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Natural History Note

Natural Hybridization between Genera That Diverged from Each Other Approximately 60 Million Years Ago

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abstract: A fern from the French Pyrenees—×*Cystocarpium roskamianum*—is a recently formed intergeneric hybrid between parental lineages that diverged from each other approximately 60 million years ago (mya; 95% highest posterior density: 40.2–76.2 mya). This is an extraordinarily deep hybridization event, roughly akin to an elephant hybridizing with a manatee or a human with a lemur. In the context of other reported deep hybrids, this finding suggests that populations of ferns, and other plants with abiotically mediated fertilization, may evolve reproductive incompatibilities more slowly, perhaps because they lack many of the premating isolation mechanisms that characterize most other groups of organisms. This conclusion implies that major features of Earth's biodiversity—such as the relatively small number of species of ferns compared to those of angiosperms—may be, in part, an indirect by-product of this slower "speciation clock" rather than a direct consequence of adaptive innovations by the more diverse lineages.

Keywords: allopolyploidy, deep hybridization, divergence-time dating, reproductive isolation, speciation, species selection.

Introduction

Gene flow among populations—hybridization broadly construed—can have a positive, creative role in speciation (Mallet 2007; Abbott et al. 2013). However, its greater impact is as a homogenizing force, reuniting populations that might otherwise have had separate evolutionary trajectories (e.g., Taylor et al. 2005; Hegde et al. 2006; Seehausen 2006). The formation of reproductive barriers between populations is thus of central importance to evolutionary biology. They facilitate local adaptation, permit phenotypic, genotypic, and ecological divergence, and ultimately result in the partitioning of life into the diversity of species that occupy this planet (Coyne and Orr 2004; Rieseberg and Willis 2007).

Earlier investigations established a positive correlation between time since divergence of two lineages and the cumulative strength of the reproductive barriers between them (Coyne and Orr 1989, 1997; Sasa et al. 1998; Presgraves 2002; Mendelson 2003; Moyle et al. 2004). Another important early observation is that the pace of accumulation of incompatibilities—the "incompatibility clock" (or "speciation clock")—varies across taxonomic groups (Prager and Wilson 1975; Edmands 2002; Coyne and Orr 2004). For example, reproductive barriers in *Drosophila* are complete within approximately 4 million years (i.e., *Drosophila* lineages that diverged more than about 4 million years ago [mya] are unable to hybridize; Carson 1976; Coyne and Orr 1997), and the oldest species pairs of African cichlids, plethodontid salamanders, hylid treefrogs, and sunfish that are still capable of producing viable hybrids diverged less than 8.5, 12, 34, and 37 mya, respectively (Mable and Bogart 1995; Bolnick and Near 2005; Smith et al. 2005; Wiens et al. 2006; Stelkens et al. 2010). Because most of these studies were done under laboratory conditions, they precluded the possible effects of most prezygotic barriers. Therefore, they likely underestimated the rate at which incompatibilities evolve in the wild, where prezygotic barriers are often strong (Kirkpatrick and Ravigné 2002; Russell 2003). Sunfish, for example, can produce viable zygotes under artificial conditions between parental lineages that diverged up to 34 mya, but the most

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divergent sunfish species reported to produce hybrids in the wild belong to lineages that separated less than 15 mya (Bolnick and Near 2005).

Although hybridization is thought to be more common in plants than animals (Mayr 1963; Mallet 2005), there is little evidence that the rate at which reproductive barriers evolve in flowering plants differs strongly from that reported for animals (reviewed in Levin 2013). For example, species of the flowering annual *Collinsia* appear to be completely incompatible after 5 million years of divergence (Baldwin et al. 2011), and the deepest flowering-plant hybridization we are aware of is between the grass genera *Hordeum* and *Secale* (Forster and Dale 1983), which diverged from each other about 14 mya (Bouchenak-Khelladi et al. 2010). Even the "exceptional" interfertile *Liriodendron* species pair (Moyle et al. 2004) diverged from each other only around 10–15 mya (Parks and Wendel 1990), well within the range reported for animals.

Given the relatively rapid evolution of reproductive isolation in animals and flowering plants, we were surprised to encounter a fern from the French Pyrenees that was morphologically intermediate between the distantly related genera *Cystopteris* and *Gymnocarpium*. These genera are very dissimilar (fig. A1; figs. A1–A5 available online) and, until recently, were placed in different subfamilies or even families (Rothfels et al. 2012*b*). Although infertile, this fern, now named ×*Cystocarpium roskamianum* (Fraser-Jenkins 2008; Fraser-Jenkins et al. 2010), propagates itself vigorously via rhizome growth and does well in cultivation.

In this study we have two primary goals. The first is to assess the hypothesis of intergeneric hybridization, using cytological and single-copy nuclear sequence data from ×*Cystocarpium* and its relatives. Next, we use a series of nested empirical Bayesian analyses of plastid sequence data to estimate the divergence time of the parental lineages.

Methods

Confirming Parentage

To confirm the parentage of ×*Cystocarpium roskamianum*, we amplified and cloned the single-copy nuclear marker *gapCp* "short" (sensu Schuettpelz et al. 2008; Rothfels et al. 2013*a*), henceforth *gapCp*. Genomic DNA was extracted from a ×*Cystocarpium* herbarium specimen (table A1; tables A1–A3 available online) with a DNeasy kit (Qiagen, Valencia, CA). As a precaution against contamination, we extracted ×*Cystocarpium* DNA twice (from the same specimen), in separate extractions conducted several weeks apart. Amplifications were performed in 21-μL reactions that used the reagent mixes of Rothfels et al. (2013*b*) and the primers (ESGAPCP8F1 and ESGAPCP11R1) and thermocycling conditions of Schuettpelz et al. (2008). Polymerase chain reaction (PCR) products were cloned according to the protocols of Schuettpelz et al. (2008), and the colony PCR products were visualized on agarose gels before sequencing with the M13 forward and M13 reverse primers supplied by Invitrogen. Sequencing was done on an ABI Prism 3700 DNA Analyzer (Applied Biosystems) at the Duke University Genome Sequencing and Analysis Core Resource, again using established protocols (Schuettpelz and Pryer 2007).

After identifying and removing PCR recombinant (chimeric) sequences (see Cronn et al. 2002), we had a total of 18 *gapCp* sequences from our x*Cystocarpium* clones (table A1). We added these data to the *gapCp* data set of Rothfels et al. (2014), which spans the diversity of Cystopteridaceae (the family containing *Cystopteris* and *Gymnocarpium*; Rothfels et al. 2012*b*) and has a particularly dense sampling of lineages that may have participated in the ×*Cystocarpium* hybridization event. These 18 ×*Cystocarpium gapCp* sequences included variants that differed from one another by a small number of substitutions that almost certainly represent PCR errors (see Grusz et al. 2009; Beck et al. 2011; Li et al. 2012; Rothfels et al. 2014; Rothfels and Schuettpelz 2014). We removed these errors, following the protocol of Rothfels et al. (2014), which resulted in a final set of four ×*Cystocarpium* alleles (table A1). Our final data set comprised 52 Cystopteridaceae *gapCp* alleles (figs. 1*A*, A2; TreeBASE study no. 16634). A phylogeny inferred from the full data (i.e., including the uncleaned sequences from both Rothfels et al. 2014 and those newly generated here) is available as figure A3.

We analyzed our final data set by using the optimal models and partitioning scheme as determined by an exhaustive PartitionFinder (Guindon et al. 2010; Lanfear et al. 2012) search. To aid in likelihood optimization, we performed this model selection step on a version of the data set with identical sequences removed (identical sequences occur in cases where multiple individuals share a subset of their alleles); these sequences were restored after model selection and were included in all subsequent analyses. The corrected Akaike information criterion (AICc) favored a five-partition model (table 1), and indels were optimized under an Mkv model (Lewis 2001). Maximum likelihood (ML) tree searches were performed with GARLI v2.0 (Zwickl 2006), with each search repeated 10 times from different random-addition starting trees. To assess support, we performed 1,000 ML bootstrap pseudoreplicates, again with GARLI, under the same settings, but with each search performed from only two random-addition starting trees (fig. 1*A*). The GARLI configuration file is available in the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.r7201 (Rothfels et al. 2015).

To further refine our inferences of parentage, we assessed the ploidy level of ×*Cystocarpium* through meiotic

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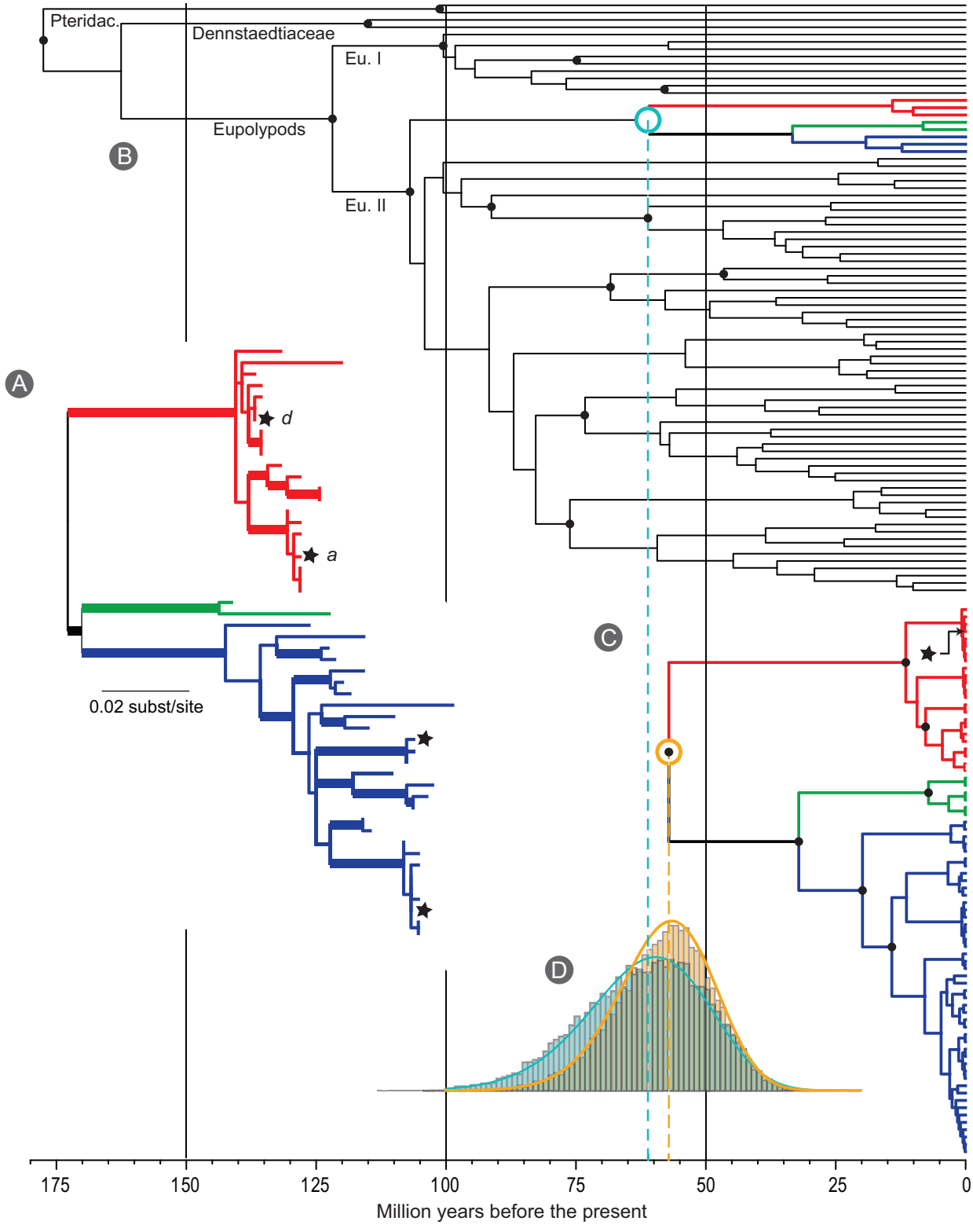

Figure 1: Evidence for the hybrid origin of ×*Cystocarpium* and a sequential empirical Bayesian analysis of divergence time. *A*, Maximum likelihood phylogram of nuclear *gapCp* data; thickened branches have ≥70% bootstrap support; "a" and "d" indicate, respectively, the

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chromosome squashes. Young fertile leaves from living plants were placed in Farmer's fixative (3 parts ethanol : 1 part glacial acetic acid) at room temperature for 24 h and stored in 70% ethanol until they were processed. Sporangia at the proper stage of development (see Windham and Yatskievych 2003) were transferred to a drop of 1% acetocarmine and broken open with the tip of a dissecting needle. When 25–40 sporangia were prepared in this manner, the stain drop was mixed with Hoyer's medium (1 : 1) and squashed by traditional methods (Manton 1950; Windham and Yatskievych 2003). Slides were scanned with a Meiji MT5310L phase contrast microscope, and representative cells were photographed with a Canon EOS T3i camera.

Sequential Empirical Bayesian Divergence Dating

To infer a date for the divergence of the progenitor lineages that we identified for ×*Cystocarpium*, we first extracted the ML divergence-time estimates (shown here in table A2) for 14 highly supported nodes from the plastid chronogram of Schuettpelz and Pryer (2009), the most comprehensive source of dating information available for ferns. We used these 14 node ages to calibrate the five-locus, 81-taxon eupolypods II plastid data set of Rothfels et al. (2012*a*), which has a much denser sampling of Cystopteridaceae and its relatives. The time to most recent common ancestor (tmrca) for each of the 14 calibrated nodes (table A2 and black circles in fig. 1*B*) was given a normal prior distribution, with a mean equal to the ML estimate from Schuettpelz and Pryer (2009) and a standard deviation equal to 10% of that mean. Taxon groups were not constrained to be monophyletic. These analyses were performed in BEAST v1.7.2 (Drummond and Rambaut 2007) under a lognormal uncorrelated relaxed-clock model (Drummond et al. 2006) and a birth-death tree prior. Each locus was permitted its own global rate, under a GTR+G substitution model with unlinked substitution parameters. Priors were left at their default values, except for the UCLD.mean parameter, which was given a lognormal distribution with a mean (in real space) of 0.0012 and a standard deviation of 1.0. This mean was selected as being slightly larger than previous estimates for this locus in ferns (Rothfels and Schuettpelz 2014), which is conservative with respect to our hypothesis of a deep hybridization event. Runs with a lognormal prior of 0.001 or a uniform prior from 0 to 0.1 yielded very similar tmrca estimates. This data set was run four times independently, each for 30 million generations, with parameter values logged every 6,000 generations. These runs converged quickly (Rambaut and Drummond 2007); the first 3 million generations were conservatively discarded as burn-in, before pooling of the four runs. Effective sample sizes (ESSs) for all parameters in the pooled postburn-in sample were above 300 (the BEAST settings file and the full posterior sample are available in the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.r7201 [Rothfels et al. 2015]; the chronogram and alignment are available in TreeBASE, study no. 16634 [http://treebase.org/treebase-web/search/study/summary.html?id=16634]).

From this pooled sample we extracted the posterior tmrca distributions for seven highly supported nodes (table A3). Inspection of these posteriors suggested that they were approximately gamma or lognormally distributed. For each posterior we found the best-fitting gamma and lognormal distributions by ML, using the R package fitdistrplus (Delignette-Muller et al. 2010; R Development Core Team 2011), and selected the best distribution by the AIC (table A3). In all cases, visual inspection of the posterior histograms and the best-fit distributions showed very close concordance. These seven full, best-fitting parametric distributions estimated from the tmrca posteriors (table A3) were used as tmrca priors on their respective nodes in our final Cystopteridaceae data set (black circles in fig. 1*C*).

This final data set comprised the three-locus, 84-taxon Cystopteridaceae plastid data set of Rothfels et al. (2013*b*), with the addition of ×*Cystocarpium* (×*Cystocarpium trnG-R* and *matk* sequences were generated via the protocols of Rothfels et al. 2013*b*; table A1); we also removed that study's outgroup taxa and one sample of *Gymnocarpium disjunctum* with limited character data, for a final total of 75 taxa. We again used BEAST v1.7.2 (Drummond and Rambaut 2007) with a lognormal uncorrelated relaxed-clock model (Drummond et al. 2006), but this time with a coalescent tree prior. The three loci were given their best-fitting substitution model, as determined by Rothfels et al. (2013*b*), with each locus permitted its own average rate.

Gymnocarpium appalachianum– and *Gymnocarpium disjunctum*–type alleles found in ×*Cystocarpium*. *B*, *C*, Maximum clade credibility plastid chronograms, with node ages at their median posterior estimates. *B*, Divergence-time estimates from Schuettpelz and Pryer (2009) applied to the data set from Rothfels et al. (2012*a*); *C*, the resulting posterior distributions applied as priors to the data of Rothfels et al. (2013*b*). Calibrated nodes are marked with black circles. Taxon colors in *A*–*C*: red for *Gymnocarpium*, green for *Acystopteris*, and blue for *Cystopteris*. Black stars indicate the position of ×*Cystocarpium* sequences. The phylogenies in *A*–*C* are reproduced with taxon labels in the appendix, available online (figs. A2, A4, and A5, respectively). *D*, Bayesian posterior distributions and mean estimates for the *Gymnocarpium-Cystopteris* divergence (values from *B* in blue, final values in orange). Eu. = eupolypods; Pteridac. = Pteridaceae; subst = substitutions. The data underlying this figure are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.r7201 (Rothfels et al. 2015).

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Table 1: Best-fit partitioning scheme (five partitions) and models of evolution for the final nuclear *gapCp* data set

Partition	Model	Exchangeability parameters[a]	State frequencies	Rate heterogeneity	Proportion invariant
First position	HKY+I	0 1 0 0 1 0	Estimated	None	Estimated
Second position	TrN+I+G	0 1 0 0 2 0	Estimated	Gamma	Estimated
Third position	SYM+G	0 1 2 3 4 5	Equal	Gamma	None
Noncoding	K81uf+G	0 1 2 2 1 0	Estimated	Gamma	None
Indels	Mkv	1 rate	Equal	None	None

[a] In the order A-C, A-G, A-T, C-G, C-T, G-T.

Priors were left at their default values except for the tmrca distributions (above), the rbcL.cg exchangeability parameter (given a gamma distribution with a shape of 4.738167 and a scale of 0.000714783), and the rbcL.gt exchangeability parameter (given a gamma distribution with a shape of 7.009774 and a scale of 0.000523609). The latter two distributions were obtained from MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003) runs on the same data (Rothfels et al. 2013*b*), fitted with fitdistrplus (Delignette-Muller et al. 2010; R Development Core Team 2011) and adopted to assist with mixing; in this data set, *rbcL* has relatively little signal, and in the absence of a Dirichlet prior on exchangeability parameters (which MRBAYES has), BEAST had difficulty sampling these two rates under its default priors. This analysis was run four times independently, each for 30 million generations. As before, the runs converged relatively rapidly (Rambaut and Drummond 2007); we very conservatively removed the first 5 million generations of each run as burn-in before pooling the samples. The effective sample sizes for all parameters in the pooled data were above 1,000 (the BEAST settings file and full posterior sample are available in the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.r7201 [Rothfels et al. 2015]; the chronogram and alignment are available in TreeBASE, study no. 16634 [http://treebase.org/treebase-web/search/study/summary.html?id=16634]).

These sequential empirical Bayesian analyses (see Carlin and Louis 1997) allowed us to combine extensive fossil calibration data with dense taxon and character sampling within the focal clade—a combination that is not possible in a single analysis or under a common model. This method improves on some previous versions of secondary-calibration analyses in that it is able to incorporate the full available data while retaining the uncertainty associated with earlier estimates when those estimates are applied to the next data set in the hierarchy. By these means, artificially precise estimates can be avoided (see Graur and Martin 2004).

Results

As in Rothfels et al. (2014), our analysis of the single-copy nuclear *gapCp* data yielded a well-supported phylogeny, showing strong differentiation among Cystopteridaceae taxa (figs. 1*A*, A2). Each of the two ×*Cystocarpium* DNA extractions yielded four distinct *gapCp* alleles, two of which fall within the *Gymnocarpium* clade and two within *Cystopteris* (figs. 1*A*, A2). Of the *Gymnocarpium*-type alleles, one is closely related to sequences from the eastern North American endemic diploid *Gymnocarpium appalachianum* (2n = 80) and the other to sequences from the primarily western North American diploid *Gymnocarpium disjunctum* (2n = 80). This combination strongly implicates the cosmopolitan allotetraploid *Gymnocarpium dryopteris* (2n = 160) as the *Gymnocarpium* parent of ×*Cystocarpium roskamianum*. *Gymnocarpium dryopteris* contains both *G. appalachianum* and *G. disjunctum* alleles (Pryer and Haufler 1993; Rothfels et al. 2014), and thus the *Gymnocarpium* contribution to ×*C. roskamianum* can be explained with a single hybridization event; furthermore, neither of the diploids occurs on the same continent as ×*Cystocarpium*, whereas *G. dryopteris* is common in the region where ×*Cystocarpium* was collected (Rothfels et al. 2013*b*). The plastid data provide further resolution by demonstrating that *Gymnocarpium* is the maternal progenitor (figs. 1*C*, A5). ×*Cystocarpium* inherited two additional *gapCp* alleles from its *Cystopteris* parent (figs. 1*A*, A2). These closely match alleles from the two included *Cystopteris fragilis* accessions from Europe, with one of the alleles being unique to these two accessions and ×*C. roskamianum* (figs. 1*A*, A2). This result strongly suggests that the other parent of ×*Cystocarpium* is a European member of the *C. fragilis* complex. Sexual tetraploid, hexaploid, and octaploid members of this complex are known from Europe (based on x = 42) and are typically referred to *C. fragilis*, *Cystopteris dickieana*, *Cystopteris diaphana*, and/or *Cystopteris alpina* (Vida and Mohay 1980; Prada 1986; Rumsey 2003; Rothfels 2012; Rothfels et al. 2013*b*). However, at least the tetraploids and hexaploids contain multiple as yet incompletely characterized lineages (Vida 1974; Rothfels et al. 2014), precluding confident species delimitation at this stage.

Chromosome squashes of ×*C. roskamianum* spore mother cells undergoing meiosis show approximately 140 stained bodies, the majority of which are unpaired uni-

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valents, although a variable number of bivalents are also present (fig. 2*A*). At metaphase I, the bivalents are pulled to the metaphase plate, whereas the univalents remain scattered in the cytoplasm (fig. 2*B*). If ×*Cystocarpium* had a complete failure of chromosome pairing, we would expect 164 univalents in a tetraploid (2 × 40 from *Gymnocarpium* and 2 × 42 from *Cystopteris*; Rothfels et al. 2012*b*). Taking into account the variable number of bivalents observed from cell to cell, we estimate the number of individual chromosomes to be about 160. This number, together with the highly irregular nature of meiosis, confirm that ×*Cystocarpium* is a tetraploid hybrid containing four divergent genomes, consistent with the four alleles recovered in the *gapCp* sequence data.

Using our sequential empirical Bayesian analyses, the posterior estimates of the time of divergence between *Cystopteris* and *Gymnocarpium* became more precise as we moved though the hierarchy (fig. 1*D*). Our final mean age estimate for the most recent common ancestor of *Cystopteris* and *Gymnocarpium*—and thus the divergence spanned by the ×*Cystocarpium* hybridization event—is 57.9 mya, with the 95% highest posterior density interval spanning 40.2–76.2 mya (fig. 1*D*). In sharp contrast to the deep divergence of the parental lineages, each of the four ×*Cystocarpium gapCp* alleles differs, at most, by a single substitution from an allele observed in one of its parents (figs. 1*A*, A2). Thus, while the divergence between the parent lineages is ancient, the hybridization event itself was very recent.

Discussion

Our results provide compelling corroboration of Fraser-Jenkins's (2008) hypothesis that ×*Cystocarpium roskamianum* arose through hybridization between the divergent fern genera *Gymnocarpium* and *Cystopteris*. On the *Gymnocarpium* side, our analysis recovered alleles grouping with the diploid species *Gymnocarpium appalachianum* and *Gymnocarpium disjunctum* (figs. 1*A*, A2; see Pryer and Haufler 1993), implicating their allotetraploid derivative (*Gymnocarpium dryopteris*) as the *Gymnocarpium* parent of ×*Cystocarpium*. Similarly, our analyses support the original hypothesis that the *Cystopteris* parent is a member of the *Cystopteris fragilis* complex (fig. 1*A*, A2; Fraser-Jenkins 2008). However, the precise identity of this tetraploid taxon is uncertain because of our limited understanding of the diverse array of diploid and allopolyploid taxa that constitute the cosmopolitan *C. fragilis* complex (Blasdell 1963; Vida 1974; Lovis 1978; Vida and Mohay 1980; Rothfels et al. 2013*b*, 2014).

To the best of our knowledge, the formation of ×*Cystocarpium* is the deepest natural hybridization yet documented in plants or animals and provides a new upper limit for the length of time it may take before reproductive barriers are complete, in this case, a cumulative total of approximately 120 million years of independent evolution (60 million years for each parent lineage). For this event to have happened, both prezygotic-isolation and hybrid-viability barriers must have remained incomplete for that duration. Yet for there to be only a single known hybrid-

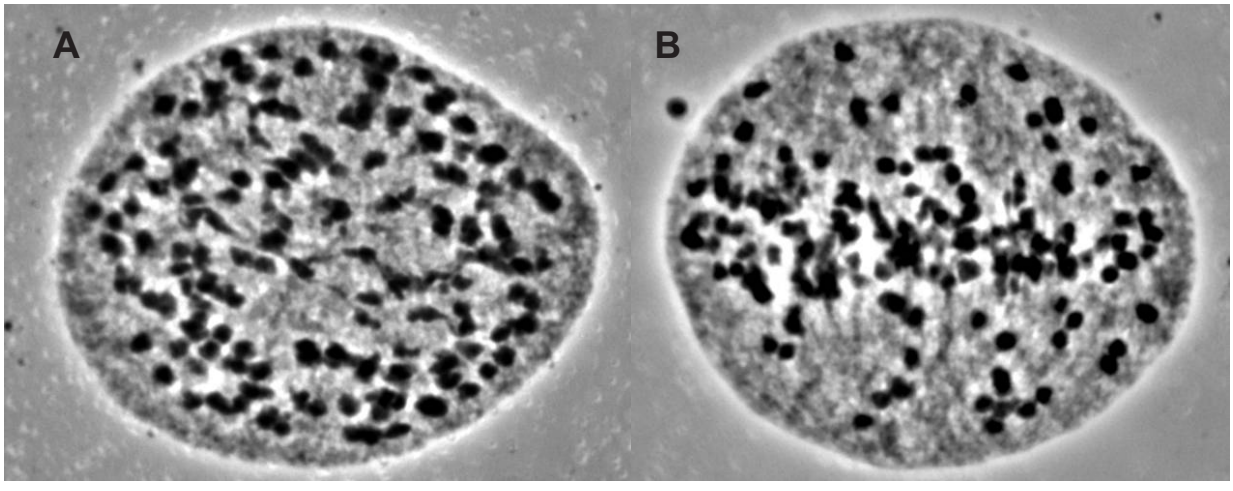

Figure 2: Meiotic chromosome squashes from ×*Cystocarpium roskamianum*. *A*, Cell at late prophase I, showing approximately 140 chromosomes, predominantly univalents but some loosely paired bivalents. *B*, Cell at metaphase I/early anaphase I, in which the bivalents are pulled to the metaphase plate while the univalents remain scattered throughout the cytoplasm.

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ization event, reproductive isolation must be strong. To explain these strong yet incomplete barriers requires that the relative strengths of these two components of reproductive isolation fall somewhere along the spectrum between strong prezygotic isolation (with little to no hybrid inviability) on the one end and no prezygotic isolation (but strong hybrid inviability) on the other. The latter extreme (no prezygotic barriers, strong hybrid inviability) would require that prezygotic barriers remain weak for tens of millions of years and that there be some mechanism by which this particular hybrid was able to escape the strong viability barriers to develop into a vigorous and apparently healthy plant. Perhaps, for example, there is a bottleneck early in development, such that only an extremely fortuitous combination of environmental conditions allows the developing hybrid to reach maturity. Alternatively, there could be segregating variation in the parental lineages, such that the right combination of parental alleles permits hybrid survival, perhaps in a manner analogous to the hybrid rescue alleles known from *Drosophila* (Watanabe 1979; Barbash et al. 2003). These circumstances are extremely restrictive, and it seems to us more tenable that the relative barrier strengths lie closer to the first extreme (strong prezygotic barriers, weak viability barriers). Prezygotic barriers are thought to evolve quickly (especially when sterile hybrids can otherwise be produced; Butlin 1987; Hopkins and Rausher 2012*b*), and this combination of barrier strengths avoids the need for awkward hypotheses for how ×*Cystocarpium* could form in the face of strong viability barriers. However, the hypothesis of weak viability barriers between *Cystopteris* and *Gymnocarpium* is still extraordinary, particularly in the context of theoretical and empirical results suggesting that the accumulation of incompatibilities between lineages should "snowball," that is, arise at a greater-than-linear rate with respect to time since divergence (Orr 1995; Matute et al. 2010; Moyle and Nakazato 2010).

Unsurprisingly, examples even approaching the magnitude of the ×*Cystocarpium* hybridization depth are extremely rare, especially outside the laboratory or cultivation. Reports of natural hybridizations potentially rivaling the phylogenetic depth of the ×*Cystocarpium* event are largely restricted to plants that rely on abiotic factors (wind, water) for their reproduction, rather than on animal intermediaries: the gymnosperm ×*Hesperotropsis* (Cupressaceae; Garland and Moore 2012; Mao et al. 2012), a recently discovered *Selaginella* hybrid (Selaginellaceae; a lycophyte; Arrigo et al. 2013), and four other fern hybrids (×*Dryostichum* and ×*Polysticalpe* in the Dryopteridaceae, *Woodsia* × *abbeae* in the Woodsiaceae, and ×*Lindsaeosoria* in the Lindsaeaceae; Wagner et al. 1992; Wagner 1993; Fraser-Jenkins 1997; Larsson 2014). The only possible exception to this pattern that we are aware of is the hybridization of guinea fowl and chickens, which are hypothesized to share a common ancestor some 30–70 mya (depending on the study; Dimcheff et al. 2002; Pereira and Baker 2006; Brown et al. 2008). The well-documented examples of this hybridization are from captivity or artificial insemination (Price and Bouvier 2002), but there are anecdotal reports of hybrids in the wild (Price 2008) that warrant further investigation.

The incomplete prezygotic isolation in these examples, despite the deep divergence of the hybridizing partners, may be because of their reliance on abiotic means of gamete transfer. They lack the behavioral or behavior-mediated features (i.e., pollination syndromes and other clear mechanisms of mate recognition) that are so integral to the speciation process in other groups (Smith 1972; Coyne and Orr 2004; Hopkins and Rausher 2012*a*). This observation corroborates earlier studies that inferred lower speciation rates in abiotically versus biotically pollinated angiosperms (Coyne and Orr 2004; Kay et al. 2006) and suggests that organisms without premating reproductive isolating mechanisms may evolve total reproductive isolation more slowly than groups with such mechanisms in place.

Slow evolution of prezygotic barriers, however, is only part of the ×*Cystocarpium* story—for the formation of deep hybrids such as ×*Cystocarpium*, the parental taxa must also have incomplete viability isolation. There are several potential mechanisms for the apparently slow evolution of these barriers. One possibility is that there is actually no general difference in the rate of evolution of viability barriers (genic incompatibilities) between groups of organisms that have premating reproductive isolating mechanisms and those that do not, but that we have the opportunity to detect examples of slow evolution only in the latter case (in the former, prezygotic isolation evolves to completion quickly, so no deep hybrids get the opportunity to form in the first place). Alternatively, it could be that the groups of nonflowering vascular plants that form deep hybrids do indeed evolve genic incompatibilities more slowly. These groups (with the exception of the gymnosperms) have both their haploid (gametophytic) and diploid (sporophytic) generations free-living and multicellular, and so perhaps they have greater developmental robustness to variations of dosage and gene-gene interactions. Further study is needed to assess these possibilities and to understand the roles of prezygotic and viability isolation in these cases. Particularly useful would be a series of laboratory crosses of ferns and other nonflowering plants to investigate the strength of viability isolation across a range of evolutionary depths in these groups.

Regardless of the precise mechanisms responsible, this pattern of slower evolution of reproductive isolation in groups with abiotic gamete dispersal implies that some

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aspects of extant diversity patterns may be driven by selection operating at levels other than that of individual organisms. In this scenario, the relative paucity of nonflowering land plant species relative to angiosperms may be due, in part, to a low birth rate of new species (a form of "species selection"; Stanley 1975; Jablonski 2008; Rabosky and McCune 2010) in these groups rather than to any particular adaptive advantages of flowering plants (Smith 1972). One reason we live in a world with more than 250,000 species of flowering plants but only around 10,000 fern species (and approximately 1,000 gymnosperms, 1,200 lycophytes, 12,000 mosses, 9,000 liverworts, and 100 hornworts) may just be that populations of nonflowering lineages take longer to achieve complete genetic separation from one another because they have fewer mechanisms to prevent the sperm of one species from encountering the egg of another.

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