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BRIEF COMMUNICATION

# Inferring the evolutionary reduction of corm lobation in *Isoëtes* using Bayesian model-averaged ancestral state reconstruction

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**PREMISE OF THE STUDY:** Inferring the evolution of characters in *Isoëtes* has been problematic, as these plants are morphologically conservative and yet highly variable and homoplasious within that conserved base morphology. However, molecular phylogenies have given us a valuable tool for testing hypotheses of character evolution within the genus, such as the hypothesis of ongoing morphological reductions.

**METHODS:** We examined the reduction in lobe number on the underground trunk, or corm, by combining the most recent molecular phylogeny with morphological descriptions gathered from the literature and observations of living specimens. Ancestral character states were inferred using nonstationary evolutionary models, reversible-jump MCMC, and Bayesian model averaging.

**KEY RESULTS:** Our results support the hypothesis of a directional reduction in lobe number in *Isoëtes*, with the best-supported model of character evolution being one of irreversible reduction. Furthermore, the most probable ancestral corm lobe number of extant *Isoëtes* is three, and a reduction to two lobes has occurred at least six times.

**CONCLUSIONS:** From our results, we can infer that corm lobation, like many other traits in *Isoëtes*, shows a degree of homoplasy, and yet also shows ongoing evolutionary reduction.

**KEY WORDS** Bayes factors; evolutionary reduction; Isoetaceae; model selection; morphological evolution; morphological simplification; RevBayes.

Interpreting the morphological evolution of *Isoëtes* L. has troubled botanists for many years. These plants have an outwardly simple, highly conserved body plan, consisting of an apical rosette of linear sporophylls on top of a reduced corm-like trunk (Engelmann, 1882; Pfeiffer, 1922; Cox and Hickey, 1984; Hickey, 1986; Taylor and Hickey, 1992; Budke et al., 2005), yet are highly variable both within (Budke et al., 2005; Liu et al., 2006) and among closely related species (Cox and Hickey, 1984; Hickey, 1986; Taylor and Hickey, 1992; Romero and Real, 2005; Bagella et al., 2011). Even characters once thought to be useful in delimiting natural groups within the genus, such as habitat (Engelmann, 1882) or megaspore morphology (Pfeiffer, 1922), have been found to be labile (Cox and Hickey, 1984; Taylor and Hickey, 1992; Budke et al., 2005; Hickey, 2007; Bagella

et al., 2011). And while some characters, such as the glossopodium (the portion of the ligule internal to the leaf), have shown some diagnostic potential (Sharma and Singh, 1984; Pant et al., 2000; Shaw and Hickey, 2005; Singh et al., 2010; Freund, 2016), actually examining and interpreting these structures requires considerable histological and computational effort, making them ill-suited for field identification. This absence of consistent, dependable characters creates a paradox: the lack of reliable traits impedes the inference of phylogenies or classifications in the genus, but without a phylogeny, examining character evolution is exceptionally difficult.

However, molecular phylogenetics has vastly improved our understanding of *Isoëtes* (Rydin and Wikstrom, 2002; Hoot et al., 2004, 2006; Schuettpehl and Hoot, 2006; Larsén and Rydin, 2016)

and has provided evidence for five major clades within the genus: Gondwanan (Clade A), Laurasian (Clade B), Italian (Clade C), Austro-Asian (Clade D), and New World or American (Clade E; Hoot et al., 2006; Larsén and Rydin, 2016). This phylogeny has completely overturned the old morphological and ecological systems of classification and has also provided a framework to begin earnestly studying character evolution in the genus. While characters such as spore ornamentation are highly homoplastic (Cox and Hickey, 1984; Larsén and Rydin, 2016), there are other changes that may be informative, such as serial reduction of the corm (Karrfalt and Eggert, 1977a, b; Pigg, 1992; Grauvogel-Stamm and Lugardon, 2001).

The isoetals have a long fossil history, spanning >300 million yr (Pigg, 1992; Gensel and Pigg, 2010; Taylor et al., 2009). Due to this long history, and the abundance of fossil representatives, there is a long-standing comparison between the corm of extant *Isoetes* and the stigmarian appendages of their extinct arborescent relatives. This comparison is supported by the unique suite of characters that are found only in the isoetalian lycophytes (Stewart, 1947; Karrfalt and Eggert, 1977a, b; Jennings et al., 1983; Pigg, 1992). These features include a unique and independently evolved form of secondary growth that is fundamentally different from the secondary growth of spermatophytes (Scott and Hill, 1900; Stokey, 1909; Pfeiffer, 1922; Stewart, 1947; Karrfalt and Eggert, 1977a; Pigg and Rothwell, 1985; Ash and Pigg, 1991; Pigg, 1992; Gensel and Pigg, 2010). Their rooting structures are also unique and have been compared to a dichotomous branching system, with their rootlets arising from modified leaves (Rothwell and Erwin, 1985). Some fossil isoetals, such as the arborescent *Protostigmaria* Jennings (Jennings et al., 1983) or geophytic *Nathorstiana* Richter (Richter, 1910; Mägdefrau, 1932; Taylor et al., 2009), have subterranean morphology comparable to that of modern *Isoetes* (i.e., not producing elongated stigmarian appendages), with the size, elaboration, or arborescent habit of the plants separating them from the contemporary taxa (Stewart, 1947; Karrfalt, 1984a; Pigg, 1992). *Protostigmaria*, while having a generally comparable morphology to extant *Isoetes*, differs by having as many as 13 lobes on its rooting axis. *Nathorstiana*, while having corms that are quite similar to those of extant *Isoetes*, shows high levels of corm lobe variability, like some extant species of *Isoetes*, yet little correlation of plant size with lobe number (some of the small plants have four lobes, and some larger plants are unlobed; Richter, 1910; Mägdefrau, 1932; Jennings et al., 1983; Rothwell, 1984; Rothwell and Erwin, 1985; Taylor et al., 2009). Extant *Isoetes*, and potentially the extinct forms, also possess a unique mechanism to penetrate into the substrate by laterally displacing the soil and then pulling the corm deeper through a combination of secondary growth and development of new rootlets. While functionally similar to the contractile roots of monocots, this mechanism does not compress the soil below the plant, allowing for deeper substrate penetration (Karrfalt, 1977). Due to these shared features, it has been hypothesized that modern *Isoetes* are the result of continuous reduction of the once arborescent isoetalian body plan, with the reduced corm lobe numbers in modern *Isoetes* continuing this trend (Stewart, 1947; Karrfalt and Eggert, 1977a, b; Jennings et al., 1983; Pigg, 1992).

These corm lobes are formed by the basal, rootlet-producing meristems (Engelmann, 1882; Stokey, 1909; Osborn, 1922; Pfeiffer, 1922; Bhambie, 1963; Karrfalt and Eggert, 1977a; Budke et al., 2005); extant plants have either a trilobate or bilobate base morphology. This morphology appears early in ontogeny and has been observed in sporelings (F. D. Freund, personal observation). In trilobate species, there are three basal furrows, which run down the lateral face of the corm and join together at the distal end (Fig. 1A–C). These basal furrows divide the corm into three sections where

secondary growth ultimately results in the formation of the triple corm lobes (Stokey, 1909; Osborn, 1922; Bhambie, 1963; F. D. Freund, personal observation). By contrast, the bilobate species have only a single furrow, which runs in a line across the base of the corm, dividing it into two halves (Fig. 1D–F). While the plants do occasionally acquire additional lobes as they age, the base morphology—the minimum number of lobes the plants have before any elaboration—is consistent (Karrfalt and Eggert, 1977a, b). Also, there are other, rarer morphologies, such as the rhizomatous, mat-forming *I. tegetiformans* Rury (Rury, 1978) and the monolobate *I. andicola* (Amstutz) L.D. Gómez (formerly treated as *Stylites andicola* Amstutz; Amstutz, 1957). However, both *I. andicola* and *I. tegetiformans* begin life with a bilobate morphology before secondarily acquiring these alternate states (Rury, 1978; Karrfalt, 1984b; Tryon et al., 1994); their base morphology is bilobate.

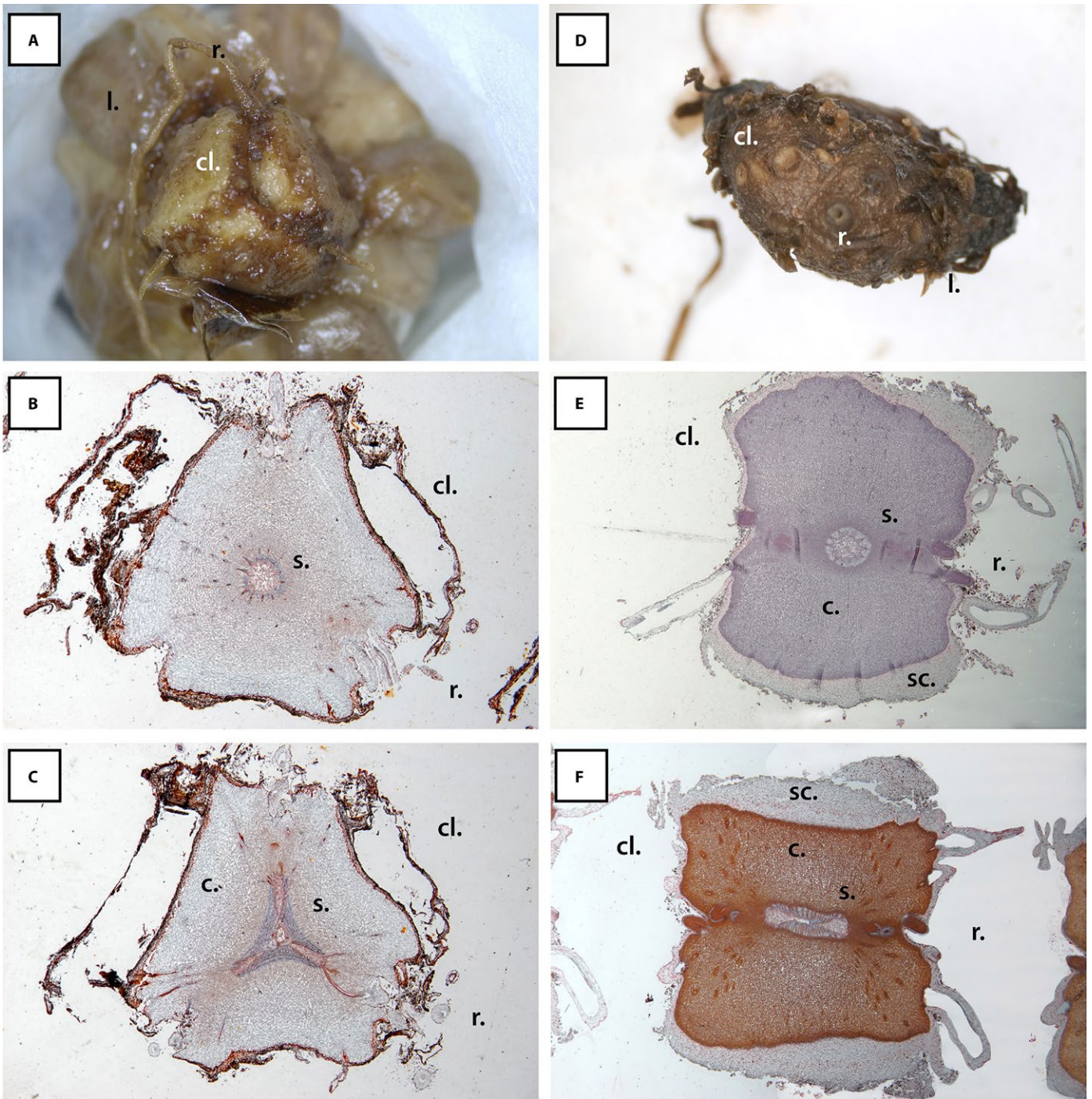
Here, we explore the evolution of corm lobation across the most recent *Isoetes* phylogeny (Larsén and Rydin, 2016). If *Isoetes* has undergone evolutionary reduction in corm lobation number as Karrfalt and Eggert (1977b) hypothesized, we expect the ancestral state for extant *Isoetes* to be trilobate, and the rate of transition from trilobate to bilobate to be higher than the rate of bilobate to trilobate. To test this hypothesis, we used reversible-jump Markov chain Monte Carlo (MCMC; Green, 1995) to explore multiple nonstationary statistical models of morphological evolution (Klopfstein et al., 2015) and to assess whether there is evidence for directional or even irreversible evolution in corm lobation morphology. Finally, we used simulations to examine our power to detect irreversible evolution on datasets of this size.

## METHODS

### Corm lobation characterization

Corm lobe numbers were collected from observations of fresh material, herbarium specimens (Appendix 1), and published descriptions (Appendix 2). We characterized lobation from fresh plants for eight species (*Isoetes appalachiana* D.F. Brunt. & D.M. Britton [four individuals]; *I. bolanderi* Engelm. [20+ individuals]; *I. eatonii* R. Dodge [three individuals]; *I. englemanni* A. Braun [four individuals]; *I. howellii* Engelm. [200+ individuals]; *I. nuttallii* A. Braun ex Engelm. [400+ individuals]; *I. occidentalis* L.F. Hend. [15+ individuals]; and *Isoetes orcuttii* A.A. Eaton [50+ individuals]). Each individual was cleaned of encrusting soil, then examined to determine the number of basal furrows and to assess the symmetry of the corm lobes. Plants of different ages were observed for each species when possible to get a sense of variability in lobation pattern as the plants aged, as well as to identify any unusual morphological outliers such as plants with asymmetric lobation or nonlinear basal furrows that may have occurred due to advanced age or damage to the corm. The *I. nuttallii* samples include two sporelings, one of which was still connected to the megaspore, which already showed distinctly trilobate arrangement of their rootlets. Taxa in which the plants have a single contiguous furrow and two symmetrical lobes were scored as “bilobate,” while species with three linear furrows with symmetrical lobation around the base of the corm were scored as “trilobate” (Fig. 1).

For lobation numbers garnered from the literature, species with a definitive, single reported corm lobation value were assigned that number, while species with a range of reported corm lobe numbers were scored as “undefined” due to the possibility that the description is of a taxon with a range of corm morphologies, such as



**FIGURE 1.** Comparative morphology of trilobate and bilobate corms. Trilobed corm: (A) *Isoetes nuttallii* (F. Freund 266, UC) in proximal view with senesced cortical tissue removed (8.1×, 29 mm field of view); (B–C) transverse sections of *I. nuttallii* (F. Freund 169, RSA796374) below apical rosette (B) and near basal furrows (C) (32.5×, 7.1 mm field of view). Bilobed corm: (D) *I. howellii* (F. Freund 266.1, UC) in proximal view (24×, 9.5 mm field of view); (E–F) cross sections of *I. bolanderi* (F. Freund 10, RSA811643) below apical rosette (E) and near basal furrow (F) (24.5×, 9.3 mm field of view). All images were adjusted in Adobe Photoshop to improve visibility by using the image levels tool and the dodge tool to even out lighting and coloration. For herbarium acronyms, see Index Herbariorum (<http://sweetgum.nybg.org/science/ih/>). Labels: c. = cortex ground tissue, cl. = corm lobe, r. = rootlets, l. = leaves, s. = stele, and sc. = senesced cortical ground tissue.

*I. tuckermanii* A. Br. (Karrfalt and Eggert, 1977b; Croft, 1980), or due to the inclusion of multiple cryptic species within a single taxon (Appendix 2).

Additionally, three accessions were coded as “unknown” for lobation due to identification uncertainties: (1) One accession of *I. savatieri* Franchet was collected in Uruguay, outside of the

currently accepted range of the species (Hickey et al., 2003), and we were unable to examine the specimen to determine its identity or lobation. (2) *Isoëtes histrix* Bory & Durieu is a known species complex (Bagella et al., 2011, 2015), and specimens fall in two different areas of our phylogeny. One is sister to *I. setacea* Lam., a position that is consistent with morphology and with existing hypotheses of their relationship (Hoot et al., 2006; Bagella et al., 2011; Troia and Greuter, 2014); we treated this accession as correctly identified. The second accession falls phylogenetically distant from the first, and presumably is misidentified; we were unable to ascertain its true identity or morphology, so we coded it as unknown. (3) *Isoëtes australis* R.O. Williams also shows up in two places in the phylogeny: Clade A and Clade D. The Clade A plant was collected and identified by Dr. Carl Taylor, an authority on *Isoëtes*, so we treated this accession as correctly identified and coded the Clade D plant as unknown. All character states and literature sources can be found in Appendix 2.

### Phylogenetic modeling

Ancestral character state reconstructions were performed on a posterior sample of 15,000 trees from Larsén and Rydin (2016) that we rooted on the bipartition between Clade A and the remainder of the genus (following Larsén and Rydin, 2016).

We employed reversible-jump MCMC (Green, 1995) in RevBayes (Höhna et al., 2016) to explore the space of all five possible continuous-time Markov models of phenotypic character evolution and to infer ancestral states. The reversible-jump MCMC sampled from the five models in proportion to their posterior probability. This approach enabled model-fit comparisons through Bayes factors (Kass and Raftery, 1995) and provided the opportunity to account for model uncertainty by making model-averaged ancestral state and parameter estimates (Madigan and Raftery, 1994; Kass and Raftery, 1995; Huelsenbeck et al., 2004; Freyman and Höhna, 2017). The five models of corm lobation evolution considered were as follows: a model with the rate of lobation gain and loss set to be equal (the 1-rate model); a model where the rates of lobation gain and loss are independent and nonzero (the 2-rate model); two irreversible models where the rate of either lobation gain or loss was fixed to zero; and lastly a model where both rates were fixed to zero. To test for directional evolution, we used nonstationary models of character evolution with root state frequencies that differed from the stationary frequencies of the process (Klopfstein et al., 2015).

Each of the five models was assigned an equal prior probability using a uniform set-partitioning prior. The root state frequencies were estimated using a flat Dirichlet prior. The rates of corm lobation gain and loss were drawn from an exponential distribution with a mean of one expected character state transition over the tree ( $\lambda = \tau/1$ , where  $\tau$  is the length of the tree).

The MCMC was run for 22,000 iterations, where each iteration consisted of 48 MCMC proposals. The 48 proposals were scheduled randomly from six different Metropolis-Hastings moves that updated the sampled tree, root frequencies, and corm lobation gain and loss rate parameters. The first 2000 iterations were discarded as burn-in, and samples were logged every 10 iterations. Convergence of the MCMC was confirmed by ensuring that the effective sample size of all parameters was over 600. The results were summarized and plotted using the RevGadgets R package (<https://github.com/revbayes/RevGadgets>). The scripts that specify our model, run the analysis, and summarize results are available in the code repository at <https://github.com/wf8/isoetes>.

### Simulations

To test how many observed characters are necessary to reliably infer irreversible evolution on a phylogeny the size of ours, we simulated 10 datasets with each of 1, 5, 10, 50, or 100 characters per tip (for a total of 50 simulations; note that our empirical dataset has a single character per tip). Each dataset was simulated under an irreversible model with the mean rate of corm lobation loss set to the value estimated by the irreversible model using the observed corm lobation data (2.39 changes per unit branch length). We performed the simulations using RevBayes over the maximum a posteriori phylogeny from the same tree distribution used to infer the ancestral states. For each of the 50 simulated datasets, an MCMC analysis was run for 11,000 iterations, with the first 1000 iterations dropped as burn-in. The model used was identical to that used for the observed corm lobation dataset, except that for the simulated datasets we fixed the maximum a posteriori phylogeny instead of integrating over the posterior distribution of trees.

### Figure editing

Figure plates of prepared slides and preserved materials were imported into Adobe Photoshop and adjusted using the levels and dodge tools to improve image definition and to reduce uneven color levels resulting from variations in background exposure. No features that were part of the original images were removed.

## RESULTS

### Model-fit comparisons

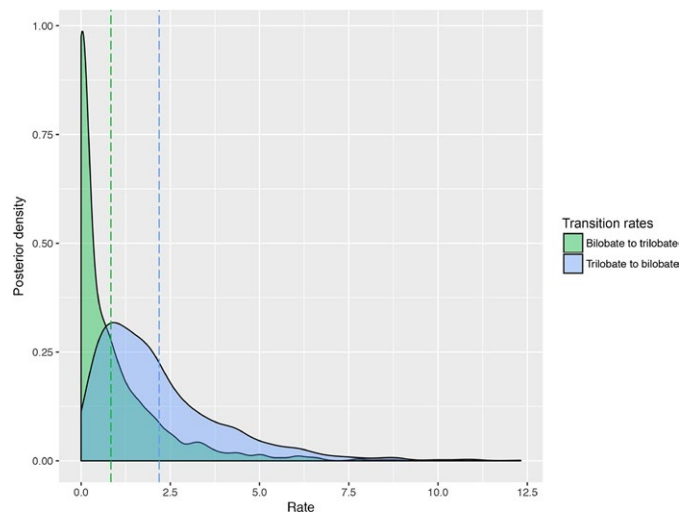
The maximum a posteriori model of corm lobation evolution was the tri- to bi- irreversible model (which did not allow transitions from the bilobate to the trilobate state) with a posterior probability of 0.38 (Table 1). This tri- to bi- irreversible model was weakly supported over the 1-rate and 2-rate reversible models (Bayes factor = 1.26 and 1.21, respectively; Kass and Raftery, 1995); however, all three models were strongly supported over the bi- to tri- irreversible model. Since the Bayes factor support for the best-supported model over the next two was negligible, we focus mostly on the model-averaged parameter estimates and ancestral states.

### Model-averaged parameter estimates and ancestral states

The model-averaged estimated rate of transition from trilobate to bilobate forms was significantly nonzero (mean = 2.17 changes per unit branch length, 95% HPD interval: 0.015–5.69), whereas the rate of bilobate to trilobate transitions was not significantly nonzero (mean = 0.82, 95% HPD interval: 0.0–3.35; Fig. 2). The model-averaged maximum a posteriori ancestral state of *Isoëtes* was trilobate with a posterior probability of 1.0 (Fig. 3). The ancestral state of the New World clade (“Clade E-2”; Fig. 3) was bilobate with a posterior probability of 0.99. The bilobate morphology arose independently in six places over the phylogeny (Fig. 3). No reversals from bilobate to trilobate were inferred. All species with unknown base corm lobation characters (*I. stevensii* J.R. Croft, *I. habbemensis* Alston, and *I. hallasanensis* H.K. Choi, Ch. Kim & J. Jung) were derived from a trilobate most recent common ancestor, with a posterior probability near 1.0.

**TABLE 1.** Comparisons of models of corm lobation evolution.

Model	Model posterior probability	Bayes factors			
		Bi- to tri- irreversible	Tri- to bi- irreversible	1-rate	2-rate
Bi- to tri- irreversible	0.0	–	<1	<1	<1
Tri- to bi- irreversible	0.38	>1000	–	1.26	1.21
1-rate	0.30	>1000	<1	–	<1
2-rate	0.32	>1000	<1	1.04	–



**FIGURE 2.** Model-averaged posterior densities of transition rates between corm lobation states. Mean values are represented by dashed lines. The rate of transition from trilobate to bilobate forms was significantly nonzero (mean = 2.17, 95% HPD interval: 0.015–5.69), whereas the rate of bilobate to trilobate transitions was not significantly nonzero (mean = 0.82, 95% HPD interval: 0.0–3.35). Transition rates are reported in changes per unit branch length.

### Simulations

For simulated datasets with a single character, the true irreversible model was at best weakly supported over the other models; the mean posterior probability of the true irreversible model was 0.37 (range: 0.22–0.44). As the number of characters increased, the posterior probability of the true model increased (Fig. 4). With 100 characters the support for the true model was strong; the mean posterior probability was 0.85 (range: 0.80–0.92).

### DISCUSSION

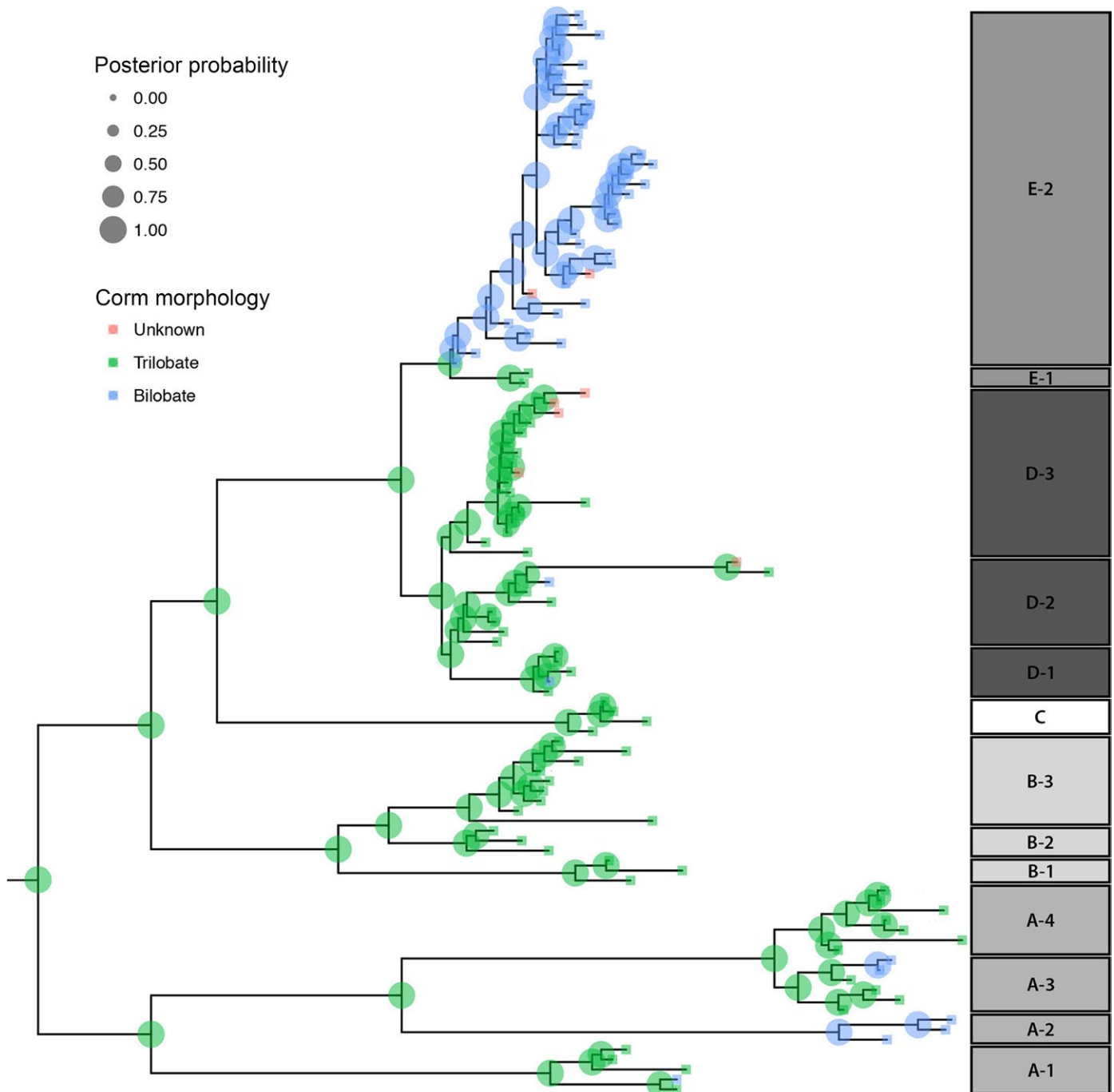
Our analyses support the hypothesis of directional reduction in lobe number over time in *Isoetes*, with the best-supported model being one of irreversible evolutionary reduction. Additionally, even when incorporating model and phylogenetic uncertainty and allowing for reversals, the model-averaged estimate of the transition rate from trilobate to bilobate was much higher than the estimate of the transition rate from bilobate to trilobate (the latter rate was not significantly nonzero). Furthermore, we found strong support for the ancestral state for all extant *Isoetes* being trilobate, indicating that there have been multiple convergent reductions of the corms to bilobate. These bilobate forms are nested deeply within

clades of trilobate plants. These results support the hypothesis that crown *Isoetes* has continued a reduction in corm morphology from the larger arborescent lycophytes. Additionally, while the bilobate form has emerged multiple times throughout the phylogeny, it is the dominant morphology in only one major clade: the “American clade” (Clade E-2, *sensu* Larsén and Rydin, 2016; Fig. 3). In this clade, nearly all species have a bilobate morphology.

The simulations demonstrate that the relatively weak support for the irreversible reduction model over the reversible models is likely due to the inherent limitation in statistical power of a single observed morphological character over a phylogeny of this size. Repeating this analysis with a larger, more densely sampled phylogeny, or one that incorporates fossil data, might find stronger support for the irreversible model of corm morphology evolution. Nevertheless, it is in cases like this, where no single model is decisively supported over others, that reversible-jump MCMC and Bayesian model-averaging demonstrate their utility for testing phylogenetic hypotheses of character evolution (Huelsenbeck et al., 2004; Freyman and Höhna, 2017).

While the bilobate morphology dominates only Clade E-2, it does occur in several other areas of the phylogeny (Fig. 2 and Appendix 2). In areas where Clade E-2 co-occurs with others, such as South America and the west coast of the United States, corm lobation is useful in determining to which clade a plant belongs. However, outside the range of Clade E-2, other bilobate taxa occur as single species nested within larger trilobate clades. As such, using corm lobation outside the Americas to assign plants with unknown phylogenetic placement is not advisable, since they may represent other independent evolutions of the character state.

When assessing corm lobation numbers, it is important to determine whether the corm lobe numbers are the base lobe numbers or additional lobes that have developed as the plant ages (Stokey, 1909; Karrfalt and Eggert, 1977a, b; F. D. Freund, personal observation). While Karrfalt and Eggert (1977b) reported a propensity for gaining additional lobes in their study of *I. tuckermanii* (68% bilobate, 30% trilobate, 2% tetralobate), other observers, working on other taxa, have not found this degree of variability (Engelmann, 1882; F. D. Freund, personal observation of >200 specimens of *I. howellii* and >400 specimens of *I. nuttallii*). In fact, we have observed a total of <10 *I. howellii* and *I. nuttallii* specimens that were not either bilobate or trilobate, respectively (F. D. Freund, personal observation). In *I. nuttallii*, the trilobate morphology was readily observable even in very young plants, including two sporelings, one of which was still attached to the megaspore (F. D. Freund, personal observation). These results suggest that the degree of variability of *I. tuckermanii* may be species-specific, and they add further support to the value of sampling multiple individuals when examining and designating corm lobation numbers within a species. If at all possible, it is additionally worthwhile to look at very young plants, especially sporelings, to determine the base lobe number.

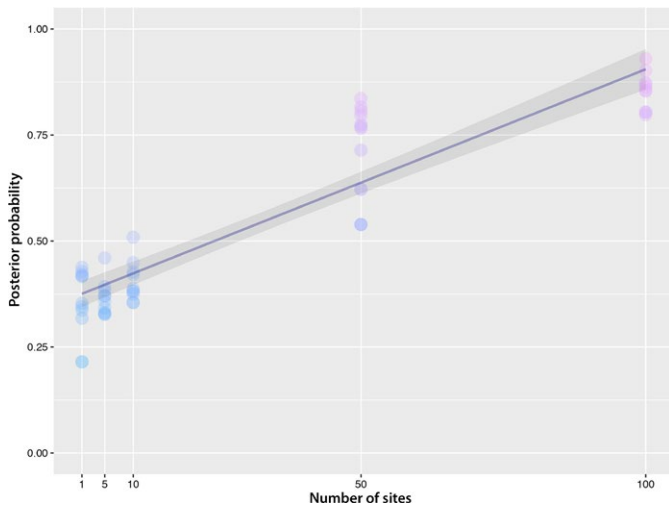


**FIGURE 3.** Bayesian model-averaged ancestral states of *Isoetes* corm lobation inferred over the posterior tree sample from Larsén and Rydin (2016). Ancestral states are summarized on the maximum a posteriori phylogenetic tree. The size of the circles at each node represents the posterior probability of the most probable ancestral state, and the color represents the state: green = trilobate corms, blue = bilobate corms, and red = unknown. Boxes on the right reflect subclade designations to broad subclade: A = Gondwanan, B = Laurasian, C = Italian, D = Austro-Asian, and E = New World or American. For specific placement of taxa into subclades, see Appendix 2.

## CONCLUSIONS

Our results support the hypothesis that crown *Isoetes* has continued an evolutionary reduction in corm morphology from the larger arborescent lycophytes, with the best-supported model being one of irreversible evolutionary reduction. However, results from our

simulation study showed that a dataset of this size only has weak statistical power to support irreversible models of character evolution, emphasizing the need for broader sampling of extant taxa. When we accounted for the uncertainty in character evolution models by making model-averaged estimates, we found strong support for the hypothesis of directional evolutionary reduction



**FIGURE 4.** Statistical power to detect irreversible evolution as a function of the number of characters available. Each point plotted represents a different simulation replicate. The y-axis shows the posterior probability of the true irreversible model of character evolution. The x-axis shows the number of simulated characters (sites or columns in the data matrix). Ten replicates were simulated for 1, 5, 10, 50, and 100-character datasets, resulting in a total of 50 simulated datasets.

in corm number, with the rate of lobe loss estimated to be much higher than the rate of lobe gain. Furthermore, we found strong support that the ancestral state for all extant *Isoetes* was trilobate, indicating that there have been multiple convergent reductions of the corms to a bilobate state.

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**APPENDIX 1. Vouchers examined for morphological determinations in this study.**

*Isoetes bolanderi*: RSA (811637, 811638, 811639, 811643). *I. howellii*: RSA (796366, 796367, 796368, 796369, 796370, 796371), UC (F. Freund 255, 266.1). *I. nuttallii*: RSA (796374, 796375, 796376), UC (F. Freund 256, 257, 258, 259, 261, 263, 264, 265, 266). *I. occidentalis*: RSA (811640, 811641, 811642). *I. orcuttii*: UC (F. Freund 254, 262). *I. storkii*: GH00021453, CM0102, NY00144272 (virtual herbarium sheets). For herbarium acronyms, see Index Herbariorum (<http://sweetgum.nybg.org/science/ih/>).

**APPENDIX 2. Corm lobation numbers for *Isoetes*. Clade designations follow Larsén and Rydin (2016). For herbarium acronyms, see Index Herbariorum (<http://sweetgum.nybg.org/science/ih/>).**

Species	Observed by F. Freund	Clade	Lobes	Locality	Sources
<i>Isoetes abyssinica</i> Choiv. – synonym of <i>I. welwitschii</i> (sensu Verdcourt and Beentje, 2002)		B-3	3	Africa, Ethiopia	Pfeiffer, 1922; Cook, 2004; Crouch et al., 2011
<i>Isoetes acadensis</i> Kott		?	2	USA (MA, ME, NH, NJ, NY, VT), CAN (NB, NF, NS)	Taylor et al., 1993
<i>Isoetes adspersa</i> A. Braun		?	3	Algeria	Pfeiffer, 1922
<i>Isoetes aequinoctialis</i> A. Braun		B-3	3	Angola, South Africa, Mali, Ghana, Tanzania, zambia, Zimbabwe, south to RSA: NAM, NC	Pfeiffer, 1922; Cook, 2004; Crouch et al., 2011
<i>Isoetes alpina</i> Kirk		D-2	3	New Zealand	Pfeiffer, 1922
<i>Isoetes alstonii</i> C.F. Reed & Verdc.		?	2	Egypt; Mozambique; Namibia; Sudan; Tanzania, United Republic of; Zambia; Zimbabwe	Crouch et al., 2011
<i>Isoetes amazonica</i> A. Braun ex Kuhn		A-3	3	Brazil (north [Pará])	Pfeiffer, 1922
<i>Isoetes anatolica</i> Prada & Rolleri		C	3	Turkey	Prada and Rolleri, 2005
<i>Isoetes andicola</i> (Amstutz) L.D. Gómez (formerly <i>Stylites andicola</i> Amstutz)	Live plants	E-2	2	Peru (Lima, Pasco, Junin, Cuzco, Puno), Bolivia	Karrfalt, 1984b; Tryon et al., 1994
<i>Isoetes andina</i> Spruce ex Hook.– synonym of <i>I. triquetra</i>		E-2	2	South America, Peru	Pfeiffer, 1922
<i>Isoetes appalachiana</i> D.F. Brunt. & D.M. Britton	Live plants	E-2	2	USA (FL, GA, NC, PA, SC, VA)	Brunton and Britton, 1997
<i>Isoetes araucaniana</i> Macluf & Hickey		?	2	Malleco (Chile)	Macluf and Hickey, 2007
<i>Isoetes asiatica</i> Makino		E-2	2	Kamchatka, Sakhalin, the Kuriles, and Japan	Yi and Kato, 2001
<i>Isoetes australis</i> R.O. Williams		A-2, D-2	2	Bruce Rock, West Australia	Williams, 1944
<i>Isoetes azorica</i> Durieu ex Milde		?	2	Islands of Azores	Pfeiffer, 1922
<i>Isoetes bolanderi</i> Engelm.	RSA (811637, 811638, 811639, 811643)	E-2	2	USA (AZ, CA, CO, ID, MT, NM, NV, OR, UT, WA, WY), CAN (AB)	Pfeiffer, 1922; Taylor et al., 1993
<i>Isoetes boliviensis</i> U. Weber		?	2	Peru (Cajamarca, San Martin, Ancash, Lima, Pasco, Junin, Ayacucho, Cuzco, Puno), Bolivia (La Paz)	Tryon et al., 1994
<i>Isoetes boryana</i> Durieu		?	3	France (Landes)	Pfeiffer, 1922
<i>Isoetes bradei</i> Herter		A-3	3	Brazil, Sudeste (São Paulo)	Brazilian Flora 2020 (in construction). Rio de Janeiro Botanical Garden. Available at <a href="http://floradobrasil.jbrj.gov.br/">http://floradobrasil.jbrj.gov.br/</a> , accessed Aug 15, 2016; Hickey, 1990
<i>Isoetes braunii</i> Unger		?	2	USA (NH, VT, MA), CAN	Pfeiffer, 1922
<i>Isoetes brevicula</i> E.R.L. Johnson		D-2	3	Western Australia	FloraBase, the Western Australian Flora, Western Australian Herbarium. <a href="https://florabase.dpaw.wa.gov.au/">https://florabase.dpaw.wa.gov.au/</a> , accessed Aug 15, 2016
<i>Isoetes brochoni</i> Motelay		?	2	France	Pfeiffer, 1922

Continued

## APPENDIX 2 Continued

Species	Observed by F. Freund	Clade	Lobes	Locality	Sources
<i>Isoëtes butleri</i> Engelm.		?	2	USA (AL, AR, GA, IL, KS, KY, MO, OK, TN, TX)	Pfeiffer, 1922
<i>Isoëtes capensis</i> A.V. Duthie		A-1	3	South Africa (Western Cape)	Cook, 2004; Crouch et al., 2011
<i>Isoëtes caroli</i> E.R.L. Johnson		D-2	3	Western Australia	FloraBase, the Western Australian Flora, Western Australian Herbarium. <a href="https://florabase.dpaw.wa.gov.au/">https://florabase.dpaw.wa.gov.au/</a> , accessed Aug 15, 2016
<i>Isoëtes caroliniana</i> (A.A. Eaton) Luebke		?	2	USA (NC, TN, VA, WV)	Taylor et al., 1993
<i>Isoëtes coreana</i> Y.H. Chung & H.K. Choi		D-3	3	Korea	Chung and Choi, 1986
<i>Isoëtes coromandeliana</i> L.f.		A-4	3	India	Pfeiffer, 1922
<i>Isoëtes cubana</i> Engelm. ex Baker		A-3	3	Cuba (Pinao del Rio), Belize, Mexico (Yucatan)	Pfeiffer, 1922
<i>Isoëtes dispersa</i> Hickey		?	2	Laguna Tembladera, Lambayeque, Peru	Tryon et al., 1994
<i>Isoëtes dixitei</i> Shende		B-3	3	Panchgani, India	Pant and Srivastava, 1962
<i>Isoëtes drummondii</i> A. Braun		D-2	3	Australia (South Australia, Western Australia, Victoria, New South Wales)	Osborn, 1922; Pfeiffer, 1922
<i>Isoëtes duriei</i> Bory		B-1	3	Algeria, Corsica, France, Italy, Turkey	Pfeiffer, 1922
<i>Isoëtes eatonii</i> R. Dodge	Live plants.	E-2	2	USA (CT, MA, NH, NJ, NY, PA, VT), CAN (ON)	Pfeiffer, 1922
<i>Isoëtes echinospora</i> Durieu		E-2	2	CAN (AB, BC, MB, NB, NT, NS, ON, PE, QC, SK, YT), USA (AK, CA, CO, ID, ME, MA, MI, MN, MT, NH, NJ, OH, OR, PA, VT, WA, WI), British Isles, Circum boreal	Pfeiffer, 1922
<i>Isoëtes elatior</i> A. Braun		?	3	Tasmania	Pfeiffer, 1922
<i>Isoëtes eludens</i> J.P.Roux, Hopper & Rhian J.Sm.		?	3	South Africa (Kamiesberg Mountains, Namaqualand)	Roux et al., 2009; Crouch et al., 2011
<i>Isoëtes engelmannii</i> A. Braun (includes <i>I. valida</i> )	Live plants.	E-2	2	CAN (ON), USA (AI, AR, CT, DE, FL, GA, IL, IN, KY, MD, MA, MI, MO, NH, NJ, NY, NC, OH, PA, RI, SC, TN, VT, VA, WV)	Pfeiffer, 1922; Taylor et al., 1993
<i>Isoëtes flaccida</i> A. Braun		E-2	2	USA (FL, GA)	Pfeiffer, 1922; Taylor et al., 1993
<i>Isoëtes flettii</i> (A.A. Eaton) N. Pfeiff.		?	2	USA (WA - Spanaway Lake)	Pfeiffer, 1922
<i>Isoëtes foveolata</i> A.A. Eaton ex R. Dodge		?	2	USA (CT, MA, NH), CAN (ONT)	Pfeiffer, 1922
<i>Isoëtes gardneriana</i> Kunze ex Mett.		?	3	Brazil (Midwest [Goias]), Paraguay	Pfeiffer, 1922
<i>Isoëtes georgiana</i> Luebke		E-2	2	USA (GA)	Taylor et al., 1993
<i>Isoëtes giessii</i> Launert		?	3	Namibia (Erongo Mountains and in seasonally wet depressions in acacia scrub)	Crouch et al., 2011
<i>Isoëtes gunnii</i> A. Braun		D-2	3	Tasmania (Lake Fenton on Mt. Field)	Pfeiffer, 1922
<i>Isoëtes habbemensis</i> Alston		D-3	(2)–3–(4)	New Guinea	Croft, 1980
<i>Isoëtes hallasanensis</i> H.K. Choi, Ch. Kim & J. Jung		D-3	3	Jeju Island, Korea	Choi et al., 2008
<i>Isoëtes hawaiiensis</i> W.C. Taylor & W.H. Wagner		E-2	2	Hawaii	Taylor et al., 1993
<i>Isoëtes heldreichii</i> Wettst.		?	3	Greece (plains of Thessaly, base of Pindus Mts.)	Pfeiffer, 1922
<i>Isoëtes herzogii</i> U. Weber		E-2	2	Bolivia	Weber, 1922
<i>Isoëtes hewitsonii</i> Hickey		?	2	Celendin, Cajamarca, Peru	Tryon et al., 1994
<i>Isoëtes histrix</i> Bory & Durieu		E-1, E-2	3	Algeria, Italy, France, islands of the Mediterranean	Pfeiffer, 1922
<i>Isoëtes howellii</i> Engelm.	RSA (796366, 796367, 796368, 796369, 796370, 796371)	E-2	2	USA (MT, ID, WA, OR, CA)	Pfeiffer, 1922; Taylor et al., 1993
<i>Isoëtes humilior</i> A. Braun		D-1	2	Tasmania (So. River Esk)	Pfeiffer, 1922; Taylor et al., 1993

Continued

## APPENDIX 2 Continued

Species	Observed by F. Freund	Clade	Lobes	Locality	Sources
<i>Isoëtes inflata</i> E.R.L. Johnson		?	2	Australia (West Australia)	FloraBase, the Western Australian Flora, Western Australian Herbarium. <a href="https://florabase.dpaw.wa.gov.au/">https://florabase.dpaw.wa.gov.au/</a> , accessed Aug 15, 2016
<i>Isoëtes japonica</i> A. Braun		D-3	3	Japan (Yokohama)	Pfeiffer, 1922
<i>Isoëtes jejuensis</i> H.K. Choi, Ch. Kim & J. Jung		D-3	3	Jeju Island, Korea	Choi et al., 2008
<i>Isoëtes kirkii</i> A. Braun		D-1	3	New Zealand	Pfeiffer, 1922
<i>Isoëtes labri-draconis</i> N.R. Crouch		?	3	South Africa (Drakensberg Range in KwaZulu-Natal)	Crouch et al., 2011
<i>Isoëtes lacustris</i> L.		E-2	2	CAN (MB, NB, NL, NT, NS, ON, OQ, SK), USA (ME, MA, MI, MN, NH, NY, VT, WI)	Pfeiffer, 1922; Taylor et al., 1993
<i>Isoëtes laosiensis</i> C. Kim & H.K. Choi		A-4	3	Laos	Kim et al., 2010
<i>Isoëtes lechleri</i> Mett.		?	2	Argentina, Ecuador, Colombia, Peru	Pfeiffer, 1922; Tryon et al., 1994
<i>Isoëtes lithophila</i> N. Pfeiff.		E-2	2	USA (TX)	Pfeiffer, 1922; Tryon et al., 1994
<i>Isoëtes longissima</i> Bory & Dur. – <i>As I. velata</i> forma <i>longissima</i> (sensu Pfeiffer, 1922)		B-3	3	Algeria	Pfeiffer, 1922
<i>Isoëtes macrospora</i> Durieu		?	2	Newfoundland to USA (MN)	Pfeiffer, 1922
<i>Isoëtes malinverniana</i> Ces. & De Not.		C	3	Italy	Pfeiffer, 1922
<i>Isoëtes maritima</i> Underw.		E-2	2	Alaska, British Columbia, Washington	Underwood, 1888
<i>Isoëtes martii</i> A. Braun ex Kuhn		E-2	2	Brazil (Southeast [Minas Gerais, Rio de Janeiro], South [Rio Grande do Sul])	Brazilian Flora 2020 (in construction). Rio de Janeiro Botanical Garden. Available at <a href="http://floradobrasil.jbrj.gov.br/">http://floradobrasil.jbrj.gov.br/</a> , accessed Aug 15, 2016
<i>Isoëtes maxima</i> Hickey, Macluf & Link-Pérez		?	2	Brazil (South [Rio Grande do Sul])	Hickey et al., 2009
<i>Isoëtes melanopoda</i> Gay & Durieu		E-2	2	USA (AL, AR, GA, IA, ID, IL, IN, KS, KY, LA, MN, MO, MS, MT, NC, NE, NJ, OK, SC, SD, TN, TX, UT, VA)	Pfeiffer, 1922; Taylor et al., 1993
<i>Isoëtes melanospora</i> Engelm.		E-2	2	USA (GA - Stone Mtn.)	Pfeiffer, 1922; Taylor et al., 1993
<i>Isoëtes mexicana</i> Underw.		E-2	2	Mexico (Chihuahua, Hidalgo, Mexico, Morelos, Michoacan)	Pfeiffer, 1922
<i>Isoëtes mongerensis</i> E.R.L. Johnson		?	3	Western Australia	FloraBase, the Western Australian Flora, Western Australian Herbarium. <a href="https://florabase.dpaw.wa.gov.au/">https://florabase.dpaw.wa.gov.au/</a> , accessed Aug 15, 2016
<i>Isoëtes muelleri</i> A. Braun		D-3	3	Eastern Australia (Rockhampton)	Pfeiffer, 1922
<i>Isoëtes muricata</i> Durieu		E-2	2	North America	Pfeiffer, 1922, as <i>I. braunii</i>
<i>Isoëtes nigriflora</i> A. Braun		?	3	Nigeria (along the Niger River, Nupe)	Pfeiffer, 1922
<i>Isoëtes novo-granadensis</i> H.P. Fuchs		E-2	2	Columbia, Peru	Tryon et al., 1994
<i>Isoëtes nuttallii</i> A. Braun ex Engelm.	RSA (796374, 796375, 796376)	B-2	3	USA (CA, OR, WA), CAN (Vancouver)	Pfeiffer, 1922; Taylor et al., 1993
<i>Isoëtes occidentalis</i> L.F. Hend.	RSA (811640, 811641, 811642)	E-2	2	USA (CA, WY, CO, ID)	Pfeiffer, 1922; Taylor et al., 1993
<i>Isoëtes olympica</i> A. Braun		B-3	3		Pfeiffer, 1922
<i>Isoëtes orcuttii</i> A.A. Eaton	Live plants	B-2	3	USA (CA)	Pfeiffer, 1922; Taylor et al., 1993
<i>Isoëtes ovata</i> N. Pfeiff.		?	3	French Guiana, Guyana	Pfeiffer, 1922
<i>Isoëtes panamensis</i> Maxon & C.V. Morton	Museo Nacional de Costa Rica (sheet no. not recorded)	A-3	2	Brazil (northeast [Maranhão, Bahia], midwest [Mato Grosso])	Brazilian Flora 2020 (in construction). Rio de Janeiro Botanical Garden. Available at <a href="http://floradobrasil.jbrj.gov.br/">http://floradobrasil.jbrj.gov.br/</a> , accessed Aug 15, 2016

Continued

## APPENDIX 2 Continued

Species	Observed by F. Freund	Clade	Lobes	Locality	Sources
<i>Isoetes parvula</i> Hickey		?	2	Laguna Yaurihuri, Ayacucho, Perú	Tryon et al., 1994
<i>Isoetes pedersenii</i> H.P. Fuchs ex E.I. Meza & Macluf		?	2	Argentina (Corrientes - Mburucuyá National Park)	Macluf et al., 2010
<i>Isoetes philippinensis</i> Merr. & R.H. Perry		D-3	3	Philippine islands	Merrill and Perry, 1940
<i>Isoetes piperi</i> A.A. Eaton		?	2	USA (WA)	Pfeiffer, 1922
<i>Isoetes pringlei</i> Underw.		?	2	Mexico (Guadalajara, state of Jalisco)	Pfeiffer, 1922
<i>Isoetes prototypus</i> D.M. Britton		?	2	CAN (NB, NS), USA (MA)	Taylor et al., 1993
<i>Isoetes pseudojaponica</i> M. Takamiya, Mits. Watan. & K. Ono		D-3	3	Japan	Takamiya et al., 1997
<i>Isoetes quiririensis</i> J.B.S. Pereira & Labiak		?	2	Brazil (Serra do Quoriri)	Pereira and Labiak, 2013
<i>Isoetes riparia</i> Engelm. ex A. Braun		?	2	CAN (southern region), USA (New England south to DE and PA)	Pfeiffer, 1922
<i>Isoetes saccharata</i> Engelm.		?	2	USA (DE, DC, MD, VA)	Pfeiffer, 1922
<i>Isoetes sampathkumarinii</i> L.N. Rao		D-2	2	India	Rao, 1944
<i>Isoetes saracochensis</i> Hickey		?	2	Laguna Saracocha, Puno, Peru	Tryon et al., 1994
<i>Isoetes savatieri</i> Franch.		E-2	2	Argentina (Puerto Bueno)	Hickey et al., 2003
<i>Isoetes schweinfurthii</i> Baker		A-4	3	South Africa (Namibia, Botswana, Limpopo), Sudan, Madagascar, Zambia	Pfeiffer, 1922; Cook, 2004; Crouch et al., 2011
<i>Isoetes setacea</i> Lam.		E-1	3	France (Montpellier, Hérault, Pyrénées-Orientales); Morocco; Portugal; Spain (Balears)	Pfeiffer, 1922
<i>Isoetes stellenbossiensis</i> A.V. Duthie		A-1	3	South Africa (western part of Western Cape province)	Cook, 2004; Crouch et al., 2011
<i>Isoetes stephansenii</i> A.V. Duthie		A-1	2	South Africa (flats around Stellenbosch in the Western Cape)	Crouch et al., 2011
<i>Isoetes stevensii</i> J.R. Croft		D-3	(2)–3–(4)	New Guinea	Croft, 1980
<i>Isoetes storkii</i> T.C. Palmer	GH00021453, CM0102, NY00144272 (virtual herbarium sheets)	E-2	2	Costa Rica	JStor Global Plants virtual herbarium. <a href="http://plants.jstor.org/search?filter=name&amp;so=ps_group_by_genus_species+asc&amp;Query=isoetes+storkii">http://plants.jstor.org/search?filter=name&amp;so=ps_group_by_genus_species+asc&amp;Query=isoetes+storkii</a> , accessed Dec 28, 2016
<i>Isoetes subinermis</i> Cesca – synonym of <i>I. histrix</i>		B-1	3		Cesca and Peruzzi, 2001
<i>Isoetes taiwanensis</i> De Vol	Live plants	D-3	3	Taiwan	Chiang, 1976
<i>Isoetes tegetiformans</i> Rury		E-2	2	USA (GA)	Rury, 1978; Taylor et al., 1993
<i>Isoetes toximontana</i> Musselman & J.P. Roux		A-1	3	South Africa (Giftberg endemic)	Cook, 2004; Crouch et al., 2011
<i>Isoetes transvaalensis</i> Jermy & Schelpe		?	3	Lesotho, South Africa (Limpopo, Mpumalanga, Free State, KwaZulu-Natal)	Cook, 2004; Crouch et al., 2011
<i>Isoetes triquetra</i> A. Braun		E-2	2	Peru	Pfeiffer, 1922
<i>Isoetes truncata</i> (A.A. Eaton) Clute		?	2	CAN (Vancouver Island), USA (Alaska)	Pfeiffer, 1922
<i>Isoetes tuerckheimii</i> Brause		E-2	2	Dominican Republic (Santo Domingo)	Pfeiffer, 1922
<i>Isoetes valida</i> (Engelm.) Clute		E-2	2	USA (NC, TN, VA, WV)	Taylor et al., 1993
<i>Isoetes velata</i> A. Braun		B-3	3	Italy (Algeria, Corsica, Sicily)	Pfeiffer, 1922
<i>Isoetes welwitschii</i> A. Braun ex Kuhn		?	3	Angola, South Africa (Mpumalanga, KwaZulu-Natal), Madagascar	Pfeiffer, 1922; Cook, 2004; Crouch et al., 2011
<i>Isoetes wormaldii</i> R. Sim		?	3	South Africa (Eastern Cape - small area between Grahamstown and East London)	Pfeiffer, 1922; Cook, 2004; Crouch et al., 2011
<i>Isoetes yunguiensis</i> Q.F. Wang & W.C. Taylor		D-3	3	China	Wang et al., 2002