LINEAGE SORTING AND PHYLOGEOGRAPHY IN *LITHOCARPUS FORMOSANUS* AND *L. DODONAEIFOLIUS* (FAGACEAE) FROM TAIWAN[1]

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Abstract

Gene genealogy of the cpDNA *atp*B-*rbc*L noncoding spacer was reconstructed to assess the phylogeographic pattern of two closely related oaks, *Lithocarpus formosanus* (Skan) Hayata and *L. dodonaeifolius* (Hayata) Hayata (Fagaceae). High levels of nucleotide and haplotype diversities but low levels of genetic differentiation between species and among populations were detected. The result is consistent with the paraphyly of this cpDNA spacer in both species suggested by a neighbor-joining analysis. A minimum spanning network of the cpDNA haplotypes identified two major clades, A and A′ (consisting of clades of B, C, and D). No clades were confined to either species or single populations. Clades B and C did not occur in two smaller populations of *L. dodonaeifolius*. The apportionment of haplotypes between species and populations indicated a lineage sorting of the cpDNA noncoding spacer. On the contrary, RAPD fingerprints revealed limited ongoing gene flow and significant genetic differentiation between species and among populations. Given low possibilities that seeds disperse across a long geographic range in the modern vegetation, high *Nm* values, estimates of number of migrants per generation deduced from the seed-carried organelle DNA marker are likely to represent historical migrations. A migrant-pool model explains the heterogeneous composition of the organelle DNA within populations and the low differentiation among populations. According to geological evidence, during the deglaciation period common ancestral populations were possibly forced to migrate into refugia at local peaks. Invading and adapting to habitats of different elevations, two oaks flower with a lag interval of about half a month, which may have triggered the reproductive isolation and speciation. Ancestral polymorphic alleles, however, prolonged the lineage sorting period within populations and species. Given a relatively short evolutionary duration since isolation, high genetic heterogeneity has made the attainment of coalescence improbable.

Key words: Fagaceae, lineage sorting, *Lithocarpus,* phylogeography, Taiwan.

One of the recent developments in plant evolutionary study is the application of phylogeography to the analysis of evolutionary events (cf. Avise, 1999). Phylogeography uses gene genealogies, which trace phylogenetic relationships among alleles within or among species, in a geographical context (Schaal, 2000). In the past, phylogeographic patterns of many taxa that evolved through glacial cycles or vicariance events have been well demonstrated based on genealogical information of organelle DNA (e.g., *Pinus,* Latta & Mitton, 1997; *Abies,* Tsumura & Suyama, 1998; *Cycas,* Huang et al., 2001; *Fagus,* Demesure et al., 1996; *Quercus,* Petit et al., 1997; *Alnus,* King & Ferris, 1998; *Kandelia,* Chiang et al., 2001; and *Beta,* Desplanque et al., 2000).

High levels of genetic variation were usually detected in these species, in part due to their long evolutionary history. However, the distribution of genetic diversity varies widely between plant species. In contrast to the distinctness between species descending from remote common ancestors (e.g., *Quercus* sects. *Lobatae* and *Cerris,* Manos et al., 1999), low genetic differentiation of organelle DNAs between closely related species has been frequently encountered (e.g., *Ipomopsis,* Wolf et al., 1997; *Quercus,* Dumolin-Lapègue et al., 1999; *Abies,* Tsumura & Suyama, 1998). Lineage sorting, as a result of a short period for coalescence, contributed to the low level of differentiation (Chiang, 2000). Nevertheless, Manos et al. (1999) showed that monophyly of organelle DNA haplotypes could be attained in some recently evolving species. In their study, cpDNA of *Quercus tomentella* (sect. *Protobalanus*) was proved monophyletic, while its most related con-sectional species *Q. cedrosensis,* as well as most other species, remained paraphyletic. Processes such as fluctuation in population size, gene migration, and selection affect the genealogical relationships among haplotypes (Schaal,

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2000). In general, coalescence of neutral alleles (such as noncoding spacers) of maternally inherited organelle DNAs (Rebound & Zeyl, 1994) is regulated by duration of isolation, population number and size, and other evolutionary agents, such as migration (cf. Hoelzer et al., 1998).

In contrast to the research frequently carried out in species of continents or oceanic islands (e.g., Hawaii, Stuessy & Ono, 1998), less attention has been paid to plants of continental islands (e.g., *Abies,* Tsumura & Suyama, 1998; *Cunninghamia,* Lu et al., 2001), which had been a part of the adjacent mainland before geographical separation. Compared to taxa of European and American continents, the distribution of most plants on a continental island such as Taiwan is constrained due to its small area and rugged landscape. In addition, in the last decades, many species of lowland areas have been under threat because of habitat destruction, a result of human economic activities. The effect of random genetic drift could be a more important factor in those island plants with reduced population sizes. Then, low levels of genetic variation within species would be expected.

To understand phylogeographic patterns and the gene genealogy of species of continental islands, oaks provide ideal material. A great number of data are available for species of continents of Europe and North America (Whittemore & Schaal, 1991; Manos et al., 1999), and the fagaceous plants share similar breeding systems and demography. In Taiwan, *Lithocarpus* species are highly diverged, with 14 species recognized (Yang et al., 1997). Like many endemic species that survived glacial cycles on the island, a large number of relictual *Lithocarpus* species, such as *L. castanopsisifolius* (Hayata) Hayata and *L. konishii* (Hayata) Hayata, are sporadically distributed with limited population sizes, partly due to biogeographic history and recent human disturbance (Lin, 1966). The taxonomy of Taiwan's oaks has been well documented (Liao, 1996; Huang et al., 1999). Some species complexes have long puzzled taxonomists, including *Lithocarpus formosanus* (Hayata) Hayata and *L. dodonaeifolius* (Hayata) Hayata. Kudô (1931: 387) recognized the latter within *L. formosanus* (as *Synaedrys* [: *Lithocarpus*] *formosana* fo. *dododaeifolia*), and Li (1953) also suggested a conspecific relationship. In contrast, recent taxonomic treatments (Liao, 1996; Yang et al., 1997) recognized two separate species.

Trees of 4–9 m in height from both taxa in *Lithocarpus* share entire leaf margins and a rounded leaf apex. The oblanceolate leaf shape of *Lithocarpus dodonaeifolius* is distinct from the elliptic leaf shape of *L. formosanus.* In addition, shorter petioles (4–8 mm), longer infructescence (3–5 cm), and tawny tomentose cupule bracts characterize *L. dodonaeifolius,* while longer petioles (10–13 mm), shorter infructescence (ca. 3 cm), and gray tomentose cupule bracts occur in *L. formosanus. Lithocarpus formosanus* and *L. dodonaeifolius* are allopatrically distributed in southern Taiwan about 30 km apart. A single extant population of *L. formosanus,* consisting of no more than 100 plants, remains in the wild, although several scattered populations were previously recorded (cf. Lu, 1996). This population is distributed along the Nanjen Stream in the Kengting National Park. Two subpopulations of *L. formosanus* occurring along ridges about 400 m alt. are separated by the Nanjen stream. In contrast, three populations of *L. dodonaeifolius* are distributed along the Central Mountain Range of the Taiwanese island: Mt. Weiliaoshan (with about 100 individuals, ca. 1200 m alt.), Chingshuiying (with about 200 individuals, ca. 1500 m alt.), and Dazen (with 9 individuals, ca. 600 m alt.). These three populations of *L. dodonaeifolius* are isolated by distances between 20 km and 60 km. Ecologically, both species usually grow on wind-facing slopes, mixing with other species of Fagaceae and Lauraceae in tropical or subtropical forests.

Organelle DNA is maternally inherited in oaks (Dumolin et al., 1995) and can only migrate across populations via seed dispersal. An "isolation by distance" model would be expected in oaks, whose seed dispersal is constrained by the migratory capability of the seed carriers. In addition, as a result of stochastic drift, coalescence of cpDNA alleles in populations with small size can be possibly reached. Nevertheless, both ongoing gene flow and historical migratory events have influenced the amount of genetic variation between and within species/populations. In this study, we looked into the stage of lineage sorting of the chloroplast DNA locus at a noncoding spacer located between *atp*B and *rbc*L genes between and within the two endemic *Lithocarpus* species. One population is limited in both population size and number, while the other possesses relatively healthy population structure. Based on genealogical relationships among alleles of the cpDNA locus, the phylogeographic pattern of the Taiwan's *Lithocarpus* species was reconstructed.

In order to determine the level of ongoing gene flow between populations, RAPD fingerprints, which are mostly amplified from the nuclear genome (Hawkins & Harris, 1998), were utilized to assess the extent of migration. In the study, several objectives are pursued: (1) the partitioning pattern of cpDNA variation within and between species; (2)

the possible migratory mode of Taiwan's oaks over the geological history; (3) the coalescence process of cpDNA alleles within species and populations; and (4) the level of ongoing gene flow between populations.

Materials and Methods

population samples

A single population of *Lithocarpus formosanus* and three populations of *L. dodonaeifolius* were surveyed (Table 1). Geographically, these populations are 20–95 km apart. Except for the complete sampling in the Dazen population of nine, about 10% of wild individuals were sampled randomly (Table 1). Both species of *Lithocarpus* grow on the wind-facing slopes. Young and healthy leaves were collected in the field, rinsed with tap water and dried in silica gel. Voucher specimens were deposited at NCKU at Cheng-Kung University. All samples were stored at −70°C until they were processed.

DNA extraction, PCR, and nucleotide sequencing

Genomic DNA was extracted from *Lithocarpus* leaf tissues following the CTAB procedure (Murray & Thompson, 1980). PCR amplification was carried out in a 100 μL reaction using 10 ng of template DNA, 10 μl of 10× reaction buffer, 10 μl $MgCl_2$ (25 mM), 10 μl dNTP mix (8 mM), 10 pmole of each primer, 10 μl of 10% NP-40, and 2 U of *Taq* polymerase (Promega, Madison, Wisconsin, U.S.A.). The reaction was programmed on an MJ Thermal Cycler (PTC 100) as one cycle of denaturation at 95°C for 4 min., 30 cycles of 45 sec denaturation at 92°C, 1 min. 15 sec. annealing at 52°C, and 1 min. 30 sec. extension at 72°C, followed by 10 min. extension at 72°C. Template DNA was denatured for 4 min. (first cycle), and cooled on ice immediately. A pair of universal primers for cpDNA *atp*B-*rbc*L spacer (Chiang et al., 1998), dNTP, and *Taq* polymerase were added to the above ice-cold mix. Reaction was restarted at the first annealing at 52°C.

PCR products were purified by electrophoresis in 1.0% agarose gel using 1 × TAE buffer. The gel was stained with ethidium bromide, and the desired DNA band was cut and eluted using agarose gel purification (QIAGEN). Eluted PCR products were directly sequenced in both directions by standard methods of the *Taq* dye deoxy terminator cycle sequencing kit (Perkin Elmer) on an Applied Biosystems Model 377A automated sequencer (Applied Biosystems).

sequence alignments and phylogenetic analyses

Nucleotide sequences of *atp*B-*rbc*L noncoding spacer of cpDNA were registered with EMBL (Table 1). Nucleotide sequences were aligned with the program Genetics Computer Group (GCG) Wisconsin Package (Version 10.0, Madison, Wisconsin) and later adjusted visually. Neighbor-joining (NJ) analysis, calculating Kimura's (1980) two-parameter distance and excluding the gaps, was performed using software MEGA (Kumar et al., 1993). Confidence of each reconstructed clade was tested by bootstrapping (Felsenstein, 1985) with 1000 replicates using unweighted characters. The nodes with bootstrap values greater than 0.70, as a rule of thumb, are significantly supported with ≥ 95% probability (Hillis & Bull, 1993). The number of mutations between haplotypes in pairwise comparisons, calculated using "number of differences" implemented in MEGA (Kumar et al., 1993), was used to construct a minimum spanning network with the aid of the MINSPNET (Excoffier & Smouse, 1994) in an hierarchical manner (Chiang & Schaal, 1999). After linking the related haplotypes into a clade, closely related clades were linked further to form higher level groups and thereby a network.

RAPD fingerprint analysis

We used the polymerase chain reaction (PCR) with arbitrary primers for obtaining RAPDs following Williams et al. (1990). We used 5 ng of DNA template, 0.3 μM primer (Operon Technologies, Alameda, California), 1 unit of Taq polymerase (Promega), 2.5 μL of PCR buffer, 2.5 mM $MgCl_2$, and 100 μM of each dNTP for each 25 μL PCR reaction, respectively.

PCR amplification took place in a PTC-100 thermal cycler (MJ Research Inc.) programmed for an initial 3 min. denaturation at 94°C, 35 cycles of 20 sec. at 94°C, 30 sec. at 36°C, and 90 sec. at 72°C, followed by a 10-min. final extension at 72°C. Forty 10-base oligonucleotide primers were screened from the Operon (Alameda, California) K and N series (OPK-01~OPK-20; OPN-01~OPN-20). RAPD products were electrophoresed on 2% Nusieve 3:1 agarose gels, stained by ethidium bromide, and photographed with Polaroid type 667 film.

A RAPD data matrix based on the presence and absence of loci was prepared by the aid of AMOVA-PREP (version 1.01; Miller, 1998). We used AMOVA version 1.55 (Excoffier et al., 1992) to deduce the significance of geographical divisions both between populations and between regions. The statis-

Table 1. Localities sampled within each species and population of Taiwan oaks. The number of individuals sampled and sequenced is indicated. Haplotype diversity (H), nucleotide diversity (*Dij*), and minimum recombination events (Rm) are indicated. H = the average number of sequence differences. *Dij* = the average number of nucleotide differences.

Species	Location	Coordinate	Symbol	Sample size	Haplotype diversity (H)	Nucleotide diversity (*Dij*) %	Chlorotypes	Minimum recombination events (Rm)	EMBL accession number
L. formosanus	Wanlider	120°51′E, 22°03′N	for	19	1	8.074 ± 1.149	A, B, C	4	AJ390792–AJ390810
L. dodonaeifolius				39	1	6.026 ± 0.702	A, B, C, D	15	
	Chingshuiying	120°45′E, 22°22′N	doda	19	1	7.039 ± 0.545	A, B, C, D	8	AJ252218, AJ252221–AJ252223 AJ252230–AJ252234 AJ390818–AJ390822, AJ390827 AJ390831–AJ390832 AJ390835–AJ390836
	Dazan	120°48′E, 22°16′N	dodb	9	1	4.560 ± 2.224	A, D	5	AJ252217, AJ252227–AJ252229 AJ390829–AJ390830 AJ390833–AJ390834, AJ390837
	Weiliaoshan	120°40′E, 22°52′N	dodc	11	1	4.554 ± 1.690	A, D	8	AJ252216, AJ252219–AJ252220 AJ252224–AJ252226 AJ390823–AJ390826, AJ390828

tics of molecular variants ΦCT (among regions), ΦST (among populations), and ΦSC (among populations within a region) were estimated (cf. Chiang et al., 2001). The significance of these F-statistic analogues was evaluated by 1000 random permutations. The UPGMA dendrogram of populations was drawn based on pairwise dissimilarities using software TFPGA (version 1.3; Miller, 1997).

POPULATION GENETIC ANALYSIS OF THE cpDNA AND mtDNA SEQUENCE VARIATION

Levels of inter- and intra-population genetic diversity were quantified by indices of haplotype diversity (*h*) (Nei & Tajima, 1983) and pairwise estimates of nucleotide divergence (*Dij*) (Jukes & Cantor, 1964) using DnaSP (Version 3.0, Rozas & Rozas, 1999). Patterns of geographical subdivision and gene flow were also estimated hierarchically with the aid of DnaSP. Recombination and gene conversion events were detected using DnaSP. Gene flow within and among regions (populations) was approximated as *Nm*, the number of female migrants per generation between populations. It was estimated using the expression $F_{ST} = 1/(1 + 2\,Nm)$, where N is the female elective population size and *m* is the female migration rate (Slatkin, 1993).

The pattern of "isolation by distance" was assessed in both cpDNA and RAPD data by plotting pairwise genetic distance (for RAPD) or log (*Nm*) values (for cpDNA) against log (geographical distance) (cf. Slatkin, 1993; Forcioli et al., 1998; Stauffer et al., 1999). The significance of the association between *Nm* and geographical distance was determined by a regression F test using the SPSS program (Norusis, 1994).

RESULTS

HAPLOTYPES, NUCLEOTIDE DIVERSITY, AND cpDNA PHYLOGENY OF *LITHOCARPUS FORMOSANUS* AND *L. DODON*

In this study, the *atp*B-*rbc*L noncoding spacer of cpDNA in *Lithocarpus formosanus* and *L. dodonaeifolius* were PCR amplified and sequenced. Length of the *atp*B-*rbc*L spacer of cpDNA varied from 730 bp (isolate *doda36*) to 935 bp (isolate *for4005*). A total of 996 bp were aligned (alignment available from authors on request), of which 201 sites (20.2%) excluding the sites with alignment gaps were polymorphic. This noncoding spacer was A+T rich (68.8%). All sequences were unique. Nineteen haplotypes ($h = 1.0$) and 39 haplotypes ($h = 1.0$) of the cpDNA noncoding spacer were determined in *L. formosanus* and *L. dodonaeifolius*, respectively, according to the DnaSP analyses. Nucleotide diversity (Jukes & Cantor, 1964) of 0.08074 ± 0.01149 and 0.06026 ± 0.00702 was estimated in the above species, respectively. In the largest population (Chingshuiying) of *L. dodonaeifolius*, nucleotide diversity as measured by pairwise estimates ($Dij = 0.07039 \pm 0.00545$) of the cpDNA was higher than that of those smaller populations ($Dij = 0.04560 \pm 0.02224$ for Dazen and $Dij = 0.04554 \pm 0.01690$ for Weiliaoshan) (Table 1).

A neighbor-joining (NJ) tree was reconstructed based on the genetic distance among aligned sequences of the cpDNA. Four groups (A–D) were identified and supported by bootstrap significantly (Fig. 1). Clades B and C were grouped further, although not significantly (bootstrap value = 0.52). The monophyly of the cpDNA noncoding spacer in either *L. formosanus* or *L. dodonaeifolius* was not suggested by the NJ analysis. Nevertheless, clade D consisted of individuals of *L. dodonaeifolius* exclusively (Table 1). Within clade A, most sequences of *L. formosanus* and *L. dodonaeifolius* were mixed, except for a subcluster A^+, which comprised sequences of the latter species only. Within the clade B, an unusually long branch leading to the *for1502* (*L. formosanus*) was identified, which differed by 49 mutational changes from its closest sequence of *for1501* (*L. formosanus*) (Fig. 2).

GENETIC DIFFERENTIATION BETWEEN *LITHOCARPUS FORMOSANUS* AND *L. DODONAEIFOLIUS*

A minimum spanning network was constructed based on mutational changes between sequences (Fig. 2). Paraphyly of the cpDNA noncoding spacer in both species agrees with the conventional cladistic analysis. Two major lineages with 98 mutational changes were determined, i.e., A and A′. Within clade A, several ancestral haplotypes nested in the network as interior nodes were identified: isolates *for203* representing *Lithocarpus formosanus*, and *doda20*, *doda1715*, *dodb008*, *dodb009*, *dodb004* for *L. dodonaeifolius*, indicating shared ancestral polymorphisms and nearly equal age of the two species. Clade B is linked to *doda20* (*L. dodonaeifolius*) via *for3903* (*L. formosanus*). Within the second clade A′, clade B is linked to clade C with 25 mutational changes, and clade D is linked to clade C with 20 mutational changes.

Low genetic differentiation of the cpDNA locus was detected between *Lithocarpus formosanus* and *L. dodonaeifolius*. An *Nm* of 43.75 and an FST of 0.00575 were deduced. Pairwise comparisons be-

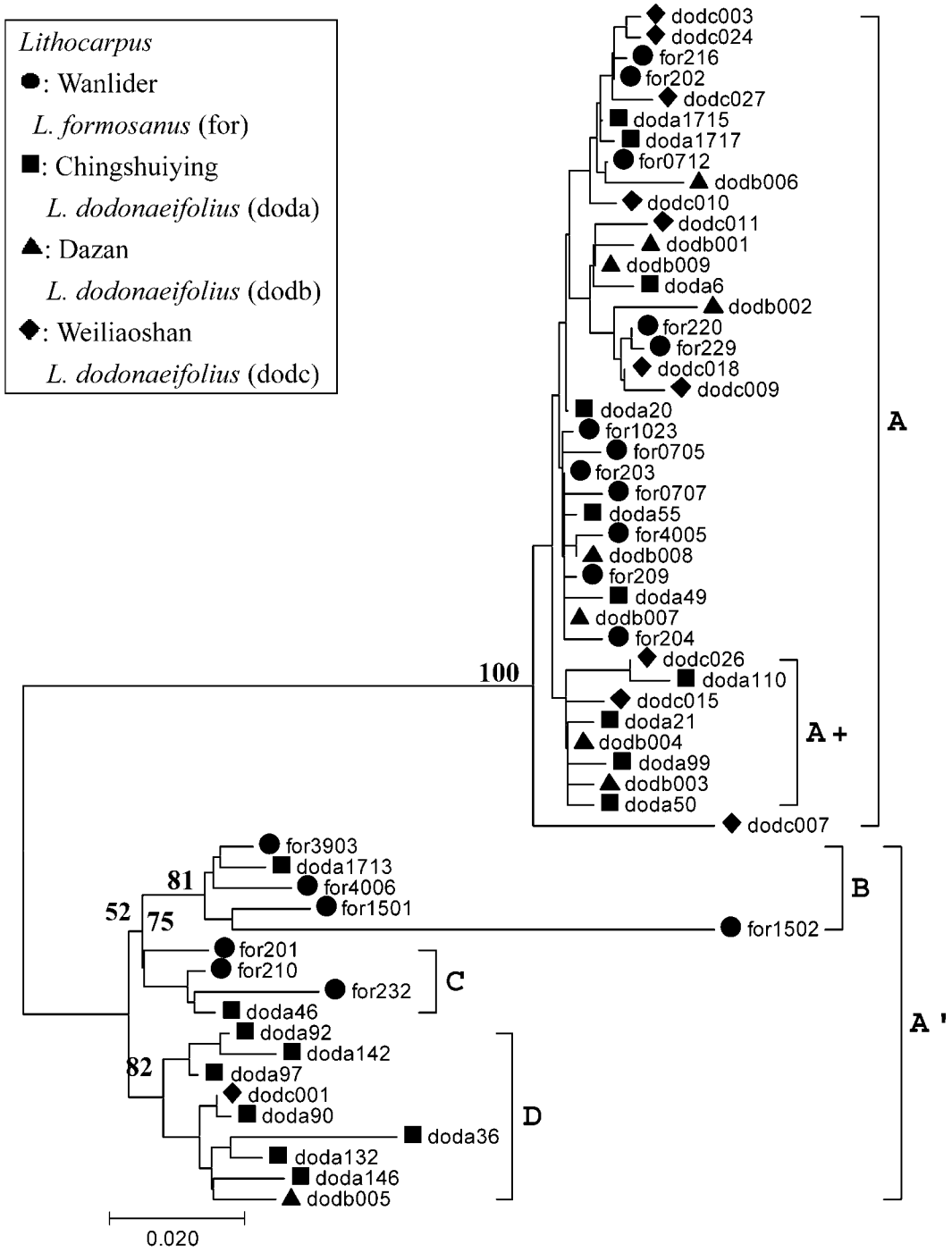

Figure 1. Neighbor-joining (NJ) tree of cpDNA of *Lithocarpus formosanus* and *L. dodonaeifolius*. Numbers in bold at nodes indicate bootstrap values. Four clades (A–D) and subtype A^+ are indicated.

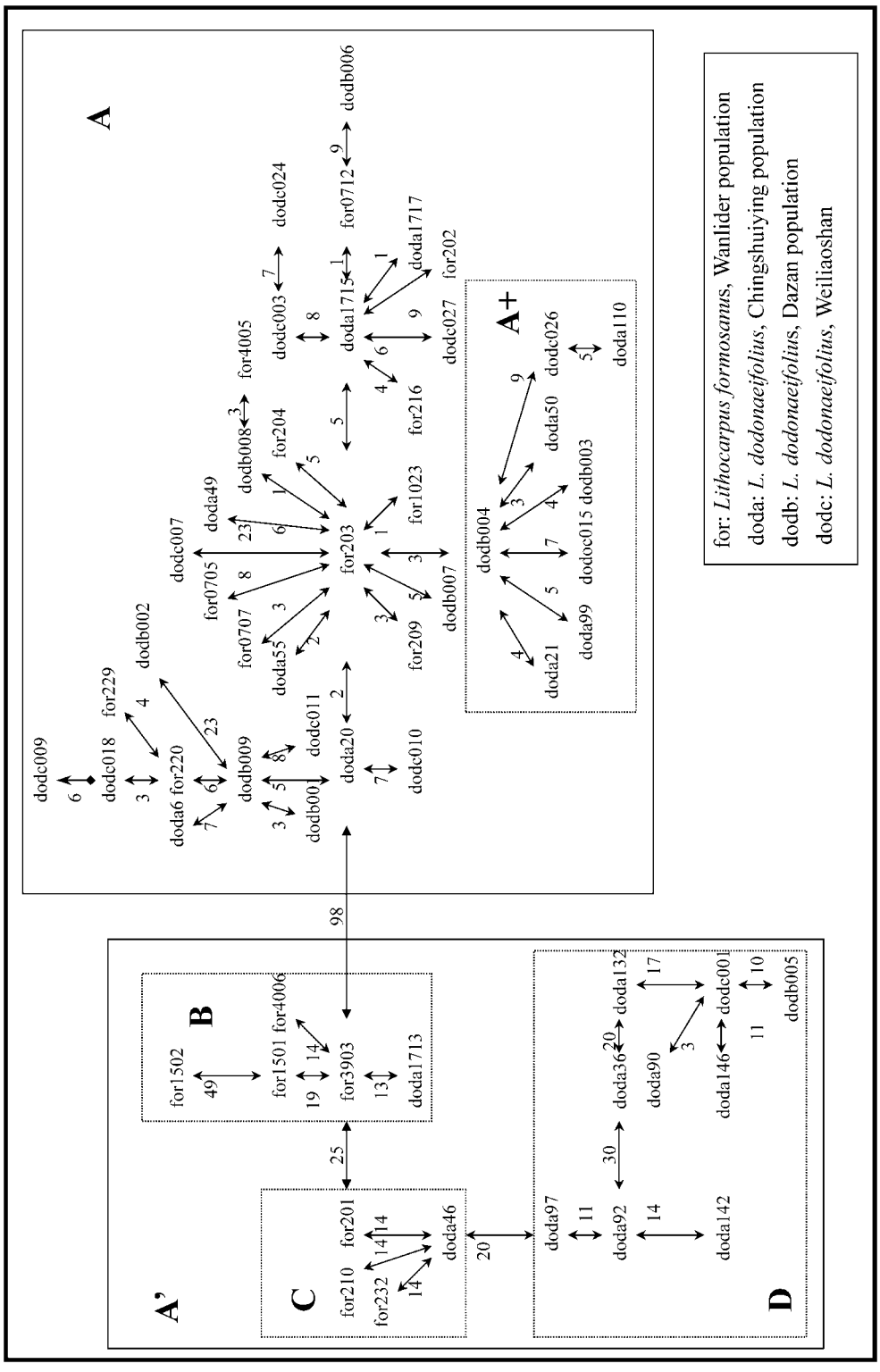

Figure 2. Minimum spanning network for two *Lithocarpus* oaks from Taiwan based on the mutational changes between cpDNA haplotypes. Clades A and A′ (consisting of B–D) were indicated. Numbers at the nodes indicate the steps of mutations.

Table 2. Pairwise *Nm* (above the diagonal) and Fst (below the diagonal) among populations deduced from chloroplast DNA sequences. For symbols for populations see Table 1. *Nm* refers to the number of female migrants per generation between populations. Fst = 1/(1+2*Nm*), where N = female effective population size, m = female migrant rate.

	L. formosanus	*L. dodonaeifolius*			
		for	doda	dodb	dodc
L. formosanus			43.74	126.89	3.14	2.34
for						
L. dodonaeifolius	0.00575					
doda		0.00197			1.49	1.22
dodb		0.07365		0.1439		4.65
dodc		0.09638		0.1390	0.05679	

tween *L. formosanus* with each population of *L. dodonaeifolius* also indicated high *Nm* values, ranging from 2.34 to 126.89, and low Fst, ranging from 0.00197 to 0.09638 (Table 2). Between populations of *L. dodonaeifolius,* the deduced *Nm* ranged from 1.22 to 4.65 and the deduced Fst ranged from 0.05679 to 0.1439. Subpopulations of *L. formosanus,* which are separated by a stream, were not differentiated based on a high value of deduced *Nm* (40.50) and low Fst (0.01250).

When lineages A and A′ were considered separately, within *L. formosanus,* both lineages were differentiated significantly, with an *Nm* of 0.11 and an Fst of 0.6937 (Table 3). Low differentiation between *L. formosanus* and each population of *L. dodonaeifolius* was suggested based on the high deduced *Nm* of the clade A (with a range of 6.83–57.29). In contrast, the much lower deduced *Nm* of the lineage A′ between *L. formosanus* and populations of *L. dodonaeifolius* was obtained, with a range between 0.06 and 2.06. Within *L. dodonaeifolius,* clade A was not differentiated between populations according to the high deduced *Nm,* with a range of 13.64 to 1015.96, while significant differentiation between populations was detected in the clade A′ based on the low deduced *Nm* (0.00–0.42). In addition, the nucleotide diversity is higher as measured by pairwise estimates in the A′ clade (*Dij* = 0.04549 ± 0.02237) than in the A clade (*Dij* = 0.01637 ± 0.01335).

Based on DnaSP analysis of sequences of both species as a whole, 18 possible minimum recombination events were detected. Four and 15 minimum recombination events occurred in *L. formosanus* and *L. dodonaeifolius,* respectively. In contrast to short DNA fragments involved in the

Table 3. Pairwise *Nm* (above diagonal) and Fst (below diagonal) between species and populations with lineages A and A′ considered separately. *Nm* refers to the number of female migrants per generation between populations. Fst = 1/(1+2*Nm*), where *N* = female effective population size, *m* = female migrant rate.

	L. formosanus for		*L. dodonaeifolius* doda		dodb		dodc	
	lineage A	lineage A′	lineage A	lineage A′	lineage A	lineage A′	lineage A	lineage A′
L. formosanus								
lineage A		0.11	42.21	0.11	6.83	2.24	57.29	0.04
for								
lineage A′	0.6937		0.07	2.06	0.16	0.06	0.08	0.14
L. dodonaeifolius								
lineage A	0.0059	0.7755		0.07	38.85	1.00	13.64	0.01
doda								
lineage A′	0.7041	0.1081	0.7894		0.13	0.05	0.07	0.42
lineage A	0.0380	0.6129	0.0064	0.6549		2.30	1015.96	0.06
dodb								
Lineage A′	0.1003	0.8193	0.2000	0.8453	0.0981		1.00	0.00
Lineage A	0.0044	0.7464	0.0180	0.7728	0.0003	0.2000		0.02
dodc								
Lineage A′	0.8766	0.6351	0.9486	0.3716	0.7992	1.000	0.9126	

genetic recombination in *L. dodonaeifolius*, two large DNA fragments, i.e., fragments of (261, 563) and (831, 950), were identified in *L. formosanus* based on DnaSP analyses (Table 1).

RAPD fingerprints and gene flow

A UPGMA dendrogram was constructed based on the deduced genetic distance among individuals (Fig. 3). All individuals of *L. formosanus* were grouped together, as were those of *L. dodonaeifolius*. Individuals of the Dazen population as well as the Weiliaoshan population were clustered together, both of which were nested within the group of the Chingshuiying population. The UPGMA dendrogram (Fig. 4) based on the TFPGA analysis, which recognized each population (instead of individual) as a unit, suggested significant differentiation, with about 54.8% dissimilarity, between *L. formosanus* and *L. dodonaeifolius*. In addition, within *L. dodonaeifolius* populations from Weiliaoshan and Dazen shared highest similarity (about 85%). Based on the deduced *Nm* (1.06) and Fst (0.19149), estimating the gene flow, the subpopulations of *L. formosanus* were barely differentiated.

Discussion

Genetic variability of the noncoding spacer between *atp*B and *rbc*L genes of cpDNA in Taiwanese oaks

Lithocarpus formosanus and *L. dodonaeifolius* seemed to possess higher levels of cpDNA haplotype diversity (19 and 39 haplotypes, respectively; Table 1) than other plants, e.g., 13 cpDNA haplotypes in *Beta vulgaris* subsp. *maritima* (Desplanque et al., 2000), 11 haplotypes in *Argania* (El Mousadik & Petit, 1996), 23 haplotypes in white oaks (Dumolin-Lapègue et al., 1997), and 13 haplotypes in *Alnus* (King & Ferris, 1998). The nucleotide diversity ($Dij = 0.06026$ and $Dij = 0.08074$) of these two Taiwanese oaks was also high, compared to that of California pines ($Dij = 0.003 \pm 0.002$) (Hong et al., 1993).

High level of genetic diversity in the Fagaceae (cf. Petit et al., 1997; Dumolin-Lapègue et al., 1997) is probably associated with their long evolutionary history, which allows genetic variation to accumulate within lineages (cf. Chiang & Schaal, 1999). Nevertheless, the higher haplotype diversity of cpDNA of *Lithocarpus* from Taiwan may be simply derived from different molecular techniques employed. Nucleotide sequencing usually detects a higher level of genetic variation than do the RFLP and PCR-RFLP techniques. In our analysis, according to the deduced restriction site map, only four chlorotypes (cpDNA polymorphisms) for 58 individual haplotypes could be identified. Within smaller populations of *L. dodonaeifolius* from Weiliaoshan and Dazen, the number of major clades was even lower (with only two), while high levels of haplotype diversity ($h = 1$) and nucleotide diversity ($Dij = 0.045$–0.046) were assessed based on the sequence variation. As stated by Desplanque et al. (2000), a substantial within-population diversity in natural plants should have existed (cf. McCauley, 1994; El Mousadik & Petit, 1996; Raspe, 1998). The detection of the existing variation surely depends not only on the conservative nature of the molecular marker itself, but also the sensitivity of the tools employed. In this study, the so-called "higher" level of genetic variation of *L. formosanus* and *L. dodonaeifolius* turned out much lower than that of other species, when nucleotide sequences were transferred to RFLP data (data not shown). Such low level of chlorotype diversity, as expected, may be ascribed to their smaller population number and size.

Interestingly, *Lithocarpus formosanus* possessed a much higher level of nucleotide diversity than did *L. dodonaeifolius*, despite fewer chlorotypes (three types) existing in the former species (vs. four types for *L. dodonaeifolius*). In general, populations with larger sizes maintained higher genetic variation compared to smaller ones, such as Chingshuiying ($Dij = 0.07039$) versus Dazen ($Dij = 0.04560$) and Weiliaoshan ($Dij = 0.04554$). According to the longest branch of the *for1502* ($P < 0.05$) in the neighbor-joining tree (Fig. 1), the unusually high nucleotide diversity in *L. formosanus* was probably ascribed to the most diverged sequence. When the sequence of *for1502* was removed from the calculation, a low level of nucleotide diversity ($Dij = 0.07369 \pm 0.01161$) was obtained. This deviation from a molecular clock ($P < 0.01$) can hardly be attributable to natural selection, whose effect is usually not intense on a noncoding spacer (Graur & Li, 2000). According to the DnaSP analysis, random genetic recombination within the chloroplast DNA marker may have resulted in the long branch of *L. formosanus* (cf. Huang et al., 2001).

As a noncoding region, the *atp*B-*rbc*L spacer has relatively low functional constraints. With nearly neutral evolution, mutations to some extent would have been retained within each lineage. However, extremely low substitution rates in the *atp*B-*rbc*L spacer have been detected in other plants (Manen & Natali, 1995; Chiang & Schaal, 2000a, b). High levels of genetic variation of the cpDNA of *Lithocarpus* in Taiwan may be in part ascribed to fre-

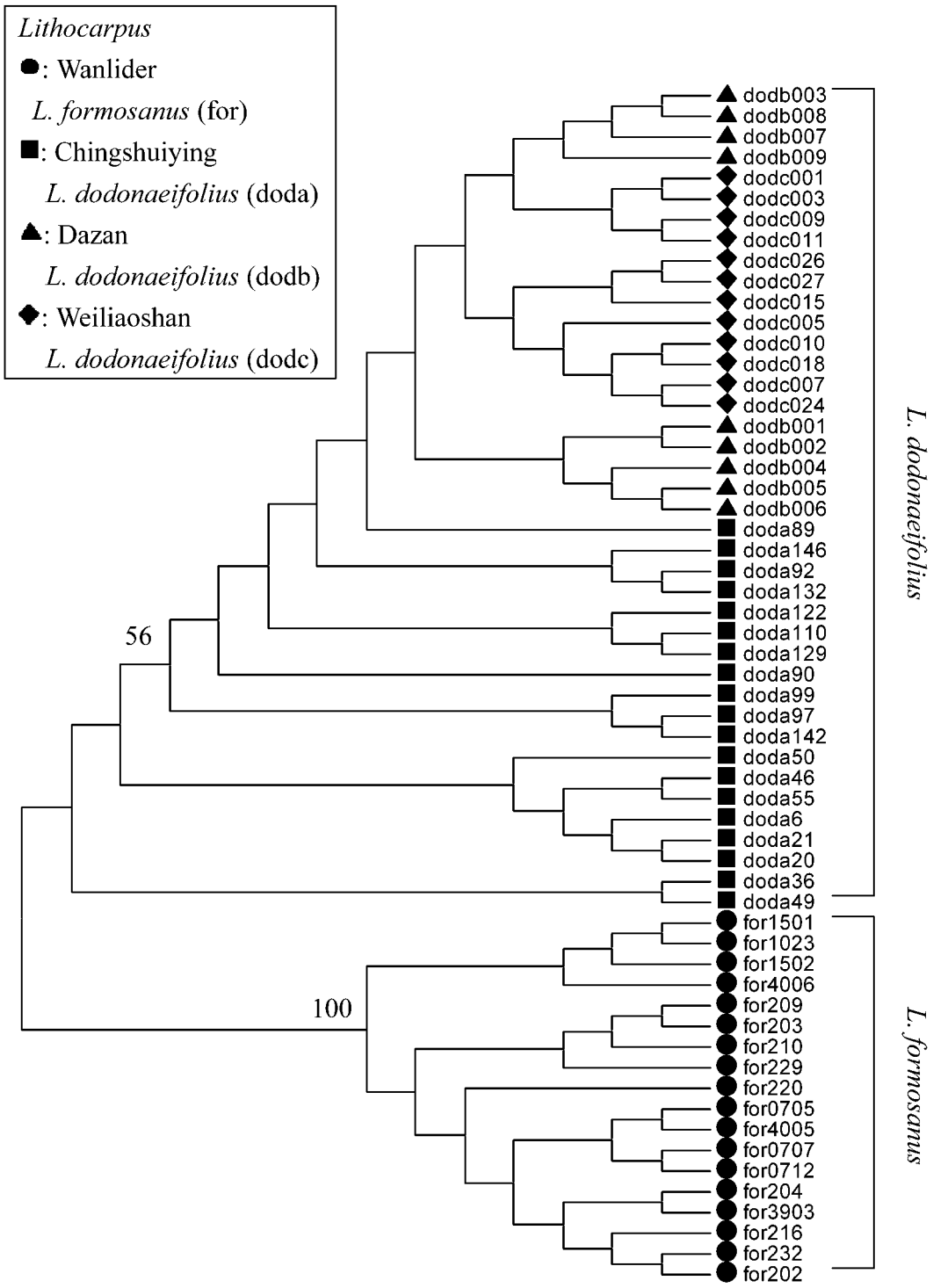

Figure 3. UPGMA tree of individuals of *Lithocarpus formosanus* and *L. dodonaeifolius* based on RAPD data. Numbers at nodes indicate bootstrap values.

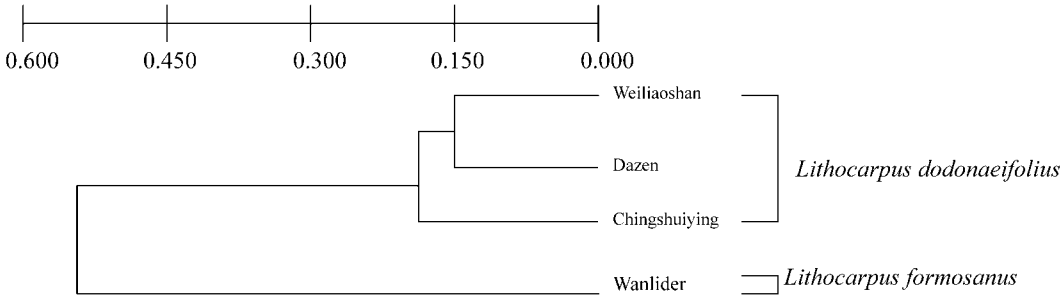

Figure 4. UPGMA tree of populations of *Lithocarpus formosanus* and *L. dodonaeifolius* based on RAPD data for: *L. formosanus* at Wanlider; doda: *L. dodonaeifolius* at Chingshuiying; dodb: *L. dodonaeifolius* at Dazen; dodc: *L. dodonaeifolius* at Weiliaoshan. The level of dissimilarity between populations is indicated.

quent intramolecular recombination, a process well documented in plant organelle DNAs (e.g., Stern & Palmer, 1984; Yesodi et al., 1997; Senda et al., 1998; Huang et al., 2001). Large DNA fragments involved in the intramolecular recombination of the cpDNA may have contributed to the longer branch leading to the *for1502*. Indel events were responsible for high polymorphisms of cpDNA as well. In contrast to random genetic recombination, indel events occurring in bp positions between 311 to 608 for the *atp*B-*rbc*L noncoding spacer appeared nonrandom.

PHYLOGEOGRAPHY OF *LITHOCARPUS FORMOSANUS*–*L. DODONAEIFOLIUS* AND GENE GENEAOLOGY OF cpDNA

RAPD fingerprints revealed significant differentiation between *Lithocarpus formosanus* and *L. dodonaeifolius* based on the UPGMA dendrogram and deduced Fst. Genetic distance between populations is strongly associated with geographical distance ($P < 0.05$, Fig. 5A), which is consistent with a model of isolation by distance (Slatkin, 1993). Interestingly, according to the differentiation between subpopulations of *L. formosanus* on the UPGMA dendrogram (Fig. 3), a ravine of narrower than 1 km in width (i.e., Nanjen stream) may become a natural barrier for pollen dispersal, although it is not significant.

In contrast to the significant genetic differentiation between species and populations indicated by RAPD fingerprinting, which represents the level of ongoing gene flow between populations, the cpDNA noncoding spacer within species and populations was non-monophyletic. The most common chlorotype A (68.9%) was predominant over other alleles (8.6% for B, 6.9% for C, and 15.6% for D) and was widespread in all populations. In contrast, chlorotype D was exclusive to *Lithocarpus dodonaeifolius*, while chlorotypes B and C were distributed in *L. formosanus* and the Chingshuiying population of *L. dodonaeifolius*. The biased distribution of alleles in populations plus low differentiation between species suggested lineage sorting of the cpDNA locus in both *Lithocarpus* species (cf. Hoelzer et al., 1998; Young, 1998). Lineage sorting results from a process of differentiated births and deaths, when ancestral polymorphisms are passed down to descendent lineages (populations) (cf. Futyuma, 1998; Chiang, 2000). It usually blurs the inference of organismal phylogeny. In this study, monophyly of the chlorotype D at species level, i.e., within *L. dodonaeifolius*, and paraphyly of other chlorotypes indicated that these chlorotypes are at different lineage sorting periods.

The extent of lineage sorting is usually determined by the genetic heterogeneity within populations and the migratory mode, which in turn is associated with the ongoing gene flow and natural hybridization as well as geological history that the species evolved through. Recurrent introgression is typically invoked to explain such sorting (cf. Manos et al., 1999). However, unexpectedly high *Nm* values between species and populations deduced from the nucleotide sequences of the cpDNA locus of *Lithocarpus* in Taiwan and the low correlation between *Nm* (up to 126.89) and the geographic distance ($P > 0.05$, Fig. 5B) indicates that the migratory mode may have deviated from the stepping-stone model, Wright's island model, or an isolation-by-distance model (cf. Hamrick & Nason, 1996).

Evidently, given limited pollen dispersal among populations (indicated by our RAPD results), the migration of *Lithocarpus* fruits or nuts across a geographic range of 20–95 km in modern habitats is even more improbable due to the discontinuity of vegetation and the constraint of migratory capabilities of any fruits- or nuts-carrying animals. Although some low levels of incidental seed dispersal among populations cannot be simply ruled out, un-

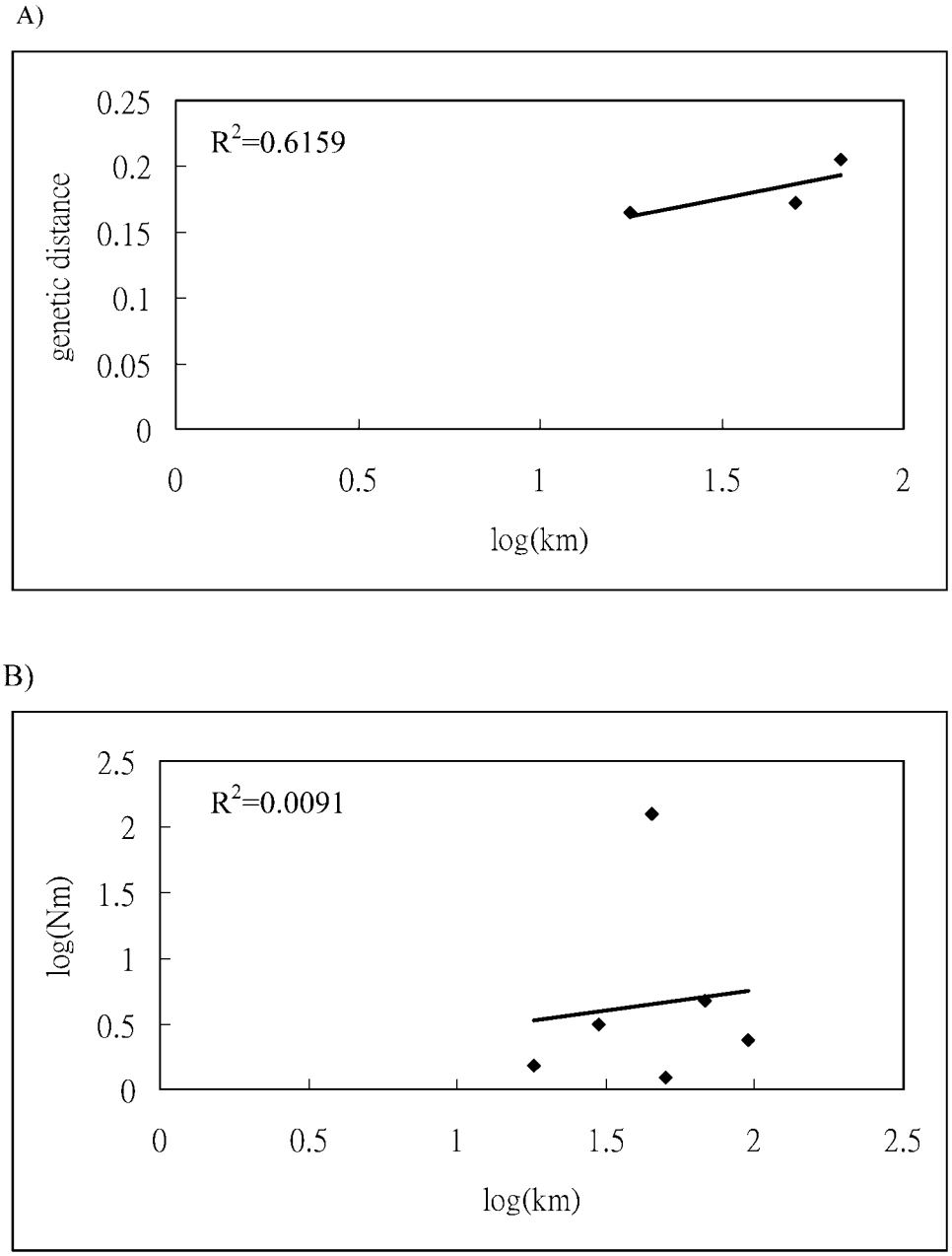

Figure 5. —A. Scatterplot of genetic distance and logarithmic scales of geographic distance among populations of *L. dodonaeifolius* based on RAPD data. Regression statistics: $R^2 = 0.6159$, $P < 0.05$. —B. Scatterplot of logarithmic scales of *Nm* and geographic distance among populations of *Lithocarpus formosanus* and *L. dodonaeifolius* based on chloroplast DNA variation. Regression statistics: $R^2 = 0.0091$, $P > 0.05$. N = female effective population size, m = female migrant rate.

usually high *Nm* values are likely to represent historical migration events instead of the current gene flow. Low genetic differentiation due to shared and heterogeneous composition of organelle DNAs within each population and the high deduced *Nm* between populations suggested a migrant-pool model, a migratory pattern with colonists recruited from a random sample of all the other populations (Wade & McCauley, 1990). Usually this model is associated with glaciation or vicariance events, which ac-

cording to fossil evidence may disturb the vegetation dramatically at a large geographic scale.

Like many angiosperm species (e.g., Fagaceae, Ferris et al., 1995, Petit et al., 1997, Dumolin-Lapègue et al., 1997; beeches, Koike et al., 1998; and beets, Desplanque et al., 2000) and gymnosperms, such as *Cunninghamia* (Lu et al., 2001) as well as *Pinus* (Strauss et al., 1993), oaks survived glacial cycles (cf. Bennett, 1990; Huang et al., 2001). According to geological record, since the late Pleistocene, Taiwan was the southeastern edge of the Asian continent before the formation of Taiwan Strait about 100,000 BP (Lin, 1966; Tsukada, 1966; Kizaki & Oshiro, 1977). This continental island was linked to the mainland via a land bridge and was not completely isolated until the last glacial retreat ca. 18,000 to 20,000 BP (Lin, 1966). Geological evidence indicates that ice ages have occurred at regular intervals of approximately 100,000 years followed by warm periods of about 20,000 years (Milankovitch cycles) (cf. Bennett, 1990; King & Ferris, 1998). During the glacial maximum many oaks and conifers previously dominant in the northern part of eastern Asia were forced to migrate into refugia in southern China and Taiwan (Chiang et al., 1999), which were mostly distributed at low elevations (cf. Tsukada, 1966). During the subsequent deglaciation, elevated global temperatures forced the lowland plants to migrate to high elevations or local peaks. Pollen records reveal the migration routes of many relictual and endemic species, which constitute a large portion of Taiwan's flora (Shaw, 1997). The current geographical distribution of *Lithocarpus dodonaeifolius* and *L. formosanus* is possibly a result of such a migration history.

According to the shared cpDNA alleles of the *atp*B-*rbc*L noncoding spacer, the speciation of *Lithocarpus dodonaeifolius* and *L. formosanus* may be recent. Like other fagaceous plants, migration via long-range seed dispersal (cf. Petit et al., 1997) of the ancestral populations of the Taiwan oaks became possible due to the dramatic change of vegetation (cf. Chiang & Hong, 1999) during deglaciation. Many novel niches were then available for plants that survived after species extinction. Seeds from different resource populations may have migrated into the refugia and settled subsequently. Such migration may have increased the heterogeneity of cpDNA composition within populations.

Extinction and re-colonization regulated by geological events, however, were thought to enhance genetic differentiation among populations (cf. Wright, 1977) owing to founder events resulting from the colonization by a small number of surviving individuals. Recently, Wade and McCauley (1990) further suggested that the results of extinction/re-colonization on genetic differentiation among populations depend on the number of founders as well as the level of heterogeneity of genetic composition within populations according to coalescence theory. When the colonist size is small and the genetic heterogeneity is low, genetic differentiation among populations will be reached fast via stochastic processes alone.

Despite the relatively small population size in both *Lithocarpus* species, no coalescence has been achieved within populations. To counter the genetic diversity loss within small populations due to genetic drift, it is likely that the genetic composition of ancestral populations, even species, prior to deglaciation as well as succeeding colonizing populations was highly heterogeneous. The four chlorotypes noted for these two *Lithocarpus* species in Taiwan may have existed long before the speciation event. In addition, recurrent genetic recombination within the chloroplast intergenic spacer may have increased the heterogeneity within species as well. Given small population sizes, the low level of genetic differentiation among *Lithocarpus* populations at the cpDNA noncoding spacer region is possibly ascribed to a short duration (since the last deglaciation) for coalescence.

Lineage sorting (or the attainment of monophyly) of chlorotypes within species and populations is assuredly regulated by the stochastic genetic drift (Chiang, 2000). The high frequency of the chlorotype A over others in both *Lithocarpus* species and the high frequency of the chlorotype B in *L. formosanus* (80%) illustrate this random effect. With a short time span for coalescence, monophyly of the cpDNA locus has not been attained within either species. The chlorotype D may have been drifted in *L. formosanus* due to its smaller population size. Obviously, the larger population (200 individuals) at the Chingshuiying site possessed higher genetic variation compared to the other populations of *L. dodonaeifolius*. Low genetic diversity in Weiliaoshan and Dazen populations for *L. dodonaeifolius* is ascribed to the lack of chlorotypes B and C. Deduced *Nm* values seemed to be indicative of lineage sorting in clades A and A′ (Table 3). Lower *Nm* among populations derived from clade A′ relative to clade A indicates a higher level of coalescence, e.g., the fixation of chlorotype D in *L. dodonaeifolius*. Drift of rare chlorotypes in smaller populations (D in *L. formosanus* as well as B and C in Weiliaoshan and Dazen populations of *L. dodonaeifolius*) thereby resulted in higher levels of genetic differentiation and genetic diversity compared to the

widespread and dominant chlorotype A across populations of both species. However, differences of deduced *Nm* values between clades A and A′ revealed problems and limitations with the interpretation of such indirect estimates of gene flow among populations (cf. Bossart & Prowell, 1998).

In contrast to the lineage sorting of the cpDNA locus, RAPD fingerprints attained "coalescence" at most loci and resulted in significant genetic differentiation between species. This coalescence at population level, however, has not been completely reached. Interestingly, subsets of smaller populations from Weiliaoshan and Dazen for *Lithocarpus dodonaeifolius* were nested within the cluster of Chingshuiying individuals, as indicated by the UPGMA tree (Fig. 3). Coherence of smaller populations versus the division seen in RAPD sampling within the larger population indicated such a random coalescence process. Incongruence between organelle DNA and RAPD fingerprints, which are usually amplified from the nuclear genome (Hawkins & Harris, 1998), may derive from the different inheritance modes between the two genomes. Genetic recombination between homologous chromosomes may have played a determining role in homogenizing the genetic differences within demes and thereby increasing the heterogeneity between populations, while crossing-over is usually lacking in organelle DNAs due to its haploid nature.

Accordingly, gene flow between *Lithocarpus dodonaeifolius* and *L. formosanus* may have been blocked since their postglacial resettlement. Adapting to habitats at different elevations as they occur, both *Lithocarpus* flower with a lag interval of about half a month. The reproductive barriers between the two species may be complete. In contrast to many European and American Fagaceae (Whittemore & Schaal, 1991; Howard et al., 1997; Manos et al., 1999; Samuel, 1999), which hybridize naturally when populations are sympatrically distributed, no interspecific hybrids have been reported in these Taiwanese *Lithocarpus.*

CONTINENT-ISLAND DISCREPANCY IN VARIATION ALLOTMENT

At the intraspecific level, patchy structure of local populations (e.g., *Aquilegia,* Strand et al., 1996; Fagaceae, Petit et al., 1997; Dumolin-Lapègue et al., 1999) or geographic subdivision between long isolated populations (e.g., northern and southern populations of *Liriodendron tulipifera* L., Sewell et al., 1996; Sarmathic–Baltic and Alpine–Central European populations of *Picea abies* (L.) H. Karst., Vendramin et al., 2000) have been documented in many tree species of continents. Low level of organelle DNA differentiation among local populations was detected in *Lithocarpus dodonaeifolius* and *L. formosanus* as well as other plants of continental islands, such as *Cycas taitungensis* Shen et al. (Huang et al., 2001), *Amorphophallus* (cf. Chiang & Peng, 1998), *Michelia formosana* (Kanehira) Masamune (Lu et al., 2002) of Taiwan as well as Japanese *Abies* (Tsumura & Suyama, 1998). Due to the limited area and available habitats of the island, the population size of Taiwan's oaks is effectively smaller than that of continental species. Under near neutrality, a long period of lineage sorting for cpDNA chlorotypes in small populations may be likely ascribed to high levels of heterogeneity in genetic composition. Interestingly, greater genetic variation has been noted in southern continental refugia (such as Italy, the Balkans, and the Iberian Peninsula) than in northern populations (e.g., Central Europe) (European oaks, cf. Dumolin-Lapègue et al., 1997, 1998; European *Alnus,* King & Ferris, 1998; *Fagus,* Demesure et al., 1996; and Japanese *Abies,* Tsumura & Suyama, 1998). The continent-island discrepancy may be associated with the fact that Taiwan, which is straddled across today's subtropics to tropics, is much more south than most areas of European and American continents geographically. Taiwan may have provided more fitting habitats for surviving plants during the glacial maximum than mainland refugia.

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