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RESEARCH ARTICLE

Assessment of genetic diversity in *Ganoderma lucidum* using RAPD and ISSR markers

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ABSTRACT: Genetic diversity of 31 isolates of *Ganoderma lucidum* from different hosts and geographic regions were analyzed using RAPD and ISSR markers. Ten polymorphic RAPDs were selected from 156 primers along with 8 polymorphic universal ISSR primers. RAPD and ISSR exhibited polymorphism of 88.2 and 78.8 per cent, respectively. RAPD markers, *viz.* S-1005, OPF-6 exhibited maximum polymorphism (93.3%) whereas ISSR- 833 showed maximum of 89.6 per cent polymorphism. Cluster analysis of 31 isolates/strains of *G. lucidum* grouped isolates in 4 subpopulation at 42 per cent (RAPD) and 5 subpopulation using ISSR markers at 40 per cent similarity coefficient. Multilocus ISSR primers clustered isolates based on their geographical origin to considerable levels and exhibited fair similarities among grouping of isolates having sessile/short stalk length than those having long stalk. Therefore, this study reports the high level of genetic diversity of *Ganoderma lucidum* from North Western Himalayas using both RAPD and ISSR techniques.

Key words: *Ganoderma lucidum*, genetic variability, RAPD, ISSR

Ganoderma lucidum (Fr.) Karst. is a wood rotting fungus belongs to phylum Basidiomycota, class Agaricomycetes, order Aphyllophorales and family Ganodermataceae. The genus comprises around 80 species worldwide and about 46 species are found in India, including *Ganoderma lucidum* and *G. philippii* (Kirk *et al.*, 2008; Ranadive *et al.*, 2011). The polyporous Ganoderma mushroom is characterized by basidiocarps (sessile or stalked) having large, perennial, woody brackets known as 'conks'. Spores are generally double-walled, truncate with yellow to brown ornamented inner layers. Fungus commonly known as Ling-zhi, Reishi, Mannentake is used to prevent and treat chronic diseases like hepatitis, bronchitis, hypertension, hypercholesterolemia and gastric cancer for over 2000 years in China, Japan, Korea and other Asian countries (Zhu *et al.*, 2007). The presence of various bioactive molecules including alkaloids, terpenoids, polysaccharides, steroids, fatty acid and proteins led it to title called 'king of herbs' (Paterson, 2006; Ihayere *et al.*, 2010). Fungus is known to grow saprophytic or parasitically on wooden logs of wide host under tropical and warm temperate conditions.

Correct identification solely based on morphological characters has remained problematic in past (Ryvarden, 1994) as fungi show overlapping phenotypic characters and specific interhybridization under different environmental conditions (Zhao and Zhang, 1994). In the absence of well-defined morphological markers, it become mandatory to utilize the environment independent DNA based molecular technique for correct identification of closely related or strains of industrial interest (Zheng *et al.*, 2009). Among these techniques, RAPD and ISSR markers based on genetic characterization of populations are the most commonly used methods. RAPD markers despite constraint of low reproducibility are popular genetic markers for inter/intraspecific variability analysis. As these short 8-12 nucleotides markers amplify both regions transcript/translated, and non-coding regions of genome at random without prior knowledge of DNA sequence information making. Other techniques like ISSR-PCR is known to generate multilocus information based on amplification of inter repeat region between two identical simple-sequence repeats (SSRs) or microsatellites that are oriented in opposite directions. The high evolutionary rate of SSR regions throughout genome enables these primers to detect significant polymorphism with high stability and good reproducibility. Therefore, the present study has been designed with the objective to study the prevailing genetic diversity of *Ganoderma lucidum* in North West Himalayas.

MATERIALS AND METHODS

Collection of *Ganoderma lucidum* isolates, their identification and maintenance

Thirty one isolates/strains of *Ganoderma lucidum* were collected from various locations of Himachal Pradesh during July-September for two consecutive years (2010 and 2011). Mycelial cultures of *G. lucidum* were collected/procured from Directorate of Mushroom Research, Solan and various other sources as mentioned (Table 1) and identified on the basis of their morphological and microscopic characteristics using standard description of the species. Isolation of the procured isolates were made on malt extract agar medium (Malt 20 g and Agar-agar 20 g/litre) and cultures were incubated at 25-35°C in BOD incubator.

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Table 1. Collection of different isolates of *Ganoderma lucidum*

Country	Location/ source	District/Place	Isolates	Host
India	Uttarakhand	Ranikhet	GL1	Dead wooden log
		Almora	GL2	Dead Oak stump
		Lanudowne	GL3	Dead wooden log
		Pantnagar	GL4	Dead Eucalyptus log
		Dawramar	GL5	Dead wooden log
	Himachal Pradesh	Billing	GL6	Dead wooden log
		Dharamshala	GL7	Dead wooden log
		Palampur I	GL8	Dead Plum tree stump
		Palampur II	GL9	Dead wooden log
		Tanda	GL10	Dead Oei stump
		Bir	GL11	Dead wooden log
		Palampur III	GL12	Living Oei
		Banuri	GL13	Living Oei
		Panchrukhi	GL14	Dead wooden log
		Bhattu	GL15	Dead wooden log
		Bandla	GL16	Dead wooden log
		Tinbad	GL17	Dead wooden log
		Andreta	GL18	Dead Oei stump
		Jaisinghpur	GL19	Dead wooden log
		Thural	GL20	Dead Oei stump
		Bhotta	GL21	Dead wooden log
		Saloni	GL22	Dead wooden log
		Nalti	GL23	Living Mango tree
		Chail	GL24	Living Deodar tree
		Summerhill	GL25	Living Deodar tree
		Sarkaghat	GL26	Dead wooden log
		Jogindernagar	GL27	Dead wooden log
		Chamba	GL28	Dead wooden log
		UHF, Nauni, Solan*	GL29	-
Korea *	DMR, Solan	-	OE52	-
Korea *	DMR, Solan	-	OE53	-

*Procured cultures

DNA Extraction

For DNA extraction, mycelium of each isolate was multiplied on malt extract broth in a shaking incubator (100 rpm), maintained at 28°C for 6-7 days using CTAB method (Murray and Thompson, 1980) with minor modifications. RNase @10 µl/ml (MBI Fermentas) was added to remove RNA and the solution was incubated for 30 min at 37°C. The DNA was stored at -80° in deep freezer (Labtech®, Daihan Lab tech Co. Pvt. Ltd.) for further use. The quantity and quality of DNA was assessed both by spectrophotometer (BioRad Smart Spec 3000) and 1.4% agarose gel, and finally stored at -80°C for further use.

RAPD analysis

A total of 156 10-mer primers were screened in replicate on two randomly selected isolates of *G. lucidum* in order to select primers showing maximum polymorphism with reproducible banding pattern (Operon Technologies Inc. Almedea, USA and Life Technologies, India Pvt. Ltd.). Ten most polymorphic and reproducible primers, *viz.* OPA-1, OPA-11, OPF-1, OPF-6, OPD-1, OPD-4, OPD-13, OPQ-1, OPBC-20 and S-1005, were finally used for genetic diversity analysis of 31 isolates of *G. lucidum.*

ISSR analysis

A total of 50 inter-simple sequence repeats (ISSR) primers were screened against two randomly selected isolates of *G. lucidum* to select the most polymorphic primers. Out of these eight ISSR primers, namely ISSR 808, ISSR 820, ISSR 831, ISSR 832, ISSR 833, ISSR 837, ISSR 839 and ISSR 854 exhibiting maximum polymorphism were finally used for genetic diversity analysis.

PCR conditions and amplification

The PCR amplification was carried out in 0.2 ml PCR tube with 25 µl reaction volume containing 2.5 µl of 10x buffer (20 mM Tris HCl, pH 8.0, 50 mM KCl) and 1.5 µl of 1.5 mM $MgCl_2$, 2 µl of 0.2 mM each dNTP mix (MBI Fermentas), 0.2 µl of Taq polymerase with 5 U/µl concentration (Merck Biosciences, India), 2 µl of DNA template (20 ng), 1.0 µl primer (10 µM) and 15.8 µl of sterilized distilled water.

Reaction mixture was vortexed and centrifuged in a microfuge (Bangalore Genei, India) for the proper mixing of the contents. Amplifications were performed using thermal cycler (2720 Thermal Cycler, Applied Biosystems, USA) programmed for initial denaturation at 94°C for 5 minutes followed by 40 cycles at 94°C for 1 minute, 37°C for 1 minute, 72°C for 2 minutes and a final extension at 72°C for 5 minutes.

The amplified PCR products obtained with ten RAPD and eight ISSR primers were resolved by electrophoresis using 1.2 per cent agarose gel in 0.5x Tris Borate EDTA buffer (0.5 M Tris, 0.05 M boric acid and 1mM EDTA, pH 8.0), visualized using ethidium bromide (0.5µg/ml) staining and gel images were captured using Alphaimager 2200 gel documentation system (Alphaimager, USA).

Data analysis

DNA fingerprints of 31 isolates of *G. lucidum* obtained with most polymorphic RAPD and ISSR primers were recorded for their presence (1) or absence (0) of DNA bands and recorded in binary matrices. Binary matrices were analyzed by NTSYS-PC software/version 2.0 (Rholf, 1998) and Jaccard's coefficient was used to construct dendrogram using SHAN clustered programme, selecting the unweighted pair group arithmetic mean (UPGMA). The dendrogram best fit to similarity matrix based on cophentic value (COPH) and matrix comparison (MXCOMP) was chosen.

RESULTS AN DISCUSSION

RAPD analysis

Data generated using 10 most polymorphic primers, *viz.* OPA-1, OPA-11, OPF-1, OPF-6, OPD-1, OPD-4, OPD-13, OPQ-1, OPBC-20 and S-1005, revealing high genetic diversity among 31 isolates of *G. lucidum.* Cluster analysis grouped isolates into two clusters (I and II) at 38 per cent similarity coefficient and comprising of 28 and 3 isolates, respectively. Cluster Ia was further subgrouped into Ia and Ib with 23 and 5 isolates respectively. In all, 31 isolates were subdivided into 4 subpopulations with a similarity coefficient of 42 per cent. There has been no study on assessment of genetic diversity in wildly grown isolates from NW Himalayas. The present study showed high diversity can be further evaluated for comparative analysis of bioactive constituents among isolates. Earlier, Hseu *et al.* (1996) has suggested the usefulness of RAPDs in systematics at the lower taxonomic levels where ITS sequence failed to discriminate within species. Singh *et al.* (2003) also characterized 61 accessions of *G. lucidum* using RAPD and AFLP analysis revealed the highly significant intraspecific variability among *G. lucidum* isolates and distinguished them into 3 different clades. Mishra (2005) in his studies using RAPD grouped wild and cultivated strains of *G. lucidum* into three clusters at 50 per cent similarity coefficient. Similar studies on *G. lucidum* through RAPD analysis were conducted by various workers, (Postnova and Skolotneva, 2009; Ghazala *et al.*, 2010; Rolim *et al.*, 2011).

ISSR analysis

Dendrogram obtained using the binary data for the presence and absence of amplicon clustered 31 isolates into two main group i.e. cluster I and II comprised of 10 and 21 isolates at 40 per cent similarity coefficient, respectively. Both the main clusters were further subgrouped into Ia and Ib accommodating 8 and 2 isolates and cluster IIa and IIb accommodating 10 and 11 isolates, respectively. Isolates with different geographical origin were grouped in separate clusters. Population was fairly distinguishable with respect to short stalk (GL8, GL9, GL10, GL17, GL26, GL27), sessile (GL15 or GL16) in cluster I, whereas those having long thin stipe grouped in cluster II. Multilocus ISSR primers generated good diversity and differentiated isolates based on their stipe length and geographic distribution. The high polymorphism shown by isolates suggests different rate of evolution at microsatellite regions of genome of 31 isolates representing different geographical locations. However, no congruence among the dendrograms was generated using both RAPD and ISSR markers. A possible explanation for the difference in resolution of RAPDs and ISSRs would be that both markers target different portions of the genome (Bhattacharya *et al.,* 2010).

High variability was also reported using 16 ISSR primers among nine *G. lucidum* (MG1-MG 9) strains, out of which, six primers produced 156 fragments and the number of fragment ranged from 6 to 17 fragments in each profile (Rueankeaw and Danuwat, 2010). Recently, use of both ISSR primer and improved RAPD (RAMP) to characterize 10 strains of *Ganoderma* spp. reported high level of genetic differences between different species and among the *G. lucidum* strains (Mei *et al.*, 2014). Few studies have been conducted using multilocus, highly reproducible ISSR marker in *Ganoderma* spp. for genetic variability studies.

Combined RAPD and ISSR analysis

Out of 245 scorable bands, 205 were found polymorphic with 83.67 per cent polymorphism with maximum polymorphism of 93.3% exhibited by OPF-6 and S-1005 (Table 2). The dendrogram generated using combined data set of both RAPD and ISSR markers separated *G. lucidum* isolates in two distinct clusters I and II with

Table 2. Number of polymorphic bands obtained by PCR amplification of *Ganoderma lucidum* DNA with operon and ISSR primers

Primer	Scorable band	Polymorphic band	Per cent Polymorphism
OPA-1	13	10	76.9
OPA-11	13	12	92.3
OPF-1	14	12	85.7
OPF-6	15	14	93.3
OPD-1	10	9	90.0
OPD-4	11	10	90.9
OPD-13	11	9	81.8
OPQ-1	12	11	91.7
OPBC-20	13	11	84.6
S-1005	15	14	93.3
ISSR-808	14	11	78.6
ISSR-820	14	12	85.7
ISSR-831	15	11	73.3
ISSR-832	14	8	57.1
ISSR-833	19	17	89.6
ISSR-837	15	13	86.7
ISSR-839	12	9	75.0
ISSR-854	15	12	80.0
Total	245	205	83.67

similarity coefficient of 44 per cent (Fig. 1). Both clusters namely cluster I and II accommodate 29 and 2 isolates, respectively. The first cluster was subdivided into Ia and Ib, having 21 and 8 isolates/strains, respectively. Whereas, cluster II comprised of two isolates having vacuolated basidiospores (Fig. 1).

Within the subcluster isolates from same environmental conditions and similar morphology were subgrouped like GL15 and GL16 (Sessile), GL24 and GL25 (sub-temperate zone); GL20, GL21, GL22 and GL23 (tropical climate); based on their geographical origin (i.e. OE52 and OE53) and location (GL1 to GL5). Close relationship between isolate GL8 and GL9 (78% similarity coefficient), was found, suggesting that they might have been collected from same location or derived from same parent.

In general, polymorphism in a given population is often due to the existence of genetic variants represented by the number of alleles at a locus and their frequency of distribution in a population (Powell *et al.*, 1996). High levels of genotypic variability are most likely due to exposure of the pathogen to diverse environments and a wide host range. Also, Wang *et al.* (2012a) combined RAPD and ISSR dendrogram to assess genetic diversity of 32 *Pleurotus eryngii* strains and found that combined analysis was better for understanding genetic relationships than RAPD and ISSR alone.

However, despite the lack of universal agreement on number of markers, their type, critical individual number and effective loci in diversity studies. Diversity analysis with more sample size and extensive surveys are useful. Because of reason that areas of high biodiversity often serve as a source for the emergence of new genotypes with novel biological characteristics for future utilization. Differential banding pattern obtained

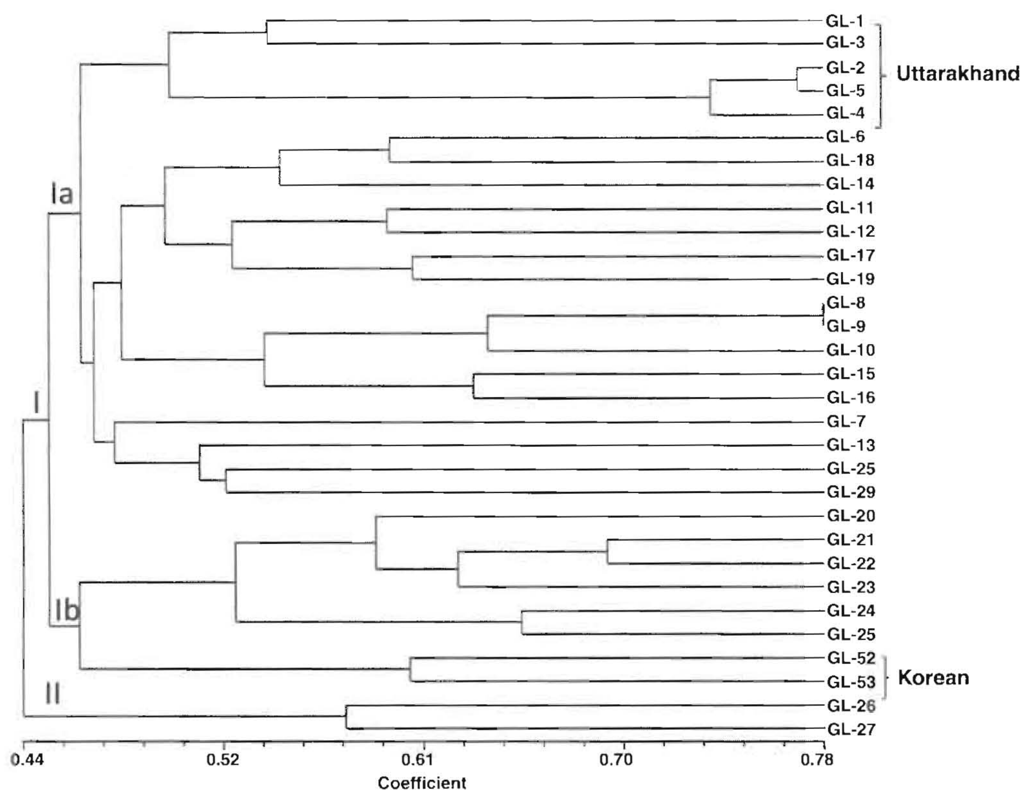

Fig. 1. Dendrogram of 31 isolates of *Ganoderma lucidum* generated by UPGMA (Unweighted pair group method arithmetic mean) analysis of DNA fingerprints obtained with RAPD and ISSR primers

by multilocus primers used by various workers suggests the accumulation of various mutations randomly at genome level.

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