Diversification and reticulation in the circumboreal fern genus Cryptogramma

Jordan S. Metzgar a,*, Edward R. Alverson b, Sinian Chen c, Alexey V. Vaganov d, Stefanie M. Ickert-Bond a

Abstract

We investigated the evolutionary complexity that resulted from cryptic diversification and polyploidy in parsley ferns (Cryptogramma). A total of 14 species were included in our data set, with six outgroup species and eight Cryptogramma species. DNA sequence data from six plastid loci (rbcL, trnL–accD, trnL–atpB, rpo4–trnS, trnG–tmr and trnP–petG) were analyzed using maximum likelihood and Bayesian methods to provide the first rigorous assessment of diversification in the genus, including testing the monophyly of the genus and sections. Cryptogramma and Coniogramme are recovered as reciprocally monophyletic sister genera. We established the monophyly of both sections within Cryptogramma. Furthermore, our sequence data reveal that described species reflect mostly allopatric reciprocally monophyletic lineages that are independent evolutionary trajectories. Using sequence data from the nuclear locus (gapCp) we find that the European C. crispa is an autotetraploid with a partially diploidized genome, while the North American tetraploid Cryptogramma sitchensis is an allopolyploid derived from C. acrostichoides and C. raddeana. Subsequent backcrossing between C. sitchensis and C. acrostichoides has allowed the introgression of C. raddeana alleles into northern populations of C. acrostichoides.

1. Introduction

The leptosporangiate fern genus Cryptogramma R.Br. is comprised of nine species in two sections. Referred to as parsley ferns for the resemblance of their foliage to that of parsley, they combine to have a circumboreal distribution with a bipolar disjunction as one taxon is present in southern South America (Alverson, 1989a; Raven, 1963). All members of the genus possess dimorphic leaves, with erect fertile leaves and shorter more finely divided sterile leaves. Fertile leaves possess false indusia and the chromosome base number for the genus is 30 (Tryon and Tryon, 1990).

Cryptogramma stelleri (S.G. Gmel.) Prantl is the only representative of section Homopteris (Rupr.) C. Chr. Found in the northern regions of North America, Asia and extreme northeastern Europe, it has a creeping rhizome, leaves with a membranous texture and is often a calciphile (Alverson, 1993; Hultén and Fries, 1986). Authors have been in wide agreement on the taxonomy of this section (Alverson, 1993; Hultén, 1968; Lellinger, 1985; Tryon and Tryon, 1990) to ten (Alverson, 1989a; Lellinger, 1985; Vaganov et al., 2010). Found across temperate and boreal regions of North America, Asia and Europe, one taxon is also found in southern South America (Tryon and Tryon, 1990). Species in sect. Cryptogramma have erect rhizomes, leaves with a more coriaceous texture and generally prefer acidic, rocky habitats (Alverson, 1989a).

Previous research has shown Cryptogramma to be closely related to Coniogramme Fée, a genus of approximately 30 species in the Old World tropics, and the monotypic Mexican genus Llavea Lag. (Prado et al., 2007; Schuettpelz et al., 2007; Zhang et al., 2005). These cryptogrammoid ferns possess an important phylogenetic position as the basal clade in the Pteridaceae (Gaston and Rollo, 1995; Prado et al., 2007; Schuettpelz and Pryer, 2007; Schuettpelz et al., 2007), comprising the subfamily Cryptogrammoideae (sensu Smith et al., 2006). This family is closely related to the large eupolypod clade (Schuettpelz and Pryer, 2007). With many active research inquiries into other subfamilies of the Pteridaceae, particularly the cheilanthoid ferns (e.g., Beck et al., 2011; Grusz et al., 2009; Kirkpatrick, 2007; Rothfels et al., 2008; Schuettpelz et al., 2008; Sigel et al., 2011) and the emerging model system Ceratopteris (e.g., McGrath and Hickok, 1999; Nakazato et al., 2007; Scott et al., 2007), there is an urgent need for a better understanding of the relationships and characteristics of the basal cryptogrammoid clade.

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No study has previously analyzed the evolution or taxonomy of the genus as a whole, with studies that are regional in scope predominating. Recent studies of the Chinese taxa reduced Cryptogramma emiensis Ching and K.H. Shing and Cr. shensiensis Ching to synonymy with Cr. brunoniana Hook. & Grev. (Zhang and Zhang, 2003). Disagreement regarding the specific status of the Asian Cr. raddeana Fomin has emerged, with some authors treating it as a variety (Zhang and Zhang, 2003) or subspecies (Fraser-Jenkins, 2008) of Cr. brunoniana, while others maintain Cr. raddeana at the species level (Zhang et al., in press). Although it was unknown if it was of allopolyploid or autopolyploid origin, the European tetraploid Cr. crispa (L.) R.Br. has been studied more extensively using breeding system evidence that suggests that its genome has been effectively diploidized (Pajarón et al., 1999). The North American members of sect. Cryptogramma were studied using a biosystematic approach (Alverson, 1989a, 1989b), which yielded the description of a new diploid species (Cr. cascaden sis E.R. Alverson; Alverson, 1989b). Allozyme and morphological evidence established that Cr. sitchensis (Rupr.) Moore is an allotetraploid (Alverson, 1988, 1989a) with Cr. acrostichoides R.Br. serving as one parent and the other hypothesized to be the Asian Cr. raddeana. In addition, these studies discovered that triploid hybrids between Cr. acrostichoides and Cr. sitchensis are common and caused much of the taxonomic confusion regarding these species (Alverson, 1988). However, a comprehensive molecular study of the group is wholly lacking, with no previous molecular evidence supporting the monophyly of the genus, sections or species.

Cryptogramma represents an excellent system to further our understanding of fern evolution, polyploidy, introgression and chromosome number evolution. Our study will be the first comprehensive examination of the genus, using multiple molecular data sets to test generic and subgeneric circumscriptions and evaluating correspondence between described species and molecular differentiation. Subtle morphological variation between putative species and sections of Cryptogramma is common and caused much of the taxonomic confusion regarding these species (Alverson, 1988). However, a comprehensive molecular study of the group is wholly lacking, with no previous molecular evidence supporting the monophyly of the genus, sections or species.

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2. Materials and methods

2.1. Taxon sampling

A total of 14 species were included in our data set, with Llavea cordifolia, five Coniogramme species and eight Cryptogramma species. Many species were represented by samples from multiple populations in an attempt to encompass potential geographic variation, with a total of 40 samples included in the data set (Table 1). An extensive effort was made to include material for as many species and populations as possible; however, no sequence data could be obtained for the recently described Cr. gorovoi E. Vaganov & Schmakov from the Russian Far East and Japan (sensu Cr. crispa var. japonica; Vaganov and Shmakov, 2007) or for the newly described Cr. bithy micna S. Jess, L. Lehm. & Bujnoch (Jessen et al., 2012) from northwestern Turkey. Llavea accessions were used as the outgroup based on previously established relationships (Prado et al., 2007; Schuettpelz et al., 2007; Zhang et al., 2005).

2.2. DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from 15 to 20 mg of silica-dried leaf tissue per sample using the Qiagen DNAeasy Plant Mini Kit (Qiagen, Valencia, California, USA). Six plastid loci (rbcL, rbcL–accD, rbcL–atpB, rps4–trnS, trnG–trnR and trnM–petG) were amplified and sequenced according to existing protocols (Table 1; Grusz et al., 2009; Korall et al., 2007).

For seven species of Cryptogramma, one exemplar accession was amplified and sequenced for the nuclear locus (gapCp) and three specimens of Cr. acrostichoides were amplified and sequenced to characterize potential introgression. Amplification protocols for this marker followed Schuettpelz et al. (2008) and PCR products were cloned using the Invitrogen TOPO TA Cloning kit (Invitrogen, Carlsbad, California, USA). Clones were amplified using Invitrogen’s M13 primer pair and the same gapCp thermocycler conditions as mentioned earlier. Alleles corresponding to the “short” copy and “long” copy of the gapCp locus were recovered (Schuettpelz et al., 2008). We did not use the gapCp “long” alleles due to a lack of taxonomic coverage (copies were recovered for only five taxa) and due to previously reported homology issues with distinguishing these alleles from the gapC locus (Grusz et al., 2009; Schuettpelz et al., 2008).

All plastid and nuclear sequencing chromatograms were corrected by eye using Sequencer version 4.10.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). For gapCp sequences, separate projects were used containing all of the sequences for a given individual. Mutations and indels were then charted through the length of the consensus sequence, allowing for the identification of separate alleles. PCR artifacts such as chimeric sequences were detected and removed during this step (Mason-Gamer et al., 2008). This study generated a combined total of 240 plastid and nuclear sequences and all were deposited in GenBank (Table 2).

2.3. Sequence alignments

For each locus, sequences were aligned using ClustalX 2.1 (Larkin et al., 2007) and the resulting alignments were then refined by eye. The aligned data matrix for the protein-coding rbcL locus lacked any insertions or deletions (indels). All of the alignments for the remaining loci included indels. Ambiguously aligned regions and indels were excluded from the final analyses, with excluded bases totaling 122 bp in rbcL–accD, 68 bp in rbcL–atpB, 324 bp in rps4–trnS, 219 bp in trnG–trnR, 249 bp in trnM–petG and 85 bp in the gapC “short” copy. Much of the non-coding portion of the rps4–trnS matrix was excluded, as the alignment in this region was extremely ambiguous between the different sections of Cryptogramma and between Cryptogramma and the other genera. We did not use any gap-coding method.

2.4. Plastid data set combinability

Bayesian Markov chain Monte Carlo (B/MCMC) analyses were implemented in MrBayes version 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012) for each single locus data set following the protocol used for the combined data matrix analysis (see Section 2.5). Majority-rule consensus topologies were calculated for each locus. These topologies were manually inspected for topological conflicts (Mason-Gamer and Kellogg, 1996) using a minimum threshold of 0.95 posterior
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<td>Rothfels 3025 (ALA, DUKE, MEXU)</td>
<td>KC700108</td>
<td>KC700148</td>
<td>KC700186</td>
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<td><em>Llavea cordifolia</em></td>
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<td>KC700150</td>
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<td>KC700151</td>
<td>KC700187</td>
<td>KC700228</td>
<td>KC700266</td>
<td>KC700302</td>
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<td><em>Coniogramme flexuinea</em></td>
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<td>Schuettpelz 836 (DUKE, REP)</td>
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<td>–</td>
<td>KC700190</td>
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<td>Schuettpelz 1052A (DUKE, TAF, BM)</td>
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<td>KC700191</td>
<td>KC700230</td>
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<td>Hieu CPC1240 (ALA)</td>
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<td>KC700153</td>
<td>–</td>
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<td>KC700133</td>
<td>KC700171</td>
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<td>KC700248</td>
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<td>KC700251</td>
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<td>KC700137</td>
<td>KC700175</td>
<td>KC700214</td>
<td>KC700252</td>
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<td>KC700139</td>
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<td><em>Cryptogramma acrostichoides</em></td>
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<td><em>Cryptogramma acrostichoides</em></td>
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<td>USA: Alaska: Taku Glacier</td>
<td>Bass s.n. (ALA)</td>
<td></td>
<td>KC700104</td>
<td>KC700144</td>
<td>KC700182</td>
<td>KC700221</td>
<td>KC700259</td>
<td>KC700295</td>
<td>–</td>
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<tr>
<td>358</td>
<td><em>sitchensis</em></td>
<td>USA: Alaska: Seward: Kenai Fjords National Park: Harding Icefield Trail</td>
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<td></td>
<td>KC700105</td>
<td>KC700145</td>
<td>KC700183</td>
<td>KC700222</td>
<td>KC700260</td>
<td>KC700296</td>
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<tr>
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<td><em>sitchensis</em></td>
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<td></td>
<td>KC700106</td>
<td>KC700146</td>
<td>KC700184</td>
<td>KC700223</td>
<td>KC700261</td>
<td>KC700297</td>
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<td>361</td>
<td><em>sitchensis</em></td>
<td>USA: Alaska: Valdez: Thompson Lake</td>
<td>Metzgar 257 (ALA)</td>
<td></td>
<td>KC700107</td>
<td>KC700147</td>
<td>KC700185</td>
<td>KC700224</td>
<td>KC700262</td>
<td>KC700298</td>
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<tr>
<td>314</td>
<td><em>stelleri</em></td>
<td>Taiwan: NanTou County, Hohuan Shelter</td>
<td>Kuo 492 (TAIF)</td>
<td></td>
<td>KC700076</td>
<td>KC700116</td>
<td>KC700154</td>
<td>KC700193</td>
<td>KC700233</td>
<td>–</td>
<td>–</td>
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<tr>
<td>375</td>
<td><em>stelleri</em></td>
<td>USA: Alaska: Alexander Archipelago: Prince of Wales Island</td>
<td>Johnson 20104 (ALA)</td>
<td></td>
<td>KC700077</td>
<td>KC700117</td>
<td>KC700155</td>
<td>KC700194</td>
<td>KC700234</td>
<td>–</td>
<td>KC700072</td>
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<tr>
<td>386</td>
<td><em>stelleri</em></td>
<td>Canada: Ontario, Thunder Bay District: Talbot Island</td>
<td>Oldham 23697 (OAC, BAB, DUKE)</td>
<td></td>
<td>KC700078</td>
<td>KC700118</td>
<td>KC700156</td>
<td>KC700195</td>
<td>KC700235</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
probability. No topological conflict among data sets was observed so all six plastid loci data sets were combined into a single data set. The combined plastid locus matrix had a length of 7143 base pairs.

2.5. Phylogenetic analyses

MrModeltest 2.3 (Nylander et al., 2004) was used to determine the optimal model of sequence evolution for each ML and B/MCMC analysis based on Akaike Information Criterion scores (Table 3).

Analyses for both the combined plastid data set and the nuclear data sets used the same procedures. Maximum parsimony (MP) searches were implemented in PAUP 4.0b10 (Swofford, 2002), with heuristic searches of 1000 random addition sequence (RAS) replicates using tree-bisection-reconnection (TBR) branch swapping to determine the most parsimonious tree(s). Subsequent MP bootstrap analyses used 500 replicates, with 10 random-addition-sequence replicates each, TBR branch swapping and the nchuc option set to 100 trees to allow the bootstrap replicates to run to completion.

Maximum likelihood (ML) analyses and ML bootstrap analyses were run using Garli 2.0 (Zwickl, 2006) and implemented on the CIPRES Science Gateway computational portal (Miller et al., 2010). To avoid introducing potential biases in sequence evolution extensive partitioning of the data set by locus and assigned each locus its own model of sequence evolution, with parameters determined separately using a B/MCMC approach in MrBayes version 3.2 with each of the plastid loci and the nuclear locus receiving its own model of sequence evolution as determined using the Akaike Information Criterion scores in MrModeltest 2.3 (Table 3). Each ML analysis was performed twice, once using random starting trees and once using stepwise addition starting trees. All ML analyses used eight replicates. ML bootstrap analyses included 100 bootstrap replicates each.

The combined plastid dataset and nuclear datasets were analyzed separately using a B/MCMC approach in MrBayes version 3.2 with each of the plastid loci and the nuclear loci receiving its own model of sequence evolution as determined using the Akaike Information Criterion scores in MrModeltest 2.3 (Table 3). Each analysis used four runs, with four chains each, for 10 million generations. Default priors were used with a sampling frequency of 1000 generations. Likelihood and generation scores were plotted to check for stationarity using Tracer v1.5 (Rambaut and Drummond, 2007). We conservatively discarded the first 2.5 million generations as the burn-in period. The remaining 30,000 trees were pooled to calculate the majority-rule consensus tree with average branch lengths and posterior probabilities.

2.6. chromEvol analysis

We also reconstructed genome evolution in the cryptogamous clad to test for ancient genome duplication events that could complicate interpretation of the nuclear gene results. The evolution of chromosome numbers in Cryptogamma was evaluated using the program chromEvol (Mayrose et al., 2010), which infers polyploidization events, chromosome gain/loss events and demipolyploidization at ancestral nodes using a series of likelihood models. In order to detect any possible biases introduced by the analytical method or process used to generate the input reference tree topology required by chromEvol, four separate topologies were used (Mayrose, pers. comm.). Two of the topologies were generated by pruning accessions from our combined plastid data matrix until each taxon was represented by a single specimen. This data set was then analyzed using the ML and B/MCMC analytical procedures outlined in Section 2.5. The two resulting topologies were used as reference trees for the chromosome number evolution reconstruction. The other two topologies were obtained by modifying the optimal ML and B/MCMC topologies inferred for the full, 40-accession combined plastid data matrix in Section 2.5. These topologies had the same excess accessions pruned so that only one sample per species remained. Branch lengths for the ML and B/MCMC topologies were then re-estimated for that data set in

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer name</th>
<th>Sequence (5'–3')</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>rbcl</td>
<td>ESRBC115A</td>
<td>ATG TCA CCACAA CAG GAG ACT AAA GC</td>
<td>Koral et al. (2006)</td>
</tr>
<tr>
<td>rbcl</td>
<td>ESRBC164F</td>
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<td>Koral et al. (2006)</td>
</tr>
<tr>
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<td>ESRBC1663R</td>
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<td>Koral et al. (2006)</td>
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<td>Koral et al. (2006)</td>
</tr>
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<td>RBCL1170F</td>
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<td>Ebihara et al. (2003)</td>
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<td>ACCDH816A</td>
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<td>Ebihara et al. (2003)</td>
</tr>
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<td>rbcL–atpB</td>
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<td>Pryer et al. (2004)</td>
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<td>Nagalingum et al. (2007)</td>
</tr>
<tr>
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<td>SYM+G</td>
<td>GCA TAY CGA GCC YGG TAG CG</td>
<td>Small et al. (2005)</td>
</tr>
<tr>
<td>rbcL–atpB</td>
<td>SYM+G</td>
<td>GCA TAY CGA GCC YGG TAG CG</td>
<td>Small et al. (2005)</td>
</tr>
<tr>
<td>rbcL–atpB</td>
<td>SYM+G</td>
<td>GCA TAY CGA GCC YGG TAG CG</td>
<td>Small et al. (2005)</td>
</tr>
<tr>
<td>rbcL–atpB</td>
<td>SYM+G</td>
<td>GCA TAY CGA GCC YGG TAG CG</td>
<td>Small et al. (2005)</td>
</tr>
<tr>
<td>rbcL–atpB</td>
<td>SYM+G</td>
<td>GCA TAY CGA GCC YGG TAG CG</td>
<td>Small et al. (2005)</td>
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<td>rbcL–atpB</td>
<td>SYM+G</td>
<td>GCA TAY CGA GCC YGG TAG CG</td>
<td>Small et al. (2005)</td>
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<td>rbcL–atpB</td>
<td>SYM+G</td>
<td>GCA TAY CGA GCC YGG TAG CG</td>
<td>Small et al. (2005)</td>
</tr>
</tbody>
</table>

- Primer used for both amplification and sequencing.
PAUP* 4.0b10 (Swofford, 2002) and MrBayes version 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012), respectively.

Chromosome number counts were compiled from the literature and species with unknown counts were coded as missing data (Table 4). Coniogramme intermedia Hieron. and Co. fraxinea (D. Don) Fée each have published counts of both n = 30 and n = 60, so they were coded as polymorphic with both counts having a 50% occurrence rate.

Chromosome number reconstruction analyses were then run in chromEvol v1.3 (Mayrose et al., 2010; http://www.zoology.ubc.ca/~mayrose/cp/chromEvol/) on the University of Alaska Fairbanks Life Science Informatics Computational Portal for all four topologies. All eight models of chromosome transition properties were evaluated for each run. Default parameters were used.

3. Results

3.1. Phylogenetic analyses of the combined plastid data set

The MP, ML and B/MCMC analyses all returned well-resolved, robust and congruent topologies (Fig. 1). The MP analysis found a single island of 300 most parsimonious trees (length = 1307 steps) on all heuristic replicates. ML analyses using random and stepwise starting trees in GARLi (Zwickl, 2006) returned identical topologies with one examplar accession per species and using topologies generating by pruning excess accessions from the optimal consensus from the ML and B/MCMC analyses and reestimating the branch lengths) had an optimal AIC score for the “Constant rate” model of chromosome evolution. All four analyses, including four analyses using different models of evolution, were supported as monophyletic group (MPBS = 95%; MLBS = 100%; B/MCMC PP = 1.00).

Accessions of the allotetraploid Cr. acrostichoides and Cr. raddeana formed a moderately supported monophyletic sister taxa with 100% MLBS support while Cr. cascadensis and Cr. acrostichoides alleles as sister clades with 100% MLBS support while MP and B/MCMC did not recover this node.

3.2. Phylogenetic analyses of the gapCp data set

The gapCp “short” copy data set yielded MP, ML and B/MCMC topologies that were most congruent with one another and with the combined plastid loci data set analyses (Fig. 2). Alleles from most species formed supported, reciprocally monophyletic clades (MPBS ≥ 70%; MLBS ≥ 100%; B/MCMC PP = 0.99). The Cr. raddeana clade contained alleles from Cr. raddeana, Cr. sitchensis and Cr. acrostichoides (MPBS = 99%, MLBS = 100% and B/MCMC PP = 1.00). Additionally, the ML analysis recovered Cr. cascadensis and Cr. acrostichoides alleles as sister clades with 100% MLBS support while MP and B/MCMC did not recover this node.

3.3. chromEvol analysis

All four permutations of the chromEvol analysis (using topologies generated by ML and B/MCMC analyses of a reduced data set with one exemplar accession per species and using topologies generating by pruning excess accessions from the optimal consensus from the ML and B/MCMC analyses and reestimating the branch lengths) had an optimal AIC score for the “Constant rate” model of chromosome evolution. All four analyses, including four analyses using different models of evolution, were supported as monophyletic group of 30 for the Llavea/Coniogramme/Cryptogramma clade (Fig. 1). One chromosome loss event was inferred; this was the loss of a single chromosome on the branch leading to Llavea. Three genome duplication events were inferred by all four chromEvol analyses: a duplication was inferred on each of the branches leading to the tetraploid Cr. crispa and Cr. sitchensis (Fig. 1) and a genome duplication event was inferred on the branch leading to Coniogramme, which was inferred to have a base number of n = 60.

4. Discussion

4.1. Intergeneric phylogenetic relationships

We have demonstrated the monophyly of both Coniogramme and Cryptogramma for the first time (Fig. 1). Although their circumscription has not generated controversy previously, their relationship as sister taxa was not suggested until the advent of molecular research (Prado et al., 2007; Schuettgelz et al., 2007; Zhang et al., 2005; but see Gastony and Rollo, 1995, for the first suggestion that Llavea and Coniogramme are closely related). Little molecular differentiation was found among accessions of Llavea cordifolia; however, our sampling was restricted to central Mexico and does not include possible variation across its geographic range (Table 1). Sampling within Coniogramme was limited and relationships and species boundaries within the genus remain enigmatic. The inferred base chromosome number of n = 30 for the cryptogrammoid clade is consistent with general patterns within Pteridaceae (Tryon and Tryon, 1990), but the inferred base number of n = 60 for Coniogramme may be an artifact of limited sampling in that genus. The inferred base chromosome number of 30 for the

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Chromosome number</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Llavea cordifolia</td>
<td>29</td>
<td>Mickle et al. (1966), Knobloch (1967)</td>
</tr>
<tr>
<td>Cr. fraxinea</td>
<td>30,60*</td>
<td>Singh and Roy (1988), Matsumoto and Nakaie (1990), Kato et al. (1992)</td>
</tr>
<tr>
<td>Co. intermedia</td>
<td>30,60*</td>
<td>Matsumoto and Nakaie (1990), Kato et al. (1992)</td>
</tr>
<tr>
<td>Co. japonica</td>
<td>60</td>
<td>Weng and Qiu (1988)</td>
</tr>
<tr>
<td>Co. wilsonii</td>
<td>Xb</td>
<td>N/A</td>
</tr>
<tr>
<td>Cr. acrostichoides</td>
<td>30</td>
<td>Taylor and Lang (1963), Löve and Löve (1976), Alverson (1989a)</td>
</tr>
<tr>
<td>Cr. brunoniana</td>
<td>30</td>
<td>Khullar et al. (1988)</td>
</tr>
<tr>
<td>Cr. cascadensis</td>
<td>30</td>
<td>Alverson (1989a)</td>
</tr>
<tr>
<td>Cr. crispa</td>
<td>60</td>
<td>Manton (1950), Löve (1970), Löve et al. (1971), Pajarón et al. (1999)</td>
</tr>
<tr>
<td>Cr. fumariifolia</td>
<td>Xb</td>
<td>N/A</td>
</tr>
<tr>
<td>Cr. raddeana</td>
<td>Xb</td>
<td>Alverson (1989a)</td>
</tr>
<tr>
<td>Cr. stelleri</td>
<td>30</td>
<td>Wagner (1963), Britton (1964), Gervais et al. (1999)</td>
</tr>
</tbody>
</table>

* See Section 2 for details on coding taxa with conflicting counts.

* No published chromosome count available.
The number for Pteridaceae is 296 or 30, but does not shed light on whether the ancestral chromosome number for Pteridaceae is 29 or 30.

4.2. Phylogenetic relationships within Cryptogramma

Our study recovers both sections within Cryptogramma as monophyletic in accordance with previous classification systems (Alverson, 1989a; Hultén, 1968; Lellinger, 1985; Tryon and Tryon, 1990; Vaganov et al., 2010) and the numerous morphological characters distinguishing the sections. Within monotypic section Homopteris, populations of Cr. stelleri from Taiwan and Alaska were found to be more closely related to one another than to a population from Ontario. However, this relationship was only well-supported in the maximum likelihood analysis and potential divergence within this taxon should be investigated with more thorough geographic sampling.

Within section Cryptogramma, the disjunct South American taxon Cr. fumarifolia (Phil.) Christ. is the earliest diverging member of the lineage although it is unclear whether this has resulted from a recent or ancient dispersal event. Most relationships are geographically unsurprising, with the Asian taxa Cr. raddiana and Cr. brunoniana being closely related and the western North American taxa Cr. acrostichoides and Cr. stichensis forming a paraphyletic grade. One exception is the close relationship of the western North American Cr. cascadensis and the European Cr. crispa.

Most published species included in the data set are found to be reciprocally monophyletic. The lone exception is Cr. acrostichoides, which is polyphyletic due to the inclusion of allotetraploid Cr. stichensis accessions. Thus, we support the recognition of all eight published Cryptogramma taxa in our data set at the species level (Fig. 1), including both tetraploid taxa as they represent reproductively autonomous, discrete genetic lineages and can be diagnosed morphologically (Barrington et al., 1989). Two taxa were not assessed in this study due to a lack of suitable material; the Turkish Cr. bithynica and the Russian Far Eastern and Japanese Cr. gorovoii (formerly Cr. crispa var. japonica) remain in need of inclusion in future molecular studies.

4.3. Deciphering the history of tetraploid Cr. crispa

Long established as a tetraploid (Manton, 1950; Löve, 1970; Löve et al., 1971; Pajarón et al., 1999), little else has been known regarding the history or formation of the European Cr. crispa. Our plastid and nuclear genetic data both indicate that this species is most closely related to only Cr. cascadensis, from northwestern North America (Fig. 1, Fig. 2). While geographically distant, Cr. cascadensis and Cr. crispa do share morphological synapomorphies such as deciduous fronds with a thin lamina (Alverson, 1989b). We hypothesize that Cr. crispa is an autotetraploid whose progenitor was the now extinct or undiscovered European diploid sister species of Cr. cascadensis. This is supported by the geographic distance separating tetraploid Cr. crispa and diploid Cr. cascadensis, as...
4.4. Deciphering the history of tetraploid Cr. sitchensis

Previous research using morphology, spore measurement and allozyme data indicated that Cr. sitchensis is an allotetraploid and one of its progenitors was Cr. acrostichoides (Alverson, 1989a). The other progenitor was hypothesized to be the Russian Cr. raddeana based on morphology (Alverson, 1989a). Our plastid results (Fig. 1) confirm the role of Cr. acrostichoides as the maternal parent in this cross, as the plastid is maternally inherited in Pteridaceae (Gastony and Yatskievych, 1992) and the nuclear data (Fig. 2) confirm the morphological hypothesis that Cr. raddeana is the other progenitor. Interestingly, all sampled accessions of Cr. sitchensis form a single clade in the plastid data analysis (Fig. 1), implying a single origin of this taxon as opposed to the multiple origins typically observed in polyploid taxa (Soltis and Soltis, 2000).

A recent origin of this allopolyploid could be implied by the short branch lengths in the Cr. sitchensis clade (Fig. 1), although estimating polyploid lineage ages is fraught with difficulty (Doyle and Egan, 2010). The Beringian distribution of allotetraploid Cr. sitchensis and one diploid progenitor (Cr. acrostichoides) combined with the proximity of the other diploid progenitor (Cr. raddeana) suggests that Cr. sitchensis is another polyploid taxon formed during glacial maxima or subsequent recolonization (Consaul et al., 2010; Garcia-Jacas et al., 2009; Schmickl et al., 2010). Increased population-level sampling coupled with divergence time estimates and ecological niche modeling will refine this hypothesis (Metzgar, in progress).

4.5. Introgression within the Cr. acrostichoides complex

Our gapCp analysis identified a clade of Cr. acrostichoides alleles and a clade of Cr. raddeana, with alleles from the allotetraploid Cr. sitchensis occurring in both clades (Fig. 2). However, the Cr. raddeana clade also contains alleles from some populations of...
Cr. acrostichoides. Lineage sorting is unlikely, as a gene duplication event would have had to occur deep in the history of section Cryptogramma (Fig. 1) and would require an un parsimonious number of losses in various taxa and populations and an unreasonably slow coalescence time (Brokaw and Hufford, 2010). Our chromosome evolution reconstruction also rejects a whole genome duplication event predating extant Cryptogramma diversity (Fig. 1). We hypothesize that the presence of gapCp alleles from two populations of Cr. acrostichoides in a clade with Cr. raddeana and Cr. sitchensis alleles (Fig. 2) results from the introgression of Cr. raddeana alleles into some Cr. acrostichoides populations via backcrossing between Cr. sitchensis and Cr. acrostichoides (Fig. 3). One Cr. acrostichoides population that does not demonstrate any evidence of introgression is from Washington State, USA (Fig. 2; Fig. 3) and its allopatry with respect to Cr. sitchensis would preclude any chance of backcrossing. A triploid hybrid bridge could be the mechanism for backcrossing between Cr. sitchensis and diploid Cr. acrostichoides, as the two species frequently form a triploid hybrid which produces some spores that appear normal and could produce viable sperm (DeBenedictis, 1969; Husband, 2004). Introgression has also been found to occur at the gapCp locus in other fern taxa (Nitta et al., 2011; Yatabe et al., 2009).

5. Conclusions

We have established the monophyly of Coniogramme and Cryptogramma, as well as both sections within Cryptogramma. Additionally, all included Cryptogramma species in our study represent monophyletic lineages (Cr. acrostichoides is monophyletic only when allotetraploid Cr. sitchensis is not considered). We support the recognition of these eight taxa at the species level. We have also confirmed or identified the parentage of allotetraploid Cr. sitchensis and autotetraploid Cr. crispa for the first time.

This study serves as the first rigorous molecular assessment of relationships within Cryptogramma. By independently analyzing monophyly and reticulation, we have identified numerous areas of interest or need for future study. The timing and direction of dispersal events and genome duplications in the genus are in need of more rigorous estimation, as well as possible correlations with climatic fluctuations such as interglacial cycles. Additionally, several Cryptogramma species would make ideal candidates for detailed molecular and ecological reconstructions of survival strategies used by free-sporing monilophytes during the Last Glacial Maximum in North America.

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