Phytotaxa 373 (1): 053–070
http://www.mapress.com/j/pt/

Article

ISSN 1179-3155 (print edition)
PHYTOTAXA
ISSN 1179-3163 (online edition)

https://doi.org/10.11646/phytotaxa.373.1.2

Molecular and cytological evidences denied the immediate-hybrid hypothesis for *Saxifraga yuparensis* (sect. *Bronchiales*, Saxifragaceae) endemic to Mt. Yubari in Hokkaido, northern Japan

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Abstract

An alpine plant *Saxifraga yuparensis* is endemic to a scree consisting of greenschist of Mt. Yubari in Hokkaido, Japan and it has been proposed as an immediate hybrid derived from two species of the same section *Bronchiales* based on morphological intermediacy: namely *S. nishidae*, a diploid species endemic to a nearby cliff composed of greenschist and tetraploid *S. rebunshirensis* comparatively broadly distributed in Japan and Russian Far East. *Saxifraga yuparensis* is red-listed and it is crucial for conservation planning to clarify whether this is an immediate hybrid and lacks a unique gene pool. The immediate-hybrid hypothesis was tested by molecular and cytological data. In nuclear ribosomal and chloroplast DNA trees based on maximum parsimony and Bayesian criteria, *S. yuparensis* and *S. rebunshirensis* formed a clade with several other congeners while *S. nishidae* formed another distinct clade. Genome-wide SNP data clearly separated these three species in principal coordinate space, placing *S. yuparensis* not in-between of *S. rebunshirensis* and *S. nishidae*. Chromosome observation indicated that *S. yuparensis* is tetraploid, not triploid directly derived from diploid-tetraploid crossing. Additionally, observation of herbarium specimens revealed that leaf apex shape of *S. yuparensis* fell within the variation of *S. rebunshirensis*. These results indicate that *S. yuparensis* is not an immediate hybrid of *S. rebunshirensis* and *S. nishidae* but a distinct lineage and an extremely narrow endemic species, that deserves for intensive conservation.

Keywords: Alpine plant, *Bronchiales*, diabasic plant, genome-wide SNP, hybrid, Japan, MIG-seq, Russia, *Saxifraga*, tetraploid

Introduction

The genus *Saxifraga* L. (1753: 398) (Saxifragaceae, e.g., Soltis *et al.* 2001) encompasses more than 440 species and is composed of at least 13 sections and nine subsections (Tkach *et al.* 2015). One of the *Saxifraga* sections, sect. *Bronchiales* DeChaine E.G. (2014: 27) was subdivided from sect. *Trachyphyllum* Koch W.D.J. (1836: 270) based on molecular phylogenetic analyses (DeChaine *et al.* 2013, DeChaine 2014). This section has approximately 20 species (DeChaine *et al.* 2013, DeChaine 2014) mostly in northern high latitudes, and among them, three species are distributed in Hokkaido, northern Japan: *S. yuparensis* Nosaka (1974: 149), *S. rebunshirensis* Sipliv. (1971: 155), and *S. nishidae*

Accepted by Nicodemo Passalacqua: 7 Sept. 2018; published: 23 Oct. 2018

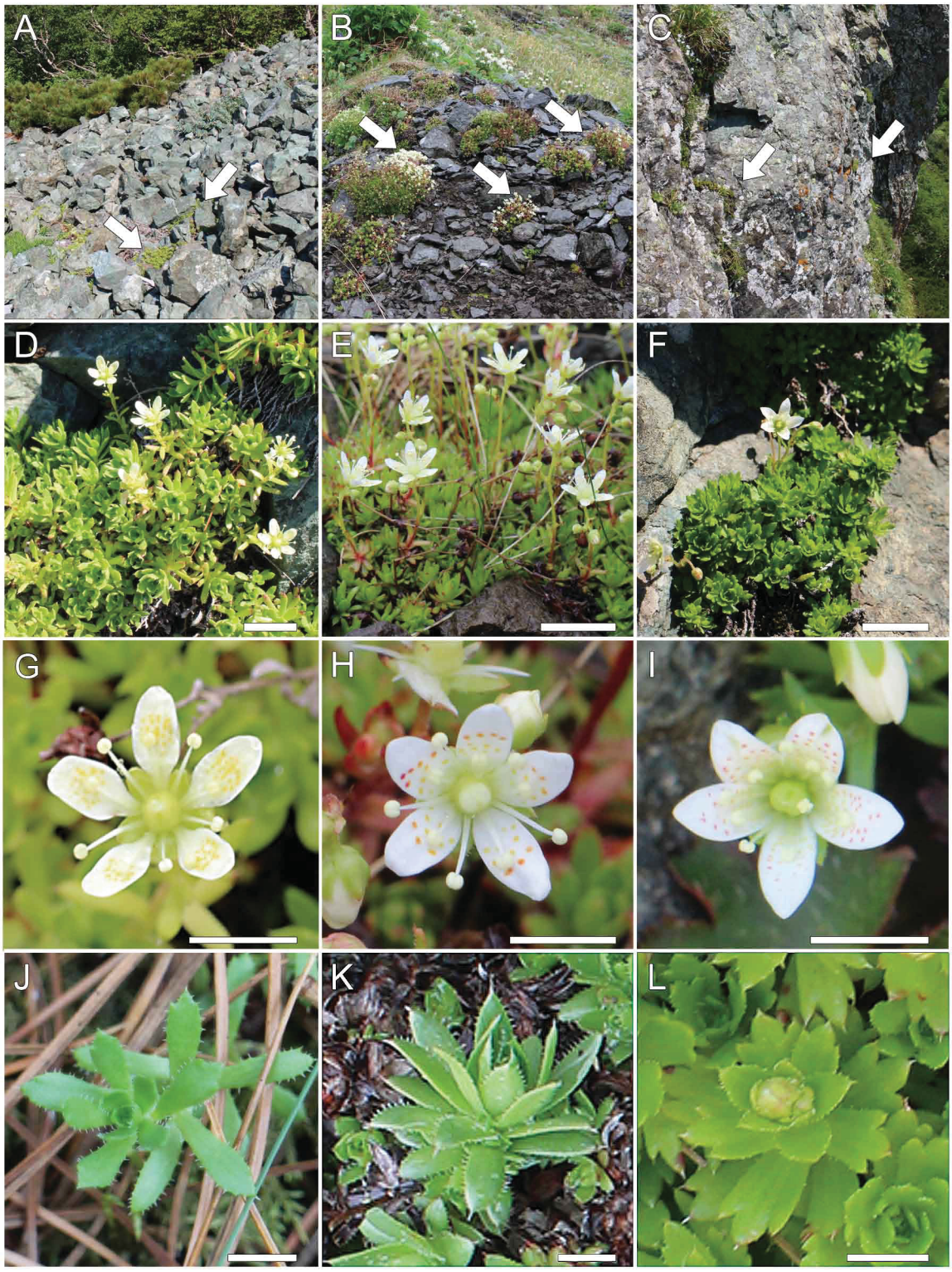

FIGURE 1. *Saxifraga yuparensis* (A, D & J 26 July 2016, G 19 July 2017, Gama-iwa at Mt. Yubari), *S. rebunshirensis* (B, E, H & K, 10 June 2016, Rebun Island), and *S. nishidae* (C, F, I & L, 26 July 2016, Gama-iwa at Mt. Yubari). A, B & C Habitat. D, E & F Habit. G, H & I Spots on petal. J, K & L Leaf. Scale bars are 1 cm for D–F and 5 mm for G–L. The figure is available in color online.

Miyabe & Kudô (1917: 170) (Fig. 1 & 2). *Saxifraga rebunshirensis* is broadly distributed in Japan (Hokkaido and high mountains in Honshu) and Russian Far East (Sakhalin and the Kuril Islands), while *S. yuparensis* is endemic to a scree consisting of greenschist at Mt. Yubari in Hokkaido and *S. nishidae* is endemic to a cliff composed of greenschist at Mt. Yubari and a cliff of schalstein at Mt. Ashibetsu, Hokkaido (Shimizu 1983, Charkevicz 1989, Sato 2007, Nakagawa & Sato 2015). Mountains composed of diabasic rocks (including greenschist, shalestein, etc.) are known to house plants

adapted to infertile soils and fragile geological features, as is the case in several endemics in Mt. Yubari (Watanabe 1971, Nosaka 1974). Diabasic plants are typically narrow endemics and grow on unstable soils (Watanabe 1971), and thereby include many endangered species. The number of individuals and patches of *S. yuparensis* are decreasing due to collapse of rocks and collection for horticultural purposes, and it is designated as an endangered species: CR (critically endangered) at the national level (Ministry of the environment of Japan 2017) and En (Endangered) at the provincial level (Hokkaido 2001).

The diagnostic characters of these three species are the color of petal spots, presence/absence of a conspicuous claw of petal base, and the shape of leaf apex. *Saxifraga yuparensis* has yellow petal spots, petal claws, and slightly tricuspidate leaves; *S. rebunshirensis* has yellow and red (irregularly only yellow) petal spots, claw-less petals, and cuspidate leaves; *S. nishidae* has yellow and red petal spots, claw-less petals, and tricuspidate leaves (Miyabe & Kudô 1917, Hara 1952, Murata 1961, Nosaka 1974, Nosaka 1980, Shimizu 1983, Ohba 1999, Nakagawa & Sato 2015). However, several authors considered that *S. yuparensis* is an immediate hybrid derived from crossing between *S. rebunshirensis* and *S. nishidae* based on morphological intermediacy in the shape of leaf apex (Toyokuni 1988, Nishikawa *et al.*1992, Iwatsuki & Kato 1994, Umezawa 2004, Shimizu *et al.* 2014, Takahashi 2015a). Additionally, seeds of *S. yuparensis* rarely germinate (Shimizu 1983) and this suggests that the species is an infertile triploid hybrid, because *S. nishidae* is diploid (2n = 26, Funamoto & Nakamura 1993) while *S. rebunshirensis* is tetraploid (2n = 48 using samples with no locality information, Sakai, 1935; 2n = 50 with samples from Nagano of central Japan, Funamoto & Nakamura 1990), although the chromosome number of *S. yuparensis* has not been reported. The hybrid-origin hypothesis, however, has not been tested by molecular analyses. On the other hand, *S. yuparensis* is sometimes treated as a synonym or variety of *S. rebunshirensis* (Shimizu 1983, DeChaine 2014, Takahashi 2015a, Okuyama 2016; note the last one is the latest Japanese flora). For planning the conservation of *S. yuparensis*, it is crucial to clarify whether this is an immediate hybrid and lacks a unique gene pool. In a preceding molecular phylogenetic study of *Saxifraga*, the species relationships were partially revealed, but the relationship among the three species remained unclear (DeChaine *et al.* 2013). The aim of this study is to elucidate the species relationships and test the immediate-hybridity of *S. yuparensis* using molecular analyses and cytological observation. In molecular analyses, DNA sequencing and genome-wide single nucleotide polymorphism (SNP) detection were employed. Collaterally, the diagnostic character of leaf apex shape was investigated using specimens of the three species to reevaluate the morphological intermediacy of *S. yuparensis*.

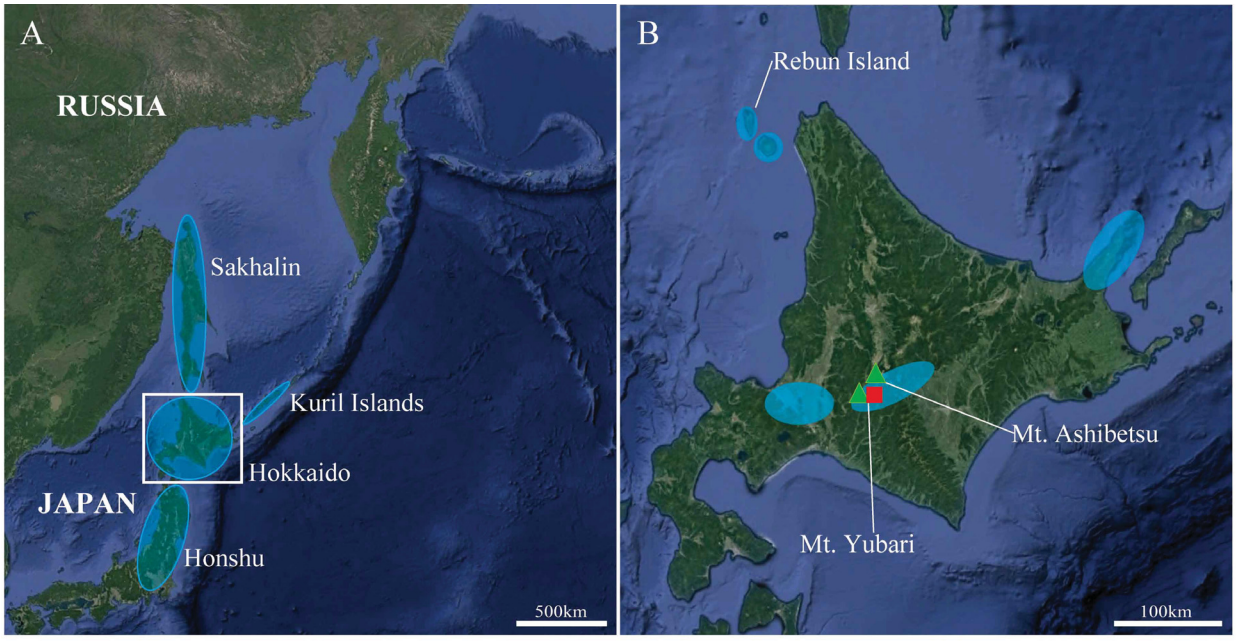

FIGURE 2. Maps of East Asia (A) showing the species range of *Saxifraga rebunshirensis* and of Hokkaido (B) showing the range of *S. yuparensis* (red; square), *S. rebunshirensis* (blue; circle), and *S. nishidae* (green; triangle). (the map image from Google Earth). The figure is available in color online.

Material & Methods

Molecular analyses

Taxon sampling

Molecular phylogenetic analyses were conducted using samples of our collection and DNA sequence data from GenBank (Table 1). *Saxifraga yuparensis* grows on a rocky scree of Mt. Yubari and has only two patches (0.3 x 0.2 m and 0.5 x 1.5 m), being located only ca. 5 m away from each other. One sample from each of the two patches (two samples in total) was collected to avoid collecting the same clones because this species can propagate vegetatively (authors' observation). DeChaine *et al.* (2013) sequenced a sample of "*S. nishidae*" (RBGE-E00295524) but we identified this specimen as *S. yuparensis* based on leaf apex morphology and found that the specimen was confused and mislabeled with genuine *S. nishidae* (RBGE-E00295525) collected on the same day on Mt. Yubari. The data of "*S. nishidae*" (RBGE-E00295524) was used for *S. yuparensis*. *Saxifraga nishidae* has more than 100 patches on a nearby rocky cliff of greenschist of Mt. Yubari. Six samples were collected from different patches with at least 5 m interval. In Mt. Yubari, *S. rebunshirensis* (*Ken Sato 85.0207* in SAPS, Appendix) had been collected more than 30 years ago from Rosoku-iwa, that is several kilometers away from the rocky place where *S. yuparensis* and *S. nishidae* grow; but this time we could not collect the species there. We did not conduct DNA extraction/PCR with the herbarium specimen because the specimen was old and we were afraid to damage the valuable specimen in vain. Instead, two samples each from two localities in Hokkaido, Rebun Island and Mt. Nishi-Kumaneshiri, were used. Also, two plants from Mt. Hakuba in central Japan were hired. GenBank data for *S. rebunshirensis* from Moneron Island to the south of Sakhalin (UBC-V164570) were also used. To elucidate the phylogenetic positions of these three species, we incorporated allied 16 species of sect. *Bronchiales* (DeChaine *et al.* 2013) in the analyses. In Russian Far East, we collected *S. ascoldica* Sipliv. (1971: 156) in Primorsky Krai and *S. cherlerioides* D.Don (1822: 382) and *S. funstonii* (Small) Fedde (1905: 613) in Kamchatka. For the other species, DNA sequence data were obtained from GenBank. Distribution of the 19 species covered the whole distribution of sect. *Bronchiales*. For outgroups, two species of sect. *Gymnopera* D.Don (1822: 343) and one species of sect. *Cymbalaria* Griseb. (1843: 336) were selected (Table 1), following the result of Tkach *et al.* (2015).

DNA extraction and sequencing

Total genomic DNA was extracted from fresh leaves using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle, 1987) with some modifications. Preceding phylogenetic studies on *Saxifraga* employed internal transcribed spacer (ITS) of nuclear ribosomal DNA (nrDNA) and *trn*L-F intergenic spacer of chloroplast DNA (cpDNA) (DeChaine *et al.* 2013, Tkach *et al.* 2015). We used primers following these preceding studies: *trn*L(UAA) and *trn*F(GAA) for *trn*L-F region (Taberlet *et al.* 1991), and 17SE and 26SE for the entire ITS1, 5.8S and ITS2 region (Sun *et al.* 1994). PCR was performed in 25 µl total volume with the following reagents: about 10 ng of genomic DNA, 1 unit of Taq DNA polymerase master mix (Ampliqon, Rødovre, Denmark), 0.4 µM of each primer, and 4 % DMSO. After an initial heating step at 94°C for 3 min, samples were incubated for 25 cycles of 94°C for 1 min, 60°C (ITS) or 52°C (*trn*L–F) for 1 min, 72°C for 2 min, with final extension at 72°C for 5 min. The cycle sequencing reaction was carried out with a Big Dye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA) with the same primers used in the PCR. Direct sequencing was performed on an ABI Prism 3130 DNA analyzer (Applied Biosystems). The sequence data were deposited in DDBJ (DNA Data Bank of Japan) database (2017, Table 1).

Phylogenetic analyses

DNA sequences were aligned using ClustalX ver. 1.8 (Thompson *et al*. 1997) implemented in BioEdit ver. 7.2.5 (Hall 1999) and then manually adjusted. Phylogenetic analyses were based on a maximum parsimony (MP) criterion using PAUP* ver. 4.0b10 (Swofford 2002) and a Bayesian approach using MrBayes ver. 3.2 (Ronquist *et al.* 2012).

In the Bayesian phylogenetic analysis, MrModeltest 2.3 (Nylander 2008) was used to estimate the appropriate evolutionary model of nucleotide substitution based on the Akaike Information Criterion (AIC). Based on the model selected, two separate runs of Metropolis coupled Markov chain Monte Carlo (MCMCMC) analyses were performed, each with a random starting tree and four chains (one cold and three heated). The MCMCMC length was one million generations, and the chain was sampled every one hundredth generation from the cold chain. The mixing and convergence of the MCMC chains of the two runs was assessed by inspection of the trace plots of parameters using Tracer ver. 1.6 (Rambaut *et al.* 2013). The first 250 sample trees (2.5% of the total 10,000 sample trees) were discarded as burn-in. After the burn-in, the effective sample sizes (ESS) of all parameters were more than 500, indicating that

TABLE 1. Samples used for ITS and cpDNA sequencing analyses. N is the number of sequenced individuals.

Taxon	Distribution*	N	Voucher	ITS accession no.	*trn*L-F accession no.
Sect. *Bronchiales*					
S. anadyrensis Losinsk.	RU: Magadan to mouth of Lena River	2	154288, 78524 (ALA)	KF196358	KF196374
S. ascoldica Sipliv.	RU: Ussuri	2	042206, 042207(SAPT)	LC314071, LC314072	LC314090, LC314091
S. austromontana Wiegand	NA: Cascade Mountains, Rocky Mountains	11	619568, 750642, 750643, 750647, 758235, 779011, 780148 (RMH), 361429, 369601 (WTU), 22800, 22801 (WWB)	KF196332–KF196347	KF196398–KF196409
S. bronchialis L.	RU: northern RU to Pacific Coast	2	283068, 282875 (WTU)	KF196360, KF196361	KF19 6396, KF196397
S. caulescens Sipliv.	RU: Siberia; Mongolia	1	00258082 (RBGE)	KF196325	KF196375
S. cherlerioides D.Don	RU: Sakhalin, Kuril Islands, Okhotsk, NA: Alaska, Yukon	8	358172, 385184, 385185 (WTU), 042200–042205 (SAPT)	KF196363, KF196364, KF196366, LC314087	KF196376–KF196378, LC314096, LC314098, LC314100, LC314105–314107
S. codyana Zhmylev	NA: Alaska, Yukon	1	212762 (UBC)	KF196327	KF196379
S. derbekii Sipliv.	RU: Far East near Sea of Okhotsk	1	164312 (UBC)	KF196367	KF196380
S. funstonii (Small) Fedde	RU: Taimyr to Chukotka, Kamchatka, NA: Alaska, Yukon, British Columbia	10	121070, 156966, 156968 (ALA), 356796 (WTU), 22807–22809 (WWB), 042197-042199(SAPT)	KF196326, KF196328–KF196331, KF196362, KF196365, LC314070, LC314088, LC314089	KF196393–KF196395, KF196410–KF196412, KF196417, LC314099, LC314101, LC314999, LC316189
S. kruhsiana Fisch. ex Ser.	RU: Kamchatka, Okhotsk	2	107779 (ALA)	KF196356, KF196357	KF196381, KF196382
S. nishidae Miyabe & Kudô	Japan: Hokkaido; Yubari Mountains, Ashibetsu Mountains	6	042191–042196(SAPT)	LC314081–LC314086	LC314108–LC314113
S. omolojensis A.P.Khokhr.	RU: Chukotka to Okhotsk	1	129124 (ALA)	KF196368	KF196384
S. rebunshirensis (Engl. & Irmsch.) Sipliv.	Japan: Hokkaido, high mountains in Honshu, RU: Sakhalin, Kuril Islands	7	164570 (UBC), 042185–042190(SAPT)	KF196323, LC314073–314078	KF196385, LC314092–314095, LC314102, LC314103
S. spinulosa Adams	RU: Urals to Chukotka, Kamchatka	2	107682, 168369 (ALA)	KF196354, KF196355	KF196386, KF196387
S. stelleriana Merk. ex Ser.	RU: Angara to Okhotsk, Zea, Lk. Baikal	1	154286 (ALA)	KF196359	KF196388
S. taylorii Calder & Savile	NA: British Columbia Haida Gwaii	3	214354, 214965 (UBC), 22805 (WWB)	KF196351–KF196353	KF196389, KF196390
S. tricuspidata Rottb.	NA: Alaska, Canada, Greenland	4	363418 (RMH), 22802–22804 (WWB)	KF196339–KF196342	KF196413–KF196416
S. vespertina (Small) Fedde	NA: Olympic Mountains, Cascade Mountains	3	284404 (WTU), 159757 (UBC), 22806 (WWB)	KF196348–KF196350	KF196391, KF196392
S. yuparensis Nosaka	Japan: Hokkaido; Yubari Mountains	3	00295524 (RBGE), 042183–042184 (SAPT)	KF196324, LC314079, LC314080	KF196383, LC314097, LC314104
Sect. *Cymbalaria*					
S. hederacea L.	–	1	–	AF261182	–
S. cymbalaria L.	–	1	–	–	AF374777
Sect. *Gymnopera*					
S. spathularis Brot.	–	1	–	AJ233858	AF374785

* NA, North America; RU, Russia.

the analyses sampled the posterior distributions of each parameter satisfactorily, and the values of Average Standard Deviation of Split Frequency (ASDSF) were below 0.008. The 50% majority rule consensus tree and Bayesian posterior probabilities (*PP*) of all the post-burn-in trees was generated using FigTree ver. 1.4.2 (Rambaut 2014).

In the MP phylogenetic analysis, indels were treated as missing data. The characters were treated as unordered, and the character transformations were weighted equally. The branch collapse option was set to collapse at a minimum length of zero. A heuristic parsimony search was performed with 1000 replicates of random additions of sequences with ACCTRAN character optimization, tree bisection-reconnection (TBR) branch swapping, and MULTREES and STEEPEST DESCENT options on. Statistical support for each clade was assessed by bootstrap analysis (Felsenstein 1985). One thousand replicates of heuristic searches, with the TBR branch swapping switched on and MULTREES options off, were performed to calculate bootstrap percentages (*BP*).

Genome-wide SNP analysis

To test the immediate hybridity and species relationships based on multiple independent nuclear loci, genome-wide SNP detection was conducted with the focal three species. The samples of our own collection used in the sequencing analysis were employed. Preceding studies (Rutledge *et al*. 2015, Karimi *et al*. 2016) based on genome-wide SNP data (observed and simulated data) successfully indicated that hybrids were plotted at the intermediate between the parental species in a principal coordinate analysis (PCA) (e.g., F1 and F2 individuals occurred at intermediate between the two parental species, and backcross individuals occurred between the F1 and the one parental species; Rutledge *et al.* 2015). For SNP detection, we employed a method MIG-seq [i.e., multiplexed inter simple sequence repeats (ISSRs) Genotyping by sequencing; Suyama & Matsuki 2015]. MIG-seq is a type of reduced representation sequencing, where ISSRs are amplified by multiplex PCR. Thereby the method is more applicable to low-quality genomic DNA than RAD-seq. Most of SNPs obtained by MIG-seq are putatively selectively neutral because these are immediately adjacent to two repeat regions (Takahashi *et al.* 2016). Highly reduced representation libraries were constructed following Suyama & Matsuki (2015) and sequenced on an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). Both ends of the fragments were read by paired-end sequencing.

Sequences of reads were quality-filtered using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), with the settings of q = 30 and p = 40. The quality-filtered reads were then bundled together to form putative loci (stacks) with the software Stacks v1.15 (Catchen *et al*. 2013) using the 'ustacks' option, with the settings of minimum depth of coverage required (m) = 20, maximum distance between stacks (M) = 2, and the deleveraging (d) and removal (r) algorithms enabled. Using the 'cstacks' option, a catalog was created for all possible loci and alleles with the parameter 'number of allowed mismatches between samples (n)' = 4. The stacks created by 'ustacks' were then searched against the catalogue using the 'sstacks' option. The data set of all the samples was considered as a single population and SNPs were retrieved using the 'populations' option, with the 'write_single_snp' option to select only the first SNP per locus to avoid the linkage between SNPs. Only loci that were shared among at least 75 % of the samples were considered. If a locus was not recovered in a sample, the genotype of the sample was treated as missing data. PCA of SNP genotypes was conducted using GENODIVE (Meirmans & Van Tienderen 2004) with the default parameter settings.

Chromosome observation

For chromosome observation, we used four individual of *Saxifraga yuparensis* from Mt. Yubari (Saya Tamura 992, 993, 994, 995, SAPT), four individuals of *S. rebunshirensis* from Mt. Nishi-kumaneshiri (Saya Tamura 434, 436, SAPT) and Rebun Island (Saya Tamura 328, 329, SAPT) of Hokkaido, and four individuals of *S. nishidae* from Mt. Yubari (Saya Tamura 996, 997, 998, 999, SAPT). Plant materials were collected in the field and cultivated in Botanic Garden, Hokkaido University. The methods for the observation of somatic chromosome numbers followed Fukuda *et al.* (2007, 2016) with some modifications. Root tips were pretreated in 0.002 M 8-hydroxyquinoline solution for 10 hours at 4°C, and fixed in Farmer's solution (glacial acetic acid: 99% ethanol = 1 : 3) for more than 16 hours at 4°C. Then the root tips were macerated in 1N HCl for 10 minutes at 60°C, stained with 1 % aceto-orcein for 10 minutes, and squashed. Chromosomes were observed under a binocular Zeiss Axio Imager A1 microscope (Carl Zeiss SpA, Italy), and photomicrographs were taken using a Anyty 3R-DKMC01 camera (3R solution corporation, Japan).

Morphological observation

A diagnostic character of leaf apex shape was observed in the three species. Typically, *S. rebunshirensis* has cuspidate leaves while *S. nishidae* has tricuspidate leaves; and *S. yuparensis* has slightly tricuspidate leaves (Shimizu 1983, Ohba

1999, Wakabayashi 2001, Shimizu 2014, Okuyama 2016). Herbarium specimens of the three species (Appendix) were examined in the herbaria of Hokkaido University Museum (SAPS), Botanic Garden, Hokkaido University (SAPT) and the National Museum of Nature and Science (TNS) to elucidate the variability of the diagnostic character especially within *S. rebunshirensis*, that has large species range.

Results

Phylogenetic relationships based on ITS

The aligned length of the ITS data was 822 bp. In *S. rebunshirensis*, five sequence types were recognized among the seven samples, and *S. nishidae* had two sequence types in the six samples. The two samples of *S. yuparensis* of our collection (SAPT-042183, 042184) showed one type of sequence with a double-peak signal (adenin and guanin) at one site (position 423 in the registered sequences) in the sequence chromatogram, while all the samples of *S. rebunshirensis* and *S. nishidae* had only adenin but not guanin at the same site. This site was coded as R in the two *S. yuparensis* samples. In the Bayesian analysis, the AIC selected the SYM+I+G model. The 50% majority rule consensus tree of all the post-burn-in trees is depicted (Fig 3). In the MP analysis, 201 nucleotide substitutions were found in 57 variable sites and 114 sites were parsimony informative among them. 15,104 equally parsimonious trees of 314 steps were obtained with a consistency index (CI) = 0.78, a retention index (RI) = 0.91, and a resealed consistency index (RC) = 0.71. The topology of the strict consensus tree was the same as that of the Bayesian tree, and therefore *BP*s of the MP analysis are plotted on the Bayesian tree (Fig 3). In the following, only clades with $PP \geq 0.95$ and/or $BP \geq 70\%$ were considered adequately supported. The phylogenetic tree had moderate resolution and supported monophyly for several species, including *S. nishidae* (*PP* = 1.00 / *BP* = 100). On the other hand, monophyly was not supported for each *S. yuparensis* and *S. rebunshirensis*; the two species were included in a clade (1.00 / 55.4) with a portion of samples (three samples) of *S. funstonii* and *S. caulescens* Sipliv. (1971: 151). This clade was nested within a clade with the other five samples of *S. funstonii* and one *S. codyana* (1.00 / 66.3). Thereby, *S. yuparensis* plus *S. rebunshirensis* were phylogenetically distinct from *S. nishidae*.

Phylogenetic relationships based on *trn*L-F

The aligned length of the *trn*L-F data was 446 bp. All the samples of *S. rebunshirensis* and *S. yuparensis* had the same sequence, that was shared with other 11 species in the same clade (*below*). In the six samples of *S. nishidae*, only one type of sequence was recovered. In the Bayesian analysis, the AIC selected the GTR model. The 50% majority rule consensus tree of all the post-burn-in trees is depicted (Fig 4). In the MP analysis, 28 nucleotide substitutions were found in 12 variable sites and 13 sites were parsimony informative among them. 574,318 equally parsimonious trees of 30 steps were obtained with CI = 1. The topology of the strict consensus tree was the same as that of the Bayesian tree, and therefore *BP*s of the MP analysis are plotted on the Bayesian tree (Fig 4). While the phylogenetic tree largely had low resolution, but nevertheless *S. nishidae* formed a well-supported clade (1.00 / 97.2), and this clade was sister to a portion of samples (three samples) of *S. austromontana* (0.97 / 63.0). In contrast, each *S. yuparensis* and *S. rebunshirensis* was not monophyletic; these two species were recovered in the same polyphyletic clade (0.98 / 61.1) with other 11 species.

Species relationships based on genome-wide SNPs

The resultant data set comprised 699 SNP loci. The result of the PCA based on the SNP genotyping data is shown (Fig. 5). The first and second principal components explained 51.6 % and 11.5 % of the variances. The SNP genotyping data clearly separated the three species in the principal coordinate space; the plots of *S. nishidae* were separated from *S. yuparensis* and *S. rebunshirensis* along the first principal coordinate, and those of *S. yuparensis* and *S. rebunshirensis* were distinguished along the second principal coordinate. Note that *S. yuparensis* was not placed in-between of *S. rebunshirensis* and *S. nishidae*.

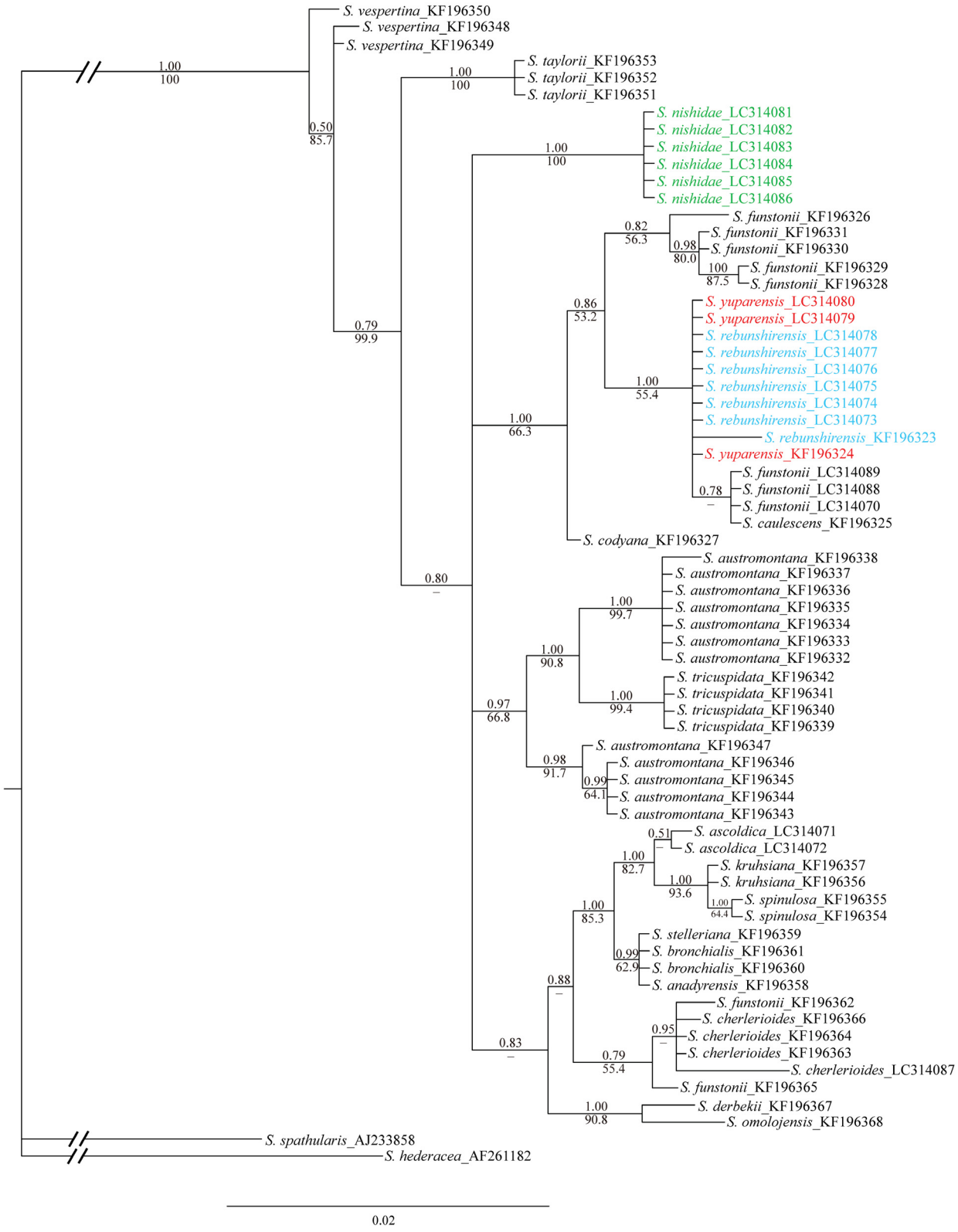

FIGURE 3. Bayesian majority-rule consensus tree of *Saxifraga* sect. *Bronchiales* based on ITS. The numerals on branches are Bayesian posterior probabilities (*PP*: upper) and bootstrap percentages (*BP*: lower) in the MP analysis. The figure is available in color online.

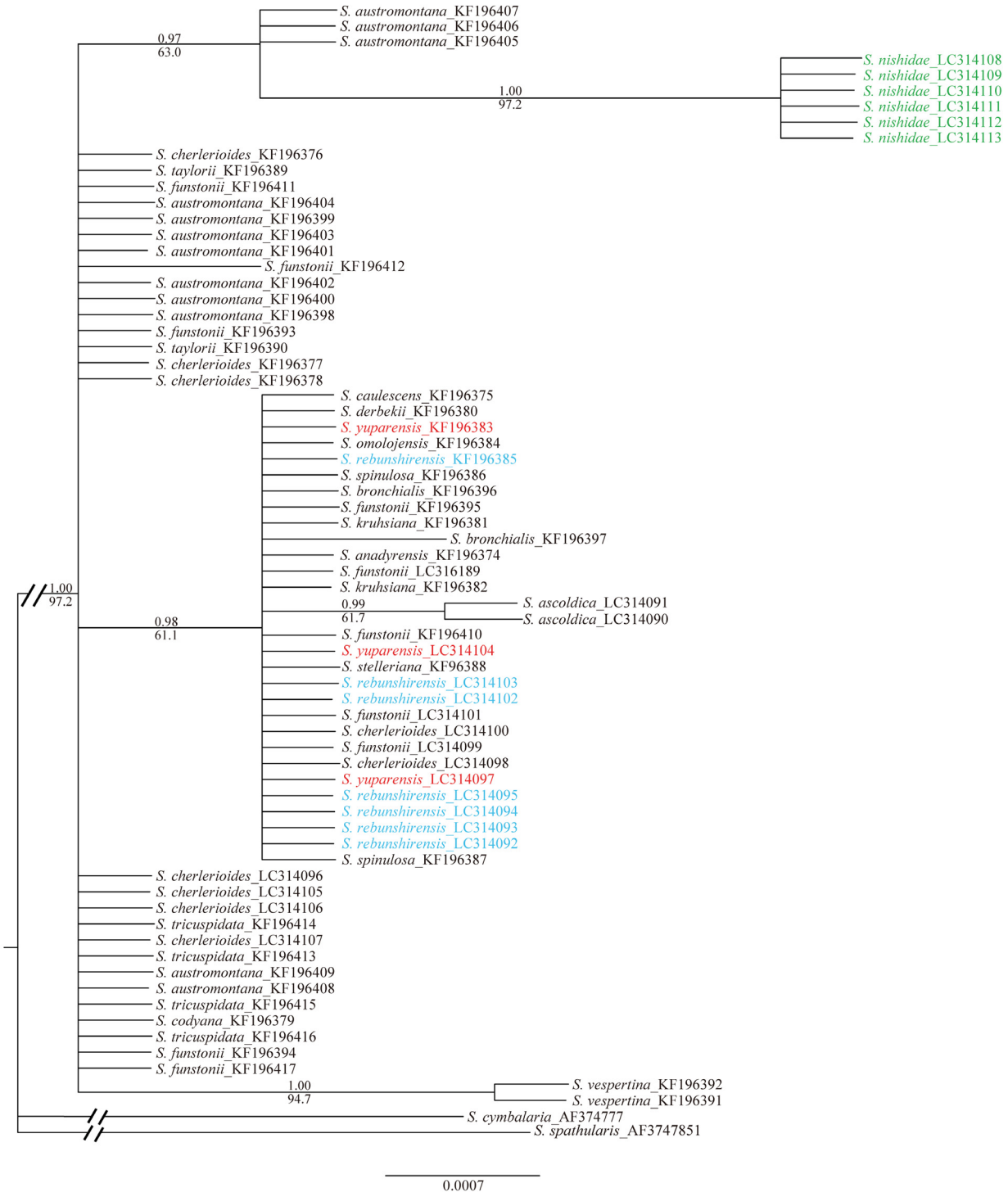

FIGURE 4. Bayesian majority-rule consensus tree of *Saxifraga* sect. *Bronchiales* based on *trn*L-F. The numerals on branches are Bayesian posterior probabilities (*PP*: upper) and bootstrap percentages (*BP*: lower) in the MP analysis. The figure is available in color online.

Chromosome number

The chromosome number of *S. yuparensis* was first determined as 2n=48 (Fig 6–A & D). In *S. rebunshirensis* the chromosome number was 2n=48 in all the three individuals examined (Fig 6–B & E) and this is the same as reported by Sakai (1935) but different from the number reported by Funamoto & Nakamura (1990) (2n = 50). The chromosome number of *S. nishidae* was 2n=26 in all the four individuals (Fig 6–C & F), and that was the same as reported by Funamoto & Nakamura (1993).

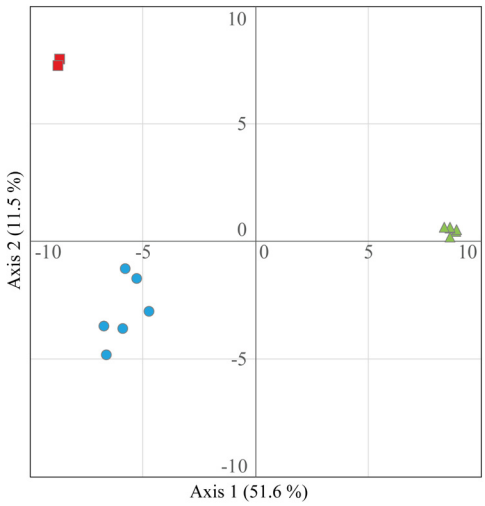

FIGURE 5. Plots of principal coordinate analysis (PCA) based on genome-wide SNPs of *Saxifraga yuparensis* (red; square), *S. rebunshirensis* (blue; circle), and *S. nishidae* (green; triangle). The figure is available in color online.

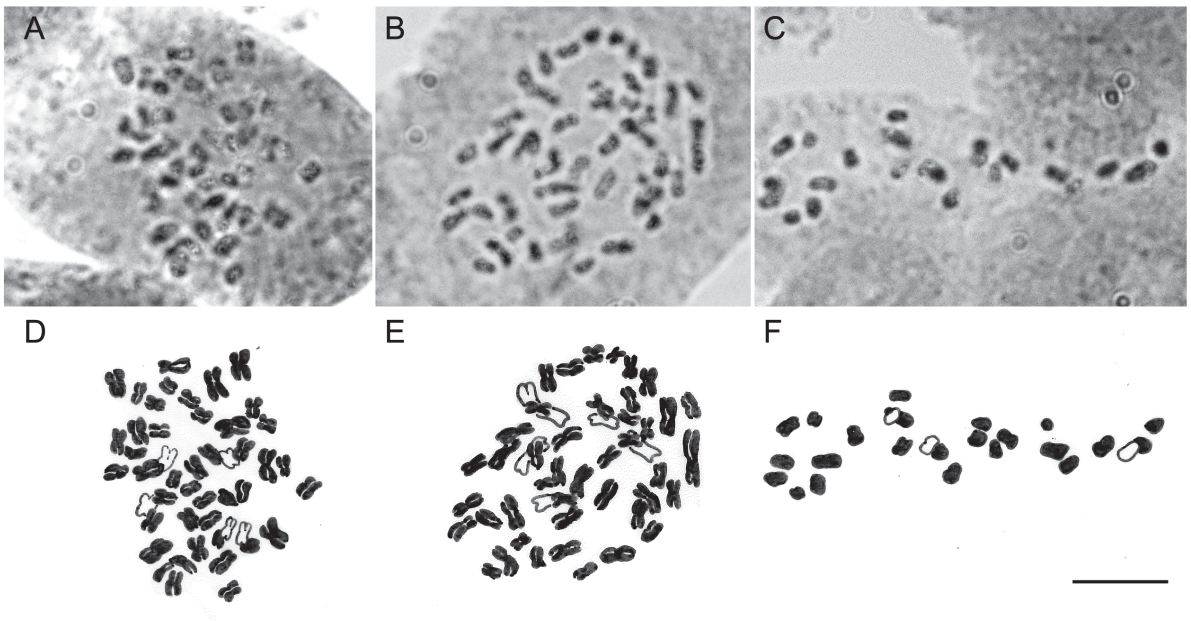

FIGURE 6. Photomicrographs of somatic metaphase chromosomes of *Saxifraga yuparensis* (A), *S. rebunshirensis* (B) and *S. nishidae* (C). D, E and F are explanatory drawings of A, B and C, respectively. Scale bar represents 5 µm.

Variability of leaf apex morphology

All the 14 specimens examined for *S. yuparensis* had slightly tricuspidate leaf apex except for leaves on flowering stems (Fig 7–A & D, Appendix). In *S. rebunshirensis*, 199 out of 213 specimens examined had cuspidate leaves (Fig 7–B & E, Appendix) but 14 specimens collected from Hokkaido, Honshu, and Sakhalin had slightly tricuspidate leaves on vegetative stems (Fig 7–F, Appendix). On the other hand, all the 26 specimens of *S. nishidae* had conspicuously tricuspidate leaf apex except for ones on flowering stems (Fig 7–C & G, Appendix), and the specimens of *S. nishidae* were clearly distinguished from the other two species.

FIGURE 7. Specimens of *Saxifraga yuparensis* (A & D), *S. rebunshirensis* (B, E & F), and *S. nishidae* (C & G). Scale bars are 5 mm for D–G. The figure is available in color online.

Discussion

In the molecular phylogenetic analyses based on ITS and cpDNA, *S. yuparensis* was not monophyletic and recovered in the same clade with *S. rebunshirensis* plus several other species (*S. funstonii* and *S. caulescens* in ITS; 11 species in *trn*L-F). On the other hand, *S. nishidae* formed a well-supported monophyletic clade in both ITS and cpDNA, and this clade was distinct from the clades including *S. yuparensis* and *S. rebunshirensis*. If *S. yuparensis* were a hybrid derivative of the two species, two distinct sequences from each of the *S. yuparensis* samples would be recovered in both the clades of *S. rebunshirensis* and *S. nishidae* in the ITS phylogeny, although it cannot be excluded that the ITS allele of one parental species (here *S. rebunshirensis*) became dominant by concerted evolution via unequal crossing-over and gene conversion through time (given that *S. yuparensis* is not an immediate but old hybrid). However, the PCA based on the SNP genotyping data clearly separated the three species and *S. yuparensis* samples were not placed in-between of *S. rebunshirensis* and *S. nishidae*. This result, being based on multiple independent nuclear loci, also negate the hybrid hypothesis of *S. yuparensis*. The result also likely negates the scenario that ITS allele of *S. rebunshirensis* became dominant by concerted evolution in *S. yuparensis*. Chromosome observation revealed that *S. yuparensis* is tetraploid. If it were an immediate hybrid derived from diploid-tetraploid crossing, it would be triploid. Thus, the chromosome data negate, at least, the immediate hybridity. Considered in a comprehensive way, all the results based on ITS and cpDNA sequences, genome-wide SNPs, and chromosome count negate the hypothesis that *S. yuparensis* is derived from hybridization between *S. rebunshirensis* and *S. nishidae*. Concerning the infertility of *S. yuparensis*, Shimizu (1983) reported that the seeds of *S. yuparensis* did not germinate, and we have not observed mature fruits in the wild population. The infertility of *S. yuparensis* is probably caused by self-incompatibility, not by triploidy. Self-incompatibility in *Saxifraga* was reported for species of the same section *Bronchiales* (Goertzen 1996). Currently there are two patches of *S. yuparensis* at Mt. Yubari but these patches were originally one patch, that were subsequently fragmented by rock debris (authors' observation), and the two patches likely contain very closely related plants such as those of the same sibling that are incompatible.

Concerning the taxonomic treatment of *S. yuparensis*, the SNP analysis suggests that *S. yuparensis* is a lineage distinct from *S. rebunshirensis*. Note, however, that *S. rebunshirensis* itself has taxonomic problems and is sometimes treated as a subspecies or variety of *S. cherlerioides*, *S. bronchialis* L. (1753: 400), or *S. funstonii* (Engler *et al.* 1919, Hara 1937, Hara 1952, Toyokuni 1975, Takahashi 2015b). The present phylogenetic analyses are not conclusive about the species relationships because monophyly was not supported for each of these species and because the trees lacked necessary resolution. The SNP analysis did not include the other species treated in the phylogenetic analyses, specifically *S. funstonii* and *S. caulescens* clustered with *S. yuparensis* and *S. rebunshirensis* in the ITS tree (and in the cpDNA tree with the other nine species, although it showed lower resolution). Full revision of their taxonomy needs more intensive analysis of the allied species using genome-wide SNPs, but it is beyond the scope of the present study (i.e., test on the hybrid hypothesis). When we follow the idea to treat *S. rebunshirensis* as an independent species (Siplivinsky 1971; DeChaine 2014), what is the diagnostic character to separate it from *S. yuparensis*? Slightly tricuspidate leaf is a character based on which *S. yuparensis* has been distinguished from *S. rebunshirensis*. In the morphological observations of specimens, it was revealed that *S. rebunshirensis* from Hokkaido, Honshu, and Sakhalin sometimes had slightly tricuspidate leaves. The leaf apex shape of *S. yuparensis* fell within the variation of *S. rebunshirensis* and thereby is not a diagnostic character. In addition, Barkalov (2009) conducted morphological comparison between *S. yuparensis* from Mt. Yubari and *S. rebunshirensis* samples from the southern Kuril Islands, where *S. yuparensis* is not reported and which should be treated as *S. rebunshirensis* (Charkevics 1989; Takahashi 2015b); he recognized no morphological difference. According to literature, another diagnostic character of *S. yuparensis* and *S. rebunshirensis* is a conspicuous claw of petal base in the former species, that is lacked in the latter species (Nosaka 1980; Shimizu *et al.* 2014), while Barkalov (2009) did not mention the character. Although it was difficult to examine the character with dried herbarium specimens, by observing living plants in wild and botanic gardens, we also recognized the difference in the two species (authors' observations, Appendix).

In conclusion, *S. yuparensis* is not an immediate hybrid of *S. rebunshirensis* and *S. nishidae* but a distinct lineage, as was revealed by the DNA sequence, genome-wide SNP, and cytological evidences. This is an extremely narrow endemic species, that is morphologically distinguished from the congeners in Hokkaido by having a claw of petal base, and deserves for intensive conservation.

Acknowledgement

We thank Seiichiro Miyamato, Minori Somada, Kenji Murakami, and Shogo Kikuta for assistance in field sampling and Hiroyuki Sato for help in herbarium work. This study was supported in part by Grants-in-Aid for Scientific Research, KAKENHI to K.N. (16K18596) and a research grant from the Mitsui & Co. Environment Fund to K.N. (R15–0067).

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Appendix

Herbarium specimens used for morphological observations. Specimens that were observed for petal shape are marked with daggers (†), and ones of *Saxifraga rebunshirensis* that had slightly tricuspidate leaves are marked with asterisks (*). Superscript letters (A-F) indicate scientific names (synonyms) or Japanese names on the labels, as shown in the footnote.

Saxifraga yuparensis: JAPAN. Hokkaido: Mt. Yubari Gamaiwa, 22-26 July 1928, *Koidzumi Hideo* 16910 (TNS). Mt. Yubari, July 1934, *Ohwi Jizaburo* 224482 (TNS). Mt. Yûpari Gama-iwa, 1 August 1967, *Mikio Tohyama s.n.* (SAPS). Mt. Yupari Gama-iwa,17 August 1973, *Shiro Nosaka* 031584, 031585 (SAPS). Hokkaido: Mt. Yubari Gamaiwa, 6 August 1991, *Ken Sato* 91.1708, 91.1328, 91.1329 (SAPS). Mt. Yubari Gama-iwa, 26 July 2016, *Saya Tamura* 582†, 586† (SAPT). Mt. Yubari Gama-iwa 19 July 2017, *Saya Tamura* 992†, 993†, 994†, 995† (SAPT).

S. rebunshirensis: RUSSIA. Sakhalin: S. Saghallen Mt. Tosso, 23 July 1927, *Hiratsuka N.*, *Nagai M. & Iwadare S. s.n.*[A] * (SAPS). Mt. Tosso, July 1927, *Sugawara s.n.*[A] (TNS). Kusu river, 18 July 1928, *Okamae Tsutomu* 252162[F] (TNS). Shirutoru,13 July 1929, *Haga T.* 13939[A] (SAPS). Mt. Tosso, 13 July 1929, *Haga T.* 13936[A] (SAPS). Shirutoru, 14 July 1929, *Haga T.* 13937[A] (SAPS). Mt. Tosso, July 1929, 20855[F] (TNS). Kasipo, 1 August 1929, 21329[F]*(TNS). Sakhalin, July 1930, *Shigezou Sugawara* 12105[C]* (SAPT). Shirutori Mt. Ishiyama, 20 July 1935, *Shigezou Sugawara* 12106[C] (SAPT). Hoe, 23 August 1935, *Nakaji Masayoshi s.n.*[A] (TNS). South of the Peninsula of Schmidt, 16 August 2001, *Fukuda T.* 2211[E]* (SAPS). Peninsula of Schmidt, 14 August 2001, *Barkarov V. Y.* 10812[D]* (SAPS). Eastern coast of the Peninsula of Schmidt, 14 August 2001, *Fukuda T.* 2117[E] (SAPS). South of the Peninsula of Schmidt, 16 August 2001, *Fukuda T.* 2198[E], 2211[E] (SAPS). Tikhaya village, 28 August 2001, *Fukuda T.* 2483[E] (SAPS). Mt. Zhdanko, 2 August 2002, *Hideki Takahashi* 30172[C] (SAPS). Mt. Ostrovskaya, 3 August 2002, *Noriyuki Fujii* 01133[C] (SAPS). Mt. Changa, 7 August 2002, *Hideki Takahashi* 30264[C], 30309[C] (SAPS). Central Sakhalin Mt. Chamga, 17 August 2006, *Ken Sato*, 06.0201 (SAPS). Central Sakhalin Mt. Vaida, 20 August 2006, *Ken Sato* 06.0316 (SAPS). Mt. Calcareous, 11 July 2007, *Takahashi H.*, *Kawahara T.*, *Kitamura K. & Taran A.*, 33.763[E] (SAPS). RUSSIA. Kuril Islands: Shikotan, 3 July 1930, *Tôno S. s.n.*[C] (SAPT). JAPAN. Hokkaido: Mt. Rishiri, 6 July 1914, *s.n.*[A] (TNS). Rebun Island, 10 July 1914, *Tamaki Seiichi* 26106[F] (TNS). Mt. Ashibetsu Ishikari, 30 July 1915, *Koidzumi Hideo* 67488[A](TNS). Kamifurano, 8 September 1915, *Koidzumi Hideo* 74161[F]*, 74162[F] (TNS). Mt. Kamifurano, 13 July 1917, *Koidzumi Hideo* 76906[F] (TNS). Mt. Rishiri, 5 August 1922, *Hiratsuka Naohide* s.n.[A] (TNS). Kamikawamura, 24-27 August 1926, *Koidzumi Hideo* 13157[F] (TNS). Mt. Chubetsuhondake, 19 August 1927, *Koidzumi Hideo* 15046[F] (TNS). Mt. Nipesotsu, 25-26 August 1927, *Koidzumi Hideo* 15339[F] (TNS). Mt. Yubari, 25 July 1928, *Koidzumi Hideo* 63705[F] (TNS). Mt. Rishiri, 23 July 1929, *Shigeo Akiyama s.n.*[A] (SAPS). Mt. Rishiri, July 1929, *Akiyama S. s.n.*[A] (SAPS). Shakotan, 3 July 1930, *Tôno S.* 010397[C] (SAPT). Mt. Rishiri, 13 August 1952, *Furuse Miyoshi* 130160[F] (TNS). Mt. Jozankei-Tengu, 3 July 1955, *Toyokuni H. s.n.*[C] (SAPS). Mt. Rishiri, 29 July 1956, *Haginiwa Joju* JH046164[C] (TNS). Mt. Taisetsu, 11 August 1956, *Toyokuni H.* 027749[C] (SAPS). Mt. Rishiri, 3 August 1957, *Satake Yoshisuke* & *Ito Eiko* 266166[C], 273271[F] (TNS). Mt. Shari, 31 July 1959, *Kawashiro Z. s.n.*[B] (SAPS). Mt. Rausu Shiretoko, 11 July 1965, *Haga S.*& *Saitou R.* 308044[B] (TNS). Mt. Rishiri, 13 August 1965, 498 (TNS). Mt. Rhishiri, 13 August 1965, *Andoh Yukio* 347[F] (TNS). Mt. Rhishiri, 13 August 1965, *Harajima Hisao* 889[F] (TNS). Mt. Rishiri, 13 August 1965, *Masuda Shinya* 487[F], 498[F], 711[B]* (TNS). Mt. Zyozankeitengu,15 June 1969, *Ken Sato* 69.0101[B] (SAPS). Mt. Zyozankei-Tengu, 18 June 1970, *Ken Sato* 70.2802[F], 70.2801[F] (SAPS). Mt.Zyozankei-Tengu, 24 June 1970, *Watanabe S.* W153[F], 154[F], 155[F], 156[F] (SAPS). Mt. Rausu, 6 July 1970, *Togashi M.* 273107[F] (TNS). Mt. Rausu, 9 september 1971, *Sato K. & Nishikawa T.* 71.5062[F] (SAPS). Mt. Yûpari at Rôsoku-iwa, 6 August 1973, *Shiro Nosaka* 031581[C]*, 031582[C]* (SAPS). Mt. Rausu, 14 August 1979, *Sato K. & Samejima J.* 79.5258[F] (SAPS). Mt. Kumano, 31 August 1979, *Ken Sato* 79.3115[F], 79.3093[F] (SAPS). Mt. Kumano, 17 July 1981, *Ken Sato* 81.0135[F], 81.0136[F] (SAPS). Pankechiebu, 24 June 1982, *Ken Sato* 82.0517[F], 82.0516[F] (SAPS). Mt. Ashibetsu, 10 July 1982, *Ken Sato* 82.0841[C] (SAPS). Mt. Kumano, 18 August 1982, *Ken Sato* 82.0337[F] (SAPS). Rebun Island, 13 July 1983, *Ken Sato* 83.0202[F] (SAPS). Mt. Kumano, 2 August 1983, *Tsuchida T.* 010396[F] (SAPT). Mt. Yubari Rosoku-iwa, 22 August 1985, *Ken Sato* 85.0207[F] (SAPS). Hoheikyo, 11 July 1986, *Matsuji Hara* 6470[C] (SAPS). Mt. Nishi-kumaneshiri-dake, 12 August 1987, *Takahashi H., Kawabata K., Kushibiki E., Kikuzawa Y.& Kudo T.* 7872[C], 7885[C] (SAPS). Mt. Tengu-dake, 24 July 1988, *Takahashi H., Hara M., Sato K. & Umezawa S.* 8548[C] (SAPS). Mt. Rausu, 7 July 1991, *Takita K.* 4766[B] (SAPT). Mt. Nipesotsu, 4 August 1991, *Takita K.* 5076[B]* (SAPT). Kamishihoro town Mt. Nishi-kumaneshiri, 27 July 1994, *Ken Sato* 94.0786, 94.0834 (SAPS). Mt. Rishiri Kutsugata-peak, 11 August 1994, *Ken Sato* 94.0990 (SAPS). Rebun Island Momo-iwa, 10 June 2016, *Saya Tamura* 328†, 329†(SAPT). Mt. Nishi-Kumaneshiri cultivated in the Botanic Garden, Hokkaido University, 1 July 2016, *Saya Tamura* 434†, 436†(SAPT). JAPAN.Honshu: Mt. Shirouma, 26 August 1902, *Yabe Yoshisada* 24445[F] (TNS). Mt. Yatsugadake Koshu, 30 August

1902, *Hayata B.* 18763[A] (SAPS). Mt. Yatsugadake Shinshu, August 1902, *Hayata B.* 18761, 18762, 18764[F] (SAPS). Mt. Amida, 25 July 1903, *Takeda H. s.n.*[A] (SAPS). Mt. Togakushiura, 19 July 1904, *Takeda H. s.n.*[A] (SAPS). Mt. Yatsugadake Shinshu, 21 July 1904, *Takeda H. s.n.*[A] (SAPS). Mt. Shirouma Shinshu, 16 August 1905, *Takeda F. s.n.* [A] (TNS). Mt. Shirouma Shinshu, 11 August 1906, *Miura Michiya s.n.*[A] (TNS). Mt. Yatsugatake, August 1906, *Sakurai Hanzaburo* 6635[F] (TNS). Mt. Yatsugatake Shinano, 26 July 1908, *Iishiba Eikichi s.n.*[C] (TNS). Mt. Ohrenge Echigo, 10 August 1908, *Nakamura Masao* 94366[F]* (TNS). Mt. Shirouma Shinano, 15-16 August 1908, *Shimura H.* 138822[F] (TNS). Mt. Yatsugatake Shinshu, 13 August 1910, *Koidzumi Hideo* 67489[A] (TNS). Mt. Hariki Shinano, 25 July 1913, *Yamakawa S.* 264[A] (TNS). Mt. Shirouma, 4 August 1913, *Yazawa Yonesaburo s.n.*[A]* (TNS). Mt. Senjo Nagano, 9 August 1913, *Takeda H.* 3663[F] (TNS). Mt. Senjogatake Shinshu, 10 August 1916, *Koidzumi Hideo* 67492[A] (TNS). Mt. Yatsugatake Shinshu, August 1916, *Koidzumi Hideo* 67491[F] (TNS). Mt. Hotaka Hida, 1 September 1916, *Koidzumi Hideo* 26206[F] (TNS). Mt. Shirouma Shinano, 27 July 1917, *Matsushima M.* 37204[C] (TNS). Mt. Shirouma, 28 July 1920, *Yazawa Yonesaburo s.n.*[A] (TNS). Mt. Shirouma Shinano, 26-30 August 1920, *Koidzumi Hideo* 785[F] (TNS). Mt. Shirouma Shinano, 21 July 1921, *Koidzumi Hideo* 45051[F] (TNS). Mt. Kaisuruga Shinano, 27 July 1921, *Koidzumi Hideo* 2035[F] (TNS). Mt. Tsuba-Kuro Shinano, 22 July 1921, *Koidzumi Hideo* 1909[F], 67367[A] (TNS). Mt. Senjo Shinano, 25 July 1922, *Koidzumi Hideo* 3516[F] (TNS). Mt. Senjo Shinano, 26 July 1922, *Koidzumi Hideo* 73067[F] (TNS). Mt. Shiomi Shinano, 28 July 1922, *Koidzumi Hideo* 3652[F] (TNS). Mt. Kogoochi Shinano, 29 July 1922, *Koidzumi Hideo* 3717[F] (TNS). Shimoina Shinano, 30 July 1922, *Koidzumi Hideo* 3725[F] (TNS). Mt. Arakawadake Shinshu, 31 July 1922, *Koidzumi Hideo* 67490[F] (TNS). Mt. Kitadake Koshu, 7 August 1922, *Koidzumi Hideo* 3882[F] (TNS). Mt. Yatsugadake Shinano, 28 August 1922, *Koidzumi Hideo* 4486[F] (TNS). Mt. Nokogiri Shinano, 29 July 1923, *Koidzumi Hideo* 5309[F] (TNS). Mt. Yatsugatake Nagano, 29 July 1923, *Koidzumi Hideo* 91244[F] (TNS). Mt. Arakawa Shinshu, 31 July 1923, *Koidzumi Hideo* 67494[A] (TNS). Kitaadzumigun Shinano, 6 August 1923, *Koidzumi Hideo* 48821[F] (TNS). Mt. Daitenjo Shinano, 6 August 1923, *Koidzumi Hideo* 64254[F] (TNS). Mt. Yari Shinano, 7 August 1923, *Koidzumi Hideo* 5087[F], 64257[F] (TNS). Mt. Kitahotaka Shinano, 7 August 1923, *Koidzumi Hideo* 48387[F] (TNS). Mt. Ohtenjo Shinano, 8 August 1923, *Koidzumi Hideo* 48274[F] (TNS). Mt. Harinoki Shinano, 18 August 1923, *Koidzumi Hideo* 5734[F] (TNS). Mt. Yari Hida, 24 August 1923, *Koidzumi Hideo* 5954[F] (TNS). Mt. Yatsugatake Gongendake Shinano, 30 August 1923, *Koidzumi Hideo* 6005[F] (TNS). Mt. Ushikubi Shinano, August 1923, *Koidzumi Hideo* 5043[F] (TNS). Mt. Kurodake Kamishinkawagun, 1923, *Koidzumi Hideo* 5859[F] (TNS). Mt. Maekogouchidake Shinano, 1923, *Koidzumi Hideo* 44023[F] (TNS). Mt. Tsugamura Suruga, 22 July 1924, *Koidzumi Hideo* 496871[F] (TNS). Mt. Shiomi, July 1924, *Yazawa Yonesaburo s.n.* [A]* (TNS). Mt. Tsurugi Yetstu, 8 August 1924, *Koidzumi Hideo* 7343[F] (TNS). Mt. Tateyama Yetstu, 9 August 1924, *Koidzumi Hideo* 7890[F] (TNS). Mt. Akaishi range Kai-Suruga, 2-9 July 1925, *Koidzumi Hideo* 9562[F], 61484[F], 51496[F] (TNS). Mt. Shirouma Shinano, July 1925, *Koidzumi Hideo* 10929[F] (TNS). Mt. Akaishi range, 8 August 1925, *Koidzumi Hideo* 9563[F] (TNS). Mt. Senjo Kai, 29 August 1925, *Ohwi Jizaburo* 224189 (TNS). Mt. Tateyama Yetsiu, July 1926, *Koidzumi Hideo*, 12136[F] (TNS). Mt. Yatsugatake, 18 July 1927, *s.n.*[A] (TNS). Mt. Yatsugatake Shinano, 7 July 1928, *Koidzumi Hideo* 88652[F] (TNS). Mt. Yatsugadake Shinshu, 28 July 1928, *Inagaki K. s.n.*[A] (SAPS). Mt. Kisokoma Shinano, 4 August 1928, *Koidzumi Hideo* 1044[F] (TNS). Mt. Hotaka Shinano, 2 September 1929, *Koidzumi Hideo* 19029[F], 19030[F], 19032[F]*, 55500[F] (TNS). Mt. Norikura Shinano, 27 July 1930, *Koidzumi Hideo & Yokouchi I.* 87496[F] (TNS). Mt. Ioh Nagano, July 1930, *Maekawa Fumio* 85567[B] (TNS). Mt. Shiomi Shinano, 3 August 1930, *Muramatsu Kouki* 3453 (TNS). Mt. Kanenashi Shinano, 23 July 1931, *Koidzumi Hideo* 45346[F] (TNS). Mt. Arakawa Suruga, 7 August 1931, *Hashimoto* 44088[F] (TNS). Mt. Akaishi Suruga, 8 August 1931, *Suzuki S. s.n.*[A] (TNS). Sannomine Echizen, 22 July 1933, *Tashiro Yoshitaro* 42366[F] (TNS). Mt. Kisokoma, 22 August 1934, *Koidzumi Hideo* 105682[F] (TNS). Mt. Kisomae, 13 August 1935, *Koidzumi Hideo* 105787[F] (TNS). Mt. Shishidake Ecchu Tateyama, 28 July 1937, *s.n.*[C] (TNS). Mt. Senjo Kai, 1 August 1938, *Okuyama Shunki* 4192[C] (TNS). Mt. Shimizu Etchu, 2 August 1938, *Hayashi*, 69396[F] (TNS). Mt. Higashi Suruga, 1 August 1948, *Hirano Hideo.* 79776[F] (TNS). Mt. Yatsugatake Shinano-Kai, 27 July 1949, *Haginiwa Joju* JH009022[C], JH009023[C], JH046160[A] (TNS). Mt. Higashi Suruga, 2 August 1949, *Hirano Hideo.* 32[F] (TNS). Mt. Kamikouchi Shinano, 26 July 1951, *Muramatsu Kouki* 3052[F] (TNS). Mt. Higashi Shinano, 29 July 1951, *Muramatsu Kouki* 3324[D] (TNS). Mt. Senjo, August 1952, *Iwatsuki Z. s.n.*[F] (TNS). Mt. Yatsugatake Shinano, 12 July 1953, *Haginiwa Joju* JH046163[C] (TNS). Mt. Yatsugatake Shinano, 28 July 1953, *Ueda Minoru* 104289[F] (TNS). Mt. Akusawa Suruga, 23 August 1953, *Haginiwa Joju* JH009021[A] (TNS). Kisawa village, 3 August 1956, *Muramatsu Kouki* 2603[D] (TNS). Yamadera-oku Yamagata, 10 June 1957, *Kato Nobuo* 133441[C] (TNS). Mt. Akaishi Shinano, 4 August 1958, *Ibaragi Yoichi* 139447[C] (TNS). Mt. Harinoki Shinano, 30 July 1959, *Okuyama Shunki & Utsumi H.* 15618[C] (TNS). Mt. Harinoki Shinano, 30 July 1959, *Okuyama Shunki* 15675[F] (TNS). Sanpusi-touge Nagano, 4 August 1962, *Kogure Kazuo* 152954[B] (TNS). Mt. Kitadake Yamanashi, 21 August 1962, *Ishizawa* 8240[F] (TNS). Mt. Kitadake Kai, 6 August 1963, *Nakamura Takehisa* 594[C] (TNS). Mt. Kitadake Kai, 7 August 1963, *Ohtake Iwao* 387, 388[B] (TNS). Mt. Kitadake Kai, 7 August 1963, *Masuda Shinya* 2395, 2424, 2455, 2530[B]

(TNS). Mt. Higashi-Arakawa Shizuoka, 1 September 1963, *Iwatsuki K.& Koyama K.* 153[E] (TNS). Mt. Kogouchi Shinano, 16 August 1964, *Masuda Shinya* 3435[B] (TNS). Otomiyama-touge Echigo, 3 August 1965, *Okuyama Shunki* 24735[C] (TNS). Mt. Senjo Shinano, 20 August 1966, *Masuda Shinya* 5289[B] (TNS). Mt. Shiomi to Mt. Kita-arakawa in Minami Alps National Park, 1 August 1967, *Konta F. & Takahashi H.* 511[B] (TNS). Mt. Yatsu Shinano, 20 August 1968, *Okuyama Shunki* 12444[C] (TNS). Asahigoya-Mt. Yukikura Toyama, 29 August 1969, *Satomi N.& Mochizuki R.* 256064[C] (TNS). Sanpukutouge-Eboshi Nagano, 7 July 1971, *Kojima Yu* 340[B] (TNS). Mt. Amakazari, 29 July 1975, *Nomura Kouhei* 39383[B] (TNS). Mt. Amakazari Niigata, 14 August 1978, *Sakuma Yoshihisa s.n.*[F] (TNS). Mt. Amakazari Niigata, 12 August 1980, *Sakuma Y*oshihisa *s.n.*[F] (TNS). Mt. Tsurugi Toyama, 1 September 1980, *Takai Yumi* 949[B] (TNS). Mt. Senmai South alps, 31 August 1984, *Konta Fumihiro.* 15688[B] (TNS). Mt. Hakuba cultivated at Hakuba Goryu Alpine Botanical Garden, 16 June 2016, *Saya Tamura* 334[†], 335[†](SAPT). Mt. Kamikouchi Suruga, *Koidzumi Hideo* 6882[F] (TNS). Mt. Kitahotaka Shinshu, *Koidzumi Hideo* 67470[F] (TNS).

S. nishidae: JAPAN. Hokkaido: Holotype (designated by Miyabe & Kudo 1917)—Mt. Yubari at Gamaiwa, 7 August 1916, *Shozo Nishida* 037669 (SAPS!). Mt. Yubari, 3 August 1921, *Takeda & Tatewaki* 016363, 016364, 016365 (SAPS). Mt. Yubari Gamaiwa, 22-26 July 1928, *Koidzumi Hideo* 16912, 16913 (TNS). Mt. Yubari, 24 July 1928, *Koidzumi Hideo* 16911 (TNS). Mt. Yubari, 1 August 1933, *Sugimoto Junichi* 45613 (TNS). Mt. Yupari Gama-iwa, 18 August 1957, *Shiro Nosaka* 031624 (SAPS). Mt. Yubari, 1 August 1987, *Hideki Takahashi* 016362 (SAPS). Mt. Yubari, 6 August 1991, *Ken Sato* 91.1327, 91.1707 (SAPS). Mt. Yubari, 22 July 1995, *Takita K.* 6312 (SAPT). Mt. Yubari, 3 August 2014, *Ken Sato* 14.200 (SAPS). Mt. Ashibetsu meotoiwa, 22 July 2014, *Nakagawa* 14019, 14020 (SAPS). Mt. Yubari Gama-iwa, 26 July 2016, *Saya Tamura* 589, 594, 610, 615, 620, 627 (SAPT). Mt. Yubari Gama-iwa 19 July 2017, *Saya Tamura* 996[†], 997[†], 998[†], 999[†] (SAPT).

A, *Saxifraga bronchialis* L.; B, *Saxifraga bronchialis* L. subsp. *funstonii* (Small) Hulten var. *rebunshirensis* Hara; C, *Saxifraga cherlerioides* D.Don var. *rebunshirensis* Hara; D, *Saxifraga rebunshirensis* (Engl. Et Irmsh.) Sipl.; E, *Saxifraga* sp.; F, Shikotanso (Japanese name)