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Low-copy nuclear sequence data confirm complex patterns of farina evolution in notholaenid ferns (Pteridaceae)

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ABSTRACT

Notholaenids are an unusual group of ferns that have adapted to, and diversified within, the deserts of Mexico and the southwestern United States. With approximately 40 species, this group is noted for being desiccation-tolerant and having "farina"—powdery exudates of lipophilic flavonoid aglycones—that occur on both the gametophytic and sporophytic phases of their life cycle. The most recent circumscription of notholaenids based on plastid markers surprisingly suggests that several morphological characters, including the expression of farina, are homoplasious. In a striking case of convergence, *Notholaena standleyi* appears to be distantly related to core *Notholaena*, with several taxa not before associated with *Notholaena* nested between them. Such conflicts can be due to morphological homoplasy resulting from adaptive convergence or, alternatively, the plastid phylogeny itself might be misleading, diverging from the true species tree due to incomplete lineage sorting, hybridization, or other factors. In this study, we present a species phylogeny for notholaenid ferns, using four low-copy nuclear loci and concatenated data from three plastid loci. A total of 61 individuals (49 notholaenids and 12 outgroup taxa) were sampled, including 31 out of 37 recognized notholaenid species. The homeologous/allelic nuclear sequences were retrieved using PacBio sequencing and the P_{URC} bioinformatics pipeline. Each dataset was first analyzed individually using maximum likelihood and Bayesian inference, and the species phylogeny was inferred using *BEAST. Although we observed several incongruences between the nuclear and plastid phylogenies, our principal results are broadly congruent with previous inferences based on plastid data. By mapping the presence of farina and their biochemical constitutions on our consensus phylogenetic tree, we confirmed that the characters are indeed homoplastic and have complex evolutionary histories. Hybridization among recognized species of the notholaenid clade appears to be relatively rare compared to that observed in other well-studied fern genera.

1. Introduction

Cheilanthoid ferns (Pteridaceae: Cheilanthoideae; PPG I, 2016) are a diverse and ecologically unusual clade that has diversified within xeric and semi-xeric habitats across the globe (Gastony and Rollo, 1998; Tryon and Tryon, 1982, 1973). One particularly remarkable subgroup of cheilanthoid ferns is the notholaenids (*sensu* Rothfels et al., 2008), a clade centered in the deserts of Mexico and the southwestern United States that comprises the genus *Notholaena* (*sensu* Yatskievych and Smith, 2003) and a few other species. Unlike most ferns, the leaves of notholaenids tend to be poikilohydric and desiccation-tolerant (Hietz, 2010; Kessler and Siorak, 2007; Proctor and Pence, 2002; Proctor and Tuba, 2002). This allows them to become dormant during the dry season, but quickly rehydrate and restore photosynthetic activity when moisture returns. Thus, notholaenids can survive droughts that kill many other associated plants. Another distinctive feature of notholaenids is the presence of "farina", powdery exudates formed by specialized glands concentrated on the abaxial surface of their leaves (Fig. 1A–C) and along the margins of their gametophytes (Fig. 1D–E).

The powdery farina present in most notholaenids is composed primarily of flavonoids—a ubiquitous and diverse class of plant secondary metabolites exhibiting a broad range of biological activities, including UV-filtering, antioxidation, pigmentation, signaling, and allelopathy (Buer et al., 2010; Peer and Murphy, 2006; Rausher, 2006). Unlike glycosylated flavonoids such as anthocyanins, which are water soluble and usually accumulate in vacuole compartments, the farina of notholaenids is composed of lipophilic flavonoid aglycones, including chalcones, dihydro-chalcones, flavanones, flavonols, dihydro-flavonols, and flavones (Wollenweber and Schneider, 2000). This farina has been hypothesized to have two possible ecophysiological functions in coping with water stress (Hevly, 1963; Wollenweber, 1984): (1) as a "boundary layer", reducing water loss from transpiration, and (2) as a

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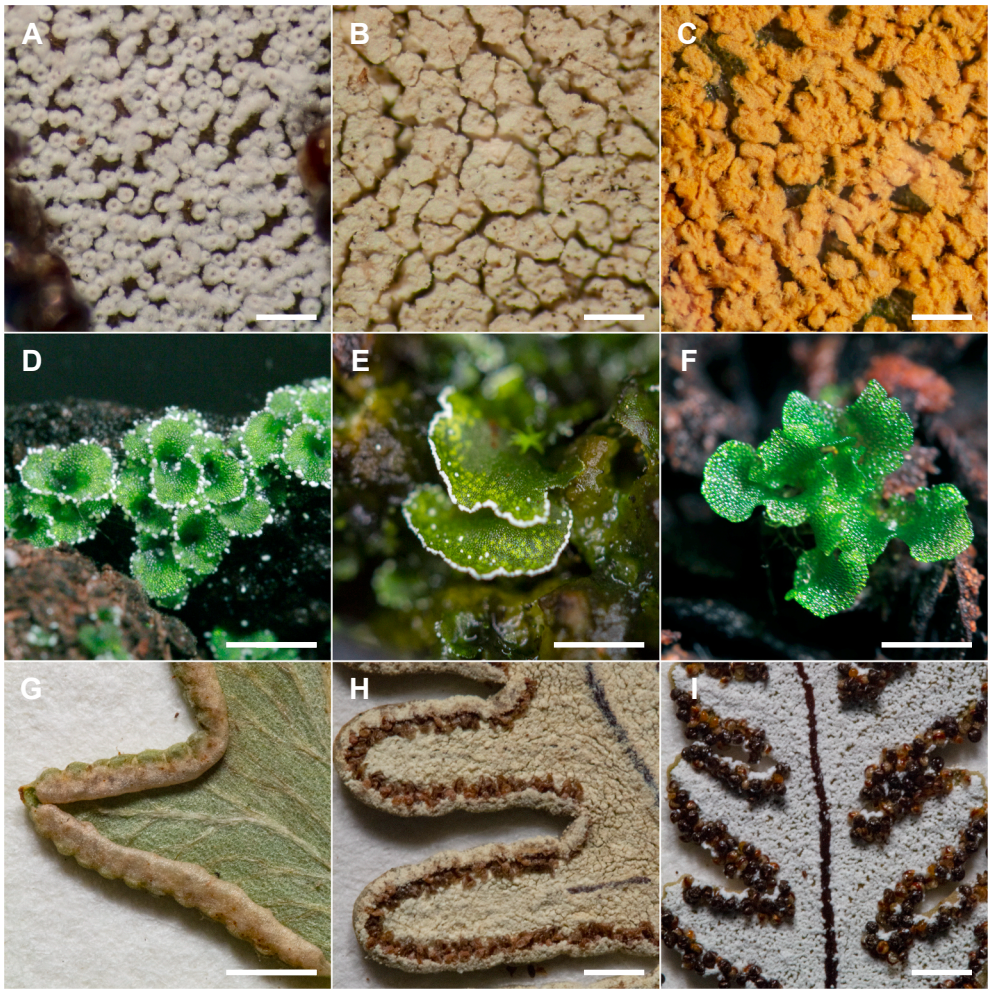

Fig. 1. Key morphological traits of notholaenid ferns. **A–C**: Sporophytic farina on abaxial leaf surface. **A.** *Notholaena candida* with white granulate farina. **B.** *N. standleyi* with yellow areolate farina. **C.** *N. ochracea* with orange rugulate farina. **D–F**: Farinose and non-farinose gametophytes. **D.** Farinose gametophytes of *N. nealleyi*. **E.** Farinose gametophytes of *N. standleyi*. **F.** Non-farinose gametophytes of *N. ochracea*. **G–I:** False indusium. **G**: Well differentiated false indusium in *Cheiloplecton rigidum*. **H:** Poorly differentiated false indusium in *N. standleyi* (leaf margin only slightly recurved, does not fully cover sori). **I.** Poorly differentiated false indusium in *N. candida*. Scale bars: **A–C**: 0.2 mm; **D–I**: 1 mm.

"sunscreen", selectively reflecting or absorbing solar radiation to protect water-stressed leaf tissue from overheating and UV damage.

In the past two decades, molecular data (mostly plastid) have been used extensively to explore intergeneric and interspecific relationships among cheilanthoid ferns (Beck et al., 2012; Eiserhardt et al., 2011; Gastony and Rollo, 1995, 1998; George et al., 2019; Grusz et al., 2009, 2014; Johnson et al., 2012; Kirkpatrick, 2007; Li et al., 2012; Link-Pérez et al., 2011; Prado et al., 2007; Rothfels et al., 2008; Schuettpelz et al., 2007, 2015; Sigel et al., 2011; Yesilyurt et al., 2015; Zhang et al., 2007). These studies identified a number of well-supported lineages within cheilanthoids, including the notholaenid clade (*sensu* Rothfels et al., 2008; Windham et al., 2009). The most recent circumscription of notholaenids is based on data from three plastid markers (*atpA*, *rbcL*, and *trnGR*) and a sampling of 29 of the 37 recognized species (Johnson et al., 2012; Rothfels et al., 2008). This plastid (i.e., strictly maternal) phylogeny is surprisingly incongruent with traditional morphological classifications (Kramer et al., 1990; Mickel and Smith, 2004). For example, "typical" notholaenids (core *Notholaena sensu* Rothfels et al., 2008, plus the morphologically very similar *N. standleyi*) are not monophyletic (Fig. 2)—nested between them are two small clades, most of whose members had not before been associated with *Notholaena* (Mickel and Smith, 2004; Tryon and Tryon, 1982) until molecular data revealed their affinities (Yatskievych and Arbeláez, 2008). There are five species in this portion of the phylogenetic tree: *Notholaena brachypus, N. ochracea* (formerly *Cheilanthes aurantiaca* (Cav.) T. Moore), *N. aureolina* (formerly *Cheilanthes aurea* Baker), *N. jaliscana* (formerly *Cheilanthes palmeri* D. C. Eaton), and *Cheiloplecton rigidum*. All have a prominent false indusium (Fig. 1G), which is poorly differentiated in typical *Notholaena* (Fig. 1H–I; Fig. 2; Rothfels et al., 2008), and none have the farinose gametophytes characteristic of that genus (Fig. 1F; Fig. 2; Johnson et al., 2012). These species are further distinguished from typical *Notholaena* in that two of them (*N. brachypus* and *C. rigidum*) lack leaf farina entirely, while the other three (*N. aureolina*, *N. jaliscana,* and *N. ochracea*) have chalcone-rich farinas rarely observed among notholaenids (Wollenweber, 1977; Wollenweber and Schneider, 2000).

Conflicts observed between the plastid phylogeny and traditional morphology-based classifications may simply be due to morphological homoplasy resulting from adaptive convergence (Rothfels et al., 2008; Sigel et al., 2011). Alternatively, the plastid phylogeny itself might be misleading, diverging from the true species tree due to hybridization, incomplete lineage sorting, or horizontal gene transfer (Maddison, 1997; Nakhleh, 2013). In fact, hybridization and allopolyploidy are very common among cheilanthoid ferns (Beck et al., 2012; Grusz et al., 2009; Schuettpelz et al., 2015) and plastid data alone cannot resolve the origin of hybrids—they can only point to the maternal progenitor (Gastony and Yatskievych, 1992; Kuo et al., 2018; Sears, 1980; Vogel et al., 1998). The best solution is to incorporate sequence data from unlinked, low-copy nuclear markers that are bi-parentally inherited (Nakhleh, 2013; Rothfels et al., 2013; Sang, 2002). Only very recently has a suite of single- and low-copy nuclear regions been developed for phylogenetic use in ferns (Rothfels et al., 2013). The development of next-generation sequencing (NGS) and associated bioinformatics pipelines now allows us to efficiently obtain considerable nuclear sequence data for phylogenetic analyses, and this is especially useful in groups that may contain allopolyploid hybrids (Rothfels et al., 2017). These new techniques provide a much-needed opportunity to assess plastid-derived hypotheses of relationships within the ferns, and increase our capacity to explore aspects of fern evolution

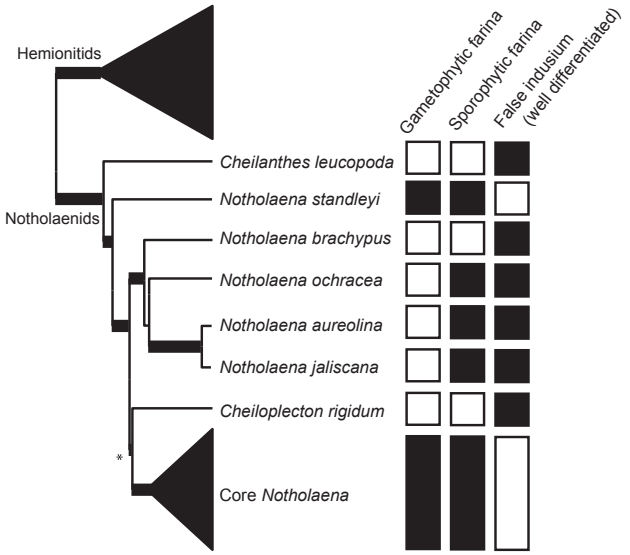

Fig. 2. Summary plastid phylogeny of notholaenid ferns based on Rothfels et al. (2008) and Johnson et al. (2012). Relationships among the early-diverging lineages are emphasized, and the branch lengths are scaled based on our own plastid phylogeny (Fig. 3E) inferred from the same three markers: *atpA*, *rbcL*, and *trnGR*. Branches with ML bootstrap support ≥70% and Bayesian posterior probability ≥0.95 are thickened; the only exception is the branch resolving *Cheiloplecton* as sister group to core notholaenids (marked with *), which has 82% ML bootstrap support, but a posterior probability of only 0.93. The absence of gametophytic farina in *Notholaena ochracea* is newly reported in this study (Fig. 1F). Character states of important taxonomic traits for each taxon or group are indicated. Black blocks = present; white blocks = absent.

previously unavailable to scientific investigation.

Here, we present a species phylogeny for notholaenid ferns, using four low-copy nuclear markers (*ApPEFP_C*, *gapCpSh*, *IBR3*, and *SQD1*) and a concatenated dataset of three plastid markers (*atpA*, *rbcL*, *trnGR*). A total of 61 individuals (12 outgroup taxa and 49 notholaenids) were sampled, including 31 out of the 37 recognized notholaenid species. Our results suggest incomplete lineage sorting among early-diverging notholaenids and reveal that *N. californica* is of hybrid origin between the two major core *Notholaena* subclades. By critically examining extant species for the presence/absence, color, and chemical composition of farina, our results confirm that the occurrence of farina is homoplastic within notholaenids, most likely due to convergence among species that employ different drought avoidance strategies in adapting to xeric environments.

2. Materials and methods

2.1. Taxon sampling

For our study, we sampled 49 individuals belonging to the notholaenid clade (Table 1), including representatives of all available species of *Notholaena* (31 out of 37 recognized species) and many of the known subspecies, variants, and chemotypes. We were especially interested in the early-diverging lineages, including *Cheilanthes leucopoda*, *Cheiloplecton rigidum*, *Notholaena standleyi*, *N. aureolina*, *N. brachypus*, *N. jaliscana*, and *N. ochracea* (Fig. 2); each of these species was represented by at least two samples. Twelve species representing major lineages of the hemionitid sister clade (*sensu* Windham et al., 2009) were selected to form the outgroup (Table 1).

2.2. DNA isolation, amplification, sequencing

Genomic DNA was isolated from silica-dried or herbarium material using either the DNeasy Plant Mini Kit (Qiagen) or the E.Z.N.A. SP Plant DNA Kit (Omega), following modifications described in Schuettpelz and Pryer (2007). Four intron-spanning (0.8–1 kb), single-/low-copy nuclear genes, *ApPEFP_C*, *gapCpSh*, *IBR3*, and *SQD1*, and three plastid markers, partial *rbcL* gene (1309 bp), *atpA* gene (~1.8 kb; with partial *atpF* gene, *atpF-atpA* intergenic spacer, and partial *atpA-trnR* intergenic spacer), and *trnGR* intergenic spacer (~1 kb; with partial *trnG* gene and partial *trnR* gene) were amplified and sequenced. Primers for *ApPEFP_C*, *gapCpSh*, and *SQD1* were newly designed for this study, using transcriptome-based alignments from Rothfels et al. (2013). All primers used in this study are summarized in Table 2.

The four nuclear markers were sequenced using the PacBio Sequel platform (Rhoads and Au, 2015), and the protocols for PCR amplification, sample pooling, and sequencing approximated Rothfels et al. (2017). The target sequences for each sample were amplified separately using barcoded primer sets, whereby the forward primer included a 16-base barcode. To minimize costs, only 48 unique barcodes were used, and they were reused for two taxonomic groups that could be easily distinguished phylogenetically within the PURC bioinformatics pipeline (Rothfels et al., 2017): *C. leucopoda* and all hemionitids as one group, and all remaining notholaenids as another group. The melting, annealing, and elongation temperatures were: *ApPEFP_C*: 94 °C, 68 °C, 72 °C; *gapCpSh* & *IBR3*: 94 °C, 58 °C, 72 °C; and *SQD1*: 94 °C, 60 °C, 72 °C, respectively. The amplification success of each PCR product was confirmed by gel electrophoresis, and DNA concentrations were visually categorized into five ranks for amplicon pooling following Rothfels et al. (2017). The pooled sample was sequenced in a single PacBio SMRT cell on Sequel platform, utilizing PacBio's circular consensus sequencing (CCS) technology (Travers et al., 2010).

The raw CCS reads were processed following the workflow recommended by Rothfels et al. (2017). The raw reads were first cleaned using USEARCH (Edgar, 2010) to remove any sequences that were < 600 bp or had > 5 expected errors. The cleaned reads were then de-multiplexed and clustered into alleles using PURC.PY and PURC_CLUSTER.PY in PURC (Rothfels et al., 2017). Because we initially lacked reliable reference sequences from across our taxon sample, we first ran PURC.PY using exon sequences from *Notholaena montieliae*, *Gaga arizonica*, and *Parahemionitis arifolia*, published by the 1KP project (Matasci et al., 2014), as reference sequences to group and annotate the reads. The resulting "alleles" (sequence clusters inferred by PURC.PY) with the most reads were selected and used as the reference sequences for the second run. These "alleles" contained both the intron and exon sequences, thereby providing greater grouping power and resolution. From the results of the second run, the "alleles" with the most reads from *Cheilanthes leucopoda*, *Cheilanthes pruinata*, *Cheiloplecton rigidum*, *Gaga hirsuta*, *Notholaena aureolina*, *N. montieliae*, *N. standleyi*, *N. sulphurea*, and *Pentagramma rebmanii* were used as the new reference sequences for the third run. The taxonomic (and locus) identity of these sequences was verified by confirming that the resulting ML trees matched earlier broad phylogenetic hypotheses (Johnson et al., 2012; Rothfels et al., 2008). This third (and final) run used eight different PURC regimes (four sets of clustering thresholds: "0.997, 0.995, 0.990, 0.997", "0.995, 0.995, 0.995, 0.995", "0.995, 0.995, 0.990, 0.990", or "0.990, 0.990, 0.990, 0.990"; and two UCHIME abskew settings: 1.9 or 1.1), and the sequences representing clusters of less than four original reads were removed. The resulting sequences from the eight analysis regimes were combined into a single alignment using MUSCLE (Edgar, 2004) implemented in ALIVIEW 1.19 (Larsson, 2014), and used to infer a maximum parsimony (MP) tree using PAUP* v.4.0a (Swofford, 2003). From this tree summarizing the results of all the regimes, a final set of allele sequences for each accession were selected (MP trees deposited in Mendeley Research Data: doi:https://doi.org/10.17632/b3kckcjpfy.1; with the selected sequences indicated by "#").

Sequence data for the three plastid markers (*atpA*, *rbcL*, and *trnGR*) were either obtained from previous studies (George et al., 2019; Johnson et al., 2012; Li et al., 2012; Pryer et al., 2010; Rothfels et al., 2008) or newly sequenced in this study. The markers were amplified following Schuettpelz et al. (2006), Schuettpelz and Pryer (2007), and Nagalingum et al. (2007), respectively. The PCR products were cleaned using ExoSAP-IT PCR cleanup kit (United States Biochemical) following Schuettpelz et al. (2015) and sequenced on a 3730xl DNA Analyzer (Applied Biosystems), using Big Dye Terminator v3.1 Cycle Sequencing reagents. Sequencing

Table 1
Taxon sampling, molecular voucher info, Fern DB #[1], number of alleles amplified for each molecular marker, spore diameter and spore # per sporangium, chromosome counts (n), and inferred ploidy of spores and sporophytes.

Species	Voucher (Herbarium)	Fern DB#	*atpA*	*rbcL*	*trnGR*	*ApPEFP_C*	*gapCp Sh*	*IBR3*	*SQD1*	Spore size[2]	Spore #[3]	n	Spore ploidy	Ploidy of sporophyte
Early diverging notholaenids														
Cheilanthes leucopoda Link	Rothfels et al. 2477-I1 (DUKE)	5345	1	1	1	2	2	2	1	56	32	30[4]	1	Diploid
	Rothfels et al. 2477-I3 (DUKE)	5481	1	1	1	–	2	2	2	–	–	–	–	
Cheiloplecton rigidum (Sw.) Fée														
var. *lanceolatum* C.C. Hall ex Mickel & Beitel	Yatskievych & Gastony 89-284 (IND)	4518	1	1	1	2	2	2	2	–	–	–	–	
	Rothfels et al. 3275 (DUKE)	–	–	–	–	–	–	–	–	90.4	32	c.90[4]	3	Autopolyploid
var. *rigidum*	Rothfels et al. 3203 (DUKE)	6617	1	1	1	2	2	2	2	83.6	32	–	–	Autopolyploid
	Rothfels et al. 3087 (DUKE)	6525	1	1	1	2	3	3	2	–	–	c.90[4]	3	Autopolyploid
Notholaena aureolina Yatsk. & Arbeláez	Yatskievych & Gastony 89-256 (IND)	4514	1	1	1	2	1	2	–	–	–	–	–	
	Rothfels et al. 08-049 (DUKE)	5139	1	1	1	1	1	1	1	–	–	–	–	
Notholaena brachypus (Kunze) J. Sm.	Windham et al. 517 (DUKE)		–	–	–	–	–	–	–	48.2	64	30[5]	1	Diploid
	Rothfels et al. 3115B (DUKE)	6548	1	1	1	1	–	1	–	57.2	64		–	
	Rothfels et al. 08-054 (DUKE)	5142	1	1	1	–	1	–	–	69.6	32	–	3*	Autopolyploid
Notholaena jaliscana Yatsk. & Arbeláez	Rothfels et al. 3118A (DUKE)	6553	1	1	1	–	1	1	2	70.8	32	–	–	Autopolyploid
	P.Rothfels et al. 4 (DUKE)	6808	1	1	1	1	1	1	1	–	–	–	–	
Notholaena ochracea (Hook.) Yatsk. & Arbeláez	Yatskievych & Gastony 89-285 (IND)	4515	1	1	1	2	1	1	2	–	–	–	–	
	Beck 1230 (DUKE)	6932	–	1	1	–	1	–	–	59.2	64	–	–	
Notholaena standleyi Maxon														
chemotype gold [G]	Kao & Windham 17-004 (DUKE)		–	–	–	–	–	–	–	54.9	32	30[4]	1	Diploid
	Schuettpelz et al. 435 (DUKE)	3142	1	1	1	1	1	1	2	57.1	32	–	1*	Diploid
	Rothfels et al. 2546 (DUKE)	5438	1	1	1	2	1	1	2	–	–	–	–	
chemotype yellow [Y]	Kao & Windham 17-016 (DUKE)									51.7	32	30[4]	1	Diploid
	Metzger et al. 129 (DUKE)	3783	1	1	1	1	1	1	–	–	–	–	–	
	Rothfels et al. 2513 (DUKE)	5389	1	1	1	2	1	1	–	–	–	–	–	
chemotype yellow-green [YG]	Windham et al. 94-161 (DUKE)	4502	1	1	1	2	2	2	–	64.1	32	–	2*	Autopolyploid
chemotype pallid [P]	Kao & Windham 17-010 (DUKE)	5419	1	1	1	2	1	2	–	63.4	32	60[4]	2	Autopolyploid
Core *Notholaena*														
Notholaena affinis (Mett.) T. Moore	C.Gonzalez & Labat 2603 (MEXU)	7089	1	1	1	2	1	–	1	43.4	64	–	1*	Diploid
Notholaena aliena Maxon	Windham & Yatskievych 761 (DUKE)	4059	1	1	1	2	2	–	–	75.0	16	–	–	Allopolyploid
Notholaena aschenborniana Klotzsch	Rothfels et al. 3441 (DUKE)	6783	1	1	1	2	2	1	2	61.5	32	–	3*	Autopolyploid
	Rothfels et al. 2992 (DUKE)	6465	1	1	1	2	3	1	2	–	–	–	3*	Autopolyploid
	Windham et al. 496 (DUKE)		–	–	–	–	–	–	–	62.6	32	90[5]	3	Autopolyploid
Notholaena brevistipes Mickel	Rothfels et al. 3048 (DUKE)	6500	1	1	1	2	1	1	–	62.4	32	–	–	
Notholaena bryopoda Maxon	Windham et al. 485 (UT)	4058	1	1	1	–	1	1	1	56.2	64	30[5]	1	Diploid
Notholaena californica D.C. Eaton														
chemotype yellow [Y]	Windham 222 (UT)		–	–	–	–	–	–	–	86.5	32	150[5]	5	Allopolyploid
	Rothfels et al. 2542 (DUKE)	5432	1	1	1	3	4	4	4	–	–	–	–	Allopolyploid
chemotype cream [W]	Rothfels et al. 2563 (DUKE)	5459	1	1	1	3	–	2	3	–	–	–	–	Allopolyploid
Notholaena candida (M. Martens & Galeotti) Hook.	Rothfels et al. 3107 (DUKE)	6539	1	1	1	2	3	2	1	46.9	64	–	1*	Diploid
	Windham et al. 521 (UT)		–	–	–	–	–	–	–	48.8	64	30[5]	1	Diploid
Notholaena copelandii C.C. Hall	Rothfels et al. 2478 (DUKE)	5347	1	1	1	1	2	1	2	48.2	64	30[4]	1	Diploid
Notholaena galeottii Fée	Rothfels et al. 3277 (DUKE)	6670	1	1	1	2	1	1	1	43.1	64	–	1*	Diploid
	Windham et al. 540 (UT)		–	–	–	–	–	–	–	65.8	32	90[5]	3	Autopolyploid
Notholaena grayi Davenp.														
ssp. *grayi*	Yatskievych & Windham 84-192a (ARIZ)	–	–	–	–	–	–	–	–	67.4	16	90[5]	3	Autopolyploid
	Schuettpelz et al. 480 (DUKE)	3187	1	1	1	2	2	2	1	–	–	–	–	
ssp. *sonorensis* Windham	Windham 784 & Yatskievych 784 (ASC)	–	–	–	–	–	–	–	–	46.6	32	30[6]	1	Diploid
	Schuettpelz et al. 490 (DUKE)	3197	1	1	1	1	1	1	–	–	–	–	–	
Notholaena greggii (Mett. ex Kuhn) Maxon	Moore et al. 1602 (UC)	10,669	–	1	1	–	–	2	1	–	–	–	–	
Notholaena jacalensis Pray	Rothfels et al. 3030 (DUKE)	6488	1	1	1	2	1	1	1	50.6	64	–	1*	Diploid
Notholaena lemmonii D.C. Eaton														

(*continued on next page*)

Table 1 (*continued*)

Species	Voucher (Herbarium)	Fern DB#	*atpA*	*rbcL*	*trnGR*	*ApPEFP_C*	*gapCp Sh*	*IBR3*	*SQD1*	Spore size[2]	Spore #[3]	n	Spore ploidy	Ploidy of sporophyte
var. *australis* R.M. Tryon	Campos 932 (MEXU)	7087	1	1	1	1	1	1	1	39.7	64	–	1*	Diploid
var. *lemmonii*	Windham & Yatskievych 229 (DUKE)	–	–	–	–	–	–	–	–	38.6	64	30[5]	1	Diploid
	Schuettpelz et al. 457 (DUKE)	3164	1	1	1	1	2	1	con-	39.5	64	–	1*	Diploid
Notholaena meridionalis Mickel	Rothfels et al. 2646 (INB)	5592	1	1	1	1	1	1	–	45.3	64	–	1*	Diploid
Notholaena montieliae Yatsk. & Arbeláez	Stevens s.n. (MO)	6182	1	1	1	1	1	1	1	49.2	64	30[4]	1	Diploid
Notholaena nealleyi Seaton ex J.M. Coult.	Rothfels et al. 2482 (DUKE)	5354	1	1	1	2	2	2	2	54	64	–	–	
Notholaena neglecta Maxon	Windham 243 (ASC)		–	–	–	–	–	–	–	64.2	32	90[5]	3	Autopolyploid
	Schuettpelz et al. 477 (DUKE)	3184	1	1	1	2	3	2	3	–	–	–	3*	Autopolyploid
Notholaena revoluta A. Rojas	Rothfels et al. 2643 (INB)	5593	1	1	1	1	2	1	–					
Notholaena rigida Davenp.	Windham et al. 491 (UT, DUKE)	4408	1	1	1	–	1	1	1	46.7	64	30[5]	1	Diploid
Notholaena rosei Maxon	Windham et al. 542 (UT)	4409	1	1	1	2	2	1	1	51	64	30[5]	1	Diploid
Notholaena schaffneri (E. Fourn.) Underw. ex Davenp.	Windham et al. 526 (DUKE)		–	–	–	–	–	–	–	44.1	64	30[5]	1	Diploid
	Rothfels et al. 3086 (DUKE)	6524	1	1	1	1	2	2	1	46.4	64	–	1*	Diploid
Notholaena sulphurea (Cav.) J. Sm.														
chemotype cream [W]	Windham et al. 488 (DUKE)	4411	1	1	1	1	1	1	1	47.6	64	30[5]	1	Diploid
chemotype orange [O]	Beck et al. 1114 (DUKE)	6933	1	1	1	2	2	2	1	48.6	64	–	1*	Diploid
chemotype yellow [Y]	Rothfels et al. 3359 (DUKE)	6728	1	1	1	2	1	1	1	46.6	64	–	1*	Diploid
Notholaena trichomanoides (L.) Desv.	Ranker & Trapp 860 (UT)	4054	1	1	1	–	2	2	–	62.8	64	60[5]	2	Autopolyploid
Number of alleles			46	49	49	68	72	66	52					
Number of taxa			46	49	49	40	45	45	33					
Outgroups														
Adiantopsis seemannii (Hook.) Maxon	Lobo et al. 14 (DUKE)	5547	1	1	1	1	1	1	–					
Cheilanthes acrostica (Balb.) Tod.	Rothfels 2735 (DUKE)	6165	1	1	1	2	–	2	2					
Cheilanthes distans (R.Br.) Mett.	Schuettpelz 320 (DUKE)	2965	1	1	1	3	2	2	3					
Cheilanthes pruinata Kaulf.	H.Forbes s.n. (UCBG 91.1232)	9564	1	1	1	2	1	1	2					
Cheilanthes tenuifolia (Burm. f.) Sw.	Kuo 394 (TAIF)	6132	–	1	1	–	2	2	2					
Gaga hirsuta (Link) F.W. Li & Windham	Rothfels et al. 08-023 (DUKE)	5133	1	1	1	2	2	3	1					
Hemionitis palmata L.	Rothfels 08-184 (DUKE)	5137	1	1	1	1	1	1	1					
Mildella fallax G.L. Nesom	Yatskievych & Gastony 89-207 (IND)	5051	1	1	1	3	2	3	2					
Negripteris scioana (Chiov.) Pic. Serm.	Rothfels et al. 4287 (DUKE)	9274	1	1	1	2	–	2	2					
Pellaea viridis (Forssk.) Prantl	T. Janssen 2701 (P)	3555	1	1	1	3	2	2	2					
Pentagramma maxonii (Weath.) Schuettp. & Windham	Rothfels 2570 (DUKE)	5468	1	1	1	1	2	2	1					
Pentagramma rebmanii (Winner & M.G. Simpson) Schuettp. & Windham	Rothfels et al. 2553 (DUKE)	5450	1	1	1	2	1	2	2					
Number of alleles			11	12	12	22	16	23	20					
Number of taxa			11	12	12	11	10	12	11					

[1] Fern Lab Database: http://fernlab.biology.duke.edu/.
[2] Average spore diameter (μm).
[3] Spore number per sporangium.
[4] New count.
[5] Voucher from Windham & Yatskievych (2003).
[6] Voucher from Windham (1993b).
* Ploidy inferred by comparing average spore diameters to vouchers of published chromosome counts of same or closely related taxa.

Table 2
Primers used in this study.

Locus	Primer	Direction	Utility	Sequence (5′ to 3′)	Reference
atpA	ESATPF412F	Forward	Amplification, Sequencing	GARCARGTTCGACAGCAAGT	Schuettpelz et al. (2006)
atpA	ESatpA535F	Forward	Sequencing	ACAGCAGTAGCTACAGATAC	Schuettpelz et al. (2006)
atpA	ESatpA557R	Reverse	Sequencing	ATTGTATCTGTAGCTACTGC	Schuettpelz et al. (2006)
atpA	ESatpA856F	Forward	Sequencing	CGAGAAGCATATCCGGGAGATG	Schuettpelz et al. (2006)
atpA	ESatpA877R	Reverse	Sequencing	CATCTCCCGGATATGCTTCTCG	Schuettpelz et al. (2006)
atpA	ESTRNR46F	Reverse	Amplification, Sequencing	GTATAGGTTCRARTCCTATTGGACG	Schuettpelz et al. (2006)
rbcL	ESrbcL1F	Forward	Amplification, Sequencing	ATGTCACCACAAACGGAGACTAAAGC	Schuettpelz and Pryer (2007)
rbcL	ESrbcL645F	Forward	Sequencing	AGAYCGTTTCYTATTYGTAGCAGAAGC	Schuettpelz and Pryer (2007)
rbcL	ESrbcL663R	Reverse	Sequencing	TACRAATARGAAACGRTCTCTCCAACG	Schuettpelz and Pryer (2007)
rbcL	ESrbcL1361R	Reverse	Amplification, Sequencing	TCAGGACTCCACTTACTAGCTTCACG	Schuettpelz and Pryer (2007)
trnGR	TRNG1F	Forward	Amplification, Sequencing	GCGGGTATAGTTTAGTGGTAA	Nagalingum et al. (2007)
trnGR	TRNG43F1	Forward	Sequencing	TGATGCGGGTTCGATTCCCG	Nagalingum et al. (2007)
trnGR	TRNG63R	Reverse	Sequencing	GCGGGAATCGAACCCGCATCA	Nagalingum et al. (2007)
trnGR	TRNGJBF	Forward	Sequencing	AGGAGCCGAATGGGCCGAAA	Sigel et al. (2011)
trnGR	TRNR22R	Reverse	Amplification, Sequencing	CTATCCATTAGACGATGGACG	Nagalingum et al. (2007)
ApPEFP_C	APPNOTHF	Forward	Amplification, Sequencing	GCAGGRCCYGGCCTTGCTGAGGA	This study
ApPEFP_C	APPNOTHR	Reverse	Amplification, Sequencing	GCAACATGAGCRGCTGGTTCACGRGG	This study
gapCpSh	NOTHGAPF	Forward	Amplification, Sequencing	GAGGRTKCTTAYAAACCAGAGATGC	This study
gapCpSh	NOTHGAPR	Reverse	Amplification, Sequencing	CTTCAGTATAACCCAAAATTCC	This study
IBR3	4321F5	Forward	Amplification, Sequencing	ATGACYGAACCAGATGTKGCDTCVTCRGATGC	Rothfels et al. (2013)
IBR3	4321R6	Reverse	Amplification, Sequencing	TGRTGGAGYCTKCCTGGGCCTA	Rothfels et al. (2013)
SQD1	SQDNOTHF	Forward	Amplification, Sequencing	CATCCTCTTACAGTYTAYGGHAAAGG	This study
SQD1	SQDNOTHR	Reverse	Amplification, Sequencing	CYCTGATRTCAAGGTATCCTCTTGT	This study

reads from each species and locus were assembled and edited using Sequencher 5.0.1 (Gene Codes Corporation, 2011). All newly obtained DNA sequences are deposited in GenBank (Appendix A).

2.3. Sequence alignment and phylogenetic analyses

For each of the seven DNA marker datasets, sequences were first aligned using MUSCLE (Edgar, 2004) in ALIVIEW 1.19 (Larsson, 2014) and then the alignment was manually refined; ambiguously aligned regions were excluded from further analysis. Two *gapCpSh* alleles were removed from the analyses at this step: a pseudogene found in *Notholaena ochracea*; and an exon-only sequence found in *N. meridionalis*. Both sequences have several indels that cause frameshifts, high non-synonymous substitution rates, and a premature stop codon on exon 11. Both species were additionally represented by the expected "normal" *gapCpSh* alleles.

Prior to model selection, each dataset was partitioned by coding/non-coding and codon position in Aliview 1.19 (Larsson, 2014), and a neighbor-joining tree was inferred using PAUP* 4.0a (Swofford, 2003). The optimal partitioning strategy, and the best model for each data subset, was then selected using the Bayesian information criterion in the "automated partitioning" or "automated model selection" tool implemented in PAUP* 4.0a (Swofford, 2003). The models evaluated include six exchangeability components (JC, F81, K80, HKY, SYM, GTR) and four among-site rate variation models (equal, +I, +G, +I+G). The best-fitting models for each dataset are provided in Table 3.

Maximum likelihood (ML) analyses were carried out using Garli 2.0 (Zwickl, 2006), with genthreshfortopoterm set to 1,000,000. The tree with the best likelihood among four replicates, each from a different random addition sequence starting tree, was selected as the best ML tree; ML bootstrap support (MLBS) was calculated from 1000 replicates, each from a single random addition sequence starting tree, with genthreshfortopoterm set to 20,000. The Bayesian analyses were carried out in MrBayes v3.2.3 (Ronquist et al., 2012) with two independent MCMC runs, each with four chains, run for 1,000,000 generations. The mixing and convergence of parameters was inspected using Tracer v1.6 (Rambaut et al., 2018). Trees were sampled every 1000 generations, and the first 25% of trees were discarded as burn-in. The resulting phylogenetic trees were rooted on the bipartition dividing the twelve hemionitid ferns from the notholaenids.

Tree topologies generated from each individual plastid region were visually inspected and compared for conflicts with bootstrap values ≥70% or Bayesian posterior probabilities ≥95% (Hillis and Bull, 1993; Mason-Gamer and Kellogg, 1996). Because no topological incongruence was observed among the plastid markers in our ML and Bayesian analyses, these regions were combined into one dataset, henceforth referred to as the "plastid dataset", and subjected to partition/model selection in PAUP* 4.0 (Swofford, 2003) with substitution schemes set as GTR, and each codon position and the non-coding data, for each locus, provided as a potential data subset. ML analyses in Garli 2.0 (Zwickl, 2006) and Bayesian analyses in MrBayes v3.2.3 (Ronquist et al., 2012) were conducted as described above.

Although we suspect that allopolyploidy is relatively rare within notholaenid ferns, one surprising result from our preliminary nuclear phylogenetic analyses was that *N. californica* (both the yellow and cream chemotypes) show clear evidence of being allopolyploids (see Results 3.1 and 3.2). Following a comparison with the plastid phylogenies, the *N. californica* nuclear alleles that grouped with *N. neglecta* are annotated as "P" (paternal) and the other group of alleles is annotated as "M" (maternal), and are treated as two different individuals when inferring species phylogenies. Species phylogenies were inferred using *BEAST (Heled and Drummond, 2010) in BEAST 2 (Bouckaert et al., 2013), either using the four nuclear datasets (*ApPEFP_C*, *gapCpSh*, *IBR3*, and *SQD1*), or these four datasets plus the plastid dataset. As above, the best partitioning scheme and associated models were selected in PAUP* 4.0 (Swofford, 2003), with each codon position and the non-coding data, for each locus, provided as a potential data subset (see Table 3). The datasets were converted to a BEAST 2 XML input file using BEAUti 2 (Bouckaert et al., 2013). The trees from different partitions within a dataset were set as linked, while different datasets were set as unlinked. Two different species settings were used: (1) each sample was treated as its own species, or (2) the species designations followed the established circumscriptions (e.g., all accessions of *N. standleyi* were considered to be of the same species). The chain length was set as 300 million generations, and the parameters and trees were logged every 0.15 million generations (i.e., sampled 2000 times). Two independent MCMC runs were carried out using BEAST 2 (Bouckaert et al., 2013) on XSEDE (Towns et al., 2014), available from the CIPRES Science Gateway (Miller et al., 2010). The mixing and convergence of parameters was inspected using Tracer v1.6 (Rambaut et al., 2018). The trees from the two independent runs were combined using LogCombiner v.2.4.6 (Bouckaert et al., 2013), and the first 25% of trees were discarded as burn-in. The maximum clade credibility tree was summarized using TreeAnnotator v.2.4.6 (Bouckaert et al., 2013) and visualized using FigTree v1.4.3. All datasets and phylogenetic trees are deposited in the Mendeley Research Data (doi:https://doi.org/10.17632/b3kckcjpfy.1).

Table 3
Partitions and evolutionary models used in phylogenetic analyses.

Datasets	Partitions	Codon positions	Model
ApPEFP_C	1	1st, 2nd codon positions	K80 + I + G
	2	3rd codon positions, introns	HKY + G
gapCpSh	1	1st codon positions	HKY + I + G
	2	2nd codon positions	JC + I
	3	3rd codon positions	K80 + G
	4	Intron	HKY + G
IBR3	1	1st, 2nd codon positions	K80 + G
	2	3rd codon positions, introns	HKY + G
SQD1	1	All	GTR + G
Plastid	1	1st codon positions of *atpA* and *rbcL*	GTR + I + G
	2	2nd codon positions of *atpA* and *rbcL*	GTR + I + G
	3	3rd codon positions of *atpA* and *rbcL*; intergenic spacers flanking *atpA*	GTR + G
	4	*trnGR*	GTR + G
4 Nuclear	1	1st, 2nd codon positions of *ApPEFP_C*, *gapCpSh*, and *IBR3*	GTR + I + G
	2	*SQD1* and 3rd codon positions and introns of *ApPEFP_C*, *gapCpSh*, and *IBR3*	GTR + G
Plastid + 4 Nuclear	1	1st codon positions of *ApPEFP_C*, *atpA*, *gapCpSh*, *IBR3*, and *rbcL*; 2nd codon positions of *IBR3*	GTR + I + G
	2	1st codon positions of *ApPEFP_C*, *atpA*, *gapCpSh*, and *rbcL*	GTR + I + G
	3	3rd codon positions of *atpA* and *rbcL*; intergenic spacers flanking *atpA*	GTR + G
	4	SQD1 and 3rd codon positions and introns of *ApPEFP_C*, *gapCpSh*, and *IBR3*	GTR + G

2.4. Assessing ploidy

Whenever possible, the ploidy of individual samples included in the phylogenetic analyses was determined based on chromosome counts. Four of our DNA samples were obtained from voucher specimens of counts previously published by Windham and Yatskievych (2003), and five other counts were taken from specimens that were part of this project. The new chromosome counts were obtained from meiotic material gathered from field collections, and protocols for fixation, preservation, and obtaining chromosome counts followed Windham and Yatskievych (2003).

The remaining individuals included in the phylogenetic analyses lacked appropriate material for chromosome counting. In samples with mature spores, the well-established correlations between spore size and ploidy (Beck et al., 2010; Grusz et al., 2009; Schuettpelz et al., 2015; Sigel et al., 2011) and between the number of spores per sporangium and reproductive mode (Gastony and Windham, 1989; Windham and Yatskievych, 2003) were used to infer ploidy and reproductive mode, respectively. The protocol for removing single sporangia, counting spore number, and measuring spore size follows Schuettpelz et al. (2015) with minor modifications. From each specimen, 1–3 mature, unopened sporangia were removed using dissecting needles under a Leica MZ 7.5 stereomicroscope. Individual sporangia were transferred to a drop of glycerol on a glass microscope slide and carefully opened with the needles to release the spores. After removing sporangial debris, the number of spores per sporangium was determined, and spore diameters were measured by using a calibrated ocular micrometer mounted on a Meiji MT5310L phase-contrast compound microscope. The mean and standard deviation of measurements from 25 spores were calculated for each sampled individual. The ploidy of these specimens was then inferred by comparison to similarly derived measurements from vouchers of published chromosome counts representing the same or closely related taxa. In a few cases (i.e., DNA vouchers that lacked chromosome counts and mature spores), maximum allele number observed among the nuclear loci was used to hypothesize whether a sample was diploid or polyploid. The origins of known or hypothesized polyploids were designated as intraspecific (autopolyploid) or interspecific (allopolyploid) depending on the phylogenetic proximity of their constituent nuclear alleles.

2.5. Farina evolution

We assessed several traits associated with farina including presence/absence of farina on gametophytes, presence/absence of farina on sporophytes, as well as the color and chemical composition of farina. Although presence/absence of gametophytic farina in notholaenid ferns had been examined previously (Johnson et al., 2012), we repeated the experiments following their protocol using additional samples. A total of 24 species was successfully germinated from spores, including two species not reported in Johnson et al. (2012), *N. ochracea* and *N. rosei*, as well as all non-core notholaenid lineages, except for *N. aureolina*. Farina presence/absence and color on the sporophyte reflect the published observations of Wollenweber (1984) and Mickel and Smith (2004), supplemented by our own observations from herbarium specimens. The chemical composition of farina was recorded from a series of studies summarized in Wollenweber and Schneider (2000). Here we simplify those observations by focusing on the major subclasses of flavonoids observed (chalcone, dihydrochalcone, flavanone, flavone, or flavanol), as well as triterpenes, and two unique substitution patterns, O-acetyl and O-butyl substitutions, reported only in notholaenids. The ancestral states of these traits were reconstructed on the species tree inferred from our nuclear and plastid dataset, using both likelihood (re-rooting method, Yang et al., 1995) and MCMC (stochastic mapping method, Huelsenbeck et al., 2003) approaches. Both analyses were carried out in the phytools v.0.6–60 package (Revell, 2012) in R (R Core Team, 2018). The character states we used in the analyses were summarized in Appendix B. When different variants within a species express different farina compositions, the prior distribution on a state for the tip is set as the proportion of the number of the variants that have the state. An equal-rates model was assumed and applied to all traits. When a species value is unknown (i.e., no farina or no chemical analyses data), the tips were dropped from the analyses.

3. Results

3.1. Phylogenetic analyses

A total of 61 individuals was included in our phylogenetic analyses. Complete voucher information and the number of alleles that were retrieved from each of the seven DNA markers are listed in Table 1. GenBank accession numbers for all sequences used in this study are listed in Appendix A, including 179 plastid sequences (107 new) and 339 new nuclear sequences. Among the 31 notholaenid species sampled, 20 have sequences from all seven DNA markers, seven species have data for six markers, two (*N. aliena* and *N. trichomanes*) have five markers, *N. greggii* has four markers and *N. leonina* yielded only three.

The unrooted phylogenies resolved from the Bayesian analyses for each of the five datasets (*ApPEFP_C*, *gapCpSh*, *IBR3*, *SQD1*, and plastid) were rooted using the hemionitid outgroup and are presented in Fig. 3A–E. The nuclear datasets yielded many more parsimony-informative characters in our final alignment (*ApPEFP_C*: 662 sites, 60%; *gapCpSh*: 465 sites, 52%; *IBR3*: 320 sites, 50%; *SQD1*: 614 sites, 49%) than did the plastid datasets

(*atpA*: 258 sites, 15%; *rbcL*: 175 sites, 13%; *trnGR*: 354 sites, 32%). The phylogeny resolved from the *BEAST analyses (rooted with the clock model) using only the nuclear datasets or using nuclear plus plastid datasets are presented in Fig. 3F and G (where each sample was set as one species), respectively; the final species tree (where the accessions were lumped together according to their morphologically-derived species designations) is presented in Fig. 4.

3.1.1. Plastid marker analyses

Our plastid phylogeny (Fig. 3E) is highly congruent with previous studies, although several branches received lower support in our analysis. Specifically, two previously strongly supported branches (in earlier studies; Rothfels et al., 2008, Johnson et al., 2012) were no longer supported in our analysis (PP < 0.95 or MLBS < 70%; indicated with arrows in Fig. 3E). These are the branches that place *Cheiloplecton rigidum* as sister to core *Notholaena* (PP = 0.93, MLBS = 82%), and *N. greggii* as sister to *N. californica* + *N. meridionalis* + *N. lemmonii* (PP = 0.64, MLBS = 53%), both of which are also not supported by the nuclear-only analyses (Fig. 3A–D, F). As in previous studies, core *Notholaena* (*sensu* Rothfels et al., 2008) is robustly supported as monophyletic (Fig. 3E; PP = 1.00, MLBS = 100%), and all species sampled from this clade produce farina on both sporophyte and gametophyte.

3.1.2. Nuclear marker analyses

Our nuclear phylogenies (Fig. 3A–D, F) are largely congruent with the plastid phylogeny (Fig. 3E), especially when comparing relationships among closely related taxa. Nevertheless, several conflicts were observed across the early-diverging lineages and among the major subgroups of core *Notholaena* (Fig. 4). Although the plastid phylogeny (Fig. 3E) resolves one of the non-farinose species, *Cheilanthes leucopoda*, as sister to the remaining notholaenid ferns (PP = 1.0, MLBS = 99%), three out of the four nuclear markers (*ApPEFP_C*, *gapCpSh*, and *SQD1*; Fig. 3A–B, D), inferred *N. standleyi* to be the earliest-diverging notholaenid (the support for this relationship in the *SQD1* analyses is not significant—PP = 0.75, MLBS = 62%—but the other two loci had strong support). On the other hand, the *IBR3* dataset suggests that all six non-core notholaenids

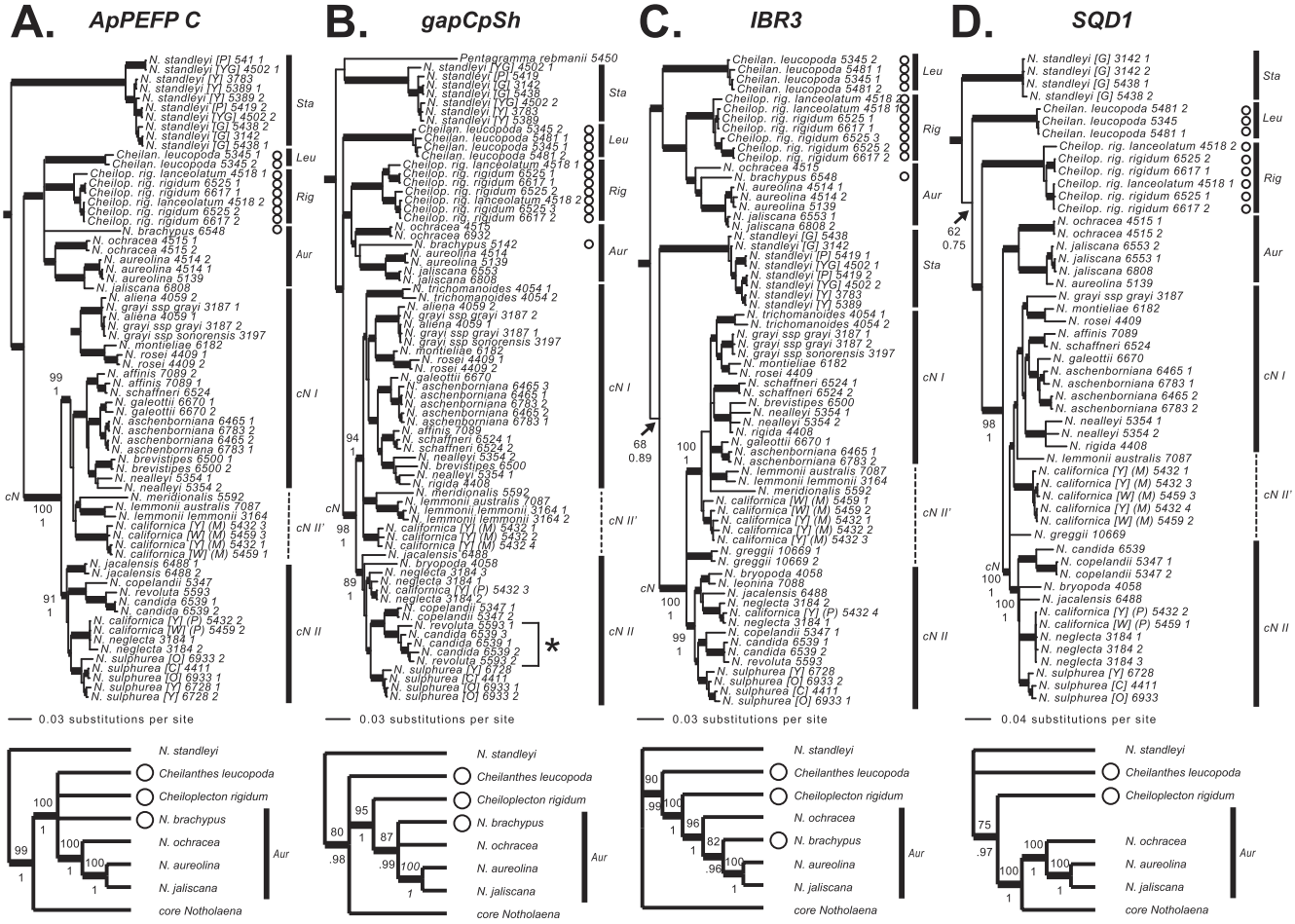

Fig. 3. Results of phylogenetic analyses inferred using Bayesian analysis. **A–D**: Low-copy nuclear gene phylograms; summary topologies are indicated at base of each gene tree (all branches with PP < 0.95 are collapsed). **A.** *ApPEFP_C*. **B.** *gapCpSh*. **C.** *IBR3*. **D.** *SQD1*. **E**: Phylogram of concatenated plastid data (*rbcL*, *atpA*, and *trnGR*). **F–G**: Maximum clade credibility trees inferred by *BEAST analyses carried out in BEAST 2 (Bouckaert et al., 2013). **F.** Species tree from the combined four-gene nuclear dataset (*ApPEFP_C*, *gapCpSh*, *IBR3*, and *SQD1*). **G.** Species tree from combined plastid and nuclear datasets (where each sample was treated as its own species). Branches with ≥0.95 posterior probability and ≥70% maximum likelihood bootstrap support (MLBS) are thickened. PP and MLBS support values for several critical branches are indicated close to those branches (MLBS above, PP below). Trees with support values for all branches are deposited in Mendeley Research Data (doi:https://doi.org/10.17632/b3kckcjpfy.1). The three non-farinose taxa—*Cheilanthes leucopoda*, *Cheiloplecton rigidum*, and *Notholaena brachypus*—are marked with open circles. The chemotypes for each sample of *N. standleyi* and *N. sulphurea* are indicated within square brackets: [W] white or cream, [G] gold, [O] orange, [P] pallid, [Y] yellow, [YG] yellow-green. The maternal alleles of *N. californica* are annotated with (M), the paternal alleles are annotated with (P). A potential gene duplication event in *gapCpSh* (Fig. 3B) at *N. candida/N. revoluta* is marked with an asterisk. Abbreviations along vertical lines: ***Sta***: *N. standleyi*; ***Leu***: *C. leucopoda*; ***Rig***: *C. rigidum*; ***Aur***: *N. aureolina* clade; ***cN***: core *Notholaena*; ***cN I***: core *Notholaena* I; ***cN II***: core *Notholaena* II (*sensu* Rothfels 2008); ***cN II'***: comprised of *N. lemmonii*, *N. meridionalis*, *N. californica* (maternal), and *N. greggii*, is a monophyletic group within cN II in the plastid phylogeny (Fig. 3E), but polyphyletic in all nuclear topologies (Fig. 3A–D, F).

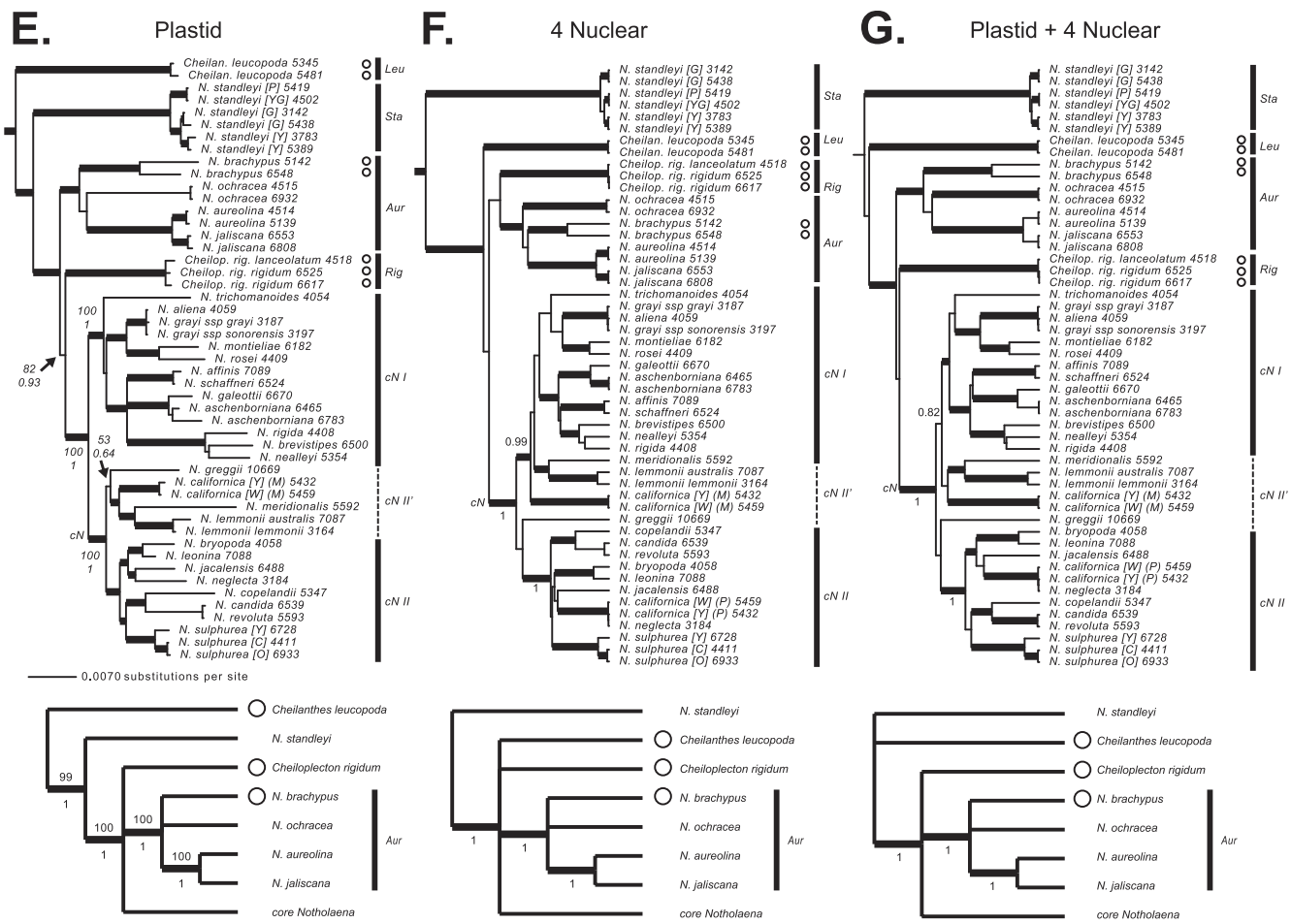

Fig. 3. (*continued*)

(*Cheilanthes leucopoda*, *Cheiloplecton rigidum*, *N. brachypus*, *N. ochracea*, *N. aureolina*, and *N. jaliscana*) form a clade sister to the rest of the notholaenids, including *N. standleyi*; however, the support for this relationship is weak (Fig. 3C, PP = 0.89, MLBS = 68%).

Notholaena brachypus, *N. ochracea*, *N. aureolina*, and *N. jaliscana* (the *N. aureolina* clade, *sensu* Rothfels, 2008), are inferred here to be a monophyletic group by all markers (Fig. 3A–F). Among them, two of the farinose species, *N. aureolina*, and *N. jaliscana*, are sister in all datasets. The relationships between *N. aureolina* + *N. jaliscana* and the other two taxa is, however, unresolved. The *ApPEFP_C* dataset (Fig. 3A) suggests that this species pair is sister to another farinose species, *N. ochracea*, whereas the *IBR3* dataset (Fig. 3C) infers that they are sister to the non-farinose species *N. brachypus*. *Cheiloplecton rigidum*, another non-farinose species, is supported here as sister to the *N. aureolina* clade by three of the nuclear datasets (*ApPEFP_C*, *gapCpSh*, and *IBR3*; Fig. 3A–C), while *SQD1* (Fig. 3D) suggests *C. rigidum* is sister to the clade formed by the *N. aureolina* clade and core *Notholeana*.

The nuclear data agree with the plastid results in recovering a monophyletic core *Notholaena* with strong support (Fig. 3A–F, PP = 1.00, MLBS > 98%). One major conflict between these datasets, however, is that the plastid divides core *Notholaena* into two well-supported (both PP = 1.00, MLBS = 100%) major groups that Rothfels et al. (2008) named core *Notholaena* I and II. All four nuclear datasets, however, strongly support (Fig. 3A–D, F; PP ≥ 0.99, MLBS ≥ 90%) the placement of three core *Notholaena* II taxa (*N. lemmonii*, *N. meridionalis*, and the maternal progenitor of *N. californica*) within core *Notholaena* I. In the plastid phylogeny (Fig. 3E), these three taxa form a well-supported monophyletic group (PP = 0.99, MLBS = 89%) sister to *N. greggii*; we label the four species as cN II' in Fig. 3. Nuclear data support *N. lemmonii* as sister to *N. meridionalis* (Fig. 3F), with these two species sister to core *Notholaena* I or *N. californica* (Fig. 3A–D, F). The placement of *N. greggii* is either unresolved (Fig. 3C–D) or is sister to other core *Notholaena* II species (Fig. 3F). Interestingly, *N. lemmonii* and *N. meridionalis* were morphologically anomalous in core *Notholaena* II in that they have linear-lanceolate leaves, rather than the ovate-pentagonal leaves characteristic of other core *Notholaena* II. All nuclear datasets point to *N. californica* being a hybrid; its paternal parent is *N. neglecta* in core *Notholaena* II. If the nuclear phylogeny is true, the newly-circumscribed core *Notholaena* I and II could be cleanly distinguished by leaf shape (except of course for the hybrid *N. californica*). However, the presence/absence of scales or non-glandular hairs would be less useful for diagnosing core *Notholaena* I, because there are too many exceptions (i.e., *N. californica*, *N. lemmonii*, *N. meridionalis*, *N. rigida*, and *N. rosei*).

3.1.3. Combined plastid and nuclear phylogeny

A species tree for notholaenid ferns was inferred using five presumably unlinked datasets, *ApPEFP_C*, *gapCpSh*, *IBR3*, *SQD1*, and "plastid" (*atpA* + *rbcL* + *trnGR*). The resultant tree (Fig. 4) suggests that *Notholeana standleyi* and/or *Cheilanthes leucopoda* is the earliest-diverging lineage of notholaenid ferns. The monotypic *Cheiloplecton* and/or the *N. aureolina* clade is sister to the monophyletic (PP = 1.00) core *Notholaena*. Most of core *Notholaena* can be sorted into two monophyletic subgroups: the group (PP = 0.98) that includes the type species of the genus, *N. trichomanoides*, shares a linear-lanceolate leaf shape; the other group (PP = 1.0) includes the most widespread species, *N. sulphurea,* and shares an ovate-pentagonal leaf shape. Relationships remain unresolved between these two major groups and the four cN II' species: *N. lemmonii*, *N. meridionalis*, *N. californica* (maternal progenitor), and *N. greggii*.

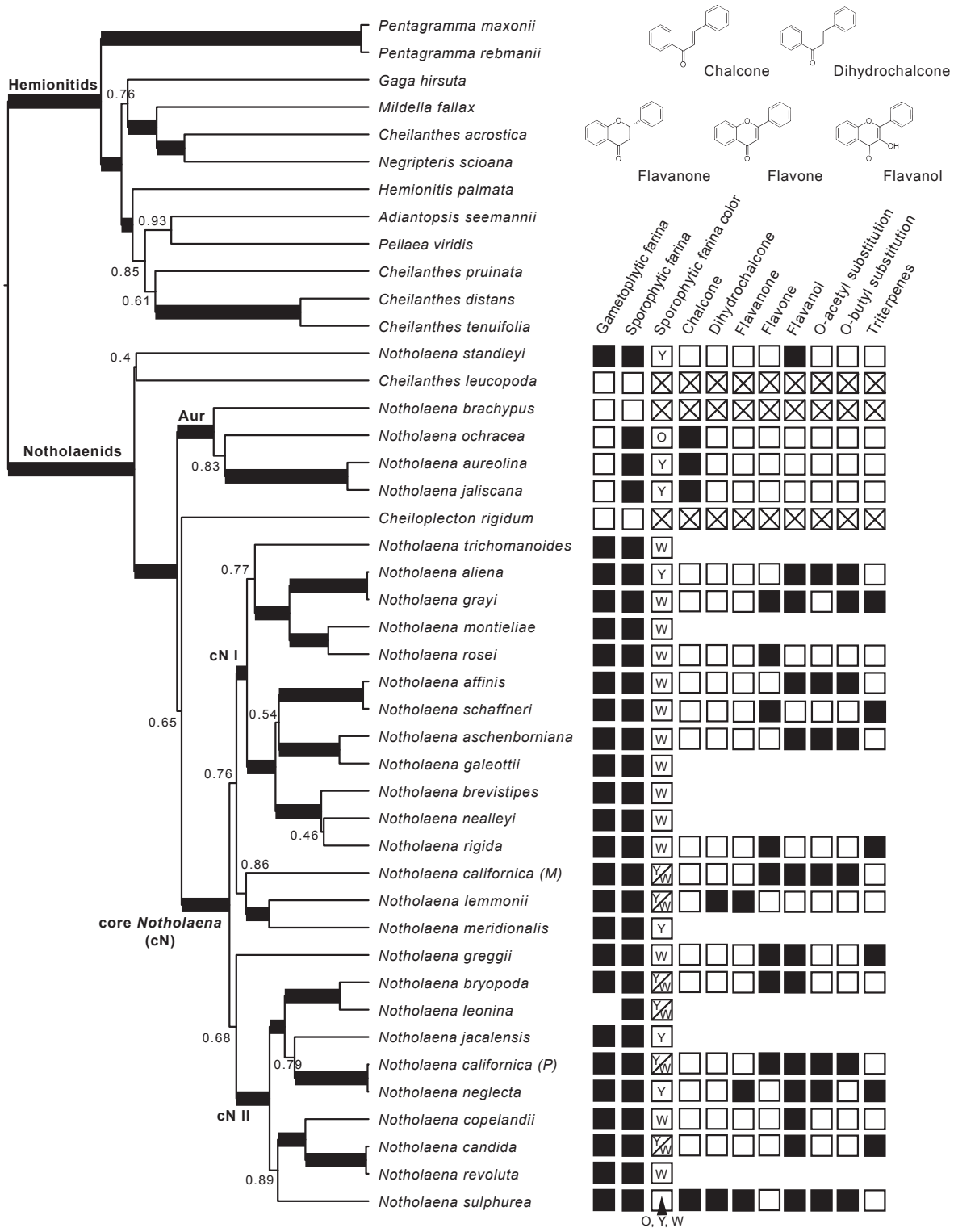

Fig. 4. Farina evolution in notholaenid ferns. The full-data species tree from Fig. 3G (where the species designations followed the established circumscriptions) is presented at the left. Branches with ≥0.95 posterior probability are thickened; all posterior probabilities < 0.95 are shown next to the branches. The maternal allele of *N. californica* is annotated with (M), the paternal allele is annotated with (P). ***Aur***: *N. aureolina* clade; ***cN***: core *Notholaena*; ***cN I***: core *Notholaena* I; ***cN II*** (without *cN II'*): core *Notholaena* II. The presence/absence of farina on gametophytes and sporophytes, color of foliar farina (O: orange, W: white or cream, Y: yellow), and the biochemical details are summarized to the right. Black blocks: present. Empty blocks: absent. No block: no data. Crossed block: not applicable (non-farinose species). Farina composition is based on earlier reports (Appendino et al., 1992; Arriaga-Giner et al., 1992b, 1992a, 1987; Scheele et al., 1987; Seigler and Wollenweber, 1983; Wollenweber, 1976; Wollenweber et al., 2001, 1993, 1978, Wollenweber, 1989, 1984, 1978, 1977; Wollenweber and Dietz, 1981; Wollenweber and Schneider, 2000; Wollenweber and Yatskievych, 1982).

3.2. Assessing ploidy

Chromosome counts are newly reported here for *C. leucopoda*, *N. copelandii, N. monteiliae,* and the gold, yellow, and pallid chemotypes of *N. standleyi*; voucher information for all specimens is provided in Table 1. All new counts were diploid ($n = 30$; Fig. 5A) except for the "pallid" chemotype of *N. standleyi*, which proved to be tetraploid ($n = 60$; Fig. 5B). Nine of the 49 notholaenid samples in the molecular analyses were derived from plants of known chromosome number, and the ploidy of 17 others could be inferred by comparing their average spore diameters to those of spores taken from vouchers of published chromosome counts representing the same or closely related taxa. Five additional specimens without mature spores were inferred to be polyploid based on the number of alleles (three or more) observed in the nuclear DNA dataset. Eighteen of the 49 notholaenid samples in the molecular analyses had insufficient data to support assignment to a particular ploidy.

Although the number of inferred nuclear alleles per locus was generally congruent with ploidy as determined from chromosome counts and inferred from correlated spore measurements, this was not an efficient approach to assessing ploidy. The number of nuclear alleles provided only a minimum estimate of ploidy (i.e., only polyploids with three or more alleles at a locus will be recognizable as such). Even the latter inference proved to be questionable when based on a single locus, as illustrated by an apparent lack of congruence between ploidy and nuclear allele number in *N. candida* (Table 1). This species was represented in the molecular dataset by a specimen exhibiting three *gapCpSh* alleles. However, spore diameter of this accession was 1.9 µm smaller than that of a known diploid specimen of *N. candida* (Table 1) and thus this sample was inferred to be diploid. We suspect this incongruence may be the result of a gene duplication event at the *gapCpSh* locus in the *N. candida* + *N. revoluta* lineage (Fig. 3B; marked with an asterisk).

Of the 31 molecular samples for which ploidy was known or inferred, 18 were classified as diploids and 13 as polyploids (Table 1). The latter were further subdivided based on allelic relationships and morphology into putative autopolyploids (derived from within a single taxonomic species) and allopolyploids (derived from interspecific hybridization). Ten of the 13 appear to be autopolyploids, with alleles that are sister to one another at various nuclear loci (Table 1, Fig. 3A–D) and morphologies nearly identical to sexual diploids assigned to the same species. One clear exception is *N. californica*; both the yellow and white chemotypes are shown here to be allopolyploids. Both have two or more alleles derived from the maternal parent (Fig. 3A–E; annotated with "(M)" after species name), and one allele from the paternal parent (Fig. 3A–D; annotated with "(P)" after species name). The paternal allele is almost identical to one of the alleles of triploid *N. neglecta*, while the maternal alleles appear to derive from a distantly related, unsampled taxon weakly supported as sister to *N. lemmonii* and *N. meridionalis*.

3.3. Farina evolution

Fig. 4 summarizes the presence/absence of farina on the sporophytes and gametophytes of notholaenids, as well as the color of leaf farina and its biochemistry. The estimated ancestral states for each trait are shown in Appendix C. Both gametophytic and sporophytic farina are homoplasious. The gametophytic farina is more likely independently evolved in core *Notholaena* and *N. standleyi* (Fig. 4; Appendix C1, C12); while the sporophytic farina is more likely to be an ancestral trait that was independently lost in *Cheilanthes leucopoda*, *Cheiloplecton rigidum*, and *N. brachypus* (Fig. 4; Appendix C2, C13). A clear pattern for the evolution of farina color and biochemistry within notholaenoids was not observed (Fig. 4; Appendix C3–11, C14–22), especially for farina color, presence of flavone, flavanol, O-acetyl group, and triterpenes, while the presence of relatively rare chemicals, like chalcone, dihydrochalcone, flavanone, and O-butyl group are more likely evolved independently in two or more clades/species.

4. Discussion

4.1. New evidence from nuclear data

Nuclear data provide the opportunity to sample multiple linkage groups, lowering the risk of obtaining misleading results due to any idiosyncracies of a single linkage group (Moore, 1995; Rothfels et al., 2015b), and, because they are biparentally inherited, can be used to identify hybrids and resolve their origins. In this study, we successfully generated long (ca. 1 kb), low-copy nuclear sequences for 49 notholaenid individuals representing 31 of the 37 species currently attributed to the clade. By combining these nuclear data with plastid data, we resolved a species phylogeny for notholaenid ferns (Fig. 4). Our results are roughly congruent with previous phylogenies inferred using only plastid data (Gastony and Rollo, 1998; Johnson et al., 2012; Rothfels et al., 2008). This includes finding that (1) core *Notholaena* is strongly supported as a monophyletic group, whereas the morphologically very similar *N. standleyi* is only distantly related to it, (2) four species (one non-farinose) assigned to *Cheilanthes* by Tryon and Tryon (1982) form a well-supported group (the "*N. aureolina*" clade) closely related to core *Notholaena*, and (3) the anomalous, non-farinose genus *Cheiloplecton* is, indeed, nested among the notholaenids.

Despite broad agreement between our results and previous studies, we did observe several incongruences between our nuclear and plastid phylogenies (Fig. 3F and 3E, respectively): (1) our nuclear phylogeny suggests *N. standleyi* is sister to all other notholaenids, whereas the

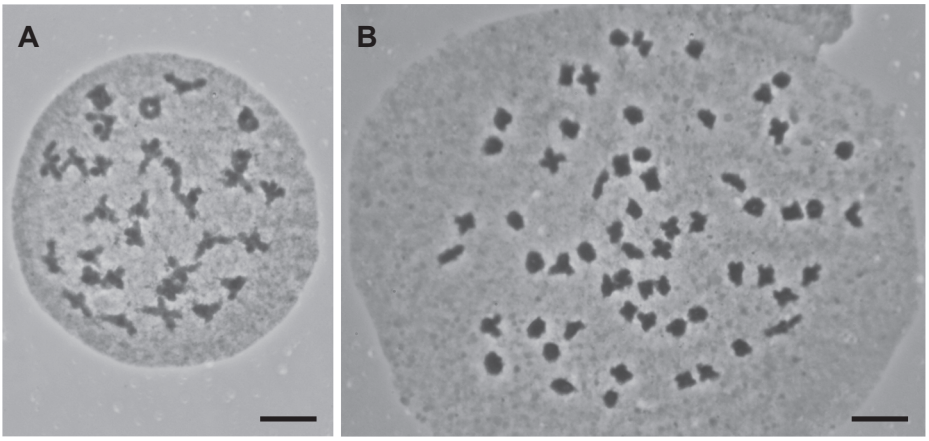

Fig. 5. Newly reported chromosome counts for *N. standleyi*. **A.** Diploid "gold" chemotype, $n = 30$. **B.** Tetraploid "pallid" chemotype, $n = 60$. Scale bars: A = 10 µm, B = 10 µm.

plastid data support *C. leucopoda* in that position; (2) three of the core *Notholaena* species—*N. meridionalis*, *N. lemmonii*, and *N. californica* (maternal progenitor)—are affiliated with core *Notholaena* I in the nuclear phylogeny, rather than with core *Notholaena* II, as in the plastid tree; and (3) nuclear data suggest *Cheiloplecton rigidum* is sister to the *N. aureolina* clade, rather than sister to core *Notholaena*. Given the relatively short branching times estimated by *BEAST (Fig. 4), these incongruences may be due to incomplete lineage sorting. In each case, the nuclear phylogenetic placements are supported by at least three out of the four nuclear loci (Fig. 3A–D), whereas the plastid placement is unique. Although we posit that the nuclear phylogeny (Fig. 3F) may better reflect the true relationships among these lineages, our current data are insufficient for strongly supporting the branching patterns throughout the clade (Figs. 3G and 4). Also, the four nuclear phylogenies do not completely agree with each other. For instance, *ApPEFP_C* and *IBR3* support *C. leucopoda* as sister to the clade formed by *Cheiloplecton* and the "*N. aureolina*" clade, but the branch is unresolved in the *gapCpSh* tree and not supported by *SQD1*. In addition, although we assume all four nuclear markers are single-copy (Rothfels et al., 2013), we do see some evidence of gene duplication in *gapCpSh* (in *N. candida* and possibly *N. revoluta*, and pseudogenes in *N. ochracea* and *N. meridionalis*).

4.2. Prevalence and origin of polyploids

Polyploidy is a major speciation process in plants, playing an important role in shaping the extant plant diversity (Rothfels and Otto, 2016). The percentage of recently-formed polyploid taxa is particularly high among ferns (ca. 33% in the meta-analysis of Wood et al., 2009), and these taxa are very distinct with regard to genetic diversity and mode of origin. One enduring debate is the relative abundance of allopolyploids (derived from interspecific hybridization) vs. autopolyploids (intraspecific polyploids), and their relative contributions to species diversity (Soltis et al., 2007). Barker et al. (2016) reviewed this topic and concluded that, although the total number of allopolyploids and autopolyploids presented in published scientific reports is roughly equal, autopolyploids are expected to be more abundant than allopolyploids because their numbers are usually underestimated and their relatively low genetic diversity makes them difficult to detect. Recent amplicon-sequencing methods now provide a cost-effective way for revisiting this issue with higher species coverage. For example, polyploids in the fern family Cystopteridaceae and the fern genus *Botrychium* appear to be overwhelmingly of hybrid origin, with little or no evidence of autopolyploidy (Rothfels et al., 2017; Dauphin et al., 2018).

Of the 49 notholaenid samples included in our molecular dataset, 30 had sufficient information to infer ploidy; 18 of these were categorized as diploid and 12 were designated as polyploids (Table 1). Nine of the 12 polyploid samples were classified as intraspecific (i.e., autopolyploids) based on the close phylogenetic relationships of their constituent alleles and a paucity of morphological features distinguishing them from their diploid relatives. However, these nine intraspecific polyploids run the gamut from strict autopolyploids (essentially no genetic or morphological divergence between the polyploid and a single diploid progenitor) to hybrids that could be considered allopolyploids if the parental taxa were justifiably treated as species.

Among the polyploid notholaenids included in our study, sample #5142 (*N. brachypus*) appears to be the best candidate for a strict autopolyploid, considering the fact that all previous morphology-based taxonomic treatments (e.g., Mickel and Smith, 2004; Tryon, 1956) have overlooked the cytogenetic diversity documented herein. The only chromosome count for *N. brachypus* is from a sexual diploid plant (Windham and Yatskievych, 2003) that exhibits 64 spores per sporangium averaging 48.2 µm in diameter (Table 1). By comparison, sample #5142 of *N. brachypus* exhibits 32 spores per sporangium that average 69.6 µm in diameter, corresponding to the values expected for an apomictic triploid (Windham et al. in prep.). Average spore diameter of a 64-spored sample (#6548) of *N. brachypus* included in the molecular analyses is intermediate between the known diploid and putative triploid, suggesting that another undetected cytotype (a sexual tetraploid) may be present in this species. In addition to *N. brachypus,* our results are congruent with hypothesized autopolyploid origins of the widespread triploids *N. aschenborniana* (Windham, 1993a), and *N. grayi* subsp. *grayi* (Gastony and Windham, 1989; Windham, 1993a, 1993b). Although these samples show somewhat greater allelic diversity, the constituent nuclear alleles form well-supported clades.

Notholaena standleyi exhibits an impressive amount of intraspecific chemical and genetic variability that may be worthy of taxonomic recognition, and thus the tetraploids newly documented within this species are difficult to classify as autopolyploid or allopolyploid. Of the six *N. standleyi* individuals sampled in this study, four represent the yellow and gold chemotypes (two samples of each) inferred to be diploid in previous studies (Benham, 1988; Knobloch et al., 1973; Windham and Schaack, 1983) and supported by the new chromosome counts presented herein. The other two samples (one representing the pallid chemotype and one belonging to the yellow-green chemotype) are known or inferred to be tetraploids (Table 1). Seigler and Wollenweber (1983) demonstrated that the yellow, gold, and yellow-green chemotypes differed in their farina color and chemistry, geographic range, and substrate preferences. The pallid chemotype, which was discovered relatively recently, has not yet been subjected to detailed chemical analyses. Like the yellow-green chemotype, it is known only from limestone outcrops, but it occupies a discrete range in southeastern Arizona and is easily distinguished by its whitish-cream farina (pers. obs.). Our plastid phylogeny indicates that the two tetraploids are sister to one another, and together they are sister to a group comprising the two diploid chemotypes (Fig. 3E). This result suggests that the two tetraploids may: (1) have diverged from an ancestor of the extant diploid lineages early in the evolutionary history of the species, or (2) share an as-yet undiscovered, calcicolous diploid progenitor. Our nuclear phylogenies (Fig. 3A–C) appear to support the latter hypothesis, suggesting that the two tetraploids may have originated from hybridization between a "missing" diploid and another lineage more closely related to the yellow chemotype. Our analyses indicate that chromosome number, spore size, and DNA sequence divergence can all be added to the list of features distinguishing the four chemotypes of *N. standleyi*. However, as pointed out by Seigler and Wollenweber (1983), there are no obvious morphological characters—other than farina composition and color—that would support their recognition as species. Considering this fact, we continue to refer to the two polyploid chemotypes as intraspecific (i.e., auto-) polyploids.

We sampled three individuals of *Cheiloplecton rigidum*, all likely to be polyploids though only two have sufficient data to support this inference (Table 1). Historically, this species was either treated within *Cheilanthes* or *Pellaea*, or in the monotypic genus *Cheiloplecton*; it was never before associated with notholaenids until plastid DNA sequence resolved it as sister to core *Notholaena* (Gastony and Rollo, 1998; Johnson et al., 2012; Rothfels et al., 2008). To explain this unexpected phylogenetic placement, we considered that *C. rigidum* might be an allopolyploid originating from intergeneric hybridization, something that is not unprecedented in ferns (Lehtonen, 2018; Rothfels et al., 2015a; Wagner et al., 1992). However, our nuclear DNA analyses support the hypothesis based on plastid data that this unique species is nested within notholaenids, without any contribution from *Pellaea*, *Cheilanthes*, or any other group (Figs. 3G and 4). As with *Notholaena standleyi, Cheiloplecton rigidum* shows a great deal of variation across its geographic range, and two varieties are generally recognized: var. *rigidum* and var. *lanceolatum* (Mickel and Smith, 2004). At their extremes, these taxa have strikingly different leaf shapes (pentagonal vs. lanceolate, respectively) and would likely be treated as different species if not for a confusing array of intermediate morphologies. All herbarium specimens examined to date have 32 large (triploid or larger) spores per sporangium, indicating that *C. rigidum* is a widespread apomictic

complex whose diploid progenitors have yet to be discovered. Our analyses include two samples classified as var. *rigidum* (#6525 and #6617), and one sample of var. *lanceolatum* (#4518). The two var. *rigidum* samples seem to be derived from reciprocal hybridization events, whereby sample #6525 always had higher reads of one haplotype (30:16 in *ApPEFP_C*; 58 (17 + 41):53 in *gapCpSh*; 499:286 in *IBR3*; and 157:19 in *SQD1*), and sample #6617 had higher reads of another haplotype (9:13 in *ApPEFP_C*; 70:285 in *gapCpSh*; 55:100 in *IBR3*; and 105:235 in *SQD1*). The specific read numbers varied when different clustering parameters were used in PURC analyses, but the general pattern remains the same. The var. *lanceolatum* sample (#4518) is a hybrid between the parent that contributed more to sample #6525 and another haplotype not observed in var. *rigidum*. This pattern suggests that *Cheiloplecton* is a complex of taxa, with at least three "missing" diploids.

The only two notholaenid species in our molecular sampling that would be considered unequivocal allopolyploids under the current taxonomy are *N. aliena* and *N. californica* (Table 1). In the plastid dataset, *N. aliena* is nearly identical to *N. grayi* (Fig. 3E). At the two nuclear loci for which we have data for *N. aliena* (Fig. 3A–B), one allele is nested within *N. grayi* while the other (designated allele 2) is slightly more divergent. Although *N. aliena* and *N. grayi* are clearly closely related, they are easily distinguished by trichome characters. Based on these morphological features, we hypothesize that *N. aliena* arose as a hybrid between *N. grayi* and *N. weatherbiana* R.M. Tryon. The latter species is very rare and was not included in our molecular analyses because we were unable to locate suitable material.

Notholaena californica is the only taxon in the current dataset that appears to be an allopolyploid hybrid between two relatively divergent lineages. Plastid loci (maternally inherited in cheilanthoid ferns; Gastony and Yatskievych, 1992) strongly support the placement of both samples (#5432 and #5459, representing the yellow and cream chemotypes, respectively) as sister to a clade comprised of *N. lemmonii* and *N. meridionalis* (Fig. 3E). In the nuclear phylogenies (Fig. 3A–D), *N. californica* always exhibits at least two alleles that cluster close to *N. lemmonii* and *N. meridionalis* (i.e., representing the clade or grade designated cN II'). At each of the four nuclear loci, the yellow chemotype of *N. californica* also shows a single allele identical (or nearly so) to an allele found in *N. neglecta*, a member of the core *Notholaena* II clade inferred to be the paternal parent. The sample representing the cream chemotype exhibits the same pattern at two loci (*ApPEFP_C* and *SQD1*; Fig. 3A, D respectively), appears to lack the *N. neglecta* allele at *IBR3* (Fig. 3C), and failed to amplify for *gapCpSh*. The failure to detect an allelic contribution from *N. neglecta* at *IBR3* in the cream chemotype is likely due to low sequencing coverage (we obtained a total of only ten *IBR3* reads from that sample), although it might reflect a real genetic difference between the two chemotypes. Our sample of the yellow chemotype came from the same population that provided the apomictic pentaploid chromosome count reported by Windham and Yatskievych (2003), and the occurrence of four alleles at three of the nuclear loci indicates that the plant contains at least four sets of chromosomes (Table 1). The cream chemotype sample (#5459) exhibits just two or three alleles at the three resolved nuclear loci, suggesting that this individual may represent a lower ploidy (probably triploid).

Of the 30 notholaenid samples in our molecular analyses for which ploidy could be inferred, 18 (60%) are identified as diploid and 12 (40%) are polyploid. If we narrow this subsample to include a single representative of each taxon (chemotype, variety, or species), two polyploid "replicates" (one *Cheiloplecton rigidum* var. *rigidum* and one *Notholaena aschenborniana*) are dropped, leaving us with a diploid to polyploid ratio of 18:10 (Table 1). Thus, polyploids constitute about 35.7% of the taxonomic diversity of notholaenids represented herein, a figure remarkably close to the average (32.86 ± 2%) reported for leptosporangiate ferns in the meta-analysis of Wood et al. (2009). What seems to distinguish notholaenids from most other well-studied fern clades is the relatively low proportion of taxonomic allopolyploids, which comprise just 30% of demonstrably polyploid taxa (Table 1). Ferns provide some of the classic examples of reticulate evolution via allopolyploidy (Manton, 1950; Wagner, 1954), and most well-studied fern clades with appropriate nuclear data show far more allopolyploids than autopolyploids (e.g., Dauphin et al., 2018; Li et al., 2012; Rothfels et al., 2017, 2014; Sessa et al., 2012). Although this imbalance may be due, in part, to the morphologically and genetically cryptic nature of autopolyploids (Barker et al., 2016), there is little doubt that allopolyploids typically predominate among the more diverse fern genera.

It might be argued that some of the polyploid notholaenid taxa we have classified as autopolyploids have sufficiently divergent parents to be considered allopolyploids. Our data highlight the fact that polyploids in this group do not fit well within a binary classification of polyploidy based on whether the parents belong to the same or different species. We agree with Doyle and Sherman-Broyles (2017) that equating "allopolyploid" with "interspecific" is problematic for a number of reasons, most notably the instability of the resultant categorization in the face of changing taxonomic concepts. However, genetic data for notholaenids (and most other plants as well) is very sparse compared to that available for *Glycine* Willd., and the taxonomy-based classification of polyploids, unsatisfactory as it is, can often provide some useful information. In the case of notholaenids, autopolyploids would still outnumber allopolyploids even if the *Cheiloplecton rigidum* complex were moved from the former category to the latter. This is in sharp contrast to the pattern evident in most fern genera (see discussion above) and is most similar to the situation observed in another cheilanthoid fern genus, *Argyrochosma* (J. Sm.) Windham. Of the eight taxa of *Argyrochosma* inferred to be polyploid by Sigel et al. (2011), at least six (75%) are likely to be autopolyploids. Two other relatively diverse cheilanthoid genera, *Pellaea* Link and *Myriopteris* Fée, also exhibit percentages of autopolyploids higher than those observed in most other fern groups (Gastony and Windham, 1989; Grusz et al., 2014). The majority of inferred autopolyploids in these four genera consist of apomictic triploids, whose reproductive biology is specially adapted to arid environments. Determining whether autopolyploidy is somehow favored under these circumstances is worthy of further investigation.

4.3. Farina presence, color, and chemistry are homoplastic

Wollenweber (1984) noted that *Notholaena* has "the greatest number of exudate-producing species and the greatest diversity from a phytochemical viewpoint". Even when *Argyrochosma* is removed from the genus (Windham, 1987), the notholaenids still include about a quarter of farinose fern diversity. The chemical composition of their farina is extraordinarily diverse, encompassing all six major classes of flavonoid aglycones produced by plants (Wollenweber and Dietz, 1981; Wollenweber and Schneider, 2000) and myriad R-groups, including hydroxyl, methyl, O-methyl, and ester derivatives that appear at various positions on the carbon backbone (Wollenweber and Schneider, 2000). We map the presence of farina and their biochemical constitutions on our species phylogeny in Fig. 4, and inferred their evolutionary history in Appendix C.

The presence of farina-producing glands on both sporophytes and gametophytes is a character shared by the early-diverging taxon *N. standleyi* and all core *Notholaena* species included in our analyses (Fig. 4). Three other species, *N. aureolina, N. jaliscana,* and *N. ochracea*, produce farina on the sporophytes but not the gametophytes (see also Johnson et al., 2012). Our ancestral state reconstruction suggests that the presence of sporophytic farina is more likely to be an ancestral state that was independently lost in *Cheilanthes leucopoda*, *Cheiloplecton rigidum*, and *N. brachypus* (Fig. 4; Appendix C2, C13); while gametophytic farina is more likely to have evolved independently in core *Notholaena* and *N. standleyi* (Fig. 4; Appendix C1, C12).

The farina constitution of notholaenids is highly diverse; previous biochemistry studies show that every species expresses a unique combination of flavonoids (Wollenweber, 1984). Several species (e.g., *N. californica*, *N. standleyi*, and *N. sulphurea*) even express different

flavonoids in different infraspecific variations (chemotypes; Seigler and Wollenweber, 1983; Wollenweber, 1989, 1984). We were unable to discern any clear phylogenetically correlated patterns, even after we summarized the results using major groups of farina constituents (Fig. 4, Appendix C). These results suggest that flavonoid synthesis may be regulated differently between even closely related notholaenid species. The farina types produced by *N. aureolina, N. jaliscana,* and *N. ochracea* are unique in that they are mainly composed of chalcones (Wollenweber, 1977; Wollenweber and Schneider, 2000). Among the other notholaenid ferns biochemically analyzed by Wollenweber, only the orange chemotype of *N. sulphurea* produced significant quantities of chalcones (Wollenweber, 1989, 1984, 1977), which likely evolved independently (Appendix C4, C15). The farina of core *Notholaena* are composed primarily of flavones and/or flavonols, sometimes with O-acetyl or O-butyl R groups; other chemical groups (chalcone, dihydrochalcone, flavanone) are relatively rare or present in trace amounts (triterpenes; Fig. 4). The farina color of the species belonging to the core *Notholaena* I clade is generally white, whereas those of core *Notholaena* II or II' can be either white or yellow (Fig. 4).

Additional gametophyte observations confirm the surprising conclusion of Johnson et al. (2012) that the occurrence of farina-producing glands on gametophytes is homoplastic as well. This character was hypothesized to be a synapomorphy for the genus *Notholaena* (Pray, 1967; Rothfels et al., 2008; Windham, 1993a), until Johnson et al. (2012) observed the trait was absent in several notholaenid species (i.e., *Cheiloplecton* and the *N. aureolina* clade). Recent gametophyte observations have further challenged the hypothesis that only members of *Notholaena* possess farinose gametophytes. Gabriel y Galán et al. (2012) report that *Argyrochosma nivea* var. *nivea*, an apogamic triploid pellaeid taxon, occasionally (~20% of the time) develops farinose hairs on the gametophytes. In this case, because farina develop only adjacent to the developing sporophyte, the gametophytic farina may be due to sporophytic transcripts. More recently, however, farinose gametophytes were reported in yet another non-notholaenid species, *Aleuritopteris squamosa* (Liu et al. 2015, see their Fig. 1), a Chinese endemic nested within the hemionitid clade of cheilanthoid ferns. Here, farinose glandular hairs were expressed more frequently on female gametophytes (96%) than on male gametophytes (12%), corroborating Giauque's (1949) observation that male-only gametophytes tend to produce fewer (or even no) glandular hairs. Revisiting Giauque (1949), we found reference to three other *Aleuritopteris* species (*A. farinosa*, *A. argentea*, and *A. anceps*) that occasionally had glandular hairs on gametophytes when cultured on soil; however, it is not clear whether those glands produced farina or not. Both recent reports (Gabriel y Galán et al., 2012; Liu et al., 2015) are from species that produce sporophytic farina, and there are still no reports of gametophytic farina in species with non-farinose sporophytes. How the production and expression of glandular hairs and farinose exudates are regulated between the two phases of the fern life cycle is a question to be more thoroughly explored in future gametophyte studies.

The isolated phylogenetic position of *N. standleyi* relative to core *Notholaena* remains an intriguing anomaly. This species shares all the clade-specific traits of core *Notholaena*, including gametophytic farina and the presence of farina on the abaxial leaf surface, and it is morphologically almost indistinguishable from *N. sulphurea* (Mickel and Smith, 2004; Tryon, 1956)—a species firmly nested within core *Notholaena* II (Johnson et al., 2012; Rothfels et al., 2008; Fig. 4). Both *N. standleyi* and *N. sulphurea* have relatively broad distributions for notholaenid ferns: *N. standleyi* extends from the southwest U.S. to southern Mexico, and *N. sulphurea* is distributed from northern Mexico to Chile (Mickel and Smith, 2004). Interestingly, both exhibit geographically correlated variations in sporophyte farina color across their ranges (Mickel and Smith, 2004; Seigler and Wollenweber, 1983). In this study, we sampled six *N. standleyi* individuals representing four chemotypes (yellow, gold, yellow-green, and pallid), as well as three diploid chemotypes of *N. sulphurea* (cream, orange, and yellow; Table 1). Surprisingly, the farina biochemistry of the two species turns out to be quite different. The farina of *N. standleyi* are mainly composed of kaempferol (a type of flavonol), with the different colors resulting from different O-methyl substitution patterns (Seigler and Wollenweber, 1983; Wollenweber, 1976). The farina of *N. sulphurea*, on the other hand, are mainly composed of chalcones, dihydrochalcones, or flavonols plus flavones (Fig. 4), which produce the orange, white, and yellow colors, respectively (Wollenweber, 1977; Wollenweber et al., 2001). The biochemical data in this case appear to support the phylogenetic separation of *N. standleyi* and *N. sulphurea*, suggesting that the morphological similarities might be the result of convergent evolution.

4.4. Function of farina – implications from their evolution within notholaenids

The function of farina in notholaenids has not been adequately investigated. Currently there are two major ecophysiology-based hypotheses relating to the production of a dense layer of flavonoid aglycones on lower leaf surfaces (Hevly, 1963; Wollenweber, 1984): (1) it may be a desiccation-avoidance strategy in which farina serve as an extra boundary layer that reduces water loss from transpiration and/or; (2) it may be a desiccation-tolerance strategy in which farina protect desiccated, curled-up leaves from UV or heat damage, allowing them to "resurrect" when water becomes available. Because our analyses infer farina to be homoplastic within notholaenids (Fig. 4), this clade provides a unique opportunity to investigate the proposed ecophysiological functions of this trait. If farina does play an important role in drought tolerance, one important question is how the three completely non-farinose species within the group (*Cheilanthes leucopoda*, *Cheiloplecton rigidum*, *N. brachypus*) deal with xeric conditions. All three are widespread in arid to semi-arid habitats in Mexico and adjacent areas, often growing with species of core *Notholaena*. *Cheilanthes leucopoda* is moderately pubescent on the lower leaf surfaces (possibly forming a very diffuse boundary layer), *Cheiloplecton rigidum* is very sparsely hairy (Fig. 6A), and *N. brachypus* is densely scaly (Fig. 6B), forming a strong boundary layer that may be functionally comparable to that of core *Notholaena* (represented in Fig. 6C by *N. grayi*). The light-colored scales covering the lower leaf surfaces of *N. brachypus* may also reflect solar radiation when the leaves are desiccated (curled-up), though it is difficult to imagine this being as effective a desiccation tolerance strategy as the very pale farina layer produced by the majority of *Notholaena* species. *Notholaena brachypus* is similar to other members of core *Notholaena* in being a classic "resurrection plant"; its leaves curl up during drought periods but become photosynthetically active again once there is sufficient moisture. Notholaenid species using this strategy typically have leaves with continuous petioles lacking pre-defined abscission zones (represented in Fig. 6D by *N. candida*), and *N. brachypus* belongs in this category. A few *Notholaena* species that commonly resurrect their leaves also produce sporadic abscission zones, including *N. standleyi* (Fig. 6E), *N. montielae* (Yatskievych and Arbeláez, 2008), *N. californica*, and *N. copelandii* (pers. obs.). An abundance of pre-defined abscission zones located just above petiole bases in cheilanthoid ferns signals an alternative approach to dealing with periodic drought, i.e., quickly dropping the old leaves with the advent of xeric conditions and replacing them with new growth when the drought ends (Hevly, 1963). Two of the three non-farinose notholaenids (*Cheilanthes leucopoda* (Fig. 6F) and *Cheiloplecton rigidum*) show clear abscission zones at the petiole bases, strongly suggesting that they are drought-deciduous and thus less dependent on other potential xeric adaptations (such as farina) to survive. Interestingly, such "fractiferous petioles" are also a common feature of the three farinose species of the *N. aureolina* clade (Mickel and Smith, 2004; Tryon and Tryon, 1982).

Other than having ecophysiological benefits, the flavonoids in farina might also function in inhibiting microbiota and herbivores (Cushnie and Lamb, 2011, 2005; Onyilagha et al., 2004). For instance, Rodriguez et al. (2018) recently reported that extractions from *N. sulphurea* farina exhibit acute toxicity to the snail *Biomphalaria peregrine*, and high to modest antifungal activities. Farina may also function as an allelopathic agent. Star (1980) found that the major chemical components of *Pityrogramma* farina will inhibit spore germination and gametophyte development of other *Pityrogramma*. This might help to reduce potential competitors and increase survivability in xeric habitats (Star,

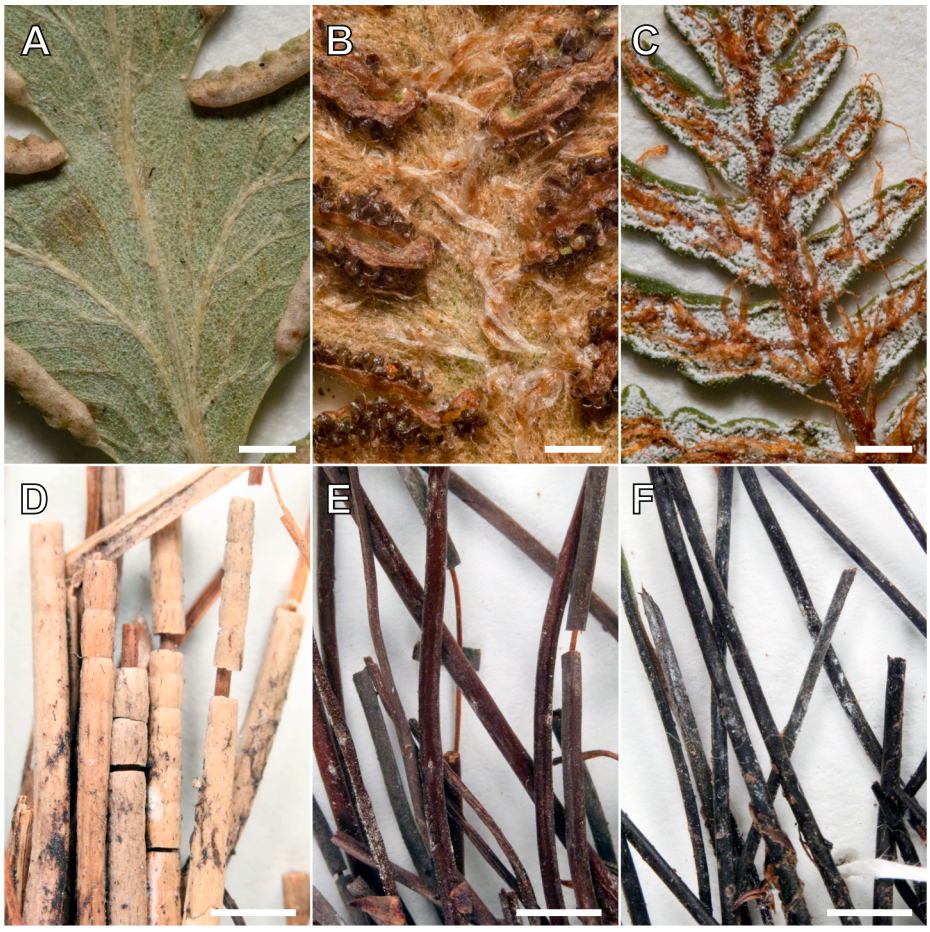

Fig. 6. Non-glandular indument on leaf abaxial surfaces (A–C) and fractiferous zones on stipes (D–F) of representative notholaenid ferns. **A.** *Cheiloplecton rigidum* is non-farinose and almost glabrous. **B.** *N. brachypus* is non-farinose and bears dense trichomes. **C.** *N. grayi* has white granulate farina and scales. **D.** *Cheilanthes leucopoda* has dense fractiferous lines at the base of stipes. **E.** *N. standleyi* with occasional scattered fractiferous lines on stipes. **E.** *N. candida* with no obvious fractiferous lines on stipes. Scale bars: 1 mm.

1980). If this hypothesis is correct, the presence of farina on gametophytes makes the story more complex and interesting, and farina could potentially be a barrier to sexual reproduction between different chemotypes. This could be a possible explanation for the distinct chemical compositions of farina between closely related species within notholaenids. Clearly the notholaenids have much to reveal about the function of farina and drought adaptation in general.

4.5. Conclusion

In this study, we used the recently developed amplicon sequencing protocol and bioinformatics pipeline PURC (Rothfels et al., 2017) to successfully retrieve long (ca. 1 kb) low-copy nuclear sequences of four loci from 49 notholaenid and 12 outgroup samples on one PacBio single molecule real time (SMRT) cell. Combining these nuclear data with sequences from three plastid loci, we were able to infer a species phylogeny for notholaenid ferns. Our principal results are roughly congruent with previous phylogenies inferred using only plastid data; however, we also observed several incongruences between them. Hybridization among recognized species of the notholaenid clade appears to be relatively rare compared to that observed in other well-studied fern genera, but we found that one species—*N. californica*—is an allopolyploid between relatively distantly-related progenitors. All characters associated with farina production in the group (presence/absence, color, and biochemistry) appear to be homoplastic and have complex evolutionary histories. It is hoped that these results will provide a framework for further exploration of the biology of this interesting group, including studies addressing the evolution and function of farina.

Declaration of Competing Interest

None.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.05.016.

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