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Identifying multiple origins of polyploid taxa: A multilocus study of the hybrid cloak fern (*Astrolepis integerrima*; Pteridaceae)[1]

James B. Beck[2,5], James R. Allison[3], Kathleen M. Pryer[4], and Michael D. Windham[4]

[2]Department of Biological Sciences, Wichita State University, 537 Hubbard Hall, Wichita, Kansas 67260 USA; [3]2241 Highway 212 SW, Conyers, Georgia 30094 USA; and [4]Department of Biology, Duke University, Box 90338 Durham, North Carolina 27708 USA

- *Premise of the study:* Molecular studies have shown that multiple origins of polyploid taxa are the rule rather than the exception. To understand the distribution and ecology of polyploid species and the evolutionary significance of polyploidy in general, it is important to delineate these independently derived lineages as accurately as possible. Although gene flow among polyploid lineages and backcrossing to their diploid parents often confound this process, such post origin gene flow is very infrequent in asexual polyploids. In this study, we estimate the number of independent origins of the apomictic allopolyploid fern *Astrolepis integerrima*, a morphologically heterogeneous species most common in the southwestern United States and Mexico, with outlying populations in the southeastern United States and the Caribbean.
- *Methods:* Plastid DNA sequence and AFLP data were obtained from 33 *A. integerrima* individuals. Phylogenetic analysis of the sequence data and multidimensional clustering of the AFLP data were used to identify independently derived lineages.
- *Key results:* Analysis of the two datasets identified 10 genetic groups within the 33 analyzed samples. These groups suggest a minimum of 10 origins of *A. integerrima* in the northern portion of its range, with both putative parents functioning as maternal donors, both supplying unreduced gametes, and both contributing a significant portion of their genetic diversity to the hybrids.
- *Conclusions:* Our results highlight the extreme cryptic genetic diversity and systematic complexity that can underlie a single polyploid taxon.

Key words: AFLP; *Astrolepis integerrima*; hybridization; Ketona Glades; multiple origins; PCO-MC; polyploidy; Pteridaceae; *trnG-trnR* intergenic spacer.

Almost 30 years ago, isozyme analyses of a small group of ferns in the genus *Asplenium* provided the first solid evidence that a single polyploid species could have multiple, independent origins (Werth et al., 1985). Subsequent molecular studies have shown that this is the rule rather than the exception among polyploid taxa scattered across the tree of life (reviewed in Soltis and Soltis, 1993, 1999). This insight poses a crucial evolutionary question: to what extent do individual lineages derived from multiple origins exhibit novel genotypes and phenotypes resulting in differing evolutionary trajectories and cryptic species (Soltis et al., 2003, 2010; Cifuentes et al., 2010)? To address this question, one must begin with an accurate inventory of the individual lineages within a focal polyploid assemblage. Establishing individual lineages can involve identifying groups of polyploid individuals that bear alternative parental diploid haplotypes (Thompson and Whitton, 2006; Grusz et al., 2009; Meimberg et al., 2009; Wu et al., 2010) or demonstrating the existence of distinct polyploid genetic clusters with multilocus data (Albach, 2007; Perrie et al., 2010; Cosendai et al., 2011; Sampson and Byrne, 2012). Unfortunately, these inferences can often be confounded by gene flow among recurrently formed polyploid lineages or between the polyploids and their diploid parents. Such post-origin gene flow is very infrequent in asexual polyploids, particularly if the transition to asexuality was concurrent with the polyploidization event or occurred shortly thereafter. Fortunately, many polyploid plants (Asker and Jerling, 1992) and animals (Suomalainen et al., 2000; Simon et al., 2003; Kearney, 2005) are asexual, providing opportunities to more confidently identify independent lineages and examine their evolutionary trajectories.

In this study, we attempt to rigorously identify independently derived lineages within the asexual polyploid fern *Astrolepis integerrima* (Hook.) D. M. Benham & Windham (Pteridaceae). As in most ferns, asexuality in *A. integerrima* involves a mature triploid sporophyte producing triploid spores through a modified, nonreductive meiosis known as Döpp–Manton sporogenesis (Gastony and Windham, 1989). These spores are dispersed and give rise to free-living triploid gametophytes, from which a new triploid sporophyte develops via mitosis. This production of nonreduced spores and the subsequent development of a sporophyte directly from a gametophyte without fertilization has been termed "apomictic alternation of generations" (Walker, 1979) and will be referred to hereafter simply as apomixis. *Astrolepis integerrima* is widespread, occurring from central

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[5]Author for correspondence (e-mail: james.beck@wichita.edu)

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Mexico to Colorado and Nevada, with disjunct populations in Alabama and the Caribbean (Benham and Windham, 1993; Allison and Stevens, 2001; Mickel and Smith, 2004). Hevly (1965) suggested a possible hybrid origin for *A. integerrima*, and Benham (1989) tested this hypothesis using comparative morphology, cytology, and isozymes. Benham (1989) concluded that populations of *A. integerrima* in the southwestern United States and northern Mexico represented an apomictic triploid hybrid between *A. cochisensis* (Goodd.) D. M. Benham & Windham and a second diploid species unknown to science. This "missing" diploid was subsequently discovered and described as *Astrolepis obscura* J. Beck and Windham (Beck et al., 2010).

Astrolepis integerrima is morphologically heterogeneous, with notable variation in plant size, pinnae size/lobing, and pinnae scale morphology (Benham and Windham, 1993; Mickel and Smith, 2004; J. B. Beck, personal observations). This variability suggests that *A. integerrima*, like many apomictic polyploids, is an assemblage of lineages resulting from multiple independent origins. Gene flow among homoploid apomictic lineages in ferns is prevented by the lack of functional archegonia on the gametophytes (Gastony and Haufler, 1976; Gastony and Windham, 1989; Morzenti, 1966; Walker, 1962; Whittier, 1965). Although functional antheridia occasionally are observed (Gastony and Haufler, 1976; Walker, 1962), these only allow unidirectional hybridization between an apomictic lineage and sexual individuals that are capable of functioning as the maternal parent. The results of such hybridizations are new, higher ploidy, apomictic lineages (see Gastony and Yatskievych, 1992), not gene flow into the respective apomictic and sexual parental taxa. The hypothesis of multiple origins of *A. integerrima* was supported by the isozyme data of Benham (1989) and a recent analysis of DNA sequence data (Beck et al., 2011). The latter study used plastid data to reconstruct evolutionary relationships among sexual diploid *Astrolepis* species and some of their apomictic polyploid offspring. Despite employing only a single, maternally inherited locus, Beck et al. (2011) identified several independently formed lineages within *A. integerrima*, suggesting that a multilocus approach could detect additional origins and also allow for the number of independent lineages in *A. integerrima* to be more rigorously estimated. Here we analyze variation in both the previously used plastid locus and a multilocus nuclear AFLP data set to determine the number of existing independent lineages in a geographically representative set of *A. integerrima* samples from the northern portion of its range.

MATERIALS AND METHODS

Sampling, DNA extraction, plastid sequencing, and AFLP genotyping— Samples were collected broadly across the range of *A. integerrima* in the United States (Benham and Windham, 1993; Allison and Stevens, 2001), including the northernmost (Las Animas Co., Colorado [CO]), westernmost (Clark Co., Nevada [NV]), and easternmost (Bibb Co., Alabama [AL]) known populations (Appendix 1). Multiple individuals were collected in each of eight populations. We collected 31 of our 33 samples in 2008 and the remaining two in 2005 and 2007. All individuals but one (#4719, see the Duke Fern Laboratory Database: http://fernlab.biology.duke.edu) were silica-dried from freshly collected material. Eighteen of the 33 samples exhibited mature spores, and both the number of spores per sporangium and mean spore diameter were determined for each of these to assess reproductive mode and ploidy level, respectively, as in our previous studies (Beck et al., 2010, 2011).

Genomic DNA was extracted following protocols in 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 and sequenced for all samples as detailed in Beck et al. (2010). The genomic DNA samples were also subjected to AFLP genotyping following a modified version of the protocol presented in Vos et al. (1995). Duplicate reactions of two samples (#5708 and #5995) were included to assess genotyping error. Restriction-ligations using the *Eco*RI and *Mse*I adapters of Vos et al. (1995) were conducted at 37°C for 12 h. Preamplifications used *Eco*RI+A and *Mse*I+A primers, and cycling conditions consisted of an initial denaturation step (72°C for 2 min) followed by 30 denaturation, annealing, and elongation cycles (94°C for 30 s, 56°C for 30 s, 72°C for 2 min) and a final elongation step (72°C for 5 min). Following the recommendation of Trybush et al. (2006), selective amplifications were performed using the Qiagen Multiplex PCR Master Mix (Qiagen, Germantown, Maryland, USA). Each of three separate selective amplifications involved an unlabeled *Mse*I+ATC primer paired with one of three labeled *Eco*RI primers: *Eco*RI+ACG (NED), *Eco*RI+AGA (6-FAM), or *Eco*RI+ATG (HEX). Reactions (10 μL total) included 5 μL Qiagen Multiplex Master Mix, 0.7 μmol/L each *Mse*I/ *Eco*RI primer, 2.6 μL water, and 1 μL (1 : 20) diluted preamplification product. Selective amplification cycling conditions consisted of an initial denaturation step (94°C for 15 min) followed by 10 cycles of (94°C for 20 s, 66°C for 30 s [−1°C per cycle], 72°C for 2 min), 20 cycles of (94°C for 20 s, 56°C for 30 s, 72°C for 2 min), and a final elongation step (60°C for 30 min). Selective amplification products were diluted (1 : 10) and sized using the 500 ROX standard on an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, California, USA) at the Duke University Genome Sequencing & Analysis Core Resource (Durham, North Carolina, USA).

Data analysis— Plastid sequence data from the 33 *A. integerrima* samples were analyzed along with those from 22 individuals representing the five sexual diploid *Astrolepis* species (Beck et al., 2010) and three outgroup samples from *Pellaea* Link. 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*. Within this clade, *Astrolepis* is sister to a small lineage of the polyphyletic genus *Pellaea* that includes *P. cordifolia* (Sessé & Moc.) A. R. Sm., *P. pringlei* Davenp., and *P. sagittata* Link. The latter two species plus a more distantly related sexual diploid, *P. truncata* Goodding, were used as outgroups. The *trnGR* data set was manually aligned in Se-Al 2.0 (Rambaut, 2002), and the analyzed matrix is available from TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S13385). All insertion/deletion events and regions of uncertain alignment were excluded from further analysis. A heuristic maximum parsimony search with 100 random-addition replicates was performed using PAUP* 4.0b10 (Swofford, 2002) with the following parameters: starting trees obtained by stepwise addition, tree-bisection-reconnection (TBR) branch swapping, MulTrees turned on, steepest descent not in effect, and zero-length branches collapsed. The resulting most parsimonious tree was drawn using FigTree 1.3 (Rambaut, 2009). One thousand bootstrap replicates, each with 100 random-addition replicates, were conducted with PAUP* 4.0b10 to obtain bootstrap support. In addition, a Bayesian Markov chain Monte Carlo analysis was performed in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). The GTR + I model of character evolution was assumed based on a previous broad analysis of *trnGR* variation in *Astrolepis* (Beck et al., 2011). All analyses comprised four independent runs, each with four chains (one cold and three heated). Flat priors were used. Chains were run for five million generations, and trees were sampled every 1000 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, 2009). These diagnostics indicated that runs reached convergence within the first 500 000 generations, and trees sampled during this period were excluded before obtaining clade posterior probabilities.

The preliminary AFLP presence-absence matrices for each *Mse*I/ *Eco*RI set were determined in GeneMarker 1.9 (SoftGenetics, State College, Pennsylvania, USA). Bins (1-bp width) were automatically constructed between 50 and 500 bp, and alleles were called using the smoothing, stutter-peak filter off, fail<1check<1pass, and 50 rfu peak height threshold settings recommended by Holland et al. (2008). To choose the most reliable subset of loci and the optimal allele-calling threshold, we analyzed these three preliminary matrices with AFLPScore (Whitlock et al., 2008) on the R platform (R Development Core Team). These criteria were used to construct enhanced allele matrices for each *Mse*I/ *Eco*RI set, and the three matrices were combined to form a total allele matrix. To visualize the relative genetic position of samples, we subjected the total allele matrix to a principal coordinates analysis (PCoA) in GENALEX 6.0 (Peakall and Smouse, 2006) using a standardized covariance matrix derived

from a binary genotypic genetic distance (Huff et al., 1993). The total matrix was then subjected to the multidimensional clustering approach employed in the principal coordinates analysis with modal clustering (PCO-MC) workflow (Reeves and Richards, 2009, 2011). Briefly, PCO-MC identifies the most genetically cohesive groups in a data set by simultaneously considering information on all axes of a PCoA, ranking each group by a "stability value" based on the density of the group in multidimensional space.

RESULTS

Reproductive mode and ploidy level—All 18 individuals with mature spores exhibited 32 spores per sporangium, which is characteristic of apomictic individuals in *Astrolepis* (Beck et al., 2010 and references therein). The range of mean spore diameters (56.1–68.7 μm) indicated that all of these samples were triploids based on previous studies correlating spore size with chromosome counts (Benham, 1989; Beck et al., 2010).

Plastid phylogeny—The analyzed *trnGR* matrix of 1115 aligned characters yielded 184 (17%) variable and 130 (12%) parsimony-informative characters. Each of the 100 random-addition replicate parsimony searches recovered the same most parsimonious tree (length = 220, CI = 0.86, RI = 0.98). This tree, along with bootstrap percentages and Bayesian posterior probabilities, is shown in Fig. 1. As in earlier studies (Beck et al., 2010, 2011), there was robust support for *Astrolepis* as a whole, for each of the five recognized sexual diploid *Astrolepis* species (*A. cochisensis*, *A. sinuata*, *A. laevis*, *A. obscura*, and *A. deltoidea*), and for several subspecific clades. The 33 *A. integerrima* samples exhibited six haplotypes (P1–P6, Fig. 1). Two haplotypes (P1, P2) were placed within the *A. cochisensis* clade, with the remaining four (P3–P6) placed in the *A. obscura* clade, which is consistent with the hypothesized parentage (*A. cochisensis* × *A. obscura*) of the northern populations of *A. integerrima* (Benham, 1989; Beck et al., 2010). Although cytoplasmic inheritance has been rigorously examined in few fern species, plastids have been shown to be maternally inherited in the closely related genus *Pellaea* (Gastony and Yatskievych, 1992). The observed plastid variability therefore suggests that both parental diploid species have served as maternal donors to *A. integerrima*. Individual origins were inferred in each case where a unique plastid haplotype was observed in an *A. integerrima* individual(s) that must have been obtained from a sampled or inferred maternal diploid individual, not another *A. integerrima* individual via de novo mutation. The six haplotypes (P1–P6; discussed below) imply a minimum of five origins of northern *A. integerrima*, as we cannot exclude the possibility that haplotype P6 was derived from P5 via post polyploid mutation based on the plastid data alone. Our inferences of these five origins are as follows.

Five samples from three populations of *A. integerrima* (Las Animas Co., CO; Howard Co., Texas [TX]; Brewster Co., TX) shared a haplotype (P1) with four samples of sexual diploid *A. cochisensis* (Figs. 1, 2A). Four samples from two populations (Pinal Co., Arizona [AZ]; Pima Co., AZ) shared an alternative *A. cochisensis* haplotype (P2; Figs. 1, 2A). Although no sampled sexual diploid individual of *A. cochisensis* exhibited haplotype P2, the existence of such a diploid can be inferred from previous work (Beck et al., 2011) that showed an apomictic autotetraploid *A. cochisensis* individual also shares haplotype P2. Neither of these two apomictic polyploid lineages could have given rise to the other; beyond exhibiting different genomic combinations, any offspring resulting from the union of their triploid and tetraploid gametes would be septaploid.

Four haplotypes attributable to the *A. obscura* clade were observed among the sampled individuals of *A. integerrima* (P3–P6; Fig. 1). Two different plants sampled from a single population (Double Glade: Bibb Co., AL) exhibited the P3 haplotype (Fig. 2A). Although no sampled sexual diploid individual of *A. obscura* shared this haplotype, the existence of such a diploid can be inferred from Beck et al. (2011), where several individuals of the apomictic trigenomic allotriploid *Astrolepis windhamii* D. M. Benham (*A. obscura* × *A. cochisensis* × *A. sinuata*) also exhibited the P3 haplotype. As in the case of P2, neither of the sampled allotriploid *Astrolepis* lineages exhibiting haplotype P3 (*A. windhamii* or *A. integerrima*) could have given rise to the other, suggesting that this haplotype was obtained from a shared diploid parent (in this case, *A. obscura*).

Eight samples of *A. integerrima* from seven populations (Val Verde Co., TX; Fern Glade: Bibb Co., AL; Otero Co., NM; Cochise Co., NM; Culberson Co., TX; Cimarron Co., Oklahoma [OK]; Eddy Co., NM) shared *trnGR* haplotype P4, an *A. obscura*-derived haplotype sister to haplotype P3 (Figs. 1, 2A). An inferred *A. obscura* diploid exhibiting the haplotype P4 also helps to explain the inferred diploid bearing haplotype P3, since the P3 haplotype exhibits a derived mutation that could not have been inherited from the apomictic *A. integerrima* P4 individuals. Four samples from three populations (Real Co., TX; Jeff Davis Co., TX; Bandera Co., TX) shared a haplotype with a sexual diploid individual of *A. obscura* (#6142) sampled from Querétaro, Mexico (haplotype P5; Figs. 1, 2A). Finally, nine samples from five populations (Clark Co., NV; Sierra Co., NM; Howard Co., TX; Doña Ana Co., NM; Val Verde Co., TX) shared an *A. obscura* haplotype (haplotype P6; Figs. 1, 2A). Based solely on our plastid data, it could be argued that haplotype P6 was derived from P5 via post polyploid mutation (and thus a single origin would be inferred for *A. integerrima* populations exhibiting both haplotypes). However, the AFLP data presented below clearly indicate that the individuals bearing haplotypes P5 and P6 are the products of separate hybridization events.

Multilocus clustering—The preliminary AFLP matrices comprised 786 total loci (*Eco*RI+ACG = 229 loci; *Eco*RI+AGA = 303; *Eco*RI+ATG = 254). AFLPScore analysis identified the locus selection and allele calling thresholds that minimized the locus mismatch percentage between each of our two duplicate samples (#5708 [P2] and #5995 [P4]). These thresholds and mismatch percentages included: *Eco*RI+ACG, locus selection threshold = 70% of grand mean peak height, allele calling threshold = 40% of locus mean peak height, average mismatch = 9.6%; *Eco*RI+AGA, 119%, 10%, 4.2%; *Eco*RI+ATG, 158%, 50%, 0%. Applying these thresholds resulted in a final total matrix of 220 loci (*Eco*RI+ACG = 120 loci; *Eco*RI+AGA = 71; *Eco*RI+ATG = 29) and an overall mismatch (error) rate of 6.6%.

The multidimensional clustering approach PCO-MC identified six groups with a stability value >10%, all but one of which are readily apparent in plots of individual scores on principal coordinates 1–3 (Fig. 3). Only plastid haplotype P5 formed a stable, coherent group suggestive of a single origin. The four remaining plastid haplotypes showed substantial diversity in the AFLP data set, suggesting that each arose through multiple, independent hybridization events.

The strongest distinction in the data set is between a group comprising three individuals collected from a single population in Pima Co, AZ (80% stability, Fig. 3A–note that the group

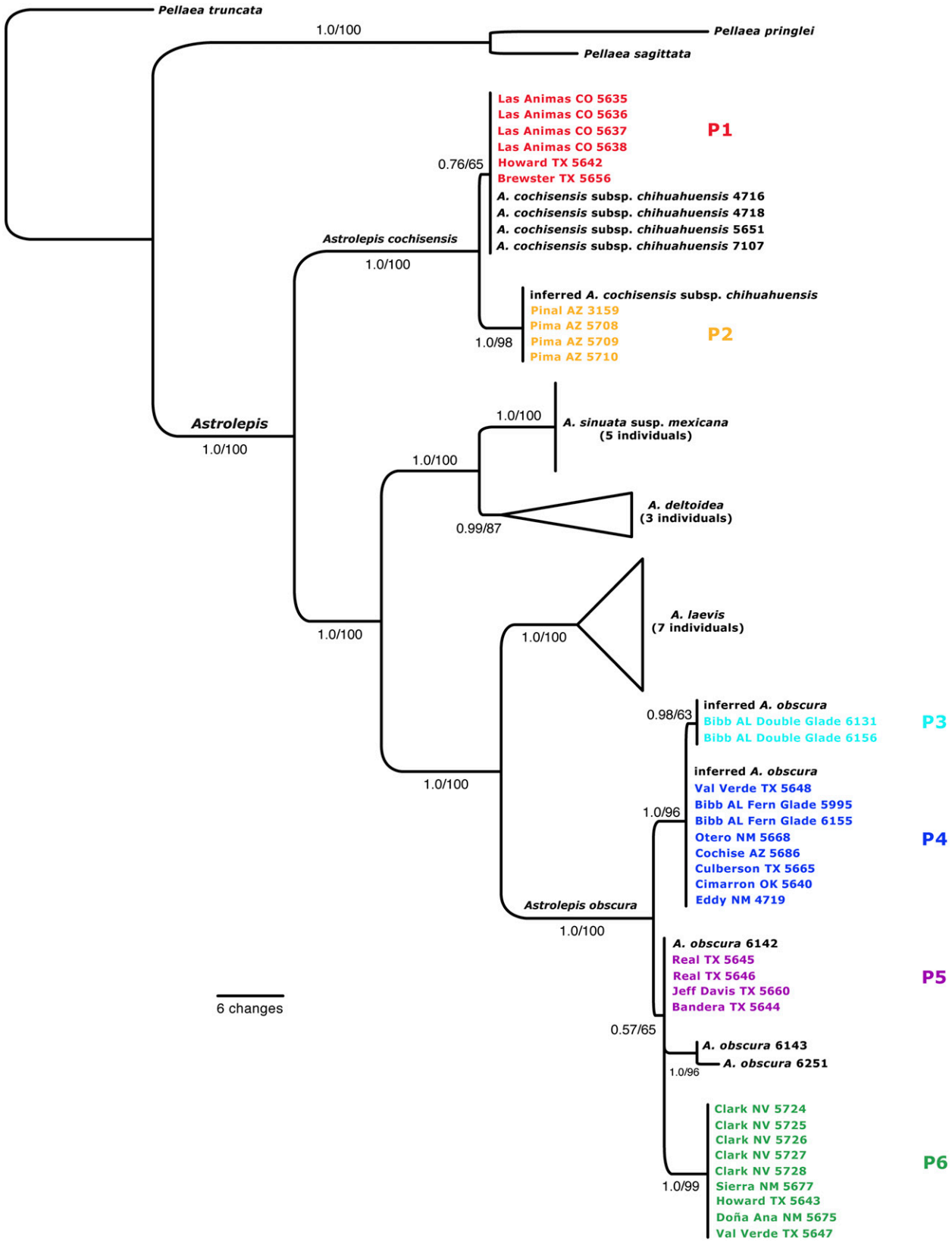

Fig. 1. The single most parsimonious tree (zero-length branches collapsed) inferred from analysis of plastid *trnGR* sequence data in apomictic triploid *Astrolepis integerrima* and the five sexual diploid *Astrolepis* species. Support values appear at each node (Bayesian posterior probability/parsimony bootstrap percentage). Each individual sample is designated with a unique ID number (see Appendix 1). Sampled or inferred diploid individuals appear in black, whereas the six plastid lineages (P1–P6) identified as *A. integerrima* appear in unique colors.

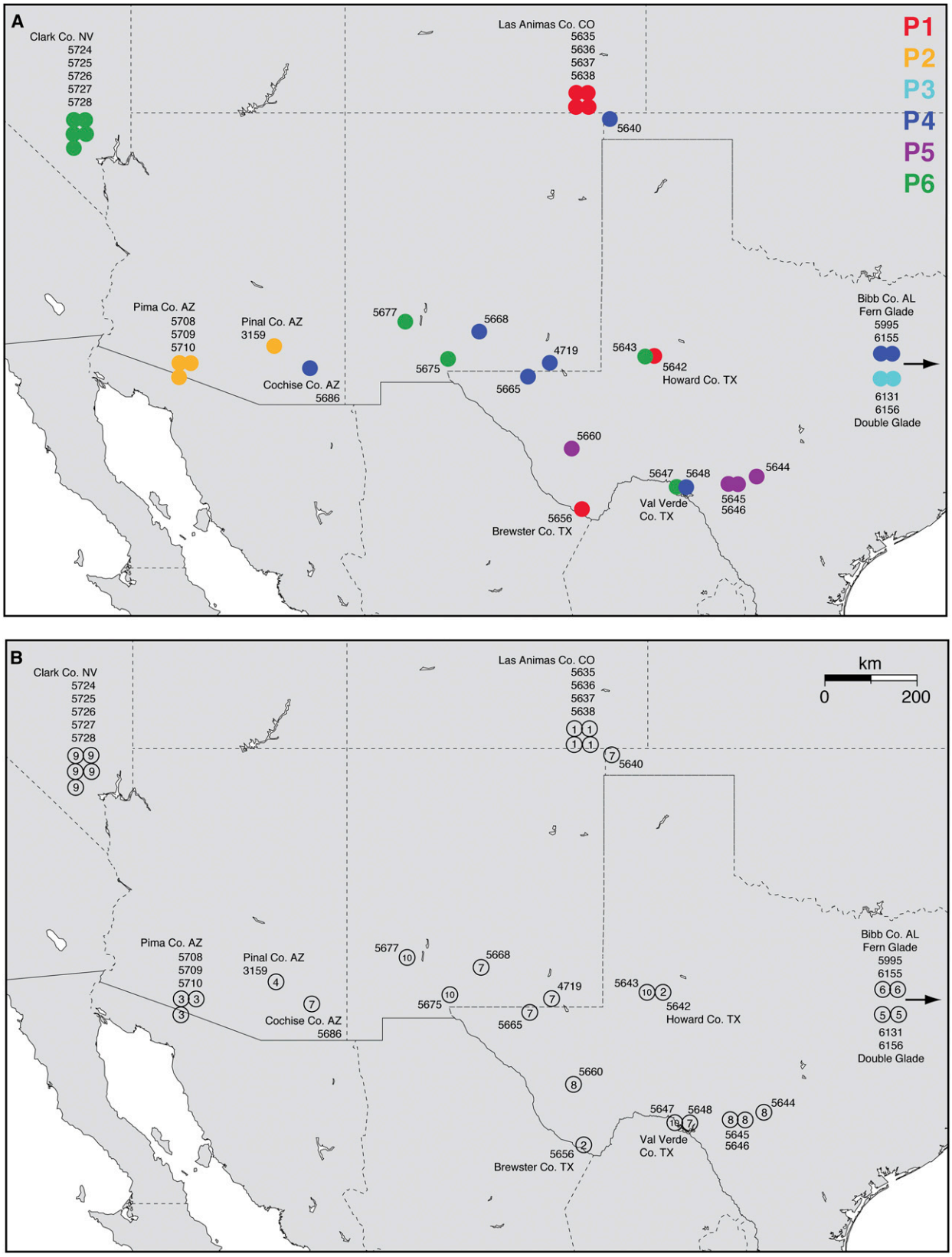

Fig. 2. Map of the southwestern United States and northern Mexico, indicating collection localities for the 33 *Astrolepis integerrima* individuals and their inferred lineage membership. Locality and sample IDs correspond to those in Fig. 1 and Appendix 1. Clusters of circles in contact indicate individuals sampled from a single population. (A) Colors (see legend in upper right) indicate membership in the six plastid lineages identified by the *trnGR* sequence data (Fig. 1). (B) Numbers indicate membership in the 10 independently formed lineages identified by the total (*trnGR* sequence + AFLP) data set.

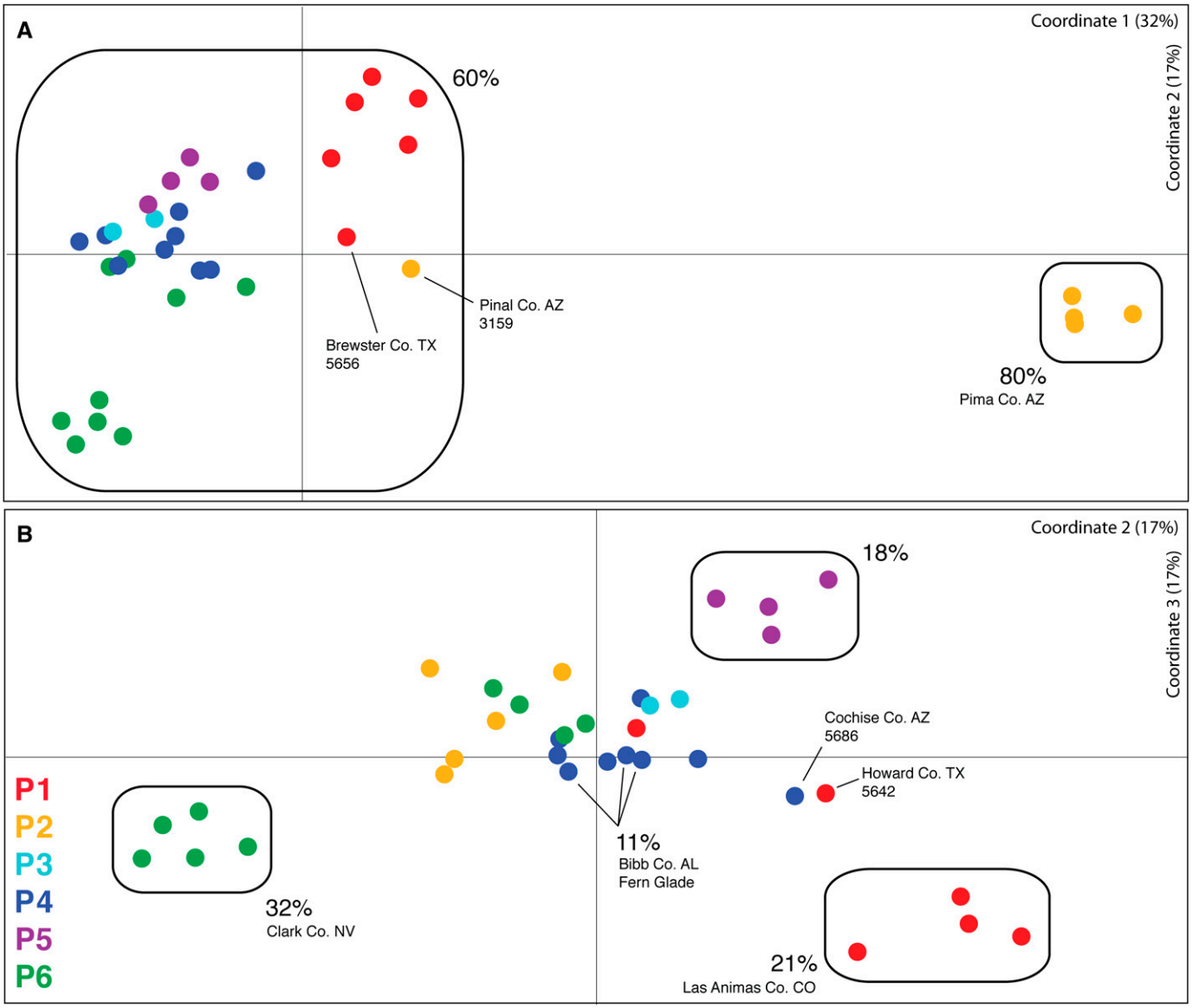

Fig. 3. Results of principal coordinate analysis (PCoA) and principal coordinates analysis with modal clustering (PCO-MC) of the AFLP data set from the 33 *A. integerrima* individuals (including two pairs of duplicates). Colors (see legend in lower left of panel B) indicate membership in the six plastid lineages identified by the *trnGR* sequence data (Fig. 1). The six most highly supported groups identified by PCO-MC are outlined and annotated with their corresponding stability values. Locality information is noted for these groups and for several individuals discussed in the text. (A) Plot of scores on principal coordinates 1 and 2. (B) Plot of scores on principal coordinates 2 and 3.

includes one duplicate reaction) and one encompassing the rest of the data set (60%, Fig. 3A). We suggest that this dramatic genetic contrast is due to differing parental dosages, with one group resulting from hybridization events involving unreduced gametes from *A. cochisensis* (CC) and the other resulting from hybridization events involving unreduced *A. obscura* gametes (OO). Regardless of the exact details, this basic partition establishes that plastid haplotype P2, which spans these groups, includes at least two independent origins.

Five individuals collected from one locality in Clark Co., NV form the next most stable group (32%, Fig. 3B). Although this Nevada population shares plastid haplotype P6 with individuals from New Mexico and Texas (Figs. 1, 2A), the AFLP data establish that the eastern populations (green dots near the center of Fig. 3B) are genetically divergent. This suggests that plastid group P6 also is the result of at least two separate origins. Four individuals collected from a single population in Las Animas Co., CO form the next most stable group (21%, Fig. 3B). Although two different samples from Texas (#5642 and #5656 in Fig. 2) share the P1 plastid haplotype with this Colorado population, the AFLP data indicate that they are genetically distinct and strongly suggest that plastid group P1 is also the result of at least two separate origins. The next most stable group (18%) includes all four individuals that exhibited plastid haplotype P5. The combined plastid and nuclear coherence of this group suggests that it may represent a single origin that has become widely established in west Texas. Finally, PCO-MC identifies a group (11%, Fig. 3B) comprising the two individuals (and the duplicate sample of one of them) collected at "Fern Glade" in Bibb Co., AL. The AFLP data establish that this group is genetically

distinct from other individuals with plastid haplotype P4 (most notably #5686 from Cochise Co., AZ; Fig. 3B), suggesting that plastid group P4 also resulted from at least two separate hybridization events.

Biogeography of independent lineages in A. integerrima— Taken together, the AFLP and plastid DNA data sets identify 10 lineages, the geographic distributions of which are presented in Fig. 2B. Six of the 10 are known from single populations, including lineage 1 (Las Animas Co., CO), lineage 3 (Pima Co., AZ), lineage 4 (Pinal Co., AZ), lineages 5 and 6 (from two different glades in Bibb Co., AL), and lineage 9 (Clark Co., NV). The other four are known from two (lineage 2), three (lineage 8), four (lineage 10) and six (lineage 7) populations, respectively. Of the eight populations from which more than one plant was sampled, six contained a single lineage, including every population at the periphery of the range of *A. integerrima* (Fig. 2B). Within-population lineage diversity was detected at two west Texas localities. Lineages 2 and 10 were found growing together in a Howard Co., TX population, and lineages 7 and 10 co-occurred in a Val Verde Co., TX population.

DISCUSSION

Astrolepis integerrima is an assemblage of many independently formed lineages— Within our relatively small sampling of *A. integerrima*, shared plastid haplotypes with observed or inferred diploid parents unambiguously identify a minimum of five independent origins (Fig. 1). Most of these plastid lineages show significant heterogeneity in the multilocus AFLP analyses (Fig. 3), and the combined data set suggests a minimum of 10 origins among the 33 sampled *A. integerrima* individuals (Fig. 2B). This is almost certainly an underestimate of the actual number of independently formed lineages in our sample, as several genetically distinctive individuals were represented by single specimens (lineage 4: #3159 Pinal Co. AZ; lineage 2: #5656 Brewster Co. TX; lineage 7: #5686 Cochise Co. AZ; lineage 2: #5642 Howard Co. TX) (Fig. 3) and could therefore not be identified as a cohesive group by PCO-MC. Further sampling from Mexico and the Caribbean undoubtedly would identify additional independent lineages. Individuals from these regions exhibit numerous morphotypes (Mickel and Smith, 2004), some of which are not observed in the United States (J. B. Beck, personal observation). The number of lineages documented in this study, combined with the extensive morphological variation observed in unsampled regions, paint a picture of *A. integerrima* as a wide-ranging, heterogeneous assemblage formed by many independent hybridization events.

Biogeographic insights from the disjunct Alabama populations— The disjunct Alabama populations of *A. integerrima* provide some intriguing biogeographic insights. These populations are found on two dolomitic "cedar glades" along the Little Cahaba River in Bibb Co., Alabama (Allison and Stevens, 2001), more than 900 km from the closest known populations in Texas. This disjunction fits a larger pattern of primarily central/western North American vascular plant species with disjunct populations in xeric eastern habitats, including glades (Erickson et al., 1942; Baskin and Baskin, 1986; DeSelm et al., 1997), granite outcrops (McVaugh, 1943), and barrens (Core, 1952; Keener, 1983; Bartgis, 1993; DeSelm, 1994; Heikens and Robertson, 1995; Webb et al., 1997). This pattern could have resulted from the regional expansion of entire xeric communities during past periods of elevated temperature, or it could reflect long-distance dispersal events by individual taxa (Palmer, 1922; McVaugh, 1943; DeSelm, 1994; DeSelm et al., 1997). Surprisingly, this notable biogeographic pattern has been the subject of only one other molecular study (Van Ee et al., 2006).

Both this and our earlier work (Beck et al., 2011) provide important insights into how and when *A. integerrima* reached the eastern United States. The data clearly establish that each of the two Alabama glades harbors a unique *A. integerrima* lineage, indicating that a single spore or otherwise genetically homogenous dispersal event is not sufficient to explain the disjunction. Rather, a genetically variable group of spores or multiple dispersal events was required. These dispersals must have occurred relatively recently, as our earlier study (Beck et al., 2011) estimated that the *A. integerrima* lineage present in the Double Glade population in Bibb Co. is ca. 0.2 Myr old, and the lineage present in the Fern Glade population is ca. 0.5 Myr old. Since these are age estimates for the lineages themselves, the dispersal of individual spores from each of these lineages to eastern North America must be more recent, perhaps far more recent. Our data therefore strongly suggest an arrival of *A. integerrima* in the eastern United States during the late Pleistocene, probably via at least two dispersal events.

Future study of recurrently formed asexual polyploids— Asexual polyploids provide an opportunity to investigate the immediate and near-term genomic and phenotypic consequences of polyploidy without the complicating effects of gene flow (Schaack, 2008; Verhoeven et al., 2010; Moritz and Bi, 2011). In these cases the oft-cited disadvantage to the asexual organism (i.e., the lack of evolutionary fluidity provided by sexuality) is clearly advantageous for the study of lineage diversity. In this study, we have demonstrated that a multilocus approach can be used to better understand the origin and geographic distribution of the independently formed lineages that compose a recurrently formed asexual polyploid. In a sample of just 33 individuals of *A. integerrima*, we found evidence for at least 10 different origins, with both putative parents functioning as the maternal donor, both supplying unreduced gametes, and both contributing a significant portion of their genetic diversity to the hybrids. This genetic heterogeneity is likely to be the rule, rather than the exception, and future studies will need to increase sampling to understand the finer details of the genetic and geographic mosaic created by hybridization, polyploidy, and asexuality.

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APPENDIX 1. Sampling information for the 33 *Astrolepis integerrima* and 22 sexual diploid *Astrolepis* individuals analyzed in the study. Herbarium acronyms follow Index Herbariorum (Thiers, 2012). Missing spore data are indicated with a dash (–). Two samples for which a duplicate AFLP reaction was performed are indicated with an asterisk.

Taxon; Duke Fern Laboratory Database number (http://fernlab.biology.duke.edu), Locality, *Collector and number*, Herbarium, *trnGR* GenBank or EMBL number, Number of spores per sporangium, Mean spore diameter (μm).

Astrolepis integerrima (Hook.) D.M. Benham & Windham; 3159, Arizona: Pinal County, *Schuettpelz 452*, DUKE, HE985191, –, –. 4719, New Mexico: Eddy Co., *Windham 3492*, DUKE, JF929949, 32, 66.281. 5635, Colorado: Las Animas Co., *Beck 1019*, DUKE, HE985192, –, –. 5636, Colorado: Las Animas Co., *Beck 1020*, DUKE, JF929915, 32, 59.328. 5637, Colorado: Las Animas Co., *Beck 1021*, DUKE, JF929914, 32, 60.731. 5638, Colorado: Las Animas Co., *Beck 1022*, DUKE, HE985193, –, –. 5640, Oklahoma: Cimarron Co., *Beck 1024*, DUKE, HE985194, –, –. 5642, Texas: Howard Co., *Beck 1026*, DUKE, HE985195, –, –. 5643, Texas: Howard Co., *Beck 1027*, DUKE, HE985196, –, –. 5644, Texas: Bandera Co., *Beck 1028*, DUKE, HE985197, –, –. 5645, Texas: Real Co., *Beck 1029*, DUKE, JF929937, 32, 60.533. 5646, Texas: Real Co., *Beck 1030*, DUKE, JF929938, 32, 64.627. 5647, Texas: Val Verde Co., *Beck 1031*, DUKE, JF929961, 32, 66.419. 5648, Texas: Val Verde Co., *Beck 1032*, DUKE, JF929950, 32, 62.866. 5656, Texas: Brewster Co., *Beck 1040*, DUKE, JF929916, 32, 75.023. 5660, Texas: Jeff Davis Co., *Beck 1044*, DUKE, JF929939, 32, 62.318. 5665, Texas: Culberson Co., *Beck 1049*, DUKE, JF929953, 32, 68.356. 5668, New Mexico: Otero Co., *Beck 1052*, DUKE, HE985198, –, –. 5675, New Mexico: Dona Aña Co., *Beck 1059*, DUKE, HE985199, –, –. 5677, New Mexico: Sierra Co., *Beck 1061*, DUKE, JF929960, 32, 64.733. 5686, Arizona: Cochise Co., *Beck 1070*, DUKE, HE985200, –, –. 5708*, Arizona: Pima Co., *Beck 1086*, DUKE, HE985201, –, –. 5709, Arizona: Pima Co., *Beck 1087*, DUKE, HE985202, –, –. 5710, Arizona: Pima Co., *Beck 1088*, DUKE, JF929925, 32, 68.665. 5724, Nevada: Clark Co., *Beck 1102*, DUKE, HE985203, –, –. 5725, Nevada: Clark Co., *Beck 1103*, DUKE, HE985204, –, –. 5726, Nevada: Clark Co., *Beck 1104*, DUKE, JF929958, 32, 62.855. 5727, Nevada: Clark Co., *Beck 1105*, DUKE, JF929959, 32, 56.138. 5728, Nevada: Clark Co., *Beck 1106*, DUKE, HE985205, –, –. 5995*, "Alabama: Bibb Co. Fern Glade, *Allison 13935*, DUKE, JF929951, 32, 63.554. 6131, "Alabama: Bibb Co. Double Glade, *Allison 13936*, DUKE, JF929948, 32, 67.639. 6155, "Alabama: Bibb Co. Fern Glade, *Allison 13935*, DUKE, HE985206, 32, 63.554. 6156, "Alabama: Bibb Co. Double Glade, *Allison 13936*, DUKE, HE985207, 32, 67.639. ***Astrolepis cochisensis*** (Goodd.) D.M. Benham & Windham **subsp. *chihuahuensis*** D.M. Benham; 4716, New Mexico: Eddy Co., *Windham 3490*, DUKE, FN565508, –, –. 4718, New Mexico: Chaves Co., *Windham 3504*, DUKE, FN565509, –, –. 5651, Texas: Terrell Co., *Beck 1035*, DUKE, FN565510, –, –. 7107, Mexico: Coahuila, *Burge 1192-1*, DUKE, JF929918, –, –. ***Astrolepis deltoidea*** (Baker) J.B. Beck & Windham; 6884, Mexico: Hidalgo, *Beck 1109*, DUKE, FN565518, –, –. 5828, Guatemala, *Hatch and Wilson 279*, US, FN565515, –, –. 6144, Mexico: Guerrero, *Fonseca 2475*, NY, FN565516, –, –. ***Astrolepis laevis*** (M. Martens & Galeotti) Mickel; 6895, Mexico: Oaxaca, *Beck 1202*, DUKE, JF929965, –, –. 5829, Mexico: Oaxaca, *Breedlove 59793*, US, JF929964, –, –. 6217, Mexico: Querétaro, *Carranza 3307*, NY, JF929966, –, –, 7167, Mexico: Zacatecas, *Lane 2448*, TEX, JF929967, –, –. 6111, Mexico: Sinaloa, *Mayfield 1674*, TEX, FN565519, –, –. 6891, Mexico: Guerrero, *Beck 1191*, DUKE, JF929973, –, –. 6892, Mexico: Guerrero, *Beck 1193*, DUKE, JF929974, –, –. ***Astrolepis obscurus*** J.B. Beck & Windham; 6142, Mexico: Querétaro, *Diaz 7481*, NY, FN565513, –, –. 6143, Mexico: Guanajuato, *Pérez 3559*, NY, FN565514, –, –. 6251, Mexico: Querétaro, *Steinmann 2500*, MO, JF929942, –, –. ***Astrolepis sinuata*** (Lag. ex Sw.) D.M. Benham & Windham **subsp. *mexicana*** D.M. Benham; 6126, Mexico: Mexico, *Mayfield 1032*, TEX, FN565512, –, –. 6212, Mexico: Chiapas, *Martinez 23874*, NY, JF929932, –, –. 7166, Mexico: Jalisco, *Holmes 9010*, TEX, JF929933, –, –. 6907, Mexico: Oaxaca, *Beck 1208*, DUKE, JF929935, –, –. 2955, Texas: Jeff Davis Co., *Schuettpelz 310*, DUKE, FN565511, –, –.