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DISENTANGLING THE PHYLOGENY OF *ISOETES* (ISOETALES), USING NUCLEAR AND PLASTID DATA

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Premise of research. The heterosporous lycopsids of *Isoetes* show limited morphological and genetic variation despite a worldwide distribution and the ancient origin of the lineage. Here major relationships within the genus are clarified, using a substantially larger sampling of species than in previous studies. A first assessment of divergence times of clades is made, and the implications for dispersal mechanisms and biogeographic distribution patterns are discussed.

Methodology. On the basis of sequences from three gene regions and 109 specimens representing 74 species of *Isoetes*, phylogeny and node ages were estimated using parsimony and Bayesian inference.

Pivotal results. Three rooting approaches (outgroup analysis, midpoint rooting, and clock rooting) coherently resolved a diverse clade containing species from South Africa, India, Australia, and South America (clade A) as sister to remaining *Isoetes*. Analysis of divergence times of clades yielded a median age of the crown group of 147 million years ago (mya) using a birth-death tree prior and 165 mya using a Yule tree prior. Clade A was dated to 111 or 125 mya, respectively. While the earliest divergences in *Isoetes* appear readily explained by ancient vicariance, patterns in younger clades are consistent with dispersal processes, sometimes over long distances. *Isoetes andicola* (Amstutz) L.D. Gómez, once hypothesized to represent a separate lineage and assigned to the genus *Stylites*, is here included in a phylogenetic study for the first time. It is closely related to some other South American species, despite its peculiar morphology with a dichotomizing stem.

Conclusions. Despite limited intrageneric variation at the molecular and morphological levels, node ages as well as species composition (phylogeny) indicate a Mesozoic origin of the extant clade. Biogeographic patterns appear complicated and intriguing but need more research. Tuberculate megaspore ornamentation (sensu Pfeiffer) is ancestral in the genus, as indicated by current knowledge. Other megaspore patterns appear restricted to two subclades.

Keywords: birth-death prior, Gondwana, Laurasia, random local clock, uncorrelated lognormal clock, Yule prior.

Introduction

Isoetes is a genus of heterosporous lycopsids that has around 150–200 extant species with a cosmopolitan distribution (Taylor and Hickey 1992; Hoot et al. 2004). It is the only remaining representative of the rhizomorphic lycopsids (Isoetales sensu DiMichele and Bateman 1996), an ancient clade characterized by pseudobipolar growth from a shootlike rootstock, stigmarian root systems with dichotomizing roots and leaflike lateral rootlets, and secondary xylem produced by a unifacial cambium (Bateman et al. 1992; DiMichele and Bateman 1996; Kenrick and Crane 1997). The fossil record of the group extends at least to the Late Devonian and includes "pseudoherbs" (e.g., *Oxroadia* and *Paurodendeon*), the famous tree lycopods from the Carboniferous period (e.g., *Sigillaria* and *Lepidodendron*), and a diversity of smaller unbranched forms that occur in both the Paleozoic (e.g., *Chaloneria*) and the Mesozoic (e.g., the Triassic *Pleuromeia* and the Cretaceous *Nathorstiana*) (Bateman et al. 1992; Pigg 1992, 2001; Bateman and DiMichele 1994; DiMichele and Bateman 1996).

Extant species have a reduced plant body with very restricted apical growth (Pigg 2001). The oldest unequivocal fossils with this morphology are from the Late Jurassic of Idaho (Ash and Pigg 1991; Pigg 2001). Additional fossil *Isoetes* occurrences are known from the Paleocene of western North America (Brown 1962; Pigg 2001). According to the traditional reduction hypothesis, *Isoetes* represents an isoetalean cone on a stigmarian base (Taylor et al. 2009). The sparsely branched *Sigillaria* is often considered the starting point of this reduction series. However, the discovery of Paleozoic lycopsids with a cormlike base, the earliest being *Otzinachsonia* (Cressler and Pfefferkorn 2005), indicate that tree forms and corm forms have evolved in parallel since the Devonian (Taylor et al. 2009). While the phylogeny and evolution of the vast Paleozoic diversity of the Isoetales has been assessed (e.g., Bateman et al. 1992; Bateman and DiMichele

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1994; DiMichele and Bateman 1996), the Mesozoic and Cenozoic fossil records are comparatively scant and poorly understood, and relationships among the fossils and modern *Isoetes* have never been resolved (Ash and Pigg 1991; Pigg 2001; Taylor et al. 2009).

Modern species of *Isoetes* occur fully submerged or in semiaquatic habitats, although a few terrestrial species also exist (Hickey 1986*a*). There is surprisingly little morphological and molecular variation between species, despite the worldwide distribution and probable Paleozoic origin of the order (Pfeiffer 1922; Pigg 1992, 2001; Retallack 1997; Rydin and Wikström 2002; Hoot et al. 2006). Species of the genus are very difficult to identify, partly because of the morphological simplicity but also because of inferred homoplasy and reticulate evolution (Taylor and Hickey 1992). During the evolution in the extant genus, repeated transitions between aquatic and terrestrial habitats are hypothesized to have occurred, and abrupt speciation through polyploidization and/or hybridization is suggested (Taylor and Hickey 1992). In the most recent monograph of the family, Pfeiffer (1922) grouped the species into four sections on the basis of megaspore morphology. Hickey (1986*a*, 1986*b*) disagreed with those results, recognized additional megaspore types, and made an evolutionary assessment based on additional characteristics, such as presence of supporting fibrous bundles in the leaves and different modifications for drought survival. He grouped *Isoetes* into three clades with a few South American species diverging first, followed by a group consisting of the Indian species and finally the remaining species (Hickey 1986*a*).

A study based on molecular data (*rbcL*; Rydin and Wikström 2002) contradicted the theories of Pfeiffer (1922) and Hickey (1986*a*) and found a basal division in the family where two South American and two West African species formed a clade separate from the other specimens in the study. Rydin and Wikström (2002) suggested that the distribution pattern of *Isoetes* reflects the current continents' positions within the ancient Gondwana landmass and that the major subclades we recognize today are older than the breakup of the supercontinent. This would, if correct, sharply contrast with the observed low sequence divergence within the family. Together with several coauthors, Hoot conducted molecular studies using the internal transcribed spacer of nuclear ribosomal DNA (nrITS) and the *atpB-rbcL* intergenic spacer to explore the genuswide phylogeny and a *LEAFY* homolog to sort through the North American hybrid complex (Hoot and Taylor 2001; Hoot et al. 2004, 2006). Their phylogeny from 2006 expanded on the hints provided by Rydin and Wikström (2002) and revealed a complex biogeography with, for example, Mediterranean species represented within several separate clades. In addition to supporting a plausible Gondwana clade, they could also add a Northern Hemisphere clade (Hoot et al. 2006).

Estimating the earliest divergence in *Isoetes* has, however, proven difficult. *Selaginella* is the closest living relative (Bateman and DiMichele 1994; Wikström and Kenrick 1997; Pryer et al. 2001; Qiu et al. 2007), but it is still very distantly related; the two lineages separated from each other 330–380 million years ago (mya; DiMichele 1980; Bateman and DiMichele 1994). *Isoetes* also seems to have a very different rate of substitutional change compared with other lycopods, leaving the clade with a relative poverty of informative characters in standard chloroplast and nuclear ribosomal markers. *Selaginella* has, instead, highly elevated substitution rates (Takamiya et al. 1996; Korall 2003).

Using outgroups to find the root of a phylogeny (Farris 1972) is the traditional and most widely used method, but with increasing genetic distance between the ingroup and the outgroup the results can be weakly supported or even misleading (Wheeler 1990; Renner et al. 2008). In an attempt to explore the rooting problem and find possible solutions, Schuettpelz and Hoot (2006) used data from previous research (selected taxa from Hoot and Taylor 2001; Rydin and Wikström 2002; Taylor et al. 2004) as well as their own preliminary analysis and tried different rooting approaches without outgroup data. Their concluding advice was to use midpoint rooting or settle for a basal trichotomy. Midpoint rooting places the root between the two most divergent groups as measured by branch length (Farris 1972). The approach has been demonstrated useful (in particular when suitable outgroups are unavailable), but it relies on the assumption that the evolutionary rate is the same across the tree (Hess and De Moraes Russo 2007). A third approach to the problem could be to use molecular clock rooting (Huelsenbeck et al. 2002; Drummond et al. 2006). This method too performs best when substitution rates are constant across lineages, but Huelsenbeck et al. (2002) showed that even when this criterion was severely violated the root of the tree was correctly identified in most cases.

The present study aims to clarify deep divergences and major relationships within *Isoetes*, using a larger sampling than in previous studies. Despite the efforts of previous researchers, phylogeny, biogeography, and divergence times of clades within *Isoetes* are still not well understood. The classification schemes and evolutionary hypotheses based on morphology have not been thoroughly tested using molecular data. Although the molecular studies mentioned above provided new information on the phylogeny, species representation was limited, especially among Old World species, and results were partly poorly supported in a statistical sense. Furthermore, basal divergences are often unresolved or uncertain in these studies (but see Rydin and Wikström 2002). We also provide a first assessment of divergence times of clades within *Isoetes*, exploring different tree priors and clock models. Implications for dispersal mechanisms and biogeographic distribution patterns are discussed.

Material and Methods

Sampling Strategy and DNA Sequencing

To better understand the major relationships within *Isoetes*, we wanted to broaden the species representation compared with that of previous studies, particularly regarding species from geographical regions other than North America. Particular effort was made to sample Old World and South American taxa to cover the worldwide distribution of the genus and complement the data provided by previous studies and available at GenBank. We also assessed the usefulness of three additional molecular markers—nuclear ribosomal 18S, chloroplast *rps4*, and mitochondrial *rps4*—but while all three were found to be alignable with the outgroups, they were almost identical across

Isoetes, as indicated by our pilot work; they were therefore not used in subsequent analyses. Instead, *rbcL*, the *rbcL-atpB* spacer, and nrITS were utilized.

Total genomic DNA was extracted from 30 specimens (appendix) using the cetyltrimethylammonium bromide method and purified using a QIAquick polymerase chain reaction (PCR) kit (Qiagen; Solna, Sweden/Hilden, Germany) following the manufacturer's instructions. Most primers have been newly constructed for the present study (table 1). Primers were designed and simulation tested using Amplify software (ver. 3.1; Engels 2005) and synthesized by Eurofins MWG Operon, Germany. PCR was conducted in an Eppendorf Mastercycler gradient (Bergman and Beving Instrument, Stockholm, Sweden), and nested PCR was performed when the initial PCR showed feeble results. The PCR program for the markers consisted of a 2-min pretreatment at 94°C; 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 50°–54°C, and extension for 1 min at 72°C; and final incubation for 7 min at 72°C after the cycles. The annealing temperatures varied for the loci and were 50°C for *rbcL* and 54°C for nrITS. In total, 49 sequences were newly produced for the present study and were analyzed together with data available at GenBank (appendix).

Phylogenetic Analysis

Sequences were assembled using the Staden package (Staden 1996) and were aligned by eye using the Se-Al sequence alignment editor (ver. 2.0; © 1996–2001, Andrew Rambaut). Phylogenetic relationships were analyzed using parsimony (PAUP* ver. 4.0; Swofford and Sullivan 2009) and Bayesian inference (MrBayes ver. 3.2.2; Ronquist et al. 2012), run on the CIPRES cluster (Miller et al. 2010). Trees were rooted according to results of rooting analyses; see below. Parsimony analyses were run using the heuristic search option, 100 random sequence additions, tree bisection and reconnection branch swapping, and the multiple trees option off. Branch support was estimated using the bootstrap option as implemented in PAUP*, with 1000 bootstrap replicates and 10 random sequence additions in each.

Prior to Bayesian analyses, the fit of different evolutionary models to the data was tested. Optimal models were selected on the basis of the Akaike information criterion and the Bayesian information criterion, both as estimated by the Perl script MrAIC (ver. 1.4; Nylander 2004), in combination with PHYML (ver. 2.4.4; Guindon and Gascuel 2003). A third strategy in the search for the optimal evolutionary model for the data set was to use the reversible-jump Markov chain Monte Carlo (RJ-MCMC) procedure (Huelsenbeck et al. 2004). There were no supported differences between the results of these approaches, and for the final analyses the RJ-MCMC procedure was used. Three partitioning schemes were tested; each marker its own partition, chloroplast versus nuclear markers, and a single partition. The latter option gave the best (most well-resolved) result and was used going forward. Flat dirichlet prior probabilities were selected for the substitution rates and the nucleotide frequencies. The prior probability of the shape parameter of the gamma distribution of rate variation was uniformly distributed in the interval (0.1, 50.0). When relevant, a prior probability for the proportion of invariable sites, uniformly distributed on the interval (0.0, 1.0), was specified. The Bayesian analyses used two parallel runs of four chains each and were run for 20 million generations with a sample frequency of 1000. Convergences of runs and suitable burn-in were assessed in Tracer (ver. 1.6; Rambaut et al. 2014) and using the online application AWTY (Are We There Yet? [Nylander et al. 2008]). Single-gene analyses were also run with settings and model selection as described above (i.e., RJ-MCMC; one partition), and results were compared and checked for inconsistencies.

Rooting Analysis

We explored three strategies to find the correct root: outgroup analysis (Farris 1972), midpoint rooting (Farris 1972), and molecular clock–based rooting (Huelsenbeck et al. 2002; Drummond et al. 2006). The outgroup analyses and experimentation with midpoint rooting were conducted in both a parsimony and a Bayesian framework and were run with settings as described above but using the species level as terminal (i.e., sequences produced from different representative individuals of a species were combined into a terminal in the analysis so as to leave as few question marks as possible in the data matrix). Only species with two or more available sequences from different gene regions were included in this analysis.

The outgroup analyses used 38 species of *Selaginella* as outgroup. Midpoint rooting was investigated for parsimony and Bayesian analyses of a data set excluding outgroups, using PAUP* and FigTree (ver. 1.4.2; © 2006–2014, Andrew Rambaut), re-

Table 1

Primer Sequences

Gene region	Primer name	Sequence (5′→3′)	Reference
nrITS forward	18SSfor	GGTGAACCTGCAGAAGGATC	This study
nrITS internal forward	ITSisoinF	GGCTCTTGCCACGATGAAGAACGC	This study
nrITS internal reverse	ITSisoinR	CATGCCCTCGGACAAGCCCCGGGCG	This study
nrITS reverse	26S1rev	GCTCGCCGTTACTAGGGAAATCC	This study
rbcL forward	IrbcL1F	CTCCTGATTATAAGACCAAAGACACCG	This study
rbcL internal forward	rbcL565F	GTTTATGCGTTGGAGAGACCG	This study
rbcL internal reverse	rbcL885R	GATCCCCACCGGACATACGCAATGC	This study
rbcL reverse	rbcL1409R	TCAAATTCAAACTTGATTTCTTTCCA	Wikström and Kenrick 1997

Note. nrITS = internal transcribed spacer of nuclear ribosomal DNA.

spectively. Molecular clock–based rooting was investigated in BEAST (ver. 1.8.1; Drummond et al. 2012) without specified age priors and using a matrix containing only *Isoetes* species. All of these approaches yielded the same topological result, and subsequent analyses (specified above) were rooted accordingly.

Analysis of Divergence Times of Clades

For estimates of divergence times of clades, a data matrix was run in BEAST; it was sampled at the species level and included 43 species of *Isoetes* and 65 outgroup species representing the remaining land plants. Numerous settings were tested to find the best for our data. The fit of the respective models to the data set was tested with path sampling and stepping-stone sampling (Baele et al. 2012, 2013). These methods produce scores of log marginal likelihood, and a difference greater than 3 between two model settings should be seen as significant (Kass and Raftery 1995). Three clock models were tested, a strict clock and two relaxed clocks: uncorrelated clock rates drawn from a lognormal distribution and random local clock (Drummond and Suchard 2010). Two tree priors were used: birth-death incomplete sampling (BDI; Kendall 1948; Stadler 2009) and a pure birth process (Yule 1925). Three partitioning schemes were tested: one for each marker (three partitions), chloroplast markers versus nuclear (two partitions), and a single partition.

A relaxed clock with uncorrelated rates drawn from a lognormal distribution and a single data partition gave the best score in the path sampling and stepping-stone sampling analyses (table 2) and were used in the final analyses, which were run for 120 million generations using the GTRIG site model and estimated base frequencies. A tree from a previous analysis was used as a starting tree. The parameters of the clock model were estimated from the data and an exponentially distributed hyperprior (ucld.stdev: initial value = 0.33, mean = 0.33, offset = 0.0; ucld.mean: initial value = 5.0, mean = 5.0, offset = 0.0) was used. Ten percent of the 120,000 generated trees were rejected as burn-in, and the remaining 108,000 trees were summarized using TreeAnnotator from the BEAST package.

Three prior distributions for the age-calibrated nodes were compared: normally, lognormally, and uniformly distributed (table 2). We also performed runs without the data to ensure that priors do not interact with each other (Heled and Drummond 2012). Uniformly distributed age priors yielded the best log marginal likelihood scores (table 2) and were in the final analyses assigned to nine nodes on the basis of fossil information (table 3). All age estimates of geological time/stratigraphy are derived from Gradstein et al. (2012), and fossils were used as described below.

It has long been suggested that the earliest evidence of land plants is dispersed spores of probable liverwort affinity (often called cryptospores) from the early Middle Ordovician (Llanvirn; see discussions in, e.g., Kenrick 2003; Wellman et al. 2003; Taylor et al. 2009). The discovery of such spores in situ in Late Ordovician fossils (Wellman et al. 2003) confirms the assumption (Kenrick 2003; Taylor et al. 2009). It can further be argued that these distinct and resistant spores would have been found in older strata if present (Kenrick 2003) and that expectation justifies a quite narrow prior distribution of the root height (node F1) of 467–485 mya. The lower (older) limit (485 mya) was also used as the lower limit of all other age priors (table 3).

Among the oldest fossils that can be assigned to crown group vascular plants (Tracheophyta, node F2), two would date the clade to the Early Devonian (Pragian; 408–411 mya): *Asteroxylon mackiei* (Kidston and Lang 1920) is an early lycopsid (Kenrick and Crane 1997) from the deposits of Rhynie chert that has been palynologically dated to the Pragian and radiometrically dated to 396 mya (Rice et al. 1995), and *Euphyllophyton bellum* (Hao 1988), from the Psongchong Formation in Yunnan, China, is a stem relative of Euphyllophytes (Kenrick and Crane 1997) that has been estimated to be of the same age (Hao 1989). But it is also possible that lycopsids already existed

Table 2

Log Marginal Likelihood (in Score Order)

Clock	Prior age distribution, tree prior, data partitions	Log marginal likelihood (stepping stone)	*Isoetes* crown, median age (mya)
Uncorrelated lognormal	Uniform, Yule, one partition	−30,899.87[a]	165
Uncorrelated lognormal	Uniform, BDI, one partition	−30,900.20[a]	147
Uncorrelated lognormal	Normal, BDI, one partition	−30,944.64	165
Uncorrelated lognormal	Lognormal, BDI, one partition	−30,944.85	170
Uncorrelated lognormal	Lognormal, Yule, one partition	−30,945.33	185
Uncorrelated lognormal	Normal, Yule, one partition	−30,948.21	185
Random local	Uniform, BDI, one partition	−30,969.92	…[b]
Strict	Uniform, BDI, one partition	−31,086.07	125
Strict	Uniform, Yule, one partition	−31,088.46	128
Random local	Uniform, BDI, two partitions	−31,360.04	…[b]

Note. BDI = birth-death incomplete sampling; mya = million years ago.

[a] Best-fitting approaches with a nonsignificant difference in fit as estimated by the log marginal likelihood values. All other approaches had a significantly worse fit to the data.

[b] Results rejected; see the text.

Table 3

Age-Calibrated Nodes

	Node	Fossil[a]	Fossil age (Ma)	Uniform prior distribution (Ma)
Embryophyta (root height)	F1	"Cryptospores"	467–485	467–485
		Euphyllophyton bellum	408–411	
Tracheophyta	F2	*Baragwanathia longifolia*	423–427	408–485
Euphyllophyta	F3	*Pertica varia*	393–402	393–485
Lycopodiophyta	F5	*Leclercqia complexa*	383–388	383–485
Isoetopsida	F6	*Cyclostigma*, *Paralycopodites*	331–383	331–485
Spermatophyta	F4	Cordaites	311–331	311–485
Selaginellaceae	F7	*Selaginella gutbieri*	307–323	307–485
Selaginella remotifolia clade	F8	*Selaginella anasazia*	201–225	201–485
Selaginella pygmaea clade	F9	*Erlansonisporites scanicus*	85–87	85–485

[a] For references and justifications, see the text.

in the late Silurian, as indicated by *Baragwanathia longifolia* (Lang and Cookson 1935), believed to be from Ludlow (423–427 mya; Garratt et al. 1984; Garratt and Rickards 1987). The upper (younger) limit of the uniformly distributed age prior of node F2 was thus set to 408 mya.

The oldest known descendant of crown group euphyllophytes (F3) is the fossil *Pertica varia* (Granoff et al. 1976, phylogenetically placed by Kenrick and Crane 1997) from the Early Devonian Battery Point Formation, Canada, which has been redated to middle to late Emsian (Hoffman and Tomescu 2013), corresponding to about 393–402 mya, the latter constituting the upper limit of the age prior for node F3.

The oldest members of crown group seed plants (Spermatophyta, F4) are the cordaites (Hilton and Bateman 2006; Doyle 2008), which are clearly documented from the mid-Pennsylvanian (upper limit of the age prior, 311 mya) but were probably already present during the Late Mississippian (Taylor et al. 2009), that is, 323–331 mya.

The age prior of clade Lycopodiophyta (F5) was set on the basis of the fossil *Leclercqia complexa* (Banks et al. 1972) from the Middle Devonian (Givetian, 383–388 Ma; upper age limit, 383 mya), which was phylogenetically placed as sister to Isoetopsida by Kenrick and Crane (1997).

The split between *Isoetes* and *Selaginella* (Isoetopsida, F6) was estimated to have occurred at least 331–383 mya (upper age limit, 331 mya) on the basis of possible crown group fossils from the Late Devonian, such as *Cyclostigma*, *Lepidosigillaria*, and *Clevelandodendron*, and the early Carboniferous fossil *Paralycopodites* (DiMichele 1980) from the early Visean, which is clearly established as a crown group member (Bateman and DiMichele 1994).

Crown group *Selaginella* (Selaginellaceae, F7) was given an upper age limit of 307 mya on the basis of three fossils. *Selaginella gutbieri* and *Selaginella zeilleri* both share synapomorphies with all species of *Selaginella* except the *selaginoides* clade—for example, flattened shoots, anisophyllous leaves, and rhizophores (Kenrick and Crane 1997)—and are dated to 307–311 mya on the basis of locality information in Thomas (1997). In addition, the megaspore *Triangulatisporites* cf. *bellus/regalis* (Cottnam et al. 2000) from the Moscovian or Bashkirian (307–323 mya) has been established as a crown group fossil (Korall 2003).

The clade comprising *Selaginella remotifolia*, *S. kraussiana*, *S. articulata*, *S. diffusa*, *S. sulcata*, and *S. fragilis* (F8) has an estimated age of at least 201–225 mya (upper age limit, 201 mya) on the basis of the Late Triassic fossil *Selaginella anasazia* (Ash 1972), phylogenetically placed by Korall and Kenrick (2002).

The clade consisting of *Selaginella pygmaea*, *S. lyalli*, *S. polymorpha*, *S. uliginosa*, and *S. gracillima* (F9) was estimated to be at least 85–87 mya (upper age limit, 85 mya) on the basis of the late Coniacian to early Santonian fossil *Erlansonisporites scanicus* (Takahashi et al. 2001), phylogenetically placed by Korall (2003).

In addition, the following clades were constrained to be monophyletic: Marchantiophyta, Bryophyta, Anthocerotophyta, Isoetaceae, and a group consisting of all land plants except Marchantiophyta.

Results

Phylogeny of Isoetes

The matrix contained 109 isoetalean terminals and 2987 characters, of which 633 were variable and 509 were informative. The three rooting approaches (outgroup analysis, midpoint rooting, and clock rooting) yielded the same result for both parsimony and Bayesian analyses—that is, the deepest split in *Isoetes* separates a diverse clade containing species from South Africa, India, Australia, and South America (clade A) from the remaining species in the genus, which in turn consists of four major clades. The subsequent Bayesian analysis with more extensive sampling in *Isoetes* and rooted in clade A (fig. 1) found the same clades, now well supported. Clades ABCDE, BCDE, CDE, and DE and clades A, B, C, D, and E all separately have posterior probabilities (pp) of 1.0.

Differences between the Combined and Single-Gene Analyses

The results of trees generated in single-gene analyses were sometimes poorly resolved, but there were no supported conflicts (here defined as $pp > 0.95$) except for the position of two specimens that varied between the nrITS and the *atpB-rbcL* spacer trees: *Isoetes longissima* was included in clade E

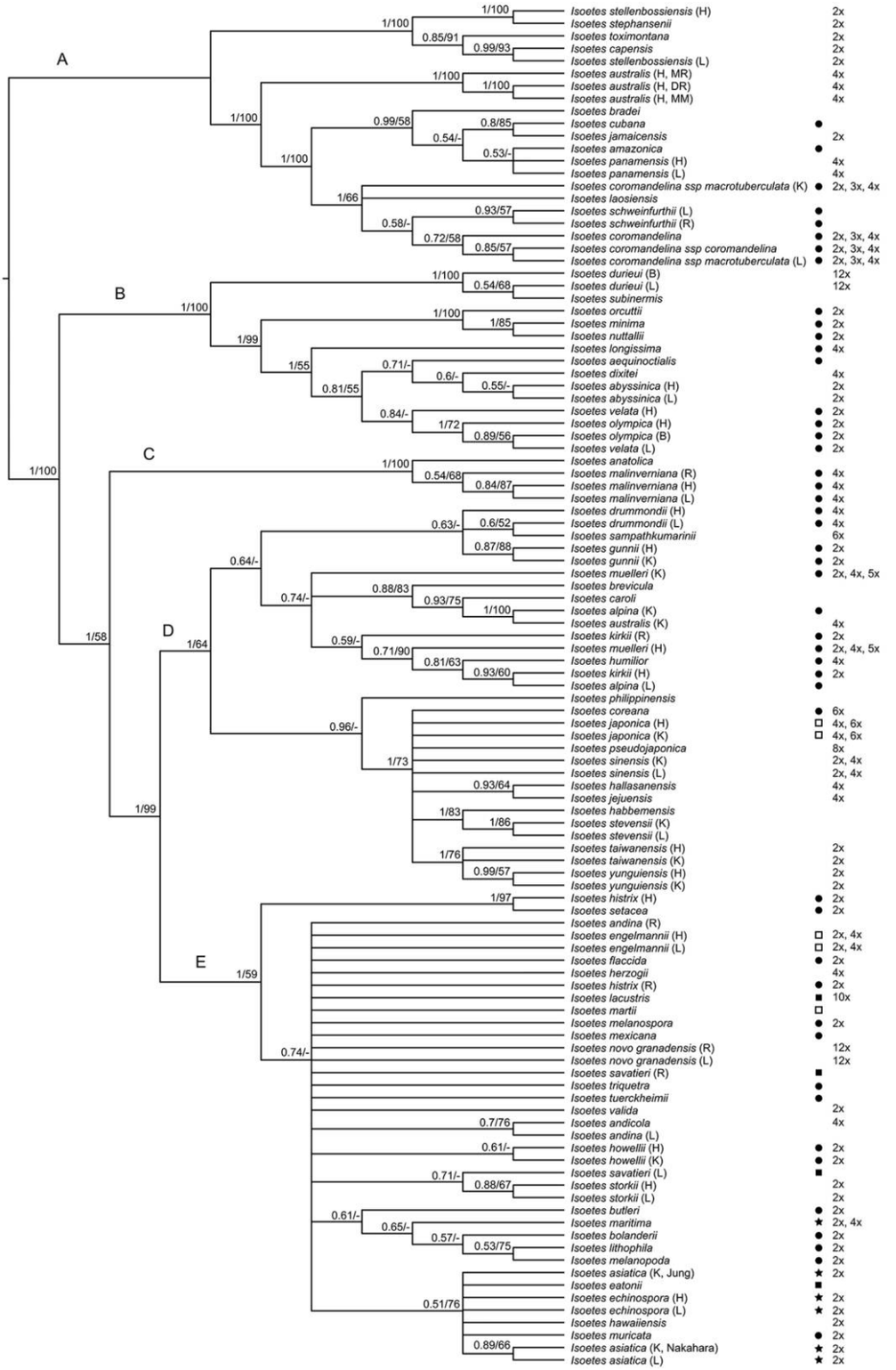

Fig. 1 Results of specimen-level Bayesian analysis with extended sampling in *Isoetes*, using clade A as outgroup. Clades A–E are discussed in the text. The letter in parentheses after some species names denotes specimen. Posterior probability (*left*) and parsimony bootstrap value (*right*) are given for each branch. In the right-hand margin, ploidy-level estimations are given (Troìa 2001; Hoot et al. 2006; Kim et al. 2009), and megaspore structure according to Pfeiffer (1922) is represented by four symbols: open box = Reticulate, filled box = Cristate, star = Echinate, and circle = Tuberculate.

(pp = 1.0) on the basis of *atpB-rbcL* spacer data but was placed in clade B (pp = 1.0) when nrITS was analyzed, and *Isoetes alpina* (K) was included in clade A (pp = 1.0) in the *atpB-rbcL* analysis but fell within clade D (pp = 1.0) when *nrITS* was examined.

Divergence Times of Clades

Two approaches had the best fit to our data as estimated by path sampling and stepping-stone sampling analyses (table 2), with only minor and nonsignificant difference in log marginal likelihood scores between the two: BDI tree prior and Yule tree prior, both with relaxed clocks with uncorrelated clock rates drawn from a lognormal distribution, a single data partition, and uniformly distributed age priors. Results from these two analyses are presented below. Age estimates (95% confidence intervals [CIs]) retrieved from these analyses were similar, although the Yule process resulted in older median ages within *Isoetes* (table 4). The chronogram in figure 2 shows the results derived from the analysis using the BDI tree prior.

All other explored approaches had significantly worse fit to the data (results interpreted in accordance with Kass and Raftery 1995). A strict clock could be rejected on the basis of log marginal likelihood scores much lower than those of the analyses using uncorrelated lognormal clock (table 2). Random local clock also did not fit the data well, as assessed by log marginal likelihood scores retrieved from path sampling and stepping-stone analyses (table 2). Random local clocks further required very long runs (>500 million generations) to reach stationarity, which is a common problem (Bellot and Renner 2014), and they delivered some unconventional phylogenetic results that neither have been otherwise observed in our results nor agree with generally accepted views of land plant phylogeny. The results from these runs were therefore rejected.

Effective age prior distributions (as assessed from the analyses without data) were in all cases consistent with the specified age priors, although uniform distributions had a tendency to shift toward normal distributions.

The tree topology retrieved from BEAST software (fig. 2) did not contradict the species-level tree yielded by MrBayes (fig. 1), and the statistical support for the major clades and their relationships was high. The earliest divergence within *Isoetes* occurred about 147 mya (95% CI, 96–215), as estimated from the analysis using the BDI tree prior, or 165 mya (95% CI, 110–235), as estimated using the Yule tree prior. This split separates clade A from all other species. The next divergence was within clade A, separating the South African clade consisting of *I. stellenbossiensis*, *I. capensis*, and *I. toximontana* 111 mya (BDI [95% CI, 63–172]; Yule, 125 mya [95% CI, 75–190]). *Isoetes australis* diverged from the remaining clade A 76 mya (BDI [95% CI, 38–129]; Yule, 86 mya [95% CI, 43–141]). Further splits within clade A are in the time range 3–23 mya (BDI [95% CI, 0–40]; Yule, 4–30 mya [95% CI, 0–55]). Clade B split from CDE around 103 mya (BDI [95% CI, 64–152]; Yule, 117 mya [95% CI, 72–171]), and divergence within the clade started around 62 mya (BDI [95% CI, 34–101]; Yule, 72 mya [95% CI, 40–114]), with the south European *I. durieui* separating from the other species, followed by American sister taxa *I. orcuttii* and *I. nuttallii*, around 38 mya (BDI [95% CI, 21–61]; Yule, 44 mya [95% CI, 24–72]). The remaining nodes within clade B diverged 4–22 mya (BDI [95% CI, 1–39]; Yule, 5–26 mya [95% CI, 1–45]). The sole species in clade C separated from clades D and E 76 mya (BDI [95% CI, 42–123]; Yule, 88 mya [95% CI, 48–143]). The diverging point between clades D and E was estimated to 32 mya (BDI [95% CI, 20–50]; Yule, 36 mya [95% CI, 22–58]), and the remaining divergence times in clade D occurred 1–15 mya (BDI [95% CI, 0–26]; Yule, 1–17 mya [95% CI, 0–29]). Within clade E, the sister taxa *I. histrix* and *I. setacea* separated from the rest of the species 22 mya (BDI [95% CI, 13–34]; Yule, 25 mya [95% CI, 15–39]), and the remaining cladogenetic events were dated to 2–14 mya (BDI [95% CI, 0–23]; Yule, 2–17 mya [95% CI, 0–26]).

Table 4

Node Ages with 95% Confidence Intervals (CIs) Retrieved from Two Analyses Using Different Tree Priors

	Birth-death incomplete sampling[a]		Yule process[a]	
	Estimated median age	95% CI	Estimated median age	95% CI
Clade A diverging (crown group age)	147	96–215	165	110–235
Within clade A, South African species diverging	111	63–172	125	75–190
Within clade A, *Isoetes australis* diverging	76	38–129	86	43–141
Within clade A, remaining internal date range	3–23	0–40	4–30	0–55
Clade B diverging	103	64–152	117	72–171
Within clade B, *Isoetes durieui* diverging	62	34–101	72	40–114
Within clade B, American species diverging	38	21–61	44	24–72
Within clade B, remaining internal date range	4–22	1–39	5–26	1–45
Clade C diverging	76	42–123	88	48–143
Clade D diverging	32	20–50	36	22–58
Within clade D, internal date range	1–15	0–26	1–17	0–29
Within clade E, *Isoetes histrix* and *Isoetes setacea* diverging	22	13–34	25	15–39
Within clade E, remaining internal date range	2–14	0–23	2–17	0–26

Note. Data are given in million years ago.

[a] These two approaches had a nonsignificant difference in fit to data as assessed by log marginal likelihood values retrieved from path sampling and stepping-stone sampling. See the text for further details.

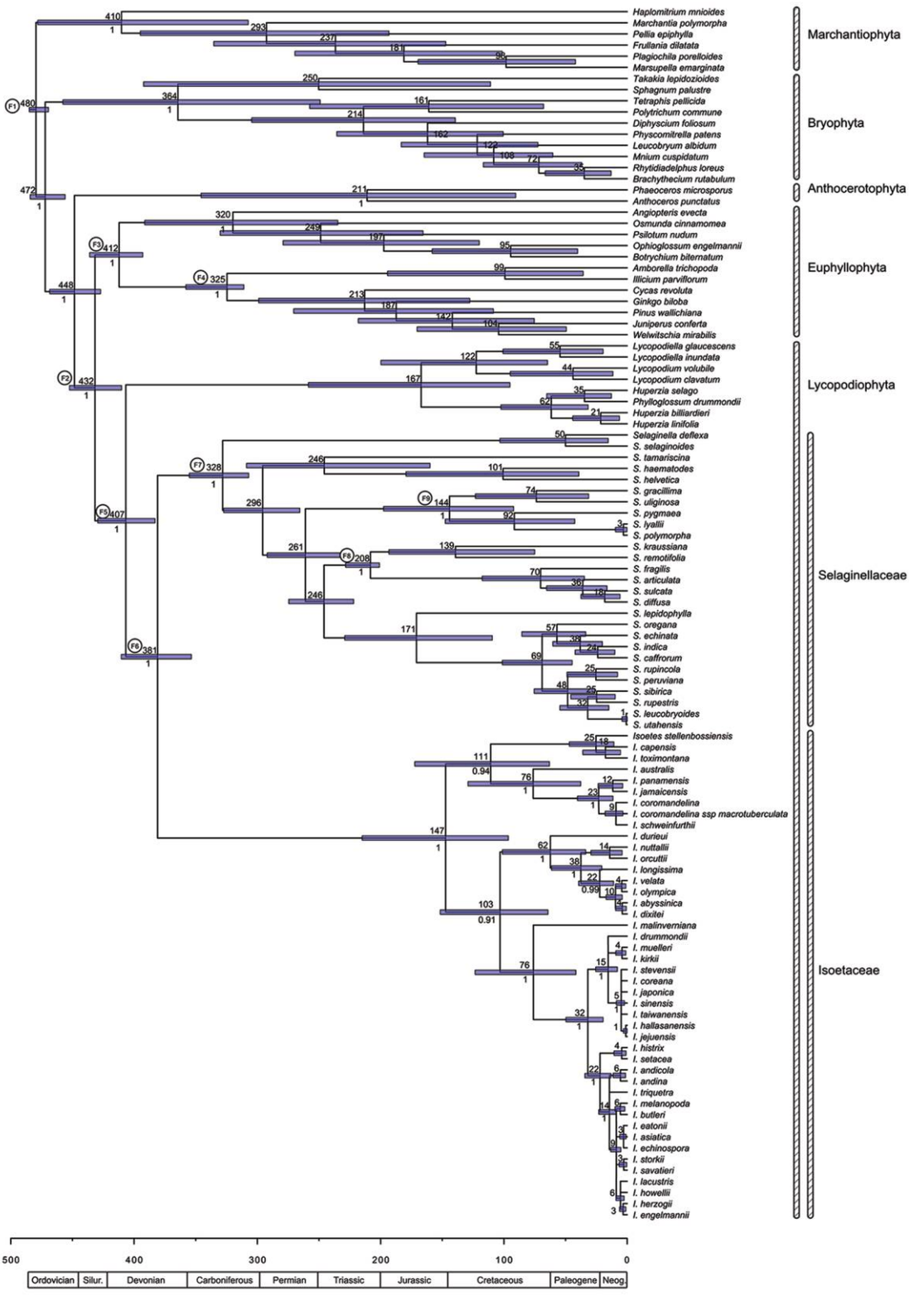

Fig. 2 Chronogram of divergence times of clades analyzed in a Bayesian framework, using a birth-death tree prior with incomplete sampling and uncorrelated lognormal clock. Median age values are given, and bars represent 95% confidence intervals of node ages. Values below the branches are posterior probabilities as estimated by BEAST. Nodes that were assigned prior age probabilities based on fossils are marked F1–F9.

Discussion

Phylogeny, Node Ages, and Biogeographical Interpretations

The divergence times of clades estimated here are much older than could be expected from the relatively low genetic (and morphological) variability in *Isoetes*. The earliest splits in the genus are here estimated to about 147 and 111 mya, respectively, when using the birth-death prior (fig. 2; table 4). Using the Yule process as tree prior yielded even older ages: 165 and 125 mya, respectively (table 4). The estimated ages of the crown group thus match the age of the oldest known fossil with the *Isoetes* habit (a reduced plant body with very restricted apical growth) remarkably well, that is, *I. rolandii* from the Late Jurassic of Idaho (Ash and Pigg 1991; Pigg 2001). The deepest splits in *Isoetes* are also in the same age range as the initial breakup of Gondwana (Seton et al. 2012). This result may help explain the existence of several well-supported clades that seemingly make little sense from a geographical point of view. For example, clade A, which is sister to the remaining *Isoetes*, contains species from all the current remains of Gondwana (except Antarctica): India, Australia, eastern South America, and southern and central Africa (fig. 3). It corresponds to a South African/South American clade in Rydin and Wikström (2002), which they tentatively suggested is a remnant of the Mesozoic, when these landmasses were adjacent and forming the Gondwana continent. Hoot et al. (2006) cautiously agreed that clade A is a good candidate for having a Gondwanan origin, although they maintained the need to find a sound rooting option for *Isoetes* in order to have a more secure foundation for the speculation. Using additional data and ample outgroup information, we have found further support for the existence of several clades in *Isoetes* that are of Mesozoic age, as estimated from absolute ages and topological results.

Clade B contains Mediterranean species and three North American species from the west coast of the United States as well as *I. dixitei* from India (fig. 4). A bold speculation is that

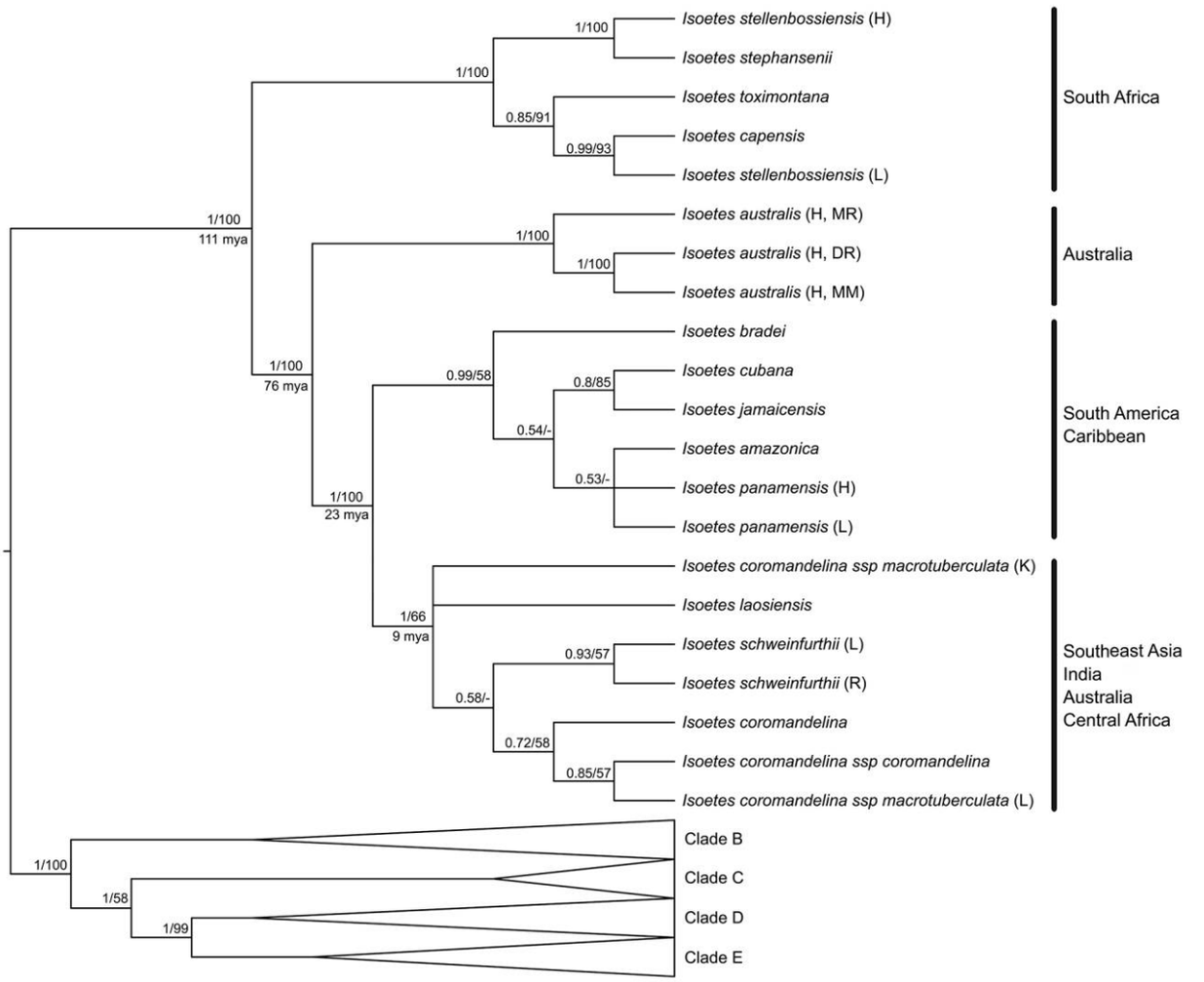

Fig. 3 *Isoetes* clade A, with median divergence times and posterior probability values followed by parsimony bootstrap values. Geographical locations of species are given to the right of the clades. mya = million years ago.

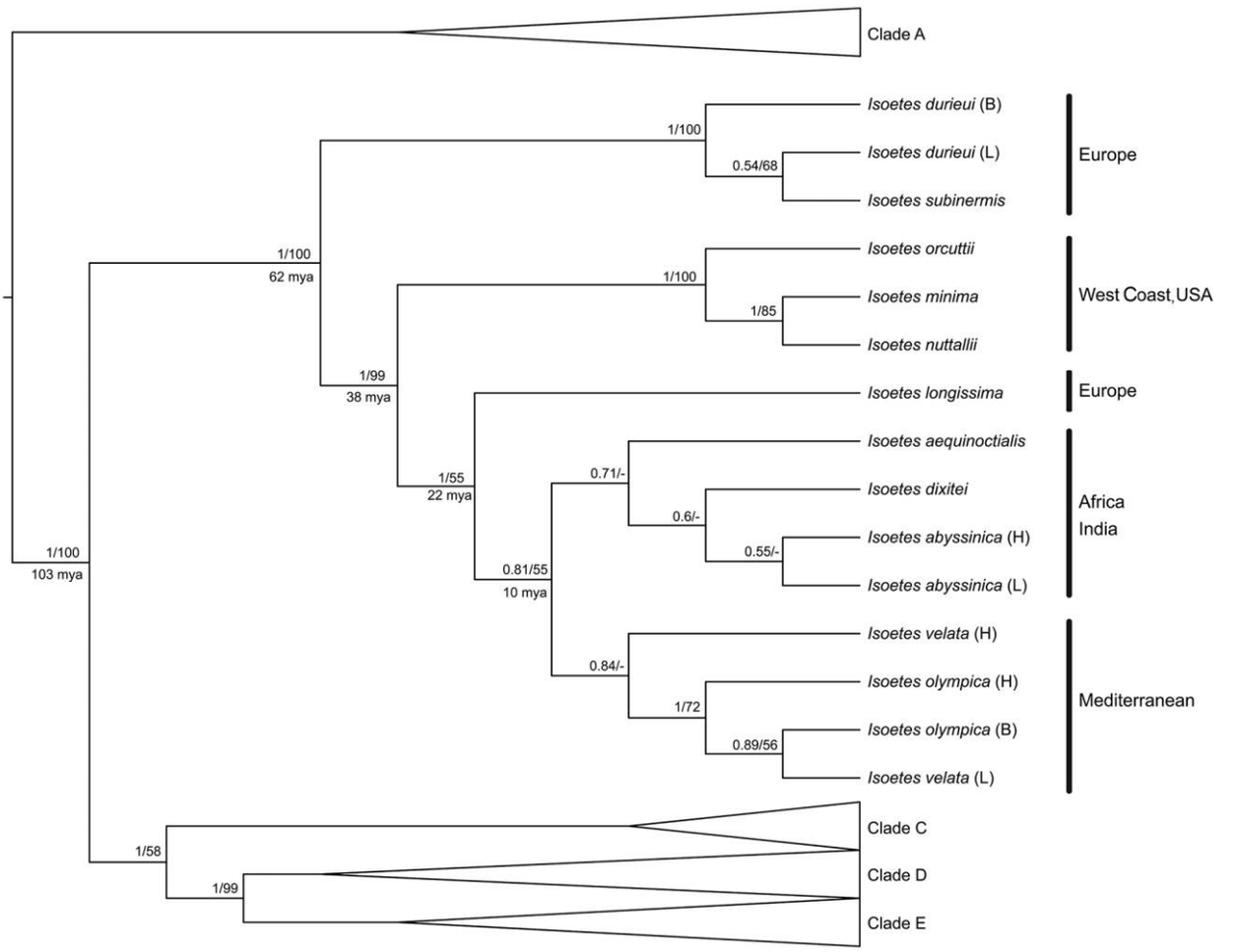

Fig. 4 *Isoetes* clade B, with median divergence times and posterior probability values followed by parsimony bootstrap values. Geographical locations of species are given to the right of the clades. mya = million years ago.

this clade represents remnants from the Laurasia and constitutes the Northern Hemisphere equivalent to the Gondwana clade. That North America species from the west coast are most closely related to mainly Mediterranean species (southern Europe, northern Africa, and the Near East) might seem surprising, but the climates are similar, at least for the Californian species (Tiffney and Manchester 2001). Closely related taxa that used to occupy the bridging areas may have died out due to climate and habitat changes in connection with such processes as the uplift of the Appalachian Mountains (Tiffney and Manchester 2001; Gallen et al. 2013). The west coast of North America also contains other relicts of the Cenozoic Tethys flora, such as redwood and giant sequoia. Clade B is dated to 62 mya (Yule process, 72 mya). This corresponds relatively well with the separation of Europe and Greenland from North America, which likely took place in the Late Cretaceous (Mosar et al. 2002).

In clade C, we find *I. malinverniana* (fig. 5), an Italian endemic that has been speculated to hail from Asia (Schneller 1982), as it only occurs in rice-growing areas, but it has not previously been linked to any Asian species, and its origin has been a mystery. Adding a nrITS sequence of *I. anatolica* from Lake Abant in northwest Turkey (Bolin et al. 2008) resolved *I. anatolica* as sister to *I. malinverniana*. Our results thus appear to confirm Schneller's (1982) speculations of a link between *I. malinverniana* and Asia, at least Asia Minor. Few studies have made a complete investigation of species distribution in the genus, but according to Underwood (1888) and Pfeiffer (1922) the genus is poorly represented in Asia, with only a handful of species occurring in Asia Minor or in the easternmost parts of the continent. Asian species described after the work by Underwood (1888) and Pfeiffer (1922) are the consequence of work in the easternmost parts of Asia (China or Japan), for example, Liu et al. (2005) and Kim et al. (2009).

Relationships in clades D and E reveal subclades from Oceania and East Asia and from the Americas and Eurasia, respectively (figs. 6, 7). However, while these clades are supported in Bayesian analyses, they do not typically retrieve any support in parsimony bootstrap analyses, which may indicate reason for caution. Some authors argue that high posterior probabilities and low bootstrap support may indicate inflated posterior

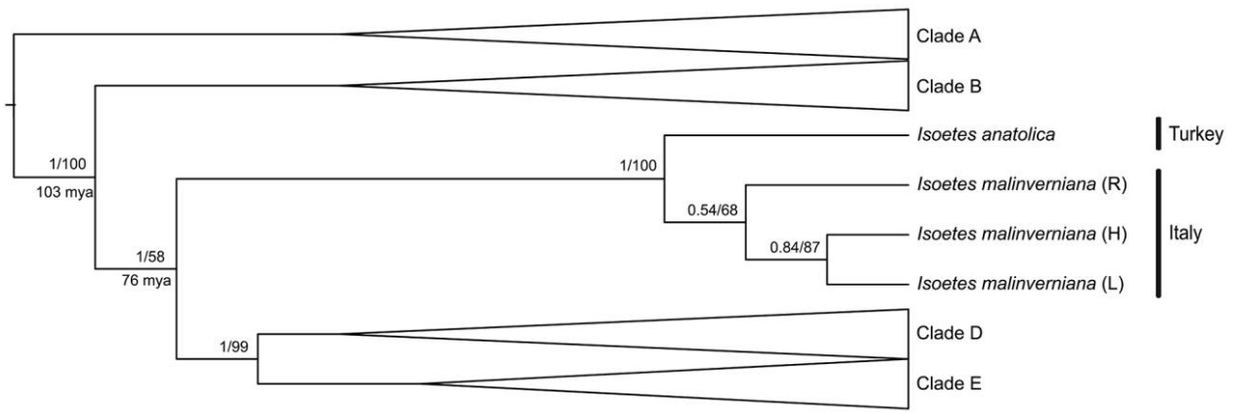

Fig. 5 *Isoetes* clade C, with median divergence times and posterior probability values followed by parsimony bootstrap values. Geographical locations of species are given to the right of the clades. mya = million years ago.

probabilities for clades that in fact are not supported by any synapomorphies (Douady et al. 2003). But as resolved here, the phylogenetic patterns and node ages in clades D and E do not indicate correlation with major Mesozoic vicariance events but appear more consistent with those of a younger clade and speciation following dispersal. It is somewhat surprising that it is not possible to separate South American species from North American species within clade E; however, on the basis of our estimated ages of clades in these groups, we agree with Hoot et al. (2006), who suggest that the relationships within this clade could be explained by recent rapid evolution. Conceivably, hybridization and/or polyploidization in combination with dispersal may have been important for evolution in these clades.

The placement of *Isoetes andicola* within clade E deserves a mention, as this species from Peru was at one point described as a new genus (and species) of the Isoetaceae, *Stylites andicola* (Amstutz 1957), mainly on the basis of a difference in stem morphology; the species has an elongated corm that branches (Amstutz 1957). This unusual feature was found to appear in later stages of development, while the young plants looked much like other species of *Isoetes* (Karrfalt 1984 and references therein). Here, we present the first, to our knowledge, molecular data from this species, and it is unquestionably positioned with some of the other South American *Isoetes* species—those of clade E. A speculative hypothesis is that this species has reverted into the ancestral state of lycopods, an elongated and dichotomizing stem, present in Lycopodiaceae, *Selaginella*, and the extinct tree lycopods of the Isoetales.

While several placements in this phylogeny might seem surprising in light of geographical location, the European species are particularly puzzling. It could be argued that it is not remarkable that the pan-European *I. setacea* and its sister, the mainly Mediterranean *I. histrix*, are part of clade E; they could be assumed to be closely related to the circumboreal *I. echinospora*, which has a partly overlapping range. But it is peculiar that they are separated with high support from the five species in clade B and from *I. malinverniana* of clade C with sympatric Mediterranean distributions, not least since some species of these clades—that is, *I. histrix* (clade E) and *I. subinermis* (clade B)—show great similarity and are difficult to tell apart (Bolin et al. 2008). We have aimed to combine as much available molecular data on the genus as possible, to give as complete a picture as possible, but we were able to include only a single specimen of several of the above-mentioned species (i.e., *I. subinermis* produced by Bolin et al. 2008 and *I. histrix* and *I. setacea* from Hoot et al. 2006). Increasing the number of representatives of those species is needed to provide a more stable foundation for phylogenetic interpretations. It should also be mentioned that with expanded knowledge of the phylogeny of *Isoetes*, it will be easier to reveal sequence contamination and potentially misidentified specimens.

Comparison with Previous Studies

The result attained here supports the findings of previous studies based on molecular data and adds further details. Clade A in Rydin and Wikström (2002) consists of *I. amazonica*, *I. bradei*, *I. kersii*, and *I. schweinfurthii*, and it constitutes one of the daughter lineages resulting from the earliest split in *Isoetes*. Rydin and Wikström (2002) did not include any species representing clade B of the present study. Our clade C, consisting of *I. malinverniana* strongly supported as sister to the remaining species (those of clade DE), is present at the same phylogenetic position in Rydin and Wikström (2002), but with low support.

In Hoot et al. (2006), the tree topology is an unresolved basal trichotomy consisting of clade A's two major subgroups and the remaining *Isoetes*. Within the latter, the position of *I. malinverniana* changes with analytical method; according to their parsimony bootstrap results, it is sister to all remaining *Isoetes* (here corresponding to clades B, D, and E), whereas their maximum likelihood analysis places it, as in our results, as sister to clade DE. In our analyses, *I. malinverniana* is always sister to clade DE, in parsimony analyses as well. Clades B, D, and E are represented in Hoot et al. (2006), and the topology within them largely corresponds to our results (although the present study has wider taxon sampling and generally higher topological resolution).

It is further interesting to note that our results to some extent support the morphology-based evolutionary scheme out-

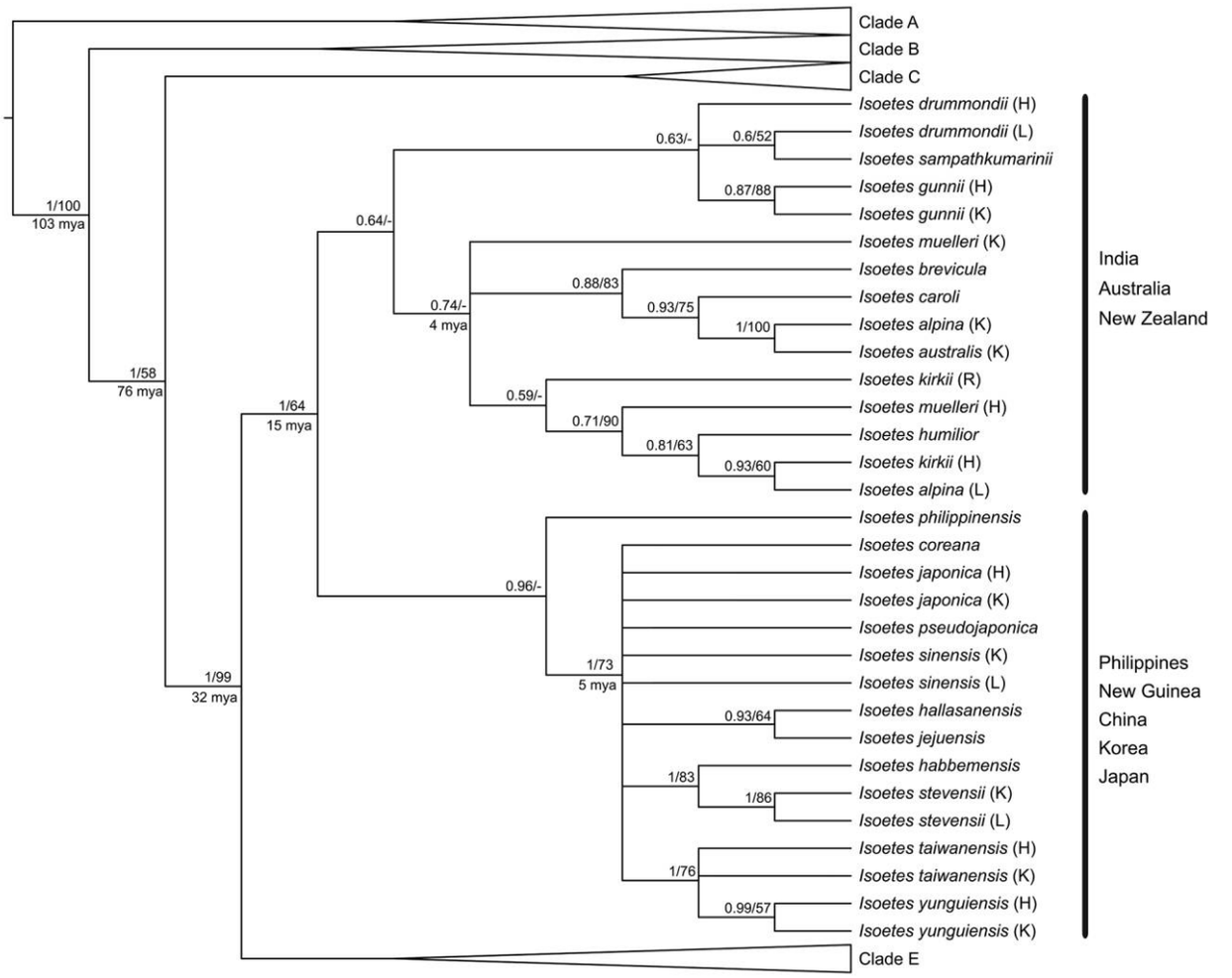

Fig. 6 *Isoetes* clade D, with median divergence times and posterior probability values followed by parsimony bootstrap values. Geographical locations of species are given to the right of the clades. mya = million years ago.

lined by Hickey (1986*a*), although some results differ between our studies, and Hickey's (1986*a*) restricted sampling prevents complete comparison. *Isoetes bradei*, here shown to belong in clade A (the sister clade of the remaining genus), was considered part of a "primitive grade" and linked to fossils of Lepidodendrales and *Isoetites* because of the presence of alate leaves (Hickey 1986*a*). Unfortunately, *I. bradei* is now most likely extinct (Taylor and Hickey 1992). Indian species were also considered outside the core clade of *Isoetes* by Hickey (1986*a*). It is unclear which Indian species he studied apart from *I. coromandelina*, which is in our results indeed part of the early-diverging clade A. However, other Indian species sampled here fall into clades B and D.

Chromosome Counts and Ploidy Levels

In a review of the available cytological information regarding *Isoetes*, Troìa (2001) found the polyploidy proportion to be high: 61% of represented species were some variant of polyploids, with the caveat that only 67 taxa were listed in the synthesis. In *Selaginella*, the closest relative, polyploidy is on the contrary quite rare (11%; Takamiya 1994). The basic chromosome number for *Isoetes* is 11 (Löve et al. 1977), which according to Troìa is low and indicates that polyploidization has not been important in the evolution of the genus until recently. As clades D and E are much more unresolved than clades A and B, we initially hypothesized that polyploidization is more common in those clades than in clades A and B. However, mapping available ploidy-level estimations on the phylogeny reveals that polyploidy is found in all major clades (fig. 1). Although diploids appear to be more common in clade E than in the other clades, it is important to note that there is a strong sampling bias. Clade E is by far the best-sampled clade in Troìa's (2001) compilation, while there has been less research conducted on species of clades A and B.

With regard to habitat and polyploidy, few patterns can be discerned, but one interesting thing is that most species that are strictly terrestrial are diploids (Taylor and Hickey 1992; Troìa 2001). Furthermore, in their study of *Isoetes* species in East Asia, Liu et al. (2004) state that diploids occur at high ele-

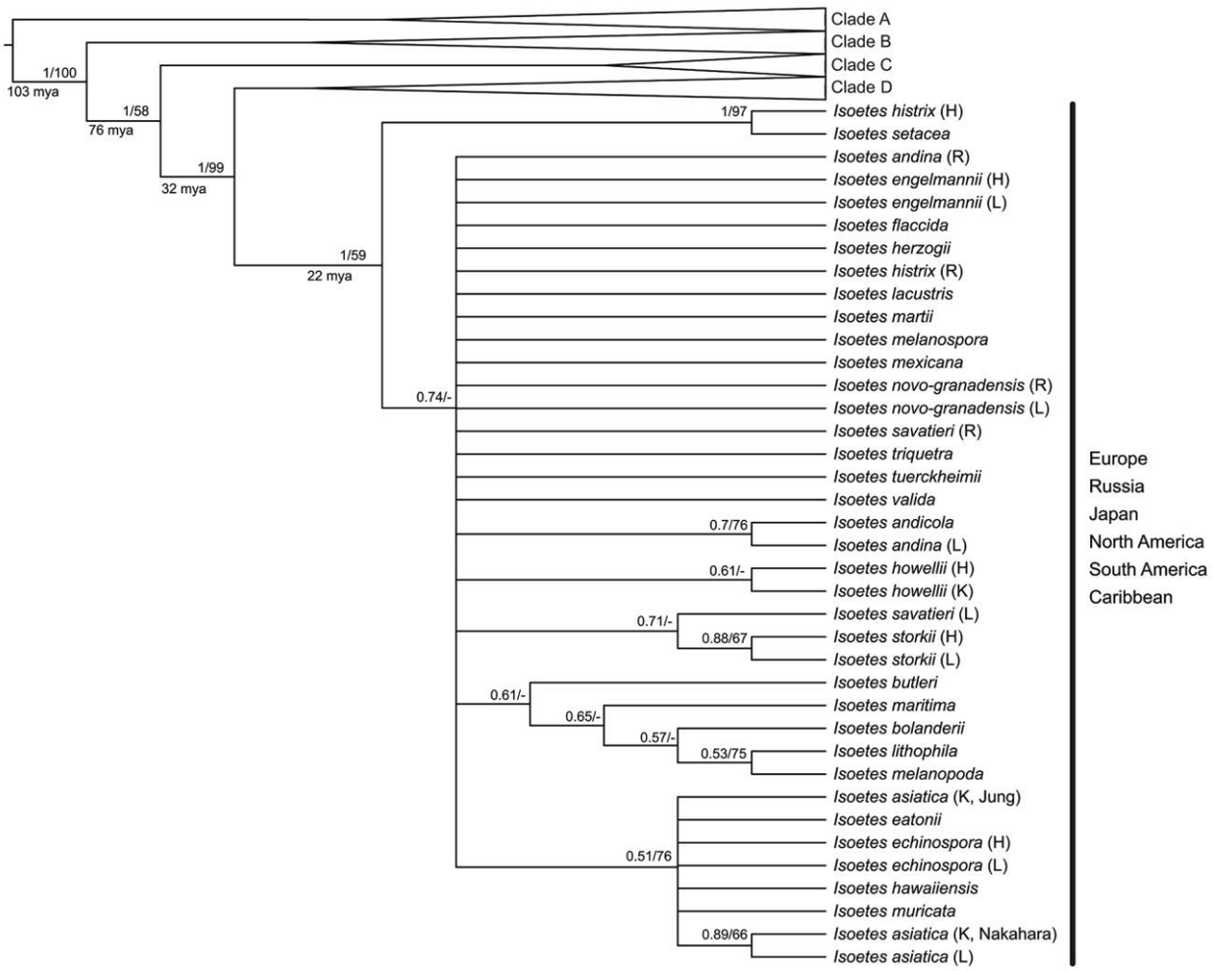

Fig. 7 *Isoetes* clade E, with median divergence times and posterior probability values followed by parsimony bootstrap values. Geographical locations of species are given to the right of the clades. mya = million years ago.

vation while polyploid species are concentrated at low elevation. Polyploid species also have wider geographical distribution and are more common within their areas than are diploids. Liu et al. (2004) speculate that this means that diploids have a higher tolerance for environmental extremes but that polyploids could be more competitive within the habitats that are more suitable for them.

Dispersal and Morphology

Although our results indicate speciation subsequent to ancient vicariance events (as well as more recent dispersal), a thorough biogeographical analysis of *Isoetes* is yet to be conducted. If the complicated phylogenetic patterns are not caused by old Gondwanan and perhaps also Laurasian ancestry but instead by more recent dispersal, it would require repeated long-distance dispersal events. Furthermore, dispersal is not enough; subsequent establishment and reproduction in the new area are also required. It should be noted that for these heterosporous lycopods both megaspores and microspores of the same species have to disperse to nearby localities in order for sexual reproduction to successfully take place in a new area. Unfortunately, there is not much information available on how *Isoetes* disperse; by waterways, by waterfowl, and even by snails and earthworms have been suggested (Duthie 1929; Jermy 1990; Taylor and Hickey 1992). Dismissing dispersal by invertebrates as too short in range for the distances we are considering, we are left with two other options. It would seem that a plant that is wholly submerged in a lake or river would inevitably have offspring (i.e., spores in this case) swept downriver, but that would still explain only dispersal within a region, and only unidirectionally so. In the third option, mega- and/or microspores are caught on the feet and legs of waterfowl and thereby spread along the travels of the bird. This seems a likely scenario, but in the only research into the subject Liu et al. (2004) did not find any evidence of birds' migratory paths within China having any correlation with *Isoetes* habitats. Even if birds and waterways are responsible for most of the dispersal of *Isoetes*—which may,

for example, be the case for species within a region, like the Mediterranean area—we are still left with the conundrum of how species would travel from, for example, South Africa to Australia. As far as we know, there are no migratory routes between those areas. Wind dispersal might be possible, and microspores are conceivably light enough to be airborne over long distances, but the bigger megaspore seems more doubtful. Could spores travel by sea currents?

It has not been within the scope of this research to study the morphology of *Isoetes*, but it would be interesting to assess whether this new phylogeny can bring some useful characteristics to light. A character that is often referred to when describing species is megaspore ornamentation, but the four groups Pfeiffer (1922) distinguished on the basis of megaspore morphology do not correspond to monophyletic groups in our results (fig. 1). All species investigated by Pfeiffer (1922) that are included in clades A, B, and C have tuberculate megaspore ornamentation, and this may be the ancestral condition in extant *Isoetes*. Also in clade D, tuberculate ornamentation is common; only one of the investigated species in clade D has reticulate ornamentation. In clade E, however, all four varieties are represented (tuberculate, echinate, cristate, and reticulate ornamentation). Hickey (1986*b*) recognized 12 types of megaspore ornamentation in *Isoetes*, but as with Pfeiffer's (1922) classification the types do not correspond to monophyletic groups of the present study.

It is possible that the diversity of megaspore ornamentation in clade E can be linked to the variability of hybrid characters, as reported by Britton and Brunton (1995). Another possibility is that spore-surface ornamentation is correlated with habitat preference, as hypothesized for spore-wall ultrastructure (Taylor 1993). Taylor (1993) found, however, that spore-wall ultrastructure was largely constant in the six studied species of *Isoetes*, and the limited variation did not correspond with habitat preference. Regarding surface ornamentation, we cannot find a clear correlation between this feature and terrestrial versus aquatic habitat preference, but the matter should be studied further. A new and full grasp of mega- and microspore ornamentation and ultrastructure in Isoetales, including extant and extinct species, would probably be worthwhile.

Intraspecific variation should also be further assessed. It is, for example, unclear how many megaspores and how many individuals Pfeiffer (1922) and Hickey (1986*b*) examined in their analyses; it may be that some species were represented by only one specimen and that undetected intraspecific variation occurs. In the sister group *Selaginella*, the ornamentation is generally consistent within species, but there are cases where the intraspecific variability is too great to make it a useful characteristic (Korall and Taylor 2006). Interestingly, this is the case for *S. selaginoides*, which has a circumboreal distribution and together with *S. deflexa* is sister to all other species of *Selaginella* (Korall and Kenrick 2002).

Many more data are needed to clearly assess how and when morphological features have evolved in the Isoetales. We hope this new phylogeny of extant species may serve as a framework for such future studies and for the formation of new ideas on character evolution in the clades. Although morphological divergence appears limited in *Isoetes*, there may be characteristics that have been previously overlooked.

Acknowledgments

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Appendix

Voucher Information and GenBank Accession Numbers

The following information is provided when possible for each specimen in the study: taxon, sample locality, voucher of *Isoetes* sequences, and GenBank accession numbers in the order of *rbcL*, *atpB-rbcL*, and nrITS. Dashes indicate missing data.

Amborella trichopoda Baill., –, –, L12628, –, –. *Angiopteris evecta* (G. Forst.) Hoffm., –, –, L11052, –, –. *Anthoceros punctatus* L., –, –, U87063, –, –. *Botrychium biternatum* Undrew., –, –, L13474, –, –. *Brachythecium rutabulum* (Hedw.) Schimp., –, –, DQ645997, –, –. *Cycas revoluta* Thunb., –, –, AY056556, –, –. *Diphyscium foliosum* (Hedw.) D. Mohr, –, –, DQ645985, –, –. *Frullania dilatata* (L.) Dumort., –, –, DQ645979, –, –. *Ginkgo biloba* L., –, –, AJ235804, –, –. *Haplomitrium mnioides* (Lindb.) R.M. Schust., –, –, DQ268971, –, –. *Huperzia billardierei* (Spring) Rothm., –, –, AJ133894, –, –. *Huperzia linifolia* L., –, –, Y07932, –, –. *Huperzia selago* L., –, –, Y07934, –, –. *Illicium parviflorum* Michx. ex Vent., –, –, L12652, –, –. *Isoetes abyssinica* Chiov., Ethiopia, Wollega Region, Gilbert, M.G. & Thulin, M. 696 (BM), KT288395, –, KT288367. *Isoetes abyssinica* Chiov., Kenya, pool in rock outcrop adjacent to Carnivore restaurant, Nairobi, Gastony 97-101 (MIL), DQ294238, DQ280350, DQ284988. *Isoetes aequinoctialis* A. Braun, Zambia, 12.50′S 28.21′E, Kornas 3453 (BR), –, –, KT288368. *Isoetes alpina* Kirk, New Zealand, Canterbury, Melville, R. 6250 (L), –, –, KT288369. *Isoetes alpina* Kirk, –, Melville 6824 (BISH), –, JN578267, JN578308. *Isoetes amazonica* A. Braun, Brazil, Harley et al. 19109 (BM), AF404491, –, –. *Isoetes anatolica* Prada & Rolleri, Turkey, Bolu province, Abant Lake, Musselman TR-2007-01 (ODU), –, –, EU444000. *Isoetes andicola* (Amstutz) L.D. Gómez, Peru, 1970, Rauh, W. 2406 (BM), KT288396, –, KT288370. *Isoetes andina* Spruce ex Hook., Colombia, Jermy, C. 17476 (BM), KT288397, –, KT288371. *Isoetes andina* Spruce ex Hook., Colombia,

Jermy, C. 17476 (BM), AF404492, –, –. *Isoetes asiatica* Makino, Russia, Kamchatka, Kharkevich S. 14 (BR), KT288398, –, KT288372. *Isoetes asiatica* Makino, –, G. Nakahara s.n. (TNS), –, JN578274, JN578314. *Isoetes asiatica* Makino, Japan, Hokkaido, J. Jung 45-1 (AJOU), FJ785182, –, FJ785161. *Isoetes australis* S. Williams (DR), Australia, rock pool, Durgacutting Rock, W. Australia, Taylor 6376 (MIL), DQ294239, DQ280351, DQ284989. *Isoetes australis* S. Williams (MM), Australia, rock pools, Mt. Madden, W. Australia, Hoot 02-30 (UWM), –, DQ479999, DQ479973. *Isoetes australis* S. Williams (MR), Australia, rock pool, Mt. Ridley, W. Australia, Seigler & Maslin 14701a (MIL), DQ294240, DQ280352, DQ284990. *Isoetes australis* S. Williams, Australia, Western, Annels and Hearn 5699 (PERTH), –, JN578275, JN578315. *Isoetes bolanderi* Engelm., USA, Colorado, Horreus de Haas, R.H. 2033 (L), –, –, KT288373. *Isoetes bradei* Herter, Brazil, São Paulo, Herter, Brade 8119 (S), AF404493, –, –. *Isoetes brevicula* E. R. L. Johnson, Australia, Western, Buehrig s.n. (PERTH), –, JN578276, JN578316. *Isoetes butleri* Engelm., USA, dolomite prairie, Des Plaines Conservation Area, Will Co., IL, Taylor 5630 (MIL), –, DQ480000, DQ479974. *Isoetes capensis* Duthie, S. Africa, seasonally moist depression, Stellenbosch Univ. Campus, Musselmann 99201 (MIL), DQ294241, DQ280353, DQ284991. *Isoetes caroli* E.R.L.Johnson, Australia, Western Australia, Lyons and Lyons 4143 (PERTH), –, JN578277, JN578317. *Isoetes coreana* Y.H.Chung & H.K.Choi, Corea, Jeonrabam-do, C. Kim 2006-73 (AJOU), FJ785176, JN578278, EU348550. *Isoetes coromandelina* L. f., India, Matkuli, foothills, Pachmarhi, Srivastava s.n. Aug-97 (MIL), DQ294242, DQ280354, DQ284992. *Isoetes coromandelina* ssp. *coromandelina* L. f., –, Cowie 11533 (DNA), –, JN578305, JN578338. *Isoetes coromandelina* ssp. *macrotuberculata* C.R.Marsden, Australia, Northern Territory, Fryxell, P.A. & Carven, L.A. s.n. (MEL), –, –, KT288374. *Isoetes coromandelina* ssp. *macrotuberculata* C.R.Marsden, –, Brennan 5641 (DNA), –, JN578306, JN578339. *Isoetes cubana* Engelm. ex Baker, Cuba, Esmeralda, Meseta de San Felipe; Camaguey Prov., Berazain s.n., 19-May-89 (MIL), –, DQ480001, DQ479975. *Isoetes dixitei* Shende, India, Wilson Point, Mahabaleswar, Maharashtra, Srivastava 50020 (MIL), –, DQ480002, DQ479976. *Isoetes drummondii* A.Braun, Australia, Western Australia, Carlquist, S.J. 5528 (L), KT288399, –, KT288375. *Isoetes drummondii* A.Braun, Australia, Grampians, West Hill Rd., Victoria, Hoot s.n. (UWM), DQ294243, DQ280355, DQ284993. *Isoetes durieui* Bory, Portugal, Penacova, Matos s.n. (BR), –, –, KT288376. *Isoetes durieui* Bory, –, Musselman TR-2007-02 (ODU), –, –, EU444001. *Isoetes eatonii* R. Dodge, Canada, Ontario, Brunton, D.F. et al. 13337 (BM), KT288400, –, KT288377. *Isoetes echinospora* Durieu, Finland, 67.49′N 23.41′E, Nurmi 97-47 (BR), KT288401, –, KT288378. *Isoetes echinospora* Durieu, USA, SW margin of Devil's Lake, Sauk Co., WI, Taylor 6951 (MIL), –, DQ480003, DQ479977. *Isoetes engelmannii* A.Braun, USA, New York, Cortland, Lawrence, G.H.M. 481 (L), KT288402, –, KT288379. *Isoetes engelmannii* A.Braun, USA, Fountain Ck., Greensville Co., VA, Taylor s.n. 18-Jul-95 (MIL), –, DQ480004, DQ479978. *Isoetes flaccida* Shuttlew., USA, Newport, E side of St. Mark's River at Hwy. 98 bridge, Wakulla Co., Taylor 5214 (MIL), –, DQ480005, DQ479979. *Isoetes gunnii* A.Braun, Australia, Central Highlands, Western Tiers, Pine Lake, Tasmania, Walsh 5733 (UWM), –, DQ480006, DQ479980. *Isoetes gunnii* A.Braun, –, J.R. Croft s.n. (BISH), –, JN578285, JN578323. *Isoetes habbemensis* Alston, Indonesia, New Guinea. Herb. spec.: Hadlow and Hope 647. Cultivated, Canberra Nat. Bot Garden, Hoot 02-38 (UWM), –, DQ480007, DQ479981. *Isoetes hallasanensis* H.K.Choi, Ch.Kim & J.Jung, Corea, Jeju-do, H.-K. Choi 2006-114 (AJOU), FJ785178, JN578288, EU348554. *Isoetes hawaiiensis* W.C.Taylor & W.H.Wagner, USA, shallow pools on tabular summit of Mt. Eke, W Maui Mtns., Maui, HI, Taylor 5658 (MIL), –, DQ480008, DQ479982. *Isoetes herzogii* U.Weber, Bolivia, lago 4, Halls, C. s.n. (BM), KT288403, –, KT288380. *Isoetes histrix* Bory & Durieu, Greece, Crete, Wanntorp NR5350 (S), –, DQ280356, DQ284994. *Isoetes histrix* Bory & Durieu, Greece, Wanntorp s.n. (S), AF404497, –, –. *Isoetes howellii* Engelm., USA, Table Mt., Butte Co., CA, Vincent & Rhode s.n. (MIL), –, DQ480009, DQ479983. *Isoetes howellii* Engelm., –, R.F. Thorne 44980 (KYO), –, KC603854, KC603855. *Isoetes humilior* A.Braun, Australia, Naas Creek, Darbyshire, P.J. 134 (L), KT288404, –, KT288381. *Isoetes jamaicensis* Hickey, Jamaica, rain pools, Harris Savannah, Clarendon Parish, Taylor 6117 (MIL), –, DQ480011, DQ479984. *Isoetes japonica* A.Braun, Japan, Imaichi Tochigi Pref., Cult. Bot. Gard., Nikko Univ. of Tokyo, Takahashi s.n., 2-Aug-90 (MIL), –, DQ480012, DQ479985. *Isoetes japonica* A.Braun, Japan, Honshu, H.-K. Choi 2006-116 (AJOU), FJ785180, –, EU348558. *Isoetes jejuensis* H.K.Choi, Ch.Kim & J.Jung, Corea, Jeju-do, H.-K. Choi 2006-104 (AJOU), FJ785179, JN578292, EU348551. *Isoetes kirkii* A.Braun, New Zealand, Lake Brunner, South Island, Woodland & Cutten s.n. (MIL), DQ294244, DQ280357, AY641100. *Isoetes kirkii* A.Braun, New Zealand, Chinnock, R.J. P 447 (BM), AF404499, –, –. *Isoetes lacustris* L., Sweden, (cult.), Larsen 20130120-3, KT288405, –, KT288382. *Isoetes laosiensis* C.Kim & H.K.Choi, –, B.Y. Sun and S.S. Choi 2039 (AJOU), –, JN578307, EU348564. *Isoetes lithophila* N.Pfeiff., USA, temporary pool, summit of Enchanted Rock, Enchanted Rock St. Park, Llano Co., TX, Taylor 4653 (MIL), –, DQ480013, DQ479986. *Isoetes longissima* Bory, Spain, edge of stream, Rio Miño, W of Lugo, Galica, Taylor 5409 (MIL), –, DQ480014, DQ479987. *Isoetes malinverniana* Ces. & DeNot., Italy, Vercelli, Raynal 20885 (BR), –, –, KT288383. *Isoetes malinverniana* Ces. & DeNot., Italy, Cultivated, Zurich Botanic Gardens. Collection site: 1 km W of Albano, Prov. di Vercelli, Piedmont, ZBG 15.9.02 (MIL), DQ294245, DQ280358, DQ284995. *Isoetes malinverniana* Ces. & DeNot., Italy, Fraser-Jenkins 930 (BM), AF404500, –, –. *Isoetes maritima* Underw., USA, Alaska, H.-K. Choi 2008-16 (AJOU), FJ785185, JN578294, FJ785173. *Isoetes martii* A.Braun, Brazil, Minas Gerais, Regn 2027 (S), AF404501, –, –. *Isoetes melanopoda* J.Gay & Durieu, USA, Moody Branch, along MS Hwy. 43, just S of Rock Branch Rd., Pearl River Co., MS, Leonard s.n. (MIL), –, DQ280359, DQ284996. *Isoetes melanospora* Engelm., USA, shallow pool on summit of Mt. Arabia, DeKalb Co., GA, Taylor 4849 (MIL), –, DQ480015, DQ479988. *Isoetes mexicana* Underw., Mexico, Morelos, Pringle 6660 (BR), –, –, KT288384. *Isoetes minima* A.A.Eaton, USA, Colockum Pass, Kititas Co., WA, Ceska 19754 (MIL), –, DQ480016, DQ479989. *Isoetes muelleri* A.Braun., Australia, Naas Creek off Boboyan Rd., ACT, Hoot 02-43 (UWM), –, DQ480017, DQ479990. *Isoetes muelleri* A.Braun., Australia, Western, Keighery and Gibson 687 (PERTH), –,

JN578295, JN578331. *Isoetes muricata* Durieu, –, J.A. Calder and R.L. Taylor 23652 (TNS), –, JN578296, JN578332. *Isoetes novo-granadensis* H.P. Fuchs, Ecuador, Carchi, Holm-Nielsen, L. B. et al. 5470 (L), KT288406, –, KT288385. *Isoetes novo-granadensis* H.P. Fuchs, Ecuador, Holm-Nielsen 5925 (S), AF404503, –, –. *Isoetes nuttallii* A.Braun., USA, seasonally moist depression, Four Corners, San Diego Co., CA, Taylor s.n., 7-Apr-96 (MIL), DQ294246, DQ280360, DQ284997. *Isoetes olympica* A.Braun., Syria, seasonally moist depression, near Saleh, Jebel Druze region, Musselmann 2007 (ODU), –, DQ480018, DQ479991. *Isoetes olympica* A.Braun., –, Al-Zein & Musselman 2009-39b (MO), –, –, GU591480. *Isoetes orcuttii* A.A.Eaton, USA, Santa Rosa Plateau, Riverside Co., CA, Taylor s.n., 5-Apr-95 (MIL), DQ294247, DQ280361, DQ284998. *Isoetes panamensis* Maxon & C.V. Morton, Costa Rica, Hepper, D.N. 171 (BM), KT288407, –, –. *Isoetes panamensis* Maxon & C.V. Morton, Costa Rica, seasonally flooded pasture along Cuejiniguil Road, Santa Elena, Guanaccaste, Taylor 6087 (MIL), DQ294248, DQ280362, DQ284999. *Isoetes philippinensis* Merr. & R.H. Perry, –, Price 500 (A), –, JN578298, JN578334. *Isoetes pseudojaponica* M. Takamiya, Mits. Watan. & K. Ono, –, R. Imaichi and Y. Hirayama 070509-1 (TNS), –, JN578299, JN578335. *Isoetes sampathkumarinii* L.N. Rao, India, Goswami, H.K. (BM), AF404504, –, –. *Isoetes savatieri* Franch., Uruguay, Hoter 95840 (BR), KT288408, –, KT288386. *Isoetes savatieri* Franch., Chile, South-West, Franch, A. 1205 (HIP), AF404505, –, –. *Isoetes schweinfurthii* Baker, Burkina Faso, Madsen, J.E. 6082 (BM), KT288409, –, KT288387. *Isoetes schweinfurthii* Baker, Nigeria, Kornas 6272 (BM), AF404506, –, –. *Isoetes setacea* Lam., Spain, Pool, Hoyo de Mauzanares, Madrid, Prada s.n., 10-Sep-88 (MIL), DQ294249, DQ280363, DQ285000. *Isoetes sinensis* Palmer, China, Zhejiang province, Jiande Hangzhou, s.n. (S), KT288410, –, KT288388. *Isoetes sinensis* Palmer, China, Wuhan, H.-K. Choi and H. Na 2005-94 (AJOU), FJ785181, JN578300, EU348563. *Isoetes stellenbossiensis* A.V. Duthie, South Africa, Rondebosch, Stauffer, H.U. 5135 (L), –, –, KT288389. *Isoetes stellenbossiensis* A.V. Duthie, S. Africa, Rondebosch Commons, Capetown, Musselmann 99204 (ODU), –, DQ480021, DQ479994. *Isoetes stephansenii* A.V. Duthie, S. Africa, rock pools, Platkip outcrop, bog at km 70 on W side of Stellenbosch, Taylor 6158 (MIL), –, DQ480022, DQ479995. *Isoetes stevensii* J.R. Croft, Papua New Guinea, Mt Giluwe, Schodde, R. 1843 (L), KT288411, –, KT288390 . *Isoetes stevensii* J.R. Croft, –, Croft and Marsh s.n. (A), –, JN578302, JN578337. *Isoetes storkii* T.C. Palmer, Panama, Mouro, A.K. & Knapp, S. 5185 (BM), KT288412, –, KT288391. *Isoetes storkii* T.C. Palmer, Costa Rica, bog at km 70 on W side of Panamerican Hwy., Cerro de la Muerte, Taylor s.n. (MIL), –, DQ480023, DQ479996. *Isoetes subinermis* Cesca, Turkey, Musselman TR-2007-03 (ODU), –, –, EU444003. *Isoetes taiwanensis* De Vol, Taiwan, Menghuan Lake, Yangming Mts., Chiou s.n., May-98 (MIL), DQ294250, DQ280364, AY641101. *Isoetes taiwanensis* De Vol, Taiwan, Taipei, H.K. Choi 2007-17 (AJOU), FJ785177, JN578303, EU348561. *Isoetes toximontana* L.J.Musselman & J.P.Roux, S. Africa, seasonally wet area along Gifberg Road, near Oubergpad, N. Cape Province, Musselmann 2001-35 (MIL), –, DQ480024, DQ479997. *Isoetes triquetra* A. Braun, Ecuador, Napo, Øllgaard, B. & Baslev, H. 10145 (BM), KT288413, –, KT288392. *Isoetes tuerckheimii* Brause, Dominican Republic, Gasong, G.J. et al. 742 (BM), KT288414, –, KT288393. *Isoetes valida* (Engelm.) Clute, USA, shore above Maury River, along Hwy. 39, N of Goshen Pass, Rockbridge Co., VA, Taylor s.n., 18-Jul-95 (MIL), –, DQ480025, DQ479998. *Isoetes velata* A.Braun., Tunisia, 37.12′N 09.14′E, Aedo et al. s.n. (BR), KT288415, –, KT288394. *Isoetes velata* A.Braun., Spain, pool, Hoyo de Mauzanares, Madrid, Prada s.n. (MIL), DQ294251, DQ280365, DQ285001. *Isoetes yunguiensis* Q.F.Wang & W.C.Taylor, China, Sha-shi-chong, Pingba Co., Guizhou Prov., Liu & Yang 5 (WH), –, DQ480026, AY641102. *Isoetes yunguiensis* Q.F.Wang & W.C.Taylor, –, Chang s.n. (PE), –, JN578304, GQ175877. *Juniperus conferta* Parl., –, –, L12573, –, –. *Leucobryum albidum* (Brid. ex P. Beauv.) Lindb., –, –, DQ645991, –, –. *Lycopodiella glaucescens* (Presl) B.Øllg., –, –, AJ133260, –, –. *Lycopodiella inundata* L., –, –, Y07938, –, –. *Lycopodium clavatum* L., –, –, Y07936, –, –. *Lycopodium volubile* G. Forst., –, –, AJ133253, –, –. *Marchantia polymorpha* L., –, –, U87079, –, –. *Marsupella emarginata* (Ehrh.) Dumort., –, –, DQ645972, –, –. *Mnium cuspidatum* (Hedw.) T. Kop., –, –, U87082, –, –. *Ophioglossum engelmannii* Prantl, –, –, L11058, –, –. *Osmunda cinnamomea* L., –, –, D14882, –, –. *Pellia epiphylla* (L.) Corda, –, –, AY688787, –, –. *Phaeoceros microsporus* (Steph.) Hässel, –, –, JX872446, –, –. *Phylloglossum drummondii* Kunze, –, –, Y07939, –, –. *Physcomitrella patens* (Hedw.) Bruch & Schimp., –, –, X74156, –, –. *Pinus wallichiana* A. B. Jacks., –, –, X58131, –, –. *Plagiochila porelloides* (Torr. ex Nees) Lindenb., –, –, AY699998, –, –. *Polytrichum commune* Hedw., –, –, U87087, –, –. *Psilotum nudum* (L.) P. Beauv., –, –, U30835, –, –. *Rhytidiadelphus loreus* (Hedw.) Warnst., –, –, AB024666, –, –. *Selaginella articulata* (Kunze) Spring, –, –, AJ295894, –, –. *Selaginella caffrorum* (Milde) Hieron., –, –, AF419070, –, –. *Selaginella deflexa* Brack., –, –, AF093253, –, –. *Selaginella diffusa* (C. Presl) Spring, –, –, AJ010852, –, –. *Selaginella echinata* Baker, –, –, AF419071, –, –. *Selaginella fragilis* A. Braun, –, –, AJ295872, –, –. *Selaginella gracillima* (Kuntze) Alston, –, –, AJ010844, –, –. *Selaginella haematodes* (Kunze) Spring, –, –, AJ010846, –, –. *Selaginella helvetica* (L.) Spring, –, –, AB574644, –, –. *Selaginella indica* (Milde) R.M. Tryon, –, –, AF419052, –, –. *Selaginella kraussiana* (Kunze) A. Braun, –, –, AJ010845, –, –. *Selaginella lepidophylla* (Hook. & Grev.) Spring, –, –, AF419051, –, –. *Selaginella leucobryoides* Maxon, –, –, AF419068, –, –. *Selaginella lyallii* (Hook. & Grev.) Spring, –, –, AJ295898, –, –. *Selaginella oregana* D.C. Eaton, –, –, AF419066, –, –. *Selaginella peruviana* (Milde) Hieron., –, –, AF419087, –, –. *Selaginella polymorpha* Badré, –, –, AJ295900, –, –. *Selaginella pygmaea* Alston, –, –, AJ295892, –, –. *Selaginella remotifolia* Spring, –, –, AB574650, –, –. *Selaginella rupestris* (L.) Spring, –, –, AF093255, –, –. *Selaginella rupincola* Underw., –, –, AF419083, –, –. *Selaginella selaginoides* (L.) P. Beauv. ex Mart. & Schrank, –, –, AF419000, –, –. *Selaginella sibirica* (Milde) Hieron., –, –, AF419076, –, –. *Selaginella sulcata* (Desv. ex Poir.) Spring ex Mart., –, –, AJ295887, –, –. *Selaginella tamariscina* (P. Beauv.) Spring, –, –, AJ295861, –, –. *Selaginella uliginosa* (Labill.) Spring, –, –, AJ010843, –, –. *Selaginella utahensis* Flowers, –, –, AF419067, –, –. *Sphagnum palustre* L., –, –, AF231887, –, –. *Takakia lepidozioides* S. Hattori & Inoue, –, –, GU295868, –, –. *Tetraphis pellicida* Hedw., –, –, U87091, –, –. *Welwitschia mirabilis* Hook. f., –, –, AJ235814, –, –.

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