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Phylogeny and Biogeography of the Genus *Lycium* (Solanaceae): Inferences from Chloroplast DNA Sequences

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Lycium **comprises approximately 70 species and is disjunctly distributed in temperate to subtropical regions in South America, North America, southern Africa, Eurasia, and Australia. Among them, only *Lycium sandwicense* A. Gray sporadically occurs widely on oceanic islands in the Pacific Ocean. To investigate phylogenetic and biogeographic relationships of the genus with emphasis on *L. sandwicense*, the coding region of *matK*, the two intergenic spacers *trnT* (UGU)–*trnL* (UAA) and *trnL* (UAA)–*trnF* (GAA), and the *trnL* (UAA) intron of chloroplast DNA (cpDNA) were sequenced. A strict consensus tree resulting from the phylogenetic analysis indicates the following: (1) New World species comprise a potentially paraphyletic assemblage; (2) southern African, Australian, and Eurasian species together are monophyletic; (3) southern African species are a paraphyletic assemblage; and (4) *L. sandwicense* is in a clade with certain New World species. The estimated biogeographic events based on the cpDNA analysis indicate that (1) *Lycium* originated in the New World, (2) all southern African, Australian, and Eurasian species have a common ancestor from the New World, (3) Australian and Eurasian species originated once from a southern African progenitor, and (4) *L. sandwicense* differentiated from the New World species.**

INTRODUCTION

The genus *Lycium* L. (Solanaceae) comprises approximately 70 species of spiny shrubs and small trees. Most species occur in arid and subarid regions, but some occur in subsaline regions or along the seacoast (e.g., Hitchcock, 1932; D'Arcy, 1979, 1991; Hunziker, 1979). *Lycium* shows two interesting features in its distribution. First, the genus is disjunctly distributed between temperate and subtropical regions: South America (ca. 30 spp.), southern Africa (ca. 20 spp.), North America (ca. 20 spp.), Eurasia (from Europe to China and Japan: ca. 10 spp.), Australia (1 sp.), and several islands in the Pacific Ocean (2 spp.) (Fig. 1). There are two biogeographic hypotheses for the establishment of the present distribution pattern of *Lycium:* those of Symon (1991) and Raven and Axelrod (1974). Based on the relatively high species diversity in South America and southern Africa, Symon (1991) hypothesized that the disjunct distribution in solanaceous genera including *Lycium* originated in the drifting of the continental masses after the break up of Gondwanaland. Raven and Axelrod (1974) supposed that the distribution pattern of *Lycium* must have attained its disjunctions by long-distance dispersal because *Lycium* is scattered in arid regions. However, no studies on the biogeography of *Lycium* have been made so far.

Second, *L. sandwicense* A. Gray is distributed widely on islands across the Pacific Ocean. This species occurs on Easter Island, the Hawaiian Islands, the Ogasawara Islands, and the Daitou Islands (Hitchcock, 1932; Yamazaki, 1991). No other plants share such a distribution pattern. Based on overall morphological features of vegetative and reproductive parts, Hitchcock (1932) considered that *L. sandwicense* was closely related to a North American species, *L. carolinianum* Walt. var. *quadrifidum* (Moç. & Sessé ex Dunal) C. L. Hitchcock. However, the relationships between *L. sandwicense* and species outside of North and South America have not been investigated. Extensive comparisons with species other than the New World species are needed to determine the origin of *L. sandwicense.*

The biogeographic history of *Lycium* could be inferred from a phylogenetic analysis of the genus. However, most previous studies of the genus were taxonomic treatments without explicit phylogenetic considerations (e.g., North and South America: Hitchcock, 1932; Barkley, 1953; Chiang-Cabrera, 1981; Eurasia: Feinbrun, 1968; Australia: Haegi, 1976; southern Africa: Joubert, 1981). No extensive worldwide studies of the genus as a whole have been made, although some phylogenetic and anatomical work has been done on the American species (e.g., Bernardello, 1987; Bernardello and Luján, 1997; Bernardello and Chiang-Cabrera, 1998), and phylogenetic relationships of the

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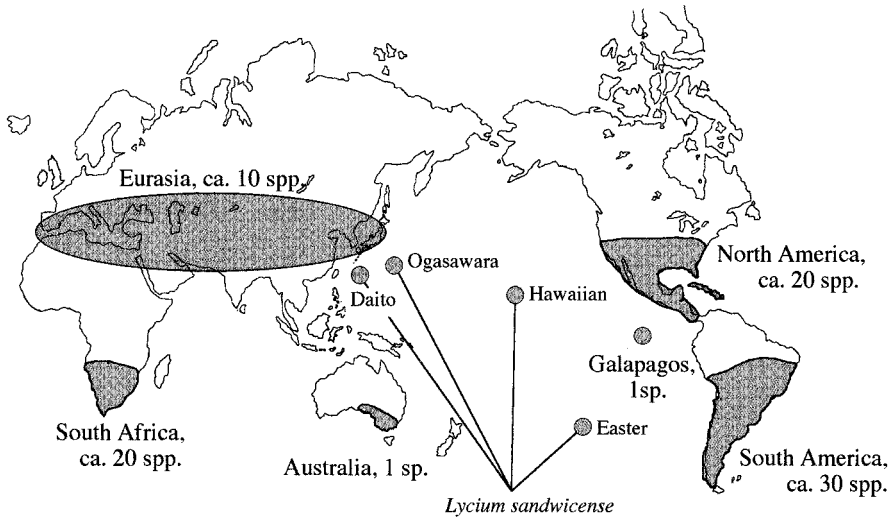

FIG. 1. Distribution map of the genus *Lycium*.

genus remain poorly understood. In recent years, chloroplast DNA (cpDNA) has been frequently used to reconstruct phylogenetic relationships of a wide range of land plants (e.g., Palmer *et al.*, 1988; Clegg and Zurawski, 1992; Chase *et al.*, 1993; Hoot *et al.*, 1995; Olmstead and Reeves, 1995). We used cpDNA sequence comparisons of the *matK* coding region, two intergenic spacers, *trnT* (UGU)–*trnL* (UAA) and *trnL* (UAA)–*trnF* (GAA), and the *trnL* (UAA) intron to provide an initial framework for studying the phylogenetic relationships of the genus. These regions have allowed evaluation of relationships to generic or species level [*matK:* Saxifragaceae *s.str.* (Johnson and Soltis, 1994); *Gilia* (Polemoniaceae: Johnson and Soltis, 1995); *Saxifraga* (Saxifragaceae: Soltis *et al.*, 1996); *trnT–trnF: Gentiana* (Gentianaceae: Gielly *et al.*, 1996); *Primula cuneifolia* (Primulaceae: Fujii *et al.*, 1995)].

This paper provides a phylogenetic reconstruction of *Lycium*. Our biogeographical investigations based on this analysis help to clarify the formation of the present disjunct distribution of the genus and the origin of *L. sandwicense* in the Pacific islands.

MATERIALS AND METHODS

Plant Materials

Twenty-nine samples were examined in this study. Their sources and voucher information are listed in Table 1. All samples used in this study were identified using the following sources: Hitchcock (1932), Feinbrun (1968), Haegi (1976), Chiang-Cabrera (1981), Joubert (1981), Yamazaki (1991), and Zhang *et al.*, (1994). *Nolana rostrata* (Lindl.) Miers and *N. albescens* (R. Phil.) I. M. Johnston were selected as outgroups because a close phylogenetic relationship of *Nolana* to *Lycium* was indicated by previous studies (Olmstead and Palmer, 1992; Olmstead and Sweere, 1994). The previously published sequence of *Nicotiana tabacum* L. (Shinozaki *et al.*, 1986) was also included as an outgroup in our analyses.

Molecular Techniques and Sequencing Strategy

Total genomic DNA was isolated from 200 to 300 μg of fresh leaf tissues or herbarium specimens based upon the modified 2 × CTAB procedure of Hasebe and Iwatsuki (1990). The isolated DNA was resuspended in 100 to 200 μL TE.

Double-stranded templates for direct sequencing were amplified by the polymerase chain reaction (PCR: Saiki *et al.*, 1988). PCR amplification was achieved using eight primers for the *matK* coding region, six primers for the two intergenic spacers *trnT–trnL* and *trnL–trnF*, and two primers for the *trnL* intron of chloroplast DNA. All primers used in this study are listed in Table 2. Eight internal primers were designed manually by comparing the sequences of *N. tabacum* and species of *Lycium*. We used the following thermocycle protocol: (94°C, 2 min) × 1 cycle, (94°C, 1.5 min; 45°C, 2 min; 60°C, 3 min) × 30 cycles, and (72°C, 15 min) × 1 cycle. PCR products were separated from other byproducts using 1% agarose gel electrophoreses. The desired bands were cut out and purified using Gene Clean Kit II (Bio 101). We sequenced the purified PCR products using the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit and Model 373A automated sequencer (Applied Bio Systems Division, Perkin–Elmer), following the manufacturer's instructions. For sequencing, we used the same primers as those used for amplification.

Data Analysis

Sequences were aligned manually by comparing with the sequence of *N. tabacum*. In *matK*, all inser-

TABLE 1

List of Taxa and Sources of Plant Materials

Area/species	Locality	Voucher
Eurasia		
Lycium barbarum L.	Ning-xia Hui-zu Zi-zhi-qu, China	X. Y. Zhu 9637 (PE)
L. chinense Mill.	Yamagata pref., Japan	A. Yokoyama and J. Yokoyama *s. n.* (TUS)
L. europaeum L.	Tohoku Univ., Japan; (cult.)	No voucher
L. ruthenicum Murr.	Astrakhan Prov., Russia	V. Sagalaev *et al., s. n.* (TUS)
Australia		
L. australe F. Muell.	Tohoku Univ., Japan; (cult.)	No voucher
South Africa		
L. afrum L.	Cape Prov., South Africa	C. Boucher 3134 (PRE)
L. cinereum Thumb.	Cape Prov., South Africa	A. A. Gubb 12801 (PRE)
L. ferocissimum Miers	Cape Prov., South Africa	J. J. Bos 267 (PRE)
L. pilifolium C. H. Wright	Cape Prov., South Africa	A. A. Gubb 12225 (PRE)
L. prunus-spinosa Dunal	Cape Prov., South Africa	A. A. Gubb 12838 (PRE)
L. schizocalyx C. H. Wright	Cape Prov., South Africa	A. A. Gubb 12489 (PRE)
L. villosum Schinz	Cape Prov., South Africa	I. McDonald 77/64 (PRE)
North America (including Central America)		
L. americanum Jacq.	Santa Marter, Netherlands Antilles	S. Marten and R. A. Howard 20731 (A)
L. andersonii A. Gray	California, Mono County, U.S.A.	J. D. Morefield and D. H. McCarty 3415 (A)
L. barlandieri Dunal		
ssp. *berlandieri*	Nuevo Leon, Mexico	Hinton *et al.* 20643 (A)
ssp. *parviflorum* (A. Gray) C. L. Hitchcock	Zacatecas, Mexico	R. C. Pollins and K. W. Rollins 74136 (TUS)
L. californicum Nutt. ex A. Gray	Isla Guadalupe, Mexico	F. Chiang 1272
L. carolinianum Walt.		
var. *carolinianum*	Florida, Monroe County, U.S.A.	W. C. Brumbach 9654 (A)
var. *quadrifidum* C. L. Hitchcock	Texas, U.S.A.	S. R. Hill 10523 (A)
L. pallidum Miers	Nevada, San Miquel County, U.S.A.	S. R. Hill and A. D. Cress 11304 (A)
South America		
L. ameghinoi Speg.	Patagonia, Argentina	A. Donat 37 (A)
L. cestroides Schlecht.	Dept. of Tarija, Bolivia	L. Bohs 2072 (A)
L. elongatum Miers	Catamerca Prov., Argentina	P. Cantino 741 (A)
L. morongii Britton	Ruta Prov., Argentina	G. E. Pieszko 6 (A)
Pacific Islands		
L. sandwicense A. Gray	Hawaiian Islands, Kaua 'i, U.S.A.	D. Herbst 2346 (A)
	Ogasawara Islands, Chichi Is., Japan	T. Fukuda and J. Yokoyama *s. n.* (TUS)
	Daito Islands, Minamidaito Is., Japan	T. Yamashiro and E. Tamaki *s. n.* (TUS)
Outgroup		
Nolana albescens	Dept. of Chanaral - Dept. of Capiano, Chile	M. Ono and K. Suzuki 274048 (TUS)
N. rostrata	Dept. of Coquinbo, Chile	M. Ono 274948 (TUS)

Note. Herbarium acronyms follow Index Herbariorum Part I (Holmgren *et al.*, 1990).

tions/deletions (indels) were positioned so that they began and ended on the first and third codon positions, respectively. We analyzed the phylogenetic relationships using both the maximum-parsimony (MP) and the maximum-likelihood (ML) methods. For MP analyses, we used PAUP 3.1.1 (Swofford, 1993). The MP analyses were conducted through a heuristic search with TBR branch swapping and MULPARS option. Multiple islands of equally most parsimonious trees (Maddison, 1991) were searched by the heuristic option with 100 random sequence additions. To place confidence levels on monophyletic groups, a bootstrap analysis (Felsenstein, 1985) with 10,000 replications was conducted to obtain repeatable bootstrap values (Hedges, 1992).

For ML analyses, we used the fastDNAml version 1.0 (Felsenstein, 1981; Olsen *et al.*, 1994). The transition/transversion (Ts/Tv) ratio used for the analyses was calculated from all pairwise comparisons of samples used in this study. We independently calculated the ratio of coding (*matK*) and noncoding (others) regions because the ratios are often considerably different among genes/DNA regions (Gielly and Taberlet, 1994; Manen and Natali, 1995). For the analysis with each Ts/Tv ratio, 10 random sequence addition searches by the Jumble option and global branch swapping by the Global option were employed to find a ML tree with the best log-likelihood. Bootstrap analyses with 100 replications were carried out by the Bootstrap option of the same program.

The biogeographic history of *Lycium* was estimated by the most parsimonious reconstruction of the distribution areas using MacClade 3.03 (Maddison and Maddison, 1992).

To estimate the divergence times among the species of *Lycium*, molecular clocks were estimated using the number of nucleotide substitutions obtained in this study. Before determining molecular clocks, the lin-

TABLE 2

Positions and Sequences of Primers Used for the Amplification of the *matK* Coding Region, the Two Intergenic Spacers *trnT–trnL* and *trnL–trnF*, and the *trnL* Intron of cpDNA

	Position (5′–3′)*	Sequence (5′–3′)	Reference
matK			
AF	3604–3580	CTATATCCACTTATCTTTCAGGAGT	Ooi *et al.* (1995)
FF	3282–3258	AATCTTATTATTTACGATCAATTCA	This study
KF	2985–2961	CCACTTTCGTCTTTCTACGGAAGGA	This study
DF	2697–2672	GGATCCATATAAACCAATTATCCAA	This study
UR	3147–3171	CAATAGCGAAGAGTTTGAACCAACA	This study
JR	2835–2859	TCCTTGAATAACCATAGGGTAACCT	This study
RR	2594–2619	TTTTCTAACATTTGGCTACGTACCA	This study
8R	2328–2351	AAAGTTCTAGCACAAGAAAGTCGA	Steele and Vilgalys (1994)
trnT–trnF (includes *trnT–trnL*, *trnL–trnF*, *trnL* intron)			
a	48546–48565	CATTACAAATGCGATGCTCT	Taberlet *et al.* (1991)
a2	48937–48960	TTTAATGGAATGGTTAGTTATAAC	This study
b	49318–49299	TCTACCGATTTCGCCATATC	Taberlet *et al.* (1991)
b2	49001–48978	TTATCTTCACTTTCGCCTGAAGGG	This study
c	49306–49325	CGAAATCGGTAGACGCTACG	Taberlet *et al.* (1991)
d	49892–49873	GGGGATAGAGGGACTTGAAC	Taberlet *et al.* (1991)
e	49862–49881	GGTTCAAGTCCCTCTATCCC	Taberlet *et al.* (1991)
f	50299–50280	ATTTGAACTGGTGACACGAG	Taberlet *et al.* (1991)

* Position indicates corresponding base pair from the first nucleotide of a large single-copy region of *Nicotiana tabacum* cpDNA (Shinozaki *et al.*, 1986).

eage relative-rate test (Wu and Li, 1985) was used to assess homogeneity of substitution rates. If a group of taxa was found to evolve at a significantly different rate, it was excluded from the set of molecular clocks and the estimation of divergence times. Because of a lack of fossil data available for the genus, we determined rates of substitutions in the cpDNA regions used in this study by comparing sequences of *rbcL*, for which substitution rate is estimated from data of a wide range of angiosperms ($2.4–2.6 \times 10^{-10}$ substitutions/site/year; Bousquet *et al.*, 1992). For the comparisons and appropriate estimations of rate differences among regions, we additionally determined *rbcL* of four species of *Lycium* (*L. andersonii, L. chinense, L. pallidum*, and *L. sandwicense*) and *N. albescens* (Accession Nos. AB051021–AB051025). *RbcL* of *L. cestroides* (Accession No. U08613; Olmstead and Sweere, 1994) was also used in the comparisons. The experimental procedures were the same as those for other regions determined in this study. Primers described by Zurawski *et al.* (1981), (1986) were used for amplification and sequencing of *rbcL*.

RESULTS

The length of the total sequence of the *matK* gene, the two intergenic spacers *trnT–trnL* and *trnL–trnF*, and the *trnL* intron is approximately 2700 bp, including 10 indels (4 in the *matK* gene, 3 in the *trnL* intron, 2 in the *trnT–trnL* spacer, and 1 in the *trnL–trnF* spacer). The length of the *matK* gene sequence varies from 1171 to 1190 bp, with 26 of the 29 sequences being 1180 bp long. In the noncoding regions, the *trnL* intron varies in length from 513 to 518 bp, the *trnT–trnL* spacer varies from 604 to 622, and the *trnL–trnF* spacer varies from 380 to 386. The sequences determined in this study have been submitted to DDBJ, EMBL, and GenBank nucleotide sequence databases under the following Accession Nos.: *matK*, AB036620–AB036648; *trnT–trnL*, AB036533–AB036561; *trnL* intron, AB036562–AB036590; *trnL–trnF*, AB036591–AB036619.

The MP analyses performed were based upon the combined sequence data of the four regions either excluding or including indels. In the analysis excluding the indels, the number of variable sites was 140, 39 of which were parsimony informative. Analysis of the 30 samples with equal character weighting generated four most parsimonious solutions of 145 steps on a single island, with a consistency index (CI) excluding autapomorphies of 0.97 and a retention index (RI) of 0.96. In the analysis including the indels, 3 of 10 indels identified from aligned sequences were informative characters. Each of the two most parsimonious trees was 161 steps long, with a CI excluding autapomorphies of 0.94 and a RI of 0.94. A strict consensus tree including indels is presented in Fig. 2. Trees based on data sets with indels have two monophyletic groups that do not appear in trees excluding indels. One consists of *L. americanum* Jacq., *L. carolinianum* Walt. var. *carolinianum*, *L. carolinianum* var. *quadrifidum* (Moç. & Sessé ex Dunal) C. L. Hitchcock, *L. elongatum* Miers,

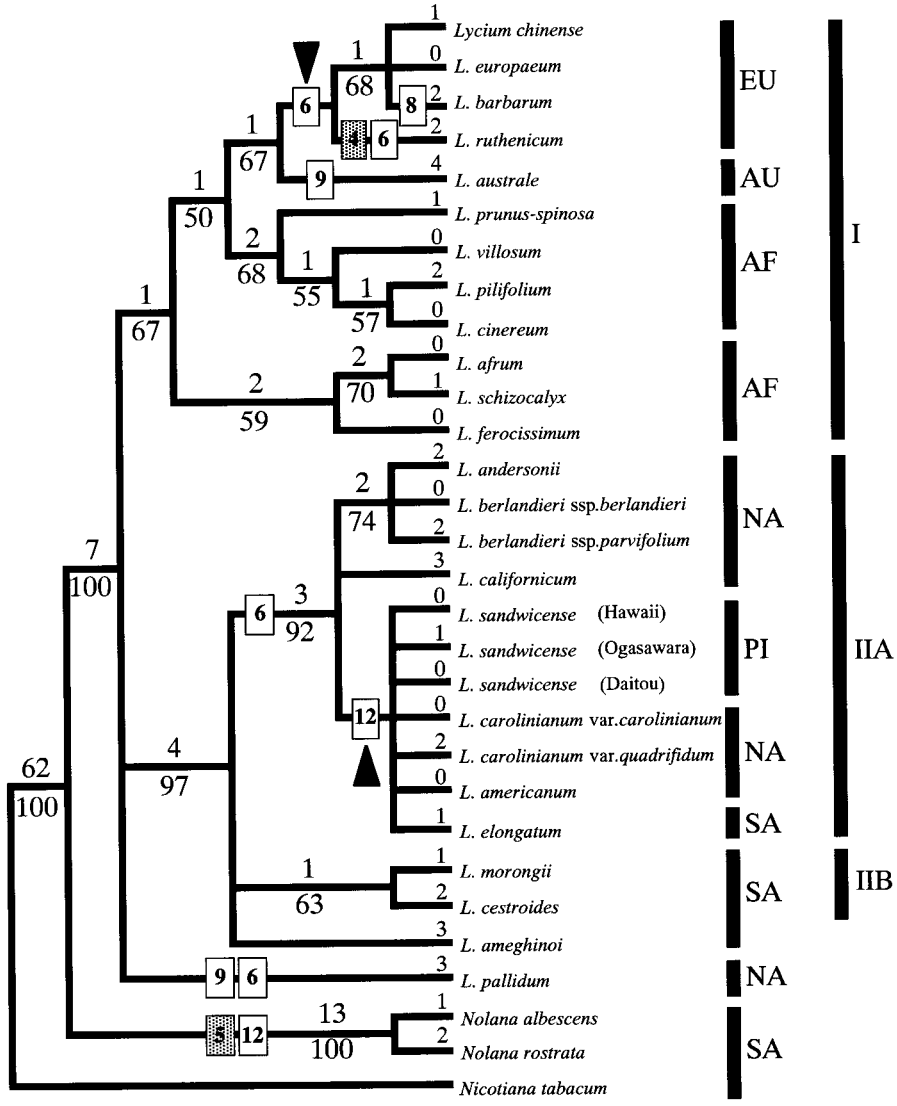

FIG. 2. A strict consensus tree of the two most parsimonious trees resulting from a phylogenetic analysis of the genus *Lycium* based on the indels and site changes in the *matK* coding region, the two intergenic spacers *trnT–trnL* and *trnL–trnF*, and the *trnL* intron of cpDNA. Branch lengths are indicated above branches; numbers below branches are bootstrap values. Numbers in white boxes represent deletions and numbers in shaded boxes indicate insertions. Arrowheads indicate the clades collapsing in the tree excluding indels. Distribution areas of each species are indicated as follows: EU, Eurasia; AU, Australia; AF, southern Africa; NA, North America; SA, South America; PI, Pacific Islands. Clades are indicated by I, IIA, and IIB.

and all samples of *L. sandwicense* A. Gray, all of which have a 12-bp deletion in the *trnT–trnL* spacer. The other comprises *L. chinese* Mill, *L. europaeum* L., *L. barbarum* L., and *L. ruthenicum* Murr., all of which have a 6-bp deletion in the *trnL–trnF* spacer as the only synapomorphy. Except for this portion, the tree topology is the same as that of trees excluding indels.

For ML analyses, we calculated Ts/Tv ratios appropriate for *Lycium* sequences. We obtained a ratio of 1.4 for *matK* and a ratio of 1.2 for noncoding regions, with 435 pairwise comparisons of samples used in this study. We conducted ML analyses with both ratios (hereafter we call ML analysis A for the Ts/Tv ratio of 1.4 and ML analysis B for the Ts/Tv ratio of 1.2). All indels were counted as missing. In the ML analysis A, each random sequence addition search produced 4846 to 16,554 different trees, and the log-likelihood of the best ML tree is −4600.76. In the ML analysis B, 4754 to 11242 trees were produced, and the log-likelihood of the best ML tree is −4599.88. The ML trees with the best log-likelihood from both analyses have the same topology (Fig. 3). This tree also has essentially the same topology as those of the MP trees.

Each of two strict consensus trees from the MP analyses has a trichotomy at the base of the ingroups, and we call the two major clades Clade I and Clade II. Both

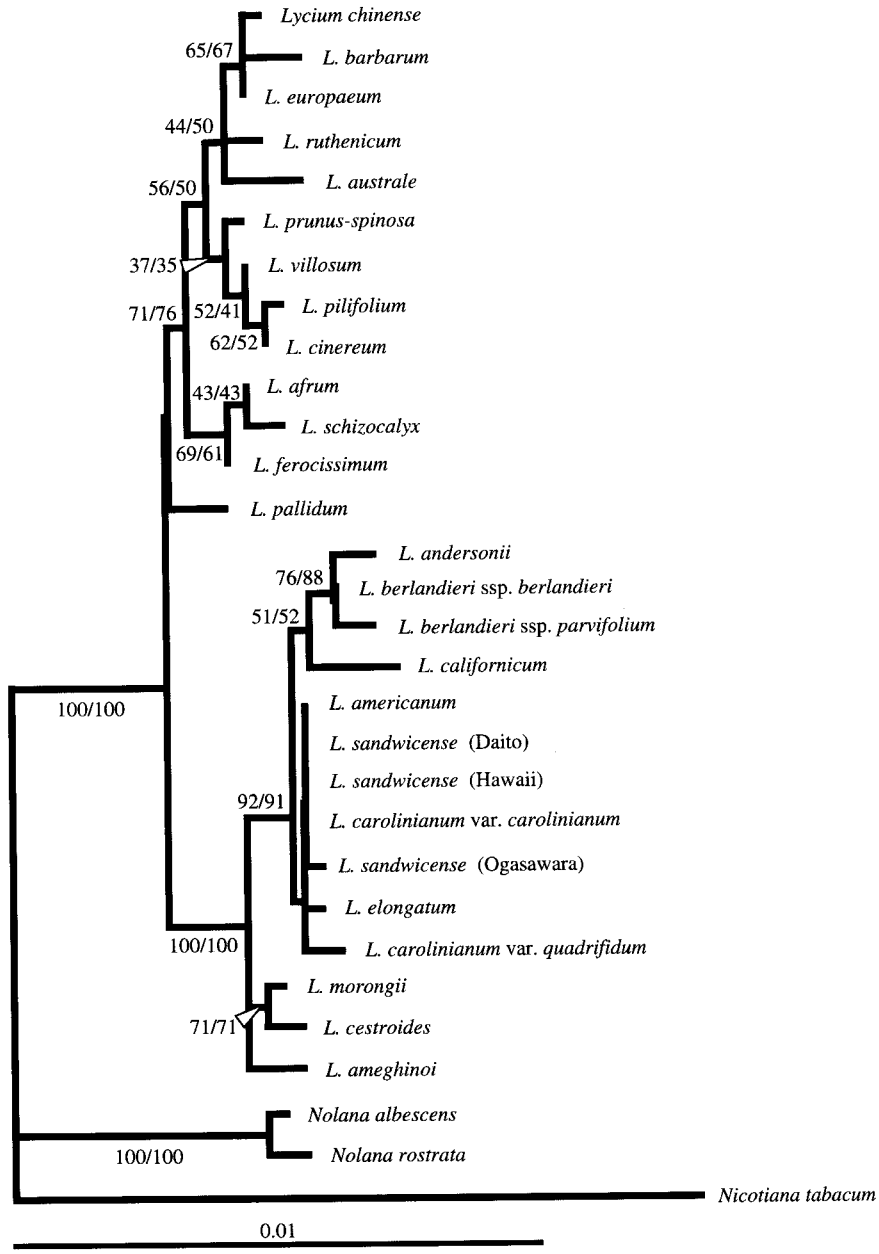

FIG. 3. The best maximum-likelihood tree with a log-likelihood of −4600.76 obtained from the ML analysis A. The tree topology was completely the same as that of the ML analysis B (log-likelihood = −4599.88). Bootstrap values are indicated for the nodes with significant branch length by a crude likelihood ratio test. For each indication of bootstrap value, the first value is from the ML analysis A and the second value is from the ML analysis B.

clades were also supported by the ML analyses. Clade I consists of all Eurasian, southern African, and Australian species. Some southern African species (*L. afrum* L., *L. ferocissimum* Miers, and *L. schizocalyx* C. H. Wright) were basally placed and the others form a monophyletic clade with the Eurasian and Australian species. The results indicate a paraphyletic assemblage of southern African species. The Australian and Eurasian species are supported as monophyletic, and all Eurasian species constitute a monophyletic group, with the Australian taxon sister to this clade (Fig. 2).

Clade II consists of South American, North American, and one Pacific species (*L. sandwicense*). It has two major subclades, and here we call them Clade IIA and Clade IIB (Fig. 2). Clade IIA consists of 11 taxa including the Pacific island species. Seven samples, *L. americanum*, *L. carolinianum* var. *carolinianum*, *L. carolinianum* var. *quadrifidum*, *L. elongatum*, and *L. sandwicense* from three Pacific island groups share a 12-bp deletion in the *trnT–trnF* spacer as previously described. The results indicate therefore that the Pacific islands species, *L. sandwicense,* is closely related

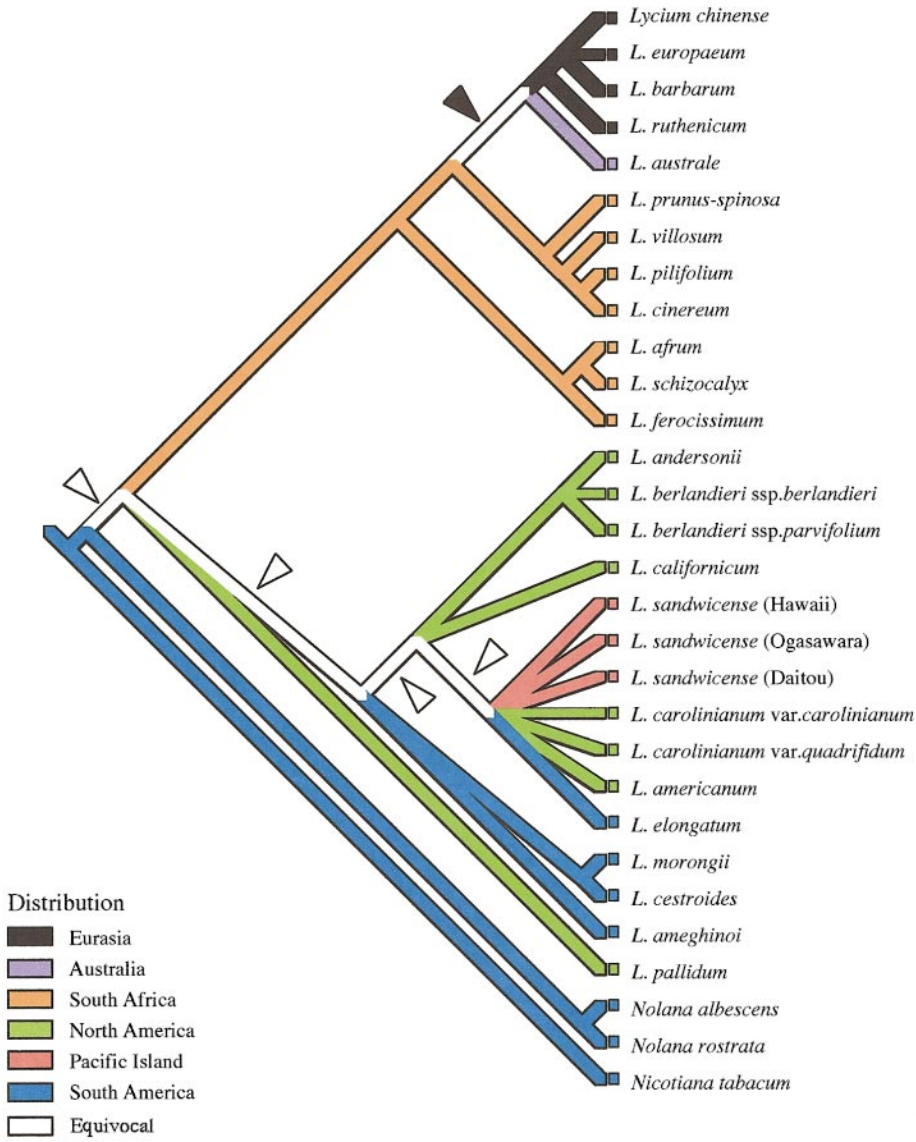

FIG. 4. An area cladgram optimized using the MacClade 3.0 software program (Maddison and Maddison, 1992). The white lines indicate equivocal (undetermined direction of character changes), distribution. The black arrow head indicates southern Africa, Australia, or Eurasia, and each of the white arrow heads indicates North or South America.

to the New World species rather than to the Eurasian, Australian, or southern African species, as suggested by Hitchcock (1932). Clade IIB consists of the South American species, *L. cestroides* and *L. morongii*. *L. ameghinoi*, which is distributed in South America, shares no substitutions or indels with species of Clade IIA or Clade IIB.

L. pallidum Miers, which is distributed in North America, shares no substitutions or indels with species of Clade I or Clade II and has a 9-bp deletion in the *matK* coding region and a 6-bp deletion in the *trnT–trnL* spacer in the sequences as autapomorphies (Fig. 2).

Figure 4 presents the strict consensus of the 24 most parsimonious reconstructions of the geographic distributions. Although we cannot determine whether the basal node of the phylogeny of *Lycium* and its outgroups is North American or South American, we suspect that the origin of *Lycium* is almost certainly in the New World. Our results also indicate that *Lycium* independently dispersed at least three times between South and North America. The southern African species are included in a monophyletic group with the Australian and Eurasian species, with the New World as the sister area. The Eurasian species are monophyletic, and Eurasia is the sister area of Australia. The southern African species arrange paraphyletically so that we propose that southern Africa is the ancestral area for Australian and Eurasian species. We cannot, however, infer the routes of dispersal from southern

TABLE 3

Comparison of the Rate of Substitution in the *matK* Gene, the Intergenic Spacers *trnT–trnL* and *trnL–trnF*, and the *trnL* Intron in *Lycium*

cpDNA region	Rate of substitution ($\times 10^{-10}$)	vs *rbcL* (times)
matK	2.9–3.9	1.2–1.5
trnT–trnL	3.1–6.5	1.3–2.5
trnL–trnF	2.2–3.1	0.9–1.2
trnL intron	2.2–3.4	0.9–1.3

Africa to Eurasia via Australia or to Australia via Eurasia or to Eurasia and Australia independently from our results.

To avoid stochastic deviations of estimated rates based on short sequence length, especially of the noncoding regions, and the shallow evolutionary history of the genus, we used percentage sequence divergence values between *N. tabacum* and three remaining species (*L. chinese, L. pallidum,* and *N. albescens*) for comparisons of evolutionary rates. Differences in substitution rates in *rbcL* and other regions are listed in Table 3. These values are lower than those reported in previous studies (*rbcL–matK:* Steele and Vilgalys, 1994; *rbcL*–noncoding regions: Gielly and Taberlet, 1994). Substitution rates of each region calculated by multiplying by a factor of those rate difference values are also listed in Table 3. Those rates yield a maximum age of 29.4 ± 9.7 mya for the average of all lineages for the recent common ancestor of the clades of *Lycium*. The divergence time of the Australian and Eurasian species was estimated at an average maximum age of 14.1 ± 3.2 mya and that of the monophyletic group with *L. sandwicense* is younger than 7.5 mya.

DISCUSSION

Comparisons of Nucleotide Substitutions

A comparison of average pairwise sequence divergence in the four sequence regions indicates that the *trnT–trnL* spacer has a higher rate of nucleotide substitution than those of the other three regions (Table 4). The *trnL* intron and the *trnL–trnF* spacer have about the same rate of nucleotide substitution and lower substitution rates than the *trnT–trnL* spacer and the *matK* coding region, suggesting that higher substitution rates should not always be expected in noncoding regions compared to coding regions of cpDNA.

Distinguishing autapomorphic, synapomorphic, and homoplastic substitutions in the four regions enables comparison of the quality of phylogenetic information yielded from the four regions (Table 4). The percentage of phylogenetically informative sites among the variable sites is highest in the *trnL–trnF* spacer (66.7%) and lowest in the *trnL* intron (25.0%). Moreover, the percentage of synapomorphic sites among informative sites is also highest in the *trnL–trnF* spacer (100.0%) and lowest in the *trnL* intron (33.3%). However, the *trnL–trnF* spacer has not only fewer informative sites (two sites; Table 4), but also a lower substitution rate than the *trnT–trnL* spacer in intrageneric comparisons of *Lycium* and its outgroup, *Nolana,* in the two intergenic spacers (Table 5). Although frequently used, the *trnL–trnF* spacer evolves most slowly, and thus its phylogenetic utility at the intrageneric level is questionable. The *trnT–trnL* spacer evolves most rapidly among the regions in this study (Tables 4 and 5) and should be a useful region for phylogenetic studies at lower taxonomic levels. The *matK* coding region, although evolving more slowly than the *trnT–trnL* spacer, has about the same number of synapomorphic sites as that of the intergenic spacer because it is approximately twice as long.

Reexamination of the Intrageneric Taxonomic Treatment of Lycium

Taxonomic studies of *Lycium* have been performed chiefly on the New World species. Hitchcock (1932) classified the species in the area into three sections: sect. *Lycium*, sect. *Selidophora*, and sect. *Sclerocarpel-*

TABLE 4

Comparison of Sequence Divergence and Phylogenetic Information from Variable Sites among the *matK* Coding Region, the Two Intergenic Spacers *trnT–trnL* and *trnL–trnF*, and the *trnL* Intron in *Lycium*

cpDNA region	Percentage sequence divergence *	Number of variable sites	Number of informative sites	Percentage informative sites **	Number of homoplastic sites	Number of synapomorphic sites	Percentage synapomorphic sites ***
matK	0.37	29	10	34.5	3	7	70.0
trnT–trnL	0.48	13	7	53.8	1	6	85.7
trnL–trnF	0.20	3	2	66.7	0	2	100.0
trnL intron	0.25	12	3	25.0	2	1	33.3

* Average of percentage pairwise sequence divergence estimated using the Jukes–Cantor model.
** Percentage of phylogenetically informative sites among the total number of variable sites.
*** Percentage of synapomorphic sites among informative sites.

TABLE 5

Average Percentage Sequence Divergence of the *matK* Coding Region, the Two Intergenic Spacers *trnT–trnL* and *trnL–trnF*, and the *trnL* Intron between and within Clade I, Clade II, and Outgroup Genus, *Nolana*

Sequence	Between *Nolana* and *Lycium*	Between Clade I and Clade II	Within Clade I	Within Clade II
matK	1.03	0.49	0.22	0.30
trnT–trnL	1.94	0.78	0.31	0.12
trnL–trnF	0.44	0.37	0.14	0.00
trnL intron	0.73	0.34	0.12	0.15

lum. Among them, sect. *Sclerocarpellum,* which consists of only two species (*L. ameghinoi* and *L. californicum*), is characterized by filaments that are neither enlarged nor glandular at the base and has a unicarpellate ovary. The former character has been considered plesiomorphic because most species of *Lycium* have the same character state. However, the latter is apomorphic because it is a unique character of sect. *Sclerocarpellum*. Our analyses indicate that the section is polyphyletic (Fig. 2). Therefore, we suspect that the ovary characters used to recognize the section are the result of convergence. Bernardello (1987) recognized four sections in South America; sect. *Lycium*, sect. *Mesocope,* sect. *Schistcalyx,* and sect. *Sclerocarpellum*. In his system, sect. *Lycium,* which consists of seven species, is characterized by a combination of the following characters; calyx without sclereids; subulate, cylindrical filaments; and an inconspicuous green basal nectary that does not protrude from the ovary wall. Although it is possible that the nectary character is apomorphic, all the others have been considered plesiomorphic because other sections also have the same character states. Our analyses indicate that three species (*L. americanum*, *L. cestroides,* and *L. elongatum*) of the seven species in the section do not form a clade, although *L. americanum* and *L. elongatum* occur as part of a polytomy within a single clade (Fig. 2). Therefore, the nectar character is also judged to be the result of convergence. Recently, Bernardello and Chiang-Cabrera (1998) presented phylogenetic reconstructions of the 51 taxa of *Lycium* in the New World based on 31 morphological characters. Results from their study are inconsistent with those presented here; for example, they suggest that *L. cestroides* is more closely related to *L. elongatum* than to *L. morongii* and that *L. andersonii* is more closely related to *L. ameghinoi* than to *L. berlandieri.* The values of the consistency index and retention index in their analyses were, however, very low (CI = 0.24, RI = 0.59), suggesting high levels of homoplasy in morphological characters.

Our results are insufficient to reconstruct the intrageneric classification of *Lycium* because of the limited number of taxa used in this study. However, it is obvious that many morphological characters in *Lycium* are the results of convergence or reversals. Further studies of both morphological and molecular characters will be necessary to revise the intrageneric classification of *Lycium*. Such studies should include the southern African, Australian, and Eurasian species because New World species do not form a monophyletic group in our results.

The Formation of the Global Distribution Pattern of Lycium

The center of diversification of *Lycium* is considered to be South America. *Grabowskia,* the sister group of *Lycium* (Hitchcock, 1932; Hunziker, 1979; Bernardello, 1987; Olmstead and Palmer, 1992), and *Nolana,* a sister group of Lycieae (Olmstead and Palmer, 1992; Olmstead and Sweere, 1994), are confined to South America. The result of the most parsimonious reconstruction of the distribution areas including outgroups in this study indicates that the common ancester of *Lycium* and the closely related genera originated in the New World (Fig. 4). Our results indicate that *Lycium* originated somewhere in the New World (whether South or North America cannot be determined) and then dispersed to southern Africa. All the Eurasian and Australian species originated from southern Africa (Fig. 5). As noted before, Symon (1991) hypothesized that the present distribution of *Lycium* was caused by drifting of the continental masses after the breakup of Gondwanaland. In contrast, Raven and Axelrod (1974) hypothesized that the distribution of *Lycium* must have attained its disjunctions by long-distance dispersal. Most present continents were nearly formed during the late Cretaceous to pre-Cenozoic (60 to 80 mya; e.g., Smith and Briden, 1977). The results of our study suggest that the maximum age of origin of *Lycium* was 29.4 ± 9.7 mya. Therefore our data do not support Symon's (1991) hypothesis. Thus, we believe that intercontinental dispersal events played significant roles in the global distribution of *Lycium,* as suggested by Raven and Axelrod (1974). Modes of seed dispersal that account for these events are discussed below.

The Formation of the Discontinuous Distribution in the New World

Neither the South nor the North American species form monophyletic groups in Clade II of our study (Fig. 2). Based on the most parsimonious reconstruction of

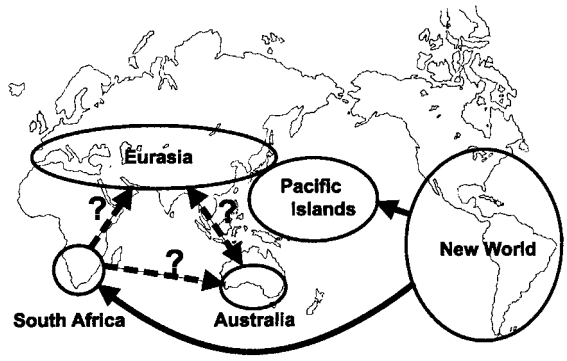

FIG. 5. The dispersal routes of *Lycium* estimated in this study.

the distribution areas, the species of *Lycium* dispersed independently at least three times between South and North America to form the present distribution of the genus (Fig. 4). Hitchcock (1932) reported that the distribution areas in South and North America were separated from each other by the tropical rain forests of the Amazon Basin. It is believed that the cycles of expansion and reduction of the rain forest approximately coincided with the glacial and interglacial climates in the Quaternary, and large areas were arid during glacial maxima (Haffer, 1969; Colinvaux, 1979; Liu and Colinvaux, 1985; Lynch, 1988). Therefore, our data, based on the phylogenetic relationships in the New World, are of particular interest for the hypothesis that the distribution of the New World species of *Lycium* were connected through the Amazonian region between South and North America during glacial times and separated into two areas during the interglacial times in the Quaternary. In Clade IIA, based on the small number of nucleotide substitutions, the estimated divergence within this clade, such as that between *L. elongatum* and *L. americanum* or *L. carolinianum* var *carolinianum*, may have mainly taken place in the Quaternary. However, Colinvaux *et al.* (1996) reported little significant forest change in the Amazon basin during the Quaternary. Moreover, the observed numbers of nucleotide substitutions between Clade IIA, Clade IIB, and *L. ameghinoi* are sufficient to estimate that these clades diverged before the Quaternary. It is, therefore, inappropriate to hypothesize that the present distribution of the New World species of *Lycium* was formed during glacial–interglacial cycles of range disruptions during the Quaternary. Instead, we believe that there is a strong possibility that the discontinuous distribution of *Lycium* in the New World was due to long-distance dispersal over the tropical rain forests of the Amazon basin during the late Tertiary.

L. sandwicense and Allied Species

Hitchcock (1932), examining the species of North and South America and the Pacific islands, believed that *L. sandwicense* (*L. carolinianum* Walt. var. *sandwicense* (Gray) C. L. Hitchcock in his treatment) was most closely related to North American *L. carolinianum* var. *quadrifidum*, due to similar morphological characters except for the smaller flower size and the absence of spines. Our results are not contradictory. A monophyletic group including *L. sandwicense* also includes both South and North American species in addition to *L. carolinianum* var. *carolinianum*, *L. carolinianum* var. *quadrifidum*, *L. americanum*, and *L. elongatum* (Figs. 2 and 3). In this monophyletic group, *L. elongatum* is morphologically distinct, with its tubular calyx and corolla, less than half-sized berry, and linear leaf; it is also distributionally distinct. *L. sandwicense* resembles *L. carolinianum* var. *carolinianum* and *L. carolinianum* var. *quadrifidum* in its reproductive and vegetative characters.

We cannot demonstrate clear relationships among these species and thus the exact origin of *L. sandwicense* cannot be determined from our study. It is obvious, however, that *L. sandwicense* of the Pacific islands originated in the New World.

Factors Contributing to Disjunction in Lycium

Birds and ocean and air currents play a role in the distribution of flowering plants over geological barriers (Carlquist, 1966, 1981; Cruden, 1966; Raven, 1972). Most flowering plants on the Pacific islands were carried by birds (Carlquist, 1967). For instance, the proportion of seed plants dispersed by birds to the Hawaiian Islands and Easter Island is estimated to be approximately 80% (Carlquist, 1974) and 70% for the Ogasawara islands (Ono and Sugawara, 1980). Species of *Lycium* have relatively small, succulent, orange, red, or blackish fruits and are probably bird-dispersed (Hitchcock, 1932; D'Arcy, 1979; Hunziker, 1979). The majority of Hawaiian plants having bird-dispersed seeds appear to have colonized from the New World (Carlquist, 1967, 1974, 1981). Flowering plants in the Hawaiian Islands having endoornithochorious seeds, such as *Cyanea* (Givnish *et al.*, 1994, 1995) and *Rubus* (Howarth *et al.*, 1997), were of North American origin based on molecular data. Our results indicate that the American species of *Lycium* overcame a barrier of at least 3900 km of open ocean to reach the Hawaiian Islands. No geological evidence exists for now vanished islands that could have served as "stepping-stones" for dispersal from North America to the Hawaiian Archipelago. The distance between North America and the Hawaiian Archipelago was even greater in the past, because North America has been gradually displaced westward by expansion of the mid-Atlantic ridge, toward the Hawaiian hot spot (Dalrymple *et al.*, 1973; Clague and Dalrymple, 1987). North American migratory bird species and accidental arrivals are regularly sighted in the Hawaiian Islands (Guppy, 1906; Carlquist, 1965; Munro *et al.*, 1989). Although most birds

could not retain seeds for long periods of time, it was reported that viable seeds could be retained in the gizzard or the intestinal tract of some migratory birds for as long as 200 to 300 h (Proctor, 1968; Vlaming and Proctor, 1968). This is long enough for a bird to cover several thousand miles. Therefore, it is possible for seeds of *Lycium* to have been dispersed from North or South America to the Hawaiian Islands (Fig. 5).

The Ogasawara Islands, in the western part of the north Pacific Ocean, are approximately 1000 km south of Honshu, Japan (Fig. 1). The Ogasawara Islands are of Paleogene origin (Kimura, 1991) and never have been connected to any land mass. Approximately 70% of the vascular plants on the islands are hypothesized to have originated in Asia (Toyoda, 1981). Plants with endoornithochorious seeds, such as *Ilex* (Aquifoliaceae) and *Ficus* (Moraceae), are considered to have originated in eastern Asia (Ono and Sugawara, 1980; Toyoda, 1981; Setoguchi and Watanabe, 2000). In contrast, our results suggest that *L. sandwicense* from the Ogasawara Islands is closely related to the New World species. The sequences from the individual sampled from the Ogasawara Islands are nearly the same as those from the Hawaiian individual in our study. This suggests that the dispersal route of *L. sandwicense* to the Ogasawara Islands is not from Asia but from the New World or the Hawaiian Islands (Fig. 4). Although close phylogenetic relationships of flowering plants in the Ogasawara Islands and the New World have not been shown, a few related taxa are shared between the Ogasawara and the Hawaiian Islands. Yamazaki (1981) suggested that some endemic vascular plants of the Ogasawara Islands, such as *Psychotria homalosperma* A. Gray, *Hedyotis grayi* Benth., and *H. mexicana* (Hook. et Arn.) Hatus. (Rubiaceae), and *Boninia glabra* Planch. and *B. grisea* Planch. (Rutaceae), are closely related to Hawaiian species based on morphological data. Knox *et al.* (1993) also demonstrated the monophyly of *Lobelia boninensis* Koidz. (Campanulaceae) of the Ogasawara Islands and its allied species, *L. hypoleuca* Hillebr and *L. gloria-montis* Rock., of the Hawaiian Islands based upon molecular data. From these results, it is probable that *L. sandwicense* of the Ogasawara Islands originated in the Hawaiian Islands rather than in the New World (Fig. 5).

The Daitou Islands are approximately 370 km east of the Ryukyu Islands, southwest of Honshu, Japan (Fig. 1). Many of the flowering plants on the Daitou Islands are closely related to plants on the Ryukyu Islands (Hatusima, 1981). However, *L. sandwicense* has almost the same nucleotide sequence as plants from the Hawaiian Islands and the Ogasawara Islands. This is the first result to demonstrate that plants on the Daitou Islands are closely related to those in the Hawaiian Islands and the New World. *L. sandwicense* in the Daitou Islands probably originated in the Ogasawara Islands, the Hawaiian Islands, or the New World (Fig. 5).

In summary, we provide a robust phylogenetic hypothesis for species of *Lycium* by analyzing the combined cpDNA data. This hypothesis, based on molecular information, contributes an unbiased interpretation of biogeographic history. It will be interesting to see whether more comprehensive sampling and additional genetic evidence (i.e., nuclear sequences such as ITS) will support the working hypothesis developed in this study. The phylogenetic basis to stimulate further studies of *Lycium* has been established.

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