DECAPLOID GAMETOPHYTE FORMATION FROM SPORES OF A PENTAPLOID CYSTOPTERIS FRAGILIS (CYSTOPTERIDACEAE) COLLECTED IN MONGOLIAN ALTAI

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ABSTRACT
Germinating spores were obtained from a pentaploid Cystopteris fragilis collected in Mongolian Altai. DNA content of nuclei in gametophytes showed for the first time that gametophytes that developed from spores of the pentaploid sporophyte had a larger number of genomes than those of the mother sporophyte, and some appeared to be decaploid. Fertile decaploid (10n) spores may be produced in the pentaploid C. fragilis and derived from monad spores observed in sporogenesis. Antheridia were produced on the 10n gametophytes, although antherozooids with swimming ability were not observed. Archegonia were not observed. Apogamous sporophytes with 10n genome content were induced from decaploid gametophytes. The monad spores produced may contribute to the formation of higher polyploid sporophyte series in ferns.

INTRODUCTION
Cystopteris fragilis (L.) Bernh. collected in Mongolian Altai showed three cytotypes, tetraploid, pentaploid and hexaploid, and although numerous univalent and bivalent chromosomes were observed in the spore mother cells of the pentaploid C. fragilis (2n = 5x = ca. 210), some germinating spores were obtained from the sporophyte (Kawakami et al., 2010). In the present paper, therefore, we aimed to investigate the genome contents of germinating spores produced and also to determine how these viable spores are produced in the pentaploid sporophyte. From the results of DNA contents of nuclei in gametophytes raised from spores of the pentaploid sporophyte, it was shown for the first time that the gametophytes produced had not the same but a larger number of genomes than those of the mother sporophyte, and surprisingly, some gametophytes had decaploid
(10n) genome contents. The results might indicate that 10n spores are produced in the sporophyte and that they derive from monad spores observed in sporogenesis. In the present study, not only decaploid spore formation but also other viable spore formation processes are discussed. Furthermore, since polyploidy is one of the outstanding features in ferns (Manton, 1950; Lovis, 1977; Walker, 1979; Takamiya, 1996; Kato, 1997), we also investigated the gametophytes cultivated on agar to determine whether or not these spores could contribute to the formation of higher polyploid sporophyte series in C. fragilis.

MATERIALS AND METHODS

Cystopteris fragilis was collected in Mongolia, west of Hovd Province, Erdeneburen sum, N 48° 38’69”, E 091° 07’74”, alt. 2450 m (Kawakami et al., 2010), and then cultivated in Japan. Spores collected were used for axenic culture. Gametophytes were cultivated on 1/4 strength of Murashige and Skoog (1962) (MS) medium supplemented with 0.75 % sucrose and 0.7 % agar. For the apogamous sporophyte formation, gametophytes were transplanted on 1/4 strength of MS medium supplemented with 3 %

Figure 1. Sporogenesis in pentaploid Cystopteris fragilis, A: sporangia on the underside of the frond; B: meiotic chromosome separation; C: two nuclei formed by chromosome separation; D: dyad spores produced in a sporangium; E: young spores produced in a sporangium, arrow indicates a monad spore; F: four spores in a tetrad; G: sterile spores produced in one sporangium; H: a large round spore with spiked ornamentation produced in a sporangium; I: spores collected from the pentaploid sporophyte, arrow indicates a fertile spore. Scale bars: A: 1 mm; B, C: 10 μm; D, E: 50 μm; F: 25 μm; G, H, I: 100 μm.
sucrose, 0.1 % casamino acid and 0.8 % agar. Cultures were maintained at 25 ℃ and illuminated by two fluorescent lamps (NEC FL 15BR) to keep 800 lux at the surface. Meiotic chromosomes were observed by fixing sporangia with 3:1 ethanol-acetic acid for 30 min at 5 ℃ and squashing them in 2 % aceto-orcein solution. The DNA contents of nuclei in fronds were estimated by flow cytometry using a Partec Ploidy Analyzer PA (Partec, Münster, Germany) (Kawakami et al., 2003).

RESULTS
The pentaploid Cystopteris fragilis produced sporangia on the underside of the frond (Figure 1A). In spore mother cells, numerous univalent and bivalent chromosomes were observed at meiotic metaphaseI. By meiotic chromosome separation (Figure 1B) two nuclei were formed (Figure 1C), and dyad spores were observed frequently in sporangia (Figure 1D). Some of these consisted of both large and small spores. Monad spores (Figure 1E) and spores in tetrads (Figure 1F) were more rarely observed. Mostly, young spores produced in sporangia did not mature and the sporangia did not develop (Figure 1G). In a few expanded sporangia, a few large spores with spiked ornamentation were observed (Figure 1H). Although spores obtained were mostly abortive, some were able to germinate (Figure 1I).

DNA contents of nuclei in 13 gametophytes derived from spores of the pentaploid sporophyte were investigated. Their genome contents were greater than those of the donor pentaploid sporophyte (Figure 2A). Two gametophytes had approximately $8n$ genome content (Figure 2B), another two had approximately $9n$ and nine gametophytes had approximately $10n$ genomes (Figure 2C).

Eight out of nine $10n$ gametophytes produced antheridia (Figure 3A), however, swimming antherozooids were not observed. Archegonia were not observed on any gametophytes during three years of culture. Apogamous sporophytes were induced from two $10n$ gametophytes after one year of culture (Figure 3B). They grew to approximately 10 mm in height but then died.

DISCUSSION
Manton (1950) made a cytological study of the pteridophyta, and following her, similar investigations on pteridophyta were carried out by many researchers (e.g. Wagner, 1954; Lovis, 1964; Sleep, 1966, 2014*; Reichstein, 1981; Pinter, 1995; Ekrt & Koutecky, 2016). In these studies, various suggestions have been proposed about differentiation and development in ferns through observations of meiosis in hybrid species.

Generally speaking, meiosis of sporophytes with many univalent chromosomes is irregular and spores produced are mostly abortive. If fertile spores could be obtained from those sporophytes, one might consider whether they are reduced spores with aneuploid chromosome numbers, as reported in triploid Osmunda regalis L. (Manton, 1950) or unreduced spores with the same chromosome number as the mother plant, produced by the pathway of Döpp-Manton or Braithwaite (Manton, 1950; Braithwaite, 1964; Walker, 1979; Kato, 1997; Kawakami et al., 2003). In the present study, however, the genome contents of gametophytes developed from spores of the pentaploid C. fragilis were greater than those of the donor pentaploid sporophyte and some gametophytes had

* Sleep examined Polystichum and proposed her ideas on the development of this genus. Her studies were not limited to Polystichum but also extended to other ferns. Because of her early death, this study (Sleep, 2014) was published posthumously.
10n contents that double the genome contents of the donor sporophyte. Since gametophytes develop directly from spores, their genome content must be considered to be the same as that of the spore from which an individual is derived. The results, therefore, suggest that spores with a higher ploidy level than the mother sporophyte are, surprisingly, produced in the pentaploid *C. fragilis*. The formation of gametophytes with genomes doubled that of the donor sporophyte is reported here for the first time.

From the observation of meiosis, it might be considered that spores with 10n genome

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**Figure 2.** Relative DNA content of gametophytes derived from spores of the pentaploid *Cystopteris fragilis*, A: distribution of relative DNA content of 13 gametophytes; B: sample of gametophytes with approximately 8n genome contents; C: Sample of gametophytes with approximately 10n genome contents.
contents are from monad spores produced in sporangia. They might be produced without meiotic cell division from the spore mother cells with doubled genome contents. When monad spores are produced, restitution nuclei might occur; and if some chromosomes are lost when they occur, monad spores with genomes smaller than $10n$, for example, $9n$ or $8n$, might be produced. However, in the formation of spores with $9n$ or $8n$ genome contents, especially in the case of $8n$ spore formation, another pathway might be considered. Since in sporogenesis dyad spores consisting of large and small spores were observed in the present study, the larger spore with $8n$ might be fertile, though the smaller spore with $2n$ might be sterile. Although dyad spores are well known to be produced in many ferns, they are unreduced spores with the same genome contents as the donor sporophyte (Walker, 1979; Kato, 1997; Kawakami et al., 2003). Why fertile $5n$ spores were not obtained in this study is unknown. Further studies of fertile spores may be required to understand sporogenesis of the pentaploid sporophyte. The present study revealed the monad spore formation in the pentaploid C. fragilis.

It is well known that polyploidy is one of the outstanding cytological features in ferns (Manton, 1950; Wagner, 1954; Lovis, 1968; Walker, 1979; Kato, 1997). The highest polyploid fern in Japan is $10x$ (Nakato, 1987; Takamiya, 1996) and the highest polyploid in the world is reported to be $16x$ (Walker, 1979). The evolutionary process of how polyploid ferns such as $10x$ or $16x$ are produced has not been investigated in detail. Since unreduced spores are well known to play a very important role for polyploid formation (Gastony, 1986; Kato, 1997), the pathway of the decaploid sporophyte ($10x$) formation could well be that they are produced by fertilization of pentaploid gametes produced from pentaploid gametophytes derived from unreduced spores produced in pentaploid plants. The present study, however, may well indicate another method of decaploid sporophyte formation: the pentaploid sporophytes produce decaploid spores and from decaploid gametophytes developed from spores, decaploid sporophytes could be induced apogamously. Furthermore, if antherozoids with an ability of fertilization could be produced from the decaploid gametophytes, though these were not observed in the present study, it might be possible to produce plants with higher polyploid levels than decaploid, such as $12x$ plants, by fertilization between decaploid male gametes and female diploid gametes derived from tetraploid sporophytes. Whether the fertile spores produced in pentaploid sporophytes can play a role in the formation of higher polyploid

Figure 3. An antheridium with antherozooids (A) and apogamous sporophytes (B) produced on gametophytes with approximately $10n$ genome content in Cystopteris fragilis. Scale bars: A: 25 μm; B: 1 mm.
C. fragilis in nature or not is quite intriguing. If decaploid C. fragilis were discovered in Mongolian Altai, it might have been produced from decaploid gametophytes apogamously. To understand the evolution of polyploidy in these ferns, further studies on C. fragilis in Mongolian Altai may be necessary.

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REFERENCES