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Geographical Differentiation in Leaf Thickness between Coastal and Freshwater Populations of the Coastal Plant *Lathyrus japonicus* (Fabaceae)

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Abstract The beach pea, *Lathyrus japonicus*, is a coastal plant that is also found along the shore of Lake Biwa, an ancient freshwater lake in Japan. These plants occur in two fundamentally different types of habitats, seacoast and freshwater lakeshores, suggesting the possibility of ecological adaptation to habitats (e.g., salt tolerance). We focused on morphological differentiation between coastal and inland populations and measured leaf blade thickness in a total of ten Japanese populations of *L. japonicus* from Lake Biwa, Ise Bay on the Pacific Ocean side, and Wakasa Bay on the Sea of Japan side. In addition, we compared transverse leaf sections between inland and coastal populations using light microscopy. An analysis of variance with a post-hoc test revealed that the inland and coastal populations comprised three major groups, with the inland populations accounting for one group and the coastal populations being assigned to the other two groups regardless of locality. Inland individuals tended to have thinner leaf blades, due to differences in the cell sizes of the palisade and spongy tissues. The succulence in *L. japonicus* at Lake Biwa may have been lost since salinity tolerance was not essential for their survival in a freshwater environment.

Key words: coastal plant, geographical variation, isolation, Lake Biwa, *Lathyrus japonicus*, leaf thickness, salinity stress.

Introduction

Coastal environments are often inhospitable, and several factors, such as soil moisture, soil nutrients, wind exposure, sand burial, salt spray, and soil salinity, likely limit plant growth on coastal sand dunes (e.g., Oosting and Billings, 1942; van der Valk, 1974; Barbour, 1978; Maun, 1994). The action of surf breakers along coasts may result in a considerable amount of salt spray on soil and plant surfaces, and deposition of airborne seawater causes damage to plant tissues (Barbour, 1954; Rozema *et al.*, 1985). Thus, salt spray permits the growth and survival of a limited number of tolerant species along the coast and indirectly controls plant communities and their development (Oosting and Billings, 1942; Oosting, 1945; Barbour, 1954; Rozema *et al.*, 1985; Sykes and Wilson, 1988; Hesp, 1991; Maun, 1994; Greipsson and Davy, 1996; Wilson and Sykes, 1999).

The beach pea, *Lathyrus japonicus* Willd. (Fabaceae), is a perennial coastal herb native to temperate coastal areas of Asia, Europe, and North and South America. The unusually extensive native range of this species is explained by the ability of the seeds to remain viable while floating in seawater for up to five years, enabling the seeds to drift nearly worldwide (Brightmore and White, 1963). Germination occurs when the hard outer seed coat is abraded by waves on sand or gravel (Kondo and Yamaguchi, 1999). *L. japonicus* inhabits the zone near the storm crest of the beach or at the seaward margin of a fixed dune but generally does not grow inland, implying that this plant grows under salinity stress from airborne salt and higher soil osmotic pressure. In Japan, *L. japonicus* is a typical coastal plant, but also occurs at Lake Biwa, an ancient freshwater lake in central Japan.

Lake Biwa, which was formed approximately four million years ago, is one of the world's few ancient lakes. The lake was initially located approximately 40 km southeast of its present position and this predecessor lake gradually moved northwest to its present position as a result of fault subsidence (Takaya, 1963; Yokoyama, 1984; Kawabe, 1989, 1994; Meyers *et al.*, 1993). Many typically coastal plants inhabit the Lake Biwa area, including *Calystegia soldanella* (L.) Roem. et Schult. (Convolvulaceae), *Vitex rotundifolia* L., *Arabis kawasakiana* Makino (Cruciferae), *Raphanus sativus* L. var *raphanistroides* Makino (Cruciferae), *Dianthus japonicus* Thunb. (Caryophyllaceae), and *Pinus thunbergii* Parl. (Pinaceae) as well as *L. japonicus* (Kitamura, 1968). It is assumed that these plants migrated to the lake from coastal populations during the period when the lake was adjacent to the sea, and that the lake populations subsequently became isolated from the coastal populations (Takaya, 1963; Kitamura, 1968). Thus, these isolated Lake Biwa plants are assumed to corroborate long-term isolation of the inland populations and intraspecific differentiation.

Furthermore, these plants occur in two fundamentally different types of habitat, seacoasts and freshwater lakeshores, suggesting the possibility of ecological adaptation, e.g., salt tolerance. Both nuclear and cytoplasmic markers indicate that *C. soldanella* and *L. japonicus* at Lake Biwa are isolated from coastal populations (Noda *et al.,* 2011; Ohtsuki *et al.,* unpublished). Isolated or fragmented habitat types are expected to promote the potential for local adaptation, and leaf succulence is correlated with salinity stress exposure in coastal plants (Boyce, 1951; Mayr, 1963; Hesp, 1991; Maun, 1994). Landlocked plants at Lake Biwa have been found to be morphologically differentiated from coastal populations; Setoguchi *et al*. (2009, 2010) found that leaf blades of *C. soldanella* and *V. rotundifolia* were thinner in inland populations than in coastal populations, indicating adaptation to a different habitat. Thus, given the geographic history of inland populations, we hypothesized that *L. japonicus* may also exhibit allopatric differentiation in morphology in relation to adaptation to its habitat. As with *C. soldanella* and *V. rotundifolia*, inland populations of *L. japonicus* have thinner leaf blades. In this study, we examined morphological differentiation in *L. japonicus* leaf-blade thickness between inland and coastal individuals. We measured the leaflet blade thickness of 410 individuals from a total of ten populations located along the shore of Lake Biwa and the coast, and discuss the effects of ecological factors and genetic background on morphology. In addition, we compared the structural differences in the transverse leaf sections of the inland and coastal populations using light microscopy.

Materials and Methods

To assess mature leaves, 410 *Lathyrus japonicus* individuals were measured in June 2010 in natural habitats on the shore of Lake Biwa (four populations), the Pacific coast (Ise Bay, three populations), and the Sea of Japan coast (Wakasa Bay, three populations). The localities and their coordinates are summarized in Table 1.

All voucher specimens were deposited in National Museum of Nature and Science, Japan (TNS).

Inland populations at Lake Biwa have been decreasing in number, and only five populations currently remain as small patches. We used electronic calipers to measure leaf blade thickness of 10 fully matured leaves from each individual. Because leaves of beach pea are compound, leaf thickness measurements were conducted on leaflets: the average thickness of each leaf was estimated based on measurements from the third proximal leaflets on either side of the rachis within a compound leaf. Additionally, we remeasured that leaflet on one side of the rachis to cor-

Table 1. Sampling localities, coordinates and number of individuals for *Lathyrus japonicus*

Region	No.	Location	Cordinates		Number of individuals
			Latitude	Longitude	
Like Biwa	1	Omimaiko	35°13′N	135°57′E	41
	2	Hiragawa	35°13′N	135°56′E	41
	3	Shinkaihama	35°13′N	136°07′E	41
	4	Imajuku	35°09′N	135°56′E	41
Pacific	5	Matsunase	34°36′N	136°34′E	41
(Ise Bay)	6	Higashioizu	34°33′N	136°40′E	41
	7	Muramatsu	34°33′N	136°41′E	41
Sea of Japan	8	Sugahama	35°39′N	139°58′E	41
(Wakasa Bay)	9	Sakajiri	35°37′N	135°58′E	41
	10	Wada	35°36′N	135°56′E	41

Table 2. Result of one-way analysis of variance (ANOVA) of leaf thickness among populations

Morphological characteristic	Source of variation	SS	dF	MS	*F*	*P*
Leaf thickness	Among groups	430028.653	9	47780.961	47.404	<0.001
	Within groups	403184.751	400	1007.962		
	Total	833213.404	409			

SS, Sum of Squares; dF, degree of Freedom; MS, Mean Squares; *F*, *F*-statics; *P*, *P*-value for the null hypothesis.

rect for measurement error. Mean values were calculated for each individual, and the dataset of 41individuals from each population was subjected to a one-way analysis of variance (ANOVA) to test for differences in leaf blade thickness among the populations. Tukey's HSD post-hoc test for multiple comparisons was used to test for significant differences between populations. Statistical analyses were performed using PASW Statistics 17.0 (WinWrap Basic). In addition, to analyze morphological structure, fully expand and mature leaflets (Biwa and Coastal population) were collected. Cross sections of those leaflets were investigated with a light microscope (BX-51 OLYMPUS). Photographs of the observed cross sections were taken with digital camera (C-4040ZOOM OLYMPUS) attached to a light microscope. Fresh and mature leaflets were sectioned with a razor blade. Cross sections of 200-350 μm in thickness were used for the observations.

Results and Discussion

Leaf blade thickness was normally distributed, and the values differed significantly among populations (ANOVA, $P<0.001$; Table 2), ranging from 226.91±3.51 μm (mean±standard error) to 315.05±4.43 μm (Fig. 1). Leaf blade thickness suggested a geographical trend; inland populations from Lake Biwa tended to have thinner blades than seashore populations (Fig. 1, Tukey's HSD). A post-hoc multiple comparison recognized all inland populations (1–4) as a single group, whereas the seashore populations were assigned to the other two groups regardless of their locality (Ise Bay or Wakasa Bay). Transverse sections of leaflets examined were shown in Fig. 2, which represented both inland and coastal population's leaflets. As has been suggested in Fig. 1, thickness of the leaflets was greater in coastal individuals compared to those in inland individuals. The difference in leaf thickness was attributed to sizes of the palisade and spongy parenchyma cells: leaves of inland individuals have smaller parenchyma cells compared to those of coastal ones. On the other hand, the thickness of epidermis and/or cuticle of the leaflets were not obviously different between inland and coastal individuals. Leaf succulence in plants inhabiting coastal dunes is induced by salinity stress (airborne salt loading on leaves and

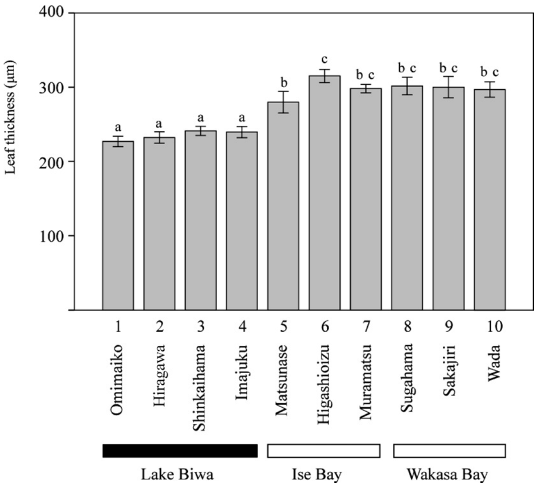

Fig. 1. Means and standard errors of leaf thickness in *Lathyrus japonicus*. Different letters above the vertical bars indicate significant differences based on Tukey's HSD post-hoc test.

branches), resulting in salt-induced hypertrophy and the doubling or tripling of leaf thickness (Boyce, 1951). In addition, different effects of salinity in structure of leaf cells and other plant tissues have been reported in several plants species (Babber *et al.,* 2000; Muthukumurasamy *et al*., 2000; Sam *et al*., 2004). Thus, *Lathyrus japonicus* in Lake Biwa may have caused the loss of leaflets succulence in the habitat environment. Noda *et al.* (2009) detected physiological differentiation between inland and coastal individuals of *Calystegia soldanella* in terms of their response to salinity stress. Inland individuals at Lake Biwa showed significantly decreased photosynthetic ability as a result of saltwater sprayed onto leaf blades, resulting in stomatal closure, whereas coastal individuals showed an insignificant decrease following the same treatment. Additionally, coastal individuals tended to have thicker leaf blades than those of inland individuals even after freshwater irrigation. Conversely, inland individuals developed thinner leaf blades than those of coastal individuals even after saltwater irrigation and saltwater spray onto leaf blades (Noda *et al.,* 2009). A preliminary study also revealed physiological differentiation in *L.*

(a) (b)

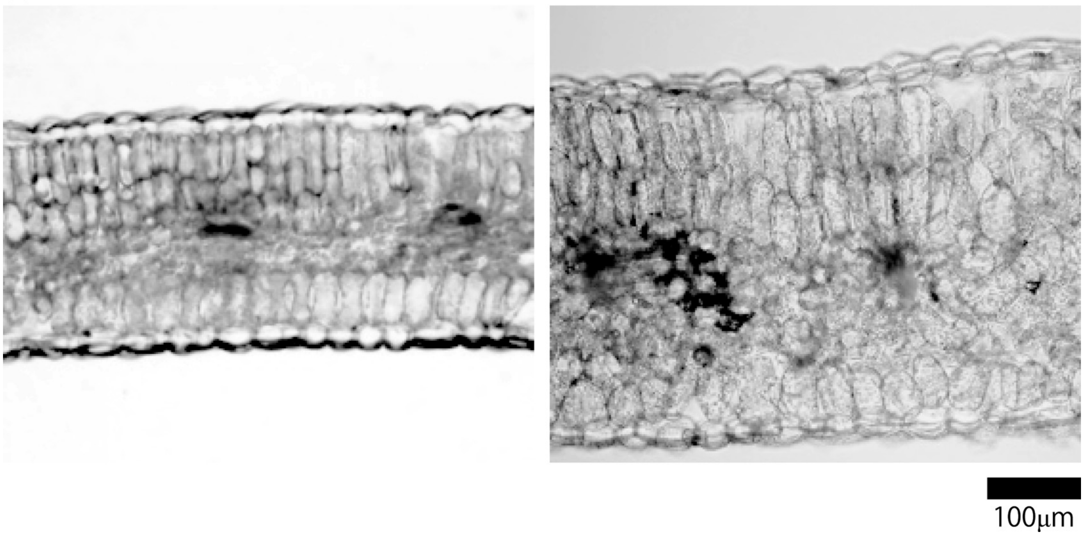

Fig. 2. Transverse leaflets sections illustrating the contrast between inland (a) and coastal populations (b). Scale bar$=100\ \mu$m

japonicus between inland and coastal habitats; flavonoid content was significantly different between inland and coastal populations, implying differentiation in physiological response, antioxidant, radical-scavenging functions, and UV-protective functions in response to changes in the surrounding environment. Additionally, These indicate that the inland plants have adapted to a freshwater environment in terms of physiological response to the surrounding environment. Thus, inland individuals from Lake Biwa had significantly thinner leaf blades, suggesting that *L. japonicus* landlocked at Lake Biwa might have adapted to the lack of salinity stress at the freshwater lakeshore accompanied with physiological differentiation.

In this study, we revealed a heterogeneous genetic structure between inland and coastal populations of *L. japonicus*, corroborating the isolation of inland populations at Lake Biwa as “land locked” as determined using molecular phylogeographic analyses (Ohtsuki *et al.,* unpublished). The same signature is exhibited by beach morning glory, *C. soldanella*; populations at Lake Biwa are genetically isolated from coastal populations (Noda *et al.,* 2011). With their loss of leaf succulence and salinity tolerance, *L. japonicus* plants at Lake Biwa appear to have morphologically differentiated from coastal plants during their inland isolation. Further studies including transplant experiments and examination of photosynthetic responses to salinity stress should be undertaken to better understand the physiological basis for this adaptive response.

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