J. Jpn. Bot. **86**: 15–25 (2011)

Origin of Hawaiian Endemic Species of *Canavalia* (*Fabaceae*) from Sea-Dispersed Species Revealed by Chloroplast and Nuclear DNA Sequences

Mohammad VATANPARAST[a], Koji TAKAYAMA[a,†], Mario S. SOUSA[b], Yoichi TATEISHI[c] and Tadashi KAJITA[a,*]

[a]Department of Biology, Graduate School of Science, Chiba University,
1-33, Yayoi, Inage, Chiba, 263-8522 JAPAN;
[b]Departamento de Botánica, Instituto de Biología, Universidad Nacional Autónoma de México,
Apartado Postal 70-367, 04510 México, D. F., MÉXICO;
[c]Faculty of Education, University of the Ryukyus,
1, Senbaru, Nishihara, Okinawa, 903-0129 JAPAN;
[†]Present address: Department of Plant Systematics and Evolution,
Institute of Botany, University of Vienna. Rennweg 14, A-1030 Wien, AUSTRIA
*Corresponding author: tkaji@faculty.chiba-u.jp

(Accepted on July 22, 2010)

To reveal the origin of the Hawaiian endemic *Canavalia* species, phylogenetic analyses of chloroplast DNA (cpDNA) and internal transcribed spacers (ITS) of nuclear ribosomal DNA (nrDNA) sequences were performed. Phylogenetic analyses of 6 cpDNA regions (6386 bp) and of nrDNA ITS (708 bp) for all 6 species of the Hawaiian endemic subgenus *Maunaloa* together with samples from the other 3 subgenera of *Canavalia* suggested that subgenus *Maunaloa* is monophyletic and more closely related to subgenus *Canavalia* than to other subgenera. Phylogenetic analyses of multiple haplotypes of the nrDNA ITS suggested that the Hawaiian endemic species of *Canavalia* originated from a sea-dispersed species of subgenus *Canavalia*, possibly *Canavalia rosea* (Sw.) DC., which is a pantropical species whose seeds are spread by sea drift. A single origin for subgenus *Maunaloa* might be also suggested.

Key words: *Canavalia*, chloroplast DNA, Hawaiian Islands, nrDNA ITS, phylogeny, seed dispersal.

Approximately 90% of native plant species of the Hawaiian Islands are endemic (Baldwin and Wagner 2010). This represents an extraordinary example of high endemism in oceanic islands and has attracted the interest of many botanists. Many studies have focused on the origin, speciation, and adaptive radiation of the endemic species in the Hawaiian Islands (reviewed in Baldwin and Wagner 2010). As the Hawaiian Islands lie several thousand km from the nearest continents, high dispersibility would be one of the key factors for the ancestors of endemic species to achieve successful migration to the Hawaiian Islands. According to the most recent review on studies of Hawaiian endemic plant species, Baldwin and Wagner (2010) have suggested that the temperate and boreal regions of North America are important sources of Hawaiian flora. This proposal is well supported by molecular phylogenetic evidence (Vargas et

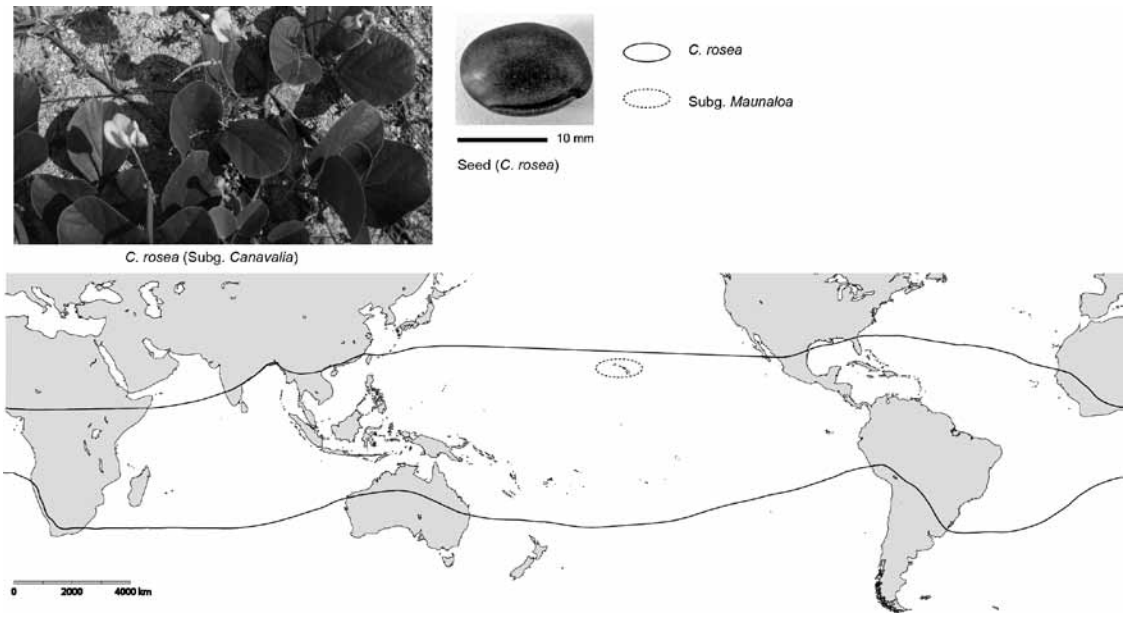

Fig. 1. Distribution range of *Canavalia rosea* and subgenus *Maunaloa* (drawn based on Sauer 1964). Plant creeping on beach (top left) and seed (top right) of *C. rosea*.

al. 1998, Lindqvist and Albert 2002). Among the species originating from North America, only a few examples (*Gossypium* (*Malvaceae*) and *Jacquemontia* (*Convolvulaceae*)) have seeds that are dispersed by the sea. In the case of the other species, the seeds are dispersed by birds or wind. Although sea dispersal is one of the most important modes of seed dispersal for shaping the littoral flora of oceanic islands, detailed studies on Hawaiian plants are lacking.

The genus *Canavalia* Adans. is a good model for investigations of the origin of Hawaiian endemic species from sea dispersed species. The genus *Canavalia* is distributed in the tropics and subtropics all over the world. According to the latest taxonomic revision, this genus is further divided into four subgenera, namely, *Catodonia* (seven species), *Wenderothia* (16 species), *Canavalia* (23 species), and *Maunaloa* (six species) (Sauer 1964, St. John 1970, Schrire 2005). Species of subgenera *Catodonia* and *Wenderothia* are distributed mostly in the New World. Species of subgenus *Canavalia* are found in both the Old and New World, including some crop species. Species of subgenus *Maunaloa* are endemic to the Hawaiian Islands. Sauer (1964) studied the morphological differences and similarities among *Canavalia* species and reported that the most primitive subgenus is probably *Wenderothia*, and subgenera *Catodonia* and *Canavalia* probably originated from this subgenus. He also proposed that subgenus *Maunaloa* would be originated from subgenus *Canavalia* because of the presence of a pantropical species, *Canavalia rosea* (Sw.) DC., in the subgenus.

Canavalia rosea is a typical member of the plant group known as the "pantropical plants with sea-drifted seeds" (Takayama et al. 2008). The members of this plant group are distributed in littoral areas of the tropics and subtropics all over the world and their main mode of seed dispersal is sea dispersal (Fig. 1). In addition to *C. rosea*, some species of the subgenus *Canavalia* also use sea dispersal to spread their seeds. *Canavalia cathartica* Thouars is distributed over Indo-West Pacific regions, *C. lineata* (Thunb.) DC. is found in South East

Asia, and *C. sericea* A. Gray is distributed in the South Pacific. The other species of *Canavalia* whose seeds are subjected to sea dispersal is *C. bonariensis* Lindley of the subgenus *Catodonia*. Other species of the genus *Canavalia* do not have sea-drifted seeds and their main mode of seed dispersal is mechanical (with the seeds being thrown by dehiscent pods) or gravity (Sauer 1964). Given the distribution ranges of the species and their modes of seed dispersal, Sauer (1964) and Carlquist (1966) suggested that the endemic species of the Hawaiian Islands might have originated from species that reached the Hawaiian Islands by sea dispersal. A good candidate species would plausibly be the pantropical species, *Canavalia rosea,* as it has native distribution in Hawaii according to a herbarium specimen (Rock s.n., ca. 1910, BISH) and has the widest distribution range among sea-dispersed species in the genus. However, this hypothesis has never been tested using modern molecular phylogenetic approaches.

To test the hypothesis about the origin of Hawaiian endemic species of *Canavalia*, we employed both nuclear and chloroplast DNA (cpDNA) markers. These markers have been successfully used to study the origin of Hawaiian endemic species in other plant groups (reviewed in Baldwin and Wagner 2010). Although a recent phylogenetic study on subtribe *Diocleinae* based on nuclear ribosomal DNA (nrDNA) ITS sequences (Varela et al. 2004) showed that subgenus *Canavalia* is a sister to the subgenus *Catodonia*, the taxon sampling of the study was not sufficient to reveal the phylogenetic relationships between the four subgenera and the origin of Hawaiian endemic species. We studied all species of the subgenus *Maunaloa* together with multiple samples of *C. rosea* collected from its wide distribution range. Other species that have sea-drifted seeds and representative species of subgenera *Wenderothia* and *Catodonia* were also included in the study.

Materials and Methods

Taxon sampling

A total of 18 species of *Canavalia* were used in this study. Data of four species were only for nrDNA ITS analyses and obtained from GenBank (Table 1). Species from all 4 subgenera according to the subgeneric delimitation of Sauer (1964) were included (Table 1). Multiple accessions were sampled for five taxa, *C. rosea* (18), *C. lineata* (2), *C. sericea* (2), *C. hawaiiensis* Degener & al. (2) and *C. galeata* Gaudich. (2). *Dioclea reflexa* Hook. f., a species from the genus *Dioclea* was used as an outgroup, based on the molecular phylogenetic tree of Varela et. al. (2004). Voucher specimens of the newly collected samples in this study were deposited in the herbarium of University of the Ryukyus (RYU). Some samples were collected from specimens preserved in the herbaria of Jardim Botânico do Rio de Janeiro (JBRJ), Universidad National Autonoma de Mexico (MEXU), and Bishop Museum (BISH).

DNA extraction, PCR and sequencing

Genomic DNA was extracted from dried leaves or seeds using the method of Doyle and Doyle (1987). The concentration of crude DNA extract was measured with a GeneQuant 100 electrophotometer (GE Healthcare, Life Sciences). For cpDNA sequences, after an initial screening of 15 cpDNA candidate regions (Shaw et al. 2007), six regions including intergenic spacers (IGS) and introns were chosen. The cpDNA regions and PCR primers used in this study were for *atpB-rbcL* IGS (Hodges and Arnold 1994), *ndhD-ndhE* (Xu et al. 2000), *trnH-psbA* IGS (Hamilton 1999), *rps*16 intron (Oxelman et al. 1997), *trnD-trnT* (Demesure et al. 1995) and *trnK*, which includes the *matK* gene (Hu et al. 2000, Lavin et al. 2000, Wojciechowski et al. 2004). Polymerase chain reactions (PCR) were performed in reaction volumes of 10–25 μL containing 1.25 units ExTaq (TaKaRa), and 0.2 mM dNTPs., 10× PCR buffer contains 1.5 mM $MgCl_2$, 0.5–1 μM

Table 1. List of *Canavalia* samples used in this study

Subgenus	Taxon	Oceanic region		Locality	Voucher specimen	DNA sample No.	nrDNA ITS**
Canavalia	*C. rosea* (Sw.) DC.	Indian Ocean	South Africa	Umdloti	T. Kajita 010509	98	cloning
			Tanzania	Dar es salaam	T. Kajita 021202	184	cloning
			Sri Lanka	Wattala, Negambo	T. Kajita 000729	31S	cloning
			Indonesia	Sumatra	T. Kajita 000812	500	direct
			Australia	Headland Harbour	Y. Tateishi 001123	1	direct
		West Pacific	Marquesas	Taipivai, Nuku Hiva	T. Kajita & Y. Tateishi 000713	31M	direct
			Tonga	Sopu	T. Kajita & al. 021024	171	direct
		East Pacific	Mexico	Sinaloa	T. Kajita & al. 1998-965	965	direct
			Panama	Veracruz	T. Kajita & Y. Tateishi 011104	202	direct
			Ecuador	Isla Jambel	T. Kajita 990720	1205	direct
		West Atlantic	Panama	Cuango, Colon	T. Kajita & Y. Tateishi 011031	109	direct
			Panama	Pina, Colon	T. Kajita & Y. Tateishi 011030	24	cloning
			Costa Rica	Puerto Viejo	T. Kajita 990821	346	cloning
			Mexico	Coatzcoalcos	T. Kajita & al. 981029	70M	cloning
			Brazil	Gaibu Pernanbuco	T. Kajita & al. 991129	70B	cloning
		East Atlantic	Senegal	Joal-Fadiout	T. Kajita 001203	107, 108	cloning
			Ghana	Busua beach	Y. Kita 980808	5G	direct
			Angola	Musul, Luanda	T. Kajita 010505	0	cloning
	C. cathartica Thouars	Pacific	Philippines	Palawan	T. Kajita & Y. Tateishi 981210	CH10	cloning
	C. lineata (Thunb.) DC.	Pacific	Taiwan	Maopi Tao	T. Kajita & Y. Tateishi 981212	CL1	direct
			Japan	Miyazaki	T. Kajita 020929	74	cloning
	C. sericea A. Gray	Pacific	Hawaii	Maui (Bishop Museum)	T. Kajita & al. 021022	42	direct
		Pacific	Tonga	Haashini-Lavengatonga	T. Kajita & al. 021024	138	cloning
	C. virosa (Roxb.) Wight & Arn.	Indian Ocean	Africa	Seed purchased	M. Vatanparast 090310	S3F8	direct
	C. brasiliensis Benth.	Atlantic	Mexico	GeneBank	(data from Valera & al. 2004)	AF467034.1	-
	C. boliviana Piper	Atlantic	Brazil	GeneBank	(data from Valera & al. 2004)	AY293838.1	-
Maunaloa	*C. hawaiiensis* Degener & al.	Pacific	Hawaii	Bishop Museum	Stemmermann 6843	6	cloning
	C. galeata Gaudich.	Pacific	Hawaii	Bishop Museum	Takeuchi 3224	7	cloning
	C. kauaiensis J.D. Sauer	Pacific	Hawaii	Bishop Museum	Lorence 1987	9	cloning
	C. molokaiensis Degener & al.	Pacific	Hawaii	Bishop Museum	Hughes 43	4	cloning
	C. napaliensis St. John	Pacific	Hawaii	Bishop Museum	Perlman 15319	5	cloning
	C. pubescens Hook. & Arn.	Pacific	Hawaii	Bishop Museum	Hobdy 1899	8	cloning
	C. hawaiiensis Degener & al.	Pacific	Hawaii	CCRT*	M. Vatanparast 010606001	C6	direct
	C. galeata Gaudich.	Pacific	Hawaii	CCRT*	M. Vatanparast 010209001	C7	direct
Catodonia	*C. parviflora* Benth.	Atlantic	Brazil	JBRJ Herbarium	H. Braga 390727	0809	direct
	C. bonariensis Lindley	Atlantic	Brazil	GeneBank	(data from Valera & al. 2004)	AY293839.1	-
Wenderothia	*C. villosa* Benth.	Atlantic	Mexico	MEXU Herbarium	Gabriel 4469	23	direct
	C. grandiflora Benth.	Atlantic	Brazil	GeneBank	(data from Valera & al. 2004)	AY293840.1	-
	C. hirsutissima J. D. Sauer	Atlantic	Mexico	MEXU Herbarium	A. Delgado 1372	11	direct
Outgroup	*Dioclea reflexa* Hook. f.	Atlantic	Costa Rica	Gandoca	T. Kajita 070113	13	direct

* Center for Conservation Research and Training, Hawaii.

** Sequence method for nrDNA ITS.

of each primer pair, and 20 ng genomic DNA. The PCR conditions were as follows: 3 min for initial denaturation at 95 °C, followed by 35 amplification cycles of 1 min denaturation at 95 °C, 1–2 min annealing at a fragment-specific temperature, 1–2 min extension at 72 °C, and a final 10 min extension at 72 °C. The PCR products were visualized by performing 0.8% agarose gel electrophoresis with ethidium bromide staining and amplified DNA was purified using either GENECLEAN III kit (Qiagen) or ExoSAP-IT (USB Corp., Cleveland, Ohio, USA) according to the manufacturer's instructions.

The cycle sequencing reactions were carried out using the ABI BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) and sequencing reaction products were purified

by ethanol precipitation. All DNA sequences were determined using an ABI 377 or ABI 3500 DNA sequencer (Applied Biosystems). For four species (*Canavalia brasiliensis* Benth., *C. boliviana* Piper, *C. bonariensis* and *C. grandiflora* Benth.), the nrDNA ITS sequences were obtained from GenBank (accession numbers AF467034.1, AY293838.1, AY293839.1 and AY293840.1, respectively). For ITS sequences in which direct sequencing yielded complicated electropherograms, the TOPO-TA cloning kit (Invitrogen) was subsequently used to obtain clones according to the manufacturer's instructions. Twelve colonies for each sample were picked, purified and amplified using the TempliPhi DNA Sequencing Template Amplification Kit (GE Healthcare). This kit utilizes bacteriophage Phi29 DNA polymerase and rolling circle amplification (RCA) technology for rapid amplification of circular template DNA (Polidoros et al. 2006). The products were then sequenced using the ABI BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). All forward and reverse strands for each sequence were assembled using Autoassembler version 2.1 (Applied Biosystems) with the default setting. Sequences were then manually edited and aligned with Se-Al version 2.0a11 (Rambaut 2002). For the ITS sequences, cloned products were aligned and verified with those obtained from direct sequencing in this study. A haplotype data matrix was made by the program DNAsp version 5.10.01 (Librado and Rozas 2009). The Phi test (Bruen et al. 2006) was employed to test the presence of recombination for cloned sequences of each sample using the program SplitsTree version 4.11.3 (Huson and Bryant 2006).

Phylogenetic inferences

Prior to the phylogenetic analyses, we performed the incongruence length difference (ILD) test (Farris et al. 1994) on the chloroplast sequences using PAUP* version 4.0b10 (Swofford 2002) to test combinability of the 6 plastid regions. A total of 100 replicates were used in the ILD test.

Maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) were employed for the phylogenetic analyses using cpDNA and nrDNA ITS sequences. For each of the sequences, the methods were conducted separately using the relevant programs. The MP analyses were conducted using PAUP* version 4.0b10 (Swofford 2002). Heuristic search were performed with a tree-bisection–reconnection (TBR) branch swapping algorithm and with indels and inversions removed. All characters were unweighted and unordered. Branch support was evaluated using the bootstrapping method of Felsenstein (1985) based on 1000 replicates.

The ML nalysis was performed using the RAxML (Randomized Axelerated Maximum Likelihood) program, version 7.2.6 (Stamatakis 2006) which implements a rapid hill-climbing algorithm. The analysis was run for the best-scoring ML tree inferences under the GTR+G model for the both cpDNA and nrDNA ITS sequences separately, with all indel and inversion characters removed. Rapid bootstrap analyses were conducted with 1000 replications using the GTRCAT (Stamatakis 2006) estimation to assess branch support.

Bayesian inference was performed using MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). For each region we selected appropriate evolutionary models using MrModeltest version 2 (Nylander 2004). The HYK (*atpB-rbcL* IGS, *trnH-psbA* IGS and *rps*16), GTR+I (*ndhD-ndhE*), HKY+I (*trnD-trnT* and *trnK*) and SYM+G (nrDNA ITS) models were selected as best-fit models based on the Akaike information criterion (Akaike 1974) using the program MrModeltest version 2 (Nylander 2004). Two independent Markov Chain Monte Carlo (MCMC) analyses with four simultaneous chains and 2,000,000 generations were run. Trees were sampled for

every 100 generations and the first 5000 trees were discarded as burn-in. The convergence of MCMC chains was visualized with Tracer version 1.5 (Rambaut and Drummond 2009) and likelihood scores for the sampled trees were inspected.

Results

Chloroplast (six regions) and nuclear DNA (ITS) molecular markers

Nucleotide sequences of the cpDNA and nrDNA regions were obtained from 40 specimens used in this study (Table 1; GenBank accsession no. HQ707397–HQ707580). Some of the cpDNA regions included parts that were difficult to align, and these were excluded from analyses. These regions include a 207 bp indel in *atpB-rbcL* IGS, four and two base pairs inversions in *ndhD-ndhE* and *trnH-psbA* IGS respectively, and all length variations containing homopolymers. Sequences were determined using cloned samples for 19 specimens from nine taxa (Table 1). The aligned length was 6386 bp for concatenated cpDNA regions and 708 bp for nrDNA.

Phylogenetic analyses based on cpDNA sequence data

The results of the ILD test for the six chloroplast regions detected heterogeneity among these regions (p value = 0.01). Although heterogeneity among regions may be expected at the 1% significance level, the six chloroplast regions were combined in the phylogenetic analyses, as we didn't find major topological conflicts among 6 trees generated from each region, and it is said that the ILD test might be too sensitive (Wiens 1998). The Hawaiian taxa, particularly, retained a clade in three trees, while they were placed in a polytomy with samples of subgenus *Canavalia* in the other trees (data not shown).

A total of 21 haplotypes, designated from C1–C21 were recognized in the aligned sequences of 6386 bp. MP analysis of the 21 haplotypes resulted in 15 most parsimonious trees with a length of 257 steps, a consistency index (CI) of 0.949 excluding uninformative characters, and a retention index (RI) of 0.879. The strict consensus of the 15 most parsimonious trees is shown in Fig. 2. Both ML and BI analyses produced topologically identical trees (not shown). Bootstrap values for MP and ML analyses and posterior probability for BI analysis are shown in the resultant tree when they exceed 70% (Fig. 2). Within the genus, three main monophyletic clades (I, II and III) were recognized with high branch supports. Clade I consists of members of the subgenera *Catodonia* and *Wenderothia*. Clade II includes members of the subgenera *Canavalia* and *Maunaloa*. Clade III includes all Hawaiian endemic species of the subgenus *Maunaloa*. Moreover, within clade I, the species of subgenus *Wenderothia* (C. *hirsutissima* J. D. Sauer and *C. villosa* Benth.) make a monophyletic group (Fig. 2). In the 10 haplotypes (C1–C10) observed in subgenus *Canavalia,* seven haplotypes (C2–C8) were exclusive to the *C. rosea,* in which all but one (C8, Mexico from Pacific region) were from the Atlantic region. The haplotype C1 was shared among *C. rosea*, *C. cathartica* and *C. lineata* from various localities in the Pacific and Indian oceanic regions. Haplotypes C9 and C10 were only obtained from *C. virosa* (Roxb.) Wight & Arn. and *C. sericea*, respectively. In Clade III, *C. kauaiensis* J. D. Sauer (C12) and *C. napaliensis* St. John (C13) form a monophyletic group and *C. molokaiensis* Degener & al. and *C. pubescens* Hook. & Arn. have same sequence haplotype (C14).

Phylogenetic analyses based on ITS sequence data

The total sequence length of the nrDNA ITS (ITS1, 5.8S, and ITS2) sequences for 40 samples ranged from 601 bp in *C. virosa* to 708 bp in *Dioclea reflexa* (an outgroup species). Aligned sequences, after excluding all gaps, yielded 514 bp that retained 35 haplotypes (H1–

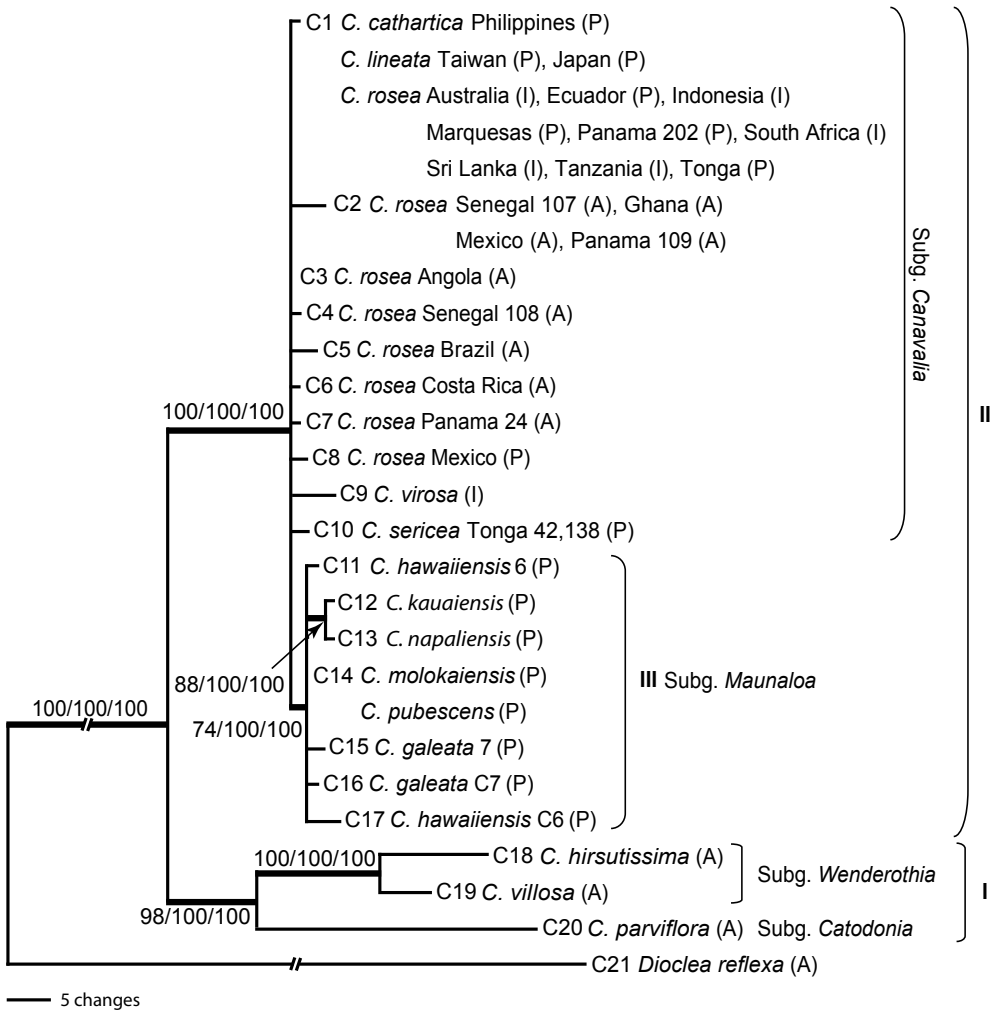

Fig. 2. The strict consensus tree of 15 equally most parsimonious trees based on 6389 bp of 6 cpDNA regions for *Canavalia* species. Tree length = 257, CI = 0.949, RI = 0.879 and RC = 0.835. Branch supports for MP/ML/BI analyses are shown along branches. *Dioclea reflexa* is used as an outgroup. Abbreviations in parenthesis. P. Pacific Ocean. A. Atlantic Ocean. I. Indian Ocean. Numbers following locality names correspond to the DNA sample No. shown in Table 1.

H35). Pairwise sequence divergence values were less than 3% substitution per site across ingroup taxa. The Phi test did not identify statistically significant evidence for recombination among cloned sequences.

The MP analysis of the nrDNA ITS sequence data resulted in 1169 most parsimonious trees with a length of 141 steps, a CI of 0.823 (excluding uninformative characters) and a RI of 0.795. The strict consensus of these trees with accompanying bootstrap values for MP and ML analyses and posterior probability for BI analysis of more than 70% are shown in Fig. 3. Both the ML and BI analyses for the nrDNA ITS produced topologically identical trees (not shown). Contrary to the cpDNA tree, the subgenus *Catodonia* was found to be more closely related to the clade comprised of samples from subgenera *Canavalia* and *Maunaloa* and supported by relatively high branch supports (Fig. 3). All samples of Hawaiian endemic taxa used in this study had 2 alleles for ITS sequence.

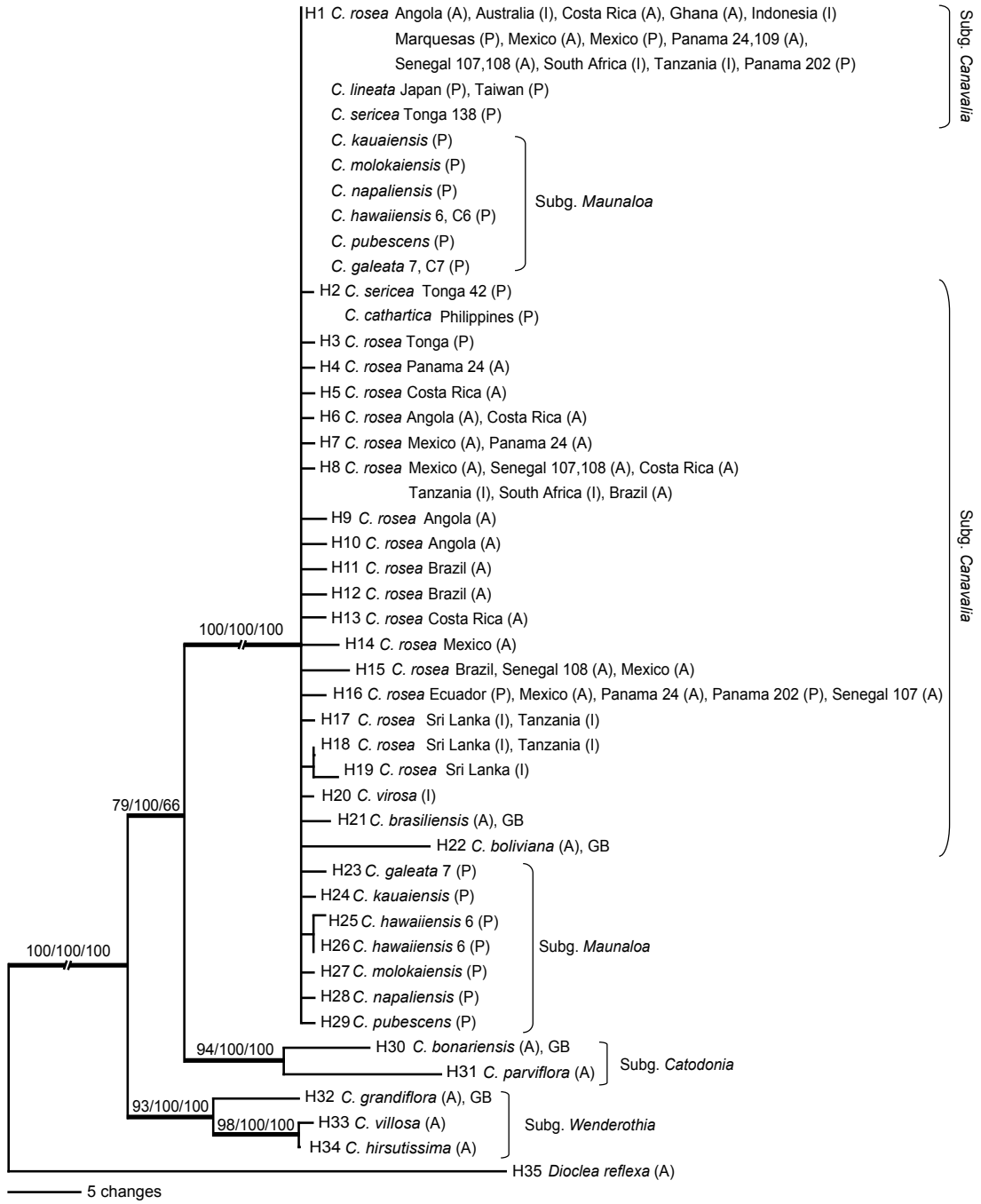

Fig. 3. The strict consensus tree of 1169 most parsimonious trees based on 708 bp of nrDNA ITS region for *Canavalia* species. Tree length = 141, CI = 0.823, RI = 0.795 and RC = 0.654. Branch supports for MP/ML/BI analyses are shown along branches. *Dioclea reflexa* is used as an outgroup. Abbreviations. P. Pacific Ocean. A. Atlantic Ocean. I. Indian Ocean. GB. GeneBank. Numbers following locality names correspond to the DNA sample No. shown in Table 1.

One was the haplotype H1 that was also shared among samples of *C. rosea*, *C. lineata* and *C. sericea*, and the others were specific to each species (H23–H29). Most of the *C. rosea* samples from the Atlantic region had more than 2 alleles (Fig. 3). *Canavalia virosa*, *C. brasiliensis* and *C. boliviana* represented distinct haplotypes.

Discussion

The origin of Hawaiian endemic species

The results of the present study suggest that the endemic Hawaiian subgenus of *Canavalia* originates from a species that reached the Hawaiian Islands by sea dispersal, and that the species would be allied to *Canavalia rosea*, a pantropical species. According to the cpDNA and nrDNA ITS phylogenetic trees, Hawaiian endemic species (subgenus *Maunaloa*) are more closely related to the subgenus *Canavalia* than to the other subgenera (Figs. 2, 3). Although the ancestral lineage of Hawaiian endemic *Canavalia* was not clearly shown, Hawaiian species share the same haplotype (H1) with *C. rosea*, *C. lineata* and *C. sericea*, all of which have seeds which are spread by sea drift. These results suggest that the Hawaiian endemic species were originated from one of the three species or from an ancestor common to them. As the haplotype H1 of *C. rosea* is widely distributed over the Pacific, Atlantic and Indian Oceans, we propose that *C. rosea* is the origin of the Hawaiian endemic species. Although *C. sericea* and another sea-dispersed species, *C. cathartica,* are in the Hawaiian Islands at present, they are not native to the islands (Sauer 1988, Whistler 1992, Wagner et al. 1999). On the other hand, *C. rosea* was present in the Hawaii according to a herbarium specimen in Bishop Museum (Rock s.n., ca. 1910, BISH), although we have no recent herbarium records of the species from Hawaii.

Speciation and Loss of dispersibility in Hawaiian *Canavalia*

Our phylogenetic analyses using cpDNA sequences showed that the 6 Hawaiian endemic species are monophyletic (Fig. 2). This result suggests that they are descended from a single species and that the speciation of the six species occurred after the colonization of their ancestor in the Hawaiian Islands. None of the species of the subgenus *Maunaloa* have seed buoyancy (Sauer 1964, Carlquist 1966). Loss of seed buoyancy in oceanic island species is a common phenomenon which occurred in many species in Hawaiian endemic plants (Carlquist 1974, Cody and Overton 1996, Baldwin and Wagner 2010). In the case of *Canavalia*, it would be associated with the habitat shift toward inland regions during the speciation process (Carlquist 1967). Similar habitat shift is also observed in *Hibiscus glaber* Matsum. that is an endemic species in the Bonin Islands and was revealed to be speciated from a pantropical plants with sea-drifted seeds, *H. tiliaceus* L. (Takayama et al. 2006). It remains unclear why the distribution of the endemic species of *Canavalia* is restricted to specific islands among the Hawaiian island chain. The endemic species might have speciated on each island after the speciation of their common ancestor that retained seed buoyancy and therefore had the ability to colonize over the Hawaiian Islands. A more detailed study using high resolution markers is needed to reveal speciation process of the Hawaiian endemic species of *Canavalia*.

Phylogenetic relationships among species

Our phylogenetic trees do not clearly show the species relationships among subgenera *Canavalia* and *Maunaloa* although we used highly variable cpDNA regions and nrDNA ITS (Figs. 2, 3). This is perhaps because the speciation within and/or among these subgenera occurred rapidly. This result made it difficult to understand the evolutionary history among the species of these subgenera. As we used rather long sequences of cpDNA for phylogenetic inference, increasing the data length of cpDNA

may not improve the resolution of phylogenetic tree. Given the difficulties in performing phylogenetic analyses for closely related species, novel nuclear markers are needed (Scherson et al. 2005), for example, a combination of single or low copy nuclear genes, or data from fragment analyses.

We thank Drs H. C. Lima, M. Bovini, A. Tidiane Ba, D. Darnaedi, I. Ibrahim, D. Neil, D. A. Madulid, E. Narvaez, I. Seneviratne, J. Flora, T. Yahara, A. Soejima, T. Ohi-Toma, K. Watanabe, Y. Mori, Y. Kita, Y. Abe, Bishop Museum (USA), JBRJ (Brazil), CNPq (Brazil), STRI (Panama), OTS (Costa Rica) and MEXU (Mexico) for their support in field sampling, and A. Yoshinaga from Center for Conservation Research and Training (CCRT), Hawaii for providing materials. We also thank Drs Y. Watano, T. Asakawa, B. Adjie, N. Wakita and A. Shirai for their useful comments on this study. This work was supported by JSPS Grants-in-Aid for Scientific Research, mainly by Nos. 19370032, 16370043 and 14740471 (to T. K.), and for sampling by 14405015 and 12575011 (to Y. T.).

References

Akaike H. 1974. A new look at the statistical model identification. IEEE T. Automat. Cont. **19**: 716–723.

Baldwin B. G. and Wagner W. L. 2010. Hawaiian angiosperm radiations of North American origin. Ann. Bot. **105**: 849–879.

Bruen T. C., Philippe H. and Bryant D. 2006. A simple and robust statistical test for detecting the presence of recombination. Genetics **172**: 2665–2681.

Carlquist S. 1966. The biota of long-distance dispersal. III. Loss of dispersibility in the Hawaiian Flora. Brittonia **18**: 310–335.

Carlquist S. 1967. The biota of long-distance dispersal. V. Plant dispersal to Pacific Islands. Bull. Torrey Bot. Club **94**: 129–162.

Carlquist S. 1974. Island Biology. Columbia University Press, New York.

Cody M. L. and Overton J. M. 1996. Short-term evolution of reduced dispersal in island plant populations. J. Ecol. **84**: 53–61.

Demesure B., Sodzi N. and Petit R. 1995. A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Mol. Ecol. **4**: 129–131.

Doyle J. and Doyle J. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry **19**: 11–15.

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.

Hamilton M. 1999. Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. Mol. Ecol. **8**: 521–523.

Hodges S. A. and Arnold M. L. 1994. Columbines: a geographically widespread species flock. Proc. Natl. Acad. Sci. U. S. A. **91**: 5129–5132.

Hu J.-M., Lavin M., Wojciechowski M. F. and Sanderson M. J. 2000. Phylogenetic systematics of the tribe *Millettieae* (*Leguminosae*) based on chloroplast *trnK*/*matK* sequences and its implications for evolutionary patterns in *Papilionoideae*. Am. J. Bot. **87**: 418–430.

Huson D. H. and Bryant D. 2006. Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. **23**: 254–267.

Lavin M., Thulin M., Labat J. and Pennington R. 2000. Africa, the odd man out: molecular biogeography of dalbergioid legumes (*Fabaceae*) suggests otherwise. Syst. Bot. **25**: 449–467.

Librado P. and Rozas J. 2009. DnaSP version 5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics **25**: 1451–1452.

Lindqvist C. and Albert V. A. 2002. Origin of the Hawaiian endemic mints within North American *Stachys* (*Lamiaceae*). Am. J. Bot. **89**: 1709–1724.

Nylander J. 2004. MrModeltest. Program distributed by the author, version 2.

Oxelman B., Lidén M. and Berglund D. 1997. Chloroplast *rps16* intron phylogeny of the tribe *Sileneae* (*Caryophyllaceae*). Plant Syst. Evol. **206**: 393–410.

Polidoros A., Pasentsis K. and Tsaftaris A. 2006. Rolling circle amplification-RACE: a method for simultaneous isolation of 5' and 3' cDNA ends from amplified cDNA templates. BioTechniques **41**: 35.

Rambaut A. 2002. Se-Al. A manual sequence alignment editor, version 2.0a11. http://tree.bio.ed.ac.uk/software/seal/.

Rambaut A. and Drummond A. 2009. Tracer. version 1.5. http://beast.bio.ed.ac.uk/Tracer.

Ronquist F. and Huelsenbeck J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19**: 1572–1574.

Sauer J. 1964. Revision of *Canavalia*. Brittonia **16**: 106–181.

Sauer J. 1988. Plant migration: the Dynamics of Geographic Patterning in Seed Plant Species.

University of California Press, Berkeley.
Scherson R., Choi H., Cook D. and Sanderson M. 2005. Phylogenetics of New World *Astragalus*: Screening of novel nuclear loci for the reconstruction of phylogenies at low taxonomic levels. Brittonia **57**: 354–366.
Schrire B. D. 2005. *Phaseoleae*. *In*: Lewis G., Schrire B., Mackinder B. and Lock M. (eds.), Legumes of the World. pp. 393–431. Royal Botanical Gardens, Kew.
Shaw J., Lickey E. B., Schilling E. E. and Small R. L. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. Am. J. Bot. **94**: 275–288.
St. John H. 1970. Revision of the Hawaiian species of *Canavalia* (*Leguminosae*). Hawaiian plant studies. Isr. J. Bot. **19**: 161–219.
Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics **22**: 2688–2690.
Swofford D. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods), version 4.0 beta 10. Sinauer Associates, Sunderland.
Takayama K., Kajita T., Murata J. and Tateishi Y. 2006. Phylogeography and genetic structure of *Hibiscus tiliaceus*–speciation of a pantropical plant with sea-drifted seeds. Mol. Ecol. **15**: 2871–2881.
Takayama K., Tateishi Y., Murata J. and Kajita T. 2008. Gene flow and population subdivision in a pantropical plant with sea-drifted seeds *Hibiscus tiliaceus* and its allied species: evidence from microsatellite analyses. Mol. Ecol. **17**: 2730–2742.
Varela E. S., Lima J. P. M. S., Galdino A. S., Pinto L. d. S., Bezerra W. M., Nunes E. P., Alves M. A. O. and Grangeiro T. B. 2004. Relationships in subtribe *Diocleinae* (*Leguminosae*; *Papilio-noideae*) inferred from internal transcribed spacer sequences from nuclear ribosomal DNA. Phytochemistry **65**: 59–69.
Vargas P., Baldwin B. G. and Constance L. 1998. Nuclear ribosomal DNA evidence for a western North American origin of Hawaiian and South American species of *Sanicula* (*Apiaceae*). Proc. Natl. Acad. Sci. U. S. A. **95**: 235–240.
Wagner W., Herbst D. and Sohmer S. 1999. Manual of the Flowering Plants of Hawaii, Revised Edition. University of Hawaii Press, Honolulu.
Whistler W. 1992. Flowers of the Pacific island seashore. University of Hawaii Press., Honolulu
Wiens J. J. 1998. Combining Data Sets with Different Phylogenetic Histories. Syst. Biol. **47**: 568–581.
Wojciechowski M., Lavin M. and Sanderson M. 2004. A phylogeny of legumes (*Leguminosae*) based on analysis of the plastid *matK* gene resolves many well-supported subclades within the family. Am. J. Bot. **91**: 1846–1862.
Xu D. H., Abe J., Sakai M., Kanazawa A. and Shimamoto Y. 2000. Sequence variation of non-coding regions of chloroplast DNA of soybean and related wild species and its implications for the evolution of different chloroplast haplotypes. Theor. Appl. Genet. **101**: 724–732.

M. VATANPARAST[a], 高山浩司[a,†], M. S. SOUSA[b], 立石庸一[c], 梶田　忠[a]：**分子データから示されたハワイ産ナタマメ属固有種の起源**

ハワイ諸島に固有のナタマメ属の *Maunaloa* 亜属の起源を明らかにするために，葉緑体 DNA と核リボソーム DNA の ITS 領域を用いた系統解析を行った．*Maunaloa* 亜属の 6 種全てと，他の 3 亜属から得られたサンプルを用いて，葉緑体 DNA の 6 領域約 6386 bp と核リボソーム DNA の ITS 領域の約 708 bp の塩基配列を用いて，それぞれ系統解析を行った結果，*Maunaloa* 亜属は他の 2 亜属よりもナタマメ亜属により近縁であることが分かった．ITS 領域について 1 サンプルから得られた複数の配列の系統関係からは，*Maunaloa* 亜属の種は，ナタマメ亜属の中でも海流散布能力を持つ 3 種により近縁であることが示唆された．地理的分布とハプロタイプの分布を考慮すると，ナガミハマナタマメ *Canavalia rosea* が *Maunaloa* 亜属の祖先種であったことが考えられる．葉緑体 DNA の系統解析から，*Maunaloa* 亜属の種は単系統であることから，ハワイ諸島における *Maunaloa* 亜属の 6 種は，共通の祖先から起源したことが示された．

([a]千葉大学大学院理学研究科,
[b]メキシコ自治大学生物学研究所植物学部門,
[c]琉球大学教育学部理科,
[†]現住所：ウィーン大学植物科学科植物系統進化学分野)