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Phylogenetic Relationships of Salicaceae Based on Analyses of Nuclear DNA Data

John Marshall Diffey
University of Southern Mississippi

The University of Southern Mississippi

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by

John Marshall Diffey

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Approved by

Mac H. Alford, Ph.D., Thesis Adviser
Associate Professor of Biological Sciences

Janet Donaldson, Ph.D., Chair
Department of Biological Sciences

Ellen Weinauer, Ph.D., Dean
Honors College

Abstract

Salicaceae are an economically and ecologically important family of flowering plants. The family includes willows and cottonwoods and was recently enlarged to include a large number of tropical species formerly placed in the family Flacourtiaceae. Relationships of these tropical relatives to willows and cottonwoods have been explored at a basic level using morphology and plastid DNA data, but to date no molecular phylogenies have been constructed with significant sampling of nuclear DNA, which sometimes results in a different picture of relationships because of its biparental inheritance. For this project, I sampled one region of nuclear DNA (GBSSI) across the family to infer relationships among the genera of Salicaceae. These results were mostly congruent with previous analyses, although sequences from some key species closely related to *Salix* and *Populus* were not obtained, possibly due to multiple copies of the gene.

Key Terms: Flacourtiaceae, nuclear GBSSI, phylogenetic systematics, Salicaceae

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Chapter 1: Introduction

Plants are vitally important to life on Earth; humans, too, could not survive without them. They provide us with oxygen that we need to breathe and carbohydrates that we need to eat. Many of the medications that we use to treat diseases are produced by plants. Plants also provide us with building materials for shelter and furniture. Beyond benefits to our species, plants are important to the ecosystems in which they reside in many important ways beyond just oxygen and chemical energy, including soil conservation. Without plants, the soil would simply wash away. In order to study plants, it is necessary to have a system that enables us to classify them and communicate unambiguously about them.

Taxonomy is the scientific field associated with classification of organisms. Providing informative taxonomy involves determining relationships between organisms, which are inferred from examining and analyzing morphological, anatomical, chemical, genetic, and fossil evidence. Knowing which organisms are most closely related and how organisms (and their features) evolved provides a plethora of useful information. For one, scientists use phylogenetic relationships to analyze factors that influence evolution of traits through time. This information also enables scientists to determine if morphological characteristics were passed through a lineage from a common ancestor or if they developed independently in different branches of an evolutionary tree. Understanding these relationships allows scientists to make predictions about inadequately studied

characteristics that can potentially apply to all plants within a family as these characteristics are discovered.

Flacourtiaceae were a plant family that included 80–95 genera and 800–1000 species of woody, pantropical plants that were difficult to identify (Chase et al., 2002). Due to new understanding of their relationships after phylogenetic analysis of DNA sequence data, the family was divided into several groups and sometimes fused with other plant families, primarily Salicaceae and Achariaceae (Chase et al., 2002), or even more finely into additional families like Samydaceae and Scyphostegiaceae (Alford, 2005).

Traditionally, Salicaceae included two genera, *Salix* and *Populus* (Cronquist, 1981; Leskinen & Alstrom-Rapaport, 1998), which have highly reduced flowers with no obvious sepals and petals and have hairy, wind- and water-dispersed seeds. While classifications within *Populus* have remained relatively stable, *Salix* has undergone several revisions due to its complexity (Leskinen & Alstrom-Rapaport, 1998). The big surprise, though, was the addition of new genera to this family from tropical Flacourtiaceae, a family with mostly "normal" flowers. This result from genetic data (Chase et al., 2002; Alford, 2005) led to the development of new questions regarding the relationships within Salicaceae.

Answering these questions is the interest of a small group of botanists, but while the number of researchers in this field is quite small, the need is great. An accurate phylogeny can greatly decrease the time involved in finding new compounds that can be used to synthesize medications. Several medications have been synthesized from extracts of species within both *Salix* and *Populus*. Perhaps the most widely known of these

medications is aspirin, which is currently one of, if not the most, widely used drugs in the world (Rainsford, 2004). In the eighteenth century, salicin, extracted from willow bark (*Salix*), was found to have antipyretic and analgesic effects. Salicylic acid was found to be the ingredient responsible for these medicinal properties. In 1897, Bayer synthesized acetylsalicylic acid from salicylic acid in 1897 and named his synthesized product Aspirin in 1899 (Glaser, 2000). Platelet aggregate inhibitors (anti-clotting agents) have also been extracted from *Populus sieboldii* (Kagawa et al., 1992). Given their close relationship, other medications with similar properties could possibly be found among species of Salicaceae, which increases the need for an accurate phylogeny of this family. In fact, antimicrobial properties have been found in *Oncoba spinosa*, a member of Salicaceae that was once a member of Flacourtiaceae (Djouossi et al., 2015). Another use is phytoremediation. Willows (*Salix*) have been used for taking up heavy metals from contaminated soils, and not surprisingly, three of the suspected relatives in the old Flacourtiaceae, *Homalium*, *Lasiochlamys*, and *Xylosma* from New Caledonia, have high recorded levels of nickel uptake (Jaffré et al., 1979).

In order to address some of the questions about taxonomy of Salicaceae, the old Salicaceae and new members of Salicaceae that were formerly classified in Flacourtiaceae will be studied. Molecular analyses will be used to determine how closely related genera from Flacourtiaceae are to *Salix* and *Populus*. The hypotheses for this study are that the relationships will be similar to previous hypotheses and that the genera that have the same sexual condition as *Salix* and *Populus* will be their closest relatives.

Chapter 2: Literature Review

Specific and accurate taxonomy is important to many areas of science, including but not limited to botany. Accurate connection of research to existing literature cannot be done without a proper binomial (Bennett and Balick, 2013). Taxonomic errors are rather common. In fact, Bennett and Balick (2013) note that taxonomic errors can be found in nearly all issues of medicinal plant journals and that the most common errors include incorrect citations of binomials, incorrect family assignments, misspelled specific epithets and generic names, and the use of synonyms rather than currently accepted names. Common names provide insufficient information; there are no rules regarding their formation, and they can be used for multiple species and vary between and within languages (Bennett and Balick, 2013). Globally, scientists use the rules of the *International Code of Nomenclature for Algae, Fungi, and Plants* (McNeill et al., 2012) which limits the scientific community to a single correct name for a taxon within a system of classification. Accuracy in taxonomy and nomenclature is vital to documentation, reproduction, and prediction (Bennet and Balick, 2013), key elements of the scientific method.

To address the issue of accuracy in taxonomy and nomenclature, the Angiosperm Phylogeny Group (APG) is used as a system for classifying flowering plants (APG, 2016). The first APG system (APG I) was published in 1998 (APG, 2016). The APG instituted a novel manner of creating a plant classification system wherein the system was not the work of a single botanist or two, but instead it was a system designed to classify flowering plants with agreement among many experts within the field (APG, 2016).

Currently, this system is in its fourth revision, APG IV (APG, 2016). This study intends to contribute to the body of work that can be found within that system.

Taxonomy has undergone large changes in recent years as a result of emerging technology. A combination of new techniques for working with DNA and computational methods which allow the comparison of large quantities of genetic information have made it possible to provide more accurate placement of species which were once difficult to classify using morphological data alone. With this major new source of data for classification, evolutionary relationships can be clarified, and reclassified species may be compared to other species which are now known to be closely related, in order to find previously unnoted morphological similarities.

Polymerase chain reaction (PCR), a method to rapidly replicate DNA sequences, was developed by Kary Mullis in 1984 (Fitzgerald-Hayes and Reichsman, 2010). The first of three steps in PCR is to heat a DNA solution to denature the double-stranded DNA. Next, an excess of primers is added, and the solution is cooled, to allow binding to the primers. Finally, a thermostable polymerase, commonly *Taq* DNA polymerase, begins synthesis at the primers. By repeating this cycle 30 or more times, a large number of copies may be made in a relatively short time (Fitzgerald-Hayes and Reichsman, 2010).

As PCR technology and reagents have improved, PCR has become cheaper and simpler, and the field of bioinformatics has provided methods for analyzing the large quantities of genetic data being produced. Bioinformatics uses computer science to analyze large quantities of biological data, such as DNA sequences (Fitzgerald-Hayes and Reichsman, 2010). The ability to compare not only large sections of DNA sequences, but

large numbers of sequences, has made the use of genetic data in taxonomy an important tool.

One area in which the use of phylogenetic analysis in taxonomy has been extremely useful is the reclassification of organisms placed in broad, uninformative taxa, such as Flacourtiaceae. In 1954, Sleumer described Flacourtiaceae as confusing and mostly unrecognizable due to combinations of common morphological features within the family occurring in different genera (Sleumer, 1954). Prior to the use of phylogenetic data, Flacourtiaceae had already undergone multiple revisions, changing the number of genera and tribes, based on several factors, including presence or absence of cyanogenic glycosides, the sexual system (individuals with both sexes, together or separate, and individuals with just one sex), and petal arrangement (Lemke, 1988). Based on phylogenetic analysis of one region of chloroplast DNA sequences, Chase et al. (2002) proposed that genera from the family be moved to other families, principally Salicaceae and Achariaceae. These changes were further modified by Alford (2005), which resulted in a large number of the genera originally placed in Flacourticeae being moved to Salicaceae (Alford, 2005, 2006).

Traditionally, Salicaceae consisted of two genera, *Salix* and *Populus* (Cronquist, 1981; Leskinen & Alstrom-Rapaport, 1998). *Populus* consists of what are commonly known as cottonwoods, poplars, and aspens, which are diploid and wind-pollinated (Leskinen & Alstrom-Rapaport, 1998). The genus *Salix*, commonly known as willows, is much more complicated, as it contains diploid and polyploid species, as well as both insect- and wind-pollinated species (Leskinen & Alstrom-Rapaport, 1998). Both genera are dioecious, meaning individual plants are either male or female, rather than having

both male and female parts on the same plant, with small flowers lacking any obvious sepals or petals and with tiny, cottony seeds (Cronquist, 1981; Leskinen & Alstrom-Rapaport, 1998). With the addition of genera formerly in Flacourtiaceae, new questions have arisen regarding the already complicated taxonomy of Salicaceae, in particular, questions about which genera are closest relatives of the unusual willows and cottonwoods.

Alford (2005) discovered that seven genera of the former Flacourtiaceae were very closely related to Salicaceae *sensu stricto* (*Salix* and *Populus*), but unfortunately, the molecular data that he used could not resolve the finer relationships among them, largely because data were not variable enough. In addition, Alford's (2005) work was based on morphology and plastid DNA sequence data; he did not sample any nuclear DNA. It is then the goal of this study to build on the previous work, collecting nuclear DNA sequence data for many species that have been moved from Flacourtiaceae to Salicaceae in order to clarify their taxonomic relationships, primarily to see if results are congruent with Alford's (2005) results and if they provide any additional resolution or more information about certain relationships that were still unclear in his study. By understanding these relationships, we can also infer what kinds of morphological changes took place that led to the tiny, unisexual flowers of *Salix* and *Populus* and their tiny cottony seeds.

Chapter 3: Materials and Methods

This study examined members of the old Salicaceae and members of the new Salicaceae that were formerly members of Flacourtiaceae to test hypotheses about which of these genera is/are the closest relative(s) of *Salix* and *Populus* and to determine if relationships are congruent with those proposed in other studies (Chase et al., 2002; Alford, 2005). In order to achieve this goal, DNA sequence data were gathered and analyzed phylogenetically. Extracted DNA was already available from Alford's (2005) study and others that Alford had extracted since then; those extractions were completed using a Qiagen DNeasy mini kit (Qiagen, Inc., Valencia, CA). Most of the same DNA samples used in that previous study (Alford, 2005) that utilized plastid DNA were used in this study. If those were not available, closely related species were used. DNA was amplified using PCR (polymerase chain reaction), sequenced, aligned, and analyzed.

A target region of DNA was amplified using the polymerase chain reaction (PCR). PCR involved the use of DNA primers, template DNA, a buffer, and DNA polymerase in order to amplify desired sequences of DNA exponentially (Fitzgerald-Hayes and Reichsman, 2010). PCR was performed in a thermal cycler machine which exposes the reagents to specific temperatures for specific periods of time, proceeding through the three steps in the PCR cycle: DNA denaturation, DNA annealing, and DNA elongation (Fitzgerald-Hayes and Reichsman, 2010).

PCR techniques here followed the instructions of Samarakoon et al. (2013). DNA denaturation occurred at 94° C. This high temperature denatured the weak hydrogen bonds holding the two strands of the DNA double helix together, providing single strands

of DNA. DNA annealing typically occurs anywhere from 45°–72° C, and it involves the bonding of the DNA primers to the DNA template sequences. The primers designate where the DNA polymerases will begin to elongate the DNA, and for this study primers for the granule-bound starch synthase (GBSSI, or *waxy*) gene were used (GBSSI-forward: 5′–ACTGTRAGCCCTTACTATGC–3′ and GBSSI-reverse: 5′–GTTCCATATCGCATAGCATGC–3′) (Mason-Gamer et al., 1998). These specific primers were developed by Dr. Mac Alford for use in Malpighiales using genomic comparisons at www.phytozome.net. At the low end of this temperature range, DNA can begin to bind to itself, or primers can bind to sites which are not perfectly complementary, thus resulting in non-target areas of DNA being amplified or no amplification at all. The primers I chose to test have melting temperatures of 59.4° C and 60.6° C in order to avoid this issue. DNA elongation occurred at 72° C. In this step, the DNA polymerases elongate the primed strands, creating more copies of the target DNA sequences.

These three steps were repeated 35 times in order to amplify the DNA. The thermal cycler was set to remain at the designated temperatures for specific periods of time for the designated number of cycles of the three steps (Samarakoon et al., 2013).

The amplified DNA was separated using gel electrophoresis, and the gel was viewed under UV light. The light allowed visualization of the DNA fragments. The fragments were compared to a DNA ladder, which acted as a standard for sequence fragments of specific molecular weights. If the desired DNA was found to be present as a single band at the right size, the DNA was used in the next step of the process.

After visualization under UV light to guarantee the DNA is present and in usable condition, the DNA was sequenced at an outside facility, MWG Operon of Louisville, KY. They returned to us sequence files, and a series of programs were used to analyze the sequence data. First, Sequencher was used to "clean" the sequences and check the computer determinations of bases (Sequencher® version 5.4.1 sequence analysis software, Gene Codes Corporation, Ann Arbor, MI USA). Next, ClustalX (Thompson et al., 1997, 1998) was used to align the sequence data, since all sequences did not start or end at exactly the same place and some sequences had gaps in them (Larkin et al., 2007). Finally, WinClada (Nixon, 1999, 2002) was used to perform phylogenetic analysis of the sequence data. The phylogenetic analysis program uses algorithms to determine the most likely evolutionary history and relationship of the species from which the DNA came using the parsimony criterion. If there were multiple most parsimonious trees, a consensus tree was determined that represented relationships found in all of the most parsimonious trees. Then, the bootstrap statistical test was performed to determine how strongly the data support the branches (Felsenstein, 1985). Bootstrap takes a sample from the original dataset many times (with replacement) and then analyzes it again and again. If a group appears in many trees, it receives high bootstrap support (maximum equals 100%), and if it appears rarely, it receives low bootstrap support (minimum equals 0%). I did 500 bootstrap replications. The Retention Index (RI), which is a value that indicates how much of the data is in agreement, was also calculated (Farris, 1989). The resulting trees were then compared to trees obtained with the other data previously collected by Alford (2005).

Table 1. Samples utilized for DNA work.

Tribe	Species	Voucher with Herbarium Code	Alford DNA Sample Number
Outgroup: Lacistemataceae	*Lacistema aggregatum*	Alford 3019 (BH)	24
Outgroup: Samydaceae	*Casearia sylvestris*	Alford 2999 (BH)	26
Saliceae	*Salix arbutifolia*	Skvor s.n. (LE)	X-1035
	Populus deltoides	Alford 3038 (BH)	57
	Populus tremuloides	Alford 3063 (BH)	48
Abatieae	*Abatia canescens*	Alford 3082 (BH)	71
Prockieae	*Banara tomentosa*	Alford 3175 (BH)	151
	Banara vanderbiltii	Alford & Lewis s.n. (BH)	41
	Hasseltia allenii	Alford 3023 (BH)	17
	Hasseltia floribunda	Alford 2990 (BH)	28
	Hasseltiopsis dioica	Alford 3010 (BH)	20
	Neosprucea paterna	Alford 3149 (BH)	109
	Pleuranthodendron lindenii	Alford 2989 (BH)	18
	Pineda incana	Alford 3124 (BH)	97
	Pineda ovata	Wood 18684 (K)	146

	Prockia costaricensis	Alford 3018 (BH)	22
	Prockia crucis	Alford 3132 (BH)	85
	Prockia flava	Michelangeli 617 (BH)	3
	Prockia pentamera	Alford 3130 (BH)	70
Bembiceae	*Bembicia axillaris*	Civeyrel 1374 (K)	C-1625
Homalieae	*Homalium racemosum*	Salazar 2410 (BH)	80
	Calantica cerasifolia	Schatz 1554 (MO)	MO-12
Flacourtieae	*Azara lanceolata*	Alford 3171 (BH)	150
	Dovyalis rhamnoides	Chase 271 (NCU)	M-271
	Hemiscolopia trimera	Chase 1280 (K)	E-1280
	Lasiochlamys reticulata	Munzinger 840 (MO)	134
	Ludia mauritiana	Robertson 6910 (EA)	144
	Oncoba spinosa	Alford 3026	37
	Scolopia mundii	Chase 6560 (K)	G-6560
	Scolopia spinosa	Chase 1288 (K)	R-1288
	Xylosma bahamensis	Alford 3031 (BH)	40
	Xylosma cordata	Alford 3126 (BH)	86
	Xylosma hispidula	Alford 3016 (BH)	19

Chapter 4: Results

DNA data from the nuclear GBSSI region were obtained for 33 species, although primers and amplification were tested for six individuals for two other nuclear regions (UBOX and one un-named, suggested by M. Olson, Texas Tech University, pers.comm.). Amplification of GBSSI, however, generally resulted in one clear band, and I chose it to focus on. Amplification of GBSSI from additional species was attempted but gave no results in two attempts. Some amplification resulted in thick bands or two bands, which was later problematic (see Discussion).

The GBSSI data created an aligned data matrix of 849 base-pairs of DNA (bp). Of these base-pairs, 212 were potentially informative substitutions, that is, they showed more than one variation at a site across the species. Phylogenetic analysis of this region using parsimony resulted in 66 most parsimonious trees of length 577 and RI of 0.75 (Figure 2).

For comparison, Dr. Alford's (2005) dataset was reduced to include the same (or closely related) species that I was able to amplify for GBSSI. This analysis included 28 species with 219 potentially parsimony informative characters from a matrix of 4429 aligned characters. Analysis of these data resulted in 8 most parsimonious trees of length 379 and RI of 0.74 (Figure 1).

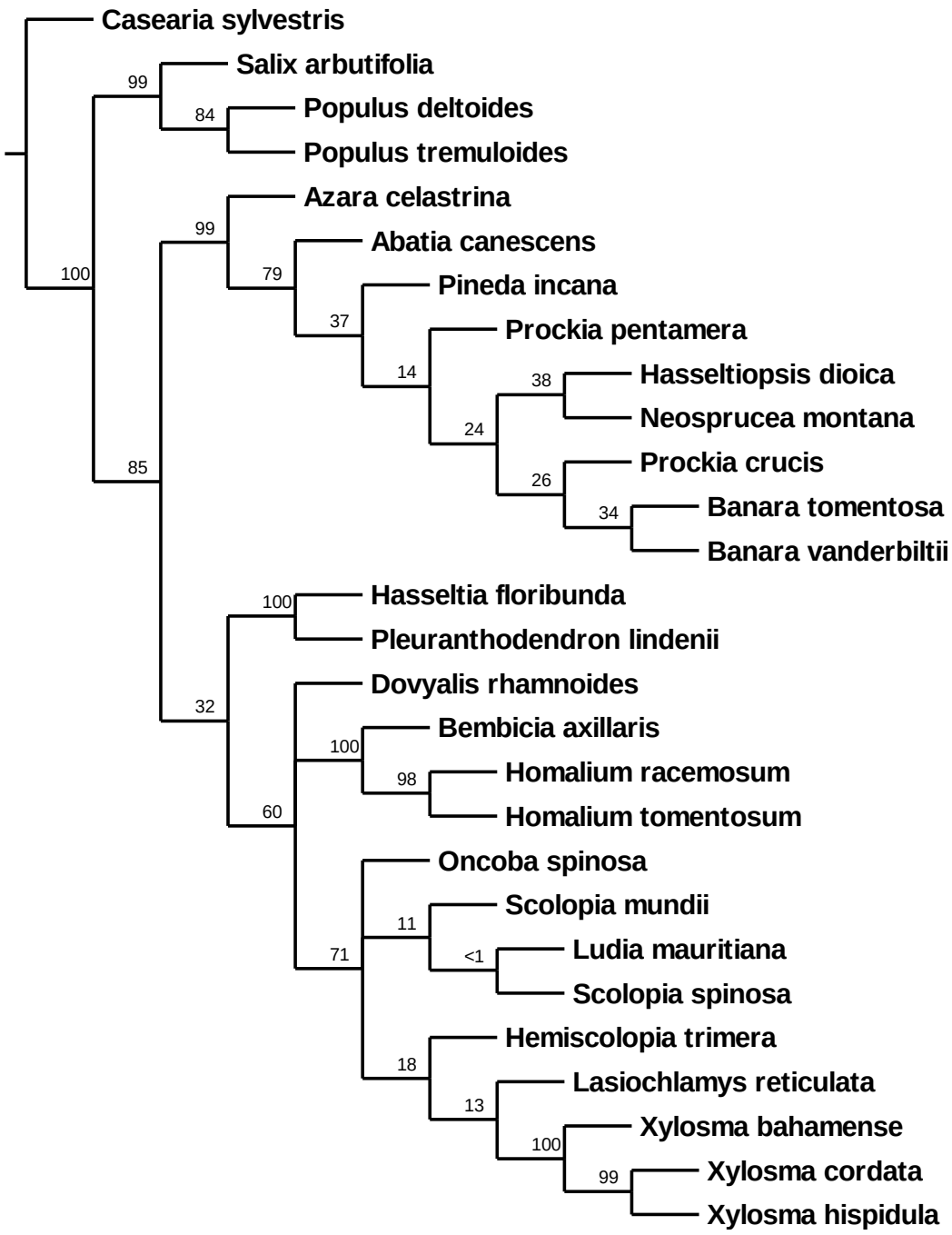

Figure 1. Strict consensus of 8 most parsimonious trees recovered in phylogenetic analysis of morphology and plastid DNA (*trnL-F* and *ndhF*), based on a subset of Alford (2005). There were 219 potentially parsimony informative characters in a matrix of 4429 aligned characters. L=379, RI=0.74. Bootstrap values are above the branches.

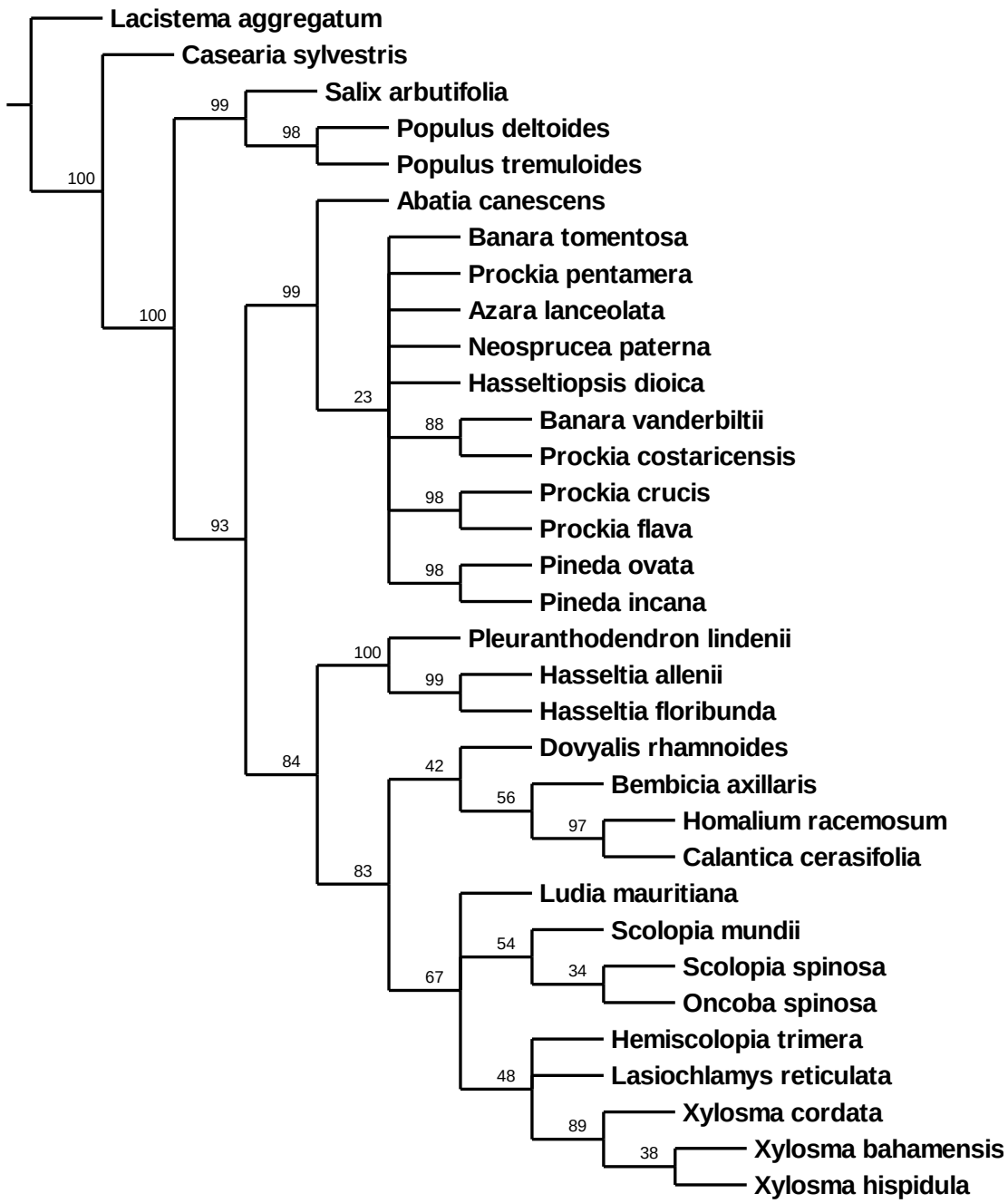

Figure 2. Strict consensus of 66 most parsimonious trees recovered in phylogenetic analysis of nuclear DNA (GBSSI) (this study). There were 212 potentially parsimony informative characters in a matrix of 849 aligned characters. L=577, RI=0.75. Bootstrap values are above the branches.

Chapter 5: Discussion

Overall, the new results from nuclear DNA (Figure 2) are congruent with the results from morphology and plastid DNA (Figure 1), although there is less resolution in most cases with the nuclear data. These new results support the conclusion that the tribes of the family (Lemke 1988; Chase et al. 2001) are not monophyletic and that several groups are well supported by DNA data and morphology: (1) a clade of commonly dioecious, thorny shrubs including *Ludia*, *Scolopia*, and *Xylosma*, (2) a clade including *Homalium* and *Bembicia* with inferior or semi-inferior ovaries, (3) a clade including *Prockia*, *Banara*, *Neosprucea*, *Pineda*, and *Abatia* with valvate sepal aestivation (sepals touch each other side to side in bud), and (4) a clade including the traditional Salicaceae, *Salix* and *Populus* (Salicaceae *sensu stricto*), that lacks obvious sepals and petals, is dioecious, and has hairy seeds. One major clue lacking from this study is the relationship of the closest relatives of *Populus* and *Salix*. Although plastid DNA data were generated for these seven genera (Alford 2005), the nuclear DNA were "dirty," perhaps reflecting two or more copies, and could not be included in the analysis. As noted in the Results above, some amplifications resulted in thick or double bands, foreshadowing that this might be a problem. This is unfortunate, because the results of Alford's (2005) original study did not conclusively show which genus or genera was/were most closely related to *Salix* and *Populus*. That would be interesting, because it would help determine the order of evolution of various features leading to the condition found in willows and cottonwoods.

The GBSSI results (Figure 2) differed in a few ways from the larger plastid and morphological results (Figure 1), but in all those cases, the bootstrap confidence values were low in one or both of the results, meaning that those differences are not strongly supported. The GBSSI data provided *more* resolution to the sister relationships of *Hasseltia* and *Pleuranthodendron*. Their broader relationships were unclear in the original study, but GBSSI data indicate with fairly strong confidence (84% bootstrap) that they are more closely related to the clade including *Dovyalis*, *Homalium*, *Scolopia*, *Xylosma*, and others than to the clade with *Banara*, *Prockia*, *Abatia*, and others. However, the original study with plastid DNA and morphology had stronger support for relationships within *Xylosma*, for *Bembicia* and *Homalium* being closely related, and for *Azara* being on the first branch separate from *Abatia*, *Pineda*, *Prockia*, *Banara*, *Hasseltiopsis*, and *Neosprucea*.

In conclusion, these data are useful in affirming Chase et al.'s (2002) and Alford's (2005) hypotheses about relationships within the family from a nuclear DNA data source and provide additional confidence about the broader relationships of *Hasseltia* and *Pleuranthodendron*.

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