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DECIPHERING THE ORIGINS OF APOMICTIC POLYPLOIDS IN THE *CHEILANTHES YAVAPENSIS* COMPLEX (PTERIDACEAE)[1]

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Deciphering species relationships and hybrid origins in polyploid agamic species complexes is notoriously difficult. In this study of cheilanthoid ferns, we demonstrate increased resolving power for clarifying the origins of polyploid lineages by integrating evidence from a diverse selection of biosystematic methods. The prevalence of polyploidy, hybridization, and apomixis in ferns suggests that these processes play a significant role in their evolution and diversification. Using a combination of systematic approaches, we investigated the origins of apomictic polyploids belonging to the *Cheilanthes yavapensis* complex. Spore studies allowed us to assess ploidy levels; plastid and nuclear DNA sequencing revealed evolutionary relationships and confirmed the putative progenitors (both maternal and paternal) of taxa of hybrid origin; enzyme electrophoretic evidence provided information on genome dosage in allopolyploids. We find here that the widespread apomictic triploid, *Cheilanthes lindheimeri*, is an autopolyploid derived from a rare, previously undetected sexual diploid. The apomictic triploid *Cheilanthes wootonii* is shown to be an interspecific hybrid between *C. fendleri* and *C. lindheimeri*, whereas the apomictic tetraploid *C. yavapensis* is comprised of two cryptic and geographically distinct lineages. We show that earlier morphology-based hypotheses of species relationships, while not altogether incorrect, only partially explain the complicated evolutionary history of these ferns.

Key words: apomixis; *Cheilanthes*; cryptic species; enzyme electrophoresis; *gapCp*; hybridization; low-copy nuclear gene; multiple origins; polyploidy; Pteridaceae.

Hybridization, polyploidy, and apomixis are each common processes in plants (Grant, 1981). When combined in a single lineage, the resulting evolutionary complexity can frustrate even the most dedicated attempts to circumscribe species and understand relationships among them. The best-known examples of this are the polyploid agamic species complexes of such angiosperm genera as *Amelanchier* (Campbell and Wright, 1996), *Antennaria* (Bayer, 1987), *Boechera* (Schranz et al., 2005), *Crataegus* (Talent and Dickinson, 2005), *Crepis* (Whitton et al., 2008), *Poa* (Soreng, 1990), *Rubus* (Einset, 1951), and *Taraxacum* (Verduijn et al., 2004). However, such taxonomic complexity is by no means limited to the angiosperms, and examples abound in the ferns as well. Polyploid agamic species complexes are especially common in the family Pteridaceae, in which members of both the pteroid (Walker, 1962) and cheilanthoid (Benham, 1989; Windham, 1993b; Windham and Rabe, 1993) lineages show extensive reticulate evolution. In this study of cheilanthoid ferns, we integrate evidence from a diverse selection of biosystematic approaches to provide a comprehensive example of the investigative depth required for complex speciation studies.

Here we investigate the evolutionary complexity that has resulted from the interplay of hybridization, polyploidy, and apomixis in the *Cheilanthes yavapensis* Reeves ex Windham complex. This complex, which is endemic to the southwestern United States and adjacent Mexico, consists of several sexual diploids, as well as apomictic and sexual polyploids, all of which hybridize to form various polyploid lineages (Windham and Rabe, 1993). In this group, apomictic triploids and tetraploids represent a major source of taxonomic confusion because they are able to hybridize with sexual taxa and form higher ploidy hybrids that are reproductively competent. A prime example of this complicated scenario is provided by *C. yavapensis* itself.

Prior to 1993, *C. yavapensis* was included within the circumscription of *C. wootonii* Maxon. The first suggestion that the latter might comprise more than one taxon came from Reeves' (1979) morphological investigation of relationships within *Cheilanthes* subg. *Physapteris*. On the basis of disparities observed in rhizome and costa scale morphologies, Reeves (1979) concluded that *C. wootonii* harbored two distinct taxa: *C. wootonii* s.s. and an entity that he tentatively called "*C. yavapensis*." Because *C. wootonii* shares many features with *C. fendleri* Hook. and *C. lindheimeri* Hook., Reeves (1979) hypothesized that *C. wootonii* arose through hybridization between these species (Fig. 1), which are sympatric over much of its range. *Cheilanthes* "*yavapensis*," on the other hand, appeared intermediate between *C. covillei* Maxon and *C. lindheimeri*, and was hypothesized to be a fertile apomictic hybrid between them (Fig. 1).

Subsequent work on this group provided additional evidence that populations referred to *C. wootonii* s.l. included at least two distinct taxa. Typical *C. wootonii* was determined to be an apomictic triploid ($n = 2n = 90$) with spores averaging <64 μm in diameter (Windham, 1983). *Cheilanthes* "*yavapensis*" proved to be an apomictic tetraploid ($n = 2n = 120$) with spores ≥64 μm

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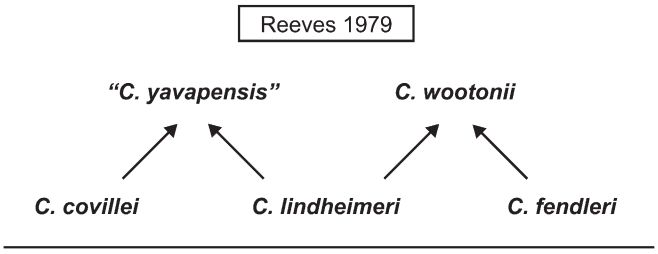

Fig. 1. Proposed relationships of taxa belonging to the *Cheilanthes yavapensis* complex, as described by Reeves (1979). Arrows point from putative parents to hypothesized offspring.

in diameter (Windham, 1993a). Results from enzyme electrophoretic analyses (Gastony and Windham, 1989) supported Reeves' (1979) hypothesis that these taxa had separate origins and distinct genetic makeups. Based on these data, Windham (1993a) formally published *C. yavapensis* Reeves ex Windham.

Despite evidence that *C. wootonii* and *C. yavapensis* are distinct entities, they remain difficult to distinguish by superficial observation of morphological traits. Although Windham and Rabe (1993) provided a suite of morphological characters (pinna pubescence, costal scale ciliation, rhizome scale color and persistence) that allowed the identification of most specimens, one of the most useful characters—spore size—requires the use of a compound microscope. Identification becomes particularly difficult in the northwestern and southeastern portions of their ranges (Nevada and Texas, respectively), where intermediate morphologies occur. Using a combination of systematic approaches—spore studies, plastid and nuclear DNA sequencing, and enzyme electrophoresis—we aim to clarify the relationships between *C. wootonii* and *C. yavapensis* and decipher the events and processes that gave rise to these apomictic polyploids.

MATERIALS AND METHODS

Taxon sampling—A total of 21 specimens representing the five members of the *C. yavapensis* complex (*C. covillei*, *C. fendleri*, *C. lindheimeri*, *C. wootonii*, and *C. yavapensis*) were selected for study (Appendix 1). Based on its position as an early-diverging member of the clade containing the *C. yavapensis* complex (i.e., the myriopterid cheilanthoids; Windham et al., in press; A.L. Grusz et al., unpublished data), *C. newberryi* was chosen as the most appropriate outgroup.

Spore number per sporangium and spore diameter—Only fertile (sporulating) specimens were included in our analyses. Intact, mature sporangia were removed from each specimen, placed in a drop of glycerol on a slide, and gently ruptured with a needle tip. These preparations were used to determine the number of spores per sporangium (64 or 32), which, in cheilanthoid ferns, is strongly correlated with reproductive mode (sexual or apomictic, respectively; Tryon, 1968; Gastony and Windham, 1989). Because spore size is a good indicator of ploidy (Barrington and Paris, 1986), the diameter of 25 spores from each individual was measured and then compared to spores from chromosome vouchers cited by Windham and Yatskievych (2003) to establish the ploidy level of each accession.

cpDNA sequencing—For each sampled individual (Appendix 1), genomic DNA was extracted from silica-dried material using the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) following the protocol described in Schuettpelz and Pryer (2007). Four plastid loci—*rbcL-atpB* (~1200 bp), *trnG-trnR* (~1138 bp), *trnP*^{UGG}*-petG* (~540 bp), and *rps4-trnS*^{GGA} (~980 bp)—were amplified by PCR using 1× PCR buffer IV containing $MgCl_2$ (ABgene, Epsom, UK), combined with 200 μM each dNTP, 100 μg/mL BSA, 50 U/mL *Taq* polymerase, 0.5 μM of each locus-specific primer pair (Table 1), and 1 μL template DNA for a 25 μL reaction. PCR amplifications entailed an initial denaturation step (94°C for 5 min) followed by 35 denaturation, annealing, and elongation cycles (94°C for 1 min, locus-specific annealing temperature for 1 min, and 72°C for 2 min), and a final elongation step (72°C for 10 min). Amplicons were visualized on a 1% agarose gel. PCR purification and sequencing followed the protocol of Schuettpelz et al. (2008). All plastid sequences (86 newly obtained) were subsequently deposited in GenBank (Appendix 1).

Nuclear DNA sequencing—Amplification, cloning, and sequencing of the low-copy nuclear locus, *gapCp*, for each individual sampled (Appendix 1) followed the protocol of Schuettpelz et al. (2008). More than one copy of *gapCp* is recovered in this group of cheilanthoid ferns, one ~900-bp copy and one ~600-bp copy. The ~900-bp copy coincides in length with a copy of nuclear *gapC*, which also occasionally amplifies using the given primers. Therefore, we chose to sequence only the ~600-bp copy (i.e., the "short copy" of *gapCp*; Schuettpelz et al., 2008).

To account for false sequence variation attributable to PCR error, consensus allele sequences were compiled for the short copy *gapCp* locus for each individual sampled. This was done by first combining all sequence clones obtained from a given *individual* into a single alignment in the program MacClade 4.08 (Maddison and Maddison, 2005). The alignments (each corresponding to a given individual) were then analyzed separately using a maximum parsimony optimality criterion with the default parsimony settings in the program PAUP* version 4 (Swofford, 2001). The resulting trees were used to determine unique alleles present in each individual. Alleles were recognized when one or more clones from a given individual were united by one or more shared characters. After identifying all sequence clones for a given allele, those sequences were combined in a single project in the program Sequencher 4.8 (Gene Codes Corp., Ann Arbor, Michigan, USA) and edited by hand using a "majority-rule" criterion to form a final consenus allele sequence; instances of PCR error and chimeras were easily identified in this way and never occurred in more than one sequence read. Consensus allele sequences

TABLE 1. Primers used to amplify and sequence DNA from members of the *Cheilanthes yavapensis* complex.

DNA region	Primer	5′-3′ Primer sequence	Primer source
rbcL-atpB	ESRBCL26R[a]	GCTTTAGTCTCCGTTTGTGGTGACAT	Korall et al., 2007
rbcL-atpB	ATPB609R	TCRTTDCCTTCRCGTGTACGTTC	Pryer et al., 2004
rbcL-atpB	ATPBSPACER703R[a]	CCAATGATCTGAGTAATSTATCC	Korall et al., 2007
trnG-trnR	TRNG1F[a]	GCGGGTATAGTTTAGTGGTAA	Nagalingum et al., 2007
trnG-trnR	TRNGR353F	TTGCTTMTAYGACTCGGTG	Korall et al., 2007
trnG-trnR	TRNG63R	GCGGGAATCGAACCCGCATCA	Nagalingum et al., 2007
trnG-trnR	TRNR22R[a]	CTATCCATTAGACGATGGACG	Nagalingum et al., 2007
trnP^{UGG}*-petG*	trnP^{UGGa}	TGTAGCGCAGCYYGGTAGCG	Small et al., 2005
trnP^{UGG}*-petG*	petG2[a]	CAATAYCGACGKGGYGATCAATT	Small et al., 2005
rps4-trnS^{GGA}	trnS^{GGAa}	TTACCGAGGGTTCGAATCCCTC	Shaw et al., 2005
rps4-trnS^{GGA}	rps4.5′[a]	ATGTCSCGTTAYCGAGGACCT	Souza-Chies et al., 1997
gapCp	ESGAPCP8F1[a]	ATYCCAAGYTCAACTGGTGCTGC	Schuettpelz et al., 2008
gapCp	ESGAPCP11R1[a]	GTATCCCCAYTCRTTGTCRTACC	Schuettpelz et al., 2008

[a] Primers used for both amplification and sequencing.

(48 newly obtained) were used in all subsequent analyses of the nuclear data set and deposited in GenBank (Appendix 1).

Sequence alignment and phylogenetic analyses—Manual alignments of the plastid *rbcL-atpB*, *trnG-trnR*, *trnP*^{UGG}*-petG*, *rps4-trnS*^{GGA}, and nuclear *gapCp* sequences were carried out using MacClade and are deposited in the database TreeBase (http://www.treebase.org; S2371: M4502, M4503). Unambiguous indels were not recoded, nor were they excluded from the alignments; ambiguous indels were excluded. A total of six data sets were analyzed: the four plastid single-gene data sets, a combined plastid four-gene data set, and the nuclear single-gene data set. The six data sets were analyzed using both a Bayesian Markov chain Monte Carlo (B/MCMC) approach employing the GTR+I+Γ model of sequence evolution, as implemented in the program MrBayes version 3.1.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), as well as a likelihood approach as implemented in the program GARLI version 0.951 (Zwickl, 2006). Conflict among the resulting phylogenies was assessed according to a 0.95 posterior probability (PP) measure for B/MCMC and a 70% maximum likelihood bootstrap (BS) criterion (Mason-Gamer and Kellogg, 1996). Because a comparison of the phylogenies that resulted from each of the four individual plastid data set analyses revealed no incongruence supported across methods (e.g., ML vs. B/MCMC) or across data sets (e.g., *rbcL-atpB* vs. *trnG-trnR*), the data from the four plastid partitions were combined into a single data set.

All B/MCMC analyses comprised four independent runs, each with four chains (one cold and three heated). Default (i.e., flat) priors were used, with the exception of the rate prior that was set to allow rates of evolution to vary among loci (ratepr = variable) in the combined analysis. Chains were run for 10 million generations, and trees were sampled from the cold chain every 1000 generations. To identify when analyses had reached stationarity, we examined the standard deviation of the split frequencies among the independent runs (as calculated by MrBayes), and the output parameter estimates were plotted using the program Tracer version 1.2.1 (Rambaut and Drummond, 2005). Based on these convergence diagnostics, the first 2.5 million generations were excluded from each analysis before obtaining a consensus phylogeny and clade posterior probabilities with the "sumt" command (contype = allcompat). In GARLI, a most likely topology was identified for each of the six data sets, and branch support was assessed using a maximum likelihood BS approach. Maximum likelihood BS analyses (500 replicates) employed the default model of sequence evolution and parameter values estimated by GARLI (Zwickl, 2006).

Enzyme electrophoresis—Fresh leaves of each ingroup taxon were obtained from populations included in the DNA and spore studies and analyzed for electrophoretically detectable enzyme markers. Samples were extracted by crushing a small section (ca. 50 mm^2) of fresh leaf tissue in ten drops of the phosphate grinding buffer-polyvinylpirrolidone solution of Soltis et al. (1983). This extract was absorbed into paper wicks that were then inserted into 12.5% starch gels for electrophoresis. Twelve enzyme loci (list available from authors) were surveyed using standard buffers and stains (Soltis et al., 1983). Following this initial survey, our work focused exclusively on the cytosolic locus of triosephosphate isomerase (*TPI-2*), which was resolved on gel/electrode buffer system 6 of Soltis et al. (1983). Stained gels were photographed using a red filter and Kodak (Rochester, New York, USA) Technical Pan 2415 high contrast film.

RESULTS

Spore number per sporangium and spore diameter—The samples of *C. fendleri* and *C. covillei* included in our analyses all had 64 spores per sporangium and spore diameters <50 μm, which is characteristic of sexual diploids. All samples of *C. wootonii* and *C. yavapensis* had 32 spores per sporangium, in agreement with previous work (Windham and Yatskievych, 2003), demonstrating that all were apomictic. Average spore diameters of *C. wootonii* ranged from 59 to 63 μm (characteristic of apomictic triploids), whereas those of *C. yavapensis* ranged from 64 to 68 μm (apomictic tetraploid). Unlike the other ingroup taxa, *C. lindheimeri* varied in both spore number per sporangium and average spore diameter. The majority of specimens had 32 spores per sporangium and spore diameters >59 μm, consistent with earlier studies (Reeves, 1979; Windham and Yatskievych, 2003) that identified *C. lindheimeri* as an apomictic triploid. However, three samples yielded 64 spores per sporangium and average spore measurements in the 47–50 μm range. These represent a rare, previously undetected, sexual diploid cytotype of *C. lindheimeri* (see Schuettpelz et al., 2008).

cpDNA phylogeny—Trees resulting from maximum likelihood and Bayesian analyses of the combined plastid four-gene data set had identical topologies. Maximum likelihood analysis resulted in a single most likely tree (ln L = −6280.9384; Fig. 2). Three well-supported clades were resolved, corresponding to the *C. covillei*, *C. fendleri*, and *C lindheimeri* genomes. The *C. covillei* clade included only accessions of diploid *C. covillei* (PP = 1.0, BS = 100%), whereas the *C. fendleri* clade (PP = 1.0, BS = 100%) included all samples of diploid *C. fendleri* plus all accessions of the apomictic triploid *C. wootonii*. The *C. lindheimeri* clade (PP = 1.0, BS = 100%) included all samples of that species (regardless of ploidy level), plus all accessions of *C. yavapensis*. Sexual diploid and apomictic triploid samples of *C. lindheimeri* have identical plastid sequences, suggesting that genetic divergence between the cytotypes is minimal.

Nuclear phylogeny—Trees resulting from maximum likelihood and Bayesian analyses of the ~600 bp short copy *gapCp* data set had identical topologies. Maximum likelihood analysis resulted in a single most likely tree (ln L = −12712.6944; Fig. 3) with three strongly supported clades corresponding to *C. covillei* (PP = 1.0, BS = 100%), *C. fendleri* (PP = 1.0, BS = 89%), and *C. lindheimeri* (PP = 1.0, BS = 99%). All consensus allele sequences obtained from the apomictic triploid samples of *C. lindheimeri* (41 clones) formed a well-supported clade with the diploid accessions (Fig. 3), supporting an autotriploid origin for the apomictic cytotype (see Schuettpelz et al., 2008). By contrast, both *C. wootonii* and *C. yavapensis* showed strong evidence of allopolyploid origins. The three accessions of triploid *C. wootonii* each yielded sequences that clustered with *C. fendleri* and *C. lindheimeri*. The situation encountered in tetraploid *C. yavapensis* was even more complex; all four accessions contained sequences derived from *C. covillei*, *C. fendleri*, and *C. lindheimeri* (Fig. 3).

Enzyme electrophoresis—Triosephosphate isomerase (TPI) was the only enzyme surveyed that provided genome specific markers. Figure 4 summarizes our findings with respect to this enzyme. In all plants sampled, TPI was expressed as two loci (*TPI-1* and *TPI-2*) active in the plastid and cytosol, respectively. As in most ferns (Gastony, 1988), the plastid locus (*TPI-1*) was represented by a highly conserved (i.e., invariant across our sample), three-banded pattern in which the fastest migrating band was distinctly fainter than the other two. This triplet generally migrated farther into the gel than products of the cytosolic locus and is most apparent in lanes 1 and 2 of Fig. 4. In contrast to the plastid locus, the cytosolic form of the enzyme (*TPI-2*) was variable across our study group, with each species yielding a distinctive band pattern.

Samples of *C. covillei* appear in lanes 1 and 2, which show a single band (labeled C-C homodimer) at the cytosolic locus. Though not represented by diploids on this gel, the marker bands for *C. fendleri* (F-F homodimer) and *C. lindheimeri* (L-L homodimer) are apparent in the samples of *C. wootonii* (lanes 5 and 6). The F-F homodimer is the fastest migrating band at the cytosolic locus and overlaps the slowest band of the plastid

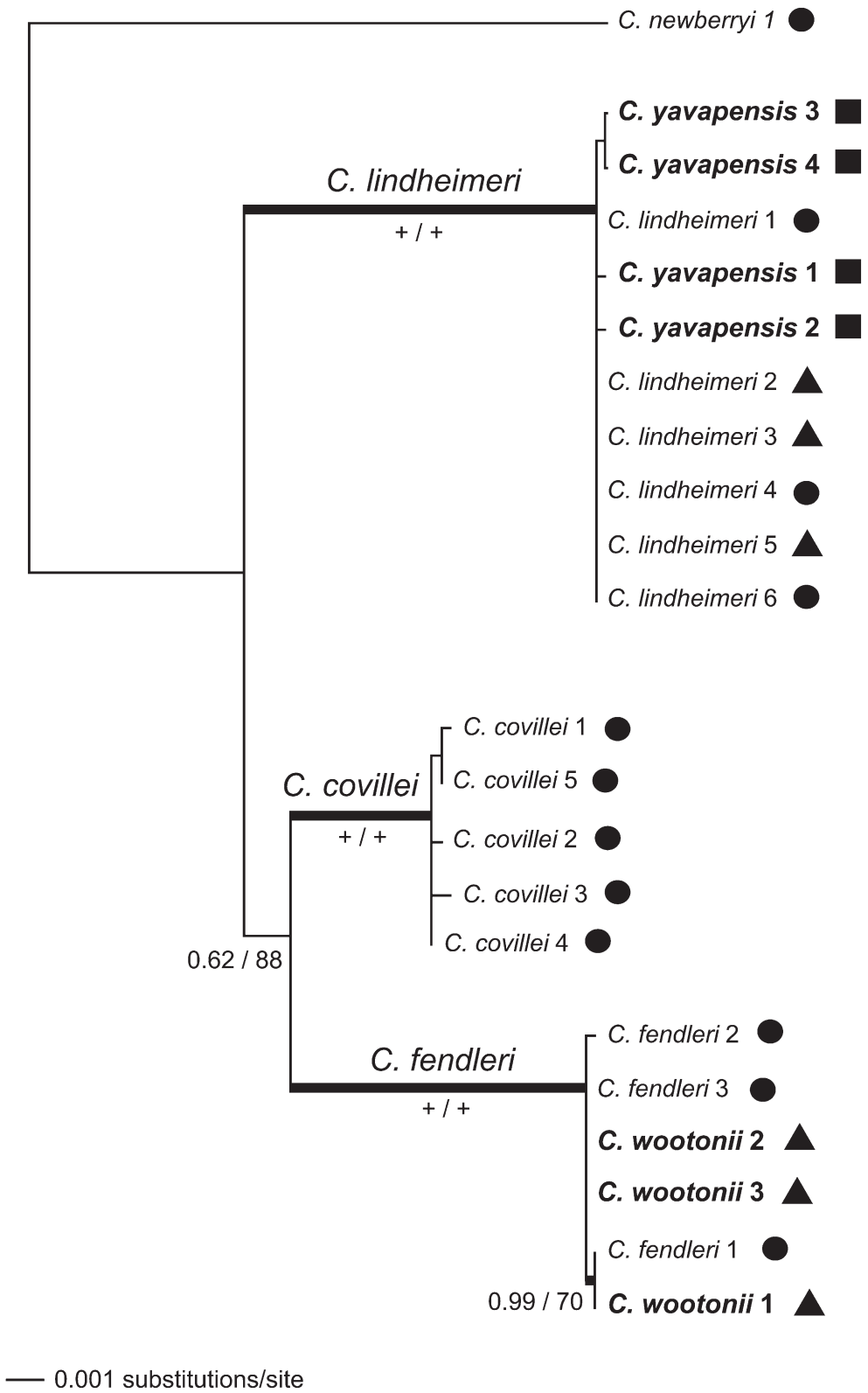

Fig. 2. Plastid phylogeny for members of the *Cheilanthes yavapensis* complex based on combined analysis of *rbcL-atpB*, *trnG-trnR*, *trnP*^{UGG}*-petG*, and *rps4-trnS*^{GGA}; the best maximum likelihood topology is shown (ln L = −6280.9384). Accessions of the apomictic allopolyploids *C. wootonii* and *C. yavapensis* are in boldface type. Following each taxon name is a unique identification number corresponding to a particular individual (Appendix 1). Circles, triangles, and squares depict ploidy level (diploid, triploid, and tetraploid, respectively). Numbers below branches correspond to posterior probability and maximum likelihood bootstrap support (PP / BS), respectively. Thickened branches indicate posterior probability ≥0.95 *and* maximum likelihood bootstrap support ≥70%. Plus signs highlight branches that have PP = 1.0 and/or BS = 100%, respectively.

locus; the L-L homodimer is the slowest migrating band. Because TPI is a dimeric enzyme, the presence of both F and L in the cytosol results in a band of intermediate mobility (F-L heterodimer; see lanes 5 and 6). This F-L heterodimer migrates the same distance as the C-C homodimer (see lanes 1 and 2). In individuals where both the F-L heterodimer and the C-C homodimer are present (see lanes 3, 4, and 7), these bands are indistinguishable, yet detectable due to the additional presence of F-C and C-L heterodimers in the cytosol. The relative intensity of the three cytosolic bands in lanes 5 and 6 is useful for determining the genomic constitution of *C. wootonii*. The F-F homodimer and F-L heterodimer are approximately equal in intensity and significantly darker than the L-L homodimer. This pattern is congruent with the 4:4:1 ratio expected in a triploid that contains two genomes of *C. fendleri* and one of *C. lindheimeri*.

Cheilanthes yavapensis shows all bands that would be expected in a hybrid containing the C, F, and L genomes (Fig. 4). The F-F and L-L homodimers are apparent, as are the F-C and C-L heterodimers. The F-L heterodimer and the C-C homodimer comigrate, but the unmistakable F-C and C-L heterodimers confirm that both are present. Despite the superficial similarity of the three separate *C. yavapensis* accessions, there are subtle differences between the samples on the left (lanes 3 and 4) and the sample on the right (lane 7). The samples in lanes 3 and 4 show enhanced expression of the L-L homodimer, indicating that these individuals may represent CFLL hybrids. In lane 7, all bands that include C are more intense, suggesting that this plant (from the type locality of *C. yavapensis*) is a CCFL hybrid.

DISCUSSION

Deciphering species relationships and hybrid origins in polyploid agamic species complexes is difficult due to the cryptic nature of the morphological characters that distinguish species. In this study of cheilanthoid ferns, we combined spore studies, plastid and nuclear DNA sequencing, and enzyme electrophoresis to resolve the contentious origin of the apomictic tetraploid hybrid *C. yavapensis* and to determine its relationship to *C. wootonii*.

Identifying maternal parents—In all ferns analyzed to date, the chloroplast genome has been shown to be maternally inherited (e.g., Gastony and Yatskievych, 1992; Vogel et al., 1998; Guillon and Raquin, 2000). Thus, it is possible to use plastid phylogenies to identify the maternal parents of hybrid ferns. In our plastid phylogeny (Fig. 2), all sampled accessions of the apomictic triploid *C. wootonii* have plastid sequences that are essentially identical to those of sexual diploid *C. fendleri*, suggesting that this species is the maternal progenitor of *C. wootonii*. Conversely, plastid sequences from all sampled accessions of the apomictic tetraploid *C. yavapensis* indicate that its maternal parent is *C. lindheimeri* (Fig. 2). These results provide unequivocal evidence that *C. wootonii* and *C. yavapensis* are distinct entities.

Other genomes present in the hybrids—The phylogeny resulting from our analysis of nuclear *gapCp* allows us to identify additional genomes (other than the maternally inherited chloroplast) present in these hybrids. All cloned sequences from accessions of the apomictic triploid *C. wootonii* group with either *C. lindheimeri* or *C. fendleri*, supporting Reeves' (1979) hypothesis regarding the hybrid origin of this taxon (Fig. 1). In contrast, the four accessions of *C. yavapensis* contained sequences derived from *C. covillei*, *C. fendleri*, and *C. lindheimeri* (Fig. 3). These data indicate that *C. yavapensis* is a trigenomic allotetraploid, contradicting earlier hypotheses (Reeves, 1979; Gastony and Windham, 1989; Windham, 1993a) that considered tetraploid *C. yavapensis* a hybrid between sexual diploid *C. covillei* and apomictic triploid *C. lindheimeri* (Fig. 1).

Within each of the three ingroup lineages identified by analysis of nuclear DNA sequences, subclades that appear to reflect allelic variation at the *gapCp* locus are evident (Fig. 3). The phylogenetic distribution of these alleles may provide additional insights into the origins of *C. wootonii* and *C. yavapensis*. In the case of apomictic triploid *C. wootonii*, each of the three accessions we sampled includes a single allele derived from *C. lindheimeri*. Samples of the other parent, *C. fendleri*, have two *gapCp* alleles, both of which are found in every sampled accession of *C. wootonii* (Fig. 3). This suggests that *C. wootonii* contains two genomes from *C. fendleri* and one from *C. lindheimeri*, which is consistent with the dosage (FFL) seen at the *TPI-2* locus (Fig. 4). In addition, the presence of a distinctive *C. lindheimeri* allele in one accession of *C. wootonii* suggests that the latter species may have arisen through multiple, independent hybridization events.

The evidence for multiple origins is even more compelling in apomictic tetraploid *C. yavapensis* (Fig. 3). This species is represented in our analyses by four separate accessions that comprise two genetically distinct forms with nonoverlapping geographic ranges. One form includes *C. yavapensis* 4 (from the type locality of *C. yavapensis*) and *C. yavapensis* 3, which were collected near the western and northern edges of the species' distribution, respectively. The other form comprises *C. yavapensis* 2 and *C. yavapensis* 1, collected near the center of the range in central and southeastern Arizona. Within these two forms, there is little or no variation in the *gapCp* sequences representing the three constituent genomes. However, a close comparison of the two forms reveals that they have incorporated different alleles from each parental species (Fig. 3), supporting multiple origins for *C. yavapensis*. These putative independent origins correspond exactly to the genetic variants encountered in the enzyme analyses discussed next.

Determining genome dosage—Nuclear DNA evidence indicates that our accessions of *C. wootonii* contain two different genomes, whereas those of *C. yavapensis* contain three (Fig. 3). Because these accessions are triploid and tetraploid, respectively, we can deduce that, in each case, one of the constituent genomes is present as two copies. To determine genome dosage, we examined the relative intensity of bands at the *TPI-2* locus (Fig. 4; Danzmann and Bogart, 1982). Turning first to triploid *C. wootonii* (Fig. 4; lanes 5 and 6), the enzyme data clearly show that it is the *C. fendleri* genome that is duplicated (note the relative intensities of the F-F homodimer and the F-L heterodimer compared to the L-L homodimer). Therefore, we conclude that the genome dosage of *C. wootonii* is two *C. fendleri* to one *C. lindheimeri* (symbolized as FFL). The two distinct origins of *C. yavapensis* apparent in the nuclear DNA phylogeny (Fig. 3) find additional support in the enzyme analyses, which indicate that this "species" encompasses two geographically correlated entities characterized by different genome dosages. These two "forms" of *C. yavapensis* are designated CFLL and CCFL in the TPI gel photo (Fig. 4). The CFLL combination is represented by lanes 3 and 4, while CCFL is seen in lane 7. These two forms have the same number of bands, but the

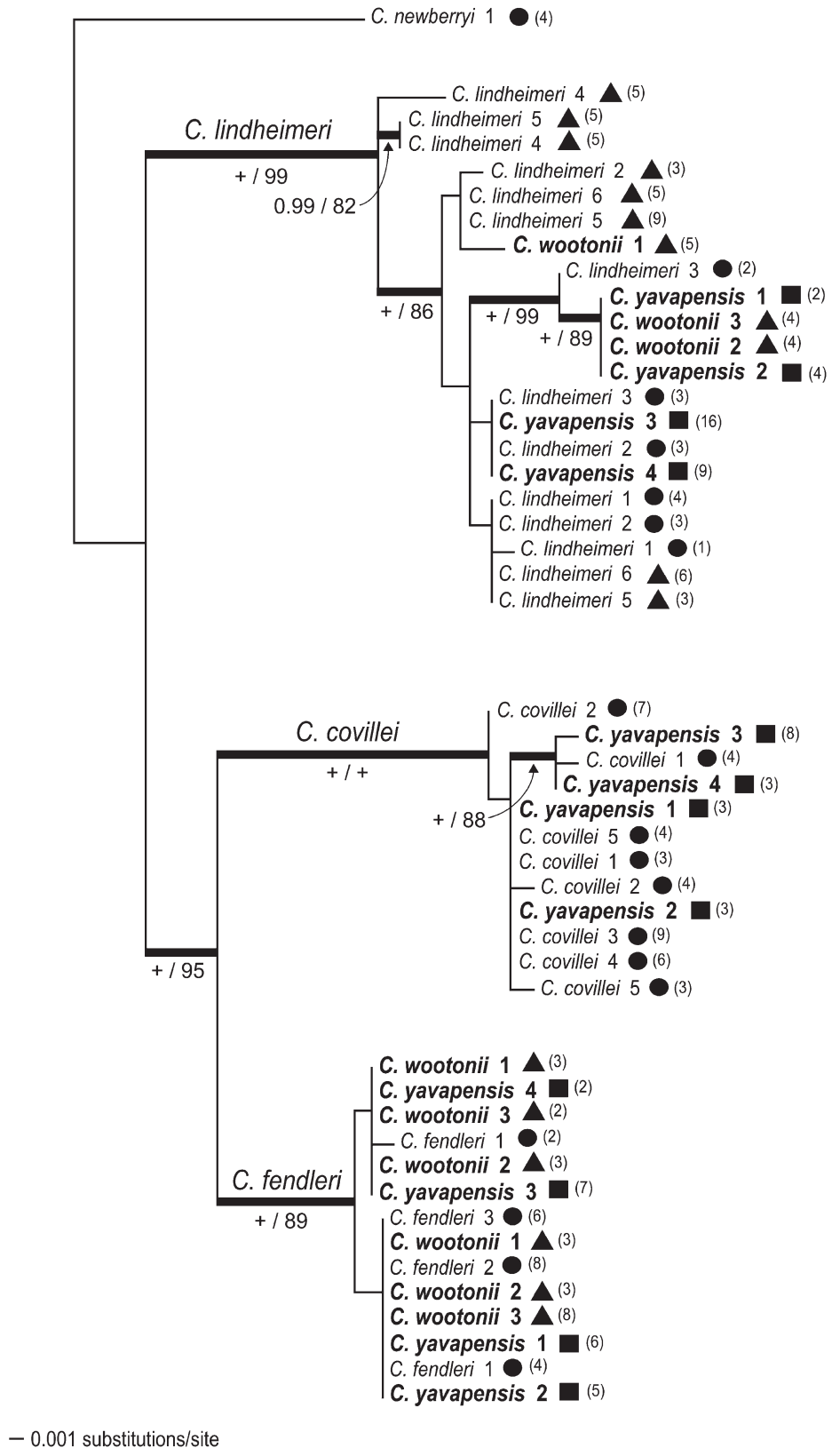

Fig. 3. Nuclear phylogeny for the *Cheilanthes yavapensis* complex based on *gapCp*; the best maximum likelihood topology is shown (ln L = −12712.6944). Consensus allele sequences from accessions of *C. wootonii* and *C. yavapensis* are in boldface type. Symbols and thickened branches are as described in Fig. 2. Numbers in parentheses (following taxon names) indicate the number of clones that were compiled to determine the corresponding consensus allele sequence.

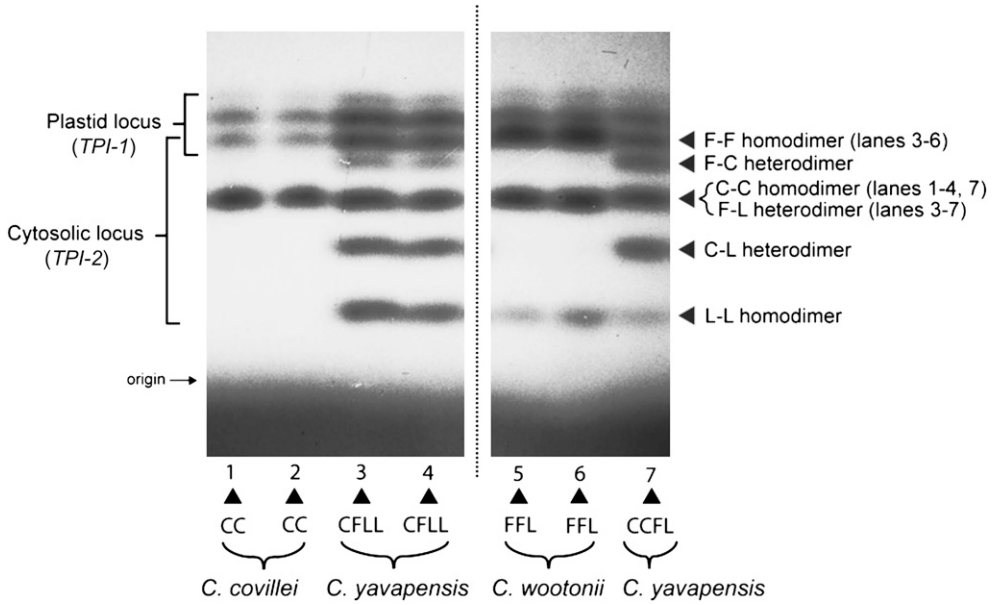

Fig. 4. Starch gel electrophoretic patterns for two loci of the TPI enzyme in *Cheilanthes yavapensis* and relatives. Lanes 1 and 2 represent diploid *C. covillei*, lanes 3 and 4 are tetraploid *C. yavapensis* (CFLL form), lanes 5 and 6 correspond to triploid *C. wootonii* (used here also as a proxy for *C. fendleri* and *C. lindheimeri*), and lane 7 is tetraploid *C. yavapensis* (CCFL form). Note the difference in genomic dosage between CFLL and CCFL (especially notable at the L-L homodimer) as well as the partial overlap of plastid and cytosolic loci. Vertical dotted line between lanes 4 and 5 indicates an excised portion of the gel not relevant to the conclusions of this study.

marker alleles are present in different dosages. This is most apparent in the L-L homodimer produced by the *C. lindheimeri* genome (the band closest to the lower edge of Fig. 4). In CFLL (lanes 3 and 4), the L-L homodimer is the darkest/thickest band; in CCFL this band is less intense (about the same intensity as in the FFL samples in lanes 5 and 6), reflecting a lower dosage of the *C. lindheimeri* genome.

Origins of the apomictic polyploids—Based on the nuclear *gapCp* data, Schuettpelz et al. (2008) demonstrated that the apomictic triploids *C. lindheimeri* and *C. wootonii* had autopolyploid and allopolyploid origins, respectively. Although these authors were able to identify the genomes (*C. fendleri* and *C. lindheimeri*) present in *C. wootonii*, their data did not permit an examination of its possible origins. Our analyses, summarized as a phyloreticulogram (Fig. 5), reveal two additional and important details about *C. wootonii*; namely, genome dosage (FFL) and the maternal parent (*C. fendleri*). Based on these data, we can offer two possible scenarios for the origin of *C. wootonii*. The simplest explanation involves direct hybridization between a normal (*n*) male gamete derived from diploid *C. lindheimeri* and an unreduced (2*n*) female gamete from diploid *C. fendleri*. A second, more complex, scenario involves the formation of a sterile homoploid hybrid between *C. fendleri* and *C. lindheimeri*, followed by the production of an unreduced gamete that backcrossed with a normal gamete of *C. fendleri*. Our ongoing studies of other likely hybrids involving these species, coupled with analyses of geographically correlated allelic variation in *C. fendleri*, may eventually reveal both how and where *C. wootonii* was formed.

Contrary to earlier morphology-based hypotheses (Reeves, 1979; Windham, 1993a), our data indicate that apomictic tetraploid *C. yavapensis* did not arise through hybridization between sexual diploid *C. covillei* and apomictic triploid *C. lindheimeri* (Fig. 1). The nuclear *gapCp* locus reveals that every sampled accession of *C. yavapensis* contains a genome derived from *C. fendleri*, in addition to the expected contributions from *C. covillei* and *C. lindheimeri* (Fig. 3). Additionally, the enzyme and *gapCp* data indicate that populations referred to *C. yavapensis* are polyphyletic; they are the result of independent hybridization events that have given rise to two entities (CCFL and CFLL) with different genomic constitutions and distinct geographic ranges.

Despite the unexpected complexity of *C. yavapensis*, it is possible to offer some robust hypotheses regarding the origins of this taxon. We have demonstrated that two distinct apomictic tetraploids are referred to *C. yavapensis*, each representing a unique genomic combination (UGC). Because each apomictic tetraploid cheilanthoid fern analyzed to date has been shown to be a hybrid between a sexual diploid and an apomictic triploid (e.g., see Gastony and Yatskievych, 1992), this is a good working hypothesis for the origin of the two UGCs referred to *C. yavapensis*. As in most organisms, hybridization between sexual and apomictic ferns is highly constrained with regard to directionality. Apomictic ferns reproduce by generating a sporophyte directly from gametophytic tissue without fertilization. Though they occasionally give rise to functional antheridia, they do not produce functional archegonia (Gastony and Yatskievych, 1992, and references therein). Therefore, when sexual fern species hybridize with apomicts, we know that the sexual taxon must be the maternal parent.

Our plastid phylogeny (Fig. 2) provides critical insight into the origins of the two UGCs included within *C. yavapensis*. Most importantly, it identifies *C. lindheimeri* as the maternal (i.e., sexual diploid) parent of all sampled accessions. By "subtracting" this maternal contribution (one L genome) from the two *C. yavapensis* UGCs identified above (CCFL and CFLL), it is possible to predict the genomic makeup of the paternal trip-

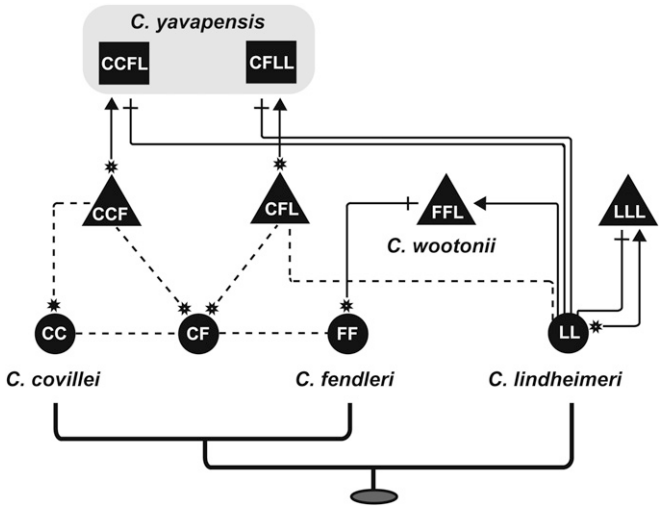

Fig. 5. Phyloreticulogram summarizing hypothesized origins of apomictic polyploids in the *Cheilanthes yavapensis* complex. Diploids, triploids, and tetraploids are represented by circles, triangles, and squares, respectively. All triploid taxa exhibit apomictic reproduction, all diploids are sexually reproducing, and the two tetraploid UGCs of *C. yavapensis* (CCFL and CFLL) are apomictic. Solid lines terminating in arrows point from the paternal parent to its offspring. Solid lines terminating in crosses point from the maternal parent to its offspring. Dashed lines represent hypothesized (or as yet unresolved) relationships, for which evidence of maternal and paternal parentage is yet to be determined. Lines originating in stars represent unreduced gametes.

loids. Based on this simple exercise, we hypothesize that the apomictic triploid (paternal) parent of the CCFL form of *C. yavapensis* had the genomic constitution CCF, whereas the sperm donor for the CFLL form was a CFL triploid (Fig. 5). Although neither of these triploids has been reported by previous authors, ongoing studies (A. L. Grusz et al., unpublished data) indicate that both exist in nature.

A full understanding of the origins of *C. yavapensis* will require tracking the various genomic components back to the sexual diploids that initiated the hybridization events. A major thrust of this effort will be to explain the origins of the two predicted triploids CCF and CFL (Fig. 5). There are two possible scenarios for the origin of CCF (analogous to the proposed origin of *C. wootonii* discussed earlier) and three distinct pathways that could yield the CFL triploid. The most likely explanation (requiring the fewest steps and no new taxa) is indicated by dashed lines in Fig. 5. In this scenario, the sexual diploids *C. covillei* and *C. fendleri* hybridize to form a sterile homoploid intermediate (CF), the existence of which has been confirmed (Windham and Rabe, 1993). Through the production of unreduced CF gametes, this homoploid hybrid could backcross to *C. covillei* (yielding CCF) and hybridize with sexual diploid *C. lindheimeri* (yielding CFL). Studies are ongoing to determine whether this is an accurate portrayal of the earliest stages of evolution in the *C. yavapensis* complex.

Despite the molecular and morphological features that seem to unite *C. yavapensis*, our study provides unmistakable evidence of independent hybridization events that gave rise to two cryptic taxa (CCFL and CFLL). *Cheilanthes wootonii* was not involved in the origin of either form of *C. yavapensis*; thus, it should continue to be treated as a separate species, despite the subtle morphological characters that distinguish it from *C. yavapensis*. As currently defined, *C. yavapensis* encompasses two genetically distinct allotetraploids that share one parent (*C. lindheimeri*) but not the other. This situation is not unusual among plants and would typically be resolved by recognizing two species. In the case of *C. yavapensis*, however, preliminary observations of the two UGCs suggest that they differ only slightly in morphology. For practical purposes of identification, they may prove impossible to differentiate. They are, however, critical pieces of the puzzle when it comes to understanding the evolution of cheilanthoid ferns in the North American Southwest.

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APPENDIX 1. Taxa sampled for DNA sequence data in this study, including voucher information and GenBank accession numbers. Taxon names are in bold italics followed by the nomenclatural authority. Numbers in boldface are identification numbers used in this study followed by corresponding Fern DNA database numbers (http://www.pryerlab.net/DNA_database.shtml). Each consensus allele sequence of nuclear *gapCp* is identified, followed by the number of clones included in that consensus, the length of the sequence reported, and its corresponding GenBank accession number. Sequence data for plastid loci (*rbcL-atpB*, *trnG-trnR*, *trnP*^{UGG}*-petG*, and *rps4-trnS*^{GGA}) for each accession are followed by their corresponding GenBank accession numbers.

Taxon Authority—**Individual number:** Fern DNA database number **(ploidy level)**, *Voucher* (Herbarium); *gapCp*: allele no., no. of clones, sequence length, GenBank accession [repeat for multiple *gapCp* alleles]; *rbcL-atpB*: GenBank accession; *trnG-trnR*: GenBank accession; *trnP*^{UGG}*-petG*: Genbank accession; *rps4-trnS*^{GGA}: GenBank accession.

Cheilanthes covillei Maxon—**1:** 3485 **(diploid)**; *Windham 2945* (UT); *gapCp*: allele 1, 4 clones, 600bp, FJ870860, allele 2, 3 clones, 600bp, FJ870861; *rbcL-atpB*:FJ870813;*trnG-trnR*:FJ870771;*trnP*^{UGG}*-petG*:FJ870791;*rps4-trnS*^{GGA}: FJ870835. **2:** 3150 **(diploid)**, *Schuettpelz 443* (DUKE); *gapCp*: allele 1, 7 clones, 600bp, FJ870857, allele 2, 4 clones, 596bp, FJ870858; *rbcL-atpB*: FJ870814; *trnG-trnR*: EU268697; *trnP*^{UGG}*-petG*: FJ870792; *rps4-trnS*^{GGA}: FJ870836. **3:** 3487 **(diploid)**, *Windham 3322* (DUKE); *apCp*: allele 1, 9 clones, 600bp, FJ870862; *rbcL-atpB*: FJ870815; *trnG-trnR*: FJ870772; *trnP*^{UGG}*-petG*: FJ870793; *rps4-trnS*^{GGA}: FJ870837. **4:** 3156 **(diploid)**, *Schuettpelz 449* (DUKE); *gapCp*: allele 1, 6 clones, 600bp, FJ870859; *rbcL-atpB*: FJ870816; *trnG-trnR*: FJ870773; *trnP*^{UGG}*-petG*: FJ870794; *rps4-trnS*^{GGA}: FJ870838. **5:** 3845 **(diploid)**, *Windham 3436* (DUKE); *gapCp*: allele 1, 4 clones, 600bp, FJ870863, allele 2, 3 clones, 600bp, FJ870864; *rbcL-atpB*: FJ870817; *trnG-trnR*: FJ870774; *trnP*^{UGG}*-petG*: FJ870795; *rps4-trnS*^{GGA}: FJ870839. ***Cheilanthes fendleri*** Hooker—**1:** 3690 **(diploid)**, *Windham 3408* (DUKE); *gapCp*: allele 1, 4 clones, 602bp, FJ870866, allele 2, 2 clones, 602bp, FJ870867; *rbcL-atpB*: FJ870818; *trnG-trnR*: FJ870775; *trnP*^{UGG}*-petG*: FJ870796; *rps4-trnS*^{GGA}: FJ870840. **2:** 3177 **(diploid)**, *Schuettpelz 470* (DUKE); *gapCp*: allele 1, 8 clones, 602bp, FJ870865; *rbcL-atpB*: FJ870819; *trnG-trnR*: FJ870776; *trnP*^{UGG}*-petG*: FJ870797; *rps4-trnS*^{GGA}: FJ870841. **3:** 3691 **(diploid)**, *Windham 3410* (DUKE); *gapCp*: allele 1, 6 clones, 602bp, FJ870868; *rbcL-atpB*: FJ870820; *trnG-trnR*: FJ870777; *trnP*^{UGG}*-petG*, FJ870798; *rps4-trnS*^{GGA}: FJ870842. ***Cheilanthes lindheimeri*** Hooker—**1:** 3490 **(diploid)**, *Windham 97-015* (DUKE, UT); *gapCp*: allele 1, 4 clones, 601bp, FJ870879, allele 2, 1 clone, 601bp, FJ870880; *rbcL-atpB*: FJ870821; *trnG-trnR*: FJ870778; *trnP*^{UGG}*-petG*: FJ870799; *rps4-trnS*^{GGA}: FJ870843. **2:** 3157 **(diploid)**, *Schuettpelz 450* (DUKE); *gapCp*: allele 1, 3 clones, 601bp, FJ870872, allele 2, 3 clones, 601bp, FJ870873; *rbcL-atpB*: FJ870822; *trnG-trnR*: FJ870779; *trnP*^{UGG}*-petG*: FJ870800; *rps4-trnS*^{GGA}: FJ870844. **3:** 3692 **(diploid)**, *Spellenberg et al. 5056* (NMC); *gapCp*: allele 1, 3 clones, 601bp, FJ870881, allele 2, 2 clones, 600bp, FJ870882; *rbcL-atpB*: FJ870823; *trnG-trnR*: FJ870780; *trnP*^{UGG}*-petG*: FJ870801; *rps4-trnS*^{GGA}: FJ870845. **4:** 3205 **(triploid)**, *Schuettpelz 498* (DUKE), *gapCp*, allele 1, 5 clones, 601bp, FJ870877, allele 2, 5 clones, 601bp, FJ870878; *rbcL-atpB*: FJ870824; *trnG-trnR*: FJ870781; *trnP*^{UGG}*-petG*: FJ870802; *rps4-trnS*^{GGA}: FJ870846. **5:** 3196 **(triploid)**, *Schuettpelz 489* (DUKE); *gapCp*: allele 1, 9 clones, 601bp, FJ870874, allele 2, 3 clones, 600bp, FJ870875, allele 3, 5 clones, 601 bp, FJ870876; *rbcL-atpB*: FJ870825; *trnG-trnR*: FJ870782; *trnP*^{UGG}*-petG*: FJ870803; *rps4-trnS*^{GGA}: FJ870847. **6:** 3147 **(triploid)**, *Schuettpelz 440* (DUKE); *gapCp*: allele 1, 5 clones, 601bp, FJ870869, allele 2, 6 clones, 601bp, FJ870870, allele 3, 3 clones, 600bp, FJ870871; *rbcL-atpB*: FJ870826; *trnG-trnR*: FJ870783; *trnP*^{UGG}*-petG*: FJ870804; *rps4-trnS*^{GGA}: FJ870848. ***Cheilanthes newberryi*** (D.C. Eaton) Domin—**1:** 3827 **(diploid)**, *Metzgar 174* (DUKE); *gapCp*: allele 1, 4 clones, 582bp, FJ870883; *rbcL-atpB*: FJ870827; *trnG-trnR*: EU268685; *trnP*^{UGG}*-petG*: FJ870805; *rps4-trnS*^{GGA}: FJ870849. ***Cheilanthes wootonii*** Maxon—**1:** 3195 **(triploid)**, *Schuettpelz 488* (DUKE); *gapCp*: allele 1, 3 clones, 602bp, FJ870884, allele 2, 3 clones, 602bp, FJ870885, allele 3, 5 clones, 601bp, FJ870886; *rbcL-atpB*: FJ870828; *trnG-trnR*: FJ870784; *trnP*^{UGG}*-petG*: FJ870806; *rps4-trnS*^{GGA}: FJ870850. **2:** 3693 **(triploid)**, *Windham 3409* (DUKE), *gapCp*, allele 1, 4 clones, 600bp, FJ870887, allele 2, 3 clones, 602bp, FJ870888, allele 3, 3 clones, 602bp, FJ870889; *rbcL-atpB*: FJ870829; *trnG-trnR*: FJ870785; *trnP*^{UGG}*-petG*: FJ870807; *rps4-trnS*^{GGA}: FJ870851. **3:** 3694 **(triploid)**, *Spellenberg* & *Mart 10407* (NMC); *gapCp*, allele 1, 4 clones, 600bp, FJ870890, allele 2, 8 clones, 602bp, FJ870891, allele 3, 2 clones, 602bp, FJ870892; *rbcL-atpB*: FJ870830; *trnG-trnR*: FJ870786; *trnP*^{UGG}*-petG*: FJ870808; *rps4-trnS*^{GGA}: FJ870852. ***Cheilanthes yavapensis*** T. Reeves ex Windham—**1:** 3489 **(tetraploid)**, *McGill 6156* (UT); *gapCp*: allele 1, 3 clones, 600bp, FJ870902, allele 2, 2 clones, 600bp, FJ870903, allele 3, 6 clones, 602bp, FJ870904; *rbcL-atpB*: FJ870831; *trnG-trnR*: FJ870787; *trnP*^{UGG}*-petG*: FJ870809; *rps4-trnS*^{GGA}: FJ870853. **2:** 3151 **(tetraploid)**, *Schuettpelz 444* (DUKE); *gapCp*: allele 1, 3 clones, 600bp, FJ870899, allele 2, 5 clones, 602bp, FJ870900, allele 3, 4 clones, 600bp, FJ870901; *rbcL-atpB*: FJ870832; *trnG-trnR*: FJ870788; *trnP*^{UGG}*-petG*: FJ870810; *rps4-trnS*^{GGA}: FJ870854. **3:** 3122 **(tetraploid)**, *Schuettpelz 415* (DUKE); *gapCp*: allele 1, 16 clones, 601bp, FJ870893, allele 2, 7 clones, 602bp, FJ870894, allele 3, 8 clones, 600bp, FJ870895; *rbcL-atpB*: FJ870833; *trnG-trnR*: FJ870789; *trnP*^{UGG}*-petG*: FJ870811; *rps4-trnS*^{GGA}: FJ870855. **4:** 3145 **(tetraploid)**, *Schuettpelz 438* (DUKE); *gapCp*: allele 1, 9 clones, 601bp, FJ870896, allele 2, 2 clones, 602bp, FJ870897, allele 3, 3 clones, 600bp, FJ870898; *rbcL-atpB*: FJ870834; *trnG-trnR*: FJ870790; *trnP*^{UGG}*-petG*: FJ870812; *rps4-trnS*^{GGA}: FJ870856.