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Serpentine Adaptation of *Ligustrum japonicum* Thunb. (Oleaceae) Based on Morphological and Anatomical Approaches

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Abstract

Serpentine soils consist of broadly skewed elemental profiles, including abundant toxic metals and low nutrient content in drought-prone, patchily distributed substrates; therefore, they are one of the most challenging settings for plant life. In this study, a comparative study was conducted using serpentine and inland populations of *Ligustrum japonicum* Thunb. (Oleaceae) to determine morphological and anatomical differences between the same species growing in the serpentine and inland areas. Longitudinal leaf sections indicated that serpentine populations had slightly thicker leaves than inland populations, contributing to the increased heights of adaxial and abaxial epidermal cells and palisade and spongy tissues. Moreover, the serpentine population had smaller stomata than the inland populations. These results suggest that the strong selective pressure under serpentine soil conditions could force leaves to restore water and avoid excessive transpiration.

Keywords: adaptation, anatomy, *Ligustrum japonicum*, morphology, serpentine area

1. Introduction

Evidence from systematic investigations of various plants has demonstrated that extreme and rapid divergence in morphological characteristics has occurred in association with ecological shifts to different environments. One such instance was observed in an environment with characteristic soil conditions. Adaptation to edaphic factors, such as physical, chemical, and biological characteristics of soil, has long been considered an important component of plant distribution, diversification, and speciation. Of these edaphic factors, serpentine soils provide an exceptional system for studying the edaphic adaptation of plants because adaptation is widespread both geographically and phylogenetically and involves similar tolerance mechanisms to unique edaphic factors (Kruckeberg, 1951, 1954; Rajakaruna et al., 2003; Wright et al., 2006). This environment is characterized by challenging crucial abiotic factors, at the boundaries of serpentine patches (Berglund et al., 2004; Sambatti & Rice, 2006; Arnold et al., 2016). These various chemical and physical characteristics together have been termed the serpentine syndrome, resulting in very low ecosystem productivity, low competition, and frequent endemism (Kruckeberg, 1951, 1954; Whittaker, 1954; Brady et al., 2005). In particular, drought, due to the low water-holding capacity of serpentine soils, could interact with major chemical factors contributing to local adaptation to specific stresses (Eskandari et al., 2017).

Serpentine soil mosaics are found in several areas of Japan. These areas are noteworthy due to the occurrence of many endemic species. Some species seem to adapt to the edaphic factors of serpentine soils and are scattered from east to west of Kochi Prefecture in Shikoku District of Japan. Endemic species such as *Aster hispidus* Thunb. ssp. *leptocladus* (Makino) Okuyama (Asteraceae), *Hypericum tosaense* Makino (Clusiaceae), and *Saussurea nipponica* Miq. ssp. *yoshinagae* Kitam. (Asteraceae) are known to occur in this area (Fujikawa, 2006). These endemic species need to possess some aspects of adaptation to this environment involving chemical components reflecting unique edaphic factors. There is a constant attraction for this area among researchers, and several studies have contributed to the evaluation of morphological and anatomical characteristics of native plants under serpentine soil conditions, especially in Japan. For example, Hayakawa et al. (2012) indicated that the narrow leaf of the serpentine-endemic *Aster hispidus* Thunb. var. *leptocladus* (Makino) Okuyama (Asteraceae) translates to fewer epidermal cells in the leaves of its closely related variant, *As. hispidus* var. *hispidus*. Moreover, Kumekawa et al. (2013) indicated that the serpentine-adapted variety *As. hispidus* var. *leptocladus* had a lower density of stomata than the inland *As. hispidus* var. *hispidus*. Aside from *As. hispidus* var. *leptocladus*, Ohga et al. (2012) reported that the stenophyllization process of the serpentine ecotype of *Adenophora triphylla* (Thunb.) A.DC. var. *japonica* (Regel) H. Hara (Campanulaceae) had narrow leaves due to the decreased number and size of epidermal cells. Additionally, this ecotype had a reduced number of stomata due to stenophyllization. Recently, Shiba et al. (2022) reported that the smaller leaves of *Eurya japonica* Thunb. (Ternstroemiaceae) in serpentines had smaller leaves by decreasing the number of cells, and their thicker leaves contributed to the increased height of epidermal cells, palisade tissue, and spongy tissue. In addition, the stomatal size of serpentine *E. japonica* was significantly smaller than that of inland areas (Shiba et al., 2022). These studies suggested that the environmental stresses to serpentine soils create severe limitations in plant growth, and some species had altered morphological and anatomical characteristics to adapt to them. The accumulation of such plant studies could reveal the common morphological and anatomical changes necessary for adapting to serpentine areas.

Ligustrum japonicum Thunb. (Oleaceae) is an evergreen shrub or small tree that grows 2–5 m tall and is native to central and southern Japan and Korea (Noshiro, 1993). Its leaves are 5–10 cm long and 2–5 cm wide, thick, and leathery with an entire margin. The upper side of the leaf is glossy dark green, whereas the lower side is paler and glaucous to yellowish green (Noshiro, 1993). This species has been subjected to conservation genetic studies with a closely related alien species, *L. lucidum* Aiton, artificially introduced from China (Yoshinaga & Kameyama, 2001; Hashimoto et al., 2003; Saito et al., 2012). Yamanaka (1959, 1974) reported that *L. japonicum* was present in some serpentine areas of Kochi Prefecture in Shikoku, Japan. These studies indicate that morphological and anatomical comparisons of *L. japonicum* in serpentine and control areas could reveal how this species has adapted to the serpentine areas. Therefore, our study aimed to clarify the morphological and anatomical differences of *L. japonicum* in adapting to serpentine environments (Figure 1).

Figure 1. *Ligustrum japonicum* Thunb. a: serpentine (Mikame), b: inland (Noichi)

2. Method

All *Ligustrum japonicum* samples examined in this study were collected directly from the field. The samples were collected from eight locations: four serpentine areas (Kagami, Ikku, Oko and Mikame) and four inland areas (Noichi, Shigeto, Taisho, and Naro). The locations are indicated in Figure 2 and Table 1. A total of 120 individuals, 30 from each population, were analyzed for serpentine and inland (control) populations.

In this study, we performed morphological and anatomical analyses of *L. japonicum* based on Shiba et al. (2021). For morphological analysis, the length, width, and thickness of the leaf blade were measured using a digital caliper and micrometer. To detect stenophyllization in the serpentine populations of *L. japonicum*, leaf index values were calculated as the ratio of leaf length to leaf width, similar to Tsukaya (2002). Leaf size was calculated from length×width/2. Five fully expanded leaves were measured randomly from one individual, and the average of these was used as the value for that individual.

For anatomical analysis, leaves were fixed overnight in a solution of formaldehyde, glacial acetic acid, alcohol (95% ethanol), and distilled water (FAA). As in the morphological analysis, five fully expanded leaves were randomly measured from one individual, and the average of these measurements was taken as the value for that individual. To measure epidermal cells and stomata on the leaf blade surface, fixed leaf surfaces were peeled using Suzuki's Universal Micro-Printing (SUMP) method (Kijima, 1962). The middle part of the leaf blade along the midrib was analyzed to determine epidermal cell size, stomatal size (guard cell area, based on guard cell pair length and width) and stomatal density. These copied SUMP images were measured twice for each leaf under an optical microscope.

To measure the thickness of each mesophyll tissues (epidermal cells, palisade tissue & spongy tissue), fixed leaves were embedded in paraffin and then longitudinal sections of 8 µm thickness were prepared using a microtome. Palisade and spongy tissues were analyzed for thickness at the widest part of the leaf blade. The samples were also examined under a light microscope.

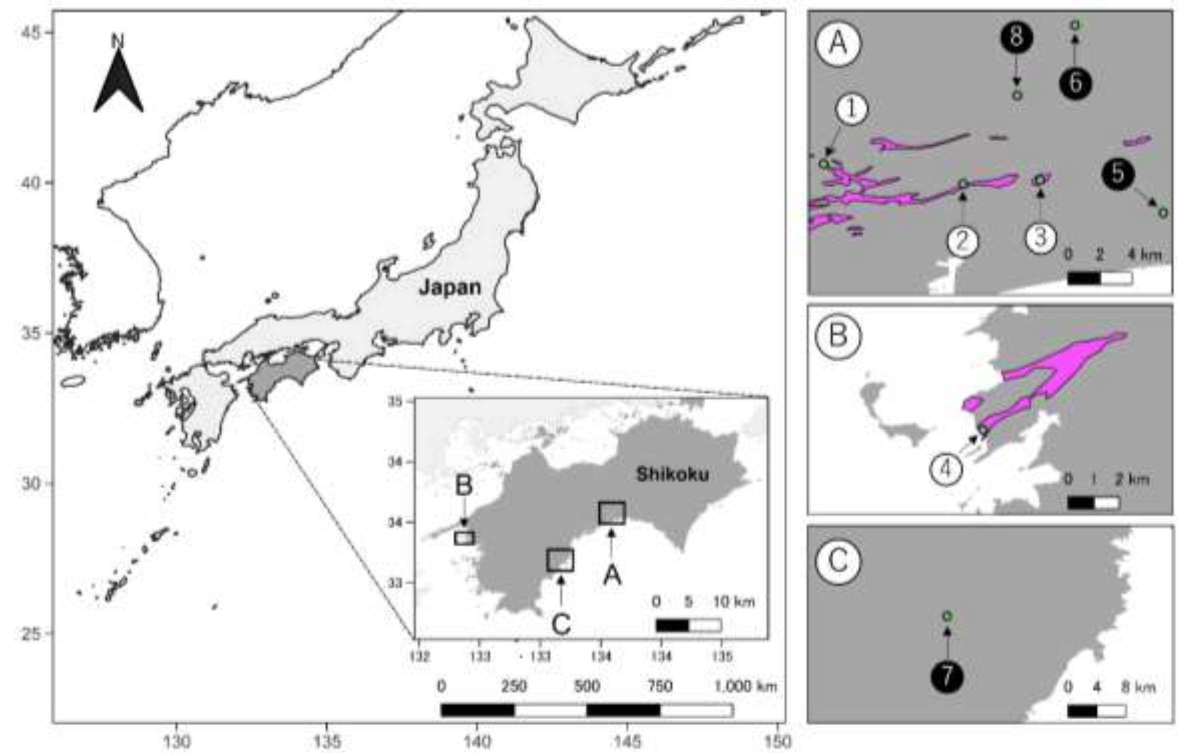

Figure 2. Sampling localities in this study

The pink areas indicate serpentinite zones, which are based on Geological Survey of Japan, AIST (2022). White and black circles indicate serpentine and inland (control) populations, respectively. Number in circles corresponds to that given in Table 1

Table 1. Sampling localities used in this study

Type	Locality name & number*		Locality	Latitude and longitude
serpentine				
	Kagami	1	Kagami-ohri, Kochi City, Kochi Pref.	33 °36N 133 °28E
	Ikku	2	Ikku, Kochi City, Kochi Pref.	33 °35N 133 °34E
	Oko	3	Oko, Nankoku City, Kochi Pref.	33 °35N 133 °37E
	Mikame	4	Mikame, Seiyo City, Ehime Pref.	33 °22N 132 °23E
Inland (control)				
	Noichi	5	Ohtani, Noichi-cho, Konan City, Kochi Pref.	33 °34'N 133 °42'E
	Shigeto	6	Kashinotani, Tosayamada-cho, Kami City, Kochi Pref.	33 °41'N 133 °39'E
	Taisho	7	Eshi, Shimanto-cho, Takaoka-gun, Kochi Pref.	33 °12'N 132 °58'E
	Naro	8	Naro, Nankoku City, Kochi Pref.	33 °39'N 133 °36'E

*: locarity number corresponds to that given in Figure 1.

3. Results

The leaf morphology of *Ligustrum japonicum* is summarized in Table 2. A comparison of individuals from serpentine and inland populations indicated that the leaf sizes of the individuals from serpentine populations tended to be smaller than those from the inland population; for example, the Mikame population's (serpentine) leaf size

Table 2. Leaf morphology (average ± standard error) of *Ligustrum japonicum*

	Serpentine							
	Kagami		Ikku		Oko		Mikame	
size (mm^2)	1,144.95±71.78	cd	1,412.9±84.35	bc	1,304.67±74.36	cd	1,024.15±37.16	d
leaf index*	1.95±0.05	cde	2.25±0.05	ab	2.06±0.04	bcd	1.90±0.04	de
thickness (μm)	373.73±28.17	bc	394.35±28.32	ab	505.08±43.81	a	344.58±15.60	bc

Table 2. (Continued)

	Inland (control)							
	Noichi		Shigeto		Taisho		Naro	
size (mm^2)	1,700.81±63.57	ab	1,784.85±70.48	a	1,393.48±79.65	bc	1,357.70±79.55	c
leaf index*	1.86±0.04	e	2.12±0.04	abc	2.31±0.06	a	2.15±0.05	abc
thickness (μm)	289.18±22.46	bcd	271.73±24.19	cd	184.65±7.10	d	210.33±21.08	d

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

*: Tsukaya (2002)

was the smallest but there was no clear difference between them. In contrast, the leaves of serpentine populations tended to be thicker than those of inland populations, and the Oko population (serpentine) had the thickest leaves. However, no significant difference was observed in leaf thickness or size between serpentine and inland populations. Previous adaptation studies have indicated that the individuals of serpentine population have narrow leaves (Hayakawa et al., 2012; Ohga et al., 2012). To detect stenophyllization in the serpentine populations of *L. japonicum*, leaf index values were calculated as the ratio of leaf length to leaf width, similar to Tsukaya (2002). However, no significant difference was detected in the leaf index between the serpentine and inland populations.

The leaf anatomy of *L. japonicum* is summarized in Tables 3-5. The mean adaxial and abaxial epidermal cell sizes were calculated using SUMP samples from all investigated localities (Figure 3 & Table 3). There was a slight change in adaxial cell size in the Mikame population, but no significant difference was found between the serpentine and inland populations. In addition, the values for the epidermal cell size, palisade tissue, and spongy tissue that contribute to the thickness of the leaves measured using longitudinal sections of leaves are shown in Figure 4 and Table 4. The results showed that the thickness of epidermal cells on the adaxial and abaxial sides, palisade tissue, and spongy tissue in serpentine populations tended to be larger than in inland populations. However, no clear differences could be observed between them.

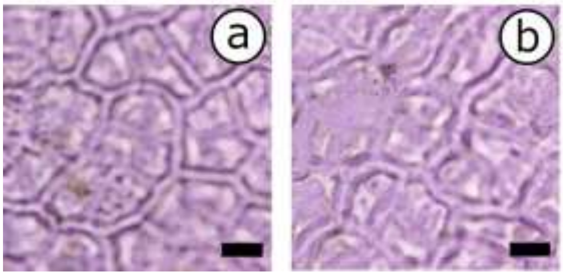

Figure 3. Epidermal cell on adaxial surface

a: serpentine (Oko), b: inland (Naro). Bar = 30 μm

We calculated the mean stomatal size and density of *L. japonicum* from serpentine and inland areas from all examined localities (Figure 5 & Table 5). Interestingly, the stomatal length, width, and size of the individuals in the serpentine populations were significantly smaller than those of the individuals in the inland populations. However, the stomatal density was not significantly different between these populations.

Table 3. Anatomical measurements (average ± standard error) of epidermal cell size (μm^2) in *Ligustrum japonicum*

	Serpentine							
	Kagami		Ikku		Oko		Mikame	
adaxial	2,372.99±86.72	bcd	2,005.12±67.74	e	2,066.99±49.39	de	2,843.17±64.01	a
abaxial	991.54±17.38	ab	1,069.13±25.11	a	898.47±12.98	b	879.24±20.83	b

Table 3. (Continued)

	Inland (control)							
	Noichi		Shigeto		Taisho		Naro	
adaxial	2,549.75±73.92	abc	2,607.31±108.76	ab	1,992.09±64.42	e	2,250.63±57.43	cde
abaxial	993.48±38.17	ab	1,013.31±50.85	ab	897.47±59.27	b	914.25±45.99	b

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

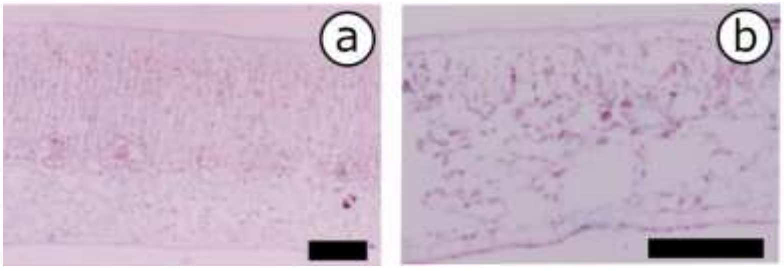

Figure 4. Longitudinal section of leaf

a: serpentine (Oko), b: inland (Naro). Bar = 100 µm

Table 4. Anatomical measurements (average ± standard error) of mesophyll tissues using longitudinal section in *Ligustrum japonicum*

	Serpentine							
	Kagami		Ikku		Oko		Mikame	
epidermal cell at adaxial side (µm)	29.03±1.13	bc	32.41±1.39	ab	35.05±1.06	a	32.55±0.50	ab
palisade tissue (µm)	155.41±16.15	b	155.11±14.21	b	219.68±23.78	a	134.98±6.89	b
spongy tissue (µm)	168.63±12.81	bc	185.13±13.39	ab	224.33±20.63	a	153.25±8.32	bc
epidermal cell at abaxial side (µm)	20.68±0.67	cd	21.73±0.65	bc	26.03±0.45	a	23.81±0.34	ab

Table 4. (Continued)

	Inland (control)							
	Noichi		Shigeto		Taisho		Naro	
epidermal cell at adaxial side (µm)	24.85±1.08	cd	24.68±0.84	d	22.83±0.54	d	24.28±0.71	d
palisade tissue (µm)	104.83±9.40	bc	104.31±11.51	bc	56.83±2.97	c	75.33±9.60	c
spongy tissue (µm)	140.01±12.09	bcd	124.08±11.83	cd	87.03±3.58	d	92.48±10.65	d
epidermal cell at abaxial side (µm)	19.51±0.85	cde	18.68±0.66	de	17.98±0.54	e	18.15±0.58	de

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

4. Discussion

Plant species adapted to the serpentine often possess morphologies distinct from closely related non-serpentine ones, such as the narrow leaves and the reduced stature (Rune, 1953), and some previous studies had shown a general tendency in the leaf morphology and anatomy of the serpentine adapted species and its closely related non-

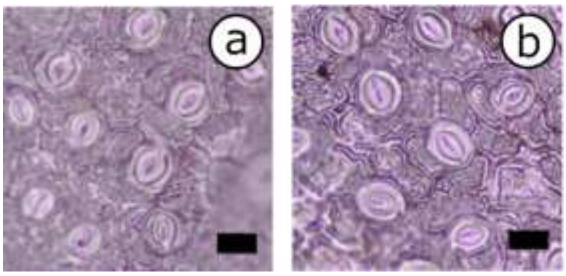

Figure 5. Stomata on abaxial surface

a: serpentine (Oko), b: inland (Naro). Bar = 20 μm

Table 5. Anatomical measurements (average ± standard error) of stomata in *Ligustrum japonicum*

	Serpentine							
	Kagami		Ikku		Oko		Mikame	
size (μm^2)	255.16±6.05	b	277.69±8.78	b	276.61±6.08	b	275.87±6.68	b
density (N/μm^2)	167.54±5.04	bc	178.78±7.23	ab	193.35±5.37	a	156.44±4.13	c

Table 5. (Continued)

	Inland (control)							
	Noichi		Shigeto		Taisho		Naro	
size (μm^2)	332.56±5.19	a	352.54±7.97	a	351.44±5.35	a	354.67±7.51	a
density (N/μm^2)	169.44±4.11	bc	159.89±3.91	bc	161.24±5.11	bc	174.63±4.61	abc

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

serpentine ones (Hayakawa et al., 2012; Ohga et al, 2012; Kumekawa et al, 2013; Shiba et al., 2022). As mentioned above, the speciation of serpentine-adapted inland species seems to be associated with leaf morphology and the stomatal density and/or size. Our morphological result was indicated that *Ligustrum japonicum* found on serpentine appear to be predominantly those also occurring on near non-serpentine area and the leaves of this species tended to be thicker in serpentine. Thicker leaves are caused by increased leaf water content and subsequent accumulation of large amounts of solute without an increase in the osmotic pressure of cells (Suárez & Sobrado, 2000). Consequently, species with thicker leaves can be found in drought-prone environments (Ohga et al., 2013; Tunala et al., 2012). The edaphic characteristics of serpentine were important in many cases, such as metal toxicity, an adverse Ca/Mg quotient, and drought (Kruckeberg, 1951, 1954). Drought tolerance is one of the essential characteristics of serpentine adaptation (Hayakawa et al., 2012; Ohga et al., 2012). In fact, previous studies on the serpentine-adapted variety of *As. hispidus* var. *leptocladus*, a serpentine ecotype of *Ad. triphylla* var. *japonica,* and the serpentine *E. japonica* reported adaptation by the acquisition of thicker leaves (Hayakawa et al., 2012; Ohga et al., 2012; Shiba et al., 2022). Similarly, serpentine *L. japonicum* also tended to have thicker leaves. Moreover, our anatomical results showed that no single parameter, such as the adaxial and abaxial epidermal cells, palisade tissue, or spongy tissue contributed to the increased leaf thickness of serpentine *L. japonicum*; however, all of them were slightly higher than plants of the inland populations. These anatomical results were also similar to those of *E. japonica* (Shiba et al., 2022), where the modification in leaf thickness, when woody species adapted from inland to serpentine areas, was related to the change in the height of each cell.

Our results indicate that *L. japonicum* in serpentine areas had reduced stomatal size rather than density. Some studies have suggested that small stomata respond rapidly to changing light intensities (McAusland et al., 2016; Lawson & Vialet-Chabrand, 2018). Moreover, Pitaloka et al. (2022) reported that smaller stomata closed significantly faster than larger stomata during the initial closure, suggesting that excessive transpiration could be avoided to resist drought conditions. In addition to the large cell size, smaller stomata were considered anatomical traits for adapting to serpentine areas. However, it remains unknown the background of the formation of small stomata. Such natural characteristics could be leveraged to discern consistent trends in the mechanisms and genetic basis of adaptation, as well as ecological speciation (Rundle & Nosil, 2005; Losos, 2011). Some studies have provided evidence for adaptation to local environments using a plant genetic model. This analysis has been facilitated by the availability of whole genome sequences, which allows for the development of genomic tools to identify gene functions and the mechanistic basis of phenotypes in model plants (Shepard & Purugganan, 2002; Irish & Benfey, 2004; Kellogg, 2004). Patterns of phenotypic diversity could indicate adaptive responses to selection. Therefore, evaluating these patterns could identify genetic polymorphisms underlying these adaptive responses. Recently, the genetic background of guard cell development has been elucidated in model plants. Some studies have identified three basic helix-loop-helix (bHLH) transcription factors that act as master regulators of stomatal development (Ohashi-Ito & Bergmann, 2006; Pillitteri et al., 2007; MacAlister et al., 2007). Three key transcription factors, SPCH, MUTE, and FAMA, mediate sequential steps of stomatal development, and their functions may be conserved in land plants (Ran et al., 2013; Harris et al., 2020). Of these, the difference in the expression time of SPCH and MUTE strongly influenced the different division patterns of meristemoids based on a comparison of terrestrial and amphibious species (Doll et al., 2021). These studies indicate that orthologous genes could potentially be isolated from *L. japonicum* and used for comparative expression and genomic analyses between serpentine and inland species, extending the study of serpentine adaptation in this species. In addition, a comparison of the molecular mechanisms involved will provide an integrated functional perspective on serpentine adaptation in *L. japonicum*.

The comprehensive understanding of serpentine adaptation is a challenging task that requires multidisciplinary investigation, but recent progress in morphological, anatomical, cytological and population genomic study has shown that understanding the basis for the interacting phenotypes which constitute serpentine adaptation is well worth the effort (Wright & Von Wettberg, 2009). Geologically, the serpentine area is unique and often a center of biodiversity. Thus, plant speciation and new serpentine endemic taxa, *As. hispidus* var. *leptocladus* f. *tubulosus* Y. Kumekawa & T. Fukuda (Asteraceae), was described recently (Kumekawa et al., 2016). It is difficult to distinguish serpentine and inland types of *L. japonicum* in the absence of gross morphological differences between them; however, they exhibit ecotypic differentiation. Therefore, there could be found plants that have not yet been described in the serpentine areas by the adaptation process as in this study.

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