Phylogeny and macroevolution in Isoetes (Isoetales)

Eva Larsén
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Abstract
This thesis has focused on the evolutionary history of Isoetes (Isoetales, Lycopsida), its phylogeny, megaspore morphology and evolutionary path through deep time. With a broadened sampling of species to include more of the worldwide diversity of the genus compared to previous work an unexpected sister to the rest of the genus was found. Isoetes wormaldi Sim is a critically endangered species from the southeastern Cape region in South Africa, which grows in ponds and slow-moving streams. There are only a few, small populations, and they are very sensitive to habitat changes. The rest of Isoetes is divided into five major clades, which show complex and not readily understandable biogeographic patterns, and by which processes the species came to live in their current locations in the world is often a conundrum. The age of the extant Isoetes is intriguing as it could potentially explain some of the baffling geographic distributions. Analyses based on whole chloroplast genomes and nuclear cistrons found that the choice of clock model and which genome dataset the dating analysis is based on matter greatly when trying to date the genus as the results were highly inconsistent. The solution to the dating analysis woes might be dependent on finding new bases, e.g., fossil evidence, for age calibrations which would require the morphology of Isoetes to be better understood. Our study of megaspore morphology found substantial differences among species of Isoetes in both ornamentation and surface texture/structure. While no major clade within the genus could be unambiguously defined by their spore morphology there are some characteristics of smaller clades and patterns of ornamentation and surface texture across the phylogeny. Our tentative hypothesis is that a pustulate megaspore ornamentation and a cobwebby texture are ancestral features in the megaspore of Isoetes.

Keywords: Bayesian inference, biogeography, Cenozoic, genomic data, fossil calibration, Isoetes, Isoetales, megaspore morphology, Mesozoic, molecular clocks, node ages, phylogeny, rhizomorphic lycopsids, scanning electron microscopy.

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List of papers

This thesis is based on the following papers, which are referred to in the text by their respective roman numerals:


Author contribution:

Paper I: EL and CR designed the research. EL carried out the laboratory work, assembled and analysed the data. EL and CR wrote the manuscript.

Paper II: EL, NW and CR designed the research. EL and AK carried out the laboratory work, EL assembled the data, EL and NW analysed the data. EL and CR wrote the manuscript with comments from NW and AK.

Paper III: EL, NW and CR designed the research. EL, AK and NW carried out the laboratory work. EL and NW assembled and analysed the data. NW and CR wrote the manuscript with comments from all co-authors.

Paper IV: EL and CR designed the research. EL carried out the scanning electron microscopy work, AK carried out the molecular laboratory work, EL assembled and analysed all data. EL wrote the manuscript with comments from CR and AK.
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Abbreviations

CDS – Coding sequence
DNA – Deoxyribonucleic acid
ILN – Independent lognormal (clock) model
NGS – Next generation sequencing
SEM – Scanning electron microscopy
TK02 – Brownian motion (clock) model described by Takeshi and Kitano (2002)
WN – White noise (clock) model
Introduction

It has always fascinated me to know that everything that lives on this earth has the same origin. Life as only appeared once (successfully) on our planet, all organisms on earth are therefore related to each other and yet there is such a diversity of form and function. If it wasn’t for life the Earth would just be minerals, water, and gases, and even what we may think of as inert geology is fundamentally altered by the presence of life. Life affects our climate and the cycles of chemical substances such as water, carbon and phosphorus. The early invention of photosynthesis can be seen as a red band in minerals where the suddenly freely available oxygen caused iron to oxidise into rust (Lyons et al., 2014) some 4 billion years ago.

Ever since life appeared on Earth it has existed as communities, as webs and connections between organisms and species. As much as it is easier to focus on one species at a time, none ever existed alone, but always as part of an ecosystem.

The first forests on Earth were communities without any seed plants. It is hard to imagine isn’t it, a forest without any flowering trees and no gymnosperms either. Those forests are now long gone and their remains are found in what is known as Coal forests (Cleal and Thomas, 2005). But some of their descendants live on as the slightly less tree-like Equisetum (horsetails) and lycophytes (dubmosses, firmosses, spikemosses and quillworts).

So how do we know anything about what life on Earth was like before we as a species existed and could observe it?

Fossils

A fossil is preserved remains or an impression of a life form from a previous geological age. There are several different ways it can be formed and in order to assess it accurately one must keep in mind how it was formed. It is rarely still composed of the same tissues as when it lived. The fossil can be preserved through compression, impressions, molds or casts, coalifications, permineralizations and petrifications and rarely as relatively unadulterated plant material (in for example amber or peat) (Taylor et al., 2009). The different preservation modes are of course limited in what information they give about the plants, and they also preserve different characteristics. It can be difficult to compare plants that are preserved in different ways so there are likely to be fossils that are described as different species just because of the preservation state. But very few individuals get preserved as fossils compared to everything that has lived on earth. The earth has a very efficient and complex recycling system for natural tissues and most plant material is decayed by aerobic bacteria and fungi. To evade this efficient composting process the organism remains need to somehow end up in an anaerobic environment, where organisms that need oxygen can’t reach it. But there are also decaying bacteria that live in anaerobic environments and for example produce methane at the bottom of lakes and in marshes. For the anaerobic decay to be limited the acidity must be high in the sediments. So it is exceedingly unlikely that any organism becomes a fossil, and should it happen it’s most likely to be one that lived in the sea, in a lake, in a flooded environment or possibly upstream from one
(Burnham et al., 1992; DiMichele and Gastaldo, 2008; Gastaldo and Demko, 2010). This makes the fossil record not only very meagre in both temporal and geographical coverage, it also makes it very biased since it favours certain environments and taxonomic groups. It is a poor proxy for historical global diversity including plant diversity (Looy et al., 2021) but the only one we have.

But scientists must be able to find the fossils as well to study them, and fossils are found wherever a fossil-bearing strata of minerals are laid open, such as mines, roadworks or simply naturally outcropping rocks initially formed by geological processes with subsequent erosion by water or wind. When fossils are found they are often difficult to reconstruct as living specimens, hampered both by the human difficulty of understanding the form of a long dead specimen, often even an extinct species, and by the fossil's imperfect and often partial preservation. "Extensive disarticulation and typically far from perfect preservation render paleobotany the most challenging of all disciplines from a taxonomic perspective [...]" (DiMichele and Bateman, 2020).

**Dating analyses**

Knowing species divergence times of clades that contained extinct or ancestral groups is not only useful to evolutionary biologists in establishing the age of a certain species group, but also particularly important for addressing a range of biological questions. Having this knowledge of evolutionary divergences allows us to place speciation events in the correct geological and environmental contexts and to gain a better understanding of speciation and dispersal mechanisms. It also allows us a perspective on the variation of species richness and species diversification rates over geological periods.

As we have seen, very few groups are likely to have a fossil of a close relative preserved. But we are still interested in the evolution and timescales of such groups, so can anything be done?

The molecular clock hypothesis that was proposed by Zuckerkandl and Pauling (1965), provides a theoretical and powerful approach to estimating divergence times. If the time of divergence between any pair of species is known, then the rate of molecular evolution can be inferred and used to date the timing of divergence between other species pairs. Under a strict clock assumption, the distance between sequences grows linearly with time, so that if the ages of some nodes are known based on the oldest fossil record from one of the pair of lineages, then the absolute rate of evolution as well as the absolute geological ages for all other nodes on the tree can be calculated (Donoghue and Yang, 2016).

This strict clock is of course making some drastic assumptions about the evolutionary rates of change across a phylogeny, it isn’t for example very likely that mutation rates have been constant over time and across the tree of life, but analysing methods are becoming increasingly sophisticated to allow estimates to be calculated using the information we have. In the potentially vain hope for new fossils to be found it provides an opportunity to make a guess that is as well-founded as possible. It could also be argued that analyses of divergence times of clades can be a way to investigate this research question using other data than the fossil record, namely molecular data. But at least one node in the tree has to be calibrated to absolute time, something that is typically done using fossil information although other sources of information could be used as well (e.g., (Hipsley and Müller, 2014).
Rhizomorphic lycopsids

The first forests that were mentioned in the introduction consisted to a large extent of isoetalean lycopsids. They have an extensive evolutionary history over vast geologic time as they already existed in the Ludlow in Silurian (Kotyk et al., 2002). They were widespread during the Late Mississippian and most of the Pennsylvanian and formed the dominant group of plants in most of the vast equatorial coal-swamp ecosystems of central and western Pangea; they were consequently the foundation for the formation of Carboniferous coal seams around the world (Phillips and Peppers, 1984). The abundant and well-preserved fossil specimens of this group are a biproduct of widespread coal-mining operations that have uncovered the Carboniferous sediments these plants became embedded in (Taylor et al., 2009).

The rhizomorphic lycopsids were not just trees; included in the group are also species with growth habits varying from small shrubs and sprawling forms to more tree-like forms (Bateman, 1992; Bateman and DiMichele, 1991). But the tree forms must have been truly majestic, exceeding 30 m in height (Thomas and Watson, 1976) and more than 2 m in basal stem diameter (DiMichele et al., 2022). These plants had long microphyllous leaves attached to characteristic diamond- or hexagonal-shaped leaf cushions, thick secondary cortical tissues, well developed stigmarian rooting systems and often monosporangiate cones with reduced numbers of megaspores (Bateman et al., 1992; Pigg, 2001).

The ecology of these plants has been extensively studied, and many constitute the focal point of ancient landscape reconstructions in natural history museums around the world.

Heterospory

Heterospory is a morphological quirk that is one of the links between the older rhizomorphic lycopsids and extant Isoetes, but it is also a characteristic of the closest living sister to Isoetes, Selaginella of the order Selaginellales. While it is likely a synapomorphy in the heterosporous lycophytes it has also evolved separately in at least two groups of ferns as well as, perhaps most famously, in seed plants.

The concept heterospory can encapsulate several distinct parts, as pointed out by Bateman and DiMichele (1994); sensu stricto it just refers to the produced spores being different. This could be used as meaning that the spores are just of different size but from the size difference there is also often an assumption of different genders of the spores and resulting gametophytes, i.e. dioicy. Some other concepts that are of importance when studying the evolution of heterospory is heterosporangy, when the spores are produced in separate sporangia, and endospory, when the gametophyte matures still inside the spore wall.

Heterospory has evolved a minimum of 8-11 times according to Bateman and DiMichele (1994), although now that Salvinia and Marsilea are in the same order (Smith et al., 2006) the lower number might be more likely. This indicates that heterospory can easily evolve but once a lineage becomes heterosporous it stays heterosporous as no reversal has been recorded (Bateman and DiMichele, 1994). But heterospory is not necessarily adaptively advantageous, it
may represent an adaptive valley rather than an adaptive peak (Chaloner and Pettitt, 1987; DiMichele et al., 1989).

Heterospory is hypothesized to have evolved in aquatic environments (Bateman and DiMichele, 1994; Kar and Dilcher, 2002). Bateman and DiMichele (1994) note that extant heterosporous lineages are most effective in aquatic and amphibious environments if they are sexually reproducing, other plants that are also very successful in these habitats rely on asexual apomictic life histories. Studies that were primarily based on Selaginella suggest that it might only be an indirect reason that it is favoured in aquatic environments, it might be more to do with being shaded which favours large nutrient reserves in spores (Petersen and Burd, 2018).

Bateman and Dimichele (1994) include a paragraph that discusses evolution in terms of a power struggle between the sporophytic and gametophytic generations and words like control, dominance and antagonism are mentioned. But I find it hard to imagine an evolutionary reason for either generation striving to control the other just for the sake of having power. While evolving together they are both dependent on the other generation, they are parts of a whole, and it’s either a hindrance to be separated or it is a boon to be able to adapt to two very different niches but in either case they are both advancing the same DNA. The move to a reduced gametophyte, which can develop fully inside it’s protective cover (the spore wall), strikes me more like an advantage, a way to persevere in otherwise unsurvivable conditions, than a sign that all control has been removed from this generation. After all, it is the gametophyte that must decide when it is a good time to spawn a new sporophyte.

Looy et al. (2021) argue that is it precisely the possibility of surviving as a spore that is rich in carbohydrates and lipids (and potentially can stay viable for 100 years in lake beds), matched with a slow growth rate and few demands on the surrounding habitats that allowed the isoetalean lycophytes to flourish when other taxa declined due to environmental perturbations. Being able to grow in nutrient-poor, largely inorganic sediments would have facilitated colonization of erosional landscapes, where more competitive taxa with greater nutrient demands would be slow to colonize (Looy et al., 2021).

**Isoetes**

There are around 200 species of *Isoetes*, the only genus within the family Isoetaceae and the only remaining representative of the rhizomorphic lycopsids (Isoetales sensu DiMichele and Bateman (1996)). The plants have a deceptively simple anatomy, appearing as a short stem which may be two- or three-lobed, covered by a tuft of elongated, linear, and smooth leaves (figure 1). The leaves have air channels, usually 4, and a sporangium sunken into the base, which in some species is covered by a velum. A small ligule sits burrowed into the leaf surface above the sporangium. Being heterosporous the sporangium of a fertile *Isoetes* leaf is either a microsporangium producing many monolete microspores or a megasporangium producing fewer and much larger trilete megaspores.

*Isoetes* shares some characteristics with extinct taxa of the same order such as pseudobipolar growth from a shoot-like rootstock, stigmarian root systems with dichotomizing roots and leaflike lateral rootlets, and secondary xylem produced by a unifacial cambium (DiMichele and Bateman, 1996; Kenrick and Crane, 1997).
Figure 1. Isoetes morphology. A – a whole plant (*Isoetes echinospora*), B – megaspores (*Isoetes schweinfurthii*), C – close up of stem (*Isoetes echinospora*), D – microspores (*Isoetes wormaldii*), E – quill-like leaves (*Isoetes lacustris*), F – megaspore with microspores attached to its distal side (*Isoetes andina*). Scale bars: 50 µm.
Species of *Isoetes* occur fully submerged or seasonally inundated, although a few terrestrial species also exist (Hickey, 1986a). They are very difficult to identify to species, partly because of the morphological simplicity but also because of inferred homoplasy and reticulate evolution (Taylor and Hickey, 1992).

**Isoetes as I found it**

The challenges in *Isoetes* research were manifold at the start of the work with this thesis. There had been few phylogenetic studies made and there was a need to increase the sampling, both of species, geographical areas, and genomic markers. The fundamental relationships and the backbone of the phylogenetic tree were enigmatic, as one of the latest studies then (Schuettpelz and Hoot, 2006) felt obliged to recommend settling for a basal trichotomy. It was also unclear how to connect the extant genus to their older isoetalean relatives. And while part of that problem relates to gaining a more complete understanding of the morphology and how it has changed through evolutionary time another question was how old the extant genus might be. An age estimate would put the biogeographical patterns into context and make plausible explanations possible to establish.
Aims

The overarching aim of this thesis is to produce a representative phylogeny of *Isoetes* (Isoetales, Lycopsida), describe the patterns of selected morphological characteristics, as well as sketch the evolutionary path the extant genus has taken through deep time.

**Paper I**

Main aims are to find a consistent rooting strategy and construct a phylogeny of *Isoetes* using newly produced as well as already separately published sequences of nrITS, *rbcL* and the *atpB*-*rbcL* spacer for a substantially increased sample of taxa compared to previous work, and finally to make a first dating analysis for the genus based on molecular data.

**Paper II**

Main aims are to improve phylogenetic results in *Isoetes* by increasing the sampling of specimens yet more with a particular focus on African species, and to use (and generate) a substantially increased set of molecular markers that are phylogenetically informative in the group. Another aim was to describe and discuss patterns of geographical distributions in the genus.

**Paper III**

Main aims are to investigate divergence times of clades in *Isoetes* and test the congruence of results when using three different clock models, as well as using entire chloroplast versus nuclear cistron DNA.

**Paper IV**

Main aims are to describe megaspore characteristics in *Isoetes* in a phylogenetic framework and explore possible clade characteristics and general evolutionary patterns.
Materials and methods

Plant material and taxon sampling

It was our aim from the very beginning to broaden the phylogenetic sampling of Isoetes species to include more of the worldwide diversity of this cosmopolitan genus. It seemed to us that there was a lot of high-quality regional work being done (Hoot et al., 2004; Kim et al., 2010; Taylor et al., 2004) but only a few studies had addressed the genus as a whole (Hoot et al., 2006; Rydin and Wikström, 2002; Schuettpelz and Hoot, 2006) and a broader species representation was clearly needed to facilitate investigations of its global evolutionary history. The majority of the DNA sequences used in this thesis was obtained from herbarium specimens and I would like to express a deep gratitude to the herbaria BM, BR, C, GB, L, MEL, NY, P, S, W, WU (Thiers, 2023), that generously allowed us access to their scientifically important and irreplaceable collections. In paper I sequences from previously published studies that were available from GenBank were complemented by our own sampling that filled in some blanks regarding the distribution of Isoetes across the world. GenBank sequences were also used to provide the land plant backbone for the dating analysis.

Papers II and III were worked on in parallel. The samples that were sent for Next Generation Sequencing (NGS) were chosen to broadly encompass the phylogenetic diversity of the genus. Twenty-five outgroup terminals were used to represent the other major land plant groups. After having assembled complete chloroplast genomes of a selection of Isoetes species, it was possible for us to find hitherto unanalysed DNA regions with high phylogenetic information across the phylogeny and these regions were used to construct the phylogeny in paper II. Through the broadened taxon sampling of for example African specimens the phylogenetic position of Isoetes wormaldii was found and it became a late addition to the NGS study (paper III).

In paper IV the goal was to study the megaspore morphology in the evolutionary context of the genus so the exact specimens should ideally have been part of the previous phylogenetic studies and placed without ambiguity. Additional, carefully selected, specimens were studied in some cases, i.e., when spores from the sequenced specimen were in a bad state, or the ornamentation unclear, as well as when spores were lacking altogether for a sequenced sample and when additional material was needed to confirm the results. For each utilized sample, spores were collected from a single sporangium from the herbarium dried specimens. Work on pollen has shown that dried material is still similar to fresh, while treatments with alcohol may alter its shape (Bolinder et al., 2016; Norbäck Ivarsson, 2013). We wanted the spores to retain their natural shape and size, and our test using freshly collected material of Isoetes lacustris showed that freshly sampled spores where identical to those extracted from old herbarium samples of the same species.
Laboratory work

DNA

For all studies extraction of total genomic DNA was done according to the cetyltrimethylammonium bromide (CTAB) method (Doyle, 1991; Doyle and Doyle, 1987), and purified using a QIAquick PCR Purification Kit (Qiagen, Sweden). For paper I, II and IV Sanger sequencing was performed by the Macrogen Sequencing Service (Amsterdam) and new primers were designed in order to successfully amplify the chosen molecular markers. In paper I, 49 new nrITS and \textit{rbcL} sequences were produced. In paper II, 702 new sequences of the markers nrITS, \textit{ndhC}-\textit{ndhK}, \textit{rbcL}, \textit{rpoC1}, \textit{ycf1}, \textit{ycf66} and \textit{trnVUAC} and its subsequent spacer were produced, and in paper IV seven new nrITS sequences were produced to complement already existing molecular data (produced during the work with paper II).

Paper III utilized a genome skimming NGS method. The sequencing was performed at the Science for Life Laboratory (SciLifeLab, Stockholm, Sweden) following the manufacturer’s instructions for the Illumina HiSeq. 2500 platform (Illumina, San Diego, CA, USA). Pair-end runs with 350-bp insert size fragments and 2 × 125 bp read lengths were performed.

SEM

Megaspores from 74 samples, representing 59 species of \textit{Isoetes} were attached to aluminium stubs with double sides tape, sputter-coated with gold and examined with a Hitachi-TM3000 SEM (Stockholm University). The spore description terminology essentially follows Hickey (Hickey, 1986a), but with some modification and additions in order to accurately describe the megaspore morphology.

Sequence assembly and alignment

In paper I sequences were assembled using software Staden (Staden, 1996; Staden et al., 1999) and aligned by eye using Se-Al sequence alignment editor (ver. 2.0; ©1996–2001, Andrew Rambaut).

In papers II and IV sequences were assembled with Geneious version 9.1.8 (Kearse et al., 2012) and version 2022.2.2 respectively, and aligned using the software MAFFT v. 7 (Katoh and Standley, 2013) with the algorithm G-INS-I with a variable scoring matrix and subsequent corrections by eye.

In paper III single raw reads from the Illumina sequencing were set in pairs, merged using the BBmerge function, and low-quality nucleotides were removed with the error probability limit 0.05 in Geneious v.10.2.6 (https://www.geneious.com). A reference-guided genome skimming approach was used to assemble the reads (Straub et al., 2012). Plastid sequences were collected from the original reads using a BLAT (BLAST-like alignment tool v36, (Kent, 2002)) search of forward and reverse reads against a reference database initially made up of the complete plastid genome of \textit{Isoetes flaccida} (NC_014675, (Karol et al., 2010)), but assembled plastids were successively added to the database once completed. Forward and reverse reads were extracted if they showed at least 70% similarity to any of the reference genomes. Following the BLAT search, reads were compiled from the original fastq data files using pulseq v.1.0.1 (github.com/bcthomas/pulseq) and made into new forward and reverse plastid data files. De novo assembly of this plastid subset of reads was executed for each taxon using ABySS v.2.3.4.
(Jackman et al., 2017; Simpson et al., 2009) and seven k-mer lengths (55, 61, 67, 73, 85, 91, 97). Generated contigs were pooled and mapped onto a reference genome using bwa v.0.7.17-r1188 (Li and Durbin, 2009) with the plastid genome of *Isoetes flaccida* (NC_014675) used as reference, resulting in complete or almost complete draft genomes. All original reads were subsequently mapped onto the draft genomes using bwa v.0.7.17-r1188 to fill unfinished gaps and evaluate sequencing depths. Generated assemblies were reviewed and edited using gap5 from the Staden Package (Bonfield and Whitwham, 2010; Staden, 1996; Staden et al., 1999).

The nuclear ribosomal DNA (rDNA) cistrons were assembled in an analogous way to that used for the plastids except that it was a bit more complicated to acquire a reference sequence. An initial reference sequence including partial external transcribed spacer (ETS), 18S gene, internal transcribed spacer 1 (ITS1), 5.8S gene, internal transcribed spacer 2 (ITS2), and 26S gene sequences was constructed based on the *Isoetes* sp. (PYHZ) transcriptome from the One Thousand Plant Transcriptomes Initiative (One Thousand Plant Transcriptomes Initiative, 2019). The transcriptome sequence was aligned against a complete 18S sequence from *Isoetes durieui* (Kranz and Huss, 1996); ITS1, 5.8S, and ITS2 sequences from *Isoetes olympica* (Bolin et al., 2011); partial 26S sequence from *Selaginella selaginoides* (Korall and Kenrick, 2004); and an rDNA cistron sequence of *Asclepias syriaca* (Straub et al., 2011).

**Phylogenetic analyses**

Bayesian analyses of phylogeny were run in papers I, II and III using software MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Paper I also used parsimony analysis (in PAUP* ver. 4.0; Swofford et al., 2003). In paper I model selection was performed using the corrected Akaike criterion, the Bayesian information criterion and, as third strategy, the reversible-jump Markov chain Monte Carlo which is a model-averaging method. Two partitioning schemes (nuclear vs plastid or each marker as a separate partition) were tried and selected based on most resolved tree. In paper III plastid protein-coding genes were concatenated into a CDS set, partitioned by codon position, and the model for each partition was chosen based on the corrected Akaike criterion both for the plastid CDS data and the nuclear rDNA data. Convergence was assessed with Tracer v 1.7 (Rambaut et al., 2018) in all three papers and additionally with AWTY (Nylander et al., 2008) in paper I.

Papers II and IV used ModelFinder (Kalyaanamoorthy et al., 2017) as implemented in the IQ-TREE web server (Trifinopoulos et al., 2016) to find the best fitting models and partitions (Chernomor et al., 2016). And maximum likelihood analyses were conducted on the IQ-TREE web server (Trifinopoulos et al., 2016). Statistic support was estimated using Ultrafast bootstrap (Hoang et al., 2018) as implemented in IQ-TREE 2 (Minh et al., 2020).

**Rooting analysis**

Rooting the phylogeny had been an issue in *Isoetes* (Schuettpelz and Hoot, 2006) due to their dissimilarity to their closest extant relatives, the Selaginellaceae, and probably also as a result of the few available and informative genetic regions. In paper I we used three different methods to find an acceptable root: outgroup analysis (with 38 species of *Selaginella*, (Farris, 1972)), midpoint rooting (Farris, 1972) and molecular clock-based rooting (in BEAST, (Drummond et al.,
A simplified dataset with as little missing data as possible was run in both parsimony and Bayesian frameworks.

## Dating analyses

### BEAST

In paper I, the software BEAST (Drummond et al., 2012, 2006) was used to run a dating analysis with 43 species of *Isoetes* and 65 outgroup species representing land plants. The fit of the different models to data were tested with path sampling and stepping stone sampling. Three clock models implemented in BEAST were applied, one strict clock, two relaxed: uncorrelated clock rates drawn from a lognormal distribution, and random local clocks. Two different tree priors were tested: 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 markers (two partitions), and a single partition for all data. The 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 and were used in the final analyses, which were run using the GTR model and estimated base frequencies.

Three prior distributions for the age-calibrated nodes were compared: normally, lognormally, and uniformly distributed, additionally runs were performed without the data to ensure that priors did not interact with each other (Heled and Drummond, 2012). Uniformly distributed age priors yielded the best log marginal likelihood scores and were in the final analyses assigned to nine nodes on the basis of fossil information.

### MrBayes

In paper III, plastid CDS and nuclear rDNA data were analysed separately with three different relaxed clocks implemented in a development version of MrBayes v.3.2.7a (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The clock models were the independent lognormal model (ILN, (Drummond et al., 2006)), the white noise model (WN, (Lepage et al., 2007)), and the Brownian motion model described by Thorne and Kishino (TK02, (Thorne and Kishino, 2002)).

Two fossil calibrations were specified as uniform prior distributions, one for the root of the phylogeny (the crown group land plants) and one for the split between extant Selaginellaceae and Isoetaceae. To enhance comparison of results, the fossil-based age constraints were chosen to align with those used by Wood et al. (2020).
Main results and general discussion

Paper I found that *Isoetes* is divided into five major clades and that a consistent rooting solution is rooting on clade A, i.e., clade A is sister to the remaining genus. This pattern was also confirmed with more data per taxon in paper III. However, papers II and III additionally showed that there was a sister outside those five clades: the rare and poorly investigated South African endemic *Isoetes wormaldii* is sister to the rest of the genus.

Phylogeny and biogeography

Since the species of *Isoetes* differ very little from each other both morphologically and in DNA sequence divergence one might assume that they are a very young group of plants, despite their old heritage. But that view is, at the very least, challenged when taking into consideration the biogeographical patterns of this genus of lycophytes.

The overall phylogeny of *Isoetes* is not easily translated into evident biogeographic patterns and processes (figure 2). Species from southern tropical Africa fall into five major clades, Indian species are resolved in three major clades, Australian and tropical Asian species are present in three and two clades, respectively, and South American species occur in at least three clades. It is a similar case for the Northern Hemisphere as European and North American species are each placed in at least three major clades.

Within clade A, there is a geographical spread which cannot easily be explained by simple dispersal. The members of this clade live in southern and tropical Africa, India, mainland Southeast Asia, South America, and Australia. It’s a distribution that has been referred to as a Gondwana-distribution.

A group of South African species are sisters to the remaining species in clade A: *Isoetes capensis, I. stellenbosiensis, I. stephanseniae* and *I. toximontana*. Several of them are seriously threatened, either declining or critically endangered. *Isoetes australis* from western Australia is not closely related to other Australian species and differs from them with a potentially paedomorphic anatomy (Williams, 1944). It also has a distichous phyllotaxy, which makes it unique in the genus. A diverse and geographically spread clade of South American and Central American species occurring from Cuba and Mexico in the north to Argentina in the south is also included in clade A. This clade is sister to a clade of Indian/tropical Asian/Australian species plus a mostly tropical African clade. It was a surprising find that a West tropical African species is nested within the otherwise Asian/Australian clade: *I. melanotheca* from Senegal. This species has to our knowledge not been included in previous phylogenetic work and its position should be confirmed with additional representatives of the species than the single sample used here.

It is hard to guess by what processes the geographical spread has come to be in subclade A. In the entirely African clade are a number of species from southern (and tropical) Africa and Madagascar (i.e., *I. welwitschii, I. schweinfurthii, I. jaegeri, I. nigritiana, I. pitotii, I. abyssinica, I. rhodesiana*), some of which are quite widespread as currently circumscribed. Our results indicate that *I. kersii* (the position of sample EL035) is included as well. But the phylogeny and species delimitations of African species of *Isoetes* need more research and presumably some alpha-taxonomic revision.
Figure 2. The geographical distribution by country of the *Isoetes* specimens sampled for this thesis. *Isoetes wormaldii* is endemic to South Africa and only present in a few localities in the Eastern Cape Region. Clade A has a broad distribution in the Southern Hemisphere and has repeatedly been referred to as the Gondwana clade. Clade B has an even broader distribution in vast areas of Europe, western North America, Africa and India. Clade C is restricted to Italy (*I. malinverniana*) and Turkey (*I. anatolica*). Clade D is distributed in South Central to South East Asia, and Oceania. Clade E is broadly distributed in the Northern Hemisphere and South America. Note that the map only shows countries of origin of samples included in my work. In some cases it would be possible to estimate the total distribution of these subclades of *Isoetes* based on our results; it is for example highly likely that clade E has representation in nearly all European countries. However, in view of the history of phylogenetic analysis in the genus, it might be risky to assign any uninvestigated species to a clade as there have been so many surprising discoveries and unintuitive relationships in *Isoetes* studies. Maps created using MapChart (www.mapchart.net).
Clade B has a nearly worldwide distribution with representatives from the Mediterranean region, North America, India, and southern (to tropical) Africa and Madagascar. The European species of clade B are resolved in two groups that correspond respectively to the Isoetes histrix group and the Isoetes longissima group of Troia et al. (2019). The biogeographical history of clade B is not straight forward to understand. Based on the phylogenetic pattern we speculated in paper I that clade B is the Laurasian equivalent to the (possibly) Gondwanan clade A. However, the inclusion of a greater sample of African species and additionally an Indian species in clade B in paper II would rather point to a Pangean origin of the clade, if we assume that vicariance is the main biogeographic process causing the pattern. But that is refuted by the hereto estimated crown age for clade B of the earliest Paleogene in paper I, or younger as estimated by Wood et al (2020) (but see also below on the difficulty in resolving node ages in Isoetes as shown in paper III). There are also more recent dispersal processes evident in the clade, such as between southern Africa and Madagascar, and between Europe and northern Africa possibly mediated by migrating birds and/or sea currents. This is what you might expect to find in a living genus, since dispersal events should be assumed to happen constantly, also in modern times. But the large-scale phylogenetic pattern in clade B may potentially indicate an older clade with substantial extinction, for example of elements of the early Cenozoic Tethys flora as discussed in paper I.

Clade C is a small clade consisting of the sisters I. malinverniana and I. anatolica is sister to the much larger clade D+E. The Italian endemic I. malinverniana is endangered and the focus of some promising conservation projects (Abeli et al., 2020).

Clade D comprises two larger subclades, one mainly Australian and the other including species from the eastern and southern Asia. In the mostly Australian clade, we find most of the Australian Isoetes, the only exceptions residing in clade A (I. australis and I. coromandelina subsp. macrotuberculata). But here are also the two species from New Zealand and perhaps more oddly an Indian species: I. sampathkumaranii. The eastern and southern Asian subclade includes species from China, Japan, Philippines, and Papua New Guinea. Except for the inclusion of the Indian species the biogeography of clade D is surprisingly understandable, for being Isoetes.

The two Andean species I. andina and I. andicola are strongly supported as sister to the remaining clade E. This result is interesting because I. andicola was once placed in its own genus Stylites based on its peculiar stem morphology (Amstutz, 1957). The remaining clade E comprises a substantial diversity of American species, and in addition some species with circumboreal distribution extending through Canada, Greenland, Scandinavia, and Russia. But poor resolution prevents us from drawing conclusions concerning relationships or evolution in this clade. The muddied phylogeny may at least partly be a result of a high prevalence of polyploids/hybrids and subsequent reticulate evolution, which appear common in Isoetes, at least in American species (Suissa et al., 2022) but may possibly also be due to a lack of suitable (sufficiently informative) molecular markers for clade E. Even so, it is a surprising result that it is not possible to see clades of South and Northern America separated from each other.

An unexpected sister to the remaining Isoetes

Isoetes wormaldii was unexpectedly shown in paper II to be sister to the rest of Isoetes. It is known only from a few localities in the Eastern Cape region of South Africa and is extremely rare, decreasing, and critically endangered (Victor and Dold, 2003). It grows submerged in
freshwater ponds and slow flowing streams in very small groups, only comprising 10-15 mature plants. Populations are reported to disappear when cattle grazing ceases and consequently it is sensitive to exploitation of habitats and has strongly declined due to agricultural cultivation expansion and urbanization (Victor and Dold, 2007). However, spores may germinate after years of dormancy, since new plants suddenly can reappear after disappearing for six years (Victor and Dold, 2007). The species was formally described by Thomas Robertson Sim in 1905 and named after W. H. Wormald who first discovered the plant in 1893 in ponds around East London, South Africa (Sim, 1905).

The leaves are relatively long for *Isoetes*, growing up to 45 cm and floating on the surface of water (Sim, 1905). According to the original description the leaves were said to have three veins, one central and two marginal, but this was clearly a misinterpretation (since lycopod leaves are microphyllous with a single unbranched vein) and subsequent work showed that leaves of *I. wormaldii* have no more than a single central vascular strand (Duthie, 1929). The leaves have characteristics that are unusual for *Isoetes*, they are “somewhat flattened” in transverse section and “hardly narrowed to the rounded point” (Sim, 1905). *Isoetes* leaves are generally described as subulate (Engelmann, 1882; Jermy, 1990), awl-shaped with reduced lamina (ala), but Hickey argued that a few South American species (*I. bacculata, I. bradei, I. gigantea*) and fossils of *Isoetites* have laminate leaves (Hickey, 1986b). The same is thus true for the South African *I. wormaldii*.

The megaspores of *I. wormaldii* are of the typical *Isoetes* type in that they are trilete with a distinct equatorial ridge, but they have an ornamentation type not seen elsewhere in the genus; foveolate. While superficially they might appear reticulate (with a light microscope for example), when viewed in SEM the walls are not formed like in reticulate ornamentation and the appearance is more as if small scoops have been taken out of a previously flat and uniform surface.

The microspores of *I. wormaldii* are monolete with a prominent proximal ridge and there are in addition two less prominent distal ridges. The microspores were said to be “3-ridged” in the original description (Sim, 1905), but this should not be misunderstood as a trilete form; all extant species of *Isoetes* have monolete microspores (Musselman, 2002), which also true for *I. wormaldii*. Trilete microspores occur in the living sister group of *Isoetes, Selaginella* (Wang et al., 2018), and in some extinct members of the isoeatalean lineage. Isoetaleans with trilete microspores existed for example in the Late Devonian *Clevelandodendron ohioensis* (Chitaley and Pigg, 1996), in the Triassic *Isoetes beestonii* (Retallack, 1997) and *Pleuromeia rossica* (Lugardon et al., 1999). Trilete microspores have rarely been reported in the Mesozoic in the Isoetales and an evolutionary trend from trilete to monolete microspores has been hypothesized (Bateman and DiMichele, 1994; Pigg, 2001, 1992). However, interpretations of spore evolution in the Isoetales might be complicated by the fact that the outermost layer of the spore, a silicified coat, may not survive fossilization (Neuburg, 1960; Pigg, 1992; Slog and Hill, 1992).

The age of *Isoetes*

The two ventures into dating the phylogeny of *Isoetes* have followed very different philosophies. In paper I the DNA data available was relatively meagre with only nrITS and the very conserved *rbcL* gene and the spacer between *atpB* and *rbcL*, but on the other hand the species sampling was comparatively large, and the outgroups were chosen to try to maximise the available fossil
calibration points. In paper III, NGS sequencing made much more data per specimen available (the entire plastid genome and the nuclear rDNA cistron) but for less species in total. Wood et al. (2020) and Periera et al. (2021) had published strongly deviating age estimates; in their analyses the crown group Isoetes was much younger compared to our results in paper I, so it interested us to follow a similar fossil calibration philosophy and use a slightly reduced taxon sampling but with much more data per specimen and then test the effect of different clock models. One important difference from previously mentioned papers is that we included Isoetes wormaldii in paper III and as sister to the remaining species in the genus it increased the average median-node depth to the Isoetes crown group by a factor of 2.55 (plastid data) or 1.95 (nuclear rDNA data).

In both papers I and III the confidence intervals of node ages are large. This might be expected when the studied group has a “broom”-distribution (a long stem branch and short crown group branches), but of course the median ages would appear less arbitrary with smaller intervals.

Furthermore, the age estimates retrieved in paper III were highly inconsistent. They differed between plastid and nuclear data and even more between analyses using different clock models. The clock models vary in how relaxed they are as concerns the assumption of rate homogeneity among branches in the tree. The independent lognormal (ILN) model (Drummond et al., 2006) draws uncorrelated rates from a lognormal distribution while the white noise (WN) model (LePage et al., 2007) is a continuous uncorrelated model of rate variation across lineages. The Brownian motion model (TK02) described by Thorne and Kishino (2002) in contrast relies on the concept of autocorrelation of rates at adjacent branches, in that way assuming that molecular clock rate is a heritable trait, which differs fundamentally from a model in which clock rates are considered uncorrelated and drawn from a distribution.

The autocorrelated TK02 model yielded the oldest median age estimates of the Isoetes crown group and both data sets yielded very similar age ranges (mid-Permian, 282 Ma, based on plastid data and Permian-Triassic border, 251 Ma, for nuclear rDNA data). The uncorrelated WN model yielded its oldest estimate with the nuclear data set but both analyses resulted in younger median node ages for the Isoetes crown group, and the ages differ more (latest Jurassic, 150 Ma based on plastid data and latest Triassic, 203 Ma, based on nuclear rDNA data). The uncorrelated ILN model provided the most recent median age estimates and also the greatest discrepancy between data sets (earliest Eocene, 54 Ma, based on plastid data and earliest Cretaceous, 134 Ma, based on nuclear rDNA data). Ingroup node ages vary in a similar way; for example, the median crown group age for clade D varies between 3 Ma (plastid data, ILN clock model) to 76 Ma (nuclear data, TK02 clock model). Our conclusion in paper III was that it is difficult to refute any of these results, and that finding evidence that can be used as a basis for a calibration point within Isoetes may be a way forward.

Spore morphology

There are substantial differences among species of Isoetes regarding the ornamentation and surface texture/structure of the megaspore. The critically endangered South African endemic Isoetes wormaldii, which was shown to be sister to the remaining Isoetes, has a unique foveolate megaspore ornamentation not seen or reported for any other species of Isoetes. This concurs with its position as standing out from other species in the genus as concerns its unusual leaf morphology and it being so molecularly divergent from the remaining genus that adding it to
phylogenetic analyses approximately doubles the average median node depth to the *Isoetes* crown group.

We intended to follow Hickey's terms for description of megaspore ornamentation (Hickey, 1986a) and we recognize nine of Hickey's 12 terms (baculate, cristate, echinate, levigate, pustulate, retate, reticulate, rugulate, and tuberculate), but we found a need to add three more (foveolate, spiculate and umbellate). The pustulate ornamentation was assessed as the most common megaspore ornamentation in *Isoetes*; it is most common in clade B, it is common in clade A and it is present in some species in the C-D-E clade. This contradicts conclusions in early work (Pfeiffer, 1922) where the majority of the species are defined as having tuberculate megaspore ornamentation, but the definition of tuberculate versus pustulate was refined by Hickey (1986a). The distinction between tuberculate (straight inclined sides with an acute apex) and pustulate (convex sides with an obtuse, rounded apex) ornamentation does not exist in Pfeiffer's work. As assessed in paper IV, actual tuberculate spore ornamentation sensu Hickey (1986a) and followed by us, occurs only in clade A.

While no major clade within *Isoetes* could be defined by their spore ornamentation there are some characteristics of smaller clades and patterns of ornamentation.

African, Indian and Australian specimens in clade A included in paper IV all have similar ornamentation, either pustulate or tuberculate and with a cobwebby surface structure. Within the South American subclade there is a greater variation, most spores have a baculate ornamentation and a surface texture described as network, but there are some more unique combinations such as the curly fibers of *Isoetes clavata* ELS121 or the extremely hairy *Isoetes pedersenii* ELS104.

In the two first clades that successionally separate away from the rest of Clade B there are some unique patterns, the ornamentation is either pustulate (*I. histrix*), retate (*I. durieui*) or spiculate (*I. orcuttii* and *I. nuttallii*), and the surface texture/structure is sponge-like, covered in thick thorns or bearing a remarkable resembles to melted cheese. Most of the remaining specimens in clade B, both African and European species, have a pustulate ornamentation and a hairy surface structure. Our tentative hypothesis is that a pustulate ornamentation is an ancestral feature in *Isoetes* and a hairy surface structure a synapomorphy of clade B, but neither of these results, nor the evolutionary conclusion, is unambiguous.

In clade C, which was only represented by *Isoetes malinverniana* in paper IV, we found a unique dimorphic megaspore ornamentation and the only occurrence of a cobweb spore surface texture outside of clade A. There are three low pustules closest to the proximal conjunction, each in their own radial area and the rest of the spore is covered in bacules that even on occasion blend into the equatorial ridge. This species also has an additional rough layer of unfinished appearance that covers the upper surface of the bacules and frequently connects them. Several specimens of *I. malinverniana* were studied to makes sure that the spores were representative and not showing an abnormal or unfinished state.

Clade D is separated into two well-supported subclades and their spore morphology also differ. The megaspores of the clade with representatives from Australia, New Zealand and India have either pustulate or rugulate ornamentation and their surface structure is hairy or occasionally a network of fibers. The diversity is greater in the clade with species representation from New Guinea, Philippines, Japan and China; its megaspores can have retate, reticulate, rugulate, or echinate ornamentation. Despite their diversity in ornamentation type they all have a similar surface structure consisting of a network of fibers.
Within clade E, the first clade to diverge from the rest of the clade is the sisters *Isoetes andicola* and *I. andina* who have a levigate and cristate spore ornamentation, respectively. In our data levigate megaspores, which are always also in possession of a dense and porous surface structure, only occur in clade E but it is likely that they also exist in its sister clade, clade D, in species we were unable to sample. The remaining species of clade E show ample diversity of morphology; pustulate, reticulate, rugulate, levigate and echinate ornamentations occur and surface structures are either hairy, dense, dense with pores or a network of fibers. But the evolutionary patterns are difficult to discuss as the phylogeny has proven challenging to resolve, most likely due to hybridisation and polyploidisation, although such patterns remain to be comprehensively studied for the clade E as a whole.

From our previous phylogenetic work based on molecular data (papers I and II), it was clear that African diversity of *Isoetes* is particularly interesting to explore further, since African species are found in several different clades, both in clades A and B and *Isoetes wormaldii* is the lone sister species of the entire family. These previous studies also found issues with species identification or species delimitation in clades A and B. One of the goals of the spore morphology study (paper IV) was therefore to explore potential differences between spores produced by different African species of *Isoetes*. Are they possible to distinguish? Are certain spore characteristics diagnostic for the different subclades? We conducted a pilot test with a small set of samples for which no molecular data had been previously produced and found indications that this is probably the case. Prior to DNA sequencing and analysis, the samples with spore-IDs ELS01, ELS02 (both *I. schweinfurthii*) and ELS04 (*I. aequinoctialis*) were hypothesised to belong to clade A based on our spore data, while sample ELS05 (*I. welwitschii*) was hypothesised to belong to clade B. Subsequent production of nrITS sequences for these samples (DNA-IDs: EL163, EL164, EL165 and EL167) and inclusion of them in phylogenetic analysis proved the hypotheses correct except for ELS04 (EL165), which turned out to belong to clade B. A possible explanation for the latter misidentification may be that there were few spores available from the specimen, and most of them not in the best condition. Overall, my conclusion from this pilot test is that megaspore morphology can be used as a first assessment of clade association of African specimens of *Isoetes*.

A solution to the age problem?

Subsequent to my investigations of spore morphology in the living clade, I made a first brief survey of megaspores in the isoetalean fossil record. There are clearly difficulties of several kinds when attempting to understand relationships among living and extinct isoetaleans, also based on megaspore data, for example that clade affinity of dispersed spores can be very difficult to assess, that isoetalean spores have an outer silicified coat that may or may not be preserved (Skog and Hill, 1992), and that the age gap to some fossils is huge, sometimes several hundred million year, making a close relationship with living species unlikely. However, I find one fossil discovery particularly interesting and relevant to assess further, namely that of Hill (1988).

Hill (1988) documents fossil remains of isoetalean leaves (named *Isoetes reticulata*) from the late Oligocene to early Miocene of Tasmania. In situ in these leaves (between the cuticles at the exact position where the sporangium would have been positioned) there were fossilized megaspores, which in my opinion share uniquely derived features with those of extant species of...
clade D. I further compared these fossil megaspores with Marsden’s (1979) SEM-images of *Isoetes neoguineensis*, for which I had no spore data, and find remarkable similarities. The fossil *Isoetes reticulata* has an equatorial ridge that is tilted upwards, which according to my data is unique to clade D. And like the megaspores of the extant *I. neoguineensis* and partly also *I. japonica* of clade D, the fossil spores have a reticulate megaspore ornamentation and a surface structure that appears somewhat “unfinished”. Using these features for phylogenetic optimization would place the fossil *Isoetes reticulata* in clade D, possibly as sister to the subclade that includes *I. neoguineensis* and *I. japonica* (figure 3). Using this information as a basis for calibration in molecular dating analyses would yield a prior node age distribution of clade D to about 20-25 Ma and may enhance calculations and convergence of results in such analyses.

*Isoetes* as I leave it (for now)

The rooting of the phylogeny of the genus is no longer an issue and there is a robustly supported backbone to the phylogeny, which consists of five major clades. Many more species have been phylogenetically placed and they have shed light on some unexpected biogeographical patterns and possibly some species delimitation issues. The dating of the extant group of *Isoetes* has been examined and analysed in several ways. That no clear answer is found is not surprising given the lack of phylogenetically placed fossils within the group, their lonely survivorship status in their order as well as the long evolutionary distance to their closest living relatives. Megaspore morphology of phylogenetically placed specimens across the genus have been studied and some patterns have been detected. There is a lot of variation that is not clade specific but instead reappears in different groups. Enough patterns are found to be able to separate the African specimens of clade A from those of clade B, as well as tentatively place the fossil *Isoetes reticulata* Hill within clade D. If the phylogenetic position of this fossil is accepted, the utilisation of it for calibration of clade D to absolute time would probably allow for more robust estimates of node ages in *Isoetes* in the future.
Figure 3. Phylogeny of Isoetes (as estimated in paper IV). The red dot indicates the position of the fossil Isoetes reticulata Hill as assessed based on phylogenetic optimisation of megaspore morphology. This information can be used to calibrate the crown group D (the yellow clade) to absolute time (about 20-25 Ma) in analyses of divergence times of clades based on molecular data.
Future studies

Low copy nuclear gene data

Most of the phylogenetically important data in this thesis has been based on the chloroplast genome, which in theory behaves more like a singular gene and it is uniparentally inherited. To understand the evolutionary history of *Isoetes* better one could study massive amounts of low copy nuclear genes, construct a phylogenetic species tree, and see if there is disparity with previous results based on for example plastid data. There is a published transcriptome (One Thousand Plant Transcriptomes Initiative, 2019) which makes constructing a library of baits possible for target capture of low copy nuclear genes. This approach could also be a foundation for studies of polyploidy in *Isoetes* in greater detail as it is possible to get different copies of the same gene from one species.

Mitochondrial data

We have mitochondrial data for the species sampled in paper III and while this genome is not usually studied in plant systematics it could be very interesting to see if it follows the same evolutionary pattern. It is often assumed that the mitochondrion is uniparentally inherited, but reality has often been murkier. Studying the mitochondrial DNA would pose its own unique difficulties as it is likely not a single molecule in *Isoetes* (Grewe et al., 2011).

African diversity

As mentioned before, it is clearly needed to study the species of *Isoetes* that occur in Africa thoroughly and delimit species and possibly describe new ones that align with the phylogenetic placements of them. This would involve field work and studying type localities, preferably in person, but first assessments could probably also be conducted based on herbarium material since ample field work across Africa would be time consuming and complicated.

Spore morphology

While paper IV is a good start regarding spore morphology in *Isoetes* in an evolutionary context, there are many species of *Isoetes* that were not represented, and they would need to be to obtain a complete picture of the spore morphology, diversity and evolution. It would also be of interest to add the microspore ornamentation to such evolutionary assessments; their morphological diversity does not appear to be as great as for megaspores but perhaps that is due to lack of study, although see (Musselman, 2002).

Furthermore, comprehensive knowledge of the whole of the living diversity of spore morphology in *Isoetes* would be a great basis for studies of the fossil spores that are of clear isoetalean origin, i.e. have been found in situ in a plant with a clear isoetalean affinity. The task would be a difficult one, but to study Cenozoic (to Cretaceous) material may be a good start.

Dating analysis

If one can arm oneself with fossil occurrences that can be phylogenetically placed it would be interesting to make new dating analyses, but the analyses would have to test a multitude of clock
models since our previous work has shown that node age estimation in *Isoetes* is a very difficult evolutionary question.

**Spore dispersal**

Knowledge of spore dispersal in *Isoetes* is patchy and incomplete, despite it being very interesting. *Isoetes* are heterosporous plants and in order for dispersal and subsequent successful reproduction to occur, both mega- and microspores have to be dispersed to the same place. Can this happen over distances longer than a meter or so? If so, how? Phylogenetic (topological) results indicate that while deep divergences in the genus probably reflect the break-up of the Gondwana continent, more recent dispersal appears evident too, sometimes over surprisingly long distances. How do these results fit with the ability of the plants to disperse? This project could test hypotheses on dispersal processes, such as spore buoyancy in sea and fresh water, microspore adherence to megaspores, and spore adherence to bird feet and feathers. An assessment of intrageneric variation regarding dispersal syndrome and hypotheses on influence of dispersal mode and spore morphology on lineage diversification would also be interesting.
Svensk sammanfattning

Det har alltid fascinerat mig att allt som lever på denna jord har samma ursprung. Liv har bara uppkommit en gång (framgångsrikt) på vår planet; alla organismer på jorden är därför släkt med varandra och ändå finns det en sådan mångfald av form och funktion. Om det inte vore för livet skulle jorden bara vara mineraler, vatten och gaser, och till och med vad vi kan tänka oss som inert geologi förändras i grunden av närvaron av liv. Livet påverkar vårt klimat och kretsloppen av kemiska ämnen som vatten, kol och fosfor. Den tidiga uppfattningen av fotosyntes kan ses som ett rött band i mineraler där det plötsligt fritt tillgängliga syret fick järn att oxidera till rost för cirka 4 miljarder år sedan.

De första skogarna på jorden var ekosystem helt utan fröväxter. Det är svårt att föreställa sig, är det inte; en skog utan några blommande träd och inga barrträd heller. Dessa skogar är nu borta för länge sedan och deras kvarlevor är delvis bevarade i form av kol. Men en del av deras ättlingar lever vidare idag som de inte speciellt trädliknande Equisetum (fräkenväxter) och lummerväxter (lumrar, mosslumar och braxengräs).


Att känna till artdivergensstider för klas som innehöll utdöda eller förfadersgrupper är inte bara användbart för evolutionära biologer för att fastställa åldern för en viss artgrupp, utan också särskilt viktigt för att ta itu med en rad biologiska frågor. Genom att ha denna kunskap om evolutionära divergenser kan vi placera artbildningsändringar i de korrekta geologiska och miljömässiga sammanhangen och att få en bättre förståelse för artbildning och spridningsmekanismer. Det ger oss också ett perspektiv på variationen av artrikedom och artdiversifieringshastigheter över geologiska perioder.

De första skogarna som jag nämnde först bestod till stor del av rhizomorfa lumrar som hörde till Isoetales. De arterna var utbredda under den sena Mississippian och större delen av Pennsylvanian och bildade den dominerande gruppen av växter i de flesta av de enorma evaktoriska kolträskekosystemen i centrala och västra Pangæa. Dessa arter var inte bara träd; i gruppen ingår också arter med växtsätt som varierar från små buskar och spretande former till mer trädliknande former. Men trädformerna måste ha varit verkligt majestätiska då de var mer än 30 m höga och mer än 2 m i stamdiameter. Heterospori är en morfologisk egenhet som är en av länkarna mellan de utlöda rhizomorfa lumrarna och nulevande Isoetes, men det är också ett kännedöme för de närmaste nulevande systern till Isoetes, Selaginella av ordningen Selaginellales. Även om det sannolikt är en synapomorf i de heterosporiga lumrarna har det också uppkommit separat i åtminstone två grupper av ormbunkar samt, kanske mest känt, i fröväxter.

Looy et al. (2021) menar att det är just möjligheten att överleva som en spor som är rik på kolhydrater och lipider (och potentiellt kan förbliva livskraftig i 100 år på sjöbottnar), tillsammans med en långsam tillväxttakt samt få krav på de omgivande livsmiljöerna som gjorde det möjligt för de rhizomorfa lumrarna att frödas när andra taxa minskade på grund av miljöförstörningar. Att kunna växa i näringsfattiga, till stor del organiska sediment skulle ha underlättat kolonisering av erosionslandskap, där mer konkurrenskraftiga taxa med större näringsbehov skulle vara långsamma att kolonisera.

Det finns omkring 200 arter av Isoetes, det enda släktet inom familjen Isoetaceae och den enda kvarvarande representanten för de rhizomorfa lumrarna. Arter av Isoetes lever av de av av handlingen påbörjades. Det hade gjorts åt fyllogenetiska studier och det fanns ett behov av att öka mängden data, både gällande antal arter, geografiska områden och DNA-regioner. De grundläggande relationerna och släkträdets utveckling var gängliga, eftersom en av de senaste studierna då (Schuettpelz and Hoot, 2006) kom till slutsatsen att vi måste acceptera att släkträdets äldsta förgreningar skulle förblivit okända. Det var också oklart hur man ska koppla det nulevande släktet till deras äldre rhizomorfa släktningar. Och medan en del av det problemet handlar om att erhålla en mer fullständig förståelse av morfologin och hur den har förändrats genom evolutionär tid var en annan fråga hur gammalt det nulevande släktet kan vara. Genom att ha en åldersuppskattning får de biogeografiska mönstren sammanhang och rimliga förklaringar.

Det var vårt mål från allra första början att bredda den fyllogenetiska provtagningen av Isoetes arter till att omfatta mer av mångfalden inom detta kosmopolitiska släkte. En bredare artrepresentation behövdes helt klart för att underlätta studier av släktets globala evolutionära historia. Majoriteten av DNA-sequenserna som används i denna avhandling erhålls från herbarieprover och jag är djupt tacksam för att herbarieerna BM, BR, C, GB, L, MEL, NY, P, S, W och WU generöst gav oss tillgång till deras vetenskapligt viktiga och oersättliga samlingar.

I den här avhandlingen fann jag att Isoetes är uppdelad i fem stora klader och att det finns en syster utanför dessa fem klader: den sällsynta och relativt okända sydafrikanska endemen Isoetes wormaldii är syster till resten av släktet.
Eftersom arterna av *Isoetes* skiljer sig mycket lite från varandra både morfologiskt och i DNA-sekvensdivergens kan man anta att de är en mycket ung grupp av växter, trots deras gamla avr. Men den uppfattnings ifrågasätts åtminstone när man tar hänsyn till de biogeografiska mönstren för detta släkte av lumrar. Den övergripande fylgenin hos *Isoetes* är inte lätt att förstå genom att jämföra med biogeografiska mönstre och processer. Arter från södra tropiska Afrika delas in i fem stora klader, indiska arter är uppdelade i tre stora klader, australiska och tropiska asiatiska arter finns i tre respektive två klader, och sydamerikanska arter förekommer i minst tre klader. Norra halvklotet utgör ett liknande fall eftersom europeiska och nordamerikanska arter är placerade i minst tre stora art. 

De två satsningarna på att datera *Isoetes* fylgeni har följt mycket olika filosoffer. I artikel I var tillgängliga DNA-data relativt magert med endast nrITS och den mycket evolutionärt bevarade rbcL-genen och spacern mellan atpB och rbcL, men å andra sidan var artprovtagningsn jämförelsevis stor, och utgrupperna valdes för att försöka maximera tillgängliga fossila kalibreringspunkter. I artikel III gjorde NGs-sekvensering mycket mer data per prov tillgänglig (hela plastidgenomet och den nukleära rDNA-citronen) men för färre arter totalt. En viktig skillnad från tidigare nämnda artiklar är att vi inkluderade *Isoetes wormaldii* i artikel III och som syster till de återstående arterna i släktet ökade den genomsnittliga median-noddjupet till *Isoetes*-krongruppen med en faktor 2,55 (plastiddata) eller 1,95 (nukleära rDNA-data).

Åldersuppskattningarna i artikel III var mycket inkonsekventa. De skilde sig mellan plastid- och nukleara data och ännu mer mellan analyser med olika klockmodeller. Vår slutsats var att det är svårt att motbevisa något av dessa resultat, och att det kanske kommer att krävas att man hittar en ny kalibreringspunkt inom *Isoetes* för att finna en väg framåt.


Vi hade för avsikt att följa Hickey's termer för beskrivning av megaspor-ornamentation (Hickey, 1986a) och vi använder nio av Hickey's termer (baculat, cristat, echinat, levigat, pustulat, retat, reticulat, rugulat och tuberculat), men vi fann att det behövdes lägga till ytterligare tre (foveolat, spiculat och umbellat). Pustulat ornamentation bedömdes vara den vanligaste hos *Isoetes*; den är vanligast i kladder B, den är vanlig i kladder A och förekommer hos vissa arter i C-D-E kladder. Även om ingen större klad inom *Isoetes* kunde definieras av deras spor-ornamentation, finns det vissa karakteristiska egenskaper hos mindre klader.


När jag nu lämnar *Isoetes* (åtminstone tillfälligt) så är roteningen av släktets fylgeni inte längre ett problem och det finns en robust fylgeni som består av fem stora klader samt *Isoetes*.
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To dad, I wish you could have gotten to read this.