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A Roadmap for Fern Genome Sequencing

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Abstract.—The large genomes of ferns have long deterred genome sequencing efforts. To date, only two heterosporous ferns with remarkably small genomes, *Azolla filiculoides* and *Salvinia cucullata*, have been sequenced. However, as sequencing technologies continue to improve and become more affordable, generating high-quality, "normal-sized" fern genomes is within reach. Here we provide new genome size data and discuss candidates for whole genome sequencing. In particular, we identified 18 species representing major branches in the fern phylogeny that are worth pursuing. We also review the current sequencing technologies and offer our opinions on the best sequencing approach for these fern species.

Key Words.—evolutionary genomics, flow cytometry, genome size, phylogeny

The past decade has witnessed a tremendous "genomic bloom," with over three hundred plant genomes published (Fig. 1; https://plabipd.de/portal/sequenced-plant-genomes). This is largely due to the rapid advancement of sequencing technologies; for example, the genome of *Arabidopsis thaliana* (L.) Heynh. that once cost US $100 million can now be obtained for less than $1,000 on a portable Nanopore MinION sequencer (Michael *et al.*, 2018).

However, the vast majority of the available plant genomes are from seed plants (Fig. 1), and only seven seed-free plant genomes have been sequenced. While such an imbalance had been predicted and cautioned by Pryer *et al.* (2002) in the early Sanger-era of genome sequencing, a large gap in the seed-free lineages still persists today. The lack of genomic information from all branches of the plant tree of life has not only limited research on the major transitions in plant evolution, but also hindered investigations into the biology of seed-free plants.

Ferns are one of the final frontiers in the exploration of plant genome space. Although fern genomes have long been regarded as too prohibitively large for sequencing, there are remarkable exceptions. Obermayer *et al.* (2002) reported a genome size of 0.75 gigabase (Gb) for *Azolla* in the heterosporous order Salviniales, which is significantly smaller than those of homosporous ferns. In our recent survey of genome sizes in Salviniales, we not only were able to confirm the *Azolla* genome size, but also discovered the *Salvinia cucullata* Roxb. genome to be merely 0.26 Gb, a size only moderately larger than *A. thaliana*. We recently completed the genomes of *Azolla filiculoides* Lam. and *S. cucullata* (Li *et al.*, 2018), which offered the first insights into fern genome space and evolution. It should be noted, however, that both of the sequenced

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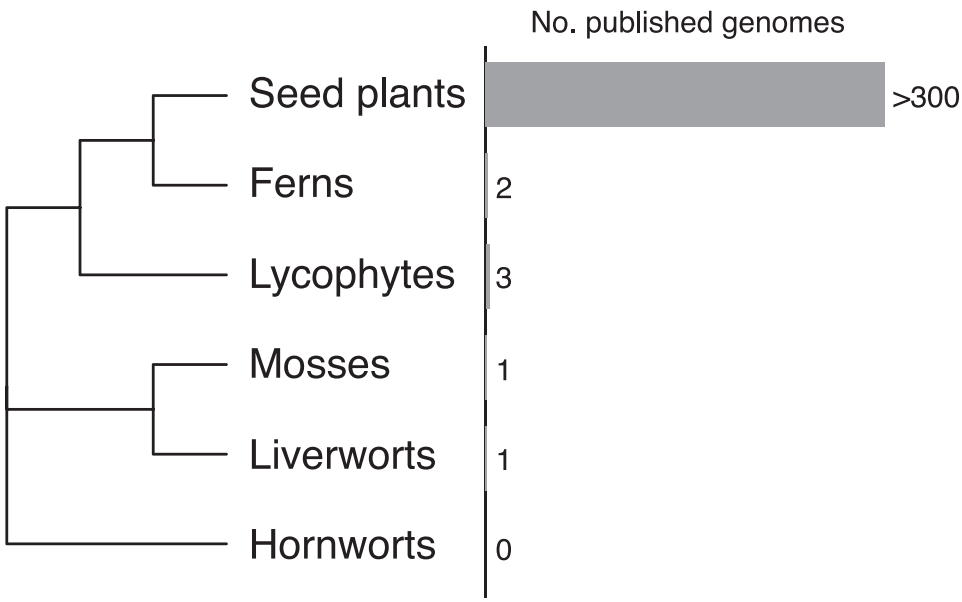

FIG. 1. The current landscape of published plant genomes.

species belong to Salviniaceae and it is unclear whether these small-sized genomes are representative of ferns as a whole. In other words, we need more fern genomes.

The goal of this paper is to identify candidates for future genome sequencing. We generated 35 new C-value estimates using flow cytometry and compiled the most up-to-date genome size data for ferns. We identified 18 species representing major branches in the fern phylogeny that are worth pursuing initially.

MATERIALS AND METHODS

Sampling.—Fresh leaf and spore materials of 35 species (covering 15 families and 20 genera) were collected from the field, the Dr. Cecilia Koo Botanic Conservation Center, or the Taipei Botanical Garden. This taxon sampling focused on the families and genera that have been reported to have relatively small genome sizes (Clark *et al*., 2016), or have little C-value information. Voucher information is provided in Table 1.

Flow cytometry.—We used flow cytometry to estimate genome sizes following the protocol of Kuo *et al*. (2017) and Kuo and Huang (2017). Various chopping buffers were used, including the Beckman, LB01, and GPB buffers. All of them have 0.5% (v/v) 2-mercaptoethanol, and RNaseA 0.1 mg/ml. 4% PVP-40 was used for Beckman and LB01, and 3% for GPB. Other details of buffer preparation and storage condition can be found in Doležel, Binarova, and Lucretti (1989), Ebihara *et al*. (2005), and Loureiro *et al*. (2007). For the

internal standard, we used *Pisum sativum* cv. "Ctirad", *Nicotiana tabacum* cv. "Xanthi", *Secale cereale* cv. "Dankovske", *Vicia faba* cv. "Inovec", or *Zea mays* "CE-777"; their genome sizes were reported by Doležel, Sgorbati, and Lucretti (1992) and Doležel *et al.* (1998), Lysak and Doležel (1998), and Johnston *et al.* (1999). Samples were run on the BD FACSCan system (BD Biosciences, Franklin Lake, New Jersey). For both the sample and internal standard, we aimed to have a coefficient of variation (CV) <5% and >1,000 particles collected for each nuclei peak. Three replicates (six for *Cibotium taiwanense* C.M. Kuo) per sample were carried out, and the average estimates are reported here.

Results and Discussion

Summary of flow cytometric analyses.—The results of our flow cytometric analyses are summarized in Table 1. All the nuclei peaks met our quality (CV) and quantity (number of particles) criteria, excepting for *Diplopterygium glaucum* (Thunb. ex Houtt.) Nakai and *D. chinensis* (Rosenst.) DeVol, which have a slightly wider average CV: 5.06 and 5.03%, respectively (Table 1).

Distribution of fern genome size.—Fern genome size (1C) ranges significantly from 0.26 Gb in *Salvinia cucullata* to 73.19 Gb in *Tmesipteris elongata* Dangeard (Fig. 2, 3). This translates into a striking 282-fold difference. Of the 48 fern families recognized in PPG I, 38 have genome size data. When considering only the minimum 1C value within each family, the median and mean estimates are 6.3 Gb and 9.4 Gb respectively (Fig. 2). Salviniales (heterosporous) have the smallest genomes—from 0.26 Gb in *S. cucullata* (Salviniaceae) to 1.4 Gb in *Marsilea minuta* L. (Marsileaceae). Among the homosporous orders, Gleicheniales stood out in particular with only 1.95 Gb in *Diplopterygium chinensis* (Gleicheniaceae) and 2.4 Gb in *Dipteris conjugata* Reinw. (Dipteridaceae; Clark *et al.*, 2016).

Ferns to sequence.—To select candidates for future genome sequencing, we focused on species that (1) occupy distinct branches in the fern phylogeny and (2) have genomes smaller than 6.5 Gb, which we believe with the current technologies is the upper limit for a genome to be realistically sequenced and assembled with less than $24,000. The only exception is *Ceratopteris richardii* Brongn., whose genome size is 11.25 Gb (Wolf *et al.*, 2015); this is because *C. richardii* is genetically trackable and has been used as a powerful model species (see Marchant, 2019 and Petlewski and Li, 2019 in this issue). A total of 18 fern species was identified (Fig. 3 and Table 2), together covering 15 families and 7 orders (Fig. 3).

Table 2 also lists our cost estimates for sequencing each of these genomes. Currently the best sequencing approach for *de novo* assembly is by using long-read technologies such as PacBio and nanopore (Li and Harkess, 2018). However, achieving high enough long-read coverage (usually 50X) can be very costly for large genomes, and one alternative is to adopt a hybrid assembly strategy that utilizes a lower long-read coverage (20X) plus ~50X Illumina short reads. While being more economical (Table 2), this hybrid approach

TABLE 1. Genome size estimates reported in this study.

Family	Species	Voucher information (herbarium)	Organ type	Buffer used	1C genome size Gb (1 standard deviation)	Sample peak CV (%)	Standard[1]	Standard peak CV (%)
Cibotiaceae	Cibotium taiwanense C.M.Kuo	Kuo4395 (TAIF); KBCC no. K017423	Spores	GPB	5.01 (0.06)	4.26	Pisum sativum cv. "Ctirad"	2.1
Dennstaedtiaceae	Dennstaedtia wilfordii (T.Moore) Christ	Kuo4430 (TAIF)	Spores	LB01	4.61 (0.01)	3.43	Nicotiana tabacum cv. "Xanthi"	2.38
Dennstaedtiaceae	Dennstaedtia hirsuta (Sw.) Mett. ex Miq.	Kuo4412 (TAIF)	Leaves	GPB	3.42 (0.02)	4.52	Nicotiana tabacum cv. "Xanthi"	3.74
Dennstaedtiaceae	Dennstaedtia scandens (Blume) T.Moore	Kuo4405 (TAIF)	Leaves	LB01	4.26 (0.02)	3.76	Nicotiana tabacum cv. "Xanthi"	3.01
Dicksoniaceae	Calochlaena straminea M.D.Turner & R.A.White	Wade2728 (TAIF)	Spores	Beckman	6.80 (0.01)	3.21	Nicotiana tabacum cv. "Xanthi"	2.16
Dipteridaceae	Cheiropleuria intergrifolia (D.C.Eaton ex Hook.) M.Kato, Y.Yatabe, Sahashi & N.Murak.	Kuo4241 (TAIF)	Leaves	Beckman	14.89 (0.01)	3.03	Nicotiana tabacum cv. "Xanthi"	4.25
Dipteridaceae	Dipteris conjugata Reinw.	Kuo4309 (TAIF)	Leaves	GPB	2.91 (0.01)	4.92	Nicotiana tabacum cv. "Xanthi"	4.02
Dipteridaceae	Dipteris lobbianta (Hook.) T.Moore	KBCC no. K038265	Leaves	GPB	5.81 (0)	3.78	Secale cereale cv. "Dankovske"	3.18
Dipteridaceae	Cheiropleuria bicuspis (Blume) C.Presl	Wade5310 (TAIF)	Leaves	LB01	18.90 (0.01)	2.91	Vicia faba cv. "Inovec"	3.31
Gleicheniaceae	Diplopterygium blotianum (C.Chr.) Nakai	Kuo4408 (TAIF)	Leaves	GPB	1.95 (0.01)	4.26	Nicotiana tabacum cv. "Xanthi"	2.68
Gleicheniaceae	Diplopterygium glaucum (Thunb. ex Houtt.) Nakai	Kuo4408 (TAIF)	Leaves	LB01	2.25 (0)	5.06	Nicotiana tabacum cv. "Xanthi"	3.08
Gleicheniaceae	Diplopterygium chinensis (Rosenst.) DeVol	Kuo4410 (TAIF)	Leaves	LB01	1.95 (0.01)	5.03	Nicotiana tabacum cv. "Xanthi"	2.81
Lindsaeaceae	Odontosoria biflora (Kaulf.) C.Chr.	Wade5051 (TAIF); KBCC no. K056946	Leaves	GPB	3.55 (0.01)	4.26	Pisum sativum cv. "Ctirad"	3.5
Lygodiaceae	Lygodium microphyllum (Cav.) R.Br.	Lu30898 (TAIF)	Leaves	GPB	5.56 (0.01)	2.45	Nicotiana tabacum cv. "Xanthi"	2.19

TABLE 1. Continued.

Family	Species	Voucher information (herbarium)	Organ type	Buffer used	1C genome size Gb (1 standard deviation)	Sample peak CV (%)	Standard[1]	Standard peak CV (%)
Lygodiaceae	Lygodium circinnatum (Burm.f.) Sw.	Hung313 (TNM)	Leaves	GPB	9.34 (0.01)	2.95	Nicotiana tabacum cv. "Xanthi"	3.64
Marattiaceae	Ptisana robusta (Alston) Senterre & Rouhan	Kuo4309 (TAIF); KBCC no. K020575	Leaves	GPB	4.43 (0.01)	2.5	Secale cereale cv. "Dankovske"	2.41
Nephrolepidaceae	Nephrolepis cordifolia (L.) C.Presl	KBCC no. K013665	Leaves	GPB	7.24 (0.15)	3.26	Nicotiana tabacum cv. "Xanthi"	3.75
Plagiogyriaceae	Plagiogyria adnata (Blume) Bedd.	Kuo4244 (TAIF)	Leaves	Beckman	23.34 (0.06)	2.08	Nicotiana tabacum cv. "Xanthi"	4.21
Plagiogyriaceae	Plagiogyria falcata Copel.	Kuo4245 (TAIF)	Leaves	Beckman	10.76 (0.04)	1.97	Nicotiana tabacum cv. "Xanthi"	2.99
Plagiogyriaceae	Plagiogyria stenoptera (Hance) Diels	Kuo4268 (TAIF)	Leaves	Beckman	12.32 (0.03)	2.46	Nicotiana tabacum cv. "Xanthi"	4.05
Plagiogyriaceae	Plagiogyria euphlebia (Kunze) Mett.	Kuo4269 (TAIF)	Leaves	Beckman	13.67 (0.1)	2.38	Nicotiana tabacum cv. "Xanthi"	2.73
Plagiogyriaceae	Plagiogyria falcata Copel.	Kuo4281 (TAIF)	Leaves	Beckman	11.03 (0.01)	3.09	Nicotiana tabacum cv. "Xanthi"	3.69
Polypodiaceae	Lepisorus pseudoussuriensis Tagawa	Kuo4411 (TAIF)	Leaves	GPB	6.36 (0.01)	2.07	Pisum sativum cv. "Ctirad"	3.19
Pteridaceae	Acrostichum aureum L.	cultivated in Taipei Botanical Garden; TFRI spore bank no. 684	Spores	Beckman	24.60 (0.08)	3.68	Nicotiana tabacum cv. "Xanthi"	4.25
Pteridaceae	Adiantum caudatum L.	Kuo4195 (TAIF); KBCC no. K023756	Leaves	Beckman	3.78 (0)	3.55	Nicotiana tabacum cv. "Xanthi"	3.19
Pteridaceae	Anogramma leptophylla (L.) Link	Kuo4453 (TAIF)	Leaves	Beckman	3.00 (0.01)	3.84	Nicotiana tabacum cv. "Xanthi"	2.31
Pteridaceae	Vaginularia junghuhnii Mett.	Lin131 (TAIF)	Leaves	GPB	10.46 (0.03)	3.02	Nicotiana tabacum cv. "Xanthi"	4.44
Pteridaceae	Cryptogramma brunoniana Wall. ex Hook. & Grev.	Kuo4458 (TAIF)	Leaves	Beckman	4.33 (0.01)	4.23	Secale cereale cv. "Dankovske"	3.12

TABLE 1. Continued.

Family	Species	Voucher information (herbarium)	Organ type	Buffer used	1C genome size Gb (1 standard deviation)	Sample peak CV (%)	Standard[1]	Standard peak CV (%)
Salviniaceae	Salvinia molesta D.S.Mitch.	KBCC no. K060545	Leaves	Beckman	2.43 (0)	3.45	Nicotiana tabacum cv. "Xanthi"	2.03
Salviniaceae	Salvinia oblongifolia Mart.	Kuo4441 (TAIF)	Leaves	LB01	2.57 (0.01)	4.78	Nicotiana tabacum cv. "Xanthi"	3.34
Salviniaceae	Salvinia natans (L.) All.	cultivated in Taipei Botanical Garden	Leaves	Beckman	1.82 (0)	3.83	Zea mays "CE-777"	2.98
Thelypteridaceae	Thelypteris palustris Schott	Kuo4440 (TAIF)	Leaves	LB01	7.25 (0.01)	3.57	Nicotiana tabacum cv. "Xanthi"	3.63
Woodsiaceae	Woodsia polystichoides D.C.Eaton	Kuo4431 (TAIF)	Spores	LB01	4.70 (0.01)	3.87	Nicotiana tabacum cv. "Xanthi"	2.9
Woodsiaceae	Woodsia manchuriensis Hook.	Kuo4435 (TAIF)	Spores	LB01	6.04 (0.02)	4.06	Nicotiana tabacum cv. "Xanthi"	3.04
Woodsiaceae	Woodsia polystichoides D.C.Eaton	Kuo4448 (TAIF)	Spores	LB01	4.20 (0.02)	4.55	Nicotiana tabacum cv. "Xanthi"	2.98

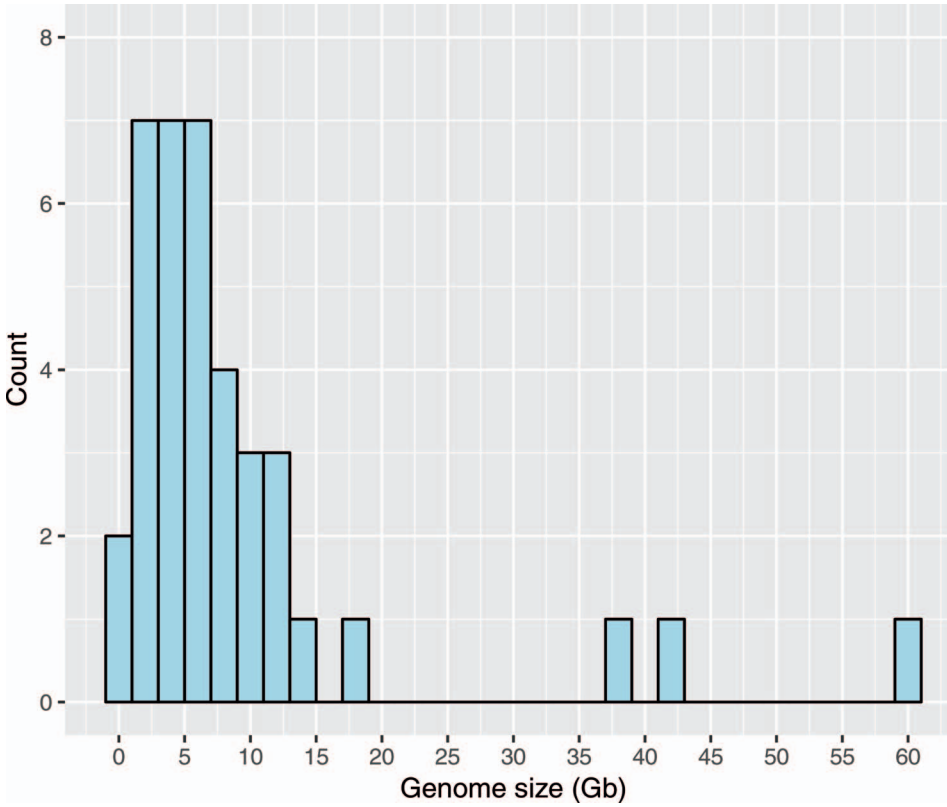

FIG. 2. The distribution of fern genome sizes at the family level. For each family only the lowest estimate was included.

typically results in a less continuous assembly than that based solely on long reads.

Our calculations of the sequencing cost were based on various online quotes and our own experience. For long reads, nanopore is probably less expensive than PacBio on a per Gb basis; we estimated $68.75 per Gb for nanopore, given a 12Gb output per flowcell and each flowcell and the associated reagents costing $825 (the cost per flowcell depends on the order size; it can be as low as $500). For Illumina, we estimated $26.5 per Gb, given that one lane of HiSeq4000 generates roughly 100Gb of data, and each lane plus library prep costs around $2,650.

Another possible sequencing strategy for large genomes is using 10X Genomics to generate long linked-reads (Li and Harkess, 2018). While this technology has been very successful in producing high-quality genomes for several animal species, it has not been widely tested in plants. Nevertheless, because of the low cost (roughly $3,000 for a 1 Gb genome), 10X Genomics could be a technology to consider in the next five years for fern genome sequencing.

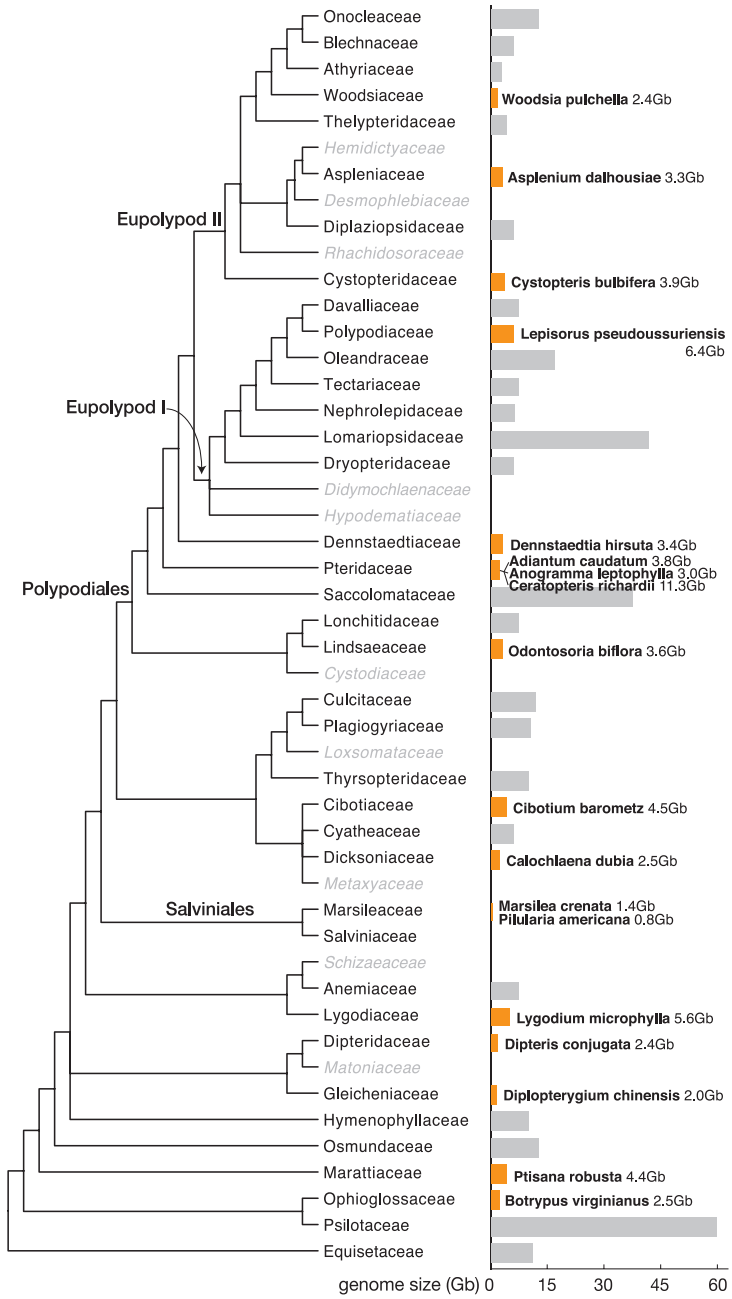

FIG. 3. Phylogenetic placement of candidate species for future whole genome sequencing efforts and their genome sizes (orange bars). Only the lowest size estimate was included for each fern family.

TABLE 2. Candidate species for future whole genome sequencing efforts and cost estimates.

Species	Family	Order	Genome size (Gb)	50X nanopore	20X nanopore + 50X Illumina
Botrypus virginianus	Ophiogolossaceae	Ohioglossales	2.5; Bai et al. (2012)	$8,594	$6,750
Ptisana robusta	Marattiaceae	Marattiales	4.4; this study	$15,125	$11,880
Diplopterygium chinensis	Gleicheniacea	Gleicheniales	2; this study	$6,875	$5,400
Dipteris conjugata	Dipteridaceae	Gleicheniales	2.4; Clark et al. (2016)	$8,250	$6,480
Lygodium microphylla	Lygodiaceae	Schizaeales	5.6; this study	$19,250	$15,120
Pilularia americana	Marsileaceae	Salviniales	0.8; Li et al. (2018)	$2,750	$2,160
Marsilea crenata	Marsileaceae	Salviniales	1.4; Li et al. (2018)	$4,813	$3,780
Calochlaena dubia	Dicksoniaceae	Cyatheales	2.5; Clark et al. (2016)	$8,594	$6,750
Cibotium barometz	Cibotiaceae	Cyatheales	4.5; Clark et al. (2016)	$15,469	$12,150
Odontosoria biflora	Lindsaeaceae	Polypodiales: Lindsaeineae	3.6; this study	$12,375	$9,720
Anogramma leptophylla	Pteridaceae	Polypodiales: Pteridineae	3; Baker et al. (2014)	$10,313	$8,100
Adiantum caudatum	Pteridaceae	Polypodiales: Pteridineae	3.8; this study	$13,063	$10,260
Ceratopteris richardii	Pteridaceae	Polypodiales: Pteridineae	11.25; Wolf et al. (2015)	$38,672	$30,375
Dennstaedtia hirsuta	Dennstaedtiaceae	Polypodiales: Dennstaedtiineae	3.4; this study	$11,688	$9,180
Lepisorus pseudoussuriensis	Polypodiaceae	Polypodiales: Polypodiineae	6.4; this study	$22,000	$17,280
Cystopteris bulbifera	Cystopteridaceae	Polypodiales: Aspleniineae	3.9; Henry et al. (2015)	$13,406	$10,530
Asplenium dalhousiae	Aspleniaceae	Polypodiales: Aspleniineae	3.3; Clark et al. (2016)	$11,344	$8,910
Woodsia pulchella	Woodsiaceae	Polypodiales: Aspleniineae	2.4; Kruk et al. (2015)	$8,250	$6,480

Additional considerations.—Although we identified 18 candidates based on genome size and phylogenetic position, future work still needs to carefully assess the availability of plant materials, feasibility to obtain high quality DNA, and degree of heterozygosity. Genome sequencing, especially in the case of long reads, requires a large quantity (>20 ug) of high molecular weight DNA (>50 kb average length). This is not a trivial task and optimization is likely needed for each species. Therefore, it would be ideal to have the target plant stably growing in a botanical garden, greenhouse, or growth chamber, so that repeated DNA extractions can be obtained from the same plant. Furthermore, transcriptomes from various organs (e.g., roots, rhizomes, and leaves) are fundamental for annotating genes in a genome assembly, which again requires a living plant.

A highly heterozygous genome would be much harder to assemble than a homozygous one. Heterozygosity can be assessed by low-coverage Illumina sequencing (20-40X) and should be done before committing the all-in, expensive long-read sequencing. Many fern species are capable of gametophytic selfing (Haufler *et al.,* 2016), which results in completely homozygous offspring. Generating such a homozygous individual might save a considerable amount of sequencing cost and make downstream bioinformatic analyses significantly easier.

It should also be noted that the currently available genome size data are not evenly distributed, and certain species/genera/families have disproportionally more estimates than others. Future work should also focus on the families that lack any genome size data and those that have very few estimates.

Summary.—With the rapid improvement of sequencing technologies and the plummeting cost, it is becoming increasingly easy to sequence plant genomes. However, careful planning is absolutely required to decide which species to sequence and how to sequence. In this paper, we compiled the most up-to-date fern genome size data, and identified 18 species with medium-sized genomes that span the fern tree of life. We anticipate this list of candidates will provide a clear roadmap going forward not only to better understand the fern genome space, but also to tackle long-standing questions on polyploidy and chromosome evolution in ferns.

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