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Molecular Phylogenetic Analyses of *Geranium robertianum* Populations Recently Found in Japan

Sachiko Nishida[1,*], Hiroshi Azuma[2], Akiyo Naiki[3] and Makoto Ogawa[4]

[1]*The Nagoya University Museum, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan. *nishida@num.nagoya-u.ac.jp (author for correspondence);*
[2]*Department of Botany, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan;*
[3]*Graduate School of Education Master's Program, Okayama University, Kita-ku, Okayama 700-8530, Japan;*
[4]*Tokushima Prefectural Museum, Bunka-no-mori Park, Hachiman-cho, Tokushima 770-8070, Japan*

Geranium robertianum L. is considered an endangered species in Japan, while it is invasive in several countries outside Japan. Recently, plants of *G. robertianum* have been found at several localities, none of which are original stations for *G. robertianum* in Japan. To determine whether the recently found plants are native Japanese plants or were naturalized from sources outside Japan, we conducted molecular phylogenetic analyses using DNA sequences of the plastid regions of the *trn L* intron, *trnL3'-trnF* and *trnH-psbA* intergenic spacer regions, and the nuclear internal transcribed spacer (ITS). Among 32 samples collected from 21 populations, we recognized three major groups, consisting of 1) recently found plants, 2) indigenous plants, and 3) plants from Britain. Our results suggest that recently discovered plants of *G. robertianum* in Japan are more likely naturalized from sources outside Japan rather than having escaped from indigenous Japanese populations. The origin of the newly found plants is not known although their origin in Britain is unlikely.

Key words: endangered species, Geraniaceae, *Geranium robertianum*, invasive plant, molecular phylogeny

An endangered species is one at risk of extinction and usually found only in limited localities or habitats. In contrast, an invasive or naturalized species is one that has extended its distribution into nonnative localities and usually grows in several habitats. We here report a case of a plant species that may be both endangered and invasive in Japan, a situation which we investigated using molecular phylogenetic analyses.

Geranium robertianum L. (Geraniaceae) is an annual or biennial plant that can be distinguished from other species of *Geranium* L. in Japan by its compound leaves, distinctive odor and glandular hairs covering most parts of the plant (Shimizu 1982, Akiyama 2001). *Geranium robertianum* originally had a restricted distribution in Japan: on Mt. Ibuki and the Suzuka mountain range running from Shiga Prefecture to Mie Prefecture through Gifu Prefecture, and on Mt. Tsurugi located between Tokushima and Kochi prefectures. These areas are both limestone regions and *G. robertianum* is considered a calcicole in Japan. Because of its narrow distribution and limited habitat, *G. robertianum* is listed as endangered in Gifu, Mie, Tokushima, and Kochi prefectures (Gifu Prefecture 2001, Tokushima Prefecture 2001, Mie Prefecture 2005, Kochi Prefecture 2010). Outside of Japan, however, *G. robertianum* is widely distributed in Europe and northwestern Asia, and is naturalized in parts of North and South America and New Zealand (Tofts 2004). In these areas, it is also found in a wide range of habitats, e.g., woodlands, hedge banks, and open habitats including on scree, rocks, and shingle

(Tofts 2004).

Recently, specimens of *Geranium robertianum* have been collected at localities in Hokkaido (#*Goda 1687* in 1994, KYO), Kyoto (#*Yamazaki 6550* in 1998, KYO), and Fukuoka (#*Koga 15820* in 2006, KYO), none of which are known to have had populations of *G. robertianum* previously. The newly found plants grow in various habitats and are indifferent to soil type (Nishida personal observation), which might be evidence that they have naturalized from outside Japan and differ from the original Japanese native *G. robertianum*. The native Japanese plants, however, are able to grow successfully in non-limestone soils under cultivation (Nishida unpublished data), so that habitat information alone cannot discriminate the origin of the newly found plants; they could be derived from original Japanese populations.

We therefore conducted molecular phylogenetic analyses on the plants from outside the originally known Japanese localities (hereafter called 'recently found plants'), comparing them to plants in the original localities (hereafter called 'indigenous plants') and plants imported as garden ornamentals from Britain (hereafter called 'British plants'). Through these analyses, we hoped to determine the origin of the newly found *Geranium robertianum*.

Materials and Methods

Plant material

DNA sequences of the plastid regions of the *trn L* intron, *trnL3′-trnF* and *trnH-psbA* intergenic spacer regions, and the nuclear internal transcribed spacer (ITS) region of 32 samples of *G. robertianum*, including six samples from indigenous plants, two samples grown from imported British seeds, and another 24 samples from 16 recently found plant populations (Table 1). A map (Fig. 1) shows the sampling locations in Japan. Since no close relatives of *Geranium robertianum* are in Japan, we used *G. thunbergii* Sieb. & Zucc. as the outgroup. *Geranium thunbergii* is the most common species of *Geranium* in Japan. Voucher specimens for this study were deposited at NUM except for the specimen from Mt. Ibuki, Shiga, which was deposited at Lake Biwa Museum.

DNA extraction, PCR amplification, and DNA sequencing

Total genomic DNA was extracted from fresh leaves or leaves dried in silica gel using the modified cetyltrimethylammonium bromide (CTAB) method of Doyle & Doyle (1987).

The *trnL* intron plus *trnL-trnF* (*trnL5′-3′-trnF* hereafter), *trnH-psbA*, and ITS regions were amplified using polymerase chain reaction (PCR). The PCR mixture (20 μL) contained 1 μL template DNA, 2 μL dNTPs (2.5 mM each), 1 μL of each primer (10 μM), 2 μL 10× *Taq* buffer (containing 20 mM $MgCl_2$), 0.5 U *Taq* polymerase (ExTaq; Takara Bio Inc., Japan). The PCR was performed with a GeneAmp PCR System 9600 or 2700 (Applied Biosystems Japan Ltd., Japan), starting at 94°C (5 min), followed by 35 cycles of denaturation at 94°C (30 s), annealing at 50°C (30 s), and extension at 72°C (30 s or 1 min), and a final extension at 72°C (7 min). After checking for a single band using electrophoresis on 1% agarose TAE gel stained with ethidium bromide, the PCR products were purified with enzyme treatment. A 2-μL mixture containing 0.3 U Exonuclease I (Takara Bio Inc.) and 0.3 U calf intestine alkaline phosphatase (Toyobo Co. Ltd., Japan) was added to each PCR tube to degrade remaining primers and dephosphorylate any remaining dNTPs. The tubes were heated at 37°C for 30 min then at 80°C for 15 min.

Direct sequencing of both strands was conducted on an ABI 3100 Genetic Analyzer (Applied Biosystems) using the BigDye Terminator version 3.1 Cyclic Sequencing Ready Reaction Kit (Applied Biosystems Japan Ltd.) following the manufacturer's protocol. Primers for the amplification and/or sequencing of *trnL5′-3′-trnF*, *trnH-psbA*, and ITS regions are listed in Table 2. We manually aligned the DNA sequences obtained on MEGA5 (Tamura *et al*. 2011).

Phylogenetic analysis

Maximum likelihood analysis (ML) was con-

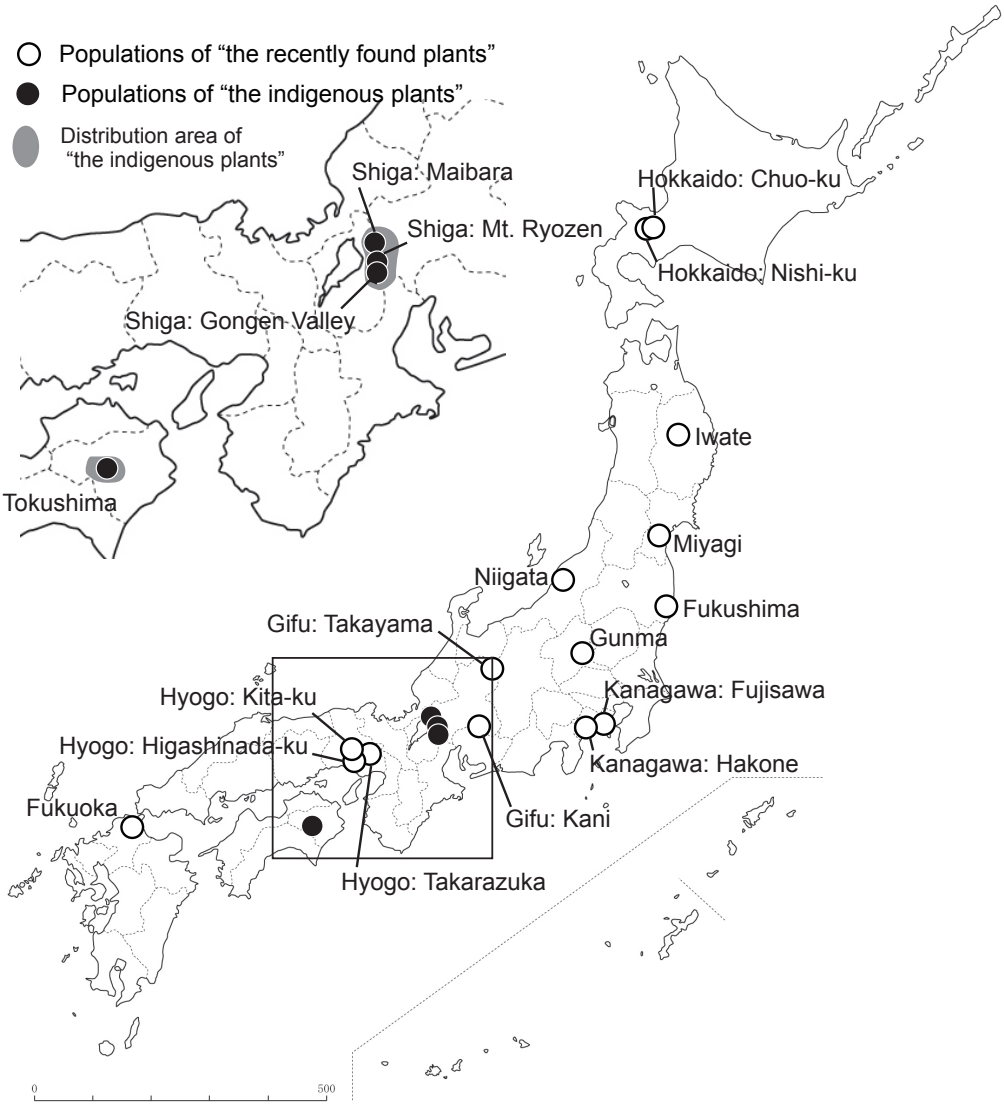

FIG. 1. Map of sampling sites of populations of *Geranium robertianum* in Japan.

ducted in MEGA5 (Tamura *et al*. 2011). The best substitution model was selected based on Bayesian information criteria (BIC) values of 24 possible combinations of substitution models and rate description, and analyzed in MEGA5. Tamura's three-parameter model, with the lowest BIC value, was chosen for all data sets, including the combined data set (*trnL5′-3′-trnF* + *trnH-psbA* and *trnL5′-3′-trnF* + *trnH-psbA* + ITS). For ML analysis, gaps were partially deleted using the 95% "site coverage cutoff" option. Nearest-neighbor interchange (NNI) was used as the ML heuristic method and the initial tree was made automatically. Five hundred bootstrap replications were performed using the same settings. Accession numbers of sequences obtained and used in this study are listed in Table 1.

TABLE 1. Sample data used in a study of *Geranium robertianum* in Japan, including locality, number of samples from each population, collector name, categorization of the plants, and accession number for sequence data at DDBJ.

Species	Locality	Number of samples	Collector	Category	Sample name in Fig. 2	Accession no.		
						ITS	*trnL5'-3'-trnF*	*trnH-psbA*
Geranium robertianum								
	Hokkaido: Sapporo, Nishi-ku	1	S. Nishida	Recently found	Hokkaido-1	AB689045	AB693025	AB689149
	Hokkaido: Sapporo, Chuo-ku	2	A. Arita	Recently found	Hokkaido-2	AB689045	AB702962	AB703074
					Hokkaido-3	AB689045	AB702963	AB703074
	Hokkaido: Sapporo, Chuo-ku, Maruyama Park	1	S. Nishida	Recently found	Hokkaido-4	AB689040	AB693020	AB689144
	Iwate: Morioka, Higashi-midorigaoka	1	M. Suzuki	Recently found	Iwate	AB689043	AB693023	AB689147
	Miyagi: Sendai, Aoba-ku	2	T. Sugano	Recently found	Miyagi-1	AB689048	AB693028	AB689152
					Miyagi-2	AB689048	AB693028	AB689152
	Niigata: Agano	1	H. Tani	Recently found	Niigata	AB689051	AB693031	AB689155
	Fukushima: Iwaki, Tokiwa-yumoto	2	H. Tanimoto	Recently found	Fukushima-1	AB689053	AB693033	AB689157
					Fukushima-2	AB689053	AB702966	AB689157
	Gunma: Kiryu, Hishi	3	T. Ohmori	Recently found	Gunma-1	AB689054	AB693034	AB689158
					Gunma-2	AB689054	AB693034	AB689158
					Gunma-3	AB703073	AB693034	AB689158
	Kanagawa: Fujisawa	2	H. Matsui	Recently found	Kanagawa-1	AB689047	AB693027	AB689151
					Kanagawa-2	AB703071	AB702964	AB689151
	Kanagawa: Hakone	1	S. Ozawa	Recently found	Kanagawa-3	AB689046	AB693026	AB689150
	Gifu: Takayama, Shin-hotaka	1	S. Nishida	Recently found	Gifu-1	AB689056	AB693036	AB689160
	Gifu: Kani, Nishi-katabira	2	H. Suga & S. Nishida	Recently found	Gifu-2	AB703072	AB693032	AB689156
					Gifu-3	AB689052	AB693032	AB689156
	Shiga: Maibara, Mt. Ibuki	2	T. Murase	Indigenous	Shiga-1	AB689055	AB693035	AB689159
					Shiga-2	AB689055	AB693035	AB689159
	Shiga: Hikone, Taga, Mt. Ryozen	1	S. Nishida	Indigenous	Shiga-3	AB689044	AB693024	AB689148
	Shiga: Hikone, Taga, Gongen Valley	2	S. Nishida	Indigenous	Shiga-4	AB689042	AB693022	AB689146
					Shiga-5	AB689042	AB702961	AB689146
	Hyogo: Takarazuka, Oharano	1	M. Mizuta	Recently found	Hyogo-1	AB689049	AB693029	AB689153
	Hyogo: Kobe, Higashinada-ku	1	S. Yuki	Recently found	Hyogo-2	AB689038	AB693018	AB689142

Hyogo: Kobe, Kita-ku	1	S. Uyemura	Recently found	Hyogo-3	AB689039	AB693019	AB689143
Tokushima: Miyoshi, Mt. Tsurugi	1	S. Nishida	Indigenous	Tokushima	AB689041	AB693021	AB689145
Fukuoka: Kitakyushu, Hirao-dai	2	K. Takakura	Recently found	Fukuoka-1	AB689050	AB702965	AB689154
				Fukuoka-2	AB689050	AB693030	AB689154
UK: (Cultivated in Japan)	2	S. Nishida	British	England-1	AB689037	AB693017	AB689058
				England-2	AB689037	AB693017	AB689058
Geranium thunbergii							
Hokkaido: Chitose, Yamato	1	S. Nishida	Outgroup	*G. thunbergii*	AB689057	AB693037	AB689161

TABLE 2. Primer sequences used in this study.

Region	Forward	Reverse	Ref.
trnL5′-3′-trnF	CGAAATCGGTAGACGCTACG	ATTTGAACTGGTGACACGAG	(1)
trnH-psbA	CGCATGGTGGATTCACAATC	AGACCTAGCTGCTATCGAAG	(2)
ITS	GGAAGTAAAAGTCGTAACAAGG	TCCTCCGCTTATTGATATGC	(3)

(1) Taberlet *et al*. (1991). (2) Azuma *et al*. (1999). (3) White *et al*. (1990).

Results

DNA sequence variation

The aligned data matrix of *trnL5′-3′-trnF* was composed of 942 characters, of which 77 sites (8.2%) were variable among all operational taxonomic units (OTUs) and seven (0.7%) varied within ingroups. The aligned data matrix of *trnH-psbA* was composed of 328 characters, of which 23 sites (7.0%) were variable among all OTUs and one (0.3%) varied within ingroups. The aligned data matrix of the ITS was composed of 644 characters, of which 89 sites (13.8%) were variable among all OTUs and six (0.9%) varied within ingroups. There was no variation within each population except for Hokkaido 2 and 3; Fukushima 1 and 2; Gunma 1, 2 and 3; Kanagawa 1 and 2; Gifu 2 and 3; Shiga 4 and 5; and Fukuoka 1 and 2 (see accession nos. in Table 1).

Phylogenetic analysis

Because no topological discordance was observed among the three phylogenetic trees (*trnL5′-3′-trnF*, *trnH-psbA*, and ITS), a combined data matrix of the three regions was used to construct a molecular phylogenetic tree. The ML tree obtained is shown in Fig. 2. We recognized three genetic groups among the *Geranium robertianum* samples, which we named Groups A–C. Group A consisted only of plants recently found in Japan, although the bootstrap value was not high (74%). Group B were plants of British lineage. Group C consisted of all remaining indigenous plants. Phylogenetic relationships among these groups are still uncertain. A subclade is recognized in Group A, consisting of three samples from Hokkaido and one from Iwate, and in Group C, consisting of two samples from Shiga, although the bootstrap support of these subclades is low (63% and 68%, respectively).

Discussion

Our results show at least three genetic lineages (Groups A–C) recognized among the *G. eranium robertianum* samples examined (Fig. 2). Based on these results, we discuss the relationships between recently found plants and indigenous plants of *G. robertianum*.

Among the samples examined in this study, all recently found plants belong to one clade (Group A) that differs from the clade containing the indigenous plants (Group C). The clear separation of the two clades suggests that the recently found plants were introduced from outside of Japan rather than derived from indigenous Japanese plants. Of course, no definitive answer can yet be given, since the possibility exists that the recently found plants are derived from an original population with an ancestral genetic type for the two clades in which we did not collect samples from the original populations. The populations from which we did collect indigenous material are actually the only two regions (the Suzuka Mountain Range and the Tsurugi Mountain Range) that are thus far known for the original distribution (Ito 1932, Kitamura 1968, Shimizu 1982, Gifu Prefecture 2001; Fig. 1). Given that the molecular analyses combined the indigenous samples from these two regions into a distinct clade, we would not expect any other original populations with very different genetic types. It would be more reasonable to conclude that the recently found plants are not derived from indigenous localities, although further studies with more samples from

different localities (especially from additional foreign countries) are needed for final determination.

If the recently found plants are from outside Japan, whether they entered by chance once or several times must be determined. They might have become naturalized from several sources, given the larger variation in sequences within Group A compared to the other two groups. This presumption, however, is premature and further studies using additional molecular regions are needed. Also, whether the subclade in Group A had a different origin from other members of the group is also unresolved. A better supported phylogenetic tree for the recently found plants is needed.

Thus far, we have found no populations outside Japan as the probable origin of the recently

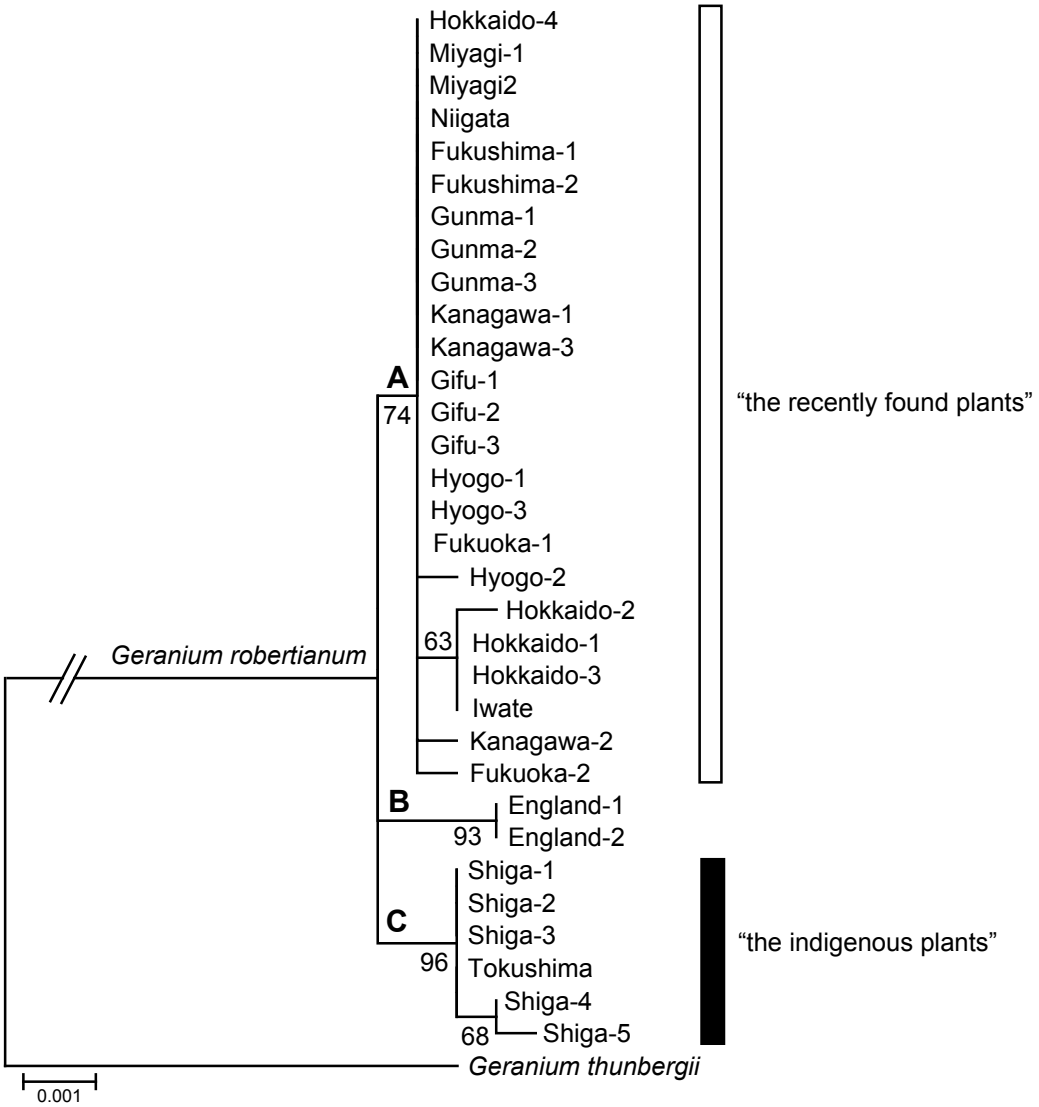

FIG. 2. Maximum likelihood tree obtained from combined data of *trnL5'-3'-trnF*, *trnH-psbA*, and ITS regions of *Geranium robertianum*. Numbers at branches indicate bootstrap percentages (> 50%). See Table 1 for abbreviations of sample names.

found plants. For populations from outside Japan, we included only plants derived from British seeds in the analyses. The seeds of *Geranium robertianum* were imported from Britain, likely for medicinal use or ornament. They were once used for skin and kidney complaints and for red-water fever in livestock in Ireland (Mabberley 2008). Our results showed that the British plants formed a distinct clade, separated from both the recently found and the indigenous plants. That the plants recently found in Japan originated from British imports is therefore unlikely. Tracing strains that have been introduced over the years is difficult. The British samples were the only seeds we were able to find in major trading markets. *Geranium robertianum* was used in Chinese medicine, but less so than other species, such as *G. thunbergii* (e.g., Kimura & Kimura 1981, Shimizu 1989). We have not found seeds or plants of *G. robertianum* in Japan that are sold as a Chinese medicine.

Of the plants studied here, none were cultivated; all were wild or (less likely) recent garden escapees, since no cultivated individuals were found in the surrounding area according to the collectors. Seeds of *Geranium robertianum* are thought to be dispersed by animals (Grime 1988). The recently found plants may have originated from one or several populations from outside of Japan, having become naturalized and widely dispersed through seeds attached to people, animals, or cars. Analysis of more samples from outside of Japan might help to identify the origin of the populations for the recently found plants. Analyzing samples from naturalized plants in the Americas or New Zealand would be informative because such naturalized plants may have a higher propensity to be invasive.

In living plants and herbarium specimens, we found no distinct differences in morphology among the three groups. The indigenous plants are relatively small, with thin leaves compared to the recently found plants and British plants. These differences, however, are more likely related to habitat, as *Geranium robertianum* is well known to vary depending on habitat; e.g., more than 10 varieties or forms have been described for various morphs, but all were combined into one subspecies by Baker (1956).

Our results suggest that plants of *Geranium robertianum* recently found in Japan are more likely to be naturalized from a source outside of Japan rather than being derived from indigenous Japanese populations, because the clade consisting of recently found plants is clearly distinct from the clade of indigenous plants. The origin of the recently found plants is unknown, but they are unlikely to have been imported from Britain.

Thus far, no evidence indicates that recently found plants have invaded indigenous populations, although some of the new introductions have been found in the same prefecture as the indigenous populations. To conserve the original biodiversity, the spread of recently found plants should be monitored to prevent their hybridization with indigenous plants.

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