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# New Cytotaxonomic Records on Threatened Fern Species in Japan

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**Abstract** Mitotic chromosome numbers of 21 fern species included in the national red list of Japan were counted, and their reproductive modes were estimated by observing the number of spores per sporangium using the living collection in Tsukuba Botanical Garden. Chromosome numbers were recorded for the first time for eight species (*Ptisana boninensis*, *Pteris kawabatae*, *Asplenium formosae*, *Athyrium palustre*, *A. spinescens*, *Arachniodes cavaleriei*, *Ctenitis microlepigera* and *Dryopteris lunanensis*).

**Key words:** *Angiopteris*, *Arachniodes*, *Arthropteris*, *Asplenium*, *Athyrium*, *Blechnum*, chromosome number, *Ctenitis*, *Deparia*, *Diplazium*, *Dryopteris*, *Microsorum*, *Pteris*, *Ptisana*, *Tectaria*, *Thelypteris*.

The latest national red list of Japan (Ministry of the Environment, Japan, 2019) has accessions of more than one-third of the native ferns and lycophytes (255 taxa) including seven extinct (EX), two extinct-in-the-wild (EW) and 82 critically endangered (CR) species. For planning their conservation strategies and judging priorities of their conservation actions, it is essential to clarify their reproductive modes (sexual, apogamous or sterile) as well as ploidy levels and identify the position they occupy in the reticulate species complex. We should also focus on the infraspecific variation of cytotypes, and observe reproductive modes and ploidy levels in as many conspecific samples as possible. Chromosome numbers have been examined for 74% of the native taxa in Japan using domestic materials (Nakato and Ebihara, 2016). In contrast, reproductive modes and ploidy levels are still unknown for a large number of threatened taxa—i.e. 108 taxa (42% of the threatened taxa in the national red list) awaiting cytotaxonomic observation. As sampling of threatened species is not easy in many cases, we utilized cultivated stocks in a botanical garden.



## Materials and Methods

Materials used for chromosome counting are listed in Table 1. Root tips were collected from cultivated stocks in Tsukuba Botanical Garden, National Museum of Nature and Science, Japan. The living stocks native to Yakushima Island were mostly collected in fieldwork supported by a joint conservation program between the Ministry of the Environment and the Japan Association of Botanical Gardens. All voucher specimens are deposited in the herbarium of National Museum of Nature and Science (TNS). Methods for counting mitotic chromosomes in root tips of living stocks followed those of Ebihara *et al.* (2014). For leptosporangiate ferns, reproductive mode was determined by counting spore number per sporangium (s/s) and by spore shape regular-

Table 1. Plant material used in this study with chromosome counts

| Name | National red list category | Chromosome number (Ploidy, base number) | Reproductive mode | Locality | Voucher [TNS-VS] | Tsukuba Botanical Garden stock No. | Fig. |
|---|---|---|---|---|---|---|---|
| Marattiaceae | | | | | | | |
| *Angiopteris fokiensis* | CR | $2n = 80$ ($2x$, $x = 40$) | unknown | Kagoshima Pref., Kawanabe-gun, Chiran-cho | 1315384 | 162222 | 1 |
| *Ptisana boninensis* | NT | $2n = 156$ ($4x$, $x = 39$) | unknown | Probably from Bonin Islands. Grown from a stipule divided from a cultivate stock in Yumenoshima Botanical Garden, Tokyo. | 1176751 | 166690 | 2 |
| Pteridaceae | | | | | | | |
| *Cheilanthes chusana* | VU | $2n = 90$ ($3x$, $x = 30$) | apogamous (32 s/s) | Kochi Pref., Kami-gun, Monobe-son | 764374 | 145595 | 3 |
| *Pteris kawabatae* | CR | $2n = 58$ ($2x$, $x = 29$) | apogamous (32 s/s) | Kagoshima Pref., Yakushima Isl. | 1309876 | 166741 | 4 |
| *Pteris setulosocostulata* | EN | $2n = 58$ ($2x$, $x = 29$) | apogamous (32 s/s) | Kagoshima Pref., Yakushima Isl., Nagata | 1309858 | 166727 | 5 |
| Cystopteridaceae | | | | | | | |
| *Acystopteris tenuisecta* | CR | $2n = 84$ ($2x$, $x = 42$) | sexual (64 s/s) | Kagoshima Pref., Yakushima Isl. | 1309841 | 166712 | 6 |
| Aspleniaceae | | | | | | | |
| *Asplenium formosae* | EN | $2n = 144$ ($4x$, $x = 36$) | sexual (64 s/s) | Okinawa Pref., Ishigakijima Isl. | 1315348 | 167453 | 7 |
| Thelypteridaceae | | | | | | | |
| *Thelypteris erubescens* | CR | $2n = 144$ ($4x$, $x = 36$) | sexual (64 s/s) | Kumamoto Pref., Amakusa-shi | 1193278 | 164667 | 8 |
| *Thelypteris gracilescens* | CR | $2n = 132$ ($4x$, $x = 33$) | sexual (64 s/s) | Kagoshima Pref., Yakushima Isl. | 1309839 | 166710 | 9 |
| Blechnaceae | | | | | | | |
| *Blechnum hancockii* | DD | $2n = 62$ ($2x$, $x = 31$) | sexual (64 s/s) | Kagoshima Pref., Suwanosejima Isl. | 1313834 | 167452 | 10 |
| Athyriaceae | | | | | | | |
| *Athyrium palustre* | VU | $2n = 160$ ($4x$, $x = 40$) | sexual (64 s/s) | Nagasaki Pref., Nishisonogi-gun, Seihi-cho | 1313416 | 167455 | 11 |
| *Athyrium palustre* | VU | $2n = 160$ ($4x$, $x = 40$) | unknown | Kagoshima Pref., Yakushima Isl. | 1309896 | — | |
| *Athyrium silvicola* | CR | $2n = 160$ ($4x$, $x = 40$) | sexual (64 s/s) | Kagoshima Pref., Yakushima Isl. | 1309848 | 166721 | 12 |
| *Athyrium spinescens* | CR | $2n = 240$ ($6x$, $x = 40$) | sexual (64 s/s) | Fukuoka Pref., Fukuoka-shi | 1315346 | 167454 | 13 |
| *Deparia longipes* | VU | $2n = 160$ ($4x$, $x = 40$) | sexual (64 s/s) | Gifu Pref., Anpachi-gun, Godo-cho | 1315385 | 159386 | 14 |
| *Deparia longipes* | VU | $2n = 120$ ($3x$, $x = 40$) | sterile (spore irregular) | Wakayama Pref., Higashimuro-gun, Nachikatsuura-cho | 1160766 | 157481 | 15 |
| *Deparia longipes* | VU | $2n = 160$ ($4x$, $x = 40$) | sexual (64 s/s) | Miyazaki Pref., Saito-shi | 1315386 | 167191 | |
| *Diplazium megaphyllum* | — | $2n = 123$ ($3x$, $x = 41$) | apogamous (32 s/s *1) | Kagoshima Pref., Tokunoshima Isl. | 1315387 | 166746 | 16 |
| Dryopteridaceae | | | | | | | |
| *Arachniodes cavaleriei* | VU | $2n = 82$ ($2x$, $x = 41$) | sexual (64 s/s) | Kagoshima Pref., Yakushima Isl. | 1309851 | 166722 | 17 |
| *Ctenitis microlepigera* | EN | $2n = 82$ ($2x$, $x = 41$) | apogamous (32 s/s) | Tokyo Metropolis, Bonin Islands, Hahajima Isl. | 1313843 | 163161 | 18 |
| *Dryopteris lunanensis* | CR | $2n = 82$ ($2x$, $x = 41$) | apogamous (32 s/s) | Nara Pref., Yoshino-gun, Totsu-kawa-mura | s.n. | *3 | 19 |
| Tectariaceae | | | | | | | |
| *Arthropteris palisotii* | CR | $2n = 164$ ($4x$, $x = 41$) | sexual (64 s/s *2) | Okinawa Pref., Kunigami-gun, Motobu-cho | 763921 | 128791 | 20 |
| *Tectaria fauriei* | CR | $2n = 160$ ($4x$, $x = 40$) | unknown | Kagoshima Pref., Tokunoshima Isl. | 764229 | 159383 | 21 |
| Polypodiaceae | | | | | | | |
| *Microsorum rubidum* | EN | $2n = 72$ ($2x$, $x = 36$) | sexual (64 s/s) | Okinawa Pref., Iriomotejima Isl. | 776991 | 157520 | 22 |

*1 Spore number counted on a herbarium specimen from the same locality (TNS VS-1291890).

*2 Spore number counted on a herbarium specimen (TNS VS-117590).

*3 A propagated stock of TBG155574 by spores.

ity in the voucher specimens or in cultivated stocks.

## Results and Discussion

Categories in the red list of Japan (Ministry of the Environment, Japan, 2019) are shown in square brackets after taxon names.

**Marattiaceae**

*Angiopteris fokiensis* Hieron.

[CR]—$2n = 80$ ($x = 40$, $2x$, reproductive mode unknown) (Fig. 1)

This is the first cytological record for the population in Japan, and the count is consistent with the records in Chinese populations ($n = 40$, Weng and Qiu, 1988; $2n = 80$, Li, 1989). Although we did not examine the reproductive mode, regular-shaped spores produced by the stock suggested its fertility.

*Ptisana boninensis* (Nakai) Yonek.

[NT]—$2n = 156$ ($x = 39$, $4x$, reproductive mode unknown) (Fig. 2)

An endemic species to Bonin Islands. This is the first chromosome count for the species, but we did not examine its reproductive mode. Our count is consistent with the estimated chromosome base number of the genus *Ptisana*, $x = 39$ (Murdock, 2008). The chromosome number of *Ptisana* has previously been counted in five species: *P. fraxinea* (Sm.) Murdock ($n = 78$; Manickam and Irudayaraj, 1988; $n = 78$ & $2n = 156$; Ninan, 1956), *P. purpurascens* (de Vriese) Murdock ($2n = 78$; Liu *et al.*, 2019), *P. salicifolia* (Schrad.) Senterre et Rouhan ($2n = 78$; Liu *et al.*, 2019), *P. salicina* (Sm.) Murdock ($2n = 78$; Brownlie, 1961; Liu *et al.*, 2019) and *P. smithii* (Mett. ex Kuhn) Murdock ($2n = 78$; Takamiya, 1995). The present count is the second record of tetraploid after *P. fraxinea*.

**Pteridaceae**

*Cheilanthes chusana* Hook.

[VU]—$2n = 90$ ($x = 30$, $3x$, apogamous) (Fig. 3)

Kawakami (1980) reported triploidy ($2n = 90$) of this species, but did not mention its reproductive mode. The chromosome number we observed completely matched that of Kawakami (1980), and ca. 32 normal-shaped spores per sporangium were observed. It is necessary to note that the "32 spores per sporangium" is not always associated with apogamy in Cheilanthoids—some sexually reproducing individuals produce 32 and/or 16 spores per sporangium (Vida *et al.*, 1971; Walker 1984; Tindale and Roy, 2002). Considering observed regular spore formation and oddploidy, it is certain that this species reproduces by apogamy.

*Pteris kawabatae* Sa.Kurata

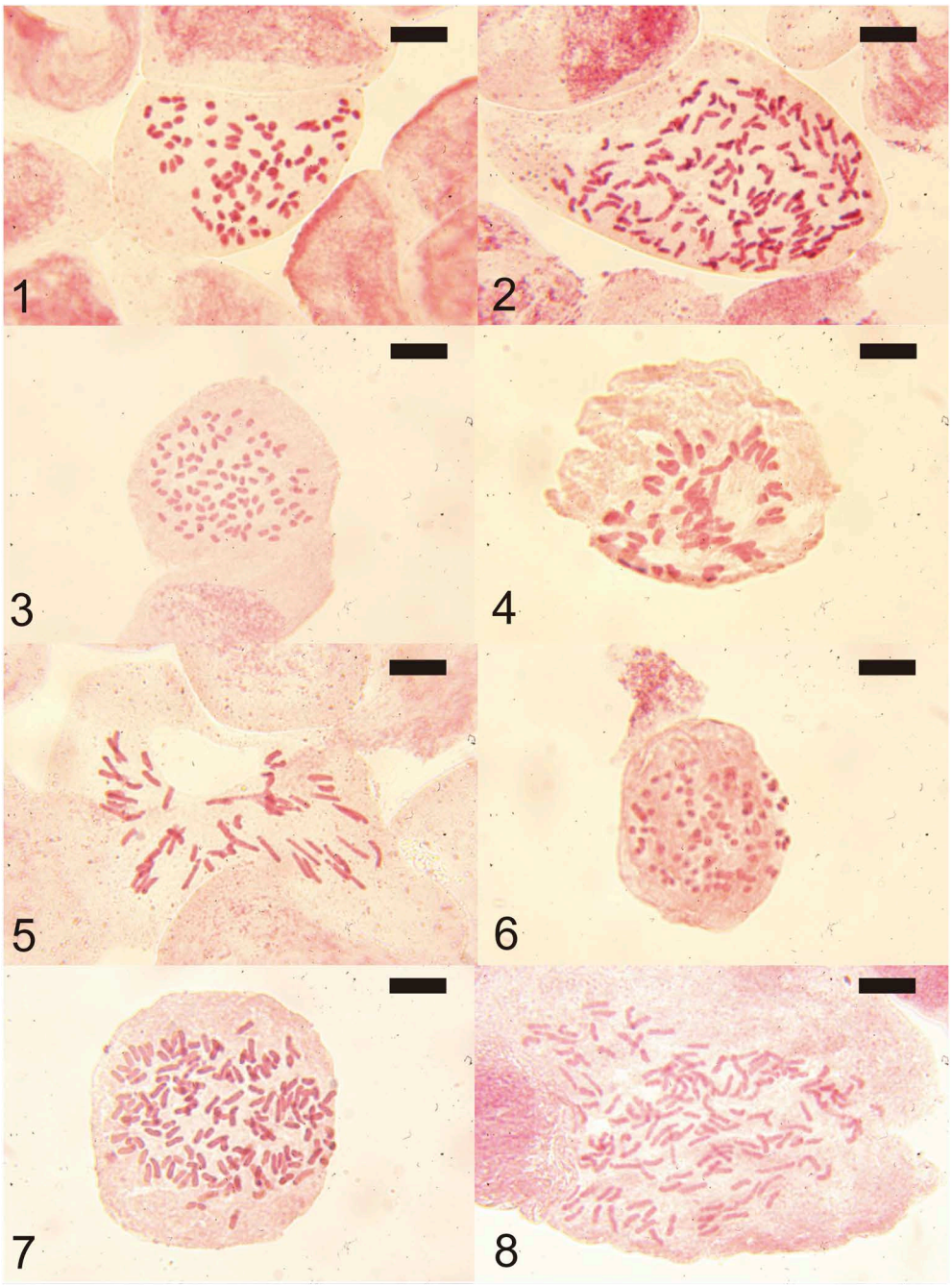
[CR]—$2n = 58$ ($x = 29$, $2x$, apogamous) (Fig. 4)

This species was once considered as endemic to Yakushima Island, southern Japan, but a recent study has clarified it is also distributed in Taiwan, China and Vietnam (Chao *et al.*, 2013). This is the first chromosome count for the species. By adding present observation on *P. kawabatae*, cytological information is available for all the nine species of *Pteris fauriei* group native to Japan—one exclusively sexual species, *P. boninensis* H.Ohba, six apogamous species (*P. kawabatae*, *P. kiuschiuensis* Hieron., *P. laurisilvicola* Sa.Kurata, *P. natiensis* Tagawa, *P. satsumana* Sa.Kurata, *P. setulosocostulata* Hayata, *P. yakuinsularis* Sa.Kurata) and two species with both reproductive modes (*P. fauriei* Hieron. and *P. oshimensis* Hieron.) (Ebihara, 2016). As noted in Ebihara and Nitta (2019), we should explore sexual progenitors on a global scale to clarify the origin of the apogamous species complex.

*Pteris setulosocostulata* Hayata

[EN]—$2n = 58$ ($x = 29$, $2x$, apogamous) (Fig. 5)

This is a Sino-Himalayan species with its distribution records in Japan limited to Yakushima Island. The present chromosome count differs from the previous one on an individual collected in Miyanoura, Yakushima Island (apogamous triploid, $2n = 87$, Mitui, 1966, 1968), hence a new cytotype [the chromosome count by Tsai (1973, 1992) is omitted from the present discus-




Figs. 1–8. Microphotographs of mitotic metaphase chromosomes. Scale bars = 10 µm. 1. *Angiopteris fokiensis* ($2n = 80$). 2. *Ptisana boninensis* ($2n = 156$). 3. *Cheilanthes chusana* ($2n = 90$). 4. *Pteris kawabatae* ($2n = 58$). 5. *Pteris setulosocostulata* ($2n = 58$). 6. *Acystopteris tenuisecta* ($2n = 84$). 7. *Asplenium formosae* ($2n = 144$). 8. *Thelypteris erubescens* ($2n = 144$).

sion due to their questionable interpretation of the result]. It is an unexpected result that two different cytotypes, diploid and triploid are present within a small number of populations in Yakushima Isl., and we should carefully re-examine their morphological differences and consider conservation methods for both cytotypes.

**Cystopteridaceae**

*Acystopteris tenuisecta* (Blume) Tagawa

[CR]—$2n = 84$ ($x = 42$, $2x$, sexual) (Fig. 6)

This species is widely distributed in Asia, though Yakushima Island is its only distribution area in Japan (Ebihara, 2016). This is the first cytological record for its population in Japan. The molecular analyses of plastid *rbcL* and nuclear *PgiC* genes performed on a sample collected in Yakushima Isl. detected no difference between the sample of Yakushima Isl. and diploid individuals of Taiwan (Ebihara *et al.*, 2019). The present result confirmed that the Yakushima population is cytologically similar to Taiwan populations. Meanwhile, tetraploid ($n = 84$: Bir in Mehra, 1961; Bir, 1971) and sterile triploid ($2n = 126$, Bir in Mehra, 1961; Bir, 1971) were reported from India, and the species as a whole is cytologically heterogeneous. Further study on cytotypic variation throughout the distribution range and its analyses are needed for clarifying the origin of polyploids.

**Aspleniaceae**

*Asplenium formosae* H.Christ

[EN]—$2n = 144$ ($x = 36$, $4x$, sexual) (Fig. 7)

This species is distributed in Taiwan, S. China and Vietnam and the locality in Japan is confined to Ishigakijima Island, Ryukyu (Ebihara, 2016). This is the first chromosome count for the species. Approximately half the number of Aspleniaceae species with known ploidy are tetraploid, while diploid ones occupies only a quarter of the species (Schneider *et al.*, 2017). "The *Neottopteris* clade" to which *A. formosae* belongs showed a notably high ratio of tetraploid (69%), and is presumed to be paleopolyploid which experienced a whole genome duplication at the stage of a common ancestor of the clade (Schneider *et al.*, 2017). Our present result, tetraploid ($2n = 144$) well fits the hypothesis.

**Thelypteridaceae**

*Thelypteris erubescens* (Wall. ex Hook.) Ching [ = *Glaphyropteridopsis erubescens* (Wall. ex Hook.) Ching]

[CR]—$2n = 144$ ($x = 36$, $4x$, sexual) (Fig. 8)

This is a Sino-Himalayan species with its distribution records in Japan limited to only two localities, Yakushima Island and a single location in Kumamoto Pref., Kyushu—the latter is the sole extant population (Ebihara, 2016). This is the first cytological record for the population in Japan, and the result of tetraploidy is consistent with the record for a population in Taiwan ($n = 72$: Mitui, 1968). On the other hand, diploidy was repeatedly reported for populations in India ($n = 36$: Verma and Loyal, 1960; Loyal 1961; Loyal in Mehra, 1961; Roy and Sakya in Fabbri, 1963; Roy *et al.*, 1971; Mehra and Khullar, 1980; Khullar *et al.*, 1983; Khullar *et al.* in Bir, 1988; $2n = 72$: Roy and Sakya in Fabbri, 1963; Roy *et al.*, 1971; $n =$ ca. 35: Irudayaraj and Manickam in Bir, 1987; $n =$ ca. 36: Manickam and Irudayaraj, 1988).

*Thelypteris gracilescens* (Blume) Ching [ = *Metathelypteris gracilescens* (Blume) Ching]

[CR]—$2n = 132$ ($x = 33$, 4x, sexual) (Fig. 9)

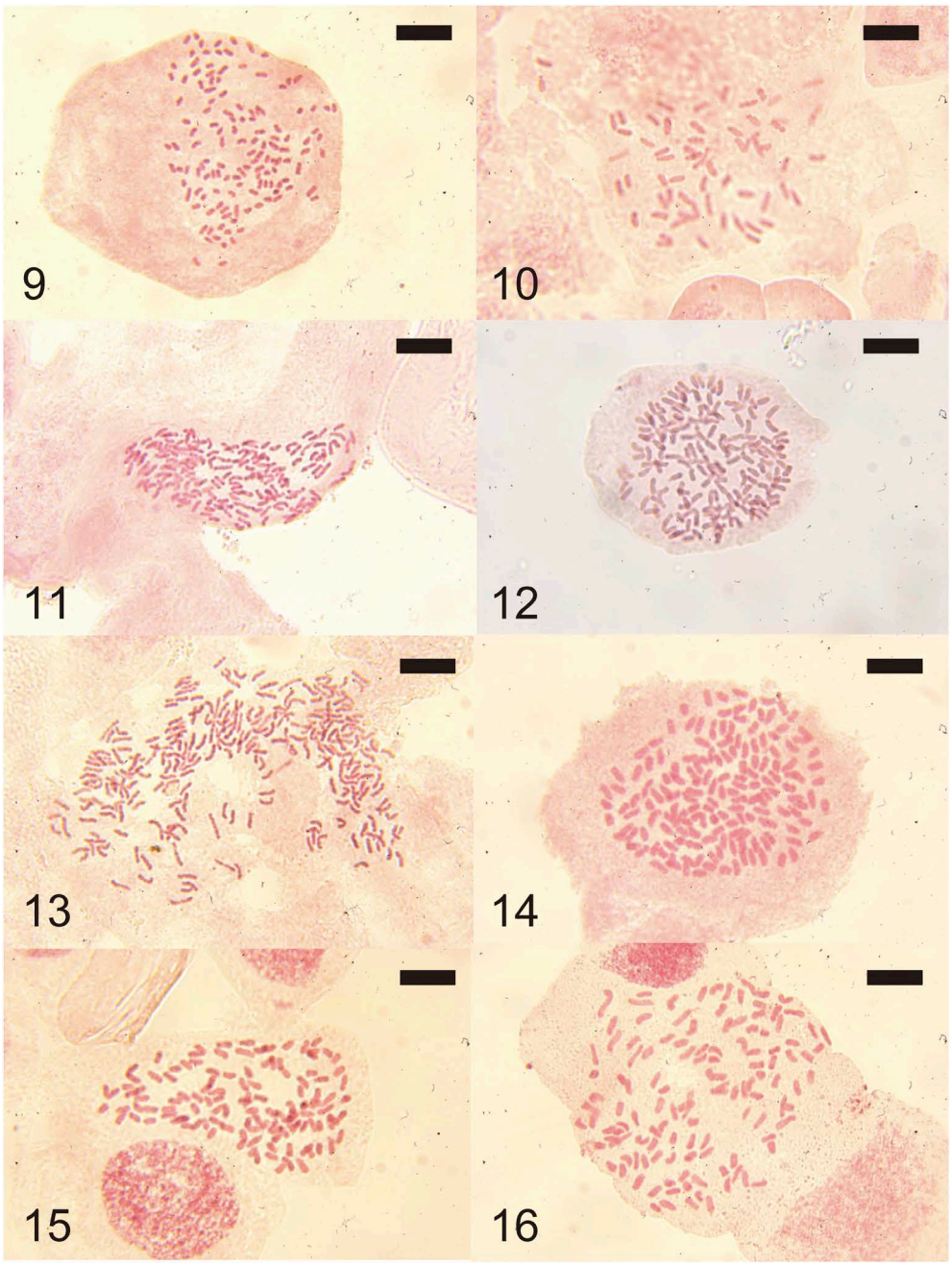
This is a widely distributed species in Asia, but all its known localities in Japan are within Yakushima Island. This is the first cytological record for the population in Japan. Our chromosome count $2n = 132$ differs from those reported for material from Taiwan ($n = 34$, Tsai and Shieh, 1978, 1983, 1985) in base number and ploidy level.

**Blechnaceae**

*Blechnum hancockii* Hance [ = *Spicantopsis hancockii* (Hance) Masam.]

[DD]—$2n = 62$ ($x = 31$, $2x$, sexual) (Fig. 10)

A species with its distribution centering in Tai-




Figs. 9–16. Microphotographs of mitotic metaphase chromosomes. Scale bars = 10μm. 9. *Thelypteris gracilescens* ($2n = 132$). 10. *Blechnum hancockii* ($2n = 62$). 11. *Athyrium palustre* ($2n = 160$, Nagasaki Pref.). 12. *Athyrium silvicola* ($2n = 160$). 13. *Athyrium spinescens* ($2n = 240$). 14. *Deparia longipes* ($2n = 160$, Gifu Pref.). 15. *D. longipes* ($2n = 120$, Wakayama Pref.). 16. *Diplazium megaphyllum* ($2n = 123$).

wan, and in Japan it is confined to Tokara Islands of Ryukyu (Ebihara, 2016). This is the first cytological record for the population in Japan, and sexual reproduction was confirmed for the first time. The present chromosome count $2n = 62$ matched that of the population in Taiwan (Ebi-

hara *et al.*, 2014).

**Athyriaceae**

*Athyrium palustre* Seriz.

[VU]—$2n = 160$ ($x = 40$, $4x$, sexual) (Fig. 11)

This species is distributed in Western Japan (Okayama Pref. of Honshu, Shikoku, Kyushu and Yakushima Island) (Ebihara, 2017) and Taiwan (Chang *et al.*, 2014). This is the first chromosome count for the species, and we confirmed that an individual from Nagasaki Prefecture is a sexual tetraploid and another from Yakushima Island also a tetraploid with unknown reproductive mode (mature frond is not available).

*Athyrium silvicola* Tagawa

[CR]—$2n = 160$ ($x = 40$, $4x$, sexual) (Fig. 12)

Widely distributed in the Sino-Himalayan region, but in Japan, it is recorded only in Mie Prefecture, northern part of Kagoshima Pref. and Yakushima Isl. (including forma *kinokuniense* Sa.Kurata). This is the first cytological record for the population in Japan. The chromosome count "$n = 40$IV" (Tsai and Shieh, 1985) based on material from Taiwan is questionable as perfect tetravalents are hardly observed in the meiosis of ferns.

*Athyrium spinescens* Sa.Kurata

[CR]—$2n = 240$ ($x = 40$, $6x$, sexual) (Fig. 13)

This species has intermediate morphology between *A. iseanum* Rosenst. and *A. vidalii* (Franch. et Sav.) Nakai and is quite similar to their sterile hybrid *A.* × *pseudospinescens* Seriz. but produces normal spores. It is known only from the type locality, Sefuri Mountains located on the border of Fukuoka and Saga Prefectures in Kyushu (Ebihara, 2017). This is the first chromosome count for the species. The hexaploidy observed in the species could not be explained by a hybridization and succeeding polyploidization that occurred between tetraploid *A. iseanum* and tetraploid *A. vidallii*, implying the involvement of an undiscovered sexual diploid.

*Deparia longipes* (Ching) Shinohara

[VU]—$2n = 160$ ($x = 40$, $4x$, sexual) (Fig. 14)/ $2n = 120$ ($x = 40$, $3x$, irregular) (Fig. 15)

This is the most recently recognized *Deparia* species in Japan, and its scattered localities have been found in Honshu and Kyushu (Ebihara, 2017). Our stocks from Gifu and Miyazaki Prefectures are sexual tetraploid, while that from Wakayama Prefecture is sterile triploid—both are new cytotypes and inconsistent with the record in the Taiwan population ($2n = 80$, sexual diploid, Shinohara *et al.*, 2006). The triploid is morphologically slightly different from the tetraploid in having narrower fronds, but more comprehensive morphological comparison between the cytotypes is necessary. It is difficult to explain the origin of triploid by hybridization between diploid and tetraploid, because of lack of diploid among the nine taxa of *Deparia* sect. *Athyriopsis* (sensu Kuo *et al.*, 2018) in Japan (Ebihara, 2017).

*Diplazium megaphyllum* (Baker) H. Christ

—$2n = 123$ ($x = 41$, $3x$, apogamous) (Fig. 16)

This is one of the most recently added species to the flora of Japan with a single population consisting of 12 mature individuals found in Tokunoshima Island (Ebihara *et al.*, 2018). We performed a cytological observation for the first time on the Japanese population. The result obtained was apogamous triploidy with $2n = 123$ chromosomes, which matched that on Taiwanese material (Chiou *et al.*, 2006).

**Dryopteridaceae**

*Arachniodes cavaleriei* (H.Christ) Ohwi

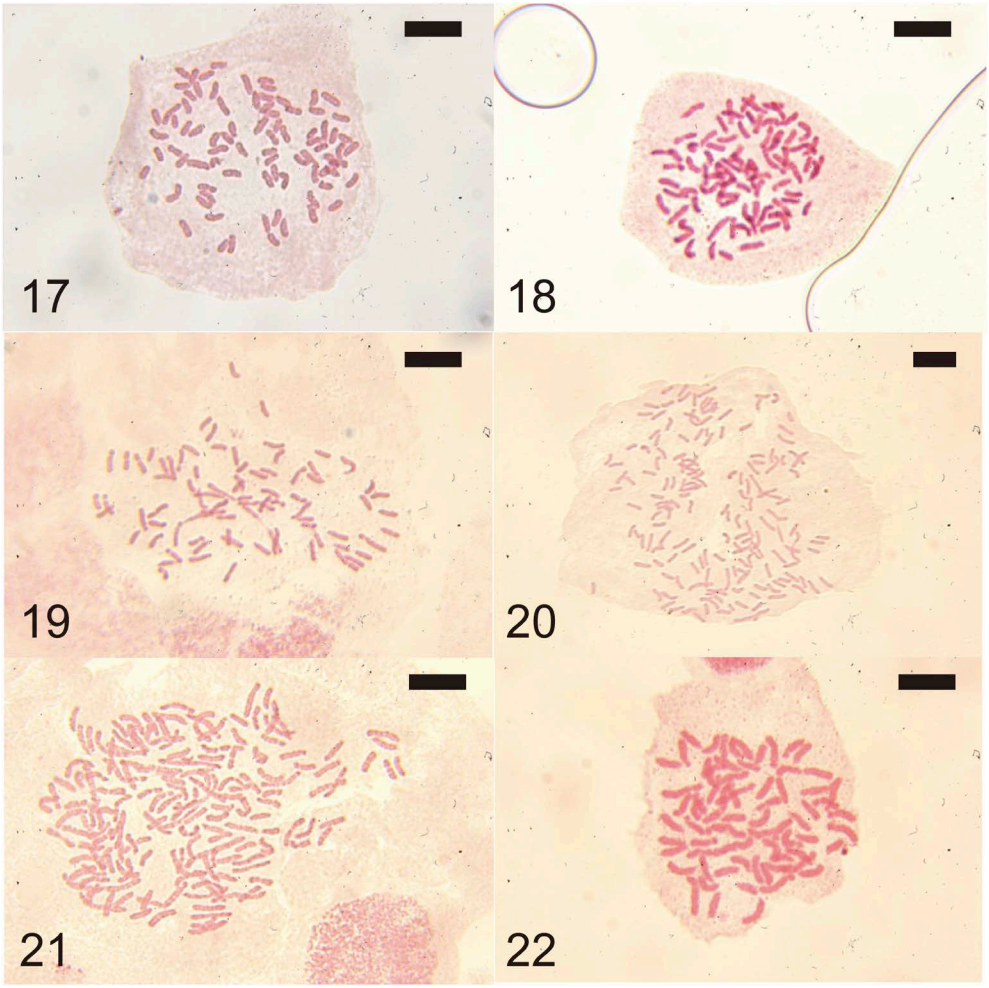
[VU]—$2n = 82$ ($x = 41$, $2x$, sexual) (Fig. 17)

A species distributed in China, Vietnam, Thailand (Lindsay and Middleton, 2012 onwards) and Yakushima Island of Japan. This is the first chromosome count for the species.

*Ctenitis microlepigera* (Nakai) Ching

[EN]—$2n = 82$ ($x = 41$, $2x$, apogamous) (Fig. 18)

Although the identity of this species endemic to Bonin Islands was once controversial, the




Figs. 17–22. Microphotographs of mitotic metaphase chromosomes. Scale bars = 10 µm. 17. *Arachniodes cavaleriei* (2*n* = 82). 18. *Ctenitis microlepigera* (2*n* = 82). 19. *Dryopteris lunanensis* (2*n* = 82). 20. *Arthropteris palisotii* (2*n* = 164). 21. *Tectaria fauriei* (2*n* = 160). 22. *Microsorum rubidium* (2*n* = 72).

recent rediscovery of a typical population (Mizunashi *et al.*, 2011) enabled us to examine its biological properties. This is the first chromosome count for the species using stocks multiplied from the stock collected by Mizunashi *et al.* (2011). We observed approximately 32 well-filled spores per sporangium, and the sizes of spores are larger than those of *C. eatonii* (Baker) Ching found in Kurata and Nakaike (1987) which is a sexual diploid (Mitui, 1968)—, these evidences suggesting it reproduces by apogamy. This is the first report of apogamous reproduction in the genus *Ctenitis* sensu PPGI (Pteridophyte Phylogeny Group, 2016).

*Dryopteris lunanensis* (H. Christ) C.Chr.
[CR]—2*n* = 82 (*x* = 41, 2*x* apogamous) (Fig. 19)

This species shows a rather scattered distribution in southern China (Zhang *et al.*, 2013), Bhutan (Fraser-Jenkins, 1998) and Ohmine Mountains of western Honshu (Nara and Mie Prefectures), Japan. This is the first chromosome count for the species, and our result is consistent with that by Hori *et al.* (2019) who estimated its apogamous reproductive mode.

**Tectariaceae**

*Arthropteris palisotii* (Desv.) Alston

[CR]—$2n = 164$ ($x = 41$, $4x$, sexual) (Fig. 20)

A widely distributed species in the paleotropics, though only a few localities are known in Ryukyu. This is the first cytological record for the population in Japan, and is a new cytotype different from the diploid previously recorded from Africa (Ghana) ($n = 41$, Manton, 1959).

*Tectaria fauriei* Tagawa

[CR]—$2n = 160$ ($x = 40$, $4x$, reproductive mode unknown) (Fig. 21)

This species is distributed in Asian tropics, and distribution records in Japan are only in three islands in Ryukyu (Tokunoshima, Okinoerabujima and Okinawa Islands) (Ebihara, 2017). This is the first cytological record for the population in Japan, and the ploidy is different from the previous record from Taiwan ($n = 40$III, Tsai and Shieh, 1985).

**Polypodiaceae**

*Microsorum rubidum* (Kunze) Copel.

[EN]—$2n = 72$ ($x = 36$, $2x$, sexual) (Fig. 22)

A widely distributed species in Asia, but the distribution in Japan is confined to southern Ryukyu. This is the first cytological record for the population in Japan. The chromosome number matched that observed in Indian material ($n = 36$, $2n = 72$, Nayar, 1963).

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