Genetic diversity of Pinus kesiya in the Central Highlands of Vietnam

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Abstract

Four *Pinus kesiya* natural populations in the Central Highland region of Vietnam, separated from one another by distances of 75 to 380km, were examined using tetranucleotide microsatellite markers to evaluate their genetic diversity and population structure. The surveyed populations displayed relatively high level of genetic variation (H_E = 0.671). Only between the Kon Tum and Dak Nong populations was the pairwise value F_{ST} significant. These two populations, separated by 300 km, also showed the greatest separation in the UPGMA cluster analysis using Nei's pairwise genetic distance. The UPGMA analysis clustered the four populations into two geographic groups (1) the Kon Tum population, which is located in the North of the Central Highlands and (2) the remaining three populations (Gia Lai, Dak Nong and Dak Lak). Within group 2, Gia Lai and Dak Lak located in the center area of the Central Highland, clustered into the same subgroup with the southern Dak Nong population a single subgroup. This topology was essentially in agreement with the geographic distribution of the studied populations. The implications for conservation and development programs for this species are also reported and discussed.

Introduction

Khasi Pine (*P. kesiya*) is a large tree, reaching heights up to 45 m and diameters at breast height up to 100 cm. It occurs in open pure stands, on steep slopes at elevations of 300-2700m, in Cambodia, China, India, Laos, Myanmar, the Philippines, Thailand and Vietnam (Turnbull et al. 1980). The Khasi Pine wood is used for various purposes, for example, construction, boxes, flooring, ceilings, furniture and resin (Hansen et al. 2003). Being a pioneer species, it can grow on soils deficient in organic matter and with low water holding capacity, and nutrient availability (Armitage and Burley 1980). The species has also performed well in provenance trials in locations beyond its natural distribution (Costa e Silva and Graudal 2008). It, therefore, holds great promise for use in afforestation programs.

In Vietnam, *P. kesiya* occurs mainly in the Central Highlands, located in the Southwest of the country. It is recognized as multipurpose species and is considered an important forest tree in Vietnam (Costa e Silva and Graudal 2008). However, the natural forests of *P. kesiya*, in both Vietnam and other parts of its natural range continue to be reduced and fragmented (Nguyen et al. 2008). Genetic conservation programs should be developed to protect the valuable genetic resources of *P. kesiya* in the remaining fragmented natural populations in Vietnam. An understanding of the population genetic structure, gene flow and mating systems of this species is important for protecting and managing the existing populations effectively (Allendorf and Luikart 2006).

It is increasingly interested in expanding the planted area of the species in Vietnam and also in other countries (Costa e Silva and Graudal 2008). Previous research suggests that *P. kesiya* sources from Vietnam could make an important contribution to breeding as they display favourable combinations of growth and wood quality traits (Costa e Silva and Graudal 2008; Hansen et al. 2003). Breeding and deployment programs for better growth, pulp wood and/or resin production are required (Costa e Silva and Graudal 2008). Knowledge of genetic diversity and mating patterns of this species would provide

essential information and fundamental guidance for breeding strategies and programs (White et al. 2007). However, to date, there are no published studies evaluating the genetic diversity and population structure of *P. kesiya* in Vietnam. Therefore, the estimate of genetic variability and relationship among the natural population of the species plays an important role in breeding programs for this species.

For population genetic studies, microsatellite or simple sequence repeat (SSR) markers are considered the molecular markers of choice, due to their abundance, high polymorphism, reproducibility and co-dominant inheritance (Li et al. 2002; Vieira et al. 2016). Advantages of SSR markers over other PC- based markers have been well established in many crop and tree species (Nowakowska 2016; Sumathi and Yasodha 2014; Tsumura 2004; Varshney et al. 2005; Vieira et al. 2016; Viruel et al. 2010). Microsatellite markers have been used to estimate genetic diversity, population structure, breeding system and parental analysis in various *Pinus* species, for example, *P. sylvestris* (Aleksandar et al. 2014; Cipriano et al. 2013), *P. caribaea* (Camacho et al. 2018; De Furlan et al. 2007); P. *merkusii* (Nurtjahjaningsih et al. 2007; Thao et al. 2013a)d *kesiya* (Cai et al. 2017; Rai and Ginwal 2018; Rai and Saha 2018).

In this study, SSR markers were used to examine the genetic diversity and population structure of four natural populations of *P. kesiya* in some provinces of Central Highland region in Vietnam. This research aimed to estimate genetic diversity at population levels, examine the genetic variation within and among populations and evaluate the genetic relationship among them.

Materials And Methods

Study sites

The study was conducted on four populations of *P. kesiya* from the Central Highland in Southwest Vietnam. These populations are located in four provinces; namely Kon Tum, Gia Lai, Dak Lak and Dak Nong. The geographic distribution of the four surveyed populations is shown in Table 1 and Appendix 1. These fragmented natural populations were selected due to their isolation, caused by deforestation, from the others by other vegetation types and also *P. kesiya* plantations.

Table 1
Sampling location and geographic distribution of *P. kesiya* populations

Sampling location	*Longitude (E)*	*Latitude (N)*	*Sample size*
Kon Tum	108.390770	14.622455	30
Gia Lai	108.042083	14.005949	30
Dak Lak	108.038735	12.650598	30
Dak Nong	107.802354	12.101364	30

Plant materials and DNA extraction

A total of 120 trees, with an age of over 20 years, were sampled to obtain fresh leaves from each Kon Tum, Gia Lai, Dak Lak and Dak Nong provinces (30 trees per province each province) (Table 1). Fresh needles were collected from the adult trees at a distance of > 100m to others in the fragmented natural stands. Needle tissue was stored at – 30^{o}C until DNA was extracted. Total genomic DNA was then extracted using the ISOPLANT II (NIPPON GENE). The extracted DNA were purified and stored at – 30^{o}C until PCR amplifications were performed.

PCR amplification

Ten polymorphic tetranucleotide microsatellite markers developed for *P. kesiya* (Thao et al. 2013b) were applied in the study (Table 2). Based on the size range and the annealing temperature of each microsatellite marker, the experiment plan was designed for efficient amplification.

Table 2
Summary data of the 10 microsatellite markers

Locus	Primer (5’ – 3’) Forward and Reverse	Size range (bp)
PK701	F: TTGGATTGGTATCTGTCCCATT	340–396
	R: TGCCAAAGTGGTTTATGGTTC	
PK704	F: ATATAATTGGGAATTTAGGGGGAC	283–295
	R: TGCACCAATGTGTGTAATCGAA	
PK827	F: AGTCATAGTACACTCCAAGGCTTA	212–228
	R: GCGAATTGATTGACTCCATTGCTT	
PK830	F: TCACAACCCTGTCAGACATCG	105–145
	R: CAAGAAGATGATTGTCTTATGCTA	
PK832	F: ATCAATTCATTGGAGCTTGGG	205–221
	R: GCAGAAAACAAGATTAAACTTCA	
PK836	F: AAAACCTTACTTAGTCAAACCCTC	411–435
	R: AAAGGACTCTCGACATAAACGTG	
K838	F: GAGCTCATGTTATTGAAAATCCTT	514–518
	R: GGCAACATACATGAACTATGCATT	
PK840	F: TATGTGCGTGTGTTTAATGGTTT	176–208
	R: TCGATTTATTTGACAGGTCTGG	
PK708	F: ACACACAAAACCTTTTATAGGCAT	225–362
	R: ATCGTTCTAATTGTCGCATATCGG	
PK711	F: ATCAATCATCGTCAACTATGCACA	311–402
	R: TGACCATGATTCACAATGCTCGAT	

The ten microsatellite markers were amplified in two multiplexes and four single reactions (Table 3). PCR reactions were performed in a 10 µL volume containing a final concentration of 40 ng of genomic DNA, 1 x Multiplex PCR Kit master mix (QIAGEN) and 0.2 µM of each primer. Amplifications were carried out on a SureCycler 8800 (Agilent Technologies). A touchdown PCR technique was applied with the conditions as follows: initial hold of 95^{o}C for 15 min, then 10 cycles of denaturation at 94°C for 30s, annealing for 90s, and extension at 72°C for 60 min. The annealing temperature decreased 0.7°C every second cycle from 7°C above the optimum annealing temperature (Ta) of the primer pair (Ta in shown in Table 3), followed by 25 cycles of 30 s at 95^{o}C, 90 s at the optimum annealing temperature and 30 s at 72^{o}C, and a final

elongation for 30 min at 60°C. The PCR products were run in an automated DNA sequencer (Applied Biosystems, ABI PRISM 3130). DNA fragments were scored with the aid of the Gene Mapper software Version 3.7 (Applied Biosystems).

Table 3
Summary data for PCR design for amplifying 4 populations of *P. kesiya*

Locus	Size range (bp)	Fluorescent dye	Annealing temperature (°C)	PCR types
PK830	105–145	FAM	50	Single
PK840	176–208	VIC	50	Single
PK711	311–402	VIC	55	Multiplex
PK836	411–435	VIC	55	
PK838	514–518	VIC	55	
PK832	205–221	PET	57	Multiplex
PK704	283–295	PET	57	
PK701	340–396	PET	57	
PK827	212–228	NED	53	Single
PK708	225–362	FAM	60	Single

Statistical analysis

The genetic variability in each population was measured using the mean number of alleles per locus (MNA), mean effective number of alleles per locus (MNE), observed (H_O) and expected (H_E) heterozygosities (Nei 1978), and inbreeding coefficient (F_{IS}). For each microsatellite locus, genetic polymorphism was assessed by calculating the total number of alleles per locus (na), effective number of alleles per locus (ne), polymorphic information content (PIC), observed (H_O) and expected (H_E) heterozygosities, and *F*-statistic (F_{IS}, F_{ST}, F_{IT}) according to Nei (1978) and gene flow (Nm). CERVUS 3.0.3 software (Kalinowski et al. 2007) was used to estimate na, H_O, H_E and PIC. Inbreeding coefficient (F_{IS}) and *F*-statistic were calculated by POPGENE 1.32 software (Yeh et al. 1997). Hardy–Weinberg Equilibrium (HWE) based on 1000 permutations the linkage disequilibrium (LD) were examined using GENEPOP software (version 4.0.10) (Raymond and Rousset 1995; Rousset 2008).

The population genetic divergence was measured by the standard method of the estimation of F_{ST} using GenAlEx version 6.5 (Peakall and Smouse 2012). The average level of gene flow (Nm = $(1 - F_{ST}) / 4F_{ST}$) among populations was indirectly estimated by a traditional method based on F_{ST} value (Slatkin and Barton 1989). Pairwise comparisons of population genetic differentiation (F_{ST}) and significance were

calculated. Using the same program, genetic variation within and among populations was further partitioned by analysis of molecular variance (AMOVA).

To build the phylogenetic tree, the POPGENE 1.32 software (Yeh et al. 1997) was adopted based on Nei's (1978) unbiased genetic distance with UPGMA method modified from NEIGHBOR procedure of PHYLIP Version 3.5.

Results

Genetic variability

Ten microsatellite markers were successfully amplified in the four *P. kesiya* populations. The genotypic data of four populations is presented in Tables 4, 5, 6 and 7. A total of 109 alleles were observed from the 120 DNA samples (Table 4). The number of alleles per locus varied from 3 (PK838) to 32 (PK708) with a global average of 10.9 alleles per locus. The majority of the markers showed relatively highly polymorphism, with three loci (PK701, PK708 and PK711) exhibiting more than 16 alleles. Only three markers (PK 704, PK 827 and PK 838) with fewer than five alleles were detected.

Table 4
Variability parameters in the four populations in Vietnam

Locus	Na	Ne	PIC	H_O	H_E	HW
PK701	16	9.12	0.881	0.810	0.896	NS
PK704	5	1.91	0.404	0.452	0.478	NS
PK827	5	3.27	0.638	0.679	0.698	NS
PK830	9	3.39	0.671	0.723	0.710	NS
PK832	6	1.40	0.263	0.125	0.290	*
PK836	9	2.92	0.622	0.583	0.662	NS
PK838	3	1.97	0.377	0.548	0.496	NS
PK840	8	4.08	0.718	0.690	0.759	NS
PK708	32	8.85	0.879	0.619	0.892	*
PK711	16	5.52	0.799	0.518	0.824	*
Average	10.9	4.24	0.625	0.575	0.671	

*(Na: total number of alleles detected per locus; Ne: effective number of alleles per locus; PIC: polymorphic information content; H_O: observed heterogygosity; H_E: expected heterogygosity; HW: test for Hardy-Weinberg equilibrium, *: significant deviation from Hardy-Weinberg equilibrium at $P \leq 0.01$; NS: no significant deviation from Hardy-Weinberg equilibrium)*

Table 5
Variability parameters in the four populations in Vietnam

Sampling location	n	MNA	MNE	H_O	H_E	F_{IS}
Kon Tum	30	7.4	3.8	0.584	0.677	0.118
Gia Lai	30	6.3	3.8	0.619	0.670	0.049
Dak Nong	30	6.2	3.7	0.558	0.650	0.121
Dak Lak	30	7.2	4.0	0.550	0.656	0.143

(N: sample size; MNA: mean number of alleles per locus; MNE: effective number of alleles per locus; F_{IS}: inbreeding coefficient)

Table 6
Summary of *F*-statistic (F_{IS}, F_{IT}, F_{ST}) and gene flow in 10 loci

Locus	F_{IS}	F_{IT}	F_{ST}	Nm*
PK701	0.0698	0.1011	0.0337	7.1685
PK704	0.0417	0.0637	0.0229	10.6589
PK827	-0.0298	0.0252	0.0534	4.4351
PK830	-0.0473	-0.0346	0.0121	20.4159
PK832	0.5631	0.5792	0.0369	6.5318
PK836	0.0798	0.0953	0.0169	14.5665
PK838	-0.1888	-0.1098	0.0664	3.5162
PK840	0.0692	0.0869	0.0190	12.8944
PK708	0.2787	0.2948	0.0224	10.9132
PK711	0.3229	0.3465	0.0348	6.9321
Average	0.1077	0.1349	0.0306	7.9320

(Nm: Gene flow estimated from F_{ST} ($Nm = 0.25(1 - F_{ST})/F_{ST}$)).*

Table 7
Matrix of pairwise comparisons of population genetic differentiation (F_{ST}) (below diagonal) and significance (above diagonal)

	Kon Tum	Gia Lai	Dak Nong	Dak Lak
Kon Tum		NS	***	NS
Gia Lai	0.027		NS	NS
Dak Nong	0.024	0.006		NS
Dac Lak	0.001	0.003	0.014	
*(***, P < 0.005; NS, not significance)*				

Over all four populations, three microsatellites (PK832, PK707 and PK711) showed significant deviations from Hardy-Weinberg equilibrium. The lowest observed heterozygosity was 1.25 for the PK832 locus and the highest was 0.810 for the PK701 locus, with a mean observed heterozygosity value of 0.575. The expected values of heterozygosity ranged from 0.290 (PK832) to 0.896 (PK701), with a mean value of 0.671 for the 10 markers. The expected heterozygosity value for each locus was higher than that of the observed heterozygosity for all but one locus (Table 4). The polymorphic information content (PIC) values ranged from 0.263 (PK832) to 0.881 (PK701), and the average value was 0.625.

Genetic variability within populations was high as indicated by the genetic variability parameters calculated for each population (Table 5). Kon Tum and Dak Lak populations exhibited a higher average number of alleles per locus; the figures were 7.4 and 7.2 respectively. The Dak Nong population showed the lowest diversity in terms of the mean number of detected alleles per locus (6.2). The observed heterozygosity for the five populations ranged from 0.550 (Dak Lak) to 0.619 (Gia Lai). The gene diversity (mean H_E) values varied from 0.650 (Dak Nong) to 0.677 (Kon Tum) (Table 5).

The inbreeding coefficient (F_{IS}) was estimated for each locus in the total population (Table 6) and individually for each population (Table 5). Table 6 also showed the overall means of F_{IS}, across all loci for each population. For the individual markers the F_{IS} values were comparatively high at three loci, PK708 and PK 711 and PK832 with the inbreeding coefficient value being 0.279, 0.323 and 0.563 respectively. An overall deficit of heterozygotes (F_{IS}) of 10.8% was found in the surveyed loci within the populations, whereas the total population F_{IT} was 13.5%. Considering the individual populations, there is a significant difference in F_{IS} among populations, with the lowest heterozygote deficit was observed in Gia Lai (0.049) and the highest was in Dal Lak (0.143).

Genetic differentiation and gene flow

The global genetic differentiation across all populations estimated by F_{ST} was 0.031 (Table 6). The indirect estimator of gene flow (Nm) among populations, inferred from F_{ST}, was 7.93. Pairwise F_{ST} values among pairs of populations ranged from 0.003 (between Gia Lai and Dak Lak) to 0.027 (between Gia Lai and Kon Tum) (Table 7). Only the pairwise value of F_{ST} between Kon Tum and Dak Nong populations was significant. These two populations have the greatest geographic separation, being at the north and south of the Central Highland. This finding is in agreement with the UPGMA analysis. At the population level, AMOVA analysis showed that 2% of the total molecular variation was attributed to inter-population differentiation, and 98% to individual differentiation within populations (Table 8).

Table 8
Analysis of molecular variance for 120 studied samples (Statistical significance is based on 1000 permutations).

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation (%)
Among Pops	3	36.205	0.199	2
Within Pops	80	633.580	7.920	98
Total	83	669.786	8.118	100

Cluster analysis

The genetic relationship among populations was examined by UPGMA cluster analysis using Nei's pairwise genetic distance. UPGMA analysis clustered 4 populations into 2 geographic groups (Fig. 2). Group 1 comprised only the Kon Tum population. Group 2 included the remaining three populations (Gia Lai, Dak Nong and Dak Lak). Within group 2, Gia Lai and Dak Lak populations were clustered into the same subgroup. These two populations are relatively close to one other (Appendix 1)/ The Dak Nong population in the south of the Central Highland formed as a single subgroup under group 2. This topology was essentially in agreement with the geographic locations of the four studied populations.

Discussion

Amplification of microsatellite markers

Ten microsatellite markers developed for *P. kesiya* were successfully amplified in this research. The majority of the markers were highly polymorphic (Table 4). Barker (1994) suggested that in diversity studies, the loci with at least four different alleles should be used in order to reduce the standard error of the estimated genetic distances. Except for one marker with 3 alleles, the total number of alleles per locus in the present study ranged from 5 to 32. The remaining markers are reliable tools for genetic studies in the species. Three markers (PK830, PK708 and PK711) showed significant deviations from the Hardy–Weinberg equilibrium, caused by a deficiency of heterozygotes (Table 4).

Genetic variation

In Vietnam, *P. skesiya* had a relatively high level of genetic variation (H_E = 0.671) in four sampled populations. These findings were in line with those in the previous research in other countries (Rai and Ginwal 2018; Rai and Saha 2018; Szmidt et al. 1996). Rai and Ginwal (2018) also determined the high level of genetic diversity (H_T=0.638) of 10 *P. kesiya* populations in India by using spSSR. Szmidt *et al.* (19996) found that *P. kesiya* possessed considerable allozyme variation and showed weak inter-population differentiation. In particular, the expected heterozygosity of *P. kesiya* was relatively high (from 0.117 to 0.160) compared to that of *P. merkusii* (from 0.016 to 0.075) (Szmidt et al. 1996). Using 7 SSR markers to evaluate the genetic diversity of 70 samples of *P. merkussi* in the Cijambu Seedling Seed Orchard (SSO) (Sumedang, West Java, Indonesia), Susilowati et al. (2018) found a moderate value of genetic diversity in the set of studied samples (H_E= 0.566), this value was also slightly lower than that of *P. kesiya* in this present study. According to Atta-Krah et al (2004), the ability of populations to respond to changing environments is influenced by genetic variation. The high value of genetic diversity of *P. kesiya* might considered as one of the reasons for the wide adaptation of the species (Costa e Silva and Graudal 2008; Hansen et al. 2003).

Nei (1978) showed gene diversity to be an appropriate measure of genetic variation within populations. The values of the observed and expected heterozygosity varied among populations, and the observed heterozygosity was lower than the expected. The majority of the loci in all populations showed heterozygote deficiency (Table 4). The heterozygote deficiency observed in the majority of loci may be related to the historical evolution of the species. Deforestation which divides natural populations into small and isolated remnants (Nguyen et al. 2008) might have led to inbreeding and genetic drift. The moderate value of F_{IS} (0.108) suggested heterozygote deficit in the loci in the total populations (Table 5). This may be a result of inbreeding in small and fragmented populations.

Gene flow

In this study, low genetic differentiation among populations was suggested by F_{ST} = 0.031. Similarly, low inter-population genetic divergence was also demonstrated by the AMOVA analysis which showed that only 2% of the total molecular variances were attributed to inter-population differentiation (Table 8). The low genetic differentiation observed suggested a correspondingly high rate of gene exchange among *P. kesiya* populations (Nm = 7.93) (Table 6). These findings were consistent with the gene flow among four *P. kesiya* populations in Thailand as indicated in Boyle et al. (1990). Pine seeds are adapted for dispersal by wind, birds, and mammals, including humans. Characterized by light wings, pine seeds can disperse far away from a parent tree by the wind. Moreover, pine pollen developed two wings, air bladders that facilitate dispersal long distances (Boyle et al. 1990; Ledig 1998; Williams 2010). These special systems of seed and pollen dispersal of pine species might greatly enhance the gene flow among populations of *P. kesiya*.

Implication for management

Genetic variation is important for a species to maintain its evolutionary potential to cope with ever-changing environments (Allendorf et al. 2013; Frankham et al. 2002). Previous studies on plant conservation mostly focused on the work about rare and endangered plants. Given the increasing deterioration of habitats, effective measures should also be taken to protect the plants which are important to ecosystems or are important genetic resources for breeding (White et al. 2007). The information gained on the levels and distribution of microsatellite variation in the natural populations of *P. kesiya* can be used to suggest appropriate management strategies. Genetic analysis showed that *P. kesiya* populations in the Central Highlands were historically in good genetic health, evidenced by the high level of genetic variation, high rate of gene exchange among populations and predominant outcrossing reproductive system.

On the other hand, the presence of inbreeding in the studied populations evidenced by a moderate value of F_{IS} (0.108) (Table 7) indicates that deforestation has affected the genetic diversity of *P. kesiya*. Considering the long-lived life history of the species, the adverse impact of human activities on genetic diversity will probably become more evident in future generations, which highlights the need for continued genetic monitoring of *P. kesiya* populations. The result that up to 98% of microsatellite variation is partitioned within populations is instructive for adopting a plan of involving fewer populations but more individuals within populations. Moreover, possessing rich genetic diversity and significant differentiation in Kon Tum population suggested that conservative resources for this population should be prioritized to protect valuable genetic resources.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Thao Van Duong and Son Le. The first draft of the manuscript was written by Thao Duong Van and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

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Figures

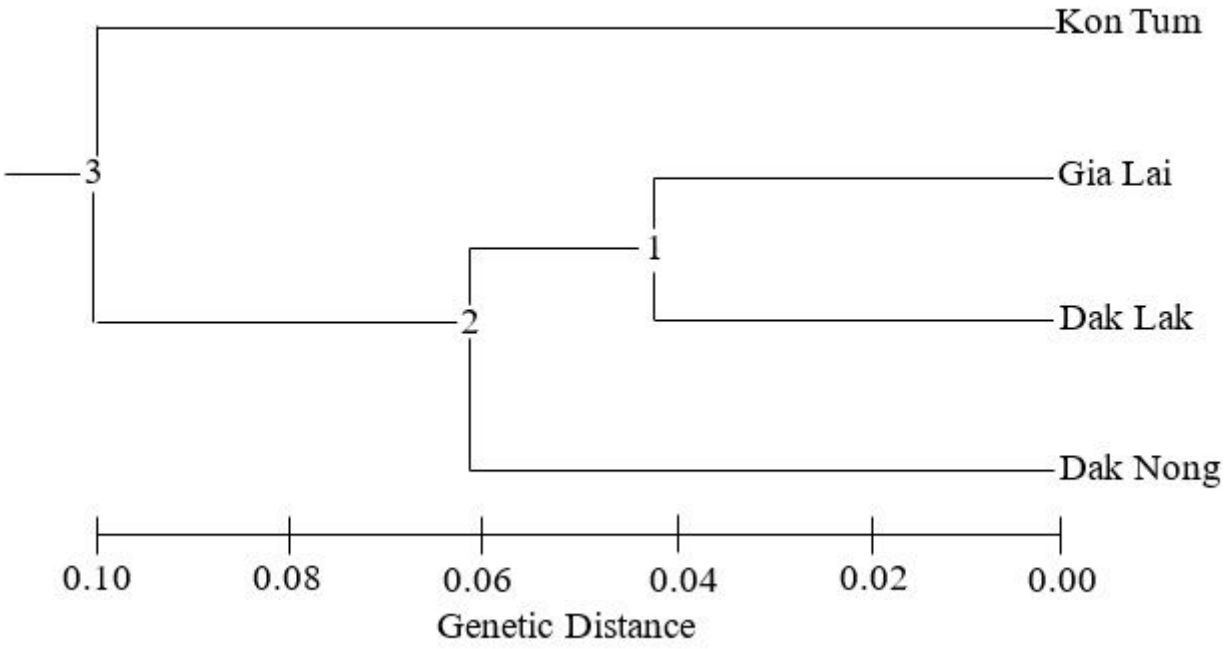

Figure 1

UPGMA dendrogram of the 4 populations based on Nei's (1978) genetic distance.

Supplementary Files

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- Appendix1.png