

Coastal Adaptation of *Adenophora triphylla* var. *japonica* (Campanulaceae)

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ABSTRACT

The comparative morphology and anatomy of leaves between the coastal ecotype and the normal type of *Adenophora triphylla* (Thunb.) A.DC. var. *japonica* (Regel) H.Hara (Campanulaceae) were examined to clarify the differences in morphological characters between the 2 groups. Morphological and anatomical analyses revealed that the coastal ecotype had a thicker leaf than the normal type, because of the increased size of epidermal and spongy cells. Thus, the main morphological change from the normal type into the coastal ecotype of *A. triphylla* var. *japonica* is the increase in leaf size, suggesting that the coastal ecotype may have evolved from the normal type via a heterochronic process.

Keywords: Adaptation; *Adenophora triphylla* var. *japonica*; Coastal; Ecotype; Heterochronic; Leaf Thickness

1. Introduction

Plant diversity has fascinated humans throughout history; the diversity is presumably plant adaptation to different natural environments, primarily due to the tremendous variations in morphological traits that exist in nature. The fitness effects of such naturally occurring variation present within or among species have driven plant evolution by natural selection [1]. In addition, considerable variation exists within many species, which likely reflects adaptations to different natural environments and is the origin of plant species differentiation [2].

One of the major challenges of plant biology is to describe and understand the morphological mechanisms behind naturally occurring variations for adaptation to various environments. Coastal regions are one type of specific environment that can lead to morphological modification. Plant species growing in coastal regions need to be adapted to an environment in which drought strongly affects plant growth [3]. Moreover, water availability is the main environmental factor limiting photosynthesis and growth even in plants well adapted to coastal conditions [3]. Another source of stress and an abiotic driver of natural selection in coastal plant communities is soil salinity [4,5]. Different levels of salt spray can result in vegetation zonation; plants adapted to salt spray grow close to the ocean and are replaced by less salt-resistant

plants further inland [6,7]. Therefore, plants in coastal regions have developed various interesting morphological adaptations including succulent tissues to store water, a pubescent epidermis and a thick cuticle to reduce transpiration and water loss, belowground structures to withstand sand burial, and an annual habit [8].

Adenophora triphylla (Thunb.) A.DC. var. *japonica* (Regel) H.Hara belonged to Campanulaceae is distributed in Japan, Korea, and Sahalin, and includes distinct morphological variations [9] to adapt to various environments. For instance, the rheophytic ecotype of this variety is found along rivers [10]. This ecotype has a narrower leaf than the non-rheophytic type because of the decreased number and size of the leaf cells [11]. The serpentine ecotype of *A. triphylla* var. *japonica* also had narrow leaves, but the leaf thickness and stomatal density was significantly different from those of the normal plants, suggesting that there were different adaptation processes between the two ecotypes of this species [12]. From these studies, it appears that *A. triphylla* var. *japonica* can easily alter its morphology and has several ecotypes in various environments. We found the coastal ecotype of *A. triphylla* var. *japonica*, which has thick leaves and stems (**Figure 1**), in some coastal areas of Kochi Prefecture in Japan (**Figure 2**), where it grows along with other coastal taxa such as *Cirsium maritimum* Makino (Asteraceae), *Dianthus japonicus* Thunb. (Caryophyllaceae), *Setaria viridis* (L.) P.Beauv. var. *pachy-*

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stachys (Franch. et Sav.) Makino et Nemoto (Poaceae), and some CAM plants of Crassulaceae. It is very important to understand the morphological modifications to adapt to a specific environment by analysing ecotypic species. Therefore, we characterise the variation of their leaves by morphological and anatomical analyses of the coastal ecotype of *A. triphylla* var. *japonica*.

2. Materials and Methods

2.1. Plant Materials

All samples of *A. triphylla* var. *japonica* examined in this study were collected from the field between August and November 2011. A total of 130 individuals representing 4 populations were sampled from across the range of the species (Table 1, Figure 2).

2.2. Morphological Analyses

For morphological analysis, individuals were measured for the following continuous macro morphological variables of leaves and stems: 1) length and width of the leaf blade; 2) leaf thickness; 3) angle of the leaf base; 4) plant height; and 5) stem width at the middle point of plant height. Measurements were made using a digi matic cal-

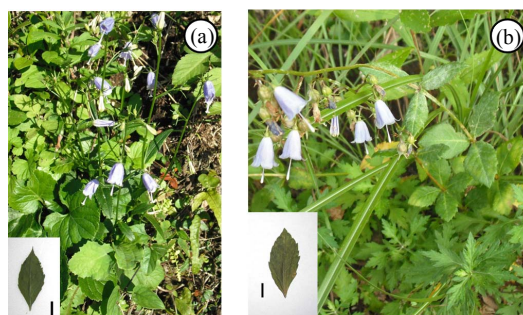


Figure 1. Plants of *Adenophora triphylla* var. *japonica*. (a) normal type; (b) coastal ecotype. Bar = 1 cm.

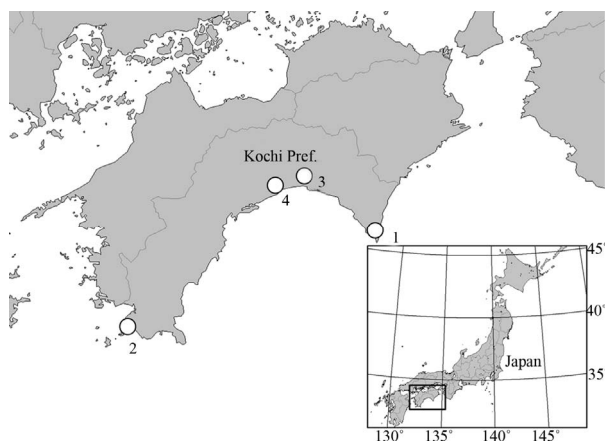


Figure 2. Sampling localities in this study. For other information, see Table 1.

iper (CD-15CXR; Mitutoyo, Kanagawa) and a digimatic outside micrometer (MDC-SB; Mitutoyo, Kanagawa). Leaf measurements were taken from a fully expanded leaf at the middle point of the plant height. The leaf size value was calculated by the following formula: leaf length \times leaf width/2. The leaf index value was calculated as the ratio of the leaf length to the leaf width, according to [13].

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 leaves were peeled off by Suzuki's Universal Micro-Printing (SUMP) method. The number of epidermal cells was calculated by following formula: leaf size/cell size. Replicas of each leaf (1 cm²) were prepared to determine the stomatal density (number/mm²) and to measure the epidermal cell size of 10 cells per leaf. These copied SUMP images were examined once for each individual using a light microscope (CX41; OLYMPUS Co., Tokyo). The leaves were fixed overnight in a solution of formaldehyde, ethanol, and acetic acid (FAA). To observe the palisade and spongy cells, the fixed leaves were dehydrated by immersion in a graded ethanol series and then embedded in Histparaffin to section at 8- μ m-thickness by using a rotary microtome (PR-50; Yamatokoki, Kanagawa). The widest part of the blade was analysed to determine the height of adaxial and abaxial epidermal cells and palisade and spongy cells (10 cells per leaf) (Figure 3). Slides were examined using a light microscope. Statistical analyses were performed using a Tukey's honestly significant difference (HSD) test and Steel-Dwass test to compare the characteristics of the 2 ecotypes.

3. Results

The average heights of *A. triphylla* var. *japonica* from the 2 coastal populations, Muroto and Otsuki, and the 2 populations of the normal type, Higashisako and Hitsuzan, were 63.34 ± 13.23 cm, 56.96 ± 22.21 cm, 47.15 ± 9.50 cm, and 40.95 ± 13.60 cm, respectively, and the average widths were 2.40 ± 0.58 mm, 2.00 ± 0.57 mm, 1.54 ± 0.31 mm,

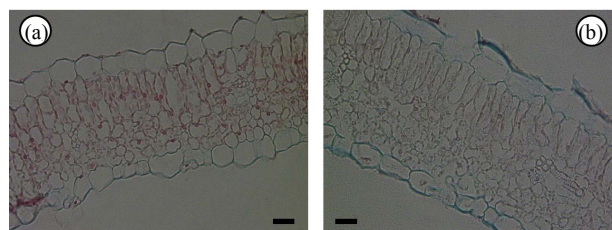


Figure 3. Transverse sections of the leaves of *Adenophora triphylla* var. *japonica*. (a) normal type; (b) coastal ecotype. Bar = 25 μ m.

Table 1. Sampling localities in this study.

Type	Locality Name	Locality No.	Number of Individuals	Locality	Latitude and longitude
Coastal	Muroto	1	34	Kochi Prefecture, Muroto City, Murotomisaki-Cho, Muroto	N 33°15' E 134°10'
	Otsuki	2	31	Kochi Prefecture, Hata-Gun, Otsuki-Cho, Issai	N 32°46' E 132°38'
Normal	Higashisako	3	30	Kochi Prefecture, Konan City, Noichi-Cho, Higashisako	N 33°35' E 133°42'
	Hitsuzan	4	35	Kochi Prefecture, Kochi City, Koishigi-Cho, Hitsuzan	N 33°32' E 133°32'

Locality numbers correspond to that shown in **Figure 2**.

and 1.71 ± 0.60 mm, respectively (**Table 2**). Although plant height was significantly different between the coastal ecotype and the normal type, the stem width was similar in each ecotype. The average leaf thickness was 230.10 ± 23.28 μm in Muroto, 228.90 ± 35.92 μm in Otsuki, 211.03 ± 19.00 μm in Higashisako, and 199.64 ± 23.28 μm in Hitsuzan. Although leaf thickness was significantly different between the coastal ecotype and the normal type, there were no significant differences in the leaf length, leaf width, or leaf size. Anatomical analyses of cell size, total number of cells, and stomatal density found no significant differences between the coastal ecotype and the normal type. Cross sections from a sample of leaves were examined to measure the height of epidermal, palisade, and spongy cells. The average heights of leaf epidermal cells of samples from Muroto, Otsuki, Higashisako, and Hitsuzan were 35.63 ± 4.99 μm , 38.38 ± 4.41 μm , 28.51 ± 1.98 μm , and 30.15 ± 3.61 μm , respectively, on the adaxial side; and 20.40 ± 1.28 μm , 21.98 ± 2.84 μm , 19.15 ± 1.78 μm , and 19.64 ± 2.90 μm , respectively, on the abaxial side. The heights of the palisade cells were 68.84 ± 19.40 μm , 96.92 ± 17.11 μm , 67.96 ± 13.36 μm , and 69.70 ± 16.79 μm , respectively, and the spongy cell height was 84.74 ± 6.81 μm , 85.52 ± 15.62 μm , 59.17 ± 8.82 μm , and 69.10 ± 11.05 μm , respectively. Of these, the adaxial epidermal cell and spongy cell heights were significantly different between the coastal ecotype and normal type.

4. Discussion

Coastal ecotypes of plants have modifications of various characters to adapt to stresses in the environment. Flavonoids are effective UV-B screening compounds that are synthesised in plants [14,15]. For the coastal ecotype of *A. triphylla* var. *japonica*, [16] indicated that the flavonoid composition was not significantly different in quality but was lower in quantity than that in the normal

(inland) type, based on chemical analyses. Further, leaf morphology rather than flavonoid composition may play an important role in adaptation to the coastal environment. In this study, the leaves of the coastal ecotype were thicker than those of the normal ecotype (**Table 2**). Moreover, the epidermal cells and spongy tissues of the coastal ecotype of *A. triphylla* var. *japonica* were larger than those of the normal type of this variety, indicating that the thick leaf of the coastal ecotype strongly contributed to the variation in epidermal cells and spongy tissues. Thus, in this ecotype, increase in leaf thickness because of the large-sized epidermal cells and photosynthetic organs such as spongy tissue might be more important for adapting to the coastal environment than that by the differences in chemical compositions. Although a previous anatomical study showed that the epidermal cells of the coastal variety of *Aster hispidus* Thunb. var. *insularis* (Makino) Okuyama (Asteraceae), were larger in size but fewer in number than those of *Aster hispidus* var. *hispidus* [17], our results indicated that the number of epidermal cells in the coastal ecotype in our study species was not lower than in the normal type. To clarify the general tendency of anatomical processes of adaptation in coastal areas, further comparative studies using another coastal ecotype need to be conducted.

In our results, the greatest morphological change from the normal type to the coastal ecotype of *A. triphylla* var. *japonica* was the increase in size. Such differences were occasionally involved in the heterochronic process, which is a model using morphological size and shape as independent variables to account for morphological differences among related taxa [18-20]. For instance, differences in leaf size and shape between the rheophytic *Osmunda lancea* Thunb. (Osmundaceae) and the closely related inland species *O. japonica* Thunb. may be related to the heterochronic process of progenesis [21]. Moreover, the leaf shapes of the species of Marsileaceae indi-

Table 2. Morphological and anatomical measurements (average \pm standard deviation) of *Adenophora triphylla* var. *japonica*.

Trait	Normal				Coastal			
	Higashisako		Hitsuzan		Muroto		Otsuki	
Morphological measurements								
Leaf length (mm)	50.58 \pm 9.88	a	39.22 \pm 13.16	b	47.84 \pm 11.31	a	44.28 \pm 14.00	ab
Leaf width (mm)	17.92 \pm 2.84	b	13.10 \pm 5.11	c	22.74 \pm 4.19	a	16.51 \pm 4.84	b
Leaf thickness(μ m)	211.03 \pm 19.00	b	199.64 \pm 23.28	b	230.10 \pm 23.28	a	228.90 \pm 35.92	a
Angle of leaf base ($^{\circ}$)	46.08 \pm 10.57	b	46.08 \pm 17.52	b	70.74 \pm 21.30	a	52.74 \pm 16.75	b
Leaf size (mm ²)	457.35 \pm 132.07	ab	275.42 \pm 176.85	c	555.84 \pm 194.36	a	375.49 \pm 179.03	bc
Leaf index ¹	2.87 \pm 0.61	a	3.22 \pm 1.10	a	2.12 \pm 0.40	b	2.84 \pm 1.27	a
Plant height (mm)	47.15 \pm 9.50	b	40.95 \pm 13.60	b	63.34 \pm 13.23	a	56.96 \pm 22.21	a
Stem width (mm)	1.54 \pm 0.31	c	1.71 \pm 0.60	bc	2.40 \pm 0.58	a	2.00 \pm 0.57	ab
Anatomical measurements								
Epidermal cell size at adaxial side (μ m ²)	2639.73 \pm 576.07	b	2912.29 \pm 563.32	ab	3290.00 \pm 666.79	a	2805.52 \pm 562.40	b
Epidermal cell number at adaxial side	141121.1 \pm 56693.7	ab	104530.0 \pm 72319.7	b	182783.2 \pm 73654.1	a	126002.5 \pm 75080.8	b
Stomatal density (N/mm ²)	306.31 \pm 74.03	a	256.63 \pm 49.75	bc	243.16 \pm 52.67	c	295.11 \pm 56.09	ab
Epidermal cell height at adaxial side (μ m)	28.51 \pm 1.98	c	30.15 \pm 3.61	c	35.63 \pm 4.99	b	38.38 \pm 4.41	a
Epidermal cell height at abaxial side (μ m)	19.15 \pm 1.78	b	19.64 \pm 2.90	b	20.40 \pm 1.28	b	21.98 \pm 2.84	a
Palisade cell height (μ m)	67.96 \pm 13.36	b	69.70 \pm 16.79	b	68.84 \pm 19.40	b	96.92 \pm 17.11	a
Spongy cell height (μ m)	59.17 \pm 8.82	c	69.10 \pm 11.05	b	84.74 \pm 6.81	a	85.52 \pm 15.62	a

Columns marked by different letters differ significantly according to the Tukey's HSD test ($p < 0.05$). ¹Nonparametric pairwise comparison was conducted "Steel-Dwass test".

cate that heterochrony plays a crucial role in leaf-shape development [22]. As for *A. triphylla* var. *japonica*, the growing areas of the coastal ecotype were warmer than those of the normal ecotype because of the proximity to the Pacific Ocean. Therefore, these differences between the coastal ecotype and normal type suggested that the coastal ecotype of *A. triphylla* var. *japonica* may have evolved from the normal type via a heterochronic process leading to pre-displacement, which is the earlier initiation of a developmental process.

Some recent studies have provided evidence for adaptation to local environments using the genetic model plant, *Arabidopsis thaliana* (L.) Heynh. [23-25]. This analysis has begun only recently thanks to 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 [26-28]. Patterns of phenotypic diversity across environmental gradients can be indicative of adaptive responses to selection, and evaluating these patterns could lead to the identification of the genetic polymorphisms underlying these adaptive responses. As for the coastal

environment, for example, a weak allele of *AtHKT1;1* that drives elevated leaf Na⁺ in this population has been previously linked to elevated salinity tolerance using genome-wide association mapping, genetic complementation, and gene expression analyses; and the geographical distribution of this allele revealed its significant enrichment in populations associated with the coast and saline soils [29]. Therefore, similar genomic comparative analyses of this allele between the coastal ecotype and the normal type of *A. triphylla* var. *japonica* will extend the study of coastal adaptation in this species. In addition, a comparison of the molecular mechanisms involved will provide an integrated functional perspective on the coastal adaptation of *A. triphylla* var. *japonica*.

Our study, along with [11,12], indicated that *A. triphylla* var. *japonica* can be categorised into serpentine, rheophytic, and coastal ecotypes in addition to the normal type. *A. triphylla* var. *japonica* f. *violacea* (H.Hara) T.Shimizu was distributed in the mountain areas of northern Honshu and Hokkaido, Japan [9]. Therefore, it will be very interesting to conduct comparative morphological and anatomical analyses of *A. triphylla* var. *ja-*

ponica f. *violacea* to understand its adaptations to mountain areas in the next stage of research.

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