high percentage of calcium probably due to its thick cell wall, which makes it suitable for growth of mineral crystals. The presence of phosphorous in the plant can also be exploited for synthesizing hydroxyapatite, thus utilizing the traditional knowledge of bone-fracture healing in advanced technique of new material synthesis.

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Molecular characterization of *Humicola grisea* isolates associated with *Agaricus bisporus* compost

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Composite compost samples were collected from Solan, Sonepat, Gangtok, Kaithal, Phagwara and Ooty, at different stages of *Agaricus bisporus* compost preparation by long and short methods using a variety of agro-waste substrates. Eight isolates of *Humicola grisea* were retrieved on yeast agar medium at 45 and 52°C. PCR amplification of internal transcribed spacer (ITS) region of 5.8S ribosomal RNA (rRNA) gene was done using ITS-1 and ITS-4 primers. An ITS fragment of approximately 550 bp was amplified from all the eight *H. grisea* isolates with no intra-specific diversity in the ITS region of 5.8S rRNA gene. RAPD genotyping was performed using five decamer primers. Combined phylogenetic analysis of RAPD profiles of *H. grisea* by five primers depicted intra-specific variation amongst the eight isolates and divided these into five distinct sub-clades. Molecular analysis carried out in the present study would suggest that isolates within this species exhibit genetic differences, which correlates well with morphological variations.

Keywords: *Agaricus bisporus*, compost microflora, genetic diversity, *Humicola grisea*, RAPD.

AGARICUS bisporus (white button mushroom) grows on compost, a product of aerobic fermentation by various microorganisms. These microorganisms convert and degrade straw to lignin humus complex, which is later utilized by the mushroom mycelium and ultimately contributes to the nutrition of *A. bisporus*[1,2]. The thermophilic fungi play a

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crucial role in determining selectivity of compost produced for growing *A. bisporus*. Amongst these *Scytalidium thermophilum*, *Humicola grisea* and *Humicola insolense* have been frequently isolated and are predominant in various composts prepared by long and short methods using a variety of agro-waste substrates[3–5]. Renard and Cailleux[6] reported better growth of *A. bisporus* on compost treated with either *S. thermophilum* or *H. grisea*. Ross and Harris[7] prepared compost by inoculating the strains and organic nitrogen mixture with *H. grisea*, *S. thermophilum* and *H. insolense* and reported hastening of composting process. *H. grisea* possess an efficient hydrolytic system and several thermostable enzyme activities such as trehalase[8], glucoamylase[9], betaglucosidase[10] and xylanase[11].

Due to its important role in *A. bisporus* growth stimulation and industrial importance, this organism has remained as the microorganism of interest for studying composting period and enhancing compost selectivity. In the present work genetic diversity amongst *H. grisea* isolates was studied. This may eventually facilitate further experimentation with selective isolates in hastening composting process.

Composite compost samples were collected from different places, namely Solan, Sonepat, Gangtok, Kaithal, Phagwara and Ooty, randomly at different stages of compost preparation by long and short methods, and using a variety of agro-waste substrates like wheat straw, paddy straw and sugarcane bagasse. *H. grisea* was isolated using serial dilutions at 45 and 52°C. One gram of compost from each sample was taken and suspended in 10 ml of sterile distilled water and agitated for 15 min on a shaker. Next 1 ml of suspension solution was diluted serially to 10^{-2}, 10^{-3} and 10^{-4} dilutions. Subsequently, 1 ml of each dilution was plated on yeast agar medium (yeast extract, 4.0 g; KH_2PO_4, 1.0g; $MgSO_4.7H_2O$, 0.5 g; soluble starch, 15 g; Agar-agar, 15 g and distilled water, 1000 ml). To make the culture medium selective, Rose Bengal and Chloramphenicol (50 ppm) were added to the basal culture medium. The inoculated petri plates were placed in BOD incubators at 45 and 52°C, in polythene bags with a beaker containing water to avoid desiccations. The polythene bags were opened twice daily to provide adequate aeration to cultures. Observations on appearance of various fungal colonies were taken up to the seventh day and CFU were calculated per gram of compost. Ten petri plates of each serial dilution were observed. Parallel controls were maintained by plating sterile distilled water with each serial dilution. *H. grisea* was isolated and identified using standard laboratory manual[12]. Pure cultures were raised on yeast starch agar medium in both petri plates and test tubes.

All the isolates were grown in liquid shake culture on yeast extract starch broth medium in 100 ml Erlenmayer flask containing 25 ml of medium at 45 and 52°C for five days. The total genomic DNA was extracted from approximately 100 mg of fungal mycelium crushed with micro-pestle in conical micro-centrifuge tubes with liquid nitrogen. DNeasy plant mini kit protocols of Qiagen, USA were followed for DNA extraction.

The polymerase chain reaction (PCR) primers ITS-1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′)[13] were used to amplify the internal transcribed spacer (ITS) of ribosomal DNA, which encompasses the 5.8S gene and the flanking ITS-1 and ITS-2 regions. Amplification by PCR was performed in a total volume of 50 µl containing 0.2 µl Taq DNA polymerase (5U μl^{-1}; Promega, USA), 5 µl $MgCl_2$ (25 mM; MBI Fermentas, USA), 4 µl dNTP mix containing 2.0 mM each A, T, C, G (MBI Fermentas), 1 µl of each ITS-1 and ITS-4 primers (Qiagen), approximately 50 ng genomic DNA and 38.5 µl nuclease free water (Ambion, USA). Two drops of mineral oil (Sigma, USA) were added before PCR. The reaction was performed in a thermal cycler (MJ Research, USA) with the following conditions, viz. 1 min initial denaturation at 95°C, 30 s annealing at 50°C, 1 min 20 s elongation at 72°C, repeated 34 times with a final elongation step of 10 min at 72°C. The PCR products were separated on 1% agarose gel in tris acetic acid EDTA (1X TAE) buffer at 80 V for 1 h. Agarose gels were stained with ethidium bromide and photographed under UV light using Syngene gel documentation system for amplified products.

Multilocus RAPD genotyping was performed using five decamer primers supplied by Operon Technologies, USA, namely OPA-1 (5′-CAG GCC CTT C-3′), OPA-4 (5′-AAT CGG GCT G-3′), OPP-6 (5’-GTG GGC TGA C-3′), OPP-14 (5′-CCA GCC GAA C-3′), and OPN-9 (5′-TGC CGG CTT G-3′). Amplification of RAPD analyses was performed in a total reaction mixture of 25 µl, each reaction mixture containing decamer primer – 2 µl (50 pM μl^{-1}), dNTP mix – 2 µl (2 mM each A, T, C, G; MBI Fermentas), $MgCl_2$ – 1 µl (25 mM, MBI Fermentas), Taq DNA polymerase – 1 µl (5U μl^{-1}; Sigma), 10X PCR buffer – 2.5 µl (100 mM Tris HCl (pH 8.3), 15 mM $MgCl_2$, 250 mM KCl; Sigma) and 16.5 µl H_2O. To this 4 µl genomic DNA (approx. 50 ng) was added. RAPD–PCR amplifications were performed in a thermal cycler (PTC100, MJ Research) with lid-heating option at 110°C and with initial denaturation step of 94°C for 3 min followed by 36 amplification cycles of 94°C for 40 s, 50 and 72°C for 2 min and final extension step at 72°C for 10 min. PCR amplification products were electrophoretically separated on 1.6% agarose gel (Sigma), prepared in 1X TAE buffer. The gel was run for 3 h at 45V. Further, the gel was stained with ethidium bromide and visualized under 300 nm UV light and photographed using Syngene gel documentation system. The gel profiles were scored for presence and absence of scorable bands with assumption of positional homology. To estimate genetic distances and similarity between isolates, similarity coefficients were calculated with the help of dendrogram constructed using UPGMA algorithm (Unweighed Pair Group Method using Arithmetic Avarages)[14,15] of the NTSYS-pc, version 2.02h software.

Eight isolates of *H. grisea* from different composite compost samples are given in Table 1. In petri plates, the fungus initially produced a dense white mycelium, which later gradually turned grey due to formation of aleuriospores and finally turned dull black; this is a characteristic of *H. grisea*. Mycelium was thick, grey, entangled, hyphae 0.5–4.5 µm in diameter. Typical sporulation was initiated by lateral swelling at right angles to hyphae. These swellings elaborated into short lateral unbranched aleuriophores with bulbous tips. Mature aleuriospores were dark brown, smooth-walled, globose and ranged from 8 to 16 µm in diameter. The morphological characters of *H. grisea* isolates studied were identical to the morphology described by Cooney and Emerson[12]. Mycelial growth and sporulation pattern of *H. grisea* isolates exhibited significant morphological differences (Figure 1). Growth and sporulation patterns were observed in all the five types. Type-1 isolates showed intermingled white mycelial growth with light grey sporulation (isolates: Hg-1, Hg-3, Hg-5). Type-2 isolates were smooth white mycelial growth in the centre followed by light grey sporulation (isolate Hg-9). Type-3 isolates were dense dark grey with sporulation in the centre and white mycelial growth at the margin (isolate Hg-4). Type-4 isolates were deep grey and with sporulation in whorls throughout the petriplate (isolates Hg-7 and Hg-8). Type-5 isolates had dense mycelial growth in the centre with sporulation in whorls at the margins (isolate Hg-6).

PCR amplification of 5.8S rRNA gene region (which encompasses ITS-1 and ITS-2 on either side) was performed using ITS-1 and ITS-4 universal primers. An ITS fragment of approximately 550 bp was amplified from all the eight *H. grisea* isolates. No intra-specific diversity in the ITS region of 5.8S rRNA gene region could be detected (Figure 2). This is attributed to the fact that 5.8S rRNA gene is known to be highly conserved. It confirmed that all the isolates belong to a single species, i.e. *H. grisea*. Functionally and evolutionarily conserved rRNA genes have been used in the various studies to determine phylogenetic relationships amongst micro-organisms[16–18]. Lyons *et al.*[5] also reported similar ITS lengths of 534 bp in *H. grisea*. The use of DNA-based tools such as direct sequencing of gene coding for production of 16S, 5.8S, 28S and 5S r-DNA has allowed assessment and comparison of phylogenetic relationship of many organisms over a wide range of taxonomic levels[19]. The ITS region and intergenic spacers of the nuclear r-DNA repeat units evolve faster and may vary among species within the genus or among populations. The revolution in this direction came with the development of universal ITS primers by White *et al.*[13]. The ITS primer makes use of conserved regions of 18S, 5.8S and 28S rRNA genes to amplify non-coding regions between them. This classical study made comparison among 5.8S rRNA sequences of *Neurospora crassa*, *Schizosaccharomyces pombe*, *Saccharomyces cerevesiae*, *Vicia faba* and *Mus musculus* to select ITS-2 and ITS-3 primers.

Of the initial primers tested, OPA-1, OPA-4, OPP-6, OPP-14 and OPN-9 were selected for further use on the basis of reproducibility of banding patterns for genotyping by random amplified polymorphic DNA (RAPD). Analysis with eight isolates of *H. grisea* was carried in triplicate with all primers to ensure reproducibility of results. Only consistently bright bands were scored for analysis and weak bands were discarded because of possible variation from one amplification to another. Distinct banding patterns were observed for each primer, which facilitated their dif-

Table 1. Source of compost, substrate, method of composting and isolation

Isolate	Place	Method	Substrate	Dilution
Hg-1	Solan	SM	WS	10^{-3}
Hg-3	Sonepat	LM	WS + SB	10^{-4}
Hg-4	Gangtok	SM	WS	10^{-3}
Hg-5	Kaithal	LM	WS	10^{-3}
Hg-6	Phagwara	LM	WS	10^{-4}
Hg-7	Solan	SM	WS	10^{-3}
Hg-8	Ooty	SM	PS + SB	10^{-3}
Hg-9	Gangtok	SM	WS	10^{-3}

SM, Short method; LM, Long method; WS, Wheat straw; PS, Paddy straw; SB, Sugarcane bagasse.

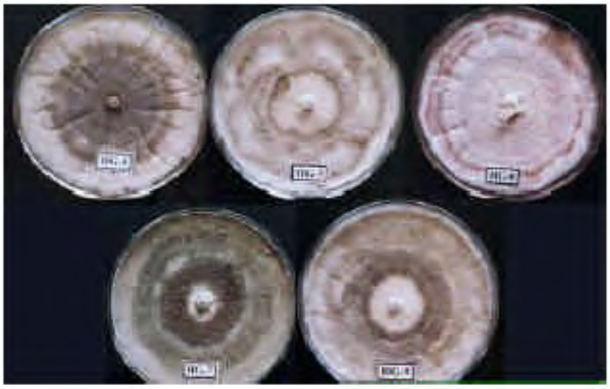

Figure 1. Cultural variations and sporulation patterns in *H. grisea* isolates.

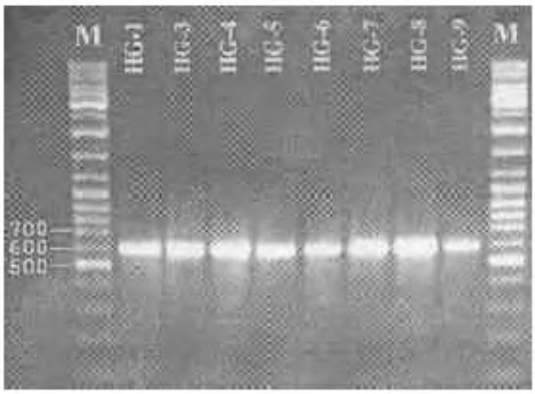

Figure 2. PCR-amplified products of ITS region 5.8S gene region in the *H. grisea* isolates.

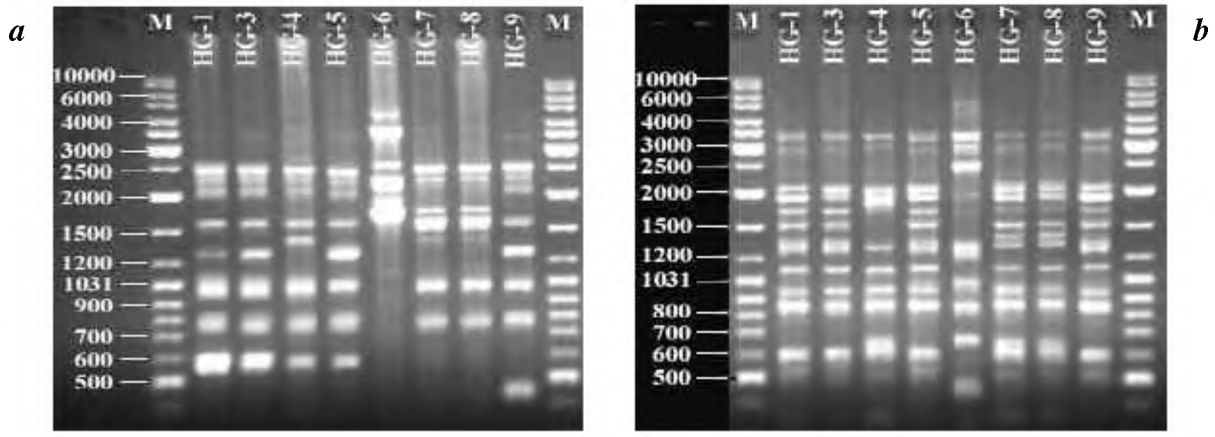

Figure 3. RAPD profiles of *H. grisea* isolates by primer OPA-1 (***a***) and primer OPA-4 (***b***).

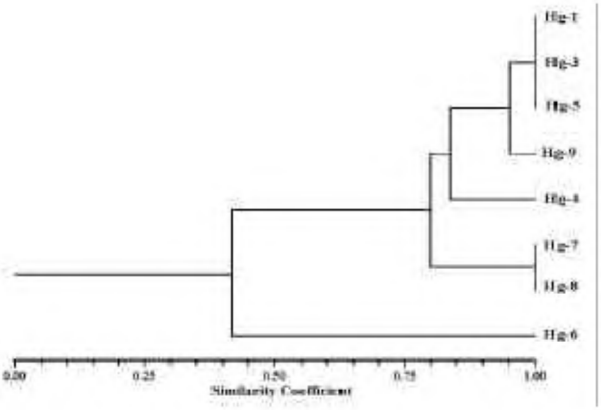

Figure 4. Combined phylogenetic tree using five primers.

ferentiation. The PCR amplified products of RAPD profiles by OPA-1 and OPA-4 are shown in Figures 3 *a* and *b*.

Combined phylogenetic analysis of RAPD profiles of *H. grisea* by five primers depicted intra-specific variation amongst the eight isolates and divided them into five distinct phylogenetic sub-clades. The biggest group included isolates Hg-1, Hg-3 and Hg-5 (Figure 4). Isolate Hg-9 was 95% similar with this group. Rest of the isolates exhibited significant phylogenetic distances from each other. Isolate Hg-6 showed maximum dissimilarity of approximately 58% from the rest of the isolates.

Lyons *et al.*[5] also reported intra-specific variations in *H. grisea* isolates from United Kingdom. Sunagawa *et al.*[20] identified polymorphism amongst eleven strains of *Lentinula edodes* and suggested that RAPD markers are a useful tool to detect interspecific variations. Using RAPD analysis, 34 isolates of *Scytalidium thermophilum* collected from different parts of the world could be separated in two distinct groups[5]. Singh *et al.*[21] distinguished seven commercial strains belonging to four genera of cultivated mushrooms using RAPD primers. The random primer used by them could distinguish mushroom strains within species, species within the genus and different genera. Yadav *et al.*[22] reported high degree of polymorphism amongst white and brown strains of *A. bisporus* using RAPD markers. Singh *et al.*[23] revealed interspecific polymorphism in eight species of *Morchella* and related genera using two 10 bp arbitrary primers. They suggested that RAPD profile using specific arbitrary primer can serve as a useful genetic marker for species identification and to improve morel systematics.

Molecular analysis carried out in the present study would suggest that isolates within this species exhibit genetic differences which correlate well with morphological variations.

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Distribution, abundance and vertical migration pattern of krill – *Euphausia superba* Dana at fishing area 58 of the Indian Ocean sector of Southern Ocean

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The First Indian Antarctic Krill Expedition (FIKEX) was an attempt to examine and obtain first-hand information pertaining to distribution, abundance and vertical migration pattern of krill – *Euphausia superba* Dana at fishing area 58 of the Indian Ocean sector of Southern Ocean. It has been ascertained that krill migrations occur between sea surface and a depth of about 100 m. Availability of food is the key factor affecting both seasonal and annual changes, and leads to krill migration. Under good feeding conditions the amplitude is maximal, and the migration cycle approaches 24 h. Adult individuals exhibit 24 h migration, whereas juveniles show lower migration, and their submergence is shallower. Water stratification may also affect krill distribution in the water column, and in certain conditions may lead to limitation of migration range.

Keywords: Antarctic krill, FIKEX, Indian Ocean, migration, Southern Ocean.

DENSE concentration, large biomass and industrial importance of Antarctic krill, *Euphausia superba* Dana play a special role in the Antarctic ecosystem. The krill has long been regarded as a key organism in the Antarctic food chain[1–3]. High krill concentrations are found in areas of intensive circulation of water mass, such as in the West Antarctic, mainly in the vicinity of the Antarctic Peninsula; the Scotia Sea; the northern part of the Weddell Sea and also close to South Georgia[1,4–9]. It occurs around the Antarctic continent and shelf waters in the south, up to the Polar Front in the north.

Except for fragmentary information regarding harvesting of finfish in the sub-Antarctic region of Kerguelen Islands, Ob and Lena sea mounts, a detailed account of exploitable krill and other living resources of (fishing area 58 according to the CCAMLR demarcations, the proposed survey area will fall in the ENDERBY–WILKES DIVISION IV; Division 58.4.4) Indian Ocean sector of Southern Ocean is not available. The present cruise was primarily aimed at studying the distribution, abundance and migration. However, the continuous hydroacoustic records yielded a wealth of field data on distribution of *E. superba* in the preferred area and periods of time. In 1995, FIKEX (First Indian Antarctic Krill Expedition) was organized to ob-

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