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Phylogeography and chromosome number variation in *Micranthes nelsoniana* and related species (Saxifragaceae) in Northeast Asia

Tomoko Fukuda[1,*], Naoko Ishikawa[2], Olga A. Chernyagina[3], Vyacheslav Y. Barkalov[4], Aleksandr A. Taran[5], Valentin V. Yakubov[4], Elena A. Marchuk[6], Elena V. Linnik[7], Ichiro Tamaki[8]

[1]College of Liberal Arts and Sciences, Mie University, Mie, Japan
[2]Botanical Gardens, Osaka City University, Osaka, Japan
[3]Kamchatka Branch of Pacific Institute of Geography FEB RAS, Petropavlovsk-Kamchatsky, Russia
[4]Federal Scientific Center of the East Asia Terrestrial Biodiversity FEB RAS, Russia
[5]Sakhalin Botanical Garden FEB RAS, Russia
[6]Botanical Garden-Institute FEB RAS, Russia
[7]State Nature Reserve Kurilskiy, Russia
[8]Gifu Academy of Forest Science and Culture, Gifu, Japan

[*]Corresponding author: Kurimamachiya-cho 1577, Tsu 514-8507, Japan. Email: fukuda@ars.mie-u.ac.jp

ABSTRACT

Micranthes nelsoniana possesses multiple different variants and numerous chromosomes. Based on the internal transcribed spacer (ITS) and chloroplast (cp)DNA sequences, the phylogeography of *M. nelsoniana* and its relatives in Northeast Asia was investigated, with extensive sampling around the Kuril Islands. The Arctic–Asian continent and a clade of marginal islands were the two main groupings that comprised the ITS phylogenetic tree. The island clade was separated into five well-supported clades: Kamchatka and Hokkaido highlands, Kuril–Aleutian Islands, southern Kuril Islands, Japanese archipelago, and Primorye region. *Micranthes fusca* was found in Japan and in the southern Kuril Islands. It is a separate species that created several types of hybrids between *M. nelsoniana* in the centre of the Kuril Islands based on a comparison of the ITS and cpDNA networks. *Micranthes nelsoniana* and *M. ohwii* appear to have hybridized in the northern Kuril Islands. Cytological investigation on the local species of *M. nelsoniana* showed that the chromosomal numbers are: $2n$ = 24, 26, 28, 30, 50, and 80. Among them, two usual numbers to this area, $2n$ = 24 and 50, appear to encourage interspecific gene exchange. The genomes of Hokkaido plants with high chromosome counts were cloned, revealing that they contained genes of both continental and marginal origins. This study revealed the crucial role of marginal islands along Northeast Asia in the genetic diversity of *M. nelsoniana* and related species.

Keywords: chromosome number; cloning; haplotype network; hybridization; Kuril Islands; *Micranthes fusca*; *Micranthes ohwii*; phylogenetic analysis

INTRODUCTION

Micranthes nelsoniana (D.Don) Small is a perennial herb that is widely distributed in the circumboreal region from Siberia to North America. It is a large species complex (Zhmylev 1995, 1996, 2004, Tkach *et al.* 2015) that includes several varieties. Phylogenetically closely related species [*M. fusca* (Maxim.) S.Akiyama & H.Ohba, *M. ohwii* (Tatew) T.Fukuda & H.Ikeda (former *Saxifraga purpurascens* Kom., Fukuda *et al.* 2016b), and *M. manchuriensis* (Engl.) Gornall & H.Ohba] emerged from the western side of the Bering Sea to Northeast Asia and appear to have experienced a complex history of migration and genetic exchange during the glacial–interglacial period around Beringia and Hultenia (Yurtsev 1974, Hewitt 2000, Eidesen *et al.* 2007, *et al.* 2014).

Numerous phylogenetic studies of the genus *Micranthes*, including of *M. nelsoniana*, have been published (e.g. Prieto *et al.* 2013, Kim *et al.* 2015, Tkach *et al.* 2015, Stubbs *et al.* 2020a, b, Folk *et al.* 2021). The findings of these studies indicate that the genus *Micranthes*, which includes 80–85 species, comprises a monophyletic group (Soltis *et al.* 1996, 2001, Prieto *et*

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al. 2013, Deng *et al.* 2015, Stubbs *et al.* 2020a, b) and that the genus is estimated to have emerged ~38.9–79.8 Mya, as assessed via molecular dating methods (Stubbs *et al.* 2020b). Regarding the phylogenetic position of *M. nelsoniana* and closely related species, Tkach *et al.* (2015) reported a well-supported clade as the section Rotundifoliatae (A.M.Johnson) Tkach, including *M. nelsoniana*, *M. fusca*, *M. purpurascens* (=*M. ohwii*), *M. manchuriensis*, and other species. Further, Stubbs *et al.* (2020a) recognized the monophyletic group Lyallii, combining Rotundifoliatae and Cuneifoliatae [e.g. *M. razshivinii* (Zhmylev) Brouillet & Gornall, *M. calyicina* (Sternb.) Gornall & H.Ohba] of Tkach *et al.* (2015) and suggested that the ancestor of Lyallii emerged in Beringia, subsequently moving to western North America and Asia (Stubbs *et al.* 2020a). However, for *M. nelsoniana*, numerous previous studies reported this species to not be monophyletic (Kim *et al.* 2015, Tkach *et al.* 2015, Stubbs *et al.* 2020a, b), and the relationship between *M. nelsoniana* and *M. fusca* and other closely related species remains unclear.

Regarding phylogeography, Kim *et al.* (2015) examined *Micranthes* spp., focusing on *M. manchuriensis* and *M. octopetala* (Nakai) Y.I.Kim & Y.D.Kim, based on significant samples from various locations. In contrast to the abundance of phylogenetic studies, however, studies regarding phylogeography encompassing the distribution area of *Micranthes* spp. are limited.

The intraspecific diversity of *M. nelsoniana* can potentially explain the difficulties encountered during its analyses. This species complex includes several varieties, with no clear boundaries among them (Hultén 1928, Voroshilov 1982). They are presented in Table 1.

Micranthes nelsoniana is also known by the variety of chromosome numbers: $2n = 26–84$ (e.g. Packer 1964, Mulligan and Porsild 1969, Zhukova and Petrovsky 1971, 1987, Zhukova *et al.* 1973, Packer and McPherson 1974, Murray and Kelso 1997) (summary in Fukuda *et al.* 2016a). Regarding the fundamental number of chromosomes, Webb and Gornall (1989) suggested that $x = 8$, 10, 11, 13, and 14 were the most prevalent gametophytic chromosome numbers in Saxifragaceae and that higher numbers are probably due to polyploidy. In *M. nelsoniana*, the number $2n = 28$ is most commonly observed (Altai and Taimyr in Siberia, south Kamchatka, Yukon), whereas $2n = 26$ is the lowest observed number and $x = 14$ is the most probable fundamental number.

The intraspecific polymorphisms of *M. nelsoniana* are considered to have resulted from complex genetic variation, hybridization, and polyploidization (Hultén 1928, Zhmylev 1995) in addition to environmental factors. Regarding hybridization, there remains debate concerning the basis of the morphological variation of this genus: e.g. *M. nelsoniana* var. *insularis* and *M. purpurascens* on the Commander Islands or *M. fusca* and *M. purpurascens* on Simushir Island (Siplivinsky 1976). Interspecific gene flow has received particular attention as one of the factors involved in the changes in inter- and intraspecific genetic composition, and hybridization is an important source of evolutionary novelty (Mallet 2007, Soltis *et al.* 2009, Folk *et al.* 2017). In addition, wide chromosome number variation reported for *M. nelsoniana* suggests the presence of high polyploidy. According to these findings, *M. nelsoniana* and its relatives appear to have undergone a complex, reticulated evolution, similar to numerous other taxa in the genus *Micranthes* (Stubbs *et al.* 2020a).

In addition to *M. nelsoniana*, we analysed samples of *M. fusca* (type: Konoma, Hokkaido), covering its distribution range from Japan (Kyushu: 52 and 75 in Fig. 1) to Simushir Island (33 in Fig. 1) in the central Kuril Islands (Barkalov 2009, Takahashi 2015; distribution based on herbarium evidence). *Micranthes fusca* is characterized by dark-red or greenish-white petals with reversed petal edges and short filaments with a size of less than half of the petal length (Hara 1939, Ohwi 1953, Voroshilov 1982, Ohba

Table 1. Characteristics and type locality of the varieties of *Micranthes nelsoniana* in NE Asia to Alaska

Taxon	Type locality	Distribution	Characteristics
Micranthes nelsoniana (D.Don) Small			
var. *nelsoniana* (D.Don) Small	Alaska, Cape Newenham	NE Russia (Okhotsk, Chukotka, N. Kurils), Alaska	Hairy leaves, glandular-villous hairs on inflorescences, a compact capitate inflorescence form
var. *reniformis* (Ohwi) S.Akiyama & H.Ohba	Sakhalin, Tosso	Sakhalin, Mt. Rishiri, the Kuril Islands, the lower banks of the Amur river, and the Shantarsky Islands	Glabrous reniform leaves, a corymbose-panicle inflorescence form, and whitish inflorescence hairs
var. *insularis* (Hultén) Gornall & H.Ohba	Aleutian Islands, Carlisle Island	The Aleutian, Commander, Kuril Islands; in the high mountainous area of Kamchatka	Large, thick glabrous leaves and dark-red capsules
var. *porsildiana* (Calder & Savile) Gornall & H.Ohba	Canada, British Columbia	Northwestern Canada and northeastern Russia (Chukotka, Kamchatka)	Small plant with small hairy leaves
var. *pacifica* (Hultén) Gornall & H.Ohba	Alaska, Juneau	Amphi-Bering	Glabrous leaves, compact inflorescences, and viscid pedicels with glandular inflorescence hairs
var. *aestivalis* (Fisch. & C.A.Mey.) Gornall & H.Ohba	Russia, Altay	Siberia (from Ural to Amur) and northeastern China	Long peduncles and pedicels

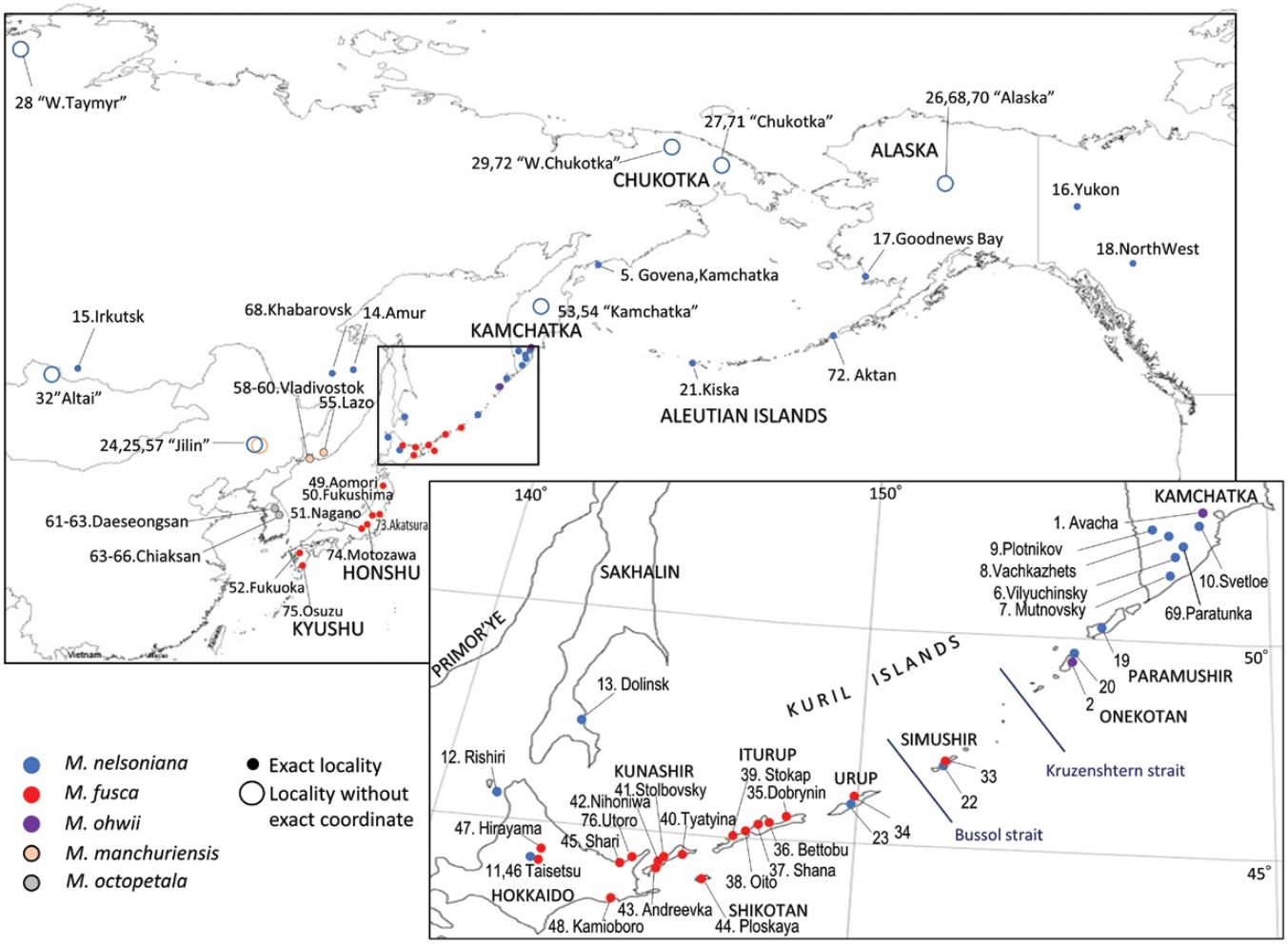

Figure 1. Map of the localities where the samples were collected. The numbers are the same as in Table 2. The species collected are indicated by coloured circles: blue for *M. nelsoniana*, red for *M. fusca*, purple for *M. ohwii*, orange for *M. manchuriensis*, and grey for *M. octopetala*. Large circles indicate a lack of information on the exact coordinates of those samples.

1989, Wakabayashi 2001, Okuyama 2016). *Micranthes ohwii* (=*Saxifraga purpurascens*) has red petals and reddish, thick, and lustrous leaves and grows at high altitudes on rocky volcanic slopes from Kamchatka to Onekotan Island in the northern Kuril Islands (Komarov 1914, 1927, Siplivinsky 1976, Charkevicz 1989, Yakubov and Chernyagina 2004, Barkalov 2009). We collected this species from Kamchatka (1 in Fig. 1) and Onekotan Island (2 in Fig. 1). In addition, we analysed *M. manchuriensis*, which grows along rivers in Primorye in Far Eastern Russia, Korea, and northeast China (55 in Fig. 1). As an outgroup, we used *M. japonica* (H.Boissieu) S.Akiyama & H.Ohba plants.

Herein, we aimed to understand the genetic relationship between *M. nelsoniana* and its closely related species in regions of the western Bering Sea to Northeast Asia. The presence of a large chromosome number hampers the performance of genome-wide fragment analyses owing to difficulties in deciding the right fragment positions in multiple gene sequences within individuals. To resolve the complexity of the reticulated evolution of *M. nelsoniana*, we adopted a detailed comparison of Sanger-sequenced nuclear and plastid phylogenetic trees and networks. Concomitantly, chromosome numbers were investigated for assumptions regarding the regional polyploidy level according to genetic characteristics. In addition, we applied the cloning method to *M. nelsoniana* samples from Hokkaido with high chromosome numbers to estimate the genetic origin of these individuals.

MATERIALS AND METHODS

Plant samples

In total, 356 individuals were collected from 40 localities as follows: *M. nelsoniana* (including five varieties: 108 samples from 24 localities); *M. fusca* (213 samples from 24 localities); *M. ohwii* (28 samples from two localities); *M. manchuriensis* (four samples from one locality); and one sample each of *M. japonica*, *M. calycina*, and *M. sachalinensis* (F. Schmidt) S.Akiyama & H.Ohba (Table 2).

DNA extraction, sequencing, and alignment

Dried leaves were ground in a ceramic mortar or homogenized using a bead crusher and washed with HEPES-buffer solution (HEPES-NaOH buffer 0.1 M, 20 mL; polyvinylpyrrolidone, 204 mg; ascorbic acid, 180 mg; and mercaptoethanol, 0.5 mL) before DNA extraction using the CTAB method [Doyle and Doyle

Table 2. Voucher information, localities, sample numbers and habitats. Sequences taken from DDBJ are indicated with accession numbers.

Taxon	Voucher information	Loc.[a]	Latitude	Longitude	Numbers analysed for[b]:				DNA sample[c]	Habitat
					ITS		cpDNA			
					Tree	Network	Tree	Network		
***Micranthes nelsoniana* (D.Don) Small**										
	var. *nelsoniana* (D.Don) Small									
	Russia, N. Kamchatka, Govena. 15 Jul. 2012, Yakubov V. (VLA)	5	60.400	166.933	3	5	3	5	218–222	Coastal meadow
	Russia, Kamchatka, Svetloe. 18 Jun. 2014, Fukuda (TI)	10	53.102	158.611	3	4	3	4	288–291	Along the stream
	Russia, along Amur, Solnechnyi, 19 Jun. 2012, Yakubov V.T041441 (VLA)	14	50.745	136.483	6	6	6	6	126–131	Near the lake
	var. *reniformis* (Ohwi) S.Akiyama & H.Ohba									
	Japan, Hokkaido, Mt. Rishiri. 18, Aug. 2013, Fukuda R2013-2 (TI)	12	45.184	141.241	4	10	3	10	269–273+	On rocky slope
	Japan, Hokkaido, Mts. Taisetsu. 25 Jul. 2015, Fukuda T2015-1 (TI)	11	43.657	142.916	1	2	1	2	327,328	At the waterhead/ rocky slope
	Russia, Sakhalin, Dolinsk. 6 Sep. 2013, Fukuda T. *et al.* S2013 (VLA)	13	47.224	142.995	6	6	7	6	252–267	Along the stream
	var. *insularis* (Hultén) Gornall & H.Ohba									
	Russia, Kuril Islands, Paramushir Is. 23 Jul 2018, Yamawaki (TI), 31 Jul 2018, Volkova (TI)	19	50.547	156.148	8	18	8	18	442–446, 464–473	On coastal rocks
	Onekotan Is. 30 Jul. 2018, Yamawaki (TI), 13 Aug. 2018, Volkova (TI)	20	49.458	154.704	3	3	3	3	358–360	On rocks at 700 m asl
	Aleutian, Kiska Quad. Bur (insularis) 13 Aug. 2006, Talbot BUL021-11A (ALA)	21	52.368	175.904	1	1	1	1	298	Meadow at 113–133 m asl
	Russia, Kuril Islands, Simushir, 8 Aug. 1999, Takahashi *et al.* 18958 (SAPS)	22	46.850	151.800	1	1	1	1	250	
	Kuril Islands, Urup Isl., 4 Aug. 1995, Takahashi H. 18536 (SAPS)	23	45.861	149.787	1	1	0	1	251	On sand at river mouth
	var. *porsildiana* (Calder & Savile) Gornall & H.Ohba									
	Canada, Yukon. 13 Jul. 2005, Bennett *et al.* 05-0503 (ALA)	16	64.719	−133.980	1	1	1	1	297	River channel
	Canada, Caribou River, 8 Aug. 2012, Bennett *et al.* 12-0359 (ALA)	18	60.937	−126.536	1	1	1	1	299	Nivean gully
	[sequence data from DDBJ]									
	LM654393 *M. porsildiana*, Kamchatka				0	1	0	0		No data
	var. *aestivalis* (Fisch. & C.A.Mey.) Gornall & H.Ohba									
	Russia, Khabarovsk, Badzhalskaya, r. Yarap, Jul. 31, 2016. Barkalov V.Yu. (VLA)	68	50.281	134.712	3	3	0	4	610–629	Near the lake

Table 2. Continued

Taxon	Voucher information	Loc.[a]	Latitude	Longitude	Numbers analysed for[b]:				DNA sample[c]	Habitat
					ITS		cpDNA			
					Tree	Network	Tree	Network		
	[sequence data from DDBJ]									
	LM654380 *M. nelsoniana* var. *aestivalis*, Russia, Altai	32			1	1	—	—		No data
	KT445991 *M. nelsoniana*, China, Jilin	25			1	1	—	—		No data
	KT445992 *M. nelsoniana*, China, Jilin	25			1	3	—	—		No data
	var. *pacifica* (Hultén) Gornall & H.Ohba									
	[sequence data from DDBJ]									
	LM654381 *M. nelsoniana* var. *pacifica*, Russia, Far East	30			1	1	—	—		No data
	[variety undetermined]									
	Russia, Kamchatka, Mt. Vilyuchinsky, 28 Jul. 2018, Fukuda (TI)	6	52.693	158.164	6	26	6	25	361–381	Along the stream/ on the slope
	Russia, Kamchatka, Mt. Mutnovsky, 2018, Chernyagina (TI)	7	52.542	158.202	4	10	4	10	447–458	On wet rocks
	Russia, Kamchatka, Mt. Vachkazhets, 1 Aug. 2018, Fukuda (TI)	8	53.053	157.934	0	0	0	1	404	Along the stream
	Russia, Kamchatka, r. Plotnikov, 16 Jun. 2014, Fukuda (TI)	9	52.912	157.501	1	1	1	1	294	Along the stream
	Russia, Kamchatka, Paratunka, 17 Jun. 2014, Fukuda (TI)	69	52.803	158.167	1	1	1	1	293	Along the stream
	Russia, Irkutsk, Bol'shie koty, Jul. 2007, Fukuda (no voucher)	15	51.905	105.073	1	1	1	1	8	By the bog
	Alaska, Goodnews Bay Quad., 15 Jun. 2004, Parker 15598 (ALA)	17	59.303	−161.490	1	1	1	1	300	Mossy terraces
	Alaska, 333161 (TNS)	70			1	1	0	0	295	No data
	Chukotka, Koryakskoye Mots., May creek Parker 4394, 9 Jul. 1993 (ALA)	71	62.483	175.867	1	1	1	1	296	Moist bars along creek
	Aleutian, Aktan Island. 1 Aug. 2019, Yamawaki K.(TI)	72	54.134	−165.779	1	1	0	1	606	On sea rocks
	[sequence data from DDBJ]									
	LM654379 *M. nelsoniana*, USA, Alaska	26			1	1	—	—		No data
	LM654376 *M. nelsoniana*, Russia, Chukotka	27			1	1	—	—		No data
	LM654377 *M. nelsoniana*, Russia, W. Taymyr	28			1	1	—	—		No data
	LM654378 *M. nelsoniana*, Russia, W. Chukotka	29			1	1	—	—		No data

Table 2. Continued

Taxon	Voucher information	Loc.[a]	Latitude	Longitude	Numbers analysed for[b]:				DNA sample[c]	Habitat
					ITS		cpDNA			
					Tree	Network	Tree	Network		
***Micranthes fusca* (Maxim.) S.Akiyama & H.Ohba**										
	var. *fusca* (Maxim.) S.Akiyama & H.Ohba									
	Japan, Hokkaido, Mts. Taisetsu, 25 Jul. 2015, Fukuda (TI)	46	43.657	142.916	2	9	2	9	442–439	At the waterhead
	Japan, Hokkaido, Mt. Hirayama, 2 Sep. 2009, Fukuda SF-2 (TI)	47	43.772	143.022	0	0	0	1	11	Along the stream
	Japan, Hokkaido, Kamioboro, 31 Aug. 2010, Fukuda (TI)	48	43.031	144.608	6	20	6	20	23, 24, 47–64	Along the stream
	Japan, Tohoku, Aomori pref. (no voucher)	49			1	1	1	1	217	Along the river
	var. *kikubuki* (Ohwi) S.Akiyama & H.Ohba									
	Japan, Fukushima, Kurohi-zawa, 14 Jul. 2012, T. Fukuda (TI)	50	37.083	139.413	4	6	4	7	132–138	Along the river
	Japan, Fukushima, Akatsura-yama, 15 Jul. 2012, T. Fukuda (TI)	73	37.141	140.011	7	8	7	10	139–148	Along the river
	Japan, Nagano, Mt. Odaka-yama, 29 Jul. 2012, T. Fukuda (TI)	51	35.438	138.030	7	8	7	8	237–244	Along the river
	Japan, Nagano, Motozawa, 2012	74	36.019	138.414	1	3	1	3	245–247	Along the stream
	Japan, Fukuoka, Mt. Inugatake. 26 Sep. 2015, T. Fukuda (TI)	52	33.520	130.997	1	1	0	1	333	Along the river
	Japan, Miyazaki, Mt. Osuzu. 27 Sep. 2015, T. Fukuda (TI)	75	32.296	131.441	1	1	1	1	336	On wet rocky slope
	var. *kurilensis* (Ohwi) T.Fukuda & H.Ikeda									
	Kuril Islands, Simushir Island, 22 Aug. 1995, Takahashi H. 19727 (SAPS)	33	46.59	152.01	0	0	0	1	440	Along the river
	Kuril Islands, Urup, Natalya bay, 7 Aug. 1995, Takahashi H. 18716 (SAPS)	34-1	46.100	150.167	0	1	0	1	77	In valley in tall herbs
	Kuril Islands, Urup, Smuglyi bay, 24 Aug. 1995, Takahashi H. 19923 (SAPS)	34-2	46.033	149.983	1	1	1	1	248	In gorge along river
	Kuril Islands, Urup, Tokotan, 28 Aug. 1995, Takahashi H. 20096 (SAPS)	34-3	45.800	149.900	0	1	0	1	249	Along the river
	Kuril Islands, Iturup, Dobrynin, Fukuda *et al.* ET-D-1 (VLA)	35	45.360	148.453	1	1	1	1	223	On coastal rocks
	Kuril Islands, Iturup, Bettobu, 1 Sep. 2012, Fukuda *et al.* (VLA)	36	45.286	148.019	1	13	1	12	225–236	On coastal rocks
	Kuril Islands, Iturup, Shana, 7 Aug. 2011, Taran A. (VLA)	37	45.258	147.886	12	20	12	20	101–121	On coastal rocks

Table 2. Continued

Taxon	Voucher information	Loc.[a]	Latitude	Longitude	Numbers analysed for[b]:				DNA sample[c]	Habitat
					ITS		cpDNA			
					Tree	Network	Tree	Network		
	Kuril Islands, Iturup, Oito, 4 Sep. 2012, Fukuda *et al.* (VLA)	38	44.990	147.509	8	20	8	24	172–195	Near river mouth
	Kuril Islands, Iturup, Stokap, 15 Aug. 2017, Taran A & Alekhin A (KAM)	39	44.844	147.285	4	12	4	12	405–421	Along the stream
	Kuril Islands, Kunashir, r.Tyatina, 12 Aug. 2016, Fukuda (VLA)	40	44.280	146.164	3	16	3	16	480–499	Along the river
	Kuril Islands, Kunashir, Stolbovsky, Fukuda *et al.* KN-2013-7 (VLA)	41	44.008	145.681	2	3	2	3	274–277	Along the river
	Kuril Islands, Kunashir, Nihoniwa, 19 Aug. 2012, Fukuda *et al.* (VLA)	42	43.943	145.562	2	1	2	5	196–205	On wet slope at seashore
	Kuril Islands, Kunashir, Andreevka, 19 Aug. 2012, Fukuda *et al.* (VLA)	43	43.887	145.624	6	12	6	22	149–171	On the river
	Kuril Islands, Shikotan, Ploskaya, 26 Aug. 2010, Fukuda *et al.* (VLA)	44	43.803	146.652	8	19	8	19	28–46	On the stream
	Japan, Hokkaido, Utoro, T. Fukuda (TI)	76	44.085	145.012	1	1	1	1	10	On the river near mouth
	Japan, Hokkaido, Shari, 27 Jul. 2015, Uchida A.(TI)	45	43.868	144.738	4	11	4	12	206–216	On the stream
	[sequence data from DDBJ]									
	LM654359 '*M. fusca*' Russia, Kamchatka	53			1	1	—	—		No data
	LM654360 '*M. fusca*' Russia, Kamchatka	54			1	1	—	—		No data
***Micranthes ohwii* (Tatew.) T.Fukuda & H.Ikeda**										
	Russia, Kamchatka, Mt. Avacha. 12 Jul. 2013, Fukuda T. (TI)	1	53.273	158.76	11	20	11	23	21–27, 65–75	On rocky slope of volcano
	Kuril Islands, Onekotan. 14, Aug. 2018, Volkova P. (TI)	2	49.479	154.742	3	5	3	5	459–463	On rocks of mountain
	[sequence data from DDBJ]									
	LM654395 '*M.purpascens*' Russia, Far East	3			1	1	—	—		No data
	LM654396 'M.purpascens' Kuril Isl., Kunashir	4			1	1	—	—		No data
***Micranthes manchuriensis* (Engl.) Gornall & H.Ohba**										
	Russia, Primorye, Lazo, 6 Jun. 2015, Fukuda T. & Bakalin V. (TI)	55	43.229	133.759	3	4	3	4	319–326	Along the river
	[sequence data from DDBJ]									
	KT445993 *M. manchuriensis*, Russia, Vladivostok	56			1	1	—	—		No data

Table 2. Continued

Taxon	Voucher information	Loc.[a]	Latitude	Longitude	Numbers analysed for[b]:				DNA sample[c]	Habitat
					ITS		cpDNA			
					Tree	Network	Tree	Network		
	KT445994 *M. manchuriensis*, China, Jilin	57			2	2	—	—		No data
	KT445995 *M. manchuriensis*, Korea	58			1	1	—	—		No data
	KT445996 *M. manchuriensis*, China, Jilin/ Russia, Vladivostok	59			18	18	—	—		No data
	KT445997 *M. manchuriensis*, Russia, Vladivostok	60			2	2	—	—		No data
***Micranthes octopetala* (Nakai) Y.I.Kim & Y.D.Kim**										
	[sequence data from DDBJ]									
	KT445985 Korea: Hwaaksan Mt. (1), Daeseongsan Mt. (1)	61			2	2	—	—		No data
	KT445986 Korea: Daeseongsan Mt. (1)	62			1	1	—	—		No data
	KT445987 Korea: Hwaaksan Mt. (14), Daeseongsan Mt. (17), Chiaksan Mt. (3)	63			34	34	—	—		No data
	KT445988 Korea: Chiaksan Mt. (1)	63			1	1	—	—		No data
	KT445989 Korea: Chiaksan Mt. (7), Sobaeksan Mt. (19)	64			26	26	—	—		No data
	KT445990 Korea: Chiaksan Mt. (1)	63			1	1	—	—		No data
***Micranthes japonica* (H.Boissieu) S.Akiyama & H.Ohba**										
	Japan, Hokkaido, Mt. Hirayama, 2 Sep. 2009, Fukuda SJ-3 (TI)	67	43.772	143.022	1	1	1	1	5	Along the stream
***Micranthes spicata* Small**										
	[sequence data from DDBJ]									
	LM654405 *M. spicata*, USA, Alaska				1	0	—	—		No data
***Micranthes lyallii* (Engl.) Small**										
	[sequence data from DDBJ]									
	LM654368 *M. lyallii* subsp. *lyallii*, Canada, British Columbia				1	0	—	—		No data
***Micranthes razshivinii* (Zhmylev) Brouillet & Gornall**										
	[sequence data from DDBJ]									
	LM654397 *M. razshivinii*, USA, Alaska				1	0	—	—		No data
***Micranthes calycina* (Sternb.) Gornall & H.Ohba**										
	Russia, Chukotka. 1991 (TNS)				1	0	—	—	284	No data
	[sequence data from DDBJ]									

Table 2. Continued

Taxon	Voucher information	Loc.[a]	Latitude	Longitude	Numbers analysed for[b]:				DNA sample[c]	Habitat
					ITS		cpDNA			
					Tree	Network	Tree	Network		
	LM654348 *M. calycina*, Russia, WChukotka				1	0	—	—		No data
***Micranthes odontoloma* A.Heller**										
	[sequence data from DDBJ]									
	LM654388 *M. odontoloma*, USA, Utah				1	0	—	—		No data
***Micranthes sachalinensis* (F. Schmidt) S.Akiyama & H.Ohba**										
	Japan, Hokkaido, Mt. Rishiri. 18, Aug. 2013 (no voucher)				—	0	1	0		On the slope of rocky mountain

[a]Locality numbers are same as in Figure 1.
[b]Number of samples used for this study. Tree: for phylogenetic analysis, network: for haplotype network analysis.
[c]A '+' means that there are additional samples.

1990: 2% CTAB, 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, and 20 mM EDTA]. Each sequence region was amplified using the polymerase chain reaction (PCR) primers listed in Table 3, and the products were purified via ExoSAP-IT (ThermoFisher Scientific). Sequencing was performed by Macrogen Japan Corp. (Kyoto, Japan) via an Applied Biosystems 3730xl DNA analyser using the primers employed for amplification. The sequences were aligned via MAFFT (Katoh *et al.* 2009) and low-quality bases at the end of the electropherograms were manually trimmed using BioEdit (Hall 1999).

Phylogenetic analysis

The phylogenetic relationships of the internal transcribed spacer (ITS) region of the nuclear ribosomal (nr)DNA (ITS1–5.8S rRNA gene–ITS2) were reconstructed using the Bayesian inference (BI) and maximum likelihood (ML) methods. In total, 268 samples (68 samples of *M. nelsoniana*, 85 samples of *M. fusca*, 16 samples of *M. ohwii*, 27 samples of *M. manchuriensis*, 65 samples of *M. octopetala*, and one sample each of *M. japonica* and *M. calycina*) were used for the ITS phylogenetic analysis. In addition, *M. spicata* Small (DDBJ accession LM654405), *M. razshivinii* (LM654397), *M. calycina* (LM654348), *M. lyallii* (Engl.) Small (LM654368), and *M. odontoloma* A.Heller (LM654388) were used as outgroups based on their close relationship with *M. nelsoniana* (Tkach *et al.* 2015, Stubbs *et al.* 2020ab). Among them, 107 samples were used from DDBJ, the accession numbers of which are listed in Table 2.

Before constructing an ITS phylogenetic tree, MODELTEST (Posada and Crandall 1998) was applied for model determination alongside the implementation of hierarchical likelihood ratio tests (hLRTs) via MrModeltest (Nylander 2004). BI analysis of the ITS sequences was conducted using MrBayes 3.2.7 (Huelsenbeck and Ronquist 2001, Ronquist *et al.* 2012). Four Markov chain Monte Carlo (MCMC) searches were run simultaneously for 320 000 generations, with sampling every 100 generations. The convergence and effective sample size of all the parameters were checked using Tracer v.1.6 (Drummond and Rambaut 2007) and the first 825 trees were discarded as burn-in.

An ML phylogenetic tree was constructed using MEGA X (Kimura 1980, Kumar *et al.* 2018) with the settings of the HKY+G (Hasegawa, Kishino, and Yano) model based on ModelTest (MEGA X), a nearest-neighbour interchange (NNI) heuristic method, and 1000 bootstrap replications (Felsenstein 1985). Furthermore, a phylogenetic tree for the plastid DNA region was constructed based on the combined sequences of the four plastid DNA loci (*trn*LF, *rbc*L, *trn*C-*rpo*B, and *mat*K) with a length of 3590 bp; 154 samples (53 samples of *M. nelsoniana*, 82 samples of *M. fusca*, 14 samples of *M. ohwii*, three samples of *M. manchuriensis*, and one sample each of *M. japonica* and *M. sachalinensis*) were used to construct the chloroplast (cp)DNA phylogenetic tree.

MODELTEST (Posada and Crandall 1998) was used for model determination by implementing the hLRTs for the plastid matrix in MrModeltest 2.4 (Nylander 2004). A BI analysis for combined cpDNA sequences was conducted using MrBayes 3.2.7 (Ronquist *et al.* 2012). Four MCMC searches were run simultaneously for 670 000 generations, with sampling every 100 generations. The convergence and effective sample size of all the parameters were assessed using Tracer v.1.6 (Drummond

and Rambaut 2007), with the first 1675 trees being discarded as burn-in.

An ML phylogenetic tree of plastid DNA sequences was also constructed using MEGA X (Kimura 1980, Kumar *et al.* 2018) with settings of the Tamura 3-parameter model based on ModelTest (MEGA X), an NNI heuristic method, and 1000 bootstrap replications (Felsenstein 1985). The samples used for nrDNA and cpDNA phylogenies were mostly the same, with a few exceptions. The differences in the number of samples between the two phylogenetic trees were attributed to the additional ITS sequences obtained from DDBJ, which registers only ITS sequences. The trees were drawn using FigTree v.1.4.4 (Rambaut 2014). The nrDNA and cpDNA phylogenetic trees were compared and each sample was connected with a line to indicate the extent of genetic agreement between these trees.

Network analysis

The ITS haplotype network was constructed using the median-joining method (Bandelt *et al.* 1999) via NETWORK 5.0.1.1. (http://www.fluxus-engineering.com/, Fluxus Technology). From the 444 available samples, 17 [four from Oito, Iturup (38 in Fig. 1), 10 from Andreevka (43 in Fig. 1), and three from Nihoniwa, Kunashir (42 in Fig. 1)] exhibited more than one heteromorphic site and were excluded from the analyses, resulting in a total of 424 samples (including 98 samples from DDBJ) as follows: 112 samples of *M. nelsoniana*, 191 samples of *M. fusca*, 27 samples of *M. ohwii*, 28 samples of *M. manchuriensis*, 65 samples of *M. octopetala*, and one sample of *M. japonica*.

Sequences with one heteromorphic site were split into two parent sequences. For example, '336f_Osuzu', which had one heteromorphic 'R' site, was split into '336f_Osuzu-A', carrying 'A', and '336f_Osuzu-G', carrying 'G'. In total, 66 sequences excluding outgroups were obtained and employed for network analysis.

The cpDNA haplotype network was constructed based on the combined sequences of the four plastid DNA loci (*trn*LF, *rbc*L, *trn*C-*rpo*B, and *mat*K) with a length of 2313 bp. In total, 344 samples were used to construct the cpDNA network: 105 samples of *M. nelsoniana*, 209 samples of *M. fusca*, 25 samples of *M. ohwii*, four samples of *M. manchuriensis*, and one sample of *M. japonica*, leading to the identification of 53 sequences, which were alphabetically named (from A to BC). The network was constructed in the same way as the ITS haplotype network. In both the networks (ITS and cpDNA), gaps and indels were also considered. Each gap and indel comprising several bases was treated as one event regardless of its length.

Statistical analyses

Based on the seven clades of the ITS phylogenetic tree, i.e. i, ii, iii, iv, and v of clade A, which were well supported, and vi and vii outwith clade A, the variation of each ITS clade was estimated using DnaSP v.6. In addition, the cpDNA sequences were grouped according to the seven ITS clades. The group of cpDNA included six groups, as it excluded the group corresponding to vii (*M. octopetala*) because all the ITS data of *M. octopetala* were collected from DDBJ and, thus, no cpDNA sequence data were available. For the six groups, population pairwise F_{ST} values were estimated using Arlequin 3.5 (Excoffier and Lischer 2010) based on the Kimura 2-parameter distance method with 1000 permutations.

Cloning

To address the origins of polyploidy, two samples of *M. nelsoniana* from Rishiri (12 in Fig. 1) and two samples from Taisetsu (11 in Fig. 1) were selected for analysis considering their phylogenetic position (in clade iv) and existing chromosome numbers ($2n = 50$ for Rishiri: Funamoto and Nakamura 1996; $2n = 80$ for Taisetsu: Fukuda and Ikeda 2019). As the cloning of PCR products sometimes generates false sequences because of PCR errors and artificial recombination (Cronn *et al.* 2002, Lihová *et al.* 2006), we attempted to prevent PCR errors by employing KOD-Plus-Neo DNA polymerase (Toyobo), thus limiting the number of PCR cycles to no more than 30 and adopting only these cloned sequences, the presence of which was confirmed in the original sequences. Two samples from each locality (four samples in total: Rishiri_1, Rishiri_2, Taisetsu_1, and Taisetsu_2)

Table 3. Primers used for amplification and sequencing.

Region	Primer name		Reference
ITS	5F	GGAAGTAAAAGTCGTAACAAGG	(White *et al.* 1990)
	4R	TCCTCCGCTTATTGATATGC	White *et al.* (1990)
*trn*L-*trn*F	LF LEFT	AACCATTTCTCCTACCCTCTCC	
	LF RIGHT	CCGACACACCATCCTCATCT	
*rbc*L	1L	CTTGGCAGCATTCCGAGTA	
	1R	CGCATAAATGGTTGGGAGTT	
*trn*C-*rpo*B	f	CACCCGGATTTGAACTGGGG	
	r	CKACAAAAYCCYTCRAATTG	
*mat*K	700F	AAGAAATCACCTATCTTTGGTTCAA	
	1400R	TGAAGATAGATTCGTATTCACATACA	
Additional primers for cpDNA phylogeny:			
*mat*K	nested_F	AAGAAATCACCTATCTTTGGTTCAA	
	nested_R	TGAAGATAGATTCGTATTCACATACA	
	1303F	TAGTCTTATTACTCCAACTC	
	1974R	CTGCATATGCGCACAAATCT	

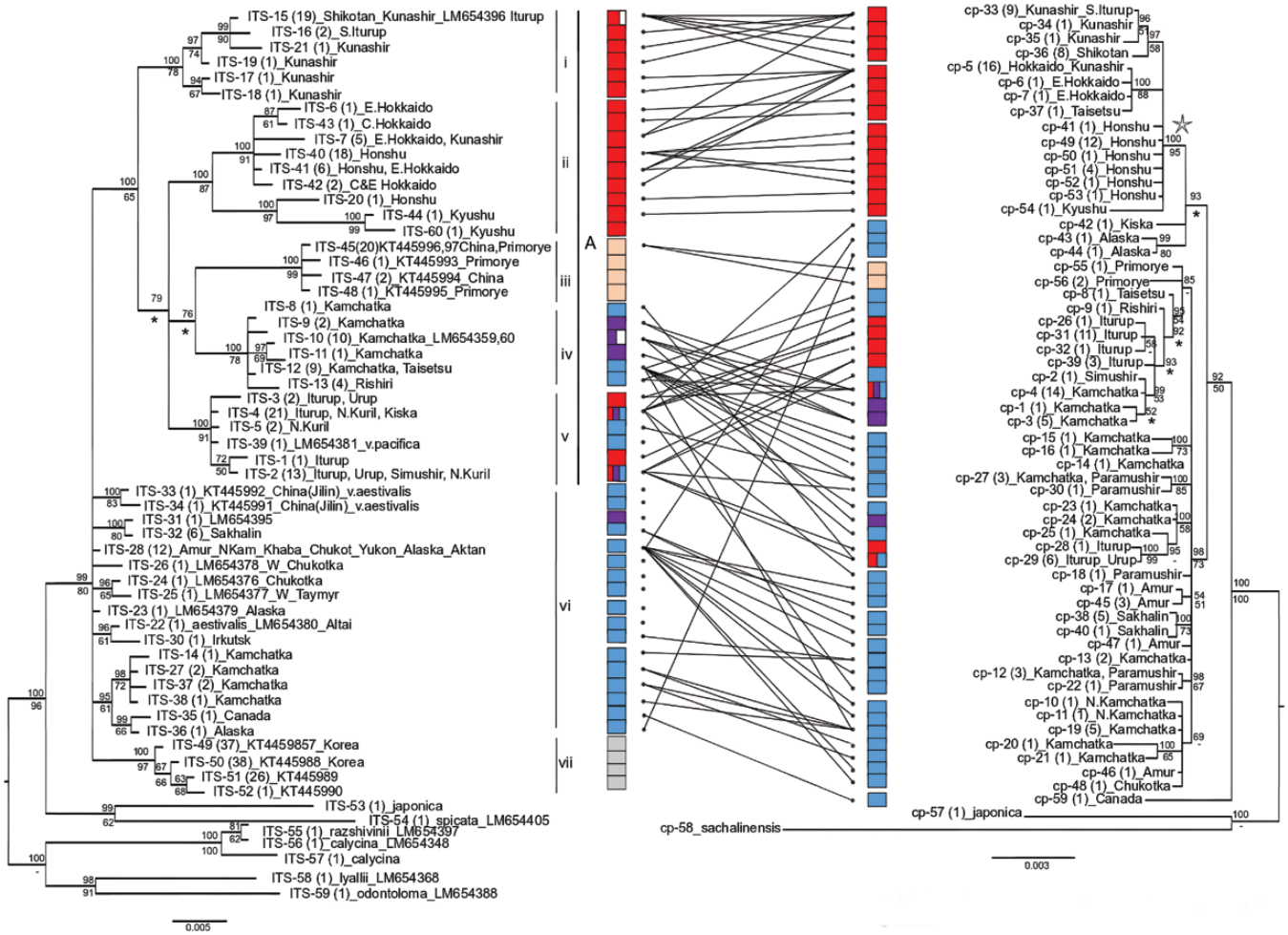

Figure 2. Bayesian inference phylogenetic tree of 59 sequences from 268 samples based on the ITS region (left), and phylogenetic tree of 59 sequences from 154 samples based on combined cpDNA regions (right). Numbers to the side of nodes indicate the posterior probability (PP, above) and bootstrap (BS, below) values by ML phylogeny (see Supporting Information). Nodes with asterisks (*) in the ITS tree have PP/BS values of < 50%. The star in the cpDNA tree indicates the clade comprising *M. fusca*, which occurred from S. Iturup to Kyushu. Numbers in parentheses indicate the number(s) of samples corresponding to the sequence. The blue bar is for *M. nelsoniana*, red for *M. fusca*, purple for *M. ohwii*, orange for *M. manchuriensis*, and grey for *M. octopetala*. The white colour in the ITS bar of the species in groups i and iv indicates possible misidentification (see Results). Lines between the ITS and cpDNA phylogenetic trees indicate the correlation of each sample between the two phylogenies.

were cloned using a Target Clone™-Plus cloning kit (Toyobo). The ribosomal ITS region, including the ITS1, ITS2, and 5.8S genes, was amplified via direct PCR using KOD-Plus Neo (Toyobo) to ensure a high PCR sequence accuracy and using the same primers as those employed for the direct sequencing of this region from other samples. The PCR conditions were as follows: 3 min at 94°C; followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and elongation at 72°C for 1.5 min; and a final extension at 72°C for 10 min. The PCR products were cloned into the pTA2 vector (Toyobo). The recombinant DNA was transferred into *Escherichia coli* competent cells, which were then plated on Luria Broth medium. The colonies were incubated overnight, and blue–white screening was performed to select colonies containing inserts. Inserts from each colony were amplified and the sequences were determined. The protocols were performed according to the manufacturer's recommendations.

To examine whether the sequences obtained were actually included in the original samples, additional primers were used for sequencing, as follows: for R1: TAAGTAATTGAGTGTTCCTTACAT and TTTAGGTCAACCACACAC; for T7: AGGTGCAAAT AATTGAATGTTCC and TCAACCACACGCAAGGAAGG; and for T9: AGCAGAAAACCTTGAGAACAAGT and CATGAGCATATTTCAAATGAT. These primers included polymorphic sites to increase the specificity of the sequences obtained. The primer sets were used for the PCR and direct sequencing of the original samples; if the PCR was successful, we considered it as a confirmation that the sequence was included in the original samples. A phylogenetic tree based on the ITS region was also constructed that included these confirmed sequences. Moreover, the ML and BI trees were drawn using the same conditions as those used to produce Figure 2, including bootstrap/posterior probabilities and other parameters.

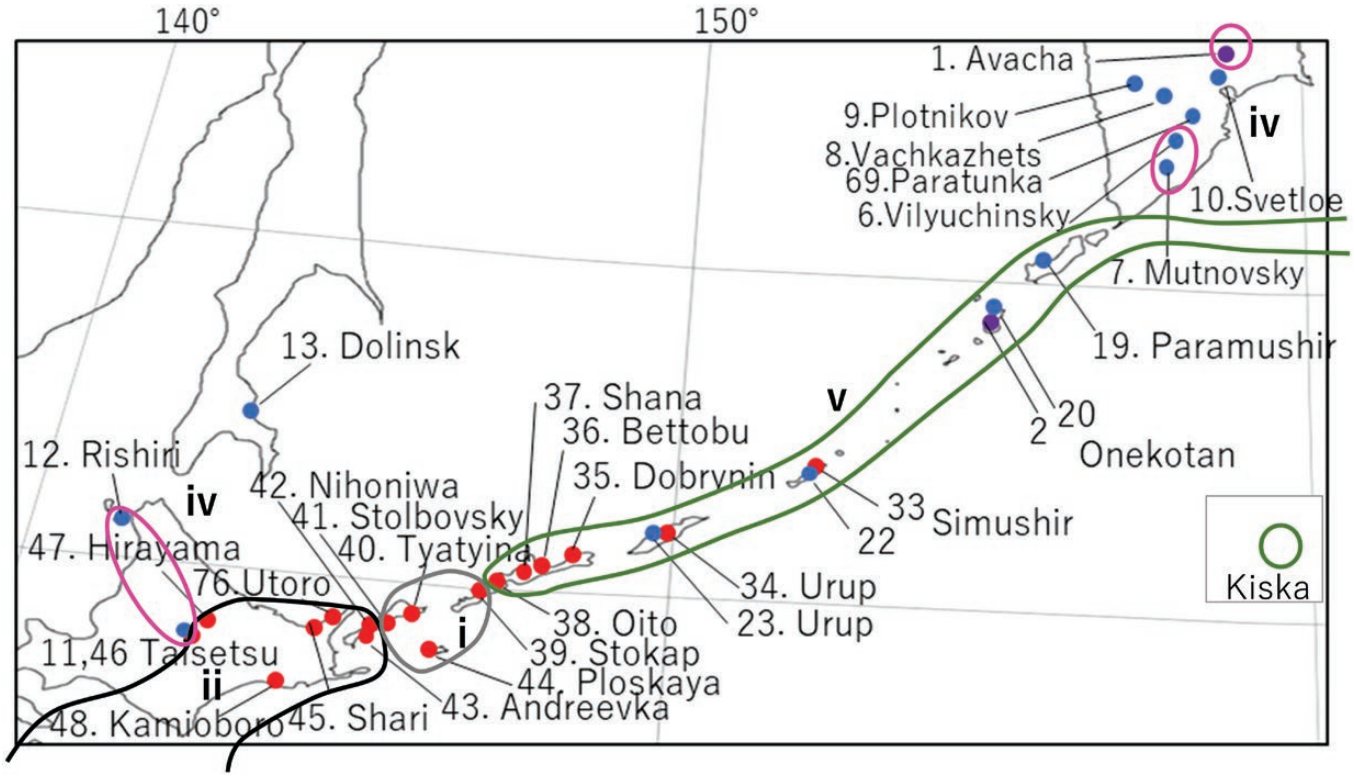

Figure 3. ITS phylogenetic groups A-i, ii, iv, and v. The collected species are indicated by coloured circles, as in Figure 1. The same groups are shown using same-coloured lines.

Chromosome numbers and karyotypes

Chromosome numbers were investigated in the *M. fusca* samples from Iturup Island and the *M. nelsoniana* sample from the Paramushir and Onekotan Islands in the northern Kuril Islands. Young root tips or germinated roots were used for analysis. Seeds were germinated on moist filter paper at 5°C in the dark. Roots were pretreated with 2 mM 8-hydroxyquinoline at 18°–25°C for 1 h and then at 5°C for 8 h. Subsequently, the roots were fixed in Farmer's fixative (ethanol/acetic acid = 3:1). The fixed roots were hydrolysed for 10 min in 1 M HCl at 60°C, stained with 1.0% aceto-orcein on the slide, slightly heated on a burner, and squashed. The slides were examined under an Axioskop2 plus microscope (Carl Zeiss). Photomicrographs of the metaphase plates were acquired using the same microscope equipped with an AxioCam HRc digital camera and using AxioVision 4.1 software (both Carl Zeiss).

Based on five or six photomicrographs at various foci for each karyotype, the karyotypes of *Micranthes nelsoniana* var. *insularis* from Paramushir Island, *M. fusca* from Bettobu, Iturup Island, and *M. nelsoniana* var. *insularis* from Onekotan Island were studied. According to Levan *et al.* (1964), the chromosome types were determined by comparing the length of the long arm to the short arm. The m type had an arm ratio from 1.0 to 1.7, the sm type had an arm ratio from 1.7 to 3.0, and the st type had an arm ratio from 3.0 to 7.0.

Herbarium study

A herbarium study was performed for three species: *M. nelsoniana*, *M. fusca*, and *M. ohwii*, focusing on the plants collected from the Kuril Islands and adjacent regions. Herbarium specimens of SAPS (Hokkaido University Museum) and VLA (Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences) were used in addition to the materials collected by us. A morphological analysis was performed primarily on the floral construction (petal forms and filament length) as well as on the characteristics of the leaves and capsules. Furthermore, flowering time and habitat were studied.

RESULTS

Phylogenetic analysis

All the studied samples are listed in Table 2 and their collection localities are indicated in Figure 1. The Bayesian phylogenetic trees are depicted in Figure 2 (left: ITS tree; right: cpDNA tree). They were obtained by applying the results of ModelTest selected by hLRT: HKY+G for ITS and GTR +I+G for combined cpDNA sequences. The ML phylogenetic trees are available in the Supporting Information. The length of the aligned matrix of all 59 unique ITS genotypes from the 268 samples was 633 bp, wherein 128 sites were variable (with 116 substitutions and 12 indels). Both the Bayesian and ML phylogenetic trees based on ITS sequences revealed that all the samples treated in this study, including those of *M. nelsoniana*, *M. fusca*, *M. ohwii*, *M. manchuriensis*, and *M. octopetala*, formed a large clade, with *M. japonica* and *M. spicata* as outgroups with a BI posterior probability (PP)/ML bootstrap value (BS) of 99/80. Within this clade, we noted clade A, which included five well-supported clades (i–v) (Fig. 2, left), In addition, outside clade A, a clade of *M. octopetala* (vii) and the rest of the samples (vi), including

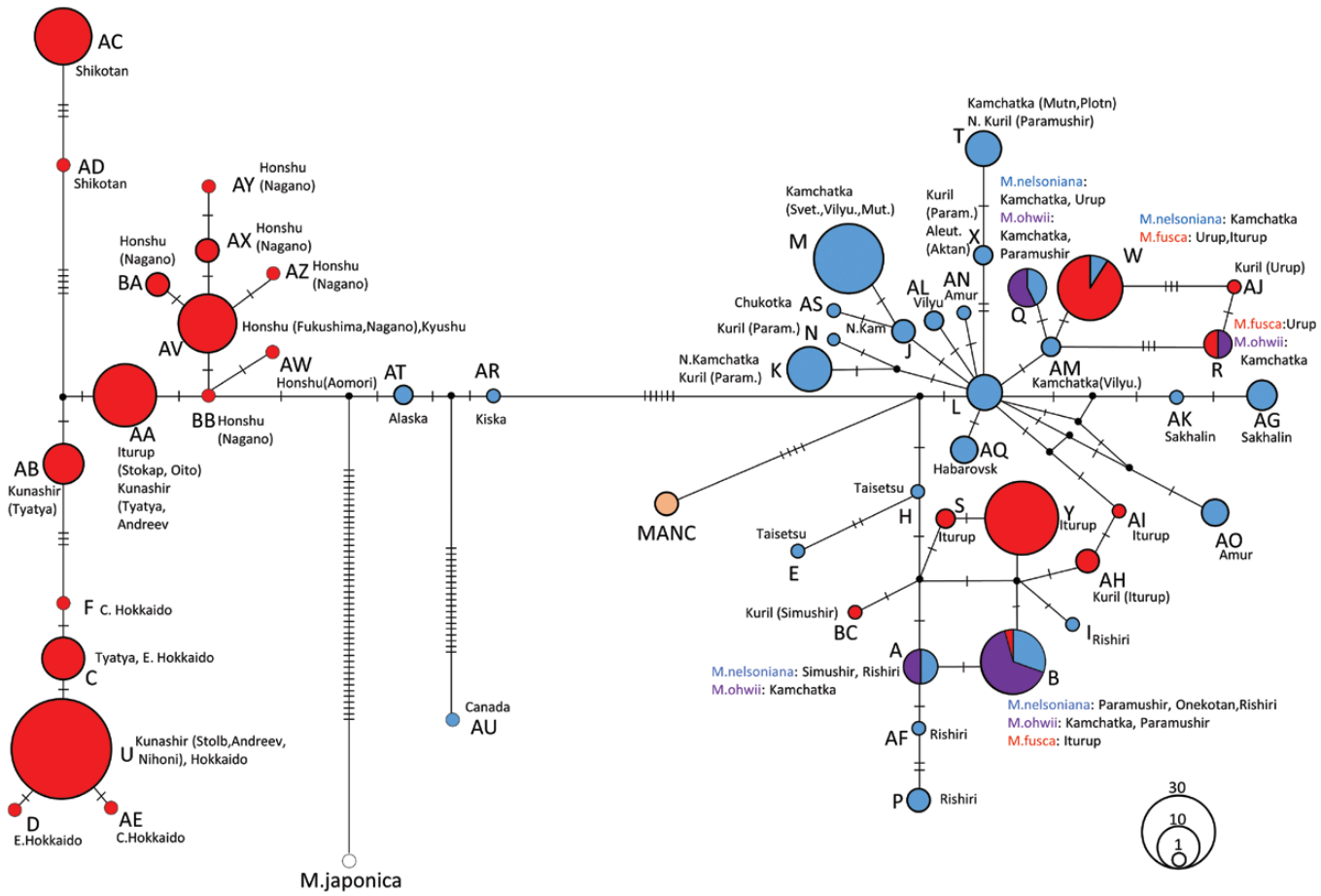

Figure 4. Haplotype network based on cpDNA. The colours are the same as in Figures 1 and 2. The short lines indicate mutations and indels.

M. nelsoniana and one sample of *M. purpurascens*, was found. In the Bayesian tree, vi + vii outside clade A did not constitute a clade; however, it formed two weak clades (BS < 50) in the ML trees: a clade of ITS-31 and ITS-32 (BS = 80) and another clade of other samples (BS < 50). In the Bayesian and ML trees, each clade of *M. manchuriensis* (iii) and *M. octopetala* (vii) formed a well-supported clade (PP/BS: 100/99 and 100/97, respectively). However, none of the other species, such as *M. nelsoniana*, *M. fusca*, and *M. ohwii*, formed monophyletic groups.

Some of the clades in clade A exhibited a distinct geographical construction (Fig. 3): i: *M. fusca* in the south Kuril Islands, with one sample of *M. purpurascens* (LM654396) from Kunashir (PP/BS: 100/78), although *M. purpurascens* may have been a misidentification because it does not occur in Kunashir; ii: *M. fusca* in Japan (partly Kunashir) (PP/BS: 100/87); and iii: *M. manchuriensis* in China and Primorye (PP/BS: 100/99). Conversely, iv exhibited a distant distribution in the highlands of Kamchatka and Hokkaido (PP/BS: 100/78), including *M. nelsoniana* from Kamchatka (Mt. Vilyuchinsky at 1000 m asl, 6 in Fig. 3: Mt. Mutnovsky at 1300 m asl, 7 in Fig. 3), *M. ohwii* from Kamchatka (Mt. Avacha at 1300 m asl, 1 in Fig. 3, on volcanic slopes), and *M. nelsoniana* from Hokkaido (Mt. Rishiri at 1700 m asl, 12 in Fig. 3; Mt. Taisetsu at 2000 m asl, 11 in Fig. 3). Samples from DDBJ, LM654359 and 654360, were identified as *M. fusca*, which may have been a misidentification because *M. fusca* does not occur in Kamchatka. Group v exhibited a large distribution, covering the Kuril Islands (from Iturup to the north) and the Kiska quadrangle in the Aleutian Islands (PP/BS: 100/91), including three species: *M. nelsoniana*, *M. ohwii*, and *M. fusca*. *Micranthes nelsoniana* var. *insularis* was included only in group v. *Micranthes fusca* was detected in three clades, i.e. i, ii, and v, and was not monophyletic. *Micranthes manchuriensis* was found along lowland rivers and comprised one clade with high BS values even after adding our samples from a different locality to those reported by Kim *et al.* (2015).

Within vi outside of clade A, some samples from the Arctic–boreal regions formed clades, such as Chukotka (ITS-24) and W. Taymyr (ITS-25) (PP/BS: 96/65), or Kamchatka (ITS-14, 27, 37, 38), Canada (ITS-35), and Alaska (ITS-36) (PP/BS: 95/61). In addition, we noted that the genotype of ITS-28 was shared among samples from distant localities, such as Amur (14 in Fig. 1), Khabarovsk (68 in Fig. 1), N. Kamchatka (5 in Fig. 1), Chukotka (71 in Fig. 1), Aktan (72 in Fig. 1: Aleutian Islands), Alaska (70 in Fig. 1), and Yukon (16 in Fig. 1).

In the Bayesian cpDNA phylogenetic tree (Fig. 2, right), the length of the aligned matrix of 59 haplotypes from 154 samples was 3590 bp, wherein 215 sites were variable (with 193 substitutions and 22 indels). In Bayesian and ML cpDNA analyses, the phylogenetic trees of the studied samples were also monophyletic (PP/BS: 100/100), with *M. japonica* and *M. sachalinensis* as outgroups. The large clade, with the exception of a sample from Canada (cp-59 haplotype), was divided into two groups: a clade

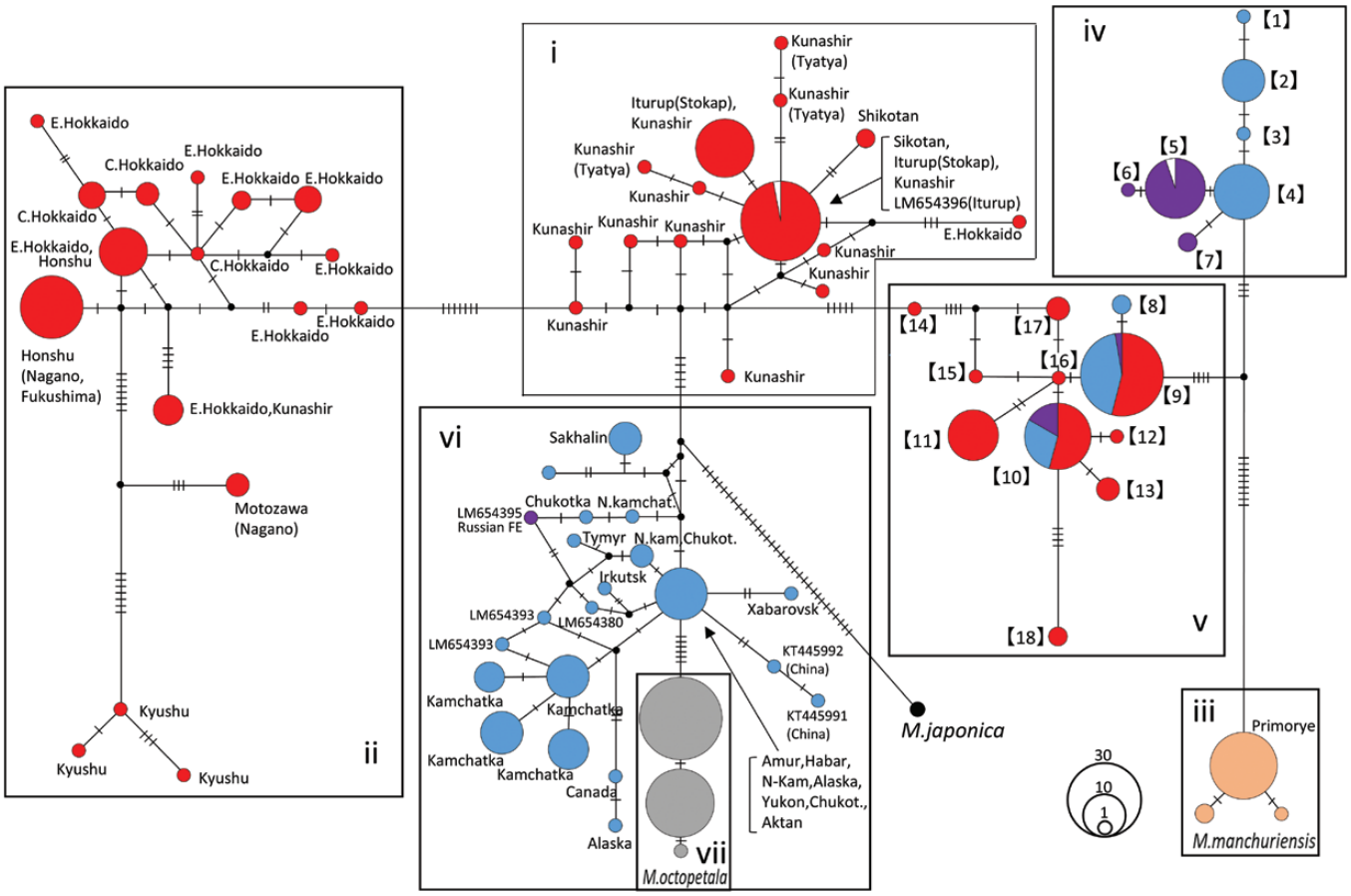

Figure 5. Haplotype network based on ITS clades. The colours are the same as in Figures 1 and 2. The short lines indicate mutations and indels. Each rectangle placed around the haplotypes indicates the range, corresponding to clade A (i, ii, iii, iv, and v) and the rest groups of the ITS phylogenetic tree (Fig. 2, left). Sectors without colour in the pie graphs in i and iv are possible misidentifications (as in Fig. 2). Numbers in square brackets, [1]–[18], correspond to the ITS haplotypes in Table 6.

comprising *M. fusca* from Honshu, Hokkaido, the south Kuril Islands, Alaska, and Kiska (PP/BS: 93/< 50) and another clade comprising *M. nelsoniana*, *M. ohwii*, *M. manchuriensis*, and a part of *M. fusca*, including samples from Iturup to Simushir (central Kuril) Islands (PP/BS: 98/73). The clade of *M. fusca* from the south of Iturup to Kyushu (star on the node in the cpDNA tree, Fig. 2) exhibited high PP/BS values (100/95). The cpDNA haplotype groups were more congruent with their geographical origin compared with the ITS groups, and most clades comprised geographically related samples.

Comparison of the ITS and cpDNA Bayesian phylogenetic trees revealed significant incongruence, as shown by lines indicating the correlation of each sample between them. In some cases, one ITS sequence corresponded to numerous chloroplast haplotypes (e.g. ITS-12 and ITS-28) and even to different species (e.g. ITS-4 and ITS-2). Conversely, there were few examples of one haplotype corresponding to several genotypes, such as cp-4 and cp-29.

Network analysis

The ITS network (see Fig. 5) revealed distinct groups corresponding to i–vii of the ITS phylogenetic tree. In the ITS network, *M. nelsoniana* of group vi also demonstrated a stellate shape, which in the central pie chart comprised samples from Amur, Khabarovsk, N. Kamchatka, Chukotka, Aktan, Alaska, and Yukon (14, 68, 5, 71, 72, 70, and 16 in Fig. 1, respectively). A large group of *M. fusca* identified via the cpDNA network on the left side (Fig. 4) corresponded to the two groups in the ITS network (Fig. 5) as follows: i: Kunashir–Shikotan/S. Iturup group near the centre and ii: Kyushu/Honshu/Hokkaido group on the left side (Fig. 5). On the right side, the highland group (iv) comprised two species: *M. nelsoniana* and *M. ohwii*; whereas the island group (v) included three species: *M. nelsoniana*, *M. fusca*, and *M. ohwii*.

Group iv of the ITS network included seven pie charts ([1]–[7]), corresponding to seven genotypes in which four types ([1]–[4]) were *M. nelsoniana* ([1]–[3] were from Rishiri). Another three types ([5]–[7]) were *M. ohwii* from Kamchatka.

Group v included 11 genotypes: three types ([8]–[10]) for *M. nelsoniana*, 10 types for *M. fusca*, and two types for *M. ohwii* ([9] and [10] from N. Kurils), two of which ([9], [10]) were shared by the three species. *Micranthes nelsoniana* in group v was mostly from the Kuril Islands, with the exception of plants from the Kiska quadrangle and Taisetsu, Hokkaido (Table 6). All *M. fusca* plants in group v were collected from Iturup (Oito: 38 in Fig. 3) to Urup (34 in Fig. 3). *Micranthes ohwii* in group v was included only in pie charts [9] and [10], and all of them were from Onekotan (2 in Fig. 3) in the northern Kuril Islands.

The cpDNA haplotype network (Fig. 4) revealed that two large groups were loosely combined. The distribution of *M. fusca* was primarily confined to the left side. Conversely, *M. nelsoniana* and other species were observed on the right side, showing a stellate shaped distribution with an L-type centre, including plants from Amur, Irkutsk, Kamchatka, and northern Kuril Islands. *Micranthes fusca* in the group on the right comprised plants from Iturup (Oito) to Simushir in the Kuril Islands. Some members of *M. nelsoniana*, *M. fusca*, and *M. ohwii* shared haplotypes.

We studied the correspondence between each sample in the ITS and cpDNA haplotype networks (Table 6). Plants in ITS groups iv and v corresponded to the right group of the cpDNA network, with the exception of one individual (haplotype AA from Oito, Iturup, in the ITS network [17]). Within the cpDNA network, the right group was roughly divided into several groups: e.g. the group with the centre of haplotype W (+AJ, R, AM, and Q) and the group with the centre of haplotype Y (+S, AH, and others). Some pie charts (W, Q, R, A, and B) were shared by both groups iv and v. When considering *M. fusca* alone, we found that *M. fusca* from Shana, Iturup (37 in Fig. 3), in the pie chart [9] and Bettobu, Iturup (36 in Fig. 3), in the pie chart [11] of the ITS network corresponded to Y and S and others (B, AJ) in the pie chart of the cpDNA network, whereas [10] and [12]–[18] (all from Oito, Iturup) corresponded to W, R, AH, and AI of the cpDNA network. The pie charts Y, S, AH, and AI were observed only in Iturup, although W and R were found in other localities.

Statistical analyses

The statistical comparison within the seven clades (i–vii) of the ITS phylogenetic tree is shown in Table 4. Clade ii ($\pi = 0.00608$) of *M. fusca* in Japan exhibited the highest nucleotide diversity. Group vi, including samples from a large area from China, eastern Siberia, Kamchatka, and Alaska, exhibited $\pi = 0.00436$, which was lower than that of group ii ($\pi = 0.00608$) of Japan. The π values obtained for *M. manchuriensis* ($\pi = 0.00045$) and *M. octopetala* ($\pi = 0.00163$) were lower than those detected in other populations.

Pairwise differences in the cpDNA populations, which were grouped according to the ITS clades (Table 5), revealed that the pairwise F_{ST} values between populations corresponding to iv (highlands of Kamchatka and Hokkaido) and v (Kuril and Aleutian Islands) were particularly low ($F_{ST} = 0.05970$, bold type in Table 5), indicating the absence of a significant genetic difference in these cpDNA populations despite the distinct differences observed in the ITS phylogenetic analysis. The pairwise value between i and ii was low (0.17075); nevertheless, both i and ii exhibited high pairwise values for other populations.

Cloning

The Bayesian phylogenetic tree obtained by applying the results of ModelTest: GTR+G (by hLRT) are shown in Figure 6. The ML phylogenetic tree based on the results of the ModelTest:HKY model had the same topology as in Supporting Information for the ML tree shown in Figure 2. The polymorphic sites in the sequences obtained by cloning are detailed in Table 7.

In the Bayesian phylogenetic trees, sequences R1 from Rishiri and T1 from Taisetsu were nested outside of all the remaining samples, forming a clade with *M. nelsoniana* from Sakhalin and *M. purpurascens* (ML654395, Russian Far East) with a PP of 60. In the ML tree, this clade was found within other samples oustide of clade A, with a BS value of 81. The R4 and R8 cloned sequences from Rishiri alongside T7 from Taisetsu were included in clade iv, the highland group from Kamchatka and Hokkaido, with PP/BS = 99/70. The T5 and T6 cloned sequences from Taisetsu were nested in group v comprising *M. fusca* (Urup, Iturup), *M. ohwii* (Onekotan), and *M. nelsoniana* (Simushir, Kiska, and Far East Russia), with PP/BS = 100/92.

Chromosome numbers

Photomicrographs of the somatic metaphase chromosomes of *M. nelsoniana* var. *insularis* from Paramushir (19 in Fig. 3) (Fig. 7A; $2n = 50$), *M. fusca* from Bettobu, Iturup (36 in Fig. 3) (Fig. 7B; $2n = 50$), and *M. nelsoniana* var. *insularis* from Onekotan (20 in Fig. 3) (Fig. 7C; $2n = 24$) and their karyotypes are shown in Figure 7. They have the following computed arm ratios: A: 24m + 1sm, B: 22m + 3sm, and C: 12m. The karyotypes in each of them steadily shrank in size without any obvious bimodality. Both of the karyotypes of *M. nelsoniana* var. *insularis* from Paramushir ($2n = 50$; Fig. 7A) and *M. fusca* from Bettobu ($2n = 50$; Fig. 7B) had sm pairs at the fourth pairs from the left of the karyotypes. For the smallest chromosome of *M. nelsoniana* var. *insularis* from Onekotan, one satellite was observed (Fig. 7C; $2n = 24$); however, owing to the small sample size, we were unable to confirm its existence in pairs.

The chromosome numbers studied and reported to date are listed in Table 8, with localities indicated using numbers, as in Figure 1. According to Table 8, high numbers of $2n = 80$–84 have been reported in Chukotka (29, 72, 27, and 71 in Fig. 1), Vrangel Island (north of Chukotka), Alaska, and Yukon. Conversely, chromosome numbers were low in south Kamchatka, Sakhalin, and partly in the northern Kuril Islands ($2n = 28$ and 30, 26, and 24, respectively). Along the Kuril Islands, $2n = 50$ was observed for *M. nelsoniana* from Paramushir Island and for *M. fusca* from Bettobu and Shana (36 and 37 in Fig. 3) of the central Iturup group. In Hokkaido and Honshu, high chromosome numbers were detected for *M. nelsoniana*: $2n = 50$ (Rishiri), $2n = 80$ (Taisetsu), and $2n = \sim100$ (99–104; central highlands in Honshu). *Micranthes fusca* from south Iturup (Stokap) to Honshu mostly had chromosome numbers of $2n = 30$, with some individuals ($2n = 45$ and 60) observed in the south Kuril Islands (Kunashir) and in one locality of Honshu ($2n = 45$).

Herbarium study

In the Kuril Islands, *M. nelsoniana* specimens were mostly found in lowlands in the central/north groups, whereas in Iturup, this species was found only in high mountainous areas: Mt. Nishi–Hitokap (Stokap), 1629 m asl; and Mt. Chirip (Bogdan–Khmeljnitskii), 1582 m asl. Among *M. nelsoniana*, we found plants that were identified as 'var. *insularis*', with red pistils and white-to-reddish petals, around the north of the Kuril Islands and in the Kiska quadrangle. *Micranthes fusca* from Japan to southern Iturup (Stokap) flowered in August–September and mostly occurred along rivers, exhibiting stable morphological characteristics, such as petals with reversed edges. *Micranthes fusca* from Iturup (Oito) to the north mostly flowered in July–August and grew in various habitats: along rivers, at the coast, on cliffs, and among tall shrubs; moreover, the plants often had large leaves with large teeth with unreversed flower petals. *Micranthes*

Table 4. Correlation of ITS with cpDNA haplotypes for groups iv and v of the ITS network.

ITS haplotype[a]	Species	cpDNA haplotype[b]	*N*	Locality	Locality no.[c]
[1]	*M. nelsoniana*	I	1	Rishiri (Hokkaido)	12
[2]	*M. nelsoniana*	A	2	Rishiri (Hokkaido)	12
	M. nelsoniana	B	2	Rishiri (Hokkaido)	12
	M. nelsoniana	P	3	Rishiri (Hokkaido)	12
	M. nelsoniana	AF	1	Rishiri (Hokkaido)	12
[3]	*M. nelsoniana*	B	1	Rishiri (Hokkaido)	12
	M. nelsoniana	Q	1	Urup (Kurils)	23
[4]	*M. nelsoniana*	E	1	Taisetsu (Hokkaido)	11
	M. nelsoniana	M	2	Mutnovsky (Kamchatka)	7
	M. nelsoniana	Q	2	Mutnovsky (Kamchatka)	7
	M. nelsoniana	T	3	Mutnovsky (Kamchatka)	7
	M. nelsoniana	T	1	Plotkinov (Kamchatka)	9
	M. nelsoniana	W	2	Mutnovsky (Kamchatka)	7
	M. nelsoniana	AL	2	Vilyuchinsky (Kamchatka)	6
	M. nelsoniana	AM	3	Vilyuchinsky (Kamchatka)	6
	M. nelsoniana	AN	1	Mutnovsky (Kamchatka)	7
[5]	*M. ohwii*	A	3	Avacha (Kamchatka)	1
	M. ohwii	B	8	Avacha (Kamchatka)	1
	M. ohwii	Q	3	Avacha (Kamchatka)	1
	M. ohwii	R	3	Avacha (Kamchatka)	1
[6]	*M. ohwii*	B	1	Avacha (Kamchatka)	1
[7]	*M. ohwii*	B	2	Avacha (Kamchatka)	1
[8]	*M. nelsoniana*	K	1	Paramushir (N. Kurils)	19
	M. nelsoniana	T	1	Paramushir (N. Kurils)	19
[9]	*M. nelsoniana*	B	1	Paramushir (N. Kurils)	19
	M. nelsoniana	B	2	Onekotan (N. Kurils)	20
	M. nelsoniana	K	8	Paramushir (N. Kurils)	19
	M. nelsoniana	L	1	Paramushir (N. Kurils)	19
	M. nelsoniana	N	1	Paramushir (N. Kurils)	19
	M. nelsoniana	T	1	Paramushir (N. Kurils)	19
	M. nelsoniana	AR	1	Kiska (Aleutian)	21
	M. fusca	S	1	Shana, Iturup (S. Kurils)	37
	M. fusca	Y	19	Shana, Iturup (S. Kurils)	37
	M. ohwii	B	1	Onekotan (N. Kurils)	2
[10]	*M. nelsoniana*	A	1	Simushir (S. Kurils)	22
	M. nelsoniana	B	1	Onekotan (N. Kurils)	20
	M. nelsoniana	H	1	Taisetsu (Hokkaido)	11
	M. nelsoniana	L	2	Paramushir (N. Kurils)	19
	M. nelsoniana	X	2	Paramushir (N. Kurils)	19
	M. fusca	R	1	Urup (S. Kurils)	34-1
	M. fusca	W	1	Urup (S. Kurils)	34-2
	M. fusca	W	9	Oito, Iturup (S. Kurils)	38
	M. fusca	AH	2	Oito, Iturup (S. Kurils)	38
	M. ohwii	B	3	Onekotan (N. Kurils)	2
	M. ohwii	Q	1	Onekotan (N. Kurils)	2
[11]	*M. fusca*	B	1	Dobrynin, Iturup (S. Kurils)	35
	M. fusca	S	1	Bettobu, Iturup (S. Kurils)	36
	M. fusca	Y	11	Bettobu, Iturup (S. Kurils)	36
	M. fusca	AJ	1	Urup (S. Kurils)	34-3
[12]	*M. fusca*	W	1	Oito, Iturup (S. Kurils)	38
[13]	*M. fusca*	W	2	Oito, Iturup (S. Kurils)	38

Table 4. Continued

ITS haplotype[a]	Species	cpDNA haplotype[b]	*N*	Locality	Locality no.[c]
	M. fusca	AH	1	Oito, Iturup (S. Kurils)	38
[14]	*M. fusca*	W	1	Oito, Iturup (S. Kurils)	38
[15]	*M. fusca*	W	1	Oito, Iturup (S. Kurils)	38
[16]	*M. fusca*	W	1	Oito, Iturup (S. Kurils)	38
[17]	*M. fusca*	W	1	Oito, Iturup (S. Kurils)	38
	M. fusca	AA	1	Oito, Iturup (S. Kurils)	38
	M. fusca	AI	1	Oito, Iturup (S. Kurils)	38
[18]	*M. fusca*	W	2	Oito, Iturup (S. Kurils)	38

[a]The numbers in square brackets are the same as in groups iv and v in the ITS network of Figure 5.
[b]The haplotypes are the same as in the cpDNA haplotype network of Figure 4.
[c]Locality numbers are the same as in Figure 1 and Table 1.

Table 5. Number of polymorphic sites, nucleotide diversity, and haplotype diversity for seven ITS clades (i–vii).

Clade no.	Species included	Locality	*N*[a]	*S*[b]	π[c]	SD	Hd[d]	SD
i	*M. fusca*	S. Kurils (Kunashir-S.Iturup)	65	18	0.00186	0.00039	0.575	0.06
ii	*M. fusca*	Japan (Kyushu-Hokkaido)	62	28	0.00608	0.00115	0.82	0.035
iii	*M. manchuriensis*	Primorye, China	28	3	0.00045	0.00019	0.267	0.107
iv	*M. ohwii, M. nelsoniana*	Kamchatka, Hokkaido	50	5	0.00208	0.00021	0.704	0.029
v	*M.fusca,M.nels.,M.ohwii*	S. Kurils – Aleutians	88	11	0.00218	0.00025	0.709	0.029
vi	*M. nelsoniana, M. ohwii*	Siberia–Kamchatka–Alaska	64	19	0.00436	0.00035	0.849	0.026
vii	*M. octopetala*	Korea	65	3	0.00163	0.00008	0.511	0.026

[a]*N*: number of the samples.
[b]S: number of variable sites.
[c]π: nucleotide diversity (per site).
[d]Hd: haplotype (gene) diversity.

Table 6. Population pairwise F_{ST} values for six cpDNA groups, defined by ITS clades (i–vi).

	i	ii	iii	iv	v	vi
i	0.00000					
ii	0.17075	0.00000				
iii	0.79787	0.83260	0.00000			
iv	0.71101	0.73914	0.53743	0.00000		
v	0.68414	0.69818	0.48807	**0.05970**	0.00000	
vi	0.74527	0.77218	0.68152	0.30320	0.25852	0.00000

Distance method: Kimura's 2-parameter. Value in bold (F_{ST} = 0.05970) indicates that pairwise F_{ST} values between populations corresponding to iv (highlands of Kamchatka and Hokkaido) and v (Kuril and Aleutian Islands) were particularly low.

fusca plants from Iturup (Oito) to the north were generally collected in August–September and exhibited well-grown capsules on the flowering branches. Only one specimen, collected from Oito, Iturup, flowered at the beginning of September and had a few inflorescence branches. This individual carried haplotype AA, the same as that of *M. fusca* from S. Iturup (Stokap) and Kunashir. *Micranthes ohwii* exhibited varying characteristics of leaf colour and lustre.

DISCUSSION

Phylogenetic analysis of the ITS sequences

Our results of phylogenetic analysis based on the rDNA ITS region in most cases revealed single sequences, which may be the result of concerted evolution (Liao 1999). Phylogenetic analyses of ITS sequences sometimes yield a poor resolution because of their limited variation (Holderegger and Abbott 2003); however, here we had sufficient intra- and interspecific variation.

Previous studies regarding the phylogenetic relationships between *M. nelsoniana* and its closely related species have indicated that this species is not monophyletic (e.g. Deng *et al.* 2015, Kim *et al.* 2015, Tkach *et al.* 2015, Stubbs *et al.* 2020a, b). Deng *et al.* (2015) reported that five species, i.e. *M. spicata*, *M. punctata*, *M. fusca*, *M. calycina*, and *M. nelsoniana* (including var. *porsildiana*), formed one clade. Tkach *et al.* (2015) found that five species, i.e. *M. nelsoniana* (including var. *porsildiana*), *M. purpurascens* (=*M. ohwii*), *M. fusca*, *M. manchuriensis*, and *M. spicata*, were monophyletic. Stubbs *et al.* (2020a, b) reported

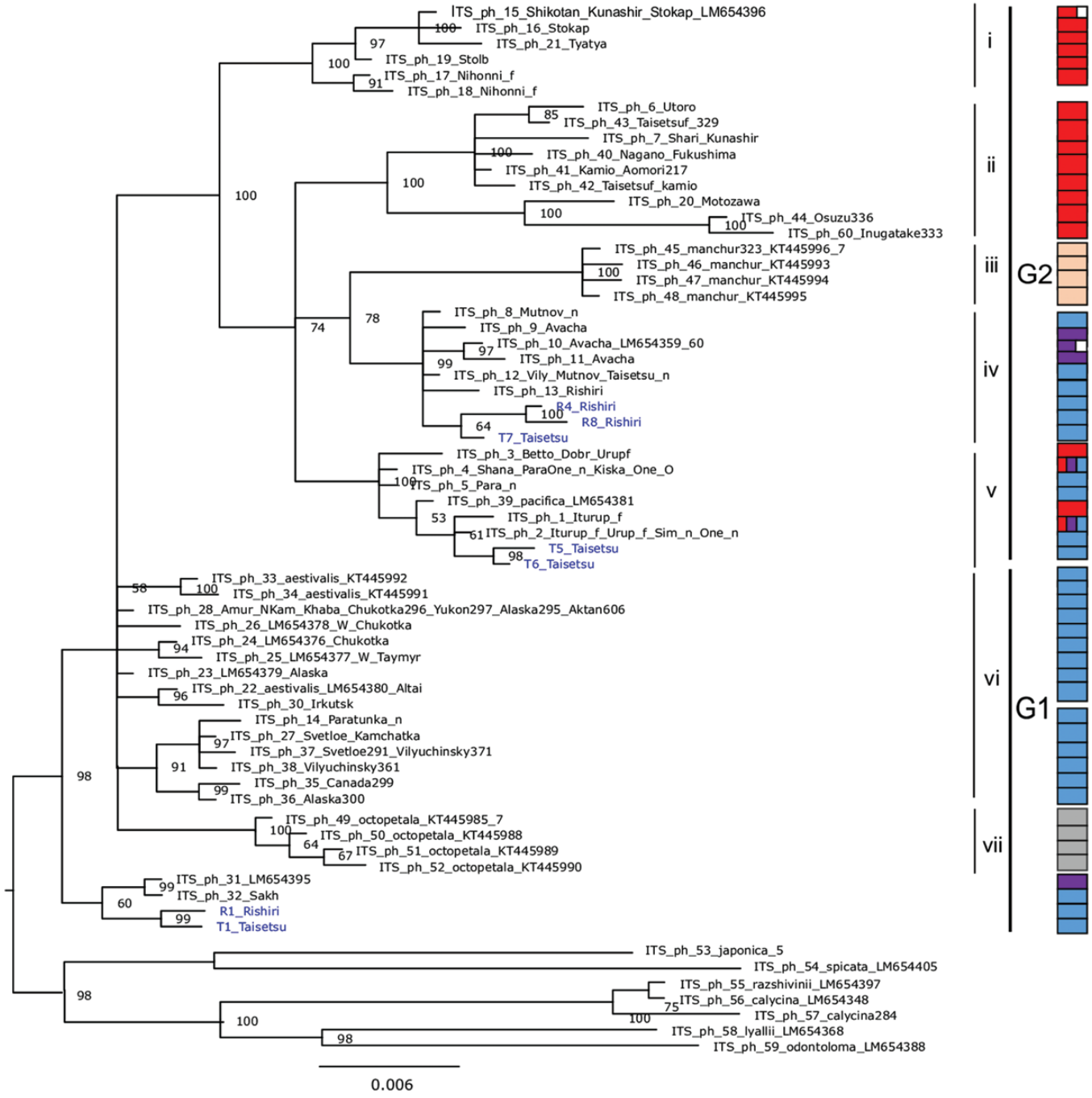

Figure 6. Bayesian phylogenetic tree based on the ITS region, with addition of the sequences obtained by the cloning of two samples from Rishiri ($2n = 50$) and two samples from Taisetsu ($2n = 80$) (shown in blue letters). This tree includes only cloned sequences, the presence of which was confirmed in the original sequences. Sequences from Rishiri are nested in iv (R4_Rishiri, R8_Rishiri) of clade A and outside this clade (R1_Rishiri). Sequences from Taisetsu are nested in iv (Taisetsu T7) and v (Taisetsu-T5, T6) of clade A and outside clade A (T1_Taisetsu). The blue bar is for M. nelsoniana, red for M. fusca, purple for M. ohwii, orange for M. manchuriensis, and grey for M. octopetala.

that *M. nelsoniana*, *M. manchuriensis*, and *M. fusca* formed one clade, with *M. spicata* and *M. japonica* as outgroups. In our Bayesian and ML phylogenetic tree based on the rDNA ITS sequence, *M. nelsoniana* and its varieties, *M. fusca*, *M. ohwii* (=*M. purpurascens*), *M. manchuriensis*, and *M. octopetala*, formed a monophyletic group, with *M. spicata* and *M. japonica* as outgroups. The phylogenetic position of the outgroups was similar to that reported by Stubbs (2020a, b) with regard to the clade of *M. calycina* and *M. razshivinii* (adding *M. unalaschensis* Micranthes unalaschcensis [Sternb.] Gornall & H.Ohba in their phylogenetic tree), *M. japonica* and *M. spicata*, and *M. lyallii* and *M. odontoloma* (Fig. 2).

Each variety of *M. nelsoniana* (Table 1) was often noted in the same clade (e.g. var. *nelsoniana* for Amur and N. Kamchatka; var. *reniformis* for Sakhalin). However, they often occurred in similar localities. In addition, var. *nelsoniana* nested in different clades (e.g. Svetloe in Kamchatka); thus, we could not confirm the genetic identity of the varieties.

Table 7. Polymorphic sites in sequences obtained by cloning, and confirmed sequences, from two samples each from Rishiri (Rishiri 1,2) and Taisetsu (Taisetsu 1,2). The polymorphic sites are drawn in green colour.

Mutation site(bp)				1	1	1	1	1	1	1	1	1	2	2	2	2	2	3	3	4	4	5	5	5	6	6	6	6	6	**Sequences obtained by cloning**		**Confirmed sequences**	
	4	5	7	2	2	3	3	6	6	7	7	9	1	3	4	6	8	0	8	3	7	4	5	8	3	5	5	5	7				
	8	7	0	3	8	6	8	1	7	0	8	9	4	7	1	5	9	0	4	7	8	7	1	6	8	1	5	8	0				
																														Rishiri_1	Rishiri_2	Rishiri_1	Rishiri_2
Rishiri-R1	A	T	AAAAAAAAAATT	A	T	T	G	G	T	G	G	A	G	-	G	G	T	T	C	-	T	T	T	A	G	G	C	G	A	3	0	○	○
Rishiri-R2	A	T	AAAAAAAAAATT	A	T	C	G	A	C	A	A	C	A	A	G	G	A	T	C	T	T	C	C	A	G	G	C	A	A	1	0	x	x
Rishiri-R3	T	T	AAAAAATATT	G	A	C	G	A	T	A	A	C	A	A	G	G	A	T	C	T	T	C	C	A	G	G	C	A	A	0	1	x	x
Rishiri-R4	T	T	AAAAAATATT	G	A	C	G	A	C	A	A	C	A	A	G	G	A	T	C	T	T	C	C	A	G	G	C	A	A	4	9	○	○
Rishiri-R5	T	T	AAAAAATATT	G	A	C	G	A	C	A	A	C	A	A	G	G	A	T	C	T	T	C	C	C	G	G	C	A	A	0	1	x	x
Rishiri-R6	T	T	AAAAAATATT	G	A	C	G	A	C	A	A	C	A	A	G	G	A	T	C	T	C	C	C	A	G	G	C	A	A	0	2	x	x
Rishiri-R7	T	T	AAAAAATATT	G	A	C	G	A	C	A	A	C	A	A	G	G	A	T	C	T	T	C	C	A	G	G	C	G	A	0	1	x	x
Rishiri-R8	T	T	AAAAAATATT	G	A	T	G	A	C	A	A	C	A	A	G	G	A	T	C	T	T	C	C	A	G	G	C	A	A	0	1	○	x
																														Taisetsu_1	Taisetsu_2	Taisetsu_1	Taisetsu_2
Taisetsu-T1	A	T	AAAAAAATTT	A	T	T	G	G	T	G	A	A	G	G	G	G	T	T	C	-	T	T	T	A	G	G	C	G	A	3	4	○	○
Taisetsu-T2	A	T	AAAAAAATT	A	T	T	G	G	T	G	A	A	G	G	G	G	T	T	C	-	T	T	T	A	G	G	C	G	A	4	1	x	x
Taisetsu-T3	A	T	AAAAAAAATT	A	T	T	G	G	T	G	A	A	G	G	G	G	T	T	C	-	T	A	C	A	C	G	C	G	A	0	1	x	x
Taisetsu-T4	A	T	AAAAAAAATT	A	T	C	A	A	C	C	A	C	A	G	T	G	T	C	C	T	T	A	C	A	C	G	C	G	A	0	1	x	x
Taisetsu-T5	T	T	AAAAAAATATT	G	T	C	A	A	C	C	A	C	A	G	T	G	T	C	C	T	T	A	C	A	C	G	C	G	A	3	3	○	○
Taisetsu-T6	T	T	AAAAAAATATT	G	T	C	A	A	C	C	A	C	A	G	T	G	T	C	C	T	T	A	C	A	G	G	C	G	A	1	4	○	x
Taisetsu-T7	T	T	AAAAAATATT	G	T	C	G	A	C	A	A	C	A	-	G	G	A	T	C	T	T	C	C	A	G	G	C	G	A	1	1	○	○
Taisetsu-T8	T	T	AAAAAATATT	G	T	C	G	A	C	A	A	C	A	-	G	G	A	T	T	T	T	C	C	A	G	G	C	G	A	1	0	x	x
Taisetsu-T9	T	A	AAAAAAATATT	G	T	C	G	A	C	G	A	C	A	G	G	-	T	T	C	T	T	A	C	C	G	A	T	G	T	0	1	x	x

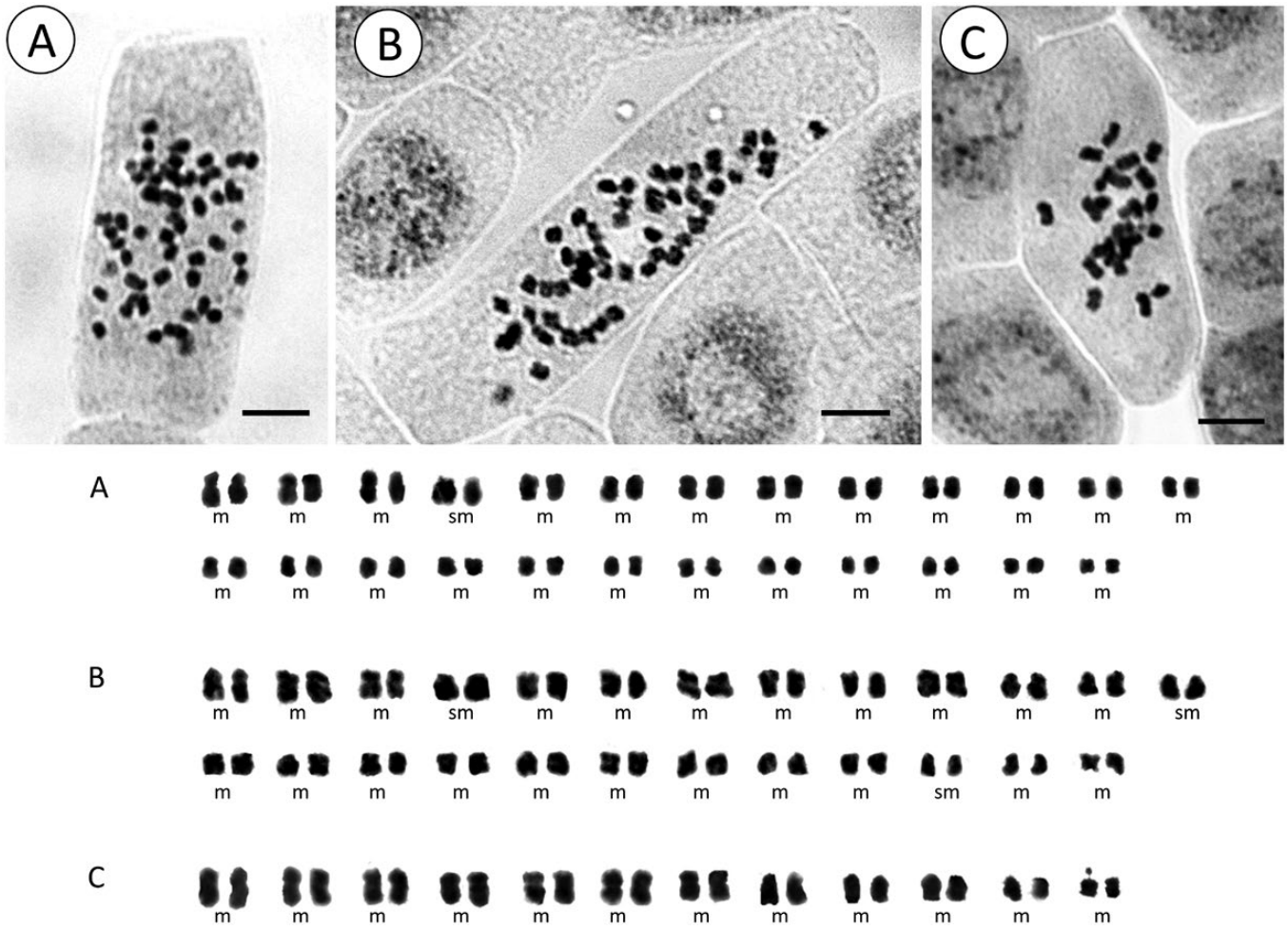

Figure 7. Photomicrographs of somatic metaphase chromosomes of *Micranthes* species. A. *Micranthes nelsoniana* var. *insularis* from Paramushir Island ($2n = 50$). B. *Micranthes fusca* from Bettobu, Iturup Island ($2n = 50$). C. *Micranthes nelsoniana* var. *insularis* from Onekotan Island ($2n = 24$). Scale bars = 5 μm. Karyotypes for A–C are shown below the photomicrographs. The arm ratio was calculated by dividing the length of the long arm by the length of the short arm; m: between 1.0 and 1.7; sm: between 1.7 and 3.0; and st: between 3.0 and 7.0.

M. nelsoniana in the continental clade

Our results indicated a large difference between continental and marginal populations within *M. nelsoniana*. In the continental group, *M. nelsoniana* is genetically distributed continuously in Siberia, lowland Kamchatka, and Alaska, whereas a different genetic group, distinct from the Arctic–continental group, occurred in the highlands of Kamchatka and Kuril/Aleutian Islands. Compared with the nucleotide diversity of *M. fusca*, that of the *M. nelsoniana* samples of the continental clade from eastern Siberia to Alaska was not high if we consider its large distribution area (Table 4). In some cases, the samples of *M. nelsoniana* from different localities from the Siberian Far East to the Aleutians shared identical ITS sequences (e.g. 'ITS-28' in Fig. 2), although they exhibited different cpDNA haplotypes. Some samples from the Arctic–boreal regions formed clades, such as Chukotka (ITS-24 in Fig. 2) and W. Taymyr (ITS-25), or Kamchatka (ITS-14, 27, 37, 38), Canada (ITS-35), and Alaska (ITS-36). Stubbs *et al.* (2020a) inferred that the ancestor of *Micranthes* had a circumboreal distribution. The extensive genetic exchanges that occurred among the populations of Arctic–continental Asia may have prevented the populations of *M. nelsoniana* from speciating as distinct species.

Distribution of the marginal group

The genetic difference of the marginal group, including the Kuril/Aleutian Islands, is reminiscent of the discussion regarding 'Hultenia (Tatewaki 1963, Yurtsev 1974)', the flora of which differs from that of the Bering region (in a narrow sense) where Arctic–continental-type plants are abundant (Yurtsev 1974). The marginal group is divided into distinct groups: the highland group; *M. fusca*; Kuril–Aleutian Islands; and Primorye.

Highland group

Although *M. nelsoniana* often grows along rivers or in wet meadows, plants of the highland group tend to occur on rocky slopes: *M. nelsoniana* from Rishiri, part of Mt. Vilyuchinsky, or on volcanic rocks for *M. ohwii* from Mt. Avacha (Table 2). The distribution of ITS group iv, comprising highland *M. nelsoniana* and *M. ohwii*, appeared to be divided by long distances in Hokkaido and Kamchatka. However, the herbarium study reported that *M. nelsoniana* from the high mountains of Iturup [e.g. Mt. Nishi–Hitokap (Stokap), 1629 m asl. and Mt. Chirip (Bogdan–Khmeljnitskii), 1582 m] are also included in this group. In addition, the highland plants of Iturup appeared

Table 8. Chromosome numbers of *M. nelsoniana* and related species

No. in Figure 1	Taxon	Locality	2*n*	Literature
—	*M. nelsoniana*	Vrangel Island	80	Zhukova *et al.* 1973, Zhukova & Petrovsky 1987
(around) 29, 27	*M. nelsoniana*	Chukotka	~60, 64, 80	Zhukova & Petrovsky 1987
(around) 29, 27	*M. nelsoniana* var. *porsildiana*	Chukotka	30	Zhukova *et al.*, 1973, 1987
(around) 28	*M. nelsoniana*	Arctic Siberia, NW Taymyr	28	Devyatov *et al.* 1997
(around) 26	*M. nelsoniana*	Alaska	~80, 84	Packer and McPherson 1974, Murray and Kelso 1997
(around) 16	*M. nelsoniana*	Yukon	84	Mulligan and Porsild 1969
(around) 16	*M. nelsoniana*	Yukon	28	Packer 1964
(around) 31	*M.nelsoniana*	Kamchatka, Azhabachiye	56	Zhukova et Petrovsky 1987
6	*M. nelsoniana*	Kamchatka, Mt.Vilyuchinsky	28	Fukuda *et al.* (2016a)
9	*M. nelsoniana*	Kamchatka, Plotnikov	28	Fukuda (unpublished)
10	*M. nelsoniana*	Kamchatka, Svetloe	28, 30	Fukuda *et al.* (2016a); unpubl.data
69	*M. nelsoniana*	Kamchatka, Paratunka	28	Fukuda (unpublished)
19	*M. nelsoniana* var. *insularis*	Kuril, Paramushir	50	Fukuda *et al.* (in this paper)
20	*M. nelsoniana* var. *insularis*	Kuril, Onekotan	24	Fukuda *et al.* (in this paper)
13	*M. nelsoniana*	Sakhalin, near Dolinsk	26	Fukuda *et al.* (2016a)
12	*M. nelsoniana*	Hokkaido, Mt. Rishiri	50	Funamoto & Nakamura 1996
11	*M. nelsoniana*	Hokkaido, Mt. Taisetsu	80	Fukuda et Ikeda 2019
(around) 51	*M. nelsoniana*	Honshu, Nagano, 3000 m asl	99–104	Fukuda et al. 2022
1	*M. ohwii*	Kamchatka, Mt. Avacha	24	Fukuda et al. 2014
35	*M. fusca*	Kuril, Iturup, Dobrynin	40–50	Fukuda (unpublished)
36	*M. fusca*	Kuril, Iturup, Bettobu	50	Fukuda *et al.* (in this paper)
37	*M. fusca*	Kuril, Iturup, Shana	50	Fukuda (unpublished)
38	*M. fusca*	Kuril, Iturup, Oito	40-50	Fukuda (unpublished)
39	*M. fusca*	Kuril, Iturup, Stokap	30	Fukuda (unpublished)
40	*M. fusca*	Kuril, Kunashir, Tyatina	30, 45	Fukuda (unpublished)
41	*M. fusca*	Kuril, Kunashir, Stolbovsky	30 (45, 60)	Fukuda *et al.* 2014
42	*M. fusca*	Kuril, Kunashir, Andreevka	30	Fukuda *et al.* 2014
44	*M. fusca*	Kuril, Shikotan	30	Fukuda *et al.* 2014
48	*M. fusca*	Hokkaido, Kamioboro	30	Fukuda *et al.* 2014
47	*M. fusca*	Hokkaido, Hirayama	30	Fukuda *et al.* 2014
—	*M. fusca*	Honshu, Iwate, Hiratsudo	30	Fukuda (unpublished)
—	*M. fusca*	Honshu, Iwate, Shizukuishi	45	Fukuda (unpublished)
50	*M. fusca*	Honshu, Fukushima	30	Fukuda *et al.* 2014
51	*M. fusca*	Honshu, Nagano	30	Fukuda *et al.* 2014
—	*M. fusca*	Honshu, Mie	30	Fukuda (unpublished)
55	*M. manchuriensis*	Russia, Primorye, Lazo	30	Fukuda et al. 2016c

to grow on mountain rocks, according to the herbarium label information.

The ITS phylogenetic tree (Fig. 2) indicates that *M. ohwii* is not monophyletic and that it splits twice from the pie chart of *M. nelsoniana* [4] of the ITS network. Conversely, Voroshilov (1982) reported that *M. purpurascens* (=*M. ohwii*) can be considered a well-delineated species as it can be identified even in sympatric occurrence with *M. nelsoniana*. Regarding the taxonomic status of *M. ohwii*, further discussion is necessary following the collection of additional samples from its central distribution area in Kamchatka.

Micranthes fusca

The distribution pattern of *M. fusca* was from Kyushu to the southern Kuril Islands, similar to the endemic pattern in Japan. Based on unique ITS and cpDNA clades, morphological traits, and constant chromosomal numbers (2*n* = 30, rarely 45 and 60), *M. fusca* from Japan to Stokap (Iturup) was considered a separate species that differed from the plants from Oito (Iturup) to the north. However, within the ITS phylogenetic tree, *M. fusca* was divided into two groups (i and ii) (Fig. 2). Morphologically, both of them were identified as *M. fusca* without essential differences. The occurrence of incomplete lineage sorting (ILS), wherein the genetic relationship does not reflect species

phylogeny, cannot be rejected. In the ITS phylogenetic tree (Fig. 2), clade ii occurred following the formation of clade i, suggesting its occurrence in the southern Kuril Islands, followed by a range expansion into the Japanese archipelago. Within *M. fusca*, large genetic distances were observed between groups i and ii (Fig. 5). The genetic contents of group ii appeared to have differentiated following isolation from group i. However, between groups i and ii, genetic exchange occurred given the the low pairwise difference between them (Table 5). Group ii exhibited high diversity. In particular, we noted the distance between Motozawa (Nagano) and Kyushu and other populations (Fig. 5). *Micranthes fusca* often occurred in the northern part of Japan (e.g. Hokkaido) or in the highlands of Japan (~1500 m asl in Honshu), and these populations appeared isolated and to have undergone genetic drift.

The northern limit for *M. fusca* remains ambiguous owing to the hybridization at the northern edge of its distribution. The northern limit of distribution for East Asian plants occurring around Japan generally fell between Urup and Simushir (at the Bussol strait, Fig. 1), according to a floristic comparison (Barkalov 2000, 2009), with many exceptional cases: as far north as Kunashir (e.g. *Acer japonicum* Thunb., *Quercus dentata* Thunb.), as Iturup (e.g. *Hydrangea paniculata* Siebold, *Quercus crispula* Blume). Nevertheless, the distribution of *M. fusca* was confirmed at least up to Stokap, Iturup Island.

Kuril/Aleutian Islands

Group v formed one large group along the Kuril/Aleutian Islands, comprising three species: *M. nelsoniana*, *M. fusca*, and *M. ohwii*. In this group, hybrid *M. fusca* with *M. nelsoniana* occurred in the southern part of the Kuril Islands (Iturup/Simushir) and *M. nelsoniana* occurred from Urup to the Kiska quadrangle. *Micranthes ohwii* with red flowers occurs in Paramushir and Onekotan of the North Kuril Islands. In the northern part of the Kuril Islands and in the Kiska quadrangle, individuals with dark-red pistils and reddish petals were found at high frequency, and were identified as *M. nelsoniana* var. *insularis*. These individuals will be discussed in the following sections.

The distribution pattern of group v was similar to that of the 'North Pacific' type (Barkalov 2009), such as *Arnica unalascensis* Less. (Asteraceae) and *Campanula chamissonis* Fed. (Campanulaceae: Roquet *et al.* 2009), which are known to be wind-dispersed. The Kuril Islands have had no continuous land bridge during their long existence (Barkalov 2009). However, the plants detected in group v in this study were distributed over the largest straits, and each cpDNA haplotype in the network revealed a large distribution area, such as haplotype B in Kamchatka–Paramushir–Onekotan–Iturup–Rishiri (Table 6), suggesting the occurrence of intensive seed dispersal along the islands.

Seed dispersal in *Saxifraga* (*sensu* Webb and Gornall 1989, including *Micranthes*) does not occur via a specialized mechanism; instead, its seeds are dispersed by wind or water, including water from melting snow (Webb and Gornall 1989). *Micranthes ohwii* (=*M. purpurascens*), occurring in Kamchatka and the north Kuril Islands, grows on the slopes of volcanic mountains and is considered a 'pioneer' plant (Barkalov 2009). The seeds of the three *Micranthes* species are similar: fusiform and < 1 mm in length with ribs (Hara 1939, Ohwi 1953, Charkevecz 1989). These facts suggest that gene exchange among the *Micranthes* species along the islands was promoted by wind dispersal.

Primorye

Micranthes manchuriensis occurs in northern China, Korea, and Primorye. In Primorye, it grows at a similar latitude as that of highland *M. nelsoniana* in Hokkaido (at 1700–2000 m asl) but occurs abundantly along lowland rivers. *Micranthes manchuriensis* was found within the marginal groups and different from *M. octopetala*, which appears divided from continental *M. nelsoniana* at its southern limit of distribution, growing on humid rocks and in the mountains of Korea (Nakai 1918). Phylogenetic and network analyses (Figs 2, 5) showed their independence from other species. Kim *et al.* (2015) discussed the genetic differences between *M. manchuriensis* and *M. octopetala* and described their phylogenetic relationship with other *Micranthes* species. Our results support their descriptions.

Relationship and hybridization among *M. nelsoniana* and related species

Comparison of the ITS and cpDNA phylogenetic trees and networks revealed that three species, i.e. *M. nelsoniana*, *M. fusca*, and *M. ohwii*, sometimes shared haplotypes, especially around the Kuril Islands. The sharing of the same or similar haplotypes among the different species suggests the possibility of hybridization or ILS (Rieseberg and Soltis 1991, Soltis and Kuzoff 1995, Comes and Abbott 2001), and it is usually difficult to distinguish between these events (Fork *et al.* 2017). However, the cpDNA network obtained here (Fig. 4) was divided into two parts: *M. fusca* on the left and *M. nelsoniana* and others on the right, which were markedly different without intermediate haplotypes. For ILS, intermediate haplotypes may not have disappeared completely. In addition, *M. fusca* in the right group with different morphological characteristics and habitats compared with the 'real' *M. fusca* in the left group occurred in contact with *M. nelsoniana* in the central Kuril Islands. Hybridization normally coincides with the sympatric occurrence of species (Goetze *et al.* 2017); thus, a hybrid origin for *M. fusca* in the right group is suggested. Based on these observations, at least some hybridization appears to have occurred in group v.

Hybrid type of Micranthes fusca

To date, *M. fusca* from southern Iturup (Oito) to Simushir has been identified as '*M. fusca*' without exception (e.g. Charkevicz 1989, Barkalov 2009). However, it differed from the 'real' *M. fusca* (from Japan to Stokap, Iturup) in morphology and in habitat. The 'real' *M. fusca* has petals with reflected margins, flowers in August–September, and grows along rivers or streams. However, the putative hybrid type has petals without reflection; has larger plant, leaf, and capsule sizes; and grows in various habitats, such as along the coast and in meadows other than along rivers. These plants have a chromosome number of $2n = 50$ at least in two localities in Iturup (Bettobu, Shana) and we observed an undefined number ($2n = 40–50$) for the remaining two localities (Oito; Dobrynin, 35 in Fig. 3).

One plant, which was collected on 4 September from Oito, was at the beginning of the flowering stage. This flowering timing was similar to that of the 'real' *M. fusca*. The specimen exhibited ITS type [17] (Fig. 5) and cpDNA haplotype 'AA'

(Fig. 4), suggesting that the maternal lineage of the hybrid was *M. fusca*. Conversely, other plants already had fruits in August and exhibited haplotypes within the right side of the network (Fig. 4), indicating that they have *M. nelsoniana* or its relatives as a maternal lineage and that they were formed due to introgressive hybridization from *M. nelsoniana* to *M. fusca*. As only one specimen of a plant with the AA haplotype was found, hybrids with *M. fusca* as the maternal lineage appear less adaptive. In addition, this specimen had fewer branches and flowers on inflorescences compared with the remaining specimens, which had a large number of capsules and numerous seeds. The latter may have greater chances of inbreeding owing to a similar flowering time (July–August) and abundant seeds.

Among the hybrid plants of *M. fusca* with *M. nelsoniana* as the maternal lineage, at least 10 ITS types ([9]–[18]; Fig. 5) and two lineages of cpDNA (haplotypes W, AJ, R/haplotypes Y, S, AH, AI; Fig. 4) were related. These hybrids inhabited various habitats. Among the plants from Iturup, those from Shana (37 in Fig. 3) with pie circle [9] and plants from Bettobu (36 in Fig. 3) with pie circle [11] shared cpDNA haplotypes Y and S (Fig. 4), which are endemic haplotypes in Iturup, with both of them occurring on coastal rocks. Conversely, plants from Oito (38 in Fig. 3), with pie circles [10] and [12]–[18], exhibited haplotypes W, R, AH, and AI (Fig. 4) and were observed along rivers. Oito is the southern limit for the hybrid *M. fusca*, and the large number of genotypes detected indicates that new hybrid combinations were intensively produced here before the occurrence of stable hybrids.

With huge capsules and numerous seeds, most hybrid *M. fusca* plants from Iturup ($2n = 50$ or 40–50) appeared viable. Consequently, they could serve as effective instances of chromosomal rearrangement following hybridization (Rieseberg 2001).

Micranthes nelsoniana and its relatives along the Kuril/Aleutian Islands

Micranthes nelsoniana var. *insularis*, which is distributed in the Kuril/Aleutian Islands, is defined by floral characteristics such as dark-red pistils and petals with white-to-reddish colours (Hultén 1936). Although its distribution range has been discussed (N. Kurils, Urup, and Iturup by Siplivinsky 1976; in all the Kuril Islands by Charkevicz 1989, Barkalov 2009), plants with such characteristics are widely observed in the northern Kuril Islands, such as Paramushir and Onekotan where *M. ohwii* with red flowers also occurs. *Micranthes nelsoniana* from Simushir to the Kiska quadrangle were included in ITS group v (Fig. 5) with many of them sharing the ITS haplotypes (pie circles [9] and [10]) with *M. fusca* and *M. ohwii*. Three *Micranthes nelsoniana* var. *insularis* individuals from Onekotan (two from [9] and one from [10]; Table 6) were included in type-B of the cpDNA network (Fig. 4), sharing a haplotype with *M. ohwii*. As two of these plants had a chromosome number of $2n = 24$ (Fig. 7C), the same as that of *M. ohwii* from Kamchatka (Mt. Avacha, 1 in Fig. 3; $2n = 24$, Table 8), it is possible that the two species undergo interbreeding. This result indirectly supports the observation of transitional forms between *M. nelsoniana* var. *insularis* and *M. ohwii* in this region (Voroshilov 1982).

Among *M. nelsoniana* var. *insularis* plants, we also found an individual with a chromosome number of $2n = 50$ (Fig. 7A; ITS type [8]), which fell into type-B of the cpDNA network. In addition, two individuals of *M. nelsoniana* from Mt. Rishiri (ITS type [2]) with $2n = 50$ shared type-B (Table 6). Moreover, haplotype B includes one hybrid *M. fusca* from Dobrynin, Iturup ($2n = 40$–50), and type-B is nested near the Y haplotype (Bettobu, Shana) of the hybrid *M. fusca*, with a chromosome number of $2n = 50$ (Table 8). The chromosome number $2n = 50$ for *M. nelsoniana* and *M. fusca* is unique around the Kuril Islands and has not been reported in other localities. It is possible that these plants with a chromosome number of $2n = 50$ and 24 promoted interspecific hybridization. The similarity of the karyotypes of *M. nelsoniana* var. *insularis* from Paramushir ($2n = 50$; Fig. 7A) and *M. fusca* from Bettobu, Iturup ($2n = 50$; Fig. 7B) also supports this possibility.

Relationship of the Hokkaido highlands with Sakhalin

Within the ITS network (Fig. 5), group iv included plants collected at high elevation in Hokkaido and Kamchatka. The results of cloning revealed that both individuals from Rishiri ($2n = 50$) and Taisetsu ($2n = 80$) in Hokkaido possessed ITS sequences similar to those from Sakhalin (R1, T1: Fig. 6) and that individuals from Taisetsu exhibited ITS sequences similar to those from the Kuril/Aleutian Islands (T5, T6: Fig.6). The results indicated that these plants had genes with different origins: continental and marginal. Although taxonomically both plants are attributed to *M. nelsoniana* var. *reniformis* (Ohwi 1933 as '*Saxifraga reniformis* Ohwii'; type: Sakhalin, Tosso; Table 1), they grow in different habitats; plants from Rishiri and Taisetsu grow in alpine habitats (1700 and 2000 m asl, respectively), whereas those from Sakhalin grow along lowland rivers under the forest line. According to the cpDNA haplotype network (Fig. 4), both plants from Rishiri (A, B, I, P, AF) and Taisetsu (E, H) were nested near haplotypes from the southern Kuril Islands, far from the haplotypes of Sakhalin (AG, AK), suggesting that the maternal plants of Rishiri and Taisetsu occurred locally or in relation to the Kuril Islands or Kamchatka highlands rather than originated due to migration from Sakhalin.

Micranthes nelsoniana in Alaska and adjacent regions

In the cpDNA haplotype network (Fig. 4), most *M. nelsoniana* plants were included in the right group alongside *M. ohwii* and the hybrid *M. fusca*. However, among *M. nelsoniana*, haplotypes AT (Alaska and Yukon) (17 and 16 in Fig. 1), AU (Canada) (18 in Fig. 1), and AR (Kiska quadrangle) (21 in Fig. 1) were found to be exceptionally close to the *M. fusca* group on the left side of the network, indicating the ancient hybridization of *M. nelsoniana* with the ancestral *M. fusca* and capturing the chloroplast genome of the latter. Ikeda *et al.* (2018) reported the range expansion and colonization of *Phyllodoce aleutica* from Japan to the Aleutian Islands and Alaska. Our results reveal that a partly similar event occurred for *M. nelsoniana*, whose population from Far East Asia affected plants of the Aleutian Islands and Alaska.

Chromosome numbers

As previously mentioned, chromosomes $2n = 24$ and 50 were found among different species in the Kuril Islands and they encouraged genetic exchange between similar and unrelated species. The varying chromosomal numbers of *M. nelsoniana* ($2n = 24$, 26, 28, 30, and others) and allied species (*M. fusca*: $2n = 30$; *M. ohwii*: $2n = 24$) and/or structural chromosome

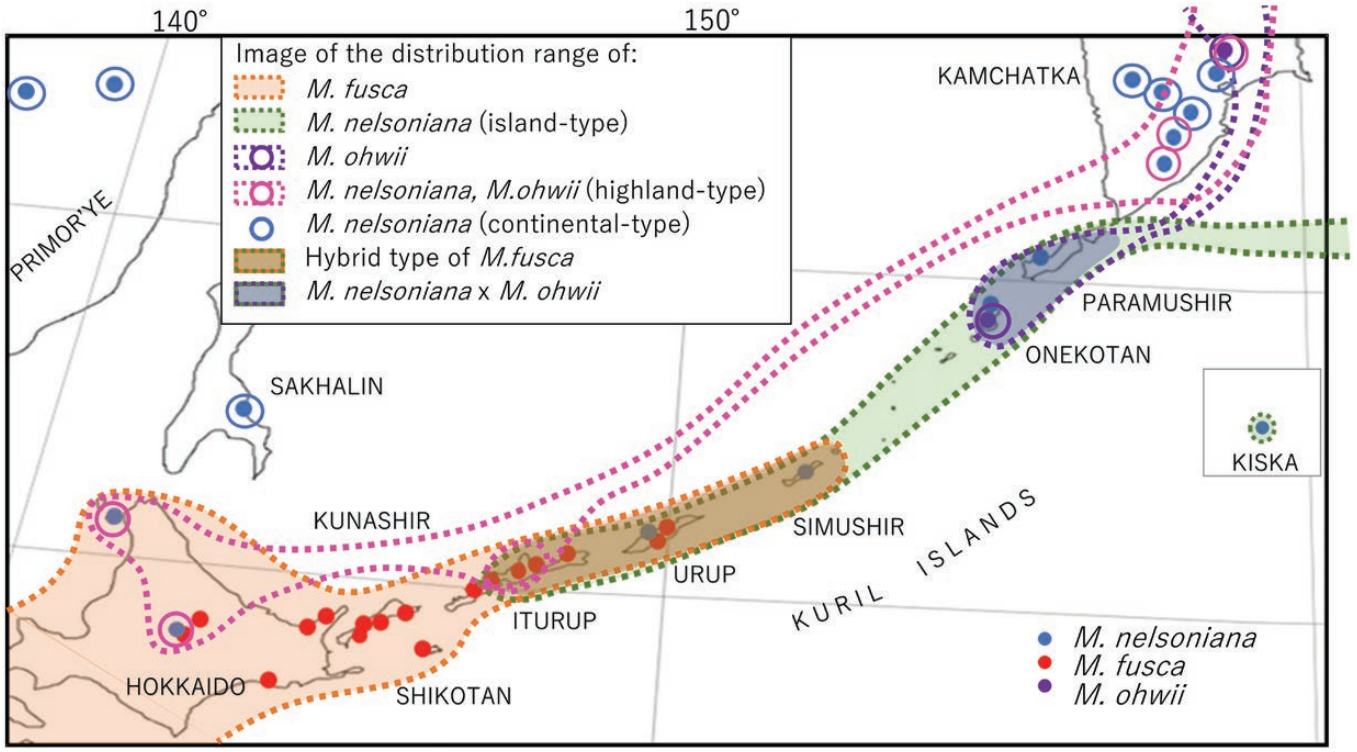

Figure 8. The distribution ranges of *M. nelsoniana* and related species/taxa. Coloured lines indicate species or intraspecific taxa: orange: *M. fusca*, green: island type of *M. nelsoniana*, purple: *M. ohwii*, pink: highland type of *M. nelsoniana* and *M. ohwii*, blue: continental type of *M. nelsoniana*, orange + green: hybrid, including various genetic assemblage, mostly introgressive hybridization from *M. nelsoniana* to *M. fusca*, green + purple: putative hybrid zone, where *M. nelsoniana* and *M. ohwii* occur, sharing cpDNA haplotypes and chromosome number ($2n = 24$).

modifications, including deletion or translocation, may help to explain the occurrence of $2n = 50$.

Study of the chromosome numbers of *M. nelsoniana* revealed that plants from Sakhalin ($2n = 26$) and southern Kamchatka ($2n = 28, 30$) were diploids, whereas larger chromosome numbers ($2n = 56, 72, 80, 84$, and others) were detected in northern Kamchatka and Chukotka (Table 8). Increases in chromosome numbers and polyploidization with increasing latitude have been reported for other plants (e.g. Brochmann *et al.* 2004). However, there are some exceptions, such as the observation of $2n = 30$ for *M. nelsoniana* var. *porsildiana* from Chukotka and Arctic regions (Zhukova *et al.* 1973, 1987) and $2n = 28$ from Taymyr, which faces the Arctic sea (Devyatov *et al.* 1997). These results indicate that an increase in chromosome numbers or polyploidization cannot be easily discussed in relation to latitude.

The occurrence of *M. nelsoniana* in Japan is highly limited, and chromosome number increases to the south: $2n = 50$ for Rishiri, $2n = 80$ for Taisetsu, and $2n = \sim100$ (99–104) for the central high mountains of Honshu. The first two individuals had genes of different origins as we have discussed in a previous section. Such changes in chromosome numbers, including different types of genes, may be adaptive for the growth of these plants, occurring at the edge of the distribution range of *M. nelsoniana*.

CONCLUSION

Micranthes nelsoniana occurs widely in the circumboreal region from Siberia to North America and appears to exhibit active gene flow around the Arctic–continental region of Eurasia/Alaska. Conversely, unique genetic groups, comprising *M. nelsoniana* and closely related species, are formed along the marginal island regions in Northeast Asia (Fig. 8): the highland group, including Kamchatka and Hokkaido; the *M. fusca* group from Japan to the southern Kuril Islands; the Kuril/Aleutian group; and Primorye. *Micranthes nelsoniana* makes contact with *M. fusca* in the central part of the Kuril Islands, resulting in several hybrid plants. A hybrid appears to occur between *M. ohwii* in the north of the Kuril Islands. Some of these hybrids shared the same chromosome numbers (e.g. $2n = 24$ and 50), which appeared to promote hybridization. Cloning experiments of the highland plants of Hokkaido with large chromosome numbers revealed that the sequences included both Arctic–continental and island elements, suggesting active genetic exchange in this region. Our study revealed a significant role of the marginal islands of Northeast Asia in the genetic diversity of *M. nelsoniana* and related species and the possible formation of hybrid taxa via genetic recombination among these species.

SUPPLEMENTARY DATA

Supplementary data are available at *Botanical Journal of the Linnean Society* Journal online.

DATA AVAILABILITY

The data underlying this article are available in the article and in its online supplementary material except for the nucleotide data, which will be shared on reasonable request to the corresponding author.

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CONFLICTS OF INTEREST

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