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## Molecular Phylogeny of the Genus *Houstonia* and Allies in Rubiaceae

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**MOLECULAR PHYLOGENY OF THE GENUS *HOUSTONIA* AND ALLIES IN RUBIACEAE**

by

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B.S. December 2011, Old Dominion University

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## ABSTRACT

### MOLECULAR PHYLOGENY OF THE GENUS *HOUSTONIA* AND ALLIES IN RUBIACEAE

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*Houstonia* (Rubiaceae) is a strictly North American genus of 24 species distributed from Mexico, throughout the United States, up to Canada. *Houstonia* has proven to be a taxonomically difficult genus since the Linnaean description of *Houstonia* and the related genera: *Hedyotis* and *Oldenlandia* in 1753. For over 250 years botanists have lumped and separated *Houstonia* from *Hedyotis* and *Oldenlandia* based on various morphological characters. The most recent circumscription of *Houstonia* (Terrell 1996) separated the genus into two subgenera with each subgenus containing two sections. Nuclear (ITS) and plastid (*trnL-F*, *rps16*) DNA sequences were used to build a molecular phylogeny depicting relationships within *Houstonia* and among the closely related genera *Stenaria* and *Stenotis*, all of which used to be considered *Hedyotis*. Separate and combined datasets show *Stenaria* is nested within the *Houstonia* lineage and therefore *Houstonia*, as currently circumscribed, is not a monophyletic lineage. These results disagree with the use of crateriform seeds to distinguish *Houstonia* (crateriform seeds) from *Stenaria* (non-crateriform seeds). It appears the most useful characters to define this group are the loss of chromosomes through the major clades as the *Houstonia-Stenaria* lineage radiated north and east in North America. Descending aneuploidy has been accompanied by slight modifications of the pollen aperture types from a simple endoaperture in *Stenotis* referred

to as colporate type A with modifications in *Houstonia-Stenaria* resulting in compound aperture types referred to as colporate type B and colpororate.

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## CHAPTER 1

### INTRODUCTION

The Rubiaceae is the fourth largest family of flowering plants with over 600 genera encompassing more than 13,000 species (Delprete and Jardim 2012). The family is composed of three subfamilies: Rubioideae Raf., Cinchonoideae Raf., and Ixoroideae Verd., with each subfamily further separated into tribes (Delprete and Jardim 2012). The focus of this work involves genera of a historically troublesome tribe in the Rubioideae known as Hedyotideae Cham. & Schltld. ex DC.. The Hedyotideae was placed basal to the tribe Spermacoceae Cham. & Schltld. ex DC and is now included in the Spermacoceae (Bremer 1996; Andersson and Rova 1999; Bremer and Manen 2000). A recent phylogenetic analysis classifying the Rubiaceae treat the Spermacoceae as a tribe with ca. 1000 species spread throughout 60 genera including most genera of the Hedyotideae (Bremer and Eriksson 2009). A complete circumscription of the tribe is taxonomically difficult due to some genera of Hedyotideae such as *Hedyotis* L. being used as what has been referred to as a repository for species that do not easily align into other genera (Wikström et al. 2013). *Houstonia* L. is one such genus that has been lumped and separated from *Hedyotis* by botanists since the Linnaean classification of *Hedyotis* and *Houstonia* in 1753 (Linnaeus 1753). The present study aims to circumscribe the North America genus *Houstonia* L. of the Hedyotideae and related genera previously defined as *Hedyotis* including *Stenaria* Terrell, *Stenotis* Terrell, and *Oldenlandiopsis* (Griseb.) Terrell and W.H. Lewis. With a molecular phylogeny constructed, characters important for speciation were studied to better understand the radiation of *Houstonia* and closely related genera throughout North America.



### *Taxonomic History of Houstonia*

In his work, *Species Plantarum*, Linnaeus (1753) described the three closely related genera: *Hedyotis*, *Houstonia*, and *Oldenlandia* L. with the type specimens designated as *Hedyotis fruticosa* L., *Houstonia caerulea* L., and *Oldenlandia corymbosa* L. Since Linnaeus' classification, several other botanists have rearranged the species of *Houstonia*, *Hedyotis*, and *Oldenlandia*. These treatments have ranged from merging all species from the three genera into *Hedyotis* to splitting the species into upward of eight genera (Terrell 1996).

Rafinesque (1820) was one of the first to propose a new treatment of *Houstonia* and *Hedyotis* after describing and assigning numerous new species to the two genera. First, he removed *Houstonia rotundifolia* Michx. and placed the species in a newly created genus, *Panetos* Raf, (Terrell 1996). He then split *Houstonia* into four subgenera: *Edrisia*, *Christimia*, *Stenaria*, and *Chamisme*. Terrell would later resurrect the name *Stenaria* and *Chamisme* with his own work circumscribing *Houstonia* and *Hedyotis*.

Torrey and Gray (1841) were the first to view these taxonomically difficult genera in an inclusive sense. They originally placed *Houstonia*, *Oldenlandia*, and *Pentodon* Hochst. in *Hedyotis* however Gray admittedly shifted his opinion on the subject throughout his career (see Terrell 1996 for a complete history of the taxonomic changes made to these genera). Gray's most notable contribution to understanding the relationships among these genera came with his work in seed morphology, a primary character still used in the most recent circumscription of *Houstonia* by Terrell (1996). Morphological differences in the seeds of *Oldenlandia*, *Houstonia*, and *Hedyotis* led Gray to his final conclusion that the three genera "equally merit restoration" (Terrell 1996). He described *Oldenlandia* as having "very numerous and small seeds angular or globular, mostly obpyramidal or trihedral, not

compressed nor hollowed on the face.” In comparison, seeds of *Houstonia* are few or moderately numerous, peltate, and hollowed or concave on their inner face (Terrell 1996).

Francis Fosberg (1943) disagreed with Gray on the importance of seed characters for classification of *Houstonia*, *Oldenlandia*, and *Hedyotis*, stating that seeds of *Hedyotis* vary to the extent that no two seeds of a capsule are alike. The seeds are essentially peltate but compression of the seeds leads to varying shapes. For this reason and other minute differences in morphological characters, Fosberg lumped species of *Oldenlandia* and *Houstonia* back into *Hedyotis* by creating five subgenera for *Hedyotis*.

Walter Lewis (1961) furthered the work of Fosberg by classifying *Houstonia* based on seed, floral, and fruit characteristics. Lewis came to the conclusion that there was not enough support based on character differences to differentiate the three genera. Therefore, he suggested merging *Houstonia* and all of the North American species of *Oldenlandia* back into *Hedyotis*, making them subgenera. Lewis (1962) continued his work on the lineage by studying the morphology and chromosome count of *Oldenlandia* and *Edrisia* which included numerous species that were previously defined as *Hedyotis* or *Houstonia*. The North American species in the subgenus *Oldenlandia* were found to have a chromosome number  $x=6$  or  $9$  and were upgraded to generic status once again (Lewis 1962). Lewis (1965) also examined pollen morphology of the North American *Hedyotis*. This led him to redefine the original six groups he first separated by chromosome number into five groups. He merged the  $x=7$  (*Houstonia rosea* (Raf.) Terrell, *Houstonia procumbens*) and  $x=8$  (*Houstonia caerulea*, *Houstonia serpyllifolia* Michx., *Houstonia pusilla* Scöpf, *Houstonia micrantha* (Shinners) Terrell) groups into one and placed *Houstonia wrightii* A. Gray ( $x=11$ ) into the  $x=9, 10^*$  (*Stenaria nigricans* (Lam.) Terrell) group (Lewis 1965).

Lewis' phylogenetic hypothesis based on pollen characters and chromosome count is the most consistent of the morphological studies with the few molecular studies conducted on the group.

Terrell et al. (1986), along with Lewis (1965), used seed and pollen morphology, and chromosome count to aid in differentiation of species. The focus of this work was *Houstonia*, but also included species of *Hedyotis*. Based on their work, Terrell formed 12 species groups, 6 lineages, and 2 basic series. The first series was composed of a Baja California ( $x=13$ ) (*Stenotis arenaria* (Rose) Terrell, *Stenotis asperuloides* (Benth.) Terrell, *Stenotis australis* (I.M. Johnst.) Terrell), *Stenotis brevipes* (Rose) Terrell, *Stenotis mucronata* (Benth.) Terrell) group from Lewis (1962) and several other species previously unstudied. The second series consisted of all other groups from Lewis' previous studies including the  $x=6,7,8,9(10^*), 11$ . Based on this work and the type specimens, Terrell reclassified the North American complex into three genera, giving *Houstonia* and *Oldenlandia* generic status once again (Terrell et al. 1986). This reclassification of the North American species resulted in 21 species of *Hedyotis*, 20 of *Houstonia*, and 9 of *Oldenlandia* (Church 2003).

Based on seed and pollen morphology and chromosome count, Terrell recognized two subgenera within *Houstonia*: subgenera *Houstonia* and *Chamisme*. *Houstonia* contained the group of species from Lewis'  $x=7,8$  groups. *Chamisme* includes the Eastern North American  $x=6$  and the  $x=11$  group from the Southwestern United States. To further his work, Edward Terrell used scanning electron microscopy to study seed morphology and different characters that could aid in defining the species of *Houstonia* and *Hedyotis*. Terrell pulled six species from the North American *Hedyotis* group and treated them generic at level. The new genus was referred to as *Stenotis* and contained the species that formed the

Baja California series ( $x=13$ ) from his previous work. There is strong support for the generic status of *Stenotis* based on geographic distribution, pollen morphology, chromosome number, and molecular analysis (Terrell 2001b).

The majority of the remaining North American species of *Hedyotis* were eventually also upgraded to generic status (Terrell 1987; Terrell 1990; Terrell 2001a; Terrell 2006; Terrell and Robinson 2009). The separation from *Hedyotis* was justified based on morphological differences from *Houstonia*, *Hedyotis*, and *Oldenlandia*. Although there has been much debate on the type specimen for *Hedyotis*, *Hedyotis fruticosa* L. or *Hedyotis auricularia* L., the group previously described by Terrell as the *Hedyotis nigricans* group differs from both of the debated type specimens. Terrell (2001a) gave this group generic status primarily based on the differences in seed morphology from *Houstonia* and chromosome number. He named the new genus *Stenaria*, from one of Rafineque's previously described subgenera of *Houstonia* (Terrell 2001a). *Stenaria* possesses non-crateriform seeds that are somewhat compressed and ellipsoid with a centric punctiform hilum whereas seeds of *Houstonia* are crateriform with a ventral depression with a linear hilar ridge or a ventral subglobose cavity without a hilar ridge. *Stenaria* also differs from *Houstonia* in having a chromosome number of  $x = 9$  or  $10$  (chromosome number is only known for *S. nigricans*) compared to chromosome numbers  $x = 6, 7, 8$ , and  $11$  in *Houstonia*. The six species comprising this genus are native to the Southwestern United States and northern Mexico, overlapping with sections of *Houstonia* (Terrell 2001a).

#### *Study Group*

*Houstonia* - *Houstonia* is comprised of 24 annual or perennial herbs, caespitose or rhizomatous, ranging from northern Mexico, throughout the United States, to eastern parts

of Canada. Characters defining the genus include opposite leaves (3-4 whorled in *Houstonia acerosa* (A. Gray) Benth. & Hook.f.), inflorescences of terminal or axillary cymes or individual flowers on elongated pedicels, homostylous or heterostylous 4-merous flowers with salverform or funnellform corollas, and biloculate capsules dehiscing loculicidally and occasionally secondarily dehiscing septicidally. These characters can aid in identifying the genus but are not the primary characters used to delimit the lineage. *Houstonia* has been split into two subgenera (*Houstonia* and *Chamisme*) with each subgenus containing two sections based primarily on seed characters, chromosome number, and pollen aperture types (Lewis 1962; Lewis 1965; Terrell 1996). Both subgenera display the crateriform seed type which has been used as a major character for separating *Houstonia* from other closely related genera of the complex. *Houstonia* is the only genus aside from *Neanotis* W.H. Lewis, Ann., to have crateriform seeds in what was previously defined as the Hedyotideae tribe (Terrell 1996). The first subgenus, *Houstonia*, is split into sections: *Houstonia* and *Mullera*. Section *Houstonia* has caeruloid seeds with a ventral cavity lacking a hilar ridge or hilar scar. Four of the five species have a chromosome number  $x=8$  with the fifth, *Houstonia procumbens*, being  $x=7$ . Pollen for all species of section *Houstonia* is defined as having the colpurate type B aperture. Section *Mullera* contains the sole species *Houstonia rosea*. This species has dorsiventrally compressed seeds with a hilar ridge, chromosome number  $x=8$ , and colpurate type B pollen apertures. *Houstonia rosea* also exhibits the smallest pollen and has 4-aperturate pollen instead of the 3-aperturate pollen characteristic of other *Houstonia* species (Terrell 1996). Subgenus *Chamisme* contains sections: *Amphiotis* and *Ericotis* and displays characteristics similar to *Houstonia rosea*. All species of the subgenus have seeds that are dorsiventrally

compressed and have a ventral depression with a narrow hilar ridge. The pollen of this subgenus is colporate, a character not found in other species of Hedyotideae. Section *Amphiotis* contains four perennial species distributed throughout central and eastern United States and Canada. Chromosome number for all four species is  $x=6$ . Section *Ericotis* comprises five perennial and five annual species distributed in the southwestern United States and Northern Mexico with a chromosome number  $x=11$ .

*Stenaria* - *Stenaria* is a genus of six perennials primarily distributed throughout Texas and northern Mexico. Many of the morphological characters of *Stenaria* such as phyllotaxy, corolla shape, and fruit type are similar to *Houstonia*, making them difficult to distinguish in the field. Primary characters used to upgrade *Stenaria* to generic rank and separate it from *Houstonia* have been seed characters (Terrell 2001a) and chromosome number (Lewis 1965). *Stenaria* possesses the more common non-crateriform seeds compared to the crateriform seeds of *Houstonia* (Terrell 2001a). Chromosome count for *Stenaria* has been recorded as  $x=9(10)$  (Lewis 1965). Of the six species, chromosome count has only been conducted on the type species, *Stenaria nigricans*. Lewis (1965) claimed that western Texas individuals of *Stenaria nigricans* had a chromosome number  $x=10$  while *Stenaria nigricans* distributed in eastern Texas was found to be  $x=9$ . The eastern distribution is more common and is considered the base chromosome number for the genus. Lewis (1965) stated although the two individuals had a difference in chromosome number, they were morphologically the same. He proposed a hypothesis of chromosome loss from *Houstonia wrightii* ( $x=11$ ) giving rise to the  $x=10$  individuals of western Texas and an additional chromosome loss resulting in the  $x=9$  group. His primary evidence for this

hypothesis was based on overlapping distribution and similar morphology between *Houstonia wrightii* and *Stenaria* (Lewis 1962).

*Stenotis* – *Stenotis* consists of four perennial (*Stenotis australis*, *Stenotis brevipes*, *Stenotis mucronata*, *Stenotis peninsularis* (Brandege) Terrell) and three annual (*Stenotis arenaria*, *Stenotis asperuloides*, *Stenotis greenii* (A. Gray) Terrell and H. Rob) herbs, all heterostylous, and primarily distributed throughout Baja California however, one species, *Stenotis greenii*, is found in the state of Arizona (Terrell 2001b). Seeds of the genus are non-crateriform, ellipsoid, with a centric punctiform hilum and some species have a prominent ventral hilar ridge (Terrell 1996). A chromosome number of  $x=13$  is known for five of the species (*Stenotis arenaria*, *Stenotis asperuloides*, *Stenotis australis*, *Stenotis brevipes*, *Stenotis mucronata*) (Lewis 1962). This number is unique to genera of the previously defined Hedyotideae and is one of the major justifications for separating *Stenotis* from *Houstonia* (Terrell 2001b). Aside from geographic distribution, seed characters, and chromosome number, the pollen type of *Stenotis* is another primary character separating the genus from *Houstonia* and *Stenaria*. *Stenotis* possesses the more common pollen with the simple colporate type A apertures whereas *Houstonia* and *Stenaria* have pollen with the compound colporate type B or colpororate aperture types (Lewis 1965).

## CHAPTER 2

### MATERIAL & METHODS

#### *Taxon Sampling*

Taxon sampling included 53 ingroup and 4 outgroup taxa from herbarium voucher specimens representing the genera: *Houstonia*, *Stenaria*, *Stenotis*, and *Oldenlandia*. To my knowledge, the highest level of sampling for previous molecular studies of *Houstonia* and its North American allies included 25 ingroup taxa (Church 2003). The present study includes 12 additional species of *Houstonia*, *Stenaria*, and *Stenotis*. Taxa were sampled to encompass all four currently recognized sections of *Houstonia* along with North American species of *Oldenlandia* formerly referred to as *Houstonia*. Taxa representing the newly recognized genera, *Stenotis* and *Stenaria*, were also sampled to analyze the validity of their generic status. These samples included 4 of 7 species of *Stenotis* and 5 of 6 species of *Stenaria*. The remaining *Stenaria* species, *Stenaria sanchezii* Lorence, is primarily located in Northern Mexico and specimen loans were unattainable. Outgroup included the Spermaceae genus, *Arcytophyllum* Wild. ex Schult and Schult. f. and two species of *Oldenlandia* known to be distantly related to the *Houstonia* lineage. A complete list of taxa with their accessions numbers is listed in the appendix A.

#### *DNA Extractions*

Total genomic DNA was extracted with the DNeasy Plant Kit (Qiagen, Valencia, California, U.S.A) following the manufacturer's protocol. Due to the difficulty of extracting DNA from herbarium specimens, an additional step was necessary to increase the likelihood of obtaining DNA. 30  $\mu$ l of Proteinase K and 30  $\mu$ l of 2-Mercaptoethanol were



added following the addition of buffer AP1 from the standard protocol. The solution was then incubated at 42°C for 12-24 hours before completing the remaining protocol.

### *Amplification and Sequencing*

One nuclear region ITS (ITS1, 5.8S, ITS2) and two plastid markers (*rps16* intron, *trnL-F* spacer) were selected for amplification. Primers for ITS, *rps16*, and *trnL-F* amplification are listed in Table 1. Sequencing reactions were completed using an ABI 2720 thermal cycler with solutions containing 12.5 µl of GoTaq Green Master Mix (Promega, Madison, Wisconsin, U.S.A), 1 µl of each 10 µM primer, 1.25 µl dimethyl sulfoxide (DMSO), 0.25 µl bovine serum albumin (BSA), 8 µl water and 1 µl of DNA extract. The amplification protocol for nuclear and chloroplast markers followed Kårehed & al. (2008). Gel electrophoresis of PCR products was used to determine the product size and amount. PCR products were purified using the AMPure PCR purification protocol (Ageencourt, Beverly, Massachusetts, U.S.A.). Sanger sequencing and sequence analyses were completed by Macrogen (Seoul, Korea) on an ABI 3730 XL.

**Table 1:** ITS, *rps16*, and *trnL-F* primers

Region	Primers	Primer Sequence from the 5' End	Reference
ITS	NY183_F	CCTTATCATTTAGAGGAAGGAG	Motley et al. (2005)
	NY43_R2	TATGCTTAAAYTCAGCGGGT	Motley et al. (2005)
<i>rps16</i>	<i>rpsF</i>	GTGGTAGAAAGCAACGTGCGACTT	Oxelman et al. (1997)
	<i>rpsR2</i>	TCGGGATCGAACATCAATTGCAAC	Oxelman et al. (1997)
<i>trnL-F</i>	“c”	CGAAATCGGTAGACGCTACG	Taberlet et al. (1991)
	“f”	ATTTGAACTGGTGACACGAG	Taberlet et al. (1991)

### *Phylogenetic Analyses*

Sequences were manually edited in the program Sequencher v. 4.8 (Gene Codes, Ann Arbor, Michigan, U.S.A). Primary alignment of individual regions was completed using the default settings for the online program, PRANK (<http://www.ebi.ac.uk/goldman-srv/webprank>; Loytynoja and Goldman 2005). Manual alignment adjustments were made using the software MacClade v.4.08a (Maddison and Maddison 2005) and Mequite version 2.72 (Maddison and Maddison 2009).

Models of nucleotide substitution for nuclear and plastid regions were evaluated with the Bayesian information criteria (BIC) using the program jModelTest v2.1.4 (Darriba et al. 2012). BIC in jModelTest supported SYM+G as the best-fit model of nucleotide substitution for ITS, GTR+I for rps16, and GTR+G for trnL-F. These models were used when running Bayesian analyses. Bayesian inference analyses were completed using MrBayes v.3.2 (Ronquist et al. 2012). Bayesian inference analyses were completed on the Cyber infrastructure for Phylogenetic Research portal (CIPRES) (<http://www.phylo.org/>; Miller et al. 2010). Each analysis was run for 100,000 generations with trees sampled every 100 generations and the first 25% of trees discarded.

## CHAPTER 3

### RESULTS

#### *Sequences and Datasets*

Sequence data were generated from three loci: nuclear- ITS and plastid- *rps16* and *trnL-F*. These regions have been shown to be potentially phylogenetically informative in the Spermaceae (Karehed et al. 2008) and *rps16* has not been used in previous studies focusing primarily on *Houstonia* and its North American allies. The combined dataset comprised 3701 base pairs after alignment (ITS: 922, *rps16*: 1230, *trnL-F*: 1548). Of the 3701 base pairs, 747 (20.2%) were variable and 453 (12.2%) were potentially parsimony informative. The nuclear ITS region was the most parsimony informative (24.3%) while the plastid *trnL-F* region was least informative (7.2%). Phylogenetic relationships indicated by the Bayesian analyses are summarized as a 50% majority-rule consensus tree with posterior probability values greater than 0.50 reported at each node. Nodes with a posterior probability of 0.90 or greater are considered supported (Manns and Bremer 2010).

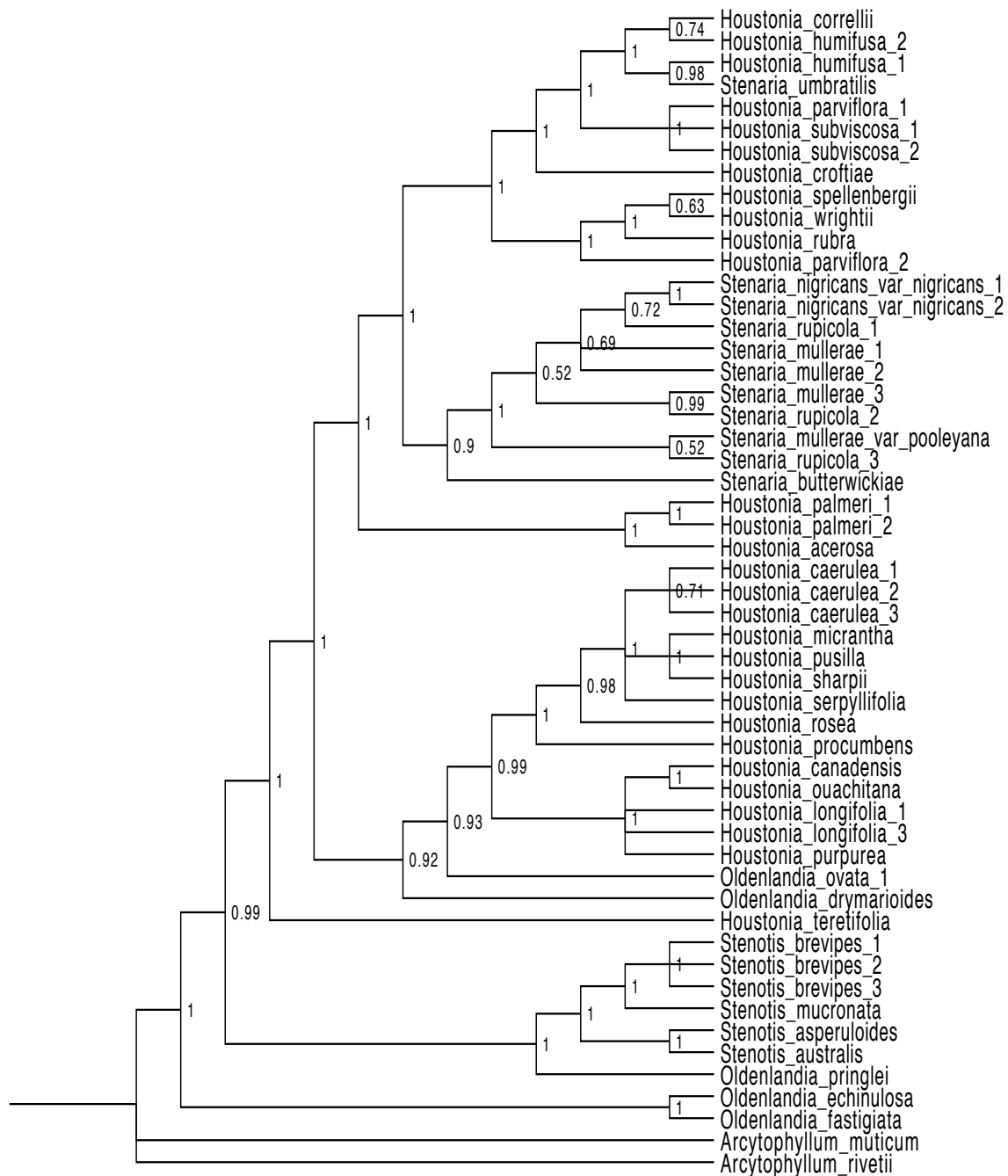
#### *Phylogenetic Relationships*

The overall topology of the phylogenetic trees obtained from the nuclear ITS region and plastid *trnL-F* and *rps16* regions are majorly congruent. General topology of the major clades in the nuclear, chloroplast, and combined trees are congruent. However, basal nodes of the plastid trees are not well resolved therefore sister relationships form polytomies in the *trnL-F* and *rps16* analyses. Results discussed here are primarily based on the combined phylogeny (Figure 1).

Analysis of both nuclear and chloroplast data separate and combined, does not support the monophyly of *Houstonia* as it is currently circumscribed. *Houstonia*, *Stenaria*, and *Stenotis* are resolved as a monophyletic lineage forming three major clades (PP = 0.99). Clade A (PP = 1.0) consists of three subclades that comprises the species of *Houstonia* designated as section *Ericotis* and the genus *Stenaria*. Subclade A1 (PP = 1.0) is formed from 8/10 species defined by Terrell as section *Ericotis* of the subgenus *Chamisme* with one species currently defined as *Stenaria* (*Stenaria umbratilis* (B.L. Rob)) nested within the section *Ericotis*. Subclade A2 (PP = 0.99) consists of the remaining *Stenaria* species sampled and is sister to subclade A1. The third subclade (labeled A3) (PP = 1.0) comprises the remaining two species of section *Ericotis* (*Houstonia acerosa* and *Houstonia palmeri* A. Gray) and is sister to the *Stenaria* clade and the remaining species of section *Ericotis*.

Clade B comprises the remaining species of *Houstonia* analyzed and is divided into two major subclades. Subclade B1 comprises 4/5 species treated by Terrell as subgenus: *Houstonia* section: *Houstonia* and one additional species (*H. sharpii* Terrell) that was considered *Hedyotis* when Terrell proposed his taxonomic treatment of *Houstonia* (PP = 1.0). The sole species designated as subgenus: *Houstonia* section: Mullera (*Houstonia rosea*) is placed sister to subclade B1 (PP = 0.98). Additionally, the remaining species of section: *Houstonia* (*Houstonia procumbens*) is sister to *Houstonia rosea* and the other species of section: *Houstonia*. Two of the *Oldenlandia* species (*Oldenlandia ovata* S. Watson, *Oldenlandia drymarioides* (Standl.) Terrell) included as ingroup taxa are placed sister to clade B and appear to be part of the North America *Houstonia* lineage. *Houstonia teretifolia* Terrell is placed basal to the *Houstonia-Stenaria* lineage (PP = 1.0)

Clade C is sister to the *Houstonia-Stenaria* lineage and comprises all species of *Stenotis* sampled (4/7) and *Oldenlandia pringlei* (PP = 0.99). *Stenotis* forms a monophyletic clade (subclade C1) (PP = 1.0) with *Oldenlandia pringlei* B.L. Rob placed sister to *Stenotis*.



**Figure 1:** Majority rule consensus tree of the *Houstonia* lineage. 50% majority rule consensus tree retrieved from the Bayesian analyses of combined dataset (ITS, *trnL-F*, *rps16*). Numbers above the branches represent Bayesian posterior probability values.

## CHAPTER 4

### DISCUSSION

Current analyses of nuclear ITS and plastid trnL-F and rps16 data does not support the monophyly of *Houstonia* as it is currently circumscribed. *Stenaria* is nested within the *Houstonia* lineage and the proper naming of the combined genera would be *Houstonia* (Anderson et al. 2002). These results support the same relationships found by other studies of *Houstonia* and its North American allies (Church 2003). Crown group placement based on the combined dataset is majorly congruent with Terrell's (1996) treatment of *Houstonia* into two subgenera with each subgenus containing two sections however there are a few discrepancies among species placement and intercladal relationships depicted in the combined phylogeny that do not agree with Terrell's circumscription (Terrell 1996). Terrell (1996) placed the  $x = 6$  and  $x = 11$  sections of *Houstonia* into the subgenus *Chamisme* based primarily on colporate pollen (compared to the colporate B pollen of subgenus *Houstonia*) and seeds that are dorsiventrally compressed with a ventral depression containing a narrow hilar ridge. The present analysis shows that the  $x = 6$  section is more closely related to the other sections of *Houstonia* ( $x = 7,8$ ) than to the section *Ericotis* ( $x = 11$ ) of which it shares a subgenus.

*Clade A*: Clade I consists of all species Terrell (1996) circumscribed as subgenus: *Chamisme* section: *Ericotis* and all species of *Stenaria* sampled. *Ericotis* contains the type specimen *Houstonia rubra* Cav. and nine other species of *Houstonia*, all occurring within the southwestern United States and Northern Mexico. The section is characterized by a chromosome number  $x = 11$  (known for all species except *Houstonia correllii* (W.H. Lewis) Terrell and *Houstonia spellenbergii* (G.L. Nesom and Vorobik) Terrell), pollen that Lewis

(1965) classified as medium-sized compared to other closely related species, 3-aperturate and colpororate. Seed characters used to define the section include slightly to strongly compressed seeds with the ventral face being “boat, saucer, or cup-shaped,” a hilar ridge in a shallow to deep depression, and reticulate testa with either distinct or alveolate areole walls (Terrell 1996). The present analysis included all 10 species with 8/10 forming the first subclade A1 and the remaining 2 (*Houstonia palmeri*, *Houstonia. acerosa*) forming subclade A3 sister to A1. Terrell (1996) recognized that *Houstonia acerosa* and *Houstonia palmeri* were closely related species based primarily on seed characters and habit.

The remaining species forming clade I with section *Ericotis* are currently treated as the genus *Stenaria* (Terrell 2001a). Five of six species of *Stenaria* were sampled for this analysis with four of the five forming a relatively well-resolved subclade (PP = 0.9) that is nested within the *Houstonia* lineage. The remaining species, *Stenaria umbratilis*, is nested within the subclade containing 8/10 species currently treated as *Houstonia* section:

*Ericotis*. *Stenaria umbratilis* is morphologically distinct from other species of *Stenaria* due to a creeping habit, only slightly woody stems at the base, and homostylous flowers (Terrell 2001a). Although, morphological dissimilarities are present for habit and floral characteristics, *Stenaria umbratilis* was treated as *Stenaria* due to the similarities in seed morphology and geographic distribution (Terrell 2001a). Molecular analyses disagree with placement of *Stenaria umbratilis* as part of the *Stenaria* group.

Clade B: Clade B comprises the remaining species of *Houstonia* sampled, two species of *Oldenlandia*, and *Houstonia teretifolia*. *Houstonia* (excluding *Houstonia teretifolia*) forms two subclades that are largely congruent with the sections defined by Terrell (1996).

Subclade B1 consists of all species in the subgenus *Houstonia* and *Houstonia sharpii*.



Subgenus *Houstonia* is split into sections *Houstonia* and *Mullera* with *Houstonia rosea* being the sole species placed in *Mullera*. In the combined phylogeny *Houstonia rosea* is nested within section *Houstonia*. This section is defined by caeruleoid seeds with a ventral orifice opening into a subglobose hilar cavity lacking a hilar ridge (Terrell 1996). *Houstonia rosea* differs from these species by having dorsiventrally compressed seeds with an open shallow concavity and a linear hilar ridge, 4-aperaturate pollen, and the smallest pollen and chromosomes relative to other species of the genus (Terrell 1996) (Lewis 1965). However, similarities in habit, flowers, capsules, and chromosome number led Terrell (1996) to place *Houstonia rosea* in subgenus *Houstonia* despite the difference in seed characters. *Houstonia rosea* has a base chromosome number of  $x = 7$  which it shares with only one species of section *Houstonia* (*Houstonia procumbens*) whereas the remaining four species of the section have a chromosome number of  $x = 8$ . In the present results, *Houstonia procumbens* is not placed together with other members of section *Houstonia*. Other than a difference in chromosome number, *Houstonia procumbens* differs from other members of section *Houstonia* in having thrum flowers with long-exserted anthers, large capsules that dehisce almost to the base, and is the only heterostylous perennial species of *Houstonia* to exhibit cleistogamous flowers (Terrell 1996). The two sections of subgenus *Houstonia* form subclade B1 and should be lumped together and considered as only subgenus *Houstonia* due to the fact that *Houstonia rosea* (section *Mullera*) is nested within section *Houstonia*.

Church (2003) concluded that it is not clear if the  $x = 7$  group is more closely related to the  $x = 6$  or  $x = 8$  group. The present analysis included both species known to have a base chromosome number of  $x = 7$  (*Houstonia procumbens*, *Houstonia rosea*) and shows that these species are more closely related to section *Houstonia* ( $x = 8$ ) than section

*Amphiotis* ( $x = 6$ ). All four species of section *Amphiotis* were sampled and placed together in subclade B2.

Three additional species sampled (*Oldenlandia ovata*, *Oldenlandia drymarioides*, *Houstonia teretifolia*) were placed separately in clade B. The two species currently recognized as *Oldenlandia* are sister to subclades B1 and B2. *Oldenlandia* is a large genus with a cosmopolitan distribution in subtropic and tropic regions (Terrell and Robinson 2006). As it is currently circumscribed, *Oldenlandia* has been found to be paraphyletic (Bremer 1996) or polyphyletic (Andersson and Rova 1999). Further molecular systematic work is necessary to resolve relationships in the genus and among other closely related genera in the Spermaceae. One specific region that requires additional attention is the North American species of *Oldenlandia*. Terrell (2006) treated the North American *Oldenlandia* by placing 4/9 (*Oldenlandia corymbosa* L., *Oldenlandia lancifolia* (Schumach) DC., *Oldenlandia uniflora* L., *Oldenlandia bosicii* (DC.) Chapm) of them into the subgenus *Oldenlandia*. Subgenera for the remaining five species (*Oldenlandia pringlei*, *Oldenlandia microtheca*, *Oldenlandia ovata*, *Oldenlandia drymarioides*, *Oldenlandia salzmannii*) are undetermined (Terrell 2006). The first four of the unplaced species listed are native to Mexico while the fifth (*Oldenlandia salzmannii*) is native to South America. The present study focused on the unresolved taxa that Terrell (2006) considered as the *Oldenlandia microtheca* group (*Oldenlandia microtheca* (Cham. & Schldl) DC., *Oldenlandia ovata*, *Oldenlandia drymarioides*). The two species included in this study (*Oldenlandia ovata*, *Oldenlandia drymarioides*) were placed in clade B and should be considered part of *Houstonia*. A primary character to lump these species into *Houstonia* is the pollen aperture type. Lewis (1965) separated species previously defined as *Hedyotis* subgenus *Edrisia* into

five palynological groups. Group one was strictly species of *Stenotis* and had a simple endoaperture type. Groups 2-4 were primarily *Houstonia* and combined the simple endoaperture with a crassimarginate endoaperture. Group 5 comprised the section *Amphiotis* (x=6) of *Houstonia* and exhibited only the crassimarginate endoaperture with the simple endoaperture lacking. The two species of the *Oldenlandia microtheca* group studied by Lewis (1965) (*Oldenlandia ovata*, *Oldenlandia drymarioides*) were placed in the second palynological group with members of subgenus *Chamisme* section *Houstonia*. Molecular data, similar pollen apertures, and overlapping distribution are all evidence for placing *Oldenlandia ovata* and *Oldenlandia drymarioides* into *Houstonia* however an extensive morphological study is necessary to fully understand the evolution of characters in the North American species of *Oldenlandia*.

*Clade C*: Clade C comprises all species of *Stenotis* sampled and *Oldenlandia pringlei*.

*Stenotis* forms a monophyletic clade (PP = 1.0) with *Oldenlandia pringlei* placed basal to the *Stenotis* clade (PP = 1.0). Terrell (2001b) updated seven species formerly recognized as *Hedyotis* and *Houstonia* to generic rank based on distribution, chromosome number, and seed characters. Six of the seven species of *Stenotis* are found in Baja California with one exception, *Stenotis greenei*, distributed only in Arizona. Molecular work is necessary to examine the placement of *Stenotis greenei* but amplification of specimen loans for this species was unattainable. The Baja California species have a base chromosome number of  $x = 13$  and ellipsoid seeds that have a centric punctiform hilum, dorsal and ventral faces either flat or convex, and reticulate testa (Terrell 2001b). The non-crateriform seeds are a primary character to separate *Stenotis* from *Houstonia* (crateriform seeds) (Terrell 2001b). However, this character was also used to separate *Stenaria* from *Houstonia* (Terrell 2001a)

and molecular data shows *Stenaria* is nested within *Houstonia* and should be considered part of *Houstonia*. *Stenotis* does form a clade separate of *Houstonia* but crateriform vs. non-crateriform seeds is not a useful character to separate the two genera. As previously mentioned, Lewis (1965) studied the pollen of 31 species of what was considered *Hedyotis* subgenus *Edrisia* and split them into five groups. Group 1 was strictly *Stenotis* and contained six of the seven species now defined as *Stenotis* (*Stenotis arenaria*, *Stenotis asperuloides*, *Stenotis brevipes*, *Stenotis mucronata*, *Stenotis peninsularis*, *Stenotis australis*). The remaining species of *Stenotis* (*Stenotis greenei*) was not included in Lewis' (1965) work. Group 1 had a simple endoaperture that was different from groups 2-4 that had combined the simple endoaperture with a crassimarginate one or group 5 which only exhibited the crassimarginate endoaperture. The difference in pollen apertures is a useful character to separate *Stenotis* from *Houstonia/Stenaria* especially when accompanied by chromosome number.

## CHAPTER 5

### CONCLUSIONS

This study presents a molecular phylogeny with the highest level of sampling of the North American genus *Houstonia* and the closely related genera *Stenaria* and *Stenotis*. *Houstonia* as it is currently recognized is not a monophyletic genus. Nuclear and plastid datasets have *Stenaria* nested within *Houstonia*. Therefore, these results are in disagreement with the use of seed characters as a defining character to separate the two genera. Descending aneuploidy as the lineage radiated north and east throughout North America has been a driving factor for speciation in *Houstonia*. A pollen aperture modification has accompanied the loss of chromosomes and is a viable character for separating *Stenotis* from *Houstonia* and *Stenaria*. *Stenotis* forms a monophyletic lineage in the separate and combined datasets. The genus is characterized by a chromosome number  $x=13$  and pollen with a simple endoaperture type. *Houstonia* and *Stenaria* have chromosome numbers ranging from  $x=6$  to  $x=11$  with *Stenaria* having a chromosome number  $x=9,10$ . Pollen modifications accompanying the descending aneuploidy have resulted in the combination of the simple endoaperture exhibited in *Stenotis* with a crassimarginate endoaperture to form a compound endoaperture in *Houstonia* and *Stenaria*. The  $x=6$  group of *Houstonia* only exhibits the crassimarginate endoaperture which is thought to be a reduced and highly advanced character.

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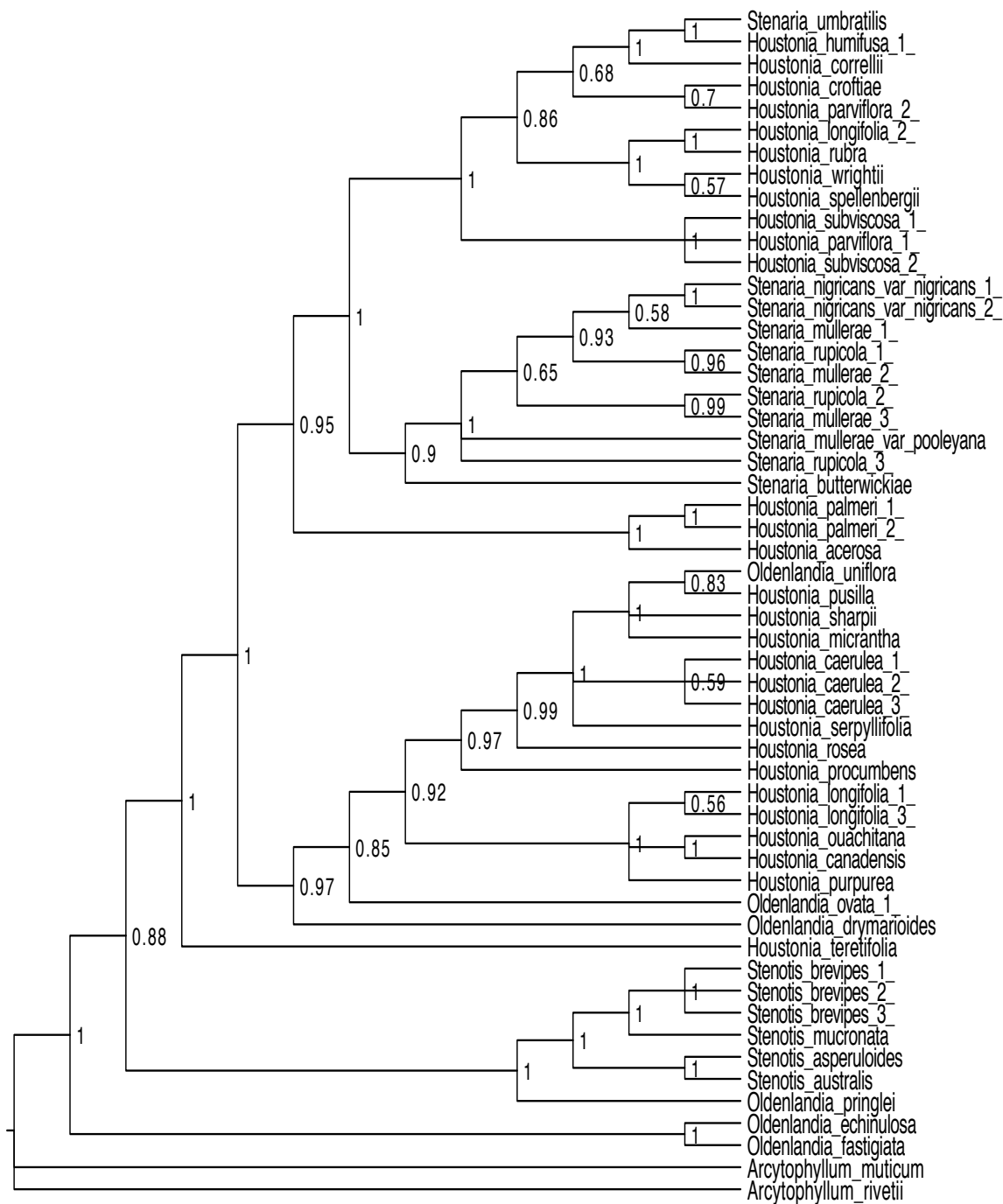
## APPENDIX A

## TAXA INCLUDED IN PRESENT STUDY WITH VOUCHER INFORMATION

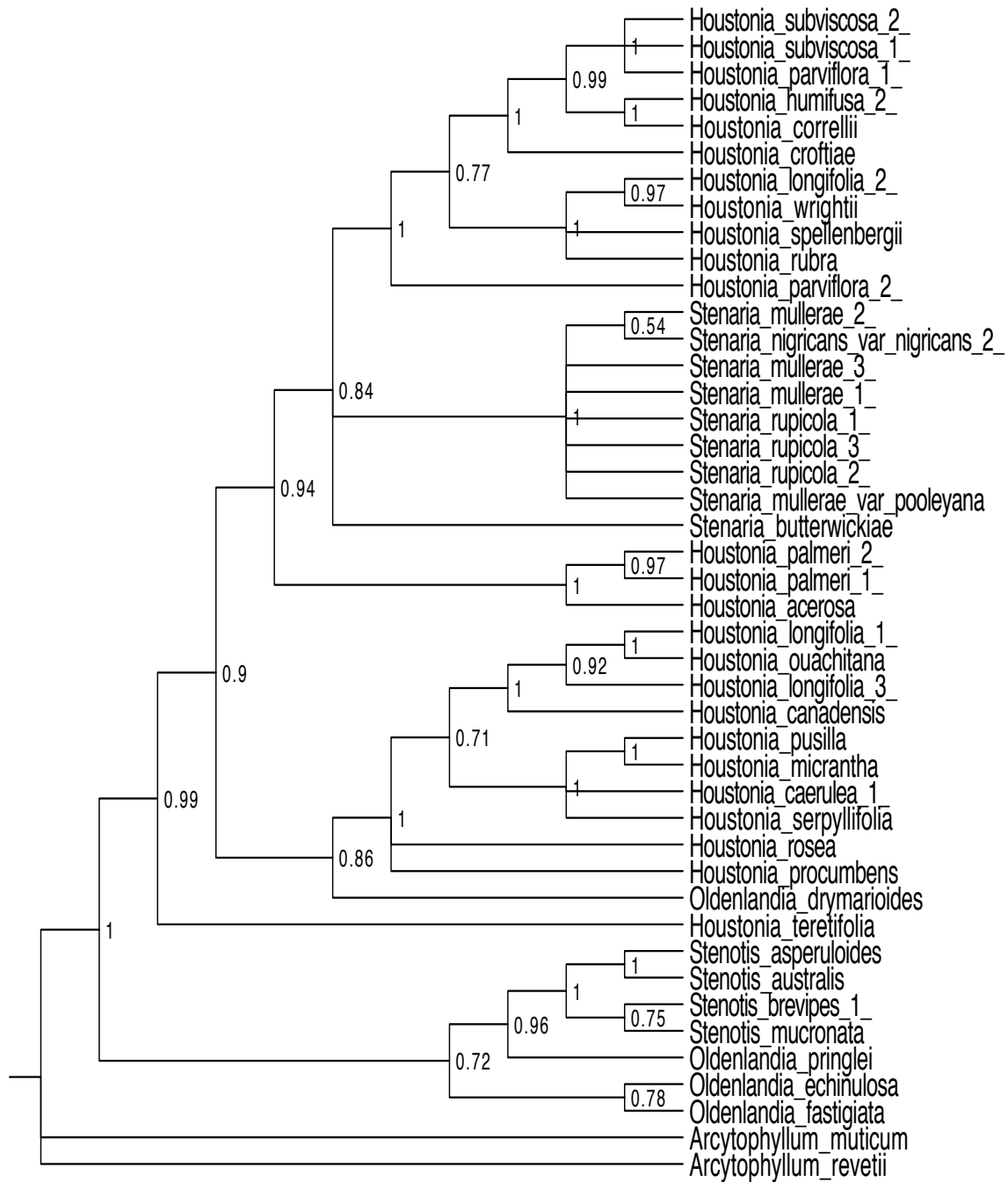
Species	Voucher #	Collector	Herbarium
<i>Houstonia acerosa</i>	4787	Unspecified	HUH
<i>Houstonia caerulea 1</i>	Unspecified	Milner	ODU
<i>Houstonia caerulea 2</i>	Unspecified	Lavergne	ODU
<i>Houstonia caerulea 3</i>	Unspecified	Thomas	ODU
<i>Houstonia canadensis</i>	24847	Pease, Ogden	HUH
<i>Houstonia correllii</i>	422831	W.R. Carr	TEX-LL
<i>Houstonia croftiae</i>	25578	W.R. Carr	TEX-LL
<i>Houstonia humifusa 1</i>	2275	Fryxell	NYBG
<i>Houstonia humifusa 2</i>	1337	Prather	NYBG
<i>Houstonia longifolia 1</i>	4056	Terrell	HUH
<i>Houstonia longifolia 2</i>	3567	E.E. and Bessie Terrell	SI
<i>Houstonia micrantha</i>	88046	R. Kral	HUH
<i>Houstonia ouachitana</i>	5081	E.E. Terrell	SI
<i>Houstonia palmeri 1</i>	18743	Unspecified	HUH
<i>Houstonia palmeri 2</i>	43	R.L. McGregor	SI
<i>Houstonia parviflora</i>	11838	W.R. Carr	TEX-LL
<i>Houstonia parviflora 2</i>	5542	E.E. and Bessie Terrell	SI
<i>Houstonia procumbens</i>	3305	M.T. Strong	SI
<i>Houstonia purpurea</i>	3274185	Terrell	SI
<i>Houstonia pusilla</i>	Unspecified	Flanders	ODU
<i>Houstonia rosea</i>	942	H.E. Moore	HUH
<i>Houstonia rubra</i>	17156	Duane Atwood	HUH
<i>Houstonia serpyllifolia</i>	16	Channell, H.F.L. Rock	HUH
<i>Houstonia sharpii</i>	2639656	A. Ventura	SI
<i>Houstonia spellenbergii</i>	11908	R. Spellenberg	NYBG
<i>Houstonia subviscosa 1</i>	18207	Steven Hill	HUH
<i>Houstonia teretifolia</i>	12191	Johnston	SI
<i>Houstonia wrightii</i>	10000	Roxana Ferris	HUH
<i>Oldenlandia drymarioides</i>	6049	Nesom	SI
<i>Oldenlandia ovata</i>	2171	Stanford	SI
<i>Oldenlandia pringlei</i>	24788	Rzedowski	SI

Species	Voucher #	Collector	Herbarium
<i>Stenaria butterwickiae</i>	40701	Butterwick and Lott	TEX-LL
<i>Stenaria mullerae 1</i>	8741	I.M Johnston	HUH
<i>Stenaria mullerae 2</i>	10925	Johnston	SI
<i>Stenaria mullerae 3</i>	8742A	Johnston	HUH
<i>Stenaria mullerae var pooleyana</i>	2527	Jackie M. Poole	TEX-LL
<i>Stenaria nigricans var nigricans 1</i>	22135	W.R. Carr	TEX-LL
<i>Stenaria nigricans var nigricans 2</i>	432010	Wendt and Collins	TEX-LL
<i>Stenaria rupicola 1</i>	19538	W.R. Carr	TEX-LL
<i>Stenaria rupicola 2</i>	2577	Reveal, Hess, Kiger	SI
<i>Stenaria rupicola 3</i>	16540	Howard	HUH
<i>Stenaria umbratilis</i>	5380	F. Ventura	SI
<i>Stenotis asperuloides</i>	2272	Annetta Carter	SI
<i>Stenotis australis</i>	3374	Annetta Carter	SI
<i>Stenotis brevipes 1</i>	2830	Annetta Carter	HUH
<i>Stenotis brevipes 2</i>	4063	Howard Gentry	HUH
<i>Stenotis brevipes 3</i>	4455	Annetta Carter	HUH
<i>Stenotis mucronata</i>	5919	Annetta Carter	SI

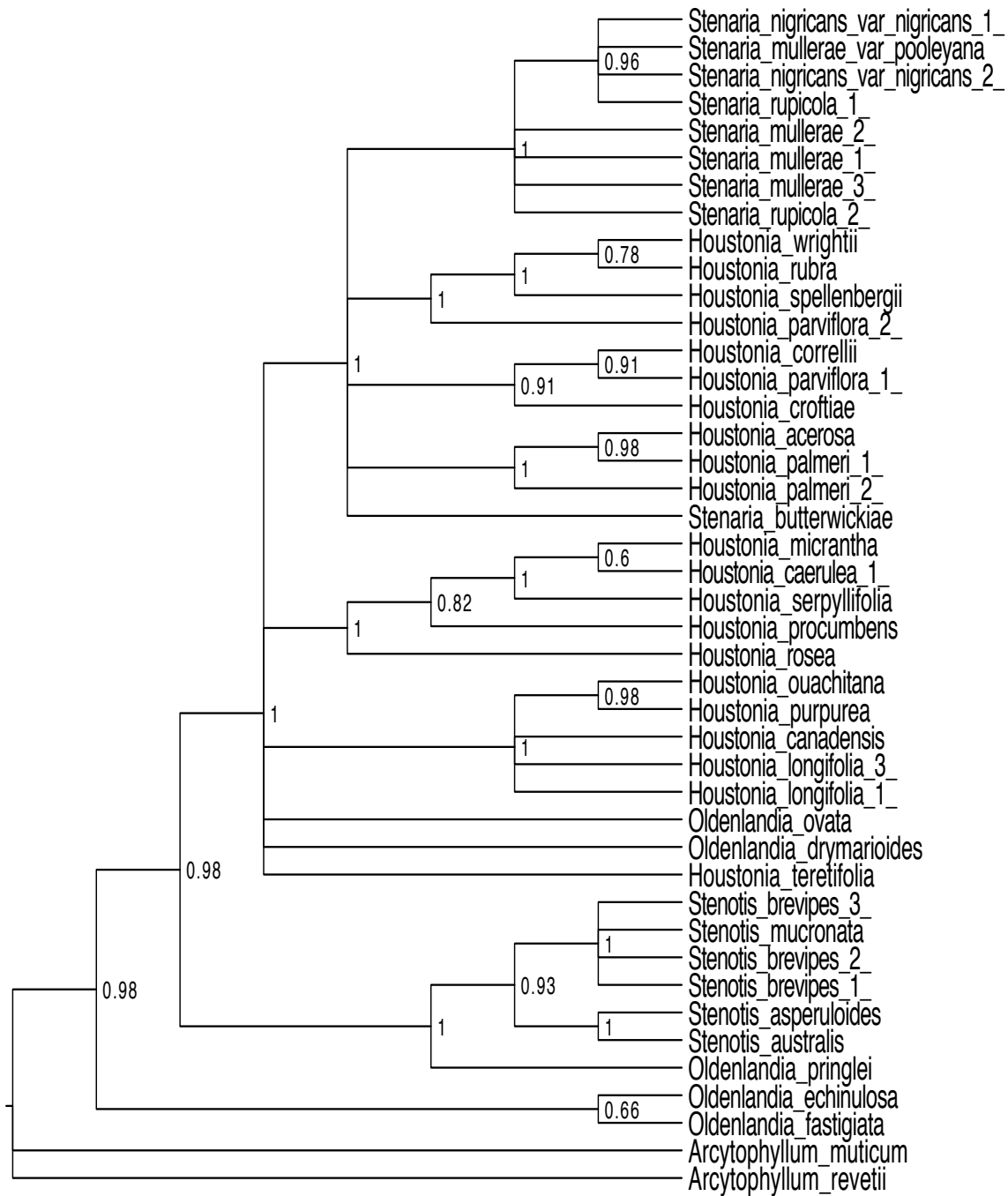
## APPENDIX B

ITS MAJORITY RULE CONSENSUS TREE OF THE *HOUSTONIA* LINEAGE

## APPENDIX C

**TRNL-F MAJORITY RULE CONSENSUS TREE OF THE *HOUSTONIA* LINEAGE**

## APPENDIX D

RPS16 MAJORITY RULE CONSENSUS TREE OF THE *HOUSTONIA* LINEAGE

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