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Reticulate evolution on a global scale: A nuclear phylogeny for New World *Dryopteris* (Dryopteridaceae)

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ABSTRACT

Reticulate, or non-bifurcating, evolution is now recognized as an important phenomenon shaping the histories of many organisms. It appears to be particularly common in plants, especially in ferns, which have relatively few barriers to intra- and interspecific hybridization. Reticulate evolutionary patterns have been recognized in many fern groups, though very few have been studied rigorously using modern molecular phylogenetic techniques in order to determine the causes of the reticulate patterns. In the current study, we examine patterns of branching and reticulate evolution in the genus *Dryopteris*, the woodferns. The North American members of this group have long been recognized as a classic example of reticulate evolution in plants, and we extend analysis of the genus to all 30 species in the New World, as well as numerous taxa from other regions. We employ sequence data from the plastid and nuclear genomes and use maximum parsimony (MP), maximum likelihood (ML), Bayesian inference (BI), and divergence time analyses to explore the relationships of New World *Dryopteris* to other regions and to reconstruct the timing and events which may have led to taxa displaying reticulate rather than strictly branching histories. We find evidence for reticulation among both the North and Central/South American groups of species, and our data support a classic hypothesis for reticulate evolution via allopolyploid speciation in the North America taxa, including an extinct diploid progenitor in this group. In the Central and South American species, we find evidence of extensive reticulation involving unknown ancestors from Asia, and we reject deep coalescent processes such as incomplete lineage sorting in favor of more recent intercontinental hybridization and chloroplast capture as an explanation for the origin of the Latin American reticulate taxa.

1. Introduction

Reticulate evolution is increasingly being recognized as a fundamental process in the evolutionary histories of organisms. The conventional depiction of evolution as a bifurcating process, leading to tree-like evolutionary patterns, is in fact not a realistic or accurate depiction of the events shaping the evolution of many groups (Linder and Rieseberg, 2004; McBreen and Lockhart, 2006; Beiko et al., 2008). Phenomena such as incomplete lineage sorting, horizontal gene transfer, hybridization, introgression, and polyploidy can lead to evolutionary scenarios that are more accurately represented as networks than as trees (Rieseberg, 1997; Huson and Bryant, 2005). Untangling the relationships among taxa in complexes that have experienced these phenomena presents a unique challenge for systematists, and an opportunity to increase our understanding of phylogenetic conflict and the historical processes it represents.

Reticulate evolution appears to be particularly common in plants (Grant, 1981; Linder and Rieseberg, 2004), and ferns in particular have long been noted for their tendency towards reticulation, frequently as a result of inter-specific hybridization and polyploidy (Barrington et al., 1989; Haufler, 1995). In the current study, we investigate phylogenetic conflict between the nuclear and plastid genomes in a fern genus, *Dryopteris*, where hybridization and polyploidy are known to be common (Manton, 1950; Hoshizaki and Wilson, 1999). *Dryopteris* is one of the largest genera in Dryopteridaceae, which is one of the most species-rich families of ferns (Smith et al., 2006). With an estimated 225–250 species worldwide (Fraser-Jenkins, 1986), members of this genus, known as the woodferns or shield ferns, are ubiquitous components of the flora in many of the world's temperate forests. The North American taxa have long been recognized as a reticulate evolutionary complex involving allopolyploid speciation (Walker, 1955; Werth, 1991; Montgomery and Wagner, 1993), although the hypothesized relationships between the polyploids and their putative parents have not yet been thoroughly tested using DNA sequence data. Little is known about the frequency of hybridization or reticulation outside of the North American species.

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A recent phylogenetic analysis of the New World species of *Dryopteris* based on the plastid genome sampled nearly half of the members of the genus (Sessa et al., 2012), and provided preliminary support for an existing hypothesis explaining relationships among the North American species. This hypothesis invokes a putatively extinct diploid progenitor called "*D. semicristata*" and is thus referred to as the "semicristata" hypothesis (Montgomery and Wagner, 1993). It was recently rejected in an analysis of the European members of the reticulate complex based on the nuclear locus *pgiC* (Juslen et al., 2011), one of the markers that we employ here. The plastid-based phylogenetic and divergence time analyses of Sessa et al. further demonstrated that none of the North American taxa, with the exception of the polyploids and their putative maternal progenitors, are each other's closest relatives; all are more closely related to Asian, European, or African taxa from which they diverged over the last 10 million years (Ma). In contrast, 14 of the 18 Central and South American species form a well-supported clade that diverged from an Asian sister clade approximately 32.3 Ma. Nothing is yet known about the ploidy of the Latin American species or whether they have experienced hybridization or polyploidization as the North American taxa have. Data from the plastid genome, which is maternally inherited in ferns (Gastony and Yatskievych, 1992; Vogel et al., 1998), is not sufficient to address these questions. The current study therefore builds on the previous, plastid-based analysis of *Dryopteris* and extends the investigation to the nuclear genome. We employ a data set based on the single-copy nuclear marker *pgiC*, as well as the plastid data set of Sessa et al. (2012), to achieve the following goals: (1) produce a nuclear phylogeny for all New World *Dryopteris* and numerous taxa from other geographic regions; (2) explore incongruence between nuclear and plastid phylogenies for the same set of taxa; and (3) determine the extent to which reticulation has influenced the evolution of the New World species, and explore the mechanisms which may be responsible for phylogenetic conflict (e.g. hybridization, incomplete lineage sorting).

2. Material and methods

2.1. Taxon sampling, DNA extraction, and plastid DNA sequencing

Taxon sampling for this study follows that of Sessa et al. (2012), with the exception of 15 species for which we were unable to amplify *pgiC*. In addition, we added seven more species that were not included in the previous plastid study, but for which *pgiC* sequences were available on GenBank. Four separate data sets were constructed based on this sampling scheme. The full *pgiC* matrix included 107 individual accessions, representing 89 *Dryopteris* species, 82 of which were in the previous plastid study (Table 1). 18 taxa were present in duplicate, with one sequence newly generated for this study and one sequence retrieved from GenBank, so that we could compare our new sequences with existing data. A new plastid matrix and a reduced *pgiC* matrix were also constructed, with identical taxon sampling (i.e. species for which we were missing either plastid or *pgiC* sequence data were excluded from these matrices). These two datasets contained the same 82 species of *Dryopteris* (Table 1), with the same 18 present in duplicate for *pgiC*. Finally, we constructed a dataset that included only species for which we found a single *pgiC* variant (i.e. putative non-reticulate taxa; see Section 3.1). This "non-reticulate" matrix included plastid and *pgiC* sequence data for 41 putatively non-reticulate *Dryopteris* species. One accession of *Polystichum andersonii* was included as an outgroup in all datasets. Ploidy levels for many, but not all, of the included *Dryopteris* taxa were obtained from a search of the available literature (Table 1).

Tissue acquisition and DNA extraction procedures are described in Sessa et al. (2012), and the plastid data employed here were generated in that study. Plastid loci include one protein-coding region (*rbcL*) and six inter-genic spacers (*psbA-trnH, trnP-petG, rps4-trnS, trnL-F, trnG-trnR*, and *rbcL-accD*). Voucher information for all taxa in this study is provided in the Appendix A.

2.2. Nuclear sequencing and assembly

We initially amplified *pgiC* from all samples using polymerase chain reaction (PCR) with the 14F and 16R primers of Ishikawa et al. (2002). These EPIC (exon-primed, intron crossing) primers are located in exons 14 and 16 of *pgiC*, resulting in amplification of portions of exons 14 and 16, all of exon 15, and the two intervening introns. Amplification occurred in 20 μL reactions containing 7.25 μL ddH_20, 4 μL 5× Colorless GoTaq Flexi buffer (Promega, Madison Wisconsin), 0.4 μL 10 mM dNTP, 1 μL 25 mM $MgCl_2$, 2 μL of each 1 mM primer, 0.25 μL GoTaq Flexi DNA polymerase (Promega, Madison, Wisconsin), and 3 μL template DNA diluted from stocks to 0.2 ng/μL. Amplifications were carried out on an Eppendorf MasterCycler Pro S (Eppendorf Scientific Inc., Hamburg, Germany) thermal cycler with the following protocol: 95 °C for 7 min, (94 °C for 30 s, 51 °C for 1 min, 72 °C for 1 min) × 40 cycles, 72 °C for 4 min. PCR products were run on a 1.2% agarose gel, from which bands were cut and DNA re-extracted using the ZymoClean Gel DNA Recovery System (Zymo Research Corp., Irvine, California). A subset of samples was submitted for direct sequencing using the amplification primers, but this resulted in chromatograms with multiple peaks for all species. We therefore opted to clone *pgiC* from all samples, using the pGEM-T Easy Vector System I (Promega, Madison, Wisconsin) and following standard protocols for cloning, colony selection, and post-cloning re-amplification with universal M13 primers. At least eight and up to 24 colonies were chosen for each individual. Several taxa were recalcitrant and ultimately yielded fewer than 8 sequences despite multiple cloning attempts. Final PCR products were purified using ExoSAP-IT (USB Corp., Cleveland, Ohio), and forward and reverse cycle-sequencing reactions carried out using BigDye Terminator 3.1 (Applied Biosystems, Foster City, California) with the 14F and 16R *pgiC* primers. Sequencing products were purified via gel filtration chromatography using Sephadex columns (Sigma–Aldrich, St. Louis, Missouri) according to the manufacturer's protocols. Sequencing occurred at the University of Wisconsin – Madison Biotechnology Center (Madison, Wisconsin).

Distinct copies or sequence variants of *pgiC* from all individuals were identified following Russell et al. (2010) and Grusz et al. (2009). Briefly, all sequences for a given accession were first pooled and observed by eye, and chimeric sequences easily identified and removed. An unrooted neighbor-joining tree was then constructed for each accession using the remaining sequences, and these trees were used along with visual inspection of the alignments to identify groups of sequences that shared at least three polymorphisms (gaps or single base pair changes). Consensus sequences were then constructed for these groups. We also retained singleton sequences that were not obviously chimeric or the result of PCR error and that also did not share at least three polymorphisms with other sequences, as they could potentially represent additional, undersampled allelic variation. All consensus and singleton sequences were deposited in GenBank (Appendix A) and used in subsequent analyses.

2.3. Sequence alignment and phylogenetic analyses

Alignment of the plastid sequences is described in Sessa et al. (2012). *PgiC* sequences were aligned using the MAFFT (Katoh et al., 2002) plugin in Geneious 5.5.3 (Drummond et al., 2011) and subsequently adjusted manually via the Geneious interface. Gaps in the alignments due to insertion/deletion events (indels)

Table 1
Species of *Dryopteris* included in this study. Number of *pgiC* sequence variants identified in the current study is indicated, and ploidy is given when known. Inclusion in our four datasets – full *pgiC*, reduced *pgiC*/plastid (these had the same sampling), non-reticulate – is indicated, and general geographic ranges of taxa are given. See Appendix A for voucher information.

Species	Ploidy level, reference	# of *pgiC* variants identified	In Full *pgiC* matrix?	In reduced *pgiC*/plastid matrix?	In non-reticulate matrix?	Distribution
Total *Dryopteris* in each matrix:			89	82	41	
D. abbreviata	2×; Manton (1950)	1	✓	✓	✓	Europe
D. aemula	2×; Manton (1950)	1	✓	✓	✓	Europe
D. affinis	2×, 3×; Fraser-Jenkins (1980)	2	✓	✓		Europe
D. aitoniana	2×; Goldblatt and Johnson (1991)	1	✓	*	*	Asia
D. alpestris	2×; Xiang (2006)	1	✓	✓	✓	Asia
D. amurensis	2×; Hoshizaki and Wilson (1999)	1	✓	*	*	Asia
D. antarctica	Unknown	3	✓	✓		Africa
D. aquilinoides	Unknown	2	✓	✓		Europe
D. arguta[a]	2×; Hoshizaki and Wilson (1999)	1	✓	✓	✓	Western North America, Central America
D. assimilis[b]	2×; Gibby et al. (1977)	1	✓	✓		Europe
D. athamantica	2×; Wíden et al. (1973)	2	✓	✓		Africa
D. austriaca[c]	2×; Gibby and Walker (1977)	1	✓	✓		Europe
D. azorica	2×; Gibby and Walker (1977)	1	✓	*	*	Europe
D. barberigera	2×; Gibby (1985)	1	✓	✓	✓	Asia
D. campyloptera[a]	4×; Walker (1961)	2	✓	✓		Eastern North America
D. carthusiana[a]	4×; Manton (1950)	2	✓	✓		Asia, Europe, Eastern North America, Western North America
D. caucasica	2×; Fraser-Jenkins and Corley (1972)	2	✓	✓		Europe
D. celsa	4×; Walker (1962)	2	✓	✓		Eastern North America
D. chinensis	4×; Nakato et al. (1995)	2	✓	✓		Asia
D. chrysocoma	2×; Gibby (1985)	3	✓	✓		Asia
D. cinnamomea[a]	Unknown	1	✓	✓	✓	Central America
D. clintoniana	6×; Walker (1962)	3	✓	✓		Eastern North America
D. costalisora	Unknown	1	✓	✓	✓	Asia
D. crassirhizoma	2×; Wíden et al. (1973)	2	✓	✓		Asia
D. crispifolia[a]	4×; Gibby et al. (1977)	2	✓	✓		Europe
D. cristata[a]	4×; Manton (1950)	2	✓	✓		Asia, Europe, Eastern North America
D. cycadina	3×; Gibby (1985)	2	✓	✓		Asia
D. cystolepidota	3×; Hoshizaki and Wilson (1999)	1	✓	✓	✓	Asia
D. dickinsii	2×; Gibby (1985)	1	✓	✓	✓	Asia
D. diffracta	4×; Fraser-Jenkins (1980)	1	✓	*	*	Asia
D. dilatata[a]	4×; Manton (1950)	2	✓	✓		Europe
D. effusa	Unknown	1	✓	*	*	Asia
D. expansa[a]	2×; Hoshizaki and Wilson (1999)	1	✓	✓	✓	Western North America
D. fatuhivensis	Unknown	1	✓	✓	✓	Pacific
D. filix-mas[a]	4×; Manton (1950)	2	✓	✓		Asia, Europe, Western North America
D. flaccisquama	Unknown	1	✓	✓		South America
D. fragrans[a]	2×; Hoshizaki and Wilson (1999)	1	✓	✓	✓	Asia, Eastern North America, Western North America
D. futura	2×; Smith (1975)	3	✓	✓		Central America
D. goldiana[a]	2×; Walker (1959)	1	✓	✓	✓	Eastern North America
D. guanchica[a]	4×; Gibby et al. (1977)	2	✓	✓		Africa
D. gymnosora	3×; Gibby (1985)	1	✓	✓	✓	Asia
D. hasseltii	2×; Tindale and Roy (2002)	1	✓	*	*	Asia
D. hondoensis	3×; Hoshizaki and Wilson (1999)	1	✓	✓	✓	Asia
D. huberi	Unknown	3	✓	✓		South America
D. inequalis	Unknown	1	✓	✓	✓	Africa
D. intermedia[a]	2×; Walker (1959)	1	✓	✓	✓	Eastern North America
D. juxtaposita	3×; Gibby (1985)	2	✓	✓		Asia
D. karwinskyana	Unknown	3	✓	✓		Central America
D. knoblochii	Unknown	3	✓	✓		Central America
D. komarovii	Unknown	1	✓	✓	✓	Asia
D. lacera	2×; Hoshizaki and Wilson (1999)	1	✓	✓	✓	Asia
D. ludoviciana[a]	2×; Walker (1959)	1	✓	✓	✓	Eastern North America
D. maderensis	2×; Gibby and Walker (1977)	1	✓	✓	✓	Europe, Africa
D. marginalis[a]	2×; Hoshizaki and	1	✓	✓	✓	Eastern North America

(*continued on next page*)

Table 1 (*continued*)

Species	Ploidy level, reference	# of *pgiC* variants identified	In Full *pgiC* matrix?	In reduced *pgiC*/plastid matrix?	In non-reticulate matrix?	Distribution
Total *Dryopteris* in each matrix:			89	82	41	
	Wilson (1999)					
D. maxonii	Unknown	1	✓	✓	✓	Central America
D. monticola	Unknown	2	✓	✓		Asia
D. muenchii	3×; Reyes-Jaramillo et al. (2008)	3	✓	✓		Central America
D. nubigena	Unknown	1	✓	✓	✓	Central America
D. odontoloma	3×; Gibby (1985)	1	✓	✓	✓	Asia
D. oligodonta[a]	2×; Gibby et al. (1977)	1	✓	✓	✓	Africa
D. oreades	2×; Wíden et al. (1996)	1	✓	✓	✓	Europe
D. pacifica	2×, 3×; Nakato et al. (1995)	2	✓	✓		Asia
D. pallida[a]	2×; Hoshizaki and Wilson (1999)	1	✓	✓	✓	Europe
D. pandae	2×; Kato et al. (1992)	2	✓	✓		Asia
D. patula	2×; Walker (1973)	1	✓	✓	✓	Central America, South America
D. pentheri	Unknown	1	✓	✓	✓	Europe
D. polita[a]	Unknown	1	✓	✓	✓	Asia
D. polylepis	2×; Wíden et al. (1996)	1	✓	✓	✓	Asia
D. pseudofilix-mas	3×; Wíden et al. (1996)	3	✓	✓		Central America
D. pycnopteroides	2×; Xiang (2006)	1	✓	✓	✓	Asia
D. reflexosquamata	2×; Tsai and Shieh (1985)	1	✓	✓	✓	Asia
D. remota	3×; Manton (1950)	2	✓	✓		Asia
D. rosea	Unknown	2	✓	✓		Central America
D. rossii	Unknown	1	✓	✓	✓	Central America
D. sacrosancta	3×; Hoshizaki and Wilson (1999)	3	✓	✓		Asia
D. saffordii	Unknown	3	✓	✓		South America
D. salvinii	Unknown	3	✓	✓		South America
D. scottii	4×; Gibby (1985)	2	✓	✓		Asia
D. sieboldii	4×; Hoshizaki and Wilson (1999)	1	✓	✓	✓	Asia
D. simplicior	Unknown	1	✓	✓	✓	Central America
D. sparsa	2×, 4×; Wíden et al. (2001)	1	✓	✓	✓	Asia
D. stenolepis	2×; Gibby (1985)	1	✓	✓	✓	Asia
D. stewartii	3×; Gibby (1985)	2	✓	✓		Asia
D. sublacera	3×; Gibby (1985)	2	✓	✓		Asia
D. subreflexipinna	Unknown	2	✓	*		Asia
D. tokyoensis	2×; Hoshizaki and Wilson (1999)	1	✓	✓	✓	Asia
D. triangularis	Unknown	3	✓	✓		Asia
D. wallichiana	2×, 3×, 4×; Gibby (1985)	2	✓	✓		Asia, Africa, Central America, South America, Pacific
D. xanthomelas	2×; Wíden et al. (1996)	1	✓	✓	✓	Africa

* Plastid data were missing, species was not included in the plastid, reduced *pgiC*, or non-reticulate matrices.
[a] Species included in duplicate in the *pgiC* matrices; one copy newly sequenced for this study, one copy from GenBank.
[b] Synonymous with *D. expansa.*
[c] Synonymous with *D. dilatata.*

were coded as present or absent using the approach of Simmons and Ochoterena (2000) as implemented in the program FastGap (Borschenius, 2009), and appended to the nucleotide data as additional characters. Congruence between the data partitions representing different portions of the plastid genome, and between the plastid and *pgiC* sequence data in the non-reticulate matrix, was assessed via the incongruence length difference (ILD) test (Farris et al., 1996), implemented as the partition homogeneity test in PAUP∗4.0d102 (Swofford, 2002). When used correctly this method can be informative (Hipp et al., 2004), though it is sensitive to a number of factors and can be prone to errors (Darlu and Lecointre, 2002), and thus should not necessarily be used as a basis for determining combinability of data (Barker and Lutzoni, 2002). This was a particular concern when considering the plastid vs. *pgiC* partitions of the non-reticulate dataset, and we therefore used the ILD to assess incongruence between these partitions, but we performed all phylogenetic analyses on the plastid and *pgiC* components of this dataset separately, as well as on the combined matrix, in order to compare topology, resolution, and support values between the combined matrix and its constituents (Xie et al., 2009).

Phylogenetic analyses were performed separately on the four data sets (full *pgiC*, reduced *pgiC*, plastid, and non-reticulate) using maximum parsimony (MP) in PAUPRat (Sikes and Lewis, 2001) and PAUP∗, maximum likelihood (ML) in Garli 2.0 (Zwickl, 2006) and RAxML 7.2.8 (Stamatakis, 2006; Stamatakis et al., 2008), and Bayesian inference (BI) in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). PAUPRat, RAxML, and MrBayes analyses were conducted on the Cyberinfrastructure for Phylogenetic Research (CIPRES) Portal 2 (http://www.phylo.org/portal2/) (Miller et al., 2010). For the non-reticulate dataset, which included plastid and *pgiC* sequences for 41 *Dryopteris,* separate analyses were conducted for the plastid and *pgiC* partitions, and for the entire combined matrix. The amount of homoplasy in the data was evaluated using consistency indices, both including (CI) and excluding (CI') autapomorphies (Givnish and Sytsma, 1997).

MP analyses with PAUPRat, based on Parsimony Ratchet (Nixon, 1999), were conducted using 1000 ratchets with 200 iterations per replicate. Support for clades was estimated using parsimony bootstrap analysis in PAUP* with 1000 replicates, TBR branch swapping, simple taxon addition with one tree held at each step, and a maximum of 100 trees saved per replicate in order to decrease the time needed to run large bootstrap replicates. All MP analyses were run both with and without the indel data included, in order to assess their effects on topology and clade support. These data were not included in the ML and BI analyses, as CIPRES does not provide a way to model standard (non-nucleotide) variables in its analyses.

For ML and BI analyses, the optimal model of molecular evolution for *pgiC* and each plastid locus was identified using jModeltest (Posada, 2008), and PhyML 3.0 (Guindon and Gascuel, 2003). The most likely phylogeny for each dataset was produced in Garli 2.0 (Genetic Algorithm for Rapid Likelihood Inference; Zwickl, 2006), using the optimal model of evolution for each partition. ML bootstrapping was executed in RAxML v. 7.2.8 (Randomized Accelerated Maximum Likelihood; Stamatakis, 2006; Stamatakis et al., 2008). The CIPRES portal allows only one model to be in place in RAxML analyses, though the dataset can be partitioned so that parameters for each partition may vary freely. Thus, for the plastid dataset, the most complex model for the set of loci was employed, and 1000 bootstrap replicates were completed. BI analyses were completed in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) on CIPRES, with different (optimal) models allowed for each region. Four independent runs of 10,000,000 generations were completed with four chains each (three heated, one cold), with a chain temp of 0.2 and uniform priors. Trees were sampled every 1000 generations. Upon completion, the first 25% of trees from each run were discarded as burn-in, and the remaining trees from the four runs combined. A majority-rule consensus of these trees showing posterior probabilities (PP) was produced with PAUP*.

2.4. Divergence time estimates

Divergence times were estimated using a Bayesian method (Drummond et al., 2006) implemented in the program BEAST 1.6.2 (Bayesian Evolutionary Analysis by Sampling Trees; (Drummond and Rambaut, 2007). This method simultaneously estimates phylogeny and molecular rates using an MCMC strategy. These analyses were performed separately on the plastid and *pgiC* portions of the non-reticulate dataset, and on the combined non-reticulate matrix including both plastid and *pgiC* components. The data sets were partitioned by gene region, and the optimal substitution model for each region was specified (though SYM is not an option in BEAST and so GTR was used instead, where appropriate). We implemented both birth–death and Yule process speciation priors, in separate analyses, to assess the effects on the results. Both are stochastic birth–death processes with a constant speciation rate (λ); the Yule model is a special case in which the extinction rate (μ) equals zero (Gernhard, 2008). Both models are appropriate for analysis of inter-species datasets (Gernhard, 2008). An uncorrelated lognormal (UCLN) model of rate change was implemented, with clock models unlinked between partitions. Analyses were run for 50,000,000 generations, with parameters sampled every 1000 generations. Tracer v1.5 (Rambaut and Drummond, 2007) was used to examine the posterior distribution of all parameters and their associated statistics, including estimated sample sizes (ESS) and 95% highest posterior density (HPD) intervals. TreeAnnotator v1.6.2 (Drummond and Rambaut, 2007) was used to summarize the set of post-burn-in trees and their parameters, in order to produce a maximum clade credibility (MCC) chronogram showing mean divergence time estimates with 95% HPD intervals. We implemented one calibration point, at the root node of *Dryopteris*, and modeled this as a lognormal prior with mean 2.0, stdev 0.5, and offset 35, in order to approximate the mean and 95% HPD intervals for the root of *Dryopteris* (42.4, 53.4–32.2 Ma) found by Sessa et al. (2012). Lognormal priors, which apply a soft maximum bound with declining probability towards older dates (Sauquet et al., 2012), are particularly appropriate for use with secondary calibration points, as the distribution can account for some of the error associated with the original estimate (Ho and Phillips, 2009; (Pirie and Doyle, 2012).

2.5. Reticulation network

A reticulation network showing inferred hybridization and/or genome merger events was constructed using the algorithm of Huber et al. (2006) implemented in the program PADRE (Lott et al., 2009a,b). The reduced *pgiC* dataset was used for this analysis, but species duplicates and all non-New World reticulate taxa were removed, in order to simplify the network and to focus on reconstructing reticulate histories for the New World taxa. The remaining matrix included the 41 putatively non-reticulate species of *Dryopteris* and 16 putatively reticulate taxa from the New World. The best ML topology for this dataset was obtained in Garli 2.0, and this multi-labeled topology was used as the input for PADRE.

3. Results

3.1. Nuclear analyses

Of the 89 *Dryopteris* species for which we sequenced *pgiC*, 50 had one distinct allelic variant (also referred to as copies), 26 had two distinct variants, and 13 had three distinct variants (Table 1). Species with more than one allelic variant are considered to be reticulate. In general, species known to be diploid based on previous research had a single sequence variant, while tetraploids had two and triploids had two or three (Table 1). The sole known hexaploid species, North American *D. clintoniana,* contained three sequence variants. There were some exceptions to these values at every ploidy level except hexaploid. Four taxa have been found to comprise a mixture of ploidy levels (Table 1), and they each produced one or two sequence variants. For 23 taxa we were unable to establish ploidy level from the literature, and these species produced one, two or three sequence variants each.

The full *pgiC* dataset consisted of 790 aligned nucleotides, of which 284 (36%) were variable, and 166 (21.0%) were parsimony-informative. Indels added an additional 87 characters, of which 40 were parsimony-informative. There were no unalignable nucleotides. The reduced *pgiC* dataset was also 790 nucleotides long, with 284 (36%) variable and 163 (20.6%) parsimony-informative sites, and the same numbers of indels. MP analysis of the full *pgiC* dataset (without indels) identified 127 most-parsimonious trees of length 504 steps. These shortest trees had a consistency index (CI) of 0.70, and CI' (excluding autapomorphies) of 0.59. The reduced *pgiC* dataset (also without indels) produced 625 most-parsimonious trees of length 502 steps, which also had a CI of 0.70 and a CI' of 0.59. MP analyses of the *pgiC* datasets produced consensus trees that were poorly resolved (34 out of 164 nodes for the full matrix and 35 out of 157 for the reduced matrix). Inclusion of indels in the MP and MP bootstrap analyses of the two *pgiC* datasets did not significantly alter topology, resolution, or clade support. These data were not included in subsequent ML and BI analyses because CIPRES does not provide a way to model them; however, the MP results indicate that additional informative characters provided by the indel data likely would not have led to additional resolution or increased support values.

The optimal model of evolution for both *pgiC* datasets was identified as SYM + Γ. ML analysis in Garli yielded a single best tree for

Fig. 1. Best maximum likelihood (ML) topology for all taxa included in this study (−ln = 4442.48), based on sequences of *pgiC*. Thickest lines indicate strong support (MP BS ⩾ 70%, ML BS ⩾ 70% and BI PP ⩾ 95%), medium lines indicate moderate support (either ML BS ⩾ 70% or BI PP ⩾ 95%), and thin lines indicate weak support (ML BS ⩽ 70% and BI PP ⩽ 95%). Support values are given as MP BS/ML BS/ BI PP. Not all weakly supported nodes are annotated, in order to preserve figure legibility. A, B, and C following taxon names indicate that a given species has multiple allelic variants of *pgiC*. Parentheses enclose the number of clones whose sequences are represented by that consensus allele sequence. Clades enclosed in gray boxes and labeled I–V refer to major clades identified in the plastid analysis of Sessa et al. (2012).

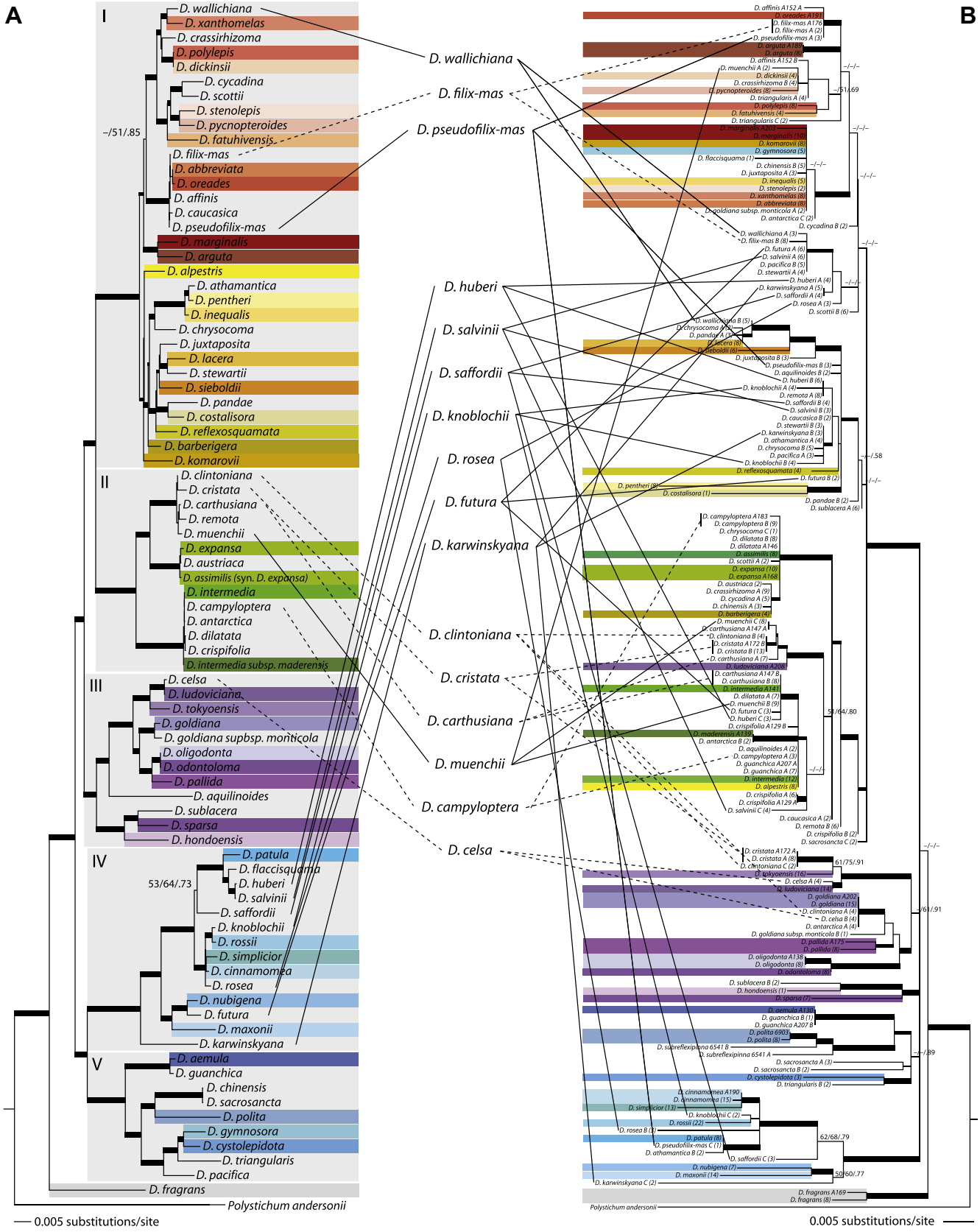

Fig. 2. Best maximum likelihood topologies for the plastid (A; −ln = 25109.34) and reduced *pgiC* (B; −ln = 4424.53) datasets. Support values and Clades I–V labeling are as in Fig. 1. Putative non-reticulate taxa, for which only a single *pgiC* sequence variant was recovered, are colored in both topologies. Reticulate taxa from the New World are listed in the alley, with lines indicating their positions in the plastid and nuclear phylogenies. Dotted lines indicate North American taxa, solid lines Latin American taxa. (For interpretation to colours in this figure, the reader is referred to the web version of this paper.)

the full *pgiC* dataset with −ln 4442.48 (Fig. 1), and for the reduced *pgiC* dataset a single best tree with −ln 4424.53 (Fig. 2B). Both trees were poorly resolved along the backbone, although several internal clades received moderate to strong support, and the two trees were nearly identical in topology with the exception of the species missing from the reduced dataset. *Dryopteris fragrans* was resolved as sister to the rest of the genus in the two best ML trees, though this relationship did not receive support in the other analyses. The topologies and support values produced by MP bootstrap, ML bootstrap, and BI analyses were highly congruent with one another for both *pgiC* datasets, with strongly supported clades generally receiving high support from all three analyses, and poorly supported clades generally not receiving support from any of them (Figs. 1 and 2). Based on the position of non-reticulate taxa, we were able to define the same five major clades as in the plastid phylogeny of Sessa et al. (2012) (Figs. 1 and 2). Clades II, IV, and V from the previous study were moderately, strongly, and weakly supported, respectively, in the *pgiC* phylogenies, while Clades I and III were each broken into two clades whose position relative to each other and to several of the other clades was unresolved.

Eighteen species were included in duplicate in the *pgiC* matrices, with one accession obtained from GenBank and one newly sequenced in the current study. Of these, 11 of 18 species produced only a single *pgiC* sequence variant. For ten of these, our newly generated sequence was either identical to the GenBank sequence or separated by only a few base pairs (and thus sister to or very nearby it in the phylogenies). For the eleventh species, *D. ludoviciana,* our sequence was placed in a totally different location in the phylogenies from the GenBank sequence; the latter was produced by Juslen et al. (2011). We recovered two sequence variants for each of the remaining seven species represented in duplicate. For four of these, our two variants were either sister to or very close to two corresponding GenBank variants, while for the final three species, only one of our two variants was present on GenBank and the other variant has apparently not previously been sequenced.

3.2. Plastid analyses

The plastid dataset consisted of 5672 aligned nucleotides, of which 1544 (27%) were variable and 1039 (18%) were parsimony-informative; no regions were unalignable. Indels added an additional 441 characters, of which 147 were parsimony-informative. MP analysis (without indels) identified 762 most-parsimonious trees of length 3038 steps, with CI 0.59 and CI' 0.50. MP analysis resulted in a strict consensus tree that was highly resolved (66 out of 82 nodes; unresolved nodes were all at the tips of the topology). As with the *pgiC* datasets, inclusion of indels did not significantly alter topology, resolution, or clade support, and these data were not included in subsequent analyses. ILD tests indicated no significant conflict between the various regions of the plastid genome ($P = 0.08$).

The optimal models of evolution for the plastid loci were identified as follows: GTR + Γ for *psbA-trnH*, *trnL-F*, *rps4-trnS*, *rbcL-accD* and *trnP-petG*, and SYM + I + Γ for *rbcL* and *trnG-trnR*. ML analysis in Garli produced a single best tree with -ln 25109.34, and MP bootstrap, ML bootstrap, and BI analyses produced highly resolved (66 out of 80 nodes), highly congruent topologies with similar support values (Fig. 2A). *D. fragrans* was resolved as sister to the remainder of the genus with strong support in all analyses, and the five major clades identified in Sessa et al. (2012) were also found here (Fig. 2A).

3.3. Non-reticulate analyses

The non-reticulate dataset consisted of 6462 aligned nucleotides, 5672 from the plastid matrix and 790 from *pgiC.* 1485 (23%) characters were variable and 812 (13%) parsimony-informative. Indels added an additional 415 characters, of which 98 were parsimony-informative. ILD tests indicated that there was significant conflict between the plastid and *pgiC* components of this dataset ($P = 0.01$). Based on visual inspection of topologies resulting from separate analyses of these partitions, conflicts between the trees were primarily caused by several species that were placed differently between them (including *Dryopteris gymnosora, D. alpestris, D. sparsa,* and *D. hondoensis;* see Figs. 3 and 4). Because of this discordance, we present results from separate analyses of the plastid and *pgiC* datasets.

The *pgiC* phylogeny generally had much lower support and resolution than the plastid phylogeny. MP analysis of the plastid component of the non-reticulate dataset produced a highly resolved strict consensus tree (36 out of 41 nodes), while the *pgiC* consensus phylogeny was poorly resolved (19 out of 41 nodes). Where the *pgiC* tree was resolved, it was largely congruent with the plastid topology, except for the above-mentioned species whose positions shifted. Analysis of both partitions together, as a combined dataset, produced a highly resolved MP tree (36 out of 41 nodes) that had the same structure as the plastid phylogeny and the well-resolved portions of the *pgiC* phylogeny. ML analyses produced single best trees for the plastid and *pgiC* datasets with −ln = 19342.35 and 3020.86, respectively. These were nearly identical in topology to the MP and ML bootstrap and BI topologies from each dataset, and to the chronograms resulting from the divergence time analyses (Section 3.4), though the chronograms are fully resolved while the best ML tree based on *pgiC* was not. Support values from all analyses for both the plastid and pgiC datasets are summarized on the chronograms in Figs. 3 and 4.

3.4. Divergence time estimates and reticulation network

After 50,000,000 generations, ESS values for all divergence time analyses (as viewed in Tracer) were well above the recommended threshold of 200 (Drummond and Rambaut, 2007), indicating that parameter space had been sufficiently sampled. The coefficient of variation indicated that the data were not evolving in a clock-like fashion (values above 0.5), and the UCLN model was thus the most appropriate model of rate variation for this set of loci. Analyses performed for each dataset using birth–death and Yule process speciation priors resulted in identical topologies with divergence time estimates that differed on average by less than one million years; we therefore present only the results from one of these analyses, using the Yule model, for each dataset. When analyzed separately, the plastid and *pgiC* portions of the non-reticulate dataset produced chronograms that differ significantly in topology (Figs. 3 and 4), and therefore also in divergence times. However, for clades that are largely congruent between the two topologies, node age estimates from the two datasets for the most recent common ancestor of a given set of taxa are generally within several million years of one another, with the *pgiC* analysis tending to propose older ages (Fig. 4). The chronogram resulting from analysis of the entire non-reticulate dataset closely resembled the plastid chronogram in both topology and clade ages. We present the results of the two separate analyses because of the degree of discordance between them (Figs. 3 and 4). Divergence time estimates within *Dryopteris* agree well with the dates found by Sessa et al. (2012), and diversification within the major clades of the genus (I–V) began between 25.6 and 12.6 Ma, based on the plastid analysis (Fig. 3).

The reticulation network produced by PADRE identified numerous genome merger events in the history of the New World taxa (Fig. 4). For several of the putatively reticulate New World species, the network analysis attributed one or more *pgiC* sequences to a single extant, non-reticulate species, while other *pgiC* sequences from reticulate species were found to be most closely related either to other reticulate species, or to groups of extant non-reticulate

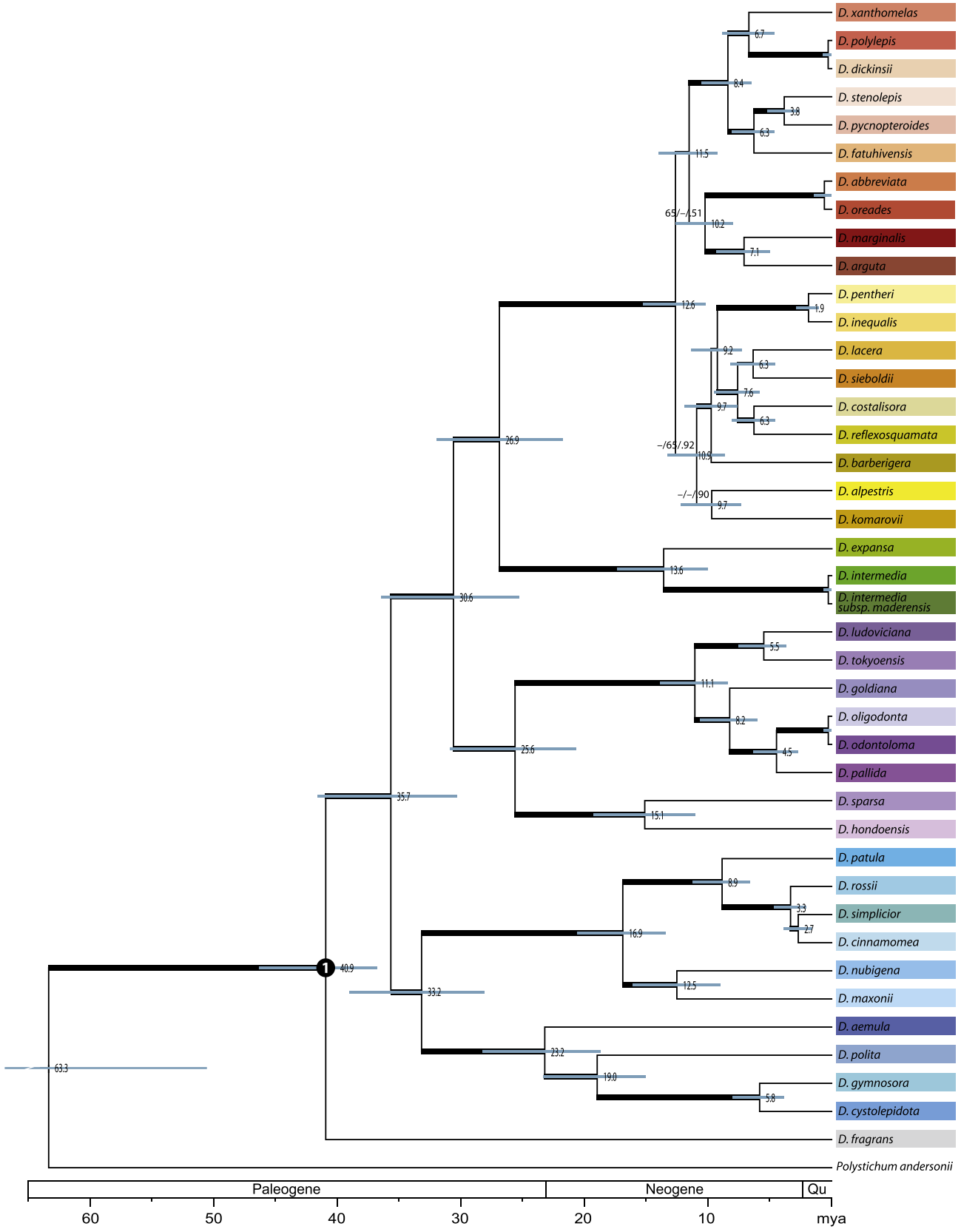

Fig. 3. Maximum clade credibility chronogram from BEAST analysis of the plastid component of the non-reticulate dataset, which included 41 species of *Dryopteris* (colored as in Fig. 2). Blue bars are 95% highest posterior density (HPD) intervals around mean divergence time estimates, which are given in millions of years (Ma) at most nodes. Black circle with 1 indicates the node used for calibration. Thick lines indicate support values as in Figs. 1 and 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

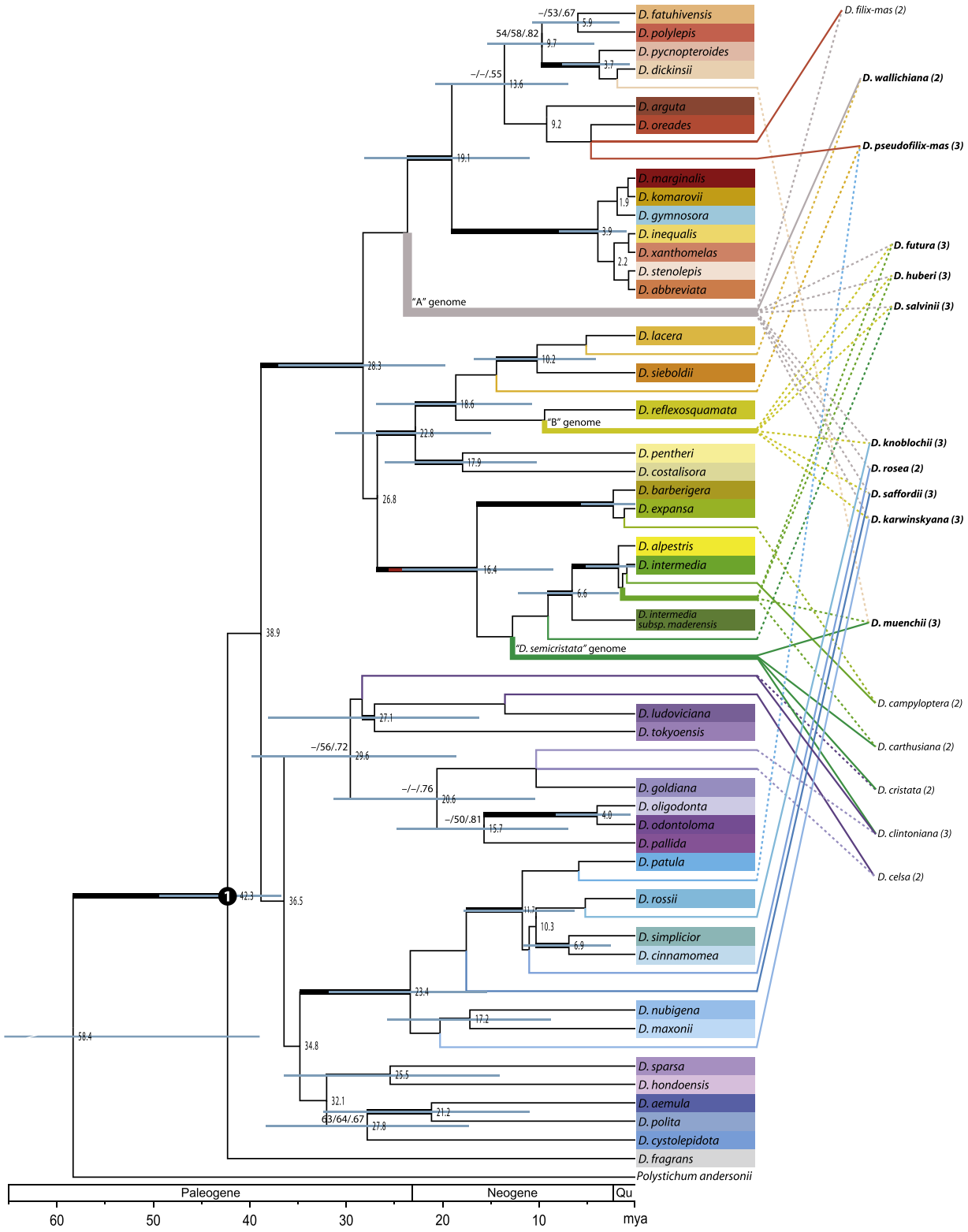

Fig. 4. Maximum clade credibility chronogram from BEAST analysis of the *pgiC* component of the non-reticulate dataset, with the reticulate network from PADRE superimposed and showing inferred genome merger events in the histories of the New World reticulate taxa, which are listed on the right with their number of *pgiC* copies indicated in parentheses. Blue HPD bars, mean ages, and calibration point are as in Fig. 3. Thick lines indicate support values as in Figs. 1–3, though nodes with mean ages but no blue HPD bar did not receive support in the MP BS, ML BS, or BI PP analyses, and were present in fewer than 50% of trees in the BEAST analysis and so were not annotated. The colored lines composing the reticulate network, which lead from the chronogram to the reticulate taxa on the right, are colored according to the genome contributions of the non-reticulate taxa; e.g. the "B" genome that is inferred to be most closely related to *D. reflexosquamata* is colored the same as that species. Sequences that are not most closely related to one particular non-reticulate taxon were given a color that is a mix of those colors assigned to the group of species to which it is most closely related. The thickness of these branches is proportional to the number of reticulate species inferred to carry a particular sequence or genome. The vertical portions of the network branches that connect to the chronogram were placed exactly halfway between each ancestral and daughter node; PADRE infers only topology, not divergence time, and so we do not mean to imply a specific date for the branching of reticulate lineages. For example, The "B" genome could have diverged from or been donated by *D. reflexosquamata* at any point between 18.6 Ma, when that species diverged from its closest relative, and the present. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

species. One such group was comprised of single *pgiC* sequences from nine of the Latin American reticulate taxa that were all most closely related to each other, and together were placed sister to a clade of extant, mostly Asian species; we labeled this group of sequences the "A" genome (Fig. 4). Six other sequences from reticulate taxa similarly formed a group sister to extant *D. reflexosquamata*; this group was labeled the "B" genome (Fig. 4). Most species had one *pgiC* sequence whose position was congruent with the species' placement in the plastid phylogeny (Fig. 2A), but three Latin American species (*D. huberi, D. salvinii,* and *D. futura*) had no *pgiC* sequence congruent with their position in the plastid phylogeny.

For the five North American allopolyploids included in the "semicristata" hypothesis (*D. campyloptera, D. celsa, D. carthusiana, D. cristata,* and *D. clintoniana*), the reticulate network combined genomes of their inferred progenitor taxa as predicted by that hypothesis. *D. campyloptera*'s two genomes were assigned to *D. expansa* and *D. intermedia*, and *D. celsa*'s to *D. goldiana* and *D. ludoviciana*. The three polyploids putatively descended from *"D. semicristata", D. clintoniana, D. cristata,* and *D. carthusiana,* shared one *pgiC* sequence in common that was not assignable to any single extant diploid taxon, and which was also found in Latin American *D. muenchii*. This group was labeled the *"D. semicristata"* genome (Fig. 4). Each of these three allopolyploids' second progenitors was identified in accordance with the "semicristata" hypothesis, including *D. intermedia* for *D. carthusiana* and *D. ludoviciana* for *D. cristata*. Two of hexaploid *D. clintoniana*'s three *pgiC* sequences were assigned to the *"D. semicristata"* genome via *D. cristata*, one of its putative parents, and the third to *D. goldiana*.

4. Discussion

The current study is one of the largest to date to examine reticulate evolution in a widespread group of ferns (only a single study of comparable geographic scope but many fewer taxa has been conducted, on *Crepidomanes* (Nitta et al., 2011)), and it is the most thoroughly sampled nuclear analysis of *Dryopteris* yet produced. The one previous study employing a nuclear marker for the genus (Juslen et al., 2011) included only 24 species, while here we sample 89. Reticulate evolution clearly runs rampant in *Dryopteris*, and we find topological incongruence indicative of reticulation among the North American and Latin American taxa, as well as among some species from Europe and Asia. As the major aim of this study was to examine reticulate evolution in the New World, we will focus our discussion on the phylogenetic relationships of the North and Latin American species.

4.1. The North American "reticulate complex"

The "reticulate complex" of North American *Dryopteris* has been the subject of extensive study historically (Walker, 1959; Walker, 1961; Peterson and Fairbrothers, 1983; Werth, 1991; Hutton and Stein, 1992; Stein et al., 2010), and numerous hypotheses to explain the parentage of the allopolyploids in this group have been put forth. The bulk of evidence from morphological, cytological, genetic, and chemical evidence thus far has supported the "*D. semicristata*" hypothesis first suggested by Walker (1955), which implicates four extant diploid species as direct participants in the parentage of five sexual allopolyploids (Table 2). The hypothesis hinges on a fifth, putatively extinct diploid taxon, "*D. semicristata*", which was invoked as a common parent of two of the allotetraploids (Table 2). As plastids are maternally inherited in ferns (Gastony and Yatskievych, 1992; Vogel et al., 1998), Sessa et al. (2012) were able to provide the first plastid sequence evidence for the maternal progenitors in this complex, and their results supported the "*D. semicristata*" hypothesis. Using a subset of the same data matrix, we replicate those results here (Fig. 2A), with each of the North American polyploids strongly supported as sister to one of its putative parents, and the putative offspring of *"D. semicristata"* forming a strongly supported clade.

The addition of nuclear data in the current study provides evidence for the second progenitor of each of the polyploids. In the *pgiC* phylogenies, one copy from each of the four tetraploids recapitulates, with strong support, the relationship found in the plastid phylogeny, while the other copy occurs with the second diploid parent as predicted by the "*D. semicristata*" hypothesis (Figs. 2B and 4, Table 2). For two of the tetraploids, *D. campyloptera* and *D. celsa,* the inferred paternal *pgiC* sequences are indistinguishable from those of diploids *D. expansa* and *D. goldiana,* respectively (Fig. 2B), to which species the reticulate network assigned one genome of each of the polyploids (Fig. 4), confirming the role of these diploids as the tetraploids' parents. The remaining two tetraploids, *D. cristata* and *D. carthusiana,* are putative offspring of "*D. semicristata"*, and our nuclear sequence data again support the existing hypothesis and the role of the missing diploid in the parentage of these species (Figs. 2B, 4). Plastid sequence data place these two taxa together in a strongly supported clade that also includes *D. clintoniana,* a putative hexaploid between *D. cristata* and *D. goldiana,* as well as triploid apomicts *D. muenchii* and *D. remota* (Fig. 2A). The reticulate network recovers a sequence group composed of these species (minus *D. remota,* which is Asian and therefore was not included in the reticulate analysis) that is not attributable to any extant diploid species (the *"D. semicristata"* genome, Fig. 4). We therefore conclude that the missing taxon was the maternal donor to all of these species (it would have been the maternal "grandmother" of *D. clintoniana* via *D. cristata).* These results accord with those from a recent study of the *"D. semicristata"* group by Stein et al. (2010), who concluded, based on isozymes and chloroplast restriction site analyses, that *"D. semicristata"* had been the maternal progenitor of *D. cristata* and *D. carthusiana.* The paternal copies for the three North American species are found with their predicted second parents in our *pgiC* phylogenies and reticulate network: *D. cristata* in a clade with *D. ludoviciana, D. carthusiana* sister to *D. intermedia,* and *D. clintoniana* with *D. goldiana. D. clintoniana's* maternal progenitor is *D. cristata,* and so the second

Table 2
Members of the North American "reticulate complex" of *Dryopteris*. Allopolyploids and their putative diploid parents (ploidy indicated) are given according to the "*D. semicristata*" hypothesis of Walker (1955); maternal and paternal designations are based on the current study (see Fig. 2). The ploidy of *"D. semicristata"* is unknown, but it is hypothesized to be diploid (Montgomery and Wagner, 1993). Dates for each putative parent indicate when they diverged from their closest living relatives; dates given are derived from a comparison of the plastid and *pgiC* divergence time analyses, but generally reflect the plastid analysis, for which the relevant nodes were better resolved and more highly supported than in the *pgiC* analyses (see Figs. 3 and 4). Hybridization and polyploidization are inferred to have happened subsequent to the later of the pair of dates for each polyploid species.

Allopolyploid	Putative maternal parent	Putative paternal parent	Inferred age of polyploid (ma)
D. campyloptera (4×)	*D. intermedia* (2×), 13.6 ma	*D. expansa* (2×) 13.6 ma	⩽13.6
D. carthusiana (4×)	"*D. semicristata*" (2×?), 13.6 ma	*D. intermedia* (2×), 13.6 ma	⩽13.6
D. celsa (4×)	*D. ludoviciana* (2×), 5.5 ma	*D. goldiana* (2×), 8.2 ma	⩽5.5
D. clintoniana (6×)	*D. cristata* (4×), 5.5 ma	*D. goldiana* (2×), 8.2 ma	⩽5.5
D. cristata (4×)	"*D. semicristata*" (2×?), 13.6 ma	*D. ludoviciana* (2×), 5.5 ma	⩽5.5

and third *D. clintoniana* copies are found sister to *D. cristata's* paternal and maternal species, which are, respectively, *D. ludoviciana* and the *"D. semicristata"* genome group (Fig. 4).

We note that the paternal copies of *D. cristata* and the third copy of *D. clintoniana* are actually sister to *D. tokyoensis* in our *pgiC* phylogenies (Figs. 1 and 2), with moderate support. This group is then sister to *D. ludoviciana,* the hypothesized donor of those sequences, plus *D. celsa,* another of its polyploid offspring. The placement of these copies from *D. cristata* and *D. clintoniana* in the reticulate network is equivocal with respect to *D. ludoviciana* and *D. tokyoensis* (Fig. 4). *D. tokyoensis* has previously been cited as a potential player in this complex (Hickok and Klekowski, 1975; Wíden and Britton, 1985), and our *pgiC* sequence data provide support for its involvement. However, the branch placing the polyploid sequences with *D. tokyoensis* is relatively short and not strongly supported (Figs. 1 and 2). Incomplete lineage sorting may therefore also account for the observed relationship, if the time between the divergence of *D. tokyoensis* and *D. ludoviciana* and the coalescence point of the *pgiC* sequences in these taxa and the polyploids was short (Joly et al., 2009; Yu et al., 2011).

D. ludoviciana and *D. tokyoensis* are sister diploid species whose estimated divergence time varies considerably between our plastid and *pgiC* dating analyses. The former places the split at approximately 5.5 Ma, congruent with the plastid analyses of Sessa et al. (2012), who attributed the divergence to vicariance via the Bering Land Bridge between North American and Asia, where the species are now restricted. The *pgiC* analysis recovers a much more ancient divergence date, at ca. 27.1 Ma (Fig. 4). Divergence times for all comparable nodes within this clade are considerably older in the *pgiC* chronogram than the plastid, which may be due to differences in substitution rate between these genomes (Soltis et al., 2002). However, given the generally poor resolution and low support of the *pgiC* phylogenies, and the large size discrepancy between the plastid and *pgiC* matrices (5672 vs. 790 characters, respectively), we are more confident in the dates based on the richer plastid dataset. Data from one or more additional nuclear markers will be essential in order to clarify whether *D. ludoviciana* or *D. tokyoensis* is the paternal progenitor of *D. clintoniana* via *D. cristata,* and to further explore relative divergence time estimates based on the plastid vs. the nuclear genome.

Despite the differences in divergence time estimates between the plastid and *pgiC* analyses, we can begin to infer approximate dates of formation for the North American allopolyploids, and we can estimate a window of existence for "*D. semicristata*". The group of sequences putatively representing maternal parentage by the "*D. semicristata*" genome is strongly supported as sister to *D. expansa* in the plastid analysis (Fig. 2A), suggesting that the latter is "*D. semicristata*"'s closest living relative. In the *pgiC* analysis, however, the "*D. semicristata*" genome is most closely related to *D. intermedia* plus *D. intermedia* subsp. *maderensis* and *D. alpestris,* though with only moderate support (Fig. 2B), and this relationship was recovered by the reticulate network (Fig. 4). Despite having slightly different species compositions between the two phylogenies, the entire *D. expansa-D. intermedia* clade has a most recent common ancestor of similar age in the two divergence time analyses: 13.6 Ma from the plastid analysis and 16.4 Ma from the *pgiC* analysis. Whether *D. expansa* or an ancestor of the *D. intermedia* group was more closely related to *"D. semicristata",* the latter would have diverged from its closest relative, hybridized with several other species to produce the extant polyploid lineages, and then gone extinct in at most the last 16 million years. Based on divergence time estimates for the second parents of each of the North American polyploids (relying largely on the better-resolved and more highly supported plastid phylogeny and chronogram; Fig. 3), approximate dates of initial formation can be inferred for each species (Table 2). Based on the inferred dates of origin of the "*D. semicristata*" genome recipients, we can then estimate a time period for the missing taxon's existence: we hypothesize that its origin was not earlier than ca. 16.4 Ma, and in order to have hybridized with *D. ludoviciana* to form *D. cristata,* it must still have been extant at least 5.5 Ma, if not more recently.

The *pgiC* sequence data newly generated for the current study provide support for the "*D. semicristata*" reticulation hypothesis, including the existence of the missing ancestor. However, a recent study also based on this marker (Juslen et al., 2011) ostensibly found evidence to reject this same hypothesis. Their conclusions were based on analysis of *pgiC* and a single plastid locus, *trnL-F.* As we do here, they also identified the sequences shared by *D. cristata* and *D. carthusiana* in the plastid phylogeny as donated by a shared maternal progenitor (which we infer to be *"D. semicristata").* However, in their nuclear phylogeny, their sole accession of *Dryopteris ludoviciana* was resolved in a different location than our accession of that species, and their *D. ludoviciana* was strongly supported as sister to *D. cristata* plus *D. carthusiana.* They therefore concluded that *D. ludoviciana* was in fact the maternal donor to those taxa. They correctly identified *D. intermedia* as the paternal progenitor of *D. carthusiana,* but were unable to identify a second parent for *D. cristata,* as the position of the putative paternal copies of *D. cristata* in their topology was unresolved. Their rejection of the "*D. semicristata*" hypothesis hinges on the single accession of *D. ludoviciana* included in their analysis, and this species' placement is at odds with its location in our analyses. Their results also conflict directly with those of Stein et al. (2010), who rejected *D. ludoviciana* as the maternal progenitor of *D. carthusiana* and *D. cristata* based on data from isozymes and chloroplast restriction site analyses. Rather than rejecting a longstanding hypothesis outright based on this single sequence, a more parsimonious explanation would seem to be contamination or other error associated with the generation of that sequence. If that were the case, it would also explain why Juslen et al. could not locate the paternal progenitor of *D. cristata:* it should be *D. ludoviciana,* which was erroneously placed elsewhere in their phylogeny. They did not sample *D. ludoviciana's* closest diploid relative, *D. tokyoensis,* in their *pgiC* analysis, which is surprising given that *D. tokyoensis* has been cited in the past as a potential participant in this complex (Hickok and Klekowski, 1975; Wíden and Britton, 1985). If they had included it, as we did, they likely would have found that the paternal copies of *D. cristata* grouped with it, not necessarily reflecting direct parentage of *D. cristata* by *D. tokyoensis,* but rather the close relationship between the latter and *D. ludoviciana.* This would perhaps have given Juslen et al. cause to question *D. ludoviciana's* placement in their nuclear phylogeny. They also did not include *D. ludoviciana* in their *trnL-F* analysis, which would have provided further insight into its placement in the phylogeny.

Clearly, further sampling of all species involved, particularly *D. ludoviciana,* is needed before a definitive conclusion on this complex can be reached. In addition, given the relatively weak support obtained in both studies employing *pgiC* to date, data from at least one additional nuclear marker will be helpful in clarifying the history of these taxa. We currently have such a study underway and hope to report those results in the near future. In the meantime, our analyses based on plastid and *pgiC* sequence data provide strong support for the "*D. semicristata*" hypothesis as an accurate reflection of the history of this group.

4.2. *D. filix-mas*

In addition to the species formally recognized as members of the "reticulate complex" in North America, the origins of *Dryopteris filix-mas* have long been a source of debate. This species is thought to be either an auto- (Wagner, 1971) or an allotetraploid (Manton, 1950), and it is widespread in northern North America as well as in

Europe and parts of Asia, though it is unclear whether the forms on the various continents are in fact the same taxon with a shared evolutionary history (Fraser-Jenkins, 1976). Suggested progenitor taxa for *D. filix-mas* have included *D. oreades* (Manton, 1950), members of the *D. villarii*/*D. pallida* species complex (Wíden et al., 1970), *D. marginalis* (Wagner, 1971), *D. abbreviata* (Wíden et al., 1971), and *D. caucasica* (Fraser-Jenkins and Corley, 1972).

We included only one North American accession of *D. filix-mas* in the current analyses, and in our plastid analysis it was strongly supported as part of a clade also containing *D. oreades* and *D. abbreviata* (Fig. 2). We found it to contain two copies of *pgiC* that were separated by only a few base pairs' difference. This was enough, however, to place them separately (though nearby one another) in our nuclear phylogenies (Figs. 1 and 2). One copy is indistinguishable from *D. oreades,* and the other is a member of the "A" genome group, according to the reticulation network (Fig. 4). Juslen et al. (2011) also found two *pgiC* variants, though in separate accessions; within accessions their sequences from clones were apparently identical. They concluded that the two variants were similar enough to be considered the same, single allele in all individuals, and so dismissed both auto- and allopolyploidy in the origins of *D. filix-mas*. Their accessions, which were identical in sequence to *D. oreades* and our *D. filix-mas* A copy (Figs. 1 and 2), were all European, while our accession was from the Pacific northwest of North America. Both studies are at this point insufficient to resolve the provenance of this taxon, but given the sequence similarity of *D. filix-mas* from Europe and North America to *D. oreades*, the latter would seem to be a likely progenitor, and these data lend support to a common origin of the Old and New World forms of *D. filix-mas*. The "A" genome lineage, a member of which we infer to be one putative parent of *D. filix-mas*, diverged from its sister group sometime between 28.3 and 19.1 Ma according to our *pgiC* divergence time analyses (Fig. 4). This is the oldest of the putative parental lineages of *D. filix-mas* (*D. oreades* being less than 1 or 9.2 Ma, according to the plastid and *pgiC* analyses, respectively) and we therefore infer that its date of first formation could not have been longer ago than ca. 28 million years.

4.3. Latin American Dryopteris

Unlike the North American species, *Dryopteris* in Latin America have historically received little attention. Almost nothing was known about their relationships prior to the work of Sessa et al. (2012), who demonstrated that 14 of the 18 Latin American species form a well-supported clade (based on analysis of the plastid genome) sister to an Asian group from which it diverged ca. 32.3 ma. The remaining four Latin American species are more closely related to North American, Eurasian, and Pacific species and presumably represent independent introductions to Central and South America. Ploidy estimates are available for only six Latin American species (Table 1): *D. arguta, D. futura,* and *D. patula* are diploids, *D. muenchii* and *D. pseudofilix-mas* are apogamic triploids, and *D. wallichiana* has been found to harbor diploid, triploid, and tetraploid individuals. We found one *pgiC* variant for each of the diploids except *D. futura,* for which we found three; we also found three variants for each of the apogamic triploids, and two variants for *D. wallichiana,* supporting a tetraploid origin for our accession. The apparent allelic variation in *D. futura* despite its status as a diploid species (based on chromosome counts; Smith, 1975) may be due to a homoploid hybrid origin (discussed below), or may be evidence that *Dryopteris* harbors allelic heterozygosity at this locus. Although most diploids appear to have a single variant of *pgiC*, tetraploids two, and triploids and hexaploids three (based on our *pgiC* sequences for the North American species, whose ploidy is well established), the potential for heterozygosity may muddle attempts to draw conclusions based on this locus. With that in mind, we will focus here on developing hypotheses for the origins of the reticulate Latin American taxa that may be tested in the future using additional nuclear markers. Reliable chromosome counts and/or genome size estimates for all of the Latin American taxa are also highly desirable, and would add greatly to our understanding of this complicated group.

Our *pgiC* data reveal a complicated reticulation scenario for the evolution of several of the Latin American species. Of the 18 *Dryopteris* from Central and South America, we found seven species to have a single *pgiC* variant, two taxa with two variants, and the remaining eight taxa with three variants (Fig. 1, Table 1). (Despite multiple rounds of cloning we were only able to obtain a single sequence for *D. flaccisquama,* and so it is excluded from subsequent discussion.) In the absence of ploidy estimates for most of these taxa, but based on our findings for the North and Latin American species for which ploidy is known, we assume that species with only a single sequence variant are diploids, though ploidy needs to be established using quantitative analysis of genome size or cytological analysis of meiotic cells. Under this assumption, however, we consider seven species that are homozygous for *pgiC* to be diploids, and the remaining ten species to be either of reticulate origin due to hybridization, incomplete lineage sorting or some other process; heterozygous at this locus; or both.

4.4. Latin American diploids

Of the seven non-reticulate, putatively diploid Latin American taxa, six belong to the well-supported Clade IV in phylogenies based on the plastid genome (Figs. 1–3): *D. cinnamomea, D. maxonii, D. nubigena*, *D. patula, D. rossii,* and *D. simplicior*. Several of these species were described by Mickel and Smith (2004) as comprising the *"Dryopteris patula"* complex in Central America, including *D. cinnamomea, D. patula, D. rossii,* and *D. simplicior*. Close relations among these species received support in a recent morphometric analysis (Hernandez-Hernandez et al., 2009), and our data indicate that these four are each others' closest diploid relatives within clade IV, forming a subclade which diverged from an ancestor shared with diploids *D. nubigena* and *D. maxonii* ca. 16.9 Ma (or 23.4 Ma according to the *pgiC* chronogram; Figs. 3 and 4).

The remaining diploid species is *D. arguta*, whose range extends from western Mexico into western North American. *D. arguta* is sister to North American diploid *D. marginalis* in our plastid phylogenies (Figs. 2A and 3), though in the *pgiC* phylogenies it is more closely related to a group of species including *D. oreades* and several other Asian taxa (Figs. 1, 2B and 4). According to the current plastid divergence time analysis and the analysis of Sessa et al. (2012), *D. arguta* and *D. marginalis* diverged ca. 7 Ma, both having descended from an ancestor that arrived independently in the New World as a result of either vicariance or long-distance dispersal from Asia (Sessa et al., 2012). The *pgiC* divergence time analyses reported here suggest that *D. arguta* diverged from its closest relative earlier than this (at least 9 million years ago). Whether *D. arguta* diverged from *D. marginalis* in North America or from *D. oreades* in Asia, it is clearly not closely related to the other diploid taxa in Central and South America. Additional sampling of *D. arguta* and all taxa potentially related to it will be required in order to resolve its exact origins.

4.5. Latin American reticulate species

The ten remaining Latin American *Dryopteris* species each produced either two or three copies of *pgiC* (labeled A–C; Figs. 1 and 2), and are therefore considered to be of reticulate origin. Nine of these species contained one *pgiC* sequence that was placed in the "A" genome group by the network analysis (Fig. 4). These sequences are more closely related to each other than to sequences

from any other species, and together they are sister to a set of species in Clade I that are mostly endemic to Asia. The clades in the *pgiC* phylogenies that include these reticulate sequences and the various Clade I taxa generally receive low support in the *pgiC* phylogenies (Figs. 1 and 2). Another large group of *pgiC* sequences from Latin American taxa constitutes the "B" genome group, which is sister to *Dryopteris reflexosquamata*, which is also a member of Clade I (Fig. 4). Together these reticulate sequences and *D. reflexosquamata* correspond to a clade that receives moderate support in the *pgiC* phylogenies (Figs. 1 and 2).

Several of the 10 putatively reticulate Latin American species followed one of two patterns. Three species have one *pgiC* copy from the "A" genome, one from the "B" genome, and one most closely related to the *D. intermedia* group (Pattern 1). The three species following Pattern 1 (*D. futura, D. huberi,* and *D. salvinii*) do not have *pgiC* sequences that are congruent with their placement in the plastid phylogeny (Figs. 2A and 4). Four species have one *pgiC* copy from the "A" genome, one from the "B" genome, and one most closely related to a putative Latin American diploid (Pattern 2). The latter copy in each species corresponds to its placement in the plastid phylogeny (Figs. 2A and 4). Species following Pattern 2 include *D. knoblochii, D. rosea, D. saffordii,* and *D. karwinskyana*, though *D. rosea* does not possess the "B" genome copy and we infer that this is due to gene loss, since it otherwise fits Pattern 2. The three final Latin American reticulate species, *D. wallichiana, D. pseudofilix-mas,* and *D. muenchii* did not adhere to either of these Patterns, though *D. wallichiana* does possess an "A" genome sequence that corresponds roughly to its placement in the plastid phylogeny (Fig. 2A).

The close affinity of the Latin American "A" and "B" genomes to Clade I taxa is surprising, as nearly all members of this clade are restricted to Asia. Our *pgiC* phylogenies lack resolution and support at many nodes within the Clade I group (Figs. 1 and 2), and the placement of species within subclades of Clade I differs between the plastid and *pgiC* phylogenies (Fig. 2). The branches at the poorly supported nodes are mostly very short, indicating that speciation likely occurred rapidly, and may have been accompanied by incomplete sorting of *pgiC* alleles. It is also possible that we have incompletely sampled variation in *pgiC* for these taxa due to PCR or cloning bias, and are considering species to be non-reticulate that in fact *are* reticulate; this may include species such as *D. barberigera, D. alpestris, D. gymnosora,* and *D. inequalis,* for which we found only one copy of *pgiC*, but whose positions are discordant between the *pgiC* and plastid topologies.

The observed distribution of *pgiC* alleles could be attributed to several mechanisms that are often invoked in reticulate evolutionary scenarios, including recent hybridization or persistent allelic diversity due to gene duplication and paralog presence, incomplete lineage sorting (ILS), or ancient introgression (Cronn et al., 2003; Mason-Gamer, 2004; Linder and Rieseberg, 2004; Meng and Kubatko, 2009). With data from only a single nuclear marker it is difficult to dismiss with certainty any of these phenomena, particularly gene duplication, introgression, and ILS, which may affect only one or a few loci and thus require data from multiple markers to diagnose (Maddison and Knowles, 2006). However, based on the *pgiC* sequences generated in the current study, we favor hybridization as the primary explanation for the origins of the Latin American reticulate *Dryopteris* taxa. Although incomplete lineage sorting and/or ancient introgression may have occurred in this group, and ILS seems particularly likely as the cause of the plastid vs. *pgiC* discordance in Clade I, neither phenomenon alone can account for sequences that are most closely related to Asian taxa (the "A" and "B" genomes) occurring in species that are restricted to the Americas. The branches leading to the divergence of the "A" and "B" genome groups are generally short (Figs. 1 and 2), and within these groups, the reticulate sequences are identical or vary by only a few nucleotides. This supports a more recent origin, rather than gene duplication or incomplete sorting of alleles that then remained unchanged or nearly so over millions of years (Holder et al., 2001; Yu et al., 2011).

Hybridization seems to best fit the available data as the most likely explanation for this group's history, though it was not necessarily straightforward. Given that we found three distinct variants of *pgiC* in both a known diploid (*D. futura*) and two known triploids (*D. muenchii* and *D. pseudofilix-mas*), there is the potential for both *pgiC* heterozygosity and hybridization, including trigenomic triploid hybridization (Laureto and Barkman, 2011), which has been documented in ferns in *Equisetum* (Bennert et al., 2005). For the seven species that adhere to either Pattern 1 or Pattern 2, we propose the following hybridization scenario: two separate Asian species, representing the "A" and "B" genomes, respectively, first hybridized in Asia. The resulting hybrid ("AB") then dispersed to the Americas, where subsequent diversification took place and genomes from various Latin American species were incorporated through hybridization events to produce the Pattern 2 taxa, and through chloroplast capture involving the *D. intermedia* group to produce the Pattern 1 taxa. The initial hybridization between species "A" and "B" could either have happened in Asia or in the Americas if these two species had first dispersed separately to the New World; the first scenario is much more parsimonious, however, as it requires only one long-distance dispersal event instead of two.

Modifications and additions to this hybridization hypothesis are needed to accommodate various species: *D. rosea* only has two *pgiC* sequences, and is missing a "B" genome sequence (Fig. 4). The most parsimonious explanation under our proposed scenario is that it lost this genome at some point following its formation. Alternatively, *D. rosea* could have formed by hybridization between its maternal parent (an unknown, Latin American species whose closest relatives are *D. rossii* plus *D. simplicior* and *D. cinnamomea*; Fig. 4) and a haploid gamete of its paternal parent that only contained the "B" genome, having lost the "A" genome prior to the formation of *D. rosea*. For the Pattern 1 taxa (*D. futura, D. huberi,* and *D. salvinii*), there is no *pgiC* sequence in a comparable position to the species' placement in the plastid phylogeny (Fig. 2A). The third *pgiC* copies for these taxa are instead most closely related either to North American *D. intermedia*, or to a group including *D. intermedia, D. alpestris*, and a European subspecies of *D. intermedia*, *D. intermedia* subsp. *maderensis*. Assuming that these sequences represent the un-accounted for maternal contributions to these species, the most likely explanation is introgression of the plastid genomes from several Latin American species into the *D. intermedia* group species prior to the hybridization events that produced the Pattern 1 taxa (Laureto and Barkman, 2011). This would account for the Pattern 1 group having plastid genomes most closely related to Latin American species, which were contributed to them via species from the *D. intermedia* group. This would have involved three separate chloroplast capture events: capture of *D. nubigena* by *D. intermedia*, which then hybridized with "AB" to produce *D. futura*, and two separate captures of *D. patula* by *D. intermedia* and a relative of the *D. intermedia* group, which then hybridized with "AB" to produce *D. huberi* and *D. salvinii*, respectively (Table 3).

Approximate dates for the events just described (Table 3) can be inferred from our divergence time analyses (Figs. 2 and 3). It is difficult to assign precise dates to the events involving the "A" and "B" genomes, as the composition of the closest relatives to these sequence groups differs between the plastid and *pgiC* topologies. According to the *pgiC* divergence time analysis, the "A" genome is most closely related to a group of species that diverged from their closest relative 28.3 Ma, and so the lineage that donated the

Table 3
Latin American reticulate taxa and their putative progenitors, based on the current study. "A", "B", and "AB" indicate species carrying the "A" and "B" genomes, and a hybrid between these species, respectively. Dates are given for the putative parents and indicate when they diverged from their closest relatives; they are generally the more recent of the dates from a comparison of the plastid and *pgiC* chronograms (see Figs. 3 and 4). Hybridization events are inferred to have happened subsequent to the later of the pair of dates for each reticulate species.

Species	Putative maternal progenitor	Putative paternal progenitor(s)	Inferred age of reticulate sp.
D. futura	*D. nubigena via D. intermedia*, 1.8 ma	"AB", 6.3 ma	⩽1.8 Ma
D. karwinskyana	*D. nubigena, D.* max*onii*, 16.9 ma	"AB", 6.3 ma	⩽6.3 Ma
D. knoblochii	*D. rossii*, 3.3 ma	"AB", 6.3 ma	⩽3.3 Ma
D. huberi	*D. patula via D. intermedia*, 1.8 ma	"AB", 6.3 ma	⩽1.8 Ma
D. muenchii	*"D. semicristata"*× *D. dickinsii*, 13.6 ma	*D. intermedia*, 1.8 ma	⩽1.8 Ma
D. pseudofilix-mas	*D. oreades hybrid*, 0.8 ma	*D. patula*, 8.9 ma	⩽0.8 ma
D. rosea	*D. patula group*, 8.9 ma	"AB", 6.3 ma	⩽6.3 Ma
D. saffordii	*D. patula group*, 16.9 ma	"AB", 6.3 ma	⩽6.3 Ma
D. salvinii	*D. patula via D. intermedia group*, 1.8 ma	"AB", 6.3 ma	⩽1.8 Ma
D. wallichiana	"A", 12.6 ma	*D. lacera*, 6.3 ma	⩽6.3 Ma

"A" genome to the Latin American species would have arisen between 28.3 and 19.1 Ma; the hybridization event with "B" could have happened any time subsequent to that. The most recent common ancestor of most of the Clade I species that are sister to the "A" genome in the plastid analysis dates to 12.6 Ma (Fig. 3). The "B" genome is most closely related to *D. reflexosquamata*, which diverged from its closest relative either 6.3 or 18.6 Ma, according to the plastid and *pgiC* analyses (Figs. 3 and 4). Thus a conservative approach suggests that the hybridization event involving "A" and "B" would have happened within the last 6.3 million years. The Latin American species that would have hybridized with "AB" to produce the Pattern 2 species were all extant by this point, according to both the plastid and *pgiC* analyses (Figs. 3 and 4). The chloroplast capture events hypothesized in the ancestry of the Pattern 1 species could have occurred prior to the arrival of "AB" in the New World, and the species whose plastids were captured (*D. nubigena* and *D. patula*) would have overlapped temporally with the capturers, members of the *D. intermedia* group, for at least the last 11.7 Ma (in the case of *D. patula*) and up to 17.2 ma (for *D. nubigena*). Although *D. intermedia* is now restricted to higher latitudes in North America, during the glacial cycles that occurred during this time period and more recently in the Pleistocene (Shafer et al., 2010), its range would likely have been pushed southward. Short-distance dispersal (Haufler, 2007) thus becomes a conceivable mechanism for bringing spores of *D. intermedia* or its relatives into contact with those of the Central American species to enable the chloroplast capture events.

The remaining three Latin American reticulate taxa, *D. muenchii, D. pseudofilix-mas,* and *D. wallichiana,* display slightly different distributions of *pgiC* alleles than the other seven species, and we propose separate origins for them. *D. wallichiana* is an intriguing taxon and deserves further study. It has a pantropical distribution (Geiger and Ranker, 2005) and is thought to be a diploid in the Old World but a tetraploid or apogamous triploid in the New World (Geiger and Ranker, 2005; Mickel and Smith, 2004). Our accession, from Costa Rica, had two *pgiC* copies, one congruent with the "A" genome and one most closely related to *D. lacera,* which diverged from its closest relative 10.2 or 6.3 Ma (Figs. 3 and 4). We infer the former to be the maternal copy, as the plastid phylogeny places *D. wallichiana* sister to *D. xanthomelas*, a member of the "A" genome's closest relative group, with strong support (Fig. 2A). We assume that our accession is tetraploid, and its lineage likely formed in Asia before dispersing to the New World. Future studies should include multiple accessions of this taxon sampled from throughout its range in order to determine relationships between lineages of different ploidy levels and how they may have formed, as well as accessions of the progenitor taxa suggested by our analyses, *D. xanthomelas* and *D. lacera*, and the other members of Clade I.

D. pseudofilix-mas has three *pgiC* copies, which were attributed to *D. oreades, D. patula*, and a lineage sister to *D. sieboldii* plus *D. lacera* in the network analysis (Fig. 4). We infer that D. oreades was the maternal donor, based on its congruent placement in the plastid phylogeny (Fig. 2A). We hypothesize that *D. oreades* hybridized with the *D. sieboldii–D. lacera* relative within the last 0.8 Ma (its date of divergence from its closest relative according to the plastid analysis; Fig. 3), and this hybrid then dispersed to Central America, where the *D. patula* genome was incorporated. Finally, *D. muenchii*'s possesses three *pgiC* copies and is known to be a triploid (Reyes-Jaramillo et al., 2008). One copy is most closely related to Asian species *D. dickinsii* (Fig. 4), one to *D. intermedia*, and one to the *"D. semicristata"* genome group. We hypothesize that *D. muenchii* likely formed as a result of hybridization first between *D. dickinsii* or its ancestor and "*D. semicristata*", which is thought to have occurred in Asia prior to its extinction (Fraser-Jenkins, 2001). "*D. semicristata*" would have been the maternal progenitor in this match, as well as in a subsequent hybridization with *D. intermedia* following a long-distance dispersal event to North America (Table 3). This last event likely occurred in the Americas because *D. muenchii* is endemic to cloud forests of southeastern Mexico (Mickel and Smith, 2004).

4.6. Reticulate taxa from other regions

As the primary focus of the current study was to investigate reticulate evolution in the New World species of *Dryopteris*, we did not attempt to exhaustively sample *pgiC* sequence variation among the non-New World species, which in some cases may have contributed to our difficulty in deciphering the history of the New World species. For the European and Asian taxa we successfully sequenced, on average, 8 clones per individual. For several species (e.g. European *D. dilatata*, which is known to be an allotetraploid [Walker, 1955; Wíden et al., 1970; Sigel, 2008], and Eurasian *D. remota,* an apogamic triploid [Manton, 1950] that appears to be another descendent of *"D. semicristata"*), this limited sampling was enough to uncover evidence of reticulation. For *D. dilatata*, in particular, our *pgiC* sequences support previous studies suggesting that this species is an allotetraploid between *D. expansa* and a member of the *D. intermedia* complex (Wíden et al., 1970; Gibby, 1983; Sigel, 2008). For the majority of the non-New World taxa included here, absence of evidence of reticulation should not be taken as evidence of absence. Further studies, with extensive sampling of the Asian taxa in particular, will be needed to uncover the full extent of reticulation in *Dryopteris* at a global scale.

5. Conclusions

The current study uncovers evidence of extensive reticulate evolution among the New World members of the genus *Dryopteris*. Our analyses, which are based on more a thorough sampling of

taxa and characters than any previous study of this group, provide support for an existing hypothesis of reticulate evolution in the North American species, and suggest that several of the Latin American species are products of hybridization involving an Asian ancestor that underwent intercontinental long-distance dispersal to reach the Americas. The hypotheses that we develop to explain the origins of the putatively reticulate species in Latin America are based on a large plastid dataset and a single nuclear marker, the latter of which produced phylogenies that have poor support and low resolution at several critical nodes. The current study thus provides a foundation from which further studies of this group must be undertaken. In particular, additional sampling of Asian *Dryopteris*, especially from Clade I, will be critical, as will sequencing of additional nuclear markers, in order to produce a highly resolved and well supported phylogeny to use as a basis for testing the hypotheses developed here. There was discordance between topology and divergence time estimates based on the plastid and nuclear analyses in the current study, even for taxa that were thought not to be of reticulate origin. Topological discordances could be due to incomplete lineage sorting, ancient hybridization or introgression, or duplication of the *pgiC* locus that has resulted in the presence of paralogs. Assessing whether any of these phenomena has contributed to the evolutionary history of this group will require data from additional nuclear markers, and a more thorough understanding of the phylogenetic relationships among these species will allow us to estimate and interpret divergence times more accurately. Confirmation of ploidy of all the Latin American *Dryopteris* species is another necessary step in a rigorous analysis of this group. Flow cytometry to estimate genome size, coupled with cytological analyses of meiotic cells, will provide an essential piece of the puzzle for interpreting the data presented here. *Dryopteris* in North, Central, and South America clearly display evidence of a complex, reticulate evolutionary history, and the group deserves further study.

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Appendix A

Voucher information and GenBank accession numbers for species of *Dryopteris* and outgroups included in this study. For species with more than one *pgiC* sequence, A, B, and/or C sequence labels are provided. Herbarium abbreviations: WIS = University of Wisconsin – Madison, Madison, Wisconsin, USA; NY = New York Botanical Garden, Bronx, New York, USA; DUKE = Duke University, Durham, North Carolina, USA; MO = Missouri Botanical Garden, St. Louis, Missouri, USA; UC = University of California-Berkeley, Berkeley, California, USA; COLO = University of Colorado Museum, Boulder, Colorado, USA. P = Muséum National d'Histoire Naturelle, Paris, France; BM = The Natural History Museum, London, UK; REU = Université de la Réunion, Saint-Clotilde, Réunion. AFSSE and BPSSE indicate taxa that were obtained as spores from the American Fern Society Spore Exchange and British Pteridological Society Spore Exchange, respectively. These spores were germinated and grown by Geiger and Ranker (2005), and DNA material later provided to us. Missing sequences are indicated by —.

Taxon; Voucher specimen (Herbarium); GenBank accessions: *trnL-trnF; rbcL-accD; rbcL; psbA-trnH; rps4-trnS; trnG-trnR; trnP-petG; pgiC* (A,B,C).

Dryopteris abbreviata (C. Presl) Kuntze; *Christenhusz 4290 (UC)*; JN189126; JN189664; JN189557; JN189448; JN189231; JN189019; JN189342; JQ670060; *D. aemula* (Ait.) Kuntze; *Väre 16353*; —;—;—;—;—;—;—; FR728934.1; *D. affinis* (Lowe) Fraser-Jenk.; *Väre 16577*; —;—;—;—;—;—;—; FR728946.1 (A), FR728945.1 (B); *D. aitoniana* Pic. Serm.; *Väre 16527*; —;—;—;—;—;—;—; FR728947.1; *D. alpestris* Tagawa ex Ching & S.K. Wu; *Heng 32147 (UC)*; JN189105; JN189645; JN189536; JN189428; JN189210; JN189000; JN189322; JQ670044; *D. amurensis* H. Christ; *Alanko 70810*; —;—;—;—;—;—;—; FR728948.1; *D. antarctica* (Baker) C. Chr.; *Hennequin 2009-R109 (REU)*; JN189141; JN189682; JN189577; JN189467; JN189250; JN189038; JN189356; JQ670026 (A), JQ670063 (B), JQ669996 (C); *D. aquilinoides* C. Chr.; *Kessler 13855 (UC)*; JN189106; JN189646; JN189537; JN189429; JN189211; JN189001; JN189323; JQ670031 (A), JQ670002 (B); *D. arguta* (Kaulf.) Maxon; *Fraser-Jenkins 13546*; —;—;—;—;—;—;—; FR728949.1; *D. arguta* (Kaulf.) Maxon; *EBS 36 (WIS)*; JN105310; JQ947924; JQ935258; JQ936649; JQ936838; JQ683022; JQ683022; JQ670052; *D. assimilis* S. Walker; *Skvortsov 1.VIII.1982 (NY)*; JN189086; JN189627; JN189517; JN189409; JN189191; JN188981; JN189303; JQ669971; *D. athamantica* (Kunze) Kuntze; *Bodenghien 2037 (UC)*; JN189107; —; JN189538; JN189430; JN189212; JN189002; JN189324; JQ669983 (A), JQ670055 (B); *D. austriaca* (Jacq.) Woyn. ex Schinz & Thell.; *Degn 25 (NY)*; JN189087; JN189628; JN189518; JN189410; JN189192; JN188982; JN189304; JQ669993; *D. azorica* (H. Christ) Alston; *Väre 16360*; —;—;—;—;—;—;—; FR728950.1; *D. barberigera* Moore; *Miehe 94-191-14 (UC)*; JN189108; JN189647; JN189539; JN189431; JN189213; JN189003; JN189325; JQ669980; *D. campyloptera* (Kunze) Clarkson; *Martineau*; —;—;—;—;—;—;—; FR728951.1; *D. campyloptera* (Kunze) Clarkson; *EBS 19 (WIS)*; JN105306; JQ947921; JQ935255; JQ936639; JQ936819; JQ683013; JQ683013; JQ670015 (A), JQ669990 (B); *D. carthusiana* (Vill.) H.P. Fuchs; *Väre 16439*; —;—;—;—;—;—;—; FR728952.1 (A), FR728953.1 (B); *D. carthusiana* (Vill.) H.P. Fuchs; *EBS 43 (WIS)*; JQ682990; JQ947938; JQ935272; JQ936647; JQ936843; JQ683026; JQ683026; JQ669989 (A), JQ670050 (B); *D. caucasica* (A. Braun) Fraser-Jenk. & Corley; *Christenhusz 4309 (UC)*; JN189109; JN189648; JN189540; JN189432; JN189214; JN189004; JN189326; JQ670038 (A), JQ670018 (B); *D. celsa* (W. Palmer) Knowlt. T.S. Palmer & Pollard ex Small; *EBS 27 (WIS)*; JN189069; JN189609; JN189499; JN189390; JN189175; JN188962; JN189284; JQ670087 (A), JQ669967 (B); *D. chinensis* Koidz.; *Zhang 2399 (UC)*; JN189110; JN189649; JN189541; JN189433; JN189215; JN189005; JN189327; JQ669985 (A), JQ670023 (B); *D. chrysocoma* (Christ) C. Chr.; *Unknown 188 (UC)*; JN189111; JN189650; JN189542; JN189434; JN189216; JN189006; JN189328; JQ670083 (A), JQ669976 (B), JQ670028 (C); *D. cinnamomea* (Cav.) C. Chr.; *Fraser-Jenkins 13499*; —;—;—;—;—;—

;—; FR728960.1; *D. cinnamomea* (Cav.) C. Chr.; *Rothfels 3099 (DUKE)*; JN189097; JN189638; JN189528; JN189420; JN189202; JN188992; JN189314; JQ669970; *D. clintoniana* (D.C. Eaton) Dowell; *EBS 8 (WIS)*; JQ683004; JQ947934; JQ935247; JQ936651; JQ936813; JQ683019; JQ683019; JQ670066 (A), JQ669966 (B), JQ670064 (C); *D. costalisora* Tagawa; *Ranker 2029 (COLO)*; JN189170; JN189710; JN189603; JN189493; JN189278; JN189063; JN189384; JQ669994; *D. crassirhizoma* Nakai; *van der Werff 14065 (UC)*; JN189112; JN189651; JN189543; JN189435; JN189217; JN189007; JN189329; JQ670016 (A), JQ669962 (B); *D. crispifolia* Rasbach Reichst. & G. Vida; *Väre 16320*; —;—;—;—;—;—;—;—; FR728961.1 (A), FR728962.1 (B); *D. crispifolia* Rasbach Reichst. & G. Vida; BPSSE; JN189164; JN189703; JN189597; JN189488; JN189272; JN189057; JN189378; JQ670027 (A), JQ670057 (B); *D. cristata* (L.) A. Gray; *Uotila 42353*; —;—;—;—;—;—;—; FR728965.1 (A), FR728964.1 (B); *D. cristata* (L.) A. Gray; *ESB 26 (WIS)*; JQ682988; JQ947940; JQ935245; JQ936665; JQ936811; JQ683010; JQ683010; JQ670037 (A), JQ670025 (B); *D. cycadina* (Franch. & Sav.) C. Chr.; *RBC TW 78 (UC)*; JN189113; JN189652; JN189544; JN189436; JN189218; JN189008; JN189330; JQ669963 (A), JQ669961 (B); *D. cystolepidota* (Miq.) C. Chr.; *AFSSE*; JN189160; JN189699; JN189593; JN189485; JN189268; JN189053; JN189374; JQ669981; *D. dickinsii* (Franch. & Sav.) C. Chr.; *BPSSE*; JN189165; JN189704; JN189598; JN189489; JN189273; JN189058; JN189379; JQ670032; *D. diffracta* (Baker) C. Chr.; *Chang 6667*; —;—;—;—;—;—;—; EU797707.1; *D. dilatata* (Hoffm.) A. Gray; *Camoletto*; —;—;—;—;—;—;—; FR728968.1; *D. dilatata* (Hoffm.) A. Gray; *Hennequin 2010-B1 (P)*; JN189139; JN189680; JN189575; JN189465; JN189248; JN189036; JN189354; JQ669975 (A), JQ670020 (B); *D. effusa* (Sw.)Urb.; *da Silva 2095 (MO)*; —;—;—; JQ936646; —;—;—; JQ670086; *D. expansa* (C. Presl) Fraser Jenk. & Jermy; *Väre & Juslen Ke11*; —;—;—;—;—;—;—; FR728973.1; *D. expansa* (C. Presl) Fraser Jenk. & Jermy; *EBS 30 (WIS)*; JN189074; JN189616; JN189506; JN189397; JN189180; JN188969; JN189291; JQ669979; *D. fatuhivensis* E. Brown; *Wood 10092 (COLO)*; JN189168; JN189707; —; JN189490; JN189275; JN189060; JN189381; JQ670010; *D. filix-mas* (L.) Schott; *Uotila 40320*; —;—;—;—;—;—;—; FR728976.1; *D. filix-mas* (L.) Schott; *EBS 38 (WIS)*; JQ683007; JQ947932; JQ935265; JQ936671; JQ936810; JQ683036; JQ683036; JQ670041 (A), JQ670040 (B); *D. flaccisquama* A. Rojas; *Fay 3152 (NY)*; JN189088; JN189629; JN189519; JN189411; JN189193; JN188983; JN189305; JQ670033; *D. fragrans* (L.) Schott; *Väre & Juslen Ke12*; —;—;—;—;—;—;—; FR728980.1; *D. fragrans* (L.) Schott; *EBS 53 (WIS)*; JQ682989; —; JQ935274; JQ936663; JQ936833; JQ683014; JQ683014; JQ669984; *D. futura* A.R. Sm.; *Quedensley 754 (UC)*; JN189103; JN189643; JN189534; JN189426; JN189208; JN188998; JN189320; JQ670070 (A), JQ670082 (B), JQ670022 (C); *D. goldiana* (Hook.) A. Gray; *Zika 1984*; —;—;—;—;—;—;—; FR728988.1; *D. goldiana* (Hook.) A. Gray; *EBS 29 (WIS)*; JN189073; JN189615; JN189505; JN189396; JN189179; JN188968; JN189290; JQ670073; *D. guanchica* Gibby & Jermy; *Zuniga & Alejandre 161.4-89*; —;—;—;—;—;—;—; FR728989.1 (A), FR728990.1 (B); *D. guanchica* Gibby & Jermy; *Hennequin 2010-C2 (P)*; JN189137; JN189678; JN189573; JN189463; JN189246; JN189034; JN189352; JQ669959 (A), JQ670076 (B); *D. gymnosora* (Makino) C. Chr.; *Unknown 94.0752 (UC)*; JN189115; JN189654; JN189546; JN189438; JN189220; JN189010; JN189332; JQ670035; *D. hasseltii* (Blume) C. Chr.; *Chang 6675*; —;—;—;—;—;—;—; EU797715.1; *D. hondoensis* Koidz.; *Moran (COLO)*; JN189149; JN189689; JN189583; JN189475; JN189257; JN189044; JN189363; JQ669965; *D. huberi* (Christ) C. Chr.; *Sperling 5841 (NY)*; JN189089; JN189630; JN189520; JN189412; JN189194; JN188984; JN189306; JQ670006 (A), JQ669995 (B), JQ670001 (C); *D. inequalis* (Schlecht.) Kuntze; *Unknown 7749* (UC); JN189117; JN189655; JN189548; JN189440; JN189222; JN189011; JN189333; JQ669988; *D. intermedia* Kuntze; *Martineau*; —;—;—;—;—;—;—; FR728993.1; *D. intermedia* Kuntze; *EBS 18 (WIS)*; —; JN189613; JN189503; JN189394; JN189178; JN188966; JN189288; JQ670071; *D. juxtaposita* Christ; *Heng 24049 (UC)*; JN189118; JN189656; JN189549; JN189441; JN189223; JN189012; JN189334; JQ670005 (A), JQ670079 (B); *D. karwinskyana* (Mett.) Kuntze; *Marcos 354 (NY)*; JN189090; JN189631; JN189521; JN189413; JN189195; JN188985; JN189307; JQ670059 (A), JQ670072 (B), JQ670009 (C); *D. knoblochii* A.R. Sm.; *Devender 98-1566 (NY)*; JN189091; JN189632; JN189522; JN189414; JN189196; JN188986; JN189308; JQ670034 (A), JQ670030 (B), JQ670085 (C); *D. komarovii* Kossinsky; *Wundisch 94-453-19 (UC)*; JN189119; JN189657; JN189550; JN189442; JN189224; JN189013; JN189335; JQ670000; *D. lacera* (Thunb.) Kuntze; *Moran (COLO)*; JN189151; JN189691; JN189585; JN189477; JN189259; JN189046; JN189365; JQ670074; *D. ludoviciana* (Kunze) Small; *Leonard 2144*; —;—;—;—;—;—;—; FR728995.1; *D. ludoviciana* (Kunze) Small; *EBSlud3 (WIS)*; JN105313; JQ947943; JQ935277; JQ936656; JQ936815; JQ683028; JQ683028; JQ670014; *D. maderensis* Alston; *Alanko 109381*; —;—;—;—;—;—;—; FR728996.1; *D. marginalis* (L.) A. Gray; *Brisson 78625*; —;—;—;—;—;—;—; FR728999.1; *D. marginalis* (L.) A. Gray; *EBS 17 (WIS)*; JN189071; JN189612; JN189502; JN189393; JN189177; JN188965; JN189287; JQ670012; *D. maxonii* Underw. & C. Chr.; *Rothfels 3197 (DUKE)*; JN189098; JN189639; JN189529; JN189421; JN189203; JN188993; JN189315; JQ669974; *D. monticola* (Makino) C. Chr.; *Togasi (COLO)*; JN189156; —;—; JN189482; JN189264; —; JN189370; JQ670019 (A), JQ670036 (B); *D. muenchii* A.R. Sm.; *EBS 54 (WIS)*; JN189104; JN189644; JN189535; JN189427; JN189209; JN188999; JN189321; JQ670042 (A), JQ670007 (B), JQ669992 (C); *D. nubigena* Maxon & C.V. Morton; *Sundue 1363 (NY)*; JN189070; JN189611; JN189501; JN189392; —; JN188964; JN189286; JQ670024; *D. odontoloma* (Moore) C. Chr.; *AFSSE*; JN189157; JN189696; JN189590; JN189483; JN189265; JN189051; JN189371; JQ670039; *D. oligodonta* Pic. Serm.; *Alanko 74198*; —;—;—;—;—;—;—; FR729000.1; *D. oligodonta* Pic. Serm.; *Hennequin 2010-C11 (P)*; JN189138; JN189679; JN189574; JN189464; JN189247; JN189035; JN189353; JQ669960; *D. oreades* Fomin; *Kukkonon 13904*; —;—;—;—;—;—;—; FR729003.1; *D. pacifica* (Nakai) Tagawa; *AFSSE*; JN189161; JN189700; JN189594; JN189486; JN189269; JN189054; JN189375; JQ670004 (A), JQ670043 (B); *D. pallida* Fomin; *Raesaenen*; —;—;—;—;—;—;—; FR729004.1; *D. pallida* Fomin; *AFSSE*; JN189158; JN189697; JN189591; —; JN189266; —; JN189372; JQ670081; *D. pandae* (Clarke) C. Chr.; *Unknown 11514 (UC)*; JN189120; JN189658; JN189551; JN189443; JN189225; JN189014; JN189336; JQ669986 (A), JQ669998 (B); *D. patula* (Sw.) Underw.; *EBS 2 (WIS)*; —; JN189610; JN189500; JN189391; JN189176; JN188963; JN189285; JQ670058; *D. pentheri* (Krasser) C. Chr.; *Hennequin 2009-R2 (BM)*; JN189140; JN189681; JN189576; JN189466; JN189249; JN189037; JN189355; JQ670069; *D. polita* Rosenst.; *Chang 6903*; —;—;—;—;—;—;—; EU797725.1; *D. polita* Rosenst.; *Ranker 2003 (COLO)*; JN189173; JN189713; JN189606; JN189496; JN189281; JN189066; JN189387; JQ670048; *D. polylepis* (Franch. & Sav.) C. Chr.; *Moran (COLO)*; JN189155; JN189695; JN189589; JN189481; JN189263; JN189050; JN189369; JQ670075; *D. pseudofilix-mas* (Fee) Rothm.; *Montgomery 04-171 (NY)*; JN189092; JN189633; JN189523; JN189415; JN189197; JN188987; JN189309; JQ670088 (A), JQ669982 (B), JQ670084 (C); *D. pycnopteroides* (Christ) C. Chr.; *Moran (COLO)*; JN189150; JN189690; JN189584; JN189476; JN189258; JN189045; JN189364; JQ669997; *D. reflexosquamata* Hayata; *Ranker 2040 (COLO)*; JN189171; JN189711; JN189604; JN189494; JN189279; JN189064; JN189385; JQ670053; *D. remota* Hayata; *Moran (COLO)*; JQ682983; JQ947922; JQ935253; JQ936655; JQ936826; JQ683042; JQ683042; JQ670080 (A), JQ670056 (B); *D. rosea* Mickel & Beitel; *Mickel 4428A (NY)*; JN189093; JN189634; JN189524; JN189416; JN189198; JN188988; JN189310; JQ670013

(A), JQ669968 (B); *D. rossii* C. Chr.; *Rothfels 3182 (DUKE)*; JN189100; JN189641; JN189531; JN189423; JN189205; JN188995; JN189317; JQ670047; *D. sacrosancta* Koidz.; AFSSE; JN189159; JN189698; JN189592; JN189484; JN189267; JN189052; JN189373; JQ670029 (A), JQ670051 (B), JQ670011 (C); *D. saffordii* C. Chr.; *Sagastegui 15507 (NY)*; JN189094; JN189635; JN189525; JN189417; JN189199; JN188989; JN189311; JQ670045 (A), JQ669999 (B), JQ670003 (C); *D. salvinii* (Baker) Kuntze; *Irwin 34351 (NY)*; JN189095; JN189636; JN189526; JN189418; JN189200; JN188990; JN189312; JQ670049 (A), JQ669969 (B), JQ670078 (C); *D. scottii* (Bed) Ching; *RBC 202 (UC)*; JN189121; JN189659; JN189552; JN189444; JN189226; JN189015; JN189337; JQ669964 (A), JQ670077 (B); *D. sieboldii* (van Houtte) Kuntze; *AFSSE*; JN189162; JN189701; JN189595; —; JN189270; JN189055; JN189376; JQ670017; *D. simplicior* Mickel & Beitel; *Breedlove 21937 (NY)*; JN189096; JN189637; JN189527; JN189419; JN189201; JN188991; JN189313; JQ670008; *D. sparsa* (D. Don) Kuntze; *Ranker 2015 (COLO)*; —; JN189708; JN189601; JN189491; JN189276; JN189061; JN189382; JQ669977; *D. stenolepis* (Baker) C. Chr.; *Unknown 99 (UC)*; JN189122; JN189660; JN189553; JN189445; JN189227; JN189016; JN189338; JQ670061; *D. stewartii* Fraser-Jenk.; *Moran (COLO)*; JN189152; JN189692; JN189586; JN189478; JN189260; JN189047; JN189366; JQ669991 (A), JQ670046 (B); *D. sublacera* Christ; *Yatskievych 18659 (UC)*; JN189123; JN189661; JN189554; JN189446; JN189228; JN189017; JN189339; JQ669987 (A), JQ670065 (B); *D. subreflexipinna* (Ogata) H. Ito; *Chang 6541*; —;—;—;—;—;—;—; EU797717.1 (A), EU797721.1 (B); *D. tokyoensis* (Matsum.) C. Chr.; *Moran (COLO)*; JN189142; JN189683; JN189578; JN189468; JN189251; JN189039; JN189357; JQ670021; *D. triangularis* Herter; *BPSSE*; JN189167; JN189706; JN189600; —;—;—;—; JQ670067 (A), JQ670062 (B), JQ669972 (C); *D. wallichiana* (Spreng.) Hyl; *EBS 1 (WIS)*; JN189067; JN189607; JN189497; JN189388; —; JN188960; JN189282; JQ669973 (A), JQ670068 (B); *D. xanthomelas* (Christ) C. Chr.; *Miehe 94-149-17 (UC)*; JN189127; JN189665; JN189558; JN189449; JN189232; JN189020; JN189343; JQ670054; *Polystichum andersonii* Hopkins; *EBS 39 (WIS)*; JN189078; JN189620; JN189510; JN189401; JN189183; JN188973; JN189295; JQ669978.

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