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DO ASEXUAL POLYPLOID LINEAGES LEAD SHORT EVOLUTIONARY LIVES? A CASE STUDY FROM THE FERN GENUS *ASTROLEPIS*

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A life-history transition to asexuality is typically viewed as leading to a heightened extinction risk, and a number of studies have evaluated this claim by examining the relative ages of asexual versus closely related sexual lineages. Surprisingly, a rigorous assessment of the age of an asexual plant lineage has never been published, although asexuality is extraordinarily common among plants. Here, we estimate the ages of sexual diploids and asexual polyploids in the fern genus *Astrolepis* using a well-supported plastid phylogeny and a relaxed-clock dating approach. The 50 asexual polyploid samples we included were conservatively estimated to comprise 19 distinct lineages, including a variety of auto- and allopolyploid genomic combinations. All were either the same age or younger than the crown group comprising their maternal sexual-diploid parents based simply on their phylogenetic position. Node ages estimated with the relaxed-clock approach indicated that the average maximum age of asexual lineages was 0.4 My, and individual lineages were on average 7 to 47 times younger than the crown- and total-ages of their sexual parents. Although the confounding association between asexuality and polyploidy precludes definite conclusions regarding the effect of asexuality, our results suggest that asexuality limits evolutionary potential in *Astrolepis*.

KEY WORDS: **Lineage-age, Mexico, phylogeny, plastid DNA, spore.**

Sexual reproduction is a pervasive feature of global biodiversity, and its preponderance over asexual alternatives is a longstanding evolutionary puzzle (Smith 1978; Bell 1982). The relative benefits of sexual and asexual reproduction have been thoroughly reviewed (Kondrashov 1993; West et al. 1999; Rice 2002; Otto and Gerstein 2006), with the general conclusion that sexuality confers distinct advantages in heterogeneous environments. In sexual lineages, recombination generates the genetic variability necessary for adaptation while simultaneously purging deleterious mutations. With little or no opportunity for recombination, theory predicts that asexual lineages are largely limited to their initial genotypes and eventually experience "mutational meltdown" as deleterious mutations accumulate (Lynch et al. 1993). These factors have been projected to raise extinction rates in asexual lineages, largely relegating them to the "tips" of the tree of life (Wagner 1970; White 1978; Whitton et al. 2008; but see Judson and Normark 1996 and Schwander and Crespi 2009).

The general absence of large, asexual-only clades among eukaryotes seems to provide a priori evidence for their macroevolutionary failure (van Dijk 2003). However, these theoretical predictions remain largely untested empirically (Janko et al. 2008), and few studies have compared the ages of asexual lineages with their closest sexual relatives. The primary obstacle to such comparisons is the strong connection between asexuality and polyploidy. Many asexual plants (Asker and Jerling 1992) and

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animals (Suomalainen et al. 2000; Simon et al. 2003; Kearney 2005) are polyploid, and various causal mechanisms for this link have been proposed (Archetti 2004; Whitton et al. 2008). Although asexuality is commonly viewed as heightening long-term extinction risk, the effects of polyploidy on lineage potential are less clear. The immediate effects of whole-genome duplication, combined with subsequent (often radical) genomic changes, have profound effects on developmental rate, fertility, and ecological tolerances, each with unpredictable fitness consequences (Levin 1983; Thompson and Lumaret 1992; Otto and Whitton 2000; Ramsey and Schemske 2002; Comai 2005; Otto 2007; Husband et al. 2008). However, if new polyploids can overcome the short-term challenges of radical genomic change, the surviving lineages can produce novel phenotypes that facilitate the exploitation of new niches (Levin 1983, 2002; Ramsey and Schemske 2002; Kearney 2005; Otto 2007). Given the combined, and potentially interacting, influences of polyploidy and sexuality, their association confounds attempts to isolate the effects of asexuality on lineage age in most asexual taxa. This caveat notwithstanding, documenting differences in the evolutionary potential of asexual polyploids versus sexual relatives (diploid or polyploid) is a clear first step in evaluating the evolutionary significance of asexuality.

In the largest study of the relative ages of sexual and asexual lineages undertaken to date, Schwander and Crespi (2009) analyzed terminal branch lengths in 14 published phylogenies and found that, on average, asexual species were not younger than closely related sexual species. However, all of the studies examined by these authors focused on invertebrate animals and little comparable data exist for other groups that comprise significant numbers of asexual taxa. This is particularly true in plants, where asexuality (here defined as the alternation of generations without fertilization, and thus excluding vegetative reproduction) is commonly observed. Not only have the relative ages of closely related asexual and sexual plant lineages never been compared, no rigorous age estimate is available for any asexual plant lineage (Neiman et al. 2009).

Among plants, asexuality is especially common in ferns, where it has been documented in approximately 10% of species (Walker 1979). Asexuality in most ferns involves an adult diploid sporophyte producing diploid spores through a modified, nonreductive meiosis. These spores are dispersed and give rise to free-living diploid gametophytes, from which the diploid sporophyte develops via mitosis. Asexuality is particularly frequent in ferns that occur in seasonally dry environments, and has traditionally been viewed as adaptive because it removes the need for a continuous film of water in which sperm swim to achieve fertilization (Moran 2004). Here we examine the relative ages of sexual and asexual lineages in the fern genus *Astrolepis* (Pteridaceae). Most *Astrolepis* species are found in xeric habitats such as cliffs, rocky slopes, and open juniper/oak/pine woodlands. The sexual, and many of the asexual, taxa occupy a core range centered in the southwestern USA (AZ, NM, TX), Mexico, and Guatemala, whereas some of the asexual lineages have undergone considerable range expansion into the southeastern USA (AL, GA), the Caribbean, and South America (Benham and Windham 1993; Mickel and Smith 2004). As with almost all ferns (Mogie 1992), asexuality is obligate in *Astrolepis*, and the group exhibits the characteristic association between ploidy and sexuality: the five sexual species of *Astrolepis* are exclusively diploid, whereas all of the asexual lineages are auto- or allopolyploids (Benham 1989; Beck et al. 2010). As mentioned above, this tight association between asexuality and polyploidy does not allow for a direct assessment of the effect of sexuality on lineage age per se. Rather, we aim to evaluate the common assertion that asexual polyploid lineages are short-lived compared to their sexual diploid relatives and provide the first rigorous age estimate of an asexual plant lineage.

An analysis of lineage age in *Astrolepis* is greatly facilitated by the strong comparative framework previously developed for the group. Both the circumscription of the sexual diploid species and the relationships among them are clearly established (Benham 1989; Benham and Windham 1992; Beck et al. 2010). The five sexual diploid species have produced an array of asexual polyploid lineages that have been well-documented using data from morphology, cytology, isozymes, and DNA sequences (Benham 1989; Windham and Yatskievych 2003; J. B. Beck et al., unpubl. data). Here, we present a well-resolved plastid phylogeny that includes both the sexual diploid species and the full array of known asexual polyploid lineages. Because plastids are maternally inherited in ferns (Gastony and Yatskievych 1992), the inferred phylogeny exhibits not only the relationships among the diploid species of *Astrolepis*, but also the relationships among the maternal diploid genomes they have contributed to their polyploid offspring. By definition, the maternal genome of any asexual polyploid cannot be older than the total-age of its maternal parent (Fig. 1). The "total-age" refers to the age of the most recent common ancestor (MRCA) of the total clade of the maternal species, which includes its ultimate ancestor and all its descendants, both living and extinct (Jeffries 1979). The "crown-age" includes the MRCA and descendants of extant members of the maternal diploid species (Jeffries 1979). Although the maternal genome of an asexual polyploid cannot be older than the total-age of its maternal sexual diploid parent, it could have arisen very early in the evolutionary history of this parent and thus could be older than the maternal sexual diploid crown-age, appearing sister to all extant sexual individuals (Fig. 1A). Alternatively, a recently derived asexual lineage would appear deeply nested within the crown group of the maternal sexual diploid species (Fig. 1B).

In this study, we attempt to determine the relative ages of a series of asexual lineages in *Astrolepis* by comparing their

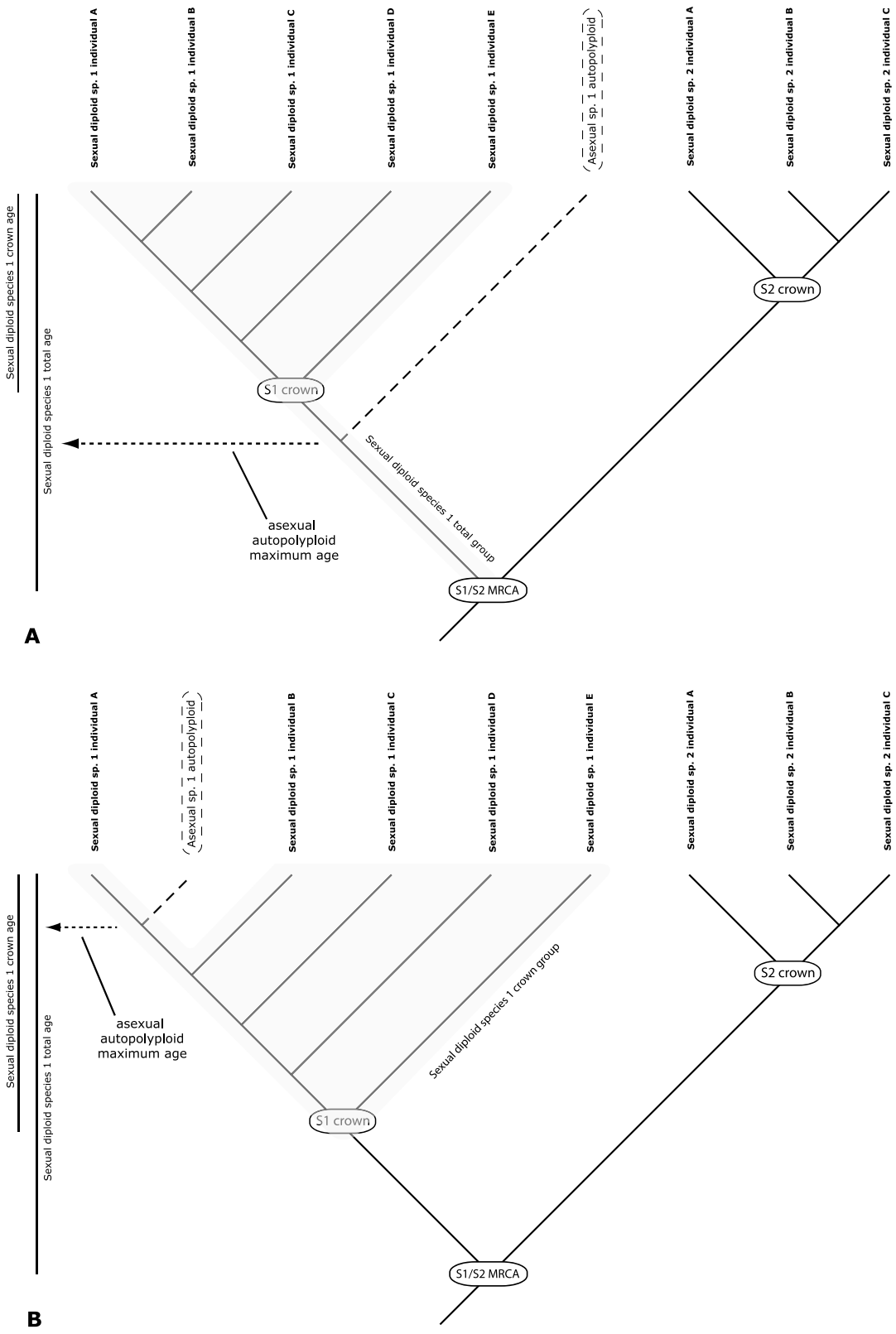

Figure 1. **Hypothetical plastid phylogeny illustrating the crown group, crown-age, total group, and total-age of two sexual diploid species, and the phylogenetic position of an asexual polyploid derived from one of the sexual diploids. (A) The asexual autopolyploid is placed in the total group, but not within the crown group of sexual diploid species one, and is therefore older than the crown-age of that species. (B) The asexual autopolyploid is nested within the crown group of sexual diploid species one, and is therefore younger than the crown-age of that species.**

phylogenetic positions and inferred ages (total- and crown-ages) to those of their sexual diploid parents. Specifically, are asexual polyploid *Astrolepis* lineages uniformly young relative to their sexual diploid counterparts? Or, are they indistinguishable in age from these close sexual relatives?

Materials and Methods

SAMPLING: DIAGNOSING DIPLOIDS AND POLYPLOIDS

Our goal was to include multiple individuals of each of the known sexual and asexual lineages of *Astrolepis*. Sampling was guided by macro-morphology, previous molecular studies, and information regarding sexuality and ploidy from an in-depth spore survey (see below). Useable DNA is easily obtained from herbarium material of *Astrolepis* (Beck et al. 2010) and fertile herbarium specimens were a critical supplement to our recent field collections, eventually comprising over 50% of our sample set.

Spore number per sporangium was used to establish reproductive mode (sexual or asexual) for over 250 morphologically diverse specimens from nine herbaria. Previous analyses of *Astrolepis*, which coupled such spore counts with chromosome counts from the same individual (Benham 1989; Windham and Yatskievych 2003), demonstrated that sporangia from sexual diploid *Astrolepis* plants were exclusively 64-spored, whereas sporangia from asexual triploids and tetraploids were exclusively 32-spored. This is in accord with earlier studies (Manton 1950; Tryon and Britton 1958) indicating that asexuality in cheilanthoid ferns (including *Astrolepis*) is associated with Döpp–Manton sporogenesis, which reduces spore number per sporangium by half. Multiple mature sporangia were removed from each specimen and placed in individual drops of glycerol; each sporangium was then opened and the spores teased apart with dissecting needles. Sporangia contained either ca. 64 or ca. 32 spores, corresponding to sexual or asexual life cycles, respectively (Manton 1950).

Using this information, we selected 72 specimens for in-depth study, choosing material that spanned the morphological and geographic disparity of the genus (Table S1). Twenty-two diploid specimens were chosen, including multiple individuals (mean 4.4) of all five sexual diploid species. These five species are clearly distinguishable by macro-morphological characters and highly supported by molecular studies, including isozyme data from three of the five diploids (Benham 1989), and both nuclear and plastid DNA sequence from all five diploids (Beck et al. 2010). Each sexual diploid was represented by individuals scattered across its geographical range: diploid *Astrolepis sinuata* (Lag. ex Sw.) D.M. Benham & Windham specimens from Texas and four Mexican states from Jalisco to Chiapas; diploid *A. cochisensis* (Goodd.) D.M. Benham & Windham from two U.S. states and the Mexican state of Coahuila; and diploid *A. laevis* (M. Martens & Galeotti) Mickel sampled in five Mexican states from Zacatecas to Oaxaca. Sampling of the two rare diploid species (*A. deltoidea* [Baker] J.B. Beck & Windham and *A. obscura* J.B. Beck & Windham) recently recognized by Beck et al. (2010) included all six known fertile specimens. The sampling of asexual lineages was similarly diverse. Multiple individuals (mean 7.8) of all six published asexual taxa were sampled, including those from different portions of their ranges (e.g., asexual *A. integerrima* from six US states, the Mexican state of Tamaulipas, and the Dominican Republic; asexual triploid *A. sinuata* specimens from Arizona, Baja California, Puerto Rico, and Peru). Several asexual individuals that did not fit published morphological descriptions (including undescribed asexual taxa recognized by Benham 1989) were also included.

Spore data also can be used to determine ploidy level in *Astrolepis*, with diploids, triploids, and tetraploids displaying statistically different and largely nonoverlapping spore diameter distributions (Benham 1989). Ploidy was inferred for the 72 sampled specimens in the following manner. A cover slip was placed over the glycerol-mounted spores from each specimen and examined at 400× magnification on a Zeiss Axioplan 2 microscope (Carl Zeiss AG, Oberkochen, Germany). Images of spores that had shed their perispore, or where the spore body could be easily distinguished from the perispore (Benham 1989), were taken with a Zeiss AxioCam HRm. Spore diameter was determined and averaged for 10–25 spores per specimen with ImageJ version 1.38 (Abramoff et al. 2004) calibrated with a slide micrometer.

A combination of nuclear genetic data, plastid genetic data, and morphology was used to estimate asexual lineage membership. Nuclear genomic composition was first established for a subset of the 72 samples. Identifying the genomes present in an individual required a large (typically 10–15) number of clone sequences per individual due to heterozygosity, gene duplication, and the presence of up to three genomes in certain allopolyploids. It was therefore not feasible to analyze all samples in this way, and 40 individuals were chosen in an attempt to include representatives from as many sexual and asexual lineages as possible. Clone sequences of the nuclear gene *gapCp* were obtained for 11 of the 22 sexual diploid samples and 29 of the 50 asexual polyploid samples using the methods outlined in Beck et al. (2010) (data not shown). That study clearly identified *gapCp* lineages that correspond to each of the five sexual diploid species, and the genomic composition of all 40 samples examined here was inferred from the phylogenetic placement of the clone sequences obtained from each individual. The genomic composition of the remaining samples was inferred using morphological comparisons to both the samples diagnosed with the *gapCp* data and those diagnosed using isozyme data in Benham (1989).

AMPLIFICATION 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 and sequenced using the protocols outlined in Beck et al. (2010).

ASTROLEPIS PHYLOGENY ESTIMATION

The *trnGR* dataset was manually aligned in Se-Al 2.0 (Rambaut 2002). Five regions of uncertain alignment totaling 112 bp were noted and excluded from further analysis. The edited *trnGR* alignment is available from TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S11489). The choice of outgroup taxa was guided by previous studies (Gastony and Rollo 1998; Kirkpatrick 2007; Schuettpelz et al. 2007) that placed *Astrolepis* in a strongly supported clade with *Pellaea* Link., *Paraceterach* Copel., and *Paragymnopteris* K.H. Shing. Within this clade, *Astrolepis* is sister to a small lineage of the polyphyletic genus *Pellaea* that includes *P. pringlei* Davenp., *P. sagittata* Link., and *P. cordifolia* (Sessé & Moc.) A.R. Sm. These three species, plus a more distantly related sexual diploid *Pellaea* (*P. truncata* Goodding), were used as outgroups. A heuristic maximum parsimony search with 100 random-addition replicates and 1×10^6 "fast" heuristic bootstrap replicates were performed using PAUP* 4.0b10 (Swofford 2002). The best-fitting model of sequence evolution (TVM + I) was identified using the Akaike information criterion in Modeltest 3.06 (Posada and Crandall 1998), and the closest available model (GTR + I) was assumed in a Bayesian Markov chain Monte Carlo (MCMC) analysis performed in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). The Bayesian analysis comprised four independent runs, each with four chains (one cold and three heated). Chains were run for 1×10^7 generations, and trees were sampled every 1000 generations. Convergence was assessed by checking for effective sample size values of >200 and interrun convergence for all parameters in TRACER version 1.5 (Rambaut and Drummond 2009). Runs converged quickly (within 100,000 generations), and the first 1×10^6 generations were conservatively discarded as burnin before obtaining clade posterior probabilities.

NODE AGE ESTIMATION

Although the phylogeny alone indicated the relative age of each asexual polyploid lineage relative to the crown- and total-ages of its sexual diploid maternal parent, all such ages were explicitly estimated using a relaxed-clock approach (Drummond et al. 2006). Briefly, rather than assuming a global substitution rate, relaxed-clock models allow the rate to vary across different portions of the tree. Using Bayesian MCMC approaches, both the phylogeny and divergence times can then be co-estimated under this relaxed-clock assumption. Because the dataset included both inter- and intraspecific sampling, a two-stage strategy was employed. First, the species-level phylogeny, the total-age of each diploid species, and the mutation rate were simultaneously estimated using the Bayesian MCMC approach implemented in BEAST version 1.5 (Drummond and Rambaut 2007). A species-level dataset was constructed comprising one sample chosen at random from each of the five sexual diploid *Astrolepis* species and one sample each from 26 other members of the *Astrolepis*/*Pellaea*/*Paraceterach*/*Paragymnopteris* clade. Regions of uncertain alignment were noted and excluded from further analysis. A normally distributed prior (22 ± 3 My) on the root height of the entire clade was constructed using the age estimate in the most likely tree and in 100 bootstrap trees evaluated by Schuettpelz and Pryer (2009). That study estimated node ages across a 400-taxon fern dataset by incorporating 24 fossil age constraints. Four independent runs were performed in BEAST, each assuming the prior on root height discussed above and the following models: GTR substitution, gamma site heterogeneity, uncorrelated lognormal clock, and a birth–death process tree. The GTR and gamma shape parameters were selected based on the most likely substitution models chosen by the Akaike information criterion in Modeltest 3.06 (Posada and Crandall 1998). Each of the four runs of the MCMC chain were run for 1×10^7 generations, sampling every 1000 generations. Convergence was assessed by checking for effective sample size values of >200 and interrun convergence for all parameters in TRACER version 1.5 (Rambaut and Drummond 2009). Runs converged quickly (within 100,000 generations), and the first 1×10^6 generations were conservatively discarded as burnin.

The mutation rate estimated in this species-level analysis was then used as a prior in "intraspecific" analyses. Preliminary analysis of the total *trnGR* dataset revealed that each of the 72 samples were placed in one of five strongly supported clades that correspond to the sexual diploid species outlined previously by Beck et al. (2010). Five datasets were assembled based on these clades, each comprising sequences from a single diploid species, autopolyploids derived from that species, and allopolyploids for which that diploid species was the maternal parent. Two independent runs were performed in BEAST for each of these datasets, each incorporating a mutation rate prior (lognormal mean 0.002, SD 0.22) developed using the species-level analysis described above, GTR substitution, gamma site heterogeneity, an uncorrelated lognormal clock, and a constant-size coalescent tree. A constant-size coalescent tree prior was chosen due to (1) the lack of demographic and life-history information that would be needed to establish appropriate priors required by more complicated models, and (2) the unusual composition of each of the five datasets. Each of these intraspecific datasets included alleles drawn from both a sexual diploid and recurrently formed asexual polyploid

lineages, and would be likely to exhibit drastically different demographic features. The number of generations needed to achieve combined effective sample size values of >200 varied among the five datasets, and each run incorporated either 2×10^8 or 5×10^8 generations. Each pair of runs was evaluated to assess interrun convergence for all parameters. Once convergence was well established, each pair of runs was combined using LogCombiner 1.5 (Rambaut and Drummond 2010a). Preliminary attempts to summarize tree statistics using TreeAnnotator version 1.5 (Rambaut and Drummond 2010b) indicated that the post-burnin combined tree files exceeded computational constraints, and each pair of post-burnin runs was subsampled (discarding 50% of trees) prior to summary.

The total-age of each sexual diploid species was inferred using the mean and 95% highest probability density (HPD) inferred at the node corresponding to the MRCA of that diploid species and its sister diploid species (see Fig. 1). The crown-age of each diploid species was inferred using the mean and 95% HPD inferred at the node corresponding to the MRCA of the sampled members of that species. The maximum age of each asexual polyploid lineage was inferred as the age of the MRCA of that polyploid and the most closely related sexual diploid individuals (Fig. 1). The age at that node is the oldest possible date for the origin of the asexual polyploid lineage, because hypothesizing that the polyploid arose even one mutational step back along the branch leading to that node requires an explanation as to how that synapomorphy is observed in both the diploid parent and polyploid offspring. Gene flow from asexual polyploids to sexual diploids is not possible in this system, as asexual polyploids typically produce unreduced spores (and thus gametes) via Döpp–Manton sporogenesis. The union of such an unreduced gamete with a reduced gamete from a sexual diploid would simply result in additional higher ploidy asexual lineages, not new sexual diploids (see Gastony and Windham 1989). Although attempts at standard reductive meiosis are observed in asexual cheilanthoid ferns, these produce abortive spores due to chromosome pairing difficulties (Manton et al. 1966; Walker 1979). Without gene flow, convergence would have to be invoked, an event that is extremely implausible at this time depth.

Results

DIAGNOSING PLOIDY AND GENOMIC COMPOSITION

Mean spore diameter for each of the 72 samples fell into one of three previously reported groups (Benham 1989; Beck et al. 2010), each of which corresponded to a distinct ploidy level (Table S1; Fig. 2). Twenty-two samples exhibited mean diameters below 50 microns, indicating that they were diploids (2*x* in Fig. 2). All 22 of these samples also produced 64 spores per sporangium, indicating that they were sexual. The remaining 50 samples exhibited mean diameters greater than 50 microns and were 32-spored, indicating that they were asexual polyploids. Forty-five samples exhibited mean diameters between 50 and 70 microns, and five samples had mean diameters greater than 71 microns, indicating that they were triploids and tetraploids (3*x* and 4*x* in Fig. 2), respectively (Benham 1989; Beck et al. 2010). The nuclear genomic composition of all 72 individuals, inferred either from *gapCp* nuclear sequence data or morphology, is detailed in Table S1 and Figure 3. The 11 sexual diploid individuals diagnosed with *gapCp* data each exhibited clone sequences only from their own diploid lineage. Twelve of the 29 asexual polyploid individuals diagnosed with *gapCp* data exhibited clone sequences from a single diploid lineage, suggesting that they were autopolyploids. The remaining 17 individuals each exhibited clone sequences from two or three diploid lineages, indicating that they were di- or trigenomic allopolyploids, respectively.

trnGR PHYLOGENY

The analyzed *trnGR* matrix of 1111 aligned characters yielded 182 (16%) variable and 138 (12%) parsimony-informative characters. Each of the 100 random-addition replicate parsimony searches recovered the same most parsimonious tree (length = 218, CI = 0.89, RI = 0.99). This tree, along with bootstrap percentages (BS) and Bayesian posterior probabilities (PP), is shown in Figure 3. Five well-supported primary clades were evident; each included all samples of just one of the five morphologically distinct sexual diploid species recognized by Beck et al. (2010) plus all asexual polyploid samples for which that diploid was the maternal parent. The plastid and nuclear/morphological data were completely congruent, the maternal parent inferred for each polyploid individual by the plastid data was also inferred as one of the components of its nuclear genome by the *gapCp* data.

PHYLOGENETIC POSITION AND AGE OF ASEXUAL POLYPLOID LINEAGES

The species-level, relaxed-clock analysis estimated the *Astrolepis* MRCA (C/S,D,O,L) at 10.5 My (Fig. 4; 95% HPD: 5.3–16.3). The earliest-diverging lineage comprised all samples of sexual diploid *A. cochisensis* plus all asexual polyploids to which this species contributed the maternal genome. Divergence events that produced the other major lineages within the genus, and thus define total-ages for the descendant species, were estimated as *A. laevis*/*A. obscura*—3.9 My (1.4–6.8) and *A. sinuata*/*A. deltoidea* —3.4 My (1.1–6.2) (Fig. 4). Asexual polyploid lineages were defined conservatively as all samples sharing both the same 1111 bp *trnGR* sequence and a nuclear genome diagnosed either by *gapCp* sequence data or morphology.

Several asexual polyploid lineages exhibited a plastid *trnGR* sequence derived from the *A. cochisensis* lineage (Fig. 3). These

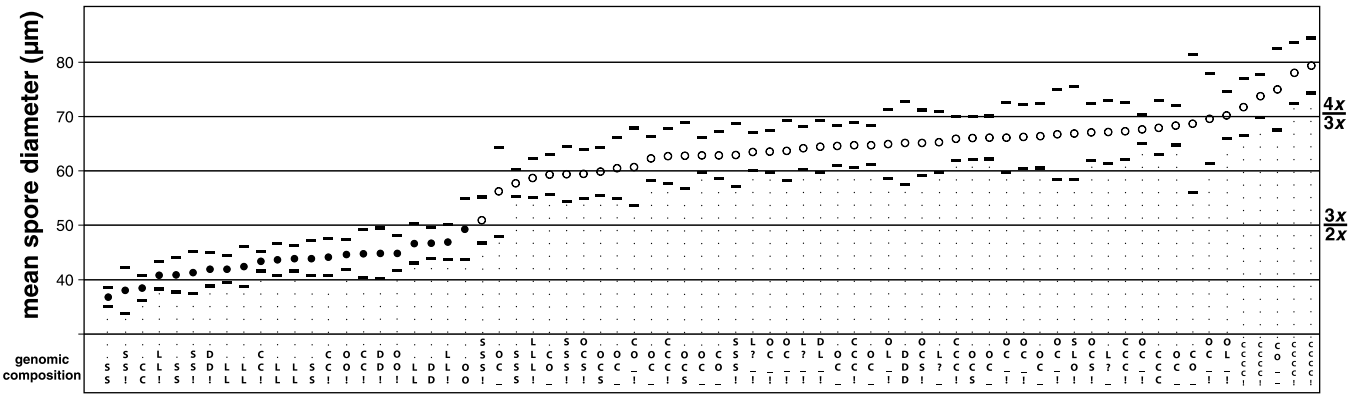

Figure 2. **Mean spore diameter in microns (± 1 SD) for the 72 sequenced *Astrolepis* specimens (arranged from smallest to largest), with the inferred genomic composition of each sample noted. Genomic composition was inferred either by plastid *trnGR* and nuclear *gapCp* data (denoted with an !) or by *trnGR* data and morphological comparisons to specimens diagnosed with plastid and nuclear data: (C = *A. cochisensis*; S = *A. sinuata*; D = *A. deltoidea* ; O = *A. obscura* ; L = *A. laevis*). Thus C/C/C/C! denotes an autotetraploid *A. cochisensis* diagnosed with both plastid and nuclear data, D/D denotes a diploid *A. deltoidea* diagnosed with plastid and morphological data, and O/L/_! denotes an *A. obscura*/*A. laevis* allotriploid diagnosed with plastid and nuclear data for which the third genomic component is unknown due to our inability to determine dosage. Filled circles indicate 64-spored individuals (sexual), open circles indicate 32-spored individuals (asexual). Notations at the right indicate previously established size ranges for diploids (2*x*), triploids (3*x*), and tetraploids (4*x*) (Benham 1989).**

lineages shared MRCAs with *A. cochisensis* sexual diploids at nodes 1 and 2. As such, they were nested within the *A. cochisensis* crown clade (node C) and are, by definition, younger than the sexual *A. cochisensis* crown- and total-ages (Figs. 3 and 4; nodes C, and C/S, D, O, L, respectively). Four asexual polyploid lineages shared the same *trnGR* sequence and an MRCA with an *A. cochisensis* sexual diploid estimated at 0.3 My (0.0–0.7; Figs. 3 and 4; node 1). One of these asexual polyploid lineages comprised three specimens diagnosed as *A. cochisensis* autotetraploids, another included five specimens diagnosed as *A. cochisensis* autotriploids, a third included two specimens diagnosed as *A. cochisensis* × *A. obscura* allotriploids, and the fourth was represented by a single specimen (5656) diagnosed as an *A. cochisensis* × *A. obscura* allotetraploid. Two additional asexual polyploid lineages shared a distinctive *trnGR* sequence and an MRCA estimated at 0.04 My (0.0–0.1) (Figs. 3 and 4; node 2). One was represented by a specimen (5001) diagnosed as an *A. cochisensis* autotetraploid, and the second by a specimen (5710) diagnosed as an *A. cochisensis* × *A. obscura* allotriploid. Although none of the four sampled *A. cochisensis* sexual diploid individuals were placed at this node, such a diploid must be inferred because neither of these asexual 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.

Two asexual polyploid lineages exhibited a *trnGR* sequence representing the *A. sinuata* lineage. These *trnGR* sequences were identical to those obtained from the five sexual diploid *A. sinuata* samples and were thus the same age as the *A. sinuata* crown-age (Fig. 3; node S) and younger than the *A. sinuata* total-age (Fig. 3; node S/D). An age estimate was not possible at this node given the lack of genetic variation within *A. sinuata* at the *trnGR* locus. One asexual polyploid lineage included four specimens diagnosed as *A. sinuata* autotriploids, and the second was represented by a single specimen (4552) diagnosed as an *A. sinuata* × *A. laevis* × *A. obscura* allotriploid.

Two asexual polyploid lineages exhibited identical *trnGR* sequences from the *A. deltoidea* lineage and an MRCA with an *A. deltoidea* sexual diploid estimated at 0.7 Mya (0.0–1.8; Figs. 3 and 4; node 4). This node was nested within the *A. deltoidea* crown clade, and these two asexual lineages were therefore younger than both the *A. deltoidea* crown- (Fig. 3; node D) and total-ages (node S/D). One was represented by a specimen (6112) diagnosed as an *A. deltoidea* × *A. laevis* allotriploid and the second by a specimen (7168) diagnosed as an *A. deltoidea* autotriploid.

Six asexual polyploid lineages exhibited a *trnGR* sequence obtained or derived from the *A. obscura* lineage. All shared MRCAs with an observed or inferred *A. obscura* sexual diploid nested within the *A. obscura* crown clade and were therefore younger than both the *A. obscura* crown- (node O) and total-ages (node O/L) (Figs. 3 and 4; nodes 5–9). Two asexual polyploid lineages exhibited identical *trnGR* sequences and an MRCA with an inferred *A. obsura* sexual diploid estimated at 0.2 My (0.0–0.5) (Figs. 3 and 4; node 5). A sexual diploid *A. obscura* was inferred at this node given that neither of the asexual polyploids at this node could have given rise to one another. One of these asexual polyploid lineages was represented by a specimen (6131) diagnosed as an *A. obscura* × *A. cochisensis* allotriploid, and the second

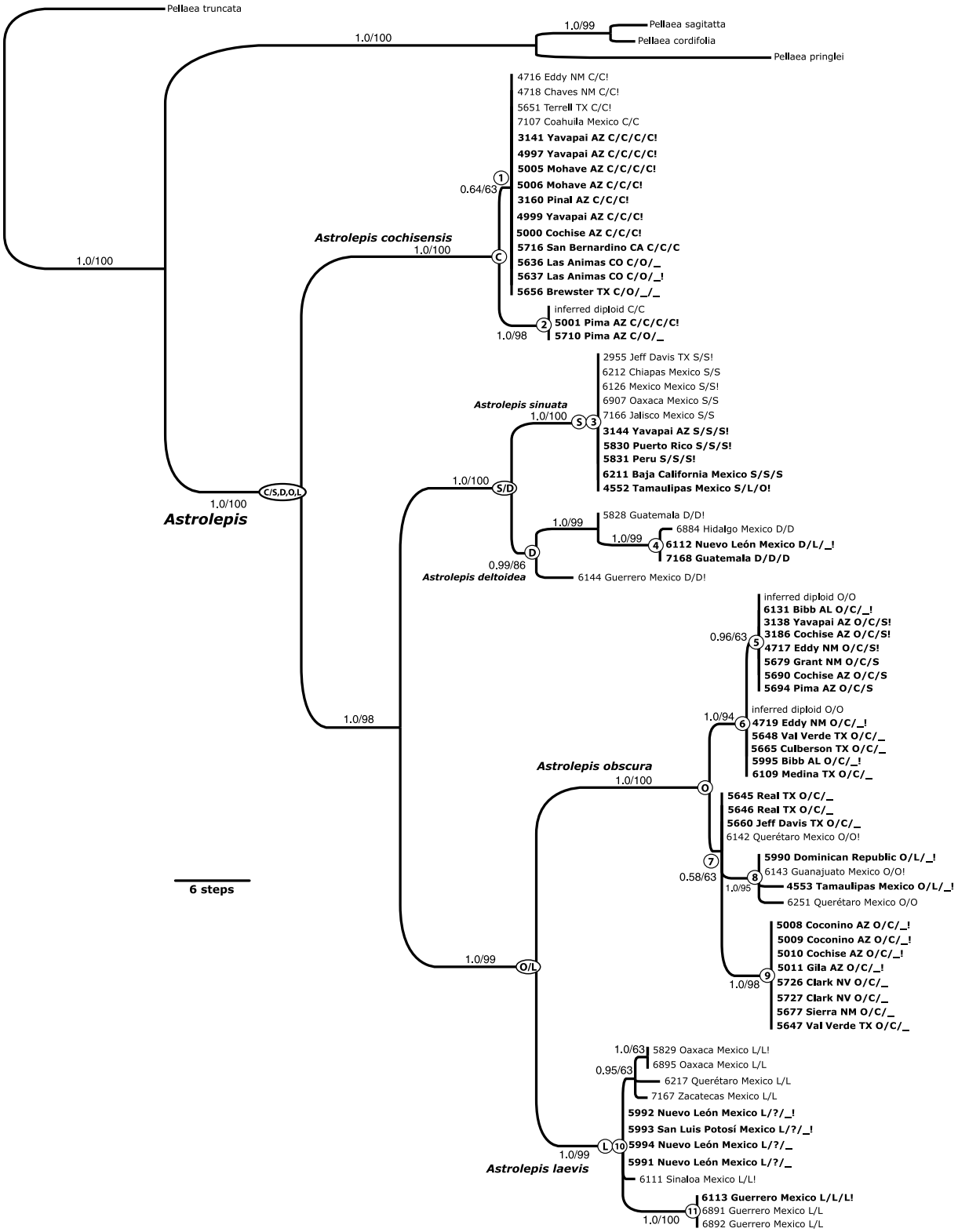

Figure 3. The single most parsimonious tree of the plastid *trnGR* sequence data. Support values appear at each node (Bayesian posterior probability/parsimony bootstrap percentage). Each sample is designated with a unique number, location name, and its inferred genomic composition (see Fig. 2 legend). Asexual samples are in bold. Crown nodes for each of the five diploid species are noted with the first letter of that species name (C = *A. cochisensis*; S = *A. sinuata* ; D = *A. deltoidea* ; O = *A. obscura* ; L = *A. laevis*). Nodes representing the MRCA of species pairs are marked with the letter designations from both species (S/D = the MRCA of *A. sinuata* and *A. deltoidea*). Numbered clades are discussed in the text. The tree was drawn using FigTree 1.3 (Rambaut 2009).

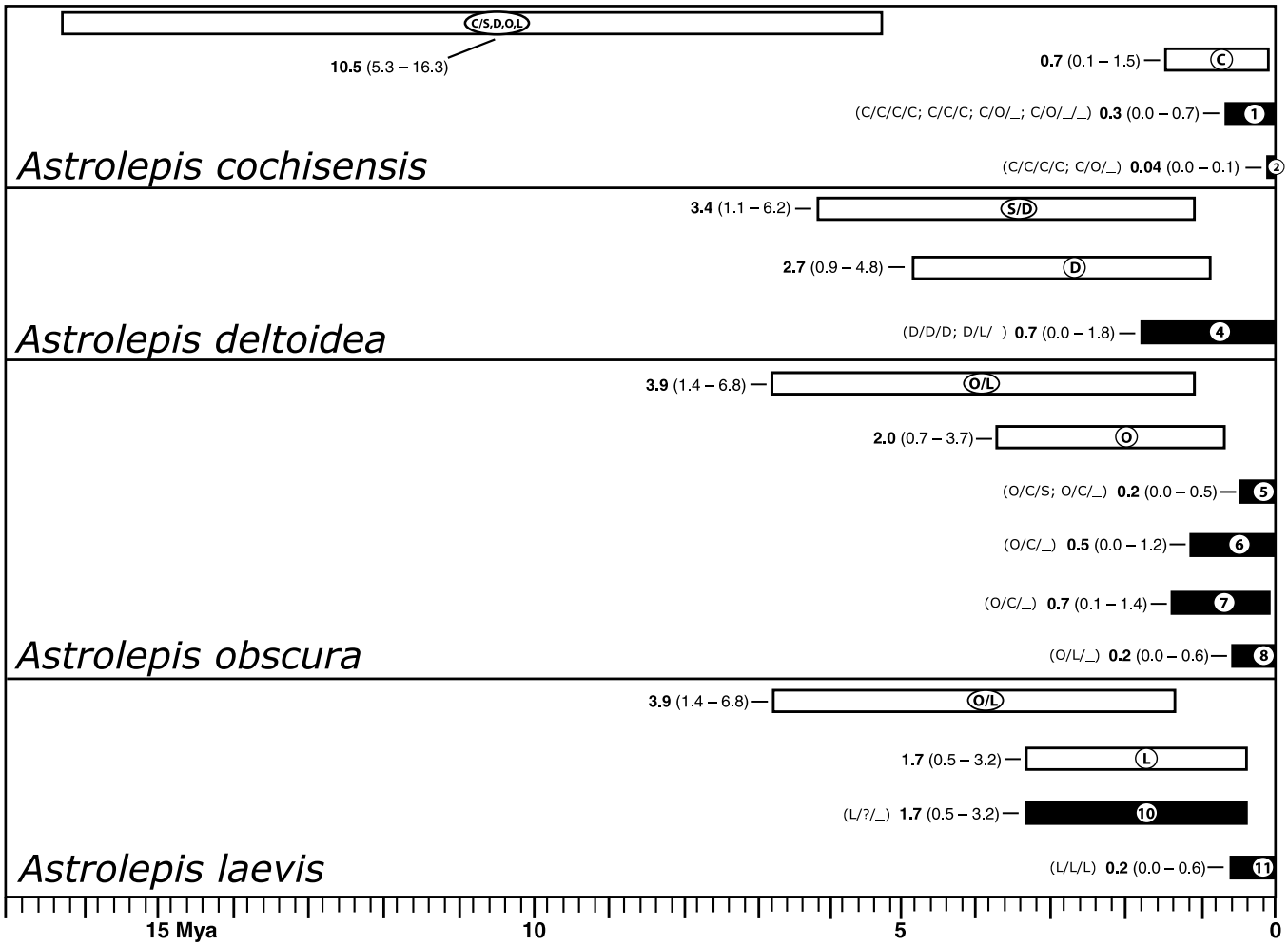

Figure 4. **Total-age and crown-age estimates [mean (95% HPD)] for each sexual diploid species except *A. sinuata* (see text) and of all nodes representing the maximum age of asexual lineages. Total-age and crown-age estimates are shown in white, asexual lineage ages are shown in black. Number or letter designations refer to nodes in Figure 3 and are discussed in the text.**

by six specimens diagnosed as *A. obscura* × *A. cochisensis* × *A. sinuata* allotriploids.

Five specimens diagnosed as *A. obscura* × *A. cochisensis* allotriploids shared a distinctive, *A. obscura*-derived *trnGR* sequence and an MRCA with an inferred *A. obscura* sexual diploid estimated at 0.5 My (0.0–1.2) (Figs. 3 and 4; node 6). A diploid *A. obscura* was also inferred at this node to explain the diploid inferred at node 5 (Fig. 3). Another asexual polyploid lineage comprising three specimens diagnosed as *A. obscura* × *A. cochisensis* allotriploids exhibited identical *trnGR* sequences and an MRCA with a sampled *A. obscura* sexual diploid estimated at 0.7 My (0.1–1.4) (Figs. 3 and 4; node 7). Two asexual polyploid lineages, each represented by a specimen diagnosed as an *A. obscura* × *A. laevis* allotriploid, shared an MRCA with *A. obscura* sexual diploids estimated at 0.2 My (0.0–0.6) (Figs. 3 and 4; node 8). Eight *A. obscura* × *A. cochisensis* allotriploid specimens represented an asexual polyploid lineage with an MRCA estimated at 0.3 My (0.0–0.8) (Fig. 3; node 9). This lineage's MRCA with a sampled *A. obscura* sexual diploid was estimated at 0.7 My (0.1–1.4) (Figs. 3 and 4; node 7).

Two asexual polyploid lineages exhibited *trnGR* sequences from the *A. laevis* lineage. One was represented by four samples that shared an MRCA with a sampled *A. laevis* sexual diploid at 1.7 My (0.5–3.2) (Figs. 3 and 4; node 10). This node coincided with the MRCA of the *A. laevis* crown clade (Figs. 3 and 4; node L), and thus was inferred to be the same age as the crown, though still younger than the total-age (Figs. 3 and 4; node O/L). Although these samples exhibited exclusively *A. laevis gapCp* clone sequences, their divergent morphology makes it unlikely that they were autotriploid offspring of diploid *A. laevis*. All other known *Astrolepis* autopolyploids are macro-morphologically identical to their sexual diploid parents, differing only in mean spore diameter. Furthermore, an asexual triploid that was morphologically

identical to sexual diploid *A. laevis* was observed (Figs. 3 and 4; node 11). Instead, these four individuals were clearly distinguishable from sexual diploid *A. laevis*, most notably in their essentially entire pinnae (the pinnae of sexual diploid *A. laevis* are strongly lobed). Our failure to recover clone sequences from the other parental species of this putative allopolyploid was likely due to mutational change at priming sites in the remaining genomes, a phenomenon previously reported for *Astrolepis* (Beck et al. 2010). The second asexual polyploid lineage was represented by a single specimen (6113) diagnosed as an *A. laevis* autotriploid, which shared an MRCA with a sexual diploid *A. laevis* at 0.2 Mya (0.0–0.6) (Figs. 3 and 4; node 11).

Discussion

ASEXUAL LINEAGE DIVERSITY GENERATED VIA MULTIPLE PATHWAYS

Molecular studies repeatedly demonstrate that even small groups of sexual species can generate an impressive array of asexual lineages. Whether arising from within a single sexual diploid (e.g., Neiman et al. 2005; Thompson and Whitton 2006; Grubbs et al. 2009) or through hybridization among such species (Whitton et al. 2008; Grusz et al. 2009), recurrently formed asexual lineages can greatly enhance observed biodiversity. *Astrolepis* provides a textbook example of the powerful combined effect of these processes. Our data conservatively identified 19 unique asexual polyploid lineages in a dataset of only 50 such samples; the future addition of individuals and nuclear loci will certainly add to this total. Although the relatively young age of these lineages suggests the macroevolutionary limitations of asexuality (see below), their diversity simultaneously highlights the remarkable success of asexual polyploid lineages over abbreviated time scales. In only a fraction of the total time-depth of the genus, asexual polyploids representing numerous genomic combinations have formed, become established, and expanded into regions often far beyond those occupied by their sexual diploid progenitors. Allopolyploid formation in particular has the potential to produce many novel, potentially fit genomic combinations in a very short time frame (Lynch 1984; Kearney 2005). Asexuality can protect these new hybrid genotypes by allowing for immediate reproductive isolation and by circumventing chromosome pairing issues with altered meiotic pathways (Kearney 2005; Hörandl 2006). Of the 19 unique asexual polyploid lineages documented in this study, 13 were allopolyploids, with all five sexual diploids involved as parents.

RELATIVE AGES OF ASEXUAL POLYPLOIDS VERSUS SEXUAL DIPLOIDS

The topological position of asexual *Astrolepis* lineages alone allowed broad conclusions to be drawn about their ages relative to the sexual species. Only three of the 19 asexual lineages occupied polytomies at the crown nodes of their maternal sexual species (Fig. 3, S/S/S, S/L/O at node 3; L/?/_ at node 10). The remaining 16 lineages were nested within the crown clade of their maternal parents and thus arose after the MRCA of that parent's extant diversity. The 95% HPDs of many age estimates were large, and the mean ages we will consider below as lineage age estimates should be viewed with an appropriate degree of caution. The age estimates of the 19 asexual polyploid lineages averaged seven times younger than their maternal parent's crown-age estimates, occupying only 35% of the time depth of their maternal parent's crown group. The age estimates for 17 of the 19 asexual polyploid lineages (excluding the two *A. sinuata*-derived asexuals) averaged 47 times younger than their respective maternal parent's total-age estimates, occupying only 10% of the time depth of their maternal parent's total group. Although it is possible that relatively old asexual lineages do exist, our broad taxonomic, morphological, and geographic sampling indicates old asexual lineages are rare in *Astrolepis*. It also should be noted that these are maximum age estimates for the asexual polyploid lineages, and that at least some of these likely had more recent origins. This is due in part to a sampling issue that is a common feature of asexual lineage age studies (Neiman et al. 2009). Sexual diploids were under-sampled relative to asexual polyploids in this study (22 vs. 50 samples), and adding sexual diploid individuals with sequences from certain clades (Fig. 3; clade 9) would shift the MRCA of those asexual lineages with a sexual lineage closer to the present. In particular, the core ranges of the sexual diploids *A. deltoidea* and *A. obscura* in central and southern Mexico were under-represented in this analysis, and additional genotypes of these taxa await discovery.

Because we were able to reconstruct a well-resolved phylogeny of both asexual lineages and their sexual progenitors, this study demonstrates that asexual polyploid *Astrolepis* lineages are not only quite recent, but they are young relative to their sexual diploid parents. Two alternative explanations could be offered to explain this age distribution. Lack of time depth among the asexual lineages could be the result of a constant turnover of extinction-prone asexual taxa that has been ongoing for millions of years, or it could be the result of recent geographic and/or ecological opportunities that have enhanced asexual lineage formation. Geographic opportunity could arise from recent range expansions that brought parental species into close proximity, thus creating the opportunity for the formation of allopolyploid hybrids. Because the historical geographic isolation of sexual diploids envisioned under this hypothesis would not impede the formation of autopolyploids, we would expect this "geographic opportunity" scenario to skew the age distributions of the asexual taxa such that autopolyploids would be significantly older, on average, than allopolyploids. No such pattern was detected in our dataset; in fact, the six asexual autopolyploid lineages documented by this

study appeared to be somewhat younger than the allopolyploids (autopolyploid mean age = 0.31 My; allopolyploid mean age = 0.48 My).

Ecological opportunity would imply that the niches of sexual and asexual lineages differ, and that the conditions favoring asexual lineage persistence have only recently come about. One traditional hypothesis is that the establishment and survival of asexual lineages has been facilitated by environmental changes linked to the advance and retreat of glacial ice, starting approximately 2.5 My and intensifying during the last one million years (Mogie and Ford 1988; Kearney 2005). Macrofossils recovered from packrat middens in the southwestern US clearly demonstrate that *Astrolepis* species in this region underwent range expansion/contraction in response to Pleistocene climatic cycles (Van Devender et al. 1984; Anderson and Van Devender 1991; Betancourt et al. 2001), and the current ranges of certain asexual genotypes (*A. sinuata* autotriploids, *A. cochisensis* autotriploids, and *A. obscura* × *A. cochisensis* allotriploids) expand upon those of their sexual progenitors in this region (Benham 1989; Benham and Windham 1993). Although one possible explanation is that asexual taxa can occupy habitats that their sexual progenitors cannot, it is also possible that these distributional patterns are a result of breeding system differences. Whereas the asexual lineages establish new populations from single spores, *Astrolepis* sexual diploids are largely, if not exclusively, outcrossers (M.D. Windham & D. M. Benham, unpubl. data). Thus, range expansion of the sexual taxa requires the synchronous arrival of two genetically compatible spores, the probability of which decreases exponentially with distance. A full evaluation of the ecological opportunity hypothesis will require phylogeographic analyses, including both population genetic data and ecological niche modeling of asexual polyploids and sexual diploids (Hickerson et al. 2010). As is common in asexual taxa, *Astrolepis* apomictic genomic combinations are often recurrently formed (Fig. 3), and seemingly broad ecological niches could simply result from the combined niches of morphologically cryptic, but evolutionary independent, asexual lineages (Hörandl 2006).

This ecological caveat notwithstanding, the absence of relatively old asexual polyploid lineages in *Astrolepis* is consistent with the dominant view of asexual taxa as short-lived offshoots of evolutionary dynamic sexual lineages (Wagner 1970). It should be stressed again that the link between asexuality and polyploidy in this study system does not allow definitive statements about the effect of asexuality per se. It is clear, however, that the commonly associated features of asexuality and polyploidy have exerted a strong effect on lineage age in *Astrolepis*. If any confounding effects of ploidy on lineage age are indeed present, these extend to a broad array of asexual lineages, including triploids, tetraploids, autopolyploids, and both di- and tri-genomic allopolyploids. Future studies should focus on groups containing sexual polyploids or asexual diploids, in which the individual effects of asexuality and polyploidy can be identified. Continuing study of the short-term success and the long-term vulnerability of asexual lineages will enhance our understanding of this important alternative to sexual reproduction, while simultaneously providing insights into the evolutionary benefits and dominance of sex.

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