

Molecular and Morphological Data Confirm the Occurrence of Two Varieties of *Helwingia japonica* subsp. *japonica* (Helwingiaceae) in Kochi Prefecture, Japan

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Abstract Twenty-six plants of *Helwingia japonica* subsp. *japonica*, including 23 plants from Kochi Prefecture and three plants from other areas of Japan with other subspecies of this species, were taxonomically compared using molecular and morphological data. The molecular phylogenetic analyses placed the 23 Kochi plants in two major clades. Student's *t*-test revealed a significant difference in the number of leaf lateral vines between the Kochi plants of the two clades. Based on taxonomy, the two groups were respectively identified as *H. japonica* subsp. *japonica* var. *japonica* and var. *parvifolia*. The present study further confirmed that two varieties are distributed in Kochi, Japan, and suggests that gene exchange between the two varieties sympatrically distributed may be hindered by certain isolation mechanism.

Key words: *Helwingia*, ITS, Kochi Prefecture, leaf lateral vine.

Introduction

The genus *Helwingia* Willd., morphologically characterized by epiphyllous inflorescences, is composed of four species in East Asia (Hara and Kurosawa, 1975; Xiang and Boufford, 2005). This genus was traditionally a member of the family Cornaceae (Noshiro, 1999), but recent molecular phylogenetic studies separated as a monotypic family Helwingiaceae (APGIII, 2009), which is thought to be closely related to *Phyllonoma* (Phyllonomaceae), having epiphyllous inflorescences and distributing in Mexico and Bolivia (Mori and Kallunki, 1977), by Tank

and Donoghue (2010), and Soltis *et al.* (2011).

In Japan, one species *Helwingia japonica* (Thunb.) F.Dietr. with two subspecies is found: *H. japonica* subsp. *liukiensis* (Hatus.) H.Hara et Kuros., is characterized by oblong–lanceolate to ovate leaves of 5–12 cm long and endemic to the Ryukyu Archipelago of Japan (Hara and Kurosawa, 1975); and *H. japonica* subsp. *japonica* is characterized by obovate to elliptic leaves of 4–15 cm long and widely distributed from Japan to Himalayas (Hara and Kurosawa, 1975; Noshiro, 1999; Fig. 1). In the later subspecies, two varieties are reorganized in Japan: *H. japonica* subsp. *japonica* var. *japonica* and var. *parvi-*



Fig. 1. Habitat of *Helwingia japonica* subsp. *japonica* in Kochi. A. Sympatric population with well-branched (arrow) and poorly branched (arrowhead) plants of *H. japonica* subsp. *japonica*. Bar = 15 cm. B. Plants that are well branched. Bar = 1 cm. C. Plants that are poorly branched. Bar = 2 cm.

folia Makino studied herein (Hara and Kurosawa, 1975; Noshiro, 1999).

The two varieties are distinguished based on leaf and stem morphologies: *H. japonica* subsp. *japonica* var. *japonica* has more than four pairs of leaf lateral vines and poorly branched habits, whereas *H. japonica* subsp. *japonica* var. *parvi-*

folia has less than four pairs of leaf lateral vines and well branched habits (Noshiro, 1999; Kobayashi, 2009) (Fig. 1). Furthermore, *H. japonica* subsp. *japonica* var. *japonica* has larger leaves than *H. japonica* subsp. *japonica* var. *parvifolia* (Yamanaka, 1996). In Japan, *H. japonica* subsp. *japonica* var. *japonica* is widely occurred from

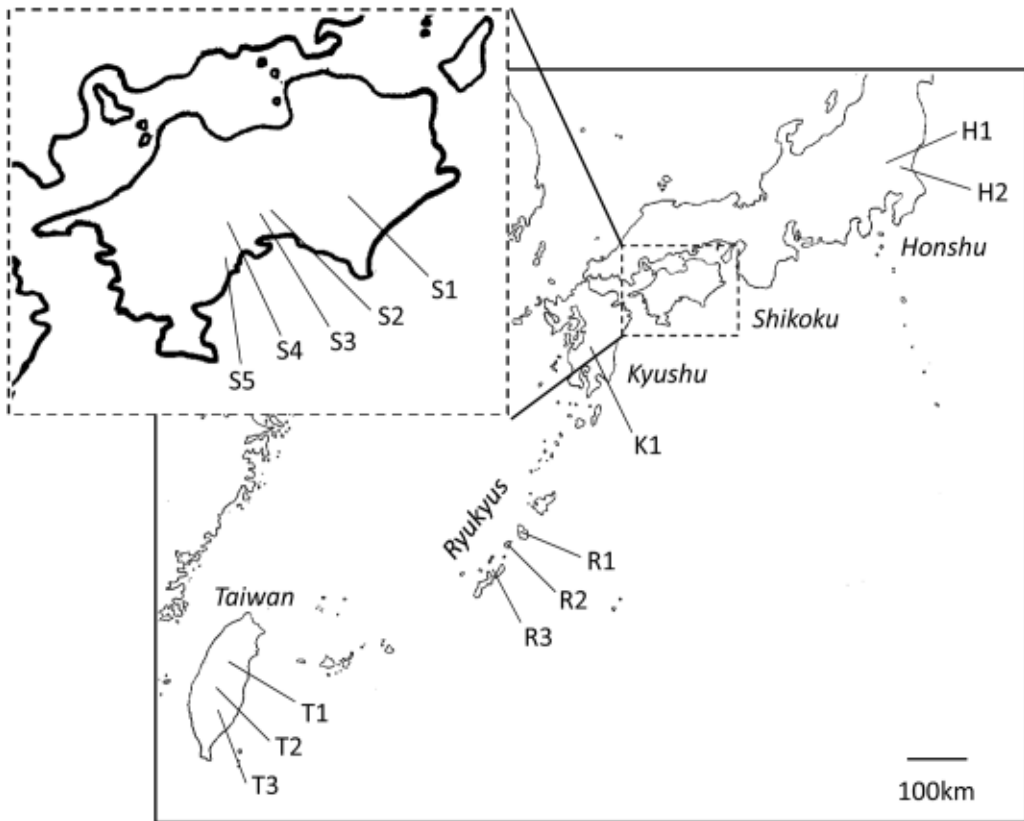


Fig. 2. Map showing the 14 collection localities of 32 individuals of *Helwingia japonica* from Japan and Taiwan. Abbreviations refer to localities are listed in Table 1.

Hokkaido to Kyushu, whereas *H. japonica* subsp. *japonica* var. *parvifolia* is comparatively narrowly distributed from southwestern Honshu to Kyushu (Hara and Kurosawa, 1975). According to Kobayashi (2009), both varieties are distributed in Kochi Prefecture of Shikoku (Fig. 2). In our botanical expedition in Kochi, we found populations with two types of *H. japonica* subsp. *japonica* plants with well-branched and poorly-branched stems (Fig. 1). Although the branching of stems is a key character to separate the two varieties, this character varies in different growth stages and habitats and thereby other salient morphological character should be examined for reliable identification of these plants. In this study, we performed molecular phylogenetic analyses using the internal transcribed spacer (ITS) region of nuclear ribosomal DNA and performed a mor-

phological comparison of leaf lateral vine number to confirm the sympatric occurrence of the two varieties of *H. japonica* subsp. *japonica* var. *japonica* and var. *parvifolia* in Kochi.

Materials and Methods

Plant materials

Information of plant materials for the present molecular phylogenetic analyses and morphological comparisons are provided in Table 1. For the molecular phylogenetic analyses, we collected 26 individuals of *Helwingia japonica* subsp. *japonica* from Honshu (two plants), Kochi of Shikoku (23 plants), and Kyushu (one plant), as well as three individuals of *H. japonica* subsp. *liukiuensis* from the Ryukyu Archipelago of Japan (Hara and Kurosawa, 1975). We also included three

Table 1. Plant materials of *Helwingia japonica* investigated in the present study and their ITS accession numbers and number of leaf lateral vine

Species	Collection locality (altitude)	Abbreviation*	Voucher no.	ITS		Mean of leaf lateral vein in three leaves ($\pm$ SD)	
				Accession no.**	Type		
INGROUP							
<i>H. japonica</i> subsp. <i>japonica</i>	Japan: Honshu, Tochigi, Mt. Amamaki (350 m)	H1	GK10511	AB981678 ^a	A	—	
	Japan: Honshu, Ibaraki, Mt. Gozen (70 m)	H2	GK14915	AB981679 ^a	A	—	
	Japan: Shikoku, Kochi, Behukyo (546 m)	S1	HU39	AB981680 ^a	F	3.00 $\pm$ 0.00	
	Japan: Shikoku, Kochi, Tosa-kuwae (128 m)	S2	HU13	AB981681 ^a	B	4.83 $\pm$ 0.37	
		S2	HU14	AB981682 ^a	F	3.33 $\pm$ 0.47	
		S2	HU15	AB981683 ^a	G	3.17 $\pm$ 0.37	
		S2	HU16	AB981684 ^a	F	3.33 $\pm$ 0.47	
		S2	HU17	AB981685 ^a	B	5.33 $\pm$ 0.47	
		S2	HU18	AB981686 ^a	F	4.00 $\pm$ 0.00	
		S2	HU19	AB981687 ^a	C	4.50 $\pm$ 0.50	
		S2	HU20	AB981688 ^a	C	5.00 $\pm$ 0.58	
		S2	HU21	AB981689 ^a	C	5.00 $\pm$ 0.58	
		S2	HU22	AB981690 ^a	B	4.67 $\pm$ 0.47	
		S3	HU1	AB981691 ^a	C	5.50 $\pm$ 0.50	
	Japan: Shikoku, Kochi, Kawanaji (213 m)	S3	HU2	AB981692 ^a	F	3.00 $\pm$ 0.00	
		S3	HU3	AB981693 ^a	F	3.67 $\pm$ 0.47	
		S3	HU5	AB981694 ^a	F	3.33 $\pm$ 0.47	
		S3	HU6	AB981695 ^a	F	4.00 $\pm$ 0.00	
		S3	HU7	AB981696 ^a	F	4.00 $\pm$ 0.00	
		S3	HU8	AB981697 ^a	D	4.33 $\pm$ 0.47	
		S3	HU9	AB981698 ^a	F	3.50 $\pm$ 0.50	
		S3	HU10	AB981699 ^a	B	5.00 $\pm$ 0.58	
		S3	HU11	AB981700 ^a	B	4.50 $\pm$ 0.50	
		S4	HU63	AB981701 ^a	F	3.33 $\pm$ 0.47	
	Japan: Shikoku, Kochi, Oohana (111 m)	S4	HU63	AB981701 ^a	F	3.33 $\pm$ 0.47	
	Japan: Shikoku, Kochi, Yusunose (66 m)	S5	HU48	AB981702 ^a	F	3.33 $\pm$ 0.47	
subsp. <i>liukiensis</i>	Japan: Kyushu, Kumamoto, Kikuchi	K1	GK17572	AB981703 ^a	E	—	
	Japan: Ryukyus, Amami Island, Asato (380 m)	R1	GK11666	AB981704 ^a	H	—	
	Japan: Ryukyus, Tokunoshima Island, Mt. Inokawa (540 m)	R2	GK9543	AB981705 ^a	H	—	
	Japan: Ryukyus, Okinawa Island, Mt. Yae (225 m)	R3	GK10747	AB981706 ^a	H	—	
	subsp. <i>taiwaniana</i>	Taiwan: Kaohsiung, Taoyuan Hsiang (2451 m)	T1	GK10871	AB981707 ^a	I	—
		Taiwan: Nantou, Chitou (1119 m)	T2	GK14991	AB981708 ^a	J	—
	Taiwan: Nantou, Meifan (2361 m)	T3	GK15097	AB981709 ^a	I	—	
OUTGROUP							
<i>Phyllonoma ruscifolia</i>				AJ492650 ^b		—	

* Referring to Fig. 1.

** ^aThe present study; ^bManen *et al.* (2002).

individuals of *H. japonica* subsp. *taiwaniana* Y.-P. Yang et H.-Y. Liu, which is endemic to Taiwan, and characterized by oblong–lanceolate to elliptic leaves of 5–12 cm long (Hara and Kurosawa, 1975). In total, 32 plant samples of *H. japonica* from Japan and Taiwan were investigated in the molecular analyses. Voucher specimens for the present molecular analyses were deposited in the herbarium of the National Museum of Nature and Science (TNS). The ITS sequence of *Phyllonoma ruscifolia* Willd. ex Schult. (Manen *et al.*, 2002) was downloaded from GenBank (<http://getentry.ddbj.nig.ac.jp/getentry/na/AJ492650>), and was used as an out-group following previous studies (Tank and Donoghue, 2010; Soltis *et al.*, 2011).

DNA extraction, PCR, and sequencing

Genomic DNA was extracted from silica gel-dried leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. The genomic DNA samples were deposited in the Molecular Biodiversity Research Center of the National Museum of Nature and Science. The ITS region was amplified using polymerase chain reaction (PCR) with an iCycler (Bio-Rad, Hercules, CA, USA). Forward primer AB101 (5'-ACG AAT TCA AGG TCC GGT GAA TGT TTC G-3') and reverse primer AB102 (5'-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3') were used for PCR (Douzery *et al.*, 1999). Amplifications were performed using the TaKaRa Sapphire Amp Fast Master Mix (TaKaRa, Otsu, Japan) with Sapphire Amp (TaKaRa). The PCR profile was 35 cycles of 5 s at 94°C, 5 s at 50°C, and 5 s at 72°C after an initial denaturing for 3 min at 94°C. PCR products were checked by electrophoresis before purification using illustra ExoProStar (GE Healthcare, Tokyo, Japan). Cycle sequencing reaction was performed with the BigDye™ Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA). Primers used for sequencing were AB101, AB102, ITS2N (5'-TCGCTGCGTTCTTCATC-3'), and ITS3N (5'-GATGAAGAACGCAGCGA-3') (Douzery *et*

al., 1999). The Sanger sequencing products were then purified using ethanol precipitation. Automated sequencing was performed on a 3130xl Genetic Analyzer (Applied Biosystems). The electropherograms were analyzed using ATGC ver. 4.01 (Genetyx Co., Tokyo, Japan). Sequence data from this study were deposited in the DDBJ database (<http://www.ddbj.nig.ac.jp/>; Table 1).

Phylogenetic analyses

DNA sequences were aligned using the program ClustalW 1.8 (Thompson *et al.*, 1994), and then manually adjusted. Phylogenetic analyses were conducted based on a Bayesian approach using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and a maximum parsimony (MP) criterion using PAUP* version 4.0b10 (Swofford, 2002). The hierarchical likelihood ratio test (hLRT) implemented in MrModeltest 2.2 (Nylander, 2004) was used to estimate the appropriate evolutionary model of nucleotide substitutions. Based on the model selected, two separate runs of Metropolis coupled Markov chain Monte Carlo (MCMCMC) analyses were performed, each with a random starting tree and four chains (one cold and three heated). The MCMCMC length was 2 million generations, and the chain was sampled every 100th generation from the cold chain. The first 5000 sample trees (25% of the total 20,000 sample trees) were discarded as burn-in after checking that the mean standard deviation of split frequencies (ASDSF) reached a stationary state at <0.01 thereafter. As a guide to convergence, the potential scale reduction factors (PSRFs) were close to 1.0 for all parameters in an output table. A 50% majority consensus tree of the output tree file from MrBayes was visualized using TreeView (Page, 1996).

In MP phylogenetic analysis, characters were treated as unordered, and character transformations were equally weighted. The branch collapse option was set to collapse at a minimum length of zero. A heuristic parsimony search was performed with 200 replicates of random additions of sequences with ACCTRAN character optimization, tree bisection–reconnection (TBR) branch

swapping, and MULTREES and STEEPEST DESCENT options on. Statistical support for each clade was assessed using bootstrap analysis (Felsenstein, 1985). A thousand replicates of heuristic searches, with the TBR branch swapping switched on and MULTREES options off, were performed to calculate bootstrap values (BS).

Comparison of leaf lateral vine number

In the morphological comparison of leaf lateral vines, 23 plants collected from Kochi were studied on herbarium specimens (Table 1). Leaf lateral vine number was examined using three leaves per individual to calculate the mean for the individual. After molecular phylogenetic analyses, if 23 plants collected from Kochi are divided into multiple clades, equality of the mean of leaf lateral vine were statistically evaluated using Student's *t*-test between (among) the clades after checking the equality of variances using the *F*-test with PRISM ver. 4 (GraphPad Software, La Jolla, CA, USA).

Results

Phylogenetic distinction based on ITS

After alignment of ITS sequences from 33 operational taxonomic units (OTUs; 32 ingroups and an outgroup member), a matrix of 790 bp was obtained. Ten ITS types (A–J) were recognized in the ingroup of *H. japonica* from Japan and Taiwan (Table 1).

The model of HKY + G was selected for the ITS data. The 50% majority rule consensus tree of all post-burn-in trees is depicted in Fig. 3 with mean branch lengths and posterior probabilities (PPs). Because the topology of the MP strict consensus tree (CI = 0.989; RI = 0.987; RC = 0.976, not shown) was compatible with that of the Bayesian tree, the bootstrap percentage (BS; 1,000 replicates) was plotted on the Bayesian

tree (Fig. 3).

The Bayesian and MP analysis (Fig. 3) showed two major clades in the ingroup (Clades I and II). Clade I consisted of 13 plants of *H. japonica* subsp. *japonica* from Kochi of Shikoku (ITS types F and G), three plants of *H. japonica* subsp. *liukuensis* (ITS type H), and three plants of *H. japonica* subsp. *taiwaniana* (ITS types I and J); and clade II consisted of 1 plant of *H. japonica* subsp. *japonica* from Honshu (ITS type A), Kochi of Shikoku (ITS types B, C and D) and Kyushu (ITS type E) of Japan. Therefore, the present molecular analyses divided 23 plants of *H. japonica* subsp. *japonica* from Kochi into two major clades.

Comparison of the leaf vine number

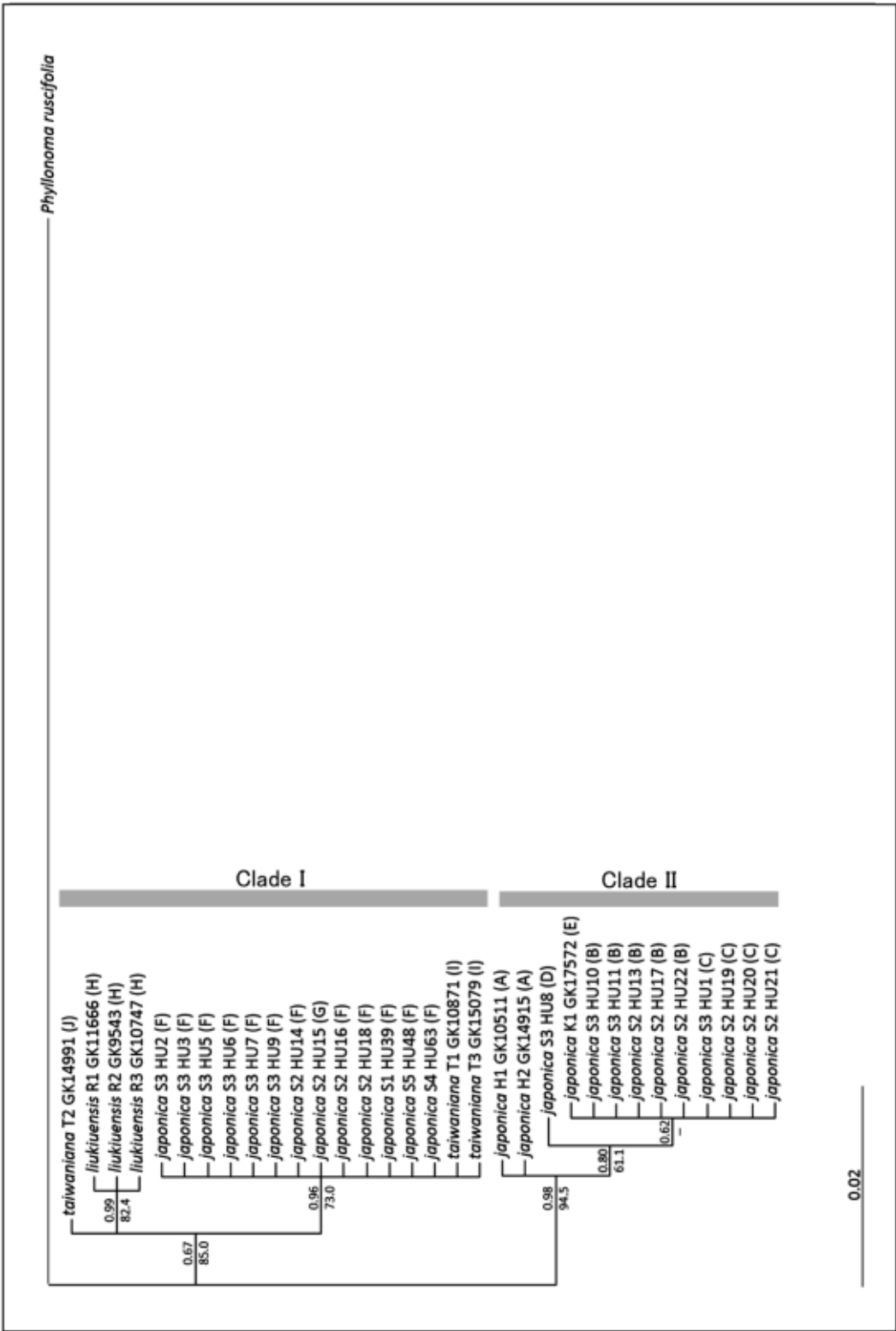
In 23 plants in Kochi of Japan, the means of leaf lateral vines number of the plants of *H. japonica* subsp. *japonica* placed in Clade I (ITS types F and G) ranged from 3.00 to 4.00, while those of plants placed in Clade II (ITS types B, C and D) ranged from 4.33 to 5.33 (Table 1, Fig. 4). The *F*-test showed no significant difference in the variance in the leaf lateral vine number between the Kochi plants in Clade I and Clade II, and thus, Student's *t*-test was applied to evaluate equality of the mean in the leaf lateral vine numbers. Student's *t*-test statistically revealed a significant difference in the mean in leaf lateral vine number between the Kochi plants placed in Clades I and II ($p < 0.005$).

Discussion

Sympatric occurrence of two varieties of Helwingia japonica in Kochi

As briefly mentioned in the introduction, Noshiro (1999) noted that *H. japonica* subsp. *japonica* var. *japonica* and var. *parvifolia* were distinguishable based on the number of lateral

Fig. 3. The Bayesian 50% majority rule consensus tree of *Helwingia japonica* based on nrITS sequences. The topology of the maximum parsimony strict consensus tree was compatible with the Bayesian tree. Alphabets in parentheses indicate nrITS types. Numerals above branches indicate Bayesian posterior probabilities (upper) and bootstrap percentages in the maximum parsimony analysis (lower; $< 50\%$).



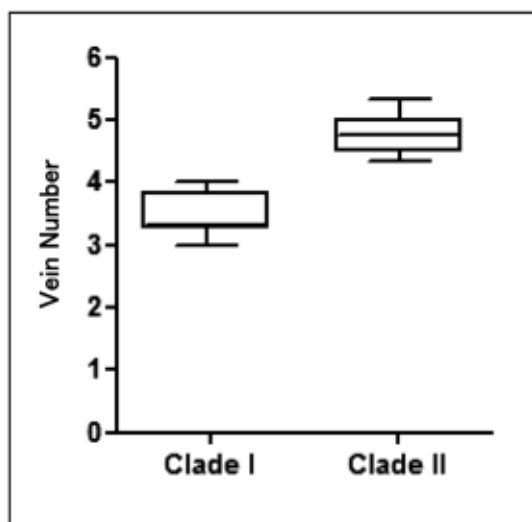


Fig. 4. Comparison of leaf lateral veins between Kochi plants of *Helwingia japonica* subsp. *japonica* placed in two clades using the box-and-whisker plots of the vein numbers.

leaf vines; the former has 4–6 pairs of leaf lateral veins, whereas the latter has 2–4 pairs. Following the taxonomic treatment of Noshiro (1999), plants with leaf lateral vein means of 3.00 to 4.00 placed in Clade I are identified as *H. japonica* subsp. *japonica* var. *parvifolia* (Fig. 1: B), and plants with leaf lateral vein means of 4.33 to 5.33 placed in Clade II are identified as *H. japonica* subsp. *japonica* var. *japonica* (Fig. 1: C). The present molecular and morphological results elucidated the occurrence of *H. japonica* subsp. *japonica* var. *japonica* and var. *parvifolia* in Kochi.

In taxonomy, however, we found that *H. japonica* subsp. *japonica* followed by the previous taxonomic concepts (e.g., Hara and Kurosawa, 1975; Noshiro, 1999) was not a monotypic taxon at subspecies level; and that leaf size and number of leaf lateral vine have parallel-evolved in *H. japonica*. Therefore, it is necessary to comprehensively reconsider these intraspecific link of *H. japonica* based on comparisons using not only leaf morphologies but also floral morphologies.

Possible hindrance of hybridization between the two varieties

To examine the hybridity of vascular plants, ITS region is often examined: a hybrid is expected to have heterozygotic nucleotide sites showing polymorphisms from putative parental species in ITS region (e.g., Saito *et al.*, 2007; Kokubugata *et al.*, 2011). In the present study, 18 segregation sites were detected between plants identified as *H. japonica* subsp. *japonica* var. *japonica* and var. *parvifolia*, from two localities Tosa-kuwae and Kawanaji in Kochi, where the two varieties occurred sympatrically within an area of ca. 4 m² (Fig. 1: A), but additive of nucleotide polymorphisms were not found. This result suggested that no hybrid were present in two localities of Kochi, and implied that certain isolation mechanisms might exist to prevent gene exchange between the two varieties in the sympatric population.

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