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Coastal adaptation of *Ligustrum japonicum* Thunb. (Oleaceae).

- A case study of stomatal adaptation pattern into coastal forests -

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Abstract: Plants in coastal areas have adapted to develop characteristic morphologies under various specific stresses. *Ligustrum japonicum* Thunb. (Oleaceae) grows from mountainous areas into coastal areas. To clarify the adaptive morphology of *L. japonicum* in coastal areas, we conducted morphological and anatomical analyses using coastal and inland populations from Kanagawa and Kochi prefectures, respectively. The results of morphological and anatomical analyses showed that stomatal size was smaller in coastal populations than in inland populations, which was common in Kanagawa and Kochi prefectures. To our knowledge, this is a remarkable result to adapt to coastal forests, suggesting that *L. japonicum* differentiated the coastal ecotype in the coastal area.

1 Introduction

There are several environmentally diverse areas in the world, and plants are affected by different stresses in each environment. Plant responses to stresses are complex, involving signal reception and transduction, followed by genetic and physiological responses. All plants are understood to be capable of perceiving and responding to stress (Bohnert et al., 1995; Bartels and Sunkar, 2005). Among them, the coastal areas experience various stresses in the form of salt spray, sand burial, dryness, high light intensity, wind exposure, soil salinity and nutrient deficiency (Greenway and Manns, 1980), and various studies have been conducted in an attempt to understand these stresses (e.g., Nakajima and Yoshizaki, 2010; Ito and Yoshizaki, 2017; Ito and Yoshizaki, 2019). In particular, the source of stress and abiotic drivers of natural selection in coastal plant communities are mainly soil salinity (Barbour, 1978; Rozema et al., 1982), and various levels of salt spray could result in vegetation zonation. Plants adapted to salt spray grow close to the ocean and are replaced by less salt-resistant plants further inland (Ignaciuk and Lee, 1980; Erickson and Young, 1995). Therefore, plants in the coastal areas have developed certain characteristic morphologies, such as succulent tissues to store water and pubescent epidermis to reduce transpiration and water loss (Hesp, 1991). Since the coastal area is a unique environment with various stresses, some coastal endemic species with unique morphological characteristics have evolved, such as *Canavalia lineata* (Thunb.) DC. (Fabaceae), *Dianthus japonicus* Thunb. (Caryophyllaceae), *Glehnia littoralis* F.Schmidt ex Miq. (Apiaceae) and *Ixeris repens* (L.) A.Gray (Asteraceae).

Regarding plants with the characteristic morphologies in the coastal areas, Tunala et al. (2012) indicated that the epidermal cells of the coastal variety of *Aster hispidus* Thunb. var. *insularis* (Makino) Okuyama (Asteraceae) were larger in size but fewer in quantity than those of *As. hispidus* var. *hispidus* and were found in succulent leaves. Moreover, Ohga et al. (2013) suggested that the coastal ecotype of *Adenophora triphylla* (Thunb.) A.DC. var. *japonica* (Regel) H.Hara (Campanulaceae) evolved from the normal type via a heterochronic process. In addition to these studies, certain anatomical characteristics developed by plants to adapt to coastal areas have also been reported. Some studies have reported that stomatal density tends to be more responsive to abiotic conditions (Ouedraogo and Hubac, 1982; Ashton and Berlyn, 1994; Richardson et al., 2001; Bucher et al., 2016; Römermann et al., 2016). Kumekawa et al. (2013) indicated that coastal endemic *As. hispidus* var. *insularis* had significantly lower stomatal density than that of *As. hispidus* var. *hispidus*. Studies of stomata under salt stress and drought stress have mainly focused on crops, herbaceous plants, and semi-shrubs (Lecoeur et al., 1995; Zhao et al., 2006; Galmés et al., 2007; Xu and Zhou, 2008), and the adaptation of stomata in trees found in coastal forests remains unclear. Consideration for these studies, a plant species with large habitats from inlands to coastal areas could have characteristic morphologies in the latter populations and be divided into different taxa. Accumulation of such studies can show that planting broad-leaf trees in coastal areas requires attention not only to the tree species but also to their origin or their collection site, resulting in the appropriate species for the coastal areas. In addition, the planting coastal areas with appropriate broad-leaf tree species could increase biodiversity, provide habitat for a variety of wildlife and enhance coastal landscape values. Therefore, we considered that it was important to describe the traits of plant species distributed from inlands to coastal areas.

Coastal forests comprise a diverse group of tree species, from pine to broad-leaved trees. Among them, *Ligustrum japonicum* Thunb. (Oleaceae) is a small tree that grows 2–5 m tall and is native to central and southern Japan and

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Korea (Noshiro, 1993). Its leaves are thick and leathery, with an entire margin 5–10 cm long, and 2–5 cm wide. The upper side of the leaf of this species is glossy dark green, whereas the lower side is paler and can range from glaucous to yellowish-green in color (Noshiro, 1993). *Ligustrum japonicum* grows under a wide range of environmental conditions, for example, Makino Memorial Foundation (2009) indicated that *L. japonicum* has the distribution from coastal to inland areas, and Noshiro (1994) and Nakajima and Yoshizaki (2018) reported that this species had grown in multiple coastal forests of a warm temperate zone in Southwest Japan, suggesting that this species could be used to compare morphological and anatomical differences between coastal and inland areas. However, the analyses in the limited coastal area could explain local adaptation of *L. japonicum*, because of its wide geographical distribution, and it is difficult to use this species to describe a common event to adapt to coastal areas. Thus, studies need to be conducted in multiple coastal regions and the results need to be compared; it could be considered as a common event if similar results were obtained in different areas. The results of our preliminary survey clarified that *L. japonicum* grew in coastal forests along the Pacific in the Shikoku and Kanto regions. Thus, the purpose of this study was to clarify the morphological changes in *L. japonicum* due to adaptation to the coastal area by comparing the populations of these two regions with inland controls.

2 Materials and Methods

Several plants in Japan exhibit geographical variations (Fujii et al., 2002; Iwasaki et al., 2010; Fukuda et al., 2011; Iwasaki et al., 2012; Sakaguchi et al., 2017); therefore, we analyzed the samples from Kochi and Kanagawa prefectures independently, because each prefecture belongs to different floristic divisions (Maekawa, 1977). All samples of *Ligustrum japonicum* examined in this study were collected from fields in Kochi and Kanagawa prefectures in the following definitions. In this study, the coastal area was defined as the area where *L. japonicum* grew with *Rhaphiolepis indica* (L.) Lindl. var. *umbellata* (Thunb.) H.Ohashi f. *ovata* (Briot) C.K.Schneid. (Rosaceae) in Kanagawa Pref. (Flora-Kanagawa Association, 2018) and with *Pittosporum tobira* (Thunb.) W.T.Aiton (Pittosporaceae) and *Eurya emarginata* (Thunb.) Makino (Ternstroemiaceae) in Kochi Pref. (Makino Memorial Foundation, 2009). Among them, Ugurushima, in Kochi Prefecture, is a small island with a circumference of only 6 km, located in the Bungo Channel on the Pacific Ocean between Kyushu and Shikoku at the westernmost tip of Kochi Prefecture. The coastal area on this island was also defined as a place where coastal forest component species. A total of 300 individuals (30 individuals per population) representing 10 populations were sampled, namely 3 (coastal: 1, inland: 2) in Kanagawa Prefecture, and 7 (coastal: 5, inland: 2) in Kochi Prefecture. These were collected from the forest margin. Those from coastal areas were collected at the margin of the coastal forest on the seaside. The collection

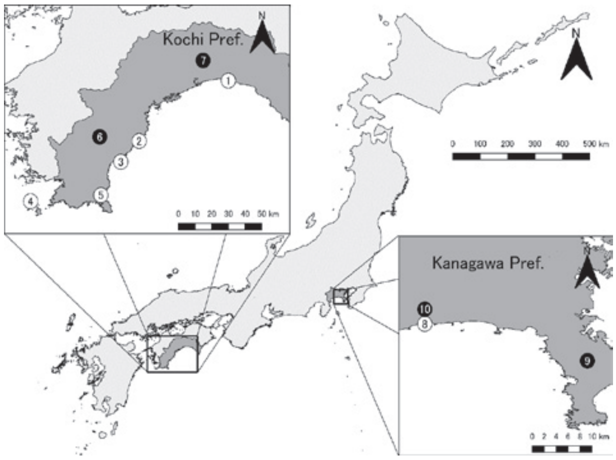

Figure 1: Sampling localities in this study. White and black circles indicate coastal and inland (control) populations, respectively. Number in circles corresponds to that given in Table 1.

locations are shown in Figure 1 and Table 1. In general, coastal environments are generally known to have higher wind velocities than inland environments (Shimizu et al., 1995). Our result of comparison of wind velocities in neighbor areas of our sampling localities (Japan Meteorological Agency) indicated that they were higher in coastal areas than those in inlands (Figure 2).

For morphological analysis, individuals were measured for the following continuous macro-morphological variables of leaves: 1) length and width of the leaf blade and 2) leaf thickness. Measurements were made using a digimatic calliper (CD-15CXR; Mitutoyo) and a digimatic outside micrometer (MDC-SB; Mitutoyo). Leaf measurements were taken from a fully expanded leaf at the midpoint of the plant height. Three leaves were selected randomly from one individual and the average value was calculated.

For anatomical analysis, fully expanded leaves were collected from each individual plant. To count the number of stomata on the blade, the abaxial surface of leaves was peeled off using Suzuki's Universal Micro-Printing (SUMP) method. The middle part of the blade along the midrib was examined to determine the number and size of the stomata. Replicas of each leaf (1 cm^2) were prepared to determine the stomatal density (number/mm^2) and to measure the stomatal size of five leaves per plant. Stomatal size was calculated using the following formula: stomatal length × stomatal width/2, based on the previous stomatal study of Kumekawa et al. (2013). These copied SUMP images were examined once for each individual using a light microscope (CX41; Olympus).

3 Results

We analyzed the leaf morphology of *Ligustrum japonicum.* Summaries of the measurements in Kochi and Kanagawa prefectures are listed in Tables 2 and 3, respectively. In the coastal areas of Kochi Prefecture (five populations: Tei, Okitsu, Inomisaki, Ugurushima and Ashizuri), leaf lengths were 56.84 ± 1.26, 52.85 ± 1.50, 49.65 ± 0.99, 57.78 ± 1.38 and 50.30 ± 1.14 mm, leaf

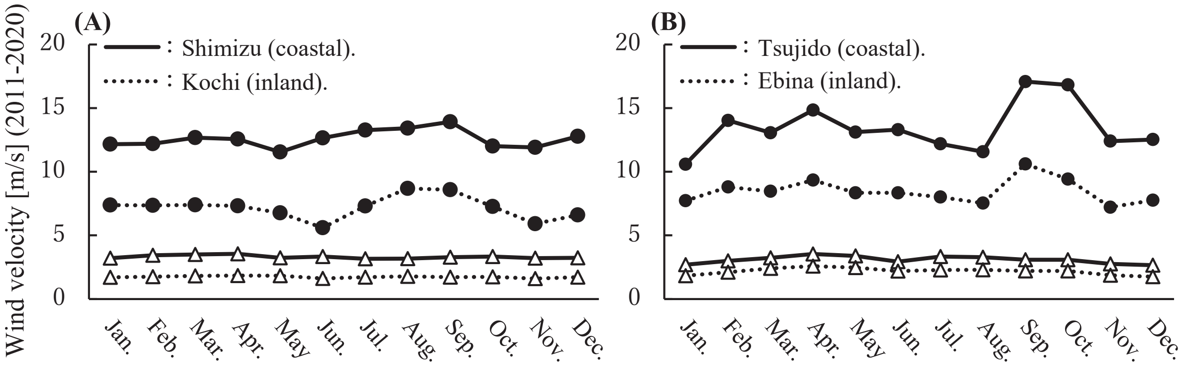

Figure 2: Comparisons of wind velocity (2011-2020) **(A)** between Shimizu (coastal) and Kochi (inland) in Kochi Pref. and **(B)** between Tsujido (coastal) and Ebina (inland) in Kanagawa Pref. Solid circles are average of maximum wind velocity, and open triangles are average of mean wind velocity.

Table 1: Sampling localities used in this study

Type	Locality name	number*	Locality	Latitude	longitude
Coastal					
	Tei	1	Tei, Yasu-cho, Konan City, Kochi Pref.	33°31'N	133°46'E
	Okitsu	2	Okitsu, Shimanto-cho, Takaoka-gun, Kochi Pref.	33°09'N	133°12'E
	Inomisaki	3	Ida, Kuroshio-cho, Hata-gun, Kochi Pref.	33°01'N	133°04'E
	Ugurushima	4	Ugurushima, Okinoshima-cho, Sukumo City, Kochi Pref.	32°47'N	132°29'E
	Ashizuri	5	Ohkinohama, Tosashimizu City, Kochi Pref.	32°49'N	132°57'E
	Sodegahama	8	Sodegahama, Hiratsuka City, Kanagawa Pref.	35°18'N	139°20'E
Inland (control)					
	Taisho	6	Eshi, Shimanto-cho, Takaoka-gun, Kochi Pref.	33°11'N	133°36'E
	Kuwanokawa	7	Naro, Nankoku City, Kochi Pref.	33°39'N	133°36'E
	Yokosuka	9	Hirasaku, Yokosuka City, Kanagawa Pref.	35°14'N	139°38'E
	Hiratsuka	10	Sengen-cho, Hiratsuka City, Kanagawa Pref.	35°20'N	139°20'E

*: locarity no. corresponds to that given in Figure 1.

Table 1: (Continued)

Type	Locality name	Distance to shoreline (m)	Elevation (m)
Coastal			
	Tei	61	18
	Okitsu	114	10
	Inomisaki	50	17
	Ugurushima	118	25
	Ashizuri	51	20
	Sodegahama	97	8
Inland (control)			
	Taisho	―	217
	Kuwanokawa	―	297
	Yokosuka	―	73
	Hiratsuka	―	9

widths were 31.22 ± 0.77, 29.05 ± 0.81, 28.45 ± 0.66, 31.27 ± 0.84 and 27.98 ± 0.62 mm, and leaf thicknesses were 243.35 ± 19.92, 287.25 ± 22.67, 244.00 ± 16.80, 238.58 ± 13.71 and 260.33 ± 18.83 μm, respectively. Inland areas (control) in Kochi Prefecture (two populations: Taisho and Kuwanokawa) had leaf lengths of 55.95 ± 1.53, and 52.83 ± 1.43 mm, leaf widths of 24.63 ± 0.82, and 25.09 ± 0.89 mm and leaf thicknesses of 184.65 ± 7.10, and 210.23 ± 21.08 μm, respectively. With the exception of Ashizuri, there were significant differences in leaf widths. Moreover, *L. japonicum* in the coastal areas tended to have thicker leaves. In Kanagawa Prefecture, the results of three populations (one coastal area [Sodegahama] and two control areas [Yokosuka and Hiratsuka]) showed that leaf lengths were 63.14 ± 1.83, 66.20 ± 1.65, and 60.06 ± 1.13 mm, leaf widths were 34.81 ± 0.82, 33.32 ± 0.96 and 35.14 ± 0.73 mm, and leaf thicknesses were 190.37 ± 7.91, 173.04 ± 7.90 and 247.10 ± 7.68 μm, respectively. No significant morphological differences were detected among leaf length, width, and thickness between the coastal and inland areas in Kanagawa Prefecture, except for leaf thickness in Hiratsuka.

We measured and calculated the mean stomatal size and density using SUMP samples from the coastal and inland areas of *L. japonicum* from all examined localities. Stomatal densities of the five coastal populations in Kochi Prefecture (142.77 ± 4.11, 180.41 ± 6.10, 228.06 ± 6.63, 162.82 ± 4.39 and 151.52 ±3.32 mm^{-2}) showed no significant difference from those of the inland populations (161.24 ± 5.11 and 174.63 ± 4.61 mm^{-2}). However, stomatal sizes (121.67 ± 3.16, 124.20 ± 2.47, 126.59 ± 2.10, 125.96 ± 3.09, and 132.27 ± 3.08 μm^2) of the coastal populations in Kochi Prefecture were significantly different from those of the inland populations (175.72 ±

Table 2: Morphological and anatomical measurements (average ± standard error) of *Ligustrum japonicum* in Kochi Pref.

	Coastal				
	Tei	Okitsu	Inomisaki	Ugurushima	Ashizuri
leaf					
length (mm)	56.84±1.26^{a}	52.85±1.50^{abc}	49.65±0.99^{c}	57.78±1.38^{a}	50.30±1.14^{bc}
width (mm)	31.22±0.77^{a}	29.05±0.81^{ab}	28.45±0.66^{ab}	31.27±0.84^{ab}	27.98±0.62^{bc}
thickness (μm)	243.35±19.92^{ab}	287.25±22.67^{a}	244.00±16.80^{ab}	238.58±13.71^{ab}	260.33±18.83^{ab}
stomata					
density (mm^{-2})	142.77±4.11^{c}	180.41±6.10^{b}	228.06±6.63^{a}	162.82±4.39^{bc}	151.52±3.32^{c}
size (μm^{2})	121.67±3.16^{b}	124.20±2.47^{b}	126.59±2.10^{b}	125.96±3.09^{b}	132.27±3.08^{b}

Columns marked by different letters differ significantly according to the Tukey's HSD test ($p < 0.05$).

Table 2: (Continued)

	Inland (control)	
	Taisho	Kuwanokawa
leaf		
length (mm)	55.95±1.53^{abc}	52.83±1.43^{abc}
width (mm)	24.63±0.82^{c}	25.09±0.89^{c}
thickness (μm)	184.65±7.10^{b}	210.23±21.08^{ab}
stomata		
density (mm^{-2})	161.24±5.11^{bc}	174.63±4.61^{b}
size (μm^{2})	175.72±2.67^{a}	177.33±3.75^{a}

2.67 and 177.33 ± 3.75 μm^2). The size of each stomata is shown in Figure 3. In Kanagawa Prefecture, the stomatal densities were 187.29 ± 3.51 (Sodegahama), 183.83 ± 3.74 (Yokosuka) and 202.05 ± 2.50 mm^{-2} (Hiratsuka) and the stomatal density of Hiratsuka was significantly higher than the other populations. The stomatal size (130.61 ± 2.61 μm^2 [Sodegahama]) of the coastal region was significantly smaller than the inland populations (174.33 ± 3.85 [Yokosuka] and 148.72 ± 1.60 μm^2 [Hiratsuka]). These morphological and anatomical results indicated that the smaller stomatal size of *L. japonicum* in the coastal areas was common between Kochi and Kanagawa prefectures compared to the inland areas.

4 Discussion

Our results indicated that the stomatal size of *Ligustrum japonicum* in the coastal areas of both Kanagawa and Kochi prefectures was significantly smaller than those of their inland areas, and our anatomical results of similar stomatal size were observed in different regions, suggesting a common event for this species to adapt into coastal areas. Moreover, we consider that the stomatal size had changed according to the coastal environments, because Frankes et al. (2009) indicated that such stomatal changes were likely due to plasticity.

The total stomatal area on the leaf surface is extremely small, and plants maintain an appropriate balance between mesophyll photosynthetic CO_2 demands and water loss (Drake et al., 2013). In particular, stomatal control of water loss allows plants to occupy habitats with

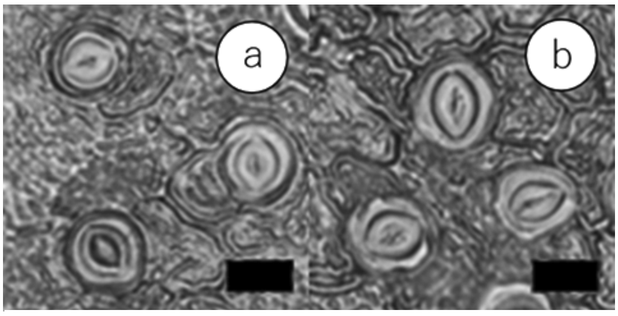

Figure 3: Suzuki's Universal Micro-Printing method (SUMP) replicas of (a) coastal (Ugurushima) and (b) inland (control) (Kuwanokawa) samples of *Ligustrum japonicum*. Bar = 20 μm.

fluctuating environmental conditions (Hetherington and Woodward, 2003). Therefore, the alteration in stomatal density or size is considered to be a key factor that influences the adaptation of plants to different environments. Of these, stomatal density has been known to vary significantly between and within species, as well as being influenced by the growth environment (Weyers and Lawson, 1997), and this has also been widely studied in a paleontological context to reconstruct past changes in atmospheric composition (Barclay et al., 2010; Franks et al., 2015; Steinthorsdottir et al., 2016). Due to the fact that the stomatal density increases in an environment where abundant water can be accessed, such as along a river, and decreases in dry environments such as serpentine areas (Hayakawa et al., 2012; Yamada et al., 2011; Ohga et al., 2012a; Ohga et al., 2012b; Ueda et al., 2012; Matsui et al., 2013), it is considered that stomatal density is likely to vary in different environments. Furthermore, the coastal endemic species *Aster hispidus* var. *insularis*, and the coastal ecotype *Adenophora triphylla* var. *japonica* adapted by decreasing the density of stomata to reduce water loss (Kumekawa et al., 2013; Ohga et al., 2013). However, the results of this study indicated that the coastal populations of *L. japonicum* had smaller stomatal size without difference in stomatal density in both coastal regions. Thus, the results of our study provide insight into whether or not the smaller stomatal size of *L. japonicum* is an adaptation in coastal areas.

It is uncertain why our results on stomatal size and

Table 3: Morphological and anatomical measurements (average ± standard error) of *Ligustrum japonicum* in Kanagawa Pref.

	Coastal	Inland (control)	
	Sodegahama	Yokosuka	Hiratsuka
leaf			
length (mm)	63.14±1.83[ab]	66.20±1.65[a]	60.06±1.13[b]
width (mm)	34.81±0.82[a]	33.32±0.96[a]	35.14±0.73[a]
thickness (μm)	190.37±7.91[b]	173.04±7.90[b]	247.10±7.68[a]
stomata			
density (mm^{-2})	187.29±3.51[b]	183.83±3.74[b]	202.05±2.50[a]
size (μm^2)	130.61±2.61[c]	174.33±3.85[a]	148.72±1.60[b]

Columns marked by different letters differ significantly according to the Tukey's HSD test ($p < 0.05$).

density differed from those of previous studies in coastal areas despite the presence of coastal environments. The main cause of such stomatal changes in *L. japonicum* could be attributed to difference between coastal and inland environments. In general, winds along the coast are stronger than those of inland areas, and some studies have been conducted on the mechanical properties of coastal trees, such as protection against coastal winds and shock absorption (Meguro and Miyawaki, 1994; Meguro et al., 1994). The increased movement of the air around a plant results in a higher transpiration rate (Ting and Loomis, 1964), and sudden strong winds from the sea could lead to excessive transpiration rates and evaporation of water from coastal plants (Greenway and Manns, 1980), but smaller stomata can respond faster to environmental changes (Drake et al., 2013). Thus, small stomata were adaptive to avoid excessive transpiration and evaporation in coastal environments, but gas exchange through small stomata was also decreased, which could lead to reduced photosynthetic efficiency, suggesting that decreasing transpiration and evaporation of *L. japonicum* in coastal areas was at the expense of photosynthetic efficiency. Therefore, the smaller stomatal size of *L. japonicum* may not always be the best adaptive strategy for colonizing coastal areas. In this case, however, decreasing stomatal density was considered to be effective in avoiding excessive transpiration and evaporation. In fact, *As. hispidus* var. *insularis*, and the coastal ecotype *Ad. triphylla* var. *japonica* had significantly reduced stomatal densities (Kumekawa et al., 2013; Ohga et al., 2013), and the former also had leaf hairs on the abaxial side to avoid wind hitting the stomata directly so as to cause less of a difference in air pressure between the inside and outside of the stomata (Sunami et al., 2013). These changes seemed to be more effective in coastal areas than in our study. Interestingly, they were herbaceous plants with limited capacity for water retention, and *L. japonicum* could retain water in the trunk under dry conditions. The clarify the stomatal relationship between trees and herbaceous plants, further analyses need to conduct to use *L. japonicum* collected from Southern areas such as Kyushu and Ryukyu archipelagos. In addition, the adaptation process against other coastal stresses such as wind-blown sand and salt spray of this species should be analyzed in the future.

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