Adaptive differentiation of *Lathyrus japonicus* (beach pea; Fabaceae) between coastal and freshwater environments based on genetic and ecophysiological analyses

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Summary

One of the major challenges in biology is elucidating the evolutionary processes that create diversity of life on earth. Geographic isolation suppresses gene exchange between populations and causes an accumulation of genetic differentiation, and subsequent speciation, which is thought to be a major driving force of species diversification. Geographic differentiation leads to habitat-specific stresses, and thus to physiological differentiation among populations within a species. Therefore, understanding intraspecific differences in physiological traits among geographically separated populations allows us to determine the mechanisms that drive intraspecific differentiation, leading to new insight into how evolutionary processes create biodiversity.

Lathyrus japonicus willd. (beach pea; Fabaceae) is a perennial coastal herb that is widely distributed in the temperate coastal parts of Asia, Europe, and North and South America. This plant inhabits sandy seashores, and the sandy shores and grasslands of freshwater lakes and/or riverbanks (e.g., the Great Lakes in North America and Lake Biwa in Japan), a distribution explained by sea-level-driven shifts in geographical distribution. The two types of habitat suggest physiological differentiation between environments, likely represented by differences in salt tolerance. Lake Biwa, which formed ~4 million years ago (MYA), is widely recognized as one of the world’s few ancient lakes. Since it is thought to have been once connected to the sea, it provides an ideal study area for intraspecific differentiation in coastal plants, as they would have undergone isolation into inland freshwater environments. However, it is unclear whether genetic differentiation has occurred between Lake Biwa and coastal populations, and the demographic history of the inland population remains unresolved. Therefore, I compared the genetics and ecophysiology of freshwater and coastal populations to test genetic and physiological differentiation. Furthermore, I elucidated the demographic history of *L. japonicus* and the adaptive differentiation on physiological traits for both types of population to each habitat.

In Chapters 1 and 2, I investigated the phylogeographical and population structures of inland and coastal populations of *L. japonicus* using plural molecular genetic markers (cpDNA sequences, neutral co-dominant nSSR loci, and nuclear sequences). Population structure analyses identified separate inland and coastal groups, suggesting a lack of gene flow between them. In addition, the

estimated demographic parameters implemented by an isolation-with-migration model (IM model) revealed a recent divergence (post glacial period) of inland populations from ancestral populations, and showed that inland populations are significantly smaller than ancestral or coastal populations. Given the demographic history of this species, my study suggests that a Holocene thermal maximum was involved in the origins of the inland populations and their genetic divergence. I concluded that ancestral migrants to Lake Biwa were derived from small founding groups which accelerated the genetic isolation of Lake Biwa populations during the relatively short-term geographic isolation.

Given the demographic history of *L. japonicus*, periods of exposure to freshwater should affect the salt tolerance because coastal environments are inhospitable due to high salinity, often in combination with other stresses. In Chapter 3, I present morphological analyses which revealed that inland individuals tend to have thinner leaf blades than those inhabiting coasts, due to differences in the cell sizes of palisade and spongy tissues. Succulence traits in *L. japonicus* at Lake Biwa may have been lost since tolerance to high salinity would not be essential for survival in freshwater environments. Flavonoid composition analyses using high-performance liquid chromatography (HPLC) revealed that all Lake Biwa individuals contained the same flavonols (glycosylates), while coastal individuals exhibited higher variation in flavonoid composition. Variation was likely lower in inland populations because of a bottleneck during landlocked periods, which is consistent with previous phylogeographic studies. A qualitative HPLC survey of flavonoid content revealed substantial variation among individuals regardless of locality. This suggests that habitat changes may have led to *L. japonicus* acclimation via alteration of the quantity and quality of flavonoids.

Salt can seriously damage plants via osmotic and ionic stresses; however, coastal plants are salt tolerant and can minimize detrimental effects using a series of physiological adaptations. *L. japonicus* has migrated from coastal to inland freshwater habitats, presumably adapting to freshwater environment. In Chapter 4, I present comparative analyses of physiological growth and ion accumulation traits, which I conducted on the freshwater and coastal populations using growth cabinet experiments with various stresses (salt, drought, and hyper osmotic stresses). Given that the length of exposure to freshwater affects physiological traits in freshwater populations, I expected to observe physiological divergence between longstanding-freshwater (Lake Biwa) and new-freshwater (Lake Kasumigaura) populations. Thus, I investigated whether physiological diversification occurred among three types of population: coastal, ancient freshwater (Lake Biwa), and recently altered freshwater (~50 years ago: Lake Kasumigaura). The Na^+ concentration in shoots was high in samples from the Lake Biwa population, whereas plants from the Lake Kasumigaura and coastal populations showed lower concentrations. In addition, a series of stress experiments revealed that the regulation of Na^+ uptake into shoots may be a key salt tolerance trait because Na^+ is a cytotoxin that disturbs ion equilibrium and affects vital cellular and physiological processes that can further decrease growth over time. Thus, *L. japonicus* at Lake Biwa has decreased its salt tolerance and retains less restriction of entry of Na^+ when artificially exposed to a coastal environment. On the other hand, samples from Lake Kasumigaura showed evidence of salt tolerance, suggesting that approximately 50 years of inland isolation is insufficient to alter physiology. In conclusion, long-term allopatric growth of *L. japonicus* in different environments caused trait divergence via irreversible physiological differentiation.

Keeping in mind the physiological differentiation between the Lake Biwa and coastal populations, I compared their ecophysiological variation in natural habitats. In Chapter 5, I show comparisons of plant growth, photosynthetic performance, and accumulation of ion and abscisic acid (ABA) between freshwater and coastal populations in different environments. To accomplish this, I used reciprocal transplant experiments at a freshwater (Lake Biwa) and coastal (Matsunase Beach) site. At the freshwater site, coastal individuals showed decreases in growth, number of flowers, and photosynthetic performance, along with lower stomatal conductance compared to the coastal site. At the coastal site, freshwater individuals exhibited low tolerance to salt and drought stresses, while showing a high Na^+ ion concentration in shoots, high ABA contents and reduction of stomatal conductance in leaves, lower survival rates, and a decreased number of flowers. The reductions in survival rate and number of the flowers can be attributed to a decrease in the photosynthetic rate. Thus, inland and coastal individuals grow ideally under conditions closest to their original habitats, whereas mutual replacement (transplanting) results in growth depression or withering. These results suggest that adaptive differentiation in physiological traits (as represented by photosynthetic traits) exist between Lake Biwa and coastal populations. In addition, physiological differentiation represented by Na^+ ion uptake between them has accumulated in the neutral process of the intraspecific differentiation. Therefore, regulation of Na^+ uptake to shoots is a key trait for maintaining normal photosynthesis in coastal environments.

In this study, I found that the physiological differentiation and a set of physiological responses of the beach pea between geographically differentiated populations to each habitat, suggesting local adaptation during intraspecific differentiation. My results also suggest the neutral divergence between geographically separated populations results in physiological differentiation. Since local adaptation is known to be a key step in species diversification, my findings increase our understanding of the importance of physiological differentiation accompanied by the process of allopatric speciation.