Systematic Botany (2011), 36(3): pp. 554–564

DOI 10.1600/036364411X583547

Species Relationships and Farina Evolution in the Cheilanthoid Fern Genus *Argyrochosma* (Pteridaceae)

Erin M. Sigel,[1,3] Michael D. Windham,[1] Layne Huiet,[1] George Yatskievych,[2] and Kathleen M. Pryer[1]

[1]Department of Biology, Duke University, Durham, North Carolina 27708 U. S. A.
[2]Missouri Botanical Garden, P.O. Box 299, St. Louis, Missouri 63166 U. S. A.
[3]Author for correspondence (erin.sigel@duke.edu)

Communicating Editor: Lynn Bohs

***Abstract*—**Convergent evolution driven by adaptation to arid habitats has made it difficult to identify monophyletic taxa in the cheilanthoid ferns. Dependence on distinctive, but potentially homoplastic characters, to define major clades has resulted in a taxonomic conundrum: all of the largest cheilanthoid genera have been shown to be polyphyletic. Here we reconstruct the first comprehensive phylogeny of the strictly New World cheilanthoid genus *Argyrochosma*. We use our reconstruction to examine the evolution of farina (powdery leaf deposits), which has played a prominent role in the circumscription of cheilanthoid genera. Our data indicate that *Argyrochosma* comprises two major monophyletic groups: one exclusively non-farinose and the other primarily farinose. Within the latter group, there has been at least one evolutionary reversal (loss) of farina and the development of major chemical variants that characterize specific clades. Our phylogenetic hypothesis, in combination with spore data and chromosome counts, also provides a critical context for addressing the prevalence of polyploidy and apomixis within the genus. Evidence from these datasets provides testable hypotheses regarding reticulate evolution and suggests the presence of several previously undetected taxa of *Argyrochosma*.

***Keywords*—**Apomixis, homoplasy, phylogeny, polyploidy, spores.

Cheilanthoid ferns, a lineage of xeric-adapted species in the family Pteridaceae, provide a prime example of how morphological homoplasy has contributed to the delineation of nonmonophyletic genera. Despite the apparent monophyly of cheilanthoids as a whole (Gastony and Rollo 1995, 1998; Schuettpelz et al. 2007), more than half of the genera have proven to be polyphyletic, including *Cheilanthes* Swartz, *Doryopteris* J. Smith, *Notholaena* R. Brown, and *Pellaea* Link (Schuettpelz et al. 2007; Rothfels et al. 2008). Difficulties in identifying discrete generic boundaries among the cheilanthoids have long been attributed to convergent evolution driven by adaptation to arid environments (Tryon and Tryon 1973). Those studying the group frequently echo the comment by Tryon and Tryon (1973: page 146) that there is "an obvious need for the development of new data which will give a better insight into the evolutionary lines within the group."

Among cheilanthoid ferns, one morphological feature that has received much attention is the presence or absence of "farina" on the leaves. In farinose species, abaxial leaf surfaces (and sometimes other parts of the leaf) are covered by a white or yellow (rarely orange) powdery substance of varying chemical composition that is excreted by glandular trichomes (Wollenweber and Schneider 2000; Fig. 1A and 1B). From a functional standpoint, farina has been hypothesized to reduce water loss in arid habitats by providing both an insulating layer over the stomata and a reflective surface to reduce overheating in high light environments (Hevly 1963; Wollenweber 1984; Wollenweber and Schneider 2000). The potential adaptive significance of farina has made it a trait of evolutionary interest, and several cheilanthoid fern genera have been defined primarily on the presence or absence of farina.

One group of xeric-adapted ferns including both farinose and non-farinose species is the genus *Argyrochosma* (J. Smith) Windham. Variation in farina within this group was the primary source of conflict among earlier taxonomic treatments. Rolla Tryon and collaborators (Tryon and Tryon 1982; Tryon et al. 1990) assigned all species of *Argyrochosma* (most of which are farinose) to the genus *Notholaena*, the remainder of which was exclusively farinose as circumscribed by these authors. In contrast, Morton (1950) advocated the placement of these same species in the genus *Pellaea* (which otherwise lacks an apparent farina) based on similarities in leaf architecture, sporangial placement, and spore ornamentation. Molecular phylogenies support *Argyrochosma* as a monophyletic group sister to core pellaeids (sensu Windham et al. 2009; see also Gastony and Rollo 1995, 1998; Kirkpatrick 2007; Schuettpelz et al. 2007; Rothfels et al. 2008), and indicate that *Argyrochosma* is more distantly related to *Notholaena*. Hence, it appears that farina has arisen independently in *Argyrochosma* and may not be homologous to farina observed in *Notholaena*. This raises questions regarding the evolution of this trait within *Argyrochosma*.

Here we reconstruct a comprehensive species-level phylogeny of *Argyrochosma*, utilizing DNA sequence data from three plastid loci (*rbcL*, *atpA*, and *trnG-trnR*). Using a combination of molecular, cytological, and spore data, we infer the relationships among all previously recognized taxa and determine whether variation in farina production reflects a single origin within the genus or a more complex evolutionary pattern. This investigation also identifies several possible cases of hybridization in the genus and contributes to an understanding of morphological homoplasy across cheilanthoid ferns as a whole.

Materials and Methods

***Taxon Sampling*—**We sampled 57 *Argyrochosma* specimens comprising 16 species, two subspecies, and three varieties (Table 1; Appendix 1). This includes all taxa assigned to the genus by Windham (1987, 1993), Ponce (1996), and Mickel and Smith (2004). *Pellaea breweri* D. C. Eaton, *Paragymnopteris delavayi* (Baker) K. H. Shing, and *Paragymnopteris marantae* (L.) K. H. Shing were selected as outgroups based on their position as the closest relatives to *Argyrochosma* in recent molecular phylogenies (Appendix 1; Kirkpatrick 2007; Rothfels et al. 2008).

***DNA Extraction, Amplification, and Sequencing*—**DNA was isolated from both silica-dried and herbarium material. Protocols for DNA extraction, amplification, and sequencing followed Nagalingum et al. (2007) and Schuettpelz and Pryer (2007). DNA sequences were obtained from 52 of the 57 samples included in this study; *A. incana* 5 and 6, *A. jonesii* 4, and *A. nivea* var. *nivea* 4 and 5 were subjected to cytogenetic study but were not sequenced. Each of three plastid regions (*rbcL*, *atpA*, and *trnG-trnR*) were entirely or partially amplified and sequenced for nearly all of the other 52 specimens (Table 1). We were unable to successfully amplify *rbcL*

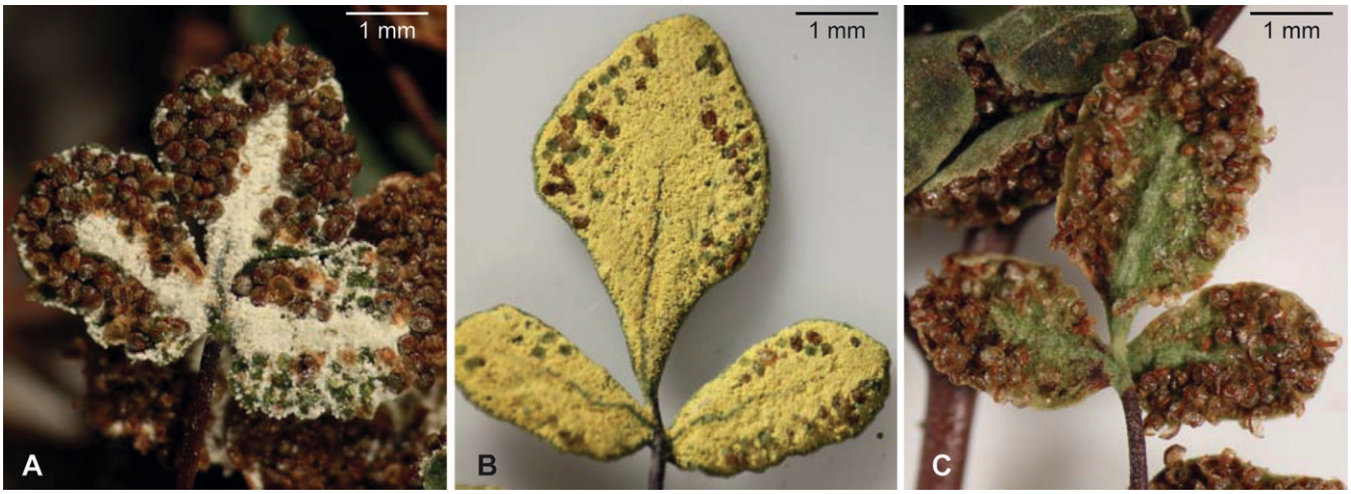

FIG. 1. Abaxial leaf surface for the three varieties of *Argyrochosma nivea*, each with a distinct farina-morph. A. *A. nivea* var. *nivea* 2 with white farina. B. *A. nivea* var. *flava* 4 with yellow farina. C. *A. nivea* var. *tenera* 2 lacking farina. Refer to Table 1 and Appendix for voucher information.

for *A. palmeri* 1 and *trnG-trnR* for *A. chilensis* and *A. jonesii* 3. Primers for amplification and sequencing were taken from Schuettpelz et al. (2006), Schuettpelz et al. (2007), Nagalingum et al. (2007), and Beck et al. (2010). New primers were developed here to amplify and sequence portions of *atpA* and *rbcL* from samples for which we were not able to amplify the entire region (Table 2).

Sequence Alignment and Datasets—DNA sequence chromatograms were manually edited and assembled using Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, Michigan). Each plastid region was manually aligned with MacClade 4.05 (Maddison and Maddison 2005). Unsequenced portions of plastid regions were coded as missing data. Unambiguous one base pair indels present in *trnG-trnR* and *atpA* were coded as a fifth character state. Ambiguously aligned regions two or more base pairs in length (also confined to *trnG-trnR* and *atpA*) were excluded prior to phylogenetic analysis. Three datasets were compiled, one for each of the three plastid regions. Datasets and phylogenetic trees are deposited in TreeBASE (study number S10649).

Phylogenetic Analyses—Separate phylogenetic analyses were conducted for each of the three plastid datasets using maximum parsimony (MP) in PAUP* 4.0b10 (Swofford 2002). All included characters were coded as unordered and equally weighted. Heuristic searches were employed with tree bisection and reconnection branch swapping (TBR) and 1,000 random addition sequence (RAS) replicates. No more than 10 trees were saved per replicate. To address topological support of the resulting MP majority-rule consensus tree, 1,000 bootstrap (MPBS) replicates with 10 RAS each were carried out.

Maximum likelihood (ML) analyses were conducted for each of the three datasets using Garli 0.951 (Zwickl 2006). A GTR + I + G substitution model with four distinct rate categories was employed. The proportion of invariant sites and state frequencies were estimated. Eight "best tree" searches with random starting trees were performed, and the single best tree was determined as that with the highest likelihood score (Table 3). Topological support for the best tree was assessed with 500 (MLBS) bootstrap replicates.

Maximum parsimony and ML trees for each plastid region were visually inspected for conflict based on a 70% bootstrap support criterion (Mason-Gamer and Kellogg 1996). No incongruence was detected between MP and ML phylogenies for the same plastid region. Additionally, no significant incongruence was detected when comparing phylogenies across different plastid regions. For this reason, the three separate datasets were combined and analyzed together using the MP and ML approaches described above (Table 3).

Reconstructing Farina Evolution—Herbarium material for each specimen was visually inspected under 10 × magnification for farina on abaxial leaf surfaces. To avoid the complication of coding inapplicable characters (Maddison 1993), each specimen was scored using one of three discrete character states: (0) absent, (1) white farina, or (2) yellow farina. To investigate the evolution of farina within the genus, we plotted farina condition on the best ML tree from the combined analysis of all three plastid regions, where branches with low support (MLBS < 75%) were collapsed. Characters were reconstructed using an unordered parsimony model in Mesquite 2.5 (Maddison and Maddison 2008).

Assessing Reproductive Mode and Ploidy Level—Species of *Argyrochosma* exhibit variability in reproductive mode, encompassing both sexual and apomictic life cycles. Among core leptosporangiate ferns, the presence of sporangia containing ~ 64 spores provides prima facie evidence of sexual reproduction. Sporangia containing reduced numbers of spores (32 or 16) generally result from nonreductive meiotic events that, when they predominate in an individual, are indicative of an apomictic life cycle (Manton 1950; Tryon and Britton 1958; Gastony and Windham 1989). Previous studies of *Argyrochosma* have confirmed the strong correlation between spore number per sporangium and reproductive mode in this genus (Windham and Yatskievych 2003). To determine mode of reproduction for the samples included here, the number of spores per sporangium was determined for all specimens with mature sporangia. Multiple, intact sporangia from each specimen were removed and opened in individual drops of glycerol. Spores from each sporangium were dispersed within the glycerol drop with a dissecting needle and counted.

Chromosome counts are available for many of the taxa in *Argyrochosma* (summarized by Windham and Yatskievych 2003). Most of the chromosome counts presented in Table 1 are derived from these published sources, but new counts are presented here for *A. limitanea* subsp. *mexicana* 1, *A. microphylla* 3, *A. nivea* var. *nivea* 4, and *A. nivea* var. *nivea* 5. Protocols for obtaining these counts followed Windham and Yatskievych (2003).

Because there is a direct correlation between spore size and sporophyte ploidy level in most ferns (Wagner 1974; Pryer and Britton 1983; Barrington et al. 1986; Guo et al. 2003; Grusz et al. 2009; Beck et al. 2010), it is possible to estimate the ploidy level of specimens that are not chromosome count vouchers by comparing their mean spore sizes to similar data derived from chromosome voucher specimens. To obtain the necessary data on spore size, the glycerol-mounted spores used to determine reproductive mode were examined at 400 × magnification on a Zeiss Axioplan 2 compound microscope or at 100 × on a Leica MZ 125 dissecting microscope. Images of 10–40 spores per specimen were obtained using a Zeiss AxioCam HRm or Canon EOS Rebel XSi. Spore length (excluding the perispore) was measured using ImageJ version 1.38 (Abramoff et al. 2004) calibrated with a slide micrometer. Mean spore length and standard deviation were calculated for each specimen. A Mann-Whitney *U* test was performed in JMP version 7 (SAS Institute Inc. 2007) to determine whether mean spore lengths of specimens representing different ploidy levels and major clades of *Argyrochosma* were significantly different.

RESULTS

Phylogeny of Argyrochosma—Of the 162 DNA sequences used in this study, 147 were generated as part of this study and have been deposited in GenBank (Appendix 1). Maximum parsimony and ML analyses of the combined dataset produced phylogenetic hypotheses of similar topology and comparable support (see Table 3 for tree statistics). The MP analysis resulted in 9,800 most-parsimonious trees of 1,252 steps; the best ML tree had a maximum log-likelihood score

TABLE 1. Synopsis of *Argyrochosma* specimens used in this study. Taxa represented by more than one specimen are numbered sequentially. Mode of reproduction: black circles = sexual, open circles = apomict, partially filled circles = sexual but producing a subset of unreduced spores, NA = not available (specimen sterile). Chromosome count (meiotic count, unless otherwise noted): §gametophyte mitotic count, ¶count taken from a duplicate specimen, [1]count previously published by Windham and Yatskievych (2003), NA = not available. Farina: A = absent, W = white, Y = yellow, *although farina absent on herbarium material, white farina observed on the first leaf of a sporophyte grown from spore; DNA sequence data: check mark = sequence data available for all three plastid genes, †missing *rbcL*, ‡missing *trnG-trnR*, NA = not available.

Argyrochosma Taxon	Voucher Information	Mode of Reproduction	Chromosome Count	Farina	DNA Sequence Data
A. chilensis (Fée & Remy) Windham	CHILE, Juan Fernández Islands, *Stottsberg 100* (MO)	•	NA	W	√ ‡
A. dealbata (Pursh) Windham	U. S. A., Kansas, *Brooks 16997* (DUKE)	•	$n = 27$[1]	W	√
A. delicatula (Maxon & Weath.) Windham	MEXICO, Nuevo León, *Windham et al. 482* (DUKE)	•	$n = 27$[1]	W	√
A. fendleri (Kunze) Windham					
A. fendleri 1	U. S. A., New Mexico, *Metzgar 120* (DUKE)	•	NA	W	√
A. fendleri 2	U. S. A., Colorado, *Windham & Beck 3529* (DUKE)	NA	NA	W	√
A. fendleri 3	U. S. A., New Mexico, *H. van der Werff 22303* (MO)	NA	NA	W	√
A. fendleri 4	U. S. A., New Mexico, *Windham 352* (MO)	•	$n = 27$[1]	W	NA
A. formosa (Leibm.)Windham	MEXICO, Oaxaca, *Windham et al. 539* (DUKE)	○	$n = 2n = 81$[1]	W	√
A. incana (C. Presl) Windham					
A. incana 1	U. S. A., Arizona, *Schuettpelz 487* (DUKE)	NA	NA	W	√
A. incana 2	U. S. A., Arizona, *Schuettpelz 491* (DUKE)	•	NA	W	√
A. incana 3	MEXICO, Sonora, *Reina & Van Devender 2005–931* (MO)	•	NA	W	√
A. incana 4	DOMINICAN REPUBLIC, San Juan, *R. A. & E. S. Howard 9113* (US)	•	NA	W	√
A. incana 5	U. S. A., Arizona, *Windham & Yatskievych 332* (MO)	•	$n = 27$[1]	W	NA
A. incana 6	U. S. A., New Mexico, *Windham & Yatskievych 374* (UT)	•	$n = 27$[1]	W	NA
A. jonesii (Maxon) Windham					
A. jonesii 1	U. S. A., California, *Windham & Pryer 3429* (UT)	NA	NA	A	√
A. jonesii 2	U. S. A., California, *Windham & Pryer 3437* (DUKE)	•	NA	A	√
A. jonesii 3	U. S. A., California, *Morefield 1664a* (UT)	•	$n = 54$[1]§	A	√‡
A. jonesii 4	U. S. A., Arizona, *Windham & Yatskievych 249* (UT)	•	$n = 27$[1]	A	NA
A. limitanea (Maxon) Windham subsp. *limitanea*					
A. limitanea subsp. *limitanea* 1	U. S. A., Arizona, *Schuettpelz 472* (DUKE)	○	NA	W	√
A. limitanea subsp. *limitanea* 2	U. S. A., Arizona, *Rothfels 2532* (DUKE)	○	NA	W	√
A. limitanea subsp. *limitanea* 3	U. S. A., Arizona, *Windham et al. 171* (UT)	○	$n = 2n = 81$[1]	W	√
A. limitanea subsp. *limitanea* 4	U. S. A., Arizona, *Windham et al. 459* (UT)	○	$n = 2n = 81$[1]	W	√
A. limitanea subsp. *limitanea* 5	MEXICO, Sonora, *Van Devender et al. 98–923* (MO)	○	NA	W	√
A. limitanea subsp. *mexicana* (Maxon) Windham					
A. limitanea subsp. *mexicana* 1	MEXICO, Coahuila, *Windham et al. 575* (DUKE)	○	$n = 2n = 81$[1] $n = 2n = 162$	W	√
A. limitanea subsp. *mexicana* 2	U. S. A., New Mexico, *Windham & Rabe 90–377* (DUKE)	○	NA	W	√
A. limitanea subsp. *mexicana* 3	U. S. A., New Mexico, *Rothfels 2514* (DUKE)	○	NA	W	√
A. lumholtzii (Maxon & Weath.) Windham	MEXICO, Sonora, *Fishbein et al. 4458* (MO)	•	NA	A	√
A. microphylla (Mett. ex Kuhn) Windham					
A. microphylla 1	MEXICO, Coahuila, *Windham et al. 576* (DUKE)	•	$n = 27$[1]	A	√
A. microphylla 2	U. S. A., New Mexico, *Worthington 34623* (DUKE)	NA	NA	A	√
A. microphylla 3	U. S. A., New Mexico, *Windham 3494* (DUKE)	•	$n = 27$	A	√
A. nivea (Poir.) Windham var. *flava* (Hook.) Ponce					
A. nivea var. *flava* 1	BOLIVIA, Chuquisaca, *Wood 8744* (UC)	○	NA	Y	√
A. nivea var. *flava* 2	ECUADOR, Azuay, *Aedo et al. 13066bis* (MO)	○	NA	Y	√
A. nivea var. *flava* 3	BOLIVIA, Chuquisaca, *Huaylla 70* (MO)	○	NA	Y	√
A. nivea var. *flava* 4	BOLIVIA, Chuquisaca, *Huaylla et al. 2207* (MO)	NA	NA	Y	√
A. nivea var. *flava* 5	PERU, Cajamarca, *Correll & Smith P912* (US)	○	NA	Y	√
A. nivea var. *flava* 6	BOLIVIA, Santa Cruz, *Kessler et al. 5323* (US)	○	NA	Y	√
A. nivea var. *nivea*					
A. nivea var. *nivea* 1	PERU, Cusco, *Galiano et al. 6865* (MO)	○	NA	W	√
A. nivea var. *nivea* 2	BOLIVIA, Oruro, *Torrico & Castillo 622* (MO)	○	NA	W	√
A. nivea var. *nivea* 3	BOLIVIA, Oruro, *Linneo 301* (MO)	○	NA	W	√
A. nivea var. *nivea* 4	PERU, *Tryon s. n.* (HUH)	○	$2n = 81$§	W	NA
A. nivea var. *nivea* 5	PERU, Junín, *Correll & Smith P759* (HUH)	○	$2n = 81$§	W	NA
A. nivea var. *tenera* (Gillies ex Hook.) Ponce					
A. nivea var. *tenera* 1	BOLIVIA, La Paz, *Reeb VR 26-V-02/12* (DUKE)	○	NA	W*	√
A. nivea var. *tenera* 2	BOLIVIA, Tarija, *Beck St. G. 23950* (UC)	◘	NA	A	√
A. nivea var. *tenera* 3	BOLIVIA, La Paz, *Sperling & King 5394* (US)	○	NA	W	√
A. nivea var. *tenera* 4	BOLIVIA, Tarija, *Serrano et al. 6182* (MO)	○	NA	W	√
A. nivea var. *tenera* 5	ARGENTINA, Jujuya, *Morrone et al. 2360* (MO)	◘	NA	A	√
A. nivea var. *tenera* 6	BOLIVIA, Chuquisaca, *Huaylla 65* (MO)	○	NA	W	√

(*Continued*)

Table 1. Continued

Argyrochosma Taxon	Voucher Information	Mode of Reproduction	Chromosome Count	Farina	DNA Sequence Data
A. pallens (Weath. ex R. M. Tryon) Windham					
A. pallens 1	MEXICO, Oaxaca, *Windham et al. 527* (DUKE)	•	$n = 27$[1]	W	√
A. pallens 2	MEXICO, Oaxaca, *Yatskievych & Gastony 89–280* (IND)	NA	NA	W	√
A. palmeri (Baker) Windham					
A. palmeri 1	MEXICO, Guanajuato, *Pray 3078* (RSA)	•	NA	W	√[†]
A. palmeri 2	MEXICO, Guanajuato, *Correll & Correll 28817* (MICH)	•	NA	W	√
A. peninsularis (Maxon & Weath.) Windham					
A. peninsularis 1	MEXICO, Baja California Sur, *de la Luz 9784* (MO)	•	NA	W	√
A. peninsularis 2	MEXICO, Baja California Sur, *Dominguez 2981* (MO)	•	NA	W	√
A. peninsularis 3	MEXICO, Baja California Sur, *de la Luz 9687* (MO)	•	NA	W	√
A. pilifera (R. M. Tryon) Windham	MEXICO, Morelos, *Yatskievych & Gastony 89–287* (MO)	•	NA	W	√
A. stuebeliana (Hieron.) Windham					
A. stuebeliana 1	PERU, Cajamarca, *Hutchison & Wright 5288* (US)	○	NA	W	√
A. stuebeliana 2	PERU, Amazonas, *Plowman 5551* (HUH)	○	NA	W	√

of -11,400.55077 (Table 3). While there were topological differences between the MP and ML trees from the combined dataset, none were well-supported (MLBS and MPBS < 70%). Here we use the best ML phylogram to show evolutionary relationships within *Argyrochosma* (Fig. 2).

The phylogeny of *Argyrochosma* has a well-supported basal dichotomy (Fig. 2), dividing the genus into a non-farinose Group 1 (MLBS = 99%, MPBS = 100%) comprising Clade A and a primarily farinose Group 2 (MLBS = 93%, MPBS = 91%) comprising Clades B, C, D, E, and F. Group 1 (= Clade A) is composed of four species, all of which lack farina. The single specimen of *A. formosa* is sister (MLBS and MPBS = 100%) to an equally well-supported clade comprising the three specimens of *A. microphylla*. The other two species form a moderately supported (MLBS = 82%, MPBS = 74%) subclade in which the only specimen of *A. lumholtzii* is sister to a well-supported (MLBS = 100%, MPBS = 99%) subclade composed of the three sampled *A. jonesii* specimens.

Within Group 2, the three main clades (B, C, and D) are each well-supported (MLBS = 100%, MPBS = 99–100%), but relationships among them are poorly resolved. Clade B is monospecific (including just the three specimens of *A. fendleri*) whereas Clade C is composed of six species. Three of the four species in Clade C represented by multiple specimens form strongly supported (MLBS = 100%, MPBS = 97–99%) groups, the only exception being *A. incana*. Although three of the four specimens identified as *A. incana* form a robust subclade (MLBS and MPBS = 100%), the geographically isolated specimen from the Dominican Republic (*A. incana* 4; Table 1) is moderately supported as sister to the only sample of *A. pilifera* (MLBS = 61%, MPBS = 82%). Although the species of Clade C are generally well-supported, relationships among them are poorly resolved by this dataset.

Clade D (MLBS = 100%, MPBS = 99%) comprises two subclades, designated Clades E and F. Clade E (MLBS = 92%, MPBS = 90%) unites three taxa, with the single specimen of *A. dealbata* moderately supported (MLBS = 77%, MPBS = 85%) as sister to the two subspecies of *A. limitanea*. Clade F

Table 2. Primers used for amplifying and sequencing *atpA*, *rbcL*, and *trnG-trnR* plastid regions. Published source of primer sequence: *Schuettpelz et al. 2006; [†]Schuettpelz and Pryer 2007; [‡]Nagalingum et al. 2007; [§]Beck et al. 2010. Primers in bold were developed specifically for this study.

	5′-primer sequence-3′
atpA	
ESATPA412F*	GARCARGTTCGACAGCAAGT
ESATPA535F*	ACAGCAGTAGCTACAGATAC
ESATPA557R*	ATTGTATCTGTAGCTACTGC
ESTRNR46F*	GTATAGGTTCRARTCCTATTGGACG
EMATPA505F	**GGGAAGATCGCCCAAATACCAG**
EMATPA1009F	**TATGACGAYCTTTCYAAACAAGC**
EMATPA102R	**GTCGGTAAGCCTGGGCTTGTTTRG**
rbcL	
ESRBCL1F[†]	ATGTCACCACAAACGGAGACTAAAGC
ESRBCL628F[†]	CCATTYATGCGTTGGAGAGATCG
ESRBCL654R[†]	GAARCGATCTCTCCAACGCAT
RBCL1361R[†]	TCAGGACTCCACTTACTAGCTTCACG
EMRBCL320F	**GATTTATTTGAGGAAGGTTCCG**
EMRBCL894R	**TGTCTATCRATRACRGCATGCAT**
trnG-trnR	
TRNG1F[‡]	GCGGGTATAGTTTAGTGGTAA
TRNG43F1[‡]	TGATGCGGGTTCGATTCCCG
TRNG63R[‡]	GCGGGAATCGAACCCGCATCA
TRNR22R[‡]	CTATCCATTAGACGATGGACG
TRNGJBF[§]	AGGAGCCGAATGGGCCGAAA

Table 3. Summary details for DNA sequence data (in base pairs) and tree statistics for each plastid region sequenced and for the combined analysis. Parsimony informative characters were calculated using an equally-weighted parsimony model. % missing data is the summed percentages of "?" and "-" in the data matrices.

	atpA	*rbcL*	*trnG-trnR*	Combined Analysis
Alignment length	1,853	1,309	1,267	4,429
Included characters	1,824	1,309	1,104	4,237
% missing data	7.23	14.41	13.07	5.59
Maximum Parsimony				
Parsimony informative characters	182	83	95	557
Consistency index	0.618	0.800	0.671	0.653
Retention index	0.887	0.945	0.892	0.889
Tree length (steps)	401	160	659	1252
No. of trees	4,691	8,304	9,990	9,800
Maximum Likelihood				
Best –ln*L* score	4,156.54738	2,928.77560	4,057.27134	11,400.55077

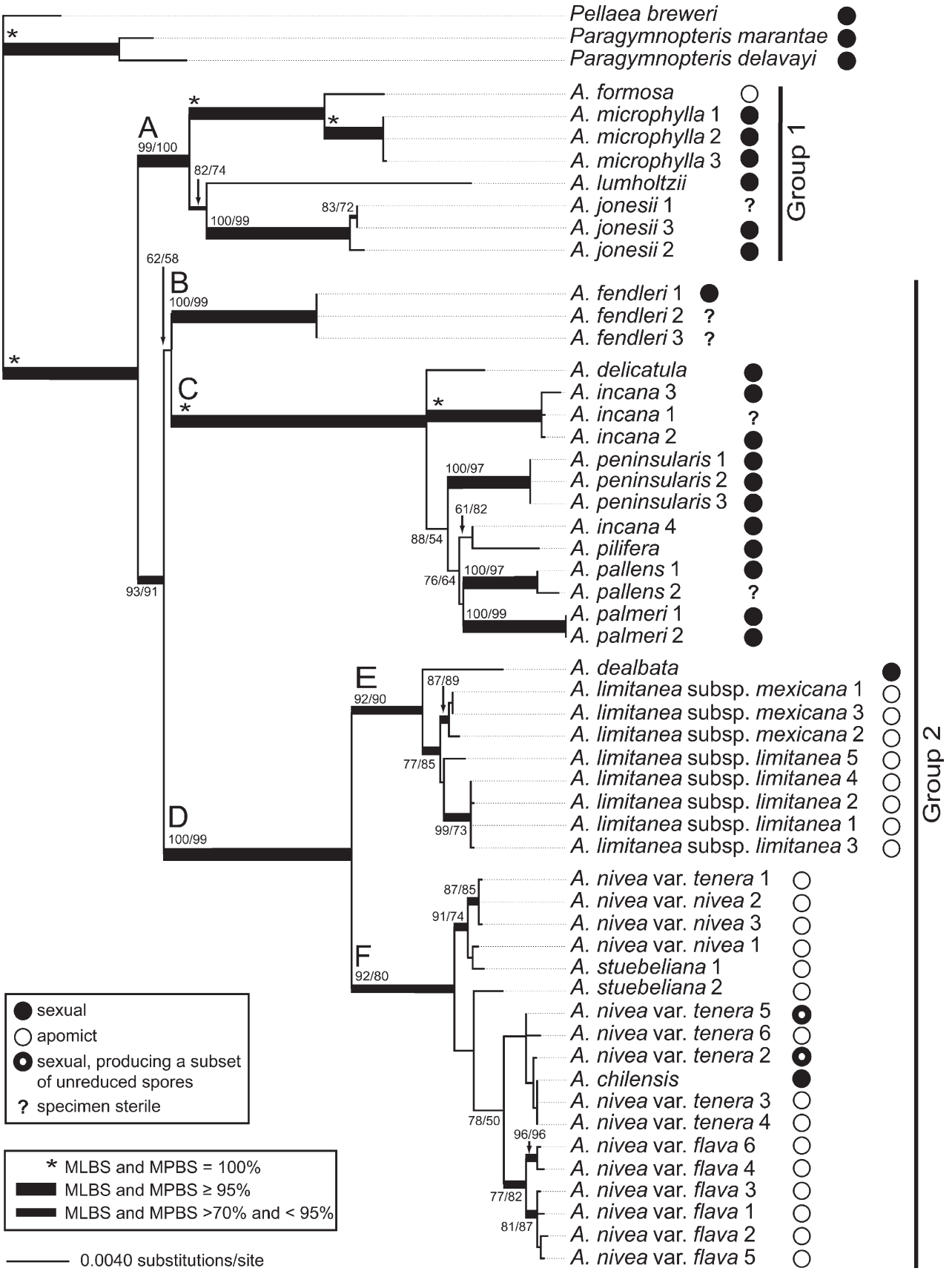

FIG. 2. The best ML phylogram from the combined analysis of *atpA*, *rbcL*, and *trnG-trnR* sequence data for 51 specimens of *Argyrochosma*, representing all currently recognized taxa (Windham 1987; Ponce 1996). The tree is rooted with *Paragymnopteris delavayi*, *P. marantae*, and *Pellaea breweri*. ML and MP bootstrap support values are given at nodes. Two groups and six major clades (A-F) are recognized in this study. Mode of reproduction is indicated for each fertile specimen (black circle = sexual, open circle = apomict, partially filled circle = sexual but producing a subset of unreduced spores, ? = sterile specimen; see Table 1). See Table 3 for tree statistics.

(MLBS = 92%, MPBS = 80%) comprises *A. chilensis*, *A. stuebeliana*, and the three varieties of *A. nivea*; relationships within this clade are only moderately to weakly supported.

Farina Evolution—A parsimony reconstruction of farina evolution in *Argyrochosma* was plotted on the best ML tree from the phylogenetic analysis (Fig. 2) and is shown in Fig. 3. Under a parsimony model, the absence of farina is the plesiomorphic state. In this reconstruction, a single origin of white farina occurred at the node uniting Clades B, C, and D, followed by two losses represented by samples *A. nivea* var. *tenera* 2 and *A. nivea* var. *tenera* 5. Yellow farina appears to be a synapomorphy uniting all specimens of *A. nivea* var. *flava*.

Mode of Reproduction and Ploidy—Figure 4 illustrates the mean spore length and standard deviations for 50 specimens; 14 of these are vouchers for meiotic (*n*) chromosome counts (Table 1). Spore measurements from sexual specimens with 64 spores per sporangium were consistently smaller than those from apomictic specimens with 32 or 16 spores per sporangium. Among the sexual specimens, two distinct and statistically different ($p < 0.0001$; Mann-Whitney *U* test) spore size groups were revealed (Fig. 4). Average spore length for the sexual specimens in Clade C (Fig. 2), with the exception of *A. incana* 4, is 40.85 µm. The mean spore length for all other sexual specimens is 49.49 µm.

Spore number per sporangium was consistent in 47 of the 50 specimens sampled. This is not surprising given the limited number of intact sporangia available for analysis. However, *A. nivea* var. *tenera* 2 and *A. nivea* var. *tenera* 5 produced both 64-spored and 32-spored sporangia, and *A. limitanea* subsp. *mexicana* 1 produced both 32-spored and 16-spored sporangia. Spores from the 16-spored sporangia were by far the largest observed in our study, with a mean spore length of 82.70 µm. Mean spore lengths, calibrated with ploidy level based on the 14 meiotic (*n*) chromosome vouchers (Table 1), confirm that 64-spored specimens are mostly sexual diploids, whereas 32-spored specimens are mostly apomictic triploids (Fig. 4). The 16-spored sporangia of *A. limitanea* subsp. *mexicana* 1 yielded a hexaploid chromosome count (Table 1).

Discussion

Phylogeny of Argyrochosma—This study provides a mostly well-resolved and robustly supported phylogeny of the New World cheilanthoid fern genus *Argyrochosma*. Based on the combined analysis of sequence data from three plastid regions (*rbcL*, *atpA*, and *trnG-trnR*), two major groups comprising six clades (Clades A-F) are resolved. Here we discuss inter- and intraspecific relationships within *Argyrochosma*, and compare our results to hypotheses proposed by previous researchers. Using our best estimate of the phylogeny, we examine the evolution of farina within *Argyrochosma*, a character of historical significance in the classification of cheilanthoid ferns.

Group 1—This group comprises a single clade (A), which unites the four non-farinose species of *Argyrochosma* (Figs. 2, 3). Three of the four species (*A. microphylla*, *A. lumholtzii*, and *A. jonesii*) have 64-spored sporangia and, hence, are inferred to reproduce sexually (Knobloch 1967; Table 1; Fig. 4). Spore

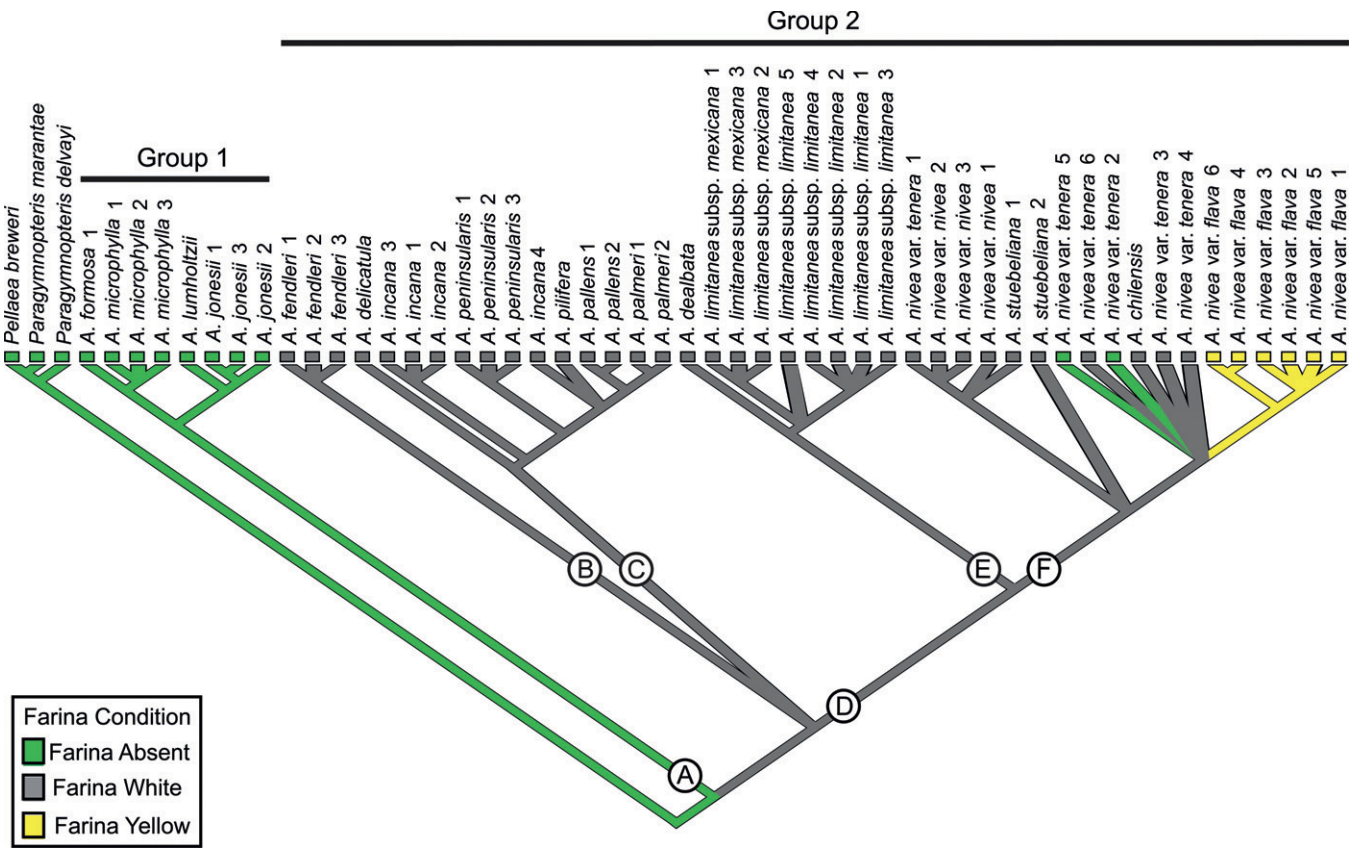

Fig. 3. Parsimony reconstruction of farina condition mapped on the best ML tree shown in Fig. 2, but where branches with low support (MLBS < 75%) were collapsed. Circled letters refer to Clades A-F in Fig. 2. Character state for each included specimen was determined using 10 × magnification. Branch color indicates farina condition (see box on figure).

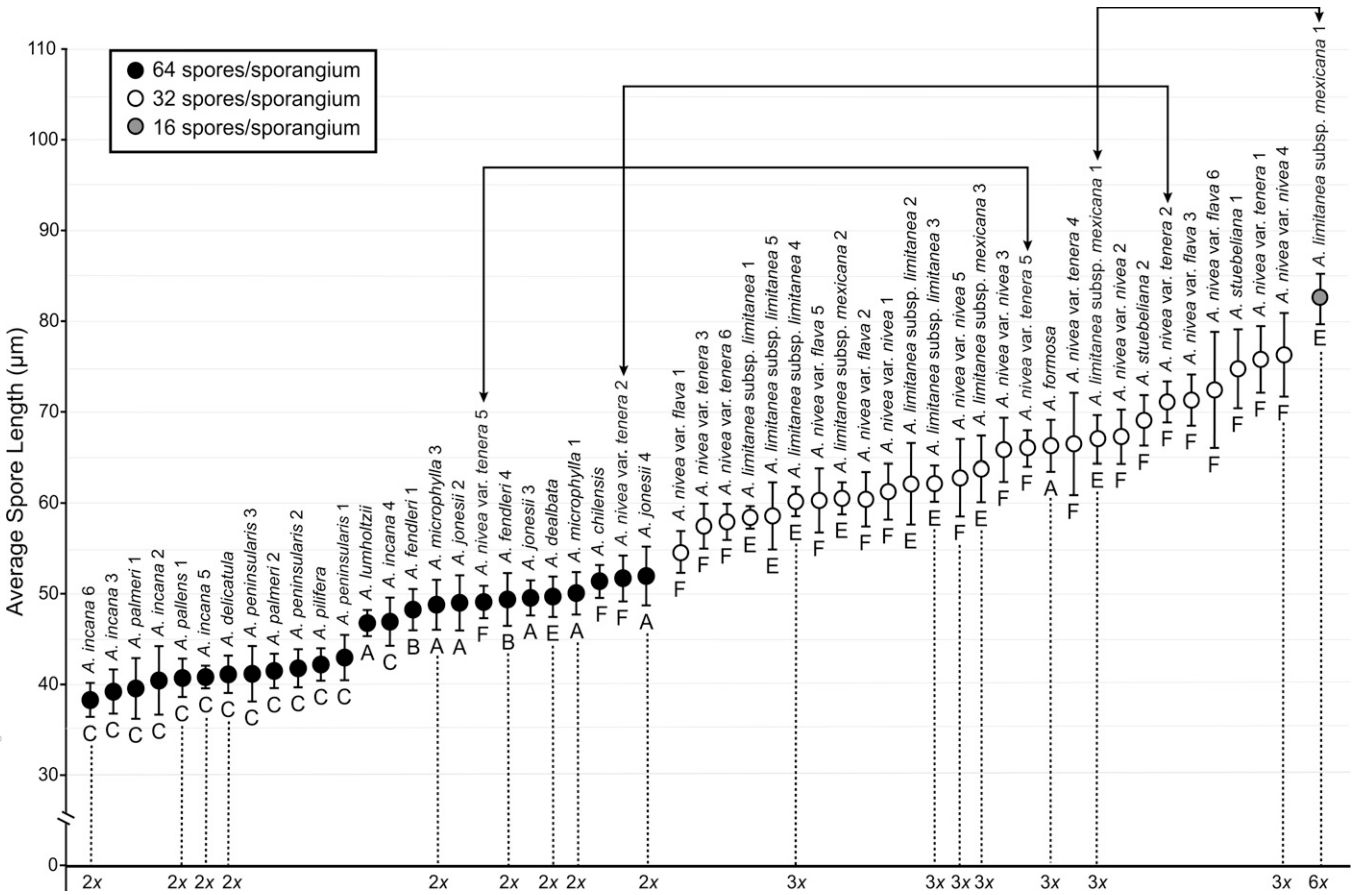

FIG. 4. Average spore lengths for specimens listed in Table 1; error bars indicate one standard deviation. Symbol shading depicts the number of spores per sporangium (see box on figure). Lines beginning and ending in arrows indicate specimens observed to have two sporangia types, each with a different number of spores per sporangium. Letters below each data point correspond to major clades indicated in Figs. 2 and 3. Ploidy is indicated for documented chromosome count vouchers listed in Table 1.

size data, in combination with meiotic chromosome counts for *A. jonesii* 4, *A. microphylla* 1, and *A. microphylla* 3, indicate that our samples of these taxa are uniformly diploid (Table 1; Fig. 4). *Argyrochosma formosa* previously has been reported to have 32 spores per sporangium (Tryon 1956), and this was confirmed for the only specimen of *A. formosa* included in our study. A published chromosome count ($n = 2n = 81$; Windham and Yatskievych 2003) for this specimen establishes this particular plant as an apomictic triploid.

A published gametophyte mitotic chromosome count of $n = 54$ (Table 1) led Windham and Yatskievych (2003) to infer that *A. jonesii* 3 was a sexual tetraploid individual. However, the spores of *A. jonesii* 3 analyzed for this study are similar in size to spores of known diploid sporophytes (Fig. 4). This apparent discrepancy may be explained by a phenomenon that occurs sporadically in many lineages of ferns. Occasionally, developing sporangia undergo mitotic nonreduction, whereby endomitosis (chromosome doubling without cytokinesis) occurs during the fourth mitotic division of the spore mother cells just prior to meiosis (Steil 1951). This leads to the production of 32 spores with a diploid complement of chromosomes. We hypothesize that *A. jonesii* 3 produced two types of sporangia: those with 64 haploid spores (common) and those with 32 diploid spores (rare). We suspect that the diploid gametophyte mitotic count reported by Windham and Yatskievych (2003) came from the latter type of sporangium, while our spore measurements represent the former. Although not directly observed in *A. jonesii* 3 (which had few intact sporangia), such bimodal spore production was observed in one specimen of *A. limitanea* subsp. *mexicana* and two specimens of *A. nivea* var. *tenera* (see discussion below in "Mode of Reproduction and Ploidy" section).

Within Group 1, two well-supported sister-species pairs are confirmed: *A. formosa* + *A. microphylla* and *A. lumholtzii* + *A. jonesii* (Fig. 2). The latter two species are similar morphologically (differing primarily in the color of the petioles and rachises), and most authors have assumed they are closely related (e.g. Tryon 1956; Mickel and Smith 2004). By contrast, *A. formosa* and *A. microphylla* are distinguished by a number of features considered important in cheilanthoid taxonomy, including shape of the rachises, texture of the rhizome scales, and shape of the ultimate leaf segments. Interestingly, *A. formosa* and A. *microphylla* show less overall sequence divergence than do *A. lumholtzii* and *A. jonesii* for the three combined plastid loci. This apparent discrepancy between morphology and molecules may provide some insight into the origin of *A. formosa*, an apomictic triploid whose sexual diploid progenitors have not been identified. Although it is possible that our sample of *A. formosa* represents an autopolyploid derived from an undetected diploid, the data suggest another possibility: that this polyploid taxon may have originated through interspecific hybridization. Under this allopolyploid scenario, the plastid genome, which is known to be maternally inherited in cheilanthoid ferns (Gastony and Yatskievych 1989), would be

derived from an *A. microphylla*-like progenitor, accounting for the unexpected sequence similarity to extant *A. microphylla*. The paternal genome, on the other hand, would be derived from an undiscovered diploid taxon morphologically more similar to polyploid *A. formosa*. It should be noted that either scenario presupposes the existence (now or in the past) of an undetected sexual diploid resembling polyploid *A. formosa*, suggesting that we have yet to fully sample the taxonomic diversity of *Argyrochosma*.

Group 2—This group comprises three well-supported clades (B, C, and D; Fig. 2), each of which is composed entirely (or primarily) of farinose taxa. Relationships among these clades are not well-supported (Fig. 2), resulting in a polytomy.

Clade B—This lineage comprises all three specimens of *A. fendleri* (Fig. 2), probably the most recognizable species of *Argyrochosma* because of the strongly angular (flexuose) branching patterns of the leaf rachises and costae (Tryon 1956; Windham 1993; Mickel and Smith 2004). Only one specimen included in our molecular analyses (*A. fendleri* 1) had the mature sporangia and spores needed to assess reproductive mode and ploidy level. This specimen had 64 spores per sporangium with an average spore length of 48 μm (Fig. 3). Similar counts and measurements were obtained from *A. fendleri* 4, a known sexual diploid not included in our molecular study (Table 1; Fig. 4).

Clade C—This clade includes six farinose species: *A. delicatula, A. incana, A. pallens, A. palmeri, A. peninsularis,* and *A. pilifera* (Figs. 2, 3). All samples in this clade had 64 spores per sporangium and are thus inferred to reproduce sexually (Fig. 2; Table 1). Although chromosome counts are available for just four of the 13 specimens sampled from this clade (Table 1), our comparison of spore sizes among fertile specimens implies that all but one of these are sexual diploids (Figs. 2, 4). With the exception of *A. incana* 4 (discussed below), mean spore lengths for taxa in Clade C are significantly smaller than those of other sexual diploids (Fig. 4), suggesting that a smaller average spore size may constitute a synapomorphy for the species of Clade C.

Three specimens of *A. incana* (1–3) collected from the southwestern U. S. A. and northern Mexico (Table 1) form a robustly supported clade (Fig. 2). However, one specimen (*A. incana* 4; Table 1) representing a disjunct population in the Dominican Republic forms a moderately supported clade with *A. pilifera*. While initially surprising, this result was not entirely unexpected. *Argyrochosma incana* has the broadest geographic distribution known for any species of *Argyrochosma* and displays wide morphological and chemical diversity (Wollenweber 1984; Windham 1993). This raises the possibility that *A. incana*, as traditionally defined, includes cryptic taxa that have been previously overlooked.

Spore length data (Fig. 4) tentatively support the notion that the Dominican population of *A. incana* may represent an undescribed taxon. Our one sampled specimen (*A. incana* 4) has an average spore length of 46.93 μm (Fig. 4), which is 7.22 μm (18%) longer than the average spore lengths of all other *A. incana* samples included in our analyses (Table 1). This approximates the expected increase in spore length associated with a doubling of chromosome number (Barrington et al. 1986), and suggests that the Dominican specimen is a sexual tetraploid. Although it is premature to reflect on the origin of this putative polyploid, its discovery has significant ramifications for estimates of taxonomic diversity in *Argyrochosma*. In this case, the increase in diversity extends beyond the discovery of a previously undetected, molecularly distinctive polyploid within *A. incana*. Regardless of whether the Dominican taxon arose through auto- or allopolyploidy, the combination of morphological, molecular, and geographic data point to the existence of another missing diploid. This (and likely other new taxa as well) would be easily detected through additional spore studies of the group.

The taxonomic status of *A. delicatula*, another member of Clade C, has been historically contentious. Citing considerable overlap in morphology and geography with *A. incana*, Tryon (1956) and Mickel and Smith (2004) suggested that *A. delicatula* perhaps should be treated as a geographic variant of that widespread species. On the basis of unpublished isozyme data, Windham (1987) argued that *A. delicatula* should be maintained as a separate species. Our phylogeny (Fig. 2) supports this latter view. Although the position of *A. delicatula* with regard to the other taxa in Clade C remains unresolved, it is clearly distinct from the strongly supported clade containing all sexual diploid specimens of *A. incana*. The remainder of Clade C consists of three well-supported subclades of *A. pallens*, *A. palmeri,* and *A. peninsularis* (Fig. 2), each with distinct geographic ranges and diagnostic morphological characters (Tryon 1956; Pray 1967; Windham 1993; Mickel and Smith 2004).

Clade D—This clade comprises five species and five subspecies divided between two well-supported subclades, E and F (Fig. 2). Although most of these taxa are strictly farinose, *A. nivea* var. *tenera*, is polymorphic for this trait (Table 1; Figs. 2, 3).

Clade E—Within Clade E, *A. dealbata* is moderately supported as sister to *A. limitanea*. The only specimen of *A. dealbata* included here is a known sexual diploid, with a published meiotic chromosome count of $n = 27$ (Windham and Yatskievych 2003; Table 1). Three of the eight specimens of *A. limitanea* have published chromosome counts of $n = 2n = 81$, indicating that they are apomictic triploids (Windham and Yatskievych 2003; Table 1). All eight specimens of *A. limitanea* included in our study produced 32 spores per sporangium, and correlations between spore size and ploidy level confirm that all are apomictic triploids (Fig. 4).

Previous authors have recognized two infraspecific taxa within *Argyrochosma limitanea*, treated as either varieties (Tryon 1956) or subspecies (Windham 1993; Mickel and Smith 2004). The geographic ranges of the two taxa overlap in parts of the southwestern U. S. A. and northern Mexico, and they can be difficult to distinguish in this region. In our phylogenetic analyses, the three specimens of subsp. *mexicana* form a monophyletic group with moderate support (Fig. 2), as do four of five specimens of subsp. *limitanea*. This result is congruent with earlier hypotheses (Windham 1993) that the two subspecies of *A. limitanea* had independent polyploid origins from one or more diploid progenitors. The unresolved placement of the specimen designated subsp. *limitanea* 5 (Fig. 2) raises the possibility of a third independent origin of *A. limitanea* s. l. Interrelationships among these polyploids have the potential to be complex and the situation cannot be fully resolved until the missing sexual diploid progenitor(s) of *A. limitanea* are found. As with *A. formosa* and *A. incana*, further spore studies of *A. limitanea* are certain to reveal additional taxonomic diversity, including undiscovered diploids critical to interpreting the evolution of the genus as a whole.

Clade F—This clade comprises all three South American species of *Argyrochosma* (Fig. 2; Table 1) and is well-supported

in our phylogenetic analyses. The apparent monophyly of this South American clade and its nested position within the tree suggest that these taxa may be descendents of a single colonizer from North or Central America. The taxonomy of this group has been contentious (Tryon 1956; Tryon and Stolze 1989), but most recent treatments recognize three species: the wide-ranging *A. nivea* and two rare endemics *A. chilensis* and *A. stuebeliana*. In addition, three infraspecific taxa currently are recognized within *A. nivea* (Fig. 1; Ponce 1996): a form with bright yellow farina (var. *flava*), one with white farina (var. *nivea*), and one without farina (var. *tenera*). Although additional sequence markers will be needed to fully resolve the relationships in Clade F, several intriguing patterns are present.

Among the three South American taxa traditionally treated as species, none is demonstrably monophyletic. *Argyrochosma chilensis*, endemic to the Juan Fernández Islands, is represented here by a single specimen and may ultimately prove to be monophyletic. However, it is clearly nested within *A. nivea* s. l. The two specimens identified as *A. stuebeliana* (a rare Peruvian endemic) are not closely related (Fig. 2). *Argyrochosma stuebeliana* 1 is nested within a well-supported clade with *A. nivea* var. *nivea*, whereas *A. stuebeliana* 2 appears sister to a clade containing specimens of *A. nivea* var. *tenera* and var. *flava*. Nonmonophyly of the South American taxa extends to the varietal level as well, with the specimen var. *tenera* 1 nested within *A. nivea* var. *nivea*. Thus, of the five *Argyrochosma* taxa traditionally recognized in South America, only *A. nivea* var. *flava*, with its unique, bright yellow farina, appears to be monophyletic (Fig. 2).

The lack of species monophyly observed among the taxa comprising Clade F can be partly explained by chromosome counts and spore data. The majority of specimens in this clade are apomictic polyploids that almost certainly were derived from sexual diploids. The morphological and genetic disparity observed among these polyploids suggests that several diploids were involved in past hybridization events, producing both autopolyploid and allopolyploid descendents. This is a common pattern in cheilanthoid ferns and has been well documented in the *Cheilanthes yavapensis* Reeves ex Windham complex (Grusz et al. 2009) and in the genus *Astrolepis* (Beck et al. 2010), both of which are closely related to *Argyrochosma*.

According to our phylogenetic reconstruction (Fig. 2), *A. nivea* var. *tenera* is paraphyletic, with the apomictic polyploid var. *tenera* 1 nested within the white farinose var. *nivea* clade. Although the herbarium voucher of var. *tenera* 1 is nonfarinose, scattered white farinose glands were observed on young sporophytes that were grown from spores (Table 1). Thus, we consider this specimen morphologically intermediate between var. *tenera* and var. *nivea* and hypothesize that it may have arisen through hybridization between diploid cytotypes of these taxa. Further examination of specimens identified as var. *tenera* revealed that only the putative sexual diploids (represented by var. *tenera* 2 and var. *tenera* 5; Figs. 2, 4) completely lack farina (Fig. 3; Table 1). The remaining specimens of var. *tenera* (3, 4, 6) are apomictic polyploids and have sparse white farina on the abaxial leaf surfaces. Although the latter specimens group with diploid var. *tenera* (as would be expected if this taxon were the maternal progenitor; Gastony and Yatskievych 1989), we hypothesize that these are probable hybrids between var. *tenera* and var. *nivea* as well.

The failure of *A. stuebeliana* specimens to form a monophyletic group (Fig. 2) may also reflect past hybridization events. Our phylogeny indicates that the apomictic polyploid designated *stuebeliana* 1 is nested within var. *nivea*. Closer examination of *stuebeliana* 1 reveals that it is morphologically intermediate (specifically in rhizome scale color and shape) between *A. nivea* var. *nivea* and *A. stuebeliana* 2. Thus, we hypothesize that *stuebeliana* 1 may have arisen through hybridization between diploid cytotypes of these two taxa. As with *A. formosa, A. incana*, and *A. limitanea*, it is necessary to hypothesize the existence of undetected sexual diploids to explain the evolutionary patterns observed in Clade F, and our understanding of this group would benefit greatly from a more thorough spore analysis.

Farina Evolution in Argyrochosma—The presence or absence of farina on abaxial leaf surfaces has been an important character used to circumscribe genera in the cheilanthoid ferns. This feature was pivotal in the decision by Tryon and Tryon (1982) to include *Argyrochosma* with other farinose cheilanthoids in *Notholaena*. Subsequent studies have demonstrated that the presence or absence of farina is a highly homoplastic character state (Gastony and Rollo 1995, 1998; Rothfels et al. 2008) that should not be the primary basis for defining genera. Indeed, we now know that *Argyrochosma* is a well supported monophyletic group that is sister to the non-farinose genus *Pellaea,* rather than the farinose genus *Notholaena* (Gastony and Rollo 1995, 1998; Kirkpatrick 2007; Schuettpelz et al. 2007; Rothfels et al. 2008). This *Argyrochosma* + *Pellaea* clade is, in turn, sister to the non-farinose myriopterid clade (Windham et al. 2009).

Here we have mapped and reconstructed farina evolution on our best ML phylogeny of *Agyrochosma* (Fig. 3). Our parsimony reconstruction indicates that the absence of farina is the plesiomorphic character state. Parsimony reconstructs a single transition to white farina in the common ancestor of Group 2 and a subsequent transition to yellow farina that occurred in the common ancestor of extant populations of *A. nivea* var. *flava.* It appears that the lack of farina in some specimens of *A. nivea* (considered to be the defining character of var. *tenera*) represents at least one evolutionary reversal within Clade F. The pattern observed here in *Argyrochosma* is similar to that documented among the notholaenid ferns (Rothfels et al. 2008), in which farina appears to have evolved once near the base of the clade, diversified into several color forms, and experienced two independent reversals (losses).

Our phylogenetic reconstruction also provides insights into the evolution of farina chemistry. *Argyrochosma fendleri,* with its distinct morphology, diverged from the other farinose species early in the evolution of the genus (Fig. 2, Clade B). According to Wollenweber (1989), its farina composition is equally distinctive, as it is dominated by a flavonoid aglycone (eriodictyol-7-methyl ether) rarely encountered in ferns. Wollenweber (1984) and Wollenweber and Schneider (2000) reported that *A. delicatula*, *A. incana*, *A. pallens*, *A. peninsularis, A. palmeri*, and *A. pilifera* have white farina in which the primary constituents are various terpenoid compounds (with trace levels of flavonoids). All of these species belong to Clade C (Figs. 2, 3), suggesting that this particular farina composition is a synapomorphy for that lineage. By contrast, most taxa in Clade D (i.e. *A. chilensis, A. dealbata, A. limitanea*, and *A. nivea* var. *nivea*) have white farina primarily composed of bibenzyl compounds, either notholaenic or isonotholaenic acid (Wollenweber et al. 1993). The unique yellow farina of *A. nivea* var. *flava* (Fig. 1) is dominated by 2′, 6′-dihydroxy-4′-methoxy chalcone and contains no (iso)notholaenic acid

(Wollenweber et al. 1993). This suggests a significant shift in farina biosynthesis that may represent a biochemical autapomorphy for var. *flava*.

Mode of Reproduction and Ploidy—Analyses of spore number per sporangium and average spore length allowed us to assess the mode of reproduction and ploidy for each taxon of *Argyrochosma* (Fig. 4). Twenty-five of the 50 specimens included in our spore study had mature, intact sporangia that produced 64 well-formed spores per sporangium, indicating that they reproduced sexually. The remainder had just 32 (or rarely 16) spores per sporangium, characteristic of an apomictic lifecycle. Among the 64-spored specimens, two statistically distinct spore size groups were revealed (Fig. 4). Both groups proved to be predominantly diploid (Table 1), indicating that the size differences do not reflect ploidy level. Instead, the two sexual spore size groups are strongly correlated with clades identified in our phylogenetic analysis. The small-spored group (mean spore length 40.85 μm) is composed entirely of species from Clade C, whereas specimens from all other clades fall in the large-spored group (mean spore length 49.49 μm). This suggests that a significant reduction in spore size may be a synapomorphy for Clade C. The only exception to this pattern is *A. incana* 4, a member of Clade C that falls in the large-spored group (Fig. 4). As discussed earlier, we hypothesize that this specimen represents an undescribed, sexual tetraploid, and the larger mean spore length is congruent with the expected increase in spore size associated with a doubling of chromosome number (Barrington et al. 1986).

We have shown that spore data, including number of spores per sporangium and mean spore length, are critical to understanding species diversification and evolution in *Argyrochosma*. These data correlate with reproductive mode and ploidy level, respectively, and are generally consistent across clades. However, in three of the 50 specimens with mature, intact sporangia, spore number per sporangium proved variable. *Argyrochosma nivea* var. *tenera* 2 and *A. nivea* var. *tenera* 5 produced both 64-spored and 32-spored sporangia, and *A. limitanea* subsp. *mexicana* 1 produced both 32-spored and 16-spored sporangia. These three examples highlight the importance of verifying spore number per sporangium for any particular specimen when attempting to use spore size as an indicator of ploidy level. The specimen designated *A. limitanea* subsp. *mexicana* 1 was previously reported to be an apomictic triploid with 32 spores per sporangium and a chromosome number of $n = 2n = 81$ (Windham and Yatskievych 2003). Although true of the majority of sporangia on the specimen, additional spore counts performed during this study revealed that a small proportion of sporangia on this specimen produced just 16 spores (Fig. 4). Chromosome preparations from these sporangia yielded counts of $n = 2n = 162$, double the chromosome number observed in the 32-spored sporangia (Table 1).

Our studies suggest that the 16-spored sporangia of this specimen underwent not one, but two rounds of endomitosis just prior to meiosis. This resulted in the production of even larger, unreduced spores with double the normal chromosome number of the triploid apomict. Spores produced by the 32- and 16-spored sporangia of *A. limitanea* subsp. *mexicana* 1 show no overlap in length, their means are separated by ~15 μm, and there is a 10 μm gap between their standard deviations (Fig. 4). Such variation in spore length and spore number per sporangium appears to be a common but understudied phenomenon in ferns (e.g. Windham 1983) and has important implications for correlating spore size with ploidy level. In the case of *A. limitanea* subsp. *mexicana* 1, the average spore length calculated for this specimen would vary significantly depending on the proportion of 32- and 16-spored sporangia sampled. Only by verifying a consistent spore number per sporangium for a particular specimen is it possible to eliminate this potential source of confusion and effectively use spore size as a proxy for ploidy level in studying fern evolution.

Acknowledgments. We thank the herbarium curators and staff at DUKE, F, HUH, IND, MICH, MO, NY, RSA, UC, US, UT for their generosity in lending their collections and for allowing destructive sampling for our DNA and spore studies. We are grateful to J. Beck, A. Grusz, C. Rothfels, E. Schuettpelz, and two anonymous reviewers for their comments, edits, and criticism. E. M. S. gratefully acknowledges F. Lutzoni and D. Swofford for their 2008 Systematic Biology course, which was the starting point for this project. This work was funded in part by NSF awards to K. M. P., M. D. W., and G. Y. (DEB-0717398 and DEB-0717430).

Literature Cited

Abramoff, M. D., P. J. Magelhaes, and S. J. Ram. 2004. Image processing with ImageJ. *Biophotonics International* 7: 36–42.

Barrington, D. S., C. A. Paris, and T. A. Ranker. 1986. Systematic inferences from spore and stomate size in the ferns. *American Fern Journal* 76: 149–159.

Beck, J. B., M. D. Windham, G. Yatskievych, and K. M. Pryer. 2010. A diploids-first approach to species delimitation and interpreting polyploid evolution in the fern genus *Astrolepis* (Pteridaceae). *Systematic Botany* 35: 223–234.

Gastony, G. J. and D. R. Rollo. 1995. Phylogeny and generic circumscriptions of cheilanthoid ferns (Pteridaceae: Cheilanthoideae) inferred from *rbcL* nucleotide sequences. *American Fern Journal* 85: 341–360.

Gastony, G. J. and D. R. Rollo. 1998. Cheilanthoid ferns (Pteridaceae: Cheilanthoideae) in the southwestern United States and adjacent Mexico—a molecular phylogenetic reassessment of generic lines. *Aliso* 17: 131–144.

Gastony, G. J. and M. D. Windham. 1989. Species concepts in pteridophytes: the treatment and definition of agamosporous species. *American Fern Journal* 79: 65–77.

Gastony, G. J. and G. Yatskievych. 1989. Maternal inheritance of the chloroplast and mitochondrial genomes in cheilanthoid ferns. *American Journal of Botany* 79: 716–722.

Grusz, A. L., M. D. Windham, and K. M. Pryer. 2009. Deciphering the origins of apomictic polyploids in the *Cheilanthes yavapensis* complex (Pteridaceae). *American Journal of Botany* 96: 1636–1645.

Guo, Q., M. Kato, and R. E. Ricklefs. 2003. Life history, diversity and distribution: a study of Japanese pteridophytes. *Ecography* 26: 129–138.

Hevly, R. H. 1963. Adaptations of cheilanthoid ferns to desert environments. *Journal of the Arizona Academy of Science* 2: 164–175.

Kirkpatrick, R. E. B. 2007. Investigating the monophyly of *Pellaea* (Pteridaceae) in the context of a phylogenetic analysis of cheilanthoid ferns. *Systematic Botany* 32: 504–518.

Knobloch, I. W. 1967. Chromosome numbers in *Cheilanthes, Notholaena, Llavea* and *Polypodium*. *American Journal of Botany* 54: 461–464.

Maddison, W. P. 1993. Missing data versus missing characters in phylogenetic analysis. *Systematic Biology* 42: 576–581.

Maddison, D. R. and W. P. Maddison. 2005. McClade 4: Analysis of phylogeny and character evolution. v. 4.08. Sunderland: Sinauer Associates.

Maddison, W. P. and D. R. Maddison. 2008. Mesquite: a modular system for evolutionary analysis. v. 2.5. http://mesquiteproject.org.

Manton, I. 1950. *Problems of cytology and evolution in the Pteridophyta*. Cambridge: Cambridge University Press.

Mason-Gamer, R. J. and E. A. Kellogg. 1996. Testing for phylogenetic conflict among molecular datasets in the tribe Triticeae (Gramineae). *Systematic Biology* 45: 524–545.

Mickel, J. T. and A. R. Smith. 2004. *The Pteridophytes of Mexico*. New York: The New York Botanical Garden Press.

Morton, C. V. 1950. Notes on the ferns of the eastern United States (concluded). *American Fern Journal* 40: 241–252.

Nagalingum, N. S., H. Schneider, and K. M. Pryer. 2007. Molecular phylogenetic relationships and morphological evolution in the heterosporous fern genus *Marsilea*. *Systematic Botany* 32: 16–25.

Ponce, M. M. 1996. Nuevas combinaciones en *Argyrochosma* (Pteridaceae). *Hickenia* 2: 177–178.

Pray, T. R. 1967. Notes on the distribution of some American cheilanthoid ferns. *American Fern Journal* 57: 52–58.

Pryer, K. M. and D. M. Britton. 1983. Spore studies in the genus *Gymnocarpium*. *Canadian Journal of Botany* 61: 377–388.

Rothfels, C. J., M. D. Windham, A. L. Grusz, G. J. Gastony, and K. M. Pryer. 2008. Towards a monophyletic *Notholaena* (Pteridaceae): resolving patterns of evolutionary convergence in xeric-adapted ferns. *Taxon* 57: 712–724.

SAS Institute Inc. 2007. *JMP user's guide*. Cary, North Carolina: SAS Institute Inc.

Schuettpelz, E. and K. M. Pryer. 2007. Fern phylogeny inferred from 400 leptosporangiate species and three plastid genes. *Taxon* 56: 1037–1050.

Schuettpelz, E., P. Korall, and K. M. Pryer. 2006. Plastid *atpA* data provide improved support for deep relationships among ferns. *Taxon* 55: 897–906.

Schuettpelz, E., H. Schneider, L. Huiet, M. D. Windham, and K. M. Pryer. 2007. A molecular phylogeny of the fern family Pteridaceae: assessing overall relationships and the affinities of previously unsampled genera. *Molecular Phylogenetics and Evolution* 44: 1172–1185.

Steil, W. N. 1951. Apogamy, apospory and parthenogenesis in the pteridophytes II. *Botanical Review* 17: 90–104.

Swofford, D. L. 2002. PAUP* Phylogenetic analysis using parsimony (*and other methods), v. 4.0 beta 10. Sunderland: Sinauer Associates.

Tryon, A. F. and D. M. Britton. 1958. Cytotaxonomic studies on the fern genus *Pellaea*. *Evolution* 12: 137–145.

Tryon, R. M. 1956. A revision of the American species of *Notholaena*. *Contributions from the Gray Herbarium of Harvard University* 179: 1–106.

Tryon, R. M. and R. G. Stolze. 1989. Pteridophyta of Peru. Part II. 13. Pteridaceae – 15. Dennstaedtiaceae. *Fieldiana*: 1–128.

Tryon, R. M. and A. F. Tryon. 1973. Geography, spores, and evolutionary relations in the cheilanthoid ferns. Pp. 45–153 in *The phylogeny and classification of ferns*. eds. A. C. Jermy, J. A. Crabbe, and B. A. Thomas. New York: Academic Press.

Tryon, R. M. and A. F. Tryon. 1982. *Ferns and allied plants with special reference to tropical America*. New York: Springer.

Tryon, R. M., A. F. Tryon, and K. U. Kramer. 1990. Pteridaceae. Pp. 404 in *The families and genera of vascular plants*, vol. 1, *Pteridophytes and Gymnosperms*. eds. K. U. Kramer and P. S. Green. Berlin: Springer.

Wagner, W. H. 1974. Structure of spores in relation to fern phylogeny. *Annals of the Missouri Botanical Garden* 61: 332–353.

Windham, M. D. 1983. The ferns of Elden Mountain, Arizona. *American Fern Journal* 73: 85–93.

Windham, M. D. 1987. *Argyrochosma*, a new genus of cheilanthoid ferns. *American Fern Journal* 77: 37–41.

Windham, M. D. 1993. *Argyrochosma*. Pp. 171–175 in *Flora of North America*, vol. 2, *Pteridophytes and Gymnosperms*, ed. Flora of North America Editorial Committee. New York: Oxford University Press.

Windham, M. D. and G. Yatskievych. 2003. Chromosome studies of cheilanthoid ferns (Pteridaceae: Cheilanthoideae) from the western United States and Mexico. *American Journal of Botany* 90: 1788–1800.

Windham, M. D., L. Huiet, E. Schuettpelz, A. L. Grusz, C. Rothfels, J. Beck, G. Yatskievych, and K. M. Pryer. 2009. Using plastid and nuclear DNA sequences to redraw generic boundaries and demystify species complexes in cheilanthoid ferns. *American Fern Journal* 99: 128–132.

Wollenweber, E. 1984. Exudate flavonoids of Mexican ferns as chemotaxonomic markers. *Revista Latinoamericana de Química* 15: 3–11.

Wollenweber, E. 1989. Exudate flavonoids in ferns and their chemosystematic implication. *Biochemical Systematics and Ecology* 17: 141–144.

Wollenweber, E. and H. Schneider. 2000. Lipophilic exudates of Pteridaceae - chemistry and chemotaxonomy. *Biochemical Systematics and Ecology* 28: 751–777.

Wollenweber, E., M. Doerr, H. Waton, and J. Favre-Bonvin. 1993. Flavonoid aglycones and a dihydrostilbene from the frond exudate of *Notholaena nivea*. *Phytochemistry* 33: 611–612.

Zwickl, D. J. 2006. *GARLI, vers. 0.951. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion*. Ph. D. dissertation. Austin: The University of Texas.

Appendix 1. Sample information. Taxon specimen number: Pryer Lab Fern DNA Database number (see http://www.pryerlab.net/DNA_database.shtml); average spore length (µm) ± one standard deviation (µm) (number of spores per sporangia); *atpA*: GenBank accession; *rbcL*: GenBank accession; *trnG-trnR*: GenBank accession. Specimen numbers are not given for taxa represented by only one specimen. NA = data not available. Nomenclatural authorities and voucher information are given for outgroup taxa; nomenclatural authorities and voucher information of ingroup taxa are provided in Table 1.

Argyrochosma chilensis: DB6385; 51.46 ± 1.81 (64); HQ846411; HQ846459; NA. ***Argyrochosma dealbata***: DB4562; 49.76 ± 2.23 (64); HQ846372; HQ846421; HQ846468. ***Argyrochosma delicatula***: DB4561; 41.15 ± 2.06 (64); HQ846371; HQ846420; HQ846469. ***Argyrochosma fendleri* 1**: DB3776; 48.31 ± 2.28 (64); HQ846363; HQ846413; FN565504. ***Argyrochosma fendleri* 2**: DB5854; NA; HQ846382; HQ846431; HQ846470. ***Argyrochosma fendleri* 3**: DB6238; NA; HQ846398; HQ846447; HQ846505. ***Argyrochosma fendleri* 4**: DB7202; 49.45 ± 2.91 (64); NA; NA; NA. ***Argyrochosma formosa***: DB4560; 66.49 ± 2.84 (32); HQ846370; HQ846419; HQ846471. ***Argyrochosma incana* 1**: DB3194; NA; HQ846361 ; HQ846412; HQ846507. ***Argyrochosma incana* 2**: DB3198; 40.46 ± 3.78 (64); HQ846362; EU268771; HQ846472. ***Argyrochosma incana* 3**: DB6243; 39.25 ± 2.43 (64); HQ846403; HQ846452; HQ846502. ***Argyrochosma incana* 4**: DB6151; 46.93 ± 2.66 (64); HQ846387; HQ846436; HQ846486. ***Argyrochosma incana* 5**: DB6232; 40.85 ± 1.25 (64); NA; NA; NA. ***Argyrochosma incana* 6**: DB7203; 38.3 ± 1.87 (64); NA; NA; NA. ***Argyrochosma jonesii* 1**: DB3839; HQ846364; HQ846414; HQ846496. ***Argyrochosma jonesii* 2**: DB3844; 49.09 ± 3.04 (64); HQ846365; EU268772; HQ846473. ***Argyrochosma jonesii* 3**: DB6328; 49.61 ± 1.94 (64); HQ846405; HQ846454; NA. ***Argyrochosma jonesii* 4**: DB7201; 52.07 ± 3.24 (64); NA; NA; NA. ***Argyrochosma limitanea* subsp. *limitanea* 1**: DB3179; 58.50 ± 1.24 (32); EU268722; EF452139; EU268668. ***Argyrochosma limitanea* subsp. *limitanea* 2**: DB5411; 62.26 ± 4.47 (32); HQ846381; HQ846430; HQ846487. ***Argyrochosma limitanea* subsp. *limitanea* 3**: DB6329; 62.31 ± 2.00 (32); HQ846406; HQ846455; HQ846462. ***Argyrochosma limitanea* subsp. *limitanea* 4**: DB6330; 60.30 ± 1.62 (32); HQ846407; HQ846456; HQ846461. ***Argyrochosma limitanea* subsp. *limitanea* 5**: DB6245; 58.72 ± 3.70 (32); HQ846404; HQ846453; HQ846501. ***Argyrochosma limitanea* subsp. *mexicana* 1**: DB4558; 82.69 ± 2.78 (16) & 67.15 ± 2.66 (32); HQ846368; HQ846417; HQ846466. ***Argyrochosma limitanea* subsp. *mexicana* 2**: DB4559; 60.56 ± 1.77 (32); HQ846369; HQ846418; HQ846460. ***Argyrochosma limitanea* subsp. *mexicana* 3**: DB5390; 63.92 ± 3.65 (32); HQ846380; HQ846429; HQ846474. ***Argyrochosma lumholtzii***: DB4974; 46.83 ± 1.44 (64); HQ846375; HQ846424; HQ846475. ***Argyrochosma microphylla* 1**: DB4557; 50.11 ± 2.34 (64); HQ846367; HQ846416; HQ846498. ***Argyrochosma microphylla* 2**: DB4583; NA; HQ846374; HQ846423; HQ846476. ***Argyrochosma microphylla* 3**: DB6228; 48.81 ± 2.75 (64); HQ846394; HQ846443; HQ846506. ***Argyrochosma nivea* var. *flava* 1**: DB5856; 54.72 ± 2.30 (32); HQ846384; HQ846433; HQ846477. ***Argyrochosma nivea* var. *flava* 2**: DB6227; 60.57 ± 3.00 (32); HQ846393; HQ846442; HQ846492. ***Argyrochosma nivea* var. *flava* 3**: DB6239; 60.43 ± 3.53 (32); HQ846399; HQ846448; HQ846500. ***Argyrochosma nivea* var. *flava* 4**: DB6241; NA; HQ846401; HQ846450; HQ846504. ***Argyrochosma nivea* var. *flava* 5**: DB6149; 60.43 ± 3.53 (32); HQ846385; HQ846434; HQ846499. ***Argyrochosma nivea* var. *flava* 6**: DB6150; 72.64 ± 6.40 (32); HQ846386; HQ846435; HQ846488. ***Argyrochosma nivea* var. *nivea* 1**: DB4975; 61.44 ± 3.08 (32); HQ846376; HQ846425; HQ846478. ***Argyrochosma nivea* var. *nivea* 2**: DB6236; 67.48 ± 2.97 (32); HQ846396; HQ846445; HQ846493. ***Argyrochosma nivea* var. *nivea* 3**: DB6240; 66.02 ± 3.52 (32); HQ846400; HQ846449; HQ846495. ***Argyrochosma nivea* var. *nivea* 4**: DB7026; 76.50 ± 4.61 (32); NA; NA; NA. ***Argyrochosma nivea* var. *nivea* 5**: DB7027; 63.02 ± 4.25 (32); NA; NA; NA. ***Argyrochosma nivea* var. *tenera* 1**: DB5335; 76.00 ± 3.67 (32); HQ846379; HQ846428; HQ846479. ***Argyrochosma nivea* var. *tenera* 2**: DB5855; 71.29 ± 2.26 (32) & 51.59 ± 2.53 (64); HQ846383; HQ846432; HQ846480. ***Argyrochosma nivea* var. *tenera* 3**: DB6152; 57.58 ± 2.49 (32); HQ846388; HQ846437; Q846489. ***Argyrochosma nivea* var. *tenera* 4**: DB6226; 66.67 ± 5.61 (32); HQ846392; HQ846441; HQ846491. ***Argyrochosma nivea* var. *tenera* 5**: DB6231; 66.12 ± 1.99 (32); & 49.13 ± 1.79 (64); HQ846395; HQ846444; HQ846483. ***Argyrochosma nivea* var. *tenera* 6**: DB6237; 58.08 ± 1.99 (64); HQ846397; HQ846446; HQ846494. ***Argyrochosma pallens* 1**: DB4556; 40.77 ± 2.11 (64); HQ846366; HQ846415; HQ846497. ***Argyrochosma pallens* 2**: DB5054; NA; HQ846377; HQ846426; HQ846481. ***Argyrochosma palmeri* 1**: DB6383; 39.62 ± 3.34 (64); HQ846409; NA; HQ846464. ***Argyrochosma palmeri* 2**: DB6384; 41.53 ± 1.89 (64); HQ846410; HQ846458; HQ846463. ***Argyrochosma peninsularis* 1**: DB6225; 43.01 ± 2.50 (64); HQ846391; HQ846440; HQ846490. ***Argyrochosma peninsularis* 2**: DB6331; 41.77 ± 2.10 (64); HQ846408; HQ846457; HQ846465. ***Argyrochosma peninsularis* 3**: DB6242; 41.21 ± 3.06 (64); HQ846402; HQ846451; HQ846503. ***Argyrochosma pilifera* 1**: DB5055; 42.25 ± 1.79 (64); HQ846378; HQ846427; HQ846482. ***Argyrochosma stuebeliana* 1**: DB6153; 74.95 ± 4.35 (32); HQ846389; HQ846438; HQ846485. ***Argyrochosma stuebeliana* 2**: DB6154; 69.25 ± 2.78 (32); HQ846390; HQ846439; HQ846484. ***Paragymnopteris delavayi* (Baker) K. H. Shing** (CHINA, Yunnan, *Yatskievych 04-90* (MO)): DB4565; NA; HQ846373; HQ846422; HQ846467. ***Paragymnopteris marantae* (L.) K. H. Shing** (CHINA, Yunnan, *Yatskievych 02-35* (MO)): DB3736; NA; EU268763; EF452161; EU268711. ***Pellaea breweri* D. C. Eaton** (U. S. A., Utah, *Windham 3447* (DUKE)): DB3930; NA; EU268764; EU268808; EU26871.