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

Goro Kokubugata¹*, Yukiko Saito¹, Koh Nakamura², Aurea Lopez-Feliciano³, Mina L. Labuguen⁴ and Masatsugu Yokota⁵

¹Department of Botany, National Museum of Nature and Science, Amakubo 4–1–1, Tsukuba, Ibaraki 305–0005, Japan  
²Biodiversity Research Center, Academia Sinica, Nangang, Taipei 115, Taiwan  
³Isabela State University, Echague, Isabela, Philippines  
⁴Community Environment and Natural Resources Office, Cauayan, Isabela, Philippines  
⁵Laboratory of Ecology and Systematics, Faculty of Science, University of the Ryukyus, Nishihara, Okinawa 903–0213, Japan  
*E-mail: gkokubu@kahaku.go.jp  
(Received 12 February 2013; accepted 25 March 2013)

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.
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...
Migration of *Lobelia zeylanica* from Taiwan

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 *trnL*3′ exon (*trnL-F*) were amplified by the polymerase chain reaction (PCR) using an iCycler (BIO RAD, California, USA). Forward primer *b*rbcLcF (5′-TGA AAA CGT GAA TTC CCA ACC GTT TAT GCG-3′) and reverse primer *b*rbcLaR (5′-CTT CTG CTA CAA ATA AGA ATC GAT CCT TTC A-3′) were used for *rbcL* (Hasebe *et al.*, 1994); and forward primer *b*trnTUGUF (TabA) (5′-CGA AAA CGT GAA TTC CCA ACC GTT TAT GCG-3′) and reverse primer *b*trnF (TabB) (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°C, 30 sec at 50°C, and 1.5 min at 72°C after an initial denaturing for 3 min at 94°C for *rbcL*; and 35 cycles of 30 sec at 94°C, 30 sec at 55°C, and 1.5 min at 72°C after an initial denaturing for 3 min at 94°C for *trnL-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* (TabF), and additional internal forward primers *trnL5*UGUF (TabC) (5′-CGA AAA CGT GAA TTC CCA ACC GTT TAT GCG-3′) and *trnL6*UGUF (TabD) (5′-ATT TGA ACT GGT GAC ACG AG-3′) and a reverse primer 3′*trnL6*UGUF (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.

---

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

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

* Referring Fig. 1.
** Deposited Sequences in the DDBJ database.
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 $8.24 \times 10^{-9}$ (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.

Acknowledgments

We thank C.-I Huang, M. Matsumura, C.-I Peng and M. Suleiman for assistances in the field trips. This study was conducted in connection with the projects 'Elucidative studies of delimitation and origin on endemic and narrow-range species in Japan' and 'Integrated research on biodiversity of interspecies relationships' managed by the National Museum of Nature and Science; was partly supported by the Mitsui & Co., Ltd. Environment Fund (No. C10-C097) and JSPS KAKENHI (Grant No. 25290085).

References


