

On the widespread capacity for, and functional significance of, extreme inbreeding in ferns

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Summary

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- Homosporous vascular plants utilize three different mating systems, one of which, gametophytic selfing, is an extreme form of inbreeding only possible in homosporous groups. This mating system results in complete homozygosity in all progeny and has important evolutionary and ecological implications. Ferns are the largest group of homosporous land plants, and the significance of extreme inbreeding for fern evolution has been a subject of debate for decades.
- We cultured gametophytes in the laboratory and quantified the relative frequencies of sporophyte production from isolated and paired gametophytes, and examined associations between breeding systems and several ecological and evolutionary traits.
- The majority of fern species studied show a capacity for gametophytic selfing, producing sporophytes from both isolated and paired gametophytes. While we did not follow sporophytes to maturity to investigate potential detrimental effects of homozygosity at later developmental stages, our results suggest that gametophytic selfing may have greater significance for fern evolution and diversification than has previously been realized.
- We present evidence from the largest study of mating behavior in ferns to date that the capacity for extreme inbreeding is prevalent in this lineage, and we discuss its implications and relevance and make recommendations for future studies of fern mating systems.

Introduction

It may be a valid generalization that many, if not most, homosporous fern taxa have the capacity to produce complete homozygotes by self-fertilization and frequently do so in nature.

Klekowski & Baker (1966)

Like all land plants, ferns experience an 'alternation of generations' in their life cycle that sets them fundamentally apart from animals and other organisms (Klekowski & Baker, 1966). This separation of phases in the life cycle creates a unique set of ecological and evolutionary challenges and opportunities for plants, especially with regard to breeding systems. While mutation is the fundamental mechanism generating variation, recombination followed by sexual reproduction is also critical for introducing novel, heritable variation into populations. The ability to sexually reproduce, and particularly to outcross, substantially increases the genetic variation available to natural selection (Charlesworth & Charlesworth, 1995). The breeding system or systems that a particular lineage employs thus have profound implications for its ecology and evolution (Goodwillie *et al.*, 2005; Neal & Anderson, 2005; Charlesworth, 2006; Johnston *et al.*, 2009), primarily by mediating genetic diversity and structure within and between populations (Soltis & Soltis, 1990; Ingvarsson, 2002; Johnston

et al., 2009). The opportunities and challenges associated with mating systems vary across plant lineages but are particularly evident in the majority of ferns and some lycophytes, which are unique among plants in having free-living gametophytes and sporophytes. If Klekowski and Baker's quote above is accurate, ferns may regularly perform a feat that has significant short-term ecological benefits, but with potentially devastating evolutionary consequences.

The alternation of generations in the plant life cycle involves cyclical transitions between haploid and diploid stages. A diploid sporophyte produces spores via meiosis that germinate into free-living haploid gametophytes capable of producing eggs and sperm via mitosis. Following fertilization and syngamy, diploidy is restored and a new sporophyte emerges from the gametophyte thallus. When plants transition from the diploid to the haploid stage (i.e. at meiosis), two alternatives are possible: homosporous and heterosporous. Homosporous is thought to be the ancestral state in land plants (Bateman & DiMichele, 1994), and in homosporous lineages, meiosis produces haploid isospores that are uniform in size and whose mitotically produced gametophyte products are (or can be) bisexual, producing both male and female gametes. Heterosporous, by contrast, involves the production of megaspores and microspores, often of unequal size, that develop into gametophytes of predetermined sexes (Klekowski &

Lloyd, 1968). Among land plants, all 350 000+ seed plants (angiosperms and gymnosperms) are heterosporous. In their sister group, the ferns, the vast majority of extant taxa (*c.* 9000 species) are homosporous, while a small group of *c.* 100 species are heterosporous (Smith *et al.*, 2006). Both heterosporous and homosporous lineages also occur in the lycophytes, which are sister to the fern plus seed plant clade.

The fundamental differences between homospory and heterospory have direct implications for the type(s) of breeding system available to a particular lineage. In homosporous plants, there are three types of sexual mating system possible (Fig. 1), in addition to asexual systems such as apogamy. These sexual systems include (1) typical outcrossing, in which eggs and sperm are produced by gametophytes derived from two different parental sporophyte individuals; (2) sporophytic selfing (SS), in which eggs and sperm are produced by gametophytes derived from different spores from the same parent individual; and (3) gametophytic selfing (GS), in which eggs and sperm are produced by a single, bisexual gametophyte. Of these three mating systems, only the first two are possible in heterosporous plants, such as seed plants (Klekowski, 1969). The third, GS, results from the fertilization of an egg by a sperm in which both are the products of a single meiotic event that produced a single isospore and, subsequently, a bisexual gametophyte. This is an extreme form of inbreeding that results in homozygosity at all loci in the daughter sporophyte (Klekowski & Lloyd, 1968). By definition, this form of inbreeding is only possible in homosporous groups; 'selfing' in heterosporous lineages such as seed plants is analogous to SS in homosporous lineages, and is quite different from the GS found in the latter. Although this form of inbreeding reduces genetic variation in the short term, the ability to undergo GS potentially increases the ecological and evolutionary flexibility of homosporous lineages. Pteridologists have long sought to understand and quantify the frequency and dynamics of GS in ferns in order to understand how this extreme breeding system may have contributed to fern evolution (Klekowski & Baker, 1966; Klekowski & Lloyd, 1968).

Gametophytic selfing offers a unique system for evaluating contrasting evolutionary and ecological strategies. Over evolutionary timescales, there are clear negative impacts for any type of selfing in diploids. In numerous plant and animal groups, selfing (in these organisms, analogous to SS in ferns; Fig. 1b) has been shown to lead to decreased fertility and survival as a result of increased homozygosity for partially recessive, detrimental mutations (Charlesworth & Willis, 2009), particularly in populations with high genetic load (Klekowski, 1982; Hedrick, 1987; Barrett & Charlesworth, 1991). Selfing is widely expected to have negative evolutionary consequences, and to limit the evolutionary potential of lineages by reducing variation and limiting adaptive potential (Lande & Schemske, 1985; Schemske & Lande, 1985; Takebayashi & Morrell, 2001; Wright *et al.*, 2013; Barrett *et al.*, 2014). In flowering plants, selfing (again analogous to SS in ferns) is largely irreversible (Takebayashi & Morrell, 2001; Goodwillie *et al.*, 2005), and has been suggested to lead to extinction as a result of loss of adaptive ability (Stebbins, 1957; Takebayashi & Morrell, 2001). Because GS in ferns is a more extreme

form of inbreeding than the selfing that occurs in flowering plants, its effects could be expected to be even more drastic (Klekowski, 1970, 1972). However, there are conditions in which selfing, even GS, may confer advantages or at least be less detrimental (Schemske & Lande, 1985). For example, it has been hypothesized that the deleterious genetic effects of GS may be lessened or nonexistent in polyploids, whose extra genome(s) can 'buffer' against accumulation of deleterious mutations by preventing or delaying exposure to selection (Klekowski & Baker, 1966). Homosporous ferns have the highest rates of polyploidy among vascular plants (Wood *et al.*, 2009), and since the 1960s it has been suggested that this high incidence of polyploidy is linked to the capacity for GS in homosporous ferns (Klekowski & Baker, 1966; Klekowski, 1973; Chapman *et al.*, 1979; Holsinger, 1987; Haufler, 1989; Soltis & Soltis, 1989; Masuyama & Watano, 1990).

The ability to undergo GS may also confer an advantage in facilitating colonization following long-distance dispersal (e.g., Baker's Law; Baker, 1955; Stebbins, 1957). Ferns have minute, dust-like spores capable of dispersing long distances (Tryon, 1970; Peck *et al.*, 1990), and ferns are often the earliest colonizers of newly available substrates such as volcanic soils and oceanic islands (Tryon, 1970; Smith, 1972). Species capable of establishing a new population from a single spore should be expected to have an advantage in these environments (Wubs *et al.*, 2010; De Groot *et al.*, 2012a,b), and several studies have demonstrated that pioneer species of ferns are often capable of GS and generally have high levels of homozygosity with very few recessive lethal alleles (e.g. Lloyd, 1974b; Crist & Farrar, 1983; Peck *et al.*, 1990; Schneller & Holderegger, 1996; Suter *et al.*, 2000). Over time, recurrent dispersal events may introduce additional genetic diversity (Dassler & Farrar, 2001; Jiménez *et al.*, 2010). Polyploidy and colonization potential might also be linked via the capacity for GS: if polyploids indeed have higher genetic tolerance for inbreeding, they should be highly successful single-spore colonizers (Tryon, 1985; Bucharová & Münzbergová, 2012). Finally, some authors have hypothesized correlations between breeding systems in ferns and various aspects of habitat preference. For example, taxa in regions with shorter growing seasons (e.g. as a consequence of latitude or water limitation) may be more likely to undergo GS in order to facilitate shorter times to fertilization (Holbrook-Walker & Lloyd, 1973; Lloyd, 1974a). This might suggest that epiphytic species, which are exposed to xeric and high-light environments (Watkins & Cardelús, 2012), should be more likely than terrestrial taxa to undergo GS. However, most epiphytic fern gametophytes are also slow growing, long lived, and tolerant of environmental stress (Watkins *et al.*, 2007a; Pittermann *et al.*, 2013; Testo & Watkins, 2013), and may therefore be able to delay sexual reproduction until a second gametophyte germinates nearby.

Despite the potential ecological advantages of GS for taxa with the aforementioned attributes, studies evaluating the genetic diversity of mature fern sporophytes clearly demonstrate that most sporophyte populations of most homosporous fern species exhibit genetic diversity and degrees of heterozygosity indicative of sporophytic outcrossing (Ranker & Geiger, 2008). One

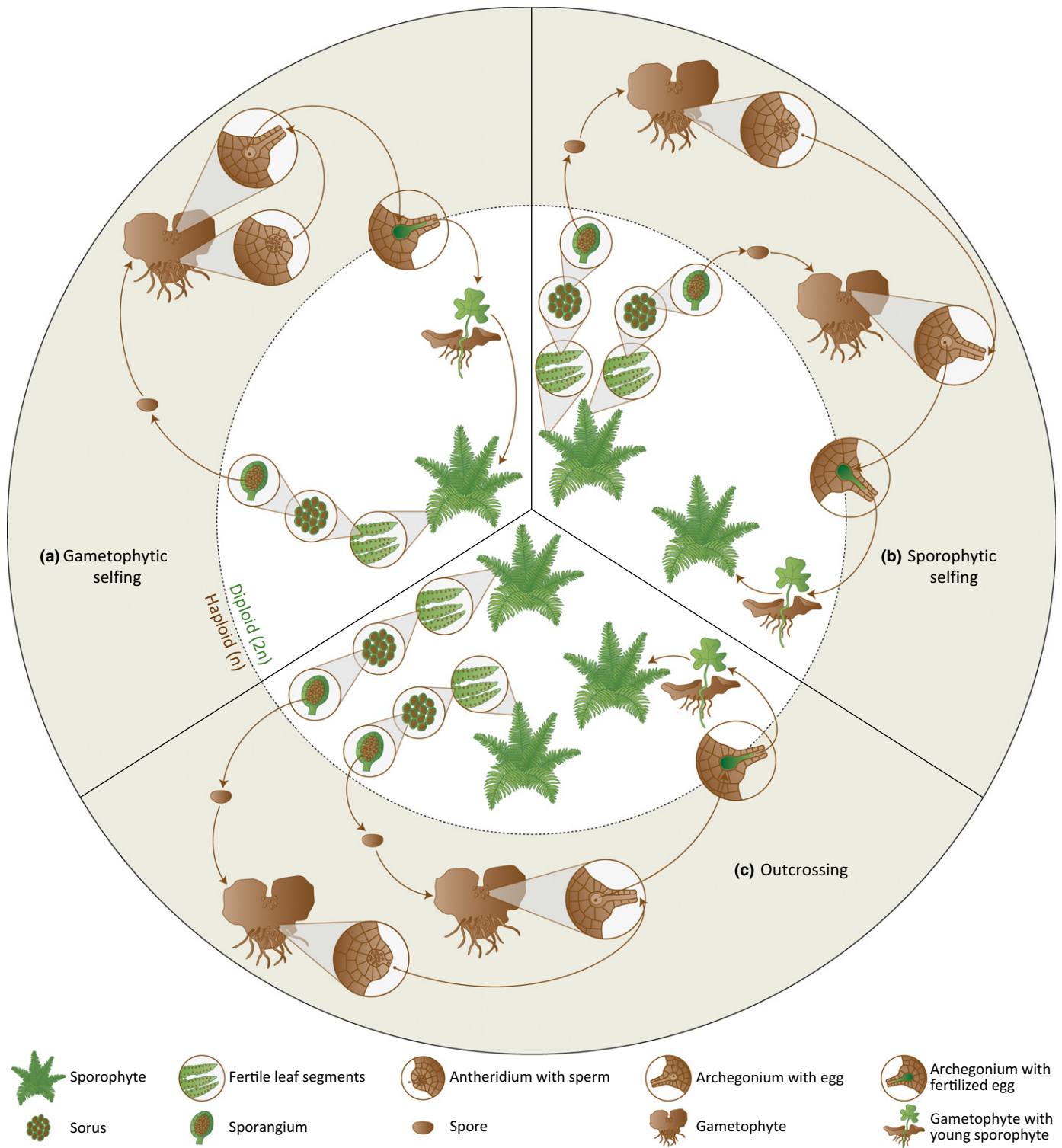


Fig. 1 Fern life cycle showing the three sexual mating systems possible in homosporous ferns. (a) In gametophytic selfing, a single spore germinates into a bisexual gametophyte that produces both eggs and sperm that undergo fertilization. This is ‘extreme inbreeding’, and the daughter sporophyte is homozygous at all genetic loci. (b) In sporophytic selfing, egg and sperm are produced by two gametophytes that developed from separate spores from the same parent sporophyte. (c) In outcrossing, egg and sperm come from two different plants. The sporophyte drawing is by Tracey Saxby and is used under a Creative Commons open license from the IAN Image Library (ian.umces.edu/imagelibrary/).

potential mechanism that promotes outcrossing is a chemical system that controls gametophytic sex expression, known as the antheridiogen system (Döpp, 1950; Näf *et al.*, 1975).

Antheridiogens are gibberellin-like pheromones that control the timing of gametangium development (Tryon & Vitale, 1977; Schneller *et al.*, 1990). Typically, the first spore to germinate

becomes an archegoniate gametophyte that releases antheridiogen compounds that stimulate antheridium production in less mature gametophytes (Hamilton & Lloyd, 1991), resulting in populations with disproportionate numbers of unisexual male and female gametophytes (Schneller *et al.*, 1990). This physiologically induced production of male and female gametangia on different but adjacent gametophytes decreases the likelihood of GS, while increasing the likelihood of SS or outcrossing, depending on the sporophytic origins of the male gametophytes compared with the female (Fig. 1).

Taken together, these observations suggest that there are clear disadvantages of GS over evolutionary time, given the widespread occurrence of antheridiogen systems (Näf, 1979; Yamane, 1998) and the predominance of outcrossing in natural populations (Ranker & Geiger, 2008), and yet GS may confer an advantage for ferns under certain ecological conditions, including single-spore colonizers of new substrates or oceanic islands, polyploids, and possibly epiphytic species. Even if GS occurs only rarely, it may nonetheless have significant consequences for gametophytes in these situations, which might otherwise leave no sporophyte offspring at all. Having the capacity for this mating system may allow diploids or polyploids to establish new populations in which heterozygosity could eventually develop, or allow single-spore colonists or isolated epiphytic gametophytes to persist long enough for a second individual to arrive (Klekowski, 1972; Dassler & Farrar, 1997, 2001), at which point outcrossing could resume.

To understand whether and how extreme inbreeding has contributed to fern evolution, we first need to know how prevalent the capacity for GS is among homosporous ferns. Numerous previous studies have gathered data germane to this question (Supporting Information Table S1), but each examined only one or a few related taxa. As a result, data on fern breeding systems are scattered across the literature, and a comprehensive evaluation of the distribution of breeding systems across ferns is lacking. In addition, different researchers have employed different approaches, and thus the comparability of results for different taxa is unclear. We addressed these issues in the current study by generating new data on GS for a large number of homosporous ferns using standardized methods. We evaluated the frequency of GS across numerous species and asked whether the ability to generate sporophytes from isolated gametophytes is correlated with habit (epiphytic versus terrestrial), region (temperate versus tropical), or ploidy level. We also review and summarize the historical data on fern breeding systems in order to draw conclusions about the frequency of extreme inbreeding based on as many taxa as possible.

Materials and Methods

Spore material

We obtained spores from close to 400 species of homosporous ferns. Many of these were collected from herbarium sheets, sometimes from collections made decades ago, and many spores were therefore found to be inviable. We were able to successfully grow and include in our experiments 115 homosporous species

representing 24 families, distributed across the fern phylogeny (Fig. 2). Spores were obtained from a variety of sources: from the American Fern Society spore exchange, from live material in the collections at Colgate University (Hamilton, NY, USA), the University of Florida (Gainesville, FL, USA), the University of Vermont (Burlington, VT, USA), and the Royal Botanic Garden Edinburgh (Edinburgh, UK), from field collections, and from herbaria (the Duke Herbarium (Duke University, Durham, NC, USA) and the Pringle Herbarium (University of Vermont, Burlington, VT, USA). For collections from living material,

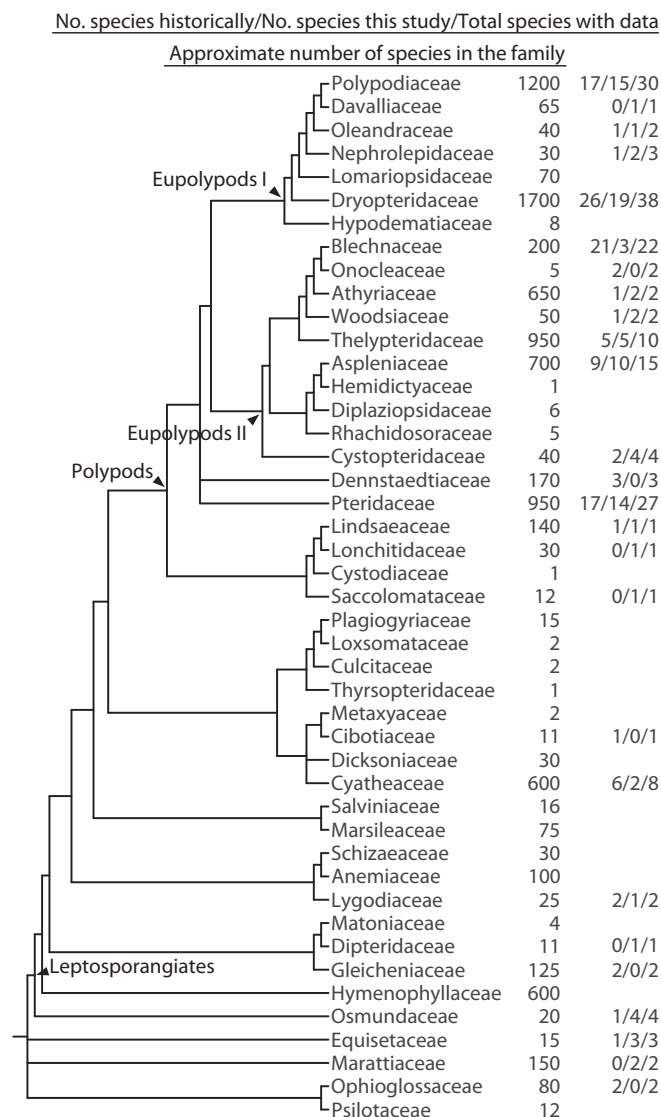


Fig. 2 Phylogeny of extant ferns, based on Smith *et al.* (2006) and Schuettpelz & Pryer (2007). For each family, estimated numbers of species in the family are given (from Smith *et al.*, 2006), as well as numbers of species with data available from previous studies and the current study, and total number of species with data now available. Thirty species in our study were included in previous studies, so in some cases the total number of species with data available is lower than the sum of the two preceding numbers. We note that, although we attempted to be exhaustive in our review of the literature, we may still have missed some relevant work, in which case the historical estimates may be underestimates of numbers of species with data available.

portions of fertile fronds were placed in glassine envelopes and allowed to dry in an air-conditioned laboratory to facilitate the release of spores. We recorded habit (epiphyte versus terrestrial) and habitat (temperate vs tropical) according to the conditions at collection or by consulting the literature. Ploidy was determined from the literature and from the databases maintained by the Index to Plant Chromosome Numbers (<http://www.tropicos.org/Project/IPCN>) and Kew Royal Botanic Gardens' Plant DNA C-values Database (<http://data.kew.org/cvalues/>). If we could not confidently determine ploidy for a given taxon, it was not included in ploidy-related analyses.

Growth conditions and experimental treatments

Culture plates were prepared using standard Bold's medium (Bold, 1957) modified with Nitsch's micronutrients (Nitsch, 1951). Spores were sown at an approximate density of 25 spores per 100 × 25 mm Petri plate (ThermoFisher Scientific, Waltham, MA, USA). Cultures were kept at 20–25°C and exposed to a 12 h : 12 h, light : dark cycle achieved with fluorescent grow bulbs (65 μmol m⁻² s⁻¹) in growth chambers (Percival Scientific, Perry, IA, USA). Cultures were established at Colgate University, the University of Vermont, and/or the University of Florida.

Mating system experimental methodology followed Testo *et al.* (2015). To determine GS rates, we transferred individual pre-sexual gametophytes to isolated chambers containing the same growth medium described in the previous paragraph. In order to determine whether each species is capable of SS, either instead of or in addition to GS, we included a second treatment in which pairs of pre-sexual gametophytes were transferred to new plates with the same medium and placed 1 cm apart. These were sibling pairs, as we had spores from a single sporophyte for each species. At least 12 replicates of each treatment were set up for each species. Both treatments were watered weekly with distilled water to facilitate fertilization, and observed weekly for up to 200 d. Sporophyte formation was determined by visual examination of cultures. The paired treatments allowed us to test for SS potential, but we did not test true outcrossing, which requires spores from separate parental sporophytes, as we lacked access to spores from multiple sporophytes. Because SS and outcrossing both involve spores generated from separate meiotic events, we consider them analogous for the purposes of the current study, in which we are primarily focused on assessing capacity for GS versus any non-gametophytic-selfing form of sexual mating.

Statistical analyses

We calculated isolate potential and sib potential following Peck *et al.* (1990) and Testo *et al.* (2015). Isolate potential (the frequency of successful GS events) is the number of sporophytes produced by isolated gametophytes divided by the total number of isolated gametophytes for that species. Sib potential (the frequency of successful SS events) is the number of sporophytes produced by paired gametophytes, divided by the total number of gametophytes in the paired treatment for each species. In ferns,

only one sporophyte is typically produced per gametophyte, even in the case of multiple fertilizations (Holsinger, 1987), so there is no danger of over-counting sporophyte production per gametophyte. Six species were present in duplicate (i.e. gametophytes were derived from spores produced by two sporophytes) and data for each of these six taxa were averaged.

We did not genotype daughter sporophytes to confirm their parentage, and so it is possible that sporophytes produced in the sib pairs were actually the result of GS, even though two gametophytes were present. To account for this potential overestimation of species capable of SS, we used an arbitrary cutoff of 10% difference between isolate potential and sibling potential to determine whether a species had probably engaged in SS. If the sporophytes in the sib pairs were in fact produced by GS, we would expect to see roughly equal rates of sporophyte production between the sib and isolate treatments. If the sporophyte production values differed by more than 10%, we assumed that SS was in fact occurring in the sibling pairs. Species that fell below the 10% difference cutoff were excluded from subsequent analyses. It is possible that our SS estimates may still be overestimates, as some sporophyte production in species with > 10% difference in sib versus isolate potential could have resulted from GS. For species that only produced sporophytes when in paired treatments, and never in isolation, we assumed that all sporophyte production in the pairs was via SS.

We used one-way between-subjects analysis of variance (ANOVA) to compare isolate potential and sib potential between groups of interest, including temperate and tropical species, epiphytes and terrestrial taxa, and diploids and polyploids, and to compare isolate and sibling potential in diploids and polyploids separately (significance at $P=0.05$, 0.01 and 0.001 indicated by *, ** and ***). For these calculations, sib potential was calculated from all species capable of SS, including those capable of both mating systems (referred to below as 'GS species' to highlight that they are capable of GS), and those that only produced sporophytes when paired (referred to below as 'SS only'). We also tested for significant differences in sib potential between species that were SS only and GS species. Because 'GS only' is difficult to understand biologically as a successful long-term strategy, we assumed that, if a species was found to undergo only GS in our experiments, it was probably not a meaningful result (see Discussion). These species were therefore not included in calculations of isolate potential. All statistical analyses were performed in R v.3.1.2 (R Development Core Team, 2008).

Results

Of the 115 species that produced sporophytes in one or both treatments, we excluded 19 that did not meet the 10% cutoff for difference between isolate percentage and sibling percentage (Table S2). Experimental results for the remaining 96 species (Table 1) showed that most species are capable of both gametophytic selfing and sporophytic selfing: 61 species (63.5%) produced sporophytes both in isolation and when paired and are referred to as 'GS species', and 31 species (32.3%) produced

Table 1 Summary of results from the current study

Mating system	No. of species	No. of epiphytic	No. of terrestrial	No. of temperate	No. of tropical	No. of diploid	No. of polyploid
SS only	31	8	23	13	18	18	2
GS species	61	9	52	32	29	21	9
Total	96	17	75	45	47	39	11

Ploidy is unknown for some taxa. SS only, species that were found to perform sporophytic selfing only and not gametophytic selfing; GS species, taxa capable of gametophytic selfing.

sporophytes only when paired and are referred to as ‘SS only’ (Fig. 3a). Four species (4.2%) produced sporophytes only in isolation, and these were not included in subsequent analyses. Isolate potential for GS species was $43.9 \pm 3.8\%$ (mean \pm SE). Sib potential was $50.1 \pm 4.3\%$ in SS only species, and $71.2 \pm 3.1\%$ in GS species; these values are significantly different ($F_{1,90} = 15.73$; $P < 0.0001$; Fig. 3b).

Among epiphytic taxa, nine species were capable of both GS and SS, and eight species were capable of SS only. Among terrestrial species, most (52 of 78 terrestrial taxa) were capable of both GS and SS, and 23 of 78 terrestrial taxa were SS only (Fig. 4a). Levels of sporophyte production did not differ significantly between epiphytic and terrestrial taxa for isolated gametophytes ($F_{1,59} = 1.281$; $P = 0.262$) (Fig. 4b), but in sib pairs, sporophyte production was significantly higher among terrestrial than epiphytic species ($F_{1,90} = 4.935$; $P = 0.0288$) (Fig. 4c). Mean isolate potential was $33.5 \pm 8.9\%$ in epiphytes ($n = 9$), and $45.7 \pm 4.2\%$ in terrestrial species ($n = 52$) (Fig. 4b). Mean sib potential was $51.7 \pm 8.1\%$ in epiphytes ($n = 17$), and $66.9 \pm 2.7\%$ in terrestrial species ($n = 75$) (Fig. 4c).

Among temperate as well as tropical taxa, most species were capable of both SS and GS (32 of 45 temperate species; 29 of 47 tropical species). Thirteen temperate species and 18 tropical species were SS only (Fig. 5a). Neither isolate potential ($F_{1,59} = 0.297$; $P = 0.588$) (Fig. 5b) nor sib potential

($F_{1,90} = 0.668$; $P = 0.416$) (Fig. 5c) varied significantly between temperate and tropical taxa. Isolate potential was $46.1 \pm 5.3\%$ in tropical species ($n = 29$) and $41.9 \pm 5.3\%$ in temperate species ($n = 32$) (Fig. 5b); sib potential was $61.9 \pm 4.1\%$ in tropical species ($n = 47$) and $66.4 \pm 3.5\%$ in temperate species ($n = 45$) (Fig. 5c).

Of the 50 species for which we were able to determine ploidy, most species were again capable of both GS and SS (21 of 39 diploid species and nine of 11 polyploid species), followed by SS only (18 of 39 diploid species and two of 11 polyploid species) (Fig. 6a). Neither isolate ($F_{1,28} = 4.028$; $P = 0.0545$) (Fig. 6b) nor sib potential ($F_{1,48} = 0.083$; $P = 0.775$) (Fig. 6c) differed significantly between ploidy levels. The difference between sporophyte production in isolates and pairs (relief of inbreeding depression) was significant in diploids ($F_{1,58} = 24.23$; $P = 7.44e^{-6}$) (Fig. 6d) but not in polyploids ($F_{1,18} = 1.107$; $P = 0.307$) (Fig. 6e). Isolate potential was $36.0 \pm 6.0\%$ in diploid species ($n = 21$) and $57.1 \pm 8.0\%$ in polyploids ($n = 9$) (Fig. 6b); sib potential was $66.7 \pm 3.3\%$ in diploids ($n = 39$) and $68.9 \pm 7.8\%$ in polyploids ($n = 11$) (Fig. 6c).

All data gathered in this study (Table S2), along with data on fern mating systems from previously published research (Table S1), are searchable by family and genus at Ferns and Lycophytes of the World (<http://www.fernssoftheworld.com>), and are available on Dryad (doi: 10.5061/dryad.b0b68).

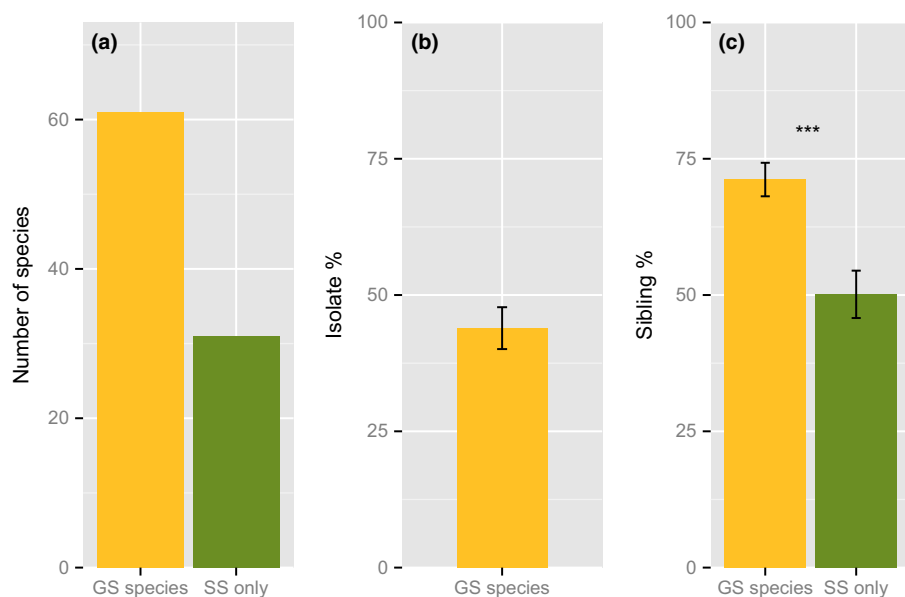


Fig. 3 Results of mating system experiments for 96 fern species. (a) Number of species found to be capable of gametophytic selfing (GS species), or that only underwent sporophytic selfing (SS only). (b) Mean isolate potential for GS species. (c) Mean sibling potential for GS species and SS only species. Error bars represent ± 1 SE. Significance: ***, $P < 0.001$.

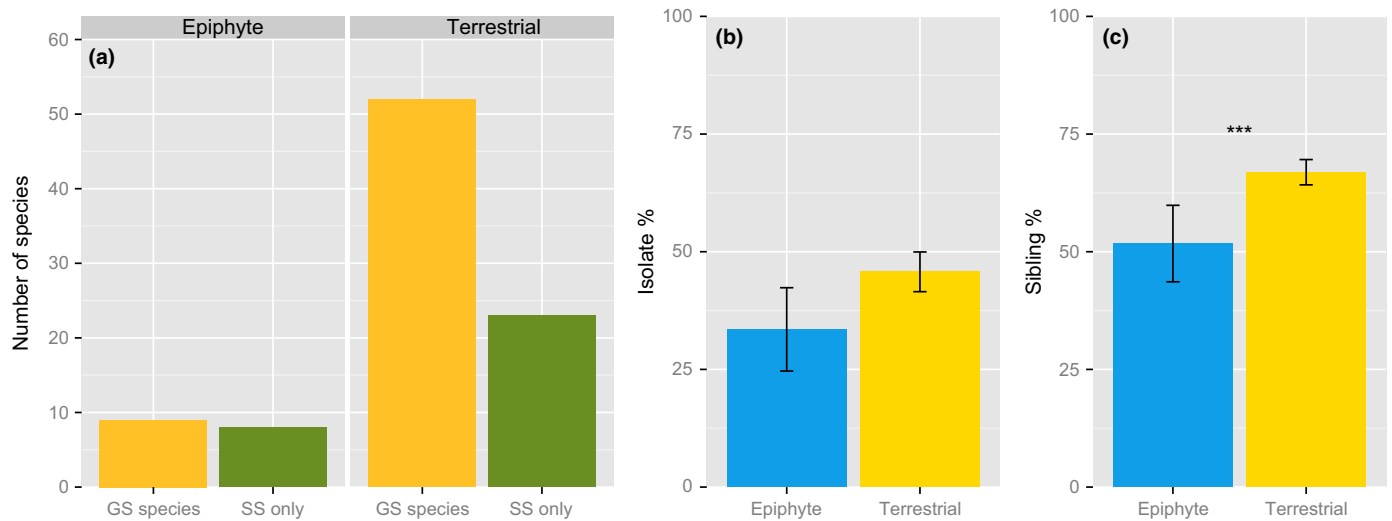


Fig. 4 Mating system results for epiphytic versus terrestrial taxa. (a) Number of gametophytic selfing (GS species) and sporophytic selfing (SS only) species among epiphytes (left) and terrestrial taxa (right). (b) Mean isolate potentials for all species capable of gametophytic selfing. (c) Mean sibling potentials for all species capable of sporophytic selfing. Error bars represent ± 1 SE. Significance: ***, $P < 0.001$.

Discussion

Of the 96 fern species included in our analyses, over 60% (63.5%, 61 species) were capable of producing sporophytes by both GS and SS (Fig. 3a). Another 32% (31 species) produced sporophytes only when in pairs (Table S2), and only four species produced sporophytes solely when gametophytes were isolated. This suggests that GS as a sole strategy is rare, an unsurprising conclusion as it is difficult to imagine this being successful as a long-term strategy on its own. These four species may have experienced some type of inter-gametophyte interaction that prevented sporophyte production in the sib pairs, or those plants at random may have reacted negatively to the laboratory conditions. While we excluded them from our analyses, these species warrant further study and we suspect that they will eventually be found to be capable of SS or outcrossing as well as GS.

We were able to locate data for 122 species from previous studies on fern mating systems (Table S1). Thirty of these species were also included in the 96 species in our data set, and so mating data are now available for a total of 188 species of homosporous ferns. For nine of the species included in previous studies, five of which we also included in our experiments, only GS was tested. We found all five of these to be capable of both GS and SS, but we cannot directly compare our results to those of the previous studies as they did not test for SS. Of the remaining 25 species included in our analyses and with existing data available, our result was the same as the previous result for 17 species. For two species we observed SS only while the previous result found them to be capable of both GS and SS or outcrossing, and for six species we found them to be capable of both (but always with very low levels of isolate percentage), while the historical analyses determined that these species were outcrossing only (five species) or selfing only (one species) (Tables S1, S2).

The relatively high level of consistency between our study and the historical survey results suggests that, in general, the data

collected by previous authors are comparable across those studies and with our newly generated results. Across all 188 species with mating data now available, 15 species (8%) were found to undergo GS only, 62 (33%) to undergo either SS or outcrossing, but not GS, and 94 (50%) to be capable of both GS and one or both of SS and outcrossing. Another four species (2%) were found historically to be capable of GS but SS and outcrossing were not tested. For a final 13 species (8%) both GS and one of the other systems were observed either in our study or in historical data, but not in both. Taken together, our data combined with the surveyed historical data strongly suggest that the capacity for GS is widespread in ferns, with up to 67% (126 of 188) of all fern species examined demonstrating the capacity for both GS and SS or outcrossing. In addition, the 19 species excluded from our analyses because they did not reach the sib potential threshold for inclusion (see Methods) were all capable of GS. This raises the total number of species capable of GS to 70% (145 of 207).

While we recovered evidence of widespread capacity for GS among ferns in the lab, this finding contrasts with genetic data from field populations. Ranker & Geiger (2008) summarized data from 49 studies that quantified levels of genetic diversity in natural populations of sporophytes, and reported that natural homosporous fern sporophyte populations were overwhelmingly outcrossing. How, then, to reconcile our results with these observations of natural populations? There are several scenarios in which the ability to undergo GS may be beneficial, and which frequently apply to ferns. Maintaining the capacity for GS, even if it occurs only infrequently, should thus confer a long-term advantage. First, ferns frequently undergo long-distance dispersal, facilitated by their microscopic spores (Tryon, 1966, 1970; Wolf *et al.*, 2001; Shepherd *et al.*, 2009; Perrie *et al.*, 2010), and ferns are known to be pioneers of new substrates and newly available habitats (Tryon, 1970; Smith, 1972; Wubs *et al.*, 2010; De Groot *et al.*, 2012a,b). The well-known migratory capabilities of

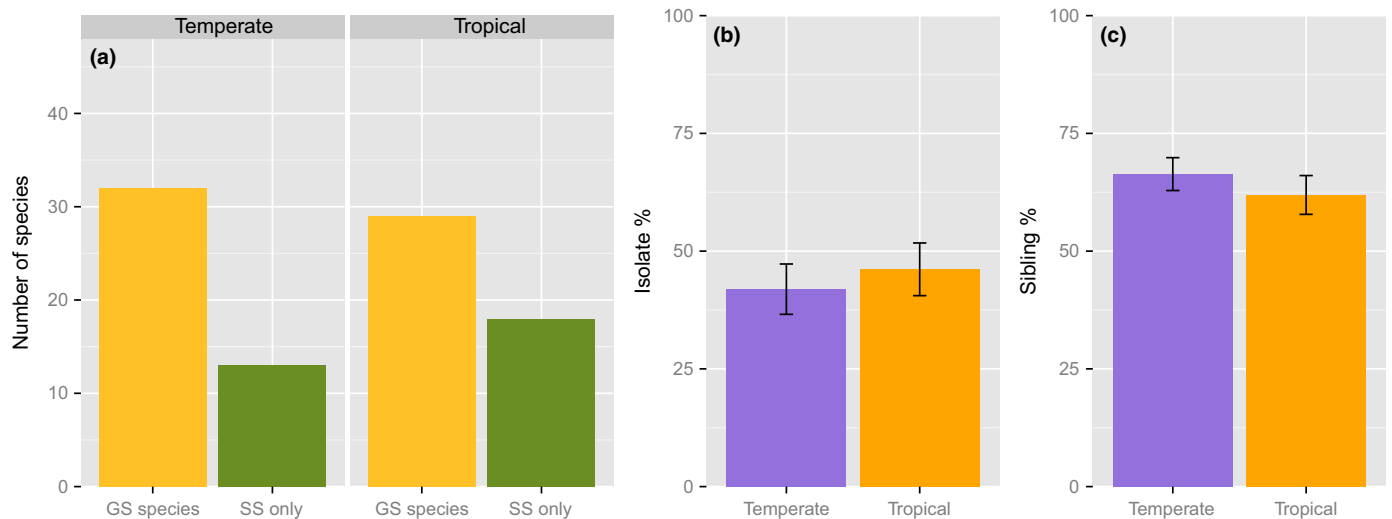


Fig. 5 Mating system results for temperate versus tropical taxa. (a) Number of gametophytic selfing (GS species) and sporophytic selfing (SS only) species among temperate (left) and tropical (right) taxa. (b) Mean isolate potentials for all species capable of gametophytic selfing. (c) Mean sibling potentials for all species capable of sporophytic selfing. Error bars represent ± 1 SE.

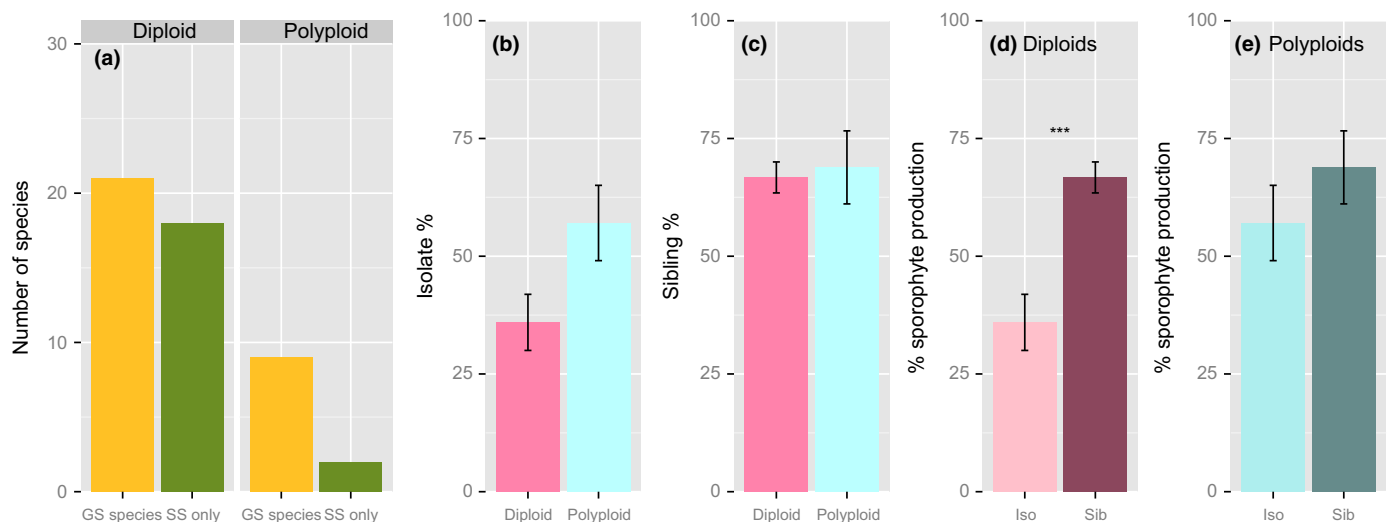


Fig. 6 Mating system results for diploid versus polyploid taxa. (a) Number of gametophytic selfing (GS species) and sporophytic selfing (SS only) species among diploid (left) and polyploid (right) taxa. (b) Mean isolate potentials for all species capable of gametophytic selfing. (c) Mean sibling (sib) potentials for all species capable of sporophytic selfing. (d) Mean isolate and sib potentials for diploids. (e) Mean isolate and sib potentials for polyploids. Error bars represent ± 1 SE. Significance: ***, $P < 0.001$.

ferns may in fact result from the ability of single-spore migrants to undergo GS, allowing them to wait until genetic variability can be restored with the arrival of later colonists (Dassler & Farrar, 2001; Pannell & Dorken, 2006; Jiménez *et al.*, 2010; De Groot *et al.*, 2012a). This may be the primary mechanism by which GS has contributed to fern evolution, given the extent to which ferns are known to engage in long-distance dispersal.

Studies on several fern species have suggested that GS is the optimal strategy in dry and/or climatically stressful environments (such as rainforest canopies), or those that are highly seasonal (more often found in temperate than tropical habitats) (Holbrook-Walker & Lloyd, 1973; Lloyd, 1974a; Lott *et al.*, 2003). Because gametophytes may not be able to tolerate such conditions for extended periods of time, rapid maturation and the capacity to self-fertilize may increase their chances of producing

and establishing a sporophyte before the gametophyte dies (Lloyd, 1974a; Lott *et al.*, 2003). We thus could have expected to see higher isolate potentials both in epiphytic compared with terrestrial species, and in temperate compared with tropical taxa. However, inner canopy systems are inherently more physically stable than terrestrial habitats in tropical rainforests (Grime, 1977), and ecologically stable environments are thought to be conducive to gametophyte longevity (Klekowski, 1979; Watkins *et al.*, 2007b). The pace of sporophyte recruitment in epiphytes could therefore be expected to be slower than in terrestrial species (Klekowski, 1979), a premise that is supported by recent work on the functional biology of epiphytic fern gametophytes (Watkins & Cardelús, 2012). However, we found no evidence that GS is more common in epiphytes or terrestrial species, or in taxa from temperate compared with tropical regions. While sib potential

was higher in terrestrial than in epiphytic taxa (Fig. 4c), isolate potential did not differ significantly between epiphytes and terrestrial species (Fig. 4b), or between temperate and tropical species (Fig. 5b,c). This lack of difference in isolate potentials between epiphytic and terrestrial taxa and between temperate and tropical taxa suggests that the benefits of maintaining the capacity for GS may apply equally to taxa with these habit/habitat preferences.

Another phenomenon that is common in ferns and for which GS may be advantageous, or at least not detrimental, is polyploidy. The extra genome(s) present following whole-genome duplication may serve as a 'buffer' against the effects of homozygosity in deleterious alleles following GS. It has therefore long been suggested that polyploids should be more tolerant of GS than diploids (Klekowski & Baker, 1966; Klekowski, 1973; Chapman *et al.*, 1979; Holsinger, 1987; Haufler, 1989; Soltis & Soltis, 1989; Masuyama & Watano, 1990). Of the species for which we were able conclusively to determine ploidy level, relatively more polyploid than diploid species were capable of GS (82% of polyploids and only 54% of diploids, although this is based on only 11 species known to be polyploid; Table 1). In accord with the frequent suggestion in the literature that polyploids should be more tolerant of selfing than their diploid relatives (see, e.g., Stebbins, 1950; Grant, 1956; Soltis & Soltis, 1987; Haufler, 1989; Masuyama & Watano, 1990; Barringer, 2007; Ozimec & Husband, 2011), we did see higher average isolate potentials in polyploid compared with diploid species, but the difference did not reach statistical significance (Fig. 6b). We also observed a significantly greater increase in sibling versus isolate sporophyte production in diploids but not in polyploids, suggesting that polyploids are buffered against expression of deleterious alleles in gametophyte selfing (Fig. 6d,e).

More data from known polyploids will be essential to further investigate this relationship, as the direction of our data supports the inference that polyploids may be more tolerant of GS than are diploids. Future data collection efforts should focus on including groups of species with polyploid series and those found to be exclusively selfing, in order to improve our understanding of the relationship between ploidy level and selfing capacity in ferns. It has also been suggested that as polyploid genomes 'diploidize' following whole-genome duplication, and return to disomic inheritance and diploid expression patterns (Dodsworth *et al.*, 2016), the fitness of selfed progeny may deteriorate and mating systems may also evolve, reverting to mixed mating or outcrossing strategies (Husband *et al.*, 2008; Ozimec & Husband, 2011). Assessing mating systems in polyploids of known age could help to test this assertion. Finally, several studies have suggested that genotype may be even more important than ploidy level in determining mating system (Klekowski, 1972; Crist & Farrar, 1983; Peck *et al.*, 1990; Suter *et al.*, 2000; Wubs *et al.*, 2010; De Groot *et al.*, 2012b). For example, de Groot and colleagues (De Groot *et al.*, 2012b) found that selfing capacity did not differ significantly between ploidy levels for two species of *Polystichum* and two species of *Asplenium* in the Netherlands, but that intraspecific variation in selfing capacity was high. In an

earlier study, Klekowski (1972) did not consider polyploidy, but found that GS and SS rates varied widely across 15 populations and five varieties of *Pteridium aquilinum*. Crist & Farrar (1983) compared isolated and paired gametophytes of *Asplenium platyneuron* from dense sporophyte populations within the principal range of the species with those from disjunct sporophytes. Isolated gametophytes from both sources readily produced sporophytes, but those from disjunct sporophytes displayed significantly higher isolate potential. Future studies of fern mating systems should therefore also strive to include multiple genotypes per species to better understand the extent to which mating system behavior may be variable within versus between species, especially at different ploidy levels.

While the data newly generated in the current study and summarized from the literature support a widespread capacity for GS among homosporous ferns, we note several caveats of these studies that should be considered. Although growing gametophytes in culture on agarose medium in the lab is a standard practice, and has been shown to resemble conditions in the field (Farrar *et al.*, 2008), others have found that gametophytes in culture have significantly different sex ratios from those found in the wild (Ranker, 2002), which could influence inferences about mating systems based on results obtained in the lab. In addition, gametophytes of some taxa can take months or years to reach sexual maturity (Chou *et al.*, 2007; Watkins *et al.*, 2007b; Testo & Watkins, 2011), and therefore would not have produced sporophytes by the time we ceased data collection. Indeed, a number of species for which gametophytes germinated in the lab never produced any sporophytes, and were therefore not included in the data we analyzed. There are several possible explanations for this: standard laboratory conditions may simply not be suitable to induce mating behavior in these species, or these species are slow to reach sexual maturity, and we did not wait long enough to observe fertilization and sporophyte production. A third option is that these species are obligate outcrossers in the strict sense, and require gametophytes derived from two separate sporophytes in order to have successful fertilization events. A final caution is that our data demonstrate the potential for GS and SS, yet may overestimate the extent to which they occur successfully in natural populations. We scored an isolate or sibling pair as producing a sporophyte if any sporophytic growth was seen. However, this essentially accounts only for sporophyte-lethal alleles that, in a homozygous sporophyte produced by GS, would result in no sporophyte formation at all. GS may produce viable young sporophytes that ultimately fail to mature and produce offspring because of sporophyte-deleterious alleles that are not expressed until later stages of development. In such cases it would seem misguided to consider those species successful inbreeders (Schneller, 1979; Peck *et al.*, 1990). Almost none of the studies that have evaluated fern mating systems in lab conditions have followed sporophytes to maturity, including ours. Notable exceptions include Peck *et al.* (1990) who demonstrated, using glasshouse grown sporophytes of *Adiantum pedatum*, that gross developmental abnormalities were present in most (67%) sporophytes produced via selfing of isolated gametophytes, compared with normal development in most (92%) sporophytes produced

by paired gametophytes, and Schneller (1987), who reexamined progeny from mating system studies conducted 8–10 yr earlier (Schneller, 1979) and that had subsequently been grown together in a common garden. Schneller reported that progeny of GS had higher mortality (Schneller, 1979) and included more dwarf plants than did progeny of SS or outcrossing. He inferred the highest amount of heterozygosity in the progeny of outcrossing, based on plant survival and leaf measurements. These results suggest that selection on sporophytes could result in outcrossed populations predominating in the wild, as demonstrated by Ranker & Geiger (2008), and strongly support the idea that following immature sporophytes through to maturity should be a central component of future mating system studies on ferns. Future work should also focus on addressing the other issues described in the preceding paragraphs, including replicating field conditions to more accurately assess mating success rates in natural settings, and including multiple individuals per species, both to test true outcrossing versus SS, and to evaluate differences in selfing potential between genotypes. Testing heterozygosity of young versus mature sporophytes of the same species in natural populations would reveal whether selection subsequent to sporophyte production can account for the discrepancy between Ranker & Geiger's (2008) observation of outcrossed natural populations and our demonstration of GS capacity. We also hope to see additional data collected on mating systems in polyploids, to determine more conclusively whether there is a relationship between ploidy and GS in ferns, as has been so long suspected. Finally, genotyping sporophytes produced in sib-pair treatments to conclusively determine whether they are the result of SS or GS was outside the scope of the current study, but would increase the robustness of results of future work. All of the taxa excluded from our analyses would have been scored as being capable of both GS and SS if we had been able to confirm that the sib-pair-produced sporophytes were indeed the products of SS. It is therefore possible that our calculation of the frequency of species capable of GS, despite being remarkably high, is still an underestimate.

The data presented here are an important step toward a more thorough understanding of the capacity for GS in homosporous ferns, and can serve as a guide for researchers wishing to know about the mating system(s) potentially in use by taxa of interest. Homospory may confer a significant advantage by increasing mating system flexibility, and we have outlined several scenarios in which maintaining the capacity for GS may have contributed substantially to fern evolution. Reproductive success is often viewed as achievable by one of two alternate, selectable strategies: one defined by evolutionary success, and the other by ecological success. The real benefit of homospory as it applies to plants with independent gametophytes is that it provides a short-term ecological advantage to taxa that are capable of GS: a unique attribute of most ferns and some lycophytes. While heterosporous lineages clearly have more effective and varied isolating mechanisms that help to retain genetic variation, homosporous lineages have greater flexibility in their mating systems and this flexibility may define their success. Taxa capable of GS can mold themselves into novel environments and in situations where GS is possible or favorable, for example when colonization of a new habitat via a

single spore has occurred, will be at a clear ecological advantage. Given that natural selection acts to maximize fitness, it is perhaps not surprising that the capacity for extreme inbreeding persists in ferns, allowing species to utilize both evolutionarily and ecologically sound strategies, as conditions require.

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Author contributions

E.B.S., W.L.T. and J.E.W. planned and designed the research, performed experiments, analyzed the data, and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1 Results of previous studies on mating systems in ferns

Table S2 Results from the current study on mating system analyses in 115 species of homosporous ferns

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