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Genetic diversity of *Populus alba* L in Kinnaur, Himachal Pradesh, India

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ABSTRACT

Populus alba L is an ecologically and economically important species of Mediterranean origin. It has been introduced in many parts of the world due to long term human interference and is still being cultivated. In Himachal Pradesh, it is mostly found in Kinnaur and its indigenous status is controversial in India. In order to understand the native status and develop strategies for breeding and conservation, the population structure and genetic diversity of *P alba* in Kinnaur region were analyzed using nuclear and chloroplast microsatellite markers. A very low value of observed (Ho) and expected heterozygosity (He), viz 0.033 and 0.062 respectively, indicated limited genetic variations in the population. During the study period, flowering was not recorded in tagged plants and only vegetative regeneration was observed. Thus it is likely to be an exotic species in Kinnaur, Himachal Pradesh. There is a need to develop strategies for conservation of the existing genetic resources of this species and introduce genetically diverse germplasm for future breeding and improvement programmes.

Keywords: *Populus alba*; genetic diversity; native status; regeneration

INTRODUCTION

Populus species (poplar) are mostly distributed in northern hemisphere and are widely cultivated due to their economic and environmental values. Of the 32 poplar species (Dickmann and Kuzovkina 2014), *Populus alba* (white poplar) is one of the important species of Mediterranean origin. Like most of the poplars, *P alba* is dioecious, wind-pollinated and produces large amounts of small cottony seeds as well (Bradshaw et al 2000). It is an important agroforestry species and cultivated in Mediterranean region, southern Europe, Russia, China, USA, Afghanistan, northwestern Himalaya and many other countries due to commercial value of its wood (Tognetti et al 2013). It is also used as an ornamental plant, source of fodder, windbreak and for dune stabilisation due to its tolerance to salt and winds. In India, this species has been reported from Jammu and Kashmir, Himachal Pradesh (Kinnaur and Lahaul and Spiti districts) and Nubra valley of Ladakh (Ramesh and Khurana 2006, Stewart and Brandis 1874).

Most of the poplars including *P alba* are regenerated through both vegetative and sexual mechanism and their success is determined by environmental conditions and genetic factors. In general, vegetative or clonal reproduction leads to generation of genetically identical individuals, whereas, sexual reproduction leads to variation. Thus the mechanism of regeneration determines the genetic structure and diversity of a species in a population (Brundu et al 2008).

Genetic diversity plays an important role in survival and adaptability of a species in the changing climatic conditions. Before initiating any improvement programme, it is very impotant to assess genetic diversity within and among the populations using metrics like proportion of polymorphism, allele frequency, heterozygosity and inbreeding. Molecular marker-based genetic analysis also provides information about the clonal structure and origin of a population. In order to confirm the origin of *P alba* in Sardinian, Brundu et al (2008) analysed the pattern of genetic variation and

observed a large number of ramets derived from single genet. Based on haplotype analysis, it was concluded that *P alba* populations are relict of native Sardinian island populations and have been spread on the island through vegetative means as a result of human activities.

Native status of *P alba* in India is very controversial (Chaturvedi and Rawat 1994, Naithani and Nautiyal 2012). Many floras have reported its distribution in wild and planted form in India. However, Stewart and Brandis (1874) has reported its wild form only along Chenab and Jhelum rivers in Kashmir.

In Himachal Pradesh, this species is mainly found in Kinnaur and rarely in Lahaul and Spiti district. Till date, no critical analysis has been carried out to understand the status of *P alba* in Himachal Pradesh. Since no previous phylogeographical and genetic study has been conducted on this species, a molecular survey was performed to evaluate its clonal structure and pattern of genetic variation in Himachal Pradesh to develop better strategy for breeding and tree improvement.

MATERIAL and METHODS

Study area and sampling

Population survey and sampling were carried out in a broad area of Kinnaur, Himachal Pradesh, India, ranging from latitude N 31 0 31′9.97′′to N 31 0 37′13.15′′and Longitude E 78 0 16′20.08′′ to E 78 0 35′38′′ in between elevations of 1,950 to 2,618 m amsl (Fig 1). In total, 15 trees were sampled from 8 villages of Kinnaur district and details of the locations are given in Table 1.

Healthy young leaves were sampled from each tree in the month of April-May 2021. The leaves were stored in plastic bags with silica gel and brought to the laboratory of Genetics and Tree Improvement Division, ICFRE – Himalayan Forest Research Institute, Panthaghati, Shimla, Himachal Pradesh and stored in deep freezer (–80^{o}C) for further analysis.

Assessment of flowering phenology

To record the flowering and seed setting behaviour of *P alba*, fifteen individual plants in district Kinnaur were tagged with aluminium tags that enabled relocating the plants in the subsequent field visits. The observations were made on flowering and seed setting of the species at monthly intervals for the period of two calendar years from March to June 2021 and 2022.

DNA isolation and PCR amplification

Genomic DNA from stored young leaves of *P alba* was extracted following CTAB method (Doyle and Doyle 1987). A total of 16 nuclear simple sequence repeats (SSRs) viz PTR1, PTR2, PTR3, PTR4, PTR5, PTR6, PTR8, PTR12 and PTR14, PMGC2679, PMGC2163 and PMGC2571 (Dayanandan et al 1998, Rahman et al 2000), WPMS14, WPMS15, WPMS18 and WPMS20 (van der Schoot et al 2000, Smulders et al 2001) and 3 chloroplast SSRs viz CCMP2, CCMP6 and CCMP10 (Weising and Gardner 1999, Brundu et al 2008) were employed (Table 2).

PCR reactions were performed using the nuclear SSR primers with the final volume of 15 ìl containing 7.5 ìl mastermix, 0.45 ìl of each forward and reverse primer and 4.6 ìl nuclease free water. Two ìl of DNA was used. The PCR cycle was: 94 C for 3 min, 35 cycles of 94 C for 1 min, Ta (varied based on the primer pair used) for 1 min and 72 C for 1 min and 72 C for 8 min and stored at 4^{o}C until used. PCR products were separated on a 6 per cent denaturing polyacrylamide gel.

Statistical analysis

Scoring of bands was done by using a software, Gel Analyzer (Version 19.1); the sizes of the amplified DNA bands were determined by comparing the migration distance of amplified fragments to the molecular weight of the 100 bp DNA ladder and accordingly an excel sheet was prepared.

The average number of alleles per locus, expected heterozygosity (He) and observed heterozygosity (Ho) for used nuclear markers were calculated using GenAlex (version 6.31ba) software, which was used to examine the genetic diversity. Fixation index or F-statistics was also calculated. PIC values were computed with the help of PowerMarker software (version 3.25).

RESULTS and DISCUSSION

Flowering and mode of *P alba* regeneration

P alba is considered a plant of old-world origin and its records from India are available in the floras written during British rule (Hooker 1894).

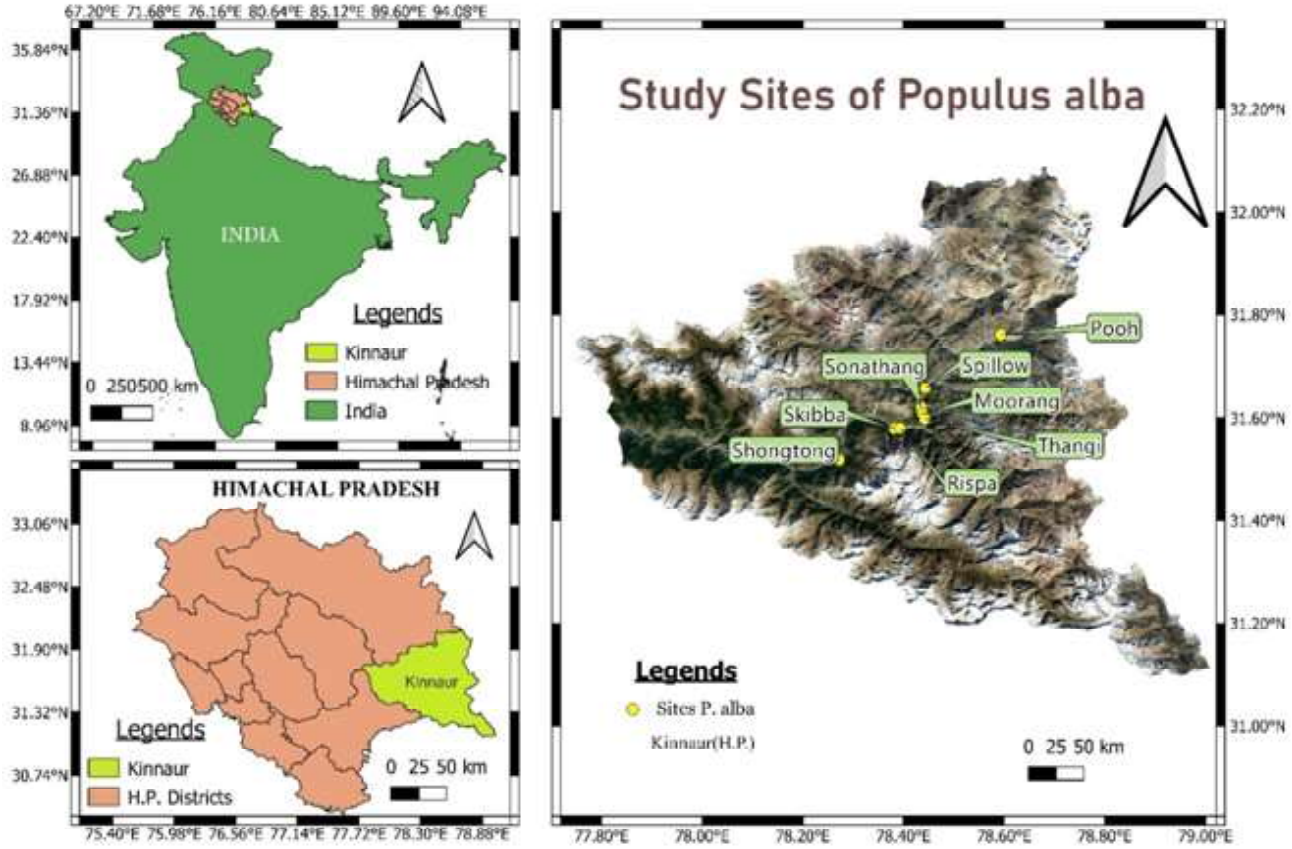

Fig 1. Sampling sites of *P alba* in Kinnaur district, Himachal Pradesh

Table 1. Sampling sites selected in district Kinnaur, Himachal Pradesh

Location	Elevation (m)	Latitude	Longitude
Pooh	2,618	31.76222	78.59389
Spillow	2,294	31.6577	78.44347
Shongtong	1,950	31.51944	78.27224
Skibba	2,270	31.57966	78.38182
Rispa	2,239	31.58019	78.3932
Sonathang	2,286	31.62032	78.43505
Moorang	2,352	31.60717	78.43815
Thangi	2,332	31.59897	78.44213

As per 'The forest flora of northwest and central India', *P alba* in Kinnaur (Kunawar) was planted only and trees with flowers and fruits were also not seen (Stewart and Brandis 1874). In this study as well, only clonally propagated plants of *P alba* were observed along the Sutlej river basin in Kinnaur. It was present in agricultural fields, as boundary plantations and in forest nurseries. Sex identification of trees was very difficult as flowering and seed formation was not observed during the study period. Flowering and fruit setting generally depend on ecological, physiological and genetic factors (López et al 2021, Kong et al 2009).

Sometimes species fail to flower and even after flowering get aborted due to mineral nutrient deficiency (Gupta and Solanki 2013). Flowering also depends on genotype variations and epigenetic modifications in response of environmental factors (Lai et al 2018, Yaish et al 2011). Therefore, genotype and environment interaction is considered very critical for flowering in plants (Navas-Lopez et al 2019).

Thus it can be assumed that the lack of flowering and seed setting in *P alba* could be the effect of either genotype or genotype environment interaction in the Kinnaur region. Furthermore, branches of this species are also heavily lopped for fodder needs. Excessive lopping generally affects the tree physiology which may result in flowering inhibition.

However, during the present study, flowering was also not recorded on the trees that were not frequently lopped. Therefore, lopping cannot be

Table 2. Primer sequences of nuclear and chloroplast SSR markers along with their annealing temperature (Ta)

Locus name	Primer sequences (5¹-3¹)	Ta (°C)
PTR1	F-AGCGCGTGCGGATTGCCATT	58
	R-TTAGTTTCCCGTCACCTCCTGTTAT	
PTR 2	F-AAGAAGAACTCGAAGATGAAGAACT	60
	R-ACTGACAAAACCCCTAATCTAACAA	
PTR3	F-CACTCGTGTTGTCCTTTTCTTTTCT	58
	R-AGGATCCCTTCCCTTTAGTAT	
PTR4	F-AATGTCGAGGCCTTTCTAAATGTCT	60
	R-GCTTGAGCAACAAACACACCAGATG	
PTR5	F-CTTCTCGAGTATAAATATAAAACACCA	58
	R-TCACATCACCCTCTCAGTTTCGC	
PTR6	F-AGAAAAGCAGATTGAGAAAAGAC	60
	R-CTAGTATAGAGAAAGAAGAAGCAGAAA	
PTR8	F-TAGGCTAGCAGCTACTACAGTAACA	60
	R-TTAAGTGCGCGTATCCCAAAGA	
PTR12	F-AATAACCATCCCTCCAATAACCTAC	60
	R-TATTTTGCACCTAAATGGCTGTTCT	
PTR14	F-TCCGTTTTTGCATCTCAAGAATCAC	60
	R-ATACTCGCTTTATAACACCATTGTC	
WPMS14	F-CAGCCGCAGCCACTGAGAAATC	60
	R-GCCTGCTGAGAAGACTGCCTTGAC	
WPMS15	F-CAACAAACCATCAATGAAGAAGAC	60
	R-AGAGGGTGTTGGGGGTGACTA	
WPMS18	F-CTTCACATAGGACATAGCAGCATC	59
	R-CACCAGAGTCATCACCAGTTATTG	
WPMS20	F-GTGCGCACATCTATGACTATCG	59
	R-ATCTTGTAATTCTCCGGCATCT	
PMGC2679	F-GGAATCCGTTTAGGGATCTG	60
	R-CGTCTGGAGAACGTGATTAG	
PMGC2163	F-CAATCGAAGGTAAGGTTAGTG	60
	R-CGTTGGACATAGATCACACG	
PMGC2571	F-TCTCGCAGATTCATGTAACCC	60
	R-GACTGTATGTTGACCATGCCC	
CCMP2	F-GATCCCGGACGTAATCCTG	63
	R-ATCGTACCGAGGGTTCGAAT	
CCMP6	F-CGATGCATATGTAGAAAGCC	58
	R-CATTACGTGCGACTATCTCC	
CCMP10	F-TTTTTTTTTAGTGAACGTGTCA	55
	R-TTCGTCGDCGTAGTAAATAG	

considered a major factor for the lack of flowering in this species.

Both clonal and sexual reproductions are used as mechanisms of population regeneration by species of poplars, including *P alba*. White poplar produces vigorous root suckers and rapidly invades new areas around trees. Therefore, it is also considered an invasive species in many parts of the world (Caudullo and de Rigo 2016). Massive root sucker formation was observed at most of the surveyed sites in Kinnaur as well. In response to genetic and ecological factors, the success of clonal and sexual reproduction in different species of poplar may vary. The regeneration mechanism that is favoured by a certain condition determines the genetic structure and clonal diversity of plants in a population (Brundu et al 2008). Any plant that reproduces only vegetatively in a particular region, can be suspected of being an exotic species (Webb 1985).

Genetic diversity of *P alba*

SSRs are highly reproducible molecular markers, therefore, widely used in species identification, genetic diversity assessment, population structure estimation, parentage analysis and molecular breeding. Both, cpSSR and Nuclear SSRs were employed in this study to analyse genetic diversity of *P alba* in Kinnaur. The fingerprint profile of cpSSR demonstrated lack of polymorphism at all the three studied loci. However, all the 16 nuclear SSRs showed positive PCR-amplification but the polymorphism was detected only at 4 loci (Figs 2, 3).

Thus the percentage of polymorphic loci (P%) was 25 per cent. The average of different alleles (Na) and effective number of alleles was 1.50 and 1.11 respectively. Observed heterozygosity was highest ie 0.267 for PTR 2 and lowest was 0.067 for both PTR 14 and PMGC2679 SSR loci (Table 3). However, the range of expected heterozygosity was from 0.553 to 0.064 with an average of 0.062. Ho explains about the actual proportion of individuals in a population heterozygous at each locus, while He is the expected heterozygosity value estimated as per Hardy-Weinberg equilibrium (HWE). Both the heterozygosity parameters (Ho and He) are widely used in estimating population genetic diversity and obtaining the information about the population structure. The average value of heterozygosity ie 0.033, indicated about the existence of very low genetic diversity in the studied population of *P alba*. However, Ho <He denoted about inbreeding in the population (Nei 1973).

Polymorphism information content (PIC) is used to measure the potential of a SSR marker to detect the polymorphism (Serrote et al 2020). Polymorphic markers with PIC >0.50 are considered as highly informative. However, PIC values in between 0.50 to 0.25 are considered as reasonably informative and PIC <0.25 as slightly informative (Fisher 1951, Huang et al 2022). In the present study, the PIC value ranged from 0.000 to 0.460 with an average 0.054. Out of studied 16 SSR markers, PIC value was zero for 13, less than 0.25 for three and in between 0.50 to 0.25 for one (PMGC2679) (Table 3).

Of the tested 16 nuclear SSR markers, 4 WPMS markers (WPMS 05, 15 18 and 20) have already been used to assess the origin of *P alba* clonal diversity in Sardinia and were found to be effective in discriminating clones of different origin (Brundu et al 2008). Other 3 PMGC markers (PMGC 2163, 2571 and 2679) used in this study were also recommended for parentage analysis of poplars including *P alba* (Khasa et al 2003). Remaining 9 PTR microsatellites (PTR 1, 2, 3, 4, 5, 6, 8, 12 and 14) developed for *P tremuloides* were used in this study to assess their transferability for the phylogenetically closely associated species *P alba*. Of the 9 tested PTR markers, only 3 were polymorphic with PIC vale <0.25. Despite the fact that most of the selected SSRs were robust and potent in discriminating *P alba* clones, significant polymorphism was not observed in the present study. Therefore, it appears that the clonal diversity of *P alba* in Kinnaur was very limited and was derived from a few genets only. In order to confirm the origin of Mediterranean island populations, Brundu et al (2008) analyzed the pattern of genetic variation in Sardinian island populations of *P alba* and obtained very similar results. They also observed that a large number of *P alba* ramets were derived from single genet at most of the surveyed locations.

CONCLUSION

There is evidence that during the course of human settlement in a new area, the introduction of non-native plant species is very common. It seems from the results that the *P alba* in Kinnaur was mostly planted and was introduced in the region. Genetic diversity and sexual regeneration are crucial for the evolution and adaptation of a species to the changing climatic conditions caused by global warming. Selection and breeding for the desired traits like yield, disease resistance and abiotic stress tolerance also depend on the variability in the population. Thus there is a need to introduce a genetically diverse germplasm of this species and develop measures to conserve the existing genetic resources.

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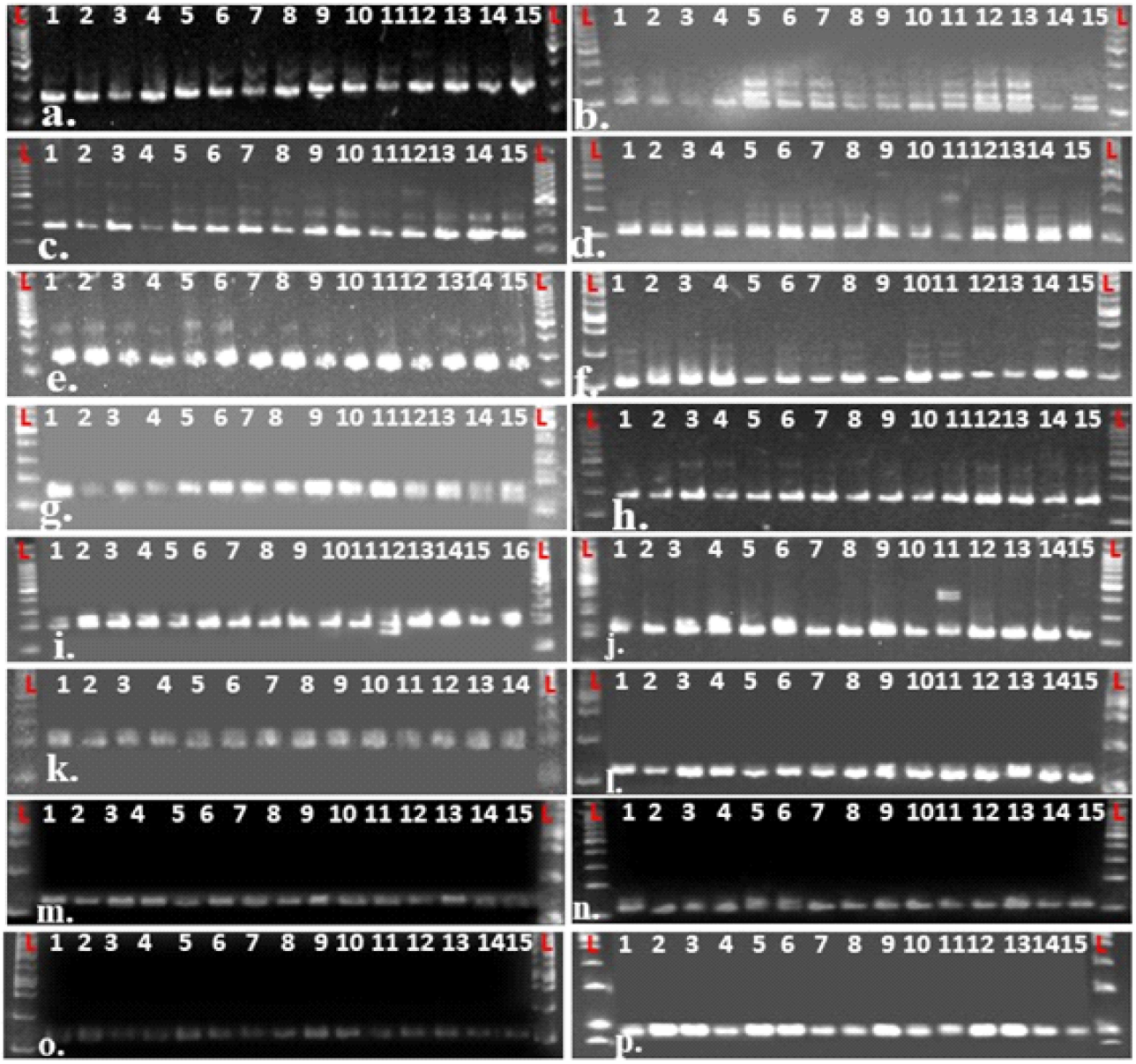
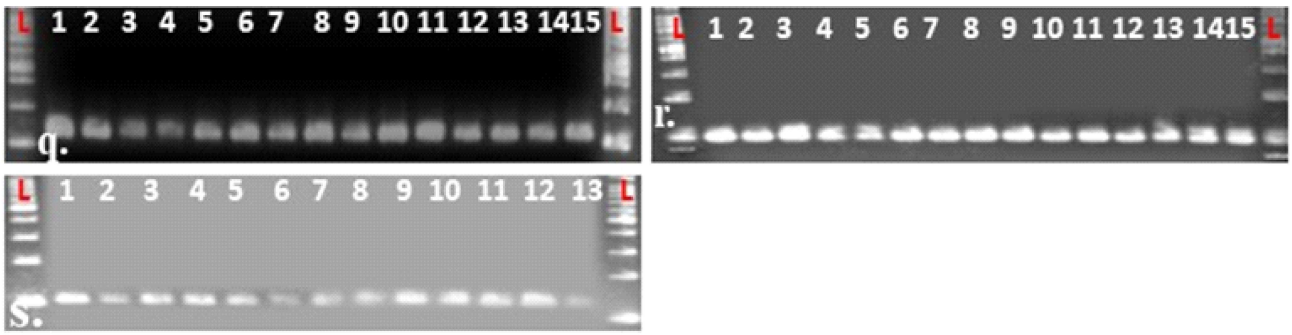


a: PTR1, b: PTR2, c: PTR3, d: PTR4, e: PTR5, f: PTR6, g: PTR8, h: PTR12, i: PTR14, j: WPMS14, k: WPMS15, l: WPMS18, m: WPMS20, n: PMGC2163, o: PMGC2571, p: PMGC2679

Fig 2. Polyacrylamide gel images showing nuclear SSR profile of *P alba*



q: CCMP2, r: CCMP6, s: CCMP10

Fig 3. Polyacrylamide gel images showing chloroplast SSR profile of *P alba*

Table 3. Summary of the genetic diversity analysis of *P alba* using nuclear SSR loci

Locus	Na	Ho	He	PIC
PTR 1	1.000	0.000	0.000	0.000
PTR 2	4.000	0.267	0.242	0.232
PTR 3	1.000	0.000	0.000	0.000
PTR 4	2.000	0.133	0.124	0.117
PTR 5	1.000	0.000	0.000	0.000
PTR 6	1.000	0.000	0.000	0.000
PTR 8	1.000	0.000	0.000	0.000
PTR 12	1.000	0.000	0.000	0.000
PTR 14	2.000	0.067	0.064	0.062
WPMS14	1.000	0.000	0.000	0.000
WPMS15	1.000	0.000	0.000	0.000
WPMS18	1.000	0.000	0.000	0.000
WPMS20	1.000	0.000	0.000	0.000
PMGC2679	4.000	0.067	0.553	0.460
PMGC2163	1.000	0.000	0.000	0.000
PMGC2571	1.000	0.000	0.000	0.000
Mean	1.500	0.033	0.062	0.054

Na: Number of different alleles, Ho: Observed heterozygosity, He: Expected heterozygosity, PIC: Polymorphism information content

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