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Isolated history of the coastal plant *Lathyrus japonicus* (Leguminosae) in Lake Biwa, an ancient freshwater lake

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Abstract

Background and aims Lake Biwa is one of the world's few ancient lakes. Formed ~4 million years ago, the lake harbours many coastal species that commonly inhabit seashores. The beach pea *Lathyrus japonicus* is a typical coastal species of this freshwater lake, but its inland populations are faced with the threat of extinction. Here, we investigated the phylogeographical and population structures of both inland and coastal populations of *L. japonicus*. We also elucidated the historical isolation of the Lake Biwa population.

Methodology In total, 520 individuals from 50 *L. japonicus* populations were sampled throughout the species distribution in Japan. Chloroplast haplotyping using intergenic spacers *psbA–trnH* and *atpI–atpH* was performed to investigate the phylogeographical structure as well as the genetic diversity of *L. japonicus*. Six nuclear microsatellite markers were also used to analyse the population structure.

Principal results Population structure analyses of chloroplast DNA (cpDNA) and nuclear DNA (nDNA) identified inland and coastal groups. Based on the genetic differentiation, inland populations exhibited a single cpDNA haplotype and significantly lower values of H_S, AR and F_{IS} than coastal populations. In addition to the presence of a bottleneck, the lack of gene flow among inland populations was supported by estimates of recent migration rates between subpopulations.

Conclusions Our data revealed that inland populations have been isolated in Lake Biwa as 'landlocked' populations since the predecessor lake was isolated from sea. This was also seen in a previous study of *Calystegia soldanella*. However, the high genetic differentiation, accompanied by a lack of gene flow among the Lake Biwa populations (according to the BAYESASS$^+$ analysis), contradicts the results with *C. soldanella*. We conclude that because of the presence of a bottleneck and low genetic diversity of the inland populations, self-sustaining population persistence may be difficult in the future. Conservation strategies must consider the genetic properties of such isolated populations.

Introduction

Ancient lakes are defined as having an uninterrupted history dating back >0.1 million years ago (MYA). These are usually seen as compact and isolated geographical entities that have undergone unique histories in a given hydrological setting (Gorther 1994). Thus, ancient lakes

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are a frequent focus of geological, biological and ecological research, and have been recognized as unique settings for evolutionary field experiments and hot spots of endemism (Wilson *et al.* 2004). Lake Biwa, which was formed ~4 MYA, is one of the world's few ancient lakes. The lake was initially located ~40 km south-east of its present position, and the predecessor lake gradually moved north-west to its present position as a result of subsidence of a fault (Takaya 1963; Yokoyama 1984; Kawabe 1989, 1994; Meyers *et al.* 1993). Lake Biwa, given that it has been an isolated inland lake for so long, harbours extensive (595 animals, 491 plant species; Mori and Miura 1990) and unique biota with many endemic species. Lake Biwa provides an experimental field site for evolutionary biology, similar to the ancient lakes Bikal and Tanganyika (Martens 1997; Sherbakov 1999). However, evolutionary studies of Lake Biwa have largely focused on gastropods and fish taxa (e.g. *Biwamelania*, flocks: Nishino and Watanabe 2000; *Urotaenia* and *Gymnogobius isaza*: Harada *et al.* 2002), whereas few studies on plants have been conducted.

Lake Biwa harbours many coastal plants that inhabit the seashore, including *Calystegia soldanella* (Convolvulaceae), *Vitex rotundifolia*, *Lathyrus japonicus* Willd, *Arabidopsis kawasakiana* Makino, *Raphanus sativus* var. *raphanistroides* Makino (Cruciferae), *Dianthus japonicus* (Caryophyllaceae) and *Pinus thunbergii* (Pinaceae) (Kitamura 1968). These plants are assumed to have migrated to the inland lake from coastal populations during the period when the lake was adjacent to the sea and the lake populations became isolated from the coastal populations (Takaya 1963; Kitamura 1968). Thus, these plants in Lake Biwa may share the same history of migration of coastal populations to the inland lake. These isolated Lake Biwa plants are assumed to share the same phylogeographical structures due to a shared history, corroborating long-term isolation of the inland populations and intraspecific differentiation.

Based on chloroplast DNA (cpDNA) haplotyping and nuclear microsatellite marker (nSSR) analysis, Noda *et al.* (2011) suggested that the population at Lake Biwa might have been isolated from coastal populations for a long period of time because the inland populations harboured mostly unique cpDNA haplotypes, lower genetic diversity and a highly differentiated genetic structure compared with those located on the coast. Therefore, we address here the question of whether coastal plant species at Lake Biwa share the same history of isolation from coastal populations by analysing the phylogeographical structure of other coastal plants.

Additionally, plants of the coast and lake occur in two fundamentally different types of habitat, i.e. sea coasts and freshwater lakeshores. This suggests the possibility of ecological adaptation to habitats (e.g. salt tolerance). Previous studies suggested morphological and physiological differentiation of *C. soldanella* and *V. rotundifolia* between lakeshore and coastal populations (Yamada 1992; Noda *et al.* 2009; Setoguchi *et al.* 2009, 2010; T. Iwashina , H. Setoguchi, Y. Murai and E. Ono, unpubl. res.). These intraspecific differentiations indicate adaptation to freshwater habitats and a history of isolation of the inland populations from those of the coast. Therefore, the study system provided by Lake Biwa and the coastal populations make an investigation of different historical influences possible.

Lathyrus japonicus (beach pea) is a perennial coastal herb that commonly occurs in temperate coastal areas of Asia, Europe, and North and South America. Its extensive distribution range is explained by seed dispersal by currents and by the seed's ability to remain viable while floating in seawater for up to 5 years (Brightmore and White 1963). Germination occurs when the hard outer seed coat is abraded by waves on sand and gravel (Kondo and Yamaguchi 1999). This species is self-incompatible and proliferates by sexual reproduction as well as clonal propagation through the elongation of rhizomes. In Japan, *L. japonicus* is a common species of sandy coastal vegetation, and this pea also occurs on the sandy shores of Lake Biwa. Thus, inland populations of *L. japonicus* could represent 'landlocked' descendants of an ancient lake population, similar to the case of *C. soldanella* (Noda *et al.* 2011). It should therefore be possible to determine whether the plants now growing at the coast or by the lake share the same phylogeographical structures due to a shared history, and to identify consequences of long-term isolation of the inland populations and intraspecific differentiation.

The inland populations of *L. japonicus* are currently threatened with extinction due to development along the shore of Lake Biwa (Fig. 1). These shores have always been flood prone, as floodplains frequently occur around the lake (Nishino and Hamahata 2005). Beach peas as well as other coastal plants periodically live in flooded habitats, but since the 1970s, the lakeshore environment has been substantially altered as a result of ongoing development (Department of Nature Conservation Bureau, Ministry of the Environment of Japan 1995; Shiga Natural Environment Preservation Division 2000; Nakajima and Azuma 2005; Nishino and Hamahata 2005). The establishment of bank protection structures has decreased the habitat of *L. japonicus* such that only five populations remain on sandy lakeshores. These populations are considered to be small [the total number of plants is ca. 1000 (ramets) above ground, but we could not evaluate the number of individuals (genets)], and each population is isolated inland (i.e.

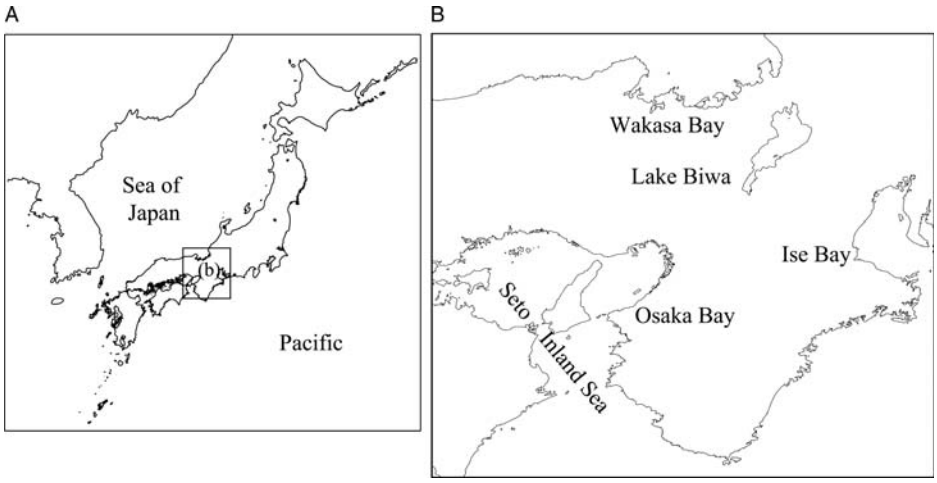

Fig. 1 Locations of the study sites. (A) Japan. Lake Biwa and the adjacent area are shown in the inset. (B) Enlargement of the inset showing Lake Biwa, and the locations of Ise Bay, Osaka Bay, Wakasa Bay and Seto Inland Sea.

lacking exposure to the lake, and seed dispersal by water cannot be accomplished), suggesting low gene flow among the extant populations. Thus, evaluating gene diversity and gene flow within and among populations would provide information that could help offer protection to the remaining populations.

cpDNA (not its haplotypes) is an informative marker due to the reduced effective population size. The merits of using cpDNA for plant phylogeographical studies have been discussed previously (e.g. Mohanty *et al.* 2001, 2002; Stehlik *et al.* 2002; Grivet and Petit 2003; Lascoux *et al.* 2003). On the other hand, neutral co-dominant nSSR loci are highly polymorphic, which enables the determination of genetic diversity and gene movement of populations. Thus, the use of both types of molecular marker should provide appropriate information to estimate the population structure and dynamics of plant species (Ennos 1994), and get both a recent and a historical perspective. In many angiosperms, the cytoplasmic genome displays maternal or maternally biased inheritance (Mogensen 1996) and cytoplasmic gene flow is restricted to seeds, whereas the nuclear genome is inherited through both seeds and pollen. Additionally, co-dominant markers with high levels of polymorphism enable accurate analyses of population genetic structure and recent demographic patterns. Thus, a comparative analysis of genetic markers derived from both genomes should provide useful information on the population structure of *L. japonicus* (e.g. gene flow, genetic structure and the presence of recent bottleneck effects).

We attempted to investigate the genetic structures of inland and coastal *L. japonicus* populations in Japan using analyses of cpDNA haplotypes and nDNA SSR loci. We expect that the Lake Biwa population has been historically isolated from coastal populations, suggesting similar geographical history to that reported for other landlocked coastal plants in Lake Biwa. The aims of this study were (i) to determine whether the Lake Biwa populations have been isolated from coastal populations for a long time; (ii) to infer genetic differentiations and the presence of a recent bottleneck by comparing the genetic diversity, gene flow and gene differentiation within and between inland and coastal populations; and (iii) to provide genetic information for conservation strategies that should consider the genetic isolation among individual populations.

Materials and methods

Site selection and sampling design

Leaf material of *L. japonicus* was sampled through most of its distribution range in Japan. Individuals were sampled at intervals of >5 m to minimize the possibility of sampling multiple ramets belonging to the same genet. Details on the sampling localities and numbers of individuals used for the analysis of cpDNA haplotype and nSSR are listed in Table 1. Leaves were dried and kept with silica gel at room temperature. In total, we used samples of 348 individuals from 50 populations for cpDNA haplotyping to cover all populations in Lake Biwa and the entire distribution range in Japan. For nSSR genotyping, we used 520 individuals from 21

Table 1 **Localities, haplotype information and sample sizes used in the analysis of cpDNA and nSSR for 50 populations of *L. japonicus* in Lake Biwa and along the sea coast of Japan.**

No.	Region		Location	Coordinates		*n*		Haplotype composition						
				Latitude	Longitude	cpDNA	nSSR	A	B	C	D	E	F	G
1	Lake Biwa		Omimaiko	35°13′N	135°57′E	13	33		13					
2			Hiragawa	35°13′N	135°57′E	13	28		13					
3			Kitahira	35°13′N	135°57′E	13	28		13					
4			Shinkaihama	35°13′N	136°07′E	8	31		8					
5			Imajuku	35°09′N	135°56′E	9	26		9					
6	Pacific	Ise Bay	Kawajiri	34°56′N	136°39′E	6					4	2		
7			Gonusi	34°38′N	136°33′E	3						3		
8			Kitahama	34°33′N	136°40′E	5	20				5			
9			Yoshizaki	34°55′N	136°39′E	7	24	1		2	4			
10			Akogigaura	34°41′N	136°31′E	6					2	4		
11			Matsunase	34°36′N	136°34′E	8	35				3	5		
12		Others	Hachinohe	40°35′N	141°28′E	11		11						
13			Ishihama	36°40′N	140°42′E	4		1			1	2		
14			Otake	36°09′N	140°35′E	10	19	2			1	4	2	1
15			Kujigawa	36°29′N	140°36′E	8								8
16			Kyochigama	36°06′N	140°36′E	8		5			1			2
17			Nijigahama	35°18′N	139°20′E	7	20				5	2		
18			Oigawako	34°28′N	138°11′E	6					3	3		
19			Nishinohama	34°48′N	137°03′E	8					8			
20			Koijigahama	34°21′N	137°06′E	7					6	1		
21			Sontaro	34°12′N	136°21′E	6					4	2		
22			Miwazaki	33°41′N	135°59′E	6		2			2	2		
23			Kushimono	33°17′N	135°26′E	9		1	5		3			
24			Sirahama	33°42′N	135°20′E	8					8			
25			Takanabe	32°12′N	131°31′E	5		3			1	1		
26			Tashiro	30°21′N	130°40′E	6					6			
27			Haruta	30°19′N	130°39′E	8	20	7			1			
28	Sea of Japan	Wakasa Bay	Echizenmisaki	35°58′N	135°57′E	8		8						
29			Sakajiri	35°37′N	135°58′E	7		6			1			

30			Sugahama	35°39′N	135°58′E	7	26	7						
31			Kehinomatsubara	35°39′N	136°03′E	6	26	5			1			
32			Suishohama	35°41′N	135°58′E	4					1	3		
33			Wada	35°36′N	135°56′E	6	29	3			3			
34		Others	Ogatomioka	44°06′N	141°39′E		27							
35			Nyudozaki	39°59′N	139°42′E	6		6						
36			Simohama	39°37′N	140°04′E	3		3						
37			Detohama	39°50′N	140°00′E	2		2						
38			Yusa	39°02′N	139°51′E	7		6	1					
39			Dekishima	40°50′N	140°16′E	6		5		1				
40			Nigatako	37°58′N	139°10′E	7	19	3	1		3			
41			Iwasehama	36°45′N	137°13′E	9		9						
42			Matsudaehama	36°50′N	137°00′E	7		2	1	4				
43			Hashi	34°57′N	132°08′E	9	28	9						
44	Seto Inland Sea	Osaka Bay	Yurahama	34°19′N	134°56′E	6	20				3	3		
45			Atsuhama	34°23′N	134°53′E	6	29				6			
46			Fukiagehama	34°13′N	132°42′E	4	15				4			
47		Others	Mihama	33°53′N	135°04′E	8		1			6	1		
48			Keinomatsubara	34°34′N	134°44′E	7					7			
49			Tsudanomatsubara	34°29′N	134°26′E	10	17	2			4	4		
50			Tsudu	34°04′N	132°12′E	5					5			
Total						348	520	110	64	7	112	42	2	11

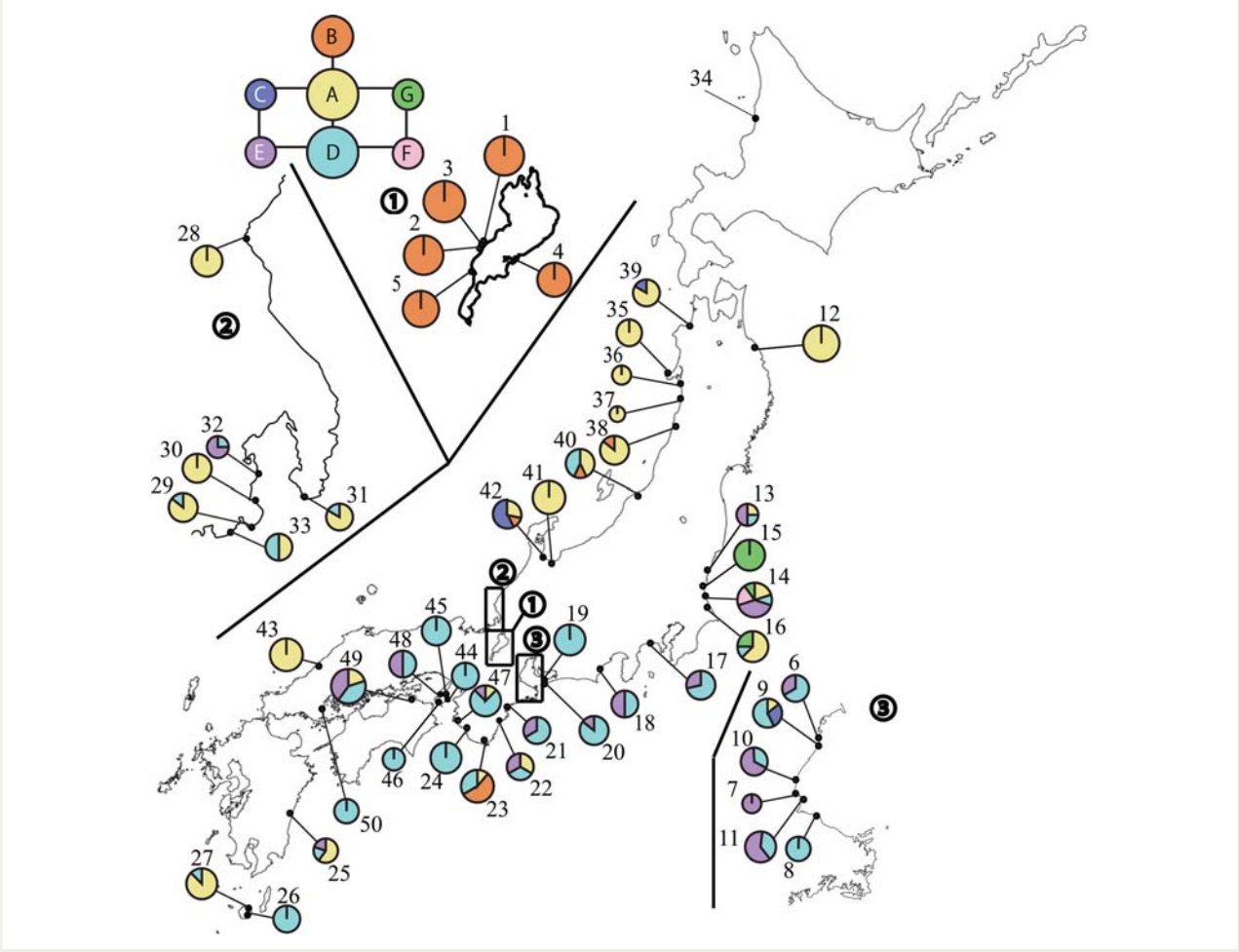

Fig. 2 Geographical distribution of cpDNA haplotypes detected in *L. japonicus*. The detailed descriptions correspond to Table 1 and the sizes of the pie charts are proportional to the number of sampled individuals per population. The three insets show Lake Biwa (1), the Wakasa Bay region (2) and the Ise Bay region (3).

populations primarily located in Lake Biwa, Wakasa Bay of the Sea of Japan, Osaka Bay of the Seto Inland Sea region and Ise Bay on the Pacific Ocean side of the island. Accordingly, we included samples from these regions to test hypotheses of the migration routes of coastal plants to Lake Biwa. We also added several populations located far from these populations to examine the geographical structure (e.g. isolation) within coastal populations (see Table 1, Fig. 2).

DNA extraction

Dried leaf materials were pulverized to a fine powder with a TissueLyser (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The leaf powder was suspended in 2-4-(2-hydroxyethyl-1-piperazinyl)ethanesulphonic acid buffer (pH 8.0) and centrifuged (10 000 rpm at 20 °C for 5 min) to remove polysaccharides (Setoguchi and Ohba 1995). Total DNA was then isolated from each pellet using cetyltrimethylammonium bromide (Doyle and Doyle 1990).

Chloroplast DNA analysis

In the preliminary screening, double-stranded DNA from 14 non-coding regions of cpDNA were amplified and sequences of ca. 12 000 bp of cpDNA were determined for eight samples of *L. japonicus* in a preliminary analysis. Each sample was selected randomly from Populations 1, 11, 33 and 44 (Table 1). Based on the results, we chose two cpDNA regions as informative markers (i.e. intergenic spacers) for further analysis: *psbA–trnH* (partial sequences of the *trnH* side: ca. 400 bp; Demesure *et al.* 1995) and *atpI–atpH* (partial sequences of the *atpH* side: ca. 700 bp; Shaw *et al.* 2007). Primers used to amplify the spacers are listed in Table 2, with primer

Table 2 Primers used for DNA sequences in this study.

Region	Name of primer	Sequence (5′–3′)	Source
psbA–trnH	psbA-*L.j*-F	AGGTATCTGGTTTACCGCGT	Developed for this study
	trnH(GUG)	ACG GGAATTGAACCCGCGCA	Demesure *et al.* (1995)
atpI–atpH	atpI	TATTTACAAGYGGTATTCAAGCT	Shaw *et al.* (2007)
	atpH	CCAAYCCAGCAGCAATAAC	Shaw *et al.* (2007)

sequences and original references. Polymerase chain reaction (PCR) was performed in a total reaction volume of 10 μL containing 6.75 μL of sterilized water, 0.08 mM dNTP mixture, 0.25 U of Takara Ex Taq (Takara Bio, Otsu, Shiga, Japan), 1.0 mM reaction buffer (Takara Ex Taq), 0.2 μM each primer and 1.0 μL of template DNA. Polymerase chain reaction was performed for 35 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C. After the PCR, products were visualized on 0.5 % TAE–agarose gels stained with ethidium bromide. The purified PCR products were sequenced using standard methods of the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an ABI Model 3130 Genetic Analyser (Applied Biosystems).

Data analysis for cpDNA

Spatial analyses of molecular variance (SAMOVA 1.0; Dupanloup *et al.* 2002) were performed to assess the genetic differentiation among 14 groups of adjacent populations to describe the geographical structure of the beach pea across its range in Japan. Given the number of groups (K), the highest differentiation among groups (F_{CT}) and the population configuration were calculated using a simulated annealing procedure. Assuming that the final configuration was influenced by the initial configuration, 600 initial conditions were used.

Parameters of population diversity (h_S, h_T, v_S, v_T) and differentiation (G_{ST}, N_{ST}) were estimated following the methods of Pons and Petit (1995, 1996) using the program PERMUT (http://www.pierroton.inra.fr/genetics/labo/Software/Permut). Parameters included mean within-population gene diversity (h_S), total gene diversity (h_T) and coefficient of genetic differentiation over all populations (G_{ST}), as well as analogous parameters (v_S, v_T and N_{ST}) obtained by taking into account the similarities between haplotypes (i.e. the number of mutations between haplotypes). Two differentiation parameters (G_{ST}, N_{ST}) were compared by a permutation test using 1000 permutations that estimated whether each population comprised closely related haplotypes. All parameters were estimated for the entire population of Japan for coastal and total populations.

Genotyping for nSSR loci

We estimated population genetic structure using nuclear markers. The genotypes of a total of 520 individuals were determined using six nSSR markers (AG36, AG13, AG19, AG12, AG16 and AG13-2; Ohtsuki *et al.*, unpubl. data) that were developed based on the methods of Lian and Hougetsu (2002) and Lian *et al.* (2006). Primer information is shown in Table 3. Using fragments containing the $(AC)_6(AG)_5$ compound SSR sequence at one end, a specific primer was designed using Primer3 v.0.4.0 (Rozen and Skaletsky 2000). Polymerase chain reaction amplifications were performed following the standard protocol of the Qiagen Multiplex PCR Kit (Qiagen) in a final volume of 6 μL. Compound SSR primers [$(AC)_6(AG)_5$] were labelled with the fluorochrome 6-FAM (Applied Biosystems). The amplification profiles included initial denaturation at 95 °C for 15 min, followed by 35 cycles of 30 s at 94 °C, 1.5 min at 56–58 °C (the annealing temperature of the primer pair) and 1 min at 72 °C, and a final extension at 60 °C for 30 min. The size of the PCR products was measured using the ABI PRISMTM 3100 Genetic Analyser (Applied Biosystems) and GeneMapper™ analysis software (Applied Biosystems).

The program MICRO-CHECKER version 2.2.3 (van Oosterhout *et al.* 2004) was used to identify putative errors of three types in our data (DeWoody *et al.* 2006): stuttering patterns, large allele dropout and null alleles on the adjusted data set (reduced to a unique number of multilocus genotypes). A modified data set was created with values set to 'missing' for loci in populations with putative nulls, and descriptive statistics were generated for the adjusted data set for comparative purposes.

Descriptive population statistics

For the nSSR analysis, the number of alleles, allelic richness (AR) (El Mousadik and Petit 1996), observed heterozygosity (H_O), gene diversity (H_S; Nei 1987) and fixation index ($F_{IS} = 1 - H_O/H_E$) were calculated for each locus

Table 3 **Characteristics of the six nSSR loci isolated from *L. japonicus*.**

Locus	Primer sequence (5′–3′)	Size range (bp)	No. of alleles	H_O	H_S	HWE
AG36	F: GTCATTAGATGCAGTTAACCGTG	115–179	29	0.944	0.755	0.001
	R: ACACACACACACAGAGAGAGAG					
AG13	F: GCTCAAGAAGATTTTGCCTG	205–263	23	0.933	0.801	0.001
	R: ACACACACACACAGAGAGAGAG					
AG19	F: CTGCTGGACTATCTCTTGA	78–122	22	0.907	0.758	0.001
	R: ACACACACACACAGAGAGAGAG					
AG12	F: GTGCCATGGACAGTTGTACCAG	310–382	26	0.906	0.780	0.001
	R: ACACACACACACAGAGAGAGAG					
AG16	F: TCAGACTCAGTAAACTTG	190–270	39	0.853	0.792	0.001
	R: ACACACACACACAGAGAGAGAG					
AG13-2	F: TGAGCCAACGCACAAAGGCT	78–138	31	0.961	0.774	0.001
	R: ACACACACACACAGAGAGAGAG					

The observed heterozygosities (H_O), gene diversities (H_S) and deviations from Hardy–Weinberg equilibrium (HWE) are shown.

and each population using the program FSTAT version 2.9.3.2 (Goudet 1995). Departures from Hardy–Weinberg equilibrium (HWE) at each locus and linkage disequilibrium between loci were tested using GENEPOP version 4.0.1 (Raymond and Rousset 1995). To assess whether populations had experienced a recent reduction of their effective population size, we used the heterozygosity excess test of Cornuet and Luikart (1996). Heterozygosity excess was estimated and tested for significance (two-tailed sign test and Wilcoxon test) under two possible mutation models, the infinite allele mutation model (IAM) and the stepwise mutation model (SMM), using the program BOTTLENECK (Piry *et al.* 1999).

Population genetic structure and gene flow

For the nSSR analysis, expected total heterozygosity (H_T) and expected within-population heterozygosity (H_S), coefficient of genetic differentiation among populations (F_{ST}; Weir and Cockerham 1984) and R_{ST} (similar to F_{ST}, except that it assumes a stepwise mutation model; Slatkin 1995) were estimated using FSTAT.

In addition, we used the program STRUCTURE ver. 2.3.3 (Pritchard *et al.* 2000) to obtain insight into how the genetic variation was organized without any prior information on the population origins based on nSSR data. To quantify the amount of variation in the likelihood for each K (the number of clusters), we performed a series of 20 independent runs for each value of K, ranging from 1 to 21. We assumed a no admixture model with correlated allele frequencies using 100 000 burn-in periods and 300 000 Markov chain Monte Carlo (MCMC) iterations.

Previous studies have shown in many cases that the posterior probability for each K increases slightly, even after the real K is reached; therefore, we used the ad hoc statistic, $\triangle K$, of Evanno *et al.* (2005) to determine the true value of K. Estimates of recent migration rates between subpopulations were determined using a molecular assignment program that relies on a non-equilibrium Bayesian method through MCMC techniques, as implemented in BAYESASS$^+$ (Wilson and Rannala 2003). This program estimates asymmetrical rates of migration between populations over the last several generations. The program was run after 3 000 000 MCMC iterations, with a burn-in of 1 000 000 iterations and a sampling frequency of 2000; delta was set to 0.15 (the default value). In addition to levels of gene flow, BAYESASS$^+$ reports 95 % confidence intervals for each estimate.

Results

Haplotype diversity and spatial distribution based on cpDNA analyses

We sequenced 1088–1103 bp corresponding to two spacers using cpDNA (395–400 bp for *psbA–trnH* and 693–703 bp for *atpI–atpH*) from 348 samples. The phylogenetic relationships among the cpDNA haplotypes were revealed in the parsimony network (Fig. 2). The polymorphic characteristics for the two spacers included two nucleotide substitutions and two indels, and seven

haplotypes were recognized among Japanese samples of *L. japonicus* (Table 4). Each of the seven haplotypes (A–G) was distinguished from adjacent haplotypes by a single substitution or a single event of an indel. These sequences were registered in DDBJ/GenBank/EMBL under accession numbers AB611010–AB611023.

The distribution of haplotypes was geographically structured between Lake Biwa and the coastal populations (Fig. 2, Table 1). All individuals in Lake Biwa were detected as haplotype B, although this haplotype was shared among four coastal populations: 23, 38, 40 and 42 (2.7 % of 292 individuals from 45 populations). Haplotypes A and D were dominant in the coastal populations (76 % of 292 individuals from 45 populations). In particular, haplotype A was dominant among populations in the Sea of Japan (78.7 % of 94 individuals from 16 populations), while haplotype D was widespread across the Pacific and the Seto Inland Sea (52.0 % of 198 individuals from 29 populations). Haplotype E was widely distributed across coastal areas in the Japanese archipelago (42 individuals and 16 populations). Haplotypes C, F and G were rare and specific to particular coastal populations.

Table 4 Haplotype composition of cpDNA in *L. japonicus*.

Haplotype	*psbA–trnH*		*atpI–atpH*	
	231	286,291	215	221
	GAAAG	C/A	G/T	ATT $(A)_7$
A	0	C	G	ATT $(A)_7$
B	0	C	G	0
C	GAAAG	C	G	ATT $(A)_7$
D	0	C	T	ATT $(A)_7$
E	GAAAG	C	T	ATT $(A)_7$
F	0	A	T	ATT $(A)_7$
G	0	A	G	ATT $(A)_7$

The results of the SAMOVA are presented in Table 5. The number of groups with the highest F_{CT} that included no single population group was three ($K = 3$). Most of F_{CT} (0.682, $P < 0.001$) can be explained by differences among populations. This configuration was consistent with the result at $K = 2$, which configured inland and coastal groups $F_{CT} = 0.603$ ($P < 0.001$). Moreover, additional groupings ($K = 4$) divided inland populations and three coastal population groups ($F_{CT} = 0.709$, $P < 0.001$). However, one of three coastal populations (Population 16) was assigned as a single group, suggesting that this group structure was invalid. Inland populations (1–5) were consistently identified as a cluster from coastal populations across $K = 2$–4. The calculated

Table 5 Result of spatial analysis of molecular variance (SAMOVA) of cpDNA sequence data from *L. japonicus* populations. All differentiations were significant ($P < 0.01$).

Source of variation	d.f.	Sum of squares	Variance components	Per cent of variance	F_{CT}
$K = 1$					
Among populations (total)	48	141.598	0.39211Va	69.56	0.696
Within populations	299	51.316	0.17163Vc	30.44	
$K = 2$ (1–5) versus (6–49)					
Among groups	1	59.277	0.60271Va	60.28	0.603
Among populations within groups	47	82.321	0.22557Vb	22.56	
Within populations	299	51.316	0.17163Vc	17.16	
$K = 3$ (1–5) versus (6–11, 13–14, 17–22, 24–26, 32, 43–49) versus (12, 15–16, 23, 27–31, 33–42)					
Among groups	2	112.056	0.51107Va	68.15	0.682
Among populations within groups	46	29.541	0.06723Vb	8.96	
Within populations	299	51.316	0.17163Vc	22.89	
$K = 4$ (1–5) versus (6–11, 13–14, 17–22, 24–26, 32, 43–49) versus (12, 15, 23, 27–31, 33–42) versus (16)					
Among groups	3	119.51	0.53010Va	70.92	0.709
Among populations within groups	45	22.087	0.04574Vb	6.12	
Within populations	299	51.316	0.17163Vc	22.96	

Table 6 Gene diversity parameters of cpDNA haplotypes within coastal populations and total populations of *L. japonicus*.

Populations	h_S	h_T	G_{ST}	v_S	v_T	N_{ST}
Coastal populations	0.325 (0.0458)	0.701 (0.0250)	0.536 (0.0603)	0.217 (0.0343)	0.516 (0.0364)	0.580 (0.0599)
Total	0.292 (0.0435)	0.747 (0.0238)	0.609 (0.0582)	0.195 (0.0322)	0.597 (0.0430)	0.674 (0.0560)

Standard error is shown in parentheses.

parameters of diversity and differentiation cpDNA haplotypes are presented in Table 6. As described above, Lake Biwa populations were fixed by a single haplotype, and we present the parameters for coastal and total populations. The value of N_{ST} (0.67) was not significantly higher than that of G_{ST} (0.61) throughout the overall populations (t-test, $P > 0.05$), and the same result was found in the coastal populations [N_{ST} (0.58) $> G_{ST}$ (0.54) (t-test, $P > 0.05$)].

Genetic diversity and genetic differentiation of populations based on nSSR analyses

The characteristics of each locus and those of loci in each population are presented in Tables 3 and 7, respectively. In total, 170 alleles were observed for the six loci across all samples and all six loci have deviated significantly from HWE at each locus. MICRO-CHECKER analysis suggested no errors from stutter or large allele dropout. Possible null alleles were indicated in Populations 4 (Locus 3, Locus 5), 9 (Locus 1, Locus 5) and 14 (Locus 4). Because we do not have segregating progeny, we cannot confirm whether nulls are truly present. The genetic diversity and allelic richness of the nSSRs observed in the 21 populations differed greatly among populations as indicated by the overall gene diversity (H_S), which ranged from 0.56 to 0.89 (Table 7). The genetic diversity of Lake Biwa populations (mean 0.62) was significantly lower than those of the coastal populations (mean of all coastal populations 0.83; t-test, $P < 0.001$). Allelic richness showed the same tendency, with the Lake Biwa populations showing significantly lower values (mean = 4.20) than those of coastal populations (mean = 8.94; $P < 0.001$). F_{IS} values for all populations ranged from −0.61 to 0.07, with a mean of −0.211 (negative value). Thus, a significant excess of heterozygotes was detected within populations. The F_{IS} values of Lake Biwa populations (mean = −0.47) were significantly lower than those of coastal populations (mean of all coastal populations = −0.12; $P < 0.001$). *Lathyrus japonicus* is self-incompatible, which decreases the probability of creating homozygote offspring because individuals cannot reproduce with themselves. An alternative result may be due to a small reproductive population size. Then, we analysed the presence of a bottleneck (Table 8). Each analysis (sign/Wilcoxon tests under the IAM/SMM) showed evidence of a recent bottleneck in inland and coastal populations. All analyses detected a recent bottleneck for inland populations 3 and 5 in harbouring significant heterozygosity excess, while no significant bottleneck was detected in inland populations 2 and 4 in any test. Approximately 88 % of the coastal populations had significant excess heterozygosity by one-fourth tests (IAM or SMM × sign or Wilcoxon test), suggesting that most coastal beach pea populations have been affected by a bottleneck.

The parameters of genetic variation within and among populations are presented in Table 9. Lake Biwa populations were highly differentiated ($F_{ST} = 0.163$, $R_{ST} = 0.268$) compared with coastal populations ($F_{ST} = 0.078$, $R_{ST} = 0.164$). A Bayesian analysis using STRUCTURE detected two appropriate clusters [K: see Additional Information] based on ad hoc $\triangle K$ statistics. The two clusters were consistent with the inland and coastal division (Fig. 3). The results of the migration rates estimated in BAYESASS$^+$ suggested a consistent level of gene flow throughout Lake Biwa (Table 10). The average migration rates in all pairwise comparisons ranged from 0.046 to 0.056. Each value represents the proportion of individuals that is derived from a corresponding source population for each generation. The migration rates among coastal populations were relatively higher than those of the Lake Biwa populations. For example, the migration rate among the mutually separated populations 34 (Sea of Japan side of northern Honshu), 11 (Pacific coast side of central Honshu) and 26 (Pacific coast side of southern Japan) ranged from 0.074 to 0.103. These data indicate a wide range of dispersal among coastal populations while the lack of gene flow among inland populations is within a narrow range.

Discussion

Analysis of within-population diversity and bottleneck

Our results suggest that the Lake Biwa populations have experienced a bottleneck effect and loss of genetic

Table 7 **Genetic diversity parameters estimated at six nSSR loci in 21 populations of *L. japonicus* in Japan.**

Region	Population	No. of samples	No. of alleles	AR	H_O	H_S	F_{IS}
Lake Biwa	1 Omimaiko	33	28	4.294	0.917	0.659	−0.391
	2 Hiragawa	28	29	4.177	0.941	0.613	−0.535
	3 Kitahira	28	24	3.855	0.970	0.654	−0.483
	4 Shinkaihama	31	32	4.159	0.758	0.561	−0.350
	5 Imajuku	26	31	4.495	0.994	0.619	−0.607
	Mean	146	29	4.196	0.916	0.621	−0.473
Pacific	8 Kitahama	20	56	8.943	0.941	0.820	−0.147
	9 Yoshizaki	24	61	8.616	0.959	0.798	−0.201
	11 Matsunase	35	88	11.013	0.865	0.874	0.010
	14 Otake	19	73	11.488	0.827	0.887	0.067
	19 Nijigahama	20	49	7.576	0.898	0.792	−0.134
	27 Tashiro	20	48	7.267	0.824	0.775	−0.063
	Mean	138	62.5	9.150	0.885	0.824	−0.078
Sea of Japan	30 Sugahama	26	58	8.400	0.934	0.829	−0.127
	31 Kehinomatsubara	26	74	10.125	0.914	0.850	−0.076
	33 Wada	29	82	11.136	0.876	0.883	0.008
	34 Ogatomioka	27	83	10.935	0.891	0.866	−0.028
	40 Nigatako	19	48	7.538	0.936	0.786	−0.190
	43 Hashi	28	57	7.940	0.928	0.789	−0.177
	Mean	155	67	9.346	0.913	0.834	−0.080
Seto Inland Sea	44 Yurahama	20	65	9.914	0.975	0.848	−0.150
	45 Atsuhama	29	53	7.305	0.971	0.799	−0.215
	46 Fukiagehama	15	45	7.500	0.989	0.807	−0.226
	49 Tsudanomatsubara	17	54	8.593	0.960	0.809	−0.187
	Mean	81	54.3	8.328	0.974	0.816	−0.195
All coastal populations		374	61.3	8.941	0.924	0.825	−0.118
Total		520	53.1	7.755	0.922	0.774	−0.211

The observed allelic richness (AR), heterozygosities (H_O), gene diversities (H_S) and fixation indices (F_{IS}) are shown.

diversity due to genetic drift. The simplicity of the cpDNA haplotype of inland populations is consistent with the nSSR data for inland populations; that is, genetic diversity, allelic richness and the fixation index were significantly lower than those of the coastal populations. Given that estimation of probability of significant heterozygosity implemented in the BOTTLENECK software can predict recent (within several generations) events in populations, historical events (e.g. migration of small ancestral populations into predecessor Lake Biwa) do not account for the apparently bottlenecked populations. Thus, our data suggest recent bottlenecks in both inland and coastal populations.

All of the five extant populations in Lake Biwa are small, and some have been under weed control (weed clipping above ground: populations 1 and 5), thus suppressing genetic diversity and promoting crossing among kin individuals within a population. No bottleneck effect, however, was detected in populations 2 and 4. This could be a reflection of recent land management; populations 2 and 4 have been managed as a campsite and a beach pea protection area, respectively.

Coastal populations are usually quite large; however, the coastline of the Japanese archipelago is frequently damaged by typhoons in summer, often seriously damaging coastal populations of *L. japonicus*. Therefore,

Table 8 Probability of a bottleneck estimated using the program BOTTLENECK. Probabilities of significant heterozygosity excess for two-tailed sign and Wilcoxon tests under the IAM and the SMM are marked with an asterisk (*$P < 0.05$,**$P < 0.01$).

Region	Population	Sign		Wilcoxon	
		IAM	SMM	IAM	SMM
Lake Biwa	1 Omimaiko	0.026*	0.490	0.016*	1.000
	2 Hiragawa	0.434	0.478	0.080	1.000
	3 Kitahira	0.025*	0.036*	0.016*	0.016*
	4 Shinkaihama	0.468	0.055	1.000	0.110
	5 Imajuku	0.041*	0.040*	0.016*	0.031*
	Total Lake Biwa	0.220	0.046*	0.047*	0.031*
Pacific	8 Kitahama	0.050	0.187	0.016*	0.156
	9 Yoshizaki	0.549	0.005**	0.156	0.016*
	11 Matsunase	0.050	0.004**	0.016*	0.016*
	14 Otake	0.241	0.480	0.078	0.679
	19 Nijigahama	0.046*	0.450	0.016*	0.844
	27 Tashiro	0.249	0.048*	0.156	0.078
	Total Pacific	0.050	0.004**	0.016*	0.016*
Sea of Japan	30 Sugahama	0.047*	0.051	0.016*	0.438
	31 Kehinomatsubara	0.565	0.209	0.438	0.110
	33 Wada	0.052*	0.191	0.016*	0.078
	34 Ogatomioka	0.566	0.006**	0.078	0.016*
	40 Nigatako	0.048*	0.041*	0.016*	0.156
	43 Hashi	0.562	0.048*	0.563	0.078
	Total Sea of Japan	0.251	0.003**	0.031*	0.016*
Seto Inland Sea	44 Yurahama	0.044*	0.543	0.016*	1.000
	45 Atsuhama	0.044*	0.043*	0.016*	0.109
	46 Fukiagehama	0.210	0.523	0.031*	0.688
	49 Tsudanomatubara	0.555	0.004**	0.563	0.016*
	Total Seto Inland sea	0.052	0.006**	0.016*	0.016*
All coastal populations		0.255	0.002**	0.031*	0.016*

Table 9 Genetic variation within and among populations of *L. japonicus* for nSSRs.

Populations	H_T	H_S	F_{ST}	R_{ST}
All populations	0.888	0.777	0.138	0.225
Within Lake Biwa	0.721	0.621	0.163	0.268
Within coastal populations	0.893	0.826	0.078	0.164
Within Sea of Japan coast	0.885	0.834	0.069	0.142
Within Pacific coast	0.886	0.824	0.082	0.194
Within Seto Inland Sea	0.870	0.816	0.082	0.129

coastal populations in the Japanese archipelago tend to be diminished (or disappear), which may trigger bottleneck effects and/or founder effects, accompanied by seed flow by currents over a large range. Seeds of *L. japonicus* are capable of germination after 7 years of dormancy (Walmsley and Davy 1997) and can easily germinate on sand after drifting (Kondo and Yamaguchi 1999). The demography of coastal populations would collapse the phylogeographical structure of *L. japonicus*, as has been reported for cpDNA haplotypes [haplotype distribution (Fig. 2), spatial analysis of molecular variance (Table 5)] and nSSRs [Bayesian clustering analysis (Fig. 3) and lower genetic variation among

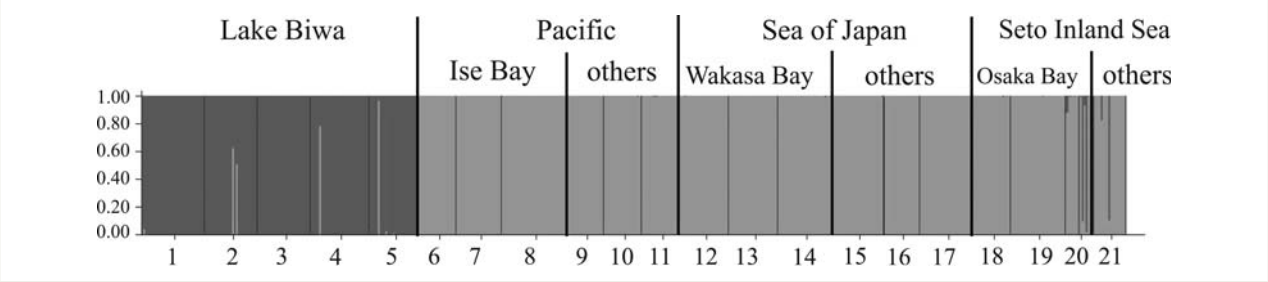

Fig. 3 Histogram of the STRUCTURE assignment test for 21 populations of *L. japonicus*, based on six nSSR loci. Each vertical bar represents an individual and its assignment proportion into one of two population clusters (K). Following a burn-in period of 100 000 iterations, 20 independent runs $K = 1–21$ were performed at 300 000 MCMC samplings. The model with $K = 2$ explained the data as satisfactory compared with models that used other K values. Regions and population numbers are shown under the bars.

Table 10 Estimates of migration rates (proportion of individuals) among subpopulations of Lake Biwa derived by BAYESASS$^+$.

Migration into	Migration from									
	1. Omimaiko		2. Hiragawa		3. Kitahira		4. Shinkaihama		5. Imajuku	
1. Omimaiko	0.812	(0.671, 0.980)	0.053	(0.000, 0.191)	0.052	(0.000, 0.191)	0.047	(0.000, 0.195)	0.050	(0.000, 0.195)
2. Hiragawa	0.046	(0.000, 0.182)	0.789	(0.670, 0.967)	0.053	(0.000, 0.199)	0.047	(0.000, 0.200)	0.050	(0.000, 0.191)
3. Kitahira	0.048	(0.000, 0.192)	0.053	(0.000, 0.196)	0.791	(0.671, 0.967)	0.050	(0.000, 0.204)	0.056	(0.000, 0.207)
4. Shinkaihama	0.047	(0.000, 0.188)	0.052	(0.000, 0.199)	0.056	(0.000, 0.202)	0.807	(0.671, 0.985)	0.051	(0.000, 0.192)
5. Imajuku	0.046	(0.000, 0.178)	0.052	(0.000, 0.203)	0.048	(0.000, 0.200)	0.049	(0.000, 0.201)	0.792	(0.672, 0.975)

Means of the posterior distributions of m, the migration rate into each population, are shown. The populations from which each individual was sampled are listed in the rows, while the populations from which they migrated are listed in the columns. Values along the diagonal are the proportion of individuals derived from the source populations for each generation. Values in parentheses below the migration rates are 95 % CI.

populations (Table 9)]. Additionally, estimating the localities of the original Lake Biwa population(s) is not possible for the coastal populations with highly disturbed demography.

Differentiation of inland versus coastal populations

Our results suggest that the genetic structures of inland populations in Lake Biwa were highly differentiated from those of coastal populations. All inland individuals shared a single haplotype (haplotype B), whereas coastal populations harboured seven haplotypes (haplotypes A–G). The results of the SAMOVA for cpDNA haplotypes consistently separated Lake Biwa populations across $K = 2–4$. Additionally, the STRUCTURE analysis suggested that the most appropriate value of K was 2, partitioning the inland and coastal populations. These results (recognizing inland populations as a unit) were consistent across $K = 2–6$ (data not shown). Thus, both cpDNA and nSSR data revealed that populations in Lake Biwa have been historically isolated as 'landlocked' populations.

A similar genetic structure was exhibited by inland populations of beach morning glory (*C. soldanella*). Specifically, the Lake Biwa populations were genetically isolated from coastal populations and harboured low genetic diversity (Noda *et al.* 2011). The previous study detected three unique cpDNA haplotypes among the four types (one haplotype was shared by inland and coastal populations), while only one haplotype (shared haplotype between inland and coastal populations) was detected in the present study. Thus, the occurrence of cpDNA haplotypes was different between *L. japonicus* and *C. soldanella*, suggesting that lakeshore *L. japonicus* have experienced a more severe bottleneck than *C. soldanella*. Although we were not able to estimate the past distribution of beach pea in Lake Biwa, its current distribution is confined to five small populations, whereas *C. soldanella* is abundantly present around the lakeshore. Thus, the current inland populations of

L. japonicus might have experienced a more severe bottleneck than *C. soldanella*, although we are unable to confirm whether the current habitats of *L. japonicus* are the result of historical events, such as the migration of ancestor populations to a predecessor lake, or the artificial altering of the lakeshore over the past 30 years.

Our results also suggest low gene flow between inland and coastal populations, which is consistent with a previous report (Noda *et al.* 2011). *Lathyrus japonicus* is an insect (bumblebee)-pollinated plant, and as such, its pollen flow is dependent on the behaviour of bumblebees. Generally, pollen flow mediated by bumblebees is assumed to occur across limited distances (Asmussen 1993), suggesting that pollen movement of *L. japonicus* might also be limited to within short distances. The drainage of the current Lake Biwa connects to the Seto Inland Sea, but no beach pea plants have been found around this basin. Additionally, we did not detect haplotype B in the Seto Inland Sea. Similar results were reported for beach morning glory; that is, no unique haplotypes of the inland populations were detected along the coasts, suggesting that seed flow between inland and coastal populations was lacking. Nuclear microsatellite marker data for *C. soldanella* also suggested isolation of inland populations from coastal ones. Thus, the present study supports the concept that the inland (Lake Biwa) and coastal populations of these coastal plants must have been separated for a long period without gene flow via seed flow and/or pollen flow. Thus, ancestral population(s) of beach pea would have migrated to a predecessor lake of Lake Biwa, and the inland population(s) might have been maintained within the freshwater lake for a long period.

Differentiation/gene flow among inland versus coastal populations

Our data also suggest that gene flow among inland populations contrasted with that of coastal populations. The estimated mean F_{ST} value across inland populations was significantly higher than that of coastal populations, indicating that each population in Lake Biwa is genetically isolated, although the mutual geographical distances were lower than those seen along the coasts (<1 km within Lake Biwa). The BAYESASS^{+} analysis also supported the lack of gene flow among inland populations. The high genetic differentiation accompanied by a lack of gene flow among the Lake Biwa population is in contrast to the patterns observed for *C. soldanella* (Noda *et al.* 2011). Specifically, inland populations exhibited lower genetic differentiation (mean $F_{ST} = 0.054$) than coastal populations (mean $F_{ST} = 0.178$). These contrasting results between the two coastal plants may be attributable to the fragmented habitat situation within Lake Biwa.

Based on our observations for *L. japonicus*, Lake Biwa populations were pollinated by bumblebees and fertile seeds were produced. However, the pollen flow distance mediated by the bumblebees is relatively limited (Asmussen 1993) and seed flow by water is unlikely because none of the habitats has ever been submerged. Compared with *C. soldanella*, *L. japonicus* grows further inland from the lakeshore [e.g. Population 1 (Omimaiko) is located ca. 80 m from the lakeshore and Population 2 is ca. 100 m from the lake (Hiragawa)]; thus, its seeds may have less of an opportunity to drift along the lake. The areas surrounding Lake Biwa have not flooded since the 1970s, when water-level controls were initiated (Nishino 1986), suggesting that the movement of beach pea seeds is restricted to within the same population. Therefore, populations in Lake Biwa may be isolated due to the current lakeshore environment.

The isolated *L. japonicus* populations in Lake Biwa might accelerate kinship within each population. The negative F_{IS} values in all of the inland populations (mean $F_{IS} = -0.473$, Table 7) indicated excess heterozygosity in each population, which could be due to a bottleneck (Mayr 1963; Cornuet and Luikart 1996). In contrast, wide-range dispersal by sea currents occurred among coastal populations. The haplotype distribution as well as the results of the STRUCTURE analysis (even after the *K* values were increased; data not shown) indicated a lack of phylogeographical structuring among the coastal populations. These results, recognizing no geographical structure across the Japanese coast, are consistent with a previous report on *C. soldanella* (Noda *et al.* 2011). The extensive seed longevity in seawater and long-distance seed dispersal may explain the lack of phylogeographical structuring of coastal plants (e.g. Arafeh and Kadereit 2006). Thus, the present study suggests that landlocked populations of beach pea in Lake Biwa harbour distinct genetic structures as a coastal plant, that is, inland isolation for long periods (possibly due to historical events) and genetic isolation among individual populations (due to a lack of current gene flow).

Conclusions and forward look

We revealed that current inland populations of beach pea within an ancient freshwater lake represent historical descendants with long-term isolation history; moreover, all inland populations exhibited low genetic diversity due to the bottleneck effect and each inland population became isolated as a result of the interference in gene flow via seed and pollen movements.

Thus, propagation appeared to be restricted to kin individuals because of the low genetic diversity within each population. Genetic diversity can be critically important for the evolutionary potential of a species in the face of environmental change, and the loss of genetic diversity has often been associated with reduced fitness (Frankham 1995; England *et al.* 2003). Thus, the current genetic diversity will need to be conserved to sustain the population persistence of this self-incompatible plant. Additionally, there is much concern that opportunities for seed flow by water are rare in current habitats due to recent changes in the lakeshore environment. Specifically, the construction of dam sites around the Lake Biwa basin (Department of Nature Conservation Bureau, Ministry of the Environment of Japan 1995; Nakajima and Azuma 2005) and drainage facilities have decreased the water level (ca. 90 cm) of Lake Biwa over the past 100 years (Nishino 1986), which reduced the sandy lakeshore and affected sand movement and vegetation. Indeed, with the exception of Population 3, all *L. japonicus* populations were located in the grasslands, which are maintained by weed clipping. These grasses are inland species that never grow by lakeshores that are subjected to periodic submergence.

The results of this study can be used to develop conservation perspectives for Lake Biwa *L. japonicus* populations. The genetic properties of *L. japonicus* should be considered and recognition given to the fact that the current populations are isolated and propagate via kin individuals. We therefore propose that sustaining *L. japonicus* populations should be fully taken into account when developing future conservation policies associated with population maintenance.

Additional information

The following additional information is available in the online version of this article –

Fig. 1: Distributions of Ln *(K)* and $\triangle K$ in the STRUCTURE analysis.

Accession numbers

cpDNA (395–400 bp for *trnH-psbA* and 693–703 bp for *atpI-atpH*) sequences were registered in DDBJ/GenBank/EMBL under accession numbers AB611010–AB611023.

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Contribution by the authors

All the authors conceived and designed the experiments. T.O. and Y.K. performed the experiments. T.O. analysed the data. T.O and H.S. prepared the manuscript.

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Conflict of interest statement

None declared.

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