RESEARCH ARTICLE

AMERICAN JOURNAL OF BOTANY

Unraveling the origin of the Appalachian gametophyte, *Vittaria appalachiana*[1]

Jerald B. Pinson[2,4] and Eric Schuettpelz[3]

PREMISE OF STUDY: Ferns and lycophytes are distinct among plants in producing two free-living life stages: a long-lived sporophyte phase and a (usually) short-lived gametophyte phase. Notably, however, some species have perennial, vegetatively reproducing gametophytes. *Vittaria appalachiana* is one of just three species in which mature sporophytes are unknown. It has a wide range throughout the Appalachian Mountains and Plateau, where it reproduces asexually via gemmae. The origin of *V. appalachiana*, however, has long been a mystery, with most previous studies suggesting it may have resulted from hybridization of two closely related *Vittaria* species (*V. graminifolia* and *V. lineata*).

METHODS: A four-gene plastid data set including 32 samples of six *Vittaria* species, plus samples of five outgroup species, was analyzed to uncover phylogenetic relationships. Additional analyses of nuclear *DET1* gene sequences allowed for the examination of hypotheses involving a hybrid origin for *V. appalachiana*.

KEY RESULTS: In the plastid phylogeny, *V. appalachiana* is well supported as monophyletic, but is embedded within *V. graminifolia*. With the exception of a single aberrant allele, this result is mirrored in the nuclear tree.

CONCLUSIONS: Through analyses of plastid and nuclear data sets, this study demonstrates that a hybrid origin for *V. appalachiana* is unlikely. Instead, it appears that this species emerged from within the *V. graminifolia* lineage. Further work is needed to fully elucidate the genetic structure within this group.

KEY WORDS Appalachian Mountains; nuclear *DET1*; hybridization; independent gametophytes; leptosporangiate ferns; plastid genes; polyploidy; Pteridaceae

Ferns represent a distinct lineage of plants (Pryer et al., 2001, 2004) characterized (along with lycophytes) by a life cycle with alternating free-living sporophyte and gametophyte phases. Fern sporophytes are generally long-lived and are thus seen as the "dominant" phase, while gametophytes are typically ephemeral (Farrar et al., 2008). There are a few fern lineages, however, in which the gametophyte phase can also be long-lived and even capable of vegetative reproduction (e.g., filmy ferns and vittarioid ferns) (Farrar, 1985). In some cases, such gametophytes may even have ranges extending well beyond those of conspecific sporophytes (Dassler and Farrar, 1997; Farrar, 1998; Rumsey and Jermy, 1998; Ebihara et al., 2009; Duffy et al., 2015).

Among fern species with long-lived gametophytes, perhaps none is as peculiar as *Vittaria appalachiana* Farrar & Mickel, commonly referred to as the Appalachian gametophyte (Farrar and Mickel, 1991). As its name suggests, this temperate member of what is an almost exclusively tropical lineage inhabits the Appalachian Mountains and Plateau of the eastern United States (Fig. 1), where it grows on porous rock outcrops, usually adjacent to water (Farrar, 1978). Most distinctive, however, is the fact that this species exists exclusively as a vegetatively reproducing gametophyte. *Vittaria appalachiana* is one of only three ferns [the others are *Crepidomanes intricatum* (Farrar) Ebihara & Weakley and *Hymenophyllum tayloriae* Farrar & Raine] in which mature sporophytes have never been observed (Raine et al., 1991; Farrar, 1992; but see Li et al., 2009). Instead, *V. appalachiana* reproduces asexually via gemmae—vegetative propagules consisting of a few cells that are produced along the margins of the gametophytes. When mature, these gemmae can separate from the gametophytes, disperse short distances, and grow into new independent, but genetically identical, individuals.

Fern gemmae are quite large in comparison to spores, typically 0.2–1.0 mm in length and are generally considered too large for

[1] Manuscript received 11 December 2015; revision accepted 25 January 2016.

[2] Department of Biology, University of Florida, Gainesville, Florida 32611-8525 USA; and
[3] Department of Botany, Smithsonian Institution, MRC 166 PO Box 37012 Washington, District of Columbia 20013-7012 USA
[4] Author for correspondence (e-mail: jbp4166@ufl.edu)

doi:10.3732/ajb.1500522

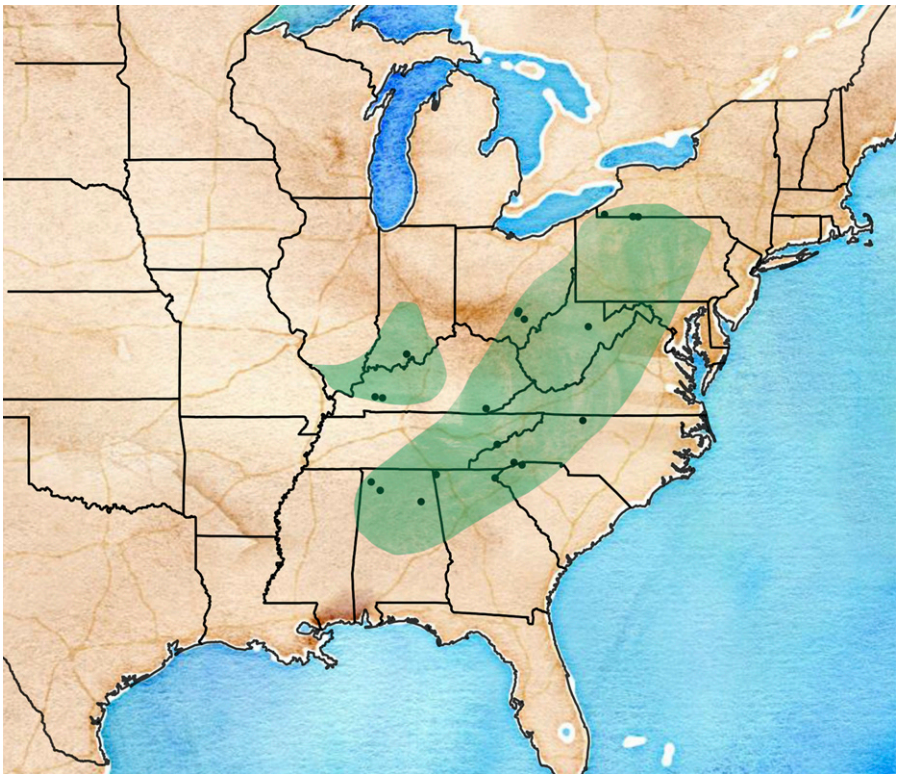

FIGURE 1 Approximate range of *Vittaria appalachiana* (light green shading; based on Farrar, 1978) and sampling locations (black dots). Samples were collected between the years of 2011 and 2013, including those provided by Sally Chambers.

long-distance wind dispersal (Farrar, 1990). Instead, gemmae are likely dispersed short distances by wind, water, or possibly animals (in bryophytes gemmae dispersal has been shown to be facilitated over short distances by slugs [Kimmerer and Young, 1995] and potentially by ants [Rudolphi, 2009]). The notion of limited dispersal capability in *V. appalachiana* is also supported by the absence of this species north of the extent of the last glacial maximum (Farrar, 1978), beyond which a transplant study has shown they are able to survive (Stevens and Emery, 2015). Even recently disturbed areas (e.g., road cuts and tunnels) or other substrates that appear suitable within the range of *V. appalachiana* frequently remain uncolonized, while the species flourishes on seemingly similar substrates close by (Farrar, 1990). Taken together, these data suggest that spore dispersal from a fully functioning sporophyte must have been responsible for the current distribution of *V. appalachiana*. The truncated range of this species in southern New York likewise indicates that the gametophytes lost their ability to produce mature, functioning sporophytes sometime before (or during) the last ice age.

In an attempt to better understand the life history of *V. appalachiana*, Farrar (1990) conducted an allozyme study, incorporating multiple loci. The results revealed population structure across the range of the species, with relatively high levels of diversity in both Ohio and Alabama, but generally low (and homogeneous) diversity elsewhere. Farrar (1990) also uncovered fixed heterozygosity for several alleles in *V. appalachiana*, a trait generally considered to be indicative of a hybrid origin (Farrar, 2006). Additionally, a chromosome count by Gastony (1977) revealed a number twice that of what was considered typical for the genus, suggesting that *V. appalachiana* is a polyploid hybrid of ancient origin (Farrar, 2006).

This study aims to elucidate the evolutionary history of *V. appalachiana* using a combination of plastid and nuclear DNA sequence data. To date, only plastid sequences from a single individual of this species have been obtained. While plastid data are sufficient for determining the affinities of species that arose via cladogenesis, these maternally inherited markers (maternal inheritance in ferns has been demonstrated by Gastony and Yatskievych [1992], Vogel et al. [1998], and Guillon and Raquin [2000]) are inadequate for assessing the origins of species that were initiated via reticulate evolution. As some hypotheses for the origin of *V. appalachiana* have invoked hybridization, we also used a biparentally inherited nuclear marker that helps to identify hybrid ancestry and characterize the progenitors involved.

MATERIALS AND METHODS

Taxonomic sampling—Eighteen samples of *Vittaria appalachiana* were included from throughout the range of the species (Appendix 1; Fig. 1); five samples were taken from cultures of wild origin maintained by S. Chambers (University of Florida), and the remaining 13 were collected directly from the wild during either the fall of 2012 or the summer of 2013. All samples for molecular analysis were dried using silica gel (Chase and Hills, 1991). Vouchers were deposited in the U. S. National Herbarium (US), the Kriebel Herbarium (PUL), or the Great Smoky Mountains National Park Herbarium (GSMNP). Fourteen samples representing five other species of *Vittaria* were also included, with multiple individuals obtained from those species with widespread ranges (Appendix 1). One individual of each of five other vittarioid genera was sampled to serve as outgroups (Appendix 1) based on earlier analyses (Crane et al., 1995; Ruhfel et al., 2008).

Extraction and amplification—Genomic DNA was extracted from all samples using a modified CTAB procedure (Beck et al., 2011). Four plastid gene regions (*atpA*, *chlN*, *rbcL*, and *rpoA*) plus one nuclear gene region (*DET1*) were then amplified from each sample using a standard PCR approach for ferns (Schuettpelz and Pryer, 2007). Each 21 μL reaction included 1 μL of sample DNA, 2 μL of 10× PCR buffer, 2 μL of 200 μM dNTPs, 0.2 μL of 10 μg/mL BSA, 0.2 μL of 5 U/mL Choice Taq polymerase (Denville Scientific, Holliston, Massachusetts, USA), and 1 μL each of the forward and reverse primers (Table 1) at a 10 μM concentration. Thermocycling conditions included an initial denaturing step at 95°C for 120 s, followed by 35 cycles, each consisting of (1) a denaturing step at 95°C for 30 s; (2) an annealing step at 55°C (*atpA*, *DET1*, and *rbcL*), 50°C (*chlN*), or 45°C (*rpoA*) for 30 s; and (3) an extension step at 71°C for either 120 s (*atpA*), 60 s (*chlN* and *rpoA*), or 90 s (*DET1* and *rbcL)*. Reactions finished with an extension step at 71°C for 300 s. The resulting PCR products were visualized on agarose gels.

TABLE 1. Amplification and sequencing primers used in this study of *Vittaria*.

Region	Primer	Direction	Primer sequence	Reference
atpA	atpA-F1	Forward	GAATCTGATAATGTTGGGGCTG	Cochran et al., 2014
atpA	atpA-R1	Reverse	AAACATCTCCNGGATAYGCTTC	Cochran et al., 2014
atpA	ESATPF412F	Forward	GARCARGTTCGACAGCAAGT	Schuettpelz et al., 2006
atpA	ESTRNR46F	Reverse	GTATAGGTTCRARTCCTATTGGACG	Schuettpelz et al., 2006
atpA	ESATPA535F[a]	Forward	ACAGCAGTAGCTACAGATAC	Schuettpelz et al., 2006
atpA	ESATPA557R[a]	Reverse	ATTGTATCTGTAGCTACTGC	Schuettpelz et al., 2006
atpA	ESATPA856F[a]	Forward	CGAGAAGCATATCCGGGAGATG	Schuettpelz et al., 2006
atpA	ESATPA877R[a]	Reverse	CATCTCCCGGATATGCTTCTCG	Schuettpelz et al., 2006
chlN	chlN-F2	Forward	CGWTAYGCRAYGGCVGAATYGSAAG	Schuettpelz et al., unpubl.
chlN	chlN-R2	Reverse	CAWATTTTTTCGATCCARGCRCGTG	Schuettpelz et al., unpubl.
rbcL	ESRBCL1F	Forward	ATGTCACCACAAACGGAGACTAAAGC	Schuettpelz and Pryer, 2007
rbcL	ESRBCL1361R	Reverse	TCAGGACTCCACTTACTAGCTTCACG	Schuettpelz and Pryer, 2007
rbcL	ESRBCL628F[a]	Forward	CCATTYATGCGTTGGAGAGATCG	Schuettpelz and Pryer, 2007
rbcL	ESRBCL654R[a]	Reverse	GAARCGATCTCTCCAACGCAT	Schuettpelz and Pryer, 2007
rpoA	rpoA-F1	Forward	TRCAYGAGTATTCYACAATAACGGG	Schuettpelz et al., unpubl.
rpoA	rpoA-R1	Reverse	AATTAAARGCTCTRGCRGGTRATTC	Schuettpelz et al., unpubl.
DET1	det1-335Ad[b]	Forward	TATGATGTGGAGTGCCCAGA	This study
DET1	det1-906AdVit[b]	Reverse	CCTCTCTGCAGAAAGGTCCAA	This study

[a]Internal sequencing primer.
[b]*Adiantum*/vittarioid-specific primer designed by Layne Huiet based on previously published *DET1* primer sequence (Rothfels et al., 2013).

Plastid gene sequencing—Plastid amplifications were cleaned with 0.5 μL of Exonuclease I (10 units/μL) and 1 μL of Shrimp Alkaline Phosphatase I (1 unit/μL; Affymetrix, Santa Clara, California, USA). Sequencing reactions were performed using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, California, USA). The PCR products were sequenced in both directions with the amplification primers, as well as internal sequencing primers in the case of *atpA* and *rbcL*, using a standard protocol (Schuettpelz and Pryer, 2007). Each 10 μL sequencing reaction contained 2 μL of the cleaned PCR product, 1.75 μL of 0.625× BigDye Terminator Sequencing Buffer, 0.5 μL of 0.375× BigDye Terminator Ready Reaction Mix, 1 μL of the appropriate primer (10 μM), and 4.75 μL of ultrapure water. Sequencing products were cleaned using the ZR-96 DNA Sequencing Clean-up Kit (Zymo Research, Irvine, California, USA) using the manufacturer's protocol. Plates were then sent to Operon (Hunstville, Alabama, USA) for sequencing.

Sequencing reads were edited and assembled in the program Sequencher version 4.5 (Gene Codes, Ann Arbor, Michigan, USA) to produce a consensus sequence for each gene, for each sample. All resulting consensus sequences were deposited in the Fern Lab Database (http://fernlab.biology.duke.edu) as well as in GenBank (Appendix 1).

Nuclear gene cloning and sequencing—To segregate the alleles present in each nuclear *DET1* amplification, we carried out a molecular cloning step using the pGEM-T kit and JM109 cells (Promega, Madison, Wisconsin, USA). Ligation reactions included 0.5 μL of PCR product, 1.25 μL 2× ligation buffer, 0.25 μL pGEM-T vector, 0.25 μL T4 ligase, and 0.25 μL ultrapure water; they were incubated at 4°C overnight. For transformations, 0.5 μL of the ligation reaction was added to 12.5 μL of JM109 cells and allowed to incubate on ice for 20 min. The cells were then heat shocked for 45 s in a water bath at 42°C and returned to ice, where they were allowed to incubate for 2 min. Each transformation tube was then supplemented with 237.5 μL of SOC broth and incubated at 37°C, shaking at 150 rpm, for 90 min. After incubation, at least 125 μL of each transformation was spread evenly onto a warm LB plate (containing ampicillin, isopropyl thiogalactopyranoside [IPTG], and 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside [X-Gal]). These plates were allowed to incubate at 37°C for at least 16 h before colonies were extracted. Following incubation, at least eight white colonies (containing an insert) from each plate were selected and used as the templates for PCR reactions, carried out as described for *DET1*, but employing the universal M13 forward and reverse primers (Promega). Visualization, cleanup, and sequencing then proceeded as described for the plastid regions above.

To identify allele sequences from the pool of clone sequences obtained from each sample, the clone sequences were first manually aligned in Mesquite version 2.75 (Maddison and Maddison, 2011). Each alignment (one per sample) was then analyzed using a heuristic maximum parsimony search in the program PAUP* version 4.0 beta 10 (Swofford, 2002), with the default settings. The most parsimonious tree (or a consensus of equally most parsimonious trees) from each analysis was then examined to identify least-inclusive clades (i.e., groups of sequences united by at least one apomorphy but not themselves containing other, similarly defined, groups of sequences) following an established approach (Grusz et al., 2009; Li et al., 2012; Schuettpelz et al., 2015). A consensus sequence for each group of clones was computed; these were deposited as alleles into the Fern Lab Database (http://fernlab.biology.duke.edu) and GenBank (Appendix 1) and then subjected to additional analyses (see below).

Phylogenetic analyses—For each gene region, sequences were manually aligned using the program Mesquite (Maddison and Maddison, 2011; Table 2). Portions of the *atpA* alignment flanking the *atpA* gene that contained large amounts of missing data and were questionably aligned, as well as two small questionably aligned regions within the *DET1* gene, were excluded from further analyses.

The plastid *atpA*, *chlN*, *rbcL*, and *rpoA* alignments were individually subjected to maximum likelihood and Bayesian analyses. The maximum likelihood analyses, employing the GTRGAMMA model, were carried out in RAxML version 8.0.3 (Stamatakis, 2014), with 1000 rapid bootstrap inferences followed by a thorough maximum likelihood search. Bayesian analyses, employing the

TABLE 2. Details concerning the alignments used in this study of *Vittaria*.

Data set	Individuals/alleles	Total characters	Included characters	Variable characters	Missing data (%)[a]
Plastid *atpA*	36	1861	1524	286	7.23
Plastid *chlN*	36	624	624	188	0.12
Plastid *rbcL*	36	1309	1309	239	0.29
Plastid *rpoA*	37	604	604	168	1.83
Plastid combined	37	4398	4061	881	5.32
Nuclear *DET1*	56	630	619	166	0.05

[a]Calculation based on included characters.

GTR+G model, were carried out in MrBayes version 3.2.1 (Huelsenbeck and Ronquist, 2001), with four simultaneous runs each incorporating four chains of five million generations. For the Bayesian analyses, trees were sampled from the cold chain every 4000 generations. To assess convergence, the standard deviation of split frequencies among runs and plots of output parameter estimates (the latter produced using Tracer 1.5; Rambaut and Drummond, 2009) were examined. Based on these diagnostics, the first 250 trees (corresponding to one million generations) were excluded from each analysis as burn-in before obtaining majority rule consensus phylogenies with clade posterior probabilities. For each plastid gene region, the resulting maximum likelihood and Bayesian trees were rooted with *Antrophyum*, based on its position as recovered in earlier studies (Crane et al., 1995; Ruhfel et al., 2008).

The plastid *atpA*, *chlN*, *rbcL*, and *rpoA* trees were then visually examined for significant conflicts (i.e., those supported by both a maximum likelihood bootstrap score greater than 70 and a Bayesian posterior probability greater than 0.95). No conflicts were found that involved *V. appalachiana* (the focus of this study), and so the plastid data sets were combined and analyzed in unison. Combined maximum likelihood and Bayesian analyses were conducted as described, but with model parameters estimated separately for each gene region. Additionally, the combined Bayesian analyses were run for 20 million generations, with trees sampled every 16 thousand generations. The maximum likelihood and Bayesian analyses of the nuclear *DET1* alignment proceeded as per the individual plastid gene regions, but with the Bayesian analyses run for 20 million generations and sampling every 16 thousand generations. As *DET1* sequences could not be obtained from *Antrophyum*, the nuclear phylogeny was rooted with the other outgroup genera.

RESULTS

Plastid phylogeny—Bayesian analysis of the combined plastid data set resulted in a phylogeny with somewhat varied levels of Bayesian posterior probability (BPP) and maximum likelihood bootstrap score (MLBS) support (Fig. 2). In some parts of the tree, relationships among and within species were fully resolved and well supported; in other parts, resolution and support were poor to nonexistent.

Within *Vittaria*, a split was resolved separating a clade including *V. bradeorum* Rosenst., *V. isoetifolia* Bory, and *V. lineata* (L.) Sm. from a clade consisting of *V. appalachiana*, *V. graminifolia* Kaulf., and *V. scabrida* Klotzsch ex Fée. Support for the former *Vittaria* clade was poor (BPP = 0.81; MLBS = 66). Within this clade, three samples of *V. lineata* were placed together in a well-supported (BPP = 1; MLBS = 100) subclade, but a fourth sample of this species was embedded in a well-supported (BPP = 1; MLBS = 96) subclade containing two samples of *V. isoetifolia* and the singular included sample of *V. bradeorum*. The other major clade resolved within *Vittaria*—containing *V. appalachiana*, *V. graminifolia*, and *V. scabrida*—was strongly supported (BPP = 1; MLBS = 100). Therein, the two samples of *V. scabrida* were resolved together (BPP = 1; MLBS = 91). *Vittaria appalachiana* was also found to be monophyletic, with the 18 included samples composing a well-supported (BPP = 1; MLBS = 86) clade with little internal structure. The samples of *V. graminifolia* were resolved in two distinct positions: two were placed with *V. scabrida* (BPP = 1; MLBS = 100) and three were resolved together (BPP = 1; MLBS = 99) as sister to *V. appalachiana* (BPP = 1; MLBS = 98).

Nuclear phylogeny—Bayesian analysis of the 56 recovered nuclear *DET1* alleles yielded a phylogeny (Fig. 3) that was mostly consistent with the plastid tree. The most notable difference involved the two primary *Vittaria* clades that were resolved as sister in the plastid analysis. In the nuclear *DET1* analysis, the components of the first clade (which was only weakly supported by the plastid data) were resolved as paraphyletic to the second clade (which was strongly supported by the plastid data). The alleles from three samples of *V. lineata* (5289, 8129, and 9340) formed a well-supported (BPP = 1; MLBS = 100) clade that was sister to the remainder of the genus (BPP = 1; MLBS = 98). The lone allele from the remaining sample of *V. lineata* (8924), which was resolved with *V. isoetifolia* in the plastid analysis, occupied an isolated position in the *DET1* tree (*DET1* sequences could not be obtained from *V. isoetifolia*). The alleles from *V. bradeorum* were most closely related to the clade containing alleles from samples of *V. appalachiana* and *V. graminifolia* (*DET1* sequences could not be obtained from *V. scabrida*).

Within the *V. appalachiana* and *V. graminifolia* clade, the alleles from samples of *V. graminifolia* were resolved in two distinct positions, consistent with what was uncovered in the plastid analysis. Alleles from samples 2395 and 9081 were resolved together (BPP = 1; MLBS = 98) and sister to a well-supported clade (BPP = 1; MLBS = 98) containing alleles from the other three samples of *V. graminifolia* (5286, 8381, and 9055) plus those from various samples of *V. appalachiana*. All alleles obtained from *V. appalachiana* were resolved together (BPP = 1; MLBS = 93), with the notable exception of one allele from sample 9331, which was found to be more closely related to alleles of *V. graminifolia* (Fig. 3). There was otherwise no structure uncovered within *V. appalachiana*, with most alleles possessing a small number of unique substitutions.

DISCUSSION

***Phylogeny of* Vittaria**—Previous analyses of vittarioid fern relationships (Crane et al., 1995; Crane, 1997; Ruhfel et al., 2008) included no more than one exemplar from any species of *Vittaria*. These

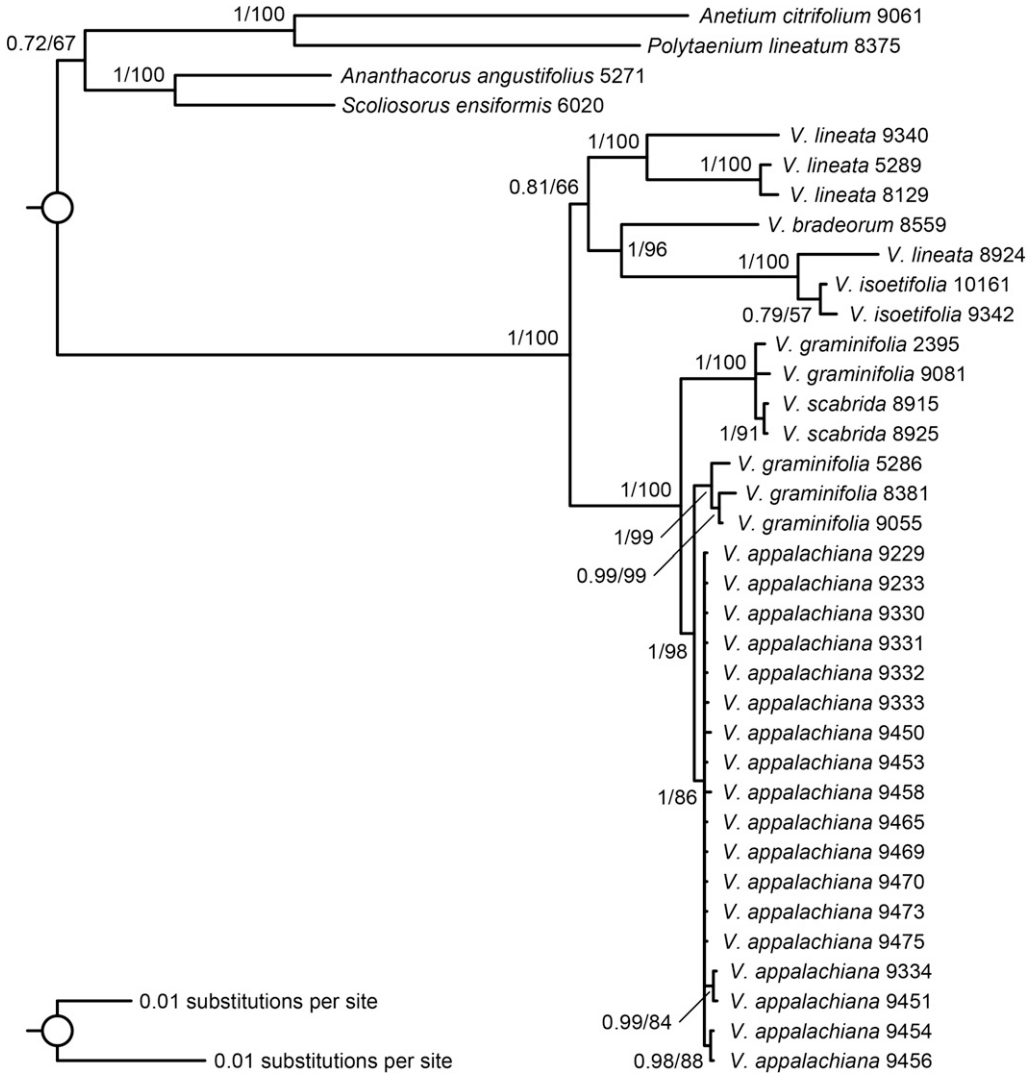

FIGURE 2 Phylogeny of *Vittaria* resulting from Bayesian analysis of a four-gene (*atpA*, *chlN*, *rbcL*, and *rpoA*) plastid data set. Numbers at nodes are Bayesian posterior probabilities and maximum likelihood bootstrap scores (BPP/MLBS). Note different scales for outgroup and ingroup branches; *Antrophyum*, used to root the phylogeny, has been pruned.

analyses uncovered two clades within the genus, one including *V. dimorpha* Müll. Berol., *V. isoetifolia*, and *V. lineata* and the other including *V. appalachiana* and *V. graminifolia*. Our results (Figs. 2, 3) are generally consistent with the earlier studies. With the inclusion of multiple accessions from each of six *Vittaria* species, however, we find that the two most widespread species in the genus (*V. lineata* and *V. graminifolia*) are not monophyletic (Figs. 2 and 3).

Three samples of *Vittaria lineata* (5289, 8129, and 9340, from Costa Rica, the United States, and Panama, respectively) form a strongly supported clade, with a Bayesian posterior probability (BPP) of 1 and a maximum likelihood bootstrap score (MLBS) of 100, in both our plastid and nuclear analyses. In our plastid analysis, however, the fourth sample of *V. lineata* (8924, from Brazil) is resolved together with *V. isoetifolia* (an African-Madagascan species) and these two species are in turn sister to *V. bradeorum* (from Costa Rica). Many close phytogeographic relationships have been noted between (or even within) neotropical and African-Madagascan fern species (Moran and Smith, 2001), and the precise nature of the relationships among *V. lineata*, *V. isoetifolia*, and *V. bradeorum* will require further study. In our nuclear phylogeny, the situation is somewhat more complicated, with *V. lineata* 8924 and *V. bradeorum* resolved as more closely related to *V. graminifolia* and *V. appalachiana* than to the remainder of *V. lineata*.

The majority of our *V. graminifolia* samples (5286, 8381, and 9055; from Costa Rica, Brazil, and Bolivia, respectively) showed strong affinities to the Appalachian gametophyte (*V. appalachiana*) in both our plastid and nuclear analyses. In the plasid analysis, however, two other accessions (2395 and 9081, from Ecuador and Bolivia, respectively) were resolved with Brazilian samples of *V. scabrida. Vittaria scabrida* was not included in the nuclear data set and the anomalous samples of *V. graminifolia* (2395 and 9081) were there resolved as sister to the larger *V. appalachiana* and *V. graminifolia* clade. Although *Vittaria scabrida* was treated as a synonym of *V. graminifolia* in the *Flora of Venezuela* (Hokche et al., 2008), the birth and death of this taxon came and went without any molecular data to support or reject its existence. Based on the

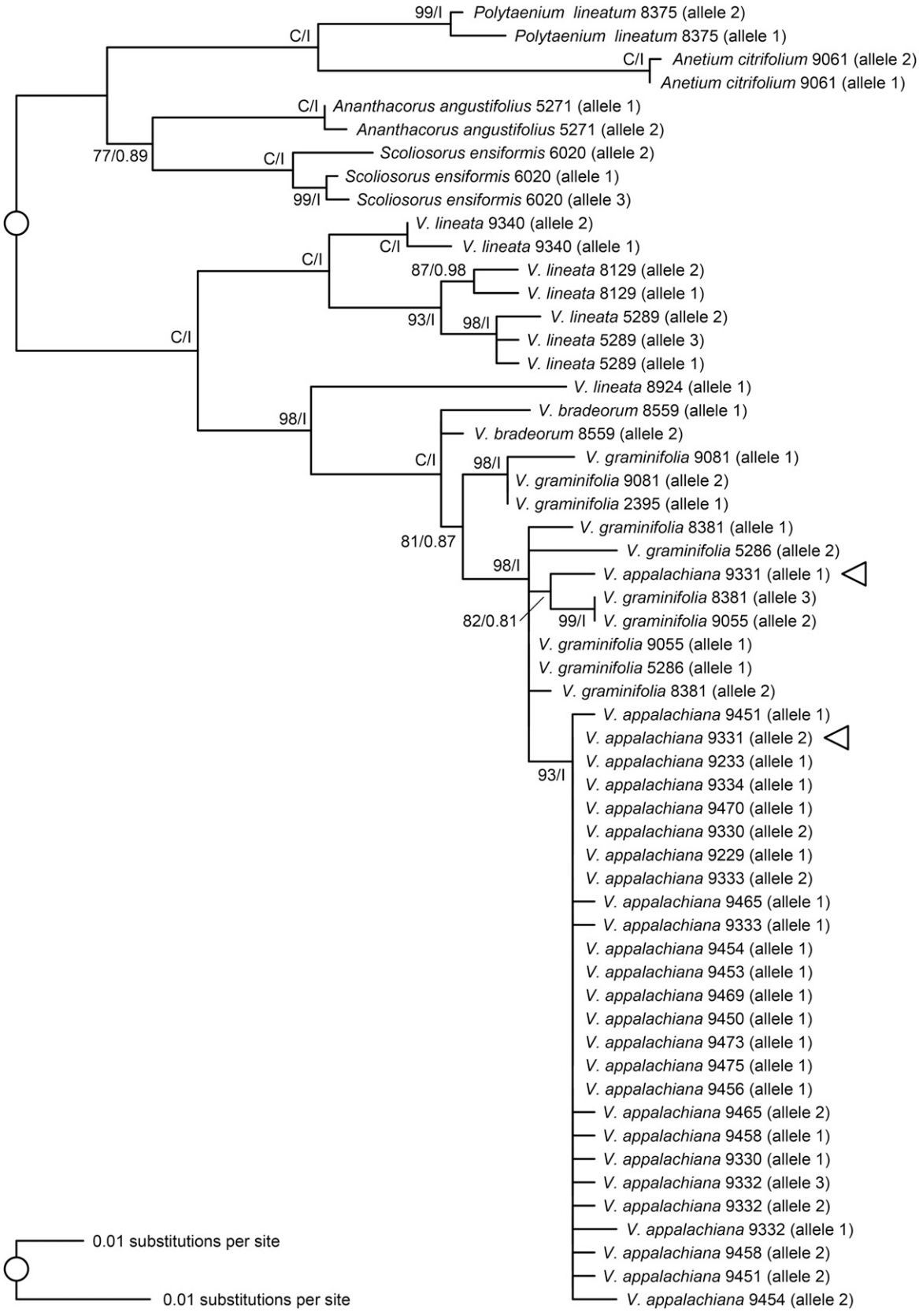

FIGURE 3 Phylogeny of *Vittaria* resulting from Bayesian analysis of a nuclear *DET1* data set. Numbers at nodes are Bayesian posterior probabilities and maximum likelihood bootstrap scores (BPP/MLBS). Note different scales for outgroup and ingroup branches. Open arrows highlight the disparate *DET1* alleles of sample 9331.

polyphyly of *V. graminifolia* in this study, this particular group may actually represent more than one species, and *V. scabrida* could be among them. Disparate chromosome counts exist for *V. graminifolia* (Gastony, 1977; Smith and Mickel, 1977), but it is unclear whether these counts correspond to particular clades.

Origin of the Appalachian gametophyte—Analyses of our plastid data set clearly indicate a strong maternal affinity of *V. appalachiana* to *V. graminifolia*, a finding that is consistent with earlier results (Crane, 1997; Ruhfel et al., 2008). Somewhat surprisingly, the nuclear data set presented here additionally suggests that *V. appalachiana* is not the product of interspecies hybridization, as previously postulated (Farrar, 1990). Instead, nearly all *DET1* alleles recovered from our *V. appalachiana* samples compose a strongly supported clade, most closely related to *V. graminifolia*. Assuming that specimens here identified as *V. graminifolia* represent just one species, we find (in both our plastid and nuclear analyses) *V. appalachiana* to be wholly nested within it.

Excluding an origin involving hybridization, we are left with two alternative explanations for the origin and evolution of *V. appalachiana*, depending on the phylogenetic distribution of cytotypes within *V. graminifolia*. The Appalachian gametophyte is known to have a chromosome count of $n = 120$ (Gastony, 1977). For *V. graminifolia*, counts of both $n = 60$ (Gastony, 1977) and $n = 120$ (Smith and Mickel, 1977) have been reported, but the phylogenetic distribution of these cytotypes is currently unknown. If the closest relatives of *V. appalachiana* turn out to be $n = 60$, genome duplication would emerge as the most likely explanation for the origin of *V. appalachiana*, with it being an autopolyploid formed from within *V. graminifolia*. If the closest relatives of *V. appalachiana*, however, are $n = 120$, it would be more parsimonious to invoke a second explanation involving divergent (sympatric or allopatric) speciation. Further work will be needed, including an extensive study of chromosome counts and/or spore sizes of *V. graminifolia* across its range, to discriminate between the possibilities.

Regardless of how the Appalachian gametophyte originated, it seems clear that it is no longer capable of sexual reproduction (Farrar, 1978). Thus, whether or not this species has a chromosome number compatible with its closest relatives, it is effectively reproductively isolated. Furthermore, previous studies (Farrar, 1974; Farrar and Mickel, 1991) have highlighted some clear morphological differences that, combined with differences in distribution, are sufficient for the continued recognition of both *V. appalachiana* and *V. graminifolia*.

The possibility that the current populations of the Appalachian gametophyte are being sustained by long-distance dispersal from some tropical sporophyte source can be rejected based on past allozyme studies (Farrar, 1990), as well as the truncated range of *V. appalachiana* in the southern portion of New York. Additionally, the monophyly of *V. appalachiana* in our plastid analysis would seem to indicate that dispersal from the tropics occurred just once, although the situation is somewhat more complicated in our nuclear tree, where one *V. appalachiana* allele is resolved outside the larger *V. appalachiana* clade.

Since the dispersal of gemmae does not appear to account for the wide range of *V. appalachiana,* it is most likely that a fully functioning sporophyte of this species existed (and quite possibly thrived) in North America when temperatures were more favorable for tropical growth in the Appalachians (Groot, 1991). In this scenario, the current distribution of *V. appalachiana* would be due solely to spore dispersal, with the sporophyte becoming extinct before or during the Pleistocene glaciations. This argument is supported by the Appalachian gametophyte's apparent inability to extend northward beyond the limit of the last glacial maximum (Farrar, 1978). If *V. appalachiana* produced sporophytes after the glaciers had receded, spore dispersal could have easily extended the range of this species further north, to mirror the distribution of, for example, *Crepidomanes intricatum*, another fern species found in eastern North America that is known only as a gametophyte (Farrar, 2006). *Vittaria appalachiana* and *C. intricatum* are two of the only species for which mature sporophytes have never been observed, and it is currently unclear how either of these species (or a third species, *Hymenophyllum tayloriae*) originated or why they lack the ability to complete their life cycles. All the same, the work presented here does indicate that *V. appalachiana* is not the result of a hybridization event, as previously postulated, and instead points to an origin involving genome duplication and/or divergent speciation.

ACKNOWLEDGEMENTS

The authors thank Marcel Van Tuinen and Brian Arbogast for their valuable input on an earlier version of this manuscript as well as two anonymous reviewers for their comments and suggestions. Sally Chambers, Michael Kessler, Fernando Matos, Joel Nitta, Jefferson Prado, Tom Ranker, Carl Rothfels, and Michael Sundue kindly provided silica-dried material for analysis. The acquisition of new material was facilitated by the U. S. National Park Service, the Kentucky Department of Parks, the U. S. Department of Agriculture, the Georgia Department of Natural Resources, the West Virginia Department of Parks and Recreation, the Ohio Division of Natural Areas and Preserves, Panama Rocks, Rock City Park, and the Kentucky State Nature Preserves Commission. Layne Huiet shared unpublished primer sequences. Funding for this research was provided in part by the U. S. National Science Foundation (grants DEB-1145925 and DEB-1405181 to E.S.).

LITERATURE CITED

Beck, J. B., P. J. Alexander, L. Allphin, I. A. Al-Shehbaz, C. Rushworth, C. D. Bailey, and M. D. Windham. 2011. Does hybridization drive the transition to asexuality in the diploid *Boechera*. *Evolution* 66: 985–995.

Chase, M. W., and H. H. Hills. 1991. An ideal material for field preservation of leaf samples for DNA studies. *Taxon* 40: 215–220.

Cochran, A. T., J. Prado, and E. Schuettpelz. 2014. *Tryonia*, a new taeniitidoid fern genus segregated from Jamesonia and Eriosorus (Pteridaceae). *PhytoKeys* 35: 23–43.

Crane, E. H. 1997. A revised circumscription of the genera of the fern family Vittariaceae. *Systematic Botany* 22: 509–517.

Crane, E. H., D. R. Farrar, and J. F. Wendel. 1995. Phylogeny of the vittariaceae: Convergent simplification leads to a polyphyletic *Vittaria*. *American Fern Journal* 85: 283–305.

Dassler, C. L., and D. R. Farrar. 1997. Significance of form in fern gametophytes: Clonal, gemmiferous gametophytes of *Callistopteris baueriana* (Hymenophyllaceae). *International Journal of Plant Sciences* 158: 622–639.

Duffy, A. M., M. C. Stensvold, and D. R. Farrar. 2015. Independent gametophytes of *Hymenophyllum wrightii* in North America: Not as rare as we thought. *American Fern Journal* 105: 45–55.

Ebihara, A., S. Matsumoto, and M. Ito. 2009. Hybridization involving independent gametophytes in the *Vandenboschia radicans* complex (Hymenophyllaceae): A new perspective on the distribution of fern hybrids. *Molecular Ecology* 18: 4904–4911.

Farrar, D. R. 1974. Gemmiferous fern gametophytes—Vittariaceae. *American Journal of Botany* 61: 146–155.

Farrar, D. R. 1978. Problems in the identity and origin of the Appalachian *Vittaria* gametophyte, a sporophyteless fern of the eastern united states. *American Journal of Botany* 65: 1–12.

Farrar, D. R. 1985. Independent fern gametophytes in the wild. *Proceedings of the Royal Society of Edinburgh* 86: 361–369.

Farrar, D. R. 1990. Species and evolution in asexually reproducing independent fern gametophytes. *Systematic Botany* 15: 98–111.

Farrar, D. R. 1992. *Trichomanes intricatum*: The independent *Trichomanes* gametophyte in the eastern United States. *American Fern Journal* 82: 68–74.

Farrar, D. R. 1998. The tropical flora of rockhouse cliff formations in the eastern United States. *Journal of the Torrey Botanical Society* 125: 91–108.

Farrar, D. R. 2006. Conservation assessment for Appalachian *Vittaria* (*Vittaria appalaciana* Farrar & Mikel). USDA Forest Service, Eastern Region, Milwaukee, Wisconsin, USA.

Farrar, D., C. Dassler, J. E. Watkins, and C. Skelton. 2008. Gametophyte ecology. *In* T. A. Ranker and C. H. Haufler [eds.], Biology and evolution of ferns and lycophytes, 222–256. Cambridge University Press, Cambridge, UK.

Farrar, D. R., and J. T. Mickel. 1991. *Vittaria appalachiana*: A name for the "Appalachian gametophyte". *American Fern Journal* 81: 69–75.

Gastony, G. J. 1977. Chromosomes of the independently reproducing Appalachian gametophyte: A new source of taxonomic evidence. *Systematic Botany* 2: 43–48.

Gastony, G. J., and G. Yatskievych. 1992. Maternal inheritance of the chloroplast and mitochondrial genomes in cheilanthoid ferns. *American Journal of Botany* 79: 716–722.

Groot, J. J. 1991. Palynological evidence for late Miocene, Pliocene and early Pleistocene climate changes in the middle U.S. Atlantic coastal plain. *Quaternary Science Reviews* 10: 147–162.

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

Guillon, J. M., and C. Raquin. 2000. Maternal inheritance of chloroplasts in the horsetail *Equisetum variegatum* (Schleich.). *Current Genetics* 37: 53–56.

Hokche, O., P. Berry, and O. Huber. 2008. Nuevo catálogo de la flora vascular de Venezuela. Fundación Instituto Botánico de Venezuela, Caracas, Venezuela.

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

Kimmerer, R. W., and C. C. Young. 1995. The role of slugs in dispersal of the asexual propagules of *Dicranum flagellare. Bryologist* 98: 149–153.

Li, F. W., K. M. Pryer, and M. D. Windham. 2012. *Gaga*, a new fern genus segregated from *Cheilanthes* (Pteridaceae). *Systematic Botany* 37: 845–860.

Li, F. W., B. C. Tan, V. Buchbender, R. C. Moran, G. Rouhan, C. N. Wang, and D. Quandt. 2009. Identifying a mysterious aquatic fern gametophyte. *Plant Systematics and Evolution* 281: 77–86.

Maddison, W. P., and D. R. Maddison. 2011. Mesquite: A modular system for evolutionary analysis, version 2.75. Available at http://mesquiteproject.org

Moran, R. C., and A. R. Smith. 2001. Phytogeographic relationships between neotropical and African-Madagascan pteridophytes. *Brittonia* 53: 304–351.

Pryer, K. M., H. Schneider, A. R. Smith, R. Cranfill, P. G. Wolf, J. S. Hunt, and S. D. Sipes. 2001. Horsetails and ferns are a monophyletic group and the closest living relatives to seed plants. *Nature* 409: 618–622.

Pryer, K. M., E. Schuettpelz, P. G. Wolf, H. Schneider, A. R. Smith, and R. Cranfill. 2004. Phylogeny and evolution of ferns (monilophytes) with a focus on the early leptosporangiate divergences. *American Journal of Botany* 91: 1582–1598.

Raine, C. A., D. R. Farrar, and E. Sheffield. 1991. A new *Hymenophyllum* species in the Appalachians represented by independent gametophyte colonies. *American Fern Journal* 81: 109–118.

Rambaut, A., and A. J. Drummond. 2009. Tracer version 1.5.0. Website http://tree.bio.ed.ac.uk/software/tracer/ [accessed 1 July 2013].

Rothfels, C. J., A. Larsson, F. W. Li, E. M. Sigel, L. Huiet, D. O. Burge, M. Ruhsam, et al. 2013. Transcriptome-mining for single-copy nuclear markers in ferns. *PLoS One* 8: e76957.

Rudolphi, J. 2009. Ant-mediated dispersal of asexual moss propagules. *Bryologist* 112: 73–79.

Ruhfel, B., S. Lindsay, and C. C. Davis. 2008. Phylogenetic placement of *Rheopteris* and the polyphyly of *Monogramma* (Pteridaceae sl): Evidence from *rbcL* sequence data. *Systematic Botany* 33: 37–43.

Rumsey, F. J., and A. C. Jermy. 1998. The independent gametophytic stage of *Trichomanes speciosum* Willd. (Hymenophyllaceae), the Killarney fern and its distribution in the British Isles. *Watsonia* 22: 1–19.

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

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

Schuettpelz, E., K. M. Pryer, and M. D. Windham. 2015. A unified approach to taxonomic delimitation in the fern genus *Pentagramma* (Pteridaceae). *Systematic Botany* 40: 629–644.

Smith, A. R., and J. T. Mickel. 1977. Chromosome counts for Mexican ferns. *Brittonia* 29: 391–398.

Stamatakis, A. 2014. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30: 1312–1313.

Stevens, S. M., and N. C. Emery. 2015. Dispersal limitation and population differentiation in performance beyond a northern range limit in an asexually reproducing fern. *Diversity & Distributions* 21: 1242–1253.

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

Thiers, B. 2016 [continuously updated]. Index herbariorum: A global directory of public herbaria and associated staff. New York Botanical Garden's virtual herbarium, Bronx, NY, USA. Available at http://sweetgum.nybg.org/science/ih/ [accessed 24 January 2016].

Vogel, J. C., S. J. Russell, F. J. Rumsey, J. A. Barrett, and M. Gibby. 1998. Evidence for maternal transmission of chloroplast DNA in the genus *Asplenium* (Aspleniaceae, Pteridophyta). *Botanica Acta* 111: 247–249.

APPENDIX 1 **Individuals sampled in this study of *Vittaria*. Herbarium acronyms are used in accordance with Index Herbariorum (Thiers, 2016). NA = not available.**

Species, Fern Lab Database (http://fernlab.biology.duke.edu) voucher number, *voucher information* (Herbarium), Place of origin, Plastid GenBank accession numbers (*atpA*, *chlN*, *rbcL*, *rpoA*), and Nuclear *DET1* clones sequenced > alleles: Nuclear *DET1* GenBank accession numbers.

Ananthacorus angustifolius (Sw.) Underw. & Maxon, 5271, *F. Matos 08-203* (DUKE), Costa Rica: Heredia, KU517449, KU517485, KU517577, KU517613, 16 > 2: KU517521/KU517522; ***Anetium citrifolium*** (L.) Splitg., 9061, *I. Jiménez 2007* (UC), Bolivia: Pando, KU517450, KU517486, KU517578, KU517614, 18 > 2: KU517523/KU517524; ***Antrophyum formosanum*** Hieron., 8409, *T. A. Ranker 2068* (COLO), Taiwan: NA, KU517451, KU517487, KU517579, KU517615, NA; ***Polytaenium lineatum*** (Sw.) J.Sm., 8375, *E. Schuettpelz 1440* (SP), Brazil: Rio de Janeiro, KU517452, KU517488, KU517580, KU517616, 17 > 2: KU517525/KU517526; ***Scoliosorus ensiformis*** (Hook.) T.Moore, 6020, *M. Sundue 1676* (DUKE), Costa Rica: Alajuela, KU517453, KU517489, KU517581, KU517617, 18 > 3: KU517527/KU517528/KU517529; ***Vittaria appalachiana*** Farrar & Mickel, 9229, *E. Schuettpelz 1482* (US), USA: North Carolina, KU517454, KU517490, KU517582, KU517618, 17 > 1: KU517530; ***Vittaria appalachiana*** Farrar & Mickel, 9233, *E. Schuettpelz 1486* (US), USA: North Carolina, KU517455, KU517491, KU517583, KU517619, 11 > 1: KU517531; ***Vittaria appalachiana*** Farrar & Mickel, 9330, *S. Stevens AL-Fern-p2s2* (PUL), USA: Alabama, KU517456, KU517492, KU517584, KU517620, 16 > 2: KU517532/KU517533; ***Vittaria appalachiana*** Farrar & Mickel, 9331, *S. Stevens KY-Clifty-s25* (PUL), USA: Kentucky, KU517457, KU517493, KU517585, KU517621, 20 > 2: KU517534/KU517535; ***Vittaria appalachiana*** Farrar & Mickel, 9332, *S. Stevens NC-p5s1* (PUL), USA: North Carolina, KU517458, KU517494, KU517586, KU517622, 34 > 3: KU517536/KU517537/KU517538; ***Vittaria appalachiana*** Farrar & Mickel, 9333, *S. Stevens OH-p1-s11* (PUL), USA: Ohio, KU517459, NA, KU517587, KU517623, 14 > 2: KU517539/KU517540; ***Vittaria appalachiana*** Farrar & Mickel, 9334, *S. Stevens NY-p5s2* (PUL), USA: New York, KU517460, KU517495, KU517588, KU517624, 9 > 1: KU517541; ***Vittaria appalachiana*** Farrar & Mickel, 9450, *J. Pinson 9* (US), USA: Tennessee, KU517461, KU517496, KU517589, KU517625, 11 > 1: KU517542; ***Vittaria appalachiana*** Farrar & Mickel, 9451, *J. Pinson 10* (US), USA: Tennessee, KU517462, KU517497, KU517590, KU517626, 14 > 2: KU517543/KU517544; ***Vittaria appalachiana*** Farrar & Mickel, 9453, *J. Pinson 12* (US), USA: Kentucky, KU517463, KU517498, KU517591, KU517627, 13 > 1: KU517545; ***Vittaria appalachiana*** Farrar & Mickel, 9454, *J. Pinson 13* (US), USA: Indiana, KU517464, KU517499, KU517592, KU517628, 9 > 2: KU517546/KU517547; ***Vittaria appalachiana*** Farrar & Mickel, 9456, *J. Pinson 15* (US), USA: Indiana, KU517465, KU517500, KU517593, KU517629, 8 > 1: KU517548; ***Vittaria appalachiana*** Farrar & Mickel, 9458, *J. Pinson 17* (US), USA: Ohio, KU517466, KU517501, KU517594, KU517630, 8 > 2: KU517549/KU517550; ***Vittaria appalachiana*** Farrar & Mickel, 9465, *J. Pinson 24* (US), USA: Ohio, KU517467, KU517502, KU517595, KU517631, 8 > 2: KU517551/KU517552; ***Vittaria appalachiana*** Farrar & Mickel, 9469, *J. Pinson 28* (US), USA: New York, KU517468, KU517503, KU517596, KU517632, 12 > 1: KU517553; ***Vittaria appalachiana*** Farrar & Mickel, 9470, *J. Pinson 29* (US), USA: West Virginia, KU517469, KU517504, KU517597, KU517633, 11 > 1: KU517554; ***Vittaria appalachiana*** Farrar & Mickel, 9473, *J. Pinson 32* (US), USA: West Virginia, KU517470, KU517505, KU517598, KU517634, 10 > 1: KU517555; ***Vittaria appalachiana*** Farrar & Mickel, 9475, *J. Pinson 34* (US), USA: Virginia, KU517471, KU517506, KU517599, KU517635, 9 > 1: KU517556; ***Vittaria bradeorum*** Rosenst., 8559, *J. Nitta 834* (UC), Costa Rica: NA, KU517472, KU517507, KU517600, KU517636, 7 > 2: KU517557/KU517558; ***Vittaria graminifolia*** Kaulf., 2395, *E. Schuettpelz 227* (DUKE), Ecuador: Zamora-Chinchipe, KU517473, KU517508, KU517601, KU517637, 8 > 1: KU517559; ***Vittaria graminifolia*** Kaulf., 5286, *C. J. Rothfels 08-065* (DUKE), Costa Rica: Cartago, KU517474, KU517509, KU517602, KU517638, 14 > 2: KU517560/KU517561; ***Vittaria graminifolia*** Kaulf., 8381, *E. Schuettpelz 1446* (SP), Brazil: Rio de Janeiro, KU517475, KU517510, KU517603, KU517639, 13 > 3: KU517562/KU517563/KU517564; ***Vittaria graminifolia*** Kaulf., 9055, *I. Jiménez 1183* (UC), Bolivia: Cochabamba, KU517476, KU517511, KU517604, KU517640, 14 > 2: KU517565/KU517566; ***Vittaria graminifolia*** Kaulf., 9081, *K. Bach 1787* (UC), Bolivia: La Paz, KU517477, KU517512, KU517605, KU517641, 9 > 2: KU517567/KU517568; ***Vittaria isoetifolia*** Bory, 9342, *F. Rakotondrainibe 3202* (MO), Madagascar: NA, NA, KU517513, KU517606, KU517642, NA; ***Vittaria isoetifolia*** Bory, 10161, *T. Janssen 2920* (MO), Madagascar: NA, KU517478, KU517514, NA, KU517643, NA; ***Vittaria lineata*** (L.) J.Sm., 5289, *C. J. Rothfels 08-177* (DUKE), Costa Rica: Heredia, KU517479, KU517515, KU517607, KU517644, 10 > 3: KU517569/KU517570/KU517571; ***Vittaria lineata*** (L.) J.Sm., 8129, *C. J. Rothfels 4008* (DUKE), USA: Florida, KU517480, KU517516, KU517608, KU517645, 9 > 2: KU517572/KU517573; ***Vittaria lineata*** (L.) J.Sm., 8924, *J. Prado 2263a* (SP), Brazil: Sao Paulo, KU517481, KU517517, KU517609, KU517646, 11 > 1: KU517574; ***Vittaria lineata*** (L.) J.Sm., 9340, *G. McPherson 20973* (MO), Panama: NA, KU517482, KU517518, KU517610, KU517647, 8 > 2: KU517575/KU517576; ***Vittaria scabrida*** Klotzsch ex Fée, 8915, *J. Prado 2249* (SP), Brazil: Sao Paulo, KU517483, KU517519, KU517611, KU517648, NA; ***Vittaria scabrida*** Klotzsch ex Fée, 8925, *J. Prado 2263b* (SP), Brazil: Sao Paulo, KU517484, KU517520, KU517612, KU517649, NA.