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Source: American Fern Journal, 109(3) : 192-211

Published By: The American Fern Society

URL: <https://doi.org/10.1640/0002-8444-109.3.192>

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Ferns: The Final Frond-tier in Plant Model Systems

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ABSTRACT.—Ferns are one of the most speciose lineages of land plants, and occupy an important phylogenetic position sister to seed plants. Despite this, ferns remain one of the last groups of land plants that do not have a fully developed model system. Here we review the biology and status of each emerging fern model. While reference genomes have been completed for *Azolla filiculoides* and *Salvinia cucullata*, they lack transformation capability. Meanwhile, other ferns including *Marsilea vestita*, *Adiantum capillus-veneris*, *Pteris vittata*, and *Ceratopteris richardii* have transformation methods, but lack genomic resources. Nevertheless, as genome sequencing becomes increasingly more affordable, we believe that *M. vestita* and *C. richardii* can become powerful fern models, which represent heterosporous and homosporous ferns, respectively.

KEY WORDS.—*Ceratopteris*, genomes, *Marsilea*, model systems, transformation

With over 12,000 species (PPG I, 2016), ferns are a diverse lineage that is integral to understanding terrestrial plant evolution and ecology. For example: How has the diversity of life cycles evolved? What genetic, molecular, and ecological mechanisms have governed major life cycle changes? What mechanisms control the tracheophyte body plan and how have they evolved through time?

The alternation of generations (phases) life cycle is common to all embryophytes. However, the life cycle of ferns and lycophytes is unique because the sporophyte and gametophyte generations are independent, and the sporophyte is generally considered the dominant phase (although gametophytes of certain species can be long-lived, e.g., *Vittaria appalachiana* (Farrar, 1967)). This contrasts with bryophytes, which produce a sporophyte that is dependent on the dominant gametophyte, and seed plants in which gametophytes are highly reduced, encased within, and completely dependent on the dominant sporophyte. The transition between the haploid-dominant and sporophyte-dominant life cycle likely had a profound impact on plant evolution (Gerrienne and Genez, 2011; Haufler *et al.*, 2016; Qiu, Taylor and McManus, 2012). Ferns are therefore an ideal system to study the mechanisms that determine sporophyte and gametophyte development in comparison to other land plants. Land plant life cycles also vary in the types of spores they produce and the sexual condition of the gametophytes. For example, the Salviniales, Selaginellales, Isoëtales, and all seed plants are heterosporous, meaning the sporophyte produces megaspores (in megasporangia) and

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microspores (in microsporangia). These spores germinate and become gametophytes that are endosporic (i.e., they develop within the spore wall) and dioicous. The vast majority of ferns, on the other hand, are exosporic and homosporous, meaning the sporophyte produces spores that are all the same size and that have the potential to develop into monoicous gametophytes. *Platyzoma microphyllum* R.Br. (Pteridaceae) is a curious outlier to these two general life cycle formats—it is heterosporous, but exosporic and can produce monoicous gametophytes (Tryon and Vida, 1967; Duckett and Pang, 2014). The transition between homospority and heterospority has occurred independently many times across land plant evolution. Ferns are an excellent system to provide insight into the mechanisms that govern this transition by comparing the biology of the homosporous ferns to the heterosporous ferns (Salviniales) and the seed plants. Furthermore, across vascular plants, homospority is correlated with a higher chromosome numbers and larger genome sizes compared to heterospority. While the reason for this correlation is not fully understood (Wolf *et al.*, 2015), it has been hypothesized that high levels of polyploidy could maintain genetic diversity in organisms with a high potential for inbreeding, linked with the production of monoicous gametophytes (Klekowski and Baker, 1966; but see Haufler and Soltis, 1986). Indeed, homosporous ferns have notoriously large genomes (average=13.82 Gb, range=1.95 - 73.19 Gb; Kuo & Li, 2019) and represent one of the final frontiers in exploring plant genome space.

Ferns are also pivotal in studying the evolutionary development of vascular plant body plans. Stem-leaf-root anatomy has evolved in the sporophyte multiple times throughout the history of land plants (Boyce, 2005; Hetherington and Dolan, 2018). To truly understand the mechanisms that govern the evolution of this anatomy, comparative genetic studies need to compare multiple lineages of land plants. For example, by focusing on a lycophyte *Selaginella kraussiana* (Kunze) A. Braun and a fern *Osmunda regalis* L., Harrison *et al.* (2005) were able to show that the same genetic pathway (in this case *KNOX-ARP* interaction) was independently recruited for the convergent evolution of leaves. Similar comparative studies in the future will provide not only new insights into the field of plant Evo-Devo, but will also reveal the genetic mechanisms that may have developed uniquely in ferns.

Although ferns are essential to understanding land plant evolution through comparisons with other lineages, they are also interesting in their own right. The sex of many homosporous ferns can be influenced by their environment, a phenomenon unknown in other plant lineages. For example, the gametophytes of proposed model *Ceratopteris richardii* may be either male or hermaphroditic. Hermaphroditic gametophytes secrete a compound called antheridiogen, which promotes development of neighboring gametophytes as exclusively antheridial (Eberle *et al.*, 1995). Other biotic and abiotic factors, such as light (Kamachi *et al.*, 2007) and soil bacteria (Ganger *et al.*, 2019), can also influence gametophyte sex expression. In addition, no other plants are known to engage in a vertically transmitted cyanobacterial symbiosis like *Azolla* (Wagner, 1997), making *Azolla* an excellent system to study symbiosis biology. And

finally, few plants can accumulate arsenic levels as high as *Pteris vittata* L. (Ma *et al.*, 2001), making it an ideal system for studying the cellular and molecular mechanisms involved in heavy metal tolerance.

Despite their utility for studying plant evolution, development, molecular biology, and ecology, a model fern has not yet been completely developed. A fully functional model requires a high-quality reference genome and efficient transformation methods. Additionally, they should not be polyploid, should be easily maintained in a laboratory environment, and have a relatively short generation time. Among ferns, only the genomes of *Azolla filiculoides* Lam. and *Salvinia cucullata* Roxb. have been sequenced (Li *et al.*, 2018), but genetic manipulation tools are lacking in these species (Table 1). On the other hand, transformation techniques have been developed for species that lack a fully sequenced genome, including: *Marsilea vestita* Hook. & Grev., *Adiantum capillus-veneris* L., *Pteris vittata*, and *Ceratopteris richardii* Brongn. (Table 1; Bui *et al.*, 2015; Kawai *et al.*, 2003; Kawai-Tooyooka *et al.*, 2004; Klink and Wolniak, 2000; Muthukumar *et al.*, 2013; Plackett *et al.*, 2014, 2015; Stout *et al.*, 2003). In this review, we describe potential model ferns, as well as the tools and resources that have already been developed for each.

AZOLLA FILICULOIDES & SALVINIA CUCULLATA

Azolla and *Salvinia* are genera of floating aquatic ferns belonging to the Salviniaceae. This family and its sister lineage Marsileaceae, are the only heterosporous ferns (Figure 2; PPG 1, 2016). The relationships of species within the genus *Azolla* require further resolution, but it likely contains five to seven species (Evrard and Van Hove, 2004; Metzgar, Schneider, and Pryer, 2007; Reid, Plunkett, and Peters, 2006). *Salvinia* consists of around twelve species, and the position of *S. cucullata*, in particular, is uncertain (Nagalingum, Schneider and Pryer, 2008).

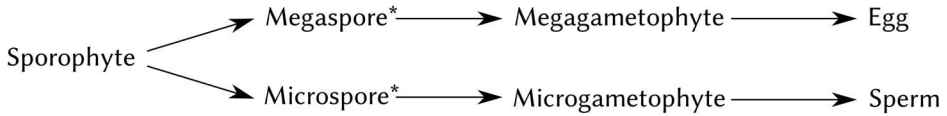
Azolla is well known for housing an obligate, vertically transmitted nitrogen-fixing cyanobacterium (*Nostoc azollae*) within specialized leaf cavities. Because of this symbiosis, *Azolla* has been used for over 1,000 years to fertilize rice paddies in Southeastern Asia (Lumpkin and Plucknett, 1980). Additionally, due to its fast growth rate and high protein content, *Azolla* has been used as supplementary feed for poultry (Basak *et al.*, 2002), fish (Abou *et al.*, 2007), and livestock (Cherryl *et al.*, 2013). *Azolla* has also been investigated as a means to treat wastewater contaminated with pollutants like arsenic (Leão *et al.*, 2017), synthetic textile dyes (Kooh *et al.*, 2016a, 2016b), swine waste (Muradov *et al.*, 2014), fluoride (Zazouli *et al.*, 2014), excess nitrogen and phosphorus (Forni *et al.*, 2001), and zinc (Zhao *et al.*, 1999). Finally, using *Azolla* as a biofuel has been pursued as extracted lipids are suitable for the synthesis of biodiesel and meet requirements on fuel density, cetane number, and iodine value (Brouwer *et al.*, 2016; Salehzadeh, Maeemi and Arasteh, 2014).

Salvinia plants grow in large mats, lack roots, have two floating leaves and a highly dissected, submerged leaf, which bears clusters of sori (Nagalingum,

TABLE 1. Summary of characteristics and available tools for each prospective model.

Species	Family	Ploidy	Generation time	Genome	RNAi	DNAi	Transformation methods		
							Agrobacterium-mediated	Particle bombardment	CRISPR/Cas9
<i>Azolla filiculoides</i>	Salviniaceae	Diploid	Unclear, but likely a few months	Li <i>et al.</i> 2018	-	-	-	-	-
<i>Salvinia cucullata</i>	Salviniaceae	Diploid	N/A (not sexually reproductive in lab)	Li <i>et al.</i> 2018	-	-	-	-	-
<i>Marsilea vestita</i>	Marsileaceae	Diploid	Unclear	-	Klink and Wolniak 2000	-	-	-	-
<i>Adiantum capillus-veneris</i>	Pteridaceae	Diploid	Unclear	-	-	Kawai-Tooyoka <i>et al.</i> 2004	-	Kawai <i>et al.</i> 2003	-
<i>Pteris vittata</i>	Pteridaceae	Tetraploid	Unclear	-	-	-	Muthukumar <i>et al.</i> 2013	-	-
<i>Ceratopteris richardii</i>	Pteridaceae	Diploid	120 days	Marchant <i>et al.</i> <i>In review</i>	Stout <i>et al.</i> 2003	Rutherford <i>et al.</i> 2004	Muthukumar <i>et al.</i> 2013; Bui <i>et al.</i> 2015	Plackett <i>et al.</i> 2014, 2015	-

Heterosporous life cycle (Salviniales, seed plants, some lycophytes)



Homosporous life cycle (most ferns, Lycopodiaceae)

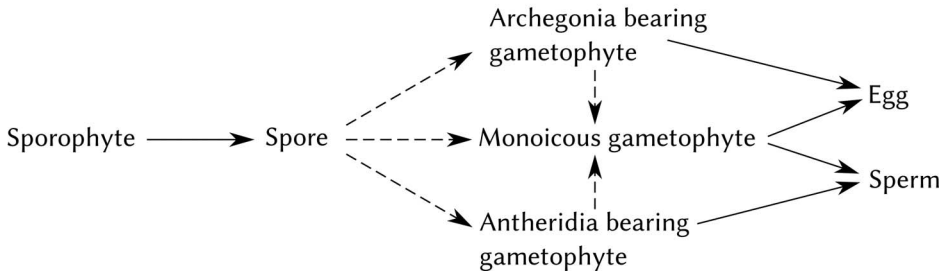


FIG. 1. Comparing the general form of homosporous and heterosporous plant life cycles. Some lycophytes, all seed plants, and Salviniales (including the prospective models *Azolla filiculoides*, *Salvinia cucullata*, and *Marsilea vestita*) are heterosporous and produce endosporic, dioicous gametophytes. The majority of the ferns (including *Adiantum capillus-veneris*, *Pteris vittata*, and *Ceratopteris richardii*) are homosporous and produce exosporic, potentially monoicous gametophytes. *Must be produced in megasporangia and microsporangia, respectively. Dashed lines represent possible paths of gametophyte development.

Schneider, and Pryer, 2006; Nagalingum, Nowak, and Pryer, 2008). In contrast to *Azolla's* reputation as a generally beneficial plant, *Salvinia* is most well known as a noxious weed. With the exception of some limited research into the phytoremediation capacity of *Salvinia* (Baral *et al.*, 2009), much of the biological research concerning this genus has involved controlling its weedy tendency (Room *et al.*, 1981; Room, 1990). In the materials sciences, *Salvinia* has garnered attention for its ability to retain air on its leaf surfaces (termed the “*Salvinia Effect*”) by means of a unique combination of hydrophilic patches on a highly hydrophobic surface (Barthlott *et al.*, 2010).

Genomic resources.—*Azolla filiculoides* and *Salvinia cucullata* both are diploid and have relatively small genomes (0.75 Gb in *A. filiculoides* and 0.26 Gb in *S. cucullata*). They are the only two ferns for which genomes have been sequenced (Li *et al.*, 2018). The assembled genomes have N50 contig sizes of 964.7 Kb for *A. filiculoides* and 719.8 Kb for *S. cucullata*. A high level of completeness was indicated for both assemblies by BUSCO (Benchmarking Universal Single-Copy Orthologs) assessment and Illumina read-mapping results. Li *et al.* (2018) identified 20,201 high confidence gene models in *A. filiculoides* and 19,914 in *S. cucullata* that were supported by transcripts or were significantly similar to other known plant proteins. Additionally, medium-coverage resequencing was done on five other *Azolla* species, laying the foundation for future pan-genome and trait association studies. Genomic

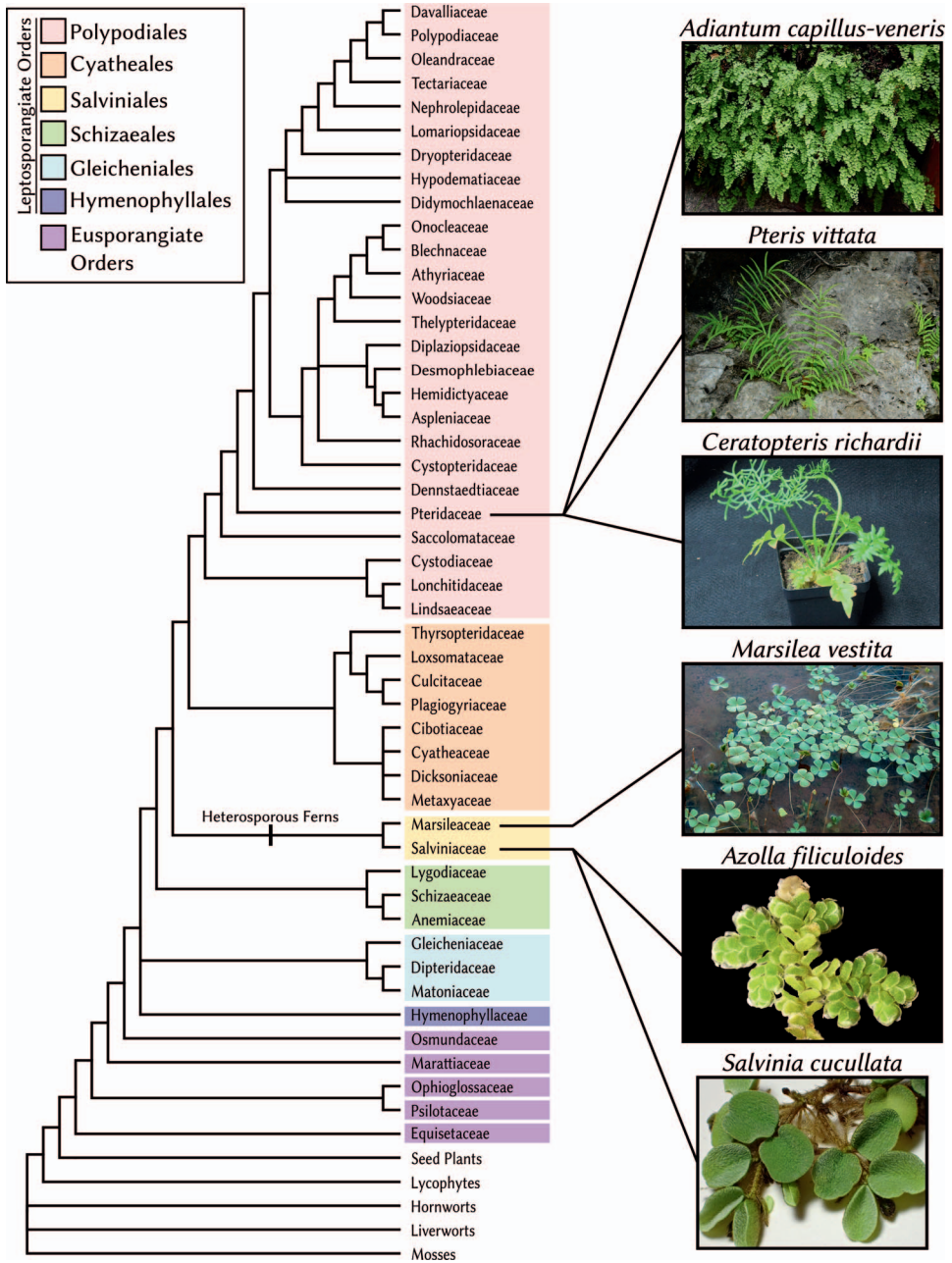


FIG. 2. Phylogeny of fern families, highlighting prospective models. Photo credits: Pi-Fong Lu (*A. capillus-veneris*, *P. vittata*, and *S. cucullata*), Chi-Lien Cheng (*C. richardii*), Wikimedia Commons (*M. vestita*).

and transcriptomic data are available at FernBase (www.fernbase.org), along with genome browsers and BLAST utilities.

Available tools and technologies.—Brouwer *et al.* (2014) described a series of methods for collecting *A. filiculoides* spores, spore germination and *in vitro* fertilization, and cryopreservation of fertilized megaspores with their *N. azollae* symbiont. Both *A. filiculoides* and *S. cucullata* are relatively easy to maintain by growing in water in a growth chamber or greenhouse.

Missing tools.—Currently no transformation method has been developed for this lineage. Past studies have shown that it is feasible to generate protoplasts and callus tissues from several *Azolla* (Redford *et al.*, 1987; Sini, Smitha, and Madhusoodanan, 2014) and *Salvinia* species (Nakamura and Maeda, 1994), which could be useful for future development of transformation methods. It should also be noted that sexual reproduction of *S. cucullata* is seldom observed in the lab environment, which is not ideal for a model system.

Assessment as a model fern.—Both *A. filiculoides* and *S. cucullata* lack significant resources required of a model organism. Although neither *A. filiculoides* nor *S. cucullata* are particularly representative of the fern lineage as a whole, *A. filiculoides* would make an excellent model for studying plant-bacterial symbioses.

MARSILEA VESTITA

Marsileaceae represents the other family of heterosporous ferns, sister to Salviniaceae (Figure 2). *Marsilea*, sometimes called the “water clover,” is a cosmopolitan genus of about 50 species (Whitten, Jacono, and Nagalingum, 2012) of aquatic to amphibious perennial ferns, which spread by rhizomes that may be floating, creeping, or subterranean (Jacono and Johnson, 2006). One particular *Marsilea* species, *M. vestita*, has been the focus of diverse developmental studies. Yet, the exact taxonomic placement of *M. vestita* remains unclear (Whitten, Jacono, and Nagalingum, 2012).

The leaves of *Marsilea* are unlike those of any other fern, consisting of four terminal leaflets in a cruciform arrangement borne on a petiole. It is the only group of ferns to exhibit true nyctinasty, or daily movement of leaf orientation (Minorsky, 2018). This leaf arrangement is in contrast to the closely related genera *Regnellidium* (with two leaflets) and *Pilularia* (with a highly reduced, filiform leaf) (Pryer and Hearn, 2009). The highly desiccation tolerant reproductive structures of *Marsilea*, called sporocarps, consist of a stalk (termed “peduncle,” “stipe,” or “pedicel”) and a sclerified wall surrounding bisporangiate sori (Nagalingum, Schneider and Pryer, 2006, 2007).

Marsilea vestita has been used as a model to study its uniquely rapid process of spermatogenesis (Wolniak *et al.*, 2011). Upon rehydration of a microspore, a microgametophyte develops endosporically and spermatogenesis completes within 12 hours, releasing 32 highly flagellated sperms. The pioneering work by Stephen Wolniak and colleagues has shown that the rapid development of microgametophytes relies on translating stored RNA from microspores, and there is little or no *de novo* gene transcription (Boothby *et al.*, 2013). At

different stages of microgametophyte development, specific pools of stored pre-mRNAs are spliced to remove introns and enable translation. Through RNAi silencing, it was further demonstrated that such stage-specific mRNA maturation is required for proper gametophyte development (Boothby *et al.*, 2013; Wolniak *et al.*, 2011). A similar process also likely underlies megagametophyte development in *M. vestita* (Kuligowski, Ferrand, and Chenou, 1991), but few follow-up studies have been done.

Available tools and technologies.—*Marsilea vestita* can be easily grown in a container of water in a growth chamber or greenhouse environment. The history of research on *M. vestita* has resulted in numerous tools that will be useful to its development as a model system. A number of methods have been developed to localize mRNA transcripts during microgametophyte development in *M. vestita* (Boothby and Wolniak, 2011; Deeb *et al.*, 2010; Kuligowski, Ferrand, and Chenou, 1991; Tsai, Van Der Weele, and Wolniak, 2004; Van Der Weele, Tsai, and Wolniak, 2007). Most importantly, *M. vestita* was the first fern in which RNAi was applied to manipulate gene expression (Klink and Wolniak, 2000, 2001, 2003; Tsai and Wolniak, 2001). This was done by directly incubating microspores in the double-stranded RNA solution. It is, however, unclear if the same approach can be readily applied in other tissue types, and whether the silencing effect can be passed beyond fertilization to the sporophyte generation.

Missing tools.—Although there are several RNA-sequencing datasets published (Boothby *et al.*, 2013; Tomei and Wolniak, 2016), no reference genome exists for *M. vestita*. Because the smallest *Marsilea* genome reported to date is only 1.34Gb (Li *et al.*, 2018; Kuo and Li, 2019), sequencing and assembling the *M. vestita* genome would likely be easier than a large homosporous fern genome. It should be noted that the generation time (from spore to spore) of *M. vestita* is unclear, as most of the past research focused on spermatogenesis that does not require the completion of life cycle.

Assessment as a model fern.—Lacking genomic data is not a significant hurdle in the effort to make *M. vestita* a model fern. Given the interest in addressing fundamental questions of life cycle evolution using ferns, a model heterosporous fern would be highly desirable, even if it is not representative of the entire fern lineage. The numerous studies conducted on *M. vestita* spermatogenesis make it a good system for studying mechanisms of cell biology, as well as a promising candidate to become a model heterosporous fern.

ADIANTUM CAPILLUS-VENERIS

Adiantum is a cosmopolitan, homosporous genus of 225 species in the Pteridaceae, nested within the leptosporangiate ferns (Figure 2; Huiet *et al.*, 2018; PPG 1, 2016). Molecular analyses revealed that *Adiantum* is sister to the vittarioids (shoestring ferns), despite stark morphological and ecological differences (Pryer *et al.*, 2016; Schuettpelz and Pryer, 2007). The sporophytes of *Adiantum* are terrestrial, shade-loving, and bear compound leaves which,

when fertile, produce sporangia only on false indusia. *Adiantum* gametophytes are determinate and cordate, with a distinct midrib and broad wings. Vittarioid sporophytes, on the other hand, are epiphytic and have simple, strap-shaped leaves with sori borne on the lamina. Vittarioid gametophytes are indeterminate, strap shaped, exceptionally long-lived, and can reproduce asexually via gemmae. Additionally, it is likely that at least one genome duplication occurred in the vittarioids after splitting from *Adiantum* (Pryer *et al.*, 2016). The stark morphological, ecological, and genomic differences that exist between these sister lineages make them ideal for comparative studies to elucidate the genetic mechanisms that control traits like leaf shape and determinate versus indeterminate growth of gametophytes.

Adiantum, in particular *A. capillus-veneris*, is perhaps most notable because it has served as a model to study photobiology. Masamitsu Wada and colleagues have used *A. capillus-veneris* to make several breakthroughs in phototropism, polarotropism, and chloroplast movement (Doi and Shimazaki, 2008; Imaizumi, 2000; Doi, Wada, and Shimazaki, 2006; Tsuboi *et al.*, 2012). Several photoreceptors have also been functionally characterized in detail, including neochrome, a phytochrome-phototropin chimeric receptor (Li and Mathews, 2016; Wada, 2013). In addition, the first complete fern plastome was generated from *A. capillus-veneris* (Wolf *et al.*, 2003), which was used to study levels of RNA-editing in the chloroplast genome (Wolf, Rowe, and Hasebe, 2004).

Available tools and technologies.—It is possible to manipulate gene expression in *A. capillus-veneris* via both gene overexpression and silencing. Genetic transformation by particle bombardment has been developed for gametophyte tissues and has been applied to rescue photoreceptor mutants (Kawai *et al.*, 2003), as well as to localize gene expression in conjunction with GUS (Tsuboi *et al.*, 2012). In addition, Kawai-Toyooka *et al.* (2004) showed that gene silencing can be achieved by bombarding gametophytes with double-stranded DNA instead of a traditional RNAi approach. This DNAi method can produce up to 90% gene silencing efficiencies.

Missing tools.—While an EST library from gametophytes (Yamauchi *et al.*, 2005) and a leaf transcriptome (Qi *et al.*, 2018) have been published, no whole genome sequence has yet been generated for *A. capillus-veneris*. The genome size is unknown, although likely to be around 4–6Gb based on the estimates from congeneric species (Bainard *et al.*, 2011; Kuo and Li, 2019).

Assessment as a model fern.—In terms of morphology, reproductive strategy, and habit, *A. capillus-veneris* is a good representative of the fern lineage. It has been a useful system to study fern photobiology, and could provide insights into fundamental evo-devo questions, especially in comparison with the vittarioids.

PTERIS VITTATA

Pteris is also a homosporous, leptosporangiate fern in the Pteridaceae (Figure 2; PPG1, 2016). The genus has now been recovered as monophyletic

and represents one of the largest fern genera, containing three subgenera and 200–250 species distributed globally (Zhang and Zhang, 2018). This diversity is reflected in the breadth of anatomy and habitat in which *Pteris* species are found.

In 2001, *Pteris vittata* was discovered to hyperaccumulate arsenic (as high as 1% dry weight; Ma *et al.*, 2001). Though some other species in *Pteris* have also been found to possess this ability, *P. vittata* is the most efficient (Luongo and Ma, 2005). Because of its potential for phytoremediation applications, much of the research conducted on *P. vittata* has focused on elucidating the mechanism of arsenic accumulation (Cesaro *et al.*, 2015; Datta *et al.*, 2017; Gu *et al.*, 2018; reviewed in Xie *et al.*, 2009). *Pteris vittata* gametophytes, in particular, were proposed as a model system for analyzing arsenic hyperaccumulation because the rapid growth rate, small size, ease of culture, and haploid genome are more conducive to research than the sporophytes (Gumaelius *et al.*, 2004). A number of transporters from *P. vittata* have been identified that mediate arsenite uptake (DiTusa *et al.*, 2016; He *et al.*, 2016; Indriolo *et al.*, 2010). Moreover, expression of a *P. vittata* arsenite antiporter *ACR3;1* was able to reduce arsenic accumulation in shoots of *Arabidopsis* and tobacco, demonstrating a potential application in crops (Chen *et al.*, 2017).

Available tools and technologies.—To test the function of *ACR3* in *P. vittata*, a gene knock-down method by RNAi was developed, in which the RNAi constructs were biolistically bombarded into gametophyte tissues (Indriolo *et al.*, 2010). Muthukumar *et al.* (2013) later showed that stable transformation can be achieved by co-incubation of spores with *Agrobacterium tumefaciens*. The transformation efficiency was reported to be around 0.05% (Muthukumar *et al.*, 2013).

Missing tools.—There is not yet a genome sequence for *P. vittata*, nor is there any published information on its genome size. Additionally, it should be noted that *P. vittata* is predominantly a tetraploid, and diploid individuals have a restricted geographical distribution (Srivastava, Ranade, and Khare, 2007). Furthermore, there is a mixture of reproductive strategies in this species, including sexual and apomictic, along with a range of ploidy levels (Chao *et al.*, 2012). The identification and collection of a sexual diploid strain would be vital to the future development of *P. vittata* as a model. Finally, there is no published guideline or manual on how to grow and maintain *P. vittata* in the lab.

Assessment as a model fern.—*P. vittata* lacks two of the most fundamental aspects of a model organism: genomic data and diploid representatives. While its development as a model organism would provide unique insight into arsenic tolerance and accumulation, significant resources would have to be dedicated to filling these gaps.

CERATOPTERIS RICHARDII

Ceratopteris is also a homosporous, leptosporangiate fern in the Pteridaceae, though the taxonomy within the genus remains somewhat unsettled (March-

ant, 2019). Polyploids and interspecific hybrids appear to be common in this genus (Lloyd, 1974), and further systematic work is needed, especially if members of this genus are to be used as model organisms.

Ceratopteris has been dubbed “the *Arabidopsis* of the fern world” (Sessa *et al.*, 2014). It has emerged as the most promising fern model species, largely because it can be readily cultured and transformed in the lab. *Ceratopteris richardii* is a diploid ($n=39$) aquatic fern that has been identified from Africa, Southeastern Asia, Japan, Australia, Fiji, and the Hawaiian Islands, although its highest concentration is in the Americas. It grows as an annual with a short, upright rhizome and slender, dimorphic leaves and divergent branches. Its short generation time (the life cycle can be completed in about 120 days) and small size make it a convenient lab model. Mature sporophytes are only about 5cm tall and spread over a diameter of less than 3cm, and gametophytes are even smaller. Thus, large numbers of plants can be cultured in a small growth chamber or greenhouse, enabling mutant screens. Plants can be vegetatively propagated easily from buds found on senescing fronds. Additionally, protoplasts can be isolated from gametophytes, and can regenerate to produce cultures (Edwards and Roux, 1998). A single mature sporophyte can produce around one million spores (per plant), which can be stored and remain viable for years (Chatterjee and Roux, 2000). Furthermore, a fast-growing cultivar strain of *C. richardii*, marketed as the “C-fern,” has been used as an educational model for K-12 and undergraduate instruction (Renzaglia and Warne, 1995). The “C-fern express” (also marketed for classroom use), on the other hand, is *C. thalictroides* (L.) Brongn., a tetraploid species (Hickok, Warne, and Fribourg, 1995).

Ceratopteris richardii is capable of intra-gametophytic self fertilization, resulting in completely homozygous sporophyte offspring (Hauffer *et al.*, 2016). At least three homozygous strains have been established (Hickok, Warne, and Fribourg, 1995). The most commonly used strain is Hn-n, which came from a spore on an herbarium specimen collected in Cuba. Two other strains are D176 from Guyana and PhiN8 from Nicaragua; they differ in leaf morphology, spore number per sporangium, as well as antheridiogen response (Hickok, Warne, and Fribourg., 1995; McGrath *et al.*, 1994). All *C. richardii* strains can be crossed with each other and yield fertile F1 progeny (Hickok, Warne, and Fribourg, 1995).

Ceratopteris richardii has enabled research on a wide variety of topics, including gametophyte development (Banks, 1999), cell wall development (Leroux *et al.*, 2013), evo-devo (e.g., Hasebe *et al.*, 1998; Sano *et al.*, 2005; Plackett *et al.*, 2018), and plant responses to gravity in space flight (Bushart *et al.*, 2013; Edwards and Roux, 1998; Salmi and Roux, 2008; Salmi *et al.*, 2011). *Ceratopteris richardii* gametophytes in particular have served as a model for elucidating mechanisms of sex determination in ferns. Several sex determination mutants have been identified (Banks, 1994; Banks, 1997a, 1997b; Eberle *et al.*, 1995; Strain, Hass, and Banks, 2001), and studies have been conducted on the hormones involved in sex determination (Atallah and Banks, 2015) as well as the effects of light (Kamachi *et al.*, 2004, 2007; Murata and Sugai, 2000;

Spiro, Torabi, and Cornell, 2004) and the soil microbiome (Ganger *et al.*, 2019) on gametophyte development and sex determination.

Ceratopteris richardii spores are the largest recorded within the homosporous ferns (~70–150 μm in diameter), making them useful for electrophysiological studies on signal transduction pathways, which could be complicated by multicellularity (Chatterjee and Roux, 2000; Chatterjee *et al.*, 2000). The development of genetically identical spores is easy to synchronize, which allows for study and manipulation of the direction of polarity and cell level gravity responses within the first 24 hours of gametophyte development (Chatterjee and Roux, 2000). In 1999, *C. richardii* became the first ever “space fern,” on board Space Shuttle Columbia as a part of the STS-93 shuttle mission (Roux *et al.*, 2003). It was found that in the spaceflight environment, around 5% of the transcripts in the *C. richardii* cDNA microarray were differentially regulated (Salmi and Roux, 2008).

Two recent studies fully explore *C. richardii*'s potential as a model organism. Bui *et al.* (2017) investigated the genetic basis of fern apogamy—a type of asexual reproduction where sporophytes develop directly from gametophyte somatic cells without fertilization. Using a combination of transcriptome-sequencing, *in situ* hybridization, gene overexpression, and RNAi knockdown, it was demonstrated that *C. richardii* *AINTEGUMENTA* is required for promoting apogamous sporophytes (Bui *et al.*, 2017). The second study focused on testing the function of *LEAFY* homologs in *C. richardii* (Plackett *et al.*, 2018). While *LEAFY* in flowering plants is a key transcription factor that marks floral meristems, its role in ferns has been unknown. Using a similar suite of tools to Bui *et al.* (2017), Plackett *et al.* (2018) showed that *C. richardii* *LEAFY* plays an important role in maintaining apical stem cells throughout both sporophyte and gametophyte development.

Available tools and technologies.—*Ceratopteris richardii* is by far the most genetically tractable fern system (Table 1). Early attempts to manipulate gene expression involved RNAi and DNA vector-based gene silencing (Rutherford *et al.*, 2004; Stout *et al.*, 2003). More recently, stable transformation has been achieved for *C. richardii* and *C. thalictroides* (“C-fern express”) using *Agrobacterium*-mediated transformation (Bui *et al.*, 2015; Muthukumar *et al.*, 2013) and particle bombardment (Plackett *et al.*, 2014; Plackett, Rabbino-witsch, and Langdale, 2015). The efficiency can be as high as 87% for transient transformants and 2.6% for stable transformants (Bui *et al.*, 2015). In addition, a genetic linkage map has been produced for *C. richardii* using 488 doubled haploid lines that were genotyped for 368 restriction fragment length polymorphisms, 358 amplified fragment length polymorphisms, and three isozyme markers (Nakazato *et al.*, 2006). This has been used to conduct quantitative trait locus (QTL) analysis to study reproductive barriers in *C. richardii* (Nakazato *et al.*, 2007).

Missing tools.—The 11.25 Gb genome of *C. richardii* has yet to be fully sequenced (Marchant, 2019). Low coverage (1.73X) genome skimming data have been published (Wolf *et al.*, 2015) and the publication of a partially assembled genome is imminent (Marchant *et al.*, 2019).

Assessment as a model fern.—A multitude of useful techniques have been developed for *C. richardii*, and it serves as the most prominent and promising prospective model fern at this point.

CONCLUSIONS AND OUTLOOK

All of the species reviewed here have the potential to become a model fern, but they all currently lack significant components of a complete model system. A genome has only been fully sequenced for *Azolla filiculoides* and *Salvinia cucullata*, yet no transformation methods have been developed for them. The closely related *Marsilea vestita*, on the other hand, lacks a genome, but extensive research has been conducted to develop RNAi methods for this species. If a genome were sequenced for *M. vestita*, which is likely to be one of the smallest genomes in ferns (Li *et al.*, 2018; Kuo and Li, 2019), much of the foundational work has already been completed to make it a promising model heterosporous fern.

Adiantum capillus-veneris has served as a model to study fern photobiology and some transformation methods have been developed; however, the genome has also not been sequenced. Similarly, *Pteris vittata* has garnered attention for its potential in the phytoremediation of arsenic. Though an *Agrobacterium*-mediated transformation method has been developed for *P. vittata*, the lack of a genome sequence and diploid strain hinder its emergence as a model.

The most developed model fern remains *Ceratopteris richardii* (also see Marchant, 2019). It has served as a model fern to tackle the genetic basis of sex determination and apogamy, as well as to elucidate the major transitions in plant evolutionary development. Efficient transformation methods via *Agrobacterium* and particle bombardment have been developed, and as exemplified by two recent studies by Bui *et al.* (2017) and Plackett *et al.* (2018), such genetic tools are powerful to link genes to specific biological functions. Importantly, the robust transformation methods also pave the way for developing CRISPR/Cas9 gene editing capacity, which has yet to be achieved in ferns (Table 1). Nevertheless, the 11.25 Gb genome, though typical of ferns, is limiting the full potential of *C. richardii* as a model organism. Fortunately, with the rapid advancement of sequencing technologies, we believe a complete *C. richardii* genome should be delivered in the near future.

It would, of course, be ideal to establish more than one model organism for ferns. *Marsilea vestita* is diploid, easy to grow in the lab, has not only a small genome that would be easy to sequence, but also numerous developed transformation techniques. Though the generation time is unclear, we envision that *M. vestita* would be the next most readily developed model and would nicely complement the homosporous *C. richardii* to serve as a heterosporous fern model. While using *M. vestita* and *C. richardii* as models will certainly not represent the whole of fern diversity, uniting resources behind these two models will enable plant biologists to study both ferns and the greater trends in land plant evolution.

ACKNOWLEDGMENTS

We thank: Jessica Nelson, Duncan Hauser, and David Wickell for constructive comments; D. Blaine Marchant for information on the *Ceratopteris richardii* genome; Li-Yaung Kuo for information on *Adiantum* genome size; Pi-Fong Lu and Chi-Lien Cheng for providing images; and Paul Wolf and Mike Barker for coordinating this special issue and the two anonymous reviewers for constructive comments.

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