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Research

Dynamics of asymmetrical hybridization in North American wood ferns: reconciling patterns of inheritance with gametophyte reproductive biology

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Summary

- Hybridization is an important evolutionary force in plants, but the mechanisms underlying it have not been well studied for many groups. In particular, the drivers of non-random patterns of interspecific gene flow (asymmetrical hybridization) remain poorly understood, especially in the seed-free vascular plants. Here, we examine patterns of asymmetrical hybridization in two widespread fern hybrids from eastern North America and study the role of gametophyte ecology in the determination of hybridization bias.
- We characterized the maternal parentage of > 140 hybrid sporophytes by sequencing a *c.* 350-bp region of chloroplast DNA (cpDNA). To identify factors contributing to patterns of asymmetrical hybridization, we cultured gametophytes of the parental species and evaluated critical aspects of their reproductive biology.
- We found that asymmetrical hybridization was prevalent across the populations of both hybrids. Reproductive traits varied across species and suggest that selfing potential, antheridiogen responsiveness, sperm dispersal capacity and gamete size all contribute to the mediation of the direction of hybridization in this group.
- Our findings suggest that asymmetrical hybridization in ferns is driven by an array of reproductive traits. This study helps to sharpen and define a mechanistic understanding of patterns of hybridization in this group and demonstrates the importance of considering gametophyte biology when studying evolutionary processes in ferns.

Introduction

The development and maintenance of reproductive isolating mechanisms are critical steps in the process of speciation, and understanding these mechanisms is a major focus of evolutionary biology (Coyne & Orr, 1998; Widmer *et al.*, 2008). Interspecific mating – contributing to a breakdown of isolating barriers – is more common among plants than animals. Such matings present a unique opportunity to examine the mechanisms that promote reproductive isolation, and to understand how and when they fail. Interspecific mating events also provide a window through which to view the evolutionary consequences of the resulting hybridization. Recent progress in the study of hybridization among angiosperms has characterized how numerous pre- and post-zygotic isolation mechanisms – including flowering time differences, pollinator specificity, pollen competition and chromosomal mismatches – influence patterns of interspecific mating (Schemske, 2000; Marques *et al.*, 2007; Pascarella, 2007; Yang *et al.*, 2007; Waelti *et al.*, 2008). Such work has provided a foundation for understanding the genetic basis of several of these mechanisms (Wulff *et al.*, 2004; Bomblies & Weigel, 2007; Bomblies *et al.*, 2007; Hoballah *et al.*, 2007), and has demonstrated that reproductive isolation is driven by a diverse set of factors with complex interactions (Tiffin *et al.*, 2001; Rieseberg & Willis, 2007; Scopece *et al.*, 2007).

In contrast with the progress made in recent years towards an understanding of the evolution of isolating mechanisms in angiosperms, our understanding of these same barriers in most other plant groups remains poor. Among these lineages, ferns are a particularly compelling study group owing to their frequent hybridization (Barrington *et al.*, 1989), which is thought to result from more limited isolating mechanisms and perceived simple reproductive strategy (Knobloch, 1976). The prevalence of hybrids within and even between some fern genera (e.g. Walker, 1961; Wagner *et al.*, 1992; Xiang *et al.*, 2000; Grusz *et al.*, 2009) has perpetuated the view that hybridization between ferns occurs relatively freely (see Barrington *et al.*, 1989).

This view is in conflict with evidence from recent studies (Vogel *et al.*, 1998a; Xiang *et al.*, 2000; Yatabe *et al.*, 2009; Hunt *et al.*, 2011) suggesting that hybridization in ferns follows patterns similar to those observed in angiosperms and that the dynamics of reproductive isolation and interspecific mating may

be comparable between these groups, including population-level variation in patterns of gene flow and high levels of hybridizational bias. Of most interest here, these studies demonstrated the occurrence of asymmetrical hybridization: a phenomenon in which reciprocal crosses involving the same two species form with different frequencies. Asymmetrical hybridization is relatively common in angiosperms (Paige *et al.*, 1991; Harder *et al.*, 1993; McConchie *et al.*, 1994; Hannan & Prucher, 1996; Hamzeh *et al.*, 2007) and is thought to arise when one species develops isolating mechanisms more rapidly than the other (Tiffin *et al.*, 2001). In extreme cases, unilateral incompatibility (i.e. one species can function as the father only) can occur; among angiosperms, this outcome has important evolutionary implications as it mediates patterns of population-level gene flow, introgression and the formation of hybrid swarms (Potts & Reid, 1985; Tiffin *et al.*, 2001; Strasburg & Rieseberg, 2008; Field *et al.*, 2011). By contrast, the evolutionary consequences of asymmetrical hybridization in ferns remain poorly understood.

The small number of studies that have examined asymmetrical hybridization in ferns suggest that the strength of hybridization bias varies considerably among taxa. Stein & Barrington (1990) reported no bias in the parentage of *Polystichum* × *potteri* Barrington, although their sample size (four individuals) was too small to be conclusive. By contrast, in a larger study of the common hybrid *Asplenium* × *alternifolium* Wulf., Vogel *et al.* (1998a) found that *Asplenium septentrionale* (L.) Hoffm. was the maternal parent of almost all plants examined. More recently, completely unidirectional hybridization was reported for another European rock-dwelling *Asplenium* hybrid, *Asplenium* × *protomajoricum* Pangua & Prada, by Hunt *et al.* (2011), and for an *Acrostichum* hybrid from southeast China (Zhang *et al.*, 2013). Xiang *et al.* (2000) reported population-level variation in the strength of asymmetrical hybridization between two common wood fern species from eastern North America, *Dryopteris intermedia* (Muhl. ex Willd.) A. Gray and *Dryopteris carthusiana* (Vill.) H.P. Fuchs. Sampling from three populations of the widespread hybrid of these species (*Dryopteris* × *triploidea* Wherry), they found that *D. carthusiana* was the maternal parent of the majority of hybrids in each population, but that the ratio of this bias ranged from weak (1.4 : 1) to nearly unidirectional (5.6 : 1) across the sites sampled. Unlike other studies, which were limited to single or undefined populations, this work provides evidence that factors acting at the population level can influence the dynamics of hybridization.

Ferns differ from seed plants in that their sexual phase is a free-living gametophyte that releases motile swimming sperm. Thus, most discussion of the factors underlying asymmetrical hybridization in ferns has focused on the basic features of gametophyte mating systems. In the two most in-depth studies of asymmetrical hybridization in ferns to date, Vogel *et al.* (1998a) and Xiang *et al.* (2000) suggested that differences in self-compatibility may influence directionality of hybridization; however, their interpretations are in conflict with each other. Vogel *et al.* (1998a) suggested that the outcrossing mating system of *A. septentrionale* was an important factor underlying that species' maternal ancestry of *A.* × *alternifolium* (*Asplenium trichomanes* × *A. septentrionale*), emphasizing synchronous gamete release in *A. septentrionale*. Conversely, Xiang *et al.* (2000) found that the predominantly self-fertilizing *D. carthusiana* tended to serve as the maternal parent in most hybridization events leading to the formation of *D.* × *triploidea* (*D. carthusiana* × *D. intermedia*). They argued that, in selfing species such as *D. carthusiana*, sperm motility may limit their potential to function as a male parent in hybridization events.

Because of their reliance on free-swimming sperm, the lack of physical barriers restricting fertilization and the potential for intragametophytic self-fertilization in some species, ferns have often been perceived as possessing simplistic mating systems (Klekowski, 1973; Singh & Roy, 1977; Traverse, 1988). Despite this view, a growing body of literature suggests that several factors play an important role in mediating reproductive success. A large proportion of fern species produce sex-determining antheridiogens (Döpp, 1950; Näf *et al.*, 1975; Haufler & Gastony, 1978; Schneller *et al.*, 1990; Chiou & Farrar, 1997; Schneller, 2008; Hollingsworth *et al.*, 2012). In nature, antheridiogen systems generate gametophyte populations comprising large female gametophytes and small male gametophytes, resulting in a disproportionate number of gametophytes that may act only as male parents relative to those in non-antheridiogen-induced populations (Tryon & Vitale, 1977; Haufler & Soltis, 1984; Schneller *et al.*, 1990; Hamilton & Lloyd, 1991). These antheridiogen systems may contribute to asymmetrical hybridization by generating unbalanced sex ratios between hybridizing species, but evidence supporting this possibility has so far been limited (Xiang *et al.*, 2000). Further, evidence suggests that, in hybridization events involving diploid and polyploid ferns, the polyploid is almost always the maternal parent (Vogel *et al.*, 1998a; Xiang *et al.*, 2000; Yatabe *et al.*, 2009; Hunt *et al.*, 2011). It is currently unclear whether this pattern relates solely to differences in breeding systems (diploids are predominantly outcrossing, whereas polyploids tend to self-fertilize; Masuyama & Watano, 1990) or is linked to ploidy-related differences in the size of gametes and gametangia, as suggested by Xiang *et al.* (2000). Other factors, such as differential success of embryos from reciprocal crosses and the relative abundance of each parental species' gametophytes at a given site, have been suggested as potential contributors, and probably play an important role (Vogel *et al.*, 1998a; Xiang *et al.*, 2000; Yatabe *et al.*, 2009; Hunt *et al.*, 2011).

Although relevant aspects of gametophyte reproductive biology have been discussed in most studies of asymmetrical hybridization in ferns, no study has attempted to reconcile the observed patterns of hybridization bias with detailed study of the mating systems of the involved species. Here, we investigate the asymmetry of inheritance in the formation of two common hybrids in the genus *Dryopteris* (Dryopteridaceae) from the northeastern USA. We used sequence data from chloroplast DNA (cpDNA), which is maternally inherited in ferns (Stein & Barrington, 1990; Gastony & Yatskievych, 1992; Vogel *et al.*, 1998b), to determine the extent of hybridization bias in four populations each of *D.* × *triploidea* (*D. carthusiana* × *D. intermedia*) and *Dryopteris* × *boottii* (Tuck.) Underw. (*Dryopteris cristata* × *D. intermedia*) from New York and Vermont, USA by comparing the genotypes of hybrids and their

progenitors. To evaluate the observed patterns of hybridization bias within the context of the progenitor species' reproductive biology, we examined gametophyte selfing and outbreeding potential, assessed intraspecific and interspecific antheridiogen production/response, measured sperm motility and compared the size of male gametes and female gametangia for the three species serving as the progenitors of the two hybrids.

Materials and Methods

Study species and collection

Among North American *Dryopteris*, the two most abundant hybrids are *D.* × *triploidea* and *D.* × *boottii*, both of which are frequently encountered with their respective parents in forests and swamps in eastern North America. *Dryopteris* × *triploidea* is a backcross triploid arising from hybridization between the widespread diploid *D. intermedia* and the allotetraploid *D. carthusiana* (Fig. 1). It is perhaps the most abundant fern hybrid in North America (Xiang *et al.*, 2000). *Dryopteris* × *boottii* is derived from a cross between *D. intermedia* and the allotetraploid *D. cristata* (L.) A. Gray, which is generally restricted to swamps and wetlands in eastern North America.

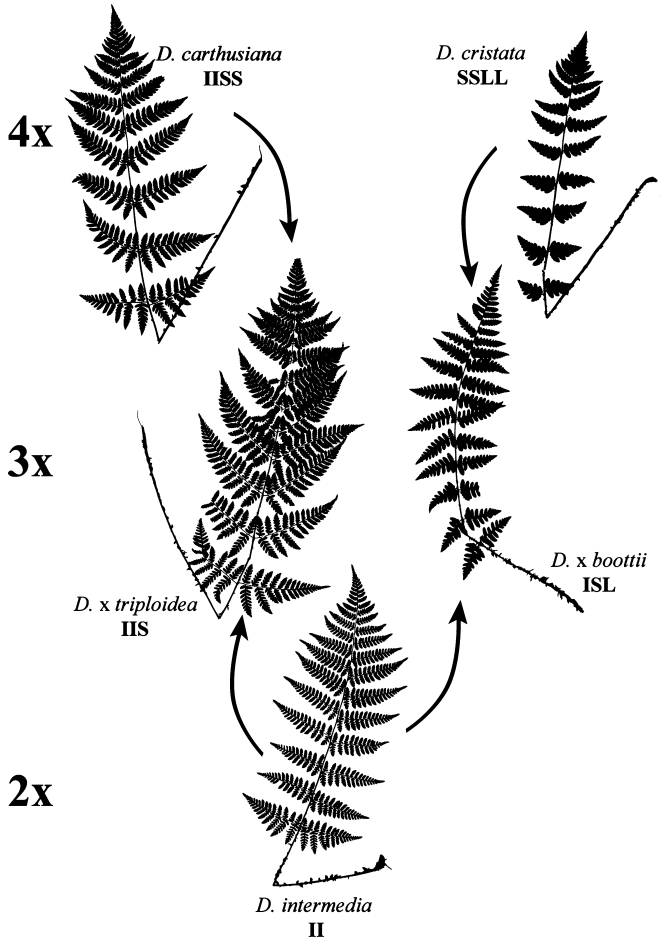

Fig. 1 Leaf silhouettes showing morphology and the hybridization scheme of the taxa examined in this study. Letters below each taxon's name denote its genomic constitution.

Leaf samples of all three progenitor species (*D. intermedia*, *D. carthusiana* and *D. cristata*) and both hybrids were collected from four sites in eastern New York and northwestern Vermont, USA. The population localities are hereafter referred to as follows: (1) Farrell Park (44°26′39″N, 73°12′14″W; Chittenden County, VT, USA); (2) LaPlatte River (44°22′43″N, 73°13′3″W; Chittenden County, VT, USA); (3) Huyck Preserve (42°31′53″N, 74°9′26″W; Albany County, NY, USA); and (4) Basic Creek (42°30′38″N, 74°2′28″W; Albany County, NY, USA). At each site, we collected leaves from several (two to four) individuals of each parental species; all hybrid plants located at each site (13–22) were sampled. All taxa were tentatively identified in the field on the basis of leaf morphology (Fig. 1) and sporangial dehiscence; specimens were later examined in the laboratory for the presence of glandular trichomes on the lamina and indusia (present in *D. intermedia* and derived hybrids) and to check spore regularity (hybrids possess abortive spores). We then compared these morphology-based determinations with genome size data obtained via flow cytometry to confirm hybrid identity. In total, nine *D. intermedia*, 12 *D. carthusiana,* 10 *D. cristata,* 73 *D.* × *triploidea* and 70 *D.* × *boottii* were collected; all vouchers (*Testo 2013-1* through *Testo 2013-174*) are deposited at VT.

Genome size estimation

We used flow cytometry to estimate genome size and to infer ploidy level in all specimens examined. Samples were prepared following the protocol of Bainard *et al.* (2011) using LB01 buffer (Dolezel *et al.*, 1989) supplemented with 1% polyvinylpyrrolidone-40 (PVP-40). Approximately 1 cm^2 of silica-dried leaf tissue from each sample was co-chopped with an equal amount of tissue of a standard of known genome size (*Pisum sativum* cv 'Ctirad', 2C = 9.09 pg) grown from seeds obtained from the Laboratory of Cytogenetics and Cytometry (Olomouc, Czech Republic) using a fresh razor blade for each sample. We chopped all samples for 1 min in 1.2 ml of ice-cold buffer with 100 µg ml^{-1} of propidium iodide and 50 µg ml^{-1} ribonuclease A added; the resulting homogenate was passed through a 30-µm filter and stained on ice in the dark for 1 h. We analyzed all samples using an EPICS XL/XL-MCL Flow Cytometry System with a 488-nm argon laser at the Vermont Cancer Center at the University of Vermont. We recorded at least 1000 events for both sample and standard peaks. The genome size (2C) was estimated by multiplying the sample peak mean fluorescence by the 2C value of the standard (9.09 pg) and dividing that value by the standard peak mean fluorescence. Genome size estimates for samples from each parental species were compared with the values reported by Bainard *et al.* (2011); the expected values for hybrids were assumed to be intermediate between those of the putative parents, as has been shown for other fern hybrids (Bennert *et al.*, 2005; Ekrt *et al.*, 2010).

DNA extraction and sequencing

We extracted total genomic DNA from fresh leaf material following a cetyltrimethylammonium bromide (CTAB) extraction

protocol (Doyle & Doyle, 1987) with the modifications of Porebski *et al.* (1997). Polymerase chain reaction (PCR) was used to amplify a *c.* 350-bp region of the chloroplast intergenic spacer *trnL-F* using the universal primers 'e' and 'f' designed by Taberlet *et al.* (1991). The region was amplified as follows: an initial denaturation cycle of 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 55°C and 60 s at 72°C, and a final extension period of 7 min at 72°C. PCR products were electrophoresed and visualized on 1% agarose gel with ethidium bromide. PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH, USA) following the manufacturer's protocols; sequencing was performed using the amplification primers employing an ABI Prism 3130x1 sequencer at the Vermont Cancer Center (Burlington, VT, USA).

Sequence analysis

All sequences were edited using Geneious v.7.0.2 (Biomatters, Ltd, Auckland, New Zealand) and aligned using the MAFFT plugin (Katoh *et al.*, 2002). Parental contributions of hybrids were determined by comparing aligned sequences of each hybrid with sequences of each progenitor from the same locality and assigning them to the parent with which they shared the *trnL-F* genotype.

Spore collection and gametophyte culture conditions

To investigate gametophyte reproductive biology of the hybridizing species, we grew gametophytes of *D. intermedia*, *D. carthusiana* and *D. cristata* in the laboratory from spores collected at the Huyck Preserve locality. Spores were collected by placing fertile pinnae in sealed glassine envelopes, allowing the pinnae to air dry for 3–5 d to allow for the release of spores. Spores were collected from three different sporophytes per species to test for individual-level variation. Spore sowing methodology, media preparation and culture conditions followed Testo & Watkins (2011, 2013) with spores of each individual sporophyte cultured in separate Petri dishes.

Gametophyte mating systems

To determine gametophyte isolate potential for each species, we transferred 60 gametophytes of each species (20 from each sporophyte) to isolated 1 × 1-cm^2 chambers in a sealed plastic container containing the same growth medium at 60 d post-sowing and observed weekly for 200 d. To determine intrasporophytic and intersporophytic crossing potentials, we transferred 60 pairs of gametophytes of each species (10 intrasporophytic pairs × 3 sporophytes and 10 intersporophytic pairs × 3 combinations) and observed for the same time period. We watered gametophytes weekly with distilled water to facilitate fertilization, and sporophyte formation was determined by examining gametophytes visually. Sporophyte production values were obtained by dividing the number of sporophytes observed at 200 d by the total number of gametophytes in the treatment ($N = 60$ for all treatments) following Peck *et al.* (1990).

Antheridiogen tests

Antheridiogen production and response were determined by sowing spores of each species in the presence of a 3-month-old archegoniate gametophyte in a 100 × 20-mm^2 Petri dish and observing the growth and sexual development of the developing young gametophytes for 3 months. Slow growth and the precocious formation of antheridia by the resulting gametophytes were interpreted as evidence of an antheridiogen response (Haufler & Gastony, 1978; Chiou & Farrar, 1997; Watkins & Farrar, 2005). Intraspecific responses were tested by sowing spores in the presence of a conspecific gametophyte, and interspecific responses were tested by sowing spores in the presence of a heterospecific gametophyte. We used two controls to assess antheridiogen responsiveness and production. As the effects of *Pteridium aquilinum* antheridiogen have been well studied (Näf *et al.*, 1975) and it is known that young gametophytes of *Onoclea sensibilis* produce antheridia only in response to antheridiogen presence (Raghavan, 1989), these species were used to assay for antheridiogen responsiveness and production, respectively. To assay for antheridiogen responsiveness, spores of all species (including *Onoclea*) were sown in the presence of 3-month-old archegoniate *Pteridium* gametophytes and observed for antheridium formation as above. To assay for antheridiogen production, spores of *Onoclea* were sown in the presence of 3-month-old archegoniate gametophytes of each species and examined for antheridium formation. An additional control was conducted by sowing spores of all species in the absence of mature gametophytes. All assays were tested with five replicate plates each.

Sperm motility

We estimated sperm motility by observing sperm longevity and calculating sperm swimming speed for all three species. To determine sperm longevity, a single antheridiate gametophyte was placed in 50 µl of distilled water on a microscope slide for 1 min to allow for sperm release. We then removed the gametophyte from the slide and transferred 20–25 µl of water (containing *c.* 20–25 spermatozoids) to another slide and added a glass coverslip. We observed sperm motility under a light microscope at 100× magnification to determine the total number of sperm present on the slide and the number of actively swimming sperm. We recorded the proportion of active sperm at 2-min intervals until all sperm ceased swimming; the microscope's lamp was turned off between observation periods to prevent overheating. Sperm from five gametophytes were observed for each species. Because sperm activity was observed at intervals rather than continually, we estimated mean sperm longevity by fitting a second-order polynomial function to a regression of the observed data and solving for the time point at which 50% of sperm remained active.

Sperm swimming speed was determined using a SPOT Insight Firewire 2.0 camera (Spot Imaging Solutions, Sterling Heights, MI, USA) mounted on a light microscope. To obtain these values, we mounted several antheridiate gametophytes on a slide in distilled water under a glass coverslip and observed spermatozoid movement at 400× magnification. To determine sperm swimming rate, we imaged individual spermatozoids five times at

1-s intervals; the distance swum during each interval was measured using ImageJ image analysis software (Abràmoff *et al.*, 2004). Measurements were recorded assuming a linear path of movement, which was consistent with the patterns of movement observed at this scale. We performed this for five spermatozoids per slide and five slides per species, for a total of 125 measurements per species. We estimated the dispersal range of an average spermatozoid for each species by multiplying that species' mean sperm swimming speed by the time of average spermatozoid longevity as calculated from the best-fit equation described in the preceding paragraph.

Gamete/gametangium measurements

To test the possibility that hybridization in these taxa is constrained by gamete/gametangium size incompatibility, we measured the diameter of spermatozoids and archegonial neck canals for all three species. We mounted gametophyte material in water on slides with a cover slip; measurements were made at 400× magnification using a light microscope. Live spermatozoids were measured after cytoplasmic release (fern sperm eject their cytoplasm before fertilization) to prevent overestimation of the functional size of the spermatozoid (Lopez-Smith & Renzaglia, 2008). Archegonial neck measurements were obtained by viewing longitudinal hand sections of the thallus cut through the archegonial cushion; in all cases, the narrowest portion of recently opened archegonial canals was measured. Measurements of ten spermatozoids and archegonia were obtained from five gametophytes of each species.

Statistical analyses

To test the statistical null hypotheses that the ratio of parental contribution among hybrids did not differ from 1 : 1 (as would be expected under non-biased hybridization), we utilized the replicated G goodness-of-fit statistic (Sokal & Rohlf, 1995). This allowed us to test for bias both within each population and among the pooled populations for each hybrid, as well as for population-level heterogeneity in hybridization bias ratios using the G_{het} test for heterogeneity. One-way ANOVA, followed by *post-hoc* Tukey tests, was used to compare gametophyte isolate potential, intra- and intersporophytic outcrossing potential, sperm swimming speed and sperm/archegonial neck sizes across species; throughout, $\alpha = 0.05$ was used. For all measurements, data were tested for normality using the Shapiro–Wilk test and log-transformed when necessary. All statistical analyses were performed using JMP Pro 10 (SAS Institute Inc., Cary, NC, USA).

Results

Genome size estimation

All samples analyzed generated well-resolved peaks with low variation; for each taxon, variance in genome size across the specimens examined was low. Three distinct ranges of genome sizes were recovered from our analyses, consistent with diploid, triploid and tetraploid plants (Table 1). In all cases, ploidy-level estimates generated by flow cytometry were consistent with our morphology-based determinations, confirming the utility of intermediate morphology coupled with spore irregularity for the identification of these hybrids. *Dryopteris* × *triploidea* and *D.* × *boottii* possessed similar-sized genomes (24.52 and 25.03 pg); these values were consistent with estimates obtained by adding the 1C values of their respective progenitor taxa. Genome-size estimates for the three parental taxa were very similar to those reported by Bainard *et al.* (2011).

Parental genotype discrimination

The target region was successfully amplified and sequenced for all samples; all individuals of *D. carthusiana* and *D. cristata* were found to share an identical genotype, consistent with earlier studies which have demonstrated that these species share a maternal progenitor (Stein *et al.*, 2010; Sessa *et al.*, 2012). All individuals of *D. intermedia* shared a genotype that differed from the *carthusiana*/*cristata* genotype by several point mutations and two indels (one 9 bp; the other 1 bp). The consistency of sequences within species and considerable differences between the *intermedia* and *carthusiana*/*cristata* genotypes demonstrate the utility of this region for discriminating between parental genotypes and their hybrids. All hybrids shared a *trnL-F* genotype with only one of their progenitors, which is consistent with the assumption of uniparental inheritance of cpDNA in ferns.

Maternal parentage of hybrids

Of the 73 *D.* × *triploidea* sequenced, 64 possessed the *carthusiana* genotype, whereas only nine possessed the *intermedia* genotype; the resulting 7.11 : 1 ratio differed very significantly from the null hypothesis of a 1 : 1 parentage ratio (Fig. 2; $G = 46.94$; $P < 0.0001$). Population-level hybridization bias differed significantly from 1 : 1 in all populations, ranging from 5.5 : 1 (LaPlatte River) to 8.5 : 1 (both Huyck Preserve and Farrell Park; Fig. 2). The degree of bias did not differ significantly across populations (G_{het} test of heterogeneity; $G = 0.26$; $P = 0.61$).

Of the 70 *D.* × *boottii* sequenced, 54 possessed the *cristata* genotype, whereas 16 possessed the *intermedia* genotype; the resulting 3.6 : 1 ratio differed very significantly from the null hypothesis of a 1 : 1 parentage ratio ($G = 33.18$; $P < 0.0001$). The strongest hybridization bias was observed in the LaPlatte River population ($G = 20.79$; $P < 0.0001$), which was fixed (15 : 0) for the *cristata* genotype. The Basic Creek population exhibited the weakest bias observed (1.5 : 1; $G = 0.81$; $P = 0.37$); this was the only population for which the observed hybridization bias did not differ significantly from the null hypothesis of a 1 : 1 ratio. A test of among-population variation in hybridization bias ratio found significant variation in the degree of bias across populations (G_{het} test of heterogeneity; $G = 9.77$; $P = 0.0018$).

Gametophyte mating systems

Gametophyte isolate potential differed significantly across species ($F_{2,179} = 649.6$; $P < 0.0001$); values (mean ± SD) ranged from

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Table 1 Genome size estimates for *Dryopteris* species and hybrids as determined by flow cytometry in this study; chromosome counts from Walker (1961)

Taxon	Published chromosome count (ploidy)	Mean 2C value (pg) (± SE)	Mean 2C value (pg) (± SE) reported by Bainard *et al.* (2011)
Dryopteris intermedia (*N* = 9)	2*n* = 82 (2*x*)	15.23 (± 0.422)	15.40 (± 0.660)
Dryopteris × *triploidea* (*N* = 73)	2*n* = 123 (3*x*)	24.52 (± 0.577)	N/A
Dryopteris carthusiana (*N* = 12)	2*n* = 164 (4*x*)	33.61 (± 0.428)	33.83 (± 0.192)
Dryopteris × *boottii* (*N* = 70)	2*n* = 123 (3*x*)	25.03 (± 0.301)	N/A
Dryopteris cristata (*N* = 10)	2*n* = 164 (4*x*)	34.01 (± 0.400)	33.85 (± 0.424)

N, number of sporophytes sampled per taxon. 2C values not obtained by Bainard *et al.* (2011) are designated 'N/A'.

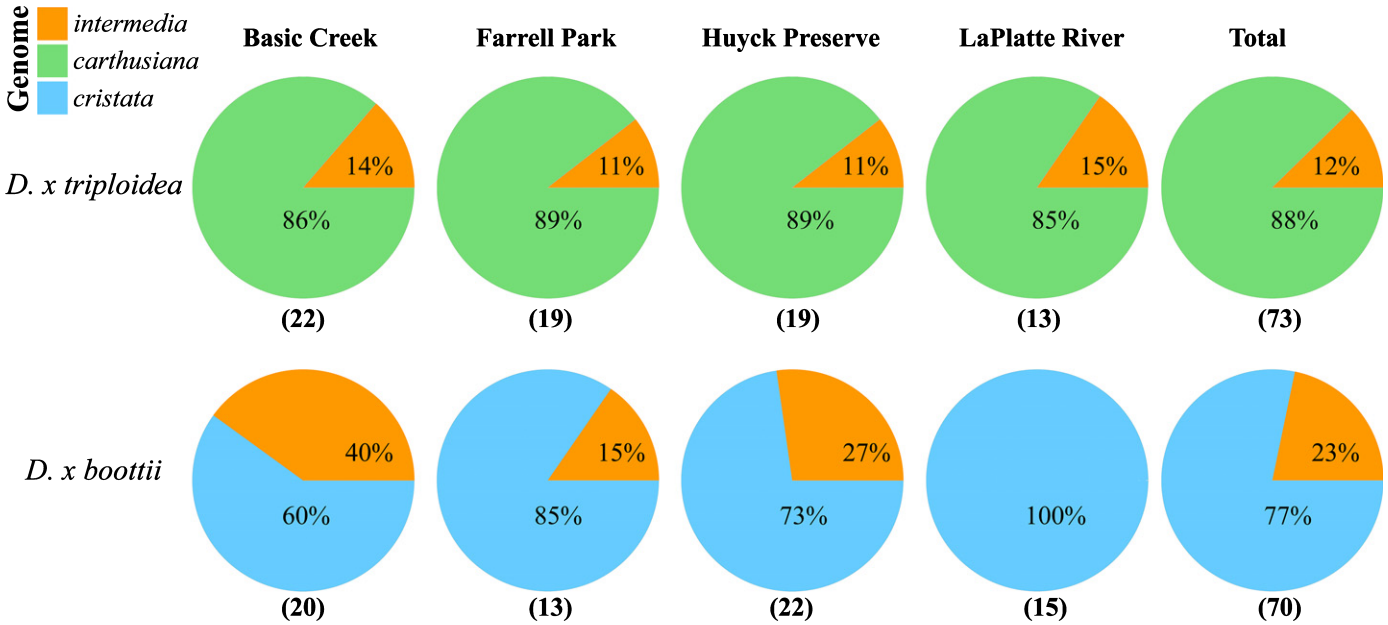

Fig. 2 Distribution of chloroplast genome inheritance in four populations of *Dryopteris* × *boottii* and *D.* × *triploidea*. Colors denote the maternal chloroplast genome inherited and percentage values denote the percentage of individuals possessing the corresponding maternally inherited genome in that population. Numbers in parentheses under each pie chart refer to the sample size at each population. Total values summed across populations are shown on the right.

21.1 ± 3.5% for *D. intermedia* to 62.8 ± 7.5% for *D. carthusiana* (Fig. 3). Sporophyte production among intrasporophytic pairs differed significantly across species ($F_{2,179} = 93.0$; $P < 0.0001$): it was highest in *D. intermedia* (67.2 ± 7.9%) and lowest in *D. cristata* (51.1 ± 7.5%). Intersporophytic gametophyte crossing potential was highest in *D. intermedia* (71.1 ± 10.8%) and lowest in *D. cristata* (46.1 ± 7.9%); differences across species were significant ($F_{1,179} = 114.0$; $P < 0.0001$). *Dryopteris intermedia* sporophyte production among isolated gametophytes was lower than that of either intrasporophytic or intersporophytic pairs ($F_{2,179} = 726.8$; $P < 0.0001$). Sporophyte production did not differ significantly among any of the treatments for either *D. carthusiana* or *D. cristata*.

Antheridiogen tests

With the exception of *D. cristata*, all species exhibited stunted gametophyte development and precocious antheridium formation when grown in the presence of archegoniate *Pteridium* gametophytes. Antheridiogen response in *Onoclea* was induced by the presence of all species, except *D. cristata*. Evidence of an active antheridiogen system was detected in *D. intermedia*, which induced antheridium formation and slowed growth in young conspecific gametophytes. No evidence of interspecific antheridiogen effects induced by mature gametophytes of *D. intermedia* was observed. Mature gametophytes of *D. carthusiana* induced stunted growth and precocious antheridium formation in gametophytes of *D. intermedia*, but not in conspecific gametophytes or in those of *D. cristata*. No evidence of an antheridiogen system was observed in *D. cristata*. No gametophytes sown in the absence of mature gametophytes exhibited evidence of an antheridiogen response. In all cases, antheridiogen responses were observed within 25 d of spore germination.

Sperm motility

Average and maximum sperm longevity were greater in *D. intermedia* (average, 15.3 min; maximum, 28 min) than in

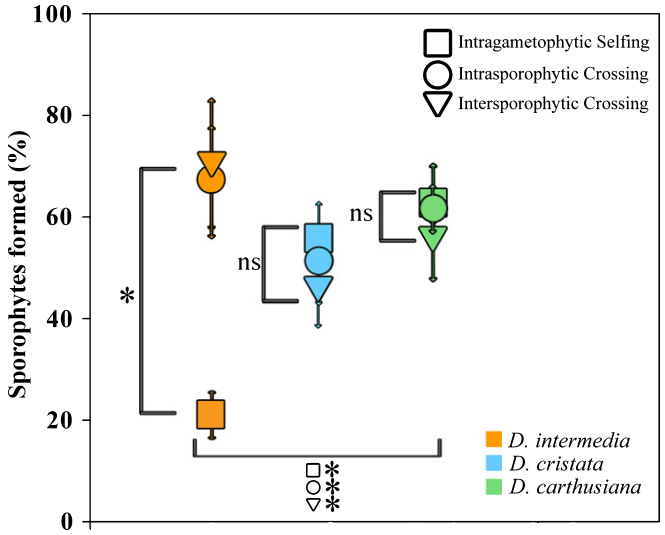

Fig. 3 Sporophyte production at 260 d across three levels of inbreeding for *Dryopteris intermedia*, *D. cristata* and *D. carthusiana*. Colors denote species and symbol shapes denote the level of inbreeding. Error bars represent ± 1SD. Brackets summarize results of one-way ANOVA and *post-hoc* Tukey tests; vertical brackets represent within-species comparisons across inbreeding levels and horizontal brackets represent across-species comparisons within a given inbreeding level. *, Significant differences in sporophyte production at $\alpha = 0.05$; ns, no significant difference. $N = 60$ throughout.

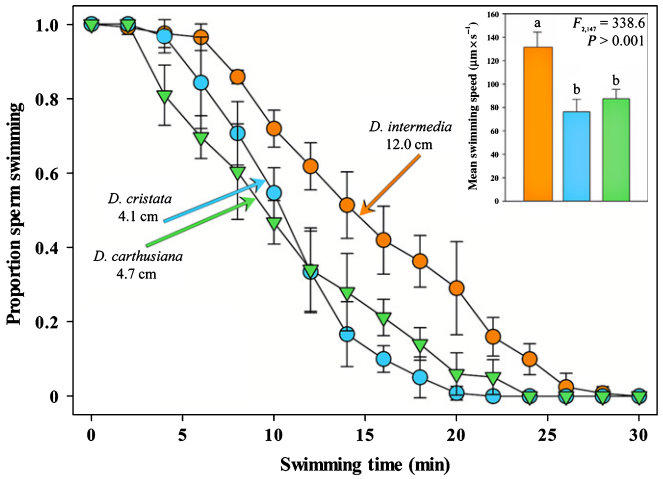

Fig. 4 Summary of sperm motility for *Dryopteris intermedia*, *D. cristata* and *D. carthusiana*. Scatter plot shows the proportion of sperm swimming at each 2-min time interval; inset bar graph shows mean sperm swimming speed. Arrows in scatter plot identify swimming viability time recovered from best-fit equation (see the Materials and Methods section) and estimated mean sperm dispersal range. Throughout, color denotes species. Error bars represent ± 1SD; lower case letters represent homogeneous subsets recovered by ANOVA and *post-hoc* Tukey tests.

either *D. carthusiana* (average, 9.1 min; maximum, 22 min) or *D. cristata* (average, 9.0 min; maximum, 20 min) (Fig. 4). Sperm of *D. intermedia* exhibited faster swimming speeds than those of either *D. carthusiana* or *D. cristata* ($F_{2,147} = 338.6$; $P < 0.0001$). The estimated mean sperm dispersal range for *D. intermedia* (12.0 cm) was nearly three times greater than that of *D. carthusiana* (4.7 cm) or *D. cristata* (4.1 cm).

Gamete/gametangium measurements

Archegonium neck canal diameter ranged from 6.77 ± 0.67 μm in *D. intermedia* to 11.39 ± 1.52 μm in *D. carthusiana* and differed significantly between *D. intermedia* and *D. carthusiana*/*D. cristata* ($F_{2,147} = 260.6$; $P < 0.0001$; Fig. 5a). Average spermatozoid diameter differed very significantly across species ($F_{2,147} = 101.8$; $P < 0.0001$) and ranged from 2.86 ± 0.24 μm in *D. intermedia* to 3.73 ± 0.33 μm in *D. carthusiana* (Fig. 5b).

Discussion

We have provided a basic mechanistic understanding of some factors that influence patterns of hybridization in ferns by assessing the extent of asymmetrical hybridization across four populations of two *Dryopteris* hybrids from eastern North America. Unlike previous studies, our approach has allowed us to ascertain the extent of biased hybridization in the formation of hybrids and to compare the degree of bias between hybrids and among populations.

For both hybrids, the majority of plants in each population possessed the chloroplast genome of their tetraploid progenitor (either *D. carthusiana* or *D. cristata*) indicating a consistent bias in inheritance across populations, with a 7.1 : 1 ratio of *carthusiana* : *intermedia* genome inheritance found for *D.* × *triploidea* and a 3.6 : 1 *cristata* : *intermedia* ratio detected for *D.* × *boottii*. These ratios are consistent with the findings of several earlier studies that reported asymmetrical hybridization in ferns, for example, Vogel *et al.* (1998a), Hunt *et al.* (2011), Zhang *et al.* (2013) and, especially, Xiang *et al.* (2000), who described biased inheritance in *D.* × *triploidea*. Unlike Xiang *et al.* (2000), we did not find evidence of population-level variation in the degree of bias for *D.* × *triploidea*, but we did encounter significant variation across populations of *D.* × *boottii*. Overall, the degrees of asymmetry detected in these *Dryopteris* hybrids are within the range previously reported for other ferns, although unidirectional hybridization, which has been reported for a number of other fern hybrids, was found in only one population of *D.* × *boottii*. Clearly, further study of hybridization bias across a greater number of populations and broader geographical ranges is needed to understand the extent and drivers of variation in asymmetrical formation of individual hybrid taxa.

The list of factors that may contribute to hybridization bias is extensive; however, aspects of the hybridizing species' breeding systems have been the focus of most speculation to date. Among angiosperms, a strong association has been demonstrated between biased hybridization and selfing potential, with self-incompatible species functioning strictly as pollen donors when hybridizing with self-compatible species (Tiffin *et al.*, 2001). In ferns, the relationship between breeding system and parental role during hybridization is less clear, with conflicting trends reported between selfing potential and the direction of bias (Vogel *et al.*, 1998a; Xiang *et al.*, 2000). We found that, in the case of both hybridizing pairs studied here, the predominantly self-fertilizing species was the maternal parent of the majority of hybrid offspring. It is also worth noting that the self-compatible

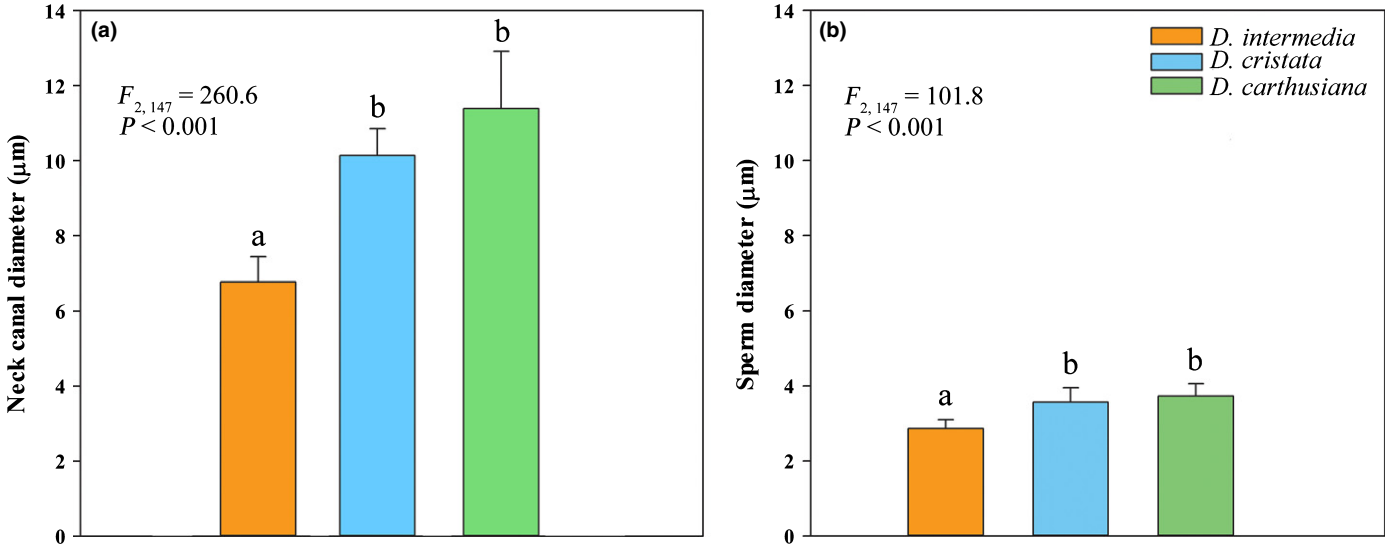

Fig. 5 Diameter of archegonial neck canals (a) and spermatozoids (b) for *Dryopteris intermedia*, *D. cristata* and *D. carthusiana*. Error bars represent ± 1SD; lowercase letters denote homogeneous subsets recovered by one-way ANOVA and *post-hoc* Tukey tests. $N = 50$.

D. carthusiana and *D. cristata* are polyploid, whereas the predominantly outcrossing *D. intermedia* is diploid.

We predicted that species with an antheridiogen system would be more likely to serve as the male parent during hybridization events because of an expected abundance of antheridiogen-induced male gametophytes. Consistent with this prediction, a strong antheridiogen system was detected in *D. intermedia*, which was the paternal donor of the majority of hybrids in all populations. Neither *D. carthusiana* nor *D. cristata* exhibited evidence of a self-influencing antheridiogen system, although mature female gametophytes of the former species did induce stunted gametophyte growth and precocious antheridium formation in *D. intermedia*. Unlike those of *D. cristata*, female *D. carthusiana* gametophytes are capable of inducing maleness in neighboring young *D. intermedia* gametophytes, thereby increasing the probability of fertilization by *D. intermedia* sperm, whilst also prohibiting nearby *D. intermedia* gametophytes from becoming female. A similar pattern of variation in antheridiogen responsiveness was reported in the genus *Cystopteris* by Haufler & Ranker (1985), and was hypothesized to play a role in the hybridization event leading to the formation of the allopolyploid *Cystopteris tennesseensis*. This interspecific antheridiogen effect may partially explain the stronger and more consistent bias detected in *D.* × *triploidea* relative to *D.* × *boottii*. Further study in other groups is needed to evaluate the evolutionary significance of antheridiogens in homosporous ferns.

Although the reliance of ferns on water for sperm dispersal is understood to be an important component of their reproductive biology (e.g. Lloyd, 1974; Willson, 1981; Barrington, 1993; Banks, 1999; Watkins & Cardelús, 2012) few studies have attempted to quantify sperm viability or dispersal within the group. Duckett & Duckett (1980) reported that sperm of *Equisetum* can swim at least 1 m; however, much shorter dispersal ranges have been reported for leptosporangiate ferns. Schneller *et al.* (1990) estimated that sperm of *Athyrium filix-femina* (L.) Roth could swim 100–200 μm s^{-1} and disperse 4–8 cm before becoming inviable. Similar swimming speeds have been reported for sperm of *Lygodium japonicum* (Thunb.) Sw. (*c.* 140 μm s^{-1}; Sakaushi *et al.*, 2003), *Marsilea vestita* Hook. & Grev. (*c.* 120 μm s^{-1}; Bilderback *et al.*, 1974) and *Pteridium aquilinum* (L.) Kuhn (*c.* 95 μm s^{-1}; Rothschild, 1951), although neither the duration of sperm viability nor the estimated dispersal distances were reported for these taxa. The sperm swimming speeds reported here for these *Dryopteris* species are generally within this range; however, the diploid sperm of *D. carthusiana* and *D. cristata* were much slower than the haploid *D. intermedia* sperm (Fig. 4). This pattern may be a result of the larger size of the former species' sperm or some as yet unidentified factor.

Dryopteris intermedia sperm exhibited greater longevity than those of either *D. carthusiana* or *D. cristata* which, coupled with faster swim speeds, yields a nearly three-fold difference in the distance an average sperm can disperse between these taxa (Fig. 4). Assuming that sperm disperse in all directions from the source gametophyte, there is a more than eight-fold difference in area of average sperm dispersal between *D. intermedia* and *D. cristata*, and a nearly seven-fold difference between *D. intermedia* and *D. carthusiana*. This substantial difference in sperm motility has important implications for the reproductive potential of these species – sperm of *D. intermedia* are able to swim long distances relative to those of either *D. carthusiana* or *D. cristata*, and thus have a greater probability of reaching a female gametophyte, including those of other species. Although differences in pollen dispersal capacity are known to influence directionality of hybridization in angiosperms (Linhart, 1973; Potts & Reid, 1988; Prentis *et al.*, 2007; Field *et al.*, 2011), this is the first study demonstrating a link between sperm dispersal capacity and biased hybridization among ferns. Beyond its relevance to the hybrid system studied here, a difference in sperm dispersability between diploid and polyploid fern species may explain an apparent tendency of tetraploids to serve as maternal parents of both

sterile hybrids (e.g. *A.* × *alternifolium*, Vogel *et al.*, 1998a; *Dryopteris* × *mickelii* J. H. Peck and *Dryopteris* × *dowellii* (Farw.) Wherry, W. L. Testo, unpublished) and allopolyploids (e.g. *Asplenium ceterach* L., Trewick *et al.*, 2002; *Dryopteris clintoniana* (D. C. Eaton) Dowell, Sessa *et al.*, 2012; *Cystopteris laurentiana* (Weath.) Blasdell, Rothfels *et al.*, 2013; *Polystichum setigerum* (C. Presl) C. Presl, S. Jorgensen & D. Barrington, unpublished) during diploid–tetraploid hybridization events. The study of hybridization involving two species of the same ploidy may provide insight into the role of sperm size in mediating the directionality of hybridization.

On reaching a suitable gametophyte, a fern sperm must successfully navigate the length of the archegonial neck canal to attain syngamy. In order to do so, the sperm must both swim through viscous mucilage produced by the archegonium and pass through the relatively long and narrow neck canal, which is typically occupied by other sperm at the same time (Bell & Duckett, 1976; Lopez-Smith & Renzaglia, 2008). The study of attempted intergeneric hybridization between *Dryopteris* and *Athyrium* gametophytes indicates that archegonial chemical secretions act to inhibit distant crosses (Schneller, 1981); however, little is known about sperm–archegonium interactions in closely related hybridizing pairs, including their potential influence on biased hybridization. Xiang *et al.* (2000) suggested that ploidy-related differences in sperm and archegonium size may influence hybridization between tetraploid *D. carthusiana* and diploid *D. intermedia*. They contended that diploid sperm of *D. carthusiana* may be too large to navigate a haploid *D. intermedia* gametophyte's archegonial neck canal, but did not measure sperm or neck canal diameter for either species. Although we found that *D. intermedia* possessed smaller sperm and narrower archegonial neck canals than either *D. carthusiana* or *D. cristata*, in no case were sperm of either tetraploid species larger than the diameter of the archegonial neck canal of *D. intermedia*. Sperm of these species are not too large to fertilize eggs of *D. intermedia*, consistent with the observation of *D. intermedia* maternal parentage at low levels in most populations of hybrids. However, a comparison of intraspecific and interspecific sperm and neck canal diameters does suggest that *D. carthusiana* and *D. cristata* sperm are probably more restricted in their movement when traveling in an archegonial neck canal of a *D. intermedia* gametophyte than when attempting to fertilize an egg of their own species. This restriction may help to explain why these species are rarely the paternal donor in these hybridization events. Conversely, the relatively large archegonial neck canals of *D. carthusiana* and *D. cristata* would provide ample room for the small sperm of *D. intermedia*, and potentially increase the number of sperm that can travel through the canal concurrently, thereby increasing the chances of successful fertilization. Although the sperm of *D. carthusiana* and *D. cristata* are not so large that they are incapable of successfully navigating the archegonial canal of *D. intermedia*, it seems probable that the reduced ratio of sperm : neck canal diameter in crosses of this direction reduces the probability of successful fertilization.

Despite the prevalence of hybridization among ferns, few studies have attempted to examine factors influencing gametophyte mating success, to characterize isolating mechanisms or to explain patterns of hybrid formation. The findings presented here begin to address these questions by providing insight into the mechanisms underlying asymmetrical hybridization in eastern North American *Dryopteris* and demonstrating a link between the direction and strength of hybridization bias and the gametophyte reproductive biology of the parental species. The predominantly outcrossing mating system of *D. intermedia* and its mediation of sex expression via antheridiogen appear to favor its predominantly paternal contribution to hybrid formation. Extending the findings of our work and earlier studies examining sex expression in these species (Cousens, 1975; Xiang *et al.*, 2000; Flinn, 2006) to a natural gametophyte community, we would expect *D. intermedia* to be represented by relatively few female gametophytes and a large proportion of antheridiogen-induced male gametophytes, whereas *D. carthusiana* and *D. cristata* would be represented primarily by self-compatible bisexual gametophytes. A significant proportion of the *D. intermedia* population would then be able to function only as sperm donors, compared with a majority of the predominantly hermaphroditic *D. carthusiana* and *D. cristata* gametophytes. Further, *D. intermedia* gametophytes are capable of dispersing sperm over a far greater area than those of either *D. carthusiana* or *D. cristata*, and can exploit a potentially favorable sperm–neck canal size difference. Taken together, these factors suggest a scenario in which fertilization of an egg of *D. carthusiana* or *D. cristata* by *D. intermedia* sperm is much more probable than the reciprocal fertilization event, consistent with the pattern of biased parentage reported here.

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