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## A Review of North American *Isoetes engelmannii* (Isoetaceae) complex hybrids, including the description of *I. x fernaldii*, *hyb. nov.* and *I. x karenae*, *hyb. nov.*

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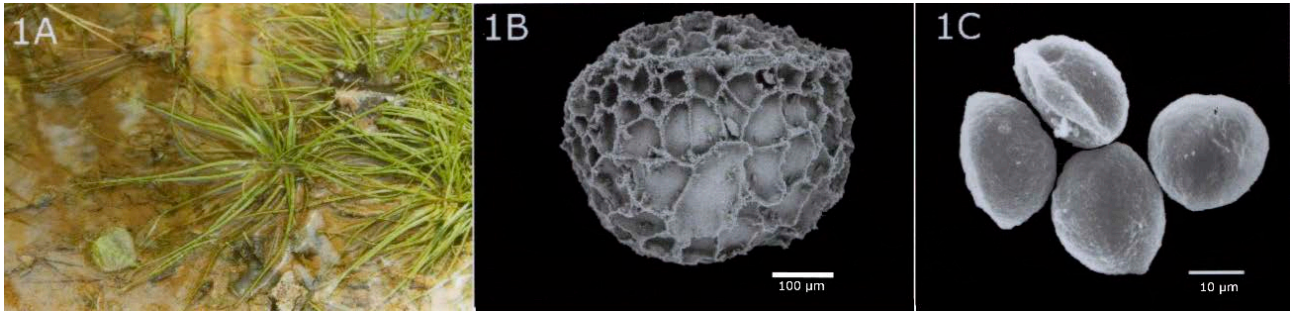
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**Abstract.** The *Isoetes engelmannii* complex of eastern North America consists of 30 taxa including 13 named species. Nine of the 17 hybrids within the complex (the largest group of *Isoetes* hybrids in the world) have been formally described. Those named hybrids are reviewed here in light of recent additions to and enhancements of the morphological and cytological evidence employed in their original description. The pedigree of three of these, *I. x brittonii*, *I. x bruntonii* and *I. x carltaylorii*, is updated and clarified. Formal descriptions are proposed for two additional taxa: *I. x fernaldii*, *hyb. nov.* (*I. engelmannii* × *I. hyemalis*) and *I. x karenae*, *hyb. nov.* (*I. appalachiana* × *engelmannii*). The potential for a further eight hybrid combinations to occur in the wild is also addressed.

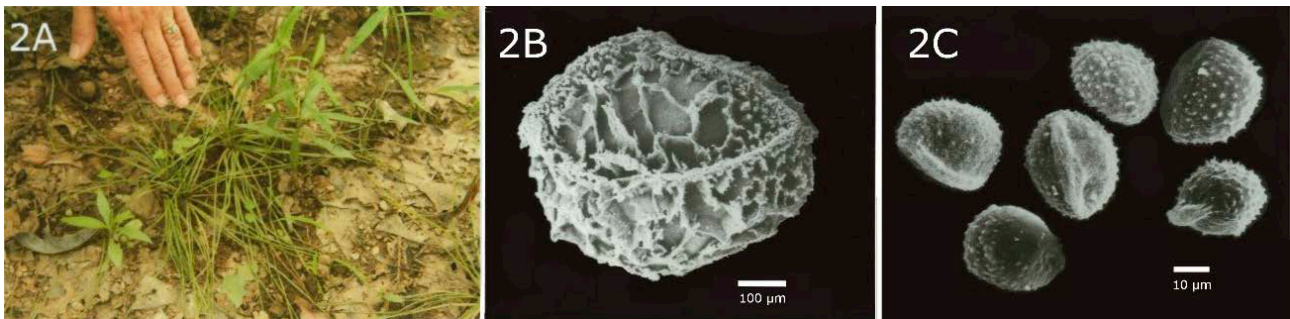
**Keywords:** *Isoetes*, hybrids, *Isoetes appalachiana*, *Isoetes x brittonii*, *Isoetes x bruntonii*, *Isoetes x carltaylorii*.

The diploid ( $2n=2x=22$ ) *Isoetes engelmannii* A. Braun is an important species within the complex of larger, mostly polyploid aquatic/semi-aquatic quillworts (Isoetaceae) of eastern North America (Taylor et al. 1993; Taylor et al. 2017; Brunton & Troia 2018) (Figure 1).

Key to the development of our contemporary interpretation of this complex was the identification of the tetraploid ( $2n=4x=44$ ) *Isoetes appalachiana* D.F. Brunton & D.M. Britton (Brunton and Britton 1997) within *I. engelmannii* sensu lato (s.l.) (Figure 2). With the identification of *I. appalachiana* as a distinct species, the potential number of hybrid combinations within this complex was virtually doubled. The two species are geographically centred on the Appalachian Mountains but are distributed (especially *I. engelmannii* sensu stricto [s.str.]) across much of the eastern United States. As per the discussion in the introduction (below) regarding the inclusive taxonomic concept employed here, the unnamed ‘northern’ and southern’ genetic entities reported within *I. appalachiana* by Hoot et al. (2004) and others is acknowledged but not investigated further.



**Figure 1.** *Isoetes engelmannii* plant and spores. 1A: plants in shallow water of flowing stream, Stanley County, NC, USA, 3 April 2016 (Photo: D.F. Brunton); 1B: megaspore lateral view, 1C: cluster of microspores (*G. Engelmann s.n.*, September 1842, Jefferson County, MO, USA (Isotype - P)).



**Figure 2.** *Isoetes appalachiana* plant and spores. 2A: emergent plants in ephemeral woodland swale, Dinwiddie County, VA, USA, 20 June 2015 (Photo: D.F. Brunton); 2B: megaspore lateral view (D.F. Brunton & K. L. McIntosh 11,171, 6 July 1992, Huntington County, PA, USA (OAC)), 2C: cluster of microspores (D.F. Brunton & K. L. McIntosh 11,176, 6 July 1992, Lycoming County, PA, USA (OAC)).

The identification of sterile hybrids has been important in determining the distinctiveness of these and other *Isoetes* species by confirming the existence of interspecific genetic barriers (Taylor et al. 1985; Brunton 2015; Taylor et al. 2016). Indeed, hybrids are recognized as an important element of the evolution and taxonomy of vascular plants in general (Plume et al. 2015; Gianguzzi et al. 2017; Goulet et al. 2017). Within the Isoetaceae, hybrids also commonly present opportunities for the development of polyploid species through allopolyploidy (Hickey et al. 1989; Troia et al. 2016; Brunton and Troia 2018).

The existence of hybrid diversity within the *Isoetes engelmannii* complex has been confirmed through recognition of a suite of often subtle but consistent and usually distinctive morphological features, often supported by cytology. Features and characteristics of the megaspores and microspores, found within sporangia at the base of fertile leaves, are critical to the identification of such plants. These include ornamentation patterns reflecting features of both putative parents.

Hybrids also have polymorphic, aborted megaspores and microspores, usually in high proportion, and a wide

range of spore sizes within individual sporangia (Taylor et al. 1985; Hickey et al. 1987; Musselman et al. 1996; Britton and Brunton 1996; Troia & Greuter 2014; Brunton 2015). Megaspores of hybrids often present uniquely congested ('brain coral') ornamentation and/or are fused together as 'dumbbell-shaped' megaspores (Jeffrey 1937; Britton 1991; Taylor et al. 2016). Hybrid plants often display hybrid vigour and are larger than putative parents at the same site (Britton 1991; Musselman et al. 1995). Cytology is an important determinant if the putative parents have different ploidy levels (Hickey et al. 1987; Taylor and Luebke 1988; Musselman et al. 1997).

*Isoetes* hybrids in the wild grow with at least one and almost always with both putative parents. They also are usually found in sites especially well suited to the mixing of spores, such as downstream of intrusions into river currents (e.g. below rapids) or in seasonally flooded creek-side swales (Britton and Brunton 1989; Britton and Brunton 1996; Musselman et al. 1996).

*Isoetes* species of environmentally stressed habitats often exhibit polymorphic spores reminiscent of sterile hybrids but still reflect only the ornamentation features

of a single species. This is most commonly encountered in species of ephemeral wetlands such as temporary pools on bedrock outcrops (Haefner and Bray 2005; Brunton 2015). Environmentally induced spore polymorphism is rare in true aquatic species, however, and is almost always expressed in such plants by only a small number of individual spores. Dumbbell-shaped megaspores are apparently not evident in plants experiencing environmentally induced polymorphism (pers. obs.).

Recognition and clarification of many of the hybrid taxa in the *Isoetes engelmannii* complex occurred in a relatively short period of time in the 1990s - early 2000s. Subsequent cytological, taxonomic and morphological data have substantially altered initial conclusions for some of these taxa. Recent molecular studies (e.g. Shafran et al. 2018) indicate multiple origins in a number (most?) of the recognized polyploid *Isoetes* of eastern North America. This is perhaps to be expected within one of the most ancient and widespread vascular plant groups on Earth (Larsen and Rydin 2016; Wood et al. 2019). Especially so when many species within it, particularly amphibious and semi-terrestrial taxa such as some of those addressed here, consist substantially or entirely of widely dispersed populations in highly stressed environments, these habitat patches having persisted unchanged for thousands or in some cases, even millions of years (Pfeiffer 1922; McVaugh 1943; Matthews and Murdy 1969; Taylor et al. 1993; Haefner and Bray 2005; Brunton and Troia 2018).

Shafran (2019) states that “using a lineage-based species concept may require the recognition of ca. 50 new species of auto- and allopolyploid *Isoetes* in eastern North America”. There is a need for the systematic investigation of morphological, ecological and phyto-geographic lines of evidence to provide consistent and repeatable collaboration with these molecular findings. This would demonstrate that genetic barriers exist between some of these newly distinguished ‘species’, and also would indicate how such taxa can be reliably distinguished in the field. In the absence of such collaboration, however, in the present study we hold to a conservative and inclusive (more traditional) taxonomic interpretation of *Isoetes* speciation. To do otherwise would preclude the identification of any sterile hybrids - indeed, the identification of many if not most populations of a particular polyploid *Isoetes* species - outside of the type location.

Clarifying the existence and status of sterile hybrids within already recognized *Isoetes* species will assist in future comprehensive, multifaceted investigations of relationships within the regionally important *Isoetes engelmannii* complex in particular and *Isoetes* in general.

## METHODS

This study is based on extensive field experience of the first author undertaken since the early 1990s throughout the range all the species addressed. Over 2,250 voucher specimens of the taxa discussed here have been examined. The largest number of vouchers studied in over 30 herbaria are in CAN, DAO, DFB, FLAS, MICH, NYS, OAC and PH (herbarium acronyms of Theirs, continuously updated).

An extensive library of Scanning Electron Microscope (SEM) images prepared by D. M. Britton before 2007 (prepared with the methods described in Brunton and Britton 2006) of microspores and megaspores of virtually all subject taxa, was reviewed. Additional SEM images have been produced since 2017 by the authors. For these new images, air dried spores were attached to SEM stubs by means of adhesive carbon discs. These were sputter coated with a gold / palladium alloy (Au/Pd) and examined with a 2017 model FEI Apreo SEM (at 15 kV and 25 pA, with a working distance of 10 mm and a spot size of 6).

The physical characteristics of specimens, especially their megaspore form, ornamentation pattern and size, were examined through a Leica Wild M3B [light] dissecting microscope at 40x magnification, with the aid of an in-mount graticule (ocular micrometer) for measurements. Megaspore sizes reported for individual specimens represent the average width (across the equatorial region) of at least 10 spores. Comparable microspore measurements are based on the average of 20 longitudinal measurements taken from SEM images of clusters of spores.

Cytological determinations of hybrid specimens reported here were made by D. M. Britton, following the techniques described in Brunton and Britton (1999, 2006). Plants from sampled populations were grown in distilled water in a growth chamber. The developing root tips were excised and pre-treated in aqueous paradichlorobenzene (PDB) at room temperature for four hours. They then were washed in distilled water, fixed in acetic alcohol (3:1 absolute ethyl alcohol to glacial acetic acid) for 30 minutes or more, hydrolysed in Warmke’s solution (1:1 concentrated HCl to absolute ethyl alcohol) for 7-10 minutes at room temperature, and stained in leucobasic fuchsin (Feulgen) for two hours. The meristems were squashed under a cover glass in 45% acetocarmine stain and examined.

## RESULTS

Nine sterile hybrid combinations in the *Isoetes engelmannii* complex are reviewed (in alphabetical order) in

light of ecological, distributional and taxonomic information developed since their original description.

Hybrid 1) *Isoetes appalachiana* × *I. engelmannii* (s.str.) (hyb. nov.)

Several individuals with classic sterile hybrid characteristics (as noted above), were found within a large mixed *Isoetes* population in Dinwiddie County, Virginia in 2015. Hundreds of *I. appalachiana* and scattered *I. engelmannii* plants were found in ephemeral swales and side channels along a permanent stream (Rowanty Creek) in seasonally flooded deciduous swamp forest (Figure 3). *Isoetes engelmannii* is common 100-200 m upstream along the creek as well (pers. obs.). Despite extensive collections in this area dating back to M. L. Fernald's investigations in the 1940s, no other *Isoetes* taxa have been found along Rowanty Creek within several kilometres of the site. *Isoetes hyemalis* D.F. Brunton occurs on an unnamed tributary of adjacent Stony Creek, however (see Hybrid 2, *Isoetes appalachiana* × *hyemalis*, below).

With both putative parents having predominantly reticulate megaspore ornamentation patterns, the range of distinctive features available for detecting this hybrid is reduced from that often available for the detection of other combinations. Nonetheless, there are several features of the Dinwiddie County material that confirm its distinctiveness.

The irregular shape, ornamentation and size of megaspores of Rowanty Creek hybrid plants are conspicuous (Figures 4A-4D). Megaspore size (514.9 µm, 1 SD 52.55 µm, N=28) was determined to be intermediate between that of diploid parent *I. engelmannii* (460-500



**Figure 3.** Deciduous swamp forest habitat (in flood) of *Isoetes* ×*karenae* (*I. appalachiana* × *engelmannii*), Rowanty Creek, Dinwiddie County, VA, USA, 20 June 2015 (Photo: D.F. Brunton).

µm) and the tetraploid parent *I. appalachiana* (520-600 µm) (Brunton and Britton 1997; Brunton 2015). Most megaspores are distorted in shape (Figures 4A-4B), with some of the fused, dumb-bell shaped megaspores that are diagnostic of hybrids, also being evident (Figure 4B). Relatively few regularly globose megaspores that are typical of non-hybrid *Isoetes* plants were evident in the suspected hybrid plants.

Microspore ornamentation is most similar to that of the smooth-spored diploid parent *Isoetes engelmannii* (Figures 4E-F), with only a subtle indication of the tuberculate ornamentation pattern of the tetraploid parent *I. appalachiana*. In keeping with this strong *I. engelmannii* expression in the hybrid, microspore size (25.4 µm, 1 SD 1.30 µm, N=40) was determined to be comparable to that diploid parent (25.5 µm) and substantially smaller than 30.4 µm length of tetraploid *I. appalachiana* microspores (Brunton and Britton 1997). A length of approximately 27-28 µm would have been more consistent with the size recorded with other triploid hybrids. This inconsistency, however, is believed to reflect the limited of microspore data (one plant) available at the Rowanty a mount Creek location.

At 17.6% (N=6, four plants as listed below), the average velum coverage of the sporangium of the hybrid is intermediate between putative parents *I. engelmannii* (10-15%) and *I. appalachiana* (20-25%) (Brunton and Britton 1997).

No fresh or live plants were available for genomic investigation or genomic analysis. That is unfortunate since the reflection of the different ploidy levels of the putative parents would have offered stronger support of the taxonomic hypothesis presented here. This necessitates the less desirable option of inferring ploidy level from spore size.

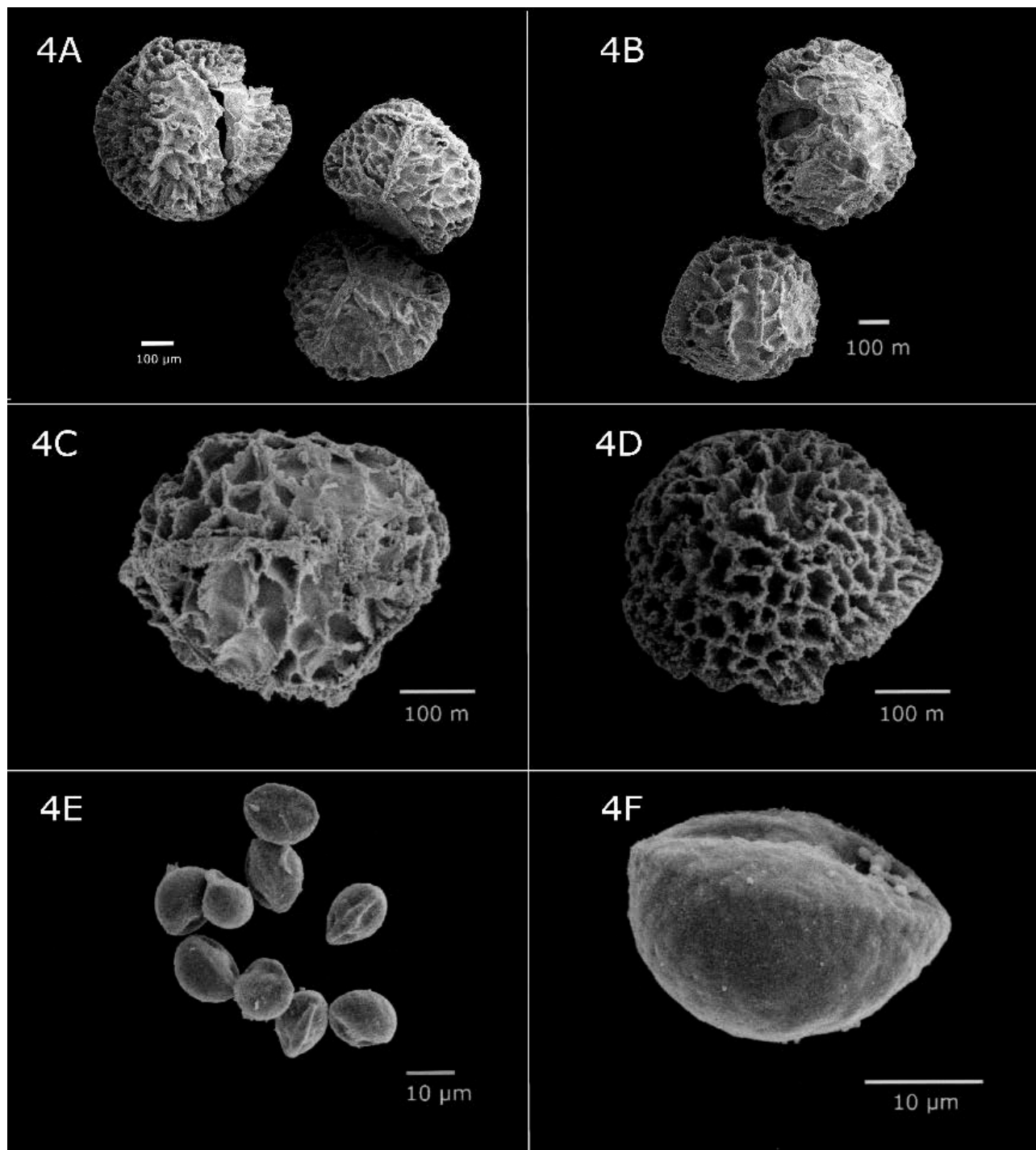
Other occurrences of this hybrid were detected in herbarium specimens showing comparable morphological characteristics (including aborted, polymorphic spores) from elsewhere in the sympatric range of the putative parents. In each case, the suspected hybrid was either a single huge plant (suggesting selective collection of individuals demonstrating such hybrid vigour) or mixed collections with one or both of the putative parents. The following binomial is proposed for this previously undescribed taxon:

***Isoetes* ×*karenae*** D.F. Brunton and P.C. Sokoloff, **hyb. nov.**

(*Isoetes appalachiana* × *I. engelmannii*) (Figures 4A-4F).

Typus: United States, Virginia, Dinwiddie County: 40 m south along west bank of Rowanty Creek from Car-





**Figure 4.** *Isoetes xkarenae* (*I. appalachiana* × *engelmannii*): 4A: cluster of polymorphic megaspores; 4B: misshaped and dumbbell-shaped megaspores; 4C: lateral view of megaspore; 4D: distal view megaspore; 4E: cluster of microspores; 4F: lateral view of microspore (D.F. Brunton & K. L. McIntosh 19,008, 20 June 2015, Dinwiddie County, VA, USA (CAN)).

son Road (CR 703), 4.1 km west northwest of Carson, 20 June 2015, D.F. Brunton & K. L. McIntosh 19,008 (holotype, CAN!)

#### *Description*

Plants – robust (more than 23 cm tall) in mixed populations with and gross form of putative parents *Isoetes*

*appalachiana* and *I. engelmannii*; Megaspores – variable in size, most aborted (misshaped) with well-formed spores of intermediate size 514.9  $\mu\text{m}$  (1 SD = 52.55  $\mu\text{m}$ ) in diameter and with densely, irregularly-reticulate ornamentation pattern formed by variable and ragged-crested muri; fused, dumb-bell shaped spores also present; Microspores – plain perispore surface obscurely verrucose, 25.4  $\mu\text{m}$  (1 SD = 1.30  $\mu\text{m}$ ) in length; Habitat – in periodically flood-scoured bank swales along deciduous swamp forest creeks; Cytology – unconfirmed (inferred  $2n=3x=33$  from megaspore size).

#### Etymology

The taxon is named in honour of Canadian field botanist Karen L. McIntosh of Ottawa, Ontario, who not only discovered the type population but has provided valuable contributions to our understanding of numerous *Isoetes* taxa during 30+ years of field investigations with the first author across North America.

#### Paratypes

United States, Pennsylvania, Berks County: Birdsboro Reservoir, 1.8 miles southwest of Birdsboro [single, robust plant], 2 October 1932, W.C. Brumbach 776-32 (PH); same site, 7 July 1934, W.C. Brumbach 205-34 [large right-hand plant on mixed sheet with *I. engelmannii*] (PH); Lancaster County: York Furnace Road on Susquehanna River [very large (>33 cm tall) plant], 4 September 1924, L. Sowden s. n. (PH).

*Isoetes*  $\times$  *karenae* is very similar in appearance to *I. engelmannii*  $\times$  *hyemalis* (below), both being triploids involving the diploid *I. engelmannii* (s. str.) as one parent. *Isoetes*  $\times$  *karenae* megaspores, however, exhibit slightly thicker muri (Figures 4C, 4D vs. Figures 8C, 8D) and a more subdued equatorial ridge (Figures 4C vs Figure 8D). It also has microspores with obscurely verrucose surfaces (Figure 4F) rather than apparently obscurely broad-based spiny perispore of *I. engelmannii*  $\times$  *hyemalis* (Figure 8F).

Given the extent of overlapping distributions of its putative parents, *Isoetes*  $\times$  *karenae* can be expected to occur in mixed *Isoetes* populations over a large area in the eastern United States. Additional occurrences may be discovered at sites from central Pennsylvania southward to at least South Carolina and northern Georgia.

#### Hybrid 2) *Isoetes appalachiana* $\times$ *I. hyemalis* (*I. $\times$ bruntonii* D. Knepper & L.J. Musselman)

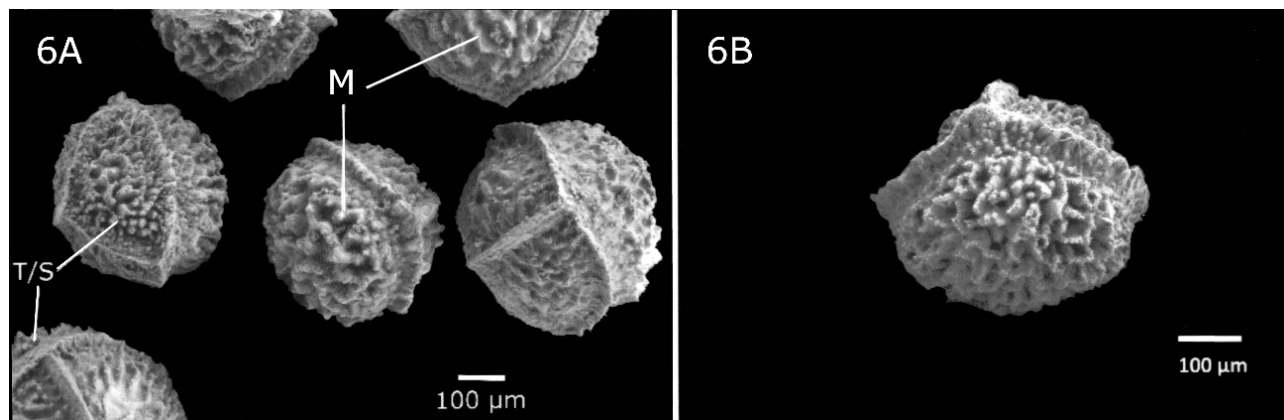
This taxon was first described as a cytologically supported triploid hybrid, thought to represent *Isoetes*

*engelmannii*  $\times$  *I. hyemalis* (Musselman et al. 1996), prior to recognition that *I. engelmannii* (s.l.) included tetraploid *I. appalachiana* (Brunton and Britton 1997). *Isoetes*  $\times$  *karenae* (above) and *I.  $\times$ bruntonii* type locations are in adjacent creek systems in Dinwiddie County, Virginia. It is at a site well suited to the mixing of *Isoetes* spores and the generation of hybrids, where trees fallen into a woodland creek created back eddies where the spores of the locally abundant *I. hyemalis* could mix with those of other taxa (Figure 5). *Isoetes appalachiana* (e.g. D. F. Brunton and K. L. McIntosh 12,557 (DFB, QFA), 14,519 (MIL), 16,097 (DFB, OAC, PH, MICH) and 19,011 (US) and *I. hyemalis* (e.g. D. F. Brunton & K. L. McIntosh 12,221 (GA, MICH, MIL, VPI, DAO), 12,557 (CAN, DFB, TRT, BM), 13,118 (MIL. OAC), 16,096 (DFB, PH) and 19,010 (DFB, US) are the only species to have been found at the type location during repeated site investigations between 1995 and 2015 (pers. obs.).

Hybrid plants from the precise *Isoetes*  $\times$  *bruntonii* type location (L.J. Musselman and R. Bray, pers. comm.) were determined to be tetraploid (D. F. Brunton, K. L. McIntosh, R. Bray & K. Haefner 13,559, 17 May 1998 (OAC). The megaspores of multiple hybrid specimens obtained from that site between June 1995 and June 2015 (in DFB) also were found to have a coarser walled, more open ornamentation pattern (Figures 6A, 6B) indicative of *I. appalachiana* rather than *I. engelmannii* (s. str.). Hybrids involving *I. engelmannii* (s. str.) such as *I.  $\times$ altonharvillii* L.J. Musselman & R. Bray, *I.  $\times$ eatonii* R. Dodge, pro sp. sensu Taylor et al. (1985), and *I.  $\times$ foveolata* A.A. Eaton, pro sp. sensu Taylor et al. (1985) all exhibit more congested megaspore ornamentation. Megaspores from the holotype (ODU), an isotype (DFB) and later cytologically determined tetraploid topotypes (D.F.



**Figure 5.** Woodland stream type location of *Isoetes*  $\times$  *bruntonii* (*I. appalachiana*  $\times$  *hyemalis*), Dinwiddie County, VA, USA, 20 June 2015 (Photo: D.F. Brunton).



**Figure 6.** *Isoetes xbruntonii* (*I. appalachiana* × *hyemalis*): 6A: cluster of polymorphic megaspores [T/S = tubercles or spines, M = interconnected muri]; 6B: lateral view of megaspore (D.F. Brunton & K. L. McIntosh 19,011B, 20 June 2015, Dinwiddie County, VA, USA (DFB)).

*Brunton & K. L. McIntosh 12,557B* (1996) and *D.F. Brunton & K. L. McIntosh 19,011B* (2015) (DFB), range in size from 500 to 623 µm, averaging 517.4 µm (1 SD 33.24, N=80). This is a larger than the megaspores size found in triploid hybrids such as *I. xkarenae* (above) or *I. engelmannii* × *hyemalis* (below), which average approximately 500 µm. No microspores from type material were available for examination in the present study.

No diploid plants, nor any triploid plants since the original cytotype report (Musselman et al. 1996), have been reported at the *Isoetes xbruntonii* type location. The isotype in DFB consists of two plants, one hybrid and one *I. appalachiana*. It is not possible to compare morphological data from the reported triploid cytotype against that of tetraploid hybrids later found at the type location as no voucher was retained of that cytotype (R. Bray, pers. comm.).

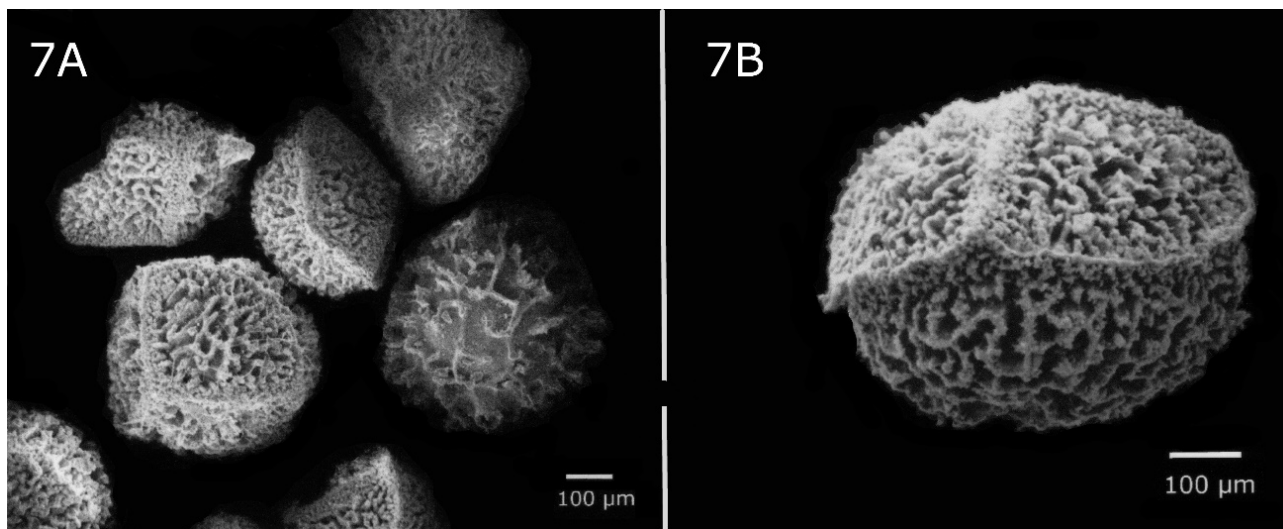
While it is technically possible that a triploid plant was originally present and has not reoccurred at the site, that ‘one-off’ occurrence would be contrary to our experience in all other regularly-producing hybrid sites. It seems most likely that either an error was made at the time of the original chromosome count for the cytotype or that the specimens did not originate from the *Isoetes xbruntonii* type location. Regardless, existing cytological vouchers from the type location are exclusively tetraploid and morphologically consistent with the type material.

While *Isoetes xbruntonii* is currently known only from eastern Virginia, it is reported (as *I. xbrittonii* D.F. Brunton & W.C. Taylor) to be frequent there (Musselman & Knepper 1994). Given the relative wide area of overlap between its putative parents, however, this hybrid could also be expected to occur in mixed *Isoetes* populations in North Carolina, South Carolina or even southern Georgia/ northern Florida.

Hybrid 3) *Isoetes appalachiana* × *I. septentrionalis* (*I. xbrittonii*)

Brunton & Taylor (1990) described this hybrid before the existence of tetraploid *Isoetes appalachiana* was recognized within *I. engelmannii* (s.l.). As with *I. xbruntonii* (above), that timing encouraged the assumption that a hybrid between a tetraploid (*I. septentrionalis* – as *I. riparia* G. Engelmann, pro parte [p.p.] and *I. engelmannii* (s.l.), would be triploid. This misunderstanding was exacerbated by the selection of a cytotype not from the type location. That specimen (*W.C. Taylor 5,128B*, 9 Sept 1984, Middlesex County, Connecticut (MIL)) is recognized as probably representing *I. engelmannii* (s. str.) × *septentrionalis* (see Table 1).

The type location for *Isoetes xbrittonii* in Lycoming County, Pennsylvania was selected because of the apparent abundance of hybrids at the site (pers. obs.). Its location at a bridge abutment intruding into the natural flow of the Susquehanna River presents ideal conditions for hybrid generation. Cytological determinations made after the Brunton and Taylor (1990) description of the taxon established that hybrid plants at the *I. xbrittonii* type location (e.g. *D. F. Brunton & K. L. McIntosh 11,176*, 6 July 1992 (OAC) were tetraploid  $2n=4x=44$  and that the associated *I. engelmannii* (s.l.) parent was tetraploid *I. appalachiana* (*D. F. Brunton & K. L. McIntosh 11,177*, 6 July 1992 (OAC), not diploid *I. engelmannii* (s. str.). Significantly as well, spore measurements from the holotype and cytologically confirmed topotypes (megaspores 542.8 µm (1 SD 55.93 µm, N=80), *W.F. Westerfold & H. A. Wahl 3,045*, 11 August 1951 (PH) and *D. F. Brunton & K. L. McIntosh 11,177* (DFB), and microspores 33.1 µm (1 SD 2.60 µm, N=11), *D. F. Brunton & K. L. McIntosh 11,177* (DFB), are more representative of tetraploids



**Figure 7.** *Isoetes* × *brittonii* (*I. appalachiana* × *septentrionalis*): 7A: cluster of polymorphic megaspores (C.E. Waters s.n., 8 July 1904, Lancaster County, PA, USA, (MICH); 7B: lateral view of megaspore (W.F. Westerfeld & H. A. Wahl 3,045, 11 August 1951, Lycoming County, PA, USA (Isotype - PH).

than triploids, which average approximately 500 µm and 28 µm, respectively. The megaspore ornamentation of *Isoetes* × *brittonii* also has a more open, less convoluted ornamentation pattern than would be expected were *I. engelmannii* (s.str.) actually the other putative parent with *I. septentrionalis* (Figures 7A, 7B).

*Isoetes* × *brittonii* remains a rare taxon, perhaps not surprisingly considering the relatively small area of overlap in the distribution of its putative parents (Brunton and Britton 1997; Brunton and McNeill 2015). It can reasonably be expected to occur elsewhere, however, in at least central and eastern Pennsylvania and adjacent New Jersey. It is presently known only in Pennsylvania from Lycoming County: West Branch Susquehanna River opposite Jersey Shore (W. F. Westerfeld and H. A. Wahl 3045, 11 August 1951 (Holotype - PAC); Union County: Lewisburg (*J. Montgomery s.n.*, 6 September 1962 (DFB)), and Lancaster County: McCall's Ferry, Susquehanna River (C. E. Waters s.n., 8 July 1904 (MICH). It was apparently extirpated at the latter site by 20<sup>th</sup> Century dam construction.

Hybrid 4) *Isoetes engelmannii* (s.str.) × *I. echinospora* M. Durieu (*I. xeatonii*, pro sp.)

The cytological criteria for hybridization in *Isoetes* were first described by Jeffrey (1937) who stated that *I. eatonii* [sic] was 'under strong suspicion of hybrid deriva-

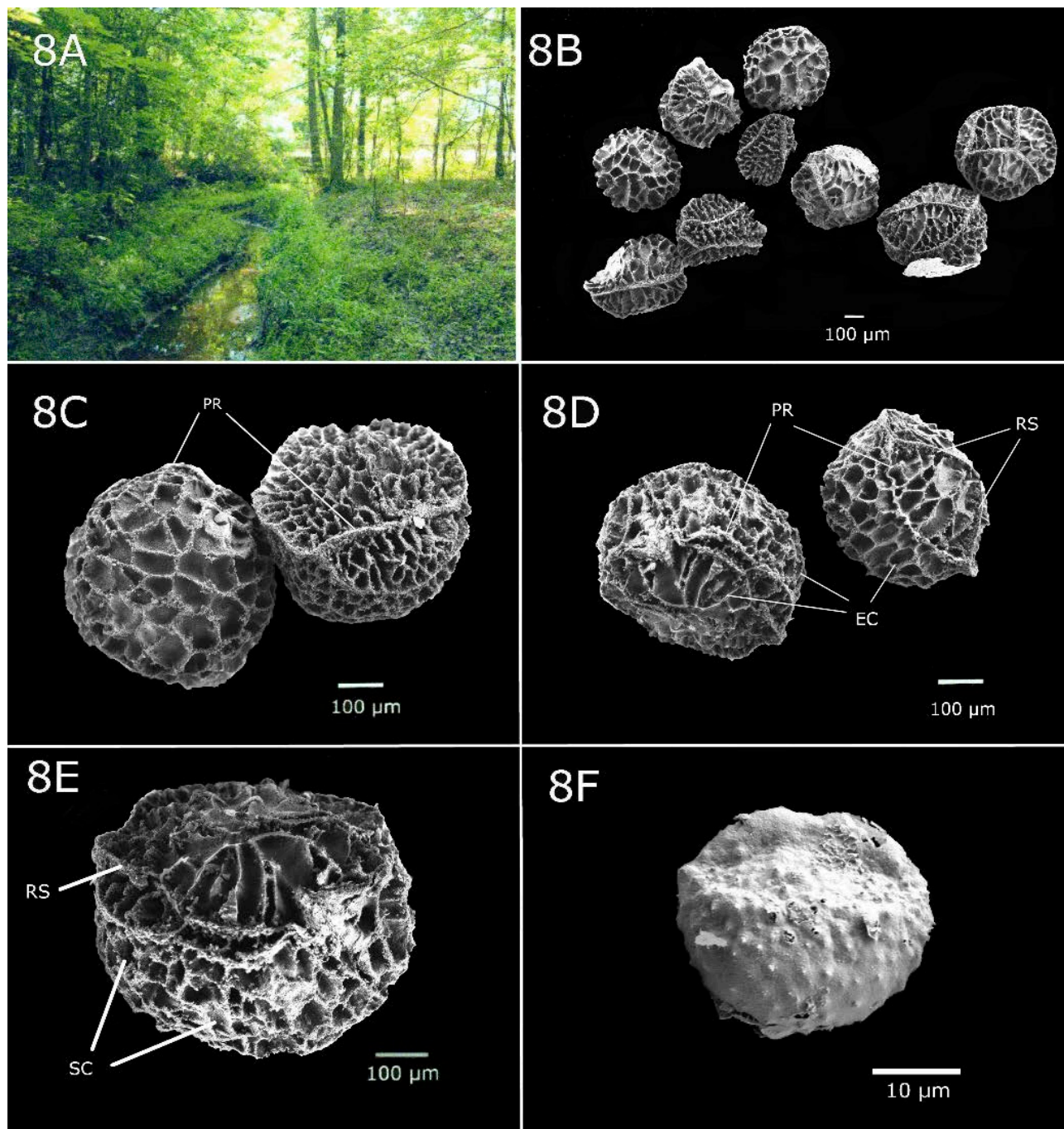
tion'. This taxon was one of the first hybrid *Isoetes* taxa to be formally recognized (Taylor et al. 1985; Hickey et al. 1987). Diploid *I. xeatonii* plants can be exceptionally large with leaves commonly over 30 cm long and some exceeding 60 cm in length (Dodg 1897). It is a true aquatic of freshwater ponds and streams, occasionally occurring in such large numbers as to hide the presence of one or both of its putative parents (Eaton 1900; Kott and Bobbette 1980). That abundance delayed recognition of its hybrid nature (Taylor et al. 1985). Its diploid hybrid status has been confirmed, however, by the morphological, ecological or cytological evidence coming forward in recent decades.

*Isoetes xeatonii* occurs in the New England region of the United States and is disjunct to the west in southern Michigan (Palmer 2018) and southern Ontario, Canada (Britton et al. 1991). The population decline of parent *I. engelmannii* (s.str.) in this region (Taylor et al. 2016) has resulted in a comparable decline in hybrid occurrences as well.

Hybrid 5) *Isoetes engelmannii* (s.str.) × *I. hyemalis* (hyb. nov.)

This hybrid combination is known from a single site in eastern Virginia where it persisted, presumably through a series of independent propagation events, for over 30 years. A large (40 cm long), mature plant of at least several years of age was collected in 1965 and

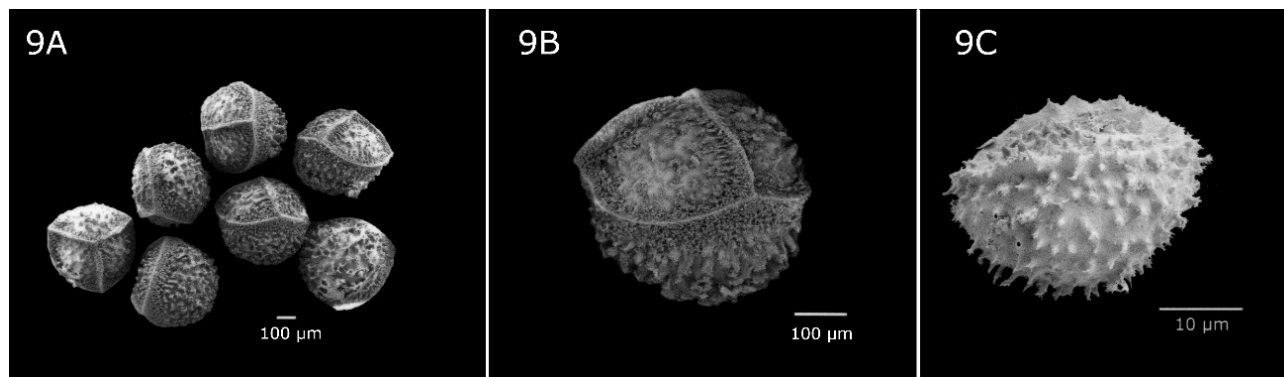




**Figure 8.** *Isoetes* × *fernaldii* (*I. engelmannii* × *hyemalis*): 8A: woodland stream habitat at type location (Gloucester County, VA, USA, 3 September 2016 (Photo: D.F. Brunton); 8B: cluster of polymorphic megaspores; 8C: distal and proximal view of megaspores with regularly reticulate cells [PR = pronounced suture ridges]; 8D: lateral view of megaspores with irregularly reticulate cells [EC = elongated cells, PR = pronounced suture ridges, RS = minute ridge spines]; 8E: lateral view of megaspore with densely reticulate ornamentation pattern [SC = small cells]; 8F: lateral view of weathered microspore (D.F. Brunton & K. L. McIntosh 14,167, 2 July 1999, Gloucester County, VA, USA (OAC).

smaller, cytologically confirmed hybrid plants were found at the same site in 1999. They were found in a small, permanently flowing creek along the edge of a

deciduous woodland (Figure 8A) growing with numerous *I. engelmannii* and *I. hyemalis* plants. In August 2006, however, the site was severely impacted by flash



**Figure 9.** *Isoetes hyemalis*: 9A: group of regular-shaped megaspores; 9B: lateral view of megaspore; 9C: lateral view of microspore (D.F. Brunton & K. L. McIntosh 11,165, 4 July 1992, Harnet County, NC, USA (topotype - DFB).

flooding associated with Hurricane Ernesto and by subsequent ditch-clearing activity. *Isoetes* plants were not evident in September 2006 nor in a subsequent site visit in June 2015 (pers. obs.).

The leaves of the hybrid plants have the gross appearance of their putative parents and a velum coverage of their sporangium of approximately 17-20% (N=4). This is centred within the 10-25% range of velum coverage of the putative parents (Brunton et al. 1994; Brunton and Britton 1997). The hybrid also exhibits conspicuously polymorphic, aborted megaspores with congested, irregular ornamentation patterns (Figure 8B). The reticulate pattern of *Isoetes engelmannii* is the dominant expression on megaspores of these hybrid plants (Figure 8C), but appear to be of a less regularly 'honeycomb' pattern than is evident with that parent (Figure 1B). Megaspores of the hybrid also have a more congested, irregularly-reticular ornamentation on the distal side than is expressed in *I. engelmannii* and individual ornamentation 'cells' are conspicuously smaller (Figure 8C, 8E). The variation amongst megaspores of the hybrid includes some having more broken-reticulate pattern of muri (walls) interspersed with very short almost tuberculate muri on the proximal side (Figure 8C). Others exhibit an ornamentation pattern of elongated, non-reticulate cells (Figures 8D) more suggestive of tetraploid putative parent *I. hyemalis* (Figure 9A, 9B).

Well-formed megaspores of hybrid plants are intermediate in size (487.8 µm (1 SD 39.90 µm, N=30) between that of the diploid *I. engelmannii* (460 µm) and tetraploid *I. hyemalis* (522 µm) (Brunton et al. 1994 and Brunton and Britton 1997, respectively). Microspores from Brunton & McIntosh 14,167 (OAC) are immature or weathered and their ornamentation pattern is somewhat obscured. Nonetheless, these microspores appear to have

a sparse ornamentation of low, squat spines (Figure 8F) intermediate between that of smooth-spored *I. engelmannii* (Figure 1C) and conspicuously broad-spiny *I. hyemalis* (Figure 9C). Well-formed microspores are 27.9 µm (1 SD 1.59 µm, N=60) in size, which is intermediate between those of putative parents *I. engelmannii* (25.5 µm - Brunton & Britton 1997) and *I. hyemalis* (30.95 µm, 1 SD 1.76 µm, N=40), Brunton and McIntosh 11,165, Harnet County, North Carolina (OAC).

The following binomial is proposed for this undescribed taxon:

***Isoetes* × *fernaldii*** D.F. Brunton and P. C. Sokoloff, **hyb. nov.**

(*Isoetes engelmannii* × *hyemalis*) (Figures 8B-8F)

**Typus:** United States, Virginia; Gloucester County: southeast side of Piney Swamp Road (SR 635) at culvert for Piney Swamp, 2 km southwest of White Marsh, Gloucester County, 2 July 1999, D.F. Brunton & K. L. McIntosh 14,167 (holotype, OAC! isotype (partial) DFB!).

#### *Description*

**Plants** - robust and exhibiting hybrid vigour (leaves to more than 40 cm tall), with gross form typical of putative parents *Isoetes engelmannii* and *hyemalis*; velum coverage of the sporangium 17-20%; **Megaspores** - variable in size, misshaped, frequently aborted; well-formed spores 487.8 µm (1 SD = 39.90 µm) in diameter with a congested, irregularly reticulate ornamentation pattern of irregularly tall muri; **Microspores** - sparsely ornamented with low, broad-based spines, 27.9 µm (1 SD = 1.59 µm) in length; **Habitat** - mixed populations growing with both putative parents in disturbed, periodically flood-scoured ditch along the edge of deciduous swamp

forest; Cytology -  $2n=3x=33$  (D.F. Brunton & K. L. McIntosh 14,167, 2 July 1999 (OAC).

#### Etymology

The epithet acknowledges the contribution made by Merritt L. Fernald (1873-1950) to our knowledge of the distribution of this genus in the southeastern United States in general and Virginia in particular. His comprehensive field investigations in the 1930s and 1940s, especially on the species-rich coastal plain (Hugo and Ware 2012), laid the foundation upon which subsequent regional floristic and taxonomic investigations such as Pease (1951) and Musselman and Knepper (1994) are based.

#### Paratype

United States, Virginia, Gloucester County; dried up creek bed in Piney Swamp, 5 miles nw of Gloucester Point, June 12, 1965. A.M. Harvill 12444 (GA!).

*Isoetes*  $\times$  *fernaldii* is similar in appearance to tetraploid *I.*  $\times$  *bruntonii*. Its smaller spore size, however, reflects its triploid status. It is even more similar in appearance to fellow triploid *I.*  $\times$  *karenae*. Compared with the latter, however, *I.*  $\times$  *fernaldii* exhibits megaspores with thinner, higher muri (Figures 8C, 8D vs. Figures 4C, 4D) and more pronounced suture ridges with a greater abundance of tiny spines along their flanks (Figures 8D vs. Figures 4C), and more evidently spiny microspores.

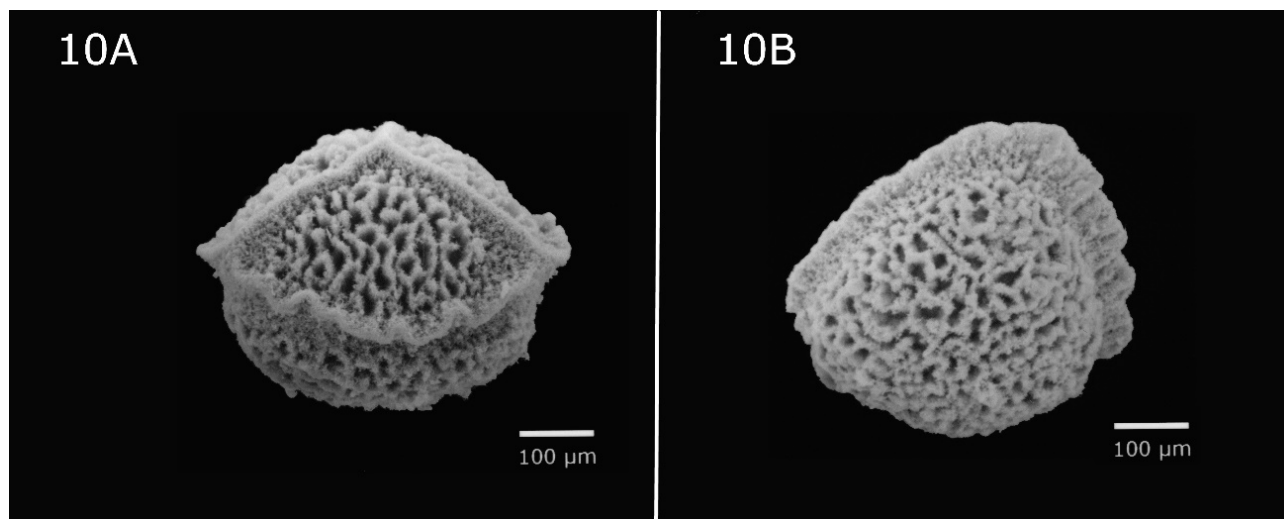
There is considerable overlap in the distributions of *Isoetes hyemalis* and *I. engelmannii* in the eastern United

States. Accordingly, additional localities for this sterile hybrid are expected to be found, particularly in Virginia and the Carolinas.

#### Hybrid 6) *Isoetes engelmannii* (s.str.) $\times$ *I. macrospora* Durieu (*I.* $\times$ *fairbrothersii* J. Montgomery & W.C. Taylor)

*Isoetes*  $\times$  *fairbrothersii* is the only hexaploid ( $2n=6x=66$ ) taxon in the *I. engelmannii* complex. Its putative 10x parent *I. macrospora* (= *I. lacustris* L., sens. auct.) is a northern species that is disjunct as far south as New Jersey. It is distinctive from other *I. engelmannii* complex hybrids by its large (>530  $\mu$ m) megaspores. Its megaspores also exhibit classic *Isoetes* hybrid characteristics, 'some [being] very large and spherical, others small and pyramidal, a few others dumbbell-shaped and some represented by wedge shaped fragments' (Montgomery and Taylor 1994).

The hybrid is known only from a single freshwater lake in New Jersey where individuals were found infrequently in mixed populations with its putative parents, most frequently with *Isoetes macrospora*. With few areas of overlapping distribution by the putative parents, the possibility of additional populations of *I.*  $\times$  *fairbrothersii* being found are limited to northern New Jersey and adjacent eastern New York. Occurrences may also be possible, however, within the disjunct western populations of *I. engelmannii* in Michigan or Ontario (Cody and Britton 1989; Taylor et al. 1993, Tryon and Moran 1997; Palmer 2018; Weldy et al. 2019).



**Figure 10.** *Isoetes*  $\times$  *foveolata* (*I. engelmannii*  $\times$  *tuckermanii*): 8A: lateral view of megaspore; 9B: distal view of megaspore (A.A. Eaton 482a, 20 August 1896, Rockingham County, NH, USA (Syntype - MICH).

Hybrid 7) *Isoetes engelmannii* (s.str.) × *I. riparia* G. Engelmann (s.str.) (*I. ×carltaylorii* L.J. Musselman)

*Isoetes ×carltaylorii* was originally described as the cytologically confirmed triploid hybrid between diploid *I. engelmannii* and tetraploid *I. acadensis* L. Kott (Musselman et al. 1997). The latter is now considered to represent *I. tuckermanii* A. Braun ssp. *acadensis* (L. Kott) D.F. Brunton (Brunton 2018)). The hybrid is known only from the tidal reaches of rivers flowing into the western side of the Chesapeake Bay in northeastern Virginia. It is unique amongst *I. engelmannii* complex hybrids in having a conspicuously subdued, reticulate megaspore ornamentation pattern of low, broad (almost vermiform) muri.

The tetraploid parent appears not to be *Isoetes tuckermanii* ssp. *acadensis* but *I. riparia* var. *reticulata* (A.A. Eaton) G. Proctor, an uncommon (now rare?) endemic of emergent tidal shores in freshwater and brackish marsh habitat on the Chesapeake Bay (Proctor 1949). *Isoetes riparia* var. *reticulata* shares many morphological similarities with *I. tuckermanii* ssp. *acadensis* (Brunton 2015), particularly in having a reticulate megaspore ornamentation pattern of low, broad (almost vermiform) muri. *Isoetes riparia* var. *reticulata* has consistently smaller megaspores with thinner, usually more densely arranged muri, however, and this intricacy is reflected in the megaspore ornamentation of the hybrid. In addition, *I. tuckermanii* ssp. *acadensis* is a non-tidal freshwater species that extends no further south than Massachusetts, 650 km from the nearest *I. ×carltaylorii* site (Taylor et al. 1993; Musselman et al. 1997; Brunton 2018).

*Isoetes ×carltaylorii* is not likely to be found outside of the Chesapeake Bay of Virginia and the adjacent District of Columbia, the only area where the putative parents are sympatric.

Hybrid 8) *Isoetes engelmannii* (s.str.) × *I. tuckermanii* (*I. ×foveolata*, pro sp.)

Along with *Isoetes ×eatonii*, this was thought to be a distinct species when described in the 19<sup>th</sup> Century (Eaton 1898) and is one of the first *Isoetes* taxa to be recognized as a hybrid (Taylor et al. 1985; Hickey et al. 1989). It is a true aquatic, found in ponds and rivers in southern New Hampshire and apparently has not been found in the wild for over a century. Megaspore ornamentation in this triploid is congested, presenting a particularly bold example of the ‘brain-coral’ pattern typical of *Isoetes* hybrids (Britton 1991) (Figures 10A, 10B).

Given the relatively large area of southern New England (Massachusetts, Connecticut, Rhode Island, New

Hampshire) where its (at least formerly) common putative parents are sympatric, the rarity of this taxon is unexpected. This may reflect an ecological separation of the parents, *Isoetes tuckermanii* apparently occurring in more acidic substrates and oligotrophic water than *I. engelmannii* (pers. obs.).

Hybrid 9) *Isoetes engelmannii* (s.str.) × *I. valida* (G. Engelmann) W.N. Clute (*I. ×altonharvillii*)

This cytologically confirmed diploid typically exhibits the classic features of *Isoetes* hybrids including hybrid vigour, brain coral megaspore ornamentation patterns, fragmented and aborted spores and occurrence in ponds, seepages or along river shores with one or (usually) both of its putative parents (Musselman et al. 1995). The ca. 50% velum coverage of the sporangia reported for the hybrid is the same as that determined for *I. valida* (Brunton and Britton 1996). This is significantly greater than would be expected of a hybrid also involving *I. engelmannii* which has a much narrower (10–15%) velum coverage (Brunton and Britton 1996, 1997). Subsequently examined specimens of *I. ×altonharvillii* from Tennessee, North Carolina and South Carolina (DFB), however, average 26.6% velum coverage (N=28, six plants), which is consistent with expectations for an *I. engelmannii* × *valida* hybrid.

*Isoetes ×altonharvillii* has been found in mixed *Isoetes* populations in ponds and streams adjacent to and within the Appalachian Mountains from Delaware to northeastern Alabama, occasionally growing in relatively high elevation sites.

Potential Hybrid Combinations

In addition to the nine formally named hybrid taxa reviewed above, there are eight hybrid combinations which remain to be confirmed in the *Isoetes engelmannii* complex. Most of these involve species with restricted range and/ habitat requirements and thus have limited potential for interaction with other *Isoetes*. Putative parental taxa include *I. boomii* N.T. Luebke, *I. georgiana* N.T. Luebke, *I. mattaponica* L.J. Musselman & W.C. Taylor and *I. microvela* D.F. Brunton. Table 1 lists and identifies the expected habitat, areas of occurrence (reflecting the sympatric range of the putative parents) and ploidy level of these presently unconfirmed hybrid combinations. The likelihood of discovery for each hybrid combination is subjectively appraised based on the apparent availability of suitable habitat and condi-



**Table 1.** Hypothesized *Isoetes engelmannii* complex hybrids.

Hybrid combinations	Ploidy	Probable habitat	Possible Area of Occurrence	Likelihood of Occurrence
<i>Isoetes appalachiana</i> × <i>I. boomii</i>	5x	ephemeral braided stream channels in deciduous swamp forest	Upper coastal plain, Georgia, northern Florida	Low
<i>Isoetes appalachiana</i> × <i>I. georgiana</i>	5x	ephemeral streams in deciduous swamp forest	Upper coastal plain, Georgia	Low
<i>Isoetes appalachiana</i> × <i>I. mattaponica</i>	3x	edges of freshwater tidal marshes or woodland freshwater stream outlets	tributaries of the Chesapeake Bay, Virginia	Low
<i>Isoetes appalachiana</i> × <i>I. microvela</i>	5x	Periodically flooded channels and stream bank swales within deciduous swamp forests	Coastal plain, North Carolina	Low
<i>Isoetes appalachiana</i> × <i>I. riparia</i> (s. str.) (coastal plain '×brittonii')	4x	ephemeral streams and emergent blackwater stream banks	Outer coastal plain, Virginia to northern North Carolina	Moderate
<i>Isoetes appalachiana</i> × <i>I. valida</i> (large-spored '×altonharvillii')	3x	Emergent, shaded streambanks and woodland seepage areas	Southern Pennsylvania to Alabama	High
<i>Isoetes engelmannii</i> × <i>mattaponica</i> (small-spored '×cartaylorii')	2x	Submerged along river shores adjacent freshwater tidal marshes	tributaries of the Chesapeake Bay, Virginia	Low
<i>Isoetes engelmannii</i> × <i>septentrionalis</i> (northern '×bruntonii')	3x	Shallow water and emergent shores of freshwater rivers and lakes	Southern New York, eastern Pennsylvania, Connecticut	High

tions for hybrid generation within the sympatric range of putative parents.

## DISCUSSION

The complicated relationship within the diverse *Isoetes engelmannii* complex is depicted in Figure 11. Thirty taxa are represented, 13 of which are species, nine representing named sterile hybrids (including the two described here) and eight (listed in Table 1) being theoretically possible hybrids.

Specimens apparently representing some of the unconfirmed hybrid combinations noted in Table 1 are known. Herbarium specimens that appear to represent triploid *Isoetes engelmannii* × *septentrionalis* from Connecticut, New Jersey, Pennsylvania, New York and Maryland, for example, are deposited in MICH, MIL, PH and PSU. Most of these are fragmentary, pre-1950s specimens with limited site data, however. Confirmation of the natural occurrence of that and other possible hybrid combination requires stronger supporting evidence. Other vouchers suspected to represent unnamed *I. engelmannii* complex hybrids are old, fragmentary and/or ambiguous. They also are deemed insufficient to independently support formal designations.

In addition to enumerating the diversity and status of hybrids within the *Isoetes engelmannii* complex, the present study illustrates that the identification of such plants by morphological features alone is relatively straightforward. A suite of reliable morphological indi-

cators of hybrid status has emerged from the investigation and description of over two dozen *Isoetes* hybrids in North America (Taylor et al. 1993; Musselman et al. 1994; Brunton and Britton 1999; Brunton 2015; Taylor et al. 2016), Japan (Takamiya 1999), eastern Russia (Mocholova et al. 2015), western Europe (Brunton et al. 2020) and India (Singh et al. 2018) over the last 25 years.

The determination of the parentage of hybrid plants in herbarium specimens on the basis of morphological features alone can be difficult. Indeed, without fully mature specimens and information on the associated *Isoetes* growing with a suspected hybrid, or in cases where voucher material is fragmentary or otherwise poorly preserved, identification may be impossible. This is particularly the case in differentiating hybrids between taxa of equal ploidy levels (and thus with spores of approximately the same size) and/ or with hybrid taxa sharing one putative parent, such as *I. ×karenae* and *I. ×fernaldii*. In these and most situations, the most compelling direct evidence of paternity can be the identity of the *Isoetes* species with which hybrid plants are growing.

Allopolyploidy involving the doubling of chromosome of interspecific hybrids is a common route of speciation in pteridophytes (Sigel et al. 2019) and is seen as an important (the most significant?) means for generating the current diversity of polyploid *Isoetes* species (Hoot 2004; Troia et al. 2016; Schafran et al. 2018). Accordingly, aligning important molecular indications of diversity with consistent phylogeographic, physiological, ecological and morphological lines of evidence is critical to the development of a coherent taxonomic understanding of

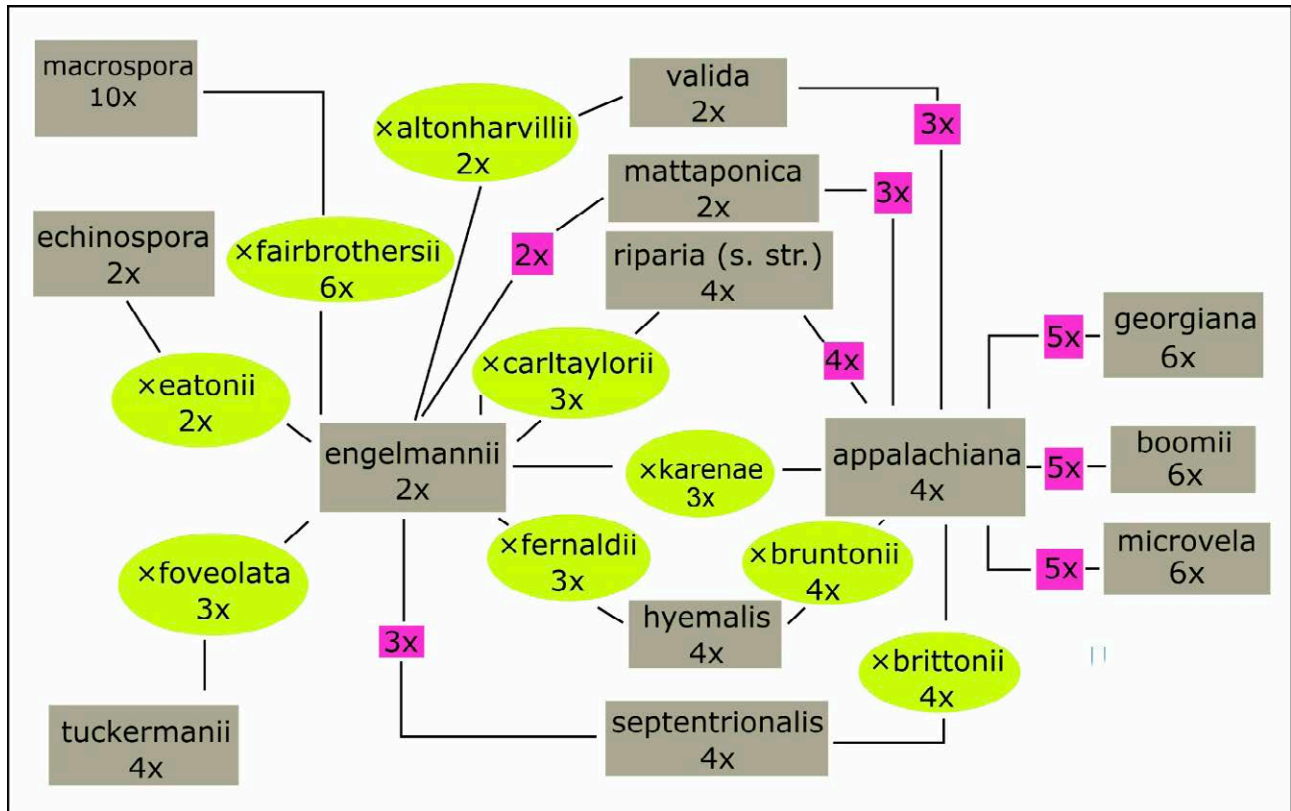


Figure 11. Relationships within the *Isoetes engelmannii* complex.

the genus. The present investigation contributes towards this by clarifying hybrid relationships within the important *I. engelmannii* complex. That process and these findings may also facilitate the identification of other sterile hybrids between populations within the dozens of undescribed, cryptic genetic entities recently reported from the eastern United States (Schafran 2019).

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