



# Genetic diversity of the endemic flat-needle pine *Pinus krempfii* (Pinaceae) from Vietnam revealed by SSR markers

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**ABSTRACT.** *Pinus krempfii* Lecomte (Pinaceae) is an endemic tree to Vietnam with restricted habitats at higher altitudes in the highlands. In this study, genetic variation of four populations of *P. krempfii* was assessed using 17 microsatellite markers (single sequence repeats). Of these 17 markers, eight were polymorphic, and among the 42 putative alleles amplified, 32 were polymorphic (accounting for 76.19%). The Cong Troi population was found to be the most genetically diverse (Shannon's information index,  $I = 0.415$ , and percentage of polymorphic bands,  $PPB = 52.95\%$ ), whereas the Hon Giao population was found to have the lowest diversity ( $I = 0.330$  and  $PPB = 47.06\%$ ). The genetic diversity at species level was also estimated ( $I = 0.414$ ,  $PPB = 76.19\%$ ). Molecular variance was found to be low among populations (11.94%) and high among individuals within the populations (88.06%). Private alleles were not detected in the Hon Giao population. The Yang Ly population

had a positive  $F_{IS}$  (inbreeding coefficient) value of 0.071, while the three remaining populations had negative values (-0.116 for Cong Troi, -0.316 for Chu Yang Sin, and -0.350 for Hon Giao). The results obtained show an excess of homozygosity in the Yang Ly population, and also suggest a deficiency of heterozygosity for this population. Several approaches and measures of conservation for *P. krempfii* are discussed and proposed.

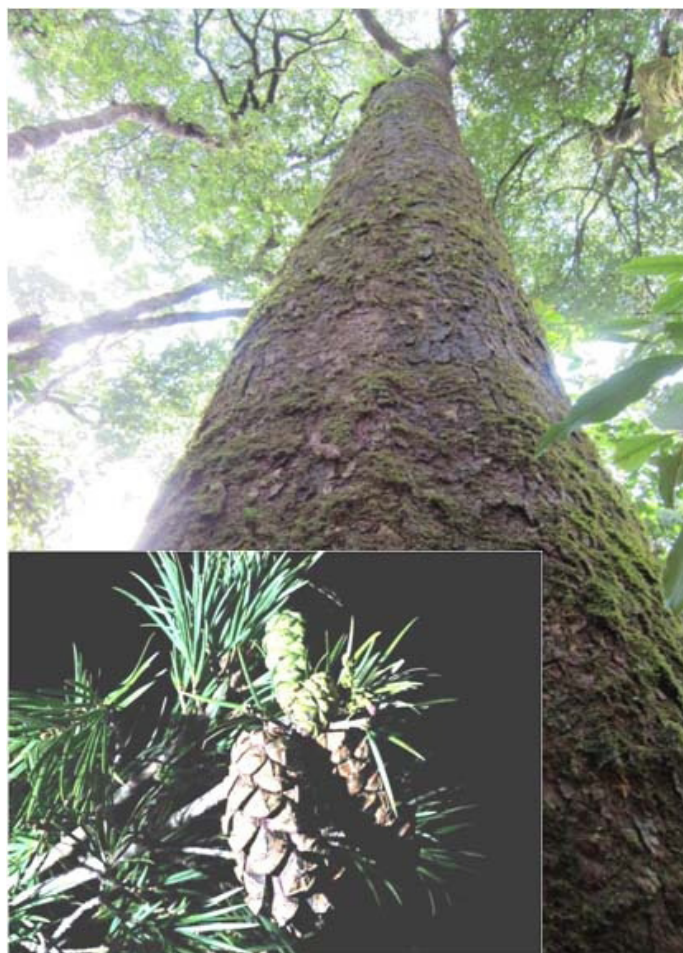
**Key words:** Endemic species; Genetic diversity; *Pinus krempfii*; Population structure; SSR markers

## INTRODUCTION

*Pinus krempfii* Lecomte is a unique flat pine species endemic to Vietnam. *P. krempfii* not only has an important role within the forest ecosystem but it is also of great commercial value, with wood, plastic, oil, and pharmaceutical applications. The chemical constituents of *P. krempfii* were reported in 2014 (Nhung et al., 2014). This species can only grow in restricted habitats at higher altitudes in the border areas of Dak Lak, Lam Dong, and Khanh Hoa Provinces. Although its estimated extent of occurrence is within the threshold for endangered species, and its area of occupancy is also likely to be less than 500 km<sup>2</sup>, there are more than five locations known and the subpopulations are not currently considered severely fragmented (Wang et al., 2000; Thomas et al., 2013). *P. krempfii* grows as a large emergent tree at altitudes between 1200-2000 m; it is always at the top or on the upper slopes of flattened ridges. The surrounding forests are typically dominated by evergreen members of the Fagaceae and Lauraceae. *P. krempfii* is often associated with *Fokienia hodginsii*, *Pinus dalatensis*, and *Podocarpus neriifolius*. *P. krempfii* is unusual amongst the pines as young trees can persist under an evergreen canopy and compete with angiosperm trees (Brodribb and Field, 2008). The rarity of this species has prevented its direct exploitation in the past. Nowadays, its presence within one of Vietnam's flagship protected areas under legal protection appears to be a good measure of conservation; however, low genetic polymorphism and restricted distribution of *P. krempfii* have put this tree under the threat of extinction. The extent of occurrence of *P. krempfii* has declined by more than 30% over the last three generations (150 years) as a result of deforestation and conversion of forests to other uses (Thomas et al., 2013). According to a recent survey by Thomas et al. (2013), only several, i.e., less than ten, natural populations of *P. krempfii* still exist in Vietnam. There is evidence of a continuing decline in the quality of habitat in some locations due to the effects of habitat fragmentation, illegal logging, and infrastructure development. On this basis, *P. krempfii* is assessed as vulnerable under criteria A2c and B1ab(iii) of the IUCN (International Union for the Conservation of Nature and Natural Resources) Red List of threatened species (Thomas et al., 2013).

In 1921, a pine-like evergreen conifer was discovered in the mountains of southern Vietnam and was described by Lecomte (1921) as *P. krempfii*. *P. krempfii* is a large tree, reaching heights of up to 30 m and with a trunk diameter up to 80 cm (Figure 1) (Nguyen and Vidal, 1996). Unlike any other pine, *P. krempfii* is characterized by having two flat leaf-like needles rather than typical pine-like needles (Lecomte, 1921). The distribution of *P. krempfii* in Vietnam is very limited. It is found only in some localities of Khanh Hoa and Lam Dong Provinces at elevations of 1200-2000 m (Nguyen, 1993). It occurs naturally in tropical mixed broadleaf forests, occasionally together with *P. dalatensis*, *Pinus merkusii*, and *Pinus kesiya* (Vu, 1996).

*P. krempfii* occurs in small groups of 10-30 trees and grows together with species of Fagaceae, Magnoliaceae, Lauraceae, *Cryptocarya* sp, *Illicium* sp, *Rhodoleia* sp, and *Podocarpus* sp, which form very dense forests (Nguyen, 1993). The most serious long term threat to *P. krempfii* is habitat fragmentation resulting from infrastructure developments, an increasing incidence of fires throughout the region, the conversion of lower altitude forests to fire-prone *P. kesiya* forests, and encroachment into protected areas for coffee production. Furthermore, illegal logging aimed at *P. krempfii* in Chu Yang Sin has recently been reported (Thomas et al., 2013).



**Figure 1.** Adult tree and twigs with cone of *Pinus krempfii* in Lam Dong Province, Vietnam (Photographs by Nguyen Tien Hiep).

Genetic markers have been used to study the phylogenetic position of *P. krempfii* and have revealed its status as a member of the subgenus *Strobos* (Willyard et al., 2007; Parks et al., 2012; Wang et al., 2000, 2014). However, only limited individuals of *P. krempfii* were included in these studies, and the levels and patterns of DNA variation within natural populations remain unclear.

Genetic variation must exist to maintain natural populations as evolutionarily viable units capable of adapting to changing environmental conditions in the long term. Thus, a genetic resource management strategy should involve investigation of genetic diversity and the extent of genetic differentiation within and between populations and have an understanding of the processes that maintain these variations. To our knowledge, there is no research to date focusing on the genetic diversity and population structure of *P. krempfii*, a species endemic to Vietnam. For this reason, the objectives of this study were to i) characterize the genetic diversity and genetic differentiation of *P. krempfii* populations using microsatellite markers (single sequence repeats, SSR) markers; ii) characterize the genetic structure in the populations, and iii) offer an effective conservation strategy for *P. krempfii*.

## MATERIAL AND METHODS

### Plant materials

This research was carried out at four sites in Vietnam, three in Lam Dong Province and one in Dak Lak Province (Table 1 and Figure 2). Leaf samples or inner bark samples of *P. krempfii* individuals were collected at random from natural stands, including from the Bidoup Nui Ba National Park, Lam Dong Province and the Chu Yang Sin National Park, Dak Lak Province.

**Table 1.** Details of *Pinus krempfii* genotypes and populations from Vietnam used in this study.

Population code	Sample size	Collection locality	Latitude (°N)	Longitude (°E)	Elevation (m)
Yang Ly	5	Yang Ly, Bidoup Nui Ba, Lam Dong	12°11'02.7"N	108°41'24.3"E	1482-1485
Cong Troi	11	Cong Troi, Bidoup Nui Ba, Lam Dong	12°05'17.9"N	108°22'13.0"E	1659-1757
HònGiáo	26	Hòn Giáo, Bidoup Nui Ba, Lam Dong	12°11'03.2"N	108°41'30.3"E	1482-1593
Chu Yang Sin	28	Hoa Son, Chu Yang Sin, Dak Lak	12°25'05.2"N	108°22'17.1"E	1110-1120



**Figure 2.** Location of the *Pinus krempfii* populations used in this study, in Vietnam.

Bidoup Nui Ba National Park is the largest of Vietnam's nature preserves and one of seven internationally important Centers of Plant Diversity in Vietnam. This park encompasses over 1000 km<sup>2</sup> and extends from eastern Cambodia into northern Dak Lak and southern Gia Lai Province in Vietnam. Most of the topography of this park is flat, with an elevation of approximately 1200-2000 m. National Park Bidoup Nui Ba is one of 28 national parks in the system of special-use forests in Vietnam. Of the 64,800 ha that comprise National Park Bidoup Nui Ba, 91% are forests or forest land, which are mainly composed of primeval forest with many different plant and animal species. Chu Yang Sin National Park is located in the territory of the Lak and Krong Bong districts of Dak Lak and is 60 km to the east of Buon Ma Thuot city. The center of the National Park is Mount Chu Yang Sin at 2442 m high, the highest peak of the mountain system of southern Truong Son. Chu Yang Sin National Park is a natural area of 58,947 ha. The topography of the national park is characterized by steep slopes and narrow valleys. At elevations below 800 m, the national park supports lowland semi-evergreen forest.

In the field, samples were placed into plastic bags with silica gel and then taken to the Department of Experimental Taxonomy and Genetic Diversity, Vietnam National Museum of Nature, and maintained at room temperature until use.

### DNA extraction

Seventy *P. krempfii* genotypes were used in this study (coded from Pk1 to Pk70). Total genomic DNA was isolated from leaves and inner bark using the method described by Porebski et al. (1997). The concentration of DNA was determined using a UV-visible light spectrophotometer (UVS 2700, Labomed, USA), and the DNA samples were diluted to 20 ng/μL and used as templates for polymerase chain reaction (PCR) amplification.

### SSR marker amplification

SSR primers were obtained from Integrated DNA Technologies, USA (Table 2). PCR amplifications were performed in a final volume of 25 μL with 50 ng DNA, 1 U *Taq* polymerase (Platinum *Taq* DNA Polymerase, Invitrogen, USA), 2.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 2.5 μL 10X PCR buffer (Invitrogen), and 0.5 mM of each primer. The PCR protocol consisted of 1 cycle at 94°C for 3 min, 40 cycles at 94°C for 30 s, 50-58°C (see Table 2) for 1 min, and 72°C for 1 min. SSR fragments were detected on a 5% polyacrylamide gel in 0.5X TBE. The gels were visualized under UV using BioDocAnalyze (Biometra, Germany).

### Data analysis

For each SSR locus, the allelic composition and total number of alleles were determined for each accession. Data were entered in the form of single-individual genotypes. Genetic polymorphism for each population was assessed using the programs GENALEX version 6 (Peakall and Smouse, 2005), FSTAT (Goudet, 1995), and POPGENE (Yeh et al., 1999). The percentage of polymorphic bands (*PPB*), Shannon's information index (*I*), the mean of expected heterozygosity ( $H_E$ ), the mean of observed heterozygosity ( $H_O$ ), and the inbreeding coefficient ( $F_{IS}$ ) were calculated. The coefficient of gene differentiation ( $F_{ST}$ ) and gene flow ( $N_m$ ) between the SSR loci were also calculated using the GENALEX 6.3 program with the following equations:

$$F_{st} = (H_T - \text{mean } H_E) / H_T \quad (\text{Equation 1})$$

$$N_m = [(I / F_{ST}) - 1] / 4 \quad \text{(Equation 2)}$$

where,  $H_E = 1 - \sum(pi)^2$ ,  $H_T =$  total expected heterozygosity  $= 1 - \sum(tpi)^2$  ( $pi$  is the frequency of the  $i^{\text{th}}$  allele,  $tpi$  is the frequency of the  $i^{\text{th}}$  allele for the total). Exact tests of deviation from the Hardy-Weinberg equilibrium for all loci and among populations were performed at the significance level ( $P$ ) of 0.001.

**Table 2.** Characteristics of 17 microsatellite (SSR) markers used to amplify sections of the *Pinus krempfii* genome.

Locus	Primer sequences	Tm (°C)	References	Size (bp)
NZPR6	5'-GGAAGAAAATGGGCCTTA-3' 3'-CTCTCTATCTCTGCCCA-5'	51	Echt et al., 1999	230
Pt79951	5'-CTTTTGTGTTTTCAACAATTGCA-3' 3'-ACATCTATCTCCCATATCGGC-5'	50	Vendramin et al., 1996	155
Pt36480	5'-TTTTGGCTTACAAAATAAAGAGG-3' 3'-AAATTCCTAAAGAAGGAAGAGCA-5'	58	Clark et al., 2000	155-157
P5	5'-GTTTCGCTAGTTTGTGATCCC-3' 3'-TCCCAGCAAATCCTTGACTC-5'	58	Soranzo et al., 1999	400
Pt9383	5'-AGAATAAACTGACGTAGATGCCA-3' 3'-ATTTCAATTCCTTTCTCC-5'	50	Clark et al., 2000	105-110
PtTX3026	5'-AATACTTGGGAGGATAC-3' 3'-AATAGCCAGTTTTGTTG-5'	52	Elsik et al., 2000	320-495
Pt26081	5'-CCCGTATCCAGATATACTCCA-3' 3'-TGGTTTGATTTCATTCGTTTCAT-5'	54	Vendramin et al., 1996	105-130
Pt30204	5'-TCATAGCGGAAGATCCTCTTT-3' 3'-CGGATTGATCCTAACCATACC-5'	52	Vendramin et al., 1996	120-165
Pt71936	5'-TTCATTGGAAATACACTAGCCCC-3' 3'-AAAACCGTACATGAGATCCC-5'	58	Vendramin et al., 1996	150-160
Pt87268	5'-GCCAGGGAAAATCGTAGG-3' 3'-AGAAGATTAGACATCCAACCC-5'	58	Vendramin et al., 1996	400
Pt15196	5'-CTTGGATGGAATAGCAGCC-3' 3'-GGAAGGGCATTAAAGGTCATTA-5'	52	Vendramin et al., 1996	160
Pnh038	5'-ACTCATTTCGGATGGTGG-3' 3'-TGGGGCTTGGACTTCAAGA-5'	50	Chiang et al., 2011	160
PeC26BGT	5'-CATCCACCAGTTCCCTACG-3' 3'-ATTGAACGCACAGAGCATT-5'	51	Mellick et al., 2009	185
PRE24	5'-GTTTTTTAAATTGGGAAGGCG-3' 3'-CGTGGGGGAGATAGTATAGAGT-5'	58	Boys et al., 2005	280-400
PtTX3020	5'-GTCGGGGAAGTGAAGTA-3' 3'-CTAGGTGCAAGAAAAGAGTAT-5'	50	Elsik et al., 2000	165-270
RPS2	5'-CATGGTGTGGTTCATTGTTCCA-3' 3'-TGGAGGCTATCAGTATGCACC-5'	50	Echt et al., 1996	140-500
RPS160	5'-ACTAAGAACTCCTCTCACC-3' 3'-TCATTGTTCCCAAATCAT-5'	56	Echt et al. 1996	250

Analysis of molecular variance (AMOVA) was conducted to calculate levels of significant variation among the four distribution regions, among populations within a region, and within populations using GENALEX 6.3. Genetic distances and identities and UPGMA cluster analysis were generated to determine the genetic association among populations based on Nei's genetic distance (Nei, 1972) using POPGENE.

## RESULTS

Of the 17 SSR markers used, 8 were found to be polymorphic and among the 42 putative genetic alleles that were amplified, 32 were polymorphic (accounting for 76.19%). Both the indices  $I$  and  $PPB$  were used to assess genetic diversity (Table 3), with the highest diversity

observed in the Cong Troi population ( $I = 0.415$  and  $PPB = 52.95\%$ ), followed by the Chu Yang Sin population ( $I = 0.390$  and  $PPB = 52.94\%$ ), the Yang Ly population ( $I = 0.373$  and  $PPB = 52.93\%$ ), and the lowest diversity in the Hon Giao population ( $I = 0.330$  and  $PPB = 47.06\%$ ). Genetic diversity at the species level was also determined ( $I = 0.414$ ,  $PPB = 76.19\%$ ).

**Table 3.** Genetic diversity parameters of the *Pinus krempfii* populations analyzed.

Population	$I$	PPB (%)
Yang Ly	0.373	52.93
Cong Troi	0.415	52.95
Hon Giao	0.330	47.06
Chu Yang Sin	0.390	52.94
Mean	0.377	51.47
At the species level	0.414	76.19

$I$  = Shannon's information index;  $PPB$  = percentage of polymorphic bands.

The degree of molecular variation was found to be low among populations (11.94%) and high among individuals within populations (88.06%), with a P value <0.001 (Table 4). To date, the genetic diversity of *P. krempfii* has not been assessed; however, in comparison to *Glyptostrobus pensilis* from Vietnam, molecular variation between populations of *P. krempfii* was lower (33.69%) but molecular variation among individuals within populations was higher (66.31%) (Tam et al., 2013).

**Table 4.** Analysis of molecular variance among/within *Pinus krempfii* populations.

Source of variance	Degrees of freedom	Sum of squares	Variance components	Total variation (%)	P value
Among population	3	19.992	0.289	11.94	<0.001
Within population	66	140.693	2.132	88.06	

Table 5 shows the genetic diversity within the four populations. The mean number of alleles per locus ranged from 1.765 (Yang Ly) to 2.118 (Chu Yang Sin), with a mean of 1.912. The effective number of alleles ranged from 1.477 (Chu Yang Sin) to 1.667 (Cong Troi), with a mean of 1.529. Only three populations were found to have private alleles; Yang Ly (0.059), Cong Troi (0.118), and Chu Yang Sin (0.294).  $H_E$  was low within the four populations (0.200-0.250) and at the species level (0.234), with the highest  $H_E$  in the Cong Troi population (0.250), and lowest in the Hon Giao population (0.200; Table 5).

**Table 5.** Number of alleles, number of haplotypes and genetic variation within *Pinus krempfii* populations.

Population	N	$N_A$	$N_E$	$A_p$	$H_o$	$H_E$	$F_{IS}$
Yang Ly	5	1.765	1.518	0.059	0.271	0.233	0.071
Cong Troi	11	1.941	1.667	0.118	0.316	0.250	-0.116
Hon Giao	26	1.824	1.455	0.000	0.292	0.200	-0.350
Chu Yang Sin	28	2.118	1.477	0.294	0.332	0.235	-0.316
Mean	17.5	1.912	1.529	0.118	0.303	0.229	-0.177
At the species level	70	2.471	1.521	-	0.310	0.234	-

N = population size;  $N_A$  = mean No. of alleles per locus;  $N_E$  = effective No. of alleles per locus;  $A_p$  = private No. of alleles per locus;  $H_o$  and  $H_E$  = mean of observed and expected heterozygosities;  $F_{IS}$  = Wright's inbreeding coefficient with  $P < 0.05$ .

At the population level, in all populations studied, only Yang Ly had a positive  $F_{IS}$  value of 0.071, while the three remaining populations had negative values (-0.116 for Cong Troi, -0.350 for Hon Giao, and -0.316 for Chu Yang Sin), with a P value <0.05 (Table 5). The obtained results suggest an excess of homozygosity and inbreeding in the Yang Ly population.

Further, of the 17 SSR markers analyzed, eight (PRE24, PtTX3020, Pt36480, Pt9383, PtTX3026, Pt26081, Pt30204, and RPS2) were found to be polymorphic (Table 6). Among these, the number of unique alleles was highest for marker Pt9383 ( $N_m = 10.796$  and  $F_{ST} = 0.023$ ), and lowest for marker Pt26081 ( $N_m = 1.813$  and  $F_{ST} = 0.121$ ) for the population *P. krempfii*. The number of different alleles at species level resulted in  $N_m$  and  $F_{ST}$  values of 4.919 and 0.064, respectively.

**Table 6.** The genetic parameters of all *Pinus krempfii* populations for eight polymorphic microsatelliteloci.

Primers	$N_A$	$N_E$	$H_O$	$H_E$	$F_{ST}$	$N_m$
PRE24	4.500	3.505	0.872	0.686	0.052	4.520
PtTX3020	3.500	2.507	0.953	0.592	0.047	5.085
Pt36480	2.000	1.361	0.181	0.240	0.076	3.049
Pt9383	2.000	1.917	0.694	0.476	0.023	10.796
PtTX3026	2.500	1.568	0.327	0.345	0.071	3.272
Pt26081	1.750	1.362	0.144	0.237	0.121	1.813
Pt30204	2.500	1.321	0.161	0.240	0.029	8.428
RPS2	3.750	2.454	0.812	0.584	0.095	2.391
Mean	2.813	1.999	0.518	0.425	0.064	4.919

$N_A$  = number of different alleles;  $N_E$  = number of effective alleles;  $H_O$  = observed heterozygosity;  $H_E$  = expected heterozygosity;  $F_{ST}$  = coefficient of gene differentiation;  $N_m$  = gene flow.

Pairwise Nei's genetic distance and identity were calculated for all populations (Table 7). The largest distance (0.098) was recorded between the populations of Yang Ly and Hon Giao and the smallest (0.030) between the populations of Hon Giao and Chu Yang Sin. The mean values of genetic distance between the studied populations ranged from 0.030 to 0.098, with a mean of 0.056. Similar to the results for genetic distance, the largest genetic identity (0.970) was found between the populations of Chu Yang Sin and Hon Giao, and the smallest (0.906) between the populations of Hon Giao and Yang Ly. The mean value of genetic identity between the populations ranged from 0.906 to 0.970, with a mean of 0.936. The obtained values of genetic distance indicate a low level of differentiation among the three populations in Lam Dong Province.

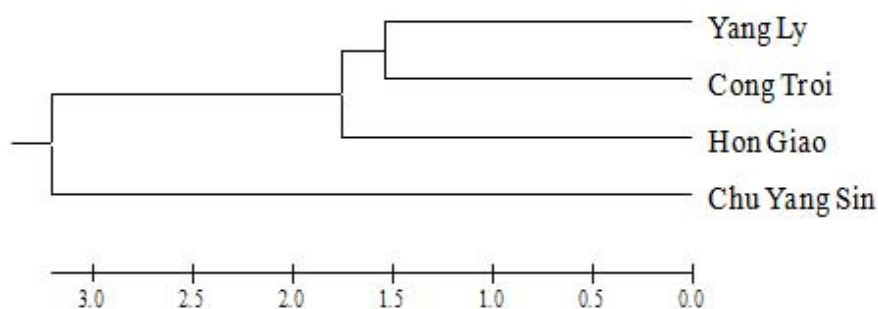
**Table 7.** Nei's genetic identity (above diagonal) and genetic distance (below diagonal) for four *Pinus krempfii* populations.

	Yang Ly	Cong Troi	Hon Giao	Chu Yang Sin
Yang Ly		0.947	0.906	0.907
Cong Troi	0.054		0.944	0.942
Hon Giao	0.098	0.058		0.970
Chu Yang Sin	0.097	0.060	0.030	

The UPGMA dendrogram (Figure 3) revealed genetic relationships among all populations investigated on the basis of the matrix of Nei's genetic distances between populations (Table 7). The four populations were divided into two major groups: the first group contained



only the Chu Yang Sin population of Dak Lak Province, and showed a relationship to the three other populations with genetic distances from 0.030 to 0.097. The second group contained the 3 populations of Lam Dong Province (Yang Ly, Cong Troi, and Hon Giao). The 2 populations of Yang Ly and Cong Troi were clustered together, with low genetic distance (0.054). Generally, populations from the same region tended to cluster together ( $P < 0.001$ ). The low distance between Yang Ly, Cong Troi, and Hon Giao is logical in Lam Dong Province, suggesting that the three populations share the same geological origin compared with the Chu Yang Sin population in Dak Lak Province.



**Figure 3.** UPGMA dendrogram based on Nei's genetic distance among four populations of *Pinus krempfii*.

## DISCUSSION

### Genetic divergence

Our first aim was to examine the genetic diversity, genetic differentiation, and population structure of *P. krempfii*. This species only grows in restricted habitats at higher altitudes in Vietnam's highlands, and is currently listed as vulnerable under the IUCN Red List of threatened species (Thomas et al., 2013). Most recently, the genetic diversity of populations of *P. krempfii* in Vietnam was also identified by Wang et al. (2014). In that study, 57 *P. krempfii* individuals were sampled from six natural populations and analyzed with respect to their sequence variation at ten nuclear gene regions (approximately 9 kb), 14 mitochondrial DNA (mtDNA) regions (approximately 10 kb), and seven chloroplast microsatellite (SSR) loci. The results showed very low nucleotide diversity at nuclear loci (0.0020) compared with *Pinus cembra* (0.0024) (Mosca et al., 2012). Furthermore, all investigated populations were monomorphic across all mtDNA regions, which have been identified as polymorphic in other pine species. Wang et al. (2014) also showed that the differentiation among *P. krempfii* populations at nuclear loci was very low (5.2%).

Compared to previous genetic diversity research in other *Pinus* species, Vietnamese *P. krempfii* showed the genetic diversity higher ( $PPB = 76.19\%$  and  $I = 0.414$ ) than *Pinus nigra* in China ( $PPB = 51.04\%$  and  $I = 0.262$ ), but lower than *Pinus sylvestris* in Portugal, Germany, and northern and southern Spain ( $PPB = 99.76\%$  and  $I = 0.690$ ) for ISSR markers (Rubio-Moraga et al., 2012; Cipriano et al., 2013).

The results obtained in the present study indicate high levels of genetic diversity within populations of *P. krempfii* in natural tropical mixed broadleaf forest in highland of Vietnam. The  $H_o$  values of 0.271-0.332, with a mean of 0.303, reported here are higher than those ob-

served for some other *Pinus* species using microsatellite markers. For example, for red pine,  $H_o$  ranged from 0.067-0.317 with a mean of 0.185 (Boys et al., 2005), and for *Pinus strobes*  $H_o$  ranged from 0.125-0.812 with a mean of 0.515 (Echt et al., 1996), but in *Pinus radiate*  $H_o$  values ranged from 0-0.85 with a mean of 0.625 (Smith and Devey, 1994). The  $H_e$  values for *P. krempfii* are much lower than the  $H_o$  values. The highest  $H_e$  values were observed in the Cong Troi population (0.250) and the lowest in the Hon Giao population (0.200; Table 5). Total genetic diversity for *P. krempfii* in this study ( $H_e = 0.229$ ) was higher than *P. nigra* ( $H_e = 0.175$ ) and *Pinus squamata* ( $H_e = 0.029$ ) (Zhang et al., 2005; Rubio-Moraga et al., 2012) and lower than some other *Pinus* species, which were similarly analyzed with ISSR markers, such as *Pinus tabulaeformis* ( $H_e = 0.4152$ ; Wang and Hao, 2010), *Pinus koraiensis* ( $H_e = 0.3477$ ; Feng et al., 2006), *Pinus sibirica* ( $H_e = 0.2699$ ; Yang et al., 2005), *P. sylvestris* ( $H_e = 0.2620$ ; Liu et al., 2005;  $H_e = 0.217$ -0.310; Labra et al., 2006; Table 5). In all populations in the current study, five of the eight loci showed significant heterozygote deficiency. Despite the lower levels of  $H_o$  and relatively few polymorphic loci used, the average of 5.25 alleles per locus (data not shown) across the eight polymorphic loci characterized for *P. krempfii* was comparable to or lower than microsatellite-based genetic studies of other pines, including six alleles per locus observed in *P. radiate* (Smith and Devey, 1994), 5.4 alleles per locus in *P. strobes* (Echt et al., 1996), and 6.7 alleles per locus in *P. sylvestris* (Soranzo et al., 1999).

This study showed that genetic diversity of *P. krempfii* was highest in the Chu Yang Sin population and lowest in the Hon Giao population. This may be related to the geographic position of the Chu Yang Sin population in Dak Lak Province compared to the three other populations in Lam Dong Province. During field expeditions, we recognized that most genotypes of the Hon Giao population were younger plants with heights of 10 m. Therefore, these may have originated from a mother tree.

The differences in the levels of genetic diversity among four populations may be related to geographic distribution, the number of populations tested, population size of the species, and the effect of climate change during the last glacial maximum.

Based on the  $F_{ST}$  and  $N_m$  values in the present study, gene flow is high and a moderate level of genetic diversity is maintained within the populations; however, this is susceptible to genetic drift. This level of differentiation is comparable to pine species with wide distribution ranges (Slatkin, 1985; Cipriano et al., 2013). Due to the lack of a geographical barrier between sampled populations of *P. krempfii*, the population differentiation in this species could have been caused by the fragmented nature of its distribution. Unlike most other pines, *P. krempfii* does not form pure stands and individual populations consist of small groups of trees dispersed among dense thickets of other tree species (Luu and Thomas, 2004). These conditions are likely to limit dispersal of its pollen and seeds, and contribute to differentiation between local populations. Furthermore, *P. krempfii* is distributed in a wet rainforest environment, which could preclude efficient wind pollination (Turner, 2001). High humidity dampens pollen grains, and heavy rains wash away pollen from the air. In summary, despite relative proximity of individual populations, the low population density of *P. krempfii* and the humid environment have limited gene flow and led to a certain degree of population differentiation. These findings indicate that even species with a very limited distribution may harbor genetically differentiated populations.

### Conservation considerations

The main objective in any plant genetic resource conservation program should be to

maintain the highest possible level of genetic variability (Karp, 1997). According to the results of our field survey, anthropogenic activities, such as overexploitation and deforestation, have undoubtedly influenced the natural habitats and reduced the area of *P. krempfii* distribution.

The very low genetic polymorphism in the Hon Giao population and the restricted distribution of *P. krempfii* suggest that the population is exposed to a considerable risk of extinction. Although most of the extant populations of *P. krempfii* are currently under legal protection in national parks in Vietnam, they face serious threat and risk of extinction by stochastic processes because of their small size. Population size is the most important of five criteria for listing species as endangered under the IUCN system (Thomas et al., 2013), and the loss of genetic variation may decrease the potential for a species to persist through biotic and abiotic changes. Thus, efforts should be made to increase the genetic diversity and population size of *P. krempfii*. *P. krempfii* does not form pure forests and typically occurs as small groups of 10-30 trees in dense subtropical forests (Luu and Thomas, 2004). We have assessed the ability of *P. krempfii* to seed, germinate, and grow of seedling at the four collected sites. The results showed a high ratio of germinated seeds and seedlings were detected in *P. krempfii* populations. However, only a very low percentage of them develop into trees with a height of 2 m (data not shown). The reason for this is a lack of nutrition, as gaps (up to 50 cm in diameter) are often formed in the humus layer, as well as topography fractures.

A loss of habitat may have decreased the population size of *P. krempfii* in the past and could prevent recovery of populations in the future. Therefore, the first effort to recover the extant *P. krempfii* populations should be the protection and restoration of the habitat that *P. krempfii* is adapted to. *In situ* conservation alone, however, cannot conserve and recover the species, because of the restricted distribution of *P. krempfii*. The results of the most recent assessment by Thomas et al. (2013) highlighted the most serious long term threat to *P. krempfii* as being habitat fragmentation resulting from infrastructure developments, increasing incidence of fires throughout the region, conversion of lower altitude forests to fire-prone *P. kesiya* forests, and encroachment into protected areas for coffee production. In addition, illegal logging aimed at *P. krempfii* in Chu Yang Sin has recently been reported (Thomas et al., 2013). Therefore, *ex situ* conservation should also be given high priority to offset habitat deterioration and fragmentation. In this regard, introductions can be designed to establish self-sustaining wild populations, and this practice should be carried out in suitable habitats. Thus, traditional breeding practices, such as controlled crosses between genetically distinct populations, even between individuals of the same stand, could be helpful to restore and enrich genetic diversity of *P. krempfii*. Controlled crosses are important genetic tools for both breeding and conservation of wild populations of economically and ecologically important plant species.

To preserve Vietnam's valuable and unique flat-needle pine and its genetic resources, given the current conservation situation, we recommend a combination of conservation measures that include *ex situ* and *in situ* conservation. First, it is necessary to closely control the growth of seedlings on site to ensure the permanent contact of their roots and the soil. Second, we recommend the construction of a core germplasm repository and the collection of germplasm resources in greater breadth and depth. This will allow the seeds to be collected and exchanged between populations, increasing the genetic diversity in each area, and conserving the scarce germplasm resources through natural regeneration. In fact, more attention should be given to *ex situ* efforts because it would be difficult to implement *in situ* conservation for all the populations due to the large distribution area and dispersal. Third, *in situ* conservation should be implemented immediately. Given their high level of genetic diversity and desirable

growth patterns in their original habitat, populations in Yang Ly, Hon Giao, and Cong Troi of Lam Dong Province and Chu Yang Sin of Dak Lak Province should be assigned high priority. Additionally, the genetic diversity of the two subgroups identified here should be protected, and a priority conservation measure should be instituted for the population in Chu Yang Sin. These measures should include the establishment of nature reserves and forest reservations, and prevention of illegal exploitation. Fourth, seed collection activity using wild resources should be promoted and controlled, and the development of cultivated *P. krempfii* resources should be encouraged.

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