Spring 5-12-2022

# Reassessment of Species Boundaries and Phylogenetic Relationships in the Desmodium ciliare complex (Fabaceae) Using Morphological and DNA Data

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# REASSESSMENT OF SPECIES BOUNDARIES AND PHYLOGENETIC RELATIONSHIPS IN THE *DESMODIUM CILIARE* COMPLEX (FABACEAE) USING MORPHOLOGICAL AND DNA DATA

by

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A Thesis
Submitted to the Graduate School,
the College of Arts and Sciences,
and the School of Biological, Environmental, and Earth Sciences
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Master of Science

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May 2022

***Published by the Graduate School***

ABSTRACT

*Desmodium* (Beggar's ticks) is a generally weedy genus of approximately 280 species in the angiosperm family Fabaceae (Subfamily Papilionoideae, Tribe Desmodieae) characterized by indehiscent loments constricted into segments. Within the Southeastern U.S., the *Desmodium ciliare* group is one of two *Desmodium* species complexes that have been historically difficult, with much argument about the limits of species and the role of hybridization. There are three commonly recognized species within the *D. ciliare* group, *D. ciliare* (Muhl. ex Willd.) DC., *Desmodium marilandicum* (L.) DC., and *D. obtusum* (Muhl. ex Willd.) DC., with two supposed hybrids in addition to the three main species involving *D. ciliare* with *D. strictum* (Pursh) DC. and *D. paniculatum* (L.) DC. To test the species boundaries, focused principally on material collected in Mississippi where all of the three putative species occur, principal component analysis (PCA) of morphological characteristics and analyses of both plastid *trnL–trnL-F* and nuclear ITS DNA were utilized. Based on phylogenetic analysis using 73 ITS and 12 *trnL–trnL-F* sequences, there was not enough resolution to circumscribe the *Desmodium* species of Mississippi, including the *D. ciliare* complex itself. However, a PCA and subsequent MANOVA of relevant vegetative/floral characters (49 used) among 33 individuals of the *D. ciliare* complex significantly distinguished all three species and two hybrid individuals (*D. ciliare* × *D. obtusum*) from each other. The three species in this complex differ morphologically by terminal leaflet length:width ratio, amount of stem/leaf pubescence, and petiole length.

ACKNOWLEDGMENTS

I'd like to thank my advisor, Dr. Mac H. Alford, for all the years of mentorship in botany and systematics and for showing me how interesting and exciting plants can be. I'd like to thank the rest of my committee, Dr. Micheal Davis and Dr. Donald Yee, for instilling a love for trees and insects while at USM. I'd like to thank Dr. Brian Kreiser for the help in molecular evolution, as well as acting as a secondary advisor towards the end of the project. I'd like to thank James Valentine and Jonathon Osborne for assisting in lab operations and sampling of *Desmodium* across Mississippi. Finally, I'd like to thank Dr. Michael Woods at Troy University (TROY) for providing herbarium samples of the *Desmodium ciliare* complex from Mississippi and Alabama.

# DEDICATION

I'd like to thank my mom for providing unconditional love and supporting me my entire academic career, my sister for encouraging and nurturing all my personal and professional passions and interests, and James Valentine for being a companion for every step of my venture into biology. Without these three individuals, my accomplishments over the past decade would not have been possible.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| *DNA* | deoxyribonucleic acid |
| *MANOVA* | Multivariate Analysis of Variance |
| *ML* | maximum likelihood |
| *MPT* | most parsimonious tree |
| *PCA* | principal component analysis |
| *SNP* | single-nucleotide polymorphism |

# CHAPTER I – INTRODUCTION

## 1.1 Introduction

*Desmodium* Desvaux 1813 (Beggar's ticks/lice, Tick-trefoils) is a genus of approximately 280–300 species in the flowering plant family Fabaceae/Leguminosae (Subfamily Papilionoideae, Tribe Desmodieae). These mostly perennial herbs are especially speciose in Eastern Asia and tropical and temperate areas around the globe, except in Europe. A largely weedy group in temperate North America, beggar's ticks hold true to their name by epizoochorous dispersal via hooked hairs on the loments that attach to vertebrate fur and human clothing (Bailey et al., 1997; Enquist, 1995; Castillo-Flores & Calvo-Irabién, 2003; Nesom, 1993; Schubert, 1980). Within the U.S., *Desmodium* consists of mostly trifoliolate, erect or sprawling herbs with 1–6 segmented loments (laterally-compressed, usually indehiscent fruit constricted into segments), and species are normally differentiated by loment, leaf, and stem indument, corolla length, and leaf and stipule characteristics (Isely, 1990; Krings, 2004; Schubert, 1950; Stone, 1970).

Like many members of the legume family, *Desmodium* is of ecological and economic importance, but also displays potential medicinal properties popular in traditional medicine in Asian and African countries (Catarino et al., 2019; Xu et al., 2015). Ecologically, *Desmodium*, as well as many other members of subfamily Papilionoideae, harbor mutualistic, nitrogen-fixing bacteria, such as *Bradyrhizobium* spp., in their root nodules (Allen & Allen, 1981; Kuypers et al., 2008; McKey, 1994; Parker et al., 2015; Sprent & James, 2007; Xu et al., 2015). Because nitrogen is an important macronutrient for plants and is often limited in certain kinds of soils, this partnership increases soil quality in the surrounding area, which benefits local flora and

wildlife. Along with other root exudates produced by *Desmodium*, this group has been useful agriculturally when implemented in crop rotation or planted alongside crops as green manure for the purposes of suppressing nematode crop pests and increasing soil-nitrogen levels and beneficial soil flora (Kimenju et al., 2008; Vijayaraghavan & Ramachandran, 1989; Wink, 2013). From a conservation standpoint, soil quality in longleaf pine (*Pinus palustris* Mill.) savannas, which historically stretched along the coastal plain of the U.S., is under intermittent (consistent, if human intervention is present) stress and removal of nutrients, such as nitrogen, by action of regular intervals of wildfires. As long as sufficient organic phosphorus is available, nitrogen fixers, such as those supported by plants like *Desmodium*, can replenish soil-nitrogen levels to a point and assist in re-establishment and persistence of the fire-dependent species within the threatened and highly diverse longleaf pine savanna communities (Ament et al., 2018; Noss et al., 2014). Furthermore, legumes are a source of fodder for local wildlife and livestock as they maintain a high nitrogen content within their tissues; however, *Desmodium* have questionable value as a substantial forage source given their variable growth, sometimes sparse leaf distribution, and intermixing among other weedy, and most likely, more suitable forage plants (Allen & Allen, 1981; Baloyi et al., 2009). One particular exception is in the southeastern U.S.: the vigorous invasive *Desmodium tortuosum* (Sw.) DC. has become a pest in peanut farms by shading out crops (Cardina & Brecke, 1991).

*Desmodium* species have been used in medical treatment of integumentary, respiratory, and circulatory diseases in Africa, and secondary compounds isolated from Asian *Desmodium* have been used in many different medical applications, such as

treating fever, cough, and blood diseases (Catarino et al., 2019; Ma et al., 2011). Understanding the evolutionary relationships between species, especially speciose groups, can be an economic way of studying the medical and economic uses of one species and extrapolating that understanding to other closely related species. *Desmodium*, one of the larger genera of Papilionoideae and the largest genus of Desmodieae, has and would be an appropriate candidate for such treatment (Woodson et al., 1980).

Within the southeastern U.S., two species complexes have been historically difficult, with unclear species boundaries: the *Desmodium paniculatum* (L.) DC. group and the *Desmodium ciliare* (Muhl. ex Willd.) DC. group, the latter being the focus of this study (Isely, 1983, 1990; Ohashi, 2013; Schubert 1950). There are three putative species (or varieties) commonly recognized within the *D. ciliare* complex: *D. ciliare*, *D. marilandicum* (L.) DC., and *D. obtusum* (Muhl. ex Willd.) DC., with 2–3 supposed hybrids in addition to the three putative species: *D. ciliare* × *D. strictum* (Pursh) DC. or *D. sessilifolium* (Torrey) Torrey & A. Gray, and *D. ciliare* × *D. paniculatum* (L.) DC. (Isely, 1990). The three putative species in this complex differ morphologically by terminal leaflet length:width ratio, degree of pubescence on stems and leaves, whether uncinate pubescent (with hooked hairs), pilose (sometimes with uncinate pubescence), or glabrate, and petiole length (Table 1.1) (Isely, 1990; Radford, Ahles, & Bell, 1968; Raveill, 2006; Woods, 2008).

Table 1.1 *Diagnostic characteristics of typical individuals of each species in the* Desmodium ciliare *complex (from Isely, 1990)*

| | *D. ciliare* | *D. marilandicum* | *D. obtusum* |
|---|---|---|---|
| *Terminal leaflet L×W* | 1.5–2.3 (–5) | 1.2–1.5 (–2) | 8–10 (–12) |
| *Pubescence amount (medial stems & petioles)* | Conspicuously to thinly | Glabrous to sparsely puberulent | Sparsely to densely puberulent |
| *Pubescence type (medial stems & petioles)* | Pilose or sometimes uncinate-puberulent | Glabrous or uncinate puberulent | Uncinate puberulent |
| *Leaflet pubescence* | Inconspicuously subappressed-pubescent to glabrate (both sides) | Glabrous or occasionally with a few hairs | Glabrate or uncinulate-puberulent above and thinly to conspicuously appressed-villous (-glabrate) below |
| *Petiole length (mm)* | 1–3 (–5) | 10–25 | 3–12 |
| *Pedicel characteristics* | 3–8 mm | 8–15 mm, filiform | 4–10 mm, often arcuate |

By emphasizing largely morphological characteristics that, at times, can overlap between species, difficulties in distinguishing between the species in the *D. ciliare* complex have been apparent in the treatments of the species in the southeastern U.S. (Isely, 1990; Ohashi, 2013; Radford et al., 1968; Woods, 2008). Hybridization has been a tentative hypothesis for the sharing of or overlapping characters for the *D. ciliare* complex, but due to a lack in molecular sampling, common garden experiments, and controlled hybridization studies for the genus, all observations have been anecdotal field- and museum-based observations (Isely, 1983, 1990; Ohashi, 2013; Woods, 2008). Wilbur

(1963), Radford et al. (1968), Gleason & Cronquist (1963), and Gray et al. (1908) treated each member of the complex as a separate species and differentiated the species by amount and type of stem and leaf pubescence and by differences in the leaflet in relation to petiole length. (Gleason & Cronquist [1963] and Gray et al. [1908], however, referred to these three species as *D. rigidum* [=*D. obtusum* as currently circumscribed], *D. obtusum* [=*D. ciliare* as currently circumscribed], and *D. marilandicum*.) Isely (1990), reinforced by Woods (2008), treated the group more thoroughly for each of the characters, added a few characteristics, such as a long terminal leaflet for *D. obtusum* and a subjectively stout pedicel character, and included keys to potential hybrids and their corresponding characteristics. Krings (2004) augmented those descriptions with more characteristics regarding pubescence length and type. In Ohashi's (2013) treatment of *Desmodium* for the *Flora of North America,* the *D. ciliare* complex was treated as one species with three varieties that possibly have hybrids among the varieties: *Desmodium marilandicum* (L.) DC. var. *marilandicum*, *D. marilandicum* var. *ciliare* (Willd.) H. Ohashi, and *D. marilandicum* var. *lancifolium* (Fernald & B. G. Schub) H. Ohashi (= *D. obtusum*). Ohashi (2013) argued that species complexes in *Desmodium* have emphasized overlapping characters too much and thus recognized the endpoints of the variation in the *D. ciliare* complex as taxonomic varieties.

Much of the genetic work that has been done in *Desmodium* has involved parsing out higher order relationships using phylogenetic analyses of DNA data at the family, subfamily, tribe, and subtribe level. Two early molecular studies of Fabaceae involved the plastid regions *rbcL*, the *rpL*2 intron, and ORF184 (Bailey & Doyle, 1997; Doyle et al., 1997; Kajita et al., 2001). Analysis of the *rbcL* gene supported the monophyly of

Fabaceae and subfamily Papilionoideae, and although showing support for several clades within Papilionoideae, showed polyphyly among several tribes, including tribe Desmodieae, which fell within part of polyphyletic tribe Phaseoleae (Doyle et al., 1997; Kajita et al., 2001). Results from ORF184 were ambiguous but the absence of the *rpL*2 intron in Desmodiinae and some Lespedezinae supported the relationships and the placement of *Desmodium* within a separate tribe, Desmodieae (Bailey & Doyle, 1997). Largely to provide foundational genetic data, a few population-level molecular studies in southern China, northern Vietnam, and India have employed random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers to identify genetic diversity and relationships among *Desmodium* and several closely related genera (Heider et al., 2009; Malgaonkar et al., 2016; Yue et al., 2010). Kajita et al. (1996) and Jabbour et al. (2018) demonstrated that *Desmodium* sensu lato was polyphyletic, and the generic limits were revised based on analyses of nuclear ITS 1 and ITS 2 and plastid *rbcL*, *psbA-trnH*, 5′ *trnK* intron, *ndhJ-trnL-trnF*, *trnT-trnL*, *trnG-trnS*, *trnQ-rps*16, *trnL-rpL*32, *rpL*16 intron, *trnC-rpoB*, and *ndhA* intron. As a result, several species within *Desmodium* were transferred to different genera still within the tribe Desmodieae; none of these genera are members of any of the American *Desmodium* complexes (Ohashi & Ohashi, 2019). Tribe Desmodieae has shown to be a clade nested within the old, paraphyletic Phaseoleae, which has now, in part, been classified as the Indigoferoid and Mellettioid clade that holds Tribes Desmodieae, Psoraleae, Phaseoleae sensu lato, and others (Hu, 2000; Jabbour et al., 2018; Wojciechowski, 2003).

Within monophyletic tribe Desmodieae, two subtribes, Desmodiinae and Lespedezinae, have been supported using nuclear and plastid sequences, such as ITS 1,

*rbcL*, *psbA-trnH*, and *matK* (Jabbour et al., 2018; Jin et al., 2019; LPWG, 2013; Stefanović, 2008). Entire genome sequencing of the plastids belonging to Desmodieae, including *Desmodium heterocarpon* (L.) DC. and *Hylodesmum podocarpum* (DC.) H. Ohashi & R.R. Mill subsp. *podocarpum*, show that Desmodieae is supported by a synapomorphic loss of the *rps*12 intron, and *Desmodium* has a highly variable and pseudogenic (problematic) *ycf*4 region (Jin et al., 2019).

**1.2 Purpose**

To address the species boundaries in the *Desmodium ciliare* complex, two approaches were undertaken: (1) collection of morphological data that were analyzed by a principal component analysis (PCA), and (2) collection of DNA sequence data from both nuclear and plastid genomes that were assessed for variation corresponding to any putative species recovered by morphological analysis and analyzed for relationships to other species of *Desmodium* in Mississippi using parsimony and Maximum Likelihood phylogenetic techniques. Given that all three putative species of the complex occur in Mississippi, sampling was focused on populations from Mississippi.

# CHAPTER II – METHODS

## 2.1 Species Concept

The *D. ciliare* complex is a widespread and common species group throughout the eastern U.S. *Desmodium marilandicum* is at the southern limit of its range in Mississippi, and *D. obtusum* is the only species of the complex generally sequestered to dry habitat. For these reasons sampling the complex, especially genetically, can provide insight into relatively unknown and complex relationships of the taxonomically difficult group (Weakley, 2019). This study will adhere to Unified Species Concept (De Queiroz, 2007) and will use morphological and molecular characters to distinguish or unify the species in the *D. ciliare* complex. Previous research and floras presented in the study separate the *D. ciliare* complex into species based on Morphological and Phylogenetic species concepts by utilizing (more or less) diagnostic combinations of vegetative characteristics to distinguish members of the complex and vegetative and floral characteristics to support the monophyly of the *Desmodium ciliare* complex (cf. Davis & Nixon, 1992; Nixon & Wheeler, 1990). These approaches of distinguishing the three species have led to issues when overlapping character states occur in individuals, leading to the proposal of hybridization in the group. De Queiroz's (2007) approach separates species concept from species delimitation and argues that species may be recognized by different kinds of data. How much and what kinds of data are sufficient to recognize species is left up to the organisms and researcher. This study will use a combination of morphological and molecular characteristics as evidence for species delimitation.

## 2.2 Sampling

*Desmodium* and the closely related *Hylodesmum* (Ohashi & Mill, 2000) were collected throughout Mississippi (MS), USA, with emphasis placed on collecting members of the *Desmodium ciliare* complex and possible hybrids between the complex and other species (Table 1.2). Care was taken to sample the four geographic provinces of MS: the Coastal Plains, the Southeastern Plains, Mississippi Alluvial Plain, and the Mississippi Loess Plains (Chapman et al., 2004), but sampling within prairie and floodplain habitats of MS were few. For the PCA, one additional specimen of *D. marilandicum* was included from the herbarium at Troy University (SERNEC: Troy000019230, *Stuart Lasseter 482*). This specimen was collected in MS and is included in the sites sampled. All specimens collected were curated and annotated as herbarium specimens at the University of Southern Mississippi Herbarium (USMS). Duplicates will be distributed to other southeastern USA herbaria.

DNA was extracted from a preliminary sample of eight individuals (two *D. ciliare*, two *D. obtusum*, two *D. marilandicum*, one *D. lineatum*, and one *D. paniculatum*), and DNA sequences were obtained and analyzed using the same genetic methods as the main genetic samples (see next paragraph). The results differed from the main analyses, so the preliminary genetic data will be presented in a table separate from the main analyses.

Based on the results of the preliminary DNA analysis, DNA regions were selected for more thorough sampling and analysis (referred to here as the main genetic samples). For each member of the *D. ciliare* complex, three to five individuals at each sample site had nuclear and plastid gene regions sampled (nuclear ITS and *H3D* and plastid *trnL-*

*trnL-F*, *trnH-psbA*, and *rpL*16). A total of 73 of these sequences (main genetic samples), six *Hylodesmum* and two to five total samples of *Desmodium paniculatum, D. perplexum* B.G. Schub., *D. nuttallii* (Schindl.) B.G. Schub., *D. laevigatum* (Nutt.) DC., *D. glabellum* (Michx.) DC., *D. viridiflorum* (L.) DC., *D. tenuifolium* Torr. & A. Gray, *D. incanum* DC., *D. lineatum* DC., *D. strictum*, *D. ciliare* × *D. marilandicum*, and *D. ciliare* × *D. obtusum* were included in the genetic analyses. There were 14 samples of the 73 resampled for the ITS region. Any samples without the label "F" or "R" at the end of the site label or gene region (e.g., LT4R and ITS5-F) were not resampled and are the ITS gene region as sequenced with only one primer. These resampled sequences were obtained using the same methodology listed as the main genetic samples except for not including TBT-PAR (Samarakoon et al., 2013) in the amplification. The volume of TBT-PAR used in the PCR amplification was substituted with additional water.

Thirty-three specimens of the *D. ciliare* complex that were also used in the genetic analysis, including three potential *D. ciliare* × *D. marilandicum* and two potential *D. ciliare* × *D. obtusum* hybrids, were characterized or measured to be included in a principal component analysis (PCA) for the morphological features listed in Chapter 2.3.

Table 2.1 Sampling sites for the *Desmodium ciliare* complex. All sites are in MS.

NF = national forest; * = Individuals from this site used in PCA

| Site | Abbreviation | Coordinates |
|---|---|---|
| Pearl River WMA (Madison Co.) * | PR | 32.5492561, -89.9334631 |
| Holmes Co. State Park * | HCP | 33.0294912, -89.9222951 |
| Desoto NF (Perry Co.) * | DS, C1–C7, NDS | 31.176639, -89.025890 |
| Ashe Lake (Desoto NF, Forrest Co.) * | BC | 31.0398, -89.18931 |
| Lil' Florida (Desoto NF, Stone Co.) | C8, C9 | 30.6675392, -89.0975175 |
| Tuxachanie Trail (Desoto NF, Harrison Co.) | TX | 30.666750, -89.133674 |
| Raleigh (Bienville NF Smith Co.) * | BV | 32.0998446, -89.5139633 |
| Sylvarena (Bienville NF, Smith Co.) | B | 32.14808, -89.42558 |
| Marathon Campground (Bienville NF, Smith Co.) | CA | 32.200764, -89.359704 |
| Bonita Reservoir (Lauderdale Co.) | BR | 32.3525836, -88.6552812 |
| Puskus Lake (Holly Springs NF, Lafayette Co.) * | HS | 34.4388726, -89.3472543 |
| Columbus (Lowndes Co.) | CL | 33.493054, -88.433228 |
| Liberty (Amite Co.) * | L | 31.2031070, -90.8018695 |
| Marion Co. State Conservation Area | MC | 31.175648, -89.733398 |
| Lake Thoreau Environmental Center (Lamar Co.) * | LT | 31.343763, -89.405988 |
| Chickasawhay WMA * | CH | 31.5227885, -88.9307374 |
| Scattertown Trail (Tombigbee NF, Winston Co.) | T | 33.230673, -88.912073 |
| Osborne Prairie (Oktibbeha Co.) | P | 33.51139, -88.73556 |

Table 2.1 Continued

| Site | Abbreviation | Coordinates |
|---|---|---|
| Wall Doxey State Park (Marshall County) (Troy University sample) * | n/a | 34.665115, -89.462401 |
| New Orleans Audobon Park (Orleans Parish) | NOLA | 29.933742, -90.122649 |

### 2.3 DNA Extraction, Amplification, Purification, and Sequencing

DNA was extracted from the plants using a DNeasy Pro kit (Qiagen, Valencia, CA). All gene regions of the main genetic samples were amplified via PCR using the method of Samarakoon et al. (2013). The PCR was carried out initially for the preliminary samples using a Thermo Sprint Thermal Cycler. The main genetic samples were amplified in a BIORAD MJ min Personal Thermal Cycler. ITS, *H3D*, *rpL*16, and *trnL-trnL-F* amplifications as well as all preliminary sample amplifications, were carried out using the following reaction conditions for 50 µL samples: Lid temperature: 95°C, 95°C for three minutes, 35 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 60 s, and 72°C for three minutes. *trnH–psbA* reactions were carried out using the following reaction conditions for 50 µL samples: Lid temperature: 94°C, 94°C for three minutes, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 50 s, and 72°C for five minutes. Due to poor amplification results, *rpL*16 and *trnL-trnL-F* were amplified twice. These second amplifications were unsuccessful as well. The second amplifications used the following parameters: *rpL*16: Lid temperature: 95°C, 96°C for three minutes, 35 cycles of 98°C for 20 s, 52°C for 45 s, and 72°C for 80 s, then 72°C for three minutes; *trnL-trnL-F*: Lid

temperature: 95°C, 95°C for three minutes, 35 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 50 s, then 72°C for three minutes.

Before samples were sent to be sequenced at Eurofins (Louisville, KY), all amplified DNA was purified using the Qiagen QIAquick® PCR Purification Kit (Hilden, Germany). The gene regions were sequenced in one direction, which is indicated in the primer names. Sequences for each sample were reviewed, and any unresolved nucleotide assignments with multiple peaks were manually resolved using Sequencher (ver. 5.2.2, 2013). All sequences have a value given in Sequencher corresponding to the quality of the base pair calls from each sequence's chromatograph. Generally, sequences with values higher than ninety after editing were used in analyses. Discordant samples were cross-referenced in GenBank using BLAST and removed from analyses if contaminated (NCBI, 2016). The sequences were aligned in Mega X using the MUSCLE Alignment (Gap open: -500.00, Gap Extend: 0, Cluster Method: UPGMA, Minimum Diagonal Length: 24) (Kumar et al., 2018) and assessed for variation among the putative species.

### 2.4 Genetic Analysis

A phylogenetic analysis of the DNA regions from Mississippi *Desmodium* with *Hylodesmum* as the outgroup included both parsimony and Maximum Likelihood optimality criteria in MegaX (Kumar et al., 2018). For the parsimony analysis, the parameters were set as followed: bootstrap method for 5,000 replications, all sites including gaps and missing data, search method using Tree-Bisection-Reconnection (TBR) with 1,000 random, initial trees, search level of one, and 5,000 max number of trees retained. To find the appropriate model for the Maximum Likelihood analysis in

MegaX (Kumar et al., 2018), a model selection analysis was used with the default parameters of automatic (neighbor-joining trees), using all sites, and no branch swap filter. For the Maximum Likelihood analysis of the ITS and *trnH-psbA* regions, the parameters were chosen based on a balance between producing a thorough analysis without taking an exorbitant amount of time given Maximum Likelihood analyses can be time extensive the more sequences are present.

The preliminary data set did not include any phylogenetic analysis. Instead, after thorough alignment using the same methodology as the main data set, each single nucleotide polymorphism (SNP) was counted and assessed for congruence with putative species boundaries (Table 3.1).

**2.5 Morphological Sampling, Principal Component Analysis, and Multivariate Analysis of Variance (MANOVA)**

The PCA was used to condense the many correlated characters, which included all vegetative and floral characteristics between each *Desmodium* individual into fewer, uncorrelated axes (i.e., PCs). Using multivariate analysis of variance (MANOVA), we then compared those PCs significantly differed among *Desmodium* species (SAS Institute., 2004). The PCA was carried out in PROC FACTOR (SAS Institute, 2004) and eigenvalues of the principal components (PCs) higher than 1.0 were selected for further analysis in the MANOVA (Hatcher & Stepansky, 1994). Retained PCs were individually analyzed among the *Desmodium* species. Standard canonical coefficients were used to determine axes that were important in explaining any multivariate effects. We used pair-wise contrasts to control for experimental-wise error rate (Scheiner, 2001; Yee & Yee, 2007).

The vegetative and floral morphological characters included in the PCA focused on characteristics historically of concern for *Desmodium*: total stem length, stem length to first flower, stem width at midstem, total leaves, stem pubescence type, stem hairs per mm at midpoint, largest leaf length, widest leaf length, largest leaf L×W, longest petiole length, largest terminal leaflet petiolule length, midpoint leaf length, midpoint leaf width, midpoint leaf L×W, midpoint petiole length, midpoint petiole pubescence, midpoint petiole hairs per mm, midpoint terminal leaflet, petiolule length, 2/3 leaf length, 2/3 leaf width, 2/3 leaf L×W, 2/3 petiole length, 2/3 terminal leaflet, petiolule length, top leaf length, top leaf width, top leaf L×W, top petiole length, top terminal leaflet petiolule length, average leaf length, average leaf width, average leaf L×W, average petiole length, average terminal leaflet petiolule length, adaxial leaflet pubescence at midpoint, adaxial leaflet pubescence per $mm^2$, abaxial leaflet pubescence at midpoint, abaxial leaflet pubescence per $mm^2$, average stipule length, stipule pubescence, inflorescence structure, fruit number (pedicels without loments attached were counted), pedicel length, pedicel hairs per mm, average calyx size (from base to tip of lobes), average article (the sections that make up one loment, usually partitioned by constrictions of the fruit) length (from one constriction to the next, adjacent constriction), loment article dissection, average stipe length, and bract length. Unless noted, all measurements were in cm. All averages were taken from 2–5 measurements from the respective character. The total leaves measurement included leaf scars in the counts. Leaf width measurements were taken from the same leaflet as the corresponding leaf length measurement. Any petiole or petiolule measurement included the pulvinus. Midpoint, 2/3, and top characters were taken in relation to the stem length to first flower measurement. Stem hairs per mm and

pedicel hairs per mm measurements only included hairs visible from one side (Fig. 3.5). Leaflet pubescence per mm measurements were taken using a 1×1 mm plot (Fig. 3.5). Pubescence character states included all possible combinations of states found in the *D. ciliare* complex (Table 2.2). Inflorescence structure was characterized by having either a raceme, a once-branching panicle (main axis with another axis coming off), or a twice-branching panicle (main axis with a secondary axis that has another axis coming off that). Average article length was measured using the constrictions between articles as endpoints and measuring along the superior portion of the article.

Table 2.2 *Character States for Desmodium PCA Matrix*

| | |
|---|---|
| Midpoint Petiole Pubescence and Stipule Pubescence | glabrous, glabrate with no uncinate hairs, glabrate with uncinate hairs, pubescent with no uncinate hairs, pubescent with only uncinate hairs, uncinate hairs with some pilose, pilose with some uncinate hairs, pilose and uncinate hairs, uncinate with some short setose hairs, short setose with some uncinate hairs, short setose, short setose and uncinate |
| Adaxial & Abaxial Leaflet Pubescence at Midpoint | glabrous, glabrate with no uncinate hairs, glabrate with uncinate hairs, pubescent with no uncinate hairs, pubescent with only uncinate hairs, uncinate hairs with some pilose, pilose with some uncinate hairs, pilose and uncinate hairs, uncinate with some short setose hairs, short setose with some uncinate hairs, short setose, short setose and uncinate, vein strigose only, vein strigose and glabrate with only uncinate hairs, vein setose and uncinate hairs |

# CHAPTER III – RESULTS

Each of the four preliminary gene regions of ITS, *trnH-psbA*, *H3D*, and *rpL*16 had various support shared between or among the preliminary sampling of *Desmodium* in the form of single nucleotide polymorphisms (SNP) (Table 3.1). Only one direction for sequencing was chosen for each region due to differential success in amplification. *trnH-psbA* had the most SNPs, three of which corresponded to *D. ciliare* and *D. obtusum* only. ITS had one SNP for *D. marilandicum* only and two shared SNPs for *D. ciliare* and *D. marilandicum*. *rpL16* had one SNP shared by *D. ciliare* and *D. marilandicum,* and a species outside the complex, *D. lineatum*. *H3D*, while a promising region (Doyle et al., 1996), shared with *rpL16* in showing the least amount of variation but had one SNP shared by *D. obtusum* and *D. marilandicum*, which none of the others did. This, and due to poor amplification, is why the gene region *H3D* was replaced in *Desmodium* sampling later with *trnL–trnL-F*. *trnL–trnL-F* displayed better amplification among preliminary samples and had SNPs for *D. marilandicum* only, *D. obtusum* only, and a shared SNP for *D. ciliare* and *D. marilandicum*.

The ITS region amplified for most of the initial 124 *Desmodium* and *Hylodesmum* extractions. After reviewing and editing the sequencing results, sequences with low confidence or too many ambiguous base pair calls were removed from the analysis, which left 69 *Desmodium* and four *Hylodesmum* in the phylogenies using the ITS region. The plastid gene region *trnH-psbA* amplified for only 25 of the 124 samples. Of the 25, only 12 were found to be of high enough quality to include in an analysis after going through the same review and editing steps as with ITS. Gene regions *trnL–trnL-F* and *H3D* did not amplify at all. Various tweaks of the amplification procedure involving

altering extension time, annealing and denaturing temperatures, and PCR mixtures presented in Samarakoon et al. (2013) by replacing TBT-PAR with water yielded no success after several failed attempts amplifying using the methodology stated earlier.

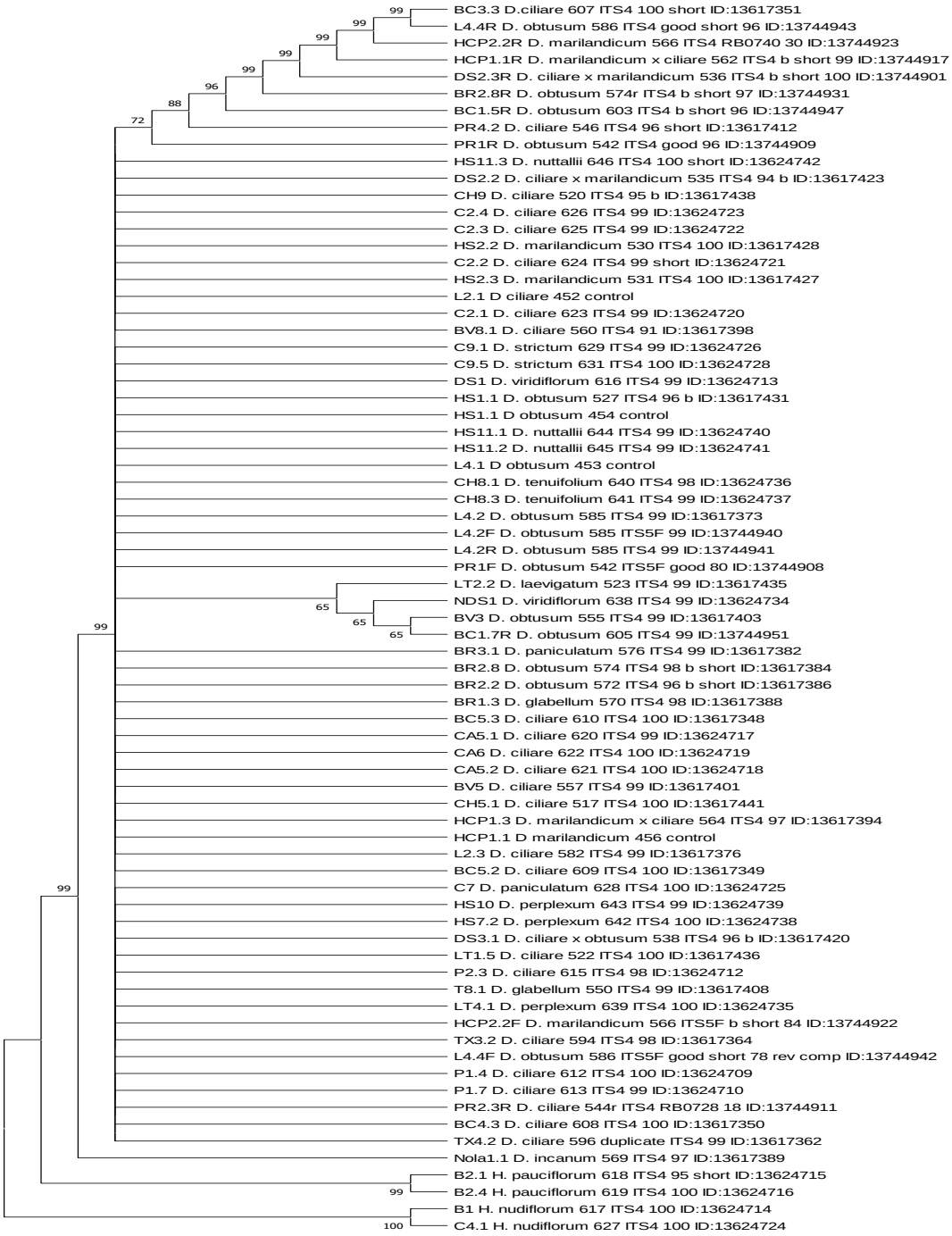
Table 3.1 *Preliminary data showing SNPs for each gene region sampled.*

| **Species** | ITS | *trnH-psbA* | *H3D* | *rpL*16 | *trnL-trnL*-F |
|---|---|---|---|---|---|
| ***D. ciliare* only** | 0 | 1 | 1 | 0 | 0 |
| ***D. obtusum* only** | 0 | 2 | 1 | 1 | 1 |
| ***D. marilandicum* only** | 1 | 0 | 0 | 0 | 1 |
| ***D. ciliare* & *D. obtusum*** | 1 | 0 | 0 | 0 | 0 |
| ***D. ciliare* & *D. marilandicum*** | 2 | 0 | 0 | 1 | 1 |
| ***D. obtusum* & *D. marilandicum*** | 0 | 0 | 1 | 0 | 0 |
| ***D. obtusum* & *D. paniculatum*** | 0 | 1 | 0 | 0 | 0 |
| ***D. ciliare* & *D. lineatum*** | 0 | 1 | 0 | 0 | 0 |
| ***D. ciliare*, *D. marilandicum*, & *D. lineatum*** | 0 | 0 | 0 | 1 | 0 |
| **Total** | 4 | 5 | 3 | 3 | 3 |

The bootstrap consensus tree using parsimony taken from 73 sequences with 660 nucleotides analyzed from the nuclear ITS region is presented in Figure 3.1. *Hylodesmum nudiflorum* served as the root taxon for all ITS trees. All lineages with bootstrap support of 65% or less were collapsed. *Hylodesmum* species and the one *D. incanum* sample had strong support from all other *Desmodium* sampled. Most other *Desmodium* sampled had little to no substantial support with the exception of one group of various *Desmodium* species and another grouping of *Desmodium obtusum, D. laevigatum,* and *D.*

*viridiflorum*. The former cluster included several samples consisting of the *D. ciliare* complex only, but these samples consisted of mostly short sequences (<300 bp) . A second consensus tree of the plastid *trnH–psbA* region (Figure 3.2) included 338 nucleotides from 12 *Desmodium* sequences. *Desmodium laevigatum* served as the root for all *trnH–psbA* trees. The bootstrap consensus tree did not yield any nodes with bootstrap values over 65%.

Figure 3.1 *Bootstrap consensus tree of the ITS4 region of MS Desmodium Using Parsimony*

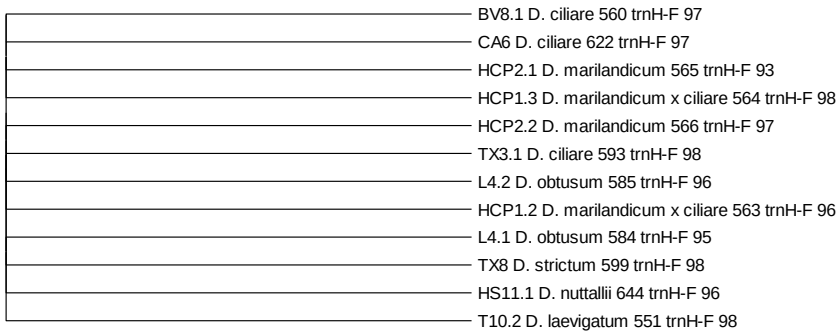



Values after ITS4 correspond to sequence quality values given from Sequencher after editing. Good = sequence with few ambiguities.

B = sequence initially with many ambiguities but edited to higher quality value. Short = sequence generally fewer than 300 base pairs.

Control = preliminary sequences included in main sampling phylogenetic analysis

Figure 3.2 *Bootstrap consensus tree of the trnH–psbA region of Mississippi Desmodium Using Parsimony*




Values after *trnH*-F correspond to sequence quality values given from Sequencher after editing.

The models used in the maximum likelihood analysis for ITS and *trnH–psbA* were based on the lowest presented Bayesian Information Criterion value (BIC). Using 146 parameters and the lowest BIC score of 5182.339, the Tamura 3-parameter (T92) using discrete Gamma distribution (+G) model was selected for the maximum likelihood analysis (Table 3.2). For the Maximum Likelihood Analysis of the ITS and *trnH–psbA* regions, the parameters were set as followed: Bootstrap method using 5,000 replications, Tamura 3-parameter model with five discrete gamma categories with gamma-distributed rates among sites, using all sites including gaps and missing data, extensive (level five) subtree-pruning-regrafting (SPR) heuristics method using a maximum parsimony initial tree, and no branch swap filter. The maximum likelihood bootstrap consensus tree (Figure 3.3) was condensed to show bootstrap values with support over 65%. As a result, the relationships for the ITS sequences are congruent with the parsimony consensus tree with the exception of having no support for any distinction among the *Desmodium* species except for *Desmodium incanum*.

For the *trnH–psbA* sequences, the Tamura 3-Parameter model using discrete Gamma distribution, was selected based on using 24 parameters and having a BIC of 1335.471 (Table 3.3). The bootstrap consensus tree (Figure 3.4) shows 12 *Desmodium* sequences with 338 nucleotides included in the analysis. All nodes less than 65% were collapsed, leaving a weakly-supported tritomy consisting of *D. nuttallii*, *D. laevigatum*, and a grouping of nine various samples within the *D. ciliare* complex and one *D. strictum*.

Table 3.2 *First 14 Maximum Likelihood fits of Nucleotide Substitution Models for ITS*

| Model | Parameters | BIC | AICc |
|---|---|---|---|
| T92 + G | 146 | 5182.339 | 3956.289 |
| T92 + I | 146 | 5184.821 | 3958.771 |
| T92 | 145 | 5185.889 | 3968.228 |
| K2 + G | 145 | 5188.400 | 3970.738 |
| K2 + I | 145 | 5190.552 | 3972.89 |
| K2 | 144 | 5191.309 | 3982.036 |
| T92 + G + I | 147 | 5191.455 | 3957.016 |
| TN93 + G | 149 | 5196.933 | 3945.717 |
| K2 + G + I | 146 | 5197.158 | 3971.108 |
| TN93 | 148 | 5197.244 | 3954.417 |
| TN93 + I | 149 | 5200.409 | 3949.193 |
| HKY + G | 148 | 5201.121 | 3958.293 |
| HKY + I | 148 | 5203.654 | 3960.827 |
| HKY | 147 | 5204.694 | 3970.255 |

MEGA output indicates: "Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. AICc value (Akaike Information Criterion, corrected) are also presented (Nei & Kumar, 2000). Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+G) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+I) (Kumar et al., 2018)."

Table 3.3 *First 14 Maximum Likelihood fits of Nucleotide Substitution Models for trnH–psbA*

| Model | Parameters | BIC | AICc |
|---|---|---|---|
| T92 + G | 24 | 1335.471 | 1187.400 |
| T92 | 23 | 1336.243 | 1194.329 |
| T92 + G + I | 25 | 1342.534 | 1188.308 |
| T92 + I | 24 | 1343.309 | 1195.238 |
| HKY + G | 26 | 1349.312 | 1188.931 |
| HKY | 25 | 1350.125 | 1195.898 |
| HKY + G + I | 27 | 1356.353 | 1189.820 |
| TN93 + G | 27 | 1356.856 | 1190.322 |
| HKY + I | 26 | 1357.270 | 1196.890 |
| TN93 | 26 | 1357.715 | 1197.334 |
| TN93 + G + I | 28 | 1363.976 | 1191.290 |
| TN93 + I | 27 | 1364.774 | 1198.240 |
| GTR + G | 30 | 1377.756 | 1192.769 |
| GTR | 29 | 1378.876 | 1200.039 |

MEGA output indicates: “Models with the lowest BIC scores (Bayesian Information Criterion) are considered to describe the substitution pattern the best. AICc value (Akaike Information Criterion, corrected) are also presented (Nei & Kumar, 2000). Non-uniformity of evolutionary rates among sites may be modeled by using a discrete Gamma distribution (+*G*) with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable (+*I*) (Kumar et al., 2018).”

Figure 3.3 *Bootstrap Consensus Tree of the ITS region of MS Desmodium Using Maximum Likelihood*

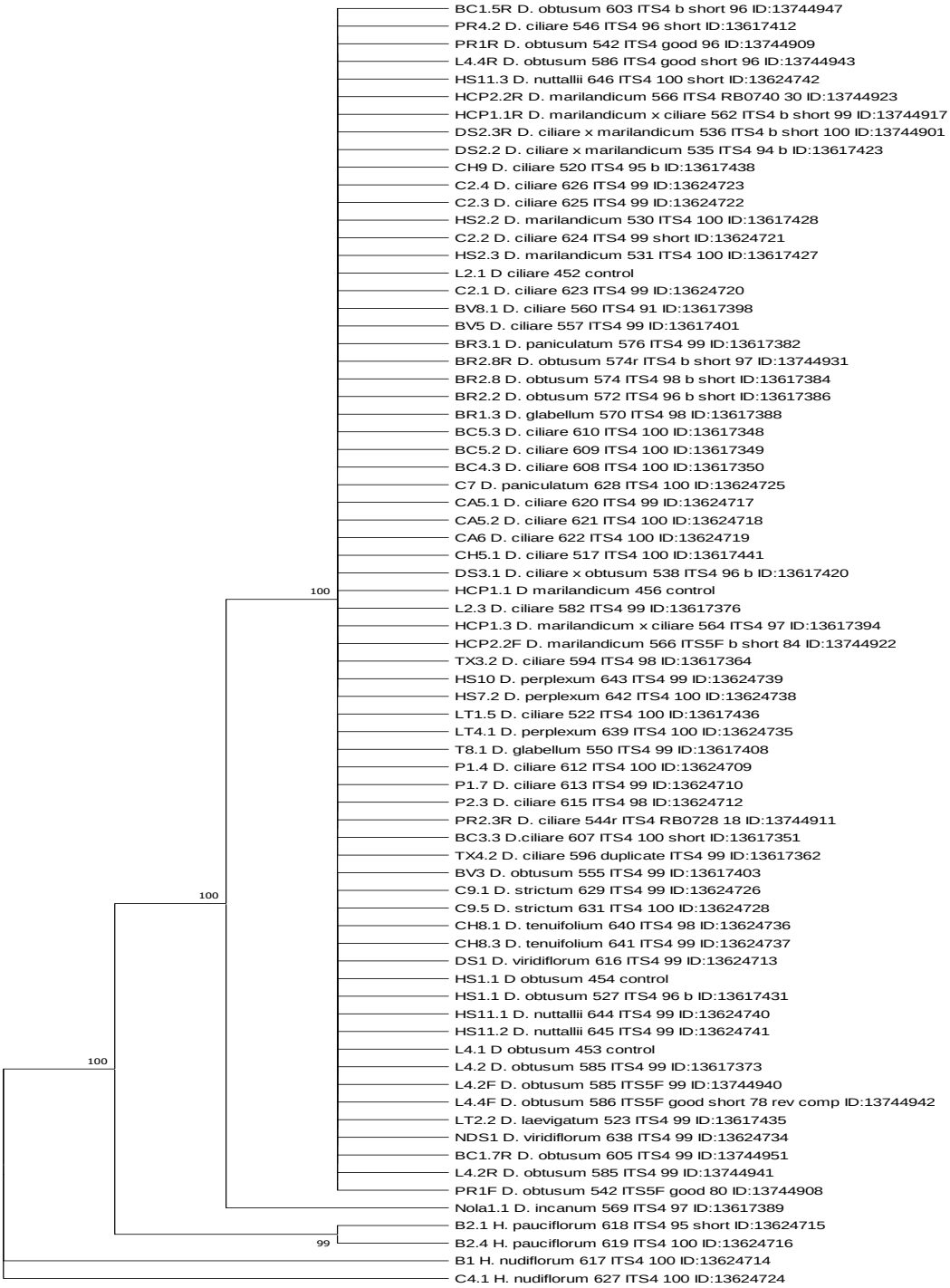
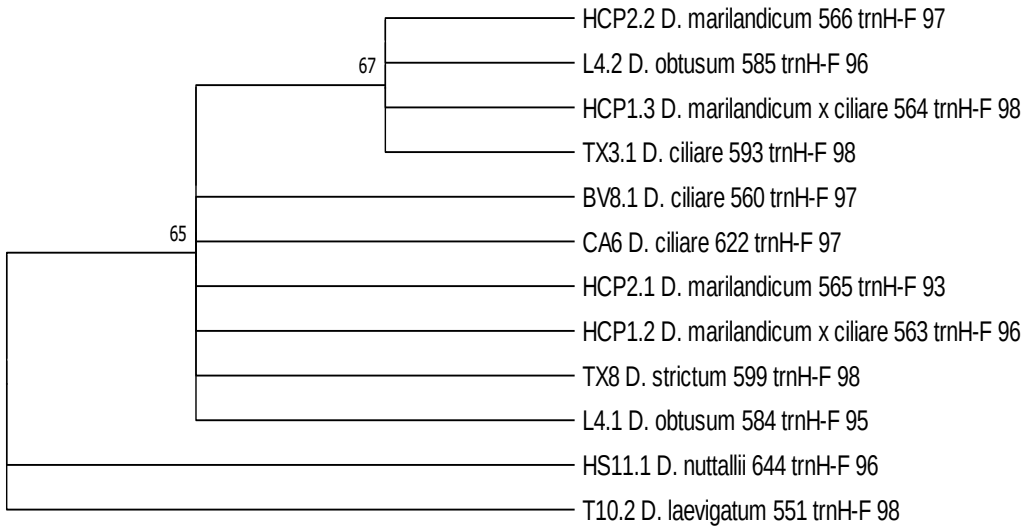


Figure 3.4 *Bootstrap Consensus Tree of the trnH–psbA region of MS Desmodium Using Maximum Likelihood*




Values after *trnH-psbA* correspond to sequence quality values given from Sequencher after editing.

Forty-nine vegetative and fruit morphological characters were summarized into 12 principal components (PCs) (Table 3.3) that explained 87.4% of the variation between the members of the *D. ciliare* complex (i.e., *Desmodium ciliare, D. obtusum, D. marilandicum, D. ciliare* × *D. obtusum,* and *D. ciliare* × *D. marilandicum*). The MANOVA yielded significant results among species (Pillai's $Trace_{48,80} = 3.24$, F-value = 7.07, $p < 0.0001$) between the 49 factors associated with each PC for the five *Desmodium* species/hybrids being considered. Based on the standardized canonical coefficients, PCs 3, 2, and 1 contributed most to the multivariate effect (Table 3.4). Characters on PC 3 (standardized canonical coefficient = 2.28) showed significant differences between *D. ciliare* × *D. obtusum* compared to other species. *Desmodium ciliare* × *D. obtusum* had significantly longer leaves, more hairs, and fewer total leaves in general than other

member of the complex (Table 3.5; Figs. 3.6 & 3.7). For PC 2 (standardized canonical coefficient = -2.19) characters displayed significant differences between *D. marilandicum* and *D. obtusum*, *D. ciliare*, and *D. ciliare* × *D. marilandicum*. In general, when compared to those three species, *D. marilandicum* had significantly longer petioles, more leaf hairs abaxially, and fewer hairs on adaxial leaves, petioles, and stipules (Table 3.5; Figs. 3.5 & 3.7). Finally, characters on PC 1 (standardized canonical coefficient = 1.37) showed *D. obtusum* as significantly different from all other *Desmodium* samples by generally having longer and wider leaves, longer pedicels, and more petiole hairs and adaxial leaflet hairs (Table 3.5; Figs. 3.5 & 3.6).

Figure 3.5 Photographs of *Desmodium* displaying pubescence characters at positions and orientations where they were measured for the PCA. Top Left: Midstem of *D. ciliare*. Top Right: Abaxial surface of *D. obtusum* showing short setose and uncinate pubescence. Bottom Left: Abaxial surface of *D. marilandicum* showing vein strigose pubescence. Bottom Right: Abaxial surface of *D. ciliare* showing short setose and uncinate hairs.

Table 3.4 *Principal Component Table showing Eigenvalues for* Desmodium ciliare *Complex Morphological Characteristics*

| PC | Eigenvalue | Proportion | Cumulative |
|---|---|---|---|
| 1 | 12.638 | 0.258 | 0.258 |
| 2 | 10.161 | 0.207 | 0.465 |
| 3 | 4.894 | 0.100 | 0.565 |
| 4 | 2.886 | 0.059 | 0.624 |
| 5 | 1.990 | 0.041 | 0.665 |
| 6 | 1.960 | 0.040 | 0.705 |
| 7 | 1.742 | 0.036 | 0.740 |
| 8 | 1.701 | 0.035 | 0.775 |
| 9 | 1.486 | 0.030 | 0.805 |
| 10 | 1.192 | 0.024 | 0.830 |
| 11 | 1.113 | 0.023 | 0.852 |
| 12 | 1.040 | 0.021 | 0.874 |

Table 3.5 *Rotated Factor Pattern values for* Desmodium *Morphological Characteristics*

| | **Factor 1** | **Factor 2** | **Factor 3** |
|---|---|---|---|
| Total Stem length | 17 | -8 | -5 |
| Stem length (Flower) | 6 | -11 | -10 |
| Stem width | 36 | 6 | 3 |
| Total Leaves | -13 | 10 | -41* |
| Stem pubescence Type | -12 | -6 | 2 |
| Stem Hairs per mm at midpoint | 27 | -24 | 71* |
| Largest Leaf length | 83* | 7 | 48* |
| Widest Leaf Length | 90* | 11 | -7 |
| Largest Leaf L×W | 15 | 5 | 87* |
| Longest Petiole length | 9 | 92* | -8 |
| Longest Terminal Leaflet Petiolule Length | 8 | 90* | 3 |
| Midpoint Leaf Length | 85* | 5 | 38 |
| Midpoint Leaf Width | 82* | -1 | 10 |
| Midpoint Leaf L×W | 37 | 10 | 41* |
| Midpoint Petiole length | 17 | 84* | -7 |
| Midpoint Petiole Pubescence | 9 | -39 | 43* |
| Midpoint Petiole Hairs per mm | 51* | -44* | 45* |

Negative numbers represent negative correlations regarding the corresponding characteristic. Asterisks correspond to retained loadings >40.

Table 3.5 Continued

| | | | |
|---|---|---|---|
| Midpoint Terminal Leaflet Petiolule Length | 20 | 82* | -2 |
| 2/3 Leaf Length | 80* | 12 | 53* |
| 2/3 Leaf Width | 91* | 19 | -9 |
| 2/3 Leaf L×W | 23 | 10 | 89* |
| 2/3 Petiole length | 3 | 88* | 2 |
| 2/3 Terminal Leaflet Petiolule Length | 19 | 88* | 10 |
| Top Leaf Length | 78* | 14 | 50* |
| Top Leaf Width | 83* | 20 | 6 |
| Top Leaf L×W | 8 | -5 | 75* |
| Top Petiole length | -4 | 71* | -11 |
| Top Terminal Leaflet Petiolule Length | 11 | 71* | 16 |
| Average Leaf Length | 85* | 10 | 49* |
| Average Leaf Width | 98* | 14 | 0 |
| Average Leaf L×W | 26 | 7 | 91* |
| Average Petiole Length | 9 | 97* | -6 |
| Average Terminal Leaflet Petiolule Length | 16 | 96* | 8 |
| Adaxial Leaflet pubescence at midpoint | 25 | -46* | 43* |
| Adaxial Leaflet pubescence per $mm^2$ | 52* | -39 | 45* |
| Abaxial Leaflet Pubescence at Midpoint | -19 | 44* | -9 |
| Abaxial Leaflet pubescence per $mm^2$ | 27 | -35 | 47* |
| Average Stipule Length | -5 | 17 | -6 |
| Stipule pubescence | 12 | -45* | 37 |
| Inflorescence structure | 0 | 1 | 7 |
| Fruit number | 19 | 8 | -6 |
| Pedicel Length | 52* | 14 | 21 |
| Pedicel | -4 | -25 | -8 |
| Pedicel Hair per mm | -16 | -25 | 5 |
| Average Calyx Size | 0 | 20 | 6 |
| Average Article Length | -8 | -1 | -28 |
| Loment Article Dissection | -10 | -27 | 7 |
| Average Stipe Length | 36 | -6 | 27 |
| Bract Length | 17 | 9 | 7 |

Figure 3.6 *PCA of PC 1 LSMEANs plotted against PC 2 LSMEANs for the* D. ciliare *complex*

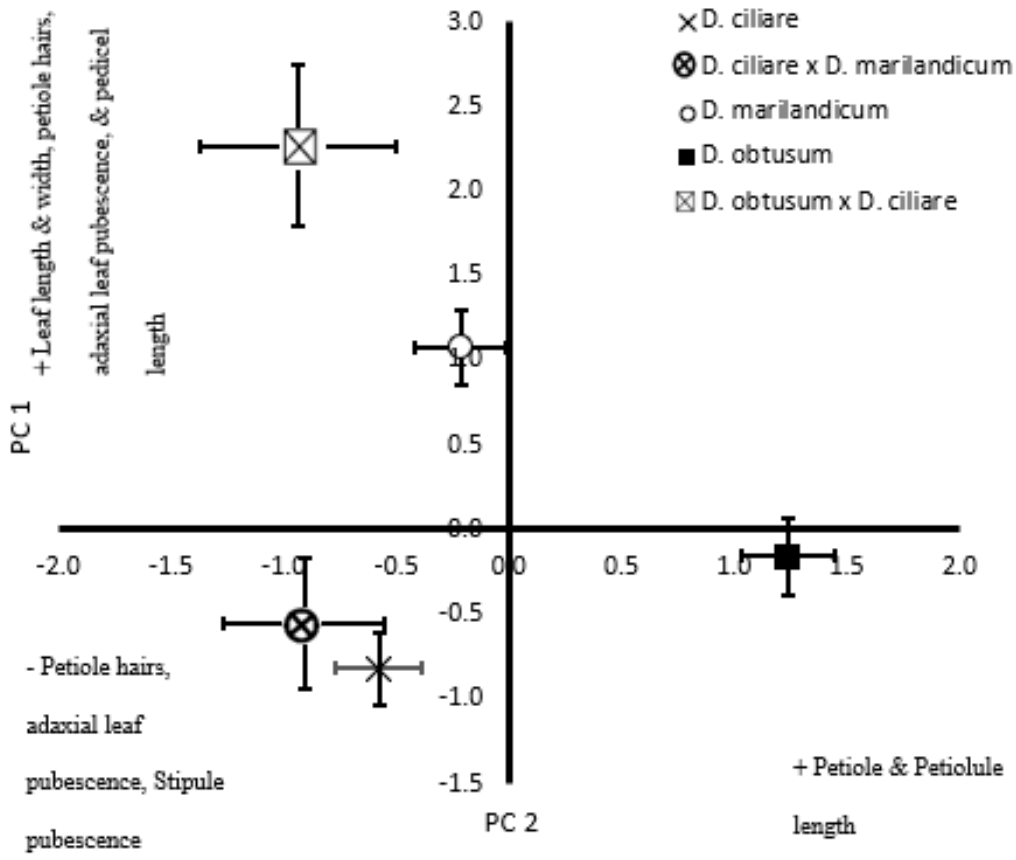


Figure 3.7 *PCA of PC 1 LSMEANs plotted against PC 3 LSMEANs for the* D. ciliare *complex*

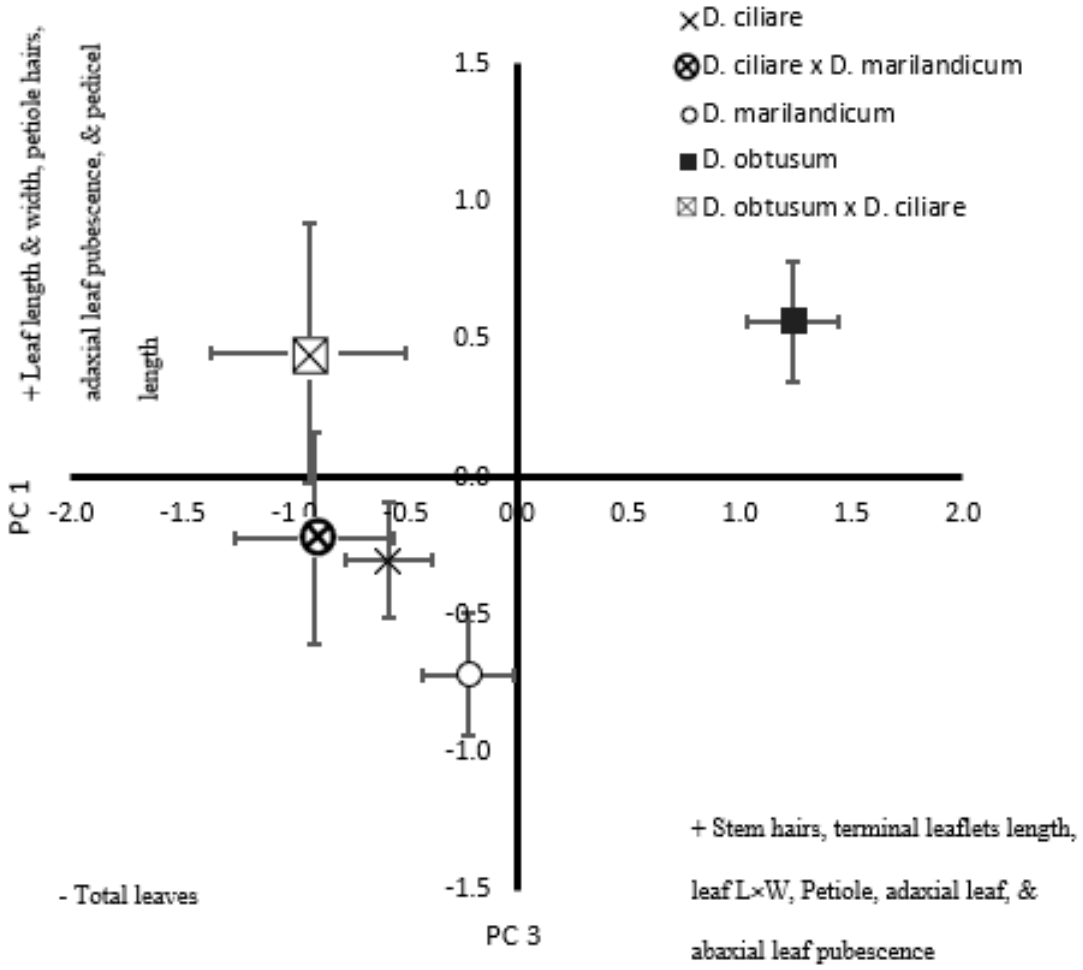
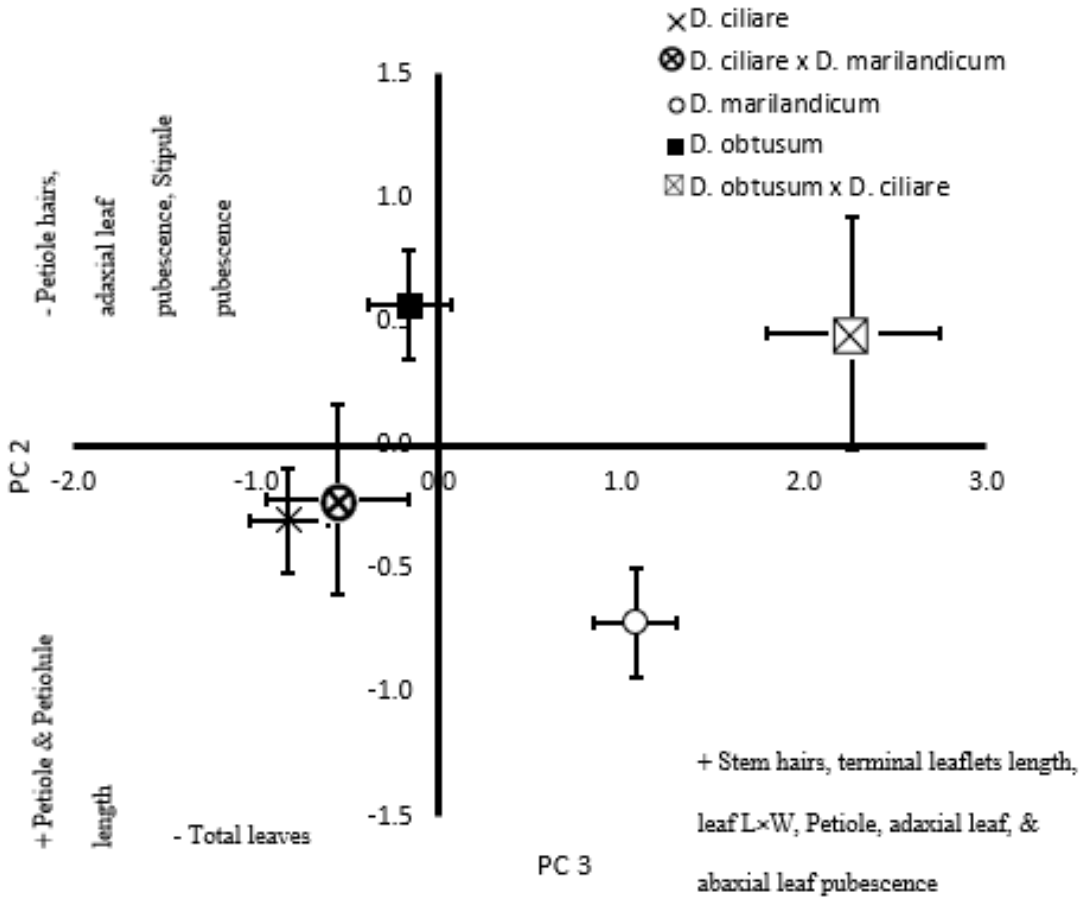


Figure 3.8 *PCA of PC 2 LSMEANs plotted against PC 3 LSMEANs for the* D. ciliare *complex*



# CHAPTER IV – DISCUSSION

The purpose of this study was to clarify the species boundaries and elucidate the evolutionary relationships among the members of the *D. ciliare* complex using morphological and DNA data. Preliminary genetic data of nuclear and plastid gene regions showed sparse variation among the *D. ciliare* complex members. The phylogenies using ITS and *trnL–trnL-F* gene regions resulted in lineages with little support and inadequate resolution for interpreting evolutionary relationships. The PCA and MANOVA using morphological data of the *D. ciliare* complex yielded significant differences.

In the preliminary samples, the plastid *trnH–psbA* gene region had the most SNPs (5), and nuclear ITS had one fewer (4). *trnH–psbA* had a SNP unique for *D. ciliare* and a SNP unique for *D. obtusum*, and for these two species, each one had a unique shared SNP with a *Desmodium* species outside of the complex. Thus, the *trnH–psbA* region may need further investigation as it may help distinguish species of the *D. ciliare* complex and possibly show which species in the complex share ancestral traits with other *Desmodium*. The ITS gene region had one of the only two SNPs specific to *D. marilandicum* making it, as well as *trnL–trnL-F*, one of the more important regions to amplify. ITS also had a shared SNP between *D. ciliare* and *D. obtusum* and two SNPs shared among *D. ciliare* and *D. marilandicum*. For this reason, this region seemed promising to potentially separate *D. marilandicum* from other members of the complex, as well as provide support to which members of the *D. ciliare* complex are sister taxa. Plastid *trnL–trnL-F*, nuclear *H3D*, and plastid *rpL*16 all had three SNPs each. *trnL–trnL-F* provided unique variation for *D. obtusum* and *D. marilandicum* individually and a shared SNP shared by *D. ciliare*

and *D. marilandicum*. *H3D* and *rpL*16 had one SNP for *D. obtusum* and each had *D. marilandicum* sharing a SNP with either *D. ciliare* or *D. obtusum*. However, due to inconsistent amplification, these regions held the least promising prospects for scaling to more samples. The preliminary genetic sampling did not support or refute the findings presented in the main *Desmodium* sampling as there wasn't any resolution between the *D. ciliare* complex found in the nuclear or plastid parsimony or maximum likelihood bootstrap consensus trees when using a broader sampling of individuals. Furthermore, shared character states among putative species in the preliminary analysis were not consistent in the relationships they suggested (e.g., ITS suggested a relationship of *D. ciliare* and *D. marilandicum*, while H3D suggested a relationships of *D. marilandicum* and *D. obtusum*). The preliminary sampling also didn't support or refute any other studies since the *D. ciliare* complex in its entirety hasn't been included in any phylogenetic study to date.

In the bootstrap consensus parsimony tree for the nuclear ITS region, both *Hylodesmum* species and *D. incanum* nodes were well supported. The rest of the phylogenetic tree was left to one well-supported clade of the rest of the *Desmodium* species. Among the polytomy were two clades with bootstrap support above 65. The top clade consisted of various *D. ciliare* complex members, but what defines the clade is that all sequences are mostly shorter than 350 bp. With that said, there are other sequences outside of this clade that exhibit at least one of these conditions, but one of those outsider sequences, BC1.7R *D. obtusum*, shows up in the middle clade in this large polytomy. This middle clade is most likely separated from the polytomy having one shared T instead of a C at site 410. This T is also shared by two *D. strictum*, two *D. tenuifolium*,

one *D. viridiflorum* (not the same *D. viridiflorum* that is already in this clade), two *D. nuttallii*, and three *D. obtusum* (also not in the clade). This clade, as well as about half of the rest of the sequences in this tree, end at about 500 bp. The other half of sequences in this analysis extend another 100 bp. To ensure that the tree wasn't influenced by the reamplified or short sequences, they were removed from a separate analysis which was analyzed in MEGA with the same parameters. The resultant tree showed exactly the same relationships and support as the larger ITS tree. This tree had well-supported *Hylodesmum* and *D. incanum* nodes with a well-supported, large polytomy with the rest of the *Desmodium*. In the ITS bootstrap consensus tree using maximum likelihood, the T92 + G evolutionary model was used as opposed to the equal rate model chosen by Ohashi (2018). Both *Hylodesmum* species, *D. incanum*, and the large *Desmodium* polytomy nodes were well supported (>99%). Unlike the bootstrap consensus parsimony tree, there were no nested clades within the *Desmodium* polytomy clade after nodes with less than 65% bootstrap support were collapsed.

Both ITS trees presented in this study reflect the relationships between *Hylodesmum*, *D. incanum*, and native Southeastern US *Desmodium* seen in the 50% majority rule trees using Bayesian inference and maximum likelihood found in Ohashi (2018). However, in the ITS tree found in Ohashi (2018), relationships between *Desmodium* species found in this study and Ohashi's paper, such as *D. viridiflorum*, *D. marilandicum*, *D. laevigatum*, and *D. tenuifolium*, have bootstrap support over 60%. Furthermore, *D. marilandicum* is the sister taxon to *D. floridanum* and *D. viridiflorum* (Ohashi, 2018), which was also seen, in part, in this study. This relationship wasn't seen consistently within this study since not all members of *D. marilandicum* and *D.*

*viridiflorum* share the same relationships within the phylogenies. Even though Ohashi's (2018) tree shows more support within *Desmodium*, relative to other members presented in the tree, including *Desmodium* not found in the Southeastern US, the Southeastern *Desmodium* presented in the Ohashi (2018) have low bootstrap support for maximum likelihood (< 75%). However, the bootstrap values for Bayesian inference are high (> 98%).

One of the reasons ITS didn't have resolved relationships for the southeastern U.S. *Desmodium* might be from the potentially recent or, in some cases incomplete, divergence among the species, especially among the *D. ciliare* complex. In the ITS consensus tree in Ohashi (2018), the number of substitutions per site expected in the Bayesian analysis for the Southeastern *Desmodium* is relatively low (~ 0.01) especially when comparing non-Southeastern US *Desmodium* to Southeastern US *Desmodium*, which had values ranging approximately 0.02 – 0.09. The low number of SNPs, whether expected or actual (as seen in the preliminary data in this study), could be the product of incomplete lineage sorting, introgression, or hybridization.

In the plastid *trnH–psbA* parsimony bootstrap consensus tree, there was no resolution among the 12 *Desmodium* sequences. In the *trnH–psbA* bootstrap consensus tree using maximum likelihood, the T92 + G evolutionary model was used as opposed to the equal rate model chosen by Ohashi (2018). The phylogeny resulted in a weakly supported clade of the *D. ciliare* complex and one *D. strictum* nested within a polytomy of *D. nuttallii* and *D. laevigatum*. Within this *D. ciliare* complex and *D. strictum* clade, another weakly supported clade of members of the *D. ciliare* complex resides. The phylogeny presented does not disagree or provide any more support for Southeastern US

*Desmodium* than what is presented in the Ohashi (2018) plastid phylogeny using several cpDNA regions, including the *rpL*16 intron and *trnL-rpL*32. It should be noted that for the plastid sequences of Southeastern US *Desmodium* presented by Ohashi, there is disagreement among the relationships of this group between nuclear and cpDNA. For instance, the sister taxa for *D. marilandicum* are *D. viridiflorum* and *D. floridanum* for ITS, but *D. tenuifolium* is sister to *D. marilandicum* in the combined plastid phylogeny. With that said, the cpDNA phylogeny has generally high (> 90%) maximum likelihood and Bayesian inference bootstrap support for the Southeastern US *Desmodium*, and the combined ITS and cpDNA phylogeny reflects the cpDNA relationships (Ohashi, 2018). As stated before, all phylogenies presented by Ohashi (2018) show a relatively low expected number of substitutions per site from the Bayesian inference, and this aligns well with the unsupported nodes present in this study's *trnH–psbA* phylogeny. Ohashi (2018) used a much larger taxonomic sampling and combined several cpDNA sequences into one analysis to provide support for the *Desmodium* phylogeny. In data presented here, there wasn't enough variation present in the approximately 300 bp *trnH–psbA* region of the *D. ciliare* complex and close relatives of the southeastern U.S. This lack of resolution could have been potentially remedied if all sequences had amplified properly in this study and if the sequences were combined into one dataset and analyzed.

For the parsimony bootstrap consensus trees, the ITS and *trnH–psbA* trees do not disagree since there isn't any resolution in the *trnH–psbA* tree. This is the same case for the maximum likelihood trees of the ITS and *trnH–psbA* trees, but the *trnH–psbA* tree provides more resolution among the *D. ciliare* complex clade and includes *D. strictum* within that clade, which provides a small amount of support, since the clade is only

supported by a bootstrap value of 65%, for anecdotal evidence that *D. strictum* and the *D. ciliare* complex hybridize (Gray et al., 1908). Even though the relationships within most of *Desmodium* genetically sampled were not well supported, it should be noted that individuals sampled at one site were not more related to other *Desmodium* sampled at the same site. This means that the relationships among the *Desmodium* sampled in this study do not differ from one another based entirely on geography.

One benefit from including the invasive *D. incanum* in the phylogenies is that it is substantially different genetically from native Southeastern US *Desmodium*, which permits its easy identification using molecular tools. *Desmodium incanum* is morphologically similar to other species of native *Desmodium*, especially without loments. If an individual is not obviously rhizomatous and creeping or if a person does not know to note that characteristic, *D. incanum* could be confused with a number of native species, especially as an immature individual of a native species.

Unlike the phylogenies, the PCA and subsequent MANOVA provided significant evidence for variation (Pillai's $\text{Trace}_{48,80} = 3.24$, F-value = 7.07, $p < 0.0001$) among members of the *D. ciliare* complex. All three species and *D. ciliare* × *D. obtusum* were shown to be significantly different from one another. The PCA showed *D. ciliare* × *D. marilandicum* consistently clustering with *D. ciliare* in all the PC plots. This supports the notion from Ohashi (2013) that the morphological characteristics delimiting the species of the *D. ciliare* complex have been historically over-emphasized as the characters that define these species (e.g., pubescence amount and type) at times overlap. *Desmodium ciliare* × *D. obtusum* displayed the opposite trend of not clustering near any of the *D. ciliare* complex, except when it overlapped only with *D. ciliare* × *D. marilandicum* (Fig.

3.6). Instead, this supposed hybrid fell well outside of the expected area between its two hypothesized parent species.

These morphological differences summarized by the individuals in this study of the *D. ciliare* complex generally coincided with the features historically used to define and differentiate the species, such as petiole length, types and amount of pubescence, and leaf length (Isely, 1990; Krings, 2004; Ohashi, 2013; Radford et al., 1968; Woods, 2008). In this case, PC3 explained the variation coinciding with putative hybrids of *D. ciliare* and *D. obtusum*. This small sampling of individuals characterized by this hybrid condition exhibited a potential over-expression of characteristics intermediate between the two parent species, which has not been noted in prior treatments (Isely, 1990). Moreover, PC3 also shed light on potential characters of interest for future identifications of potential *D. ciliare* and *D. obtusum* hybrids, such characteristics as fewer leaves and more stem, petiole, abaxial leaf, and adaxial leaflet pubescence.

*Desmodium marilandicum* differs from other members in the complex by having more leaf hairs abaxially at midpoint and fewer adaxial leaflet at midpoint, petiole, and stipule hairs, which has not been seen in all taxonomic keys in the literature (Ohashi, 2013; Weakley, 2019). However, Wilbur (1963) does emphasize pubescence characteristics of the leaf surface and petiole to differentiate members of the *D. ciliare* complex. Out of the nine *D. marilandicum* in the study, one individual had glabrous stipules, four had uncinate glabrate stipules, and two had uncinate pubescence with some short setose hairs, and two shared the condition that the rest of the *Desmodium* possessed, (i.e., stipules with short setose and uncinate hairs). This, however, was a qualitative characteristic due to the difficulty in quantifying an uneven and small surface, such as a

*D. ciliare* complex stipule, which is characteristically small compared to most other *Desmodium*. The leaf pubescence characters significant to *D. marilandicum* also were both qualitative characters. Four *D. marilandicum* had glabrous adaxial leaves and five had uncinate glabrate adaxial leaf pubescence. The other *Desmodium*, except for one *D. ciliare* with a glabrous condition and one with uncinate glabrate condition, had "regular" pubescence with some combination of uncinate, pilose, or setose hairs. All *D. marilandicum* and *D. ciliare* × *D. marilandicum* were the only taxa with strigose hairs only on veins accompanied by an abaxial leaf surface that was glabrous, glabrate with uncinate hairs, or pubescent with uncinate hairs. All other taxa had variable amounts of pilose, uncinate, and/or setose hairs, none of which were restricted to the leaf veins. The midpoint petiole pubescence of *D. marilandicum*, unlike the previous traits, was measured quantitatively (visual counts of hairs along one side of the petiole for one mm) and had significantly fewer hairs than the other taxa. *Desmodium marilandicum* averaged four hairs per mm among all individuals with a range of 3–11. *Desmodium ciliare* × *D. marilandicum* averaged eight hairs per mm among all individuals with a range of 6–9. *Desmodium ciliare* averaged 11 hairs per mm among all individuals with a range of 6–19. Finally, *D. obtusum* and *D. ciliare* × *D. obtusum* combined (since the values fell within each other's ranges) averaged 20 hairs per mm among all individuals with a range of 9–32.

The PCA (Table 3.5) identified several morphological characteristics not used in Southeastern U.S. (Isely, 1990; Krings, 2004; Ohashi, 2013; Radford et al., 1968; Woods, 2008). These characteristics include *D. obtusum* having fewer leaves, longer pedicels, more and different petiole and adaxial leaf hairs, and more abaxial leaf hairs. *Desmodium*

*obtusum* had an average 0.48 leaves per cm of stem (measured up to the first flower) with a range of 0.18–0.81 (*D. ciliare* = 0.86 with 0.37–2.83; *D. marilandicum* = 1.16 with 0.35–2.2). The pedicel length for *D. obtusum* in this study averaged 12.16 mm with a range of 10.38–15.64 (*D. ciliare* = 7.66 with 6.07–10.38; *D. ciliare* and *D. ciliare* × *D. marilandicum* = 7.89 with 5.63–12.19; *D. marilandicum* = 10.93 with 8.96–13.1). *Desmodium obtusum* had uncinate hairs with some short setose hairs or uncinate and short setose hairs on the adaxial side of leaves. The other species are described in the previous paragraph. Unlike PC2, *D. obtusum* can be differentiated based on quantitative measurement of leaf hairs on both sides. The adaxial pubescence on *D. obtusum* averaged 38 hairs per $mm^2$ with a range of 23–50 (*D. ciliare* = 21 with 0–42; *D. ciliare* and *D. ciliare* × *D. marilandicum* = 18 with 0–42; *D. marilandicum* = 3 with 0–8). *Desmodium obtusum* had an average of 32 hairs per $mm^2$ on the abaxial leaf surface with a range of 17–46 (*D. ciliare* = 30 with 9–57; *D. ciliare* and *D. ciliare* × *D. marilandicum* = 25 with 5–57; *D. marilandicum* = 12 with 3–30).

Based solely on the DNA variation and phylogenies inferred from DNA data presented in this study, there is not enough data to support or refute the various circumscriptions of species in the *D. ciliare* complex. At most, there were only a few instances of weakly-supported clades held together by one or two base pairs. Both phylogenies (nuclear and plastid) presented here also failed to provide any substantial insight into the evolutionary relationships in the genus as well. However, the PCA and MANOVA provided clearer insight into the delineation of species in the *D. ciliare* complex and provided potential characteristics to differentiate these species in the future. Based on the morphological analyses in this study, all three members of the *D. ciliare*

complex were significantly different from one another with two individuals of one hybrid (i.e., *D. ciliare* × *D. obtusum*) being significantly different from all individuals of the complex in this study. *Desmodium ciliare* × *D. marilandicum* individuals in this study were indistinguishable from *D. ciliare* based on the PCA and MANOVA. In light of the Unified Species Concept (de Queiroz, 2007), this research has found one line of evidence (morphological data, as analyzed by PCA and tested by MANOVA) that supported the recognition of all three commonly recognized species. DNA evidence, however, was ambiguous. Until more extensive DNA studies occur, using a generalist approach towards delimiting the *D. ciliare* complex should be used, where over-emphasizing one or two characteristics of an individual within this complex would be ill advised. Instead, species identification for individuals within the complex should gravitate towards what the majority of characteristics suggest. For example, instead of calling an individual *D. ciliare* × *D. marilandicum*, based on having glabrous to glabrate pubescence along the stem and having one, or maybe two, longer petioles (approximately one cm), the individual is probably better identified as *D. ciliare* because of the presence of uncinate hairs, most of which likely fell off with age, and the majority of the petioles being shorter than one cm.

To more accurately represent the evolutionary history of the species of the *D. ciliare* complex, a larger sampling of individuals across the range of the putative species and more DNA data are needed. Because there were only 33 individuals used in the PCA in this study, and as the PCA was the most successful part in delineating the *D. ciliare* complex, more samples of the *D. ciliare* complex, especially more problematic individuals identified as hybrids, should be included in the analysis. In an ideal scenario,

individuals of the *D. ciliare* complex, including tentative hybrids, would be sampled throughout their range as well.

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