MOLECULAR SYSTEMATICS OF *ISOËTES* (ISOËTACEAE) IN EASTERN NORTH AMERICA

by

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ABSTRACT

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Isoëtes (Isoëtaceae, Isoetales, Lycopodiophyta) is a cosmopolitan genus of aquatic lycophytes, occurring on every continent except Antarctica. Of approximately 200 total taxa, about half are in a clade of species mostly occurring in North and South America. Eastern North America accounts for 22% of global taxonomic diversity, containing 32 fertile taxa and 16 named hybrids. This taxonomic diversity is built upon relatively little morphological difference, and even combined with phylogenetic analysis using several nuclear and chloroplast DNA markers, no well-resolved systematic treatment within this clade exists.

This study aims to clarify the relationships between all species and subspecies of *Isoëtes* in eastern North America using a phylogenomic approach. Subsets of taxa were analyzed separately depending on their presumed mode of evolution: fertile diploid taxa thought to have originated through allopatric speciation, and fertile allopolyploids derived from whole genome duplication following primary hybridization. Phylogenies inferred from whole chloroplast genome DNA sequences using maximum-likelihood and Bayesian inference were fully resolved with high support. Ancestral state reconstruction of megaspore and microspore ornamentation, megaspore color, and seasonality of spore maturation found that more than 80% of these character state transitions occurred on terminal tips of the tree, and that some shared morphological characters are the result of homoplasy. Only *I. '*graniticola-NC'*, I. 'laurentiana', I. septentrionalis,* and *I.*

tuckermanii showed very strong relationships indicating a clear maternal ancestor, with other polyploids suggesting ancestral or unknown diploid progenitors often in conflict with nuclear phylogenetic data.

Parentage of polyploid taxa was inferred by comparing DNA sequences of a low-copy nuclear marker (*LEAFY* intron 2) to all diploid taxa present in the eastern US under phylogenetic and similarity criteria. Some hypotheses based on previous work, such as *I. engelmannii* and *I. valida* as parents of *I. appalachiana* and *I. engelmannii* and *I. echinospora* as parents of *I. septentrionalis,* were validated, but most polyploid taxa were found to be derived from different sets of parental species. Using a lineage-based species concept may require the recognition of ca. 50 new species of auto- and allopolyploid *Isoëtes* in eastern North America.

This dissertation is dedicated to the memory of Timothy J. Motley (1965 – 2013) and Rebecca D. Bray (1939 –2018)

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CHAPTER 1

INTRODUCTION

The genus *Isoëtes* (Isoëtaceae, Isoetales, Lycopodiophyta), established by Linnaeus in 1753, was taxonomically overlooked in North America for almost a century (Engelmann, 1882). Collections of *Isoëtes* in North America were called *I. lacustris* – the only taxon in the genus for 30 years – until the 1840s when other species were recognized (Engelmann, 1882; Pfeiffer, 1922). The first monographic work in this continent was Engelmann's *The Genus Isoëtes in North America* (1882). In this he recognized 14 species from the mainland and 1 from the Caribbean islands, plus several infraspecific varieties. While this was a significant advancement in *Isoëtes* research, Engelmann admitted that only a small portion of the northeastern and mid-Atlantic United States had been thoroughly explored (Engelmann, 1882). The next comprehensive work would not take place for another 40 years, Pfeiffer's Monograph of the Isoëtaceae (1922). This global conspectus includes 21 species found in North America. Possibly the most significant contribution of Pfeiffer's monograph is her emphasis on megaspore ornamentation for species delineation, a character that is still used today. The most recent treatment of *Isoëtes* of North America is in the Flora of North America (Taylor et al., 1993). Twenty-four species are included, primarily distinguished by megaspore ornamentation, geography, and habitat.

General Morphology

The morphological similarity of all species of *Isoëtes* makes their identification and taxonomy exceedingly difficult. Even the particularly observant Engelmann (1882) remarked that "the species of *Isoëtes* are the simplest vascular plants known". The photosynthetic body consists of the elongate, acicular-filiform tips of the sporophylls. These sporophylls, often referred to as leaves, are arranged in a whorl on the subterranean rootstock. In cross section, sporophylls are roughly triangular to quadrangular, varying in shape from trapezoidal to sub-hemispheric, and in their interior are four large air cavities (lacunae) separated by cross-shaped septa. Transverse septa also divide the lacunae at irregular intervals. All *Isoëtes* feature a prominent adaxial groove running the length of the sporophyll.

Stomata occur in certain taxa, especially toward the sporophyll tips; this feature was used at one time for classification (Engelmann, 1882). Presence of stomata generally coincides with the presence of peripheral bast bundles (peripheral strands), small, thicker collenchyma cells associated with the larger parenchyma of the epidermis and septa (Engelmann, 1882; Pfeiffer, 1922). These bundles apparently serve as mechanical support, having no vascular function (Pfeiffer, 1922). They most commonly occur in four places, one at each adaxial angle of the sporophyll, and one at each end of the septum connecting the adaxial and abaxial walls (Engelmann, 1822; Pfeiffer, 1922). However, six bundles are found in at least one taxon (*I. cubana* Engelm.) and only three in another (*I. nutallii* A. Braun ex Engelm.) (Engelmann, 1882). Smaller bundles in addition to the primary four-six may also occur in the epidermis of some rigid-leaved plants (e.g. *I. melanopoda*), these referred to as accessory bundles (Engelmann, 1882; Pfeiffer, 1922). Sporophyll number per plant, length, and coloration are, in many cases, variable among and even within populations.

In fertile plants, a sporangium is borne at the base of each sporophyll on the adaxial side where it meets the rootstock. The sporophyll base is widened to accommodate the sporangium, which sits inside the nearly spherical to ovoid fovea (depending on the shape of the sporangium). On the adaxial side of the sporangium, a one cell thick tissue, the velum, may extend over the sporangial wall. The degree to which the velum encloses the sporangium varies from 0-100%, and it is considered taxonomically useful for certain species (Engelmann, 1882; Pfeiffer, 1922). On both lateral sides of the sporangium the sporophyll is broadened into thin alae, wing-like projections that are imbricate with those of neighboring sporophylls. On the adaxial surface of the sporophyll just above the sporangium is the ligule, a two-parted structure consisting of an embedded glossopodium and emergent tongue (Shaw and Hickey, 2005). The shape of the glossopodium is taxonomically useful in certain species (Sharma and Singh, 1984; Shaw and Hickey, 2005). The function of the ligule is unknown, though its ability to secrete a protein and polysaccharide-rich mucilage has caused speculation that it could prevent desiccation of young leaves, physically protect leaf primordia, or transport solutes (Kristen and Biedermann, 1981; Shaw and Hickey, 2005). Some authors have even proposed that the physiological similarity of the secretory mechanism in *Isoëtes* to modern carnivorous plants suggests the ligule is a vestigial organ of ancient carnivorous lycopsids (Kristen et al., 1982).

The sporangia are distinct organs, almost completely separate from the tissue of the sporophylls except along the central vascular bundle. Their shapes range from round to elongate, often varying considerably on the same individual (Engelmann, 1882). The exposed adaxial surface of the sporangial wall is sometimes flecked with brown schlerenchymatous cells, which can give a colored appearance to the sporangia (Pfeiffer, 1922). The interior of a sporangium is crossed with several diaphragms, the trabeculae (Pfeiffer, 1922). Separate mega- and

microsporangiate sporophylls occur in whorls, with the outermost generally producing megaspores and the inner producing microspores (*I. butleri* is an exception, with all plants being functionally dioecious) (Engelmann and Butler, 1878). Spore production is seasonal – megaspores begin developing first, then microspores (in a long growing season, a second set of megaspores may be produced following the microspores) (R.D. Bray, pers. comm.).The sporangia have no sutures or other method of dehiscence; spores are released by decay of the sporangial wall (Pfeiffer, 1922). The trabeculae may play some role in spore dispersal over time (L.J. Musselman, pers. comm.)

The megaspores (synonymous with macrospores or gynospores in older literature) of *Isoëtes* are the female reproductive units. The physical appearance of the mature megaspores was first emphasized by Pfeiffer (1922) and has remained one of the most important taxonomic features. Each is small and subglobose, ranging from 250 to 900 um in diameter across the genus, though variation is much less within species (Pfeiffer, 1922). Megaspore diameter is positively correlated with ploidy level, and the relationship has been used taxonomically (Kott, 1983; Taylor et al. 1993; Brunton 2015; Pereira et al. 2015). Some data suggest that within species megaspore diameter may be influenced by environmental conditions (Cox and Hickey, 1984). They are marked by several prominent ridges; an equatorial ridge encircling the spore above the middle, and three proximal ridges extending from the equatorial ridge and connecting at the proximal pole of the spore (Figure 1). A girdle adjacent to the equatorial ridge on its distal side is apparent in some species. The girdle is considered obscure when this area is textured similarly to the rest of the distal surface (Figure 2; Taylor et al., 1993). Coloration is most commonly white, but it can grade to gray/black (*I. melanospora*; Engelmann, 1877) and even green (*I. toximontana*; Musselman and Roux, 2002). The ornamentation of the siliceous exterior

spore wall is highly variable and distinctive, particularly among the diploid taxa (Hickey, 1986; Taylor, 1993; Taylor et al., 1993). Main categories of ornamentation include tuberculate, echinate, cristate, and reticulate (corresponding with Pfeiffer's (1922) division of the genus into the sections Tuberculatae, Echinatae, Cristae, and Reticulatae, respectively).

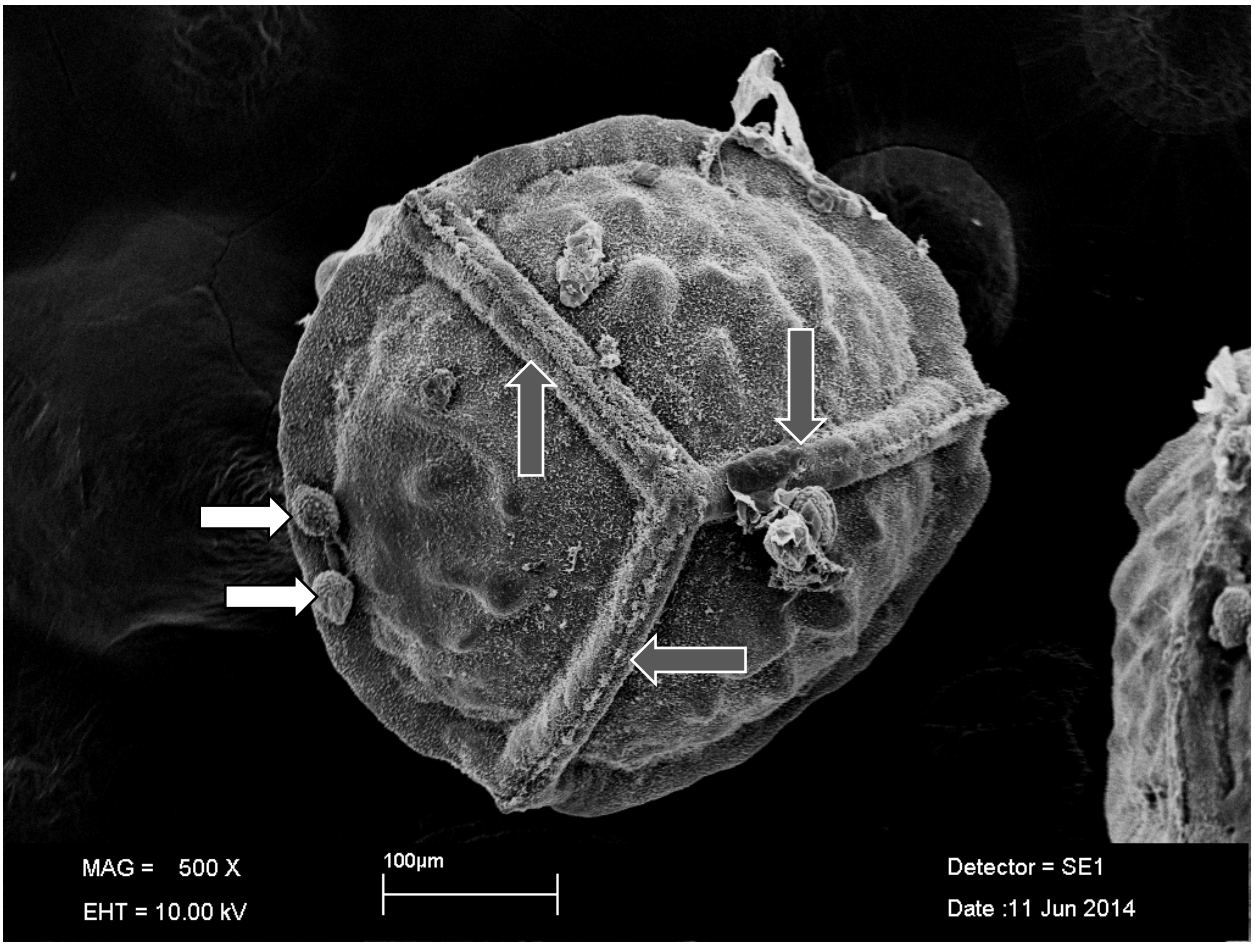


FIGURE 1. Megaspore of unnamed *Isoëtes*. Microspores on equatorial ridge marked by white arrows. Proximal ridges marked by gray arrows.

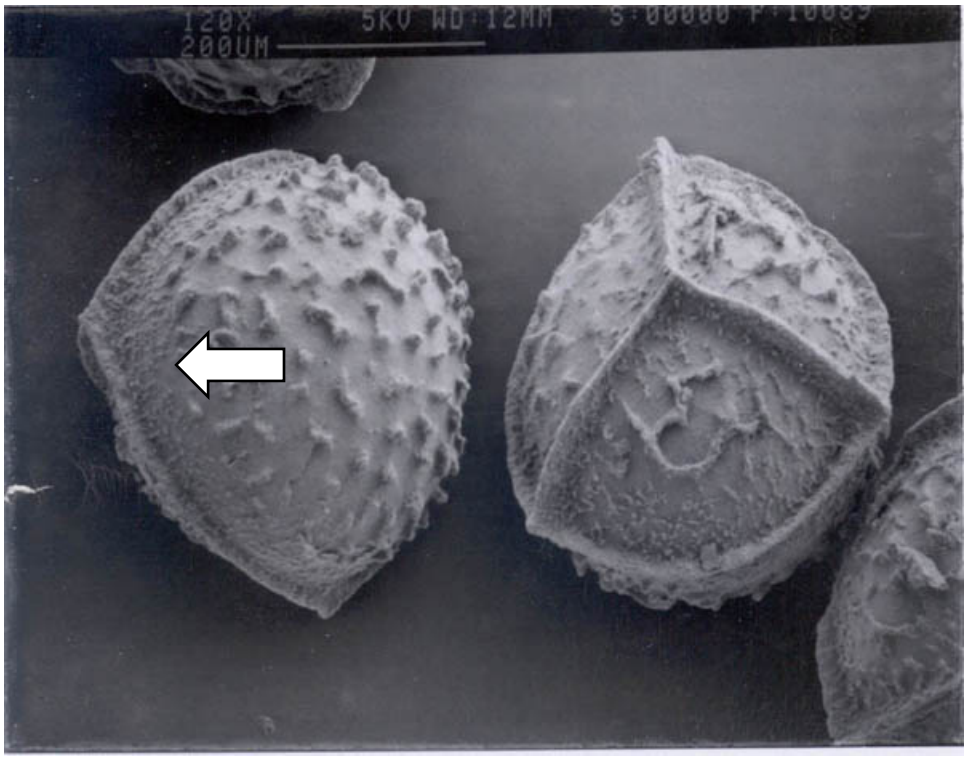


FIGURE 2. Megaspores of *I. piedmontana s.l.* Girdle area marked by arrow. Image retrieved from ODU Plant Site (www.odu.edu/plant).

Microspores (=androspores) are considerably smaller at 20-50 um in length and at most 25 um wide (Pfeiffer, 1922; Taylor et al., 1993). The spores are monolete, reniform in shape, and when dry appear gray, brown, or black in mass (Taylor et al., 1993). They have a single proximal ridge running the length of the sharply angled proximal surface, and two distal ridges parallel to the proximal ridge at approximately the equator, at the margins of the curved distal surface (Figure 3; Musselman, 2002). Microspore macro-ornamentation generally falls into the categories of echinate, cristate, psilate, aculeate, or laevigate. Micro-ornamentation on these

spores may also be filamentose, bacillate, fimbriate, or granular. As with megaspores, there tends to be a positive correlation between microspore size and ploidy level (Musselman, 2002). While potentially informative characters, no geographical, ecological, or systematic trends are apparent based on microspore morphology and they have yet to be used in any taxonomic treatments (Musselman, 2002).

The rootstock (alternatively called corm, tuber, and trunk by other authors) is the subterranean, stem-like organ that produces both sporophylls and roots (Kott and Britton, 1985). Sporophylls emerge in a whorled pattern (distichous in a few taxa) from the center of the rootstock's upper surface with the shoot apex most central, while roots grow from the fossa, a planar depression between basal lobes (Engelmann, 1882; Pfeiffer, 1922; Kott and Britton, 1985). Different species of *Isoëtes* may have two to three lobes; those of the eastern United States generally have two lobes so this character has not been informative (Kott and Britton, 1985; Budke et al., 2005). In rare cases, a three-lobed specimen of a generally two-lobed species occurs (Pfeiffer, 1922). Shape of the rootstock varies from rectangular to subglobose (Engelmann, 1882; Kott and Britton, 1985). Older tissue is found at the margins of the rootstock lobes and is sloughed off in layers—sometimes referred to as abscission caps-- defying any attempts to age the plants based on this outward growth (Osborn, 1922; Karrfalt, 1977). Roots are stigmarian in form, branching dichotomously as they elongate (Kott and Britton, 1985). Growth of new roots initiates along the fossa. As the rootstock grows, roots are translocated along with the tissue of the rootstock. This is evidenced by the growth of new roots only along the fossa and the change in root color from white to brown from the center to the margins (Engelmann, 1882; Kott and Britton, 1985).

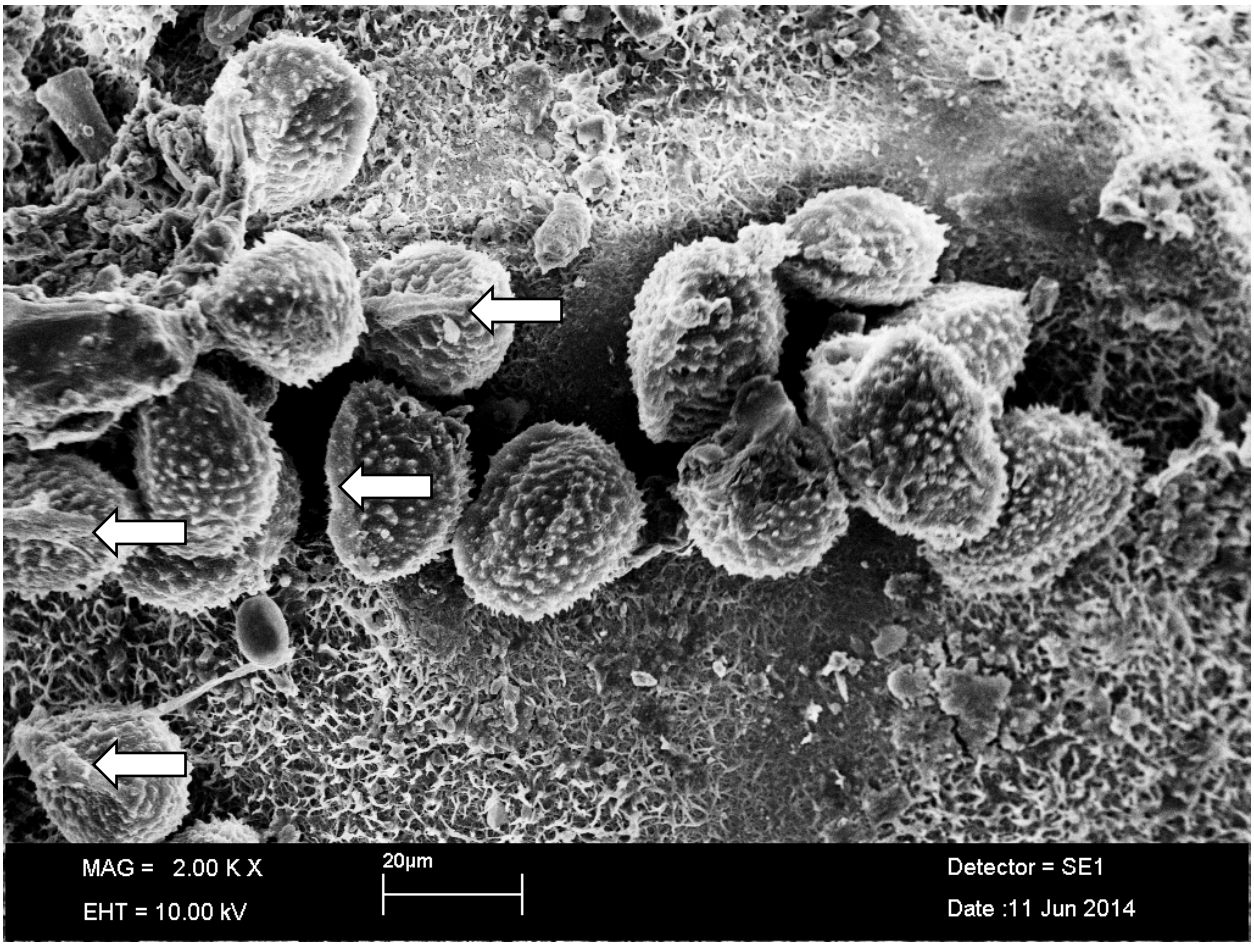


FIGURE 3. Microspores of unnamed *Isoëtes*. Proximal ridges marked by arrows.

Ecology

Despite their nearly global ubiquity, much is still unknown about the ecology of *Isoëtes*. They are obligate wetland plants, though their tolerance for dryness varies tremendously by species. Some may occur permanently submerged in lakes and rivers (e.g. *I. echinospora*, *I. lacustris*), while others are essentially terrestrial (e.g. *I. melanopoda*) (Engelmann, 1882; Pfeiffer, 1922). Other species may occur in freshwater tidal and non-tidal rivers, braided

swamps, upland depressions, alkali flats, and ephemeral pools on rock outcrops (Engelmann, 1882; Taylor et al., 1993). In at least one species, *I. butleri* (and possibly another undescribed one), specific edaphic properties seem to control occurrence (Taylor et al., 1993). Seasonal development varies among species depending on habitat and water availability. Generally, in areas where soil remains moist throughout the summer, plants grow through the summer and spores mature in early autumn. In habitats where the soil is desiccated during hot summer months, plants mature in late spring and then die back to the rootstock until soil becomes moistened again, usually in the autumn (Engelmann, 1882; Pfeiffer, 1922; Taylor et al., 1993). How the mature spores are distributed remains a partial mystery. Given the aquatic nature of most plants, it is assumed that spores are dispersed by water either by regular currents or extreme storm events. The young sporelings and corms have both been proposed as propagules distributed by this vector (Musselman et al., 2014). However, another dispersal mechanism must be present (or have previously existed) for *Isoëtes* to arrive in habitats where dispersal by water alone could not move the spores (e.g. mountain tops in *I. melanospora*). Dispersal by waterfowl has been proposed but is only supported by anecdotal evidence. Pfeiffer (1922) recounted observations by Durieu that ducks in North Africa consume the corms of *I. histrix*. She records the same information from an unnamed observer somewhere in the eastern United States. Fish, pigs, and muskrats are other animals she states (through others' observations) will consume *Isoëtes* (Pfeiffer, 1922). No studies have been conducted to test any hypotheses about natural dispersal of spores or other plant parts.

Classification and Systematics

The first classification of *Isoëtes* by Engelmann (1882) combined the morphological and niche species concepts predominant at the time. He ultimately settled on a classification combining morphology and habitat, although admitting it is "by no means a faultless one":

1. Trunk bi-lobed
    a. Submerged species -- *I. lacustris, I. pygmaea, I. tuckermani, I. echinospora, I. bolanderi*
    b. Amphibious species
        i. Without peripheral bast bundles
            1. Velum incomplete – *I. saccharata, I. riparia*
            2. Velum complete – *I. melanospora*
        ii. With peripheral bast bundles
            1. Velum incomplete – *I. engelmanni*, *I. howelli*
            2. Velum complete – *I. flaccida*
    c. Terrestrial species
        i. Velum incomplete – *I. melanopoda, I. butleri*
        ii. Velum complete – *I. nutallii*
2. Trunk tri-lobed – *I. cubana*

Pfeiffer (1922) stressed the importance of megaspore ornamentation, using four general categories to delineate sections within the genus. She created the Tuberculatae, Echinatae, Cristatae, and Reticulatae based on those respective ornamentation types. However, within these sections the characters she used for classification were mostly the same as ones Engelmann used:

velum coverage, habitat, trunk lobing, peripheral bundles, and stomata presence (Pfeiffer, 1922). This emphasis on the spores was not without controversy, as some authors believed this resulted in a "rather distorted presentation of the relationships of the species" (Reed, 1945). Nevertheless, megaspore morphology has remained an important feature in recent classification (Taylor et al., 1993). The only taxonomic feature that has become important since Pfeiffer's monograph is chromosome number. This has been instrumental in separating both basic diploids and hybrids (Musselman et al., 1995; Musselman et al., 1996; Brunton and Britton, 1997; Brunton and Britton, 1998; Hoot et al., 2004).

The introduction of chromosome number as an important characteristic in *Isoëtes* systematics has significantly added to the understanding of reticulate evolution and allopolyploid origin of several taxa. As recounted in Hickey et al. (1989b), contention surrounding the existence of hybridization has persisted since the early days of *Isoëtes* research. Dodge (1896) observed specimens that seemed to intergrade between species, suggesting hybridization. Eaton (1900) countered by claiming that hybridity is extremely rare in the genus based on the lack of hybrid formation in crossing experiments. More recently, Boom's (1980) artificial crosses and the ease of their formation suggested that hybridization is common in nature. However, Kott and Britton (1983) still argued that not enough evidence existed to show *in situ* hybridization is common. Continued work eventually settled the debate, and hybridization between species is accepted as the rule in *Isoëtes,* rather than the exception (Luebke and Taylor, 1985).

Recognizing the role of hybridization within the genus illuminated the importance of allopolyploid speciation in the systematics of the group, starting with hybridization between existing species whose offspring may then undergo whole genome doubling to create the various polyploids in existence today (Luebke and Taylor, 1989; Taylor and Hickey, 1992). These new

species may then backcross with either parent to form a second round of derived taxa, a pattern often referred to as reticulate evolution (Luebke and Taylor, 1989; Taylor and Hickey, 1992). Once thought to be just "evolutionary noise" (Wagner, 1970), ancient hybridization and genome duplication are now thought to be associated with diversification in some of the largest extant groups of plants (Soltis et al., 2014).

The introduction of molecular biological techniques in the 1980s provided a new avenue for evaluating the systematics of *Isoëtes*. Hickey et al. (1989a) demonstrated the enzyme triose phosphate isomerase (TPI) as a phylogenetically useful marker. Presence of a particular form of TPI is thought to be synapomorphic in certain Neotropical *Isoëtes* (Hickey et al., 1989a). TPI is also informative on smaller scales, showing variation between and among populations of *I. piedmontana* and *I. melanopoda* (Heafner and Bray, 2005). In the past decade, DNA sequencing of the second intron of the *LEAFY* gene has been used to unravel phylogenetic relationships of *Isoëtes* in the southeastern United States (Hoot and Taylor, 2001; Hoot et al., 2004). While other gene regions such as nuclear ribosomal ITS and *rbcL* have proved useful at resolving more basal nodes, they do not have the signal necessary to differentiate among taxa in eastern North America (Hoot and Taylor, 2001; Rydin and Wikstrom, 2002; Taylor et al., 2004). The nucleotide sequences of the entire chloroplast genomes provide additional variability to make it a useful marker. The chloroplast genomes of *I. flaccida* (Karol et al., 2010) and *I. melanopoda* (Duff and Schilling, 2000) have been sequenced and provide a scaffold for genomic analysis of other species. These whole plastome sequences can be used to identify gene regions with phylogenetic signal or used in their entirety (Shaw et al., 2007; Koral et al., 2010).

From the last review of the genus in the United States and Canada (Taylor et al., 1993) until the outset of this project, molecular systematics and cytology had explained further species

relationships and reticulate evolution within the group. A large number of new taxa were named in North America alone based in part on these molecular and cytological techniques (Brunton et al., 1994; Musselman et al., 1995; Musselman et al., 1996; Brunton and Britton, 1997; Brunton and Britton, 1998; Brunton and Britton, 1999; Musselman et al., 2001; Luebke and Budke, 2003; Rosenthal et al., 2014). Since Engelmann's (1882) first treatment, the number of species in North America increased from 14 to 34 (Table 1). The phylogeny of *Isoëtes* was in many respects still in its preliminary stages, having been applied only to a few select groups of taxa with three gene regions: nuclear ribosomal ITS, a chloroplast *atpB-rbcL* spacer, and the second intron of the *LFY* homolog (Hoot and Taylor, 2001; Rydin and Wikström, 2002; Hoot et al., 2004; Taylor et al., 2004). To date only the *LEAFY* gene was useful in resolving species in the Americas, and even then not completely (Hoot et al., 2004). Many questions remained unanswered regarding the phylogeny of *Isoëtes* and its biogeography.

TABLE 1. Recognized *Isoëtes* taxa in the United States and Canada. Spellings follow the respective literature.

	Engelmann (1882)	**Pfeiffer (1922)**	**Taylor et al. (1993)**	**Currently Accepted (2019)**
1	*I. bolanderi* Engelm.	*I. bolanderi*	*I. acadiensis* Kott	*I. acadiensis*
2	*I. butleri* Engelm.	*I. braunii* Durieu	*I. bolanderi*	*I. appalachiana* D.F. Brunt. & D.M. Britton
3	*I. echinospora* Durieu	*I. butleri*	*I. boomii* Luebke	*I. bolanderi*
4	*I. engelmanni* A. Braun	*I. eatoni* R. Dodge	*I. butleri*	*I. boomii*

TABLE 1. Continued.

	Engelmann (1882)	Pfeiffer (1922)	Taylor et al. (1993)	Currently Accepted (2019)
5	*I. flaccida* Shuttlew.	*I. engelmanni*	*I. caroliniana* (A.A. Eaton) Luebke	*I. butleri*
6	*I. howellii* Engelm.	*I. flaccida*	*I. echinospora*	*I. echinospora*
7	*I. lacustris* L.	*I. flettii* (A.A. Eaton) N. Pfeiff.	*I. engelmannii*	*I. engelmannii*
8	*I. melanopoda* J. Gay & Durieu	*I. foveolata* A.A. Eaton ex R. Dodge	*I. flaccida*	*I. flaccida*
9	*I. melanospora* Engelm.	*I. howellii*	*I. georgiana* Luebke	*I. georgiana*
10	*I. nuttallii* A. Braun ex Engelm.	*I. lithophila* N. Pfeiff.	*I. howellii*	*I. howellii*
11	*I. pygmaea* Engelm.	*I. macrospora* Durieu	*I. lacustris*	*I. hyemalis* D.F. Brunt.
12	*I. riparia* Engelm. ex A. Braun	*I. melanopoda*	*I. lithophila*	*I. junciformis* D.F. Brunt. & D.M. Britton
13	*I. saccharata* Engelm.	*I. melanospora*	*I. louisianensis* Thieret	*I. lacustris*
14	*I. tuckermani* A. Braun ex Engelm.	*I. nuttallii*	*I. maritima* Underw.	*I. lithophila*
15		*I. occidentalis* L.F. Hend.	*I. melanopoda*	*I. louisianensis*
16		*I. orcuttii* A.A. Eaton	*I. melanospora*	*I. maritima*
17		*I. piperi* A.A. Eaton	*I. nuttallii*	*I. mattaponica* Musselman & W.C. Taylor
18		*I. riparia*	*I. occidentalis*	*I. melanopoda*
19		*I. saccharata*	*I. orcuttii*	*I. melanospora*

TABLE 1. Continued.

	Engelmann (1882)	Pfeiffer (1922)	Taylor et al. (1993)	Currently Accepted (2019)
20		*I. truncata* Clute	*I. prototypus* D.M. Britton	*I. microvela* D.F. Brunt.
21		*I. tuckermani*	*I. riparia*	*I. minima* A.A. Eaton
22			*I. tegetiformans* Rury	*I. nuttallii*
23			*I. tuckermanii*	*I. occidentalis*
24			*I. virginica* N. Pfeiff.	*I. orcuttii*
25				*I. prototypus*
26				*I. riparia*
27				*I. saccharata*
28				*I. septentrionalis* D.F. Brunt.
29				*I. tegetiformans*
30				*I. tenneseensis* Luebke & Budke
31				*I. texana* Singhurst, Rushing & W.C. Holmes
32				*I. tuckermanii*
33				*I. valida* (Engelm.) Clute
34				*I. virginica*

Under the tenets of the most modern biological species concepts, all members of a species must be defined by a unique set of characteristics and be descendants of a single common ancestor (Baum and Donoghue, 1995; Luckow, 1995). This has caused considerable disruption in the systematics of *Isoëtes,* as molecular data show morphologically indistinct plants with polyphyletic populations and polyploid taxa with varying parentage scenarios (Bolin et al., 2008; W.C. Taylor, unpublished data). This throws several taxa into confusion and raises the questions:

1) What are the phylogenetic relationships among the basic diploids of *Isoëtes*?
2) What are the maternal and paternal lineages of polyploid species?
3) Do polyphyletic taxa represent cryptic species?
4) Are multi-parentage scenarios common within polyploid species?
5) Can the current taxonomy be reconciled with a molecular phylogeny?

In similar cases where reticulate evolution plays a significant role, a "diploids first" approach to phylogeny is efficacious, creating a framework which can then be used to investigate polyploid lineages (Beck et al., 2010). Chapter 2 formally describes *I. mississippiensis,* one of the few undescribed diploid taxa in the study region diagnosable based on morphology alone, a hypothesis to be tested by the molecular phylogeny. Extrapolating from apparent cryptic speciation identified by Hoot et al. (2004), Heafner and Bray (2005), and Bolin et al. (2008), I expect that a molecular phylogeny including numerous representatives of each diploid taxon will show a classification unlike those based solely on a morphological and ecological classification. Comparison of DNA sequences across several populations -- particularly in wide ranging taxa such as *I. engelmannii* and *I. melanopoda* -- may show polyphyly indicating that cryptic species are present. In Chapter 3, low-copy nuclear markers are developed which can be used to test the

relationships inferred from the LEAFY marker, and potentially provide phylogenetic resolution where LEAFY is insufficient. The maternal lineages of diploid taxa in the southeastern US, are presented as a whole chloroplast genome phylogeny in Chapter 4. Several taxonomically informative characters are mapped to the phylogeny and ancestral states determined to identify the most likely locations of state transitions within the phylogeny. Again, based on prior work (Hoot et al., 2004; Heafner and Bray, 2005; Bolin et al., 2008) parentage analyses of polyploid species are expected to show that across populations, the diploid parents of a polyploid species vary. Chapter 5 tests this with combined plastid and nuclear data. A whole chloroplast genome phylogeny including diploid and polyploid taxa illustrates several species that are not monophyletic, as well as which polyploids have clear or uncertain maternal diploid progenitors. Likewise, a nuclear phylogeny identifies the likely diploids involved in the formation of the polyploid individuals sampled, and where individuals presently treated as one species exhibit different parentages.

CHAPTER 2

ISÖETES MISSISSIPPIENSIS: A NEW QUILLWORT FROM MISSISSIPPI, USA

INTRODUCTION

Isoëtes (Isoëtaceae) is a cosmopolitan genus of heterosporous lycophytes containing 200-300 species (Hickey et al., 2003; Troia et al., 2016). Lycophytes have an extensive fossil record dating from the Devonian and a morphology so conserved that members of the genus *Isoëtes* are recognized in the Triassic (Pigg, 2001). Extant species are widely distributed from the tropics to the sub-arctic (Troia et al., 2016). They range in habitat from evergreen aquatics to seasonal terrestrials. Resembling a tuft of chives or grass, they are easily overlooked in the field.

In spite of their antiquity, widespread distribution, and diverse ecological adaptations, *Isoëtes* species are remarkably uniform in their morphology. Plants appear simple in form with a lobed subterranean rootstock producing a tuft of linear sporophylls above and below roots along a groove between the lobes. This apparent morphological simplicity makes it easy to recognize a member of the genus, but difficult to distinguish species. Earlier taxonomists relied primarily on habitat, megaspore texture, and megaspore size to separate taxa (Engelmann, 1882; Pfeiffer, 1922; Reed, 1965; Boom, 1982). More recently, chromosome counts and molecular markers have been used to further define taxa and infer their phylogeny (Taylor et al., 1993; Hoot et al., 2004; Heafner and Bray, 2005; Rosenthal et al., 2014).

Ornamentation and size of megaspores and microspores are important morphological features used to identify species of *Isoëtes.* Pfeiffer (1922) erected four sections based on the megaspore ornamentation types cristate, echinate, reticulate, and tuberculate. While these sections are no longer recognized as having phylogenetic value in the genus, the emphasis on macro-ornamentation for identification remains (Brunton, 2015). Several categories for megaspores (cristate, echinate, laevigate, psilate, reticulate, rugulate, and tuberculate) and microspores (aculeate, cristate, echinate, laevigate, and psilate) are accepted, though there can be gradation between categories (Taylor et al., 1993; Musselman, 2002). Micro-ornamentation of megaspores and microspores is sometimes recognized but has not been included in any recent taxonomic treatments of the genus (Reed, 1965; Boom, 1982; Taylor et al., 1993; Brunton, 2015). Generally, megaspore size increases with ploidy level (Pereira et al., 2015; Brunton, 2015).

The habitat of species of *Isoëtes* can be fairly specific and is often used in taxonomic treatments (Engelmann, 1882; Reed, 1965; Taylor et al., 1993; Brunton, 2015). Species are generally segregated as aquatic, amphibious, or terrestrial, based on the proportion of their growing season spent in water (Engelmann, 1882; Taylor et al., 1993). Some species occur only in certain habitats, such as rock pools, calcareous glades, oligotrophic lakes, and swamp forests. Widespread species such as *I. melanopoda* Gay & Durieu (*s.s.*) and *I. engelmannii* Braun have more varied habitat preference (Taylor et al., 1993; Brunton, 2015).

Characteristics of sporophylls and rootstocks of *Isoëtes* may also provide taxonomic information, though the utility of some of these features is questionable. Velum coverage of the sporangium, sporangium shape, sporangium wall coloration, and sporophyll length, number,

color, and shape are sometimes used for species identification, but these character states can be subtle and it is unclear how they may be influenced by environmental conditions (Engelmann, 1882; Pfeiffer, 1922; Reed, 1965; Boom, 1982; Taylor et al., 1993; Brunton, 2015). Cultivated plants often appear different than those *in situ*, and spore development, photosynthetic pathways, and gene expression are significantly altered by water conditions (Brunton, 2015; Yang and Liu, 2015; Yang and Liu, 2016). However, the *gestalt* formed from the combination of these characters usually leads experts to accurate field identification.

While searching for populations of *Isoëtes louisianensis* in southwestern Pearl River Co., MS, in the spring of 1996, one of us (Leonard) discovered a population of *Isoëtes* that did not appear to be *I. louisianensis* or any other known species. These plants had very long and numerous sporophylls bearing megaspores with a smooth surface rather than an irregularly reticulate texture that is typical of *I. louisianensis* megaspores. In addition, the megaspores of this plant were noticeably smaller than those of *I. louisianensis*. Further investigation turned up a second population downstream in Lotts Creek. Both of these waterways are tributaries of the Pearl River, converging near Picayune, MS.

METHODS

Field work was performed in 1996, 1998, and 2013 to obtain specimens for further study. Specimens were deposited in the Old Dominion University herbarium (ODU). Length and width of the rootstock, sporophylls, and sporangia were measured for 10 individuals. Megaspores and microspores were removed from sporangia, cleaned by sonication in distilled water for 90 seconds, and dried on a slide warmer at maximum temperature (approximately 60°C). Light

images were captured using a Nikon SMZ800 stereomicroscope with attached Digital Sight camera, and measurements made within the Digital Sight control panel. Spores were prepared for scanning electron microscopy by coating with 25 nm of gold-palladium using a Cressington high resolution sputter coater (Cressington Scientific Instruments Ltd.). Imaging was performed on a Zeiss EVO MA 15 scanning electron microscope. Chromosome counts were determined by root tip squashing as described in Heafner and Bray (2005). Site descriptions were prepared and lists of associated species were made.

RESULTS

Analysis of morphological characters, chromosome counts, and ecological evaluation leads us to conclude our collections represent an undescribed species of *Isoëtes*.

Isoëtes mississippiensis S.W. Leonard, W.C. Taylor, Musselman and R.D. Bray sp. nov. TYPE: U. S. A. Mississippi: Lotts Creek (30.57396°N, 89.76196°W, elevation 14 m), 18 June 2013, P. Schafran MS-08 L. Musselman, S. Leonard, W. Taylor, M. Alford, and D. McNair (holotype: US; isotypes: MO, NY, ODU, USMS).

Description

Plants amphibious in and along persistent streams. Rootstock subglobose, bilobed, brown, 0.5−1.0 cm long, 1.0−1.5 cm wide. Roots dichotomously branched. Sporophylls (leaves) linear, bright green, darkening with age, pale toward base, spirally arranged, erect to spreading, up to 40 cm long and 2.0 mm wide at mid-length, in tufts of ca. 20, semi−terete with adaxial surface flattened, becoming more terete distally, with translucent alae ca. 1 mm wide extending

along lateral edges from base to ca. one-quarter leaf length, tapering gradually toward apex, abruptly dilated and spatulate toward base where streaks of brown pigmented cells are often evident on pale outer surface of leaf base. Ligule triangular, ca. 1 mm long. Sporangium ovate, most 4−10 mm long, most 4−5 mm wide, adaxial wall spotted to streaked with scattered clusters of brown pigmented cells. Velum incomplete, covering less than one third of sporangium wall. Megaspores globose, white, trilete, macro-ornamentation laevigate with echinate micro-ornamentation, ca. 280−380 μm in diameter, averaging ca. 340 μm. Microspores broadly fusiform, macro-ornamentation echinate with bacillate micro-ornamentation, pale brown in mass, monolete, 25−30 μm long.

Morphology

Rootstocks of all specimens examined vary in length from 0.5−1.0 cm and in width from 1.0−1.5 cm. All rootstocks are subglobose in shape and bilobed. Sporophylls reach a maximum length of 40 cm and maximum width of 2.0 mm at mid-length. Sporangia are 4−10 mm long and 4−5 mm wide. Megaspores are laevigate with echinate micro-ornamentation (Figures 4, 5, 6). Diameter of megaspores varies from 280−380 μm, with an average of 340 μm. Microspores are echinate with bacillate micro-ornamentation and are 25−30 μm long (Figure 4).

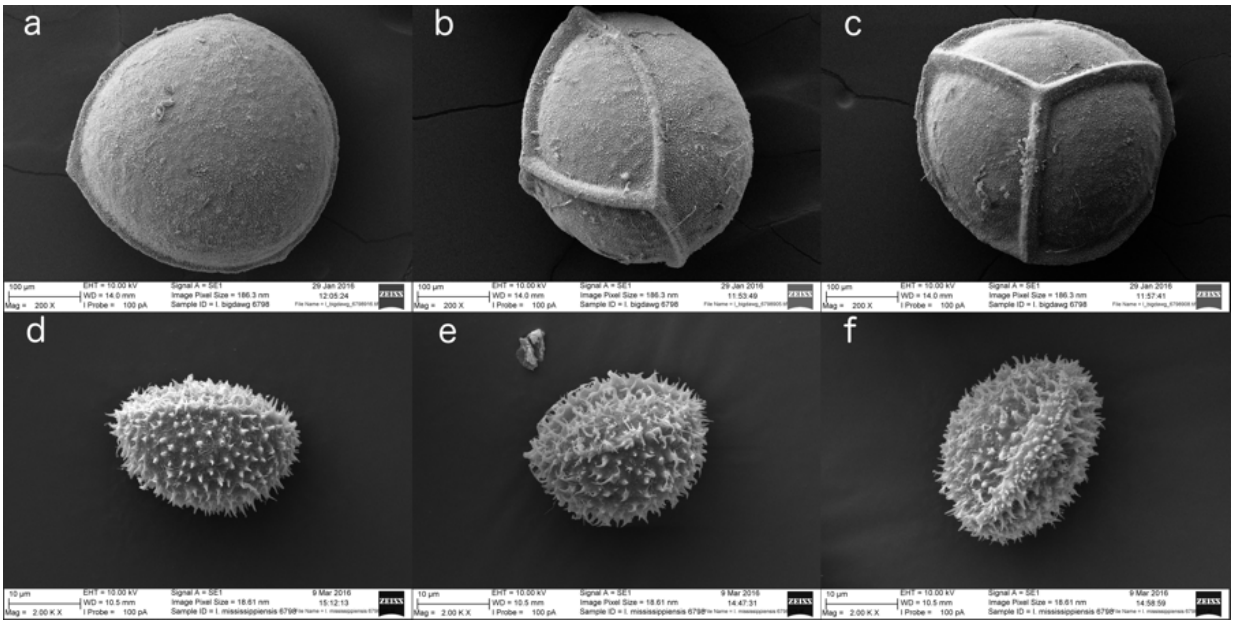


FIGURE 4. SEMs of megaspores (a,b,c) and microspores (d,e,f) of *I. mississippiensis* displaying distal (a,d), equatorial (b,e), and proximal (c,f) views. Megaspores from Schafran MS-08, microspores from Taylor 6798. Megaspore magnification 200X; microspore magnification 2000X.

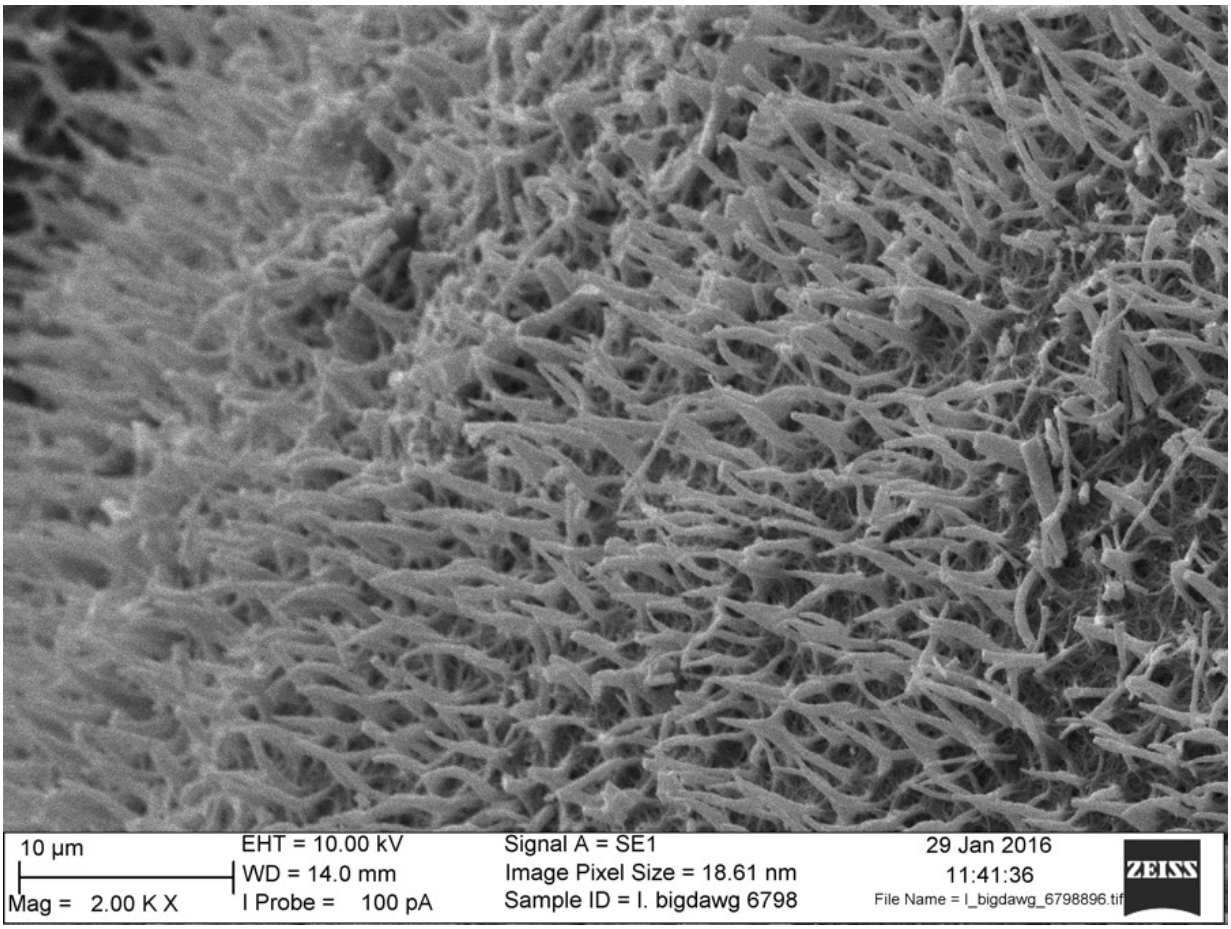


FIGURE 5. SEM detail of megaspore micro-ornamentation. Magnification 2000X.

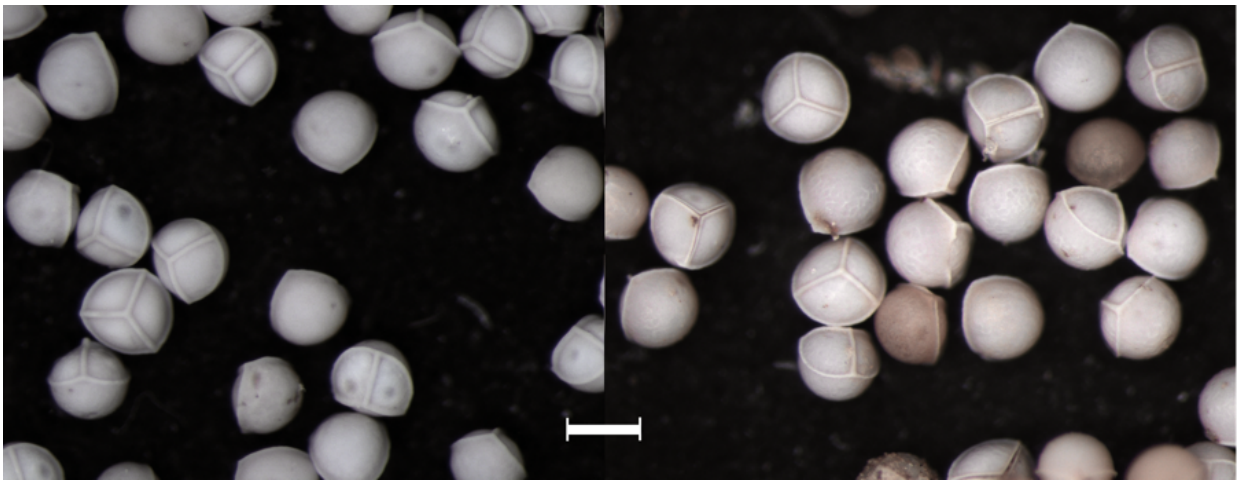


FIGURE 6. Light microscope image of megaspores of *I. mississippiensis* from Schafran MS-07 (left) and MS-08 (right). Magnification 63X. Scale bar = 0.3 mm.

Cytology

Chromosome counts show individuals of *Isoëtes mississippiensis* to be diploid (2n=22).

Ecology

Isoëtes mississippiensis occurs in sluggish, persistent streams in southern Mississippi (Figure 7). At the Moody Branch locality, the maintained right-of-way of Mississippi Highway 43 allows abundant sunshine to reach the stream and adjacent wetlands. Small bushes and saplings of titi (*Cyrilla racemiflora*) and red maple (*Acer rubrum*) are periodically cut down and allowed to fall in the stream. Sediment and detritus provide anchors for herbaceous growth of sedges, rushes, and coarse grasses (*Rhynchospora inexpansa*, *Juncus* spp., *Erianthus giganteus*, *Panicum* spp.). In the shallow water stream margin is *Iris virginica*. The woodland edge is suitable habitat for crossvine (*Bignonia capreolata*) and rattan vine (*Berchemia scandens*). Upstream where a defined channel is present the overstory consists of swamp black gum (*Nyssa*

biflora), laurel oak (*Quercus laurifolia*), red maple, and encroaching loblolly pines (*Pinus taeda*). Shrubs in the understory are Elliott's blueberry (*Vaccinium elliottii*), yaupon (*Ilex vomitoria*), and titi. In the upper reaches of Moody Branch, the channel is braided and the water sluggish, more typical of a swamp black gum forest with Rankin's jessamine (*Gelsemium rankinii*), Virginia willow (*Itea virginica*), and dog hobble (*Viburnum nudum*).

After flowing west for several kilometers, Moody Branch turns sharply south just west of Mississippi Highway 43 and eventually merges with Lotts Creek. The forested wetland adds pond cypress (*Taxodium ascendens*) and a dense shrub understory with *Smilax laurifolia*. At the Walkiah Bluff Road crossing of Lotts Creek disturbance has been severe, yet *I. mississippiensis* has revegetated new habitat in the roadside ditch north of the road and on sandbars.

Etymology

This species is named for the state of Mississippi, its only known locality.

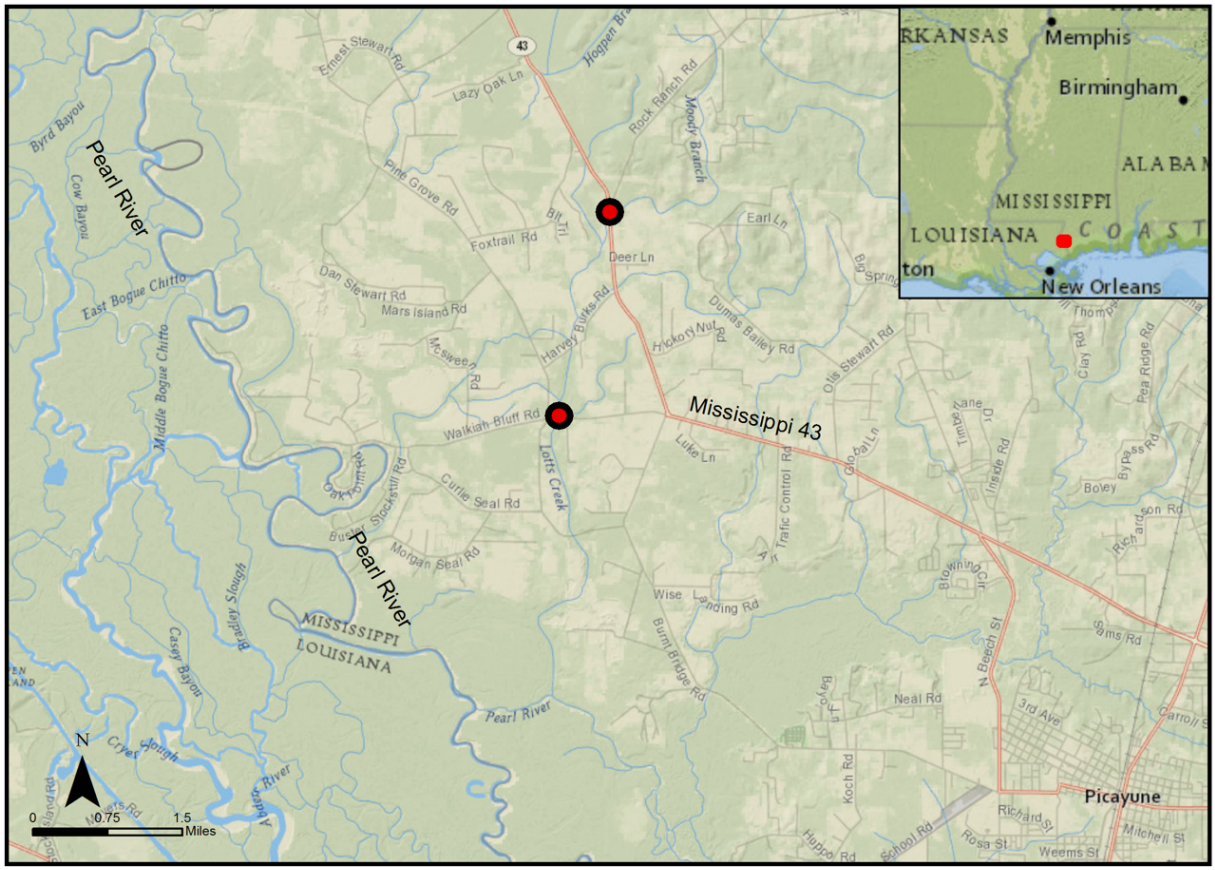


FIGURE 7. Map showing two localities of *I. mississippiensis*. Inset: Map of Mississippi with detail area highlighted. Map created using ArcGIS software (Esri).

Specimens Examined

Leonard 9393, 9 March 1996 (MMNS); Leonard 9395, 22 March 1996 (MMNS); Leonard 9831, 2 June 1997 (MMNS); Leonard 12405, 12 May 2011(ODU); Leonard 12406, 12 May 2011 (ODU); Musselman with Taylor, 98908, 17 October 1998 (ODU); Bolin JB-MS-01, 9 January 2009 (ODU); Schafran MS-07, 18 June 2013 with Musselman, Leonard, Taylor, and Alford (MO; NY; ODU; USMS); Schafran MS-08, 18 June 2013 with Musselman, Leonard,

Taylor, and Alford (US; ODU); Taylor 6798, 18 June 2013 with Musselman, Leonard, Schafran, and Alford(US);

DISCUSSION

Evaluation of the morphological and cytological features of *I. mississippiensis* shows it to be distinct from all other taxa in the southeastern US. In the coastal plain of the Gulf Coast states, nine other species are known: *I. appalachiana, I. boomii, I. flaccida s.l., I. hyemalis, I. louisianensis, I. melanopoda s.l., I. microvela, I. texana*, and *I. valida* (Singhurst et al., 2011; Brunton, 2015; Weakley, 2015). A basic diploid chromosome count (2n=22) plus laevigate megaspore ornamentation separates *I. mississippiensis* from all these taxa except *I. texana* and occasionally *I. melanopoda*. These species may be further separated by presence/absence of phyllopodia, difference in megaspore size, and velum coverage (Table 2). Additionally, the habitats of these species are quite different. *Isoëtes mississippiensis* occurs along persistent streams, while *I. texana* is found in freshwater ponds and interdunal swales and *I. melanopoda* grows in wet prairies, soil pockets on rock outcrops, and woodland depressions (TABLE 2; Taylor et al., 1993; Singhurst et al., 2011).

TABLE 2. Comparisons of Gulf Coastal Plain *Isoëtes.*

Character	***I. mississippiensis***	***I. texana***	***I. flaccida s.l.***	***I. melanopoda s.l.***	***I. valida***
Ploidy	2n=22	2n=22	2n=22	2n=22	2n=22
Habitat	Persistent streams	Persistent freshwater ponds, interdunal swales	Springs, stream bottoms, river bottoms, ditches	Ephemeral wet prairies, open graminoid swales, woodland pools, soil pockets on rock outcrops	Woodland seepages
Megaspore Ornamentation	Laevigate	Smooth to obscurely rugulose	Low tubercules to broad, interconnected mounds	Low tubercles or ridges	Broken reticulate
Megaspore Size (μm)	280-380 ($\bar{x}$=340)	350-405 (no mean reported)	250-500 (no mean reported)	280-440 ($\bar{x}$=380-410)	$\bar{x}$=450
Microspore Ornamentation	Spinulose/ echinate	Papillose	Papillose	Spinulose/ echinate	Spinulose/ echinate
Microspore Size (μm)	25-30	25-30	25-33	20-30	27
Velum Coverage (%)	15-33	100	80-100	5-15	45-70

TABLE 2. Continued.

Character	*I. louisianensis*	*I. hyemalis*	*I. appalachiana*	*I. boomii*	*I. microvela*
Ploidy	2n=44	2n=44	2n=44	2n=66	2n=66
Habitat	Creeks, streams	Blackwater streams	Creek banks, woodland pools, lakes	Slow-flowing woodland streams	Persistent streams in deciduous swamp forests
Megaspore Ornamentation	Irregularly reticulate	Broken reticulate to sub-echinate	Broken reticulate	Cristate to reticulate	Densely reticulate with irregular crests and thin tubercles
Megaspore Size (μm)	500-625 (no mean reported)	400-580 ($\bar{x}$=522)	450-611 ($\bar{x}$=534)	460-610 (no mean reported)	$\bar{x}$=527
Microspore Ornamentation	Spinulose/ echinate	Spinulose/ echinate	Psilate to low tuberculate	Papillose/aculeate	Psilate to low tuberculate
Microspore Size (μm)	25-35	20-31	29-32	25-30	30
Velum Coverage (%)	<50	10-20	20-25	30-50	10

Key to the Diploid Species of Isoëtes of the Gulf Coastal Plain of the United States

1. Megaspores psilate to laevigate, rarely low tuberculate or low rugulate
    2. Plants at least sometimes with darkened, often sclerified, brown-black leaf bases; velum coverage generally <15%……. *I. melanopoda s.l.*
    2. Plants never with darkened leaf bases; velum coverage usually >15%.
        3. Megaspores 280-380 μm; velum coverage 15-30%..... *I. mississippiensis*

3. Megaspores 350-405 μm; velum coverage 100%..... *I. texana*

1. Megaspores tuberculate, reticulate, cristate, or rugulate
    4. Velum coverage 75-100%; microspores papillose…… *I. flaccida s.l.*
    4. Velum coverage less than 75%; microspores echinate
        5. Megaspore ornamentation of tubercles or ridges; velum coverage less than ca. 25%..... *I. melanopoda s.l.*
        5. Megaspore ornamentation broken reticulate; velum coverage between ca. 25 and 75%..... *I. valida*

Conservation

Isoëtes mississippiensis is known from only two locations along approximately 2 miles of the Lotts Creek—Moody Branch waterway. Neither of these populations is located on preserved land. Extensive field work is needed to search for additional populations in the nearby Pearl River Wildlife Management Area and Bogue Chitto National Wildlife Refuge.

CHAPTER 3

LOW-COPY NUCLEAR MARKERS IN *ISOËTES* L. (ISOËTACEAE) IDENTIFIED WITH TRANSCRIPTOMES

INTRODUCTION

Isoëtes L. (Isoëtaceae, Lycopodiophyta) is a cosmopolitan genus of ca. 250 recognized species. These heterosporous lycophytes consist of a 2-3 lobed rootstock that bears linear, quill-like, microphyllous leaves or sporophylls. All microphylls have the potential to develop into sporophylls (Foster and Gifford, 1974). Mega- and microsporangia are produced at the base of sporophylls, in some species covered by a layer of tissue called a velum. Traditionally, spore ornamentation and velum coverage were taxonomically important. Though species inhabit a variety of ecological niches, from obligate aquatic to ephemeral terrestrial habitats, their morphology is extremely conserved. Phylogenetic studies in closely related clades of *Isoëtes* have been limited by a dearth of morphological features and molecular markers. Hoot and Taylor (2001) identified the nuclear ribosomal gene internal transcribed spacer (ITS), a LEAFY homolog nuclear gene intron (*LFY*), and the plastid *atpB-rbcL* spacer region as informative markers in *Isoëtes*. However, while these markers and the plastid *rbcL* gene show utility in large scale, global phylogenies, they generally lose resolution at the regional level (Rydin and Wikström, 2002; Hoot et al., 2006; Larsen and Rydin, 2016). *LFY* is more variable than the other three markers and is fairly informative in recently diverged species groups (Hoot et al., 2004; Taylor et al., 2004). With only a single informative nuclear marker within groups such as the

eastern North American clade, it is difficult to fully test phylogenetic hypotheses of reticulate evolution and incomplete lineage sorting

Transcriptomes provide a valuable tool for marker selection and PCR primer design in the absence of a sequenced genome, as is the case in *Isoëtes*. Databases such as the 1000 Plants project (http://www.onekp.com; Matasci et al., 2014) contain transcriptomes across all major lineages of land plants, allowing identification of unique marker regions for a group of interest. Here we describe use of transcriptome data to develop PCR primers for phylogenetically informative low-copy nuclear markers in *Isoëtes.*

METHODS

Markers of interest were selected based on a literature search of reportedly low-copy nuclear markers in ferns and mosses (Table 3; Szövényi et al., 2006; Schuettpelz et al., 2008; Rothfels et al., 2013). Nucleotide sequences for these markers were obtained from the NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/; Clark et al., 2016) or TreeBASE (http://www.treebase.org; Sanderson et al., 1994) databases. Transcriptomes for three taxa of *Isoëtes* were provided by S. Hetherington (*I. echinospora;* University of Oxford) and the 1000 Plants project (http://www.oneKP.com)(*I. tegetiformans* and *I. sp.*; pers. comm.). Using the BLAST+ 2.4 software package (Camacho et al., 2009), local BLAST databases were constructed from each *Isoëtes* transcriptome. The sequences of selected fern (Rothfels et al., 2013) and moss (Szövényi et al., 2006) low-copy nuclear markers were BLASTed against the transcriptome databases to identify those markers present as single-copy in *Isoëtes*. These single-copy marker regions were extracted from their respective transcriptome and aligned with marker sequences

from the literature using Geneious version 7 (Kearse et al., 2012). Primer sequences from the literature were modified to match the *Isoëtes* transcriptome sequences.

TABLE 3. Primers designed for low-copy markers identified in *Isoëtes* transcriptomes.

Marker ID	Primer Names (Forward, Reverse)	Primer Sequences (Forward, Reverse)	PCR Annealing Temperature (°C)
pgiC	pgiC_1156F pgiC_1900R	5'— GGTCTCCTAAGTGTCTGGAATGT —3' 5'— GTTCTCCAAAATCAATTTCTCC —3'	55
IBR3_1	IBR3_2F IBR3_6R	5'— CTCAAATCAGCTCATGCAATTG —3' 5'— AGCTCCCAATCCAACACAGC —3'	60
IBR3_2	IBR3_13F IBR3_16R	5'— CAATGACTGAACCGCAAGTTG —3' 5'— GACCCAACGAGTCTCATGCAG —3'	60
Transducin_1	Transducin_1F Transducin_1R	5'— GATGTGGTTGGTGAGTCTGG —3' 5'— CACTTCATTGAACCTCAG —3'	55
Transducin_2	Transducin_2F Transducin_2R	5'— GGAACAAAAGCAGGGACATTAG —3' 5'— CATCAGAAGAGATGTCCATAC —3'	55
gapC_short	gapC_5F gapC_7R	5'— GAATCTACTGGTGTCTTCAC —3' 5' —TTCTGGTTTATATTCATGCTCG —3'	55
gapC_long	gapC_5F gapC_9R	5'— GAATCTACTGGTGTCTTCAC —3' 5'— ATGGTCCATCAACAGTYTTCTG —3'	55

Plants were collected from the field and leaf tissue desiccated with silica gel. Voucher specimens have been stored at the Old Dominion University herbarium (ODU) and/or the U.S. National Herbarium (US). DNA was extracted from approximately 200 mg of dried tissue with the Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, California, USA) or AutogenPrep 965 (Autogen Inc., Holliston, Massachusetts, USA) using standard protocols. Sixteen diploid taxa of *Isoëtes* and one species of *Lycopodium* (one individual per taxon) were selected from available DNAs to represent various levels of divergence (Table 4).

TABLE 4. Collection locations, vouchers, and GenBank accessions for taxa included in this chapter.

Taxon	Phylogenetic Clade (per Larsén and Rydin 2016)	Collection Locality	Voucher (Herbarium)	GenBank Accessions		
				pgiC	*IBR3*	*gapC*
Isoëtes butleri Engelm.	Clade E	Texas, USA	*Schafran 47* (ODU)	KY243331	KY270816	KY270832
I. echinospora Durieu	Clade E	New York, USA	*Schafran NY—4* (ODU)	KY243333	KY270818	KY270835
I. engelmannii A. Braun	Clade E	Tennessee, USA	*Schafran 46* (ODU)	KY243334	KY270819	—
I. flaccida var. *chapmanii* Engelm.	Clade E (= *I. flaccida*)	Florida, USA	*Bolin JB_FL_01* (ODU)	KY243332	KY270817	KY270833
I. flaccida Shuttlew.	Clade E	Florida, USA	*Schafran FL—01* (ODU)	KY243335	KY270820	KY270836
I. histrix Bory & Durieu	Clade E	Sicily, Italy	*A. Troia s.n.*	KY243347	—	—
I. lithophila Pfeiff.	Clade E	Texas, USA	*Schafran 61* (ODU)	KY243336	KY270822	KY270838
I. longissima Bory	Clade B (= *I. velata*)	Sicily, Italy	*A. Troia s.n.*	KY243348	KY270823	KY270839
I. melanopoda J. Gay & Durieu ssp. *melanopoda*	Clade E	Mississippi, USA	*Taylor 6796* (US)	KY243338	KY270825	KY270841
I. melanopoda ssp. *silvatica* D.F. Brunt. & D.M. Britton	Clade E (= *I. melanopoda s.l.*)	North Carolina, USA	*Schafran NC—05* (ODU)	KY243342	KY270828	KY270845
I. melanospora Engelm.	Clade E	Georgia, USA	*Schafran 12* (ODU)	KY243339	KY270826	KY270842
I. nuttallii A. Braun ex Engelm.	Clade B	California, USA	*Taylor 6734* (US)	KY243351	—	—
I. piedmontana (N. Pfeiff.) C.F. Reed	—	Georgia, USA	*Schafran 18* (ODU)	KY243341	KY270827	KY270844
I. storkii T.C. Palmer	Clade E	Costa Rica	*Taylor 6760* (US)	KY243352	KY270829	KY270846
I. tegetiformans Rury	—	Georgia, USA	*Schafran 19* (ODU)	KY243343	KY270830	KY270847
I. valida (Engelm.) Clute	Clade E	Pennsylvania, USA	*Schafran 37* (ODU)	KY243344	KY270831	—
Lycopodium clavatum L.	—	New York, USA	*Schafran s.n.*	MG434746	—	—

Note: One individual sampled per taxon.

Markers were amplified by PCR on an Applied Biosystems (ABI) 2720 thermocycler, with a reaction mixture of 12.5 µL of 2X GoTaq PCR master mix (Promega Co., Madison, Wisconsin, USA), 0.5 µL of 0.1mg/mL bovine serum albumin, 1.0 µL each of 10 µM forward and reverse primer, 7.5 µL of sterile distilled water, and 2.5 µL of DNA template (10—60 ng). PCR reactions were carried out with an initial melting period at 94°C (5 min.), followed by 35 cycles of 94°C (30 sec.), annealing at 55-60°C (30 sec.), and extension at 72°C (1 min.), with a final extension at 72°C (7 min.). Amplification success was confirmed by electrophoresis using a 1.5% sodium boric acid-based agarose gel.

Markers were selected for Sanger sequencing based on their producing a single band across all samples and for a maximum size of ~1000bp. PCR products were treated with ExoSAP-IT PCR cleanup enzyme mix (Affymetrix Inc., Santa Clara, California, USA) before cycle sequencing with BigDye Terminator v3.1 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The labeled sequencing fragment were read on an ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific Inc.) and the resulting chromatograms were edited and analyzed using Geneious (Kearse et al., 2012).

RESULTS

Initial screening of primers showed that all amplify in at least some of the eastern North American taxa. Gel electrophoresis revealed that IBR3_1 and Transducin_2 are too long (~2000bp) while Transducin_1 has short and long copies in some individuals (~500bp and ~1000bp), making these poor candidates for a Sanger sequencing approach without needing molecular cloning or gel extraction. While *gap*C_short readily amplified, it is contained within *gap*C_long, making sequencing of the shorter fragment redundant. *pgi*C, *IBR3*_2 (hereafter

IBR3), and *gap*C_long (hereafter *gap*C) were selected for PCR and sequencing of the full taxa list (Table 5).

TABLE 5. Amplification and sequence quality of markers across taxa

Taxon	Amplification			Sequencing		
	pgiC	*IBR3*	*gapC*	*pgiC*	*IBR3*	*gapC*
Isoëtes butleri	+	+	+	+	+	+
I. echinospora	+	+	+	+	+	+
I. engelmannii	+	+	+	+	+	—
I. flaccida var. *chapmanii*	+	+	+	+	+	+
I. flaccida	+	+	+	+	+	+
I. histrix	+	—	—	+	NA	NA
I. lithophila	+	+	+	+	+	+
I. longissima	+	+	+	+	+	+
I. melanopoda	+	+	+	+	+	+
I. melanopoda ssp. *silvatica*	+	+	+	+	+	+
I. melanospora	+	+	+	+	+	+
I. nuttallii	+	—	—	+	NA	NA
I. piedmontana	+	+	+	+	+	+
I. storkii	+	+	+	+	+	+
I. tegetiformans	+	+	+	+	+	+
I. valida	+	+	+	+	+	—
Lycopodium clavatum	+	—	+	+	NA	—

Note: + = successful amplification or sequence quality > 85%; — = no amplification or sequence quality < 85%; NA = sequencing not attempted.

pgiC

This primer pair is rooted in exons 14 and 16, and amplifies across introns 14, 15, and exon 15 of this locus (Rothfels et al., 2013). The region amplified easily across all taxa of *Isoëtes* and *Lycopodium clavatum and* generated consistently high-quality sequence data. All sequences

aligned well, with a total alignment length of 466 bp and pairwise identity of 83%. Excluding *L. clavatum*, alignment length decreases to 357 bp and pairwise identity increases to 89%. Sequence length between these species of *Isoëtes* ranges from 310 to 347 bp, with a mean of 324 bp (Table 6). This is approximately half the length of the same region in ferns tested by Rothfels et al. (2013).

gapC

*gap*C encodes cytosolic glyceraldehyde-3-phosphate and is part of the GAPDH gene family (Strand et al., 1997; Wall, 2002; Szövényi et al., 2006). Primers designed by Szövényi et al. (2006) are rooted in exons 5 and 9 and amplify all exons and introns in between. However, given concern that the resulting marker in *Isoëtes* may be too long for Sanger sequencing, the primers designed for this study were rooted in exons 5 and 8, amplifying introns 5, 6, 7, and exons 6 and 7.

This marker showed the least ability to routinely generate high quality sequence data. Though not detected in any of the transcriptomes available, it is possible this results from off-target amplification of other members of the GADPH gene family (i.e. *gap*Cp or an unnamed *gap*C/*gap*Cp relative) (Schuettpelz et al., 2008; Rothfels et al., 2013). The *Isoëtes*-only alignment is 561 bp and has a pairwise identity of 85% (Table 6).

TABLE 6. Alignment statistics for all sequences with quality scores >85%.

	Isoëtes					***Lycopodium* + *Isoëtes***				
Marker	Amplicon Length Range (Mean)	Alignment Length	Pairwise % Identity	Identical Sites (%)	PIS (%)	Amplicon Length Range (Mean)	Alignment Length	Pairwise % Identity	Identical Sites (%)	PIS (%)
pgiC	310—347 (324)	357	89	240 (67)	80 (22)	310—458 (331)	466	83	192 (41)	82 (18)
IBR3	587—682 (659)	700	87	415 (59)	111 (16)	—	—	—	—	—
gapC	443—543 (507)	561	85	304 (54)	95 (17)	—	—	—	—	—

Note: PIS = Parsimony Informative Sites

IBR3

Unlike *pgi*C and *gap*C, this marker does not have an extensive history of use as a phylogenetic marker. This gene is thought to encode an indole-3-butyric acid-specific peroxisomal enzyme related to acyl-CoA dehydrogenases (Zolman et al., 2007). Rothfels et al. (2013) showed it to be single-copy throughout selected fern lineages, and this also appears to be the case in *Isoëtes*. Primers from *IBR3* amplify most species of *Isoëtes* easily, with the exception of two members of the Mediterranean clade (*I. histrix* and *I. nuttallii*). Alignment of *Isoëtes* sequences is 700 bp long with 87% pairwise identity (TABLE 6).

DISCUSSION

Transcriptome-mining is shown to be a useful tool for identification of putative low-copy markers for primer design. Despite having access to transcriptomes of just three species of *Isoëtes* in the North American clade, primers could be designed for regions that show phylogenetic signal across widely divergent clades in the genus, and potentially across all Lycopodiophyta. Although techniques such as target enrichment allow for generation of datasets orders of magnitude larger (Mandel et al., 2014), design of primers for Sanger sequencing is still more time- and cost- efficient in taxonomic groups where just a few markers may be needed to infer well-resolved phylogenies.

CHAPTER 4

A WHOLE CHLOROPLAST GENOME PHYLOGENY OF DIPLOID SPECIES OF *ISOËTES* (ISOËTACEAE, LYCOPODIOPHYTA) IN THE SOUTHEASTERN UNITED STATES

INTRODUCTION

Isoëtes (Isoëtaceae, Lycopodiophyta) is a cosmopolitan genus of ca. 200 described species (Troia et al., 2016). Their common names, "quillwort" and "Merlin's grass", originate from their morphology, generally consisting of many linear, sub-terete sporophylls (leaves) borne on a subterranean rootstock. All species of *Isoëtes* reproduce through production of mega- and microspores in sporangia on separate sporophylls (occasionally separate plants, e.g. *I. butleri*).

The evolutionary history of *Isoëtes* is notoriously difficult to infer. Early attempts were hampered by the dearth of variable character states between species and the phenotypic plasticity that occurs within some characters (Hickey, 1986; Taylor and Hickey, 1992). Some sub-generic classifications were proposed such as subgenus *Euphyllum*, characterized by alate leaves, and subgenus *Isoëtes*, comprised of species with non-alate leaves (Hickey, 1990). Within subgenus *Isoëtes*, sections *Coromandelina* and *Isoëtes* were proposed (Taylor and Hickey, 1992). Despite fairly strong morphological phylogenetic hypotheses, none of these subgeneric classifications were supported by molecular data (Hoot and Taylor, 2001; Rydin and Wikström, 2002; Hoot et al., 2006; Schuettpelz and Hoot, 2006; Larsén and Rydin, 2016). This suggests numerous changes

and reversions in character states and habitat preference, hindering any phylogenetic inference based on non-molecular data (Taylor and Hickey, 1992).

Among molecular phylogenies of species in the genus, the clade containing taxa of the southeastern United States is consistently difficult to resolve (Hoot and Taylor, 2001; Hoot et al., 2006; Larsén and Rydin, 2016). Currently ca. 25 taxa are recognized in the region comprising Delaware, Maryland, Virginia, West Virginia, Kentucky, Tennessee, North Carolina, South Carolina, Georgia, Florida, Alabama, Mississippi, Arkansas, Louisiana, Oklahoma, and Texas (Brunton, 2015). Of these, 15 are thought to be basic diploids, hypothesized to have evolved through vicariance. The remaining taxa are hybrids and allopolyploids ranging in ploidy level from diploid to octoploid (Brunton, 2015). One of the shortcomings of previous phylogenies is the use of few markers with low phylogenetic signal sufficient to discriminate among the southeastern US species. Common markers, plastid *rbcL* and the *atpB-rbcL* spacer, as well as nuclear ribosomal ITS, cannot resolve relationships within the southeastern American clade (Rydin and Wikström, 2002; Hoot et al., 2006; Larsén and Rydin, 2016).

The plastid genome (plastome) has long been a source of phylogenetic markers for plant systematics. Studies have employed coding and noncoding regions of the plastome to evaluate the relationships from the infraspecific level to the backbone of all multicellular plants (Shaw et al., 2005, 2007, 2014). With a high selective pressure on the photometabolic genes encoded by the plastome, its nucleotide sequence and structure are relatively slowly evolving (Wicke and Schneeweiss, 2015). Protein coding regions show greater conservation than non-coding introns and intergenic spacers, but rates of mutation vary across these categories and across lineages (Wicke and Schneeweiss, 2015). Therefore, typical studies that use few (≤6) chloroplast markers often cannot resolve low level phylogenies (Shaw et al., 2014). With the increasing ease of

whole plastome assembly from next generation sequencing data, however, phylogenomic studies can generate robust phylogenies at all taxonomic levels (Wicke and Schneeweiss, 2015).

To infer the phylogeny of *Isoëtes* in the southeastern United States, we employ a diploids-first approach, wherein a phylogeny of the basic diploid taxa provides a framework to infer the parentage of hybrids and allopolyploids (Beck et al., 2010; Burgess et al., 2015). Here we present a phylogeny of southeastern diploid *Isoëtes* based on plastome data.

METHODS

Sample Collection

Plants were collected from type localities whenever possible (Table 7). If a taxon was no longer extant at its type locality, another representative population was selected. For two species, *I. texana* and *I. mattaponica*, material for DNA extraction could not be obtained. For most species, approximately five sporophylls from a single plant were stored in silica gel for DNA extraction. *Isoëtes tegetiformans* and *I. melanospora* were too small to acquire enough material from a single plant, so sporophylls from several plants in the same population were pooled. *Isoëtes nuttallii* was selected as the best outgroup from material available for DNA extraction due to its placement well outside the "American" clade in previous molecular phylogenies (Larsén and Rydin 2016, Hoot et al. 2006), despite its actual occurrence in western North America. Voucher specimens were deposited in the Old Dominion University (ODU) and US National (US) herbaria.

TABLE 7. List of taxa and specimens included in chapter 4.

Taxon	Voucher (Herbarium)	Locality	Date	GenBank Accession
I. butleri Engelm.	Schafran 47 (ODU)	Ft. Worth Nature Center, TX	18 April 2015	MG668891
I. echinospora Durieu	Schafran 32 (ODU)	Cleveland Lake, Lewis Co., NY	16 July 2014	MG668903
I. engelmannii A. Braun	Schafran 46 (ODU)	Hiawassee River, Reliance, TN	5 April 2015	MG668892
I. flaccida Shuttlew.	Taylor 6770 (US)	St. Marks River, Newport, FL	26 Jan. 2013	MG668893
I. flaccida var. *chapmanii* Engelm.	Bolin JB_FL_01 (ODU)	Chipola River, Marianna, FL	13 Sept. 2009	MG599108
I. lithophila N. Pfeiff.	Schafran 61 (ODU)	Enchanted Rock, Llano Co., TX	20 April 2015	MG668894
I. melanopoda J. Gay & Durieu	Taylor 6940 (US)	Giant City State Park, Jackson Co., IL	26 April 2015	MG668895
I. melanopoda ssp. *silvatica* Brunton and Britton	Schafran NC-05 (ODU)	Mecklenburg Co., NC	10 March 2013	MG668896
I. melanospora Engelm.	Schafran 12 (ODU)	Summit of Stone Mountain, GA	15 May 2014	MG668897
I. mississippiensis S.W. Leonard, W.C. Taylor, Musselman, and R.D. Bray	Schafran MS-08 (US)	Lotts Creek, Picayune, MS	18 June 2013	MG668898
I. nuttallii A. Braun ex Engelm.	Taylor 6734 (US)	Vernal Fall, Mariposa Co., CA	14 June 2012	MG668899
I. piedmontana (N. Pfeiff.) C.F. Reed	Schafran 18 (ODU)	Heggie's Rock, Appling, GA	17 May 2014	MG668900
I. tegetiformans Rury	Schafran 19 (ODU)	Heggie's Rock, Appling, GA	17 May 2014	MG668901
I. valida (Engelm.) Clute	Schafran 37 (ODU)	Michaux State Forest, Cumberland Co., PA	1 Nov. 2014	MG668902

DNA Extraction, Library Preparation, and Sequencing

Total genomic DNA (gDNA) was extracted from approximately 200 mg of silica-dried leaf tissue using either the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, California) or the Gene Prep instrument (Autogen Inc., Holliston, Massachusetts) using the manufacturers' instructions. gDNA was quantified with a Qubit 2.0 fluorometer (ThermoFisher Scientific, Waltham, Massachusetts) and quality assessed by measuring the 260 nm:280 nm absorption ratio with an Epoch spectrophotometer (BioTek Instruments Inc., Winooski, Vermont). For each sample an aliquot of 300 ng of gDNA was sheared into approximately 500 bp fragments with a Q800R2 sonicator (Qsonica LLC, Newtown, Connecticut). Fragmentation to this size range was confirmed by separating sonicated DNAs on a 2% agarose gel by electrophoresis. Fragmented gDNA was cleaned using Agencourt AMPure XP beads (Beckman Coulter Inc., Brea, California) to size select for approximately 500 bp fragments.

Fragmented gDNA was prepared for sequencing on a MiSeq sequencer (Illumina Inc., San Diego, California) using the NEBNext Ultra DNA Library Prep Kit and Multiplex Oligos for Illumina (New England Biolabs Inc., Ipswich, Massachusetts). End repair, adaptor ligation, indexing for multiplexing, and PCR enrichment were done according to the manufacturers' instructions as follows.

End Repair

For each sample, 55.5 µL fragmented gDNA (initial concentration 3 ng/µL) was combined with 3.0 µL End Prep Enzyme Mix and 6.5 µL End Repair Reaction Buffer (10X). The reagents were thoroughly pipette-mixed and incubated on a thermocycler at 20°C for 30 minutes, followed by 65°C for 30 minutes.

Adaptor Ligation

To the End Prep reaction mixture, 15 µL Blunt/TA Ligase Master Mix, 1.0 µL Ligation Enhancer, and 2.5 µL NEBNext Adaptor for Illumina were added and pipette-mixed well. The mixture was incubated on a thermocycler at 20°C for 15 minutes. 3.0 µL USER Enzyme was added, pipette-mixed, and returned to a thermocycler at 37°C for 15 minutes. The reaction mixture was cleaned using AMPure XP beads at a 1:1 ratio by volume, with a final elution volume of 15 µL. DNA concentration was measured by Qubit.

PCR Enrichment

25 µL NEBNext Q5 HotStart HiFi PCR Master Mix, 15 µL Adaptor Ligated DNA fragments, 5 µL Index Primer (unique per sample), and 5 µL Universal Primer were combined and thoroughly pipette-mixed. PCR was performed using 1 denaturation cycle at 98°C for 30 seconds, followed by 8 cycles of denaturation at 98°C for 10 seconds and annealing/extension at 65°C for 75 seconds. A final extension cycle was performed at 65°C for 5 minutes. PCR products were cleaned using AMPure XP beads at a 1:1 ratio by volume, with a final elution volume of 33 µL.

Appropriate length and quantity of the libraries was confirmed with an Agilent 2200 Tapestation (Agilent Technologies, Santa Clara, California). Libraries were diluted or concentrated to 4 nM and 5 µL of each was added into one pool. A BluePippin instrument (Sage Science Inc., Beverly, Massachusetts) was used to size select for 400 – 550 bp fragments. The size selected 4 nM library pool then was submitted to the Smithsonian Laboratories of Analytical Biology sequencing facility.

Data Processing and Chloroplast Genome Assembly

Sequencing reads were downloaded from the Illumina BaseSpace database, having already been separated by primer indices into individual samples. A custom Python wrapper script was used to remove adaptor contamination and low-quality bases with Trimmomatic 0.33 (Bolger et al., 2014), and to combine paired-end reads with PEAR 0.9.6 (Zhang et al. 2014). Putative chloroplast reads were extracted by comparing all reads to a reference plastome of *I. flaccida* (Karol et al., 2010) using Bowtie2 (Langmead and Salzberg, 2012). The putative chloroplast reads were reference-assembled to the *I. flaccida* plastome with the reference assembler in Geneious R10 (Biomatters Ltd., Auckland, New Zealand). Reads in the reference-based assembly were manually corrected around repeat regions. In addition, the putative chloroplast reads were *de novo* assembled using SPAdes 3.6.0 with k-mer lengths of 21, 33, 55, 77, 99, and 127 bp (Bankevich et al., 2012). Scaffolds from the *de novo* assembly were aligned to the *I. flaccida* plastome, and the consensus sequence was compared to the reference-based assembly. Any disagreements between assemblies, generally in repeat regions, were either manually corrected based on the raw data, or excluded from phylogenetic analysis. Plastome assemblies were annotated by comparison with the annotated *I. flaccida* plastome using Geneious with a cutoff of 90% similarity.

Phylogenetic Analyses

MAFFT 7 (Katoh and Standley, 2013) was used to align all the consensus plastome assemblies. An optimal evolutionary model for each alignment was selected with PAUP* v.4 (Swofford 2002) based on corrected Akaike information criterion (AICc) values. Less optimal models and rate variations were also evaluated to determine the effect on tree topology. MrBayes

3.2.6 (Huelsenbeck and Ronquist, 2001) and RAxML 7.3.0 (Stamatakis 2006) were used for phylogenetic analysis. Gaps in the alignment were treated as missing data. Parsimony analysis was performed in PAUP*. As was done for total plastome alignments, gene introns and protein coding sequences were extracted separately using Geneious and those alignments were analyzed with MrBayes.

Using the automated model selection implemented in PAUP*, the generalized time reversible model (GTR; Tavaré, 1986) of evolution was selected for whole plastome, coding sequence, and intron alignments including *I. nuttallii* (alignments without the outgroup were not analyzed). For the whole plastome, rate variation was best modeled using gamma distributed rate variation and a proportion of invariable sites (GTR+I+G), while the optimal rate variation for the coding sequence and intron alignments included only the proportion of invariable sites parameter (GTR+I). In addition, other iterations of two common evolutionary models, Jukes-Cantor (JC; Jukes and Cantor, 1969) and Hasegawa-Kishino-Yano (HKY; Hasegawa et al., 1985) and rate variations equal, proportion of invariable sites alone(+I), and gamma distributed sites (+G) were applied to all alignments to test the sensitivity of tree topology to model selection. Coding sequence and intron alignments were analyzed both as concatenated and partitioned matrices.

All MrBayes analyses were run for 50 million generations, sampling every 1000 generations, with 1 cold chain and 3 heated chains. Runs were assumed to be converged when split standard deviations were less than 0.001, and potential scale reduction factors equaled 1.000 +/- 0.001. MCMC output was visualized in Tracer 1.6 to check for problems with run convergence (Rambaut et al., 2014). All trees were rooted with *I. nuttallii*.

Megaspore and microspore ornamentation type, megaspore color, and seasonality of spore maturation were selected as taxonomically informative characters and character states were

mapped to the phylogeny for each taxon. Ancestral character states were inferred using parsimony in Mesquite 3.31 (Maddison and Maddison, 2017). All characters were treated as categorical and unordered.

RESULTS

Assembly and Alignment

Following filtering of low-quality reads and merging of paired-end reads, the total number of reads per sample ranged from 310,119 to 2,058,639 (mean=1,345,289). Of the putative chloroplast reads that passed reference-based filtering with Bowtie2, there was a range from 7479 to 89,260 reads (mean=43,527). The efficiency of genome skimming to collect chloroplast data is variable, with the percent of putative chloroplast reads varying from 0.94% to 8.65% of total reads, with a mean of 3.45%. This may represent inherent variation in the number of chloroplasts present in the collected plant tissues. This does not consider any variation of potential mitochondrial contamination that can pass through this filtering process. Between 6 and 22 scaffolds were constructed for each sample during *de novo* assembly, with a median of 12.5 scaffolds. N50 values ranged from 14,545 to 91,749 bp (median=26,025.5) and the sum length of scaffolds varied from 132,080 to 155,618 bp (median=138,690.5).

The final plastome assemblies displayed very little variation in size. Unaligned plastome length ranged from 144,680 to 145,294 bp (mean=145,102.8) with a percent standard deviation of 0.1%. The alignment of entire plastome sequences for thirteen southeastern *Isoëtes* diploids plus *I. nuttallii* yielded a matrix of 147,946 sites, of which 10,218 (6.9%) were variable (Table 8). Excluding *I. nuttallii*, the alignment length decreases to 146,006 sites and the proportion of variable sites decreases to 3,129 (2.0%). Pairwise identity between whole plastome alignments

with and without the outgroup were 98.8% and 99.6%, respectively, and the percentages of parsimony informative sites were 0.21% and 0.19%, respectively.

Alignments of all 80 coding regions showed similar lengths and pairwise identities, 69,476 characters and 99.6% including *I. nuttallii*, and 69,372 characters and 99.8% excluding it (Table 8). The number of variable and parsimony informative sites decreased by excluding *I. nuttallii*, from 2.7% to 1.3% variable sites and 0.18% to 0.16% parsimony informative sites.

Non-coding intron alignments were 16,425 (with outgroup) and 15,986 characters (without outgroup) long, consisting of 20 separate regions. The regions showed the highest proportions of variable and parsimony informative sites, 7.1% and 0.22%, respectively, including *I. nuttallii*. Excluding *I. nuttallii*, there were 2.3% variable sites and 0.19% parsimony informative sites. Intron alignments also displayed the lowest pairwise identities, 98.8% with *I. nuttallii*, and 99.5% without.

TABLE 8. Alignment statistics with (and without) the outgroup *I. nuttallii.*

	Total Length (characters)	**Variable Sites (%)**	**Parsimony Informative Sites (%)**	**Pairwise Identity (%)**	**Optimal Evolutionary Model**
Whole Plastome	147946 (146006)	6.9 (2.0)	0.21 (0.19)	98.8 (99.6)	GTR+I+G
Coding Sequences	69476 (69372)	2.7 (1.3)	0.18 (0.16)	99.6 (99.8)	GTR+I
Introns	16425 (15986)	7.1 (2.3)	0.22 (0.19)	98.8 (99.5)	GTR+I

Whole Plastome Phylogeny

Across all model and rate iterations tested (GTR+I+G, GTR+I, GTR+G, GTR, HKY+I+G, HKY+I, HKY+G, HKY, JC+I+G, JC+I, JC+G, JC) there was no change in tree topology and little change in posterior probabilities (data not shown). Bayesian, maximum likelihood (ML), and parsimony-based trees shared the same topology. Support for several nodes was lower in ML compared with Bayesian analyses, although most were still >90 (Figure 8). The basal node of clade A, while well supported in the Bayesian tree, is significantly weaker (bootstrap value of 69%) in the ML tree (Figure 8). Henceforth, all references will be to the Bayesian phylogeny inferred under the GTR+I+G model. All nodes were well supported, with posterior probabilities of 100. Two major clades (Figure 9, clades A and B) are evident at the deepest node of the tree. Clade A consisted of *I. melanospora, I. engelmannii, I. melanopoda* ssp. *silvatica, I. tegetiformans*, and *I. piedmontana*, while *I. butleri, I. mississippiensis, I. flaccida* var. *chapmanii, I. melanopoda* ssp. *melanopoda, I. lithophila, I. flaccida* var. *flaccida*, *I. echinospora,* and *I. valida* comprised clade B. Within clade B were two sister groups, clade C (*I. mississippiensis, I. flaccida var. chapmanii, I. melanopoda* ssp. *melanopoda*) and clade D (*I. lithophila, I. flaccida var. flaccida, I. echinospora, I. valida*). Branch lengths ranged from 3.22e-4 to 5.65e-5 substitutions per site.

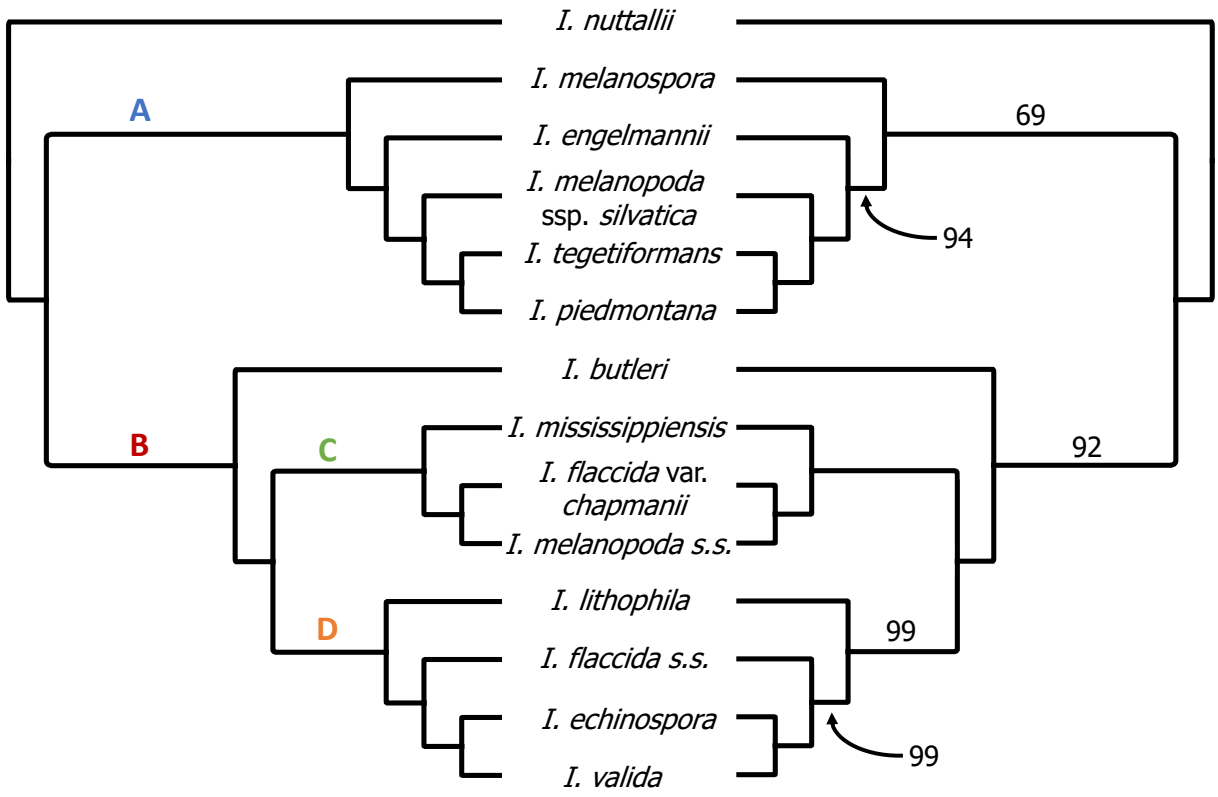


FIGURE 8. Bayesian (left) and maximum-likelihood (right) cladograms of the whole plastome alignment. All nodes were supported by posterior probabilities or bootstrap values of 100 except where noted. Letters label major clades.

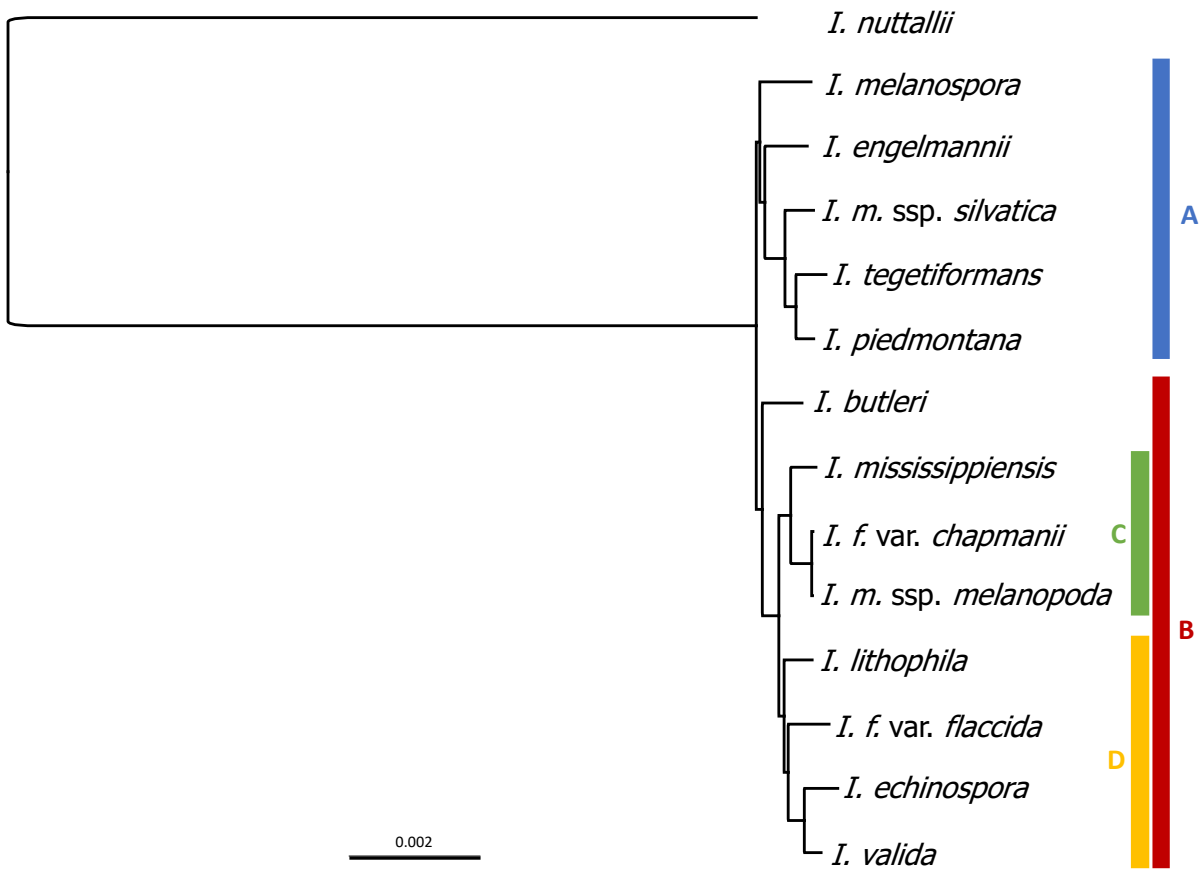


FIGURE 9. Bayesian phylogram of the whole plastome alignment. All nodes were supported with posterior probabilities of 100. Colored bars/letters represent major clades. Scale bar represents substitutions per site.

Protein Coding Sequence and Intron Phylogenies

In general, the phylogenies inferred from coding sequence and intron alignments separately supported the whole plastome phylogeny, though topologies and support values varied. Most of the relationships within clades A, B, C, and D were supported by both datasets. The placement of *I. butleri* or *I. melanospora* as the most basal branch was weakly supported by the coding sequence and intron phylogenies, respectively (Figure 10). Both of these topologies

conflicted with that of the whole plastome, where neither species was sister to the remaining taxa (Figure 9). Except for the placement of *I. melanospora*, the remainder of clade A was supported by both coding sequences and introns. Likewise, clade B was supported by these data, except for the placement of *I. butleri*. Clade C was well supported, with no conflicts in topology among any analysis. Clade D was mostly well supported, but the intron data produced a polytomy at its backbone, rather than placing *I. lithophila* as the most basal branch of the clade (Figure 10).

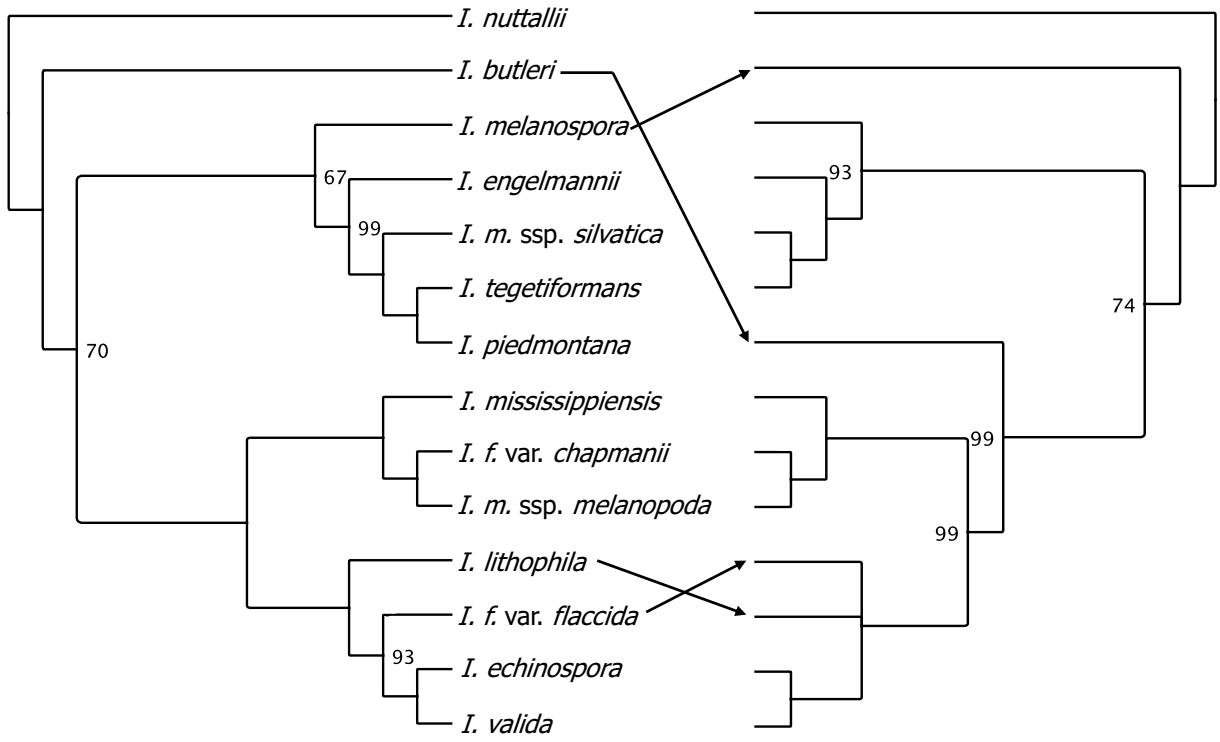


FIGURE 10. Bayesian cladograms based on coding sequence (left) and intron (right) alignments with arrows indicating changes in topology. All nodes were supported by posterior probabilities of 100 except where noted.

Character State Reconstruction

Within clade A, spores that mature in the spring before plants enter their summer desiccation-dormancy is common to several taxa: *I. melanopoda* ssp. *silvatica*, *I. melanospora, I. tegetiformans*, and *I. piedmontana* (Figure 11). Only individuals of *I. engelmannii* persist through the summer to produce their spores in the fall. Megaspore ornamentation is fairly consistent among all members of this clade. All taxa have tuberculate megaspores (occasionally pseudo-reticulate to cristate in *I. piedmontana*), except *I. engelmannii* which has strongly reticulate megaspores. Gray-black megaspore coloration occurs in *I. melanospora* and *I. tegetiformans,* while all other have taxa are white megaspores.

In clade B, most taxa (*I. flaccida* var. *chapmanii, I. flaccida* var. *flaccida, I. echinospora,* and *I. valida*) have spores that mature in late summer or autumn. *Isoëtes butleri, I. melanopoda* ssp. *melanopoda*, *I. mississippiensis*, and *I. lithophila* have spores that mature in late spring. Most taxa in this clade have spinulose microspores, except for *I. butleri*, which has aculeate microspores, and *I. flaccida s.l.*, which has papillose microspores. Most taxa in clade B have tuberculate megaspores. Only *I. echinospora* and *I. valida* differ, with echinate and reticulate-cristate ornamentation, respectively. *Isoëtes lithophila* is the only taxon in this clade with gray-black megaspore coloration.

Ancestral character state reconstruction places white, tuberculate megaspore ornamentation, papillose microspore ornamentation, and springtime maturation at the basal node of the southeastern clade (Figure 11). Of eleven nodes within clades A and B, tuberculate megaspore ornamentation is inferred at ten (one has more than one most parsimonious state) and white coloration is found at eleven nodes. Microspore ornamentation is inferred to be spinulose at seven nodes, while two nodes have papillose ornamentation (two have multiple most

parsimonious states). A phenology of spores maturing in spring is the most parsimonious state at nine nodes, with two nodes having autumn-maturing spores.

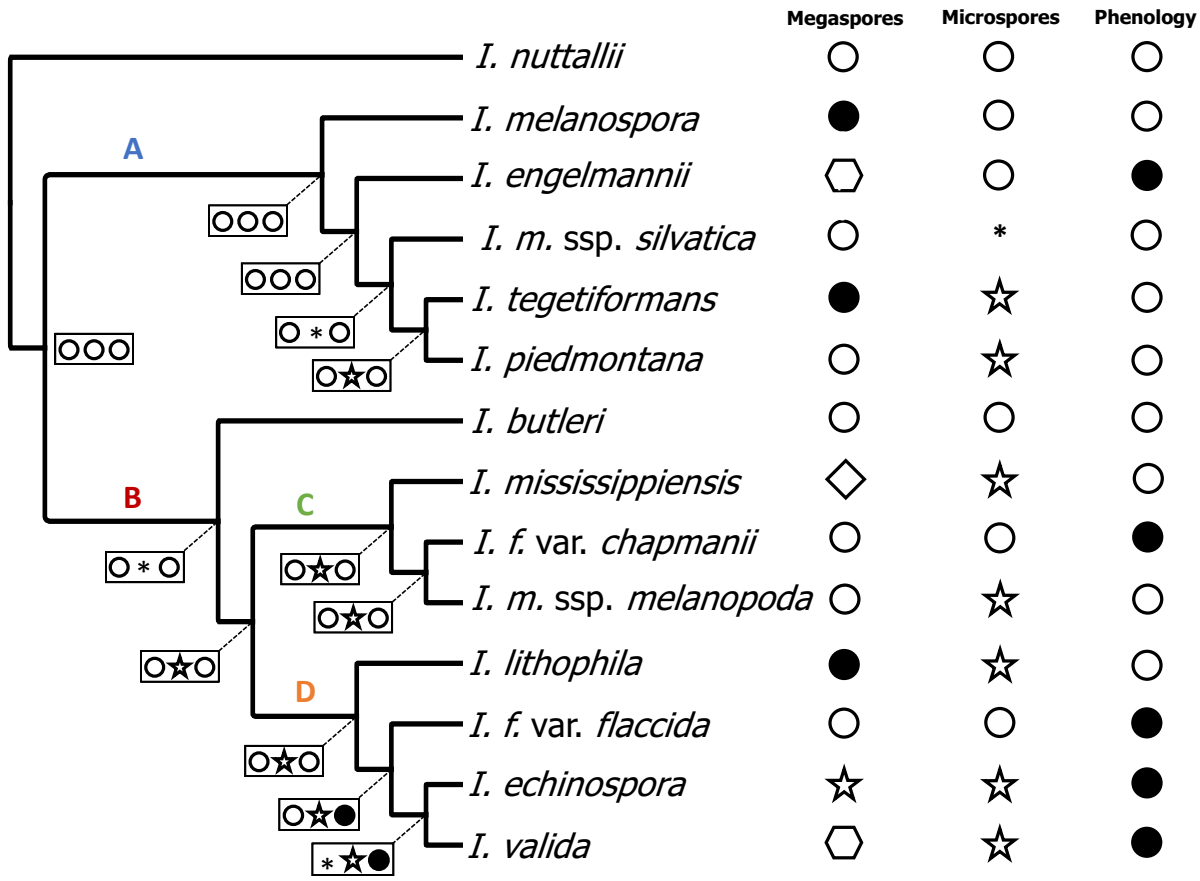


FIGURE 11. Bayesian whole plastome cladogram with character states. Megaspore key: circle = tuberculate; hexagon = reticulate; star = echinate; diamond = laevigate; open = white spores; filled = gray-black spores. Microspore key: circle = papillose; star = spinulose; triangle = aculeate. Phenology key: open = spores mature in spring; filled = spores mature in summer-autumn. * = character state unknown or more than one most parsimonious state. Node labels follow the order of the table.

DISCUSSION

Our analyses indicate that plastome DNA sequences are useful for resolving species relationships in closely related groups of *Isoëtes*. Comparison of phylogenies suggests that for the same dataset, evolutionary model selection has little effect on resulting tree topology and support. Algorithm selection (i.e. MrBayes vs. RAxML) appears to have little effect on topology but can result in different levels of support. Comparison of coding sequence and intron-based phylogenies suggests that different topologies may be inferred depending on marker selection. This should serve as a cautionary note for other phylogenetic studies of *Isoëtes* based on relatively short markers, as selection for a certain type of marker region may influence the resulting phylogeny, especially among closely related taxa.

The phylogenetic relationships and inference of ancestral character states supports the hypothesis that most of the extant morphological diversity in southeastern *Isoëtes* is relatively recent, and many similar traits are the result of convergence rather than descent (Hickey, 1986; Taylor and Hickey, 1992). Parsimony indicates that eleven of fourteen character state transitions in the southeastern clade occurred on the terminal branches of the tree. Only three are inferred more deeply in the phylogeny: spinulose microspore ornamentation uniting the clades containing *I. tegetiformans* and *I. piedmontana* (potentially including *I. melanopoda* ssp. *silvatica*) as well as clades C and D (potentially all of clade B), and autumn phenology uniting *I. flaccida* var. *flaccida*, *I. echinospora*, and *I. valida*. These results suggest that species groupings based on these features (e.g. Pfeiffer, 1922) do not accurately represent evolution in the genus. Further work should examine these apparently labile characters and what roles they may play in *Isoëtes* speciation, especially where species are adapted to particular environmental conditions (Taylor et al., 1993).

While a complete analysis of the evolution of the plastome in southeastern *Isoëtes* is not presented here, we note that all genes and transfer RNA and ribosomal RNA coding regions are retained in all taxa. Almost all taxa display numerous autapomorphic sites as inferred from branch lengths, and the number of site differences between pairwise taxa (excluding *I. nuttallii*) is generally several hundred (mean=572; median=607; Figure 12). One exception occurs between *I. flaccida* var. *chapmanii* and *I. melanopoda* ssp. *melanopoda*, which have only 15 site differences – approximately the same variation observed between two individuals of *I. flaccida* var. *flaccida*. This does not appear to be a misidentification, as nuclear DNA sequences from the same individuals of *I. flaccida* var. *chapmanii* and *I. melanopoda* ssp. *melanopoda* do not indicate a close relationship (see Chapter 5). Instead, this may represent a hybrid origin of the chloroplast within *I. flaccida* var. *chapmanii* through chloroplast capture. A few structural differences appear in the *I. nuttallii* plastome relative to the southeastern US taxa. Two small inversions are present, one in the *atpB*-*rbcL* spacer that is 344 bp long, with the other in the *trnK*-UUU-*psbA* spacer that is 24 bp long. In addition, the entire *atpF* intron has been lost in *I. nuttallii*. This does not appear to be a problem of assembly, as sequence reads clearly span the site of the intron.

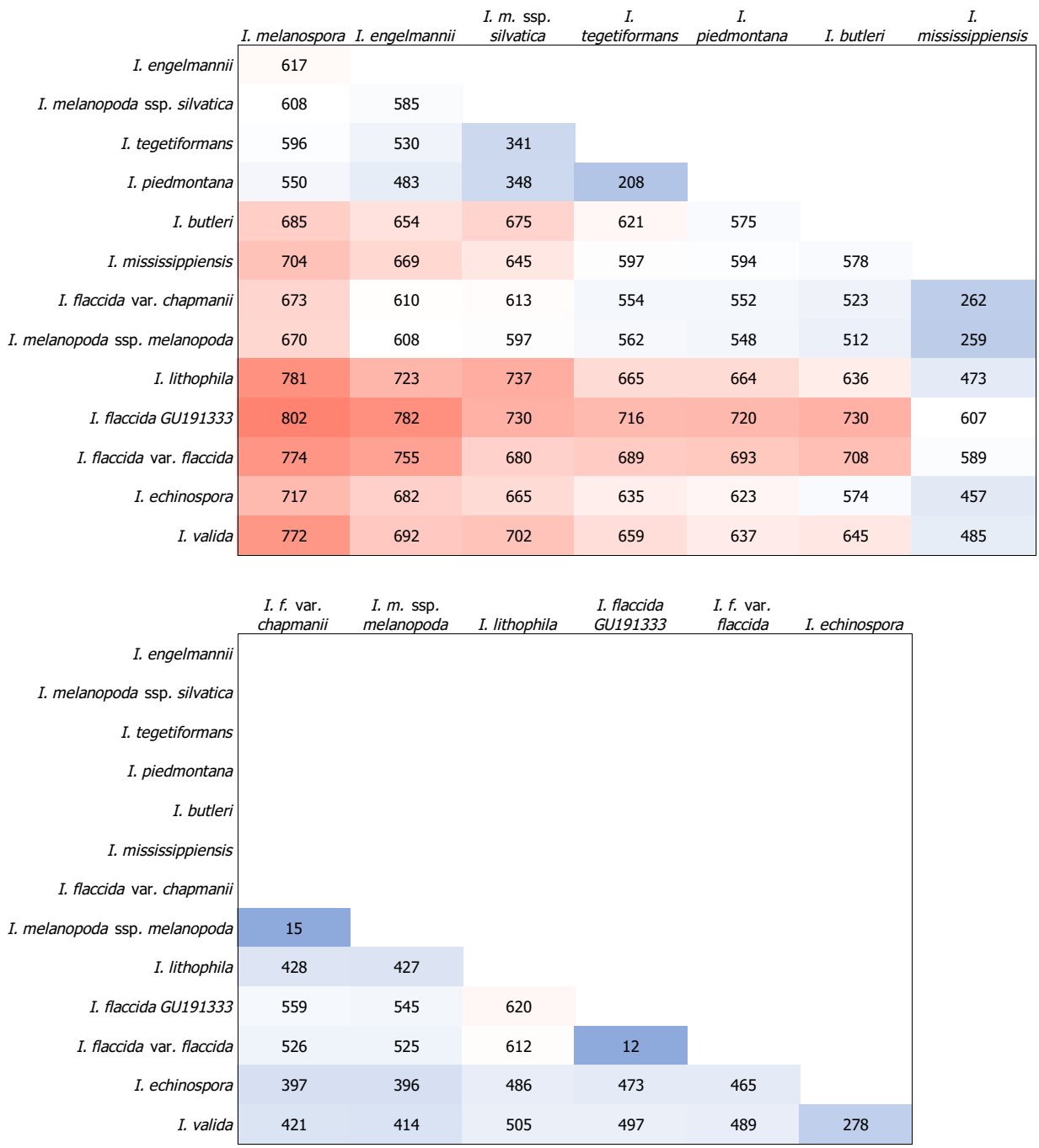


FIGURE 12. Heat map of number of pairwise differences from the whole plastome alignment, excluding *I. nuttallii.* Includes the reference *I. flaccida* (GU191333) from Karol et al. (2010). Colors scaled from blue (minimum) to white (median) to red (maximum).

While this study represents a significant advancement in our understanding of the phylogeny of *Isoëtes* in the southeastern US, it also highlights many areas needing further research. The plastome represents only one evolutionary lineage and may conflict with nuclear and mitochondrial genomes, causing incongruence between genome phylogenies and species phylogenies. Preliminary nuclear data from *Isoëtes* of the Southeast indicate different species phylogenies are inferred from different genomes (unpublished data). The inheritance and evolution of plastomes, often represented as uniparentally-inherited and non-recombinational, can be complicated by heteroplasmy, recombination, and intracellular horizontal gene transfer in many plant taxa (Wolfe and Randle, 2004; Scarcelli et al., 2016). More complex models of molecular evolution are needed to compensate for intra-individual and intra-species variation in plastomes (Wicke and Schneeweiss, 2015). The level of genetic variation between populations of *Isoëtes* and the vectors by which populations interbreed are very poorly known for most species. In terrestrial habitats with no obvious spore dispersal mechanisms, populations of *Isoëtes* are assumed to be reproductively isolated (Taylor and Hickey, 1992). In these cases, small population size and genetic drift may be driving speciation. As more is learned about overlooked morphological characters, molecular phylogenies may gain additional support (Freund, 2016; Bray et al., 2018). The addition of unsampled (i.e. *I. texana* and *I. mattaponica*) and potentially unrecognized taxa (e.g. a unique population of *I. melanospora* in South Carolina, Taylor et al., 1993) to this phylogeny may further enhance our understanding of the evolution of diploid *Isoëtes* in the southeastern US. Finally, this study indicates polyphyly of both *I. flaccida s.l.* and *I. melanopoda s.l.*, supporting the raising of *I. flaccida* var. *chapmanii* and *I. melanopoda* ssp. *silvatica* each to the species level, and highlighting the need to update the taxonomy of the genus based on molecular phylogenetic data.

CHAPTER 5

PARENTAGE OF POLYPLOID *ISOËTES* IDENTIFIED USING WHOLE CHLOROPLAST GENOMES AND SINGLE MOLECULE AMPLICON SEQUENCING

INTRODUCTION

Of ca. 30 recognized species of *Isoëtes* in eastern North America, 14 are thought to be allopolyploids derived from hybridization between two parental species and subsequent whole genome duplication of the sterile F_1 hybrid (Taylor et al., 1985; Taylor and Hickey, 1992; Taylor et al., 1993). The hybrid taxa have long been recognized as distinct based on their production of polymorphic, abortive megaspores and generally vigorous appearance (Dodge, 1897; Eaton, 1900). A combination of morphology, chromosome number, *in vitro* crosses, and enzyme electrophoresis data allowed Taylor et al. (1985) to propose that these abnormal individuals are sterile hybrids derived from crosses between fertile species. Using various combinations between diploids and fertile tetraploids, reticulate evolution was hypothesized to explain the origin of fertile species of *Isoëtes* at any polyploid level (Taylor et al., 1985; Taylor and Hickey, 1992).

Possibly the best studied polyploid *Isoëtes* taxon is *I. riparia*. Based on the megapore ornamentation of *I. riparia* (4x) as appearing intermediate between that of *I. echinospora* (2x) and *I. engelmannii* (2x), Taylor et al. (1985) proposed those diploids as the progenitors of the tetraploid. They also found that *I.* × *eatonii* (2x) and *I.* × *gravesii* (2x), while generally producing abortive megaspores, occasionally produced megaspores with similar ornamentation to *I. riparia*. Additionally, *I.* × *eatonii* and *I.* × *gravesii* occur almost exclusively in the region of New England where *I. echinospora* and *I. engelmannii* are sympatric, while *I. riparia* occurs in

this region and adjacent states/provinces where *I. echinospora* and *I. engelmannii* are allopatric (records of *I. riparia* south to North Carolina likely represent another cryptic taxon). These data suggested that *I. riparia* was derived from a genome duplication of *I.* × *eatonii* or *I.* × *gravesii,* which are homoploid hybrids between *I. echinospora* and *I. engelmannii* (Taylor et al., 1985). The hypothesis was supported by *in vitro* crosses with both parentage scenarios of the fertile diploids (i.e. microspores of *I. echinospora* crossed with megaspores of *I. engelmannii,* and *vice versa*) resulting in the production of sporophyte offspring, indicating that diploid hybrids can be created between fertile species (Taylor et al., 1985). Finally, enzyme electrophoresis patterns showed similar banding between *I.* × *eatonii*, *I.* × *gravesii,* and *I. riparia* that were additive between *I. echinospora* and *I. engelmannii* (Taylor et al., 1985; Taylor and Hickey, 1992).

Caplen and Werth (2000a, 2000b) continued studying the *I. riparia* complex through isozyme screening of additional diploid species and tetraploid populations across eastern North America. They found that six more northerly tetraploid populations in Ontario, Quebec, and Maryland matched the Taylor et al. (1985) hypothesis for the formation of *I. riparia*, while ten populations from New Jersey to Georgia suggested other diploid species as parents. *Isoëtes mattaponica* × *I. valida* and *I. mattaponica* × *I. flaccida* were found to be the most likely parents of the other tetraploids, though standardized likelihood scores showed that alternative parentage scenarios were nearly as likely in many populations (Caplen and Werth, 2000b).

DNA sequence data have also been utilized to infer relationships between diploid and polyploid *Isoëtes.* Hoot and Taylor (2001) documented that nuclear ribosomal ITS and intron 2 of a *LEAFY* homolog (hererafter LEAFY) could differentiate between diploid species in eastern North America, and by comparing heterozygous sites in sequences from *I.* × *eatonii* and *I. riparia*, they again found support that *I. echinospora* and *I. engelmannii* are the diploid

progenitors of the diploid hybrid and tetraploid. Expanding on their work with *I. riparia*, Hoot et al. (2004) applied the same techniques to other tetraploid species of *Isoëtes – I. acadiensis, I. appalachiana, I. azorica, I. hyemalis, I. louisianensis, I. maritima, and I. tuckermanii*. Their results identified several new parentage hypotheses, though several invoked unknown diploid progenitors where DNA sequences were dissimilar to any sampled diploid – an approach common in studies of polyploid origins (Sessa *et al.,* 2012; Brassac and Blattner, 2015, Luo *et al.* 2017). Perhaps most importantly, they found evidence that different populations of *I. appalachiana* were derived from different parents, suggesting that morphological identification is insufficient to reconstruct the patterns of reticulate evolution (Hoot et al., 2004).

The recognition of reticulate evolution in *Isoëtes* inspired evaluation of the species concepts applied in the genus. Hickey et al. (1989) reviewed the predominance of the morphological species concept in delineating taxa of *Isoëtes*. Most workers have identified species, subspecies, and varieties based on the uniqueness of a set of characters often incorporating size, shape, color, and ornamentation of spores, size, shape and color of leaves, amount of coverage of the velum over the sporangia, habitat, and cytology. While apparently sufficient in most cases to delineate species under a modern species concept (*sensu* de Quieroz, 2007), the few cases that have explicitly tested phenotypic plasticity in *Isoëtes,* such as leaf length (Boom, 1982) and various morphological features of the plants (Hickey et al., 1989), produced evidence supporting the hypothesis that clinal variation in morphology resulted in oversplitting of species. Despite recognition of the importance of allopatry and allopolyploidy in the "rapid and continuing speciation…that often confuses taxonomic boundaries under the restraints of a morphological species concept" (Hickey et al., 1989), taxonomic work over the following 30 years has largely utilized the morphological species concept (including a ploidy

level component), at best using evidence of evolutionary lineages to support *a priori* morphological distinctiveness (e.g. Rosenthal et al., 2014).

DNA sequences from multiple genomic compartments can be used to infer maternal and paternal parentage of a polyploid. Generally, the chloroplast is assumed to be maternally inherited (e.g. Grusz et al., 2009; Sessa et al., 2012; Sigel et al., 2014; Pereira et al., 2017; Dauphin et al., 2018), though heteroplasmy (Ramsey and Mandel, 2019) or paternal inheritance (Neale and Sederoff, 1989) of the chloroplast occur in some plants. Likewise, mitochondrial genomes are usually maternally inherited, but they are less useful for shallow-level phylogenetics due to their conserved nucleotide sequences in genic regions (Wicke and Schneeweiss, 2015). Single-copy nuclear homeologues provide evidence for multiparental lineages in polyploids. Using either molecular cloning (Hoot et al., 2004) or single-molecule sequencing (Dauphin et al., 2018), nucleotide sequences from the different homeologue copies present in a hybrid or polyploid individual can be generated. By comparing the phylogenetic position of each DNA sequence to those from non-hybrid diploids, parentage can be inferred. Typically, one nuclear homeologue will show a relationship to the same diploid taxon as the chloroplast, which is interpreted to be the maternal lineage. Relationships present only in the nuclear phylogeny are interpreted as paternal lineages (Hoot et al., 2004; Grusz et al., 2009; Sessa et al., 2012; Sigel et al., 2014; Pereira et al., 2017; Dauphin et al., 2018). Occasionally, single sequences or clades with no clear relationship to one diploid species are interpreted as being derived from extinct or unsampled taxa (Hoot et al., 2004; Sessa et al., 2012; Pereira et al., 2019).

To further test the parentage hypotheses of hybrid and polyploid *Isoëtes* in eastern North America, the approaches described above were employed with expanded sampling to incorporate many populations of each taxon, with particular emphasis on the southeastern United States.

METHODS

Sample Collection

Records of hybrid and polyploid *Isoëtes* individuals were retrieved from specimen data available in spring 2017 through the Southeast Regional Network of Expertise and Collections (SERNEC), the Milwaukee Public Museum (MIL), C.V. Starr Virtual Herbarium (NY), New York State Museum (NYS), Global Biodiversity Information Facility (GBIF), and personal collections of A. Cressler, D.F. Brunton, J.F. Bolin, L.J. Musselman, R.D. Bray, S. Leonard, and W.C. Taylor. From May-October 2017, these localities were visited in states between Mississippi to New York, and Ontario and Nova Scotia (Appendix A). Depending on rarity of a taxon, in each population between 0-5 individuals were collected (0 meaning only leaf material was collected), and no more than 10% of individuals in a population were harvested. Leaf tissue was surface cleaned and preserved in silica gel. Whole plants were transported to Catawba College (Salisbury, NC) for genome size measurement by flow cytometry.

DNA Extraction and Sequencing

Due to the young clade age (Larsén and Rydin, 2016; Pereira et al., 2017) and low divergence between chloroplast DNA sequences (Schafran et al., 2018b), a whole chloroplast genome (plastome) was assembled for a representative individual of each taxon, using a topotype collection when possible. DNAs were isolated from 1 cm^2 of dried, macerated leaf tissue using

the Autogen Gene Prep system following the manufacturer's protocol (Autogen Inc., Holliston, MA). Plastomes were generated following methodology in Schafran et al. (2018b). Read depth was used to further filter *de novo* contigs by removing any contigs with coverage more than 3 standard deviations less than maximum coverage for any contig, which represents the dominant plastome haplotype in an individual. Separate consensus sequences were constructed by mapping *de novo* contigs to a reference (*I. flaccida* NC014675), and by mapping putative chloroplast reads to the same reference. Consensus sequences were aligned and examined for disagreement. In general, disagreement occurred in length of dinucleotide repeats and where low coverage resulted misassembly in *de novo* contigs (unpublished data). *De novo* scaffolds were considered more accurate in repeat regions, while mapped reads were preferred in low coverage regions. To fill gaps or ambiguous regions, reads were mapped back to the consensus of combined *de novo* and mapping-based assemblies. Outgroup taxa were selected based on Larsén and Rydin (2016) to represent at least one taxon from each major clade (clades A-D in Larsén and Rydin), and rooted with the clade containing *I. toximontana*, *I. cangae*, and *I. serracarajensis* following Pereira et al. (2017).

Sequences of the LEAFY marker (Hoot and Taylor, 2001; Hoot et al., 2004) were generated using targeted sequencing on the Pacific Biosciences RSII platform (Rothfels et al., 2017; Dauphin et al. 2018). Primer sequences from Hoot and Taylor (2001) were modified by attaching a unique 16 bp nucleotide sequence ("barcode") to the 5' end of the forward and reverse primer sequences. 96 barcode sequences supplied by Pacific Biosciences (retrieved from https://www.pacb.com/products-and-services/analytical-software/multiplexing/) were filtered using the Thermo Fisher Scientific Multiple Primer Analyzer (https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-

biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html) to remove any barcode+primer combinations likely to form primer-dimers. Combining 20 unique forward barcodes and 20 unique reverse barcodes allows for 400 individuals to be sampled in one sequencing run. Barcoded LEAFY amplicons were generated by PCR using a unique barcode pair for each individual. 25 μL PCR reactions were carried out with a combination of 12.5 μL 2× GoTaq Hot Start Master Mix (Promega Co., Madison, WI), 0.5 μL of bovine serum albumin (0.1 mg/mL H_2O), 1.0 μL each of forward/reverse barcoded primers (10 μM in H_2O), approximately 100 ng template DNA (volume various by sample), with the remainder composed of PCR-grade H_2O (generally 2-8 μL). Amplification occurred on an ABI 2720 thermocycler (Thermo Fisher Scientific Inc., Waltham, MA) with an initial melting period of 5 min at 94°C, followed by a touch-down phase of 10 cycles of 94°C for 30 s, annealing at 60°C for 30 s (decreasing 0.5°C each cycle), and extension at 72°C for 1.5 min. The touch-down phase was followed by 20 cycles using the same parameters except for an annealing temperature of 55°C for each cycle. A final extension step of 72°C for 7 min was used. Correct size of the amplicon was confirmed by electrophoresis on a 1.5% sodium boric acid agarose gel with Hi-Lo DNA marker (Bionexus, Oakland, CA). Amplicons were cleaned using KAPA Pure beads (Kapa Biosystems, Wilmington, MA) and eluted into PCR-grade H_2O. DNA quantity and quality were measured using an Epoch microplate spectrophotometer (BioTek Instruments, Winooski, VT). Amplicons were pooled based on DNA quantity and the estimated ploidy level of each plant to target equal coverage for each homeologue copy. Amplicons were sent to Duke University Sequencing and Genomic Technologies (Durham, NC) for sequencing. Sequence files containing circular consensus sequences (CCS) were filtered using Geneious Prime 2019 (http://www.geneious.com/) to

remove all CCS with more than 1% low quality base calls, then processed with the Pipeline for Unraveling Reticulate Complexes (PURC, Rothfels et al., 2017) to demultiplex samples, cluster sequences into operational taxonomic units (OTUs), and generate consensus sequences for each OTU. Based on negative controls and PCR replicates, OTUs that were identified by PURC but were composed of fewer than 10% of the total reads in a sample were assumed to be spurious – the result of sequencing error, PCR error, chimaera formation, or contamination. The threshold of 10% is supported by other projects employing the same protocol (J. Nelson and F.-W. Li, pers. comm.). These OTUs were removed from the dataset.

Phylogenetic Analysis

Plastomes were aligned with MAFFT 7 (Katoh and Standley, 2013) and visually inspected with Geneious Prime 2019. Given their identical nucleotide sequences, one copy of the inverted repeat region was removed. Areas in the alignment with missing data and highly variable repeat regions were also removed manually, particularly where variation in motif copy number was evident within an individual or where read depth was low. Phylogenies were inferred using maximum likelihood-based (ML) algorithms RAxML 7.3 (Stamatakis, 2006) and IQ-TREE 1.6 (Nguyen et al., 2015). Due to the large number of samples, Bayesian inference as implemented in MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001) could not run to completion.

Model selection for the trimmed alignment was performed using ModelFinder (Kalyaanamoorthy et al., 2017) implemented in IQ-Tree. The best-scoring model capable of being implemented in each program was used. RAxML was run using parameters for rapid bootstrapping and searching for best likelihood tree (e.g. raxmlHPC -f a -p 12345 -s alg -x 12345 -# 100 -m GTRGAMMA -n TEST). IQ-TREE was run utilizing ModelFinder to determine the

best fitting model of evolution, then a tree search was performed with ultrafast bootstrap approximation (Hoang et al., 2018) and SH-like approximate likelihood ratio test (SH-alrt; Guindon et al., 2010) (e.g. iqtree -s example.phy -m MFP -alrt 5000 -bb 5000).

Given the growing recognition that phylogenomic datasets can generate inaccurately large support values (Kumar et al., 2012) and that the common model of the chloroplast genome as uniparentally inherited and free from recombination is both biologically inaccurate and phylogenetically misleading (Gonçalves et al., 2019, Ramsey and Mandel, 2019), the concatenated alignment above was also analyzed with multispecies coalescent (MSC) methods suitable for dealing with incongruence due to incomplete lineage sorting (ILS). The trimmed plastome alignment was partitioned into 5k bp, 10k bp, and 15k bp segments, and each was analyzed using ML (IQ-TREE), a gene tree summary method (ASTRAL-III; Zhang et al., 2018), and a fully coalescent model (SVDquartets implemented in PAUP* v4.0a build 165; Swofford 2002; Chifman and Kubatko, 2014). ASTRAL-III was performed using the resulting ML trees. Trees were visualized and manipulated with R packages 'ape' (Paradis and Schliep, 2018) and 'phytools' (Revell, 2012), FigTree 1.4.4 (Rambaut, 2018), and Geneious Prime 2019.

Using MAFFT, LEAFY sequences from hybrids and polyploids were aligned with sequences from all described basic diploid taxa found in eastern North America and LEAFY sequences from any other species of *Isoëtes* available on GenBank. Model selection and phylogenetic analysis was performed as above with IQ-TREE. After generating the phylogeny, where PCR replicates or multiple individuals from the same population appeared to be effectively identical (each with the same number of OTUs and sequences within each OTU >99% identical) these sequences were collapsed and replaced with a majority consensus sequence. Names of collapsed samples were modified to include either "all" for PCR replicates

or “pop” for individuals from the same population, and the “size” measurement of the number of reads per OTU was replaced with “n” number of individuals/replicates that were collapsed into the new tip. With a new DNA alignment containing these collapsed samples, the phylogeny was inferred again using the same parameters. The distance matrix from the IQ-TREE ML phylogeny was processed by a custom Python script to extract information about the nearest diploid to each OTU. Given a user-supplied list of diploid tips, every other tip was compared to all diploid tips to find the diploid with the shortest patristic distance to each putative polyploid tip. The identity of the nearest diploid for each tip belonging to the same sample was then combined and output as a table of all unique diploid combinations and which samples contained each combination.

To estimate similarity between polyploid individuals, the ML distance matrix from the LEAFY phylogeny was exported with Geneious Primer, and all sequences from each sample were extracted. Pairwise comparisons of patristic distance for LEAFY OTUs were made between all pairs of samples with more than one OTU. For each pairwise comparison between samples, the Hungarian method (Kuhn, 1955, as implemented in the Python package ‘scipy’ v1.2.1; Oliphant et al. 2001) was used to optimize the distance matrix to obtain the minimum total distance by pairing the most similar OTUs from both samples. Each minimum total distance was entered into a pairwise matrix of all putative polyploid samples. The R package ‘phangorn’ (Schliep, 2011) was used to construct a Neighbor Joining tree from the sample-pairwise distance matrix. The UpSet figure was created with UpSetR (Conway et al. 2017).

Flow Cytometry

Fresh *Isoëtes* leaf and two standards (*Raphanus sativus* ‘saxa’ and *Glycine max* ‘Polanka’) were chopped in LB01 buffer and stained with propidium iodide. A BD Accuri C6

flow cytometer (North Carolina Research Campus, Kannapolis, NC) was used to measure nuclei fluorescence. Fluorescence signals were screened with a FL-2, 580/20 nm-bandpass filter and a FL-3, 670-nm longpass filter. Analysis of unfiltered homogenate was based on light-scatter (SSC-A) vs. fluorescence signals (FSC-A). Genome size was calculated using the equation: Sample 2C DNA content = (Sample G1 peak mean / Standard G1 peak mean) X Standard 2C DNA content (pg DNA).

RESULTS

Plastome Sequencing and Assembly

Across all Illumina sequencing runs, a total of 890,712,744 paired-end reads passed adapter and quality trimming, with a mean of 16,494,680 reads per sample (one standard deviation = 23,198,935; minimum = 505,556; maximum = 121,258,596). Approximately 1.8% of total reads (15,689,244 reads) were filtered as putative chloroplast reads (per sample mean 2.0%; standard deviation = 1.6%; minimum = 0.3%; maximum = 8.4%). Plastome coverage averaged 269X, but with a wide range (standard deviation = 459X; minimum = 4X; maximum = 2981X). There was little correlation between sequencing depth and % chloroplast reads recovered (linear regression $r^2 = 0.17$). *De novo* assembly of the putative chloroplast reads yielded contigs with a mean N50 of 37812 (standard deviation = 35542; minimum = 539; maximum = 103874). Final assembled plastomes showed little variation in size. Average length was 145,076 bp with a standard deviation of 250.8 bp (minimum = 144077 bp; maximum = 145481 bp). The number of ambiguous basecalls was low (median = 0.0%, mean = 0.4%), with three high outliers (3.3, 5.4% and 9.7%, see below).

Two samples, *I. butleri* Taylor 7001 and *I. junciformis* Brunton 17608 showed high levels of missing or ambiguous bases, 9.7% and 3.3%, respectively. This was due to very low coverage (4X and 7X) of these plastomes. *Isoëtes boomii* Schafran 73-1 had 5.5% missing/ambiguous bases despite 499X coverage. Examination of the *de novo* scaffolds showed disproportionately short contigs (N50 = 808), with multiple contigs mapping to many loci in the reference plastome. Contigs at each locus showed an approximately 30X difference in coverage, so they were separated and assembled into separate high coverage and low coverage plastomes. Phylogenetic analysis resolved the high coverage plastome in the North America clade, while the low coverage plastome fell into a clade with the South American species *I. pallida*, *I. cangae*, and *I. serracarajensis* with high support (data not shown). While extracting DNA of *I. boomii*, the Autogen DNA extraction platform suffered a malfunction. Adjacent wells to *I. boomii* contained *I. clavata* and *I. triangula* from French Guiana. Based on the phylogenetic evidence, it is likely that the equipment malfunction resulted in a small amount of contamination from *I. clavata/I. triangula*. The high coverage plastome is subsequently treated as *I. boomii.*

Alignment of all 59 plastomes, including outgroups and samples from GenBank, resulted in a matrix of 152,737 sites with 83.2% identical sites and 98.4% pairwise identity. 4.8% of the matrix was represented by gaps and 0.3% ambiguous sites. Alignment of the ingroup (American clade) of 51 plastomes resulted in a matrix of 148,554 sites with 94.6% identical sites and 99.5% pairwise identity. 2.0% of the matrix was represented by gaps and 0.5% ambiguous sites. Starting from the complete alignment, manual removal of one copy of the inverted repeat, repeat regions that displayed poor alignment, and alignment positions that consisted only of ambiguous bases resulted in an alignment with 136,406 sites with 3.5% gaps and 0.3% ambiguous bases.

LEAFY Sequencing and Filtering

Across five sequencing runs on Pacific Biosciences platforms, 1,278,437 CCS were generated. After quality trimming and size selection to the 900—1400 bp range, 658,325 sequences remained. 124,259 sequences could be demultiplexed and annotated by PURC. Clustering identified 1,225 OTUs from 568 samples (including replicates and negative controls), which was reduced 10% to 1,102 OTUs following contaminant removal. The number of sequences after contaminant removal decreased to 122,765.

Flow Cytometry

A total of 301 individuals were measured, including 7 individuals of various ploidy levels where 2-3 repeated measures were taken from the same individual. Based on the maximum coefficient of variation (CV) observed from the individual replicates, populations with CVs less than 6 were treated as having a single ploidy level (Figure 13). These represented 94% of sampled populations. Only 4 populations had CVs >6, ranging from 14-27; these were interpreted as populations containing individuals of mixed ploidy levels. Excluding these 4 outliers, there was no significant difference in CVs between individual and population level replicates ($p=0.999$).

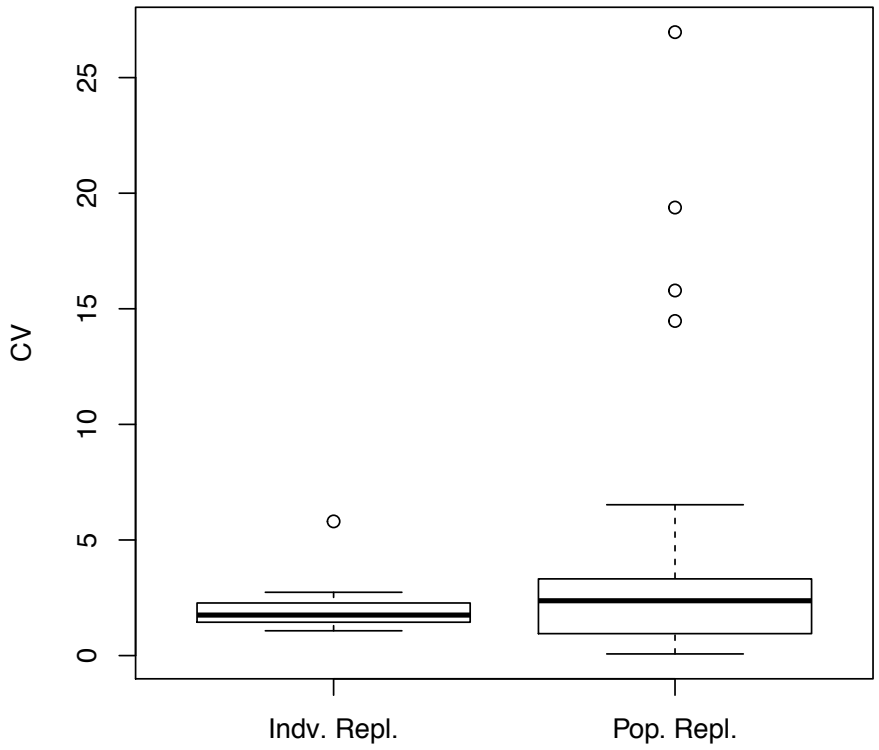


FIGURE 13. Boxplots of coefficients of variation for individual and population level replicates.

In agreement with Bolin et al. (2018), a strong significantly positive correlation was obtained between C-value and the number of LEAFY OTUs recovered per individual (r^2=0.78, p<2e-16). Though their interquartile ranges are separated, overlaps of minimum—maximum range between individuals with different numbers of OTUs precludes the use of C-value as a strong predictor of ploidy level (Figure 14). Hereafter, samples with C-values within the interquartile range for a given number of OTUs are treated as putative members of that ploidy level (e.g. an individual with 1 LEAFY OTU and a C-value of 2.2 is a putative diploid, and an individual with 2 LEAFY OTUs and a C-value of 3.8 is a putative tetraploid).

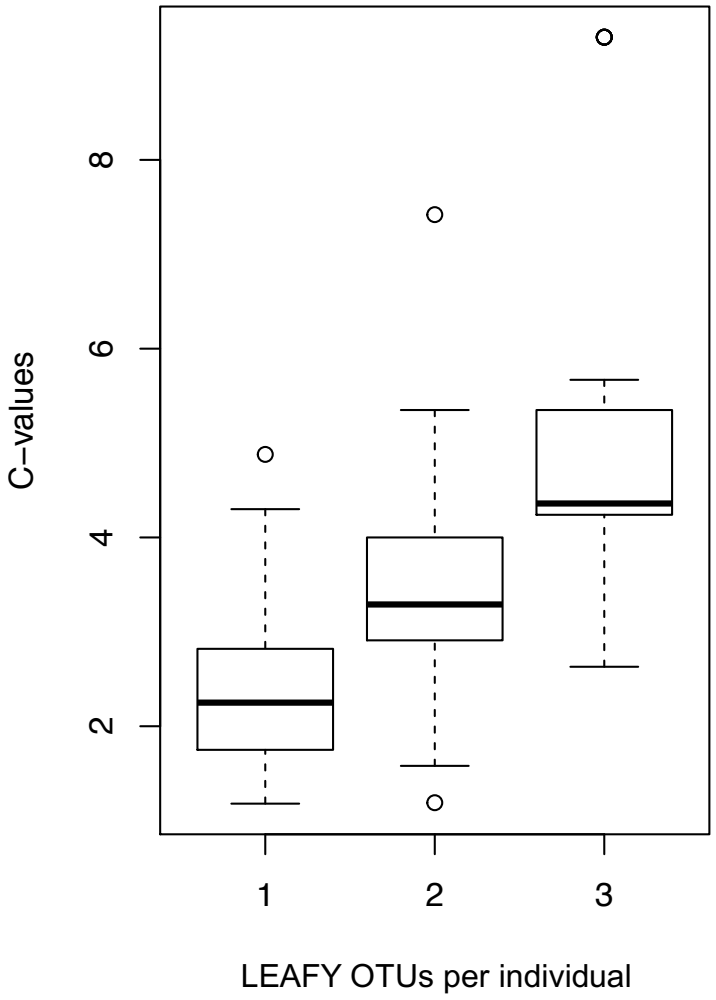


FIGURE 14. Boxplots of C-values binned by the number of LEAFY OTUs recovered from each individual.

Isoëtes engelmannii is the best represented species in the C-value dataset that was supported as a single taxon by phylogenetic data. Across 6 populations in North Carolina and Virginia, C-values ranged from 1.7-1.95 and ANOVA results showed that most variation in

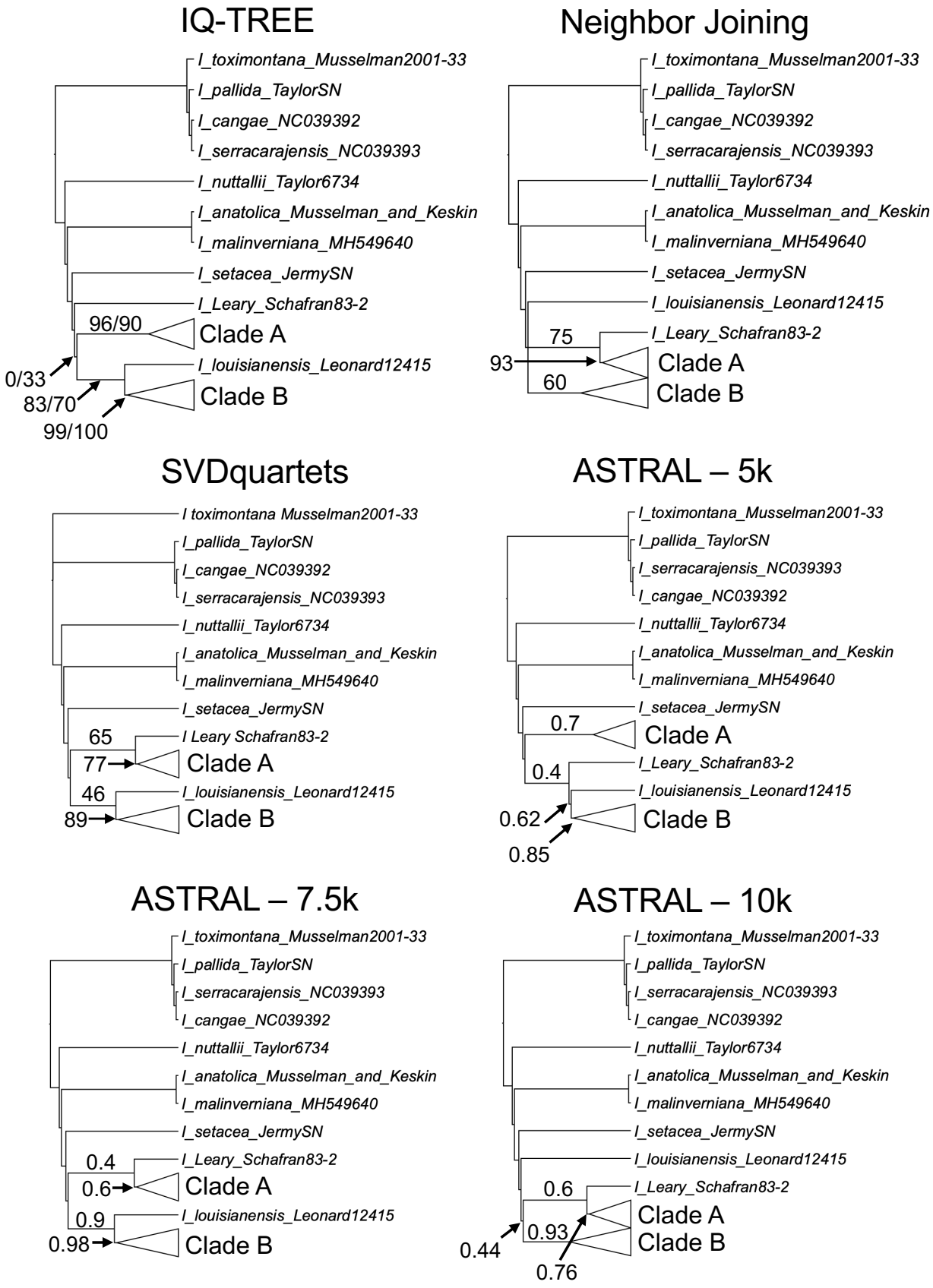
genome size occurred at the population level (p=0.0002, F=12.9). *Isoëtes appalachiana*, a tetraploid whose parentage of *I. engelmannii* × *I. valida* was well supported by the phylogenetic data, showed additivity between genome sizes of its parents. C-values from individuals of *I. appalachiana* (range: 3.7-4.0) mostly overlapped with the sum of the minimum--maximum range for the diploid species (*I. engelmannii*: 1.7-1.95, *I. valida*: 2.18-2.26). Other tetraploid complexes showed less agreement. *Isoëtes septentrionalis* (*I. engelmannii* × *I. echinospora*: C-value 2.56) fit a model of additivity in some cases (*Schafran 151:*C-value 4.52), but not others (*Schafran 160, 161*; C-values 2.74-2.91). Additional populations of *I. echinospora* need to be sampled to determine what range of genome sizes exist.

Plastome Phylogenetic Analysis

Plastome tree topologies were generally consistent between analyses, with the exception of some taxa that had variable, poorly-supported positions. Relationships between the outgroups were highly supported and agreed with Larsén and Rydin (2016) and Pereira et al. (2017; Figure 15). *Isoëtes setacea* was found to be sister to the clade containing all the individuals collected from North America (except *I. nuttallii*). The majority of North American samples occurred in two clades (Clades A and B in Figures 15, 16) that were strongly supported, each with internally consistent sets of taxa and topologies that agreed between the majority of analyses. *Isoëtes* 'Leary', a potential undescribed diploid from Georgia, and one individual of *I. louisianensis* (*Leonard 12415*) occupied weakly supported (BS < 90) basal positions within the American clade, though position varied between analyses. Depending on analysis, *Isoëtes* 'Leary' was placed sister to all other North American individuals (IQ-TREE), sister to Clade A (neighbor joining, SVDquartets, ASTRAL-7.5k, ASTRAL-10k) or sister to (*I. louisianensis* (*Leonard*

12415) + Clade B) (Figure 15). *Isoëtes louisianensis* (*Leonard 12415*) most often occurred sister to Clade B (IQ-TREE, SVDquartets, ASTRAL-5k, ASTRAL-7.5k), but was also placed on a polytomy with Clade B and (Clade A + *I.* 'Leary') (neighbor joining) and sister to all other North American individuals (ASTRAL-10k).

FIGURE 15. Outgroups and variable positions of *I. 'Leary'* and *I. louisianensis Leonard 12415* between plastome analyses. Numbers in ASTRAL subtitles indicate basepair length of alignments used to generate maximum likelihood trees. All branches with perfect support (e.g. bootstrap = 100, local posterior probability = 1.0) except where noted. Branch lengths not to scale.


IQ-TREE
l_toximontana_Musselman2001-33
l_pallida_TaylorSN
l_cangae_NC039392
l_serracarajensis_NC039393
l_nuttallii_Taylor6734
l_anatolica_Musselman_and_Keskin
l_malinverniana_MH549640
l_setacea_JermySN
l_Leary_Schafran83-2
96/90
Clade A
l_louisianensis_Leonard12415
0/33
83/70
99/100
Clade B
Neighbor Joining
l_toximontana_Musselman2001-33
l_pallida_TaylorSN
l_cangae_NC039392
l_serracarajensis_NC039393
l_nuttallii_Taylor6734
l_anatolica_Musselman_and_Keskin
l_malinverniana_MH549640
l_setacea_JermySN
l_louisianensis_Leonard12415
75
l_Leary_Schafran83-2
93
Clade A
60
Clade B
SVDquartets
l toximontana Musselman2001-33
l_pallida_TaylorSN
l_cangae_NC039392
l_serracarajensis_NC039393
l_nuttallii_Taylor6734
l_anatolica_Musselman_and_Keskin
l_malinverniana_MH549640
l_setacea_JermySN
65
l Leary Schafran83-2
77
Clade A
46
l_louisianensis_Leonard12415
89
Clade B
ASTRAL – 5k
l_toximontana_Musselman2001-33
l_pallida_TaylorSN
l_serracarajensis_NC039393
l_cangae_NC039392
l_nuttallii_Taylor6734
l_anatolica_Musselman_and_Keskin
l_malinverniana_MH549640
l_setacea_JermySN
0.7
Clade A
l_Leary_Schafran83-2
0.4
l_louisianensis_Leonard12415
0.62
Clade B
0.85
ASTRAL – 7.5k
l_toximontana_Musselman2001-33
l_pallida_TaylorSN
l_serracarajensis_NC039393
l_cangae_NC039392
l_nuttallii_Taylor6734
l_anatolica_Musselman_and_Keskin
l_malinverniana_MH549640
l_setacea_JermySN
0.4
l_Leary_Schafran83-2
0.6
Clade A
0.9
l_louisianensis_Leonard12415
0.98
Clade B
ASTRAL – 10k
l_toximontana_Musselman2001-33
l_pallida_TaylorSN
l_serracarajensis_NC039393
l_cangae_NC039392
l_nuttallii_Taylor6734
l_anatolica_Musselman_and_Keskin
l_malinverniana_MH549640
l_setacea_JermySN
l_louisianensis_Leonard12415
0.6
l_Leary_Schafran83-2
Clade A
0.93
Clade B
0.44
0.76

The plastome phylogeny indicated several cases of polyphyletic diploid and polyploid species (Figure 16). *Isoëtes piedmontana*, long suspected to be a species complex (Heafner and Bray, 2005), appeared in two places within Clade A (colored green, Figure 16). The sample *Schafran NC01* and an individual from GenBank (MH549641) were placed sister to *I. graniticola* (*Taylor 6776*) – unsurprising given that all three were collected from granite outcrops near Salisbury, NC – and with *I. engelmannii* as the nearest diploid lineage. The sample *Schafran 18,* placed sister *to I. graniticola Schafran 14* and *I. tegetiformans,* is treated as *I. piedmontana s.s.* since it was collected from a paratype locality (the holotype population believed to be extirpated). *Schafran NC01* and the *I. piedmontana* individual from GenBank are referred to hereafter as *I.* 'piedmontana-NC'.

Isoëtes graniticola, a species recently established as a tetraploid member of the *I. piedmontana* complex (Brunton, 2016), seemed to have three maternal origins (colored blue, Figure 16). *Taylor 6998,* placed sister to a clade containing several diploid (*I. mattaponica*, *I. melanopoda* ssp. *silvatica*, *I. viridimontana*) and polyploid taxa (*I. georgiana*, *I. hyemalis*, *I. louisianensis*), was collected from the holotype locality so represents *I. graniticola s.s. Taylor 6776*, placed sister to *I.* 'piedmontana-NC', is hereafter treated as *I.* 'graniticola-NC', and *Schafran 14* sister to *I. piedmontana s.s*. is treated as *I.* 'graniticola-GA'.

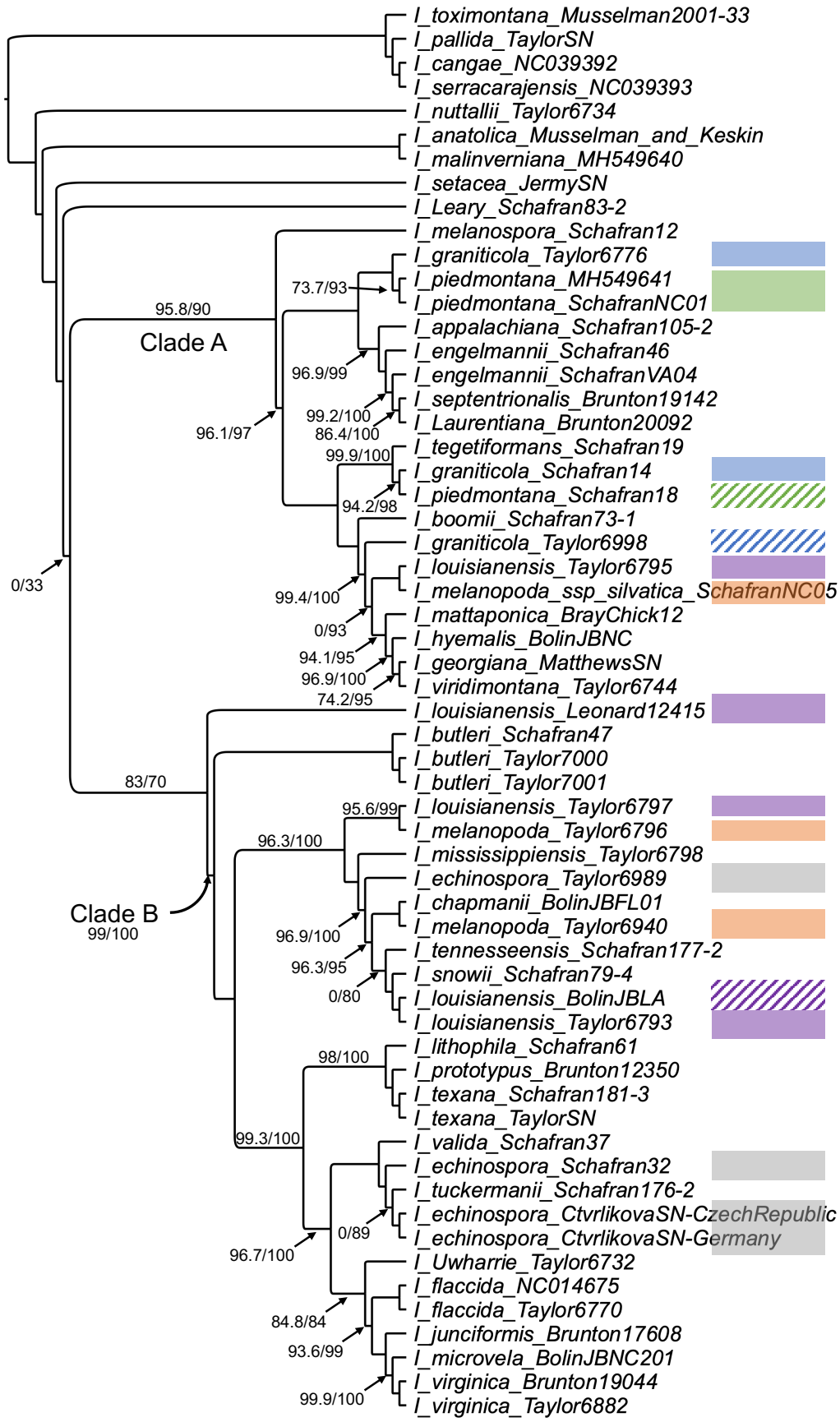
Samples of *I. louisianensis* appeared on three distinct lineages (colored purple, Figure 16). *Isoëtes louisianensis Bolin JBLA* was a topotype collection from Thigpen Creek, Washington Parish, LA, and is treated as *I. louisianensis s.s.* One additional sample, *I. louisianensis Taylor 6793*, was placed sister to *Bolin JBLA,* with the undescribed diploid *I.* 'snowii' sister to this pair. The sample *I. louisianensis Taylor 6797* was sister to *I. melanopoda Taylor 6796*, this pair on a branch sister to the subclade containing *I. louisianensis s.s., I.*

melanopoda Taylor 6940, I. mississippiensis, I. 'snowii', *I. flaccida var. chapmanii* (= *I. chapmanii*), *I. tennesseensis*, and *I. echinospora Taylor 6989* (but see potential issues with this sample below). One sample of *I. louisianensis*, *Taylor 6795*, was placed in Clade A sister to *I. melanopoda* ssp. *silvatica*. As noted above, the position of *I. louisianensis Leonard 12415* varied by analysis.

Isoëtes melanopoda (including *I. melanopoda* ssp. *silvatica*) occurred in three separate lineages. *Isoetes m.* ssp. *silvatica* was placed in Clade A, sister to a clade containing the diploids *I. mattaponica* and *I. viridimontana*, while samples of *I. melanopoda s.s.* (i.e. those not *I. m*. ssp. *silvatica*) were found in Clade B (colored orange, Figure 16). The samples of *I. melanopoda s.s*. were not resolved together, made polyphyletic by the diploid individuals *I. mississippiensis* and *I. echinospora Taylor 6989* (but see issues with this sample below).

Most samples of *I. echinospora* formed a single clade (including *I. tuckermanii*, a tetraploid presumably derived from *I. echinospora*), except for *Taylor 6989*. None of these species was sampled from topotypes or type specimens, so none are assumed to best represent their taxon. The plastome of *Taylor 6989*, collected in Vancouver, Canada, shared high similarity with *I. bolanderi* (Jacob Suissa, unpublished data), suggesting that *Taylor 6989* could be a misidentified specimen of *I. bolanderi* or *I. maritima,* an allotetraploid derived from *I. bolanderi* and *I. echinospora*. Given the possibility that *Taylor 6989* represents a misidentification, it was excluded from further analysis.

FIGURE 16. Maximum likelihood cladogram noting polyphyletic taxa based on *a priori* morphological identifications. Similar colored tips represent samples with the same taxon identification. Hashed colored blocks indicate a topotype specimen. Branch support values are approximate likelihood ratio test/ultrafast bootstrap values and are both 100 except where noted.


I_toximontana_Musselman2001-33
I_pallida_TaylorSN
I_cangae_NC039392
I_serracarajensis_NC039393
I_nuttallii_Taylor6734
I_anatolica_Musselman_and_Keskin
I_malinverniana_MH549640
I_setacea_JermySN
I_Leary_Schafran83-2
I_melanospora_Schafran12
I_graniticola_Taylor6776
I_piedmontana_MH549641
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I_appalachiana_Schafran105-2
I_engelmannii_Schafran46
I_engelmannii_SchafranVA04
I_septentrionalis_Brunton19142
I_Laurentiana_Brunton20092
I_tegetiformans_Schafran19
I_graniticola_Schafran14
I_piedmontana_Schafran18
I_boomii_Schafran73-1
I_graniticola_Taylor6998
I_louisianensis_Taylor6795
I_melanopoda_ssp_silvatica_SchafranNC05
I_mattaponica_BrayChick12
I_hyemalis_BolinJBNC
I_georgiana_MatthewsSN
I_viridimontana_Taylor6744
I_louisianensis_Leonard12415
I_butleri_Schafran47
I_butleri_Taylor7000
I_butleri_Taylor7001
I_louisianensis_Taylor6797
I_melanopoda_Taylor6796
I_mississippiensis_Taylor6798
I_echinospora_Taylor6989
I_chapmanii_BolinJBFL01
I_melanopoda_Taylor6940
I_tennesseensis_Schafran177-2
I_snowii_Schafran79-4
I_louisianensis_BolinJBLA
I_louisianensis_Taylor6793
I_lithophila_Schafran61
I_prototypus_Brunton12350
I_texana_Schafran181-3
I_texana_TaylorSN
I_valida_Schafran37
I_echinospora_Schafran32
I_tuckermanii_Schafran176-2
I_echinospora_CtvrlikovaSN-CzechRepublic
I_echinospora_CtvrlikovaSN-Germany
I_Uwharrie_Taylor6732
I_flaccida_NC014675
I_flaccida_Taylor6770
I_junciformis_Brunton17608
I_microvela_BolinJBNC201
I_virginica_Brunton19044
I_virginica_Taylor6882
Clade A
Clade B
95.8/90
73.7/93
96.9/99
96.1/97
99.2/100
86.4/100
99.9/100
94.2/98
0/33
99.4/100
0/93
94.1/95
96.9/100
74.2/95
83/70
95.6/99
96.3/100
99/100
96.9/100
96.3/95
0/80
98/100
99.3/100
0/89
96.7/100
84.8/84
93.6/99
99.9/100

Patristic distances between diploids and polyploids highlighted clear relationships of some polyploids to putative maternal diploids, while other polyploids had similar distances to multiple diploid species (Table 9). Three tetraploids found in the northeastern US and Canada, *I.* '*laurentiana*', *I. septentrionalis*, and *I. tuckermanii*, showed strong relationships to single species. *Isoëtes* '*laurentiana*' and *I. septentrionalis* had relatively low distance to *I. engelmannii* and high distance to the next-nearest diploid taxon*, I. '*piedmontana-NC' (ratio of distances 31.1 and 39.9, respectively, Table 9). *Isoëtes tuckermanii* displayed a distance ratio of 37.1 between its closest diploid species, *I. echinospora*, and the second closest diploid, *I. valida* (Table 9). Intermediate distance ratios were observed for *I.* 'graniticola-NC' (11.3, closest to *I.* 'piedmontana-NC' and *I. engelmannii Schafran 46*) and *I. louisianensis Taylor 6795* (6.4, closer distance to *I. melanopoda Taylor 6940*, *I. chapmanii Bolin JBFL01*, and *I. mississippiensis Taylor 6798* despite sister position to *I. melanopoda Taylor 6796*). Low distance ratios (<3.0) were found for all other polyploids, indicating very little difference in distance between a polyploid and two or more diploid species.

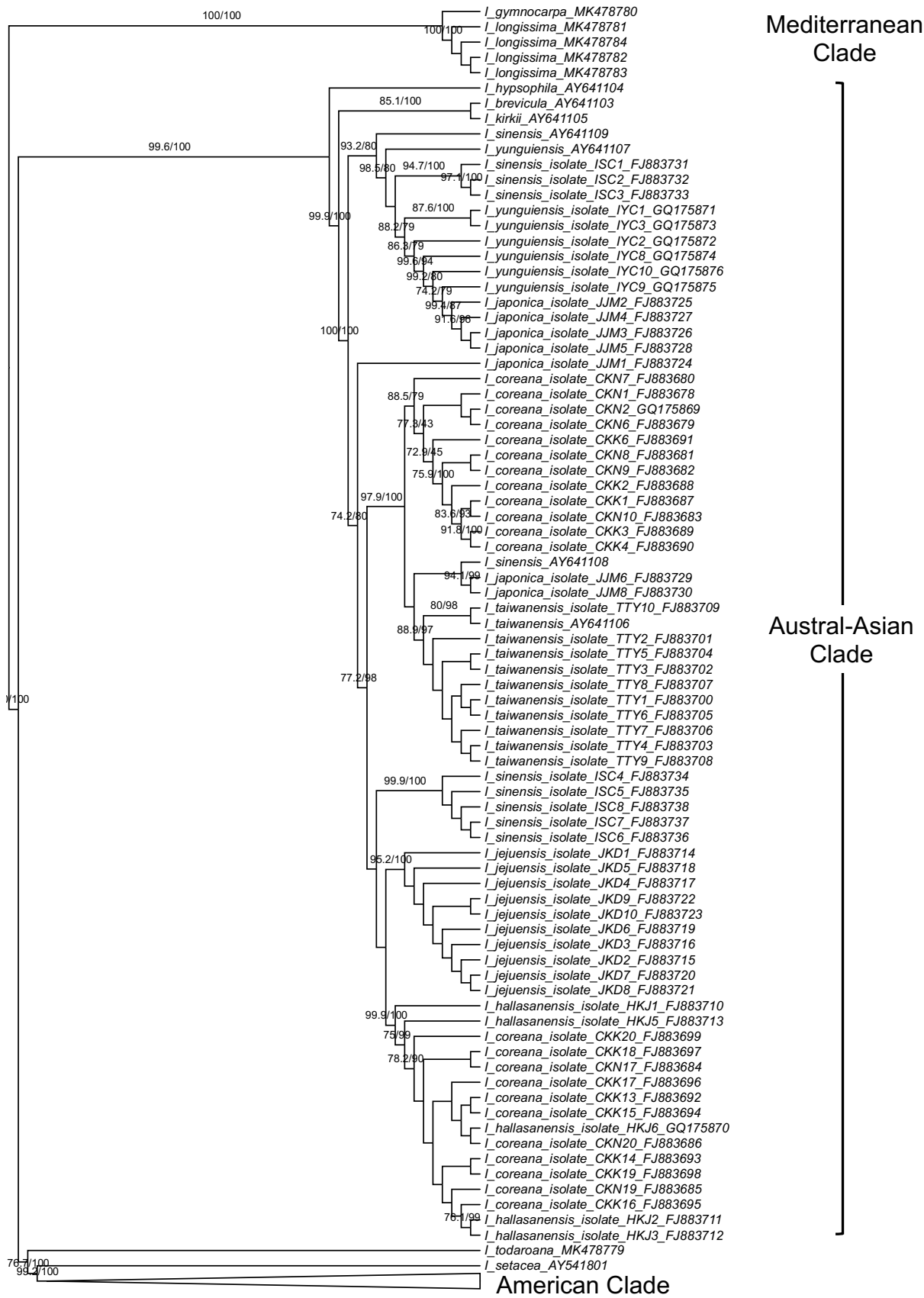
TABLE 9. Distances of polyploid *Isoëtes* to first and second-closest diploid in the plastome maximum likelihood phylogeny and second-closest:closest distance ratio.

	Patristic Distance		
	Closest Diploid Species	Second-Closest Diploid Species	Distance Ratio
I. appalachiana Schafran 105-2	0.000367	0.00045	1.2
I. boomii Schafran 73-1	0.000437	0.000459	1.1
I. georgiana Matthews s.n.	0.000107	0.000236	2.2
I. graniticola Schafran 14	0.000237	0.000635	2.7
I. graniticola Taylor 6776	0.000046	0.000519	11.3
I. graniticola Taylor 6998	0.00017	0.000192	1.1
I. hyemalis Bolin JBNC	0.000061	0.000176	2.9
I. laurentiana Brunton 20092	0.000017	0.000528	31.1
I. louisianensis Bolin JBLA	0.000361	0.000622	1.7
I. louisianensis Leonard 12415	0.001316	0.001327	1.0
I. louisianensis Taylor 6793	0.000361	0.000622	1.7
I. louisianensis Taylor 6795	0.000038	0.000244	6.4
I. louisianensis Taylor 6797	0.001057	0.00109	1.0
I. microvela Bolin JBNC201	0.000764	0.000789	1.0
I. septentrionalis Brunton 19142	0.00001	0.000399	39.9
I. tennesseensis Schafran 177-2	0.00053	0.000621	1.2
I. tuckermanii Schafran 176-2	0.000017	0.000631	37.1
I. virginica Brunton19044	0.000783	0.000789	1.0
I. virginica Taylor 6882	0.000784	0.000795	1.0

LEAFY Phylogenetic Analysis

Rooted with *I. gymnocarpa* and *I. longissima* as the outgroup, the Austral-Asian clade is resolved as sister to I. *setacea* + the American clade in agreement with prior studies (Larsén and Rydin, 2016; Pereira et al., 2017; Figure 17). The American clade was generally characterized by short, weakly supported internal branches and well-supported clades containing one diploid taxon or a complex of several taxa.

FIGURE 17. LEAFY maximum likelihood cladogram with outgroup clades expanded and American clade collapsed. Branch support values are approximate likelihood ratio test (ALRT)/ultrafast bootstrap (UFBS). Support values removed where ALRT and UFBS were less than 50. Branch lengths not to scale.


Mediterranean Clade
Austral-Asian Clade
American Clade
I_gymnocarpa_MK478780
I_longissima_MK478781
I_longissima_MK478784
I_longissima_MK478782
I_longissima_MK478783
I_hypsophila_AY641104
I_brevicula_AY641103
I_kirkii_AY641105
I_sinensis_AY641109
I_yunguiensis_AY641107
I_sinensis_isolate_ISC1_FJ883731
I_sinensis_isolate_ISC2_FJ883732
I_sinensis_isolate_ISC3_FJ883733
I_yunguiensis_isolate_IYC1_GQ175871
I_yunguiensis_isolate_IYC3_GQ175873
I_yunguiensis_isolate_IYC2_GQ175872
I_yunguiensis_isolate_IYC8_GQ175874
I_yunguiensis_isolate_IYC10_GQ175876
I_yunguiensis_isolate_IYC9_GQ175875
I_japonica_isolate_JJM2_FJ883725
I_japonica_isolate_JJM4_FJ883727
I_japonica_isolate_JJM3_FJ883726
I_japonica_isolate_JJM5_FJ883728
I_japonica_isolate_JJM1_FJ883724
I_coreana_isolate_CKN7_FJ883680
I_coreana_isolate_CKN1_FJ883678
I_coreana_isolate_CKN2_GQ175869
I_coreana_isolate_CKN6_FJ883679
I_coreana_isolate_CKK6_FJ883691
I_coreana_isolate_CKN8_FJ883681
I_coreana_isolate_CKN9_FJ883682
I_coreana_isolate_CKK2_FJ883688
I_coreana_isolate_CKK1_FJ883687
I_coreana_isolate_CKN10_FJ883683
I_coreana_isolate_CKK3_FJ883689
I_coreana_isolate_CKK4_FJ883690
I_sinensis_AY641108
I_japonica_isolate_JJM6_FJ883729
I_japonica_isolate_JJM8_FJ883730
I_taiwanensis_isolate_TTY10_FJ883709
I_taiwanensis_AY641106
I_taiwanensis_isolate_TTY2_FJ883701
I_taiwanensis_isolate_TTY5_FJ883704
I_taiwanensis_isolate_TTY3_FJ883702
I_taiwanensis_isolate_TTY8_FJ883707
I_taiwanensis_isolate_TTY1_FJ883700
I_taiwanensis_isolate_TTY6_FJ883705
I_taiwanensis_isolate_TTY7_FJ883706
I_taiwanensis_isolate_TTY4_FJ883703
I_taiwanensis_isolate_TTY9_FJ883708
I_sinensis_isolate_ISC4_FJ883734
I_sinensis_isolate_ISC5_FJ883735
I_sinensis_isolate_ISC8_FJ883738
I_sinensis_isolate_ISC7_FJ883737
I_sinensis_isolate_ISC6_FJ883736
I_jejuensis_isolate_JKD1_FJ883714
I_jejuensis_isolate_JKD5_FJ883718
I_jejuensis_isolate_JKD4_FJ883717
I_jejuensis_isolate_JKD9_FJ883722
I_jejuensis_isolate_JKD10_FJ883723
I_jejuensis_isolate_JKD6_FJ883719
I_jejuensis_isolate_JKD3_FJ883716
I_jejuensis_isolate_JKD2_FJ883715
I_jejuensis_isolate_JKD7_FJ883720
I_jejuensis_isolate_JKD8_FJ883721
I_hallasanensis_isolate_HKJ1_FJ883710
I_hallasanensis_isolate_HKJ5_FJ883713
I_coreana_isolate_CKK20_FJ883699
I_coreana_isolate_CKK18_FJ883697
I_coreana_isolate_CKN17_FJ883684
I_coreana_isolate_CKK17_FJ883696
I_coreana_isolate_CKK13_FJ883692
I_coreana_isolate_CKK15_FJ883694
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I_todaroana_MK478779
I_setacea_AY541801

The basal-most lineage in the American clade contains sequences from *I. andicola*, a South American tetraploid. Sister to all other North American sequences is the "Butner clade" containing OTUs from several polyploid taxa identified as *I. piedmontana s.l.*, *I. hyemalis s.l.*, and *I. microvela s.s*. There are no diploids present in this clade, so the origin of these sequences within the polyploids is unclear. All samples represented in this clade were collected in North Carolina (Figure 18).

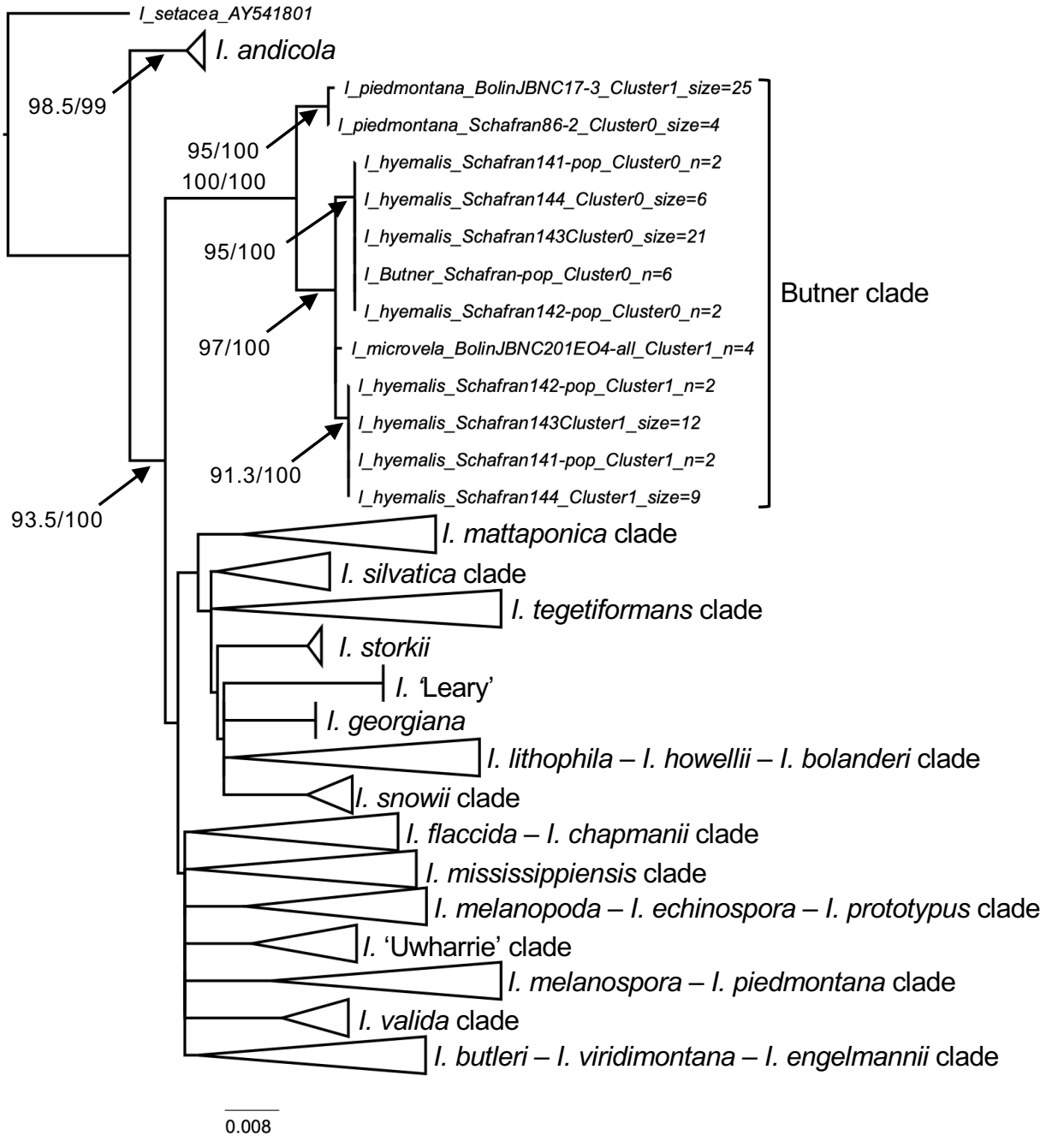
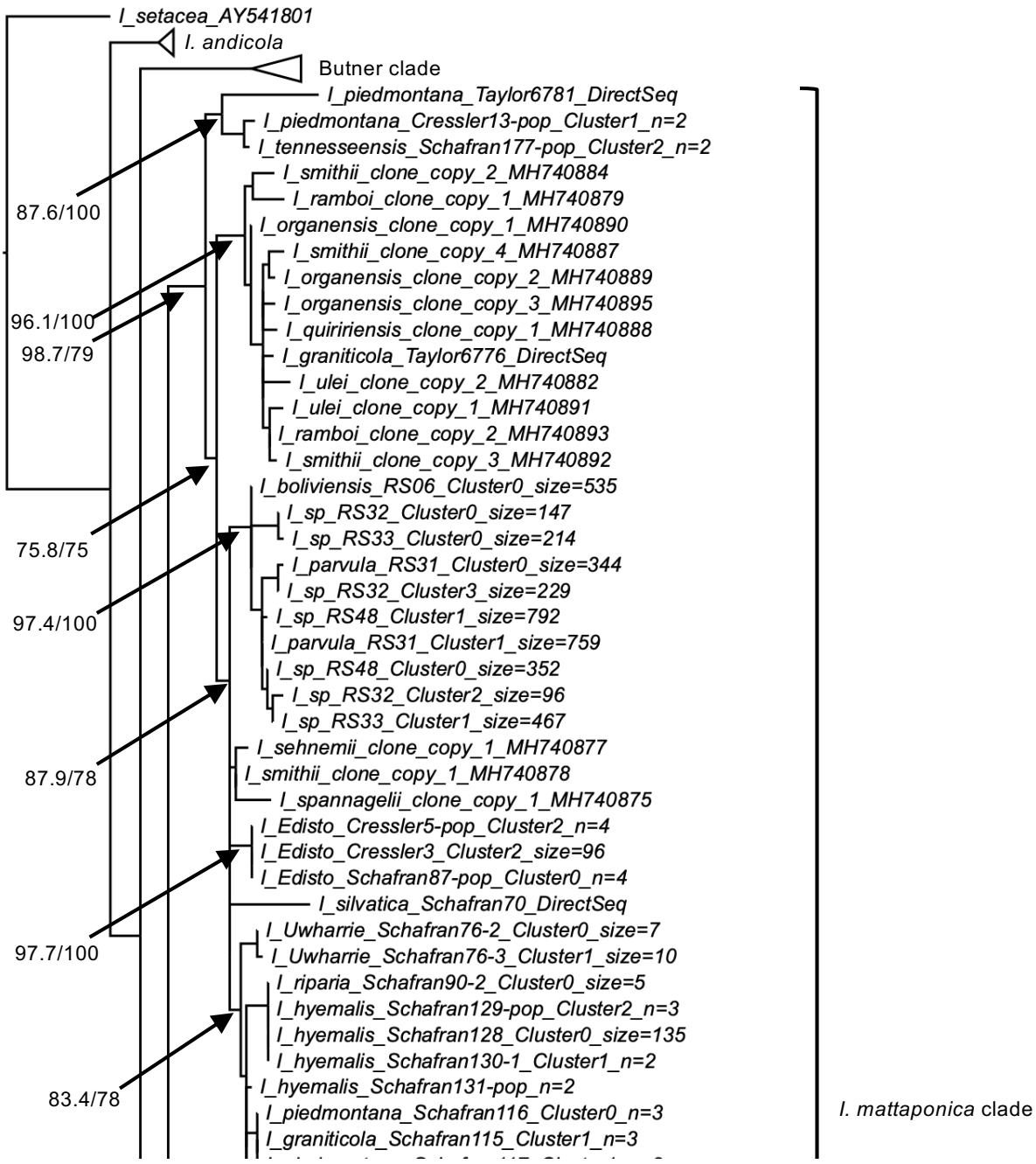


FIGURE 18. "Butner clade" expanded in the LEAFY maximum likelihood phylogeny. Branch support values are approximate likelihood ratio test (ALRT)/ultrafast bootstrap (UFBS). Support values shown only within and adjacent to focal clade. Scale equals expected number of substitutions per site.

The "*I. mattaponica* clade" contains sequences from several South American taxa in addition those from numerous North American species (Figure 19). The North American plants are predominantly nested in one moderately supported clade with a single diploid member, *I. mattaponica*. Other diploid individuals in this clade were identified as *I. piedmontana* and *I. melanopoda* ssp. *silvatica*, though those species *sensu stricto* occur in other clades. Polyploids that were identified as *I. hyemalis s.l.* and *I. riparia s.l.* are also prominent in this clade. Sister to the *I. mattaponica* clade is a group of individuals collected from the Andean Mountains in South America, including *I. boliviensis*, *I. parvula*, and sister to that is a group of predominantly Brazilian taxa. The basal-most sequences of the clade are three individuals from North America. Their relationship to *I. mattaponica* is unlikely, given the South American taxa interspersed in the phylogeny.

FIGURE 19. "*I. mattaponica* clade" expanded in the LEAFY maximum likelihood phylogeny. Branch support values are approximate likelihood ratio test (ALRT)/ultrafast bootstrap (UFBS). Support values shown only within and adjacent to focal clade. Scale equals expected number of substitutions per site. Red tip labels indicate specimens recognized as *I. mattaponica s.s.*


I_setacea_AY541801
I. andicola
Butner clade
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I_tennesseensis_Schafran177-pop_Cluster2_n=2
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I_ramboi_clone_copy_1_MH740879
I_organensis_clone_copy_1_MH740890
I_smithii_clone_copy_4_MH740887
I_organensis_clone_copy_2_MH740889
I_organensis_clone_copy_3_MH740895
I_quiririensis_clone_copy_1_MH740888
I_graniticola_Taylor6776_DirectSeq
I_ulei_clone_copy_2_MH740882
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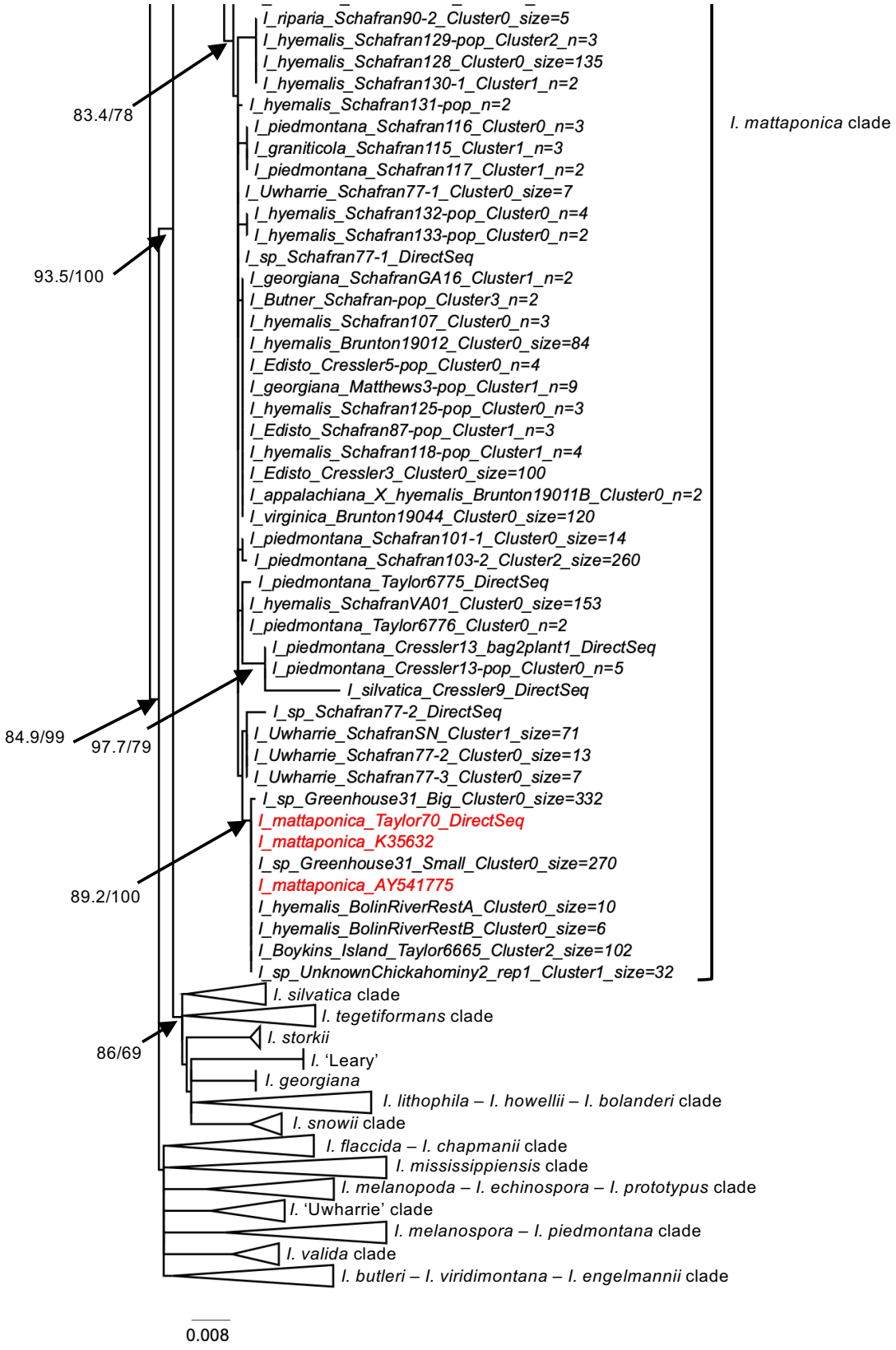
I_sp_RS32_Cluster0_size=147
I_sp_RS33_Cluster0_size=214
I_parvula_RS31_Cluster0_size=344
I_sp_RS32_Cluster3_size=229
I_sp_RS48_Cluster1_size=792
I_parvula_RS31_Cluster1_size=759
I_sp_RS48_Cluster0_size=352
I_sp_RS32_Cluster2_size=96
I_sp_RS33_Cluster1_size=467
I_sehnemii_clone_copy_1_MH740877
I_smithii_clone_copy_1_MH740878
I_spannagelii_clone_copy_1_MH740875
I_Edisto_Cressler5-pop_Cluster2_n=4
I_Edisto_Cressler3_Cluster2_size=96
I_Edisto_Schafran87-pop_Cluster0_n=4
I_silvatica_Schafran70_DirectSeq
I_Uwharrie_Schafran76-2_Cluster0_size=7
I_Uwharrie_Schafran76-3_Cluster1_size=10
I_riparia_Schafran90-2_Cluster0_size=5
I_hyemalis_Schafran129-pop_Cluster2_n=3
I_hyemalis_Schafran128_Cluster0_size=135
I_hyemalis_Schafran130-1_Cluster1_n=2
I_hyemalis_Schafran131-pop_n=2
I_piedmontana_Schafran116_Cluster0_n=3
I_graniticola_Schafran115_Cluster1_n=3
87.6/100
96.1/100
98.7/79
75.8/75
97.4/100
87.9/78
97.7/100
83.4/78
I. mattaponica clade

FIGURE 19. Continued.



The "*I. silvatica* clade" contains one major clade centered around *I. melanopoda* ssp. *silvatica s.s*. (Figure 20) Other diploids misidentified as *I. piedmontana s.l.* and *I. mattaponica s.l*. are found in this group. *Isoëtes melanopoda* ssp. *silvatica* is involved with several polyploid taxa including *I. hyemalis*, *I. virginica*, *I. graniticola*, *I. riparia*, *I. boomii*, *I. microvela*, *I. louisianensis*, *I. 'laurentiana'*, and *I. georgiana*.

FIGURE 20. "*I. silvatica* clade" expanded in the LEAFY maximum likelihood phylogeny. Branch support values are approximate likelihood ratio test (ALRT)/ultrafast bootstrap (UFBS). Support values shown only within and adjacent to focal clade. Scale equals expected number of substitutions per site. Red tip labels indicate specimens recognized as *I. silvatica s.s.*

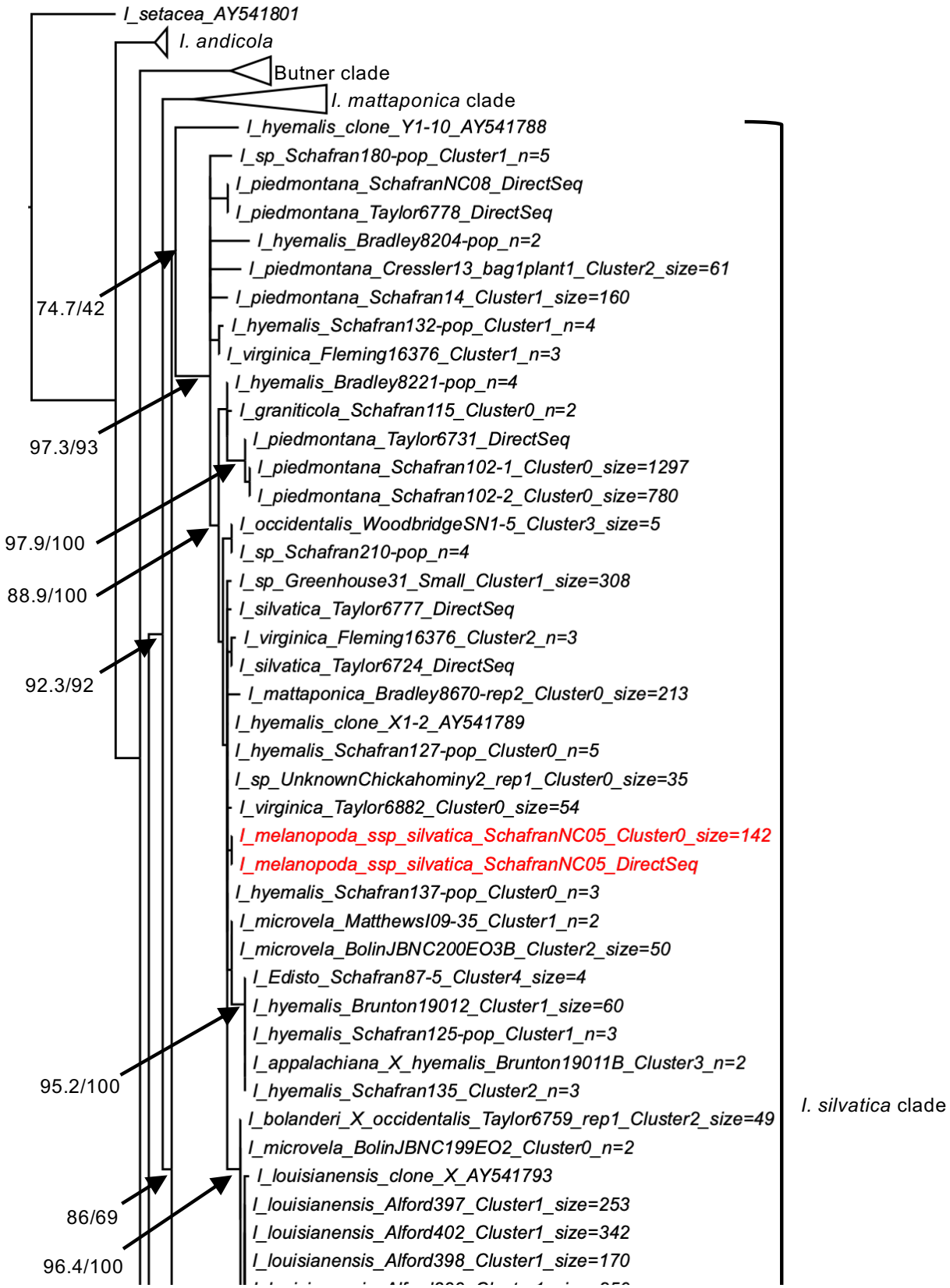
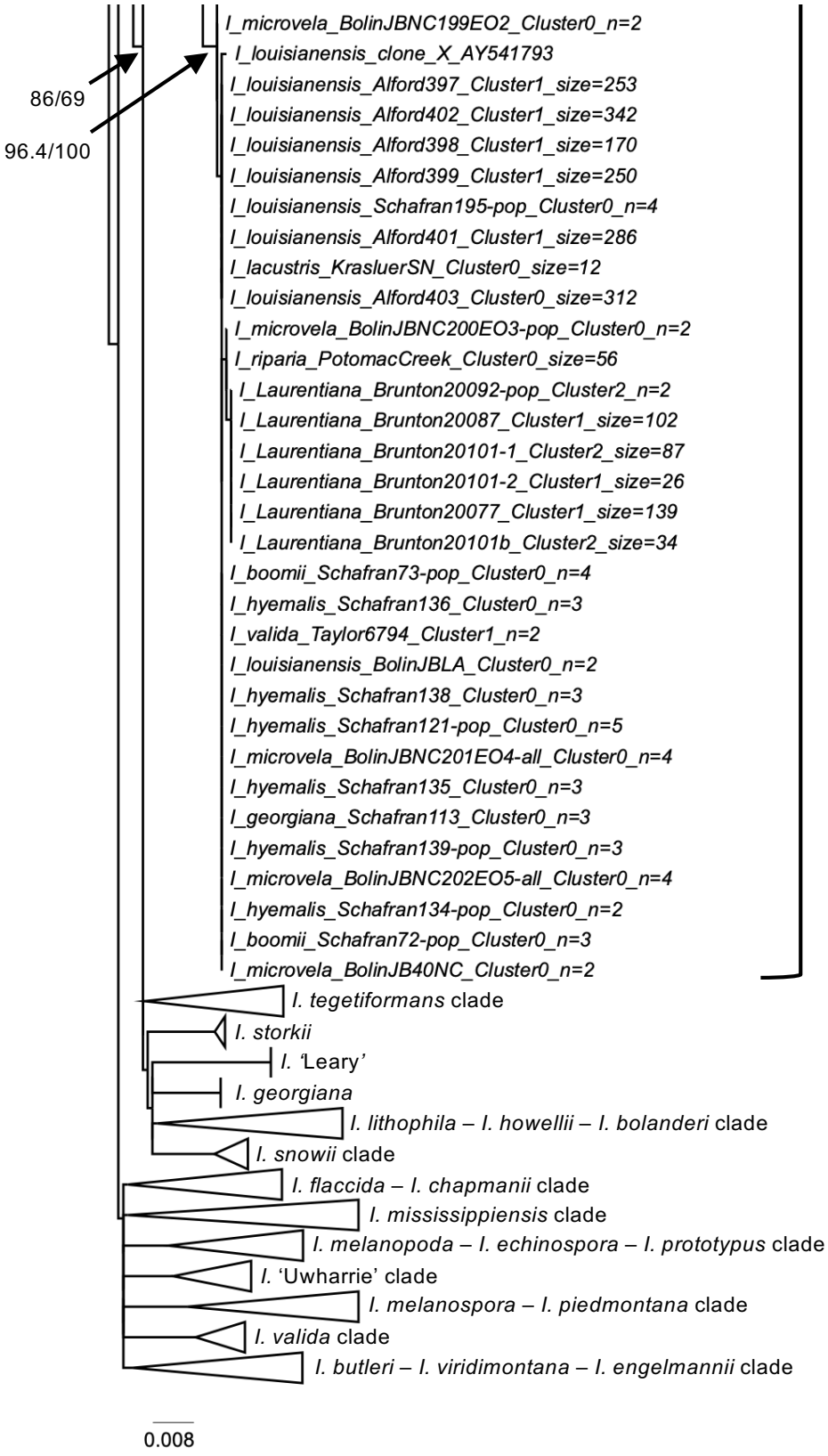

I_setacea_AY541801
I. andicola
Butner clade
I. mattaponica clade
I_hyemalis_clone_Y1-10_AY541788
I_sp_Schafran180-pop_Cluster1_n=5
I_piedmontana_SchafranNC08_DirectSeq
I_piedmontana_Taylor6778_DirectSeq
I_hyemalis_Bradley8204-pop_n=2
I_piedmontana_Cressler13_bag1plant1_Cluster2_size=61
I_piedmontana_Schafran14_Cluster1_size=160
I_hyemalis_Schafran132-pop_Cluster1_n=4
I_virginica_Fleming16376_Cluster1_n=3
I_hyemalis_Bradley8221-pop_n=4
I_graniticola_Schafran115_Cluster0_n=2
I_piedmontana_Taylor6731_DirectSeq
I_piedmontana_Schafran102-1_Cluster0_size=1297
I_piedmontana_Schafran102-2_Cluster0_size=780
I_occidentalis_WoodbridgeSN1-5_Cluster3_size=5
I_sp_Schafran210-pop_n=4
I_sp_Greenhouse31_Small_Cluster1_size=308
I_silvatica_Taylor6777_DirectSeq
I_virginica_Fleming16376_Cluster2_n=3
I_silvatica_Taylor6724_DirectSeq
I_mattaponica_Bradley8670-rep2_Cluster0_size=213
I_hyemalis_clone_X1-2_AY541789
I_hyemalis_Schafran127-pop_Cluster0_n=5
I_sp_UnknownChickahominy2_rep1_Cluster0_size=35
I_virginica_Taylor6882_Cluster0_size=54
I_melanopoda_ssp_silvatica_SchafranNC05_Cluster0_size=142
I_melanopoda_ssp_silvatica_SchafranNC05_DirectSeq
I_hyemalis_Schafran137-pop_Cluster0_n=3
I_microvela_MatthewsI09-35_Cluster1_n=2
I_microvela_BolinJBNC200EO3B_Cluster2_size=50
I_Edisto_Schafran87-5_Cluster4_size=4
I_hyemalis_Brunton19012_Cluster1_size=60
I_hyemalis_Schafran125-pop_Cluster1_n=3
I_appalachiana_X_hyemalis_Brunton19011B_Cluster3_n=2
I_hyemalis_Schafran135_Cluster2_n=3
I_bolanderi_X_occidentalis_Taylor6759_rep1_Cluster2_size=49
I_microvela_BolinJBNC199EO2_Cluster0_n=2
I_louisianensis_clone_X_AY541793
I_louisianensis_Alford397_Cluster1_size=253
I_louisianensis_Alford402_Cluster1_size=342
I_louisianensis_Alford398_Cluster1_size=170
74.7/42
97.3/93
97.9/100
88.9/100
92.3/92
95.2/100
86/69
96.4/100
I. silvatica clade

FIGURE 20. Continued.



Isoëtes tegetiformans was resolved on a short, weakly supported branch sister to many sequences from putative hexaploid plants identified as *I. georgiana*, *I. boomii*, and *I. microvela* (Figure 21). Poor support and relatively long branch lengths makes it unlikely that *I. tegetiformans* was involved in the formation of these polyploids.

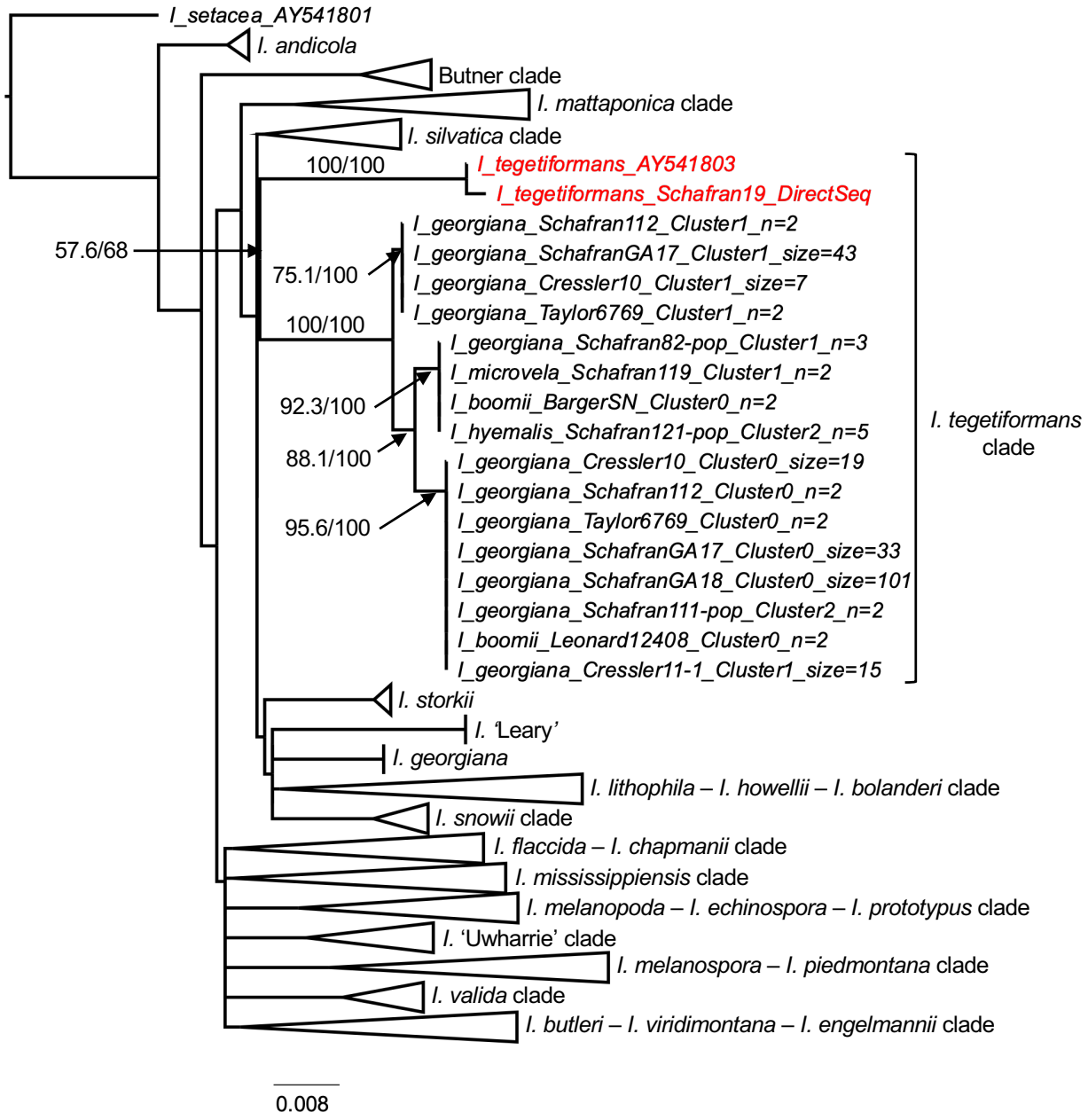


FIGURE 21. "*I. tegetiformans* clade" in LEAFY maximum likelihood phylogeny. Branch support values are approximate likelihood ratio test (ALRT)/ultrafast bootstrap (UFBS). Support values removed where ALRT or UFBS was less than 50. Scale equals expected number of substitutions per site. Red tip labels indicate specimens recognized as *I. tegetiformans s.s.*

The Costa Rican *I. storkii* is sister to a polytomy containing several North America taxa, including diploids *I. lithophila, I. howellii*, *I. bolanderi*, and *I. snowii* (Figure 22). Several clades represent groups of sequences from *I*. 'Leary' (separate from the *I.* 'Leary' clade below), *I. georgiana, I. hyemalis, I. virginica, I. occidentalis,* and *I. lacustris*. There are no diploids included with any of these groups. The presumed diploid parent of the clade including *I. occidentalis* and *I. lacustris* was described as Unknown Z by Hoot et al. (2004). *Isoëtes lithophila* assumed a sister position to a clade of taxa from western North America, including the diploids *I. bolanderi* and *I. howellii*, tetraploid *I. maritima*, and hybrids including any of those species. The sequence from *I. butleri Taylor 6788,* collected from Georgia, is presumed to be erroneous.

FIGURE 22. "*I. storkii--I. lithophila--I. bolanderi--I. howellii* clade" in LEAFY maximum likelihood phylogeny. Branch support values are approximate likelihood ratio test (ALRT)/ultrafast bootstrap (UFBS). Support values shown only within and adjacent to focal clade. Scale equals expected number of substitutions per site. Red tip labels indicate diploid specimens recognized as their respective species *sensu stricto.*

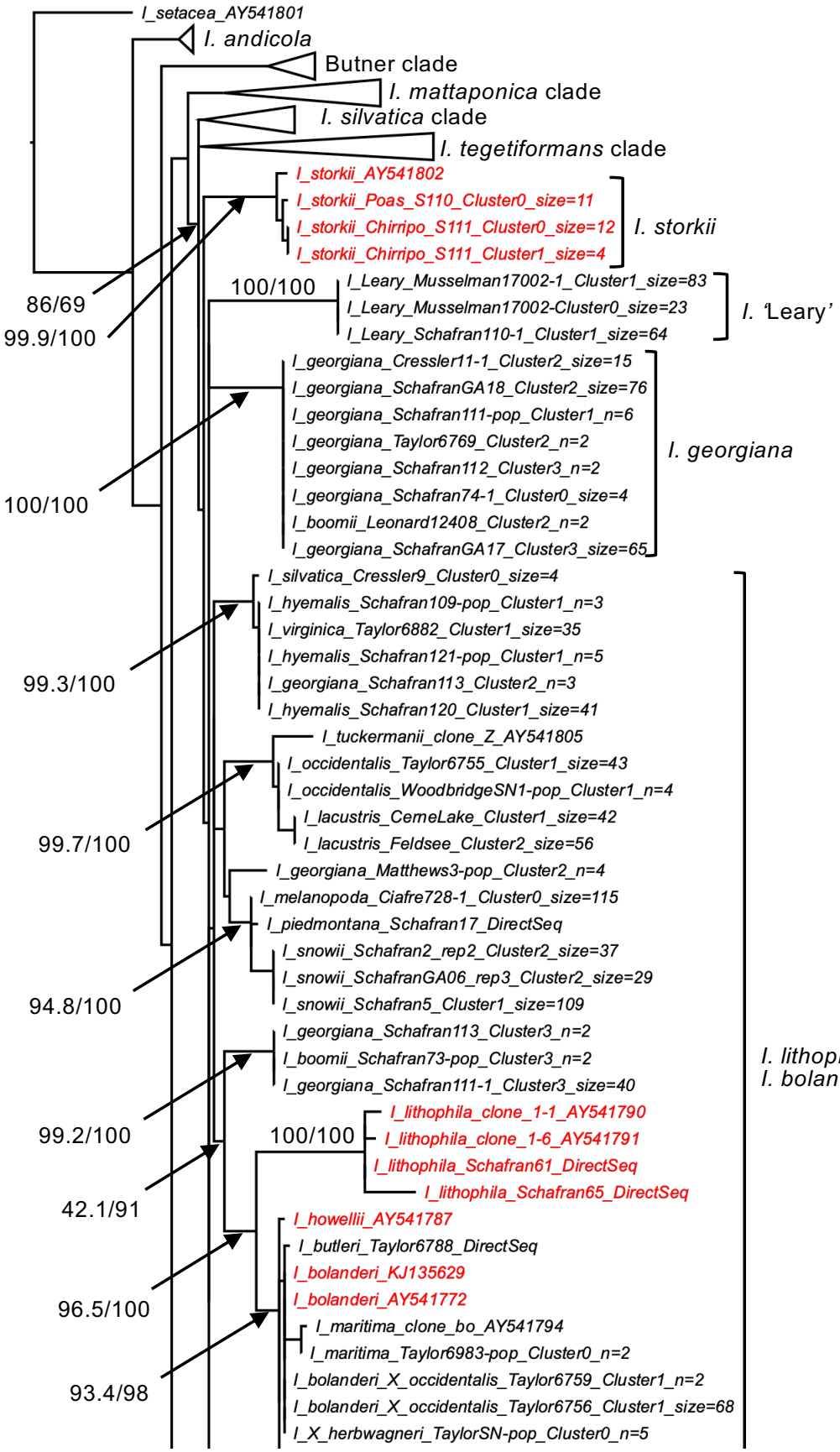
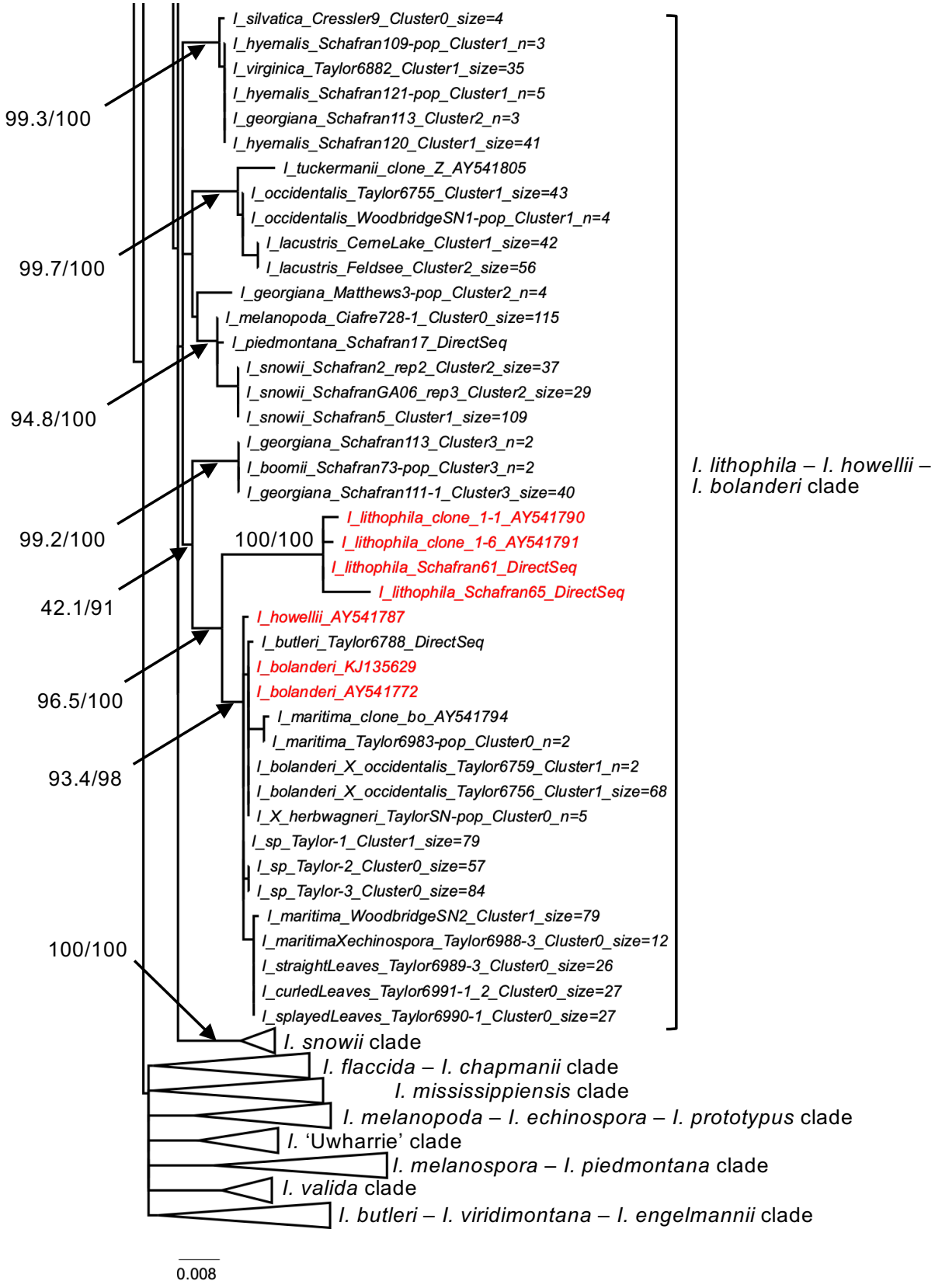

I_setacea_AY541801
I. andicola
Butner clade
I. mattaponica clade
I. silvatica clade
I. tegetiformans clade
I_storkii_AY541802
I_storkii_Poas_S110_Cluster0_size=11
I_storkii_Chirripo_S111_Cluster0_size=12
I_storkii_Chirripo_S111_Cluster1_size=4
I. storkii
86/69
99.9/100
100/100
I_Leary_Musselman17002-1_Cluster1_size=83
I_Leary_Musselman17002-Cluster0_size=23
I_Leary_Schafran110-1_Cluster1_size=64
I. 'Leary'
I_georgiana_Cressler11-1_Cluster2_size=15
I_georgiana_SchafranGA18_Cluster2_size=76
I_georgiana_Schafran111-pop_Cluster1_n=6
I_georgiana_Taylor6769_Cluster2_n=2
I_georgiana_Schafran112_Cluster3_n=2
I_georgiana_Schafran74-1_Cluster0_size=4
I_boomii_Leonard12408_Cluster2_n=2
I_georgiana_SchafranGA17_Cluster3_size=65
I. georgiana
100/100
I_silvatica_Cressler9_Cluster0_size=4
I_hyemalis_Schafran109-pop_Cluster1_n=3
I_virginica_Taylor6882_Cluster1_size=35
I_hyemalis_Schafran121-pop_Cluster1_n=5
I_georgiana_Schafran113_Cluster2_n=3
I_hyemalis_Schafran120_Cluster1_size=41
99.3/100
I_tuckermanii_clone_Z_AY541805
I_occidentalis_Taylor6755_Cluster1_size=43
I_occidentalis_WoodbridgeSN1-pop_Cluster1_n=4
I_lacustris_CerneLake_Cluster1_size=42
I_lacustris_Feldsee_Cluster2_size=56
99.7/100
I_georgiana_Matthews3-pop_Cluster2_n=4
I_melanopoda_Ciafre728-1_Cluster0_size=115
I_piedmontana_Schafran17_DirectSeq
I_snowii_Schafran2_rep2_Cluster2_size=37
I_snowii_SchafranGA06_rep3_Cluster2_size=29
I_snowii_Schafran5_Cluster1_size=109
94.8/100
I_georgiana_Schafran113_Cluster3_n=2
I_boomii_Schafran73-pop_Cluster3_n=2
I_georgiana_Schafran111-1_Cluster3_size=40
99.2/100
I_lithophila_clone_1-1_AY541790
100/100
I_lithophila_clone_1-6_AY541791
I_lithophila_Schafran61_DirectSeq
I_lithophila_Schafran65_DirectSeq
42.1/91
I_howellii_AY541787
I_butleri_Taylor6788_DirectSeq
I_bolanderi_KJ135629
I_bolanderi_AY541772
96.5/100
I_maritima_clone_bo_AY541794
I_maritima_Taylor6983-pop_Cluster0_n=2
93.4/98
I_bolanderi_X_occidentalis_Taylor6759_Cluster1_n=2
I_bolanderi_X_occidentalis_Taylor6756_Cluster1_size=68
I_X_herbwagneri_TaylorSN-pop_Cluster0_n=5
I. lithophila – I. howellii –
I. bolanderi clade

FIGURE 22. Continued.

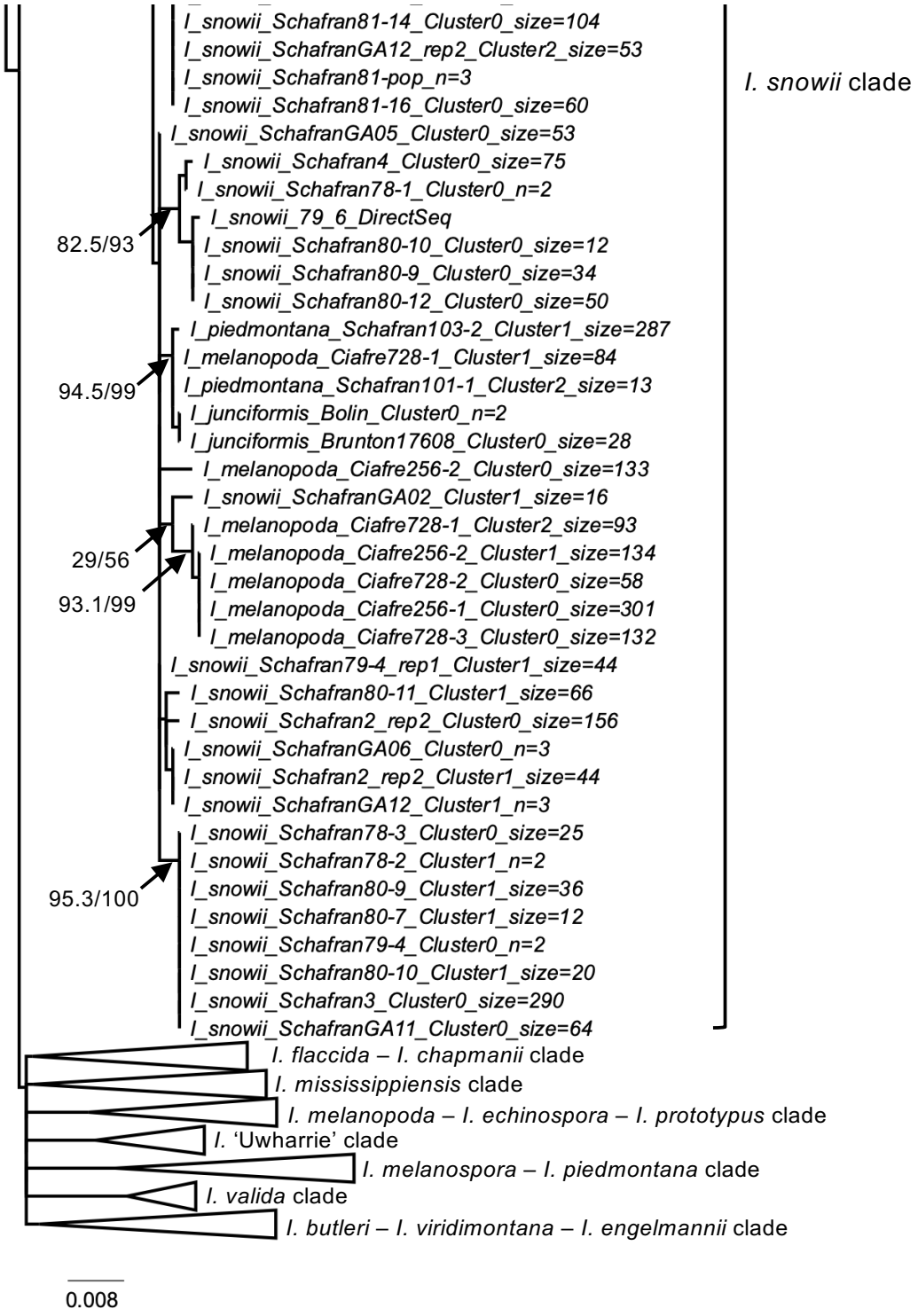


Isoëtes "snowii" represents undescribed diploid and tetraploid taxa present on sandstone outcrops in southeastern Georgia (Figure 23). Sequences from these individuals form a single clade, but the number of OTUs identified in diploid individuals was variable and greater than observed for any other diploid species. While diploid individuals occur only in one locality, sequences from *I. junciformis*, *I. melanopoda s.l., I. piedmontana s.l.,* and *I. '*Leary*'* appeared in this clade. The presence of one cluster from a single replicate of *I. bolanderi* × *occidentalis Taylor 6759* is presumed to be a contaminant.

FIGURE 23. "*I. snowii* Clade" in LEAFY maximum likelihood phylogeny. Branch support values are approximate likelihood ratio test (ALRT)/ultrafast bootstrap (UFBS). Support values shown only within and adjacent to focal clade. Scale equals expected number of substitutions per site.

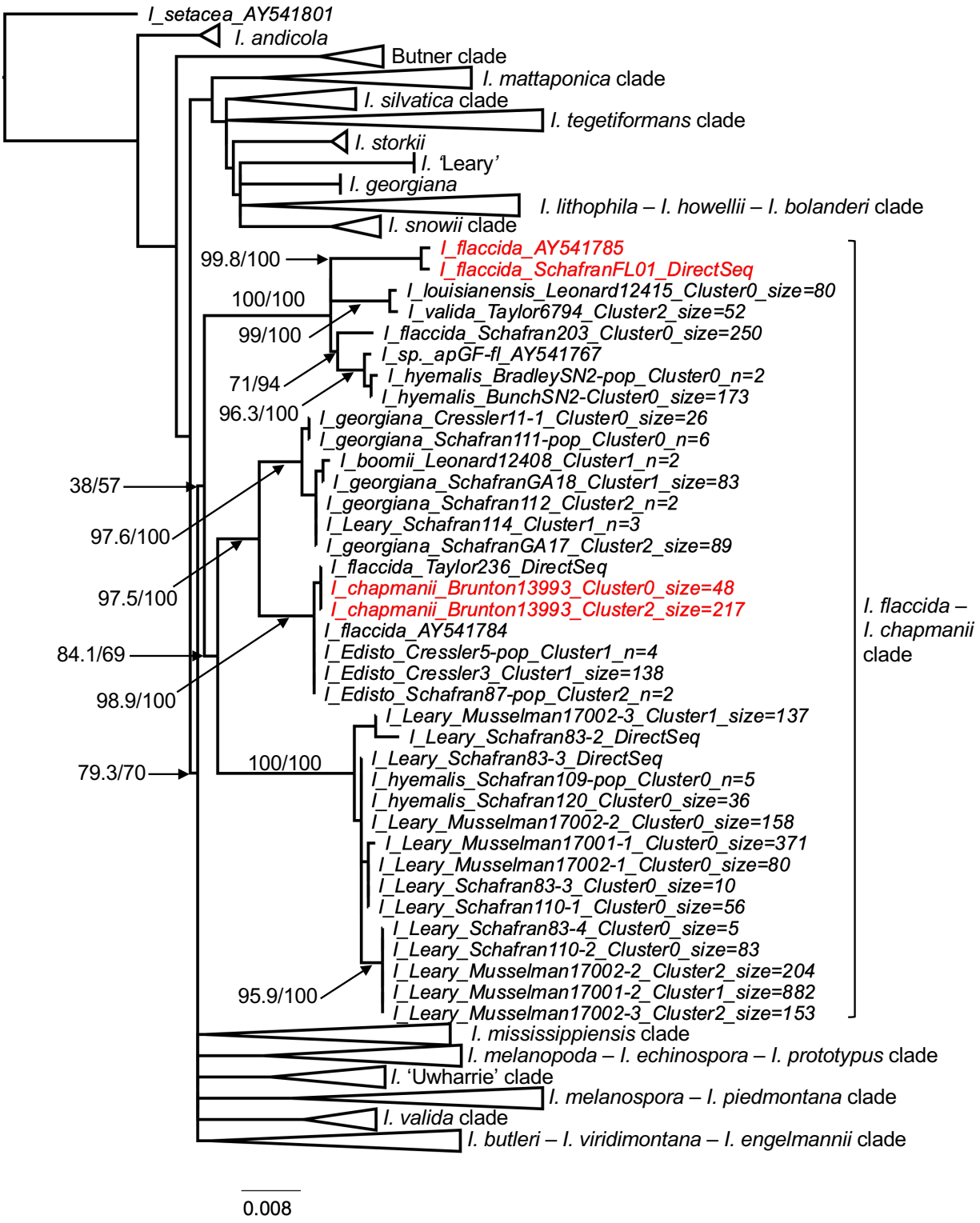

I_setacea_AY541801
I. andicola
Butner clade
I. mattaponica clade
I. silvatica clade
I. tegetiformans clade
I. storkii
I. 'Leary'
I. georgiana
I. lithophila – I. howellii – I. bolanderi clade
I_snowii_Schafran80-8_Cluster0_size=19
I_snowii_Schafran2_Cluster0_alignment_extraction_n=2
I_snowii_Schafran80-11_Cluster0_size=43
I_Leary_Schafran114_Cluster0_n=3
I_snowii_Schafran78-2_Cluster0_n=2
I_snowii_Schafran8-pop_Cluster0_n=2
I_piedmontana_Schafran102-3_Cluster2_size=508
I_piedmontana_Schafran102-3_Cluster1_size=286
I_piedmontana_Schafran103-1_Cluster0_size=319
I_junciformis_Schafran104_rep1_Cluster2_size=12
I_piedmontana_Schafran101-2_Cluster0_size=7
I_piedmontana_Schafran101-3_Cluster0_size=8
I_junciformis_Cressler12_Cluster0_size=20
I_junciformis_Schafran104_Cluster2_n=2
I_snowii_SchafranGA03_Cluster0_size=109
I_junciformis_Bolin_Cluster1_n=2
I_junciformis_Brunton17608_Cluster3_size=54
I_snowii_Schafran80-7_Cluster0_size=7
I_snowii_SchafranGA05_Cluster1_size=11
I_snowii_Schafran5_Cluster0_size=168
I_snowii_Schafran6_Cluster0_size=369
I_snowii_SchafranGA13_Cluster0_size=62
I_snowii_SchafranGA14_Cluster0_n=2
I_snowii_SchafranGA15_Cluster0_size=579
I_snowii_SchafranGA02_Cluster0_size=14
I_snowii_Schafran81-13_Cluster0_size=24
I_snowii_Schafran5_Cluster0_n=2
I_snowii_Schafran6_Cluster0_n=2
I_snowii_SchafranGA01_Cluster0_n=2
I_snowii_Schafran81-14_Cluster0_size=104
I_snowii_SchafranGA12_rep2_Cluster2_size=53
I_snowii_Schafran81-pop_n=3
I_snowii_Schafran81-16_Cluster0_size=60
I_snowii_SchafranGA05_Cluster0_size=53
77.6/68
77.2/92
90.7/100
100/100
I. snowii clade

FIGURE 23. Continued.



Three clades were weakly united containing *I. flaccida*, *I. chapmanii* (=*I. flaccida* var. *chapmanii*), and *I.* 'Leary', an undescribed diploid from western Georgia (Figure 24). The *I. flaccida s.s.* clade included few sequences from polyploids, one collection each of *I. hyemalis* and *I. appalachiana*, and two collections of *I. louisianensis* (one misidentified as *I. valida*). Sequences from *I. chapmanii* (including two lumped in *I. flaccida*) were closely placed to OTUs from collections of a suspected hexaploid from the Edisto River, South Carolina. A well supported sister clade to *I. chapmanii* + *I.* 'Edisto' contained OTUs from *I. georgiana*, *I. boomii*, and a single individual of *I.* 'Leary'. Sister to the *I. chapmanii* + *I.* 'Edisto' + *I. georgiana-I. boomii* clade is a clade containing the majority of sequences from *I.* 'Leary' representing putatively undescribed diploid and polyploid taxa. All samples in this clade originated in southwest Georgia, including two identified as *I. hyemalis*.

FIGURE 24. "*I. flaccida*--*I. chapmanii* clade" in LEAFY maximum likelihood phylogeny. Branch support values are approximate likelihood ratio test (ALRT)/ultrafast bootstrap (UFBS). Support values shown only within and adjacent to focal clade. Scale equals expected number of substitutions per site. Red tip labels indicate specimens recognized as respective species *sensu stricto*.


I_setacea_AY541801
I. andicola
Butner clade
I. mattaponica clade
I. silvatica clade
I. tegetiformans clade
I. storkii
I. 'Leary'
I. georgiana
I. lithophila – I. howellii – I. bolanderi clade
I. snowii clade
99.8/100
I_flaccida_AY541785
I_flaccida_SchafranFL01_DirectSeq
100/100
I_louisianensis_Leonard12415_Cluster0_size=80
I_valida_Taylor6794_Cluster2_size=52
99/100
I_flaccida_Schafran203_Cluster0_size=250
I_sp._apGF-fl_AY541767
71/94
I_hyemalis_BradleySN2-pop_Cluster0_n=2
I_hyemalis_BunchSN2-Cluster0_size=173
96.3/100
I_georgiana_Cressler11-1_Cluster0_size=26
I_georgiana_Schafran111-pop_Cluster0_n=6
I_boomii_Leonard12408_Cluster1_n=2
38/57
I_georgiana_SchafranGA18_Cluster1_size=83
I_georgiana_Schafran112_Cluster2_n=2
97.6/100
I_Leary_Schafran114_Cluster1_n=3
I_georgiana_SchafranGA17_Cluster2_size=89
I_flaccida_Taylor236_DirectSeq
97.5/100
I_chapmanii_Brunton13993_Cluster0_size=48
I_chapmanii_Brunton13993_Cluster2_size=217
I_flaccida_AY541784
84.1/69
I_Edisto_Cressler5-pop_Cluster1_n=4
I_Edisto_Cressler3_Cluster1_size=138
98.9/100
I_Edisto_Schafran87-pop_Cluster2_n=2
I_Leary_Musselman17002-3_Cluster1_size=137
I_Leary_Schafran83-2_DirectSeq
79.3/70
100/100
I_Leary_Schafran83-3_DirectSeq
I_hyemalis_Schafran109-pop_Cluster0_n=5
I_hyemalis_Schafran120_Cluster0_size=36
I_Leary_Musselman17002-2_Cluster0_size=158
I_Leary_Musselman17001-1_Cluster0_size=371
I_Leary_Musselman17002-1_Cluster0_size=80
I_Leary_Schafran83-3_Cluster0_size=10
I_Leary_Schafran110-1_Cluster0_size=56
I_Leary_Schafran83-4_Cluster0_size=5
I_Leary_Schafran110-2_Cluster0_size=83
95.9/100
I_Leary_Musselman17002-2_Cluster2_size=204
I_Leary_Musselman17001-2_Cluster1_size=882
I_Leary_Musselman17002-3_Cluster2_size=153
I. flaccida – I. chapmanii clade
I. mississippiensis clade
I. melanopoda – I. echinospora – I. prototypus clade
I. 'Uwharrie' clade
I. melanospora – I. piedmontana clade
I. valida clade
I. butleri – I. viridimontana – I. engelmannii clade
0.008

Two weakly supported branches united one clade with OTUs from *I. andicola*, a South American tetraploid, two OTUs from *I. hyemalis*, and a large clade centered around *I. mississippiensis* (Figure 25). The *I. mississippiensis* clade contained many individuals that were identified as *I. piedmontana*, *I. melanopoda*, *I. louisianensis*, and *I. snowii*. The Unknown W from Hoot et al. (2004) appeared in this clade.

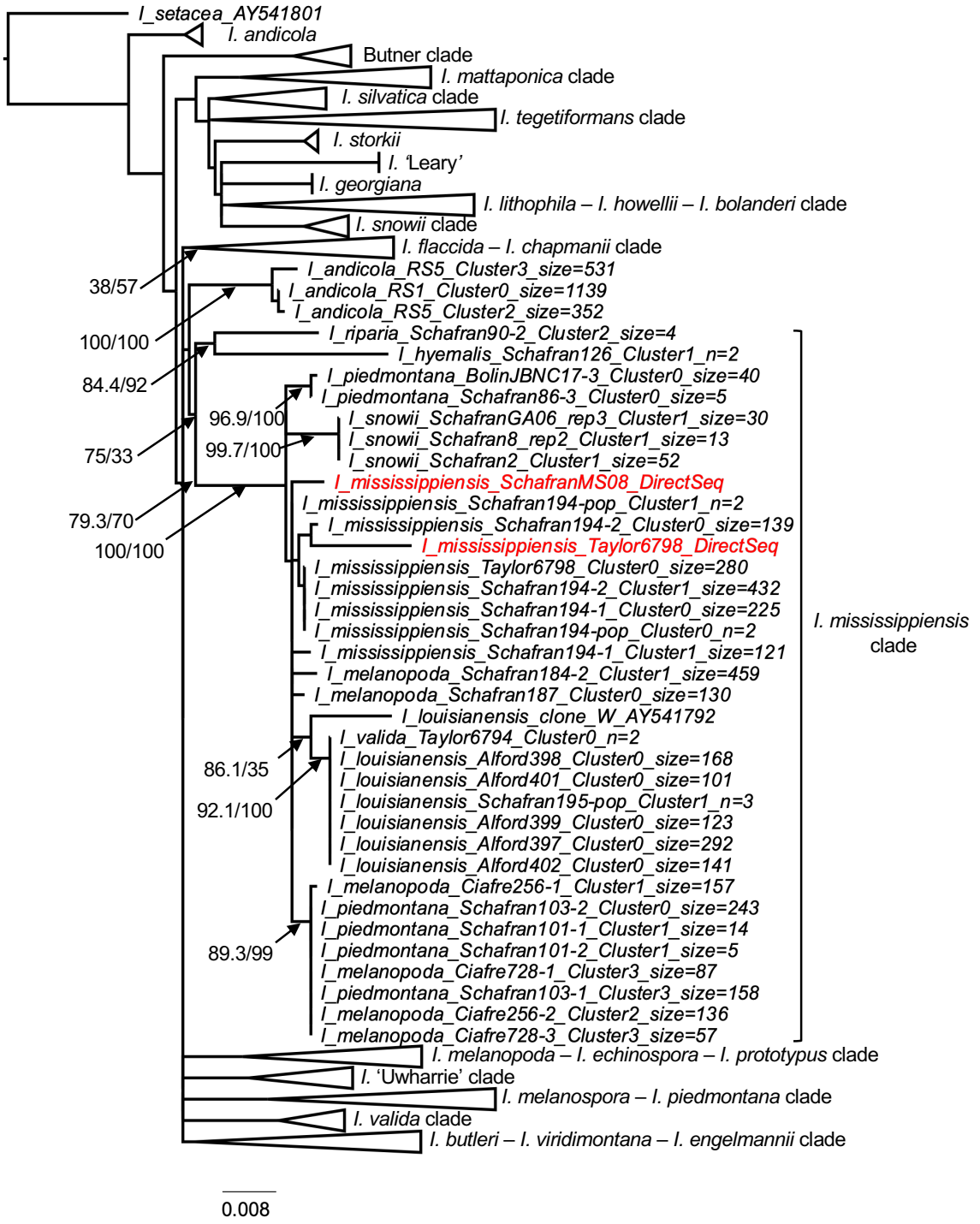


FIGURE 25. "*I. mississippiensis* clade" in LEAFY maximum likelihood phylogeny. Branch support values are approximate likelihood ratio test (ALRT)/ultrafast bootstrap (UFBS). Support values removed where ALRT or UFBS was less than 50. Scale equals expected number of substitutions per site. Red tip labels indicate specimens recognized as *I. mississippiensis s.s.*

Isoëtes melanopoda s.s. formed several well supported clades along a polytomy that also included *I. prototypus* and *I. echinospora* (Figure 26). *Isoëtes melanopoda Schafran 188* with *I. melanopoda s.l.*, *I. piedmontana s.l.*, and *I. junciformis* fell into a clade sister to the *I. melanopoda* + *I. echinospora* + *I. prototypus* polytomy. From this polytomy, three well supported clades arise each containing diploid individuals of *I. melanopoda*. Different polyploid taxa tended to segregate within these clades. The largest clade contained the diploid *I. melanopoda Taylor 6796* and OTUs from *I. microvela*, *I. hyemalis*, *I. virginica, I. riparia, I. junciformis, I.* '*laurentiana*', and *I.* 'Butner'. The second largest clade with diploid *I. melanopoda Smith 36037* and *I. melanopoda Schafran 188* also included putative polyploid individuals of *I. melanopoda*, *I.* 'Butner', and *I. virginica*. *Isoëtes prototypus* formed a moderately supported clade only slightly divergent from the *I. melanopoda* polytomy. Species in this clade were generally collected from the northern United States and Canada – *I. occidentalis*, *I. lacustris*, *I. tuckermanii s.l.*, *I. acadiensis s.l.*, and several hybrids. Likewise, *I. echinospora* occurred in a weakly supported, slightly divergent clade from the *I. melanopoda* polytomy. All individuals of *I. echinospora* occurred in this clade, in addition to polyploids and hybrids such as *I. riparia*, *I. septentrionalis*, *I. tuckermanii*, *I. maritima*, *I. occidentalis*, *I.* ×*eatonii*, *I.* ×*dodgei, and I.* ×*herbwagneri.*

FIGURE 26. "*I. melanopoda--I. prototypus--I. echinospora* clade" in LEAFY maximum likelihood phylogeny. Branch support values are approximate likelihood ratio test (ALRT)/ultrafast bootstrap (UFBS). Support values shown only within and adjacent to focal clade. Red tip labels indicate specimens recognized as respective species *sensu stricto*.

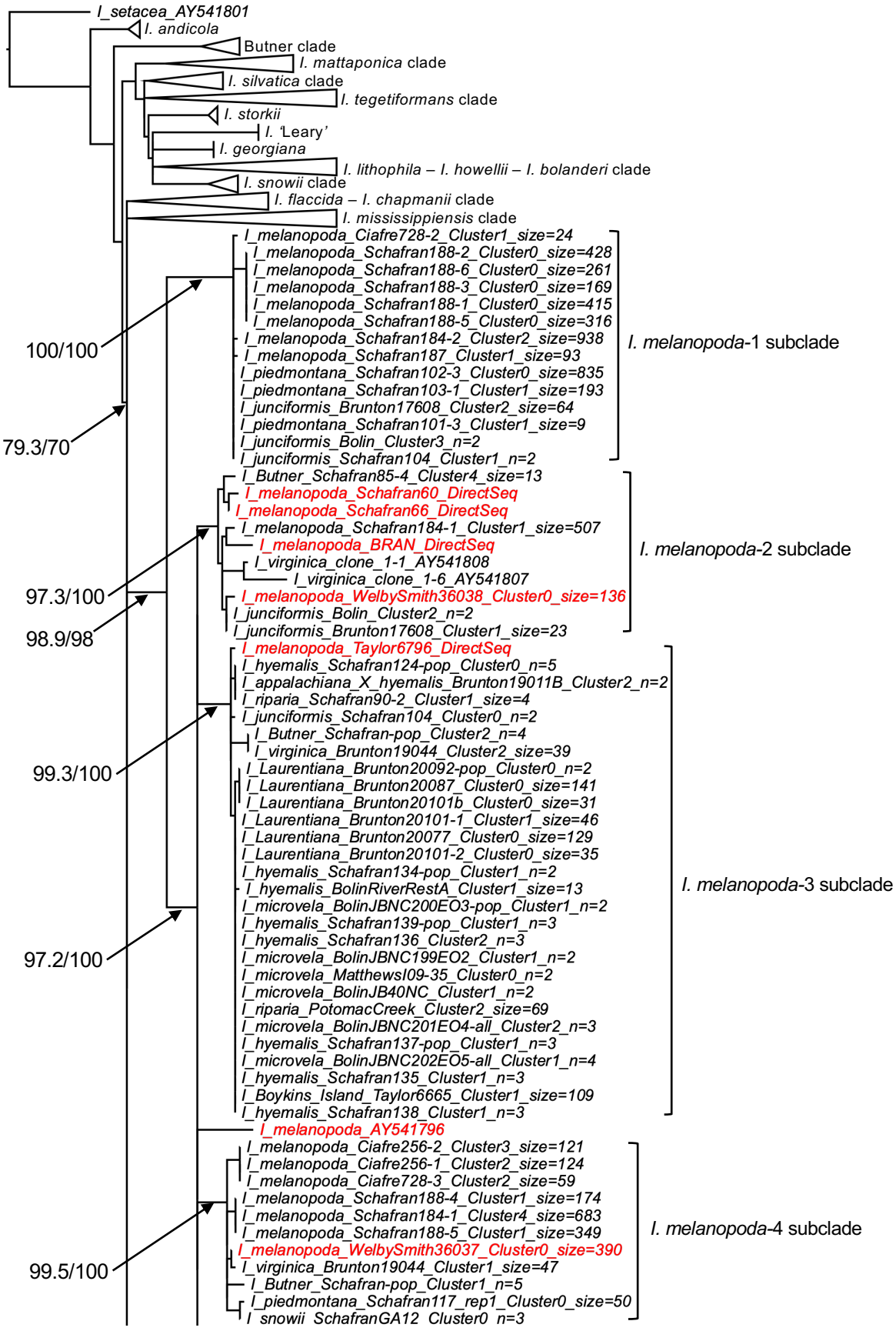
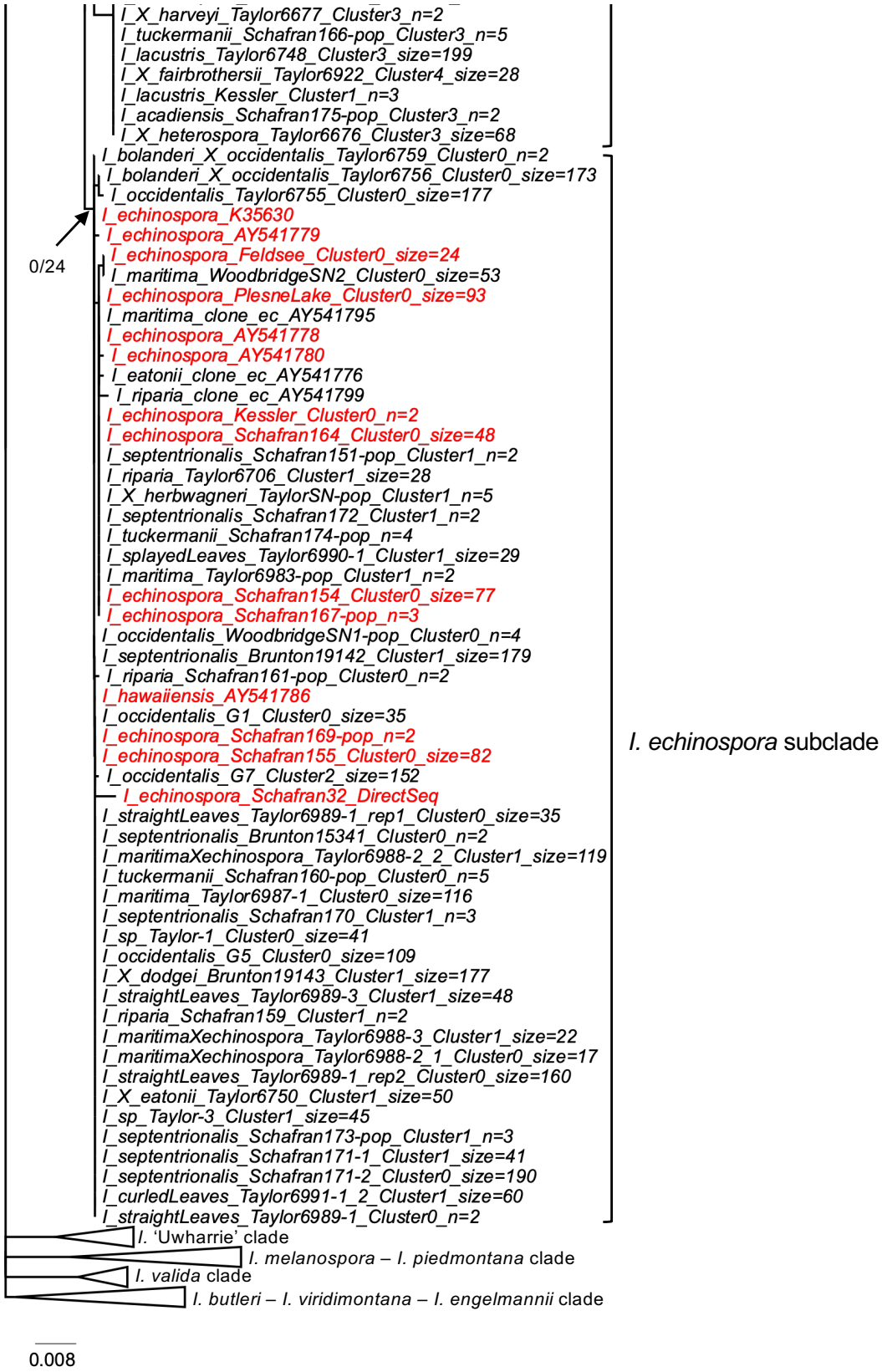

I_setacea_AY541801
I. andicola
Butner clade
I. mattaponica clade
I. silvatica clade
I. tegetiformans clade
I. storkii
I. 'Leary'
I. georgiana
I. lithophila – I. howellii – I. bolanderi clade
I. snowii clade
I. flaccida – I. chapmanii clade
I. mississippiensis clade
I_melanopoda_Ciafre728-2_Cluster1_size=24
I_melanopoda_Schafran188-2_Cluster0_size=428
I_melanopoda_Schafran188-6_Cluster0_size=261
I_melanopoda_Schafran188-3_Cluster0_size=169
I_melanopoda_Schafran188-1_Cluster0_size=415
I_melanopoda_Schafran188-5_Cluster0_size=316
I_melanopoda_Schafran184-2_Cluster2_size=938
I_melanopoda_Schafran187_Cluster1_size=93
I_piedmontana_Schafran102-3_Cluster0_size=835
I_piedmontana_Schafran103-1_Cluster1_size=193
I_junciformis_Brunton17608_Cluster2_size=64
I_piedmontana_Schafran101-3_Cluster1_size=9
I_junciformis_Bolin_Cluster3_n=2
I_junciformis_Schafran104_Cluster1_n=2
I. melanopoda-1 subclade
I_Butner_Schafran85-4_Cluster4_size=13
I_melanopoda_Schafran60_DirectSeq
I_melanopoda_Schafran66_DirectSeq
I_melanopoda_Schafran184-1_Cluster1_size=507
I_melanopoda_BRAN_DirectSeq
I_virginica_clone_1-1_AY541808
I_virginica_clone_1-6_AY541807
I_melanopoda_WelbySmith36038_Cluster0_size=136
I_junciformis_Bolin_Cluster2_n=2
I_junciformis_Brunton17608_Cluster1_size=23
I. melanopoda-2 subclade
I_melanopoda_Taylor6796_DirectSeq
I_hyemalis_Schafran124-pop_Cluster0_n=5
I_appalachiana_X_hyemalis_Brunton19011B_Cluster2_n=2
I_riparia_Schafran90-2_Cluster1_size=4
I_junciformis_Schafran104_Cluster0_n=2
I_Butner_Schafran-pop_Cluster2_n=4
I_virginica_Brunton19044_Cluster2_size=39
I_Laurentiana_Brunton20092-pop_Cluster0_n=2
I_Laurentiana_Brunton20087_Cluster0_size=141
I_Laurentiana_Brunton20101b_Cluster0_size=31
I_Laurentiana_Brunton20101-1_Cluster1_size=46
I_Laurentiana_Brunton20077_Cluster0_size=129
I_Laurentiana_Brunton20101-2_Cluster0_size=35
I_hyemalis_Schafran134-pop_Cluster1_n=2
I_hyemalis_BolinRiverRestA_Cluster1_size=13
I_microvela_BolinJBNC200EO3-pop_Cluster1_n=2
I_hyemalis_Schafran139-pop_Cluster1_n=3
I_hyemalis_Schafran136_Cluster2_n=3
I_microvela_BolinJBNC199EO2_Cluster1_n=2
I_microvela_MatthewsI09-35_Cluster0_n=2
I_microvela_BolinJB40NC_Cluster1_n=2
I_riparia_PotomacCreek_Cluster2_size=69
I_microvela_BolinJBNC201EO4-all_Cluster2_n=3
I_hyemalis_Schafran137-pop_Cluster1_n=3
I_microvela_BolinJBNC202EO5-all_Cluster1_n=4
I_hyemalis_Schafran135_Cluster1_n=3
I_Boykins_Island_Taylor6665_Cluster1_size=109
I_hyemalis_Schafran138_Cluster1_n=3
I. melanopoda-3 subclade
I_melanopoda_AY541796
I_melanopoda_Ciafre256-2_Cluster3_size=121
I_melanopoda_Ciafre256-1_Cluster2_size=124
I_melanopoda_Ciafre728-3_Cluster2_size=59
I_melanopoda_Schafran188-4_Cluster1_size=174
I_melanopoda_Schafran184-1_Cluster4_size=683
I_melanopoda_Schafran188-5_Cluster1_size=349
I_melanopoda_WelbySmith36037_Cluster0_size=390
I_virginica_Brunton19044_Cluster1_size=47
I_Butner_Schafran-pop_Cluster1_n=5
I_piedmontana_Schafran117_rep1_Cluster0_size=50
I_snowii_SchafranGA12_Cluster0_n=3
I. melanopoda-4 subclade
100/100
79.3/70
97.3/100
98.9/98
99.3/100
97.2/100
99.5/100

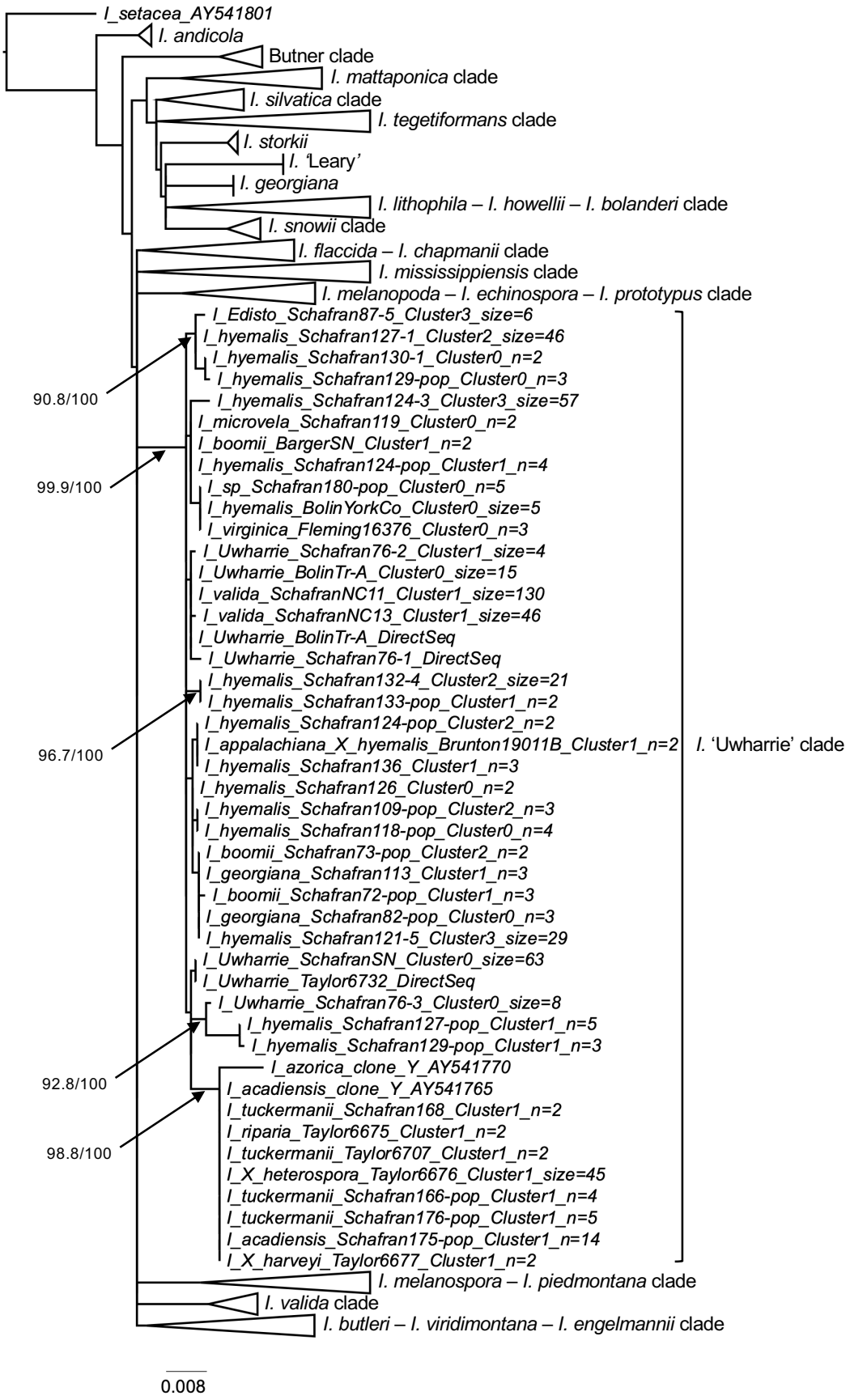
FIGURE 26. Continued.

FIGURE 26. Continued.



The “Uwharrie clade” contained only one diploid taxon, *I.* ‘Uwharrie’, an undescribed diploid collected in the Uwharrie Mountains of North Carolina (Figure 27). This is equivalent to Unknown Y from Hoot et al. (2004). This clade contains a variety of polyploid taxa including *I. hyemalis*, *I. microvela, I. virginica*, *I.* ‘Edisto’, *I. boomii*, *I. georgiana*, *I. tuckermanii*, *I. azorica, I. acadiensis*, and *I. riparia*. There is relatively little distance between members of this clade, with the exception of a subclade containing the taxa occurring in the northern US, Canada, and the Açores (*I. acadiensis*, *I. azorica*, *I. tuckermanii*, etc.).

FIGURE 27. “Uwharrie clade” in LEAFY maximum likelihood phylogeny. Branch support values are approximate likelihood ratio test (ALRT)/ultrafast bootstrap (UFBS). Support values shown only within and adjacent to focal clade. Scale equals expected number of substitutions per site.


I_setacea_AY541801
I. andicola
Butner clade
I. mattaponica clade
I. silvatica clade
I. tegetiformans clade
I. storkii
I. 'Leary'
I. georgiana
I. lithophila – I. howellii – I. bolanderi clade
I. snowii clade
I. flaccida – I. chapmanii clade
I. mississippiensis clade
I. melanopoda – I. echinospora – I. prototypus clade
I_Edisto_Schafran87-5_Cluster3_size=6
I_hyemalis_Schafran127-1_Cluster2_size=46
I_hyemalis_Schafran130-1_Cluster0_n=2
I_hyemalis_Schafran129-pop_Cluster0_n=3
90.8/100
I_hyemalis_Schafran124-3_Cluster3_size=57
I_microvela_Schafran119_Cluster0_n=2
I_boomii_BargerSN_Cluster1_n=2
I_hyemalis_Schafran124-pop_Cluster1_n=4
99.9/100
I_sp_Schafran180-pop_Cluster0_n=5
I_hyemalis_BolinYorkCo_Cluster0_size=5
I_virginica_Fleming16376_Cluster0_n=3
I_Uwharrie_Schafran76-2_Cluster1_size=4
I_Uwharrie_BolinTr-A_Cluster0_size=15
I_valida_SchafranNC11_Cluster1_size=130
I_valida_SchafranNC13_Cluster1_size=46
I_Uwharrie_BolinTr-A_DirectSeq
I_Uwharrie_Schafran76-1_DirectSeq
I_hyemalis_Schafran132-4_Cluster2_size=21
I_hyemalis_Schafran133-pop_Cluster1_n=2
I_hyemalis_Schafran124-pop_Cluster2_n=2
96.7/100
I_appalachiana_X_hyemalis_Brunton19011B_Cluster1_n=2
I. 'Uwharrie' clade
I_hyemalis_Schafran136_Cluster1_n=3
I_hyemalis_Schafran126_Cluster0_n=2
I_hyemalis_Schafran109-pop_Cluster2_n=3
I_hyemalis_Schafran118-pop_Cluster0_n=4
I_boomii_Schafran73-pop_Cluster2_n=2
I_georgiana_Schafran113_Cluster1_n=3
I_boomii_Schafran72-pop_Cluster1_n=3
I_georgiana_Schafran82-pop_Cluster0_n=3
I_hyemalis_Schafran121-5_Cluster3_size=29
I_Uwharrie_SchafranSN_Cluster0_size=63
I_Uwharrie_Taylor6732_DirectSeq
I_Uwharrie_Schafran76-3_Cluster0_size=8
I_hyemalis_Schafran127-pop_Cluster1_n=5
I_hyemalis_Schafran129-pop_Cluster1_n=3
92.8/100
I_azorica_clone_Y_AY541770
I_acadiensis_clone_Y_AY541765
98.8/100
I_tuckermanii_Schafran168_Cluster1_n=2
I_riparia_Taylor6675_Cluster1_n=2
I_tuckermanii_Taylor6707_Cluster1_n=2
I_X_heterospora_Taylor6676_Cluster1_size=45
I_tuckermanii_Schafran166-pop_Cluster1_n=4
I_tuckermanii_Schafran176-pop_Cluster1_n=5
I_acadiensis_Schafran175-pop_Cluster1_n=14
I_X_harveyi_Taylor6677_Cluster1_n=2
I. melanospora – I. piedmontana clade
I. valida clade
I. butleri – I. viridimontana – I. engelmannii clade
0.008

Isoëtes piedmontana s.s. and *I. melanospora* formed a clade with only two polyploid individuals, *I. piedmontana s.l*. (treated as *I. graniticola* in the plastome phylogeny) and *I. louisianensis* (Figure 28). There is no clear phylogenetic separation between *I. piedmontana* and *I. melanospora*.

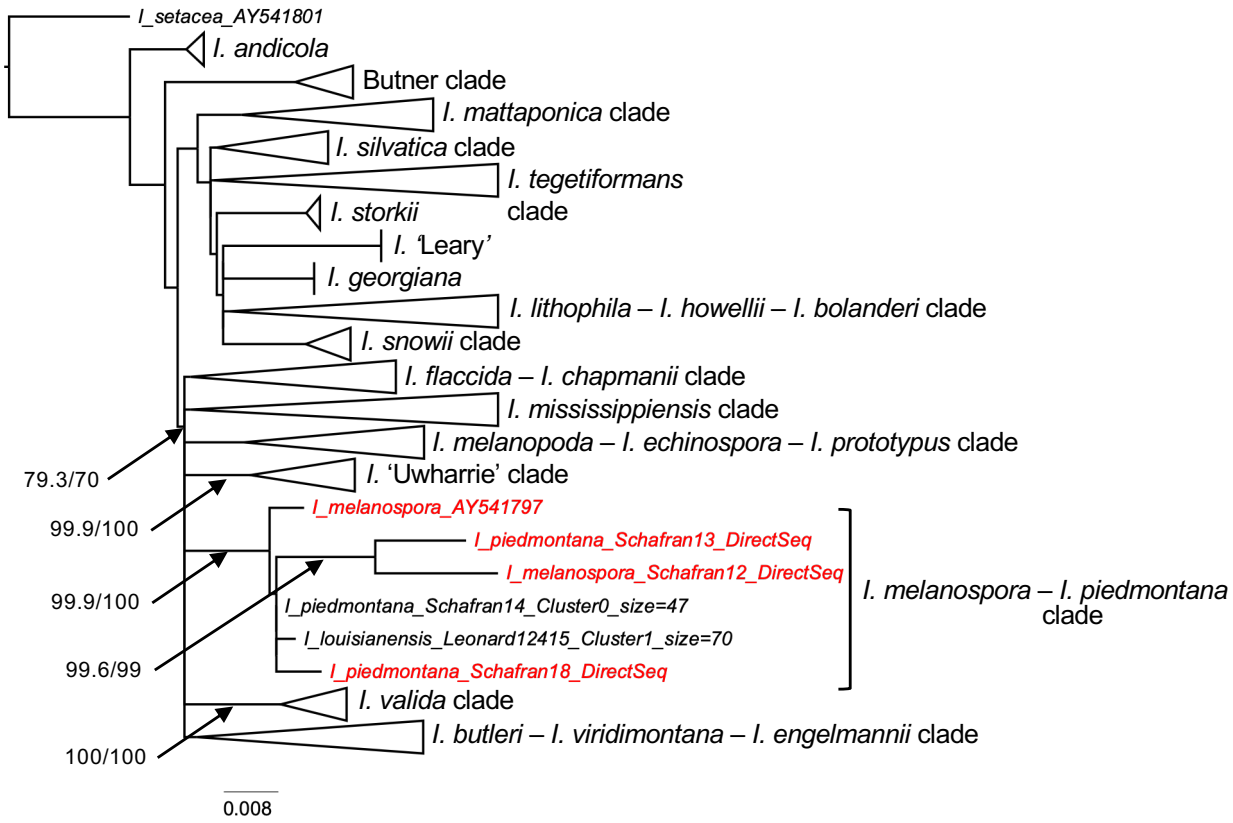
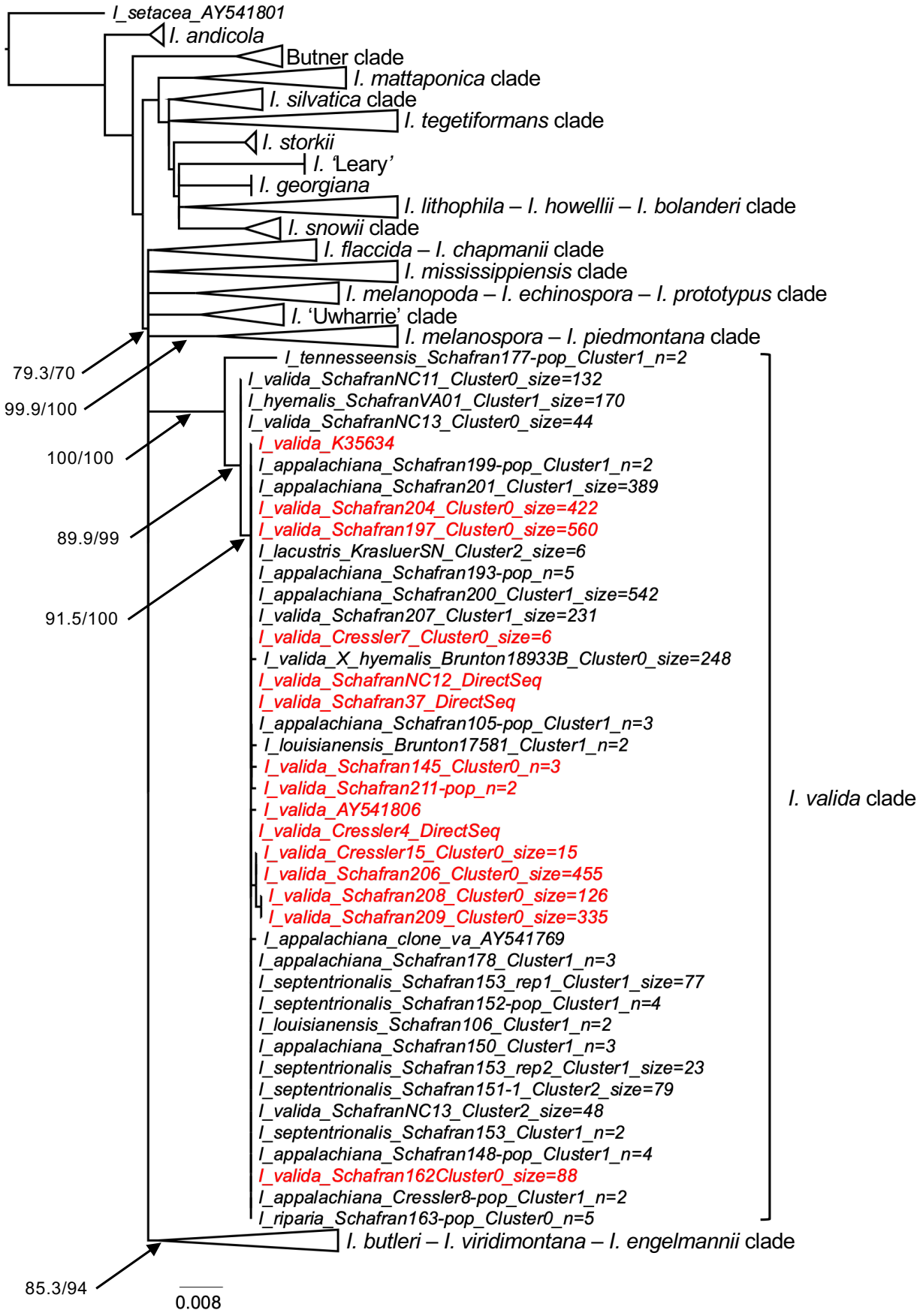


FIGURE 28. "*I. melanospora—I. piedmontana* clade" in LEAFY maximum likelihood phylogeny. Branch support values are approximate likelihood ratio test (ALRT)/ultrafast bootstrap (UFBS). Support values removed where ALRT or UFBS was less than 50. Scale equals expected number of substitutions per site. Red tip labels indicate specimens recognized as respective species *sensu stricto.*

Isoëtes valida formed a mostly flat clade, predominantly with *I. appalachiana*, but also individuals of *I. hyemalis*, *I. septentrionalis*, *I. riparia*, and *I. louisianensis* (Figure 29). OTUs from *I. tennesseensis* resolved sister to the rest of the *I. valida* clade.

FIGURE 29. "*I. valida* clade" in LEAFY maximum likelihood phylogeny. Branch support values are approximate likelihood ratio test (ALRT)/ultrafast bootstrap (UFBS). Support values shown only within and adjacent to focal clade. Scale equals expected number of substitutions per site. Red tip labels indicate specimens recognized as *I. valida s.s.*


I_setacea_AY541801
I. andicola
Butner clade
I. mattaponica clade
I. silvatica clade
I. tegetiformans clade
I. storkii
I. 'Leary'
I. georgiana
I. lithophila – I. howellii – I. bolanderi clade
I. snowii clade
I. flaccida – I. chapmanii clade
I. mississippiensis clade
I. melanopoda – I. echinospora – I. prototypus clade
I. 'Uwharrie' clade
I. melanospora – I. piedmontana clade
79.3/70
99.9/100
100/100
89.9/99
91.5/100
I_tennesseensis_Schafran177-pop_Cluster1_n=2
I_valida_SchafranNC11_Cluster0_size=132
I_hyemalis_SchafranVA01_Cluster1_size=170
I_valida_SchafranNC13_Cluster0_size=44
I_valida_K35634
I_appalachiana_Schafran199-pop_Cluster1_n=2
I_appalachiana_Schafran201_Cluster1_size=389
I_valida_Schafran204_Cluster0_size=422
I_valida_Schafran197_Cluster0_size=560
I_lacustris_KrasluerSN_Cluster2_size=6
I_appalachiana_Schafran193-pop_n=5
I_appalachiana_Schafran200_Cluster1_size=542
I_valida_Schafran207_Cluster1_size=231
I_valida_Cressler7_Cluster0_size=6
I_valida_X_hyemalis_Brunton18933B_Cluster0_size=248
I_valida_SchafranNC12_DirectSeq
I_valida_Schafran37_DirectSeq
I_appalachiana_Schafran105-pop_Cluster1_n=3
I_louisianensis_Brunton17581_Cluster1_n=2
I_valida_Schafran145_Cluster0_n=3
I_valida_Schafran211-pop_n=2
I_valida_AY541806
I_valida_Cressler4_DirectSeq
I_valida_Cressler15_Cluster0_size=15
I_valida_Schafran206_Cluster0_size=455
I_valida_Schafran208_Cluster0_size=126
I_valida_Schafran209_Cluster0_size=335
I_appalachiana_clone_va_AY541769
I_appalachiana_Schafran178_Cluster1_n=3
I_septentrionalis_Schafran153_rep1_Cluster1_size=77
I_septentrionalis_Schafran152-pop_Cluster1_n=4
I_louisianensis_Schafran106_Cluster1_n=2
I_appalachiana_Schafran150_Cluster1_n=3
I_septentrionalis_Schafran153_rep2_Cluster1_size=23
I_septentrionalis_Schafran151-1_Cluster2_size=79
I_valida_SchafranNC13_Cluster2_size=48
I_septentrionalis_Schafran153_Cluster1_n=2
I_appalachiana_Schafran148-pop_Cluster1_n=4
I_valida_Schafran162Cluster0_size=88
I_appalachiana_Cressler8-pop_Cluster1_n=2
I_riparia_Schafran163-pop_Cluster0_n=5
I. valida clade
I. butleri – I. viridimontana – I. engelmannii clade
85.3/94
0.008

Isoëtes butleri was inferred as sister to the *I. viridimontana* + *I. engelmannii* clade with moderate support (Figure 30). There were no sequences from any other taxa included in the *I. butleri* clade. The *I. viridimontana* clade, sister to the *I. engelmannii* clade, contained sequences from several polyploids and hybrids: *I. lacustris*, *I. tuckermanii*, *I. riparia*, *I. acadiensis*, *I.* ×*heterospora*, *I.* ×*harveyi,* and *I.* ×*fairbrothersii.* Included are sequences from Hoot et al. (2004) identified as originating from *I. engelmannii*. Subtending the polytomy on which *I. viridimontana* appeared are sequences from *I. boomii* and *I. piedmontana* (treated as *I. graniticola* in plastome phylogeny). The *I. engelmannii* clade is comprised of two moderately supported subclades, each containing diploid individuals representative of *I. engelmannii* s.s. One clade included sequences from *I. septentrionalis*, *I.* '*laurentiana*', *I. tuckermanii, I. riparia, I. appalachiana, I.* ×*dodgei, I.* ×*eatonii*, and *I.* ×*fairbrothersii*. The other included *I. appalachiana, I. louisianensis, I. hyemalis, I. tennesseensis, I. georgiana, I. melanopoda*, and *I.* ×*eatonii.*

FIGURE 30. "*I. butleri—I. viridimontana—I. engelmannii* Clade" in LEAFY maximum likelihood phylogeny. Branch support values are approximate likelihood ratio test (ALRT)/ultrafast bootstrap (UFBS). Support values shown only within and adjacent to focal clade. Scale equals expected number of substitutions per site. Red tip labels indicate specimens recognized as respective species *sensu stricto.*

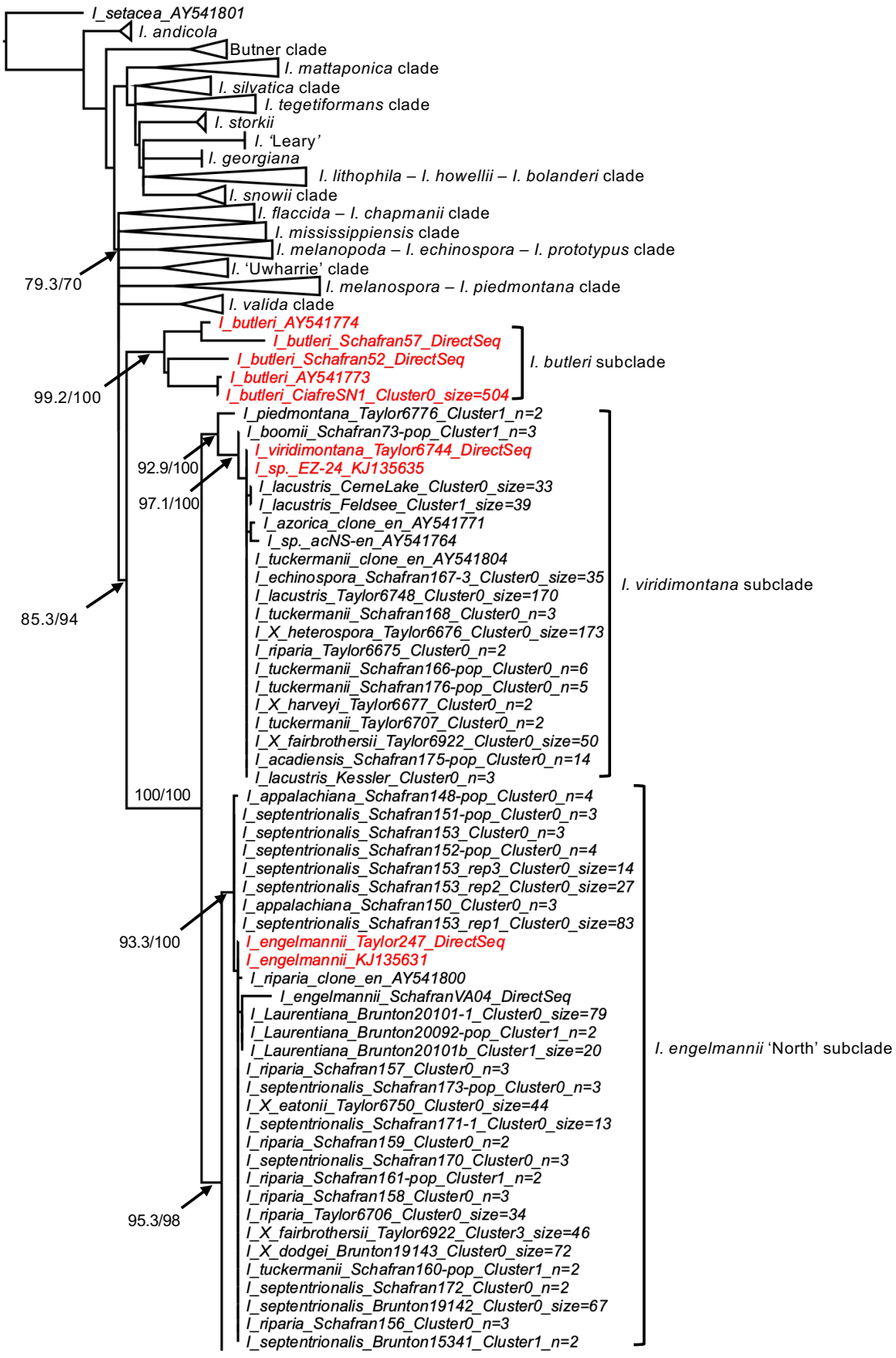
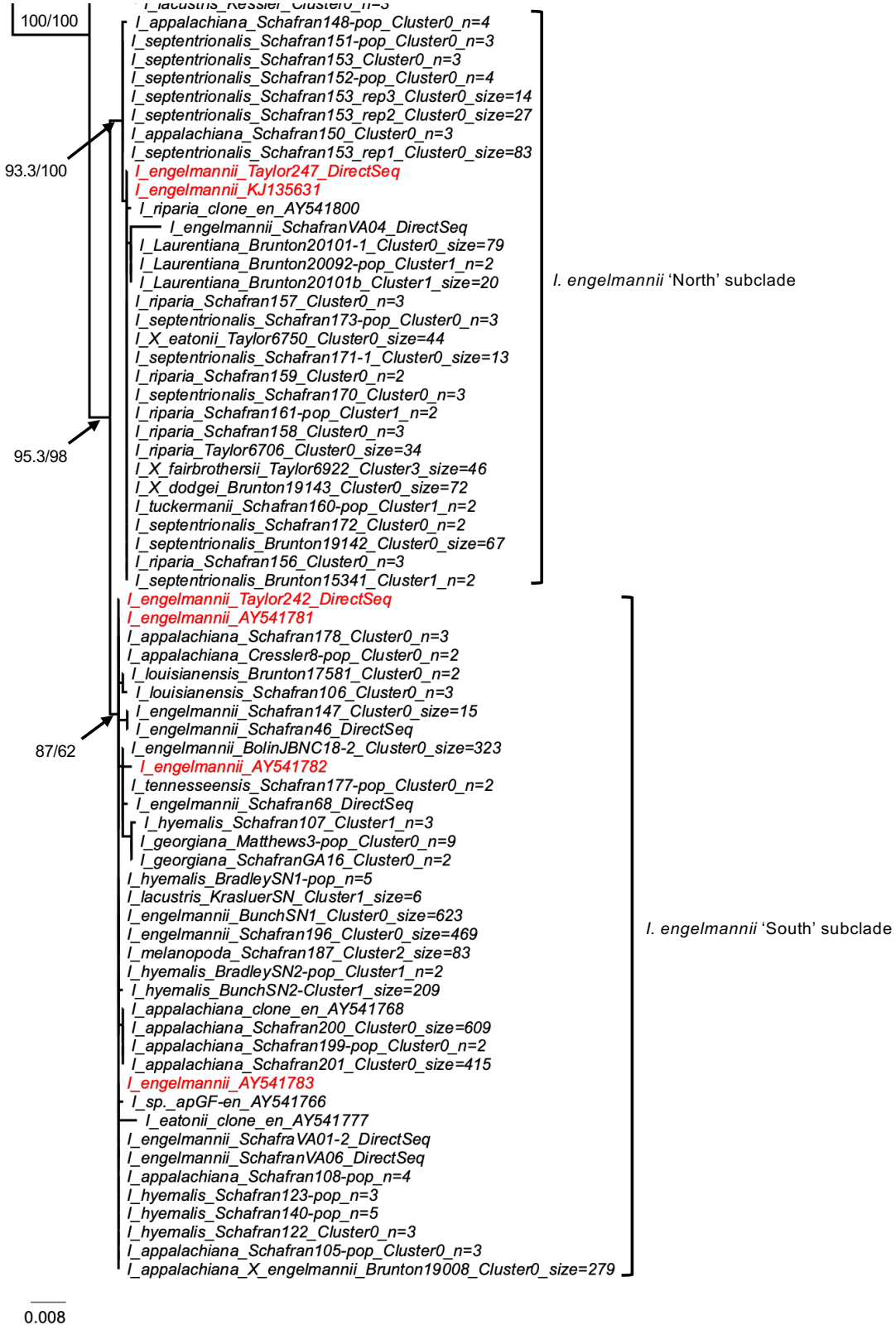

I_setacea_AY541801
I. andicola
Butner clade
I. mattaponica clade
I. silvatica clade
I. tegetiformans clade
I. storkii
I. 'Leary'
I. georgiana
I. lithophila – I. howellii – I. bolanderi clade
I. snowii clade
I. flaccida – I. chapmanii clade
I. mississippiensis clade
I. melanopoda – I. echinospora – I. prototypus clade
I. 'Uwharrie' clade
I. melanospora – I. piedmontana clade
I. valida clade
79.3/70
I_butleri_AY541774
I_butleri_Schafran57_DirectSeq
I_butleri_Schafran52_DirectSeq
I_butleri_AY541773
I_butleri_CiafreSN1_Cluster0_size=504
I. butleri subclade
99.2/100
I_piedmontana_Taylor6776_Cluster1_n=2
I_boomii_Schafran73-pop_Cluster1_n=3
I_viridimontana_Taylor6744_DirectSeq
I_sp._EZ-24_KJ135635
I_lacustris_CerneLake_Cluster0_size=33
I_lacustris_Feldsee_Cluster1_size=39
I_azorica_clone_en_AY541771
I_sp._acNS-en_AY541764
I_tuckermanii_clone_en_AY541804
I_echinospora_Schafran167-3_Cluster0_size=35
I_lacustris_Taylor6748_Cluster0_size=170
I_tuckermanii_Schafran168_Cluster0_n=3
I_X_heterospora_Taylor6676_Cluster0_size=173
I_riparia_Taylor6675_Cluster0_n=2
I_tuckermanii_Schafran166-pop_Cluster0_n=6
I_tuckermanii_Schafran176-pop_Cluster0_n=5
I_X_harveyi_Taylor6677_Cluster0_n=2
I_tuckermanii_Taylor6707_Cluster0_n=2
I_X_fairbrothersii_Taylor6922_Cluster0_size=50
I_acadiensis_Schafran175-pop_Cluster0_n=14
I_lacustris_Kessler_Cluster0_n=3
I. viridimontana subclade
92.9/100
97.1/100
85.3/94
100/100
I_appalachiana_Schafran148-pop_Cluster0_n=4
I_septentrionalis_Schafran151-pop_Cluster0_n=3
I_septentrionalis_Schafran153_Cluster0_n=3
I_septentrionalis_Schafran152-pop_Cluster0_n=4
I_septentrionalis_Schafran153_rep3_Cluster0_size=14
I_septentrionalis_Schafran153_rep2_Cluster0_size=27
I_appalachiana_Schafran150_Cluster0_n=3
I_septentrionalis_Schafran153_rep1_Cluster0_size=83
I_engelmannii_Taylor247_DirectSeq
I_engelmannii_KJ135631
I_riparia_clone_en_AY541800
I_engelmannii_SchafranVA04_DirectSeq
I_Laurentiana_Brunton20101-1_Cluster0_size=79
I_Laurentiana_Brunton20092-pop_Cluster1_n=2
I_Laurentiana_Brunton20101b_Cluster1_size=20
I_riparia_Schafran157_Cluster0_n=3
I_septentrionalis_Schafran173-pop_Cluster0_n=3
I_X_eatonii_Taylor6750_Cluster0_size=44
I_septentrionalis_Schafran171-1_Cluster0_size=13
I_riparia_Schafran159_Cluster0_n=2
I_septentrionalis_Schafran170_Cluster0_n=3
I_riparia_Schafran161-pop_Cluster1_n=2
I_riparia_Schafran158_Cluster0_n=3
I_riparia_Taylor6706_Cluster0_size=34
I_X_fairbrothersii_Taylor6922_Cluster3_size=46
I_X_dodgei_Brunton19143_Cluster0_size=72
I_tuckermanii_Schafran160-pop_Cluster1_n=2
I_septentrionalis_Schafran172_Cluster0_n=2
I_septentrionalis_Brunton19142_Cluster0_size=67
I_riparia_Schafran156_Cluster0_n=3
I_septentrionalis_Brunton15341_Cluster1_n=2
I. engelmannii 'North' subclade
93.3/100
95.3/98

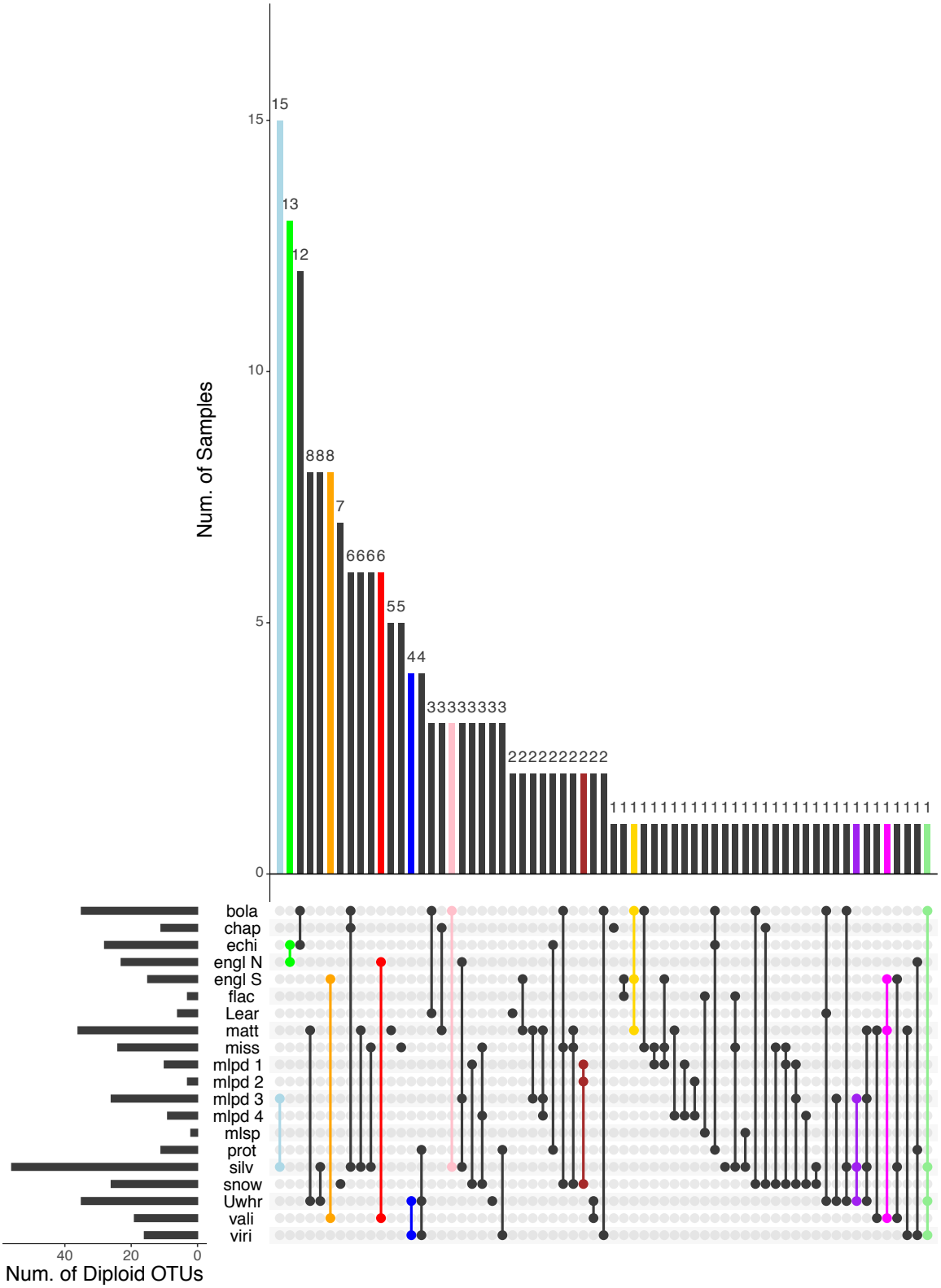
FIGURE 30. Continued.



Classification of LEAFY OTUs

Patristic distance was used as a metric of similarity between each sequence in the LEAFY phylogeny and a set of representative diploid individuals. 200 samples in the phylogeny were represented by a single sequence or cluster (OTU), while 184 samples had multiple OTUs that ranged from 2-4 OTUs per sample. Measuring patristic distances of polyploid OTUs to diploids and subsequently combining the diploids matched to each sample identified 76 unique combinations from 22 diploid clades (Figure 31; Table 10). Thirty-four (45%) of diploid combinations were each found in a single individual. An additional 13 (17%) were identified from multiple individuals, but all from the same locality. The remaining 38% of diploid combinations were identified from more than locality. Approximately 9% (7 combinations) contained two OTUs that were nearest to the same diploid, while the remainder had at least two different diploids present. Half of polyploid taxa represented by type samples had genotypes that were only present at one locality and half were represented at multiple localities. Two polyploid species, *I. acadiensis* and *I. tuckermanii,* contained the same diploid OTUs.

FIGURE 31. UpSet plot showing summary of combinations between diploid clades. Diploids on Y-axis coded by first four letters of specific epithet except *I. melanopoda* (mlpd) and *I. melanospora* (mlsp). Colored sets denote combinations with type representatives (bolded samples in Table 10). Light blue = *I.* '*laurentiana*'; green = *I. septentrionalis*; orange = *I. appalachiana* 'South'; red = *I. appalachiana* 'North'; dark blue = *I. tuckermanii*; pink = *I. virginica*; brown = *I. junciformis*; gold = *I. georgiana*; purple = *I. microvela*; pink = *I. tennesseensis*; light green = *I. boomii.*


Num. of Samples
15
13
12
888
7
6666
55
44
33333333
2222222222
1111111111111111111111111111111111111
bola
chap
echi
engl N
engl S
flac
Lear
matt
miss
mlpd 1
mlpd 2
mlpd 3
mlpd 4
mlsp
prot
silv
snow
Uwhr
vali
viri
Num. of Diploid OTUs

Comparing taxonomic identifications with genotypes, the level of agreement varied among taxa. Some samples with single OTUs (putative diploids) were in close agreement with other members of the clade. For example, of 20 samples morphologically identified as *I. valida*, 17 were supported as putative diploids and placed within the "*I. valida* clade", and no putative diploids were placed in the "*I. valida* clade" that weren't previously identified as *I. valida* (Figure 29, Appendix B). In contrast, of 4 samples identified as *I. mattaponica*, 1 was placed in the "*I. silvatica* clade". In the "*I. mattaponica* clade", of 17 putative diploids, only 3 were morphologically identified as *I. mattaponica* – the others identified as *I. melanopoda* ssp. *silvatica*, *I. piedmontana*, *I.* 'Uwharrie', or unknown *Isoëtes* (Figure 19, Appendix B). This pattern of uncertainty also occurred in the putative polyploids. Of 10 samples morphologically identified as *I. appalachiana*, every plant contained one OTU in the "*I. valida* clade" and one OTU in the "*I. engelmannii* clade". But *I. hyemalis* samples occurred with 12 different combinations of diploids. The relative placement of samples from GenBank was consistent with Pereira et al. (2019), except where undescribed diploids were included in this study (e.g. placement of *I. acadiensis clone Y AY541765* with *I.* 'Uwharrie' rather than Unknown Y).

TABLE 10. Samples in LEAFY phylogeny assigned to nearest diploid taxon (taxa) based on patristic distance(s).

Diploid[A]	Samples[B]	Likely Error[C]	Population/ Replication Outlier[D]
I. 'Leary'	*I. 'Leary' Musselman17001-1, I. 'Leary' Musselman17001-2, I. 'Leary' Schafran110-2, I. 'Leary' Schafran83-2 DirectSeq, I. 'Leary' Schafran83-3, I. 'Leary' Schafran83-4*		
I. 'Uwharrie'	*I. 'Uwharrie' BolinTr-A, I. 'Uwharrie' BolinTr-A DirectSeq, I. 'Uwharrie' Schafran76-1 DirectSeq, I. acadiensis clone Y AY541765, I. azorica clone Y AY541770*	*I. hyemalis BolinYorkCo*	
I. bolanderi	*I. 'Leary' Musselman17002-, I. bolanderi KJ135629, I. butleri Taylor6788 DirectSeq, I. maritima clone bo AY541794, I. piedmontana Schafran17 DirectSeq, I. silvatica Cressler9, I. sp Taylor-2, I. tuckermanii clone Z AY541805*	*I. georgiana Schafran74-1*	*I. georgiana Schafran111-1*
I. butleri	*I. butleri AY541773, I. butleri CiafreSN1, I. butleri Schafran52 DirectSeq, I. butleri Schafran57 DirectSeq*		
I. chapmanii	***I. chapmanii Brunton13993**, I. flaccida Taylor236 DirectSeq*		
I. echinospora	*I. eatonii clone ec AY541776, I. echinospora AY541778, I. echinospora AY541780, I. echinospora Feldsee, I. echinospora KJ135630, I. echinospora Kessler, I. echinospora PlesneLake, I. echinospora Schafran154, I. echinospora Schafran155, I. echinospora Schafran164, I. echinospora Schafran167-pop n 3, I. echinospora Schafran169-pop n 2, I. echinospora Schafran32 DirectSeq, I. hawaiiensis AY541786, I. maritima Taylor6987-1, I. maritima clone ec AY541795, I. occidentalis G1, I. occidentalis G5, I. riparia clone ec AY541799, I. septentrionalis Schafran171-2, I. straightLeaves Taylor6989-1, I. straightLeaves Taylor6989-1 rep1, I. straightLeaves Taylor6989-1 rep2, I. tuckermanii Schafran174-pop n 4*	*I. maritima × echinospora Taylor6988-2_rep1*	
I. engelmannii 'North'	*I. engelmannii KJ135631, I. engelmannii SchafranVA04 DirectSeq, I. riparia Schafran156, I. riparia Schafran157, I. riparia Schafran158, I. riparia clone en AY541800, I. septentrionalis Schafran153 rep3*		

TABLE 10. Continued.

Diploid[A]	Samples[B]	Likely Error[C]	Population/ Replication Outlier[D]
I. engelmannii 'South'	*I. appalachiana Schafran108-pop n 4, I. appalachiana × engelmannii Brunton19008, I. appalachiana clone en AY541768, I. eatonii clone en AY541777, I. engelmannii AY541781, I. engelmannii AY541783, I. engelmannii BolinJBNC18-2, I. engelmannii BunchSN1, I. engelmannii SchafraVA01-2 DirectSeq, I. engelmannii Schafran147, I. engelmannii Schafran196, I. engelmannii Schafran46 DirectSeq, I. engelmannii Schafran68 DirectSeq, I. engelmannii SchafranVA06 DirectSeq, I. engelmannii Taylor242 DirectSeq, I. hyemalis BradleySN1-pop n 5, I. hyemalis Schafran122, I. hyemalis Schafran123-pop n 3, I. hyemalis Schafran140-pop n 5, I. sp. apGF-en AY541766*		
I. flaccida	*I. flaccida Schafran203, I. flaccida SchafranFL01 DirectSeq, I. sp. apGF-fl AY541767*		*I. valida Taylor6794_rep 1*
I. lithophila	*I. lithophila Schafran65 DirectSeq, I. lithophila clone 1-1 AY541790, I. lithophila clone 1-6 AY541791*		
I. mattaponica	*I. 'Uwharrie' Schafran77-1, I. 'Uwharrie' Schafran77-2, I. 'Uwharrie' Schafran77-3, I. hyemalis BolinRiverRestB, I. hyemalis Schafran128, I. hyemalis Schafran131-pop n 2, I. mattaponica KJ135632, I. mattaponica Taylor70 DirectSeq, I. piedmontana Cressler13 bag2plant1 DirectSeq, I. piedmontana Schafran116, I. piedmontana Taylor6775 DirectSeq, I. piedmontana Taylor6781 DirectSeq, I. silvatica Cressler9 DirectSeq, I. silvatica Schafran70 DirectSeq, I. sp Greenhouse31 Big, I. sp Schafran77-1 DirectSeq, I. sp Schafran77-2 DirectSeq*	*I. graniticola Taylor6776 DirectSeq*	
I. mississippiensis	*I. louisianensis clone W AY541792, I. mississippiensis Taylor6798, I. mississippiensis Taylor6798 DirectSeq, I. snowii Schafran8 rep2*		
I. melanopoda 1	*I. melanopoda Schafran188-1, I. melanopoda Schafran188-2, I. melanopoda Schafran188-3, I. melanopoda Schafran188-4, I. melanopoda Schafran188-6*		

TABLE 10. Continued.

Diploid[A]	Samples[B]	Likely Error[C]	Population/ Replication Outlier[D]
I. melanopoda 2	*I. Butner Schafran85-4, I. melanopoda BRAN DirectSeq, I. melanopoda Schafran60 DirectSeq, I. melanopoda WelbySmith36038, I. virginica clone 1-1 AY541808, I. virginica clone 1-6 AY541807*		
I. melanopoda 3	*I. melanopoda Taylor6796*		
I. melanopoda 4	*I. melanopoda WelbySmith36037*		
I. melanospora	*I. melanospora Schafran12 DirectSeq, I. piedmontana Schafran13 DirectSeq*		
I. prototypus	*I. melanopoda AY541796, I. occidentalis G10, I. piedmontana Cressler14-pop n 2, I. prototypus KJ135633*		
I. melanopoda ssp. silvatica	*I. hyemalis Bradley8204-pop n 2, I. hyemalis Bradley8221-pop n 4, I. hyemalis clone X1-2 AY541789, I. hyemalis clone Y1-10 AY541788, I. louisianensis Alford403, I. louisianensis BolinJBLA, I. louisianensis clone × AY541793, I. mattaponica Bradley8670, I. melanopoda ssp silvatica SchafranNC05, I. piedmontana Schafran102-1, I. piedmontana Schafran102-2, I. piedmontana SchafranNC08 DirectSeq, I. piedmontana Taylor6731 DirectSeq, I. piedmontana Taylor6778 DirectSeq, I. silvatica Taylor6724 DirectSeq, I. silvatica Taylor6777 DirectSeq, I. sp Schafran210-pop n 4*	*I. occidentalis WoodbridgeSN 1-5*	*I. microvela BolinJBNC200 EO3B*
I. snowii	*I. snowii SchafranGA15, I. junciformis Cressler12, I. junciformis Schafran104 rep1, I. snowii Schafran2, I. snowii Schafran3, I. snowii Schafran4, I. snowii Schafran5, I. snowii Schafran6, I. snowii Schafran78-1, I. snowii Schafran78-3, I. snowii Schafran79-4, I. snowii Schafran79-4 rep1, I. snowii Schafran8-pop, I. snowii Schafran80-12, I. snowii Schafran80-8, I. snowii Schafran81-13, I. snowii Schafran81-14, I. snowii Schafran81-16, I. snowii Schafran81-pop n 3, I. snowii SchafranGA01, I. snowii SchafranGA03, I. snowii SchafranGA06, I. snowii SchafranGA11, I. snowii SchafranGA13, I. snowii SchafranGA14*		*I. snowii SchafranGA12 rep 2*

TABLE 10. Continued.

Diploid[A]	Samples[B]	Likely Error[C]	Population/ Replication Outlier[D]
I. storkii	*I. storkii Poas S110*		
I. tegetiformans	*I. tegetiformans Schafran19 DirectSeq*		
I. valida	*I. appalachiana Schafran193-pop n 5, I. appalachiana clone va AY541769, I. riparia Schafran163-pop, I. valida Cressler15, I. valida Cressler4 DirectSeq, I. valida Cressler7, I. valida KJ135634, I. valida Schafran145, I. valida Schafran162, I. valida Schafran197, I. valida Schafran204, I. valida Schafran206, I. valida Schafran207, I. valida Schafran208, I. valida Schafran209, I. valida Schafran211-pop n 2, I. valida Schafran37 DirectSeq, I. valida SchafranNC12 DirectSeq, I. valida × hyemalis Brunton18933B*		
I. viridimontana	*I. azorica clone en AY541771, I. sp. EZ-24 KJ135635, I. sp. acNS-en AY541764, I. tuckermanii clone en AY541804*		
I. 'Leary' × I. 'Leary'	*I. 'Leary' Musselman17002-2, I. 'Leary' Musselman17002-3*		
I. 'Leary' × I. 'Uwharrie' × I. bolanderi	*I. hyemalis Schafran109-pop*		
I. 'Leary' × I. bolanderi	*I. 'Leary' Musselman17002-1, I. 'Leary' Schafran110-1, I. hyemalis Schafran120*		
I. 'Uwharrie' × I. 'Uwharrie'	*I. hyemalis Schafran126, I. hyemalis Schafran141-pop, I. hyemalis Schafran142-pop, I. hyemalis Schafran143, I. hyemalis Schafran144*		
I. 'Uwharrie' × I. 'Uwharrie' × I. mattaponica	*I. hyemalis Schafran129-pop*		

TABLE 10. Continued.

Diploid[A]	Samples[B]	Likely Error[C]	Population/ Replication Outlier[D]
I. 'Uwharrie' × I. 'Uwharrie' × I. melanopoda-3	*I. hyemalis Schafran124-pop*		
I. 'Uwharrie' × I. bolanderi × I. bolanderi × I. silvatica	*I. georgiana Schafran113*		
I. 'Uwharrie' × I. bolanderi × I. silvatica × I. viridimontana	***I. boomii Schafran73-pop***		
I. 'Uwharrie' × I. mattaponica	*I. 'Uwharrie' Schafran76-2, I. 'Uwharrie' Schafran76-3, I. 'Uwharrie' SchafranSN, I. hyemalis Schafran118-pop, I. hyemalis Schafran130-1, I. hyemalis Schafran133-pop*		
I. 'Uwharrie' × I. mattaponica × I. melanopoda-3		*I. riparia Schafran90-2*	
I. 'Uwharrie' × I. mattaponica × I. melanopoda-3 × I. prototypus	*I. 'Butner' Schafran-pop*		
I. 'Uwharrie' × I. mattaponica × I. melanopoda-3 × I. silvatica	*I. appalachiana × hyemalis Brunton19011B*		
I. 'Uwharrie' × I. melanopoda-3 × I. silvatica	*I. hyemalis Schafran136,* ***I. microvela BolinJBNC201EO4-all***		

TABLE 10. Continued.

Diploid[A]	Samples[B]	Likely Error[C]	Population/ Replication Outlier[D]
I. 'Uwharrie' × I. mississippiensis	*I. piedmontana BolinJBNC17-3*		
I. 'Uwharrie' × I. prototypus × I. prototypus × I. viridimontana	*I. × harveyi Taylor6677, I. × heterospora Taylor6676, I. tuckermanii Schafran166-pop*		*I. acadiensis Schafran175-4*
I. 'Uwharrie' × I. silvatica	*I. boomii BargerSN, I. boomii Schafran72-pop, I. hyemalis Schafran127-pop, I. microvela Schafran119, I. sp Schafran180-pop*	*I. 'Edisto' Schafran87-5, I. georgiana Schafran82-pop*	
I. 'Uwharrie' × I. silvatica × I. silvatica	*I. virginica Fleming16376*		
I. 'Uwharrie' × I. valida	*I. valida SchafranNC11*		
I. 'Uwharrie' × I. valida × I. valida	*I. valida SchafranNC13*		
I. 'Uwharrie' × I. viridimontana	***I. acadiensis Schafran175-pop**, I. riparia Taylor6675, I. tuckermanii Schafran168, **I. tuckermanii Schafran176-pop**, I. tuckermanii Taylor6707*		
I. bolanderi × I. chapmanii × I. silvatica	*I. boomii Leonard12408, I. georgiana Cressler11-1, I. georgiana Schafran111-pop, I. georgiana SchafranGA18*		
I. bolanderi × I. chapmanii × I. silvatica × I. silvatica	*I. georgiana Schafran112, I. georgiana SchafranGA17*		

TABLE 10. Continued.

Diploid[A]	Samples[B]	Likely Error[C]	Population/ Replication Outlier[D]
I. bolanderi × *I. echinospora*	*I.* × *herbwagneri TaylorSN-pop, I. bolanderi* × *occidentalis Taylor6756, I. bolanderi* × *occidentalis Taylor6759, I. curledLeaves Taylor6991-1 2, I. maritima* × *echinospora Taylor6988-3, I. maritima Taylor6983-pop, I. maritima WoodbridgeSN2, I. occidentalis Taylor6755, I. sp Taylor-1, I. sp Taylor-3, I. splayedLeaves Taylor6990-1, I. straightLeaves Taylor6989-3*		
I. bolanderi × *I. echinospora* × *I. prototypus*	*I. occidentalis WoodbridgeSN1-pop*		
I. bolanderi × *I. engelmannii 'South'* × *I. mattaponica*	***I. georgiana Matthews3-pop***		
I. bolanderi × *I. mississippiensis*		*I. snowii SchafranGA06 rep3*	
I. bolanderi × *I. mississippiensis* × *I. snowii*	*I. snowii Schafran2*		
I. bolanderi × *I. mississippiensis* × *I. snowii* × *I. snowii*	*I. melanopoda Ciafre728-1*		
I. bolanderi × *I. silvatica*	***I. virginica Taylor6882***		

TABLE 10. Continued.

Diploid[A]	Samples[B]	Likely Error[C]	Population/ Replication Outlier[D]
I. bolanderi × *I. silvatica* × *I. silvatica*	*I. hyemalis Schafran121-pop, I. georgiana Taylor6769-all*		
I. bolanderi × *I. snowii*	*I. snowii Schafran5*		
I. bolanderi × *I. viridimontana*	*I. lacustris CerneLake, I. lacustris Feldsee*		
I. chapmanii × *I. mattaponica* × *I. mattaponica*	*I. 'Edisto' Cressler3, I. 'Edisto' Cressler5-pop, I. 'Edisto' Schafran87-pop*		
I. chapmanii × *I. snowii*	*I. 'Leary' Schafran114*		
I. echinospora × *I. engelmannii 'North'*	*I. × dodgei Brunton19143, I. × eatonii Taylor6750, I. riparia Schafran159, I. riparia Schafran161-pop, I. riparia Taylor6706, I. septentrionalis Brunton15341,* ***I. septentrionalis Brunton19142****, I. septentrionalis Schafran151-pop, I. septentrionalis Schafran170, I. septentrionalis Schafran171-1, I. septentrionalis Schafran172, I. septentrionalis Schafran173-pop, I. tuckermanii Schafran160-pop*		
I. echinospora × *I. prototypus*	*I. maritima* × *echinospora Taylor6988-2 2, I. occidentalis G7*		
I. engelmannii 'North' × *I. melanopoda-3* × *I. silvatica*	*I. laurentiana Brunton20092-pop, I. laurentiana Brunton20101-1, I. laurentiana Brunton20101b*		

TABLE 10. Continued.

Diploid[A]	Samples[B]	Likely Error[C]	Population/ Replication Outlier[D]
I. engelmannii 'North' × I. prototypus × I. prototypus × I. viridimontana	*I. × fairbrothersii Taylor6922*		
I. engelmannii 'North' × I. valida	***I. appalachiana Schafran148-pop**, I. appalachiana Schafran150, I. septentrionalis Schafran152-pop, I. septentrionalis Schafran153*		
I. engelmannii 'South' × I. flaccida	*I. hyemalis BradleySN2-pop, I. hyemalis BunchSN2*		
I. engelmannii 'South' × I. mattaponica	*I. georgiana SchafranGA16, I. hyemalis Schafran107*		
I. engelmannii 'South' × I. mattaponica × I. valida	***I. tennesseensis Schafran177-pop***		
I. engelmannii 'South' × I. mississippiensis × I. melanopoda-1	*I. melanopoda Schafran187*		
I. engelmannii 'South' × I. silvatica × I. valida		*I. lacustris KrasluerSN*	

TABLE 10. Continued.

Diploid[A]	Samples[B]	Likely Error[C]	Population/ Replication Outlier[D]
I. engelmannii 'South' × I. valida	*I. appalachiana Cressler8-pop, I. appalachiana Schafran105-pop, I. appalachiana Schafran178, I. appalachiana Schafran199-pop, I. appalachiana Schafran200, I. appalachiana Schafran201, I. louisianensis Brunton17581, I. louisianensis Schafran106*		
I. flaccida × I. melanospora	*I. louisianensis Leonard12415*		
I. flaccida × I. mississippiensis × I. silvatica	*I. valida Taylor6794*		
I. mattaponica × I. mattaponica	*I. piedmontana Cressler13-pop*		
I. mattaponica × I. melanopoda-3	*I. Boykins Island Taylor6665, I. hyemalis BolinRiverRestA*		
I. mattaponica × I. melanopoda-4	*I. graniticola Schafran117*		
I. mattaponica × I. melanopoda-3 × I. prototypus	*I. virginica Brunton19044*		
I. mattaponica × I. mississippiensis × I. snowii	*I. piedmontana Schafran101-1, I. piedmontana Schafran103-2*		

TABLE 10. Continued.

Diploid[A]	Samples[B]	Likely Error[C]	Population/ Replication Outlier[D]
I. mattaponica × I. silvatica	*I. graniticola Schafran115, I. hyemalis Brunton19012, I. hyemalis Schafran125-pop, I. hyemalis Schafran132-pop, I. sp. Greenhouse31 Small, I. sp. UnknownChickahominy2*		
I. mattaponica × I. valida	*I. hyemalis SchafranVA01*		
I. mattaponica × I. viridimontana	*I. piedmontana Taylor6776*		
I. melanopoda-1 × I. melanopoda-2 × I. snowii × I. snowii	***I. junciformis Bolin, I. junciformis Brunton17608***		
I. melanopoda-1 × I. melanopoda-3 × I. snowii × I. snowii	*I. junciformis Schafran104*		
I. melanopoda-1 × I. melanopoda-4	*I. melanopoda Schafran188-5*		
I. melanopoda-1 × I. snowii	*I. melanopoda Ciafre728-2, I. piedmontana Schafran101-3*		
I. melanopoda-1 × I. snowii × I. snowii	*I. piedmontana Schafran102-3*		

TABLE 10. Continued.

Diploid[A]	Samples[B]	Likely Error[C]	Population/ Replication Outlier[D]
I. melanopoda-2 × I. melanopoda-4	*I. melanopoda Schafran184-1*		
I. melanopoda-3 × I. silvatica	*I. laurentiana Brunton20077, I. laurentiana Brunton20087,* ***I. laurentiana Brunton20101-2****, I. hyemalis Schafran134-pop, I. hyemalis Schafran137-pop, I. hyemalis Schafran138, I. hyemalis Schafran139-pop, I. microvela BolinJB40NC, I. microvela BolinJBNC199EO2, I. microvela BolinJBNC200EO3-pop, I. microvela BolinJBNC202EO5-all, I. microvela MatthewsI09-35, I. riparia SchafranPotomacCreek*		
I. melanopoda-3 × I. silvatica × I. silvatica	*I. hyemalis Schafran135*		
I. melanopoda-4 × I. snowii	*I. snowii SchafranGA12*		
I. melanospora × I. silvatica	*I. piedmontana Schafran14*		
I. mississippiensis × I. melanopoda-1	*I. melanopoda Schafran184-2*		
I. mississippiensis × I. melanopoda-4 × I. snowii	*I. melanopoda Ciafre256-1, I. melanopoda Ciafre728-3, I. piedmontana Schafran103-1*		
I. mississippiensis × I. melanopoda-4 × I. snowii × I. snowii	*I. melanopoda Ciafre256-2*		

TABLE 10. Continued.

Diploid[A]	Samples[B]	Likely Error[C]	Population/ Replication Outlier[D]
I. mississippiensis × *I. mississippiensis*	*I. mississippiensis Schafran194-pop*		
I. mississippiensis × *I. silvatica*	*I. louisianensis Alford397, I. louisianensis Alford398, I. louisianensis Alford399, I. louisianensis Alford401, I. louisianensis Alford402, I. louisianensis Schafran195-pop*		
I. mississippiensis × *I. snowii*	*I. piedmontana Schafran101-2*		
I. prototypus × *I. prototypus* × *I. viridimontana*	*I. echinospora Schafran167-3, I. lacustris Kessler, I. lacustris Taylor6748*		
I. silvatica × *I. silvatica*		*I. georgiana Cressler10*	
I. silvatica × *I. snowii*		*I. bolanderi* × *occidentalis Taylor6759 rep1*	
I. snowii × *I. snowii*	*I. snowii Schafran78-2, I. snowii Schafran80-10, I. snowii Schafran80-11, I. snowii Schafran80-7, I. snowii Schafran80-9, I. snowii SchafranGA02, I. snowii SchafranGA05*		

[A] Diploid taxa and subclades used for distance comparisons with all samples

[B] Samples from LEAFY phylogeny categorized by the minimum distance from each OTU to a diploid taxon or subclade. Bolded samples represent topotype collections.

[C] Samples where number of sequences per OTU was less than 10 and results disagree with other data (morphological ID, genome size, biogeography, results from other samples in population)

[D] Samples whose diploid assignment disagreed with the majority of its population, but cluster sizes were large enough to be reliable (generally > 20 reads per OTU).

NOTE: Some diploid assignments (particularly to *I. bolanderi* and *I.* 'Uwharrie') represent distant clades lacking diploids, so samples with the same diploid combinations do not necessarily represent the same taxon.

The neighbor joining tree from the polyploid sample-based distance matrix provided support for taxa that were identified from the classification based on combination of individual polyploid OTUs assigned to pre-assigned diploid clades. Species such as *I. appalachiana* (both 'North' and 'South' clades), *I. georgiana*, *I. septentrionalis*, *I. acadiensis*, and *I. tuckermanii* were reconstructed as clades containing the same individuals as above (Figure 32 compared with Table 10). In some cases, this method better represented the diversity of genotypes that was discovered. One example included a grouping of *I. 'hyemalis'* (*Schafran 126, 141, 142, 143, 144*). Because these samples contained OTUs dissimilar from all described diploids, they were characterized as *I.* 'Uwharrie × *I.* 'Uwharrie' using assignment. However, visual inspection of the ML phylogenies showed that divergent OTUs were misleadingly lumped under *I.* 'Uwharrie'. The neighbor joining tree based on the distance comparison better represented the difference between these taxa, separating *Schafran 126* into a part of the tree away from the others (Figure 32).

Besides those groups of samples that appeared to represent well defined species, other groupings seemed to display geographic signal, with all or most of the individuals occurring in the same physiographic region. *Isoetes 'hyemalis'* and I. 'Leary' from extreme southwestern Georgia, clustered together, while *I. 'piedmontana'* and *I. 'hyemalis'* from the Piedmont region of North Carolina formed another group. Another was composed of *I. 'hyemalis'* and *I.* 'Uwharrie' from the North Carolina Sandhills and adjacent Uwharrie Mountains (Figure 32). With the exception of the recently discovered *I.* '*laurentiana*' in Quebec, the clade including *I. microvela s.s.* also contained *I. 'hyemalis'* and *I. 'riparia'* from the Coastal Plain of North Carolina and Virginia (Figure 32). Two other groupings primarily from southern Georgia seemed to represent hexaploid *I. 'georgiana'* and *I. 'boomii',* and a large group of putative polyploids

with variable genome sizes that may represent tetraploid and hexaploids (Bolin, pers. comm.). The latter assemblage combines *I. junciformis*, *I. 'piedmontana'* and *I. 'snowii'*, and collections of *I. melanopoda* from central Tennessee.

FIGURE 32. Neighbor joining tree based on minimized distances of pairwise polyploid comparisons. Topotype specimens colored red, tips that represent likely sequence error colored gray. Some clades marked where several specimens appear to clearly represent certain taxa.

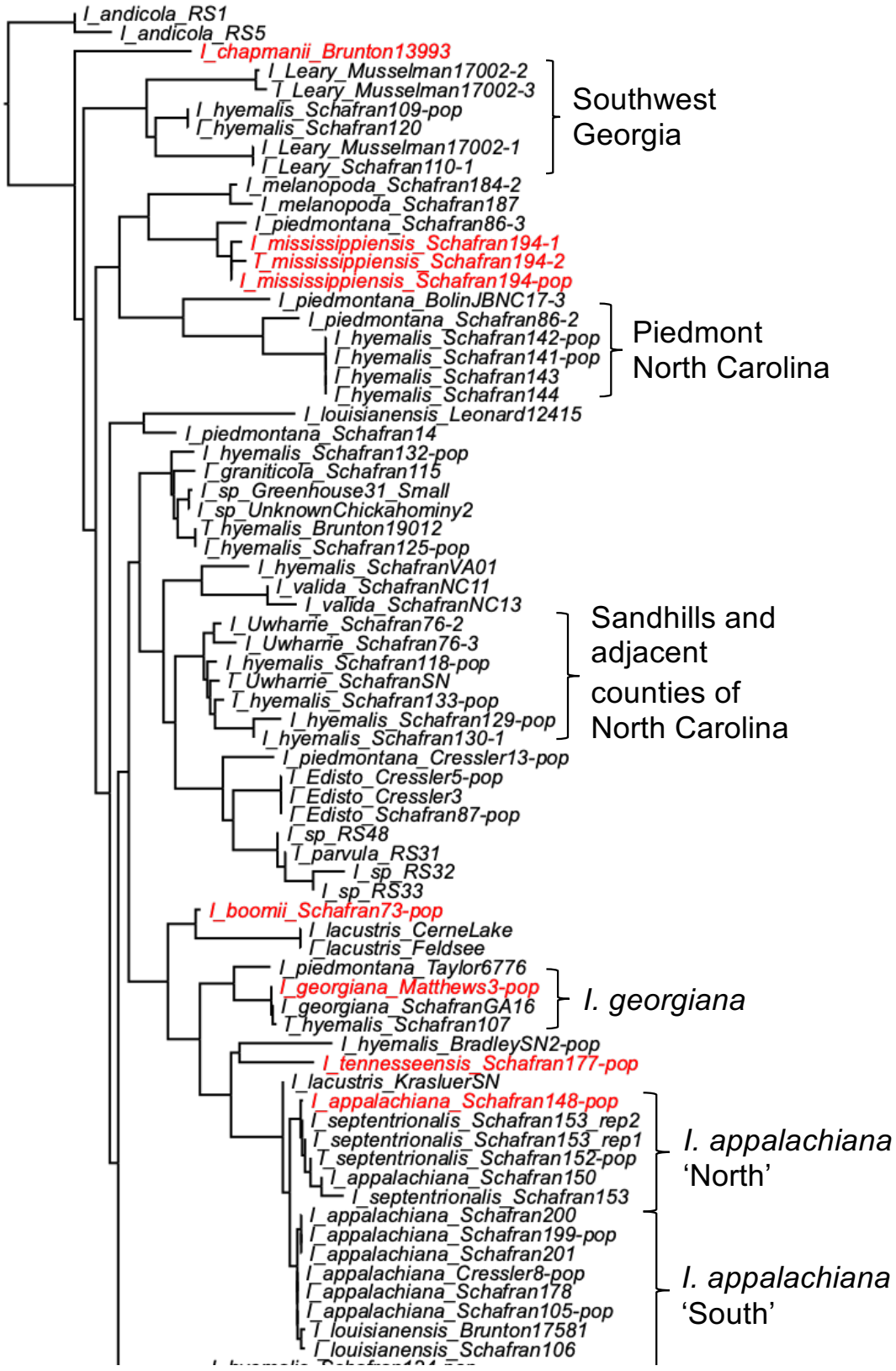

l_andicola_RS1
l_andicola_RS5
l_chapmanii_Brunton13993
l_Leary_Musselman17002-2
l_Leary_Musselman17002-3
l_hyemalis_Schafran109-pop
l_hyemalis_Schafran120
l_Leary_Musselman17002-1
l_Leary_Schafran110-1
Southwest Georgia
l_melanopoda_Schafran184-2
l_melanopoda_Schafran187
l_piedmontana_Schafran86-3
l_mississippiensis_Schafran194-1
l_mississippiensis_Schafran194-2
l_mississippiensis_Schafran194-pop
l_piedmontana_BolinJBNC17-3
l_piedmontana_Schafran86-2
l_hyemalis_Schafran142-pop
l_hyemalis_Schafran141-pop
l_hyemalis_Schafran143
l_hyemalis_Schafran144
Piedmont North Carolina
l_louisianensis_Leonard12415
l_piedmontana_Schafran14
l_hyemalis_Schafran132-pop
l_graniticola_Schafran115
l_sp_Greenhouse31_Small
l_sp_UnknownChickahominy2
l_hyemalis_Brunton19012
l_hyemalis_Schafran125-pop
l_hyemalis_SchafranVA01
l_valida_SchafranNC11
l_valida_SchafranNC13
l_Uwharrie_Schafran76-2
l_Uwharrie_Schafran76-3
l_hyemalis_Schafran118-pop
l_Uwharrie_SchafranSN
l_hyemalis_Schafran133-pop
l_hyemalis_Schafran129-pop
l_hyemalis_Schafran130-1
Sandhills and adjacent counties of North Carolina
l_piedmontana_Cressler13-pop
l_Edisto_Cressler5-pop
l_Edisto_Cressler3
l_Edisto_Schafran87-pop
l_sp_RS48
l_parvula_RS31
l_sp_RS32
l_sp_RS33
l_boomii_Schafran73-pop
l_lacustris_CerneLake
l_lacustris_Feldsee
l_piedmontana_Taylor6776
l_georgiana_Matthews3-pop
l_georgiana_SchafranGA16
l_hyemalis_Schafran107
I. georgiana
l_hyemalis_BradleySN2-pop
l_tennesseensis_Schafran177-pop
l_lacustris_KrasluerSN
l_appalachiana_Schafran148-pop
l_septentrionalis_Schafran153_rep2
l_septentrionalis_Schafran153_rep1
l_septentrionalis_Schafran152-pop
l_appalachiana_Schafran150
l_septentrionalis_Schafran153
I. appalachiana 'North'
l_appalachiana_Schafran200
l_appalachiana_Schafran199-pop
l_appalachiana_Schafran201
l_appalachiana_Cressler8-pop
l_appalachiana_Schafran178
l_appalachiana_Schafran105-pop
l_louisianensis_Brunton17581
l_louisianensis_Schafran106
I. appalachiana 'South'

FIGURE 32. Continued.

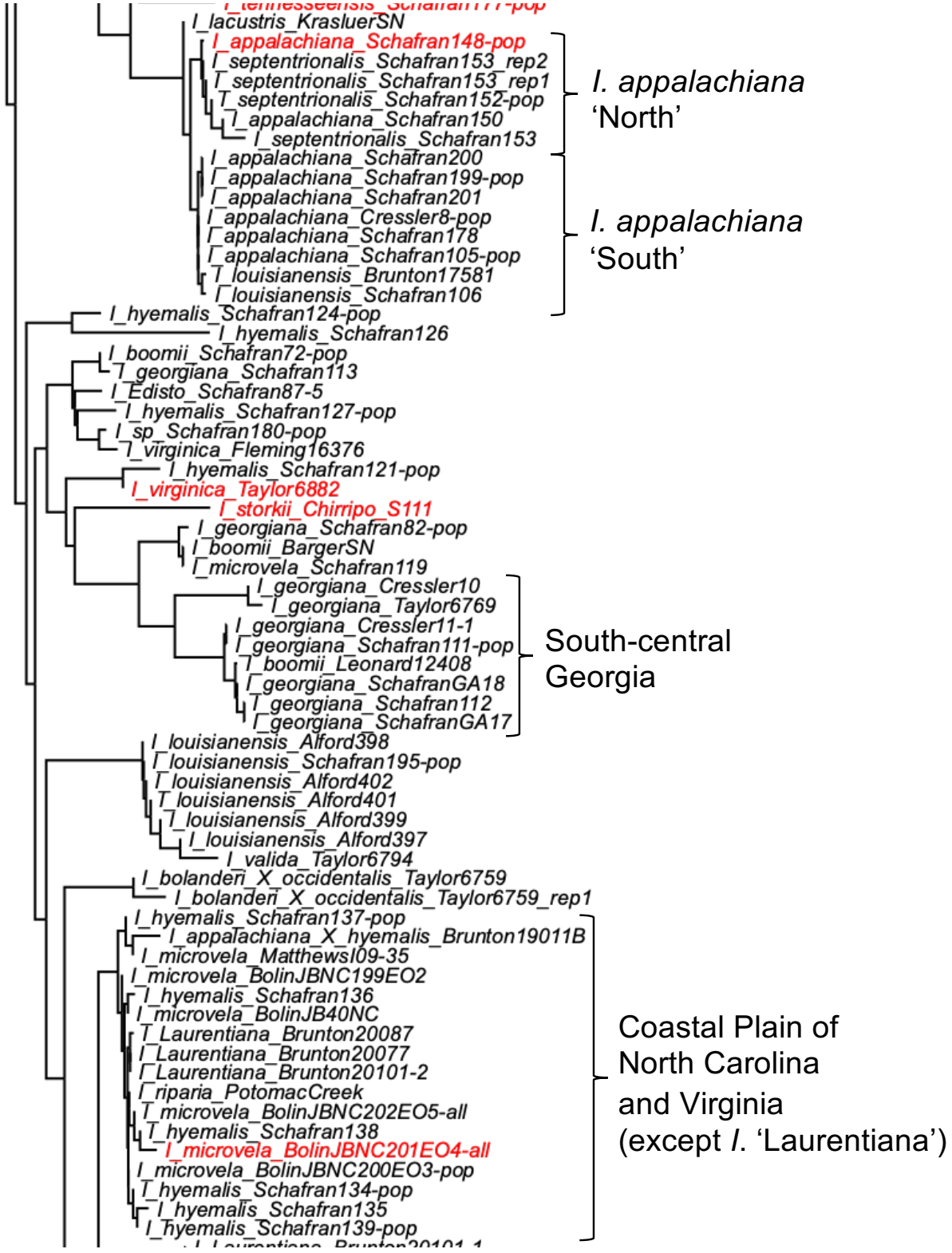


FIGURE 32. Continued.

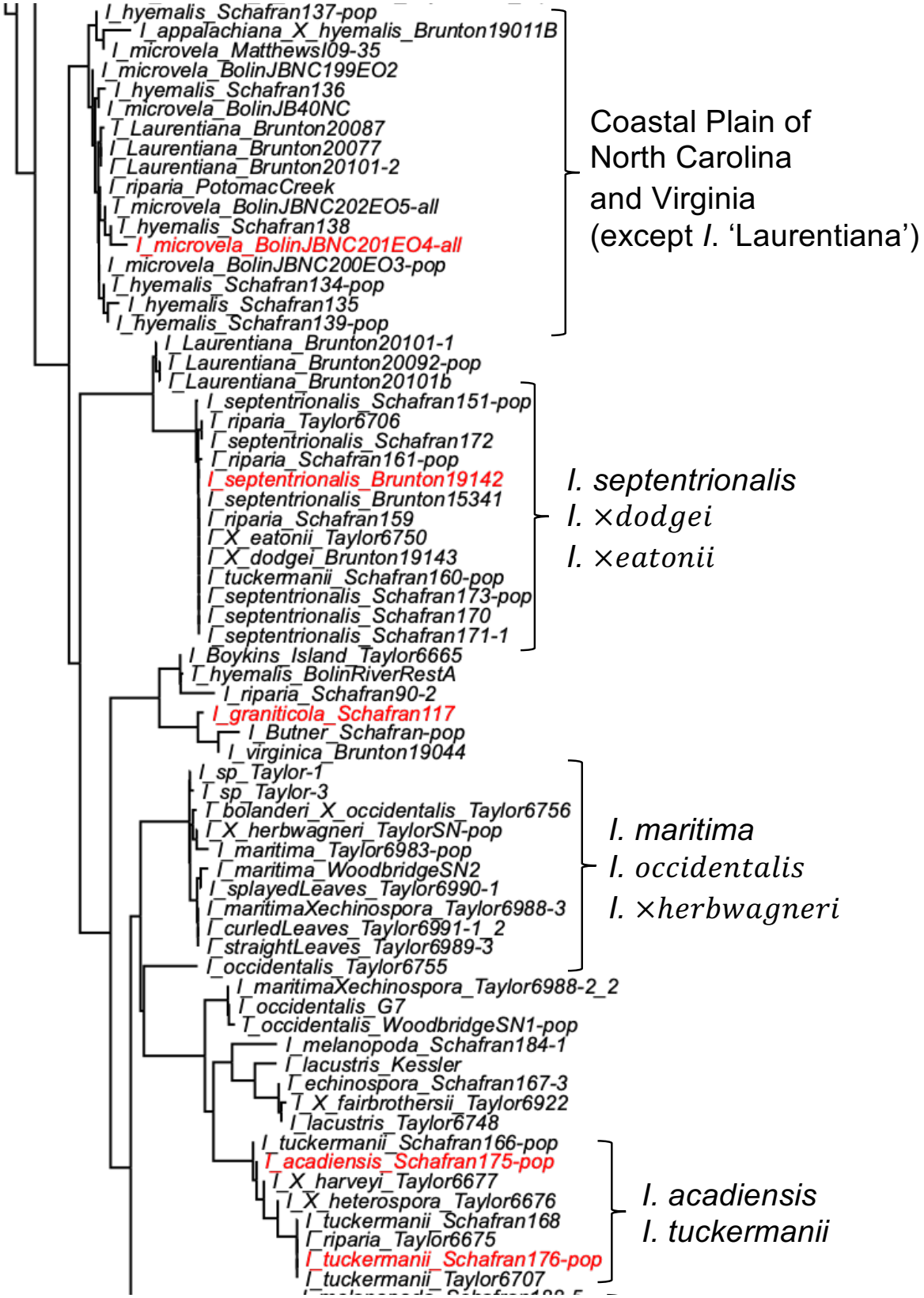
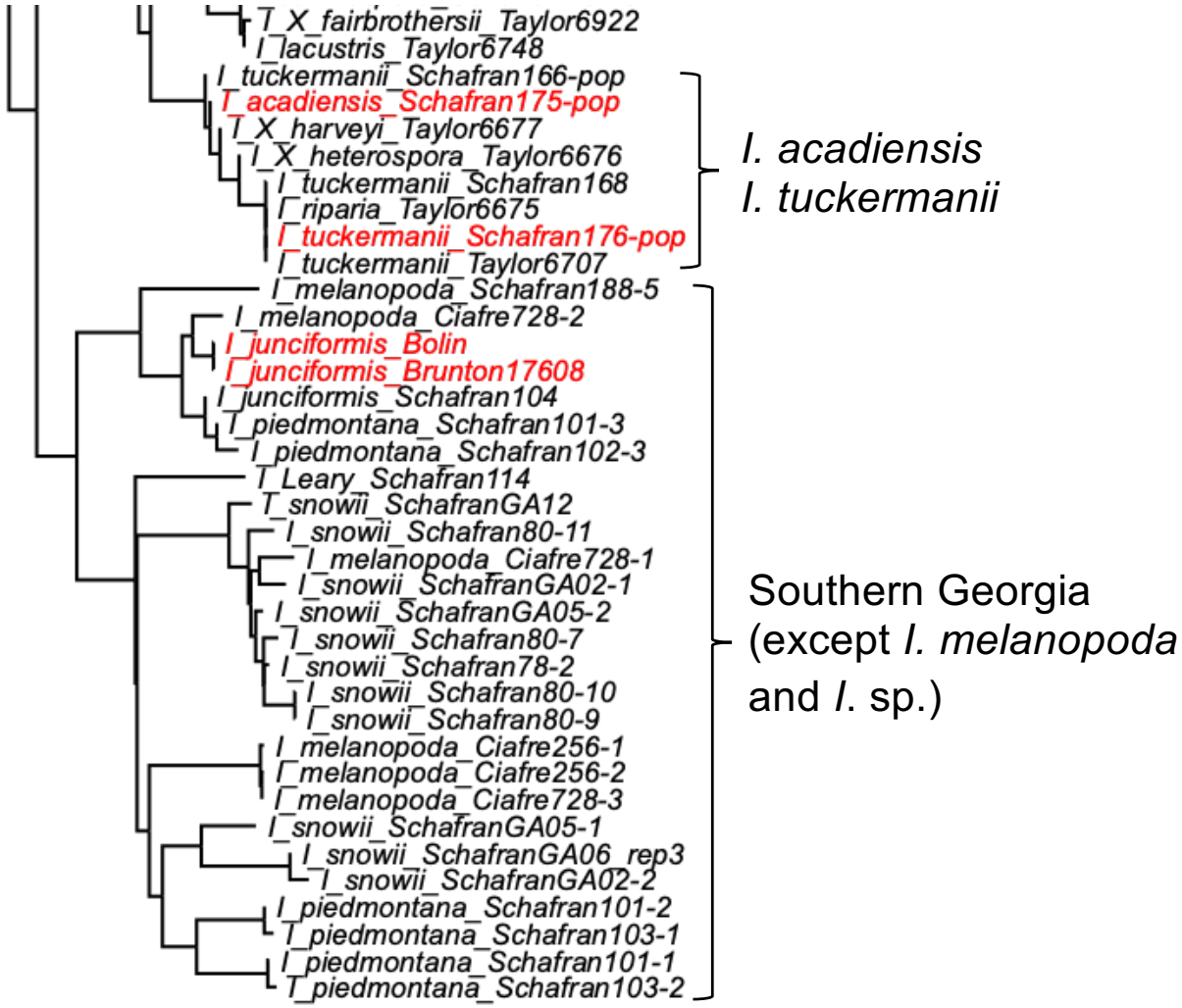


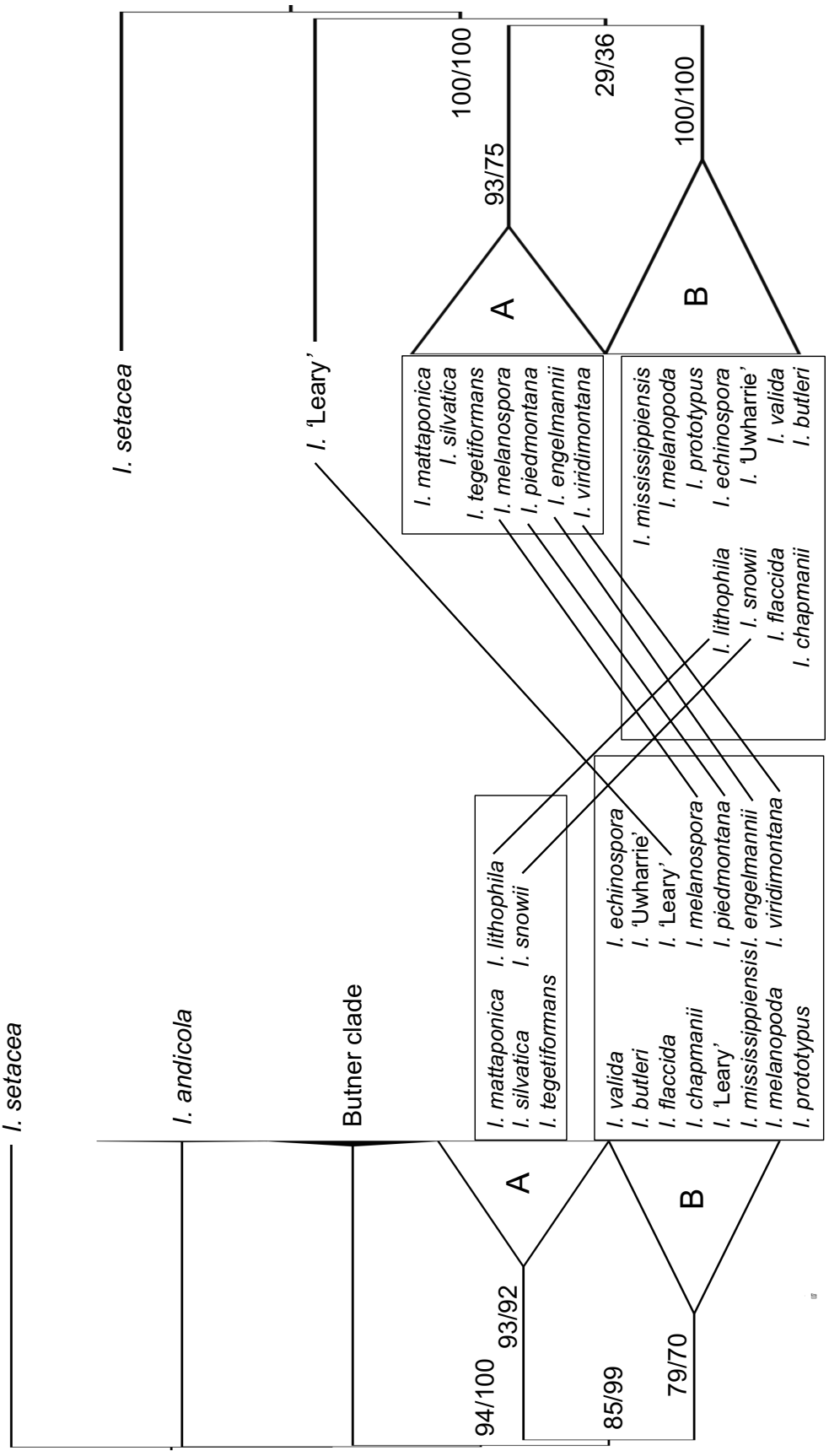
FIGURE 32. Continued.



Nuclear-Plastid Incongruence

There is low agreement in tree topology between nuclear and plastid phylogenies (Figure 33). The LEAFY phylogeny generally did not resolve strong relationships between diploid species and where strong support for a multi-diploid clade was found (e.g. *I. echinospora* + *I. melanopoda* + *I. prototypus*), a similarly strong relationship was not recovered in the plastome phylogeny. Collapsing clades to the basal-most split between North American diploids (excluding the *I.* 'Butner' clade in the LEAFY phylogeny and *I.* 'Leary' in the plastome phylogeny) formed moderately to highly supported clades in both phylogenies with similar diploid species composition in clade B, though several species were resolved in clade A of the LEAFY phylogeny and clade B of the plastome phylogeny or *vice versa* (Figure 33).

FIGURE 33. Comparison of LEAFY (left) and plastome (right) ML phylogenies highlighting incongruence between diploids in major clades. Branch support values (approximate likelihood ratio test/ultrafast bootstrap) shown for major clades above branch or at node near respective branch.


I. setacea
I. andicola
Butner clade
I. mattaponica
I. silvatica
I. tegetiformans
I. lithophila
I. snowii
A
I. valida
I. butleri
I. flaccida
I. chapmanii
I. 'Leary'
I. mississippiensis
I. melanopoda
I. prototypus
I. echinospora
I. 'Uwharrie'
I. 'Leary'
I. melanospora
I. piedmontana
I. engelmannii
I. viridimontana
B
94/100
93/92
85/99
79/70
I. setacea
I. 'Leary'
I. mattaponica
I. silvatica
I. tegetiformans
I. melanospora
I. piedmontana
I. engelmannii
I. viridimontana
A
100/100
93/75
I. mississippiensis
I. melanopoda
I. prototypus
I. echinospora
I. 'Uwharrie'
I. valida
I. butleri
B
29/36
100/100

DISCUSSION

These results expand on previous studies of both the general phylogeny of *Isoëtes* (Hoot and Taylor 2001, Larsén and Rydin 2016, Pereira et al., 2017) and the origin of allopolyploid taxa (Caplen and Werth, 2000b, Hoot et al., 2004, Bolin et al., 2008, Pereira et al., 2019), all of which suggested polyphyletic polyploid taxa and the presence of unknown diploid progenitors. While previous work has focused on either one polyploid complex or used one to few representatives for many polyploid complexes, this study is the first to infer systematic relationships for many representatives from all polyploid complexes in eastern North America. Under the assumption that a polyploid with a unique combination of diploid parents represents a different formation event and thus an independent lineage (Soltis and Soltis, 2009), both plastid and nuclear datasets indicate conflicting origins for polyploid species as currently treated in eastern North America. The occurrence of morphologically identified polyploid species with a broad assemblage of diploid parentages indicates an inability of visible characteristics to reliably separate taxa that represent evolutionary patterns in *Isoëtes.*

Disagreement between plastome and LEAFY phylogenies shows the utility of different markers at various phylogenetic depth. The LEAFY phylogeny generally recovered strongly supported clades that represent infraspecific and neopolyploid relationships, while failing to resolve many interspecific relationships. Therefore, any polyploid sequences with sister positions and some sequence divergence from diploid clades should be interpreted with caution. Topologies of plastome phylogenies inferred by various methods were mostly consistent, with the exception of the placement of *I.* 'Leary' and *I. louisianensis Leonard 12415* near the base of the American clade. This uncertainty may be due to a lack of sampling from South American taxa if these two samples are more closely related to species outside eastern North America

(Heath et al. 2008). Some minor variation occurred in placement of polyploids in more terminal clades, for example *I. tennesseensis* and *I. hyemalis.* This had little effect on inference of the diploid parent, since these polyploids had similar patristic distances to multiple diploid taxa. The high support and consistency of diploid relationships within plastome phylogenies suggest it is the best model of tree-like evolution at the intraspecific level.

Using both plastid and nuclear data, it is possible to identify some patterns of reticulate evolution that resulted in the diversity of allopolyploids observed today. The three tetraploids that occur in the plastid *I. engelmannii* clade, *I. appalachiana, I. septentrionalis*, and *I.* '*laurentiana*', also occurred in the LEAFY *I. engelmannii* clade, strongly suggesting *I. engelmannii* as the maternal ancestor of each polyploid. The other LEAFY sequences from topotype specimens (*I. appalachiana Schafran 148*, *I. septentrionalis Brunton 19142, I.* '*laurentiana*' *Brunton 20092*) occurred with various diploids. *Isoëtes appalachiana Schafran 148*, with 2 LEAFY OTUs, had a parentage of *I. engelmannii* and *I. valida. Isoëtes septentrionalis Brunton 19142,* also with 2 LEAFY OTUs, had a parentage of *I. engelmannii* and *I. echinospora*. *Isoëtes* '*laurentiana*' *Brunton 20092*, while a putative tetraploid based on megaspore and genome size measurements (D.F. Brunton and J.F. Bolin, unpublished data), returned 3 LEAFY OTUs, one matching *I. engelmannii*, one matching *I. melanopoda* ssp. *silvatica*, and one matching *I. melanopoda s.s.* The presence of 3 copies of LEAFY suggests *I.* '*laurentiana*' is either a hexaploid, or somehow violates the fixed heterozygosity model typically applied in polyploid *Isoëtes,* potentially through a mechanism such as crossing over of homeologous chromosomes (Udall et al. 2005) or multisomic inheritance of chromosomes in allopolyploids of closely related species (Ramsey and Schemske 2002). These 3 LEAFY copies are found in multiple individuals, making it unlikely these results are due to sequencing error.

Other individuals identified as *I.* '*laurentiana*' (*Brunton 20077, 20087, 20101-2*) only contained the *I. melanopoda* ssp. *silvatica* and *I. melanopoda s.s.* LEAFY copies, adding confusion to the true identity and origin of this taxon.

Other polyploids with congruence between plastid and nuclear data were *I. georgiana* and *I. boomii*, though with less certainty than the OTUs noted above. The plastome of *I. georgiana* was most similar to *I. viridimontana*, but only about twice as distant to *I. mattaponica* (Table 9). The relatively low SH-like approximate likelihood ratio test (SH-alrt) value of 74 (Figure 16), as well as low support for this branch in other analyses (SVDquartets bootstrap value of 13, tree not shown) could indicate the sister relationship of *I. georgiana* and *I. viridimontana* is erroneous. Support for the more inclusive clade containing *I. georgiana, I. viridimontana, I. mattaponica*, and *I. hyemalis* is more highly supported (SH-alrt value of 94, SVDquartets bootstrap value of 86). Of 3 LEAFY OTUs found in *I. georgiana*, one occurred with *I. mattaponica*, one with *I. engelmannii*, and one in a small clade lacking any diploid taxa weakly supported as sister to Unknown Z from Hoot et al. (2004). The combination of nuclear and plastid data could suggest *I. mattaponica* as the maternal diploid parent, if it is assumed that the sister placement of *I. georgiana* and *I. viridimontana* is erroneous.

The plastome of *I. boomii* was closest to *I. melanopoda* ssp. *silvatica* but had a similar distance to *I. viridimontana.* One of the four LEAFY OTUs identified in the topotype population (*Schafran 73*) occurred with *I. melanopoda* ssp. *silvatica*, while a second OTU was placed sister to *I. viridimontana*, leaving doubt about the maternal lineage of this species. The other OTUs occurred with the diploid *I.* 'Uwharrie' and in a small clade weakly supported as sister to *I. lithophila, I. bolanderi*, and *I. howellii*. Individuals from a nearby population in the same watershed *(Schafran 72*) returned only 2 LEAFY OTUs, *I. melanopoda* ssp. *silvatica* and *I.*

‘Uwharrie’. Variability in the number of OTUs was likely caused by low sequencing depth of both populations, suggesting that some OTUs could represent error or were too under-sequenced for the clustering algorithm to identify them.

Many species displayed incongruence between their plastid and nuclear data. *Isoëtes microvela s.s.* (*Bolin JBNC201*) had a plastome with *I.* ‘Uwharrie’, *I. flaccida*, *I. valida*, *I. lithophila*, *I. texana*, and *I. prototypus* as close diploids. However, the 3 LEAFY OTUs matched *I. melanopoda* ssp. *silvatica*, *I. melanopoda s.s.*, and the ‘Butner’ clade lacking any diploids. The *I. virginica* samples (*Taylor 6882* and *Brunton 19044*) had identical plastome sequences in the trimmed alignment excluding gaps and were very similar to *I. microvela*, sharing the same relationships to diploids. But the two *I. virginica* samples had completely dissimilar LEAFY OTUs, neither fitting the phylogenetic placement of the plastomes. *Taylor 6882* had one OTU matching *I. melanopoda* ssp. *silvatica*, another in a clade sister to clades containing Unknown Z (Hoot et al., 2004), *I. lithophila*, *I. bolanderi*, and *I. howellii*. *Brunton 19044* contained an OTU in the *I. mattaponica* clade, and its second in one of the *I. melanopoda s.s.* clades. The plastome of *I. tennesseensis* resolved near *I. melanopoda, I. chapmanii*, and *I. snowii,* but its 3 LEAFY OTUs appeared with *I. engelmannii, I. valida*, and sister to the predominantly South American clade (including *I. mattaponica*). *Isoëtes tuckermanii*, whose plastome suggested a clear relationship to *I. echinospora*, had 2 LEAFY OTUs nested within *I. viridimontana* and *I.* ‘Uwharrie’ clades. *Isoëtes graniticola* had a plastome nearest *I. melanopoda* ssp. *silvatica*, but 2 LEAFY OTUs in separate clades with *I. melanopoda s.s.* and *I. mattaponica*. *Isoëtes junciformis* presented a particularly difficult taxon to interpret. Its plastome was in a clade subtended by *I. flaccida* and *I.* ‘Uwharrie’. Despite being recognized as a tetraploid, 4 LEAFY OTUs were recovered, two in the *I. snowii* clade, and two in separate *I. melanopoda s.s*. clades.

These levels of incongruence make inference of parentage questionable when sequences from polyploids are similar to multiple diploids, or where plastid and nuclear data disagree. In some cases, disagreement may have resulted from chloroplast capture (Tsitrone et al. 2003). Genome-wide nuclear data generated by the GoFlag project (NSF DEB 1541506) suggest a hybrid origin of *I. chapmanii*, where the nuclear genome strongly matches *I. flaccida* (P.W. Schafran et al., unpublished data), while this study shows that the plastome matches *I. melanopoda* with an intraspecific level of similarity. Within many diploid species there was gene tree disagreement, suggesting incomplete lineage sorting and hybridization may have formed the diploid genomes (P.W. Schafran et al., unpublished data).

Samples with only nuclear data cannot be used to infer maternal vs. paternal diploid progenitors but remain useful for highlighting disagreement within traditionally circumscribed species. This is easily observed by the tetraploid nomenclature occurring throughout the LEAFY phylogeny. Comparison of samples to those treated as types or otherwise representative of a species illustrated cases of misidentification and cryptic speciation. Misidentification occurred where the parentage of a polyploid individual matched a representative sample of another species. For example, samples identified as *I. louisianensis* (*Brunton 17581* and *Schafran 106*) and *I. septentrionalis* (*Schafran 152, 153*) all had LEAFY sequences matching *I. valida* and *I. engelmannii,* which characterizes *I. appalachiana*. Using patristic distances from the LEAFY phylogeny to estimate the nearest diploid for each polyploid OTU and estimates of ploidy level based on genome size, hypothetical taxon names can be applied for an individual or population. Following are summaries of recognized and major hypothetical taxa suggested by the data:

Molecular Descriptions of Diploids

Isoëtes bolanderi (2x)

Isoëtes bolanderi and *I. howellii* form a tight clade well supported as sister to *I. lithophila.* The presence of OTUs from *I.* ×*herbwagneri, I. maritima,* and *I. occidentalis* in this clade supports hypotheses that *I. bolanderi* is one diploid parent in the *I. occidentalis* complex. Because western North America was outside the scope of this study, sampling is insufficient to completely disentangle the relationships of these taxa. Based on patristic distances, numerous samples from eastern North America were assigned to *I. bolanderi*, but these OTUs appeared on well supported branches with lengths typical of other diploids, suggesting these OTUs actually represent unknown species.

Isoëtes butleri (2x)

Despite being abundant and widespread throughout the Midwest and occasional in the Southeast, *I. butleri* does not interact with any other species. There are no putative polyploids containing sequences in the *I. butleri* clade. In addition, there were no misidentifications of other taxa as *I. butleri* or *vice versa*. This is likely to due to its fairly unique habitat on limestone glades, reproductive biology (Turner et al. 2005), and spore morphology (Taylor et al. 1975). Relatively long branch lengths within the *I. butleri* clade may suggest isolation and population structure.

Isoëtes chapmanii (=*I. flaccida* var. *chapmanii*) (2x)

Topotype Sample: *Brunton 13993*

One sequence (*I. flaccida Taylor 236*) appeared to be another diploid representative of *I. chapmanii*, likely collected from one of its few known populations (Taylor et al., 1993). Despite its very limited range, *I. chapmanii* is involved with several putative hexaploids in Georgia and South Carolina (*I. 'boomii' Leonard 12408, I. 'georgiana' Cressler 11, I. 'georgiana' Schafran 112, I. 'Edisto' Cressler 3*) and one tetraploid (*I. 'Leary' Schafran 114*).

Isoëtes echinospora (2x)

Isoëtes echinospora is one of the most widespread species of *Isoëtes*, distributed circumboreally across North America and Europe, but displays remarkably little sequence divergence across its range. LEAFY sequences from populations in the US (*Schafran 32, Schafran 154*) and Europe (*Feldsee, Kessler, Plesne Lake*) formed a single, flat clade, suggesting high population connectivity or rapid range expansion. Though a specimen from near the type locality in central France (Durieu 1861) was not sampled, the similarity between North American and European individuals gives some confidence that this clade represents *I. echinospora s.s.* As found in previous work, *I. hawaiiensis* shows no divergence from *I. echinospora* (Hoot et al., 2004). Considerable variation in gross morphology has resulted in confusion in identification and taxonomy in *I. echinospora s.l.* (Taylor et al., 1993). This dataset appears to have identified individuals from mixed populations (*I. 'septentrionalis' Schafran 171-2*) and entire misidentified populations (*I. 'tuckermanii' Schafran 174*). Samples identified as *I. maritima* (*Taylor 6987-1*) and *I. occidentalis* (*Grinter 1, 5*) may represent misidentifications or an unrecognized autotetraploid and autohexaploid, respectively.

Isoëtes echinospora commonly forms hybrids with other taxa in its range. In the Pacific Northwest with *I. bolanderi* it forms members of the *I. occidentalis* complex (*I.* ×*'herbwagneri'*

Taylor s.n., I. 'maritima' Taylor 6983, I. 'occidentalis' Taylor 6755, I. 'occidentalis' Woodbridge s.n.). In eastern North America, *I. echinospora* forms *I. septentrionalis s.s.* (*Brunton 19142*), *I.* ×*'dodgei*' (Brunton 19143) and *I.* ×*'eatonii'* (Taylor 6750) with *I. engelmannii.*

Isoëtes engelmannii (2x)

Numerous putative diploid individuals of *I. engelmannii* were identified by presence of a single *I. engelmannii* OTU per sample. Several were previously identified as other species, including *I. 'riparia'* (*Schafran 156, 157, 158*), *I. 'appalachiana'* (*Schafran 108*), and *I. 'hyemalis'* (*Schafran 122*). Genome size measurements from some of these populations (*Bradley s.n.1, Schafran 108, 122, 123, 140*) with C-values ranging from 3.9-4.9 strongly suggest the existence an autopolyploid of *I. engelmannii*. Diploid individuals have C-values ca. 1.5-2.0 (Bolin et al. 2018, Bolin unpublished data).

As expected for one of the most widespread and abundant species of *Isoëtes* in eastern North America, *I. engelmannii* sequences occurred in several putative polyploids. Two subclades of *I. engelmannii* in the LEAFY phylogeny are divided into 'North' and 'South' based on the geographic location of the associated polyploids. Putative polyploid OTUs in the 'North' clade originated from Pennsylvania northward, while those in the 'South' clade originated from Virginia south and southwest. *Isoëtes septentrionalis s.s. Brunton 19142* and *I. appalachiana s.s. Schafran 148* were formed by *I. engelmannii* 'North' crossing with *I. echinospora* and *I. valida*, respectively.

Isoëtes flaccida (2x)

These results support the traditional circumscription of *I. flaccida* as a diploid restricted to Florida (*Schafran FL01*) and extreme southern Georgia (*Schafran 203*). It is involved in few putative polyploids, such as *I. 'appalachiana'* (*I. sp. apGF-fl AY541767*, Hoot et al., 2004), *I. 'hyemalis'* (*Bradley s.n .2*), and *I. louisianensis* (*Leonard 12415*). *Schafran 108*, collected from the same locality as *I. sp. apGF-fl* (Hoot et al., 2004), did not display any OTUs from *I. flaccida*, but appeared to be an autotetraploid of *I. engelmannii*.

Isoëtes lithophila (2x)

Isoëtes lithophila is apparently isolated from other species of *Isoëtes*. Despite growing in physical proximity to *I. melanopoda* (e.g. *Schafran 60* and *61*), there are no indications of hybridization or introgression.

Isoëtes mattaponica (2x)

Topotype Sample: *Taylor 70*

Though previously treated as a rare endemic diploid species of freshwater tidal marshes in the Chesapeake Bay, these data suggest *I. mattaponica* is widespread throughout the Southeast where it has been confused with *I. melanopoda* ssp. *silvatica* and *I. piedmontana*. It occurs on granite rock outcrops (*Cressler 13*, *Schafran 116, Taylor 6675*) and small forested wetlands (*Cressler 9, Schafran 77*) in North Carolina and Georgia. C-values of 1.4-1.7 strongly suggest a diploid. Other individuals found in freshwater tidal marshes in Georgia (*Bradley 8670*) were misidentified and do not represent *I. mattaponica*. This species is involved in the formation of 16 hypothetical polyploid taxa ranging from southeastern Alabama (*I. 'hyemalis' Schafran 118*) to

northern Virginia (*I. 'BoykinsIsland' Taylor 6665*). Autotetraploids of *I. mattaponica* may exist as individuals with C-values 2.9-3.2, typical of other tetraploid taxa, but with only the *I. mattaponica* OTU present (*I. 'hyemalis' Schafran 128, 131*). Its phylogenetic position with numerous South American species in the LEAFY tree suggests a possible South American origin of *I. mattaponica*.

Isoëtes mississippiensis (2x)

Holotype Samples: *Schafran MS08, Taylor 6798*

The diploid *I. mississippiensis* is known from only one area in southern Mississippi, but it appears in numerous putative polyploids from Louisiana to North Carolina. Unknown W from *I. louisianensis* in Hoot et al. (2004) appears to be *I. mississippiensis*. Other populations of *I. 'louisianensis'* also have *I. mississippiensis* as a parent (*Alford 397, 398, 399, 401, 402, Schafran 195, Taylor 6794*), as well as populations of *I. 'melanopoda'* (*Ciafré 256, 728, Schafran 184, 187*) and *I. 'piedmontana'* (*Bolin JBNC17-3, Schafran 101, 103*).

Isoëtes melanopoda s.s. (excluding *I. melanopoda* ssp. *silvatica*) (2x)

Samples thought to represent *I. melanopoda s.s.* resolved in several separate clades along a polytomy with *I. prototypus* and *I. echinospora*, raising about the true identity of *I. melanopoda*. Unfortunately, locations of type populations near Athens, Illinois and Clinton, Iowa are unknown or extirpated, and type specimens were unavailable for sequencing. The four clades of *I. melanopoda* were treated independently, given that each apparent lineage is involved with the formation of separate putative polyploids, such as *I. 'melanopoda'* (*Ciafré 256, 728, Schafran 184, 187*), *I. junciformis* (*Brunton 17608*), *I. microvela* (*Bolin JBNC201EO4*), and *I.*

'laurentiana' (*Brunton 20101*). The presence of individuals with genome sizes typical of tetraploids and LEAFY OTUs from two *I. melanopoda* clades (*Schafran 184-1, 188-5*) could support the existence of cryptic species.

Isoëtes melanospora (2x)

Topotype Sample: *Schafran 12*

Samples of *I. melanospora* were intermixed in a clade with *I. piedmontana*. Given the lack of any clear segregation between the two, assignments of polyploid OTUs to either species is considered tenuous. A population in South Carolina sometimes identified as *I. melanospora* (Taylor et al., 1993) occurred in the *I. silvatica* clade (*Schafran NC08*).

Isoëtes prototypus (2x)

Little sequence divergence separated *I. prototypus* and *I. echinospora* in the LEAFY phylogeny, though this was contradicted in the plastome phylogeny. Most of the putative hybrids and polyploids -- *I. ×harveyi Taylor 6677, I. ×heterospora Taylor 6676, I. ×fairbrothersii 6922*, *I. 'tuckermanii' Schafran 166, I. 'echinospora' Schafran 167-3, I. 'lacustris' Taylor 6748* -- occurred within or near the range of *I. prototypus* in Maine, New Brunswick, and Nova Scotia. Many of these contained two OTUs within the *I. prototypus* clade, suggesting there could be heterozygous LEAFY alleles and undiscovered variation in diploid populations. Surprisingly, OTUs from individuals far outside the *I. prototypus* range appeared in this clade. From the Pacific Northwest, *I. 'occidentalis' Grinter 10*, *I. 'occidentalis' Woodbridge s.n.,* and *I. 'maritima×echinospora' Taylor 6988-2* were found with *I. prototypus*, as were *I. 'virginica' Brunton 19044* and *I. 'piedmontana' Cressler 14* from the Southeast.

Isoëtes silvatica (=*I. melanopoda* ssp. *silvatica*) (2x)

Topotype Sample: *Schafran NC05*

Like *I. mattaponica*, *I. silvatica* is comprised of individuals found in forested wetlands (*Schafran NC05*), freshwater tidal marshes (*Bradley 8670*), and granite outcrops (*Schafran NC08, Taylor 6731*) from southern Virginia to western Georgia. Given their overlapping ranges and habitats, its unsurprising there are likely polyploid derivatives (*I. 'graniticola' Schafran 115, I. 'hyemalis' Brunton 19012, I. sp. UnknownChickahominy2*). In addition to *I. silvatica* × *I. mattaponica*, another 17 combinations makes *I. silvatica* the diploid that participates in the most hypothetical polyploids in the study region. An autotetraploid of *I. silvatica* (C-values 3.3-3.4) may exist in 2 populations (*Bradley 8204, 8221*).

Isoëtes snowii (2x)

Topotype Sample: *Schafran 79-2*

Sequences from individuals of *I. snowii*, all from sandstone outcrops in southern Georgia, suggest complex population genetics among diploids, tetraploids and triploid hybrids (Musselman 2001, Bolin unpublished data). Some plants displayed slightly variable LEAFY alleles while others had only one, with no apparent correlation to morphology (unpublished data). This may suggest the tetraploids are autotetraploids, with occasional gene flow between ploidy levels allowing both divergence and passage of variant LEAFY alleles. *Isoëtes snowii* appears to have had a role in the formation of *I. junciformis s.s.* (*Brunton 17608*), *I. 'piedmontana'* and *I.* 'Leary' in western Georgia (*Schafran 101, 102, 103, 114*), and *I. 'melanopoda'* in central Tennessee (*Ciafré 256, 728*).

Isoëtes storkii (2x)

The Costa Rican *I. storkii* appears nested within the clade of North American *Isoëtes* but is not involved in the formation of any of the sampled polyploids.

Isoëtes tegetiformans (2x)

Topotype Sample: *Schafran 19*

Isoëtes tegetiformans did not appear to be involved with any polyploid taxa.

Isoëtes valida (2x)

Specimens of *I. valida* largely agreed with their identification, with a few exceptions in Alabama (*Brunton 18993B*, *Schafran 193*) and Maryland (*Schafran 163*). These results confirm *I. valida* as a parent of *I. appalachiana s.s.* (Hoot et al., 2004, Pereira et al., 2019), but also identify new putative polyploids derived from *I. valida* including *I. 'hyemalis'* (*Schafran VA01*), *I. 'lacustris'* (*Kessler s.n.*), *I. tennesseensis* (*Schafran 177*), and individuals previously identified as *I. 'valida'* (*Schafran NC11, NC13*).

Isoëtes viridimontana (2x)

Topotype Sample: *Taylor 6744*

Despite its single occurrence in Vermont, *I. viridimontana* is widely represented in polyploid taxa. *Isoëtes acadiensis s.s.* (*Schafran 175*)*, I. azorica* (*Jermy 21018*, Hoot et al., 2004)*,* and *I. tuckermanii s.s.* (*Schafran 176*) all contained *I. viridimontana*, replicating Pereira et al. (2019). Other hybrids and putative polyploids were found with *I. viridimontana* OTUs, including *I. '×harveyi' Taylor6677, I. '×heterospora' Taylor6676, I. '×fairbrothersii', I.*

'lacustris' (*Kessler s.n., Taylor 6748, Cerne Lake, Feldsee*), and *I. 'riparia'* (*Taylor 6675*). In the Southeast, *I. boomii s.s*. (*Schafran 73*) and *I. piedmontana* (*Taylor 6776, =I. 'graniticola-NC'* in plastome phylogeny).

Isoëtes 'Leary' (2x)

Topotype: *Schafran 83, 110*

Individuals previously treated as *I. flaccida* and *I. cf. junciformis* from forested wetlands in the region around Leary, Calhoun Co., Georgia, appear to represent a previously undescribed taxon. Genomes with a C-value around 2 and with a single LEAFY OTU support the presence of a diploid, and its phylogenetic placement and patristic distance far from any of the described diploids in eastern North America strongly suggests it represents an undescribed species. Some individuals occurring with *I.* 'Leary' had larger genome sizes (C-values 2.1-2.8) and 2 LEAFY OTUs, but all had at least one OTU present in the core *I.* 'Leary' clade. Its poor resolution in the plastome phylogeny may support the hypothesis of tropical origin sometimes ascribed to *I. flaccida* (Boom 1982).

Isoëtes 'Uwharrie' (2x)

Topotype: *Taylor 6732*

This putative undescribed species, a confirmed diploid collected from the Uwharrie National Forest in North Carolina (Taylor, pers. comm.), appears to represent the Unknown Y of Hoot et al. (2004) and Pereira et al. (2019). No other putative diploid individuals have been identified outside the Uwharrie National Forest, but this taxon appears in numerous polyploids. Hoot et al. (2004), Pereira et al. (2019), and this study all identify *I. acadiensis s.s.* (*Schafran*

175), *I. azorica* (*Jermy 21018*, Hoot et al., 2004), and *I. tuckermanii s.s.* (*Schafran 176*) in the *I.* 'Uwharrie' clade. Other polyploids including *I.* 'Uwharrie' are *I. boomii s.s.* (*Schafran 73*) and *I. microvela s.s.* (*Bolin JBNC201EO4*). Diploid assignment by patristic distance overrepresents the polyploid OTUs in the *I.* 'Uwharrie' clade due to the presence of other well supported clades without diploid representatives that likely represent unknown taxa.

Molecular Descriptions of Polyploids

Isoëtes acadiensis (4x)

Topotype Sample: *Schafran 176*

Nuclear Parentage: *I. 'Uwharrie'* × *I. viridimontana*

Chloroplast Donor: N/A

These results agree with the finding in Pereira et al. (2019) that *I. acadiensis* is *I. 'Uwharrie'* (=Unknown Y) × *I. viridimontana.* Other samples identified with the same genotype were *I. 'riparia' Taylor 6675, I. tuckermanii Schafran 168, I. tuckermanii s.s. Schafran 176,* and *I. tuckermanii Taylor 6707.* The inclusion of *Schafran 176,* selected as the closest available topotype of *I. tuckermanii s.s*., suggests a close relationship between both tetraploids and supports a recent proposal to treat *I. acadiensis* as *I. tuckermanii* ssp. *acadiensis* (Brunton, 2019). No chloroplast data were available to determine the maternal lineage.

Isoëtes appalachiana (4x)

Topotype Sample: *Schafran 148*

Nuclear Parentage: *I. engelmannii* × *I. valida*

Chloroplast Donor: *I. engelmannii*

Confirming Hoot et al. (2004) and Pereira et al. (2019), this study finds *I. engelmannii* and *I. valida* as parents of *I. appalachiana*. *Isoëtes appalachiana* in Pennsylvania is the result of *I. engelmannii* 'North' × *I. valida* (*Schafran 148, 150, 152, 153*), while in in Tennessee, Mississippi, and Alabama *I. appalachiana* is derived from *I. engelmannii* 'South' × *I. valida* (*Brunton 17581, Cressler 8, Schafran 105, 106, 178, 199, 200, 201*). That the *I. engelmannii* 'North' and 'South' genomes within *I. appalachiana* are more similar to diploid individuals of *I. engelmannii* than to each other very likely indicates multiple formation events in this tetraploid species. The maternal lineage in *Schafran 148* is *I. engelmannii,* but could vary in other populations if *I. appalachiana* is derived from multiple hybridization events.

Isoëtes boomii (6x)

Topotype: *Schafran 73*

Nuclear Parentage: *I. 'Uwharrie'* × *I. 'bolanderi'* × *I. silvatica* × *I. viridimontana*

Chloroplast Donor: Uncertain; likely *I. silvatica*, *I. mattaponica*, or *I. viridimontana*

This hexaploid was identified by four different diploid OTUs present at the type locality. Sequences were binned from all individuals due to low coverage for all samples, making the assumption that all individuals in the population were identical. The OTU identified as *I. 'bolanderi'* does not represent the species *sensu stricto*, but is a separate well supported clade sister to *I. bolanderi* + *I. howellii* + *I. lithophila*. Phylogenetic placement of the plastome was not close to one single diploid, but was similarly distant to *I. melanopoda ssp. silvatica*, *I. mattaponica*, and *I. viridimontana.* This genotype was not found in any other specimens. A population of *I. 'boomii'* (*Schafran 72*) very close to the type locality had only *I. 'Uwharrie'* × *I. silvatica.*

Isoëtes georgiana (6x)

Topotype: *Matthews 3*

Nuclear parentage: *I. 'bolanderi'* × *I. engelmannii* 'South' × *I. mattaponica*

Chloroplast Donor: Uncertain; close to *I. viridimontana* and *I. mattaponica*

Isoëtes georgiana had three nuclear genotypes consistent with a hexaploidy under the assumed model of fixed heterozygosity. One genotype was closest to *I. bolanderi* but occurred in one of several well supported clades with no other near diploids, so this OTU likely represents an unsampled diploid. The other parents were well supported as *I. engelmannii* and *I. mattaponica*. The plastome occurred in an uncertain position, closest to *I. viridimontana* but only slightly more distant to *I. mattaponica*. This genotype occurred only at the type locality, though some a similar genotype was found in the same geographic region (*I. 'hyemalis' Schafran 107, I. 'georgiana' Schafran GA16*, both *I. engelmannii 'South'* × *I. mattaponica*).

Isoëtes graniticola (4x)

Topotypes: *Schafran 117, Taylor 6998*

Nuclear Parentage: *I. mattaponica* × *I. melanopoda-4*

Chloroplast Donor: Uncertain; similar distances to *I. silvatica*, *I. mattaponica*, *I. viridimontana*

Treating *I. graniticola s.l.* as the tetraploid form of *I. piedmontana,* four individuals were included in this study. At the type locality in Alabama (*Schafran 117*), *I. mattaponica* and *I. melanopoda-4* were identified in the nuclear genome, while a population in Georgia (*Schafran 14*) was derived from *I. melanospora/I. piedmontana* × *I. silvatica*, and populations in North Carolina (*Bolin JBNC17, Taylor 6776*) were *I.* 'Butner' × *I. mississippiensis* and *I. mattaponica* × *I. viridimontana*, respectively. The widespread disagreement in the nuclear genome suggests

numerous cryptic tetraploids occurring on granite rock outcrops, corroborating isozyme data from Heafner and Bray (2005). Plastid data show a similar amount of dissimilarity among populations. At the type locality, *I. graniticola* has a plastome with similar distance to *I. silvatica*, *I. mattaponica*, and *I. viridimontana*. In Georgia the plastome is sister to *I. piedmontana*, supporting the nuclear data. The *Taylor 6776* specimen from North Carolina has a plastome most similar to *I. 'piedmontana'* in the same locality. The diploid *I. 'piedmontana'* taxon in North Carolina also appears in the *I. mattaponica* clade in the LEAFY phylogeny, so is probably the source of the *I. mattaponica* OTU in *Taylor 6776*.

Isoëtes hyemalis (4x)

Topotype: *Bolin JBNC*

Nuclear Parentage: N/A

Chloroplast Donor: Uncertain; similar distance to *I. viridimontana, I. mattaponica,* and *I. silvatica*

Only plastid data from *I. hyemalis s.s.* could be generated because DNA from older collections did not amplify. The plastome placement was near to *I. viridimontana, I. mattaponica,* and *I. silvatica,* and the position of *I. hyemalis* moved slightly within the clade depending on the type of analysis. Using the two nearest populations to the type locality (all in Harnett Co., NC), *I. 'Uwharrie'* × *I. mattaponica* is assumed to represent the nuclear genotype of *I. hyemalis s.s.* With this circumscription, *I. hyemalis* occurs only near the Sand Hills region of North Carolina (*Schafran 76, 130, 133*) except for a disjunct population in Alabama (*Schafran 118*). Other populations throughout the Southeast display a variety of diploid relationships. Samples from near Raleigh, North Carolina (*Schafran 141, 142, 143, 144*), each had two OTUs

in the *I.* 'Butner' clade, suggesting an auto- or allotetraploid with one or two unknown species. The finding of *I. 'applachiana'* from Hoot et al. (2004) identified as *I. engelmannii* × *I. flaccida* was replicated in populations of *I. 'hyemalis'* in the Coastal Plain of South Carolina (*Bradley s.n. 2*) and Virginia (*Bunch s.n. 2*). In tributaries of the Chesapeake Bay*, I. 'hyemalis'* had *I. mattaponica* × *I. melanopoda-3* as its parentage (*Bolin RiverRestA, Taylor 6665*). Several other diploid combinations were identified in single populations. These results suggest numerous cryptic taxa lumped in *I. hyemalis s.l.*

Isoëtes junciformis (4x)

Topotypes: *Brunton 17608, Bolin s.n.*

Nuclear Parentage: *I. melanopoda-1* × *I. melanopoda-2* × *I. snowii* × *I. snowii*

Chloroplast Donor: Uncertain; sister to *I. flaccida* and *I.* 'Uwharrie'

The rare tetraploid *I. junciformis* displayed a surprising number of nuclear genotypes, conflicting with the typical allopolyploid model that predicts two nuclear genotypes in a tetraploid. That the genotypes in *I. junciformis* occur as similar pairs might suggest two heterozygous *I. snowii* and *I. melanopoda* parents. Other collections with similar genotypes that may be involved in the *I. junciformis* complex include *I. 'junciformis' Schafran 104* (*I. melanopoda-1* × *I. melanopoda-3* × *I. snowii* × *I. snowii*), *I. 'piedmontana' Schafran 102* (*I. melanopoda-1* × *I. snowii* × *I. snowii*), and *I. 'melanopoda' Ciafré 728* (*I. melanopoda-1* × *I. snowii*) among others. Estimation of the maternal parent of *I. junciformis* was hindered by a low quality plastome assembly, but its phylogenetic position based on available data was not near any of the parents suggested by the LEAFY phylogeny. This same trend is observed with

the other plastid genomes in the clade with I. junciformis (*I. microvela, I. virginica*), raising the hypothesis of a chloroplast capture event in a shared parent of all three polyploids.

Isoëtes lacustris (10x)

Topotype: N/A

Nuclear Parentage: Various

Chloroplast Donor: N/A

Type materials were unavailable for sampling, but collections of *I. lacustris s.l.* from North American and Europe showed variable parentage, though not segregated by continent. Collections from Germany (*Ctvrlikova s.n. Feldsee Lake*) and the Czech Republic (*Ctvrlikova s.n. Černé Lake*) identified Unknown Z (called *I. bolanderi* by patristic distance) × *I. viridimontana* as putative parents, but samples from Switzerland (*Kessler s.n.*), Vermont, (*Taylor 6748*), and Ontario (*Schafran 167-3*) found *I. prototypus* × *I. prototypus* × *I. viridimontana.* Results from *I. 'lacustris' Krasluer s.n.* (Pniewo, Poland) identifying three diploid parents from the southeastern US are likely erroneous due to low coverage.

Isoëtes '*laurentiana*' (4x)

Topotype: *Brunton 20092*

Nuclear Parentage: *I. engelmannii* × *I. melanopoda-3* × *I. silvatica*

Chloroplast Donor: *I. engelmannii*

The putative new tetraploid from the St. Lawrence River in Quebec displays a genome size typical of tetraploids (C-value 3.6 - 4.1) but varied in genotype among individuals. The combination of I. *melanopoda-3* × *I. silvatica* was present in all individuals, but half of those

sampled also included *I. engelmannii* 'North', which appears to be the maternal parent. Given the lack of typical indicators of hybridization (deformed megaspores, heterosis), it is unclear what mechanism is generating the variability observed in the nuclear genotype. The *I. 'laurentiana'* genotype *I. melanopoda-3* × *I. silvatica* is a combination shared with *I. 'hyemalis'* and *I. 'microvela'* from many populations in southeastern North Carolina.

Isoëtes louisianensis (4x)

Topotype: *Bolin JBLA*

Nuclear Parentage: *I. silvatica*

Chloroplast Donor: Uncertain; *I. snowii, I. melanopoda, I. mississippiensis*

The federally endangered *I. louisianensis* displayed considerable variation between populations based on their plastome sequences. *Isoëtes louisianensis s.s. Bolin JBLA* and *Taylor 6793* form a clade sister to *I. snowii*, but several other diploids are about twice the distance: *I. melanopoda*, *I. chapmanii*, and *I. mississippiensis. Isoëtes 'louisianensis' Taylor 6795* is closely related to *I. melanopoda* ssp. *silvatica,* while *Taylor 6797* is sister to *I. melanopoda Taylor 6796*, but due to a long terminal branch of *Taylor 6796* and short internal branches in this clade, *I. louisianensis Taylor 6797* is less distant to *I. melanopoda Taylor 6940*, *I. chapmanii*, and *I. mississippiensis*. *Isoëtes 'louisianensis' Leonard 12415* occupies an uncertain position near the base of the American clade. The poor support values may suggest that it is more closely related to unsampled clades in Central or South America.

No nuclear data were obtained from many of the above samples due to low quality of the DNAs and specimens, resulting in poor amplification of the LEAFY marker. Only one OTU was recovered from the topotype *Bolin JBNC,* matching *I. silvatica*. Coverage was high enough that

this result appears reliable, though in conflict where the plastid relationships. *Leonard 12415* showed a nuclear parentage of *I. flaccida* × *I. melanospora.* A population lacking plastid data (*Alford 397, 398, 399, 401, 402, Schafran 195*) was found to be *I. mississippiensis* × *I. silvatica.* Combined with some level of morphological variability (Taylor, pers. comm.) these results support the existance of cryptic species within *I. louisianensis*.

Isoëtes microvela (6x)

Topotype: *Bolin JBNC201EO4*

Nuclear Parentage: *I.* 'Butner′ × *I. melanopoda-3* × *I. silvatica*

Chloroplast Donor: Uncertain

Isoëtes microvela sensu stricto occurs only at its type locality (Jones Co., North Carolina). Though one other sample (*Schafran 136*) is combined with *I. microvela s.s*. based on distance to known diploids, it does not contain the 'Butner' genotype based on the phylogeny. Most other populations treated as *I. microvela* (*Bolin JBNC40, JBNC199EO2, JBNC200EO3, JBNC202EO5, Matthews I09-35*) share the *I. melanopoda-3* × *I. silvatica* parentage with *I. microvela s.s.* but lack the 'Butner' genotype. Other populations identified as *I. 'hyemalis'* and *I.* '*laurentiana*' also share the *I. melanopoda-3* × *I. silvatica* genotype. The maternal ancestor is uncertain, as the plastome is similarly distant to *I.* 'Uwharrie', *I. flaccida*, *I. valida*, *I. lithophila*, *I. texana*, and *I. prototypus*.

Isoëtes 'riparia' (4x)

Topotype: N/A

Nuclear Parentage: N/A

Chloroplast Donor: N/A

No reliable data were obtained for *I. riparia s.s.* Historic type populations from the Delaware River near Philadelphia, as well as populations on the Delmarva Peninsula, are mostly extirpated. A putative tetraploid collected from the Eastern Shore of Maryland (*Schafran 180*) occurred in a different habitat than described for *I. riparia* and had a genotype matching *I. 'hyemalis'* (*Schafran 127*), *I. 'boomii'* (*Schafran 72*), and *I. microvela* (*Schafran 119*). Most populations in the dataset identified as *I. riparia* have been reclassified as *I. septentrionalis* (Brunton and McNeill 2015).

Isoëtes septentrionalis (4x)

Topotype: *Brunton 19142*

Nuclear Parentage: *I. echinospora* × *I. engelmannii*

Chloroplast Donor: *I. engelmannii*

Results for *I. septentrionalis* (=*I. riparia s.l.* in previous studies) largely confirm previous work (Caplen and Werth, 2000b, Hoot et al., 2004, Pereira et al., 2019). The combination *I. echinospora* × *I. engelmannii* was found in the topotype specimen as well as numerous others identified as *I. septentrionalis* or *I. riparia* (*Schafran 151, 159, 160, 161, 170, 171, 172, 173, Taylor 6706*). The hybrids *I.* ×*dodgei* (*Brunton19143*) and *I.* ×*eatonii* (*Taylor 6750*), suspected members of the *I. septentrionalis* complex, were supported with the same genotype at *I. septentrionalis*. The *I. riparia* sample from Hoot et al. (2004), which was resolved in the *I. engelmannii* 'South' clade in their analysis, was strongly supported in the 'North' clade in this study. The presence of this genotype as far south as Maryland, where the sample in question

originated, suggests that *I. riparia s.s.* could be equivalent to *I. septentrionalis*. In contrast, two samples of *I.* ×*eatonii* (*Taylor 6750* and *Taylor s.n.* from Hoot et al., 2004) had *I. engelmannii* genotypes from the 'North' and 'South' clades, respectively. As with *I. appalachiana*, this could predict multiple origins of *I. septentrionalis/I. riparia* from the same diploid parents.

Isoëtes tennesseensis (8x)

Topotype: *Schafran 177*

Nuclear Parentage: *I. engelmannii* 'South' × *I. mattaponica* × *I. valida*

Chloroplast Donor: Uncertain; similar distances to *I. melanopoda*, *I. chapmanii*, and *I. snowii*

The only octoploid taxon in North America, its parentage appears to involve three diploids from the Southeast. While historically treated as *I. lacustris* or *I. macrospora,* the genetic dissimilarity supports its treatment as a unique endemic taxon of the Southern Appalachians and suggests these high level polyploids formed completely independently from each other. However, the plastome did not closely match any of the species present in the nuclear data, raising questions about its origin.

Isoëtes tuckermanii (4x)

Topotype: *Schafran 176*

Nuclear Parentage: *I.* 'Uwharrie' × *I. viridimontana*

Chloroplast Donor: *I. echinospora*

Results from this study conflict with Hoot et al. (2004) and Pereira et al. (2019) about the parentage of *I. tuckermanii*. The individual used in previous studies, collected from Nova Scotia, was interpreted as Unknown Z × *I. viridimontana. Schafran 176* was collected much closer to

the type locality in Boston, Massachusetts, so it is assumed to more likely represent *I. tuckermanii s.s.* A recent collection from the same location as the Hoot et al. (2004) specimen was found to be *I. echinospora* (*Schafran 174*). The plastome from *I. tuckermanii* strongly matched *I. echinospora*, which is surprising since it is not one of the components of the nuclear genome, but *I. echinospora* and *I. tuckermanii* are known to hybridize so a plastome origin through chloroplast capture may be possible. The nuclear genotypes of *I. tuckermanii* and *I. acadiensis* were identical, supporting the recent taxonomic change lowering *I. acadiensis* to *I. tuckermanii* ssp. *acadiensis* (Brunton, 2019).

Isoëtes virginica (4x)

Topotype: *Taylor 6882*

Nuclear Parentage: *I. 'bolanderi'* × *I. silvatica*

Chloroplast Donor: Uncertain

Isoëtes virginica is a rare taxon with an unclear phylogenetic history. Hoot et al. (2004) found two LEAFY clones with greater similarity to each other than any diploid. Even with much greater sampling, in this study these sequences still formed a clade with each other, nested in the larger *I. melanopoda-2* clade. *Taylor 6882*, collected from what is believed to be the type locality, and *Brunton 19044* from North Carolina, contributed more confusion. The parentage of *Taylor 6882* included *I. silvatica* and an OTU in one of the clades near *I. bolanderi* that likely represent unsampled diploids, while *Brunton 19044* was *I. mattaponica* × *I. melanopoda-3* × *I. prototypus.* Across all three individuals of *I. 'virginica'*, their sequences do not appear in any of the same clades. The only evidence for any shared evolutionary history is that the plastomes of *Taylor 6882* and *Brunton 19044* are nearly identical, and also very similar to *I. microvela*. These

samples are isolated in the plastome phylogeny without a clear diploid relative, instead similarly distant to *I. flaccida*, *I. valida*, *I. lithophila*, *I. texana*, and *I. prototypus*. The incongruence between plastid and nuclear genomes as well as between populations indicates much further study needed to understand these taxa.

CHAPTER 6

SUMMARY

This study examined the diversity and systematics of *Isoëtes* in eastern North America, arguably the best studied group of species in the genus, but which still present considerable taxonomic and systematic uncertainty. Herein data were presented that document the utility of some morphological characteristics in practical taxonomy, of whole chloroplast genomes and single-copy nuclear markers for resolving interspecies relationships, and of these data for inferring evolutionary origins of polyploid taxa.

The paucity of taxonomically informative morphological characters resulted in the same sets of features being analyzed in every study of *Isoëtes* through the 19th-20th centuries. In the latter 20th century, general consensus found spore ornamentation, spore size, velum coverage, habitat, and ploidy level to be the most informative characters for making taxonomic changes to the genus. A new diploid species, *Isoëtes mississippiensis*, was described based on laevigate megaspore ornamentation, spinulose microspore ornamentation, velum coverage 15-33%, and a diploid number of chromosomes, a combination of character states not observed in any other taxa in eastern North America. Further phylogenomic analysis supported the distinctiveness of *I. mississippiensis*, highlighting that even in a genus with great morphological similarity, unique character sets can identify undescribed taxa.

Use of whole chloroplast genome sequences resolved relationships within the American clade of *Isoëtes* for the first time. All described diploid species from eastern North America showed highly supported relationships. Ancestral character state analysis of taxonomic

characters such as megaspore ornamentation and color showed 79% of transitions occurred on the terminal branches. Some shared features, such as black megaspores or cristate-reticulate ornamentation, were found to be convergent. This suggests that while morphology may be sufficient to delineate taxa, it poorly represents patterns of evolution. Inclusion of polyploid chloroplast genomes in the phylogeny clearly indicated a maternal lineage from one diploid to some polyploid species, but for many polyploids there was no clear diploid ancestor. Cladistic placement and patristic distance for some polyploids suggests descent from a common ancestor of several extant diploids, but in several, especially *I. junciformis, I. microvela*, and *I. virginica*, the lack of any clear diploid ancestor – plus general disagreement with the nuclear phylogeny – indicates a more complicated origin.

Expanding primarily on work by Caplen and Werth (2000), Hoot et al. (2004) and Bolin et al. (2008), inference of polyploid parentage using biparentally inherited nuclear markers supported several prior hypotheses, such as *I. appalachiana* and *I. septentrionalis*, but found that most polyploids had taxonomy that disagreed with evolutionary lineages. For 15 described polyploids in eastern North America, at least 70 different combinations of diploid genomes were identified. Well supported clades of polyploid sequences like *I.* 'Butner' likely represent unsampled diploids – species that are undiscovered, extinct, or occur outside the study's range.

How to reconcile the taxonomy of *Isoëtes* with phylogenetic evidence remains a significant challenge. Starting with a biological species concept evidenced by individuals with deformed spores, supported by DNA sequences and cytology as F_1 hybrids between sterile species, then each taxon contributing to the (presumably) sterile hybrid must represent a species. As formation of unreduced gametes by F_1 hybrids is the only proposed pathway to genome duplication in *Isoëtes*, any polyploid with genomic contributions from two parental species must

derive from a unique hybridization event. Though homoploid hybrids in *Isoëtes* are undocumented outside of diploids (they have been hypothesized based on spore morphology), the same barriers to successful chromosome pairing would presumably prevent development of fertile spores between, for example, two tetraploids with different diploid progenitors. In addition, hybrids between individuals at two different ploidy levels, regardless of genome composition, would fail to produce viable spores (Takamiya et al., 1999). Under this simplistic model of allopolyploidy, each polyploid with different parental genomes represents a lineage that is intersterile with its closest relatives, *i.e.* a species (Soltis and Soltis 2009). From the results presented here, approximately 50 species are unaccounted for in the current treatment of *Isoëtes*, an increase of 300% over the number of described polyploids.

However, preliminary genome-wide data from several of the species included in this study show disparity in tree topologies across loci (Schafran et al., unpublished data). Phylogenetic models incorporating incomplete lineage sorting showed only slight improvement over concatenation, suggesting that lateral gene transfer between some diploid species may have occurred. These data also strongly support a hybrid origin of *Isoëtes chapmanii,* with the nuclear genome from *I. flaccida* but the chloroplast captured from *I. melanopoda*. Some confirmed or suspected diploids in this study, such as *I. snowii Schafran 78-2, I. melanopoda Schafran 188-5*, and *I. mississippiensis Schafran 194,* also showed what appeared to be heterozygosity of the LEAFY marker, which though uncommon may invalidate the strict single-copy status of this marker as previously employed in *Isoëtes* systematics, or indicate some level of interbreeding between divergent populations or taxa. Ultimately, it may be possible that some level of gene flow between species of occurs through hybrid intermediaries, resulting in the complex mixture of genotypes observed in this study (Harrison and Larson, 2014). It is as true today as 30 years

ago that “all of the species of *Isoëtes* that have been intensively investigated readily conform to a dynamic interpretation of the biological species concept” (Hickey et al. 1989). Results of ongoing target-enrichment and whole genome sequencing should elucidate the degree of genetic interchange between taxa, illuminating aspects of reproductive biology and thus more appropriate species boundaries.

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APPENDICES

APPENDIX A. SAMPLES INCLUDED IN PLASTOME PHYLOGENY AND PLASTOME ASSEMBLY STATISTICS

Taxon	Ploidy	Collection #	Locality	Post-Trim Paired-End Reads	Chloroplast Reads	Percent Chloroplast Reads	Assembled Plastome Size (bp)	Plastome Coverage	*De novo* Assembly N50	Percent Missing/ Ambiguous Sites
I. 'laurentiana'	44	Brunton 20092[+]	Parc du Haut Fond, QC	21,458,826	130,698	0.61	145,066	124	91,759	0
I. 'Leary'	22	Schafran 83-2 (ODU)	Leary, GA	1,531,700	23,916	1.56	145,134	30	24,754	0.2
I. 'Uwharrie'	22	Taylor 6732 (US)	Uwharrie Natl. Forest, NC	530,564	25,574	4.82	145,084	33	14,458	0.5
I. anatolica	22	Musselman and Keskin TR-2001-01 (ODU)	Vicinity of Abant Lake, Bolu Province, Turkey	13,284,644	465,270	3.5	144,829	453	91,572	0
I. appalachiana	44	Schafran 105-2 (ODU)	Lick Branch, Riggins, MS	55,753,654	584,186	1.05	145,059	567	27,199	0
*I. boomii**	66	Schafran 73-1 (ODU)	Tributary of Long Branch, Cadwell, GA	54,679,216	557,016	1.02	145,399	499	808	5.4
I. butleri	22	Schafran 47 (ODU)	Ft. Worth, TX	7,141,820	227,022	3.18	144,912	235	33,226	0
I. butleri	22	Taylor 7000 (US)	Alabama	1,428,800	15,250	1.07	145,029	20	23,423	0.4
I. butleri	22	Taylor 7001 (US)	Alabama	505,556	3,196	0.63	145,078	4	1,503	9.7
*I. chapmanii**	22	Bolin JBFL01 (ODU)	Tributary of Chipola River, Marianna, FL	5,042,822	65,178	1.29	145,096	64	24,803	0
I. echinospora	22	Schafran 32 (ODU)	Cleveland Lake, Watson, NY	1,805,678	16,298	0.9	145,186	18	9,252	0.4
I. echinospora	22	Ctvrlikova s.n.(ODU)	Plesne Lake, Czech Republic	24,811,300	478,444	1.93	145,248	463	91,834	0
I. echinospora	22	Ctvrlikova s.n. (ODU)	Feldsee, Germany	29,903,446	94,120	0.31	145,254	88	91,994	0

Taxon	Ploidy	Collection #	Locality	Post-Trim Paired-End Reads	Chloroplast Reads	Percent Chloroplast Reads	Assembled Plastome Size (bp)	Plastome Coverage	*De novo* Assembly N50	Percent Missing/ Ambiguous Sites
I. echinospora	22	Taylor 6989 (US)	Comox Lake, Cumberland, BC	16,915,102	160,070	0.95	145,090	152	91,693	0
I. engelmannii	22	Schafran 46 (ODU)	Hiawassee River, Reliance, TN	5,201,790	197,188	3.79	144,817	200	24,776	0
I. engelmannii	22	Schafran VA04 (ODU)	Maury River, Goshen, VA	30,187,436	306,706	1.02	145,479	294	18,064	0.3
I. flaccida	22	Taylor 6770 (US)	St. Marks River, Newport, FL	1,142,646	25,114	2.2	145,126	23	10,661	0
*I. georgiana**	66	Matthews s.n. (ODU)	Little Abrams Creek, Doles, GA	26,421,740	359,464	1.36	145,341	349	91,901	0
I. graniticola	44	Schafran 14 (ODU)	Quarry near Culverton, GA	7,214,036	151,162	2.1	145,118	154	91,891	0
I. graniticola	44	Taylor 6776 (US)	Woodleaf, Rowan Co., NC	1,938,518	105,664	5.45	145,029	134	24,788	0.1
*I. graniticola**	44	Taylor 6998 (US)	Flat Rock Park, Lineville, AL	1,041,602	23,054	2.21	145,127	31	24,778	0.2
*I. hyemalis**	44	Bolin JBNC[+]	BBQ Creek, NC	3,445,230	22,894	0.66	145,133	20	3,659	0.2
*I. junciformis**	44	Brunton 17608[+]	Tributary of Whiddons Mill Creek, Chula, GA	1,863,074	7,092	0.38	145,405	7	1,853	3.3
*I. lithophila**	22	Schafran 61 (ODU)	Enchanted Rock, TX	5,804,420	102,728	1.77	144,978	192	2,615	0.1

Taxon	Ploidy	Collection #	Locality	Post-Trim Paired-End Reads	Chloroplast Reads	Percent Chloroplast Reads	Assembled Plastome Size (bp)	Plastome Coverage	*De novo* Assembly N50	Percent Missing/ Ambiguous Sites
*I. junciformis**	44	Brunton 17608[+]	Tributary of Whiddons Mill Creek, Chula, GA	1,863,074	7,092	0.38	145,405	7	1,853	3.3
*I. lithophila**	22	Schafran 61 (ODU)	Enchanted Rock, TX	5,804,420	102,728	1.77	144,978	192	2,615	0.1
I. louisianensis	44	Taylor 6793 (US)	Gator Branch, DeSoto Natl. Forest, MS	3,948,286	18,098	0.46	145,152	16	24,776	0.1
I. louisianensis	44	Taylor 6795 (US)	Okey Branch, DeSoto Natl. Forest, MS	8,319,706	40,308	0.48	145,119	35	14,454	0.1
I. louisianensis	44	Taylor 6797 (US)	Moody Branch, Picayune, MS	5,738,068	33,230	0.58	145,041	59	3,120	0.1
*I. louisianensis**	44	Bolin JBLA[+]	Thigpen Creek, Washington Parish, LA	7,703,752	49,102	0.64	145,156	47	24,800	0.1
I. louisianensis	44	Leonard 12415 (ODU)	Hell Hole Creek, Avera, MS	40,792,900	259,812	0.64	145,209	104	539	0
*I. mattaponica**	22	Bray Chick12[!]	Mattaponi River, Aylett, VA	36,708,442	887,828	2.42	145,065	393	8,643	0
I. melanopoda	22	Taylor 6940 (US)	Giant City State Park, Makanda, IL	3,617,226	304,620	8.42	145,075	321	24,804	0
I. melanopoda	22	Taylor 6796 (US)	Shubuta, MS	5,235,012	48,406	0.92	145,481	48	24,841	0.1
*I. melanopoda ssp. silvatica**	22	Schafran NC05 (ODU)	Mecklenburg Co., NC	7,783,990	365,396	4.69	145,109	391	91,903	0

Taxon	Ploidy	Collection #	Locality	Post-Trim Paired-End Reads	Chloroplast Reads	Percent Chloroplast Reads	Assembled Plastome Size (bp)	Plastome Coverage	*De novo* Assembly N50	Percent Missing/ Ambiguous Sites
*I. melanospora**	22	Schafran 12 (ODU)	Stone Mountain, GA	6,257,620	250,822	4.01	145,045	260	27,338	0
*I. microvela**	66	Bolin JBNC201[+]	White Oak River, Maysville, NC	35,439,412	807,522	2.28	145,190	783	27,240	0
*I. mississippiensis**	22	Taylor 6798 (US)	Lotts Creek, Picayune, MS	6,717,478	145,454	2.17	145,061	149	27,360	0
I. nuttallii	22	Taylor 6734 (US)		905,504	42,342	4.68	144,680	53	4,625	0
I. pallida	22	Taylor s.n.[+]		15,539,904	336,378	2.16	144,077	328	90,694	0
*I. piedmontana**	22	Schafran 18 (ODU)	Heggie's Rock, GA	2,058,638	22,294	1.08	144,931	36	24,811	0
I. piedmontana	22	Schafran NC01 (ODU)	Litaker, NC	1,345,338	45,156	3.36	145,034	56	24,791	0.1
*I. prototypus**	22	Brunton 12350[+]	Holland Lake, Harvey, NB	11,341,756	199,714	1.76	145,090	193	91,869	0
*I. septentrionalis**	44	Brunton 19142[+]	Ottawa River, Ottawa, ON	34,924,668	590,046	1.69	145,120	565	91,795	0
I. setacea	22	Jermy s.n.[!]	Lesvos, Greece	13,594,120	82,288	0.61	144,261	78	103,874	0
*I. snowii**	22	Schafran 79-4 (ODU)	Broxton Rocks, GA	20,921,388	807,526	3.86	145,145	781	91,716	0
*I. tegetiformans**	22	Schafran 19 (ODU)	Heggie's Rock, GA	2,530,964	84,678	3.35	144,996	82	7,029	0
*I. tennesseensis**	88	Schafran 177-2 (ODU)	Hiawassee River, Reliance, TN	32,479,800	516,576	1.59	145,147	498	27,222	0

Taxon	Ploidy	Collection #	Locality	Post-Trim Paired-End Reads	Chloroplast Reads	Percent Chloroplast Reads	Assembled Plastome Size (bp)	Plastome Coverage	*De novo* Assembly N50	Percent Missing/ Ambiguous Sites
*I. texana**	22	Taylor s.n.[+]	Falcon Point Ranch, TX	4,490,246	69,500	1.55	145,475	70	38,286	0.1
*I. texana**	22	Schafran 181-3 (ODU)	Powderhorn Ranch, TX	121,258,596	3,105,306	2.56	145,137	2,981	1,392	0
*I. toximontana**	22	Musselman 2001-33 (ODU)	Gifberg, South Africa	10,346,972	329,548	3.18	144,491	320	91,560	0
I. tuckermanii	44	Schafran 176-2 (ODU)	Ponkapoag Pond, MA	27,039,080	136,998	0.51	145,269	130	91,996	0.1
I. valida	22	Schafran 37 (ODU)	Michaux State Forest, PA	3,451,420	55,602	1.61	145,132	72	24,817	0
I. virginica	44	Brunton 19044[+]	Oxford, NC	2,344,776	28,046	1.2	145,170	31	10,083	0.2
*I. virginica**	44	Taylor 6882 (US)	Lofton, Augusta Co., VA	2,064,278	29,444	1.43	145,128	36	11,329	0.3
*I. viridimontana**	22	Taylor 6744 (US)	Haystack Pond, VT	96,759,784	1,849,900	1.91	145,117	1,476	878	0

*Topotype collection

[+]Specimen held in personal collection of the collector

[!]No voucher specimen known

APPENDIX B. SAMPLES INCLUDED IN LEAFY PHYLOGENY AND PACBIO ANALYSIS STATISTICS

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_acadiensis_Schafran175-1_rep1	Shubenacadie Grand Lake, NS	4	—	173	2	TRUE
Isoetes_acadiensis_Schafran175-1_rep2	Shubenacadie Grand Lake, NS	4	—	446	2	TRUE
Isoetes_acadiensis_Schafran175-1_rep3	Shubenacadie Grand Lake, NS	4	—	150	2	TRUE
Isoetes_acadiensis_Schafran175-2_rep1	Shubenacadie Grand Lake, NS	4	—	48	2	TRUE
Isoetes_acadiensis_Schafran175-2_rep2	Shubenacadie Grand Lake, NS	4	—	69	2	TRUE
Isoetes_acadiensis_Schafran175-2_rep3	Shubenacadie Grand Lake, NS	4	—	98	2	TRUE
Isoetes_acadiensis_Schafran175-3_rep1	Shubenacadie Grand Lake, NS	4	—	24	2	TRUE
Isoetes_acadiensis_Schafran175-3_rep2	Shubenacadie Grand Lake, NS	4	—	95	2	TRUE
Isoetes_acadiensis_Schafran175-4_rep1	Shubenacadie Grand Lake, NS	4	—	25	2	TRUE
Isoetes_acadiensis_Schafran175-4_rep2	Shubenacadie Grand Lake, NS	4	—	201	4	FALSE
Isoetes_acadiensis_Schafran175-4_rep3	Shubenacadie Grand Lake, NS	4	—	136	4	FALSE
Isoetes_acadiensis_Schafran175-5_rep1	Shubenacadie Grand Lake, NS	4	—	54	2	TRUE
Isoetes_acadiensis_Schafran175-5_rep2	Shubenacadie Grand Lake, NS	4	—	212	2	TRUE
Isoetes_acadiensis_Schafran175-5_rep3	Shubenacadie Grand Lake, NS	4	—	114	2	TRUE
Isoetes_andicola_RS1	Peru	4	—	2382	2	TRUE
Isoetes_andicola_RS5	Peru	4	—	1923	4	FALSE
Isoetes_appalachiana_Cressler8-1	Franklin State Forest, TN	4	—	36	2	TRUE
Isoetes_appalachiana_Cressler8-2	Franklin State Forest, TN	4	—	43	2	TRUE
Isoetes_appalachiana_Schafran105-1	Riggins, MS	4	—	47	2	TRUE
Isoetes_appalachiana_Schafran105-2	Riggins, MS	4	—	50	2	TRUE
Isoetes_appalachiana_Schafran105-3	Riggins, MS	4	—	30	2	TRUE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_appalachiana_Schafran108-1	Ochlocknee River, FL	4	3.94	238	1	FALSE
Isoetes_appalachiana_Schafran108-3	Ochlocknee River, FL	4	3.94	177	1	FALSE
Isoetes_appalachiana_Schafran108-4	Ochlocknee River, FL	4	3.94	248	1	FALSE
Isoetes_appalachiana_Schafran108-5	Ochlocknee River, FL	4	3.94	221	1	FALSE
Isoetes_appalachiana_Schafran148-1	Juniata River, PA	4	3.93	29	2	TRUE
Isoetes_appalachiana_Schafran148-2	Juniata River, PA	4	3.93	31	2	TRUE
Isoetes_appalachiana_Schafran148-4	Juniata River, PA	4	3.93	201	2	TRUE
Isoetes_appalachiana_Schafran148-5	Juniata River, PA	4	3.93	170	2	TRUE
Isoetes_appalachiana_Schafran150_rep1	Black Moshannon State Forest, PA	4	3.7	223	2	TRUE
Isoetes_appalachiana_Schafran150_rep2	Black Moshannon State Forest, PA	4	3.7	24	2	TRUE
Isoetes_appalachiana_Schafran150_rep3	Black Moshannon State Forest, PA	4	3.7	17	2	TRUE
Isoetes_appalachiana_Schafran178_rep1	Evergreen, AL	4	—	51	2	TRUE
Isoetes_appalachiana_Schafran178_rep2	Evergreen, AL	4	—	128	2	TRUE
Isoetes_appalachiana_Schafran178_rep3	Evergreen, AL	4	—	87	2	TRUE
Isoetes_appalachiana_Schafran199_big	Cades Cove, TN	4	3.99	1031	2	TRUE
Isoetes_appalachiana_Schafran199_small	Cades Cove, TN	4	3.99	1843	2	TRUE
Isoetes_appalachiana_Schafran200	Cades Cove, TN	4	—	1151	2	TRUE
Isoetes_appalachiana_Schafran201	Cades Cove, TN	4	—	804	2	TRUE
Isoetes_appalachiana_X_engelmannii_Brunton19008	Carson, VA	3	—	189	1	FALSE
Isoetes_appalachiana_X_hyemalis_Brunton19011B_rep1	Stony Creek, VA	4	—	68	4	FALSE
Isoetes_appalachiana_X_hyemalis_Brunton19011B_rep2	Stony Creek, VA	4	—	49	4	FALSE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_bolanderi_X_occidentalis_Taylor6756	El Dorado Co., CA	4	—	115	2	TRUE
Isoetes_bolanderi_X_occidentalis_Taylor6759_rep1	El Dorado Co., CA	4	—	612	4	FALSE
Isoetes_bolanderi_X_occidentalis_Taylor6759_rep2	El Dorado Co., CA	4	—	69	2	TRUE
Isoetes_boliviensis_RS06	Peru	4	—	535	1	FALSE
Isoetes_boomii_BargerSN_rep1	Conecuh National Forest, AL	6	—	72	2	FALSE
Isoetes_boomii_BargerSN_rep2	Conecuh National Forest, AL	6	—	82	2	FALSE
Isoetes_boomii_Leonard12408_rep1	Tift Co., GA	6	—	157	3	TRUE
Isoetes_boomii_Leonard12408_rep2	Tift Co., GA	6	—	72	3	TRUE
Isoetes_boomii_Schafran72-1	Cadwell, GA	6	—	31	2	FALSE
Isoetes_boomii_Schafran72-3	Cadwell, GA	6	—	15	2	FALSE
Isoetes_boomii_Schafran72-5	Cadwell, GA	6	—	22	2	FALSE
Isoetes_boomii_Schafran73-1	Cadwell, GA	6	—	24	4	FALSE
Isoetes_boomii_Schafran73-2	Cadwell, GA	6	—	4	1	FALSE
Isoetes_boomii_Schafran73-4	Cadwell, GA	6	—	20	3	TRUE
Isoetes_boomii_Schafran73-5	Cadwell, GA	6	—	36	3	TRUE
Isoetes_butleri_CiafreSN1	Flat Rock Cedar Glade State Natural Area, TN	2	—	504	1	TRUE
Isoetes_Butner_Bolin-1	Butner, NC	2	—	42	2	FALSE
Isoetes_Butner_Bolin-2	Butner, NC	2	—	54	2	FALSE
Isoetes_Butner_Schafran85-1	Butner, NC	2	—	46	4	FALSE
Isoetes_Butner_Schafran85-2	Butner, NC	2	—	60	2	FALSE
Isoetes_Butner_Schafran85-3	Butner, NC	2	—	43	3	FALSE
Isoetes_Butner_Schafran85-4	Butner, NC	2	—	51	3	FALSE
Isoetes_Butner_Schafran85-5	Butner, NC	2	—	28	2	FALSE
Isoetes_chapmanii_Brunton13993_rep1	Mariana, FL	2	3.27	68	1	TRUE
Isoetes_chapmanii_Brunton13993_rep2	Mariana, FL	2	3.27	48	1	TRUE
Isoetes_curledLeaves_Taylor6991-1_2	Comox Lake, BC	4	—	87	2	TRUE
Isoetes_echinospora_Feldsee	Feldsee Lake, Germany	2	—	24	1	TRUE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_echinospora_Kessler_rep2	Switzerland	2	—	10	1	TRUE
Isoetes_echinospora_Kessler_rep3	Switzerland	2	—	7	1	TRUE
Isoetes_echinospora_PlesneLake	Plesne Lake, Czech Republic	2	—	93	1	TRUE
Isoetes_echinospora_Schafran154	Shehawken Lake, PA	2	—	77	1	TRUE
Isoetes_echinospora_Schafran155	Starlight Lake, PA	2	—	82	1	TRUE
Isoetes_echinospora_Schafran164	Yankee Lake, NY	2	—	48	1	TRUE
Isoetes_echinospora_Schafran167-2	Gordon Bay, ON	2	—	114	1	TRUE
Isoetes_echinospora_Schafran167-3	Gordon Bay, ON	2	—	115	3	FALSE
Isoetes_echinospora_Schafran167-4	Gordon Bay, ON	2	—	79	1	TRUE
Isoetes_echinospora_Schafran167-5	Gordon Bay, ON	2	—	140	1	TRUE
Isoetes_echinospora_Schafran169-1	Boshkung Lake, ON	2	—	180	1	TRUE
Isoetes_echinospora_Schafran169-3	Boshkung Lake, ON	2	—	157	1	TRUE
Isoetes_Edisto_Cressler3	Orangeburg, SC	6	—	128	3	TRUE
Isoetes_Edisto_Cressler5-1_rep1	Denmark, SC	6	—	36	3	TRUE
Isoetes_Edisto_Cressler5-1_rep2	Denmark, SC	6	—	78	3	TRUE
Isoetes_Edisto_Cressler5-2_rep1	Denmark, SC	6	—	39	3	TRUE
Isoetes_Edisto_Cressler5-2_rep2	Denmark, SC	6	—	156	3	TRUE
Isoetes_Edisto_Schafran87-1	Denmark, SC	6	—	15	2	FALSE
Isoetes_Edisto_Schafran87-2	Denmark, SC	6	—	59	3	TRUE
Isoetes_Edisto_Schafran87-4	Denmark, SC	6	—	5	1	FALSE
Isoetes_Edisto_Schafran87-5	Denmark, SC	6	—	26	5	FALSE
Isoetes_engelmannii_BunchSN1	Cumberland State Forest, VA	2	—	623	1	TRUE
Isoetes_engelmannii_JBNC18-2	Badin Lake, NC	2	—	323	1	TRUE
Isoetes_engelmannii_Schafran147	Reliance, TN	2	1.78	15	1	TRUE
Isoetes_engelmannii_Schafran196	Elizabeth Furnace Campground, VA	2	—	469	1	TRUE
Isoetes_flaccida_Schafran203	Boston, GA	2	2.72	250	1	TRUE
Isoetes_georgiana_Cressler10	Chula, GA	6	—	26	2	FALSE
Isoetes_georgiana_Cressler11-1	Chula, GA	6	—	56	3	TRUE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_georgiana_MatthewsSN-1_rep1	Sylvester, GA	6	—	116	2	FALSE
Isoetes_georgiana_MatthewsSN-1_rep2	Sylvester, GA	6	—	216	2	FALSE
Isoetes_georgiana_MatthewsSN-2_rep2	Sylvester, GA	6	—	107	3	TRUE
Isoetes_georgiana_MatthewsSN-3_rep1	Sylvester, GA	6	—	160	2	FALSE
Isoetes_georgiana_MatthewsSN-3_rep2	Sylvester, GA	6	—	59	3	TRUE
Isoetes_georgiana_MatthewsSN-4_rep1	Sylvester, GA	6	—	223	2	FALSE
Isoetes_georgiana_MatthewsSN-4_rep2	Sylvester, GA	6	—	125	3	TRUE
Isoetes_georgiana_MatthewsSN-5_rep1	Sylvester, GA	6	—	97	2	FALSE
Isoetes_georgiana_MatthewsSN-5_rep2	Sylvester, GA	6	—	180	3	TRUE
Isoetes_georgiana_Schafran111-1	Sylvester, GA	6	5.35	363	3	TRUE
Isoetes_georgiana_Schafran111-2	Sylvester, GA	6	5.35	243	2	FALSE
Isoetes_georgiana_Schafran111-3	Sylvester, GA	6	5.35	175	3	TRUE
Isoetes_georgiana_Schafran111-4	Sylvester, GA	6	5.35	112	3	TRUE
Isoetes_georgiana_Schafran111-5	Sylvester, GA	6	5.35	194	2	FALSE
Isoetes_georgiana_Schafran111-6	Sylvester, GA	6	5.35	222	2	FALSE
Isoetes_georgiana_Schafran112_rep1	Norman Park, GA	6	5.59	356	4	FALSE
Isoetes_georgiana_Schafran112_rep2	Norman Park, GA	6	5.59	31	4	FALSE
Isoetes_georgiana_Schafran113_rep1	Cadwell, GA	6	5.03	357	4	FALSE
Isoetes_georgiana_Schafran113_rep2	Cadwell, GA	6	5.03	25	4	FALSE
Isoetes_georgiana_Schafran113_rep3	Cadwell, GA	6	5.03	21	3	TRUE
Isoetes_georgiana_Schafran74-1	Ashburn, GA	6	—	4	1	FALSE
Isoetes_georgiana_Schafran82-1	Broxton, GA	6	—	4	1	FALSE
Isoetes_georgiana_Schafran82-2	Broxton, GA	6	—	7	1	FALSE
Isoetes_georgiana_Schafran82-3	Broxton, GA	6	—	9	2	FALSE
Isoetes_georgiana_Schafran82-4	Broxton, GA	6	—	8	2	FALSE
Isoetes_georgiana_SchafranGA16_rep1	Sylvester, GA	6	—	143	2	FALSE
Isoetes_georgiana_SchafranGA16_rep2	Sylvester, GA	6	—	42	2	FALSE
Isoetes_georgiana_SchafranGA17	Ashburn, GA	6	—	81	4	FALSE
Isoetes_georgiana_SchafranGA18	Ashburn, GA	6	4.95	113	3	TRUE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_georgiana_Taylor6769_rep1	Turner Co., GA	6	—	34	3	TRUE
Isoetes_georgiana_Taylor6769_rep2	Turner Co., GA	6	—	49	3	TRUE
Isoetes_graniticola_Schafran115_rep1	Chattahoochee Bend State Park, GA	4	4.88	199	2	TRUE
Isoetes_graniticola_Schafran115_rep2	Chattahoochee Bend State Park, GA	4	4.88	8	1	FALSE
Isoetes_graniticola_Schafran115_rep3	Chattahoochee Bend State Park, GA	4	4.88	17	2	TRUE
Isoetes_graniticola_Schafran117_rep1	Flat Rock Park, Lineville, AL	2	3.2	139	2	FALSE
Isoetes_graniticola_Schafran117_rep2	Flat Rock Park, Lineville, AL	2	3.2	7	1	TRUE
Isoetes_graniticola_Schafran14	Sparta, GA	4	—	83	2	TRUE
Isoetes_graniticola_Taylor6776_rep1	Rowan Co., NC	4	—	63	2	TRUE
Isoetes_graniticola_Taylor6776_rep2	Rowan Co., NC	4	—	22	2	TRUE
Isoetes_hyemalis_BolinRiverRestA	Mt. Airy, VA	4	—	23	2	TRUE
Isoetes_hyemalis_BolinRiverRestB	Mt. Airy, VA	4	—	6	1	FALSE
Isoetes_hyemalis_BolinYorkCo	York Co., VA	4	—	5	1	FALSE
Isoetes_hyemalis_Bradley8204-1	Carolina Sandhills Natl. Wildlife Refuge, SC	4	3.27	139	1	FALSE
Isoetes_hyemalis_Bradley8204-2	Carolina Sandhills Natl. Wildlife Refuge, SC	4	3.27	523	1	FALSE
Isoetes_hyemalis_Bradley8221-1	Carolina Sandhills Natl. Wildlife Refuge, SC	4	3.35	298	1	FALSE
Isoetes_hyemalis_Bradley8221-2	Carolina Sandhills Natl. Wildlife Refuge, SC	4	3.35	411	1	FALSE
Isoetes_hyemalis_Bradley8221-3	Carolina Sandhills Natl. Wildlife Refuge, SC	4	3.35	20	1	FALSE
Isoetes_hyemalis_Bradley8221-4	Carolina Sandhills Natl. Wildlife Refuge, SC	4	3.35	293	1	FALSE
Isoetes_hyemalis_BradleySN1-1	Yemassee, SC	4	4.46	905	1	FALSE
Isoetes_hyemalis_BradleySN1-2	Yemassee, SC	4	4.46	897	1	FALSE
Isoetes_hyemalis_BradleySN1-3	Yemassee, SC	4	4.46	1674	1	FALSE
Isoetes_hyemalis_BradleySN1-4	Yemassee, SC	4	4.46	936	1	FALSE
Isoetes_hyemalis_BradleySN1-5	Yemassee, SC	4	4.46	846	1	FALSE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_hyemalis_BradleySN2-1	Ernest F. Hollings ACE Basin Natl. Wildlife Refuge, SC	4	—	158	2	TRUE
Isoetes_hyemalis_BradleySN2-2	Ernest F. Hollings ACE Basin Natl. Wildlife Refuge, SC	4	—	662	2	TRUE
Isoetes_hyemalis_Brunton19012	St. George, SC	4	—	59	2	TRUE
Isoetes_hyemalis_BunchSN2	Southampton Co., VA	4	—	382	2	TRUE
Isoetes_hyemalis_Schafran107_rep1	Esto, FL	4	5.67	196	2	TRUE
Isoetes_hyemalis_Schafran107_rep2	Esto, FL	4	5.67	12	2	TRUE
Isoetes_hyemalis_Schafran107_rep3	Esto, FL	4	5.67	54	2	TRUE
Isoetes_hyemalis_Schafran109-1	Iron City, GA	4	3.88	143	2	TRUE
Isoetes_hyemalis_Schafran109-2	Iron City, GA	4	3.88	52	3	FALSE
Isoetes_hyemalis_Schafran109-3	Iron City, GA	4	3.88	197	2	TRUE
Isoetes_hyemalis_Schafran109-4	Iron City, GA	4	3.88	181	2	TRUE
Isoetes_hyemalis_Schafran109-5	Iron City, GA	4	3.88	195	2	TRUE
Isoetes_hyemalis_Schafran118-1	Cottonwood, AL	4	3.03	254	2	TRUE
Isoetes_hyemalis_Schafran118-2	Cottonwood, AL	4	3.03	215	2	TRUE
Isoetes_hyemalis_Schafran118-4	Cottonwood, AL	4	3.03	293	2	TRUE
Isoetes_hyemalis_Schafran118-5	Cottonwood, AL	4	3.03	256	2	TRUE
Isoetes_hyemalis_Schafran120-1	Colquitt, GA	4	4	77	2	TRUE
Isoetes_hyemalis_Schafran121-1	Bullard Creek WMA, GA	4	5.03	156	3	FALSE
Isoetes_hyemalis_Schafran121-2	Bullard Creek WMA, GA	4	5.03	117	3	FALSE
Isoetes_hyemalis_Schafran121-3	Bullard Creek WMA, GA	4	5.03	204	3	FALSE
Isoetes_hyemalis_Schafran121-4	Bullard Creek WMA, GA	4	5.03	164	3	FALSE
Isoetes_hyemalis_Schafran121-5	Bullard Creek WMA, GA	4	5.03	187	4	FALSE
Isoetes_hyemalis_Schafran122_rep1	Awendaw, SC	4	4.88	154	1	FALSE
Isoetes_hyemalis_Schafran122_rep2	Awendaw, SC	4	4.88	410	1	FALSE
Isoetes_hyemalis_Schafran122_rep3	Awendaw, SC	4	4.88	363	1	FALSE
Isoetes_hyemalis_Schafran123-1	Huger, SC	4	3.93	112	1	FALSE
Isoetes_hyemalis_Schafran123-2	Huger, SC	4	3.93	46	1	FALSE
Isoetes_hyemalis_Schafran123-3	Huger, SC	4	3.93	153	1	FALSE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_hyemalis_Schafran124-1	Berkeley Co., SC	4	3.15	236	2	TRUE
Isoetes_hyemalis_Schafran124-2	Berkeley Co., SC	4	3.15	216	3	FALSE
Isoetes_hyemalis_Schafran124-3	Berkeley Co., SC	4	3.15	173	3	FALSE
Isoetes_hyemalis_Schafran124-4	Berkeley Co., SC	4	3.15	203	2	TRUE
Isoetes_hyemalis_Schafran124-5	Berkeley Co., SC	4	3.15	279	2	TRUE
Isoetes_hyemalis_Schafran125-1	St. George, SC	4	3.37	107	2	TRUE
Isoetes_hyemalis_Schafran125-2	St. George, SC	4	3.37	57	2	TRUE
Isoetes_hyemalis_Schafran125-3	St. George, SC	4	3.37	88	2	TRUE
Isoetes_hyemalis_Schafran126_rep1	Johnsonville, SC	4	—	250	2	TRUE
Isoetes_hyemalis_Schafran126_rep2	Johnsonville, SC	4	—	241	2	TRUE
Isoetes_hyemalis_Schafran127-1	Pireway, NC	4	3.29	135	3	FALSE
Isoetes_hyemalis_Schafran127-2	Pireway, NC	4	3.29	239	2	TRUE
Isoetes_hyemalis_Schafran127-3	Pireway, NC	4	3.29	137	2	TRUE
Isoetes_hyemalis_Schafran127-4	Pireway, NC	4	3.29	31	2	TRUE
Isoetes_hyemalis_Schafran127-5	Pireway, NC	4	3.29	98	2	TRUE
Isoetes_hyemalis_Schafran128-1	Carvers Creek State Park, NC	4	2.94	135	1	FALSE
Isoetes_hyemalis_Schafran129-1	Lillington, NC	4	3.23	190	3	FALSE
Isoetes_hyemalis_Schafran129-2	Lillington, NC	4	3.23	219	3	FALSE
Isoetes_hyemalis_Schafran129-3	Lillington, NC	4	3.23	115	3	FALSE
Isoetes_hyemalis_Schafran130-1_rep1	Lillington, NC	4	3.21	160	2	TRUE
Isoetes_hyemalis_Schafran130-1_rep2	Lillington, NC	4	3.21	117	2	TRUE
Isoetes_hyemalis_Schafran131-1	Vass, NC	4	3.19	172	1	FALSE
Isoetes_hyemalis_Schafran131-2	Vass, NC	4	3.19	100	1	FALSE
Isoetes_hyemalis_Schafran132-1	Scotland Co., NC	4	3.2	239	2	TRUE
Isoetes_hyemalis_Schafran132-2	Scotland Co., NC	4	3.2	85	2	TRUE
Isoetes_hyemalis_Schafran132-3	Scotland Co., NC	4	3.2	131	2	TRUE
Isoetes_hyemalis_Schafran132-4	Scotland Co., NC	4	3.2	130	3	FALSE
Isoetes_hyemalis_Schafran133-1	Scotland Co., NC	4	3.12	126	2	TRUE
Isoetes_hyemalis_Schafran133-2	Scotland Co., NC	4	3.12	211	2	TRUE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_hyemalis_Schafran134-1	Burgaw, NC	4	4.31	92	2	TRUE
Isoetes_hyemalis_Schafran134-2	Burgaw, NC	4	4.31	64	2	TRUE
Isoetes_hyemalis_Schafran135_rep1	Atkinson, NC	4	4.6	192	3	FALSE
Isoetes_hyemalis_Schafran135_rep2	Atkinson, NC	4	4.6	257	3	FALSE
Isoetes_hyemalis_Schafran135_rep3	Atkinson, NC	4	4.6	520	3	FALSE
Isoetes_hyemalis_Schafran136_rep1	Pender Co., NC	4	—	39	3	FALSE
Isoetes_hyemalis_Schafran136_rep2	Pender Co., NC	4	—	173	3	FALSE
Isoetes_hyemalis_Schafran136_rep3	Pender Co., NC	4	—	125	3	FALSE
Isoetes_hyemalis_Schafran137-1	Columbus Co., NC	4	4.34	141	2	TRUE
Isoetes_hyemalis_Schafran137-2	Columbus Co., NC	4	4.34	192	2	TRUE
Isoetes_hyemalis_Schafran137-3	Columbus Co., NC	4	4.34	109	2	TRUE
Isoetes_hyemalis_Schafran138_rep1	Duplin Co., NC	4	4.24	100	2	TRUE
Isoetes_hyemalis_Schafran138_rep2	Duplin Co., NC	4	4.24	191	2	TRUE
Isoetes_hyemalis_Schafran138_rep3	Duplin Co., NC	4	4.24	98	2	TRUE
Isoetes_hyemalis_Schafran139-1	Beulaville, NC	4	4.36	165	2	TRUE
Isoetes_hyemalis_Schafran139-2	Beulaville, NC	4	4.36	192	2	TRUE
Isoetes_hyemalis_Schafran139-3	Beulaville, NC	4	4.36	96	2	TRUE
Isoetes_hyemalis_Schafran140-1	Croatan Natl. Forest, NC	4	4.3	159	1	FALSE
Isoetes_hyemalis_Schafran140-2	Croatan Natl. Forest, NC	4	4.3	170	1	FALSE
Isoetes_hyemalis_Schafran140-3	Croatan Natl. Forest, NC	4	4.3	26	1	FALSE
Isoetes_hyemalis_Schafran140-4	Croatan Natl. Forest, NC	4	4.3	102	1	FALSE
Isoetes_hyemalis_Schafran140-5	Croatan Natl. Forest, NC	4	4.3	117	1	FALSE
Isoetes_hyemalis_Schafran141-1	Hillsborough, NC	4	—	16	2	TRUE
Isoetes_hyemalis_Schafran141-2	Hillsborough, NC	4	—	18	2	TRUE
Isoetes_hyemalis_Schafran142-1	Hillsborough, NC	4	2.85	10	2	TRUE
Isoetes_hyemalis_Schafran142-2	Hillsborough, NC	4	2.85	25	2	TRUE
Isoetes_hyemalis_Schafran143	Hillsborough, NC	4	—	33	2	TRUE
Isoetes_hyemalis_Schafran144	Hillsborough, NC	4	2.85	15	2	TRUE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_hyemalis_SchafranVA01	Zuni, VA	4	3.61	135	2	TRUE
Isoetes_junciformis_BolinSN_rep1	Chula, GA	4	—	177	4	FALSE
Isoetes_junciformis_BolinSN_rep2	Chula, GA	4	—	66	4	FALSE
Isoetes_junciformis_Brunton17608	Chula, GA	4	—	53	4	FALSE
Isoetes_junciformis_Cressler12	Chula, GA	4	—	20	1	FALSE
Isoetes_junciformis_Schafran104_rep1	Ft. Benning, GA	4	—	37	4	FALSE
Isoetes_junciformis_Schafran104_rep3	Ft. Benning, GA	4	—	18	3	FALSE
Isoetes_lacustris_CerneLake	Cerne Lake, Czech Republic	10	—	75	2	FALSE
Isoetes_lacustris_Feldsee	Feldsee Lake, Germany	10	—	95	2	FALSE
Isoetes_lacustris_Kessler_rep1	Switzerland	10	—	70	3	FALSE
Isoetes_lacustris_Kessler_rep2	Switzerland	10	—	434	3	FALSE
Isoetes_lacustris_Kessler_rep3	Switzerland	10	—	219	3	FALSE
Isoetes_lacustris_KrasluerSN_rep2	Pniewo Lake, Poland	10	—	24	3	FALSE
Isoetes_lacustris_Taylor6748	Grand Isle Co., VT	10	—	217	3	FALSE
Isoetes_laurentiana_Brunton20077	Sainte-Pétronille, QC	4	—	268	2	TRUE
Isoetes_laurentiana_Brunton20087	Sainte-Pétronille, QC	4	—	243	2	TRUE
Isoetes_laurentiana_Brunton20092-1	Parc du Haut-Fonds, QC	4	4.1	214	3	FALSE
Isoetes_laurentiana_Brunton20092-2	Parc du Haut-Fonds, QC	4	4.1	71	3	FALSE
Isoetes_laurentiana_Brunton20101-1	Marais Provancher, QC	4	3.6	212	3	FALSE
Isoetes_laurentiana_Brunton20101-2	Marais Provancher, QC	4	3.6	61	2	TRUE
Isoetes_laurentiana_Brunton20101b	Marais Provancher, QC	4	3.6	85	3	FALSE
Isoetes_Leary_Musselman17001-1	Leary, GA	2	1.95	371	1	TRUE
Isoetes_Leary_Musselman17001-2	Leary, GA	2	1.95	882	1	TRUE
Isoetes_Leary_Musselman17002-1	Chickasawhatchee WMA, GA	2	2.07	163	2	FALSE
Isoetes_Leary_Musselman17002-2	Chickasawhatchee WMA, GA	2	2.03	362	2	FALSE
Isoetes_Leary_Musselman17002-3	Chickasawhatchee WMA, GA	2	1.97	290	2	FALSE
Isoetes_Leary_Schafran110-1	Leary, GA	2	2.82	120	2	FALSE
Isoetes_Leary_Schafran110-2	Leary, GA	2	2.82	83	1	TRUE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_Leary_Schafran114_rep1	Terrell Co., GA	2	2.57	128	2	FALSE
Isoetes_Leary_Schafran114_rep2	Terrell Co., GA	2	2.57	15	2	FALSE
Isoetes_Leary_Schafran114_rep3	Terrell Co., GA	2	2.57	16	2	FALSE
Isoetes_Leary_Schafran83-3	Leary, GA	2	1.98	10	1	TRUE
Isoetes_Leary_Schafran83-4	Leary, GA	2	1.98	5	1	TRUE
Isoetes_louisianensis_Alford397	DeSoto Natl. Forest, MS	4	—	545	2	TRUE
Isoetes_louisianensis_Alford398	DeSoto Natl. Forest, MS	4	—	338	2	TRUE
Isoetes_louisianensis_Alford399	DeSoto Natl. Forest, MS	4	—	373	2	TRUE
Isoetes_louisianensis_Alford401	DeSoto Natl. Forest, MS	4	—	387	2	TRUE
Isoetes_louisianensis_Alford402	DeSoto Natl. Forest, MS	4	—	483	2	TRUE
Isoetes_louisianensis_Alford403	DeSoto Natl. Forest, MS	4	—	312	1	FALSE
Isoetes_louisianensis_BolinJBLA_rep1	Thigpen Creek, LA	4	—	198	1	FALSE
Isoetes_louisianensis_BolinJBLA_rep2	Thigpen Creek, LA	4	—	16	1	FALSE
Isoetes_louisianensis_Brunton17581_rep1	Butler Co., AL	4	—	159	4	FALSE
Isoetes_louisianensis_Brunton17581_rep2	Butler Co., AL	4	—	83	2	TRUE
Isoetes_louisianensis_Leonard12415	Greene Co., MS	4	—	58	2	TRUE
Isoetes_louisianensis_Schafran106_rep1	Conecuh Co., AL	4	—	113	2	TRUE
Isoetes_louisianensis_Schafran106_rep2	Conecuh Co., AL	4	—	7	1	FALSE
Isoetes_louisianensis_Schafran106_rep3	Conecuh Co., AL	4	—	17	2	TRUE
Isoetes_louisianensis_Schafran195-1	DeSoto Natl. Forest, MS	4	3.38	724	1	FALSE
Isoetes_louisianensis_Schafran195-2	DeSoto Natl. Forest, MS	4	3.38	1391	2	TRUE
Isoetes_louisianensis_Schafran195-3	DeSoto Natl. Forest, MS	4	3.38	1518	2	TRUE
Isoetes_louisianensis_Schafran195-4	DeSoto Natl. Forest, MS	4	3.38	284	2	TRUE
Isoetes_maritima_Taylor6983-1_rep1	Somas River, BC	4	—	188	2	TRUE
Isoetes_maritima_Taylor6983-1_rep2	Somas River, BC	4	—	146	2	TRUE
Isoetes_maritima_Taylor6987-1	Spider Lake, BC	4	—	116	1	FALSE
Isoetes_maritima_WoodbridgeSN2	Unalaska, AK	4	—	132	2	TRUE
Isoetes_maritimaXechinospora_Taylor6988-2_rep1	Spider Lake, BC	3	—	17	1	FALSE
Isoetes_maritimaXechinospora_Taylor6988-2_rep2	Spider Lake, BC	3	—	140	2	FALSE
Isoetes_maritimaXechinospora_Taylor6988-3	Spider Lake, BC	3	—	34	2	FALSE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_mattaponica_Bradley8670_rep2	Georgetown Co., SC	2	1.04	213	1	TRUE
Isoetes_melanopoda_Ciafre256_1	Algood, TN	2	3.89	582	3	FALSE
Isoetes_melanopoda_Ciafre256_2	Algood, TN	2	3.89	524	4	FALSE
Isoetes_melanopoda_Ciafre728_1	Algood, TN	2	—	379	4	FALSE
Isoetes_melanopoda_Ciafre728_2	Algood, TN	2	—	82	2	FALSE
Isoetes_melanopoda_Ciafre728_3	Algood, TN	2	—	248	3	FALSE
Isoetes_melanopoda_Schafran184_1	Angelina Natl. Forest, TX	2	3.33	1190	2	FALSE
Isoetes_melanopoda_Schafran184_2	Angelina Natl. Forest, TX	2	3.33	1397	2	FALSE
Isoetes_melanopoda_Schafran187	Angelina Natl. Forest, TX	2	—	306	3	FALSE
Isoetes_melanopoda_Schafran188_1	Newton Co., TX	2	—	415	1	TRUE
Isoetes_melanopoda_Schafran188_2	Newton Co., TX	2	—	428	1	TRUE
Isoetes_melanopoda_Schafran188_3	Newton Co., TX	2	—	169	1	TRUE
Isoetes_melanopoda_Schafran188_4	Newton Co., TX	2	—	174	1	TRUE
Isoetes_melanopoda_Schafran188_5	Newton Co., TX	2	—	665	2	FALSE
Isoetes_melanopoda_Schafran188_6	Newton Co., TX	2	—	261	1	TRUE
Isoetes_melanopoda_ssp_silvatica_SchafranNC05	Mecklenburg Co., NC	2	1.37	51	1	TRUE
Isoetes_melanopoda_WelbySmith36037	Touch-the-Sky Natl. Wildlife Refuge, MN	2	—	390	1	TRUE
Isoetes_melanopoda_WelbySmith36038	Pipestone Natl. Monument, MN	2	—	136	1	TRUE
Isoetes_microvela_BolinJB40NC_rep1	Pender Co., NC	6	—	238	2	FALSE
Isoetes_microvela_BolinJB40NC_rep2	Pender Co., NC	6	—	19	2	FALSE
Isoetes_microvela_BolinJBNC199EO2_rep1	Brunswick Co., NC	6	—	78	2	FALSE
Isoetes_microvela_BolinJBNC199EO2_rep2	Brunswick Co., NC	6	—	102	2	FALSE
Isoetes_microvela_BolinJBNC200EO3A	Brunswick Co., NC	6	—	139	2	FALSE
Isoetes_microvela_BolinJBNC200EO3B	Brunswick Co., NC	6	—	221	3	TRUE
Isoetes_microvela_BolinJBNC201EO4A_rep1	Onslow Co., NC	6	3.77	107	3	TRUE
Isoetes_microvela_BolinJBNC201EO4A_rep2	Onslow Co., NC	6	3.77	12	2	FALSE
Isoetes_microvela_BolinJBNC201EO4B_rep1	Onslow Co., NC	6	3.77	182	3	TRUE
Isoetes_microvela_BolinJBNC201EO4B_rep2	Onslow Co., NC	6	3.77	66	3	TRUE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_microvela_BolinJBNC202EO5A_rep1	Brunswick Co., NC	6	4.21	139	2	FALSE
Isoetes_microvela_BolinJBNC202EO5A_rep2	Brunswick Co., NC	6	4.21	21	2	FALSE
Isoetes_microvela_BolinJBNC202EO5B_rep1	Brunswick Co., NC	6	4.21	99	2	FALSE
Isoetes_microvela_BolinJBNC202EO5B_rep2	Brunswick Co., NC	6	4.21	58	2	FALSE
Isoetes_microvela_MatthewsI09-35_rep1	Brunswick Co., NC	6	—	229	3	TRUE
Isoetes_microvela_MatthewsI09-35_rep2	Brunswick Co., NC	6	—	229	2	FALSE
Isoetes_microvela_Schafran119_rep1	Conecuh Co., AL	6	4.89	249	2	FALSE
Isoetes_microvela_Schafran119_rep2	Conecuh Co., AL	6	4.89	19	2	FALSE
Isoetes_mississippiensis_Schafran194-1	Picayune, MS	2	1.74	346	2	FALSE
Isoetes_mississippiensis_Schafran194-2	Picayune, MS	2	1.74	571	2	FALSE
Isoetes_mississippiensis_Taylor6798	Picayune, MS	2	—	117	2	FALSE
Isoetes_occidentalis_G1		6	—	35	1	FALSE
Isoetes_occidentalis_G10		6	—	119	1	FALSE
Isoetes_occidentalis_G5		6	—	109	1	FALSE
Isoetes_occidentalis_G7		6	—	276	2	FALSE
Isoetes_occidentalis_Taylor6755	El Dorado Co., CA	6	—	98	2	FALSE
Isoetes_occidentalis_WoodbridgeSN1-2	Unalaska, AK	6	9.3	561	3	TRUE
Isoetes_occidentalis_WoodbridgeSN1-3	Unalaska, AK	6	9.3	352	3	TRUE
Isoetes_occidentalis_WoodbridgeSN1-4	Unalaska, AK	6	9.3	123	3	TRUE
Isoetes_occidentalis_WoodbridgeSN1-5	Unalaska, AK	6	9.3	14	3	TRUE
Isoetes_parvula_RS31	Peru	2	—	1103	2	FALSE
Isoetes_piedmontana_BolinJBNC17-3	Rolesville, NC	2	2.91	65	2	FALSE
Isoetes_piedmontana_Cressler13_bag1plant1	Youth, GA	2	—	125	2	FALSE
Isoetes_piedmontana_Cressler13_bag1plant2	Youth, GA	2	—	168	1	TRUE
Isoetes_piedmontana_Cressler13_bag1plant3	Youth, GA	2	—	63	2	FALSE
Isoetes_piedmontana_Cressler13_bag1plant4	Youth, GA	2	—	145	2	FALSE
Isoetes_piedmontana_Cressler13_bag2plant1	Youth, GA	2	—	97	1	TRUE
Isoetes_piedmontana_Cressler14-1	Fort Payne, AL	2	—	324	1	TRUE
Isoetes_piedmontana_Cressler14-2	Fort Payne, AL	2	—	776	1	TRUE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_piedmontana_Schafran101-1	Columbus, GA	2	—	41	3	FALSE
Isoetes_piedmontana_Schafran101-2	Columbus, GA	2	—	12	2	FALSE
Isoetes_piedmontana_Schafran101-3	Columbus, GA	2	—	17	2	FALSE
Isoetes_piedmontana_Schafran102-1	Columbus, GA	2	1.79	1297	1	TRUE
Isoetes_piedmontana_Schafran102-2	Columbus, GA	2	2.63	780	1	TRUE
Isoetes_piedmontana_Schafran102-3	Columbus, GA	2	—	1629	3	FALSE
Isoetes_piedmontana_Schafran103-1	Columbus, GA	2	3.69	670	3	FALSE
Isoetes_piedmontana_Schafran103-2	Columbus, GA	2	2.69	790	3	FALSE
Isoetes_piedmontana_Schafran116_rep1	Franklin, GA	2	1.7	88	1	TRUE
Isoetes_piedmontana_Schafran116_rep2	Franklin, GA	2	1.7	24	1	TRUE
Isoetes_piedmontana_Schafran116_rep3	Franklin, GA	2	1.7	15	1	TRUE
Isoetes_piedmontana_Schafran86-2	Rolesville, NC	2	—	4	1	TRUE
Isoetes_piedmontana_Schafran86-3	Rolesville, NC	2	—	5	1	TRUE
Isoetes_riparia_Schafran156_rep1	Callicoon, NY	4	—	93	1	FALSE
Isoetes_riparia_Schafran156_rep2	Callicoon, NY	4	—	18	1	FALSE
Isoetes_riparia_Schafran156_rep3	Callicoon, NY	4	—	26	1	FALSE
Isoetes_riparia_Schafran157_rep1	Callicoon, NY	4	—	202	1	FALSE
Isoetes_riparia_Schafran157_rep2	Callicoon, NY	4	—	25	1	FALSE
Isoetes_riparia_Schafran157_rep3	Callicoon, NY	4	—	25	1	FALSE
Isoetes_riparia_Schafran158_rep1	Hancock, NY	4	—	164	1	FALSE
Isoetes_riparia_Schafran158_rep2	Hancock, NY	4	—	17	1	FALSE
Isoetes_riparia_Schafran158_rep3	Hancock, NY	4	—	26	1	FALSE
Isoetes_riparia_Schafran159_rep1	Schuylerville, NY	4	—	92	2	TRUE
Isoetes_riparia_Schafran159_rep2	Schuylerville, NY	4	—	31	2	TRUE
Isoetes_riparia_Schafran161-1	Sand Lake, NY	4	2.74	216	2	TRUE
Isoetes_riparia_Schafran161-2	Sand Lake, NY	4	2.74	162	2	TRUE
Isoetes_riparia_Schafran163-1_rep1	Deep Creek Lake, MD	4	—	160	1	FALSE
Isoetes_riparia_Schafran163-1_rep2	Deep Creek Lake, MD	4	—	443	1	FALSE
Isoetes_riparia_Schafran163-1_rep3	Deep Creek Lake, MD	4	—	31	1	FALSE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_riparia_Schafran163-2_rep1	Deep Creek Lake, MD	4	—	112	1	FALSE
Isoetes_riparia_Schafran163-2_rep2	Deep Creek Lake, MD	4	—	244	1	FALSE
Isoetes_riparia_Schafran90-2	Seaford, DE	4	—	13	3	FALSE
Isoetes_riparia_SchafranSN-PotomacCreek	Crows Nest Natural Area Preserve, VA	4	2.43	48	2	TRUE
Isoetes_riparia_Taylor6665	Westmoreland Co., VA	4	—	86	2	TRUE
Isoetes_riparia_Taylor6675_rep1	Hancock Co., ME	4	—	86	2	TRUE
Isoetes_riparia_Taylor6675_rep2	Hancock Co., ME	4	—	13	1	FALSE
Isoetes_riparia_Taylor6706	Hancock Co., ME	4	—	23	2	TRUE
Isoetes_septentrionalis_Brunton15341_rep1	Marmora, ON	4	—	59	2	TRUE
Isoetes_septentrionalis_Brunton15341_rep2	Marmora, ON	4	—	65	2	TRUE
Isoetes_septentrionalis_Brunton19142	Ottawa, ON	4	—	109	2	TRUE
Isoetes_septentrionalis_Schafran151-1	Jersey Shore, PA	4	4.52	175	2	TRUE
Isoetes_septentrionalis_Schafran151-2	Jersey Shore, PA	4	4.52	110	2	TRUE
Isoetes_septentrionalis_Schafran151-3	Jersey Shore, PA	4	4.52	32	2	TRUE
Isoetes_septentrionalis_Schafran152-1	Lycoming Co., PA	4	4	175	2	TRUE
Isoetes_septentrionalis_Schafran152-2	Lycoming Co., PA	4	4	153	2	TRUE
Isoetes_septentrionalis_Schafran152-3	Lycoming Co., PA	4	4	117	2	TRUE
Isoetes_septentrionalis_Schafran152-4	Lycoming Co., PA	4	4	152	2	TRUE
Isoetes_septentrionalis_Schafran153_rep1	Lycoming Co., PA	4	—	160	2	TRUE
Isoetes_septentrionalis_Schafran153_rep2	Lycoming Co., PA	4	—	50	2	TRUE
Isoetes_septentrionalis_Schafran153_rep3	Lycoming Co., PA	4	—	14	1	FALSE
Isoetes_septentrionalis_Schafran170_rep1	Tweed, ON	4	—	40	2	TRUE
Isoetes_septentrionalis_Schafran170_rep2	Tweed, ON	4	—	139	2	TRUE
Isoetes_septentrionalis_Schafran170_rep3	Tweed, ON	4	—	91	2	TRUE
Isoetes_septentrionalis_Schafran171-1	Calabogie, ON	4	—	58	2	TRUE
Isoetes_septentrionalis_Schafran171-2	Calabogie, ON	4	—	190	1	FALSE
Isoetes_septentrionalis_Schafran172_rep1	Fitzroy Harbour, ON	4	—	56	2	TRUE
Isoetes_septentrionalis_Schafran172_rep2	Fitzroy Harbour, ON	4	—	186	2	TRUE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_septentrionalis_Schafran173-1	Fitzroy Harbour, ON	4	—	133	2	TRUE
Isoetes_septentrionalis_Schafran173-2	Fitzroy Harbour, ON	4	—	35	2	TRUE
Isoetes_septentrionalis_Schafran173-3	Fitzroy Harbour, ON	4	—	156	2	TRUE
Isoetes_silvatica_Cressler9	Elbert Co., GA	2	—	4	1	TRUE
Isoetes_snowii_Schafran2_rep1	Broxton, GA	2	—	40	1	TRUE
Isoetes_snowii_Schafran2_rep2	Broxton, GA	2	—	95	2	FALSE
Isoetes_snowii_Schafran3	Broxton, GA	2	—	120	1	TRUE
Isoetes_snowii_Schafran4	Broxton, GA	2	—	30	2	FALSE
Isoetes_snowii_Schafran5_rep1	Broxton, GA	2	—	151	1	TRUE
Isoetes_snowii_Schafran5_rep2	Broxton, GA	2	—	149	1	TRUE
Isoetes_snowii_Schafran6_rep1	Broxton, GA	2	—	17	1	TRUE
Isoetes_snowii_Schafran6_rep2	Broxton, GA	2	—	181	1	TRUE
Isoetes_snowii_Schafran78-1_rep1	Broxton, GA	2	—	33	1	TRUE
Isoetes_snowii_Schafran78-1_rep2	Broxton, GA	2	—	7	1	TRUE
Isoetes_snowii_Schafran78-2_rep1	Broxton, GA	2	—	70	2	FALSE
Isoetes_snowii_Schafran78-2_rep2	Broxton, GA	2	—	29	2	FALSE
Isoetes_snowii_Schafran78-3	Broxton, GA	2	—	25	1	TRUE
Isoetes_snowii_Schafran79-4_rep1	Broxton, GA	2	—	119	2	FALSE
Isoetes_snowii_Schafran79-4_rep2	Broxton, GA	2	—	81	1	TRUE
Isoetes_snowii_Schafran8	Broxton, GA	2	—	19	2	FALSE
Isoetes_snowii_Schafran8	Broxton, GA	2	—	166	1	TRUE
Isoetes_snowii_Schafran80-10	Broxton, GA	2	—	32	2	FALSE
Isoetes_snowii_Schafran80-11	Broxton, GA	2	—	109	2	FALSE
Isoetes_snowii_Schafran80-12	Broxton, GA	2	—	50	1	TRUE
Isoetes_snowii_Schafran80-7	Broxton, GA	2	—	19	2	FALSE
Isoetes_snowii_Schafran80-8	Broxton, GA	2	—	19	1	TRUE
Isoetes_snowii_Schafran80-9	Broxton, GA	2	—	70	2	FALSE
Isoetes_snowii_Schafran81-13	Broxton, GA	2	—	24	1	TRUE
Isoetes_snowii_Schafran81-14	Broxton, GA	2	—	104	1	TRUE
Isoetes_snowii_Schafran81-16	Broxton, GA	2	—	60	1	TRUE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_snowii_SchafranGA01_rep1	Broxton, GA	2	—	4	1	TRUE
Isoetes_snowii_SchafranGA01_rep2	Broxton, GA	2	—	6	1	TRUE
Isoetes_snowii_SchafranGA02_rep1	Broxton, GA	2	—	30	2	FALSE
Isoetes_snowii_SchafranGA03	Broxton, GA	2	—	109	1	TRUE
Isoetes_snowii_SchafranGA05	Broxton, GA	2	—	64	2	FALSE
Isoetes_snowii_SchafranGA06_rep1	Broxton, GA	2	2.96	85	1	TRUE
Isoetes_snowii_SchafranGA06_rep2	Broxton, GA	2	2.96	137	1	TRUE
Isoetes_snowii_SchafranGA06_rep3	Broxton, GA	2	2.96	82	2	FALSE
Isoetes_snowii_SchafranGA11	Broxton, GA	2	—	64	1	TRUE
Isoetes_snowii_SchafranGA12	Broxton, GA	2	—	123	2	FALSE
Isoetes_snowii_SchafranGA12_rep1	Broxton, GA	2	—	217	2	FALSE
Isoetes_snowii_SchafranGA12_rep2	Broxton, GA	2	—	217	3	FALSE
Isoetes_snowii_SchafranGA13	Broxton, GA	2	—	62	1	TRUE
Isoetes_snowii_SchafranGA14_rep1	Broxton, GA	2	—	15	1	TRUE
Isoetes_snowii_SchafranGA14_rep2	Broxton, GA	2	—	13	1	TRUE
Isoetes_snowii_SchafranGA15	Broxton, GA	2	—	239	1	TRUE
Isoetes_sp_Greenhouse31_Big	Chickahominy River, VA	2	—	332	1	TRUE
Isoetes_sp_Greenhouse31_Small	Chickahominy River, VA	2	—	578	2	FALSE
Isoetes_sp_RS32	Peru	4	—	472	3	FALSE
Isoetes_sp_RS33	Peru	4	—	681	2	TRUE
Isoetes_sp_RS48	Peru	4	—	1144	2	TRUE
Isoetes_sp_Schafran180_1	Girdletree, MD	4	—	891	2	TRUE
Isoetes_sp_Schafran180_2	Girdletree, MD	4	—	390	2	TRUE
Isoetes_sp_Schafran180_3	Girdletree, MD	4	—	950	2	TRUE
Isoetes_sp_Schafran180_4	Girdletree, MD	4	—	665	2	TRUE
Isoetes_sp_Schafran180_5	Girdletree, MD	4	—	571	2	TRUE
Isoetes_sp_Schafran210_1	Eden, NC	2	—	963	1	TRUE
Isoetes_sp_Schafran210_2	Eden, NC	2	—	2326	1	TRUE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_sp_Schafran210_3	Eden, NC	2	—	326	1	TRUE
Isoetes_sp_Schafran210_4	Eden, NC	2	—	52	1	TRUE
Isoetes_sp_TaylorSN-1	Swan Lake, MT	4	—	120	2	TRUE
Isoetes_sp_TaylorSN-2	Swan Lake, MT	4	—	57	1	FALSE
Isoetes_sp_TaylorSN-3	Swan Lake, MT	4	—	129	2	TRUE
Isoetes_sp_UnknownChickahominy2_rep1	Chickahominy River, VA	4	—	30	2	TRUE
Isoetes_sp_UnknownChickahominy2_rep2	Chickahominy River, VA	4	—	96	3	FALSE
Isoetes_splayedLeaves_Taylor6990-1	Comox Lake, BC	4	—	56	2	TRUE
Isoetes_storkii_Chirripo_S111	Costa Rica	2	—	16	2	FALSE
Isoetes_storkii_Poas_S110	Costa Rica	2	—	11	1	TRUE
Isoetes_straightLeaves_Taylor6989-1_rep1	Comox Lake, BC	4	—	35	1	FALSE
Isoetes_straightLeaves_Taylor6989-1_rep2	Comox Lake, BC	4	—	160	1	FALSE
Isoetes_straightLeaves_Taylor6989-3	Comox Lake, BC	4	—	74	2	TRUE
Isoetes_tennesseensis_Schafran177-1_rep2	Reliance, TN	8	7.42	352	3	FALSE
Isoetes_tennesseensis_Schafran177-1_rep3	Reliance, TN	8	7.42	109	3	FALSE
Isoetes_tennesseensis_Schafran177-2_rep1	Reliance, TN	8	7.42	187	3	FALSE
Isoetes_tennesseensis_Schafran177-2_rep2	Reliance, TN	8	7.42	440	3	FALSE
Isoetes_tennesseensis_Schafran177-2_rep3	Reliance, TN	8	7.42	230	3	FALSE
Isoetes_tuckermanii_Schafran160-1	Grafton, NY	4	2.91	320	1	FALSE
Isoetes_tuckermanii_Schafran160-2	Grafton, NY	4	2.91	138	1	FALSE
Isoetes_tuckermanii_Schafran160-3	Grafton, NY	4	2.91	45	2	TRUE
Isoetes_tuckermanii_Schafran160-4	Grafton, NY	4	2.91	96	2	TRUE
Isoetes_tuckermanii_Schafran160-5	Grafton, NY	4	2.91	57	1	FALSE
Isoetes_tuckermanii_Schafran166-1	Wahwashkesh, ON	4	—	146	3	FALSE
Isoetes_tuckermanii_Schafran166-2	Wahwashkesh, ON	4	—	136	4	FALSE
Isoetes_tuckermanii_Schafran166-3	Wahwashkesh, ON	4	—	153	3	FALSE
Isoetes_tuckermanii_Schafran166-4	Wahwashkesh, ON	4	—	201	4	FALSE
Isoetes_tuckermanii_Schafran166-5	Wahwashkesh, ON	4	—	102	4	FALSE
Isoetes_tuckermanii_Schafran166-6	Wahwashkesh, ON	4	—	128	3	FALSE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_tuckermanii_Schafran168-1_rep1	Dorset, ON	4	—	54	1	FALSE
Isoetes_tuckermanii_Schafran168-1_rep2	Dorset, ON	4	—	195	2	TRUE
Isoetes_tuckermanii_Schafran168-1_rep3	Dorset, ON	4	—	121	2	TRUE
Isoetes_tuckermanii_Schafran174-1	Newville Lake, NS	4	—	145	1	FALSE
Isoetes_tuckermanii_Schafran174-2	Newville Lake, NS	4	—	104	1	FALSE
Isoetes_tuckermanii_Schafran174-3	Newville Lake, NS	4	—	163	1	FALSE
Isoetes_tuckermanii_Schafran174-4	Newville Lake, NS	4	—	123	1	FALSE
Isoetes_tuckermanii_Schafran176-1	Ponkapoag Pond, MA	4	—	124	2	TRUE
Isoetes_tuckermanii_Schafran176-2	Ponkapoag Pond, MA	4	—	37	2	TRUE
Isoetes_tuckermanii_Schafran176-3	Ponkapoag Pond, MA	4	—	157	2	TRUE
Isoetes_tuckermanii_Schafran176-4	Ponkapoag Pond, MA	4	—	85	2	TRUE
Isoetes_tuckermanii_Schafran176-5	Ponkapoag Pond, MA	4	—	46	2	TRUE
Isoetes_tuckermanii_Taylor6707_rep1	Rockingham Co., NH	4	—	82	2	TRUE
Isoetes_tuckermanii_Taylor6707_rep2	Rockingham Co., NH	4	—	37	2	TRUE
Isoetes_Uwharrie_BolinTr-A	Troy, NC	2	1.37	15	1	TRUE
Isoetes_Uwharrie_Schafran76-2	Troy, NC	2	1.37	11	2	FALSE
Isoetes_Uwharrie_Schafran76-3	Troy, NC	2	1.37	18	2	FALSE
Isoetes_Uwharrie_Schafran77-1	Troy, NC	2	1.37	7	1	TRUE
Isoetes_Uwharrie_Schafran77-2	Troy, NC	2	1.37	13	1	TRUE
Isoetes_Uwharrie_Schafran77-3	Troy, NC	2	1.37	7	1	TRUE
Isoetes_Uwharrie_SchafranSN	Troy, NC	2	—	63	2	FALSE
Isoetes_valida_Cressler15	Young Harris, GA	2	—	15	1	TRUE
Isoetes_valida_Cressler7	Young Harris, GA	2	—	6	1	TRUE
Isoetes_valida_Schafran145_rep1	Reliance, TN	2	2.26	20	1	TRUE
Isoetes_valida_Schafran145_rep2	Reliance, TN	2	2.26	35	1	TRUE
Isoetes_valida_Schafran145_rep3	Reliance, TN	2	2.26	18	1	TRUE
Isoetes_valida_Schafran162	Shenandoah Natl. Park, VA	2	—	88	1	TRUE
Isoetes_valida_Schafran193-1	Autauga Co., AL	2	2.2	980	1	TRUE
Isoetes_valida_Schafran193-2	Autauga Co., AL	2	2.2	1202	1	TRUE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_valida_Schafran193-3	Autauga Co., AL	2	2.2	1307	1	TRUE
Isoetes_valida_Schafran193-4	Autauga Co., AL	2	2.2	837	1	TRUE
Isoetes_valida_Schafran193-5	Autauga Co., AL	2	2.2	1241	1	TRUE
Isoetes_valida_Schafran197	Fort Valley, VA	2	—	560	1	TRUE
Isoetes_valida_Schafran204	Greenbrier Co., WV	2	—	422	1	TRUE
Isoetes_valida_Schafran206	Great Smoky Mountains Natl. Park, NC	2	—	455	1	TRUE
Isoetes_valida_Schafran207	Great Smoky Mountains Natl. Park, NC	2	—	231	1	TRUE
Isoetes_valida_Schafran208	Great Smoky Mountains Natl. Park, NC	2	—	126	1	TRUE
Isoetes_valida_Schafran209	Great Smoky Mountains Natl. Park, NC	2	—	335	1	TRUE
Isoetes_valida_Schafran211-4	Nachitoches Parish, LA	2	—	12	1	TRUE
Isoetes_valida_Schafran211-5	Nachitoches Parish, LA	2	—	6	1	TRUE
Isoetes_valida_SchafranNC11	Newton, NC	2	—	127	2	FALSE
Isoetes_valida_SchafranNC13	Hickory, NC	2	—	72	3	FALSE
Isoetes_valida_Taylor6794_rep1	Wayne Co., MS	2	—	93	3	FALSE
Isoetes_valida_Taylor6794_rep2	Wayne Co., MS	2	—	50	2	FALSE
Isoetes_valida_X_hyemalis_Brunton18933B	Troy, AL	3	—	109	1	FALSE
Isoetes_virginica_Brunton19044	Person Co., NC	4	—	75	3	FALSE
Isoetes_virginica_Fleming16376_rep1	Disputanta, VA	4	—	39	3	FALSE
Isoetes_virginica_Fleming16376_rep2	Disputanta, VA	4	—	246	3	FALSE
Isoetes_virginica_Fleming16376_rep3	Disputanta, VA	4	—	173	3	FALSE
Isoetes_virginica_Taylor6882	Augusta Co., VA	4	—	41	2	TRUE
Isoetes_X_dodgei_Brunton19143	Ottawa, ON	3	—	90	2	FALSE
Isoetes_X_eatonii_Taylor6750	Rutland Co., VT	2	—	40	2	FALSE
Isoetes_X_fairbrothersii_Taylor6922	Sussex Co., NJ	6	—	79	4	FALSE
Isoetes_X_harveyi_Taylor6677_rep1	Hancock Co., ME	7	—	120	3	FALSE
Isoetes_X_harveyi_Taylor6677_rep2	Hancock Co., ME	7	—	65	4	FALSE

Sample	Locality	Estimated Ploidy (x)	C-Value	Total CCS Reads	Clusters (OTUs)	Fits Fixed Heterozygosity Model Based on Morpho-ID?
Isoetes_X_herbwagneri_Taylor-1	Twin Lakes, MT	3	—	124	2	FALSE
Isoetes_X_herbwagneri_Taylor-2	Twin Lakes, MT	3	—	126	2	FALSE
Isoetes_X_herbwagneri_Taylor-3	Twin Lakes, MT	3	—	119	2	FALSE
Isoetes_X_herbwagneri_Taylor-4	Twin Lakes, MT	3	—	41	2	FALSE
Isoetes_X_herbwagneri_Taylor-5	Twin Lakes, MT	3	—	30	2	FALSE
Isoetes_X_heterospora_Taylor6676	Hancock Co., ME	7	—	130	4	FALSE

APPENDIX C. COPYRIGHT RELEASE FOR CHAPTER 4

CASTANEA
The Journal of The Southern Appalachian Botanical Society

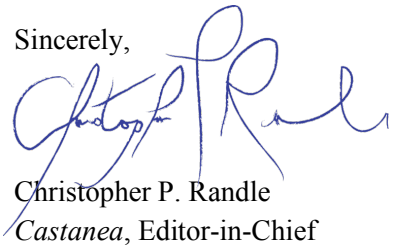
5 November 2019

Peter W. Schafran
Department of Biological Sciences
Old Dominion University
Norfolk, VA, 23529

Dear Mr. Schafran:

This letter serves as permission to republish your article, "A Whole Chloroplast Genome Phylogeny of Diploid Species in *Isoëtes* (Isoëtaceae, Lycopodiophyta) in the Southeastern US" (*Castanea* 83(2): 224-235) as part of your Ph.D. dissertation. Congratulations on nearing completion of your Ph.D. research and best of luck in your future endeavors.

Sincerely,



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Two first author, four co-author invited lectures at scientific meetings.