

Phytogeographic aspects of *Lysionotus pauciflorus sensu lato* (Gesneriaceae) in the China, Japan and Taiwan regions: phylogenetic and morphological relationships and taxonomic consequences

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Received: 19 April 2010 / Accepted: 20 December 2010
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Abstract Comparison of leaf morphometric variation and phylogenetic analyses based on nrITS and partial *trnC-D* cpDNA sequences were conducted for samples of *Lysionotus pauciflorus sensu lato* collected from China, Japan and Taiwan. In the morphometric comparison, leaves of plants from Japan proper and those from Taiwan proper were not significantly distinguishable by their leaf length to width ratio. In phylogenetic analyses, monophyly of *L. pauciflorus s. l.* samples was strongly supported, and Japanese and Taiwanese samples fell into a clade separate from Chinese samples. In the Japan–Taiwan clade, plants from Japan proper formed a sister clade to those from Okinawa Island (Japan) and Taiwan. The present results suggest that (1) the *L. pauciflorus s. l.* samples from China, Japan and Taiwan must be treated as a single species; (2) *L. pauciflorus s. l.* originated in China; (3) a single dispersal event, dispersal via a land-bridge during low sea levels in a Pleistocene

glacial maximum, from China to Taiwan and Japan is proposed; (4) three oversea dispersal events, probably through anemochory, to Japan proper, across the Tokara Gap, to Okinawa islands crossing the Kerama Gap, and from Taiwan to Lanyu Island are proposed. The study gives further insight into the biogeographical dynamics between the Ryukyus and surrounding areas.

Keywords Gesneriaceae · *Lysionotus* · Leaf morphology · Phytogeography · Ryukyus

Introduction

The Ryukyu Archipelago (the Ryukyus), situated between Japanese Kyushu and Taiwan, is composed of about 140 islands (Fig. 1), and is climatologically classified as subtropical. In the Ryukyus, there are two specific biogeographical barriers preventing dispersal of terrestrial animals and plants that are not adapted for hydrochory or anemochory across the Tokara Gap situated between Japan proper (including Yakushima Island) and more southern islands including Okinawa Island, and the Kerama Gap situated between Okinawa Island and more southern islands (e.g. Hotta 1974; Fig. 1). These two gaps biogeographically divide the Ryukyus into three major areas, the northern Ryukyus including the Yakushima Island, the central Ryukyus including Okinawa Island, and the southern Ryukyus, with each region having characteristic biota (e.g. Tagawa and Miyagi 1991; Ota 1998; Yokota et al. 2007). Most recently, however, Nakamura et al. (2009) suggested that the effect of the historical barrier of the Kerama Gap is rather insignificant, because the floristic differentiation across the Kerama Gap is not greater than could be explained solely by geographical distance, and

Electronic supplementary material The online version of this article (doi:10.1007/s00606-010-0410-2) contains supplementary material, which is available to authorized users.

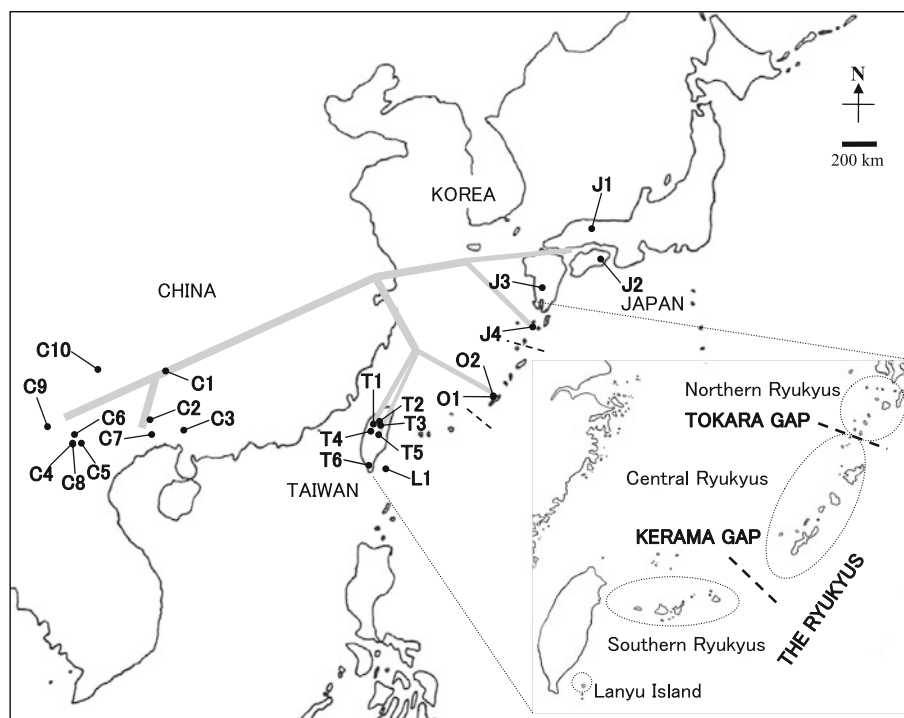
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Fig. 1 Map of NE Asia showing the localities of the 47 accessions of *L. pauciflorus sensu lato* investigated. Letter/number combinations refer to localities listed in Table 1. Superimposed lines correspond to phylogenetic relationships between *L. pauciflorus sensu lato* populations in China, Taiwan and Japan based on combined ITS and *trnC-D* data

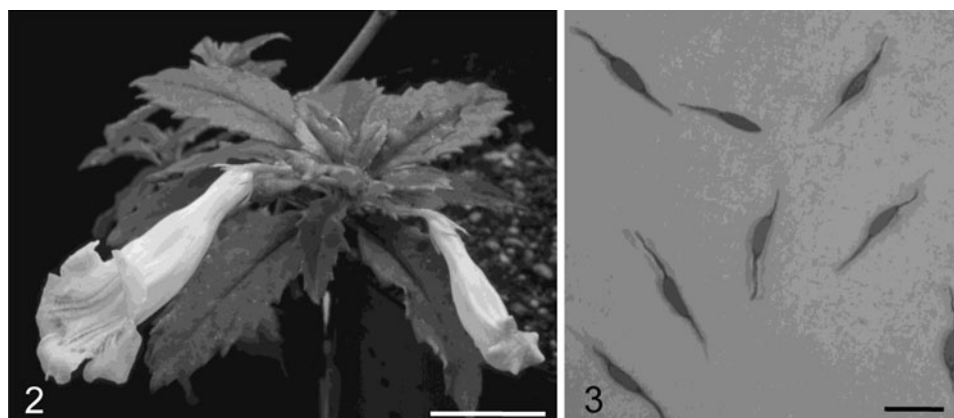


suggested caution in discussions of biogeography with excessive emphasis only on the role of geographic gaps.

Until now, only a few molecular studies have been carried out on phylogeographic and phylogenetic relationships between taxonomic entities on the Ryukyus and their allied entities in China, Japan proper and Taiwan, for instance *Lilium* (Liliaceae; Hiramatsu et al. 2001), *Ophiorrhiza* (Rubiaceae; Nakamura et al. 2006), *Caridandra* (Hydrangeaceae; Setoguchi et al. 2006), *Pieris* (Ericaceae; Setoguchi et al. 2008) and *Lysimachia* (Myrsinaceae; Kokubugata et al. 2009). Although these studies provided initial insights into the phylogeography and insular endemisms of the Ryukyus and its allied regions, further work is required to test whether common patterns can be detected for biogeographical relationships between the Ryukyus and its surrounding areas.

Lysionotus pauciflorus, described by Maximowicz (1874) from a type specimen collected from the western part of Japan, is an evergreen herbaceous subshrub in the family Gesneriaceae (Fig. 2). In the wider taxonomic sense this species is distributed widely in the southern part of China (Anhui, Fujian, Guangdong, Guangxi, Guizhou, Hainan, S. Henan, Hubei, Hunan, S. Jiangsu, Jiangxi, S. Shaanxi, Sichuan, Yunnan, Zhejiang), with outliers in Japan and Taiwan and extension to the northern parts of Vietnam (Li and Kao 1998; Wang et al. 1998). In Japan, *L. pauciflorus* has been known from western parts of Japan proper and two nearby islands, including Yakushima Island in the northern Ryukyus (Ohwi 1984). In 1994, however, a population of *Lysionotus* plants was discovered at the summit of a mountain on Okinawa Island in the central Ryukyus (Fig. 1). Following Yokota and Tomiyama (2006)

Figs. 2–3 Plant and seed morphology of *L. pauciflorus sensu lato*. Figure 2 plant collected from Okinawa Island of Japan (GK735) and cultivated at the National Museum of Nature and Science. Figure 3 seeds of a plant collected in Taiwan (GK4077)



only two populations are known now on Okinawa Island, and as a consequence the species is listed as a critically endangered species by the Japanese Ministry of Environment (2007).

Taxonomically, Yamazaki (1993, 1994) treated the Okinawa plants as *L. apicidens* (Hance) T. Yamazaki. He also regarded the Chinese and Taiwanese plants as *L. apicidens*, while he circumscribed *L. pauciflorus* as a species endemic to Japan only, excluding those of the Okinawa Island, thereby disagreeing with most taxonomists (e.g. Kao and Devol 1978; Li and Kao 1998; Wang et al. 1990, 1998; Hatusima and Amano 1994). Following Yamazaki (1993, 1994), the two species are distinguishable in their leaf shape, with the leaves of *L. pauciflorus* being narrower than those of *L. apicidens*, a rather minor difference. On the other hand, for *Lysionotus* plants in Lanyu Island of Taiwan (Fig. 1), Hatusima (1970) described *L. ikedae* as an independent insular-epidemic species, but most taxonomists regard it as a variety of *L. pauciflorus* var. *ikedae*, characterized by broader leaves (Kao and Devol 1978; Li and Kao 1998; Wang et al. 1990, 1998).

The aims of the present study were to consider the taxonomic and phylogenetic relationships among plants of *L. pauciflorus sensu lato* (*s. l.*) collected from China, Japan and Taiwan, utilizing leaf morphometric comparisons and molecular phylogenetic analyses of sequences of the internal transcribed spacer (ITS) of nuclear ribosomal DNA (nrDNA) and part of the *trnC*-D spacer region of chloroplast DNA (cpDNA). The results are discussed in the light of possible dispersal patterns of this species in Japan and Taiwan.

Materials and methods

Plant materials

For leaf-morphometric comparisons 42 individuals of *L. pauciflorus s. l.* were measured: 9 individuals from four populations in Japan proper including a type specimen of *L. pauciflorus* deposited in the herbarium of the Komarov Botanical Institute of the Russian Academy of Sciences (LE) using the photograph in Yang (2006), 7 individuals from two populations in Okinawa Island (Japan), 14 individuals from five populations in Taiwan proper, 4 individuals from one population in Lanyu Island (Taiwan) including a type specimen of *L. ikedae* Hatus. deposited in the herbarium of Kagoshima University (KAG) using the photograph in Hatusima (1970), and 8 individuals collected from eight populations in China (Table 1).

For molecular analyses, 48 plants of *L. pauciflorus s. l.* were included, 30 of those used for morphometric studies plus 18 additional samples used for molecular studies only; 8 plants from four populations in Japan proper, 6 plants from two populations in Okinawa Island (Japan), 12 plants

from five populations in Taiwan proper, 2 plants from one population in Lanyu Island (Taiwan) and 20 plants from ten populations in China (Table 1). Eight samples of four additional species of *Lysionotus* from China were included as additional ingroup taxa (Table 1). Three species each of the two genera, *Aeschynanthus* and *Hemiboea*, closely related to *Lysionotus* (Möller et al. 2009), were included as outgroup taxa and the phylogenetic trees rooted on species of the first genus according to results from Möller et al. (2009) (Table 1).

Voucher specimens for morphometric investigation and molecular analyses were deposited in the herbaria of Royal Botanic Garden Edinburgh (E) and the National Museum of Nature and Science (TNS).

In the present paper, we tentatively treated all plants from China, Japan and Taiwan as *L. pauciflorus s. l.*, and reconsider their taxonomic status based on the present data in the discussion.

Leaf-morphometric analysis

For morphometric comparisons, the leaf length and leaf width of three leaves in each individual were measured on herbarium voucher specimens, and the leaf ratio calculated as leaf length/leaf width.

Equality of variances in the leaf ratio were statistically evaluated using Bartlett's test among five geographic groups: (1) China, (2) Japan proper including Yakushima Island, (3) Okinawa Island (Japan), (4) Taiwan proper, and (5) Lanyu Island (Taiwan). The leaf ratio data were further analyzed by the Kruskal-Wallis test using PRISM ver. 4 (GraphPad Software, La Jolla, CA).

DNA extraction, PCR and sequencing

DNA was extracted from silica gel-dried leaves using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocols. The genomic DNA samples were deposited in the Molecular Biodiversity Research Center of the National Museum of Nature and Science, Tsukuba. The entire ITS region, including ITS1, 5.8S and ITS2, and part of the *trnC*-D cpDNA spacer were amplified by polymerase chain reaction (PCR) using an iCycler (Bio-Rad, Hercules, CA). Forward primer 'AB101' (5'-ACG AAT TCA AGG TCC GGT GAA GTG TTC G-3') and reverse primer 'AB102' (5'-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3') (Douzery et al. 1999) were used for ITS, and the forward primer 'trnC^{GCA}F' (5'-CCA GTT CRA ATC YGG CTT ACT ATA TCC AT-3') and reverse primer 'trnD^{GUC}R' (5'-GGG ATT GTA GYT CAA TTG GT-3') (Shaw et al. 2005) were used for the *trnC*-D spacer. Amplifications were performed using Takara Ex Taq (Takara, Otsu, Japan) with Ampdirect Plus (Shimadzu, Kyoto, Japan). The PCR profile was 35 cycles of 30 s at

Table 1 Samples of *Lysionotus* subjected to morphological and molecular analysis, and their localities, codes, voucher numbers, leaf morphologies, and ITS and *trnC-D* GenBank accession numbers

Species	Locality	Code ^a	Voucher ^b	ITS			<i>trnC/D</i>	
				Accession no. ^c	Type	Poly-C ^d	Accession no. ^c	Type
Ingroup								
<i>L. pauciflorus sensu lato</i>	Japan, Honshu, Misasa, Tottori, Misasa, Tsuga	J1	GK11864	AB514440	A	12	AB547266	a
		J1	GK11865	AB547191	A	12	AB547267	a
	Japan, Shikoku, Tokushima, Naka-cho, Kito	J2	GK10476	AB514441	A	12	AB547268	a
		J2	GK12179	AB547193	B	14	AB547269	a
	Japan, Kyushu, Miyazaki, Ebinoi, Iino	J3	GK9162	AB498573	C	7	AB547270	a
		J3	GK12180	AB547194	D	13	AB547271	a
	Japan, Ryukyus, Kagoshima, Yakushima Is.	J4	GK11642	AB498574	E	15	AB547272	b
		J4	GK12181	AB547192	A	12	AB547273	b
	Japan, Ryukyus, Okinawa, Okinawa Is., Motobu	O1	GK690	–	–	–	–	–
		O1	GK735	AB498562	F	7	AB547274	c
		O1	GK12182	AB547195	F	7	AB547275	c
	Japan, Ryukyus, Okinawa, Okinawa Is., Motobu	O2	GK11880	AB547196	G	7	AB547276	c
		O2	GK11882	AB514557	H	7	AB547277	c
		O2	GK11883	AB547197	G	7	AB547278	c
		O2	GK12280	AB547198	H	7	AB547279	c
		O2	GK12280	AB547198	H	7	AB547279	c
	Taiwan, Hsinchu, Wufen Hsiang	T1	GK5113	AB498563	G	7	AB547280	c
		T1	GK5122	–	–	–	–	–
		T1	GK12184	AB547199	G	7	AB547281	c
	Taiwan, Hsinchu, Chienshih Hsiang	T2	GK5209	AB498564	G	7	AB547282	c
		T2	GK12185	AB547200	G	7	AB547283	c
	Taiwan, Hsinchu, Chienshih Hsiang	T3	GK4077	AB514442	G	7	AB547284	c
		T3	GK12186	AB547201	G	7	AB547285	c
	Taiwan, Taichung, Hopping Hsiang	T4	GK11541	AB514443	G	7	AB547286	c
		T4	GK12188	AB547202	G	7	AB547287	c
	Taiwan, Nantou, Jenai Hsiang	T5	GK11585	AB514444	G	7	AB547293	d
		T5	GK12189	AB547203	G	7	AB547294	d
	Taiwan, Pingtung, Hengchun Hsiang	T6	GK3702	–	–	–	–	–
		T6	GK3704	AB498566	G	7	AB547289	c
		T6	GK12187	AB547204	G	7	AB547290	c
	Taiwan, Taitong, Lanyu Hsiang, Lanyu Island	L1	GK11643	AB498567	G	7	AB547291	c
		L1	GK11982	AB547205	G	7	AB547292	c
		L1	GK11983	–	–	–	–	–
	China, Guizhou, Xinjiang	C1	MMO03-356B	AB498575	I	7	AB547295	e
		C1	MMO03-356C	AB547206	I	7	AB547296	e
	China, Guizhou, Libo	C2	MMO07-1091A	AB498577	I	7	AB547297	e
C2		MMO07-1091B	AB547207	I	7	AB547298	e	
China, Guangxi, Dayao Shan	C3	MMO06-889A	AB498576	I	7	AB547309	f	
	C3	MMO06-889B	AB547208	I	7	AB547310	f	
China, Guangxi, Bai He	C4	MMO06-841A	AB498579	J	7	AB547299	e	
	C4	MMO06-841B	AB547209	J	7	AB547300	e	
China, Guangxi, Jing Xi	C5	MMO07-1118A	AB498581	J	7	AB547301	e	
	C5	MMO07-1118B	AB547210	J	7	AB547302	e	
China, Guangxi, Napo, Po He	C6	MMO06-836A	AB498583	J	7	AB547303	e	
	C6	MMO06-836B	AB547211	J	7	AB547304	e	

Table 1 continued

Species	Locality	Code ^a	Voucher ^b	ITS			<i>trnC/D</i>	
				Accession no. ^c	Type	Poly-C ^d	Accession no. ^c	Type
	China, Guangxi, Ma Shan	C7	MMO06-797A	AB498584	J	7	AB547311	g
		C7	MMO06-797C	AB547212	J	7	AB547312	g
	China, Yunnan, Funing, Tian Peng	C8	MMO06-938A	AB498580	J	7	AB547305	e
		C8	MMO06-938B	AB547213	J	7	AB547306	e
	China, Yunnan, Xichou, Far Dou	C9	MMO01-123_2	AB498582	J	7	AB547307	e
		C9	MMO01-123_3	AB547214	J	7	AB547308	e
	China, Yunnan, Weixin	C10	MMO07-1018A	AB498585	K	7	AB547313	h
		C10	MMO07-1018B	AB547215	K	7	AB547314	h
<i>L. chingii</i> W.Y. Chun ex W.T. Wang	China, Yunnan, Jinping	Lc1	MMO06-983A	AB498569		7	AB547316	
	China, Yunnan, Jinping	Lc1	MMO06-983B	AB547216		7	AB547317	
<i>L. denticulosus</i> W.T. Wang	China, Guangxi, Napo, Ping Men	Ld	MMO06-855A	AB498572		7	AB547321	
			MMO06-855B	AB547217		7	AB547322	
<i>L. oblongifolius</i> W.T. Wang	China, Guangxi, Napo, Nong Biming	Lo1	MMO01-142	AB498571		7	AB547318	
	China, Yunnan, Funing, Tian Peng	Lo2	MMO06-937A	AB498570		7	AB547319	
			MMO06-937B	AB547218		7	AB547320	
<i>L. serratus</i> D. Don	China, Yunnan, Xichou	Ls	MMO01-100	AB498587		7	AB547323	
Outgroup								
<i>Aeschynanthus acuminatus</i> A.P. de Candolle	Japan, Ryukyus, Okinawa, Iriomote Is.		GK11644	AB498561				
<i>A. austroyunnanensis</i> W.T. Wang	China, Yunnan, Xishuangbanna (cult RBGE 19951561)		A. Reid & J. Fernie 004	AB000000				
<i>A. longicalyx</i> Ridl.	Malaysia, ex cult RBGE 1968624		Paddy 600	AB000000				
<i>Hemiboea cavaleriei</i> H. Léveillé	China, unknown locality		Gu G3	FJ501355				
<i>H. subcapitata</i> C. B. Clarke	China, Chengdu-Chongqing		Wang 11306	FJ501357				
<i>H. bicornuta</i> (Hayata) Ohwi	Unknown origin, cult. RBGE		19951207	FJ501356				

^a Referring to the text and Fig. 1.

^b GK G. Kokubugata (TNS), MMO M. Möller (E).

^c ITS sequences registered in the DDBJ database.

^d Length variations of the poly-C tracts at the beginning of ITS2.

94°C, 30 s at 60°C, and 1.5 min at 72°C after an initial denaturing for 3 min at 94°C for ITS, and 35 cycles of 30 s at 94°C, 30 s at 65°C, and 2.5 min at 72°C after an initial denaturing for 3 min at 94°C for *trnC-D*.

The PCR products were checked by electrophoresis before purification using ExoSAP-IT (USB Corporation, Cleveland, OH). Cycle sequencing was carried out using a BigDye Terminator cycle sequencing kit ver. 3.1 (Applied Biosystems, Foster City, CA), using the PCR primers, and additional internal reverse primer 'N2' (5'-GGC GCA ACT TGC GTT CAA-3') and forward primer 'N3' (5'-GCT CTC GCA GCA TCG ATG AAG-3') designed by T. Yukawa (TNS, personal communication) for ITS, and an additional internal primer 'ycf6F' (5'-ATG GAT ATA GTA AGT CTY GCT TGG GC-3'), 'psbMF' (5'-AGC AAT AAA

TGC RAG AAT ATT TAC TTC CAT-3') and 'psbMR' (5'-ATG GAA GTA AAT ATT CTY GCA TTT ATT GCT-3') for the *trnC-D* spacer (Shaw et al. 2005). The Sanger sequencing products were then purified by ethanol precipitation. Automated sequencing was carried out on an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems). The electropherograms were assembled using ATGC ver. 4.01 (Genetyx, Tokyo, Japan). Sequence data from this study were deposited to DDBJ (Table 1).

Phylogenetic analyses

The sequenced DNA regions were aligned using ClustalX 1.8 (Thompson et al. 1994), then manually adjusted. Two analyses were performed; an ITS analysis including all 62

Table 2 Results of the Kruskal-Wallis test of the leaf ratios of *L. pauciflorus s. l.* investigated in the present study

	No. of samples	Average ratios (\pm SD)	Japan without Okinawa Island	Okinawa Island	Taiwan without Lanyu Island	Lanyu Island	China
Japan without Okinawa Island	9	4.54 (\pm 0.95)	–	$P < 0.001$	n.s.	$P < 0.001$	$P < 0.05$
Okinawa Island	7	2.10 (\pm 0.19)		–	$P < 0.001$	n.s.	n.s.
Taiwan without Lanyu Island	14	3.26 (\pm 0.68)			–	$P < 0.05$	n.s.
Lanyu Island	4	1.78 (\pm 0.17)				–	n.s.
China	8	2.65 (\pm 0.58)					–

n.s. no significant.

in- and outgroup samples, and a combined ITS and *trnC-D* analysis on 56 samples comprising all *L. pauciflorus s. l.* samples rooted on the other *Lysionotus* species. The *trnC-D* sequences were added to obtain further resolution among the *L. pauciflorus s. l.* samples. The combinability of ITS and *trnC-D* sequences was assessed using the incongruence length difference (ILD) test (Farris et al. 1994), implemented as the partition homogeneity test in PAUP* 4.0b10 (Swofford 2002). Phylogenetic analyses were conducted based on maximum parsimony (MP) using PAUP* 4.0b10 (Swofford 2002).

In the MP phylogenetic analyses, indels were treated as missing data. Characters were treated as unordered, and character transformations were weighted equally. The branch collapse option was set to 'amb-' (if minimum length is zero). A heuristic parsimony search was performed with 200 replicates of random additions of sequences, with the ACCTRAN character optimization, tree bisection–reconnection (TBR) branch swapping, MULTREES, and STEEPEST DESCENT options on. Statistical support for each clade was assessed by bootstrap analysis (Felsenstein 1985). To calculate bootstrap values, 10,000 replicates of heuristic searches, with the TBR branch swapping on and MULTREES options off, were performed.

Estimation of divergence time

Because no macro fossils are known from Gesneriaceae, we used rates of sequence evolution to estimate divergence times within *L. pauciflorus*. We used the averaged rate of ITS nrDNA evolution of 4.13×10^{-9} substitutions per site per year for herbaceous plants of Kay et al. (2006). Because the pair-wise genetic distance based on the ITS region between lineages of *L. pauciflorus* was very low, multiple hits and reversals would unlikely have occurred (Sanderson 2002), and we thus used uncorrected ITS distances (cf. Hughes et al. 2005).

Results

Leaf morphometric characters

In *L. pauciflorus s.l.* collected from China, Japan and Taiwan, the length and width of the leaf blade ranged from

14.9 to 82.7 mm and from 5.1 to 42.0 mm, respectively. In the bar chart the leaf length/width ratios of the plants from Japan proper including Yakushima Island tended to be higher (3.6–6.2), with the two plants from Yakushima (J4-GK 11642 and 12181) having the highest ratio (Fig. 4).

Bartlett's test showed a significant difference in variances in the leaf ratio among the five geographic groups ($P < 0.05$). While the Kruskal-Wallis test showed that the leaf ratio of the Japan proper samples was not significantly different from those of Taiwan proper, it was significantly different from those of China ($P < 0.05$), Okinawa (Japan) and Lanyu (Taiwan) (both $P < 0.001$). The samples from Taiwan proper were different from those of Okinawa ($P < 0.001$) and Lanyu ($P < 0.05$) (Table 2).

Variation in the nrITS and cpDNA *trnC-D* sequences in *L. pauciflorus s. l.*

Eleven ITS types ('A' to 'K' in Table 1) were detected among the *L. pauciflorus s. l.* samples investigated. The ITS sequences of plants from four populations in Japan proper were identical in nucleotides, with only variations in the poly-C tracts at the beginning of ITS2, from 15 bp in one of the two Yakushima plants (J4-GK11642) to 7 bp in the Miyazaki plant (J3-GK 9162) (ITS types 'A' to 'E'). A single ITS type was detected among the 14 plants in the seven populations of Taiwan including Lanyu Island ('G'). Three ITS types were found among the 20 plants in ten populations from China ('I' to 'K' in Table 1). On Okinawa Island, the ITS sequence for two plants in population O2 (O2-GK11880 and 11883) was identical with that of the Taiwanese plants. Sequences of two plants in population O1 (O1-735 and 12182) differed by a transition from 'T' to 'G' at position 128 ('F'). Two other plants in the same population (O2-GK11882 and 12280) showed both nucleotides 'T' and 'G' at position 128, visible as double peaks in the respective electropherograms ('H').

Eight cpDNA haplotypes were detected in *trnC-D* among the *L. pauciflorus s. l.* samples investigated here ('a' to 'h' in Table 1). In Japan proper, the sequences of two Yakushima plants ('b') differed from the others ('a') by a single transition (J4-GK11642 and 12181). The plants from

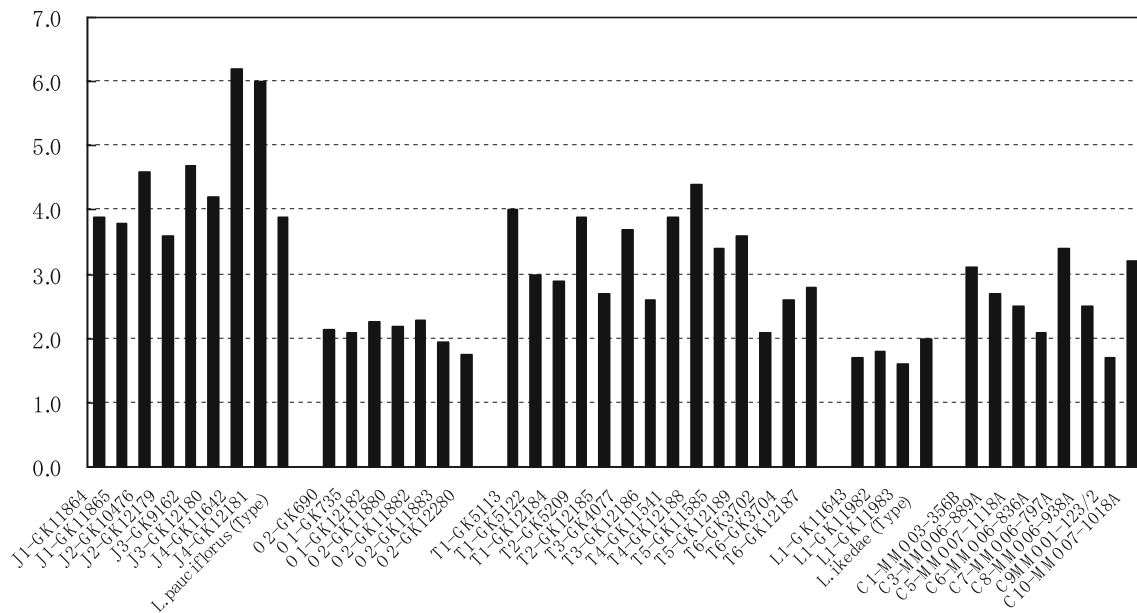


Fig. 4 Leaf ratios of samples of *L. pauciflorus s. l.* analyzed morphologically in the present study

Taiwan and Okinawa Island shared identical sequences ('c'), except two plants from the Taiwanese population T5 ('d') (T5-GK11585 and 12189). In China four haplotypes were detected ('e' to 'h' in Table 1).

Verification of monophyly of *L. pauciflorus s. l.* samples based on ITS

The ITS matrix was 687 bp long and contained 40 uninformative and 149 parsimony-informative characters. The MP analysis of the ITS dataset resulted in four most parsimonious trees of 261 steps length and a consistency index (CI) of 0.84, retention index (RI) of 0.93 and rescaled consistency index (RC) of 0.78. The *L. pauciflorus s. l.* sampled formed a highly supported clade (bootstrap support 99%) with the other *Lysionotus* species forming species clades in grades with *L. serratus* sample closest to the *L. pauciflorus s. l.* clade (BS 98%), followed by *L. chingii* (BS 100%), and a clade of *L. oblongifolius* + *L. denticulosus* (BS 93%). The 48 samples of *L. pauciflorus s. l.* formed three groups, with the samples from China falling on a polytomy, with the remaining samples from Japan proper (BS 63%) and Taiwan + Okinawa (BS 95%) as sister clades (Fig. 5).

Inter- and intraspecific phylogenetic relationships based on ITS and *trnC-D* sequences

The ILD test confirmed the presence of significant congruence between the ITS and *trnC-D* sequences in the reduced *Lysionotus* dataset ($P = 0.41$) and the data were deemed combinable. The combined matrix had 87

parsimony-informative and 17 uninformative characters across the 2,389 characters. The MP analysis yielded eight equally most parsimonious trees of 113 steps with high character fit values (CI 0.93, RI 0.98, RC 0.91; Fig. 6). The samples of *L. denticulosus* and *L. oblongifolius* formed a monophyletic clade (BS 100%), followed by those of *L. chingii* (BS 100%) and *L. serratus* as closest to the *L. pauciflorus s. l.* samples (BS 100%). The 48 samples of *L. pauciflorus s. l.* formed a highly supported clade (BS 98%; clade A in Fig. 6).

Among *L. pauciflorus s. l.*, the Japanese and Taiwanese samples together formed a strongly supported subclade (BS94%; clade D in Fig. 6). The samples from China were split, with four samples collected from Yunnan (MMO 07-1018A & B) and Guangxi (MMO 06-889A & B) forming a sister clade to the Japanese/Taiwanese subclade (BS 86%; together clade C in Fig. 6). Within this subclade plants from Japan proper (BS 87%; clade E in Fig. 6) were sister to samples from Okinawa Island and Taiwan (BS 99%; clade F in Fig. 6). Among the Taiwanese samples GK11585 and GK12189 formed a weakly supported clade (BS60%).

Estimation of divergence times

The divergence time based on the ITS sequence divergence between Chinese and Japanese + Taiwanese plants (clade C in Fig. 6) was estimated to be about 1.1 ± 0.5 MYA, and the split between samples from Japan proper (clade E in Fig. 6) and those from Okinawa Island and Taiwan (clade F in Fig. 6) was estimated to have occurred about 0.9 ± 0.4 MYA. Both dates correspond to the middle Pleistocene epoch.

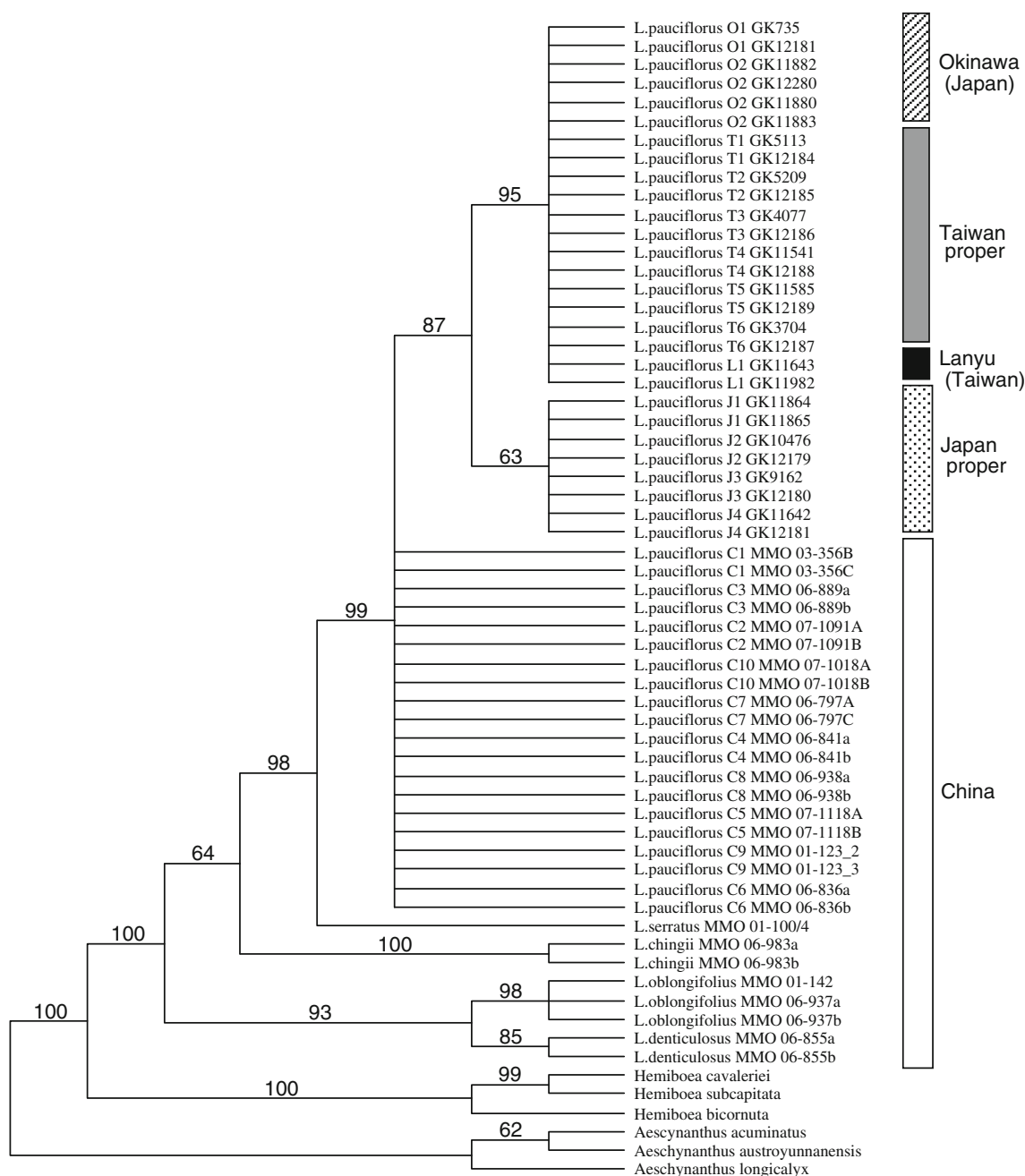


Fig. 5 Strict consensus tree based on four most parsimonious trees for 62 samples of five species of *Lysionotus* plus three samples each of *Aeschynanthus* and *Hemiboea* as outgroups, based on ITS sequences. Numbers above the branches indicate bootstrap percentages

Discussion

Validation of taxonomic classification using leaf-shape data

The present leaf-morphometric analysis showed no significant difference in leaf ratio ($P > 0.05$) between *Lysionotus* plants from Japan proper, i.e. *L. pauciflorus sensu* Yamazaki (1993, 1994), and those from Taiwan proper, i.e. *L. apicidens sensu* Yamazaki (1993, 1994). This result

indicates that the plants from Japan proper are not clearly distinguishable from those of Taiwan proper by their leaf shape, thus disagreeing with Yamazaki (1993, 1994).

In the present study, although the eight plants from Japan proper fell into a separate clade and had narrower leaves, their leaf ratios were not significantly different from those of the plants from Taiwan proper (i.e. they were also shorter). These two clades also formed sister clades. This further supports their taxonomic closeness. However, the leaf ratio of the *L. pauciflorus s. l.* samples was not always

differences, fully correlating with the phylogeny are found in future studies.

L. pauciflorus dispersal in Northeast Asia

From our present phylogenetic analysis it is clear that the origin of *L. pauciflorus* lies in China, as the lineages branching off first among the *L. pauciflorus* samples come from China, as do most of the species in the genus, such as the four representative species included here, *L. serratus* (widely distributed in the southern part of China, Guangxi, Guizhou, Xizang and Yunnan, and in Bhutan, India, Myanmar, Nepal, Thailand and Vietnam), *L. chingii* (Guangxi and Yunnan in China, and Vietnam), *L. oblongifolius* (Guangxi in China) and *L. denticulosus* (Guangxi and Yunnan in China) (Wang et al. 1998).

Following the evolution of *L. pauciflorus* in China, the species must have dispersed into regions of Taiwan and Japan (Fig. 6; Electronic Supplementary Material). Taiwan proper is thought to have emerged during the late Miocene (9 MYA) due to folding of the sea floor of the Philippine Sea plate (Sibuet and Hsu 2004), and was repeatedly connected to the Eurasian Continent during glacial–interglacial cycles in the late Pleistocene (0.2–0.02 MYA) (Boggs et al. 1979). The estimated divergence time of 1.1 MYA between plants of *L. pauciflorus* in China and Taiwan here fits a dispersal route from China to Taiwan by land, and current isolation by raised sea levels (i.e. vicariance).

The two biogeographical barriers for terrestrial plant and animal dispersal, namely the Tokara Gap and Kerama Gap, in the Ryukyus (Fig. 1) are important for *Lysionotus* studied here. The Kerama Gap has been varyingly estimated to have finally formed during the late Pleistocene (0.02 MYA) (Ujiié 1990) to the mid Pleistocene (1 MYA) (Kizaki and Oshiro 1977). However, no variation in the ITS and *trnC-D* sequences were found between some plants from Taiwan and Okinawa. This suggests a recent dispersal of *L. pauciflorus* between these islands, and that this dispersal occurred across water. The absence of *L. pauciflorus* in the southern Ryukyus (Yokota and Tomiyama 2006), situated between Okinawa Island and Taiwan (Fig. 1), supports such a long-distance dispersal event between Taiwan and Okinawa Island.

The Tokara Gap (Fig. 1) is thought to have existed at least since the early Pleistocene (1.5 MYA) (Kizaki and Oshiro 1977; Ota 1998). We calculated a divergence time of 0.9 MYA between plants from Japan proper including Yakushima (clade E in Fig. 5) and those of Okinawa Island and Taiwan (clade F in Fig. 5). This is too recent to explain dispersal of *L. pauciflorus* across the gap via a land-bridge.

Lanyu Island (Taiwan) is an oceanic island that has never been connected to the continent or other islands (e.g.

Shen and Tsai 2002). In view of our data here, *L. pauciflorus* must have dispersed from Taiwan proper to Lanyu Island across the sea. The identical ITS sequence of plants from Lanyu and Taiwan proper suggests that this was a relatively recent dispersal event.

Thus, it seems that habitat expansion of *Lysionotus* across the Ryukyus involved repeated dispersals across water. Hydrochory is an unlikely mechanism by which these plants cross water; the seeds are very small with a thin seed testa. Exozoochory or anemochory, on the other hand, are more likely scenarios. Seeds of *L. pauciflorus* are 0.5 mm long with 2 mm long hair-like appendages on both the micropylar and hilar end (Fig. 3) (Wang et al. 1998). These hair-like appendages could aid their attachment to the feet of birds, or, more importantly, reduce the specific weight of the seeds that could aid sea-crossings by anemochory. It is well known that Taiwan and the eastern part of Japan including the Ryukyus experience several typhoons annually with strong winds (e.g. Hatusima 1975), and thus seed dispersal by wind is the most likely scenario. This way, *L. pauciflorus* seems to have dispersed across the Kerama Gap and Tokara Gap.

Direction of dispersal: south–north or north–south?

Different dispersal patterns from China to Japan and Taiwan have been proposed: Setoguchi et al. (2006, 2008) recently suggested that *Caridiandra* (Hydrangeaceae) and *Pieris* (Ericaceae) showed a northward dispersal pattern from China to Japan proper through land-bridges of Taiwan and the Ryukyus. Conversely, Hatusima (1975) speculated that certain temperate plant taxa, for example *Disporum*, *Polygonatum* (Liliaceae) and *Ajuga* (Lamiaceae), dispersed southwards from Japan proper to the Ryukyus through a land-bridge crossing the Tokara Gap. In view of the current distribution pattern of *L. pauciflorus* in southern parts of China, it is tempting to speculate a northward dispersal route from China to Japan proper via Taiwan and Okinawa Islands. Our molecular phylogenetic results are somewhat equivocal as to the dispersal direction of *L. pauciflorus*. The origin of the species in China is undisputable (Figs. 5 and 6; Electronic Supplementary Material). The major split among the samples involves Japan proper on one side and Taiwan and Okinawa on the other and involves either a crossing of the Tokara Gap from the south (prior to the formation of the Kerama Gap), or an occupation of Japan proper from the north. Previously, major north–south dispersal routes of terrestrial plants from China to Japan proper suggested routes through the Ryukyus: one is from Siberia to the northern part of Japan proper through Sakhalin. Another route is from the Korean Peninsula to western parts of Japan proper (Sauer 1998). The opening of the Tsushima Strait in the early Pliocene (1.7–1.5 MYA)

severing the land connection between the Korean Peninsula and Japan proper (Kitamura et al. 2001), would roughly fit the divergence times for the major split here (given the high SE). However, there is no evidence that suggests that *Lysionotus* species have in the past distributed or are currently dispersing in the Korean Peninsula and adjacent regions of China. Following the current distribution patterns of *Lysionotus* species, a hypothetical north to south dispersal in Japan of *L. pauciflorus* is thus less likely.

At the moment, there is not enough data to completely clarify the dispersal direction within the regions of Japan and Taiwan for *Lysionotus*, but a south–north movement is more likely. Further analyses using more fine-scale DNA markers, such as microsatellites or AFLPs, are necessary to fully address this issue.

Acknowledgments We thank C.-I. Huang (HAST), T. Minamitani, M. Morimoto, the Motobu Municipal Museum and S. Azuma for providing plant materials. We also thank K. Nakamura (HAST) for valuable suggestions. This study was conducted in connection with projects on the “Integration of Systematics and Molecular Phylogenetics in All Groups of Organisms” and “Taxonomic and Conservation Studies on Endangered Plant Species in Japan” managed by the National Museum of Nature and Science, and supported in part by a Grant-in-Aid for Scientific Research Program C (JSPS; no. 20510220; G.K.) and 21st Century COE program of the University of the Ryukyus (M.Y.). RBGE is supported by the Scottish Government Rural and Environment Research and Analysis Directorate (RERAD). The field work of M.M. was supported by the Percy Sladen Memorial Fund, Oleg Polunin Memorial Fund, and Davis Expedition Fund of the University of Edinburgh.

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