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Molecular characterization of specialty mushrooms of western Rajasthan, India

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Eighteen specialty mushroom germplasm accessions were collected from Udaipur (Rajasthan) and characterized using DNA fingerprinting and ribosomal rRNA gene sequencing. Phylogenetic analyses based on RAPD profiles and nucleotide sequence of 5.8S rRNA gene along with its spacer regions revealed variation of inter-generic and intra-species isolates among accessions. Based on ITS sequence polymorphism, seven isolates were identified as *Podaxis pistillaris*, four as *Phellinus igniarius*, one as *Gymnopilus subearlei* and six as *Phellorinia herculea*. The similarity matrix revealed very high intra-species homology (99.5–100%) and significant inter-generic diversity (21.2–37.7%). *G. subearlei* and *P. herculea* have been discovered as new additions to the Indian basidiomycetes biodiversity.

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Keywords: ITS, molecular taxonomy, mushroom, phylogenetic analysis, sequences polymorphism.

MUSHROOMS supplement and complement the nutritionally deficient cereals and are regarded as the highest producers of protein per unit time and area. High demand for mushrooms in Europe and America makes them an important horticultural crop and an important foreign-exchange earner. Mushroom mycelia and spores are often microscopic and usually filamentous with very few phenotypic markers that can be used to differentiate between individuals in a population. This limitation has hampered the studies of their population biology. In the past, plant pathologists had to rely on phenotypic markers such as vegetative compatibility, mating type or specific avirulent gene to differentiate individuals[1,2].

The term 'specialty mushroom' is commonly used to encompass all mushrooms that are less common in a particular area or country. In the Indian context, all edible mushrooms other than the common button mushroom, *Agaricus* are grouped under the specialty mushrooms[3]. Different authors have used different criteria for taxonomic classification of specialty mushrooms belonging to Homobasidiomycetes[4,5]. Lack of common and unifying criteria has contributed to the difficulty of studying these mushrooms in a systematic manner[6–8].

Phellorinia inquinans and *Podaxis pistillaris* have been reported as wild edible mushrooms from Rajasthan, Punjab and Haryana[9–11]. *Phellinus ignarius* often found associated with Esca disease of grapevine[12] and wood decay of apple orchards[13], is also being used as a traditional medicine[14]. The genus *Gymnopilus* represents an important component of fungal biodiversity on wood containing more than 200 lignicolous species[8].

Molecular markers, specially DNA techniques are quick and reliable to establish the identities of wild collections and are helpful in mushroom taxonomy. Randomly Amplified Polymorphic DNA (RAPD) uses only one short primer with an arbitrary sequence and binds at many complementary sites in the genomic DNA, amplifying a variety of differentially sized fragments that can be separated by electrophoresis to give a specific banding pattern. It is widely used to distinguish strains and individuals in a given population, including mushrooms[15–17]. The ribosomal RNA genes (rDNA) of fungi are located on a single chromosome and are present as repeated subunits of a tandem array of transcribed and non-transcribed stretches of DNA, which appeared highly conserved[18].

This communication reports molecular characterization of specialty mushroom germplasm of western Rajasthan, India. Eighteen specialty mushroom germplasm accessions were collected under the activities of All India Coordinated Mushroom Improvement Project (AICMIP), Rajasthan College of Agriculture, Maharana Pratap University of Agricultural Sciences, Udaipur (Table 1). Pure cultures were raised in petri plates on malt extract agar culture medium for 10 days to obtain uniform mycelial growth.

For DNA extraction mycelial cultures were raised in liquid culture medium (malt extract 10 $g\,l^{-1}$; glucose – 5 gl^{-1}) for eight days at 25°C. Genomic DNA was extracted from 100 mg of fungal mycelium crushed in liquid nitrogen in micro-centrifuge tubes. DNeasy plant mini kit protocols of QIAGEN were followed for DNA isolation. DNA was quantified using calf thymus DNA as the standard.

Amplification of 5.8S rRNA gene for assessing ITS length variation was done using primer ITS-1 and ITS-4 developed by White *et al.*[19] following Singh *et al.*[17].

RAPD reactions were performed using four decamer arbitrary primers supplied by Operon Technologies, namely OPA-1 ((5′-CAG GCC CTT C-3′), OPA-4 (5′-AAT CGG GCT G-3′) OPA-9 (5′-GTG GTC CGC A-3′), OPN-15 (5′-CAG CGA CTG T-3′). Each amplification was performed in a total reaction volume of 25 µl containing decamer primer, 2 µl (50 pmol μl^{-1}); dNTP mix, 2 µl (2 mM each); $MgCl_2$, 1 µl (25 mM); Taq DNA polymerase, 1 µl (6U μl^{-1}); 10X PCR buffer, 2.5 µl (100 mM, Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 250 mM KCl) and 16.5 µl of dH_2O. To this, 4 µl genomic DNA (approx 50–60 ng) was added. RAPD–PCR amplification was performed in a thermal cycler with initial denaturation step of 94°C for 3 min followed by 36 amplification cycles of 94°C for 40 s, 50°C for 40 s and 72°C for 2 min and final elongation at 72°C for 10 min. PCR amplification products were electrophoretically separated on 1.6% agarose gel prepared in 1X TAE. The gel was run for 3 h at 45 V. Staining was done with ethidium bromide and photographed.

To establish the genetic relationship among the isolates, similarity coefficients were calculated between isolates and a dendrogram drawn using unweighted pair group method using arithmetic averages algorithm (UPGMA) of the NTSYS-pc, version 2.02 h (ref. 20).

PCR product of the ITS-amplified region containing ITS-1, 5.8S rDNA and ITS-II was directly sequenced using ITS-1 (forward primer) and ITS-4 (reverse primer) by Big dye terminator method with the ABI prism DNA sequencer at Delhi University. The sequence data were assembled and analysed. Nucleotide sequence comparisons were performed with Basic Local Alignment Search Tool (BLAST) network services using National Center for Biotechnology Information (NCBI), USA database. Molecular identification up to species level was done and the species designated to the sequenced cultures, analysis based on similarity with the best-aligned sequence of BLAST search. The 5.8S rRNA gene sequence alignments were performed using Clustal X 1.83 software.

Based on ITS lengths, three fragment length groups could be visualized on gel electrophoresis. Group 1 included all accessions of *P. pistillaris* and *P. herculea* with ITS lengths of approximately 750 bp. Whereas Group 2 included all accessions of *P. igniarius* with ITS lengths of approximately 600 bp and Group 3 had only one accession

Table 1. Per cent similarity matrix of nucleotide sequences of specialty mushrooms of western Rajasthan generated using Clustal X 1.8

	Phellorinia herculea						*Podaxis pistillaris*							*Gymnopilus subearlei*	*Phellinus igniarius*			
Isolate	RA-1e	RA-1b	RA-1a	RA-1d	RA-1g	RA-1c	Pod/04	RA-2C	RA-2B	RA-2E	RA-2H	RA-2G	RA-2K	RA-2M	RA-2A	RA-2F	RA-2D	RA-2I
P. herculea																		
RA-1e	100	99.8	100	99.8	100	100	78.1	78.3	77.5	77.5	77.5	77.5	77.6	69.3	62.9	62.9	62.9	62.9
RA-1b		100	99.7	100	99.8	99.8	77.7	78.2	76.9	77.8	76.8	76.9	77.3	67.6	62.5	62.5	62.5	62.6
RA-1a			100	99.5	100	100	78.5	78.6	77.5	78.0	77.5	77.4	77.1	68.0	62.4	62.4	62.4	62.4
RA-1d				100	99.8	99.8	78.7	78.8	77.6	78.2	77.5	77.5	77.0	68.0	62.3	62.3	62.3	62.3
RA-1g					100	100	78.6	78.7	77.6	78.0	77.6	77.6	77.3	68.5	62.5	62.5	62.5	62.5
RA-1c						100	78.3	78.4	77.7	77.7	77.7	77.7	77.7	69.3	63.1	63.1	63.1	63.2
P. pistillaris																		
Pod/04							100	100	99.7	100	100	100	100	69.9	64.5	64.5	64.5	64.6
RA-2C								100	99.7	100	100	100	100	70.4	64.5	64.5	64.5	64.5
RA-2B									100	99.7	99.7	99.7	99.7	69.6	64.6	64.6	64.6	64.6
RA-2E										100	100	100	100	70.9	64.7	64.7	64.7	64.8
RA-2H											100	100	100	69.8	64.7	64.7	64.7	64.7
RA-2G												100	100	69.9	64.7	64.7	64.7	64.7
RA-2K													100	71.8	64.7	64.7	64.7	64.7
G. subearlei																		
RA-2M														100	64.5	64.5	64.5	64.3
P. igniarius																		
RA-2A															100	100	100	100
RA-2F																100	100	100
RA-2D																	100	100
RA-2I																		100

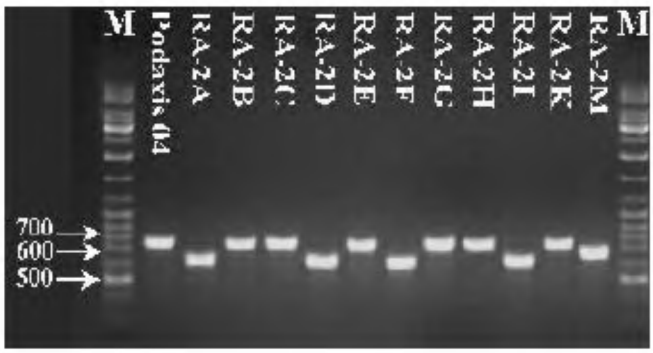

Figure 1. ITS profiles of 12 specialty mushroom germplasms.

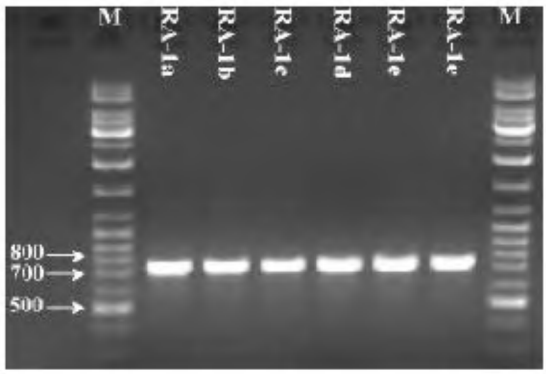

Figure 2. ITS profiles of *Phellorinia herculea* accessions.

(RA-2M) of *G. subearlei* with ITS length of approximately 650 bp (Figures 1 and 2). All the accessions of *P. herculea* exhibited similarity in ITS lengths with *P. pistillaris* accessions and could not be distinguished from each other.

Direct sequencing of PCR-amplified ITS regions facilitated molecular identification of all the germplasm accessions. When the nucleotide sequences of eighteen accessions were subjected to BLAST analysis using NCBI databases, seven were molecularly identified as *P. pistillaris*, six as *P. herculea*, four as *P. igniarius*, and one as *G. subearlei*. Per cent similarity matrix of all the 18 nucleotide sequences was generated using Clustal X (version 1.8) software (Table 1). The matrix revealed high intra-species homology amongst the accessions of *P. herculea* (99.5–100%) and *P. pistillaris* (99.7–100%). All the four accessions of *P. igniarius* exhibited 100% identity in nucleotide sequences. Nevertheless, significant inter-generic diversity among the four genera could be detected. Very low levels of similarity in the nucleotide sequences of *P. herculea* vs (*P. pistillaris* 76.8–78.8%; *G. subearlei* 67.6–69.3; *P. igniarius* 62.3–63.2%), *P. pistillaris* vs (*G. subearlei* 69.6–70.4%; *P. igniarius* 64.5–64.7%), and *G. subearlei* vs (*P. igniarius* 64.3–64.5%) could be recorded.

Pairwise sequence alignment exhibited single nucleotide polymorphisms at intra-species level and facilitated grouping of accessions within species. Accordingly, two types of nucleotide sequences were obtained in *P. herculea* accessions. Group 1 included accessions RA-1b, RA-1c and RA-1d while Group 2 included accessions RA-1a, RA-1e and RA-1g. Both the groups differed by a single nucleotide base pair at two positions in ITS-I (positions 17 and 119). Similarly, *P. pistillaris* accessions of Group 1 included *Podaxis*-04, RA-2C, RA-2E, RA-2H, RA-2G and RA-2K) and Group 2 included RA-2B, whereas in the ITS-1 region nucleotide G of Group 1 was replaced by A (position 132) and in 5.8s rRNA conserved gene region C was replaced by T at position 388 (Figure 3). However, all the four accessions identified as *P. igniarius* were found to have identical ITS sequences. The nucleotide sequences have been assigned GenBank accession numbers from DQ311082 to DQ 311087 by NCBI and are available in the public domain for comparisons.

RAPD profiles of specialty mushroom accessions generated using four decamer primers exhibited significant polymorphism in scorable bending patterns. RAPD profiles amplified using random primers are presented in Figures 4 and 5.

Phylogenetic analysis of the RAPD scorable prominent bands exhibited both inter- and intra-specific polymorphism in 12 specialty mushroom accessions (Figure 6). All the seven accessions of *P. pistillaris* could be distinguished from each other and formed a separate genetic cluster exhibiting 67 to 93% similarity with each other. Although genetically quite distinct, *G. subearlei* was more closely related to *P. pistillaris* than *P. igniarius* accessions. Out of the four accessions of *P. igniarius,* RA-2D and RA-2I exhibited 100% similarity with each other, whereas accessions RA-2A and RA-2F showed maximum genetic distances both at inter-generic and intra-species level ranging from 70 to 78%. Nevertheless, *P. herculea* collections exhibited 85 to 100% similarity among all the six accessions and were phylogenetically distinguished into four distinct subclades (Figure 7).

The present study validates the existence of intra-specific diversity in *P. herculea*, *P. pistillaris* and *P. igniarius*. Phylogenetic relationships of *P. herculea* in Phellriniaceae[7], *P. pistillaris*[6] within *Lapiota* and *G. subearlei* in genus *Gymnopilus*[8] based on ITS rDNA sequences have been studied. Some genera, e.g. *Amanita*[21] have many morphological characters that have been used to support infra-generic groups. Unfortunately, this is not the case with *Gymnopilus*, where only the presence or absence of a partial membranous veil and the size of basidiospores have been used. Both characters are shown to be highly homoplastic and of little value at this taxonomic level. Guzman-Davalos *et al.*[8] suggested that the traditional infra-generic classification of *Gymnopilus* is not supported by ribosomal DNA sequence data.

Under the present study, phylogenetic analysis of RAPD profiles proved more useful in revealing both inter-generic and intra-species variability than ITS multiple sequence alignment alone. This is because multiple sequence

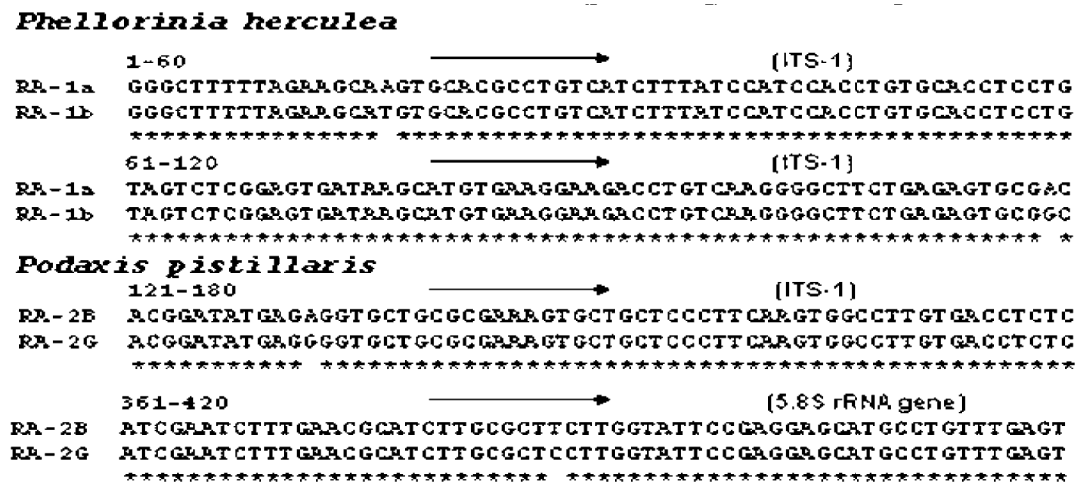

Figure 3. Pairwise alignment of nucleotide regions of *Podaxis pistillaris* and *Phellorinia herculea* showing single nucleotide polymorphism. Alignment done using Clustal X 1.83 version.

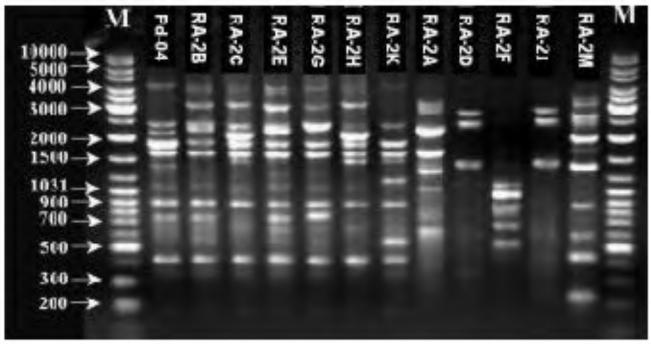

Figure 4. RAPD profiles of 12 specialty mushroom accessions using OPP-9 primer.

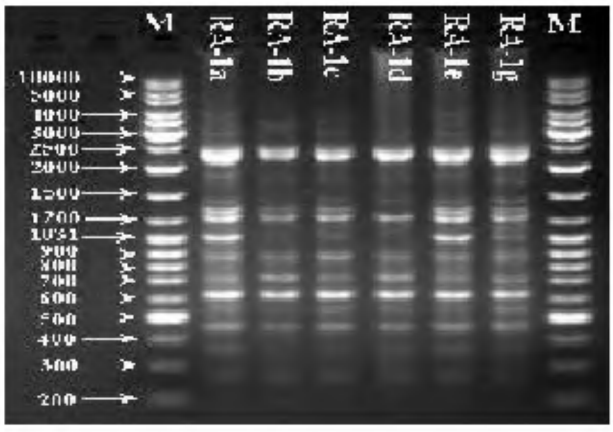

Figure 5. RAPD profiles of *Phellorinia* sp. group of accessions using OPA-1 primer.

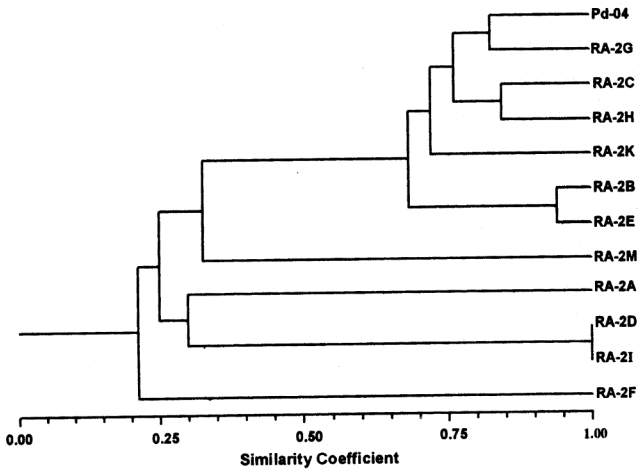

Figure 6. Phylogenetic tree showing intra-species diversity in 12 specialty mushroom accessions.

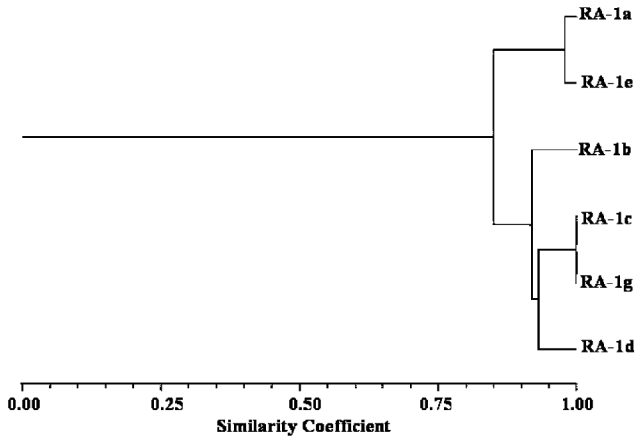

Figure 7. Phylogenetic tree showing intra-species diversity in *P. herculea* accessions.

alignment of a single conserved gene region utilizes and compares negligible portion of genomic DNA compared to combined phylogenetic analysis of several random primers which scan the genomic DNA for target arbitrary sequences. Molecular identification results of the ITS sequences of 5.8S rRNA gene and published mushroom records of India validate that *P. herculea* and *G. subearlei* are new additions to the Indian Basidiomycetes.

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RT–PCR detection and molecular characterization of *Onion yellow dwarf virus* associated with garlic and onion

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Onion yellow dwarf virus **(OYDV) is an important pathogen of onion and garlic, causing severe losses in onion seed crop and garlic clones. Due to variability in the N-terminal region of coat protein (CP) of different isolates, and difficulty in OYDV-specific antibody production, ELISA may not be a preferred method for its detection. As an alternative a rapid and reliable detection protocol of RT–PCR was standardized. Primers designed from conserved RNA-dependent RNA polymerase and 3′-UTR region were used for detection of OYDV in garlic and onion. The amplified product was cloned and sequence analysis showed that it was 1111 bp long. Amino acid sequences of CP gene of Delhi isolate showed sequence identity in the range 74.9 to 96.1% with different isolates of OYDV from other countries. Sequence analysis also indicated that OYDV is a garlic-type potyvirus.**

Keywords: Garlic, *Onion yellow dwarf virus*, onion, RT–PCR.

ONION yellow dwarf virus (OYDV), an aphid-borne potyvirus, is one of the major viral pathogens of onion and garlic. It restricts seed production in onion[1]. Leaves of OYDV-infected onion show irregular yellow striping to almost complete yellowing and also downward curling,

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