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REGULAR PAPER

Chloroplast DNA sequencing and detailed microsatellite genotyping of all remnant populations suggests that only a single genet survives of the critically endangered plant *Rehmannia japonica*

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Abstract *Rehmannia japonica* (Thunb.) Makino ex T. Yamaz. is an endangered perennial herb species in Japan. Although earlier the Japanese considered it a variety of *R. glutinosa*, recent Japanese taxonomists have consistently regarded it as an independent species. According to the historical literature, *Rehmannia japonica* seems to have been known in China and Japan in the past. However, Chinese taxonomists do not recognize *R. japonica* at present. In Japan, only two populations are known, and although these populations flower every year, seed reproduction has not been observed. In this study, we aimed to reveal the phylogenetic relationships and levels of genetic diversity of *R. japonica*. A haplotype network based on two chloroplast DNA regions (*trnL-trnF* and *rps16*) showed that the sequences of *R. japonica* were distinguishable by three or four sites of indels from the most closely related species, *R. chingii*, consistent with the separate species status of *R. japonica*. An analysis of genetic diversity using twelve microsatellite loci showed that all of the ramets of *R. japonica* collected from two geographically isolated populations had an identical multilocus genotype, including identical heterozygous genotypes at six loci. This result indicated asexual origin of all sampled ramets. This study also suggests that the absence of sexual reproduction of *R. japonica* is explained by self-incompatibility combined with only a single genet remaining in the *R. japonica* populations.

Keywords Clonal plant · Conservation · Historical transplantation · Microsatellite · *Rehmannia chingii* · *Rehmannia japonica*

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Introduction

In Japan, many endangered plants occur in small populations in rural landscapes, and conservation activities have been conducted for them. However, some of these endangered plant species may originate from introduction by humans. For example, *Loropetalum chinense* (R. Br.) Oliv. (Hamamelidaceae) is a common evergreen tree species in China, but it is endangered in Japan and strictly protected by the local governments or landowners. However, genetic analysis of all wild individuals in Japan revealed only two or three genets in each population, indicating artificial propagation such as cutting and transplanting (Isagi and Kaneko 2014). Therefore, reexamination of the origin and conservation value of "endangered" species growing in rural landscapes is recommended in order to avoid spending limited conservation resources on human-introduced populations.

Although *Rehmannia japonica* (Thunb.) Makino ex T. Yamaz. is listed as critically endangered in the Japanese red list (Ministry of Environment of Japan 2015), its taxonomic status has been unclear. The genus *Rehmannia* had been included in the family Scrophulariaceae. Its current

taxonomic placement is under question due to the disintegration of Scrophulariaceae (Mabberley 2008; Olmstead et al. 2001; Oxelman et al. 2005), but recent phylogenetic studies showed that a monophyletic group, including *Rehmannia* is sister to Orobanchaceae (Albach et al. 2007; Xia et al. 2009). In general, six species, *R. elata* N. E. Br., *R. piasezkii* Maxim., *R. henryi* N. E. Br., *R. solanifolia* P. C. Tsoong and T. L. Chin, *R. glutinosa* (Gaertn.) Libosch. ex Fisch. and C. A. Mey., and *R. chingii* H. L. Li are recognized in the genus *Rehmannia* (Albach et al. 2007; Chin 1998; Xia et al. 2009) and these six species are endemic to China (Chin 1998).

In addition to the six Chinese species of *Rehmannia*, *Rehmannia japonica* has been recognized in Japan. Although earlier Japanese taxonomists such as Matsuda (1918), Makino (1932), and Hara (1949) considered it as a variety of *R. glutinosa*, recent Japanese taxonomists have consistently regarded it as an independent species (e.g., Yamazaki 1993; Yonekura 2012). *Rehmannia japonica* is very similar to *R. chingii* in its flower size and shape (Yamazaki 1961), but it is distinguished by several morphological characters (Yamazaki 1993). *Rehmannia japonica* is considered to have been widely cultivated as a horticultural plant in the past in Japan (Yamazaki 1961), and historical Japanese names of *R. japonica*, "Senrigoma" or "Komenmou," are mentioned in the traditional medical books of China and Japan. In China, "Komenmou" was found in the historical Chinese medical book, "*Compendium of Materia Medica"*, which was published in 1596 (https://books.google.co.jp/books?id=zVueCQAAQBAJ). In Japan, "Komenmou" or "Senrigoma" was illustrated in "*Kai*" (http://dl.ndl.go.jp/info:ndljp/pid/2555607/1) and "*Honzou zufu*" (http://dl.ndl.go.jp/info:ndljp/pid/1287126/1), which were published in 1765 and 1828, respectively. In the three books, "Komenmou" was distinguished from *R. glutinosa,* which is a famous medical plant species. Although taxonomic identification of *R. japonica* and "Komenmou" in the historical literature is needed, *Rehmannia japonica* may have been known in China and Japan in the past. However, *Rehmannia japonica* is not presently reported from China (Hong et al. 1998; Yamazaki 1961, 1993), and the taxonomic status and exact origin of this species is still uncertain.

Rehmannia japonica is extremely rare now. Only two populations are known in Japan. These two populations are found in Shizuoka Prefecture. Because of the limited number of populations, *R. japonica* is classified as "critically endangered" in the Japanese Red Data List (Ministry of Environment of Japan 2015). Old stone walls of terraced fields are the main habitats of *R. japonica*, and renovations of these walls and the resulting habitat destruction has directly led to the decreasing numbers of populations of this species. In addition to the limited number of populations, both *R. japonica* populations appear to have serious problems with sexual reproduction. Despite flowering every year, *Rehmannia japonica* does not produce seeds (Yamazaki 1993; Kaneko personal observation). Thus, this species depends on vegetative reproduction via rhizome elongation, and continued loss of habitat by the renovation of stone walls could cause irreversible damage to the continued existence of the populations.

In this study, we aimed to reveal the phylogenetic relationships and levels of genetic diversity of *R. japonica*. Previous studies inferred molecular phylogenies of the six Chinese species of *Rehmannia* using chloroplast DNA regions but did not sample *R. japonica* (Albach et al. 2007; Xia et al. 2009). A molecular phylogenetic analysis including *R. japonica* may clarify its taxonomic identity. The results might also demand a reconsideration of the conservation status of *R. japonica*. If *R. japonica* is genetically identical to one of the other *Rehmannia* species and is an introduced species from China, it is not appropriate that the conservation agencies treat it as an endangered species. On the other hand, if *R. japonica* is genetically distinct, its populations in Japan should be conserved as the only remaining populations of this species. In addition to the molecular phylogenetic analysis, the genetic diversity of these remnant populations was evaluated using microsatellite markers developed in the present study. Detailed genetic analysis can assess the genetic diversity and number of genets in remnant populations (e.g., Isagi and Kaneko 2014). The results also allow insights into a possible reason for the lack of sexual reproduction in *R. japonica* and provide the basic information needed for developing an effective conservation program for this species.

Materials and methods

Study site

Only two populations are known for *R. japonica*. They occur in Shizuoka Prefecture, Japan (Population 1: ca. 300 flowering ramets, N35°10′, E137°53′, Population 2: ca. 100 flowering ramets, N35°08′, E137°54′). These two populations are separated by about 3 km. The habitat of this species is old stone walls of terraced fields on steep slopes. Although the age of construction of these stone walls is not known, population 2 has been known since the 1960s (Ohba personal communication). Local famers have not been made aware that this plant species is listed as critically endangered in the Red List (Ministry of Environment of Japan 2015). We also investigated a population of *R. chingii*, which is the putative sister taxon of *R. japonica* (Yamazaki 1961) at Mt. Tian-mu, Zhejiang, China (N30°19′, E119°26′).

Table 1 Variable sites of seven *Rehmannia* species in the *trnL* intron and *trnL–trnF* intergenetic spacer

No.	Species	GenBank no. (references)	*trnL* intron and *trnL–F* (840 bp)											
					2	4	4	5	7	7	7	7	8	
			6	7	8	5	9	6	0	2	9	9	1	
			2	0	3	9	9	9	0	0	5	7	8	
1	*R. japonica*	LC152782 (This study)	A	–	————	C	AAAA	A	C	T	C	C	G	
2	*R. chingii*	LC152783 (This study)	A	–	————	C	——	A	C	T	C	C	G	
3	*R. chingii*	DQ856494 (Albach et al. 2007)	–	–	————	C	——	A	C	T	C	C	G	
4	*R. chingii*	EF363679 (Xia et al. 2009)	A	–	————	C	——	A	C	T	C	C	G	
5	*R. glutinosa*	DQ856493 (Albach et al. 2007)	A	–	————	C	AAAA	A	C	C	C	T	G	
6	*R. glutinosa*	EF363680 (Xia et al. 2009)	A	–	————	C	AAAA	A	C	C	C	T	G	
7	*R. solanifolia*	DQ856492 (Albach et al. 2007)	A	–	————	C	AAAA	A	C	C	C	T	G	
8	*R. solanifolia*	EF363678 (Xia et al. 2009)	A	–	————	C	AAAA	A	C	C	C	T	G	
9	*R. henryi*	DQ856497 (Albach et al. 2007)	A	–	————	C	AAAA	A	T	C	C	T	G	
10	*R. henryi*	EF363677 (Xia et al. 2009)	A	–	————	T	AAAA	A	C	C	T	T	A	
11	*R. piasezkii*	DQ856495 (Albach et al. 2007)	A	–	————	C	AAAA	T	C	C	C	T	G	
12	*R. piasezkii*	EF363676 (Xia et al. 2009)	A	–	————	C	AAAA	T	C	C	C	T	G	
13	*R. elata*	DQ856496 (Albach et al. 2007)	A	A	GAAGAAAGAATCAAATATT	C	AAAA	T	C	C	C	T	G	

Sampling and DNA extraction

Plant materials for molecular analysis were sampled from two populations of *R. japonica* and one population of *R. chingii*. In *R. japonica*, leaf samples of 39 and 19 ramets were collected from population 1 and 2, respectively, in 2009. Samples were collected across the distribution of each population, from flowering ramets and small ramets with only one or two small leaves above ground. These samples were stored at −30 °C for later analysis. Voucher was deposited in the Herbarium of the Faculty of Symbiotic Systems Science, Fukushima University (FKSE), Fukushima, Japan. In *R. chingii,* we collected leaf samples from 15 ramets from Mt. Tian-mu population in 2010. These samples were desiccated using silica gel and stored at room temperature before analysis. Total genomic DNA was extracted using a modified CTAB method (Milligan 1992).

Chloroplast DNA analysis

We amplified and sequenced the *trnL* intron, *trnL* exon, and *trnL–trnF* spacer region (referred to here as the *trnL–F* region) and *rps16* region of chloroplast DNA for four individuals of *R. japonica* and two individuals of *R. chingii*. The *trnL–F* region was amplified with primers "c" and "f" of Taberlet et al. (1991). The *rps16* intron was amplified with primers rps16-2F and rps16-R3 (Bremer et al. 2002). The PCR amplification was performed in a thermal cycler GeneAmp PCR System 2700 (Applied Biosystems, Foster City, California, USA). A High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) was used, and the purified products were sequenced directly with an ABI BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems) on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Both strands of the amplified PCR products were sequenced. Electropherograms were assembled with FinchTV (http://www.geospiza.com/finchtv/). The assembled sequences of each locus were aligned with those obtained from DDBJ/EMBL/GenBank (Tables 1, 2) for *R. elata*, *R. piasezkii*, *R. henryi*, *R. solanifolia*, *R. glutinosa*, and *R. chingii* using CLUSTAL X (Thompson et al. 1997). All consecutive indels were treated as one-point mutations. Based on these aligned sequences, a parsimony network of chloroplast DNA haplotypes was drawn using the program TCS1.06 (Clement et al. 2000).

Microsatellite marker development and analysis

Microsatellite markers were developed using the improved technique for isolating codominant compound microsatellite markers of Lian and Hogetsu (2002) and Lian et al. (2006). An adaptor-ligated, restricted DNA library for *R. japonica* was constructed as follows: genomic DNA was digested with the blunt-end restriction enzyme *Ssp*I. The restriction fragments were then ligated with a specific blunt adaptor (consisting of the 48-mer: 5′-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3′ and an 8-mer with the 3′-end capped with an amino residue: 5′-ACCAGCCC-NH_2-3′) using the Takara DNA ligation kit (Takara, Otsu, Japan).

Table 2 Variable sites of seven *Rehmannia* species in *rps16* intron

No.	Species	GenBank no. (references)	*rps16* intron (680 bp)															
							1	1	1	2	2	3	3	5	5	5	5	
			2	2	3	9	2	4	9	7	8	6	8	7	8	8	9	
			0	3	4	9	9	5	1	7	4	0	7	4	0	9	5	
1	*R. japonica*	LC152780 (This study)	G	G	G	CA	G	G	AACTTC	T	A	T	G	C	G	–	A	
2	*R. chingii*	LC152781 (This study)	G	G	G	—	G	G	AACTTC	T	A	T	G	C	G	A	A	
3	*R. chingii*	DQ856488 (Albach et al. 2007)	G	G	G	—	G	G	AACTTC	T	A	T	G	C	G	A	A	
4	*R. chingii*	FJ172696 (Xia et al. 2009)	G	G	G	—	G	G	AACTTC	T	A	T	G	C	G	A	A	
5	*R. glutinosa*	DQ856487 (Albach et al. 2007)	–	G	A	—	A	G	AACTTC	C	A	T	A	T	G	–	C	
6	*R. glutinosa*	FJ172697 (Xia et al. 2009)	–	G	A	—	A	G	AACTTC	T	A	T	A	T	G	–	C	
7	*R. solanifolia*	DQ856486 (Albach et al. 2007)	–	G	A	—	A	G	AACTTC	C	A	T	A	T	G	–	C	
8	*R. solanifolia*	FJ172695 (Xia et al. 2009)	–	G	A	—	A	G	AACTTC	C	A	T	A	T	G	–	C	
9	*R. henryi*	DQ856491 (Albach et al. 2007)	–	G	A	—	G	T	AACTTC	T	A	T	T	C	G	–	C	
10	*R. henryi*	FJ172694 (Xia et al. 2009)	–	G	A	—	G	G	AACTTC	T	A	T	A	C	G	–	C	
11	*R. piasezkii*	DQ856489 (Albach et al. 2007)	–	G	A	—	G	G	AACTTC	T	A	C	A	C	C	–	C	
12	*R. piasezkii*	FJ172693 (Xia et al. 2009)	–	G	A	—	G	G	AACTTC	T	A	C	A	C	C	–	C	
13	*R. elata*	DQ856490 (Albach et al. 2007)	–	A	A	—	G	G	———	T	–	T	A	C	C	–	C	

Fragments from the *Ssp*I DNA library were amplified by PCR using a compound SSR primer, $(AC)_6(AG)_7$, $(AC)_6(TC)_7$, or $(TC)_6(AC)_7$, and an adaptor primer (5′-CTATAGGGCACGCGTGGT-3′). The amplified fragments, ranging from 400 to 800 bp, were then separated on a 1.5% LO3 agarose gel (Takara) and purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, California, USA). The purified DNA fragments were cloned using the Qiagen PCR Cloning plus Kit (Qiagen), following the manufacturer's instructions. The cloned fragments were amplified using the M13 forward and reverse primers. Amplified fragments were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). For each fragment containing a compound SSR sequence at one end, a specific primer was designed from the sequence flanking the compound SSR using Primer 3 (version 0.4.0; Rozen and Skaletsky 2000).

Polymorphism of the developed microsatellite markers was evaluated for 58 individuals of *R. japonica* and 15 individuals of *R. chingii*. Amplifications followed the standard protocol of the Qiagen Multiplex PCR Kit (Qiagen) in a final volume of 10 µL, which contained 5 ng of extracted DNA, 5 µL of 2×Multiplex PCR Master Mix, and 0.2 µM of each multiplexed primer. Compound SSR primers ($(AC)_6(AG)_7$, $(AC)_6(TC)_7$, or $(TC)_6(AC)_7$) were labeled with fluorochromes FAM, PET, or VIC (Applied Biosystems), respectively. Amplifications were performed with the GeneAmp PCR System 2700 thermal cycler using the following conditions: initial denaturation at 95 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing 57 °C for 1 min 30 s, extension at 72 °C for 1 min, and a final extension at 60 °C for 30 min. The sizes of the PCR products were measured using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and GENESCAN and GENOTYPER software (Applied Biosystems). The loci showing polymorphism were tested for deviation from Hardy–Weinberg equilibrium and linkage disequilibrium between loci using FSTAT (version 2.9.3; Goudet 1995). Significance levels were adjusted using Bonferroni correction for multiple testing.

Results

Chloroplast DNA analysis

The aligned sequences consisted of 1,520 bp from the two regions of chloroplast DNA: *trnL–F* (840 bp) and *rps16* (680 bp). In *trnL–F,* seven nucleotide substitutions and four indels were observed across *Rehmannia* (Table 1). In *rps16*, ten nucleotide substitutions and five indels were observed (Table 2). Sequences for these two DNA regions were identical for the four samples of *R. japonica*. The sequences obtained in this study were deposited in the DDBJ (accession numbers LC152768-LC152779). A haplotype network based on the nucleotide substitutions and indels of the two chloroplast DNA regions showed that the sequence of *R. japonica* was different from the other six *Rehmannia* species (Fig. 1). The most similar sequences to *R. japonica* belong to *R. chingii* (No. 2 and 4), but the sequences of *R. chingii* were distinguished from *R. japonica* by three or four sites of indels (Tables 1, 2). The sequences of the other

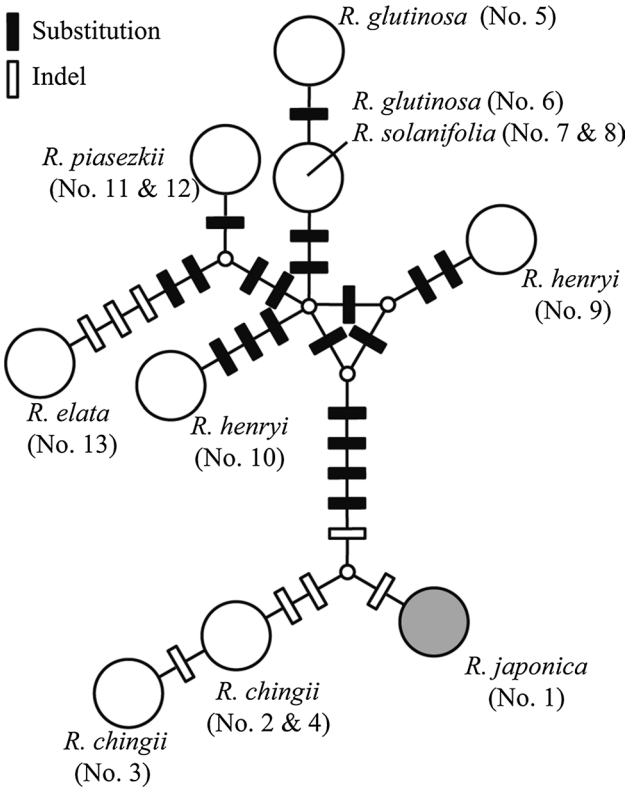

Fig. 1 Parsimony network of the nine cpDNA haplotypes of *trnL-F* and *rps16* sequence data found in the seven species of *Rehmannia*. *Solid* and *open bars* represent nucleotide substitutions and indels, respectively

five species (*R. elata*, *R. piasezkii*, *R. henryi*, *R. solanifolia*, and *R. glutinosa*) were clearly different from that of *R. japonica*.

Microsatellite analysis

Twelve loci were identified that showed a clear, strong, single band for each allele (Table 3). Levels of polymorphism were evaluated for 58 ramets from the two populations of *R. japonica*. The 58 samples collected from two geographically-isolated populations showed same multilocus genotype at the twelve loci (Table 4). Only the single alleles were observed for the six loci (*Reja052*, *Reja075*, *Reja087*, *Reja152*, *Reja171*, and *Reja185*), while two alleles were observed for the other six loci (*Reja042*, *Reja057*, *Reja083*, *Reja156*, *Reja163*, and *Reja167*). In *R. chingii,* five out of the twelve loci were amplified, and the 15 samples showed 15 multilocus genotypes at these five loci (Table 4). The number of alleles per locus ranged from 4 to 12 with an average of 8.0. The observed and expected heterozygosities (H_O and H_E) ranged from 0.25 to 0.73 and from 0.52 to 0.88, respectively, with averages of 0.73 for both. A significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$), which is likely due to the existence of null alleles, was observed at four loci (*Reja057*, *Reja075*, *Reja087*, and *Reja156*). There was no evidence of significant linkage disequilibrium ($P < 0.05$).

Discussion

Phylogenetic identity and origin of *R. japonica*

The present results indicate that *R. japonica* may be genetically distinct from the other six *Rehmannia* species, as is also suggested by their morphology. Yamazaki (1961) suggested that the most closely-related species to *R. japonica* is *R. chingii* based on morphological characteristics, and *R. japonica* and *R. chingii* are distinguished morphologically by the length of petiole, density of pilose hairs on leaf, and short granular of filament (Chin 1998; Yamazaki 1961, 1993). These taxonomic conclusions based on morphological comparisons were supported by the present analysis of chloroplast DNA sequences. The sister taxon of *R. japonica* was *R. chingii* and these two species were distinguishable by the chloroplast DNA haplotypes. In the sequences of the *trnL–F* and *rps16* regions, a number of substitutions and indels were variable among the seven species of *Rehmannia*. However, taxonomically-distinct *R. glutinosa* and *R. solanifolia* shared an identical haplotype, and thus, the three indels that distinguish *R. japonica* and *R. chingii* could potentially confirm their identities as different species.

The microsatellite analysis also suggested genetic differentiation between *R. japonica* and *R. chingii.* Seven of twelve microsatellite markers developed from *R. japonica* did not amplify in *R. chingii,* and significant deficiency of observed heterozygosity was observed at four loci in *R. chingii*. These unamplified loci and samples probably contain null alleles, likely due to nucleotide substitutions, insertions and deletions in the primer annealing sites between *R. japonica* and *R. chingii*. Although transferability of microsatellite markers among different species varies among taxa and loci, it decreases with increasing genetic distance among taxa in general. Microsatellite markers developed for various plant species using the same protocol as in the present study showed clearly higher transferability among related species than that seen between *R. japonica* and *R. chingii* (e.g., Izuno et al. 2011; Kaneko et al. 2011; Mori et al. 2008). This fact is consistent with the assertion that *R. japonica* and *R. chingii* are genetically distinct enough to treat as different species.

The present findings also suggested the need for extensive morphological and genetic analysis about *Rehmannia* species in China. In order to discuss the origin of *R. japonica*, an understanding of the genetic variation of each

Table 3 Characteristics of 12 compound microsatellite loci for *Rehmannia japonica*

Locus	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	Size of alleles (bp)		DDBJ no.
				R. japonica	*R. chingii*	
Reja042	$(AC)_6(AG)_9$	ACACACACACACAGAGAGAGAG TCAGCTGAAAATGAATCTGC	57	121, 129	–	LC152768
Reja052	$(AC)_6(TC)_8$	ACACACACACACTCTCTCTCTC CATGTATGCACTGCAGATTAAA	57	97	–	LC152769
Reja057	$(AC)_6(TC)_8$	ACACACACACACTCTCTCTCTC AAAATAGCATGACTGGCTCA	57	144, 148	142–157	LC152770
Reja075	$(AC)_6(TC)_{11}$	ACACACACACACTCTCTCTCTC TTTCAAATGGGCTAGCTGTT	57	197	183–197	LC152771
Reja083	$(AC)_6(TC)_7$	ACACACACACACTCTCTCTCTC AAAGCTAGCGAACGAACAGT	57	94, 108	–	LC152772
Reja087	$(AC)_6(TC)_7$	ACACACACACACTCTCTCTCTC ATTCCCACATTCAATTCCAC	57	103	120–132	LC152773
Reja152	$(TC)_6(AC)_8$	TCTCTCTCTCTCACACACACAC GAGCCATAAGGGATTGCTAA	57	167	163–169	LC152774
Reja156	$(TC)_6(AC)_8$	TCTCTCTCTCTCACACACACAC CCCCATGATTGTGATGTTC	57	100, 109	97–121	LC152775
Reja163	$(TC)_6(AC)_7$	TCTCTCTCTCTCACACACACAC AATTTGGTGCAAAATCTGGT	57	131, 147	–	LC152776
Reja167	$(TC)_6(AC)_{15}$	TCTCTCTCTCTCACACACACAC GTACTTGGTCACGCTTCCTT	57	90, 96	–	LC152777
Reja171	$(TC)_6(AC)_7$	TCTCTCTCTCTCACACACACAC ATCCAAATGAGGTTGGAAAA	57	207	–	LC152778
Reja185	$(TC)_6(AC)_7$	TCTCTCTCTCTCACACACACAC GATGGAGCCTCCTAAAATGA	57	297	–	LC152779

*T*a annealing temperature of primer pair

Rehmannia species is needed. Although we could not determine the origin of *R. japonica* based on the present and previous studies, background genetic information of Chinese *Rehmannia* is quite limited at present. In addition, Chinese and Japanese taxonomists are separately investigating the *Rehmannia* species, and most Chinese taxonomists probably have not recognized the possibility that *R. japonica* could exist in China. Therefore, the extensive analysis of *Rehmannia* species, especially *R. chingii*, may help identify the original populations of *R. japonica*.

Single genet remaining in *R. japonica*

Detailed genotyping of the two remnant populations using microsatellite markers showed that all sampled ramets of *R. japonica* are an identical genet that presumably originated from vegetative reproduction. The microsatellite analysis indicated remarkable differences in genetic diversity for *R. japonica* and *R. chingii*. All 58 ramets of *R. japonica* showed an identical multilocus genotype at the twelve loci, whereas the 15 ramets of *R. chingii* showed 15 different multilocus genotypes with high allelic polymorphism at the five amplified loci (Table 4). These results suggest that the microsatellite markers developed here will be valid for estimating the genetic diversity of *R. japonica.* In addition, all samples of *R. japonica* showed the identical heterozygous genotype for six loci. This result indicates that not only is the genetic diversity of *R. japonica* populations very low but also that all sampled ramets were produced without the recombination of sexual reproduction.

With the assumption of self-incompatibility, absence of sexual reproduction of *R. japonica* (Yamazaki 1993; Kaneko personal observation) may be explained by the only genet remaining in the *R. japonica* populations. Although there is no report of self-incompatibility in *R. japonica*, self-incompatibility was reported in *R. glutinosa* (Zhou et al. 2010). If *R. japonica* were a self-incompatible plant like *R. glutinosa*, seeds would not be produced in populations composed of a single genet. In the habitat of *R. japonica*, small ramets with only one or two small leaves were found that looked like seedlings. However, we found that these small ramets were physically connected by rhizome

Table 4 Variability of 12 microsatellite loci in the two populations of *Rehmannia japonica* in Japan and the *R. chingii* population in China

	R. japonica Population 1 (n = 39, G = 1)				*R. japonica* Population 2 (n = 19, G = 1)				*R. japonica* Population 1&2 (n = 58, G = 1)				*R. chingii* Population (n = 15, G = 15)		
	A	Genotype	H_O	H_E	*A*	Genotype	H_O	H_E	*A*	Genotype	H_O	H_E	*A*	H_O	H_E
Reja042	2	(121, 129)	1.00	0.50	2	(121, 129)	1.00	0.50	2	(121, 129)	1.00	0.50	*X*	–	–
Reja052	1	(97, 97)	0.00	0.00	1	(97, 97)	0.00	0.00	1	(97, 97)	0.00	0.00	*X*	–	–
Reja057	2	(144, 148)	1.00	0.50	2	(144, 148)	1.00	0.50	2	(144, 148)	1.00	0.50	10	0.64*	0.88
Reja075	1	(197, 197)	0.00	0.00	1	(197, 197)	0.00	0.00	1	(197, 197)	0.00	0.00	8	0.25*	0.80
Reja083	2	(94, 108)	1.00	0.50	2	(94, 108)	1.00	0.50	2	(94, 108)	1.00	0.50	*M*	–	–
Reja087	1	(103, 103)	0.00	0.00	1	(103, 103)	0.00	0.00	1	(103, 103)	0.00	0.00	6	0.41*	0.64
Reja152	1	(167, 167)	0.00	0.00	1	(167, 167)	0.00	0.00	1	(167, 167)	0.00	0.00	4	0.73	0.52
Reja156	2	(100, 109)	1.00	0.50	2	(100, 109)	1.00	0.50	2	(100, 109)	1.00	0.50	12	0.43*	0.83
Reja163	2	(131, 147)	1.00	0.50	2	(131, 147)	1.00	0.50	2	(131, 147)	1.00	0.50	*X*	–	–
Reja167	2	(90, 96)	1.00	0.50	2	(90, 96)	1.00	0.50	2	(90, 96)	1.00	0.50	*X*	–	–
Reja171	1	(207, 207)	0.00	0.00	1	(207, 207)	0.00	0.00	1	(207, 207)	0.00	0.00	*X*	–	–
Reja185	1	(297, 297)	0.00	0.00	1	(297, 297)	0.00	0.00	1	(297, 297)	0.00	0.00	*X*	–	–
Average	1.5		0.50	0.25	1.5		0.50	0.25	1.5		0.50	0.25	8.0	0.73	0.73

M (*Reja083*) and *X* (*Reja042*, *Reja052*, *Reja163*, *Reja167*, *Reja171*, and *Reja185*) indicate that the locus showed more than two alleles per sample and the locus was not amplified, respectively

n number of ramets genotyped, G number of multilocus genotypes, *A* number of alleles per locus, H_O observed heterozygosity, H_E expected heterozygosity

An asterisk (*) indicates a significant deviation from Hardy–Weinberg equilibrium expectations ($P < 0.05$)

with flowering ramets, and the multilocus genotypes of all sampled ramets (small or flowering) were identical. Therefore, the probability of the existence of ramets having different genotypes is small. Most likely, only a single genet survives in the *R. japonica* populations in Japan.

Implications for conservation

The present findings suggest that *R. japonica* has high conservation value due to morphological and genetic differentiation from the other six species of *Rehmannia* and its extremely limited number of the populations. *Rehmannia japonica* is likely to remain as a single genet, and we should consider the genetic and ecological circumstances of this species when planning conservation measures. In general, it is thought that clonal plant without genetic variation can be particularly vulnerable to permanent and sudden environmental changes (Callaghan et al. 1992), and *R. japonica* in Japan may be unable to adapt against environment change due to its limited number of ramets and lack of genetic variation. The fact that only two populations are remaining also leads to high vulnerability to the catastrophic disturbance of habitat. The stone walls which have been the habitat of *R. japonica* are old and likely not sustainable for a long time. These habitats are located on steep slopes and landslide-prone terrain. Each population could disappear easily with just one landslide. Therefore, monitoring and conservation measures such as maintenance of these stone walls are required to prevent the extinction of *R. japonica* in the in situ environment, and the construction of ex situ populations is needed to prevent unexpected extinction by catastrophic events.

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