Analysis of morphological and anatomical characteristics of Isoetes using Isoetes tennesseensis

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Budke, J. M., R. J. Hickey & K. D. Heafner (Miami University, Department of Botany, 316 Pearson Hall, Oxford, OH 45056, U.S.A.; email: jessica.budke@uconn.edu). Analysis of morphological and anatomical characteristics of *Isoetes* using *Isoetes tennesseensis*. Brittonia 57: 167–182. 2005.—The three known populations of *Isoetes tennesseensis* were examined to document and analyze their morphological and anatomical characters. Characters examined included velum coverage, lacunae, leaf form and size, sporangial wall cells, and ligule and labium morphology. Three types of morphological patterns were found: stable, variable, and dimorphic. The discovery of dimorphism between mega- and microsporophylls for velum coverage, velum length, sporangium size, and ligule shape is of particular interest. This study presents a model for future work designed to complement current species descriptions and it establishes a baseline for morphological comparisons with other *Isoetes*.

Key words: *Isoetes*, morphological characters, lycopod, pteridophyte, dimorphism.

Introduction

Pfeiffer (1922) published the first and only worldwide monograph of *Isoetes* L. She proposed a new sectional system based on megaspore surface morphology. The strengths of this taxonomic system were its simplicity and utility; even herbarium specimens with little or no information regarding habitat could be readily classified to section. However, Pfeiffer stated that megaspore surface morphology can vary within species or within individuals, thus other characters should be used to definitively separate species. Despite her warnings, most *Isoetes* species described after 1922 have been distinguished primarily on this character. Hickey (1986a, 1986b, 1986c) strengthened Pfeiffer's cautions by showing that megaspore surface morphology is subject to convergence as well as within-species, within-plant, and within-spore variation. In spite of such variation, megaspores continue to be the dominant source of taxonomic characters, whereas other sources of morphological variation remain largely neglected.

Only a few papers on North American *Isoetes* have focused on comparative morphological variation. Two notable examples are the studies by Matthews and Murdy (1969) and Kott and Britton (1985). They concluded that most vegetative characters are environmentally plastic, developmentally dependent, or invariant. Specifically, Kott and Britton (1985) strongly supported species identification in *Isoetes* based primarily on spore characteristics; whereas Matthews and Murdy (1969) concluded that characteristics examined in their study were continuously variable across populations. Therefore, these papers may have discouraged analyses of morphological characters

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such as velum coverage, sporangial pigmentation, leaf form and size, and ligule and labium morphology in *Isoetes* research. Consequently, authors rarely stress their taxonomic usefulness. In the North American literature, Boom (1979) and Reed (1965) stand apart in maintaining an emphasis on vegetative morphology and ecological data in distinguishing taxa. Their data, and those of Hickey (1986a), suggest that intensive analyses of species morphologies will yield stable taxonomic characters.

There are several examples of *Isoetes* characterizations from outside North America which thoroughly integrate morphological characters. A good example is the work of Rauh and Falk (1959a, 1959b) on *Stylites gemmifera* Rauh and Falk (=*I. andicola* (Amstutz) L. D. Gómez). In 159 pages, they fully describe the anatomy, morphology, development, and ecology of this Andean species. Hall (1971) demonstrated the utility of a number of novel anatomical features in delineating *Isoetes* of Ghana by documenting variability among characters. Additionally, Prada (1979) examined leaf anatomical characteristics of *Isoetes* species from Spain including *I. lacustris* and *I. echinospora*, species that are both present in North America. Well-documented, infraspecific variability for selected anatomical and spore characters has also been published for the African *I. melanotheca* Alston (Pitot, 1959) and *I. jaegeri* Pitot (Pitot, 1968). Recently, Takamiya et al. (1997) utilized morphological characters to differentiate among both divergent and closely related species in Japan. Similarly, Hickey (1986a, 1986b, 1986c) and Small and Hickey (2001) have shown that morphology can be successfully used to differentiate taxa in South America. Despite these studies, neither the methodologies nor the morphological characters espoused have been used to circumscribe North American *Isoetes* species with much regularity.

During the 20th century the number of recognized species in *Isoetes* has increased from 64 (Pfeiffer, 1922) to an estimated 350 (Hickey et al., 2003). In North America the numbers have increased from 19 (Pfeiffer, 1922) to 23 (Taylor et al., 1993), with several more added subsequent to 1993. Typically, these new species are distinguished based on cytology and megaspore ornamentation. With species of different ploidy levels, cytology is highly informative. Unfortunately, cytological data are not available for most collections. In order to thoroughly embrace the variability within a taxon, the organism's entire morphology needs to be examined and documented. For *Isoetes*, this includes the microphylls, sporangia, corm, ligules, and labia. Research in our laboratory indicates that such morphological characters are of value in defining *Isoetes* taxa and can provide an important complement to published species descriptions.

The goal of this study is to document and analyze morphological and anatomical characters using *Isoetes tennesseensis* Luebke and Budke as a model against which other currently recognized *Isoetes* species can be compared. This recently described species is the only known *Isoetes* octaploid ($2n = 88$) in North America. Its unique chromosome number provided a definitive method of species confirmation for the populations used in our study.

Methods

During the summer of 2001, living material was collected from the three known, extant populations of *Isoetes tennesseensis* all of which are in the Hiwassee River, Polk County, Tennessee (Table I). Identities of specimens from all three populations were confirmed cytologically following procedures modified from Luebke and Budke (2003). Only confirmed octaploids were used in this study.

Morphological characters were measured using either a flexible ruler, a Wild M3C dissecting microscope, or an Olympus BHS compound microscope. For both microscopes an ocular micrometer was used. Specifically, leaf width was measured at the mid-subula length and ala width was measured at the widest point above the sporangium. Velum length was measured as the distance from the apex of the sporangium, down the middle, to the edge of the velum. The velum coverage of the sporangium was then calculated as a percentage of sporan-

TABLE I
POPULATIONS STUDIED OF *Isoetes tennesseensis*. ALL WERE COLLECTED FROM THE HIWASSEE RIVER, POLK CO., TENNESSEE, U.S.A

Population	Numbers of individuals collected and examined	Voucher Data
1	9	24 June 2001, *J. Budke, K. Heafner, E. Lickey, P. Cox,* & *J. Shaw 1* (MU)
2	12	13 July 2001, *J. Budke, K. Heafner, E. Lickey,* & *K. Gustafson 8* (MIL, MU)
3	9	15 July 2001, *J. Budke, K. Heafner, E. Lickey* & *K. Gustafson 17* (MIL, MU; type collection)

gium length. Microspores were mounted onto glass slides in Hoyer's medium (Willey, 1971), and their lengths measured. The diameters of dry megaspores were measured using the dissecting microscope. To examine sporangial wall cells, a portion of the exposed adaxial wall was dissected and mounted in Hoyer's medium, and cell lengths were measured using the compound microscope. All specimens examined had sporangia that were fully mature. The standard by which we determined this was spore maturity and completeness of ornamentation.

Specimens for histological analyses were fixed in FAA (formalin-aceto-alcohol), dehydrated in a graduated tertiary butyl alcohol series, and embedded in paraffin (Johansen, 1940). Specimens were sectioned using a rotary microtome at a thickness of 12 μm, fixed to the slide using Mayer's adhesive (Johansen, 1940) and stained with a 0.2% solution of toluidine blue. All specimens were examined using the compound microscope. Leaf cross sections were made from the middle of the leaf subula.

For scanning electron microscopy (SEM), megaspores and microspores were air-dried, whereas leaf material was fixed in FAA, dehydrated in an ethanol series to 100%, and critical point dried. All samples were mounted onto SEM stubs using double-sided tape and sputter-coated with ca. 21 nm of gold-palladium. The SEM analyses were carried out using a Jeol T-200 (Tokyo, Japan) scanning electron microscope.

Standard descriptive statistics, including mean, median, and standard deviation were calculated for each character using MINITAB® 13.32 (Minitab Inc., 2000). Dimorphic characters and variation among populations were assessed with two-sample *t*-tests using MINITAB.

Results

The corms of *Isoetes tennesseensis* were consistently bilobed, and in our collections had a maximum size of 23.0 mm in height, 21.0 mm in diam. Corms as small as 1.0 mm in height and 5.0 mm across were also collected. Dichotomous roots, typical for the genus, were produced along the circumbasal fossa.

Leaf length in *Isoetes tennesseensis* was quite variable with mean leaf lengths from 78.2 mm to 124.1 mm across the populations. Standard deviations for this character across the three populations were consistent, but high in all cases (Table II). The mean leaf length for the species was 104.3 mm. Alae lengths showed similar interpopulational variation (Table II). Ala to leaf length ratios were calculated for all specimens examined (Table II). No scales or phyllopodia were observed in this species.

In cross section, the mid-subulae of *Isoetes tennesseensis* were most commonly half-terete with flattened adaxial and rounded abaxial sides, some were weakly trapezoidal with the adaxial and abaxial sides flattened and parallel, while a few were terete in outline (Fig. 1A; Table II). The adaxial surface was broader than the abaxial, an asymmetry paralleled internally by statistically significant differences (in all cases $p < 0.001$) in the widths and depths of the adaxial and abaxial lacunae (Table III). No statistically significant within-character differences were found across populations. The lacunae were separated from each other by parenchymatous cell layers that were consistently two to three cell layers thick

TABLE II
LEAF CHARACTERS MEASURED FOR *Isoetes tennesseensis*. POPULATION NUMBERS REFER TO THOSE LISTED IN TABLE I

Character	Pop. 1	Pop. 2	Pop. 3	Pop. 1–3 combined
Leaf length, mm				
Mean	120.1	78.2	124.1	104.3
SD [N]	21.02 [16]	23.02 [25]	25.21 [20]	31.79 [61]
Range	82–148	42–118	91–159	42–159
Ala length, mm				
Mean	30.7	17.5	30.6	25.2
SD [N]	6.25 [16]	6.96 [25]	8.08 [20]	9.62 [61]
Range	18–39	6–34	18–45	6–45
Ala width, mm				
Mean	3.4	2.6	3.1	3.0
SD [N]	0.86 [16]	0.60 [25]	0.82 [20]	0.80 [61]
Range	1.7–4.8	1.2–3.3	1.9–4.3	1.2–4.8
Ala/leaf length				
Mean	0.257	0.226	0.245	0.238
SD [N]	0.045 [16]	0.045 [25]	0.029 [20]	0.042 [61]
Range	0.190–0.352	0.097–0.312	0.190–0.310	0.097–0.352
Subula (leaf) width, mm				
Mean	2.00	1.47	1.86	1.74
SD [N]	0.592 [16]	0.314 [25]	0.262 [20]	0.449 [61]
Range	1.2–3.8	1.0–2.2	1.5–2.4	1.0–3.8
Subula (leaf) W/D ratio (serial section data)				
Mean	1.28	1.31	1.33	1.31
SD [N]	0.265 [20]	0.230 [25]	0.266 [21]	0.244 [66]
Range	0.91–1.88	0.80–1.84	0.83–1.73	0.80–1.88

and from the outside environment by the epidermis as well as three to four cell layers of parenchyma. There was no evidence of either stoma or peripheral fibrous bundles. Internally, the leaves of *Isoetes tennesseensis* had a central veinal region (Fig. 1A, B) with either one (64 samples) or two (two samples) small intrastelar canals. These canals were elliptic in sectional view (Table IV). Secondary wall thickenings were not present within the veinal canals.

The lacunae of the leaves were traversed by diaphragms of stellate parenchyma (Fig. 1C–E). In *Isoetes tennesseensis*, these diaphragms were consistently two to three cells in thickness. The cells were flattened in a plane parallel to the diaphragm and each had five to seven radial arms that abut similar arms of adjacent cells. In longitudinal view, the main bodies of the cells were vertically aligned and tightly appressed. Small hair-like protuberances covered the cell surfaces. At the junctures of the radial arms, the protuberances were larger, clavate and formed paired whorls, with one whorl per cell (Fig. 1D, E).

Ligules (Fig. 2A–K) in *Isoetes tennesseensis* degrade as leaves approach full maturity. As a result, mature, intact ligules were difficult to obtain and characterize. Most ligules associated with megasporophylls were depressed-ovate whereas those of microsporophylls were deltate (Table V). Ligule ratios between mega- and microsporophylls were significantly different ($p = 0.001$). In all cases the ligules showed a slightly auriculate base.

The labia of *Isoetes tennesseensis* were generally spatulate, often with a forked or bilabiate apex (Fig. 2A–K). Lengths, widths and length to width ratios were determined for the labia (Table VI). No significant differences in the lengths ($p = 0.344$), widths ($p = 0.846$), or ratios ($p = 0.724$) were found between labia of mega- and microsporophylls.

The sporangia of *Isoetes tennesseensis* were basal and broadly elliptic. Noticeable

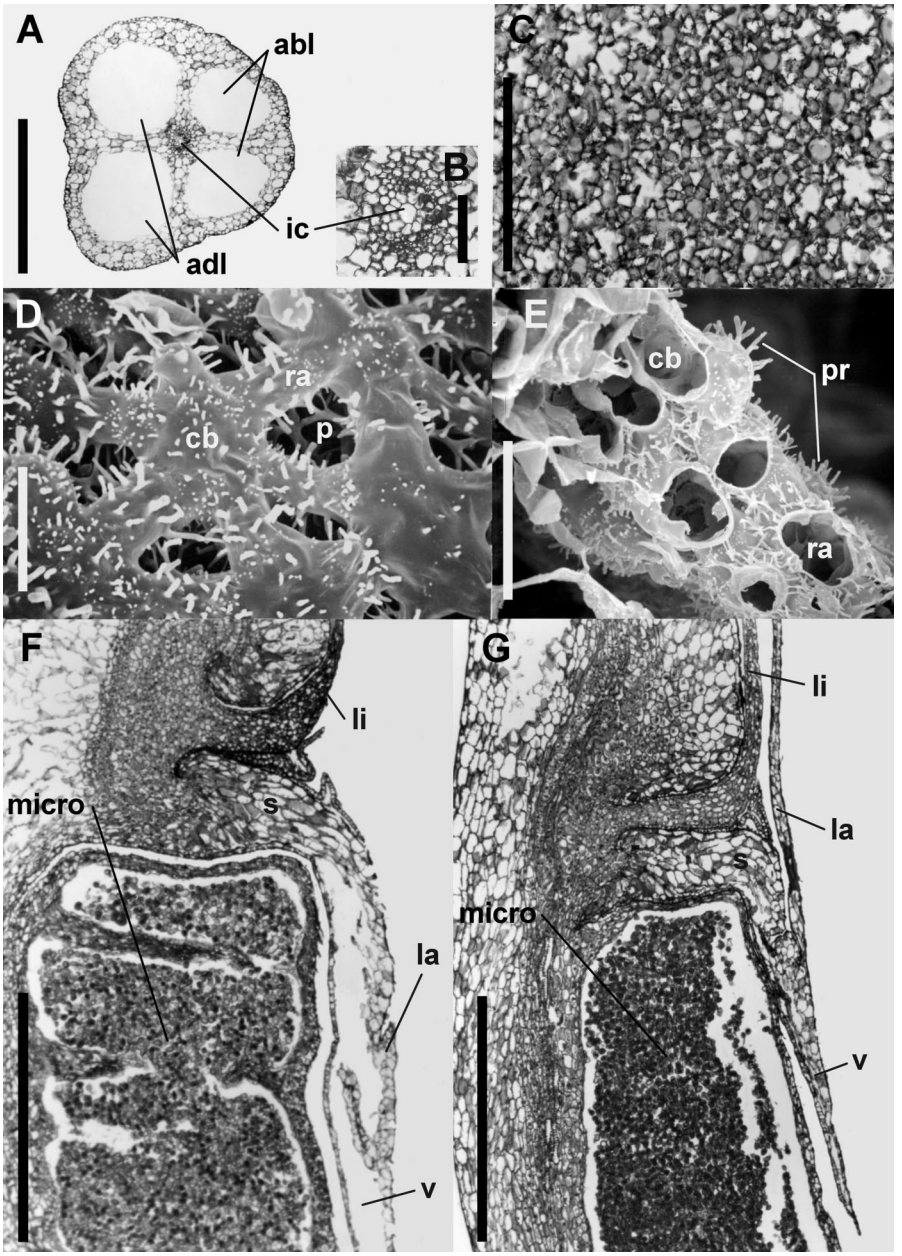

FIG. 1. Morphological and anatomical features of *Isoetes tennesseensis*. **A.** Cross section of a microphyll, showing the difference in size between the abaxial and adaxial lacunae. **B.** Intrastelar canal. **C.** Cross-sectional view of the stellate diaphragm cells, triangle shaped pores, and protuberances. **D.** SEM photograph of the stellate diaphragm cells from a cross-sectional cut. **E.** SEM photograph of longitudinal section of a leaf showing the stacked stellate diaphragm cells. **F.** Median longitudinal section of microsporangia showing the velum where the labium is just starting to form. **G.** Median longitudinal section of microsporangia showing the velum where the labium is fully developed. Specimen A, B and G (*Budke et al. 17*); C and F (*Budke et al. 1*); D and E (*Budke et al. 8*). Abbreviations: abl = abaxial lacunae; adl = adaxial lacunae; cb = cell body; ic = intrastelar canal; li = ligule; la = labium; micro = microsporangium; p = pores; pr = protuberances; ra = radial arm; s = sella; v = velum. Scale bars in A, F and G = 1 mm; scale bar in B = 100 μm; scale bar in C = 200 μm; scale bar in D = 30 μm; scale bar in E = 50 μm.

TABLE III
LACUNAE CHARACTERS FROM LEAF CROSS SECTIONS OF *Isoetes tennesseensis*. POPULATION NUMBERS REFER TO THOSE LISTED IN TABLE I

Character	Pop. 1	Pop. 2	Pop. 3	Pop. 1–3 combined
Abaxial lacuna width, μm				
Mean	632	571	686	625
SD [N]	110.2 [40]	122.5 [52]	140.6 [42]	133.1 [134]
Range	450–850	380–950	450–1000	380–1000
Abaxial lacuna depth, μm				
Mean	591	509	606	564
SD [N]	125.0 [40]	94.8 [52]	92.4 [42]	112.4 [134]
Range	350–900	300–730	430–750	300–900
Abaxial lacuna W/D ratio				
Mean	1.11	1.15	1.16	1.14
SD [N]	0.303 [40]	0.297 [52]	0.288 [42]	0.294 [134]
Range	0.64–1.88	0.66–1.93	0.71–1.73	0.54–1.93
Adaxial lacuna width, μm				
Mean	796	735	890	802
SD [N]	181.9 [40]	172.0 [52]	231.5 [42]	204.6 [134]
Range	480–1130	430–1180	480–1380	430–1380
Adaxial lacuna depth, μm				
Mean	650	591	641	625
SD [N]	128.0 [40]	125.3 [52]	117.3 [42]	125.6 [134]
Range	400–900	350–940	410–930	350–940
Adaxial lacuna W/D ratio				
Mean	1.25	1.28	1.44	1.32
SD [N]	0.327 [40]	0.333 [52]	0.459 [42]	0.382 [134]
Range	0.80–2.28	0.68–2.16	0.58–2.51	0.58–2.51

differences exist between the absolute sizes of megasporangia and microsporangia. Megasporangia averaged 5.2 × 4.1 mm whereas the microsporangia averaged 3.6 × 2.8 mm. The sporangia differed significantly in both length ($p = 0.005$) and width ($p < 0.001$), but not in length to width ratio ($p = 0.268$). These data, along with separate population statistics, are provided in Table VII. Cross sections of sporangia showed that the placentae were flat to convex based on the depth to width ratio.

TABLE IV
INTRASTELAR CANAL CHARACTERS OF *Isoetes tennesseensis*. POPULATION NUMBERS REFER TO THOSE LISTED IN TABLE I

Character	Pop. 1	Pop. 2	Pop. 3	Pop. 1–3 combined
Intrastelar canal width, μm				
Mean	38.3	30.8	37.3	35.0
SD [N]	7.67 [19]	5.56 [26]	6.69 [21]	7.41 [66]
Range	22.5–55.0	18.8–41.3	27.5–52.5	18.8–55.0
Intrastelar canal depth, μm				
Mean	33.0	26.5	29.3	29.3
SD [N]	6.03 [19]	6.05 [26]	5.09 [26]	6.27 [66]
Range	25.0–50.0	13.8–37.5	20.0–42.0	13.8–50.0
Intrastelar canal W/D ratio				
Mean	1.18	1.22	1.3	1.24
SD [N]	0.227 [19]	0.313 [26]	0.391 [26]	0.321 [66]
Range	0.67–1.63	0.70–1.86	0.71–2.21	0.67–2.21

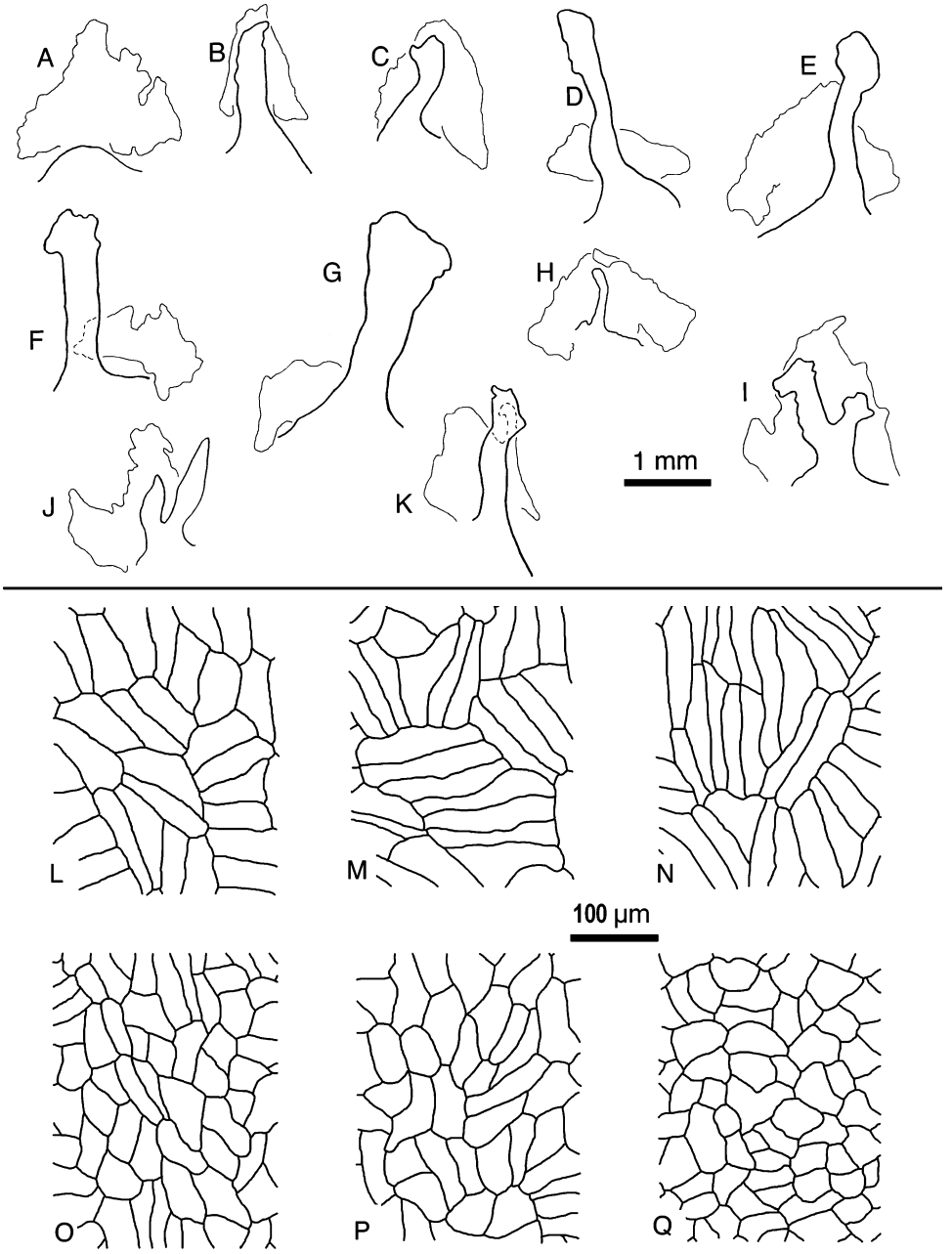

FIG. 2. Leaf base characters of *Isoetes tennesseensis*. **A–K.** Variation in ligule and labium shape and size. Labia in the foreground, ligules in the background. Most ligules partially or extensively degraded; **A, C, E, H.** Have the least amount of ligule degradation. **I, J.** Note bifid labia. **L–Q.** Variation in sporangial cell wall shape and size; **L–N.** Wall cells of megasporangia. **O–Q.** Wall cells of microsporangia. All drawings to scale. Specimens A–D, F, G, L, M, O and P (*Budke et al. 17*); H–J (*Budke et al. 1*); K, N and Q (*Budke et al. 8*).

TABLE V
LIGULE CHARACTERS FOR *Isoetes tennesseensis*. POPULATION NUMBERS REFER TO THOSE LISTED IN TABLE I

Character	Pop. 1	Pop. 2	Pop. 3	Pop. 1–3 combined
Ligule length, mm				
Mean	1.46	0.88	1.57	1.34
SD [N]	0.549 [8]	0.503 [6]	0.485 [8]	0.570 [22]
Range	0.57–2.30	0.31–1.60	0.65–2.30	0.31–2.30
Ligule width, mm				
Mean	1.61	1.05	1.85	1.55
SD [N]	0.439 [8]	0.210 [6]	0.463 [8]	0.504 [22]
Range	0.80–2.10	0.81–1.30	1.10–2.50	0.80–2.50
Ligule L/W ratio				
Mean	1.05	0.82	1.88	0.92
SD [N]	0.789 [8]	0.379 [6]	0.296 [8]	0.530 [22]
Range	0.44–2.88	0.31–1.23	0.41–1.27	0.31–2.88
Ligule length, mm—megasporophylls				
Mean	1.29	0.37	1.29	1.11
SD [N]	0.638 [4]	0.085 [2]	0.433 [4]	0.591 [10]
Range	0.57–2.10	0.31–0.43	0.65–1.60	0.31–2.10
Ligule width, mm—megasporohylls				
Mean	1.75	0.94	1.98	1.68
SD [N]	0.332 [4]	0.092 [2]	0.287 [4]	0.479 [10]
Range	1.30–2.00	0.87–1.00	1.60–2.30	0.87–2.30
Ligule L/W ratio—megasporophylls				
Mean	0.73	0.40	0.64	0.63
SD [N]	0.353 [4]	0.130 [2]	0.167 [4]	0.262 [10]
Range	0.44–1.24	0.31–0.49	0.41–0.80	0.31–1.24
Ligule length, mm—microsporophylls				
Mean	NA	1.13	1.85	1.49
SD [N]	NA	0.397 [4]	0.387 [4]	0.526 [8]
Range	NA	0.69–1.60	1.40–2.30	0.69–2.30
Ligule width, mm—microsporophylls				
Mean	NA	1.10	1.73	1.41
SD [N]	NA	0.241 [4]	0.613 [4]	0.545 [8]
Range	NA	0.81–1.30	1.10–2.50	0.81–2.50
Ligule L/W ratio—microsporophylls				
Mean	NA	1.03	1.12	1.07
SD [N]	NA	0.246 [4]	0.160 [4]	0.198 [8]
Range	NA	0.69–1.23	0.92–1.27	0.69–1.27

Sporangial wall cells are uniformly thin and the cells generally lack pigment. Occasionally cells with dark cellular contents were noted. These were observed in only one out of nine sporangial mounts. Epidermal cells of megasporangia are elongate (Fig. 2L–N; Table VIII). Cells in a similar position on microsporangia are more isodiametric (Fig. 2O–Q; Table VIII). Cell length-to-width ratios were found to be significantly different between mega- and microsporangia ($p < 0.001$).

Velum coverage, measured as percent vertical coverage of the sporangium, ranged from 0 to 59% with significant differences between mega- and microsporophylls ($p < 0.001$). However, as indicated by the high standard deviations, the ranges for the two types of sporophylls overlap broadly (Table VII). Mean velum length also resulted in a significant difference between mega- and microsporophylls ($p = 0.004$).

Megaspores of *Isoetes tennesseensis* were reticulate distally with occasional regions of open muri that approached a cristate condition. Proximally, megaspores

TABLE VI
LABIUM CHARACTERS FOR *Isoetes tennesseensis*. POPULATION NUMBERS REFER TO THOSE LISTED IN TABLE I

Character	Pop. 1	Pop. 2	Pop. 3	Pop. 1–3 combined
Labium length, mm				
Mean	1.13	1.49	1.83	1.44
SD [N]	0.473 [11]	0.467 [8]	0.345 [8]	0.514 [27]
Range	0.36–1.90	0.74–2.30	1.40–2.40	0.36–2.40
Labium width, mm				
Mean	0.40	0.42	0.63	0.47
SD [N]	0.229 [11]	0.220 [8]	0.294 [8]	0.259 [27]
Range	0.12–0.77	0.23–0.83	0.32–1.20	0.12–1.20
Labium L/W ratio				
Mean	3.3	4.1	3.6	3.7
SD [N]	1.63 [11]	1.65 [8]	1.92 [8]	1.69 [27]
Range	1.8–6.8	2.3–7.2	1.5–7.5	1.5–7.5
Labium length, mm—megasporophylls				
Mean	1.27	1.16	1.92	1.54
SD [N]	0.565 [3]	0.314 [4]	0.354 [6]	0.517 [13]
Range	0.81–1.90	0.74–1.40	1.40–2.40	0.74–2.40
Labium width, mm—megasporophylls				
Mean	0.49	0.31	0.70	0.53
SD [N]	0.325 [3]	0.105 [4]	0.304 [6]	0.302 [13]
Range	0.12–0.74	0.23–0.46	0.32–1.20	0.12–1.20
Labium L/W ratio—megasporophylls				
Mean	3.7	4.0	3.4	3.7
SD [N]	2.65 [3]	1.19 [4]	2.24 [6]	1.92 [13]
Range	1.8–6.8	2.8–5.2	1.5–7.5	1.5–7.5
Labium length, mm—microsporophylls				
Mean	NA	1.87	1.55	1.74
SD [N]	NA	0.404 [3]	0.071 [2]	0.336 [5]
Range	NA	1.50–2.30	1.50–1.60	1.50–2.30
Labium width, mm—microsporophylls				
Mean	NA	0.58	0.40	0.50
SD [N]	NA	0.297 [3]	0.021 [2]	0.233 [5]
Range	NA	0.25–0.83	0.38–0.41	0.25–0.83
Labium L/W ratio—microsporophylls				
Mean	NA	4.1	3.9	4.0
SD [N]	NA	2.70 [3]	0.39[2]	1.92 [5]
Range	NA	2.3–7.2	3.7–4.2	2.3–7.2

were echinate to echinate-cristate. Also, there was a narrow girdle in which the reticulations of the distal hemisphere gradually diminished to form weakly developed papillae. Proximal and equatorial ridges were broad, bold, and generally devoid of ornamentation (e.g., Luebke & Budke, 2003). Megaspores ranged in diam. from 625 μm to 1025 μm, with a mean value of 801 μm. Microspores averaged 42.36 μm in length and were consistently laevigate (Table VII).

Discussion

Based on our analyses of both morphological and anatomical characters of *Isoetes tennesseensis*, it was determined that the characters can be characterized as: stable, variable, or dimorphic. Stable characters showed relatively little variation, whereas variable characters were defined as those whose morphology ranged widely from the mean. Dimorphic characters were defined as statistically significant differences be-

TABLE VII
SPORANGIUM, VELUM, AND SPORE CHARACTERS FOR *Isoetes tennesseensis*. POPULATION NUMBERS REFER TO THOSE LISTED IN TABLE I

Character	Pop. 1	Pop. 2	Pop. 3	Pop. 1–3 combined
Sporangium length, mm				
Mean	3.9	3.6	5.3	4.3
SD [N]	1.16 [5]	1.66 [17]	1.75 [16]	1.81 [38]
Range	2.5–5.2	1.1–6.7	2.7–8.5	1.1–8.5
Sporangium width, mm				
Mean	3.3	2.9	4.0	3.4
SD [N]	0.92 [5]	1.22 [17]	1.00 [16]	1.20 [38]
Range	2.0–4.3	1.2–5.4	2.4–5.7	1.2–5.7
Sporangium L/W ratio				
Mean	1.20	1.26	1.32	1.28
SD [N]	0.216 [5]	0.190 [17]	0.198 [16]	0.195 [38]
Range	0.89–1.50	0.79–1.59	0.93–1.74	0.89–1.74
Megasporangium length, mm				
Mean	3.9	5.2	5.9	5.2
SD [N]	1.16 [5]	1.60 [4]	1.75 [10]	1.71 [19]
Range	2.5–5.2	3.4–6.7	3.6–8.5	2.5–8.5
Megasporangium width, mm				
Mean	3.3	4.5	4.4	4.1
SD [N]	0.92 [5]	0.96 [4]	0.86 [10]	0.98 [19]
Range	2.0–4.3	3.5–5.4	3.3–5.7	2.0–5.7
Megasporangium L/W ratio				
Mean	1.12	1.13	1.32	1.25
SD [N]	0.216 [5]	0.132 [4]	0.204 [10]	0.200 [19]
Range	0.89–1.50	0.97–1.29	1.00–1.74	0.89–1.74
Microsporangium length, mm				
Mean	NA	3.3	4.4	3.6
SD [N]	NA	1.39 [13]	1.45 [6]	1.47 [19]
Range	NA	1.1–5.6	2.7–6.3	1.1–6.3
Microsporangium width, mm				
Mean	NA	2.5	3.3	2.8
SD [N]	NA	0.82 [13]	0.92 [6]	0.92 [19]
Range	NA	1.2–4.1	2.4–4.6	1.2–4.6
Microsporangium L/W ratio				
Mean	NA	1.28	1.32	1.32
SD [N]	NA	0.232 [13]	0.205 [6]	0.187 [19]
Range	NA	0.79–1.59	0.93–1.55	0.93–1.59
% Velum coverage—megasporophylls				
Mean	7.8	22.3	14.0	14.1
SD [N]	9.96 [5]	10.2 [4]	8.9 [9]	10.3 [18]
Range	0–24	12–36	4–24	0–36
% Velum coverage—microsporophylls				
Mean	NA	39.5	28.9	35.4
SD [N]	NA	10.2 [11]	13.2 [7]	12.3 [18]
Range	NA	26–59	13–50	13–59
Velum length—megasporophylls, mm				
Mean	0.39	1.11	0.83	0.77
SD [N]	0.52 [5]	0.49 [4]	0.63 [9]	0.60 [18]
Range	0.0–1.25	0.61–1.54	0.21–1.87	0.0–1.87
Velum length—microsporophylls, mm				
Mean	NA	1.34	1.44	1.38
SD [N]	NA	0.37 [11]	0.84 [7]	0.58 [18]
Range	NA	0.47–1.74	0.35–2.39	0.35–2.39

TABLE VII
CONTINUED

Character	Pop. 1	Pop. 2	Pop. 3	Pop. 1–3 combined
Megaspore diameter, μm				
Mean	783	799	815	801
SD [N]	67.0 [20]	95.7 [30]	95.0 [30]	89.9 [80]
Range	650–875	625–1025	625–1025	625–1025
Microspore length, μm				
Mean	NA	41.43	44.22	42.36
SD [N]	NA	2.12 [100]	2.57 [50]	2.63 [150]
Range	NA	37.49–45.65	39.12–48.90	37.49–48.90

tween mega- and microsporophyll associated characters.

STABLE CHARACTERS

Corm lobing has had a long history of use in *Isoetes* taxonomy. It was considered by early workers (Braun, 1864; Weber, 1922) to be a reliable and consistent character and even recent workers (Kott & Britton, 1985) have suggested that it is generally stable, although of reduced taxonomic utility in northeastern North America because of its interspecific uniformity. Nonetheless, the genus does show considerable variation and it is important to note the stability of this character in *I. tennesseensis*.

Lacunar wall thickness, uniform in *Isoetes tennesseensis*, is potentially correlated with habitat and the presence or absence of stoma. In species with stoma, walls vary in thickness from several cells thick down to just the epidermis in regions adjacent to the guard cells. In species lacking stoma, wall thickness appears to be consistent within species but may vary among taxa. *Isoetes savatieri* Franchet, for example, has robust lacunar walls, which add significantly to the rigidity of the leaf. Variation in lacunar wall thickness within species appears to be rare, but has been reported for *I. wormaldii* Sim (Duthie, 1929).

Hall (1971) and Takamiya et al. (1997) showed that veinal (intrastelar) canal number varied among *Isoetes* species and can serve as a good taxonomic character. These canals have long been a source of interest to morphologists due to the repeated reports of isolated secondary wall thickenings located within the canals. These thickenings have been interpreted to be remnants of protoxylem tracheids and the canals have often been compared to protoxylem lacunae of other taxa (e.g., *Equisetum*; Bierhorst, 1958, 1971). Recently, Romeo et al. (2000) showed that the cells surrounding these lacunae are endodermal in nature and have a well-developed casparian strip, in accord with earlier observations of Scott and Hill (1900) and Williams (1943). In *I. tennesseensis* and in species with multiple veinal canals, the canals are usually each bound by a distinct endodermal layer, which excludes the majority of tracheary elements of the leaf. One published exception appears to be *I. sinensis* T. C. Palmer var. *sinensis* in which adjacent intrastelar canals share a short common endodermal region. West and Takeda (1914) commented on the cells surrounding these canals and suggested that they do not represent a true endodermis and coined the term pseudo-endodermis in light of the anomalous position and form of the cells. Our data show that veinal canal number is stable within *I. tennesseensis*, showing only rare variation in number (Fig. 1A, B; Table IV). The condition of this character in other North American species is unknown.

Historically, little attention has been paid to the nature and development of the diaphragms of the lacunae, other than to note their presence. Hall (1971) described these translacunar diaphragms as being, ''two or three cells in thickness, perforated by pores which are triangular, or circular, to elliptical.'' In *Isoetes tennesseensis*, the air spaces between the diaphragm cells are typically triangular in surface view (Fig. 1C, D). Ad-

TABLE VIII
SPORANGIAL WALL CELL CHARACTERS FOR *Isoetes tennesseensis*. POPULATION NUMBERS REFER TO THOSE LISTED IN TABLE I

Character	Pop. 1	Pop. 2	Pop. 3	Pop. 1–3 combined
Megasporangium cell length, μm				
Mean	NA	138.8	117.5	128.1
SD [N]	NA	47.25 [12]	26.67 [12]	39.06 [24]
Range	NA	65.0–240.0	75.0–160.0	65.0–24.0
Megasporangium cell width, μm				
Mean	NA	27.9	28.8	28.3
SD [N]	NA	7.22 [12]	9.08 [12]	8.03 [24]
Range	NA	20.0–40.0	20.0–45.0	20.0–45.0
Megasporangium cell L/W ratio				
Mean	NA	5.42	4.57	5.00
SD [N]	NA	2.48 [12]	1.86 [24]	2.18 [24]
Range	NA	1.86–9.25	2.11–6.75	1.86–9.25
Microsporangium cell length, μm				
Mean	NA	56.3	72.5	64.4
SD [N]	NA	13.51 [12]	16.86 [12]	17.09 [24]
Range	NA	40.0–85.0	40.0–95.0	40.0–95.0
Microsporangium cell width, μm				
Mean	NA	35.4	28.3	31.9
SD [N]	NA	6.89 [12]	9.13 [12]	8.70 [24]
Range	NA	25.0–45.0	15.0–45.0	15.0–45.0
Microsporangium cell L/W ratio				
Mean	NA	1.63	2.91	2.27
SD [N]	NA	0.42 [12]	1.47 [12]	1.25 [24]
Range	NA	1.00–2.40	1.38–6.33	1.00–6.33

ditionally, the diaphragms of *I. tennesseensis* are composed of two to three layers of stellate parenchyma cells. The cells of these layers are distinctly stacked one upon the other in vertical rows (Fig. 1E). This arrangement of diaphragm cells has not been noted in other species, perhaps because most analyses of the diaphragms have concentrated on transverse rather than longitudinal views. The surface of the diaphragm cells have been observed as having spines (protuberances) on the surface in African species (*I. abyssinica*, and *I. nigritiana* A. Br. ex Kuhn) by Hall (1971) and in Australian species (*I. coromandelina*, *I. drummondii* A. Br., and *I. muelleri* A. Br.) by Marsden (1979). *Isoetes tennesseensis* has numerous protuberances across the surfaces of the stellate cells of the diaphragms, but these are enlarged and much more noticeable near the juncture of adjacent arms (Fig. 1D). At that point the protuberances are arranged in two encircling whorls, one whorl per arm. Marsden (1979) also noted that these were well developed near the junctures of the stellate arms of the diaphragm cells in Australian species. Additionally these protuberances have been found in several *Isoetes* species of the Iberian Peninsula, including the circumboreal *I. lacustris* (Prada, 1979; Prada & Rolleri, 2003).

Hall (1971) used epidermal papillae, internal (lacunar) hairs, and subepidermal tubules to differentiate among *Isoetes* species in Ghana. Internal hairs not associated with the diaphragm cells but projecting into the lacunae have been noted in the African *I. abyssinica* Chiovenda and *I. melanotheca* Alston (Pitot, 1959) and in the Indian *I. coromandelina* L.f., *I. indica* Pant and Srivastava, and *I. pantii* Goswami and Arya (Bhambie, 1963). Epidermal papillae are known from *I. tenuifolia* A. C. Jermy (Hall, 1971), and these as well as epidermal striations of questionable similarity have been found in some Australasian species (Marsden, 1979). Epidermal papillae, internal

hairs, and subepidermal tubules are lacking in *I. tennesseensis*.

A sporangial character mentioned frequently in the *Isoetes* literature is pigmentation. Unfortunately, few authors have discriminated among the characters that are routinely subsumed under this general category. Specifically, there appear to be two potential sources of sporangial coloration: cell wall pigmentation and lumen pigmentation. In the former, the walls of the exposed (not covered by vela) epidermal cells may be discolored by a non-lignin pigment (Hall, 1971). A second form of pigmentation appears to be due to discoloration within the lumen itself. Although this has not been definitely shown to be vacuolar, it would appear to be so. The limited spotting seen in *I. tennesseensis* appears to be of this latter type. In addition to pigmentation, sporangial epidermal cells may show variation in cell wall thickness. The walls in *I. tennesseensis* are uniformly thin. Previous descriptions of sporangia pigmentation in American *Isoetes* have not been adequately discussed relative to pigment position or wall thickness to make comparisons with other species.

Megaspore surface morphology is uniform among populations and among individuals within populations. Individual spores show variation in surface ornamentation between proximal and distal surfaces; a phenomenon that is common in *Isoetes*. The species is identifiable on the basis of megaspore morphology depending on the experience of the worker. The consistently laevigate microspores are characteristic of most species with predominately reticulate megaspores.

Variable Characters

The velum is of interest in this species due to its complex structure. In *Isoetes tennesseensis,* it is composed of velum proper, sella and, in some cases, labium tissue (Fig. 1F, G). No mention of similar variation appears in the literature, although the origin of the labium from an apparently proximal side of the sella in *I. nigritiana* is of interest and deserves additional study. Complex vela of different forms have been reported. Palmer (1932), for example, describes a complex velum in *I. lechleri* Mett. var. *anomala* Palmer (=*I. karstenii* A. Br. var. *anomala* (Palmer) Small & Hickey) composed of a thickened outer layer and an inner delicate layer. Palmer notes that these two layers were ''not always coincident.'' Examinations in our laboratory of the type material (*Rimbach 171,* US) of this taxon have failed to find a true double velum. We suspect that what Palmer noted was a separation of the inner epidermis of the velum from the outer epidermis and associated internal parenchyma (see Fig. 1F). Because of textural differences in these layers, it is not surprising that they would have undergone differential shrinking during specimen preparation. In longitudinal sections of various species of *Isoetes,* especially those with extensive velum coverage, we have seen separation of these layers. Finally, Hall (1971) describes velum pleats on the surface of the nearly complete velum in *I. tenuifolia*. These pleats appear to be vertically-elongate outgrowths of the velum, and although they do not appear to be artifacts of preservation, no indication is given about the frequency of their occurrence. Velum pleats were not observed in *I. tennesseensis*.

The morphology of the labium varies within *Isoetes tennesseensis*. Some leaves lack labia, some have just a short ridge, but most leaves have a spatulate labium, often with bifid apices. No dimorphism in labium morphology exists between the mega- and microsporophylls and the full extent of variation in this character can be found within a single plant. In general, North American *Isoetes* have small (depressed triangular) labia and hence *I. tennesseensis* stands out among these species with its unique labium morphology (Fig. 2A–K). Only *I. weberi* Herter of southeastern Brazil shows a similar morphology (Hickey, 1985). The spatulate, bifid labium is quite extraordinary in *Isoetes*. The high degree of variation in this character exhibited by *I. tennesseensis* may be related to a mixing of genomes in this high polyploid species (Luebke & Budke, 2003).

Luebke and Budke (2003) described the leaves of *Isoetes tennesseensis* as terete in cross section. Our analyses, however, sug-

gest that cross sections of the subulae can vary from half-terete to weakly trapezoidal to terete, with the half-terete shape being the most common. This shape is dictated by the strong asymmetry of lacunae as seen in sectional view (Fig. 1A). The relative sizes of these adaxial and abaxial lacunae have been used by Takamiya et al. (1997) to differentiate between two varieties of *I. sinensis* in Japan. Leaf cross-sectional shape has also been used as a key character for identifying and differentiating a number of neotropical (Hickey, 1994; Small & Hickey, 2001) and Papuasian (Croft, 1980) species. Lacunae characterizations of other North American species have not been previously published. However, ongoing work in our laboratory suggests that lacunae and subula cross-sectional shape will be of diagnostic value. For example, species such as *I. butleri* Engelm. have distinctly triquetrous leaves that can immediately be recognized in the field tactilely. In these species the abaxial lacunae are triangular in outline whereas the adaxial lacunae are quadrate.

Dimorphic Characters

Megasporangia and microsporangia of *Isoetes tennesseensis* are dimorphic in size, yet maintain a consistent length to width ratio. To our knowledge, the only other indication of such dimorphism is in *I. australis* S. Williams, in which megasporangia are 0.75–1.25 × 0.5–0.75 mm whereas microsporangia are 1–1.5 × 0.5–0.6 mm (Williams, 1943). However, it is not known if these are significantly different. Kott and Britton (1985) showed considerable overlap in sporangium size for eight taxa in northeastern North America. High infraspecific variation and size overlap among species led them to conclude that sporangium size was not a useful taxonomic character. For similar reasons, very few *Isoetes* taxonomists have used this character. Alston (1959) is perhaps the last to use it with any real confidence. The presence of a statistically significant bimodality in sporangium size in a single species, however, suggests that sporangium size should not be immediately ruled out as a potential character. If previous studies compared variously mixed samples of mega- and microsporangia, then significant interspecific differences may have been overlooked. However, we recognize that sporangia in *Isoetes* are particularly difficult characters to utilize taxonomically due to ontogenetic and developmental variation. Our unpublished studies have shown that megaspore number and megasporangium size in *I. andicola* (Amstutz) L. D. Gómez show a large amount of ontogenetic variation. Young plants produce as few as four spores per sporangium, and with age this number increases to eight, twelve, and higher. Sporangia increase in size accordingly.

Size dimorphism in sporangial wall cells was also noticed while examining cell shape. Wall cells of the megasporangia average 4.99× longer than wide, whereas the wall cells of the microsporangia are only 2.27× longer than wide. This within-plant variation is reminiscent of the interspecific variation documented by Hall (1971): sporangial wall cells of *Isoetes tenuifolia* are depicted as being 6–10× longer than wide whereas those in *I. abyssinica* are only 3–5× longer. Hall (1971) did not indicate from which types of sporangia he obtained his data, nor if they were consistent across specimens and sporangia types. Critical analyses of cellular patterns of the sporangium epidermis in other species may lead to insights about relationships and provide additional taxonomic characters.

Dimorphism was also identified in velum coverage between microsporangia and megasporangia. Initially, we ascribed the higher velum coverage to incomplete enlargement of sporangia on inner microsporophylls; a strong case for not using percent velum coverage of sporangia for just such a reason was made by Kott and Britton (1985). Upon closer examination, however, it was found that the variation in percent velum coverage in *Isoetes tennesseensis* reflects differences in velum length. Our data show that microsporangia (with mean lengths of 3.62 mm and mean percent velum coverage of 35.4%) had a mean velum length of 1.2 mm, whereas the older megasporangia (with a mean length of 5.20 mm and mean velum coverage of 14.1%) had a mean velum length of 0.7 mm. Thus, not

only are there significant differences in velum coverage, but in overall velum length as well. Kott and Britton's (1985) argument was based on the premise that velum length was constant and that variation in percent cover was a reflection of differential realized growth of the sporangia. This is not the case in *I. tennesseensis*.

The ligule is an ephemeral structure that when young outpaces the growth of the young leaf (Smith, 1900), but with age, the ligule becomes dwarfed by the elongating leaf. In *Isoetes tennesseensis* shape differences in ligules were found between those of the micro- and megasporophylls. Ligules of the megasporophylls tended to be depressed-ovate whereas those of the microsporophylls were deltate. We suspect that these differences are artifacts associated with greater ligule degradation in the older megasporophyll leaves. The ligules of the microsporophylls are likely more representative of nearly entire ligules. Hence, this does not represent a true dimorphism. Ligule form in *I. tennesseensis* needs further study in order to determine if this dimorphism is solely degradation related.

Dimorphism in characters associated with megasporophylls and microsporophylls has rarely been addressed in the *Isoetes* literature. To the best of our knowledge, other than spore differences, the only references to such phenomena are those of Hickey (1985) with regard to sporangial pigmentation. In that work it was noted that *I. gardneriana* Mett. and *I. triangula* U. Weber have black megasporangial walls and pale tan, diaphanous microsporangial walls. Additional observations on the nature of this sporangial pigmentation are wanting. Our observations of character dimorphism in sporangial size, velum coverage, and cell wall pattern, suggest that these characters need to be reevaluated on a case by case basis before they are discarded from taxonomic work as suggested by some researchers (e.g., Matthews & Murdy, 1969).

Conclusions

A wide variety of morphological and anatomical characters have been examined in our study. Unfortunately, we have not been able to address all aspects of the internal anatomy, specifically in association with the corm and roots. These two structures may provide additional sources of characters and show unknown variation in *Isoetes*.

Though previous taxonomic research on North American *Isoetes* has de-emphasized morphological characters, we have found that these characters are potentially significant. Other researchers have concluded that vegetative characters are environmentally plastic, developmentally dependent, or invariant (Matthews & Murdy, 1969; Kott & Britton, 1985). We concur that there is a sizeable amount of variability in some characters. However, we find that such variation can be quantified and can have biological and taxonomic importance. For example, even when absolute character measurements may not be discriminating, ratio data may show distinctive developmental patterns (Small & Hickey, 2001) or the degree of variation present may be statistically distinct among species (Hickey, 1978). Furthermore, the dimorphic patterns observed here suggest an additional source of information that may have been overlooked in previous analyses.

This study establishes a baseline for morphological comparisons with other *Isoetes*. We hope that these data will encourage researchers to examine the morphological and anatomical characters of *Isoetes* species not only in North America, but also worldwide. Such work will complement and expand our knowledge of previously published species. This should result not only in a thorough evaluation of the potential taxonomic value of morphological characters, but also in a better understanding of morphological evolution in *Isoetes*.

Acknowledgments

We would like to thank Pat Cox, Karl Gustafson, Ed Lickey, and Joey Shaw for collection assistance, Paul Russell for assistance with SEM, and Michael Barker for assistance with SEM and figure composition. We also acknowledge funding from the Undergraduate Summer Scholars Pro-

gram at Miami University and the Willard S. Turrell Herbarium (MU).

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