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Genetic relatedness between some saprophytic and parasitic macrofungi of Darjeeling Hills

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Abstract
Eight dominant saprophytic and parasitic macro fungi collected from Darjeeling hills [N 26°31'27.13' - E 87°59'-88.53'] of North Bengal region were studied using internal transcribe spacer (ITS) and RAPD PCR. rDNA region of saprophytic and parasitic macro fungi with ITS1 and ITS4 primers produced range between 400-800bp products. The genetic relatedness among these macro fungi were analyzed with four random primers. RAPD profiles showed genetic diversity among the isolates with the formation of two clusters. Analysis of dendrogram revealed that similarity coefficient ranged from 0.34 – 0.86.

Keywords: Saprophytic , parasitic macro fungi , rDNA, RAPD

The fungi are an immensely diverse group of organisms, encompassing a huge range of forms from microscopic single celled yeasts to large macrofungi, as exemplified by the well-known mushrooms and toadstools and the largest of fruitbodies, the giant puffball. An estimates for the number of fungal species as reported by Hawksworth et al., 1995 revealed that 72,065 species spread across 11 phyla in 7745 genera . New fungal species continue to be described, and a year 2009 estimate based on the description rate in The Index of Fungi would be of the order of 80–1,00, 000 species of fungi. The great majority of the 80 000+ fungal species so far named and described are likely to occur in the soil environment at some stage in their life-cycle. Fungi therefore have many different functions in soils, which include both active roles, such as the degradation of dead plant material and inactive roles where propagules are present in the soil as resting states. The development of molecular techniques has provided a new range of tools that can provide clear insights into specific interactions and activities in soil environments. The combination of broad spectrum polymerase chain reaction (PCR) detection, coupled with RAPD and single strand conformation polymorphisms (SSCP) or denaturing gradient gel electrophoresis (DGGE), can give more accurate answers to fundamental questions on ecosystem diversity. Macrofungi are defined here to include ascomycetes and basidiomycetes with large, easily observed spore-bearing structures that form above or below ground. Although macrofungi have perhaps the longest history of diversity studies of any group of fungi, they are nevertheless understudied over most of the world. More data are available from North America and Europe than from any other region, but knowledge of macrofungal diversity is incomplete even for these regions. Macrofungi are distinguished by having spore-bearing structures visible to the naked eye (mushrooms, brackets, puffballs, false-truffles, cup fungi, etc.). Most macrofungi are Ascomycota or Basidiomycota, but a few are Zygomycota.

The peak mushrooms and macrofungi season for each region is differ from each ecological climate. Each year is a little different; the season may be early or late depending on rainfall and temperature (Arora. 1991). Most terrestrial macrofungi are saprobes or mycorrhizal symbionts, but some are pathogens of plants or fungi. Fungi fruiting on woody substrata are usually either saprobes or plant pathogens.

In the present investigation two types of macrofungi, (a) saprophyte and (b) parasitic on insects were considered for their molecular characterization through RAPD. There is no organic matter which is not attacked and destroyed by fungi and bacteria. Everything that goes to form organic substances comes from nature and reenters the natural "economic" cycle because of the action of microorganisms. The breakdown of organic substances is achieved by fungi in the mycelial stage, i.e. as mold, even though, in many cases, this mold will produce a fruit-body. .

In natural conditions the breakdown of given types of matter is carried out exclusively by specific species of saprophytic fungi. (Pacioni, 1981). There are numerous associations between insects and fungi. Of particular importance among them, because of their possi-ble practical implications, are those which are established with the *entomopathogenic* fungi, i.e. fungi which are parasitic on insects: these fungi could well offer mankind an alternative weapon in the war against insects. In temperate climates members of the genus *Cordyceps* are relatively common; these usually have thread- or club-shaped carpophores, often brightly col-ored. The commonest species, *Cordyceps militaris* forms orange-red clubs on members of Lepidoptera and Co-leoptera. *Cordyceps sinensis*, parasitic on Lepidoptera, has been used in China for medicinal purposes since earliest times. (Pacioni, 1981). Four dominant saprophytic fungi and four macrofungi parasitic on insects collected form Darjeeling hills were selected for analysis of their genetic relatedness.

Materials and methods :

Fungal strain and culture

Macrofungi were collected from the forest area of Darjeeling. The specimens were collected,

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Fig 1. A. *Clavariadelphus pistillaris* (DG 26 MF 4), B. *Russula ochroleuca (22-6-006MF 5)* C. *Inocybe fastigiata* (22-06-007 MF 6) D. *R. cyanoxantha* (GL-201MF 3) E. *Cordyceps sp.* (IS-001 MF1) F. *Cordyceps sp.* (IS-004 MF 2) G. *Cordyceps sp.* (IS-007 MF 10) H. *Cordyceps sp.* (IS-009 MF 11)

photograpghs were taken and kept in plastic bags. The locations of collection area of macrofungi were recorded by GPS tools (Garmin). These fungi grow on top of dead and decomposed leaves and logs. Specimens at different stages of development were collected by digging (not pulling) them up so as not to damage their bases. For those attached on dead logs and woods, efforts were made to scrape a piece of the wood or bark on which the specimen was attached. A paper slip indicating the place where it is collected and placed in each plastic bag. Upon returning from the collecting trip, the materials were examined and morphological characteristics were recorded. The specimens were measured, noted down for the shape, size, color and other characteristics. The information on characters affected by drying was also noted down. Each specimen was identified following comparison with the literatures. Fungal tissue cultures from each of eight selected fungi from the somatic tissue of saprophytes (IS-001MF1, IS-004MF2, IS-007 MF10, IS-009MF11) and macrofungi parsitic on insects GL-201MF3, DG 26 MF 4, 22-6-006MF 5, 22-06-007 MF6) were prepared aseptically and culture stored in 4°C for future work.

Genomic DNA Extraction

Isolation of genomic DNA from each of macrofungi was done by growing the fungi for 3-4 days in malt dextrose agar. The mycelia were incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mM NaCl and 2% SDS, for 1 hr at 60°C followed by centrifugation at 12,000 rpm for 15 min. The supernatant was then extracted with equal volume of water saturated phenol and further centrifuged at 12,000 rpm for 10 min; the aqueous phase was further extracted with equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) and centrifuge at 12,000 rpm for 15 min; the aqueous phase was then transferred in a fresh tube and the DNA was precipitated with chilled chanol (100%). DNA was pelleted by centrifuging at 12000 rpm for 15 min and washed in 70% ethanol by centrifugation. The pellets were air dried and suspended in TE buffer (pH 8.0).

Qualitative and quantitative estimation of DNA

The extraction of total genomic DNA from the isolates as per the above procedure was followed by RNAase treatment. Genomic DNA was re suspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse (60µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

PCR amplification of ITS region

All isolates of Macro fungi were taken up for ITS-PCR amplification. Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec and extension at 70 °C for 2 min and the final extension at 72 °C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 ml) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis.

RAPD of Macro fungi isolates

For RAPD, random primers i.e. BAS 359, OPA-1; OPD-6; OPA-4; were selected . PCR was programmed with an initial denaturing at 94°C for 4 min. followed by 35cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 70°C for 90 s and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 ml) containing 0.25 % bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination by horizontal electrophoresis.

Scoring and data analysis

The image of the gel electrophoresis was documented through Kodac gel documentation system and NTSYSPc analysis software. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed *In Silico* into similarity matrix using NTSYSpc (Numerical Taxonomy System

Table 1: The nucleotide sequence used for ITS and RAPD PCR

Primer	Primer Seq 5'-3'	Mer	TM	% GC
	ITS-Primers pairs			
ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57%
ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45%
	RAPD primers			
BAS 359	AGGCAGACCT	10	31.0	60%
OPA-4	AATCGGGCTG	10	39.3	60%
OPD6	GGGGTCTTGA	10	32.8	83%
OPA1	CAGGCCCTTC	10	38.2	70%

Table 2: Analysis of the polymorphism obtained with RAPD markers in macro fungi isolates

Seq Name	Total no RAPD bands	Band size (bp). (approx.)		Poly-morphic bands	Poly-morphic (%)
		Min	Max.		
BAS359	07	100	2000	07	100
OPA-4	06	100	1000	06	100
OPD-6	05	100	1000	05	100
OPA1	04	100	1000	04	100
Total	22			04	100

Biostastiscs, version 2.11W) The SIMQUAL program was used to calculate the Jaccard's coefficients. The RAPD patterns of each isolate was evaluated, assigning character state "1" to indicate the presence of band in the gel and "0" for its absence in the gel. Thus a data matrix was created which was used to calculate the Jaccard similarity coefficient for each pair wise comparison. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc (Rohlf, 1993.).

Result and Discussions:

Morphological characteristic of macrofungi.

The detail descriptions of four sprophytes are as follows:

Clavariadelphus pistillaris (DG 26 MF 4)

GIS Location : N26°50'.181''E88°13'.876'']

Source : Forest floor

Description : Carpophore 8-25 cm high with a maximum d ameter of 2.5 cm, club-shaped, slender or stout, surface smooth then sulcate lengthwise, light yellow tending to grayish brown. Flesh white, bruising brownish (Fig:1 A)

Russula ochroleuca (22-6-006MF 5)

GIS Location : N26°50'.323''E88°13'.431''

Source : Forest floor and trees of *Castronopsis* of eastern himalaya

Description : Carpophore 4-11 cm, yellow but variously tinged, pale lemon-yellow, yellowish ochre spotted with orange or brownish at center, late in season often light olive-yellow, gray at center, convex-umbilicate then flat, slightly depressed, cuticle half detachable, moist and shiny, margin thick, curved, sometimes lobate. Gills pale cream-colored or faintly pale yellow with a few small brownish markings with age, averagely crowded, unequal, intervenose, ventricose, slightly obtuse frontwards. Stipe 3-7 x 1.5-2.5 em, white, slightly grayish, spotted with brownish yellow from base upward, cylin-drical, sometimes club-shaped, flared beneath gills, full, soft, slightly pithy at top, Flesh white, grayish at top of stipe, thick, soft then tough. Odor pleasant, flavor varying from piquant to sweet. Spores white, ovoid, aculeate, 7-9 X 6.3-9 microns, amyloid. (Fig :1 B)

Inocybe fastigiata (22-06-007 MF 6)

GIS Location : N26°50'.276''E88°13'.765''

Source : Forest floor of *Picea* trees of eastern himalaya

Carpophore 2-7 cm, pales straw, conical then raised at the edge; tinged with olive, then brownish, crowded, narrow, with lighter edges. Stipe 3-8X0.4-1 cm whitish, cylindrical at the base, tapering, fibrillose. Flesh whitish, fibrous in the stipe. Spermatic odor, no flavor. Spores brownish, elliptical, smooth, 7-10x4-5 microns. (Fig:1 C)

Russula cyanoxantha (GL-201MF 3)

GIS Location : N26°50'.921''E88°13'.434''

Source : Forest floor

Description : Carpophore 5-15 cm, blackish-violet, pale purple at edge and conspicuous green at disc, varying to slate-gray with lighter areas, or bluish violet or even a uniform green when mature, rounded then convex, flat, fairly depressed, cuticle two-thirds detachable, thin, viscous in damp weather, shiny, with radial fibrils and grooves, margin curved inward then ob-tuse, sometimes striate when mature. Gills white tinged bluish green, fairly crowded, unequal, forked, intervenose, ventri-cose. Stipe 5-10 X 1.5-4 cm, white, sometimes tinged lilac or reddish, with brownish markings, sturdy, even, narrowing and rooting at base, fleshy, soft then spongy, pruinous, slightly ru-gose. Flesh white, sometimes grayish when mature, thick, soft, moist. Odor pleasant, flavor first sweet then unpleasant. Spores white, elliptical, with small isolated warts, 7-10 X 6-7.5 microns, amyloid. (Fig :1 D)

The detail descriptions of four macrofungi parasitic on insects are as follows:-

***Cordyceps sp.* (IS-001 MF1)**

GIS Location : N26°50'.621''E88°13'.575''

Source : Insect larva from forest floor

Description : concentrically zoned with contrasting shades of yellow, red, brown, zones alternately velvety and smooth. Tubes: white to pale yellow; pores 3-5 per mm, angular. Stalk: absent. (Fig:1 E)

***Cordyceps sp.* (IS-004 MF 2)**

GIS Location : N26°50'.633''E88°13'.555''

Source : Insect larva from forest floor

Description : Whitish, , smooth; base white-hairy., edges fringed. Stalk: very short, compressed, hairy, elliptical, smooth, odourless. (Fig:1 F)

***Cordyceps sp.* (IS-007 MF 10)**

GIS Location : N26°50'.311''E88°13'.785''

Source : Wasp from forest floor

Description : Concentric bands of color ; tough and leathery, becoming brittle and hard when dry; velvety with different tomentum from one color zone to another, reflecting different growth conditions. Tubes up to 3 mm deep; cinnamon to rusty brown. (Fig:1 G)

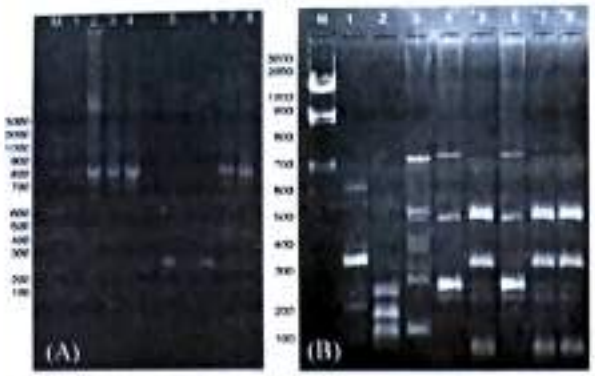

Fig 2 (A) 1% Gel electrophoresis of PCR of ITS region and (B) RAPD PCR with primer BAS359. Lane M : Low range DNA marker, Lane 1: *Russula cyanoxantha* (GL-201MF 3), 2: *Clavaridelphus pistillaris* (DG 26 MF 4), 3: *Russula ochroleuca* 22-6-006MF5, 4: *Inocybe fastigiata* (22-06-007 MF 6), 5:*Cordyceps sp.* (IS-001MF1), 6: *Cordyceps sp.* (IS-004MF2), 7: *Cordyceps sp.* (IS-007 MF 10), 8:*Cordyceps sp.* (IS-009MF 11) in both the photograph

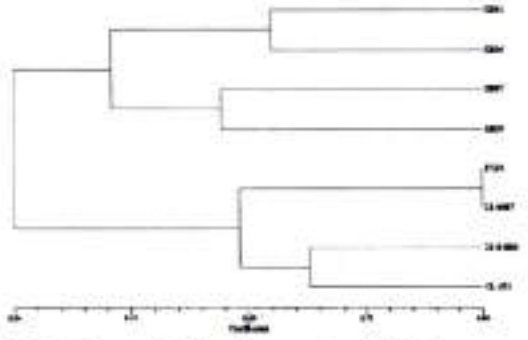

Fig 3: Dendrogram showing the genetic relationships among eight microfungi based on RAPD analysis.

***Cordyceps sp.* (IS-009 MF 11)**

GIS Location : N26°50'.201" E88°13'.445"

Source : Insect larva from forest floor

Description : Concentric bands of color whitish ; tough and leathery, becoming brittle and hard when dry; velvety with different tomentum from one color zone to another, reflecting different growth conditions. (Fig:1 H)

For the study of molecular characterization the ribosomal RNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome [Kindermann et. al. 1998). They also occur in multiple copies with up to 200 copies per haploid genome [Lieckfeldt, E. and K.A. Seifert 2000, Taylor et. al. 1999] arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species [Druzhinina et. al. 2004]. In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies. In the present study, we focused on the ITS regions of ribosomal genes for the construction of primers that can be used to identify macrofungi. ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 primers. Amplified products of size in the range of 400-800bp was produced by the primers (Fig 2A). The ITS PCR has helped to detect polymorphism at ITS region of rDNA among the macrofungi isolates.

The genetic relatedness among eight isolates of macrofungi were analyzed by four random primers BAS359, OPA-1, OPD-6 and OPA-4 to generate reproducible polymorphisms. All amplified products with the primers had shown polymorphic and distinguishable banding patterns which indicate the genetic diversity of macrofungi isolates. A total of 22 reproducible and scorable polymorphic bands ranging from approximately 100bp to 2000bp were generated with four primers among the eight macrofungi isolates (Table 2).

RAPD profiles showed that primer BAS359 scored highest bands which ranged between 100bp to 2000bp (Fig 2B). Relationships among the isolates was evaluated by cluster analysis of the data based on the similarity matrix.

The Dendogram was generated by unweighted pair-group methods with arithmetic mean (UPGMA) using NTSYSpc software (Fig 3). Based on the results obtained all the nineteen isolates can be grouped into two main clusters. One cluster represents the isolates saprophyte macro fungi (GL-201MF3, DG 26 MF 4, 22-6-006MF 5 and 22-06-007 MF6) and other parasitic macro fungi (IS-001MF1, IS-004MF2, IS-007 MF10, and IS-009MF11) . The similarity coefficient ranges from 0.34 – 0.86. There is formation of two distinct group at 0.34 similarity.

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Gaysina LA, Purina ES, Safiullina LM and Bakieva GR (2009) Resistance of *Klebsormidium flaccidum* (Kützing) Silva, Mattox & Blackwell (Streptophyta) to heavy metals. NBU J Pl Sc 3: 39-41.

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