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Full Length Research Paper

Nucleotide variation at the methionine synthase locus in an endangered tree species, *Fokienia hodginsii* (*Cupressaceae*) in Vietnam

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Nucleotide variation at the methionine synthase (*MetE*) locus within and among populations of an endangered forest tree *Fokienia hodginsii* in Vietnam was investigated in the present study. A total of 12 populations were sampled across Vietnam. The length of the sequenced locus varied from 1567 to 1559 bp. A total of 42 polymorphic sites were detected among samples. Overall, nucleotide diversity was estimated to be 0.00499 and 0.00692 at the total (π_{tot}) and silent sites (π_{silent}) in the pool, respectively. Nucleotide diversity within populations varied from 0.00300 to 0.00521 at the total and 0.00357 to 0.00666 at silent sites. The estimates of nucleotide diversity were lower in the 4 populations located in central and southern Vietnam (0.00300 to 0.00380) in comparison with the northern populations (ranging from 0.00399 to 0.00543). Overall estimates of genetic differentiation among 12 populations were low (F_{ST} = 0.093 and K_{ST}^* = 0.078), even though both values were highly significant (P < 0.001). Pairwise analysis among 12 populations showed significant genetic differentiation as evaluated by F_{ST} and S_{nn} but not significant as evaluated by K_{ST}^*. Analysis of genetic clustering using BAPS provided the best support for all 144 sequences belonging to the same genetic cluster. The implication of the results revealed in this study in the genetic conservation of *F. hodginsii* was discussed.

Key words: Population genetics, conservation, forest, methionine synthase (MetE), structure.

INTRODUCTION

Fokienia hodginsii is a monotypic, ancient, 'living fossil' and a member of the *Cupressaceae* family. It is endemic to Laos, Vietnam and southern China where it is widespread and is currently listed as globally near-threatened (IUCN, 2004). *F. hodginsii* is highly valued both economically and culturally. *F. hodginsii* is widely distributed in Vietnam. It occurs in montane evergreen forests on granite or limestone derived soils in provinces of Ha Giang, Lao Cai, Dien Bien, Yen Bai, Son La, Phu Tho, Hoa Binh, Thanh Hoa, Nghe An, Ha Tinh, Thua Thien Hue, Kontum, Gia Lai, Dac Lac, Lam Dong, Ninh Thuan and Khanh Hoa (Luu and Thomas, 2000). This conifer species prefers high humidity ranging from 81% in December to 86% in September (Van et al., 2000). In Vietnam, *F. hodginsii* is being extensively harvested to supply lucrative domestic, and possibly foreign markets, leading to severe population fragmentation and local

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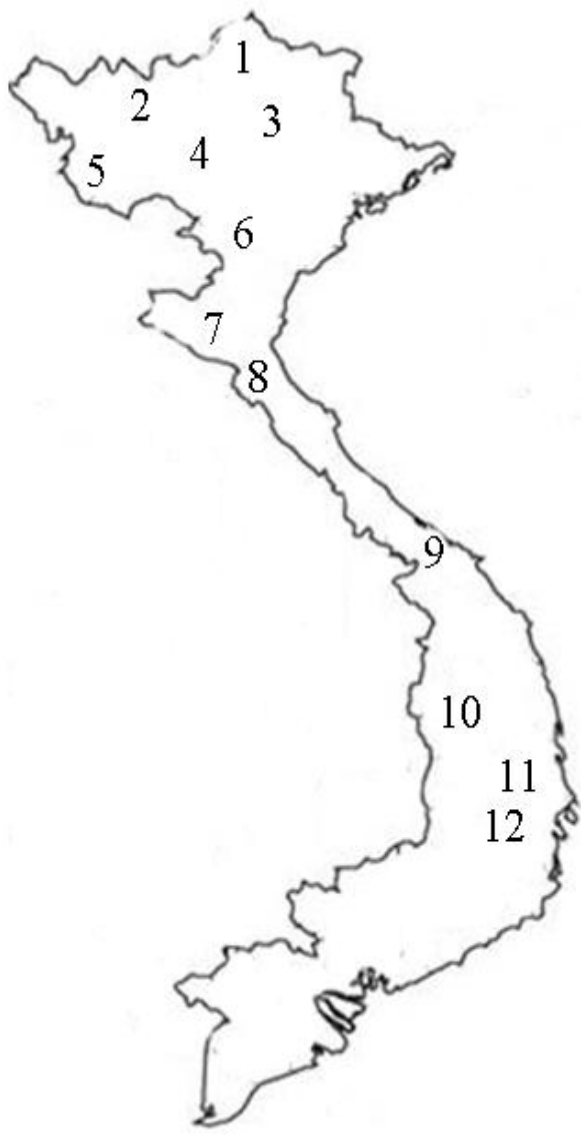

Figure 1. Location of the 12 sampled populations of F. *hodginsii* in Vietnam. Population codes (1 to 12) are those given in Table 1.

extinction. *F. hodginsii* was listed in Vietnam Red Book in 1996 and the Government decided to close the *F. hodginsii* forests.

Population genetics plays an important role in conservation biology and ecology in general. The assessment of genetic diversity in animal and plant populations, especially of endangered species is now pervasive. Good methods for DNA analyses are being increasingly used to estimate the extent and organization of genetic diversity within populations, to infer the causes of its spatio-temporal dynamics and to suggest strategies for conservation and the wise use of genetic resources. In this sense, in a period of dramatic human exploitation and consumption of natural biological resources and concomitant development of biotechnologies, the emerging field of conservation genetics can help to guide the necessary harmony between economic development and nature preservation. Conservation genetics hence provides important tools for the assessment of biodiversity according to the biodiversity convention of the United Nations (Rio convention).

Molecular identification and populations genetics studies of *F. hodginsii* in Vietnam were recently performed by Nguyen et al. (2011, 2012). These studies employed inter-simple sequence repeat (ISSR)S marker to measure genetic diversity in populations distributed in northern Vietnam and used 18S-rRNA sequence to identify *Cupressaceae* species in Vietnam. The research investigated phylogenetic relationships of nine *Cupressaceae* species and revealed that *F. hodginsii* is within the genus *Calosedus*. Population genetics study using nucleotide polymorphism has not been reported for *F. hodginsii*. In this study, we aim to investigate sequence polymorphism at a nuclear functional locus, methionine synthase (MetE) to study nucleotide variation within and among populations of *F. hodginsii* collected across Vietnam. Information on genetic variation at nucleotide level will be valuable for the conservation of *F. hodginsii* in Vietnam.

MATERIALS AND METHODS

F. hodginsii seeds were collected from 12 populations across Vietnam (Figure 1 and Table 1). We collected seeds from trees growing at least 20 m apart. Seeds collected were then stored in silica gel and carried to the laboratory in Hanoi. Seeds were sown immediately after they arrived at Hanoi.

DNA extraction

In gymnosperms, megagametophytes are of maternal origin and are haploid. We extracted DNA from megagametophyte of *F. hodginsii* for use in this study. Since DNA samples are haploid, direct sequencing is used to obtain nucleotide sequences and directly determine haplotype sequences. *F. hodginsii* seeds were sown on wet paper in a Petri plate. After germination, we removed all seed coats and embryos. Using a pestle, we collected a fresh megagametophyte in a 2-ml tube. Haploid genomic DNA was extracted from megagametophytes using DNsaesy plant mini kit (QIAGEN, Valencia, CA). Total DNA was determined using a fluorometer and diluted to 5 ng/µl.

Polymerase chain reaction amplification

Polymerase chain reaction was carried out in 25 µl solution consisting of 2.5 µl $MgCl_2$ (25 mM), 2 µl dNTP (8 mM), 10 pmol of each primer, 1.25 units Tag DNA polymerase (Invitrogen) and 1.5 µl of template DNA. The reaction mixture was subjected to amplification in the Gene Amp PCR System 2400, under the following thermal cycler: an initial denaturing step at 94°C for 5 min, followed by 40 cycles consisting of 1 min at 94°C, 30 s at 52°C, 1 min extension at 72°C and 10 min at 72°C for a final cycle to complete the extension of any remaining products before holding the samples at 4°C until they were analysed. The sequence of primers used for PCR amplification and internal primers for sequencing the *MetE* locus in this study are listed in Table 2.

Table 1. Geographical location of the sampled populations.

Population	Code	Altitude (m)	Latitude (N)	Longitude (E)	Number of chromosomes sampled
Ha Giang	1	1700	22°02′	105°09′	12
Lao Cai	2	1500	22 °30′	104°22′	12
Bac Can	3	1450	22 °20′	106°33′	12
Yen Bai	4	1550	21 °30′	105°29′	12
Dien Bien	5	1470	21 °15′	100°35′	12
Son La	6	1280	20 °15′	105°48′	12
Nghe An	7	1900	19 °31′	105°27′	12
Ha Tinh	8	1850	18 °21′	106°28′	12
Da Nang	9	1900	16 °45′	107°35′	12
Gia Lai	10	1780	15 °32′	108°31′	12
Khanh Hoa	11	1890	13 °47′	109°45′	12
Lam Dong	12	1970	13 °10′	109°11′	12

Table 2. List of primers used in this study.

Primer code	Primer sequence (5′ to 3′)	Annealing temperature (°C)	Reference
Met1F	CCATGGCTAGAGGAAATGCC	52	Chiang et al., 2002
Met4R	GCCCAGATGTTCCTTCCATC	52	Chiang et al., 2002
Met1F1	ATGTTACATTTCCGACGATA	Internal primer	This study
Met1F2	TATTGGCACGTACGATGGCA	Internal primer	This study
Met4R1	AATGCCATGGATCATCGTTA	Internal primer	This study

DNA sequencing

PCR products were sequenced using the BigDye terminator sequencing kit (Applied Biosystems, Foster City, CA) on an ABI310 automated sequencer (Applied Biosystems).

Data analysis

The obtained sequences were aligned manually using a sequence alignment editor, SE-Al v. 2.0a11 (Rambaut, 1996). Intron positions were determined on the basis of homologous genes in other plants and the GT–AG rule. All indels were excluded from the analysis. DnaSP 4.10.0 (Rozas et al., 2003) was used to estimate the standard population genetic parameters (pairwise nucleotide diversity and nucleotide polymorphism).

F_{ST} statistics (Hudson et al., 1992) were used to measure the amount of genetic differentiation among populations. Significance levels of the nearest-neighbor statistic (S_{nn}; Hudson, 2000) and a weighted measure of the ratio of the average pairwise differences within populations to the total average differences (K_{ST}*; Hudson et al., 1992b) were calculated using 10,000 permutation tests.

Tajima's D (Tajima, 1989) statistic (D_T) was used to test for deviations from neutrality. This test measures skews in the frequency spectrum, where a negative D_T suggests an excess of low-frequency polymorphisms and a positive D_T indicates an excess of intermediate-frequency polymorphisms.

Linkage disequilibrium (LD) analyses were performed using DnaSP. The 2 indices of LD, D' and R^2 (squared allele frequency correlation) were estimated across all informative sites. D' has a potential range from -1 to 1, with the magnitude of disequilibrium being indicated by a departure from zero in either direction. Furthermore, the percentage of pairs of sites in significant LD was also calculated; the statistical significance of each pairwise test was evaluated using the χ^2 test and statistical significance was determined at a level of $P < 0.05$. Pairwise analyses are not fully independent because of LD itself; hence, the proportion of significant pairwise tests and the mean $|D'|$ and R^2 were compared for pairs of sites separated by different molecular map distances (<400 or >400 bp). Comparison of these values over the two separate distance ranges was carried out by χ^2 analysis.

RESULTS

Nucleotide variation

The length of *MetE* locus sequenced in this study varied from 1567 to 1559 bp. Four indels were found and excluded from all analyses. Estimates of nucleotide variation within populations and in the pool (the whole samples) are shown in Table 3. A total of 42 polymorphic sites were detected in 144 sequences generated from 12 populations of *F. hodginsii* collected across Vietnam. The estimate of overall nucleotide diversity was 0.00401 and 0.00519 at the total (π_{tot}) and silent sites (π_{silent}), respectively. Nucleotide diversity within populations varied from 0.00300 to 0.00543 at the total and from 0.00357 to 0.00666 at silent sites. On average, approximately 85 and 78% of the nucleotide diversity in the pool was found within populations at total and silent

Table 3. Estimates of nucleotide variation within populations.

Population	Polymorphic site	θ_W (±SD)	π_{total} (±SD)	π_{silent} (±SD)	D_T
1	14	423 ± 111	521 ± 137	601 ± 128	0.43
2	17	398 ± 139	432 ± 122	589 ± 110	0.56
3	13	410 ± 126	399 ± 165	597 ± 129	- 0.74
4	13	436 ± 108	459 ± 110	609 ± 178	0.45
5	14	442 ± 120	543 ± 129	666 ± 156	0.67
6	14	398 ± 100	440 ± 198	568 ± 173	0.44
7	18	498 ± 119	490 ± 121	609 ± 111	- 0.21
8	14	377 ± 154	467 ± 198	592 ± 176	0.49
9	15	329 ± 100	320 ± 117	499 ± 119	- 0.43
10	13	228 ± 139	300 ± 166	357 ± 132	0.55
11	12	381 ± 154	380 ± 154	413 ± 195	- 0.22
12	11	210 ± 138	330 ± 141	421 ± 132	0.45
Within-population mean		378 ± 85	423 ± 79 (85%)	543 ± 97 (78%)	
Pool	42	446 ± 123	499 ± 57	692 ± 64	

θ and π values and their standard deviations were multiplied by a factor of 10^5. Within-population means were calculated from individual estimates of 12 within populations; numbers in parentheses indicate the percentages of means of within populations relative to those estimated from the pool of 12 populations.

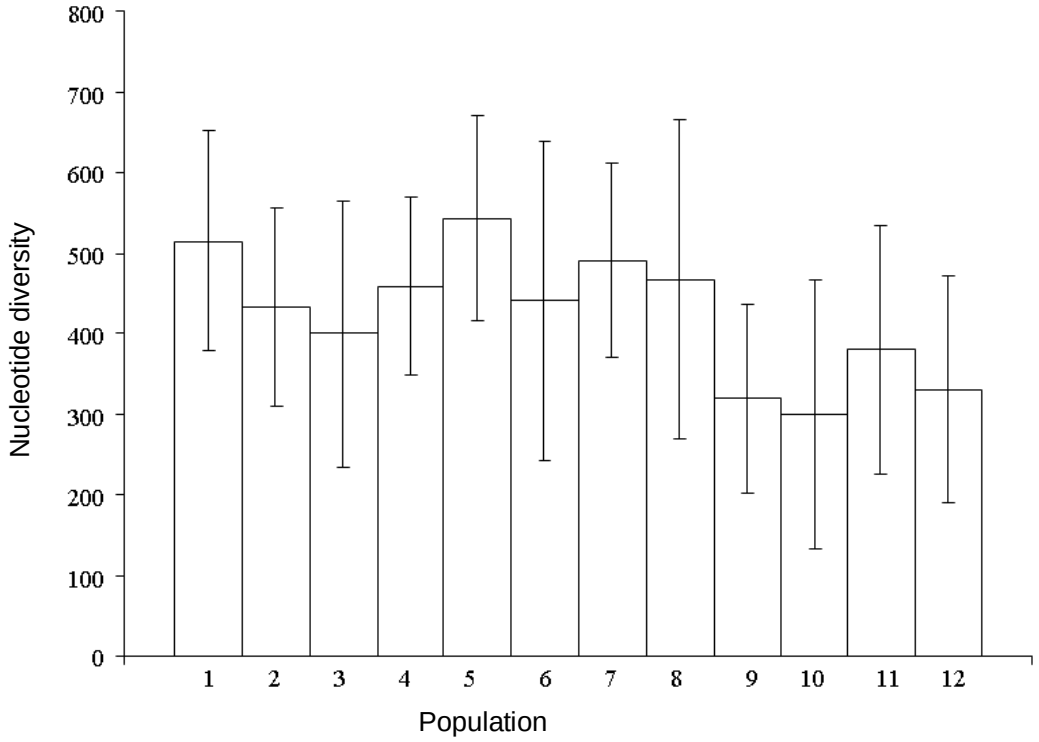

Figure 2. Comparison of total nucleotide diversity among populations. Values of nucleotide diversity were multiplied by 100 000.

sites, respectively.

The estimates of total nucleotide diversity for the four southern populations (9 to 12) ranged from 0.00300 to 0.00380 which were lower than those for the eight populations (1 to 8) (0.00399-0.00543) (Table 3, Figures 1 and 2). The difference was also observed in the estimates of nucleotide diversity at silent sites between the 2 groups of populations (Table 3).

Tajima's *D* (Tajima, 1989a) statistic (D_T) was used to test for deviations from neutrality. The estimates of D_T

Table 4. Linkage disequilibrium analysis for *MetE*.

Parameter	*N*	|*D'*|	R^2	Significant pairwise test (%)
<400 bp	233	0.799	0.212	33
>400 bp	255	0.681	0.019	16

N (pairs): Number of pairwise comparisons between sites.

Table 5. Analyses of population differentiation.

Population	1	2	3	4	5	6	7	8	9	10	11	12
1		0.021	0.001	0.012	0.045*	0.033	0.061	0.009	0.081**	0.004	0.001	0.013
2	0.044		0.000	0.025	0.033	0.045	0.001	0.017**	0.027	0.011*	0.097	0.055*
3	0.098	0.022**		0.004	0.036	0.077	0.098	0.003	0.005	0.079*	0.000	0.029
4	0.123**	0.091	0.077		0.006	0.076	0.001	0.000	0.098**	0.032*	0.054	0.099
5	0.165*	0.039	0.125**	0.145*		0.000	0.091	0.023***	0.054	0.000	0.012*	0.003
6	0.098*	0.141***	0.011	0.033	0.165**		0.099	0.025*	0.014*	0.019	0.091	0.023*
7	0.088	0.204***	0.223	0.047*	0.160	0.020		0.009	0.043	0.004	0.067	0.009
8	0.002	0.009	0.083*	0.055	0.098**	0.024*	0.083*		0.045	0.098**	0.007	0.098
9	0.054**	0.011	0.072	0.049	0.012	0.044	0.248*	0.067		0.000	0.013	0.043**
10	0.079	0.021	0.034*	0.009	0.098**	0.056	0.223***	0.022*	0.125*		0.041	0.055*
11	0.099***	0.018*	0.067	0.141**	0.055***	0.099*	0.141*	0.072*	0.085	0.039		0.000
12	0.077	0.067	0.001	0.098*	0.086	0.011	0.123**	0.034*	0.141**	0.044*	0.008	

Upper diagonal is K-ST (Hudson et al., 1992), lower diagonal is FST (Hudson et al., 1992); significance of FST was evaluated by the permutation test (10 000 permutations of Snn [Hudson, 2000]); *P < 0.05; **P < 0.01 and ***P < 0.001.

are shown in Table 3. None of the D_T estimates were significant, suggesting that there was no significant excess of low-frequency and intermediate-frequency alleles within populations of *F. hodginsii*. The ratios of nonsynonymous to synonymous polymorphisms ($\theta_a/\theta s$) were estimated to be 0.2445, indicating that the *MetE* locus is generally under strong purifying selection.

Linkage disequilibrium

The 3 statistics of linkage disequilibrium were estimated and are shown in Table 4. Linkage disequilibrium between polymorphic sites was estimated and compared between the 2 groups of sites <400 and >400 bp apart. The estimates of the means |*D*'| and R^2 among sites <400 bp were significantly higher than those among sites >400 bp apart (P < 0.05, *t*-test). The percent of significant pairwise tests was also significantly lower in sites >400 bp apart (P < 0.05, χ^2-test). The results suggest that linkage disequilibrium at MetE locus declined significantly among sites >400 bp apart. Recombination plays a role in generating nucleotide diversity in *MetE* of *F. hodginsii*.

Population differentiation

The overall estimates of genetic differentiation among 12 populations were low; F_{ST} = 0.093 and K_{ST}* = 0.078, even though both values were highly significant (P < 0.001). Analysis of genetic clustering using BAPS provided the best support for all the 144 sequences belonging to the same genetic cluster. Pairwise analysis of genetic differentiation among the 12 populations is shown in Table 5. Among the populations analyzed, many pairs of populations showed significant genetic differentiation, as evaluated by F_{ST} and

Table 6. Summary of nucleotide diversity in conifers.

Species	Number of loci	π_{tot}	π_{sil}	Reference
Fokienia hodginsii	1	0.00401	0.00519	This study
Pinus sylvestris	1	0.00140	0.00490	Dvornyk et al., 2002
P. sylvestris	2	0.00040	0.00040	Garcia-Gil et al., 2003
P. pinaster	10	0.00240	NR	Pot et al., 2005
P. radiata	10	0.00186	NR	Pot et al., 2005
Cryptomeria japonica	7	0.00252	0.00319	Kado et al., 2003
Chamaecyparis obtusa	10	0.00240	NR	Kado et al., 2007
C. pisifera	10	0.00298	NR	Kado et al., 2007

NR, Not reported.

S_{nn}. However, only a few pairs showed significant differentiation when evaluated by K_{ST}*. Most of the significant F_{ST} and K_{ST}* values were very low.

DISCUSSION

The estimates of nucleotide diversity at total and silent sites of *MetE* locus in *F. hodginsii* in the present study were comparable to those of *pal1* locus in an European conifer, *Pinus sylvestris* (Dvornyk et al., 2002). The estimates were generally higher than several other conifers previously reported such as *Pinus* (Garcia-Gil et al., 2003; Neale and Savolainen, 2004; Pot et al., 2005; Pyhäjärvi et al., 2007), *Cryptomeria* (Kado et al., 2003), *Chamaecyparis* (Kado et al., 2008) and *Picea* (Heuertz et al., 2006) (Table 6). Nucleotide diversity in conifers was generally lower than that in broad-leaved tree species such as *Populus* (Ingvarsson, 2005), *Quercus* (Quang et al., 2008), *Zanthoxylum* (Kamiya et al., 2008) and *Betula* (Järvinen et al., 2003). The present study revealed a low level of among-population differentiation for all local populations of *F. hodginsii* in Vietnam. Previous studies employing nuclear sequence polymorphism also revealed low and very low levels of genetic differentiation among populations of outcrossing forest trees such as oaks and conifers (Quang et al., 2008; Järvinen et al., 2003; Dvornyk et al., 2002; Garcia-Gil et al., 2003; Neale and Savolainen, 2004; Pot et al., 2005; Pyhäjärvi et al., 2007; Kado et al., 2003, Kado et al., 2008, Heuertz et al., 2006).

A recent study (Nguyen et al., 2011) using ISSR markers to assess the genetic diversity within and differentiation among populations of *F. hodginsii* across Vietnam revealed a low level of diversity in comparison with other tree species previously reported. Nguyen et al. (2011) showed the possibility of habitat fragmentation to explain the very low level of genetic diversity observed in populations of *F. hodginsii* in Vietnam. In accordance with their report, the present study also revealed lower level of nucleotide diversity in populations located in central and southern Vietnam. Those populations may have been strongly influenced by habitat fragmentation and small sizes. In Vietnam, *F. hodginsii* forests were greatly fragmented by human activities such as logging and clearing and they form small forest patches. In central Vietnam, most of the populations of *F. hodginsii* were especially small in size, varying from 50 to 150 adult individuals per forest (Nguyen et al., 2011). Small population size and forest fragmentation may have caused increased levels of inbreeding and diversity loss (Ellstrand and Elam, 1993; Barrett and Kohn, 1991).

Lower level of nucleotide diversity was found in the central and southern parts of Vietnam. *F. hodginsii* forests in Vietnam have been overexploited. These forest areas are typically home to the ethnic minority groups. These people often have a low living standard and education. They are heavily reliant on forest products both for subsistence and to provide income through trade. To secure genetic diversity of this species in Vietnam, it is necessary to establish a collection bank of seeds and seedlings of *F. hodginsii* from populations distributed across Vietnam and especially from the populations in central and southern Vietnam where *F. hodginsii* forests are more fragmented and heavily exploited. On the conservation point of view, the populations in the south are especially important because they are adaptive to the climate conditions in the southern distribution limit of the species and are sensitive to the loss of genetic diversity when habitat fragmentation occurs.

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