J Plant Res (2006) 119:401–405
Digital Object Identifier (DOI) 10.1007/s10265-006-0283-1

SHORT COMMUNICATION

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Phylogeography of the genus *Cardiandra* based on genetic variation in cpDNA sequences

Received: September 20, 2005 / Accepted: March 8, 2006 /

Abstract We investigated the phylogenetic relationships within the genus *Cardiandra* based on plastid DNA sequences. The phylogenetic tree showed that *Cardiandra* populations from the Ryukyu Islands (Japan) and Taiwan were monophyletic (Ryukyu–Taiwan clade), whereas taxa from China and mainland Japan were sisters to this clade. The divergence time between the Ryukyu–Taiwan clade and the other species was estimated to be 0.082 MYA, i.e., the late Pleistocene. The infrageneric and/or infraspecific differentiation of *Cardiandra* is estimated to have depended largely on allopatric differentiation caused by the presence or division of the past landbridge of the Ryukyu Islands, which connected mainland Japan to the Asian Continent during the Quaternary.

Key words *Cardiandra* · Eastern Asia · Hydrangeaceae · Phylogeography · Quaternary · Ryukyu Islands

The genus *Cardiandra* is a small genus in the Hydrangeaceae that comprises only two species (Ohba 1985). These perennial herbs are distributed in an arc ranging from mainland Japan to southern China via the Ryukyu Islands and Taiwan (Fig. 1). In the only published taxonomic revision of the genus *Cardiandra*, Ohba (1985) recognized two species, *Cardiandra alternifolia* Sieb. et Zucc. and *C. amamiohsimensis* Koidz., based on floral morphologies. *Cardiandra alternifolia* has ornamental flowers with styles <1.2 mm in length, whereas *C. amamiohsimensis* has nonornamental flowers with longer styles ranging from 1.5–1.7 mm.

Cardiandra alternifolia is widespread across Japan, the Ryukyu Islands (Iriomotejima), Taiwan, and southern China. Three intraspecific taxa are allopatrically distributed (Fig. 1). The subspecies *alternifolia* is confined to mainland Japan, whereas subspecies *moellendorffii* var. *moellendorffii* Ohba is distributed on the island of Iriomotejima and in southern China, and subspecies *moellendorffii* var. *binata* F. Maekawa occurs in Taiwan. The other species, *C. amamiohsimensis*, is endemic to the island of Amamioshima in the Ryukyus.

The archipelago between the Japanese Islands and Taiwan via the Ryukyus is the product of the repeated formation and division of the landbridge to the Asian continent; it continues on through Taiwan, the Ryukyu Islands, and mainland Japan due to transgression and regression resulting from climatic oscillation in the Pliocene and Quaternary periods (Kizaki and Oshiro 1977; Ujiie 1990; Kimura 1996, 2000). The phylogeographic structure of insular *Cardiandra* is thought to have been influenced by the paleogeographic dynamics of the continental island system. Intra- and infraspecific phylogeny of the genus is expected to reflect the geographical history of the Ryukyu Islands.

In this study, we aimed to elucidate the phylogenetic relationship within the genus *Cardiandra* based on molecular data and discuss the phytogeography of the genus, with special reference to the plants distributed in the Ryukyu Islands. For this purpose, we examined the *rbcL* and *matK* genes, as well as two spacer regions of chloroplast DNA.

Cardiandra samples were collected from their natural habitats, cultivation stocks at botanical gardens, and herbarium sheets from the herbarium at Kyoto University (KYO). The specimens and their collection data are listed in Table 1.

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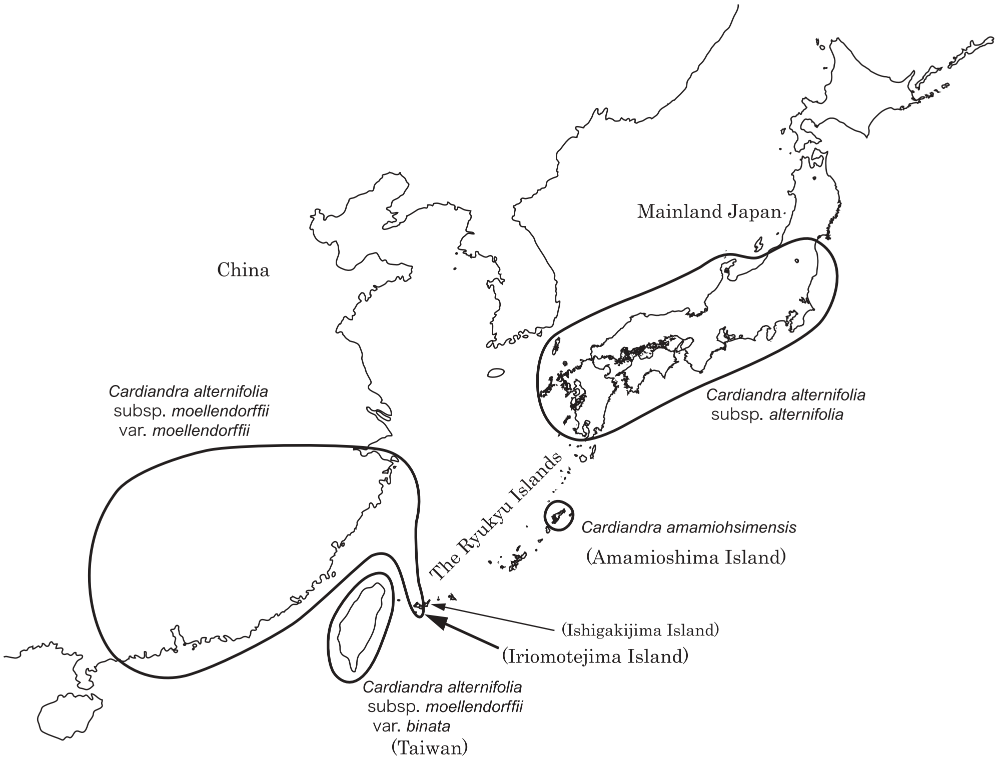

Fig. 1. Geographical distribution of the genus Cardiandra, showing the distribution of *C. alternifolia* subsp. *alternifolia*, *C. alternifolia* subsp. *moellendorffii* var. *moellendorffii*, *C. alternifolia* subsp. *moellendorffii* var. *binata*, and *C. amamiohsimensis*. The distributions are based on Ohba (1985)

Table 1. Materials used in the present study and vouchers

Taxa	Distribution	Voucher specimens
Cardiandra alternifolia Sieb. et Zucc.		
Subsp. *alternifolia*	Mainland Japan	A. Sogo 9906-3 (KYO)
Subsp. *moellendorffii* (Hance) Hara et H. Ohba		
Var. *moellendorffii*	Iriomotejima Island	K. Yasuda 2257 (KYO)
	China	Liu, no number (KYO)
Var. *binata* F. Maekawa	Taiwan	Setoguchi 04T-M2303 (KYO)
Cardiandra amamiohsimensis Koidz.	Amamioshima Island	Cultivated at Tsukuba Botanical Garden [TBG84096]
Deinanthe bifida Maxim.	Mainland Japan	Cultivated at Kyoto Botanical Garden [no number]
Hydrangea paniculata Sieb.	Mainland Japan – China	Chia 534 (HAST)
Hydrangea macrophylla (Thunb.) Ser.	Mainland Japan and the Izu Islands	Setoguchi H20-05 (KYO)

KYO Herbarium of Kyoto University; *HAST* Herbarium of the Academia Sinica, Taiwan

Voucher specimens were deposited in the KYO herbarium. The leaves were dried and kept in silica gel for DNA extraction. *Deinanthe bifida* and two species of *Hydrangea* in the Hydrangeaceae were chosen as outgroups based on the reconstructed phylogeny of the family (Hufford et al. 2001).

Silica-gel-dried or fresh samples were frozen using liquid nitrogen and ground to a fine powder. Before DNA extraction, the leaf powder was suspended in HEPES buffer (pH 8.0) and centrifuged at 10,000 rpm at 20°C for 5 min to remove sticky polysaccharides (Setoguchi and Ohba 1995). Total DNA was isolated from the pellets using the CTAB method of Doyle and Doyle (1987).

Double-stranded DNA from two coding regions, the *rbcL* and *matK* genes, the *trnK* intron (5′ and 3′ sides of the *matK* gene), and the *trnS* (GCU)–*trnG* (UCC) spacer of chloroplast DNA was examined. Primers were synthesized based on Hasebe et al. (1994), Ooi et al. (1995), Demesure et al. (1995), and Hamilton (1999) respectively.

Each region was amplified through 30 cycles of symmetric polymerase chain reaction (PCR). PCR cycle conditions followed those of Setoguchi and Watanabe (2000), with modification of the annealing temperature to each Tm value. The PCR products were purified by Sephadex G-100 (Amersham Pharmacia Biotech, Uppsala, Sweden) following the method of Miikeda and Yukawa (2001). Purified DNA was sequenced using the standard methods of the Taq Dye Deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), using the primers above on a Model 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequence data were aligned manually with the GENETYX program (The Software Development Co., Tokyo, Japan). Insertions and/or deletions (indels) were generally placed to increase the number of matching nucleotides in a sequence position. After machine-aligning the sequences, we manually fine-tuned the alignment.

Sequences of the *rbcL* gene (1,268 bp), *trnK* intron (2,421 bp) plus 1,514 bp of the *matK* gene, and the *trnS* (GCU)–*trnG* (UCC) spacer (~ 755 bp) were determined for samples of five plants of *Cardiandra* and for the three outgroup taxa. All DNA sequences were deposited in the DDBJ/EMBL/GenBank databases (accession numbers AB236023–AB236045).

Parsimony analysis of the branch-and-bound search was performed using PAUP* version 4.0*beta v. 10 (Swofford 2002). Gaps in DNA sequences were treated as missing data in the analysis. Phylogenetic analysis based on the combined data of the *rbcL* and *matK* genes, the *trnK* intron, and the *trnS*–*trnG* spacer (ca. 4,460 bp) generated a single most-parsimonious (MP) tree of 199 steps with a consistency index of 0.927 (excluding uninformative characters) and a retention index of 0.948 (Fig. 2). The bootstrap values (Felsenstein 1985) of 1,000 replicates and decay indices (Bremer 1988, 1994) are presented below each branch in Fig. 2. The analysis supported the genus *Cardiandra* as a monophyletic group with a bootstrap value of 100% and a decay index >5.

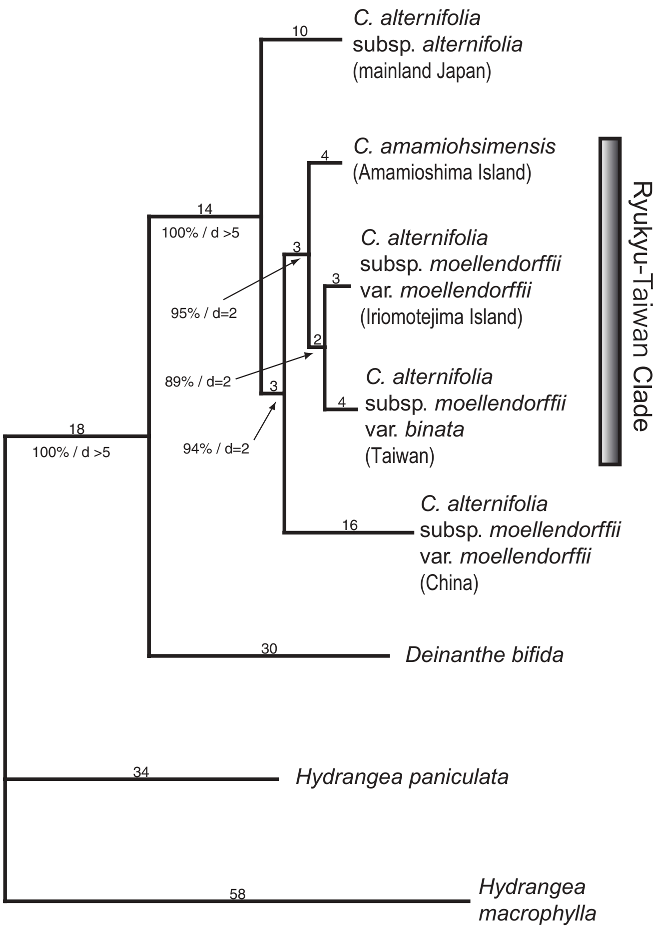

Fig. 2. Single most parsimonious tree of *Cardiandra* derived from the analysis of the combined data of *rbcL*, *matK*, *trnK* intron, and *trnS*–*trnG* spacer (tree length = 199 steps; CI = 0.927; RI = 0.948; excluding uninformative characters). *Numbers above branches* indicate branch length, and *numbers below branches* indicate bootstrap percentages of 1,000 replicates (*left*) and decay indices (*right*). The distribution or locality of sample materials of *Cardiandra* is shown in *parentheses* below the OTU

Within the genus *Cardiandra*, the analysis indicated that *C. alternifolia* subsp. *alternifolia* from mainland Japan was a sister to the remaining groups (Fig. 2). *Cardiandra alternifolia* was suggested to be a paraphyletic group; the clade of the remaining samples included *C. amamiohsimensis* and *C. alternifolia* from southern China, Taiwan, and Iriomotejima (supported by a bootstrap value of 94% and a decay index of 2). Moreover, *C. alternifolia* subsp. *moellendorffii* was revealed to be paraphyletic, i.e., the monophyletic group of subsp. *moellendorffii* from Iriomotejima and Taiwan was clustered with the insular species endemic to Amami-oshima, *C. amamiohsimensis* (supported by a higher bootstrap value at 95% and a decay index of 2). Thus, our results suggest that *Cardiandra* from the Ryukyu Islands and Taiwan are monophyletic (Ryukyu–Taiwan clade: Fig. 2). The phylogenetic tree indicated that subsp. *moellendorffii* from southern China was sister to the Ryukyu–Taiwan clade.

We calculated the number of synonymous substitutions in the *rbcL*- and *matK*-gene-coding regions to estimate the divergence time between the Ryukyu–Taiwan clade and its sister taxa distributed in China and mainland Japan. One and three synonymous substitutions were found in the *matK*-gene-coding region between the Ryukyu–Taiwan clade and its sister taxa in China, and between taxa of mainland Japan and remaining samples, respectively. The divergence time for the two groups was calculated by $T = D_A/2\lambda$ (Nei 1987). For the *matK* gene of *Cardiandra*, we used the rate of synonymous nucleotide substitutions per site year (λ) of *Fagopyrum*, which is 4.0×10^{-9} substitutions synonymous site^{-1} year^{-1} (Yamane et al. 2003) as a reference rate for perennial herbs. The estimated divergence time between the Ryukyu–Taiwan clade and its sister taxa was about 0.082 MYA, i.e., the late Pleistocene of the Quaternary. Moreover, the estimated divergence time between *C. alternifolia* in mainland Japan and the remaining samples was estimated to be about 0.246 MYA, i.e., the middle Pleistocene of the Quaternary. During that period, the Ryukyu Islands underwent repeated formation and division of the landbridge from southeastern China to Japan via Taiwan and the Ryukyu Islands by means of transgression and regression (Kizaki and Oshiro 1977; Ujiie 1990; Kimura 1996, 2000). The phytogeographical structure of island plants is related to the paleogeographic dynamics of the continental island system, i.e., distributions of plant taxa showed repeated glacial range expansions (formation of landbridges) and interglacial range contractions (fragmentation into islands) in the Pliocene and Quaternary periods. In fact, phylogeographical structures of some plant taxa and traces of introgressive hybridization between allopatric species in the Ryukyu Islands have been reported (e.g., Setoguchi and Watanabe 2000; Chiang et al. 2001; Hiramatsu et al. 2001). Thus, it is possible that the common ancestor of the Ryukyu–Taiwan clade expanded its distribution through the landbridge of the Ryukyu Islands.

The topogeographic proximity observed within phylogenetic relationships in insular *Cardiandra* species suggests their speciation, in association with the configuration of the past landbridge resulting from climate oscillations during the Pliocene and Quaternary era. *Cardiandra* plants on the islands shared a common ancestor found on the continuous landmass of the landbridge running across Taiwan to Japan on glaciers during the period. In the postglacial period, the ancestral species was geographically isolated into two lineages, Iriomotejima + Taiwan and Amamioshima, according to the configuration of the landbridge at that time. The phylogeographic adhesiveness of insular plants, representing Iriomotejima + Taiwan, has also been demonstrated in *Lilium longifolium* (Hiramatsu et al. 2001). Later, the populations on each island (Taiwan, Iriomotejima, and Amamioshima) in these two landmasses became geographically isolated, owing to transgression in the postglacial age in the Pleistocene. The estimated divergence time, after 0.082 MYA, for insular differentiation within the Ryukyu–Taiwan clade agrees well with the period of the Quaternary configuration of the landbridge suggested by paleogeographic findings (Kizaki and Oshiro 1977; Ujiie 1990; Kimura 1996, 2000). The differentiated populations on each island may have had several opportunities for range expansion of their distribution via the glacial landbridge (the last landbridge formation is estimated to be ca. 0.02 MYA); however, *Cardiandra* contracted its distribution range into the present one. Paleobotanical data of *Cardiandra* are needed for further discussion on the past geographical movement of this plant taxon.

The phylogenetic relationships within the genus *Cardiandra* suggest that the genus continually expanded its distribution across southeastern China to mainland Japan via the landbridge of the Ryukyu Islands, and that the division of the landbridge isolated each population of *Cardiandra*. Thus, the infrageneric and/or infraspecific differentiation of *Cardiandra* is estimated to have depended largely on the presence or division of the Ryukyu–Taiwan landbridge that connected Japan to the Asian Continent.

Moreover, our results suggest that *Cardiandra alternifolia* is paraphyletic. Therefore, the morphological characteristics that characterize this species, i.e., presence of ornamental flowers and short styles (< 1.2 mm), could be considered to be plesiomorphies (and/or convergences). The *C. amamiohsimensis* characteristic of lacking ornamental flowers may have some ecological significance, perhaps involving some adaptation to their pollinators. Kato (2000) reported a unique and rich anthophilous insect community on Amamioshima. However, no observations of insect visitors to *C. amamiohsimensis* have been reported. A survey of *Cardiandra* pollinators could improve our understanding of the ecological significance of ornamental flowers in this genus.

Another interpretation of the paraphyletic status of *C. alternifolia* is that of introgressive hybridization between *C. amamiohsimensis* and *C. alternifolia* from Iriomotejima and Taiwan. Setoguchi and Watanabe (2000) reported introgressive hybridization between insular endemics of *Ilex* on the Ryukyu Islands and the Bonin Islands, oceanic islands located in the north Pacific. The insular endemic *Ilex* species on Amamioshima and Ishigakijima (a neighboring island to Iriomotejima) in the Ryukyu Islands were found to have undergone gene transfer. The introgression was estimated to have been caused by ecological features of insular endemics, i.e., limits of distribution range or sympatric distribution across a small land area. Further study is needed to discuss the evolutionary history of the paraphyletic species *Cardiandra alternifolia*.

Acknowledgments We thank Mr. Shigeru Matsutari (Kyoto Botanical Garden) and Ms. Keiko Yasuda (RIHN) for providing plant samples. We also thank Mr. Chien-I Hunag at the Academia Sinica, Taiwan, for kindly supporting plant collections in the field. This study was supported by a Grant-in-Aid for Scientific Research (#13575011 and 15405014) from the Japan Ministry of Education, Culture, Science, Sports, and Technology, and by Research Project (#3-2) from the Research Institute for Humanity and Nature.

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