

Stomatal Study of Introduced Species, *Ligustrum lucidum* Aiton (Oleaceae), in Coastal Areas of Japan

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Abstract

Alien, introduced, and non-native species expand their distribution through the development of various transportation networks. In Japan, *Ligustrum lucidum* Aiton (Oleaceae) was introduced in the mid-1800s for use as roadside trees, but has escaped from the planting area and spread everywhere. This species also invades coastal areas and has adapted to develop its characteristic features under various stress conditions. To clarify the adaptive features of *L. lucidum* in coastal areas, we conducted morphological and anatomical analyses. We found that the stomatal size of *L. lucidum* was smaller in the coastal populations than in inland populations, which is similar to the coastal adaptation pattern of *L. japonicum* Thunb., a closely related native species. Our results suggest that strong selective pressure on coastal area conditions could force their leaves to avoid excessive transpiration, and such an adaptation pattern of *L. lucidum* could expand its distribution to various coastal areas.

Keywords: adaptation, coastal areas, introduced species, leaf, *Ligustrum lucidum*, stomata

1. Introduction

The number of non-native species introduced worldwide has more than doubled compared with estimates from approximately three decades ago (Williamson & Fitter, 1996a, b). This is predominantly due to the growth in global trade and human mobility (Sala et al., 2000). Biological invasions are considered a major environmental issue of public concern. The introduction of a non-native species into an ecosystem is likely to present an ecological risk if the species can integrate successfully into the ecosystem (Gozlan & Newton, 2009). This can result in interactions that are detrimental to native species or even cause dysfunction of the whole ecosystem (Mack et al., 2000; Pejchar & Mooney, 2009; Bellard et al., 2016). These invasions are on the rise and human modification of ecosystems may provide them with new niche spaces (Tilman & Lehman, 2001; Inouye, 2008; Blois et al., 2013; Harte et al., 2015). When dispersed into new environments, introduced species can thrive by filling vacant niches or by outperforming native plants (Davis & Pelsor, 2001; Daehler, 2003). For example, the Japanese knotweed (*Fallopia japonica* (Houtt.) Ronse Decr. (Polygonaceae)), one of the world's 100 most invasive species (Roy et al., 2018), is particularly invasive throughout North America, Europe, Australia, and New Zealand (Nentwig et al., 2018), and exists as a female clone in the United Kingdom from a single introduction (Bailey & Conolly, 2000; Hollingsworth & Bailey, 2000). Kudzu (*Pueraria montana* (Lour.) Merr. (Fabaceae)) is an invasive leguminous vine native to Asia, and the promotion of the vine as a forage crop and soil stabilization agent during the early 20th century facilitates its firm establishment across the southeastern part of the United States (Forseth & Innis, 2004). In Japan, introduced species invade many sites because of increasing human activities, rapidly increasing after the latter half of the nineteenth century; today, about one-quarter of vascular plants are alien and naturalized species (Shimizu & Konta, 2003). There has been a marked increase in the problems arising from the accidental or deliberate escape of introduced plant species into the wild; introduced species such as *Festuca arundinacea* (Schreb.) Darbysh. (Poaceae), *Pyracantha angustifolia* M. Roem. (Rosaceae), and *Trifolium repens* L. (Fabaceae) can affect ecosystems via competition for pollinators, hybridization, reduction of native species, habitat modification, and transmission of novel diseases (Ishida et al., 1998; Nakatsubo, 1998; Okubo & Oka, 2005). There are many such examples, some of which cause serious

consequences for biodiversity conservation (Yoshioka, 2005).

Ligustrum lucidum Aiton belongs to the Oleaceae family and is an evergreen shrub that reaches approximately 8 m in height (Noshiro, 1993). This species is native to central and southern China and has been introduced in not only Japan but also in southern North America and Australia (Uyemura et al., 2010). In Japan, this species was introduced in the mid-1800s and was used as a roadside tree because of its resistance to air pollution. It has also been used as a garden shrub and planted in many parks and schools (Yoshinaga & Kameyama, 2001; Ito & Fujiwara, 2007). Recently, there have been increasing problems from the accidental or deliberate escape of introduced plant species into the wild, and *L. lucidum* has escaped from the planting area of this species and spread into urban areas (Yoshinaga & Kameyama, 2001), riverbeds (Hashimoto et al., 2003) and landfills (Iijima & Sago, 2006). Furthermore, *L. lucidum* reportedly has many seedlings of this species in forest communities and urban areas (Ishida et al., 1998; Fujita & Shinohara, 2001), suggesting the possibility of colonies of this species in the future. *L. lucidum* has been reported to grow sympatrically with the native *L. japonicum* Thunb. (Hashimoto et al., 2003; Kameyama et al., 2012; Saito et al., 2012) and could pose a considerable ecological threat through not only direct competition but also hybridization with this native species (Yoshinaga & Kameyama, 2001), but Saito et al. (2012) indicated that natural hybridization between them could not be detected in the field based on PCR-RFLP analyses using chloroplast and nuclear DNA. This result was supported by genetic studies using different sympatric populations (Kameyama et al., 2012), however, Kameyama et al. (2012) indicated that a small number of seeds were produced by artificial experimental crosses between *L. lucidum* and *L. japonicum*, suggesting that the difference in flowering phenology between both species prevents natural hybridization in the field, and both species produce hybrids when flowering time is shifted.

Nakajima and Yoshizaki (2016) reported the occurrence of *L. lucidum* in the coastal forests of the Chubu district of Japan, and we confirmed its occurrence in different coastal forests in the Kanto district (Figure 1). The occurrence of *L. lucidum* in these two different coastal forests suggests that this species has invaded various coastal areas in Japan. Coastal forests consist of species that are well-adapted to withstand the stresses of high salt spray exposure, wind damage, and limited fresh water, and various studies have been conducted to understand these stresses (Nakajima & Yoshizaki, 2010; Ito & Yoshizaki, 2017; Ito & Yoshizaki, 2019). Natural selection promotes the adaptation of plants for survival and acts on their resulting phenotypes (Bartels & Sunkar, 2005), and such characteristic environments have allowed various endemic species, such as *Quercus phillyreoides* A. Gray (Fagaceae), *Pittosporum tobira* (Thunb.) W. T. Aiton (Pittosporaceae), *Daphniphyllum teijsmannii* Zoll. ex Kurz, and (Daphniphyllaceae), to evolve in coastal areas. Moreover, many plants in inland areas cannot easily invade coastal areas; therefore, characteristic flora is formed in these areas (Flowers et al., 1977; Médail & Quézel, 1997; Khan et al., 2002). However, some studies have reported that plants with a wide distribution from inland to coastal areas have undergone various morphological and anatomical changes to adapt to coastal areas. For example, Tunala et al. (2012) indicated that epidermal cells in the coastal variety of *Aster hispidus* Thunb. var. *insularis* (Makino) Okuyama (Asteraceae) were larger in size but fewer in number than those in *As. hispidus* var. *hispidus*, and were involved in succulent leaves to store water. Sunami et al. (2013) reported that leaf hair on the abaxial side of leaves was correlated with the stomatal density of *As. hispidus* var. *insularis* and the less hair on the leaf, the lower the stomatal density to avoid transpirational water loss. Ohga et al. (2013) suggested that the coastal population of *Adenophora triphylla* (Thunb.) A. DC. var. *japonica* (Regel) H. Hara (Campanulaceae) has evolved relatively thick leaves via a heterochronic process to store water. Shiba et al. (2022b) indicated that the coastal population of *Eurya japonica* Thunb. (Ternstroemiaceae) had smaller stomata and larger epidermal cells on the adaxial and abaxial sides to reduce transpiration during gas exchange and to retain water in the leaves, respectively. Thus, although coastal areas can only be invaded by species with morphological and anatomical traits that could avoid environmental stresses there, it is surprising and interesting that *L. lucidum* have grown in large numbers in coastal areas in a short period o after its introduction in Japan. Therefore, a better understanding of the invasive ability and future invasive potential of *L. lucidum* can be achieved through morphological and anatomical analysis.

Recently, Takizawa et al. (2022) suggested, based on morphological and anatomical analyses using inland and coastal populations, that the coastal ecotype of *L. japonicum* was accompanied by a reduction in stomatal size. As *L. japonicum* is closely related to *L. lucidum* based on molecular phylogenesis (Saito et al., 2012), the adaptation process of *L. lucidum* to coastal areas is concerning. Some studies have indicated that patterns of morphological and anatomical changes were similar between the invasive plants and the closely related native species (Davidson et al., 2011; Palacio-López & Gianoli, 2011) and that *L. lucidum* adapted to coastal areas with similar morphological and anatomical changes as the coastal ecotype of *L. japonicum*. However, it is doubtful whether *L. lucidum* has invaded coastal areas through the same process as the adaptation pattern of *L. japonicum*,

even though only 150 years have passed since the introduction of *L. lucidum* in Japan. The short-term coastal adaptation of *L. lucidum* may have acquired a more specialized form in coastal environments than that of *L. japonicum*. Therefore, it is important to reveal the morphological and anatomical adaptation processes of *L. lucidum* to clarify the factors that enable introduced species to expand their distribution into coastal areas. This study aimed to investigate the adaptive leaf traits of *L. lucidum* by comparing individuals from coastal and inland areas using morphological and anatomical approaches.



Figure 1. *Ligustrum lucidum* Aiton (Oleaceae)

(A1) coastal forests in Chigasaki, (A2) coastal (Chigasaki), (B) inland (Shimomiyata).

2. Materials and Methods

All *L. lucidum* samples examined in this study were collected from the field. The collection locations are shown in Figure 2 and Table 1.

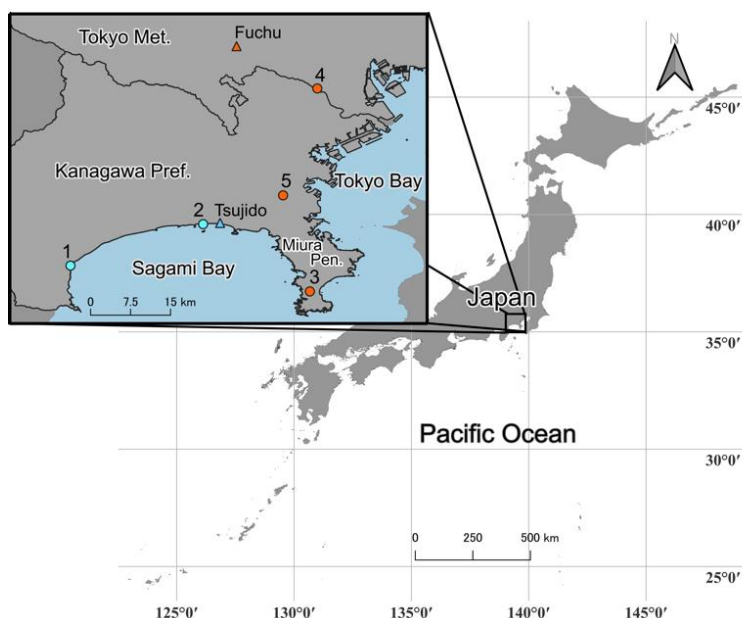


Figure 2. Sampling localities in this study

Blue and red circles indicate coastal and inland (control) populations, respectively. Number in circles corresponds to that given in Table 1. Blue and red triangles indicate the locations of coastal (Tsujido) and inland (Fuchu) meteorological instruments. Wind velocity data from meteorological observations were shown in Figure 3.

The inland populations near each coastal area were used as controls. A total of 150 individuals (30 individuals per population) representing five populations involved in two coastal areas and three populations were sampled. *L. lucidum* had been grown together with the following coastal endemic species: *Pittosporum tobira* (Thunb.) W. T. Aiton (Pittosporaceae) and *Eurya emarginata* (Thunb.) Makino (Ternstroemiaceae) in coastal forests.

Moreover, according to the Japan Meteorological Agency, our coastal sampling areas showed stronger winds than inland areas (Figure 3).

For morphological analysis, individual plants 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 caliper (CD-15CXR; Mitutoyo) and digimatic outside micrometer (MDC-SB; Mitutoyo) based on a previous study by Shiba et al. (2021). Leaf measurements were obtained from a fully expanded leaf at the middle of the total plant height. Three leaves were randomly selected from each individual, and their average values were calculated.

Table 1. Sampling localities in this study

Type	Locality name and number*	Locality	Latitude and longitude	
Coastal				
	Odawara 1	Hayakawa, Odawara City, Kanagawa Pref.	35°23'N	139°14'E
	Chigasaki 2	Higashikaiganminami, Chigasaki City, Kanagawa Pref.	35°31'N	139°42'E
Inland (control)				
	Shimomiyata 3	Shimomiyata, Hassemachi, Miura City, Kanagawa Pref.	35°18'N	139°64'E
	Tamazutsumi 4	Tamazutsumi, Setagaya-ku, Tokyo Pref.	35°60'N	139°65'E
	Konandai 5	Konandai, Konan-ku, Yokohama City, Kanagawa Pref.	35°38'N	139°58'E

* : locality number corresponds to that given in Figure 2.

Table 1. (Continued)

Type	Locality name	Distance to shoreline (m)	Elevation (m)
Coastal			
	Odawara	93	41
	Chigasaki	180	5
Inland(control)			
	Shimomiyata	—	11
	Tamazutsumi	—	10
	Konandai	—	34

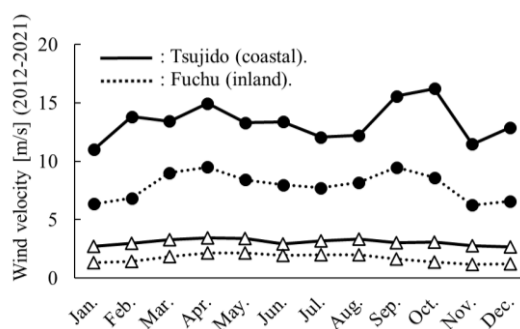
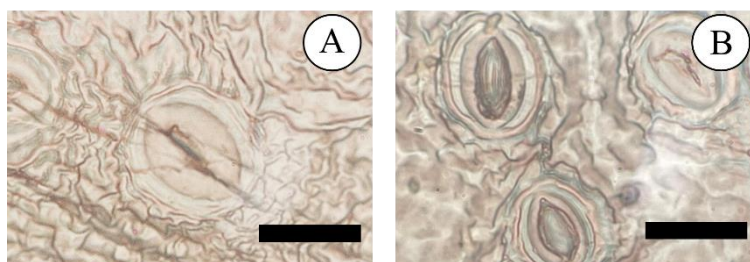


Figure 3. Comparison of wind velocity in coastal and inland areas

Comparisons of wind velocity (2012-2021) between Tsujido (coastal) in Kanagawa Pref. and Fuchu (inland) in Tokyo Met. Locality of Tsujido and Fuchu corresponds to that given in Figure 2. Solid circles are average of maximum wind velocity, and open triangles are average of mean wind velocity.

Fully expanded leaves were collected from each individual for anatomical analysis. The middle part of the blade along the midrib was analyzed to determine the number and size of the stomata. A Dino-Lite AF4515 Digital Microscope (ANMO Electronics Corporation, Taiwan) was used to photograph the dissection of each leaf (Figure 4). To count the number of stomata and measure stomatal size, we used the DinoCapture 2.0 software (ANMO Electronics Corporation, Taiwan).

Figure 4. Stomata of *Ligustrum lucidum*

Suzuki's Universal Micro-Printing method (SUMP) replicas of (A) coastal (Chigasaki) and (B) inland (Tamazutsumi) samples (Kijima, 1962). Bar = 20 μm .

3. Results

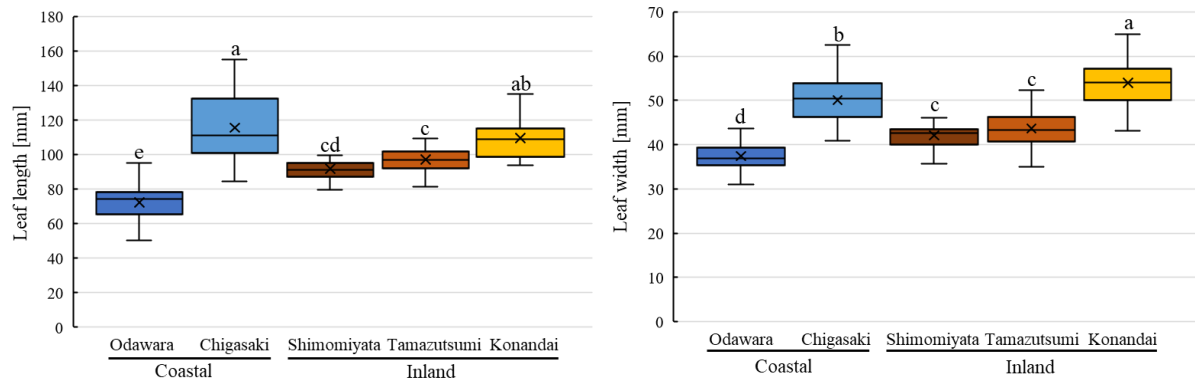
We analyzed the leaf morphology of *Ligustrum lucidum*. A summary of the measurements is presented in Table 2. In the coastal areas (two populations: Odawara and Chigasaki), leaf lengths were 72.23 ± 2.06 and 115.56 ± 3.45 mm (Figure 5), leaf widths were 37.39 ± 0.67 and 50.10 ± 0.99 mm (Figure 5), and leaf thickness were 128.87 ± 3.95 and 201.67 ± 6.59 μm (Figure 6), respectively. In the inland (control) areas (three populations: Shimomiyata, Tamazutsumi, and Konandai), leaf lengths were 91.75 ± 1.43 , 97.04 ± 1.43 (Figure 5), and 109.57 ± 2.11 mm, leaf widths were 42.08 ± 0.65 , 43.69 ± 0.73 , and 53.92 ± 1.05 mm (Figure 5), and leaf thickness were 234.72 ± 9.52 , 152.87 ± 7.21 , and 160.46 ± 3.10 μm (Figure 6), respectively. There were no significant morphological differences between the coastal and inland areas.

Table 2. Morphological and anatomical measurements (average \pm standard error) of *Ligustrum lucidum* in Kanagawa Pref.

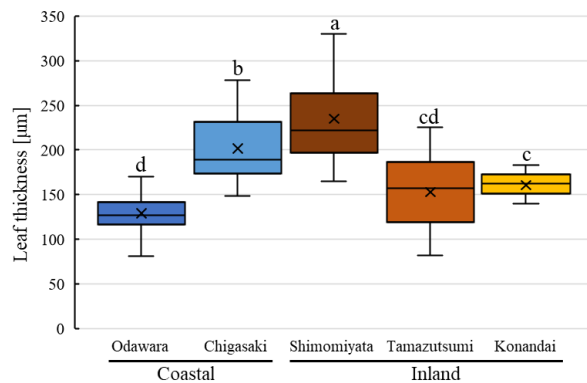
	Coastal		Inland (control)		
	Odawara	Chigasaki	Shimomiyata	Tamazutsumi	Konandai
leaf					
length (mm)	72.23 ± 2.06^e	115.56 ± 3.45^a	91.75 ± 1.43^{cd}	97.04 ± 1.43^c	109.57 ± 2.11^{ab}
width (mm)	37.39 ± 0.67^d	50.10 ± 0.99^b	42.08 ± 0.65^c	43.69 ± 0.73^c	53.92 ± 1.05^a
thickness (μm)	128.87 ± 3.95^d	201.67 ± 6.59^b	234.72 ± 9.52^a	152.87 ± 7.21^{cd}	160.46 ± 3.10^c
stomata					
density (N/mm^2)	235.29 ± 3.00^b	217.28 ± 3.31^c	240.15 ± 2.96^b	197.36 ± 3.14^d	253.64 ± 2.99^a
size (μm^2)	310.82 ± 3.03^e	484.38 ± 4.95^d	639.52 ± 6.06^a	589.98 ± 6.16^b	513.36 ± 4.96^c

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

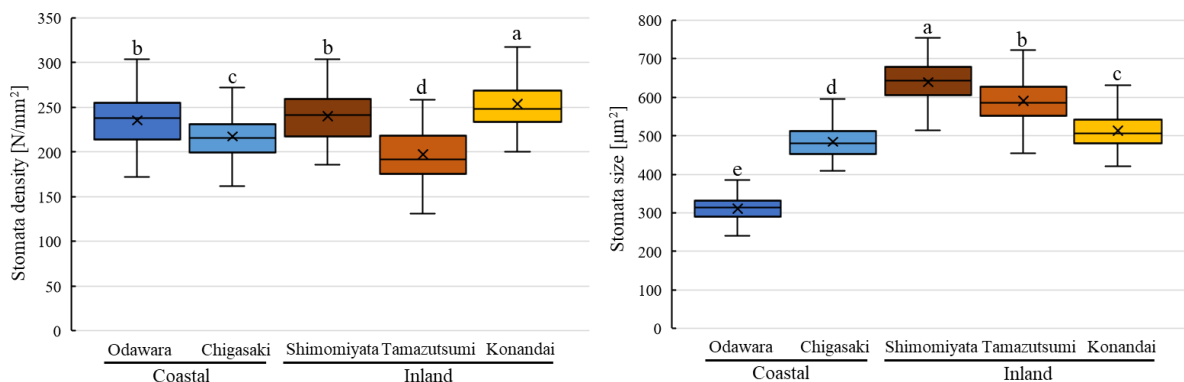
We measured and calculated the mean stomatal size and density of *L. lucidum* from all examined localities. The stomatal density of the leaves from Odawara and Chigasaki in coastal populations (235.29 ± 3.00 , and 217.28 ± 3.31 N/mm^2) were not considerably different from those of Shimomiyata, Tamazutsumi, and Konandai in inland populations (240.15 ± 2.96 , 197.36 ± 3.14 , and 253.64 ± 2.99 N/mm^2) (Figure 7). However, the stomatal size (310.82 ± 3.03 and 484.38 ± 4.95 μm^2) of the leaves from the coastal populations were significantly different from those of the inland populations (639.52 ± 6.06 , 589.98 ± 6.16 , and 513.36 ± 4.96 μm^2) (Figure 7). These morphological and anatomical results showed that the reduction in the stomatal size of *L. lucidum* was a common factor in coastal areas.

Figure 5. Leaf length and width of *Ligustrum lucidum*

Boxplots of leaf length and width measurements corresponding to Table 2.

Figure 6. Leaf thickness of *Ligustrum lucidum*

Boxplots of leaf thickness measurements corresponding to Table 2.

Figure 7. Stomata density and size of *Ligustrum lucidum*

Boxplots of stomata density and size measurements corresponding to Table 2.

4. Discussion

Stomata balance gas exchange between the leaf and the atmosphere to maximize CO₂ uptake for photosynthetic carbon assimilation and to minimize water loss through transpiration. Kume-kawa et al. (2013) showed that stomatal size and density adapt to various environments. Our results showed that the stomatal size of the coastal populations of *L. lucidum* was smaller than those of the inland populations, indicating that this species

experienced stomatal size reduction to adapt to coastal areas. Moreover, it was interesting that the process of adaptation of the introduced species, *L. lucidum*, to the coastal areas was similar to that of the native species, *L. japonicum*, supporting Davidson et al. (2011) and Palacio-López and Gianoli (2011), who claimed that introduced species show similar adaptation patterns to closely related native species. However, which coastal stresses led to common stomatal changes in *L. lucidum* and *L. japonicum* remains unknown. A recent analysis of many accessions in the model plant *Arabidopsis* described significant correlations between stomatal size and water use efficiency (Dittberner et al., 2018), indicating that stomatal size is an adaptive trait that contributes to the optimization of water stress. Coastal plants suffer from dehydration caused by wind, intense sunlight with diffused reflection, and sand-loaded salt spray, with salt spray being specific to coastal environments (Boyce, 1954); therefore, the transpiration of their water reserves must be minimized. According to the Japan Meteorological Agency, and as shown in Figure 3, the wind velocity near the coastal area where *L. lucidum* was growing was faster than at inland, and the former received greater stress from the wind than the latter. Such stresses may have contributed to the reduction in the stomatal size of *L. lucidum* and *L. japonicum* in coastal areas, such that they exhibited similar adaptation patterns in coastal areas. In the future, there is a risk that such adaptation patterns of *L. lucidum* will enable the expansion of its distribution into various coastal areas, and therefore, further investigation and careful management plans of the ecosystem in Japan are required to achieve conservation.

Whether the variation in stomatal size occurred due to genetic factors or plasticity, that is, growth under different environmental conditions remains unclear. In general, a species can be constrained by selective pressures acting in their particular native climate; therefore, species exposed to more environmental variation in their distribution range are reported to have more phenotypic plasticity than those exposed to restricted environmental variation in their ranges (Valladares et al., 2006; Valladares et al., 2014). For example, Franks et al. (2009) concluded that stomatal size was a plastic variation because *Eucalyptus* species (Myrtaceae) had different stomatal sizes in various environments. Moreover, the stomatal size of potato (*Solanum tuberosum* L.: Solanaceae) plants has also been observed in plastic developmental responses to environmental changes (Sun et al., 2014). Plant traits that allow introduced species to become successful invaders remain a challenging question in conservation ecology (van Kleunen et al., 2010), and previous studies have emphasized how high phenotypic plasticity in specific functional traits in introduced species is important for exhibiting favorable phenotypes in a wide range of environments (Richards et al., 2006; Davidson et al., 2011). Moreover, these are considered potential factors that facilitate the successful colonization of different environments (Matesanz et al., 2010). Godoy et al. (2012) reported that morphological and physiological leaf traits are usually associated with invasiveness. Moreover, the leaves of introduced species compared to the native seem to have a high plastic response to light levels (Yamashita et al., 2000). Nascimento et al. (2015) suggested that functional traits that play critical roles in survival and development are susceptible to change and adapt to different environments. Considering these studies, *L. lucidum* has invaded coastal areas by changing stomatal size, suggesting that *L. lucidum* may have the capacity for acclimation owing to phenotypic plasticity. The question of how *L. lucidum* maintains phenotypic plasticity in stomatal size remains. Bradshaw (1965) indicated that phenotypic plasticity can be explained as a change in the phenotypic expression of a genotype in response to environmental factors. Stomatal anatomical traits have been determined during organ growth when stomata are gradually formed across the developing epidermis (Geisler & Sack, 2002; de Marcos et al., 2016) under the influence of internal and environmental factors (Casson & Hetherington, 2010; Qi & Torii, 2018). The developmental process of stomata has been extensively studied in *A. thaliana* (Ohashi-Ito & Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007). Based on the results from other model plants, three key transcription factors mediate the sequential steps of stomatal development, and their functions are conserved in land plants (Liu et al., 2009; Ran et al., 2013; Raissig et al., 2016; Ortega et al., 2019; Wang et al., 2019; Harris et al., 2020). Recently, Doll et al. (2021) indicated that the different expression times of two of the three transcription factors have a strong influence on the different division patterns of meristemoids, suggesting that the difference in the expression time of these transcription factors may influence the stomatal size of *L. lucidum*. In the future, isolation and comparative expression analyses of homologues of *SPEECHLESS* and *MUTE* from *L. lucidum* will reveal factors in the adaptation of *L. lucidum* to coastal areas.

In this study, we indicated that *L. lucidum* had invaded into the coastal forest by changing smaller stomatal size (Tables 1, 2 and Figure 7). But we could not find this species in the shoreline side of coastal forests because Nakajima and Yoshizaki (2016) suggested that the shoreline side of the coastal forest had the highest selective pressure owing to wind and soil conditions, and these environmental effects decreased from the shoreline side to inland. Therefore, *L. lucidum* could grow in coastal forests by varying its stomatal size, but this stomatal variation alone would not enable it to grow along the shoreline side of coastal forests. Nakajima and Yoshizaki

(2016) reported that *Cinnamomum yabunikkei* H. Ohba (Lauraceae) and *Ilex integra* Thunb. (Aquifoliaceae) grew widely from the shoreline to the inland side of the coastal forest, where *L. lucidum* has been reported. These plants may have hidden morphological and anatomical characteristics that allow them to grow close to the coastline of the coastal forest. Therefore, further morphological and anatomical comparisons of the shoreline and inland populations may reveal adaptation traits on the shoreline side of coastal forests.

Recently, Shiba et al. (2022a) reported that *L. japonicum* can be grown on serpentine soils. Serpentine soils occur in small patches along fault lines where igneous rocks appear and are characterized by a suite of challenging crucial abiotic factors, such as low calcium-to-magnesium ratio, high heavy metal concentrations, nutrient deficiency, and low moisture retention, leading to sharp transitions in abiotic conditions at the boundaries of serpentine patches (Brady et al., 2005). Adaptation to edaphic factors has long been considered an important component in plant distribution, diversification, and speciation (de la Vega, 1996; Shiba et al., 2022c). Serpentine soils provide an exceptional system for studying the edaphic adaptation of plants (Kruckeberg, 1951, 1954; Rajakaruna et al., 2003; Wright et al., 2006, 2009). Serpentine soil mosaics exist in several areas of Japan, and many endemic species occur in each serpentine area, including *Aster hispidus* Thunb. var. *leptocladus* (Makino) Okuyama, *Hypericum tosaense* Makino, and *Saussurea nipponica* Miq. ssp. *yoshinagae* Kitam. (Toyokuni, 1955; Hayakawa et al., 2012). Interestingly, the adaptation of *L. japonicum* to serpentine areas showed a reduction in stomatal size (Shiba et al., 2022a), suggesting that this stomatal variation pattern was similar to the adaptation of *L. japonicum* to coastal areas (Takizawa et al., 2022). Therefore, it is possible that *L. lucidum* also adapted to and invaded serpentine areas with similar anatomical change patterns as *L. japonicum*; however, there has been no report of *L. lucidum* growth in serpentine areas.

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