

Rheophytic Adaptation of *Eurya japonica* Thunb. (Ternstroemiaceae)

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Abstract

Plants along rivers have narrow leaves to avoid the stress caused by the river's flow during flooding. Plants that have undergone such morphological leaf modifications to adapt to rivers are called rheophytes. Some populations of *Eurya japonica* Thunb. (Ternstroemiaceae) were grown on riversides so that the comparative morphology and anatomy of leaves between riverside and inland (control) populations could be examined to confirm their rheophytic speciation. Our morphological and anatomical analyses revealed that the leaf of *E. japonica* in the riverside populations was significantly smaller than that of the inland populations due to the decreased number of cells; therefore, the pattern of rheophytic differentiation in riversides was not the stenophyllization but the miniaturization of the leaf. Moreover, our results indicated that this species in the riverside populations had thicker leaves and higher density of stomata than those of inland populations, suggesting that *E. japonica* had been morphologically modified in response to the light and water environments along the river.

Keywords: adaptation, *Eurya japonica*, leaf, morphology, rheophyte

1. Introduction

Diverse environments have led to plants evolving various traits to adapt to them, such as the dwarf plants in the alpine and the succulent leaves in the drylands. Among them, riverside environments have been selected for plants with narrow lanceolate or cuneate leaves, called rheophytes, to adapt to sudden flooding and strong river currents after heavy rain (van Steenis, 1981). Therefore, artificial alterations, such as dams, to avoid flooding of rivers could affect the vegetation along the riverside because the selective pressure changes in the area downstream (Saito, Hoshino, Yoshikawa, & Hoshino, 2019); in fact, Azami, Saitou, Kodama and Watanabe (2001) reported that the change in communities along riversides from *Phragmites japonicus* Steud. (Poaceae) to *Lolium arundinaceum* (Schreb.) Darbysh. (Poaceae), *Pueraria lobata* (Willd.) Ohwi (Fabaceae) and *Rosa multiflora* Thunb. (Rosaceae) was observed downstream of the Miharu Dam on the Ohtakine River in Fukushima Prefecture.

Similar adaptive modifications of leaves in rheophytes have occurred independently in various plant families (van Steenis, 1981). In fact, some comparative studies using rheophytic and closely related species have been conducted as model cases of leaf modification. To elucidate the formation of these leaves, for example, a comparative anatomical study of fern rheophytes, *Osmunda lancea* Thunb. (Osmundaceae) and its closely related dryland species, *O. japonica* Thunb. indicated a strong correlation between the gross morphology and anatomy of leaves (Imaichi & Kato, 1992). In angiosperms, Usukura, Imaichi and Kato (1994) concluded that the narrow leaves of the rheophyte *Farfugium japonicum* (L. fil.) Kitam. var. *luchuense* (Masam.) Kitam. (Asteraceae) evolved because of the decreased number of leaf cells across the width of the leaf. Moreover, the rheophytic species of *Rhododendron indicum* f. *otakumi* and *R. ripense* Makino (Ericaceae) were involved only in the decreased number of leaf cells (Setoguchi & Kajimura, 2004; Ueda et al., 2012), and the narrow leaves of *Viola mandshurica* W. Becker var. *ikedaeana* (W. Becker ex Taken.) F. Maek. (Violaceae) were also induced by a decrease in number of cells (Matsui et al., 2013). Although their narrow lanceolate or cuneate leaves were formed by changes in the number of leaf cells, Tsukaya (2002a) and Yamada et al. (2011) reported a variation in leaf width in the rheophyte *Dendranthema yoshinaganthum* (Makino ex Kitam.) Kitam. and *Aster microcephalus* (Miq.) Franch. et Sav. var. *ripensis* Makino in Asteraceae were involved in both the size and number of leaf cells. In addition, Ohga et al. (2012) indicated that the rheophytic ecotype of *Adenophora triphylla* (Thunb.) A. DC. var. *japonica* (Regel) H. Hara (Campanulaceae) has also been involved in both, the size and number of leaf cells. In this way, it has been clarified that the formation process of narrow leaves in rheophytes was categorized into the following two patterns: one was

formed by only decreasing the number of cells, and the other was involved in decreasing not only the number but also the size of leaf cells.

In addition to cell size and number of leaves, some anatomical studies reported that rheophytic species had seen an increase in their stomatal size density, suggesting that they had differentiated the use of solar radiation and water for photosynthesis from closely related inland species (Ohga et al., 2012; Ueda et al., 2012; Matsui et al., 2013). These observed morphological and anatomical traits of rheophytic species could be explained by certain aspects of the riparian environment, such as high irradiation and frequent flooding after heavy rainfall-to which they were exposed. Above mentioned studies of rheophytic differentiation have clarified these traits by comparison with the putative rheophyte and its closely related inland species or ecotypes. However, in the case where the species growing in a mountainous area invade the riversides or when it grows continuously from a mountain to a riverside, it is doubtful whether the plant possessed the above-mentioned morphologies.

Eurya japonica Thunb. (Ternstroemiaceae) is approx. 1-3.5 m tall, has broad-leaved, evergreen woody perennials, and is widely distributed in Japan from Honshu to Ryukyu and Taiwan, southern China and Korea, and inhabits forests dominated by *Pinus* spp and *Quercus* spp. in Japan. This species has been found in various riparian forests in Japan (Yamaguchi, 2004; Shimada & Tani, 2005; Ito et al., 2008; Fujimaki et al., 2015), and our preliminary survey confirmed that *E. japonica* also grew in areas that sustain flash floods along the following rivers in southern Shikoku: Shimanto, Yusuhara, Yoshino, Naka, Ioki, and Asemi rivers (Figure 1). Moreover, it is interesting that *E. japonica* grew sympatrically with the rheophytic *R. ripense* at all points along the riversides mentioned above in our preliminary survey. In addition to the rheophytic *R. ripense*, we confirmed that *E. japonica* was sympatric with *D. yoshinaganthum* at the survey site of the Naka River, the rheophytic ecotype of *Ad. triphylla* var. *japonica* in the Shimanto and Yoshino rivers, *V. mandshurica* var. *ikedaeana* in the Asemi River and *As. microcephalus* var. *ripensis* in the Ioki and Yusuhara Rivers. Therefore, it was very interesting that *E. japonica* on these riversides may have experienced the rheophyteic modification because it grew together with the above mentioned rheophytes under selective pressure of sudden flooding and strong river flow after heavy rain, but no research has been conducted so far focusing on such morphological and anatomical differences found in between varying environments. A comparison of these riverside *E. japonica* populations with their inland populations could reveal some differentiation for this species to adapt to riversides. Therefore, we conducted comparative morphological and anatomical analyses to investigate the differentiation between them.

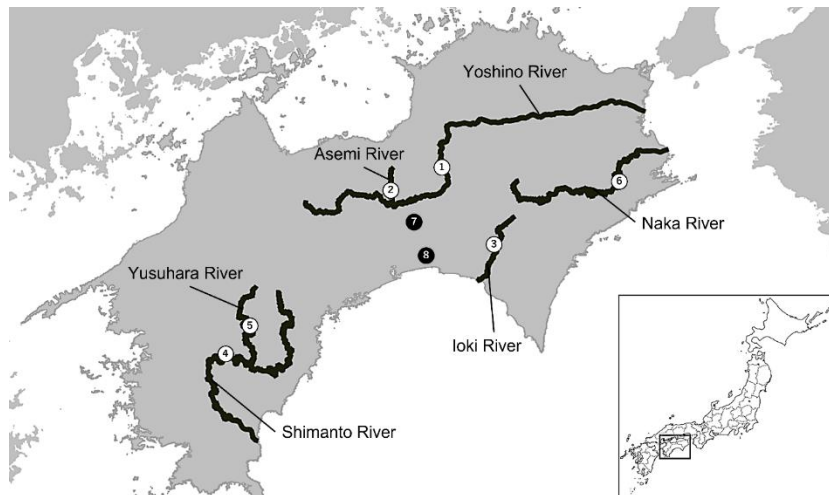


Figure 1. Sampling localities in this study. White and black circles indicate riverside and inland (control) populations, respectively. Number in circles corresponds to that given in Table 1

Table 1. Sampling localities used in this study

Type	Locality name (River) & number*	Locality	Latitude and longitude
riverside			
	Oboke (Yoshino River) 1	Kamimyo, Yamashiro-cho, Miyoshi City, Tokushima Pref.	33°53'N 133°46'E
	Motoyama (Asemi River) 2	Sougauchi, Motoyama-cho, Nagaoka-gun, Kochi Pref.	33°47'N 133°33'E
	Koi (Ioki River) 3	Koi, Aki City, Kochi Pref.	33°37'N 133°59'E
	Tokawa (Shimanto River) 4	Tokawa, Shimanto-cho, Takaoka-gun, Kochi Pref.	33°14'N 132°52'E

Yusuhara (Yusuhara River)	5	Nakahira, Yusuhara-cho, Takaoka-gun, Kochi Pref.	33°20'N	132°58'E
Naka (Naka River)	6	Momae, Naka-cho, Naka-gun, Tokushima Pref.	33°50'N	134°30'E
Inland (control)				
Shigeto	7	Kashinotani, Tosayamada-cho, Kami City, Kochi Pref.	33°41'N	133°39'E
Noichi	8	Ohtani, Noichi-cho, Konan City, Kochi Pref.	33°34'N	133°42'E

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

2. Method

All samples of *Eurya japonica* examined in this study were collected from the field. We collected samples from eight localities (six localities on the riversides and two in the inland). These localities were named as follows: six riverside areas including (Oboke [Yoshino River], Motoyama [Asemi River], Koi [Ioki River], Tokawa [Shimanto River], Yusuhara [Yusuhara River], and Naka [Naka River]), and two inland areas (Shigeto [Experimental Forest in Kochi University], and Noichi [Mt. Sanpo]). Localities are indicated in Figure 1 and Table 1. *E. japonica* was analyzed in a total of 210 individuals on the riversides (40 from Oboke, 30 from Motoyama, 38 from Koi, 31 from Tokawa, 30 from Yusuhara, and 41 from Naka) and 63 individuals (33 from Shigeto and 30 from Noichi) from the inland areas.

For morphological analysis, individuals were measured for the following continuous macromorphological variables of leaves: length and width of the leaf blade, and angle of the leaf base were measured with a digital caliper and a protractor. Fully expanded leaves were analyzed. Five leaves were randomly measured from one individual, and their values averaged of them was used as the value of that individual.

For anatomical analysis, fully expanded leaves were collected from each individual. The leaves were fixed in a solution of formaldehyde, ethanol, and acetic acid (FAA) overnight. To count the number of cells on the blade, the surface of the fixed leaves was peeled off using Suzuki's Universal Micro-Printing (SUMP) method (Kijima, 1962). The middle part of the blade along the midrib was analyzed to determine the number and size of the epidermal cells. Replicas of each leaf (1 mm²) were used to measure stomatal density. These copied SUMP images were examined twice for each leaf under a light microscope.

To measure the thicknesses of the epidermal cell, palisade tissue, and spongy tissue, the fixed leaves were dehydrated by immersion in a graded ethanol series and then embedded in paraffin. Cross-sections of leaves were 8 µm thick. The widest part of the blade was analyzed to determine the size of the palisade cells. The samples were also examined under a light microscope.

3. Results

In the Results section, summarize the collected data and the analysis performed on those data relevant to the discourse that is to follow. Report the data in sufficient detail to justify your conclusions. Mention all relevant results, including those that run counter to expectation; be sure to include small effect sizes (or statistically nonsignificant findings) when theory predicts large (or statistically significant) ones. Do not hide uncomfortable results by omission. Do not include individual scores or raw data with the exception, for example, of single-case designs or illustrative examples. In the spirit of data sharing (encouraged by APA and other professional associations and sometimes required by funding agencies), raw data, including study characteristics and individual effect sizes used in a meta-analysis, can be made available on supplemental online archives.

We analyzed the leaf morphology of *Eurya japonica* using morphological and anatomical approaches. A summary of the measurements of leaf morphology is presented in Table 2. We calculated the mean leaf length, width, thickness, and angle of the leaf base for each individual. Leaf length and width in the riverside populations were smaller than those in the inland populations. To detect stenophyllization, which means the process of narrow leaves, in riverside populations, leaf index values were calculated as the ratio of leaf length to leaf width based on Tsukaya (2002b). If the results of comparing these leaf index values between riverside and inland populations show a significant difference, this indicates that the population with the large value has narrow leaves. No significant difference between the riverside and inland populations was detected in the leaf index. Moreover, there was no significant difference in the angle of the leaf base between them, indicating that these populations have similar leaf silhouettes, although the leaf in the riverside populations was significantly smaller than that of the inland plants.

We calculated the mean epidermal cell size using the length and width of the cell measured from the SUMP samples of this species (Table 3). The results showed that the cell length, cell width, and cell size were not significantly different between the riverside and inland populations. These results of epidermal cells of leaves indicated that the difference in leaf size between riverside and inland populations was caused not by the difference in cell size, but by the difference in the

number of cells. In addition, the results of measuring the tall of epidermal cells, palisade tissue, and spongy tissue, which contributes to the thickness of the leaves using longitudinal sections of leaves, are shown in Table 4. The results showed that the thickness of epidermal cells on both sides was significantly different between riverside and inland populations, but palisade and spongy tissues showed no clear differences between them. In palisade tissue, samples from Koi (Ioki River) had similar thicknesses to those of inland populations, but samples from other riversides were significantly thicker than those from inland populations. For spongy tissue, however, samples from Oboke (Yoshino River) and Tokawa (Shimanto River) were thicker than those from the inland areas.

Table 2. Leaf morphology (average \pm standard error) of *Eurya japonica*

	Riverside						Inland (control)	
	Oboke	Motoyama	Koi	Tokawa	Yusuhara	Naka	Shigeto	Noichi
length (mm)	31.92 \pm 0.78 ^d	42.02 \pm 0.95 ^{bc}	46.47 \pm 1.23 ^b	38.16 \pm 0.88 ^c	40.63 \pm 0.83 ^c	33.36 \pm 1.02 ^d	55.00 \pm 0.90 ^a	54.01 \pm 1.13 ^a
width (mm)	12.24 \pm 0.32 ^f	16.28 \pm 0.28 ^c	15.92 \pm 0.30 ^{cd}	13.89 \pm 0.34 ^e	14.69 \pm 0.24 ^e	13.42 \pm 0.31 ^e	20.84 \pm 0.33 ^b	22.92 \pm 0.48 ^a
leaf index*	2.64 \pm 0.06 ^{abc}	2.58 \pm 0.05 ^{bcd}	2.93 \pm 0.07 ^a	2.78 \pm 0.06 ^{ab}	2.77 \pm 0.03 ^{ab}	2.49 \pm 0.05 ^{cd}	2.66 \pm 0.05 ^{abc}	2.37 \pm 0.04 ^d
angle of leaf base (°)	50.41 \pm 1.32 ^c	56.81 \pm 1.51 ^b	56.04 \pm 1.52 ^b	54.52 \pm 1.22 ^{bc}	51.06 \pm 0.58 ^{bc}	54.18 \pm 1.12 ^{bc}	55.10 \pm 1.02 ^{bc}	62.89 \pm 1.66 ^a
thickness (μ m)	315.05 \pm 11.30 ^a	20.85 \pm 17.40 ^{bc}	228.33 \pm 5.55 ^{cd}	297.88 \pm 16.78 ^{ab}	243.00 \pm 12.10 ^{cd}	270.90 \pm 11.35 ^{abc}	191.43 \pm 8.90 ^{de}	173.00 \pm 7.90 ^e

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

*: Tsukaya (2002b)

Table 3. Anatomical measurements (average \pm standard error) of epidermal cell in *Eurya japonica*

	Riverside						Inland (control)	
	Oboke	Motoyama	Koi	Tokawa	Yusuhara	Naka	Shigeto	Noichi
adaxial surface								
length (μ m)	20.51 \pm 0.15 ^a	20.37 \pm 0.18 ^a	20.65 \pm 0.19 ^a	20.36 \pm 0.46 ^a	20.02 \pm 0.22 ^a	19.92 \pm 0.16 ^a	20.13 \pm 0.27 ^a	19.93 \pm 0.25 ^a
width (μ m)	20.60 \pm 0.11 ^a	20.62 \pm 0.19 ^a	20.94 \pm 0.20 ^a	20.41 \pm 0.47 ^a	20.22 \pm 0.22 ^a	20.23 \pm 0.15 ^a	19.91 \pm 0.20 ^a	20.03 \pm 0.32 ^a
size (μ m ²)	401.69 \pm 7.76 ^a	423.77 \pm 7.73 ^a	436.06 \pm 8.32 ^a	432.15 \pm 18.61 ^a	406.93 \pm 9.10 ^a	404.48 \pm 6.26 ^a	401.69 \pm 7.76 ^a	411.94 \pm 9.59 ^a
abaxial surface								
length (μ m)	18.86 \pm 0.18 ^a	19.24 \pm 0.14 ^a	19.20 \pm 0.07 ^a	19.42 \pm 0.11 ^a	19.69 \pm 0.14 ^a	19.34 \pm 0.07 ^a	19.70 \pm 0.66 ^a	19.85 \pm 0.22 ^a
width (μ m)	17.48 \pm 0.14 ^{ab}	17.19 \pm 0.14 ^{bc}	17.25 \pm 0.09 ^b	17.68 \pm 0.11 ^{ab}	17.94 \pm 0.12 ^a	18.05 \pm 0.11 ^a	18.01 \pm 0.22 ^a	17.99 \pm 0.22 ^a
size (μ m ²)	332.33 \pm 3.52 ^c	332.78 \pm 4.52 ^{bc}	333.25 \pm 2.57 ^{bc}	344.90 \pm 3.83 ^{abc}	354.76 \pm 4.52 ^{ab}	349.78 \pm 2.47 ^{abc}	354.63 \pm 12.45 ^{ab}	356.44 \pm 3.66 ^a

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

Table 4. Anatomical measurements (average \pm standard error) of mesophyll tissues using longitudinal section in *Eurya japonica*

	Riverside						Inland (control)	
	Oboke	Motoyama	Koi	Tokawa	Yusuhara	Naka	Shigeto	Noichi
epidermal cell at adaxial side (μ m)	25.70 \pm 1.07 ^a	20.80 \pm 1.22 ^{bc} d	20.88 \pm 0.65 ^{bc}	26.33 \pm 0.65 ^a	24.35 \pm 0.67 ^{ab}	25.38 \pm 0.77 ^a	13.28 \pm 0.70 ^e	17.18 \pm 0.58 ^d
palisade tissue (μ m)	105.63 \pm 4.48 ^a	98.15 \pm 8.26 ^a	61.40 \pm 2.91 ^b	103.60 \pm 6.25 ^a	88.23 \pm 6.49 ^a	93.45 \pm 5.61 ^a	63.20 \pm 3.90 ^b	59.88 \pm 3.36 ^b
spongy tissue (μ m)	161.60 \pm 6.70 ^a	124.30 \pm 8.12 ^b c	127.30 \pm 3.31 ^b c	147.73 \pm 9.63 ^a b	111.88 \pm 6.08 ^c d	130.03 \pm 5.45 ^b c	105.38 \pm 6.33 ^{cd} e	82.30 \pm 5.26 ^e
epidermal cell at abaxial side (μ m)	22.13 \pm 0.81 ^a	17.60 \pm 0.82 ^b	18.75 \pm 0.66 ^b	20.23 \pm 0.59 ^{ab}	18.55 \pm 0.47 ^b	22.05 \pm 0.44 ^a	9.58 \pm 0.49 ^d	13.65 \pm 0.34 ^c

Table 5. Anatomical measurements (average \pm standard error) of stomata in *Eurya japonica*

	Riverside						Inland (control)	
	Oboke	Motoyama	Koi	Tokawa	Yusuhara	Naka	Shigeto	Noichi
length (μm)	19.05 \pm 0.19 ^c	20.00 \pm 0.13 ^{abc}	20.25 \pm 0.17 ^{ab}	19.99 \pm 0.17 ^{abc}	19.26 \pm 0.13 ^{bc}	20.00 \pm 0.10 ^{abc}	20.10 \pm 0.18 ^{abc}	20.83 \pm 0.61 ^a
width (μm)	5.24 \pm 0.08 ^a	5.18 \pm 0.04 ^a	5.12 \pm 0.02 ^a	5.08 \pm 0.02 ^a	5.22 \pm 0.04 ^a	5.10 \pm 0.02 ^a	5.22 \pm 0.05 ^a	5.22 \pm 0.07 ^a
size (μm^2)	100.41 \pm 2.38 ^a	103.61 \pm 1.09 ^a	103.89 \pm 1.16 ^a	101.56 \pm 1.04 ^a	100.66 \pm 1.24 ^a	102.15 \pm 1.06 ^a	104.75 \pm 1.34 ^a	107.02 \pm 1.93 ^a
density (N/ μm^2)	511.90 \pm 7.69 ^a	393.20 \pm 7.78 ^b	303.40 \pm 4.88 ^c	412.15 \pm 7.79 ^b	386.37 \pm 14.53 ^b	411.90 \pm 9.27 ^b	243.33 \pm 6.49 ^d	233.26 \pm 5.67 ^d

We measured and calculated the mean stomatal size (guard cell area, based on guard cell pair length and width) and density using SUMP samples from the riverside and inland areas for *E. japonica* from all the localities we examined (Table 5). The stomatal length, width, and size of riverside populations were not significantly different from those of the inland populations. However, the stomatal density of the riverside populations was significantly higher than those of the inland populations.

4. Discussion

Our results indicated that *Eurya japonica* had morphological and anatomical differentiation of leaves under the selective pressure of rivers, similar to other previously reported rheophytes, suggesting that strong selection pressures such as a high frequency of flooding affect the leaf form in these riversides. Many plants have shown that the change in habitat from inland to riversides has been accompanied by stenophyllization; therefore, riverside plants have been distinguished as being different species and having different ecotypes (Imaichi & Kato, 1993; Usukura et al., 1994; Tsukaya, 2002a; Ueda et al., 2012; Ohga et al., 2012). However, our results revealed that the riverside population of *E. japonica* did not experience stenophyllization events based on the leaf index (Table 2), although plants that grew sympatrically, such as *Rhododendron ripense*, *Viola mandshurica* var. *ikedaeana*, *Aster microcephalus* var. *ripensis*, and *Adenophora triphylla* var. *japonica* had narrow leaves. Our results suggest that the riverside population of *E. japonica* was not seem to be adapted along the river, but its leaf length and width were smaller along all the riversides we studied. Therefore, our results concluded that the morphological differentiation from inland to riverside populations of *E. japonica* was not stenophyllization but the miniaturization of leaves. However, it is doubtful whether the miniaturization of leaves played an important role in avoiding the stress caused by the river flow during flooding, even though this species had smaller leaves in all riverside populations. One of the answers was that both, stenophyllization and the miniaturization of leaves lead to a reduction in leaf width. Therefore, we considered that the reduction of leaf width was the key to morphological change to adapt along the river, regardless of whether this occurred by stenophyllization or miniaturization of leaves. To the best of our knowledge, this is the first report of rheophytic adaptation by the miniaturization of leaves.

Our anatomical results indicated that rheophytic adaptation by miniaturization of leaves in this species had experienced a decrease in the number of leaf cells because there was no significant difference in leaf cell size between riverside and inland populations (Table 3). This anatomical differentiation of the rheophytic *E. japonica* was similar to that of *R. indicum* f. *otakumi*, *R. ripense*, and *V. mandshurica* var. *ikedaeana* (Setoguchi & Kajimura, 2004; Ueda et al., 2012; Matsui et al., 2013), although *Dendranthema yoshinaganthum*, *As. microcephalus* var. *ripensis* and *Ad. triphylla* var. *japonica* have adapted along the river by forming narrow lanceolate or cuneate leaves with not only the number of cells but also the cell size (Tsukaya, 2002a; Yamada et al., 2011). *Eurya japonica* had a closer phylogenetic relationship with species belonging to Ericaceae than those of Asteraceae and Campanulaceae, and occupied a more ancestral position than the latter families (Chase et al., 1993; Soltis et al., 2000; Savolainen & Chase, 2003; Soltis & Soltis, 2004). These results suggest that the rheophytic modification of leaves resulted in a decreased number of cells in all angiosperms, but rheophytic leaves in the most advanced lineage were formed by changing not only the number of cells but also the cell size based on the angiosperm phylogeny (Chase et al., 1993; Soltis et al., 2000; Savolainen & Chase, 2003; Soltis & Soltis, 2004).

Leaves are the major organs for photosynthesis; light-demanding plants can survive and grow only under high light, and the plant can modify its morphology, anatomy, and physiological characteristics because adapting to different light environments is an important factor in increasing net carbon gain (Augspurger, 1984; Kitajima, 1994; Aleric & Kirkman, 2005). Our morphological and anatomical results indicated that leaves of rheophytic *E. japonica* were thicker than those of inland populations (Table 2), and leaf thickness varied to adapt to fluctuating light conditions by changing the cell size in the adaxial–abaxial direction (Talbert & Holch, 1957; Yano & Terashima, 2001; Gotoh et al., 2018). In our study, all survey areas along the river were located in a large gap in the riparian forest because each river was relatively wide, and there was an environment in which a lot of sunlight radiated onto this species, suggesting that such growing environments for riverside populations of *E. japonica* contributed to the thickening of the leaves. Previous studies have reported that the

rheophytic *R. ripense*, *V. mandshurica* var. *ikedaeana*, and *Farfugium japonicum* var. *luchuense* had thicker leaves than those of closely related dryland species of *R. macrosepalum*, *V. mandshurica*, and *F. japonicum*, respectively (Nomura, Setoguchi, & Takaso, 2006; Ueda et al. 2012; Matsui et al. 2013). Therefore, the thick leaves induced along rivers were the general tendency in rheophyte plants because this modification was common to different plant lineages. From the anatomical point of view of the leaves, moreover, our results showed that the increase in leaf thickness of riverside populations of *E. japonica* was associated with elongation of epidermal cells, palisade tissue, and spongy tissue in the dorsoventral direction (Table 4). Li et al. (2014) reported that the intensity of light affected the thickness of cell size in a leaf, and recent morphological and physiological studies using the model plant *Arabidopsis thaliana* indicated that the intensity of light was involved in the thickness of the leaves, contributing to the elongation of palisade tissues in the dorsoventral axis of leaves (Hoshino et al., 2019). Therefore, our results suggest that the morphological and anatomical differentiation of leaves in the riverside populations of *E. japonica* reflected the unique light conditions along each river. In the future, it will be necessary to clarify the relationship between the amount and/or the intensity of light and the thickness of the leaves of rheophytic *E. japonica*.

Stomata play an important role in the regulation of gas exchange by leaves. Stomatal conductance of water vapor and CO₂ is determined primarily by the size and density of stomata for a species in a certain environment, and some studies have reported that stomatal size and density are controlled by both, genetic and environmental factors (Lake, Woodward, & Quick, 2002; Hetherington & Woodward, 2003; Casson & Gray, 2008; Yan, Sun, Song, & Liu, 2012; Zhang et al., 2012). Our results indicated that the stomatal density of riverside populations was significantly higher than that of the inland populations (Table 5). The increase in stomatal density was not, however, a specific differentiation, and some similar cases have been reported in rheophytic adaptation of various angiosperms (Nomura et al., 2006; Ueda et al., 2012; Matsui et al., 2013). Some studies have indicated that changes in stomatal density in response to soil moisture appears to correspond to environmental conditions (Bakker, 1991; Miyazawa, Livingston, & Turpin, 2006; Xu & Zhou, 2008; Sun, Yan, Cui, & Liu, 2014), and several approaches to improve water-use efficiency through the modification of stomatal traits have been tested in model plants (Büßis, von Groll, Fisahn, & Altmann, 2006; Liu, Ohashi-Ito, & Bergmann, 2009; Dow, Berry, & Bergmann, 2014; Dittberner et al., 2018). Soils along the riversides have been reported to have a higher moisture content (Collison & Anderson, 1996; Andreassian, 2004), suggesting that the stomatal density in the riverside population of *E. japonica* must vary under such environmental conditions.

Whether some morphological modifications in the riverside populations of *E. japonica* were genotypically adapted to specific environmental conditions or this was a result of phenotypic plasticity, was unclear. In general, species may be constrained by the selective pressures acting in their particular native climate, and thus a species exposed to more environmental variation in their distribution range has more phenotypic plasticity than species exposed to restricted environmental variation (Valladares, Gianoli, & Gómez, 2007; Valladares et al. 2014). It has been reported that *E. japonica* grows not only along riverside but also in special environments such as coastal and serpentine areas (Itow, Hotta, & Kawasato, 1974; Yamanaka, 1974). The fact that *E. japonica* grew in these special environments, even though many plants could not easily invade these areas because of their severe selection pressure, is considered to be possible because of the plasticity of this plant. Therefore, we hypothesized that *E. japonica* had the greatest capacity for acclimation as a result of increased phenotypic plasticity. To verify this hypothesis, artificial transplanting of *E. japonica* seeds between inland and riverside areas and seedling analyses would be the most obvious way to determine if it has genetic differentiation or phenotypic plasticity.

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