Hybridizations and genetic relationships among *Lindernia* species (Scrophulariaceae): *L. procumbens* and two subspecies of *L. dubia*

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Abstract

Lindernia procumbens and *L. dubia* are common annual weeds in flooded rice fields of Japan. Two subspecies of *L. dubia*, subsp. *major* and subsp. *dubia*, are usually recognized in Japan but they are both regarded as synonyms of *L. dubia* elsewhere. In a cluster analysis based on AFLP, most *L. dubia* subsp. *major* formed a separate cluster from *L. dubia* subsp. *dubia* although 11% of haplotypes classified using AFLP were not coincident with classification using the shape of leaf bases, which is the commonly used identification trait. Artificial F_1 plants between *L. procumbens* and *L. dubia* subsp. *major*, and between *L. procumbens* and *L. dubia* subsp. *dubia* did not produce seed. Forty percent of capsules produced by F_1 plants from these two subspecies were slimmer and 80% pollen were sterile in slimmer capsules. However, seed number of most F_1 capsules was not different from that of self-fertilized plants, suggesting that there was no complete reproductive isolation between the subspecies. Natural hybridization of these subspecies may have occurred but we are not aware of it because F_1 plants are rare and F_2 plants are indistinguishable from these subspecies.

Keywords: Lindernia dubia ssp. *major*, *Lindernia dubia* ssp. *dubia*, naturalized species, paddy weed, genetic diversity, *Lindernia procumbens*, hybridization, AFLP

1. Introduction

Lindernia procumbens (Krock.) Borbas and *L. dubia* Pennell are very common annual weeds in flooded rice fields in Japan. Genus *Lindernia* all. is a member of the family Linderniaceae (Rchb.) Borsch, K. Müller, and Eb. Fisch., as distinct from the family Schophulariaceae Juss in APG III (Rahmanzadeh et al., 2005; the angiosperm phylogeny group, 2009). *Lindernia procumbens* is an archaeophyte species in Japan (Yamazaki, 1993), whereas *L. dubia*, native to North America, was listed as a naturalized plant in 1954 (Yamazaki, 1954). *L. dubia* has recently increased rapidly (Yoshino *et al.*, 2006a) and is now more common than the native species. In the last fifteen years, biotypes of these weeds, resistant to sulfonylurea herbicides, have been frequently found and their distribution in Japan has increased (Uchino and Watanabe, 2002; Uchino, 2003).

Two distinct subspecies of *L. dubia* were described by Pennell (1935), *L. dubia* subsp. *major* and *L. dubia* subsp. *dubia.* This classification of subspecies is now commonly applied in Japan. These subspecies are distinguished typically by the shape of the base of their leaves; subsp. *major* has a cuneate leaf base while subsp. *dubia* has a round leaf base Morita (1994). This classification of subspecies, however, is apparently not used outside of Japan and the names of subspecies are regarded as synonyms of *L. dubia* (Copperrider and McCready, 1975; Delipavlov & Cheshmejiev, 1984; Carretero, 1985; Chaw & Kao, 1989; Conesa & Recasens, 1989; Kallen, 1994; Seliskar et al., 1995; López, 1997; Lewis, 2000). Nonetheless, the two subspecies seem to be distinct from each other in Japan because very few intermediate or mixed types are found, although they have been distributed sympatrically in rice fields in Japan since the 1960s (Yoshino *et al.*, 2006a).

In this study, we verified the possibility of hybridization among the *Lindernia* weeds including a native species, *L. procumbens*, by artificial hybridizations, and surveyed the genetic relationships among them by Amplified Fragment Length Polymorphism (AFLP) analysis to assess the validity of the classification of *Lindernia* weeds in Japan. In addition, the genetic distance based on AFLP analysis was analyzed in relation to geographical distance, and we discussed the differentiations of *Lindernia* weeds from the point of view of naturalization.

2. Materials and methods

2.1. Sample collection for AFLP

Lindernia procumbens, *L. dubia* subsp. *major* and *L. dubia* subsp. *dubia* were sampled across Japan from 9, 10 and 10 populations, respectively. Each population consisted of 2 or 3 individuals. *Lindernia procumbens* plants were sampled at Sobetsu (42°33N, 140°46E), Nagai (38°6N, 140°2E), Tsukuba (36°5N, 140°6E), Kyoto (35°5N, 135°47E), Uji (34°54N, 135°46E), Bizen (34°45N, 134°13E), Hongo (34°25N, 132°59E), Katsuyama (33°36N, 130°35E) and Hiyoshi (31°15N, 130°28E). *Lindernia dubia* subsp. *major* plants were sampled in Taiwa (38°15N, 140°55E), Asahi (38°19N, 140°9E), Kawanishi (38°0N, 140°3E), Yabuki (37°12N, 140°20E), Tsukuba, Otsu (35°4N, 135°52E), Kyoto, Uji, Yamaga (33°26N, 131°31E) and Makurazaki (31°16N, 130°18E). *Lindernia dubia* subsp. *dubia* plants were sampled in Yokohama (Aomori, 41°5N, 141°15E), Moriyoshi (39°43N, 140°16E), Yabuki, Tsukuba, Yatomi (35°6N, 136°44E), Kyoto, Uji, Hongo, Katsuyama and Makurazaki. The collection was obtained in three ways: seeds, seed banks in soil samples and fresh leaves. Seed samples were collected from mature plants at each field site and these seeds were planted and grown

on the Kyoto University farm; fresh leaves were collected about 60 d after sowing. Soil samples collected at each paddy field site were irrigated in pots and germinated plants were collected about 60 d after irrigation commenced. Fresh leaves were collected directly from plants and refrigerated at each sampling site. We identified *L. dubia* with cuneate leaf bases as *L. dubia* subsp. *major* and those with round leaf base as *L. dubia* subsp. *dubia*. All leaves were treated with liquid nitrogen and stored at -80 °C before DNA extraction.

2.2. AFLP fingerprinting

Leaf samples of about 100 mg were crushed using a multi-beads shocker (Yasui Kikai, Osaka, Japan) at 2,000 (smash intensity) for 5 s after freezing in liquid nitrogen. Genomic DNA was extracted according to a partly modified CTAB method of Doyle and Doyle (1990). AFLP fingerprinting was conducted using a partly modified method of Vos et al. (1995) using a thermal cycler (Sequi-Gen GT, Bio-Rad, California, U.S.). The restriction enzyme digestion and the ligation were conducted using AFLP Core Reagent Kit (Invitrogen, California, U.S.). PCR products were separated by polyacrylamide gel electrophoresis using Sequi-Gen GT Sequencing Cell (Bio-Rad, 165-3863, 38 × 50 cm, California, U.S.) and Electrophoresis Power Supply EPS 3500 XL (Amersham Bioscience, U.K.), using their partially-modified manuals. After electrophoresis, gels were stained by a silver staining method with Silver SequenceTM DNA Sequencing System (Promega, Wisconsin, US).

The genotypes were visually scored within 100 to 500 bp. Genetic distance between each pair of individuals was calculated using the Dice distance coefficient obtained as (1-Dice similarity index) (Dice, 1945). The genetic distances obtained were used for

cluster analysis using UPGMA with 1000 bootstraps by using the Clustering Calculator Program (Brzustowski, 2002) and TreeView 1.6.6 (Page, 1996). Genetic diversities of total populations (H_T), within each population (H_S) and among populations (G_{ST}) were calculated using Nei's (1978) unbiased genetic distance with POPGENE 1.32.

2.3. Artificial hybridization

Lindernia procumbens, *L. dubia* subsp. *major* and *L. dubia* subsp. *dubia* were sampled in the paddy field at the Kyoto University farm and served as parents for artificial hybridization. They were transplanted into 430 mL plastic cups filled with paddy soil and grown in flooded conditions, then covered with 2 mm mesh cloth to avoid pollination by insects. Hand pollination was conducted after flowering under an anatomic microscope between 8:00 a.m. and 10:00 a.m. on sunny mornings. After ripening of seeds, we counted the number of seeds in the capsules obtained by hand pollination and these seeds were stored in the refrigerator at 7 °C. Selfing progeny were also made and saved in the same way as hybridized plants.

F_1 plants were grown from this seed in a greenhouse and in a growth chamber. In the greenhouse on the Kyoto University farm, we planted F_1 seeds in 430 mL plastic cups filled with paddy soil and F_1 plants were grown under natural temperature and light conditions. In the growth chamber NC-220S(C) (Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan), plants were grown in plastic boxes with 24 cells (3 cm × 3 cm) filled with peat moss (Golden peat ban, Sakata Seed Corporation, FH-180) at 30 °C under standard fluorescent light condition for 16 hours and 25 °C dark condition for 8 hours. In both greenhouse and chamber experiments, 30 seeds from one capsule were sown in the same cup or box. Germinated plants were counted on the 30th

day after sowing. After counting, plants were thinned, allowing the most vigorous plants to remain. The growth of F_1 plants was evaluated for the following five categories.

The pollen staining was conducted with 10 μL fixing solution (ethanol: acetic acid = 3:1) and 10μL of stain solution (1% (w/v) blue cotton; 50% glycerin solvent). Pollen morphology was observed under an optical microscope and the number of pollen grains was counted. Based on three morphological traits (Yoshino *et al.* 2007), F_1 plants were divided into three types: those like *L. dubia* subsp. *major*, those like *L. dubia* subsp. *dubia* and an intermediate type between these subspecies. All F_1 plants grown in both greenhouse and growth chamber were used for seed counting, pollen staining and examining potentially diagnostic morphological traits.

3. Results

3.1. Genetic relationships based on AFLP fingerprinting and Relationships between genetic and geographical distance

Lindernia procumbens populations had more loci than *L. dubia* subsp. *major* and *L. dubia* subsp. *dubia* populations (Table 1). *Lindernia procumbens* formed a single cluster that was completely separated from another cluster consisting of the two subspecies of *L. dubia* in the cluster analysis based on AFLP fingerprinting (Fig. 1).

The *L. dubia* cluster included two sub-clusters. Group A included most of *L. dubia* subsp. *major* and group B included most of *L. dubia* subsp. *dubia*. Only two (2031 and 2091) of the 27 *L. dubia* subsp. *major* belonged to group B, and four (3103, 3153, 3181 and 3183) of 24 *L. dubia* were located in group A.

The genetic distance between *L. procumbens* plants was correlated with the geographical distance of their collection site, although no such correlation was observed

in *L. dubia* subsp. *major* and *L. dubia* subsp. *dubia* (Fig. 2). The genetic distance of *L. dubia* subsp. *dubia* had two peaks regardless of their geographical distance.

3.2. Artificial hybridization

The mean seed number was significantly higher in the combinations of *L. dubia* subsp. *major* and *L. dubia* subsp. *dubia* than the other combinations including *L. procumbens* (Table 2). The combination of *L. dubia* subsp. *dubia* × *L. procumbens* did not produce any seeds. In F_1 plants, only the progeny of the combination of the two subspecies formed capsules while any other combinations including *L. procumbens* did not produce capsules. The rate of F_1 plants that produce capsules was not significantly different from that of self-fertilized plants by chi-test ($p > 0.05$, data not shown). However, 40% of the produced capsules were slimmer in the F_1 plants while 97% of capsules of selfing progeny of their parents were normal. The slimmer capsules had fewer seeds, 68% of slimmer capsules had less than 100 seeds per capsule while 94% of normal capsules had more than 101 seeds per capsule (data not shown).

3.3. Pollen fertility and morphology of F_1 plants between L. dubia subsp. major and L. dubia subsp. dubia

Pollen of F_1 offspring of crosses between *L. dubia* subsp. *major* and *L. dubia* subsp. *dubia* were classified as fertile uniformly stained circular, and sterile unstained and/or non-circular. The anthers of plants which formed narrower capsules contained 20% fertile pollen, while normal capsules contained 91%, showing a significant difference between narrower and normal capsules ($p<0.001$ by t test).

Upon visual inspection, F_1 plants were clearly different from either of their parents

and either of selfing progeny of the parents. Above ground plant length was longer in F_1 plants than in the selfing progeny of their parents and F_1 plants bent at their nodes and crept on the ground (data not shown).

The leaf shape (b in Table 3) and the shape of leaf base (c in Table 3) of F_1 plants were the same as *L. dubia* subsp. *dubia* and the depth of the leaf teeth (d in Table 3) and floral arrangement (e in Table 3) of F_1 plants were the same as *L. dubia* subsp. *major*. Petals of F_1 plants had purple spots like *L. dubia* subsp. *dubia* but these spots were paler than *L. dubia* subsp. *dubia* (a in Table 3).

4. Discussion

Lindernia procumbens is a prehistoric-naturalized plant to Japan and hence might have become geographically differentiated in Japan. Despite the earlier naturalization of *L. dubia* subsp. *major* than *L. dubia* subsp. *dubia*, its genetic diversity was smaller than *L. dubia* subsp. *dubia*. The genetic diversity of *L. dubia* subsp. *dubia* was the same as *L. procumbens*, suggesting the multiple invasion of *L. dubia* subsp. *dubia* to Japan.

Lindernia procumbens formed an independent cluster by AFLP and F_1 between *L. procumbens* and *L. dubia* did not produce seed. These results were consistent with the notion that *L. procumbens* and *L. dubia* are different species.

Most classification decisions based on the shape of the leaf base of *L. dubia* agreed with the results of AFLP analysis. However, the results showed that the classification based on leaf base shape was insufficient to classify *L. dubia* subsp. *major* and *L. dubia* subsp. *dubia*. The round leaf base of *L. dubia* subsp. *dubia* appeared to be dominant over the cuneate leaf base of *L. dubia* subsp. *major*, and therefore the plants with the round leaf bases seem to include the hybrid plants between these subspecies. Most of

the plants whose haplotypes did not coincide with classification by leaf bases were expected to have a round leaf base. Therefore, identification not only by leaf base shape, but also by other taxonomic characters, e.g. pedicel length, might be necessary to distinguish them (Yoshino *et al.* 2006b).

From the results of the artificial hybridization, there was no complete reproductive isolation between *L. dubia* subsp. *major* and *L. dubia* subsp. *dubia*, while *L. procumbens* is genetically isolated from two subspecies of *L. dubia*. It is possible that hybrids between these subspecies are distributed in Japan.

The morphology of F_1 Plants was not affected by the reciprocal crosses. Inheritance mode of petal color pattern, paler petal spots, seemed to be codominant in *L. dubia*. The codominant inheritance mode in flowers color was similar to related species such as *Antirrhinum*, *Mimulus* and *Torenia* (Bradshaw et al., 1995; Aida et al., 2000; Jones et al., 2001). Plant length of F_1 plants showed heterosis by hybridization but did not show reproductive success because F_1 plants were creeping, which would not seem to confer a selective advantage in a rice field. Narrow capsules were the result of decreased seed number, due to sterile pollen, and capsule shape itself did not seem to change by genetic factors.

As plants with the same morphological character to artificial F_1 plants were not found in either fields or herbarium specimens, F_1 plants were thought to exist rarely under natural conditions because *L. dubia* is a self-fertilizing species with cleistogamous flowers which normally self-pollinates (Ikeda and Miura, 1994; Morita, 1994). However, hybridization of these subspecies has accidentally occurred, and some F_1 plants have had fertile seeds because they are distributed sympatrically in many rice fields in Japan (Yoshino et al., 2006a). F_2 and later progeny would be expected to have

the same reproductive ability as their parents.

In most of these subspecies samples, classification based on leaf base shape was consistent with the results of AFLP analysis, and the haplotypes were different between the two subspecies. Therefore, we conclude that it is appropriate to distinguish the two subspecies taxonomically and the infraspecific rank such as subspecies or variety would appear adequate, because of the rare possibility of natural hybridization between them. Distributions of these subspecies overlapped extensively in United Status and Japan (Pennell, 1935; Yoshino et al., 2006a). ‘Variety’ may be more appropriate than ‘subspecies’ because naturally occurring plants with overlapping distributions have tended to be classified as varieties (Hamilton and Reichard, 1992).

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Figure legends

Fig. 1. UPGMA cluster of *L. procumbens* (1 ---), *L. dubia* subsp. *major* (2 ---) and *L. dubia* subsp. *dubia* (3 ---) in Japan. Bar shows the genetic distance. Italic figures show the 1000 times trials of the bootstrap value. Sampling sites were 1011, 1013: Sobetsu; 3021～3023: Yokohama; 2031～2033: Taiwa; 3041～3043: Moriyoshi; 1051～1053: Nagai; 2061～2063: Asahi; 2071～2073: Kawanishi; 2081～2083, 3081～3083: Yabuki; 1091, 2091, 2092, 3091: Tsukuba; 3101～3103: Yatomi; 2111～2113: Otsu; 1121～1123, 2121～2123, 3121～3123: Kyoto; 1131～1133, 2131～2133, 3131～3133: Uji; 1141～1143: Bizen; 1151～1153, 3151～3153: Hongo; 1161, 1163, 3161～3163: Katsuyama; 2171～2173: Yamaga; 2181～2183, 3181～3183: Makurazaki; 1191, 1192: Hiyoshi.

Fig. 2. The relationship between genetic and geographical distance of *L. procumbens*, *L. dubia* subsp. *major* and *L. dubia* subsp. *dubia*. Regression equation and coefficient of determination for *L. procumbens*, *L.dubia* subsp. *major* and *L. dubia* subsp. *dubia* were $y = 11.45 \times 10^{-5}x + 0.11$, $R^2 = 0.3618$, $y = 0.76 \times 10^{-5}x + 0.08$, $R^2 = 0.0052$, $y = 8.66 \times 10^{-5}x + 0.11$ $R^2 = 0.1141$, respectively. *Lindernia procumbens* and *L. dubia* subsp. *dubia* were significant ($p = 0.001$), though *L.dubia* subsp. *major* was not significant ($p > 0.05$).

Fig. 1 –Yoshino *et al.* – Top ↑

Fig. 2 –Yoshino *et al.* – Top ↑

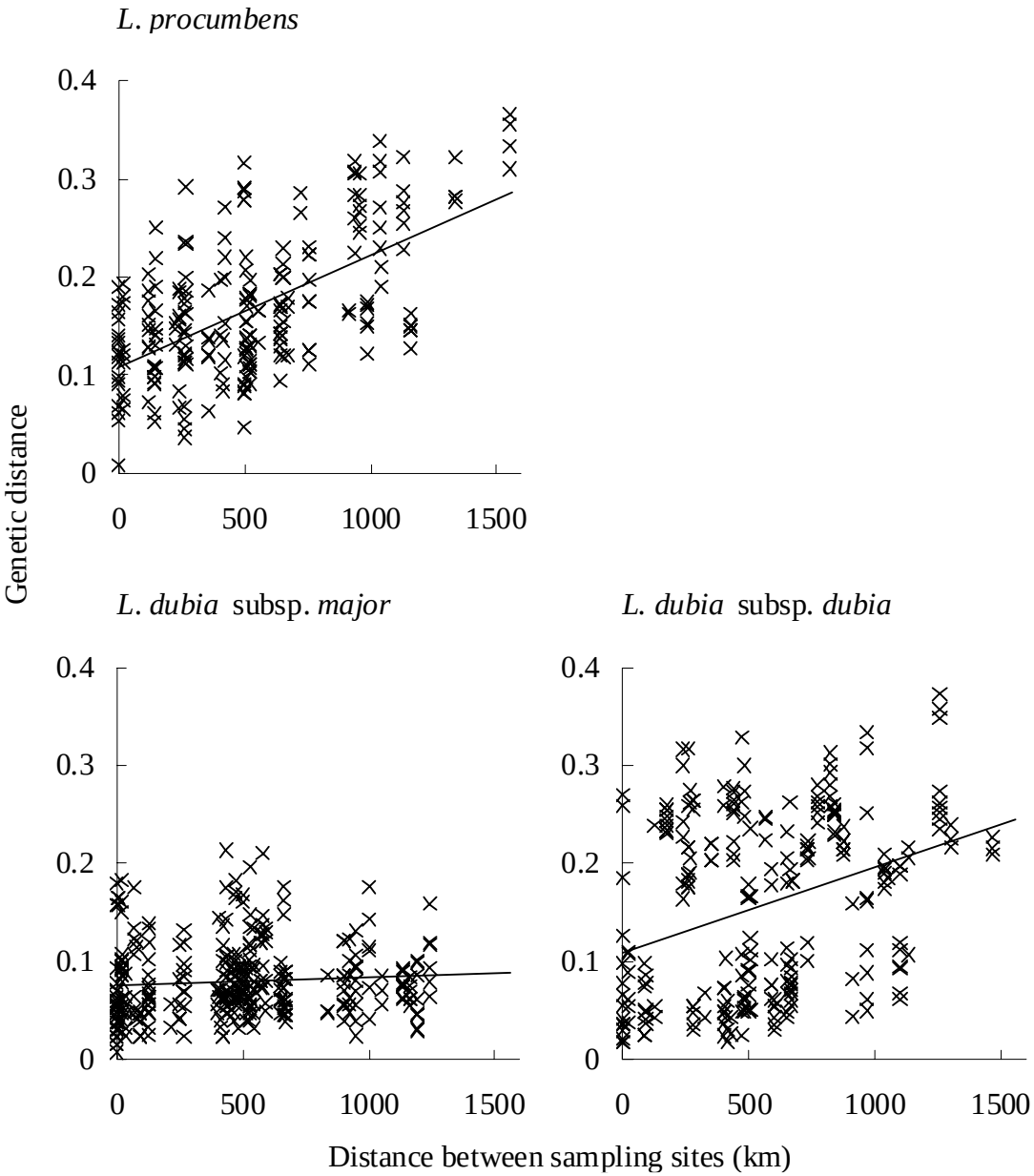

Table 1 Genetic distance and genetic diversity of *L. procumbens*, *L. dubia* subsp. *major* and *L. dubia* subsp. *dubia* from AFLP fingerprinting.

		L. procumbens	*L. dubia* subsp. *major*	*L. dubia* subsp. *dubia*	Total
Number of individuals		22.0	27.0	23.0	74.0
Number of populations		9.0	10.0	10.0	30.0
Number of alleles		145.0	116.0	98.0	279.0
Number of unique alleles		86.0	22.0	12.0	
Number of polymorphic alleles		55.0	36.0	25.0	167.0
(%)		37.9	31.0	25.5	59.9
Average number of bands		76.7	69.7	72.1	72.4
Genetic distance (minimum - maximum)	*L. procumbens*	0.012-0.133	0.685-1.024	0.664-0.872	
	L. dubia subsp. *major*		0.003-0.090	0.134-0.253	
	L. dubia subsp. *dubia*			0-0.113	
Genetic diversity	All populations (H_T)	0.23	0.19	0.24	
	Each population (H_S)	0.20	0.15	0.21	
	Coefficient of differentiation (G_{ST})	0.13	0.21	0.13	

Table 2 Growth of F_1 and self-fertilization plants of *L. procumbens*, *L. dubia* subsp. *major* and *L. dubia* subsp. *dubia*.

Combination		Number of Seeds		% of plants					p value of chi test	
				No germination (1)*	No flower (2)	No seeds (3)	A few seeds (4)	Normal (5)	Vs. reciprocal crosses	Vs. self fertilization
F_1	*L. procumbens* × *L. dubia* subsp. *major*	189	ab**	33%	67%				0.22	0.01
	L. dubia subsp. *major* × *L. procumbens*	67	b	148 a**	67%	33%				
	L. procumbens × *L. dubia* subsp. *dubia*	19	ab		100%				-	0.89
	L. dubia subsp. *dubia* × *L. procumbens*	0		4 a	-***	-	-	-		
	L. dubia subsp. *major* × *L. dubia* subsp. *dubia*	171	b		11%	53%	11%	5%	19%		
	L. dubia subsp. *dubia* × *L. dubia* subsp. *major*	295	a	218 b	4%	44%	9%	11%	31%	0.30	< 0.01
	F_1 total				10%	66%	11%	3%	10%		< 0.01
Self fertilization	*L. procumbens*					75%	13%		13%		
	L. dubia subsp. *major*				2%	67%		2%	30%		
	L. dubia subsp. *dubia*				2%	59%			39%		
	Self fertilization total				1%	67%	4%	1%	27%		

* No germination (1): No germination occurred, No flower (2): More than one seed germinated but no plant bore flowers before withering, No seeds (3): More than one plant bore flowers but seeds were produced by less than 11 per one capsule in any flower, A few seeds (4): 11 to 100 seeds per one capsule were produced in any flowers, Normal(5): more than 101 seeds were produced in any flowers.

** Different letters indicate significant difference ($p<0.05$)(Student t test).

*** Not surveyed because the seeds were not produced.

Table 3 Morphological traits of F_1 and selfing plants of *L. dubia* subsp. *major* and *L. dubia* subsp. *dubia*. See Table 1 for details of each type.

Morphological traits		% of plants			p value of chi test			
		Type A*	Type B	Type C	reciprocal crosses	Type A: Type B: Type C 1:2:1	3:0:1	1:0:3
Petal color pattern (a)	*L. dubia* subsp. *major*×*L. dubia* subsp. *dubia*	17%	54%	29%	0.98	0.89	0.01	0.09
	L. dubia subsp. *dubia*×*L. dubia* subsp. *major*	15%	56%	30%				
	L. dubia subsp. *major*	63%	33%	4%				
	L. dubia subsp. *dubia*	19%	19%	63%				
Leaf shapes (b)	*L. dubia* subsp. *major*×*L. dubia* subsp. *dubia*	5%	8%	87%	0.78	0.01	0.00	0.16
	L. dubia subsp. *dubia*×*L. dubia* subsp. *major*	3%	11%	86%				
	L. dubia subsp. *major*	59%	28%	13%				
	L. dubia subsp. *dubia*	4%	4%	93%				
Shape of leaf bases (c)	*L. dubia* subsp. *major*×*L. dubia* subsp. *dubia*	13%	8%	79%	0.32	0.05	0.00	0.50
	L. dubia subsp. *dubia*×*L. dubia* subsp. *major*	6%	17%	78%				
	L. dubia subsp. *major*	69%	25%	6%				
	L. dubia subsp. *dubia*	4%	4%	93%				
Leaf margin (d)	*L. dubia* subsp. *major*×*L. dubia* subsp. *dubia*	57%	37%	7%	0.70	0.43	0.22	0.00
	L. dubia subsp. *dubia*×*L. dubia* subsp. *major*	46%	43%	11%				
	L. dubia subsp. *major*	80%	15%	5%				
	L. dubia subsp. *dubia*	0%	61%	39%				
Pedicels (e)	*L. dubia* subsp. *major*×*L. dubia* subsp. *dubia*	89%	6%	6%	0.70	0.00	0.56	0.16
	L. dubia subsp. *dubia*×*L. dubia* subsp. *major*	81%	9%	9%				
	L. dubia subsp. *major*	96%	0%	4%				
	L. dubia subsp. *dubia*	28%	0%	72%				

* Type A: type like *L. dubia* subsp. *major*, Type B: intermediate type between *L. dubia* subsp. *major* and L. *dubia* subsp. *dubia*, Type C: type like *L. dubia* subsp. *dubia*. Petal color pattern(a) - Type A: Margin: pink, spot: none, Type B: Margin: pale, spot: exist or none, Type C: Margin: deep purple, spot: exist. Leaf shapes (b) - Type A: Oblanceolate, Type B: Narrow elliptic ~ lanceolate , Type C: Ovate. Leaf bases (c) - Type A: Attenueate ~ cuneate , Type B: Obtuse ~ elliptic , Type C: Rotund. Leaf margin (d) - Type A: Obviously serrate , Type B: Slightly obviously serrate ~ unobviously serrate , Type C: Nearly entire ~ entire. Pedicels (e) - Type A: Spiral at all nodes , Type B: Opposite at some nodes (not at all nodes) , Type C: Opposite at all nodes.