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Morphological, Genetic, and Environmental Characterization of an Unusual Population of *Isoetes* (Isoetaceae, Lycopodiophyta)

Shannon L. Walker
University of Southern Mississippi

The University of Southern Mississippi

Morphological, Genetic, and Environmental Characterization of an Unusual Population of *Isoetes* (Isoetaceae, Lycopodiophyta)

by

Shannon Walker

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Approved by

Mac H. Alford, Ph.D., Professor and Thesis Advisor
Department of Biological Sciences

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

Ellen Weinauer, Ph.D., Dean
Honors College

Abstract

A large and unusual population of *Isoetes* (Isoetaceae, Lycopodiophyta) in the DeSoto National Forest, Wayne County, Mississippi, was studied to determine if the individuals there represent a new species or if they represent part of the variation of the one primary species of the longleaf pine belt of Mississippi, *Isoetes louisianensis*, which it most closely resembles. The unusual population and specimens of known *Isoetes louisianensis* were examined comparatively based on morphology, megaspore ornamentation, examination of habitat characteristics, and phylogenetic analysis of DNA sequence data from the nuclear internal transcribed spacer 1 and 2 (ITS) and the 5.8S ribosomal gene. No differences were discovered between the populations in DNA sequence data, small differences were discovered in megaspore ornamentation and habitat, and more significant differences were discovered in morphology, although all of the differences were based on small sample size. Thus, the results of this study are inconclusive as to the species status of the unusual *Isoetes* population. However, the detailed environmental data collected in the drainage do show that senescence (loss of leaves) closely follows water levels, not temperatures or seasons, as some have hypothesized. This study augments scientific understanding of *Isoetes louisianensis*, considering that much about this species is still unknown due to many new, recent discoveries of populations, similarities in appearance with other species of *Isoetes*, natural occurrences in *Isoetes* of cross-fertilization with the production of sterile hybrids, variations in ploidy level, and the need for scanning electron microscopy to carefully observe megaspore ornamentation. Plans have been made to continue assessing the unusual *Isoetes* population and typical *Isoetes louisianensis* populations based on chromosome numbers, vegetative anatomy, and phylogenetic analysis of additional DNA regions.

Key Words: *Isoetes*, *Isoetes louisianensis*, Louisiana Quillwort, megaspores, nuclear internal transcribed spacer (ITS), species delimitation

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List of Abbreviations

bp = base-pair(s)

BSA = bovine serum albumin

Cat. No. = catalog number

DNA = deoxyribonucleic acid

ESEM = environmental scanning electron microscope

ITS = (nuclear) internal transcribed spacers 1 and 2 plus 5.8S (of the ribosomal genes), a region of DNA

ITS4 = reverse primer for amplifying ITS

ITS5 = forward primer for amplifying ITS

LAI = leaf area index

LFY = (second intron of the) nuclear LEAFY (homolog), a region of DNA

MPT(s) = most parsimonious tree(s) (from phylogenetic analysis using the parsimony criterion)

PCR = polymerase chain reaction

SEM = scanning electron microscopy

TBT-PAR = PCR enhancer developed by Samarakoon et al. (2013)

USDA = United States Department of Agriculture

USGS = United States Geological Survey

UV = ultraviolet light

Chapter I—Purpose of Study

The purpose of this thesis is two-fold: to determine whether a population of *Isoetes* (quillworts, Isoetaceae, Lycopodiophyta) in the DeSoto National Forest in Wayne County, Mississippi, is a new species or a variation of the one primary species of the longleaf pine belt, *Isoetes louisianensis*, and to provide more information about *I. louisianensis*, as there is little morphological, genetic, and environmental data on this recently described species. *Isoetes louisianensis* is an endangered species of quillwort found in southeastern Louisiana, southeastern Mississippi, and southwestern Alabama (Leonard 2011). *Isoetes louisianensis* inhabits sandy substrate along temporary, shallow streams in upland pine forests and pine flatwoods (Landry and Thieret 1973). Although it is still listed as endangered, a process is underway to delist the species due to its much greater abundance and wider distribution than previously known (S. Leonard, pers. comm.).

At first observation, the Wayne County population of *Isoetes* appears rather unusual in that the population differs greatly from *I. louisianensis* in the size of its leaves, or sporophylls. The sporophylls of the common species are generally 15–40.5 cm (6–16 inches) in length, while sporophylls of the unusual individuals can reach 91.5 cm (36 inches) in length. Additionally, there appears to be a difference in habitat between the typical populations of *I. louisianensis* and the unusual Wayne County population. While *I. louisianensis* often occupies scoured marginal areas of shallow, temporary streams, the Wayne County population occupies a deeper temporary stream and occurs in denser patches in the flowing water. Based on this difference in habitat and the dissimilarity in size, it is possible that this population of *Isoetes* is a new species. The null hypothesis is that this population merely represents a different phenotype of the typical *I. louisianensis*, perhaps due to the noted environmental differences. The alternative hypothesis is that this population represents a new species. Similarities in morphology, megaspore ornamentation, and DNA sequence data would support the null hypothesis. Differences in these data would support the alternative hypothesis. Environmental data will provide context for the morphological and DNA data and may provide reasons for the phenotypic differences if the two populations represent the same species or evidence for occupation of different niches if the two populations represent different species.

The genus *Isoetes* is generally difficult to study, as many species are similar in appearance, readily cross-fertilize to form sterile hybrids, and exhibit polyploidy (Gifford and Foster 1989). Morphological characterization of the megaspores has been used traditionally to identify different species of *Isoetes* (see the taxonomic key in the *Flora of North America*, Taylor et al. 1993, or Brunton 2015), but even the megaspores of separate species are similar in appearance and are therefore challenging to differentiate. Plants of the genus *Isoetes* also vary widely depending on environmental factors, and a single species can exhibit a range of morphologies depending on external factors (Gifford and Foster 1989). These challenges have led to great debate over acceptance of certain *Isoetes* species. Even if the unusual Wayne County population of *Isoetes* is not a new species, an examination of this population will contribute to the limited body of knowledge currently available.

Chapter II—Literature Review

Isoetes, commonly called quillworts, are part of the phylum Lycopodiophyta, an early branch of the first vascular plants. Lycopodiophyta are commonly referred to as lycopods, which do not produce seeds but instead produce spores and have simple vascular tissue (Raven et al. 2005). The extant genus is likely descended from tree-like ancestors that occurred approximately 320 million years ago during the upper Carboniferous period of the Paleozoic era (Hoot et al. 2004). These ancestors were not trees *per se* but large lycopods which sometimes measured greater than 30 meters, or 100 feet, in height (Wnuk 1989).

Figure 1. *Isoetes* individual from the unusual population in Wayne County at the beginning of the growing season.

Extant *Isoetes* are relatively small, possessing a short axis, called a corm, from which the roots and long, cylindrical leaves (sporophylls) develop (Gifford and Foster 1989), which is the source of their common name (Figure 1). Each sporophyll of a quillwort possesses four air chambers, which are used to transport gases needed for respiration. Quillworts are found around the world, in either permanent sources of water or in seasonal pools. Like bryophytes and ferns, quillworts need water for reproduction, which provides the sperm a way to travel to the egg for fertilization. Quillworts are heterosporous, meaning that they produce two kinds of spores within the same plant: male spores, called microspores, and female spores, called megaspores. The spore-bearing structures, called sporangia, are found at the base of the leaves, with microsporangia (male) and megasporangia (female) occurring on separate sporophylls. Microsporangia produce microspores, which germinate into microgametophytes that produce sperm. Megasporangia produce megaspores, which germinate into small megagametophytes that have egg-containing structures. Spores are released as sporophylls decay at the end of the season. Identification of species of *Isoetes* is typically done by characterizing megaspores based on texture, size, and other decorations (Hickey 1986).

Isoetes louisianensis is a species that was discovered in southeastern Louisiana in 1972 by Garrie Landry (Landry and Thieret 1973). It was listed by the U. S. Fish and Wildlife Service as an endangered species on October 28, 1992 (U. S. Fish and Wildlife Service 1992). Locally known as the Louisiana Quillwort, *Isoetes louisianensis* occurs in sandy or gravely substrate in temporary pools and streams (U. S. Fish and Wildlife Service 1996). Due to its method of reproductive dispersal, spores in water, it occurs in patches (U. S. Fish and Wildlife Service 1992). It is a tetraploid, meaning that it has four sets of chromosomal DNA, and is likely the result of hybridization between two different quillworts. This species has been considered one of the most endangered quillworts in North America, although its distribution has been enlarged in recent years and is now known to occur in parts of Louisiana, Mississippi, and Alabama (Leonard 2011) (Figure 2).

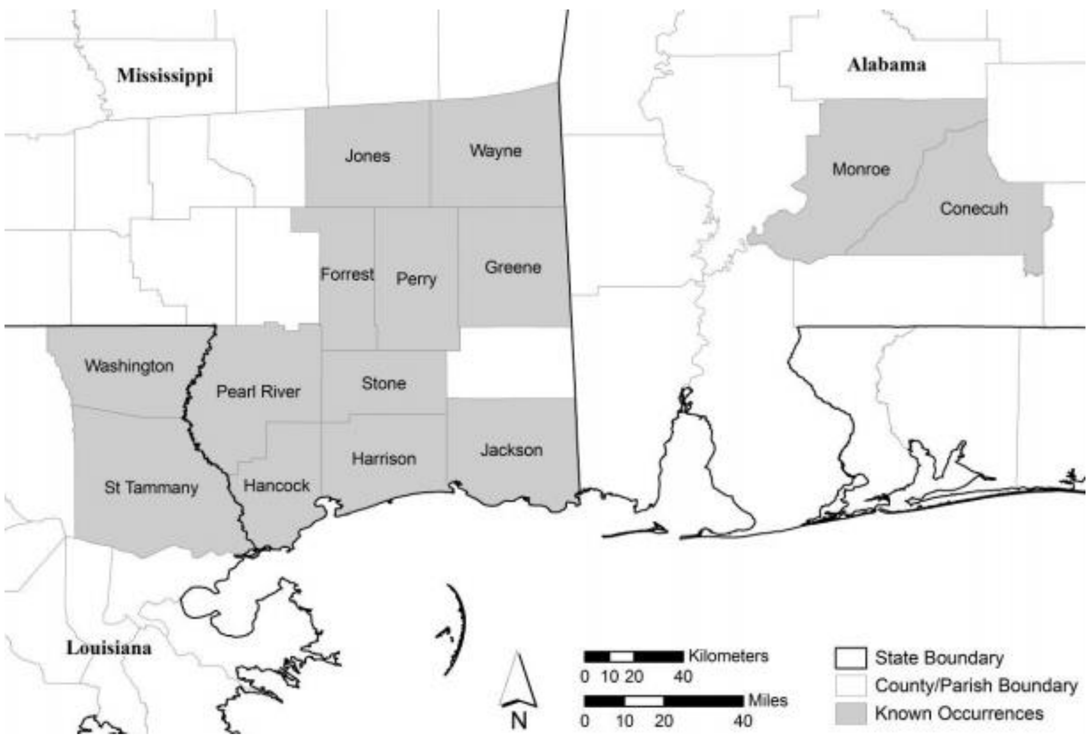

Figure 2. Current distribution map of *Isoetes louisianensis*, adapted from the U. S. Fish and Wildlife Service, Louisiana quillwort (*Isoetes louisianensis*) 5-year Review. Retrieved from https://www.fws.gov/southeast//pdf/five-year-reviews/louisiana-quillwort.pdf.

Changes to the characteristics of the streams which *I. louisianensis* inhabits can eliminate suitable habitat and have a negative impact upon populations (U. S. Fish and Wildlife Service 1996). Alterations to habitats caused by humans are the greatest threats to the Louisiana Quillwort, and these include damage caused by ditching, road construction, mining, timber removal, and the use off-road vehicles.

After its original identification by Garrie Landry, there was dispute about the species status of *I. louisianensis* (Landry and Thieret 1973). Landry and Thieret described its similarity to the species *Iseotes engelmannii*, but the status of *I. louisianensis* as a separate species from *I. engelmannii* was disputed by Boom, who hypothesized that *I. louisianensis* was a hybrid of *I. engelmannii* and *I. melanopoda* (Boom 1980, 1982). Further analysis of reproduction and megaspore morphology, however, provided evidence that *I. louisianensis* is not a hybrid form (U. S. Fish and Wildlife Service 1996).

For identification purposes, *I. louisianensis* is described as having broad, dark green to light green leaves (Brunton 2015). The plants are generally rooted deeply in the substrate, and the velum, a flap of tissue that covers the sporangia, generally covers about 30% of the sporangial surface. The megaspores are irregularly networked with both long muri, patterned ridges found on the megaspore surface, and shorter, isolated muri (Figure 3). In all populations found in Mississippi, *I. louisianensis* can be found in seasonal channels that are dry during some part of the year, generally in summer (U. S. Fish and Wildlife Service 1996). During this time, the sporophylls will die back and only underground structures, such as the corm and roots, will remain. When the weather once again provides enough water for overflow channels to flood, generally in mid- to late winter, *I. louisianensis* will once again grow aboveground sporophylls.

Differentiation between species of *Isoetes* has been traditionally difficult due to their similar appearance and megaspore morphology (Gifford and Foster 1989). Species of *Isoetes* will also readily form sterile hybrids, which can be confused for either a new species or for one of the parent species. Additionally, individuals within a species are highly variable depending on environmental factors (Gifford and Foster 1989).

Figure 3. Cluster of megaspores in an *Isoetes louisianensis* individual (photograph courtesy of Daniel McNair).

Chapter III—Materials and Methods

Detailed locations recorded on herbarium specimens and of populations observed in this study are not included here in accordance with current practices to protect rare and threatened species. Museum specimens referenced here are all housed in the herbarium (USMS) of the University of Southern Mississippi, and locality information of the studied populations has been recorded by the Chickasawhay District of the DeSoto National Forest, the U. S. Fish and Wildlife Service, and the Mississippi Natural Heritage Program of the Mississippi Department of Wildlife, Fisheries, and Parks.

In order to determine whether the unusual population of *Isoetes* in Wayne County is a new species, preliminary morphological data were collected from five individuals located in the unusual Wayne County population and from two positively identified and typical individuals of *Isoetes louisianensis* located in a tributary about one mile west of the unusual population. These individuals were marked with flags for long-term observation (e.g., Figure 18). Other data were collected from a herbarium specimen of *I. louisianensis* (*Steve Leonard #9511*) and an unmounted specimen from the unusual Wayne County population (*Jennifer Lamb s.n.*). Data were compared across the two populations (one "normal" *Isoetes louisianensis*, one "unusual") to determine whether the unusual Wayne County population is a new species. The methods used in this assessment were:

1. Comparison of DNA sequences,
2. Comparison of morphology and megaspore ornamentation, and
3. Comparison of habitat characteristics.

Differences in the sequences of the ITS region between *I. louisianensis* and the Wayne County population would be significant because these are noncoding, rapidly evolving regions of plant DNA (Baldwin et al. 1995). These regions are significant in that changes within these regions have no known adverse effect on the plants and so do not experience negative selective pressures. Over time, the amount of variation in these sequences between separated populations increases. Discrete species and even populations can then be distinguished based on the amount of variation within these sequences (Baldwin et al. 1995).

Analysis of megaspore morphology was also conducted on megaspores isolated from the two preserved specimens. While not of known phylogenetic significance, megaspore morphology is a character traditionally used to identify and differentiate species of *Isoetes* (e.g., Brunton 2015, Taylor et al. 1993). Morphological analysis consisted of measurements of sporophyll length at the peak of the growing season between individuals from the two populations of *Isoetes* in Wayne County. Sporophyll length was then compared between the two populations.

Lastly, environmental data including soil particle size distribution, soil compaction, plant community composition, presence/absence of aboveground parts of *Isoetes*, and water depth and temperature were collected. Habitat is often a distinguishing feature of *Isoetes* (Taylor et al. 1993). Data collected in this study may not be extensive enough to provide compelling evidence for the full description of a unique habitat, but these data may emphasize environmental variables that are worthy of further study. Additionally, there is little ecological data for many southeastern *Isoetes*, especially *I.*

louisianensis, and thus this portion of this thesis represents a source of information which has been lacking in many previous studies.

1. <u>Comparison of DNA Sequences</u>

Segments of leaf for DNA analysis were collected from the *Lamb s.n.* specimen from the unusual Wayne County population. DNA was isolated from that leaf material according to the procedures for the Qiagen DNeasy Plant Mini Kit (Cat. No. 69104, Valencia, CA), using a mortar and pestle in the first step.

Copies of the nuclear ITS region were then made from this template DNA using polymerase chain reaction (PCR). Primers ITS5F and ITS4R were used to amplify the ITS 1, 2, and 5.8S ribosomal gene (Hoot and Taylor 2001, White et al. 1990). The components of the reaction were 25 µL TaKaRa Premix Taq (Version 2.0, Cat. No. R004A, TAKARA BIO INC., Otsu, Japan), 10 µL TBT-PAR (see below), 8 µL water, 2.5 µL forward primer, 2.5 µL reverse primer, and 2.0 µL template (*Isoetes*) DNA. The polymerase chain reaction (PCR) mixture was enhanced by the addition of TBT-PAR as described by Samarakoon et al. (2013). TBT-PAR was prepared as a 5× solution containing 750 mM trehalose, 1 mg/mL nonacetylated BSA, 1% Tween-20, and 8.5 mM Tris hydrochloride, pH 8.0. PCR amplification of the nuclear ITS regions was performed by initial denaturation for three minutes at 95˚C followed by 35 cycles of 98˚C for 10 seconds, 55˚C for 30 seconds, and 72˚C for one minute. This was followed by a final three minutes of extension at 72˚C.

The products of the PCR amplification were then separated in a 2% agarose gel using gel electrophoresis run at 100 V and then stained with ethidium bromide (0.3125 mg/L) for 20 minutes and visualized with UV (ultraviolet) light (example shown in Figure 4).

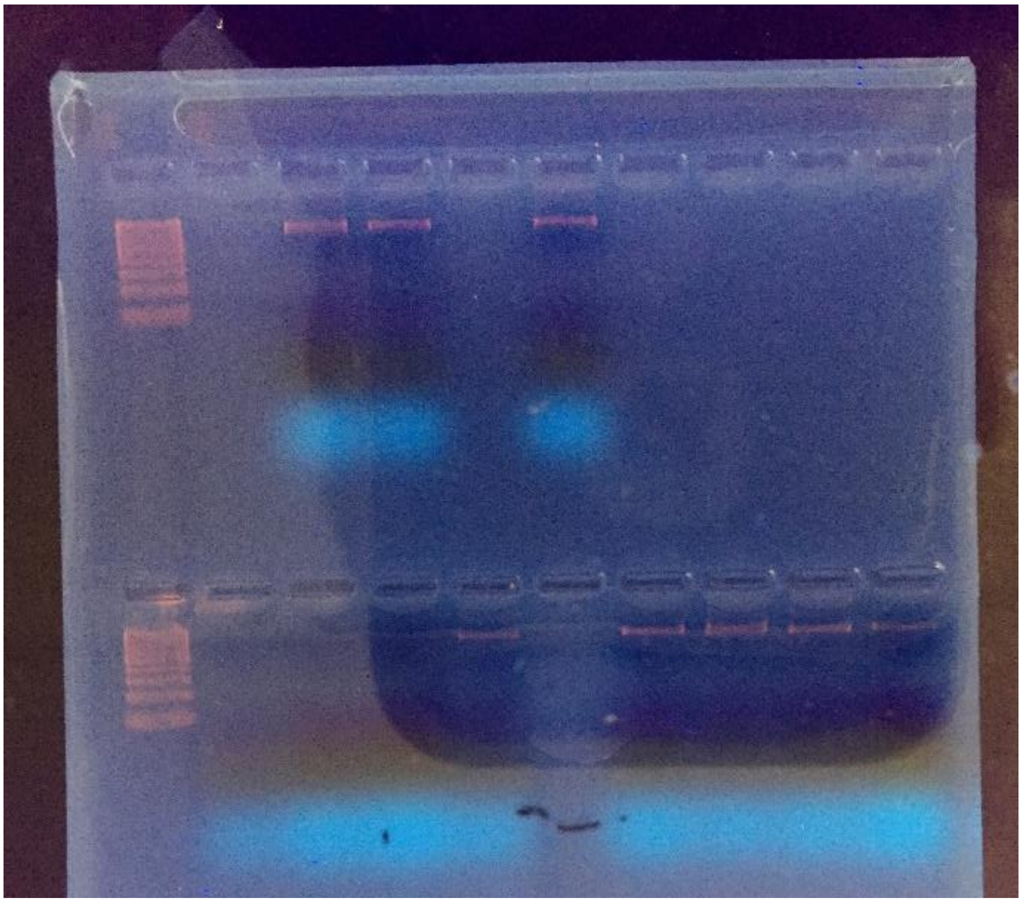

Figure 4. Agarose gel during UV fluorescence showing DNA ladders on the left and bands of isolated DNA of about 750 bp long.

Following positive results for presence and correct size of amplified DNA, original PCR amplified materials were sent to Eurofins Genomics in Louisville, KY, for sequencing. The sequences were returned as .ab1 files and were reviewed for quality using Sequencher version 5.4.1 (Gene Codes Corporation, Ann Arbor, MI). Forward and reverse sequences were assembled automatically in Sequencher using the default settings, and then the consensus sequence was exported as a fasta file.

In addition to the sequences obtained here, sequence data from other species of *Isoetes* were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and from unpublished studies kindly shared by Peter Schafran (Old Dominion University, pers. comm.). The sequence files were opened in ClustalX 2.1 **(**Thompson et al. 1997, 1998, Larkin et al. 2007) to align sequences using the default parameters. After alignment, sequences were imported into WinClada (Nixon 2002) for phylogenetic analysis using parsimony. Most parsimonious trees were generated using the heuristics search option, and individual branches of the phylogeny were assessed for their strength by a bootstrapping analysis of 500 replications.

2. <u>Comparison of Morphology and Megaspore Ornamentation</u>

Megaspores were isolated from the base of mature female sporophylls from the *Leonard* and *Lamb* preserved specimens. Damage to the specimens was minimized by sampling a maximum of two sporophylls per specimen and using forceps and a dissecting needle to remove the megaspores from the specimen. Slides were created by adhering megaspores to aluminum stubs with double-sided carbon-rich tape. The megaspores were then coated in silver using a sputter coater and allowed to dry. Megaspores were visualized under a FEI Quanta 200 environmental scanning electron microscope (ESEM) (Thermo Fischer Scientific, Materials and Structural Analysis Division, Hillsboro, OR) at 1.5–10.0 kV. The large range for voltage used in these analyses was to compensate for large amounts of charging on the megaspore surface. Due to the sharp ridges of the megaspores, voltage needed to be adjusted along a broad range in order to obtain clear images. Images were analyzed for distinctive patterns of bands and ridges on the megaspore surface.

Sporophyll length was measured for five individuals of the unusual Wayne County population and two individuals of *I. louisianensis* during the peak of the growing season (June 8, 2017). The longest sporophyll present on each plant was measured from the base of the sporophyll to the tip using a ruler. These seven individuals were marked with flags and observed throughout the duration of the study for growth patterns.

3. <u>Comparison of Habitat Characteristics</u>

Data for soil compaction, particle size analysis, and water temperature and level was collected for the two Wayne County sites. United States Geological Survey (USGS) soil data for Wayne County were first obtained for each site via the USGS web soil survey (Soil Survey Staff) based on the Wayne County Soil Survey (U. S. Department of Agriculture 2009). In order to verify the classification given by the USGS, soil samples were collected from each site using a soil corer and stored in capped plastic soil cores. Soil was collected from three sites in the unusual Wayne County population area—two in the dry stream bed and one outside of the flood zone. Soil was also collected from the nearby tributary with the positively identified *I. louisianensis* individuals. One sample was taken in the stream bed and one sample from outside the flood zone.

Samples were analyzed for particle size as described by Dr. Franklin Heitmuller based on the procedures of Gee and Bauder (1986). For hydrometer analysis, soil samples were individually mixed and then 100 g were dried overnight in a 105°C oven. Each sample was disaggregated by hand using a mortar and pestle and then 50.0 g of each were deflocculated by adding 250 mL of distilled water and 100 mL of 5% sodium hexametaphosphate (Calgon) and then allowed to sit overnight. A milkshake mixer was then used to further disaggregate the sediments for five minutes. Sediments were then added to Bouyoucos tubes, and distilled water was added to bring the mixture up to 1,000 mL.

A control tube was prepared by adding 900 mL of distilled water to 100 mL of 5% sodium hexametaphosphate. A thermometer was left in the control tube for the duration of the experiment for use as a reference temperature. A stopper was inserted into the Bouyoucos tubes, and the samples were shaken vigorously for one minute. The tubes were placed on the counter, and a stopwatch was immediately started. A hydrometer was placed into the tubes, and specific gravity was recorded at various time increments. At each time increment, specific gravity and temperature was recorded for each tube, including the control. This portion of the analysis was used to assess the proportion of the sediment that was in the clay and silt fractionation.

After hydrometer assessment, sediments were analyzed via sieve analysis to obtain data for larger particles. Sediment samples were poured over a stack of sieves measuring phi –1, 0, 1, 2, 3, and 4, which correspond to mesh sizes from large to small. Dry 150 mL beakers were weighed and labeled according to sample number and mesh size. A squeeze bottle was used to remove sediments from each mesh size into the appropriately labeled beaker for each sample. Beakers were dried in an oven at 105°C overnight to remove excess water and then weighed. The weight obtained was divided by the original 50.0 g to obtain the sand fractionation.

The hydrometer analysis was then performed using formulas in an Excel spreadsheet provided by Dr. Franklin Heitmuller based on the procedure of Gee and Bauder (1986). The spreadsheet based calculations on Stoke's Law: $w = \frac{2\,(\rho_p - \rho_f) g r^2}{9\mu}$ where w is the settling velocity, ρ is density (with p and f subscripts denoting particle and fluid respectively), g is the acceleration due to gravity, r is the radius of the particle, and μ is the dynamic viscosity of the fluid. This calculation is associated with some error as it assumes that each particle is spherical. However, it is widely used in particle size analysis and provides reasonably accurate results despite its assumptions. The provided spreadsheet then incorporated each point into a particle size distribution curve. Data from sieve analysis was then added to produce a particle size distribution curve for the entirety of each soil sample fractionation. These data were then compared to the 12 United States Department of Agriculture (USDA) soil textural classes using the soil textural class triangle (U. S. Department of Agriculture, National Soil Survey Handbook, Figure 9) to determine the soil classification (Gee and Bauder 1986).

Soil compaction was measured in both Wayne County sites using a DICKEY-john Soil Compaction Tester (Churchill Industries, Minneapolis, MN). The ¾ inch tip for soft soil was used. Readings were taken three inches to either side of each field-observed individual in parallel with the stream flow, and the presence, thickness, and strength (in psi) of each compaction layer was recorded. The data were then compared between the two sites.

Water temperature and depth were taken using a HOBO U20L water level and temperature data logger (Onset Computer Corporation, Bourne, MA). The HOBO water level logger was attached to a metal fencepost using wire and zip ties, which was subsequently embedded in the deepest part of the unusual Wayne County population stream. In order to protect the sensors from damage, the loggers were attached to the fencepost along the face that was not directly against the flow of water. Two water level loggers were attached, one measuring subaqueous temperature and pressure, and the other measuring barometric temperature and pressure. The logger collecting barometric data was attached at the apex of the fencepost, well above the height of flood waters. The logger collecting subaqueous data was attached ~5 cm above the bottom sediments. This was done to prevent potential sediment accretion from disrupting the sensor. Data were recorded every ten minutes between May 5, 2016, and March 1, 2017. The data were quantified and graphed using HOBOware Pro software (Onset Computer Corporation, Bourne, MA) and then matched with field observations of presence, senescence, and absence of aboveground features in the unusual Wayne County population over the same period.

In addition to quantitative measurements, observations of the species composition of the plant community were made. An individual species possesses a range of environmental tolerances within which they function and will respond differently to different environmental conditions (Gleason 1917, 1926). The composition of species found in a particular community is indicative of certain environmental conditions based on the assessment of environmental tolerances of each species composing that community (Gurevitch et al. 2006). Thus, the physical conditions of a site may be inferred from the composition of the plant community.

Chapter IV—Results and Data

1. Comparison of DNA Sequences

The aligned matrix of nuclear ITS DNA sequence data consisted of 747 base pairs (bp). Of these, 142 bp were variable and potentially parsimony informative, indicating that there was variation (19%) between species which may be useful in phylogenetic analysis. A heuristic parsimony analysis was performed, holding 1000 total trees with 500 replications, with two trees saved per replication. Parsimony analysis resulted in 142 most parsimonious trees (MPTs) of length 272 and RI = 0.92, and a strict consensus tree was calculated that visually showed relationships that were supported by all of the MPTs (Figure 5). A bootstrap analysis (Felsenstein 1985) was then completed on the aligned matrix with 500 replications, with each replication having five starting points and holding two trees. The percentage of times that clades in the strict consensus tree appeared in the bootstrap replicates was mapped on the branches of the tree (Figure 5). The values reveal how often the same result was returned with a random subset (with replacement) of the original data, with 0 indicating no support and 100 indicating strong support.

The reconstructed phylogeny reveals several well-supported clades, including a clade of North American species, of which both *I. louisianensis* and the unusual *Isoetes* were a part. Within this clade, the ITS region was not useful in differentiating species, that is, it was not variable (Figure 6). There were no changes to the sequence within the clade that could be used to differentiate between *I. louisianensis*, the unusual *Isoetes*, and several other closely related North American species, including a newly described species (*Isoetes mississippiensis*: Schafran et al., 2016) and one of the putative parents suggested by Boom (1980, 1982), *Isoetes melanopoda* (Figure 6). The ITS region was useful, however, in determining relationships in other parts of the genus. Bootstrap values also support these relationships, as demonstrated in Figure 5.

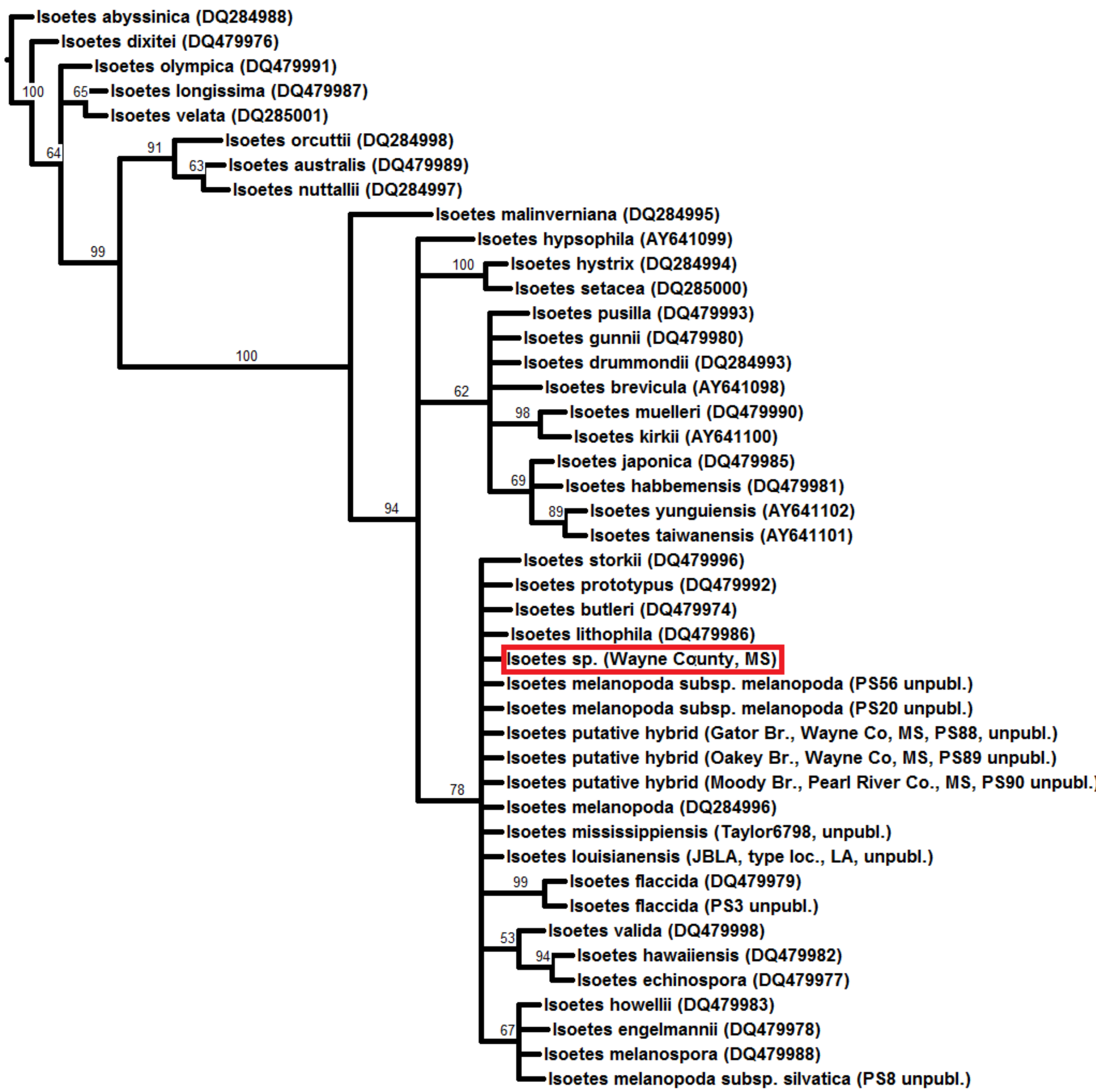

Figure 5. Strict consensus of the 142 most parsimonious trees (MPTs) generated from parsimony analysis of the nuclear ITS region showing bootstrap values supporting each branch. The unusual population is marked in red. Accession numbers are given for data downloaded from GenBank.

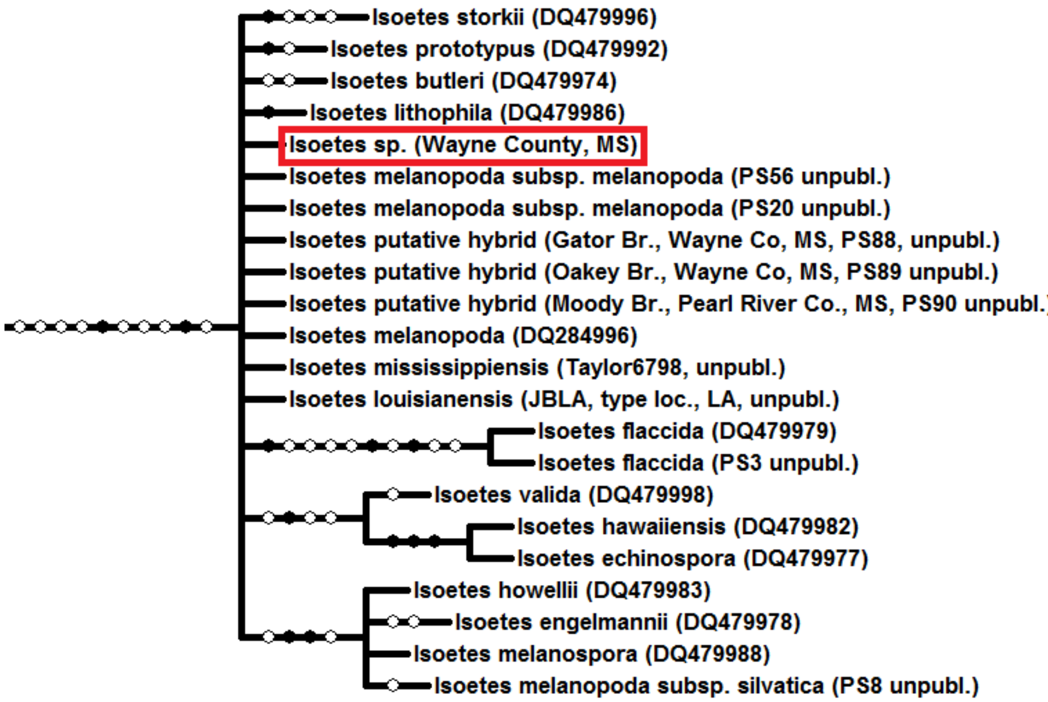

Figure 6. The isolated North American clade of the strict consensus tree of the MPTs generated from the nuclear ITS region showing the number of bp differences in each branch.

2. Comparison of Morphology and Megaspore Ornamentation

Descriptions of the ornamentation of the megaspores follows the terminology described by Punt et al. (2007). The ornamentation of megaspores isolated from the *Leonard #9511* specimen ("typical" *Isoetes louisianensis*) showed reticulate ornamentation with high-ridged muri which connect to form ellipsoid lumina. The apexes of the muri were sharply ridged with strongly pronounced equatorial bands exceeding the height of the muri (Figure 7).

Ornamentation of the megaspores from the unusual population (*Lamb s.n.*) was hamulate to weakly striate with muri that do not connect—thus no lumina were observed. Apexes of muri were rounded and wider than those observed in the *Leonard* specimen.

Megaspore diameter for *I. louisianensis* averaged ~440 µm while the diameter of megaspores of the unusual *Isoetes* averaged ~430 µm, which were very similar. These dimensions are smaller than the 545–600 µm given for *I. louisianensis* as described in *The Flora of North America* (Taylor et al. 1993).

The following selected images were obtained from SEM analysis of *Isoetes louisianensis* (*Leonard #9511*).

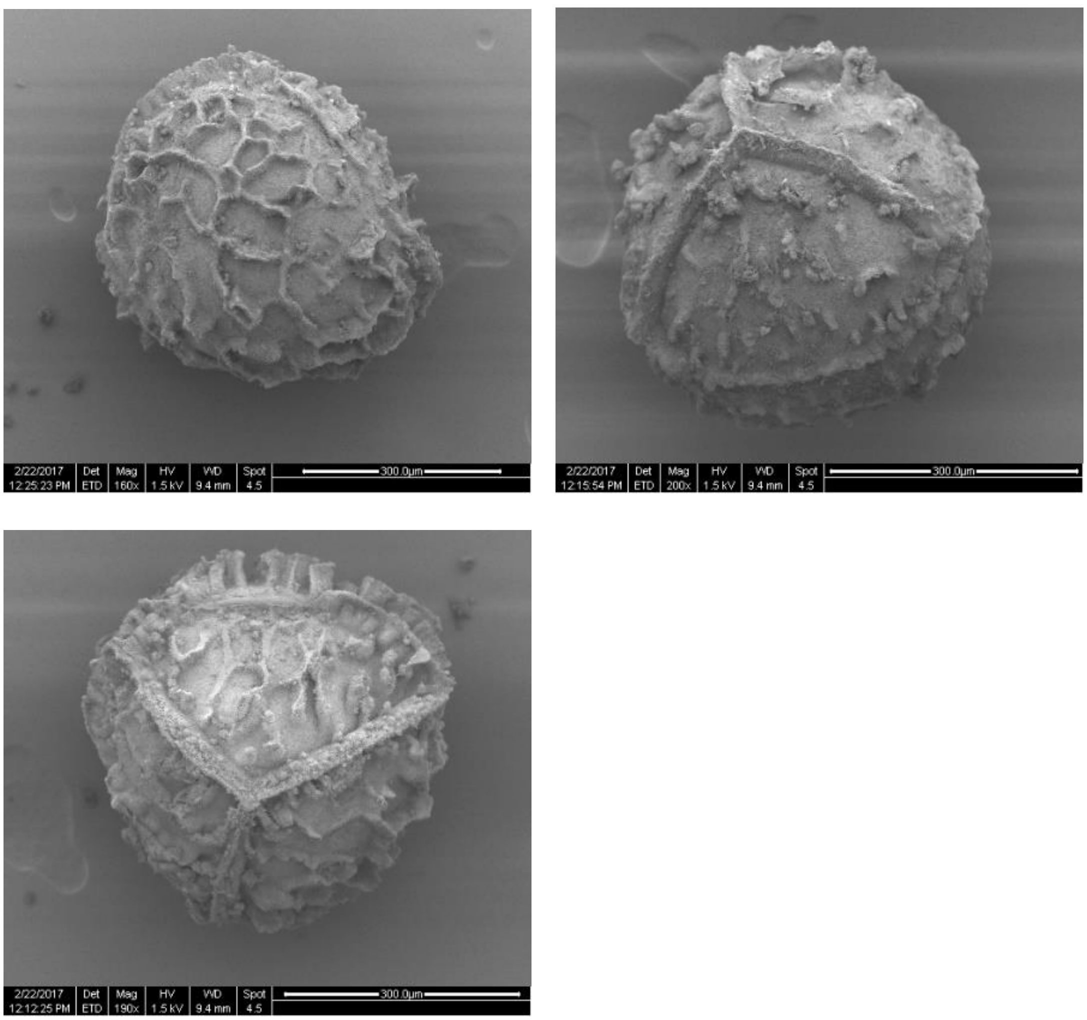

Figure 7. Selected SEM images of megaspores isolated from the Perry County herbarium specimen of *Isoetes louisianensis*. Scale bars are 300 µm.

The following selected images were obtained from SEM of the megaspores from the unusual Wayne County *Isoetes* population (*Lamb s.n.*).

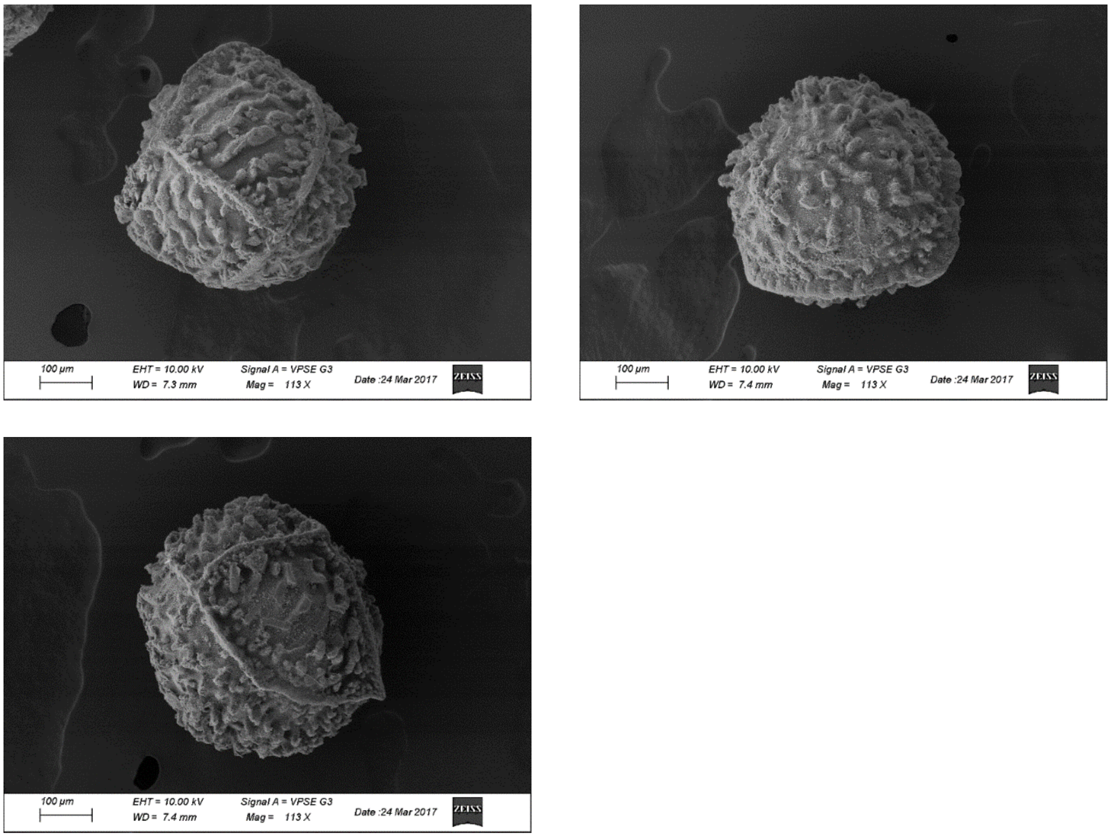

Figure 8. Selected SEM images of megaspores isolated from the preserved specimen of the unusual *Isoetes*. Scale bars are 100 μm.

Sporophylls for the unusual Wayne County *Isoetes* population were consistently longer than those of the positively identified *I. louisianensis* population. On average, the five specimens of the unusual *Isoetes* had maximum sporophyll lengths of 28.56 cm while the two specimens of positively identified *I. louisianenesis* averaged sporophyll lengths of 11.8 cm. On average, then, maximum sporophyll length at the peak of the growing season (June 8, 2016) was 41.3% greater in the unusual *Isoetes* population than in the population of *I. louisianensis*. Field observations confirmed that these specimens were representative of the average sporophyll lengths of the two populations. Despite the very small sample size, a *t*-test indicates that these means are significantly different at the 0.02 level.

Specimen	Sporophyll length at peak growth (cm)
LA #1	14.5
LA #2	9.1
WC #1	33
WC #2	36.3
WC #3	22.5
WC #4	36.5
WC #5	14.5

Table 1. Sporophyll length at the peak of the growing season (June 8, 2016) in the seven field specimens. (LA=*I. louisianensis*, WC=unusual population)

3. <u>Comparison of Habitat Characteristics</u>

I. Soil analyses

Soil compaction tests revealed the presence of compacted soil layers at both the unusual *Isoetes* site and the *I. louisianensis* site (Table 2). At the unusual *Isoetes* site, sample #1 possessed a compaction layer at a depth of 51–56 cm (20–22 inches) below the surface and measuring strength greater than 300 psi. The soil at sample #2 was continuously compacted at 35.5 cm (14 inches) and below with a strength greater than 300 psi. No compaction layer was detected for samples #3 and #4. At the *I. louisianensis* site, the soil of sample #1 was continuously compacted starting 38 cm (15 inches) below the surface with a strength of 250 psi. Sample #2 at the *I. louisianensis* site possessed two separate compaction layers, one possessing a strength of 250 psi and spanning 23–30.5

cm (9–12 inches) below the surface and the other having a strength greater than 300 psi and spanning 30.5–40.5 cm (12–16 inches) below the surface.

Site	Sample Number	Compaction Layer Present	Depth of Compaction Layer (inches)	Strength of compaction layer (psi)
Unusual *Isoetes* site	1	Yes	20–22	>300
Unusual *Isoetes* site	2	Yes	>14	>300
Unusual *Isoetes* site	3	No	NA	NA
Unusual *Isoetes* site	4	No	NA	NA
Unusual *Isoetes* site	5	Yes	>15.5	150
I. louisianensis site	1	Yes	>15	250
I. louisianensis site	2	Yes	9–12 12–16	>300 250

Table 2. Soil compaction data for each site.

The USGS web soil survey, constructed based on the Wayne County Soil Survey (U. S. Department of Agriculture 2009), currently classifies the soils in both streams as Bibb-Iuka complex, 0–1% slopes, and frequently flooded, with the map unit symbol of BkA (Soil Survey Staff). These soils are texturally classified as fine sandy loams. Soil texture for both the Wayne County Soil Survey and this study were assessed based on the USDA classification of soil texture where clay particles have a diameter less than 0.002 mm, silt particles have a diameter between 0.002 mm and 0.05 mm, sand particles have a diameter between 0.05 and 2.0 mm, and gravel has a diameter greater than 2.0 mm (U. S. Department of Agriculture, National Soil Survey Handbook, U. S. Department of Agriculture 2009).

Comparing the results of the particle size analysis (Table 3) to the USDA soil triangle of the 12 major soil textural classes (U. S. Department of Agriculture, National Soil Survey Handbook, Figure 9), the soils of both streams can be classified as loams, which does not support the USGS soil survey assessment.

Sample	% Clay	% Silt	% Sand
I. louisianensis site (#1)	18.0	49.3	32.7
I. louisianensis site (#2)	18.0	51.1	30.9
Unusual *Isoetes* site (#3)	19.0	45.8	35.2
Unusual *Isoetes* site (#4)	13.0	45.2	41.8
Unusual *Isoetes* site (#5)	12.0	47.5	40.5

Table 3. Textural fractionation as a percent of each soil sample.

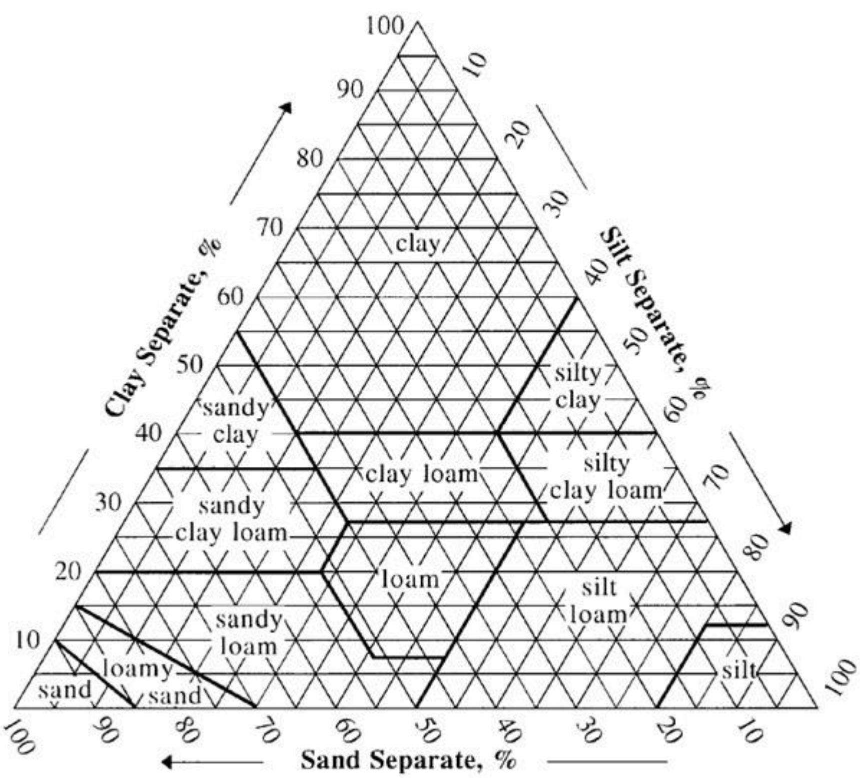

Figure 9. The USDA soil triangle of the 12 major soil textural classes.

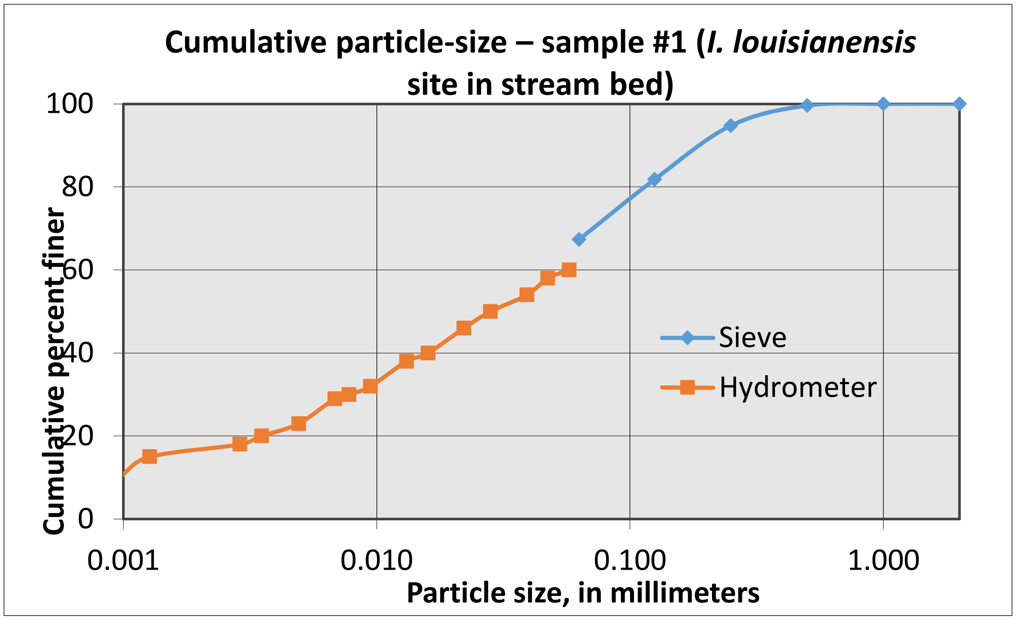

Figure 10. Graph of cumulative particle size for soil sample #1 from the *Isoetes louisianensis* site as a percent finer than the remaining sample.

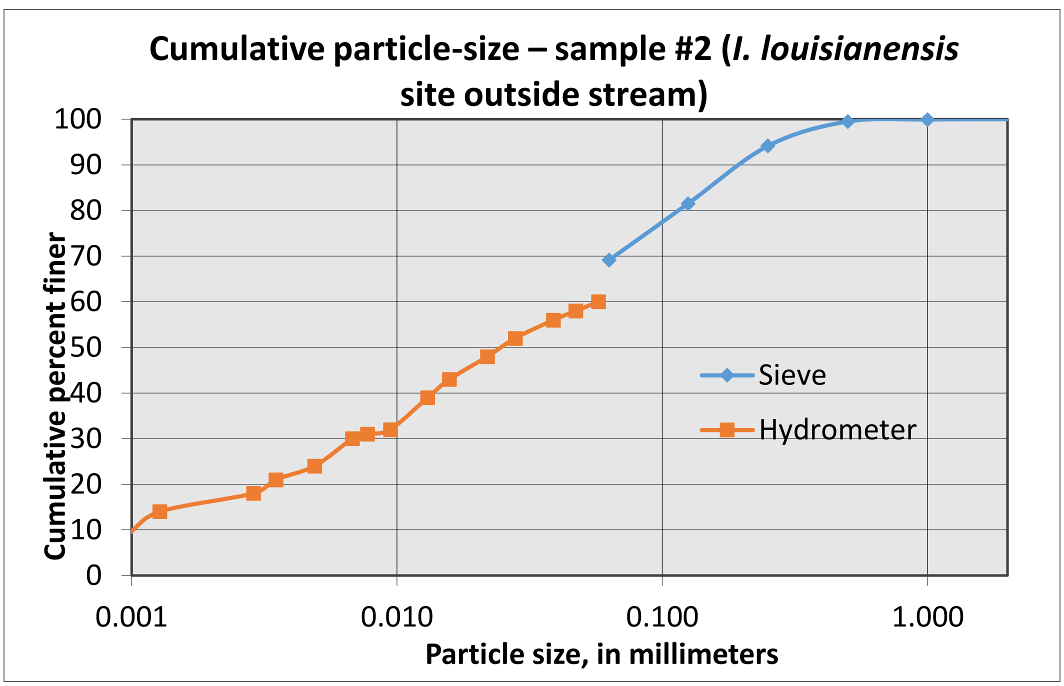

Figure 11. Graph of cumulative particle size for soil sample #2 from the *Isoetes louisianensis* site as a percent finer than the remaining sample.

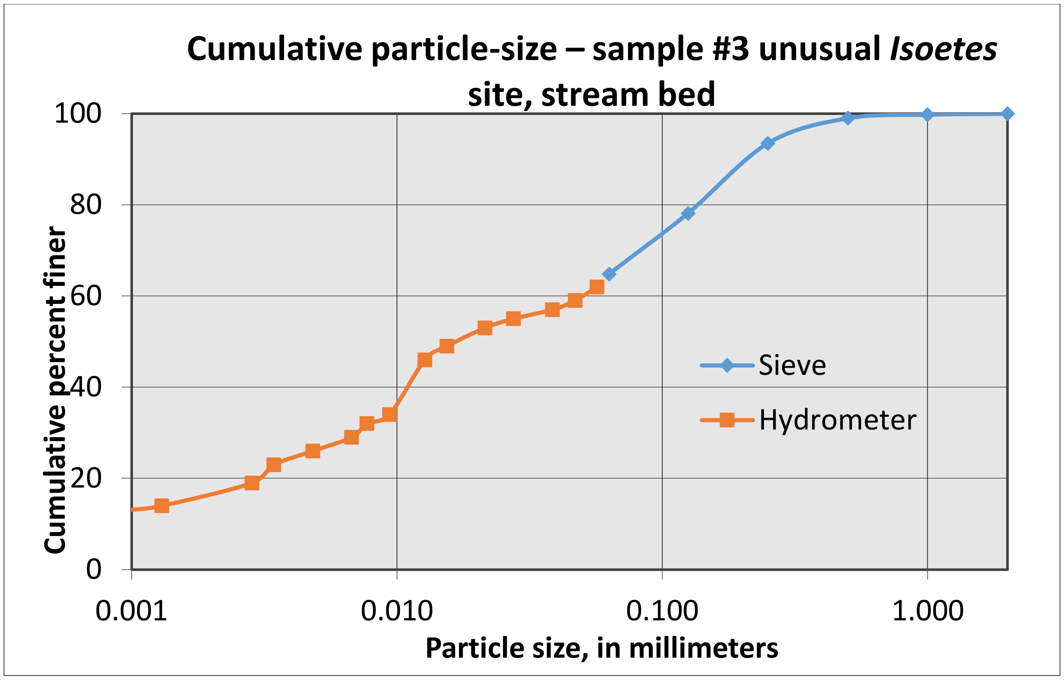

Figure 12. Graph of cumulative particle size for soil sample #3 from the unusual *Isoetes* site as a percent finer than the remaining sample.

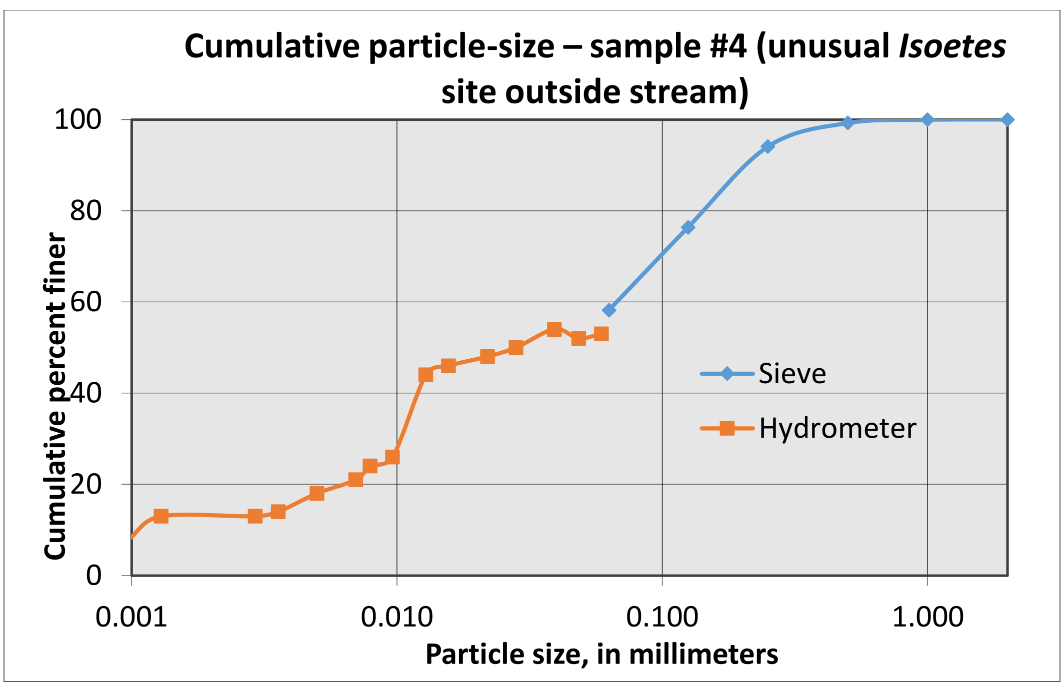

Figure 13. Graph of cumulative particle size for soil sample #4 from the unusual *Isoetes* site as a percent finer than the remaining sample.

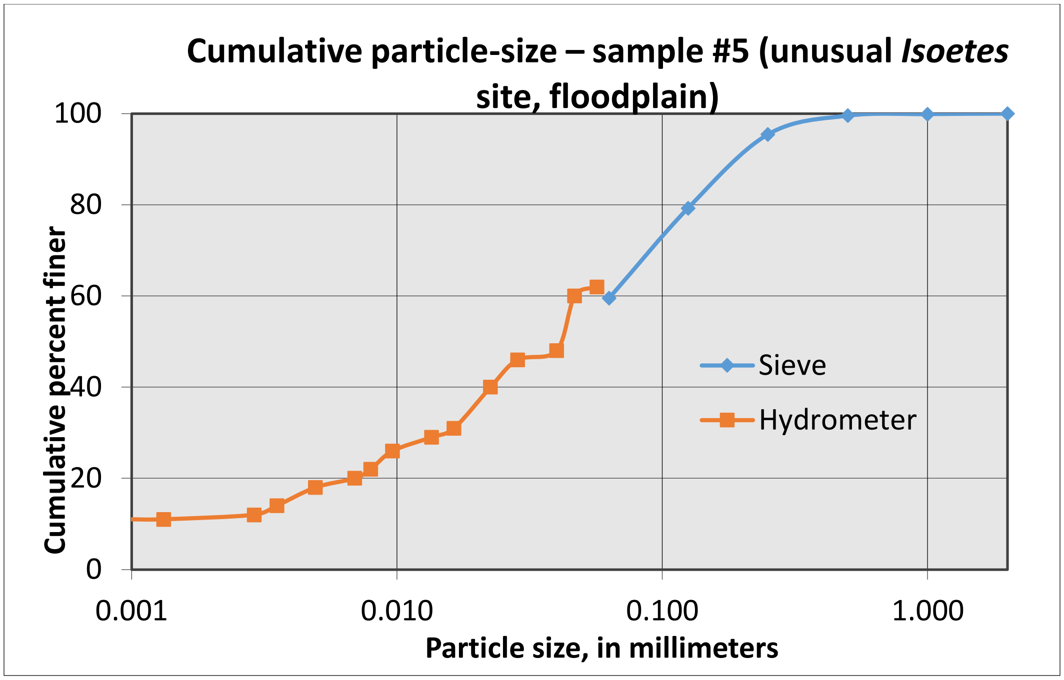

Figure 14. Graph of cumulative particle size for soil sample #5 from the unusual *Isoetes* site as a percent finer than the remaining sample.

II. Composition of the Plant Community

The following pages provide tables (Tables 4 and 5) which list the species composition of the plant community for both the unusual *Isoetes* and the *I. louisianensis* sites in Wayne County. Both communities had certain species in common such as *Nyssa biflora* (Swamp Tupelo) and *Cyrilla racemiflora* (Swamp Titi). These species are water-loving and reinforce observations that these areas flood with water during parts of the year. Because *Arundinaria gigantea*, *Rubus trivialis*, *Saururus cernuus*, and *Toxicodendron radicans* occur at the *I. louisianensis* site and not at the unusual *Isoetes* site this could indicate differences in certain abiotic and biotic factors between the two populations. Images of each site taken on the same day during the growing season (June 8, 2016) (Figures 15 and 16) have also been provided for visual reference.

Angiosperms
Acer ruburm (Red Maple)
Carex louisianica (Louisiana Sedge)
Cyrilla racemiflora (Swamp Titi)
Hypericum hypericoides (St. Andrew's Cross)
Itea virginica (Virginia Sweetspire)
Liquidambar styraciflua (Sweetgum)
Magnolia virginiana (Sweetbay Magnolia)
Morella cerifera (Wax Myrtle)
Nyssa biflora (Swamp Tupelo)
Quercus laurifolia (Laurel Oak)
Vaccinium arboreum (Tree Sparkleberry)
Vaccinium elliottii (Elliott's blueberry)

Gymnosperms
Pinus palustris (Longleaf pine)

Bryophytes
Sphagnum sp.
Atrichum sp.

Table 4. List of plant species found at the unusual *Isoetes* site.

Figure 15. Unusual *Isoetes* site (1) on March 26, 2017.

Angiosperms
Acer rubrum (Red Maple)
Arundinaria gigantea (Switch Cane)
Bignonia capreolata (Crossvine)
Cyrilla racemiflora (Swamp Titi)
Dioscorea sp. (Wild Yam)
Ilex opaca (Virginia Holly)
Liquidambar styraciflua (Sweetgum)
Magnolia virginiana (Sweetbay Magnolia)
Parthenocissus quinquefolia (Virginia Creeper)
Quercus laruifolia (Laurel Oak)
Rubus trivialis (Dewberry)
Saururus cernuus (Lizard's Tail)
Toxicodendron radicans (Poison Ivy)

Pteridophytes
Lorinseria areolata (Virginia Chain Fern)

Bryophytes
Atrichum sp.

Table 5. List of plant species found at the *I. louisianensis* site.

Figure 16. *Isoetes louisianensis* site on March 26, 2017.

III. Water Level and Presence/Absence of Aboveground Features

Water level logger data and field observations indicated that the presence of above-ground structures in the unusual *Isoetes* population corresponded with both the presence of warmer temperatures and water in the stream. Senescence was associated with a lack of water in the stream coupled with relatively consistent warm to hot air temperatures. Because air temperatures do not fluctuate significantly before senescence, it is more likely that water level rather than temperature triggered senescence in this population. During the majority of the period when aboveground features were absent, the stream was dry and air temperatures steadily decreased. The last weeks before aboveground structures were observed, data were punctuated by three major spikes in water level in mid-November and broad fluctuations in air temperature. Following these events, aboveground features were once again observed in the field.

The following pictures (Figures 17 and 18) illustrate the water level at the unusual *Isoetes* site at separate times during the year.

Figure 17. Unusual *Isoetes* site (2) on March 26, 2017. The green plants in the stream are all *Isoetes* individuals.

Figure 18. Unusual *Isoetes* stream with individual #3 flag in view on September 25, 2016. Note the absence of aboveground *Isoetes* structures in this picture.

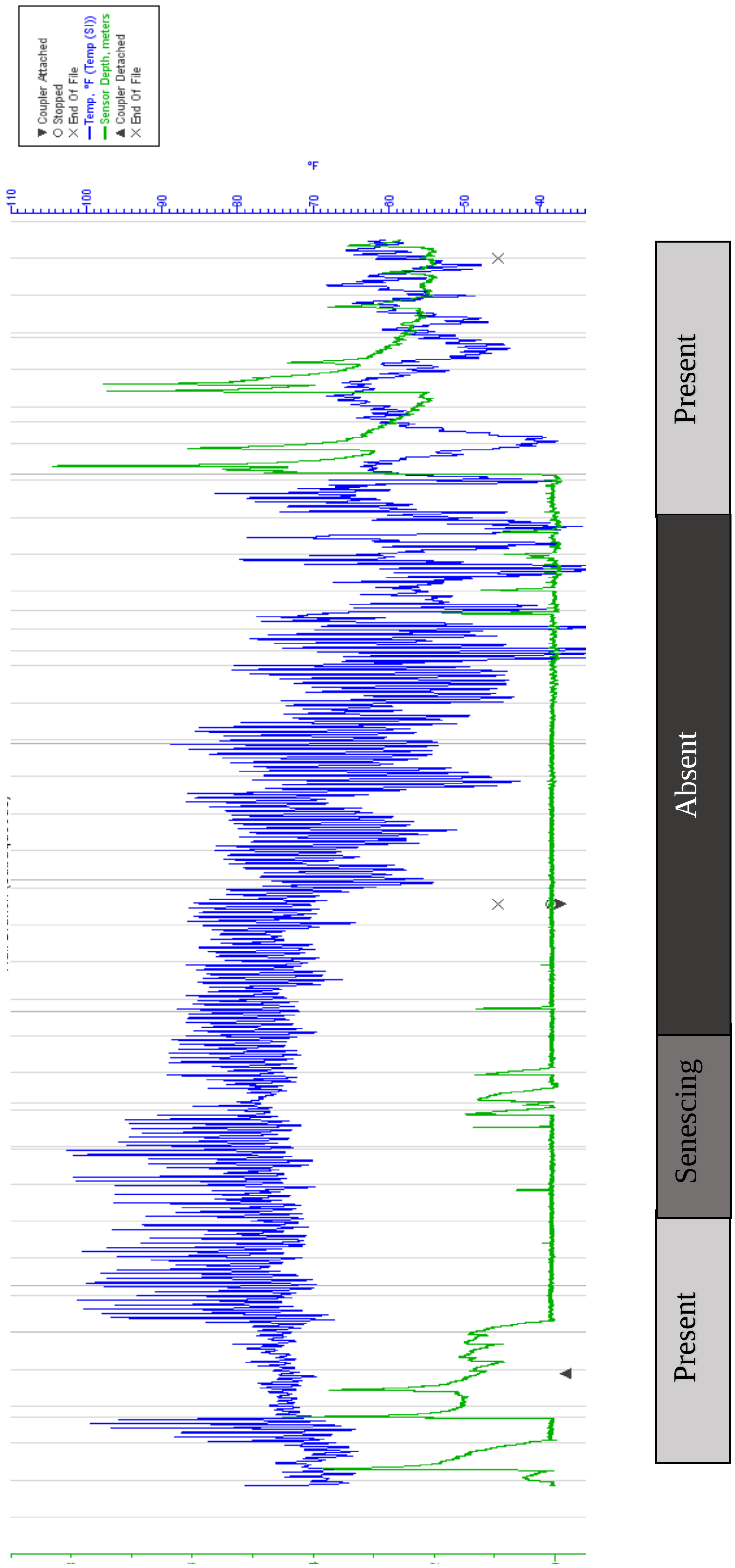

Figure 19. Water depth and temperature matched with observed patterns of presence/absence of aboveground features at the unusual *Isoetes* site.

Chapter V—Discussion

Given the above results, neither the null hypothesis that the unusual *Isoetes* population merely represents a different phenotype of the typical *I. louisianensis* nor the alternative hypothesis that this population represents a new species is strongly supported. Megaspore ornamentation, environmental data, and plant community are slightly different between the two populations, but sampling size is small. Sporophyll length is significantly different between the two populations, but DNA sequences are indistinguishable. Despite the ambiguity conveyed by these data, this study does provide insights into several aspects of these two populations and *Isoetes* in the southeastern United States.

Data collected in this study are significant because, prior to this investigation, continuous water depth data had not been matched with observations of presence/absence of sporophylls. These data corroborate field observations made by Steve Leonard, who noted that senescence appeared to be coupled with prolonged dry periods in streams inhabited by quillworts in Louisiana (U. S. Fish and Wildlife 1996). This may also explain the distribution of *I. louisianensis* within its range, as Mississippi populations are restricted to ephemeral streams, such as the Wayne County streams monitored in this study (U. S. Fish and Wildlife 1996).

While the nuclear ITS region was not useful in differentiation between most *Isoetes* species in North America, it did support several separate clades. The bootstrap values for these clades indicate that they are well supported. Thus, although the nuclear ITS region was not useful at the species level for the North American clade, it could provide utility in assessing the relatedness of these clades to each other, a result supported by Hoot and Taylor (2007).

The lack of variation in this region could be due to rapid speciation and radiation of the North American clade (Hoot and Taylor 2007). Analysis of two sets of combined DNA sequences, both of which contained the nuclear ITS region, supported this hypothesis. When the ITS region was combined with the second intron of the LEAFY (LFY) homolog, it allowed for the resolution of a sister group relationship between *Isoetes hawaiiensis* and *Isoetes echinospora* (Hoot and Taylor 2007).

An additional difficulty associated with the analysis of the nuclear ITS region is that the rate of evolution in this region is different for different lineages and appears to be associated with plant life-form (Baldwin et al. 1995). The nuclear ITS region has been useful in the assessment of angiosperm species (Baldwin et al. 1995). However, it does not appear useful in the analysis of the North American clade of *Isoetes*, which could be attributed to a different rate of nuclear ITS evolution between the two groups.

The second intron of the LFY homolog is four times more variable than the ITS region and shows promise in assessment of the relationships between species in the North American clade (Hoot and Taylor 2007). This region would provide the necessary variation for phylogenetic analysis at the species level. During this study, several attempts were made to amplify this region in the sample from the unusual *Isoetes*; however, these attempts did not return sufficient DNA for analysis. There could be several reasons for this. It is possible that the primer used did not anneal properly during PCR. This is most likely the case due to the positive results of subsequent amplification of the ITS region. Future attempts will be made to find a solution to this problem so that the relationship between the unusual *Isoetes* population and *I. louisianensis* may be assessed by variable DNA sequence data.

SEM revealed differences between the ornamentation pattern of the unusual *Isoetes* in Wayne County and the typical *Isoetes louisianensis* population. The megaspores of the unusual *Isoetes* population demonstrated irregular arrangement of muri with distinctively rounded apexes that do not connect to form lumina. When compared to the sharply ridged muri which form distinct lacunae in the megaspores of the typical *I. louisianensis*, differences between the megaspores of the two are readily distinguishable.

While differences in megaspore ornamentation may not be of taxonomic significance given the current sample size, the difference in the ornamentation may be used to differentiate between the unusual *Isoetes* and the general population of *I. louisianenis*. Megaspores from both sites were similar in size; however, they were much smaller than the average size for *I. louisianensis* recorded in *The Flora of North America*, possibly indicating that these megaspores were immature (Taylor et al. 1993). This would have affected descriptions of megaspore ornamentation. Collections of fresh materials from both sites around decaying sporophyll bases at the end of the season would increase the sample size and ensure that fully mature megaspores are collected (Taylor et al. 1993).

The difference in the sporophyll lengths between the two populations of *Isoetes* was notable. Average sporophyll length was greater by 41.3% in the unusual *Isoetes* population than in the population of *I. louisianensis* and statistically significant based on at *t*-test at the 0.02 level. It is possible that environmental differences were the key driver of the differences between the two populations.

Different environmental conditions for the two sites were indicated by the plant community composition. The positively identified *I. louisianensis* site contained a greater number of sun-tolerant species, including *Saururus cernuus*, *Rubus trivialis*, and *Arundinaria gigantea.* Many of the species at this site were also less tolerant of flooded conditions, such as *Toxicodendron radicans* and *Rubus trivialis*. These were present at the edges of the stream area, while more flood tolerant species, such as *Saururus cernuus, Arundinaria gigantea, Cyrilla racemiflora,* and *Quercus laurifolia*, were present a short distance within the stream bed. Notably, the *I. louisianensis* population was not present upstream in an area which had much greater shade. This could indicate that this population of *I. louisianensis* does not tolerate low light levels or that another factor is present which excludes them from inhabiting that portion of the stream. Additionally, *I. louisianensis* did not form dense, monotypic stands within the streambed as did the unusual *Isoetes* population, and instead co-occurred with species such as *Saururus cernuus*. This could indicate that the *I. louisianensis* population was experiencing greater interspecific competition with other plant species than the unusual *Isoetes*.

With increased light exposure, it is often seen that total leaf area will decrease in plants and vice versa with decreased light (Gurevitch et al. 2006). Competition between species in a given environment may also result in decreased growth and overall production due to a decrease in available resources (Gurevitch et al. 2006). These two factors—increased light and competition—in the *I. louisianensis* stream could have reduced the maximum sporophyll length in this population, while sporophyll lengths of the unusual *Isoetes* population may be greater due to a decrease in available light below the canopy and less interspecific competition within the stream.

Plant community assessment also revealed a relationship between *Carex louisianica* and the unusual *Isoetes*. At this site, *Carex louisianica* represented the major understory plant. The transition zone between *C. louisianica* and *Isoetes* was very abrupt, often with the separation of the two species only centimeters wide with *C. louisianica* occupying areas just above the level of persistent inundation and *Isoetes* occupying the more continuously inundated zones. This could be due either to competitive exclusion of *C. louisianica* by the unsual *Isoetes* population, or due to the increased environmental tolerance of the *Isoetes* to persistent inundation during parts of the year. Understanding of the relationship between these two species may be useful in understanding the success of the unusual *Isoetes* in this stream and in evaluating how *Isoetes* interact with other species in their environment. Increased observations coupled with greenhouse experiments are needed to assess this relationship.

Compaction in soils can interfere with root function in a variety of ways. Compaction may impede function by limiting the availability of water and gases necessary for a variety of processes (Brady and Weil 2010), especially respiration and uptake of mineral nutrition. Additionally, if layers are highly compacted, dry soil may exhibit strengths above 2000 kPa or 290 psi, which can resist root penetration (Brady and Weil 2010).

No compaction layers were found in either stream that were dense and shallow enough to interfere with root function of *Isoetes*. The presence of compaction layers in each stream, however, could be partially responsible for the persistence of water in the streams over several weeks following a rain event. Compacted layers are less permeable to water (Brady and Weil 2010). Thus, when water collects within streams possessing compacted soil layers it takes longer to percolate through the soil, resulting in the retention of that water over a longer period of time. Further investigations may reveal that these moderately shallow and highly compacted layers are essential in the promotion of suitable habitat for *Isoetes* in the region. If this is the case, then one potential method of conservation may be limiting the activities in areas with known *Isoetes* populations which effect the presence, strength, and depth of compacted layers in the soil. Activities such as logging, the use of heavy machinery and off-road vehicles, and construction affect compaction layers in soils, and thus these activities should be limited in areas with known *Isoetes* populations (Brady and Weil 2010).

Particle size analysis and subsequent comparison with the USDA soil triangle resulted in a loam textural class for all samples at both sites. This is contrary to the USGS soil survey for Wayne County. This could have been caused by the deposition and subsequent eluviation of different sediments through time or through differences between techniques used in soil analysis. The soil survey maps are also based on representative samples, not measurements from all possible localities. For soil samples in this study, soil horizons were mixed, likely resulting in a high fraction of fine particles from the organics-rich and depositional upper soil horizons. This would have increased the percentage of clays, with a diameter of <0.002 mm (U. S. Department of Agriculture, National Soil Survey Handbook), and reduced the overall percentage of the rest of the soil particle fraction. For the Wayne County, MS Soil Survey, a variety of techniques and characteristics were used in their assessment of the soil taxonomy (U. S. Department of Agriculture 2009). Selected samples were chosen for assessment, and then maps were constructed based on a combination of these samples and field observations.

The Department of Agriculture recognizes that only a select number of laboratory samples may be assessed when conducting the survey and that variation will occur, especially in areas of gradation (U. S. Department of Agriculture 2009). Differences in the textural classification between the assessment made in this study and by the Wayne County Soil Survey then are more likely due to differences in sampling and assessment than to actual differences in soil texture. Additionally, the functional difference between the textural class of loam and sandy loam is slight regarding plant growth (F. Heitmuller, pers. comm.). Thus, the soil particle size analysis and textural classification conducted in this study do not support the hypothesis that Wayne County populations of *Isoetes* inhabit streams with substantially different substrates than those generally observed for *I. louisianensis.*

The coupling of water level and temperature with observations of presence, absence, and senescence of aboveground features is significant because these data had not been collected before. The dynamic shifts in water level of the stream during the growing season was notable, with water level fluctuating by >0.5 m in only a few days. In some cases, there is evidence of rapid flooding events which quickly subsided, leaving a virtually dry streambed only a few days later.

A continuous assessment of the overall variability in water level had not been previously conducted. This is noteworthy, as these data resolve a question about senescence. Louisiana quillworts are known to senesce and lose their leaves in the summer, but whether that was due to heat/summer season or lack of water was uncertain. These data indicate the sporophyll senescence follows a time period with lack of water, supporting unpublished observations made by S. Leonard who observed that *Isoetes* populations in Louisiana are facultatively evergreen (U. S. Fish and Wildlife 1996). Louisiana individuals from the same population may eliminate aboveground structures if inhabited streams become dry but may retain those structures through the dry season if provided with water (U. S. Fish and Wildlife 1996). These observations and data collected during this study indicate that it is reduced water availability, not temperature or season, that is responsible for the senescence of sporophylls during parts of the year.

Additionally, the variability of this stream could be significant in the success of the Louisiana Quillwort in occupying these areas, as well as a reason for their scattered distribution over their range. The variability in the availability of water and the inconsistency in the water depth during the wet season likely conveys a great deal of stress to vegetation within the stream. The presence of a monoculture of *Isoetes* within the stream (as seen in Figure 17) could indicate that *Isoetes* is one of the few species that can tolerate these conditions. As previously stated, observations have been made of quillworts from Louisiana either retaining or eliminating aboveground structures depending on water availability during parts of the year (U. S. Fish and Wildlife Service 1996), which was supported by the water level data collected during this study. This ability to survive extended wet-dry cycles through the senescence of aboveground structures could provide an explanation for the dominance of the unusual *Isoetes* in the Wayne County stream. This may also be significant in explaining the distribution of *I. louisianensis*, as Mississippi populations may only be found in these ephemeral streams (U. S. Fish and Wildlife Service 1996). Further investigations, including greenhouse experiments, mesocosm studies, and continued observation at many sites are necessary to address this hypothesis.

Overall, the results of this study are inconclusive as to the species status of the unusual *Isoetes* population. Results such as the difference in megaspore size and ornamentation, sporophyll length, and inference of different light regime preference could indicate that the unusual *Isoetes* population represents an ecotype that may have developed in response to environmental pressures. If this is the case, then this population may be undergoing allopatric change as a result of its separation from other *I. louisianensis* populations. This would present a unique opportunity to monitor this population for the impact of environmental variation on evolution of *Isoetes*.

Alternatively, this population could be representative of the great capacity for phenotypic plasticity seen in *Isoetes* in response to environmental conditions (Gifford and Foster 1989). If this is the case, then the dramatic increase in the lengths of the sporophylls in the unusual *Isoetes* population could be attributed to light availability, increased nutrients, differences in soil and water chemistry, or decreased interspecific competition.

Additional field observations were made during this project that merit further study. The growth rate of the unusual *Isoetes* population appeared to be more rapid than that of the *I. louisianensis* population. Additionally, the number of individuals inhabiting the unusual *Isoetes* site vastly exceeded the number of individuals at the *I. louisianensis* site. These two differences could be the result of interspecific competition, light and nutrient availability, soil and water chemistry, or genetic differences between the two populations. Further studies could include analyses of leaf area index (LAI) of the two habitats to assess the differences in light availability, nutrient and pH analyses of soil and water chemistry to infer possible positive or negative effects on growth in the two populations, and continued observations of interspecific competition between *Isoetes* and other species. Greenhouse experiments could be conducted in these assessment areas as well to augment field studies.

Some difficulties were encountered while conducting this project which prevented the acquisition of some data. First, the barometric HOBOware water level logger was stolen from the field at some point during the last period of data collection. No external sources of barometric data could be found for the study area in Wayne County, MS, so the subaqueous data could not be matched with any barometric data to provide water level during that assessment period. While the data provided in this thesis are still sufficient for assessment of annual growth patterns and water depth, they are not as extensive as previously hoped.

Additionally, a permit from the U. S. Fish and Wildlife Service for the collection of fresh plant materials from both the unusual *Isoetes* and *I. louisianensis* site is still pending (permit identification #TE61573C-0). This permit is necessary for the collection of materials on public land, as *I. louisianensis* is an endangered plant. Once the permit is approved, plans have been made to collect material for analysis of internal leaf anatomy, chromosome number, variation in both the nuclear ITS region and the second intron of the LFY homolog, and morphological characters such as corm diameter and lobing and sporophyll number across several individuals in the same population. These assessments would provide greater insights into population variation and the status of the unusual *Isoetes* population as a potential species.

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