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Origin and Genetic Variation of Agamosporous Ferns[1)]

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Abstract Two agamosporous fern species, *Pteris cretica* and *Dryopteris yakusilvicola* as well as some sexual species related to them were cytologically and enzyme electrophoretically analysed in order to elucidate their genetic variation and its origin. *Pteris cretica* in Japan has been reported to contain diploid and triploid agamosporous races. Our allozyme data strongly suggested that the agamosporous triploid originated through recurrent hybridization between the diploid agamosporous race of *P. cretica* and the related sexual species, *P. kidoi*. Recurrent hybridization with related sexual species can be considered to contribute to increase the genetic variation of this agamosporous species. On the other hand, triploid agamosporous species, *D. yakusilvicola* was suggested to be of hybrid origin between diploid sexual *D. sabaei* and the tetraploid sexual race of *D. sparsa* based on our allozyme data. Moreover, 56 individuals of *D. yakusilvicola* from various localities which were electrophoretically analyzed showed the same banding pattern. This genotypic uniformity suggests that this species was recently derived through a single hybridization event.

Key words: agamospory, genetic variation, hybrid origin, *Pteris cretica, Dryopteris yakusilvicola*.

About 700 fern species are distributed in Japan. Mitui (1975) counted 17% of them as agamosporous or including agamosporous races. Thus, agamosporous taxa are not rare in pteridophytes, especially in Japan. Agamosporous reproduction is genetically identical with vegetative reproduction because all progenies are genetically identical with their mother. Therefore, agamosporous species can be expected to have less genetic variation than normally sexual species. But, some agamosporous fern taxa (*Cyrtomium, Dryopteris, Pteris*, etc.) show very complicated morphological variation and often cause taxonomic problems. Why do some agamosporous fern species show such complicated morphological variation? Several factors such as (1) recurrent origin from the sexual ancestor (Gastony and Gottlieb, 1985), (2) recurrent hybridization between agamosporous and sexual species (Walker, 1962), (3) segregation by occasional homoeologous pairing (Klekowski, 1973) or uneven chromosome allocation in meiosis (Gustafsson, 1947), (4) somatic mutation (Stebbins, 1971), have been considered to contribute to their variation. Among these factors, we speculated (2) is especially important. Walker (1962) demonstrated that agamosporous triploids of *Pteris* species can be experimentally induced by hybridization between sexual diploids and agamosporous diploids, and that agamosporous reproduction is inherited with dominance. He showed that agamosporous gametophytes can produce active spermatozoids from their antheridia and act as male parents. If an agamosporous race hybridizes with the progenitor sexual race and/or other related sexual species, divergence within the agamosporous race increases enormously. We can easily expect such agamosporous race to show more morphological variation than normal sexual species.

When a diploid agamosporous race hybridizes with a diploid sexual race, the derived hybrid is triploid since the spermatozoid of an agamosporously reproducing gametophyte is diploid and the egg of the sexual gametophyte is haploid. It is

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worth noting that when such hybridization takes place, intraspecific polyploidy in the agamosporous race should be observed. Gastony and Gottlieb (1985) investigated *Pellaea andromedifolia* which contains sexual diploid, agamosporous triploid, and agamosporous tetraploid races using enzyme electrophoresis. They discussed the likelihood that the agamosporous tetraploid may be of hybrid origin between the sexual diploid and the agamosporous triploid of the species, but their allozyme data did not support this hypothesis. Watano and Iwatsuki (1988) analyzed the *Asplenium unilaterale* complex containing triploid agamosporous species *Asplenium hondoense* and diploid sexual species *A. cataractarum*, using allozymic comparison. In the course of their study, they found the agamosporous tetraploid hybrid between *A. hondoense* and *A. cataractarum* which additively combines their banding patterns. They could show the existence of a natural hybrid between agamosporous and sexual species with reliable electrophoretic evidence but they could not demonstrate recurrent hybridization that can increase the intraspecific variation in an agamosporous species because they found only three hybrid individuals of an identical genotype.

Recurrent hybridization between agamosporous and sexual races may contribute to complicated variation of some agamosporous taxa, but these processes have not yet been well demonstrated in wild population of ferns. Therefore, we tried to get allozymic evidence of such hybridization processes. In this study, two fern groups, the *Pteris cretica* complex and the *Dryopteris sparsa* complex, including agamosporous species *D. yakusilvicola*, are selected for materials because they have both sexual and agamosporous species and because intraspecific polyploidy within agamosporous species has also been reported in both groups by previous workers and our own cytological research.

The variation within an agamosporous taxon can be understood as a mixture of clones with different genotypes. Thus, in this study we first attempted to recognize such clones by means of cytological observations and enzyme electrophoretic analyses. Then the genotypes of the recognized clones were compared with each other and with those of the related sexual species in order to consider their origins.

Materials and Methods

Plant materials for cytological and electrophoretic analyses were collected from various wild populations of the *Pteris cretica* complex and the *Dryopteris sparsa* complex in Japan. For the *D. sparsa* complex, plants were collected mainly on Yakushima Island.

For cytological examinations of meiotic chromosomes, small portions of leaves with young sporangia were fixed in acetic acid-ethanol (1 : 3) solution, and chromosome counting was made on spore mother cells at meiosis with the ordinary aceto-carmine squash method (Manton, 1950). For observations of mitotic chromosomes, active root tips were pretreated with 0.02M 8-hydroxyquinoline solution at 20°C for 3 hours and fixed in 45% acetic acid for 10 minutes. They were then macerated in 1M hydrochloric acid at 60°C for a few minutes and squashed in 2% aceto-orcein solution.

Thin-layer horizontal polyacrylamide gel (4.75% acrylamide, 0.25% Bis, $0.1 \times 8.7 \times 17$ cm) was used for electrophoresis. Both gel and ellectrode buffer systems are modified #7 and #8 of Soltis et al. (1983). Staining of enzymes was conducted also following the methods of Soltis et al. (1983). Analyzed enzymes were aspartate aminotransferase (AAT), hexokinase (HK), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), shikimate dehydrogenase (SKDH), and triosephosphate isomerase (TPI). When more than one isozyme was present for an enzyme, the most anodal isozyme was designated as 1 and the next most anodal 2. Allozymes at individual loci were given letters, with the fastest migrating allozyme designated as a, the second fastest as b. For details, see Suzuki and Iwatsuki (1990) for the investigations of *P. cretica* and Darnaedi, Kato and Iwatsuki (1990) for those of *D. yakusilvicola*.

Recurrent Hybrid Origin of Agamosporous Triploid of *Pteris cretica*

Pteris cretica is widely distributed in subtropical and temperate regions of the world and intraspecific polyploidy has been reported from this agamosporous species. Manton (1950) found three agamosporous cytotypes in this species: diploid ($2n=58$), triploid ($2n=87$), and tetraploid ($2n=116$). Agamosporous diploid and triploid cytotypes have been reported also from Japan by Walker (1962), Mitui (1965) and Nakato (1975). Momose (1967) observed antheridia on the gametophyte of this species and spermatozoids from it. Therefore, this species can be expected to have the potential to hybridize

with sexual taxa.

We collected 44 living stocks of *P. cretica* from 23 localities in Japan and mitotic observations were made using their root tips. Both diploid (2n=58) and triploid (2n=87) sporophytes were detected by our cytological observation. We counted the spore number per sporangium in order to determine their reproductive mode. The sporangia of normally sexual ferns usually have 64 spores and agamosporous sporangia usually have 32 spores. Both meiosis and mitosis of *P. cretica* in Japan were observed by Nakato (1975) and this agamosporous species was confirmed to contain 32 spores per sporangium. All 44 individuals of which we observed mitosis had 32 spores per sporangium and they were supported to have an agamosporous reproductive mode. Of the 44 individuals cytologically examined, 26 individuals were determined to be agamosporous diploids, and the other 18 individuals were agamosporous triploid.

These 44 individuals were examined also by enzyme electrophoresis. The following 5 enzymes, AAT, HK, PGI, PGM and SKDH were analyzed and polymorphisms were detected. The obtained zymograms are shown in Fig. 1. Individuals showing the same band pattern for all five enzymes were considered to belong to the same clone. Among 44 individuals, totally 11 clones were recognized. Five clones were detected in the diploid and the other six were in the triploid. Additionally, 291 other individuals from 51 localities were analyzed only by enzyme electrophoresis, but each of them showed the same phenotype as one of these eleven clones. Of the six triploid clones, four clones had unique alleles, Hk^d and $Pgi\text{-}2^a$, which were not found in the 2x clones. Based on these data, triploid clones can be divided into two groups: group 3xI which lacks alleles Hk^d and $Pgi\text{-}2^a$, and group 3xII with Hk^d and $Pgi\text{-}2^a$.

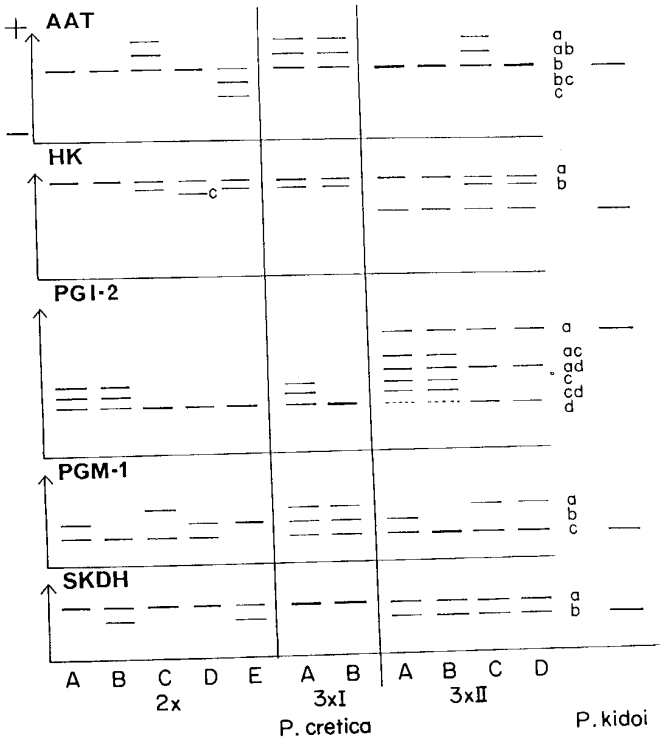

Fig. 1. Schematic zymograms of *Pteris kidoi* and eleven clones of *P. cretica*. The upper side of the figure is more anodal. Each band is given letters as described in the materials and methods. In dimeric enzymes (AAT and PGI-2), the homodimeric band is symbolized by single letter and the heterodimeric one by two letters.

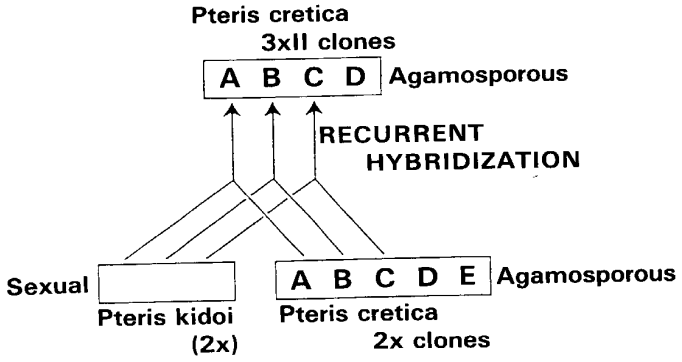

Fig. 2. Recurrent hybridization of agamosporous species *Pteris cretica* with the related diploid sexual species, *P. kidoi*.

Cytological observation supports this grouping. The 3xII clones had more numerous short chromosomes (ca. 2 micron) than 3xI clones.

The absence of alleles Hk^d and $Pgi\text{-}2^a$ of the 3xII clones from the diploid clones suggests that the 3xII clones include genomes different from those of the 2x clones. *Pteris kidoi* Kurata, known only as a diploid sexual species, is related to *P. cretica* and is a potential donor to the 3xII genomes. Nine individuals of *P. kidoi* collected from three different localities were analyzed by enzyme electrophoresis. Two unique alleles, Hk^d and $Pgi\text{-}2^a$, were detected in this diploid sexual species. If 3xII clones are of hybrid origin between the 2x clones and *P. kidoi*, three of the four genotypes of 3xII clones can be explained without assuming any mutations or the presence of other, as yet undetected, 2x clone. Thus, if 2xA, B, and C clones of *P. cretica* hybridize with *P. kidoi*, the expected genotype is identical with that of 3xIIA, B, and C clone of *P. cretica*, respectively (see Fig. 1). These results also mean that hybridization between the 2x clone of the agamosporous species *P. cretica* and diploid sexual species *P. kidoi* took place

at least three times to produce the agamosporous triploid 3xII clones (Fig. 2). In this work, recurrent hybrid origin of triploid agamosporous ferns are first demonstrated in natural populations of pteridophytes, and this process can be considered to contribute at least in part to the genetic variation of agamosporous *P. cretica*.

Hybrid Origin of Triploid Agamosporous Species *Dryopteris yakusilvicola* between Diploid Sexual Species *D. sabaei*, and Sexual Tetraploid Race of *D. sparsa*

Dryopteris yakusilvicola was first described from the montane forests of Yakushima Island by Kurata in 1967 and is now considered endemic to this island. Kurata noted that in many features *D. yakusilvicola* is intermediate between *D. sparsa* and *D. sabaei*. We made more detailed morphological comparisons among these three species of the *D. sparsa* complex (Darnaedi and Iwatsuki, 1987). The results obtained are shown in Table 1. Thus, the morphological data suggest that *D. yakusilvicola* is of hybrid origin between *D. sparsa* and *D. sabaei*.

The *D. sparsa* complex has been cytologically examined by Kurita (1966) and Hirabayashi (1974). A sexual diploid was reported from *D. sabaei*, an agamosporous triploid from *D. yakusilvicola*, and both an agamosporous triploid and a sexual tetraploid were reported from *D. sparsa*, but only a few individuals were examined for each species of *D. sparsa* complex.

We made an intensive cytological study of this complex on Yakushima Island, the unique place where all three species are distributed. We collected 18 plants of *D. sabaei*, 29 plants of *D. sparsa* and 20 plants of *D. yakusilvicola*, and both mitosis and meiosis were observed. The results of our cytological observation for 18 plants of *D. sabaei* and 20 plants of *D. yakusilvicola* confirm that the former is a diploid sexual and the latter is a triploid agamosporous species. *Dryopteris sparsa* on Yakushima Island has three cytotypes, an agamosporous diploid, an agamosporous triploid and a sexual tetraploid. The agamosporous diploid of *D. sparsa* was first found by our observation.

A total of 260 plants from 14 population sites of the *D. sparsa* complex (83 of *D. sabaei*, 56 of *D. yakusilvicola* and 116 plants of *D. sparsa*) were electrophoretically analyzed. To elucidate their ploidy level, 67 of the 260 plants were also cytologically examined. The zymograms obtained are shown in Fig. 3. Six enzymes coded by eight interpretable gene loci (*Pgm-1, Pgm-2, Lap, Hk, Aat, Idh, Tpi-1, Tpi-2*) were electrophoretically resolved. Polymorphic banding patterns were observed for all these enzymes. All the alleles found in the diploid and triploid agamosporous races of *D. sparsa*, were observed in the sexual tetraploid race.

For *D. sabaei* and *D. sparsa*, species specific alleles were found at three loci, *Pgm-1, Aat* and *Idh*. At *Pgm-1*, allele *a* is fixed in *D. sabaei* and allele *b* is in *D. sparsa*. *Dryopteris yakusilvicola* is fixed heterozygous for alleles *a* and *b* at *Pgm-1*. The situation is the same at *Aat* and *Idh*. At *Idh*, allele *a* is fixed in *D. sabaei*, *b* in *D. sparsa* and heterozygous *a* and *b* in *D. yakusilvicola*. At *Aat*, allele *c* has been found only in *D. sabaei*, and allele *e* is only in *D. sparsa*. *Dryopteris yakusilvicola* has both *c* and *e*. Also for the other loci, *D. yakusilvicola* shares

Table 1. Comparison of diagnostic morphological characters among *Dryopteris sparsa, D. yakusilvicola* and *D. sabaei*.

Character	*D. sparsa*	*D. yakusilvicola*	*D. sabaei*
scale shape	lanceolate	ovate-lanceolate	ovate
scale texture	dull	more or less shiny	shiny
scale persistency	deciduous	persistent	persistent
scale distribution	lower half of stipe	lower half to all part of stipe	all part of stipe
sorus distribution	all pinnae soriferous	all pinnae soriferous or basal pinnae sterile	basal pinnae sterile
glandular hairs	dense	rather dense	sparse to absent
secondary spore ornamentation	reticulate	spiny-reticulate	spiny

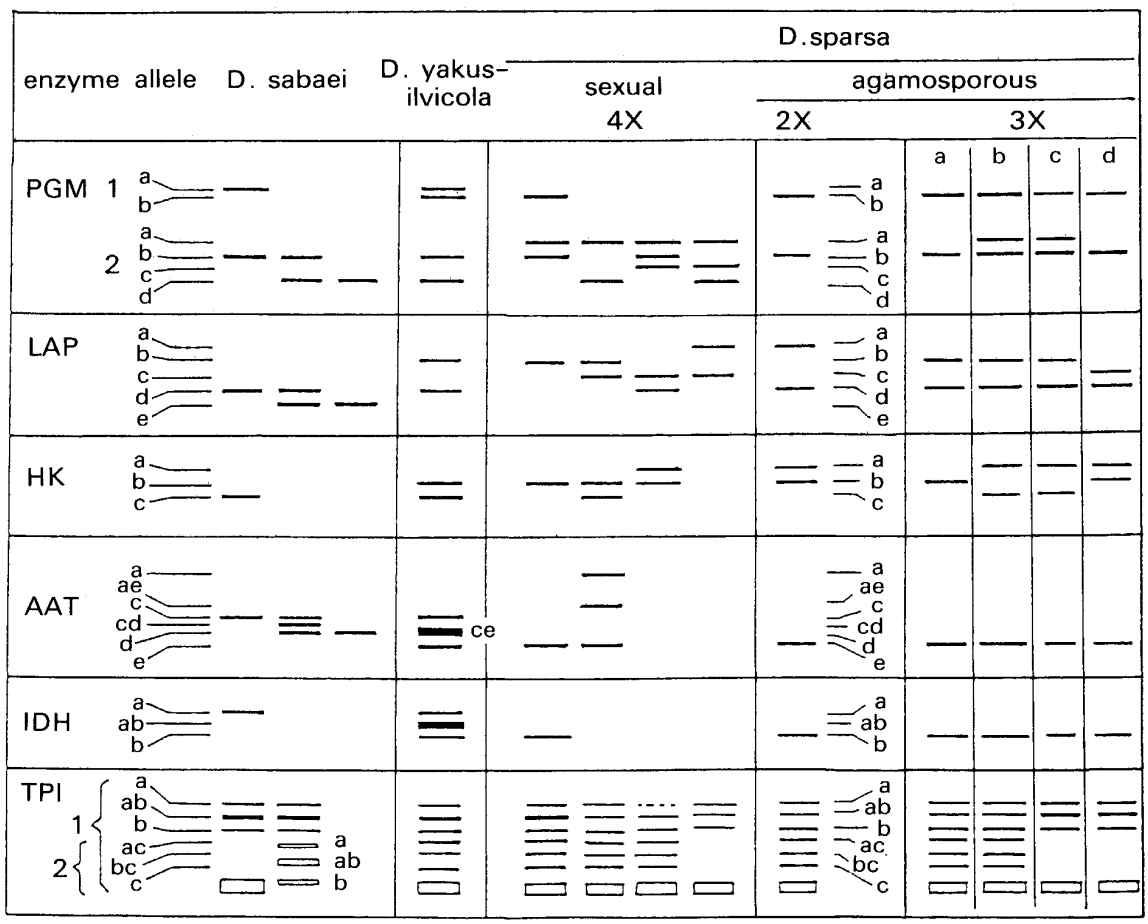

Fig. 3. Schematic zymograms of *Dryopteris sabaei, D. yakusilvicola* and *D. sparsa*. Designation of each band is the same as in Fig. 1. For sexual races, observed band patterns are shown at each locus.

alleles with both *D. sabaei* and *D. sparsa*. Therefore, the hybrid origin of *D. yakusilvicola* between *D. sabaei* and *D. sparsa* is strongly supported by the enzyme electrophoretic data.

Which cytotype of *Dryopteris sparsa* is the parent of *D. yakusilvicola*? In light of the cytological variation of *D. sparsa*, two hypotheses on the origin of *D. yakusilvicola* are possible. (1) *Dryopteris yakusilvicola* might have originated through hybridization between sexual diploid *D. sabaei* and agamosporous diploid *D. sparsa*. (2) *Dryopteris yakusilvicola* might have originated through hybridization between sexual diploid *D. sabaei* and sexual tetraploid *D. sparsa* and acquired the agamosporous reproductive mode afterward.

Among the alleles involving in these hypotheses *Lap-a* and *Hk-a* of agamosporous diploid *D. sparsa* were not found in *D. yakusilvicola*. Thus it is very unlikely that agamosporous diploid *D. sparsa* was involved in the origin of *D. yakusilvicola*. On the other hand, the genotype of *D. yakusilvicola* is easily explained if it is assumed to be of hybrid origin between *D. sabaei* and sexual tetraploid *D. sparsa*. Therefore, hypothesis (2) is more plausible at this moment.

All 56 plants of *D. yakusilvicola* from various places on Yakushima Island exhibit the same banding pattern, with no detectable genetic variation for the six enzymes examined. Its genetic uniformly contrasts with the genetic variable of its putative parental species and suggests that *D. yakusilvicola* was derived through a single hybridization event between *D. sparsa* and *D. sabaei*. It also indicates that neither somatic mutation nor recombination for the loci examined have taken place since the origin of *D. yakusilvicola* and suggests that *D. yakusilvicola* is a neo-endemic of Yakushima Island. This assumption is supported by the facts that the distribution of *D. yakusilvicola* is confined to Yakushima Island and that the parental species have a much wider distribution but coexist with *D. yakusilvicola* on Yakushima Island.

Concluding Remarks

Walker demonstrated that new agamosporous races can be experimentally induced by hybridization betweeen sexual species and agamosporous species of lower ploidy. In this work, wild agamosporous triploids of *P. cretica* are suggested to originate through recurrent hybridization between agamosporous diploid *P. cretica* and diploid sexual *P. kidoi*. Thus, the morphological variation of agamosporous *P. cretica* may be explained at least in part by its multiple hybrid origins.

The present study also demonstrates another type of speciation in whcih agamosporous *D. yakusilvicola* has probably been derived from the two different sexual species, *D. sabaei* and *D. sparsa*. It indicates that *D. yakusilvicola* has newly acquired the agamosporous reproductive mode in the course of its speciation. Moreover, genetic uniformity of *D. yakusilvicola* suggests that this agamosporous species has been derived through a single event of hybridization, although *Dryopteris yakusilvicola* is morphologically variable. This result indicates that morphological variability of agamosporous species need not correlate with detectable electrophoretic variation. Therefore we should analyze other morphologically variable agamosporous species such as *Dryopteris erythrosora, D. varia* and *Pteris fauriei* genetically using allozymic comparison.

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