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Migration of *Lobelia zeylanica* (Campanulaceae) from Taiwan to the Ryukyu Archipelago Inferred from Chloroplast DNA Data

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Abstract Molecular phylogenetic analysis of *Lobelia zeylanica*, broadly distributed in tropical Asia, were conducted to elucidate its migration route to the northernmost populations in subtropic Yonaguni Island, the Ryukyu Archipelago of Japan. The present analysis using *rbcL* gene and *trnL-F* intergenic spacer region of chloroplast DNA revealed that all two Yonaguni plants and three of four Taiwanese plants shared the identical sequences in the two markers. They formed a well-supported clade with a Taiwanese remainder and were separated from plants from Malesian regions. The present study suggests that *L. zeylanica* have likely migrated from Taiwan to Yonaguni Island.

Key words: *Lobelia*, migration, phylogeography, Ryukyus, Taiwan.

Introduction

The Ryukyu Archipelago (the Ryukyus) is situated between Kyushu Island of Japan and Taiwan, and comprises about 140 islands (Fig. 1). The Ryukyus is biogeographically divided into three major areas, namely, the northern, central and southern Ryukyus, each of which has a characteristic biota (e.g., Tagawa and Miyagi, 1991; Ota, 1998; Nakamura *et al.*, 2009). Yonaguni Island studied herein belongs to the southern Ryukyus, and is about 130 km away from Taiwan Island (Fig. 1). Yonaguni and Taiwan islands share many floristic elements including those presumably originated in Malesian region (Hatusima, 1975), but few phylogeographical study has been conducted to elucidate migratory history of such plant taxa.

Lobelia zeylanica L. (Campanulaceae, sect. *Delostemon*) (Fig. 2) is a perennial herb, and widely distributed from New Guinea and Sri Lank (Lammers, 2007) to Yonaguni Island of the Ryukyus (Hatusima, 1975). In Japan, this species is known from only three populations in Yonaguni Island (unpublished data), which marks the northern limit of its distribution range. Thus *L. zeylanica* is treated as a critically endangered species in the Japanese plant redlist (Japanese Ministry of the Environment, 2012). In this study, we conducted molecular phylogenetic analysis on *L. zeylanica* collected from Yonaguni Island, Taiwan Island, Luzon Island of the Philippines and Borneo Island of Malaysia, using *rbcL* gene and *trnL-F* intergenic spacer region of chloroplast DNA for discussing its migration route to Yonaguni Island.

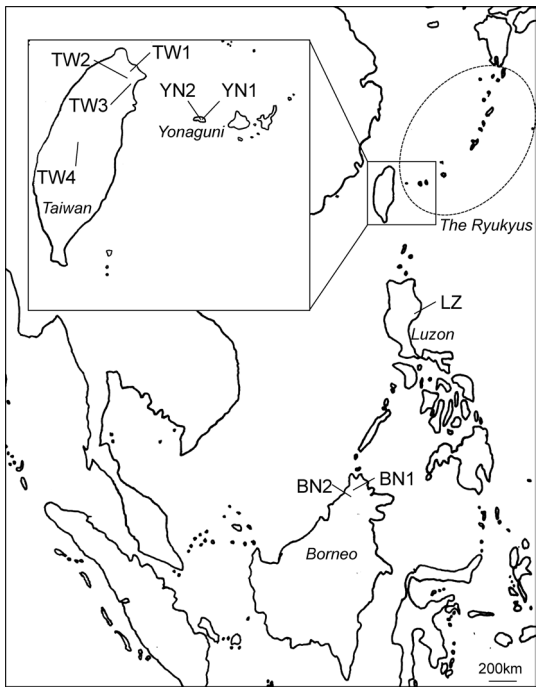

Fig. 1. Map showing nine localities where *Lobelia zeylanica* plants were collected. Broken lines circle the islands of the Ryukyus. For abbreviations for collection localities, refer Table 1.

Materials and Methods

Plant materials

For the present molecular analysis, a plant of *Lobelia zeylanica* was collected from each population at two localities in Yonaguni Island, four localities in Taiwan Island, one locality in Luzon Island of the Philippines, and two localities in Borneo Island (Malaysian territory), in total nine plants were obtained (Table 1 and Fig. 1). Voucher specimens for the molecular analysis were deposited in the herbarium of the National Museum of Nature and Science (TNS).

Lobelia aquatica Cham. closely related to *L. zeylanica* (Kokubugata *et al.*, 2012), was selected as an outgroup taxon, and its sequences of *rbcL* (EF141029) and *trnL-F* (DQ356182) reported by Antonelli (2008) were obtained from the DDBJ/EMBL/GenBank database (http://www.ncbi.nlm.nih.gov/gquery/) (Table 1).

DNA extraction, PCR and sequencing

Genomic DNA was extracted from silica gel

Fig. 2. Habit of *Lobelia zeylanica* in Yonaguni Island (*GK14127*; photographed on October 11, 2011).

Table 1. Nine plants of *Lobelia zeylanica* investigated in the present study

Species	Collection locality	Abbreviation*	Voucher no.	*rbcL*		*trnL-F*	
				Accession no.**	Type	Accession no.**	Type
L. zeylanica	Japan, Ryukyus, Okinawa, Yonaguni (1)	YN1	*GK14126*	AB818960	A	AB793518	a
	Japan, Ryukyus, Okinawa, Yonaguni (2)	YN2	*GK14127A*	AB818961	A	AB793519	a
	Taiwan, New Taipei, Pingchi	TW1	*GK8775*	AB645971	A	AB793522	a
	Taiwan, New Taipei, Shihting	TW2	*GK15118*	AB818963	A	AB793521	a
	Taiwan, Ilan, Toucheng	TW3	*GK14959*	AB818962	A	AB793520	a
	Taiwan, Nantou, Yuchih	TW4	*GK11617*	AB818964	A	AB793523	b
	Philippines, Luzon, Isabela, Dinapigue	LZ	*GK13156*	AB818965	B	AB793524	c
	Malaysia, Borneo, Sabah, Kinabaru	BN1	*GK7894*	AB645970	C	AB793525	d
	Malaysia, Borneo, Sabah, Crocker	BN2	*GK7880*	AB645969	C	AB793526	e

* Referring Fig. 1.

** Deposited Sequences in the DDBJ database.

dried leaves using the DNeasy Plant Mini Kit (Qiagen, Qiagen, Valencia, CA) 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, Japan. The sequence of 1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbcL*) and intergenic spacer region between *trnL* and *trnF* genes including *trn*L3′exon (*trnL-F*) were amplified by the polymerase chain reaction (PCR) using an iCycler (BIO RAD, California, USA). Forward primer '*rbcL*cF' (5′-TGA AAA CGT GAA TTC CCA ACC GTT TAT GCG-3′) and reverse primer '*rbcL*aR' (5′-CTT CTG CTA CAA ATA AGA ATC GAT CTC TCC A-3′) were used for *rbcL* (Hasebe *et al.*, 1994); and forward primer '$trnT^{UGU}$F (TabA)' (5′-CAT TAC AAA TGC GAT GCT CT-3′) and reverse primer '$trnF^{GAA}$ (TabF)' (5′-ATT TGA ACT GGT GAC ACG AG-3′) were used for *trnL-F* (Taberlet *et al.*, 1991). Amplifications were performed using Takara Ex *Taq* (Takara, Otsu, Japan) with Ampdirect Plus (Shimadzu, Kyoto, Japan). The PCR profile was 35 cycles of 30 sec at 94℃, 30 sec at 50℃, and 1.5 min at 72℃ after an initial denaturing for 3 min at 94℃ for *rbcL*; and 35 cycles of 30 sec at 94℃, 30 sec at 55℃, and 1.5 min at 72℃ after an initial denaturing for 3 min at 94℃ for *trnT-F*. The PCR products were checked by electrophoresis before purification using ExoSAP-IT (USB Corp., Ohio, USA). Cycle sequencing was carried out using the BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA); using the PCR primers for *rbcL*, and using PCR primer of '$trnF^{GAA}$ (TabF)', and additional internal forward primers '$trnL59^{UAA}$F (TabC)' (5′-CGA AAT CGG TAG ACG CTA CG-3′) and $39trnL^{UAA}$F (TabE) (5′-GGT TCA AGT CCC TCT ATC CC-3′) and a reverse primer '$39trnL^{UAA}$R (TabD) (5′-GGG GAT AGA GGG ACT TGA AC-3′) for *trnL-F* (Taberlet *et al.*, 1991). The Sanger sequencing products were then purified by ethanol precipitation. Automated sequencing was carried out on an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, California, USA). The electropherograms were assembled using ATGC ver. 4.01 (GENETYX Co., Tokyo, Japan). Sequence data from this study were deposited to DDBJ (http://www.ddbj.nig.ac.jp/) (Table 1).

Phylogenetic analysis

DNA sequences were aligned using ClustalW 1.8 software (Thompson *et al.*, 1994) and then manually adjusted. The combinability of the two cpDNA regions was assessed using the incongruence length difference (ILD) test (Farris *et al.*, 1994). Phylogenetic analysis were conducted based on a maximum parsimony (MP) criterion

using PAUP* version 4.0b10 (Swofford, 2002).

In the MP phylogenetic analysis, indels were treated as missing data. Characters were treated as unordered, and character transformations were weighted equally. 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 by bootstrap analysis (Felsenstein, 1985). Ten thousand replicates of heuristic searches, with the TBR branch swapping, and MULTREES option off, were performed to calculate bootstrap values (BS).

Results and Discussion

Phylogenetic relationships based on rbcL and trnL-F

In *rbcL* sequence, three haplotypes were recognized (types A–C) in *Lobelia zeylanica*, including type A in two plants from Yonaguni Island and the four plants from Taiwan Island, type B in the plant from Luzon Island, and type C in the two plants from Borneo Island (Table 1 and Fig. 3). In *trnL-F* sequence, five haplotypes a–e were recognized (types a–e), including type a in the two plants from Yonaguni Island and three of the four plants from Taiwan Island, type b in the other plant from Taiwan Island, type c in the plant from Luzon Island, type d in the plant from Borneo Island, and type e in the other plant from Borneo Island (Table 1 and Fig. 3).

After alignment of the sequences of the nine plants of *L. zeylanica* with the outgroup taxon of *L. aquatica* (in total 10 OTUs), the sequence length of 867 bp in *rbcL* and 972 bp in *trnL-F* were obtained. The ILD test did not show significant incongruence between the two markers ($p = 1.00$). The MP analysis was therefore conducted using the combined data of 1839 (867 + 972) bp. In the MP analysis, 4 of 28 (3 of 7 in *rbcL* and 1 of 21 in *trnL-F*) variable characters were parsimony-informative. A single parsimonious tree of 28 steps was obtained with a consistency index (CI) of 1.00 and retention index (RI) of 1.00.

Nine plants of *L. zeylanica* were divided to three clades (I to III in Fig. 3): clade I was composed of plants from Yonaguni and Taiwan islands, clade II was composed of the plant from Luzon Island, and clade III was composed of the two plants from Borneo Island.

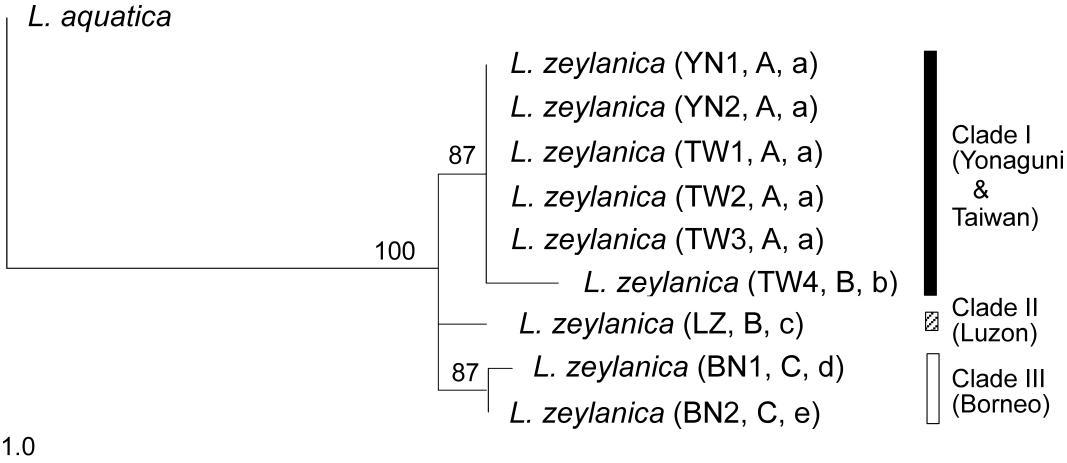

Fig. 3. The most parsimonious tree for nine plants of *Lobelia zeylanica* with an outgroup taxon of *L. aquatica*. Numbers above the branches indicate bootstrap percentage. Parenthetic abbreviations and alphabets indicate collection locality (refer Table 1 and Fig. 1) and haplotypes of *rbcL* (A–C) and *trnL-F* (a–e).

Implication of dispersal event of L. zeylanica *from Taiwan to Yonaguni Island*

As mentioned in the introduction, *L. zeylanica* primarily distributes in the Asian tropics (e.g., Lammers, 2007), with extending subtropical Yonaguni Island as northern limit of its distribution range (Hatusima, 1975). Therefore, it is more reasonable to consider that this species has dispersed from south to north in the north hemisphere. Our MP analysis revealed that the plants of *L. zeylanica* from Yonaguni Island were more closely related to those from Taiwan Island than those from Luzon and Borneo islands. Based on these results, we conclude that *L. zeylanica* has likely migrated from Malesian region to Yonaguni Island via Taiwan Island.

Lobelia zeylanica is known to bear dehiscent dry fruits and seeds with no attachment like a wing (Lammers, 1998). Therefore, this species is not likely to cross over sea migratory birds (fruit) or winds (seed). Geohistorically, Yonaguni Island is thought to have been finally separated from Taiwan Island in early Pleistocene (1.5–0.7 MYA) based on geological and palaeo-oceanological data (Koba, 1992; Osozawa *et al.*, 2011). Substitution rate of *trnL-F* spacer region in annual or perennial herbs was estimated as 8.24×10^{-9} (substitution/site/year) (Richardson *et al.*, 2001). The timing of the final disconnection between Yonaguni and Taiwan islands and the identical sequences of *trnL-F* of *L. zeylanica* between Yonaguni Island and Taiwan Island are compatible with vicariance scenario. Therefore, we can hypothesize that *L. zeylanica* may have dispersed through land bridge before early Pleistocene in a case of migration from Taiwan Island to Yonaguni Island.

However, *L. zeylanica* widely distribute in tropical Asia (Lammers, 2007), and it is difficult to explain all dispersal events through land bridge for this species. It is reported that certain *Lobelia* species have both of dry and berry fruits, for example fruits of *L. angulata* G. Forst. were varied from berry to capsule (Moelino and Tuyn, 1960); and berry and dry capsule fruits of *L. fluviatilis* R.Br. (= *Hypsela sessiliflora* F.E. Wimmer) were respectively reported by Wimmer (1953) and Heenan *et al.* (2008). Therefore, it is possible to assume that *L. zeylanica* may rarely have had berry fruit, and have been dispersed oversea by migrate birds as the step-wise colonization outlined by Chiang and Schaal (2006). If the dispersal event has happened in this species, we can also assume oversea migration from Taiwan Island to Yonaguni Island after separation of Yonaguni Island from Taiwan Island. Further fruit morphological surveys must be necessary for discussing this issue.

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References

Antonelli, A. 2008. Higher level phylogeny and evolutionary trends in Campanulaceae subfam. Lobelioideae: molecular signal overshadows morphology. Molecular Phylogenetics and Evolution 46: 1–18.

Chiang, T.-Y. and Barbara A. Schaal, B. A. 2006. Phylogeography of plants in Taiwan and the Ryukyu Archipelago. Taxon 55: 31–41.

Farris, J. S., Källersjö, M., Kluge, A. G. and Bult, C. 1994. Testing significance of incongruence. Cladistics 10: 315–319.

Felsenstein, J. 1985. Confidence limits on phylogenies–an approach using the bootstrap. Evolution 39: 783–791.

Hasebe, M., Omori, T., Nakazawa, M., Sano, T., Kato, M. and Iwatsuki, K. 1994. *RbcL* gene sequences provide evidence for the evolutionary lineages of leptosporangiate ferns. Proceedings of the National Academy of Sciences of the USA 91: 5730–5734.

Hatusima, S. 1975. Flora of the Ryukyus, added and corrected. Okinawa Association of Biology Education, Naha (in Japanese).

Heenan, P. B., Knox, E. B., Courtney, S. P., Johnson, P. N and Dawson, M. I. 2008 Generic placement in *Lobelia* and revised taxonomy for New Zealand species previously in *Hypsela* and *Isotoma* (Lobeliaceae). New Zealand Journal of Botany 46: 87–100.

Japanese Ministry of the Environment. 2012. Red Data List (Plants). http://www.biodic.go.jp/rdb/rl2012/redList2012_ikansoku.csv (in Japanese) [Accessed and verified on February 9, 2012]

Koba, M. 1992. Influx of Kuroshio Current into the Okinawa Trough and inauguration of Quaternary coral-reef building in the Ryukyu Island Arc, Japan. Quaternary Research 31: 359–373.

Kokubugata, G., Nakamura, K., Forster, P. I., Hirayama Y. and Yokota, M. 2012. Antitropical distribution of *Lobelia* species (Campanulaceae) between the Ryukyu Archipelago of Japan and Oceania as indicated by molecular data. Australian Journal of Botany 60: 417–428.

Lammers, T. G. 1998. Campanulaceae. In: Editorial Committee of the Flora of Taiwan (ed.) Flora of Taiwan, second edition. Editorial Committee of the Flora of Taiwan, Taipei, pp. 775–800.

Lammers, T. G. 2007. World Check List and Bibliography of Campanulaceae. Royal Botanic Gardens, Kew.

Moelino, B. and Tuyn, P. 1960. Campanulaceae. Flora Malesiana series I. 6: 107–141.

Nakamura, K., Suwa, R., Denda T. and Yokota, M. 2009. Geohistorical and current environmental influences on floristic differentiation in the Ryukyu Archipelago, Japan. Journal of Biogeography 36: 919–928.

Osozawa, S., Shinjo, R., Armid, A., Watanabe, Y., Horiguchi, T. and Wakabayashi, J. 2011. Palaeogeographic reconstruction of the 1.55 Ma synchronous isolation of the Ryukyu Islands, Japan, and Taiwan and inflow of the Kuroshio warm current. International Geology Review 54: 1369–1388.

Ota, H. 1998. Geographic patterns of endemism and speciation in amphibians and reptiles of the Ryukyu Archipelago, Japan, with special reference to their paleogeographical implications. Researches on Population Ecology 40: 189–204.

Richardson, J. E., Pennington, R. T., Pennington, T. D. and Hollingsworth, P. M. 2001. Rapid diversification of a species-rich genus of neotropical rain forest trees. Science 293: 2242–2245.

Swofford, D. L. 2002. PAUP*: Phylogenetic Analysis using Parsimony (*and other methods), ver. 4. Sinauer Associates, Massachusetts.

Taberlet, P., Gielly, L., Pautou, G. and Bouvet, J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Molecular Biology 17: 1105–1109.

Tagawa, H. and Miyagi, Y. 1991. Vegetation of the southwest islands and its features and conservation. In: Natural Conservation Bureau of the Environment Agency (ed.) Report on Essential Conditions for Conservation of Wild Species in Southwest Islands in Fiscal 1990. WWF Japan, Tokyo, pp. 122–183 (in Japanese).

Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673–4680.

Wimmer, F. E. 1953. Campanulaceae–Lobelioideae, II Teil. In: Stubbe, H. and Noach, K. (eds.) Das Pflanzenreich, IV. 276b, pp. 1–813, Akademie Verlag, Berlin.