RESEARCH ARTICLE

Genetic diversity and population structure of *Litsea glutinosa* (Lour.) in Central India

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Abstract *Litsea glutinosa* (Lour.), one of the most dwindling forest species in central India, is represented by highly fragmented populations that have been drastically reduced for the last 40 years, promulgating government ban on its extraction. For the first time with the help of ISSR markers, we investigated genetic variation and population structure of *L. glutinosa* in central Indian states. A total of 84 genotypes from 10 populations covering the entire potential pockets of the species in central India were collected. The percentage of polymorphic loci ranged from 44.79% (Rewa) to 94.79% (Marvahi) with a mean value of 70.10%. The sampled populations harbored high level of genetic diversity (mean $h = 0.294$ and $I = 0.424$) that was partitioned more within populations (73%) than between populations (27%). Bayesian structure analysis revealed the existence of four admixed genetic pools in *L. glutinosa*. The unsustainable extraction rather than genetic factor seems to be responsible for population fragmentation and dwindling status of this species. The dioecious nature of the species advocates an in-situ conservation to be the most suited approach for which Chhindwara, Jagdalpur, Balaghat and Jabalpur populations are appropriate.

Keywords Conservation · Genetic diversity · Population structure · *Litsea glutinosa*

Introduction

Importance of germplasm conservation in reversing the genetic erosion of a scarce and dwindling species is beyond doubt. For successful planning and implementation of conservation strategies, a knowledge about the genetic diversity and population structure of the target species is extremely important (Kajund and Jaaska 2010; Gordon et al. 2012; Lopes et al. 2014; Wu et al. 2015). Conservation of genetic diversity positively affects the survival of a dwindling species by providing buffering capacity against the changing environment (Frankham et al. 2002). Small populations are highly prone to extinction due to genetic drift, inbreeding depression and climate change (Falconer and Mackay 1996). Genetic drift and inbreeding decreases the heterozygosity leading to decline of genetic diversity and subsequently loss of fitness and adaptability (Lacy 1997; Frankham 2005). This necessitates an assessment of genetic diversity within and among different populations for successful conservation and management of species with small population size.

Litsea glutinosa (Lour.), commonly known as Indian laurel is an evergreen tree occurring throughout India ascending up to an altitude of 1400 m in the Himalayas (Dev 2006). It is native to India, Southern China, Malaysia, Australia and Western Pacific islands (GISD 2012). The bark of *L. glutinosa* is mucilaginous, feebly balsamic and mildly astringent and is sold as ‘Maida Lakri’- a common demulcent drug in Indian market. In cure of diarrhea, rheumatism, sap of fresh bark or its decoction is used and it is also aids in longevity. The bark is also used as a plaster in cases of sprain, bruises, wounds, inflammation, back pain, gouty joints and bone fractures, etc. It is reported to have analgesic, antiseptic and emollient effects (Kirtikar and Basu 1981; Devi and Meera 2010). In China, seed oil is

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used for manufacture of soap (Huang Puhua et al. 2008). In a recent study, *Litsea glutinosa* has also been reported as a source of essential oils, arabinoxylans and other components with antiseptic properties (Prusti et al. 2008; Qin WenHui et al. 2012; Das et al. 2013).

In India, the bark of *Litsea glutinosa* is commercially used for joss stick manufacturing, which is valued around 1000 crores rupees industry providing employment to approximately 1.5 million peoples with an annual growth rate of 10–15%. This industry consumes almost the whole production of the bark of *L. glutinosa* (Rath 2004). The indiscriminate over exploitation of the bark has resulted in irreparable loss to its wild germplasm, making the species scarce and dwindling in various states of India (Rath 2003; Dubey et al. 2007). To the best of our knowledge, no investigation has unfortunately been carried out for a wide scale genetic diversity analysis of this multipurpose forest tree species populations of Central India.

Therefore, we assessed the genetic diversity and population structure of *L. glutinosa*, employing low cost, easy accessible and highly polymorphic inter simple sequence repeats (ISSR) markers. The new information would help to formulate conservation strategies for existing scarce natural populations of this species.

Materials and methods

Survey and collection: Working plans of the forest divisions of Madhya Pradesh and Chhattisgarh were consulted to identify potential pockets. The help of forest staff/officials of the identified forest divisions were secured to locate them in the field for collection of propagules (Fig. 1). The sparse distribution of *L. glutinosa* compelled to resort to purposive sampling for collection of propagating material that was established as ex-situ germplasm bank at Tropical Forest Research Institute, Jabalpur (Madhya Pradesh). The tender young leaves were collected from different accessions conserved in germplasm bank for genomic DNA extraction as detailed in Table 1.

ISSR genotyping: Genomic DNA extraction was carried out using CTAB procedure (Doyle and Doyle 1987). The concentration and purity of the extracted genomic DNA was checked before setting ISSR assay. Fifteen ISSR primers from University of British Columbia, Canada were selected for this study based on presence of clear, repeatable and polymorphic bands after screening fifty-two primers (Table 2). The PCR amplification performed in a 20 µl reaction volume consisted of 0.1 mM each of dNTPs, 1 U Taq polymerase, 1X of Taq polymerase buffer, 1.6 mM MgCl₂ (Promega, USA), 0.8 µM primer and 50 ng genomic DNA. DNA amplification was performed in a

Fig. 1 Map displaying the location of *L. glutinosa* collected from different forest divisions of Central India

Table 1 Details of the investigated populations of *Litsea glutinosa* Lour

Forest division	Code	Latitude N	Longitude E	Sample size in analysis ^a	Population size
Kondagaon	KDG	19°59′	81°66′	8	10
Jagdarpur	JDB	19°11′	82°03′	8	10
Jabalpur	JBP	23°09′	79°98′	9	10
Marvahi	MRV	22°63′	81°78′	7	10
Balaghat	BGT	21°53′	80°63′	9	26
Bhanupratapur	BHN	20°28′	81°07′	9	10
Dhamtari	DTR	20°73′	81°80′	8	10
Seoni	SEO	21°87′	79°52′	9	09
Rewa	REW	24°82′	81°45′	8	08
Chhindwara	CWA	22°42′	78°61′	9	31

^aSamples analysed were taken from the Germplasm Conservation Bank of Tropical Forest Research Institute, Jabalpur (Madhya Pradesh) which housed the accessions from different localities as indicated in table

gradient palm cycler (Corbett Research, Australia) programmed for an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C (denaturation) for 30 s, 50 °C (annealing) for 30 s and 72 °C (extension) for 1 min. Final extension for 10 min at 72 °C was provided at the end of 30 cycles. The amplified products were size fractionated on 2% agarose gel stained with 0.5 µg ml⁻¹ ethidium bromide along with DNA ladder. The fractionated amplified products were visualized and captured using gel documentation system (Alfa Innotech, USA).

Data analysis: Reproducible and well defined bands obtained after PCR amplification using each ISSR primers was scored as the 1 (presence) or 0 (absence) for construction of binary matrix for genetic diversity analyses.

Total number of fragments, polymorphic fragments, percent polymorphism and polymorphic information content (PIC) was estimated using GDDom (Abuzayed et al. 2016) software. POPGENE version 1.32 (Yeh et al. 2000) was used for calculation of observed number of alleles (N_a), effective number of alleles (N_e), Nei's (1973) gene diversity (h), Shannon's Information index (I) and percentage of polymorphic loci (P). Analysis of molecular variance (Excoffier et al. 1992) was carried out using GenAlEx ver 6.2 (Peakall and Smouse 2006; 2012) to partition genetic variation among and within populations.

The Bayesian clustering method was used to find out the population structure using STRUCTURE 2.2.3 software (Pritchard et al. 2000; Falush et al. 2003). STRUCTURE performs Bayesian assignments of genotypes to a given number of genetically homogenous population clusters (K). In the present investigation, twenty-five replications of each proposed k value (from $k = 1$ to 13) were investigated following admixture ancestry and correlated allele frequencies model. Length of burn-in period and number of Markov Chain Monte Carlo (MCMC) repeats after burn-in were set at 50,000 and 100,000 respectively. For

determination of optimal k , delta k was estimated as described (Evanno et al. 2005) employing STRUCTURE HARVESTOR software (Earl and von Holdt 2012). Bar chart for the proportion of the member coefficient of each individual/population for best k was prepared using Structure Plot v 2.0 (Ramasamy et al. 2014).

Furthermore, DARwin version 6 (Perrier and Jacquemoud-Collet 2006) was also employed to construct a bootstrapped Neighbor-joining tree.

Results

Out of the fifty two ISSR primers screened, fifteen primers amplifying highly polymorphic well distributed fragments with good distinction were subsequently employed for final investigation on the 84 genotypes belonging to 10 populations of *Litsea glutinosa*. Primer set amplified a total of 97 loci that ranged from 200 to 1500 bp. The polymorphism varied from 80 to 100% with an average of 98% per primer (Table 2). Polymorphic information content (PIC) of ISSR markers ranged from 0.2174 (UBC-812) to 0.4649 (UBC-854) with an average of 0.3478.

Estimates of genetic diversity indices of each population are summarized in Table 3. The polymorphic loci (P) ranged from 44.79% in the REW population to 94.79% in the MRV population, with a mean value of 70.10%. The values of genetic diversity estimates across the ten populations were 1.701 for mean observed number of alleles (N_a), 1.537 for effective number of alleles (N_e), 0.294 for Nei's (1973) gene diversity (h) and 0.424 for Shannon's Information index (I). The MRV population ($h = 0.414$, $I = 0.591$) followed by JDB population ($h = 0.332$, $I = 0.475$) topped the list of the sampled populations.

Analysis of molecular variance (AMOVA) revealed that *L. glutinosa* populations had a highly significant genetic

Table 2 Polymorphism and PIC statistics of inter simple sequence repeats (ISSR) marker used in investigation

Sr.	Code	Primer seq 5'-3'	Number of polymorphic fragments/total no. fragments	Percent polymorphism	PIC value
1	UBC-852	5'-TCTCTCTCTCTCTCTCAA-3'	5/5	100	0.4314
2	UBC-854	5'-TCTCTCTCTCTCTCTCGG-3'	5/5	100	0.4649
3	UBC-856	5'-ACACACACACACACACCA-3'	10/10	100	0.4239
4	UBC-859	5'-TGTGTGTGTGTGTGTGGC-3'	6/6	100	0.4015
5	UBC-866	5'-CTCCTCCTCCTCCTCCTC-3'	4/4	100	0.2842
6	UBC-848	5'-CACACACACACACACAGG-3'	5/5	100	0.3895
7	UBC-868	5'-GAAGAAGAAGAAGAAGAA-3'	7/7	100	0.3788
8	UBC-827	5'-ACACACACACACACACG-3'	7/7	100	0.4581
9	UBC-829	5'-TGTGTGTGTGTGTGTGC-3'	6/6	100	0.3762
10	UBC-830	5'-TGTGTGTGTGTGTGTGG-3'	6/6	100	0.3881
11	UBC-808	5'-AGAGAGAGAGAGAGAGC-3'	5/5	100	0.2218
12	UBC-810	5'-GAGAGAGAGAGAGAGAT-3'	8/8	100	0.2809
13	UBC-811	5'-GAGAGAGAGAGAGAGAC-3'	9/10	90	0.2493
14	UBC-812	5'-GAGAGAGAGAGAGAGAA-3'	8/8	100	0.2174
15	UBC-835	5'-AGAGAGAGAGAGAGAGCC-3'	4/5	80	0.2508

Table 3 Genetic diversity parameters for the 10 populations of *Litsea glutinosa* Lour

Population	Code	Genetic cluster ^a	N_a	N_e	h	I	P (%)
Kondagaon	KDG	Cluster-II	1.792	1.593	0.327	0.473	79.17
Jagdapur	JDB	Cluster-IV	1.760	1.613	0.332	0.475	76.04
Jabalpur	JBP	Cluster-II	1.781	1.591	0.326	0.471	78.12
Marvahi	MRV	Cluster-IV	1.948	1.773	0.414	0.592	94.79
Balaghat	BGT	Cluster-IV	1.740	1.589	0.322	0.461	73.96
Bhanupratapur	BHN	Cluster-I	1.656	1.482	0.268	0.389	65.62
Dhamtari	DTR	Cluster-I	1.583	1.458	0.250	0.359	58.33
Seoni	SEO	Cluster-III	1.729	1.522	0.290	0.422	72.92
Rewa	REW	Cluster-III	1.448	1.341	0.188	0.271	44.79
Chhindwara	CWA	Cluster-III	1.573	1.411	0.229	0.332	57.29

^aGenetic clusters as revealed by Bayesian based structure analysis N_a Observed number of alleles, N_e Effective number of alleles [Kimura and Crow (1964)], h Nei's (1973) gene diversity, I Shannon's Information index [Lewontin (1972)], P percentage of polymorphic loci

differentiation that comprised two hierarchical levels, 73% within populations and the remainder 27% between populations (Table 4). Nei's (1978) unbiased measure of genetic

identity and genetic distance between different populations ranged from 0.074 to 0.363 with the highest genetic distinction between the population pair CWA and DTR,

Table 4 Analysis of molecular variance for 10 populations of *Litsea glutinosa* Lour

Source of variation	df	Sum of squares	Variation components	Percentage of variation
Among Pops	9	485.865	4.859	27
Within Pops	74	976.706	13.199	73
Total	83	1462.571	18.058	100

Table 5 Nei's (1978) Unbiased Measures of genetic identity and genetic distance (Above diagonal-Nei's genetic identity and below diagonal- Nei's genetic distance)

Pop ID	KDG	JDB	JBP	MRV	BGT	BHN	DTR	SEO	REW	CWA
KDG	****	0.8856	0.8487	0.8702	0.8193	0.8027	0.77	0.8222	0.7348	0.7507
JDB	0.1215	****	0.8518	0.8349	0.8355	0.7408	0.7594	0.8161	0.7709	0.7495
JBP	0.1641	0.1604	****	0.8947	0.8441	0.8436	0.835	0.8735	0.8014	0.7905
MRV	0.139	0.1805	0.1113	****	0.9245	0.8527	0.8582	0.8676	0.8141	0.7757
BGT	0.1993	0.1797	0.1695	0.0785	****	0.8373	0.8349	0.8488	0.8416	0.7728
BHN	0.2198	0.3	0.1701	0.1594	0.1775	****	0.8867	0.8131	0.7691	0.7099
DTR	0.2614	0.2752	0.1804	0.1529	0.1804	0.1202	****	0.8387	0.7903	0.6956
SEO	0.1958	0.2033	0.1353	0.142	0.1639	0.207	0.176	****	0.9287	0.8908
REW	0.3082	0.2602	0.2214	0.2057	0.1725	0.2625	0.2353	0.074	****	0.8554
CWA	0.2867	0.2884	0.2351	0.254	0.2577	0.3426	0.363	0.1156	0.1562	****

followed by CWA and BHN and the lowest genetic distinction observed between population pair REW and SEO, followed by MRV and JBP (Table 5).

Neighbor-joining dendrogram of 84 genotypes based on pair wise distance produced four major clusters each of two populations that subscribed the genotypes of BHN and DTR populations in the first cluster, KDG and JDB populations in the second cluster, REW and CWA in the third cluster and MRV and BGT in the fourth cluster. Genotypes of SEO and JBP populations get intermixed in these four clusters (Fig. 2).

Further, Bayesian model based STRUCTURE analysis (Figs. 3, 4) also detected four clusters ($K = 4$) indicating existence of four genetic pools in *L. glutinosa* populations of Central India. The 10 sampled populations displayed variable proportions of membership drawn from the four Bayesian clusters (Table 6). The computed divergence in allelic frequencies among the inferred four clusters demonstrated the highest divergence between cluster 2 and 4 followed by cluster 1 and 2 (Table 7). Expected heterozygosity among individuals of the cluster 2 was higher than that among the rest three clusters (Table 8).

Discussion

In forest tree species with no prior genomic information, the dominant marker system like ISSR is routinely employed for assessment of genetic diversity and population fragmentation (Junqiu et al. 2006; Ci et al. 2008; Panda et al. 2015). ISSR marker system employed in the present investigation has exhibited high PIC value that

indicates its suitability and informativeness (Botstein et al. 1980). The adequacy of polymorphism detected by a marker system is essential for estimation of population genetic parameters, for this, it should sample optimum polymorphic loci representing the entire genome (Staub et al. 2000; Mariette et al. 2002; Luikart et al. 2003; Singh et al. 2015).

Our study reveals the existence of high level of genetic diversity (P : 44.79–94.79%, h : 0.188–0.414 and I : 0.2706–0.5915) in the sampled populations of *L. glutinosa* compared to those of endangered species, e.g. *Commiphora wightii* (Haque et al. 2010), *Mentha cervina* (Rodrigues et al. 2013), *Breonadia salicina* (Gaafar et al. 2014). This confirms that *L. glutinosa* populations are dwindling in their natural habitats of Central India but still harbor adequate genetic diversity to be utilized for chalking out effective strategy for their conservation and reversing fragmentation. Else, the fragmentation would widen and the species be drifted to endangered status.

With the help of ISSR markers, Junqiu et al. (2006) have also reported higher values of diversity indices (P : 87.01%, h : 0.2466 and I : 0.3826) in *Litsea szemaois*, an endangered species of the same genus. Ci et al. (2008) have enlisted several possible reasons for high estimates of genetic diversity in Lauraceae including mating system, life history and habitat. *Litsea glutinosa* harbors a dioecious mating system that possibly confers such high genetic variation to its sampled populations (Atwell et al. 1999). In fact, high levels of genetic diversity can be maintained in some rare and endangered species at small population sizes (Rossetto et al. 1995; Ci et al. 2008; Gordon et al. 2012; Zhao et al. 2012). Wu et al. (2015) have also reported high genetic

Fig. 2 Bootstrapped neighbor joining tree of 84 *Litsea glutinosa* (Lour.) genotypes constructed using ISSR loci

Fig. 3 Bar chart for the proportion of the member coefficient of each individual/population for best k (K = 4) prepared using Structure Plot v 2.0

diversity in critically endangered *Rhododendron protistum* var. *Giganteum*. The observed high estimates of genetic diversity indices are, however, in contrast to the established relationship between genetic variation and population size (Frankham 1996; Leimu et al. 2006) and also to the earlier

reports in other endangered tree species such as *Metrosideros boninensis* (Kaneko et al. 2008), *Ostrya rehderiana* (Li et al. 2012), *Aquilaria malaccensis* (Singh et al. 2015), *Pulsatilla patens* (Szczecinska et al. 2016).

Fig. 4 Population structure estimation in a set of *Litsea glutinosa* genotype. ΔK calculated by the Evanno et al. (2005) method. Maximum value is observed at $K = 4$

Table 6 Proportion of membership of each pre-defined population in each of the 4 clusters i.e. population Q matrix

Given population	Inferred clusters				No of individual
	1	2	3	4	
KDG	0.077	0.564	0.006	0.354	8
JDB	0.039	0.280	0.047	0.634	8
JBP	0.168	0.398	0.100	0.334	9
MRV	0.053	0.165	0.068	0.713	7
BGT	0.062	0.062	0.012	0.864	9
BHN	0.893	0.062	0.014	0.031	9
DTR	0.758	0.031	0.024	0.187	8
SEO	0.161	0.112	0.686	0.042	9
REW	0.039	0.014	0.920	0.027	8
CWA	0.031	0.173	0.791	0.004	9

Analysis of molecular variance (AMOVA) showed that 27% of genetic variation occurred between the 10 populations, whereas 73% of genetic variation occurred within these populations indicating weak differentiation between the sampled populations. It may be reiterated that *Litsea glutinosa* is a dioecious species and therefore invariably out crossing species. This may have contributed to the higher percentage of within population variation. It indicated weak genetic differentiations of populations, which is common feature in predominantly out crossing tree species (Hogbin and Peakall 1999; Mohapatra et al. 2009; Vaishnav et al. 2015). Further, Nei's unbiased measures of genetic identity and genetic distance also indicated towards the less genetic divergence between sampled populations.

Table 7 Allele-freq. divergence among inferred populations (Net nucleotide distance), computed using point estimates of P

	Cluster-1	Cluster-2	Cluster-3	Cluster-4
Cluster-1	–	0.2252	0.1924	0.1420
Cluster-2	0.2252	–	0.2013	0.2432
Cluster-3	0.1924	0.2013	–	0.1511
Cluster-4	0.1420	0.2432	0.1511	–

Table 8 Average distances (expected heterozygosity) between individuals in same cluster

Cluster no.	Avg. distance
Cluster 1	0.2284
Cluster 2	0.2807
Cluster 3	0.1781
Cluster 4	0.1805

Knowledge about the relationship among populations/subpopulation provides an invaluable aid in formulating an effective conservation strategy. Population structure analysis helps in understanding genetic diversity in a given species, its fragmentation and identification of appropriate sub-populations enabling to capture maximum genetic diversity for conservation and improvement. Our assessment of population structure using Bayesian model based STRUCTURE analysis revealed that four genetic pools of *Litsea glutinosa* exists in central India. However, none of the *L. glutinosa* populations constituted a distinct group but remained intermixed in four pools. This result indicates towards the existence of continuous population of this species in recent past that became fragmented and rendered dwindling in the forest of central Indian states due to unscientific and unchecked extraction by the traders (Rath 2004). The overexploitation of *L. glutinosa* appears to have occurred with a rapid pace in the recent past that has anthropogenically fragmented the population. However, the rapid overexploitation of the species could set only a weak genetic drift and mating isolation in the fragmented population that takes a prolonged period (Ranker 1994). Consequently, the inferred four clusters exhibit a weak structure with admixing of sampled genotypes. This is also supported by the Neighbor-joining clustering and analysis of molecular variance to a great extent.

Conservation implications

In consonant with the above discussion, the estimates of diversity indices indicate high level of genetic diversity in *L. glutinosa*. However, existence of limited number of

mature tree corroborated its dwindling status in the Central India. Therefore, suitable conservation strategies should be devised and deployed for its conservation, gainfully employing the information obtained from the present investigation. Looking to dioecious nature of this non-domesticated forest species, the in-situ conservation may probably be the most viable approach. As genetic structure revealed existence of four admixing genetic pools, an emphasis should be given on diversity, population size and representation to all four inferred genetic pools. For the purpose of conservation, *L. glutinosa* genetic resources from Chhindwara, Jagdalpur, Balaghat, Jabalpur, Bhanupratapur would be appropriate.

For ex-situ conservation which is complementary to in-situ approach and sometimes obligatory strategy for rescuing the species at the verge of extinction, the broad-based sampling methodology needs to be followed to capture maximum diversity as the targeted species harbors major variation proportions within population. Considering a great demand for the bark powder, the concerned state forest departments are advised to include *L. glutinosa* in their perennial large plantation programmes such as Hariyali Prasar Yojna, National Afforestation Programme, etc.

Conclusion

In conclusion, our investigation suggests that: (1) high levels of genetic diversity exist primarily within rather than between populations of *L. glutinosa*, (2) unsustainable extraction has led to widespread population fragmentation rather than genetic factors, and (3) only four genetic pools of *L. glutinosa* exist in central India.

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