Microsatellite analysis to study genetic diversity in Khasi Pine (*Pinus kesiya* Royle ex. Gordon) using chloroplast SSR markers

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Abstract

Pinus kesiya (Khasi pine) is the principal pine species in northeast India having high commercial value. Chloroplast microsatellites (cpSSR) were used to study the genetic diversity and population genetic structure of 10 populations of *P. kesiya* covering entire natural range of distribution in India. A total of 33 primer pairs (cpSSRs) of *P. thunberghii* and *P. sylvestris* were tested in *P. kesiya* for their transferability, out of which 18 chloroplast primers showed positive amplification and 10 were found polymorphic. A total of 250 individuals from 10 different populations were genotyped using the selected 10 cpSSRs. When alleles at each of the 10 loci were jointly analysed a total of 36 size variants were discovered, which combined to designate 90 haplotypes among 250 individuals. None of the haplotype was found common among the populations as they were population specific. The cpSSR indicated that *P. kesiya* populations have maintained a moderately high genetic diversity (H_T=0.638) which is typical in most coniferous species. However, the inter-population genetic diversity was higher than the intra population diversity and the genetic differentiation between populations was also found to be very high (F_{ST}=0.47). A Bayesian cluster analysis separated the populations into six clusters where most of the individuals were found in single population clusters with minor admixtures. The distribution of genetic diversity and sub structuring of *P. kesiya* reflect week pollen mediated gene flow due to geographic isolation and genetic drift. The study has revealed useful cpSSR markers for *P. kesiya*, which were lacking earlier and also added an insight into the state of Khasi pine forest in the region, which can be useful for the better management and future conservation programs.

Keywords: : *Simple sequence repeats, cpSSR, genetic diversity, Khasi Pine, P. kesiya.*

Introduction

The genus *Pinus* is evolutionarily an ancient genus (Mirov, 1967; Miller, 1982) and is native to tropic as well as the temperate climate. They are widespread in South-East Asia and in India, they are found abundantly in the Himalayas with five species occurring naturally, viz., *Pinus roxburghii* Sargent*, P. wallichiana* Jackson*, P. gerardiana* Wall*, P. kesiya* Royle ex Gordon and *P. merkusii* Jungh and de Vriese. *P. kesiya* locally known as Khasi pine in India has a natural range of distribution in the Khasi and Jaintia hills in Meghalaya andoccurs in small patches in the states of Manipur, Nagaland and Arunachal Pradesh. It is the only sub-tropical pine that grows in the eastern Himalayas atelevations ranging from 800 to 2000 m (Chaudhary and Bhattacharyya, 2002). It is a major source of timber in the region and the locals exploit this species for resin extraction, firewood, etc. Also this fast-growing three-needle pine has high commercial value for its pulp and oleoresin and is subjected to overexploitation. Furthermore, fast paced urbanization in otherwise less populated habitats and unchecked developmental activities are having a negative impact on this species due to fragmentation of its habitats. Due to this, the pine forests are at a risk of losing their genetic base which is important for their sustenance and survival.

In recent years, special emphasis is given to forest conservation owing to the rapid loss of forest cover due to unrestrained urbanization and climate change. In order to conserve these fast diminishing natural forests, it is important to understand the extent of genetic diversity of the species under threat. Molecular marker techniques have helped immensely in assessing the genetic resources of plants (Porth and El-Kassaby, 2014) and levels of genetic diversity in tree species. It has helped to generate information and database which will be a step forward in pin pointing those populations which require special attention in terms of conservation and management.

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Microsatellite markers are frequently used for assessing the levels of genetic diversity as well as the genetic structuring at both population and species level in order to develop suitable conservation strategies (Frankham et al., 2002; Lopez-Vinyallonga et al., 2011). They are becoming an extremely useful tool in population genetics due to their high variability and co-dominance (Provan et al., 1999). Moreover, high mutation rate of SSRs results in large standing allelic diversity (Ellegren 2004) and provides every individual with a unique genotype (Queller et al. 1993) using only a few loci (Ganea et al., 2015). Simple sequence repeats (SSRs) have been used extensively for characterizing the genetic diversity of Pine species all over the world. Recently, Cai et al. (2017), developed microsatellite markers for *P. kesiya* var. *langbianensis*, but still, very few microsatellite markers have been developed and reported in *P. kesiya* species. Since the cost and effort to procure SSRs is still significantly high, it is common practise to cross amplify primers designed from different sources in the target species (Moreno et al., 2011). Although *P. kesiya* is one of the principle species in the region having multiple uses, very little attention is given with regard to the conservation of its genetic resources mainly due to the unavailability of information regarding the genetic diversity of this species. The present work was undertaken not only to disclose the genetic diversity and population structure of *P. kesiya* in this region but also to identify cpSSR primer pairs for this species through trans-specific amplification from other species (i.e. *P. sylvestris*, Provan et al. 1998; and *P. thunberghii*, Vendramin et al. 1996).

Chloroplast microsatellite markers are highly variable having a relatively high rate of sequence evolution (Provan et al., 1999; Kelchner 2000; Diekmann et al., 2012).Bayer et al.(1999), reported that as chloroplast genomes show uniparental inheritance and do not recombine during sexual reproduction, different microsatellite loci are linked together and individual haplotypes can be easily detected by applying a set of different chloroplast microsatellite markers. The small genome size of cpDNA and the absence of sexual recombination make cpSSRs ideal markers for population and ecological genetics studies (Morgante et al., 1996).

In this paper, we have used chloroplast microsatellites to study 1) the levels of haplotypic diversity existing in populations and (2) the population genetic structure of the existing populations of *P. kesiya* along the whole distributional area in North East India. Ten cpSSR loci from 250 trees from 10 sites in the core distribution range of the species across Himalayan Mountains covering four states viz. Meghalaya, Arunachal Pradesh, Manipur and Nagaland were analysed. The information will be useful to guide gene conservation strategies for the species.

Materials and Methods

Plant material

Needles were collected from 10 geographically distinct populations from their natural range of distribution in Northeast India, covering 4 states (Fig. 1). Twenty five adult individuals were randomly sampled in each population. Trees considered for collection were well spaced and separated from each other by at least 100 m. This was done in order to avoid sampling individuals arising from the same maternal line. The samples were then stored at -80°C prior to DNA isolation.

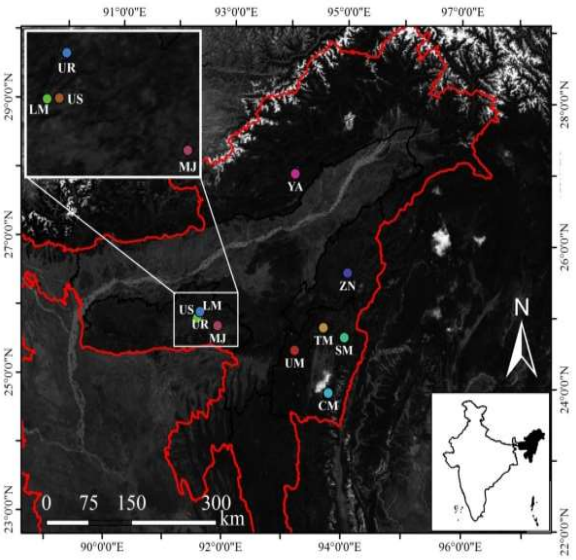

Figure 1
Map showing the geographical location of *P. kesiya* populations

DNA extraction and PCR amplification

Genomic DNA was extracted from needles of sampled trees using a modified CTAB method given by Doyle and Doyle (1990) and Stange et al. (1998). DNA quality was determined by gel electrophoresis using 0.8 % agarose gel and the concentration was measured on a Biophotometer (Eppendorf-6131, Germany). The genomic DNA samples were diluted to a final concentration of 15ng/µL for setting up the PCR reaction.

For trans-specific amplification, a total of 33 chloroplast SSR markers were used where 20 cpSSRs were from *P. thunberghii* (Vendramin et al., 1996) and 13 were from *P. sylvestris* (Provan et al., 1998). These primers were tested for transference in *P. kesiya*. The protocol by Vendramin et al. (1996) with some modifications amplified the microsatellite loci in all populations of *P. kesiya*. Using gradient Polymerase chain reaction (PCR) the optimum annealing temperature was standardized. PCR amplifications were performed using 15-µL reaction volumes containing 15 ng genomic DNA, 1x Taq buffer (Bangalore Genei Ltd.) , 3.0 mM of $MgCl_2$(Bangalore Genei Ltd.), 0.2 mM of dNTP (Bangalore Genei Ltd.), 0.2 µM of forward and reverse primer (SIGMA-ALDRICH, USA) and 5 units of Taq Polymerase(Bangalore Genei Ltd.). Samples were amplified using Thermal cyclerwith thefollowing profile: initial denaturation (94°C, 5 min), followed by 35 cycles of denaturation (94°C, 1 min), annealing (locus-specific temperature, 1 min), extension step (72°C, 1 min) and a final extension (72°C, 8 min).

After PCR amplification, the amplicons were resolved on 8 % polyacrylamide gel (Wang et al., 2003)and theresolved bands were visualised under UV light trans-illuminator and the gel image was captured using a digital camera (Canon, EOS 400D). The details of the primers used for the study are given in Table 1.

Scoring and analysis of data

Scoring of each band was done manually and an input file was then prepared for statistical analysis.

Haplotype distribution

Since chloroplast has a haploid genome that is paternally inherited in pines (Neal and Sederoff, 1989; Watano et al., 1996) and does not undergo recombination, therefore it can be considered as a single locus. Each individual chloroplast haplotype was defined as the combination of the 10 polymorphic fragments obtained in *P. kesiya*. The following population genetic parameters were computed for each population: haplotype frequency (*p*i), the effective number of haplotypes (*n*e) = 1/ ($\sum pi^2$) and the unbiased haplotypic diversity H_e = [n/ (n-1)]*(1-$\sum pi^2$), where n is the number of individuals analysed (Nei, 1987; Avise, 1994; Vendramin et al., 1998).

Genetic diversity estimation

The software POPGENE version 1.32 (Yeh et al., 1999) was used to calculate the total gene diversity (H_T) and diversity within population (H_S). The gene flow (*N*m) among populations was estimated by applying McDermott and McDonald's (1993) formula Nm= 0.5(1-G_{ST})/G_{ST} for the studied populations.

Analysis of Molecular Variance (AMOVA)

The analysis of molecular variance (AMOVA) was carried out in Arlequin software version 3.11 (Excoffier et al., 2005) to calculate the partitioning of genetic variation between and within the ten populations of *P. kesiya*.

Structure analysis

To estimate population structure among the individuals, we used a Bayesian analysis method using the software BAPS6 (Corander et al., 2013). Through this software individuals are clustered intogenetically distinguishable groups on the basis of allelefrequencies and linkage disequilibrium (Schug et al., 2007). Hidden population structure within populations is identified using this approach (Corander and Marttinen, 2006) as it first infers the most likely individual clusters in the sample population and then performs the most likely admixture of genotypes (Corander et al., 2003).

For estimating the individual clustering we used K values ranging from 5-20 and the results obtained were used in an admixture analysis with 100 iterations to estimate the admixture coefficients for the individuals. The analysis was performed multiple times for each K value with 5-10 iterations and in each simulation were used 200 reference individuals and 100 iterations to estimate the admixture coefficients of the reference individuals.

Results

In the present study, the cpSSR primers used were derived from microsatellites developed from *P. thunberghii* and *P. sylvestris*. Thirty-three cpSSR primers were used to PCR-amplify ten populations of *P. kesiya* out of which only 18 amplified consistently under standard conditions. Ten of the 18 cpSSR loci analysed (Pt15169, Pt107517, Pt45002, Pt51873, Pt63718, Pt100783, PCP9434, PCP36567, PCP45071, PCP71987) were polymorphic in all the *P. kesiya* populations investigated. The detail of these 10 primers has been presented in Table 1.

Table 1
The detail of chloroplast microsatellite primers that were used for the study.

S. No.	Primer	Sequences (5'-3')		Tm (°C)	Product size (bp)	Repeat motif	Reference
1	Pt15169	Forward	CTTGGATGGAATAGCAGCC	58.3	115-118	$(C)_8(T)_8(AT)_8$	Vendramin *et al.*, 1996
		Reverse	GGAAGGGCATTAAGGTCATTA				
2	Pt107517	Forward	AAAGCTTTTATTGCGGCC	54.6	95	$(T)_{11}$	Vendramin *et al.*, 1996
		Reverse	ATGGCAGTTCCAAAAAAGC				
3	Pt45002	Forward	AAGTTGGATTTTACCCAGGTG	56.2	161-167	$(T)_{15}$	Vendramin *et al.*, 1996
		Reverse	GAACAAGAGGATTTTTTTCTCATACA				
4	Pt51873	Forward	AATCTTTCTACGGAACGGAAA	58.2	86	$(T)_{10}$	Vendramin *et al.*, 1996
		Reverse	ATCATTTTGTGCTATGCAATGA				
5	Pt63718	Forward	CACAAAAGGATTTTTTTTCAGTG	52.9	92-93	$(T)_{10}$	Vendramin *et al.*, 1996
		Reverse	CGACGTGAGTAAGAATGGTTG				
6	Pt100783	Forward	ATACGTATGTATCCCCTAACTGTCA	54.6	113	$(T)_{10}$	Vendramin *et al.*, 1996
		Reverse	TCAATTTTTGCCATATCCTGA				
7	PCP9434	Forward	AAACTGACGTAGATGCCATGG	58.5	131	$(A)_{10}$	Provan *et al.*, 1998
		Reverse	GCGGTATGAGGGAAGAAGC				
8	PCP36567	Forward	AAAAGAGGAGGAAAAACACCTT	58.5	115	$(T)_{11}$	Provan *et al.*, 1998
		Reverse	AAGAGCAGACAAGTAAGGGGC				
9	PCP45071	Forward	ACTGGTCTGATCGACCCAAT	58.5	149	$(T)_{15}$	Provan *et al.*, 1998
		Reverse	TTCTACACTTGCGGAAACCC				
10	PCP71987	Forward	TCTTTGCAAGAAGGATGGCT	53.4	113	$(T)_{16}$	Provan *et al.*, 1998
		Reverse	GGGGAGTAATCCGTGGAATT				

Lane M: 100 bp DNA ladder

Figure 2
Gel image showing alleleic variation at locus Pt100783 between the individuals of the populations of *P. kesiya*

Haplotype distribution

A total of 36 size variants were detected among the 250 individuals analysed.The variants were combined to designate 90 haplotypes and none of the haplotype was found common

among the populations. Therefore, they were considered as population specific haplotypes. Table 2 shows the genetic characteristics of chloroplast haplotypes based on ten cpSSR loci in ten *P. kesiya* populations analysed. The highest value of haplotypic diversity was observed in MJ (H_e = 0.869) and the lowest in CM (H_e = 0.260). Estimates of the effective number of haplotypes (n_e) and haplotypic diversity (H_e) averaged across all the populations were 3.369 and 0.654, respectively.

Table 2
Haplotypic distribution of ten populations of *P. kesiya*

Populations	Location	N	Latitude/Longitude	Alt. (m)	*ne*	*He*
US	Upper Shillong, Meghalaya	25	25°35'2.5"N/91°54'0.95"E	1725	3.521	0.746
LM	Lumparing, Meghalaya	25	25°35'3.6"N/91°52'10.7"E	1380	4.412	0.806
UR	Umiam, Ri-Bhoi, Meghalaya	25	25°40'36.4"N/91°55'37.2"E	874	4.261	0.797
MJ	Mookyndur, Jaintia Hills, Meghalaya	25	25°27'3.24"N/92°12'32.0"E	1350	6.050	0.869
YA	Yachuli, Arunachal Pradesh	25	27°30'54.7"N/93°47'0.65"E	493	4.900	0.829
ZN	Zunheboto, Nagaland	25	26°0'33.41"N/94°31'25.5"E	1788	4.545	0.813
SM	Senapati, Manipur	25	25°5'43.38"N/94°21'41.9"E	1600	1.563	0.375
TM	Tamenglong, Manipur	25	25°16'3.82"N/94°1'15.66"E	1290	2.000	0.521
UM	Ukhrul, Manipur	25	24°59'16.5"N/93°29'43.0"E	1200	2.000	0.521
CM	Chandel, Manipur	25	25°19'34.3"N/94°0'2.16"E	790	1.333	0.260
Mean					3.369	0.654

N, sample size; *He*, Haplotypic diversity; *ne*, effective number of haplotypes

Genetic diversity estimation

The effective number of alleles (n_e) ranged from 1.668 for Pt51873to 4.485 for Pt15169.The gene diversity (H_T) levels for ten polymorphic loci ranged from 0.472 for Pt51873 to 0.768 for Pt15169. The within-population diversity (H_S) ranged from 0.149 for PCP36567to 0.367 for PCP45071 with a mean of 0.251. The gene flow (N_m) was equal to 0.324 which shows that gene flow was low for this species (Table 3).

AMOVA analysis

Analysis of Molecular variance (AMOVA) was carried out to calculate the partitioning of genetic variation between and within the ten populations of *P. kesiya* using software Arlequin version 3.11 (Excoffier et al., 2005). The analysis revealed a more or less equal proportion of total variation distributed within and among populations (52.81 % of the total variation within populations and 47.19 % among populations). However, the populations revealed relatively high genetic differentiation (F_{ST} = 0.47) and inter-population diversity.

Table 3
Genetic diversity indicators for the ten chloroplast SSR markers

Locus	n_a	n_e	H_T	H_S	N_m
Pt15169	6.000	4.485	0.768	0.316	0.350
Pt107517	5.000	2.316	0.519	0.249	0.460
Pt45002	4.000	2.231	0.617	0.193	0.228
Pt51873	5.000	1.668	0.472	0.176	0.291
Pt63718	4.000	2.828	0.658	0.235	0.277
Pt100783	4.000	2.142	0.566	0.365	0.910
PCP9434	7.000	5.122	0.808	0.237	0.208
PCP36567	4.000	3.101	0.696	0.149	0.136
PCP45071	4.000	2.252	0.588	0.367	0.829
PCP71987	6.000	3.106	0.692	0.227	0.244
Mean	4.900	2.925	0.638	0.251	0.324

n_a = observed number of alleles; n_e = effective number of alleles; H_T = Total genetic diversity; H_S = diversity within population; N_m = gene flow.

Table 4
Analysis of molecular variance (AMOVA) of P. kesiya for Chloroplast SSR markers

Source of Variation	Df	Sum of squares	Variance components	Percentage of variation	F-statistics
Among Populations	9	415.16	1.76 Va	47.19	F_{ST}=0.47
Within Populations	240	474.40	1.97 Vb	52.81	
Total	249	889.56	3.74		

***Significant at 0.1% level of probability

Population Structure analysis

The Bayesian analysis methodology takes the number of populations as well as the allele frequencies in each population as random variables thereby detecting the hidden population substructure by clustering sampled populations into a panmictic group (Corander et al., 2004; Eliades et al., 2011). The BAPS analysis revealed that the highest posterior probabilities for the individual level analysis stabilized at K=6. The software divided the ten populations into 6 independent clusters for both the individual and admixture models. These groups to some extent correspond with the natural geographical distribution of *P. kesiya*. The result is shown in Figure 3. At K=6, most of the populations are clustered independently with low levels of admixture in all the 250 individuals. Populations from Meghalaya (US – MJ) were divided into three groups, where US and UM were included in a common group (Pink). The population from Arunachal Pradesh (YA) was clustered separately while populations from Manipur showed admixtures. In case of population from ZN (Red), it displays a genotype (Pink) which

is shared with individuals from US and UR. Similarly TM and UM also show genotypes common in US and UR with admixtures. SM has one independent genotype (Green), a second genotype (Pink) and a third (Red) common to ZN. The population from CM has one independent genotype (Yellow), which it shares with LM a second genotype found in US, UR and UM.

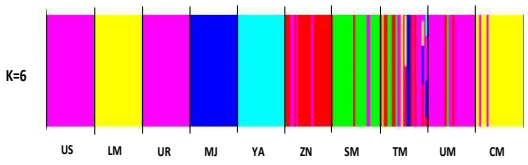

Figure 3-
Inferred clusters of individual genotypes with highest posterior probabilities of likelihood values obtained at K=6. The colours represent the most likely ancestry of the cluster from which the genotype or partial genotype was drawn. The populations are separated by black vertical lines.

Discussion

Genetic diversity and gene flow

Genetic diversity is the basis of the evolutionary potential of species to respond to environmental changes and therefore becomes an essential pillar in conservation genetics (Toro and Caballero, 2005). The development of SSR markers is still inefficient, laborious and costly, principally in organisms with large and complex genomes, such as conifers. A number of researches have highlighted the ability of cross amplification in *Pinus* species (Villalobos-Arámbula et al., 2014; Xu et al., 2013; Chauhan et al., 2010; Xiang- Xiang and Ji-sen, 2005; Echt et al, 1998). In the present study, the population genetic parameters for ten populations of *P. kesiya* were estimated through transspecific amplification of chloroplast microsatellite markers from *P. thunberghii* (Vendramin et al. 1996) and *P. sylvestris* (Provan et al. 1998). The study demonstrated that the cpSSR primer pairs of other pine species can be successfully transferred in *P. kesiya* and can be utilized for population genetic analysis in this species. To date, no nuclear SSR markers have been developed for the *P. kesiya* species.

The cpSSRs showed a relatively high transfer rate in *P. kesiya* (subgenus Pinus) from *P. thunberghii* with 60 % transfer rate followed by *P. sylvestris* with 46 % transfer rate. A study by Cato and Richardson (1996) reported the successful transfer of chloroplast sequences from *P. thunberghii* to ten pine species. Chauhan et al. (2010) also successfully transferred 95 % microsatellites from *P. thunberghii*, 100 % from *P. sylvestris*, 20 to 28.57 % from *P. taeda*, 33.33 % from *P. resinosa*, 80 % from *P. merkusii* and 42.85 % from *P. densiflora* in *P. roxburghii*. Out of the primers which successfully transfer in *P. kesiya* ten loci were polymorphic.

The products amplified with the ten polymorphic primer pairs were in the expected size range (Table 1). A total of 36 size variants were found and these combined into 90 haplotypes with their frequencies ranging from 0.1 to 0.8. None of the haplotype was found common or being shared among the populations. Therefore, they were considered as population specific haplotypes (Vendramin et al., 1998, 2000). The results indicate that *P. kesiya* in the region has maintained a high level of haplotypic diversity (mean H_e= 0.654), which has been observed in most coniferous species based on cpSSRs (Tereb et al., 2006; Vendramin et al., 1998; Ribeiro et al., 2001; Gómez et al., 2003; Hansen et al., 2005; Clark et al., 2000). The haplotypic diversity was highest in population from Jowai, Meghalaya (H_e = 0.869) indicating that this population should be considered for genetic conservation programs (Echt et al., 1998).

Overall total gene diversity at the ten chloroplast loci was high (mean H_T=0.638) which is in confirmatory to those reported by other authors (Gómez- Garay et al., 2010; Echt et al., 1998). However, the inter-population genetic diversity (H_T - H_S) was higher than the diversity within populations (mean H_S=0.251). This is also being supported by the high population genetic differentiation (F_{ST} = 0.47). The gene flow estimate (mean N_m = 0.324) was relatively low for pines which is generally between the range of 4.6 and 17.2 (Ledig et al., 2001). However, the value is similar to the one found in *P. roxburghii* with average N_m = 0.581 (Chauhan, 2011). This indicates that there is little genetic exchange among the populations of *P. kesiya* resulting in high differentiation among populations. This may be due to the spatial distance of the geographical localities from where the populations were collected and lesser to do with the fragmentation of the pine forest resulting from anthropogenic activities although this possibility cannot be completely ruled out. Inbreeding and the presence of a subpopulation structure, where gene flow is prevented by habitat fragmentation, both cause the loss in heterozygosity (Hartl and Clark, 2007). This, in turn, results in increased genetic diversity among populations. Geographically widespread species generally show a significantly higher intra-population genetic diversity estimate compared to locally confined species, but the latter showed higher genetic diversity among populations (Hamrick 1992).

Genetic differentiation

Wright (1951) suggested that the F_{ST} values above 0.25 indicate very high genetic differentiation (Hartl and Clark, 1997). Therefore, results for the cpSSRs in this study showed that the genetic differentiation was pronounced with F_{ST} value of 0.47 indicating highly differentiated populations. The values are higher than those reported in most conifers such as Scottish *P. sylvestris* with F_{ST} value of 0.37 (Sinclair et al., 1997), *P. rzedowskii* with F_{ST} value of 0.175 (Delgado et al., 1999) and *P. pinceana* with a value of 0.247 based on isozyme (Ledig et al., 2001). The low gene flow values due to geographical isolation resulting in higher chance of inbreeding within isolated populations may explain why the results of the present study revealed a high population differentiation. Moreover, restricted gene flow among

populations (high F_{ST}) may limit migration of genes or genotypes to new suitable habitats and may lead to local extirpation of populations and reduced genetic diversity in *P.kesiya* through the loss of rare alleles as they are affected by changing climate and anthropogenic pressure.

The Bayesian analysis identified 6 independent ancestries and some of them appeared within populations as well. Furthermore, the clusters showed very limited and in some populations no admixture indicating that they were highly differentiated probably due to weak pollen-mediated gene flow between populations. This may be due to the geographical boundaries that may act as barriers to gene flow and also as a result of the sporadic distribution of the populations under investigation. According to Perry (1991), genetic differentiation among populations is related to the species distribution on rugged mountain ranges where topography could act as a natural barrier to gene flow. Due to over exploitation of *P. kesiya* forests during the last several decades, it is presently restricted only in isolated patches of the Khasi and Jaintia hills in Shillong and in Manipur. The restricted gene flow may have prevented the exchange of alleles among *P. kesiya* populations resulting in the high genetic differentiation. Since the populations collected were geographically far apart it is possible that the populations have not admixed in recent history which is evident from the data that shows distinct clusters both within and among populations.

Genetic diversity provides the foundation for adaptation to changing environments (Aitken et al., 2008). Protection of genetic diversity both within and among populations is essential for species with more disjunct populations (Falk et al., 2006). To capture and maintain the genetic diversity for ex situ conservation of *P. kesiya*, we recommend making collections from as many populations as practical across the range, due to the moderate population structure and existence of uncommon population specific unique haplotypes. High genetic diversity areas such as Meghalaya region should be given special attention with regard to genetic conservation programs for the sustainable management of Khasi Pine forest. An integrated management plan including in-situ and ex-situ conservation strategies based on genetic, ecological and demographic data could maximize the maintenance of the existing genetic diversity in the long run and adaptation of *P. kesiya* to new threats and environmental changes.

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