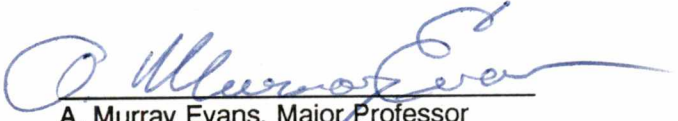
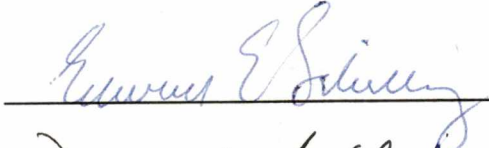


To the Graduate Council:

I am submitting herewith a thesis written by Robert Joel Duff entitled "An Electrophoretic Study of Two Closely Related Species of *Isoetes* L. from the Southern Appalachians." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Botany.


A. Murray Evans, Major Professor

We have read this thesis
and recommend its acceptance:




Accepted for the Council:


Vice Provost
and Dean of The Graduate School

**AN ELECTROPHORETIC STUDY OF TWO CLOSELY RELATED SPECIES
OF *ISOETES* L. FROM THE SOUTHERN APPALACHIANS**

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Robert Joel Duff

December 1991

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ABSTRACT

Allozyme electrophoresis, spore morphology, and vegetative morphology were used to analyze the relationship of *Isoetes caroliniana* (A. Eaton) Taylor and Leubke to *I. engelmannii* A. Braun.

Allozyme electrophoresis was applied to twenty-nine populations of *I. engelmannii* and *I. caroliniana* and related taxa. It was found that sixteen populations of *I. caroliniana* can be distinguished from six populations of *I. engelmannii* by four loci; AAT-1, 6-PGD, LAP-1, and TPI-2. Three populations of what were putatively thought to represent *I. engelmannii* were determined to be probable examples of natural hybrids between *I. engelmannii* and *I. caroliniana*, and *I. engelmannii* and *I. melanopoda*.

Descriptions of spore ornamentation and vegetative morphology are recorded and discussed for each of the populations collected for electrophoresis. Examination of spore ornamentation revealed that *I. caroliniana* could be distinguished from *I. engelmannii* with the former having cristate to echinate ornamentation whereas the latter has a reticulate ornamentation.

The amount of variability of morphological characters is discussed with respect to the other available data and the two taxa are described within a range of that observed variability.

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CHAPTER I

INTRODUCTION

The genus *Isoetes* L. is the only representative of the family; *Isoetaceae*. This genus contains about 150 recognized species, commonly referred to as "quillworts", in worldwide distribution of which 22 are currently recognized in North America (Taylor, et. al., *manusc.*). Others have placed the number of species in North America as high as 50-60 (Tryon and Tryon, 1982). As recently as the early 1980's, there were thought to be only 60 species worldwide; nineteen of which were present in North America (Lellinger, 1985). This newfound diversity among the genus is due to two things: first, an increase of interest in this genus has increased the amount of collecting which has led to the discovery of new taxa; and secondly, new systematic techniques have been developed which have allowed for the separation of taxa that were previously thought to be conspecific.

Members of *Isoetes* usually inhabit very moist to completely aquatic habitats although a few are known to exhibit a terrestrial habit. They may be found from the tropics to the near-sub-arctic latitudes (Bierhorst, 1971). They are rush-like perennials whose leaves are joined at the base to a corm-like rhizome which is usually located just below the surface of the soil. It is at the base of the sporophylls, found below the soil surface, that the mega- and microspores are produced in sporangia. The morphology and reproduction of *Isoetes* are reviewed extensively by Bierhorst (1971).

Classification within *Isoetes* has followed several paths as outlined by Pfeiffer (1922) in her "Monograph of the Isoetaceae." Linnaeus (1754) first characterized *Isoetes* by relating spore-producing structures to the structures of seed plants. Later researchers, recognizing the fern-like characteristics of the life cycle, attempted to create infrageneric classification on the basis of the relationships of the plants to water (Engelmann, 1882). Pfeiffer (1922) recognized some basic divisions within the genus based solely on megaspore morphology as viewed by light microscope.

Clute (1908, 1928) and Reed (1965) have used morphological and ecological characters to characterize taxa. Since then the emphasis has been on the morphology of the megaspores and, to a lesser degree, of the microspores. Recent taxonomic classifications of the members of this genus have relied heavily on spore morphology (Small, 1964; Evans, 1968; Boom, 1979, 1982; Taylor et al., 1975; Kott and Britton, 1983; Lellinger, 1985; Hickey, 1986; and Taylor et al., *manusc.*). This has proved to be a practical method for species sorting and species identification, but has not proved to be a suitable basis for subdivisions of the genus. Only recently, Hickey (1990) proposed a new subgenus, *Euphyllum*, for three extant species. This division of the genus *Isoetes* into two subgenera, *Isoetes* and *Euphyllum*, was based on unique morphological characters and anatomy rather than spore ornamentation affinities. In fact, the three taxa representing the new subgenus exhibit a wide variety of spore morphology. One of the major problems that lies ahead is the development of an infrageneric classification based on evolutionary relationships.

Recent examinations of *Isoetes* in the southeastern United States have resulted in several approaches to classification of the 15 taxa in the region. Reed (1965) divided *Isoetes* up into several "species-complexes." In the southeastern United States he recognized three such complexes: the *Isoetes riparia* complex, the *Isoetes engelmannii* complex, and the *Isoetes melanopoda* complex. These complexes seem to represent ecological groupings rather than phylogenetic ones. Boom (1979, 1982) brought together a synopsis of the *Isoetes* of the southeastern United States. In his thesis (1979) he examined all the species that Reed (1965) had recognized plus three others: *I. louisianensis* Thieret (Landry and Thieret, 1973), *I. tegetiformans* Rury (1978), and *I. macrospora* Dur. (Dennis et al., 1979). Boom produced two artificial taxonomic keys for the southeastern *Isoetes*; one was based on ecological and morphological characters and the other was based solely on spore characteristics.

Specific Problem:

Braun (1846) originally described *Isoetes engelmannii* A. Br. (Engelmann's quillwort) from specimens collected by Engelmann in 1842 from a Missouri pond. Later Engelmann (1867, 1882) described several varieties of the species: var. *valida* (= var. *engelmannii*) from Pennsylvania, var. *gracilis* from "southern New England," and var. *georgiana* from Floyd County, Georgia. Eaton (1900a,b, 1905) described the range of *I. engelmannii* to encompass most of the east coast and named two more varieties (*caroliniana* and *fontana*).

Isoetes engelmannii var. *caroliniana* A. Eaton was described by Eaton (1900a) from Mitchell County, North Carolina, based on distinctive features in leaf and spore detail. This variety of *I. engelmannii* has been most commonly recognized in the literature with interpretations of other varieties varying to a great extent. Clute (1928) recognized the variety as having "leaves and an indusium that are slightly broader, and megaspores which are marked by thin and broken crests which often look like the spinules of *Isoetes echinospora*." In her monograph of the *Isoetaceae*, Pfeiffer (1922) recognized only two varieties of *I. engelmannii*; var. *engelmannii* and var. *caroliniana*. She characterized var. *caroliniana* as having slightly finer leaves than var. *engelmannii*, in contrast to Clute's (1928) description, and having spores with "high reticulate ridges, much crisped and cut with an irregular margin, producing (a) somewhat spiny effect." Reed (1965) recognized three varieties of *Isoetes engelmannii* in his *I. engelmannii* complex: *I. engelmannii* var. *engelmannii*, var. *caroliniana*, and var. *georgiana* Engelmann. He found that var. *caroliniana* had leaves that were shorter and stouter than var. *engelmannii*, which tended to spread out or sprawl. Boom (1979) didn't recognize either var. *caroliniana* or *georgiana*, attributing them to "suspected instances of natural hybridizations."

Recently, var. *caroliniana* and var. *georgiana* of *I. engelmannii* have been elevated to species in a manuscript by Taylor et al. (manusc.) based on spore ornamentation differences compared to *I. engelmannii* var. *engelmannii* and a few preliminary tests using starch gel

electrophoresis (Taylor, pers. comm.). Few localities are known for *I. caroliniana*, most of them being in North Carolina. Variety *georgiana* has also been raised to species level in the same publication (Taylor et al., manusc.). This species is only known from one location and is probably the result of hybridization (Taylor et al., manusc.) of *I. flaccida* and *I. engelmannii* and a subsequent doubling event to produce a polyploid with forty-four chromosomes and intermediate spore characters. This taxon was not included as part of this study.

In light of the recent elevation of *I. caroliniana* to species level it seemed that a more intensive study of the evolutionary relationship between *I. engelmannii* and *I. caroliniana* would be in order. The primary purposes of this study were to address the validity of *I. caroliniana* as a separate taxon from *I. engelmannii* and to better understand its habitat and distribution. It is postulated that *I. caroliniana* is derived by geographical separation from populations of *I. engelmannii* but this is not necessarily the case. If simple separation for a long period of time were the only necessity, then the very large range of *I. engelmannii* would be difficult to explain. This was one of the questions addressed in this study. If the taxonomic separation is not considered valid then the differences between the various populations examined would have to be explained as falling within the total range of variability of *I. engelmannii*.

It was proposed that the use of electrophoretic techniques would help clarify the relationship of *I. engelmannii* to *I. caroliniana*. Electrophoretic data can be especially helpful in taxa which exhibit few morphological characters and has been used in *Isoetes* for both populational and taxonomic studies. Hickey (pers. comm.; Hickey et al., 1989) has been actively involved in using electrophoresis to examine the population biology and relationships of many species around the world, especially in South America. Taylor (pers. comm.) has compared some taxa of *Isoetes*, including *I. caroliniana* and *I. engelmannii*, by this method.

Although electrophoresis is a very useful tool for comparing taxa genetically it would not be acceptable to use this tool as the sole means of making taxonomic decisions. It seems necessary to demonstrate that any electrophoretic dissimilarity between taxa is correlated with

some morphological divergence before they are considered to be distinct. This correlation would allow for the recognition of taxa from morphological examination alone which is still an important process in the practice of species identification. With that in mind, this study included the integration of morphological and ecological investigations with the information gathered from the electrophoretic study. Because of the traditional emphasis of botanists on spore ornamentation within this genus, the spore ornamentation of collected populations of *Isoetes* was documented and a herbaria study of megaspores from preserved plants was also made part of this study. Several types of data were used to help clarify the relationships of the two taxa in question and to demonstrate the potential for such information to head to a better understanding of other taxa within this unique genus.

CHAPTER II

ELECTROPHORESIS

Introduction:

The classic studies of Harris (1966), Hubby and Lewontin (1966), and Lewontin and Hubby (1966) in the 1960's established protein electrophoresis as a valuable tool for investigating the genetics of animals at a molecular level. In the early 1970's these techniques were applied to plants on a wide scale by Gottlieb (1971, 1976, 1977) and in the 1980's by D. Crawford (1983, 1985, 1989), C. Haufler (1985, 1987), D. Soltis (1985, 1986) and many others. Further references of electrophoretic studies may be found in Crawford (1990), Hillis and Moritz (1990), and Soltis and Soltis (1989).

Crawford (1990) suggests that electrophoretic data may be particularly useful for characterizing taxa when the morphological boundaries between species are obscured by phenotypic plasticity. Wheelar and Guries (1982), Crawford (1975, 1979), Reynolds and Crawford (1980), and Crawford and Wilson (1979) have used electrophoresis to distinguish species of *Chenopodium* which are weedy annual plants that exhibit a high degree of phenotypic plasticity. Recent studies with some fern-allies (Soltis and Soltis, 1988) and other plants with reduced morphology (For example: Nickrent et al., 1984; and Nickrent, 1986; *Arceuthobium* (dwarf mistletoes) and Soltis et al., 1987 (Bromeliaceae)) have also shown that electrophoresis may be especially useful for taxa which have few distinguishing morphological characteristics. *Isoetes* exhibits few morphological characters which are useful taxonomically and many of those characters exhibit marked phenotypic plasticity.

Electrophoretic data has also been used to infer the phylogeny of recently diverged taxa and to suggest means by which speciation may have taken place (Gottlieb, 1973, 1984; Crawford and Bayer, 1981; and Crawford et al., 1987). Little is known about the phylogeny of many taxa of *Isoetes* and the means by which speciation has taken place. *Isoetes engelmannii* and

I. caroliniana are presumed to be closely related, with *I. engelmannii* being the progenitor species, but this can not be established with morphological data alone.

The advantages and disadvantages of electrophoresis are discussed by Avise (1974), Buth (1984), Crawford (1985), Gottlieb (1977, 1984), and Kephart (1990). The most important advantage that electrophoresis brings to this study is the presumed independence of the allozyme characters from many of the highly variable morphological characters. This allows one to compare electrophoretic data with morphological characters.

Only recently have the techniques of electrophoresis been applied to *Isoetes*. Taylor (pers. comm.) has compared taxa of *Isoetes* using eight enzyme systems (TPI, 6-PGD, MDH, IDH, PGM, SKDH, LAP and AAT; Table 2) and Hickey (1989) has been using electrophoresis to examine the relationships and population biology of many species worldwide, especially those from South America. Information gathered from this study will be useful in further describing the population biology and mode of speciation of the examined taxa and by inference within the genus as a whole.

Materials:

Populations representing the two taxa to be studied were collected from the field. Population locations were mostly identified based on collections represented in various herbaria (Appendix 1). Looking for these plants even at a known locality often proved to be frustrating, because of the necessity of ideal conditions such as the right lake and river levels and presence of enough plants to be noticed among the many similar-appearing plants with which they grow. Only six newly discovered populations are included in this work (Table 1). Every citation of a location in the eastern Tennessee and western North Carolina area that could be found was followed up and many previously cited localities from West Virginia, western Virginia, northern Alabama, and northern Georgia were also visited. Appendix 2 lists populations which were recorded in the literature but couldn't be relocated. It is not known if failure to relocate them was

because of loss of habitat, or because water levels were too high, or because the plants were not yet visible. Both appendices are included as a future reference for those interested in locating populations of *Isoetes* in this study region.

Samples of 25 plants were collected at sites at which the plants were abundant. Usually these 25 samples included three entire plants for transplant to aquaria at the University of Tennessee and for herbarium specimens, and leaves from 22 other plants collected at random from as wide an area as possible. Many populations were very small and subsampling was impossible. In such cases, all plants found were sampled (leaves taken). Samples were kept on ice until they could be stored in a cold room. Population structure and morphological description are reviewed in Chapter IV and data are included in Table 8.

Dr. Carl Taylor, of the Milwaukee Public Museum, sent samples of populations of several species. Some of these samples were included directly in the electrophoretic data (CONN and VERM) and others were used for comparative purposes only. All of the populations observed electrophoretically are listed in Table 1.

Tissue sample preparation for electrophoresis:

Tissues of actively growing leaves were used for allozyme electrophoresis. These tissues were kept cold and were ground with pestle in mortar (glass test tubes and twelve depression spot plates) with 0.5 ml of cold extraction buffer (Phosphate grinding buffer-PVP solution; Soltis et al., 1983). Tissue samples were prepared for enzyme electrophoretic analysis as described in Werth (1985) and Soltis et al. (1983). Samples were used within 24 hours of preparation after several attempts to freeze the extracts proved to be ineffective.

Methods:

Leaf samples taken from the field were stored in a cold room at 0-5°C for as long as a month without apparent loss of metabolic activity of the enzymes. Samples of large populations

TABLE 1: Populations of *Isoetes* surveyed for electrophoretic data.

Taxon	Code	State	Voucher¹
<i>I. butleri</i>	BUTL	GA	Catoosa Co.: Evans (cultivated)
<i>I. caroliniana</i>	ASHE	NC	Ashe Co.: Duff 9012 (TENN)
	BRPA	VA	Franklin Co.: Evans 5001 (TENN)
	CATA	NC	Haywood Co.: Evans/Duff 9105 (TENN)
	COWE	NC	Macon Co.: Duff 9008 (TENN)
	EVAN	NC	Haywood Co.: Evans/Duff 9106 (TENN)
	GILE	VA	Giles Co.: Duff 9009 (TENN)
	HUMP	NC	Avery Co.: Evans 4981 (TENN)
	JOHN	TN	Washington Co.: Duff 9018/9103 (TENN)
	MCAM	TN	Polk Co.: Duff 9112 (TENN)
	MLBS	VA	Giles Co.: C. Werth (TENN)
	OSWD	TN	Polk Co.: Duff 9110 (TENN)
	PARK	TN	Polk Co.: Duff 9007/9015 (TENN)
	POND	NC	Ashe Co.: Duff 9011 (TENN)
	RNMT	NC	Mitchell Co.: Evans (cultivated)
	SHVA	TN	Johnson Co.: Duff 9013 (TENN)
	WILD	NC	Avery Co.: Duff 9010 (TENN)
<i>I. engelmannii</i>	CDSP	TN	Franklin Co.: Duff 9109 (TENN)
	CEME	TN	Putnam Co.: Duff 9001 (TENN)
	COFF	TN	Coffee Co.: Duff 9005/9016 (TENN)
	FCFR	TN	VanBuren Co.: Duff 9003/9101 (TENN)
	MAYP	TN	Coffee Co.: Duff 9004 (TENN)
	MONT	TN	Putnam Co.: Duff 9002 (TENN)
	WATR	TN	Washington Co.: Duff 9117 (TENN)
	CONN ²	CT	Tolland Co.: C. Taylor (cultivated)
	ALAB ²	AL	Tallapoosa Co.: C. Taylor (cultivated)
	VERM ²	VT	Strafford Co.: C. Taylor (cultivated)
<i>I. louisianensis</i>	LOUS	LA	Washington Pa.: Boom, TN Greenhouse

Table 1 (cont.)

Taxon	Code	State	Voucher¹
<i>I. macrospora</i>	MACR	TN	Polk Co.: Duff 9014 (TENN)
<i>I. melanopoda</i>	EXME	TN	Grundy Co.: Duff 9006/9107 (TENN)
	MLOU ²	LA	Louisiana: C. Taylor (cultivated)
	MELA ²	TX	Enchanted Rock: C. Taylor (cultivated)

¹Populations locations are reported in more detail in Appendix A.

²Samples from these populations were sent by Carl Taylor from plants cultivated at the Milwaukee Public Museum.

usually included many whole plants which could generally be stored for several months because of their continued growth. Comparisons of newly collected plants, plants stored for more than a month, and plants grown in aquaria revealed no detectable differences in the intensity of enzymatic activity in those enzymes examined. One notable exception to this was the occurrence of greatly reduced enzymatic activity in those plants grown in aquaria under fluorescent light. It was found that this troublesome problem was caused by the adverse effects of the high pH of the water in which the plants had been grown. The pH of the water in the three tanks ranged from pH 8.0 to pH 8.3. The enzymatic activity of *I. butleri*, which naturally occurs in calcareous habitats, was unaffected, but *I. engelmannii* and *I. caroliniana*, which were always found growing in soils with pH's between 5.2 and 6.8 (Table 8), had much reduced enzymatic activity. When water in the tanks was kept at a pH between 6.0 and 6.5 using copper sulfate and sphagnum moss there was a noticeable increase in detectable enzymatic activity even in those leaves which had developed under the previous basic condition (morphological changes due to environmental effects are discussed in Chapter IV and V).

It was determined in limited tests that seasonal variation could be considered an insignificant factor in the analysis of the data. Some populations (FCFR, COFF, PARK, and JOHN) of both *I. engelmannii* and *I. caroliniana* were collected several times during different times in the year and the electrophoretic zymograms produced from those plants revealed no visible differences in enzymatic activity or mobility. Roots and megaspores of some plants were examined separately and these revealed no activity for most loci.

Enzyme electrophoresis and detection:

Eight enzyme systems were assayed by horizontal starch gel electrophoresis, using six different buffer systems (Table 2) on 12% starch gels. The methods used for enzyme electrophoresis, histochemical staining, and enzyme detection were essentially those described in Werth (1985) and Soltis et al. (1983). Some modifications were made, including the adjustment

TABLE 2: Enzyme systems assayed and the number of encoding loci scored from populations of *Isoetes* examined.

Enzyme	Abbreviation	E. C. Code ²	Buffer System ¹	Loci Scored
Aldolase	ALD	4.1.2.13	#7	1
Aspartate Aminotransferase	AAT ^{3,6}	2.6.1.1	#7	2
Glucose-6-phosphate Isomerase	GPI ^{4,7}	5.3.1.9	#6	-
Isocitrate Dehydrogenase	IDH	1.1.1.42	Morph ⁵ ,#9,#5	1
Leucine Aminopeptidase	LAP	3.4.11.1	#7	2
Malic Enzyme	ME ⁴	1.1.1.40	#7,#9	-
Malate Dehydrogenase	MDH	1.1.1.37	Morph ⁵ ,#9,#1	3
Phosphoglucomutase	PGM	2.7.5.1	Morph ⁵ ,#5,#6	1
6-Phosphogluconate Dehydrogenase	6-PGD	1.1.1.44	Morph ⁵ ,#9,#5	1
Shikimate Dehydrogenase	SKDH ⁴	1.1.1.25	Morph ⁵ ,#9,#5	-
Triosephosphate Isomerase	TPI	5.3.1.1	#7	2

¹ The buffer systems used are described in Werth (1985) and Soltis *et al.* (1983).

² Enzyme commission code

³ This enzyme was formerly known as GOT (Glutamate oxaloacetate transaminase).

⁴ These enzymes were not scored because of problems in interpretation or poor resolution but are included because they do exhibit differences between the specimens being examined.

⁵ Morpholine buffer (Werth, 1985) was adjusted to pH 8.2.

⁶ Stain was adjusted to a pH of 7.0.

⁷ This enzyme was formerly known as PGI (Phosphoglucoisomerase).

of the pH of the gel and electrode buffer solutions, and are noted in Table 2. Information received from the lab of Carl Taylor (pers. comm.) was invaluable in the search for appropriate techniques and gel systems to be used. Several enzyme systems that were stained were not used in the final analysis because they were either unreadable or were uninterpretable. These enzymes included: ACPH (Acid Phosphatase), EST (Esterase), ME (Malic enzyme), GPI (Glucose-6-phosphate Isomerase (=PGI)), and SKDH (Shikimate Dehydrogenase).

The genetic bases of the various enzyme patterns observed were interpreted on the basis of the banding patterns, by comparisons of several buffer systems, with information obtained from personal communications with other researchers familiar with the enzyme systems of *Isoetes*, and by reference to the recorded nature of the enzyme systems (Gottlieb, 1977, 1982; Weeden and Wendel, 1989). Allelic comparisons between different gels were accomplished by running control plants on each gel. These control plants were grown in the lab throughout the research period and included several plants from populations COFF, MACR, RNMT, JOHN, and MONT.

Data Analysis:

The BIOSYS package provided by Swofford and Selander (1981) was used to calculate pairwise genetic distances of Nei (1972, 1978), Prevosti (Wright, 1978), and Rogers (1972) and genetic similarities of Nei (1978) and Rogers (1972) from the estimated allele frequencies (Table 3) for each of the 29 populations examined and between each species. The matrices of Nei (1978) and Rogers (1972) were subjected to analysis by the unweighted pair-group method with arithmetic averaging (UPGMA) procedure of Sneath and Sokal (1973) to produce phenograms.

The Wagner distance procedure (Farris, 1972), also provided by the BIOSYS package, was performed on the matrix of Rogers' (1972) distances and Prevosti (Wright, 1978) distances to produce Wagner trees representing phylogenetic relationships. The Wagner parsimony method imposes minimal constraints on permissible character-state changes. It assumes that characters are measured on an interval scale and allows for free reversibility so that the tree may

be rooted at any point with no change in tree length. Trees were produced which were unrooted because the extreme divergence of many of the outgroup taxa did not allow character state polarization. Optimization of branch lengths was also implemented in each case.

Results:

Eight enzyme systems coded by 17 putative loci were resolved electrophoretically. Of the seventeen loci, four were excluded because they were either too poorly resolved or were uninterpretable (ALD-2, 6-PGD-2, and PGM-2). Mean allele frequencies of scored loci are listed by population in Table 3. Loci were numbered sequentially beginning with the most anodal form. Alleles were lettered beginning with the most anodal form (all systems migrated anodally). Representative electrophoretic patterns for each locus are depicted in Figure 1 and discussed in the following paragraphs.

All of the enzymes surveyed showed variability among at least one of the six putative species examined. Three loci (AAT-1, LAP-1, and TPI-2) exhibited fixed allelic differences between *I. engelmannii* and *I. caroliniana*. When the MONT and CEME populations were excluded from the analysis the loci 6-PGD and SKDH also represented fixed allelic differences between the taxa. Populations MONT and CEME were fixed for unique alleles for loci AAT-1 and AAT-2. The CDSP population was fixed for unique alleles for AAT-2 and 6-PGD. Numerous allelic differences were found between populations LOUS, BUTL, MACR, and EXME, all of which represent separate distinct taxa, and for the COFF population, which probably is an interspecific hybrid between *I. engelmannii* and *I. melanopoda*. No locus was represented by more than two alleles within a population and only four loci (LAP-1, AAT-1, PGM-1 and TPI-1) revealed any polymorphisms within a population. Determination of electrophoretic banding patterns and results of comparisons among populations are as follows:

AAT. Two regions of activity were detected for this dimeric enzyme and were interpreted as being the result of the action of two loci. Often a lightly-stained third band was seen between

PGM	LDH	LAP-1	LAP-2	LOUS L
				MACR M
				BUTL B
				GILE C
				MLBS C
				BRPA C
				SHVA C
				JOHN C
				RNMT C
				HUMP C
				EVAN C
				CATA C
				ASHE C
				POND C
				WILD C
				COWE C
				MCAM C
				OWSD C
				PARK C
				MAYP X
				EXME P
				CONN E
				VERM E
				WATR E
				CDSP E
				COFF E
				FCFR E
				CEME E
				MONT E

Figure 1. Representative banding patterns of loci for all populations examined. *Isoetes engelmannii* (E), *I. caroliniana* (C), *I. melanopoda* (P), *I. macrospora* (M), *I. butleri* (B), *I. louisianensis* (L), and *I. engelmannii* x *I. melanopoda* (X).

							LOUS L
							MACR M
							BUTL B
							GILE C
							MLBS C
							BRPA C
							SHVA C
							JOHN C
							RNMT C
							HUMP C
							EVAN C
							CATA C
							ASHE C
							POND C
							WILD C
							COWE C
							MCAM C
							OWSD C
							PARK C
							MAYP X
							EXME P
							CONN E
							VERM E
							WATR E
							CDSP E
							COFF E
							FCFR E
							CEME E
							MONT E

Figure 1. (cont.)

			LOUS L
			MACR M
			BUTL B
			GILE C
			MLBS C
			BRPA C
			SHVA C
			JOHN C
			RNMT C
			HUMP C
			EVAN C
			CATA C
			ASHE C
			POND C
			WILD C
			COWE C
			MCAM C
			OWSD C
			PARK C
			MAYP X
			EXME P
			CONN E
			VERM E
			WATR E
			CDSP E
			COFF E
			FCFR E
			CEME E
			MONT E

Figure 1. (cont.)

TABLE 3: Mean allele frequencies within populations. Populations are described in Table 1. Sample sizes for each enzyme studied are included in parenthesis ().

LOCUS	MONT	CEME	FCFR	COFF	CDSP	WATR	VERM	CONN
AAT-1	(3)	(7)	(20)	(40)	(16)	(7)	(3)	(3)
A	1.000	1.000	-----	-----	-----	-----	-----	-----
B	-----	-----	1.000	1.000	1.000	1.000	1.000	1.000
C	-----	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	-----	-----	-----	-----	-----	-----
E	-----	-----	-----	-----	-----	-----	-----	-----
AAT-2	(3)	(7)	(20)	(40)	(16)	(7)	(1) ^a	(1) ^a
A	1.000	1.000	-----	-----	-----	-----	-----	-----
B	-----	-----	-----	-----	1.000	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	1.000	1.000	-----	1.000	1.000	1.000
ALD-1	(3)	(7)	(20)	(40)	(16)	(7)	(3)	(3)
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	-----	-----	-----	-----	-----	-----
IDH-1	(3)	(7)	(21)	(40)	(16)	(20)	(3)	(3)
A	-----	-----	-----	-----	-----	-----	-----	-----
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
C	-----	-----	-----	-----	-----	-----	-----	-----
LAP-1	(3)	(7)	(19)	(40)	(16)	(20)	(3)	(3)
A	-----	-----	-----	-----	-----	-----	-----	-----
B	-----	-----	-----	-----	-----	-----	-----	-----
C	1.000	1.000	1.000	0.950	1.000	1.000	1.000	1.000
D	-----	-----	-----	-----	-----	-----	-----	-----
E	-----	-----	-----	0.050	-----	-----	-----	-----
F	-----	-----	-----	-----	-----	-----	-----	-----
G	-----	-----	-----	-----	-----	-----	-----	-----
LAP-2	(3)	(7)	(19)	(40)	(16)	(20)	(3)	(3)
A	-----	-----	-----	-----	-----	-----	-----	-----
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
6-PGD-1	(3)	(7)	(22)	(38)	(15)	(14)	(3)	(3)
A	-----	-----	-----	-----	1.000	-----	-----	-----
B	1.000	1.000	-----	-----	-----	-----	-----	-----
C	-----	-----	1.000	1.000	-----	1.000	1.000	1.000
D	-----	-----	-----	-----	-----	-----	-----	-----
E	-----	-----	-----	-----	-----	-----	-----	-----

Table 3. (cont.)

LOCUS	MONT	CEME	FCFR	COFF	CDSP	WATR	VERM	CONN
PGM-1	(3)	(9)	(22)	(17)	(15)	(17)	(3)	(3)
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	-----	-----	-----	-----	-----	-----
E	-----	-----	-----	-----	-----	-----	-----	-----
F	-----	-----	-----	-----	-----	-----	-----	-----
G	-----	-----	-----	-----	-----	-----	-----	-----
MDH-1	(3)	(7)	(21)	(40)	(16)	(20)	(3)	(3)
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	-----	-----	-----	-----	-----	-----
MDH-2	(3)	(7)	(21)	(40)	(16)	(20)	(3)	(3)
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	-----	-----	-----	-----	-----	-----
MDH-3	(3)	(7)	(21)	(40)	(16)	(20)	(3)	(3)
A	1.000	1.000	-----	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	1.000	-----	-----	-----	-----	-----
D	-----	-----	-----	-----	-----	-----	-----	-----
E	-----	-----	-----	-----	-----	-----	-----	-----
TPI-1	(2)	(2)	(3)	(3)	(15)	(7)	(3)	(3)
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
TPI-2	(2)	(2)	(3)	(3)	(15)	(7)	(3)	(3)
A	-----	-----	1.000	1.000	1.000	-----	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	1.000	1.000	-----	-----	-----	1.000	-----	-----

Table 3. (cont.)

LOCUS	EXME	MAYP	PARK	OSWD	MCAM	COWE	WILD	POND
AAT-1	(5)	(10)	(50)	(18)	(11)	(20)	(17)	(20)
A	-----	-----	-----	-----	-----	-----	-----	-----
B	1.000	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	0.900	1.000	-----	-----	-----	-----
E	-----	1.000	0.100	-----	1.000	1.000	1.000	1.000
AAT-2	(1) ^a	(10)	(50)	(18)	(11)	(20)	(17)	(20)
A	-----	-----	-----	-----	-----	-----	-----	-----
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	1.000	-----	-----	-----	-----	-----	-----
D	1.000	-----	1.000	1.000	1.000	1.000	1.000	1.000
ALD-1	(9)	(10)	(50)	(18)	(11)	(30)	(27)	(18)
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	-----	-----	-----	-----	-----	-----
IDH-1	(10)	(10)	(52)	(20)	(11)	(30)	(27)	(18)
A	1.000	-----	-----	-----	-----	-----	-----	-----
B	-----	1.000	1.000	1.000	1.000	1.000	1.000	1.000
C	-----	-----	-----	-----	-----	-----	-----	-----
LAP-1	(10)	(10)	(48)	(20)	(11)	(20)	(27)	(35)
A	-----	-----	-----	-----	-----	-----	-----	-----
B	-----	-----	-----	-----	-----	-----	-----	-----
C	1.000	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	1.000	1.000	1.000	1.000	1.000	0.986
E	-----	1.000	-----	-----	-----	-----	-----	-----
F	-----	-----	-----	-----	-----	-----	-----	0.014
G	-----	-----	-----	-----	-----	-----	-----	-----
LAP-2	(10)	(10)	(48)	(20)	(11)	(20)	(27)	(35)
A	-----	-----	-----	-----	-----	-----	-----	-----
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
6-PGD	(8)	(10)	(45)	(20)	(11)	(30)	(28)	(18)
A	-----	-----	-----	-----	-----	-----	-----	-----
B	-----	1.000	1.000	1.000	1.000	1.000	1.000	1.000
C	1.000	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	-----	-----	-----	-----	-----	-----
E	-----	-----	-----	-----	-----	-----	-----	-----

Table 3. (cont.)

LOCUS	EXME	MAYP	PARK	OSWD	MCAM	COWE	WILD	POND
PGM-1	(10)	(10)	(56)	(18)	(11)	(30)	(32)	(18)
A	0.700	0.500	0.714	0.861	0.545	0.500	1.000	0.528
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	-----	0.500	-----	-----	-----	-----	-----	-----
E	-----	-----	-----	-----	-----	-----	-----	-----
F	0.300	-----	0.286	0.139	0.455	0.500	-----	0.472
G	-----	-----	-----	-----	-----	-----	-----	-----
MDH-1	(10)	(10)	(52)	(20)	(11)	(30)	(27)	(18)
A	-----	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	1.000	-----	-----	-----	-----	-----	-----	-----
MDH-2	(10)	(10)	(52)	(20)	(11)	(30)	(27)	(28)
A	-----	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	1.000	-----	-----	-----	-----	-----	-----	-----
MDH-3	(10)	(10)	(52)	(20)	(11)	(30)	(27)	(28)
A	-----	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	-----	-----	-----	-----	-----	-----
E	1.000	-----	-----	-----	-----	-----	-----	-----
TPI-1	(4)	(4)	(7)	(19)	(11)	(13)	(11)	(5)
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
TPI-2	(4)	(4)	(7)	(19)	(11)	(13)	(11)	(5)
A	-----	-----	-----	-----	-----	-----	-----	-----
B	-----	1.000	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	1.000	-----	1.000	1.000	1.000	1.000	1.000	1.000

Table 3. (cont.)

LOCUS	ASHE	CATA	EVAN	HUMP	RNMT	JOHN	SHVA	BRPA
AAT-1	(34)	(4)	(7)	(4)	(6)	(31)	(26)	(4)
A	----	----	----	----	----	----	----	----
B	----	----	----	----	----	----	----	----
C	----	----	----	----	----	----	----	----
D	----	----	----	1.000	----	----	----	----
E	1.000	1.000	1.000	----	1.000	1.000	1.000	1.000
AAT-2	(34)	(4)	(7)	(4)	(6)	(31)	(26)	(4)
A	----	----	----	----	----	----	----	----
B	----	----	----	----	----	----	----	----
C	----	----	----	----	----	----	----	----
D	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ALD-1	(24)	(18)	(10)	(4)	(6)	(17)	(23)	(4)
A	1.000	1.000	1.000	1.000	----	1.000	1.000	1.000
B	----	----	----	----	1.000	----	----	----
C	----	----	----	----	----	----	----	----
D	----	----	----	----	----	----	----	----
IDH-1	(24)	(18)	(8)	(4)	(6)	(17)	(18)	(4)
A	----	----	----	----	----	----	----	----
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
C	----	----	----	----	----	----	----	----
LAP-1	(31)	(7)	(8)	(4)	(6)	(16)	(18)	(4)
A	----	----	----	----	----	----	----	----
B	----	----	----	----	----	----	----	----
C	----	----	----	----	----	----	----	----
D	0.984	1.000	0.813	1.000	1.000	1.000	0.250	0.750
E	----	----	----	----	----	----	----	----
F	0.016	----	0.187	----	----	----	0.750	0.250
G	----	----	----	----	----	----	----	----
LAP-2	(31)	(7)	(8)	(4)	(6)	(16)	(18)	(4)
A	----	----	----	----	----	----	----	----
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
6-PGD	(30)	(17)	(11)	(4)	(6)	(31)	(22)	(4)
A	----	----	----	----	----	----	----	----
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
C	----	----	----	----	----	----	----	----
D	----	----	----	----	----	----	----	----

Table 3. (cont.)

LOCUS	ASHE	CATA	EVAN	HUMP	RNMT	JOHN	SHVA	BRPA
PGM-1	(28)	(15)	(11)	(4)	(6)	(30)	(26)	(4)
A	0.929	0.500	1.000	1.000	1.000	0.500	1.000	0.625
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	-----	-----	-----	-----	-----	-----
E	-----	-----	-----	-----	-----	-----	-----	-----
F	0.071	0.500	-----	-----	-----	0.500	-----	0.375
G	-----	-----	-----	-----	-----	-----	-----	-----
MDH-1	(24)	(18)	(10)	(4)	(6)	(16)	(18)	(4)
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	-----	-----	-----	-----	-----	-----
MDH-2	(24)	(18)	(10)	(4)	(6)	(16)	(18)	(4)
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	-----	-----	-----	-----	-----	-----
MDH-3	(24)	(18)	(10)	(4)	(6)	(16)	(18)	(4)
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	-----	-----	-----	-----	-----	-----	-----	-----
E	-----	-----	-----	-----	-----	-----	-----	-----
TPI-1	(12)	(3)	(3)	(1)	(6)	(3)	(18)	(1)
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
TPI-2	(12)	(3)	(3)	(1)	(6)	(3)	(18)	(1)
A	-----	-----	-----	-----	-----	-----	-----	-----
B	-----	-----	-----	-----	-----	-----	-----	-----
C	-----	-----	-----	-----	-----	-----	-----	-----
D	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Table 3. (cont.)

LOCUS	MLBS	GILE	BUTL	MACR	LOUS
AAT-1	(3)	(11)	(7)	(14)	(2)
A	----	----	----	----	----
B	----	----	----	----	----
C	----	----	----	1.000	1.000
D	----	----	----	----	----
E	1.000	1.000	1.000	----	----
AAT-2	(3)	(11)	(7)	(1) ^a	(1) ^a
A	----	----	----	----	----
B	----	----	----	----	----
C	----	----	1.000	----	----
D	1.000	1.000	----	1.000	1.000
ALD-1	(3)	(11)	(4)	(14)	(2)
A	1.000	1.000	----	1.000	----
B	----	----	----	----	----
C	----	----	----	----	1.000
D	----	----	1.000	----	----
IDH-1	(3)	(11)	(4)	(14)	(2)
A	----	----	----	----	----
B	1.000	1.000	----	1.000	1.000
C	----	----	1.000	----	----
LAP-1	(3)	(11)	(6)	(11)	(2)
A	----	----	----	1.000	----
B	----	----	----	----	1.000
C	----	----	----	----	----
D	1.000	1.000	----	----	----
E	----	----	----	----	----
F	----	----	----	----	----
G	----	----	1.000	----	----
LAP-2	(3)	(11)	(6)	(1) ^a	(1) ^a
A	----	----	1.000	----	----
B	1.000	1.000	----	1.000	1.000
6-PGD	(3)	(15)	(5)	(13)	(2)
A	----	----	----	----	----
B	1.000	1.000	----	----	1.000
C	----	----	----	----	----
D	----	----	----	1.000	----
E	----	----	1.000	----	----

Table 3. (cont.)

LOCUS	MLBS	GILE	BUTL	MACR	LOUS
PGM-1	(3)	(15)	(5)	(13)	(2)
A	1.000	1.000	-----	0.654	0.500
B	-----	-----	0.500	-----	-----
C	-----	-----	-----	0.346	-----
D	-----	-----	-----	-----	-----
E	-----	-----	-----	-----	0.500
F	-----	-----	-----	-----	-----
G	-----	-----	0.500	-----	-----
MDH-1	(3)	(11)	(4)	(14)	(2)
A	1.000	1.000	-----	1.000	-----
B	-----	-----	1.000	-----	-----
C	-----	-----	-----	-----	1.000
D	-----	-----	-----	-----	-----
MDH-2	(3)	(11)	(4)	(14)	(2)
A	1.000	1.000	-----	1.000	-----
B	-----	-----	1.000	-----	-----
C	-----	-----	-----	-----	1.000
D	-----	-----	-----	-----	-----
MDH-3	(3)	(11)	(4)	(14)	(2)
A	1.000	1.000	-----	1.000	-----
B	-----	-----	1.000	-----	-----
C	-----	-----	-----	-----	-----
D	-----	-----	-----	-----	1.000
E	-----	-----	-----	-----	-----
TPI-1	(2)	(1)	(2)	(3)	(2)
A	1.000	1.000	0.250	-----	1.000
B	-----	-----	-----	1.000	-----
C	-----	-----	0.750	-----	-----
TPI-2	(2)	(1)	(2)	(3)	(2)
A	-----	-----	-----	-----	-----
B	-----	-----	-----	-----	-----
C	-----	-----	1.000	1.000	-----
D	1.000	1.000	-----	-----	1.000

^a These loci were not detected and so the values recorded are for calculation purposes only.

the bands of the two loci and this band was interpreted to be an interlocus heterodimer. AAT-2 was not detected from samples of *I. louisianensis*, *I. macrospora*, or *I. melanopoda*. Locus AAT-1 was monomorphic for allele B in *I. engelmannii* except for the MONT and CEME populations, and was monomorphic for either allele D or E in all populations of *I. caroliniana*. Locus AAT-2, which did not always stain consistently, was monomorphic for allele D in all populations of *I. caroliniana* and all populations of *I. engelmannii* except for the CDSP population. Both AAT loci exhibit distinctive phenotypes for both the MONT and CEME populations.

ALD. This enzyme is cited as being tetrameric in structure (Soltis and Soltis, 1989) but was monomorphic for all populations examined. Both *I. engelmannii* and *I. caroliniana* were monomorphic for allele A except for the RNMT population which was monomorphic for allele B. ALD was found to be a good marker enzyme for *I. butleri* and *I. louisianensis*. When the stain was applied for several hours other bands became apparent in the area just above the origin but were difficult to discern.

IDH. This dimeric enzyme produced what appeared to be a multiple-banded phenotype (similar to what would be expected from a heterozygote) in all populations but was interpreted as being a single monomorphic locus with ghost bands or degradation products above and below a single darkly stained band (Hickey, pers. comm.). The single locus for this enzyme was monomorphic for the same allele in all populations of *I. engelmannii* and *I. caroliniana*. This enzyme may be used as a good marker for *I. melanopoda*, *I. butleri*, and *I. louisianensis*. The sample of *I. melanopoda* exhibited bands of distinct mobility with all buffer systems used but only buffer system #9 (Soltis et al., 1988) allowed the distinction of *I. butleri* and *I. louisianensis* from other taxa.

LAP. Two loci of this monomeric enzyme were detected. Initially the zymograms of this enzyme were interpreted as a single locus with two widely separated alleles but the presence of high variability among the fastest migrating alleles, the occurrence of an occasional heterozygote in the upper banding region, and the occurrence of polymorphism in the upper band between

individuals within a single population lead to the interpretation that two loci were being observed. Allele C of LAP-1 was present exclusively in all populations of *I. engelmannii* and allele D was present only in populations of *I. caroliniana*. LAP-2 was fixed for allele B for all populations examined except BUTL (*I. butleri*) and was not detected from samples of *I. macrospora* and *I. louisianensis*. LAP was a good marker enzyme for all species examined except *I. melanopoda* which had the same banding patterns as *I. engelmannii*.

6-PGD. All populations of *I. engelmannii* and *I. caroliniana* exhibited two bands with similar mobilities. Because 6-PGD is a dimeric enzyme several interpretations can be advanced to explain these 2-banded phenotypes: it could represent a case of two monomorphic loci; a single locus with a second band representing the product of a post-translational modification; one band could represent the product of a duplication; or a single locus could be present that is heterozygous for all individuals, with a third band remaining undetected because it is produced by a null allele. Because samples of *I. butleri* and *I. macrospora* occasionally revealed clear heterozygotes in the same region (gel buffer system #5, Soltis et al. 1983), and because populations of *I. engelmannii* and *I. caroliniana* showed different mobilities for the two bands in synchronous fashion (figure 3) the interpretation that two loci are present became less credible. If the bands represent the products of two independent loci the parallel changes in mobility would be difficult to explain. Gene duplication doesn't adequately describe this phenomenon because it would require the duplication of the locus in both species after they had diverged. The interpretation of one band representing a post-translational modification of a single locus seems to be the best interpretation with the available information. This interpretation is also the most conservative with respect to its eventual influence on the statistical analysis. Other interpretations resulting in the scoring of two loci would only add to the differences between *I. caroliniana* and *I. engelmannii*.

The 6-PGD locus as interpreted was monomorphic for allele B in all populations of *I. caroliniana* and was monomorphic for alleles A or C in populations of *I. engelmannii*. Populations

MONT and CEME were monomorphic for allele B which is not present in any other population of *I. engelmannii*. A third band was observed very close to the origin in populations of *I. caroliniana* and could possibly be considered a second locus (two loci are the usual number of loci recorded for this enzyme (Soltis and Soltis, 1989)).

PGM. This monomeric enzyme is often noted as having two loci (cytosolic and plastid) and was observed to have several areas of activity if the stain was left on the gel for a long period of time. The first visible bands to be detected are clearly the product of a single locus because many populations were represented by only a single monomeric band and others by two bands interpreted as being heterozygotes of PGM-1. Population EXME had both homozygotes for alleles A and F. A second locus appeared occasionally but was too inconsistent to score reliably. Allele A was detected in every population examined except BUTL, and a slower allele (F) was detected as part of a heterozygote in some populations of *I. caroliniana* (PARK, OSWD, MCAM, COWE, POND, ASHE, CATA, JOHN, and BRPA). Other slow alleles were detected in MACR, LOUS, BUTL, and EXME.

MDH. This dimeric enzyme was originally interpreted as being a heterozygote for all individuals examined because of the presence of three distinct bands with very similar mobilities (figure 3) but James Hickey of Miami University (Ohio) has found (pers. comm.) that the three bands represent three individual loci. The three loci were all monomorphic for the same phenotype in all populations of *I. engelmannii* and *I. caroliniana* except for the population FCFR which was fixed for a slower migrating phenotype for MDH-3. All three loci were also found to be good markers for *I. louisianensis*, *I. butleri*, and *I. melanopoda* (see figure 1).

TPI. This dimeric enzyme exhibited the most complexity and the most variation among populations. TPI-1 was quite clear and was monomorphic for all populations except BUTL. It exhibited the same phenotype for all populations of *I. engelmannii* and *I. caroliniana*. The banding patterns of most of the populations examined appeared to be the result of the activity of three loci, but recent work with this enzyme by Hickey et al. (1989a) revealed through genetic crosses and

examination of many species that in fact the third region of activity closest to the origin is the result of postranslational modifications and *I. engelmannii* has been observed to have this third region of activity. All populations were observed to have at least two regions of activity (gel buffer system #5 (Table 2) revealed many fewer bands for all populations but always revealed two regions of activity) with several populations exhibiting other regions of activity which are difficult to explain without extrapolating differences by unconventional methods. Some populations of *I. caroliniana* and the MONT, CEME, and WATR populations could be interpreted as having two widely separated loci with the extraneous bands between them caused either by postranslational modifications or interlocus heterodimers which are frequent in this enzyme system. Five populations revealed two strongly stained bands that were quite close together and the most anodal was interpreted to be TPI-1 and the second band, TPI-2, despite its close proximity to the first band. Some populations exhibited banding patterns that were difficult to interpret (CDSP, MAYP, MACR, and LOUS) when compared with all of the other populations which were included in the interpretations. These difficulties resulted in a conservative interpretation that attempted only to reflect broad differences among banding patterns and not every banding pattern observed. The result is that the apparent differences between some populations of *I. engelmannii* and *I. caroliniana* contribute in the statistical analysis while differences found within populations of *I. engelmannii* and some other populations (MACR and LOUS), which would result in lower identities among populations of *I. engelmannii*, were not included because it could not be certain what the origin of those differences was.

Some of the enzyme systems which were not used in the statistical analysis did exhibit noticeable variation among populations. ACPH (Acid Phosphatase) was much too variable and inconsistent to interpret, but observations of the readable bands showed similar patterns among populations of *I. engelmannii* and *I. caroliniana* except for the MONT, CEME, and RNMT populations, which were clearly different. SKDH (Shikimate Dehydrogenase) exhibited a banding pattern remarkably similar to that of 6-PGD with the COFF, VERM, CONN, WATR, and CDSP

populations being dissimilar from the populations of *I. caroliniana* and MONT and CEME populations. GPI (Glucose-6-phosphate Isomerase (=PGI)) appeared to produce variation among several of the more distinct taxa examined but appeared similar for both *I. engelmannii* and *I. caroliniana*. This enzyme was not scored because of the apparent overlapping of two loci which made interpretation very difficult. ME (Malic Enzyme) also appears to show dissimilar banding pattern between populations of *I. engelmannii* and those of *I. caroliniana*.

Pairwise genetic distances of Nei (1978), Prevosti (Wright, 1978), and Rogers (1972) and genetic similarities of Nei (1978) and Rogers (1972) were calculated from the estimated allele frequencies presented in Table 3 and the identity (similarity) index of Nei (1978) and pairwise genetic distances of Rogers (1972) are presented in Table 4 (all of the other measures of similarity/distance produced similar values and are not included). Table 5 shows the average genetic identities (Nei, 1978) between taxa with and without the CEME and MONT populations included.

Examination of the tables reveals that populations interpreted as *I. caroliniana* have high identities between populations (0.843-1.000) with an average conspecific identity of 0.954 (Nei, 1978). Taken together, the eight populations of *I. engelmannii* show much less similarity among populations; the values ranging from 0.615 to 1.000 with a mean of 0.813. Examination of Table 3 reveals that two populations, CEME and MONT, are responsible for the lowest interpopulational identity values because of their many unique alleles. Because these two populations seem somewhat distinctive they were removed from the pool of data for *I. engelmannii* and the identities recalculated. This caused the range of similarities of the six populations of *I. engelmannii* to be much reduced (0.769-1.000) although still quite broad. The mean genetic identity for these six remaining populations was 0.897.

The mean genetic identity between *I. engelmannii* and *I. caroliniana* was 0.690 (0.538-0.781) with the MONT and CEME populations included as *I. engelmannii* and 0.668 (0.538-0.781) when the MONT and CEME populations were removed from *I. engelmannii*. The mean identity

Table 4: Calculated genetic distances and identities. Nei (1978) unbiased genetic distance above diagonal, Rogers (1972) genetic distance below diagonal.

Population	MONT	CEME	FCFR	COFF	CDSP	WATR	VERM	CONN	EXME	MAYP
1. MONT	*****	1.000	0.615	0.691	0.692	0.769	0.692	0.692	0.446	0.667
2. CEME	0.000	*****	0.615	0.691	0.692	0.769	0.692	0.692	0.446	0.667
3. FCFR	0.385	0.385	*****	0.923	0.769	0.846	0.923	0.923	0.603	0.510
4. COFF	0.312	0.312	0.081	*****	0.845	0.923	1.000	1.000	0.601	0.595
5. CDSP	0.308	0.308	0.231	0.158	*****	0.769	0.846	0.846	0.446	0.589
6. WATR	0.231	0.231	0.154	0.081	0.231	*****	0.923	0.923	0.681	0.589
7. VERM	0.308	0.308	0.077	0.004	0.154	0.077	*****	1.000	0.603	0.589
8. CONN	0.308	0.308	0.077	0.004	0.154	0.077	0.000	*****	0.603	0.589
9. EXME	0.562	0.562	0.408	0.412	0.562	0.331	0.408	0.408	*****	0.268
10. MAYP	0.346	0.346	0.500	0.419	0.423	0.423	0.423	0.423	0.726	*****
11. PARK	0.249	0.249	0.403	0.324	0.403	0.249	0.326	0.326	0.536	0.333
12. OSWD	0.241	0.241	0.395	0.317	0.395	0.241	0.318	0.318	0.551	0.342
13. MCAM	0.266	0.266	0.420	0.341	0.420	0.266	0.343	0.343	0.550	0.268
14. COWE	0.269	0.269	0.423	0.344	0.423	0.269	0.346	0.346	0.554	0.269
15. WILD	0.231	0.231	0.385	0.306	0.385	0.231	0.308	0.308	0.562	0.269
16. POND	0.267	0.267	0.420	0.342	0.420	0.267	0.343	0.343	0.551	0.268
17. ASHE	0.236	0.236	0.389	0.311	0.389	0.236	0.313	0.313	0.555	0.266
18. CATA	0.269	0.269	0.423	0.344	0.423	0.269	0.346	0.346	0.554	0.269
19. EVAN	0.225	0.225	0.379	0.300	0.379	0.225	0.302	0.302	0.555	0.263
20. HUMP	0.231	0.231	0.385	0.306	0.385	0.231	0.308	0.308	0.562	0.346
21. RNMT	0.308	0.308	0.462	0.383	0.462	0.308	0.385	0.385	0.638	0.346
22. JOHN	0.269	0.269	0.423	0.344	0.423	0.269	0.346	0.346	0.554	0.269
23. SHVA	0.223	0.223	0.377	0.298	0.377	0.223	0.300	0.300	0.554	0.262
24. BRPA	0.252	0.252	0.406	0.327	0.406	0.252	0.329	0.329	0.537	0.258
25. MLBS	0.231	0.231	0.385	0.306	0.285	0.231	0.308	0.308	0.562	0.269
26. GILE	0.231	0.231	0.385	0.306	0.385	0.231	0.308	0.308	0.562	0.269
27. BUTL	0.970	0.970	0.970	0.969	0.970	0.970	0.970	0.970	0.960	0.804
28. MACR	0.488	0.488	0.488	0.409	0.488	0.411	0.411	0.411	0.717	0.496
29. LOUS	0.577	0.577	0.654	0.652	0.731	0.577	0.654	0.654	0.649	0.577

Table 4 (cont.)

Population	PARK	OSWD	MCAM	COWE	WILD	POND	ASHE	CATA	EVAN	HUMP
1. MONT	0.765	0.766	0.749	0.745	0.769	0.749	0.769	0.746	0.779	0.769
2. CEME	0.765	0.766	0.749	0.745	0.769	0.749	0.769	0.746	0.779	0.769
3. FCFR	0.607	0.611	0.592	0.589	0.615	0.592	0.614	0.589	0.623	0.615
4. COFF	0.689	0.691	0.673	0.670	0.695	0.673	0.694	0.670	0.704	0.695
5. CDSP	0.607	0.611	0.592	0.589	0.645	0.592	0.614	0.589	0.623	0.615
6. WATR	0.765	0.766	0.749	0.745	0.769	0.749	0.769	0.746	0.779	0.769
7. VERM	0.686	0.688	0.671	0.667	0.692	0.670	0.691	0.667	0.701	0.692
8. CONN	0.686	0.688	0.671	0.667	0.692	0.670	0.691	0.667	0.701	0.692
9. EXME	0.448	0.446	0.441	0.439	0.446	0.441	0.447	0.439	0.452	0.446
10. MAYP	0.680	0.668	0.743	0.741	0.746	0.743	0.748	0.741	0.756	0.667
11. PARK	*****	0.998	0.934	0.932	0.930	0.933	0.933	0.932	0.927	0.993
12. OSWD	0.019	*****	0.914	0.911	0.921	0.913	0.922	0.911	0.918	0.999
13. MCAM	0.082	0.101	*****	1.000	0.985	1.000	0.990	1.000	0.983	0.906
14. COWE	0.086	0.105	0.003	*****	0.981	1.000	0.986	1.000	0.979	0.902
15. WILD	0.091	0.088	0.035	0.038	*****	0.983	1.000	0.981	0.998	0.923
16. POND	0.085	0.104	0.002	0.003	0.037	*****	0.988	1.000	0.981	0.905
17. ASHE	0.087	0.083	0.031	0.034	0.007	0.031	*****	0.986	0.998	0.922
18. CATA	0.086	0.105	0.003	0.000	0.038	0.003	0.034	*****	0.979	0.903
19. EVAN	0.106	0.102	0.049	0.053	0.014	0.050	0.019	0.053	*****	0.920
20. HUMP	0.030	0.011	0.112	0.115	0.077	0.114	0.084	0.115	0.091	*****
21. RNMT	0.168	0.465	0.112	0.115	0.077	0.114	0.084	0.115	0.091	0.154
22. JOHN	0.086	0.105	0.003	0.000	0.038	0.003	0.034	0.000	0.053	0.115
23. SHVA	0.149	0.145	0.093	0.096	0.058	0.093	0.062	0.096	0.043	0.135
24. BRPA	0.095	0.114	0.025	0.029	0.048	0.026	0.041	0.029	0.034	0.125
25. MLBS	0.091	0.088	0.035	0.038	0.000	0.037	0.007	0.038	0.014	0.077
26. GILE	0.091	0.088	0.035	0.038	0.000	0.037	0.007	0.038	0.014	0.077
27. BUTL	0.953	0.965	0.881	0.881	0.894	0.881	0.890	0.881	0.887	0.970
28. MACR	0.406	0.408	0.416	0.419	0.411	0.417	0.408	0.419	0.405	0.411
29. LOUS	0.487	0.496	0.421	0.423	0.423	0.422	0.420	0.423	0.417	0.500

Table 4 (cont.)

Population	RNMT	JOHN	SHVA	BRPA	MLBS	GILE	BUTL	MACR	LOUS
1. MONT	0.692	0.745	0.781	0.769	0.769	0.769	0.020	0.521	0.434
2. CEME	0.692	0.745	0.781	0.769	0.769	0.769	0.020	0.521	0.434
3. FCFR	0.538	0.589	0.625	0.610	0.615	0.615	0.020	0.521	0.355
4. COFF	0.618	0.670	0.705	0.692	0.695	0.695	0.020	0.602	0.357
5. CDSP	0.538	0.589	0.625	0.610	0.615	0.615	0.020	0.521	0.276
6. WATR	0.692	0.745	0.781	0.769	0.769	0.769	0.020	0.600	0.434
7. VERM	0.615	0.667	0.703	0.690	0.692	0.692	0.020	0.600	0.355
8. CONN	0.615	0.667	0.703	0.690	0.692	0.692	0.020	0.600	0.355
9. EXME	0.368	0.439	0.453	0.451	0.446	0.446	0.020	0.276	0.350
10. MAYP	0.667	0.741	0.757	0.760	0.746	0.746	0.184	0.506	0.423
11. PARK	0.852	0.932	0.884	0.933	0.930	0.930	0.029	0.599	0.522
12. OSWD	0.843	0.911	0.876	0.915	0.921	0.921	0.020	0.598	0.513
13. MCAM	0.906	1.000	0.940	1.000	0.985	0.985	0.102	0.588	0.586
14. COWE	0.902	1.000	0.936	0.999	0.981	0.981	0.102	0.586	0.584
15. WILD	0.923	0.981	0.957	0.989	1.000	1.000	0.100	0.600	0.592
16. POND	0.905	1.000	0.940	1.000	0.983	0.983	0.102	0.588	0.586
17. ASHE	0.922	0.986	0.958	0.994	1.000	1.000	0.101	0.600	0.593
18. CATA	0.903	1.000	0.936	0.999	0.981	0.981	0.102	0.586	0.584
19. EVAN	0.920	0.979	0.976	0.994	0.998	0.998	0.102	0.607	0.600
20. HUMP	0.846	0.902	0.879	0.909	0.923	0.923	0.020	0.600	0.513
21. RNMT	*****	0.902	0.879	0.909	0.923	0.923	0.100	0.521	0.592
22. JOHN	0.115	*****	0.936	0.999	0.981	0.981	0.102	0.586	0.584
23. SHVA	0.135	0.096	*****	0.974	0.957	0.957	0.102	0.609	0.601
24. BRPA	0.125	0.029	0.067	*****	0.989	0.989	0.104	0.603	0.600
25. MLBS	0.077	0.038	0.058	0.048	*****	1.000	0.100	0.600	0.592
26. GILE	0.077	0.038	0.058	0.048	0.000	*****	0.100	0.600	0.592
27. BUTL	0.894	0.881	0.886	0.875	0.894	0.894	*****	0.082	0.103
28. MACR	0.488	0.419	0.404	0.405	0.411	0.411	0.894	*****	0.268
29. LOUS	0.423	0.423	0.415	0.412	0.423	0.423	0.881	0.726	*****

TABLE 5: Matrix of Nei's (1978) genetic identity averaged for species of *Isoetes*. Bold numbers denote averages from calculations if the MONT and CEME populations are treated as a separate taxon.

Species	Number of Populations	1	2	3	4
1. <i>I. engelmannii</i>	8	0.813 (0.615-1.000)			
	6	0.897 (0.769-1.000)			
2. <i>I. melanopoda</i>	1	0.554 (0.446-0.681)	*****		
	1	.589 (0.446-0.681)	*****		
3. <i>I. engelmannii</i> x <i>I. melanopoda</i> ¹	1	0.600 (0.510-0.667)	0.268 (0.268-0.268)	*****	
	1	.577 (.510-.667)		*****	
4. <i>I. caroliniana</i>	16	0.690 (0.538-0.781)	0.440 (0.368-0.453)	0.728 (0.667-0.760)	.954 (.843-1.000)
	16	0.668 (0.538-0.781)			
5. <i>I. butleri</i>	1	0.020 (0.020-0.020)	0.020 (0.020-0.020)	0.184 (0.184-0.184)	0.087 (0.020-0.104)
	1	.020 (.020-.020)			
6. <i>I. macrospora</i>	1	0.561 (0.521-0.602)	0.276 (0.276-0.276)	0.506 (0.506-0.506)	0.592 (0.521-0.609)
	1	0.574 (0.521-0.602)			
7. <i>I. louisianensis</i>	1	0.375 (0.276-0.434)	0.350 (0.350-0.350)	0.423 (0.423-0.423)	0.577 (0.513-0.601)
		0.356 (.276-.434)			
8. MONT/CEME	2	0.692 (0.625-0.769)	0.446 (0.446-0.446)	0.667 (0.667-0.667)	0.758 (0.692-0.781)

Table 5 (cont.)

Species	Number of Populations	5	6	7	8
5. <i>I. butleri</i>	1	*****			
6. <i>I. macrospora</i>	1	0.082 (0.082-0.082)	*****		
7. <i>I. louisianensis</i>	1	0.103 (0.103-0.103)	0.268 (0.268-0.268)	*****	
8. MONT/CEME	2	0.020 (0.020-0.020)	0.521 (0.521-0.521)	0.434 (0.434-.434)	1.000 (1.000-1.000)

¹ This is the MAYP population.

between the MONT and CEME populations and the six populations of *I. engelmannii* was 0.692 (0.615-.769) and with those of *I. caroliniana* was 0.758 (0.692-0.781).

There were several populations in which the activity of some loci was difficult to detect or not detected at all. Because of the inability of the BIOSYS computer package (Swofford and Selander, 1981) to accept missing loci, all loci which could not be detected in a population were recorded as a single fixed allele representing the most common allele detected in other closely related populations. These loci (alleles) are denoted in Table 3 and were recorded as a single individual representing a population to reduce its influence on the statistical analysis as much as possible. This practice has the tendency to cause the affected populations to have an over-estimated similarity to other populations. For example: the populations VERM and CONN were poorly resolved for all of the loci stained for on the #7 (Soltis et al., 1983) gel and the AAT-2 locus was not observed. Rather than assign a "dummy" locus which would act as an unique allele, allele D was specified as being fixed in this population (one plant). This decision was arrived at by comparing all other loci and finding that they all had the same banding pattern as the plants of the COFF population and so the same allele that the COFF population was used for the VERM and CONN populations. This created similarity values of 1.00 among the three populations, which may be inaccurate, especially considering the geographical distance between Tennessee and New England, but was at least biased toward creating greater identities among populations rather than greater distances.

The leaf samples from the population from Tallapoosa Co. AL (ALAB) were not included in the electrophoretic data because too many loci were not detected. The loci (AAT-1, 6-PGD, PGM, IDH, PGM, and MDH) that were detected did seem to show the same patterns as plants interpreted to be *I. caroliniana*. This would be surprising considering the location and reticulate spore ornamentation of these plants. The plants themselves were not observed by the investigator and only leaves were received for electrophoresis so any conclusion about this particular population would be considered premature.

Figures 2 and 3 represent two phenograms produced by cluster analysis using the unweighted pair-group method with arithmetic averaging (UPGMA). Phenograms produced from the five different coefficients of similarity/distance revealed no differences in the way groupings occurred at any level of the phenograms. The phenograms show that both *I. engelmannii* and *I. caroliniana* are distinct from all of the outgroups and that they are more similar to each other than to any other species. The populations of *I. caroliniana* generally form a much more cohesive group compared to those of *I. engelmannii*. The MAYP population and the MONT and CEME populations seem to form distinct groups. All of the outgroups are shown to be considerably distinct from one another with *I. butleri* exhibiting the most dissimilarity to taxa being studied.

The sixteen populations interpreted initially as *I. caroliniana* based on spore ornamentation and geographical location did in fact form a cohesive group in all phenograms produced. Eleven of the sixteen formed a cluster at the level of 0.951 similarity with the other five (HUMP, OSWD, PARK, RNMT, and SHVA) added at lower levels of similarity. Each of these five populations was distinguished by the presence of a single unique phenotype for either AAT-1 or ALD-1.

Populations of *I. engelmannii* did not form as cohesive a group as did those of *I. caroliniana*. Six of the populations (CDSP, CONN, COFF, FCFR, VERM, and WATR) formed a cluster with a similarity of only 0.815, with the population from Franklin Co., TN (CDSP) being the most dissimilar. The two populations from Putnam Co., TN (MONT, CEME) were identical to each other but grouped separately from the other populations of *I. engelmannii* and were in fact more closely grouped with *I. caroliniana*. These two populations share two alleles restricted otherwise to *I. caroliniana* (6-PGD, TPI-2) and another restricted to *I. engelmannii* (LAP-1) suggesting they maybe the result of past interspecific hybridization between *I. caroliniana* and *I. engelmannii*, but they also exhibit unique alleles for some of the loci examined (AAT-1, AAT-2) suggesting possible long term isolation from any of the examined populations.

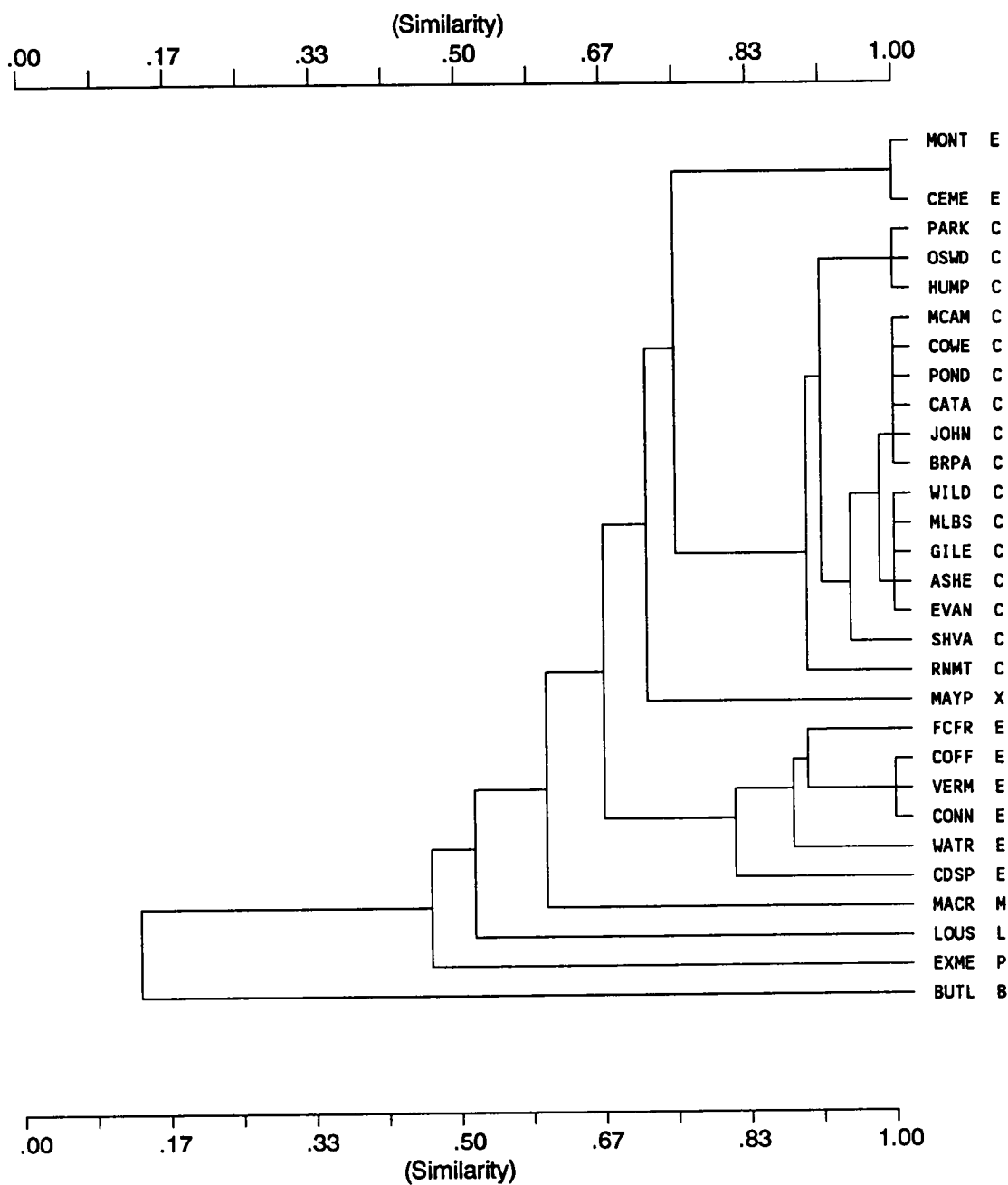


Figure 2. UPGMA cluster phenogram based on Nei's (1978) genetic identity. Population codes are given in Table 1. Farris (1972) $\Phi^* = 14.164$, cophenetic correlation = .974. *Isoetes engelmannii* (E), *I. caroliniana* (C), *I. melanopoda* (P), *I. macrospora* (M), *I. butleri* (B), *I. louisianensis* (L), and *I. engelmannii* x *I. melanopoda* (X).

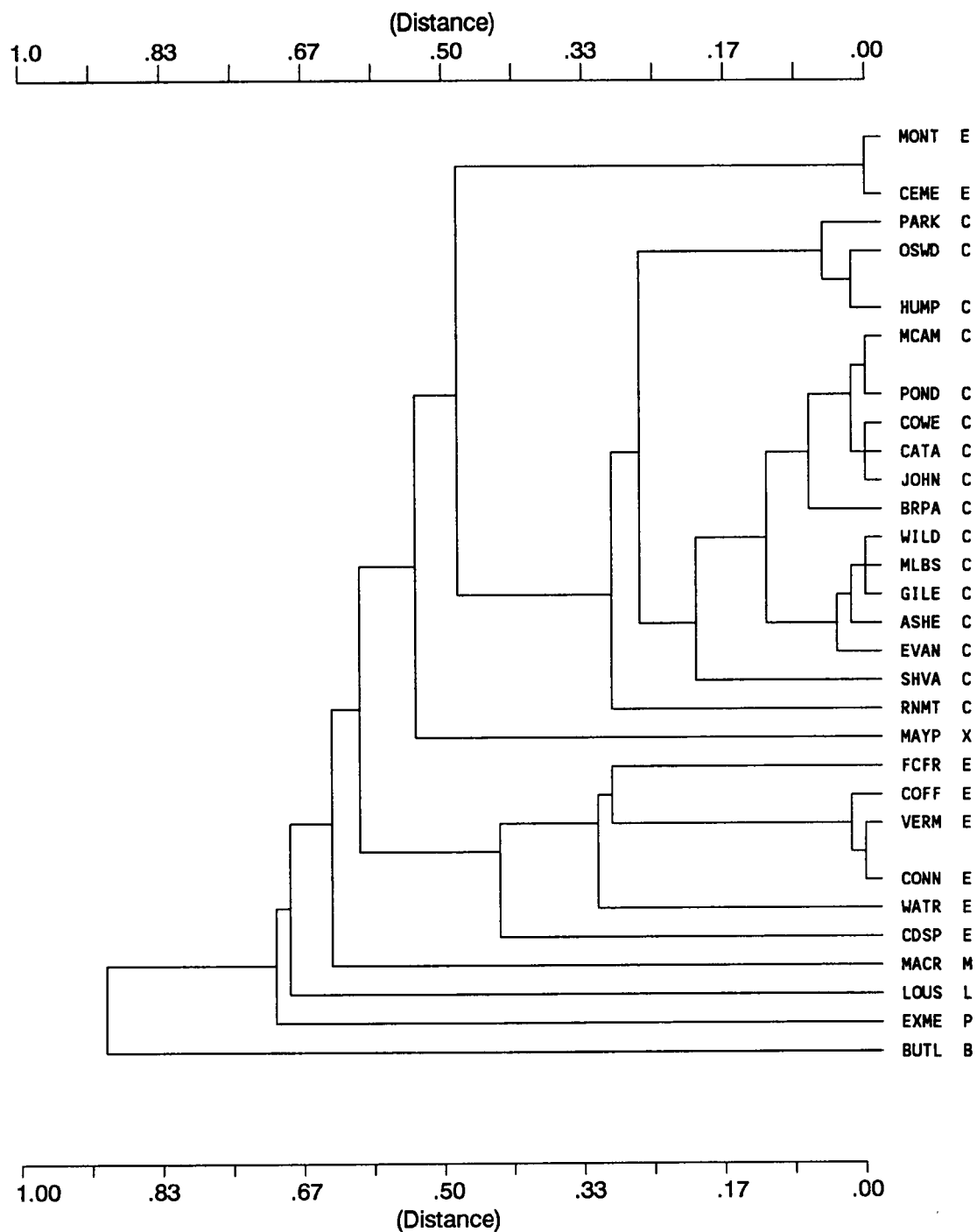


Figure 3. UPGMA cluster phenogram based on Rogers' (Wright, 1978) modified distance. Population codes are given in Table 1. Farris (1972) $\Phi^* = 12.209$, cophenetic correlation = .985. *I. engelmannii* (E), *I. caroliniana* (C), *I. melanopoda* (P), *I. macrospora* (M), *I. butleri* (B), *I. louisianensis* (L), and *I. engelmannii* X *I. melanopoda* (X).

The population from near Manchester, TN (MAYP) clustered with only a 0.761 similarity to the next closest group which was the cluster of the two populations from Putnam Co. TN. It is believed, based on spore ornamentation and vegetative morphology (Chapter IV), that this population represents the result of natural hybridization between *I. engelmannii* and *I. melanopoda*. There was no effect on the grouping patterns when this population was removed and the cluster analysis run again. Also, cluster analysis was run with several partial data sets with one or two loci from the original data set missing without affecting the general pattern of the phenogram with respect to the relationship between the species. Only the position of the MONT, CEME, and MAYP populations was affected, inducing them to group together with either *I. engelmannii* or *I. caroliniana*.

Figures 4 and 5 represent the Wagner distance trees derived from Rogers'(1972) matrix of distances among populations (Table 4). Prevosti (Wright, 1978) and modified Rogers distance (Wright, 1978) matrices were also used to create Wagner distance trees and they showed identical branching patterns. The allozymic distinctiveness of each population is indicated by the number of character state changes defining a terminal population and is proportional to the terminal branch lengths (Swofford, 1981). Branch lengths of *I. melanopoda* (EXME), *I. macrospora* (MACR), *I. butleri* (BUTL), *I. louisianensis* (LOUS), and the Coffee Co. TN (COFF) population revealed them to be distinctive taxonomic entities. Again the populations of *I. engelmannii* clumped together except the MONT and CEME populations, and had variable branch lengths with the Franklin Co. TN (CDSP) population having the longest branch length (most dissimilar). The populations of *I. caroliniana* were all derived from the same node with the exception of three populations (PARK, OSWD, and HUMP) which were derived from the node shown in the populations of *I. engelmannii*. All of the populations of *I. caroliniana* had very short branch lengths indicating a lack of distinctiveness on the part of individual populations.

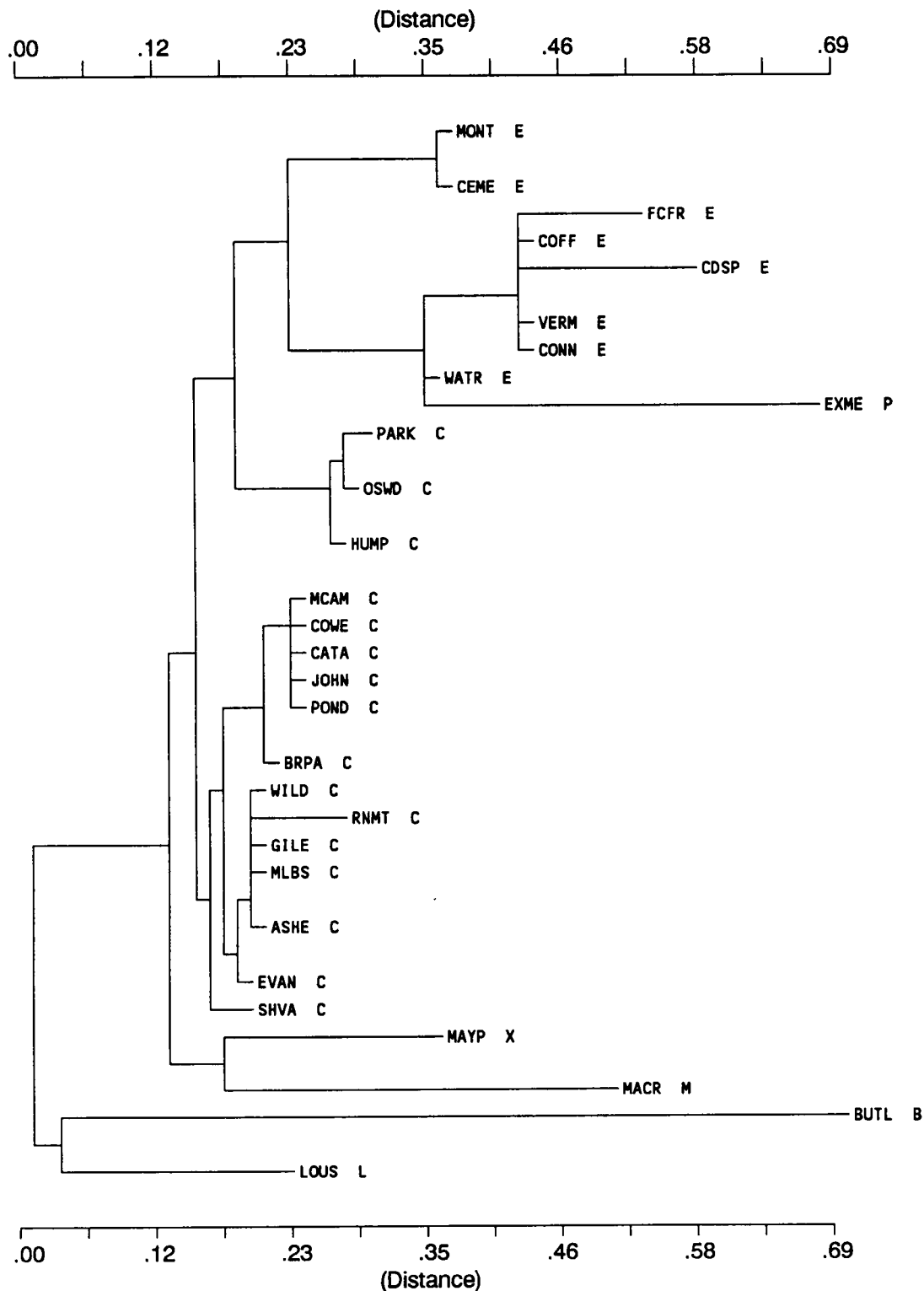


Figure 4. Wagner distance tree based on Rogers' (1972) genetic distance matrix. Total length of tree = 2.860. Cophenetic correlation = .978. *Isoetes engelmannii* (E), *I. caroliniana* (C), *I. melanopoda* (P), *I. macrospora* (M), *I. butleri* (B), *I. louisianensis* (L), and *I. engelmannii* x *I. melanopoda* (X).

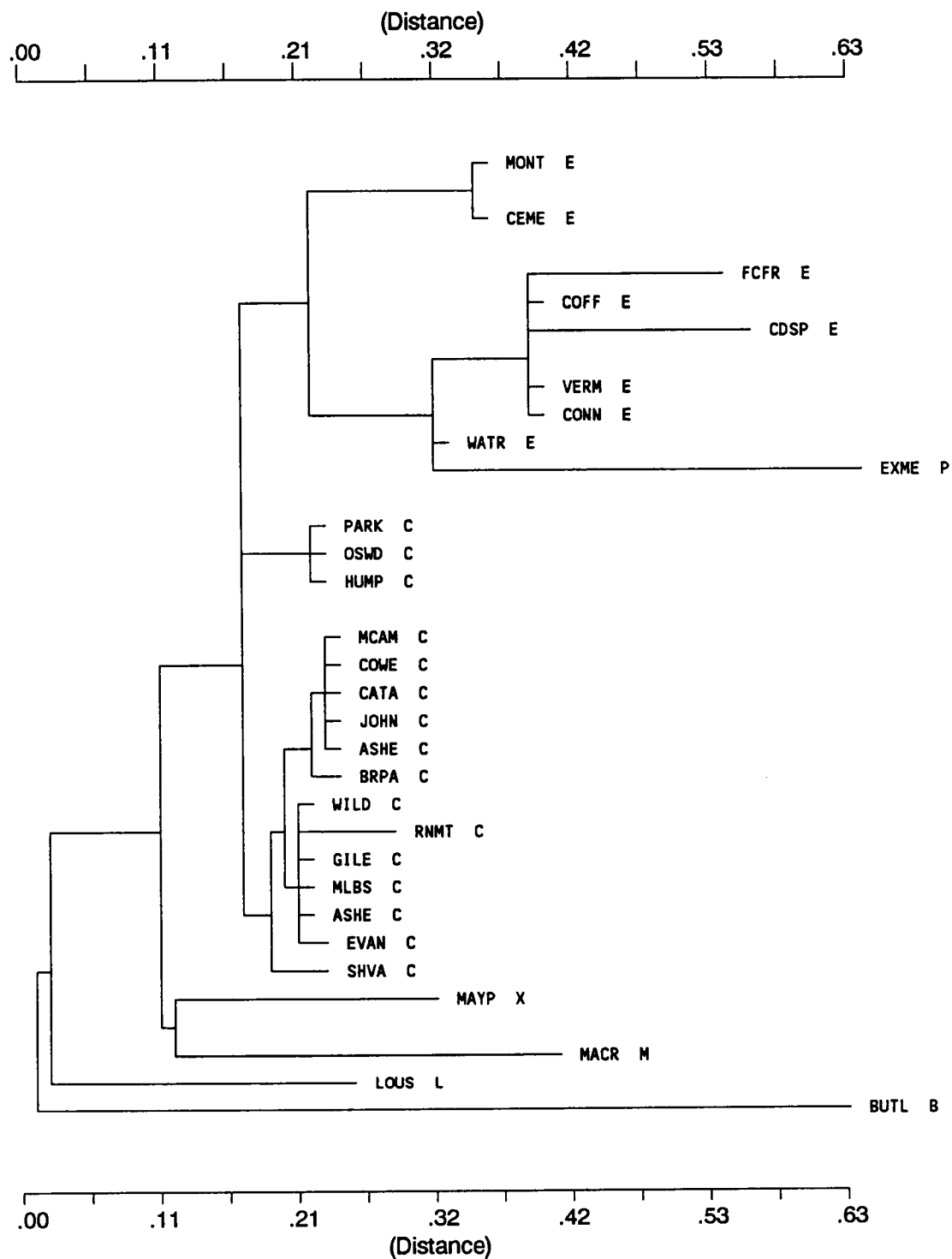


Figure 5. Wagner distance tree based on Rogers' (1972) genetic distance matrix (Table 4) with optimization of branch lengths. Total length of tree = 2.723. Cophenetic correlation = .986, Farris (1972) π^* = 9.908. *Isoetes engelmannii* (E), *I. caroliniana* (C), *I. melanopoda* (P), *I. macrospora* (M), *I. butleri* (B), *I. louisianensis* (L), and *I. engelmannii* x *I. melanopoda* (X).

Discussion:

Electrophoretic data show that *I. caroliniana* is distinct from *I. engelmannii*. Three loci (AAT-1, LAP-1, and TPI-2) may be used to make this distinction and the locus 6-PGD also may be used a marker for *I. caroliniana*. Previously AAT and TPI-2 had been used to distinguish the two taxa (C. Taylor, pers. comm.) but this was based on a limited number of samples of both taxa. In this study many more populations were examined and the distinction between the two taxa has remained consistent.

Interpopulational relationships within the two taxa were quite different. The sixteen populations of *I. caroliniana* were shown to form a coherent group (Figure 4). The RNMT, PARK, OSWD, and HUMP populations were the most dissimilar but were separated by no more than an allelic difference at a single locus (AAT-2 or ALD). The populations interpreted as *I. engelmannii* did not exhibit the coherency that *I. caroliniana* did. The populations initially interpreted as *I. engelmannii* (CDSP, CEME, COFF, CONN, FCFR, VERM, and WATR) did not form a single group (Figures 4 and 5). The MONT and CEME populations formed their own group. The CDSP and WATR populations, although part of the *I. engelmannii* group, were not closely linked with the other members of the group and were more dissimilar to the others than any population of *I. caroliniana* was from one another.

Many electrophoretic studies involving a large portion of the total diversity of the plant and animal kingdoms have been done over the past twenty years which have resulted in a better understanding about the genetic relationship among and within taxa. Reviewing data from the 1970's, Gottlieb (1977) found that congeneric species averaged much lower genetic identities (0.670) than populations of the same species (0.950). Since that time those numbers have changed very little although the range of mean genetic values for congeneric species has increasingly broadened such that it is very important that an investigator have a knowledge of the specimens being examined for the greatest accuracy in interpreting biochemical data.

The genetic identities between populations of *I. engelmannii* and *I. caroliniana* were quite low (avg.=0.690; range of 0.538-0.781) reflecting values of the level often associated with congeneric species (Gottlieb, 1977). The identities of the populations within each taxon also reflect the hypothesized evolution of these species in that the mean genetic identity for populations of *I. engelmannii* is much lower (0.813) than that for *I. caroliniana* (0.954). This is what we would expect of a progenitor-derivative relationship. The relatively low mean genetic identities of *I. engelmannii* and *I. caroliniana* are understandable despite the close relationship of the two taxa: the population biology of these plants is such that interpopulation gene exchange is unlikely and so populations of *I. caroliniana* which are geographically isolated have been evolving separately for a long time. The reduced variability among populations may also be the result of the initial populations being derived from a small number of plants representing a subset of the total gene pool found among all populations of *I. engelmannii* (founder effect).

The genetic identities among populations of *I. engelmannii* (0.813) were far below the expected genetic identities of many congeneric species. The low genetic identity of populations of this species is probably the result of the age of the taxon, the lack of interpopulational gene flow, and interspecific hybridization. *Isoetes engelmannii* is a wide-ranging species in the eastern and coastal United States, and is thought to be the progenitor taxon for many of the other taxa found in the southeastern United States and so represents one of the oldest taxa of *Isoetes* in North America. Lack of gene flow could result in the separate evolution of each population with changes caused primarily by neutral mutations in the gene code and random genetic drift. Lack of specimens from across the whole range does limit the scope of observations that may be derived from this study with respect to population biology. *Isoetes engelmannii* may have been found to be more similar genetically if specimens had been acquired from throughout its entire range. Another possibility is that *I. engelmannii* as we know it today may include more than one species.

Only three loci (AAT-1, PGM-1, and LAP-1) were found to exhibit heterozygosity in any of specimens examined and only three other loci were polymorphic within a single taxon (AAT-2, 6-PGD, and TPI-2). The extremely low number of polymorphic loci and heterozygotes found among individuals may be the result of: 1) the high frequency of inbreeding among individuals within the genus, 2) the very localized nature of the populations and small population size, 3) genetic bottlenecks in the past, and 4) some sample bias made during collection. Inbreeding and isolation from other populations are probably the most important influences on the population genetics of these plants. Two populations found in lakes were very large (WILD, CDSP) and so large samples were taken ($N > 40$). Neither of these populations revealed any polymorphic loci, heterozygotes, or any rare alleles. Analysis of twenty or more individuals from every population would have been desirable but many populations were very small ($N < 15$). The remarkable uniformity of plants within the populations that were collected on a large scale serves to increase the confidence of data derived from such small populations because even analysis of a single plant was likely to reveal most of the possible phenotypes to be found in the whole gene pool of the population.

CHAPTER III

SPORE MORPHOLOGY

History:

Descriptions of spore morphology have historically been the most useful means of characterizing the species of *Isoetes*. Pfeiffer (1922) classified members of *Isoetes* according to megaspore surface ornamentation. Pfeiffer recognized four basic megaspore surface patterns and used those descriptions to establish sections within the genus. Boom, in his thesis (1979), also provides a key for recognizing the southeastern United States *Isoetes* based on spore ornamentation alone. Recently it has been acknowledged that there are many more types of ornamentation than can be described by four simple patterns, and Hickey (1986) has proposed a "more discriminatory megaspore terminology circumscribing twelve defined morphological types." Hickey acknowledges that "although megaspore surface morphology is unreliable in delineating natural subdivision, within *Isoetes*, it does provide a ready means for species sorting and is of great importance in species identification." It has been within these guidelines that all subsequent descriptions of taxa have used this terminology (Kott and Britton, 1983; Taylor et al, manusc.).

Isoetes engelmannii var. *caroliniana* has long been recognized as being distinct from *I. engelmannii* based on spore ornamentation. Clute (1928) recognizes that *I. engelmannii* var. *caroliniana* is distinguished by "megaspores (that) are marked by thin and broken crests which often present spinules like those of *I. echinospora* and have few if any reticulations." Boom (1979) doesn't recognize the North Carolina variety of *I. engelmannii* but feels that those specimens annotated as such are most likely the result of hybridizations of *I. engelmannii* with the Carolina coastal species, *I. riparia* (the taxonomic history of the two taxa is reviewed in Chapter I).

Materials and methods:

All collections made for the electrophoretic study were also collected with the retrieval of spores in mind and mature megaspores were retrieved from most sites (mature spores can be gathered at almost any time in the year by taking soil from around the base of the plants in which spores from the previous growing season can usually be found). Also Carl Taylor graciously sent samples of spores of both *I. engelmannii* and *I. caroliniana* from various locations in the eastern U.S. (Appendix A) and specimens from the University of Tennessee Herbarium (TENN) were also examined. Examination of spores was by light dissection microscope (spore sizes ranged from 350 μ m to 600 μ m) and all measurements of spores were made by measuring the width across the equatorial crest. Figure 6 is a diagram of a generalized *Isoetes* megaspore, as adapted from Boom (1979). Often the proximal and distal faces of the megaspore revealed differences in their ornamentation (for example: MLBS, OSWD, MONT, and CEME).

Results:

Table 6 lists the size and ornamentation of the spores from populations collected in which more than one plant was examined per population. These data reveal that spore size was quite variable from one population to another among both *I. engelmannii* and *I. caroliniana* and that populations of *I. engelmannii* had the greatest variability between and within populations. Despite the variability in size there was no apparent difference in the size of spores between *I. engelmannii* and *I. caroliniana*.

Table 7 lists observations of spore ornamentation from some of the collected populations (some collected populations did not reveal any mature megaspores) and from examined herbarium specimens. All terminology regarding megaspores was taken from Hickey's (1986) recent examination of the importance of spore morphology in this genus. Specimens other than those recorded in Table 7 were examined and are recorded in Appendix I. Figures 7 and 8 show

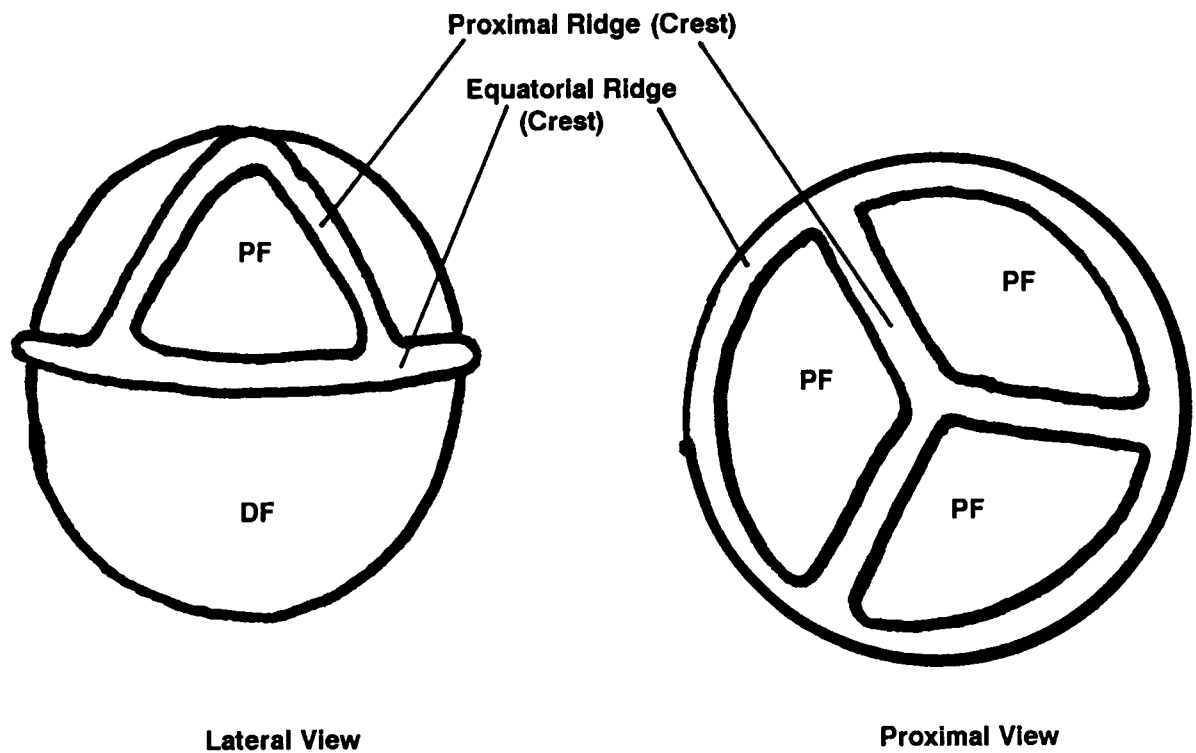


Figure 6. Generalized diagram of the megaspores of *Isoetes*. Redrawn from Boom (1979) and using terms from both Kott and Britton (1983) and Boom (1979). PF = proximal face (hemisphere); DF = distal face (hemisphere).

TABLE 6: Measured megaspore sizes, standard deviations, and characterization of megaspore ornamentation of *Isoetes* species using terminology established by Hickey (1986).

Species	Population ¹	N	Ave.(μ m)	SD	Megaspore Ornamentation
<i>I. caroliniana</i>	COWE	10	491	± 22.2	Cristate
	WILD	16	404	21.8	Cristate-Echinate
	ASHE	15	450	5.2	Cristate-Echinate
	JOHN	10	420	52.1	Cristate
	BRPA	10	378	23.6	Cristate-Reticulate
	CATA	20	520	111.1	Reticulate-Cristate
	WATR	20	494	31.0	Cristate
	MCAM	10	415	20.0	Cristate-Reticulate
	OSWD	20	495	25.1	Cristate
	POND	10	469	20.7	Cristate
	GILE	20	449	28.3	Cristate-Echinate
	MLBS	5	400	30.0	Cristate-Reticulate
	PARK	7	411	18.2	Cristate-Reticulate
<i>I. engelmannii</i>	MAYP ²	11	490	35.2	Reticulate-Tuberculate
	MONT	10	450	10.0	Reticulate
	CEME	5	450	5.0	Reticulate
	FCFA	20	488	56.5	Reticulate
	CDSP	20	558	40.6	Reticulate

¹ Population abbreviations as used in Table 2 on back page.

² This population is believed to be a hybrid of *I. engelmannii* x *I. melanopoda*.

TABLE 7: Observed megaspore ornamentation of collected specimens and/or herbarium materials of *Isoetes*. All terminology conforms to terminology established by Hickey (1986).

County ¹	State	Spore Size ²	Spore Ornamentation,Etc....
<i>I. caroliniana</i>			
Heywood(CATA)	NC	*520 μ m	Densely Echinata-Cristate
Stoke	NC	450	Cristate, Jagged crests
Avery(WILD)	NC	*454	Cristate-Echinata
Ashe(ASHE)	NC	*450	Cristate-Echinata
Ashe(POND)	NC	*469	Cristate-Echinata
Macon	NC	453	Cristate-proximal face, Reticulate-distal face
Macon(Coweeta)	NC	*491	Cristate
Monongalia	WV	400	Cristate-Echinata
Giles	VA	450	Cristate, Jagged crests
Giles(MLBS)	VA	*400	Cristate-distal face, Reticulate-proximal face
Giles(GILE)	VA	475	Cristate-Reticulate
Franklin(BRPA)	VA	*378	Cristate-Reticulate
Allegheny	VA	450	Cristate, Jagged crests
Sevier	TN	500	Cristate-Reticulate, Spore size varies greatly
Washington(JOHN)	TN	*420	Cristate
Polk(OSWD)	TN	*495	Cristate-proximal face, Reticulate-distal face
Polk(PARK)	TN	*411	Cristate-Reticulate
Polk(MCAM)	TN	*415	Cristate-Reticulate
<i>I. engelmannii</i>			
Putnam(MONT)	TN	*490	Reticulate-distal face, Echinata-proximal face
Putnam(CEME)	TN	*450	Reticulate-distal face, Echinata-proximal face
Marion	TN	400	Finely Reticulate, Spores are dull yellow
Marion(Snake Pd.)	TN	500	Reticulate, High equatorial crest
Fentress	TN	500	Reticulate
Coffee(COFF)	TN	450	Reticulate
Coffee(MAYP)	TN	*490	Retuclulate-Tuberculate

Table 7: (cont.)

County ¹	State	Spore Size ²	Spore Ornamentation, Etc....
<i>I. engelmannii</i> cont.			
VanBuren(FCFR)	TN	*488 μ	Reticulate
VanBuren	TN	450	Reticulate
Grundy(Reservoir)	TN	450	Reticulate
Grundy	TN	525	Reticulate
Cumberland	TN	500	Reticulate
Franklin	TN	500	Reticulate
Franklin(CDSP)	TN	*558	Reticulate
Sullivan	TN	500	Sparsely Reticulate
Gadsden	FL	580	Reticulate
Conecuh	AL	500	Echinate-Cristate, highly variable spore size
Marshall	AL	471	Reticulate
Freeman	AL	475	Reticulate
Tolland	CT	525	Reticulate
Luzerin	PA	475	Reticulate
Allegan	MI	550	Reticulate
<i>I. louisianensis</i>			
Washington Parish	LA	550	Reticulate (dense ridges), brown-spotted sporangial wall
<i>I. macrospora</i>			
Polk	TN		Reticulate
<i>I. melanopoda</i>			
Cleburn	AR	400	Tuberculate, unpigmented sporangia
Piatt	IL	375	Tuberculate, black etching on sporangial wall
Franklin Parish	LA	325	Tuberculate, grey sporangial wall
Ouachita Parish	LA	375	Tuberculate, brown spotted sporangial wall
Grundy(EXME)	TN	*400	Reticulate-Tuberculate

Table 7 (cont.)

¹ More complete citations are given in Appendix A.

² Spore sizes that come directly from Table 3 are denoted by an asterisk (*), all other spore sized are approximations made by examination of herbaria material.

the distribution of collected populations for electrophoresis and specimens examined from herbarium material for megaspore characteristics.

Examination of the megaspore ornamentation of *I. caroliniana* showed almost none of the organization that is so characteristic of *I. engelmannii*. The spore exines of *I. caroliniana* appeared very rough and disorganized with respect to *I. engelmannii* and fell within a range of variation that went from the presence of ridges that form weakly organized areoles (cristate-reticulate), to the presence of ridges that appear as thin muri that are taller than they are broad and don't form organized areoles creating a scattered appearance (cristate), to the presence of symmetrical projections that are taller than they are broad mixed with thin muri (cristate-echinate). The spores of *I. engelmannii* exhibited even more variability in appearance from population to population especially with respect the density of the reticulations. The variability observed within populations was also much greater than those of *I. caroliniana* which had little to no observable variation in spore ornamentation within a single population. The crests of the megaspores of *I. caroliniana* were often uneven or jagged in appearance in contrast to those of *I. engelmannii* which were always observed to have even crests.

The megaspores of *I. engelmannii* are generally characterized as being textured with a thin, uniform reticulum with distinct proximal and equatorial crests. Their megaspores are white and are born in occasionally pigmented sporangia. None of the sporangia of those plants interpreted as *I. caroliniana* were observed to have any pigmentation. Figure 9 shows pictures of the megaspores of *I. engelmannii* and *I. caroliniana*.

Several specimens of *I. caroliniana* exhibited different ornamentations on distal or proximal faces of the megaspores (Macon Co.: NC; MLBS; Cades Cove, TN; and OSWD). The MONT and CEME populations also exhibited this dual ornamentation pattern and in this case the difference was striking because the proximal face was echinate (ridges were as wide as they were long and so appeared as spikes) and the distal face was reticulate (organized ridges). For these two

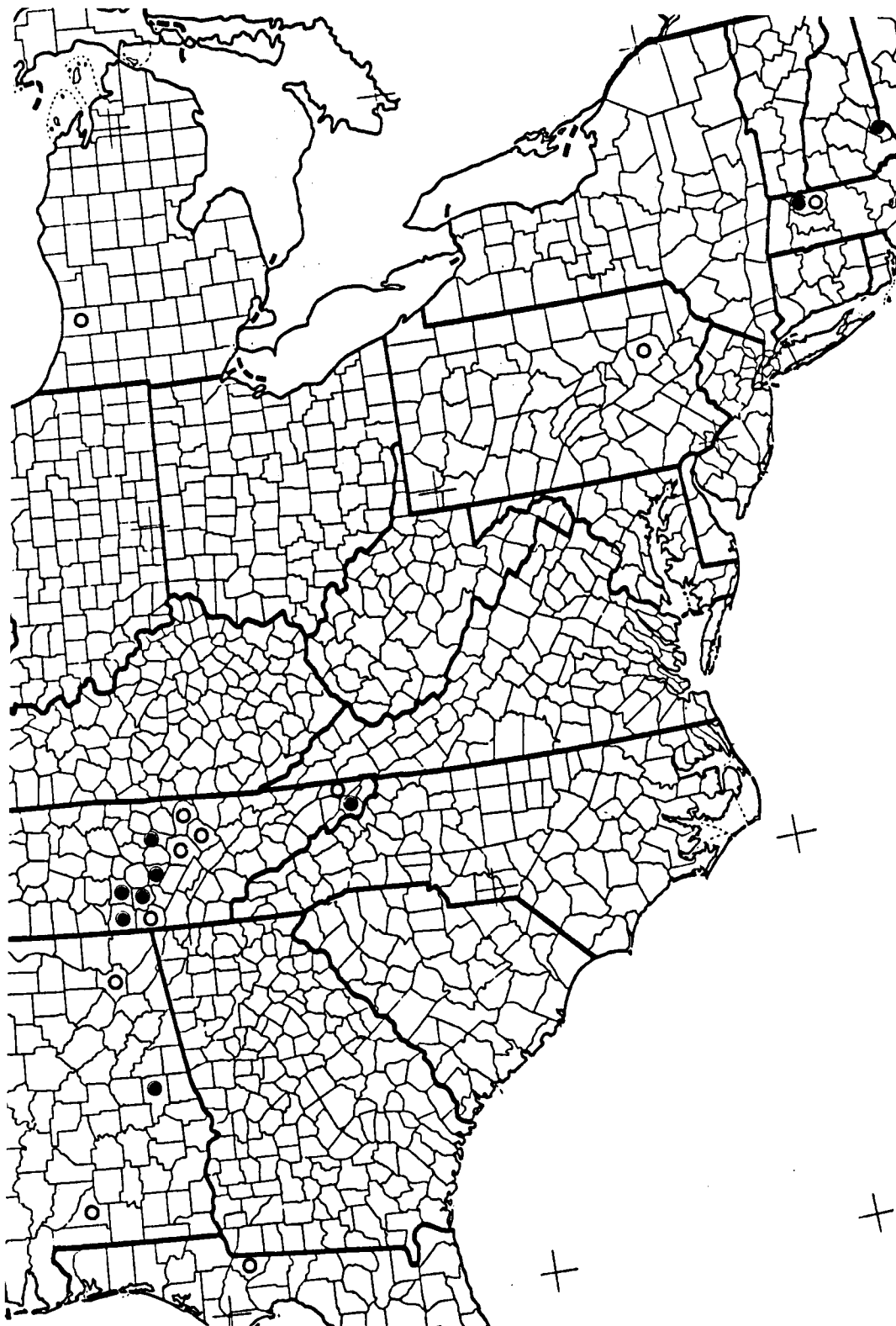


Figure 7. Distribution of collected populations of *Isoetes engelmannii* for electrophoresis (●), and specimens examined for megaspore ornamentation (○).

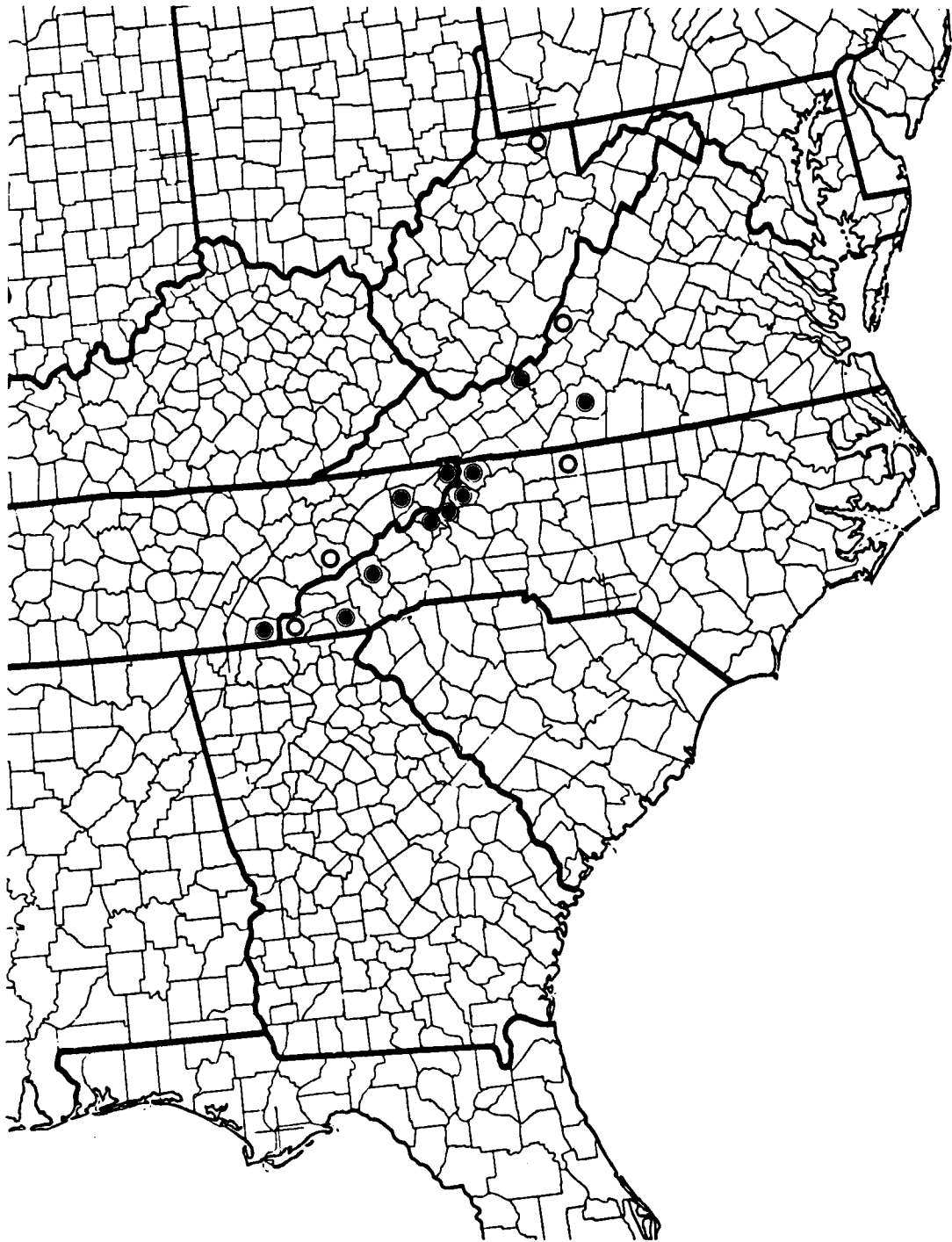


Figure 8. Distribution of collected populations of *Isoetes caroliniana* for electrophoresis (●), and specimens examined for megaspore ornamentation (○).

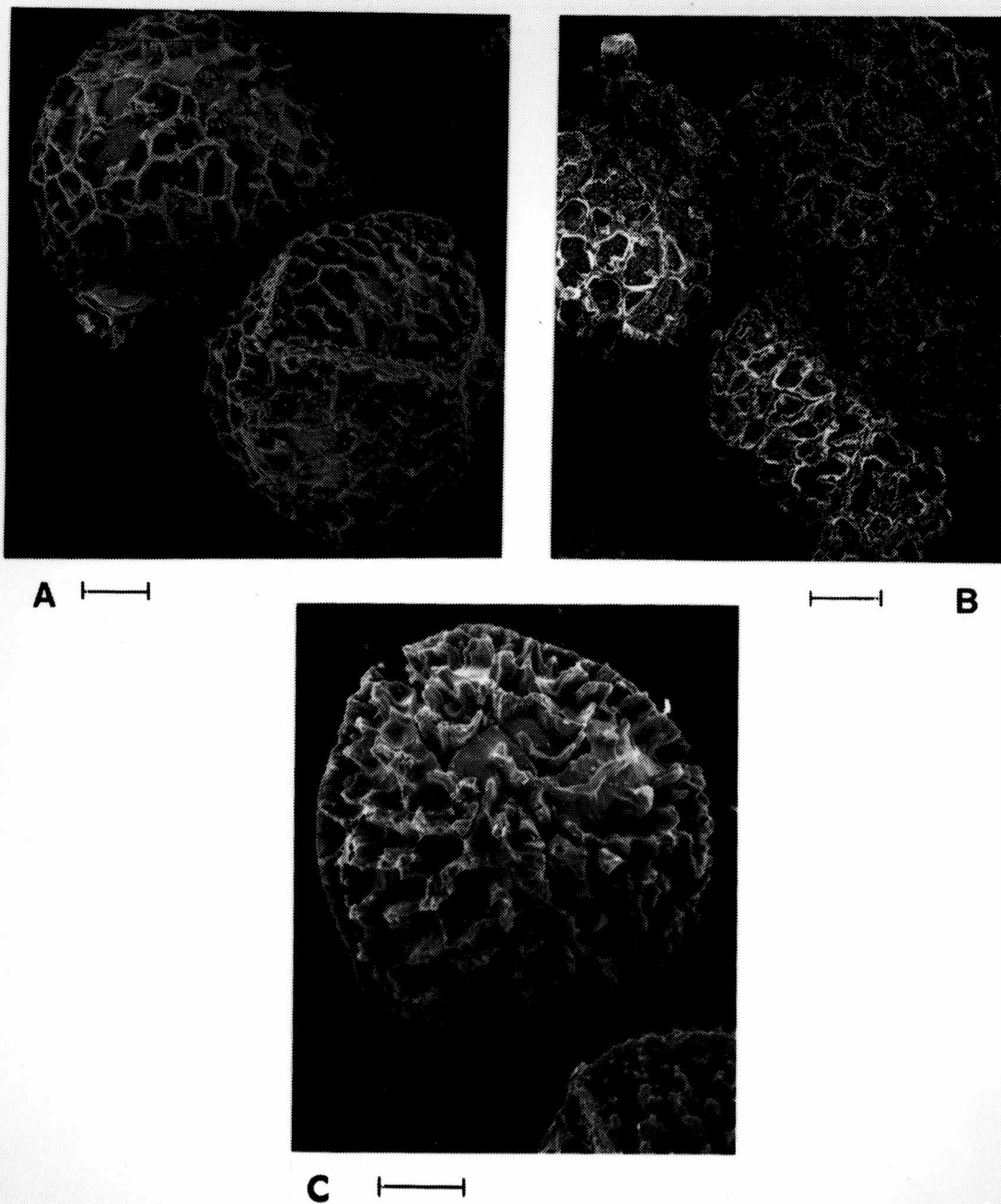


Figure 9. SEM micrographs of megaspores of *Isoetes engelmannii* and *I. caroliniana*. (A) *Isoetes engelmannii* from Fall Creek Falls Reservoir, VanBuren Co.: TN (FCFR), (B) *I. engelmannii* from the Watauga River in Washington Co.: TN (WATR) and (C) *I. caroliniana* from Stokes Co.: NC. Lines represent 100μm.

populations the dual ornamentation pattern is postulated to be the result of hybridization between *I. engelmannii* and *I. caroliniana*.

Discussion:

Megaspore-based classifications have provided for the most efficient means of differentiation among taxa of *Isoetes*. The presence of consistent differences in megaspore ornamentation of populations of *I. engelmannii* and *I. caroliniana* provide a traditional basis for confirming the taxonomic separation of these two taxa. It also provides for a method of identification that doesn't rely on extensive laboratory work.

The use of megaspore ornamentation is complicated by several factors. First, the use of immature spores may lead to the incorrect identification of a plant. Immature megaspores have a mealy outer wall which is easily broken or removed and will rupture upon contact with any dissection instrument (Matthews and Murdy, 1969). The silica wall of the immature spore may also exhibit a different ornamentation than the mature spore. Collectors are cautioned to collect late in the growing season of each taxon or preserve soil about the "corm" which often will contain leaf bases and spores from the previous year. Secondly, variation in megaspore size and ornamentation indicates that megaspore morphology is liable to exhibit convergence of characters. Recent studies by Taylor *et al.* (1985), Boom (1979), Cox (1983), Kott and Britton (1983), Hickey (1985, 1986), and Cox and Hickey (1984) have postulated that convergence may result from limited genetic potential, selection by similar habitats, and hybridization. *Isoetes caroliniana* could show very consistent megaspore morphology because of similar habitat rather than because of genetic uniformity and the variability among *I. engelmannii* populations could be the result of different environmental (selectional) pressures although allozyme data indicate that this is probably not the case.

Thirdly, the megaspore ornamentation of *I. engelmannii* has been documented (Eaton, 1900b; in Hickey, 1986) to exhibit variability along a north-south transect. Lastly, hybridization has

been reported (Boom, 1979; Taylor and Leubke, 1986, 1988; Leubke and Taylor, 1985; Taylor et al, 1985) to occur between almost any two taxa of *Isoetes*. This can create confusion because of the intermediate spore characters that it may introduce into a taxon.

The occurrence of different megaspore ornamentation, although not without flaws, is a convenient means of species identification. The consistent differences observed between the megaspores of *I. engelmannii* and *I. caroliniana* may be used effectively to distinguish the two taxa although these differences alone are not the sole reason for the distinction of the two taxa.

CHAPTER IV

MORPHOLOGY AND ECOLOGY

Introduction:

Kott and Britton (1983) began their paper on spore morphology and taxonomy of *Isoetes* by saying: "to accept any concept of species in the genus *Isoetes* L. one must first accept that perhaps the only constant in this group is its variation." This sentiment has been echoed by many other investigators. All of the relatively few morphological features of the quillworts such as habit, leaf morphology, ligule, velum, sporangium, and ecological habitat have at one time or another been used to circumscribe and describe the range of species variation with some degree of success. The reason for the failure of morphological data in many cases has been that so little is known about the effect of water depth, episodic drying, temperature, pH, or nutrients on the morphology of *Isoetes* plants, including their spores.

Isoetes engelmannii has been characterized as living in acidic soil with many growth forms ranging from aquatic to emergent, to practically a terrestrial habit. Leaf shape, color and texture can vary tremendously between and even within populations [Taylor et al., (manusc.) may be referred to for the most recent description of specific morphological features]. Clute (1928) recognized *I. engelmannii* as being part of his group of amphibious quillworts. He used the term amphibious to describe those taxa which may be most commonly found along rivers and streams where receding waters may leave them exposed to the air for at least part of the year. He describes *I. engelmannii* as being very diverse in their habitat and morphology. When found in the "quiet waters of lakes and ponds their leaves are inclined to be short and arranged in a distinct rosette; but in rivers where the species are exposed to more or less current the leaves are likely to be longer..."

As a variety of *I. engelmannii*, *I. caroliniana* was described with emphasis on why it should be considered different from *I. engelmannii* var. *engelmannii* and that emphasis usually took the

form of descriptions of spore morphology with little emphasis placed on habitat preference and leaf morphology. The range of variability in other morphological features that has been attributed *I. engelmannii* has never been attributed to *I. caroliniana*. The variety has been described most commonly as having robust erect leaves [except Pfeiffer (1922) who describes the leaves as being thinner than those of var. *engelmannii*] that are dark green with visible cross-hatching in the leaves and inhabiting the bogs, seeps, and ditches in upland North Carolina and southern Virginia.

Observations:

Observations including pH of ground water, soil condition, plant habit and biotic factors were made at each site as collections were being taken for electrophoresis. Some of these observations are recorded in Table 8. Plants from many of the collected populations of *I. engelmannii* and *I. caroliniana* were grown in aquaria for up to eighteen months. The plants which were grown exhibited different morphologies or rates of growth depending on the season (amount of light), pH of the water, and the amount of water present.

Clute's (1928) observations of the phenology of *I. engelmannii* were also confirmed during the time of this study. It is clear that the plants of *I. engelmannii* an annual cycle of producing long flaccid leaves in the spring and a set of shorter recurved leaves later in the fall. Usually the spring leaves bear the sporangia and the leaves decay readily upon lowering of water levels in the summer at which time the shorter leaves with numerous stomata replace them. Most of the observed populations of *I. engelmannii* exhibit some version of this pattern depending on their particular habitat. Seasonal changes in leaf morphology in plants from the MONT and CEME populations were observed. They were growing completely emergent, and they maintained erect leaves all year but alternated between longer luxuriant leaves and shorter recurved darker-green leaves arranged in distinct circles. All of the other populations of *I. engelmannii* that were grown

TABLE 8: Habitat and plant descriptions of plants from collected sites. Population codes are explained in Table 1.

Population	Habitat Description	Plant Habit	pH
<i>I. caroliniana</i>			
COWE	Ditch with slow moving water, wet year-round, numerous rushes	Emergent from edges of water, corms up to seven centimeters below surface	5.6
GILE	Plants at the edge of an open sphagnum bog in small stream	Emergent from water and from moist areas at edges	6.1
MLBS	Small man-made lake	Emergent and submerged in lake	6.2
CATA	Plants growing in open areas leading away from a seep.	Emergent from water, flaccid leaves in winter	6.0
EVAN	Sphagnum bog with pine overstory	Leaves in relaxed position growing from sphagnum moss	5.9
RNMT	Open seep on the side of Roan Mt., elev. 5600 ft.	Leaves short, emergent from sphagnum moss	5.9
HUMP	Open seep on the side of Hump Mt., elev. 5000 ft.	Emergent from edge of seep, leaves shorter than avg.	5.7
WILD	Manmade lake with sandy bottom, extensive population	Completely submerged under one to five feet of water	5.8
POND	Farmpond with constant water level, muddy water on both visits	Most plants completely submerged but some emergent	6.5
ASHE	Small cement fish pond in open lawn area	Leaves lax in shallow water, spring and fall	6.4
SHVA	Ditch between road and pasture area, dry in the summer, thick grass late in the year	Emergent from thick algal mat, leaves long	6.1
JOHN	Seep in the center of trail underneath white pine trees	Leaves long and lax, covered with leaves in the fall	6.0
PARK	Hard clay on tail end of a small island, plants very local below low water mark	Plants small and completely submerged	5.6

Table 8 (cont.)

Population	Habitat Description	Plant habit	pH
<i>I. caroliniana</i> cont.			
OSWD	Madmade lake with sandy bottom, elev. 3800 ft., no plants growing above the low water mark	Emergent from edge of lake when water level is low	6.1
MCAM	Calm waters of confluence of stream with reservoir, rooted in heavy mud	Leaves flaccid where shaded, and emergent where open	6.2
BRPA	In stream leading away from seep, shaded area	Leaves flaccid as plants grow from water edge	6.1
<i>I. engelmannii</i>			
CEME	Ditch along road with hard clay and gravel bottom, plants rooted along edge with many grasses, dry in the summer	Emergent from waters edge,	5.2
MONT	Abandoned quarry in wet depression which fills with water in the spring, probably never dries out completely	Emergent or submerged	5.7
FCFR ¹	Open area along the edge of reservoir in area just below the low water mark to about ten feet under the water	Completely submerged, small plants with short leaves	5.5
MAYP ²	Ditch with abundant grasses, dry in the summer	Leaves long and flaccid in moving water, only large plants found in the summer	6.0
COFF	Large depression in roadside ditch with water lilies, dries out in the summer	Leaves long and flaccid in spring, plants not found in late summer	6.3
CDSP	Manmade pond fed by spring, plants abundant	Leaves long and flaccid in water, a few plants emergent near springs edge	6.1

Table 8 (cont.)

Population	Habitat Description	Plant Habit	pH
<i>Isoetes macrospora</i>			
MACR	Rocky bottom in the fast moving waters of the Hiawassi River	Leaves short and thick, completely submerged,	6.7
<i>Isoetes melanopoda</i>			
EXME	Stagnant water in roadside ditch with many grasses, dries out in the summer	Leaves twisted and wiry, emergent from waters edge, not found in the summer	6.8

¹ This population was not submerged when the reservoir was intentionally lowered in January 1991 at which time the plants exhibited a relaxed nature; the larger leaves decayed and only the newest leaves remained active.

² This populations is believed to be a hybrid of *Isoetes melanopoda* x *Isoetes engelmannii*.

in culture were grown completely submerged and had distinctly lax wiry leaves which could not support themselves if the water level was lowered.

Stomatal formation is also variable among and within taxa of *Isoetes*. Leaves of both *I. engelmannii* and *I. caroliniana* grown as emergents had more stomata in their leaves, especially those of *I. caroliniana*. Leaves grown submerged were found to quickly dry and shrivel up when exposed to the air. *Isoetes macrospora* plants, lacking stomata, shriveled up soon after being exposed to the air and did not exhibit new leaf growth.

Relative to *I. engelmannii*, populations of *I. caroliniana* exhibit a much more robust growth habit and their leaves are a dark green dilated toward the base and gradually tapered to the tip of the leaf. The leaves have a visible cross-hatching caused by the presence of peripheral fibrous bundles. Most populations of this species found in North Carolina and Virginia were found at sites which one could broadly characterize as being seeps, bogs, or ditches although a few were found in small ponds (ASHE and POND). In all cases the opportunity existed for the plants to attain an emergent habit. Only in populations ASHE and POND were the plants completely submerged at the time of collection and upon contact with the atmosphere the leaves of these plants dried up but new leaves developed when cultivated as emergents. This sort of habit in which the plants may grow either under or out of the water is referred to as being "aquatic-emergent". This is contrasted with most of the collected populations of *I. engelmannii* from the Cumberland Plateau of Tennessee which were unable to maintain an erect leaf habit even when new leaves were allowed to grow out from the water. *Isoetes engelmannii* seems to be primarily an aquatic taxon with the genetic capacity to grow or adapt to an emergent, or even seasonal, environment when necessary.

Although the morphological variability of *Isoetes* in general is quite subtle and the full range of the variability of *I. engelmannii* has not been personally observed, it is apparent that there are distinct genetic differences between many of the populations such that the variability can not be simply explained as being phenotypic adaptations. This is readily apparent when the

plants from many different populations are grown together in an aquarium under very similar conditions. Many populations maintained distinct morphologies such as the flaccid versus the more erect leaf habit or the leaves being longer or shorter, light green or dark green, wiry and thin or broad and thick.

Almost every population of *I. caroliniana* was observed to grow in very specific habitats: plants from the COWE population were found only in a short part of a ditch which was both open and wet all year; plants from the GILE population were found only at the edge of an open sphagnum bog growing in a small stream; and plants from the CATA population were found only in open places in the slow moving water where few grasses were growing; the EVAN population was found not far from the CATA population in a boggy wooded spot without moving water in sphagnum moss. When found in ponds and lakes the plants were found at a more specific depth or location along the banks than specimens of *I. engelmannii*. Other examples of ecological specificity are given in Chapter V.

The COFF population is a good example of the adaptability that some populations of *I. engelmannii* exhibit that is not observed in populations of *I. caroliniana*. The site at which the COFF population was found was a depression beside Highway 41 in Coffee Co., TN. This site undergoes many changes throughout the period of a couple of years. When the site was visited for the second time in August of 1990 it was completely dry whereas the ditch had been filled with several feet of standing water in the spring. The plants were found growing only in the deepest depressions where the moisture would be retained for the longest period of time but a period of several weeks without rain could dry the site out completely. By digging through the dry clay many hardened corms were retrieved. Upon introduction into the aquaria in the laboratory the corms produced many new leaves in a matter of a few days. After two weeks, each of the plants had developed more than thirty new leaves. This initial burst of growth was followed by six months of almost no new growth which ended with the decay of the leaves and the releasing of spores after which new leaves began to develop. In nature, the plants of this population are

probably adapted to quick reproduction when there is water available and then the storage of food in the corm for the next wet period. It is suspected that not all populations of *I. engelmannii* are as well adapted to as diverse a ground water cycle as those of the COFF population. No other collected population exhibited the woody corm development that is present in the COFF population. None of the sites where *I. caroliniana* was found is likely to ever experience periods of desiccation and so are not expected to have acquired this characteristic, but experimental dessication of plants or long term site observations were not explored.

The growth of plants in culture represents a very limited venture which was not done under highly controlled conditions but it does provide insight into the phenology and development of the plants. No in-depth observations need be made to realize that the variability observed in the field is not just a response to the environment but is also established in genetical variability.

CHAPTER V

DISCUSSION

A total of 29 populations of *Isoetes* was examined to determine the taxonomic status of the proposed species; *I. caroliniana*. Electrophoresis, spore ornamentation, and plant habit all support the distinction of *I. caroliniana* from *I. engelmannii*. This same conclusion may not have been made ten years ago with only morphological data but the additional evidence of electrophoresis and a greater understanding of the phylogeny of *Isoetes* support the recognition of *I. caroliniana* as a separate taxon.

Several questions need to be addressed concerning the species concept in *Isoetes* and the relationship of electrophoretic and morphological data. The first question raised in this study is about the apparent morphological variability exhibited by many populations, especially of *I. engelmannii*. Is this variability simply the result of phenotypic plasticity in response to environmental change, and therefore an unreliable set of characters to be used for taxonomic purposes, or is the variability the result of direct genetic differences among the plants? Secondly, how has speciation occurred and do the data presented here support the current concepts of speciation within the genus?

Genotypic verses Phenotypic variation:

The low genetic identity among all populations of *I. engelmannii* (0.813, see Tab. 5) correspond well with the observations that they are more variable morphologically than populations of *I. caroliniana* (0.954). Both electrophoretic and morphological data exhibit similar patterns of variability, but the relationship of electrophoretic data to morphological data is not always so apparent. There are several examples discussed below that demonstrate some perplexing relationships of morphology, electrophoresis, and environmental influence.

In Polk Co., TN three populations were found (PARK, OSWD, and MCAM) all of which

consistently revealed electrophoretic banding patterns, and megaspore ornamentation, which were identical to populations of *I. caroliniana* in North Carolina, except for the AAT-1 locus in PARK and OSWD populations. Yet these populations exhibited distinctly different morphologies in the field even though no population was found more than three miles from another. Plants from OSWD were found at the border of a manmade lake and were growing mostly submerged and those leaves which were emergent were rising erectly from the water. Plants from the Parksville reservoir, Greasy Creek outlet (PARK), downstream from OSWD, were completely submerged and were not found in areas which would be exposed for any time during the year except for when the reservoirs' water level is drawn down in the winter by TVA (Tennessee Valley Authority). Plants from PARK were all quite diminutive each time that the population was observed (May, August, March, and June). These plants were only found in an area of hard clay at one end of an island where the current of the stream would not disturb them. Plants from MCAM, also found in the Parksville reservoir, were rooted in heavy mud in the quiet waters at the confluence of the McCamy branch and the Parksville reservoir. These plants were some of the largest observed from any of the collected populations in this study. They had long leaves (up to 60 cm) which lay lax in the water with only a few of the youngest leaves growing erect from the water.

These three populations demonstrate the difficulties facing the taxonomist in this genus. As previously mentioned all of these populations exhibit very similar electrophoretic banding patterns which is not unexpected because of their proximity to one another and especially because they are all found within the same water drainage system. The three populations appear to be distinct morphologically upon first inspection but several observations can be made. The plants from OSWD were found emergent from the lake and the plants from MCAM were almost completely lax in the water but their condition doesn't necessarily represent genetic differences because the water level in the Parksville reservoir was quite low when the MCAM plants were observed. The leaves which were lax developed under completely submerged conditions. When submerged the peripheral fibrous bundles of the leaves which would aid them to remain erect

might be less well developed. Plants such as those at OSWD apparently have been growing close to the surface of the water or emerged for some time and so may have been stimulated to strengthen the bundles which would allow them to grow in such an erect manner. Also MCAM plants were growing under partial shade and the OSWD and PARK plants were in complete sunlight. The Parksville plants were found completely submerged at all four visits. The clay substrate in which they were growing was distinctly different from the soils of the other two sites, and that could possibly explain the small stature of the plants.

The morphological differences between these populations could be explained as being ecologically induced phenotypic variation and thus be the result of different expressions of plants with the same genetic capacity. When representative of these populations are grown in an aquarium under the same soil, light, pH, and water level conditions we would expect that these plants might exhibit more similar growth habits. This was not the case. For more than a year the plants from PARK never grew much larger and in fact did not grow well in the aquarium. The MCAM plants became more robust but new leaves never grew as robust as those of OSWD although the leaves grew out to a much longer length. In short, the plants retained many of the distinctive features that were present in the habitats from which they came.

This brings up the question of whether the differences observed are ecologically induced or are directly related to the genetic differences. In this case, if the differences are the result of changes the amino acid sequence of the enzymes themselves, electrophoresis was unable to detect this change and we must assume that the set of enzymes that were analyzed in this electrophoretic study are not important with respect to the morphological characters which are observed.

Several observations were made which support the concept of morphological differences based solely on ecological conditions. The plants from the JOHN population were found in a boggy area underneath a thick cover of white pines. The plants had long lax leaves which lay in the muddy substrate. When some plants were transferred to the lab aquarium the new leaves

which grew from these plants were extremely robust and grew up to a length of 50 cms. It was likely that lack of sunlight was what led the plants in the field to exhibit the lax leaf condition. Other examples of this same phenomenon were those plants from CATA, EVEN, and BRPA. In the case of the ASHE, POND, and WILD populations the plants were all found submerged in ponds but when grown in the lab the new leaves became more robust and grew emergent. All of these populations have been demonstrated to represent members of *I. caroliniana*.

Some of the populations of *I. engelmannii* also exhibited a lax leaf habit such as those from COFF and FCFR and when the plants were transferred to the lab they maintained the same form and did not grow erect or emergent under any of the laboratory conditions. These plants have obviously lost or never had the genetic ability to produce the anatomical structures which would allow them to produce erect leaves.

While the generalization has been made that all the plants of the taxon *I. caroliniana* have been found to be able to produce an emergent nature, no single generalization can be made for those plants of *I. engelmannii*. This fits well with the assumption that *I. caroliniana* has split from the parental taxon, *I. engelmannii*. *Isoetes engelmannii* exhibits more variability on all levels studied, (electrophoresis, spore ornamentation, leaf morphology) than *I. caroliniana*. All the leaf characteristics of *I. caroliniana* can be found somewhere in the range of variability of *I. engelmannii* and, as previously discussed, the patterns observed in *I. caroliniana* can be explained as being a partial set of that of *I. engelmannii*.

Species concept and *I. caroliniana*:

Many species of *Isoetes* that consist of only a few populations usually have distinct morphological and spore ornamentation characters. The distinctiveness of these species, such as *I. butleri*, *I. melanospora*, and *I. peidmontana*, is usually a result of adaptations to specific environmental pressures (granite outcrops, limestone soils, periodic desiccation, etc.). Taxa such as *I. engelmannii*, *I. caroliniana*, *I. echinospora*, *I. riparia*, and others have much larger ranges and

exhibit much more phenotypic variability. *Isoetes engelmannii* has a very large range (the largest of the eastern U.S. taxa) and is one of the most variable taxa morphologically and this study has shown that the populations are as variable electrophoretically as they are morphologically. *Isoetes caroliniana* with its reduced range and less broad ecological and morphological limits of variability was shown to have much less allozyme variation than *I. engelmannii*. The divergence of *I. caroliniana* and *I. engelmannii* has most likely been the result of long term isolation resulting in little morphological change but allowing for allozyme divergence to take place. This is in direct contrast to highly endemic taxa in which selectional pressures and hybridization, which create relatively quick divergence among genetic characters and mating systems but perhaps fewer changes among allozymes.

Additional evidence of allopatric speciation comes from hybridization studies done by Boom (1979). Boom was able to produce artificial hybrids of every possible combination of *I. macrospora* ($n=110$), *I. engelmannii* ($n=22$), *I. riparia* ($n=22$), and *I. flaccida* ($n=22$). This suggests that reproductive barriers between species are not acquired for a long time after morphological divergence has occurred. Hybridization of species has been invoked by many investigators to explain some collections that apparently have intermediate characters (Taylor and Leubke, 1986; Matthew and Murdy, 1969; Boom, 1979).

Although reproductive barriers are not observed in the laboratory, in natural circumstances, taxa are generally well isolated by geographic, phenological, or ecological barriers and so the frequency of gene exchange between taxa or even widely separated populations of a single taxon is likely to be very low and so the integrity of species may be maintained despite lack of reproductive barriers. Natural hybridization apparently occurs occasionally and Boom (1979) reports several suspected cases of these natural hybrids, but many times natural hybrids may be very difficult to detect electrophoretically because of introgression with the parental plants. Introgression may be a real factor behind the considerable variability that is present in many

widespread species where the confusing characters are often found in populations at the edges of the range of a species where two species are in sympatry with each other.

Plants from the MAYP population represent an apparent case of natural hybridization between *I. engelmannii* and *I. melanopoda*. Electrophoresis placed the population well outside either the *I. engelmannii* or *I. caroliniana* clusters in the phenograms produced. Members of this population exhibited three unique alleles (AAT-2, LAP-1, and PGM-1). All other loci exhibited alleles present in either *I. engelmannii*, *I. caroliniana*, or *I. melanopoda*. The morphology of these plants provided the strongest evidence of their hybrid origin. The wall of the sporangia of these plants have brown to black etchings across the whole surface and the leaf bases are sclerified. Both of these characteristics are similar to *I. melanopoda*. *Isoetes engelmannii* is usually without any pigmentation in any part of the leaf base or sporangium. The variable megaspore size and ornamentation is also indicative of other known interspecific hybrids (Taylor and Leubke, 1986).

The populations MONT and CEME may represent intraspecific hybridization between *I. caroliniana* and *I. engelmannii*. Electrophoresis supports (Chapter II) past hybridization because of the many common alleles to both *I. engelmannii* and *I. caroliniana* and the occurrence of unique alleles found only in these two populations. These populations seem to be adapted, like *I. engelmannii*, to episodic drying and have megaspores with reticulate-cristate ornamentation.

Boom (1979) has attributed the occurrence of *I. caroliniana* to instances of natural hybridizations of *I. engelmannii* with *I. riparia*, but the present study sheds doubt on that hypothesis because of the demonstrated allozymic uniformity among the populations of *I. caroliniana* over a large area. If hybridization of *I. engelmannii* with *I. riparia* were occurring along the coastal plain of North and South Carolina one would have expected more variability. The possibility of introgression of some genes from *I. riparia* into *I. caroliniana* is not discounted and this could have been determined if *I. riparia* had been included in the allozyme analysis. Attempts to locate populations of *I. riparia* for study were unsuccessful.

James Hickey has done the most to develop a species concept for *Isoetes* that is practical but at the same time reflect phylogenetic relationships. Because recent studies have suggested that megaspore variation may be as plastic as vegetative characters, Hickey has found (1986) that little emphasis has been placed on the correlation of the vegetative morphology, anatomy, and other physiological characteristics of *Isoetes* as a means of "supporting or refuting the evolutionary relationships among species as indicated by megaspore morphology." He suggests that "megaspore characters, when used in concert with phenology and habitat preference, are useful for delimiting natural species alliances."

Hickey et al. (1989) finds that a "dynamic" interpretation of the biological species concept is necessary for understanding *Isoetes*. He states that "available data also suggest that the genus is currently undergoing rapid and continuing speciation...a situation that often confuses taxonomic boundaries under the restraints of a morphological (or any other non-dynamic) species concept." This speciation seems to be occurring as the result of two mechanisms. First, Hickey et al. (1989) states that species have "evolved gradually (*I. caroliniana*) as a result of spatial isolation and genetic divergence," and secondly, "species have formed abruptly by interspecific hybridization followed by chromosome doubling."

This study has demonstrated that the use of several types of data when used in "concert" with one another are more effective in revealing taxonomic differences among species which are very closely related. *Isoetes caroliniana* most likely has arisen as the result of allopatric speciation in which enzymatic and some morphological divergence has taken place with presumably few reproductive barriers having been created although artificial hybridizations were not tested.

CHAPTER VI

SUMMARY

In an effort to assess the validity of *I. caroliniana* (A. Eaton) Taylor and Leubke as a taxonomic species, twenty-five populations of what were thought to represent *I. engelmannii* and *I. caroliniana* were collected and analyzed. Along with specimens of several other species (*I. melanopoda*, *I. louisianensis*, *I. butleri*, and *I. macrospora*), investigations involving allozyme electrophoresis and spore ornamentation were undertaken supplemented by field ecological observations. Additional specimens were received from Carl Taylor (Milwaukee Public Museum) and were included in the electrophoretic investigation and herbarium specimens were examined for spore ornamentation.

Electrophoresis was employed to study allozyme differences among populations of *I. engelmannii* and *I. caroliniana*. A total of eight enzyme systems coding for thirteen loci were used to compare populations of *I. engelmannii* and *I. caroliniana*. It was found that sixteen populations of *I. caroliniana* can be distinguished from six populations of *I. engelmannii* by four loci; AAT-1, 6-PGD, LAP-1, and TPI-2. Three populations thought to represent *I. engelmannii* were determined to be possible examples of natural hybrids between *I. engelmannii* and *I. caroliniana* (MONT and CEME) and *I. engelmannii* and *I. melanopoda* (MAYP).

Examinations of megaspore ornamentation was undertaken as a means of comparing the results of the electrophoretic survey with the traditional means of species identification. It was found that megaspores of *I. caroliniana* could be distinguished from *I. engelmannii*; the former have cristate to echinate ornamentation whereas those of the latter have a reticulate ornamentation.

Isoetes caroliniana can generally be characterized in the field by its robust leaves in the spring that are up to 10mm wide at the base, and that often lack pigmentation at the base. The leaves have easily observed cross-hatching of white lines perpendicular to the leaf that are

caused by the development of peripheral bundle fibers. The plants are usually found in bogs, seeps, and in ditches and farm ponds that are wet year-round. The megaspores, always found in unpigmented sporangia, are cristate to echinate with some having very jagged crests. The range of the species is from eastern Tennessee to western Virginia to western North Carolina and may extend into West Virginia, South Carolina, and Georgia.

Isoetes engelmannii was not observed over its full range as part of this study and so only a sample of its total variability was examined. In the southeastern United States *I. engelmannii* may inhabit several different habitats, such as lakes and rivers, and may exhibit variable morphologies. Most populations are characterized by leaves which are much less robust than those of *I. caroliniana* and are found in completely aquatic habitats whereas other populations may be found in sites which undergo periodic desiccation and are adapted to survival through dry periods. The megaspores, unlike *I. caroliniana*, are sometimes found in partially pigmented sporangia, and in most populations of *I. engelmannii* are reticulate with some variation possibly due to hybridization with *I. melanopoda*. The range of *I. engelmannii* is now known to extend from Missouri to the eastern coast and from Georgia to eastern Canada.

Isoetes engelmannii is seen as being the progenitor species for *I. caroliniana* and this is supported by the fact that *I. engelmannii* is much more variable electrophoretically and morphologically. Inbreeding, small population size, and probable bottle-necking of individual populations can all be invoked as reasons for the amount of variability among populations of *I. engelmannii* and the great degree of divergence shown by the other species examined (identities <.500).

Additional work needs to be done both electrophoretically and morphologically with both of these species and the other taxa in the southeastern United States including the possible hybrid populations, MAYP, MONT, and CEME. Environmental influences on spore and leaf morphology and cytological work are also needed to understand better the relationships of many of these morphologically similar taxa.

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APPENDICES

APPENDIX A
SPECIES CITATIONS
***ISOETES BUTLERI* Engelmann**

SPECIES EXAMINED:

ALABAMA: CHEROKEE CO.: Ca. 6 mi. S of Centre on AL 9, sandy clay of savannah pasture swale, Kral 35013, 5 June 1969 (TENN).

GEORGIA: CATOOSA CO.: Chickamauga National Military Park, 34°54'20"N, 85°14'48"W, cedar glades in moist shallow soil over limestone, Boom 63, 20 Apr 1978 (TENN), Boom 64, 20 May 1978 (TENN).

KENTUCKY: WARREN CO.: E of US 231 ca. 0.5 mi. N of Simpson-Warren Co. line, pastured cedar glade, Ste. Genevieve Limestone, Baskin & Baskin 1648, 12 Apr 1973 (TENN).

TENNESSEE: DAVIDSON CO.: Cedar glades near Mt. View School, hwy. 1S, Shaver 2346, 2347, 31 Mar 1935 (TENN); Cedar barrens, Lavergne near Nashville, Hesler 114, 31 Mar 1934 (TENN). RUTHERFORD CO.: Limestone barren opposite Stones River, Military Park N of Murfreesboro on Nashville hwy., Sharp & Koelling 23142, 10 May 1958 (TENN). WILLIAMSON CO.: Hillboro Road, ca. 6.2 mi. S of Davidson Co. across from Cecil Sim's home, Shaver 10962, 11 May 1952 (TENN). WILSON CO.: Cedar glade on N side of county road, ca. 2 mi. E of Vesta and ca. 4 mi. SE of Gladeville in moist depression, Webb 883, 26 May 1977 (TENN); Cedars of Lebanon State Park, ca. 2 mi. behind pool. Boom 107, 25 May 1978 (TENN).

ISOETES CAROLINIANA (A. A. Eaton) Taylor and Luebke

SPECIMENS COLLECTED:

NORTH CAROLINA: ASHE CO.: Small fish pond in front of residence on US 221 N 0.2 mi. of jct. with NC state rt. 88, Duff 9012, 1 Aug 1990, and Duff 9117, 9 May 1991 (TENN); Just off the W side of US rt. 221 N of Deep Run just N of the Ashe Co./Watauga Co. line in pasture pond accross from Church. Plants found in the SW end of the pond, emergent and submerged, Duff 9011, 1 Aug 1990 (TENN). AVERY CO.: Ca. 3 mi. S of Banner Elk at Wildcat Lake near Grandfather Home for Children, abundant, Duff 9010, 1 Aug 1990 (TENN); In a small Carex-Juncus seep, ca. 5,100 ft elev. on S side of Hump Mt., just W of an indistinct grown-over jeep trail that appears to coming from Houston Ridge to the Appalachian Trail toward the summit of Hump Mt., Evans 4981, 23 July 1990 (TENN). HEYWOOD CO.: Edge of seep W of the Cataloochee River 0.4 miles N of first bridge going N of Cataloochee, GSMNP, Evans 4973 and Duff 9104, 2 Feb 1991 (TENN); In ditch and spagnum bog accross the road from a parking area just on the N side of the second bridge going N from Cataloochee, GSMNP, Evans 4977 and Duff 9105, 2 Feb 1991 (TENN). MACON CO.: Small ditch off of rd. in front of the office of the Coweeta Experimenta Station. Few plants in sluggish water, Duff 9008, 30 May 1990 (TENN).

TENNESSEE: JOHNSON CO.: In ditch along the E side of state rt. 91 about 1.05 mi. S of intersection with US Rt 421 in Shady Valley (five plants found), Duff 9013, 1 Aug 1990, Duff 9117, 7 May 1991 (TENN). POLK CO.: Submerged in Parksville Lake inlet along rt. 30 on S end of small island 30 feet out from a picnic area, Duff 9007, 30 Jun 1990, and Duff 9112, 8 May 1991 (TENN), and Duff 9015, 2 Sep 1990 (TENN); In manmade lake on the top of Oswald Dome, Duff 9111, 8 May 1991 (TENN); McCamy Branch just upstream of the bridge on rt. 30 where the stream enters Parksville Lake, plants on the E side of the stream, Duff 9113, 8 May 1991 (TENN).

VIRGINIA: FRANKLIN CO.: Smart View Picnic area, Blue Ridge Parkway, in open water course in seepy area drainage into small pond, A. M. Evans 5001, 24 Sep 1990 (TENN); GILES CO.: NW corner of sphagnum bog 0.4 miles E of rt. 804 on S side of rt. 537, in running water draining out of the bog into a nearby culvert, Duff 9009, 26 Nov 1990 (TENN).

SPECIMENS EXAMINED:

NORTH CAROLINA: ASHE CO.: Pool near jct. of US 221 and NC 88 E of Jefferson, Radford 45227, 17 Sep 1966 (TENN). MACON CO.: Horse Cove, Highlands. Stream in swamp, J. K. Underwood, M. B. Wilson, and Mrs. Lott. 2796, 24 Aug 1934 (TENN). MECKLENBURG CO.: Margin of lake shore, Lake Norman, growing in mud, under alders, Latta Historical Park, E of "beach" area, J. Mathews, R. Brown, and J. Hicklin. 12 Sep 1979 (TENN). MITCHELL CO.:

Bakersville Quad., wet ditch on N side of For. Serv. Rd. from S of Roan High Bluff to Eagle Cliff, Due S of Roan High Bluff. 5950 ft. elev., P. Somers, with E. Shell, D. Horn, and D. Fulcher. 3 Jul 1974 (TENN).

TENNESSEE: CARTER CO.: Along the Watauga River, ca. 1.5 mi. below Wilbur Dam, W. Michael Dennes, R. Young, and Paula Collier, 18 June 1980 (TENN). POLK CO.: Mud Flat along hwy. 64, E. end of bridge, E. of Parksville; emergent or submerged, Brian Boom 317, 17 Nov 1978 (TENN); Mouth of Maddens Branch, tributary of the Ocoee River. Just upstream from bridge of hwy. 64 (Old Copper Road), Abundant, Brian Boom 425, 11 July 1979 (TENN); Shallow water along lake margin, McCamy Branch of Chilhowee Lake in Cherokee National Forest, ca. 7 mi. NE of Ocoee reservoir. A. Lean Bates, 3 Dec 1981 (TENN); In shallow stream near confluence with Ocoee River; along hwy. 64 near Ocoee Dam No. 2. Locally abundant, B. Wofford with B. Boom 79-179, 26 Jun 1979 (TENN).

VIRGINIA: ALLEGHANY CO.: Edge of Craig Co.; Potts Mt. in dried up pond on Mt. top. Pond apparently intermittant, Harvel 23, Aug 1966 (TENN). GILES CO.: Mt. Lake Biological Station along margins of pond behind laboratory building. Barely emergent to submerged; abundant, A. M. Evans, 24 Jul 1978, and Evans 4247, 15 Jul 1980 (TENN); Along road to Cascades ca. 1 mi. W of Mountain Lake Hotel, the roadside ditches near the Ashby House at Little Meadows, W. H. Wagner 62210, 5 Aug 1962 (TENN); "Little Meadows" on SW side of rt. 714, 0.5 mi. towards Cascades in front of Ashby House, A. M. Evans 2220, 14 Jul 1966 (TENN).

WEST VIRGINIA: MONONGALIA CO.: Along edge of Tibbs Run Reservoir, Elizabeth Bartholomew #Mo-53, 13 Aug 1946 (TENN).

ISOETES ENGELMANNII A. Br.

SPECIMENS COLLECTED:

TENNESSEE: COFFEE CO.: US 41 0.5 mi. E of Asbury Road in ditch next to the road just E of the Bait and Beer Shop and across from an auto junkyard (infrequent), site dries out in the summer, Duff 9004, 24 June 1990 (TENN); In ditch on SW side of US 41 just 50 ft. NW of Beans Creek (ditch dries out in the summer), Duff 9005, 23 June 1990 (TENN) and Duff 9016, 20 Dec 1990 (TENN). PUTNAM CO.: Shallow quarry pond, ca. 7 miles NW of Monterey, ca. 2.0 miles NW of Shady Grove Church (only one plant found), Duff 9002, 18 June 1990; Ditch across from cemetery next to Shady Grove Church, ca. 7 miles NW of Monterey, Duff 9001, 18 June 1990 (TENN). VAN BUREN CO.: Submerged 100 yards from parking lot next to dam of Fall Creek Falls Reservoir on eastern side of the lake, Duff and Evans 9003, 24 June 1990 (TENN), and Duff 9101, 20 Dec 1990 (TENN).

SPECIMENS EXAMINED:

TENNESSEE: COFFEE CO.: In a stream along the bordering edges of the May Prairie, H. R. DeSelm and M. Edwards, 12 June 1972 (TENN); Along hwy 41 ca. 3 mi. SE of Manchester; moist ditch on E. side of hwy; uncommon, Brian Boom 277, 1 July 1978 (TENN). CUMBERLAND CO.: Obed River at Potter's Ford, Frank Robinson, 31 Aug 1955 (TENN). FRANKLIN CO.: Cumberland Plateau surface above Wolf Cove. Submerged in shallow water on the S end of the Plateau Pond. 1800 ft. Pitcher Ridge Quad. R. K. Clements and B. E. Wofford 201, 12 June 1985 (TENN). GRUNDY CO.: Immersed in shallow water of old swimming pool, about 2 mi. S of Tracy City, rd. 56. Jamison and Shaver 4160, 28 Aug 1943 (TENN); One mile N of Tracy City; margin of man-made dam, Ken Rogers 44214, 16 Aug 1969 (TENN). HAMILTON CO.: Mudflats at the upper end of Sale Creek on Chickamauga Reservoir; ca. 1.5 mi. E of Sale Creek, TN., David Webb and Doug Murphy 3693, 28 Oct 1980 (TENN). HARDIN CO.: On mud and in shallow water around the margins of Bloody Pond in Shiloh National Military Park; ca. 7 mi. SW of Savannah, David Webb 4338 26 Sept 1981 (TENN). MARION CO.: Submerged in deep water, Snake Pond, Cumberland Plateau, approx. 10 mi. SE of Sewanee, Alt. 1800 ft., H. K. Svenson 9511, 13 July 1938 (TENN); Cummings Lake on Nickajack reservoir along shoreline where small creek enters lake, TRM 442.7. Buddy Price and Doug Murphy 2204 TVA, 20 Aug 1979 (TENN). VAN BUREN CO.: In moist sand above Cane Creek Falls, J. K. Underwood and A. J. Sharp 2938, 19 Aug 1935 (TENN).

ALABAMA: CONECUH CO.: Evergreen city limits. County hwy 30, 0.5 mi. S of I-65. Rooted heavy mud at swamp margin, J. W. Short with J. D. Freeman 822, 16 Sept 1976 (TENN).

MARSHALL CO.: Shallow ditch in low woods on S side of County Rd. at SW end of Browns Creek embayment on Guntersville Reservoir, ca. 6 mi. SW of Guntersville, David Webb 4757, 12 April 1983 (TENN); Margin of small pond on the Murphy Hill Coal Gasification site; ca. 2.5 mi. NNW of Five Point and 1.3 mi. E of the main channel of the Tennessee River at TRM 396.4, David Webb and Doug Murphy 3716, 23 Jan 1981 (TENN).

FLORIDA: GADSDEN CO.: Shallow pool near US 27 bridge over Ochlockonee River, NW of Tallahassee, S. W. Leonard 6251, 7 Apr 1976 (TENN).

***ISOETES LOUISIANESIS* Thieret**

LOUISIANA: WASHINGTON PARISH: Aquatic in Thigpen Creek at La. 16, 1 mi. E of Ennon, Sec. 50, T4S, R11E, Dale Thomas and Lindsay Tyser 37614, 4 Nov 1973 (TENN); Aquatic in Thigpen Creek at La. 16, 1 mi. E of Ennon, Sec. 50, T4S, R11E, Dale Thomas 35493, 23 June 1973 (TENN).

***ISOETES MACROSPORA* Dur.**

SPECIMENS COLLECTED:

TENNESSEE: POLK CO.: Just upstream of the bridge at the jct. of hwy. 30 and state rt. 315, on the N side of the Hiwassee R. Rooted in gravel in fast moving water, Duff 9015, 2 Sept 1990 (TENN).

SPECIMENS EXAMINED:

TENNESSEE: MONROE CO.: Little Tennessee River at Jones Ferry (abandoned), Wofford & Dennis 78-133, 26 July 1978 (TENN), Boom 318, 17 Nov 1978 (TENN); Little Tennessee River at SW end of Davis Island, Wofford & Dennis 78-135, 26 July 1978 (TENN). POLK CO.: Shallow shoals of Hiwassee River, 1.6 road ni. NW of jct. of hwy. 30 and State Road 2518, Wofford & Evans 78-169, 21 Aug 1978 (TENN).

VIRGINIA: SHENANDOAH CO.: Passage Creek, shallow water, Evans s.n., 8 Nov 1978 (TENN).

***ISOETES MELANOPODA* Gay & Dur.**

SPECIMENS COLLECTED:

TENNESSEE: GRUNDY CO.: In ditch (plants uncommon) on the NE side of US 41 just S of Elk Creek (near Monteagle), Duff 9006, 23 June 1990 (TENN).

SPECIMENS EXAMINED:

ARKANSAS: CLEBURN CO.: Alluvial soil, sand bars, and pools along Little Red River at the end of Ark. 210 at Cow Shoals E of Heber Springs and Ark. 110, Dale Thomas, Eric Sundell, and Carl Amason, 21 Oct 1990 (TENN).

ILLINOIS: PIATT CO.: Dried up swamp along railroad, NW of Monticello, H. E. Ahles 7389, 9 June 1953 (TENN).

LOUISIANA: OUACHITA PARISH: Wet area in lawn S of La. 134 and W of railroad at gas compression plant at Fairbanks; Sec. 10, T19N, R4E, R. Dale Thomas, Scott Thomas, and Kurt Keene 103571, 20 March 1988 (TENN). FRANKLIN PARISH: Edge of oat field W of dirt road S of La. 132 and Old Mixon School in Murphy Woods, Sec. 15, T16N, R8E. Abundant, Dale Thomas 88417, 8 May 1984 (TENN).

APPENDIX B
CITATIONS¹ UNABLE TO LOCATE

***ISOETES CAROLINIANA* (A. A. Eaton) Taylor and Luebke**

NORTH CAROLINA: CLAY CO.: Bog, Chatuge Lake, 3.5 mi. E of Hayesville, Radford 16329, 21 Aug 1949 (FSU); Shallow pool near Buch Creek along hwy.64, Leonard & Radford 2310, 3 April 1969 (MEM, NLU). HENDERSON CO.: Bog at East Flat Rock, Benedict 2658, 29 Jul 1933 (VPI).

TENNESSEE: POLK CO.: Mud flat along the N side of the Ocoee River along hwy. 64, E end of bridge, E. of Parksville; emergent of submerged. Brian Boom, 317, 17 Nov. 1978 (TENN).

***ISOETES ENGELMANNII* A. Braun**

ALABAMA: MARSHALL Co.: Margin of small pond on the Murphy Hill Coal Gasification site, ca. 2.5 miles NNW of Five Points and 1.3 miles E of the main channel of the TN River at TRM 346.4. David Webb and Doug Murphy 3716, 23 Jan 1981 (TENN), Shallow ditch in low woods on S side of County Rd. at SW end of Browns Creek embayment on Guntersville Reservoir, ca. 6.0 miles SW of Guntersville. David Webb 4757, 12 Apr 1983 (TENN).

GEORGIA: Catoosa Co.: Shallow water of boggy area near side of outlet of Blue Springs 7.0 mi. W 20°S of Ringold. Duncan 22334, 2 May 1964 (TENN).

TENNESSEE: CUMBERLAND CO.: Obed River at Potters Ford, Robinson s.n., 31 Aug 1955 (TENN). FRANKLIN CO.: Cumberland Plateau surface above Wold Cove. Submerged in shallow water on the south end of the Plateau Pond. 1800 ft. Pitcher Ridge Quad. (Pond was half filled because of recent logging and no plants were found, 5-7-1991) R. K. Clements and B. E. Wofford 201, 12 June 1985 (TENN). GRUNDY CO.: 1 mi. N of Tracy City, margin of manmade dam, Rogers 44214, 16 Aug 1969 (FSU, SMU, VDB). HAMILTON CO.: Mudflats at the upper end of Sale Creek on Chickamauga Reservoir; ca. 1.5 miles E of Sale Creek, TN. (Not found because of high lake level) David Webb and Doug Murphy 3693, 28 Oct 1980 (TENN). MARION CO.: Cummings Lake on Nickajack Reservoir along shoreline where small creek enters lake, TRM 442.7. (Not found when lake was high) Buddy Price and Doug Murphy, 2204 TVA MORGAN CO.: Clear Fork River, Rugby, Jennison 1118, 31 May 1934 (TENN). VAN BUREN CO.: In moist sand above Cane Creek Falls at Fall Creek Falls State Park, Sharp & Underwood 4009, 19 Jul 1935 (TENN).

WEST VIRGINIA: GREENBRIER CO.: Along Big Draft Road near Camp Wood in Anthony's Creek, Clarkson 2967, 29 Jul 1959 (WVA). MONONGALIA CO.: Along edge of Tibbs Run Reservoir, Bartholomew Mo-53, 13 Aug 1946 (GA, KY, SMU, VPI, WVA).

***ISOETES MELANOPODA* Gay & Dur.**

TENNESSEE: RUTHERFORD CO.: E Clark Avenue, Murfreesburo (area is well developed now), Svenson s.n., 3 Jun 1954 (TENN, VDB).

¹Citations from University of Tennessee herbarium specimens, Boom (1979), and personal communications.

VITA

Robert Joel Duff was born on 12 January, 1967, in Elmhurst, Illinois. He attended elementary schools in Grand Junction, Colorado and in Spring Valley, California and attended high school in Port Hueneme, CA where he earned many academic and music awards before earning his high school diploma in June, 1985. The following August he entered Calvin College in Grand Rapids, MI where he earned a Bachelor of Science degree in biology in May of 1989.

In August, 1989, Mr. Duff accepted a teaching assistantship and enrolled in the University of Tennessee, Knoxville to pursue a Master of Science degree in botany, with research in systematics, under the tutelage of Dr. A. Murray Evans. While there, he earned the Science Alliance Upgrade Award For Excellence In Teaching And Service and the Holton Teaching Award. Mr. Duff will receive his Master of Science in Botany in December, 1991.

After receiving his Master of Science degree, he plans to continue his studies at the University of Tennessee, Knoxville to pursue a Doctor of Philosophy with a major in Botany.