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Large scale phylogenomic analysis resolves a backbone phylogeny in ferns

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Abstract:	Background: Ferns, originated about 360 million years ago, are the sister group of seed plants. Despite the remarkable progress in our understanding of fern phylogeny, with conflicting molecular evidences and different morphological interpretations, relationships among major fern lineages remain controversial. Results: With the aim to obtain a robust fern phylogeny, we carried a large scale phylogenomic analysis using high-quality transcriptome sequencing data which covered 69 fern species from 38 families and 11 orders. Both coalescent-based and concatenation-based methods were applied to both nucleotides and amino acids sequences in species tree estimation. Among the mainly consistent and strongly supported topologies, topologies yielded from applying coalescent-based method and concatenation-based method, respectively, to nucleotides sequence are congruent except one position. Conclusions: Our result confirmed that Equisetales is sister to the rest of ferns, and Dennstaedtiaceae is sister to eupolypods. Moreover, our result strongly supported some relationships different from the current view of fern phylogeny, including that Marattiaceae may be sister to the monophyletic clade of Psilotaceae and Ophioglossaceae; Gleicheniaceae and Hymenophyllaceae form a monophyletic clade which is sister to Dipteridaceae; and that Aspleniaceae is sister to the rest groups in eupolypods II. These results were interpreted with morphological traits, especially sporangia characters, and a new evolutionary route of sporangial annulus in ferns was suggested. This backbone phylogeny in ferns sets a foundation for further studies in biology and evolution in ferns, and therefore in plants.
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Response to Reviewers:	Reviewer #1: page 3, line 60, change "basing on" to "based on" R: "Basing on" has been changed to "based on" as suggested (page 3, line 61). page 4, line 66, change "basing on" to "based on", and change "analysis" to "analyses" R: "Basing on" has been changed to "based on" , "analysis" has been changed to "analyses" (page 4, line 67). page 4, line 74, change "close" to "closely" R: "Close" has been changed to "closely" (page 4 line 77). page 5, line 91, change "Schezaeles" to "Schizaeales" R: "Schezaeles" has been changed to "Schizaeales" (page 5, line 92). page 5, line 101, change "Genbank" to "NCBI" R: "Genbank" has been changed to "NCBI" (page 5, line 102). page 5, line 103, change "filtration" to "filtering" R: "Filtration" has been changed to "filtering" (page5, line 104). page 5, line 107, change "lineage" to "lineages" R: "Lineage" has been changed to "lineages" (page5, line 108). page 6, line 127-130, suggested wording: "For each combination of reconstruction methods (coalescent-based or concatenation-based) and sequence types (nucleotides or amino acids), Matrix 1 and Matrix 2 always yielded the same topology. In general, the four cladograms (Figure 3, Figure S1, S2, S3) from a combination of methods and sequence types are consistent except six positions (Table 2)." R: It has been changed as suggested (page 6, line 127-131), thank you. page 6, line 130-132, what do you mean by "most agreed"? R: This sentence has been changed as “Among the topologies, the one estimated by applying coalescent-based method to nucleotide sequence (Figure 3) and the one applying concatenation-based method (Figure S2) are most congruent”(page 6, line 131-133). page 6, line 133, change "evolution" to "evolutionary" R: "Evolution" has been changed to "evolutionary" (page 6, line 134). page 7, line 149, change "among close related taxa" to "at shallow phylogenetic scale" R: It has been changed as suggested (page 6, line 150), thank you. page 7, line 152-154, suggested wording: "..., and are often the controversial nodes from past studies based on different genes, we suggest such inconsistency might be caused partially by LIS and reticulate evolution." R: It has been changed as suggested (page 7, line 153-154), thank you. page 8, line 158-159, Rothfels et al (2015) is not the first to report Equisetum being

	sister to the rest. See Rai and Graham (2010, AJB), and Kuo et al (2011, MPE). Also change "basing" to "based" R: It has been changed as “This topology confirmed the results reported by Rai & Graham [12], and Kuo et al. [33] based on plastid genes, and has been accepted by the PPG I [3] in 2016” (page 8 , line 158-160). page 8, line 172, change "view of mainstream" to "mainstream" R: "View of mainstream" has been changed to "mainstream" (page 8, line 175). page 9, line 179, no need to say "forking ferns" and "filmy ferns" again here. R: "Forking ferns" and "filmy ferns" have been deleted here, thank you (page 9, line 182). page 9, line 181, change "Differently" to "On the other hand" R: "Differently" has been changed to "On the other hand" (page 9, line 184). page 9, line 185, change "may form a sister lineage to" to "may be sister to" R: "May form a sister lineage to" has been changed to "may be sister to" (page 9, line 187). page 9, line 186, change "the Gleicheniales order" to "Gleicheniales" R: "The Gleicheniales order" has been changed to "Gleicheniales" (page 9, line 189). page 9, line 195, remove "the disputation of inner" R: "The disputation of inner" has been removed (page 9, line 197). page 9, line 200, change "in agree with" to "in agreement with", and references are needed for this sentence. R: It has been changed as “Our results showed that eupolypods are divided into two major lineages, eupolypods I and eupolypods II in agreement with the consensus opinion [3]” (page 10, line 201-202). page 10, line 202, change "new" to "different" R: "New to" has been changed to "different from" (page 10, line 204). page 10, line 206, both "phylogram" and "cladogram" are used in this manuscript, and in a seemly interchangeable way. I'd prefer "topology". R: Both "phylogram" and "cladogram" have been changed to “topology” in this manuscript. Thank you. (e.g., page 10, line 208). page 10, line 208, change "more close to" to "more closely related to" R: "More close to" has been changed to "more closely related to" (Page 10, line 210). page 10, line 206-216, NO MORE USE OF "PRIMITIVE"!!! Everthing extant is equally "advanced". R: Although extant species are equally “advanced”, their characters can be “primitive”, “original”, or “derived”. This sentence has been changed as “In Pteridaceae, the unstable structure of spherical sporangia, including variable annulus and short sporangial stalk, indicates these characters of sporangia are relatively original and are close to those with oblique annulus in early leptosporangiate. We also noticed that the characters of spherical sporangia with slightly oblique annulus in Monachosorum should be more primitive than the flattened sporangia with typical vertical annulus in other genera of Dennstaedtaceae” (page 10, line 211-216). page 10-11, "The evolution of sporangia annulus in ferns". I'm still having trouble understanding how the authors deduce the "routes" of annulus evolution. The "two subroutes" is particularly confusing - it would only make sense if Schizaeales and Salviniales are monophyletic, which they are not. And again, the ladderized thinking - with Polypodiales having the advanced, final states while the others being the primitive intermediates - is not correct. R: Thanks to your advice. In the former manuscript, the "two subroutes" was indeed incongruent with the reconstructed evolutionary history of sporangia annulus in ferns as in Figure 4. We have re- interpreted the evolutionary history and avoided the “ladderized thinking”.

	This paragraph has been changed as “By observing the character of sporangial annulus of abundant samples in each fern group, and combining these characters with our well-resolved backbone phylogeny (Figure 3), we reconstructed the evolutionary history of sporangial annulus in ferns (Figure 4). According to the results, we infer that ex-annulus sporangia, as in Equisetaceae, Psilotaceae, and Ophioglossaceae, is the ancient state in ferns; rudimentary multiseriate annulus, which is inverse U-shaped in Marattiaceae, and U-shaped in Osmundaceae; equatorial transverse-oblique uniseriate annulus, as in Gleicheniaceae and Hymenophyllaceae; oblique annulus as in Cyatheales (tree ferns), and vertical annulus as synapomorphy in polypods, have been derived from the ex-annulus state. Both Apical annulus as in Lygodium and Schizaea, and vestige or disappeared annulus as in Salviniales (aquatic ferns) are likely to be specialized in parallel from oblique annulus” (page 11, line 220-231). page 11, the monophyly of eusporangiate ferns is highlighted in Conclusion, but this is also one of the inconsistent relationship between the ASTRAL and concatenation analyses. I suggest perhaps discuss the incongruence and potential pitfalls in phylotranscriptomics instead. R: Since the monophyly of eusporangiate ferns are supported by coalescent-based method, but not by concatenation-based method, here we only say that the monophyly of eusporangiate ferns is possible in both the abstract (page 2, line 30-31) and the conclusion (page 11, ling 240-242). In discussion, we say “The incongruence between the results based on coalescent and concatenation methods may be caused by strong ILS effect, which is a main pitfall when using concatenation method [21]”(page 8, line 168-171). page 14, line 312, there are way more fossils that can be used as calibrations. Why only included two? R: Fossil ages reflect the minimum times of divergence, which is more recent than the true divergence time. Given the small possibility in finding the earliest fossil for a lineage, and the difficulties in fossil species identification and correct dating, perfect fossils for calibration are rarely available. It is not always better to use more fossil dates as calibrations in estimation the divergence times for a phylogeny. Here we use two fossils (Archaeocalamites Senftenbergia: 354 MY, Grammatopteris: 280 MY) as the minimum ages of monilophytes and leptosporangiate ferns, which are relatively high in quality. When we use 8 fossil dates in calibration, the result did not improved remarkably. change "sporangia annulus to "sporangial annulus" R: "sporangia annulus” has been changed to "sporangial annulus" in this manuscript, thank you. Reviewer #2: The revised manuscript deals with and addresses many of the methodological comments from the previous reviews. Importantly, data and scripts are all deposited on FigShare and can be accessed. They have also added summary methods in addition to their concatenation analyses before. My remaining concerns about this manuscript are mostly related to individual sentences. These need to be revised for improved accuracy, clarity, or both. Minor comments: 1- Page 4: " Moreover, due to the fact that amino-acid sequence is more conserved than nucleotide sequence, it may be more suited to estimate relationships among distant taxa. " - The main issue with nucleotides is the fact that they tend to have compositional bias, especially in the third codon position. The discussions about what form of data is suited for "distant" or "recent" taxa is a bit hand-wavy, and in my view, misplaced. The important distinction between AA and nucleotide data is model fit. R: We agree with your comment, and thanks for pointing out our misunderstanding. We have deleted this sentence in our revised manuscript, and made an careful interpretation about the advantage and disadvantage for protein and DNA data in phylogeny: “Nucleotide sequence, with higher variability than amino acid sequence, usually brings more useful information in phylogeny reconstruction, especially for closely related taxa. However, the compositional bias in nucleotide sequence, especially in the third codon position, may lead to a deviation from the true phylogeny.

Here, both nucleotide and amino-acid sequences are used in phylogeny reconstruction” (page 4, line 73-78).

2. Calling parts of the tree "sites" is rather confusing. Sites typically are used for alignments. I would use another term (e.g., parts, areas, relationships, etc.).
R: “Sites” has been changed to “positions” in the revised manuscript (e.g. page 6, line 131).

3. Why authors keep referring to their estimated phylogenetic trees as cladograms and/or phylogram is unclear and non-standard. Why not just call them phylogenetic trees?
R: “Cladograms” and “phylogram” were used in the former manuscript to infer the phylogenetic trees without time calibration, they have been changed to “topology” instead (e.g. page 6, line 129).

4. Page 6: "Among the cladograms, the one estimated by applying coalescent-based method to nucleotide sequences (Figure 3) is the most agreed."
- I have no idea what this sentence means. What does it mean to say a tree is most agreed? Agreed with what?
- Also, if the authors clarify what they mean, still, whether agreement between different analyses using the same tool means anything is not clear. That ASTRAL trees are more consistent among different analyses does not indicate higher quality of ASTRAL trees, and the authors should not imply that.
R: Thank you for the comments. “Most agreed” was to say that “the topology estimated by applying coalescent-based method to nucleotide sequences (Figure 3) is more consistent among different analyses”. Since we have no evidence that the topology estimated by applying coalescent-based method to nucleotide sequences (Figure 3) is necessarily more reliable than the one estimated by applying concatenation-based method, we have changed this sentence as “Among the topologies, the one estimated by applying coalescent-based method to nucleotide sequence (Figure 3) and the one applying concatenation-based method (Figure S2) are most congruent” (page 6, line 131-133).

5. Page 13: "To reduce the complexity of each group, we removed all sequences of the species that had more than 10 sequences in this group. "
- Again, it's not clear what is meant by "complexity" here. Phylogenetic analyses greatly benefit from increased taxon sampling, and if the authors have completely removed taxa from their analyses, that is not a good practice. The only justified reason I can think of is that with extra sequences, analyses would be infeasible computationally.
R: The de novo assembly by trinity results in many sequences with high similarity, which includes both paralogs and isoforms (Haas, 2013). The complexity mentioned here meant that if a clustered gene family contains too many sequences (eg. more than 10), the risk of contamination of isoforms instead of true paralogs will be raised. In addition, as commented by the reviewer, it is indeed infeasible computationally when the sequence number grows larger, since phylogeny trees are built for each gene family. Concerning the taxa number, the reviewer worried that if we remove all the sequence from the taxa in the gene family will reduce the taxa number in use. We set the threshold value for taxa cover degree that is 75% and 90%, so this process will not influence taxon sampling.
The de novo assembly by trinity produces many sequences with high similarity, which includes both paralogs and isoforms (Haas, 2013). The complexity mentioned here meant that if a clustered gene family contains too many sequences (eg. more than 10), the risk of contamination of isoforms instead of true paralogs will be raised. In addition, as commented by the reviewer, it is indeed infeasible computationally when the sequence number grows larger, since phylogeny trees are built for each gene family.
Concerning the taxa number, the reviewer worried that if we remove all the sequence from the taxa in the gene family will reduce the taxa number in use. We set the threshold value for taxa coverage that is 75% and 90%, so this process will not influence taxon sampling.
This paragraph has been changed as “As the de novo assembly by Trinity produces many sequences with high similarity, which contain both paralogs and isoforms [47], when a clustered gene family contains too many sequences (eg. more than 10), the risk of contamination of isoforms rises, along with the computational infeasibility.

	Hence, when a species has more than 10 sequences in a gene family, we remove all sequences in this gene family of this species" (page 13, line 272-277). 6. Page 13: "Out of 69 samples in total, 65 samples (that is 94.2% of total) were defined to have a relatively higher gene coverage degree. " - Please be more specific. What if any threshold was used? R: This sentence has been changed as "Out of the 69 samples in total, the gene coverage of 65 samples (94.2%) exceeded 82%, with at least 251 complete genes identified" (page 14, line 299-300). 7. Page 14: "Statistically consistency was estimated from unrooted gene trees under the multi-species coalescent model." - This sentence has no meaning whatsoever. Authors don't seem to know what "statistical consistency" is and they should avoid making any claim about it. Remove or rephrase, please. R: This sentence has been removed in the revised manuscript. Reviewer #3: The authors satisfactory addressed all points raised in the previous round of review. The inclusion of a coalescent-based method to estimate the phylogeny is a very welcome addition and the differences in the obtained phylogenies are succinctly presented. R: Thank you for the comments.
Additional Information:	
Question	**Response**
Are you submitting this manuscript to a special series or article collection?	No
Experimental design and statistics Full details of the experimental design and statistical methods used should be given in the Methods section, as detailed in our Minimum Standards Reporting Checklist. Information essential to interpreting the data presented should be made available in the figure legends. Have you included all the information requested in your manuscript?	Yes
Resources A description of all resources used, including antibodies, cell lines, animals and software tools, with enough information to allow them to be uniquely identified, should be included in the Methods section. Authors are strongly encouraged to cite Research Resource Identifiers (RRIDs) for antibodies, model organisms and tools, where possible. Have you included the information requested as detailed in our Minimum Standards Reporting Checklist?	Yes

Availability of data and materials All datasets and code on which the conclusions of the paper rely must be either included in your submission or deposited in publicly available repositories (where available and ethically appropriate), referencing such data using a unique identifier in the references and in the “Availability of Data and Materials” section of your manuscript. Have you have met the above requirement as detailed in our Minimum Standards Reporting Checklist?	Yes

Large scale phylogenomic analysis resolves a backbone phylogeny in ferns

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Abstract

Background: Ferns, originated about 360 million years ago, are the sister group of seed plants. Despite the remarkable progress in our understanding of fern phylogeny, with conflicting molecular evidences and different morphological interpretations, relationships among major fern lineages remain controversial.

Results: With the aim to obtain a robust fern phylogeny, we carried a large scale phylogenomic analysis using high-quality transcriptome sequencing data which covered 69 fern species from 38 families and 11 orders. Both coalescent-based and

concatenation-based methods were applied to both nucleotides and amino acids sequences in species tree estimation. Among the mainly consistent and strongly supported topologies, topologies yielded from applying coalescent-based method and concatenation-based method, respectively, to nucleotides sequence are congruent except one position.

Conclusions: Our result confirmed that Equisetales is sister to the rest of ferns, and Dennstaedtiaceae is sister to eupolypods. Moreover, our result strongly supported some relationships different from the current view of fern phylogeny, including that Marattiaceae may be sister to the monophyletic clade of Psilotaceae and Ophioglossaceae; Gleicheniaceae and Hymenophyllaceae form a monophyletic clade which is sister to Dipteridaceae; and that Aspleniaceae is sister to the rest groups in eupolypods II. These results were interpreted with morphological traits, especially sporangia characters, and a new evolutionary route of sporangial annulus in ferns was suggested. This backbone phylogeny in ferns sets a foundation for further studies in biology and evolution in ferns, and therefore in plants.

Key Words: phylogenomic, monilophytes, evolution, sporangium, transcriptome

Background

Phylogeny, which reflects natural history, is fundamental to understanding evolution and biodiversity. Ferns (monilophytes), originated about 360 million years (MY) ago, are the sister group of seed plants [1, 2]. With estimated 10,578 extant living species globally [3], they are the second most diverse group in vascular plants. Phylogenetic studies for ferns, especially based on molecular evidences, have been widely carried

in recent decades. These studies have revolutionized our understanding of the evolution in ferns, among which the milestones being setting ferns as the sister group of seed plants [1, 2], placing Psilotaceae and Equisetaceae within ferns [2, 4, 5], and revealing a major polypods radiation following the rise of angiosperms [6, 7]. Resolution at shallow phylogenetic depth among families or genera have also been improved remarkably [8-14].

However, previous researches on fern phylogeny have mostly relied on plastid genes [10, 12, 13], some combined with a few nuclear genes [4, 5, 14] or morphological traits [5, 11]. Due to incomplete lineage sorting (ILS), genes from different resources often show conflicting evolutionary patterns, especially when based on a limited number of samples, some deep relationships in fern phylogeny remain controversial (Figure 1). In the latest PPG I system [3], which has derived from many recent phylogenetic studies, some important nodes remain uncertain, such as (i) what are the relationships among Marattiales, Ophiglossales and Psilotales? (ii) are Hymenophyllales and Gleicheniales sister groups? and (iii) what are the relationships among families in eupolypods II?

Transcriptome sequencing (RNA-Seq) represents massive transcript information from the genome. Phylogenetic reconstructions based on RNA-Seq are more efficient and cost-effective than traditional PCR-based or EST-based methods when lacking whole-genome data [15]. Successful cases in recent years include mollusks [16], insects [17], the grape family [18], angiosperms [19], and land plants including six

ferns [20]. Here, with the aim to reconstruct the framework of fern phylogeny, we sampled abundant fern species representing all important linages and applied latest phylogenomic analyses based on RNA-Seq.

To reconstruct a robust and well-resolved phylogeny in ferns, applying multiple methods of phylogenomic analysis is extremely important. Since concatenation-based estimations of species tree usually have good accuracy under low level of ILS, while coalescent-based methods are developed to overcome the effect of ILS, but are sensitive to gene tree estimation error [21], so both concatenation-based and coalescent-based estimations are applied. Nucleotide sequence, with higher variability than amino acid sequence, usually brings more useful information in phylogeny reconstruction, especially for closely related taxa. However, the compositional bias in nucleotide sequence, especially in the third codon position, may lead to a deviation from the true phylogeny. Here, both nucleotide and amino-acid sequences are used in phylogeny reconstruction.

In the aspect of morphology, fern sporangium is an organ for enclosing and dispersing spores, most of which functions like a unique catapult with annulus [22]. During the last centuries, Bower's hypothesis on the evolution of sporangia with a focus on annulus [23] had been one of the most important cornerstones to fern phylogeny based on morphology [24, 25]. However, this hypothesis has been challenged by somewhat conflicting frameworks of fern phylogeny [4, 10, 12, 14, 26]. A robust framework in fern phylogeny which reflects the evolutionary history will

improve our understanding for the evolution of fern sporangia as well as other characters.

Data description

Taxa sampling and RNA-Seq

We chose 69 fern species from 38 families according to PPG I system (totally 48 fern families), covering all the 11 orders (Equisetales, Psilotales, Ophioglossales, Marattiales, Osmundales, Hymenophyllales, Gleicheniales, Schizaeales, Salviniales, Cyatheales, and Polypodiales). Information about the location and time for sampling is given in Table S1. All the sampled species were collected under the permissions of the natural reserves and Shanghai Chenshan Botanical Garden in China.

Sporophyll or/and trophophyll were collected and frozen in liquid nitrogen immediately, and preserved in Ultra-low temperature refrigerator at -80°C before RNA extraction. Total RNA was extracted using TRIzol (Life Technologies Corp.) according to the manufacturer's protocols. The RNA concentration was determined using a NanoDrop spectrophotometer, and RNA quality was assessed with an Agilent Bioanalyzer. Paired-end reads were generated by Majorbio Company (Shanghai, China) using the HiSeq 2500 system. Raw reads were deposited in NCBI [27].

Transcriptomes assembly and orthology assignment

Transcriptomes data were generated from 69 fern species (Table 1). After filtering, about 2,726.9 million pair-end DNA sequence reads (about 313 Gbp) were retained. We assembled these reads *de novo* and obtained a total of 5,449,842 contigs [28].

In order to obtain a reliable phylogenetic relationship, we selected four species as the outgroup, representing the main lineages of land plants: *Amborella trichopoda*

(representing angiosperms), *Picea abies* (representing gymnosperms), *Selaginella moellendorffii* (representing lycophytes), *Physcomitrella patens* (representing bryophytes). The translated ORF (protein) sequences of these four species were downloaded from Phytozone [29] and used in the following analysis.

To ensure the consistency of phylogenomic analysis, we used a phylogenetic-based ortholog selection method, and obtained two subsets of “one to one” orthologous genes that differed in gene number and species occupancy rate, named “Matrix 1” and “Matrix 2” [30]. Matrix 1 consists of 2391 genes that are present in at least 52 taxa (that is 75% of the 69 taxa in total), resulted in 2,024,565 nucleotide and 674,855 amino acid positions, the gene and character occupancy were 88% and 85% respectively. Matrix 2 consists of 1334 genes that are present in at least 62 taxa (that is 90% of the 69 taxa in total), resulted in 1,171,332 nucleotide and 390,444 amino acid positions, the gene and character occupancy reached 94% and 90% in each. For each orthologues gene set, coalescent-based and concatenation-based methods were applied separately to both nucleotides and amino acids sequences. A working flow diagram showing the major processes in this study is given in Figure 2.

Results

Species tree estimated in 69 ferns

For each combination of reconstruction methods (coalescent-based or concatenation-based) and sequence types (nucleotides or amino acids), Matrix 1 and Matrix 2 [31, 32] always yielded the same topology. In general, the four topologies (Figure 3, Figure S1, S2, S3) from a combination of methods and sequence types are consistent except six positions (Table 2). Among the topologies, the one estimated by applying

coalescent-based method to nucleotide sequence (Figure 3) and the one applying concatenation-based method (Figure S2) are most congruent.

Reconstruction of the evolutionary history of sporangial annulus

Our reconstruction of the evolution of sporangial annulus (Figure 4) showed that ex-annulus sporangia are inferred to be the ancestral state (proportional likelihood [PL]: 1), and the rest of annulus states are likely derived from ex-annulus sporangia. Vertical annulus is suggested as synapomorphy for all polypod ferns (PL > 0.99). Both oblique annulus and rudimentary annulus have experienced parallel evolution.

Discussion

Comparison of topologies estimated by various methods

By comparing topologies estimated by coalescent-based and concatenation-based method using both nucleotide and amino-acid sequences (Table 2), we find that the topologies yielded from coalescent-based and concatenation-based methods using nucleotide sequence are mostly consistent, except the position of *Angiopteris fokiensis.* Topologies yielded from coalescent-based method using nucleotide sequence and amino-acid sequence showed three positions of inconsistency, all of which belong to eupolypods. Since eupolypods have experienced rapid evolutionary radiation in Cenozoic (Figure 3), and nucleotide sequences usually provide more information to reconstruct relationships at shallow phylogenetic scale, we consider the topology yielded from nucleotide sequence maybe more reliable. However, the inconsistent positions among topologies often show relatively lower supporting values, and are often controversial nodes from past studies based on different genes, we suggest such inconsistency might be caused partially by LIS and reticulate evolution.

Relationships of eusporangiate ferns

Which clade is sister to the remaining taxa in ferns is a long-debated question (Figure 1). Our results strongly supported that Equisetales (horsetails) are the sister group to all other monilophytes. This topology confirmed the results reported by Rai & Graham [12], and Kuo *et al.* [33] based on plastid genes, and has been accepted by the PPG I [3] in 2016. Distinct from most fern phylogeny based on molecular evidences (Figure 1), our results based on coalescent method revealed that Psilotales (whisk ferns), Ophioglossales (moonworts), and Marattiales (king ferns) form a monophyletic clade as ((Psilotales, Ophioglossales), Marattiales), which is sister to Leptosporangiate ferns. The monophyletic origin of Psilotales, Ophioglossales, and Marattiales, which belong to eusporangiate ferns, is supported by the structure of sporangia. Being different from the Leptosporangiate type, sporangia of eusporangiate ferns have no sporangiophore, they are thick in wall and large in volume, produce a large amounts of spores, and have no sporangial annulus or only a few thickened cells. The incongruence between the results based on coalescent and concatenation methods may be caused by strong ILS effect, which is a main pitfall when using concatenation method [21].

Relationship of early leptosporangiates

Within early leptosporangiates, our results revealed a new monophyletic clade that Gleicheniaceae (forking ferns) is sister to Hymenophyllaceae (filmy ferns), which is different from the mainstream [3, 10, 12-14, 34]. Similar but still different from the topology (((Dipteridaceae, Matoniaceae), Gleicheniaceae), Hymenophyllaceae) reported by Pryer *et al.* in 2004 [5], in our results, *Cheiropleuria*, which belongs to

Dipteridaceae and formerly placed in Gleicheniales [2, 5, 12, 26, 35, 36], is sister to the monophyletic clade of (Gleicheniaceae, Hymenophyllaceae).

This new relationship is supported by sporangia character. Early leptosporangiates [36] are characterized with diverse sporangia and annulus. However, both Gleicheniaceae and Hymenophyllaceae have spherical sporangia with transverse-oblique annulus, as well as short sporangial stalk connecting to prominent receptacle [37]. On the other hand, flattened sporangia with slightly oblique annulus are found in *Cheiropleuria.* Moreover, long sporangial stalk and inapparent receptacle are common in *Cheiropleuria*, *Dipteris* and *Matonia*. We suggest Dipteridaceae, probably together with its sister lineage Matoniaceae [5, 12], may be sister to the clade of (Gleicheniaceae, Hymenophyllaceae). According to our results, Gleicheniales, which is comprised of Dipteridaceae, Matoniaceae, and Gleicheniaceae [26], is no longer a monophyletic lineage, but a paraphyletic one.

Relationships within polypod ferns

Polypods include more than 80% of living ferns, and their phylogeny remains somewhat controversial and elusive [26, 35, 36]. Our results strongly supported that Dennstaedtiaceae instead of Pteridaceae, is sister to eupolypods. This pattern confirmed the topology suggested recently by Rothfels *et. al* basing on 25 low-copy nuclear genes [14] and Lu *et. al* basing on plastid genes [13], as well as PPG I system [3]. In our result, relationships of Pteridaceae [34, 36, 38] and Dennstaedtiaceae [36] are also well resolved. Notably, *Monachosorum* is sister to the rest members in

Dennstaedtiaceae, rather than being sister to the lineage of Peridium, Hypolepis and Histiopteris [36].

Our results showed that eupolypods are divided into two major lineages, eupolypods I and eupolypods II in agreement with the consensus opinion [3]. Within eupolypods II, our results supported that Aspleniaceae is the sister group to the rest members, which is different from the current viewpoints [26, 36, 39]. Within eupolypods I, our result strongly supported that Lomariopsidaceae and Nephrolepidaceae form a paraphyletic group, rather than a monophyletic clade based on plastid genes [10, 26, 36].

Our new topology confirmed the morphology-based hypothesis that Dennstaedtiaceae with two indusial, rather than Pteridaceae with one false indusium, is more closely related to eupolypod ferns [40]. In Pteridaceae, the unstable structure of spherical sporangia, including variable annulus and short sporangial stalk, indicates these characters of sporangia are relatively original and are close to those with oblique annulus in early leptosporangiate [23]. We also noticed that the characters of spherical sporangia with slightly oblique annulus in *Monachosorum* should be more primitive than the flattened sporangia with typical vertical annulus in other genera of Dennstaedtaceae. For distinguishing eupolypods I and eupolypods II, the number and shape of the vascular bundles at the base of petiole have been demonstrated to be a powerful diagnostic character [36, 39].

The evolution of sporangial annulus in ferns

By observing the character of sporangial annulus of abundant samples in each fern group, and combining these characters with our well-resolved backbone phylogeny (Figure 3), we reconstructed the evolutionary history of sporangial annulus in ferns (Figure 4). According to the results, we infer that ex-annulus sporangia, as in Equisetaceae, Psilotaceae, and Ophioglossaceae, is the ancient state in ferns; rudimentary multiseriate annulus, which is inverse U-shaped in Marattiaceae, and U-shaped in Osmundaceae; equatorial transverse-oblique uniseriate annulus, as in Gleicheniaceae and Hymenophyllaceae; oblique annulus as in Cyatheales (tree ferns), and vertical annulus as synapomorphy in polypods, have been derived from the ex-annulus state. Both Apical annulus as in Lygodium and Schizaea, and vestige or disappeared annulus as in Salviniales (aquatic ferns) are likely to be specialized in parallel from oblique annulus. Inconsistent with Bower's hypothesis [23], our results showed that sporangia with apical annulus as in Schizaeales are no longer the primitive type in ferns but a specialized one. Correspondingly, the oldest fossils of Schizaeaceae is now believed to appear in Jurassic (201-145 Ma BP) rather than formerly thought Carboniferous (359-252 Ma BP) [41].

Conclusion

Our results confirmed that Equisetales is sister to all the other monilophytes, and Dennstaedtiaceae is sister to eupolypods which have been reported previously. Moreover, our results revealed some new relationships, such as eusporangiate ferns except Equisetales may form a monophyletic clade as ((Psilotaceae, Ophioglossaceae), Marattiaceae); while Gleicheniaceae and Hymenophyllaceae form

a monophyletic clade which is sister to Dipteridaceae; and Aspleniaceae is sister to the rest groups in eupolypods II. Most of these results are supported by sporangia characters, and a new evolutionary route of sporangial annulus in ferns is suggested.

Potential implications

Here, we present a robust fern phylogeny yielded from a largescale phylogenomic analysis based on a high-quality RNA-seq dataset set covering 69 fern specie. This backbone phylogeny in ferns sets a foundation for further studies in biology and evolution in ferns and therefore in plants, especially when fern genomes are not available.

Methods

De novo transcriptome assembly

For each paired-end library, we first removed the Illumina adapter of raw reads using Scythe [42] and trimmed the poor quality bases using DynamicTrim Perl script of the SolexQA package with default parameters [43]. Next, *de novo* transcriptome assembly of each species was conducted using the Trinity package (version: trinityrnaseq_r20140413) with default parameters [44]. To discard the duplicated sequences, the obtained contigs were clustered using CD-HIT-EST (v4.6.1) to generated a non-redundant contigs. All contigs with lengths greater than 200 bp were used for downstream analysis. We used the transDescoder, a program in the Trinity package, to identify the candidate coding sequences (CDSs) from the contigs with default criteria. Finally, the translated protein sequences of CDSs were searched by BLASTP against the NCBI nr protein database with an e-value threshold of 1E-5. These BLASTP hit sequences were used for further analysis.

Orthology assignment, alignment, and alignment masking

The orthology assignment for the 69 sample assemblies together with the four outgroup species employed a phylogenetic based clustering method described previously [16]. In short, all-vs-all BLAST search of amino acid sequence was performed among every species, the BLAST results were clustered using MCL [45] software with the parameters '-I 2–tf 'gq(20)". Optimization of the inflation parameter (I) was conducted as described previously [46], the default value 2.0 was selected ultimately. As the de novo assembly by Trinity produces many sequences with high similarity, which contain both paralogs and isoforms [47], when a clustered gene family contains too many sequences (eg. more than 10), the risk of contamination of isoforms rises, along with the computational infeasibility. Hence, when a species has more than 10 sequences in a gene family, we remove all sequences in this gene family of this species. Then, groups with at least 35 (50%) ferns species were aligned using einsi command, implemented in MAFFT [48], and trimmed by Gblocks with default parameters [49]. Next, for each group, homologous gene tree was built with RAxML software (version: 8.0.20) by implementing the maximum likelihood method (ML) [50]. To infer orthologous genes, we used treeprune.pyscript in the agalma [51] package to mask the monophyletic sequences. We pruned the paralogous subtrees from the homologous gene trees until only one monophyletic subtree retained. Next, the resulted orthologous gene trees were further filtered by the criteria that each species should be represented by only one sequence, this resulted subset genes were referred to "one to one orthologs", which were largely free of gene duplication. Then, we extracted both the CDSs (nucleotide sequence) and translated amino acid sequence from the each orthologous gene group, followed by aligning with MAFFT and trimming with Gblocks. The alignment which with coding and corresponding translated sequences lengths greater than 150 bp (or 50 amino acids) were kept for the further analysis.

BUSCO analysis

The Basic Universal Single Copy Orthologs (BUSCOs), which employ a core set of orthologs conserved in all eukaryotic species to determine the gene coverage of each assembly [52], was employed to assess the completeness of the transcriptome assembly we obtained (Table S2) [53]. A total of 303 BUSCOs were employed to blast against by translated amino acid of the assemblies using BLASTP. Then the number of complete and partially matched gene from each assembly was counted respectively. Out of the 69 samples in total, the gene coverage of 65 samples (94.2%) exceeded 82%, with at least 251 complete genes identified. Unexpectedly, among our total assemblies, 1 sample (*Aleuritopteris chrysophylla*, named RS_72) presented extremely low gene coverage degree, in which only 72 (23.8%) complete housekeeping genes were found (Supplementary Table 2). However, when the sample is deleted from the matrix used to construct the backbone of the phylogenetic tree, the topology remains unchanged, indicating that the lower completeness in this sample doesn't affect our results (data not shown).

Phylogenetic analysis

The coalescent-based species tree was reconstructed by ASTRAL v4.10.4 [54], carried out 100 replicates of multi-locus bootstrapping [55]. Each gene tree was constructed with the PROJTT model by RAxML v8.2.4 [50], performed 100 random replicates to calculate bootstrap value. For the concatenation analysis, we preformed the maximun likelihood analyses (ML) for each matrix using RAxML softwore (version: 8.0.20). The branch support was evaluated using 100 bootstrap replicates. We used the "GTR + Γ4 + I" model for DNA matrices, and the JTTF model for the corresponding protein matrices, selected by "ProtienModelselection.pl" [56]. To estimate the divergence times, we used the concatenated alignment of orthologs, calibrated with ages of two fossils (*Archaeocalamites Senftenbergia*: 354 MY, *Grammatopteris*: 280 MY [6, 57]) as the minimum ages of monilophytes and leptosporangiate ferns, respectively, and a maximum-age constraint of 500 MY for land plants, in a Bayesian

relaxed clock method using MCMCTREE [58] on the coalescent species tree.

Reconstruction of the evolution of sporangial annulus

Characters of sporangial annulus of the sampled species were observed using a polarized light microscope (Axio Scope.A1, ZEISS) after the fresh and mature sporangia were treated with sodium hypochlorite (NaClO) solution. The evolution of sporangial annulus was reconstructed with likelihood method implemented in Mesquite v2.7.5 [59]. All character states (i.e., vertical annulus, oblique annulus, rudimentary annulus, ex-annulus, apical annulus, transverse annulus, and vestigial annulus) were treated as unordered and equally weighted. To reconstruct character evolution, a maximum likelihood approach using Markov k-state 1 parameter model [60] was applied. To account for phylogenetic uncertainty, the “Trace-characters-over-trees” command was used to calculate ancestral states at each node including probabilities in the context of likelihood reconstructions. To carry out these analyses, characters were plotted onto 100 trees that were sampled in the ML analyses of the combined dataset using RAxML v7. The results were finally summarized as percentage of changes of character states on a given branch among all 100 trees utilizing the option of “Average-frequencies-across-trees”.

Declarations

List of abbreviations

BUSCOs, the basic universal single copy orthologs;

ILS, incomplete lineage sorting;

MY, million years;

PPG, the pteridophyte phylogeny group;

RNA-Seq, transcriptome sequencing.

Additional files

Additional file1: Tables S1 to S2 and Figures S1 to S3.

Availability of data and materials

Raw reads of RNA-Seq for 69 fern species were deposited in GenBank under Bioproject accession number PRJNA281136.

Transcriptome datasets for 69 fern species:
https://figshare.com/s/0f773861b6813f97ff63;

datasets of coalescent-based species tree:
https://figshare.com/s/e5e70c2fd3990e5176d8;

Datasets of concatenation based phylogenetic tree:
https://figshare.com/s/8af236b660f61078e40b;

Alignments: https://figshare.com/s/f835735cb66911ff1ffd;

BUSCO results: https://figshare.com/s/bf999173d04b4c311d46;

Scripts: https://figshare.com/s/b28085ee6a7b69f758e9.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

YHY and HShen conceived of and oversaw the study. YHY, HShen designed, ML, JPS, RW, DMJ and LL implemented the data analyses. YHY, HShen, HJW, XLZ, HShang and YFG collected the specimens. HShen, RZ and YFG prepared the specimens for sequencing. XLZ provides the anatomical data. DMJ, HShen, YHY, JPS, ML, RW, HShang, XLZ and XCZ wrote the manuscript.

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Ethics approval and consent to participate

Not applicable

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Figure legends

Figure 1. Topologies (a-f) adapted from published results [5, 12-14, 26, 33]. Branches with support < 75% were shown using dotted lines; and taxa which differ in their phylogeny locations were shown in different colors.

Figure 2. A working flow diagram showing the major processes of data production and analysis in this study. Three major processes are De novo transcriptome assembly, one-to-one orthologs prediction, and phylogenetic analysis. The rectangles represent the main results and the ellipses represent the main methods and analysis.

Figure 3. Phylogeny of ferns reconstructed by coalescent-based method using nucleotide sequence with divergence times calculated. Support values for the main phylogeny (a) calculated from Matrix 1/Matrix 2 are listed as percentages; * indicates 100%/100%. Representative leave(s), sporangium and the corresponding lineage are labeled with a same number. Simplified topology (b) shows the main linages as in Figure 1. Species in phylogeny (a) and the corresponding lineage in topology (b) are shown in a same color.

Figure 4. Reconstructed evolutionary history of sporangial annulus in ferns. Sampled species with seven types of sporangial annulus are shown in different colours. For each ancient node, percentage of character state of sporangial annulus is shown.

Table 1. Sequencing and assembly information of the transcriptome data. The number of ortholog genes used in Matrix 1 and Matrix 2 were shown.

ID	Species	Clean data (G)	Total reads (clean)	Q30%	Number of contigs	N50 (bp)	Mean (bp)	Genes in Matrix 1	Genes in Matrix 2
RS1	*Pronephrium simplex*	4.7	38045864	91.24	151319	887	581.07	2,168	1,254
RS10	*Antrophyum callifolium*	4.0	32745384	91.76	64107	1819	998.73	2,226	1,305
RS101	*Oleandra musifolia*	4.5	36487068	91.45	37075	1493	919.3	2,093	1,248
RS103	*Woodsia polystichoides*	3.9	31465870	90.91	47812	1348	811.3	2,287	1,310
RS107	*Equisetum diffusum*	4.4	35693238	90.21	88932	1154	655.64	1,811	1,254
RS108	*Oreogrammitis dorsipila*	4.6	37037324	90.57	266540	591	485.1	2,141	1,273
RS11	*Vandenboschia striata*	4.8	38639790	90.3	261724	460	422.76	1,959	1,276
RS111	*Pleurosoriopsis makinoi*	4.8	38983796	90.13	98187	1145	632.29	2,182	1,277
RS112	*Azolla pinnata subsp. asiatica*	4.4	35735206	90.57	78295	1348	777.92	1,418	839
RS114	*Taenitis blechnoides*	4.1	32898682	90.98	70495	1262	711.3	2,186	1,278
RS115	*Gymnogrammitis dareiformis*	3.9	31630988	89.81	119483	569	449.38	1,996	1,220
RS116	*Schizaea dichotoma*	4.5	36668734	89.6	67422	1350	826.92	2,035	1,285
RS119	*Botrychium japonicum*	4.8	38603000	90.28	85236	1477	846.97	1,866	1,283
RS122	*Goniophlebium niponicum*	4.8	38786214	90.82	54152	1663	951.92	2,279	1,300
RS123	*Arthropteris palisotii*	4.4	35646740	91	50700	1454	891.67	2,286	1,311
RS124	*Matteuccia struthiopteris*	4.2	34080998	90.44	57514	1345	776.52	2,290	1,313
RS127	*Salvinia natans*	4.2	33780056	91.17	79393	1379	767.14	1,905	1,173
RS128	*Woodwardia prolifera*	5.1	40967322	91.63	69931	1557	859.72	2,328	1,328
RS14	*Diplazium viridescens*	4.0	32320416	90.46	88236	1434	780.87	2,269	1,310
RS16	*Bolbitis appendiculata*	4.7	37503336	91.66	201426	802	556.39	2,226	1,288
RS17	*Dryopteris pseudocaenopteris*	4.1	33136196	91.23	102751	723	514.92	2,236	1,298
RS18	*Dicranopteris pedata*	4.2	33942120	92.04	74011	1193	684.09	2,031	1,304
RS19	*Haplopteris amboinensis*	4.2	42772168	94.17	47603	1713	1041.8	2,249	1,307
RS21	*Psilotum nudum*	8.5	85199034	93.6	66212	1739	927.19	1,741	1,223
RS24	*Cyclopeltis crenata*	4.6	37158058	91.5	29668	600	491.82	2,146	1,279
RS25	*Asplenium formosae*	4.6	46629754	93.5	73318	1722	989.84	2,273	1,312
RS27	*Lomariopsis spectabilis*	4.1	33233594	91.77	98030	1466	750.42	2,225	1,304
RS28	*Cheiropleuria bicuspis*	5.1	41617294	91.35	99411	1435	832.82	2,022	1,295
RS31	*Plagiogyria japonica*	5.7	46472760	91.92	89532	1258	733.9	2,036	1,222
RS34	*Alsophila podophylla*	4.9	48768608	93.43	66254	1580	904.62	2,195	1,289
RS35	*Histiopteris incisa*	4.3	43115390	93.81	61231	1749	985.03	2,319	1,316
RS36	*Pteris vittata*	4.1	41212858	94.37	76666	1868	1021.13	2,296	1,312
RS37	*Cibotium barometz*	4.1	33263550	91.92	85555	1612	891.87	1,790	1,099
RS38	*Osmunda japonica*	4.1	33485274	92.05	58612	1730	901.28	1,732	1,159
RS39	*Loxogramme chinensis*	3.9	31392952	92.16	84796	1065	651.88	2,240	1,305
RS4	*Microlepia hookeriana*	4.0	40561422	94.49	95951	1610	874.06	2,262	1,301
RS41	*Pteridium aquilinum*	4.6	46157134	93.51	55615	1742	960.37	2,321	1,316

RS42	*Hypolepis punctata*	4.4	43828154	93.56	59717	1371	833.68	2,277	1,308
RS43	*Dicksonia antarctica*	3.9	31210608	91.69	56494	1533	902.96	2,045	1,213
RS45	*Rhachidosorus mesosorus*	4.4	35348994	91.98	80069	1541	835.92	2,300	1,315
RS46	*Drynaria bonii*	4.5	36017548	92.02	68132	1077	643.93	2,176	1,279
RS47	*Platycerium bifurcatum*	4.1	33209740	91.62	40456	1097	694.56	2,148	1,283
RS48	*Angiopteris fokiensis*	4.4	35120302	91.12	57637	1629	932.57	1,917	1,306
RS5	*Diplaziopsis brunoniana*	4.3	34698846	91.35	70184	822	541.31	2,040	1,234
RS50	*Dennstaedtia pilosella*	4.5	45618446	93.63	84813	1582	831.56	2,308	1,313
RS51	*Monachosorum henryi*	4.1	41658504	93.42	87832	1465	803.17	2,255	1,288
RS52	*Acystopteris japonica*	5.5	44662146	91.15	57118	1507	873.59	1,222	677
RS53	*Monachosorum maximowiczii*	4.8	48497004	93.58	101448	1817	899.54	2,257	1,294
RS54	*Dennstaedtia scabra*	5.1	51360716	93.47	92158	1565	845.44	1,818	1,056
RS56	*Arachniodes nigrospinosa*	5.1	50929362	94.47	57168	1623	916.1	2,332	1,319
RS69	*Cheilanthes chusana*	5.2	51851066	94.18	49449	1727	1012.63	2,317	1,324
RS7	*Elaphoglossum mcclurei*	4.1	32800248	92.31	57330	1398	846.79	2,267	1,299
RS70	*Lomagramma matthewii*	4.4	35218876	91.21	65170	1748	947.18	2,258	1,307
RS71	*Osmolindsaea odorata*	4.6	46808646	94.13	113778	1521	845.96	2,257	1,312
RS72	*Aleuritopteris chrysophylla*	4.8	47955674	94.18	61637	1669	929.63	2,307	1,322
RS77	*Marsilea quadrifolia*	4.3	34724432	91.76	65227	1607	930.31	2,188	1,299
RS8	*Humata repens*	4.5	36606746	91.17	68932	1267	690.35	2,264	1,315
RS81	*Tectaria subpedata*	4.2	42539482	94.43	57384	1326	797.83	2,128	1,242
RS84	*Ophioglossum vulgatum*	4.4	35637330	91.77	71821	1226	741.62	1,631	1,179
RS85	*Nephrolepis cordifolia*	5.0	40063236	90.81	55207	1530	842.63	2,302	1,319
RS86	*Microlepia platyphylla*	4.6	46324294	94	74956	1763	945.87	2,267	1,295
RS88	*Lygodium flexuosum*	4.2	34098316	91.44	66751	1514	867.82	2,064	1,296
RS89	*Hypodematium crenatum*	4.1	32711798	91.58	52813	1416	852.57	2,298	1,319
RS90	*Acrostichum aureum*	5.4	43422574	90.69	46189	1729	1043.2	2,303	1,319
RS91	*Adiantum caudatum*	5.1	51062204	94.23	51145	1575	950.49	2,323	1,327
RS92	*Parahemionitis cordata*	4.1	33309450	91.72	47508	1456	894.42	2,306	1,317
RS93	*Microlepia speluncae*	4.4	44124842	94.55	94980	1720	917.59	2,292	1,308
RS97	*Stenochlaena palustris*	4.7	37887642	91.81	58416	1655	945.83	2,300	1,316
RS98	*Ceratopteris thalictroides*	3.9	31741082.0	91.4	74728	1610	912.26	2,231	1,296

Table 2. Inconsistent topologies using different methods and sequences.

Site	Coalescent-based method		Concatenation-based method	
	nucleotide	amino-acid	nucleotide	amino-acid
A	**(Anfo,(Pnu,(Ovu,Bja)))**	**(Anfo,(Pnu,(Ovu,Bja)))**	((Pnu,(Ovu,Bja)),(Anfo,#))	((Pnu,(Ovu,Bja)),(Anfo,#))
B	**(Cbi,(Dpe,Vst))**	**(Cbi,(Dpe,Vst))**	**(Cbi,(Dpe,Vst))**	((Dpe,Vst),(Cbi,#))
C	**(Asfo,(Aja,(Dbr,#)))**	**(Asfo,(Aja,(Dbr,#)))**	**(Asfo,(Aja,(Dbr,#)))**	(Asfo,((Aja,Dbr),#))
D	**(Dvi,(Mst,(Spa,Wpr)))**	((Dvi,Mst),(Spa,Wpr))	**(Dvi,(Mst,(Spa,Wpr)))**	**(Dvi,(Mst,(Spa,Wpr)))**
E	**(Bap,(Emc,Lma))**	(Emc,(Bap,Lma))	**(Bap,(Emc,Lma))**	(Emc,(Bap,Lma))
F	**(Nco,((Tsu,Apa),#))**	(Nco,(Tsu,(Apa,#)))	**(Nco,((Tsu,Apa),#))**	**(Nco,((Tsu,Apa),#))**

(A) Anfo: *Angiopteris fokiensis*, Pnu: *Psilotum nudum*, Ovu: *Ophioglossum vulgatum*, Bja: *Botrychium japonicum*; (B) Cbi: Cheiropleuria bicuspis, Dpe: *Dicranopteris pedata*, Vst: *Vandenboschia striata*; (C) Asfo: Asplenium formosae, Aja: *Acystopteris japonica*, Dbr: *Diplaziopsis brunoniana*; (D) Dvi: *Diplazium viridescens*, Mst: *Matteuccia struthiopteris*, Spa: *Stenochlaena palustris*, Wpr: *Woodwardia prolifera*; (E) Bap: *Bolbitis appendiculata*, Emc: Elaphoglossum mcclurei, Lma: *Lomagramma matthewii*; (F) Nco: *Nephrolepis cordifolia*, Tsu: *Tectaria subpedata*, Apa: *Arthropteris palisotii*. # indicates other sampled species within this lineage. Topologies consistent with the one yielded from coalescent-based method and nucleotide sequences are shown in bold.

Figure 1

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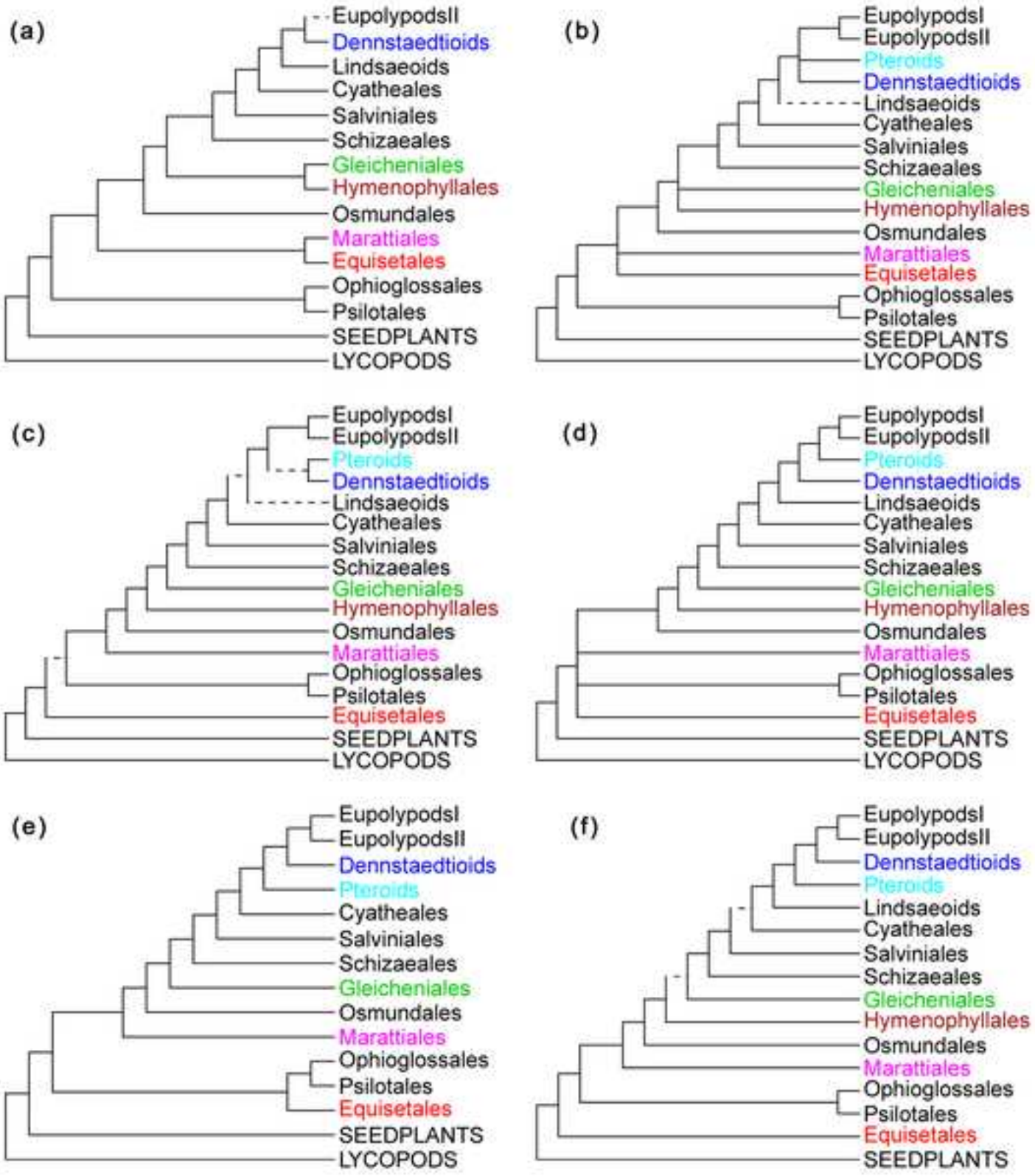

Figure 2

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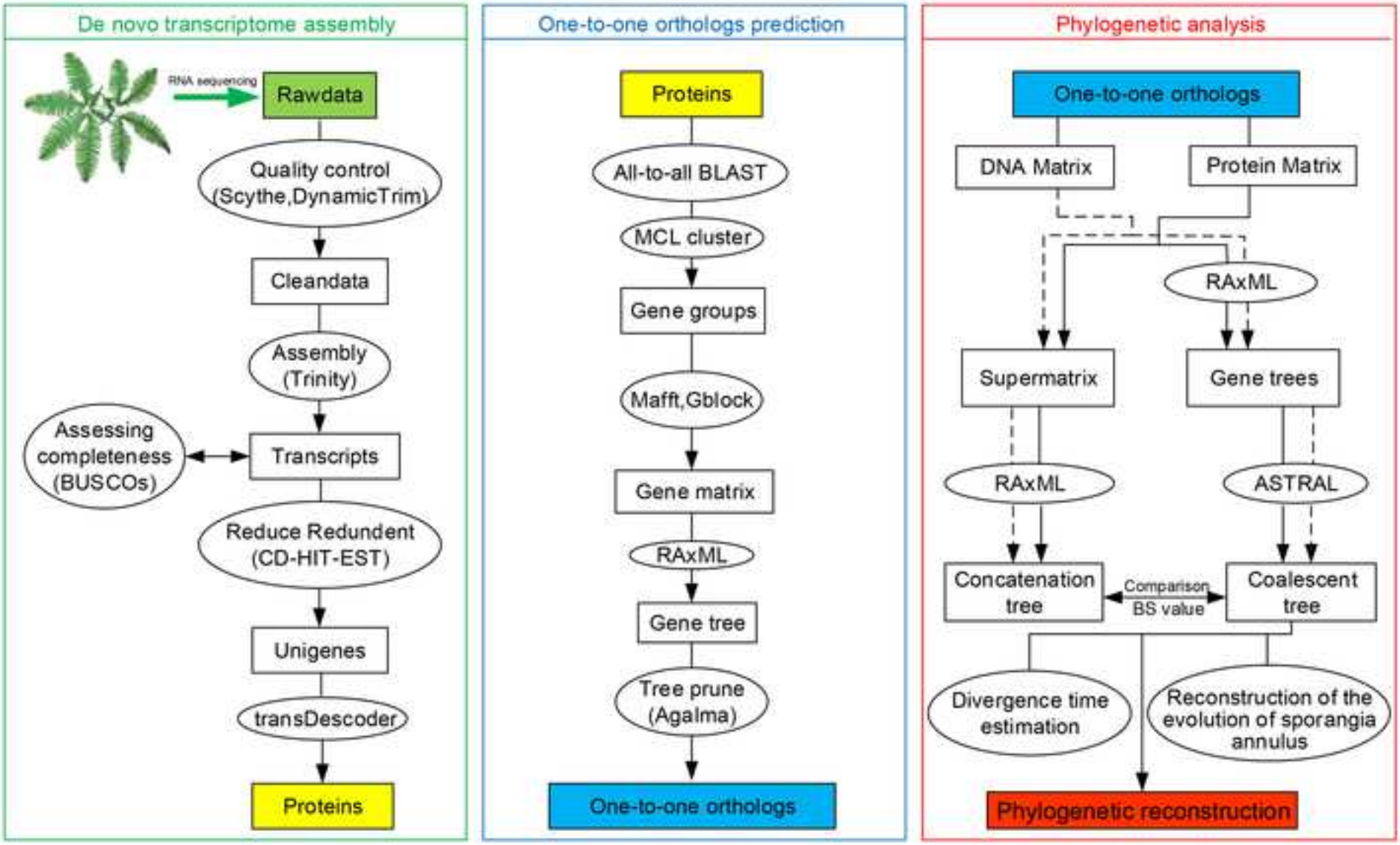

Figure 3

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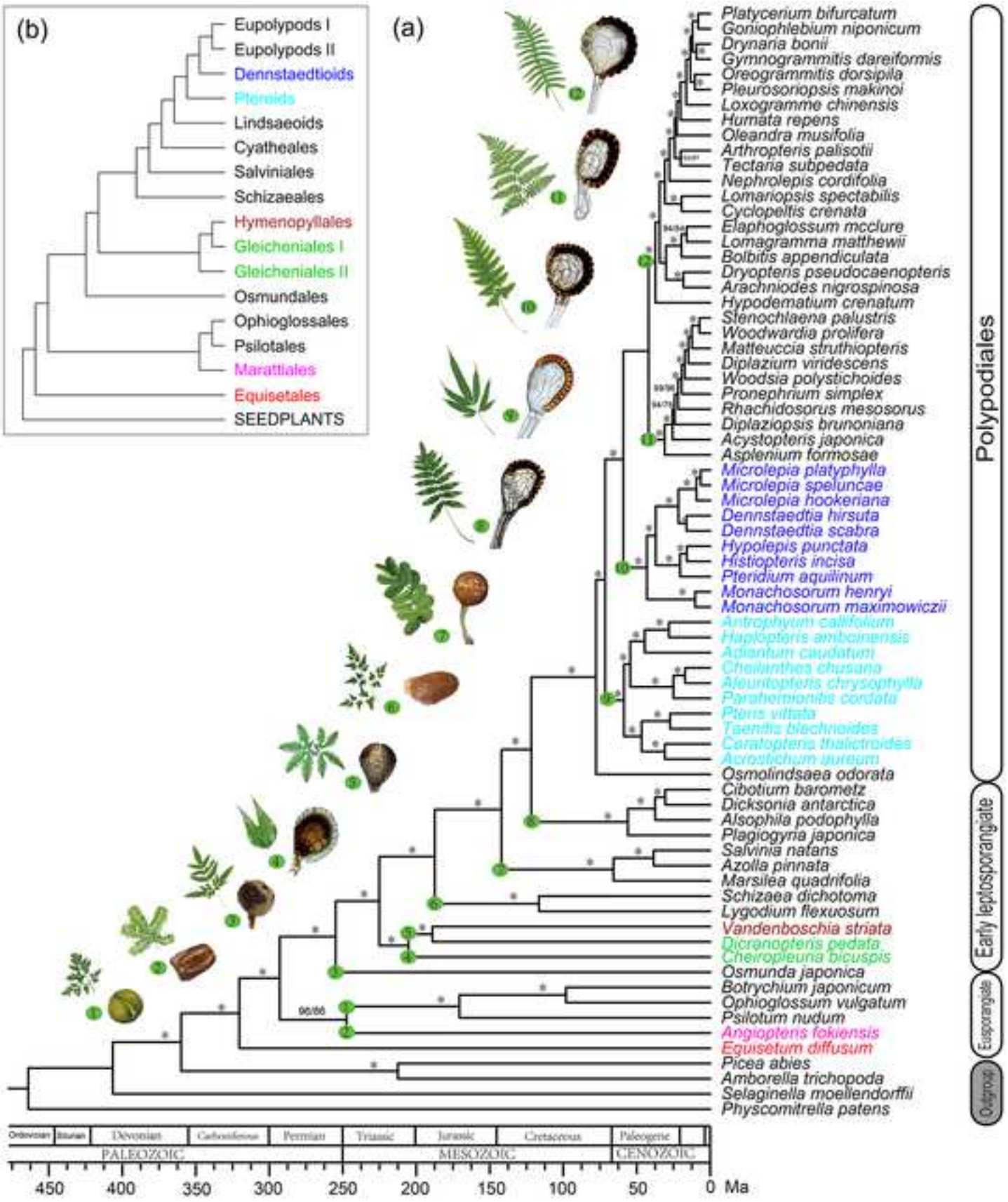

Figure 4

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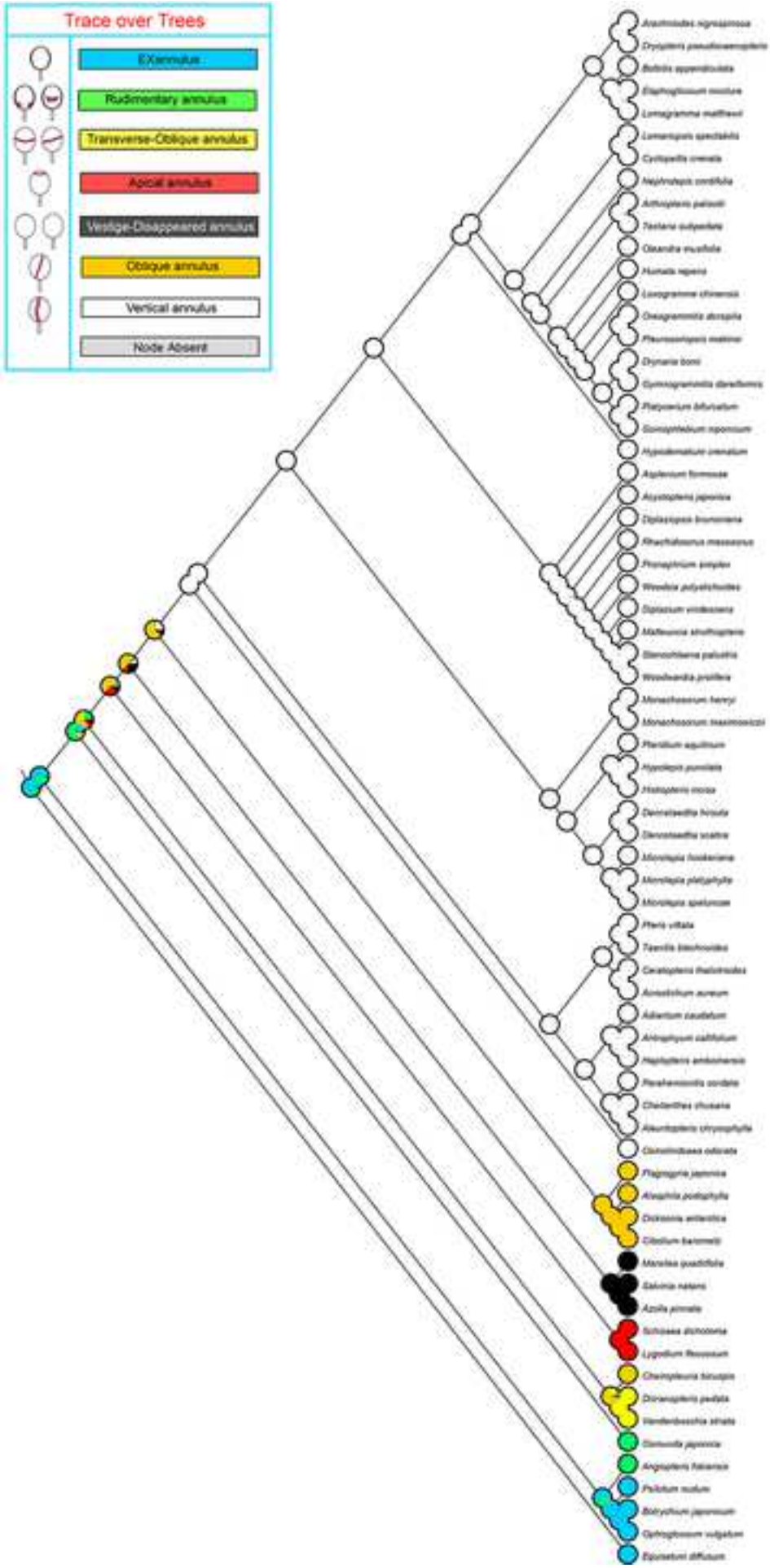

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Sep. 1st, 2017

Dear Editor for GigaScience:

We have revised a manuscript entitled "**Large scale phylogenomic analysis resolves a backbone phylogeny in ferns**" (formerly manuscript number: GIGA-D-17-00009) for your consideration to be published in **GigaScience**. The materials in the manuscript have not been published, nor are under consideration for publication elsewhere.

All the suggestions and comments by the reviewers have been carefully considered and mostly adopted in our revised manuscript. The major revisions includes:

1. We re-interpreted the evolutionary history of sporangial annulus in ferns rigidly according to the results of reconstructed evolutionary history as in Figure 4, and the "ladderized thinking" are carefully avoided (Page 11, line 220-231);
2. We made a careful discussion about the inconsistencies between the results yielded from concatenation-based and coalescent-based methods using DNA and Protein sequences (Page8, Line 168 to Line171).

Thank you very much for handling our manuscript. I am looking forward to hearing your decision soon.

Sincerely yours,
Yue-Hong Yan