



Phylogenetic analysis reveals the origins of tetraploid and hexaploid species in the Japanese *Lepisorus thunbergianus* (Polypodiaceae) complex

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Abstract

The Japanese *Lepisorus thunbergianus* complex contains diploid and tetraploid races of *L. thunbergianus* and a hexaploid species, *L. mikawanus*. Here, we performed molecular phylogenetic analysis on this complex to delimit species and to elucidate the evolutionary origins of tetraploid and hexaploid species. Chloroplast DNA (cpDNA) phylogeny supported the monophyly of the complex. Based on a single-copy nuclear gene (*PgiC*) tree, the tetraploid *L. thunbergianus* samples could be classified into two variants: an allotetraploid of hybrid origin between diploid *L. thunbergianus* and Japanese *L. angustus* and another allotetraploid of hybrid origin between diploid *L. thunbergianus* and an unknown diploid race of *L. tosaensis*. These variants can be recognized morphologically and distinguished from their parent species. Hence, here we described these allopolyploids as new species, *L. nigripes* and *L. kuratae*, respectively. The hexaploid species *L. mikawanus* has three types of *PgiC* alleles, each of which was derived from diploid *L. thunbergianus*, *L. tosaensis*, and Japanese *L. angustus*, while cpDNA shows that it is included in Japanese *L. thunbergianus* clade. Based on the cpDNA phylogeny and *PgiC* nucleotide sequences, we therefore concluded that *L. mikawanus* is an allohexaploid that originated through hybridization between tetraploid species, *L. nigripes* and an unknown ancestral diploid race of *L. tosaensis*.

Keywords Allopolyploidy · Fern · Hybrid origin · *Lepisorus* · Species Complex · Taxonomy

Introduction

Hybridization and polyploidy play important roles in speciation and diversification within the plant kingdom (Soltis and Soltis 2009). These processes are very common, even in seedless vascular plants (Barrington et al. 1989; Sigel 2016). For example, it is estimated that polyploidy is involved in

30% of fern speciation (Wood et al. 2009). Recent advances in single- or low-copy nuclear DNA marker development in ferns (Ishikawa et al. 2002; Rothfels et al. 2013; Schuettelpelz et al. 2008) have provided opportunities to elucidate the hybridization and polyploidization processes in many polyploid complexes (Adjie et al. 2007; Chang et al. 2013; Ebihara et al. 2005; Hori et al. 2014; Jaruwattanaphan et al. 2013; Nitta et al. 2011; Rothfels et al. 2014; Sessa et al. 2012; Shepherd et al. 2008).

Lepisorus (J.Sm.) Ching is a genus that is distributed mainly through the Old World tropics and subtropics and comprises 40–70 species (Hennipman et al. 1990; Lin 2000; Zink 1993). Species delimitation within *Lepisorus* is very confusing to researchers due to the paucity of taxonomically informative characters and the plasticity of the characters used in species diagnosis. *Lepisorus thunbergianus* (Kaulf.) Ching is one of the most taxonomically problematic species in the genus *Lepisorus*. Wang et al. (2010) investigated the phylogenetic relationships within the genus using chloroplast DNA (cpDNA) sequences from 54 species, including one Japanese and two Chinese samples of *L. thunbergianus*.

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The results indicated that the *L. thunbergianus* sample collected in Japan was sister to a subclade, including the two *L. thunbergianus* samples from China, suggesting that *L. thunbergianus* is not monophyletic. Many intraspecific cytotypes have been reported: $2n = 50, 51, 75, 76, 100, 101$ and 102 (Takamiya 1996). Nakato et al. (1983) concluded that the $2n = 50, 100$ and 102 cytotypes were stable strains, based on the observation of normal spores in these cytotypes. Takei (1978, 1982) carried out karyotype analysis and proposed that both tetraploid cytotypes ($2n = 100$ and 102) originated from hybridization between diploid *L. thunbergianus* ($2n = 50$) and a closely related species, *L. onoei* (Franch. et Sav.) Ching ($2n = 50$). On the other hand, Mitui et al. (1987) hypothesized that the $2n = 100$ cytotype had an autopolyploid origin of diploid *L. thunbergianus*, while the $2n = 102$ cytotype originated through allopolyploidization between diploid *L. thunbergianus* and *L. angustus* Ching ($2n = 52$), in order to account for the difference in their chromosome numbers. Shinohara et al. (2010) conducted allozyme analysis using samples from a part of its distribution (Okutama, Tokyo) and hypothesized that both tetraploid and hypertetraploid cytotypes of *L. thunbergianus* ($2n = 100$ and 102) originated through allopolyploidization between diploid *L. thunbergianus* and *L. angustus*. Although the genetic evidence proposed by Shinohara et al. (2010) was convincing, an additional study using samples from the entire distribution area (from Kyushu to Tohoku) of tetraploid *L. thunbergianus* in Japan (Mitui et al. 1987) is needed to elucidate whether other diploid species were involved.

Serizawa and Aman (2012) paid attention to stipe (leaf stalk) color variation of *L. thunbergianus* in Aichi Prefecture, Japan, and tentatively classified *L. thunbergianus* into two stipe color types: green and blackish types. Their DNA ploidy analysis using flow cytometry revealed that the nuclear DNA contents of the Green type corresponded to diploid, triploid and tetraploid levels, and those of the Blackish type to tetraploid and hexaploid levels. Interestingly, they also showed that the nuclear DNA contents of the Green type were distinctly larger than those of the Blackish type at the same tetraploid level, suggesting that the two stipe color types of tetraploids could be reproductively isolated from each other. Furthermore, Serizawa (2015) redescribed hexaploid plants of the Blackish type as *L. mikawanus* Sa.Kurata because the broad lanceolate lamina of the hexaploid and its distribution range (Aichi and Shizuoka prefectures) are consistent with the description of *L. mikawanus* Sa.Kurata, which has not been reported since it was first described (Kurata 1965).

The aims of the present study were (1) to test the hypothesis of Shinohara et al. (2010) of the origin of tetraploid *L. thunbergianus* using a molecular phylogenetic approach and samples collected across the entire distribution range in Japan, (2) to elucidate the evolutionary origin of the

hexaploid species *L. mikawanus*, and (3) to delimit species by assessing genomic constitutions of polyploids in the complex. We ultimately recognize two new allotetraploid species with different pairs of parental diploid taxa, and their taxonomic treatments are provided.

Materials and methods

Sampling of materials

We define the *Lepisorus thunbergianus* complex as the group composed of diploid and tetraploid *L. thunbergianus* in Japan and hexaploid *L. mikawanus*. Since Wang et al. (2010) showed that the Chinese *Lepisorus* plants identified as *L. thunbergianus* were phylogenetically distinct from Japanese ones, they are excluded from the complex. A total of 24 plants of the complex were collected at 18 sites that covered its known distribution ranges in Japan. (Table 1; Fig. 1). Two samples of *L. angustus*, one of *L. onoei*, one of *L. hachijoensis* Sa.Kurata and two of *L. tosaensis* (Makino) H.Itô were obtained as candidates of parental diploid taxa of the polyploids in the complex (Table 1; Fig. 1). *Lepisorus uchiyamae* H.Itô and *L. affinis* Ching were used as outgroup taxa based on the reported phylogenetic relationships of the *L. thunbergianus* complex and closely related species (Wang et al. 2010). Shinohara et al. (2010) considered that triploid cytotypes ($2n = 75, 2n = 76$) of *L. thunbergianus* were F_1 hybrids between diploid ($2n = 50$) and tetraploid ($2n = 100, 2n = 102$) cytotypes of *L. thunbergianus* based on the intermediate chromosome numbers and allozyme band patterns. The triploid cytotypes were reported to be sterile because their spores were entirely abortive (Nakato et al. 1983). To avoid interploidal and/or interspecific hybrids, samples with abortive spores were removed based on the observation of spores. The detailed locations of the collection sites and the voucher information are listed in Table 1 and Table S1, respectively.

Ploidy level determination

We determined the ploidy level of each specimen in three ways: chromosome number counting, spore length measurement, and genome size estimation by flow cytometry. Mitotic chromosome numbers of 14 representative samples were counted (Table 1). Root tips were pretreated with 2 mM 8-hydroxyquinoline for 8 h at approximately 17 °C. After fixation in Carnoy's solution (3:1 ethanol:acetic acid) for 30 min, the root tips were macerated in a 3:1 mixture of 1 N HCl and 45% acetic acid at 60 °C for 1 min and then squashed in 2% aceto-orcein. Chromosomes were observed under a Nikon LABOPHOT microscope (Nikon, Tokyo, Japan). Spore size is frequently used to estimate

Table 1 Locations, ploidy levels, chromosome numbers and genomic DNA amounts of *Lepisorus* samples collected in this study

Taxon and sample ID	Locality (locality number)	Ploidy	Chromosome number	Genome size (pg/C)
<i>Lepisorus angustus</i>				
<i>L. angustus</i> _1	Kitashiobara, Yama-gun, Fukushima (4)	2x	–	–
<i>L. angustus</i> _2	Sudama, Hokuto, Yamanashi (11)	2x	2n = 52	6.7
<i>L. hachijoensis</i>	Hachijo Is., Tokyo (9)	2x [#]	–	–
<i>L. mikawanus</i>	Hasso, Inuyama, Aichi (13)	6x	2n = 152	20.6
<i>L. onoei</i>	Mt Ihara, Itojima, Fukuoka (22)	2x	–	–
<i>L. thunbergianus</i> (2x)				
<i>L. thunbergianus</i> _1	Yayoi-cho, Inage, Chiba, Chiba (6)	2x	2n = 50	7.9
<i>L. thunbergianus</i> _2	Minato, Futtsu, Chiba (7)	2x	–	–
<i>L. thunbergianus</i> _3	Kawadu-cho, Kamo, Shizuoka (10)	2x	2n = 50	8
<i>L. thunbergianus</i> _4	Ichihara, Kikuchi, Kumamoto (23)	2x	–	–
<i>L. thunbergianus</i> _5	Satuma-cho, Satuma, Kagoshima (24)	2x	–	–
<i>L. thunbergianus</i> (4x) variant 1 (<i>L. nigripes</i> sp. nov.)				
<i>L. thunbergianus</i> _6	Fukaura-cho, Aomori (1)	4x	2n = 102	14.5
<i>L. thunbergianus</i> _7	Tazawa, Senhoku, Akita (2)	4x	2n = 102	14.4
<i>L. thunbergianus</i> _8	Genbi-machi, Ichinoseki, Iwate (3)	4x	–	14.0
<i>L. thunbergianus</i> _9	Minato, Futtsu-shi, Chiba (7)	4x	–	–
<i>L. thunbergianus</i> _10	Juunisho, Kamakura, Kanagawa (8)	4x	–	14.1
<i>L. thunbergianus</i> _11	Nagasaka, Hokuto, Yamanashi (12)	4x	2n = 102	14.0
<i>L. thunbergianus</i> _12	Kouyasan, Kouya, Wakayama (15)	4x	2n = 100	–
<i>L. thunbergianus</i> _13	Nagaredani, Kwachinagano, Osaka (16)	4x	–	–
<i>L. thunbergianus</i> _14	Kokuhu-cho, Takayama, Gifu (18)	4x	2n = 102	14.0
<i>L. thunbergianus</i> _15	Higatani, Miyadu, Kyoto (19)	4x	2n = 102	–
<i>L. thunbergianus</i> _16	Mt. Kubote, Buzen, Fukuoka (21)	4x	2n = 102	14.3
<i>L. thunbergianus</i> _17	Mt. Ihara, Itojima, Fukuoka (22)	4x	2n = 100	13.9
<i>L. thunbergianus</i> (4x) variant 2 (<i>L. kuratae</i> sp. nov.)				
<i>L. thunbergianus</i> _18	Torinome, Nasusiobara-shi, Tochigi (5)	4x	–	15.4
<i>L. thunbergianus</i> _19	Inage-ku, Chiba-shi, Chiba (7)	4x	2n = 100	15.7
<i>L. thunbergianus</i> _20	Juunisho, Kamakura, Kanagawa (9)	4x	–	–
<i>L. thunbergianus</i> _21	Toyama, Toyama-shi, Toyama (18)	4x	–	–
<i>L. thunbergianus</i> _22	Hasso, Inuyama, Aichi (13)	4x	–	15.8
<i>L. thunbergianus</i> _23	Nagaredani, Kwachinagano, Osaka (17)	4x	–	–
<i>L. tosaensis</i>				
<i>L. tosaensis</i> _1	Momozaki, Kumano-shi, Mie (15)	4x	2n = 100	17.4
<i>L. tosaensis</i> _2	Yabakei-machi, Nakatsu-shi, Oita (21)	4x	–	–

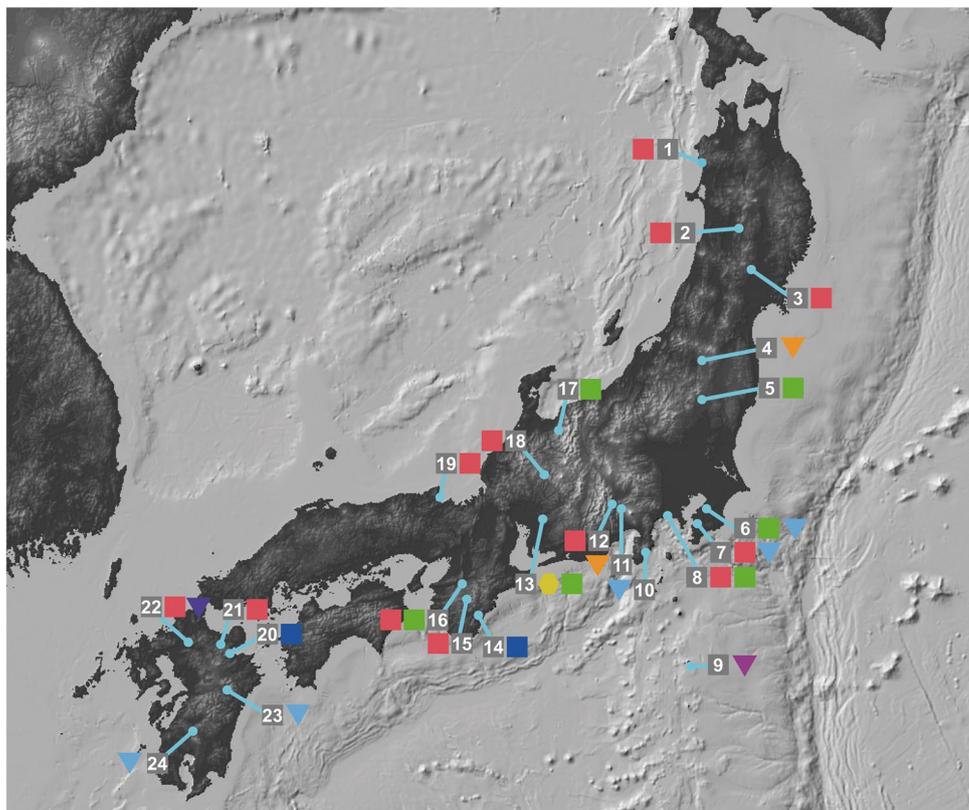
Locality numbers in parentheses correspond to those in Fig. 1. The ploidy level of each sample was estimated based on spore size measurements and chromosome counts

[#], ploidy level of *L. hachijoensis* was based on Mitui (1971)

ploidy levels among closely related fern species (Barrington et al. 1986; Dyer et al. 2012; Huang et al. 2006). We examined the spores of each specimen to infer ploidy levels. The length (distance between the extreme points of a central longitudinal section of a monolete spore) of 15–20 randomly selected spores of each specimen was measured using a Nikon LABOPHOT microscope with a calibrated ocular micrometer. Flow cytometry with propidium iodide (PI) was conducted on 16 samples for which fresh leaves were available (Table 1). A piece of

the leaf blade of a *Lepisorus* sample was co-chopped with that of *Petroselinum crispum* L. (2C = 4.5 pg, Obermayer et al. 2002) as an internal standard, using a razor blade in 1.2 mL chopping buffer (1.0% [v/v] Triton X-100, 140 mM 2-mercaptoethanol, 50 mM NaHSO₃, 50 mM Tris-HCl (pH 7.5), 25 µg mL⁻¹ PI). Following the addition of 0.6 mL chopping buffer, the mixture was filtered through a 30 µm nylon mesh. The extract was centrifuged for 2 min at 4,000×g. After removal of the supernatant, 0.5 mL of chopping buffer was added. The fluorescence intensities

Fig. 1 Collection sites of *Lepisorus thunbergianus* and related species in Japan. Numbers on this map correspond to locality numbers in Table 1. Symbols and their colors represent respective ploidy levels and species examined in this study. Symbols: hexagon = hexaploid, square = tetraploid, triangle = diploid. Colors: yellow = *L. mikawanus*, red = tetraploid *L. thunbergianus* variant 1 [= *L. nigripes*], light green = tetraploid *L. thunbergianus* variant 2 [= *L. kuratae*], blue = *L. tosaensis*, orange = *L. angustus*, light blue = diploid *L. thunbergianus*, dark purple = *L. onoei*, purple = *L. hachijoensis*



of the samples were analyzed on a Cell Lab Quanta SC (Beckman Coulter, Tokyo, Japan).

Chloroplast and nuclear DNA sequencing

Total genomic DNA was extracted from silica gel-dried samples using the CTAB method (Doyle and Doyle 1987). Two chloroplast DNA regions, *rbcL* and an intergenic spacer between *rps4* and *trnS* (hereafter *rps4-trnS*), were separately amplified using the standard PCR protocol. The following primer pairs were used: ESRBCL1F and ESRBCL1361R for *rbcL* (Schuettelpelz and Pryer 2007) and *rps4_PTER_F* (5'-CTCGCTACCGAGGACCTCG-3') and *trnS_PTER_R1* (5'-CTACCGAGGGTTCGAATC-3') for *rps4-trnS*, which were newly designed based on the sequence of *Pteridium aquilinum* L. (accession number, NC014348). We designed new primers, PGIC_LEP_15F (5'-TTGCCAGGCATTAGA GAAGC-3') and PGIC_LEP_16R (5'-GCCTTCTATTGA AACCCCTTTC-3'), for the single-copy nuclear gene *PgiC* based on the sequence of *Lepisorus thunbergianus* (JQ806503) determined by Wang et al. (2012). PCR thermocycling conditions involved initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 45 s, 56 °C for 45 s (*rbcL* and *rps4-trnS*) or 52 °C for 45 s (*PgiC*), 72 °C for 90 s, and a final extension at 72 °C for 7 min.

In order to separate allelic variants of the nuclear *PgiC* gene, we conducted single-strand conformation polymorphism (SSCP) gel electrophoresis of the PCR products, generally following the methods of Jaruwattanaphan et al. (2013) and Fujiwara et al. (2017). A part of the DNA band separated on SSCP gel was cut out, and DNA extracted from the band was used as a template for further PCR amplification (Jaruwattanaphan et al. 2013).

For DNA sequencing, the PCR products were purified using Illustra ExoStar 1-Step (GE Healthcare, Chicago, Illinois, USA) and were used as templates for direct sequencing. Cycle sequencing was conducted with a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, California, USA). The reaction mixtures were analyzed by an ABI3500 genetic analyzer (Applied Biosystems) and also partly by Eurofins Genomics (Tokyo, Japan). The resulting nucleotide sequences were deposited into the International Nucleotide Sequence Databases (INSD) with the following accession numbers: LC331914 to LC331943 for *rbcL*, LC332083 to LC332112 for *rps4-trnS*, and LC332113 to LC332166 for *PgiC*.

Phylogenetic analysis

CpDNA and *PgiC* gene nucleotide sequences obtained from 24 samples of the *L. thunbergianus* complex and six of its

related species collected in the present study (Table 1), as well as some sequences determined by Wang et al. (2010, 2012), were assembled and phylogenetically analyzed. The sequences of *Lepisorus uchiyamae* (Makino) H.Itô and *Lepisorus affinis* Ching were used as outgroups based on the reported phylogenetic relationships of the *L. thunbergianus* complex and its closely related species (Wang et al. 2010, 2012). INSD accession numbers of the cpDNA and *PgiC* datasets are shown in Table S1. DNA sequences were aligned in Bioedit (Hall 1999) using the ClustalW algorithm (Thompson et al. 1994), followed by manual editing. Indels generated by insertion or deletion events were coded as binary data using the “simple index coding” method of Simmons and Ochoterena (2000) using the IndelCoder option of SeqState 1.4.1 software (Müller 2005). Binarized characters were added to the alignments, and the corresponding sites with gaps were excluded as missing data. Only one sequence for each haplotype was included in each dataset. For phylogenetic analysis, a combined dataset of the two cpDNA regions and a *PgiC* dataset were separately analyzed by the maximum likelihood (ML) method using Garli 2.01 (Zwickl 2006) and Bayesian inference (BI) using MrBayes version 3.1.2 (Ronquist et al. 2012). The best fitting substitution model for each DNA region was determined using the Akaike information criterion (AIC) (Akaike 1974) in

jModelTest (Posada 2008). The Mkv model (Lewis 2001) was applied to binary-coded data. In the ML analysis, eight independent runs were performed with random starting trees and ‘genthreshfortopoterm’ set to 100,000. The bootstrap support (BS) values were assessed using 1,000 bootstrap pseudo-replicate datasets. The consensus tree of the bootstrap replicates was obtained using SumTrees (Sukumaran and Holder 2010). In the BI analysis, four MCMC chains were run for 3,000,000 generations with samples taken every 100 generations. Tracer 1.6 (Rambaut and Drummond 2013) was used to evaluate convergence. The first 500,000 generations were discarded as burn-in. The cpDNA and nuclear gene alignments and the ML and BI trees were deposited in TreeBase (treebase.org; study: S22707).

Results

Chromosome counts and ploidy analysis

The chromosome numbers counted in the present study are shown in Table 1. A chromosome number of $2n=52$ ($2x$) was counted for *Lepisorus angustus* (Fig. 2b, g). Three cytotypes, $2n=50$ ($2x$) (Fig. 2a, f), $2n=100$ ($4x$) (Fig. 2c, e, h, j), and $2n=102$ ($4x$) (Fig. 2d, i), were observed in *L.*

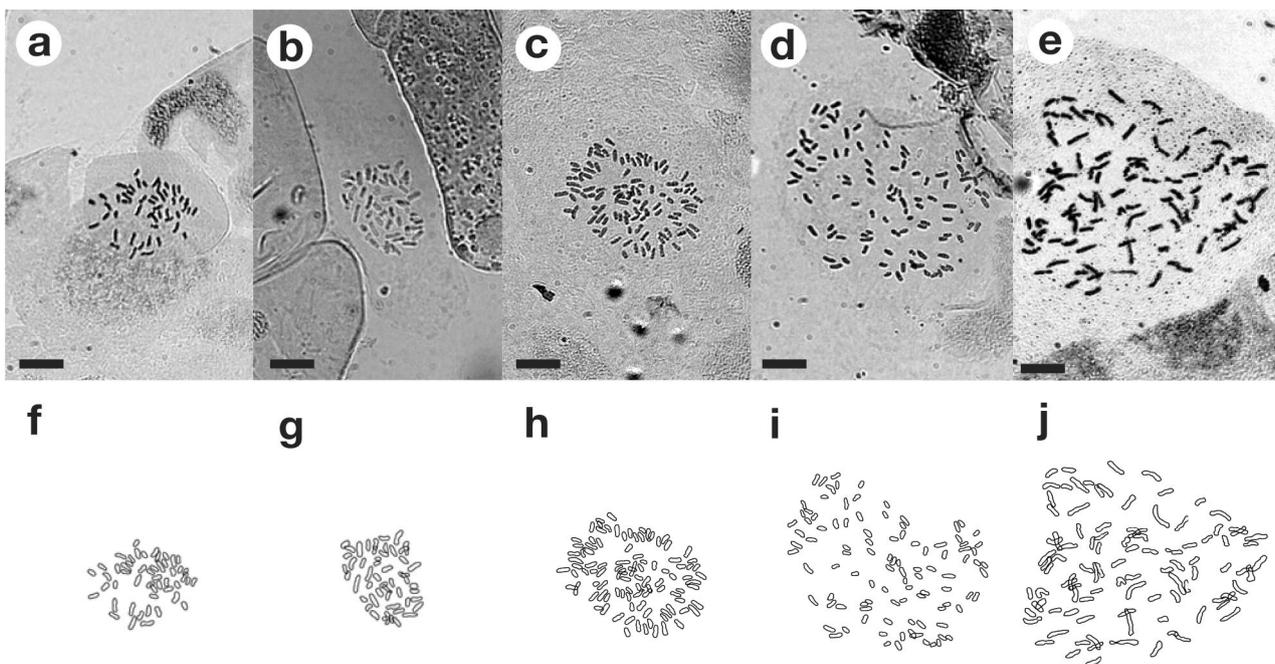


Fig. 2 Mitotic metaphase chromosomes of *Lepisorus thunbergianus* and *L. angustus*. Chromosome counts, sample ID and voucher specimen in TNS are given in parentheses. **a, f** Diploid *Lepisorus thunbergianus* ($2n=50$, *L. thunbergianus*_1, T. Fujiwara 1603163); **b, g** *L. angustus* ($2n=52$, *L. angustus*_2, T. Fujiwara 15111510); **c, h** tetraploid *L. thunbergianus* variant 1 [= *L. nigripes*] ($2n=100$, *L. thun-*

*bergianus*_12, T. Fujiwara 1604107); **d, i** tetraploid *L. thunbergianus* variant 1 [= *L. nigripes*] ($2n=102$, *L. thunbergianus*_13, T. Fujiwara 16040921); **e, j** tetraploid *L. thunbergianus* variant 2 [= *L. kuratae*] ($2n=100$, *L. thunbergianus*_19, T. Fujiwara 16040927). Scale bar 10 μ m

thunbergianus. The mitotic chromosome number of an *L. tosaensis* sample collected in Mie Pref. was $2n = 100$ ($4x$) (Fig. 3a, c). The chromosome number of *L. mikawanus* was $2n = 152$ ($6x$) (Table 1; Fig. 3b, d).

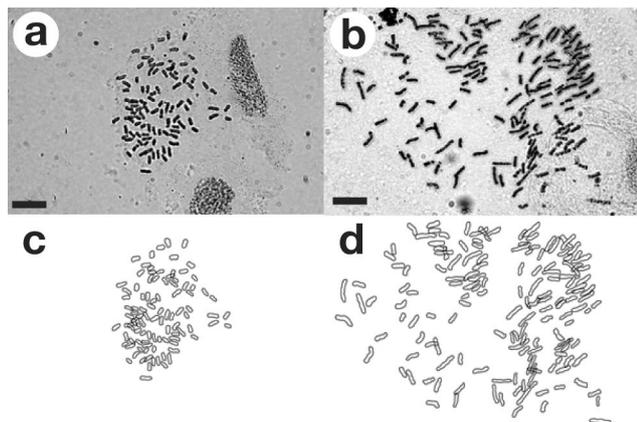
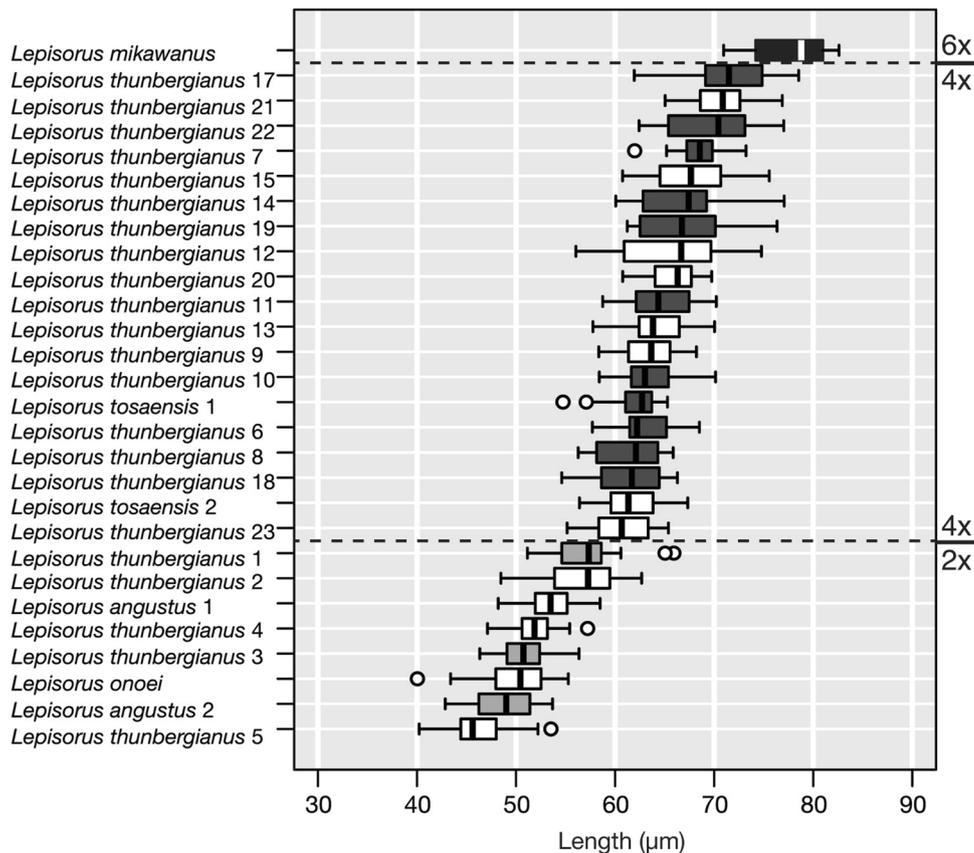


Fig. 3 Mitotic metaphase chromosomes of *Lepisorus tosaensis* and *L. mikawanus*. Chromosome counts, sample ID (if multiple samples were collected for the species) and voucher specimen in TNS are given in parentheses. **a, c** *Lepisorus tosaensis* ($2n = 100$, *L. tosaensis_1*, T. Fujiwara 16120210); **b, d** *L. mikawanus* ($2n = 152$, T. Fujiwara 16120817). Scale bar 10 μm

Fig. 4 Boxplots demonstrating the spore length of each specimen. In each boxplot, a thick line, edges of the box, whiskers, and circles indicate the median value, the lower and upper quartiles, the minimum and maximum values, and outliers, respectively. The samples of which ploidy levels were determined by chromosome counts or flow cytometry are shown by boxes with light gray (diploid), dark gray (tetraploid), or black color (hexaploid)



Spore size measurements were conducted on all samples except those which sporangia were too immature to examine (*L. hachijoensis* and *L. thunbergianus_16*). Figure 4 shows a clear correlation between chromosome number and spore size in the *L. thunbergianus* complex. Samples of diploid species, *L. angustus* and *L. onoei*, and $2n = 50$ ($2x$) samples of *L. thunbergianus* had smaller spores than the tetraploid sample of *L. tosaensis* and the $2n = 100$ and 102 ($4x$) samples of *L. thunbergianus*. The spores of hexaploid *L. mikawanus* were the largest among the samples examined. Based on the correspondence of ploidy levels and spore sizes, samples were divided into diploid, tetraploid and hexaploid groups (Fig. 4).

The 1C DNA amounts of the two diploid *L. thunbergianus* samples with $2n = 50$ were 7.9 and 8.0 pg (Table 1). One *L. angustus* sample with $2n = 52$ had a slightly smaller DNA amount (1C = 6.7 pg) than diploid *L. thunbergianus*. The 1C values of the *L. thunbergianus* samples assigned to the tetraploid group based on spore size (Fig. 4) ranged from 13.9 to 15.8 pg, consistent with the result of ploidy determination by spore size. The tetraploid sample of *L. tosaensis* that was found in the present study had a larger DNA amount (1C = 17.4 pg) than tetraploid *L. thunbergianus*. The hexaploid *L. mikawanus* sample had the largest DNA amount (1C = 20.6 pg).

cpDNA phylogeny

The *rbcL* and *rps4-trnS* sequence datasets consisted of 1134 nucleotide sites without gaps and of 995 nucleotide sites and 14 binary-coded indel sites, respectively (Table 2). GTR (for *rbcL*) and TPM1uf + G (for *rps4-trnS*) were selected as the best-fitting models. The ML tree for the combined dataset of *rbcL* and *rps4-trnS* is presented in Fig. 5. BI analysis of the combined dataset yielded a tree topology identical to that of ML.

All samples of Japanese *L. thunbergianus* and *L. mikawanus* formed the highly supported clade ‘Japanese *L. thunbergianus*’, with 100% maximum likelihood BS and 1.0 Bayesian posterior probability (PP). Within the clade, some tetraploid *L. thunbergianus* samples (red-shaded samples in Fig. 5) and *L. mikawanus* (yellow) formed a monophyletic group with weak support (BS = 58%, PP = 0.99). The haplotype sequence from the remaining tetraploid *L. thunbergianus* samples (green) was identical to one of the two haplotypes of diploid *L. thunbergianus* (light blue). All of the *L. tosaensis* samples, including the sequence deposited in INSD, formed the ‘*L. tosaensis*’ clade (BS = 79%, PP = 1.0). The haplotype of Chinese *L. angustus* (Z. H. Shen S25, Wang et al. 2010) was included in the clade sister to ‘Japanese *L. thunbergianus*’, in contrast to Japanese *L. angustus* (orange).

Nuclear gene phylogeny

The aligned dataset of *PgiC* consisted of 611 nucleotide sites and 32 binary-coded indel sites (Table 2). The ML and BI analyses yielded an identical tree topology. In the ML tree (Fig. 6), the *PgiC* sequences obtained from Japanese *L. thunbergianus* samples were grouped into two highly supported clades, ‘Japanese *L. thunbergianus*’ (BS = 99%, PP = 1.0) and ‘*L. tosaensis*’ (BS = 85%, PP = 1.0), and one branch named ‘Japanese *L. angustus*’. The samples of tetraploid *L. thunbergianus* in Japan can be classified into two variants, variant 1 (red-shaded samples) and variant 2 (green), based on their allele combinations. Each of the tetraploid *L.*

thunbergianus variant 1 individuals (*L. thunbergianus* 6–17) had two types of alleles; one was included in the ‘Japanese *L. thunbergianus*’ clade, and the other was identical to the allele of Japanese *L. angustus*. Each tetraploid *L. thunbergianus* variant 2 individual (*L. thunbergianus* 18–23) also had two alleles. Although one of the two alleles was included in the ‘Japanese *L. thunbergianus*’ clade, the other was in the ‘*L. tosaensis*’ clade, which included all of the alleles from the tetraploid *L. tosaensis* samples (deep blue). Hexaploid *L. mikawanus* had three alleles. The first allele belonged to the ‘Japanese *L. thunbergianus*’ clade, the second to the ‘*L. tosaensis*’ clade, and the third was identical to that of Japanese *L. angustus*.

Discussion

Ploidy of Japanese *Lepisorus thunbergianus* complex

In Japanese *Lepisorus thunbergianus*, many intraspecific cytotypes have been reported: $2n = 50, 51, 75, 76, 100, 101$ and 102 (Takamiya 1996). Nakato et al. (1983) concluded that the $2n = 50, 100$ and 102 cytotypes were stable strains, based on the observation of normal spores in these cytotypes. In this study, the only samples with normal spores were used and three cytotypes, $2n = 50, 100$ and 102 were observed in *L. thunbergianus*, consistent with the conclusion of Nakato et al. (1983). Wang et al. (2010) pointed out two types of basic chromosome number (BCN) in the genus *Lepisorus*: type I ($x = 35, 36, 37$) and type II ($x = 23, 25, 26$). Furthermore, they showed that the species with the BCN of $x = 23, 25, 26$ form a monophyletic group in their cpDNA tree. We newly obtained a mitotic chromosome number of $2n = 152$ ($6x$) for *L. mikawanus* (Fig. 3b, d). Therefore, all members of the *L. thunbergianus* complex proved to be in the group with $x = 23, 25, 26$. This suggests that candidates for parental diploid taxa contributed their genomes to the tetraploid cytotypes of *L. thunbergianus*, and hexaploid *L. mikawanus* would be confined to the group with the BCN of $x = 23, 25, 26$. Based on this finding, we also included

Table 2 Statistics and best-fitting model for each dataset used in phylogenetic analyses

Dataset	No. of sequences	Included sites	Variable sites	Model for Garli	Models for MrBayes
<i>rbcL</i>	57	1,134	64	GTR	GTR
<i>rps4-trnS</i>	57	1,009	111	partitioned	partitioned
Nucleotide sites		995	97	TPM1uf + G	SYM + G
Indel sites		14	14	Mkv	Mkv
cpDNA combined	57	2,143	175	Partitioned	Partitioned
<i>PgiC</i>	65	643	171	Partitioned	Partitioned
Nucleotide sites		611	139	GTR + I	GTR + I
Indel sites		32	32	Mkv	Mkv

Fig. 5 The maximum likelihood tree for the combined cpDNA dataset of *rbcL* and *rps4-trnS*. The thickest lines indicate strong support (BS \geq 70 and PP = 1.0), moderately thick lines indicate moderate support (BS \geq 70 and PP = 0.99), and thin lines indicate weak support (BS < 70 or BPP < 0.99). Support values are given as BS/PP above branches. The symbols before the sample IDs (Table 1) denote ploidy levels: triangle = diploid, square = tetraploid, and hexagon = hexaploid. The samples collected in this study are colored according to the legend given



the cpDNA and *PgiC* sequences of the *Lepisorus* species of $x = 23, 25, 26$ from previous studies (Wang et al. 2010, 2012) in our datasets.

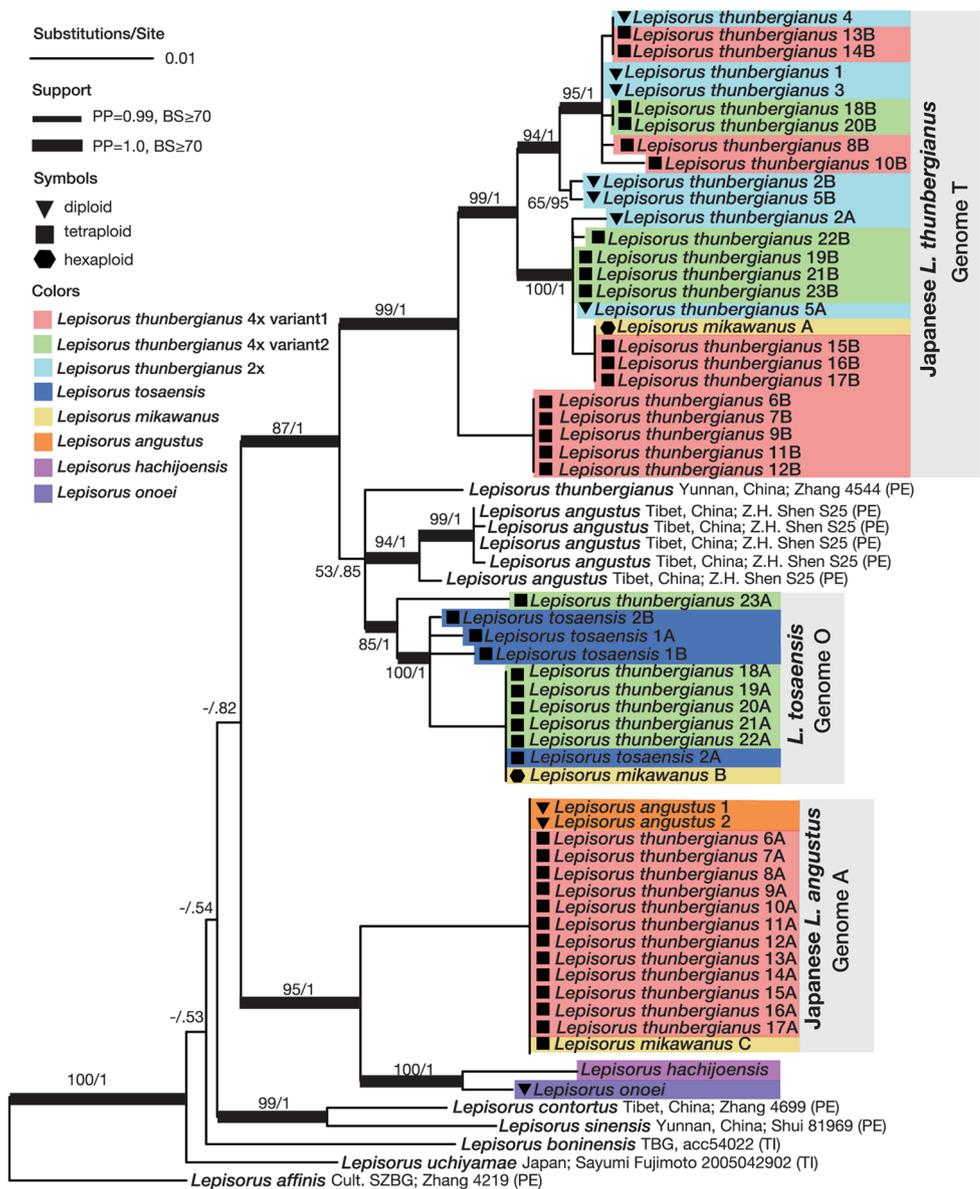
Two allotetraploid species of *L. thunbergianus* and their origins

The *PgiC* phylogeny (Fig. 6) revealed that each of the tetraploid *L. thunbergianus* samples had two distinct alleles involved in two different clades: the ‘Japanese *L. thunbergianus*’ clade and ‘Japanese *L. angustus*’ clade (variant 1: *L. thunbergianus*_6–17), or ‘Japanese *L. thunbergianus*’ clade and ‘*L. tosaensis*’ clade (variant 2: *L. thunbergianus*_18–23).

Because tetraploid *L. thunbergianus* samples do not have any *PgiC* alleles closely related to those of *L. onoei*, the involvement of *L. onoei* in the origin of tetraploid *L. thunbergianus* (Takei 1982) could be excluded.

Regarding the tetraploid *L. thunbergianus* variant 1, one *PgiC* allele was included in the ‘Japanese *L. thunbergianus*’ clade, and the other allele was identical to that of the Japanese *L. angustus* samples. In the ‘Japanese *L. thunbergianus*’ clade of *PgiC* phylogeny (Fig. 6), one allele from some samples of variant 1 (*L. thunbergianus*_6, 7, 9, 11 and 12) was not intermingled with the alleles of diploids and was positioned at the base of the clade. This might suggest that the allele was derived from the diploid species that

Fig. 6 The maximum likelihood tree of the single-copy nuclear gene, *PgiC*. The thickest lines indicate strong support (BS >= 70, PP = 1.0), moderately thick lines indicate moderate support (BS >= 70, PP = 0.99), and thin lines indicate weak support (BS < 70 or PP < 0.99). Support values are given as BS/PP above branches. The symbols before the sample IDs (Table 1) denote ploidy levels: triangle = diploid, square = tetraploid and hexagon = hexaploid. “A”, “B” and “C” at the end of sample names are given to distinguish multiple alleles detected in each individual (Table 1). The samples are shown with the same coloration pattern used in the cpDNA tree (Fig. 5)



is closely related to but different from Japanese *L. thunbergianus*. However, cpDNA phylogeny showed that the cpDNA haplotypes of *L. thunbergianus*_6, 7, 9, 11 and 12 were intermingled with those of the other variant 1 samples in the subclade of variant 1 and *L. mikawanus* (Fig. 5). Therefore, we concluded that tetraploid *L. thunbergianus* variant 1 is an allotetraploid that originated through allopolyploidization between diploid *L. thunbergianus* in Japan and Japanese *L. angustus* (Fig. 7). Given the maternal inheritance of cpDNA in ferns (Gastony and Yatskievych 1992; Vogel et al. 1998), diploid *L. thunbergianus* distributed in Japan is plausibly the maternal ancestor of the tetraploid *L. thunbergianus* variant 1. This hypothesized parentage supports the conclusion of Shinohara et al. (2010). The tetraploid *L. thunbergianus* variant 1 includes two cytotypes,

$2n=100$ and $2n=102$ (Table 1). The chromosome number of $2n=102$ can be explained by the combination of the chromosome numbers of the hypothesized parents: diploid *L. thunbergianus* ($2n=50$) in Japan and Japanese *L. angustus* ($2n=52$) (Table 1; Fig. 2a, b). On the other hand, the chromosome number of $2n=100$ appears to be inconsistent with the hypothesized parentage. As suggested by Shinohara et al. (2010), the cytotype of $2n=100$ may have originated secondarily from the cytotype of $2n=102$, possibly through aneuploid gamete formation. The stipe colors in most samples of tetraploid *L. thunbergianus* variant 1 were dark brown or blackish (Fig. 8). Based on this morphological feature, we concluded that this variant corresponds to *L. thunbergianus* ‘Blackish type’ in Serizawa and Aman (2012). It should be noted, however, that both of the

Fig. 7 Hypothesis of reticulate evolution in the *Lepisorus thunbergianus* complex in Japan. Solid lines indicate the nuclear DNA contribution, and the dotted lines indicate the cpDNA contribution

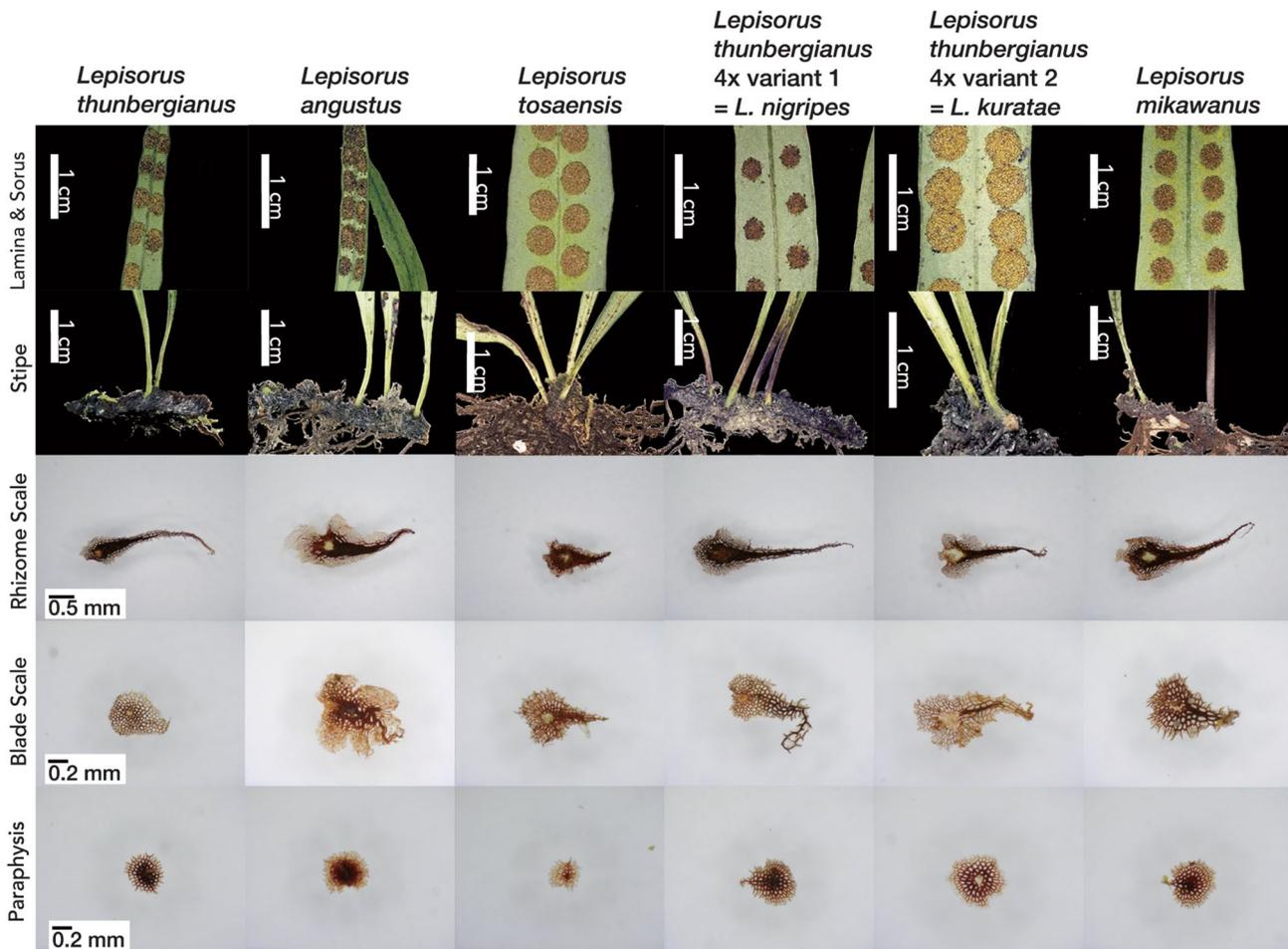
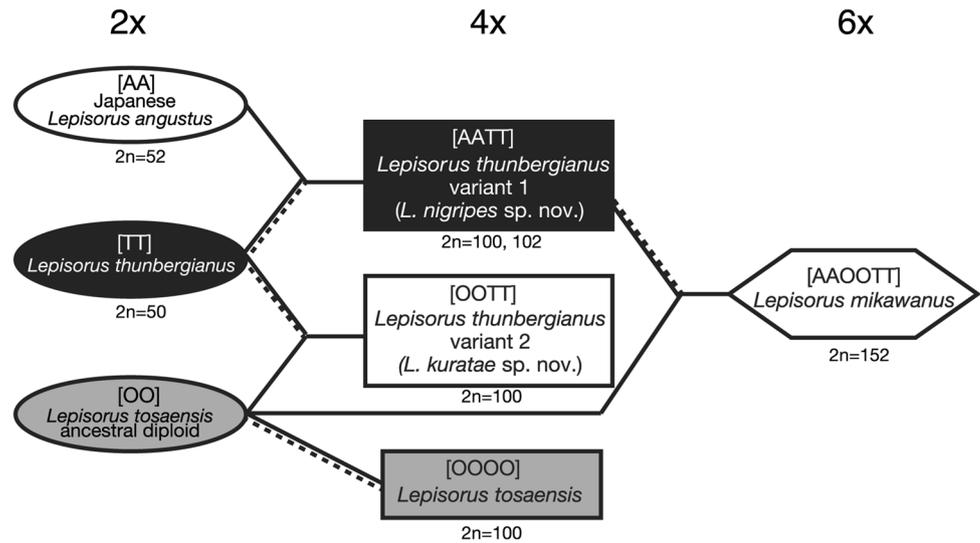


Fig. 8 Morphological characters of each *Lepisorus* species in this study. From left, diploid *L. thunbergianus* (*L. thunbergianus*_4, T. Fujiwara 1503173), *L. angustus* (*L. angustus*_2, T. Fujiwara 15111510), *L. tosaensis* (*L. tosaensis*_1, T. Fujiwara 16120210),

tetraploid *L. thunbergianus* variant 1 [= *L. nigripes*] (*L. thunbergianus*_12, T. Fujiwara 1604107), tetraploid *L. thunbergianus* variant 2 [= *L. kuratae*] (*L. thunbergianus*_20, T. Fujiwara 1502215), *L. mikawanus* (T. Fujiwara 16120817)

parental taxa of tetraploid *L. thunbergianus* variant 1 are taxonomically problematic. Although the non-monophyly of *L. thunbergianus* was pointed out by Wang et al. (2010), the present study showed that *L. angustus* is also not monophyletic; the cpDNA sequence of Japanese *L. angustus* was placed in the ‘Japanese *L. angustus*’ clade, while that of Chinese *L. angustus* was in a clade sister to the ‘Japanese *L. thunbergianus*’ clade (Fig. 5). The circumscriptions of both *L. thunbergianus* and *L. angustus* need to be revised in a future study.

The *PgiC* alleles of tetraploid *L. thunbergianus* variant 2 were located in two clades, the ‘Japanese *L. thunbergianus*’ clade and the ‘*L. tosaensis*’ clade (Fig. 6). The tetraploid cytotype of *L. tosaensis* is a new addition to this species, in which only $n=75$ ($6x$) has been reported so far (Mitui 1971). Because all alleles of the tetraploid *L. tosaensis* samples were included in the ‘*L. tosaensis*’ clade, it is suggested that the *L. tosaensis* samples examined in this study would have an autotetraploid origin. Therefore, tetraploid *L. thunbergianus* variant 2 is an allotetraploid between a diploid *L. thunbergianus* in Japan and an unknown diploid of *L. tosaensis* (Fig. 7). Based on the cpDNA phylogeny (Fig. 5), diploid *L. thunbergianus* distributed in Japan is the maternal ancestor of the tetraploid *L. thunbergianus* variant 2. This hypothesis is supported morphologically; *L. tosaensis* and the tetraploid *L. thunbergianus* variant 2 share some morphological features, thick and broad lanceolate lamina and caespitose leaves (Fig. 8). In addition to these morphological features, all samples of the tetraploid *L. thunbergianus* variant 2 have green-colored stipules. Therefore, this variant may correspond to *L. thunbergianus* ‘Green type’ in Serizawa and Aman (2012). As shown in Serizawa and Aman (2012), the 1C DNA amounts (15.4–15.8 pg) of tetraploid *L. thunbergianus* variant 2 (=tetraploid Green type) are higher than those (13.9–14.5 pg) of tetraploid *L. thunbergianus* variant 1 (=tetraploid Blackish type) (Table 1).

Shinohara et al. (2010) concluded that tetraploid *L. thunbergianus* is a segmental allopolyploid in which some sets of chromosomes behave as in an autopolyploid in meiosis, based on the fact that some tetraploid samples showed homozygosity for the allele from a single parental species at some of the six allozyme loci examined. In this study, however, each of the samples from both tetraploid *L. thunbergianus* variant 1 and variant 2 had two distinct *PgiC* alleles that were derived from different parent species and showed no indication of loss of homeologous loci. In order to test whether the two variants of the tetraploid *L. thunbergianus* are segmental allopolyploid, many more samples need to be analyzed using multiple nuclear gene markers.

Multiple origins of polyploidy species are common in the plant kingdom (Soltis et al. 2014; Symonds et al. 2010) and have also been reported in ferns (Beck et al. 2012; Perrie et al. 2010; Sigel et al. 2014; Werth et al. 1985). In the

present study, we also confirmed that both of the allotetraploid *Lepisorus* species contained genetic variation that was derived from independent allopolyploidization events between respective parental species pairs. With respect to tetraploid *L. thunbergianus* variant 1, its *PgiC* alleles (red-shaded ones) in the ‘Japanese *L. thunbergianus*’ clade were separated into three distinct groups, of which two include alleles from diploid *L. thunbergianus*. It seems likely that each of the three groups could reflect an independent allopolyploidization event (Fig. 6). For the same reason, the *PgiC* phylogeny suggests at least two independent origins for tetraploid *L. thunbergianus* variant 2.

Origins of hexaploid species, *L. mikawanus*

Lepisorus mikawanus Sa.Kurata was described in Kurata (1965) based on the sample collected in Aichi Pref., Japan. Unfortunately, however, the type locality was lost through estate development, and a detailed taxonomic study on this species was not conducted until Serizawa (2015) redescribed *L. mikawanus*.

In the present study, we showed that *L. mikawanus* is a hexaploid species with $2n=152$ (Fig. 3). In the *PgiC* phylogeny (Fig. 6), three alleles obtained from *L. mikawanus* were split into the ‘Japanese *L. thunbergianus*’ clade, the ‘*L. tosaensis*’ clade and the branch of ‘Japanese *L. angustus*’, suggesting that *L. mikawanus* has three different genomes. Based on the result, two scenarios for the origin of *L. mikawanus* are presented here. The first hypothesis is that *L. mikawanus* originated through allopolyploidization between Japanese *L. angustus* and tetraploid *L. thunbergianus* variant 2. The other hypothesis is that *L. mikawanus* has a hybrid origin between an unknown diploid race of *L. tosaensis* and tetraploid *L. thunbergianus* variant 1. The cpDNA phylogeny revealed that the sequence of *L. mikawanus* formed a clade together with those of tetraploid *L. thunbergianus* variant 1 (Fig. 5). Furthermore, *L. mikawanus* shares two *PgiC* alleles with some individuals of tetraploid *L. thunbergianus* variant 1 (*L. thunbergianus*_15, 16 and 17, Fig. 6). Therefore, the latter hypothesis that *L. mikawanus* originated from the hybrid between the diploid race of *L. tosaensis* and tetraploid *L. thunbergianus* variant 1 is more plausible (Fig. 7), although the diploid race of *L. tosaensis* remains unknown.

Taxonomy of allotetraploid species in Japanese *Lepisorus thunbergianus* complex

Lepisorus thunbergianus has been treated as a species complex containing diploid and tetraploid races (Ebihara 2017; Iwatsuki 1992). The present study revealed that these are morphologically distinguishable from each other and also from related species based on some diagnostic characters, such as lamina shape, stipule color and scales (Fig. 8). The

holotype of *L. thunbergianus* (Kaulf.) Ching (UPS-THUNB 24513; <http://cpthunberg.ebc.uu.se/specimens/24513>) that was collected in Japan can be identified as a diploid cytotype because it has more clustered leaves and a straw-colored stipe, compared with tetraploid *L. thunbergianus* variant 1, and narrower lanceolate laminae and a longer rhizome, compared with tetraploid *L. thunbergianus* variant 2. Thus, the plants of tetraploid *L. thunbergianus* variant 1 and variant 2 are described as belonging to new species, *L. nigripes* and *L. kuratae*, respectively, in the [taxonomic treatments](#) section.

Taxonomic treatments

Lepisorus kuratae T.Fujiw. et Seriz. sp. nov.

An allotetraploid species that originated from the hybridization between *L. thunbergianus* and the ancestral diploid race of *L. tosaensis*; differs from the former in having thinner laminae and dentate blade scales and from *L. tosaensis* in having thicker rhizomes, thicker laminae and medial sori.

Epiphytic perennials. Rhizomes shortly creeping, 2–3 mm in diameter; rhizome scales dark brown, lanceolate and with broad and transparent wings on both sides of the base, 2–4 mm long and 1 mm wide, dentate on the margin. Leaves clustered; stipes straw colored or greenish, 0.3–1 cm long and 1–1.2 mm in diameter; laminae simple, linear or narrowly lanceolate, 9–20 cm long and 0.6–1.5 cm wide, usually widest at the middle, light green, thick cartaceous or thin leathery; blade scales broadly lanceolate, dentate on the margin, acuminate at the apex. Sori medial, orbicular, suborbicular or oblong, 2.5–3 mm in diameter, sometimes confluent with the adjacent ones; paraphyses orbicular, brown or dark brown and clathrate, sometimes opaque in the central parts. Chromosome number $2n = 100$.

Japanese name: Fuji-nokisinobu.

Holotype: JAPAN, Osaka Pref, Kawachinagano-shi, Nagaredani, alt. ca. 300 m (T. Fujiwara no. 16040927, TNS, Fig. 9a–d, *L. thunbergianus*_23 in this study).

Distribution: Japan (Honshu, Shikoku).

Specimens examined: **JAPAN: Iwate Pref.**, Yasakae, Ichinoseki-shi, Jul. 25, 1980, *T. waku n.s.* (TNS404301); **Fukushima Pref.**, Sanyuzan, Nihonmatsu-shi, Sep. 14, 2008, *S. Watanabe 4883* (TNS01256253); **Tochigi Pref.**, Tanuma-machi, Sano-shi, Jun. 4, 1978, *T. Waku n.s.* (TNS331179); **Gunma Pref.**, Itakura-machi, Oura-gun, Jul. 12, 1983, *S. Waku n.s.* (TNS595390); **Saitama Pref.**, Niikura, Wako-shi, Sep. 15, 1980, *T. Waku 10261* (TNS413304); **Chiba Pref.**, Higashimatsuzaki, Katori-shi, Jan. 12, 1980, *Y. Koike n.s.* (TNS385969); **Tokyo Pref.**, Itsukaichi, Akiruno-shi, May. 6, 1978, *K. Yoshizawa 69141* (TNS385969); **Kanagawa Pref.**, Kojiri, Hakone-shi, Apr. 20, 1980, *A. Sato n.s.* (TNS405652); **Gifu Pref.**, Kuze-mura, Ibi-gun, Jan. 17, 1979, *M. Muramatsu 79–767*

(TNS01248198); **Shizuoka Pref.**, Mt. Fuji, Shizuoka-shi, Jul. 10, 1986, *J. Haginiwa JH014076* (TNS964076); **Aichi Pref.**, Ishihara-cho, Okazaki-shi, Feb. 10, 1980, *K. Inukai 2983* (TNS387179); **Shiga Pref.**, Shigaraki-cho, Kouga-shi, Feb. 14, 1977, *A. Yamamoto n.s.* (TNS409113); **Kyoto Pref.**, Minamiyamashiro-mura, Souraku-gun, Jun. 10, 1979, *Y. Kimizuka n.s.* (TNS396025); **Nara Pref.**, Shimoichi-cho, Yoshino-gun, Nov. 11, 2015, *F. Kasetani 1099-5* (TNS01256286); **Wakayama Pref.**, Hatsushima-machi, Arita-shi, Jan. 16, 1977, *H. Masago n.s.* (TNS416715); **Kagawa Pref.**, Nishitani, Okawa-gun, Nov. 10, 1974, *Y. Koike n.s.* (TNS416850); **Ehime Pref.**, Uchiko-cho, Kita-gun, Dec. 25, 1981, *A. Minami 27430* (TNS01233840).

Lepisorus nigripes T.Fujiw. et Seriz. sp. nov.

An allotetraploid species that originated from the hybridization between *L. thunbergianus* and the Japanese *L. angustus*; differs from the former in having rather distant leaves and longer and usually blackish stipes and from the latter in having thicker rhizomes, lanceolate and narrowly acuminate rhizome scales and broader lamina.

Epiphytic perennials Rhizomes rather widely creeping, 2–3 mm in diameter; rhizome scales dark brown, lanceolate and with narrow transparent wings on both sides of the base, 3–5 mm long and about 1 mm wide, slightly dentate on the margin. Leaves rather distant; stipes dark brown or rarely straw colored, 0.5–4 cm long and 1–1.5 mm in diameter; laminae simple, linear or narrowly lanceolate, 7.5–24 cm long and 0.5–1.2 cm wide, usually widest at or above the middle, dark green, leathery or thick leathery; blade scales lanceolate, dentate on the margin, acuminate at the apex, darkened in the apical parts. Sori medial, orbicular or suborbicular, 1–2.5 mm in diameter; paraphyses orbicular or suborbicular, dark brown and clathrate, opaque in the central parts. Chromosome number $2n = 100, 101$ or 102 .

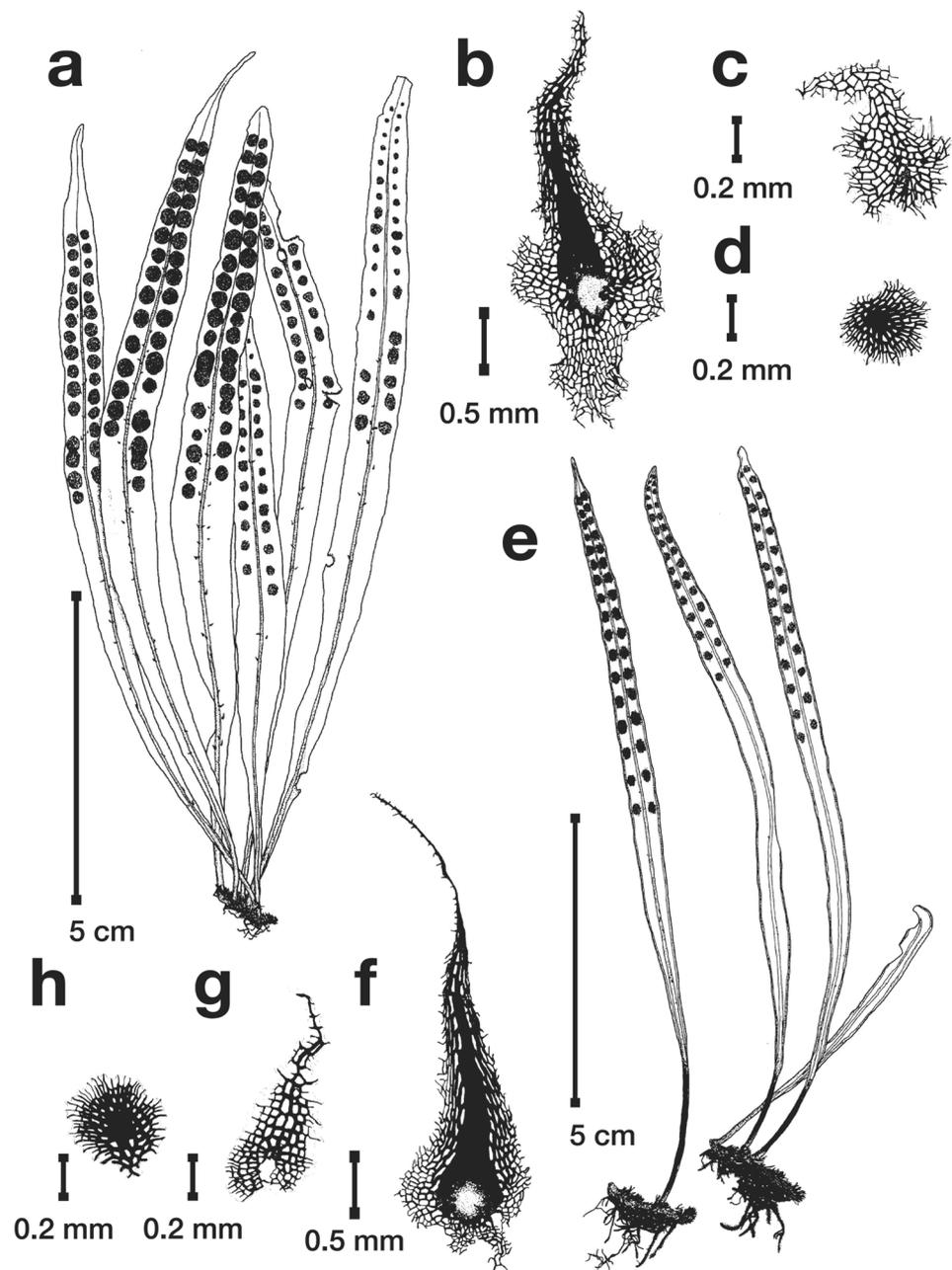
Japanese name: Kuro-nokisinobu.

Holotype: JAPAN, Yamanashi Pref., Hokuto-shi, Nagasaki, alt. ca. 690 m (T. Fujiwara no. 16040605, TNS, Fig. 9e–h, *L. thunbergianus*_11 of this study, $2n = 102$).

Distribution: Japan (Hokkaido, Honshu, Shikoku, Kyushu) and the Ryukhus (Isl. Yaku).

Specimens examined: **JAPAN: Hokkaido Pref.**, Asabu-cho, Jul. 3, 1978, *M. Hara n.s.* (TNS399395); **Aomori Pref.**, Takaiwa, Hachinohe-shi, Jul. 12, 2012, *S. Fujinami SF20120712-4* (TNS01167818); **Yamagata Pref.**, Kanayama, Mogami-gun, Oct. 7, 1975, *N. Kato n.s.* (TNS396011); **Fukushima Pref.**, Koshioe, Sudagawa-shi, Jul. 24, 1979, *T. Okubo n.s.* (TNS398603); **Miyagi Pref.**, Miya-machi, Shiogama-shi, Aug. 22, 1978, *M. Takeuchi n.s.* (TNS409615); **Tochigi Pref.**, Mt. Sano, Sano-shi, Sep. 5, 1980, *T. Tashiro n.s.* (TNS936338); **Gunma Pref.**, Onoko, Shibukawa-shi, Sep. 2, 1985, *S. Waku n.s.* (TNS01246629); **Saitama Pref.**,

Fig. 9 **a–d** *Lepisorus kuratae* T.Fujiw. et Seriz (T. Fujiwara 16040927). **a** Habit; **b** Rhizome scale; **c** Blade scale; **d** Paraphysis. **e–h** *Lepisorus nigripes* T.Fujiw. et Seriz (T. Fujiwara 16040605). **e** Habit; **f** Rhizome scale; **g** Blade scale; **h** Paraphysis



Higashichichibu, Chichibu-gun, Jul. 10, 1988, *T. Iwata* 8604 (TNS01150527); **Tokyo Pref.**, Nippara, Okutama-shi, Nov. 18, 1978, *H. Kobayashi n.s.* (TNS399076), **Ishikawa Pref.**, Tsuguu, Hakusan-shi, Nov. 23, 1978, *M. Hashimoto* 6677 (TNS272494), **Yamanashi Pref.**, Mt. Kushigata, Minamikoma-gujn, Jul. 15, 1979, *Y. Nagasawa n.s.* (TNS415697); **Gifu Pref.**, Natsuyake, Gero-shi, Nov. 17, 1978, *Y. Yamamoto n.s.* (TNS405124); **Shizuoka Pref.**, Haruno, Hamamatsu-shi, Jun. 6, 1978, *I. Yamashita n.s.* (TNS405124); **Aichi Pref.**, Nohara, Toyota-shi, Nov. 22, 1979, *K. Inukai* 2607 (TNS413300); **Mie Pref.**, Kabuto, Kameyama-shi, Oct. 8, 1978, *S. Yamauchi n.s.* (TNS404331); **Kyoto Pref.**,

Shino-cho, kameoka-shi, Jul. 12, 1992, *S. Tsugaru & M. Sawada* 165507 (TNS597656); **Wakayama Pref.**, Shirahama, Nishimuro-gun, Aug. 14, 1971, *H. Masago n.s.* (TNS416746); **Okayama Pref.**, Toyonaga-akouma, Niimi-shi, Oct. 2, 1977, *T. Watanabe n.s.* (TNS399013); **Tokushima Pref.**, Iyakei, Miyoshi-shi, Jun. 17, 1979, *T. Nakamura n.s.* (TNS406898); **Kagawa Pref.**, Mt. Otaki, Takamatsu-shi, Jun. 23, 1974, *S. Mitani n.s.* (TNS416851), **Ehime Pref.**, Uwa-cho, Seiyo-shi, Mar. 5, 1978, *M. Hyoudo n.s.* (TNS413806), **Kumamoto Pref.**, Kukiko, Minamata-shi, Feb. 14, 1965, *M. Shiroto n.s.* (TNS410959); **Kagoshima Pref.**, Yaku Is., Jul. 27, 1961, *H. Ito n.s.* (TNS01044064).

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