

Three Independent Migrations of *Dodonaea viscosa* (Sapindaceae) to Japan Archipelago Revealed by Molecular Data

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Abstract To investigate the migration of *Dodonaea viscosa* to the Japanese Archipelago, we conducted molecular phylogenetic analyses using internal transcribed spacer (ITS) and partial external transcribed spacer (ETS) sequences from plants collected on the Bonin Islands and the Ryukyu Archipelago. *Dodonaea viscosa* occurring on the Bonin Islands, the northern and central Ryukyu Islands, and the southern Ryukyu Islands were placed in separate clades. This suggests that these three lineages must have migrated independently to the Japanese Archipelago.

Key words : Bonin Islands, disjunctive distribution, Ryukyu Islands.

Introduction

Dodonaea Mill. comprises 68 species of trees and shrubs, 65 of which are endemic to Australia. Out of the remaining three species, *D. polyandra* Merr. et L.M.Perry is found in both Australia and Papua New Guinea, *D. madagascariensis* Radlk. is endemic to Madagascar, and *D. viscosa* Jacq. (Fig. 1) is widely distributed throughout the subtropical and tropical regions. The biogeographic range of *D. viscosa* includes Australia, South America, and Africa (West, 1984) extending to Southeast Asia, China, Japan, and Taiwan (Ohashi, 2016).

In Japan, *D. viscosa* has a disjunct distribution in the Bonin Islands and the Ryukyu Archipelago

(hereafter, the Ryukyus; Fig. 2). Within the Bonin Islands, it occurs on islands of Chichi-jima, Ani-jima, and Muko-jima (Toyoda, 2003). On the northern Ryukyus, it occurs at Tanega-shima Island (Hatusima, 2003), and it is widespread in the central Ryukyus, occurring on the islands of Takara-jima, Amami-ohshima, Tokuno-shima, Okinawa-jima, Iheya-jima, Kume-jima, and Zamami-jima (Hatusima and Amano, 1994). On the southern Ryukyus, it is found on island of Miyako-jima, Ishigaki-jima, and Iriomote-jima (Hatusima and Amano, 1994). Molecular phylogenetic analyses have not yet been applied to examine the phylogenetic relationships among Japanese populations of *D. viscosa*.

Here, we present the results of molecular phylogenetic analyses of *D. viscosa* plants from Japan. To determine phylogenetic relationships



Fig. 1. Habit of *Dodonaea viscosa* in Iriomote Island. A. plant. B. fruit. C. male flower.

among Japanese populations, we utilized sequences of the internal transcribed spacer (ITS) region and a portion of the external transcribed spacer (ETS) region of nuclear ribosomal DNA.

Materials and Methods

Sampling and sequence data from DNA database

In this study, 12 plants of *D. viscosa* were collected from the Bonin Islands and Ryukyus: from

Chichi-jima of the Bonin Island (3 plants of a population), Tanega-shima of the northern Ryukyus (2 plants of a population), Tokunoshima (1 plant of a population) and Zamami-jima (1 plant of a population) of the central Ryukyus, and Ishigaki-jima (3 plants of a population) and Iriomote-jima (2 plants of a population) of the southern Ryukyus (Table 1 and Fig. 2). Additionally, we collected plants from Taiwan (4 plants of 3 populations) and from the Philippines (3 plants

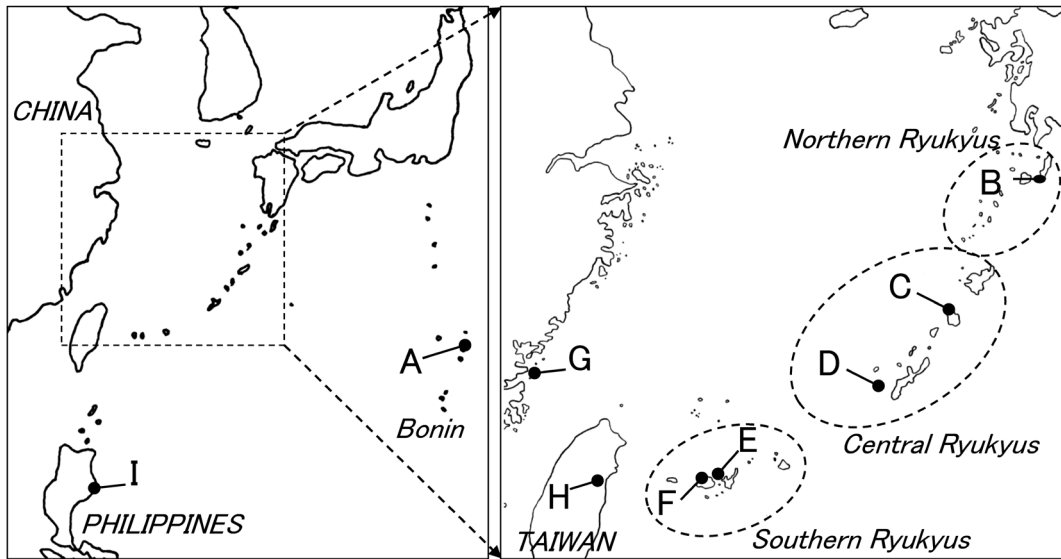


Fig. 2. Map of Japan and adjacent regions showing seven localities, where *Dodonaea viscosa* was collected (see Table 1 for codes of collection localities).

Table 1. Plants of *Dodonaea viscosa* collected and investigated in the present study and their voucher specimens, collection areas and accession number of ITS and ETS sequences (DDBJ).

Individual	Locality*	Accession no.	
		ITS	ETS
GK18081	JAPAN, Bonin Islands, Chichi-jima (A)	LC494534	LC494553
GK18082		LC494535	LC494554
GK18083		LC494536	LC494555
GK15546	JAPAN, Northern Ryukyus, Tanegas-shima Is. (B)	LC494537	LC494556
GK19192		LC494538	LC494557
GK12177	JAPAN, Central Ryukyus, Tokuno-shima Is. (C)	LC494539	LC494558
GK12266	JAPAN, Central Ryukyus, Zamami-jima Is. (D)	LC494540	LC494559
GK21411	JAPAN, Southern Ryukyus, Ishigaki-jima Is. (E)	LC494541	LC494560
GK21412		LC494542	LC494561
GK21413		LC494543	LC494562
GK14838	JAPAN, Southern Ryukyus, Iriomote-jima Is. (F)	LC494544	LC494563
GK14848		LC494545	LC494564
GK13244		LC494546	LC494565
GK13245	TAIWAN, Matsu Islands, Nangan Is. (G)	LC494547	LC494566
GK13246		LC494548	LC494567
GK14371		LC494549	LC494568
GK21453	TAIWAN, Hualian, Taroko (H)	LC494550	LC494569
GK21454		LC494551	LC494570
GK21455		LC494552	LC494571

* Codes in parentheses refer to the localities shown in Fig. 2.

from a population) (Table 1). All voucher specimens were deposited in the herbarium of the Department of Botany, National Museum of Nature and Science (TNS), Tsukuba.

To elucidate the phylogenetic background of

the 19 Japanese plants of *D. viscosa*, published sequence data of 49 accessions of *D. viscosa* (including intraspecific taxa) and its closely related three species, namely *D. biloba* J.G. West, *D. procumbens* F.Muell., *D. camfieldii*

Table 2. Genebank accession number of ITS and ETS referred from a previous study (Harrington and Gadek, 2009).

Taxon	Collection area	Collection number (Herbarium)	Accession no.	
			ITS	ETS
INGROUP				
<i>Dodonaea biloba</i> J.G.West	Australia	Telford & Carroll 875 (CBG)	FJ546929	FJ546876
<i>D. procumbens</i> F.Muell.	Australia, New South Wales	J.G. West 5502 (CANB)	FJ546931	FJ546878
<i>D. viscosa</i> Jacq.				
ssp. <i>angustifolia</i> (L.f.) J.G.West	Australia, New South Wales	M.D.Crisp 4017 (CBG)	FJ546963	FJ546911
ssp. <i>angustissima</i> (DC.) J.G.West	Australia, Victoria	M. Harrington 299 (JCT)	FJ546965	FJ546913
ssp. <i>burmaniana</i> (DC.) J.G.West	Australia, Queensland	M. Harrington 406 (JCT)	FJ546967	FJ546916
ssp. <i>cuneata</i> (Sm.) J.G.West	Australia, New South Wales	J.G. West 5467 (CANB)	FJ546969	FJ546918
ssp. <i>mucronata</i> J. G.West	Australia, Central desert	R. Connors 977 (CANB)	FJ546970	FJ546919
ssp. <i>spatulata</i> Smith	Australia, Victoria, near Horsham	Canning 5646 (CBG)	FJ546968	FJ546917
ssp. <i>viscosa</i>	China, southern central part	Hyland14946 (CANB)	FJ546938	FJ546885
	Taiwan, Taipei	C. Huang 1897 (HAST)	FJ546960	FJ546908
	Taiwan, Taipei	C. Huang 1909 (HAST)	FJ546961	FJ546909
	Mariana, Pagan	T. Roberts s.n. (JCT)	FJ546953	FJ546901
	India, New Tehri	S. Phartyal s.n. (JCT)	FJ546944	FJ546892
	New Caledonia, Taitoula River	Biffin & Craven 115 (JCT)	FJ546946	FJ546894
	New Caledonia, Mont Dore	E.A. Brown 03/134 (NSW)	FJ546947	FJ546895
	Australia, Queensland	P.R. Sharpe & G. Batianoff 4029 (CANB)	FJ546971	FJ546920
	Australia, Queensland	M. Harrington 325 (JCT)	FJ546972	FJ546921
	Australia, Queensland	R.L. Jago 4870 (JCT)	FJ546974	FJ546923
	Australia, Queensland	M. Harrington 407 (JCT)	FJ375187	FJ372732
	New Zealand, North Island, Lower Hutt	<i>Living collection (RBGE19981090)</i>	FJ546948	FJ546896
	New Zealand South Island, north coast	J. G. West 5458 (CANB)	FJ546949	FJ546897
	New Zealand South Island, east coast	J. G. West 5283 (CANB)	FJ546950	FJ546898
	New Zealand South Island, Canterbury	S. J. Wagstaff & M. I. Dawson s.n. (CANB)	FJ546951	FJ546899
	Oman	J.R. Maconochie 3481 (CBG)	FJ546952	FJ546900
	South Africa, KwaZulu-Natal	K. Edwards 234 (JCT)	FJ546955	FJ546903
	South Africa, Cederberg Western	P. Linder s.n. (JCT)	FJ546954	FJ546902
	Hawaii, Kauai	K. Edwards 270 (JCT)	FJ546943	FJ546891
	Hawaii, Kona	Morden 1136 (BISH)	FJ546942	FJ546890
	Hawaii, Pohakuloa	Morden 1309 (BISH)	FJ546941	FJ546889
	Hawai, Maui, Poli Poli	Morden 1787 (BISH)	FJ546940	FJ546888
	Hawai, Maui, Ulupalakua	PCMB B80/687878	AY864896	FJ546887
	USA, Arizona	Yatskievych 05-03 (MO)	FJ546933	FJ546880
	USA, Arizona	Yatskievych 05-04 (MO)	FJ546934	FJ546881
	Brazil	Wasum & Wasum182 8 (CBG)	FJ546936	FJ546883
	Bolivia, Acevedo	P. Acevedo 11144 (USI)	FJ546935	FJ546882
	Colombia, Paramo	FB/S778 (NPB)	FJ546937	FJ546884
	China, southern central part	Hyland14946 (CANB)	FJ546938	FJ546885
	Taiwan, Taipei	C. Huang 1897 (HAST)	FJ546960	FJ546908
	Taiwan, Taipei	C. Huang 1909 (HAST)	FJ546961	FJ546909
	Mariana, Pagan	T. Roberts s.n. (JCT)	FJ546953	FJ546901
	India, New Tehri	S. Phartyal s.n. (JCT)	FJ546944	FJ546892
OUTGROUP				
<i>D. camfieldii</i> Maiden & Betche	Australia, New South Wales	Orme, A. 194 (NSW)	FJ546930	FJ546877

Maiden et Betche (Harrington and Gadek, 2009) were used in the present analysis (Table 2). For the outgroup, we employed *D. camfieldii*, based on Harrington and Gadek (2009).

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).

PCR amplifications were conducted by primer sets of 17SE (5'-ACG AAT TCA TGG TCC GGT GAA GTG TTC G-3') and reverse primer modified 26SE (5'-G AAT TCC CCG GTT CGC TCG CCG TTA C-3') for ITS (Sun *et al.*, 1994); and by a primer set of ETS-18S (5'-GAG CCA TTC GCA GTT TCA CAG-3') and ETS-9bp (5'-CAT GGG CGT GTG AGT GGT GA-3') for a part of ETS (Wright *et al.*, 2001). PCR comprised 35 cycles of 30sec at 94°C, 30sec at 55°C, and 1.5min at 72°C using ExTaq (Takara, Kusatsu,

Japan) with Ampdirect Plus (Shimazu, Kyoto, Japan), followed by a hold at 4°C to terminate the reaction, and was performed using a GeneAmp PCR System 9700 (Thermo Fisher Scientific, Tokyo, Japan).

Cycle sequencing was performed with a Big-Dye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Waltham, MA, USA) using the PCR primer set and internal primers of ITS2N (5'-GGC GCA ACT TGC GTT CAA-3') and ITS3N (5'-GCT CTC GCA GCA TCG ATG AAG-3') (Yukawa, T., personal communication) for ITS, and the PCR primer set for ETS. Cycle sequencing products were purified by ethanol precipitation. Automated sequencing was carried out on an Applied Biosystems 3500xl Genetic Analyzer. The electropherograms were assembled using the ATGC ver. 4.01 software (Genetyx Co., Tokyo, Japan). Sequence data gained were deposited in the DNA Data Bank of Japan (DDBJ) database (<http://www.ddbj.nig.ac.jp/>).

Phylogenetic analyses

DNA sequences were aligned using the ClustalW 1.8 software (Thompson *et al.*, 1994) and then manually adjusted. Phylogenetic analyses were conducted based on a Bayesian approach using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) and a maximum parsimony (MP) criterion using PAUP* version 4.0b10 (Swofford, 2002). The combinability of ITS and a part of ETS sequences was assessed using the incongruence length difference (ILD) test (Farris *et al.*, 1994).

In the Bayesian phylogenetic analysis, MrModeltest 2.2 (Nylander, 2004) was used to estimate the appropriate evolutionary model of nucleotide substitutions. Based on the model selected, two separate runs of Metropolis coupled Markov chain Monte Carlo (MCMCMC) analyses were performed, each with a random starting tree and four chains (one cold and three heated). The MCMCMC length was one million generations and the chain was sampled every one-hundredth generation from the cold chain. The mixing and convergence of the MCMCMC

chains of the two runs were assessed by inspection of the trace plots of parameters using TRACER version 1.5.0 (Drummond and Rambaut, 2007); the first 2,500 sample trees (25% of the total 10,000 sample trees) were discarded as burn-in. After the burn-in, the effective sample sizes (ESS) of all parameters were >200, indicating that the analyses sampled the posterior distributions of each parameter satisfactorily, and the average standard deviation of split frequencies (ASDSF) were <0.01. The potential scale reduction factors (PSRFs) were ascertained to be reasonably close to 1.0 for all parameters in an output table. A 50% majority consensus tree of the output tree file from MrBayes was generated using TREEVIEW (Page, 1996).

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 using bootstrap analysis (Felsenstein, 1985). Ten thousand replicates of heuristic searches, with the TBR branch swapping switched on and MULTREES options off, were performed to calculate bootstrap values.

Results

*Field surveys of *Dodonaea viscosa**

We conducted field surveys of *D. viscosa* in Chichi-jima, the Bonin Islands and the Ryukyus, excluding Takara-jima, using reference material and herbarium information to locate populations. We confirmed that populations occurred on Chichi-jima, Tanega-shima, Tokuno-shima, Zamami-jima, Ishigaki-jima, and Iriomote-jima, but did not locate any populations on Amami-ohshima, Okinawa-jima, Kume-jima and Miyako-jima.

Differentiation of ITS and ETS sequences in Japanese plants of D. viscosa

We confirmed that ITS and ETS sequence data could be appropriately combined after ILD test, and of 61 operational taxonomic units (OTUs) obtained a 1,044bp matrix of ITS (612bp) and partial ETS (432) sequences.

The ITS and partial ETS (hereafter, ITS-ETS) sequences were identical among three plants of *D. viscosa* collected from Chichi-jima (Bonin Islands); among four plants collected from Tanega-shima, Tokuno-shima and Zamami-jima (central Ryukyus). In the five plants collected from two islands of the southern Ryukyus, one nucleotide substitution was found at the 514bp position between *GK21413* from Ishigaki-jima (guanine) and the other four plants, i.e., *GK21411* and *GK21412* from Ishigaki-jima and *GK14848* and *GK14838* from Iriomote-jima (adenine). A single base pair indel was found at the 443bp position between *GK21412* and the four other plants. Polymorphic signals were found at the 425bp position between *GK21412* (cytosine and thymine) and the other four plants (cytosine) and at the 514bp position between *GK14838* and *GK14848* (cytosine and guanine), and *GK21411*, *GK21412*, and *GK21413* (cytosine).

We found 26 nucleotide substitutions in the ITS region (15 nucleotide substitutions) and the portion of the ETS region (11 nucleotide substitutions) among plants collected from Chichi-jima of the Bonin Islands, the northern and central Ryukyus, and the southern Ryukyus (Table 3).

Phylogenetic analyses based on combined ITS-ETS sequences

In the Bayesian analysis, the GTR + G model was selected. The 50% majority rule consensus tree of all post-burn-in trees was depicted with Bayesian posterior probabilities (PPs) (Fig. 3). In the maximum parsimony (MP) analysis, 67 of 119 variable characters were parsimony informative, and 6,927 equally most parsimonious trees of 144 steps were obtained with a consistency index (CI) of 0.854, a retention index (RI) of

Table 3. Variable sites in the ITS and a part of ETS sequences of 12 Japanese plants of *Dodonaea viscosa* investigated in the present study.

Locality	Individual	Sequence position																									
		ITS													ETS												
		37	111	393	411	425	465	493	496	509	510	515	546	548	577	612	66	123	156	239	287	292	346	353	355	372	374
Bonin Islands, Chichi-jima	GK18081	T	C	G	A	T	C	C	C	C	C	C	G	T	C	C	T	T	C	C	C	A	C	C	A	T	A
	GK18082	T	C	G	A	T	C	C	C	C	C	C	G	T	C	C	T	T	C	C	C	A	C	C	A	T	A
	GK18083	T	C	G	A	T	C	C	C	C	C	C	G	T	C	C	T	T	C	C	C	A	C	C	A	T	A
	GK15546	G	C	G	G	C	A	T	C	C	C	C	G	T	C	T	T	C	T	T	T	A	C	T	G	C	G
Northern Ryukyus, Tanega-shima	GK19192	G	C	G	G	C	A	T	C	C	C	C	G	T	C	T	T	C	T	T	T	A	C	T	G	C	G
	GK12177	G	C	G	G	C	A	T	C	C	C	C	G	T	C	T	T	C	T	T	T	A	C	T	G	C	G
	GK12266	G	C	G	G	C	A	T	C	C	C	C	G	T	C	T	T	C	T	T	T	A	C	T	G	C	G
	GK21411	T	T	A	G	C	A	T	C	T	C	T	A	C	T	C	C	T	T	T	T	A	C	T	G	T	A
Central Ryukyus, Tokuno-shima	GK21412	T	T	A	G	C	A	T	C	T	C	T	A	C	T	C	C	T	T	T	T	G	T	C	G	T	A
	GK21413	T	T	A	G	C	C	T	T	T	C	T	A	C	T	C	C	C	T	T	T	G	T	C	G	T	A
	GK14838	T	T	A	G	C	C	T	T	T	C	T	A	C	T	C	C	C	T	T	T	G	T	C	G	T	A
	GK14848	T	T	A	G	C	C	T	T	T	C	T	A	C	T	C	C	C	T	T	T	G	T	C	G	T	A

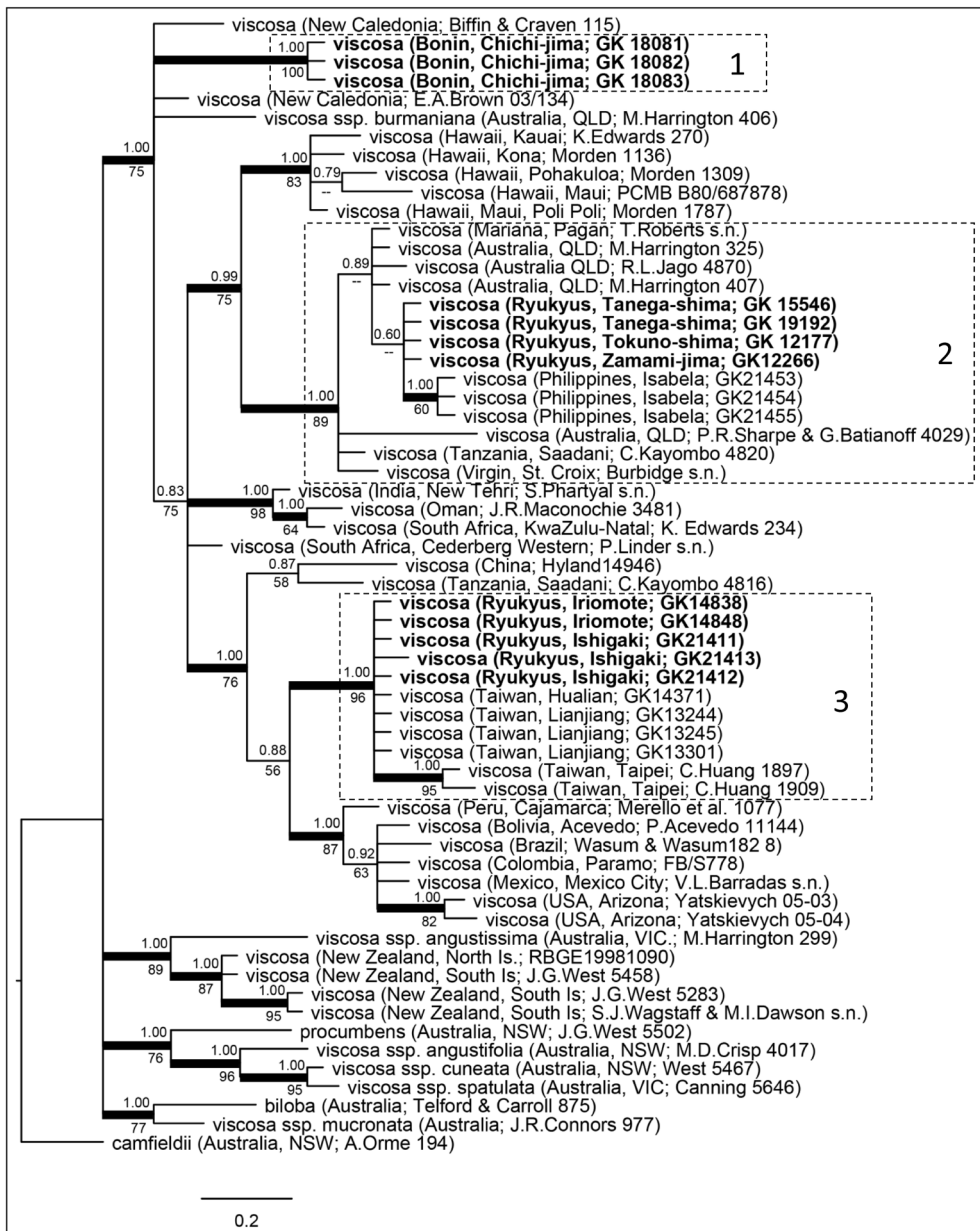


Fig. 3. Bayesian phylogenetic tree of 61 OTUs of *Dodonaea viscosa* and allied species based on ITS-ETS sequences. Bayesian posterior probabilities (upper) and bootstrap percentages in the MP analysis (lower) are shown on branches. Bold lines indicate $PP \geq 0.99$ and $BS \geq 60\%$.

0.946, and a rescaled consistency index (RC) of 0.808. The topology of the strict consensus tree was highly compatible with that of the Bayesian tree, except that the clades with low bootstrap percentages (BS; $< 50\%$) collapsed to polytomy. Thus, BS were plotted on the Bayesian tree (Fig.

3). Only clades supported by $PP = 1.00$ and/or $BS > 70\%$ are discussed below.

Both the Bayesian and MP analyses (Fig. 3) demonstrated that plants of *D. viscosa* from Chichi-jima formed a well-supported clade ($BS/PP = 100/1.00$; Clade 1), but the closest clade to

the Chichi-jima plants was not clear from our analyses. Plants from the northern and central Ryukyus formed a clade with those from the Philippines, Tanzania, U.S. Virgin Islands, Hawaii, Australia, Mariana Islands (89/1.00; Clade 2), and Clade 2 connected to a clade from Hawaii (75/0.99). Plants from the southern Ryukyus formed a clade with those from Taiwan (96/1.00; Clade 3).

Discussion

Our analysis determined that the plants of *D. viscosa* occurring on Chichi-jima of the Bonin Islands (Clade 1), the northern and central Ryukyus (Clade 2), and the southern Ryukyus (Clade 3) form three distinct clades, with strong statistical support, and Clades 2 and 3 include OTUs from different areas. This suggests that three independent migrations of *D. viscosa* to Japan have occurred over time.

Previous research indicated that *D. viscosa* seeds are too large to be dispersed by wind over long distances (Schimper, 1891). West (1980) conducted floatation experiments with *D. viscosa* capsules in seawater and found that 30% remained afloat after 100 days, and 79% of seeds germinated after soaking in seawater for 6 months, suggesting that long distance seed dispersal on surface ocean currents may be possible in this species. Guppy (1906) suggested that granivorous birds are a more likely dispersal vector for *D. viscosa*. We suggest that surface ocean currents or granivorous birds might have facilitated the three migrations of *D. viscosa* to Japan.

We were unable to determine which population of *D. viscosa* was most closely related to those occurring on Chichi-jima. It has been reported that the Bonin Islands share mutual or closely related species with the Hawaiian Islands, such as *Lycium sandwicense* A.Gray (Solanaceae; Fukuda *et al.*, 2001) and *Lobelia boninensis* Koidz. (Campanulaceae; Konx *et al.*, 2017). However, the Chichi-jima plants of *D. viscosa* are not closely related to the populations from the Hawaiian examined. Further, the Chi-

chi-jima plants are not related to those from the Mariana Islands, located south of the Bonin Islands. Notably, the clade formed by plants from the northern and central Ryukyus is related to populations from the Philippines, although this relationship had weak statistical support ($>50/0.66$). Additional phylogenetic analyses using plant material from other regions may help to clarify these relationships.

It is thought that the southern Ryukyus and Taiwan share a portion of their terrestrial flora, which is believed to be the result of species migration from Taiwan to the southern Ryukyus (Hatusima, 1975). Therefore, the plants of *D. viscosa* may migrate through a land bridge between the two regions. The southern Ryukyus and Taiwan are suggested to have physically separated in the early Pleistocene (1.5–0.7 million years ago; Koba, 1992; Osozawa *et al.*, 2011). In the present study, we were unable to estimate divergence time for *D. viscosa* in the southern Ryukyus and Taiwan, because plants from the two regions had almost identical ITS-ETS sequences. Further analyses using other DNA regions in *D. viscosa* may clarify direction of the migration, and whether this species crossed between Taiwan and the southern Ryukyus after the two regions separated, or was dispersed over a land bridge prior to separation.

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