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RESEARCH ARTICLE

Postglacial population expansion of *Dacrydium pectinatum* (Podocarpaceae) in Hainan, southern China, based on cpDNA *trn*L-F nocoding sequence data

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Abstract This study determined the sequences of chloroplast DNA (cpDNA) *trn*L-F non-coding regions of individuals of a tropical coniferous species, *Dacrydium pectinatum*, collected from 12 natural populations located in Hainan Province, southern China. Sequence length varied from 868 bp to 876 bp, indicating length polymorphism. Base composition in the sequences was high in A+T content between 64.17% and 64.95%, and no recombination event occurred (Rm = 0). Thirty haplotypes were identified based on statistical parsimony algorithm by running the TCS program. Populations of *D. pectinatum* in Hainan were lacking genetic differentiation. Such a deduction was supported by the observed F_{ST} values (0.00), AMOVA (24.17% of molecular variance attributed to difference among populations, P>0.05), high values of *Nm* (ranging from 1.92 to 2.50) and the branching structure in neighbor-joining (NJ) tree constructed from haplotypes. A 'star-like' pattern was exhibited in the TCS network of *trn*L-F haplotypes, and majority of the haplotypes coalesced near the tips in NJ tree. Gene genealogies of cpDNA haplotypes proposed a recent population expansion of *D. pectinatum* in Hainan, which was further supported by the results from Tajima's *D* test and mismatch distribution analysis. Our data, in conjunction with geological and palynological evidences, showed that in the Holocene, due to global warming, refugee populations of *D. pectinatum* in Hainan might experience a range expansion.

Keywords *Dacrydium pectinatum* de Laubenfels, cpDNA *trn*L-F noncoding sequence, haplotype, genetic differentiation, population expansion

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1 Introduction

Dacrydium pectinatum de Laubenfels, an evergreen dioecious tree, which is up to 30 m tall and 3 m d.b.h. (diameter in breast height), is the sole species of genus *Dacrydium* (Podocarpaceae) occurring in China (Fu et al., 1999). Formerly, *D. pectinatum* constituted as the dominant species of the rainforests in Hainan. But after more than 20 years of excessive logging, it is now a vulnerable species in China. Fossil pollen records show that historically distribution of *D. pectinatum* might extend to 22°–24° N during the late Tertiary (20 000–40 000 years before present) (Zheng, 1991). Until the glacial fastigium about 15 000 years BC, it did not completely retreat from the Chinese mainland due to drastic climatic and sea-level changes (Zheng, 1991). Since then, natural populations of *D. pectinatum* in China became narrow-ranged, and have merely survived in the humid mountain rainforests of middle Hainan Province, such as Diao Luo Shan, Ba Wang Ling, and Jian Feng Ling. These sites provided valuable materials for the assessment of large-scale historical events on the distribution and subdivision of conifers.

Demographic history of forest trees can be traced backward in time by using phylogeographical methods to assess genetic variability and population differentiation based on sequence data (Otto, 2000; Huang et al., 2002; Burban and Petit, 2003). Chloroplast DNA (cpDNA) non-coding regions are suitable markers in deciphering the spatio-temporal dynamics of plants. Their uniparental inheritance, low frequency of recombination, and moderate to high level of evolution rate have proved to be appropriate for inferring the migratory routes of species in association with glaciation events (Ferris wt al., 1995; Petit et al., 1997). At the intraspecific level, sequence variations of cpDNA non-coding regions have been used to reconstruct phylogeographical patterns in some coniferous species, e.g.,

Taiwan fir (Lu et al., 2001; Huang et al., 2003) and Maritime pine (Burban and Petit, 2003).

Geographic variation of the cpDNA was comparatively studied early in conifers, particularly in pines (Wagner et al., 1987, 1989, 1991). It was suggested that conifers generally tend to display a relatively high degree of genetic variability and a low degree of population differentiation (Loveless and Hamrick, 1984; Ledig, 1986; Hamrick et al., 1992). However, an exception was verified in some coniferous taxa, such as *Pinus resinosa*, in which low genetic diversity and strong genetic differentiation were identified (Walter and Epperson, 2001). *D. pectinatum* is a coniferous tree with considerable ecological significance for the tropical forest. But little is known about its population, genetic structure, and demographic history. Therefore, the phylogeography of *D. pectinatum* in Hainan Province, southern China, was inferred based on cpDNA *trn*L-F sequence variation.

2 Materials and methods

2.1 Plant materials

A total of 12 natural populations of *D. pectinatum* were sampled from Hainan Province, China. Samples were collected individually from populations located in Dong Si Fen Shui Ling (FS, altitude 1 000 m), Lao Dian Xiao Lu (LD, altitude 1 050 m), Dong San Shan Ji (SS, altitude 1 100 m), Dong San Jiu Gong Lu (SG, altitude 1 000 m), and Dong Si Fa Qu (FQ, altitude 1 000 m) of Ba Wang Ling Natural Reserve, and Da Diao Luo (DD, altitude 960 m), Diao Luo Hou Shan at altitudes of 938 m (LHA), 1 000 m (LHB) and 1 080 m (LHC), Bai Shui Ling at altitudes of 610 m (BSA), 626 m (BSB), and 980 m (BSC) of Diao Luo Shan Natural Reserve. Leaves from young shoots were sampled and immediately preserved in silica gel. All samples were stored at −20°C until the time they are processed.

2.2 DNA preparation and PCR amplification

Total genomic DNA was extracted from the ground tissue following the modified CTAB protocols (Su et al., 1998). DNA concentration and purity were determined by measuring UV absorption by using a Pharmacia 2000 UV/Visible spectrophotometer. Its intactness was checked through 0.8% agarose gel electrophoresis. PCR was performed in a reaction volume of 100 mL using 50 mmol/L KCl, 10 mmol/L Tris–HCl, 1.5 mmol/L $MgCl_2$, 0.1% Triton X-100, 0.2 mmol of each dNTP, 50ng template DNA, 2U Taq polymerase, 40 pmol of each primer. Primers designed based on Taberlet et al. (1991) were used to amplify *trn*L-F non-coding sequence. Primer c: 5′-CGAAATCGGTAGACGCTACG-3′, Primer f: 5′-ATTTGAACTGGTGACACGAG-3′. Primers were synthesized by Shanghai Bioasia Biotech Ltd. The thermocycling profile consisted of 3 min at 94ºC, 30 cycles of 45 s at 94ºC, 60 s at 55ºC, 90 s at 72ºC, and an additional extension for 7 min at 72ºC. The size of PCR products was determined by agarose electrophoresis.

2.3 Cycle sequencing

Five-microliters of PCR products were applied to cycle sequencing by using the same primers as in the PCR reaction.

2.4 Recovery of PCR products using low-melting-point agarose

PCR products were purified by running through a 1.0% low melting agarose gel. The desired DNA band was cut and recovered from the gel using UNIQ-10 kit (Shanghai Bioengineering Ltd., China).

2.5 DNA cloning and sequencing

A purified PCR product was ligated by a pMD18-T vector and then was used to transform competent *Escherichia coli* cells DH-5α. A positive clone was identified by blue/white selection and was ascertained by PCR. Purified plasmid DNA was sequenced in both directions by standard methods on an ABI 377 automated sequencer. Primers M13F and M13R located on pMD18-T vector were utilized for sequence determination.

2.6 Data analysis

Genbank accession numbers of the determined *trn*L-F non-coding sequences of haplotypes are AY534768-AY534797. Sequences were aligned with the program CLUSTAL X (Thompson et al., 1997). Length variation and nucleotide composition were calculated using BioEdit (Hall, 1999). Haplotype diversity (h) and nucleotide diversity (D_{ij}) were quantified using DnaSP (Rozas and Rozas, 1999). Minimum number of recombination events was estimated by Hudson and Kaplan's Rm parameter (Hudson and Kaplan, 1985). Tests of neutrality were performed using Tajima's D test (Tajima, 1989). The mismatch distribution analysis (Rogers and Harpending, 1992) was used to test for demographic signatures of population expansions. Neighbor-joining analysis by calculating the Kimura 2-parameter distance was conducted using PHYLIP (Felsenstein, 1995). *Pinus thungergii* was chosen as outgroup, and confidence of the reconstructed clades was tested by bootstrapping with 1000 replicates. Network was constructed based on statistical parsimony algorithm with the aid of the TCS (Templeton et al., 1992). Gene flow within and among populations was approximated as Nm, the number of female migrants per generation between populations. Nm was estimated using the expression $F_{ST}=1/(1+2\ Nm)$, where N was the female effective population size and m is the female mi-

gration rate. A Mantel test was implemented to determine whether pairwise values of *Nm* were related to geographic distances between populations (Mantel, 1967). Software Arlequin (Schneider et al., 2000) was used to deduce the molecular variance partition within and among populations based on square Euclidean distances.

3 Results

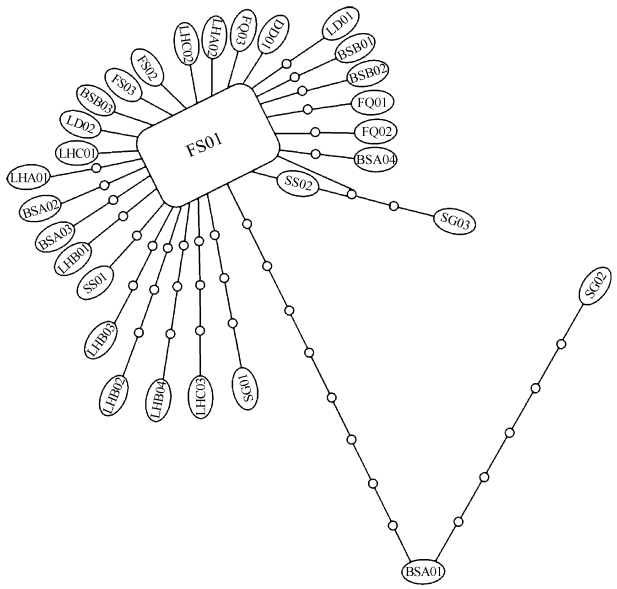
3.1 Haplotype diversity and nucleotide diversity

In this study, cpDNA *trn*L-F non-coding sequences of *D. pectinatum* were determined by cycle sequencing. For samples incapable of being determined by this strategy, PCR-amplified *trn*L-F fragments were cloned into T vector, and then sequenced. Sequence sizes varied from 868 bp to 876 bp, showing length polymorphism. Insertions at sites 112–119 made the spacer longer in haplotypes BSA01 and SG02. Nucleotides A and T are common in the chloroplast sequence, with contents ranging from 64.17% to 64.95%, which was consistent with the nucleotide composition of most non-coding regions and pseudogenes. Minimum number of recombination events (Rm) is zero. Thirty haplotypes of cpDNA *trn*L-F were identified in *D. pectinatum* by running the TCS program.

High level of haplotype diversity (h=0.821) were detected within the whole species of *D. pectinatum* (Table 1). Across all populations, haplotype diversity ranges from 0.545 to 0.909, with the population FS as the lowest while BSB and LHB were the highest. Conversely, low level of nucleotide diversity (D_{ij}=0.002 52) occurred in *D. pectinatum*. At the population level, D_{ij}-values vary from 0.00070 to 0.00534, with populations FS as the lowest whereas SG was the highest.

Table 1 Haplotype diversity (h) and nucleotide diversity of cpDNA *trn*L-F non-coding sequences of *D. pectinatum*

Population	Localities	Sample size /n	Haplotype diversity /h	Nucleotide diversity /D_{ij})
D. pectinatum		138	0.821	0.002 52 ± 0.000 24
FS	109°13′E, 18°25′N	12	0.545	0.000 70 ± 0.000 22
LD	109°14′E, 18°26′N	10	0.800	0.002 05 ± 0.000 43
BSA	109°52′E, 18°41′N	10	0.889	0.002 87 ± 0.000 41
BSB	109°52′E, 18°41′N	12	0.909	0.002 79 ± 0.000 37
BSC	109°52′E, 18°41′N	12	0.545	0.001 05 ± 0.000 35
SS	109°15′E, 18°28′N	12	0.727	0.001 40 ± 0.000 35
SG	109°16′E, 18°27′N	10	0.889	0.005 34 ± 0.001 12
DD	109°54′E, 18°44′N	12	0.727	0.001 05 ± 0.000 23
FQ	109°14′E, 18°22′N	12	0.848	0.002 44 ± 0.000 43
LHA	109°51′E, 18°43′N	12	0.545	0.001 05 ± 0.000 35
LHB	109°51′E, 18°43′N	12	0.909	0.005 24 ± 0.000 68
LHC	109°51′E, 18°43′N	12	0.727	0.002 10 ± 0.000 65



Missing intermediates were indicated by circles;
Each branch between two (sampled or missing) haplotypes indicates a single mutational step

Fig. 1 Network relating haplotypes of cpDNA *trn*L-F non-coding regions in populations of *D. pectinatum* by statistical parsimony algorithm

3.2 Neutrality test and mismatch distribution

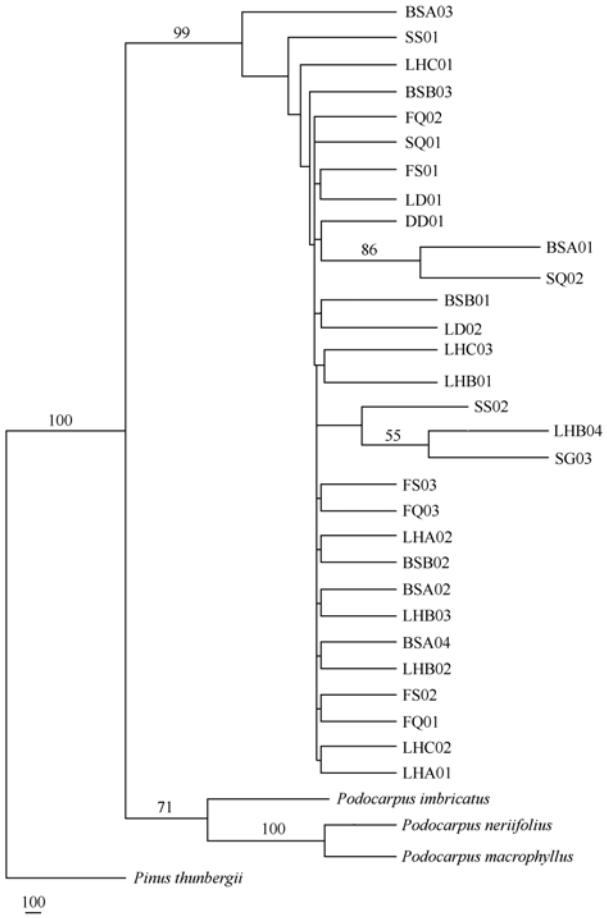
Neutrality test by Tajima's *D* (−2.62202, $P<0.001$) demonstrated the negative values were significantly different from zero, indicating an excess of rare alleles over than what was expected for null neutral hypothesis in an equilibrium population.

The mismatch distribution of cpDNA *trn*L-F non-coding spacer sequences of *D. pectinatum* closely matched to the expectations under the sudden-expansion model (Fig. 3). The raggedness index (Tajima, 1989) was low and not significantly different from expectation ($R=0.022$, $P=0.0899$). This showed evidence of recent population expansions.

3.3 Gene genealogies of cpDNA haplotypes

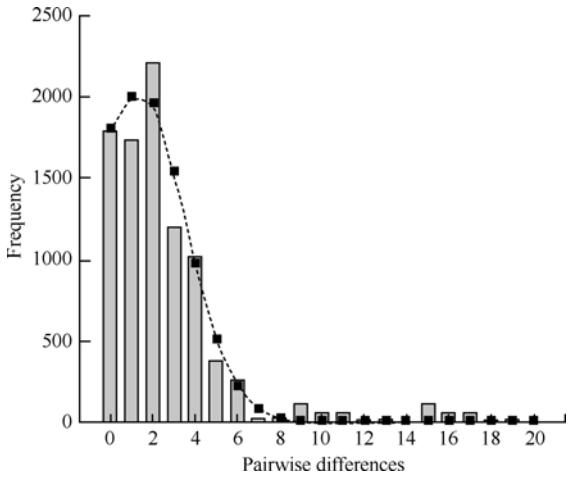
Templeton et al. (1992) established the statistical parsimony algorithm to reconstruct the genealogy. The method first defines the uncorrected distance above which the parsimony criterion is violated with more than 5% probability (parsimony limit). Then, all connections are linked among haplotyes starting with the smallest distances and ending either when all haplotyes are connected or the distance corresponding to the parsimony limit has been reached. A network, exhibiting 'star-like' pattern, was inferred from *trn*L-F haplotypes of *D. pectinatum* by statistical parsimony algorithm using the TCS program (Fig. 1). FS01 was identified as an ancestral haplotype, it occurred in populations FS, LD, SS, SG, FQ, LHA, and LHC, whereas no sharing was found for those derived haplotypes. Majority of the haplotypes coalesced to central haplotype FS01 with short branch length of only one to four mutational steps, except BSA01 and SG02 which remained at a relatively isolated position.

Rooted at *Pinus thungergii*, a neighbor-joining (NJ) tree was recovered based on the nucleotide variation of the *trn*L-F sequences of *D. pectinatum* haplotypes and its closely related species, *Podocarpus imbricatus* Bl., *Podocarpus neriifolius* D. Don, and *Podocarpus macrophyllus* (Thunb.) D. Don (Fig. 2). Haplotypes of *D. pectinatum* formed a monophyletic group which was strongly supported by a bootstrap value of 99% (Fig. 3). Except BSA03, SS01, and LHC01, most of the haplotypes coalesced near the tips in the tree. Interestingly, sequences of the same populations were never grouped into a monophyletic clade. Consequently, the branches from different locations were highly mixed, indicating efficient gene flow between them.



Numbers above branches indicate the bootstrap values of 1000 replicates

Fig. 2 Neighbor-joining tree based on cpDNA *trn*L-F non-coding sequences of haplotypes of *D. pectinatum* and its closely related species, *Podocarpus imbricatus*, *P. neriifolius*, and *P. macrophyllus*, rooted using *Pinus thungergii* as outgroup



The abscissa represents the number of pairwise differences and the ordinate the frequency of observations;
The vertical bars are the observed distribution of mismatches and the dotted line represents the expected distribution under the sudden-expansion model of Rogers (1995) as modified by Schneider and Excoffier (1999)

Fig. 3 Mismatch distribution across populations of *D. pectinatum* based on cpDNA *trn*L-F non-coding sequences

3.4 Population genetic differentiation

With regard to DNA divergence of *trn*L-F non-coding sequences between populations of *D. pectinatum*, the average number of nucleotide differences and the average number of nucleotide substitution per site ranged from 0.833 to 5.033, and 0.00096 to 0.00454, respectively (Table 2). Divergence between population FS and populations BSC, DD as well as LHA appears the lowest, whereas that between population SG and LHB was the highest (Table 2).

Non-significant differentiation among populations was revealed by the estimates of F_{ST} (= 0.00) and *Nm* (varying from 1.92 to 2.50) (Table 3). Pairwise estimates of *Nm* between populations showed non-significant relationship to geographic distance (R=0.06, P=0.778). Hierarchical analyses of sequence difference under AMOVA indicated that 75.83% of molecular variance was attributed to the difference within populations, whereas merely, 24.17% of molecular variance attributed to difference among populations (P>0.05).

4 Discussion

In this study, cpDNA *trn*L-F non-coding sequences were examined by using phylogeographical approaches in conjunction with nucleotide diversity, neutral test, and mismatch analysis to deduce population genetic structure and demographic history of *D. pectinatum* in Hainan Province, China.

Recombination did not occur in the *trn*L-F region of *D. pectinatum* (Rm=0). In the analysis of the *trn*D-*trn*T spacer in *Cunninghamia konishii* (Cupressaceae), only one possible recombination was detected (Lu et al., 2001).

Table 2 Average number of nucleotide differences (above diagonal) and average number of nucleotide substitution per site (below diagonal) between populations of *D. pectinatum*

	FS	LD	BSA	BSB	BSC	SS	SG	DD	FQ	LHA	LHB	LHC
FS		1.333	1.733	1.667	0.833	1.000	2.933	0.833	1.500	0.833	2.833	1.333
LD	0.001 54		2.400	2.333	1.500	1.667	3.600	1.500	2.167	1.500	3.500	2.000
BSA	0.002 00	0.002 76		2.733	1.900	2.067	3.920	1.900	2.567	1.900	3.867	2.400
BSB	0.001 92	0.002 69	0.003 15		1.833	2.000	3.933	1.833	2.500	1.833	3.833	2.333
BSC	0.000 96	0.001 73	0.002 19	0.002 11		1.167	3.100	1.000	1.667	1.000	2.944	1.500
SS	0.001 15	0.001 92	0.002 38	0.002 30	0.001 34		3.167	1.167	1.833	1.167	3.111	1.667
SG	0.003 39	0.004 16	0.004 53	0.004 54	0.003 58	0.003 66		3.100	3.767	3.100	5.033	3.600
DD	0.000 96	0.001 73	0.002 19	0.002 11	0.001 15	0.001 34	0.003 58		1.667	1.000	3.000	1.500
FQ	0.001 73	0.002 50	0.002 96	0.002 88	0.001 92	0.002 11	0.004 35	0.001 92		1.667	3.667	2.167
LHA	0.000 96	0.001 73	0.002 19	0.002 11	0.001 15	0.001 34	0.003 58	0.001 15	0.001 92		3.000	1.500
LHB	0.003 26	0.004 03	0.004 45	0.004 42	0.003 39	0.003 58	0.005 81	0.003 46	0.004 22	0.003 46		3.500
LHC	0.001 54	0.002 31	0.002 77	0.002 69	0.001 73	0.001 92	0.004 16	0.001 73	0.002 50	0.001 73	0.004 04	

Table 3 Pairwise comparisons of *Nm* between populations of *D. pectinatum* based on cpDNA *trn*L-F non-coding sequences

	FS	LD	BSA	BSB	BSC	SS	SG	DD	FQ	LHA	LHB	LHC
FS		2.03	1.99	2.50	2.50	2.50	1.92	2.50	2.50	2.50	2.50	2.50
LD			2.00	2.32	2.10	2.16	2.00	2.10	2.29	2.10	2.45	2.25
BSA				2.24	2.04	2.09	2.22	2.04	2.21	2.04	2.50	2.18
BSB					2.50	2.50	2.11	2.50	2.50	2.50	2.50	2.50
BSC						2.50	1.96	2.50	2.50	2.50	2.81	2.50
SS							2.37	2.50	2.50	2.50	2.79	2.50
SG								1.96	2.08	1.96	2.41	2.06
DD									2.50	2.50	2.50	2.50
FQ										2.50	2.50	2.50
LHA											2.50	2.50
LHB												2.50

Very low levels of recombination in the chloroplast genome were also recorded in other plants (Despanque et al., 2000). Because of the non-existence of recombination between molecules, each cpDNA haplotype has just one ancestor in the previous generation, which enables the genetic relationship among haplotypes to be inferred in light of coalescent theory (Beebee and Rowe, 2004).

Populations of *D. pectinatum* in Hainan Province, China, are lacking genetic differentiation. Such a deduction was supported by F_{ST} values (= 0.00), AMOVA (24.17% of molecular variance attributed to difference among populations, $P>0.05$) as well as high values of *Nm* (1.92–2.50). The evident ongoing gene flow was also reflected in the branching pattern of the NJ tree as haplotypes characteristic of one locality were frequently found in another (Fig. 2). The results are understandable because of the effects on genetic structure among populations given by ecological and life history traits of *D. pectinatum*, such as outcrossing breeding system, long-distance dispersion of seeds, abundant production of wind-disseminated pollens, and long-lived life cycle, all of which prevent population genetic divergence (Loveless and Hamrick, 1984; Allnutt et al., 1999; Fu et al., 1999). Recently, based on cpSSR data, relatively high gene flow and low genetic structure have also been identified among populations of white bark pine (*Pinus albicaulis*) which is with bird-dispersed seeds and wind-disseminated pollen (Richardson et al., 2002). In addition, the paternal gene flow mediated by long-range pollen transport in conifers would also decrease the level of population differentiation (Vendramin et al., 1999).

Nm values in the Mantel test and geographic distances demonstrated that the migrations of *D. pectinatum* did not match the model of "isolation by distance" (Wright, 1943). In contrast, "island" model might account for the exchanges of alleles since the same magnitude of pairwise values of *Nm* were detected between populations with different geographical distances (Table 3) (Jobling et al., 2004). Again, long-distance pollen and seed dispersal might play a major role in such a migration pattern. An ancestral haplotype, FS01, was found to be present in seven of the twelve investigated populations in this study. It should be of the same origin since no sharing was detected for those derived haplotypes (Hwang et al., 2003). If this be the case in *D. pectinatum*, it implies that cpDNA *trn*L-F haplotypes could exchange between populations with a geographical distance as far as 110 km (e.g., populations FS *vs.* LHA). Distant cpDNA haplotype migration has also been demonstrated in the Taiwan fir (Hwang et al., 2003).

High level of haplotype diversity (h = 0.821) and low nucleotide diversity (D_{ij} = 0.002 52) were revealed in *D. pectinatum*, which represented a signal of rapid demographic expansion from small effective population size (Avise, 2000). Phylogenetic pattern of *trn*L-F haplotypes demonstrats a 'star-like' feature which is characterized by most haplotypes linked to central haplotype with short branch length (Fig. 1). This relatively simple pattern means that is possible that populations of *D. pectinatum* preserved in refugia have experienced population expansion after glaciation, and since then, there have been insufficient time to form a more complicated population structure (Pages and Holmes, 1998). In the NJ tree, majority of the haplotypes coalesced near the tips of the tree (Fig. 2), which suggests the coalescence events just recently occurred. Furthermore, significantly negative Tajima's *D* and examination of frequency distributions of pairwise differences of *trn*L-F sequences (Fig. 3) coincidentally proposed a recent demographic expansion across populations of *D. pectinatum* (Aris-Brosou and Excoffier, 1996; Hundertmark, 2002).

Geological and palynological evidences have shown that during the Late Wurm glaciation, adverse climate and glacier advancement forced *D. pectinatum* to totally retreat from the Chinese mainland, and only survived in the mountain areas above the altitude of 500 m in the Hainan island (extreme south of China) with more favourable climate conditions (Zheng, 1991). Nonetheless, in the following Holocene, due to global warming, refugee populations of *D. pectinatum* might recolonize habitats in the north or at higher altitudes where they could grow for range expansion. The inferred extension of distribution, however, would be restricted in the Hainan Province since the island's connection with the Chinese mainland was already obstructed because of shifts in the location of regional land masses due to plate tectonics and subsequent division by rising sea levels (Xing et al., 1995). And it is almost impossible for the seeds of *D. pectinatum* to disperse across the Qiongzhou Strait with the width of 20–40 km.

Recent molecular analyses indicated that migration from glacial forest refugia still imprinted detectable effects on the extant patterns of genetic variation in plants (Allnutt et al., 1999; Richardson et al., 2002). Because of their relatively long generation times, trees could be of particular value for investigating such processes. However, compared with temperate trees, relatively few studies have been made on tropical tree species using DNA techniques (Chalmers et al., 1992; Dawson et al., 1995; Gillies et al., 1997). By examining *D. pectinatum*, a dominant coniferous species in tropical rainforest, our data produces evidences to suggest that molecular phylogeographical approaches are capable of providing insights into historical events, such as range expansion and patterns of post-glacial migration, within trees of tropical areas (Newton et al., 1999).

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