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Morphological and Anatomical Variations in Rheophytic Ecotype of Violet, *Viola mandshurica* var. *ikedaeana* (Violaceae)

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ABSTRACT

We compared the leaf morphology and anatomy of the putative rheophytic ecotype of *Viola mandshurica* W. Becker var. *ikedaeana* (W. Becker ex Taken.) F. Maek. and its closely related variety, *V. mandshurica* var. *mandshurica*. We showed that the leaf of the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* was narrower than that of *V. mandshurica* var. *mandshurica*. Moreover, the leaf thickness and guard cell size of the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* were significantly larger than those of *V. mandshurica* var. *mandshurica*. We further showed that leaves of the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* contained fewer cells than did those of *V. mandshurica* var. *mandshurica*. Our results suggest that the narrower leaves of *V. mandshurica* var. *ikedaeana* are caused by a decrease in the number of cells. A narrower leaf may enable the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* to resist the strong flow of water that occurs after heavy rainfall, while a thicker leaf may enhance tolerance to desiccation and high-intensity light.

Keywords: Ecotype; Leaf; Rheophyte; Stenophyllization; *Viola mandshurica*

1. Introduction

Irregular flooding can be an important stress factor for plants that do not inherently possess or are not able to develop traits enabling survival under submerged conditions. Spring and summer floods, which occur after strong rainfall [1], have a particularly strong impact on plant survival [2-4]. Plants that occur in the environment along the river, called rheophytes, are subjected to flash floods as a strong selective pressure. Morphological and anatomical differences in vegetative characters between a rheophyte and its closely related species appear, at least in part, to be correlated with differences in ecological setting [5]. Rheophytic species generally have narrow lanceolate or cuneate leaves [6-8], suggesting that similar adaptive modifications may have occurred independently within various plant families [5]. Some anatomical studies of angiosperm rheophytes have been conducted on the basis of morphological modifications. In the Asteraceae, the narrow leaves of the rheophyte *Farfugium japonicum* (L. fil.) Kitam. var. *luchuense* (Masam.) Kitam. were shown to evolve through a decrease in the number of cells across the width of the leaf [9]. Moreover, the variations of leaf-width in the rheophyte *Dendranthema yoshinaganthum* (Makino ex Kitam.) Kitam. and *Aster microcephalus* (Miq.) Franch. et Sav. var. *ripensis* Makino were found to involve the size and number of leaf cells [10,11]. In the Campanulaceae, evolution of the rheophytic type of *Adenophora triphylla* (Thunb.) A.DC. var. *japonica* (Regel) H. Hara was also shown to involve the size and number of leaf cells [12]. Meanwhile, the rheophytic types of *Rhododendron indicum* (L.) Sweet f. *otakumi* T. Yamaz. and *R. ripense* Makino (Ericaceae) were found to evolve through a decrease in the number of leaf cells [13,14]. All of these anatomical studies were conducted on plants in the asterid group, according to angiosperm phylogeny [15-18]. A compre-

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hensive understanding of the general tendency among angiosperms requires investigation of rheophytes belonging to plant groups other than the Asterid Group. In the present study, we focused on members of the Violaceae belonging to the rosid group of core dicotyledons.

Viola mandshurica W.Becker var. *ikedaeana* (W. Becker ex Taken.) F. Maek. is distributed in mountains on the Japanese mainland (western Honshu, Shikoku, and Kyushu), and also in Korea and Taiwan [19,20]. In addition, we newly found the variety on riversides (**Figure 1**), and it has hastate leaves that are narrower than the triangular- and oblong-lanceolate leaves of its closely related variety, *V. mandshurica* var. *mandshurica*. Comparative morphological and anatomical analyses, using the putative rheophytic ecotype of *V. mandshurica* var. *ikedaeana*, have not previously been conducted, and the leaf modification process of *V. mandshurica* var. *ikedaeana* (for example, a decrease in cell number and/or size) remains to be elucidated. A widely used indicator for rheophytic leaf size is the leaf index, *i.e.*, the ratio of leaf length to leaf width [21], which has been shown to differ significantly between rheophytes and closely related taxa. In the present study, we investigated the morphological and anatomical differentiation of the putative rheophytic ecotype of *V. mandshurica* var. *ikedaeana*, by conducting comparative morphological and anatomical analyses between this species and its closely related variety, *V. mandshurica* var. *mandshurica*.

2. Materials and Methods

2.1. Plant Materials

All samples of *V. mandshurica* var. *mandshurica* and the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* examined in this study were collected from the field. We collected samples from 6 localities (2 localities for *V. mandshurica* var. *mandshurica* and 4 localities for the rheophytic ecotype of *V. mandshurica* var. *ikedaeana*). The collection localities are shown in **Figure 2** and **Table 1**. In this study, we collected all individuals of *V. mandshurica* var. *ikedaeana* ranged from typical morphology of the variety to intermediate morphology with *V. mandshurica* var. *mandshurica* from riversides to investigate whether the rheophytic ecotype under floods and to evaluate the morphological and anatomical differentiation. We analysed 48 individuals of *V. mandshurica* var. *mandshurica* (Asakura, 18; Noichi, 30), and a total of 115 individuals of *V. mandshurica* var. *ikedaeana*, representing 2 populations of the Shimanto (Towa, 25; Nakahira, 30) and Yoshino rivers (Nanato, 30; Oboke, 30).

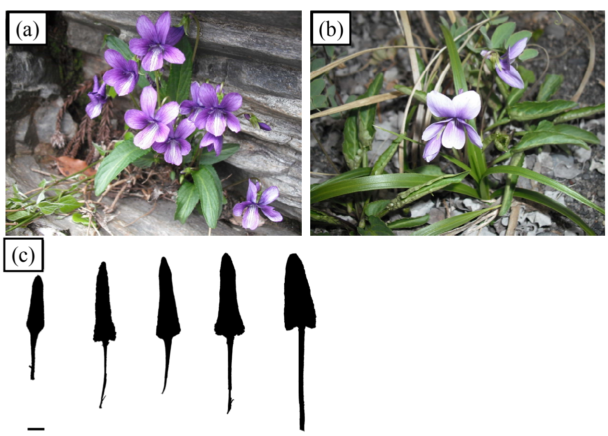

Figure 1. *Viola mandshurica* varieties. (a) Rheophytic ecotype of *V. mandshurica* var. *ikedaeana*; (b) *V. mandshurica* var. *mandshurica*; (c) Selected leaf silhouettes of *V. mandshurica* var. *ikedaeana* showing wide range of leaf shape. Bar = 1 cm.

2.2. Morphological Analyses

For morphological analysis, individuals were measured for the following continuous macromorphological leaf variables: 1) length and width of the leaf blade; 2) leaf thickness; and 3) angle of the leaf base. Measurements were made using a digimatic calliper (CD-15CXR; Mitutoyo, Kanagawa, Japan) and a digimatic outside micrometer (MDC-SB; Mitutoyo, Kanagawa, Japan). The leaf size was calculated by using the following formula: (leaf length × leaf width)/2. The leaf index was calculated as the ratio of the leaf length to the leaf width, according to [21].

2.3. Anatomical Analyses

For anatomical analysis, fully expanded leaves were collected from each individual. To count the number of cells on the blade, the adaxial surface of the leaves was peeled

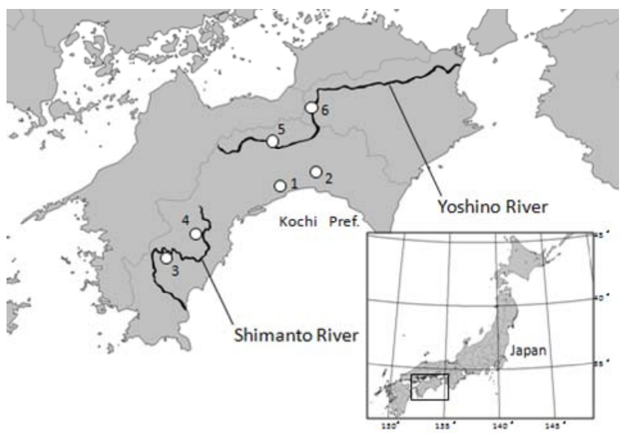

Figure 2. Sampling localities used in this study. Additional information is provided in Table 1.

Table 1. Sampling localities used in this study.

Species	Locality name	Locality no.	Locality	Latitude and longitude
V. mandshurica var. *mandshurica*	Asakura	1	Kochi Prefecture, Kochi-City, Akebono-Cho, Asakura	33˚55'N 133˚49'E
	Noichi	2	Kochi Prefecture, Kounan-City, Noichi-Cho, Nishino	33˚56'N 133˚69'E
V. mandshurica var. *ikedaeana*	Towa	3	Kochi Prefecture, Takaoka-Gun, Shimanto-Cho, Towa	33˚22'N 132˚83'E
	Nakahira	4	Kochi Prefecture, Takaoka-Gun, Yusuhara-Cho, Nakahira	33˚33'N 132˚96'E
	Nanato	5	Kochi Prefecture, Nagaoka-Gun, Motoyama-Cho, Nanato	33˚82'N 133˚55'E
	Oboke	6	Tokushima Prefecture, Miyoshi-City, Yamashiro-Cho, Kamimyo	33˚88'N 133˚76'E

Locality no. corresponds to that given in **Figure 2**.

off by using Suzuki's Universal Micro-Printing (SUMP) method. The number of epidermal cells was calculated by using following formula: leaf size/cell size. Replicas of each leaf (1 cm^2) were prepared to determine the stomatal density (number per mm^2) and to measure the epidermal cell size of 10 cells per leaf. These copied SUMP images were examined once for each individual, with a light microscope (CX41; Olympus Co., Tokyo, Japan). Statistical analyses were performed using Tukey's honestly significant difference (HSD) test and the Steel-Dwass test to compare the characteristics of *V. mandshurica* var. *mandshurica* and the rheophytic ecotype of *V. mandshurica* var. *ikedaeana*.

3. Results

3.1. Morphological Analyses of *V. mandshurica* var. *mandshurica* and the Rheophytic Ecotype of *V. mandshurica* var. *ikedaeana*

The leaf lengths of *V. mandshurica* var. *mandshurica* (2 populations—Asakura and Noichi) and *V. mandshurica* var. *ikedaeana* (4 populations—Towa, Nakahira, Nanato and Oboke) were 34.77 ± 0.97 mm, 59.79 ± 1.16 mm, 31.13 ± 1.99 mm, 31.65 ± 1.84 mm, 32.94 ± 1.62 mm, and 35.62 ± 1.68 mm, respectively; the leaf widths were 21.73 ± 2.14 mm, 29.93 ± 1.57 mm, 8.35 ± 0.37 mm, 10.77 ± 0.55 mm, 10.64 ± 0.31 mm, and 11.95 ± 0.51 mm, respectively; and the average angles of the leaf base were 156.49 ± 9.64 degrees, 180.94 ± 1.55 degrees, 72.97 ± 2.67 degrees, 125.72 ± 10.00 degrees, 103.91 ± 3.08 degrees, and 105.47 ± 3.85 degrees, respectively (**Table 2**). The leaf thicknesses were 191.83 ± 5.22 µm, 219.29 ± 4.12 µm, 311.52 ± 10.02 µm, 246.84 ± 4.02 µm, 274.87 ± 5.04 µm, and 252.74 ± 4.30 µm, respectively. The resulting leaf sizes for *V. mandshurica* var. *mandshurica* and the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* were 377.80 ± 60.63 mm^2, 894.75 ± 60.54 mm^2, 129.98 ± 19.72 mm^2, 170.41 ± 21.80 mm^2, 175.28 ± 17.26 mm^2, and 212.86 ± 21.37 mm^2, respectively. With the exception of leaf length, all of the macromorphological leaf traits differed significantly between *V. mandshurica* var. *mandshurica* and the rheophytic ecotype of *V. mandshurica* var. *ikedaeana*.

The leaf index of *V. mandshurica* var. *mandshurica* (1.60 ± 0.07 [Asakura], 2.04 ± 0.05 [Noichi]) was significantly lower than that of the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* (3.70 ± 0.12 [Towa], 2.94 ± 0.08 [Nakahira], 2.34 ± 0.12 [Nanato], 3.27 ± 0.10 [Oboke]).

3.2. Anatomical Analyses of *V. mandshurica* var. *mandshurica* and the Rheophytic Ecotype of *V. mandshurica* var. *ikedaeana*

The cell sizes of *V. mandshurica* var. *mandshurica* (2 populations—Asakura and Noichi) and *V. mandshurica* var. *ikedaeana* (4 populations—Towa, Nakahira, Nanato and Oboke) were 6585.97 ± 362.66 µm^2, 8241.96 ± 279.70 µm^2, 5241.65 ± 117.68 µm^2, 6346.67 ± 271.50 µm^2, 6252.58 ± 145.25 µm^2, and 6911.77 ± 650.03 µm^2. The cell size did not differ significantly between *V. mandshurica* var. *mandshurica* and the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* (**Table 2**). Based

Table 2. Morphological and anatomical measurements of *V. mandshurica* var. *mandshurica* and *V. mandshurica* var. *ikedaeana*.

Trait	*V. mandshurica* var. *mandshurica*		*V. mandshurica* var. *ikedaeana*			
	Asakura	Noichi	Towa	Nakahira	Nanato	Oboke
Leaf length (mm)	34.77 ± 0.97[b]	59.79 ± 1.16[a]	31.13 ± 1.99[b]	31.65 ± 1.84[b]	32.94 ± 1.62[b]	35.62 ± 1.68[b]
Leaf width (mm)	21.73 ± 2.14[b]	29.93 ± 1.57[a]	8.35 ± 0.37[d]	10.77 ± 0.55[cd]	10.64 ± 0.31[cd]	11.95 ± 0.51[c]
Leaf size (mm^2)	377.80 ± 60.63[b]	894.75 ± 60.54[a]	129.98 ± 19.72[c]	170.41 ± 21.80[c]	175.28 ± 17.26[c]	212.86 ± 21.37[c]
Leaf index[1]	1.60 ± 0.07[e]	2.04 ± 0.05[d]	3.70 ± 0.12[a]	2.94 ± 0.08[b]	2.34 ± 0.12[b]	3.27 ± 0.10[ab]
Leaf thickness (μm)	191.83 ± 5.22[c]	219.29 ± 4.12[b]	311.52 ± 10.02[a]	246.84 ± 4.02[a]	274.87 ± 5.04[a]	252.74 ± 4.30[a]
Angle of leaf base (degrees)	156.49 ± 9.64[b]	180.94 ± 1.55[a]	72.97 ± 2.67[d]	125.72 ± 10.00[c]	103.91 ± 3.08[c]	105.47 ± 3.85[c]
Epidermal cell size (μm^2)	6585.97 ± 362.66[ab]	8241.96 ± 279.70[a]	5241.65 ± 117.68[c]	6346.67 ± 271.50[bc]	6252.58 ± 145.25[bc]	6911.77 ± 650.03[ab]
Epidermal cell number	73544.4 ± 7440.3[a]	84750.8 ± 6098.7[a]	32302.3 ± 4208.3[b]	30373.9 ± 2768.2[b]	39203.6 ± 2677.5[b]	36797.0 ± 3319.9[b]
Stomatal density (number per mm^2)	122.88 ± 4.64[b]	158.02 ± 4.42[a]	121.27 ± 3.74[b]	172.99 ± 10.41[a]	144.10 ± 6.03[ab]	173.02 ± 8.83[a]
Guard cell size (μm^2)	122.45 ± 5.51[c]	132.81 ± 4.91[c]	250.39 ± 4.02[b]	281.95 ± 8.40[a]	285.19 ± 6.56[a]	266.67 ± 3.51[ab]

All measurements are represented as mean ± standard deviation. Columns marked by different letters showing significant differences according to Tukey's HSD test ($p < 0.05$); [1]Nonparametric pairwise comparison was conducted by using the Steel-Dwass test.

on the leaf size and cell size, we calculated the number of epidermal cells per leaf. The cell numbers for *V. mandshurica* var. *mandshurica* and the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* were 73544.4 ± 7440.3, 84750.8 ± 6098.7, 32302.3 ± 4208.3, 30373.9 ± 2768.2, 39203.6 ± 2677.5, and 39797.0 ± 3319.9, respectively. Leaves of *V. mandshurica* var. *mandshurica* contained a significantly higher number of cells than did those of the rheophytic ecotype of *V. mandshurica* var. *ikedaeana*. The stomatal density of *V. mandshurica* var. *mandshurica* (122.88 ± 4.64 and 158.02 ± 4.42) did not differ significantly from that of the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* (121.27 ± 3.74, 172.99 ± 10.41, 144.10 ± 6.03, and 173.02 ± 8.83). By contrast, the stomatal size (250.39 ± 4.02 μm^2 [Towa], 281.95 ± 8.40 μm^2 [Nakahira], 285.19 ± 6.56 μm^2 [Nanato], and 266.67 ± 3.51 μm^2 [Oboke]) of the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* was significantly larger than that of *V.* var. *mandshurica* (122.45 ± 5.51 μm^2 [Asakura] and 132.81 ± 4.91 μm^2 [Noichi]).

4. Discussion

The speciation of inland rheophyte species appears to be associated with stenophyllization, and rheophytes have been shown as scattered among various taxa, from bryophytes to angiosperms [5]. In the present study, we have demonstrated that rheophytic ecotype of *V. mandshurica* var. *ikedaeana* has a significantly narrower leaf than *V. mandshurica* var. *mandshurica*, indicating that *V. mandshurica* var. *ikedaeana* is the rheophytic species. Some other rheophytic species, such as *Osmunda lancea* Thunb. (Osmundaceae) [7,8] and *Astilbe japonica* (C. Morren et Decne.) A. Gray (Saxifragaceae) [22], were found in our sampling localities along the Shimanto and Yoshino rivers of Shikoku (data not shown). Moreover, rheophytes of *R. ripense*, *Adenophora triphylla* var. *japonica*, and *Aster microcephalus* var. *ripensis* were previously collected from neighbouring areas along these rivers [11-13]. Taken together, these findings suggest that strong selection pressures caused by high flooding frequency affects the leaf form along these riversides, thereby leading to the selection of rheophytic species.

Our results indicate that a decrease in the number of cells in a leaf contributes to stenophyllization of the rheophytic ecotype of *V. mandshurica* var. *ikedaeana*. This anatomical mechanism of stenophyllization is similar to that previously reported for *R. ripense* [13], *R. indicum* (L.) Sweet f. *okutami* T. Yamaz. [14], and *F. japonicum* var. *luchuense* [9,23]. These species are of different lineages from *V. mandshurica* var. *ikedaeana*, suggesting that the same stenophyllization process operates independently among rheophytic taxa. Previous anatomical studies have shown a decreased number of cells in all angiosperm rheophytes [9-14,23], indicating that this is a common mechanism contributing to stenophyllization. Based on angiosperm phylogeny [15-18], variation of cell size in the rheophytic *Ad. triphylla* var.

japonica and *As. microcephalus* var. *ripensis* may be an additional process of stenophyllization in the advanced lineage of the Asterid Group. By contrast, alterations in the cell-elongation process appear to be responsible for the narrow, thickened leaves of fern rheophytes [6,7]. Therefore, the stenophyllization process of rheophytes differs between angiosperms and ferns.

Our morphological data for the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* indicate that the angle of the leaf base is strongly correlated with the length and width of a leaf. Moreover, the decreasing angle of the leaf base results in a lanceolate leaf, indicating that stenophyllization of leaves of the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* occurred with the transition from triangular- and oblong-lanceolate leaves to hastate leaves. The general tendency for plants growing closer to streams to have lanceolate leaves may be caused by selection pressures of habitats along a gradually decreasing flooding frequency, from streambed to inland [24]. Therefore, based on its macromorphological characteristics and lanceolate leaves, the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* appears to be tolerant of swift-running streams.

In the present study, we further demonstrated that the leaf thickness and stomatal size of the rheophytic ecotype of *V. mandshurica* var. *ikedaeana* were significantly larger than those of *V. mandshurica* var. *mandshurica*. Previous studies of *F. japonicum* var. *luchuense* and *R. ripense* showed that individuals growing on riverbanks had larger leaf thicknesses than did those of inland plants [9,14,23]. Taken together, these findings indicate that increased leaf thickness is a general tendency among rheophytes—*V. mandshurica* var. *ikedaeana*, *R. ripense*, and *F. japonicum* var. *luchuense* show a similar pattern of differentiation but are widely separated phylogenetically. Moreover, leaf thickness and stomatal size are positively correlated with the mean solar radiation during leaf expansion [25,26]. In general, stomata are the main gates for gaseous exchange of leaves [27-29]. The guard cells that surround the stomata contain chloroplasts, and are therefore able to increase their sugar concentration; this, in turn, causes water absorption and swelling of the cells [30,31]. In addition, stomatal conductance depends on leaf characteristics such as size, number, and stomatal density [32-34]. The rheophytic ecotype of *V. mandshurica* var. *ikedaeana* grows mainly in riverside habitats—on sunny, moist rocks and on riverbanks—in southern Shikoku, Japan; therefore, the condition of high irradiance along the riverside may lead to increased leaf thickness and stomatal size.

In summary, we have analysed the evolution of the rheophytic ecotype of *V. mandshurica* var. *ikedaeana*, by using morphological and anatomical data. The results provide an unbiased interpretation of the rheophytic evolution of angiosperms. Our data clearly indicate the rheophytic process of *V. mandshurica* var. *ikedaeana*, but do not provide a definitive interpretation of the rheophytic pattern of adaptation. In the Violaceae, for example, [35] reported that *V. grypoceras* A. Gray var. *ripensis* N. Yamada et Okamoto had a rheophytic distribution on some riversides in central to western Japan, and that the leaves were smaller and narrower than those of its closely related variety, *V. grypoceras* var. *grypoceras*. However, whether the process is caused by a decrease in the size or number of leaf cells remains to be determined. Further studies based on samplings that are more comprehensive and additional taxa are required to support our working hypothesis of cell alteration in rheophytes. Thus, we believe that our morphological and anatomical data will stimulate future studies of diversity in rheophytes.

5. Acknowledgements

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