Mobile elements shape plastome evolution in ferns

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Abstract

Plastid genomes display remarkable organizational stability over evolutionary time. From green algae to angiosperms, most plastid genomes are largely collinear, with only a few cases of inversion, gene loss, or, in extremely rare cases, gene addition. These plastome insertions are mostly clade-specific and are typically of nuclear or mitochondrial origin. Here, we expand on these findings and present the first family-level survey of plastome evolution in ferns, revealing a novel suite of dynamic mobile elements. Comparative plastome analyses of the Pteridaceae expose several mobile open reading frames that vary in sequence length, insertion site, and configuration among sampled taxa. Even between close relatives, the presence and location of these elements is widely variable when viewed in a phylogenetic context. We characterize these elements and refer to them collectively as Mobile Open Reading Frames in Fern Organelles (MORFFO). We further note that the presence of MORFFO is not restricted to Pteridaceae, but is found across ferns and other plant clades. MORFFO elements are regularly associated with inversions, intergenic expansions, and changes to the inverted repeats. They likewise appear to be present in mitochondrial and nuclear genomes of ferns, indicating that they can move between genomic compartments with relative ease. The origins and functions of these mobile elements are unknown, but MORFFO appears to be a major driver of structural genome evolution in the plastomes of ferns, and possibly other groups of plants.
Introduction

Plastid genomes (plastomes) are a rich source of molecular sequence data and have proven to be especially useful in explorations of plant evolutionary history. From single-gene analyses to full plastome phylogenomics, important evolutionary insights can be gleaned from these relatively small, highly conserved, and minimally repetitive chromosomes (Taberlet et al. 1991; Chaw et al. 2004; Yoon et al. 2004; Pryer et al. 2004; Shaw et al. 2005; Leliaert et al. 2012; Ruhfel et al. 2014; Givnish et al. 2010; Moore et al. 2010; Gitzendanner et al. 2018). Plastomes contain high proportions of protein coding genes compared to plant nuclear genomes, with many of these genes being essential to photosynthesis (Wicke et al. 2011). Consequently, plastomes experience relatively low nucleotide substitution rates, especially in the inverted repeats, making them extremely stable over evolutionary time (Wolfe et al. 1987; Li et al. 2016; Zhu et al. 2016).

The plastomes of land plants seem to be especially resistant to changes in gene content, which, along with gene order, generally varies little between distantly related lineages—even after hundreds of millions of years (Palmer 1985). Relatively few plastome genes have been lost, except in heterotrophic lineages in which photosynthetic genes are typically not required for survival (Bungard 2004). Even rarer is the acquisition of new genes (Timmis et al. 2004). Since the gain of ycf1 and ycf2 in the algal ancestors, very few new genes have been incorporated into land plant plastomes (Timmis et al. 2004; de Vries et al. 2015). However, some groups do show exceptional variability in plastome structure, even among closely related taxa (Chumley et al. 2006; Guisinger et al. 2011; Cai et al. 2008; Haberle et al. 2008; Hirao et al. 2008). Notable among these exceptional lineages is Campanulaceae, within which a prolific group of inserted ORFs (Open Reading Frames) appear to have driven over 125 large inversions across the family (Knox 2014).
Overall stability in plastome structure across land plants contrasts, strikingly, with punctuated and/or persistent genomic rearrangements that are apparent in certain lineages (Chumley et al. 2006; Guisinger et al. 2011; Cai et al. 2008; Haberle et al. 2008; Hirao et al. 2008; Mower & Vickrey 2018). Ferns are among these, showing evidence of multiple genomic inversions since their initial diversification (Labiak & Karol 2017; Zhu et al. 2016; Stein et al. 1992; Wolf et al. 2015). An increasing number of fern plastome sequences are beginning to reveal a dynamic organellar genome, shaped in large part by structural inversions and/or shifts in gene content from single copy regions into the inverted repeat (Wolf et al. 2010). Genomic inversions like these have been associated with shifts in molecular evolutionary rate (Li et al. 2016; Zhu et al. 2016; Blazier et al. 2016) and may be moderated by selective constraints related to gene synteny and gene expression (Wicke et al. 2011; Cui et al. 2006).

Despite the great strides that have been made in our understanding of plastome evolution in ferns over the last decade (Mower & Vickrey 2018), dense taxonomic sampling is almost always lacking. Instead, studies of plastome evolution in ferns have focused on disparate groups of deeply divergent taxa (Gao et al. 2011; Wei et al. 2017), or on comparisons of only a few closely related species (Labiak & Karol 2017). Here, we aim to bridge this gap with the first family-scale comparative analysis of plastome structure and content in ferns.

Our focus is on Pteridaceae, an early-diverging family of polypod ferns that comprises roughly 1,200 species and accounts for well over 10% of extant fern diversity (PPG I 2016). The family is cosmopolitan in distribution and occupies a wide array of niches, from shaded forests to xeric and even aquatic habitats (Tryon 1990). Members exhibit a range of reproductive modes and some groups are noteworthy for undergoing frequent whole genome duplication. Among the most striking evolutionary patterns in the family is a dramatic shift in molecular evolutionary rate that has been documented across plastid and nuclear genomes of the so-called “vittarioid” ferns (Rothfels & Schuettpelz 2014; Grusz et al. 2016). In this study, we leverage genome skimming data to assemble and analyze 27 new plastomes from
across the Pteridaceae in an effort to: (1) examine plastome variation in Pteridaceae; (2) gain insight into genomic shifts within members of the Vittarioideae; and (3) reevaluate the phylogenetic relationships among major lineages comprising the family. Our data expose a massive plastome inversion and a group of mobile elements—newly characterized here—that appear to be a particularly dynamic component of fern plastomes, as evidenced from within Pteridaceae and beyond.

Materials and Methods

Taxon Sampling

Taxonomic sampling included 29 ingroup species, representing all major clades within the Pteridaceae (Schuettpelz et al. 2007), as well as three outgroup taxa (Table 4). Increased sampling from within subfamily Vittarioideae was undertaken in an effort to better understand the molecular evolutionary rate heterogeneity between the two main subclades therein, *Adiantum* and the vittarioid ferns.

DNA Extraction, Library Prep, and Sequencing

Whole genomic DNA for all newly sampled ingroup taxa (27 total) was extracted from silica-dried leaf tissue using the Qiagen DNeasy Plant Mini Kit (Germantown, Maryland) following the manufacturer’s protocol. Whole genomic DNA for 26 samples (all except *Vittaria appalachiana*) was sent to the Duke University Center for Genomic and Computational Biology for in-house library preparation and sequencing. There, individual genomic libraries (~300 bp) were prepared using the Kappa Hyper Prep Kit (Wilmington, Massachusetts). In total, 32 samples (26 included in this study) were multiplexed and pooled over one complete flowcell (8 lanes) on the Illumina HiSeq 2000/2500 platform for 125 bp paired-end sequencing. The *V. appalachiana* DNA was sent to BGI (Shenzhen, China) and
sequenced on the Illumina HiSeq 2000 platform, generating 5 Gb of 100 bp paired-end reads from an ~800 bp library.

**Genome Assembly and Annotation**

Raw sequence reads from *V. appalachiana* were assembled using Velvet version 1.2.03 (Zerbino & Birney 2008) according to previously described procedures (Guo et al. 2014; Sigmon et al. 2017). Genome assembly for all other ingroup taxa was performed using NOVOPlasty (Dierckxsens et al. 2017). NOVOPlasty implements a seed-based, *de novo* genome assembly, which can lessen structural assembly biases that may otherwise mask inferred rearrangements. NOVOPlasty employs the seed to retrieve a given sequence from the target genome, which is then extended and circularized (Dierckxsens et al. 2017).

In most cases, the *rbcL* gene from *Adiantum capillus-veneris* (NC_004766; Table 4) was used as a seed sequence, but in select cases, if *rbcL* had inadequate coverage, the entire plastome of *A. capillus-veneris* was used instead. Raw, unfiltered Illumina reads were subsampled to ≤ 30 million reads to reduce memory requirements. The default k-mer of 39 was used unless there was low organellar genome coverage (< 1%), in which case the k-mer was reduced to 23–30. In cases involving long repetitive regions, higher k-mers of 45–55 were used. For *Jamesonia brasiliensis* and *Cheilanthes bolborrhiza*, complete assembly of plastomes was not possible, but we were able to get contigs of considerable size, which have been included in this study.

Following assembly, genomes were annotated in Geneious 11.1 (Kearse et al. 2012), using the gene sequences of *A. capillus-veneris* as a reference. Putative RNA editing sites were annotated to retain conserved open reading frames (Wolf et al. 2003, 2004). Intergenic sequences that differed dramatically from *A. capillus-veneris* were queried against the NCBI Nucleotide database using BLAST (Altschul et al. 1990) to ensure that they did not result from the false assembly of mitochondrial or nuclear sequences.
Assembly errors were further assessed by mapping raw reads to the newly assembled genome using Bowtie2 (Langmead & Salzberg 2012), looking for dips in read depth. Additionally, overall plastome assembly quality was assessed for each sample using Pilon (Walker et al. 2014). In all cases, changes proposed by Pilon were relatively minor (< 10 nucleotides) suggesting that the quality and accuracy from NOVOPlasty assembly was high.

**Plastome phylogenomic analyses**

In total, 32 plastomes were included in our phylogenomic analyses; 26 were new to this study and the remaining 6 were obtained from Genbank (Table 4). Each annotated plastome was opened in Geneious and all CDS/gene regions were extracted in FASTA format. Using these taxon-specific FASTA files, containing all CDS/gene regions, we then compiled a FASTA file for each locus. Sequences for each region were aligned using MAFFT 7.394 (Kuraku et al. 2013; Katoh et al. 2017) and alignments of all loci concatenated using Sequence Matrix (Vaidya et al. 2011). The resulting concatenated matrix, comprising 76 loci and 68,047 nucleotide sites, partitioned by gene, was processed through PartitionFinder2 (Lanfear et al. 2017, 2012) on the CIPRES Science Gateway version 3.3 (Miller et al. 2010) using the following settings: branchlengths=linked, models=GTR, GTR+G, GTR+I+G, and model_selection=AICc. PartitionFinder2 identified 36 unique model partitions spanning the concatenated matrix.

Phylogenomic analyses of the concatenated, partitioned dataset were implemented using maximum likelihood and Bayesian optimality criteria on the CIPRES Science Gateway version 3.3 (Miller et al. 2010). Maximum likelihood searches were conducted in RAxML V.8 using multiparametric bootstrapping (-b; 1000 replicates) and our previously described partitioned model (-q). Bayesian inference was performed using MrBayes 3.2.6 and comprised four independent runs, each with four chains (one cold, three heated) and otherwise default (i.e., flat) priors, with the exception that rates of
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evolution were allowed to vary among loci (ratepr = variable). Chains were run for 10 million generations and trees were sampled from the cold chain every 1,000 generations. To determine at which point the analysis had reached stationarity, the standard deviation of split frequencies among the independent runs (as calculated by MrBayes) was examined and the output parameter estimates were plotted using Tracer v1.6 (Rambaut et al. 2015). Based on convergence diagnostics, the first 2.5 million generations were excluded before obtaining a consensus phylogeny and clade posterior probabilities with the “sumt” command (contype = allcompat).

Characterizing MORFFO elements

To search for MORFFO like sequences in GenBank, all MORFFO insertions found in Pteridaceae were aligned using Geneious, and a consensus sequence was generated. All BLAST (Altschul et al. 1990) (Madden 2013) queries for MORFFO were performed using this consensus sequence. This consensus sequence was also used when querying both VecScreen (NCBI Resource Coordinators 2017) and RepeatMasker (Smit et al. 2013). Three main search strategies were employed when using NCBI Nucleotide BLAST. Initial queries for MORFFO like sequences were performed with BLASTN using the default parameters. Then, BLASTX and TBLASTN searches were performed using the default parameters and a word size of 3. Additional searches using specific MORFFO sequences rather than a consensus returned equivalent results.

To evaluate the level of selective constraint on the three MORFFO genes, estimates of $d_N/d_S$ were calculated for five species that contained all three MORFFO genes (*Bommeria hispida*, *Hemionitis subcordata*, *Notholaena standleyi*, *Tryonia myriophylla*, and *Vaginularia trichoidea*). First, codon-based alignments were generated using the ClustalW-Codons option in MEGA version 7.0.18 (Kumar et al. 2016). Alignments were trimmed using Gblocks version 0.91b (Castesana 2000) in codon mode with a relaxed set of parameters ($t$=c, $b2$=3, $b5$=half). For each trimmed gene alignment, branchwise estimates of
\( d_s/d_S \) were calculated for each species using the GA-branch model (Kosakovsky Pond & Frost 2005), which uses a genetic algorithm to optimize the number of \( d_s/d_S \) rate classes across the tree and maximum likelihood to optimize branch length and substitution rates, as implemented on the Datamonkey web server (Delport et al. 2010). For the analysis, the HKY rate matrix was chosen as the substitution rate model based on the Datamonkey model selection tool. To evaluate the influence of the tree topology on \( d_s/d_S \) estimates, the analysis was run using either a NJ tree or a user-defined tree that matched organismal relationships shown in Figure 1.

Phylogenetic relationships among MORFFO sequences were estimated using maximum likelihood best tree and bootstrap searches, implemented in RAxML V.8 (Stamatakis 2014) with multiparametric bootstrapping (-b; 1000 replicates).

Results

Genome Assembly and Annotation

We assembled and annotated 25 complete plastomes from previously unsampled species, representing all major clades within Pteridaceae (Schuettpelz et al. 2007), plus two partial plastome sequences for Jamesonia brasiliensis and Cheilanthes bolborhiza (139,531 bp and 39,380 bp, respectively). The average length of complete plastid genome sequences was 153,153 bp (range 145,327 bp to 165,631 bp) with an average GC content of 41.46% (range 36.7–45.3%; Table 1). Gene content remained largely stable across samples, with no losses of protein coding genes relative to Adiantum capillus-veneris. We did, however, detect a loss of \( trnT \) in all vittarioid ferns sampled (Fig 1), as well as a loss of \( trnV \) in Onychium japonicum, Ceratopteris cornuta, plus all vittarioids with the exception of Haplopteris elongata. Across all samples, there were 82 protein coding genes, 33–35 tRNA genes and 4 rRNA genes. Gene order was unchanged across the family—with the exception of a 7,000 bp genomic inversion within the inverted repeats of all vittarioid species except Vaginularia trichoidea.
Several plastid DNA insertions were recovered from multiple clades within Pteridaceae. The most prominent of these comprised a suite of genomic insertions, here referred to as Mobile Open Reading Frames in Fern Organelles (MORFFO), that were detected in most of the plastomes sampled. These MORFFO clusters are characterized by three large and distinct open reading frames (ORFs) that are variably absent, or present in a number of different arrangements (Fig 1). One ~1,300 bp ORF (morffo1) is flanked by inverted repeats of ~40 bp that are often in the motif TGT CGA TAG, repeated 3–5 times. The amino acid sequences of morffo1 do not bear similarity to any characterized proteins in GenBank, but do bear similarity to a hypothetical protein found in the early-diverging fern Mankuya chejuensis and the green alga Roya anglica (Table 2). A larger ORF (morffo2) of ~1,700 bp has domains similar to primases associated with mobile elements in cyanobacteria and archaea (DN_5 superfamily) when queried using BLASTX (Altschul et al. 1990; Table 2). A smaller ORF (morffo3) of ~630 bp has no significant similarity to any known genes or proteins; it is found less frequently than the two larger ORFs, but is still prevalent. Often, but not always, morffo1 is found inserted in frame with morffo2 to form a larger ORF of ~3,500 bp. Importantly, morffo1, morffo2, and morffo3 can be found in a variety of different arrangements, but when present they are always found immediately adjacent to one another (Fig 1).

The location of MORFFO elements varied across the genomes sampled, being included in the Large Single Copy (LSC), the Inverted Repeat (IR), or the Small Single Copy regions (SSC; Fig 2). As a whole, MORFFO sequences (morffo1, morffo2, morffo3) were similar among species, ranging from 92% to 45% sequence identity (Table 3). For those species with a full set of three MORFFO sequences, the genes appear to encode functional proteins. The coding sequences are intact (no internal stop codons or frameshifting indels) and exhibit nonsynonymous (dS) to synonymous (dS) substitution rate ratios that are consistent with selective constraint (dS/dS << 1, ranging from 0.17 to 0.43), with the exception of morffo1 from Tryonia myriophylla, which has at least two frameshifting indels and a dS/dS approaching 1 (S1...
Table). A weak association was observed between the genomic location of MORFFO elements and phylogenetic position among species sampled (Table 3).

Chromosome-wide read depth analyses revealed no shifts in coverage spanning MORFFO insertions or insertion boundaries, indicating that these inserts are not an artifact of genomic library preparation or genome misassembly. Furthermore, MORFFO insertions were detected in *Vittaria appalachiana*, which was sequenced and assembled in a separate lab, using an alternative assembly protocol. We also examined each member of the MORFFO cluster using VecScreen (NCBI Resource Coordinators 2017) and RepeatMasker (Smit et al. 2013), neither of which yielded matches to any known vectors or transposable elements. We searched for MORFFO sequences against current draft assemblies of nuclear genomes of the ferns *Azolla* and *Salvinia* on FernBase (Li et al. 2018) as well as scaffolds for the draft genomes of *Ceratopteris*, and while they were not detected *Azolla* or *Salvinia*, we did observe the presence of *morffo1* in *Ceratopteris* scaffolds. In addition, we searched for the presence of MORFFO in available transcriptomes from members of Pteridaceae in the 1 kp project (Wickett et al. 2014; Matasci et al. 2014; Xie et al. 2014; Johnson et al. 2012), and found no evidence of transcription of MORFFO.

To test whether MORFFO elements could be of mitochondrial origin, we filtered plastid reads using the mitochondrial option in NOVOPlasty (Dierckxsens et al. 2017), and then assembled the remaining reads using *morffo1* as a seed. This did not generate an assembly of any known mitochondrial sequence. Instead, a seemingly circular 2,139 bp contig was inferred in *Adiantum tricholepis*, containing *morffo1* and *morffo2*, but no known mitochondrial sequences. Furthermore, this contig lacked the flanking inverted repeats normally associated with *morffo1*. A control assembly using the mitochondrial genes *atp1* and *cox1* as seed sequences was also generated using non-plastid raw reads from this species. The assemblies based on mitochondrial genes had markedly lower average coverage depth (86.5) than that of the *morffo1* based assembly (556) and the plastome (359), suggesting that the MORFFO cluster in
Adiantum tricholepis exists as an independent mobile element that is not an integrated component of the mitochondrial genome. Where this element resides within the cell is unclear.

Relationships among MORFFO sequences were estimated using a maximum likelihood optimality criterion and 1000 bootstrap replicates. Each MORFFO element—morffo1, morffo2, morffo3—comprises a monophyletic clade, within which some sequences are united with moderate to high bootstrap support (Supplementary Figure 1). However, relationships among sequences within each morffo clade were not congruent with the accepted species tree.

**Plastome phylogenomic analyses**

Our final, concatenated plastome alignment included 68,047 sites spanning 76 plastid loci for 31 taxa, including three outgroups. PartitionFinder2 returned a most favorable partition model with 36 subsets (AICc: 1211937.74576), from which partition blocks were assigned in RAxML (Stamatakis 2014) and MrBayes (Ronquist et al. 2012). Trees inferred using maximum likelihood (ML) and Bayesian optimality criteria were in full topological agreement with maximum support on all branches, with the exception of the branch subtending Onychium japonicum, Tryonia myriophylla, Jamesonia brasiliensis, Gastionella chaerophylla, and Pityrogramma trifoliata, which was supported by an ML bootstrap of 95 and a posterior probability of 1.0 (Fig 1).

**Discussion**

Comparative analyses of plastomes over the past two decades have dramatically improved our understanding of their evolution across land plants. Early data painted a picture of structural and organizational stability among deeply divergent embryophyte plastomes, punctuated by relatively few large-scale inversions (Hoot & Palmer 1994; Ogihara et al. 1988; Wolf et al. 2010). Recent evidence,
however, has begun to expose the plastome as a dynamic molecule that in some lineages undergoes frequent changes in DNA content and structure (Knox 2014; Guisinger et al. 2011; Cremen et al. 2018; Lin et al. 2012). As more information has come to light, many highly rearranged plastomes have also been found to host sizeable insertions, occasionally including open reading frames (ORF) of unknown homology (Knox 2014; Cremen et al. 2018). In some cases, these inserted ORFs appear to encode functional proteins, whereas in others they resemble conserved domains that have undergone extensive rearrangements and/or pseudogenization, comparable to what has been observed within some plastid genes (e.g., *ndhK*, *clpP*, and *ycf2*; (Haberle et al. 2008; Smith 2014; Sun et al. 2016; Lin et al. 2012). Several studies have determined that similar, undescribed plastid ORFs are the result of horizontal transfer from mitochondria to plastids (Rabah et al. 2017; Goremykin et al. 2009; Iorizzo et al. 2012; Ma et al. 2015; Burke et al. 2016).

Plastome ORF insertions like these—with no known sequence homology—have not been characterized in ferns, although previous authors have reported large intergenic expansions and insertions in some taxa (Logacheva et al. 2017; Gao et al. 2011). It was speculated that some of these intergenic expansions originated via intracellular transfer from the mitochondrion (Logacheva et al. 2017), but until now, limited sampling in previous studies has obscured the highly mobile nature of these peculiar sequences. Here, we take a focused phylogenetic approach, targeting the fern family Pteridaceae, to reveal a suite of highly mobile ORFs (MORFFO) within a broad sampling of plastomes from across the family. Preliminary analyses indicate that MORFFO elements, which are frequently associated with extensive genomic rearrangements, may be present in lineages well-removed from ferns.

**Characterization of MORFFO elements**

Logacheva et al. (2017) established that ‘hypervariable’ sequences of significant length, are found in the inverted repeat (IR) of *Woodwardia unigemmata* as well as the LSC of *Plagiogyria*. Our results are
consistently with their findings, and further expose the dynamic nature of these sequences (MORFFO) among a collection of closely-related fern plastomes. Searches for MORFFO-like sequences outside of Pteridaceae returned similar, putatively homologous regions in many ferns (Table 2,3), but not in seed plants. Significantly, an 8 Kbp region in the plastome of the fern *Mankyua chejuensis* (Ophioglossaceae) contains an expanded complement of the MORFFO cluster. Additional searches for MORFFO-like sequences outside of vascular plants revealed similar conserved domains in several cyanobacteria plastomes (Table 2), as well as domains in the plastomes of the green algae *Prasiola crispa*, *Roya obtusa*, and *Roya anglica* (Table 2).

Within ferns, we note that MORFFO elements are frequently found adjacent to inferred sites of genomic inversion. For example: (1) *morffo1* is found adjacent to the border of one of two hypothesized inversions in the region spanning *rpoB-psbZ* which occurred in a common ancestor of the core leptosporangiates (Fig 3; PPG I 2016); (2) *morffo3* is found within a 9.7 Kbp inversion that characterizes leptosporangiate ferns (Kim et al. 2014); (3) *morffo1* and *morffo2* are found inserted adjacent to the 7 Kbp inversion seen in the plastomes of vittarioid ferns; (4) and *morffo1* and *morffo2* also appear adjacent to an inversion described in filmy ferns (Fig 3; Wolf et al. 2011; Kim et al. 2014; Gao et al. 2011; Kuo et al. 2018). Although MORFFO insertions are frequently associated with inversions, we are unsure why. One possibility is that MORFFO may target nucleotide sites that are prone to inversion. Conversely, the insertion of MORFFO could be directly influencing inversion events. In several taxa, we observe a proliferation of the inverted repeats flanking *morffo1*, possibly caused by replication slippage, or possibly by the repeated insertion and excision of *morffo1*. In other groups, plastome reorganization has been similarly associated with the presence of small dispersed repeats like these (Wicke et al. 2011). Likewise, the relationship between MORFFO insertion sites and inversions is not unlike the insertions seen in other dynamic embryophyte plastomes (Knox 2014).
The variable presence, location, and configuration of MORFFO observed in a phylogenetic context suggests that these ORFs are mobile elements. With a few notable exceptions, plastid genes are not frequently gained or lost, yet our results indicate that MORFFO moves into, out of, and across the plastome in relatively short evolutionary timescales. While MORFFO sequences have been observed in mitochondrial contigs (Logacheva et al. 2017), it is important to note that they are not found in either of the currently available complete mitochondrial genomes of ferns (Guo et al. 2017). Additionally, the location of these insertions in mitochondrial genomes seems to vary as much as in those of plastids, making it difficult to pinpoint a potential mitochondrial origin. Furthermore, we have also noted the presence of MORFFO in the nuclear genome of *Ceratopteris*. Thus, MORFFO appears to be moving across genomes as readily as within them. However, this does not explain the origin of the elements nor the mechanisms of their movement.

Plasmid-like sequences have been observed in the plastomes of diatoms, green algae, dinoflagellates, and red algae (Zhang et al. 1999; Cremen et al. 2018; Ruck et al. 2014; La Claire & Wang 2000; Lee et al. 2016). Although we are not aware of previous work describing chloroplast plasmids in land plants, this is a plausible mechanism to explain the variable presence and location of MORFFO elements. It would also explain the variability in order and direction of MORFFO insertions (Fig 1). As noted above (see results section), we were able to assemble a circular sequence containing *morffo1* and *morffo2* from *Adiantum tricholepis*, which did not have the MORFFO insert in its plastome. The coverage analysis for this sequence indicates that MORFFO is likely a high copy-number sequence that is independent of both the plastome and the mitochondrial genome. This, combined with the fact that *morffo2* has regions that share similarity to conserved domains (DN_5 superfamily) associated with primase genes found in mobile elements of cyanobacteria, strongly suggests that these sequences could be of plasmid origin, possibly from a plastid plasmid.
Alternatively, MORFFO elements could be of viral origin. In addition to being similar to plasmid primases, the conserved domain found in \textit{morffo2} also resembles primase genes found in phages. Viral origins could explain why MORFFO is found frequently but irregularly in fern plastomes. Likewise, many of the above arguments in favor of a plasmid or plasmid-like origin for MORFFO sequences can also be attributed to viral origin.

The structural similarities that \textit{morffo1} shares with bacterial insertion sequences is noteworthy, especially because insertion sequences are known to cause inversions (Darmon & Leach 2014). This, along with the clear mobility of these sequences strongly suggest that \textit{morffo1} could be a previously undescribed insertion sequence. The case for \textit{morffo1} being an insertion sequence is made stronger by the fact that of all the MORFFO sequences, it appears to display the most independence. In non-plastid DNA, it is almost always found without the other MORFFO sequences. It is also more frequently seen independent of additional MORFFO sequences in the plastomes of ferns outside of Pteridaceae. Furthermore, copies of \textit{morffo1} were detected in the nuclear genome of \textit{Ceratopteris} and the mitochondrial genome of \textit{Asplenium nidus}, suggesting that it may be a particularly promiscuous mobile element. The relationship of \textit{morffo2} and \textit{morffo3} to \textit{morffo1}, however, remains unclear. If \textit{morffo1} is an independent insertion sequence, then how are \textit{morffo2} and \textit{morffo3} inserted?

Phylogenetic analysis of MORFFO elements reveals three strongly-supported, monophyletic groups: \textit{morffo1}, \textit{morffo2}, and \textit{morffo3} elements. Relationships within each MORFFO clade do not reflect the accepted species phylogeny, but phylogenetic similarity across clades may reflect shared histories of degradation among MORFFO elements (Supplementary Figure 1.)

We also note that whereas MORFFO elements are pervasive in Pteridaceae, they appear to be less common in most other groups of ferns. In part, this may be an artifact of the historical reliance on reference-based assemblies, which can be biased towards assembling genomes that appear more similar to their reference, thus reducing the likelihood of detecting significant rearrangements. Based on the few
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sequences available in GenBank, it would seem that MORFFO elements may be prevalent in Plagiogyria
and Ophioglossaceae; however, it remains to be determined how widespread this cluster of ORFs is
among other lineages of ferns. More studies at the family level are needed to understand the extent to
which MORFFO sequences are moving throughout fern genome space. The current paucity of fern
nuclear and mitochondrial genomes makes it difficult to determine the source of these inserts. At the time
of writing there are only two fern mitochondrial sequences (Guo et al. 2017) available in GenBank, and
no nuclear genomes, although several are in preparation. As more genomes are published in the coming
years, the reservoir from which MORFFO clusters are migrating should become clear.

Plastome variation across Pteridaceae

Pteridaceae is an ecologically and morphologically diverse family comprising more than 10% of
extant fern species (Schuettpelz et al. 2007). Within this group, subfamily Vittarioideae, comprising the
genus Adiantum and the so-called vittarioid ferns, is especially noteworthy. High levels of molecular
substitution rate heterogeneity has been detected between members of the genus Adiantum and the
vittarioid ferns, in both plastid and nuclear DNA sequences (Rothfels & Schuettpelz 2014; Grusz et al.
2016). As noted above, we also find variation in plastome structure across the family, with MORFFO
elements (morffo1, morffo2, and morffo3) being repeatedly gained, lost, and/or rearranged, even among
closely related taxa (Fig 1).

The physical position of MORFFO cluster insertions is relatively conserved within the 5 major
clades comprising Pteridaceae (Fig 1), but in some cases the location and composition of these clusters
varies widely, even between congeneric relatives (e.g., Myriopteris lindheimeri vs. M. scabra and Vittaria
appalachiana vs. V. graminifolia; Fig 1). Based on our sampling, we find no evidence of MORFFO
elements within Cryptogrammoideae or Parkerioideae. However, unique insertions of MORFFO
sequences have taken place in some members of the Pteridoideae, including Jamesonia brasiliensis
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(between trnN and cef2), *Tryonia myriophylla* (between trnD and trnY), and *Pteris vittata* (between psbM and petN). MORFFO elements were notably absent in the species of *Gastioniella*, *Pityrogramma*, and *Onychium* sampled (Fig 1). Compared to the Pteridoideae, subfamilies Vittarioideae and Cheilanthoideae exhibit relative stability in their MORFFO insertion sites (Fig 1). Altogether, we find at least nine unique MORFFO cluster insertions across the Pteridaceae (there are almost certainly more), not including a multitude of species-specific rearrangements, gains, and losses of individual MORFFO elements (*morffo1*, *morffo2*, and *morffo3*) following cluster insertions.

Within each independent MORFFO cluster insertion, the presence and position of *morffo1*, *morffo2*, and *morffo3* are highly variable among species sampled. For example, a variety of insertions, rearrangements, and losses of all three elements can be found in the MORFFO cluster found between *rpoB* and *trnD* within subfamily Vittarioideae (Fig 1). Likewise, the MORFFO cluster between *rps12* and *rrn16* in Cheilanthoideae shows insertions and losses of all three MORFFO elements, including a duplication of *morffo2* in *Hemionitis subcordata* (Fig 1).

Interestingly, vittarioid ferns do not appear to have experienced expansion or contraction of the IR, which have been associated with extensive genomic rearrangements, gene loss, and the proliferation of repetitive regions in other groups (Zhu et al. 2016). In addition to the variable presence of MORFFO elements, we find that their insertion into the ancestral vittarioid IR (Fig 1) may have also coincided with a loss of *trnT*. Most vittarioid ferns share an additional gene loss (*trnV*)—with the exception of *Haplopteris elongata*, in which *trnV* is found intact. Given that *Vaginularia trichoidea* and *H. elongata* are inferred to be successively sister to the remaining vittarioid ferns sampled (Fig 1; Schuettpelz et al. 2016), this topology implies the gain of *trnV* into the plastome of *H. elongata*. Nevertheless, a shared ~7 Kbp inversion between *rrn16* and *rrn5* in all vittarioid ferns except *V. trichoidea* further supports our phylogenetic inferences based on DNA sequence data (Figs 1 and 3).
The vittarioid ferns are characterized by high levels of plastome rearrangement, elevated molecular substitution rates, a shift to epiphytism, morphological reduction, and shared ancestral whole genome duplication (Pryer et al. 2016). This array of shared traits leads one to ask which (if any) might have driven these changes in vittarioid plastome structure and expression. Similarly, frequent rearrangements, insertions, and losses of the MORFFO suite within the plastomes of subfamily Cheilanthoideae coincide with adaptations to extreme xeric environments, extensive whole genome duplications, hybridization, and apomixis—any of which may relate to the changes we detect in plastome structure across this subfamily.

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Table 1: Summary of basic genomic features of plastomes used in this study. IR = Inverted Repeat, LSC = Large Single Copy, SSC = Small Single Copy

| Species                | Inverted Repeat Size | LSC Size | Genome Size | SSC Size | %GC | MORFFO |
|------------------------|----------------------|----------|-------------|----------|-----|--------|
| *Adiantum aleuticum*   | 26,289               | 83,345   | 157,519     | 21,596   | 45  | 1,2    |
| *Adiantum capillus-veneris* | 23,448             | 82,282   | 150,568     | 21,390   | 41  | Absent |
| *Adiantum tricholepis* | 23,233               | 82,740   | 150,667     | 21,461   | 42  | Absent |
| *Antrophyum semicostatum* | 20,977            | 87,492   | 150,274     | 21,390   | 41  | Absent |
| *Bommeria hispida*     | 23,142               | 83,623   | 149,424     | 21,227   | 37  | Absent |
| *Calciptilopteris ludens* | 20,977             | 87,492   | 150,274     | 21,390   | 41  | Absent |
| *Ceratopteris cornuta* | 22,020               | 83,178   | 148,444     | 21,226   | 35  | Absent |
| *Ceratopteris richardii* | 22,020            | 83,178   | 148,444     | 21,226   | 35  | Absent |
| *Cheilanthes bolborrhiza* | ~25,000          | na       | 39,380*     | na       | 44  | 1,2    |
| *Cheilanthes micropteris* | 23,306            | 88,393   | 157,567     | 22,562   | 41  | 1,3    |
| *Cryptogramma acrostichoides* | 22,652         | 83,690   | 150,162     | 21,168   | 42  | Absent |
| *Cystopteris chinensis* | 26,671              | 83,093   | 155,548     | 21,321   | 42  | Absent |
| *Dryopteris decipiens* | 23,456              | 82,462   | 150,978     | 21,604   | 42  | Absent |
| *Gastoniella caerophylla* | 22,657            | 81,918   | 148,099     | 20,867   | 40  | 2      |
| *Haplopteris elongata* | 30,921              | 82,607   | 156,631     | 21,182   | 43  | 1,2,3  |
| *Hemionitis subcordata* | 22,704             | 84,531   | 159,531     | 20,941   | 41  | 1      |
| *Jamesonia brasiliensis* | 23,208             | 81,944   | 149,387     | 21,027   | 42  | Absent |
| *Llavea cordifolia*    | 25,567              | 83,039   | 155,548     | 21,321   | 42  | Absent |
| *Myriopteris covillei* | 27,115              | 82,874   | 162,051     | 24,947   | 42  | 1      |
| *Myriopteris lindheimeri* | 27,115            | 82,874   | 162,051     | 24,947   | 42  | 1      |
| *Myriopteris scabra*   | 27,261              | 83,769   | 159,556     | 21,265   | 42  | 1,2,3  |
| *Onychium japonicum*   | 23,419              | 82,289   | 150,156     | 21,029   | 41  | Absent |
| *Pellaea truncata*     | 23,240              | 82,685   | 150,713     | 21,368   | 42  | Absent |
| *Pentagramma triangularis* | 23,378           | 85,675   | 153,445     | 21,014   | 42  | 1,3    |
| *Pityrogramma trifoliata* | 22,465            | 82,321   | 148,156     | 20,905   | 40  | Absent |
| *Pteridium aquilinum*  | 25,275              | 82,604   | 154,108     | 20,954   | 42  | 2      |
| *Pteris vittata*       | 27,115              | 82,874   | 162,051     | 24,947   | 42  | 1      |
| *Scoliosorus ensiformis* | 21,078            | 82,358   | 145,327     | 20,813   | 40  | Absent |
| *Tryonia myriophylla*  | 20,977              | 87,238   | 156,327     | 20,807   | 40  | 1,2,3  |
| *Vaginularia trichoida* | 21,185             | 84,330   | 149,531     | 20,831   | 40  | 1      |
| *Vittaria graminifolia* | 22,066             | 86,058   | 151,035     | 20,845   | 40  | 1,2    |
Table 2: Summary of hits to MORFFO sequences in NCBI blast, using either BLASTX or TBLASTN. * = putative DN_5 superfamily conserved domain

| Species                        | Organism group and genome compartment | search strategy | MORFFO match | Length of match (aa) | % identity | Accession number |
|--------------------------------|---------------------------------------|-----------------|--------------|----------------------|------------|------------------|
| Actinostachys pennula         | Fern plastome                         | TBLASTN         | morffo2      | 231                  | 34         | KU764518.1       |
| Alsophila spinulosa           | Fern plastome                         | TBLASTN         | morffo2      | 78                   | 44         | FJ556581.1       |
| Angiopteris angustifolia      | Fern plastome                         | TBLASTN         | morffo2      | 332                  | 49         | KP099647         |
| Angiopteris evecta            | Fern plastome                         | TBLASTN         | morffo2      | 331                  | 47         | DQ821119.1       |
| *Asplenium nidus mitochondrial group* | Fern mitochondrial genome             | TBLASTN         | morffo1      | 260                  | 52         | AM600641.1       |
| Asplenium prolongatum         | Fern plastome                         | TBLASTN         | morffo2      | 110/59               | 77/64      | KY427332.1       |
| Chondrocystis(plasmid)*       | Cyanobacterium                        | TBLASTN         | morffo2      | 233                  | 27         | AP018284.1       |
| Crocosphaera watsonii*        | Cyanobacterium                        | BLASTX          | morffo2      | 365                  | 43         | WP_00731007.2    |
| Dryopteris fragrans           | Fern plastome                         | TBLASTN         | morffo1/morffo2 | 323/355            | 46/49      | KX418656.2       |
| Helminthostachys zeylanica    | Fern plastome                         | TBLASTN         | morffo2      | 226                  | 70         | KM817788.2       |
| Huperzia lucidula             | Lycopod plastome                      | TBLASTN         | morffo2      | 114                  | 35         | AY660566.1       |
| Huperzia serrata              | Lycopod plastome                      | TBLASTN         | morffo2      | 114                  | 35         | KX426071.1       |
| Lepisorus clathratus          | Fern plastome                         | TBLASTN         | morffo1/morffo2 | 183/208           | 50/56      | KX419704.1       |
| Lygodium japonicum            | Fern plastome                         | TBLASTN         | morffo2      | 152                  | 36         | HM021803.1       |
| Myxosarcina spp.*             | Green alga plastome                   | BLASTX          | morffo2      | 497                  | 27         | WP_05205595.1    |
| Nostoc punctiforme*           | Cyanobacterium                        | TBLASTN         | morffo2      | 245                  | 28         | CP001037.1       |
| Ophioglossum californicum     | Fern plastome                         | TBLASTN         | morffo1/morffo2/morffo3 | 120/169/60/60  | 56/41/42    | KC117178.1       |
| Polypodium glycyrrhiza        | Fern plastome                         | TBLASTN         | morffo3      | 162                  | 53         | KP136832         |
| Prasiola crispa               | Green alga plastome                   | TBLASTN         | morffo2      | 416                  | 25         | KR017750.1       |
| Roya anglica                  | Green alga plastome                   | TBLASTN         | morffo1      | 202                  | 30         | NC_024168        |
| Roya obtusa                   | Green alga plastome                   | TBLASTN         | morffo1      | 202                  | 30         | KU646496.1       |
| Volvox carteri                | Green alga plastome                   | TBLASTN         | morffo1/morffo2 | 200/303           | 28/25      | EU755299.1       |
| Woodwardia unigemmata         | Fern plastome                         | TBLASTN         | morffo1/morffo2 | 247/105           | 51/77      | KT599101.1       |
Table 3: Summary of matches for MORFFO sequences within ferns, using BLASTN. IR = Inverted Repeat, LSC = Large Single Copy, SSC = Small Single Copy

| Species                        | MORFFO present         | Length of match (bp) | % identity | Region | Accession number |
|--------------------------------|------------------------|----------------------|------------|--------|------------------|
| Adiantum aleuticum             | morffo1/ morffo2       | 1316/ 1851           | 88/67      | IR     | MH173079         |
| Alsophila podophylla           | morffo1/morffo2/morffo3| 1250/1778/631        | 78/76/81   | IR     | MG262389         |
| Antraphyllum semicostatum      | morffo1/ morffo3       | 1323/ 629            | 53/ 57     | LSC    | MH173087         |
| Asplenium prolongatum          | morffo2                | 265                  | 81         | IR     | KY427332         |
| Bommeria hispida               | morffo1/ morffo2/ morffo3| 1296/1708/ 1708    | 89/87/84   | SSC    | MH173074         |
| Calciophilopteris ludens       | morffo1/ morffo2       | 1284/928             | 78/ 51     | IR     | MH173084         |
| Cheilanthes bolborrhiza        | morffo1/ morffo2       | 1304/1859            | 92/90      | IR     | MH173073         |
| Cheilanthes micropteris        | morffo1/ morffo3       | 1292/ 630            | 50/ 50     | LSC    | MH173078         |
| Cibotium barometz              | morffo1/ morffo2       | 1296/1850            | 77/50      | IR     | NC_037893        |
| Dicksonia squarrosa            | morffo1                | 1321                  | 50         | IR     | KJ569698         |
| Diplopterygium glaucum         | morffo3                | 639                   | 55         | LSC    | KF225594         |
| Drynaria roosii                | morffo1                | 1314                  | 51         | LSC    | KY075853         |
| Haplopteris elongata           | morffo1/ morffo2       | 1336/1859            | 56/ 55     | IR     | MH173086         |
| Hemionitis subcordata          | morffo1/ morffo2/ morffo3| 1296/ 1863/ 665    | 91/75/92   | IR     | MH173072         |
| Hymenasplenium unilaterale     | morffo1/ morffo3       | 252/ 643             | 65/61      | IR/LSC | KY427350         |
| Jamesonia brasiliensis         | morffo1                | 1310                  | 51         | IR     | MH173077         |
| Mankyua chejuensis             | morffo1/ morffo2       | 193/186               | 68/67      | IR     | KP205433         |
| Myriopteris covillei           | morffo2                | 1886                  | 49         | IR     | MG953517         |
| Myriopteris lindheimeri        | morffo2                | 852                   | 67         | IR     | HM778032         |
| Myriopteris scabra             | morffo1                | 1310                  | 98         | IR     | MH173083         |
| Notholaena standleyi           | morffo1/ morffo2/ morffo3| 1312/ 1866/ 665    | 89/86/91   | IR     | MH173067         |
| Pentagramma triangularis       | morffo2/ morffo3       | 1860/ 630             | 83/84      | LSC    | MH173070         |
| Plagiogyria glauca             | morffo1                | 1305                  | 49         | LSC    | KP136831         |
| Plagiogyria glauca             | morffo2                | 1856                  | 46         | Mito   | Wolf et al. (2014) |
| Plagiogyria japonica           | morffo2                | 1295                  | 50         | LSC    | HQ658099         |
| Pteridium aquinulum            | morffo2                | 280                   | 65         | LSC    | HM535629.1       |
| Pteris vittata                 | morffo2                | 1172                  | 80         | LSC    | MH173068         |
| Rhachidosorus consimilis       | morffo2                | 430                   | 70         | IR     | KY427356         |
| Tryonia myriophylla            | morffo1/ morffo2/ morffo3| 1326/ 1854/ 641    | 89/84/86   | LSC    | MH173076         |
| Vaginularia trichoidea         | morffo1/ morffo2/ morffo3| 1315/ 1836/ 630    | 53/ 52/ 62 | LSC    | MH173085         |
| Vittaria appalachiana          | morffo1                | 1316                  | 68         | LSC    | MH173091         |
### Table 4. Taxonomic sampling and voucher information for samples used in this study.

| Taxon                                           | Voucher or citation                  | Genbank Accession |
|-------------------------------------------------|--------------------------------------|-------------------|
| Adiantum aleuticum (Rupr.) C. A. Paris          | Rothfels 4097 (DUKE)                 | MH173079          |
| Adiantum capillus-veneris L.                     | Wolf et al. 2004                     | NC004766          |
| Adiantum tricholepis Fée                         | Rothfels 08-094 (DUKE)               | MH173071          |
| Antrophyum semicostatum Blume                   | Schuettpelz 1561 (BO)                | MH173087          |
| Bommeria hispida (Mett. ex Kuhn) Underw.        | Beck 1130 (DUKE)                     | MH173074          |
| Calciphilopteris ludens (Wall. ex Hook.) Yesilyurt & H. Schneid. | Huiet s.n. (DUKE) | MH173084          |
| Ceratopteris cornuta (P. Beauv.) Lepr.           | Rothfels 4298 (DUKE)                 | MH173082          |
| Ceratopteris richardii Brongn.                  | Marchant et al. unpub.               | KM052729          |
| Cheilanthes bolborhiza Mickel & Beitel         | Rothfels 3294 (DUKE)                 | MH173073          |
| Cheilanthes micropteris Sw.                     | Prado 2132 (DUKE)                    | MH173078          |
| Cryptogramma acrostichoides R. Br.              | Rothfels 4195 (DUKE)                 | MH173081          |
| Cystopteris chinesis (Ching.) X. C. Zhang & R. Wei | Wei et al. 2017                     | KY427337          |
| Dryopteris decipiens (Hook.) Kunze              | Wei et al. 2017                      | KY427348          |
| Gastoniella chaerophylla (Desv.) Li Bing Zhang & Liang Zhang | Prado 2178 (SP) | MH173080          |
| Haplopteris elongata (Sw.) E. H. Crane           | Schuettpelz 1559 (BO)                | MH173086          |
| Hemionitis subcordata (D. C. Eaton ex Davenp.) Mickel | Rothfels 3163 (DUKE) | MH173072          |
| Jamesonia brasiliensis Christ                   | Schuettpelz 1444 (SP)                | MH173077          |
| Llavea cordifolia Lag.                          | Schuettpelz 1744 (US)                | MH173088          |
| Myriopteris covillei (Maxon) Á. Löve & D. Löve  | Schuettpelz 443 (DUKE)               | MG953517          |
| Myriopteris lindheimeri (Hook.) J. Sm.          | Schuettpelz 450                      | NC014592          |
| Myriopteris scabra (C. Chr.) Grusz & Windham    | Windham 3495 (DUKE)                  | MH173083          |
| Notholaena standleyi Maxon                      | Schuettpelz 435 (DUKE)               | MH173067          |
| Onychium japonicum (Thunb.) Kanze               | Schuettpelz 1057 (DUKE)              | MH173069          |
| Pellaea truncata Goodd.                         | Schuettpelz 430 (DUKE)               | MH173066          |
| Pentagramma triangularis (Kauf.) Yatsk., Windham & E. Wollenw. | Schuettpelz 1332 (DUKE) | MH173070          |
| Pityrogramma trifoliata (L.) R. M. Tryon        | Rothfels 3658 (DUKE)                 | MH173075          |
| Pteridium aquilinum (L.) Koon.                  | Der et al. unpub.                    | NC014348          |
| Pteris vittata L.                               | Schuettpelz 893 (DUKE)               | MH173068          |
| Scoliosorus ensiformis (Hook.) T. Moore         | Schuettpelz 1782 (US)                | MH173090          |
| Tryonia myriophylla (Sw.), Schuetpp., J. Prado & A. T. Cochran | Schuettpelz 1434 (SP) | MH173076          |
| Vaginularia trichoidea Fée                       | Schuettpelz 1553 (BO)                | MH173085          |
| Vittaria appalachiana Farrar & Mickel           | Stevens OH-p1-s11 (PUR)              | MH173091          |
Figure 1. Distribution of MORFFO elements across the Pteridaceae phylogeny. Topology results from maximum likelihood analysis of plastome data (-lnL = 609991.403586); thickened branches indicate bootstrap/posterior probability support = 100/1.0. Symbols highlight shared insertion sites, with empty squares signifying evident lack of a MORFFO insertion. Short arrows flanking morffo1 indicate short inverted repeats. Novel insertions and losses, as inferred by maximum parsimony, are depicted as arrows or crossed-out circles, respectively.

Figure 2. Detected insertion sites in plastomes of Pteridaceae, relative to Adiantum capillus-veneris. Light gray bar denotes inverted repeat region.

Figure 3. Major inversion events uncovered in fern plastomes. A) Depiction of the two inversion events necessary to explain gene order differences between Angiopteris and Adiantum and their relationship to morffo1. B) Depiction of the inversion events seen in early leptosporangiate and vittarioid ferns, highlighting relationship of morffo1 to the event.

Supplementary Table 1. dN/dS analysis of MORFFO.

Supplementary Figure 1. Maximum likelihood reconstruction of Pteridaceae MORFFO elements. Thickened branches signify strong bootstrap support (lightly thickened branches $\geq$ 70%, heavily thickened branches = 100% support).
