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THE ORIGIN OF FERN *ATHYRIUM CHRISTENSENIANUM* (KOIDZ.) SERIZ. (ATHYRIACEAE)

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Athyrium christensenianum is considered an apogamous fern species that has originated from a hybrid of diploid sexual *A. crenulatoserrulatum* and tetraploid sexual *A. decurrentialatum*. There have been recent reports on tetraploid sexual *A. christensenianum*. In this study, I attempted to understand the relationships between triploid and tetraploid *A. christensenianum*. It appeared that tetraploid sexual *A. christensenianum* is of a hybrid origin between ancestral diploid sexual *A. decurrentialatum* and *A. crenulatoserrulatum*. In addition, triploid *A. christensenianum* did not seem to be of a hybrid origin between diploid sexual *A. crenulatoserrulatum* and tetraploid sexual *A. decurrentialatum*, rather of a hybrid origin between tetraploid sexual *A. christensenianum* and diploid sexual *A. crenulatoserrulatum*.

Keywords: *AK1*, Athyriaceae, chromosome, ferns, hybrid, *PgiC*

INTRODUCTION

In the sexual lineages of ferns, meiosis produces 64 haploid spores per sporangium following four mitotic divisions of spore mother cells, each of which has half the number of chromosomes of parental chromosome numbers (Manton, 1950). In contrast, apogamous ferns produce only 32 spores per sporangium by following one of these alternative spore-generating pathways to yield chromosomally unreduced diplospores: premeiotic endomitosis (Döpp, 1932; Manton, 1950) and meiotic first division restitution (Braithwaite, 1964). Most apogamous ferns produce spores by following the former pathway (Manton, 1950).

However, there is another scheme of apogamous reproduction in *Athyrium christensenianum* (Koidz.) Seriz. *A. christensenianum* (Koidz.) Seriz., a triploid apogamous species, is considered a hybrid of diploid sexual *A. crenulatoserrulatum* Makino and tetraploid sexual *A. decurrentialatum* (Hook.) Copel. (Kurita, 1964; Hirabayashi, 1970; Park and Kato, 2003). Park and Kato (2003) reported that *A. christensenianum* produces 16 or 64 rather regularly shaped apogamous spores per sporangium, which can produce sporophytes without fertilization. The mechanism of sporogenesis has not been resolved well yet, and all the previous studies reported irregular meiosis in young sporangium (Kurita, 1964; Hirabayashi, 1970; Park and Kato, 2003). In addition, a recent study on tetraploid sexual *A. christensenianum* suggested a more complicated origin of *A. christensenianum* (Hori and Murakami, 2019). This study attempted to examine and comprehend the relationship between triploid and tetraploid *A. christensenianum*, using nuclear and plastid DNA analysis.

MATERIAL AND METHODS

PLANT MATERIAL

Several samples of *A. christensenianum*, *A. decurrentialatum*, and *A. crenulatoserrulatum* were collected (Supplementary material S1). In molecular analysis, four species of the genus *Deparia* and one *Diplazium* were used as outgroups. Living plant and voucher specimens were maintained in the herbaria of Kochi Prefectural Makino Botanical Garden. Voucher information about these samples is listed in Suppl. 1. These materials included an individual of tetraploid sexual *A. christensenianum* (*Hori 2974*) (Hori and Murakami, 2019). Moreover, I observed the mitotic chromosomes of an additional individual (*Hori 2980*) of *A. christensenianum*, following the method reported by Hori and Murakami (2019).

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MOLECULAR ANALYSIS OF PLASTID AND NUCLEAR MARKERS

For molecular analysis, total DNA was extracted from silica-dried leaves using cetyltrimethylammonium bromide solution, as described by Doyle and Doyle (1990). Plastid *rbcL* was used as the maternally inherited cpDNA marker (Gastony and Yatskievych, 1992; Hori et al., 2018), and *AK1* and *PgiC* (15F-16R) were used as the biparentally inherited nrDNA markers (Ishikawa et al., 2002; Hori et al., 2018).

PCR–SINGLE-STRAND CONFORMATION POLYMORPHISM (SSCP) ANALYSIS

PCR-SSCP was used to determine allelic variations at the nuclear locus for each individual, according to the method described by Hori et al. (2018). Further, electrophoresis was performed using gels containing 2% glycerol at 15 °C for 15 h at 300 V for *AK1* and *PgiC*, followed by silver staining. Sequencing of the bands separated on the SSCP gels was performed by drying polyacrylamide gel following silver staining by sandwiching the gel between Kent paper and a cellophane sheet on an acrylic back plate at 55 °C for 4 h. For DNA extraction, a piece of the DNA band was peeled from the dried gel using a cutter knife and then incubated in 50 μL TE buffer (10 mM Tris-HCl and 1 mM EDTA; pH 8.0) at 25 °C overnight. The obtained supernatant was used as a template for further PCR amplification using the same primer set that was used for the original PCR amplification. The PCR products were purified using ExoSAP-IT (USB, Ohio, USA) or Illustra ExoStar 1-Step (GE Healthcare, Wisconsin, USA) and then used as templates for direct sequencing. For sequencing, the reaction mixtures were prepared using a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) and subsequently analyzed using an ABI 3130 Genetic Analyzer (Applied Biosystems). All plant samples were classified based on their PCR-SSCP banding patterns, and the genomic constitution of each pattern was identified by determining the nucleotide sequence of each DNA band separated on the SSCP gel.

PHYLOGENETIC ANALYSIS

For phylogenetic analysis, only one sequence representing each allele of the nuclear gene loci (i.e., *AK1* and *PgiC*) and each haplotype of cpDNA (i.e., *rbcL*) was used in the datasets. Sequences of all genes were aligned using the MUSCLE program (Edgar, 2004) and analyzed using different methods, including Bayesian inferense (BI) analysis using MrBayes 3.2.6 (Ronquist et al., 2012) and maximum parsimony (MP) analysis using MEGA X software (Kumar et al., 2018). In BI analysis, the best fitting model of sequence evolution for each DNA region was selected using jModelTest 2.1.10 (Darriba et al., 2012). The *rbcL* was constructed using the SYM+I+ model, *AK1* and the *PgiC* tree was constructed using the HKY model. Four chains of Markov chain Monte Carlo (MCMC) were run simultaneously and sampled every 100 generations for a total of 10 million generations. Tracer 1.7.1 (Rambaut et al., 2018) was used to examine the posterior distribution of all parameters and their associated statistics, including estimated sample sizes. The first 25,000 of the sample trees from each run were discarded as a burn-in period. The MP tree was obtained using the subtree pruning-regrafting algorithm (Swafford et al., 1996) at the search level 1, at which the initial trees were obtained by random addition of sequences (10 replicates). Indels were treated as missing characters in the MP and BI analyses. Therefore, they were not distinguished on the molecular trees. The bootstrap method with 1,000 replications was employed to estimate the confidence level of the monophyletic groups.

RESULTS AND DISCUSSION

CYTOLOGICAL STUDY

The mitotic metaphase chromosome number observed in an individual of *A. christensenianum* from Tokyo pref. (*Hori 2980*) was 2n = 120 (Fig. 1). The basic chromosome number of the genus *Athyrium* is x = 40; accordingly, these samples were determined to be triploid.

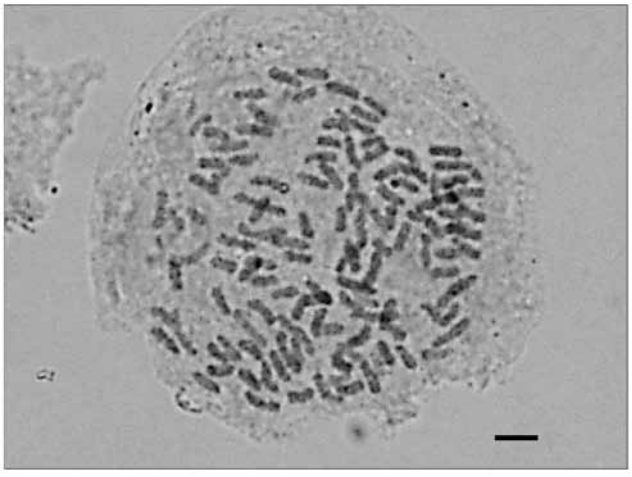

Fig. 1. Photograph of mitotic metaphase chromosomes of triploid (2*n* = 120) *Athyrium christensenianum* (*Hori2980*). Scale bar = 4 μm.

MOLECULAR PHYLOGENETIC TREES ACCORDING TO THE NUCLEOTIDE SEQUENCES OF THE TWO NUCLEAR AND PLASTID *RBCL* MARKERS

In most samples, several alleles were detected by SSCP. In total, 13 and 12 distinct sequences were identified at the *AK1* and *PgiC* loci, respectively. After editing, the data matrix for phylogenetic analyses included 614 and 358 characters, respectively, of which 81 (13%) and 47 (13%) characters were polymorphic and 31 (5%) and 22 (6%) were parsimoniously informative, respectively. The MP trees according to the sequences of *AK1* and *PgiC* with Bayesian posterior probabilities (PP) and bootstrap percentages (BPs) of the MP analyses are partially shown in Figs. 2a,b.

In each of the molecular trees of the nuclear markers, the sequences obtained from *A. crenulatoserrulatum* and *A. decurrentialatum* could be distinguished (Type A = *A. crenulatoserrulatum* and Type B = *A. decurrentialatum*) as belonging to the monophyletic groups.

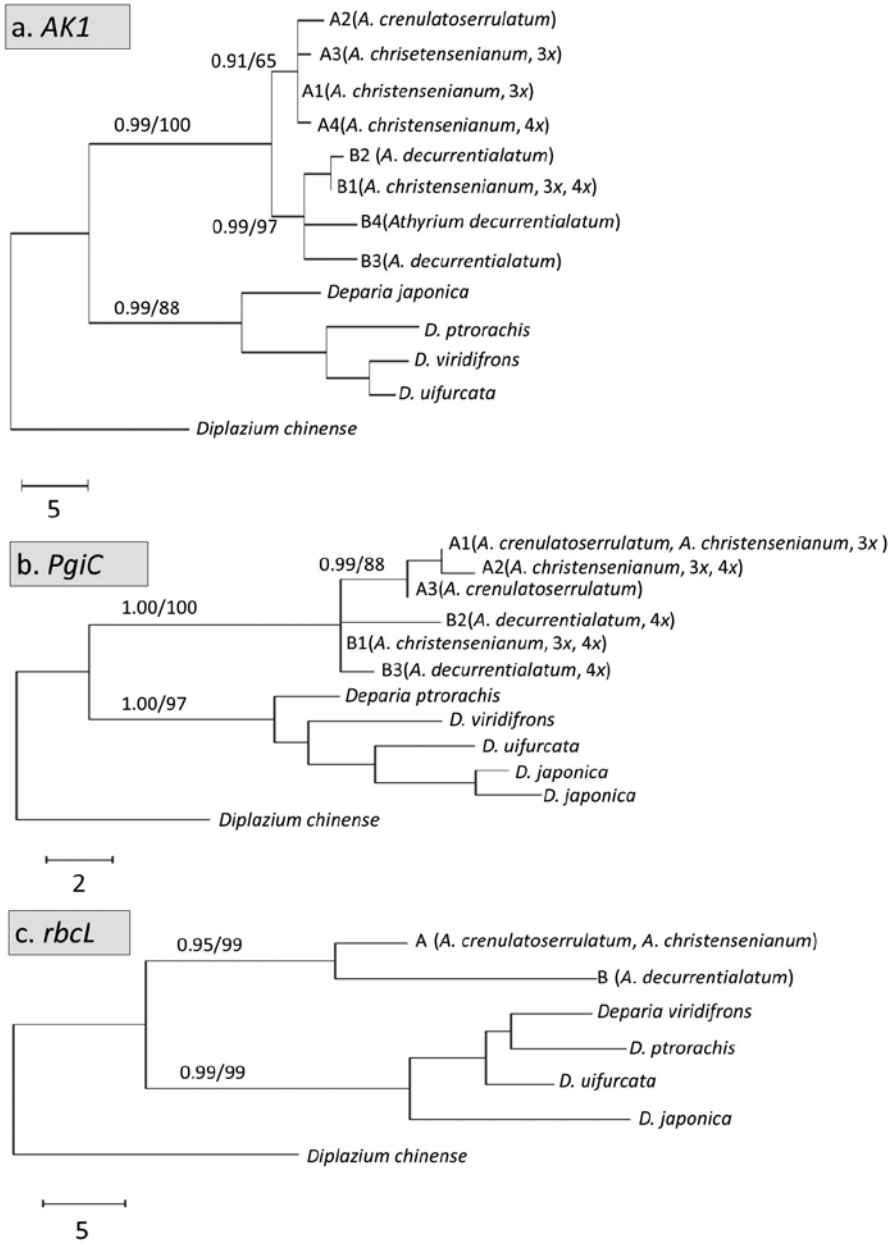

Fig. 2. (**a**) MP tree based on sequence variation of gene *AK1* with PP (> 0.90) and BPs (> 50) of MP analyses on each branch; (**b**) *PgiC*; (**c**) *rbcL*.

The combination of the alleles of each sample in the complex was the same between the two nuclear loci: *A. crenulatoserrulatum*, A; *A. decurrentialatum*, B; triploid *A. christensenianum*, AAB; and tetraploid *A. christensenianum*, AB (Suppl. S1).

Two types of plastid *rbcL* sequences (haplotypes A and B) were recognized from the materials except the outgroups. After editing, the data matrix for phylogenetic analyses included 1,205 characters, of which 89 (7%) characters were polymorphic and 37 (3%) characters were parsimoniously informative. The BI and MP analyses resulted in the creation of phylogenetic trees with similar topologies. The MP tree is shown in Fig. 2c. *A. crenulatoserrulatum* and *A. christensenianum* had haplotype A, whereas *A. decurrentialatum* had haplotype B.

The allelic constitutions of triploid and tetraploid *A. christensenianum* are not concordant with the hypotheses reported in previous studies (Kurita, 1964; Hirabayashi, 1970; Park and Kato, 2003). Tetraploid sexual *A. christensenianum* had one allele of *A. crenulatoserrulatum* and *A. decurrentialatum*, each in nuclear *AK1* and *PgiC*. Otherwise, triploid *A. christensenianum* had two alleles of *A. crenulatoserrulatum* and one allele of *A. decurrentialatum*. These allelic constitutions suggest alternative hypotheses: (1) tetraploid sexual *A. christensenianum* originates from the hybridization of diploid sexual *A. crenulatoserrulatum* (maternal, supported by *rbcL* haplotype) and an ancestral or extinct diploid *A. decurrentialatum*; (2) triploid apogamous *A. christensenianum* originates from the hybridization of diploid sexual *A. crenulatoserrulatum* and tetraploid sexual *A. christensenianum*.

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