Analyses of morphological and genetic variability in *Ganoderma* isolates collected from north west Himalayas

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Abstract
The morphological and genetic variability of thirty one Ganoderma isolates collected from their natural habitat were assessed by macroscopic / microscopic characters and RAPD molecular markers respectively. Based on the surface texture of fruiting bodies, isolates in the present study were broadly classified as non-laccate and laccate Ganoderma.

Out of 10 OPA primers (Operon Technologies, Inc.) used for the RAPD analyses, six showed good polymorphism (i.e. OPP-7, OPP-8, OPP-14, OPP-18, OPP-19 and OPP-20). The clustering based on similarity coefficients allowed the separation of isolates in two groups.

Keywords: Edible mushroom, RAPD, Mycelial growth.

Introduction

Ganoderma is a cosmopolitan white rot fungal genus famous for its medicinal properties. The fruit body of *Ganoderma* for its perceived health benefits has gained wide popular use as a dietary supplement in different parts of the world. It is used to prevent and treat immunological diseases like hypertension, tumorigenesis etc. *Ganoderma* species have a worldwide distribution in ecosystems both in tropical and temperate geographical regions. They are usually found in subtropical and tropical regions since they can survive under hot and humid conditions.

Ganoderma species grow as a facultative parasite that can live as saprobes on rotting stumps and roots[16]. Basidiocarps, in *Ganoderma*, grow from a living, or more commonly, from a dead trunk or branch of a tree in the form of a bracket. One of the two types of basidiocarps is produced depending on the species: a laccate fruiting body with a shiny upper surface or a non – laccate fruiting body with a dull upper surface 2.[16,24]

Species and collections of *Ganoderma* have been misnamed. This is due to the presence of heterogenic forms, taxonomic obstacles and inconsistencies in the way the genus has been subdivided[14]. Since *Ganoderma* species are genetically heterogeneous, a wide range of genetic variation has been reported and caused by out crossing over generations and different geographical origins[13,16]. This has led to variation in their listed morphological characteristics even within same species[6]. The taxonomy of the genus is traditionally based on the morphological characteristics. Though they are really useful for differing species, there are still difficulties for distinguishing the close groups such as the populations or strains of the same species. Environmental factors, variability, interhybridization and morphological propensity make the accurate identification hard for *Ganoderma* species[32].

However, the new techniques using molecular markers have turned easy the identification not only for *Ganoderma* species, but also for other organisms which have the same taxonomic difficulties. The use of DNA as identification tool allowed to differentiate the subgroups, as the various *G. lucidum* strains, making easy to distinguish mainly the commercial strains and strains of industrial interest[31].

Different techniques for molecular studies have been used for analyzing the genetic diversity in basidiomycetes such as isoenzymes[11], AFLP (*Amplified Fragment Length Polymorfism*)[17], RFLP (*Restriction Fragment Length Polymorfism*)[15], ITS (*Internal Transcribed Spacers*)[9] and RAPD (*Random Amplified Polymorphic DNA*)[27]. Among these techniques, RAPD is still one of the cheapest and quickest methods for accessing the variability at DNA level, being especially useful on intraspecific analysis. These markers have the advantage of amplifying both regions of the genome which may be transcript/translated. This is important when the objective is to evaluate the variation along the biggest part of the species genome[5,28].

On the other hand, RAPD has limitations for its low experimental reproducibility, although this problem may be overcome with the use of many primers and with more strict criteria in analyzing the results[2]. This work aimed to select the RAPD molecular markers for evaluating *Ganoderma* isolates genetic similarity.

Culture source/collection of *Ganoderma* isolates: A total of twenty three *Ganoderma* isolates were collected from different regions of Himachal Pradesh and Punjab. Six mycelial cultures of *Ganoderma* were procured from Directorate of Mushroom Research (DMR), Solan (Himachal Pradesh) and two cultures were procured from University of Horticulture and Forestry (UHF).

Identification of isolates: Collected samples were identified on the basis of macroscopic and microscopic traits of basidiocarp and cultural characteristics. Various macroscopic traits (morphological features and dimensions) such as shape, size and colour of pileus, presence or absence of stipe, context colour, length and thickness of stipe of fresh fruit bodies were recorded for accurate identification of isolates. Basidiospores size, colour and shape were also recorded with the help of projection microscope (Radical RXLr-4)[25].

Maintenance of pure cultures: Potato dextrose agar (PDA) was used to raise the pure cultures of isolates.

Analysis of genetic variability: Genetic variability among 31 isolates was characterized using RAPD primers based on polymerase chain reaction (PCR) technique.

DNA extraction: DNA extraction from *Ganoderma* isolates was done as per the protocol. DNA of thirty one isolates was extracted from mycelial threads after multiplication on potato dextrose medium at 25°C for seven days. Mycelial mat was centrifuged for 5 min at 10000 rpm followed by washing with 500 µl TE buffer. The resulting palette was grinded in extraction buffer (200 mm Tris-HCl, pH-8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) with a conical grinder followed by addition of 150 µl of 3M sodium acetate (pH-5.2). The lysates were incubated at -20°C for 10 min followed by centrifugation.

DNA was precipitated from the supernatant by adding equal volumes of isopropanol and resultant pallet was washed with 70% ethanol. The DNA palette was air dried and dissolved in 20 µL TE buffer. Individual PCR reactions were performed for each isolate in 0.2 ml tubes using 10 random decamer primers synthesized from Operon Technologies, USA.

Amplification was performed in 25 µl reactions containing PCR buffer, 5 mM each of the deoxyribonucleotide triphosphate, 10 pmol each of the appropriate primers, DNA having concentration of 15 ng/µl and 1unit of Taq polymerase. The amplification protocol includes initial denaturation at 94°C for 5 min followed by 40 cycles of amplification, 95°C for 1 min (denaturing), 36°C for 1 min (annealing), 72°C for 2 min (extension) and final extension step at 72°C for 10 min.

Gel electrophoresis and RAPD data scoring: The amplicons after PCR were analyzed by electrophoresis on 1.2% (W/V) agarose gels by running in 0.5X TBE buffer. After staining with ethidium bromide, the gels were visualized under a UV transilluminator and photographed using Bio-Rad gel documentation system. The amplification products generated by each RAPD primer were scored as ''1'' or ''0'' for presence or absence of specific allele respectively. To estimate the similarity and genetics distance among different *Ganoderma* isolates, cluster analysis based on Nei's unweighted pair-group with arithmetic average (UPGMA) was performed using the Popgen-32 software and a dendrogram was constructed.

Results and Discussion

A total of twenty three *Ganoderma* isolates designated as GL1, GL2, GL3, GL4, GL5, GL6, GL7, GL8, GL9, GL10, GL11, GL12, GL13, GL14, GL15, GL16, GL17, GL18, GL19, GL20, GL21, GL22 and GL23 were collected from different regions of Himachal Pradesh and Punjab . Six mycelial cultures of *Ganoderma* were procured from Directorate of Mushroom Research (DMR), Solan (Himachal Pradesh) and two cultures were procured from University of Horticulture and Forestry (UHF).

During mycofloristic survey of different regions of Himachal Pradesh and Punjab, *Ganoderma* fruit bodies were seen growing on logs of decaying woods and living hosts as well as on humus soil (Table 1). *Ganoderma* fruit bodies were found on both living and dead trees, mostly in hot places. 34.75% of these hosts were not identifiable as those were highly decomposed. Most common identifiable hosts were *Dalbergia sissoo, Acacia* (khair) and *Albizia chinensis* (13.04%) followed by *Cedrus deodara* and *Acacia nilotica* (8.69%). Mango and *Prunus* were also identified as *Ganoderma* hosts.

Similar to these observations, Singh et al[22] have also reported saprophytic growth of *Ganoderma* near stumps of oak and broad leaved trees in summer and autumn at a temperature of 25-30°C in wild. Elliot and Broschat[3] also confirmed that some species of *Ganoderma* can be found on live trees, the same results were obtained in this study.

The collected *Ganoderma* isolates were identified to be of *Ganoderma*taceae family because of typical characteristics they had in common with other members of this family. All basidiocarps possessed tiny pores underside their cap which contained reproductive spores, these traits were similar to the description given by Van der Westhuizen and Eicker[24] for *Ganoderma.*The colour of the collected *Ganoderma* basidiocarps varied from brown to yellow-brownish and reddish-brown. The collected isolates exhibited ear, finger-shaped, flattened to discoid fruiting bodies in wide ranges of sizes. The fruiting bodies of collected *Ganoderma* isolates were found to be thick, woody and tough hard to break with hands.

Based on the surface texture of fruiting bodies (Table 2), isolates in the present study were broadly classified as non-laccate *Ganoderma* (GL2, GL5, GL7, GL9, GL15, GL17 and GL20) comprising 30.43% of isolates and laccate *Ganoderma* (GL1, GL3, GL4, GL6, GL8, GL10, GL11, GL12, GL13, GL14, GL16, GL18, GL 19, GL21, GL22 and GL23) comprising 69.57% of isolates. A great variation was observed in microscopic and macroscopic traits of basidiocarps and basidiospores of different *Ganoderma* isolates. Similar to our observations, variations in

basidiocarp morphology of naturally produced basidiocarps have also been observed by many other researchers[22,26].

This variation may be due to difference in environmental conditions, type (hardwood/conifer) and nature of host (living/dead) and differential developmental stages of mushroom.[10,19,30] The size of basidiospores in different *Ganoderma* isolates varied from 7.3-9.9×4.8-6.5 µm to 9.5-11.1×6.5-7.2µm. The basidiospore size recorded in the present study also matched with the observations of other researchers from different parts of world. Seo and Kirk[20] reported the size of naturally grown *G. lucidum* basidiospores from Japan and Korea to be 8.5-11.0 × 6.5-8.5 µm and 8.5-13.0 × 5.5-7.0 µm respectively which is more or less similar to our results.

Table 1
Collection details of *Ganoderma* isolates from different regions of (Himachal Pradesh and Punjab) North- Western Himalaya

S.N.	Isolate	Location	Collection origin	Host lant
1.	GL1	Ghagas	31°22'32"N 76°48'17"E	Dead stump of unidentified tree
2.	GL2	Bilaspur	31.33°N 76.75°E	Dead stump of unidentified tree
3.	GL3	Bilaspur	31.33°N 76.75°E	Dead *Acacia catechu* (Khair)
4.	GL4	Bilaspur	31.33°N 76.75°E	Dead stump of unidentified tree
5.	GL5	Swarghat	31.33°N 76.75°E	Dead *Acacia catechu* (Khair)
6.	GL6	Hamirpur	31.68°N 76.52°E	Dead stump of unidentified tree
7.	GL7	Nalti	31.68°N 76.52°E	Dead *Mangifera indica* (Mango)
8.	GL8	Hamirpur	31.68°N 76.52°E	Dead stump of unidentified tree
9.	GL9	Hamirpur	31.68°N 76.52°E	Living *Dalbergia sissoo* (Sheesham)
10.	GL10	Hamirpur	31.68°N 76.52°E	Dead stump of unidentified tree
11.	GL11	Hoshiarpur	31.53°N 75.92°E	Dead stump of unidentified tree
12.	GL12	Hoshiarpur	31.53°N 75.92°E	Dead *Acacia catechu* (Khair)
13.	GL13	Bathinda	30°13′48″N 74°57′07″E	Dead *Acacia nilotica* (Babul)
14.	GL14	Hoshiarpur	31.53°N 75.92°E	Dead *Dalbergia sissoo* (Sheesham)
15.	GL15	Patiala	30.34°N 76.38°E	Living *Acacia nilotica* (Babul)
16.	GL16	Palampur	32.1167°N 76.5333°E	Living *Albizia chinensis* (Oyi)
17.	GL17	Dharamshala	32 .222°N 76.317°E	Living *Prunus* (Plum)
18.	GL18	Palampur	32.1167°N 76.5333°E	Dead *Albizia chinensis* (Oyi)
19.	GL19	Palampur	32.1167°N 76.5333°E	Dead stump of unidentified tree
20.	GL20	Dehra	31.8819125°N 76.2153554°E	Dead *Dalbergia sissoo* (Sheesham)
21.	GL21	Tanda	32°5'56"N 76°17'58"E	Dead *Albizia chinensis* (Oyi)
22.	GL22	Kufri	31.10°N 77.25°E	Living *Cedrus deodara* (Deodar)
23.	GL23	Chail	30.9697°N 77.1975°E	Living *Cedrus deodara* (Deodar)
24.	GL24	DMR, Solan	Unknown*	Unknown*
25.	GL25	DMR, Solan	Unknown*	Unknown*
26.	GL26	DMR, Solan	Unknown*	Unknown*
27.	GL27	DMR, Solan	Unknown*	Unknown*
28.	GL28	UHF, Solan	Unknown*	Unknown*
29.	GL29	DMR, Solan	Unknown*	Unknown*
30.	GL30	UHF, Solan	Unknown*	Unknown*
31.	GL31	DMR, Solan	Unknown*	Unknown*

Table 2
Variations observed in morphological characters of *Ganoderma* isolates collected from different geographical regions

Isolate	Basidiocarp				Basidiospore	
	Texture	Shape	Pileus (cm)	Stipe (cm)	Size (µm)	Q value/ Spore index*
GL1	Laccate	Flattened	5.4- 6.5×3.6-4.2	0.5-1.0×0.8-1.5	8.3-10.1×5.1-6.2	1.3
GL2	Non-laccate	Flattened	3.0- 3.5×4.5- 5.3	2.5- 2.8×0.8x1.2	8.6-11.4×6.0-7.1	1.6
GL3	Laccate	Auriform	6.7-7.5×5.2-5.7	1.2-1.6×0.9-1.1	8.8-10.7×5.8-6.7	1.3
GL4	Laccate	Auriform	6.5-8.1×3.6-4.8	0.5-1.4× 0.5-0.9	8.3-10.1×5.6-6.9	1.7
GL5	Non-laccate	Auriform	6.5-7.3×3.4-4.3	1.5-2.2× 0.7-1.1	9.5-11.5×6.1-7.2	1.4
GL6	Laccate	Flattened	9.2-12.2×5.4-5.7	1.2-1.8× 1.2-1.4	9.3-11.7×6.5-7.8	1.7
GL7	Non-laccate	Discoid	5.6-6.3× 5.1-5.5	3.9-4.3× 0.9-1.2	7.3-9.9×4.8-6.5	1.7
GL8	Laccate	Auriform	9.4-13.6×5.2-5.8	0.5- 0.7×1.0-1.3	8.1-10.2×5.7-6.6	1.6
GL9	Non-laccate	Flattened	9.5-13.5×8.1-8.5	0.5-0.9×0.5-1.0	8.2-10.2×5.5-6.5	1.3
GL10	Laccate	Discoid	3.1-6.4× 2.5-6.3	2.5- 3.5×1.2-1.5	9.1-11.6×5.1-6.8	1.6
GL11	Laccate	Auriform	10.0-12.5×5.2-5.9	0.5- 1.0×0.9-1.6	9.0-11.3×5.5-6.9	1.7
GL12	Laccate	Finger shaped	3.2- 3.8×2.2- 2.6	3.5- 4.0×0.8- 1.2	8.2-10.6×4.9-6.5	1.3
GL13	Laccate	Discoid	2.5- 4.5×2.2-2.6	1.5- 1.8× 0.5-0.9	9.2-11.2×5.3-6.9	1.4
GL14	Laccate	Flattened	9.2-12.5×5.0-6.5	0.5- 1.0×0.9- 1.4	8.4-10.1×6.4-7.1	1.4
GL15	Non-laccate	Flattened	4.5- 6.7× 3.5-6.0	3.9-4.3×0.8- 1.3	9.1-11.1×5.7-7.3	1.6
GL16	Laccate	Flattened	4.5- 7.1×3.9-5.4	3.5- 4.7×0.7- 1.4	8.8-10.2×5.3-6.6	1.5
GL17	Non-laccate	Discoid	3.7-10.3×3.1-4.6	1.7- 2.6×1.1- 1.6	8.2-10.5×5.5-6.8	1.7
GL18	Laccate	Discoid	4.5- 6.4×3.5-4.2	2.5- 3.0×1.0- 1.3	8.6-10.6×5.6-7.1	1.6
GL19	Laccate	Flattened	8.0-11.7×4.1-5.6	Absent	8.6-11.3×6.1-6.9	1.5
GL20	Non-laccate	Discoid	4.3- 5.5×2.5-3.5	3.5- 4.0×1.2-1.5	8.3-10.3×6.7-7.4	1.5
GL21	Laccate	Flattened	6.5-8.8 ×4.0-5.5	2.7- 3.0×1.0- 1.5	8.5-10.7×5.4-6.8	1.3
GL22	Laccate	Flattened	8.0-12.8×4.6-6.6	Absent	9.1-10.9×5.8-7.3	1.4
GL23	Laccate	Flattened	8.0-11.0×4.1-6.4	Absent	9.5-11.1×6.5-7.2	1.5

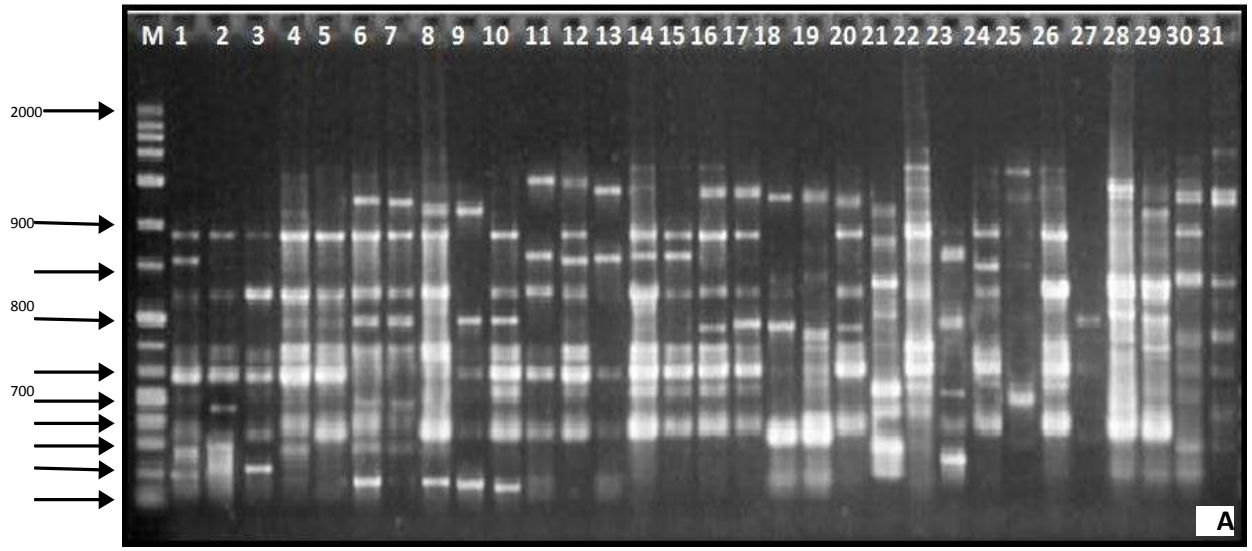
RAPD Analysis: DNA amplification was obtained in all the isolates of *Ganoderma*. Out of 10 decamer primers (i.e. OPP-7, OPP-8, OPP-9, OPP-10, OPP-14, OPP-16, OPP-17, OPP-18, OPP-19 and OPP-20, Operon Technologies Inc. (Alameda, CA, USA), only six primers (i.e. OPP-7, OPP-8, OPP-14, OPP-18, OPP-19 and OPP-20) could yield polymorphism and four primers (OPP-9, OPP-10, OPP-16 and OPP-17) resulted in either sub-optimal or non distinct amplification products. All the six primers gave consistent results and produced reasonable number of distinguishable and polymorphic bands. Banding pattern obtained after PCR amplification of 31 isolates with six most polymorphic decamer primers is shown in figure 1.

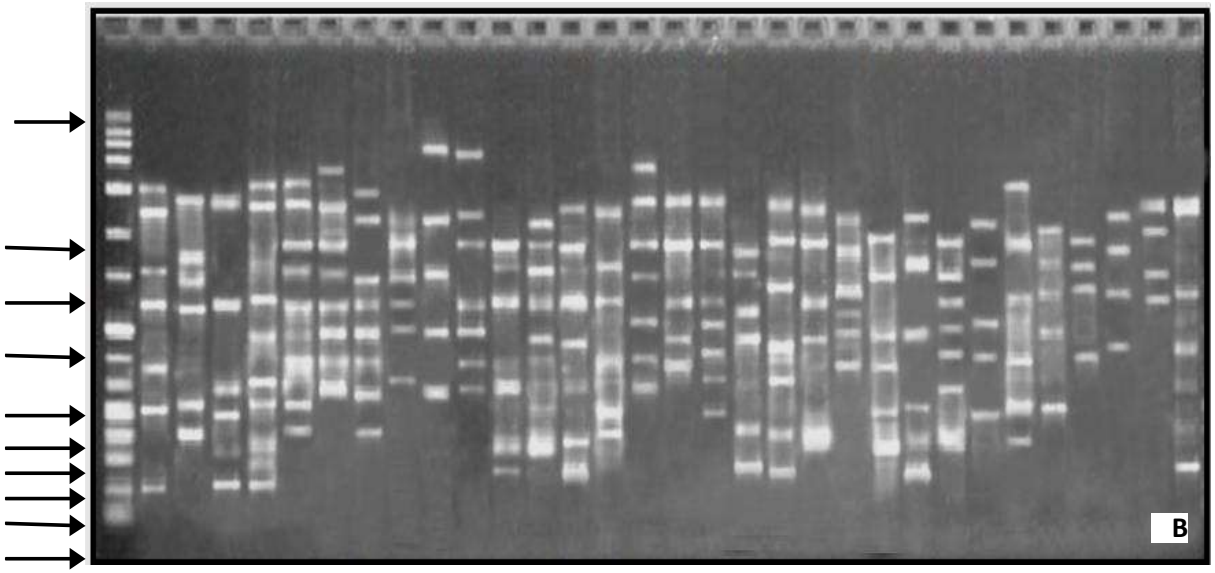
The similarity matrix was constructed based upon the presence or absence of the RAPD fragments. Cluster analysis of data generated a dendrogram using MVSP version 3.22 (Figure 2). Results revealed high genetic diversity in tested isolates. Thirty one isolates were grouped in two clusters (I and II) with a similarity coefficient of approximately 25%. Cluster I comprised of 2 isolates viz. GL25 and GL21 with 31% similarity amongst them and the other cluster contained only 29 isolates. Cluster II was further divided into two sub-clusters (IIa and IIb) with IIa comprised of single isolate (GL29) which was in separate lineage from rest 28 isolates of lower (IIb) sub-cluster. Lower sub-cluster (IIb) further clustered 28 isolates into 7 groups. First group consisted of 2 isolates (GL30 and GL28) with 33% similarity amongst them. Second and third groups comprised of single isolates GL26 and GL27 respectively. Eleven isolates (GL24, GL20, GL18, GL19, GL17, GL16, GL7, GL9, GL10, GL8 and GL6) were clustered together in fourth group with 34% similarity amongst them.

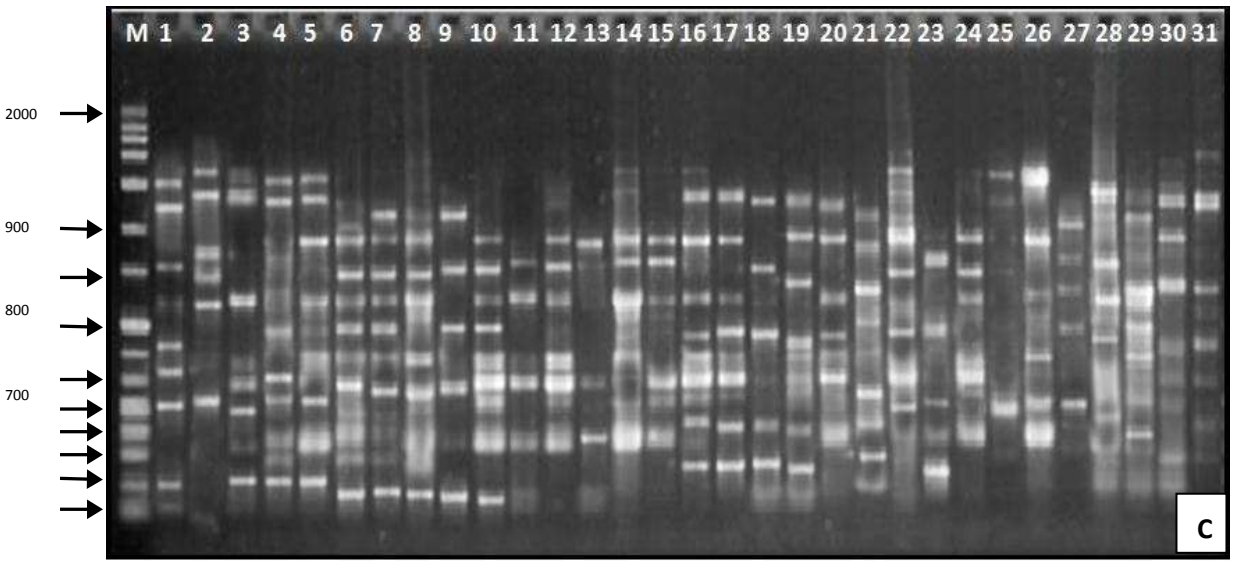
Fifth group consisted of single isolate (GL31) and sixth group comprised of two isolates (GL23, GL22) sharing 48% similarity. Ten isolates were present in seventh group, among them isolates GL13 and GL11 showed maximum similarity (60%) and isolates GL5 and GL1 were 58% similar with each other. Data obtained after RAPD analysis suggests that the genetic variation may depend upon the geographical origin of the mushroom.

These results are in agreement with Idris et al[7] who analyzed RAPD patterns of 40 different *Ganoderma* isolates from different geographical areas and reported similarity of 4.8% to 69.2% among specimen taken for analysis, which also indicated heterogeneity of the *Ganoderma* isolates. The

present findings are in agreement with the work of Keypour et al[8] who studied genetic diversity of two laccate species of *Ganoderma lucidum* and *Ganoderma* resinaceum using RAPD marker and found wide range of inter-specific diversity (61.48 % and 40.16 %) of *G. lucidum* and *G. resinaceum* respectively.







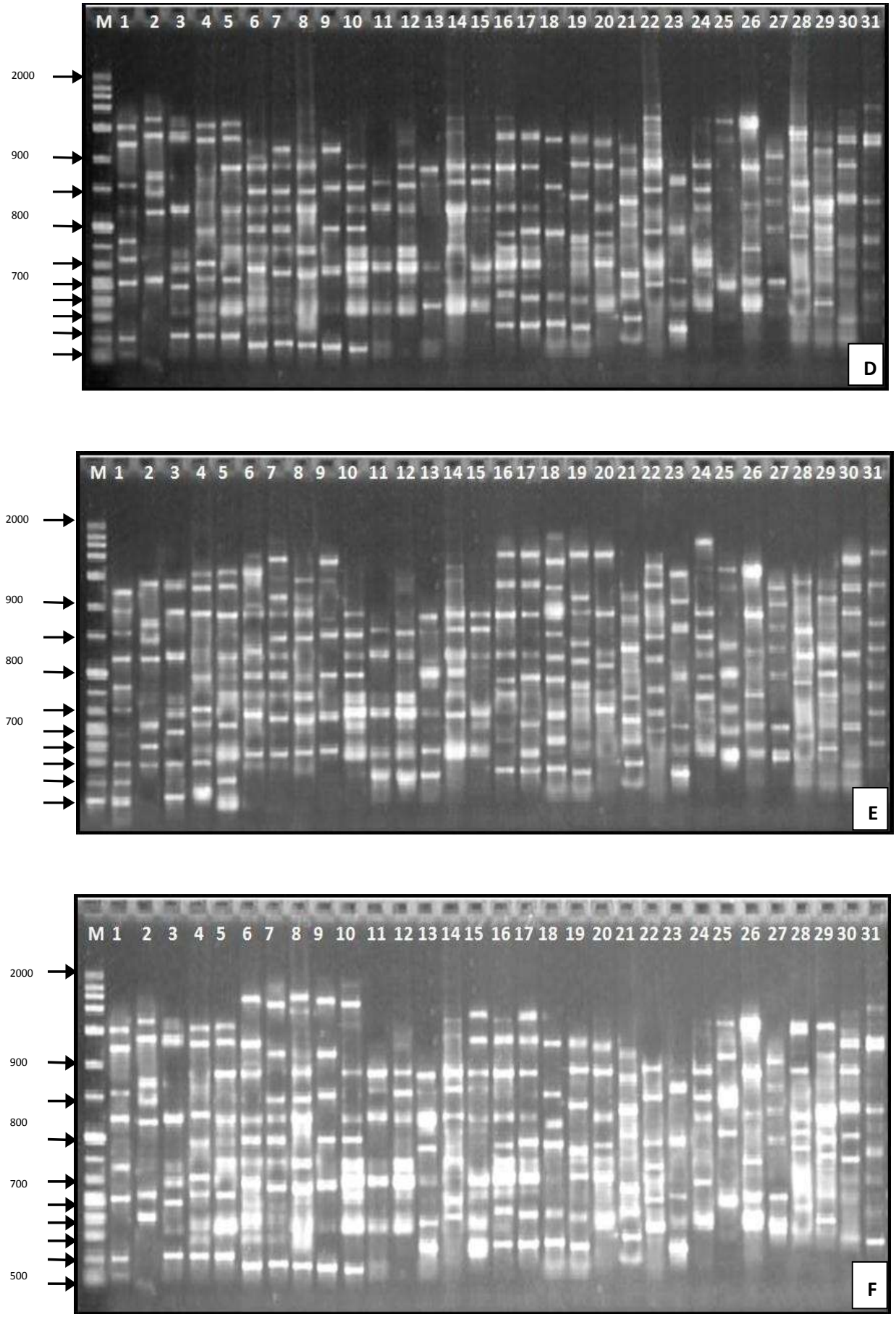


Figure 1: Random amplified polymorphic DNA fragment patterns generated on agarose gel in 31 isolates of *Ganoderma* species using A) OPP-07, B) OPP-08, C) OPP-14, D) OPP-18, E) OPP-19 and F) OPP-20 primer

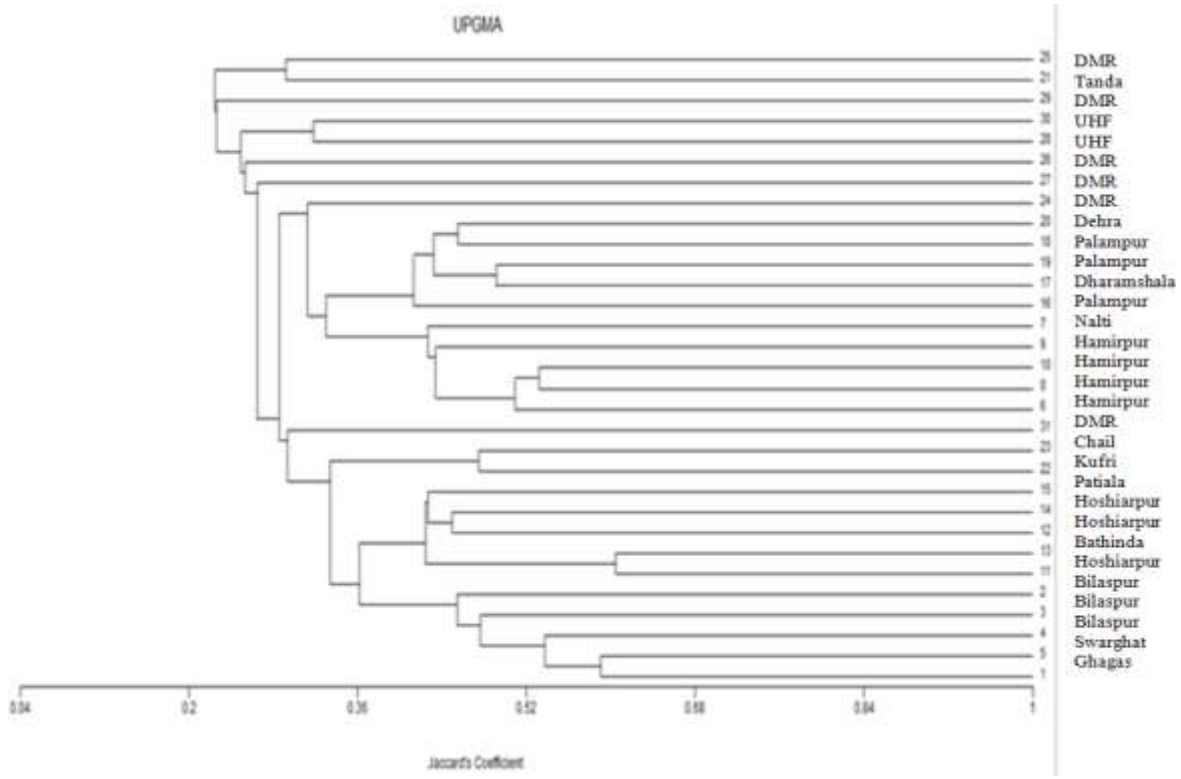


Figure 2: Dendrogram of 31 isolates of *Ganoderma* generated by UPGMA (Unweighted pair group method arithmetic mean) analysis of DNA fingerprints obtained with six RAPD primers

Conclusion

Familoni et al[4] found genetic relatedness and genetic diversity among studied wild *P. ostreatus* indigenous to two sates in Southwestern Nigeria using RAPD markers. Data obtained after RAPD analysis suggests that the genetic variation may depend upon the geographical origin of the mushroom.

Additionally, Zakariah et al[30] stated that genetic diversity within *Ganoderma* species can be a result of adaptation to various geographical regions, adaptation to exploit different hosts or may be the genus had evolved from an ancestor with a wide genetic base. Lastly, high level of genetic diversity in *Ganoderma* species perhaps may be a result of horizontal gene transfer which usually happens between fungus and their host through natural selection fungus tending to evolve relative ways of gene acquisition from their hosts for better adaptation to their hosts.[12]

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