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Molecular Evidence for Hybridization Events Involved in the Origin of *Ardisia walkeri* (Primulaceae) Revealed by Nuclear and Chloroplast DNA Sequence Data

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Abstract We compared the ITS1 of nrDNA and *psbA-trnH* intergenic spacer of cpDNA sequences of *Ardisia walkeri*, a putative hybrid, with those of the hypothesized parental species of *A. japonica* and *A. pusilla*. The ITS1 comparison revealed that two plants from Chiba of Japan treated as *A. walkeri* based on morphology must be of hybrid origin between *A. japonica* and *A. pusilla* in agreement with previous reports. Furthermore, the *psbA-trnH* comparison revealed that the two Chiba plants of *A.* × *walkeri* could be a hybrid originated from maternal species of *A. japonica*. On the other hand, two plants morphologically identified as *A. walkeri* from Tokuno-shima Island of the Ryukyus might not be hybrids of the two *Ardisia* species.

Key words: ITS1, Japan, leaf morphology, *psbA-trnH*, Ryukyu Islands.

Introduction

Ardisia Swartz comprises 400 to 500 species of trees, shrubs, suffrutescent and rarely herbs, and distributes mostly in East and Southeast Asia (Jie and Pipoly III, 1996). In Japan, eight species are known (Yamazaki, 1993). Out of the eight species, *A. japonica* (Hornsted) Blume, distributing in China, Japan, Korea, and Taiwan (Yamazaki, 1993) (Fig. 1A), and *A. pusilla* de Candolle, distributing in China, Japan, Korea, and Taiwan (Yamazaki, 1993) (Fig. 1B), are commonly assigned to *Ardisia* sect. *Bladhia* (Thunberg) Mez ex Walker. *Ardisia walkeri* Yuen P. Yang was formally described on a type specimen collected from Shizuoka Prefecture of Japan proper (Yang and Dwyer, 1989): it was previously treated as *A. japonica* var. *montana* Miq. or *A. montana* (Miq.) Siebold ex Franch. et Sav., but Yang and Dwyer (1989) noticed that the epithet of "*montana*" has been already applied to other *Ardisia* species. *Ardisia walkeri* was reported to distribute from Kanto to Kyushu District of Japan proper, Izu Islands and Tokuno-shima Island of Ryukyu Archipelago (hereafter the Ryukyus) (Koyama and Kokubugata, 1998).

Regarding the phylogenetic background of *A. walkeri*, it has been hypothesized to be a natural hybrid between *A. japonica* and *A. pusilla*, because *A. walkeri* has a morphological intermediate between the putative parent species (Walker, 1954; Yang and Dwyer, 1989; Yamazaki, 1993). In contrast, other studies have postulated that *A. walkeri* is unlikely to be a hybrid of these two *Ardisia* species, because *A. walkeri* does not always co-occur with both of *A. pusilla* and *A. japonica* (Nakai, 1922; Shimabuku, 1997). For example, in Amami and Tokuno-shima islands in the Ryukyus, *A. walkeri* and *A. pusilla* are present, but *A. japonica* is

Fig. 1. Herbarium specimens of the three *Ardisia* species investigated. A: *A. japonica* (*G.Kokubugata 11320* from Nakano-shima Island, the Ryukyus, Japan). B: *A. pusilla* (*G.Kokubugata 8980* from Okinawa-jima Island, the Ryukyus, Japan). C: *A. walkeri* (*J.Hagiwara 38574* from Chiba, Japan proper). D: *A. walkeri* (*G.Kokubugata 12570* from Tokuno-shima Island, the Ryukyus, Japan). Scale bar = 5 cm. Arrows indicate leaves on creeping stems of *A. pusilla* and *A. walkeri*.

absent (Hatusima, 1975; Walker, 1976; Shimabuku, 1997).

In a review of molecular methods for analysis of hybrid speciation in plants, Hegarty and Hiscock (2005) suggested that relying solely on one source of data, for example the internal transcribed spacer (ITS) of nuclear DNA (nrDNA), may be insufficient because of concerted evolution (Wendel *et al.*, 1995) and intraindividual copy variation (Alvarez and Wendel, 2003; Bailey *et al.*, 2003). However, Sang *et al.* (1995) reported ITS nucleotide additivity in hybrids at positions where the parents differed, and the molecular techniques were useful for investigating hybrid origins. Indeed, ITS sequence additivity has also been demonstrated for other families, namely in Asteraceae (Saito *et al.*, 2006, 2007) and Laminae (Kokubugata *et al.*, 2011), suggesting the suitability of the ITS for hybrid analysis. Furthermore, addition of certain chloroplast DNA (cpDNA) data can assist detection and indicate the direction of hybridization in plants (Rieseberg and Ellstrand, 1993; Schwarzbach and Rieseberg, 2002; Saito *et al.*, 2007).

These molecular techniques, however, have not been applied to examine the hypothesized hybrid origin of *A. walkeri*, which was recommended by Koyama and Kokubugata (1998). In the present study, the TS1 of nrDNA and *psbA-trnH* intergenic spacer (*psbA-trnH*) of cpDNA sequences from plants identified as *A. japonica*, *A. pusilla*, and *A. walkeri* by morphological characteristics were compared to determine whether the first two species were the parents of *A. walkeri*.

Materials and Methods

Morphological identification and plant materials

Taxonomic identification using morphological characteristics of the three *Ardisia* species followed Kitamura and Murata (1981): plants having no leaves on creeping stems and chartaceous leaves were treated as *A. japonica* (Fig. 1A); plants having leaves on creeping stems and subcoriaceous leaves were treated as *A. pusilla* (Fig. 1B); and plants having leaves on creeping stems and chartaceous leaves were treated as *A. walkeri* (Fig. 1C and D).

In this study, a plant of *A. japonica* was collected from each of six Japanese localities and a Taiwanese locality; a plant of *A. pusilla* was collected from each of two Japanese localities and a Taiwanese locality, and two plants morphologically identified as *A. walkeri* were collected from a locality on Tokuno-shima Island of the central Ryukyus, Japan (*G.Kokubugata 12570* and *12580*) (Table 1; Fig. 2). Genomic DNA was isolated from two herbarium specimens morphologically identified as *A. walkeri* from Chiba (*J.Hagiwara 38573* collected in 1985; and *H.Koyama 10231* in 1997 ; Table 1; Fig. 2) being near its type locality of Shizuoka (star in Fig. 2) in Japan proper. In total, 14 plants of three *Ardisia* species from 13 localities in Japan and Taiwan were investigated in the present study. All voucher specimens were deposited in the herbarium of the National Museum of Nature and Science (TNS).

DNA extraction, polymerase chain reaction, and sequencing

Total genomic DNA was isolated from leaf tissue using a DNeasy Plant Mini Kit (Qiagen Hilden, Germany) following the manufacturer's instructions with some modifications. Extracted DNA was used as a template for polymerase chain reaction (PCR).

The internal transcribed spacer 1 (ITS1) of nrDNA was amplified by PCR using 17SE (5′-ACGAATTCATGGTCCGGTGAAGTGTTCG-3′; Sun *et al.,* 1994) as the forward primer and 26SE (5′-GAATTCCCCGGTTCGCTCGCCGTTAC-3′; Topik *et al.*, 2005; modified 26SE in Sun *et al.*, 1994) as the reverse primer. The *psbA-trnH* intergenic spacer (*psbA-trnH*) of cpDNA was amplified using psbAF (5′-GTTATGCATGAACGTAATGCTC-3′) as the forward primer and trnHR (5′-CGCGCATGGTGGATTCACAAATC-3′) as the reverse primer (Sang *et al.*, 1997). PCR comprised 30 cycles of 30 s at 94℃, 30 s at 55℃, and 1 min at 72℃, followed by a

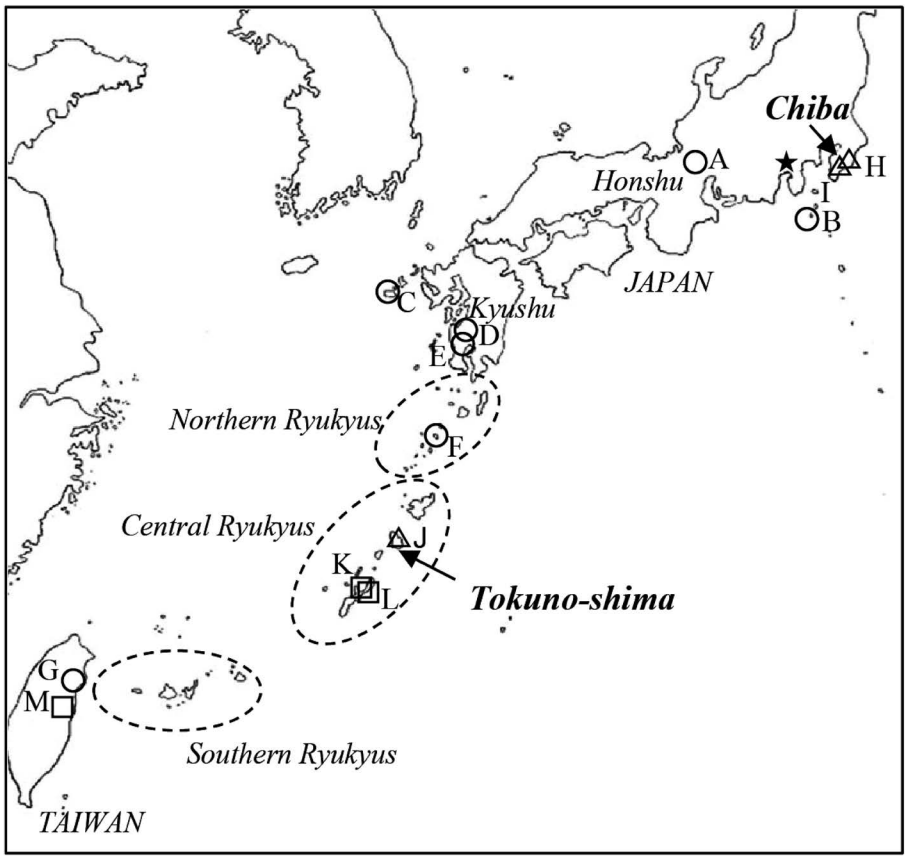

Fig. 2. Map of Japan and Taiwan showing 12 localities from which three *Ardisia* species were collected (see Table 1 for abbreviations of collection localities). ○: *A. japonica*. □: *A. pusilla*. △: *A. walkeri*. Star indicates the type locality of *A. walkeri*.

hold at 4℃ to terminate the reaction, and was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Waltham, MA, USA).

Amplification was performed using EmeraldAmp PCR Master Mix (Takara, Otsu, Japan). The PCR profile comprised 35 cycles of 30 s at 94℃, 30 s at 55℃, and 1.5 min at 72℃, after an initial denaturation for 3 min at 94℃. PCR products were examined using electrophoresis before purification with an Illustra™ ExoProStar (GE Healthcare UK Ltd., England). Cycle sequencing was performed with a BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Waltham, MA, USA) using the PCR forward primer 17SE for the ITS1 sequence and the PCR primers psbAF and trnHR for the *psbA-trnH* sequence. Cycle sequencing products were purified by ethanol precipitation. Automated sequencing was carried out on an Applied Biosystems 3130xl Genetic Analyzer. The electropherograms were assembled using the ATGC ver. 4.01 software (Genetyx Co., Tokyo, Japan). The ITS1 and *psbA-trnH* sequences were compared among the three *Ardisia* species. Sequence data from this study were deposited in the DNA Data Bank of Japan (DDBJ) database (http://www.ddbj.nig.ac.jp/).

Results

The sequence lengths of the ITS1 region of seven *A. japonica* plants and two *A. walkeri* plants from Tokuno-shima Island were 249 base pairs (bp), while the length of three *A. pusilla*

Table 1. Variable sites in the nuclear ribosomal ITS1 and *psbA-trnH* sequences of three *Ardisia* taxa in the 14 accessions from 12 localities (*abbreviations in parentheses refer to the localities reported in Fig. 2)

Individual	Locality	Abbreviations for locality*	ITS1 sequence position					*psbA-trnH* sequence position				Accession no. (DDBJ)	
			49	74	90	94	210	165	187	321	322	ITS	*psbA-trnH*
A. japonica													
G.Kokubugata8724	Japan, Honshu, Gifu, Ibikawa.	A	G	G	T	C	C	T	T	A	T	LC437964	LC437978
A.Ebihara & Y.Saito 2834	Japan, Honshu, Tokyo, Kozu Island.	B	G	G	T	C	C	T	T	A	T	LC437965	LC437979
K.Fuse37	Japan, Kyushu, Nagasaki, Fukue-jima Island.	C	G	G	T	C	C	T	T	A	T	LC437966	LC437980
G.Kokubugata9130	Japan, Kyushu, Kumamoto, Itsuki.	D	G	G	T	C	C	T	T	A	T	LC437967	LC437981
G.Kokubugata9088	Japan, Kyushu, Kagoshima, Isa.	E	G	G	T	C	C	T	T	A	T	LC437968	LC437982
G.Kokubugata11320	Japan, northern Ryukyus, Nakano-shima Island.	F	G	G	T	C	C	T	T	A	T	LC437969	LC437983
C.Tsutsumi604	Taiwan, Ilan, Nannao.	G	G	G	T	C	C	T	T	A	T	LC437970	LC437984
A walkeri													
J.Hagiwara38574	Japan, Honshu, Chiba, Isumi.	H	A/G	C/G	C/T	C/T	C/T	T	T	A	T	LC437971	LC437985
H.Koyama10231	Japan, Honshu, Chiba, Kamogawa.	I	A/G	C/G	C/T	C/T	C/T	T	T	A	T	LC437972	LC437986
G.Kokubugata12570	Japan, the central Ryukyus, Kamogawa Tokuno-shima Island.	J	G	G	T	C	C	T	T	A	T	LC437973	LC437987
G.Kokubugata12580	Japan, the central Ryukyus, Kamogawa Tokuno-shima Island.	J	G	G	T	C	C	T	T	A	T	LC437974	LC437988
A. pusilla													
G.Kokubugata8980	Japan, the central Ryukyus, Okinawa, Okinawa-jima Island.	K	A	C	C	T	T	G	G	C	G	LC437975	LC437989
G.Kokubugata9030	Japan, the central Ryukyus, Okinawa, Okinawa-jima Island.	L	A	C	C	T	T	G	G	C	G	LC437976	LC437990
G.Kokubugata10463	Taiwan, Hualien, Hsiulin.	M	A	C	C	T	T	G	G	C	G	LC437977	LC437991

plants was 248 bp due to a single base pair indel at the 230-bp position in *A. pusilla*. In contrast, the ITS sequence of two plants identified as *A. walkeri* from Chiba was clearly read until the 229 position, but was unreadable after the 230-bp position because of sequence wave disorder.

Comparison of the ITS1 sequences of nrDNA indicated no intraspecific polymorphic variation in the seven *A. japonica* plants and three *A. pusilla* plants, respectively. Five variable nucleotide positions were found between *A. japonica* and *A. pusilla*: 49, 74, 90, 94 and 210 (5′–3′) (Table 1). The two plants identified as *A. walkeri* from Chiba exhibited polymorphic signals at the five sites differentiating *A. japonica* and *A. pusilla*, thus showing sequence additivity. In contrast, the ITS1 sequences of the two plants identified as *A. walkeri* from Tokuno-shima Island were identical to those of *A. japonica* (Table 1).

Seven variable nucleotide positions were found in the *psbA-trnH* sequences of cpDNA, four of which, positions 181, 203, 337 and 338 (5’–3’), were useful for differentiating *A. japonica* and *A. pusilla* (Table 1). The four nucleotide positions of the plants identified as *A. walkeri* from Chiba and Tokuno-shima Island were identical to those of *A. japonica*.

Discussion

Plants treated as A. walkeri *in Chiba*

The present results suggest that the *A. walkeri* plants from two localities of Chiba in Japan proper could be hybrids between *A. japonica* and *A. pusilla*, in agreement with Walker (1954), Yang and Dwyer (1989), and Yamazaki (1993). The ITS1 sequence disorder of the two plants of *A. walkeri* from Chiba after 230 bp could be caused by a single base pair indel at position 230 in *A. pusilla*.

In Japan, *A. japonica* distributes from Hokkaido, Japan proper to the northern Ryukyus (Fig. 1), and *A. pusilla* from the Kanto District of Japan proper to the southern Ryukyus. The two localities in Chiba, from which two plants were collected for the present study (*J.Haginiwa*

38573 and *H.Koyama 10231*), are situated in an area of overlap between the distribution ranges of *A. japonica* and *A. pusilla* (Yamazaki, 1993). Indeed, the two species have similar moist and shaded habitats in forests, and occasionally occur sympatrically (Koyama and Kokubugata, 1998). Furthermore, the two species flower from late June to early August (Yamazaki, 1993), and are assigned to the same *A.* section of *Bladhia* (Walker, 1940). Therefore, it is possible that a hybrid between the two species exists in the western part of Japan proper. *Ardisia* × *walkeri* is thought to be a Japanese-endemic species (Hatusima, 1975; Walker, 1976; Kitamura and Murata, 1981; Yang and Dwyer, 1989). However, the distribution ranges of *A. japonica* and *A. pusilla* also overlap in China, Jeju Island of South Korea, and Taiwan. Although *A.* × *walkeri* was not reported in these other overlapping regions, it is possible that hybrid plants might also occur there.

Plastids in angiosperms are usually maternally inherited (Mogensen, 1996); therefore, haplotype tracing enables determination of the maternal parent of hybrid plants. The presence of *psbA-trnH* haplotypes of *A. japonica* in the two *A.* × *walkeri* plants supports their parent status, and suggests that they originate from crosses of maternal *A. japonica* and paternal *A. pusilla*.

Ardisia × *walkeri* is rare in Japan; therefore, it is treated as an endangered (EN) species by the Japanese Ministry of the Environment (2018). For conservation of *A.* × *walkeri*, its taxonomic status and phylogenetic background, including detection of simple F_1 hybrids or independent taxa with fertility through polypoidization, must be clarified using molecular and cytological techniques.

Plants treated as A. walkeri *in Tokuno-shima Island of the Ryukyus*

In the Ryukyus, plants of *A. pusilla* are abundant, while those of *A. walkeri* are reported to occur only on Amami and Tokuno-shima islands; *A. japonica* has not been recorded (Hatusima, 1975; Walker, 1976; Shimabuku, 1997; Hotta, 2013). The two plants collected from Tokuno-shima Island (*G.Kokubugata 12570* and *12580*) were morphologically identifiable as *A.* × *walkeri*, and differed from those of *A. japonica* in Japan proper in having chartaceous leaves on the creeping stems. However, the two Tokuno-shima plants possessed the ITS1 and *psbA-trnH* sequences identical to those of *A. japonica*. Therefore, the Tokuno-shima plants might not be natural hybrids, at least F_1 hybrids, between the two *Ardisia* species. Alternatively, they may have originated from an introgressive hybridization of *A. japonica* from *A. pusilla* if *A. japonica* was present on Tokuno-shima Island in the past. Further molecular investigation using microsatellite makers would enable determination of the taxonomic status and origin of *Ardisia* plants in Tokuno-shima Island.

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