Genetic variation in an apogamous fern species
*Cyrtomium fortunei* (Dryopteridaceae) and
its acquisition mechanisms

無配生殖をおこなうヤブソテツ類（オシダ科）にみられる
遺伝的多様性とその獲得メカニズムの解明（英文）

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Summary

Apogamy in ferns is a type of asexual reproduction in which unreduced spores are formed, and the resultant gametophytes produce the sporophytes of the next generation without fertilization. Thus, all offspring from a parent are expected to be clonal. However, apogamous species frequently show a large amount of morphological variations, and often some genetic variation as well. *Cyrtomium fortunei* (Dryopteridaceae) and its relatives, which are distributed throughout Japan, are reported to be apogamous triploids, but show large and continuous morphological variation. Four varieties (var. *fortunei*, var. *clivicola*, var. *intermedium* and var. *atropunctatum*) of this species have been recognized. However, it is difficult to distinguish these varieties only by morphology because their morphological variations are large and continuous.

In Chapter 1, I examined whether the apogamous fern species, *Cyrtomium fortunei* have genetic variation, and if so, whether this variation relates to morphological variation within local populations of the species. Among 224 individuals growing in four distantly located populations in Japan (Moroyama, Kawazu, Kobe and Fuchu populations), where several varieties grow together, two *rbcL* types (α and β) and eight allozyme types (A–H) were identified. Several different genetic clones were detected in all the local populations examined. Only individuals, which could be morphologically classified as var. *intermedium* based on bicolored indusia, had *rbcL* β and allozyme type H, and thus, were genetically differentiated by their nuclear and plastid genomes from the other three varieties of *C. fortunei*. The other three varieties shared the same *rbcL* (α) and significant correlation between allozyme types and morphological variation was not observed.

It was also shown in Chapter 1 that considerable genetic diversity was observed in
all local populations of the apogamous fern species so far examined. Several hypotheses have been proposed to explain the increased genetic variation within an apogamous fern species: (1) hybridization with closely related sexual species, (2) unequal meiosis, and (3) genetic segregation by homoeologous chromosome pairing. Because only apogamous triploids were reported from the apogamous fern species in Japan, I considered that hybridization with closely related sexual species or unequal meiosis should not contribute to produce intra-specific or intra-populational genetic variation in the apogamous species.

Thus in Chapter 2, I hypothesized that genetic segregation has occurred in *Cyrtomium fortunei*, and I made both genetic and cytological comparison between a parental sporophyte and its offspring. Of 732 progenies examined (250 gametophyte offspring and 482 sporophyte offspring), obtained from a parental sporophyte K220, which had both *pgiC* a and b sequences, 11 (4.4%) gametophytes and 8 (1.7%) sporophytes showed only the a sequence. Cytologically, offspring sporophyte (100 samples) so far examined was all shown to be triploid, which are the same as that of their parent sporophyte. Therefore, I could show that genetic segregation has occurred in apogamous *C. fortunei*.

This is the second evidence after the first one by Ishikawa et al. (2003a), showing genetic segregation in apogamous ferns. However, my results showed the frequencies much more accurately than the first one. Moreover, I could first show that the frequency of genetic segregates in offspring gametophytes is significantly higher than that in sporophytes of the next generation ($\chi^2 = 4.90\; P = 0.027$). These data suggest the existence of deleterious genes in apogamous fern species, which express during the morphogenesis and growth of sporophytes. It is one of the most important findings in the present study.
The life cycle of ferns is characterized by sporogenesis and existence of the free-living gametophyte (prothallium). In most of diploid ferns, motile sperms from the prothallium must swim through water to reach the eggs in the matured archegonia of a different prothallium growing nearby because fertilization within a gametophyte (intra-gametophytic selfing) cannot occur (Soltis and Soltis 1990, Watano and Iwatsuki 1988). Therefore, the fertilization of ferns is a highly risky process, especially during dry conditions. However, this only applies to sexual reproduction.

Apogamy or agamospory in ferns is a type of asexual reproduction in which unreduced spores are formed, and the resultant gametophytes produce the sporophytes of the next generation without fertilization (Manton 1950). Apogamous reproduction is common in ferns. About 10% of all fern species (Lovis 1977) and approximately 15% of Japanese fern taxa are reported to have apogamous reproduction (Takamiya 1996). Differing from other asexual reproductive modes, such as vegetative reproduction through gemma or adventitious buds, apogamous reproduction involves the production of spores that can tolerate dry conditions. Therefore, apogamous reproduction is advantageous especially for long distance dispersal and the formation of new populations from small numbers of spores.

On the other hand, apogamous reproduction has a serious drawback in the production of genetic variation within species and their populations, because the processes of normal meiosis and fertilization, which produce and maintain intra-specific genetic variation in sexual reproduction, are lacking. The production and maintenance of genetic variation by sexual reproduction has evolutionarily important. In the case of apogamous reproduction, only genetic clones of the parent individual are produced, and eventually genetic variation
within the population will disappear. In such a situation, if there is extreme environmental change or an epidemic of infectious diseases, the extinction risk of the populations is high.

Apogamy fern species are expected to demonstrate small amount of genetic variation. As a matter of fact, Darnaedi et al. (1990) reported that *Dryopteris yakusilvicola* Kurata, a triploid apogamous species of recent hybrid origin, endemic to Yaku Island, did not show any allozyme variation within species. However, this is not common. In spite of the clonal nature of apogamous reproduction, many apogamous fern species show large morphological and some genetic variation (Watano and Iwatsuki 1988, Suzuki and Iwatsuki 1990, Lin et al. 1995, Takamiya et al. 2001).

Previous studies analyzing genetic variation in an apogamous fern species using enzyme electrophoresis have reported 45, 4, and 14 different clones from *D. nipponensis* Koidz. (Ishikawa et al. 2003a), *Asplenium unilaterale* Lam. (Watano and Iwatsuki 1988), and *D. bissetiana* (Baker) C.Chr. (Lin et al. 1995), respectively. Furthermore, genetic variations in combination with cytological variation have been reported in apogamous ferns. Six different triploid and five diploid clones have been reported in *Pteris cretica* (Suzuki and Iwatsuki 1990) and one tetraploid and four triploid clones have been reported in *Diplazium doederleinitii* (Takamiya et al. 2001).

Thus, in spite of the clonal nature of apogamous reproduction, many apogamous fern species show large morphological variation. In fact, several apogamous fern species have been reported to show some genetic variation as noted above. Several hypotheses have been proposed to explain the increased genetic variations within apogamous fern species: (1) hybridization with closely related sexual species (Walker 1962, Suzuki and Iwatsuki 1992), (2) unequal meiosis (Lin et al. 1992), and (3) genetic segregation by homoeologous chromosome pairing (Klekowski 1973, Ishikawa et al. 2003 a, b). Details of these hypotheses
are as follow.

(1) The crossing ability of apogamous ferns with closely related sexual species was first demonstrated by Walker (1962) with artificial crossing experiments between apogamous and sexual races of *Pteris* species. He reported that the reduced egg of the sexual diploids is fertilized by the unreduced sperm of the apogamous triploid to produce tetraploid hybrids, which can reproduce apogamously. Suzuki and Iwatsuki (1992) supported this hypothesis by suggesting that wild populations of apogamous *P. cretica* in Japan were derived through hybridization between the apogamous parents of the species and the closely related sexual species *P. kidoi*.

(2) Unequal meiosis reported by Lin et al. (1992) may also cause different genotypes within the progeny of apogamous ferns. They performed cytological and genetic studies using *Dryopteris pacifica*, from which both diploid and triploid apogamous races had been recorded, to elucidate the origin of its intra-specific cytological variation. They reported that two (1.3%) of the 150 gametophytes cytologically examined by them, were diploid. And one (0.9%) of the 110 sporophyte offspring examined, was diploid. They also observed unequal meiosis in the triploid apogamous sporophytes, producing diploid spores. Thus, they could show that diploid spores and the gametophytes arising from them, as well as diploid apogamous sporophyte offspring, are occasionally formed from parental triploid apogamous sporophytes. However, they could not clarify whether this process truly contributes to the genetic variation observed in wild populations of apogamous ferns.

(3) Klekowski (1973) first proposed that the paring of homoeologous chromosomes could be one of the mechanisms leading to genetic variation in apogamous ferns, although no positive evidence was provided by his study. The sporogenesis pathway in most apogamous ferns follows the Döpp–Manton scheme (Manton 1950). In most sexual ferns, the archesporial
cell undergoes four mitotic divisions giving rise to 16 spore mother cells in each sporangium. They undergo meiosis and 64 spores are formed. In the Döpp–Manton scheme, however, the fourth mitosis is terminated at early anaphase, and a restitution nucleus is formed. The resultant eight spore mother cells possess doubled sets of chromosomes. For example, if the genotype of the parental triploid apogamous sporophyte is \( abc \), the genotype of the spore mother cells of the apogamous fern should be \( aabbcc \). In the usual process of apogamous reproduction, the genotype of all offspring from such a sporophyte would also be \( abc \) because only homologous chromosome paring (a-a, b-b, c-c) occurs in the spore mother cells. In contrast, if homoeologous chromosome pairing (\( a-b \), \( a-b \), \( b-c \), \( c-b \)) occurs, gametophytic progeny with \( aac \) and \( bcc \) genotypes will be produced in addition to those with the \( abc \) genotype. In the same way, if homoeologous chromosome pairing (\( a-c \), \( a-c \), \( b-b \)) occurs, progeny with \( aab \) and \( bcc \) genotypes will also be produced. Thus, genetic segregation through homoeologous chromosome paring can generate genetic variation within apogamous fern species.

Ishikawa et al. (2003a) first reported positive evidence supporting genetic segregation through homoeologous chromosome paring in apogamous ferns using \( D. nipponensis \). They compared genotypes of the parent sporophyte and its progeny using the enzyme electrophoretic method. Among 250 progeny gametophytes developed from spores of a parental sporophyte of \( D. nipponensis \) with the \( Pgi-2 \) genotype \( abc \), three showed different genotypes from that of the parent (three \( aac \) and one \( bcc \) gametophytes). Similarly, among 34 sporophyte offspring of the parent sporophyte with \( abc \) genotype, one showed \( bcc \) genotype, whereas the other 33 showed \( abc \). Thus, Ishikawa et al. (2003a) clearly showed the existence of occasional genetic segregation possibly through homoeologous chromosome paring in ferns.

Meanwhile, the genus \textit{Cyrtomium} C. Presl (Dryopteridaceae), which is
characterized by peltate indusia and anastomosing veins that form areolae with included veinlets, is an Asiatic fern genus comprising approximately 40 species. This genus contains many apogamous species, such as *C. macrophyllum* Tagawa (Kurita 1967, Hirabayashi 1970), *C. caryotideum* C. Presl (Kurita 1966, Mitsuta 1986, Matsumoto 1976), and *C. fortunei* J. Sm (Kurita 1960, Mitui 1968, 1980, Hirabayashi 1970, Matsumoto and Shimura 1985). In terms of reproductive modes and cytotypes of *C. fortunei*, Nakato et al. (1995) reported a diploid sexual type, which has 82 somatic chromosomes and 64 spores in each sporangium, in addition to the triploid apogamous type, which has 123 somatic chromosomes and 32 spores in each sporangium, from China. However, until date only apogamous triploids have been recorded from *C. fortunei* in Japan (Takamiya 1996).

In the most recent *Flora of Japan* by Iwatsuki et al. (1992), *Cyrtomium fortunei* sensu lato was characterized by pinnae thin-chartaceous to papyraceous, dentate at margin in distal portion; number of lateral pinna pairs, usually more than 10 pairs; width of each pinna less than 4 cm. In *C. fortunei*, four varieties: var. *fortunei*, var. *clivicola*, var. *intermedium*, and var. *atropunctatum* have been recognized based on the differences in the following three morphological characters: (1) number of lateral pinna pairs, (2) color of indusium, and (3) presence or absence of auricle at anterior base of the pinna (Table 1). Among the four varieties of *C. fortunei*, var. *fortunei* has the highest number of lateral pinna pairs (15–30 pairs), whereas var. *clivicola*, var. *intermedium*, and var. *atropunctatum* have 5–20, 10–15, and 10–20 pairs, respectively. The color of var. *intermedium* and var. *atropunctatum* indusia is dark brown in the central portions and grayish white in the other portions, whereas those of the other two varieties are completely grayish white. Var. *intermedium* has an auricle at the anterior base of each pinna, but it is absent in var. *atropunctatum*. Var. *fortunei* and var. *clivicola* sometimes have auricles, but not always.
Anyway, the number of lateral pinna pairs overlaps among the four varieties. Thus morphological variation is continuous and individuals with intermediate morphology or various combinations of the abovementioned morphological characteristics exist. Therefore, it is very difficult to distinguish these four varieties from one another. Furthermore, even in each local population of *Cyrtomium fortunei* in Japan, large and continuous morphological variation is observed. As this species is apogamous, it is expected to have low or discontinuous intraspecific variation. However, actual conditions are very different from the expectation.

Because apogamous *Cyrtomium fortunei* demonstrate large and continuous morphological variation and it is difficult to classify the varieties of *C. fortunei* only by morphological characteristics, we need to examine whether they have different genetic backgrounds. Furthermore, it is important to clarify amount of genetic variation within the apogamous species. If this species have large genetic variation even though only apogamous cytotype is contained in it, I considered that we need to elucidate mechanisms producing gengetic diversity within the apogamus fern species.

In this study, I carefully investigated several local populations of *C. fortunei* in Japan to clarify its genetic variation (Chapter 1). Because I could clarify that large and continuous genetic variation exist in all local populations of *C. fortune*, I studied genetic segregation of the apogamous fern species as a possible mechanism for producing genetic diversity in each population (Chapter 2).
Chapter 1. Genetic variation in the apogamous fern *Cyrtomium fortunei* (Dryopteridaceae)

1.1. Introduction

As mentioned in the General Introduction, apogamy in ferns is a type of asexual reproduction in which unreduced spores are formed, and the resultant gametophytes produce the sporophytes of the next generation without fertilization (Manton 1950). In apogamous ferns, all offspring from a sporophyte are genetically same unless mutations occur during reproduction. Therefore, the amount of genetic variation within an apogamous species is expected to be very low, especially in the case that the apogamous species is not of recent and recurring origin.

*Cyrtomium fortunei* was all reported to be apogamous in Japan, but still show large and continuous morphological variation. Therefore, we can expect that also large and continuous genetic variation might be observed in the apogamous species. I considered that we need to examine genetic variation especially in population level using genetic markers.

In Chapter 1, I collected fresh leaf samples from mature individuals of *C. fortunei* in four distantly located populations in Japan. In all populations, individuals that could be classified into the different varieties of *C. fortunei* grew together. I first determined the genotypes of all samples and distinguished clones using the *rbcL* sequence and allozyme variation as genetic markers. In asexually reproducing organisms, the minimum biological unit is a genetic clone. Thus, I considered that the most efficient method of analyzing apogamous species such as *C. fortunei* was to identify clones growing within local populations using genetic markers. Such intensive population genetic analyses have never
been performed in any apogamous fern.

Allozyme variation is a powerful codominant marker for population genetic analyses of wild plants. Nucleotide sequence variation of \textit{rbcL}, which is encoded in chloroplast DNA and maternally inherited in ferns, is a useful tool for determining maternal races in local fern populations (Yatabe et al. 1998, Murakami et al. 1999). Lu et al. (2005) sequenced the chloroplast DNA \textit{rbcL} and \textit{trnL-F} regions of 19 species of the genus \textit{Cyrtomium}, including \textit{C. fortunei}, and eight species of related genera to establish the molecular phylogeny. However, they did not analyze varieties of \textit{C. fortunei}. Detailed analysis of \textit{rbcLs} from four varieties of \textit{C. fortunei} has not been performed. After identifying genetic clones, I compared morphological variation between the same or different genetic types within each local population and between different populations.

In Chapter 1, each genetic type that could be distinguished by \textit{rbcL} and/or allozyme markers are tentatively termed a separate clone. It should be noted that genetic types differing in any genetic marker belong to different clones, but types with identical genotypes do not necessarily belong to the same clone.

In Chapter 1, the following four questions were specifically considered: (1) Do several different genetic clones exist in each population where several morphological varieties are observed together? (2) If several genetic clones do exist in each local population, how many clones are present in each population? (3) Are the same clones (individuals showing the same genetic type) shared, even among remote populations, or are different clones distributed in each local population? (4) Is genetic differentiation observed among the varieties that have been identified according to their morphological characteristics or do the four varieties correspond to different genetic clones?
1.2. Materials and Methods

Study sites and sample collection

Cyrtomium fortunei plant materials used in this study were collected from four populations in Japan: Moroyama, Saitama Pref.; Kawazu, Shizuoka Pref.; Kobe, Hyogo Pref.; and Fuchu, Hiroshima Pref. (Fig. 1). In each population, many morphological variants, which could be classified into several different varieties of *C. fortunei*, grew together.

In general, whole fresh leaf samples were collected from mature individuals in each population. In the Moroyama, Kobe, and Fuchu populations, quadrats were set and location of each individual was recorded before sample collection. In the Moroyama population, 51 *Cyrtomium fortunei* mature individuals were examined in an area of 60 × 20 m. In the Kobe population, mature leaves were collected from 83 individuals in an area of 100 × 20 m and from an additional 24 nearby individuals. In the Fuchu population, leaf samples from 55 individuals were collected in an area of 50 × 50 m. Quadrat size differed among populations because quadrats were set such that total number of samples in each quadrat was approximately 50, despite different growing densities. The growing density of *C. fortunei* in the Kawazu population was too low to set a quadrat. In every population, growing environments such as the amount of direct sunlight (amount of shade), moisture in the soil, and topographical features were observed and recorded for each sample.

We identified the four varieties of *C. fortunei* based on the distinctive morphological characters shown in Table 1. We tentatively identified the fern samples with more than 15 pinna pairs as var. *fortunei*, and those with 15 or less pairs as var. *clivicola* according to Iwatsuki (1992). *Cyrtomium fortunei* var. *clivicola* and var. *intermedium* were found in the Moroyama and Kobe populations, although these varieties were not always distinguishable due to continuity of their morphological variation. Only var. *fortunei* and var.
clivicola were recognized in the Fuchu population, and var. intermedium was not found. In the Kawazu population, in addition to var. fortunei and var. clivicola, var. atropunctatum was also observed.

Two pinnae were picked from each leaf sample. One was maintained as a fresh leaf sample at 4°C in the refrigerator until it was used for allozyme and ploidy analyses. The other was dried with silica gel and kept in a small plastic bag at room temperature until it was used for DNA extraction. Furthermore, fresh root samples were collected from representative individuals in the Kobe population. The remaining parts of the leaf samples were dried in newspapers and maintained as voucher specimens. All voucher specimens have been deposited in the Makino Herbarium of Tokyo Metropolitan University.

DNA extraction and sequencing of rbcL gene

Total DNA was extracted from silica gel dried leaf samples using 2× hexadecyltrimethylammonium bromide (CTAB) solution according to the method of Doyle and Doyle (1990) with some modifications. In brief, 100 mg of dried leaves were ground into a fine powder using a TissueLyser (Retsch QIAGEN, Germany). Next, 500 µl of 2× CTAB solution was added, mixed, and heated at 55°C for 20 min. After chloroform–isoamyl alcohol (24:1) extraction, isopropanol precipitation was performed. The DNA pellet obtained was washed with 70% ethanol, air dried, and redissolved in 50 µl TE buffer. PCR amplification of the rbcL gene was performed using Nova Taq Hot Start DNA Polymerase (Novagen, Madison, WI), 1× Ampdirect Plus Buffer (Shimazu, Kyoto, Japan), and the af and cr primers of Hasebe et al. (1994). In addition, four original internal primers dF (5’-GGTGTGTGATTCAAGCTGGT-3’), dR (5’-GAGCCTGTACGCAAAGCTTCT-3’), e2F (5’-GCGGTGGACTTTGATTTCCACA-3’), and e2R (5’-GACAATTGGTGCAACCCAACC-3’).
were developed in this study and used for sequencing. The conditions for PCR amplifications were as follows: initial denaturation at 95°C for 10 min, 45 cycles at 94°C for 30 s, at 58°C for 30 s, and at 72°C for 90 s. The PCR products were incubated at 37°C for 30 min and 80°C for 20 min with 5% ExoSAP-IT (USB, Cleveland, Ohio) to remove single-stranded DNA. For cycle sequencing reactions, a BigDye Terminator kit version 3.1 (Applied Biosystems, Foster City, CA) was used. The reaction mixtures were analyzed on an automated DNA sequencer model 3100 (Applied Biosystems). The nucleotide sequences obtained were aligned using ClustalX2 (Larkin et al. 2007).

Molecular phylogenetic analysis

To compare our rbcL sequences obtained from Japanese Cyrtomium fortunei with those obtained by Lu et al. (2005) from Chinese materials, maximum parsimony (MP) analysis was performed using MEGA 5 software (http://www.megasoftware.net/). The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 1 in which the initial trees were obtained with random addition of sequences (10 replicates). Analysis involved the two nucleotide sequences of rbcL type from Japanese materials as well as the 16 rbcL sequences reported by Lu et al. (2005) from C. macrophyllum, C. omeiense, C. urophyllum, C. caryotideum, C. aequibasis, C. fortunei, C. devexiscapulae, C. falcatum, C. nephrolepioides, C. shingianum, C. hemionitis, C. grossum, C. chingianum, C. lonchitoides, and C. guizhouense from China. There were a total of 1237 positions in the final dataset of the rbcL sequences. Polystichum lonchitis was used as an outgroup. Bootstrap analysis with 1000 replicates was performed to evaluate internal supports of the trees obtained.

Allozyme analysis
Fresh pinnae were ground in 1 ml of cold extraction buffer (pH = 7.5) containing 0.1 mM Tris-HCl, 1 mM EDTA-4Na, 10 mM KCl, 10 mM MgCl2, 0.4% 2-mercaptoethanol, and 10% polyvinylpyrrolidone. Enzymes were resolved on 6% polyacrylamide gels following the procedures of Shiraishi (1988). We examined phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PG), hexokinase (HK), and leucine aminopeptidase (LAP), following the procedures of Shiraishi (1988). Loci were numbered, with the most anodal locus as 1 and progressively more cathodal locus with higher numbers. Alleles were similarly indicated at each locus, with the most anodal form designated “a” and progressively slower forms “b,” “c,” and so on.

Estimation of reproductive mode

The reproductive mode (apogamous or sexual reproduction) in most homosporous ferns can be estimated simply by counting the number of spores in a sporangium. Sexual ferns usually have 64 spores per sporangium, whereas apogamous ones have 32 (Manton 1950). We counted the number of spores per sporangium in the voucher specimens from the above four localities to determine the reproductive mode.

Cytological observation and ploidy analysis using flow cytometry

Mitotic chromosomes were observed for materials collected from six plants, which showed different genetic types, in the Kobe population. In this population, the largest number of different genetic types were identified by rbcL sequencing and allozyme analysis. Therefore, cytological observation and ploidy analysis of C. fortunei was performed for this population. Root tips were pretreated with 0.002 M 8-hydroxyquinoline for 6 h at approximately 20°C. After fixation in 45% acetic acid for 15–30 min, the root tips were
hydrolyzed in 1 N HCl at 60°C and then squashed in 2% aceto-orcein. Chromosomes were observed with an Olympus BX-41 microscope and photographed with an Olympus DP-50 digital camera.

Furthermore, to examine the ploidy level of individuals growing in the Kobe population in detail, DNA content of each nuclei extracted from fresh pinnae samples was measured by flow cytometry using a CyFlow Ploidy Analyzer (Partec, Munster, Germany) as well as a Cystain UV Precise P kit (Partec, Munster, Germany). A sample of leaf segment approximately 5 × 5 mm was finely chopped with a razor blade in 0.5 ml of nuclei extraction buffer from the kit, then filtered through a 50 µm mesh, and stained with 1.5 ml of staining buffer containing 4′, 6′-diamidino-2-phenylindole hydrochloride from the kit. Fresh leaf tissues of a diploid C. fortunei individual collected from China (Nakato et al. 1995) and another triploid individual from the Kobe population, whose ploidy was determined by chromosome counting in this study, were used as controls. First, DNA content per nuclei was measured for the diploid and triploid controls. I then analyzed 80 samples collected from the Kobe population under the same conditions.

Morphological observation

After identifying allozyme types, morphological characters among the different genetic types were observed and compared. The characters observed were (1) the number of lateral pinna pairs, (2) color of indusium, and (3) presence or absence of auricle at the anterior base of the pinna. Differences in the character (1) among the allozyme types were statistically tested using Steel-Dwass multiple range test.
Quantification of genetic variation

To quantify the clonal diversity detected in each population as well as within *C. fortunei* species, Simpson’s diversity index (*D*) was calculated for every population as well as for the entire species:

\[ D = 1 - \sum \frac{[n_i(n_i - 1)]}{[N(N - 1)]}, \]

where \( n_i \) is the number of individuals of variant i and \( N \) is the number of individuals collected.
1.3. Results

Nucleotide sequence variation of \textit{rbcL} gene

I determined the 1317-bp nucleotide sequence of the \textit{rbcL} gene for 224 individuals of \textit{Cyrtomium fortunei} from the four populations. Two types of \textit{rbcL} sequences were obtained, hereafter referred to as \textit{rbcL} sequence \( \alpha \) and \( \beta \). These sequences have been deposited in the DNA Database of Japan under accession numbers AB598689 and AB598690, respectively. Seven base pair substitutions were observed between the two sequences. Leaf samples collected from the Moroyama and Kobe populations showed both \( \alpha \) and \( \beta \) \textit{rbcL} sequences (Table 2). In contrast, samples collected from the Kawazu and Fuchu populations showed only sequence \( \alpha \).

Molecular phylogenetic analysis

Molecular phylogenetic analysis of \textit{rbcL} sequences obtained from the genus \textit{Cyrtomium} was performed by the MP method to infer the phylogenetic position of the sequences \( \alpha \) and \( \beta \). One of the 30 most parsimonious trees (length = 78) obtained in this study is shown in Fig. 2 with bootstrap probabilities. Its consistency index was 0.72 and retention index was 0.80. The \textit{rbcL} sequence \( \beta \) from Japanese \textit{C. fortunei} was the same as that from \textit{C. aequibasis}, and \textit{C. aequibasis} was more closely related to \textit{C. macrophyllum}. On the other hand, the \textit{rbcL} sequence \( \alpha \) differed in two base pairs from the Chinese \textit{C. fortunei} reported by Lu \textit{et al.} (2005), but still formed a clade with it (Fig.2).

Allozyme variation

I performed electrophoretic analysis of four enzyme systems and determined the
genotype of 224 individuals of C. fortunei. I could resolve six loci in total. For PGM and 6PG enzyme systems, two loci (Pgm-1 and Pgm-2, and 6pg-1 and 6pg-2) each were detected. However, 6pg-1 showed no allelic variation and was excluded from further analysis. For HK and LAP enzyme systems, one locus each was detected.

In total, eight electrophoretically distinguishable types were recognized (Fig. 3 and Table 2), and they were named allozyme types A–H. Genotypes and the number of individuals of each allozyme type observed in the four populations examined are shown in Table 3. In all four populations examined in this study, individuals with several allozyme types were observed. Therefore, several different clones grew together in these populations (Fig. 4). In the Moroyama and Kobe populations, three and five types were identified, respectively. Three allozyme types (A, E, and H) are common between these two populations, although they are remote populations separated by approximately 500 km (Table 3). Only individuals of allozyme type H had rbcL sequence β, whereas those of all other allozyme types had the rbcL sequence α. Table 3 summarizes the results of Simpson’s diversity index (D).

Reproductive mode

Reproductive mode was estimated by counting the number of spores per sporangium for 140 individuals (46 from the Moroyama population; 11 from Kawazu; 46 from Kobe; and 43 from Fuchu). At least two individuals for each of the eight clones recognized in this study were included in analyses. All individuals examined had 32 spores in each sporangium. This suggested that individuals of all eight allozyme types were apogamous.
Morphology

On the basis of my genetic analyses and morphological observations, individuals of allozyme type H with rbcL sequence β had bicolored indusia (Fig. 5). Furthermore, those of allozyme type B in the Kawazu population had bicolored indusia, but their rbcL sequence was α. Individuals of all other allozyme types (A, C–G) had uniformly light gray indusia, although they varied in leaf morphology.

The relationship between allozyme types and number of lateral pinna pairs is shown in Table 4. In addition, variation in number of lateral pinna pairs in each allozyme type is shown in Fig. 6. In this figure, it can be seen that the allozyme type D is significantly higher than other allozyme types (A, B, C, E, F and H) in number of lateral pinna pairs. However, the type G is not significantly different from the types A to F, including the type D. The type H tends to be lower in the number, but it is not significant from other types (A, B, C, E and F). Anyway, large variation in the number of pinna pairs was observed in each allozyme type, although it is still possible that several different clones were contained in one allozyme type. The relationship between the varieties of C. fortunei and allozyme types is shown in Table 5.

Cytological analysis

The chromosome numbers of a few individuals of each of the five allozyme types from the Kobe population were counted (Fig. 7). All allozyme types showed 2n = ca. 123. Therefore, all the genetic types detected in the population were identified as triploid.

Flow cytometry showed that the triploid control had 1.5 times more DNA per nuclei than the diploid control. This diploid individual of C. fortunei originating from China had rbcL α. Thus, its close relationship to the Japanese triploid C. fortunei could be supposed. DNA content per nuclei in the 80 individuals from the Kobe population was identical to that
of the triploid control and 1.5 times that of the diploid control. Therefore, all 80 individuals were identified as triploid. We concluded that individuals of *C. fortunei* in the Kobe population are all apogamous triploids.
1.4. Discussion

In this study, two \textit{rbcL} types (\(\alpha\) and \(\beta\)) and eight allozyme types (A–H) were detected in \textit{Cyrtomium fortunei}. On the basis of morphology, four varieties were identified, however, a greater number of genetic clones were observed in \textit{C. fortunei}. In the Kobe and Moroyama populations, individuals of allozyme type H had \textit{rbcL} sequence \(\beta\), whereas those of all the other types had \textit{rbcL} sequence \(\alpha\). This shows that \textit{rbcL} \(\beta\) type was genetically different from \textit{rbcL} \(\alpha\) type even in nuclear genomes (allozyme loci). Furthermore, only individuals with \textit{rbcL} \(\beta\) and the H allozyme pattern had clearly bicolored indusia, which is one of the morphological characteristics peculiar to \textit{C. fortunei} var. \textit{intermedium}. Therefore, it was concluded that \textit{C. fortunei} var. \textit{intermedium} is genetically different in its nuclear or plastid genomes from other varieties of \textit{C. fortunei}. Seven base pairs were different between \textit{rbcL} \(\alpha\) and \(\beta\) and the differences were sufficiently large to be considered as those between independent species (Yatabe \textit{et al.} 2009). However, zymograms of the H type are similar to the other types (Fig. 3). In order to clarify evolutionary origin of var. \textit{intermedium}, careful comparison of the variety using several molecular markers with \textit{Cyrtomium macrophyllum} group with the \textit{rbcL} \(\beta\) is necessary.

As for the other allozyme types, the type B corresponded to \textit{C. fortunei} var. \textit{atropunctatum}, which is characterized also by having bicolored indusia, although the color of their central portion is not so dark as that of the allozyme type H or var, \textit{intermedium} (Fig. 5). For var. \textit{fortunei} and var. \textit{clivicola}, both having grayish white indusia, we could not separate the allozyme types A, C, D, E, F and G into two groups so as to correspond to these two varieties.

On the basis of our \textit{rbcL} and allozyme data, \textit{C. fortunei} var. \textit{fortunei} and var.
*clivicola* were found to be morphologically and genetically indistinguishable. Using genetic markers, I could determine that at least seven genetic clones were present in the apogamous fern species *C. fortunei* even after var. *intermedium*, which is genetically and morphologically distinct, was excluded from it. Thus, how did this apogamous species acquire such considerable genetic variation? Several hypotheses have been proposed to explain the increased genetic variation within an apogamous fern species: (1) hybridization with closely related sexual species (Suzuki and Iwatsuki 1992), (2) unequal meiosis (Lin et al. 1992), (3) recurring origin (Watano and Iwatsuki 1988), and (4) genetic segregation by homoeologous chromosome pairing (Klekowski 1973, Ishikawa et al. 2003a, b). Hypotheses (1) and (2) cannot explain the process of genetic variation observed in *C. fortunei* because only apogamous triploids have been recorded in *C. fortunei* in Japan. Even in the case of *C. fortunei*, a possibility of hypothesis (3), the recurring origin of triploid apogamous types from related sexual types in the past remains. However, it seems that genetic variation would not be maintained for long periods (thousands of generations) in local populations, usually consisting of at most several hundreds individuals. Natural selection and genetic drift inevitably operate, even in apogamous species or their populations. Moreover, gene flow among their populations does not seem high, as we saw that constitutions and frequencies of allozyme types were considerably different among the populations. As a result, several different clones do not seem to be maintained in apogamous species unless genetic variation is constantly generated. Therefore, I considered that novel genetic clones should be produced from an apogamous lineage of *C. fortunei*.

A series of band patterns were observed on zymograms obtained from the samples collected from the Kobe population that could have been easily generated through segregation during homologous chromosome pairing. For example, band patterns of the clone F can be
explained by segregation from those of clone E, especially at the 6pg-2 loci. The frequency of segregation by homologous paring in *C. fortunei* should be examined in future studies. For this purpose, we need to analyze and compare the genetic composition of offspring derived from an individual parent of the apogamous species.

If genetic segregation by homologous chromosome pairing occurs in an apogamous fern species, it is expected that homozygosity will increase and heterozygosity will disappear. However, heterozygous allozyme patterns were still observed in *C. fortunei*. I considered that deleterious genes were expressed in some of the segregated individuals and were removed from the populations.

Another interesting question related to the relatively high genetic variation in *C. fortunei* is how genetic diversity is maintained in many local populations of apogamous fern species. Value of Simpson’s diversity index (*D*) calculated for *C. fortunei* was high enough, even comparing with those of *Dryopteris nipponensis* and *D. pacifica* from which genetic segregation and unequal meiosis were reported, respectively (Table 6). This suggests that several different clones grow together in each local population of *C. fortunei*. Two hypotheses can explain this situation: (1) niche differentiation occurs in each clone, and thus, several clones can coexist for a long time in each local population, and (2) new clones are always generated by mutation and/or genetic recombination faster than they are removed from the populations by selection and/or random genetic drift. The first hypothesis predicts that each clone grows in different environments. In fact, niche differentiation, or at least diversity related to the amount of shade and soil moisture among allozyme types, was not observed in this study, although we located each genetic type in the three different local populations. The second hypothesis predicts that new clones with different genotypes will be generated at a relatively high rate. Of course, these two hypotheses are not mutually exclusive. However,
more detailed ecological comparison among the allozyme types in each population and
examination of genetic segregation frequency are necessary to understand the high genetic
variation observed in populations of the apogamous fern species *C. fortunei*. 
Chapter 2. Evidence of genetic segregation in the apogamous fern species

*Cyrotomium fortunei* (Dryopteridaceae)

2.1. Introduction

In the study of Chapter 1, the examined samples of *Cyrotomium fortunei* were all apogamous triploids, but still showed a considerable amount of genetic diversity in every local population. Therefore, I hypothesized that new clones are always generated by genetic recombination faster than they are removed from the populations by selection and/or random genetic drift. This hypothesis predicts that new clones will be generated at a relatively high rate.

Three hypotheses have been proposed to explain the increased genetic variation within apogamous fern species, as mentioned in General Introduction. Among them, (1) hybridization with closely related sexual species and (2) unequal meiosis, cannot explain constant production of genetic variation in *C. fortunei* because neither sexual reproduction nor intra-specific polyploidy has been recorded in this species in Japan. Thus, I considered that new clones should be generated by genetic segregation through homoeologous chromosome pairing.

Ishikawa *et al.* (2003a) first reported positive evidence supporting genetic segregation through homoeologous chromosome pairing in apogamous ferns using *Dryopteris nipponensis*. They compared genotypes of the parent sporophyte and its progeny using the enzyme electrophoretic method. Among 250 progeny gametophytes developed from spores of a parental sporophyte of *D. nipponensis* with the Pgi-2 genotype *abc*, three showed different genotypes from that of the parent (three *aac* and one *bbc* gametophytes). Similarly, among 34
sporophyte offspring of the parent sporophyte with \(abc\) genotype, one showed \(bcc\) genotype, whereas the other 33 showed \(abc\).

However, in the \textit{D. erythrosora} complex, including \textit{D. nipponensis}, closely related sexual and apogamous types exist and hybrids between different reproductive types have also been reported in the complex (Hirabayashi 1970). Therefore, the genetic segregation observed in apogamous \textit{D. nipponensis} might be due to recent hybridization with closely related sexual types. Moreover, the frequency of genetic segregation, especially observed in sporophyte offspring from an apogamous parent, might not be accurate because the number of examined sporophyte offspring is too low. Hence, Ishikawa \textit{et al.} (2003a) could not examine whether genetic segregation frequencies are different between gametophytic and sporophytic generations.

In the study of Chapter 2, I aimed to accurately estimate the frequency of genetic segregation in \textit{C. fortunei}. If genetic segregation by homoeologous chromosome pairing occurs, offspring have only a portion of the genetic information of their parent and their ploidy level is the same as their parent. In order to clarify the occurrence and frequency of genetic segregation, I performed both genetic and cytological comparison between a parent and its offspring.

In the genetic comparison, I used \textit{pgiC} region. The \textit{pgiC} is believed to be a single-copy gene in ferns (Ishikawa \textit{et al.} 2002). I determined nucleotide sequences of \textit{pgiC} and estimated genotype of each sample. For the cytological comparison, I used chromosome counting and flow cytometry.

In Chapter 2, I will specifically address the following questions: (1) Does genetic segregation occur in apogamous \textit{Cyrtomium fortunei}? If so, what is its frequency? (2) Is there a difference in the frequencies of genetic segregates between gametophytic and sporophytic
generations?
2.2. Materials and methods

Plant materials

Silica gel dried leaf sample for DNA extraction and spores of *Cyrtomium fortunei* were collected from “K220” sporophyte originally growing in the Kobe population (Kita-ku, Hyogo Pref. Japan), from which largest amount of intra-populational genetic variation was observed in my previous study (Ootsuki *et al.* 2011). In order to collect large quantities of fresh spores from the plant and also to avoid contamination of other naturally growing individuals of *C. fortunei*, the plant was transplanted to Suginami, Tokyo in 2007 and cultivated. Its spores were collected in June 2009 after its growing condition became stable. A part of a fertile leaf of the parental sporophyte was washed carefully with flowing water, then spores were collected by drying at room temperature in a clean envelope. Fragments of empty sporangia, scales of leaves and other unnecessary matters were removed on a powder paper. Finally obtained spores were dispensed to 1.5ml Eppendorf tube and stored at -20°C until use.

Cultivation of gametophytes and offspring sporophytes

Spores were sown on inorganic nutrient agar media in petri dishes of 60 mm in diameter, containing 1% agar with a mixture of EDTA (13.8 M EDTA 2Na, 7.7 M FeSO₄), Knop's solution (0.24 mM Ca (NO₃)₂, 0.14 mM KNO₃, 0.06 mM MgSO₄ and 0.1 mM KH₂PO₄) and Niche's solution (0.001 mM H₂SO₄, 2.2 mM MnSO₄, 0.1 M ZnSO₄, 3 mM H₃BO₃, 0.6 mM CuSO₄, 0.6 mM Na₂MoO₄, 3.3 mM CoCl₂, pH 5.6-5.8), and cultured in a growth chamber following the method of Masuyama (1996). Until germination, I incubated in
a condition of 25°C and 24-hr light. After germination, each small gametophyte was transplanted onto the same inorganic nutrient agar media in each hole of 96-well microtiter plates (no. 3861-096; Iwaki, Tokyo, Japan) and cultivated under the same condition for about 6 months until the gametophytes or sporophytes from them became 2-5 mm. One hundred sporophytes were transplanted from the plates to the sterile vermiculite in 60mm×60mm×100mm pots, then I gave water containing 0.1% Hyponex 7-4-4 (Hyponex Japan, Osaka, Japan) to them twice a week.

DNA extraction

Total DNA was extracted from plant samples of gametophytes or sporophytes using 2 × hexadecyltrimethylammonium bromide (CTAB) solution according to the method of Doyle and Doyle (1990) with some modifications. Each gametophyte or sporophyte sample was carefully picked up one by one using a needle under a stereomicroscope, and transferred to a 2 ml Eppendorf tube containing zirconia beads (5 mm in diameter). When gametophyte and sporophyte tissue was connected, they were carefully separated using a razor blade. The needle and the razor were washed with 100% ethanol and burned in each time of use. Then the 2 ml Eppendorf tube was freezed at -20°C and the plant samples were ground into fine powder using a TissueLyser (Retsch QIAGEN, Germany). Next, 500 µl of 2 × CTAB solution was added, mixed, and heated at 60°C for 1 day. After chloroform–isoamyl alcohol (24:1) extraction, isopropanol precipitation was performed. The DNA pellet obtained was washed with 70% ethanol, air-dried, and re-dissolved in 50 µl of TE buffer.

Total DNA was also extracted from about 4 mg of leaf tissue of the parental K220 plant for cloning using a DNeasy Plant Mini kit (Qiagen, Chatsworth, CA, USA).
Sequencing of \textit{pgiC} gene of the parental sporophyte K220

Commonly, the PCR profile consisted of an initial denaturation at 96°C for 10 min, followed by 45 cycles of denaturation at 96°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1.5 min, with a final extension at 72°C for 5 min on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). However, the PCR conditions and DNA polymerase were optimized in each experiment. Before sequencing, 3 µl 0.05 × ExoSAP-IT (USB, Ohio, USA) was added to the remaining 10 µl of PCR products that contained a positive insert, and the mixture was incubated at 37°C for 30 min to remove extraneous oligonucleotide and dNTPs, and then incubated at 80°C for 20 min to deactivate the enzymes contained in ExoSAP-IT (USB). Labeling reaction was performed using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) following the manufacturer’s protocols. Sequencing was performed on a 3100 Genetic Analyzer (Applied Biosystems). The resulting sequence files were analyzed using the phrap/phred/consed software package. (http://www.phrap.org)

In the preliminary amplification from the total DNA of K220 using Ampdirect Plus Kit (Shimazu, Kyoto, Japan), I could amplify \textit{pgiC} fragment using the combination of PCR primers, 14F (5’- GTGCTTCTGGGTCTTTTGAGTG -3’) (Ishikawa \textit{et al.} 2002) and 17R (5’-CGGGCTGTGGCTCTCAATAATTCC-3’) (Ishikawa \textit{et al.} unpublished data). Therefore, in the next step, the 14F-17R \textit{pgiC} fragments for cloning were amplified using PrimeSTAR Max DNA Polymerase (TaKaRa, Kyoto, Japan). The PCR products were purified following the manufacturer’s protocol for QIAquick Gel Extraction kit (Qiagen). Then, they were cloned using Qiagen PCR Cloningplus kit (Qiagen). Colonies were grown on LB medium (1.5% agar, 1% Bacto tryptone, 0.5% Bacto yeast extracts, 1% NaCl, pH 7.0) containing 0.01% ampicillin, 0.004% XGAL and 0.012% IPTG at 37°C overnight, and then
incubated at 4°C for at least 4 h. Positive (white) colonies were harvested and mixed with 50 µl sterilized MilliQ water and heated at 95°C for 5 min. To check for the presence of an insert, 0.5 µl of the colony suspension was utilized as a PCR template. Then, I performed colony PCR with sequencing primers M13-Forward-HT (5’-CGCCAGGGTTTTCCCAGTCACGAC-3’) and M13-Reverse-HT (5’-CGGATAACAATTTCACACAGGAAAC-3’). Using obtained partial sequences of pgic region, the specific PCR primers for Cyrtomium fortunei were designed using AmplifX 1.5.4 (http://ifrjr.nord.univ-mrs.fr/AmplifX).

Detection of genetic segregation

Offspring gametophytes and sporophytes with segregated genotypes were searched by PCR amplification and direct sequencing of pgic gene. In order to remove artifacts during PCR amplification, pgic fragments were amplified independently three times, each using different set of primers.

In the fist trial, nested PCR amplification was performed using 732 DNA samples extracted from offspring of the K220 parental plant as templates. In the PCR of the first trial, the 14F - 16R2 primer set and Ampdirect Plus kit (Shimazu) was used. The conditions for the PCR amplification were as follows: initial denaturation at 96°C for 10 min, 60 cycles at 94°C for 30 s, at 50°C for 30 s, and at 70°C for 60 s on a thermal cycler of GeneAmp PCR System 9700 (Applied Biosystems). In the case of a nested PCR reaction, 5 µl of the first PCR products diluted 1/50 - 1/1000, was served as the template for the second reaction. The 43F (5’-GGTCACCCAGCAGCGGTATGGAATATT-3’) – 459R (5’-TCAAGCCCCGACCCAAACCACGTGAATCTC-3’) primers and Ampdirect Plus kit (Shimazu) were used for the second PCR in the first trial. The conditions for nested PCR
amplifications were as follows: initial denaturation at 96°C for 10 min, 45 cycles at 94°C for 30 s, at 65°C for 30 s, and at 72°C for 60 s on a GeneAmp PCR System 9700 (Applied Biosystems). Sequencing was performed in the same way as for sequencing pgiC gene of the parental plant. Genotype of each sample was determined based on the wave signals in all base substitution sites.

The second and third PCR amplifications were performed only for the DNA samples, which showed segregated genotype. They were performed using 14F-244R (5’-ATGATATGGTGACGCTTTCCC-3’) and 269F (5’-CTTCGTTTTGACCGCTTTAGAATGC-3’)-432R (5’-ACGGAAACTTTTCACCTAGCCT-3’) primer sets, respectively, both using Ampdirect Plus kit (Shimazu Japan). The conditions for these PCR amplifications were as follows: initial denaturation at 96°C for 10 min, 60 cycles at 94°C for 30 s, at 50°C for 30 s, and at 70°C for 60 s on a GeneAmp PCR System 9700 and 2720 thermal cyclers (Applied Biosystems). In the second and third analyses, nested PCR was not done. Sequencing procedures for them were completely the same as those described above for the first analysis.

Cytological observation and ploidy analysis using flow cytometry

Cytological observation was performed for the K220 plant as well as for a few individuals of offspring gametophytes and sporophytes. For observation of the parental and offspring sporophytes, root tips were pretreated with 0.002 M 8-hydroxyquinoline for 6 h at approximately 17 - 20°C. After fixation in 70% ethanol and 45% acetic acid (3:1) for 15 – 30 min, these root tips were hydrolyzed in 1 N HCl at 60°C for 1 – 3 min and then squashed in 2% aceto-orcein solution. For the observation of gametophyte, pre-treatment using 8-hydroxyquinoline was not performed. The other observation procedures were the same as
those for the observation of the sporophytes. Then, chromosomes were observed under a microscope (Olympus model M or Nikon model S) and photographed with a Canon 20D or Nikon coolpix 4500 digital camera.

To examine the ploidy level of the offspring sporophytes from the K220 parent sporophytes more extensively, DNA content of each nuclei extracted from fresh pinna samples was measured by flow cytometry using a CyFlow Ploidy Analyzer (Partec, Munster, Germany) as well as a Cystain UV Precise P kit (Partec, Munster, Germany). After cultivation of the offspring sporophytes for one year or more, approximately 5 × 5 mm of a pinnae segment was chopped finely with a razor blade in 0.5 ml of Nuclei Extraction Buffer from the kit, then filtered through a 50 µm mesh and stained in 1.5 ml of Staining Buffer from the DNA kit containing 4’, 6’-diamidino-2-phenylindole hydrochloride. Fresh leaf tissues of a diploid *Cyrtomium fortunei* individual collected from China (2n = 82, Nakato *et al.* 1995) as well as those of the K220 parent, whose ploidy was determined by chromosome counting in this study, were used as controls. Initially, the amount of DNA per nuclei was measured for the diploid and triploid controls, and then 100 samples of the offspring sporophytes were analyzed under the same conditions.
2.3. Result

Nucleotide sequences of *pgiC* gene observed in the parent K220 sporophyte

I could detect two types of nucleotide sequence (867 bp) of the *pgiC* gene in the parent K220. These sequences correspond to the partial sequences in *pgiC* exon 14-17. The two obtained sequences have been deposited in the DNA Database of Japan (DDBJ) under accession numbers AB601622 and AB601623, respectively. Hereafter, they are referred as *pgiC* sequences “a” and “b” (Fig. 8). Six base substitutions were observed between the two sequences (Table 7); one substitution in the 14th intron and the remaining five in the 15th intron of *pgiC*. No base substitution was observed in any exons or the 16th intron.

Detection of genetic segregation in the offspring gametophytes and sporophytes

Among 250 individuals of the gametophytes developed from the spores of the K220 parent sporophyte with *pgiC* sequences “a” and “b”, 11 gametophytes showed only the sequence “a” (Fig. 9). Remaining 239 gametophytes showed combined wave patterns (double peaks) of the *pgiC* sequences “a” and “b”. It means that 4.4% of the offspring gametophytes had a segregated genotype (Fig. 10).

On the other hand, among 482 progeny sporophytes examined, 8 (1.7%) showed only the sequence “a”, or the segregated genotypes which were different from that of the parental sporophyte K220, while the other 476 showed the same genotype of the parent (Fig. 10).

Cytological observation and ploidy analysis

I could count 123 somatic chromosomes in the parent K220, as well as in one
offspring gametophytes and eight sporophytes (Fig.11). In addition, the flow cytometry analyses showed that the triploid control contained about 1.5 times more DNA per nuclei than the diploid control. This diploid sexual individual of *Cyrtomium fortunei* originated from China. The amount of DNA per nuclei in the 100 individuals of the offspring sporophytes was identical to that of the triploid control and 1.5 times that of the diploid control (Fig.12). Therefore, all the 100 offspring were identified as triploid. I concluded that individuals of the offspring sporophytes were all apogamous triploids.
**2.4. Discussion**

In this study, I first hypothesized that genetic segregation through homoeologous chromosome pairing has occurred in considerable frequencies in apogamous *Cyrtomium fortunei*. The hypothesis will be supported if both of the following evidences are obtained: (1) Progeny with segregated genotypes are detected, (2) Ploidy of all offspring is the same as that of the parent sporophyte. It means that unequal meiosis does not contribute to diversify the offspring genetically.

Such the evidence could be obtained by the genetic and cytological comparison between the parent and its offspring. If genetic segregation by homologous chromosome pairing occurs in the apogamous fern species, offspring that had only a portion of genetic information of their parent and their ploidy level is the same as their parent. According to the results of the genetic comparison, partial progeny (4.4% of the offspring gametophytes and 1.7% of the offspring sporophytes) showed only one peak of the *pgiC* sequence ("a"), whereas their parent had double peaks derived from the two sequences ("a" and "b"). Because offspring with only "b" sequence were not detected, the genotype of the parent K220, which was shown as a triploid by chromosome counting, might be *aab*.

The second evidence could be obtained by cytological analysis. The unequal meiosis observed by Lin *et al.* (1992) can also be a possible cause, which generated genetic variation in the progeny of the apogamous ferns. However, the cytological observation just indicated that offspring sporophytes and gametophytes are triploid of *2n = ca. 123* and *'n' = ca. 123*, respectively. In addition, amount of DNA per nuclei measured for the 100 offspring sporophytes was identical to that of the triploid control and approximately 1.5 times that of the diploid control. Therefore, all the examined offspring were identified as triploid.
Therefore, I concluded that genetic segregation not through unequal meiosis but maybe through homoeologous chromosome paring has occurred in *C. fortunei*. This is the second evidence after the first one by Ishikawa *et al.* (2003a), showing genetic segregation of apogamous ferns. However, Ishikawa *et al.* (2003a) did not examine ploidy of the offspring, but estimated their triploid genotypes based only on uneven allozyme patterns.

Another novelty of the present study is about more accurate estimation of genetic segregation frequency. Ishikawa *et al.* (2003a) reported that the frequency of homoeologous chromosome paring in *Dryopteris nipponensis* is 1.76%, based on their combined data on gametophytes and sporophytes. However, their data, especially the segregation frequency observed in offspring sporophytes was not so reliable because the number of the analyzed offspring sporophytes was too small. It was also impossible to compare the segregation frequencies between gametophyte and sporophyte generations. In this study, I made genetic and cytological analyses of the high enough number of both offspring gametophytes and sporophytes so as to estimate segregation frequency more accurately and also to be able to compare the frequencies between gametophytic and sporophytic generations.

As the result, I could show that the genetic segregation rates observed in apogamous *C. fortunei* is as high as 4.4% and 1.7% in offspring gametophytes and sporophytes, respectively. Moreover, I could first show the significant difference in the frequencies between gametophytic and sporophytic generations ($\chi^2=4.90$ $P=0.027$). It means that frequencies of offspring with segregated genotypes are higher in gametophytes than sporophytes.

These results suggested the possible existence of lethal or deleterious genes, which express during morphogenesis and growth of the offspring sporophytes. It is natural that there are many genes that express and work only in sporophytes or during its formation, but not
work in spores or gametophytes, because sporophytes of fern have much more complicated structure than gametophytes. If deleterious mutations occur in essential genes for developing sporophytes or their survivorship, I can see spores and gametophytes of the mutants, but cannot see their sporophytes. This is the first report that detected deleterious genes in apogamous ferns. If genetic segregation occurs in every generation at considerable frequencies in apogamous ferns, I can easily expect that they will quickly lose heterozygosity within individual and finally even in their populations. However, according to my former study (Ootsuki et al. 2011), all local populations of apogamous *C. fortunei* so far examined showed intra-populational genetic variability and contained individuals with highly heterozygous allozyme patterns. Deleterious genes in the apogamous fern might take important roles to prevent from losing heterozygosity.

In the present study, I did not investigate frequency of genetic segregation at spore stage. If I can elucidate the frequency in the future, it should be even higher than that in gametophytes because many genes that do not express in spores must be working in gametophytes, and the frequency is closer to the accurate frequency of genetic segregation during meiosis in apogamous *Cyrtomium fortunei*. Now, it is possible to examine genetic segregation frequency even at the spore stage.

In this study, I used the K220 plant of *C. fortunei*, which is triploid but has only two kinds of *pgiC* genes, as the parent sporophyte to collect spores. Actually, I could not find any matured sporophytes with three kinds of *pgiC* genes in Kobe population (unpublished data). Moreover, I used PCR and direct sequencing techniques to detect offspring with segregated genotype. Using these methods, I cannot distinguish offspring with *abb* genotype from that with *aab*. Therefore, I could not detect many offspring with segregated genotype in the present study. For estimating genetic segregation rate of apogamous ferns even more
accurately in future, I should try to find parent sporophyte with three kinds of nuclear gene
markers. Moreover, such the segregation rates might be different by genomic region. In the
next step, it is necessary to examine the frequency of genetic segregation by genome wide
mapping using many genetic markers that cover at least all chromosomes. Anyway, the results
of the present research give important suggestions to the mechanism for apogamous ferns
species to acquire and retain considerable intra-specific and intra-populational genetic
diversity.
General Discussion

In Chapter 1 of this thesis, I examined the genetic variation within the apogamous fern species *Cyrtomium fortunei* found in Japan. I identified two *rbcL* (α and β) and eight allozyme (A–H) types (Fig. 2 and Fig. 3). Because all allozyme types had *rbcL* α, except allozyme type H, which had *rbcL* β, I recognized only seven genetic types within *C. fortunei*. Furthermore, I demonstrated that only individuals with *rbcL* β and allozyme H pattern had clearly bicolored indusia, which is one of the unique morphological character in *C. fortunei*. Therefore, I concluded that *C. fortunei* var. *intermedium* is distinct from the other varieties of *C. fortunei*. Furthermore, significant genetic differentiation was not observed among the other three varieties, i.e., var. *fortunei*, var. *clivicola*, and var. *atropunctatum*.

In Chapter 1, I also carefully examined the genetic variation within the four distantly located populations, where several morphological varieties of *C. fortunei* grow together. In the three local populations (Moroyama, Kobe, and Fuchu), where more than 50 samples were collected and genetically analyzed, as many as 2–4 different allozyme types were recognized, even after removing the peculiar allozyme type H. I detected genetic variation in all of the populations examined until date, although only apogamous triploids were detected in the collected samples. Therefore, I hypothesized that novel genetic clones may constantly be produced because of genetic segregation through homoeologous chromosome paring from an apogamous lineage of *C. fortunei* in these populations.

In Chapter 2, in order to clarify the mechanisms producing genetic variation within the apogamous fern populations, I performed both genetic and cytological comparison between a parent and its offspring. I detected two types of *pgiC* (a and b) in the parent K220 sporophyte (Fig. 1). Then, I examined 732 progenies (250 gametophyte offspring and 482
sporophyte offspring) from the parental sporophyte expressing both \( pgIC_a \) and \( b \). Among them, 11 (4.4%) gametophyte offspring and 8 (1.7%) sporophyte offspring exhibited genotypes (with only \( pgIC_a \)) different from that of the parent sporophyte (Fig 2). Moreover, all the examined offspring were identified as triploid. Therefore, I obtained data indicating that genetic segregation occurs in the apogamous \( C. fortunei \). In addition, significant differences were observed in the frequencies of genetic segregation between gametophytic and sporophytic generations (\( \chi^2 = 4.90, P = 0.027 \)), indicating that frequencies of offspring with segregated genotypes are higher in gametophytes than in sporophytes. This strongly suggested that lethal or deleterious genes are expressed only in the sporophytic generation of the apogamous fern species. This is one of the most important findings of my thesis.

Finally, by combining data obtained in Chapters 1 and 2, I will discuss whether the obtained frequencies of genetic segregation 2 for the apogamous \( C. fortunei \) can explain the relatively high degree of genetic variation observed in each local population. In comparison to other angiosperms, ferns have a much higher number of chromosomes (mean haploid chromosome number is 57.05 in ferns and 15.99 in other angiosperms) (Klekowski and Baker 1966). Therefore, such a high number of chromosomes may contribute to the relatively high genetic variation observed in apogamous ferns. We can calculate the percentage of genetically different offspring produced by genetic segregation (\( y \)) as follows:

\[
y = 1 - (1 - r)^x \times 100%,
\]

where \( r \) is the genetic segregation rate in one chromosome and \( x \) is the base number of chromosomes. In \( C. fortunei \), the base number is 41. This formula suggests that genetic segregation rates are the same in any chromosome. Fig. 13 shows the graphs obtained using
this formula. For example, even if the genetic segregation rate is only 1%, this graph predicts that as much as one-third of the offspring should be genetically different from each other. Therefore, the obtained genetic segregation rates of 4.4% among gametophyte offspring and 1.7% among sporophyte offspring in Chapter 2 can easily explain the acquisition and retention of the considerable levels of intraspecific and intrapopulational genetic diversity observed in the local populations of *C. fortunei*.

In addition, the advantage of the high chromosome number in apogamous ferns regarding high genetic variation is shown in Fig 14. In this figure, I overlaid various curves obtained when the base number was 2, 10, 41, or 100. As the base number increased, the effect of genetic segregation also dramatically increased. Thus, a high chromosome number is advantageous for apogamous ferns to obtain and retain genetic diversity. However, most apogamous ferns are reported to be triploids, and higher polyploids such as tetraploids or pentaploids are rare (Takamiya 1996). At present, it is unclear why most apogamous ferns are triploids.
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Finally, I would like to thank all members of Makino Herbarium, Tokyo Metropolitan University for their encouragements.
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sequences provide evidence for the evolutionary lineages of leptosporangiate ferns.


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homoeologous chromosome pairing in the apogamous fern species \textit{Dryopteris nipponensis}

Ishikawa, H., M. Ito, Y. Watano & S. Kurita, 2003b. Extensive electrophoretic variation in the


Geobot. 54: 59-68.


Kodansha, Tokyo.


Table 1. Four varieties of *Cyrtomium fortunei sensu lato* distributed in Japan and their distinctive morphological characteristics (Iwatsuki 1992).

<table>
<thead>
<tr>
<th>Variety</th>
<th>Number of lateral pinna pairs</th>
<th>Color of central partion of indusium</th>
<th>Presence or absence of auricle at the anterior base of the pinna</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cyrtomium fortunei</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>var. fortunei</td>
<td>15–30</td>
<td>grayish white</td>
<td>present/absent</td>
</tr>
<tr>
<td>var. clivicola</td>
<td>5–20</td>
<td>grayish white</td>
<td>present/absent</td>
</tr>
<tr>
<td>var. intermedium</td>
<td>10–15</td>
<td>dark brown</td>
<td>present</td>
</tr>
<tr>
<td>var. atropunctatum</td>
<td>10–20</td>
<td>dark brown</td>
<td>present</td>
</tr>
</tbody>
</table>
Table 2. Distribution of the two $rbcL$ types ($\alpha$ and $\beta$) in the four local populations of *Cyrtomium fortunei sensu lato*.

<table>
<thead>
<tr>
<th>Population</th>
<th>$rbcL$-type</th>
<th>Total number of individuals examined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$</td>
<td>$\beta$</td>
</tr>
<tr>
<td>Moroyama</td>
<td>28</td>
<td>23</td>
</tr>
<tr>
<td>Kawazu</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Kobe</td>
<td>85</td>
<td>22</td>
</tr>
<tr>
<td>Fuchu</td>
<td>55</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 3. Distribution of eight allozyme types and the numbers of individuals in each allozyme type found in the four populations of *Cyrtomium fortunei sensu lato* and Simpson’s diversity index ($D$) calculated based on the allozyme variation in each population.

<table>
<thead>
<tr>
<th>Population</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>Simpson’s Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moroyama</td>
<td>1</td>
<td></td>
<td></td>
<td>27</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td>0.53</td>
</tr>
<tr>
<td>Kawazu</td>
<td>6</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.55</td>
</tr>
<tr>
<td>Kobe</td>
<td>37</td>
<td>26</td>
<td>11</td>
<td>11</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td>0.77</td>
</tr>
<tr>
<td>Fuchu</td>
<td>51</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.14</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>6</td>
<td>5</td>
<td>51</td>
<td>55</td>
<td>11</td>
<td>13</td>
<td>45</td>
<td>0.82</td>
</tr>
</tbody>
</table>
Table 4. Relationship between number of lateral pinna pairs and allozyme types.

<table>
<thead>
<tr>
<th>Number of lateral pinna pairs</th>
<th>Allozyme type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>1–5</td>
<td>0</td>
</tr>
<tr>
<td>6–10</td>
<td>2</td>
</tr>
<tr>
<td>11–15</td>
<td>7</td>
</tr>
<tr>
<td>16–20</td>
<td>0</td>
</tr>
<tr>
<td>20–25</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 5. Relationship between morphological varieties and allozyme types.

<table>
<thead>
<tr>
<th>Varieties</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>var. fortunei</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>var. clivicola</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>var. intermedium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>var. atropunctatum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 6. Comparison of Simpson’s diversity index ($D$) calculated based on the allozyme variation in six apogamous species *Cyrtomium fortunei*, *Dryopteris bissetiana*, *D. nipponensis*, *D. pacifica*, *D. sacrosancta* and *D. varia*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Simpson's Index $D$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. fortunei</em></td>
<td>0.82</td>
<td>This study</td>
</tr>
<tr>
<td><em>D. bissetiana</em></td>
<td>0.81</td>
<td>Lin <em>et al.</em> 1995</td>
</tr>
<tr>
<td><em>D. nipponensis</em></td>
<td>0.81</td>
<td>Ishikawa <em>et al.</em> 2003a</td>
</tr>
<tr>
<td><em>D. pacifica</em></td>
<td>0.91</td>
<td>Lin <em>et al.</em> 1995</td>
</tr>
<tr>
<td><em>D. sacrosancta</em></td>
<td>0.33</td>
<td>Lin <em>et al.</em> 1995</td>
</tr>
<tr>
<td><em>D. varia</em></td>
<td>0.90</td>
<td>Lin <em>et al.</em> 1995</td>
</tr>
</tbody>
</table>
Table 7. Variable sites in the nucleotide sequences of *pgiC* obtained from the parent K220 sporophyte of *Cyrtomium fortunei.*

<table>
<thead>
<tr>
<th><em>pgiC</em> sequences</th>
<th>Nucleotide position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>115</td>
</tr>
<tr>
<td>a</td>
<td>-G-</td>
</tr>
<tr>
<td>b</td>
<td>-A-</td>
</tr>
</tbody>
</table>
Fig. 1. Location of the four study sites and number of individuals examined in each local population.
Fig. 2. One of the most parsimonious trees based on rbcL sequence of Japanese *Cyrtomium fortunei* and Chinese species of the genus *Cyrtomium* reported by Lu *et al.* (2005) (length = 78 steps; consistency index = 0.72; retention index = 0.80). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) and number of base pair substitution are shown above and below each branch, respectively.
Fig. 3. Zymograms of the Japanese *Cyrtomium fortunei sensu lato*. Band patterns of four enzyme systems (PGM, HK, 6PG, and LAP) are shown for each of the eight allozyme types (A–H).
Fig. 4. Maps showing distribution of the eight allozyme types in the Moroyama (Saitama Pref.), Kobe (Hyogo Pref.) and Fuchu (Hiroshima Pref.) populations.
Fig. 5. Indusia of the four varieties of *Cyrtomium fortunei* 1, var. *fortunei*; 2, var. *clivicola*; 3, var. *atropunctatum*; 4, var. *intermedium* (scale bar = 1 cm).
Fig. 6. Variation in number of lateral pinna pairs in each allozyme type.

Horizontal lines indicate minimum, first quartile, median (thick line), third quartile and maximum, respectively from the bottom. The circles (○) indicate outlier points. Sample size is in parentheses. Different alphabetical letters (a, b, c and d) indicate significant difference at \( P < 0.05 \) by Steel-Dwass’s multiple range test.
Fig. 7. Somatic chromosomes in individuals with allozyyme types A, E, F, G, and H in the Kobe population (scale bar = 50 µm), and their explanatory diagrams (right). All the clones had 2n = ca. 123.
Fig. 8 Two *pgiC* sequences “a” and “b” observed in the parent K220 sporophyte of *Cyrtomium fortunei*. Variable nucleotide positions are highlighted with boxes. Positions of PCR primers used in the present study are indicated by arrows.
Fig. 9 Observed patterns of signal peaks in the output data from DNA automated sequencer. Upper is the pattern for the offspring with segregated genotype, which shows only single peaks(indicated by red arrows). Lower is that for the parent K220 sporophyte and offspring with original genotype, which shows double peaks.
Fig. 10. The number and frequency of offspring gametophytes and sporophytes with the segregated genotype (■) and unsegregated parental genotype (□).
Fig. 11 Somatic chromosomes: A, parent K220 sporophyte (2n = ca. 123); B, offspring gametophyte (‘n’ = 123); C, offspring sporophyte (2n = ca. 123).
Fig. 12 Distribution of rates from the flowcytometer for diploid (standard, from China) and triploid parent and/or offspring of *Cyrtomium fortunei*. The horizontal scale indicates the ratio of the triploid/diploid peak from flowcytometer. The black histograms indicate the ratio of the parent K220 or sporophytes of the next generation, which were cytologically observed as triploids.
Fig. 13. The relationship between the genetic segregation rate per chromosome per generation (x-axis) and the expected percentage of genetically different offspring produced from a parent sporophyte (y-axis) in apogamous ferns. This graph assumes that genetic segregation rates are the same in each of the chromosome and the chromosome base number is 41 as observed in the genus *Cyrtomium*.
Fig. 14. Effects of increasing the chromosome base number from 2 to 100. The graph structure is same as that shown in Fig 1.
要旨

シダ植物の無配生殖種の生活環においては、胞子体上に非減数の胞子が形成され、次世代の胞子体は、受精を経ずに配偶体の体細胞の一部から分化して形成される。このような無配生殖種では、遺伝的多様性を生み出す上で重要な、受精と正常な減数の2つの過程が抜け落ちていることから、子孫はすべて親個体と遺伝的に同一のクローンになるはずである。しかし、実際には無配生殖種内に形態的・遺伝的多様性がみられる例の方がむしろ一般的である。日本産ヤブソテツ（Cyromonium fortunei）とその変種群（以下、ヤブソテツ類とよぶ）は、国内ではこれまで3倍体で無配生殖をおこなうものしか知られていない（Takamiya 1996, 岩槻 1992）。しかし、著しい形態学的変異を示し、また、しばしば複数の変種が同じ地域集団内で共存することが知られていた。そこで本研究では、まずヤブソテツ類の各地域集団内、あるいは地域集団間で遺伝的多様性がどのように見られるかを明らかにすることを第一の研究目的とした。

ヤブソテツ類の地理的に離れた4つの地域集団（埼玉県毛呂山、静岡県河津、兵庫県神戸、広島県府中）から葉のサンプルを採取し、アロザイム酵素多型解析、rbcL遺伝子の塩基配列解析、生殖様式・倍数性の確認、形態観察等を行った。rbcL遺伝子の塩基配列解析の結果、7塩基の置換が見られる2種類の配列（αとβ）が見出された。4酵素種（PGM,HK,6PG,LAP）によるアロザイム酵素多型解析の結果、バンドパターンの違いから8種類（A～H）のアロザイムタイプが認識された。生殖様式の確認は、胞子囊中の胞子数の計数によって行った。A～Hいずれのアロザイムタイプも無配生殖型であることが確かめられた。そして、最も多くのアロザイムタイプが共存していた神戸集団において、染色体数の計数とフローサイトメータによる各個体の倍数性の推定を行った。その結果、観察したいずれの個体も3倍体であることが確かめられた。

以上のように、分子マーカーを用いたクローン識別の結果、従来は4変種と
見なされていたヤプソテツ類に8種類の異なる遺伝子型をもつ個体、すなわち少なくとも8種類のクローンが含まれていること、どの地域集団にも複数のクローンが共存し、それらの一部は集団を越えて共有されていること等が明らかになった。基本的に3倍体無配生殖型しか存在しないヤプソテツ類のどの地域集団にも少ながらぬ遺伝的多様性が見られたことは、興味深い事実である。


もし、無配生殖種において遺伝的分離が起これば、次世代の個体の中に、親の遺伝情報の一部しかもっていない子孫が存在しているはずである。本研究では遺伝的分離をした個体を効率よく検出するために、核にコードされ、シダ植物ではシングルコピーであるとされる pgIC 遺伝子を遺伝マーカーとして用いた。最初にヤプソテ
ツ類から PCR 増幅した pgIC 遺伝子をクローニングして, その塩基配列決定を行った。その結果, 親株として選定した K220 株から 6 塩基に置換が見られる a, b の 2 種類の配列が見出された。次に, この親株より胞子を採取して, その胞子に由来する配偶体を培養した。そして, その pgIC 遺伝子を解析した。調べた 250 個体の配偶体のうち, 11 個体 (4.4%) は a のみの pgIC 遺伝子しかもっていなかった。さらにこれらの配偶体上に生じた次世代胞子体を 482 個体解析したところ, a のみの pgIC 遺伝子しかもっていない次世代胞子体が 8 個体 (1.7%) 見つかった。配偶体世代と次世代胞子体世代との間で, 遺伝的分離を起こした結果, 親個体の一部の遺伝子しかもたないようになったと考えられる個体の頻度を比較したところ, 両者には有意差がみられた (χ² = 4.90, P = 0.027)。

一方, 親株である K220 株, およびそれに由来する子孫配偶体と次世代胞子体について倍数性の確認を行ったところ, 調べた限り全て 3 倍体であった。このことから, 無配生殖をおこなうヤブソテツ類において不等減数分裂ではなく, おそらく同祖性染色体の対合によると考えられる遺伝的分離によって新たな遺伝子型をもつクローニングが生じていると考えられる。また, 次世代の胞子体世代では配偶体世代よりも, そのような親個体の一部の遺伝子しかもたない子孫個体の頻度が有意に低かった。これは, 胞子が発芽でき, 配偶体は形成されるものの, 次世代の胞子体が形成できずに枯死する個体が少なくなく存在することを示していると考えられる。すなわち, 胞子体形成に関わる有害遺伝子が無配生殖をするヤブソテツ類には存在し, それが遺伝的分離の結果, 発現したことが示唆された。無配生殖種は通常, 遺伝的分離をしないので, 無配生殖種が有害遺伝子をもつことを示した研究はこれまでなかった。したがって, これは本研究における重要な研究成果である。