs slightly from the other two taxa in that it appeared to have noticeable tubercles developing on branches. The other two specimens that were observed did not appear to have tubercles developing on branches. In addition, MB06 was an accession from the Allerton Garden (National Tropical Botanical Garden, Kaua'i Island, Hawaii), and was previously identified as *P. pudica*. However, it did not demonstrate the columnar growth that is typical of *P. pudica*, nor did it have the subsessile petioles that taxa of *P. pudica* possess. Thus, this specimen was included to verify its designation as a *P. caracasana*, which is reflected in its clustering with the other taxa of *P. caracasana*.

P. rubra clusters (Ped_WES, Rub_Cel_WES, and Rub_Die_FCBG) are also grouped on acuminate leaf tip, conduplicate leaf ptyxis, and oblanceolate leaf shape characters (Fig. 2.5). However, their distinguishing features are entire leaf margins and absence of tomentose midribs. There is some dispersion among the clusters due to

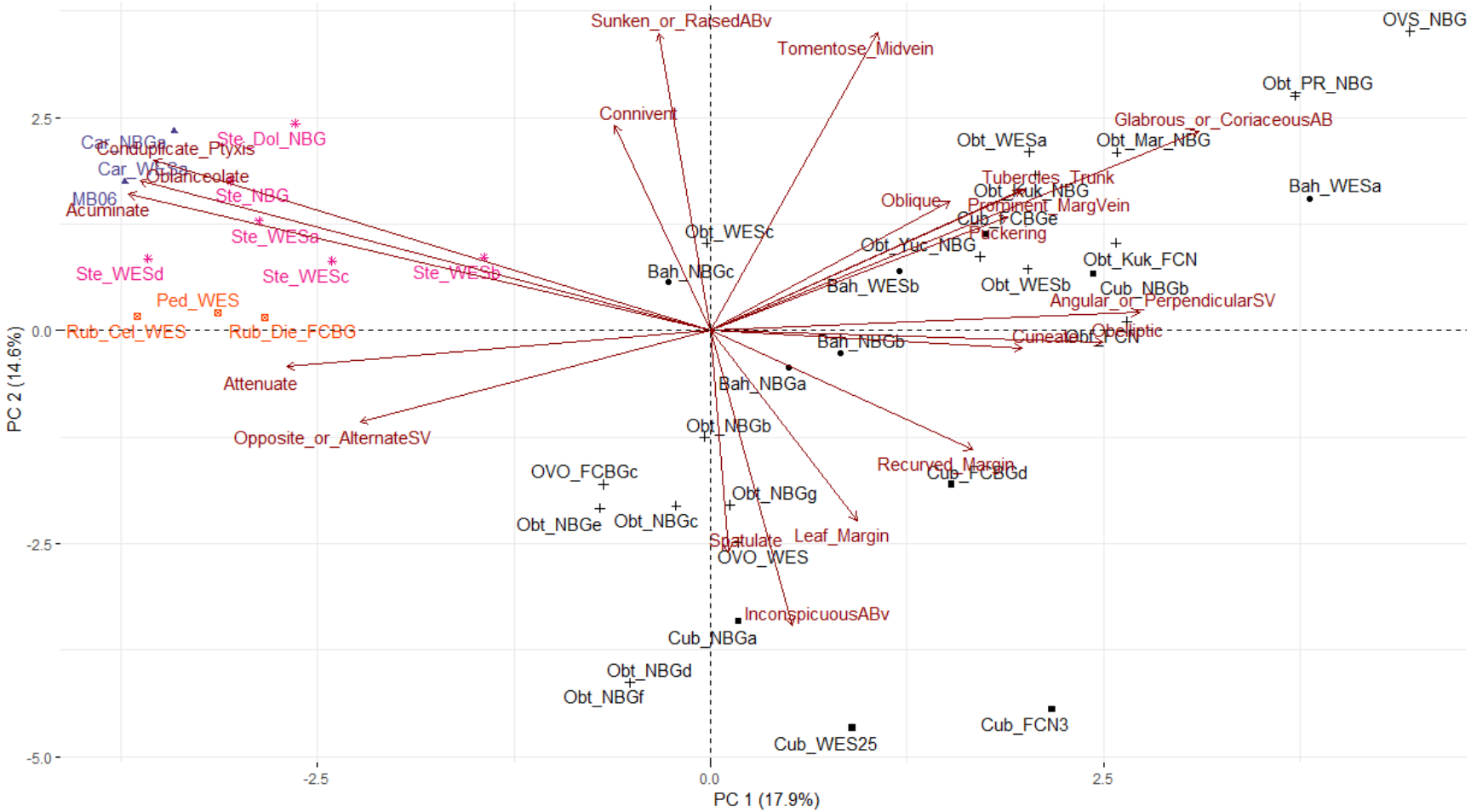


Figure 2.5. Fourth iteration PCA. A biplot of individuals and variables associated with PC1 and PC2. Taxa of *P. caracasana* (Car), *P. rubra* (Rub & Ped), and *P. stenopetala* (Ste) are colorized to highlight placement of these taxa into clusters.

differences in angular or perpendicular secondary venation and follicle occurrence characters among the three accessions.

Not all taxa of *P. stenopetala* (Ste_WESa,b,c,d, Ste_NBG, and Ste_Dol_NBG) formed a distinct cluster, as evidenced by their dispersion along the negative axis of PC1 but tended to cluster near one another (Fig. 2.5). Aside from the presence of oblanceolate leaf shape, acuminate leaf apex, conduplicate leaf ptyxis, and attenuate leaf bases, these taxa also possessed tomentose midveins and mucronulate leaf apices. However, due to variability in leaf apex characters, leaf bases, leaf margins, abaxial surface, secondary venation, tubercles, and follicle production, the clustering among these taxa were more dispersed (see Appendix A).

The remaining samples represent members of the *P. obtusa* complex (Fig. 2.5). Taxa of *P. bahamensis* (Bah), *P. cubensis* (Cub), *P. obtusa* (Obt), *P. obtusa* var. *obtusa* (OVO), *P. obtusa* var. *sericifolia* (OVS) do not show any noticeable clustering, even when other PCs were explored. Their dispersion among PC1 and PC2 alone is indicative of the morphological variability both between and within these named species. They are simply too morphologically variable, given the current set of characters analyzed.

DISCUSSION

The over-arching goal of this study was to identify descriptive morphological characters that were easy to score, which could then be used to update an existing dichotomous key to expedite on-site identification of *Plumeria* species. To my knowledge, this was the first investigation since Woodson (1938a) to evaluate descriptive morphology with a specific focus on this genus. The objective was to determine combinations of morphological characters that would aid in identifying *Plumeria* taxa. The distinguishing characters and their relevance to prior classification for the species identified in this study are discussed below for each taxon.

P. clusioides Griseb.

Woodson (1938a) subsumed this species under *P. obtusa* var. typica, and Govaerts et al. (2003) also regard this as a synonym of *P. obtusa*. Although both

species are naturally found in Cuba (Britton, 1915; Leon and Alain, 1957), individuals identified as *P. clusioides* were clearly distinguishable from other *P. obtusa* taxa in this study (Fig. 2.3 B). In contrast to Woodson (1938a) and Govaerts et al. (2003), Britton (1915) recognized *P. clusioides* as a distinct species and described it has having short petioles, glabrous leaves, obovate or oblanceolate leaf shape, and obscure lateral venation, which is in agreement with the findings of this study.

Characters such as inconspicuous abaxial venation, flat leaf orientation, raised adaxial secondary venation, obtuse leaf tips, cuneate leaf bases, and pink leaf margins and midveins are useful in distinguishing taxa of *P. clusioides* (Fig. 2.6 A-C). Pink midveins and accompanied pink leaf margins are one of the most diagnostic features of live specimens of *P. clusioides*, and these characters are most noticeable in newly developing leaves.

***P. stenophylla* Urb.**

Taxa of *P. stenophylla* can be identified by the presence of oblanceolate leaf shape, attenuate leaf bases, sunken abaxial venation, lack of tomentose midveins, and glabrous abaxial surfaces (Fig. 2.6 D-F), which has been confirmed via PCA analysis. In addition, mucronate leaf apices and frequently produced follicles can also distinguish *P. stenophylla* (Appendix A). Alain and Leon (1957) describe leaves of this particular species as having lanceolate-linear to sublinear shape, acuminate apices, attenuate leaf bases, and ascending secondary venation. A similar description of this species is provided by Urban (1924). However, *P. stenophylla* was considered to be a synonym of *P. filifolia* Griseb. by Woodson (1938a), Govaerts et al. (2003), and Acevedo-Rodríguez and Strong (2012). In addition, the latter authors did not consider the distinct filiform leaves of *P. filifolia*, which *P. stenophylla* clearly lacks as well as other details that are distinct in the morphologies of the two species when both live specimens and *exsiccatae* are considered. *In lieu* of the fact that both species are naturally distributed in Cuba (Urban, 1924) and can look morphologically similar, it seems reasonable that botanists would regard both species as synonymous. However, *P. stenophylla* should be recognized as a species on the basis of characters that place accessions of this species into its own cluster in the PCA, although additional work will be needed to

assess characters that distinguish it from *P. filifolia* as direct comparisons could not be made in this study.

***P. subsessilis* A. DC.**

This species is endemic to Hispaniola, present-day Haiti and Dominican Republic (Woodson, 1938b; Moscoso, 1943), is clearly distinguishable from most other species in this study, and is also recognized as a legitimate species (Govaerts et al., 2003; Acevedo-Rodríguez and Strong, 2012). PCA analyses showed that *P. subsessilis* is characterized by acuminate leaf apices, pink midveins and margins, conduplicate leaf ptyxis, and subsessile leaf attachment (Fig. 2.4 A). In addition, although loadings for decurrent secondary venation was not highly correlated with the first two PCs, this additional feature can be used to identify *P. subsessilis*. In live specimens, these characters are noticeable, whereas pink midveins and pink margins are more prominent on younger foliage (Fig. 2.6 G-J). In his recognition of this species, Woodson (1938a; 1938b) also noted the presence of subsessile petioles, secondary venation entering the midrib at decurrent angles, and pronounced venation on both leaf surfaces. Similar foliar descriptions are also given by De Candolle (1844). Hence, a combination of these characters is sufficient to identify specimens of *P. subsessilis*.

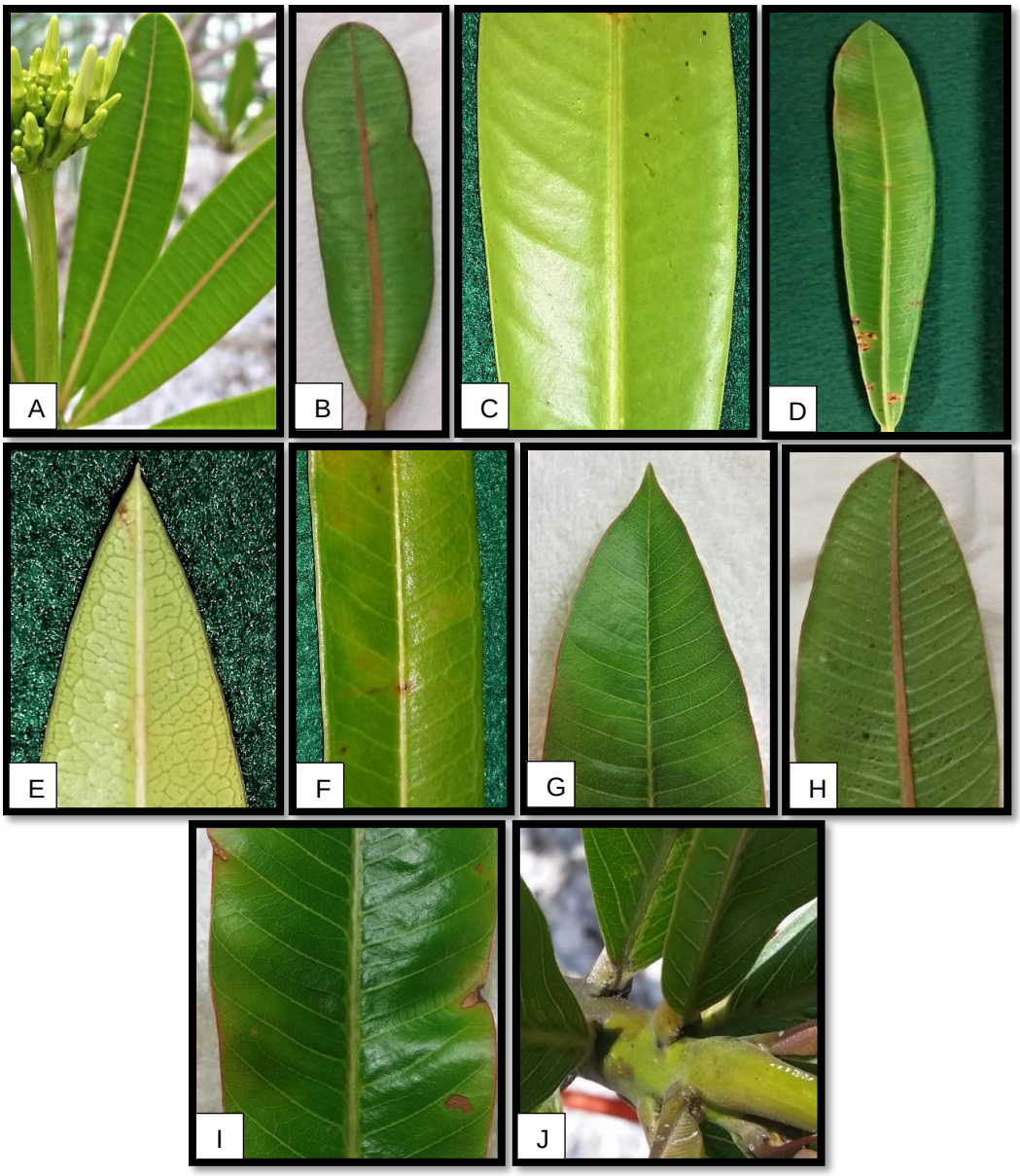


Figure 2.6. Morphological characters that distinguish *P. clusioides*, *P. stenophylla*, and *P. subsessilis*. A: Flat leaf deflection and raised adaxial secondary venation of *P. clusioides*. B: Adaxial surface of a young *P. clusioides* leaf showing pink midveins, obtuse leaf tip, and cuneate leaf bases. C: Inconspicuous abaxial venation of a *P. clusioides* leaf. D: Oblanceolate leaf shape with attenuate leaf bases of *P. stenophylla*. E: Glabrous abaxial leaf surface, mucronate leaf tip and sunken abaxial venation of *P. stenophylla*. F: Adaxial surface of a *P. stenophylla* leaf showing alternate secondary venation. G: Acuminate leaf apex and pink leaf margins of *P. subsessilis*. H: Abaxial leaf surface of *P. subsessilis*, showing a pink midvein. I: Adaxial view of *P. subsessilis* showing secondary venation entering the midrib at decurrent angles. J: Subsessile leaf attachment of *P. subsessilis*. Photos by Kauahi Perez.

***P. pudica* Jacq.**

This species is recognized by Jacquin (1763), Woodson (1938a), Gleason and Killip (1939), Govaerts et al. (2003), and Acevedo-Rodríguez and Strong (2012), and is naturally distributed from Panama to northern Venezuela and extends as far as Martinique in the Lesser Antilles (Woodson, 1938a; Govaerts et al., 2003). Jacquin (1763) first described this species in Curaçao, making note of its erect growth habit, which is referred to as "columnar" in this chapter. Woodson (1938b) described this species as having scarcely manifested (subsessile) petioles, cochleate or pandurate leaf shape, obtuse to shortly acuminate leaf apices, cuneate bases, glabrous adaxial surfaces, and pilose to glabrate abaxial leaf surfaces. My observations of these characters are similar (see Appendix A). PCA showed that conduplicate ptyxis, cordate-acuminate leaf apices, columnar growth habit, angular secondary venation, and subsessile leaf attachment provided distinguishable characters that identify specimens of *P. pudica* (Fig. 2.5). These features were easily scoreable in live specimens that were assessed (Fig. 2.7 A-D). During personal observations, many live specimens of *P. pudica* also possessed a distinctive leaf shape. The overall leaf shape is referred to as spatulate, but Woodson (1938b) described the leaf shape as cochleate or pandurate. Thus, pandurate leaf shape should be the preferred leaf shape descriptor of this species since it is more accurate. Included as part of the overall pandurate leaf shape is a distinctive leaf apex that is referred to as cordate-acuminate (heart-shaped) leaf tip. In short, these findings agree with those of previous workers.

***P. caracasana* J.R. Jhonst.**

Plumeria caracasana was first described in Johnston's collections from La Guaira, Venezuela (Johnston, 1912). Named as "*Plumiera caracasana*" in his description, Johnston described this taxon as having spatulate or oblanceolate leaf shape, acute or obtuse at the apex, glabrous above, either glabrous or pilose at the main veins below, and blades with entire margins. Woodson (1938a) and Govaerts et al. (2003), however, considered this taxon as a synonym of *P. pudica*. This is understandable as both species share similar morphological characters. Even in review of online herbarium specimens, *P. caracasana* and *P. pudica* show a similar gross

morphology of leaves. In this study, taxa of *P. caracasana* clustered close to those of *P. pudica* (Fig. 2.3 B), which indicated some degree of morphological similarity. However, *P. caracasana* remained distinct from *P. pudica* primarily based on oblanceolate leaf shape and acuminate leaf apices (Fig. 2.5). Moreover, the live specimens examined typically had recurved leaves with undulate leaf margins and pronounced petioles, but lacked the columnar growth, pandurate leaf shape, and subsessile petioles that were diagnostic of *P. pudica* (Fig. 2.7 E-G). Furthermore, *P. caracasana* readily sets seed in Hawaii's environment whereas seed set is rare in *P. pudica*. Based on these lines of evidence, it is suggested that the name *P. caracasana* be considered valid.

***P. alba* L.**

Plumeria alba is naturally distributed from Puerto Rico to the Windward Islands of the Lesser Antilles (Govaerts et al., 2003; Acevedo-Rodríguez and Strong, 2012). Among all of the descriptions of this species, the lanceolate leaf shape and revolute leaf margins stand out (Linné and Salvius, 1753; Jacquin, 1763; Grisebach, 1864; Standley, 1924; Stahl, 1937; Howard, 1989). Woodson (1938a) and De Candolle (1844) also noted coriaceous abaxial leaf surfaces, and Stahl (1937) noticed the perpendicular secondary venation on the abaxial surface of leaves. These easy-to-score characters were also apparent in *P. alba* accessions that were sampled (Fig. 2.7 H-K). Results of PC3 and PC4 showed that *P. alba* is recognizable by recurved leaves, puckering of the laminal surface, oblique leaf bases, and revolute leaf margins (Fig. 2.4 B). In addition, although clustering of these taxa was not distinct from other taxa that assembled on PC1 and PC2, taxa of *P. alba* also assembled on the characters for coriaceous abaxial leaf surfaces and perpendicular secondary venation (Fig. 2.4 A). Thus, results of this study confirm that these morphological characters are useful for identifying live specimens of *P. alba*.

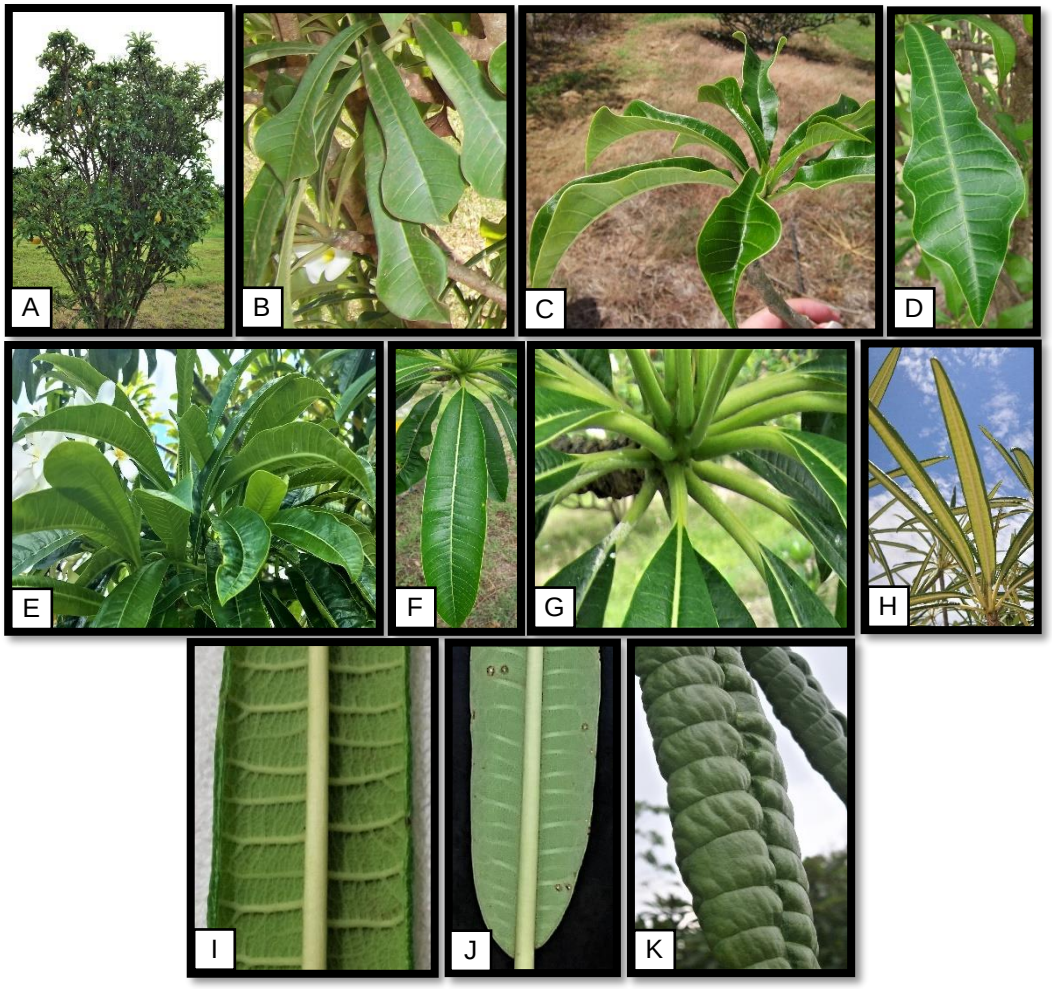


Figure 2.7. Morphological characters that distinguish *P. pudica*, *P. caracasana*, and *P. alba*. A: Columnar (erect) growth habit of *P. pudica*. B: Cordate-acuminate leaf tips of *P. pudica*. C: Conduplicate leaf ptyxis of *P. pudica*. D: Pandurate leaf shape of *P. pudica*. E: Recurved leaves, conduplicate ptyxis, and acuminate leaf apices of *P. caracasana*. F: Oblanceolate leaf shape of *P. caracasana*. G: Petiolate leaf attachment of *P. caracasana*. H: Revolute leaf margins of *P. alba*. I: Coriaceous abaxial leaf surface and perpendicular venation of *P. alba*. J: Oblique leaf bases of *P. alba*. K: Puckered laminar surface of *P. alba* (photo by R. Criley). Photos A-G, I by Kauahi Perez and H, K by Richard Criley.

***P. rubra* L.**

The natural distribution of *P. rubra* is from Mexico to Venezuela and the Greater Antilles (Liogier and Martorell, 1982; Govaerts et al., 2003). This is the most highly cultivated species of the genus and is now found worldwide in tropical locations (Criley, 2009; Acevedo-Rodríguez and Strong, 2012). Numerous authors have provided descriptions of the leaves of *P. rubra*, many of which have little in common (Jacquin, 1763; De Candolle, 1844; Grisebach et al., 1863; Britton and Millspaugh, 1920; Standley, 1924; Stahl, 1937; Woodson et al., 1970; Howard, 1989). Due to the extensive variation found among these descriptions it has made it impossible to accurately identify this species based on leaf characters alone. On the other hand, there is general agreement upon petiolate leaf attachment, glabrous adaxial and abaxial leaf surfaces, acuminate leaf apex, and attenuate leaf bases as defining characters, which agree with my own observations of live specimens of *P. rubra* (Fig. 2.8 A-E). PCA showed that *P. rubra* representatives formed a cluster based on acuminate leaf tips, conduplicate leaf ptyxis, oblanceolate leaf shape, and attenuate leaf bases (Fig. 2.5). Thus, findings of this study corroborate with previous descriptions of this species.

***P. stenopetala* Urb.**

The original description of this taxon was based on a late 19th century collection from Haiti (Urban, 1902). Urban (1902) provided the first Latin description of this species. Leaves were described as having petiolate leaf attachment, oblong-elliptic to oblong-spatulate (oblanceolate) leaf shape, rounded (obtuse) to shortly acuminate leaf apices, acute leaf bases, lamina that is often folded longitudinally (conduplicate ptyxis), glabrous on the adaxial surface with fine pubescence on the abaxial surface. The morphological leaf descriptors used in this study are similar, and these characters have been observed on live specimens of *P. stenopetala* as well as on dried specimens (Fig. 2.8 F-I). However, Woodson (1938a) regarded *P. stenopetala* as a potential hybrid between *P. rubra* and *P. subsessilis*, attributing anthropogenic influences as a source of hybridization between these two species. Govaerts et al. (2003) and Acevedo-Rodríguez and Strong (2012) also recognize this as a hybrid and regard the name *Plumeria* x *stenopetala* as acceptable. Taxa of putative *P. stenopetala* in PCA analyses did not group together tightly in a distinct cluster but did group near one another (Fig.

2.5). Clustering was based on the presence of oblanceolate leaf shape, acuminate leaf apex, conduplicate leaf ptyxis, and attenuate leaf bases, tomentose midveins, and mucronulate leaf apices.

However, the extensive morphological variation in other characters provide a reasonable explanation for greater dispersion on the PCA plot than found in most other taxa. It is interesting to note, though, that these taxa share morphological characters with both *P. rubra* and *P. subsessilis*, such as acuminate leaf apices and attenuated leaf bases (Appendix 2). Furthermore, the fact that *P. stenopetala* taxa were positioned in between *P. rubra* and *P. subsessilis* in the third iteration of PCA (Fig. 2.5) could substantiate that *P. stenopetala* is indeed a hybrid. On the other hand, the growth habit of *P. stenopetala* and branch thickness are quite distinct from *P. rubra*. It is difficult to make a definitive statement as to the potential hybrid origin of this taxon at this time due to the variability shown in this study.

Remaining Taxa of the *P. obtusa* Complex

Numerous descriptions of *P. obtusa* (originally collected from the Antilles) can be found in the literature (Linné and Salvius, 1753; De Candolle, 1844; Grisebach et al., 1863; Britton and Millspaugh, 1920; Stahl, 1937; Woodson, 1938b; Leon and Alain, 1957). Among these are multiple differing descriptions of leaf size, shape, and texture. These reflect what can easily be seen simply by looking at leaf shape alone, for instance, as leaves range from obovate or oblong-obovate to lanceolate or oblong-oblanceolate (Fig. 2.9 A). Along with *P. obtusa*, Britton and Millspaugh (1920) mention a species known as *P. bahamensis*, and describe it as having lanceolate or linear-lanceolate leaf shape, glabrous with acute or acuminate leaf apices, attenuated bases, and lateral veins straight ascending. Additionally, Urban (1925) recognized a species he called *P. cubensis* that shares many of the characters of both *P. obtusa* and *P. bahamensis*. On the other hand, Woodson (1938a) recognized only two types of *P. obtusa*—var. *typica* and var. *sericifolia*—using abaxial pubescence as the defining character of taxa belonging to *P. obtusa* var. *sericifolia*. Discounting the minor subtleties of leaf shapes, he placed eleven previously described species under *P. obtusa* var. *sericifolia* and subsumed over 20 previously described species under *P.*

obtusa var. *typica*, including *P. bahamensis* and *P. cubensis*. Ironically, many of the *P. bahamensis* and some of the *P. cubensis* that were examined have pubescence on the lower leaf surface (Appendix A).

Taxa of the *P. obtusa* complex, which included specimens of *P. cubensis*, *P. bahamensis*, *P. obtusa* and several named varieties of this species, were the most challenging specimens to analyze. Even with refining the data set through multiple iterations of PCA, these taxa did not form any distinct clusters. One reasonable explanation for this dispersion is the inherent morphological variation of individual specimens (Fig. 2.9 A-F). For example, even among specimens of *P. bahamensis* there is noticeable variation in leaf, branch, and trunk characters (Appendix A). When such variation was noticed on a specimen, multiple leaves were examined to determine the most common character states on each specimen. Even with great effort, however, such variation made it difficult to score some specimens of this complex, which eventually made it difficult to verify species. Given that these taxa fail to form distinct clusters indicates that a more intensive study is required to determine whether these are separate species or not. Other growth habit characters, follicle size and shape, branch thickness, and flower size are all distinguishing characters that were not weighed in this determination.

Further, there is some evidence that the *P. obtusa* commonly grown in Hawaii and across the Pacific may be a hybrid as the infrequent seedling offspring vary in leaf characters. These *P. obtusa* differ in subtle ways from the Caribbean *P. obtusa* observed at NBG and FTBG collections. Hence, it is difficult to determine whether *P. cubensis*, *P. bahamensis*, and *P. obtusa* are valid species because the current descriptive morphology used in this study, especially presence or absence states, is simply not enough.

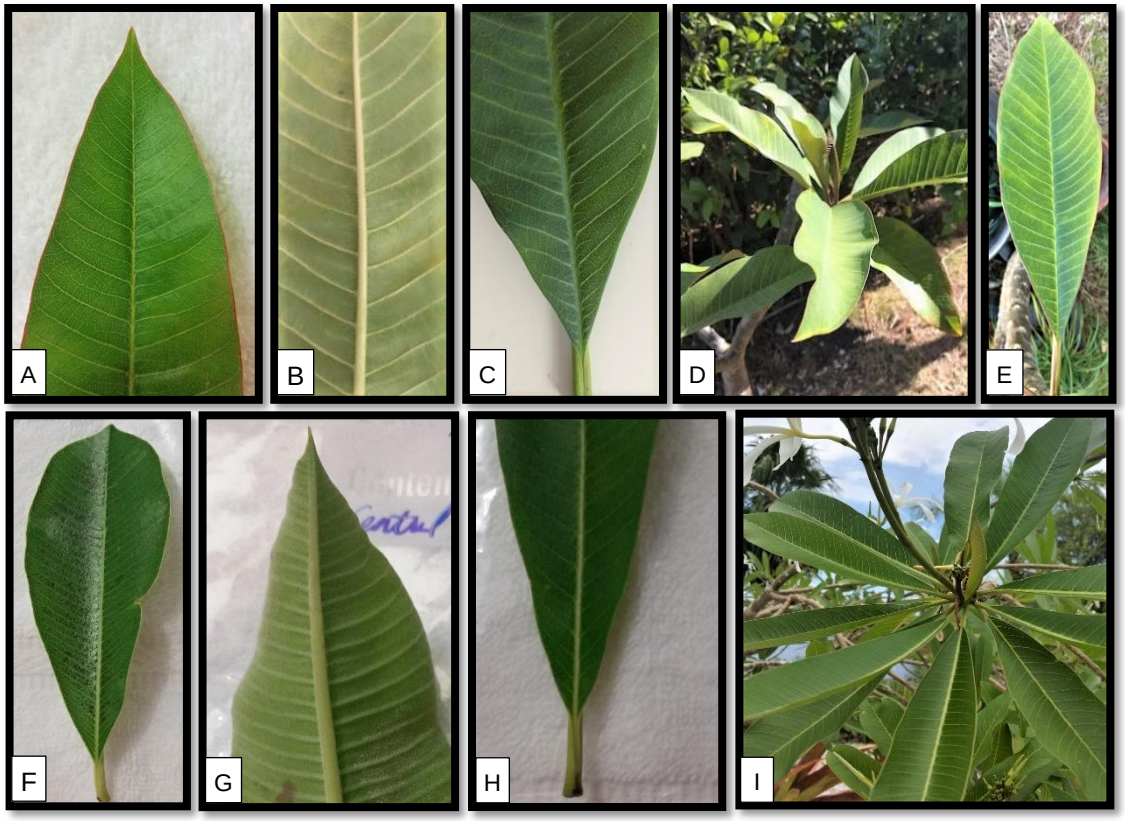


Figure 2.8. Morphological characters that distinguish *P. rubra* and *P. stenopetala*. A: Glabrous adaxial leaf surface and acuminate leaf tip of *P. rubra*. B: Glabrous abaxial leaf surfaces of *P. rubra*. C: Attenuate leaf base of *P. rubra*. D: Conduplicate leaf ptyxis of *P. rubra*. E: Oblanceolate leaf shape of *P. rubra*. F: Oblanceolate leaf shape of *P. stenopetala*. G: Acuminate leaf apex (abaxial view) of *P. stenopetala*. H: Attenuate leaf bases of *P. stenopetala*. I: Conduplicate leaf ptyxis of *P. stenopetala*. Photos by Kauahi Perez.

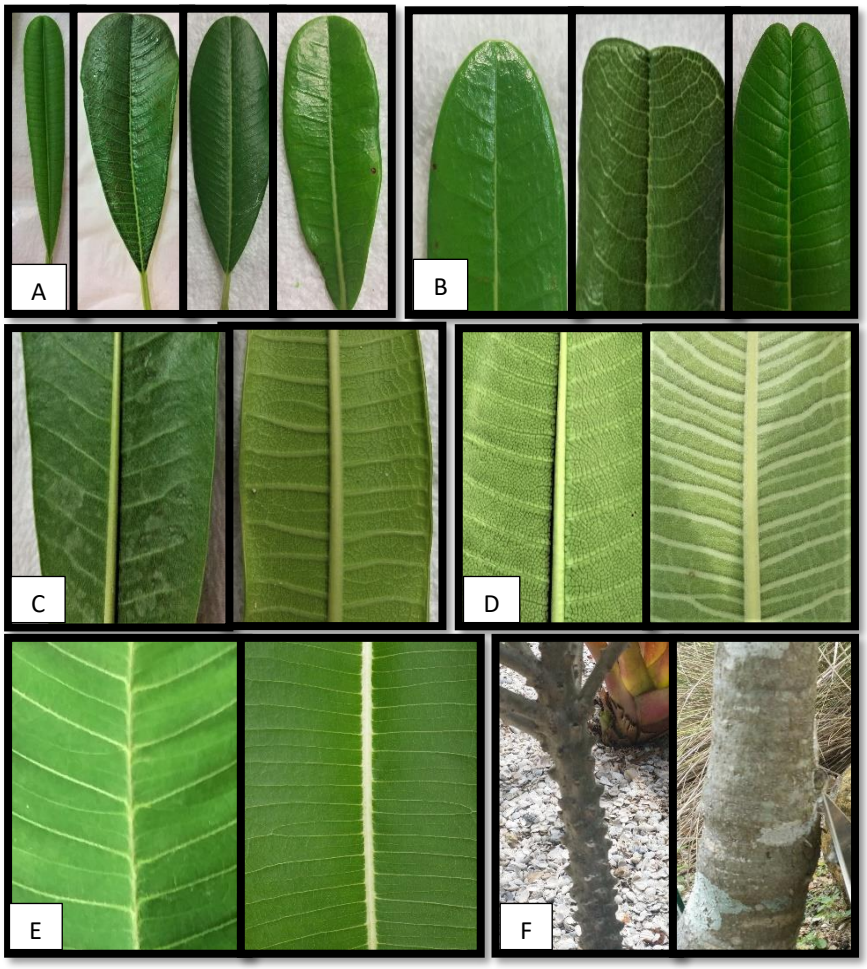


Figure 2.9. The diversity of morphological characters of taxa within the *P. obtusa* complex. A: Marked differences in leaf shape. B: Differences in leaf apices and surface textures. C: Angled and perpendicular secondary venation. D: Glabrous or coriaceous abaxial leaf surfaces. E: Decurrent or direct secondary venation entering the midrib. F: Specimens of *P. obtusa* showing trunks with and without tubercles. Photos by Kauahi Perez.

Utility and Limitations of Descriptive Foliar Characters

Overall, this study highlights the merits and limitations of using descriptive morphology to identify species of *Plumeria*. While these characters are not necessarily novel they do provide alternative means to evaluate vegetative characters as historically applied (Jacquin, 1763; De Candolle, 1844; Urban, 1898, 1902, 1920, 1924; Woodson, 1938a; Woodson et al., 1970).

Pink midveins and margins are novel characters included in this study as a result of including live specimens. These two characters, used in combination with other morphological characters, can be used to visually identify species in the field. Pink midveins and pink leaf margins, in addition to inconspicuous abaxial venation, flat leaf orientation, raised adaxial secondary venation, obtuse leaf tips, and cuneate leaf bases are characteristic features of *P. clusioides*. On the other hand, pink midveins and leaf margins in combination with acuminate leaf apices, pink midveins and margins, conduplicate leaf ptyxis, and subsessile leaf attachment visually characterize *P. subsessilis*, and allowed for the detection of a possibly misidentified specimen (Fig. 2.4 A).

Columnar growth habit is a character that distinguishes *P. pudica* from other species as only *P. pudica* showed this character. Given a complex of informative characters in analyses, *P. pudica* also remained separate from *P. caracasana*. Thus, this provides evidence that *P. caracasana* should not be considered a synonym of *P. pudica*. Instead, *P. caracasana* should be given its own status as a species.

Other characters that are valuable in the overall identification of *Plumeria* spp. include leaf attachment (subsessile or petiolate) and inconspicuous abaxial venation. Subsessile leaf attachment is a character that can be used to distinguish *P. pudica* and *P. subsessilis* from other species of *Plumeria*. Additionally, inconspicuous abaxial venation was a character that Grisebach et al. (1863) used to describe *P. obtusa*. However, this character is consistent in all specimens of *P. clusioides* that were observed, as well. As inconspicuous venation is known to occur in other species of this genus (De Candolle, 1844; Grisebach et al., 1863; Leon and Alain, 1957), it is recommended to use this character as a supplement in combination with more robust characters that define *P. clusioides*.

Other characters of use include sunken or raised secondary venation of both leaf surfaces. On one hand, raised venation on the upper surface of leaves is only present on *P. clusioides* and *P. stenophylla*. On the other hand, sunken venation was found on lower leaf surfaces of *P. clusioides*, *P. stenophylla*, and on some specimens of *P. cubensis* and *P. obtusa*. Hence, sunken or raised secondary venation on leaf surfaces should be used in combination with other characters. Similarly, decurrent secondary venation was used by Woodson to describe the way in which secondary venation entered the midrib in leaves of *P. subsessilis* and *P. rubra*. This character is consistent for both species. However, this character was also occasionally observed in leaves of *P. obtusa* and *P. stenopetala*. Thus, sunken or raised secondary venation can be used to identify taxa of *P. subsessilis* and *P. rubra*, but only in combination with other characters that are unique to either species.

Of questionable utility in distinguishing species, however, are the angle of the secondary veins. De Candolle (1844) and Urban (1898, 1925) report angles to describe the secondary venation in relation to the midveins, varying from acute to oblique. Perpendicular secondary venation was also noted in various descriptions of *Plumeria* (Grisebach, 1864; Britton, 1910, 1915; Britton and Millspaugh, 1920; Stahl, 1937). This character is most prominent in *P. alba*, as verified in this study. Beyond this one species, though, the reliability of either perpendicular or angled secondary venation is questionable because it is highly variable, especially when live specimens are examined.

Leaf orientation was another character of limited value. In live collections sampled, leaf orientation on some plants was planar—what is termed “flat leaf orientation” in this study. On other plants, leaves were in an orientation such that the upper surfaces on a leaf were facing each other. This character is known as conduplicate leaf ptyxis, and this was a novel character found in this study. This character is stable in *P. caracasana*, *P. pudica*, *P. stenopetala*, *P. stenophylla*, *P. subsessilis*, and *P. rubra*, which suggests that it is useful in diagnosing live specimens of these species. However, this was also found in live specimens of *P. obtusa*, which suggests that this is not a reliable character for diagnosing specimens of *P. obtusa*.

Presence of tubercles (cicatrices), formed from fallen leaves, was a character that De Candolle (1844) and Urban (1924) used to describe branches of various *Plumeria* specimens. In my observations, however, the presence of tubercles on branches is not a reliable character as multiple species possessed this character and this character was sometimes difficult to score. The occurrence of tubercles on the main trunk of certain accessions of *P. obtusa* was also noticed. However, this character was also difficult to score due to variability in presence or absence of tubercles even among the same named accessions. Therefore, it is not recommended to use either of these characters to diagnose live specimens.

Other characters that were also highly variable and difficult to reliably assess include leaf shape, leaf apices and bases, leaf margin characters, follicle occurrence, and arrangement of secondary venation along the midrib (alternate vs. opposite). The variability of these characters is at the heart of conflicting descriptions of many botanists who have attempted to accurately describe the species within *Plumeria* (Linné and Salvius, 1753; Jacquin, 1763; De Candolle, 1844; Urban, 1902; Britton and Millspaugh, 1920; Standley, 1924; Leon and Alain, 1957; Woodson et al., 1970).

Prospects

Studies have shown that a combination of quantitative and qualitative measurements on both vegetative and reproductive traits have proven useful at answering taxonomic questions in various plant groups (Lens et al., 2008; Smitha et al., 2018; Viera Barreto et al., 2018). Other characters that have been used to evaluate taxa within Apocynaceae include exocarp color and number of seeds per follicle (Alvarado-Cárdenas, 2007), pubescence on petioles and inflorescences (Woodson, 1938a), presence of extrafloral nectaries (Grisebach, 1864; Woodson and Moore, 1938), and aestivation or contortion of floral buds (Endress et al., 2007b; Livshultz et al., 2007). In addition, studies have shown that a combined approach using morphological data and molecular data helped in resolving species relationships (Ronblom and Anderberg, 2002; Endress et al., 2007b; Simões et al., 2010; Pettengill and Neel, 2011; Steele and Pires, 2011). Therefore, the inclusion of such characters and approaches to studies of *Plumeria* shows promise for verifying and delineating species boundaries.

CONCLUSION

Although not all the taxa included in this study could be verified, based on descriptive morphology, it has been shown that it is possible to recognize most species based solely on a combination of descriptive morphological characters that I feel are diagnostic of these species. Furthermore, though a sampling of species in this genus was not comprehensive, this study represents a modern study of the genus since its last taxonomic revision by Woodson. More work is needed to reach a definitive answer of how many species of *Plumeria* exist in the world today.

The hypothesis that a combination of characters is needed to distinguish a species has been verified, thereby substantiating the use of qualitative morphological characters for identifying distinct morphological species. An additional data set using quantitative measurements, including image analyses, and molecular characters would make a nice accompaniment to this existing data set. This would provide more evidence as to how the species boundaries of *Plumeria* are defined and enhance our understanding of this genus.

ACKNOWLEDGEMENTS

I would like to thank Dr. Chad Husby (Fairchild Tropical Botanic Garden), Dr. Alan Meerow (Miami USDA), Tex Norwood and Carol Vannoorbeeck (Florida Colors Nursery), Hetty Ford (Naples Botanical Garden), Donald Hodel (University of California), Carlo Moricci (The Palmetum of Santa Cruz de Tenerife), and Mike Ferrero for providing us with plant material for this study. This study on *Plumeria* has been supported by research grants from the Southern California Plumeria Society, the Plumeria Society of America, UH Manoa Graduate Student Organization, and private funding.

CHAPTER 3

EVALUATING NUCLEAR AND PLASTID REGIONS TO DELIMIT *PLUMERIA* SPECIES

ABSTRACT

The genus *Plumeria* is comprised of taxa known for their cultural, ornamental, cosmetic, and ethnomedicinal uses. However, disagreement among collectors and taxonomists over species designations, combined with lack of unambiguous morphological descriptors or molecular markers, has made it difficult to identify species. Therefore, five molecular regions (*ITS2*, partial *matK*, *psbJ-petA*, *trnH-psbA*, and *rpl32-trnL*) were evaluated to determine their efficacy in identifying groups of taxa as true species within this genus. Maximum likelihood (ML) and Bayesian inference (BI) methods were employed on separate and combined molecular data to determine the phylogenetic utility and species discriminatory abilities of these markers. Molecular analyses revealed that *rpl32-trnL* provided the best phylogenetic signal and ability to discriminate species but was still not able to identify all the species that were tested. In addition, the *rpl32-trnL* and *trnH-psbA* regions contained indels that were unique to different species, and suitable for distinguishing most *Plumeria* species. Concatenating *rpl32-trnL* with *ITS2*, *trnH-psbA*, and *psbJ-petA* enhanced phylogenetic signal resulting in a well resolved tree topology. Although some of the molecular regions can be used as markers to distinguish certain *Plumeria* species, no single marker can distinguish all species, and further study may reveal other molecular regions with greater resolving power for phylogenetic studies of this genus. Furthermore, the use of quantitative morphological, reproductive, and perhaps anatomical characters will likely enhance proper diagnosis of species. In conclusion, the molecular markers used in this study can identify the majority of distinct species.

INTRODUCTION

The family Apocynaceae (dogbane family) is one of the largest angiosperm families and is comprised of over 5,500 species within 410 genera (The Plant List, 2013; Fishbein et al., 2018). Genera are grouped within one of five subfamilies, 25 tribes, and 49 subtribes (Endress et al., 2014). Most taxa within Apocynaceae are distributed throughout the tropics with a few found in temperate regions (Endress and Bruyns, 2000; Sennblad and Bremer, 2002; Endress et al., 2007a). Although members of this family produce poisonous cardiac glycosides and various alkaloids, many are used for medicinal purposes (Staples and Herbst, 2005; Judd et al., 2008). Furthermore, many members of this family find use as widespread ornamentals, including *Adenium* (desert rose), *Alyxia* (maile), *Asclepias* (milkweed), *Carissa* (Natal plum), *Nerium* (oleander), *Vinca* (periwinkle), and *Plumeria* (frangipani) (Judd et al., 2008).

The genus *Plumeria* (syn. *Plumiera*, *Plumieria*) (Woodson, 1938a) belongs to the subfamily Rauvolfioideae (Britton, 1915; Simões et al., 2007) and is comprised of taxa known for their cultural, ornamental, cosmetic, and ethnomedicinal uses. They are morphologically distinguishable from other Rauvolfioids by thick, succulent branchlets with pronounced leaf scars, spiral to alternate phyllotaxis, waxy and salverform or infundibuliform corollas with narrow bases and sinistrorse aestivation, stamens deeply included and adnate to the corolla tube, subinferior ovaries that are bicarpellate and apocarpous, bifollicular dehiscent fruit that are basally united, and basally winged seeds with a thin endosperm (Woodson, 1938a; Woodson et al., 1970; Leeuwenberg, 1994). However, there is no accurate count of the number of species in this genus due to ambiguous species diagnoses and disagreement among specialists in the field.

Although 12 species are currently recognized in the genus (The Plant List, 2013), disagreements exist among authors regarding legitimacy of these species (Table 3.1; see also Appendix F for additional references therein). For instance, Acevedo-Rodríguez and Strong (2012) recognize *P. clusioides* Griseb. as a legitimate species, whereas Govaerts et al. (2003) do not. These disagreements are further compounded by the many disparities in species descriptions within numerous sources of literature, especially in the treatment of synonymous names for *Plumeria* spp. (Urban, 1898, 1902; Britton, 1910; Johnston, 1912; Britton, 1915; Britton and Millspaugh, 1920; Urban, 1920;

Hollick, 1922; Britton, 1923; Urban, 1924; Woodson, 1938a; Woodson et al., 1970; Williams, 1996), many of which are misused or unconfirmed among collectors (Criley, 2009). The poor state of knowledge of species delineations and a need for a clear taxonomy are the impetus for this study.

To date the only taxonomic treatment of the genus comes from Woodson (1938a), and is based solely on his morphological analyses of *exsiccatae* from American and European herbaria. Woodson recognized seven species and several botanical varieties, primarily based on floral shape and secondarily on leaf characters. He synthesized the prior work by Grisebach et al. (1863), Grisebach (1864, 1866), Urban (1898, 1902, 1920, 1924), Britton (1910, 1915, 1923), Britton and Millspaugh (1920), Johnston (1912), and Hollick (1922), in which numerous species were described primarily on the basis of leaf characters. Many previously described species (i.e., *P. bahamensis*, *P. clusioides*, *P. cubensis*) were placed under one of two varieties of *P. obtusa*—var. *typica* or var. *sericifolia*. Woodson's justification for combining these previously described species into a "*P. obtusa* complex" was due to the difficulty of further separating these taxa from each other and the type species, arguing that even the morphological variability of specimens representing *P. obtusa* rendered the use of leaf characters alone as inadequate. In addition, he sank other morphologically distinct taxa such as *P. caracasana*, *P. stenophylla*, and *P. stenopetala* within the species *P. pudica*, *P. filifolia*, and *P. rubra* x *P. subsessilis*, respectively. A serious limitation of Woodson's work was that he was not able to view live specimens which can take on a dramatically different appearance compared to counterparts in herbaria.

The Search for Informative Molecular Characters

Using morphological characters to identify *Plumeria* spp. is difficult as evidenced by the disagreements in prior treatments as described above. This can be compounded by variability at the individual plant level due to a number of factors such as phenotypic plasticity, genetic variation, somatic mutations, and genotype-by-environment interactions (Schlichting, 1986). Furthermore, seedlings of *Plumeria* spp. can be so morphologically variable that reliable species recognition is impossible.

Table 3.1. Taxonomic status and natural geographic distribution data of *Plumeria* taxa, as recognized by the World Checklist of Selected Plant Families (WCSP) and other sources of literature. Column one is considered the synonym or the invalid name while the name in column three is the accepted name in the literature you surveyed (column four).

Taxon	Reference	Accepted By	Not Accepted By	Natural Distribution
P. pudica Jacq.	Enum. Syst. Pl.: 13 (1760)	Woodson, R.E. (1938a) Govaerts, R. (2003) Acevedo-Rodríguez, P. & M.T. Strong (2012)		Panama to N. Venezuela
P. caracasana J.R. Johnst.	Contr. U.S. Natl. Herb. 12: 108 (1908)	synonym of *P. pudica.*	Woodson, R.E. (1938a) Govaerts, R. (2003)	Venezuela (Caracas to La Guaira)
P. alba L.	Sp. Pl.: 209 (1753)	Woodson, R.E. (1938a) Govaerts, R. (2003) Acevedo-Rodríguez, P. & M.T. Strong (2012)		Puerto Rico to Windward Is.
P. obtusa L.	Sp. Pl.: 210 (1753)	Woodson, R.E. (1938a) Govaerts, R. (2003) Acevedo-Rodríguez, P. & M.T. Strong (2012)		Florida Keys, Caribbean, SE. Mexico to Guatemala
P. bahamensis Urb.	Symb. Antill. 1: 387 (1899)	synonym of *P. obtusa.*	Woodson, R.E. (1938a) Govaerts, R. (2003)	Bahamas Acklins Island
P. clusioides Griseb.	Cat. Pl. Cub.: 171 (1866)	Acevedo-Rodríguez, P. & M.T. Strong (2012)	Woodson, R.E. (1938a) Govaerts, R. (2003)	Cuba
P. cubensis Urb.	Repert. Spec. Nov. Regni Veg. 21: 2019 (1925)	Acevedo-Rodríguez, P. & M.T. Strong (2012)	Woodson, R.E. (1938a) Govaerts, R. (2003)	Cuba

Table 3.1. (Continued) Taxonomic status and natural geographic distribution data among *Plumeria* taxa, as recognized by the World Checklist of Selected Plant Families (WCSP) and other sources of literature.

Taxon	Reference	Accepted By	Not Accepted By	Natural Distribution
P. ekmanii Urb.	Symb. Antill. 9: 239 (1924)	Acevedo-Rodríguez, P. & M.T. Strong (2012)	Woodson, R.E. (1938a) Govaerts, R. (2003)	Cuba
P. filifolia Griseb.	Pl. Wright. 2: 519 (1862)	Woodson, R.E. (1938a) Govaerts, R. (2003) Acevedo-Rodríguez, P. & M.T. Strong (2012)		E. Cuba
P. montana Britton & P. Wilson	Bull. Torrey Bot. Club 50: 46 (1923)	Acevedo-Rodríguez, P. & M.T. Strong (2012)	Woodson, R.E. (1938a) Govaerts, R. (2003)	Cuba
P. rubra L.	Sp. Pl.: 209 (1753)	Woodson, R.E. (1938a) Govaerts, R. (2003) Acevedo-Rodríguez, P. & M.T. Strong (2012)		Mexico to Venezuela
P. *stenophylla* Urb.	Symb. Antill. 9: 237 (1924)	synonym of *P. filifolia.*	Woodson, R.E. (1938a) Govaerts, R. (2003) Acevedo-Rodríguez, P. & M.T. Strong (2012)	Cuban (Palmarito de Cauto)
P. tuberculata G. Lodd.	Bot. Cab. 7: t. 681 (1823)	Woodson, R.E. (1938a) Acevedo-Rodríguez, P. & M.T. Strong (2012)	Govaerts, R. (2003)	Bahamas to Hispaniola
P. subsessilis A.DC.	Prodr. 8: 393 (1844)	Woodson, R.E. (1938a) Govaerts, R. (2003) Acevedo-Rodríguez, P. & M.T. Strong (2012)		Hispaniola
P. x *stenopetala* Urb.* (*P. obtusa* x *P. subsessilis*)	Symb. Antill. 3: 335 (1902)	Woodson, R.E. (1938a) Govaerts, R. (2003) Acevedo-Rodríguez, P. & M.T. Strong (2012)		Hispaniola

*Named as *P. stenopetala* in this study.

Additionally, since many clones are relatively infertile when selfed or crossed in Hawaii, hybrids may be formed rendering species barriers unclear at best.

While morphology is difficult to use in identifying *Plumeria* species molecular markers show promise and have been the popular tool of choice more recently for identifying plant species, characterizing germplasm, and answering various evolutionary and ecological questions (Meerow, 2005; Keeley et al., 2007; Korotkova et al., 2011; Cantley et al., 2014; Yang et al., 2019). Such markers can also aid in the exchange of germplasm material at local, national, and international botanical gardens, authenticating specimens, identifying duplicates, and uncovering cryptic species (Ford-Lloyd, 2001; Bickford et al., 2007). However, identifying suitable molecular gene and non-coding regions is requisite before any of these applications can occur.

Recent studies have shed light on the phylogenetic utility of different coding and noncoding chloroplast DNA (cpDNA) and nuclear DNA (nrDNA) regions at various taxonomic levels (Sennblad and Bremer, 1996; Shaw et al., 2005; Shaw et al., 2007; Simões et al., 2007; Fishbein et al., 2011; Qiu et al., 2013). Chloroplast intergenic spacer regions, namely *psbJ-petA* and *rpl32-trnL*, consistently appear to have "potentially informative characters" that may be useful for low-level taxonomic studies (Shaw et al., 2007; Shaw et al., 2014). The *matK* region has also proven useful at resolving phylogenetic relationships among various plant groups (Soltis et al., 2001; Hilu et al., 2003; Qiu et al., 2013), including within the Apocynaceae (Endress et al., 2007b; Livshultz et al., 2007; Simões et al., 2007).

The most popular regions for distinguishing among taxa are *rbcL*, *matK*, and *trnH-psbA* of the chloroplast and the nuclear internal transcribed spacer (*ITS*) (Fišer Pečnikar and Buzan, 2014). The *matK* region has received mixed reviews as a molecular marker to identify taxa within plant groups because it contains informative nucleotide regions in certain plant groups, but not in others (CBOL Plant Working Group, 2009; Wong et al., 2013; Ma et al., 2014; Hosein et al., 2017). In Apocynaceae, Mahadani et al. (2013), Tripathi et al. (2013), and Cabelin and Alejandro (2016) reported that *matK* could discriminate among species, but Selvaraj et al. (2015) have found otherwise.

For some groups, on the other hand, *trnH-psbA* has been shown to have high discriminatory power (Tripathi et al., 2013; Wu et al., 2019; Yang et al., 2019), whereas studies in other angiosperm groups show that it does not (Kim et al., 1999; Selvaraj et al., 2015; Cabelin and Alejandro, 2016). Of all the most commonly used regions the nuclear non-coding *ITS* region appears to be the most effective in identifying genera and species when used on its own and in conjunction with other molecular regions (Cheng et al., 2016; Liu et al., 2016; Wu et al., 2019). Additionally, it was found that using only partial regions of the *matK* gene (Sivalingam et al., 2016) plus the *ITS* [*ITS2*] (Chen et al., 2010; Yao et al., 2010; Pang et al., 2012) allowed successful identification of a number of plant species. Given the disparities in effectiveness among these regions, I chose to evaluate the following regions for their ability to distinguish *Plumeria* species and their utility in constructing a phylogeny: partial *matK*, intergenic spacer regions *trnH-psbA*, *psbJ-petA*, *rpl32-trnL*, and nuclear *ITS2*.

There is a distinction between recognizing species by particular gene/spacer regions (commonly referred to as DNA barcoding) and phylogenetic analyses. Whereas DNA barcoding aims to identify species using relatively short segments of DNA (Fazekas et al., 2012), phylogenetic analyses aim to understand and resolve evolutionary relationships between and among taxa at various taxonomic levels (Judd et al., 2008). Korotkova et al. (2011) have shown that the ability of a marker to discriminate species does not necessarily correlate with phylogenetic utility. Conversely, although a molecular marker may not identify closely related species, it may still provide phylogenetic insight toward an understanding of *Plumeria* taxonomy.

The taxonomic problem with distinguishing species in the genus *Plumeria* is two-fold. First, it is difficult to determine species boundaries based on morphology because of overlapping morphological character states and the differences inherent in the appearance of dried and fresh material of individual species (Fig. 3.1), and it is not clear how more recent taxonomists delineate these species. Second, molecular characterization of *Plumeria* spp. is hampered by limited sampling of species and scarcity of comparable DNA sequences in public databases coupled with the fact that different DNA regions were surveyed.

Figure 3.1. Leaf morphologies of *Plumeria* accessions from the University of Hawaii Waimanalo Research Station. Accessions used in this study are indicated in boldface. Accession names and location (row number-plot number) are as follows: Left column— ***P. stenophylla*** (1-28), ***P. bahamensis*** (1-17), ***P. alba*** (1-30), ***P. pudica*** (10-14), *P. stenophylla* 'Cuba' (1-26), ***P. caracasana*** (9-11), ***P.* sp. 'Isabella'** (1-21), ***P. stenophylla*** (1-27), ***P. bahamensis*** (10-21), *P. cubensis* (1-25). Middle column— ***P. rubra* 'Pedasi'** (10-20), ***P. obtusa*** (1-11), *P.* sp. (1-16), *P. obtusa* (1-24), ***P. alba*** (1-1), ***P. obtusa*** (1-2), ***P. obtusa* var. *obtusa*** (1-5). Right column— *P.* sp. (10-24), *P.* sp. seedling from Yucatan (1-19), *P. obtusa* (1-15), Narrow leaf *P.* sp. from Cuba (1-20), ***P. stenopetala*** (1-10), ***P. stenopetala*** (1-9), ***P. stenopetala*** (1-8), ***P. stenopetala*** (1-7), *P. stenophylla* (1-22), ***P. montana*** (1-29).

This makes direct sequence comparisons among taxa virtually impossible (Table 3.2). Tripathi et al. (2013) reported similar limitations in that barcode loci for their taxonomic groups in India were poorly represented in popular databases, such as the Barcode of Life Database (BOLD) and National Center for Biotechnology Information (NCBI). Clearly, an enhanced sampling of taxa and use of the same molecular regions for comparisons will be necessary to elucidate species limits and phylogenetic relationships of members in *Plumeria*.

Table 3.2. Publicly available *Plumeria* sequences within BOLD and NCBI Databases (Ratnasingham and Hebert, 2007; Benson et al., 2016).

Database	Taxon	Molecular Regions
BOLD Systems v4	*P. alba*	*matK*, *rbcL*, *trnH-psbA*
	P. cubensis	*matK*, *rbcL*
	P. inodora	*rbcL*
	P. obtusa	*matK*, *rbcL*, *trnH-psbA*
	P. pudica	*rbcL*
	P. rubra	*matK*, *rbcL*, *rpoC1*, *ITS2*
NCBI GenBank Database	*P. alba*	*trnL*, *trnL-trnF*, *ITS1*, 5.8S, *ITS2*, *matK*, *rbcL*, 5.8S, 18S, 28S, *trnH-psbA*
	P. cubensis	*trnL*, *trnL-trnF*, *rbcL*, *rpl16*, *rps16*, *trnK*, *matK*
	P. inodora	*rbcL*
	P. obtusa	atpB, *trnL-trnF*, *trnL* intron, *matK*, *rbcL*, *trnH-psbA*
	P. pudica	*matK*, *rbcL*, 16S, *hrcR*, *hrcT*, *ITS1*, 5.8S, *ITS2*
	P. rubra	*ITS1*, 5.8S, *ITS2*, *CytP450*, *rbcL*, *matK*, *rpoC1*, *trnL-trnF*, *trnH-psbA*

Therefore, the objective of this study is to evaluate DNA sequences for their effectiveness in distinguishing *Plumeria* taxa and for their phylogenetic utility. The aim is to answer the following questions: 1) What individual molecular markers (chloroplast and/or nuclear) can be used to distinguish *Plumeria* species? 2) Does some combination of chloroplast and/or nuclear DNA markers better distinguish among taxa? 3) Are these regions phylogenetically informative? It is hypothesized that no single region will be sufficient to distinguish species, but rather a combination of molecular regions will be required. Further, some of these molecular regions may be useful when applied in a phylogenetic context.

MATERIALS AND METHODS

Sample Collection, DNA Extraction, and Purification

A total of 92 taxa representing accepted and synonymized *Plumeria* spp. were sampled from collections at two sites in Hawaii (University of Hawaii Waimanalo Research Station on O'ahu, National Tropical Botanical Garden [PTBG] on Kaua'i), and four sites in Florida (Florida Colors Nursery, Naples Botanical Garden, Miami USDA-ARS-SHRS National Germplasm Repository, and Fairchild Tropical Botanic Garden). Total genomic DNA was extracted from fresh or silica gel-dried leaf materials (Chase and Hills, 1991) using Isolate II Plant DNA Kits (Bioline, Taunton, Massachusetts, USA). Voucher and accession data are given in Appendix D. For material from which DNA was difficult to extract, a CTAB protocol (Doyle and Doyle, 1987), DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA), or E.Z.N.A. HP Plant DNA Mini Kit (Omega Bio-tek, Norcross, Georgia, USA) was used following the manufacturers' protocols but with extended incubation time at 65°C for 70 minutes. DNA quality was verified by gel electrophoresis and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). For some samples that yielded poor quality genomic DNA, due to degraded leaf tissues, the Isolate Fecal DNA Kit (Bioline) was used following the manufacturer's protocol. In cases where the final DNA elution step yielded opaque or light-brown solutions, additional purification using the QIAquick PCR Purification Kit (Qiagen) or Monarch PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, Massachusetts, USA) was used.

Outgroup taxa included those that were basal to *Plumeria* but still within the subfamily Rauvolfioideae *sensu lato* Simões et al. (2007). Accession data for outgroup taxa that were sequenced in this study and GenBank accession numbers for sequences that were downloaded are also given in Appendix D.

DNA Amplification, Sequencing, and Alignment

Molecular regions were amplified via polymerase chain reaction (PCR) in 25-µL reaction volumes containing 12.5 µL of MyTaq 2X Red Master Mix (Bioline, Taunton, Massachusetts, USA), 9.5 µL of nuclease-free water, 1.0 µL of 10 µM forward or

reverse primer, and 1-2.5 µL of template DNA. DNA templates were not standardized to specific concentrations prior to PCR, so the amount of template DNA was adjusted, as necessary, to generate enough PCR products for sequencing.

Primer sequences for *rpl32-trnL* and *psbJ-petA* regions were adapted from Shaw et al. (2007). Primers for PCR and sequencing of *trnH-psbA*, partial *matK*, and nuclear *ITS2* regions were designed using the PrimerQuest Tool (Integrated DNA Technologies, www.idtdna.com) on primer design templates consisting of *Plumeria* sequences available on GenBank (Clark et al., 2016). Primer sequences and respective PCR protocols are given in Table 3.3. Amplifications were carried out on an iCycler v.4.006 (BIO-RAD, Hercules, California, USA). PCR products were verified via gel electrophoresis to confirm successful PCR reactions.

Unincorporated dNTPs and primers were removed from PCR products using ExoSap-IT (Applied Biosystems, Foster City, California, USA) following the manufacturer's protocol. Sequencing reactions of 3.0 µL of purified PCR product, 2µL (each) of 1.6 µM forward- or reverse-primer, and 2.0 µL nuclease-free water were then submitted to the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) Lab at the University of Hawaii at Mānoa campus for bidirectional Sanger sequencing using BigDye terminator chemistry on an Applied Biosystems 3730XL DNA Analyzer.

Sequences were checked and edited manually as needed by comparing forward and reverse electropherograms, and contigs were assembled using Geneious Prime v.1.1 (Biomatters, Auckland, NZ). Multiple sequence alignments for each region were done using the Geneious Alignment algorithm as implemented in Geneious Prime and were also scanned by eye to manually exclude regions of ambiguity. Review of alignments from individual regions resulted in the identification of potentially diagnostic (polymorphic) characters (Appendix E). These were scored as transition, transversion, insertion, and deletion (gap) characters. Summary statistics were calculated for each region in MEGA X (Knyaz et al., 2018).

Table 3.3. Forward (F) and reverse (R) primer sequences of nuclear and chloroplast regions tested and their associated PCR protocols.

Region	Forward and reverse sequence
ITS2	***ITS2*-F**: 5' – TTG CGC CCA AAG CCA TTA – 3'
	***ITS2*-R**: 5' – GCT TAA ACT CAG CGG GTA GTC – 3'
matK	***matK*-F**: 5' – CTT CGG AAG AAC GTA AAG – 3'
	***matK*-R**: 5' – CAC AAG AAA GTC GAA GTA T – 3'
rpl32-trnL	**rpl32-F**: 5' – CAG TTC CAA AAA AAC GTA CTT C – 3'
(Shaw et al., 2007)	***trnL*^{UAG}-R**: 5' – CTG CTT CCT AAG AGC AGC GT – 3'
psbJ-petA	**psbJ-F**: 5' – ATA GGT ACT GTA RCY GGT ATT – 3'
(Shaw et al., 2007)	**petA-R**: 5' – AAC ART TYG ARA AGG TTC AAT T – 3'
trnH-psbA	**trnH-F**: 5' – CTG CTG TAG AAG CTC CAT CTA TC – 3'
	psbA-R: 5' – CCT TGA TCC ACT TGG CTA CAT – 3'
	PCR Protocol
	Initial denaturation at 95°C for 5 min.
ITS2	30 cycles of: 95°C, 30 sec; 58°C, 30 sec; 72°C 30 sec
matK	35 cycles of: 95°C, 45 sec; 48°C, 45 sec; 72°C 45 sec
trnH-psbA	30 cycles of: 95°C, 30 sec; 56°C, 30 sec; 72°C 30 sec
psbJ-petA	35 cycles of: 95°C, 45 sec; 55°C, 45 sec; 72°C 45 sec
rpl32-trnL	35 cycles of: 95°C, 45 sec; 58°C, 30 sec; 72°C 30 sec
	Final extension at 72°C for 10 min.

Maximum Likelihood and Bayesian Analyses

The resulting alignments were analyzed using phylogenetics programs available on the CIPRES Science Gateway v.3.3 (Miller et al., 2010). Evolutionary models and parameters were selected based on the Bayesian Information Criterion (BIC) in jModelTest2 on XSEDE 2.1.6 (Darriba et al., 2012). PartitionFinder2 on XSEDE 2.1.1 (Guindon et al., 2010; Lanfear et al., 2012; Lanfear et al., 2016) was used to determine if a partitioning scheme was also needed within individual regions. The best-fit models as determined by jModelTest2 for separate and combined (partitioned) data sets were: *ITS2*, HKY+G; *matK*, TPM1uf+G; *rpl32-trnL*, TVM+G; *trnH-psbA*, F81+G; *psbJ-petA*, TPM1uf+G. Molecular regions were analyzed separately and in combination (concatenated), if individual regions yielded similar tree topologies. Loci that did not yield similar tree topologies were excluded from the concatenated data set.

Maximum Likelihood (ML) analyses were conducted using RaxML-HPC2 v.8 on XSEDE (Stamatakis, 2014), applying models indicated above to corresponding regions. Robustness of nodes was assessed by non-parametric bootstrapping with 1,000 replications. Branches with bootstrap support (BS) values ≥ 95% were considered highly supported, ≥ 70% as well-supported, and anything less than that was considered moderately to less supported. ML trees were imported into MEGA X and consensus trees were constructed. Branch support values less than 50% were considered to have poor support for a given relationship between taxa and were collapsed at their corresponding nodes.

Bayesian Inference (BI) analyses were performed using MrBayes on XSEDE (3.2.6) (Ronquist et al., 2012). Analyses consisted of two independent runs using the Markov Chain Monte Carlo analysis with four chains sampled every 100 generations. The first 25% of trees were discarded as burn-in, each analysis running for ten million generations. Excluding burn-in, all trees from independent runs were saved to construct 50% majority-rule consensus trees. Branches with posterior probabilities (PP) ≥ 0.95 were considered strongly supported, ≥ 0.70 as well-supported, and anything less than that was considered less supported. Trees were first imported to iTOL (Letunic and Bork, 2016), converted to Newick format, and imported into MEGA X where branches supported by posterior probabilities less than 0.50 were collapsed at their nodes.

DNA Barcoding Gap Analysis

As a method of assessing the value of individual regions as DNA barcodes to identify species of *Plumeria*, regions were analyzed for barcoding gaps. Uncorrected p-distances were calculated using the partial deletion option in MEGA X, which then allowed us to find maximum intraspecific and minimum interspecific (nearest neighbour) sequence divergences to determine the presence of a barcoding gap (Meier et al., 2008; Srivathsan and Meier, 2012). Discontinuity between levels of intra- and interspecific p-distances were indicative of a barcode gap.

Operational Taxonomic Units as Species Clusters

The concept of operational taxonomic units (OTUs) is used in this study as a basis for calculations of intraspecific and interspecific variability and species identification potential of markers since species limits within *Plumeria* are unclear. There are several taxa that have been described as species but have later been regarded as a synonym or reduced to a botanical variety or vice versa. Consequently, we did not assume that all presently accepted species names reflect true species. Hence, clusters of taxa that are identified in analyses are referred to as OTUs.

Limitations

All samples were collected from specimens within botanical gardens, the Plumeria collection at the University of Hawaii Waimanalo Experiment Station, or from nurseries. Thus, there may be some concern from reviewers because none of the specimens were collected from the wild. However, at some point in time these specimens were still collected from the wild and may currently be harder to collect from the wild. Additionally, although most samples were not vouchered, many are still maintained at their respective institutions.

RESULTS

Sequence Characteristics

Summary statistics for each region are shown in Table 3.4. Sequences of the *ITS2*, *psbJ-petA*, and *matK* regions were straightforward and contained relatively few gaps. The *trnH-psbA* region contained short ambiguous alignments and mononucleotide repeats of varying lengths among the same taxa, rendering it difficult to align. This is a commonly reported problem in the DNA barcoding literature for this region, but is circumvented by excluding these nucleotides prior to analysis (Fazekas et al., 2012). The *rpl32-trnL* region was the longest and most problematic region to align, sometimes requiring re-sequencing of samples. Multiple alignment algorithms (Geneious, MUSCLE, ClustalW, MAFFT) were compared to resolve ambiguous regions. There was also a low percentage of missing data for individual regions, mostly for the outgroup taxa that were downloaded from GenBank, when they were concatenated with other regions for which sequences for a region were not available. Sampling of the *matK* region was also discontinued prior to formal ML and BI analyses because there was little sequence variation in existing samples, resulting in only 68 samples being analyzed.

The most useful diagnostic characters were found in the *trnH-psbA* and *rpl32-trnL* regions (Appendix E). For these regions, some characters were unique to a species, whereas other characters were found to be shared among only a few species. The polymorphic molecular characters identified from these regions were adequate and sufficient to distinguish at least 8 OTUs of *Plumeria*.

DNA Barcode Gap Analysis

To determine if a barcoding gap was present, the maximum intraspecific (within species) sequence divergence was compared to the minimum interspecific (between species) sequence divergence for each region (Table 3.5). A barcode gap was considered to exist if the maximum sequence variation within an OTU was less than the minimum variation between OTUs. Among all five regions, maximum intraspecific sequence divergences ranged from 0.00000 – 0.04846, while minimum interspecific sequence divergences ranged from 0.00000 – 0.030370 among all OTUs. Although

barcode gaps existed within each region, as indicated in boldface (Table 3.5), these were not found among all OTUs. *TrnH-psbA* and *rpl32-trnL* were the regions within which the most informative barcoding gaps were found, rendering these regions potentially useful as barcodes for *Plumeria*. Even a comparison of average maximum intraspecific and minimum interspecific sequence divergences for each region showed that the *ITS2*, *matK*, and *psbJ-petA* regions had more variation within OTUs as compared to the variation between them, indicating limited utility for determining species (Fig. 3.2). In contrast, the *trnH-psbA* and *rpl32-trnL* regions had less variation within each OTU as compared to the variation between them, again highlighting their potential to identify *Plumeria* OTUs.

Table 3.4. Summary statistics of individual and combined molecular regions of *Plumeria* taxa. Numbers represent base pairs, unless indicated as a percent (%). The number of conserved, variable, and singleton sites correspond to base positions in alignments of each region.

Parameter	***ITS2***	***matK***	***trnH-psbA***	***psbJ-petA***	***rpl32-trnL***	***Combined***
Range of sequence length	208 – 316	829 – 914	362 – 393	919 – 935	839 – 1,006	1,442 – 2,542
Average length (excluding gaps)	230	829	374	927	987	2,425
Aligned length (gaps included)	232	836	422	942	1,007	2,603
No. of conserved sites	199 (86%)	816 (98%)	388 (92%)	922 (98%)	965 (96%)	2479 (89%)
No. of variable sites	33 (14%)	14 (2%)	27 (6%)	20 (2%)	41 (4%)	116 (4%)
No. of singleton sites	3 (1%)	0 (0%)	4 (1%)	4 (0.4%)	8 (1%)	19 (1%)
No. of taxa sampled	93	64	90	86	83	82

Table 3.5. Maximum intraspecific (Within) and minimum interspecific (Between) sequence divergences (p-distances) of molecular regions analyzed. Numbers in boldface denote instances wherein the maximum intraspecific sequence variation is less than the minimum interspecific sequence variation.

Taxon	***ITS2***		***matK***		***trnH-psbA***		***psbJ-petA***		***rpl32-trnL***	
	Within	Between	Within	Between	Within	Between	Within	Between	Within	Between
P. alba	**0.00000**	**0.03070**	0.00000	0.00000	**0.00000**	**0.00541**	0.00109	0.00109	**0.00200**	**0.00699**
P. bahamensis	0.00441	0.00000	0.00000	0.00000	0.00000	0.00000	0.00108	0.00000	0.00407	0.00000
P. caracasana	0.00439	0.00000	**0.00000**	**0.00241**	**0.00000**	**0.00831**	0.00324	0.00000	**0.00000**	**0.00500**
P. clusioides	0.00000	0.00000	0.00000	0.00000	0.00261	0.00000	0.00000	0.00000	0.00000	0.00000
P. cubensis	0.02203	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00100	0.00000
P. ekmanii	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
P. filifolia	0.00881	0.00000	0.00000	0.00000	0.00000	0.00000	0.00108	0.00000	0.00000	0.00000
P. sp. 'Isabella'	0.00439	0.00000	**0.00000**	**0.00120**	**0.00000**	**0.00806**	**0.00000**	**0.00108**	**0.00000**	**0.00799**
P. montana	0.00000	0.00000	0.00000	0.00000	0.00538	0.00000	0.00000	0.00000	0.00000	0.00000
P. obtusa	0.04846	0.00000	0.00241	0.00000	0.01639	0.00000	0.00217	0.00000	0.00908	0.00000
P. pudica	0.00000	0.00000	**0.00000**	**0.00241**	**0.00265**	**0.00831**	0.00216	0.00000	**0.00000**	**0.00500**
P. rubra	0.01754	0.01316	0.00483	0.00000	**0.00000**	**0.00850**	0.00856	0.00000	**0.00800**	**0.00813**
P. obtusa var. *sericifolia*	0.00441	0.00441	0.00121	0.00120	**0.00000**	**0.00272**	0.00216	0.00000	**0.00000**	**0.00476**
P. stenopetala	0.01310	0.00881	0.00483	0.00000	0.01344	0.00000	0.00324	0.00000	0.00476	0.00000
P. stenophylla	0.02203	0.00441	0.00241	0.00000	0.00543	0.00538	0.00539	0.00000	0.00238	0.00000
*P. subsessilis**	**0.00000**	**0.00873**	N/A	N/A	0.00000	0.00000	N/A	N/A	0.00000	0.00000

**P. subsessilis* samples were either deliberately omitted from PCR sampling, as is the case for the *matK* region, or failed to yield PCR product, as is the case for the *psbJ-petA* region due to low quality DNA.

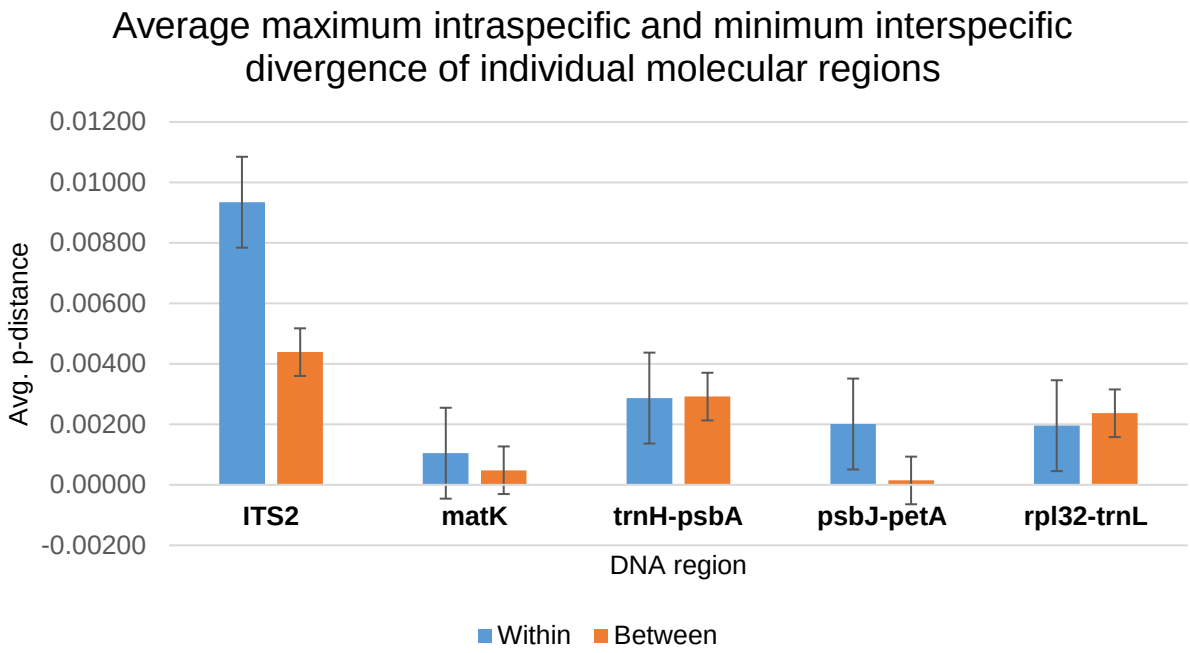


Figure 3.2. Average sequence divergences among the five molecular regions tested. Averages were obtained from pooling maximum intraspecific (within) p-distances and minimum interspecific (between) p-distances among all taxa from each region (see Table 3.5).

Analysis of *ITS2*

The *ITS2* region in members of Apocynaceae ranges from 210–300 bp and has been reported to correctly identify 89–100% of genera and 78–98% of species within Apocynaceae (Selvaraj et al., 2015). In *Plumeria*, PCR amplicons ranged from 208-316 bp (Table 3.4) with an average length of 263 bp. In proportion to its size, this region contained the most variable sites. The final alignment of this region resulted in 232 positions. Only *P. alba* could be identified by five unique *ITS2* sequences (Appendix E). All other taxa lacked any unique genetic signatures for this region.

ML and BI analyses distinguished a highly supported branch for taxa of *P. alba* (BS=98%, PP=0.99). Taxa of *P. pudica*, *P. caracasana*, and *P. sp.* 'Isabella' all form a highly supported grouping of OTUs in both ML (BS=96%) and BI (PP=1.00) analyses. *Plumeria stenopetala* OTUs form a well-supported Stenopetala grouping in the ML analysis (BS=79%), and a highly supported grouping in the BI analysis (PP=0.94). *Plumeria subsessilis* also forms a well-supported relationship with these taxa in both ML and BI analyses (BS=79%, PP=0.84) indicating that *P. subsessilis* and *P. stenopetala* share a high degree of sequence similarity. Taxa of *P. obtusa* var. *sericifolia*, *P. tuberculata*, and *P. obtusa* WES1-24 form a moderately supported grouping as the Sericifolia cluster in the ML consensus tree (BS=63%), and this is echoed in the BI consensus tree with high branch support (PP=0.99), which provides support that *P. tuberculata* and *P. obtusa* var. *sericifolia* are synonymous. The Rubra cluster is not clearly identified in the ML analysis, but its placement as sister to most other taxa is suggested (BS=53%). On the other hand, a well-supported (PP=0.71) cluster of Rubra was recovered in the BI analysis, and as in the ML analysis was placed as sister to most other taxa. ML analysis showed that samples of *P. cubensis, P. clusioides*, *P. ekmanii*, *P. montana*, *P. filifolia*, *P. stenophylla*, *P. obtusa*, and *P. obtusa* var. *sericifolia* formed a complex but well-supported Obtusa grouping (BS=78%). This species complex (Obtusa complex) was also recovered with high branch support in the BI analysis (PP=1.00). Unfortunately, not all taxa could be grouped into clusters. These taxa include samples of *P. bahamensis*, *P. cubensis*, *P. obtusa*, and *P. stenophylla*. This disconnect with the Obtusa complex can be attributed to high intraspecific sequence variation (Table 3.5).

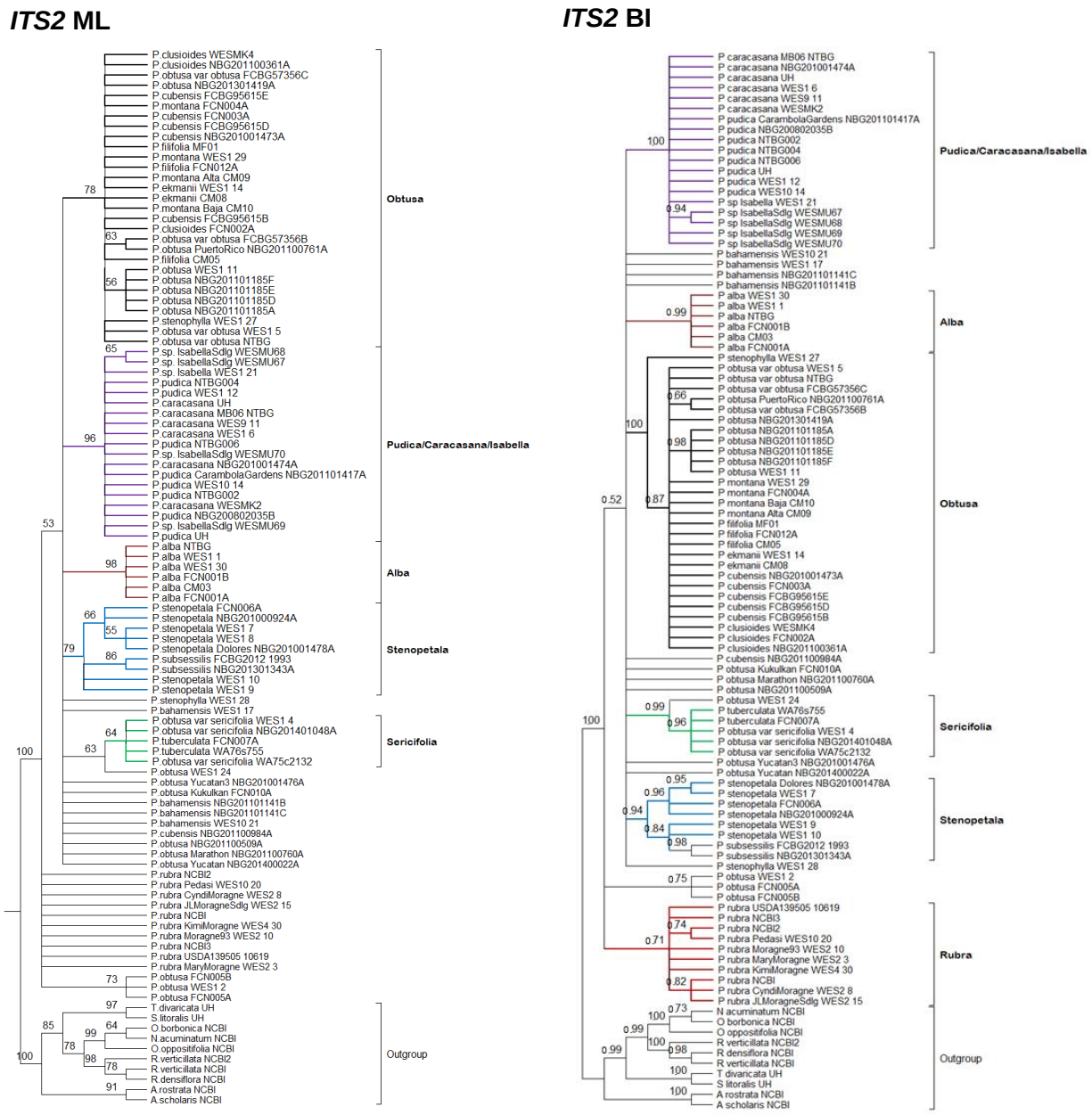


Figure 3.3. Topology-only cladograms of majority rule consensus trees based on maximum likelihood (ML) and Bayesian inference (BI) analyses of the *ITS2* region. Bootstraps (BS) of the ML tree and posterior probabilities (PP) of the BI tree (ln*L* = -1746.54) are indicated above the branches. Branch support values <50% are collapsed to corresponding nodes. Colorized branches denote OTU groupings and are also denoted by brackets and boldface text.

Analysis of *matK*

The entire *matK* region is ~1500 bp (Simões et al., 2007), but Mahadani et al. (2013) found that using an internal portion of the *matK* gene was sufficient for distinguishing most species within Apocynaceae. Primers were developed to amplify an internal portion of the *matK* gene within *Plumeria*. PCR products for this region yielded sequences ranging from 829 to 914 bp long with an average of 866 bp. Relative to the other sequenced loci, the *matK* region contained one of the highest percentages of conserved sites (Table 3.4), fewest gaps, and the lowest amount of intraspecific sequence variation (Table 3.5 and Fig. 3.2). Nevertheless, diagnostic characters were found for *P. obtusa* var. *sericifolia* (syn. *P. tuberculata*), *P. caracasana*, *P. pudica*, and *P.* sp. 'Isabella' (Appendix E). The final alignment including gaps resulted in 836 positions.

In the ML tree, five OTUs were recovered (Fig. 3.4). Taxa of *P. rubra* form a clade that is moderately supported (BS=70%) as sister to all other taxa. *P. tuberculata* and *P. obtusa* var. *sericifolia* form the Sericifolia cluster that also includes one accession of *P. stenophylla,* which is moderately supported (BS=64%). *P. caracasana* and *P. pudica* form a weakly supported clade (BS=66%) that is further subdivided into the Pudica and Caracasana clusters with higher branch support. *P.* sp. 'Isabella' taxa also form a distinct cluster, albeit with weak support (BS=61%). The BI consensus tree of the *matK* region also supports the findings of 5 clusters. The Rubra clade is maintained as sister to all other taxa (PP=0.69), and the same taxa are recovered in the Sericifolia clade with higher branch support (PP=0.97). However, resolution is slightly enhanced as multiple taxa are grouped to form a moderately supported Obtusa species complex (PP=0.70). On the other hand, *P. caracasana* and *P. pudica* form a highly supported grouping (PP=0.99), but *P. pudica* is nested within *P. caracasana*. Different from the ML analysis is the inclusion of *P.* sp. 'Isabella' grouping (PP=0.99) within a highly supported Stenopetala cluster (PP=0.91). Still, internal relationships among species groupings that are sister to *P. rubra* could not be determined as evidenced by the internal polytomies.

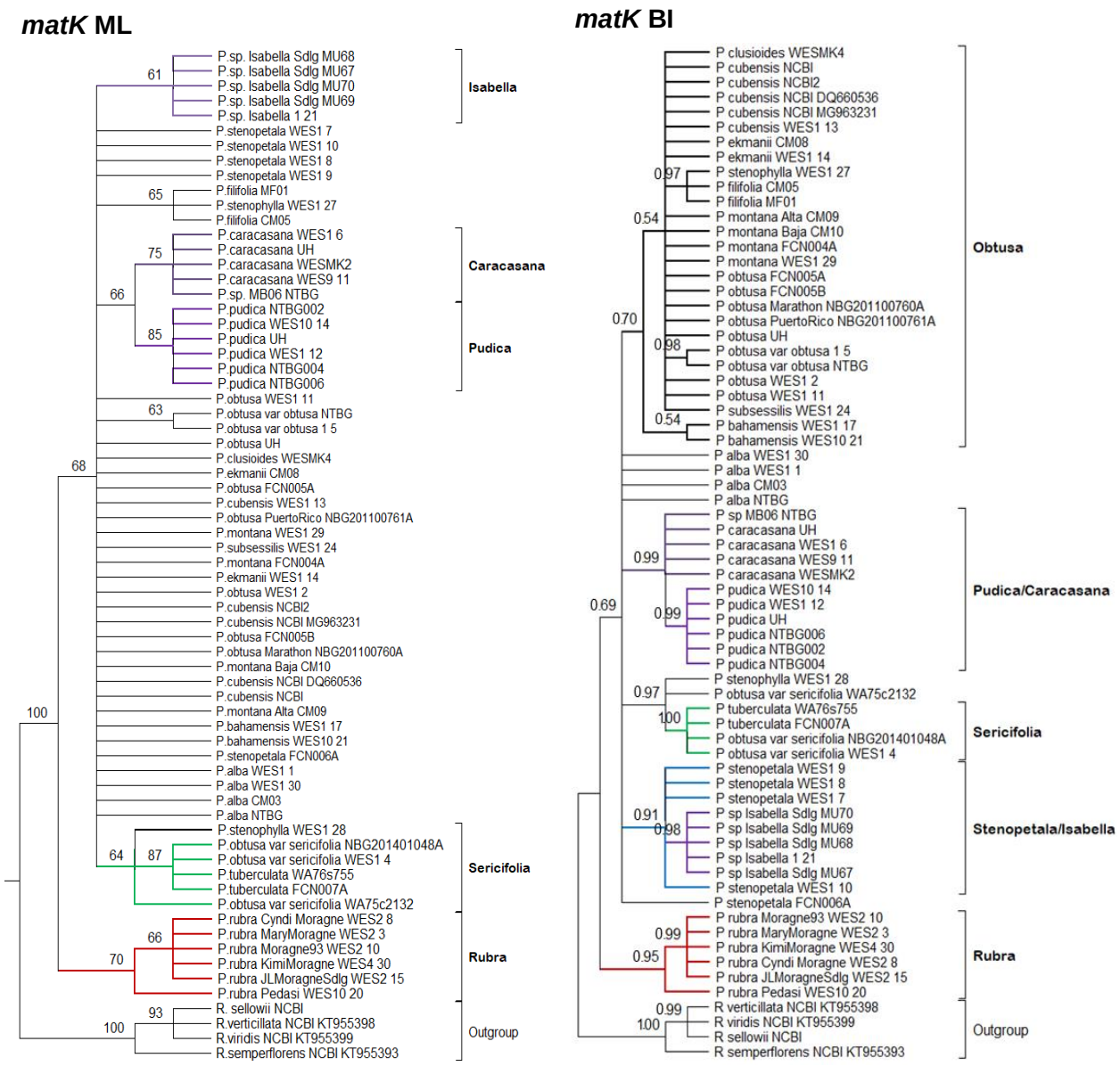


Figure 3.4. Topology-only cladograms of majority rule consensus trees based on maximum likelihood (ML) and Bayesian inference (BI) analyses of the *matK* region. Bootstraps (BS) of the ML tree and posterior probabilities (PP) of the BI tree (ln*L* = -1659.92) are indicated above the branches. Branch support values <50% are collapsed to corresponding nodes. Colorized branches denote OTU groupings and are also denoted by brackets and boldface text.

Analysis of *trnH-psbA*

The size of the *trnH-psbA* region varies in Apocynaceae from 280-467 bp (Selvaraj et al., 2015). In *Plumeria*, sequences ranged from 362-393 bp with an average length of 374 bp (Table 3.4). The final alignment contained 422 positions. A portion of this region also contained a variable number of poly-A/T nucleotide repeats, sometimes among accessions of the same species, resulting in ambiguous regions that were later excluded from analysis since they were difficult to align. To the exclusion of other taxa, a DNA barcoding gap was found for six species, thus highlighting this region's value as a DNA barcode for the genus (Table 3.5). Moreover, this region contained one of the most potentially informative diagnostic characters, consisting of single nucleotide polymorphisms (SNPs), gaps and inserts, by which nine OTUs could be identified (Appendix E).

Maximum likelihood and Bayesian analyses of this region allowed for the detection of seven and six species clusters, respectively (Fig. 3.5). Topologies of ML and BI consensus trees were similar, in that nearly the same groupings of taxa were recovered. *P.* sp. 'Isabella' OTUs formed a moderately-supported clade in the ML tree (BS=72%) whereas the BI consensus tree showed them as part of an internal polytomy. *Plumeria alba* formed a well- to highly supported grouping in the ML (BS=90%) and BI (PP=0.99) trees, respectively. *Plumeria stenopetala* formed a group with one member of *P. stenophylla*, but this relationship is only weakly supported in both ML and BI trees. The ML analysis showed that the Sericifolia grouping, which also includes taxa of *P. subsessilis* and *P. stenophylla* within this cluster, is moderately supported (BS=77%). These relationships are highly supported in the BI analysis (PP=1.00). Taxa of *P. pudica* and *P. caracasana* form a weakly supported branch (BS=54%) as sister OTUs, and this relationship is maintained in the BI tree with higher branch support (PP=0.87). Most taxa of *P. rubra* are recovered in the Rubra grouping in the ML tree (BS=69%), whereas all the *P. rubra* taxa are grouped together to form the highly supported Rubra cluster in in the BI tree (PP=97%). All the other taxa, especially *P. obtusa*, failed to cluster into any distinct clade in either ML or BI analysis.

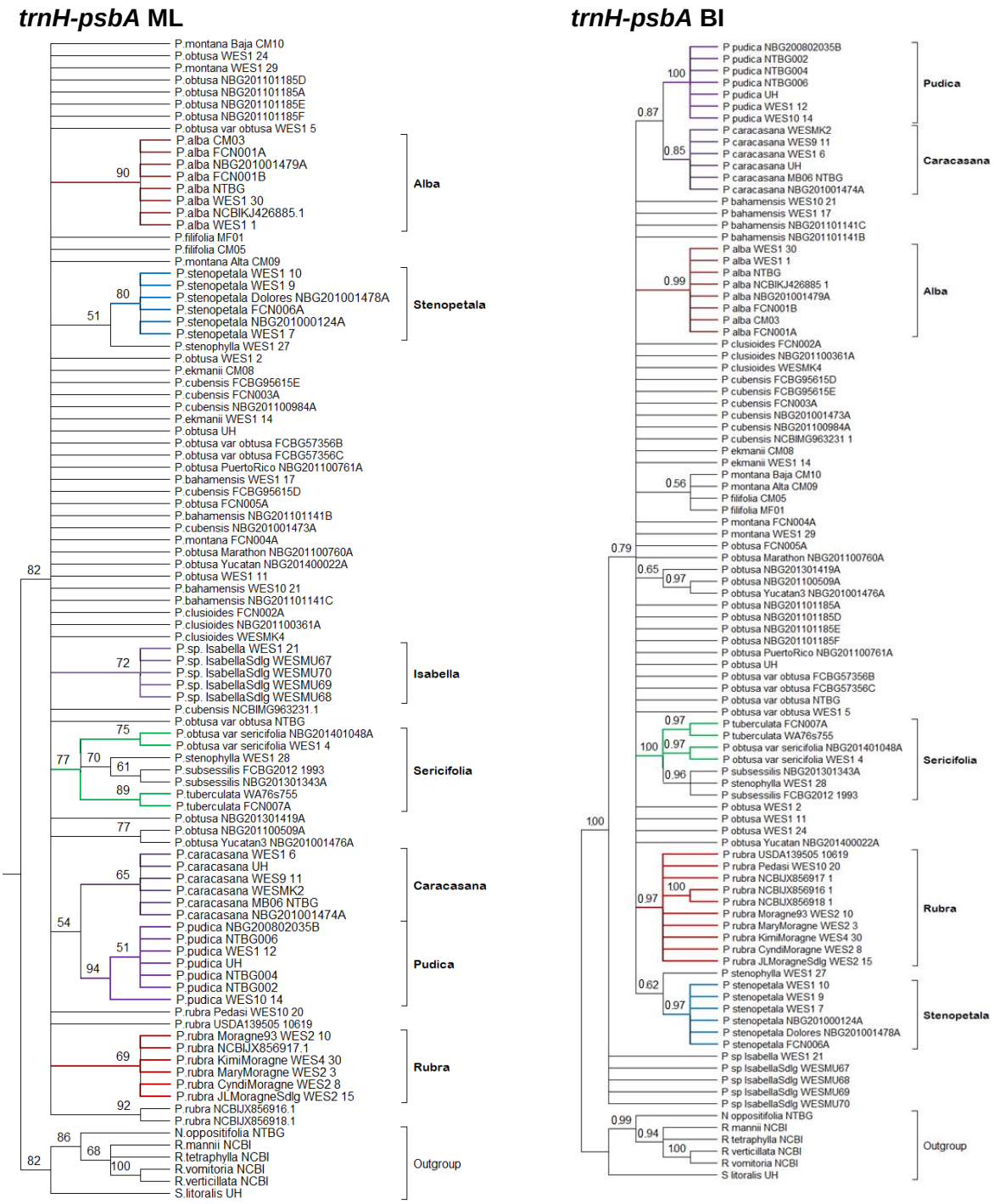


Figure 3.5. Topology-only cladograms of majority rule consensus trees based on maximum likelihood (ML) and Bayesian inference (BI) analyses of the *trnH-psbA* region. Bootstraps (BS) of the ML tree and posterior probabilities (PP) of the BI tree (ln*L* = -1607.34) are indicated above the branches. Branch support values <50% are collapsed to corresponding nodes. Colorized branches denote OTU groupings and are also denoted by brackets and boldface text.

Analysis of *psbJ-petA*

The *psbJ-petA* intergenic spacer is located in the large single copy region of the chloroplast genome (Shaw et al., 2007) and is reported to have an average length of 1,040 bp. Within the genus *Plumeria*, sequence length ranged from 919-935 bp with an average length of 927 bp (Table 3.4). The final alignment resulted in 942 positions, of which 20 sites were variable. Although average maximum intraspecific sequence divergences were greater than average minimum interspecific sequence divergences (Fig. 3.2), some diagnostic characters were found for at least three OTUs (Appendix E).

The ML analysis of this region resulted in a large polytomy and allowed only four clusters of *Plumeria* taxa to be identified, two of which were lumped as one (Fig. 3.6). *Plumeria pudica* and *P. caracasana* form a well-supported Pudica/Caracasana group (BS=82%). As in other analyses, the Sericifolia cluster is comprised of *P. tuberculata* and *P. obtusa* var. *sericifolia* and is highly supported (BS=94%). *Plumeria* sp. 'Isabella' forms a weakly supported (BS=63%), but distinct cluster. Except for one accession, all other taxa of *P. rubra* form the Rubra cluster (BS=93%). The BI analysis resulted in better resolution of an Obtusa species complex (PP=0.84) that is comprised of accessions of *P. bahamensis*, *P. clusioides*, *P. cubensis*, *P. ekmanii*, *P. filifolia*, *P. montana*, *P. stenophylla*, *P. obtusa*, and *P. obtusa* var. *obtusa*. However, this gain of resolution among the Obtusa grouping came with an associated loss of resolution in other parts of the tree. A relationship comprised of *P. alba* with *P. tuberculata* and *P. obtusa* var. *sericifolia* is moderately supported (PP=0.78), but the Sericifolia grouping is still maintained (PP=1.00). Additionally, the Rubra cluster found in ML analysis was recovered in the BI analysis, albeit with less branch support (PP=0.78). Unlike findings in the ML analysis, the BI analysis places taxa of the Isabella cluster with taxa that form the Pudica/Caracasana cluster (PP=0.91), but the Isabella cluster is still maintained as distinct (PP=0.99) from the Pudica/Caracasana grouping (PP=1.00). Unlike analyses of previous regions, neither ML nor BI analysis of *psbJ-petA* produced a *P. stenopetala* cluster.

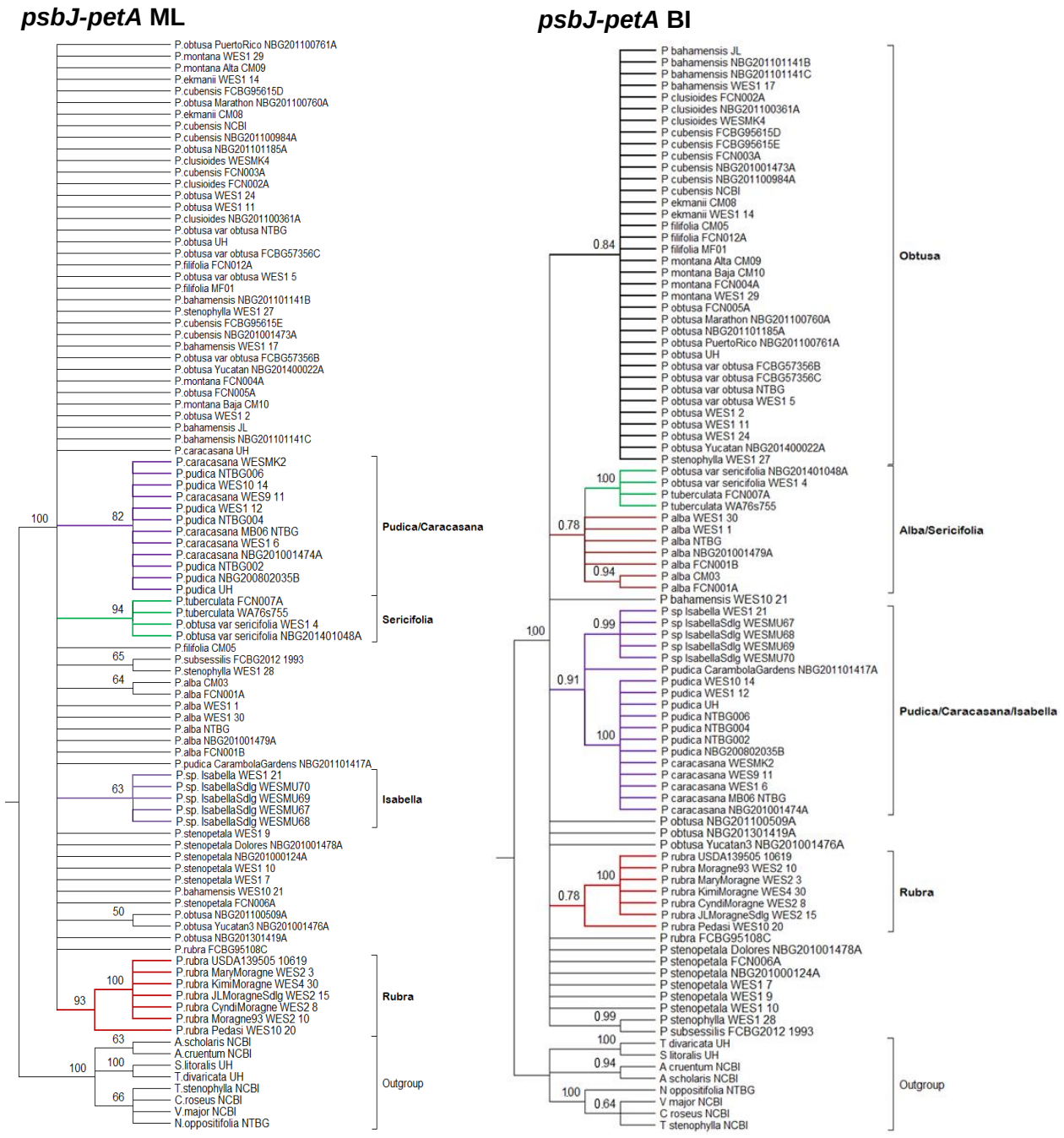


Figure 3.6. Topology-only cladograms of majority rule consensus trees based on maximum likelihood (ML) and Bayesian inference (BI) analyses of the *psbJ-petA* region. Bootstraps (BS) of the ML tree and posterior probabilities (PP) of the BI tree (ln*L* = -2852.92) are indicated above the branches. Branch support values <50% are collapsed to corresponding nodes. Colorized branches denote OTU groupings and are also denoted by brackets and boldface text.

Analysis of *rpl32-trnL*

The *rpl32-trnL* region is in the small single copy region of the chloroplast genome and is an average of 1,018 bp in length (Shaw et al., 2007). In *Plumeria*, sequences ranged from 839-1,006 bp, with an average of 987 bp (Table 3.4). The final alignment of this region resulted in 1,007 positions. Similar to the *trnH-psbA* region, this region demonstrated relatively low maximum intraspecific sequence variation in comparison with minimum interspecific variation for at least six OTUs (Table 3.5, Fig. 3.2). Initial inspection of nucleotide sequences resulted in the identification of 41 diagnostic characters that could distinguish seven species (Appendix E).

This region provided the greatest separation of species clusters of all sequence regions tested. Although the internal relationships were still poorly resolved—as indicated by internal polytomies—both ML and BI analyses showed clustering of eight OTUs (Fig. 3.7), and all but two accessions fell into an OTU grouping. *Plumeria rubra* accessions were moderately supported as a cluster in the ML analysis (BS=67%). This relationship was also well-supported in the BI analysis (PP=0.91) but was located as a sister group to all other OTUs. Other highly supported OTUs in both ML and BI analyses include the Stenopetala, Alba, Pudica, Caracasana, and Isabella clusters. As in other analyses, the Sericifolia grouping contained *P. subsessilis* and an accession of *P. stenophylla* (BS=63%, PP=0.96). The remaining taxa mostly fell into the Obtusa species complex, with low to moderate branch support in the ML and BI analyses, respectively (BS=54%, PP=0.82).

***rpl32-trnL* ML** ***rpl32-trnL* BI**

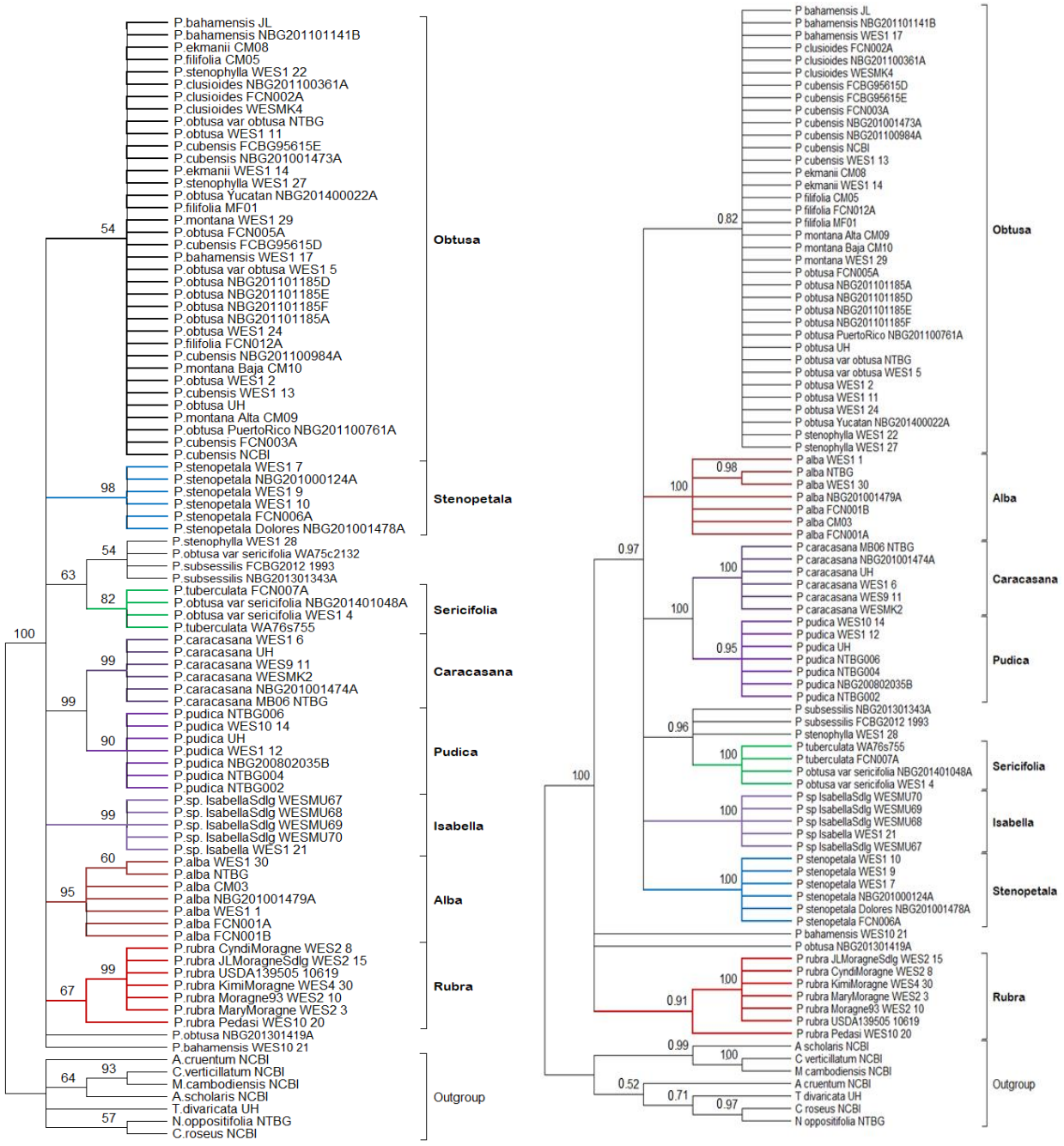


Figure 3.7. Topology-only cladograms of majority rule consensus trees based on maximum likelihood (ML) and Bayesian inference (BI) analyses of the *rpl32-trnL* region. Bootstraps (BS) of the ML tree and posterior probabilities (PP) of the BI tree (ln*L* = -3799.25) are indicated above the branches. Branch support values <50% are collapsed to corresponding nodes. Colorized branches denote OTU groupings and are also denoted by brackets and boldface text.

Analysis of Combined Data

No single region could fully resolve the genetic relationships among the *Plumeria* taxa sampled as shown by the presence of large internal polytomies (Figs. 3.2-3.6). Of the five regions surveyed, the following regions were concatenated: *ITS2*, *trnH-psbA*, *psbJ-petA*, and *rpl32-trnL*. The *matK* region was excluded from concatenation due to a lower number of sampled taxa. Although data sets were subjected to a partition homogeneity test (i.e. Incongruence Length Difference Test), the following rationale for combining data sets is provided: 1) similar groupings of taxa were recovered among the different regions and analyses, 2) similar branch support values for those recovered groupings were given between ML and BI analyses, and 3) attempts were made to resolve the internal polytomies by finding a suitable combination of regions. Furthermore, preliminary analyses of 2- and 3-region concatenated sets still yielded hard polytomies, so only the 4-region data set was subjected to further analyses. The combined data set resulted in 2,603 positions, 2,479 of which were conserved, 116 of which were variable, and a total of 74 sampled *Plumeria* taxa (Table 3.4).

The species clusters found in the analyses of the *rpl32-trnL* region were recovered in the analysis of the combined data set (Figs. 3.8 & 3.9). In comparison with tree topologies obtained from the *rpl32-trnL* region (Fig. 3.7), the ML tree of the combined regions showed a loss of resolution in the Obtusa grouping as all the taxa that were previously recovered into the *P. obtusa* species complex were now collapsed into polytomies. In comparison with findings from other regions, the Rubra, Alba, and Stenopetala accessions formed distinct groupings that are highly supported (for Rubra BS=94%, for Stenopetala BS=99%, and for Alba BS=100%). There is also moderate support (BS=89%) for a sister relationship between Sericifolia and Subsessilis taxa, which was previously recovered in analyses of *rpl32-trnL* and *trnH-psbA* regions. Groupings of Pudica and Caracasana taxa are placed in a highly supported sister relationship to each other (BS=100%), and the Isabella cluster was placed as sister to Pudica and Caracasana (BS=96%) thereby indicating strong support for the relatedness among these three OTUs. Unfortunately, the concatenated loci did not fully resolve the internal relationships among OTUs as evidenced by the large internal polytomy.

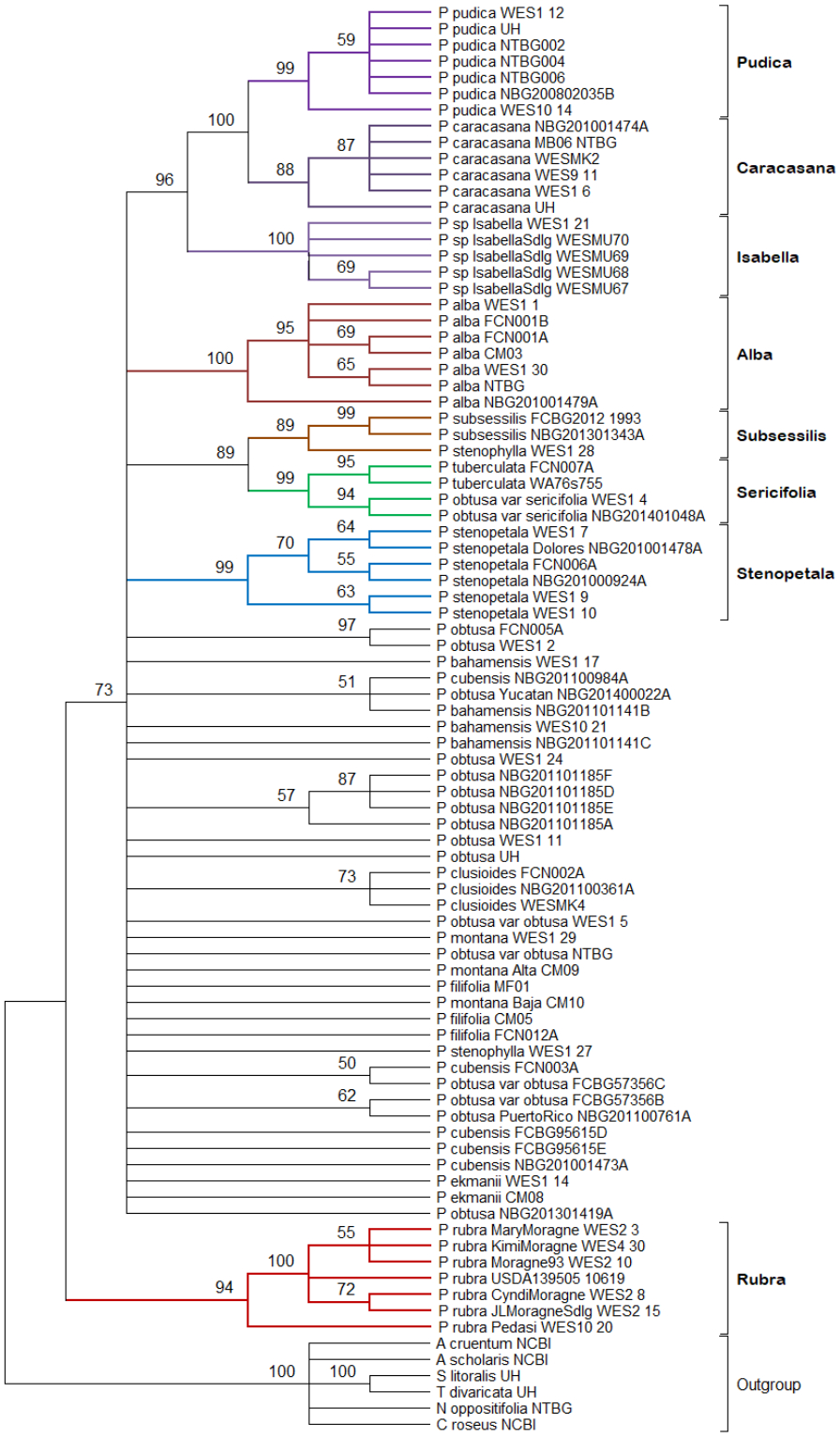


Figure 3.8. Majority rule consensus tree (cladogram) based on maximum likelihood (ML) analysis of the 4-region concatenated data set. Bootstraps (BS) are indicated above the branches. Branch support values <50% are collapsed to corresponding nodes. Colorized branches denote OTU groupings and are also denoted by brackets and boldface text.

However, concatenation resulted in a more resolved tree topology in the BI analysis (Fig. 3.9). Rubra OTUs were placed as sister to all other taxa and comprised a well-resolved monophyletic clade (PP=0.99). Pudica, Caracasana, and Isabella were again recovered as a monophyletic clade (PP=1.00) that is basal to the remaining OTUs. Moreover, the association of Isabella with Caracasana and Pudica groupings is highly supported in the BI analysis (PP=1.00), as it was in the ML analysis. Alba formed a distinct and highly supported clade (PP=1.00) that is also basal to the remaining OTUs (PP=0.99). Stenopetala taxa are highly supported as a distinct OTU, but their placement in relation to the remaining OTUs is only moderately supported (PP=0.66). Similarly, Sericifolia and Subsessilis OTUs comprised a highly supported clade (PP=1.00), but their relationship to the Obtusa species complex is only moderately supported (PP=0.60). OTUs that form the Obtusa species complex were well-supported (PP=0.99). Beyond this grouping, it is difficult to make any further claims regarding the relationships among the taxa within this complex as several taxa failed to group with their conspecifics. This could be a result of high sequence variation among these taxa (Table 3.5) or potential misidentification.

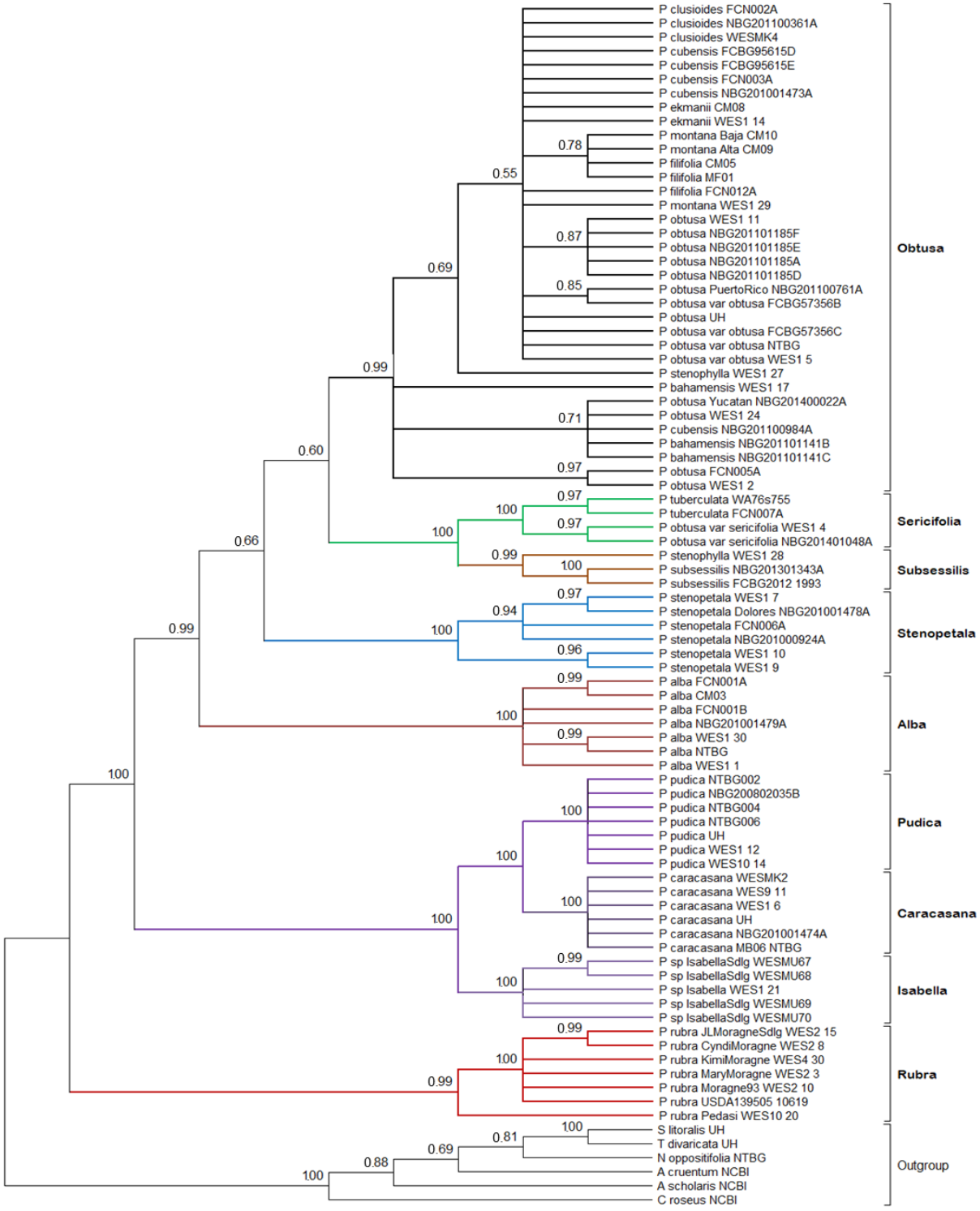


Figure 3.9. Majority rule consensus tree based on Bayesian analysis of the 4-region concatenated data set (lnL = -8244.16). Posterior probabilities (PP) are indicated above the branches. Branch support values <0.50 are collapsed to corresponding nodes. Colorized branches denote OTU groupings and are also denoted by brackets and boldface text.

DISCUSSION

Phylogenetic utility of regions

While no single region was able to fully resolve the relationship of these congeneric species, the *rpl32-trnL* region provided the best phylogenetic signal from a single region (Fig. 3.7). Combining four out of the five regions provided enough phylogenetic resolution to indicate likely species relationships within this genus. As a result, an initial phylogenetic framework was established from which we can begin to understand evolutionary relationships among *Plumeria* species.

DNA Barcodes for *Plumeria*

DNA barcoding is an attractive tool that has found utility in identifying species in various plant groups including Apocynaceae (Pettengill and Neel, 2010; Wong et al., 2013; Selvaraj et al., 2015). The minimum requirement for a DNA region to be deemed useful for barcoding is that there be a barcoding gap by which all members of one species can be clearly distinguished from members of other species [interspecific variation], while allowing for some variation among members within the same species [intraspecific variation] (Fišer Pečnikar and Buzan, 2014). What constitutes a suitable barcode gap is not well defined. However, the distance analysis criteria of Meier et al. (2008) was followed by comparing the maximum sequence variation within a species (intraspecific variation) to the minimum sequence variation between species (interspecific variation) and assessed whether a local barcode gap existed for each region. Although no single region could identify all the species we sampled, DNA barcoding gaps were identified that were unique to each species within each region (Table 3.5). Furthermore, diagnostic characters that were able to identify OTUs were discovered (Appendix E). At best, the *rpl32-trnL* or *trnH-psbA* regions provide enough variation to identify at least eight OTUs (Fig. 3.10).

Reports on the various successes or failures of to identify species using a single region are well documented in the literature (Mahadani et al., 2013; Wu et al., 2019; Yang et al., 2019). Moreover, no single region can be applied to all plant groups and be expected to perform the same (Kelchner, 2000). The context in which these regions are used is especially important. For instance, Selvaraj et al. (2015) report on the

exceptional ability of the *ITS2* region to discriminate genera and species of Apocynaceae. However, their sampling was done at the generic level. At this level, it seems reasonable that enough genetic variation would exist to distinguish among genera. Likewise, given the inherent genetic diversity of species in such a large family as the Apocynaceae, it is expected that they would find a high species discriminating ability among such a diverse sampling of taxa. In my study, however, only six OTUs were identified using the *ITS2* region (Fig. 3.3), and at best only eight out of the 16 putative species were identified using the *rpl32-trnL* region.

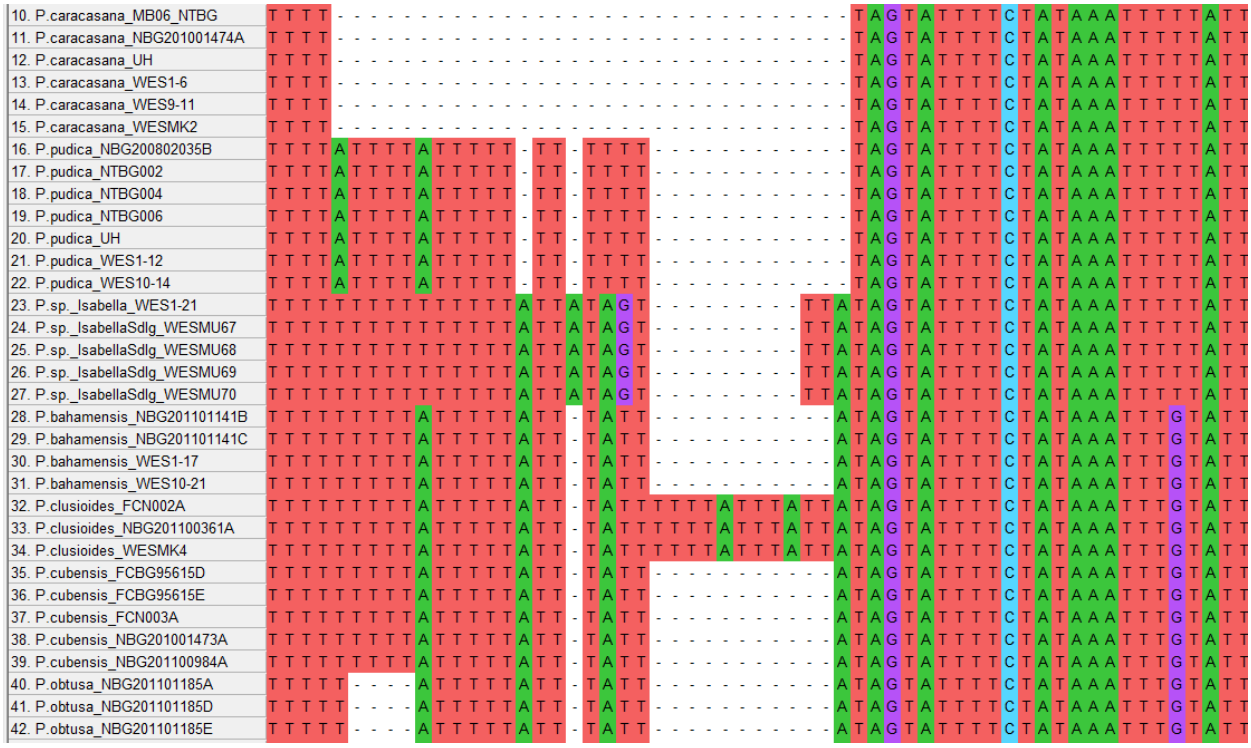


Figure 3.10. A portion of the *trnH-psbA* region showing examples of potentially diagnostic nucleotides among several closely related taxa. *P. caracasana* is missing a region that is present among its sister taxa, *P. pudica* and *P.* sp. 'Isabella'. *Plumeria* sp. 'Isabella' accessions contain indels not present in either *P. caracasana* or *P. pudica*. *Plumeria clusioides* can also be distinguished from other taxa of the *Obtusa* complex by the presence of an 11-bp insertion not found in other members of this complex. The full suite of diagnostic characters is available in Appendix E.

For the most part, molecular findings support the traditional taxonomy of *Plumeria* spp. based on morphological evidence. Findings of this study also provide evidence that certain taxa, currently considered synonymous, are in fact distinct from one another. The discussion that follows elaborates on these points.

P. rubra L.

P. rubra was one of the most consistently identified OTUs in all five regions surveyed, and their relationships were highly supported in ML and/or BI analyses, unequivocally validating their uniqueness as a legitimate species. The placement of this group was initially unclear from analyses of individual regions. Rubra groupings were placed as basal in analyses of *ITS2*, *matK*, and *rpl32-trnL* regions, but not in others. However, BI analysis of the combined data suggests that this clade is basal to all other OTUs (Fig. 3.9). If this is true, then this could have potential evolutionary implications regarding the loss of petal color and scent as all other *Plumeria* species have flowers that are white-petaled with relatively little fragrance.

P. alba L.

P. alba taxa were identified as an OTU in the analyses of the *ITS2*, *trnH-psbA*, and *rpl32-trnL* regions (Figs. 3.3, 3.5, and 3.7). They could also be characterized using these three regions due to the presence of a barcode gap (Table 3.5). Coincidentally, this species can also be identified morphologically by the presence of lanceolate leaves and “puckered-revolute” leaf margins (Woodson, 1938a). From analyses of single regions, its relationship to other OTUs was unclear. However, in the combined data set (Fig. 3.9) it appears closely related to the Stenopetala, Isabella, Caracasana, and Pudica OTUs.

P. subsessilis A. DC. and *P. stenopetala* Urb.

P. subsessilis is clearly distinguishable from most other species in this study and has also been recognized as a legitimate species by Acevedo-Rodríguez and Strong (2012) and Govaerts et al. (2003). Urban (1902) provided the first Latin description of *P. stenopetala*, based on a specimen collected in Haiti. However, *P. stenopetala* was

later considered by Woodson (1938b) as a “doubtful species” and was instead subsumed under *P. subsessilis* as a supposed hybrid between *P. subsessilis* and *P. rubra*, *P. obtusa* or *P. obtusa* var. *sericifolia*, based upon other specimens collected from Haiti. In analyses of the *ITS2* region, there is some support for the relationship between *P. stenopetala* and *P. subsessilis* (Fig. 3.3). On the other hand, analyses from the *trnH-psbA* and *rpl32-trnL* regions placed taxa of *P. stenopetala* in their own OTU group (Figs. 3.5 and 3.7) and this is further substantiated in the combined analyses (Figs. 3.8 and 3.9). More importantly, in the analyses of the *trnH-psbA* and *rpl32-trnL* regions there is strong support for a relationship between *P. subsessilis* and *P. obtusa* var. *sericifolia*. What is interesting here is the highly supported relationship between these OTUs even though they have drastically different morphologies. Thus, at this time it is unclear how *P. subsessilis*, *P. obtusa* var. *sericifolia*, and *P. stenopetala* are related to one another, and more research will need to be conducted to verify Woodson’s claim of the hybrid origin of *P. stenopetala*.

The *P. obtusa* Complex and the Sericifolia Group

In his treatment of *P. obtusa*, Woodson (1938a) collapsed many previously described *Plumeria* spp. under *P. obtusa*, including several of the taxa sampled in this study: *P. clusioides*, *P. bahamensis*, *P. montana*, *P. ekmanii*, and *P. cubensis*. Woodson also mentioned the existence of a *P. obtusa* species complex, alluding to the morphological variation in leaves among type specimens of *P. obtusa*. I concur with this interpretation as it seems especially likely given the wide geographic distribution of *P. obtusa*—Bahamas, Cuba, Hispaniola (Haiti and Dominican Republic), Jamaica, Puerto Rico, Yucatan, and British Honduras (Belize)—and overlapping collection sites of the taxa that Woodson included in his work.

There are consistent lines of evidence from analyses of multiple regions that verify the occurrence of a complex of taxa that form the *P. obtusa* complex, many of which are accepted by Acevedo-Rodríguez and Strong (2012) but treated as synonyms of *P. obtusa* by Govaerts et al. (2003) and Woodson (1938a). What is interesting is that some of these taxa are morphologically distinct but share high sequence similarity with many other species that fall into the species complex. For instance, *P. ekmanii* has

noticeably thicker and rounder leaves as compared with *P. obtusa*, yet consistently falls into the *P. obtusa* complex in molecular analyses. This phenotypic variation highlights the difficulty of using foliar morphology to identify species limits, which can be exceptionally challenging when dealing with a species complex as is the case with this study.

The relationship between *P. filifolia* and *P. stenophylla* is equivocal, based on findings from this study. According to the World Checklist of Plants (WCSP, 2019), *P. filifolia* is an accepted plant name. *P. stenophylla*, first described by Urban (1924), is considered a synonym of *P. filifolia* (Govaerts et al., 2003; Acevedo-Rodríguez and Strong, 2012) likely due to a high degree of morphological similarity and geographical distribution. Analyses of the *ITS2* region (Fig. 3.3) show a highly supported relationship between these two species, suggesting *P. stenophylla* could be a synonym of *P. filifolia*. However, this relationship was not recovered in analyses from other regions. On the other hand, samples of both species fall into the *P. obtusa* complex, suggesting a relationship between these two species, but this remains unresolved. In a similar vein, the tendency of *P. filifolia* and *P. stenophylla* to group with taxa in the *P. obtusa* complex is difficult to understand in the analyses of the *ITS2*, *matK*, *psbJ-petA*, and *rpl32-trnL* regions. A possible explanation for this is lack of interspecific variation within these regions (Table 3.5).

Another interesting finding regarding taxa of the *P. obtusa* var. *sericifolia* is their grouping with respect to the *P. obtusa* complex. Given their propensity to form their own cluster in analyses of multiple data sets, the pre-existing notion among collectors that *P. tuberculata* and *P. obtusa* var. *sericifolia* are synonyms has been verified. Moreover, given that it does not group with the *P. obtusa* complex, it may be worth reconsidering the taxonomic status of Sericifolia taxa. It is currently accepted as a synonym of *P. obtusa* (Govaerts et al., 2003), while Woodson (1938a) treated this as a botanical variety of *P. obtusa*. There is evidence from four out of the five regions evaluated to suggest that this OTU should be reconsidered as a separate species from *P. obtusa*, or at least considered a botanical variety (ssp.) of *P. obtusa* as opposed to a synonym. Given its placement within a polytomy next to the Obtusa grouping, more analyses are needed to determine the taxonomic status of the Sericifolia taxa.

P. pudica Jacq., *P. caracasana* J.R. Jhonst., and *P.* sp. 'Isabella'

From this study, it is unclear whether *P. pudica*, *P. caracasana*, and *P.* sp. 'Isabella' are synonymous or if they are cryptic species (Kress et al., 2015). *Plumeria pudica* and *P. caracasana* were considered synonymous by Woodson (1938a), Acevedo-Rodríguez and Strong (2012), and Govaerts et al. (2003) on the basis of morphology and geographic distribution. On the other hand, evidence from this study shows that these two taxa are distinguishable by molecular analyses. It is reasonable, however, to regard *P. pudica* and *P. caracasana* as synonymous given their overlapping morphological characters and geographical distribution (northern Venezuela) (Johnston, 1912; Gleason and Killip, 1939). Regardless of this possibility, molecular analyses of the *matK* (Fig. 3.4), *trnH-psbA* (Fig. 3.5), *rpl32-trnL* (Fig. 3.7), and combined regions (Figs. 3.8 and 3.9) point to a sister group relationship, as evidenced by high bootstrap support and posterior probabilities. Evidence is also provided that *P.* sp. 'Isabella,' which is not formally described in the scientific literature deserves potential recognition as a species because of the highly supported relationship as a sister taxon to *P. pudica* and *P. caracasana*. Furthermore, all three taxa contain unique genetic signatures that allow them to be distinguished from one another (Appendix E). Hence, a focused study that incorporates molecular, morphological, and ecological data should be conducted to establish the separate species nature of *P. caracasana*, *P. pudica*, and *P.* sp. 'Isabella'.

Future Directions

Although not all the taxa included in this study could be verified, I have shown that it is possible to recognize most species and have established a basis for which evolutionary relationships can be studied based on a combination of molecular characters that I feel are diagnostic of these species. Furthermore, though I did not include a complete sampling of species in this genus, this study represents a modern study of the genus since its last taxonomic revision by Woodson. A more comprehensive geographic sampling along with the use of more vouchered specimens (Funk et al., 2018) would be needed for each species to determine the full utility of regions, such as *trnH-psbA* and *rpl32-trnL*, and finally determine species boundaries and phylogenetic relationships of *Plumeria*.

CONCLUSION

The objective of this study was to evaluate molecular regions for their effectiveness in distinguishing *Plumeria* taxa and their phylogenetic utility. No single region was adequate to distinguish species, but the *rpl32-trnL* region followed by the *trnH-psbA* region provided the best ability to discriminate species. A combination of molecular regions was required to sort out the maximum amount of species. Yet, the identity of all sampled taxa could not be verified to accepted species, especially those belonging to the *P. obtusa* complex. Rather, the species within this complex will require other molecular regions to identify them. Nevertheless, the regions evaluated have allowed for the ability to distinguish most of the species and their concomitant diagnostic molecular characters, indicating the differential abilities of each region to sort out different species. To this end, the hypothesis is confirmed that no single region was able to distinguish all species. However, given the level of success in this study additional molecular data is expected to provide more information to define species boundaries and enhance our understanding of phylogenetic relationships within the genus *Plumeria*.

ACKNOWLEDGEMENTS

I would like to thank Dr. Chad Husby (Fairchild Tropical Botanic Garden), Dr. Alan Meerow (Miami USDA), Tex Norwood and Carol Vannoorbeeck (Florida Colors Nursery), Hetty Ford (Naples Botanical Garden), Donald Hodel (University of California), Carlo Moricci (The Palmetum of Santa Cruz de Tenerife), and Mike Ferrero for providing us with plant material for this study. Many thanks also goes to Elizabeth Winnicki, Mona Al-Tamimi, Preksha Bapna, and Sunisa Kogar for their assistance. This study on *Plumeria* has been supported by research grants from the Southern California Plumeria Society, the Plumeria Society of America, UH Manoa Graduate Student Organization, and private funding.

CHAPTER 4

SYNTHESIS, PROSPECTS, AND CONCLUSION

INTRODUCTION

Plumeria L. is a genus within the family Apocynaceae, subfamily Rauvolfioideae, and is comprised of taxa that have been valued for ornamental, cultural, and ethnomedicinal purposes (Judd et al., 2008; Criley, 2009; Shinde et al., 2014). Although twelve species are currently recognized in the genus (The Plant List, 2013), disagreements exist regarding legitimacy of these species, the number of valid species and their delimitation. These disagreements are further compounded by the many disparities in species descriptions, especially in the treatment of synonymous names for *Plumeria* spp. (Urban, 1898, 1902; Britton, 1910; Johnston, 1912; Britton, 1915; Britton and Millspaugh, 1920; Urban, 1920; Hollick, 1922; Britton, 1923; Urban, 1924; Woodson, 1938a; Woodson et al., 1970; Williams, 1996), many of which are misused or unconfirmed among collectors (Criley, 2009). For instance, Acevedo-Rodríguez and Strong (2012) recognize *P. clusioides* Griseb. as a legitimate species, whereas Govaerts et al. (2003) do not. The poor state of knowledge of species delineations and a need for a clearer taxonomy were the impetus for this study.

SYNOPSES OF CHAPTERS

Chapter 1

Chapter 1 introduces the current issues associated with the taxonomy of *Plumeria* and proposes two approaches to address these problems, morphological and molecular studies. The overall hypothesis was that morphological and molecular analyses of *Plumeria* accessions would allow clear recognition and verification of species, establish genetic relationships and provide criteria for the delimitation of currently unrecognized ones.

Chapter 2

In Chapter 2, the objective was to identify *Plumeria* spp. using qualitative morphological characters that were easy to score using the following question: *What qualitative foliar characters can be used to distinguish Plumeria spp.?*

To answer this question, *Plumeria* accessions from botanical gardens were assessed for the presence or absence of 43 descriptive foliar (morphological) characters. An iterative approach to principal component analysis (PCA) showed that a combination of leaf shape, margin, midvein, apex, base, ptyxis, secondary venation, and surface characters were useful for distinguishing most species. Some specimens formed distinct clusters, suggesting that certain taxa, such as *P. caracasana* should be recognized as legitimate species, and verified that descriptive morphological characters will suffice for most of the recognized *Plumeria* spp. and putative species examined. However, not all taxa were identified by distinct (unique) clustering, especially those belonging to the *P. obtusa* complex. While the hypothesis that a combination of morphological characters can provide species identification was confirmed for most species (eight out of the 11 putative species sampled), other characters (quantitative, reproductive, and anatomical) will be needed to sort out taxa, especially in a species complex.

Chapter 3

In Chapter 3, the objective was to identify DNA loci that could delineate species and resolve genetic relationships in *Plumeria* by examining separate and combined molecular regions. The questions posed were:

1) *What individual molecular markers (chloroplast and/or nuclear) can be used to distinguish Plumeria spp.?*
2) *Does some combination of chloroplast and/or nuclear DNA markers better distinguish among taxa?*
3) *Are these regions phylogenetically informative?*

To answer these questions, accessions were sampled and five molecular regions comprising nuclear and chloroplast loci were evaluated for their suitability in

distinguishing species and their utility in revealing phylogenetic relationships. Results showed that although a single region could be used to differentiate most species through a DNA barcoding approach, the combined data of four regions was required to generate enough phylogenetic signal to elucidate the internal genetic relationships among species. The *rpl32-trnL* region followed by the *trnH-psbA* region could discriminate most species, but the polytomy of the *P. obtusa* species complex made it difficult to separate taxa within this group. Even when four loci were combined this species complex remained intact. On the other hand, it was possible to draft a phylogenetic reconstruction of species and build a case for recognizing currently subsumed taxa such as *P. caracasana*, *P.* sp. 'Isabella', and *P. obtusa* var. *sericifolia* (syn. *P. tuberculata*). However, no single region was able to distinguish all species. Given the level of success in this study it is likely that adding additional gene regions will provide the needed resolving power to separate these taxa and enhance our understanding of phylogenetic relationships within this genus.

SYNTHESIS

From this research, the following question can be addressed:
How well does the molecular data reflect the morphological data regarding the identification of Plumeria spp.?

Descriptive morphology, as traditionally applied, serves as a basis for species' identifications and provides the first level of documentation to verify the identity of species (De Candolle, 1844; Grisebach, 1864; Urban, 1898; Woodson, 1938a). Here, I set out to evaluate characters based upon descriptions in literature and empirical observation. Characters from literature references, such as leaf shape, revolute margins, and leaf surface texture were found to be informative. However, it was also found that novel characters such as conduplicate leaf ptyxis, recurved leaves, and secondary venation characters were also useful for discriminating among species. This resulted in the discovery of the value of employing (unique) combinations of old and new foliar characters to correctly identify species. The value of these characters would no doubt increase when combined with additional sources of data such as quantitative

and geographic data from the literature, beyond the scope of this study (Grisebach, 1864; Woodson and Moore, 1938; Woodson, 1938a; Leon and Alain, 1957).

The use of chloroplast regions is merited for its ability to identify accessions in botanical gardens, authenticate plants in medicinal products, and identify units of conservation in ecology-based applications (Ford-Lloyd, 2001; Chen et al., 2010; Muscarella et al., 2014; Kress et al., 2015). Five commonly cited DNA regions were evaluated for their efficacy at delimiting species and phylogenetic utility (Kress et al., 2005; Bieniek et al., 2015; Selvaraj et al., 2015). When used alone, none of the five loci were sufficient to identify all accessions we surveyed. However, their resolving power was greatly increased when they were combined allowing clear recognition of most of the commonly accepted species and also the identification of new taxa that should be recognized as genetically distinct and separate species.

There is considerable agreement in species recognition in the morphological and molecular analyses, although they do not coincide exactly in some cases. This agreement was clear for *P. subsessilis*, *P. stenopetala*, *P. alba*, *P. pudica*, *P. caracasana*, and *P. rubra*. However, the identification of *P. clusioides* and *P. stenophylla* in the morphological analyses did not coincide with the molecular data and the identification of *P. obtusa* var. *sericifolia* accessions in molecular analyses were incongruent with morphological data. This supports the need to incorporate quantitative data, at the very least, in combination with the use of other loci, and will help build a better case for taxonomic revision for this genus.

PROSPECTS

Morphological and molecular data have proven useful at resolving species and phylogenetic relationships among plants at various levels. Studies have shown that a combination of quantitative and qualitative morphological characters of both vegetative and reproductive traits have proven useful to clarify taxonomic questions in a variety of plant families and genera (Lens et al., 2008; Smitha et al., 2018; Viera Barreto et al., 2018), even within the Apocynaceae (Grisebach, 1864; Woodson and Moore, 1938; Woodson, 1938a; Alvarado-Cárdenas, 2007; Endress et al., 2007b; Livshultz et al., 2007).

Additionally, studies have shown that combining morphological data with molecular data helped in resolving taxonomic and phylogenetic relationships among closely related taxa (Ronblom and Anderberg, 2002; Endress et al., 2007b; Simões et al., 2010; Pettengill and Neel, 2011; Steele and Pires, 2011). Even more, with the powerful tool of whole genome sequencing becoming more commonplace, it is now possible to scan entire genomes between taxa to identify regions with potential species discriminating abilities and phylogenetic resolving power to the extent that individuals within populations can be identified (Coissac et al., 2016). Therefore, the inclusion of such characters and approaches to studies of *Plumeria* shows promise for further verifying and delineating species boundaries, and allowing for more in-depth studies on evolutionary histories of taxa within this genus.

CONCLUSION

The current taxonomy of the genus *Plumeria* is in need of clarification given the conflicting delimitations and extensive elevation and demotion of species as unique. The current study has shed light on the validity and limitations of both morphological and molecular approaches shown to be effective in disentangling the taxonomy of *Plumeria* spp. Two main conclusions were reached: First, descriptive leaf morphology is useful in discerning most of the currently recognized species of *Plumeria* but should be supplemented with quantitative measurements and other data, such as reproductive, physiological, and ecological characters, to solidify species boundaries. Second, combining molecular regions is not only useful in verifying most of the currently recognized *Plumeria* species but is also useful in understanding the phylogenetic relationships among these species. However, other molecular regions in the chloroplast or nuclear genome may exist that may be more informative for identifying species and understanding evolutionary relationships in this genus. To this end, a total evidence approach that incorporates foliar and floral characters (qualitative and quantitative), physiological, ecological, and geographic data, and other molecular regions is bound to enhance the current findings and elucidate the taxonomic boundaries and phylogenetic relationships among extant *Plumeria* species.

APPENDICES

Appendix A. Accession data for morphological analyses, including collector, propagule, and voucher (institution) information. Locality abbreviations are as follows: NBG – Naples Botanical Garden, WES – Waimanalo Experiment Station (University of Hawaii), PTBG – Pacific Tropical Botanical Garden, FCN – Florida Colors Nursery, FTBG – Fairchild Tropical Botanic Garden. Abbreviated Name column corresponds to taxa names on principal component analysis graphs.

Taxon	Locality	Institutional Accession Number	Abbreviated Name	Provenance
P. alba	NBG	201001479*A	Alb_NBG	Roatan, Honduras
P. alba	WES	WES1-1	Alb_WESa	Oka Nursery, Waimanalo, HI
P. alba	WES	WES1-30	Alb_WESb	Santa Barbara, CA
P. bahamensis	NBG	201101141*A	Bah_NBGa	*Unknown*
P. bahamensis	NBG	201101141*B	Bah_NBGb	*Unknown*
P. bahamensis	NBG	201101141*C	Bah_NBGc	*Unknown*
P. bahamensis	WES	WES1-17	Bah_WESa	Haleiwa, HI
P. bahamensis	WES	WES10-21	Bah_WESb	Haleiwa, HI
P. caracasana	NBG	201001474*A	Car_NBG	*Unknown*
P. caracasana	WES	WES9-11	Car_WES	*Unknown*
P. caracasana	PTBG	MB06	MB06	*Unknown*
P. clusioides	FCN	FCN002	Clu_FCN	Honduras
P. clusioides	NBG	201100361*A	Clu_NBG	Honduras
P. clusioides	WES	WESMK4	Clu_WES	Antilles
P. cubensis	FTBG	95615 D	Cub_FCBGa	Havana, Cuba
P. cubensis	FTBG	95615 E	Cub_FCBGb	Havana, Cuba
P. cubensis	FCN	FCN003A	Cub_FCN3	Honduras
P. cubensis	NBG	201001473*A	Cub_NBGa	Honduras
P. cubensis	NBG	201100984*A	Cub_NBGb	Mt. Coot-tha Botanical Garden, Australia
P. cubensis	WES	WES1-25	Cub_WES	Mt. Coot-tha Botanical Garden, Australia

Appendix A. (Continued) Accession data for morphological analyses.

Taxon	Locality	Institutional Accession Number	Abbreviated Name	Provenance
P. obtusa	FCN	FCN005A	Obt_FCN	*Unknown*
P. obtusa	NBG	201101183*A	Obt_NBGb	*Unknown*
P. obtusa	NBG	201101185*A	Obt_NBGc	*Unknown*
P. obtusa	NBG	201101185*D	Obt_NBGd	*Unknown*
P. obtusa	NBG	201101185*E	Obt_NBGe	*Unknown*
P. obtusa	NBG	201101185*F	Obt_NBGf	*Unknown*
P. obtusa	NBG	201301419*A	Obt_NBGg	*Unknown*
P. obtusa	WES	WES1-2	Obt_WESa	Hong Kong, China
P. obtusa	WES	WES1-11	Obt_WESb	Nong Nooch Tropical Garden, Thailand
P. obtusa	WES	WES1-15	Obt_WESc	*Unknown*
P. obtusa 'Kukulkan'	FCN	FCN010A	Obt_Kuk_FCN	Progreso (Merida), Yucatan
P. obtusa 'Kukulkan'	NBG	201400022*B	Obt_Kuk_NBG	Progreso (Merida), Yucatan
P. obtusa 'Marathon'	NBG	201100760*A	Obt_Mar_NBG	*Unknown*
P. obtusa 'Puerto Rico'	NBG	201100761*A	Obt_PR_NBG	Puerto Rico
P. obtusa 'Yucatan 3'	NBG	201001476*A	Obt_Yuc_NBG	Merida, Mexico
P. obtusa var. *obtusa*	FTBG	57356C	OVO_FCBG	Veradero, Cuba
P. obtusa var. *obtusa*	WES	WES1-5	OVO_WES	Nong Nooch Tropical Garden, Thailand
P. obtusa var. *sericifolia*	NBG	201401048*A	OVS_NBG	*Unknown*
P. pudica	NBG	200802035*B	Pud_NBG	*Unknown*
P. pudica	WES	WES1-12	Pud_WESa	Puerto Rico
P. pudica	WES	WES10-14	Pud_WESb	Puerto Rico
P. pudica 'Carambola Gardens'	NBG	201101417*A	Pud_CG_NBG	Honduras
P. rubra 'Celadine'	WES	WES7-14	Rub_Cel_WES	*Unknown*
P. rubra 'Dieudonne'	FTBG	2006-0776A	Rub_Die_FCBG	Unknown

Appendix A. (Continued) Accession data for morphological analyses.

Taxon	Locality	Accession Number	Abbreviated Name	Provenance
P. rubra 'Pedasi'	WES	WES10-20	Rub_Ped_WES	Panama
P. stenopetala	NBG	201000924*A	Ste_NBG	Bangkok, Thailand (purchased)
P. stenopetala	WES	WES1-10	Ste_WESd	Dominican Republic
P. stenopetala	WES	WES1-7	Ste_WESa	Queen Kapiolani Garden, Honolulu, HI
P. stenopetala	WES	WES1-8	Ste_WESb	Ho'omaluhia Botanical Garden, Kaneohe, HI
P. stenopetala	WES	WES1-9	Ste_WESc	Ho'omaluhia Botanical Garden, Kaneohe, HI
P. stenopetala 'Dolores'	NBG	201001478*A	Ste_Dol_NBG	Dominican Republic
P. stenophylla	WES	WES1-22	Sph_WESa	Nong Nooch Tropical Garden, Thailand
P. stenophylla	WES	WES1-26	Sph_WESb	Nong Nooch Tropical Garden, Thailand
P. stenophylla	WES	WES1-27	Sph_WESc	Nong Nooch Tropical Garden, Thailand
P. subsessilis	FTBG	2012-1993	Sub_FCBGa	La Vega, Dominican Republic
P. subsessilis	NBG	201301343*A	Sub_NBG	La Vega, Dominican Republic
P. subsessilis	WES	WES1-24	Sub_WES	Nong Nooch Tropical Garden, Thailand

Appendix A. (Continued) Collector, propagule, and voucher (institution) information.

Taxon & Accession No.	Collector	Propagule	Voucher No. & Institution
P. alba 201001479*A	L. Vannoorbeeck	Graft	*No voucher*
P. alba WES1-1	Staples, G.W. (1062)	Cutting	645803 (Bishop Museum)
P. alba WES1-30	J. Thielmann	Cutting	*No voucher*
P. bahamensis 201101141*A	*Unknown*	Seedling	*No voucher*
P. bahamensis 201101141*B	*Unknown*	Seedling	*No voucher*
P. bahamensis 201101141*C	*Unknown*	Seedling	*No voucher*
P. bahamensis WES1-17	J. Little	Seedling	774055 (Bishop Museum)
P. bahamensis WES10-21	J. Little	Seedling	*No voucher*
P. caracasana 201001474*A	*Unknown*	Graft	*No voucher*

Appendix A. (Continued) Collector, propagule, and voucher (institution) information.

Taxon & Accession No.	Collector	Propagule	Voucher No. & Institution
P. caracasana WES9-11	*Unknown*	Cutting	*No voucher*
P. caracasana MB06	*Unknown*	*Unknown*	*No voucher*
P. clusioides FCN002	M. Ferrero	Cutting	*No voucher*
P. clusioides 201100361*A	M. Ferrero	Cutting	*No voucher*
P. clusioides WESMK4	M. Ferrero	Cutting	775446 (Bishop Museum)
P. cubensis 95615 D	S. Zona	Cutting	*No voucher*
P. cubensis 95615 E	S. Zona	Cutting	*No voucher*
P. cubensis FCN003A	M. Ferrero	Cutting	*No voucher*
P. cubensis 201001473*A	M. Ferrero	Graft	*No voucher*
P. cubensis 201100984*A	R. Criley	Seed	*No voucher*
P. cubensis WES1-25	R. Criley	Graft	774048 (Bishop Museum)
P. obtusa FCN005A	*Unknown*	*Unknown*	*No voucher*
P. obtusa 201101183*A	*Unknown*	*Unknown*	*No voucher*
P. obtusa 201101185*A	*Unknown*	Seedling	*No voucher*
P. obtusa 201101185*D	*Unknown*	Seedling	*No voucher*
P. obtusa 201101185*E	*Unknown*	Seedling	*No voucher*
P. obtusa 201101185*F	*Unknown*	Seedling	*No voucher*
P. obtusa 201301419*A	H. Ford	Cutting	*No voucher*
P. obtusa WES1-2	J. Little	Cutting	774063 (Bishop Museum)
P. obtusa WES1-11	R. Eggenberger	Cutting	774057 (Bishop Museum)
P. obtusa WES1-15	R. Criley	Seedling	*No voucher*
P. obtusa ‘Kukulkan’ FCN010A	L. Vannoorbeeck	Cutting	*No voucher*
P. obtusa ‘Kukulkan’ 201400022*B	L. Vannoorbeeck	Cutting	*No voucher*
P. obtusa ‘Marathon’ 201100760*A	H. Ford	Seedling	*No voucher*
P. obtusa ‘Puerto Rico’ 201100761*A	L. Vanoorbeeck	Seedling	*No voucher*

Appendix A. (Continued) Collector, propagule, and voucher (institution) information.

Taxon & Accession No.	Collector	Propagule	Voucher No. & Institution
P. obtusa 'Yucatan 3' 201001476*A	L. Vannoorbeeck	Cutting	*No voucher*
P. obtusa var. *obtusa* 57356C	D. Seibert	Cutting	*No voucher*
P. obtusa var. *obtusa* WES1-5	R. Criley	Cutting	774062 (Bishop Museum)
P. obtusa var. *sericifolia* 201401048*A	*Unknown*	Graft	*No voucher*
P. pudica 200802035*B	*Unknown*	Graft	*No voucher*
P. pudica 201001478*A	H. Lazinger	Cutting	*No voucher*
P. pudica 201301343*A	H. Lazinger	Cutting	*No voucher*
P. pudica 'Carambola Gardens' 201101417*A	L. Vannoorbeeck	Graft	*No voucher*
P. rubra 'Celadine' WES7-14	R. Criley	Cutting	*No voucher*
P. rubra 'Dieudonne' 2006-0776A	L. Vannoorbeeck	Cutting	*No voucher*
P. rubra 'Pedasi' WES10-20	G. Hawkins	Cutting	774043 (Bishop Museum)
P. stenopetala 201000924*A	M. Ferrero	Graft	*No voucher*
P. stenopetala WES1-10	J. Lau	Seedling	774058 (Bishop Museum)
P. stenopetala WES1-7	R. Criley	Seedling	774061 (Bishop Museum)
P. stenopetala WES1-8	R. Criley	Seedling	774060 (Bishop Museum)
P. stenopetala WES1-9	R. Criley	Seedling	774059 (Bishop Museum)
P. stenopetala 'Dolores' 201001478*A	J. Fondeur	Cutting	*No voucher*
P. stenophylla WES1-22	K. Leonhardt	Cutting	774050 (Bishop Museum)
P. stenophylla WES1-26	M. Ferrero	Cutting	774047 (Bishop Museum)
P. stenophylla WES1-27	R. Criley	Graft	774046 (Bishop Museum)
P. subsessilis 2012-1993	J. Lopez	Cutting	*No voucher*
P. subsessilis 201301343*A	J. Lopez	Cutting	*No voucher*
P. subsessilis WES1-24	R. Criley	Graft	774049 (Bishop Museum)

Appendix B. Morphological matrix of taxa and characters used in principal component analyses. Character numbers are defined in Appendix C.

Taxa	Accession Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
P. alba	201001479*A	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1	0	1	0	0
P. alba	WES1-1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1	0	1	0	0
P. alba	WES1-30	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1	0	1	0	0
P. bahamensis	201101141*A	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	1	0
P. bahamensis	201101141*B	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	0	1	0
P. bahamensis	201101141*C	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0
P. bahamensis	WES10-21	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0
P. bahamensis	WES1-17	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1
P. caracasana	201001474*A	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0
P. caracasana	WES9-11	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0
P. caracasana	MB06	0	0	1	0	0	0	1	0	1	0	0	0	0	0	0	0	0	1	0
P. clusioides	201100361*A	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1
P. clusioides	WESMK4	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1
P. clusioides	FCN002	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1
P. cubensis	95615 D	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1
P. cubensis	95615 E	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1
P. cubensis	FCN003A	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1
P. cubensis	201001473*A	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	0
P. cubensis	201100984*A	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1
P. cubensis	WES1-25	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
P. obtusa	FCN005A	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0
P. obtusa	201101183*A	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	1	0
P. obtusa	201101185*A	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0

Appendix B. (Continued) Morphological matrix of taxa and characters used in principal component analyses.

Taxa	Accession Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
P. obtusa	201101185*D	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0
P. obtusa	201101185*E	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0
P. obtusa	201101185*F	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0
P. obtusa	201301419*A	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0
P. obtusa	WES1-11	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0
P. obtusa	WES1-15	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0
P. obtusa	WES1-2	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1
P. obtusa var. *obtusa*	57356C	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0
P. obtusa var. *obtusa*	WES1-5	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	0
P. obtusa var. *sericifolia*	201401048*A	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0
P. obtusa 'Puerto Rico'	201100761*A	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0
P. obtusa 'Yucatan3'	201001476*A	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
P. obtusa 'Kukulkan'	FCN010A	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0
P. obtusa 'Kukulkan'	201400022*B	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	0
P. obtusa 'Marathon'	201100760*A	0	0	0	0	1	0	1	0	0	0	0	1	0	0	1	0	0	1	1
P. pudica	200802035*B	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	1	0
P. pudica	WES10-14	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	1	0
P. pudica	WES1-12	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	1	0

Appendix B. (Continued) Morphological matrix of taxa and characters used in principal component analyses.

Taxa	Accession Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
P. pudica 'Carambola Gardens'	201101417*A	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	1	0
P. rubra 'Celadine'	WES7-14	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0
P. rubra 'Dieudonne'	2006-0776 A	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0
P. rubra 'Pedasi'	WES10-20	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0
P. stenopetala	201000924*A	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
P. stenopetala	WES1-10	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
P. stenopetala	WES1-7	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0
P. stenopetala	WES1-8	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0
P. stenopetala	WES1-9	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0
P. stenopetala 'Dolores'	201001478*A	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0
P. stenophylla	WES1-26	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
P. stenophylla	WES1-22	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
P. stenophylla	WES1-27	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	1
P. subsessilis	2012-1993	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0
P. subsessilis	201301343*A	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0
P. subsessilis	WES1-24	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0

Appendix B. (Continued) Morphological matrix of taxa and characters used in principal component analyses.

Taxa	Accession Number	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
P. alba	201001479*A	1	1	0	1	0	0	1	0	0	1	1	1	0	1	1	1	0
P. alba	WES1-1	1	1	0	1	0	0	1	0	0	1	1	1	0	1	1	1	0
P. alba	WES1-30	1	1	0	1	0	0	1	0	0	1	1	1	0	1	1	1	0
P. bahamensis	201101141*A	1	1	0	1	0	1	0	0	0	1	0	1	0	1	1	1	0
P. bahamensis	201101141*B	1	1	0	1	0	1	0	0	0	1	0	1	0	1	1	1	0
P. bahamensis	201101141*C	1	1	0	1	0	1	0	0	0	1	0	1	0	1	1	1	0
P. bahamensis	WES10-21	1	1	0	0	0	1	0	0	1	1	0	1	0	1	0	1	0
P. bahamensis	WES1-17	1	1	0	1	0	1	0	0	1	1	1	1	0	1	1	1	0
P. caracasana	201001474*A	1	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0
P. caracasana	WES9-11	1	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0
P. caracasana	MB06	1	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0
P. clusioides	201100361*A	0	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	1
P. clusioides	WESMK4	0	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	1
P. clusioides	FCN002	0	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	1
P. cubensis	95615 D	1	1	0	1	0	0	1	0	0	0	0	0	0	1	1	1	0
P. cubensis	95615 E	1	1	0	0	0	0	1	0	0	1	0	1	0	1	1	1	0
P. cubensis	FCN003A	0	1	0	1	1	0	0	0	0	0	0	0	0	1	1	1	0
P. cubensis	201001473*A	1	1	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0
P. cubensis	201100984*A	1	1	0	0	0	1	0	0	0	1	0	1	0	1	1	1	0
P. cubensis	WES1-25	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	1	0
P. obtusa	FCN005A	1	1	0	1	1	0	0	0	0	1	0	1	0	1	1	0	0
P. obtusa	201101183*A	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0
P. obtusa	201101185*A	1	1	0	1	0	1	0	0	0	0	0	0	0	1	1	1	0

Appendix B. (Continued) Morphological matrix of taxa and characters used in principal component analyses.

Taxa	Accession Number	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
P. obtusa	201101185*D	1	1	0	1	0	1	0	0	0	0	0	0	0	1	0	1	0
P. obtusa	201101185*E	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0
P. obtusa	201101185*F	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0
P. obtusa	201301419*A	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0
P. obtusa	WES1-11	1	1	0	1	0	0	1	0	0	1	0	1	0	1	1	1	0
P. obtusa	WES1-15	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
P. obtusa	WES1-2	1	1	0	1	0	0	0	1	0	1	0	1	0	1	0	1	0
P. obtusa var. *obtusa*	57356C	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0
P. obtusa var. *obtusa*	WES1-5	0	1	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0
P. obtusa var. *sericifolia*	201401048*A	1	1	0	0	0	1	0	0	0	1	0	1	0	1	1	0	0
P. obtusa 'Kukulkan'	FCN010A	1	1	0	1	0	1	0	0	1	1	0	1	0	1	1	0	0
P. obtusa 'Kukulkan'	201400022*B	1	1	0	0	0	1	0	0	1	1	0	1	0	1	0	1	0
P. obtusa 'Marathon'	201100760*A	1	1	0	0	0	0	1	0	0	1	1	1	0	1	1	0	0
P. obtusa 'Puerto Rico'	201100761*A	1	1	0	1	0	0	1	0	0	1	1	1	0	1	1	0	0
P. obtusa 'Yucatan3'	201001476*A	1	1	0	1	0	0	1	0	0	1	0	1	0	1	0	1	0

Appendix B. (Continued) Morphological matrix of taxa and characters used in principal component analyses.

Taxa	Accession Number	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
P. pudica	200802035*B	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0
P. pudica	WES10-14	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0
P. pudica	WES1-12	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0
P. pudica 'Carambola Gardens'	201101417*A	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0
P. rubra 'Celadine'	WES7-14	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0
P. rubra 'Dieudonne'	2006-0776 A	1	1	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0
P. rubra 'Pedasi'	WES10-20	1	1	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0
P. stenopetala	201000924*A	1	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0
P. stenopetala	WES1-10	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
P. stenopetala	WES1-7	1	1	0	1	0	0	0	1	0	0	0	1	0	1	0	1	0
P. stenopetala	WES1-8	1	1	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0
P. stenopetala	WES1-9	1	1	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0
P. stenopetala 'Dolores'	201001478*A	1	1	0	0	0	0	0	1	0	1	0	1	0	1	0	1	0
P. stenophylla	WES1-26	1	1	0	1	0	0	0	1	0	0	0	0	0	1	0	1	1
P. stenophylla	WES1-22	1	1	0	1	0	0	0	1	0	0	0	0	0	1	0	1	1
P. stenophylla	WES1-27	1	1	0	1	0	0	0	1	0	0	0	0	0	1	0	1	1
P. subsessilis	2012-1993	1	0	1	0	0	0	0	1	0	0	0	1	1	0	0	1	0
P. subsessilis	201301343*A	1	0	1	0	0	0	0	1	0	0	0	1	1	0	0	1	0
P. subsessilis	WES1-24	1	1	0	1	0	0	1	0	0	0	0	1	0	0	0	1	0

Appendix B. (Continued) Morphological matrix of taxa and characters used in principal component analyses.

Taxa	Accession Number	37	38	39	40	41	42	43
P. alba	201001479*A	1	0	0	0	0	0	1
P. alba	WES1-1	1	0	0	0	0	0	1
P. alba	WES1-30	1	0	0	0	0	0	1
P. bahamensis	201101141*A	1	0	0	0	0	0	1
P. bahamensis	201101141*B	1	0	0	0	0	0	1
P. bahamensis	201101141*C	1	0	0	0	0	0	1
P. bahamensis	WES10-21	1	0	0	0	0	0	1
P. bahamensis	WES1-17	1	0	1	0	1	0	1
P. caracasana	201001474*A	1	0	0	0	1	0	0
P. caracasana	WES9-11	1	0	0	0	0	0	1
P. caracasana	MB06	1	0	0	0	0	0	1
P. clusioides	201100361*A	0	1	0	0	1	0	0
P. clusioides	WESMK4	0	1	0	0	1	0	0
P. clusioides	FCN002	0	1	0	0	1	0	0
P. cubensis	95615 D	1	1	0	0	0	0	0
P. cubensis	95615 E	1	0	0	0	1	0	0
P. cubensis	FCN003A	0	1	0	0	0	0	1
P. cubensis	201001473*A	0	1	0	0	0	0	1
P. cubensis	201100984*A	1	0	0	0	1	0	1
P. cubensis	WES1-25	0	1	0	0	0	0	1
P. obtusa	FCN005A	1	0	0	0	1	0	1
P. obtusa	201101183*A	1	0	0	0	1	0	1
P. obtusa	WES1-11	1	0	1	1	1	0	1

Appendix B. (Continued) Morphological matrix of taxa and characters used in principal component analyses.

Taxa	Accession Number	37	38	39	40	41	42	43
P. obtusa	WES1-15	1	0	1	0	0	0	0
P. obtusa	WES1-2	1	0	1	1	1	0	0
P. obtusa	201101185*A	1	0	0	0	1	0	1
P. obtusa	201101185*D	0	1	0	0	1	0	1
P. obtusa	201101185*E	1	0	0	0	0	0	1
P. obtusa	201101185*F	0	1	0	0	1	0	1
P. obtusa	201301419*A	0	0	0	0	1	0	1
P. obtusa var. *obtusa*	57356C	1	0	0	0	0	0	1
P. obtusa var. *obtusa*	WES1-5	1	0	0	0	0	0	1
P. obtusa var. *sericifolia*	201401048*A	1	0	1	1	1	0	0
P. obtusa ‘Kukulkan’	FCN010A	1	0	0	0	0	0	1
P. obtusa ‘Kukulkan’	201400022*B	1	0	1	0	1	0	1
P. obtusa ‘Marathon’	201100760*A	1	0	0	0	1	0	1
P. obtusa ‘Puerto Rico’	201100761*A	1	0	0	1	1	0	1
P. obtusa ‘Yucatan3’	201001476*A	1	0	0	0	0	0	0
P. pudica	200802035*B	1	0	0	0	0	1	0
P. pudica	WES10-14	1	0	0	0	0	1	0
P. pudica	WES1-12	1	0	0	0	0	1	0
P. pudica ‘Carambola Gardens’	201101417*A	1	0	0	0	0	1	0
P. rubra ‘Celadine’	WES7-14	1	0	0	0	0	0	1
P. rubra ‘Dieudonne’	2006-0776 A	1	0	0	0	0	0	0
P. rubra ‘Pedasi’	WES10-20	1	0	0	0	0	0	1
P. stenopetala	201000924*A	1	0	0	0	1	0	1

Appendix B. (Continued) Morphological matrix of taxa and characters used in principal component analyses.

Taxa	Accession Number	37	38	39	40	41	42	43
P. stenopetala	WES1-10	1	0	0	0	1	0	1
P. stenopetala	WES1-7	1	0	0	0	1	0	1
P. stenopetala	WES1-8	1	0	0	0	0	0	1
P. stenopetala	WES1-9	1	0	0	0	0	0	1
P. stenopetala 'Dolores'	201001478*A	1	0	0	0	0	0	0
P. stenophylla	WES1-26	0	0	0	0	0	0	1
P. stenophylla	WES1-22	0	0	0	0	0	0	1
P. stenophylla	WES1-27	0	0	0	0	0	0	1
P. subsessilis	2012-1993	1	0	0	0	0	0	0
P. subsessilis	201301343*A	1	0	0	0	0	0	0
P. subsessilis	WES1-24	1	0	0	1	1	0	1

Appendix C. Morphological characters, character states, and definitions of terminology used, based on Harris and Harris (2011) and Staples and Herbst (2005). Numbers and abbreviations in parentheses correspond to how characters are named in the morphological matrix (Appendix B).

Leaf Shape Characters

(1) Obelliptic leaf shape (Obelliptic): 0=absence, 1=presence
Almost in the shape of a narrow oval, but with the distal end somewhat larger than the proximal end.

(2) Lanceolate leaf shape (Lanceolate): 0=absence, 1=presence
Lance-shaped, but much longer than wide, with the widest point below the middle of the leaf near the proximal end (near the petiole).

(3) Oblanceolate leaf shape (Oblanceolate): 0=absence, 1=presence
Inversely lanceolate, with the attachment at the narrower end.

(4) Oblong leaf shape (Oblong): 0=absence, 1=presence
Leaves two to four times longer than broad with nearly parallel sides.

(5) Elliptical leaf shape (Elliptical): 0=absence, 1=presence
In the shape of an ellipse or narrow oval; broadest at the middle and narrower at the two equal ends.

(6) Spatulate leaf shape (Spatulate): 0=absence, 1=presence
Leaves shaped like a spatula with a rounded blade above gradually tapering to the base.

(7) Recurved leaves (Recurved_Lf): 0=absence, 1=presence
Entire leaves curved downward.

Leaf Apex Characters

(8) Acute leaf apex (Acute): 0=absence, 1=presence
Leaf tips that taper to a pointed apex with more or less straight sides.

(9) Acuminate leaf apex (Acuminate): 0=absence, 1=presence
Gradually tapering to a sharp point and forming concave sides along the tip.

(10) Obtuse leaf apex (Obtuse): 0=absence, 1=presence
Blunt or rounded at the apex, with the sides coming together at the apex at an angle greater than 90 degrees.

(11) Emarginate leaf apex (Emarginate): 0=absence, 1=presence
Leaf apex with a notch at the tip.

(12) Retuse leaf apex (Retuse): 0=absence, 1=presence
Leaf apex with a shallow notch in a round or blunt apex.

(13) Cordate-acuminate leaf apex (Cordate-acuminate): 0=absence, 1=presence
A special condition of *Plumeria* leaves, specifically of *P. pudica*, in which the outline of leaf apices resemble an inverted heart-shaped in appearance, but with elongated points. A vast majority of *P. pudica* specimens that were examined appear to have cordate leaf apices, yet in some herbarium specimens leaf apices are more acuminate than cordate.

(14) Mucronate leaf apex (Mucronate): 0=absence, 1=presence
A protrusion at the leaf apex, straight and stiff, and sharply pointy to the touch.

(15) Mucronulate leaf apex (Mucronulate): 0=absence, 1=presence
A protrusion at the leaf apex, broader than long; straight and blunt to the touch.

Leaf Base Characters

(16) Equilateral leaf bases (Equilateral): 0=absence, 1=presence
Leaf bases that are equal-sided.

(17) Oblique leaf bases (Oblique): 0=absence, 1=presence
Leaf bases with unequal sides, somewhat slanting.

(18) Attenuate leaf bases (Attenuate): 0=absence, 1=presence
Leaf bases tapering gradually to a narrow base.

(19) Cuneate leaf bases (Cuneate): 0=absence, 1=presence
Wedge-shaped, triangular and tapering to a point at the base.

Appendix C. (Continued) Morphological characters, character states, and definitions of terminology used.

(20) Connivent at leaf base (Connivent): 0=absence, 1=presence
Adaxial lamina appearing to almost touch (converge) at the petiole juncture. Johnston (1912) describes this character state as decurrent into the petiole.

(21) Leaf attachment (Leaf_Attachment): 0=subsessile, 1=petiolate
Some *Plumeria* taxa possess petioles that are almost sessile (subsessile), while other taxa have petiolate leaf attachment. In his descriptions of *Plumeria* spp., De Candolle (1844) uses this character to describe the leaf attachment of *P. subsessilis*. Woodson (1938a) used this character to describe *P. pudica*.

Leaf Margin Characters

(22) Pink leaf margins (Pink_Margin): 0=absence, 1=presence
The younger leaves of *P. subsessilis* and *P. clusioides* leaves appear to possess this character, which fades with over time becoming almost absent at maturity. Some specimens of *P. rubra* also possess this character.

(23) Leaf margins (Leaf_Margin): 0=undulate, 1=entire
Many live specimens that were examined, especially those belonging to Woodson's "*P. obtusa* complex" appeared to have entire margins, whereas other taxa display leaf margins that are shallowly and smoothly indented (undulate) when viewed in a vertical orientation.

(24) Flat leaf orientation (Flat_Orientation): 0=absence, 1=presence
Leaf margin is not deflected upwards or downwards.

(25) Recurved leaf margins (Recurved_Margin): 0=absence, 1=presence
Sometimes referred to as reduplicate or declined leaf margins. Portions of the lamina are deflected downward, toward the underside of the leaf but not rolled under. Many of the taxa that Woodson (1938) subsumed under his evaluation of *P. obtusa* show this character, especially toward the apex of leaves.

(26) Revolute leaf margins (Revolute_Margin): 0=absence, 1=presence
Leaf margin is curled downwards and generally rolled inwards to the midrib of the leaf. Revolute leaf margins are a constant character in descriptions of *P. alba*, but we have observed this character on other species.

(27) Conduplicate leaf ptyxis (Conduplicate_Ptyxis): 0=absence, 1=presence
Sometimes referred to as incurved leaf orientation. Leaf margin is deflected upwards so that laminar surfaces on both sides of the midrib are oriented toward each other. To our knowledge, this is the first time this character has been used to evaluate species in the genus *Plumeria*.

Leaf Surface Characters

(28) Adaxial leaf texture (Glabrous_or_ScabrousAD): 0=glabrous, 1=scabrous
Adaxial leaf surfaces are either smooth to the touch (glabrous) or rough to the touch (scabrous).

(29) Abaxial leaf texture (Glabrous_or_CoriaceousAB): 0=glabrous, 1=coriaceous
Some taxa have abaxial leaf surfaces that are smooth to the touch (glabrous), devoid of trichomes, whereas the abaxial surfaces of other taxa have the texture of leather (coriaceous), due to the presence of trichomes. Many species of *Plumeria* have been described based on the presence or absence of leaf indument (Standley, 1924). Britton (1915) referred to this character as lanate.

(30) Puckering of adaxial surface (Puckering): 0=absence, 1=presence
Puckering is a character used to describe laminal surfaces between secondary veins, giving a raised or bubbling appearance to adaxial surfaces of leaves. Woodson (1938) observed this feature on *P. alba* specimens, but we also have observed this feature in taxa of *P. obtusa*.

Leaf Venation Characters

(31) Tomentose midvein (Tomentose_Midvein): 0=absence, 1=presence
Midveins covered with dense, interwoven trichomes occurred on midveins of some taxa. In his description of *Plumeria*, Woodson (1938b) described this character as pilosulous.

Appendix C. (Continued) Morphological characters, character states, and definitions of terminology used.

(32) Midvein pink in coloration (Pink_Midvein): 0=absence, 1=presence
A character that exists in only *P. subsessilis* and *P. clusioides*. This feature is most prominent in younger leaves but fades with maturity, appearing faintly or completely absent in mature leaves.

(33) Secondary venation entry to the midrib (Decurrent_or_DirectSV): 0=decurrent, 1=direct
Decurrent secondary venation was a character that Woodson (1938a) used to describe the way in which secondary veins entered the midrib in leaves of *P. subsessilis* and *P. rubra*. Occasionally, this character was also observed in leaves of other taxa.

(34) Angle of secondary venation to primary vein (Angular_or_PerpendicularSV): 0=angular, 1=perpendicular
The angle of secondary venation appeared to be an important feature among species within this genus (Grisebach, 1864; Britton, 1910; Britton and Millspaugh, 1920; Stahl, 1937). We acknowledge two types. Perpendicular venation is a character state in which secondary veins are oriented in a perpendicular angle to the midvein. Venation patterns that were not deemed perpendicular were considered as angular secondary venation.

(35) Arrangement of secondary venation (Opposite_or_AlternateSV): 0=opposite, 1=alternate
This character describes the secondary venation patterns along the midrib. Some taxa appear to have opposite venation patterns in a manner similar to opposite phyllotaxis, whereas other live specimens that we examined appear to have an alternating secondary venation pattern.

(36) Sunken vs. raised adaxial venation (Sunken_or_RaisedADv): 0=sunken, 1=raised
Secondary venation of certain taxa appears to have a sunken or appressed appearance of veins on the adaxial surface. Other specimens appear to have raised adaxial venation.

(37) Sunken vs. raised abaxial venation (Sunken_or_RaisedABv): 0=sunken, 1=raised
A similar occurrence of raised or sunken venation on the abaxial surfaces were observed. It should be noted that the character for sunken vs. raised venation on the upper (adaxial) surface was not correlated with sunken vs. raised venation on the lower (abaxial) surface. That is, secondary venation can appear to be sunken or appressed on both laminal surfaces on some taxa.

(38) Inconspicuous abaxial venation (InconspicuousABv): 0=absence, 1=presence
Some leaves of *Plumeria* taxa, such as *P. clusioides*, do not show definitive raised or sunken venation (Grisebach et al., 1863; Leon and Alain, 1957) on the abaxial surface of leaves, but still show venation that is translucent. Other accessions, as in some samples of *P. cubensis*, show secondary venation that is appears appressed, and not as definitively raised as the venation of *P. obtusa* and *P. rubra*. We collectively refer to these venation characters as inconspicuous abaxial venation.

(39) Prominent marginal vein on abaxial surface (Prominent_MargVein): 0=absence, 1=presence
Certain taxa of the *P. obtusa* complex appear to possess this character in which secondary veins anastamose to a common marginal vein that is very prominent on the underside of leaves.

Trunk and Growth Characters

(40) Prominent tubercles on main trunk axis (Tubercles_Trunk): 0=absence, 1=presence
Tubercles are formed from leaf scars (cicatrices), which give many species of this genus a knobbed appearance to branches. However, only a few taxa in this genus possess tuberculous protrusions, giving trunks a densely studded appearance.

(41) Prominent tubercles on branches (Tubercles_Branch): 0=absence, 1=presence
Tubercle-like projections can also occur on the stems/branches of certain *Plumeria* taxa.

(42) Growth habit (Growth_Habit): 0=open, 1=columnar
Most taxa in this genus tend to exhibit a sprawling growth pattern, resulting from lateral branching, which produces a spreading growth habit. On the other hand, taxa of *P. pudica* appear to have a more upright, columnar growth habit.

(43) Follicle Occurrence (Follicle_Occurrence): 0=rare, 1=frequent
On certain specimens, developing or dehisced seedpods were rarely observed. On other specimens, multiple seedpods (developing and/or dehisced) were observed to occur on a plant. Sometimes multiple seedpods were observed developing from a single inflorescence.

Appendix D. Accession data for molecular analyses, including live plant materials and Genbank accession numbers for downloaded sequences. Collection locality abbreviations are as follows: UH = University of Hawaii at Manoa campus; FCN = Florida Colors Nursery (Homestead, FL); NTBG = National Tropical Botanical Gardens (PTBG) (Koloa, Kaua'i); WES = Waimanalo Experiment Station (Waimanalo, O'ahu); NBG = Naples Botanical Garden (Naples, FL); FTBG = Fairchild Tropical Botanic Garden (Coral Gables, FL); WA = Waimea Arboretum (Pupukea, O'ahu).

Taxon	Accession No.	Collection Locality	Provenance Data
P. alba	CM03	UH	Collected by C. Morici in the Canary Islands.
P. alba	FCN001A	FCN	Collected by L. Vanoorbeeck from Roatan, Honduras.
P. alba	FCN001B	FCN	Collected by M. Ferrero from the wild.
P. alba	NTBG970401003	NTBG	Origin unknown; seedling of Waimea Arboretum (Oahu) accession no. 97s73.
P. alba	WES1-1	WES	Collected by G.W. Staples & R. Criley (coll. No. 1062) from Waimanalo (containerized tree); Voucher 645803 in Bishop Museum.
P. alba	NBG201001479A	NBG	Collected as grafted plant from Florida Colors Nursery (Florida).
P. alba	WES1-30	WES	Collected by G. Thielman in Santa Barbara, CA.
P. bahamensis	NBG201101141B	NBG	Collector and origin unknown; received on Aug. 30, 2011.
P. bahamensis	NBG201101141C	NBG	Collector and origin unknown; received on Aug. 30, 2011.
P. bahamensis	WES1-17	WES	Collected by G. Stokes from Nassau, The Bahamas, given to J. Little, given to R. Criley; Voucher 774055 in Bishop Museum.
P. bahamensis	WES10-21	WES	
P. caracasana	NTBGMB06	NTBG	Unknown
P. caracasana	NBG201001474A	NBG	Collector likely L. Vanoorbeeck from Florida Colors Nursery (Florida); received on Aug. 31, 2010.
P. caracasana	UH	UH	
P. caracasana	WES1-6	WES	Most likely collected from J. Little from Nong Nooch Tropical Garden (Thailand).
P. caracasana	WES9-11	WES	
P. caracasana	WESMK2	WES	Collected by R. Criley from Nong Nooch Tropical Garden (Thailand); Voucher 775447 in Bishop Museum.
P. clusioides	FCN002A	FCN	Collected by L. Vanoorbeeck likely from Cuba.
P. clusioides	NBG201100361A	NBG	Received from Florida Colors Nursery on Apr. 19, 2011.
P. clusioides	WESMK4	WES	Collected as cutting by R. Criley from Florida Colors Nursery (Florida); Voucher 775446 in Bishop Museum.
P. cubensis	FTBG95615B	FTBG	Collected by S. Zona from Jardin Botanico Nacional (Cuba)
P. cubensis	FTBG95615D	FTBG	Collected by S. Zona from Jardin Botanico Nacional (Cuba)
P. cubensis	FTBG95615E	FTBG	Collected by S. Zona from Jardin Botanico Nacional (Cuba)
P. cubensis	FCN003A	FCN	Collected by M. Ferrero from Cuba in the wild.

Appendix D. (Continued) Accession data for live plant materials.

Taxon	Accession No.	Collection Locality	Provenance Data
P. cubensis	NBG201001473A	NBG	Received as a grafted plant from Florida Colors Nursery on Aug. 31, 2010.
P. cubensis	NBG201100984A	NBG	Received from University of Hawaii as seed on Aug. 20, 2011.
P. cubensis	WES1-25	WES	Collected by R. Criley from Mt. Coot-tha Botanic Garden (Australia); Duplicate accession at Waimea Arboretum no. 8255; Voucher 774048 in Bishop Museum.
P. ekmanii	CM08	UH	Collected by C. Morici from the Canary Islands.
P. ekmanii	WES1-14	WES	Collected by C. Morici from Palmetum en Tenerife, originally collected from Mt. El Yunque, Cuba.
P. filifolia	CM05	UH	Collected by C. Morici from the Canary Islands.
P. filifolia	FCN012A	FCN	Collected by M. Ferrero from Cuba in the wild.
P. filifolia	MF01	UH	Collected by M. Ferrero from the wild.
P. montana 'Alta'	CM09	UH	Collected by C. Morici from the Canary Islands.
P. montana 'Baja'	CM10	UH	Collected by C. Morici from the Canary Islands.
P. montana	FCN004A	FCN	Collected by M. Ferrero from Cuba in the wild.
P. montana	WES1-29	WES	Collected by M. Ferrero from Nong Nooch Tropical Garden (Thailand); Voucher 774044 in Bishop Museum.
P. obtusa	FCN005A	FCN	Unknown
P. obtusa	FCN005B	FCN	Unknown
P. obtusa 'Kukulkan'	FCN010A	FCN	Collected by C. Vanoorbeeck from Progresso, Mexico.
P. obtusa 'Marathon'	NBG201100760A	NBG	Collected as seed by H. Ford on July 13, 2011.
P. obtusa	NBG201100509A	NBG	Origin unknown; received in 2008.
P. obtusa	NBG201101185A	NBG	Unknown
P. obtusa	NBG201101185D	NBG	Unknown
P. obtusa	NBG201101185E	NBG	Unknown
P. obtusa	NBG201101185F	NBG	Unknown
P. obtusa	NBG201301419A	NBG	Collected by H. Ford as a plant on Aug. 13, 2013.
P. obtusa 'Puerto Rico'	NBG201100761A	NBG	Received as a seedling from Florida Colors Nursery (Florida) on Jul. 13, 2011.
P. obtusa var. *obtusa*	FTBG57356B	FTBG	Collected by Dr. Seibert from Veradero, Cuba

Appendix D. (Continued) Accession data for live plant materials.

Taxon	Accession No.	Collection Locality	Provenance Data
P. obtusa var. *obtusa*	FTBG57356C	FTBG	Collected by Dr. Seibert from Veradero, Cuba
P. obtusa var. *obtusa*	NTBG860059	NTBG	Collected in 1986 from Jardin Botanico Nacional (Cuba).
P. obtusa var. *obtusa*	WES1-5	WES	Collected by R. Criley from Nong Nooch Tropical Garden (Thailand); Voucher 774062 in Bishop Museum.
P. obtusa var. *sericifolia*	NBG201401048A	NBG	Received as a grafted plant from Florida Colors Nursery (Florida) on Mar. 11, 2014.
P. obtusa var. *sericifolia*	WA75c2132	WA	Collected by J. Lau from the Dominican Republic; accession no. 75c2132 at Waimea Arboretum; Voucher 533676 in Bishop Museum.
P. obtusa var. *sericifolia*	WES1-4	WES	Collected by C. Morici from seed from Palmetum Tenerife; Duplicate accession at Waimea Arboretum no. 76S775. Previously named *P. tuberculata* in WES.
P. obtusa	WES1-2	WES	Collected by J. Little from a hotel planting in Hong Kong, China; Voucher 774063 in Bishop Museum.
P. obtusa	WES1-24	WES	Collected by M. Ferrero from Nong Nooch Tropical Garden (Thailand); accessioned in NNTG as *P. subsessilis*; Duplicate accession at Waimea Arboretum no. 15940
P. obtusa 'Yucatan'	NBG201400022A	NBG	Collected by L. Vannoorbeeck from Progreso, Yucatan; received as a plant in 2009.
P. pudica 'Carambola Gardens'	NBG201101417A	NBG	Received as a grafted plant from L. Vannoorbeeck; most likely an F1 of P. pudica; received on Dec. 6, 2011.
P. pudica	NBG200802035B	NBG	Origin unknown; received as a grafted plant in 2006.
P. pudica	NTBG50318002	NTBG	Collected by D. Orr from Waimea Arboretum (Oahu) accession no. 01p82 in Aug. 25, 2005, received at Waimea Arboretum from J. Little.
P. pudica	NTBG50318004	NTBG	Collected by D. Orr from Waimea Arboretum (Oahu) accession no. 01p82 in Aug. 25, 2005, received at Waimea Arboretum from J. Little.
P. pudica	NTBG50318006	NTBG	Collected by D. Orr from Waimea Arboretum (Oahu) accession no. 01p82 in Aug. 25, 2005, received at Waimea Arboretum from J. Little.
P. pudica	UH	UH	Collected from seed by R. Criley.

Appendix D. (Continued) Accession data for live plant materials.

Taxon	Accession No.	Collection Locality	Provenance Data
P. pudica	WES1-12	WES	Collected by J. Little from Nong Nooch Tropical Garden (Thailand).
P. pudica	WES10-14	WES	Collected by H. Lazinger from Puerto Rico.
P. rubra 'CyndiMoragne'	WES2-8	WES	
P. rubra 'JLMoragne' seedling	WES2-15	WES	Collected as a seedling by R. Criley from J. Little.
P. rubra 'KimiMoragne'	WES4-30	WES	
P. rubra 'MaryMoragne'	WES2-3	WES	
P. rubra 'Moragne93'	WES2-10	WES	
P. rubra 'Pedasi'	WES10-20	WES	Collected by J. Thielman, possibly from Panama.
P. rubra	USDA139505-10619	USDAMIA	
P. sp. 'Isabella'	WES1-21	WES	Collected by R. Criley from Nong Nooch Tropical Garden (Thailand).
P. sp. 'Isabella' seedling	WESMU67	WES	Seedling of *P.* sp. 'Isabella' collected by R. Criley (WES1-21).
P. sp. 'Isabella' seedling	WESMU68	WES	Seedling of *P.* sp. 'Isabella' collected by R. Criley (WES1-21).
P. sp. 'Isabella' seedling	WESMU69	WES	Seedling of *P.* sp. 'Isabella' collected by R. Criley (WES1-21).
P. sp. 'Isabella' seedling	WESMU70	WES	Seedling of *P.* sp. 'Isabella' collected by R. Criley (WES1-21).
P. stenopetala 'Dolores'	NBG201001478A	NBG	Received from L. Vanoorbeeck on Aug. 31, 2010; formerly from D. Fugina; originally collected by J. Fondeur from the Dominican Republic.
P. stenopetala	FCN006A	FCN	Collected by C. Vanoorbeeck purchased from Bangkok, Thailand.
P. stenopetala	NBG201000924A	NBG	Received from Florida Colors Nursery as a grafted plant on Jun.4, 2010; originally from a cultivated plant in Bangkok, Thailand.
P. stenopetala	WES1-7	WES	Collected by R. Criley from Queen Kapiolani Garden, Waikiki, Oahu; Voucher 774061 in Bishop Museum.

Appendix D. (Continued) Accession data for live plant materials.

Taxon	Accession No.	Collection Locality	Provenance Data
P. stenopetala	WES1-8	WES	Collected as seed by R. Criley from Ho'omaluhia Botanical Garden (Oahu); Voucher 774060 in Bishop Museum.
P. stenopetala	WES1-9	WES	Collected as seed by R. Criley from Ho'omaluhia Botanical Garden (Oahu); Voucher 774059 in Bishop Museum.
P. stenopetala	WES1-10	WES	Collected as seed by R. Criley from Ho'omaluhia Botanical Garden (Oahu); Voucher 774058 in Bishop Museum.
P. stenophylla	WES1-27	WES	Collected by R. Criley from Mt. Coot-tha Botanic Garden (Australia) in 2007; formerly accessioned as *P. filifolia;* Duplicate accession at Waimea Arboretum no. 15931; Voucher 774046 in Bishop Museum.
P. stenophylla	WES1-28	WES	Collected by C. Elhardt from Guantanamo, Cuba in 2006; Voucher 774045 in Bishop Museum.
P. subsessilis	FTBG2012-1993	FTBG	Collected by J.F. Lopez from La Vega, Dominican Republic in July 2012; Duplicated in Naples Botanical Garden (Florida).
P. subsessilis	NBG201301343A	NBG	Collected from seed by J. Lopez (Fairchild Tropical Botanic Garden) from the wild in Dominican Republic; received on Aug. 6, 2013.
P. tuberculata	FCN007A	FCN	Unknown
P. tuberculata	WA76s755	WA	
Stemmadenia litoralis	UH	UH	
Tabernaemontana divaricata	UH	UH	
Neisosperma oppositifolia	NTBG050364007	NTBG	Collected by D. Orr from Waimea Arboretum (Oahu) accession no. 01p82 in Aug. 25, 2005, received at Waimea Arboretum from J. Little.

Appendix D. (Continued) Genbank accession data for sequences downloaded from NCBI.

	Genbank Accession Numbers				
NCBI Taxon Sampled	***ITS2***	***matK***	***rpl32-trnL***	***psbJ-petA***	***trnH-psbA***
Alstonia rostrata	KR531723.1				
Alstonia scholaris	KR531723.2		MG963247	MG963247	
Neisosperma acuminatum	KR531723.3				
Ochrosia borbonica	KR531723.4				
Ochrosia oppositifolia	KR531723.5				
Plumeria rubra	KR531723.10				
Plumeria rubra	KR531723.11				
Rauvolfia verticillata	KR531723.14				
Rauvolfia densiflora	KR531723.12				
Plumeria rubra	KR531723.9, KR531723.10, KR531723.11	Z70191			
Rauvolfia verticillata	KR531723.13	KT955398			
Plumeria alba		FJ754255			KJ426885
Plumeria cubensis		DQ660536, MG963231			
Rauvolfia sellowii		DQ660537			
Rauvolfia semperflorens		KT955393			
Rauvolfia viridis		KT955399			
Vinca minor		KX911166			
Tonduzia longifolia		DQ660552			
Craspidospermum verticillatum			MG963267		
Lacmellea panamensis			MG963264		
Aspidosperma cruentum			MG963248	MG963248	
Catharanthus roseus			KC561139	KC561139	
Vinca major			MG963228	MG963228	
Tonduzia stenophylla			MG963272	MG963272	

Appendix E. Diagnostic molecular characters of *Plumeria* taxa, including transition, transversion, and insertion/deletion (indel) characters based on nucleotide positions in sequence alignments of individual molecular regions. Data in boldface indicate characters that are unique and thus diagnostic to individual species but may also be used in combination with other characters associated with the respective species.

Region	Taxa	Diagnostic Character	Type	Position in Alignment*
ITS2				
	P. alba	**C**	**Transition**	**118**
		C	**Transversion**	**181**
		A	**Transversion**	**213**
		G	**Transition**	**214**
		G	**Transition**	**221**
matK				
	P. obtusa *(syn. P. obtusa var. sericifolia)*	**T**	**Transversion**	**192, 644**
		A	**Transition**	**299**
	P. caracasana	T	Transversion	732
	P. pudica	**C**	**Transition**	**27**
		T	**Transition**	**305**
		T	Transversion	732
	P. sp. 'Isabella'	**T**	**Transition**	**514**
psbJ-petA				
	P. rubra	**G**	**Transition**	**35**
		7 bp gap	**Deletion**	**480-486**
		15 bp insert	**Insertion**	**818-832**
	P. alba	**T**	**Transversion**	**379**
		8 bp gap	**Deletion**	**839-846**
	P. sp. 'Isabella'	**A**	**Transversion**	**498**
trnH-psbA				
	P. alba	**A**	**Transversion**	**86**
		T	**Transversion**	**264**
		22 bp insert	**Insertion**	**369-390**
	P. caracasana	**T**	**Transition**	**75**
		gap	**Deletion**	**87-117**
		8 bp insert	Insertion	438-445

*Relative to position in the alignment used in this study. Alignments acquired from other data sets using comparable regions may not necessarily coincide with the alignment positions featured in this study.

Appendix E. (Continued) Diagnostic molecular characters of *Plumeria* taxa.

Region	Taxa	Diagnostic Character	Type	Position in Alignment*
trnH-psbA				
	P. pudica	**T**	**Insertion**	**101**
		gap	**Deletion**	**117**
		T	Insertion	137
		G	**Transition**	**177**
		G	**Transition**	**299**
		T	**Transversion**	**391**
		8 bp insert	Insertion	438-445
	P. sp. 'Isabella'	T	Transversion	92
		A	**Insertion**	**101**
		G	**Transversion**	**104**
		TT	**Insertion**	**106-107**
		T	Insertion	137
		A	**Transversion**	**344**
	P. clusioides	**11 bp insert**	**Insertion**	**106-116**
	P. obtusa var. *sericifolia*	C	Transversion	205
		T	Transversion	239
	P. rubra	**A**	**Transversion**	**75**
		A	**Transversion**	**80**
		C	**Transversion**	**117**
		T	Transversion	137
	P. stenopetala	**T**	**Transversion**	**392**
		T	**Transversion**	**462**
	P. subsessilis	A	Transversion	84
		T	Transversion	92
		C	Transversion	205
		T	Transversion	239
rpl32-trnL				
	P. alba	**G**	**Transition**	**121**
		C	Transversion	258
		A	Transversion	522
		G	**Transversion**	**544**
		T	**Transversion**	**688**

Appendix E. (Continued) Diagnostic molecular characters of *Plumeria* taxa.

Region	Taxa	Diagnostic Character	Type	Position in Alignment*
rpl32-trnL				
	P. caracasana	T	Transversion	53
		T	Transversion	86
		A	Transversion	374
		A	**Transversion**	**515**
		A	Transition	516
		A	Transversion	552
		G	**Transversion**	**634**
		A	Transversion	700
		ATAAAT	**Insertion**	**729-734**
		T	**Transversion**	**898**
		G	Transversion	920
	P. pudica	**A**	**Transition**	**53**
		T	Transversion	86
		A	Transversion	374
		A	Transition	516
		A	Transversion	552
		A	**Transversion**	**582**
		A	Transversion	700
		G	Transversion	920
	P. sp. 'Isabella'	A	Transversion	166
		T	**Transversion**	**516**
		A	Transversion	522
		T	Transversion	1,003
		A	**Transversion**	**1,052**
	P. obtusa var. *sericifolia*	A	Transversion	374
		G	Transversion	624
		177 bp gap	Deletion	645-821
		T	Transversion	1,003
	P. rubra	G	Transversion	393
		T	Transversion	498
		A	**Transition**	**686**
		G	Transition	809
	P. stenopetala	**C**	**Transversion**	**98**
		A	**Transversion**	**166**
		A	**Transversion**	**766**
		C	**Transversion**	**805**

Appendix F. Taxonomic status and natural geographic distribution of *Plumeria*, as derived from the World Checklist of Selected Plant Families (WCSP) and other sources of literature. This is not a comprehensive list of extant taxa. More information is available online at: http://wcsp.science.kew.org/home.do.

Taxon	Reference	Accepted By	Not Accepted By	Synonyms	Natural Distribution
P. pudica Jacq.	Enum. Syst. Pl.: 13 (1760)	Govaerts, R. (2003) Acevedo-Rodríguez, P. & M.T. Strong (2012) Hokche, O., P.E. Berry, & O. Huber (2008) Davidse, G. et al. (2009) Baksh-Comeau, Y. et al. (2016)		*P. caracasana* J.R.Johnst., Contr. U.S. Natl. Herb. 12: 108 (1908) *P. cochleata* S.F.Blake, Contr. Gray Herb., n.s., 53: 47 (1918)	Panama to N. Venezuela
P. caracasana J.R. Johnst.	Contr. U.S. Natl. Herb. 12: 108 (1908)		Govaerts, R. (2003)	Accepted as a synonym of *P. pudica*.	Venezuela (Caracas to La Guaira)
P. alba L.	Sp. Pl.: 209 (1753)	Welsh, S.L. (1998) Govaerts, R. (2003) Acevedo-Rodríguez, P. & M.T. Strong (2012) Baksh-Comeau, Y. et al. (2016)		*P. revolutifolia* Stokes, Bot. Mat. Med. 1: 501 (1812) *P. hypoleuca* var. *angustifolia* Gasp., Ann. Civili Regno Due Sicilie 1: 122 (1833) *P. alba* var. *jacquiniana* A.DC. in A.P.de Candolle, Prodr. 8: 392 (1844)	Puerto Rico to Windward Is.
P. obtusa L.	Sp. Pl.: 210 (1753)	Cirilo, N. & G.R. Proctor (1994) Govaerts, R. (2003) Nelson Sutherland, C.H. (2008) Davidse, G. et al. (2009) Morales, J.F. (2009) Acevedo-Rodríguez, P. & M.T. Strong (2012)		*P. bahamensis* Urb., Symb. Antill. 1: 387 (1899) *P. obtusa* var. *sericifolia* (C.Wright ex Griseb.) Woodson, Ann. Missouri Bot. Gard. 25: 214 (1938)	Florida Keys, Caribbean, SE. Mexico to Guatemala
P. bahamensis Urb.	Symb. Antill. 1: 387 (1899)		Govaerts, R. (2003)	Accepted as a synonym of *P. obtusa*.	Bahamas Acklins Island
P. clusioides Griseb.	Cat. Pl. Cub.: 171 (1866)	Acevedo-Rodríguez, P. & M.T. Strong (2012)	Govaerts, R. (2003)		Cuba
P. cubensis Urb.	Repert. Spec. Nov. Regni Veg. 21: 2019 (1925)	Acevedo-Rodríguez, P. & M.T. Strong (2012)	Govaerts, R. (2003)		Cuba

Appendix F. (continued) Taxonomic status and natural geographic distribution data among *Plumeria* taxa.

Taxon	Reference	Accepted By	Not Accepted By	Synonyms	Natural Distribution
P. ekmanii Urb.	Symb. Antill. 9: 239 (1924)	Acevedo-Rodríguez, P. & M.T. Strong (2012)	Govaerts, R. (2003)	*P. obtusa* var. *parviflora* Griseb., Pl. Wright. 2: 519 (1862) *P. clusioides* var. *parviflora* (Griseb.) M.Gómez, Anales Soc. Esp. Hist. Nat. 23: 273 (1894)	Cuba
P. filifolia Griseb.	Pl. Wright. 2: 519 (1862)	Govaerts, R. (2003) Acevedo-Rodríguez, P. & M.T. Strong (2012)		*P. stenophylla* Urb., Symb. Antill. 9: 237 (1924)	E. Cuba
P. montana Britton & P.Wilson	Bull. Torrey Bot. Club 50: 46 (1923)	Acevedo-Rodríguez, P. & M.T. Strong (2012)	Govaerts, R. (2003)		Cuba
P. rubra L.	Sp. Pl.: 209 (1753)	Welsh, S.L. (1998) Govaerts, R. (2003) Nelson Sutherland, C.H. (2008) Davidse, G. et al. (2009) Morales, J.F. (2009) Acevedo-Rodríguez, P. & M.T. Strong (2012)		Plumeria rubra f. typica Woodson, Ann. Missouri Bot. Gard. 25: 211 (1938) Heterotypic synonyms can also be found online (WCSP).	Mexico to Venezuela
P. stenophylla Urb.	Symb. Antill. 9: 237 (1924)		Govaerts, R. (2003) Acevedo-Rodríguez, P. & M.T. Strong (2012)	Accepted as a synonym of *P. filifolia*.	Cuban (Palmarito de Cauto)
P. tuberculata G. Lodd.	Bot. Cab. 7: t. 681 (1823)	Acevedo-Rodríguez, P. & M.T. Strong (2012)	Govaerts, R. (2003)	*P. domingensis* Urb., Symb. Antill. 3: 338 (1902) *P. gibbosa* Urb., Symb. Antill. 3: 338 (1902)	Bahamas to Hispaniola
P. subsessilis A.DC.	Prodr. 8: 393 (1844)	Govaerts, R. (2003) Acevedo-Rodríguez, P. & M.T. Strong (2012)		*P. berteroi* A.DC. in A.P.de Candolle, Prodr. 8: 393 (1844) *P. jaegeri* Müll.Arg., Linnaea 30: 397 (1860)	Hispaniola

Appendix F. (continued) Taxonomic status and natural geographic distribution data among *Plumeria* taxa.

Taxon	Reference	Accepted By	Not Accepted By	Synonyms	Natural Distribution
P. x *stenopetala* Urb.* (*P. obtusa* x *P. subsessilis*)	Symb. Antill. 3: 335 (1902)	Govaerts, R. (2003) Acevedo-Rodríguez, P. & M.T. Strong (2012)		*Plumeria* × *biglandulosa* Urb., Symb. Antill. 3: 337 (1902) *Plumeria* × *pauliniae* Urb., Symb. Antill. 3: 336 (1902) *Plumeria* × *discolor* Urb. & Ekman, Ark. Bot. 20A (5): 36 (1926) *Plumeria* × *longiflor*a Urb. & Ekman, Ark. Bot. 20A (5): 38 (1926) *Plumeria* × *trouinensis* Urb. & Ekman, Ark. Bot. 20A (5): 37 (1926)	Hispaniola

* Treated in analyses as *P. stenopetala*

REFERENCES

Acevedo-Rodríguez, P., and M. T. Strong. 2012. Catalogue of seed plants of the West Indies. *Smithsonian Contributions to Botany* 98: 1-1192.

Aguoru, C. U., O. P. Abah, and J. O. Olasan. 2015. Systematic descriptions and taxonomic studies on three (3) species of *Plumeria* in North Central Nigeria. *International Journal of Innovation and Applied Studies* 17: 403-411.

Allred, K. W., and F. W. Gould. 1983. Systematics of the Bothriochloa saccharoides Complex (Poaceae: Andropogoneae). *Systematic Botany* 8: 168-184.

Alvarado-Cárdenas, L. O., Ochoterena, H. 2007. A phylogenetic analysis of the Cascabela-Thevetia species complex (Plumerieae, Apocynaceae) based on morphology. *Annals of the Missouri Botanical Garden* 94: 298-323.

Baksh-Comeau, Y., S. S. Maharaj, C. D. Adams, S. A. Harris, D. L. Filer, and W. D. Hawthorne. 2016. An annotated checklist of the vascular plants of Trinidad and Tobago with analysis of vegetation types and botanical 'hotspots'. *Phytotaxa*: 1-431.

Benson, D. A., M. Cavanaugh, K. Clark, I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, and E. W. Sayers. 2016. GenBank. *Nucleic Acids Research* 45: D37-D42.

Bickford, D., D. J. Lohman, N. S. Sodhi, P. K. L. Ng, R. Meier, K. Winker, K. K. Ingram, and I. Das. 2007. Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution* 22: 148-155.

Bieniek, W., M. Mizianty, and M. Szklarczyk. 2015. Sequence variation at the three chloroplast loci (matK, rbcL, trnH-psbA) in the Triticeae tribe (Poaceae): Comments on the relationships and utility in DNA barcoding of selected species. *Plant Systematics and Evolution* 301: 1275-1286.

Boal, C. W., F. C. Sibley, T. S. Estabrook, and J. Lazell. 2006. Insular and migrant species, longevity records, and new species records on Guana Island, British Virgin Islands. *The Wilson Journal of Ornithology* 118: 218-224.

Britton, N. L. 1910. Studies of West Indian Plants-III. *Bulletin of the Torrey Botanical Club* 37: 345-363.

Britton, N. L. 1915. Studies of West Indian Plants-VII. *Bulletin of the Torrey Botanical Club* 42: 487-517.

Britton, N. L. 1923. Studies of West Indian Plants-XI. *Bulletin of the Torrey Botanical Club* 50: 35-56.

Britton, N. L., and C. F. Millspaugh. 1920. The Bahama flora, 333-335. Published by the authors, New York, New York, USA.

Brundu, G., and I. Camarda. 2004. The exotic flora of Chad: A first contribution. *Weed Technology* 18: 1226-1231.

Brussell, D. E. 2004. A medicinal plant collection from Montserrat, West Indies. *Economic Botany* 58: S203-S220.

Cabelin, V. L., and G. J. Alejandro. 2016. Efficiency of matK, rbcL, trnH-psbA, and trnL-F (cpDNA) to molecularly authenticate Philippine ethnomedicinal Apocynaceae through DNA barcoding. *Pharmacognosy Magazine* 12: S384-388.

Calonje, M., S. Martín-Bravo, C. Dobeš, W. Gong, I. Jordon-Thaden, C. Kiefer, M. Kiefer, J. Paule et al. 2009. Non-coding nuclear DNA markers in phylogenetic reconstruction. *Plant Systematics and Evolution* 282: 257-280.

Cantley, J. T., N. G. Swenson, A. Markey, and S. C. Keeley. 2014. Biogeographic insights on Pacific Coprosma (Rubiaceae) indicate two colonizations to the Hawaiian Islands. *Botanical Journal of the Linnean Society* 174: 412-424.

CBOL Plant Working Group. 2009. A DNA barcode for land plants. *Proceedings of the National Academy of Sciences* 106: 12794-12797.

Chase, M. W., and H. H. Hills. 1991. Silica gel: an ideal material for field preservation of leaf samples for DNA studies. *Taxon* 40: 215-220.

Chen, S., H. Yao, J. Han, C. Liu, J. Song, L. Shi, Y. Zhu, X. Ma et al. 2010. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLOS ONE* 5: e8613.

Cheng, T., C. Xu, L. Lei, C. Li, Y. Zhang, and S. Zhou. 2016. Barcoding the kingdom Plantae: New PCR primers for ITS regions of plants with improved universality and specificity. *Molecular Ecology Resources* 16: 138-149.

Chinn, J. T., and R. A. Criley. 1982. Plumeria cultivars in Hawaii, Hawaii Institute of Tropical Agriculture and Human Resources Research Bulletin, 51 p. College of Tropical Agriculture and Human Resources, University of Hawaii, Honolulu, Hawaii, USA.

Choudhary, M., V. Kumar, and S. Singh. 2014. Phytochemical and pharmacological activity of genus *Plumeria*: An updated review. *International Journal of Biomedical and Advance Research* 5: 266-271.

Cirilo, N., and G. R. Proctor. 1994. Vascular plants of the Caribbean Swan islands of Honduras. *Brenesia* 41-42: 73-80.

Clark, K., I. Karsch-Mizrachi, D. J. Lipman, J. Ostell, and E. W. Sayers. 2016. GenBank. *Nucleic Acids Research* 44: D67-D72.

Coissac, E., P. M. Hollingsworth, S. Lavergne, and P. Taberlet. 2016. From barcodes to genomes: Extending the concept of DNA barcoding. *Molecular Ecology* 25: 1423-1428.

Criley, R. A. 1992. Hawaii's lei flower industry. *HortScience* 27: 490-492.

Criley, R. A. 2005. Plumeria in Hawaii. *College Tropical Agriculture and Human Resources, University of Hawaii at Manoa* OF-31: 1-29.

Criley, R. A. 2009. *Plumeria rubra*: An old ornamental, a new crop. *Acta Horticulturae* 813: 183-190.

da Silva, T. S. R. 2017. Species descriptions and digital environments: Alternatives for accessibility of morphological data. *Revista Brasileira de Entomologia* 61: 277-281.

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

Davidse, G., et al. 2009. Flora Mesoamericana, vol. 1, 1-855. Universidad Nacional Autónoma de México, Mexico City, Mexico.

De Candolle, A. P. 1844. Sistens Corolliflorarum Ordines XIII. *In* A. de Candolle [ed.], Prodromus systematis naturalis regni vegetabilis, vol. 8. Sumptibus Sociorum Treuttel et Würtz, Paris, France.

Doyle, J. J., and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11-15.

Duke, J. A. 1965. Keys for the identification of seedlings of some prominent woody species in eight forest types in Puerto Rico. *Annals of the Missouri Botanical Garden* 52: 314-350.

Eggenberger, R. M., and M. H. Eggenberger. 2005. The handbook on Plumeria culture. Fourth Edition ed. Golden Bridge Publications, Lodi, California, USA.

Endress, M., and P. Bruyns. 2000. A revised classification of the Apocynaceae s.l. *Botanical Review* 66: 1-56.

Endress, M. E., S. Liede-Schumann, and U. Meve. 2007a. Advances in Apocynaceae: The enlightenment, an introduction. *Annals of the Missouri Botanical Garden* 94: 259-267.

Endress, M. E., S. Liede-Schumann, and U. Meve. 2014. An updated classification for Apocynaceae. *Phytotaxa* 159: 175–194.

Endress, M. E., R. W. J. M. van der Ham, S. Nilsson, L. Civeyrel, M. W. Chase, B. Sennblad, K. Potgieter, J. Joseph et al. 2007b. A phylogenetic analysis of Alyxieae

(Apocynaceae) based on rbcL, matK, trnL Intron, trnL-F spacer sequences, and morphological characters. *Annals of the Missouri Botanical Garden* 94: 1-35.

Fazekas, A. J., M. L. Kuzmina, S. G. Newmaster, and P. M. Hollingsworth. 2012. DNA barcoding methods for land plants. *In* W. J. Kress AND D. L. Erickson [eds.], DNA Barcodes: Methods and Protocols 10.1007/978-1-61779-591-6 10.1007/978-1-61779-591-6, 223-252. Humana Press, New York, New York, USA.

Fišer Pečnikar, Ž., and E. V. Buzan. 2014. 20 years since the introduction of DNA barcoding: From theory to application. *Journal of Applied Genetics* 55: 43-52.

Fishbein, M., D. Chuba, C. Ellison, R. J. Mason-Gamer, and S. P. Lynch. 2011. Phylogenetic relationships of *Asclepias* (Apocynaceae) inferred from non-coding chloroplast DNA sequences. *Systematic Botany* 36: 1008-1023.

Fishbein, M., T. Livshultz, S. C. K. Straub, A. O. Simões, J. Boutte, A. McDonnell, and A. Foote. 2018. Evolution on the backbone: Apocynaceae phylogenomics and new perspectives on growth forms, flowers, and fruits. *American Journal of Botany* 105: 495-513.

Ford-Lloyd, B. V. 2001. Genotyping in plant genetic resources. *In* R. J. Henry [ed.], Plant genotyping: The DNA fingerprinting of plants, 59-81. CABI Publishing, New York, New York, USA.

Funk, V., R. Edwards, and S. Keeley. 2018. The problem with(out) vouchers. *Taxon* 67: 1-3.

García-Lara, S., R. Grether, I. Ramírez-Morillo, and R. D. de Stefano. 2015. Testing a species hypothesis with morphometric analysis: *Pithecellobium insigne* (Leguminosae, Mimosoideae, Ingeae). *Journal of the Torrey Botanical Society* 142: 314-324.

Gleason, H. A., and E. P. Killip. 1939. The flora of Mount Auyan-Tepui, Venezuela. *Brittonia* 3: 141-204.

Govaerts, R., D. Goyder, and A. Leeuwenberg. 2003. World checklist of Apocynaceae. Facilitated by the Royal Botanic Gardens, Kew, Published on the Internet; http://wcsp.science.kew.org/.

Grisebach, A. 1864. Flora of the British West Indian Islands, 410-411. L. Reeve, London, England.

Grisebach, A. 1866. Catalogus plantarum cubensium exhibens collectionem Wrightianam aliasque minores ex insula Cuba missas. Apud Gulielmum Engelmann, Lipsiae (Leipzig), Saxony, Germany.

Grisebach, A., A. Gray, and C. Wright. 1863. Plantae Wrightianae e Cuba Orientali, Pars II. (Monopetalae et Monocotyledones). *Memoirs of the American Academy of Arts and Sciences* 8: 503-536.

Guindon, S., J.-F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk, and O. Gascuel. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* 59: 307-321.

Gupta, M., U. Mazumder, P. Gomathi, and V. T. Selvan. 2006. Antiinflammatory evaluation of leaves of *Plumeria acuminata*. *BMC Complementary Alternative Medicine* 6: 36.

Haber, W. A. 1984. Pollination by deceit in a mass-flowering tropical tree *Plumeria rubra* L. (Apocynaceae). *Biotropica* 16: 269-275.

Harris, J. G., and M. W. Harris. 2011. Plant identificaion terminology: An illustrated glossary. Second ed. Spring Lake Publishing, Spring Lake, Utah, USA.

Harshberger, J. W. 1903. Notes on the strand flora of Great Inagua, Haiti and Jamaica. *Torreya* 3: 67-70.

Hilu, K. W., T. Borsch, K. Muller, D. E. Soltis, P. S. Soltis, V. Savolainen, M. W. Chase, M. P. Powell et al. 2003. Angiosperm phylogeny based on matK sequence information. *American Journal of Botany* 90: 1758-1776.

Hochbach, A., J. Schneider, and M. Röser. 2015. A multi-locus analysis of phylogenetic relationships within grass subfamily Pooideae (Poaceae) inferred from sequences of nuclear single copy gene regions compared with plastid DNA. *Molecular Phylogenetics and Evolution* 87: 14-27.

Hodel, D. R., L. M. Ohara, and G. Arakelian. 2017. A new and serious leafhopper pest of *Plumeria* in southern California. *PalmArbor, University of California, Cooperative Extension* 5: 1-19.

Hokche, O., P. E. Berry, and O. Huber. 2008. Nuevo catálogo de la flora vascular de Venezuela. Fundación Instituto Botánico de Venezuela, Caracas, Venezuela.

Hollick, A. 1922. A review of the fossil flora of the West Indies, with descriptions of new species. *Bulletin of the New York Botanical Garden* 12: 259-324.

Hosein, F. N., N. Austin, S. Maharaj, W. Johnson, L. Rostant, A. C. Ramdass, and S. N. Rampersad. 2017. Utility of DNA barcoding to identify rare endemic vascular plant species in Trinidad. *Ecology and Evolution* 7: 7311-7333.

Howard, R. A. 1989. Flora of the Lesser Antilles : Leeward and windward islands. Arnold Arboretum, Jamaica Plain, Massachusetts, USA.

Iezzoni, A. F., and M. P. Pritts. 1991. Applications of principal component analysis to horticultural research. *HortScience* 26: 334-338.

Jacquin, N. J. 1763. Selectarum stirpium Americanarum historia, 35-37. Ex officina Krausiana, Vindobonae, Rome.

Johnston, J. R. 1912. A collection of plants from the vicinity of La Guaira, Venezuela. *Contributions from the United States National Herbarium* 12: 105-111.

Joulain, D. 2008. Flower scents from the Pacific. *Chemistry & Biodiversity* 5: 896-909.

Judd, W. S., C. S. Campbell, E. A. Kellog, P. F. Stevens, and M. J. Donoghue. 2008. Plant systematics. 3rd ed. Sinauer Associates, Sunderland, Massachusetts, USA.

Kassambara, A., and F. Mundt. 2017. Factoextra: Extract and visualize the results of multivariate data analyses. R package version 1.0.5. website: https://CRAN.R-project.org/package=factoextra [accessed 31 October 2018].

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, A. Cooper et al. 2012. Geneious basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28: 1647-1649.

Keeley, S. C., Z. H. Forsman, and R. Chan. 2007. A phylogeny of the "evil tribe" (Vernonieae: Compositae) reveals Old/New World long distance dispersal: Support from separate and combined congruent datasets (trnL-F, ndhF, ITS). *Molecular Phylogenetics and Evolution* 44: 89-103.

Kelchner, S. A. 2000. The evolution of non-coding chloroplast DNA and its application in plant systematics. *Annals of the Missouri Botanical Garden* 87: 482-498.

Kim, S.-C., D. J. Crawford, R. K. Jansen, and A. Santos-Guerra. 1999. The use of a non-coding region of chloroplast DNA in phylogenetic studies of the subtribe Sonchinae (Asteraceae: Lactuceae). *Plant Systematics and Evolution* 215: 85-99.

Knudsen, J., R. Eriksson, J. Gershenzon, and B. Ståhl. 2006. Diversity and distribution of floral scent. *Botanical Review* 72: 1-120.

Knyaz, C., G. Stecher, M. Li, S. Kumar, and K. Tamura. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* 35: 1547-1549.

Korotkova, N., T. Borsch, D. Quandt, N. P. Taylor, K. F. Müller, and W. Barthlott. 2011. What does it take to resolve relationships and to identify species with molecular markers? An example from the epiphytic Rhipsalideae (Cactaceae). *American Journal of Botany* 98: 1549-1572.

Kress, W. J., C. García-Robledo, M. Uriarte, and D. L. Erickson. 2015. DNA barcodes for ecology, evolution, and conservation. *Trends in Ecology & Evolution* 30: 25-35.

Kress, W. J., K. J. Wurdack, E. A. Zimmer, L. A. Weigt, and D. H. Janzen. 2005. Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences of the United States of America* 102: 8369-8374.

Lanfear, R., B. Calcott, S. Y. W. Ho, and S. Guindon. 2012. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* 29: 1695-1701.

Lanfear, R., T. Senfeld, P. B. Frandsen, A. M. Wright, and B. Calcott. 2016. PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution* 34: 772-773.

Lawton, J. R. S., and E. E. J. Akpan. 1968. Periodicity in Plumeria. *Nature* 218: 384-386.

Le, S., J. Josse, and F. Husson. 2008. FactoMineR: An R package for multivariate analysis 25: 1-18.

Leeuwenberg, A. J. M. 1994. Pollination of Apocynaceae. *Wageningen Agricultural University Papers* 94: 61-81.

Lens, F., M. E. Endress, P. Baas, S. Jansen, and E. Smets. 2008. Wood anatomy of Rauvolfioideae (Apocynaceae): A search for meaningful non-DNA characters at the tribal level. *American Journal of Botany* 95: 1199-1215.

Leon, H., and H. Alain. 1957. Flora de Cuba IV, 173-176. Contribuciones ocasionales del Museo de Historia Natural del Colegio de La Salle No. 16, Havana, Cuba.

Letunic, I., and P. Bork. 2016. Interactive tree of life (iTOL) v3: An online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Research* 44: W242-W245.

Linné, C. v., and L. Salvius. 1753. Species Plantarum, 209-210. Impensis Laurentii Salvii, Holmiae, Sweden.

Liogier, H. A., and L. F. Martorell. 1982. Flora of Puerto Rico and adjacent islands : A systematic synopsis, 140. Editorial de la Universidad de Puerto Rico, Puerto Rico.

Little, J. 2006. Growing Plumeria in Hawaii. Mutual Publishing, Honolulu.

Liu, J., H.-F. Yan, and X.-J. Ge. 2016. The use of DNA barcoding on recently diverged species in the genus *Gentiana* (Gentianaceae) in China. *PLoS ONE* 11: e0153008.

Livshultz, T., D. J. Middleton, M. E. Endress, and J. K. Williams. 2007. Phylogeny of Apocynoideae and the APSA clade (Apocynaceae s.l.). *Annals of the Missouri Botanical Garden* 94: 324-359.

Ma, H.-l., Z.-b. Zhu, X.-m. Zhang, Y.-y. Miao, and Q.-s. Guo. 2014. Species identification of the medicinal plant *Tulipa edulis* (Liliaceae) by DNA barcode marker. *Biochemical Systematics and Ecology* 55: 362-368.

Mahadani, P., G. D. Sharma, and S. K. Ghosh. 2013. Identification of ethnomedicinal plants (Rauvolfioideae: Apocynaceae) through DNA barcoding from northeast India. *Pharmacognosy Magazine* 9: 255-263.

Marr, K. L., R. J. Hebda, and E. A. Zamluk. 2011. Morphological analysis and phytogeography of native Calamagrostis (Poaceae) from British Columbia, Canada and adjacent regions. *Madroño* 58: 214-233.

Mead, R., R. N. Curnow, and A. M. Hasted. 2003. Statistical methods in agriculture and experimental biology. Third ed., 403-418. Chapman and Hall/CRC, New York, New York, USA.

Meerow, A. W. 2005. Molecular genetic characterization of new floricultural germplasm. *Acta Horticulturae* 683: 43-63.

Meier, R., G. Zhang, and F. Ali. 2008. The use of mean instead of smallest interspecific distances exaggerates the size of the "barcoding gap" and leads to misidentification. *Systematic Biology* 57: 809-813.

Merrill, E. D. 1937. On the significance of certain Oriental plant names in relation to introduced species. *Proceedings of the American Philosophical Society* 78: 111-146.

Miller, M. A., W. Pfeiffer, and T. Schwartz. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees, Gateway Computing Environments Workshop (GCE), 1-8, New Orleans, Louisiana, USA.

Morales, J. F. 2009. Estudios en las Apocynaceae neotropicales XXXIX: revisión de las Apocynoideae y Rauvolfioideae de Honduras. *Anales del Jardin Botanico de Madri* 66: 217-262.

Moscoso, R. M. 1943. Catalogus florae Domingensis. Parte I. Spermatophyta, 496-498. L. & S. Printing, New York, New York, USA.

Murashige, T. 1966. The deciduous behavior of a tropical plant, *Plumeria acuminata*. *Physiologia Plantarum* 19: 348-356.

Muscarella, R., M. Uriarte, D. L. Erickson, N. G. Swenson, J. K. Zimmerman, and W. J. Kress. 2014. A well-resolved phylogeny of the trees of Puerto Rico based on DNA barcode sequence data. *PLOS ONE* 9: e112843.

Nazar, N., D. J. Goyder, J. J. Clarkson, T. Mahmood, and M. W. Chase. 2013. The taxonomy and systematics of Apocynaceae: Where we stand in 2012. *Botanical Journal of the Linnean Society* 171: 482-490.

Nelson, S. 2009. Plumeria Rust, Cooperative Extension Service, College of Tropical Agriclture and Human Resources, University of Hawaii, Honolulu.

Nelson Sutherland, C. H. 2008. Catálogo de las plantes vasculares de Honduras, 1-1576. Secretaría de Recursos Naturales y Ambiente, Tegucigalpa, Honduras.

Pang, X., C. Liu, L. Shi, R. Liu, D. Liang, H. Li, S. S. Cherny, and S. Chen. 2012. Utility of the trnH–psbA intergenic spacer region and its combinations as plant DNA barcodes: A meta-analysis. *PLoS One* 7: e48833.

Pettengill, J. B., and M. C. Neel. 2010. An evaluation of candidate plant DNA barcodes and assignment methods in diagnosing 29 species in the genus *Agalinis* (Orobanchaceae). *American Journal of Botany* 97: 1391-1406.

Pettengill, J. B., and M. C. Neel. 2011. A sequential approach using genetic and morphological analyses to test species status: The case of United States federally endangered *Agalinis acuta* (Orobanchaceae). *American Journal of Botany* 98: 859-871.

Potgieter, K., and V. A. Albert. 2001. Phylogenetic relationships within Apocynaceae s.l. based on trnL intron and trnL-F spacer sequences and propagule characters. *Annals of the Missouri Botanical Garden* 88: 523-549.

Qiu, X., H. Zhang, H. Jian, Q. Wang, N. Zhou, H. Yan, T. Zhang, and K. Tang. 2013. Genetic relationships of wild roses, old garden roses, and modern roses based on internal transcribed spacers and matK sequences. *HortScience* 48: 1445-1451.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. website: http://www.R-project.org/.

Rahman, H., V. B. Reddy, S. Ghosh, S. K. Mistry, G. Pant, and G. Sibi. 2014. Antioxidant, cytotoxic and hypolipidemic activities of Plumeria alba L. and Plumeria rubra L. *American Journal of Life Sciences* 2: 11-15.

Ratnasingham, S., and P. D. N. Hebert. 2007. BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). *Molecular Ecology Notes* 7: 355-364.

Ronblom, K., and A. A. Anderberg. 2002. Phylogeny of Diapensiaceae Based on Molecular Data and Morphology. *Systematic Botany* 27: 383-395.

Ronquist, F., M. Teslenko, P. van der Mark, D. L. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu et al. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61: 539-542.

Sánchez-Sánchez, O., and G. A. Islebe. 2002. Tropical forest communities in southeastern Mexico. *Plant Ecology* 158: 183-200.

Schlichting, C. D. 1986. The evolution of phenotypic plasticity in plants. *Annual Review of Ecology and Systematics* 17: 667-693.

Seifriz, W. 1943. The plant life of Cuba. *Ecological Monographs* 13: 375-426.

Selvaraj, D., R. K. Sarma, D. Shanmughanandhan, R. Srinivasan, and S. Ramalingam. 2015. Evaluation of DNA barcode candidates for the discrimination of the large plant family Apocynaceae. *Plant Systematics and Evolution* 301: 1263-1273.

Sennblad, B., and B. Bremer. 1996. The familial and subfamilial relationships of Apocynaceae and Asclepiadaceae evaluated with rbcL data. *Plant Systematics and Evolution* 202: 153-175.

Sennblad, B., and B. Bremer. 2002. Classification of Apocynaceae s.l. according to a new approach combining Linnaean and phylogenetic taxonomy. *Systematic Biology* 51: 389-409.

Seth, M. K. 2003. Trees and their economic importance. *Botanical Review* 69: 321-376.

Shaw, J., E. B. Lickey, E. E. Schilling, and R. L. Small. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: The tortoise and the hare III. *American Journal of Botany* 94: 275-288.

Shaw, J., H. L. Shafer, O. R. Leonard, M. J. Kovach, M. Schorr, and A. B. Morris. 2014. Chloroplast DNA sequence utility for the lowest phylogenetic and phylogeographic inferences in angiosperms: The tortoise and the hare IV. *American Journal of Botany* 101: 1987-2004.

Shaw, J., E. B. Lickey, J. T. Beck, S. B. Farmer, W. Liu, J. Miller, K. C. Siripun, C. T. Winder et al. 2005. The tortoise and the hare II: Relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* 92: 142-166.

Shinde, P. R., P. S. Patil, and V. A. Bairagi. 2014. Phytopharmacological review of *Plumeria* species. *Scholars Academic Journal of Pharmacy* 3: 217-227.

Simões, A. O., M. E. Endress, and E. Conti. 2010. Systematics and character evolution of Tabernaemontaneae (Apocynaceae, Rauvolfioideae) based on molecular and morphological evidence. *Taxon* 59: 772-790.

Simões, A. O., T. Livshultz, E. Conti, and M. E. Endress. 2007. Phylogeny and systematics of the Rauvolfioideae (Apocynaceae) based on molecular and morphological evidence. *Annals of the Missouri Botanical Garden* 94: 268-297.

Simões, A. O., M. E. Endress, T. van der Niet, L. S. Kinoshita, and E. Conti. 2004. Tribal and intergeneric relationships of Mesechiteae (Apocynoideae, Apocynaceae): evidence from three noncoding plastid DNA regions and morphology. *American Journal of Botany* 91: 1409-1418.

Sivalingam, D., R. Rajendran, and K. Anbarasan. 2016. DNA barcoding of highly threatened sacred plant - *Aegle marmelos* L. *International Journal of Current Research in Biosciences and Plant Biology* 3: 88-96.

Sloan, S. A., J. K. Zimmerman, and A. M. Sabat. 2007. Phenology of Plumeria alba and its herbivores in a tropical dry forest. *Biotropica* 39: 195-201.

Smitha, K., A. Paton, and P. Sunojkumar. 2018. Re-establishment of Plectranthus bishopianus (Lamiaceae) based on morphological and micromorphological data. *Plant Systematics and Evolution* 304: 807-816.

Soltis, D. E., P. S. Soltis, P. K. Endress, and M. W. Chase. 2005. Phylogeny and Evolution of Angiosperms. Sinauer Associates, Sunderland, MA.

Soltis, D. E., R. K. Kuzoff, M. E. Mort, M. Zanis, M. Fishbein, L. Hufford, J. Koontz, and M. K. Arroyo. 2001. Elucidating deep-level phylogenetic relationships in Saxifragaceae using sequences for six chloroplastic and nuclear DNA regions. *Annals of the Missouri Botanical Garden* 88: 669-693.

Srivathsan, A., and R. Meier. 2012. On the inappropriate use of Kimura-2-parameter (K2P) divergences in the DNA-barcoding literature. *Cladistics* 28: 190-194.

Stahl, A. 1937. Flora de Puerto Rico III. 3rd ed., 182-184. Federal Emergency Relief Administration, San Juan, Puerto Rico.

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

Standley, P. C. 1924. Trees and shrubs of Mexico. *Contributions from the United States National Herbarium* 23: 1147-1150.

Staples, G. W., and D. R. Herbst. 2005. A tropical garden flora: Plants cultivated in the Hawaiian islands and other tropical places, 116-124. Bishop Museum Press, Honolulu, Hawaii, USA.

Steele, P. R., and J. C. Pires. 2011. Biodiversity assessment: State-of-the-art techniques in phylogenomics and species identification. *American Journal of Botany* 98: 415-425.

The Missouri Botanical Garden. 1938. Volume information. *Annals of the Missouri Botanical Garden* 25: 841-853.

The Plant List. 2013. Version 1.1. Published on the Internet. Website http://www.theplantlist.org/ [accessed 18 Oct 2018].

Tohar, N., M. A. Mohd, I. Jantan, and K. Awang. 2006. A comparative study of the essential oils of the genus *Plumeria* Linn. from Malaysia. *Flavour and Fragrance Journal* 21: 859-863.

Tripathi, A. M., A. Tyagi, A. Kumar, A. Singh, S. Singh, L. B. Chaudhary, and S. Roy. 2013. The internal transcribed spacer (ITS) region and trnhH-psbA are suitable candidate loci for DNA barcoding of tropical tree species of India. *PLOS ONE* 8: e57934.

Urban, I. 1898. Symbolae Antillanae I, 387-388. Reprint: A. Asher & Co., Amsterdam, Holland.

Urban, I. 1902. Symbolae Antillanae III, 335-336. Reprint: A. Asher & Co., Amsterdam, Holland.

Urban, I. 1920. Symbolae Antillanae VIII, 538-539. Reprint: A. Asher & Co., Amsterdam, Holland.

Urban, I. 1924. Symbolae Antillanae IX, 237-240. Reprint: A. Asher & Co., Amsterdam, Holland.

Urban, I. 1925. Repertorium specierum novarum regni vegetabilis. Selbstverlag des Herausgebers, Berlin, Germany.

Viera Barreto, J., P. Pliscoff, M. Donato, and G. Sancho. 2018. Disentangling morphologically similar species of the Andean forest: Integrating results from multivariate morphometric analyses, niche modelling and climatic space comparison in Kaunia (Eupatorieae: Asteraceae). *Botanical Journal of the Linnean Society* 186: 259-272.

Watson, D. P., J.T. Chinn, H.F. Clay, and J. L. Brewbaker. 1965. Hawaiian Plumerias. University of Hawaii at Manoa, Honolulu, Hawaii, USA.

WCSP. 2019. *World Checklist of Selected Plant Families*. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet. Website http://wcsp.science.kew.org/ [accessed 03 Jan 2019].

Welsh, S. L. 1998. Flora societensis: a summary revision of the flowering plants of the Society Islands: Mehetia, Tahiti, Moorea, Tetiaroa (iles du vent); Huahine, Raiatea, Tahaa, Bora Bora, Tupai, Maupiti, and Mopelia (iles sous le vent), vol. IV, 420 p. E.P.S., Orem, Utah, USA.

Whistler, W. A. 1988. Ethnobotany of Tokelau: The plants, their Tokelau names, and their uses. *Economic Botany* 42: 155-176.

Wickham, H. 2016. ggplot2: Elegant graphics for data analysis.

Wiens, J. J. 2004. The role of morphological data in phylogeny reconstruction. *Systematic Biology* 53: 653-661.

Williams, J. K. 1996. The Mexican genera of the Apocynaceae (sensu A. DC.), with key and additional taxonomic notes. *SIDA Contributions to Botany* 17: 197-213.

Wong, K. L., P. P. But, and P. C. Shaw. 2013. Evaluation of seven DNA barcodes for differentiating closely related medicinal Gentiana species and their adulterants. *Chinese Medicine* 8: 16.

Wong, M. 2008. Xeriscape plants. University of Hawaii at Manoa Cooperative Extension Service, Honolulu, Hawaii, USA.

Woodson, R. E. 1938a. Studies in the Apocynaceae. VII. An evaluation of the genera *Plumeria* L. and *Himatanthus* Willd. *Annals of the Missouri Botanical Garden* 25: 189-224.

Woodson, R. E. 1938b. North American flora. New York Botanical Garden, New York, New York, USA.

Woodson, R. E., and R. J. Seibert. 1938. Contributions toward a flora of Panama. II. Miscellaneous collections during 1936-1938. *Annals of the Missouri Botanical Garden* 25: 823-840.

Woodson, R. E., and J. A. Moore. 1938. The vascular anatomy and comparative morphology of Apocynaceous flowers. *Bulletin of the Torrey Botanical Club* 65: 135-166.

Woodson, R. E., R. W. Schery, and J. W. Nowicke. 1970. Flora of Panama. Part VIII. Family 162. Apocynaceae. *Annals of the Missouri Botanical Garden* 57: 59-130.

Wu-Yang, H., C. Yi-Zhong, X. Jie, C. Harold, and M. Sun. 2007. A potential antioxidant resource: Endophytic fungi from medicinal plants. *Economic Botany* 61: 14-30.

Wu, F.-F., Q. Gao, F. Liu, Z. Wang, J.-L. Wang, and X.-G. Wang. 2019. DNA barcoding evaluation of *Vicia* (Fabaceae): Comparative efficacy of six universal barcode loci on abundant species. *Journal of Systematics and Evolution* doi: 10.1111/jse.12474.

Yang, J., L. Feng, M. Yue, Y.-L. He, G.-F. Zhao, and Z.-H. Li. 2019. Species delimitation and interspecific relationships of the endangered herb genus *Notopterygium* inferred from multilocus variations. *Molecular Phylogenetics and Evolution* 133: 142-151.

Yao, H., J. Song, C. Liu, K. Luo, J. Han, Y. Li, X. Pang, H. Xu et al. 2010. Use of ITS2 region as the universal DNA barcode for plants and animals. *PLoS One* 5.