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Genetic Diversity of *Saxifraga acerifolia* and *S. fortunei* Based on Nuclear and Chloroplast Microsatellite Markers

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Abstract *Saxifraga acerifolia* is a perennial herb endemic to rock wall surfaces of waterfalls with splashing water located in two gorges in Japan. The unique habitat of this plant caused population fragmentation and population size contraction leading to a bottleneck effect. This species is designated as an endangered plant: Category II on the Japanese Red List. Based on molecular phylogenic study, the sister species were *Saxifraga fortunei* distributed wider range over Japan Archipelago. To evaluate their genetic diversity, we developed nuclear and chloroplast microsatellite markers based on the genomic DNA sequence and the reconstructed chloroplast genome sequence of *Saxifraga acerifolia*. Four polymorphic nuclear markers and seven polymorphic chloroplast markers were obtained. Analyses using the seven chloroplast microsatellite markers, six and 13 haplotypes were detected in *Saxifraga acerifolia* and *S. fortunei*, respectively. The lower haplotype diversity in *Saxifraga acerifolia* would be due to the narrower distribution range compared with *S. fortunei* and/or the past bottleneck effect of the extant small population.

Key words: chloroplast genome, endangered species, haplotype network, microsatellite, population genetics, *Saxifraga*.

Introduction

The genus *Saxifraga* sect. *Irregulares* is well characterized by zygomorphic flowers with two elongated petals, whereas other sections have actinomorphic flowers with five isometric petals. A molecular phylogeny supports the monophyly of this section comprised of approximately 13 species, which ranges from southwestern China to Sakhalin through the Japanese islands (Tkach *et al.*, 2015).

Saxifraga acerifolia Wakabayashi et Satomi is a perennial herb that is confined to two gorges in Fukui and Ishikawa Prefectures in Japan at an elevation of 500–600 m. This species inhabits rocks in waterfalls with splashing water. The unique growing environment of this plant has led to population fragmentation, that may cause a genetic bottleneck effect on isolated populations. Due to its very narrow range, *Saxifraga acerifolia* has been designated as a Category II endangered plant (Ministry of the Environment, 2017) and as "Critically Endangered" on the regional Red Lists (Ishikawa Prefecture, 2010; Fukui Prefecture, 2016). Therefore, investigating its genetic diversity parameters, and evaluating the

genetic differences between the populations in the two gorges, will provide valuable information for its conservation and to understand the population demography of this endangered plant with a unique habitat.

Based on our molecular phylogenetic study (Magota *et al.*, unpublished), *Saxifraga acerifolia* forms a clade together with its sister species of *S. fortunei*, a wide-ranging and common species across the Japanese islands and adjacent regions. Thus, the phylogenetic relationship can provide an opportunity to compare the genetic diversity levels and spatial genetic structures of these species with contrasting distribution ranges. To do so, there is a need for development of genetic markers that can be cross-amplified between the species. Here, we report a set of novel nuclear and chloroplast microsatellite (SSR; simple sequence repeat) markers for *Saxifraga acerifolia* based on its genomic DNA sequence, and detected chloroplast haplotypes to investigate the genetic diversity in *S. acerifolia* and *S. fortunei*. Evaluation of haplotype diversity of the endangered *Saxifraga acerifolia* will provide valuable information to its protection.

Materials and Methods

DNA extraction and Ion PGM sequencing

Saxifraga acerifolia leaf material was collected from an individual cultivated at Yoshida Campus, Kyoto University, Kyoto, Japan (voucher accession number KYO 00037497, deposited in Kyoto University Herbarium). Genomic DNA was extracted from dried leaf samples using the cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987), after washing the leaf powder twice with HEPES buffer (pH = 8.0; Setoguchi and Ohba, 1995). A total of 50 ng of DNA was used to construct DNA fragment libraries using the Ion Xpress Plus Fragment Library Kit, following the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). Template ion sphere particles were prepared using an Ion PGM Hi-Q OT2 Kit on the Ion OneTouch 2 system (Thermo Fisher Scientific). The Ion OneTouch ES system was used to enrich template-positive particles. The particles were run on Ion 318 chips and sequenced using an Ion PGM Sequencer (Thermo Fisher Scientific).

Reconstruction and annotation of the chloroplast genome sequence of Saxifraga acerifolia

A total of 271,064 raw reads (average 190.3 bp) were imported into CLC Genomics Workbench version 7.5.1 software (CLC bio, Aarhus, Denmark), and 271,063 cleaned reads (average 179.8 bp) were obtained after quality-based trimming (quality limit = 0.03). The cleaned reads were assembled using MITObim version 1.8 software (Hahn *et al.*, 2013) with the complete chloroplast genomic sequence of *Sedum sarmentosum* (GenBank accession no. NC023085) as the reference. The annotation analysis was performed using the CPGAVAS Anno Genome module (Liu *et al.*, 2012) with a cut-off BLASTN *E*-value of 1×10^{-10}. Inverted repeat sequences were detected using REPuter with default parameters (Kurtz *et al.*, 2001). A circular map was obtained using OGDRAW (Lohse *et al.*, 2013).

Development of SSR markers

To develop nuclear SSR markers, we screened microsatellite regions including ≥5 dinucleotide, ≥5 trinucleotide, and ≥4 tetranucleotide repeats, using MSATCOMMANDER (Faircloth, 2008). A total of 421 microsatellite motifs were found: 271 of dinucleotide (5–20 repeats), 127 of trinucleotide (5–17 repeats), and 23 of tetranucleotide (4–6 repeats) (Fig. 1), suggesting low genetic diversity. We designed 120 PCR primers using MSATCOMMANDER with the following conditions: primer size of 15–30 bp, annealing temperature of 57–62°C, GC content of 30–70%, and an expected amplicon size of 50–450 bp.

To develop chloroplast SSR markers, we screened chloroplast microsatellite regions including ≥10 mononucleotide repeats, using MSATCOMMANDER, and found 35 loci. We designed 20 PCR primer pairs for these regions using Primer3 (Rozen and Skaletsky, 2000) with the fol-

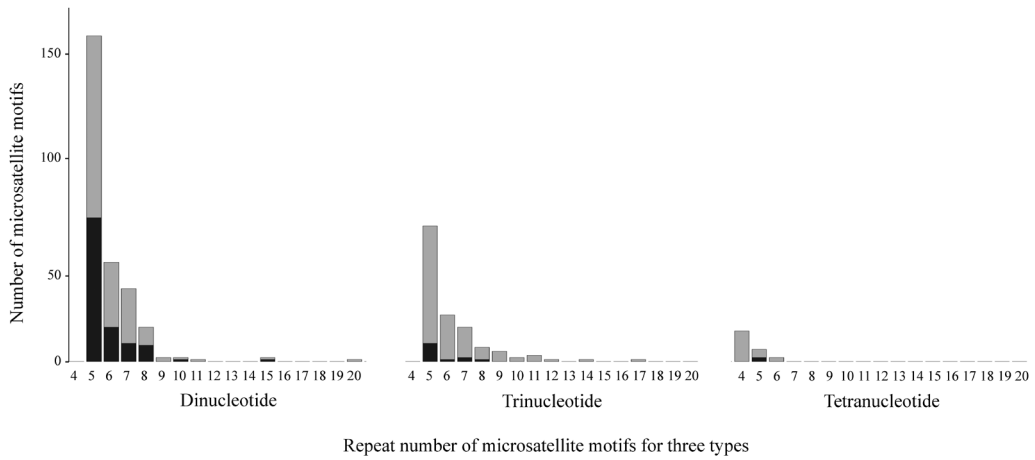

Fig. 1. Number of microsatellite motifs selected to develop PCR primers on the genome of *Saxifraga acerifolia*. 107 of 271 dinucleotide repeat, 13 of 127 trinucleotide repeat, and two of 13 tetranucleotide repeat motifs were selected to develop PCR primers. The vertical line shows the number of microsatellite motifs and the horizontal line shows the repeat number of microsatellite motifs. The light gray and dark gray bars indicate the number of all detected microsatellite motifs and the motifs that were selected for developing PCR primers, respectively.

lowing conditions: primer size of 18–20 bp, annealing temperature of 58–62°C, GC content of 30–70%, and an expected amplicon size of 100–400 bp. An M13-tail sequence (5′-CACGACGTTGTAAAACGAC-3′, 5′-TGTGGAATTGTGAGCGG-3′, 5′-CTATAGGGCACGCGTGGT-3′, or 5′-CGGAGAGCCGAGAGGTG-3′) was added to all forward primers to construct multiplex sequences, and a PIG-tail sequence (5′-GTTTCTT-3′) was added to all reverse primers.

We used 16 *Saxifraga acerifolia* individuals from the two populations to evaluate polymorphisms of these microsatellite loci. Furthermore, we used 15 *Saxifraga fortunei* individuals ranging across Japan (Table 1) to check the versatility of the designed markers. The total PCR reaction volume was 5 µl, containing approximately 0.5 ng DNA, 2.5 µl of 2× QIAGEN Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 0.01 µM of forward primer, 0.2 µM of reverse primer, and 0.1 µM of fluorescence-labeled M13 primer. The PCR thermal profile was set as follows: an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 58°C for 3 min, 68°C for 1 min, and then a final extension at 68°C for 20 min. Amplified PCR products were loaded onto an ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA), and the fragment length was determined using GeneMapper software (Applied Biosystems). To evaluate the polymorphisms of the markers and the genetic diversity, we calculated the number of alleles per locus, the observed heterozygosity (H_O), the expected heterozygosity (H_E) for the nuclear markers, and the number of alleles per locus and unbiased diversity (*uh*) for the chloroplast markers, using GenAlex version 6.503 soft-

Table 1. Localities of *Saxifraga fortunei* samples used in this study. The geographic information of the two *S. acerifolia* populations is not shown here, because the species is threatened by illegal digging.

	Sampling locality	Latitude	Longitude
1	Yufutsu, Hokkaido	42°33′49″N	142°12′52″E
2	Tsuruoka, Yamagata	38°31′52″N	139°57′23″E
3	Kimitsu, Chiba	35°06′55″N	139°35′35″E
4	Ina, Nagano	35°32′58″N	138°07′05″E
5	Hakuba, Nagano	36°39′53″N	137°48′50″E
6	Okazaki, Aichi	34°33′17″N	137°14′39″E
7	Sakai, Fukui	36°08′05″N	136°22′30″E
8	Matsuzaka, Mie	34°20′44″N	136°08′52″E
9	Higashimuro, Wakayama	33°40′34″N	135°53′16″E
10	Nantan, Kyoto	35°18′37″N	135°43′00″E
11	Takahama, Fukui	35°18′06″N	135°17′24″E
12	Fukuchiyama, Kyoto	35°15′17″N	135°05′29″E
13	Muroto, Kochi	33°20′26″N	134°07′51″E
14	Koyu, Miyazaki	32°10′24″N	131°16′53″E
15	Yakushima Island, Kagoshima	30°18′17″N	130°34′13″E

ware (Peakall and Smouse, 2006). Deviations from Hardy-Weinberg equilibrium (HWE) were assessed for each nuclear locus using GenAlex version 6.503. In addition, we detected haplotypes with chloroplast SSR markers, and calculated median joining network of *Saxifraga acerifolia* and *S. fortunei* using NETWORK version 5.0.0.3 (Bandelt *et al*., 1999).

Results and Discussion

Structure of the chloroplast genome of Saxifraga acerifolia

The chloroplast genome length reconstructed with MITObim was 151,395 bp (GenBank accession no. AP018459), nearly identical to 150,448 bp of *Sedum sarmentosum*'s one. When the cleaned reads were mapped to the assembled genome sequence, the average read depth was 22 across the genome. The nearly complete chloroplast genome with 941 bp of undetermined sites was composed of an 82,807-bp large single-copy (LSC) region, a 14,844-bp small single-copy (SSC) region, and 53,744 bp of a pair of inverted

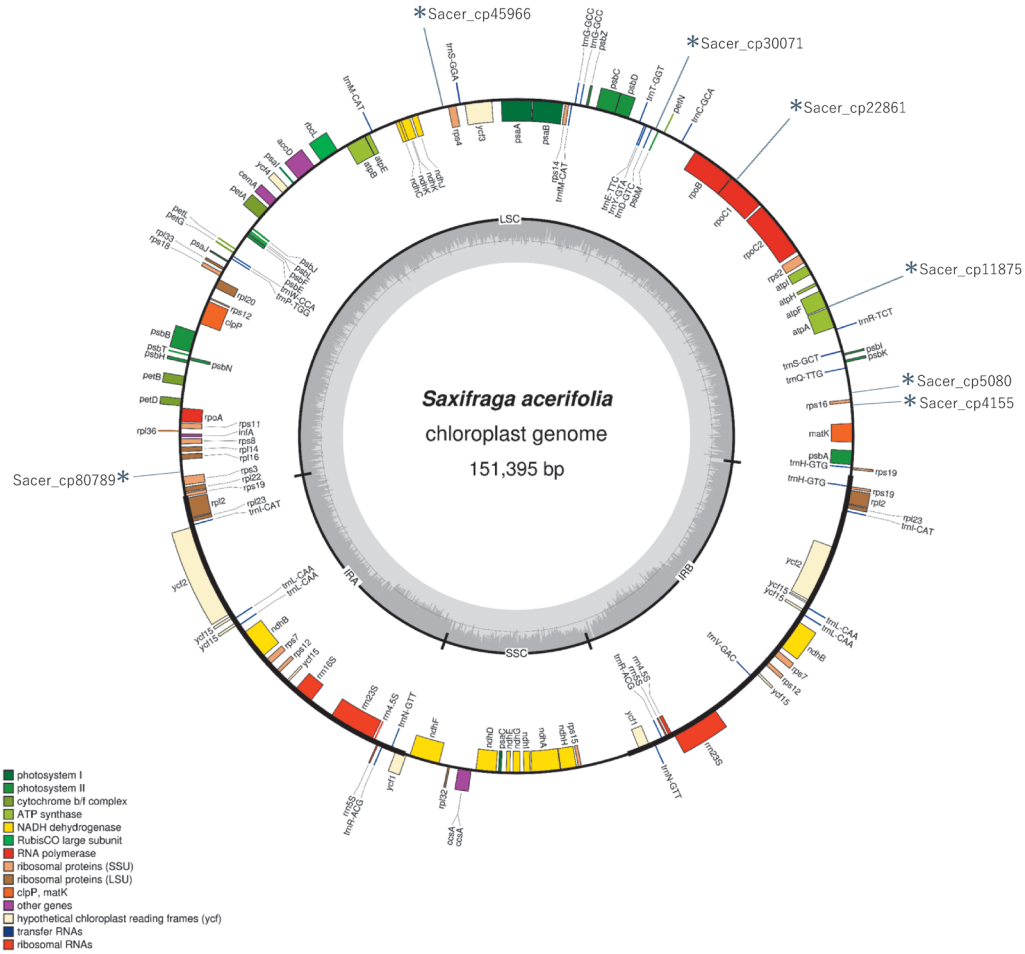

Fig. 2. Distribution of genes on the chloroplast genome of *Saxifraga acerifolia*. The whole genome size of the chloroplast DNA was estimated to be 151,395 base pairs (bp) with a large single-copy region (85,493 bp), small single-copy region (17,226 bp), and a pair of inverted repeat regions (48,676 bp). The dark-gray and light-gray on the inner circle correspond to GC content and AT content, respectively. The positions of polymorphic microsatellite loci are indicated with asterisks.

Table 2. Functions of genes annotated in chloroplast sequence

Functions	Family name	Genes
Genes for photosynthesis	Subunits of ATP synthase	*atpA*, *atpB*, *atpE*, *atpF*, *atpH*, *atpI*
	Subunits of NADH-dehydrogenase	*ndhA*, *ndhB*, *ndhC*, *ndhD*, *ndhE*, *ndhF*
	Subunits of cytochrome b/f complex	*petA*, *petB*, *petD*, *petG*, *petL*, *petN*
	Subunits of photosystem I	*psaA*, *psaB*, *psaC*, *psaI*, *psaJ*
	Subunits of photosystem II	*psbA*, *psbB*, *psbC*, *psbD*, *psbE*, *psbF*, *psbH*, *psbI*, *psbJ*, *psbK*, *psbL*, *psbM*, *psbN*, *psbT*, *psbZ*
	Subunit of rubisco	*rbcL*
Self-replication	rRNA genes	*rrn4.5S*, *rrn4.5S*, *rrn5S*, *rrn5S*, *rrn16S*, *rrn23S*
	tRNA genes	*trnC-GCA*, *trnD-GTC*, *trnE-TTC*, *trnfM-CAT*, *trnG-GCC*, *trnH-GTG*, *trnI-CAT*, *trnL-CAA*, *trnM-CAT*, *trnN-GTT*, *trnP-TGG*, *trnQ-TTG*, *trnR-ACG*, *trnR-TCT*, *trnS-GCT*, *trnS-GGA*, *trnT-GGT*, *trnV-GAC*, *trnW-CCA*, *trnY-GTA*
	Large subunit of ribosome	*rpl2*, *rpl14*, *rpl16*, *rpl20*, *rpl22*, *rpl23*
	Small subunit of ribosome	*rps2*, *rps3*, *rps4*, *rps7*, *rps8*, *rps11*, *rps12*, *rps14*, *rps15*, *rps16*, *rps18*, *rps19*
	DNA-dependent RNA polymerase	*rpoA*, *rpoB*, *rpoC1*, *rpoC2*
Other genes	Subunit of Acetyl-CoA-carboxylase	*accD*
	c-type cytochrom synthesis gene	*ccsA*
	Envelop membrane protein	*cemA*
	Protease	*clpP*
	Translational initiation factor	*infA*
	Maturase	*matK*
Genes of unknown function	Conserved open reading frames	*ycf1*, *ycf2*, *ycf3*, *ycf4*, *ycf15*

repeat (IR) regions. A total of 132 genes were annotated, including 46 genes for photosynthesis, 67 genes for self-replication, 7 genes for other functions, and 12 genes for unknown functions (Fig. 2, Table 2). The overall GC content was 37.7%, and IRs (42.0%) holds greater GC content than LSC (36.0%) and SSC (31.8%) regions.

Development of nuclear SSR markers and genetic diversity

Among the 120 primer pairs tested, 57 showed clear allelic peaks with expected product lengths, 3 of which (Sacer_1601, Sacer_9094, and Sacer_13684) were polymorphic (Tables 3, 4, and 6). The number of alleles per locus was two, the H_O ranged from 0.125 to 0.250, and the H_E ranged from 0.219 to 0.500. In *Saxifraga fortunei*, 33 out of 120 loci were amplified, and two of them (Sacer_10700 and Sacer_13684) were polymorphic (Tables 3, 4, and 6). One locus (Sacer_13684) was polymorphic in both species, and ten alleles were detected in *Saxifraga fortunei*, whereas the other locus (Sacer_10700) harbored two alleles. The H_O was 0.000 and 0.400, and the H_E was 0.124 and 0.862 in the respective loci. Two loci (Sacer_9094 and Sacer_13684) in *Saxifraga acerifolia*, and two loci (Sacer_10700 and Sacer_13684) in *Saxifraga fortunei* were deviated from HWE ($P<0.05$). The significant deviations from HWE in the latter species are likely due to the fact that samples from isolated populations, which would be assigned to independent panmictic groups, are combined for the tests.

Development of chloroplast SSR markers and genetic diversity

In *Saxifraga acerifolia*, 13 of the 20 loci were amplified and 3 of them were polymorphic (Tables 3, 5, and 6). In *Saxifraga fortunei*, all 13 markers amplified in *S. acerifolia* showed clear peaks, and 7 of them were polymorphic. Three loci (Sacer_cp4155, Sacer_cp5080, and Sacer_cp11875) were polymorphic in both species, whereas the other loci (Sacer_cp22861, Sacer_cp30071, Sacer_cp45966, and Sacer_cp80789) were polymorphic only in *Saxifraga fortunei*, with two to four alleles (Table 5). Among the seven markers, six were located in intergenic

Table 3. Characteristics of four nuclear and seven chloroplast microsatellite markers for *Saxifraga acerifolia* and *S. fortunei*

Organella	Locus name	Repeat-motif	Primer sequence (5′-3′)	BLASTX top hit description	E-value	GenBank accession no.
nuclear	Sacer_1601	$(AG)_6$	F: TGAAGTTGCCAGTGTTACAAGCCTATAGGGCACGCGTGGT R: GTTTCTTCCCAAGCACGATAATGAAATTGC	CRCB domain-containing protein, partial [Cephalotus follicularis]	3.0E-04	LC360662
nuclear	Sacer_9094	$(AACG)_5$	F: TGTGGAATTGTGAGCGGATTCGGTCTCTTCGTCCATG R: GTTTCTTTGGACGGCTGAGATCATGTC	No significant hit	0	LC360663
nuclear	Sacer_10700	$(AT)_5$	F: CTATAGGGCACGCGTGGTTTGGTCTGATGAGTTCCCGG R: GTTTCTTCAAGCTCTTCTGACATGACCTG	No significant hit	0	LC360664
nuclear	Sacer_13684	$(AG)_6$	F: AGACAGAACCAACAGTCAATCGCGGAGAGCCGAGAGGTG R: GTTTCTTAGAGGATCATGAAGAGAGTGCC	Hypothetical protein PENVUL_c176G00998 [Penicillium vulpinum]	2.2	LC360665
chloroplast	Sacer_cp4155	$(A)_{22}$	F: TGTGGAATTGTGAGCGGTGCATGACCCAATCAAAACA R: GTTTCTTAGCTGACGGGTTCGTTGA	—	—	LC360649
chloroplast	Sacer_cp5080	$(C)_{10}$	F: CGGAGAGCCGAGAGGTGCGGTAGACCGCTCATTGG R: GTTTCTTCTCGAGCCGTACGAGGAG	—	—	LC360650
chloroplast	Sacer_cp11875	$(A)_{10}$	F: CGGAGAGCCGAGAGGTGAGCAATGCCATCGCCTAC R: GTTTCTTTTGGGGCGATGAAAGAAA	—	—	LC360651
chloroplast	Sacer_cp22861	$(T)_{10}$	F: CTATAGGGCACGCGTGGTTCCCGACTTCACCTCGAC R: GTTTCTTGCTCGGAATTGTGGGTGT	—	—	LC360652
chloroplast	Sacer_cp30071	$(T)_{11}$	F: TGTGGAATTGTGAGCGGTCAAATCGATTCATCGTCCA R: GTTTCTTTACCCCGAAGGCGGTAGT	—	—	LC360653
chloroplast	Sacer_cp45966	$(A)_{10}$	F: TGTGGAATTGTGAGCGGTGGGACAAACGGGAGTAAA R: GTTTCTTGCTCAGGATTGCCCATTTT	—	—	LC360654
chloroplast	Sacer_cp80789	$(T)_{14}$	F: TGTGGAATTGTGAGCGGTGTGAAGCGATGAGTTGGTT R: GTTTCTTGCTGCCAGCGATGGAATA	—	—	LC360655

regions (*matK-rps16*, *rps16-trnQ* (TTG), *atpA-atpF*, *psbM-trnD* (GTC), *rps4-ndhJ*, and *rpl16-rps3*) and the remainder was in an intron (*rpoC1*). All loci were located in the LSC region (Fig. 2). In *Saxifraga acerifolia*, the number of alleles ranged from two to three, and *uh* ranged from 0.233 to 0.675. In *Saxifraga fortunei*, the number of alleles ranged from two to six, and *uh* ranged from 0.248 to 0.867 (Table 5).

Saxifraga acerifolia showed polymorphism at fewer loci than wide-ranging *S. fortuei* (Table 5), likely owing to population size reduction accompanied with bottleneck effect(s) over its history that sculptured its current distribution into two gorges. This unique habitat may have also decreased the allelic diversity among and within populations. High rate of successful cross-amplification of chloroplast markers in *Saxifraga fortunei* should be attributed to its being a sister taxon of *S. acerifolia* (Tables 4, 5).

Table 4. Genetic diversity of four nuclear markers in *Saxifraga acerifolia* and *S. fortunei*. *A*, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity. *Deviation from Hardy-Weinberg equilibrium ($P<0.05$).

Locus name	*S. acerifolia* (n = 16)				*S. fortunei* (n = 15)				Total (n = 31)			
	A	H_O	H_E	Size range (bp)	*A*	H_O	H_E	Size range (bp)	*A*	H_O	H_E	Size range (bp)
Sacer_1601	2	0.125	0.219	251–253	—	—	—	—	2	0.125	0.219	251–253
Sacer_9094	2	0.125*	0.305	212–220	—	—	—	—	2	0.125	0.305	212–220
Sacer_10700	1	0.000	0.000	256	2	0.000*	0.124	260–264	3	0.000	0.062	256–264
Sacer_13684	2	0.250*	0.500	87–89	10	0.400*	0.862	65–103	10	0.325	0.681	65–103
Average	1.8	0.063	0.256		6.0	0.200	0.493		4.3	0.144	0.317	

Table 5. Genetic diversity of seven chloroplast markers in *Saxifraga acerifoia* and *S. fortune*. *A*, number of alleles; *uh*, unbiased diversity

Locus name	Region	*S. acerifolia* (n = 16)			*S. fortunei* (n = 15)			Total (n = 31)		
		A	*uh*	Size range (bp)	*A*	*uh*	Size range (bp)	*A*	*uh*	Size range (bp)
Sacer_cp4155	*matK-rps16*	3	0.675	261–263	6	0.867	250–255	9	0.771	250–263
Sacer_cp5080	*rps16-trnQ* (TGG)	2	0.233	104–105	2	0.248	100–101	4	0.240	100–105
Sacer_cp11875	*atpA-atpF*	2	0.400	229–230	3	0.257	229–234	3	0.329	229–234
Sacer_cp22861	*rpoC1*; Intron	1	0.000	264	3	0.590	264–266	3	0.295	264–266
Sacer_cp30071	*psbM-trnD* (GTC)	1	0.000	165	3	0.533	164–166	3	0.267	164–166
Sacer_cp45966	*rps4-ndhJ*	1	0.000	420	4	0.619	417–431	5	0.310	417–431
Sacer_cp80789	*rpl16-rps3*	1	0.000	301	2	0.248	305–318	3	0.124	301–318
Average	Average	1.6	0.187		3.3	0.480		4.3	0.341	

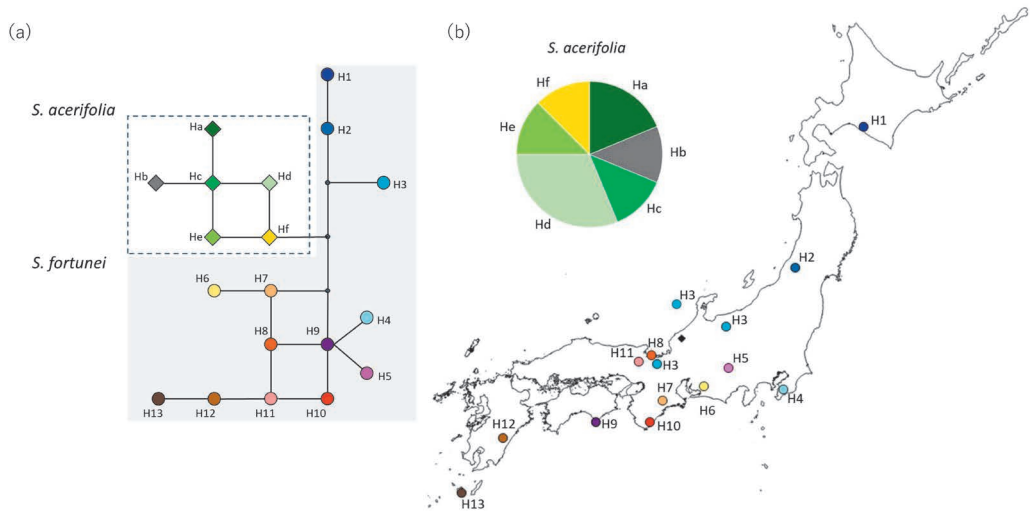

Fig. 3. (a) Haplotype network of *Saxifraga acerifolia* and *S. fortune*i based on seven chloroplast microsatellite markers. Six haplotypes (Ha–Hf) are in *Saxifraga acerifolia* (within dotted line) and 13 haplotypes (H1–H13) are in *S. fortunei* (in the shadow). (b) Distribution of *Saxifraga acerifolia* and *S. fortunei* in the Japanese Archipelago. The sampled points are shown as coloured dots suggesting the haplotypes. In *Saxifraga acerifolia*, the ratio of each haplotype was shown.

Chloroplast haplotype network and distribution in Japanese Archipelago

Based on seven chloroplast SSR markers, we detected six haplotypes in *Saxifraga acerifolia* and 13 haplotypes in *S. fortunei*. The relationship of each haplotype was shown in a network (Fig.

Table 6. Amplified microsatellite markers

Organella	Locus name	Repeat motif	Primer sequence (5′-3′)	BLASTX top hit description	E-value	GenBank accession no.	Allele size range (bp)	
							S. acerifolia	*S. fortunei*
nuclear	Sacer_327	$(CT)_5$	F: TGTGGAATTGTGAGCGGCCG GGTTGTGGAGAAGTTTC R: GTTTCTTCAGCTAGCAGTTC-TAATTTGATATCAC	No significant hit	0	LC360666	247	251
nuclear	Sacer_533	$(AT)_5$	F: CGGAGAGCCGAGAGGT-GCTGCCTACATTTAACCGCCC R: GTTTCTTCCTCAGCTCCTCC-ACCATC	No significant hit	0	LC360667	322	322
nuclear	Sacer_534	$(AT)_5$	F: CTATAGGGCACGCGTGGTCT-GCCTACATTTAACCGCCC R: GTTTCTTCGGTGAGGTTAG-TGGTTTGC	Hypothetical protein [*Beta vulgaris*]	2.00E-20	LC360668	431	431
nuclear	Sacer_712	$(AT)_5$	F: TGTGGAATTGTGAGCGGTC-ACCGAAAGAGCTGAAATCATG R: GTTTCTTGCTGGACTTGCGA-GATTTATAAG	No significant hit	0	LC360669	214	—
nuclear	Sacer_861	$(AG)_5$	F: CGGAGAGCCGAGAGGTGTC-TGTTATGTATTTAAGAGCCGAG R: GTTTCTTGGGATGTACTCTA-CCCTAGCC	No significant hit	0	LC360670	140	140
nuclear	Sacer_883	$(AT)_5$	F: TGTGGAATTGTGAGCGGAA-AGCAAGCGATCACCCATG R: GTTTCTTAGGAAGGAAGTG-GAGCGAAG	PHD domain-containing protein [*Cephalotus follicularis*]	2.00E-28	LC360671	382	382
nuclear	Sacer_956	$(CT)_5$	F: CGGAGAGCCGAGAGGTGCT-TAACTGACATGAGAAATTTAT-AGAAACC R: GTTTCTTTGTGTGAAAGCTT-GTGACGG	Uncharacterized protein [*Asparagus officinalis*]	2.00E-11	LC360672	233	—
nuclear	Sacer_1339	$(AG)_5$	F: CGGAGAGCCGAGAGGTGCC-AGTAGTTTGACGTTCGGC R: GTTTCTTCAAAGCTCGACA-CTGCTAGC	No significant hit	0	LC360673	235	247
nuclear	Sacer_1571	$(AG)_5$	F: CGGAGAGCCGAGAGGTGAG-CTAGCAGTTCTAAATATTAATT-CAAGC R: GTTTCTTTTGACGCGGTGAG-TAGGATC	No significant hit	0	LC360674	147	144
nuclear	Sacer_2425	$(AT)_5$	F: CACGACGTTGTAAAACGAC-AGCTTGGAAATAGTACAGA-ATGC R: GTTTCTTTGTCGTATCAGTT-TGAAGTTGG	No significant hit	0	LC360675	141	—
nuclear	Sacer_2567	$(AT)_6$	F: CTATAGGGCACGCGTGGTA-AAGAGGGTGAGAAGTAACGAC R: GTTTCTTTGTAACGAGTCAG-GAGGTAAAC	No significant hit	0	LC360676	105	—
nuclear	Sacer_2806	$(AG)_5$	F: CGGAGAGCCGAGAGGTGGT-GATGATGAATATATAGGAGA-ATTTAGGG R: GTTTCTTAGGCAGTTGGTTG-TAAGAAGG	No significant hit	0	LC360677	159	159
nuclear	Sacer_2919	$(AG)_5$	F: CTATAGGGCACGCGTGGTCC-AAGGAGGGCTAGCTAGTC R: GTTTCTTCAAATGCGGCAAC-CTGGTG	No significant hit	0	LC360678	124	124
nuclear	Sacer_3393	$(AG)_5$	F: TGTGGAATTGTGAGCGGTCA-AGGACAATTTCTTAGCTATCTCC R: GTTTCTTACTTCGTCAACAA-ACCCTGC	No significant hit	0	LC360679	153	—
nuclear	Sacer_3512	$(ATT)_5$	F: CGGAGAGCCGAGAGGTGTC-ACATAAGCCGTCATAAAGTG R: GTTTCTTGATTCCCTCGAGC-ACTTAGTTC	No significant hit	0	LC360680	186	—

Table 6. Continued.

Organella	Locus name	Repeat motif	Primer sequence (5′-3′)	BLASTX top hit description	E-value	GenBank accession no.	Allele size range (bp)	
							S. acerifolia	*S. fortunei*
nuclear	Sacer_3600	$(AT)_5$	F: CACGACGTTGTAAAACGACCTCGGTAATGCTGTTGTAGGAG R: GTTTCTTTGAAATTATGTGAGGAATCAATGATGC	Uncharacterized protein [*Ipomoea nil*]	3.00E-09	LC360681	121	—
nuclear	Sacer_3910	$(ATT)_5$	F: CTATAGGGCACGCGTGGTGCTTGTCAGGTATTACTCTTTCCC R: GTTTCTTAGCATATTATTGAATCAACCCAACC	No significant hit	0	LC360682	153	—
nuclear	Sacer_3935	$(AT)_5$	F: CTATAGGGCACGCGTGGTAGGTACCATCCATGACCCTTC R: GTTTCTTTGCTGAACTAAGGCACCAAG	E3 ubiquitin-protein ligase COP1 [*Fragaria* x *ananassa*]	3.80E-02	LC360683	162	—
nuclear	Sacer_4065	$(AT)_5$	F: CACGACGTTGTAAAACGACCGTGACCGTTGGATTAAATCATAG R: GTTTCTTACCATTGGATATACTCGCATTCAC	No significant hit	0	LC360684	238	—
nuclear	Sacer_4360	$(AT)_5$	F: CGGAGAGCCGAGAGGTGAAGCATTTGTTCTCGCTCCG R: GTTTCTTAGACGCCTAAGTTGACCTGG	No significant hit	0	LC360685	279	275
nuclear	Sacer_5168	$(AT)_5$	F: TGTGGAATTGTGAGCGGACGCATTTAACATAAACAACGC R: GTTTCTTTGTTAGGTTTAATTATTCAGTGAAGTGTG	No significant hit	0	LC360686	282	—
nuclear	Sacer_5195	$(GT)_8$	F: CTATAGGGCACGCGTGGTAAGATGTTCCAGTTCAGCATCG R: GTTTCTTGACTTTACTTCTCATTTGCGCC	No significant hit	0	LC360687	216	—
nuclear	Sacer_5212	$(AT)_8$	F: TGTGGAATTGTGAGCGGGGTTTATTGCTACCTGTTCCC R: GTTTCTTAAGAACTTGGGAAGGGCATTTG	Rust resistance kinase Lr10-like, partial [*Juglans regia*]	2.00E-18	LC360688	281	—
nuclear	Sacer_5285	$(AT)_5$	F: CACGACGTTGTAAAACGACGCCGTGACTTCGACTTTGAG R: GTTTCTTGTGTTCTGTTCACGCGCTAC	No significant hit	0	LC360689	385	385
nuclear	Sacer_5290	$(TA)_5$	F: CACGACGTTGTAAAACGACCAAATTGGCCGCGTGAAATC R: GTTTCTTCATACACTGCCCACCACATG	No significant hit	0	LC360690	229	—
nuclear	Sacer_5392	$(AT)_5$	F: TGTGGAATTGTGAGCGGTGATCTTCACGAATAGATATGTTACC R: GTTTCTTATCAACCCAGTCTCGCAATG	No significant hit	0	LC360691	160	160
nuclear	Sacer_5945	$(AC)_5$	F: CACGACGTTGTAAAACGACATTCCAGCCACTAGATACTCCG R: GTTTCTTTTCGGGATGAATTGGATGCAC	Hypothetical protein [*Dorcoceras hygrometricum*]	7.00E-10	LC360692	204	204
nuclear	Sacer_6260	$(AAT)_5$	F: CGGAGAGCCGAGAGGTGACGAAGATGATGACGGGAGAG R: GTTTCTTAGCATCAAACAACAAATATGACATAC	No significant hit	0	LC360693	196	—
nuclear	Sacer_6327	$(AG)_6$	F: CTATAGGGCACGCGTGGTGGTTTAAAGAGTGGCATCAGGG R: GTTTCTTCTACCACTACCTCCTACGCTG	Uncharacterized protein [*Vitis vinifera*]	5.00E-34	LC360694	198	—
nuclear	Sacer_6443	$(GA)_6$	F: CTATAGGGCACGCGTGGTTGTCATGTGTAACCCGTTATAAGAG R: GTTTCTTATCAATTGTCGGCGTAACGG	No significant hit	0	LC360695	180	181

Table 6. Continued.

Organella	Locus name	Repeat motif	Primer sequence (5′-3′)	BLASTX top hit description	E-value	GenBank accession no.	Allele size range (bp)	
							S. acerifolia	*S. fortunei*
nuclear	Sacer_7179	$(AG)_5$	F: TGTGGAATTGTGAGCGGGTC-GGTTACTTAGCTACCACTTATC R: GTTTCTTTCTTGAGGCGGTG-AGTAGAC	No significant hit	0	LC360696	183	—
nuclear	Sacer_7308	$(CT)_5$	F: CACGACGTTGTAAAACGAC-ACGGAGTCGAACATCGTCAC R: GTTTCTTCCAACAAATTTCA-GCTAGCAACC	No significant hit	0	LC360697	133	133
nuclear	Sacer_7483	$(GT)_6$	F: CTATAGGGCACGCGTGGTAC-TACGAAATGACATTCAGGACG R: GTTTCTTAGGTTGTGTTGAA-TTAGTTTGTTTGG	No significant hit	0	LC360698	183	183
nuclear	Sacer_7935	$(CT)_5$	F: CTATAGGGCACGCGTGGTGC-TGTTCCCATAGCGTTACG R: GTTTCTTCAAAGAATAGGCT-GCGTCCG	No significant hit	0	LC360699	210	—
nuclear	Sacer_8212	$(AT)_5$	F: CTATAGGGCACGCGTGGTCT-CTGTAACCAATGCGAGCC R: GTTTCTTGATTGCGCTATGG-GATGAGC	Uncharacterized protein [*Chenopodium quinoa*]	2.00E-05	LC360700	162	162
nuclear	Sacer_8233	$(AG)_7$	F: TGTGGAATTGTGAGCGGCTT-GATCTCTCTTGCCCAGTTG R: GTTTCTTAGGACTCTTCAAC-GGACTCTTC	No significant hit	0	LC360701	281	—
nuclear	Sacer_8417	$(TC)_7$	F: CGGAGAGCCGAGAGGTGCA-TCTCTATTGCGGCATACCTC R: GTTTCTTACAAGAAGCCTGG-AGATATGGC	No significant hit	0	LC360702	189	189
nuclear	Sacer_8431	$(CT)_5$	F: CGGAGAGCCGAGAGGTGAA-ACACCCGCGTACCTTC R: GTTTCTTCCAAAGATTTCAG-CTAGCAGTTC	No significant hit	0	LC360703	112	112
nuclear	Sacer_9231	$(AC)_5$	F: CACGACGTTGTAAAACGAC-CCTTGATCATGGTCGTCTGC R: GTTTCTTCCCAGAAGAGGA-GGATGCTC	Hypothetical protein [*Dorcoceras hygrometricum*]	5.00E-18	LC360704	281	281
nuclear	Sacer_9434	$(CT)_5$	F: CGGAGAGCCGAGAGGTGCC-AATAAACACCTGCCGGAG R: GTTTCTTAGTCAAAGTCCTG-ATATGGTTTAAC	No significant hit	0	LC360705	142	—
nuclear	Sacer_9837	$(AG)_5$	F: CGGAGAGCCGAGAGGTGTT-TCGTGCAATGGTAGGTCG R: GTTTCTTTGAACATCGT-CACCATATCACG	No significant hit	0	LC360706	171	171
nuclear	Sacer_10540	$(AT)_5$	F: CGGAGAGCCGAGAGGTGTT-TCACTCGTCGACCCTTCC R: GTTTCTTACACGTGCTTCTT-TGCTACC	Putative AC9 transposase [*Apostasia shenzhenica*]	4.00E-02	LC360707	202	210
nuclear	Sacer_11457	$(AT)_8$	F: CGGAGAGCCGAGAGGTGGC-GTCAATTTATGGTTGGATGC R: GTTTCTTACTTGTTTAGGCT-GTACATGGC	No significant hit	0	LC360708	213	206
nuclear	Sacer_11739	$(AT)_5$	F: CGGAGAGCCGAGAGGTGAG-TCCCTCAGATTTCCCACG R: GTTTCTTGGTACGACCGGAC-AACTACC	NADH-quinone oxidoreductase protein [*Medicago truncatula*]	4.00E-39	LC360709	312	308
nuclear	Sacer_13136	$(AT)_5$	F: CACGACGTTGTAAAACGACT-TGTAGACTGGGCGTGGATG R: GTTTCTTCACACAACTACCC-ATGGCAC	No significant hit	0	LC360710	154	—
nuclear	Sacer_13233	$(AG)_5$	F: CACGACGTTGTAAAACGAC-GTCTTCTCTTCAAAGCTAGCCG R: GTTTCTTGATTCCGTGAAAG-AAACCTCCC	No significant hit	0	LC360711	268	268

Table 6. Continued.

Organella	Locus name	Repeat motif	Primer sequence (5′-3′)	BLASTX top hit description	E-value	GenBank accession no.	Allele size range (bp)	
							S. acerifolia	*S. fortunei*
nuclear	Sacer_13410	$(AT)_5$	F: CACGACGTTGTAAAACGACCAAGACACAAGGCTAGGCTTG R: GTTTCTTGGGTGTCAATAAATCCAGGAGG	Uncharacterized mitochondrial protein AtMg00810-like, partial [Phoenix dactylifera]	1.00E-41	LC360712	251	—
nuclear	Sacer_13472	$(AT)_5$	F: TGTGGAATTGTGAGCGGTCATGAACACAAACTAAATGACAGTC R: GTTTCTTAAGATATGCACATTGTTCATTCAC	No significant hit	0	LC360713	139	139
nuclear	Sacer_13541	$(AT)_6$	F: CACGACGTTGTAAAACGACTTCGCATGACAAACTTACTCCC R: GTTTCTTGCTCATTAGTCAGTTGCCTACG	No significant hit	0	LC360714	154	146
nuclear	Sacer_13615	$(AC)_5$	F: CTATAGGGCACGCGTGGTCCATCTTGCACAATTAAATTTATAACGTG R: GTTTCTTTGGTGGCTCTTTATTTCATGTAAG	Hypothetical protein [Prunus persica]	5.00E-19	LC360715	82	82
nuclear	Sacer_13650	$(AG)_7$	F: TGTGGAATTGTGAGCGGAGAACAGAGTGAATTTGAAGGG R: GTTTCTTCTCCAAATTTAGAATTGGTTATATACAGTG	No significant hit	0	LC360716	159	—
nuclear	Sacer_14280	$(AG)_5$	F: CACGACGTTGTAAAACGACTGGTGGTAGATCGAAACTTGG R: GTTTCTTTCATCGTGTTCTTTCATTTCATAGC	No significant hit	0	LC360717	157	—
nuclear	Sacer_14931	$(CT)_5$	F: TGTGGAATTGTGAGCGGCTTAACTGACATGAGAAATTTATAGAAACC R: GTTTCTTAGGCACGTATGGACTTGAAAG	Uncharacterized protein [Erythranthe guttata]	1.00E-13	LC360718	170	170
chloroplast	Sacer_cp16184	$(T)_{11}$	F: CACGACGTTGTAAAACGACCCCGCTTCCATCATCTCT R: GTTTCTTTTCGAGGGGGAAATGAGA	—	—	LC360656	402	399
chloroplast	Sacer_cp26106	$(T)_{11}$	F: CACGACGTTGTAAAACGACTTCGTCGACCAACCCTTC R: GTTTCTTCGGTCTATACGGGCACCA	—	—	LC360657	388	389
chloroplast	Sacer_cp39842	$(C)_{11}$	F: CACGACGTTGTAAAACGACCCCCTCTTCCAGGTCCAT R: GTTTCTTCATGCTTTAGCGCCTGGT	—	—	LC360658	302	302
chloroplast	Sacer_cp43270	$(A)_{10}$	F: CTATAGGGCACGCGTGGTCGCTCTAGTGCCCGAAAA R: GTTTCTTGCCCCGCTTCAGTTCATA	—	—	LC360659	353	351
chloroplast	Sacer_cp52987	$(T)_{10}$	F: CGGAGAGCCGAGAGGTGAATTCGCCCAAGGGTAGC R: GTTTCTTCTGATCCTGGGGTTTCCA	—	—	LC360660	332	332
chloroplast	Sacer_cp60664	$(A)_{10}$	F: CGGAGAGCCGAGAGGTGTTTGAATGTGGGGGCTGT R: GTTTCTTTCCGATGGATCCGCTATG	—	—	LC360661	418	415

3a). In *Saxifraga fortunei*, two groups among 13 haplotypes were detected: northern group (H1–H3) and central and southern group (H4–H13) (Fig. 3a, b). These two groups were distinguished by four missing mutation steps in the network. *Saxifraga acerifolia* was derived from the missing haplotype between the two group of *S. fortunei*, suggesting the possibility that *S. acerifolia* might have been diverged from *S. fortunei* as has been indicated by molecular phylogeny. Another

explanation would be incomplete lineage sorting of chloroplast DNA haplotypes between the two species.

Saxifraga fortunei with wide distribution range harbored the more number of haplotypes and the higher genetic diversity than *S. acerifolia* with narrow distribution range (Tables 4, 5 and Fig. 3a). Thereby, nuclear and chloroplast SSR markers indicated *Saxifraga acerfolia* has lower genetic diversity than its sister taxon, *S. fortunei*, suggesting limited habitat and narrow distribution range would have decreased the genetic diversity. Higher genetic diversity found in *Saxifraga fortunei* would be attributed to the wide distribution range nevertheless the limited numbers of samples, only one representative individuals collected for each population. Further study using more population samples covering the whole distribution range would reveal the genetic diversity of *Saxifraga fortunei* and the evolutionally history of *S. acerifolia.*

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