*Systematic Botany* (2010), 35(2): pp. 223–234


# A Diploids-First Approach to Species Delimitation and Interpreting Polyploid Evolution in the Fern Genus *Astrolepis* (Pteridaceae)

**James B. Beck,[1,3] Michael D. Windham,[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 (james.beck@duke.edu)

*Communicating Editor: Javier Francisco-Ortega*

***Abstract*—**Polyploidy presents a challenge to those wishing to delimit the species within a group and reconstruct the phylogenetic relationships among these taxa. A clear understanding of the tree-like relationships among the diploid species can provide a framework upon which to reconstruct the reticulate events that gave rise to the polyploid lineages. In this study we apply this "diploids-first" strategy to the fern genus *Astrolepis* (Pteridaceae). Diploids are identified using the number of spores per sporangium and spore size. Analyses of plastid and low-copy nuclear sequence data provide well-supported estimates of phylogenetic relationships, including strong evidence for two morphologically distinctive diploid lineages not recognized in recent treatments. One of these corresponds to the type of *Notholaena deltoidea*, a species that has not been recognized in any modern treatment of *Astrolepis*. This species is resurrected here as the new combination ***Astrolepis deltoidea***. The second novel lineage is that of a diploid initially hypothesized to exist by molecular and morphological characteristics of several established *Astrolepis* allopolyploids. This previously missing diploid species is described here as ***Astrolepis obscura***.

***Keywords*—***Astrolepis*, "diploids-first," *gapCp*, missing diploid, polyploidy, *trnG-trnR*.

Examples of mixed-ploidy species complexes are found in every major lineage of vascular plants, including the lycophytes (Kim et al. 2009), ferns (Grusz et al. 2009), gymnosperms (Ickert-Bond and Wojciechowski 2004), monocots (Fortune et al. 2008), and eudicots (Doyle et al. 2004). Polyploidy adds considerable complexity to phylogeny reconstruction in these groups, particularly in lineages derived through interspecific hybridization (allopolyploids). Even if the constituent genomes of allopolyploids can be successfully identified, the reticulate phylogenetic patterns in a mixed-ploidy group depart from the typical bifurcating patterns produced by cladogenesis (Cronquist 1987; McDade 1990). The common view of evolution in these situations (Wagner 1970) is of a diploid common ancestor giving rise to multiple diploid descendant lineages via cladogenesis, followed by reticulation among these diploids to produce allopolyploid lineages. This can be followed by further reticulation in the form of allopolyploids backcrossing to one parent lineage (Xiang et al. 2000), hybridizing with another, more distantly related diploid (Haufler et al. 1995), or undergoing cladogenesis (Werth and Windham 1991). To deal with this complexity, some authors have promoted focusing on the diploid species within a genus before addressing the polyploid lineages (Brown et al. 2002; Windham and Al-Shehbaz 2006). They reason that if the circumscription of diploids and the tree-like relationships among them are known, then both the reticulate origins of allopolyploids and their secondary reticulate and/or bifurcating relationships can be more accurately reconstructed.

Here we apply this "diploids-first" approach to the fern genus *Astrolepis* Benham & Windham (Pteridaceae), a group initially circumscribed (Benham and Windham 1992) to include three extant diploid taxa and an array of auto- and allopolyploid lineages. This group of xeric-adapted ferns has a range centered in the southwestern U. S. A. (Arizona, New Mexico, Texas), Mexico, and Guatemala, although some of the apomictic lineages can be found in the southeastern U. S. A. (Alabama, Georgia), the Caribbean, and South America. *Astrolepis* species were historically treated as a single morphologically variable taxon, *Notholaena sinuata* (Lag. ex Sw.) Kaulf., comprising a number of varieties (Weatherby 1943; Tryon 1956). Hevly (1965) recognized several of these varieties as species, and hypothesized that one of them, *Notholaena integerrima* (Hook.) Hevly, was a hybrid derived from two divergent diploids. Benham (1989) conducted the first comprehensive biosystematic study of the group, utilizing evidence from macro- and micromorphology, cytology, and isozymes. Based on that study, Benham and Windham (1992) proposed the generic name *Astrolepis* for the group after identifying several synapomorphies—most notably a base chromosome number of $x = 29$ and two vascular bundles in the petiole that clearly distinguish *Astrolepis* from hypothesized relatives in *Notholaena* R. Br. or *Cheilanthes* Sw.

In addition to confirming the existence of at least three sexual diploids in *Astrolepis*, Benham and Windham (1992) discovered a complex assortment of auto- and allopolyploids (Fig. 1). Their results also indicated that each of the three diploid ($2n = 58$) taxa; *Astrolepis sinuata* (Lag. ex Sw.) D. M. Benham & Windham subsp. *mexicana* D. M. Benham, *Astrolepis cochisensis* (Goodd.) D. M. Benham & Windham subsp. *chihuahuensis* D. M. Benham, and *Astrolepis laevis* (M. Martens & Galeotti) Mickel (as *A. beitelii* Mickel)—had produced apomictic autotriploids ($n = 2n = 87$) and in the case of *A. cochisensis*, an apomictic autotetraploid ($n = 2n = 116$). Although macromorphologically similar to their respective diploid progenitors, these autopolyploids could be easily distinguished from the sexual diploids by having 32 (rather than 64) spores per sporangium and significantly larger average spore diameters. Citing these distinctions as well as the biological significance of the rare diploid populations, Benham (1992) recognized the different ploidy levels within each species as subspecies.

Each of the diploid species of *Astrolepis* sampled by Benham (1989) exhibited a distinctive isozyme profile. Each of the autopolyploids mentioned above contained marker alleles derived from a single sexual diploid species. Other individuals combined alleles characteristic of morphologically divergent diploids and were considered allopolyploids (Fig. 1). Notably, each of the allopolyploid taxa contained alleles derived from one (or occasionally two) of the known diploids, plus distinctive "orphan" alleles not observed in any of the sampled diploids. These orphan alleles implied the existence of a missing diploid taxon, an increasingly common hypothesis in detailed studies of allopolyploid taxa (Werth and Lellinger 1992; Hoot et al. 2004; Windham and Yatskievych 2005; Holloway et al.

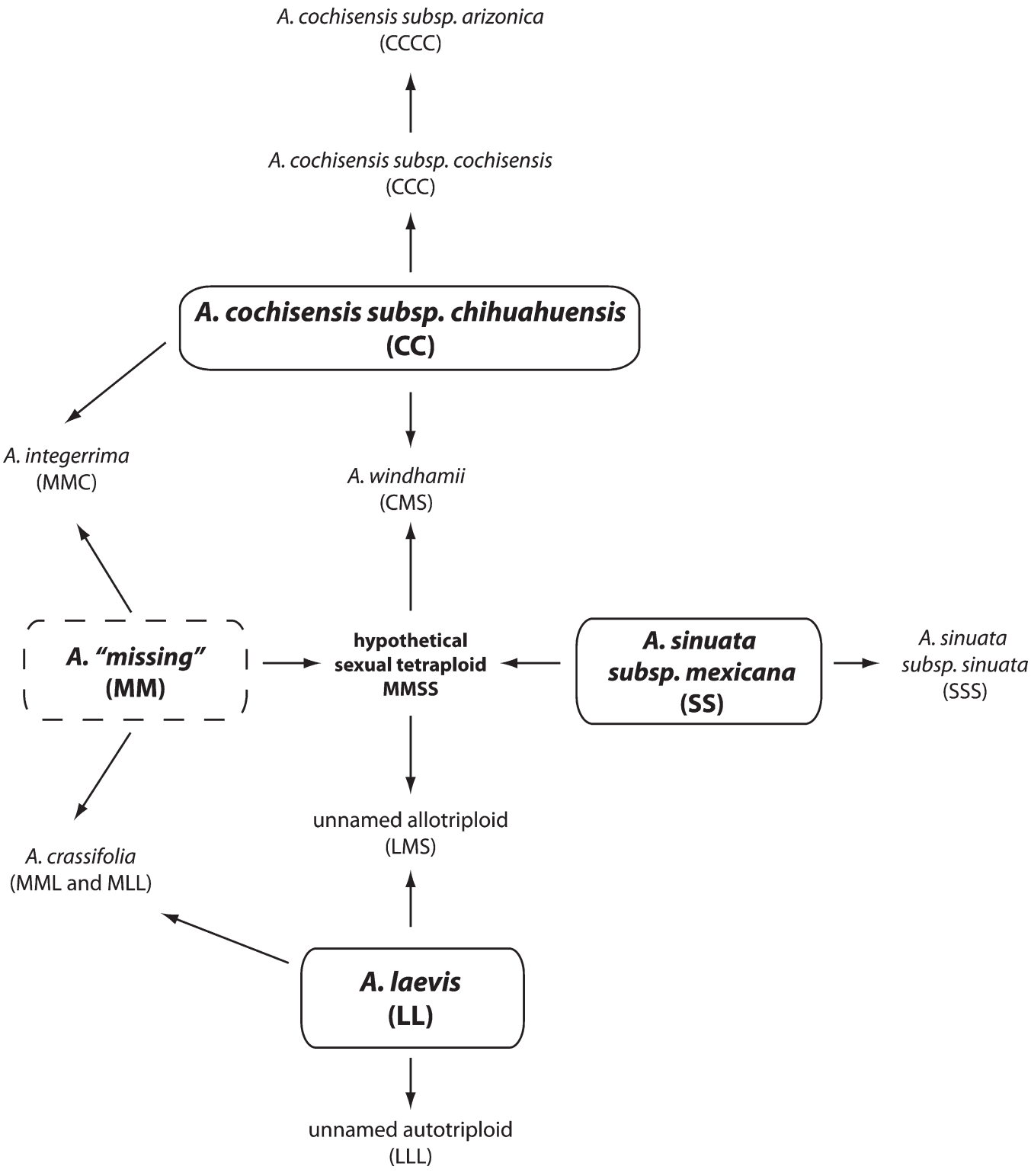


FIG. 1. Reticulate relationships within *Astrolepis* sensu Benham (1989). Diploid taxa are outlined with boxes and their genomes are denoted with letters (*A. cochisensis* = C, *A. sinuata* = S, *A. laevis* = L, *A. "missing"* = M); the genomic components that comprise all other taxa are represented by these same letters. Note a hypothesized sexual tetraploid that is as yet unobserved.

2006; Slotte et al. 2006; Kim et al. 2008). Benham (1989) viewed the extensive involvement of the missing diploid in hybridization events within *Astrolepis* as evidence that it was extant, and encouraged future workers to search for this taxon.

Given what is already known about *Astrolepis* (Benham 1989; Benham and Windham 1993; Mickel and Smith 2004), a more rigorous evaluation of its diploid members is likely to result in the discovery of novel diversity. In addition, any further understanding of the origin and composition of the known allopolyploids relies on a thorough understanding of the diploids. In this study, we investigate morphology and DNA sequence variation (both at a rapidly evolving plastid

region and a low copy nuclear locus) in a diverse sampling of diploid *Astrolepis*. These data are then used to address the following questions: 1) do the diploid species as currently circumscribed correspond to monophyletic groups?; 2) is the putative missing diploid extant?; and 3) what are the phylogenetic relationships among these diploid taxa?

## Materials and Methods

***Identifying Diploids Using Spore Number and Size***—Our primary sampling strategy involved targeting specimens that best fit the apparent description of the missing diploid (Benham 1989), as well as multiple, geographically-distinct individuals from each of the three known diploid taxa, *A. laevis*, *A. cochisensis* subsp. *chihuahuensis*, and *A. sinuata* subsp. *mexicana*. From this point forward, the subspecies names designating diploid *A. cochisensis* and diploid *A. sinuata* will not be used, and unless otherwise noted all references to *Astrolepis* samples refer to putative diploids. Herbarium specimens from across the range of the genus were examined. For each fertile specimen, the number of spores per sporangium was determined (Barrington et al. 1986; Grusz et al. 2009) and the mean spore diameter was measured to assist in the identification of diploids. Multiple mature sporangia were removed from each specimen and placed in individual drops of glycerol, and each sporangium was opened and the spores teased apart with dissecting needles. Sporangia contained either ca. 64 or ca. 32 spores, corresponding to a sexual or apomictic life cycle, respectively (Manton 1950; Tryon and Britton 1958). Earlier studies on these ferns, which coupled sporangium spore counts with chromosome counts from the same individual, demonstrated that sporangia from sexual diploid *Astrolepis* plants are exclusively 64-spored, whereas sporangia from apomictic triploids and tetraploids are exclusively 32-spored (Knobloch et al. 1973; Knobloch and Tai 1978; Benham 1989; Windham and Yatskievych 2003). Additionally, Benham (1989) demonstrated that mean spore size was a strong predictor of ploidy, with diploids, triploids, and tetraploids displaying statistically different and largely nonoverlapping spore size distributions. A count of 64 spores per sporangium, combined with the small size of diploid spores that was apparent even under the dissecting scope, therefore provided a strong initial signal for diploidy.

As part of our larger study of both diploid and polyploid lineages within *Astrolepis* we documented spore number per sporangium in this way for 165 specimens from nine herbaria. We then selected a core group of 44 specimens for in-depth study, choosing material that spanned the morphological, geographical, and presumed cytological diversity of the genus. Mean spore size was then determined for each of these. A cover slip was placed over the glycerol-mounted spores from each specimen and examined at 400 × magnification on a Zeiss Axioplan 2 microscope. Images of spores that had shed their perispore, or in which the spore body could be easily distinguished from the perispore (Benham 1989) were taken with a Zeiss AxioCam HRm. Spore diameter was determined for 10–25 spores per specimen with ImageJ version 1.38 (Abramoff et al. 2004) calibrated with a slide micrometer. Twelve putative diploid individuals that exhibited both a sexual life history (64 spores per sporangium) and a mean spore diameter that corresponded to those of diploids identified in Benham (1989) were chosen for molecular analysis (see Appendix 1).

***Amplification, Cloning, and Sequencing***—Genomic DNA was extracted from herbarium or silica-dried leaf material following Schuettpelz and Pryer (2007). A portion of the plastid genome spanning the *trnG* intron, one of the two *trnG* exons, and a portion of the *trnG-trnR* intergenic spacer (hereafter referred to as *trnGR*) was amplified with the primers "TRNG1F" and "TRNR22R" (Nagalingum et al. 2007) and sequenced with the primers "TRNG1F," "TRNG43F1," TRN63R," (Nagalingum et al. 2007) and "TRNGJBF" (5'–AGGAGCCGAATGGGCCGAAA–3'—this study). Polymerase Chain Reaction (PCR) conditions included an initial denaturation step (95°C for 2 min) followed by 35 denaturation, annealing, and elongation cycles (95°C for 30 sec, 45°C for 30 sec, 71°C for 1 min) and a final elongation step (71°C for 5 min). A portion of the nuclear gene *gapCp* was amplified under the thermocycling conditions and using the primers "ESGAPCP8F1" and "ESGAPCP11R1" specified in Schuettpelz et al. (2008). Amplified *gapCp* products were cloned using either TOPO TA (Invitrogen, Carlsbad, California) or pGEM-T (Promega, Madison, Wisconsin) cloning kits. In order to alleviate PCR bias (Polz and Cavanaugh 1998) in any single amplification, four separate amplifications per sample were typically pooled prior to ligation. Comparisons of initial cloned sequences to data from Schuettpelz et al. (2008) indicated that the "8F1" and 11R1" primers amplified both the focal *gapCp* "short" and the *gapCp* "long" copies discussed therein. A *gapCp* short specific primer, "JBGAPCPSR" (5'-TGTCRTACCAYGACACCAGC-3') was designed and used in tandem with the 8F1 primer to successfully amplify only *gapCp* short (referred to as *gapCp* hereafter).

***Cloned Sequence Selection***—A number of factors, both biological and artifactual, can contribute to diversity in a set of cloned sequences from a diploid individual. These include allelism at a locus, gene duplication of that locus, PCR error, and PCR-mediated recombination (chimerism) between the two alleles at a single locus or between alleles at duplicated loci (Cronn et al. 2002). To account for this diversity, a target of eight clones per individual (once clear recombinants between alleles at duplicated loci had been deleted) was set as a minimum for further consideration. This minimum clone number per individual was achieved for all but one sample from a relatively old (1966) herbarium specimen, and these clones (mean 10.6 clones per individual) were subject to further consideration. Fine scale recombination, such as that between two alleles at a locus in a heterozygous individual, as well as PCR error, were then assessed with the aid of haplotype (allele) networks created using TCS 1.21 (Clement et al. 2000) for the set of considered clones per individual. Fine-scale recombination would result in identical character state change on multiple branches of the network; however, this pattern was not observed in any of our samples. PCR error was apparent, as 11 of 12 individuals exhibited more than two putative alleles per network. The most unequivocal of these errors were identified as any clone that met these three criteria: 1) was observed only once; 2) differed by a single character-state change from a clone observed multiple times; and 3) exhibited a character-state change that was not observed in an aligned matrix of 617 *gapCp* clones from 43 *Astrolepis* diploids and polyploids. These clones were eliminated from further analysis. Both the initial number of clones considered and those ultimately analyzed per individual appear in Appendix 1.

***Sequence Alignment and Phylogenetic Analysis***—The *trnGR* and *gapCp* data sets were manually aligned in Se-Al 2.0 (Rambaut 2002) and each aligned matrix was exported as a NEXUS file. A combined analysis was not performed, as each dataset in isolation identified well-supported phylogenetic relationships that were consistent with each other (see Results). In addition, combining datasets would necessitate choosing single cloned nuclear sequences to represent individuals in the *gapCp* dataset, a difficulty further compounded by two putative instances of gene duplication at this locus (see Results). All insertion/deletion (indel) events, both autapomorphic and synapomorphic, were scored using the "simple gap coding" method of Simmons and Ochoterena (2000) and added as additional binary characters to the end of the NEXUS file. Regions of uncertain alignment were noted and excluded from further analysis. Both datasets are available from TreeBASE (study number S2535). For each dataset, a heuristic maximum parsimony search with 100 random-addition replicates was performed using PAUP* 4.0b10 (Swofford 2002). Ten thousand bootstrap replicates, each with 100 random-addition replicates, were conducted with PAUP* 4.0b10 to obtain bootstrap support for the *trnGR* dataset. Due to computational constraints, ten thousand "fast heuristic" bootstrap replicates were conducted with PAUP* 4.0b10 to obtain bootstrap support for the *gapCp* dataset. The best-fitting model of sequence evolution for each DNA region (excluding the binary indel partition) was identified using the Akaike Information Criterion in Modeltest 3.06 (Posada and Crandall 1998), and a Bayesian Markov Chain Monte Carlo analysis was performed on each dataset in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). For both the *trnGR* and *gapCp* analyses, the indel characters were assigned the binary model of character evolution (Nst = 1, Coding = Variable) as recommended in the MrBayes documentation. All Bayesian analyses comprised four independent runs, each with four chains (one cold and three heated). Flat priors were used, with the exception of the rate prior that was set to allow rates to vary among partitions. Chains were run for ten million generations, and trees were sampled every 1,000 generations. Convergence was evaluated by examining the standard deviation of split frequencies among runs and by plotting the log-likelihood values from each run using Tracer 1.4 (Rambaut and Drummond 2007). These diagnostics indicated that runs reached convergence within the first two million generations, and trees sampled during this period were excluded before obtaining clade posterior probabilities. The choice of outgroup taxa was guided by previous studies (Gastony and Rollo 1998; Kirkpatrick 2007; Schuettpelz and Pryer 2007; Rothfels et al. 2008), which placed *Astrolepis* in a clade with *Paragymnopteris* K. H. Shing, and *Pellaea* Link.

## Results

***Spore Analyses***—Mean spore diameters for the 44 core specimens are shown in Fig. 2. Measurements from specimens with 64-spored sporangia (putative diploids) were consistently

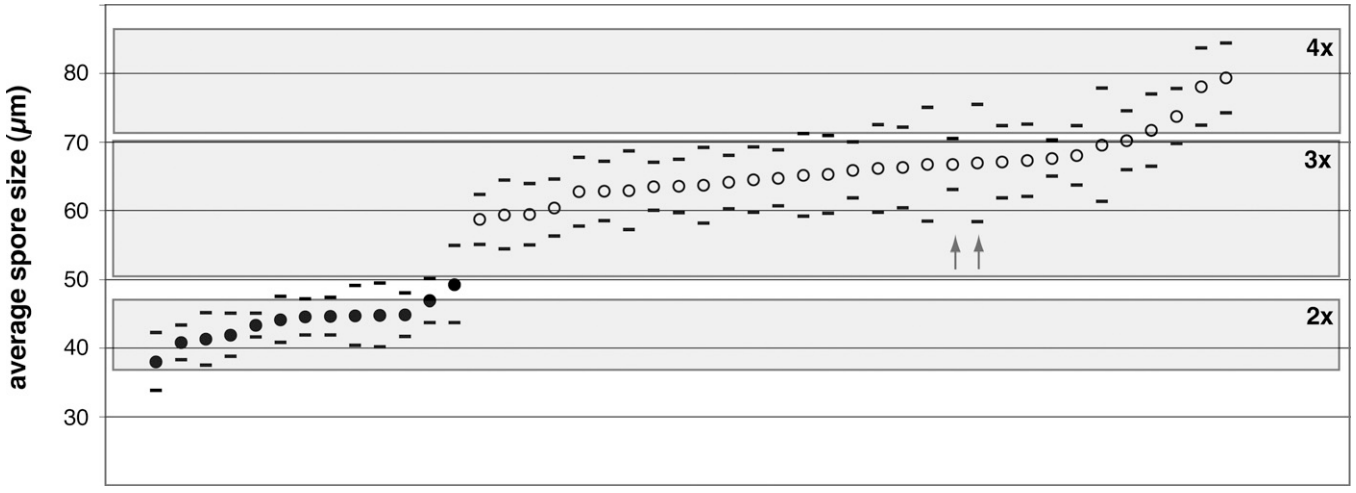


FIG. 2. Mean spore diameter (+/− 1 SD) for the 44 core *Astrolepis* specimens (arranged from smallest to largest). Filled circles indicate 64-spored individuals, open circles indicate 32-spored individuals. The three shaded boxes indicate the size ranges previously reported for diploid, triploid, and tetraploid individuals of *A. sinuata* and *A. cochisensis* (Benham 1989). The arrows indicate two specimens that have been documented as triploids via chromosome counts (Windham, unpubl. data).

smaller than those from specimens with 32-spored sporangia (putative triploids and tetraploids), and these two groups are statistically different ($p < 0.0001$; Mann-Whitney *U* test). The spore sizes we observed (Fig. 2) closely corresponded to the ranges for each ploidy level previously reported by Benham (1989), and the correlation was further strengthened by two triploid chromosome counts (Windham unpubl. data) that were made for specimens with spore sizes in the triploid range. These results strongly indicated that those specimens with 64-spored sporangia and mean spore diameters less than 50 μm are diploids, and 12 of these putative diploid specimens were chosen for DNA sequencing (Appendix 1). This study ideally would have included more individuals, but sampling was limited by the time and expense involved in generating a robust set of *gapCp* clone sequences per individual.

***Plastid Sequence Variation and Phylogeny***—The analyzed *trnGR* matrix of 1,119 aligned characters yielded 217 (19%) variable and 126 (11%) parsimony-informative characters. This character total included 16 indels, of which eight (50%) were parsimony-informative. The matrix contained 917 (5%) data cells coded as either missing or ambiguous. Each of the 100 random-addition replicate parsimony searches recovered the same most parsimonious tree (length = 278, CI = 0.85, RI = 0.87). This tree, along with bootstrap percentages (BS) and Bayesian posterior probabilities (PP), is shown in Fig. 3. Support for the monophyly of *Astrolepis* (1.0 PP, 100% BS) was strong, as was support (1.0 PP, 87% BS) for a clade comprising *Astrolepis*, *Pellaea pringlei* Davenp., and *Pellaea sagittata* Link. Within *Astrolepis*, clades comprising samples of each of the diploid species recognized herein received maximum support, with the exception of *A. deltoidea* (0.99 PP, 87% BS). The bifurcating relationships among these species all received maximum support. *Astrolepis cochisensis* is sister to a main clade exhibiting two subclades, one comprising *A. obscura* and *A. laevis*, with the other comprising *A. sinuata* and *A. deltoidea*.

***Nuclear Sequence Variation and Phylogeny***—A set of 127 *gapCp* clones was generated from the 12 diploid *Astrolepis* samples. A final set of 62 clones was chosen for analysis after excluding both clear cases of PCR error (see Materials and Methods) and identical sequences from a single sample. The *gapCp* dataset was rooted with *Astrolepis cochisensis* (based on results from the plastid portion of this study) to avoid deleting characters due to uncertain alignments when compared to more distantly related taxa. The analyzed *Astrolepis gapCp* matrix of 648 aligned characters yielded 194 (30%) variable and 132 (20%) parsimony-informative characters. This character total included 21 indels, of which 17 (81%) were parsimony-informative. The matrix contained 197 (0.5%) data cells coded as either missing or ambiguous. The 100 random addition-replicate parsimony searches recovered 31,937 most parsimonious trees (length = 251, CI = 0.88, RI = 0.97). One of these trees, rooted with *Astrolepis cochisensis* and with bootstrap percentages and Bayesian posterior probabilities, is shown in Fig. 4.

Although complicated by two apparent instances of gene duplication, the nuclear *gapCp* topology was essentially congruent with the plastid *trnGR* tree. The *A. obscura*/*A. laevis* (1.0 PP, 95% BS) and *A. sinuata*/*A. deltoidea* clades (0.93 PP, 61% BS) received either strong or moderate support. Four strongly supported (1.0 PP, 99–100% BS) subclades were evident within the *A. obscura*/*A. laevis* clade, and each comprised either only *A. obscura* or *A. laevis* clones. Two of these, "*A. obscura* 1" and "*A. laevis* 1," were strongly supported (1.0 PP, 94% BS) as sister to one another. The other two subclades, "*A. obscura* 2" and "*A. laevis* 2," formed a polytomy along with the *A. obscura*/*A. laevis* 1 clade. Clone sequences from both diploid *A. obscura* samples were placed only in each of the *A. obscura* 1 and 2 clades. Likewise, clone sequences from the two *A. laevis* samples were found only in the *A. laevis* 1 and 2 clades, although clone sequences from the Sinaloa individual were not observed in *A. laevis* 2. Taken together, these patterns strongly suggest that these four subclades resulted from a gene duplication that preceded the most recent common ancestor of *A. obscura* and *A. laevis*. Our failure to recover *A. laevis* 2 clone sequences from the Sinaloa individual could be due either to an insufficient number of sequenced clones, or loss of this duplicate gene in this *A. laevis* individual. Consistent with the latter interpretation, the two *A. laevis* 2 clones recovered from the Oaxaca *A. laevis* sample were clearly pseudogenes, as both exhibit a 144-bp insertion and two (11 and 13 bp) deletions in *gapCp* exon 9, a two-bp deletion in exon 10, and a one-bp

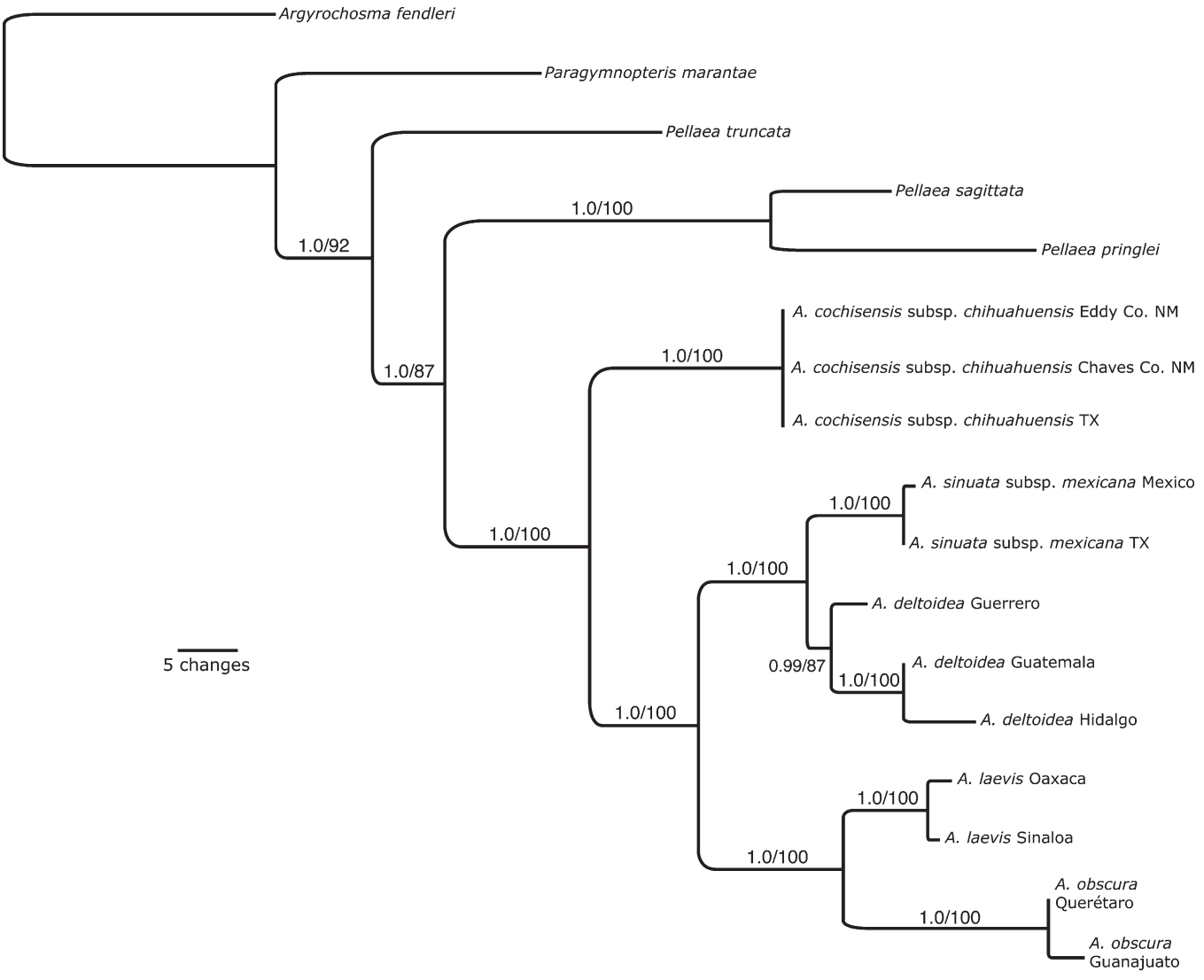


FIG. 3. The single most parsimonious tree resulting from analysis of the plastid *trnGR* dataset. Support values appear at each node (Bayesian posterior probability/parsimony bootstrap percentage). The tree was drawn using FigTree 1.1 (Rambaut 2008).

deletion in exon 11. This pseudogenization process could have led to sufficient change at priming sites or outright loss of this *gapCp* copy in the Sinaloa *A. laevis* individual.

A similar pattern is evident in the *A. sinuata*/*A. deltoidea* clade, with two strongly supported subclades, *A. sinuata*/*A. deltoidea* 1 and *A. sinuata*/*A. deltoidea* 2 (1.0 PP, 89% BS and 0.99 PP, 82% BS respectively). Clone sequences from each of the two *A. sinuata* samples were found in both clades, as were clone sequences from the Hidalgo *A. deltoidea* individual. Again, the failure to recover *A. sinuata*/*A. deltoidea* clade 1 sequences from the Guerrero and Guatemala *A. deltoidea* individuals could be due either to an insufficient number of sequenced clones, or loss of this presumably duplicate gene in some *A. deltoidea* individuals. Although *A. deltoidea* was both morphologically distinctive and well supported by the *trnGR* data, incomplete sorting of its alleles relative to those of *A. sinuata* was evident at both *gapCp* copies.

## DISCUSSION

Our data both clarify the number of diploid *Astrolepis* lineages and the bifurcating relationships among them, which are the two primary goals of the "diploids-first" approach. In particular, the genetic distinctiveness of each diploid (Figs. 3, 4) now provides a clear framework for identifying the origin and composition of the complex array of *Astrolepis* polyploids, through the examination of the plastid (maternal) and nuclear (biparental) constitution of each auto- and allopolyploid lineage.

***Phylogeny of Diploid Astrolepis***—Previous plastid phylogenetic analyses incorporating multiple *Astrolepis* species (Gastony and Rollo 1998; Kirkpatrick 2007; Rothfels et al. 2008) supported the monophyly of the genus and placed it in a "pellaeoid" clade including part of *Pellaea* Link, [sections *Pellaea* and *Platyloma* (J. Sm.) Hook. & Baker], *Paraceterach* Copel., and *Paragymnopteris* K. H. Shing. These earlier studies were unanimous in rejecting the monophyly of *Pellaea* (*sensu* Tryon et al. 1990), and those with relevant sampling (Gastony and Rollo 1998; Kirkpatrick 2007) suggested that *Astrolepis* was sister to a portion of *Pellaea*. Although it was not our primary goal to examine relationships within this larger pellaeoid clade, our *trnGR* data (Fig. 3) are congruent with previous plastid studies in clearly demonstrating that *Astrolepis* is monophyletic and sister to a clade that includes *Pellaea pringlei* Davenp. and *P. sagittata* Link.

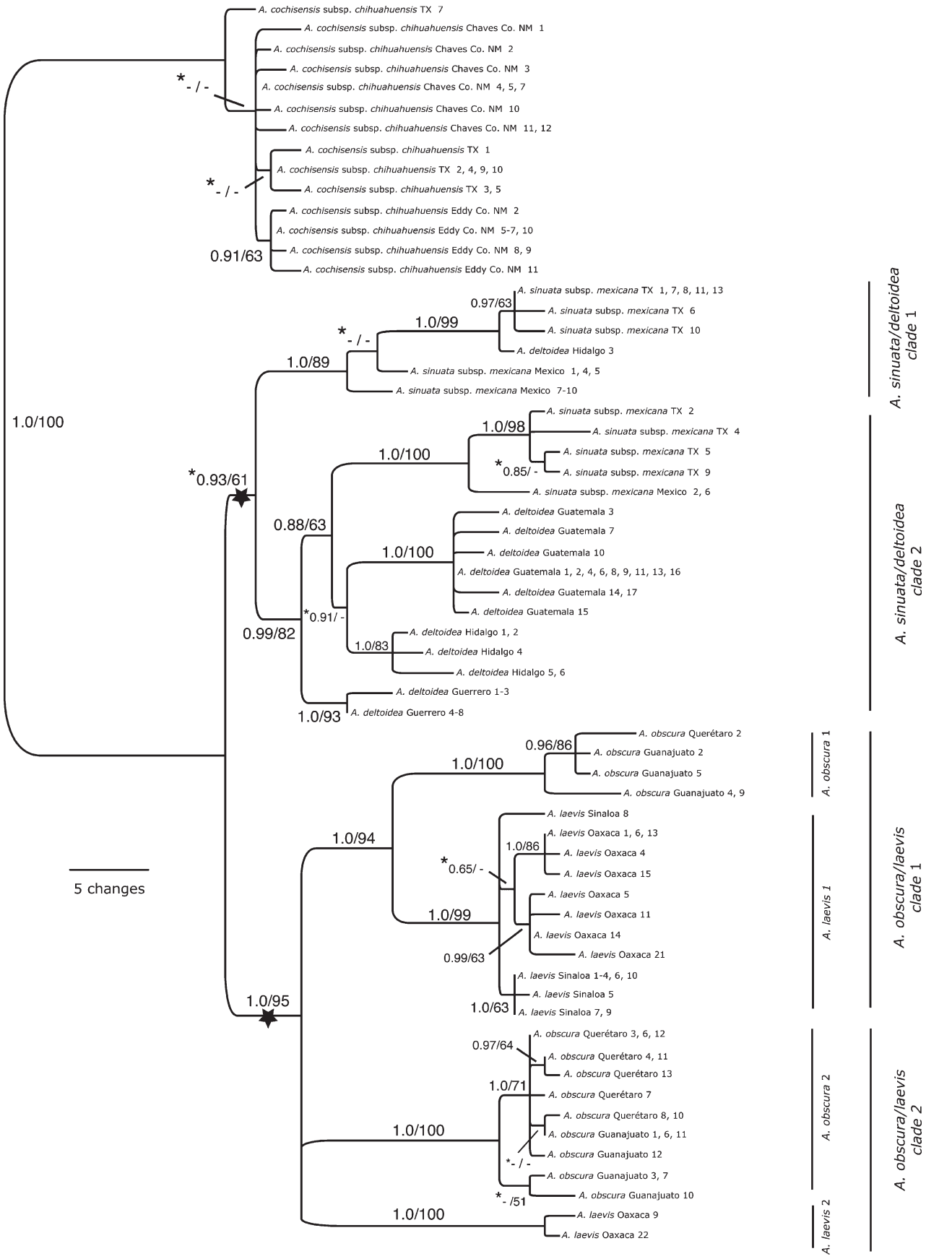


FIG. 4. One of 31,937 most parsimonious trees resulting from analysis of the nuclear *gapCp* dataset, rooted with *A. cochisensis*. Support values appear at each node (Bayesian posterior probability/parsimony bootstrap percentage). Nodes not present in all most parsimonious trees are noted with an asterisk. Numbers following sample names refer to individual clone sequences, multiple numbers indicating that this sequence was recovered in multiple clones from that individual. Two putative gene duplication events are noted with stars.

Due to the complex patterns of reticulate evolution encountered in *Astrolepis* (Fig. 1) and the relatively recent delimitation of diploid lineages (Benham 1989 and discussion below), previous workers have said little regarding bifurcating relationships within the genus. The plastid phylogeny presented here (Fig. 3) provides a strongly supported hypothesis for these relationships, and the *gapCp* tree is consistent with this proposed evolutionary history. *Astrolepis cochisensis* is strongly placed as sister to the rest of the genus with maximum support in the *trnGR* tree (Fig. 3), and the 14 clone sequences analyzed from the three *A. cochisensis* individuals form a clade receiving maximum support in the *gapCp* tree (Fig. 4). This phylogenetic position is consistent with the apparent morphological, chemical, and biogeographic distinctiveness of the species. *Astrolepis cochisensis* exhibits the smallest pinnae (4–7 vs. 8–28 mm long), has distinctive ovate-elliptic (vs. lanceolate-elongate) abaxial pinna scales (Fig. 5), and is the only member of the genus known to be toxic to livestock (Mathews 1945). Additionally, *A. cochisensis* appears to be the only diploid *Astrolepis* that does not occur south of the "arid semiarid" ecological zone (Toledo and Ordóñez 1993) of northern and central Mexico. The sister clade to *A. cochisensis* contains four morphologically and genetically divergent diploid taxa forming two subclades. These subclades, comprising the species pairs *A. sinuata*/*A. deltoidea* and *A. obscura*/*A. laevis*, all receive maximum support in the *trnGR* tree (Fig. 3) and moderate to strong support in the *gapCp* tree (Fig. 4). Although morphological synapomorphies uniting either of these sister species pairs are not apparent, the species themselves are rather easily distinguished using a combination of character states (see key below). The morphological clarification of these diploid lineages, combined with our new understanding of their evolutionary relationships, will provide the framework necessary to understand the diversity and history of *Astrolepis* polyploids.

***Apparent Gene Duplication at gapCp***—Both gene duplication and incomplete lineage sorting/hybridization appear to have complicated bifurcating patterns in the nuclear *gapCp* topology (Fig. 4). Gene duplications are a common feature of many gene families (Lynch and Conery 2000; Demuth and Hahn 2009), and have been observed at relatively shallow time scales in a wide range of nuclear genes (Kramer et al. 2003; Archambault and Bruneau 2004; Gardiner et al. 2008), including *gapCp* (Petersen et al. 2003). The potential for gene duplication and loss presents a major challenge for phylogeny reconstruction, particularly in the case of polyploids, which initially harbor completely duplicated genomes that can undergo complicated patterns of gene loss (Feldman et al. 1997; Fortune et al. 2006; Lu et al. 2009; Small and Wendel 2000). Both gene duplication and a lack of reciprocal monophyly due to either incomplete lineage sorting or hybridization are evident in the *A. sinuata*/*A. deltoidea gapCp* clade (Fig. 4). Both plastid (Fig. 3) and morphological evidence strongly suggest that *A. sinuata* and *A. deltoidea* are independent evolutionary lineages. The lack of reciprocal monophyly of clone sequences from these two lineages at either of the putatively *gapCp* duplicated loci (Fig. 4) could be due to incomplete lineage sorting and/or hybridization (Nichols 2001; Mallet 2005). Distinguishing between these two scenarios is an especially difficult problem (Maddison 1997; Sang and Zhong 2000), and further evaluation of the integrity and evolutionary history these two putative species will require the analysis of additional nuclear loci.

***Updating the Classification of Diploid Astrolepis: Previously Known Taxa***—The number of recognized *Astrolepis* species has varied widely through time due to differing taxonomic philosophies, access to comparative material, and new insights from cytology and molecular techniques. Eight species had been described before the first comprehensive treatment of taxa now included in *Astrolepis* (Tryon 1956, as *Notholaena*). At that time, only apomicts with 32-spored sporangia were known, and Tryon (1956) was thus unaware of the capacity for polyploidy to produce a grade of morphologically intermediate forms. Tryon therefore treated the group as a single species (*Notholaena sinuata*) with three varieties. Hevly (1965) was the first to report individuals with 64-spored sporangia, putatively sexual populations of *A. sinuata* and *A. cochisensis*. He noted that these two taxa are "sharply set apart" and treated them and a putative allopolyploid hybrid between them as three distinct species (of *Notholaena*). Benham (1989) and Benham and Windham (1992) verified the existence of diploid populations of *A. sinuata* and *A. cochisensis* cytologically, and hypothesized the existence of diploid *A. laevis* (as *A. beitelii*) based on spore size/spore number per sporangium and isozyme banding patterns. Their revision recognized three species (*A. beitelii, A. cochisensis*, and *A. sinuata*) encompassing diploid and autopolyploid populations, and three exclusively polyploid hybrids. The most recent treatment of the genus (Mickel and Smith 2004) recognized all three of the diploid-centered taxa (and two of the allopolyploids) as separate species.

***The Resurrection of A. deltoidea and the Discovery of A. obscura***—In addition to the three previously known diploid *Astrolepis* species discussed above, our analyses revealed two additional diploid taxa. Both exhibit exclusive morphological and genetic character states, suggesting that they represent distinct evolutionary lineages. These unique character states would be sufficient to warrant species status under several of the species concepts (Monophyletic, Phylogenetic, Quantitative, and Phenetic) outlined by Luckow (1995). This is noted only to be explicit regarding our species criterion (McDade 1995), and not to suggest that this criterion is superior to others. Species are segments of evolutionary lineages (de Queiroz 1998; Hey 2006), and future workers may choose to redefine *Astrolepis* species based on new evidence and/or other species criteria.

The resurrection of *A. deltoidea* began with the discovery of a putatively diploid specimen with 64-spored sporangia from Guatemala (*Hatch & Wilson 279*—US) recently annotated as *A. laevis* by D. M. Benham. Although morphologically most similar to *A. laevis*, *trnGR* and *gapCp* sequences from this specimen suggested a sister relationship with *A. sinuata* (Figs. 3,4). The Guatemalan specimen is unusually robust for an *Astrolepis*, with leaves at least 80 cm long and deeply pinnatifid pinnae nearly 3 cm long. This drew our attention to the closing note of the *Astrolepis sinuata* treatment in *The Pteridophytes of Mexico* (Mickel and Smith 2004), where the authors noted a "very large specimen from Guerrero" (*Fonseca & Velazquez 2475*—NY), reaching 1 m in length with deeply cut pinnae. Spore analysis of this specimen revealed that it, too, was a probable diploid with 64 spores per sporangium. Our plastid sequence data revealed that this Guerrero individual, the original Guatemala specimen, and a third specimen with 64-spored sporangia from Hidalgo (*Mears 278*—TEX-LL) form a strongly supported clade sister to diploid *A. sinuata* (Fig. 3); they are easily distinguished from that species based

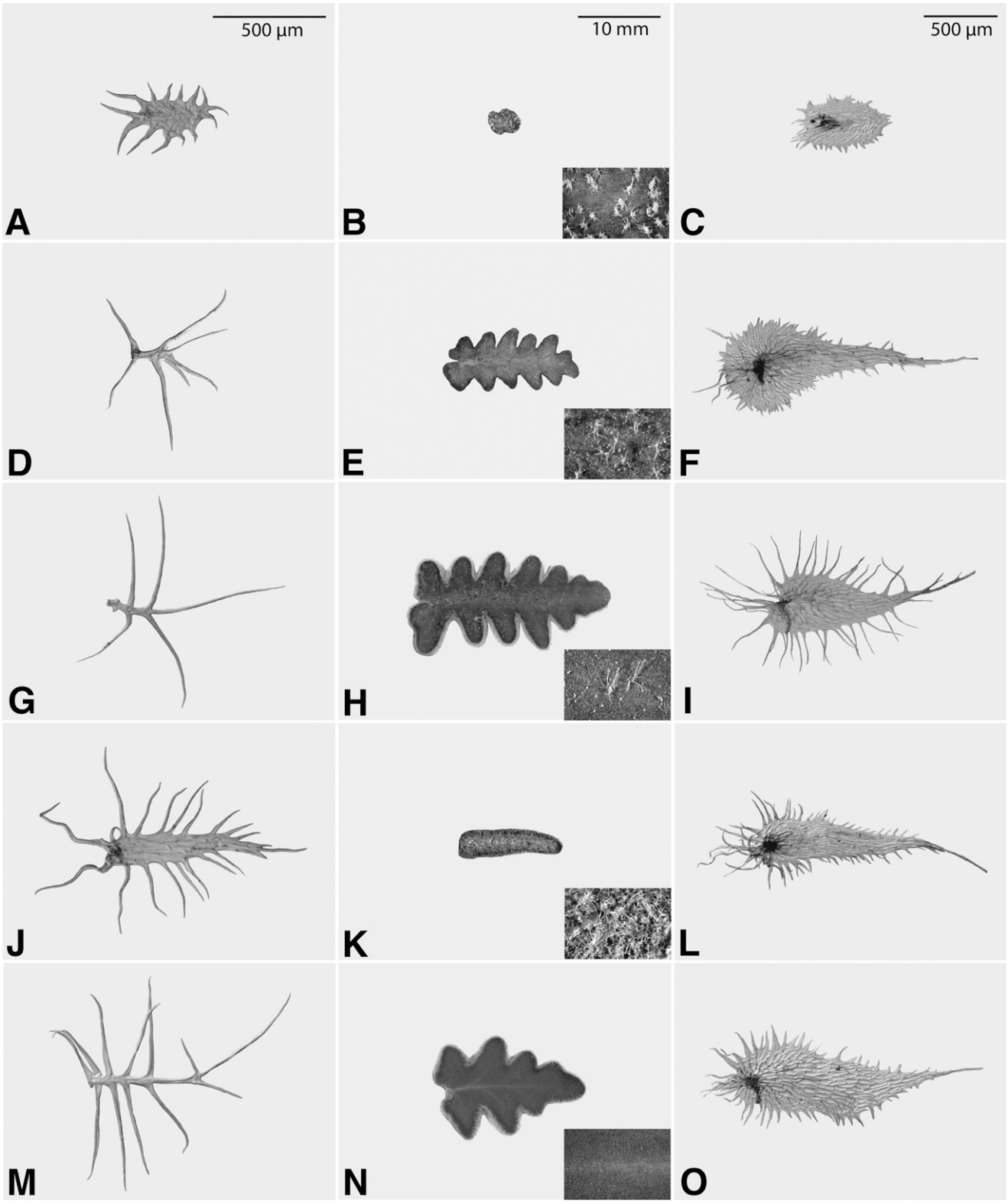


Fig. 5. Representative adaxial pinnae scales (left column), pinnae (center column), and abaxial pinnae scales (right column) of the five diploid *Astrolepis* species. A–C. *A. cochisensis* subsp. *chihuahuensis* (*Windham 3490*); D–F. *A. sinuata* subsp. *mexicana* (*Mayfield et al. 1032*); G–I. *A. deltoidea* (*Hatch & Wilson 279*); J–L. *A. obscura* (*Steinmann et al. 2500*); M–O. *A. laevis* (*Mayfield 1674*).

on characteristics of the rhizome scales and abaxial pinna scales (Fig. 5; see key below). While examining type specimens previously synonomized under *A. laevis* and *A. sinuata* (Mickel and Smith 2004), we discovered that the distinctive pinna scales of these specimens closely matched those of the type of *Notholaena deltoidea* Baker. Although this specimen (*Leibold s. n.*—K) comprises a single juvenile, nonsporulating individual, it matches the three specimens discussed above

in every observable character, and we propose the name *Astrolepis deltoidea* for this diploid lineage.

The search for the taxon herein called *A. obscura* was motivated by the isozyme evidence of Benham (1989), which established that a missing genome was involved in the origins of at least five unique allopolyploid combinations (see Fig. 1). Based on the morphologies of these hybrids and of each of their known parents, Benham (1989) hypothesized that the missing diploid would exhibit elongate, entire pinnae with a dense covering of scales on the adaxial pinnae surface. This suggested to us that the missing diploid might be masquerading as *Astrolepis integerrima* (Hook.) D. M. Benham & Windham, a morphologically variable allotriploid known from isozyme studies to contain the missing genome (Benham 1989). Based on this reasoning, a search for individuals with 64-spored sporangia that were previously identified as *A. integerrima* was initiated. Collections from three U. S. A. herbaria archiving large numbers of *A. integerrima* specimens (NY, TEX-LL, and US) were examined to determine spore number per sporangium. Two individuals with 64 spores per sporangium, as opposed to the 32 spores encountered in the allotriploid apomict *A. integerrima*, were identified. These two samples yielded *trnGR* sequences that formed a well-supported lineage sister to *A. laevis* (Fig. 3).

Our results are completely congruent with Benham's (1989) isozyme data and with the topological placement of *gapCp* clones obtained from various allopolyploids containing the missing genome (Beck et al. unpubl. data). Furthermore, the morphology of these two samples corresponded exactly to Benham's (1989) predictions; they exhibited either entire (*Pérez & Zamudio 3559*—NY) or slightly lobed (*Díaz & Carranza 7481*—NY) pinnae with an extremely dense covering of adaxial scales (Fig. 5). A recent survey of *Astrolepis* specimens at the Missouri Botanical Garden (MO) yielded another specimen with 64-spored sporangia (*Steinmann et al. 2500*—MO) of similar morphology, bringing the number of known samples of *A. obscura* to three.

We hope our discovery of this formerly unknown diploid in *Astrolepis* inspires the search for missing taxa that are implied by the genetic constitutions of allopolyploids in other groups. Such efforts represent a critical step in efforts to understand the evolutionary history and taxonomy of any genus showing significant polyploidy.

## Taxonomic Treatment

***Note*—**The following key is intended for use with sexual diploid collections that produce 64 small (< 50 μm diam) spores per sporangium. See Fig. 5 for representative images of adaxial pinnae scales, pinnae, and abaxial pinnae scales. Each of the five diploid taxa recognized here have been involved in the origins of di-genomic and trigenomic allopolyploids, the taxonomy of which will be addressed in upcoming papers.

### Key to the Diploid Species of *Astrolepis*

1. Largest pinnae < 7 mm long, adaxial pinna scales ovate-elliptic to orbicular ..... *A. cochisensis* subsp. *chihuahuensis*
1. Largest pinnae > 8 mm long, adaxial pinna scales lanceolate to substellate ..... 2
   2. Rhizome scales mostly < 6 mm long, adaxial pinna scales < 0.6 mm long ..... *A. sinuata* subsp. *mexicana*
   2. Rhizome scales mostly > 6 mm long, adaxial pinna scales > 0.6 mm long ..... 3
      3. Pinnae entire or with 2–5 shallow lobes, adaxial surface of pinnae densely covered with scales; adaxial pinna scales ciliate, with a wide central axis ..... *A. obscura*
      3. Pinnae with 7–13 lobes, dissected ca. halfway to the costae, adaxial surface with scattered, largely deciduous scales; adaxial pinna scales highly dissected, with a narrow central axis ..... 4
         4. Abaxial pinnae scales ciliolate, adaxial surface of the pinna essentially glabrous at maturity ..... *A. laevis*
         4. Abaxial pinnae scales strongly ciliate, pinna with scattered adaxial scales, particularly on the costa ..... *A. deltoidea*

**Astrolepis deltoidea** (Baker) J. Beck & Windham, comb. nov.
*Notholaena deltoidea* Baker, Syn. Fil., ed. 2, 514. 1874.—TYPE. MEXICO. *s. loc.*, *Leibold s. n.* (K!).

Emended description (extreme values in parentheses from Liebold's juvenile, nonsporulating holotype): Rhizomes, erect, compact; rhizome scales uniformly brown, 7–10 × 0.16–0.6 mm, margins dentate; leaves (8–)75–100 cm; blades linear, (4–)65–80 cm, pinnate-pinnatifid; rachis scales dimorphic, the larger ones lanceolate, the main body 2.5–4.0 mm × 130–700 μm, the margins ciliate; pinnae (5–)60–80, mostly alternate, the largest (14–)24–30 × (10–)12–17 mm wide, ovate-oblong with broadly rounded apices, ± symmetrically lobed approximately 2/3 the way to the costae, lobes (4–)9–13, the terminal lobe clearly larger than the adjacent lobes; abaxial surfaces concealed by a dense covering of scales, these scales lanceolate, the main body 1.0–2.1 mm × 300–600 μm, the margins long-ciliate throughout; adaxial surfaces with scattered scales these usually ± along the costae; adaxial scales substellate, with linear main body 0.6–1.2 mm × 30–70 μm, long-ciliate, longest cilia 300–600 μm long, typically at least one half the length of the main body, 7–14 cilia per scale; sporangia containing 64 spores averaging ca. 45 μm in diam.

***Distribution*—**The label on the type specimen of *A. deltoidea* simply states that it was collected in Mexico. Two more recent collections from Mexico are from the Sierra Madre Oriental in western Hidalgo and a montane forest site (2,500 m) in the Sierra Madre del Sur of central Guerrero. The two specimens from Guatemala indicate dry cliffs and mountain slopes (2,100 m) along the northern edge of Lake Altitlán in the Chiapan-Guatemalan Highlands. *Astrolepis deltoidea* therefore spans the Isthmus of Tehuantepec, a relatively low-lying area between the Mexican and Chiapan-Guatemalan Highlands that has been shown to be a biogeographic barrier in a variety of taxa (Sullivan et al. 1997; Cortés-Rodríguez et al. 2008; Castoe et al. 2009).

***Morphological Affinities*—***Astrolepis deltoidea* can be distinguished from all other diploid *Astrolepis* by its distinctive abaxial pinna scales, which are long-ciliate throughout (Fig. 5). *Astrolepis deltoidea* is further distinguished from its sister

species *A. sinuata* by its relatively long (> 6 mm) rhizome scales and expanded terminal pinna lobes, which are noticeably larger than the adjacent lobes. It is morphologically most similar to *A. laevis*, but can be distinguished from this taxon by the greater number of pinna lobes (> 8), and the persistence of some adaxial scales along the pinna costae. The specimen from Guerrero (*Fonseca & Velazquez 2475*—NY) is distinct from the remaining three known specimens in a number of respects, including its relatively large (to 44 mm) pinnae, denticulate (vs. ciliate) rachis scales, and ovate (vs. lanceolate) abaxial scales. This Guerrero individual was also genetically differentiated from the other sequenced *A. deltoidea* specimens (Figs. 3, 4). Further investigation is needed to fully evaluate the distinctiveness of this specimen; therefore, its unique morphological characteristics were not incorporated into the description presented here.

***Additional Specimens Examined***—GUATEMALA. Rio Panajachel, near Lake Atitlán, 10 Aug. 1936, *Hatch & Wilson 279* (US); Above Lake Atitlán, ca. 2–5 km W of Panajachel, 2,100 m, 6 Dec. 1963, *Williams et al. 25325* (NY).

MEXICO. Hidalgo: 2 mi N of Posada del Rey, 5 July 1966, *Mears 278* (TEX-LL)

**Astrolepis obscura** J. Beck & Windham, sp. nov.—TYPE: MEXICO. Guanajuato: 10 km NW of Xichú, 4 Feb. 1997, *Pérez & Zamudio 3559* (holotype: NY!).

*Astrolepis integerrimae* persimilis sed pagina adaxiale pinnarum squamis persistentibus obtecta, squamis paginae adaxialis 18–36 (haud 10–15) ciliis praeditis, sporangiis 64 (haud 32) sporas parvas (45–49, haud 59–70 mu diametro) continentibus recedit.

Rhizomes, erect, compact; rhizome scales uniformly brown, 5–10 × 0.12–0.25 mm, margins denticulate; leaves 14–22 cm; blades linear, 5–15 cm, 1-pinnate; rachis scales dimorphic, the larger ones lanceolate, the main body 1.5–2.4 mm × 250–370 µm, the margins ciliate; pinnae 30–60, mostly alternate, the largest 7–14 × 2–5 mm wide, oblong-linear to elliptic with narrowly to broadly rounded apices, entire or slightly asymmetrically lobed, lobes 2–5; abaxial surfaces concealed by a dense covering of scales, these scales lanceolate, the main body 1.4–2.7 mm × 240–500 µm, the margins long-ciliate proximally and ciliolate distally; adaxial surfaces concealed by a dense covering of persistent scales, providing a silvery appearance; adaxial scales with a lanceolate main body 0.7–2.6 mm × 80–180 µm, long-ciliate, longest cilia 200–550 µm long, typically less than one half the length of the main body, 18–36 cilia per scale; sporangia containing 64 spores averaging spore ca. 45–49 µm in diam.

***Distribution***—Two of the three known collections of *A. obscura* are from submontane limestone slopes in the Sierra Madre Oriental of northwestern Querétaro (1,400 m) and eastern Guanajuato (1,000 m). Given the frequency of such habitats, this taxon is likely to be more broadly distributed in this region. The morphologically distinctive collection from central Querétaro (*Díaz & Carranza 7481*) is from a xeric riverbank along the Rio Moctezuma (950 m).

***Etymology***—The specific epithet refers to both the difficulty in discovering this elusive taxon, and the dense covering of adaxial pinnae scales that largely obscure the pinnae surface.

***Morphological Affinities***—*Astrolepis obscura* can be easily distinguished from all other diploid *Astrolepis* species by its entire to shallowly lobed pinnae with a dense, persistent covering of lanceolate scales on the adaxial surface. Although some collections identified as *A. integerrima* approach this morphology, *A. obscura* is clearly differentiated from the type of *A. integerrima* (*Leibmann s. n.*—K) in that it exhibits a nearly continuous covering of persistent scales on adaxial pinna surfaces, adaxial pinna scales with a greater number of cilia (18–36 vs. 10–15), and 64 (vs. 32) spores per sporangium that are significantly smaller (45–49 vs. 59–70 µm in diam). As a whole, *A. integerrima* comprises a morphologically variable assemblage of apomictic allotriploids, and *A. obscura* can be distinguished from these plants based on spore size (45–49 vs. 63–70 µm), breeding system (64 vs. 32 spores per sporangium), and genetics. One of the *A. obscura* specimens from Querétaro (*Díaz & Carranza 7481*) is distinct from the remaining two known specimens in a number of respects, exhibiting shallowly lobed (vs. entire) pinnae, relatively long (to 2.6 mm) adaxial pinnae scales, and more extensive pigmentation at the base of the midrib scales. Further investigation is needed to evaluate the distinctiveness of this potentially unique lineage.

***Additional Specimens Examined***—MEXICO. Querétaro, near Cadereyta, 950 m, 17 Nov. 1993, *Díaz & Carranza 7481* (NY); Querétaro, La Vuelta, 21.2875° N 99.2089° W, 1400 m, 10 June 2002, *Steinmann et al. 2500* (MO).

Acknowledgments. The authors would like to thank the curators of ASC, BM, K, MO, NY, RB, TEX-LL, US, and UTEP for herbarium loans and permission to sample material for spore studies and molecular analysis. Special thanks go to Ihsan Al-Shehbaz and Henk van der Werff for help with the Latin diagnosis and to Greg McKee (US), Robbin Moran (NY), Rusty Russell (US), Harald Schneider (BM), and Ben Torke (NY) for facilitating herbarium studies. Comments and criticism from Amanda Grusz, Layne Huiet, Carl Rothfels, Eric Schuettpelz, Erin Sigel, and two anonymous reviewers greatly improved the manuscript. This study was funded in part by NSF awards DEB-0717398 (to KMP and MDW) and DEB-0717430 (to GY), and a Systematics Research Fund Award from the Linnean Society of London and the Systematics Association (to JBB).

## Literature Cited

Abramoff, M. D., P. J. Magelhaes, and S. J. Ram. 2004. Image processing with ImageJ. *Biophotonics International* 11: 36–42.

Archambault, A. and A. Bruneau. 2004. Phylogenetic utility of the LEAFY/FLORICAULA gene in the Caesalpinioideae (Leguminosae): gene duplication and a novel insertion. *Systematic Botany* 29: 609–626.

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.

Benham, D. M. 1989. *A biosystematic revision of the fern genus* Astrolepis *(Adiantaceae)*. M. S. thesis, Northern Arizona University.

Benham, D. M. 1992. Additional taxa in *Astrolepis*. *American Fern Journal* 82: 59–62.

Benham, D. M. and M. D. Windham. 1992. Generic affinities of the star-scaled cloak ferns. *American Fern Journal* 82: 47–58.

Benham, D. M. and M. D. Windham. 1993. *Astrolepis*. Pp. 140–143 in *Flora of North America*. ed. Flora of North America Editorial Committee. New York: Oxford University Press.

Brown, A. H. D., J. L. Doyle, J. P. Grace, and J. J. Doyle. 2002. Molecular phylogenetic relationships within and among diploid races of *Glycine tomentella* (Leguminosae). *Australian Systematic Botany* 15: 37–47.

Castoe, T. A., J. M. Daza, E. N. Smith, M. M. Sasa, U. Kuch, J. A. Campbell, P. T. Chippindale, and C. L. Parkinson. 2009. Comparative phylogeography of pitvipers suggests a consensus of ancient Middle American highland biogeography. *Journal of Biogeography* 36: 88–103.

Clement, M., D. D. Posada, and K. A. Crandall. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9: 1657–1659.

Cortés-Rodríguez, N., B. E. Hernández-Baños, A. G. Navarro-Sigüenza, A. T. Peterson, and J. García-Moreno. 2008. Phylogeography and population genetics of the amethyst-throated hummingbird (*Lampornis amethystinus*). *Molecular Phylogenetics and Evolution* 48: 1–11.

Cronn, R., M. Cedroni, T. Haselkorn, C. Grover, and J. F. Wendel. 2002. PCR-mediated recombination in amplification products derived from polyploid cotton. *Theoretical and Applied Genetics* 104: 482–489.

Cronquist, A. 1987. A botanical critique of cladism. *Botanical Review* 53: 1–52.

de Queiroz, K. 1998. The general lineage concept of species, species criteria, and the process of speciation. Pp. 57–75. in *Endless forms: species and speciation*. eds. D. J. H oward and S. H. Berlocher. Oxford: Oxford University Press.

Demuth, J. P. and M. W. Hahn. 2009. The life and death of gene families. *BioEssays* 31: 29–39.

Doyle, J. J., J. L. Doyle, J. T. Rauscher, and A. H. D. Brown. 2004. Evolution of the perennial soybean polyploid complex (*Glycine* subgenus *Glycine*): a study of contrasts. *Biological Journal of the Linnean Society* 82: 583–597.

Feldman, M., B. Liu, G. Segal, S. Abbo, A. A. Levey, and J. M. Vega. 1997. Rapid elimination of low-copy DNA sequences in polyploid wheat: a possible mechanism for differentiation of homoeologous chromosomes. *Genetics* 147: 1381–1387.

Fortune, P. M., K. A. Schierenbeck, A. K. Ainouche, J. Jacquemin, J. F. Wendel, and M. L. Ainouche. 2006. Evolutionary dynamics of *Waxy* and the origin of hexaploid *Spartina* species (Poaceae). *Molecular Phylogenetics and Evolution* 43: 1040–1055.

Fortune, P. M., N. Pourtau, N. Viron, and M. L. Ainouche. 2008. Molecular phylogeny and reticulate origins of the polyploid *Bromus* species from section *Genea* (Poaceae). *American Journal of Botany* 95: 454–464.

Gardiner, A., D. Barker, R. K. Butlin, W. C. Jordan, and M. G. Ritchie. 2008. *Drosophila* chemoreceptor gene evolution: selection, specialization and genome size. *Molecular Ecology* 17: 1648–1657.

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.

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.

Haufler, C. H., M. D. Windham, and E. W. Rabe. 1995. Reticulate evolution in the *Polypodium vulgare* complex. *Systematic Botany* 20: 89–109.

Hevly, R. H. 1965. Studies of the sinuous cloak-fern (*Notholaena sinuata*) complex. *Journal of the Arizona Academy of Science* 3: 205–208.

Hey, J. 2006. On the failure of modern species concepts. *Trends in Ecology & Evolution* 21: 447–450.

Holloway, A. K., D. C. Cannatella, H. C. Gerhardt, and D. M. Hillis. 2006. Polyploids with different origins and ancestors form a single sexual polyploid species. *American Naturalist* 167: E88–E101.

Hoot, S. B., N. S. Napier, and W. C. Taylor. 2004. Revealing unknown or extinct lineages within *Isoetes* (Isoetaceae) using DNA sequences from hybrids. *American Journal of Botany* 91: 899–904.

Huelsenbeck, J. P. and F. Ronquist. 2001. MrBayes: Bayesian inference of phylogeny. *Bioinformatics* 17: 754–755.

Ickert-Bond, S. M. and M. F. Wojciechowski. 2004. Phylogenetic relationships in *Ephedra* (Gnetales): evidence from nuclear and chloroplast DNA sequence data. *Systematic Botany* 29: 834–849.

Kim, C., H. Shin, and H. K. Choi. 2009. Genetic diversity and population structure of diploid and polyploid species of *Isoetes* in East Asia based on amplified fragment length polymorphism markers. *International Journal of Plant Sciences* 170: 496–504.

Kim, S.-T., S. E. Sultan, and M. J. Donoghue. 2008. Allopolyploid speciation in *Persicaria* (Polygonaceae): insights from a low-copy nuclear region. *Proceedings of the National Academy of Sciences USA* 105: 12370–12375.

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. and W. Tai. 1978. The chromosome number of *Notholaena cochisensis*. *American Fern Journal* 68: 63.

Knobloch, I. W., W. Tai, and T. A. Ninan. 1973. The cytology of some species of the genus *Notholaena*. *American Journal of Botany* 60: 92–95.

Kramer, E. M., V. S. Di Stilio, and P. M. Schlüter. 2003. Complex patterns of gene duplication in the APETALA3 and PISTILLATA lineages of the Ranunculaceae. *International Journal of Plant Sciences* 164: 1–11.

Lu, F., J. S. S. Ammiraju, A. Sanyal, S. L. Zhang, R. T. Song, J. F. Chen, G. S. Li, Y. Sui, X. Song, Z. K. Cheng, A. C. de Oliveira, J. L. Bennetzen, S. A. Jackson, R. A. Wing, and M. S. Chen. 2009. Comparative sequence analysis of MONOCULM1-orthologous regions in 14 *Oryza* genomes. *Proceedings of the National Academy of Sciences USA* 106: 2071–2076.

Luckow, M. 1995. Species concepts - assumptions, methods, and applications. *Systematic Botany* 20: 589–605.

Lynch, M. and J. S. Conery. 2000. The evolutionary fate and consequences of duplicate genes. *Science* 290: 1151–1155.

Maddison, W. P. 1997. Gene trees in species trees. *Systematic Biology* 46: 523–536.

Mallet, J. 2005. Hybridization as an invasion of the genome. *Trends in Ecology & Evolution* 20: 229–237.

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

Mathews, F. P. 1945. A comparison of the toxicity of *Notholaena sinuata* and *N. sinuata* var. *cochisensis*. *Rhodora* 47: 393–395.

McDade, L. 1990. Hybrids and phylogenetic systematics I. Patterns of character expression in hybrids and their implications for cladistic analysis. *Evolution* 44: 1685–1700.

McDade, L. 1995. Species concepts and problems in practice: insights from botanical monographs. *Systematic Botany* 20: 606–622.

Mickel, J. T. and A. R. Smith. 2004. *Astrolepis*. Pp. 132–135 in *The Pteridophytes of Mexico* eds. J. T. Mickel and A. R. Smith. New York: The New York Botanical Garden.

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.

Nichols, R. 2001. Gene trees and species trees are not the same. *Trends in Ecology & Evolution* 16: 358–364.

Petersen, J., H. Brinkmann, and R. Cerff. 2003. Origin, evolution, and metabolic role of a novel glycolytic GAPDH enzyme recruited by land plant plastids. *Journal of Molecular Evolution* 57: 16–26.

Polz, M. F. and C. M. Cavanaugh. 1998. Bias in template-to-product ratios in multitemplate PCR. *Applied and Environmental Microbiology* 64: 3724–3730.

Posada, D. and K. A. Crandall. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14: 817–818.

Rambaut, A. 2002. Se-Al, version 2.0a11. Available at: http://tree.bio.ed.ac.uk/software/seal.

Rambaut, A. 2008. FigTree, version 1.1. Available at: http://tree.bio.ed.ac.uk/software/figtree.

Rambaut, A. and A. J. Drummond. 2007. Tracer, version 1.4. Available at: http://beast.bio.ed.ac.uk/tracer.

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

Sang, T. and Y. Zhong. 2000. Testing hybridization hypotheses based on incongruent gene trees. *Systematic Biology* 49: 422–434.

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

Schuettpelz, E., A. L. Grusz, M. D. Windham, and K. M. Pryer. 2008. The utility of nuclear *gapCp* in resolving polyploid fern origins. *Systematic Botany* 33: 621–629.

Simmons, M. P. and H. Ochoterena. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* 49: 369–381.

Slotte, T., A. Ceplitis, B. Neuffer, H. Hurka, and M. Lascoux. 2006. Intrageneric phylogeny of *Capsella* (Brassicaceae) and the origin of the tetraploid *C. bursa-pastoris* based on chloroplast and nuclear DNA sequences. *American Journal of Botany* 93: 1714–1724.

Small, R. L. and J. F. Wendel. 2000. Copy number lability and evolutionary dynamics of the *Adh* gene family in diploid and tetraploid cotton (*Gossypium*). *Genetics* 155: 1913–1926.

Sullivan, J., J. A. Markert, and C. W. Kilpatrick. 1997. Phylogeography and molecular systematics of the *Peromyscus aztecus* species group (Rodentia: Muridae) inferred using parsimony and likelihood. *Systematic Biology* 46: 426–440.

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

Toledo, V. M. and M. D. Ordóñez. 1993. The biodiversity scenario of Mexico: a review of terrestrial habitats. Pp. 757–777 in *Biological diversity of Mexico: origins and distribution*. eds. T. P. Ramamoorthy, R. Bye, A. Lot, and J. Fa. New York: Oxford University Press.

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

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

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. Jr. 1970. Biosystematics and evolutionary noise. *Taxon* 19: 146–151.

Weatherby, C. A. 1943. Polypodiaceae. Pp. 308–324 in Plants of Coahuila, Eastern Chihuahua and adjoining Zacatecas and Durango I. ed. I. M. Johnston. *Journal of the Arnold Arboretum* 24: 306–339.

Werth, C. R. and D. B. Lellinger. 1992. Genomically preserved plants and their nomenclature. *Taxon* 41: 513–521.

Werth, C. R. and M. D. Windham. 1991. A model for divergent, allopatric speciation of polyploid pteridophytes resulting from silencing of duplicate gene expression. *American Naturalist* 137: 515–526.

Windham, M. D. and I. A. Al-Shehbaz. 2006. New and noteworthy species of *Boechera* (Brassicaceae) I: sexual diploids. *Harvard Papers in Botany* 11: 61–88.

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. and G. Yatskievych. 2005. A novel hybrid *Polypodium* (Polypodiaceae) from Arizona. *American Fern Journal* 95: 57–67.

Xiang, L. L., C. R. Werth, S. N. Emery, and D. E. McCauley. 2000. Population-specific gender-biased hybridization between *Dryopteris intermedia* and *D. carthusiana*: evidence from chloroplast DNA. *American Journal of Botany* 87: 1175–1180.

Appendix 1. Sample information. Taxon, collection site, collector (voucher location) (year collected), Pryer Lab Fern Database # (see http://www.pryerlab.net/DNA_database.shtml), average spore diameter, *trnGR* EMBL accession #, *gapCp* EMBL accession #, number of *gapCp* clones analyzed (number of *gapCp* clones initially considered).

***A. cochisensis*** (Goodd.) D. M. Benham & Windham subsp. ***chihuahuensis*** D. M. Benham, U. S. A., New Mexico, Eddy Co., *Windham 3490* (DUKE) (2007), DB4716, 43.34 µm, FN565508, FN568525–FN568528, 4 (9); U. S. A., New Mexico, Chaves Co., *Windham 3504* (DUKE) (2007), DB4718, 44.75 µm, FN565509, FN568529–FN568534, 6 (10); U. S. A., Texas, Terrell Co., *Beck 1035* (DUKE) (2008), DB5651, 44.16 µm, FN565510, FN568535–FN568538 4 (8). ***A. laevis*** (M. Martens & Galeotti) Mickel, Mexico, Oaxaca, Chicahauxtla, *Breedlove & Almeda 59793* (US) (1983), DB5829, 40.81 µm, FN565518, FN568574–FN568582, 9 (14); Mexico, Sinaloa, El Palmito, *Mayfield 1674* (TEX-LL) (1993), DB6111, 46.92 µm, FN565519, FN568583–FN568586, 4 (10). ***A. sinuata*** (Lag. ex Sw.) D. M. Benham & Windham subsp. ***mexicana*** D. M. Benham, U. S. A., Texas, Jeff Davis Co., *Schuettpelz 310* (DUKE) (2004), DB2955, 41.30 µm, FN565511, FN568539–FN568545, 7 (14); Mexico, Mexico, Ixtapen de la Sal, *Mayfield et al. 1032* (TEX-LL) (1991), DB6126, 38.03 µm, FN565512, FN568546–FN568548, 3 (9). ***A. obscura*** J. Beck & Windham, Mexico, Querétaro, Cadereyta, *Díaz & Carranza 7481* (NY) (1993), DB6142, 44.62 µm, FN565513, FN568549–FN568554, 6 (12); Mexico, Guanajuato, Xichú, *Pérez & Zamudio 3559* (NY) (1997), DB6143, 44.85 µm, FN565514, FN568555–FN568561, 7 (11). ***A. deltoidea*** (Baker) J. Beck & Windham, Mexico, Guerrero, Carrizal, *Fonseca & Velázquez 2475* (NY) (1998), DB6144, 41.92 µm, FN565516, FN568568–FN568569, 2 (8); Guatemala, Lake Atitlán, *Hatch & Wilson 279* (US) (1936), DB5828, 44.82 µm, FN565515, FN568562–FN568567, 6 (16); Mexico, Hidalgo, Posada del Rey, *Mears 278* (TEX-LL) (1966), DB6110, all dehisced, FN565517, FN568570–FN568573, 4 (6). ***Argyrochosma fendleri*** (Kunze) Windham, U. S. A., New Mexico, Bernalillo Co., *Metzgar 120* (DUKE) (2006), DB3776, NA, FN565504, NA. ***Paragymnopteris marantae*** (L.) K. H. Shing, China, Yunnan Province, Jianchuan, *Yatskievych 02-35* (MO) (2002), DB3736, NA, EU268711, NA. ***Pellaea sagittata*** Link., Mexico, Michoacán, *Steinmann 1939* (MO) (2001), DB5029, NA, FN565507, NA. ***Pellaea pringlei*** Davenp., Mexico, Michoacán, *Steinmann 4728* (MO) (2004), DB5028, NA, FN565506, NA. ***Pellaea truncata*** Goodd., U. S. A., Texas, Culberson Co., *Windham & Yatskievych 763* (DUKE) (1985), DB4911, NA, FN565505, NA.