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Article

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Thelypteris sylva-nipponica, a new allotetraploid species in the *Parathelypteris* group (Thelypteridaceae)

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Abstract

A broad-leaved form of *Thelypteris nipponica*, usually called "var. *borealis*", was identified as an allotetraploid originated from hybridization between two diploid species *T. nipponica* and *T. musashiensis* based on chromosome number, plastid *rbcL* sequence and nuclear *gapCp* "short" sequence. We found that the original material of *Thelypteris nipponica* var. *borealis* morphologically corresponds not to the plant widely known as "var. *borealis*" but to diploid *T. nipponica*. Therefore, we described the tetraploid distributed in Japan and China as a new species, *Thelypteris sylva-nipponica*.

Keywords: lectotype, new species

Introduction

Thelypteris nipponica var. *borealis* (Hara 1934: 695) Hara (1938: 621) (hereafter called "var. *borealis*") is a taxon recognized as a northerly distributed variety of *T. nipponica* (Franchet & Savatier 1879: 646) (Iwatsuki *et al.* 1995), distinguished by the absence of ciliate hair on soral indusia (Hara 1934). Hara originally noticed the taxon during his study on the flora of Mt. Apoi in southern Hokkaido, Japan. It is distributed in the northeastern part of Japan (Hokkaido and northeastern Honshu, Iwatsuki *et al.* 1995) and China (Lin *et al.* 2013) according to the recent flora. It is often distinguished from *T. nipponica* var. *nipponica,* which has lanceolate lamina, by its broader (i.e. deltate-oblong) lamina without distinctly reduced basal pinnae, rather than by the character of its indusial hair which tends to be invisible due to shrinkage of indusia in mid-summer to autumn (A. Ebihara, *pers. obs.*). On the other hand, individuals of "var. *borealis*" closely resemble both *T. musashiensis* (Hiyama 1951: 155) Nakato, Sahashi & Kato (2004: 103) and *T. japonica* f. *formosa* (Christensen 1934: 86) Nakato, Sahashi & Kato (2004: 102). We aim to clarify the entire picture of "var. *borealis*" based on both cytotaxonomic information and DNA sequences using material collected mostly in Japan.

Thelypteris nipponica including "var. *borealis*" is sometimes classified into the genus *Parathelypteris* together with *T. musashiensis* and *T. japonica* (Baker 1891: 318) Ching (1936: 312) when we adopt the system by Holttum (1971) or Pteridophyte Phylogeny Group (2016). However, *Parathelypteris* is evidently non-monophyletic (Fawcatt 2018; Patel *&* *al.* 2019; Pteridophyte Phylogeny Group 2016), and *T. nipponica* and its closely related species (e.g. *T. castanea* (Tagawa 1935: 132) Ching (1936: 315), *T. japonica* and *T. musashiensis*) belong to an independent clade from *T. glanduligera* (Kunze 1837: 44) Ching (1936: 320), the type species of the genus. Although several globally sampled phylogenetic analyses (e.g. Almeida *et al.* 2015; He *et al.* 2012) suggested a sister and close relationship between *Thelypteris glanduligera* and *T. nipponica*, the results are likely artifacts due to misidentified material they employed. The phylogenetic tree based on eleven plastid and nuclear loci (Fawcatt 2018) suggested that *Parathelypteris* species including *Thelypteris*. *castanea*, *T. chinensis* (Ching 1936: 311), *T. japonica*, *T. musashiensis* and *T. nipponica* merged

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into the *Coryphopteris* clade. We, thus, tentatively adopt *Thelypteris* in a broader sense, considering a generic revision of the family is still awaiting publication (Fawcett 2020).

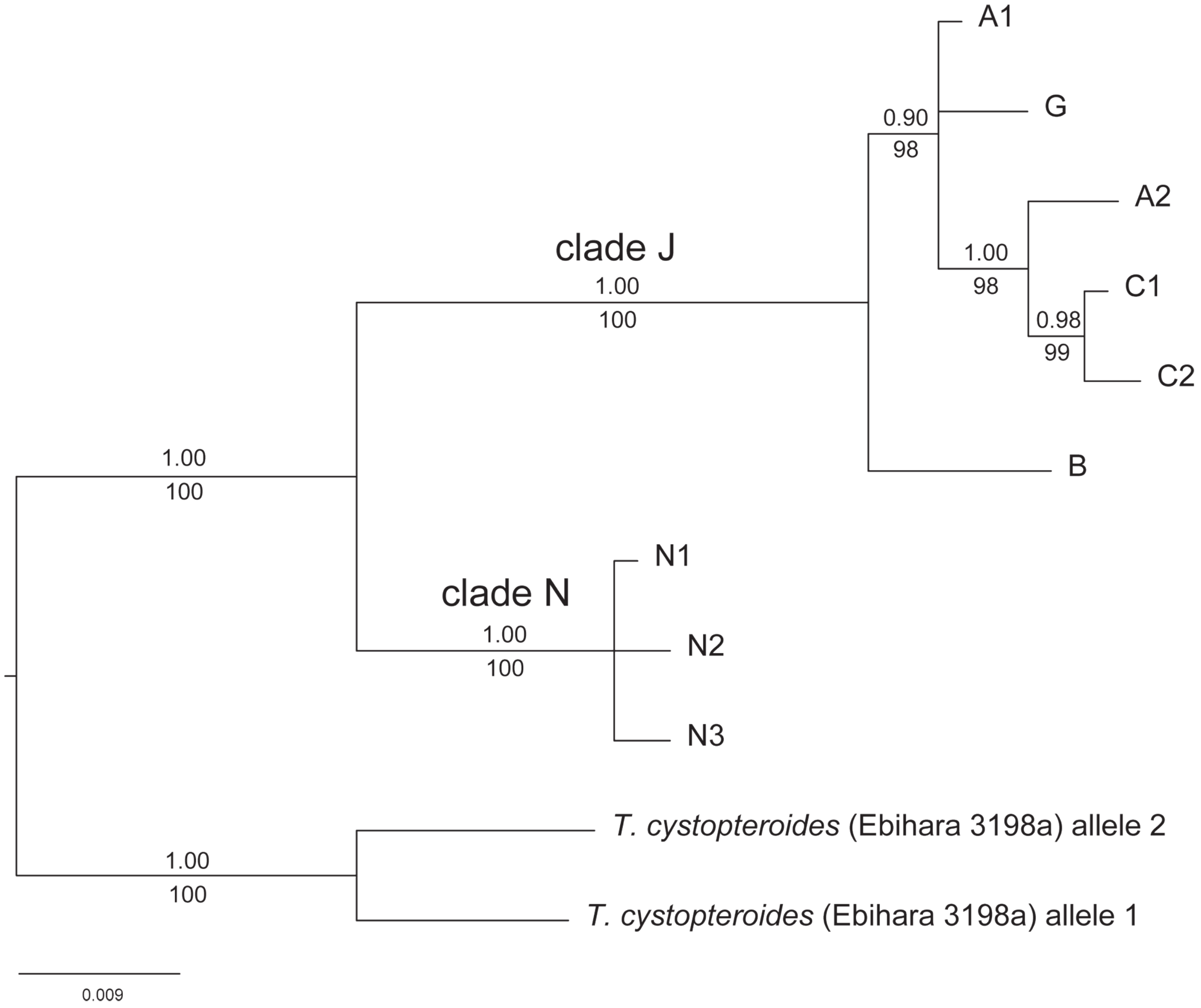

FIGURE 1. A majority-rule consensus tree of nuclear *gapCp*Sh region of *Thelypteris nipponica* and related species inferred by the Bayesian method rooted with *T. cystopteroides*. Each OUT (A1, A2, B, C1, C2, G, N1, N2 and N3) corresponds to an allele identified/isolated from single or multiple samples, which appears in Table 2. Support values of nodes: posterior probabilities of Bayesian inference / bootstrap values (%) in maximum likelihood analysis (>50% are shown).

Material & methods

Materials

A total of 13 living materials of *T. nipponica* var. *nipponica*, "var. *borealis*" and their intermediate forms were collected from six distantly separated areas in northern and central Honshu, Japan (Table 1). The *Parathelypteris* species included in the same clade (e.g. *T. castanea*, *T. japonica*, *T. musashiensis* and their putative hybrids) were added (Table 1). DNA samples of those species collected for our DNA barcoding project (Ebihara *et al.* 2010) were also used in the present study. *Thelypteris cystopteroides* (Eaton 1858: 110) Ching (1936: 316) belonging to the *Parathelypteris* s.s. clade was used as an outgroup taxon for phylogenetic analyses. Voucher specimens were deposited in the herbarium of National Museum of Nature and Science (TNS).

Chromosome and spore observation

Chromosome number was counted in the metaphase of root tip cells following the methods by Ebihara *et al.* (2014) with an Olympus DF microscope. Shapes of the spores collected from fresh leaves or voucher specimens of our material were observed under the microscope. Spores were mounted in Bioleit (Ohken Co., Tokyo), and shape regularity and ornamentation were observed in all the samples with mature spores. Spore size (the length along the long axis excluding perispore) was measured in 20 spores derived from multiple sporangia per specimen for *T. nipponica* var. *nipponica* and "var. *borealis*", and mean and standard deviation (SD) were calculated for each sample.

TABLE 1. Materials used for chromosome observation and DNA analyses in this study.

Sample name	Voucher specimen No. [TNS VS-]	Locality
Thelypteris nipponica var. *nipponica*		
S. Fujimoto et al. NN2915	1193086	Japan: Tochigi Pref., Nikko-shi
S. Fujimoto et al. NN2916	1193081	Japan: Tochigi Pref., Nikko-shi
S. Fujimoto et al. NN2904	1193085	Japan: Nagano Pref., Omachi-shi
S. Fujimoto et al. NN2906	1193087	Japan: Nagano Pref., Kitasaku-gun, Tateshina-machi
M. Muramatsu NN2908	1193138	Japan: Nagano Pref., Shimoina-gun. Anan-cho
Thelypteris nipponica var. *borealis*		
A. Ebihara 3325	1193131	Japan: Akita Pref., Yurihonjo-shi
A. Ebihara 3326	1193130	Japan: Akita Pref., Yurihonjo-shi
A. Ebihara 3600	1286249	Japan: Miyagi Pref., Kami-gun, Kami-cho
A. Ebihara 3601	1286250	Japan: Miyagi Pref., Kami-gun, Kami-cho
T. Narushima AE1975	776977	Japan: Yamanashi Pref., Minamitsuru-gun, Narusawa-mura
T. Oka AE1951	776388	Japan: Nagano Pref., Matsumoto-shi
M. Muramatsu NN2912a	1193134	Japan: Nagano Pref., Shimoina-gun. Anan-cho
M. Muramatsu NN2913	1193133	Japan: Nagano Pref., Shimoina-gun. Anan-cho
Y. Saito NN2919	1193077	Japan: Fukui Pref., Katsuyama-shi
Y. Saito NN2921	1193080	Japan: Fukui Pref., Katsuyama-shi
Y. Saito NN2922	1193075	Japan: Fukui Pref., Katsuyama-shi
Y. Saito NN2924	1193070	Japan: Fukui Pref., Katsuyama-shi
Y. Saito NN2926	1193076	Japan: Fukui Pref., Katsuyama-shi
Y. Saito NN2927	1193073	Japan: Fukui Pref., Katsuyama-shi
Thelypteris nipponica var. *nipponica* × *T. nipponica* var. *borealis*		
S. Fujimoto NN2905	1193083	Japan: Nagano Pref., Omachi-shi
S. Fujimoto 070907-2	766632	Japan: Nagano Pref., Kamiminochi-gun, Togakushi-mura
Thelypteris musashiensis		
A. Ebihara et al. 1933	776398	Japan: Saitama Pref., Chichibu-shi
H. Hatta & S. Matsumoto s.n.	1176796	Japan: Tokyo Pref., Mikurajima Isl., cultivated in Tsukuba Botanical Garden [TBG stock No. 15445]
N. Nakato 2863	1193065	Japan: Tokyo Pref., Nishitama-gun, Okutama-machi
N. Nakato 2866	1193068	Japan: Tokyo Pref., Nishitama-gun, Okutama-machi
Thelypteris japonica		
N. Nakato 2850	1176853	Japan: Tokyo Pref., Higashiyamato-shi
N. Nakato 2851	1176855	Japan: Tokyo Pref., Ome-shi
S. Fujimoto 080811-2	776368	Japan: Tokyo Pref., Hachioji-shi
Y. Saito NN2930	1193074	Japan: Fukui Pref., Katsuyama-shi
S.-J. Lin et al. WY2006-14	762119-762120	China: Fujian Prov.

......continued on the next page

TABLE 1 (Continued)

Sample name	Voucher specimen No. [TNS VS-]	Locality
Thelypteris japonica ｆ. *formosa*		
S. Fujimoto s.n.	764365	Japan: Mie Pref., Minamimuro-gun, Kiho-cho [TBG stock No. 144731]
Thelypteris japonica × *T. musashiensis*		
S. Matsumoto s.n.	763917	Japan: Ibaraki Pref., Tsukuba-shi [TBG stock No. 79149]
N. Nakato 2862	1193062	Japan: Tokyo Pref., Nishitama-gun, Okutama-machi
M. Muramatsu NN2909	1193137	Japan: Nagano Pref., Shimoina-gun. Anan-cho
M. Muramatsu NN2910	1193136	Japan: Nagano Pref., Shimoina-gun. Anan-cho
Thelypteris japonica × *T. nipponica* var. *borealis*		
M. Muramatsu NN2914	1193132	Japan: Nagano Pref., Shimoina-gun. Anan-cho
Thelypteris musashiensis × *T. nipponica* var. *borealis*		
Y. Saito NN2917	1193079	Japan: Fukui Pref., Katsuyama-shi
Y. Saito NN2925	1193072	Japan: Fukui Pref., Katsuyama-shi
Thelypteris castanea		
A. Ebihara et al.060930-01	759279	Japan: Okinawa Pref., Iriomote Isl.
Thelypteris cystopteroides (outgroup taxon)		
A. Ebihara 3198a	1209664	Japan: Kagoshima Pref., Amami-oshima Isl.

DNA analyses

DNA extraction was performed using DNeasy Plant Mini Kit (Qiagen) following the provided protocol. 1205 bp of plastid *rbcL* sequences were used as a marker of maternal lineage, and the method of amplification and sequencing followed those in Ebihara *et al.* (2010). For a biparently inherited DNA marker, we used nuclear *gapCp* "short" region ("*gapCp*Sh"). Approximately 700 bp fragments of the *gapCp*Sh region including introns were amplified with two primers (SgGapCp8.2F: 5'-GAGTGCCTACTCCCAATGTTTCTG-3`, and ESGAPCP11R1, Schuettpelz *et al.* 2008). The sequence polymorphism of the *gapCp*Sh region was detected and isolated by Single Strand Conformation Polymorphism (SSCP) method under the following electrophoresis condition: 15℃, 12h, 350V, MDE (Cambrex, USA) gel containing 2% glycerol). Methods of the protocols of SSCP analyses and succeeding DNA sequencing generally followed those in Ebihara *et al.* (2005, 2019). Obtained sequences were aligned with MUSCLE (Edgar 2004), and the sites with indels were removed. An appropriate substitution model was selected using MrModeltest2 (Nylander 2004). The posterior probability of Bayesian inference was determined using MrBayes 3.2.7a (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Two simultaneous runs involved four chains (10000000 generations each), in which each chain was sampled every 1000 generations. The first 25% of a sample was discarded as burn-in, and the remainder was used to calculate the 50% majority-rule consensus tree. RAxML 8.2.10 (Stamatakis 2014) was used to reconstruct the maximum likelihood (ML) phylogeny, and 1000 replicates were run for calculating bootstrap values.

Results

Chromosome and spore observation

Chromosome numbers $2n = 64$ (diploid) and $2n = 126$ (tetraploid) were observed in *T. nipponica* var. *nipponica* and in morphologically typical individuals of "var. *borealis*", respectively (Fig. 2, Table 2). These individuals produced normal regular spores with alate perispore (corresponding to "membranous" in Nakato *et al.* 2004), and the sizes tend to be slightly larger in "var. *borealis*" than in var. *nipponica* (Table 2). Four individuals growing near *T. nipponica* var. *nipponica* and/or "var. *borealis*" were observed to produce irregular-shaped spores and showed triploid chromosome number, 2*n* = 94 or 95. The sample "*M. Muramatsu NN2914*" sympatrically growing with "var. *borealis*" showed tetraploid chromosome number, 2*n* = 125, and produced irregular spores. We also observed chromosome numbers 2*n* = 124, 2*n* = 62, 2*n* = 93 in *T. japonica*, *T. musashiensis* and *T. japonica* × *T. musashiensis*, respectively (Fig. 2, Table 2). These counts matched those reported by a previous study (Nakato *et al.* 2004). Approximately 64 or at least more than 32 spores were observed per sporangium except in the individuals producing irregular spores.

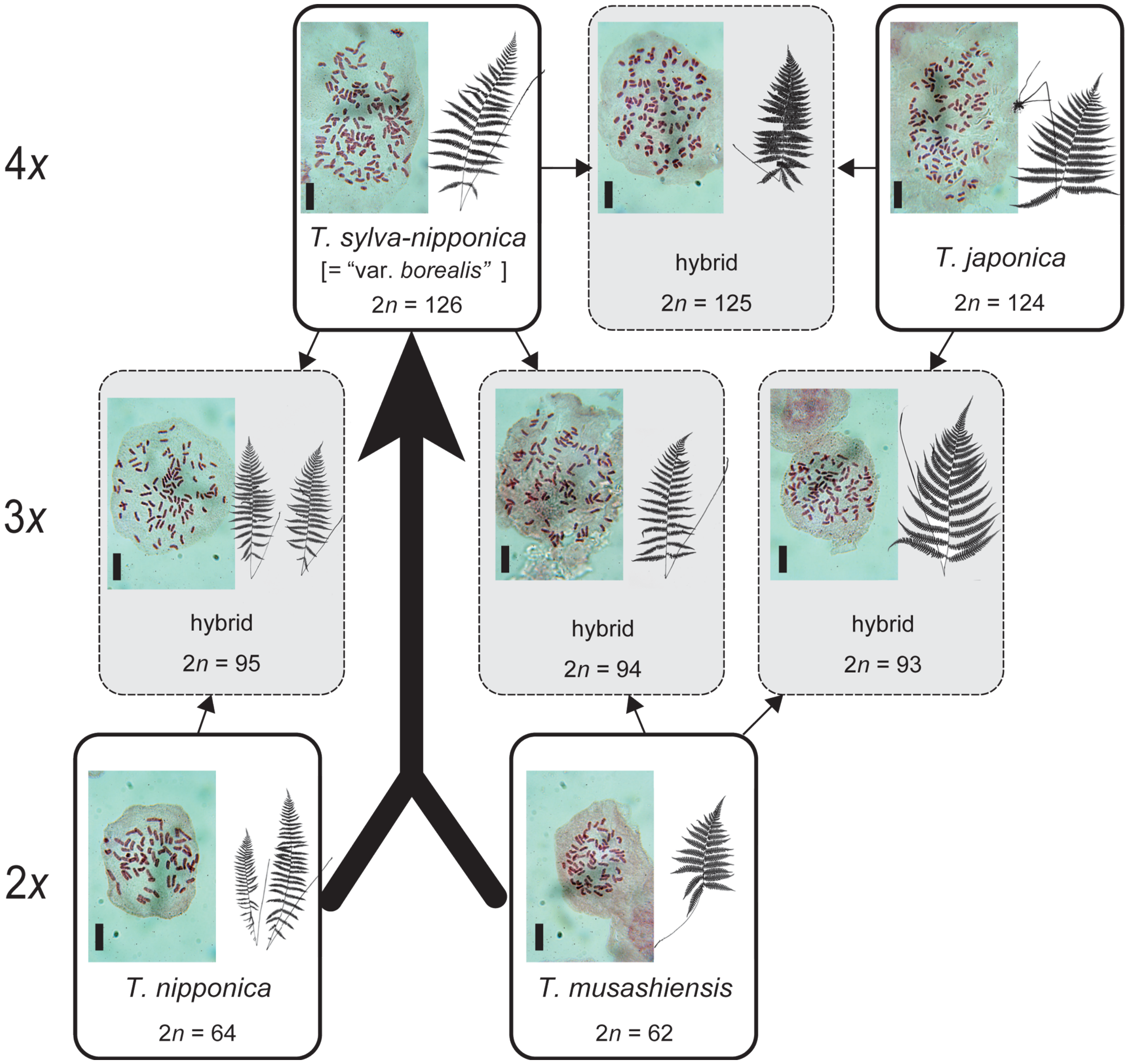

FIGURE 2. An explanatory figure of reticulate relationships of *Thelypteris nipponica* and related species. Microphotographs of chromosomes were taken in the following samples—*T. japonica*: *Nakato 2850*; *T. musashiensis*: *Nakato 2863*; *T. nipponica*: *Fujimoto et al. NN2915*; *T. sylva-nipponica*: *Saito NN2922*; *T. japonica* × *T. musashiensis*: *Nakato 2862*; *T. japonica* × *T. sylva-nipponica*: *Muramatsu NN2914*; *T. nipponica* × *T. sylva-nipponica*: *Fujimoto NN2905*; *T. musashiensis* × *T. sylva-nipponica*: *Saito NN2917.* Scale bar = 10 µm.

Plastid *rbcL* sequences

A single *rbcL* haplotype each was found in diploid *T. nipponica* var. *nipponica* (haplotype n1) and tetraploid "var. *borealis*" (haplotype n2, Table 2), and they differ from each other by one base pair. We obtained the same sequences as those deposited by our previous study (Ebihara *et al.* 2010, Ebihara & Nitta 2019) in *T. japonica* (haplotype a) and *T. musashiensis* (haplotype c), and putative hybrid individuals between the two species always showed the haplotype c. Haplotypes of other individuals which produce irregular spores varied from haplotype n1, n2 to c.

FIGURE 3. Lectotype of *Thelypteris nipponica* var. *borealis* deposited in TI. A. Whole the sheet. Scale bar = 5 cm. B. A ciliate hair on an indusium. Scale bar = 100 µm. C. Spores in a sporangium. Scale bar = 50 µm.

Nuclear gapCpSh *sequences*

A majority of the samples showed two or more alleles in the nuclear *gapCp*Sh region (Table 2). Two major clades were found in the phylogenetic tree based on isolated sequences named "N" and "J" (Fig. 1). *Thelypteris nipponica* var. *nipponica* is included in clade N, while *T. japonica*, *T. musashiensis* and *T. castanea* are in clade J. "Var. *borealis*" and the triploid sample possess two or more alleles belonging to both N and J clades.

TABLE 2. A summary of results of spore morphology, chromosome count, plastid *rbcL* haplotypes and *gapCp*Sh genotypes.

Voucher specimen	Spore shape	Spore ornamentation	Spore size mean (SD) [µm]	Chromosome number	Ploidy level	*rbcL* haplotype	*gapCp*Sh allele	*gapCp*Sh genotype
Thelypteris nipponica var. *nipponica*								
S. Fujimoto et al. NN2915	N/A	N/A		$2n = 64$	$2x$	n1	N1/N2	N1N2
S. Fujimoto et al. NN2916	Normal	Alate	41.1 (2.6)	$2n = 64$	$2x$	n1	N1	N1N1
S. Fujimoto et al. NN2904	N/A	N/A		-	-	n1	N1	N1N1
S. Fujimoto et al. NN2906	Normal	Alate	40.9 (2.9)	-	-	n1	N3	N3N3
M. Muramatsu NN2908	Normal	Alate	42.5 (2.4)	$2n = 64$	$2x$	n1	N1/N2	N1N2
Thelypteris nipponica var. *borealis*								
A. Ebihara 3325	N/A	N/A		-	-	n2	C1/N3	C1C1N3N3
A. Ebihara 3326	Normal	Alate		$2n = 126$	$4x$	n2	C1/N3	C1C1N3N3
A. Ebihara 3600	Normal	Alate		$2n = 126$	$4x$	n2	C1/N3	C1C1N3N3
A. Ebihara 3601	Immature	N/A		$2n = 126$	$4x$	n2	C1/N3	C1C1N3N3
T. Narushima AE1975	Immature (normal)	N/A		-	-	n2	C1/N3	C1C1N3N3
T. Oka AE1951	Immature	N/A		-	-	n2	C1/N3	C1C1N3N3
M. Muramatsu NN2912a	N/A	N/A		$2n = 126$	$4x$	n2	C1/N3	C1C1N3N3
M. Muramatsu NN2913	Normal	Alate	48.8 (2.6)	$2n = 126$	$4x$	n2	C1/N3	C1C1N3N3
Y. Saito NN2919	Normal	Alate	44.6 (1.8)	$2n = 126$	$4x$	n2	C1/N3	C1C1N3N3
Y. Saito NN2921	Normal	Alate	46.6 (2.4)	$2n = 126$	$4x$	n2	C1/N3	C1C1N3N3
Y. Saito NN2922	Immature	N/A		$2n = 126$	$4x$	n2	C1/N3	C1C1N3N3
Y. Saito NN2924	Normal	Alate	47.9 (3.0)	$2n = 126$	$4x$	n2	C1/N3	C1C1N3N3
Y. Saito NN2926	Normal	Alate	46.4 (1.5)	$2n = 126$	$4x$	n2	C1/N3	C1C1N3N3
Y. Saito NN2927	N/A	N/A		$2n = 126$	$4x$	n2	C1/N3	C1C1N3N3
Thelypteris nipponica var. *nipponica* × *T. nipponica* var. *borealis*								
S. Fujimoto NN2905	Irregular	N/A		$2n = 95$	$3x$	n1	N3	N3N3
S. Fujimoto 070907-2	Irregular	N/A		-	-	n2	C1/N3	C1N3N3
Thelypteris musashiensis								
A. Ebihara et al. 1933	Normal	Echinate		-	-	c	C1	C1C1
H. Hatta & S. Matsumoto s.n.	Normal	Echinate		-	-	c	C1/C2	C1C2
N. Nakato 2863	Normal	Echinate		$2n = 62$	$2x$	c	C1	C1C1
N. Nakato 2866	Normal	Echinate		$2n = 62$	$2x$	c	C1	C1C1
Thelypteris japonica								
N. Nakato 2850	Normal	Alate		$2n = 124$	$4x$	a	A1/B	A1A1BB
N. Nakato 2851	Normal	Alate		$2n = 124$	$4x$	a	A1/B	A1A1BB
S. Fujimoto 080811-2	Normal	Alate		-	-	a	A1/B	A1A1BB
Y. Saito NN2930	Normal	Alate		-	-	a	A1/B	A1A1BB
S.-J. Lin et al. WY2006-14	Normal	Alate		-	-	a	A1/B	A1A1BB

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TABLE 2 (Continued)

Voucher specimen	Spore shape	Spore ornamentation	Spore size mean (SD)	Chromosome number	Ploidy level	*rbcL* haplotype	*gapCp*Sh allele	*gapCp*Sh genotype
Thelypteris japonica ｆ. *formosa*								
S. Fujimoto s.n.	Normal	Alate		-	-	a	A1/B	A1A1BB
Thelypteris japonica × *T. musashiensis*								
S. Matsumoto s.n.	Irregular	N/A		-	-	c	A2/B/C1	A2BC1
N. Nakato 2862	Irregular	N/A		$2n = 93$	$3x$	c	A1/B/C1	A1BC1
M. Muramatsu NN2909	Irregular	N/A		$2n = 93$	$3x$	c	A1/B	A1B（C）
M. Muramatsu NN2910	Irregular	N/A		$2n = 93$	$3x$	c	A1/B	A1B（C）
Thelypteris japonica × *T. nipponica* var. *borealis*								
M. Muramatsu NN2914	Irregular	N/A		$2n = 125$.	$4x$	n2	A1/B/C1/N3	A1BC1N3
Thelypteris musashiensis × *T. nipponica* var. *borealis*								
Y. Saito NN2917	Immature	N/A		$2n = 94$	$3x$	n2	C1/N3	C1C1N3N3
Y. Saito NN2925	N/A	N/A		$2n = 94$	$3x$	c	C1/C2/N3	C1C2N3
Thelypteris castanea								
A. Ebihara et al.060930-01	Normal	Echinate		-	-	g	G	GG

Discussion

The samples of “var. *borealis*” producing regular spores are exclusively tetraploid and are distinct from diploid var. *nipponica*. Although both diploid ($n = 32$, $2n = 64$, Mitui 1980) and tetraploid ($n = 62$, Hirabayashi 1970) were reported for *T. nipponica* in previous studies, the tetraploid material is highly likely to correspond to “var. *borealis*”. The results of nuclear *gapCp*Sh sequencing show that “var. *borealis*” always possesses heterozygous sequences identical to *T. nipponica* var. *nipponica* and *T. musashiensis*, suggesting it is an allotetraploid species of hybrid origin. Morphologically, “var. *borealis*” strikingly exhibits intermediate states between the two species, *T. nipponica* var. *nipponica* and *T. musashiensis*, in frond shape, indusium size, indusium persistency, and the amount of indusium hair (Table 3). Contrary to its protologue “Indusia glanduloso-papilosa nunqum ciliata”, indusia of the tetraploid “var. *borealis*” are sparsely ciliate. The habitat of “var. *borealis*” is usually terrestrial in the understory of deciduous forest, considerably different from that of var. *nipponica* which exclusively occurs in sunny marshy places. Another progenitor species, *T. musashiensis* grows in shady places, but particularly on rocks (Nakato *et al.* 2004), and never occurs sympatrically with *T. nipponica* var. *nipponica*. There is no variation in plastid *rbcL* sequences or nuclear *gapCp*Sh sequences among 14 individuals of “var. *borealis*” from six localities. We found that each allele constituting the *gapCp*Sh genotype of “var. *borealis*” matches one of the variable alleles of the progenitors. These results suggest that “var. *borealis*” is an allotetraploid originated from a single hybridization between *T. nipponica* var. *nipponica* (as maternal parent, considering maternal inheritance of plastid DNA in ferns, Kuo *et al.* 2018) and *T. musashiensis*. We have not yet identified the place of origin of the allotetraploid. To track its evolutionary process, further exploration of infraspecific genetic variation is necessary especially in China where the distribution of the two progenitor species overlaps.

TABLE 3. A comparison of *Thelypteris sylva-nipponica* and closely related species in the *Parathelypteris* group.

	T. nipponica (var. *nipponica*)	*T. sylva-nipponica* (= "*T. nipponica* var. *borealis*")	*T. musashiensis*	*T. japonica*
Ploidy level	2*x*	4*x*	2*x*	4*x*
Lamina outline	Lanceolate	Deltate-oblong	Deltate to deltate-oblong	Deltate
Persistency of indusia (in summer)	Almost shrinked	Covering sorus	Covering sorus	Covering sorus
Hairs on indusia	Sparse	Sparse	Sparse	Pubescent
Spore ornamentation	Alate	Alate	Echinate	Alate
Hairs on abaxial rachis	Pubescent	Pubescent	Almost glabrous	Almost glabrous
Habitat	Sunny marshy place	Terrestrial in forest floor	Epilithic in forest floor	Terrestrial in forest floor and forest edge

We found that the traditional circumscription of *T. nipponica* var. *nipponica* included sterile triploids which were most likely derived from hybridization between var. *nipponica* and "var. *borealis*". This hybrid seems not to be rare on the periphery of the localities of *T. nipponica* var. *nipponica*, and its frequent formation probably has hindered the recognition of "var. *borealis*". Triploid individuals with irregular-shaped spores which are presumably sterile (Whittier & Wagner 1971) were also occasionally found among the specimens originally identified as "var. *borealis*", and evidence obtained from nuclear *gapCp*Sh sequences and chromosome number suggested that they are hybrids between "var. *borealis*" and *T. musashiensis*. *M. Muramatsu NN2914*, an individual collected at the periphery of "var. *borealis*" in Nagano Prefecture having reddish-purple stipe was found to be tetraploid hybrid between two tetraploid parents, "var. *borealis*" and *T. japonica*. A reticulate relationship concerning *T. nipponica* in broader sense was inferred from our DNA sequences, ploidy levels, chromosome numbers and spore shapes (Fig. 2). These data were synthesizable without discrepancy except in two samples *M. Muramatsu NN2909* and *M. Muramatsu NN2910*—their *rbcL* haplotype is "c" (the *T. musashiensis* type), but the C allele corresponding to *T. musashiensis* was not detected in *gapCp*Sh, which was presumably caused by the presence of hardly amplifiable "C" allele of *gapCp*Sh. Even though relationships of the three species in the clade J have not been clarified, neither of the two diploid species (*T. castanea* and *T. musashiensis*) seems to be a progenitor of tetraploid *T. japonica*.

It is often difficult to identify these hybrids based solely on frond morphology, instead, spore shapes and chromosome numbers are useful distinguishing characters. Chromosome basic number in Thelypteridoid ferns is known to vary from 27 to 36 (Smith 1990), and it is different even between *T. nipponica* var. *nipponica* ($x = 32$) and *T. musashiensis* ($x = 31$) within the *Coryphopteris* clade sensu Fawcatt (2018). In the case of *T. nipponica s.l.*, this difference can work as a useful key for identification of the members involved in a polyploid complex derived from reticulate evolution (Fig. 2).

Since the type material of *T. nipponica* var. *borealis* has not been identified yet, we explored collections, including unmounted materials, in the herbarium of the University of Tokyo (TI) where Hiroshi Hara's first set of specimens is deposited. His only specimen having consistent label information with the original description is a mounted one collected on Aug. 8, 1933 at the foot of Mt. Apoi and identified as "*Dryopteris thelypteris* A.Gray var. *yezoensis* Hara; *D. yezoensis* Hara MSS" (Fig. 3). The name clearly suggests that the author recognized it as a new taxon and that he thought of it as an infrataxon of *D. thelypteris* (Linnaeus 1753: 1071) Gray (1848: 630) (≡ *T. palustris* Schott 1834: t. 10) at an early stage, but the frond and soral morphology falls within the range of *T. nipponica* in broader sense and never within *T. palustris*. Thus, we consider the specimen as the original material of *T. nipponica* var. *borealis*—presumably Hara noticed that the specimen belongs to *T. nipponica s.l.* after preparing the label. The specimen consists of a single fertile frond without rhizome, and its remaining spores are immature but confirmed as normal shaped (Fig. 3C). However, the narrowly lanceolate lamina does not in any way match the plant currently called "var. *borealis*" but perfectly match var. *nipponica*. Although it is not easy to observe hairs on shrunken indusia of the specimen, we confirmed scattered ciliate hairs on indusial (Fig. 3B) after careful observation under a microscope. Considering these observations, the name *T. nipponica* var. *borealis* was given to a plant that falls into the current range of *T. nipponica* var. *nipponica*, and accordingly there is no name applicable to the tetraploid plant "var. *borealis*".

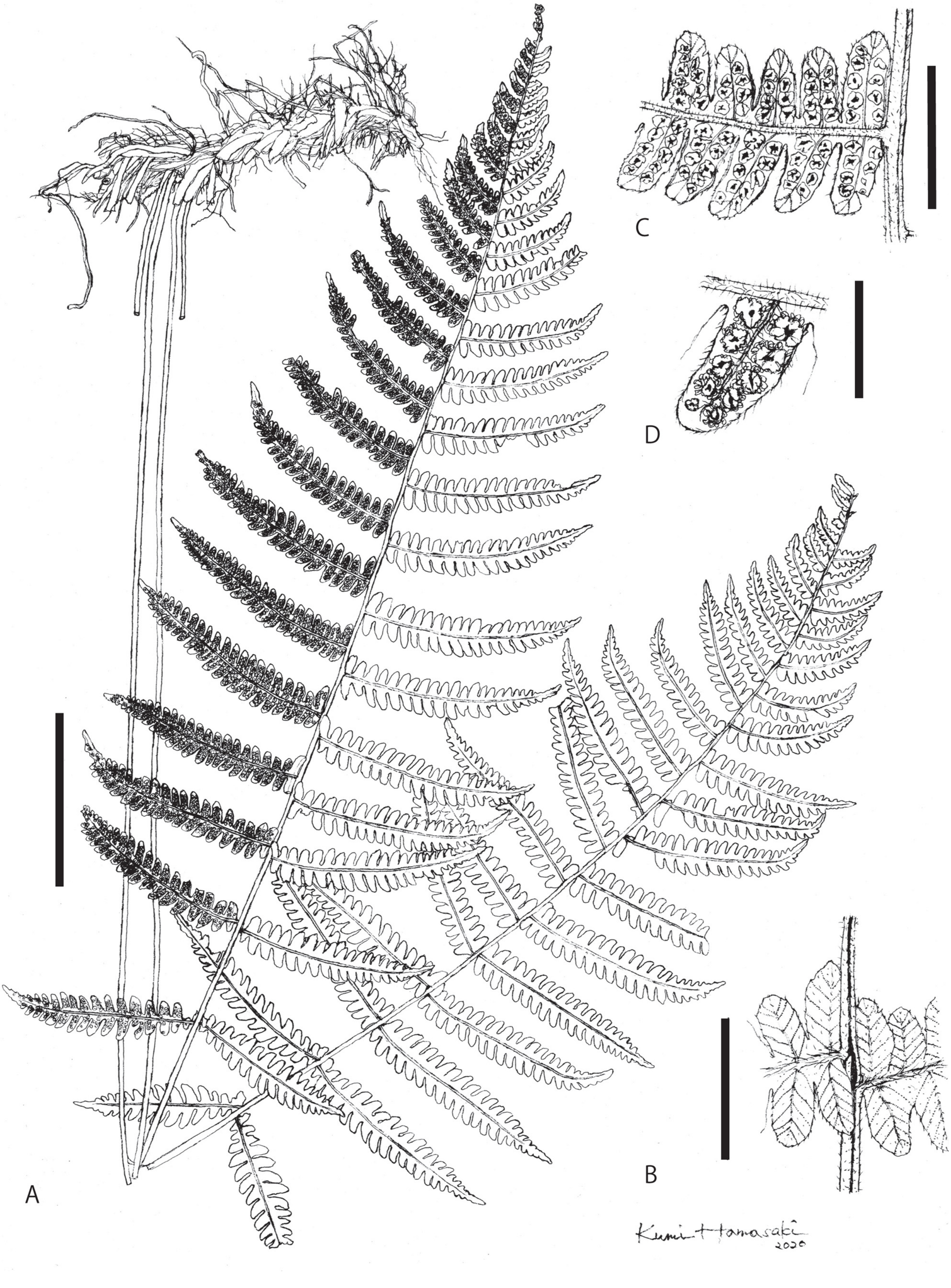

FIGURE 4. An illustration of holotype of *Thelypteris sylva-nipponica*. A. Whole the sheet. Scale bar = 5 cm. B. A basal part of lateral pinnae (adaxial view). Scale bar = 1 cm. C. A basal part of a lateral pinna with sorus (abaxial view). Scale bar = 1 cm. D. A segment with sorus (abaxial view). Scale bar = 5 mm.

Taxonomic treatment

Thelypteris nipponica (Franchet & Savatier 1879: 646) Ching (1936: 309). TYPE:—JAPAN, “in tractu Nikô” [Tochigi Pref., Nikko], 1866, *Savatier 2429* (holotype P-00643916!).

Homotypic synonym:—*Aspidium nipponicum* Francher & Savatier (1979: 636). *Dryopteris nipponica* (Franch. & Sav.) Chresitensen (1905: 279). *Lastrea nipponica* (Franch. & Sav.) Copeland (1947: 139). *Parathelypteris nipponica* (Franch. & Sav.) Ching (1963: 302). *Wagneriopteris nipponica* (Franch. & Sav.) Löve & Löve (1977: 325).

Heterotypic synonym:—*Dryopteris nipponica* (Franch. & Sav.) C.Chr. var. *borealis* Hara (1934: 695). *Thelypteris nipponica* (Franch. & Sav.) Ching var. *borealis* (H.Hara) Hara (1938: 621). *Lastrea nipponica* (Franch. & Sav.) Copel. var. *borealis* (H.Hara) Tagawa (1959: 222). *Parathelypteris nipponica* (Franch. & Sav.) Ching var. *borealis* (H.Hara) Nakaike (1992: 842). *Parathelypteris borealis* (H.Hara) Shing (1999: 37). TYPE: JAPAN. [Hokkaido] Prov. Hidaka, foot of Mt. Apoi, 8 August 1933. *H. Hara s.n.* (Fig. 3, lectotype TI! **designated here**).

Distribution and habitat:—Japan (Hokkaido and eastern half of Honshu), Russia (Far East), Korea and China. In sunny marshy places.

Thelypteris sylva-nipponica Ebihara & Nakato, *sp. nov.* (Fig. 4)

—*Thelypteris nipponica* var. *borealis* auct. non H.Hara, Iwatsuki *et al.* (1995) 188.

Type:—JAPAN. Aichi Pref., Kitashitara-gun, Shitara-cho, Tsugu, Orimoto. 19 September 2010, *M. Muramatsu 25388* (holotype TNS VS-1226664!)

Diagnosis:—Similar to *Thelypteris nipponica* but differs in having broader lamina with non-narrowed basal part and persistent indusia (vs. caducous in *T. nipponica*). Also similar to *T. musashiensis* and *T. japonica* f. *formosa*, but differs in having pubescent rachis on abaxial side (vs. glabrous).

Summer-green fern. Rhizome short-creeping, (1.3–)2.0–2.6(–3.2) mm in diameter; Leaves almost monomorphic, stipe (14–)23–31(–33) cm long, pale green, brownish at base, sparsely hairy with transparent, unicellular hairs, scales present only on basal portion, light brown, deltate-oblong, (3.2–)3.8–4.6(–5.6) mm long; Lamina deltate-oblong, (17–)23–28(–30) × (8.9–)11–14(–17) cm, bipinnatisect, apex acute to acuminate, herbaceous, pale yellowish green, yellow glands present on abaxial surface, rachis pale green, more or less densely hairy on abaxial side with transparent short hairs, lateral pinnae (18–)23–26(–27) pairs, oblong-lanceolate to narrowly oblong-lanceolate, (2.8–)3.9–5.6(–7.2) × (0.7–)0.8–1.1(–1.2) cm in basal ones, almost sessile, ultimate segments 2–3(–4) mm wide, round to obtuse at apex, margin entire to undulate, sparsely hairy; Sorus almost medial, forming two rows on both sides of costule, indusiate, indusia reniform, 0.6–0.8(–1.0) mm in diameter, stalked glands present, short transparent unicellular hairs present rather sparsely; Spore monolete, longer diameter 44–50 µm excluding perispore. Tetraploid with chromosome number $2n = 126$.

Distribution and habitat:—Japan (Hokkaido and eastern half of Honshu) and China (Anhui, Fujian, Guangxi, Guizhou, Hunan, Jiangxi, Shaanxi and Sichuan). Usually occurs in the forest floor layer of deciduous broad-leaf forests.

Etymology:—The species resembles *T. nipponica*, and usually occurs in the forests.

Key to the ‘*Parathelypteris*’ species included in the *Coryphopteris* clade in East Asia (excluding sterile hybrids)

1	Lowest pinna remarkably shortened (less than quarter of the longest one)	*T. nipponica*
-	Lowest pinna not remarkably shortened	2
2	Abaxial side of rachises glabrous or almost glabrous	3
-	Abaxial side of rachises hairy	4
3	Lamina lanceolate	*T. chinensis*
-	Lamina deltate to deltate-oblong	5
4	Stipe castaneous	*T. castanea*
-	Stipe pale-green	*T. sylva-nipponica*
5	Spores with echinate perispore	*T. musashiensis*
-	Spores with alate perispore	*T. japonica*

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APPENDIX 1. GenBank accession numbers of *rbcL* and *gapCp* short (*gapCp*Sh) sequences used in this study.

rbcL haplotype		
a	AB575027	(Ebihara *et al.* 2010)
c	AB575031	(Ebihara *et al.* 2010)
n1	LC484386	(Ebihara & Nitta 2019)
n2	AB575033	(Ebihara *et al.* 2010)
*gapCp*Sh allele		
A1	LC571591	
A2	LC571592	
B	LC571593	
C1	LC571594	
C2	LC571595	
G	LC571596	
N1	LC571597	
N2	LC571598	
N3	LC571599	
T. cystopteroides 1	LC571600	
T. cystopteroides 2	LC571601	