A Plastid Phylogeny of the Cosmopolitan Fern Family Cystopteridaceae (Polypodiopsida)

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Abstract—Among the novel results of recent molecular phylogenetic analyses are the unexpectedly close evolutionary relationships of the genera *Acystopteris*, *Cystopteris*, and *Gymnocarpium*, and the phylogenetic isolation of these genera from *Woodsia*. As a consequence, these three genera have been removed from Woodsiaceae and placed into their own family, the Cystopteridaceae. Despite the ubiquity of this family in rocky habitats across the northern hemisphere, and its cosmopolitan distribution (occurring on every continent except Antarctica), sampling of the Cystopteridaceae in phylogenetic studies to date has been sparse. Here we assemble a three-locus plastid dataset (*matK, rbcL, trnG-R*) that includes most recognized species in the family and multiple accessions of widespread taxa from across their geographic ranges. All three sampled genera are robustly supported as monophyletic, *Cystopteris* is strongly supported as sister to *Acystopteris*, and those two genera together are sister to *Gymnocarpium*. The *Gymnocarpium* phylogeny is deeply divided into three major clades, which we label the *disjunctum* clade, the *robertianum* clade, and core *Gymnocarpium*. The *Cystopteris* phylogeny, similarly, features four deeply diverged clades: *C. montana*, the *sudetica* clade, the *bulbifera* clade, and the *fragilis* complex. *Acystopteris* includes only three species, each of which is supported as monophyletic, with *A. taiwaniana* sister to the *japonica/tenuisecta* clade. Our results yield the first species-level phylogeny of the Cystopteridaceae and the first molecular phylogenetic evidence for species boundaries. These data provide an essential foundation for further investigations of complex patterns of geographic diversification, speciation, and reticulation in this family.

Keywords—Cosmopolitan species, *Cystopteris*, fern phylogeny, *Gymnocarpium*, intralinkage incongruence, species complex.

Cystopteris Bernh. and *Gymnocarpium* Newman—including the bulblet fern, bladder ferns, fragile ferns, and oak ferns (see Fig. 1A–C, E–H)—are among the most frequently encountered and familiar ferns in the northern hemisphere, occurring in most forested and rocky habitats in North America, Europe, and Asia. However, despite their familiarity, their phylogenetic relationships have been contentious. *Acystopteris* Nakai (Fig. 1D) and *Cystopteris* have long been considered close relatives, with many authors historically treating them together under a broad concept of *Cystopteris* (e.g. Tagawa 1935; Blasdell 1963). Prior to the proliferation of molecular evidence, however, most taxonomists did not consider *Cystopteris* s.l. and *Gymnocarpium* to be closely allied. Instead, these two taxa usually were assigned to the dryopteroid and athyrioid fern lineages, respectively, with the caveat that they were each morphologically anomalous within those lineages and that their phylogenetic positions were thus uncertain (e.g. Sledge 1973). This confusion regarding the affinities of *Cystopteris* and *Gymnocarpium* to other polypod ferns has continued until very recently. For example, when naming the family Cystopteridaceae, Schmakov (2001) included *Pseudocystopteris* Ching (which belongs in the Athyriaceae; Kato 1977; Sano et al. 2000; Fraser-Jenkins 2008; Liu 2008; Rothfels et al. 2012b). Quite recently, Z. R. Wang (1997), M. L. Wang et al. (2004) and Smith et al. (2006) each advocated familial concepts that grouped *Cystopteris* and *Gymnocarpium* with very distantly related taxa (Rothfels et al. 2012b).

Wolf et al. (1994) and Hasebe et al. (1995) provided the first molecular evidence that *Cystopteris* and *Gymnocarpium* are, indeed, closely allied to one another, and that they are evolutionarily distinct from both the dryopteroid (Dryopteridaceae sensu Smith et al. (2006)) and athyrioid ferns (Athyriaceae sensu Rothfels et al. (2012b)). Subsequent molecular phylogenetic studies—those with greater taxon and character sampling—have yielded an increasingly clear understanding of this group, culminating in the resurrection and recircumscription of the Cystopteridaceae (Rothfels et al. 2012b). This

family occupies a critical position sister to the rest of the large eupolypod II clade (Sano et al. 2000; Schuettpelz and Pryer 2007; Kuo et al. 2011; Rothfels et al. 2012a) and represents a deeply isolated lineage within eupolypod ferns—it last shared a common ancestor with other extant fern lineages approximately 100 million years ago (Schuettpelz and Pryer 2009; Rothfels et al. 2012a).

The Cystopteridaceae consists of at least three genera—*Gymnocarpium*, *Acystopteris*, and *Cystopteris*—and possibly *Cystoathyrium* Ching (Rothfels et al. 2012a, 2012b). The latter includes a single species from China that is known only from the type specimen at PE and may be extinct (Rothfels et al. 2012b). Although *Cystoathyrium* has not been included in any molecular phylogenetic study to date, the limited morphological information available suggests it may be a member of Cystopteridaceae (Rothfels et al. 2012b; Sundue and Rothfels, unpubl.). The other three genera comprise approximately 37 species: seven in *Gymnocarpium* (Sarvela 1978; Pryer 1993), three in *Acystopteris*, and about 27 in *Cystopteris* (Blasdell 1963; Rothfels 2012).

Although the circumscription of Cystopteridaceae and its placement within the fern tree of life are now relatively well established, species boundaries and relationships among the included taxa remain unclear. *Cystopteris* s.l. was last monographed 50 yr ago (Blasdell 1963; he included *Acystopteris* in his generic concept), and the closest we have to a global monograph of *Gymnocarpium* is a six-page synopsis (Sarvela 1978). Both *Cystopteris* and *Gymnocarpium* have extremely broad geographical distributions (Fig. 2). Populations of *G. dryopteris*, for example, are scattered across the northern half of both North America and Eurasia (Fig. 2A), and *Cystopteris fragilis* s.l. is perhaps the most widely distributed fern in the world, ranging from the high arctic to Tierra del Fuego in the Americas, blanketing most of Eurasia, occurring in eastern and southern Africa, eastern Australia, New Zealand, Hawai'i, and many isolated rocky oceanic islands (Fig. 2C, D; the "*C. fragilis* complex"). The few biosystematic investigations undertaken to date have revealed that the
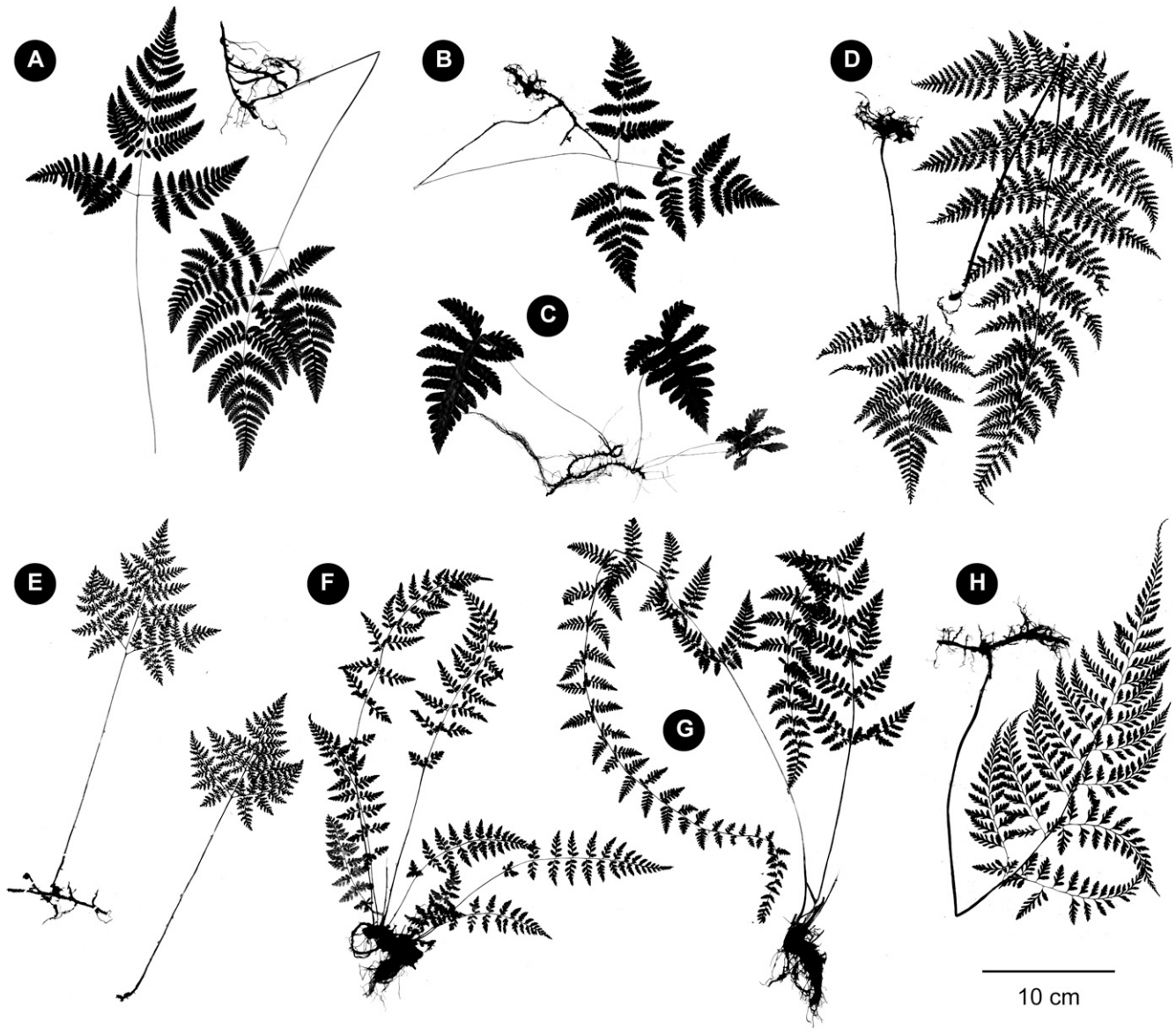
Fig. 1. Silhouettes of representative Cystopteridaceae species. A. *Gymnocarpium robertianum* [*Larsson 282* (DUKE), Norway, north of Fauske]. B. *G. dryopteris* [*Rothfels 4048.3* (DUKE), U. S. A., Vinalhaven Island, Maine]. C. *G. oyamense* [*Nakato s. n.* (DUKE), from cultivation; originally collected from Okutama-Machi, Tokyo, Honshu, Japan]. D. *Acystopteris japonica* [*Sino-American Guizhou Botanical Expedition 1961* (MO), China, vicinity of Lengjiaba, Guizhou]. E. *Cystopteris montana* [*Windham 670 & Haufler* (DUKE), U. S. A., Summit County, Colorado]. F. *C. tenuis* [*Rothfels 3927 & Lewer* (DUKE), Canada, Halton Region, Ontario]. G. *C. bulbifera* [*Rothfels 3951 & P. Rothfels* (DUKE), Canada, Leeds and Grenville County, Ontario]. H. *C. pellucida* [*Boufford 27439, Donoghue, & Ree* (CAS), China, Luding Xian, Sichuan].

widespread species of both *Cystopteris* and *Gymnocarpium* include multiple independent lineages; these often differ in ploidy level and typically involve reticulate evolutionary histories (Vida 1972, 1974; Sarvela 1978; Pryer et al. 1983; Haufler et al. 1990; Haufler and Windham 1991; Pryer and Haufler 1993).

In order to explore the intriguing patterns of diversification and phylogeography within these species complexes, we first need to understand their basic phylogenetic relationships. Here, we assemble a three-locus plastid dataset that includes most recognized species of Cystopteridaceae, including multiple accessions from across the geographic ranges of many taxa (especially the polyploids). Our primary goal is to establish the first robust phylogeny for the family—focusing on the branching (divergent) relationships as a necessary prerequisite for investigating species boundaries

and reticulate evolution within Cystopteridaceae (Rothfels et al., unpubl.).

Materials and Methods

Taxonomic Sampling—We analyzed an ingroup sample of 75 accessions of Cystopteridaceae, selected to maximize the inclusion of named taxa appearing in published taxonomic works (Tagawa 1935; Blasdell 1963; Mickel 1972; Bir and Trikha 1974; Sarvela 1978; Sarvela et al. 1981; Moran 1983; Pryer et al. 1983; Haufler et al. 1993; Pryer and Haufler 1993; Pellinen et al. 1998; Mickel and Tejero-Díez 2004; Wang 2008). Based on the list of 37 species tentatively accepted by Rothfels (2012; his Appendix E), our study includes all three species of *Acystopteris*, all seven species of *Gymnocarpium*, and 19 of the 27 species of *Cystopteris*. For widespread species, we attempted to include multiple accessions scattered across their known geographic ranges. To root the tree, we included an outgroup of nine species scattered throughout the remainder of Eupolypods II—the sister group to Cystopteridaceae (Schuettpelz and
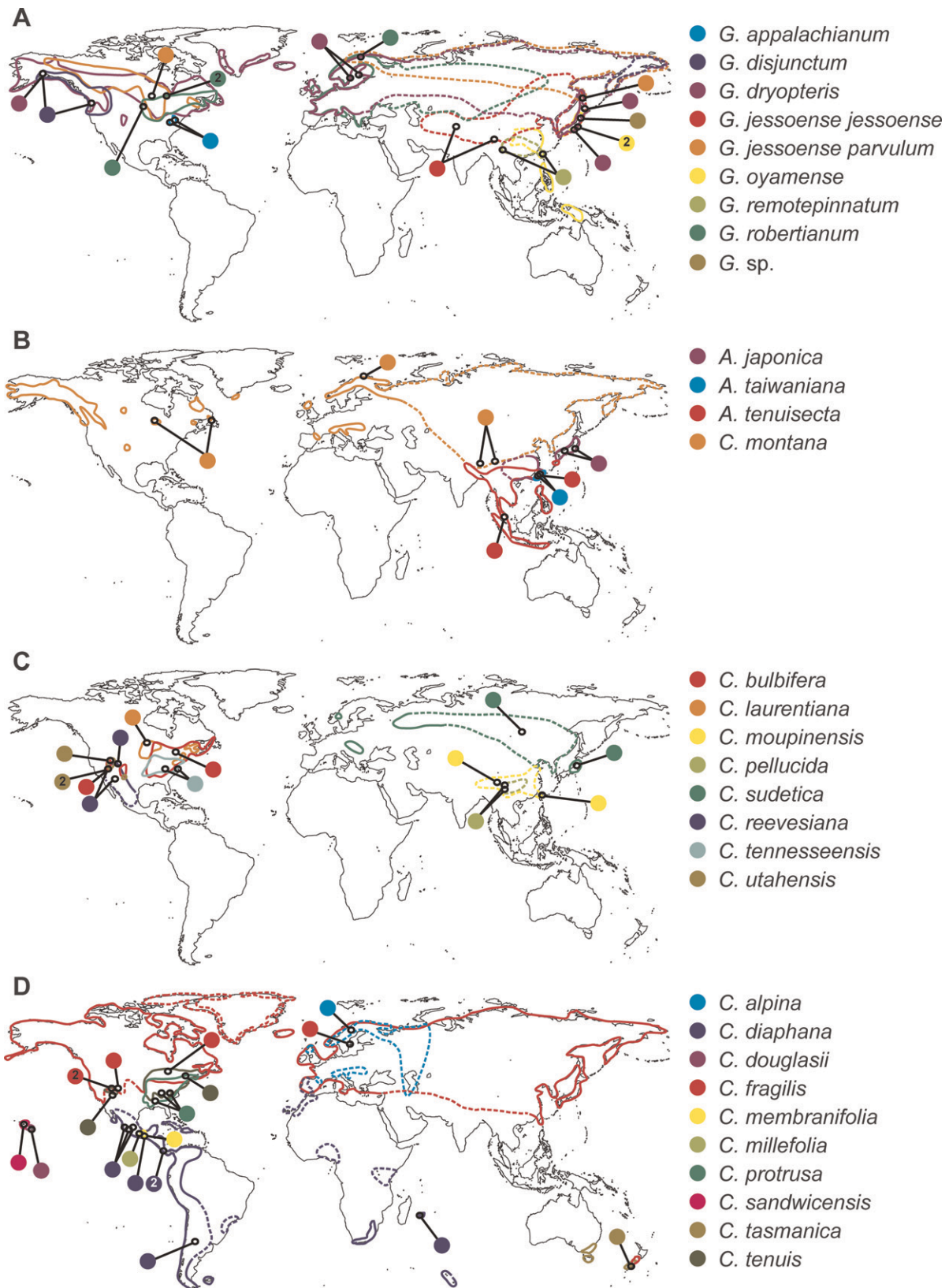
FIG. 2. Geographic ranges of sampled taxa of Cystopteridaceae. Ranges are approximated by the colored polygons, with dotted lines indicating uncertain range limits. Colored circles indicate the collection location of the vouchers used in this study; a numeral inside the colored circle indicates the number of accessions from that location. A: *Gymnocarpium*. B: *Acystopteris* and *Cystopteris montana*. C: The *C. bulbifera* clade, the *C. sudetica* clade, and *C. laurentiana* and *C. reevesiana*. D: The *C. fragilis* complex (excluding *C. laurentiana* and *C. reevesiana*, which are presented in panel C). Range boundaries determined from published floristic works (Tagawa 1935; Mickel 1972; Bir and Trikha 1974; Sarvela 1978; Sarvela et al. 1981; Moran 1983; Britton et al. 1984; Fraser-Jenkins 1986; Lobin 1986; Prada 1986; Salvo and Otermin 1986; Breckle 1987; Haufler et al. 1990; Haufler and Windham 1991; Haufler et al. 1993; Pryer 1993; Denk 1998; Latorre 2000; Velayos et al. 2001; Mickel and Tejero-Díez 2004; Murphy and Rumsey 2005; Fraser-Jenkins 2008; Wang 2008; Crouch et al. 2011; Japanese Society for Plant Systematics 2012).

TABLE 1. Primers used in amplification and sequencing (F = forward; R = reverse).

Locus	Primer		Sequence (5′ – 3′)	Reference
matK	AJmatKf1	F	GTATTACAKAAAAGTGRAGRGCTTAG	This study
matK	AJmatKf3	F	TGGAAAGGTYAYTCAGTTYCGGTCTTGG	This study
matK	AJmatKr1	R	ATYTCAATCTACGCAATCCAT	This study
matK	AJmatKr3B	R	CGATTTCGTAMATGTARAAATTTCG	This study
rbcL	ESRBCL1F	F	TCAGGACTCCACTTACTAGCTTCACG	(Schuettpelz and Pryer 2007)
rbcL	ES645F	F	ATGTCACCACAAACGGAGACTAAAGC	(Schuettpelz and Pryer 2007)
rbcL	ESRBCL663R	R	TACRAATARGAAACGRTCTCTCCAACG	(Schuettpelz and Pryer 2007)
rbcL	ESRBCL1361R	R	TCAGGACTCCACTTACTAGCTTCACG	(Schuettpelz and Pryer 2007)
trnG-R	trnG1F	F	GCGGGTATAGTTTAGTGGTAA	(Nagalingum et al. 2007)
trnG-R	CRcysTRNGf1	F	GCTAYACGACCAARACGTAAGC	This study
trnG-R	CRcysTRNGr1	R	GTGGCATCCATAAAATCYATGTCAG	This study
trnG-R	trnR22R	R	CTATCCATTAGACGATGGACG	(Nagalingum et al. 2007)

Pryer 2007; Rothfels et al. 2012a). Our total sample includes 84 accessions (Appendix 1).

DNA Isolation, Amplification, and Sequencing—DNA was extracted from herbarium specimens or silica-dried material in the Fern Lab Silica Archive (http://fernlab.biology.duke.edu/) using a 96-well modification (Beck et al. 2011; doi:10.5061/dryad.11p757m0) of a standard CTAB protocol (Doyle and Dickson 1987), or using DNeasy kits (Qiagen, Valencia, California, USA). Three plastid loci were selected for analysis: *matK*, *rbcL*, and the *trnG-trnR* intergenic spacer (henceforth "*trnG-R*"). Primer sequences and associated data are provided in Table 1. The full lengths of *matK* and *trnG-R* were amplified and sequenced in two overlapping fragments, using the primers *AJmatKf1+AJmatKr3B* and *AJmatKf3+AJmatKr1* for *matK* and *trnG1F+CRcysTRNGr1* and *CRcysTRNGf1+trnR22R* for *trnG-R*. Most *rbcL* sequences were amplified in one piece using the primer pair *ESRBCL1F+ES1361R*. For some herbarium specimens with degraded DNA, *rbcL* was amplified in two pieces, using the primer pairs *ESRBCL1F+ES633R* and *ES645F+ES1361R*. Loci were amplified in 21 μl reactions consisting of 2 μl Denville buffer (10x), 2 μl dNTPs (each 2mM), 0.2 μl BSA (10 mg/ml), 0.2 μl Denville Choice taq (5 U/μl), 1 μl of each primer (10 μM), 1 μl of DNA, and 13.6 μl of water. Our thermal cycling program for *matK* consisted of an initial denaturation step (94°C for 3 min), 35 denaturation, annealing, and elongation cycles (94°C for 45 sec, 50°C for 30 sec, 72°C for 1.5 min), and a final elongation step (72°C for 10 min). For *rbcL* and *trnG-R* we used the same program, except that the annealing temperature was 45°C for *rbcL* and 55°C for *trnG-R*. PCR products were purified using Shrimp Alkaline Phosphatase (USB, Cleveland, Ohio) following established protocols (Rothfels et al. 2012a) and sequenced on an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Carlsbad, California) at the Duke University Genome Sequencing and Analysis Core Resource, again using established protocols (Schuettpelz and Pryer 2007). Chromatograms were assembled and edited in Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, Michigan), and the resulting 169 newly generated sequences are deposited in GenBank (Appendix 1).

Sequence Alignment and Phylogenetic Analysis—Sequences for each locus were manually aligned in Mesquite v2.72 (Maddison and Maddison 2009). Ambiguously aligned regions (limited to *trnG-R*) were excluded prior to analysis. A total of four datasets were analyzed: the three single-locus datasets (to test for incongruence) and a combined three-locus dataset (Table 2). The single-locus datasets were analyzed under maximum likelihood (ML) in Garli v2.0 (Zwickl 2006), using the best model as determined by the small-sample correction for the Akaike Information Criterion (AICc; Akaike 1974; Hurvich and Tsai 1989; Burnham and Anderson 2004) in jModeltest v0.1.1 (Posada 2008; see Table 2). For each locus, ML tree searches were performed on 500 bootstrap pseudoreplicate datasets, each

searched from two different random-addition starting trees; other settings were left at their default values. The majority-rule consensus trees from each pool of bootstrap trees were compared for highly supported (≥70% bootstrap support) incompatible splits (Mason-Gamer and Kellogg 1996).

The combined three-locus dataset was analyzed under both ML and Bayesian frameworks. The ML analyses used the same settings as the single-locus analyses (above), in a single Garli (Zwickl 2006) run with the data partitioned by locus, substitution parameters unlinked among partitions, and each partition permitted its own average rate (subset-specificrates = 1). The search for the ML best tree started from each of 20 different random-addition starting trees, and support was assessed with 1,000 bootstrap pseudoreplicates, each searched from two random-addition starting trees. The Bayesian analyses were performed in the parallel version of MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003; Altekar et al. 2004), with parameters unlinked among the three partitions. The best-fitting models for *rbcL* and *trnG-R* (see Table 2) were implemented with the nst=mixed, statefreqpr=dirichlet(1,1,1,1) and rates=invgamma settings; *rbcL* differed in that base frequencies were fixed at 0.25 each (statefreqpr=fixed(equal)). The model settings for *matK* were more straightforward (nst=6 rates=gamma statefreqpr=dirichlet(1,1,1,1)). The average rates for each partition were allowed to be different (ratepr=variable); other priors were left at their default values. Four independent runs, each with four chains (one cold, three heated), were run for 50 million generations with a sample taken every 7,500 generations. The resulting sample parameter traces were visualized in Tracer v1.5 (Rambaut and Drummond 2007). The runs each converged (and to the same area of parameter space) well before 500,000 generations; to be very conservative, we excluded the first 5 million generations of each run as burn-in, prior to summarizing the posterior. Our final pool included 24,000 samples; effective sample sizes for all parameters were greater than 300. Our final three-locus dataset is available from TreeBASE (accession S13449).

RESULTS

Phylogenetic Analyses—Four datasets were analyzed in this study: one for each locus individually, and one of the three loci combined. While the differing numbers of sequences among the datasets make precise comparisons difficult, *matK* appears to be more informative (i.e. it has a higher percentage of strongly supported bipartitions) than *trnG-R*, which in turn outperforms *rbcL* (Table 2).

There are two well-supported conflicts among the loci. The first of these conflicts is relatively minor: *trnG-R* supports a

TABLE 2. Statistics for the datasets analyzed in this study. Missing data includes both uncertain bases (?, N, R, Y, etc.) and gaps (-); MLBS: maximum likelihood bootstrap support. *The combined dataset was analyzed under a partitioned model, with each locus given its own best-fitting model.

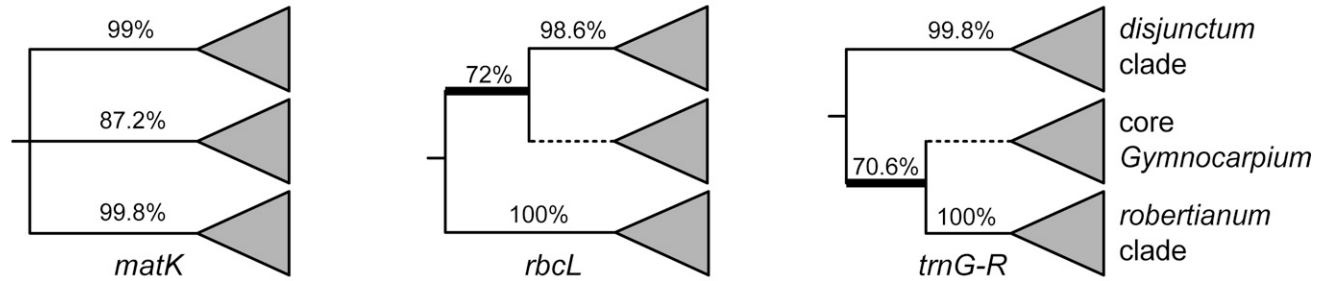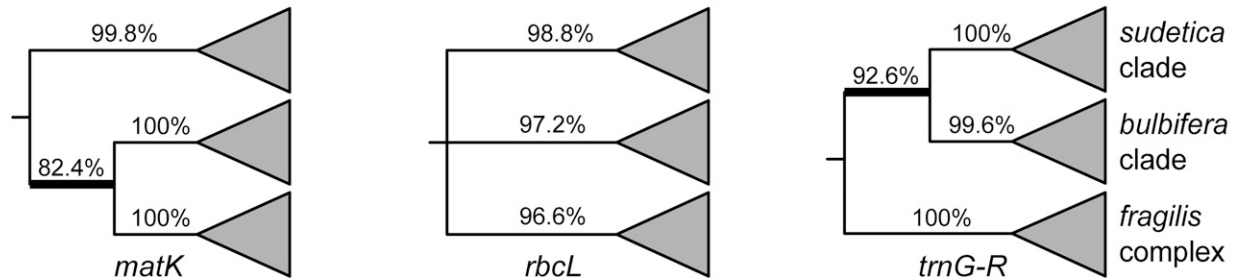| Dataset	Taxa	Included sites	Variable sites	Missing data (%)	Best-fitting model	MLBS	
						Mean	Partitions >70%
matK	74	1211	559	1.6	GTR+G	90%	85%
rbcL	61	1309	256	6.2	TrNef+I+G	81%	67%
trnG-R	84	1119	538	4.9	TVM+I+G	87%	80%
combined	84	3639	1353	17.4	–*	90%	85%

A: *Gymnocarpium*

B: *Cystopteris*

Fig. 3. Intralinkage incongruence. Simplified, rooted three-taxon trees showing support for the two conflicting relationships in our dataset, one in *Gymnocarpium* (A) and the other in *Cystopteris* (B). Numbers above branches are maximum likelihood bootstrap support values, dashed branches indicate an absence of support (<70%), and thickened branches indicate supported relationships that conflict with a relationship supported by another locus in our dataset (in each case, there is a single such conflict).

large *Gymnocarpium* clade that includes all our accessions except for those in the *disjunctum* clade, whereas *rbcL* supports a clade of all our *Gymnocarpium* accessions except for those in the *robertianum* clade (Fig. 3A). In this case, the conflicting bootstrap support is only marginally greater than our 70% cut-off (70.6% for *trnG-R* and 72% for *rbcL*). The second case of intralinkage incongruence is more substantial. Here, the conflicting relationships are near the base of the *Cystopteris* crown group (following the divergence of *C. montana*) and the discordance is much stronger. The *matK* locus supports a sister relationship between the *bulbifera* clade and the *fragilis* complex with 82.4% bootstrap support, whereas *trnG-R* supports a sister relationship between the *bulbifera* and *sudetica* clades with 92.6% bootstrap support (*rbcL* is equivocal; Fig. 3B). This incongruence is not the result of misidentification or lab error (the same extractions were used for all loci, and multiple accessions were involved in each case). Errors of alignment inference remain a possibility, but only one locus (*trnG-R*) has areas of ambiguous alignment, and they were excluded prior to analysis. Furthermore, careful review of the alignments failed to uncover any regions that could possibly be contributing to this result. The combined dataset includes 3639 sites for 84 taxa; additional dataset characteristics are provided in Table 2.

Cystopteridaceae Phylogeny—Analysis of the combined dataset inferred strong support for the vast majority of internodes across the tree, under both ML bootstrapping and Bayesian analysis (Fig. 4; Table 2). The monophyly of the family as a whole is maximally supported (1.0 posterior probability and 100% ML bootstrap support), as is the monophyly of the three genera. *Cystopteris* and *Acystopteris* are maximally supported as sister genera, and they, together, are sister to *Gymnocarpium* (Fig. 4).

The *Gymnocarpium* phylogeny features three deeply diverged "major clades": the *disjunctum* clade, the *robertianum* clade,

and core *Gymnocarpium*. Though each of these is strongly supported, relationships among them are uncertain. The *disjunctum* clade includes the diploid *G. disjunctum*, the widespread allotetraploid *G. dryopteris*, and the triploid hybrid between them, *G.* × *brittonianum*. The *G. robertianum* clade includes all accessions of that species, as well as a Japanese accession of uncertain identity. The remainder of the genus comprises the core *Gymnocarpium* clade, including the morphologically anomalous *G. oyamense*, the eastern North American *G. appalachianum*, two taxa currently treated as subspecies of *G. jessoense* (subsp. *jessoense* and subsp. *parvulum*), and the east Asian *G. remotepinnatum*.

Within the *Acystopteris* clade, each of the three recognized species is maximally supported as monophyletic. *Acystopteris japonica* and *A. tenuisecta* are sister (1.0 posterior probability and 100% ML bootstrap support), and they, together, are sister to *A. taiwaniana*.

The first split within *Cystopteris* is maximally supported, separating *C. montana* from the rest of the genus. The next branch is not well supported—similar to the situation at the base of *Gymnocarpium*. Consequently, relationships among three highly supported clades (*sudetica*, *bulbifera*, and the *fragilis* complex) remain uncertain. The *sudetica* clade is predominantly Asian; of its three recognized species, *C. pellucida* and *C. moupinensis* are exclusively East Asian, while *C. sudetica* extends west across Eurasia. In contrast, the *bulbifera* clade, which includes diploid *C. bulbifera* and related allopolyploids, is limited to North America. The bulk of *Cystopteris* species belong to the remaining clade (informally known as the *C. fragilis* complex), which is maximally supported as monophyletic. The first divergence within the *C. fragilis* clade robustly separates *C. protrusa* from the rest of the complex. Relationships among the remaining *C. fragilis* complex species are convoluted, with many taxa (including *C. fragilis* s.s.) appearing in multiple subclades.

Woodsia obtusa
Diplaziopsis javanica
Hemidictyum marginatum
Pseudophegopteris cruciata
Deparia lancea
Pseudocystopteris atkinsonii
Athyrium otophorum
Onocleopsis hintonii
Blechnum schomburgkii

* / *
78/ *
87/.96
49/.54
92/ *
71/.88
90/ *
* / *

Gymnocarpium

G. disjunctum (4710): **USA**. Alaska
G. disjunctum (7751): **USA**. Washington
G. dryopteris (7981): **Japan**. Hokkaido
G. dryopteris (4601): **USA**. Alaska
G. dryopteris aokigaharaense (7984): **Japan**. Yamanashi
G. dryopteris (7059): **Sweden**. Uppsala
G. x brittonianum (7806): **USA**. Colorado
G. dryopteris (5837): **Finland**. Varsinais-Suomi.
G. robertianum (5852): **USA**. Minnesota
G. robertianum (7945): **Norway**. Nordland
G. robertianum (7197): **Canada**. Ontario
G. robertianum (7178): **Canada**. Ontario
G. sp. (7979): **Japan**. Iwate
G. oyamense (6399): **Japan**. Tokyo
G. oyamense (7983): **Japan**. Tokyo
G. appalachianum (7800): **USA**. Virginia
G. appalachianum (7639): **USA**. Virginia
G. jessoense parvulum (8053): **Canada**. Ontario
G. jessoense parvulum (8120): **Russia**. Sakhalin
G. remotepinnatum (3066): **China**. Yunnan
G. remotepinnatum (4862): **Taiwan**. Nantou
G. jessoense jessoense (7069): **Pakistan**. Astor/Nanga Parbat
G. jessoense jessoense (6059): **China**. Tibet

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63/ *
* / *
-- /.89
90/ *
94/ *
99/ *
90/ *
* / *
* / *
96/ *

Acystopteris

A. taiwaniana (4870): **Taiwan**. Nantou
A. taiwaniana (6137): **Taiwan**. Chial
A. tenuisecta (4225): **Malaysia**. Pahang
A. tenuisecta (4831): **Taiwan**. Nantou
A. japonica (7978): **Japan**. Tokyo
A. japonica (7097): **Japan**. Kyoto

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* / *
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* / *

Cystopteris

C. montana (7072): **China**. Tibet
C. montana (7071): **China**. Tibet
C. montana (5839): **Canada**. Newfoundland
C. montana (7943): **Norway**. Finnmark
C. montana (6969): **Canada**. Ontario

* / *
98/.96
* / *

C. pellucida (6055): **China**. Yunnan
C. pellucida (6060): **China**. Yunnan
C. moupinensis (4861): **Taiwan**. Nantou
C. moupinensis (7074): **China**. Tibet
C. sudetica (7096): **Russia**
C. sudetica (7980): **Japan**. Nagano

* / *
98/ *
86/ *

C. bulbifera (7650): **Canada**. Ontario
C. tennesseensis (7990): **USA**. Tennessee
C. utahensis (6848): **USA**. Utah
C. utahensis (5834): **USA**. Arizona
C. utahensis (5832): **USA**. Arizona
C. bulbifera (5836): **USA**. Arizona
C. tennesseensis (4513): **USA**. North Carolina

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* / *
61/.35

C. protrusa (6362): **USA**. Virginia
C. protrusa (5838): **USA**. Mississippi
C. protrusa (5841): **USA**. Kentucky
C. alpina (7920): **Sweden**. Vøsterbotten
C. tasmanica (6379): **New Zealand**. Otago
C. sandwicensis (6208): **USA**. Hawai'i
C. douglasii (6206): **USA**. Hawai'i
C. tenuis (6387): **USA**. Vermont
C. fragilis "brittonii" (5847): **Canada**. Ontario
C. fragilis (5950): **USA**. Colorado
C. fragilis "haufleri" (7034): **USA**. Utah
C. tenuis (5833): **USA**. Arizona
C. reevesiana (6342): **USA**. Colorado
C. laurentiana (8081): **Canada**. Ontario
C. fragilis (7103): **Sweden**. Uppsala
C. reevesiana (5844): **USA**. Arizona
C. fragilis (5851): **USA**. Utah
C. reevesiana (3126): **USA**. Arizona
C. diaphana (6513): **Mexico**. Guanajuato
C. diaphana (6592): **Mexico**. Nayarit
C. diaphana (5622): **Costa Rica**. San José
C. diaphana (6560): **Mexico**. Jalisco
C. membranifolia (6732): **Mexico**. Oaxaca
C. millefolia (6761): **Mexico**. México
C. diaphana (5316): **Costa Rica**. San José
C. diaphana (5845): **Mexico**. Oaxaca
C. diaphana (6380): **Argentina**. San Luis
C. diaphana (6386): **France**. Reunion

56/.81
85/ *
48/.58
70/.97
* / *
* / *
83/ *
99/ *
* / *
95/ *
99/ *
96/ *
67/ *
61/.99
81/ *
--/.79
60/.99

0.02 subst'ns/site

disjunctum clade
robert'um clade
core Gymnocarpium
Acystopteris
C. montana
sudetica clade
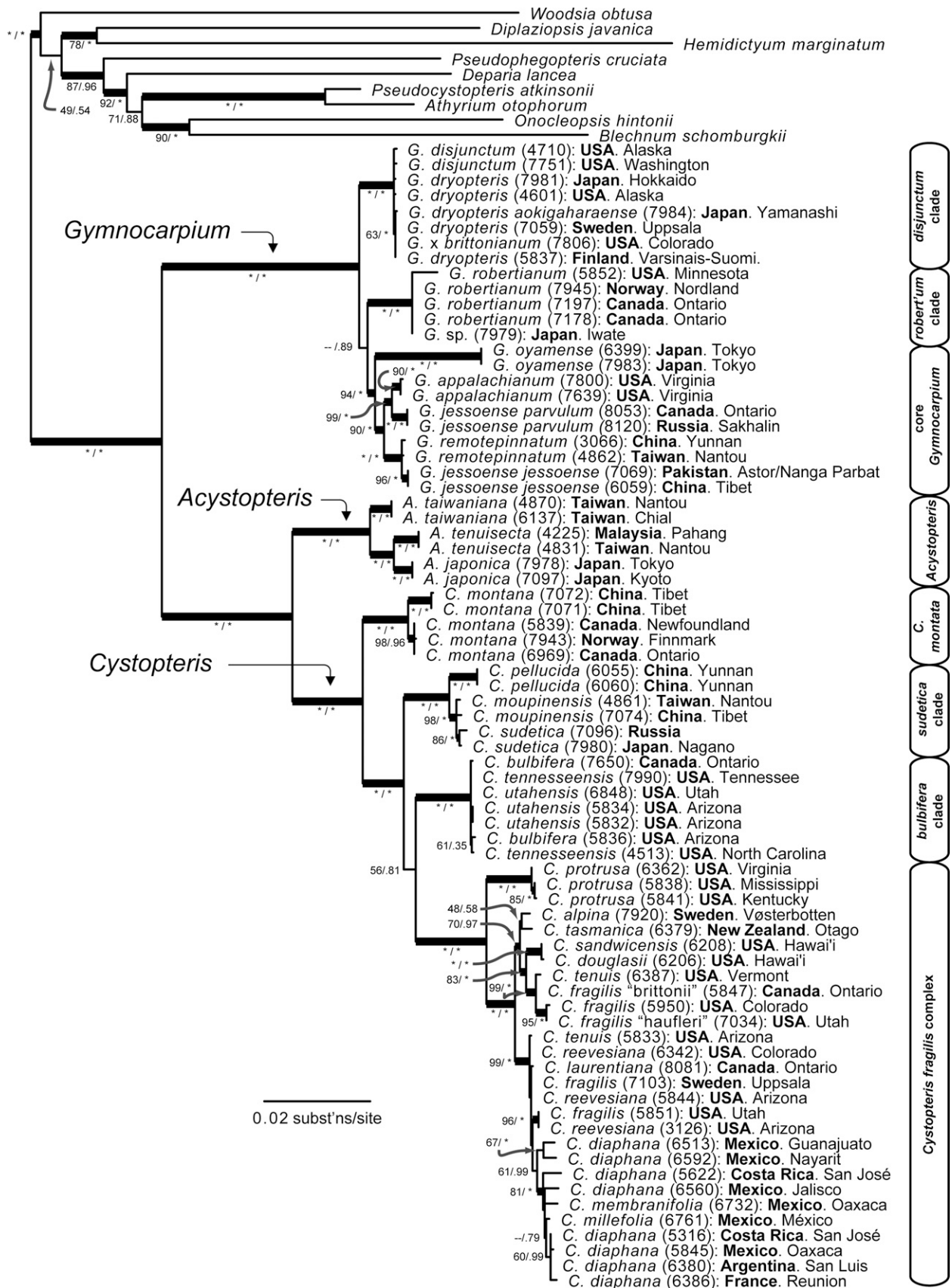bulbifera clade
Cystopteris fragilis complex

FIG. 4. Maximum likelihood phylogram of the concatenated plastid data. Support values (maximum likelihood bootstrap support/Bayesian posterior probability) appear below the branches; values of 100% and 1.0, respectively, are indicated by asterisks. Bold branches are highly supported (≥70% maximum likelihood bootstrap support and ≥0.95 posterior probability). Numbers in brackets following the species' names are accession numbers from the Fern Lab database (http://fernlab.biology.duke.edu/); see Appendix 1 for further voucher information.

Discussion

Phylogenetic Analyses and Intralinkage Incongruence— The relative informativeness of the loci examined in our dataset (*matK* outperforming *trnG-R*, which was superior to *rbcL*) is consistent with other molecular phylogenetic analyses of ferns across a variety of evolutionary depths (Kuo et al. 2011; F.-W. Li et al. 2011; Rothfels et al. 2012a). An unexpected result, however, was the appearance of well-supported conflicts among the individual loci (Fig. 3). Because all three loci are in the plastid genome, they should constitute a single non-recombining linkage group with a common evolutionary history. To the contrary, our data reveal two cases of intralinkage incongruence where at least one locus has ≥70% bootstrap support for a relationship that is incompatible with a relationship well-supported by another locus (again, with at least 70% support; Fig. 3; Mason-Gamer and Kellogg 1996). We suspect that this incongruence is due to the failure of our phylogenetic inference methods to fully capture the idiosyncrasies of molecular evolution in these taxa, as has been seen in other multi-region studies of single linkage groups (Rothfels et al. 2012a; Weisrock 2012). As with the mitogenomic data of Weisrock (2012), the conflicts in our data could be due to different patterns of selection operating on the individual loci, biases in base composition across loci and taxa, slight changes in taxon representation, or unusual variance in the stochastic substitution process.

Cystopteridaceae Phylogeny—Our broad results, demonstrating that both the family and its constituent genera are monophyletic and that *Cystopteris* + *Acystopteris* is sister to *Gymnocarpium,* are consistent with the high support inferred for these relationships in earlier molecular phylogenies (Sano et al. 2000; Liu 2008; C. Li et al. 2011; Rothfels et al. 2012a). The monophyly of the family, and its deep divergence from its closest relatives (Rothfels et al. 2012a), further emphasizes the need to recognize these three genera as comprising their own family, rather than including them in a broad Athyriaceae sensu Wang et al. (2004) or Woodsiaceae sensu Smith et al. (2006).

Gymnocarpium Phylogeny—While *Gymnocarpium* is maximally supported as monophyletic, the deepest divergence within the genus is uncertain (Fig. 4). Data from *trnG-R* support the *G. disjunctum* clade as sister to the remainder of the genus (71% bootstrap support), whereas *rbcL* places *G. robertianum* as the earliest diverging lineage (with 72% bootstrap support; Fig. 3A). On its own, *matK* resolves the same relationship as *trnG-R*, but without support, which is the same result obtained from the combined data (Fig. 4). Despite the addition of substantial amounts of data relative to previous studies (the largest previous sample of *Gymnocarpium* was the three accessions included in Rothfels et al. (2012a)), the early evolutionary history of *Gymnocarpium* remains enigmatic.

Of the three major groups that comprise *Gymnocarpium*, the *disjunctum* clade is the most straightforward to describe. In our sampling, it includes both accessions of the diploid species *G. disjunctum*, all samples of the cosmopolitan tetraploid *G. dryopteris*, and the only accession of triploid *G.* × *brittonianum*, which is hypothesized to be a hybrid between the other two taxa (Pryer and Haufler 1993; Fig. 4). The grouping of *G. disjunctum* and *G. dryopteris* is expected, given that isozyme analyses suggest that *G. dryopteris* is an allopolyploid between *G. disjunctum* and *G. appalachianum* (Pryer

and Haufler 1993). Our data further support this hypothesis by indicating that *G. disjunctum* is the maternal parent of all *G. dryopteris* populations sampled. Another striking feature of this clade is the genetic uniformity observed across all loci—the sequences of *G. dryopteris* are nearly identical regardless of whether they are from Alaska, Scandinavia, or Japan (Fig. 4). This genetic similarity of *G. dryopteris* accessions across loci extends to the lone accession of *G. dryopteris* var. *aokigaharaense*, providing no support to its recognition at the varietal level. However, in naming the variety, Nakaike (1969) pointed out that it was somewhat intermediate between *G. dryopteris* and *G. jessoense*. Thus, nuclear data will be necessary to corroborate or refute a possible hybrid origin of this taxon.

Our results for the *robertianum* clade have three noteworthy elements. First, our single accession of *G. robertianum* from the southern part of its North American range (5852: USA, Minnesota; see Appendix 1; Fig. 2A) is somewhat divergent from the other accessions. Second, as in tetraploid *G. dryopteris*, the remaining accessions in this clade have near-identical sequences across loci, whether they are from North America, Scandinavia, or Japan (Fig. 4). And finally, one of these accessions is from far beyond the recognized range of *G. robertianum* (7979: Japan, Iwate; see Fig. 2A). Plants of this morphology in that region are typically treated as *G. jessoense* subsp. *jessoense*. Our two samples of the latter from China and Pakistan are well-supported members of the core *Gymnocarpium* clade and thus quite divergent from the *robertianum* clade. Nuclear data will be needed to determine whether this Japanese accession represents an unrecognized range extension of *G. robertianum* or is, instead, an unrecognized allopolyploid with *G. robertianum* as the maternal parent. Future conclusions regarding the identity of these plants could have important consequences for *Gymnocarpium* nomenclature, because *G. jessoense* is typified on Japanese material (Koidzumi 1936).

The third major clade of *Gymnocarpium* contains most of the named taxa, including *G. oyamense,* which is sometimes recognized as the segregate genus *Currania* Copel. (e.g. Copeland 1909; Lloyd and Klekowski 1970). The inclusion of this species within core *Gymnocarpium* is somewhat surprising given its anomalous morphology (including elongate sori and pinnatifid leaf dissection; Fig. 1C). It provides yet another example of a pattern seen elsewhere in Eupolypods II (e.g. Aspleniaceae, Onocleaceae, Blechnaceae; Rothfels et al. 2012a), where certain strongly apomorphic taxa are embedded within a larger group that has an apparently conserved, pleisiomorphic morphology. *Gymnocarpium oyamense* also is noteworthy because it is on a much longer branch, suggesting a strongly elevated rate of molecular evolution. By comparison, all other species in the genus display remarkably clock-like rates of evolution. Such elevated rates of substitution have been seen in other groups of ferns (Des Marais et al. 2003; Schuettpelz and Pryer 2006; F.-W. Li et al. 2011; Rothfels et al. 2012a; Rothfels and Schuettpelz, unpubl.). In these studies, however, the elevated rates characterized significant portions of the phylogeny, not single, isolated species.

Within the core *Gymnocarpium* clade, *G. oyamense* is sister to the remaining species. The southeastern Appalachian endemic diploid *G. appalachianum* is sister to a well-supported clade of *G. jessoense* subsp. *parvulum* accessions from North America and East Asia (Fig. 4). These two taxa are, in turn, sister to accessions of *G. jessoense* subsp. *jessoense* from

mainland Asia + the East Asian *G. remotepinnatum* (Fig. 4). The placement of *G. jessoense* subsp. *jessoense* (diploid) and *G. jessoense* subsp. *parvulum* (tetraploid) in different well-supported clades suggests that these taxa should be treated as distinct species. The taxonomy and nomenclature of Asian *Gymnocarpium* species is particularly complex, and one problem that our dataset does not address is the uncertain identity of *G. fedtschenkoanum* Pojark. (Pojarkova 1950). Fraser-Jenkins (2008) assigns the majority of Himalayan *Gymnocarpium* populations to *G. fedtschenkoanum*, restricting the name *G. jessoense* to more eastern populations. Future taxonomic work on *Gymnocarpium* will need to emphasize a global approach, with a concerted focus on Asian taxa.

Acystopteris Phylogeny—*Acystopteris* has long been recognized as a distinctive element, either as a subgenus of *Cystopteris* (Blasdell 1963; Kato 1977) or as a separate genus (Nakai 1933; Smith et al. 2006; Wang 2008; Rothfels et al. 2012b; Japanese Society for Plant Systematics 2012). It shares with *Cystopteris* a base chromosome number of $x = 42$ (Mitui 1975) and a distinctive, hood-like indusium, but differs in having catenate scales and tuberculate light-tan spores (Blasdell 1963; Rothfels et al. 2012b; Sundue and Rothfels, unpubl.), as well as an unusual, low-elevation, tropical distribution (Fig. 2B; Wang 2008). Our *Acystopteris* sampling includes two accessions from each of the three named species; each is monophyletic, as is the genus as a whole (Fig. 4). The well-supported position of *Acystopteris* as sister to *Cystopteris* permits either its continued recognition as a separate entity, or its merger into a broader concept of *Cystopteris*. Given that *Acystopteris* is easily diagnosed and has unique ecological and biogeographical features, as well as a relatively deep divergence from *Cystopteris*, we favor recognizing it at the generic level.

Within *Acystopteris*, *A. tenuisecta* is strongly supported as sister to *A. japonica*, and this clade is then sister to the Taiwan endemic *A. taiwaniana*. This result is surprising, given that *A. taiwaniana* is frequently treated as a variety of *A. japonica* and these two taxa are rarely, if ever, confused with *A. tenuisecta*. Wang (2008) suggested that *A. taiwaniana* might be an allopolyploid formed by hybridization between *A. japonica* and *A. tenuisecta*; however, this hypothesis is not supported by our data.

Cystopteris Phylogeny—Our phylogenetic hypothesis for *Cystopteris* includes four highly supported "major" clades (Fig. 4): *montana*, *sudetica*, *bulbifera*, and the *C. fragilis* complex (including *C. protrusa*). A clade comprising all *C. montana* accessions, including samples from Canada, Norway, and China, is strongly supported as sister to the remainder of the genus. This topology would permit the recognition of the genus *Rhizomatopteris* A.P. Khokhr. (typified on *C. montana*; Khokhrjakov 1985), while still retaining a monophyletic *Cystopteris*. However, in naming *Rhizomatopteris*, Khokhrjakov (1985) included *C. sudetica* (and by extension, *C. moupinensis* and *C. pellucida*) in his generic concept. *Rhizomatopteris* sensu Khokhrjakov, then, includes all taxa with broadly deltate-to-pentagonal leaves and widely spaced internodes on long-creeping rhizomes (see Fig. 1E, H). This assemblage is not monophyletic, and there seems little value in recognizing a monotypic *Rhizomatopteris* (containing only *C. montana*).

Blasdell's (1963: 80) "evolutionary tendencies" diagram (an early tree-like visualization inferred using elements of Wagner's (1980) "groundplan divergence scheme") is remarkably similar to our molecular phylogeny. His diagram, based entirely on morphological characters, includes a basal division between *Acystopteris* and *Cystopteris*, followed by a subsequent split with one branch largely corresponding to our *C. fragilis* complex clade (but excluding *C. diaphana*), and another branch with *C. montana* at its base, *C. bulbifera* next, and *C. sudetica* and *C. pellucida* grouped together. Indeed, if the incongruent position of *C. diaphana* is ignored, and the extant species that he included on internal branches are moved to branch tips, then his diagram perfectly anticipates our four-clade (plus *Acystopteris*) result. On the other hand, Blasdell's (1963) proposed classification of *Cystopteris* s.s. into two sections (*Emarginatae* and *Cystopteris*) is incompatible with certain aspects of our phylogeny. His *Cystopteris* section *Emarginatae* constitutes a paraphyletic grade comprised of *C. montana*, the *sudetica* and *bulbifera* clades, plus *C. diaphana*. In our phylogeny, the latter is deeply nested within the *C. fragilis* complex (Fig. 4), rendering Blasdell's (1963) *Cystopteris* section *Cystopteris* paraphyletic as well.

CYSTOPTERIS MONTANA, AND THE SUDETICA AND BULBIFERA CLADES—The position of *C. montana* as sister to the rest of the genus is very strongly supported. Within the *C. montana* lineage, there is a shallow but highly supported split separating the two high-elevation Tibetan accessions from the North American and Scandinavian samples (Fig. 4). Although Blasdell (1963) reports both diploid and tetraploid cytotypes for *C. montana* based on spore size differences, the species is cytologically documented only as a tetraploid (Haufler et al. 1993). Further investigations are necessary to determine if cryptic taxa exist within *C. montana*, and whether the Chinese taxon *C. modesta* Ching (not included in this study) is distinct from *C. montana* (see Fraser-Jenkins 2008).

Relationships among the three other major clades of *Cystopteris*—the *sudetica* clade, the *bulbifera* clade, and the *C. fragilis* complex—are not highly supported in our combined analysis. This lack of support is not caused by a lack of signal in our data, but rather by conflicting signals among the different partitions (see Intralinkage Incongruence discussion, above, and Fig. 3). Our sampling includes three species from the *sudetica* clade: *C. pellucida*, *C. moupinensis*, and *C. sudetica*. These species are primarily Asian; only *C. sudetica* extends west into Europe (Fig. 2C). Members of this clade are morphologically somewhat intermediate between *C. montana* (with which they share long-creeping rhizomes, widely-spaced leaves, and a tendency towards expanded basal pinnules on the lowermost pinnae) and *C. bulbifera* (with which they share an elongate-deltate leaf shape; see Fig. 1). In our sample, *C. pellucida* is sister to the rest of the *sudetica* clade (Fig. 4). An enigmatic species with a restricted range in central China (Fig. 2C), it differs from *C. moupinensis* in having more membranous leaves and a coarser leaf division (Wang 2008). *Cystopteris moupinensis* was recognized as a variety of *C. sudetica* by Blasdell (1963), and our analysis indicates that the two taxa are, indeed, very closely related. Our two accessions of *C. sudetica* are resolved as monophyletic, but these are very slightly diverged from—and form a polytomy with—our two *C. moupinensis* accessions (Fig. 4). The two species are allopatric (or very nearly so; Fig. 2C), and differ chiefly in the presence (*C. sudetica*) or absence (*C. moupinensis*) of glands on the indusia.

In our analysis, the *bulbifera* clade comprises all accessions of three species: *C. bulbifera*, *C. tennesseensis*, and *C. utahensis*. A limestone specialist of eastern North America with disjunct populations in the west, diploid *C. bulbifera* is hypothesized

to be involved in the origin of tetraploid *C. tennesseensis* (through hybridization with *C. protrusa*; Shaver 1950; Haufler et al. 1990) and tetraploid *C. utahensis* (through hybridization with *C. reevesiana*; Haufler and Windham 1991). In our dataset, *C. bulbifera*, *C. tennesseensis,* and *C. utahensis* have nearly identical sequences (Fig. 4), confirming that *C. bulbifera* is the maternal parent of both tetraploids, and that they formed recently (as suggested by Haufler et al. (1990)). Our only sample of *C. laurentiana*, an allohexaploid also thought to contain a *C. bulbifera* genome (Wagner and Hagenah 1956), derived its plastid from a member of the *C. fragilis* complex (Fig. 4). Molecular confirmation that *C. bulbifera* was involved in the formation of *C. laurentiana* will therefore require data from the nuclear genome.

Cystopteris fragilis Complex—The *Cystopteris fragilis* complex has a special place in fern systematics, with Lovis (1978: 356) describing it as "perhaps the most formidable biosystematics problem in the ferns." This species complex occurs on every continent except Antarctica (Fig. 2C, D), and includes ploidy levels ranging from diploid to octaploid (Blasdell 1963). Our plastid data, though preliminary, make some important new contributions. First, they permit a robust circumscription of the *C. fragilis* complex as the inclusive clade encompassing *C. fragilis* and *C. protrusa*, but not *C. bulbifera* or *C. sudetica* (Fig. 4). Second, they strongly support the position of the eastern North American, forest-dwelling diploid *C. protrusa* as sister to the rest of the complex (a position anticipated by Blasdell (1963) in his groundplan divergence scheme tree).

The bulk of the *C. fragilis* complex (i.e. excluding *C. protrusa*) is distinguished by a basal dichotomy. One side of the split is significantly supported, but barely so: it has 70% bootstrap support and 0.97 posterior probability (Fig. 4). This clade contains an interesting assemblage of taxa, including the alpine Eurasian hexaploid *C. alpina,* the endemic Australasian tetraploid *C. tasmanica*, both Hawaiian endemics (*C. sandwicensis* and *C. douglasii*), an eastern North American accession of *C. tenuis*, and three North American accessions of *C. fragilis* (including two unnamed hexaploids mentioned by Haufler and Windham (1991)). Most *Cystopteris* specimens with rugose spores—often called *C. dickieana* R. Sim. (Alston 1951; Bir and Trikha 1974; Nardi 1974; Wang 1983; Prada 1986; Parks et al. 2000) or *C. fragilis* subsp. *dickieana* (R. Sim) Hyl. (Fraser-Jenkins 2008)—also fall in this clade.

The sister clade is highly supported, and includes our only western North American accession of *C. tenuis*, all samples of *C. reevesiana* (a diploid), the North American putative allohexaploid *C. laurentiana,* various collections of *C. fragilis* from Europe and North America, *C. diaphana*, and single accessions identified as *C. membranifolia* and *C. millefolia* (Fig. 4). The latter three taxa form a weakly supported (61% bootstrap support and 0.99 posterior probability) clade that includes all Latin American samples of the *C. fragilis* complex, providing some support for Blasdell's (1963) broad application of the name *C. diaphana* to plants from this region.

Our preliminary results for the *C. fragilis* clade confirm that its reputation for systematic complexity is well deserved. Except for *C. protrusa*, none of the species for which we included multiple accessions is monophyletic (Fig. 4). Each of the three known diploid members—*C. protrusa*, *C. reevesiana*, and a diploid cytotype of *C. diaphana* (Blasdell 1963)—lacks the morphological distinctiveness characteristic of species in the other clades of *Cystopteris*, making it difficult to diagnose

polyploid taxa or to infer parentage based on morphological data alone. Though plastid DNA provide critical information, they tell only part of the story. Any progress on defining species limits in this group will depend on coordinated cytological and biparental molecular analyses, which are currently underway (Rothfels et al., unpubl.).

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APPENDIX 1. List of accessions sampled in this study, presented in the following format: Species, Voucher (HERBARIUM ACRONYM), Fern Lab database number (http://fernlab.biology.duke.edu/), Provenance, and GenBank numbers (with citations for previously published sequences) for *trnG-R, rbcL, matK* (in that order). The first instance of a taxon is in bold, with authority included. Missing data are indicated by "–".

Acystopteris japonica (Luerss.) Nakai. *Tsugaru & Takahashi 25409* (MO), 7097, JAPAN. Kyoto: JX874071,–, JX873970. *Acystopteris japonica, Ebihara 060728-01* (TNS:763998), 7978, Tokyo: Nishitama-gun, JX874072, AB574893 (Ebihara et al. 2010), JX873971. *Acystopteris taiwaniana* (Tagawa) A. Löve & D. Löve. *Schuettpelz 1127A* (DUKE), 4870, TAIWAN. Nantou: JF832188 (Rothfels et al. 2012a), JF832052 (Rothfels et al. 2012a), JX873972. *Acystopteris taiwaniana, Kuo 175* (TAIF), 6137, Chial: JX874073, JF303968 (Kuo et al. 2011), JF303925 (Kuo et al. 2011). *Acystopteris tenuisecta* (Bl.) Tagawa. *Schuettpelz 807* (DUKE), 4225, MALAYSIA. Pahang: JX874074,–, JX873973. *Acystopteris tenuisecta, Schuettpelz 1088A* (DUKE), 4831, TAIWAN. Nantou: JF832189 (Rothfels et al. 2012a), JF832053 (Rothfels et al. 2012a), JX873974. *Cystopteris alpina* Desv. *Larsson 242 & Rautenberg* (DUKE), 7920, SWEDEN. Västerbotten: Storuman, JX874075, JX874032, JX873975. *Cystopteris bulbifera* (L.) Bernh., *Windham 94-189* (DUKE), 5836, U. S. A.: Arizona: Coconino County, JX874076, JX874033, JX873976. *Cystopteris bulbifera, Rothfels 3929 & Lewer* (DUKE), 7650, CANADA. Ontario: Hamilton Region, JX874077,–, JX873977. *Cystopteris diaphana* (Bory) Blasdell, *Matos 08-147* (DUKE), 5316, COSTA RICA. San José: Canton Villa Mills, JX874078, JX874034, JX873978. *Cystopteris diaphana, Rothfels 2620* (DUKE), 5622, Canton Perez Zeledon, JX874079, JX874035, JX873979. *Cystopteris diaphana, Windham 560* (DUKE), 5845, MEXICO. Oaxaca: JX874080, JX874036,–. *Cystopteris diaphana, Arana 889* (DUKE), 6380. ARGENTINA. San Luis: JX874081, JX874037,–. *Cystopteris diaphana, Grangaud 1875bis* (DUKE), 6386, FRANCE. Ile de la Reunion: Cilaos, JX874082, JX874038, JX873980. *Cystopteris diaphana, Rothfels 3073* (DUKE), 6513, MEXICO. Guanajuato: Municipio Cortázar, JX874083, JX874039, JX873981. *Cystopteris diaphana, Rothfels 3123* (DUKE), 6560, Jalisco: Municipio Tequila, JX874084, JX874040, JX873982. *Cystopteris diaphana, Rothfels 3171* (DUKE), 6592, Nayarit: Municipio Xalisco, JX874085,–, JX873983. *Cystopteris douglasii* Hook. *Oppenheimer #H100823* (DUKE), 6206, U. S. A. Hawai'i: Makawao District, JX874086, JX874041, JX873984. *Cystopteris fragilis* (L.) Bernh. *Kelsey s. n.* (DUKE), 5851, Utah: Salt Lake County, JX874087, JX874042,–. *Cystopteris fragilis, Smith 2* (DUKE), 5950, Colorado: Park County, JX874088, JX874043, JX873985. *Cystopteris fragilis, Larsson 21* (DUKE), 7103, SWEDEN. Uppsala JF832204 (Rothfels et al. 2012a), JF832062 (Rothfels et al. 2012a), JF832266 (Rothfels et al. 2012a). *Cystopteris fragilis* "brittonii", *Windham 865* (DUKE), 5847, CANADA. Ontario: Manitoulin District, JX874089, JX874044,–. *Cystopteris fragilis* "haufleri", *Windham s. n.* (DUKE), 7034, U. S. A.. Utah: Salt Lake County, JX874090,–, JX873986. *Cystopteris laurentiana* (Weath.) Blasdell. *Oldham 17525* (DUKE), 8081, CANADA. Ontario: Thunder Bay Region, JX874091,–, JX873987. *Cystopteris membranifolia* Mickel. *Rothfels 3365* (DUKE), 6732, MEXICO. Oaxaca:

Municipio Santa Maria Teopoxco, JX874092, JX874045, JX873988. *Cystopteris millefolia* Mickel. *Rothfels 3411* (DUKE), 6761, MEXICO. México: Municipio Ocuilan, JX874093, JX874046, JX873989. *Cystopteris montana* (Lam.) Berhn. ex Desv. *LeBlond 6448* (DUKE), 5839, CANADA. Newfoundland: St. Barbe North District, JF832205 (Rothfels et al. 2012a), JF832063 (Rothfels et al. 2012a), JX873990. *Cystopteris montana, Harris 09-073* (DUKE), 6969, Ontario: Thunder Bay District, JX874094,–, JF832267 (Rothfels et al. 2012a). *Cystopteris montana, Dickoré 11796* (UC), 7071, CHINA. Tibet: southeast Tibet, JX874095,–, JX873991. *Cystopteris montana, Miehe & Wündisch 94-79-23* (UC), 7072, Tibet: south Tibet, JX874096, JX874047, JX873992. *Cystopteris montana, Larsson 315* (DUKE), 7943, NORWAY. Finnmark: Alta, JX874097,–, JX873993. *Cystopteris moupinensis* Franch. *Schuettpelz 1118A* (DUKE), 4861, TAIWAN. Nantou County, JF832206 (Rothfels et al. 2012a), JF832064 (Rothfels et al. 2012a), JX873994. *Cystopteris moupinensis, Dickoré 11892* (UC), 7074, CHINA. Tibet: SE Tibet, JX874098, JX874048, JX873995. *Cystopteris pellucida* (Franch.) Ching ex C.Chr. *Hoffmeister et al. 22* (MO), 6055, Yunnan: De Qin County, JX874099,–, JX873996. *Cystopteris pellucida, Yatskievych et al. 02-57* (MO), 6060, Zhongdian County, JX874100, JX874049, JX873997. *Cystopteris protrusa* (Weatherby) Blasdell. *Alford 2088* (DUKE), 5838, U. S. A. Mississippi: Wilkinson County, JX874101, JX874050, JX873998. *Cystopteris protrusa, Murrell 1582* (DUKE), 5841, Kentucky: Warren County, JX874102, JX874051, JX873999. *Cystopteris protrusa, Rothfels 2879* (DUKE), 6362, Virginia: Grayson County, JX874103, JX874052, JX874000. *Cystopteris reevesiana* Lellinger. *Schuettpelz 419* (DUKE), 3126, U. S. A. Arizona: Coconino County, JX874104, EF452149 (Schuettpelz et al. 2007), JX874001. *Cystopteris reevesiana, Windham 462* (DUKE), 5844, Cochise County, JX874105, JX874053,–. *Cystopteris reevesiana, Smith 12* (DUKE), 6342, Colorado: Fremont County JX874106, JX874054, JX874002. *Cystopteris sandwicensis* Brack. *Wood 9009* (pers. herb. Daniel D. Palmer), 6208, U. S. A. Hawai'i: Kauai, JX874107, JX874055, JX874003. *Cystopteris sudetica* A. Braun & Milde. *Unknown* (MO 4378105), 7096, RUSSIA. JX874108,–, JX874004. *Cystopteris sudetica, Ueno 1314* (TNS:766629), 7980, JAPAN. Nagano: Matsumoto-shi, JX874109, AB574939 (Ebihara et al. 2010), JX874005. *Cystopteris tasmanica* Hook. *Thorsen 192/07* (DUKE), 6379, NEW ZEALAND. Otago, JX874110, JX874056, JX874006. *Cystopteris tennesseensis* Shaver. *Rothfels 2441* (DUKE), 4513, U. S. A. North Carolina: Jones County, JX874111, JX874057, JX874007. *Cystopteris tennesseensis, Windham 81-13* (DUKE), 7990, Tennessee: Putnam County, JX874112,–, JX874008. *Cystopteris tenuis* (Michx.) Desv. *Ring 6374* (DUKE), 5833, U. S. A. Arizona: Coconino County, JX874113, JX874058, JX874009. *Cystopteris tenuis, Barrington 2373* (DUKE), 6387, Vermont: Chittenden County, JX874114, JX874059, JX874010. *Cystopteris utahensis* Windham & Haufler. *Rink 6566* (DUKE), 5832, U. S. A. Arizona: Coconino County, JX874115, JX874060, JX874011. *Cystopteris utahensis, Windham 92-380* (DUKE), 5834, Coconino County, JX874116, JX874061,–. *Cystopteris utahensis, Rothfels 2973* (DUKE), 6848, Utah: Utah County, JX874117,–, JX874012. *Gymnocarpium appalachianum* Pryer & Haufler. *Rothfels & Zylinski 3914* (DUKE), 7639, U. S. A. Virginia: Highland County, JX874119,–, JX874014. *Gymnocarpium appalachianum, Rothfels & Zylinski 3897* (DUKE), 7800, Page County, JX874120, JX874062, JX874015. *Gymnocarpium disjunctum* (Ruprecht) Ching. *Metzgar 224* (DUKE), 4710, U. S. A. Alaska: Kenai Peninsula Borough, JX874121, JX874063, JX874016. *Gymnocarpium disjunctum, Sigel & Miles 2010-82* (DUKE), 7751, Washington: Snohomish County, JX874122,–,–. *Gymnocarpium dryopteris* (L.) Newm. *Metzgar 209* (DUKE), 4601, U. S. A. Alaska: Kenai Peninsula Borough, JX874123, JX874064, JX874017. *Gymnocarpium dryopteris, Christenhusz 3758* (DUKE), 5837, FINLAND. Varsinais-Suomi Archipelago: Jurmo, JX874124, JX874065,–. *Gymnocarpium dryopteris, Larsson 6* (DUKE), 7059, SWEDEN. Uppsala: JF832218 (Rothfels et al. 2012a), JF832068 (Rothfels et al. 2012a), JF832277 (Rothfels et al. 2012a). *Gymnocarpium dryopteris, Ebihara & Kadota HK2007-815* (TNS), 7981, JAPAN. Hokkaido: Uryu-gun, JX874125, AB574992 (Ebihara et al. 2010), JX874018. *Gymnocarpium dryopteris* var. *aokigaharaense* Nakaike. *Okegawa 1976* (TNS), 7984, JAPAN. Yamanashi: Minamitsuru-gun, JX874126, AB574993 (Ebihara et al. 2010), JX874019. *Gymnocarpium jessoense* (Koidz.) Koidz. *Boufford et al. 29916* (MO), 6059, CHINA. Tibet: Bomi Xian, JX874127, JX874020. *Gymnocarpium jessoense, Dickoré 12767* (UC), 7069, PAKISTAN. Astor/Nanga Parbat, JX874128,–, JX874021. *Gymnocarpium jessoense* subsp. *parvulum* Sarvela. *Brinker 1628* (DUKE), 8053, CANADA. Ontario: Kenora District, JX874130, JX874067, JX874023. *Gymnocarpium jessoense* subsp. *parvulum, Legler 877* (NY), 8120, RUSSIA. Sakhalin Region, JX874131,–, JX874024. *Gymnocarpium oyamense* (Baker) Ching. *Nakato s. n.* (DUKE), 6399, JAPAN. Tokyo: JF832219 (Rothfels et al. 2012a), JF832069 (Rothfels et al. 2012a),–. *Gymnocarpium oyamense, Fujimoto 071023* (TNS), 7983, Nishitama-gun, JX874132, AB574995 (Ebihara et al. 2010), JX874025. *Gymnocarpium remotepinnatum* (Hayata) Ching. *Yatskievych 02-31*

(MO), 3066, CHINA. Yunnan: Jianchuan County, JF832220 (Rothfels et al. 2012a), EF463317 (Schuettpelz and Pryer 2007), JX874026. *Gymnocarpium remotepinnatum*, *Schuettpelz 1119A* (DUKE), 4862, TAIWAN. Nantou County, JX874133, JX874068, JX874027. **Gymnocarpium robertianum** (Hoffm.) Newm. *Pryer s. n.* (DUKE), 5852, U. S. A. Minnesota: Clearwater County, JX874134,–, JX874028. *Gymnocarpium robertianum*, *Gregory s. n.* (TRTE), 7178, CANADA Ontario: Cochrane District, JX874135,–, JX874029. *Gymnocarpium robertianum*, *Oldham & Bakowsky 28524* (DUKE), 7197, Cochrane District, JX874136,–, JX874030. *Gymnocarpium robertianum*, *Larsson 282* (DUKE), 7945, NORWAY. Nordland: Fauske County, JX874137, JX874069, JX874031. **Gymnocarpium sp.** *Ebihara et al. TH2007-996* (TNS), 7979, JAPAN. Iwate: Shimohei-gun, JX874129, AB574994 (Ebihara et al. 2010), JX874022. **Gymnocarpium × brittonianum** (Sarvela) Pryer & Haufler. *Smith 212.1* (DUKE), 7806, U. S. A. Colorado: Grand County, JX874118,–, JX874013. **OUTGROUP:** *Athyrium otophorum* (Miq.) Koidz. *Smith s. n.* (UC), 3744, Cult., JF832195 (Rothfels et al. 2012a), EF463305 (Schuettpelz and Pryer 2007),–. *Athyrium otophorum*, *Ebihara et al. 070210-02* (TNS),–, JAPAN. Shizuoka:–,–, JF832258 (Rothfels et al. 2012a). **Blechnum schomburgkii** (Klotzsch) C. Chr. *Schuettpelz 242* (DUKE), 2410, ECUADOR. Zamora-Chinchipe: JF832198 (Rothfels et al. 2012a), EF463160 (Schuettpelz and Pryer 2007), JF832261 (Rothfels et al. 2012a). **Deparia** *lancea* (Thunb.) R. Sano. *Schuettpelz 298* (DUKE), 2558, Cult. (Duke U. Greenhouse), JF832207 (Rothfels et al. 2012a), EF463306 (Schuettpelz and Pryer 2007),–. *Deparia lancea*, *Kuo 112* (TAIF),–, TAIWAN,–,–, JF303940 (Kuo et al. 2011). **Diplaziopsis javanica** (Blume) C. Chr. *Schuettpelz 1220A* (DUKE), 4967, TAIWAN. Ilan: JF832212 (Rothfels et al. 2012a), JF832066 (Rothfels et al. 2012a),–. *Diplaziopsis javanica*, *Kuo 138* (TAIF),–, TAIWAN.–,–, JF303928 (Kuo et al. 2011). **Hemidictyum marginatum** (L.) C. Presl. *Christenhusz 2476* (DUKE), 3054, FRENCH GUIANA. Montagnes Tortue, JF832221 (Rothfels et al. 2012a), EF463318 (Schuettpelz and Pryer 2007), JF303927 (Kuo et al. 2011). **Onocleopsis hintonii** Ballard. *Rothfels 3360 et al.* (DUKE), 6729, MEXICO. Oaxaca: JF832230 (Rothfels et al. 2012a), JF832077 (Rothfels et al. 2012a), JF832281 (Rothfels et al. 2012a). **Pseudocystopteris atkinsonii** (Bedd.) Ching. *Schuettpelz 1094* (DUKE), 4837, TAIWAN. Nantou County, JF832235 (Rothfels et al. 2012a), JX874070,–. *Pseudocystopteris atkinsonii*, *Kuo 477* (TAIF),–, Nantou County,–,–, JF832285 (Rothfels et al. 2012a). **Pseudophegopteris cruciata** (Willd.) Holttum. *Janssen 2724* (P), 3559, FRANCE. Ile de la Reunion, JF832236 (Rothfels et al. 2012a), EF463279 (Schuettpelz and Pryer 2007), JF832286 (Rothfels et al. 2012a). **Woodsia obtusa** (Spr.) Torrey *Schuettpelz 328* (DUKE), 2973, Cult.: originally from U. S. A. Texas: Burnet County, JX874138, EF463319 (Schuettpelz and Pryer 2007),–.