

Genetic differentiation and polyploid formation within the *Cryptogramma crispa* complex (Polypodiales: Pteridaceae)

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Abstract: The tetraploid fern *Cryptogramma crispa* (L.) R.Br. ex Hook. is distributed across alpine and high latitude regions of Europe and western Asia and is sympatric with the recently described octoploid *C. bithynica* S.Jess., L.Lehm. & Bujnoch in north-central Turkey. Our analysis of a 6-region plastid DNA sequence dataset comprising 39 accessions of *Cryptogramma* R.Br., including 14 accessions of *C. crispa* and one accession of *C. bithynica*, revealed a deep genetic division between the accessions of *C. crispa* from western, northern, and central Europe and the accessions of *C. crispa* and *C. bithynica* from Turkey and the Caucasus Mountains. This legacy likely results from Pleistocene climate fluctuations and appears to represent incipient speciation between the eastern and western clades. These plastid DNA sequence data also demonstrate that the western clade of *C. crispa*, specifically the western Asian clade, is the maternal progenitor of *C. bithynica*. Our analysis of DNA sequence data from the biparentally inherited nuclear locus *gapCp* supports an autopolyploid origin of *C. bithynica*, with *C. crispa* as the sole progenitor.

Key words: *Cryptogramma*, ferns, autopolyploidy, phylogeography, glacial refugium

1. Introduction

The repeated range contractions and expansions caused by Pleistocene climate oscillations have long been recognized as an important driver of genetic diversity and differentiation in European biota (Hewitt, 2004). Several regions have been repeatedly identified as important refugia for taxa during glacial maxima, such as the Iberian Peninsula, the Italian Peninsula and the Balkans (Taberlet et al., 1998; Hewitt, 2004; Schmitt, 2007), and each region may comprise numerous additional small-scale refugia (see Médail and Diadema, 2009). Some organisms also show evidence of refugia in Turkey and/or the Caucasus Mountains (King and Ferris, 1998; Michaux et al., 2004; Gömöry et al., 2007; Grassi et al., 2008; Ansell et al., 2011). Glaciation and associated climate shifts also appear to increase the rate of polyploid formation in refugial areas and subsequent contact zones (Parisod et al., 2010). European fern taxa have also been shown to use these same refugia and also show an increase in the formation of polyploid lineages in response to glacial climate cycles (Vogel et al., 1999; Trewick et al., 2002).

The fern genus *Cryptogramma* R.Br. (Pteridaceae) consists of ten mainly boreal species, of which three are known to be polyploid taxa (i.e. *C. bithynica*, *C. crispa*,

and *C. sitchensis*; Metzgar et al., 2013). Previous research on the genus using a combined plastid and nuclear DNA dataset has shown diploid taxa to be genetically distinct, mostly allopatric lineages and revealed that the Beringian tetraploid *Cryptogramma sitchensis* is an allopolyploid (Metzgar et al., 2013). The European and southwestern Asian tetraploid *C. crispa* was shown to be an autopolyploid based on all its nuclear alleles being recovered in a single clade with no other taxon's alleles present (Figure 1; Metzgar et al., 2013). The recently described southwestern Asian octoploid *C. bithynica* S.Jess., L.Lehm. & Bujnoch (Jessen et al., 2012) is endemic to north-central Turkey and has never been included in a previous phylogenetic or molecular study, including that of Metzgar et al. (2013). Therefore, it is unknown if *C. bithynica* is an auto- or allopolyploid lineage and which species were involved in its formation. Sympatry and some shared morphological characters (i.e. deciduous leaves) suggest that *C. crispa* may have been involved in the formation of *C. bithynica*, but several other morphological characters such as leaf thickness vary and are used to distinguish these two taxa (Jessen et al., 2012).

Cryptogramma has not been previously used to study phylogeographical patterns in Europe and southwestern

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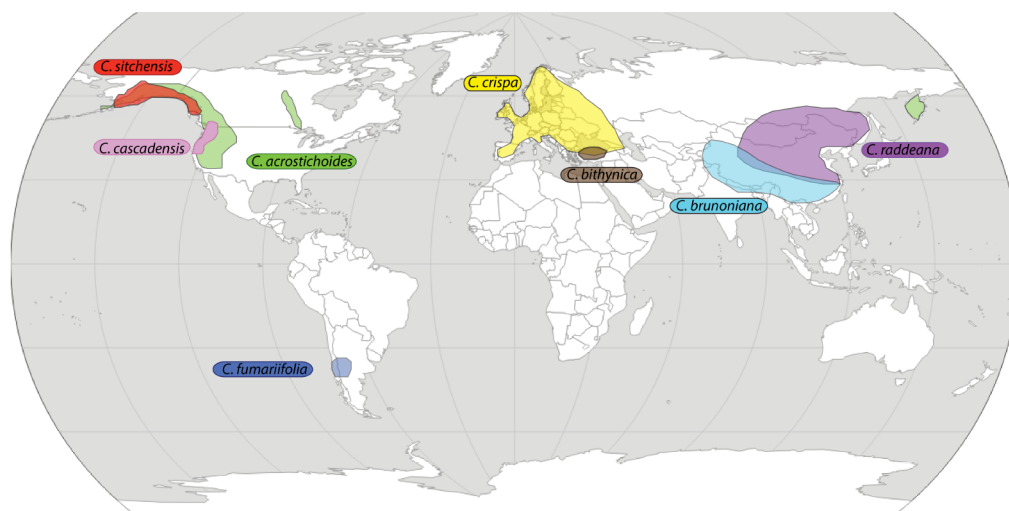


Figure 1. Approximate distributions of species of *Cryptogramma* sect. *Cryptogramma*. Colors reflect those used in plastid and nuclear phylogenies for each of the taxa (Figures 2 and 3).

Asia, although its distribution patterns and the frequent occurrence of polyploidy make it well suited to an examination of the role of Pleistocene refugia on genetic divergence, incipient speciation, and polyploid formation in a free-sporing vascular plant lineage. Here we characterize genetic diversity across the range of *C. crispa*, identify possible Pleistocene refugia, and identify the progenitor species of *C. bithynica*. This study expands on the nuclear and plastid datasets previously used to study phylogenetic relationships within *Cryptogramma* (Metzgar et al., 2013).

2. Materials and methods

2.1. Taxon sampling

The phylogenetic position of *Cryptogramma* within the Pteridaceae is well established (Zhang et al., 2005; Prado et al., 2007; Schuettpelz et al., 2007; Metzgar et al., 2013) and the genus has two reciprocally monophyletic sections, *Homopteris* and *Cryptogramma*, with one and nine species, respectively (Metzgar et al., 2013). Due to the well-established intergeneric relationships within the cryptogrammoid ferns (Zhang et al., 2005; Prado et al., 2007; Schuettpelz et al., 2007; Metzgar et al., 2013) and the strongly supported position of *Cryptogramma fumariifolia* (Phil.) Christ as the sister lineage to all remaining *Cryptogramma* sect. *Cryptogramma* taxa (Metzgar et al., 2013), we only included *Cryptogramma* sect. *Cryptogramma* accessions in the current study and used *C. fumariifolia* as the outgroup. *Cryptogramma stelleri*, the sole taxon in section *Homopteris*, was not included in this study as it is genetically isolated from all other *Cryptogramma* species and its exclusion greatly

reduced the amount of excluded data in the sequence alignments. The current study included 39 accessions from nine species of *Cryptogramma*, including 14 accessions of *C. crispa* and one accession of *C. bithynica* (Table 1).

2.2. DNA amplification and sequencing

Six plastid DNA regions were used in this study (*rbcl*, *rbcl-accD*, *rbcl-atpB*, *rps4-trnS*, *trnG-trnR*, and *trnP-petG*), and the *gapCp* “short” nuclear locus (henceforth *gapCp*) was sequenced for a subset of accessions (Table 1). The Invitrogen TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) was used to clone nuclear PCR products, and clones were amplified using the Invitrogen M13 primer pair. *Cryptogramma crispa* accessions each had 25 clones and the *C. bithynica* accession was sequenced for 44 clones. Primers, PCR conditions, cloning, sequencing, and matrix construction followed established protocols (Metzgar et al., 2013). For *gapCp* sequences, the sequence correction procedure first involved the examination of contigs formed by all sequences from a single accession in Sequencher version 4.10.1 (Gene Codes Corporation, Ann Arbor, MI, USA). All mutations and indels were mapped across the length of the putative allele and compared to identify and remove chimeric sequences and Taq error, with the resulting consensus sequence(s) exported as separate alleles (Grusz et al. 2009; Metzgar et al. 2013). Of the 254 sequences used here, 63 were generated expressly for this study and were deposited in GenBank (Table 1).

2.3. Phylogenetic analyses

Sequences were added to existing datasets (Metzgar et al., 2013) and aligned by eye using MacClade 4.08 (<http://macclade.org>). We excluded a total of 331 base pairs due to ambiguously aligned portions of the 6827 bp plastid

Table 1. Sampling data for specimen vouchers, including locality, herbarium (*sensu* Thiers, 2014), and GenBank accession numbers.

DNA ext. no.	Taxon	Locality	Collector/no. (herbarium acronym)	GenBank accession no.	<i>rbcL</i>	<i>rbcL-atpB</i>	<i>rps4-trnS</i>	<i>trnG-trnR</i>	<i>trnT-petG</i>	<i>gapCp</i> "short"
277	<i>Cryptogramma acrostichoides</i> R.Br.	USA, Alaska, Kodiak, near the transient boat harbor	Stuedebaker 09-473 (ALA)	KC700093	KC700133	KC700171	KC700210	KC700248	KC700284	-
278	<i>Cryptogramma acrostichoides</i>	USA, Utah, Salt Lake County, Little Cottonwood Canyon, near Snowbird	Rolfjels 2979 (ALA, DUKE, NHIC)	KC700094	KC700134	KC700172	KC700211	KC700249	KC700285	-
280	<i>Cryptogramma acrostichoides</i>	USA, Washington, Mason County, N of Lake Cushman along the Mt. Ellinor trail in the Olympic Mtns.	Windham 3624 (DUKE, UT)	KC700095	KC700135	KC700173	KC700212	KC700250	KC700286	-
281	<i>Cryptogramma acrostichoides</i>	USA, Oregon, Linn Co., Horse Rock Ridge, SW of Crawfordville.	Pryer 06-04 (DUKE)	KC700096	KC700136	KC700174	KC700213	KC700251	KC700287	-
296	<i>Cryptogramma acrostichoides</i>	USA, Oregon, Lane County, trail to Proxy Falls	Alverson s.n. (ALA)	KC700097	KC700137	KC700175	KC700214	KC700252	KC700288	-
353	<i>Cryptogramma acrostichoides</i>	USA, Washington, King County, Source Lake Lookout Trail, above Source Lake, Cascade Range	Zika 25403 (ALA)	KC700098	KC700138	KC700176	KC700215	KC700253	KC700289	KC700066, KC700071
359	<i>Cryptogramma acrostichoides</i>	USA, Alaska, Seward, Kenai Fjords National Park, Harding Icefield Trail	Metzgar 247 (ALA)	KC700099	KC700139	KC700177	KC700216	KC700254	KC700290	-
362	<i>Cryptogramma acrostichoides</i>	USA, Alaska, Southeast Alaska, 10 miles northwest of Juneau, Mendenhall Lake, behind Mendenhall Glacier Visitor Center	Anderson 745 (ALA)	KC700100	KC700140	KC700178	KC700217	KC700255	KC700291	KC700070, KC700058
365	<i>Cryptogramma acrostichoides</i>	USA, Alaska, Sitkalidak Island, Sitkalida Lagoon, cliffs along east side of lagoon	Stuedebaker 10-61 (ALA)	KC700101	KC700141	KC700179	KC700218	KC700256	KC700292	-
497	<i>Cryptogramma acrostichoides</i>	Russia, Kamchatka, north of Kamchatka peninsula, near Karaginskij	Chernyagina s.n. (ALA)	KC700102	KC700142	KC700180	KC700219	KC700257	KC700293	KC700059, KC700067
582	<i>Cryptogramma bilyynica</i> S.	Turkey, Uludağ, silicate scree slope on NNE side of mountain	Jessen SJ-3820 (ALA)	KT000629*	KT000649*	KT000639*	KT221146*	KT221156*	KT221165*	KT000619*, KT000620*
313	<i>Cryptogramma brunoniana</i>	Taiwan, NanTou County, Mt. ShihMen	Kuo 455 (TAIF)	KC700081	KC700121	KC700159	KC700198	KC700238	KC700273	KC700061
457	<i>Cryptogramma brunoniana</i>	China, Xizang (Tibet) Province, Baxoi Xian, Aujitu La (pass), N of Rawu (Raog)	Boufford 29733 (GH)	KC700082	KC700122	KC700160	KC700199	KC700239	KC700274	-
458	<i>Cryptogramma brunoniana</i>	China, Gansu Province, Wen Xian, Motianling Shan, Baishui Jiang Nature Reserve	Boufford 37747 (GH)	KC700083	KC700123	KC700161	KC700200	KC700240	KC700275	-
298	<i>Cryptogramma cascadenis</i>	USA, Oregon, Deschutes/Linn County boundary, McKenzie Pass	Alverson s.n. (ALA)	KC700086	KC700126	KC700164	KC700203	KC700241	KC700277	-
354	<i>Cryptogramma cascadenis</i>	USA, Washington, King County, Source Lake Lookout Trail, above Source Lake,	Zika 25404 (ALA)	KC700087	KC700127	KC700165	KC700204	KC700242	KC700278	KC700064, KC700065
282	<i>Cryptogramma crispata</i> (L.) R.Br. ex Hook	Norway, Hordaland, Bergen	Reeb VR4-VIII-02/11 (DUKE)	KC700088	KC700128	KC700166	KC700205	KC700243	KC700279	KC700062, KC700063
376	<i>Cryptogramma crispata</i>	Spain, Madrid Province, Sierra de Guadarrama, Siete Picos	Pajarón s.n. (ALA)	KC700089	KC700129	KC700167	KC700206	KC700244	KC700280	-
377	<i>Cryptogramma crispata</i>	Spain, Soria Province, Sierra de Urbión, Laguna Negra, cracks and between blocks of sandstone	Pajarón s.n. (ALA)	KT000624*	KT000644*	KT000634*	KT221141*	KT221151*	KT221160*	-

Table 1. (Continued).

389	<i>Cryptogramma crispa</i>	Norway, Troms Skjervoy County, Storfjellet, Aarviksand. In rocky depression on northern part of bare mountain region	Larsson 307 (DUKE, UPS)	KT000625 ^a	KT000645 ^a	KT000635 ^a	KT221142 ^a	KT221152 ^a	KT221161 ^a	-
390	<i>Cryptogramma crispa</i>	Sweden, Norrbotten Gällivare County, Dundret, Gällivare	Larsson 333 (DUKE, UPS)	KC700090	KC700130	KC700168	KC700207	KC700245	KC700281	-
391	<i>Cryptogramma crispa</i>	Austria, Steiermark, Niedere Tauern/Seckauer Alpen, Maierangerkogel – Vorwitzerattel	Pflugheih 111847 (ALA)	KC700091	KC700131	KC700169	KC700208	KC700246	KC700282	-
450	<i>Cryptogramma crispa</i>	Italy, northwest of Brunico, Astnerberg	Shimakov s.n. (ALTB)	KC700092	KC700132	KC700170	KC700209	KC700247	KC700283	-
489	<i>Cryptogramma crispa</i>	Spain, Ávila Province, Sierra de Gredos, Arroyo y Circo de los Pozas	Pajarón s.n. (ALA)	KT000626 ^a	KT000646 ^a	KT000636 ^a	KT221143 ^a	KT221153 ^a	KT221162 ^a	-
580	<i>Cryptogramma crispa</i>	Switzerland, Tessin, Val Scrdena bei Isone, S-Abhang der Cima Calasco	Jessen Sf. 3892 (ALA)	KT000627 ^a	KT000647 ^a	KT000637 ^a	KT221144 ^a	KT221154 ^a	KT221163 ^a	-
581	<i>Cryptogramma crispa</i>	Bulgaria, Pirin Mountains, south of Dautovo Lake	Jessen Sf. 3891 (ALA)	KT000628 ^a	KT000648 ^a	KT000638 ^a	KT221145 ^a	KT221155 ^a	KT221164 ^a	-
584	<i>Cryptogramma crispa</i>	Russia, Dombai, North Caucasus	Jessen Sf. 3099 (ALA)	KT000630 ^a	KT000650 ^a	KT000640 ^a	KT221147 ^a	KT221157 ^a	KT221166 ^a	KT000621 ^a , KT000622 ^a , KT000623 ^a
585	<i>Cryptogramma crispa</i>	France, Pyrenees Mountains, Cirque de Troumouse	Jessen Sf. 2920 (ALA)	KT000631 ^a	KT000651 ^a	KT000641 ^a	KT221148 ^a	KT221158 ^a	KT221167 ^a	-
597	<i>Cryptogramma crispa</i>	Russia, North Ossetia, Irvaskii region	Shitnikov s.n. (LE)	KT000632 ^a	KT000652 ^a	KT000642 ^a	KT221149 ^a	KT221159 ^a	KT221168 ^a	-
601	<i>Cryptogramma crispa</i>	Russia, Dagestan, Samur River	Popova 695 (LE)	KT000633 ^a	-	KT000643 ^a	KT221150 ^a	-	KT221169 ^a	-
396	<i>Cryptogramma funariifolia</i>	Chile, Provincia de Nuble, Comuna de Pinto, Shangri-La	Larrazin 34009 (ALA, CONC)	KC700079	KC700119	KC700157	KC700196	KC700236	KC700271	KC700073, KC700074, KC700075
397	<i>Cryptogramma funariifolia</i>	Chile, Provincia de Nuble, Comuna de Pinto, Shangri-La	Larrazin 34010 (ALA, CONC)	KC700080	KC700120	KC700158	KC700197	KC700237	KC700272	-
451	<i>Cryptogramma nuddeana</i>	Russia, Republic of Buryatia, Severo-Muiskiy range, Samokuyva	Naumov 1989 (NS)	KC700084	KC700124	KC700162	KC700201	-	-	KC70005
452	<i>Cryptogramma nuddeana</i>	Russia, Khabarovsk Krai, 30 km north of Sofiysk	Neitshuev s.n. (NS)	KC700085	KC700125	KC700163	KC700202	-	KC700276	-
355	<i>Cryptogramma stichensis</i> (Rupr.) T.Moore	USA, Alaska, between Portage and Whittier, Bering Glacier	Metzgar 248 (ALA)	KC700103	KC700143	KC700181	KC700220	KC700258	KC700294	KC700057, KC700056, KC700060, KC700068
356	<i>Cryptogramma stichensis</i>	USA, Alaska, Taku Glacier	Bas s.n. (ALA)	KC700104	KC700144	KC700182	KC700221	KC700259	KC700295	-
358	<i>Cryptogramma stichensis</i>	USA, Alaska, Seward, Kenai Fjords National Park	Metzgar 246 (ALA)	KC700105	KC700145	KC700183	KC700222	KC700260	KC700296	-
360	<i>Cryptogramma stichensis</i>	USA, Alaska, Palmer, Hatcher Pass	Metzgar 249 (ALA)	KC700106	KC700146	KC700184	KC700223	KC700261	KC700297	-
361	<i>Cryptogramma stichensis</i>	USA, Alaska, Valdez, Thompson Lake	Metzgar 257 (ALA)	KC700107	KC700147	KC700185	KC700224	KC700262	KC700298	-

^a: Indicates sequences generated for this study.

alignment (66 bp in *rbcl-accD*, 15 bp in *rbcl-atpB*, 67 bp in *rps4-trnS*, 84 bp in *trnG-trnR*, and 88 bp in *trnP-petG*). The resulting *gapCp* alignment was 599 bp long with no excluded characters. Alignments are available in TreeBASE (study ID 17439; <http://treebase.org>).

For model-based phylogenetic analyses, the appropriate model of sequence evolution was selected using Akaike information criterion scores calculated in MrModeltest 2.3 (Nylander et al., 2004). Prior to combining the six plastid region datasets, each region was analyzed separately using Bayesian Markov chain Monte Carlo (B/MCMC) and maximum parsimony bootstrap (MPBS) methods. The B/MCMC analyses were conducted in MrBayes version 3.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003; Ronquist et al., 2012). Each of these analyses was run for 10 million generations and implemented using default priors on four runs with four chains a piece. Tracer v1.5 (<http://beast.bio.ed.ac.uk/Tracer>) was used to inspect parameter convergence with the first 2 million generations discarded as the burn-in. The majority-rule consensus tree, posterior probabilities, and average branch lengths were calculated from the resulting 32,000 trees. The MPBS analyses consisted of 500 bootstraps replicated with 10 random addition sequence replicates implemented in PAUP* 4.0b10 (Swofford, 2002). The majority-rule consensus trees for the six plastid datasets were then inspected for supported (PP \geq 0.95; MPBS \geq 70) topological conflicts (Mason-Gamer and Kellogg, 1996). One conflict was detected with the *rbcl-atpB* B/MCMC topology supporting the inclusion of *C. crispa* + *C. bithynica* in a clade with *Cryptogramma brunoniana* Wall. ex Hook. & Grev. + *Cryptogramma raddeana* Fomin (PP = 0.98) rather than as the sister lineage to *Cryptogramma cascadiensis* E.R. Alverson. This relationship was not significantly supported in the *rbcl-atpB* MPBS analysis, so all six plastid datasets were combined into a single 6827 bp alignment.

Phylogenetic analyses of both the *gapCp* alignment and the combined plastid alignment were conducted using maximum parsimony (MP), maximum likelihood (ML), and B/MCMC. MP tree searches were run for 1000 heuristic replicates, using the random addition sequence (RAS) starting tree and tree-bisection-reconnection (TBR) branch swapping options in PAUP* 4.0b10 (Swofford, 2002). MP support values were calculated using 500 bootstrap replicates, each with 10 random addition sequence replicates.

ML analyses used region-specific models of sequence evolution implemented in Garli 2.0 (Zwickl, 2006) on the CIPRES Science Gateway computational portal (Miller et al., 2010). ML analyses were ran twice for eight replicates, using random starting trees and using stepwise addition starting trees. All ML bootstrap (MLBS) analyses were run

for 100 replicates. B/MCMC analyses were as previously described.

2.4. Spore measurements

Spores were removed from five herbarium specimens and mounted in glycerol on slides and examined using a Nikon Eclipse 80i compound microscope. From each specimen, 18–46 spores were measured at 400 \times magnification. Each spore was measured along its longest axis (Alverson, 1989) and both standard deviation and mean spore size were calculated for each specimen. Additional spore measurements values were culled from the primary literature (Table 2).

3. Results

3.1. Plastid DNA analyses

The combined six-region plastid dataset contained 185 variable sites and 172 parsimony informative characters. MP, ML, and B/MCMC analyses recovered congruent, well-supported (PP \geq 0.95; MLBS \geq 90%; MPBS \geq 90%) phylogenies for the combined six-region plastid dataset (Figure 2). The MP analysis identified two equally most parsimonious trees with a length of 205. The ML analysis identified a most-likely topology with a likelihood of -10406.891151 , with no topological differences between searches using random or stepwise starting trees. The B/MCMC topology was calculated from 32,000 post-burn-in trees and was well resolved with strong support for most relationships (Figure 2). All three methods recovered all diploid species as monophyletic (including polyploid progeny where relevant) with strong support. The *C. bithynica* accession was consistently recovered in a clade of *C. crispa* haplotypes from eastern Europe and western Asia. These inferred relationships are all congruent with previous phylogenetic assessments of *Cryptogramma* (Metzgar et al., 2013).

3.2. Nuclear DNA analyses

Tree topologies recovered from our MP, ML, and B/MCMC analyses of the *gapCp* dataset were congruent with one another and with previous research (Metzgar et al., 2013). We found eight most parsimonious trees in the MP search with a length of 60. The optimal ML topology had a likelihood of -1233.560927 , with identical topologies recovered using random or stepwise starting trees. The B/MCMC analysis generated a strongly supported topology that was congruent with the MP and ML analyses (Figure 3). Alleles of *C. bithynica* were recovered in a clade containing alleles of European and Caucasian *C. crispa* specimens with strong support (Figure 3).

3.3. Spore measurements

The mean spore lengths in *C. crispa* from the Caucasus Mountains (48–57 μ m) fell within the observed range of variation for other *C. crispa* samples from across Europe

Table 2. Mean spore size and chromosome numbers in the *Cryptogramma crispera* complex. Sample size for species measured by the present study range from 18–46 spores/specimen. Missing data indicated by dashes.

Sample no.	Species	Locality	Mean spore size (μm)	Standard deviation	Chromosome count	Number of spores measured	Reference
1	<i>Cryptogramma crispera</i>	Great Britain, Wales	-	-	$n = 60$	-	Manton, 1950
2	<i>C. crispera</i>	Iceland	-	-	$2n = 120$	-	Löve, 1970
3	<i>C. crispera</i>	England	54.8	2.23	-	26	Alverson, unpub.
4	<i>C. crispera</i>	England	57.5	1.56	-	25	Alverson, unpub.
5	<i>C. crispera</i>	England	53.7	1.60	-	25	Alverson, unpub.
6	<i>C. crispera</i>	Finland	58.6	2.20	-	25	Alverson, unpub.
7	<i>C. crispera</i>	France	49.8	1.30	-	25	Alverson, unpub.
8	<i>C. crispera</i>	France	54.1	1.79	-	25	Alverson, unpub.
9	<i>C. crispera</i>	Germany	50.9	1.72	-	25	Alverson, unpub.
10	<i>C. crispera</i>	Switzerland	49.8	1.30	-	25	Alverson, unpub.
11	<i>C. crispera</i>	Switzerland	55.3	1.75	-	25	Alverson, unpub.
12	<i>C. crispera</i>	Spain, Huesca Province; Scotland, Central Region	49.3–54.8	-	$2n = 120$	10	Pajarón et al., 1999
13	<i>C. crispera</i>	Switzerland, Tocino; Turkey, Artvin Province	48-57	-	$2n = 120$	-	Jessen et al., 2012
582	<i>C. bithynica</i>	Turkey, Uludag	70.87	-	$2n = 240$	-	Jessen et al., 2012
376	<i>C. crispera</i>	Spain, Madrid Province	47.1	3.98	-	37	Present study
377	<i>C. crispera</i>	Spain, Soria Province	49.4	5.39	-	18	Present study
489	<i>C. crispera</i>	Spain, Ávila Province	53.3	4.94	-	46	Present study

(47.1–58.6 μm) from the current and previous studies and were considerably smaller than in *C. bithynica* (70.87 μm) (Jessen et al., 2012; Table 2).

4. Discussion

4.1. Formation of *C. bithynica*

Our results suggest that the Turkish octoploid *C. bithynica* originated as an autopolyploid within the Caucasian clade of *C. crispera* (Figures 2–4). In the phylogeny derived from our plastid DNA data (Figure 2), *C. bithynica* was nested within a well-supported clade of *C. crispera* accessions from the Caucasian Mountains (Figure 4). Since plastids in ferns are typically inherited maternally (Gastony and Yatskievych, 1992), western Asian *C. crispera* probably acted as the maternal parent of *C. bithynica*. In our phylogeny based on the biparentally inherited nuclear locus *gapCp*, *C. bithynica* was nested within *C. crispera* (Figure 3), indicating an autopolyploid origin of *C. bithynica*. An

autopolyploid origin of *C. bithynica* is also concordant with current distributional patterns, as *C. crispera* is its only sympatric congener (Figure 1; Metzgar et al., 2013). Shared morphological characters such as deciduous leaves, ovate leaf segments, and sterile leaf shape also support this hypothesis, although *C. crispera* and *C. bithynica* can be distinguished based on leaf size, spore length (Table 2), and some subtle leaf characteristics such as leaf thickness and size (Jessen et al., 2012). The spore measurements and chromosome counts of *C. crispera* accessions across Europe and western Asia suggest that it is consistently tetraploid, $2n = 4x = 120$, with spore measurements (47.1–58.6 μm) easily distinguished from the isolated octoploid *C. bithynica* lineage (70.87 μm ; Manton, 1950; Löve, 1970; Pajarón et al., 1999; Jessen et al., 2012; Alverson, unpublished data; Table 2). Spore measurement data, when assessed in a phylogenetic context, have been used previously as a proxy for ploidy in Pteridaceae (Barrington

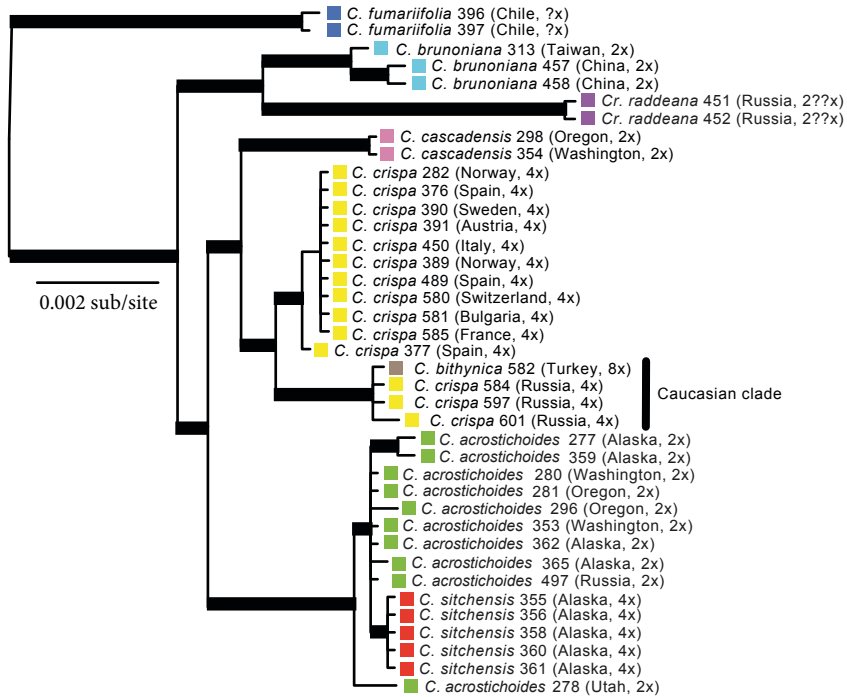


Figure 2. Phylogeny of *Cryptogramma* sect. *Cryptogramma* generated using a Bayesian Markov chain Monte Carlo (B/MCMC) analysis of a combined 6-region plastid dataset. Sixty-three of the 254 sequences used here were generated expressly for this study and were deposited in GenBank (Table 1). Interspecific relationships are congruent with previous research (Metzgar et al., 2013), but new sequence data has expanded sampling within the *C. crispa* clade. Strongly supported relationships (B/MCMC PP = 1.00; MLBS ≥ 95 %; MPBS ≥ 91 %) are depicted with thickened branches. Numbers following taxon names refer to extraction numbers (Table 1). The Caucasian clade of *C. crispa* and *C. bithynica* is marked by a vertical black bar. Colors as in Figure 1.

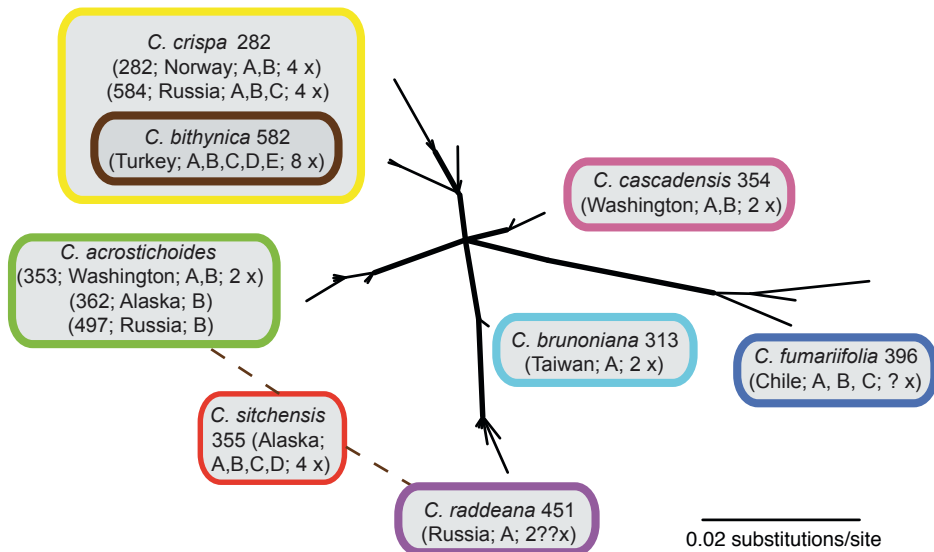


Figure 3. Unrooted phylogeny of nuclear *gapCp* “short” alleles of species of *Cryptogramma* sect. *Cryptogramma* analyzed by Bayesian Markov chain Monte Carlo (B/MCMC). Branches that represent strongly supported relationships (B/MCMC PP = 0.99; MLBS ≥ 100 %; MPBS ≥ 70 %) are thickened. Taxa present in clades are noted in color-coded bubbles along with taxon name, DNA extraction number, allele identifier, and estimated ploidy level. Colors as in Figure 1.



Figure 4. Distribution and sampling localities for *C. crispa* and *C. bithynica* with plastid clades (Figure 2) indicated by red dotted lines. Accessions sampled for DNA analysis are shown as colored circles with solid borders. Accessions included in spore size analysis (Table 2) are shown as colored circles with dashed borders. The Caucasus Mountains are depicted by inverted triangles.

et al. 1986; Grusz et al. 2009; Beck et al., 2011), although it is not reliable in at least one fern lineage (*Asplenium* L.; Dyer et al., 2013).

4.2. Genetic partitioning within *Cryptogramma crispa*

A sharp plastid DNA division is apparent within *C. crispa* (Figure 2), with the accessions from Turkey and the Caucasus Mountains clearly distinct from those of western and central Europe (Figure 1). The considerable sequence divergence observed between these clades is nearly equivalent to that separating sister species in *Cryptogramma*, although there appears to be no morphological distinction (including spore size; Table 2) between them (Figure 2; Metzgar et al., 2013). This genetic division could be indicative of incipient speciation occurring within *C. crispa*. Diversification following climate-induced range shifts have been commonly documented in temperate plants in general (e.g., Qiu et al., 2009) and in temperate ferns specifically (Haufler et al., 2008). Sequencing of additional loci would quantify gene flow between the two clades. Analysis of the nuclear locus *gapCp* revealed no division into eastern and western clades among *C. crispa* and *C. bithynica* alleles (Figure

3). The reason for this discrepancy between plastid and nuclear sequence data is unclear, but additional nrDNA sequencing of additional accessions of *C. bithynica* and *C. crispa* could be beneficial in resolving it.

The genetic distinctness of the Turkey + Caucasus Mountains clade (Figure 2) suggests a second Pleistocene refugium. Numerous plant and animal lineages show evidence of surviving climatic fluctuations in Turkish and/or Caucasian refugia (King and Ferris, 1998; Petit et al., 2002; Seddon et al., 2002; Rokas et al., 2003; Dubey et al., 2005; Kučera et al., 2006; Challis et al., 2007; Gömöry et al., 2007; Naydenov et al., 2007; Grassi et al., 2008; Ansell et al., 2011). The recent history of *C. crispa* appears to be most similar to that of *Vitis vinifera* L. subsp. *silvestris* (C.C.Gmel.) Hegi (Grassi et al., 2008) and *Arabis alpina* L. (Ansell et al., 2011). All three taxa display shared Turkish and Caucasian haplotypes that have not recolonized any additional regions.

The restricted range of eastern *C. crispa* haplotypes suggests that the Caucasus Mountains could have served as a barrier to recolonization, similar to other lineages (Figure 4; Seddon et al., 2002; Dubey et al., 2005), but

future research efforts sampling accessions north and northwest of the Caucasus Mountains would be needed to better evaluate this possibility. Phylogeographic patterns involving Turkey have been previously characterized into several broad categories (Bilgin, 2011) based on the geographic boundaries of genetic diversity. *Cryptogramma crispa* is an example of Bilgin's (2011) "Pattern I" with western and eastern clades that are divided between the Balkans and Anatolia. This divide between Anatolian and Balkan accessions was likely caused by the Sea of Marmara (Ansell et al., 2011) or western Anatolia (Bilgin, 2011).

This clear geographic separation of plastid genetic diversity within *C. crispa* probably reflects use of at least two different refugia during the Pleistocene glaciations. There is little differentiation between accessions across western, northern, and central Europe in the plastid phylogeny (Figure 2), with the exception of one moderately supported basal divergence in Spain. Iberia has commonly been inferred as a refugium for other vascular plant lineages (Taberlet et al., 1998; Hewitt, 2004; Schmitt, 2007), including ferns (Trewick et al., 2002; Jiménez et al., 2009). Our results could be suggestive that Iberia was a source for recolonization of deglaciated regions, but other potential sources cannot be eliminated. Unlike the pattern previously shown for some European ferns (Vogel et al., 1999; Trewick et al., 2002), the higher ploidy lineage (*C. bithynica*) is geographically restricted to a glacial refugium rather than having recolonized deglaciated regions. The

small geographic range of *C. bithynica* suggests that it has formed recently, although future research could assess this hypothesis using divergence time estimation.

This study illustrates the genetic isolation and incipient speciation that can result from climate change cycles. The use of the Caucasus Mountains as refugia is a novel finding for a free-sporing fern lineage. The octoploid *C. bithynica* will benefit from additional research, especially to determine if it has arisen multiple times.

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