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Molecular characterization of the keratinophilic fungi isolated from high altitude regions of Kashmir

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Abstract

Keratinophilic fungi is an ecologically important group that cycle one of the most abundant and highly stable animal protein on the earth 'Keratin'. The keratin degrading ability of these fungi has been attributed to the production of the extracellular proteases known as keratinases. They have great potential in wool and silk cleaning, leather industry, developing cost effective feather by-products, valorization of the keratin containing wastes, bioremediation and curing skin diseases. In addition, prospective application in prion degradation can revolutionize the protease world in the near future. In the present study, we focussed on the isolation of keratinophilic fungi from the soils of high altitude areas of Kashmir. The sites selected were Khanyar (5173 ft) and Tangmarg (8900 ft). Nineteen isolates of keratinophilic fungi were isolated from these soils by keratin bait technique. These were purified and identified by studying the micro and morphological characters by using relevant literature. Molecular characterization offers more discrimination in fingerprinting an organism and studying its lineage, we thereby relied on PCR based RAPD technique. It is a sensitive and rapid molecular tool for species identification as many fungi do not produce characteristic spores. For molecular characterization, genomic DNA from fungal isolates were isolated and purified. These were then amplified using twenty-one RAPD primers for detecting the polymorphism. PCR products were then separated on the agarose gel. The data was analysed using RAPD-PLOT, PHYLIP and TREE VIEW softwares. Dendrogram generated divided the isolated keratinophiles into three main groups. This data supported the morphological analysis to a noticeable extent.

Keywords: *Dendrogram*, *Keratinophilic*, *Polymorphism*, *Phylogeny*, *RAPD*, *Fungi*

Introduction

Keratin is a rigid scleroprotein, fibrous in nature and insoluble in water. Its main sources are feathers, hair, wool, nails, horns, hooves *etc*. Its structure comprises of α and β chains with molecular weight around 20,000-25,000 Da. Cysteine is a major constituent of this protein (18-24%) (Powell *et al*., 1995). Keratin is used as a source of energy by insects, bacteria, actinomycetes and fungi. Amongst them the largest group of organisms that utilize keratin as the sole source of carbon is the keratinophilic fungi. The term '*keratinophilic*' is derived from a greek word meaning 'keratin loving' but may not hold true always. It refers to the specialized group of fungi, for which keratinized substrates are the natural habitats. They have been reported to be both saprophytic as well as pathogenic (Aho, 1988). Another class of fungi that occur commonly in the soil as kertain decomposers is called 'Keratinolytic fungi' and generally belong to the groups Dueteromycetes and the Ascomycetes (Kaul and Sumbali, 1997; Marchisio *et al*., 1994). Some potential pathogens with keratinolytic activity include dermatophytes belonging to the genera *Trichophyton*, *Microsporum* and *Epidermatophyton* (Buchta and Hejtmanek, 1985; Aho, 1988). The keratin degrading ability of these fungi is attributed to their potential of producing an extracellular protease known as 'keratinases'. These keratinases are of high commercial importance due to its numerous applications (Bockle *et al*., 1995; Lin *et al*., 1995; Gupta and Ramnani, 2006). They are being exploited in the detergent and leather industries, wool and silk cleaning industries, personal care products and more recently in prion degradation (Okoroma *et al*., 2009).

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Keeping the above in mind, in the present study we aimed at the isolation and characterization of such fungi from high altitude regions which may have native enzymatic properties. PCR based characterization is rapid, sensitive, specific and hence highly promising. DNA polymorphism is a common trait of many fungal species and can be studied using specific molecular markers. We used the RAPD (Random Amplified Polymorphic DNA) approach which has proved to be potent and useful technique in the taxonomical classification of the fungi which are morphologically indistinguishable. Another prominent reason for the application of these molecular tools is for the species identification as many fungi do not produce characteristic spores which are key to fungal species identification.

Materials and Method

Sample collection and fungus isolation- The soil samples were taken from Khanyar and Tangmarg regions of the Kashmir in the pre-sterilized polythene bags. It was sieved and pH was checked by pH meter. For the isolation of the keratinophilic fungi, the 'Tokawa' hair baiting method was used with slight modifications (Benedek, 1962). Briefly, 10-20 grams of the soil samples were taken in autoclaved petridishes and tyndallized baits (hair, chicken feathers) were used for isolation (Table 1). The samples were incubated and observed for the growth of fungal mycelia.

Morphological characterization-After pure culturing, the slides were made using methylene blue stain to observe the spore shape, hyphal bearing and mycelium type. Further spore size was calculated using ocular micrometery.

Soil sample	Set	Subset	Bait	Incubation temperature
A	IA	AHI	Hair	37 °C
	IIA	AH2	Hair	18 °C
		AF1	Feather	37 °C
		AF2	Feather	18 °C
B	IB	BH1	Hair	37 °C
	IIB	BH2	Hair	18 °C
		BF1	Feather	37 °C
		BF2	Feather	18 °C

Molecular characterization- Total genomic DNA was isolated following the protocol of Maroof *et al*. (1984). The DNA hence obtained was purified by treatment with 1µl of RNAse per sample for 2 hours at 37 °C. DNA quantification was done on 0.7% agarose gel by comparing with λ DNA marker (300 µg/µl). The DNA was diluted to a uniform concentration of 40 ng/µl for the PCR amplification.

PCR amplification- The DNA was PCR amplified using the ingredients as shown in Table- 2. The sequences of the RAPD primers are shown in Table 3. The PCR was carried out in Eppendorf master cycler gradient thremalcycler. The final optimized condition for amplification was initial denaturation at 94 °C for 5 minutes, denaturation at 94 °C for 1 minute, annealing temperature at 45 °C for 2 minutes and extension at 72 °C for 1 minute. The program was repeated for 40 cycles and final extension was done at 72 °C for 10 minutes. The PCR products were electrophoresed on a 2 % agarose gel and visualized under UV trans illuminator.

S. No	Ingredients	Reaction mixture	Stock concentration	Working concentration
1	Buffer	2.0 µl	10X	1X
2	dNTPS	2.5 µl	1mM	-
3	$MgCl_2$	3.0 µl	25 mM	2 mM
4	Taq pol	0.4 µl	6 U/µl	1.8 U/µl
5	Primers	2.0 µl	0.5 M	0.4 M
6	DNA	3.0 µl	-	40 ng/µl
7	MilliQ	7.1 µl	-	-
	Total	20.0 µl		-

Table-2: Optimised PCR assay

S. No.	Primer code	Primer sequence
1	OPK3	CCAGCTTAGG
2	OPK4	CCGCCCAAAC
3	OPK5	TCTGTCGAGG
4	OPM14	AGGGTCGTTC
5	OPA3	AGTCAGCCAC

Table-3: Sequences of the RAPD primers

Phylogenesis- For the analysis of the phylogenetic relatedness amongst the fungal isolates, a dendrogram was generated using the RAPD-PLOT, PHYLIP and TREE VIEW softwares.

Results and Discussion

A noteworthy number of keratinophilic fungal species were found to be present in the collected soil samples. Although some of them showed similar macroscopic characteristics, a significant polymorphism was revealed in the molecular analysis. After sieving soil samples, the pH of the soil sample 'A' was found to be 8 while that of sample 'B' was 7.5 indicating a slightly high alkaline nature of soil sample 'A'. Nineteen pure cultures of the keratinophilic fungi were isolated using the keratin bait technique (Figure-1).

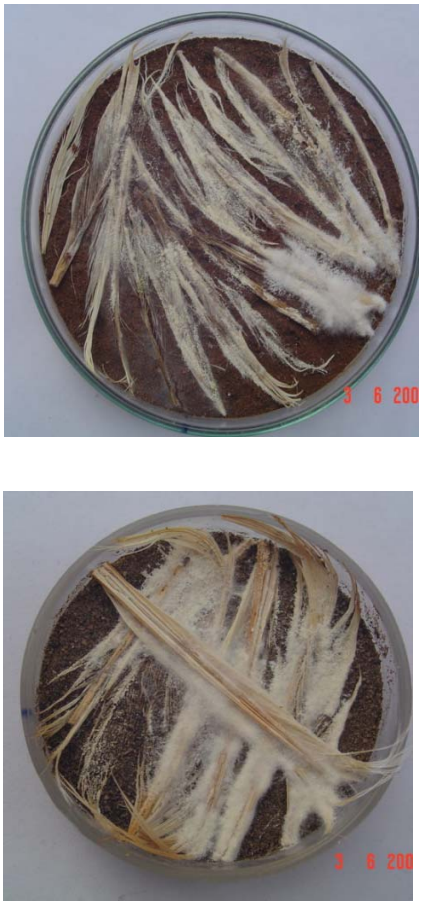

Fig. 1: Plate showing culture

These were purely cultured on the SDA medium and then subcultured (Figure-1). Nine cultures out of the total isolates were selected for the further work. These fungal cultures were all white in colour, but showed variations in the texture and reverse colony characteristics. Ac1 culture had a velvety texture with small oval shaped spores of size (7-4) × (2-4) µm, while Ac2 had a puffy growth and thin walled oval spores of size (7-5) × (2-4) µm. Ac3 showed the presence of pyriform shaped arthospores of (9-14) × (7-9) µm, showing a powdery white texture on the plates. Ac4 showed absence of sporulation, while Ac5 too had rough walled pyriform spores of size (7-10) × (4-8) µm on a racquet hyphae. Ac3 and 5 were identified as members of *Chrysosporium* species. The spores of Ac6 were thin walled and elongated doughnut shape of size (9-14) × (5-7) µm. The Ac7 had thin walled oval spores of size (9-10) × (2-3) µm. Ac8 had (5-7) × (3-4) µm sized oval spores, while Ac9

had pyriform spores of size (10-11) × (2-3) µm and velvetty white texture.

Molecular analysis: About twenty one RAPD primers were used for PCR amplification, of which five primers showed proper polymorphism amongst the selected fungal samples (Figure 2 and 3). The PCR amplified products were directly scored from the electrophoresis gel for the presence and absence of the bands. Each band was treated as RAPD marker. The presence of the band was scored as 1 while absence of the band was scored as 0.

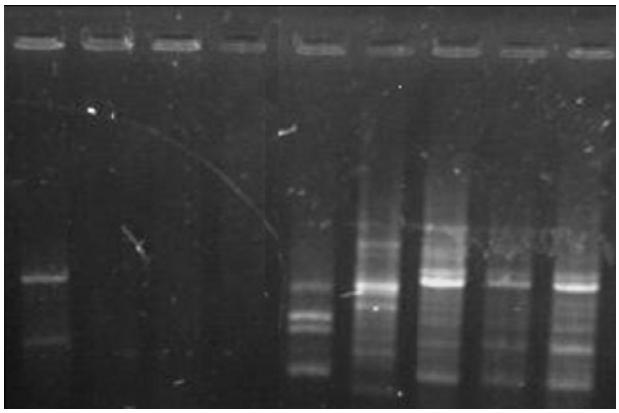

Fig. 2: RAPD polymorphism using primer pair 1-2

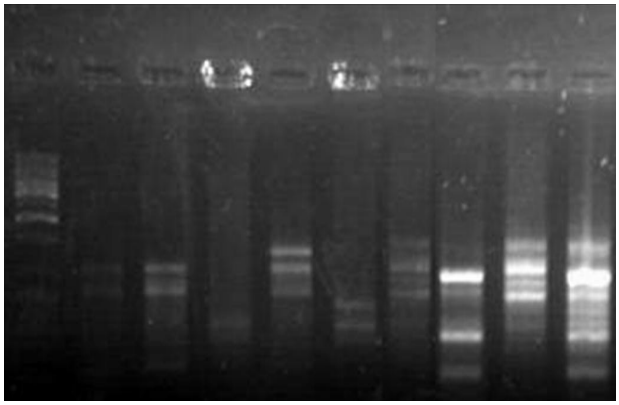

Fig. 3: RAPD polymorphism using primer pair 3-4 (wells 1-4) and 4-5 (wells 5-9)

The generated dendrogram divided the fungal isolates into three main groups (Figure-4). Acession numbers 3, 4, 5, 9, 6, 7 were placed in one group, wherein Ac6 and 7 were more closely related. Ac9 was closer to this subgroup followed by Ac5. Ac4 was close to Ac3 which was distant from other members. Ac1 and Ac2 formed another cluster present near Ac6 and Ac7. Ac8 formed a separate branch distant from the other two clusters.

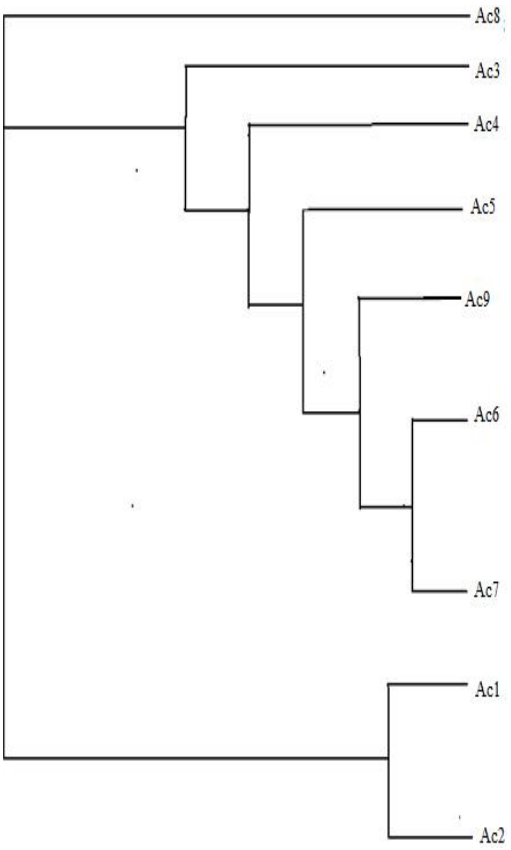

Fig. 4- Dendrogram generated using RAPD PLOT and PHYLIP

Till date, fungal systematics is chiefly based on the morphological criteria and identified basically by their phenotypes. Numerous alternative approaches have been developed like nutritional and physiological studies, secondary metabolites, serological testing, molecular markers *etc*, but they are used as complimentary tools of morphological data. A number of molecular targets like mitochondrial cytochrome B gene (Yokoyama *et al*., 2001), aflR gene (Chang, 2001), TOP2 gene (Kanbe *et al*., 2002), β tubulin gene and rRNA genes (Iwen *et al*., 2002) *etc* for identification of various fungi have been developed. Kaul and Sumbali (1997) have made a special study on the keratinolytic fungi in the Jammu region and have added new fungi like *M. chrysosporodia* and *M. flava* to the list. Prevalence of the keratinophilic fungi in the alkaline soils has been reported and alkaline pH of our soil samples was an early indication of the presence of the keratinophilic fungi (Kaul and Sumbali, 1999; Mercantini *et al.*, 1980). Research on the soil samples collected from

the glacier banks of Gulmarg, Khilanmarg, Sonamarg and Tangmarg regions of the Kashmir valley revealed the prevalence of the keratinophilic fungi and related dermatophytes (Deshmukh, 2002). *Chrysosporium keratinophilum* (3.7 %), *Chrysosporium tropicum* (5.6 %), *Ctenomyces serratus* (11.2 %), *Geomyces pannnorum* (2.8 %), *Microsporum nanum* (1.9 %), *Trichophyton ajelloi* (15 %). There was a significant difference in the microscopic characteristics of the fungal isolates as discussed above. These differences were confirmed by the molecular characterization where marked polymorphism was observed among the isolates. A study reported the intraspecific variation in the *Metarhizium anisopliae* populations by RAPD and ITS primer based approach (Velasquez *et al*., 2007). Moreover, RAPD and RFLP assays were performed for the characterization of the obligate biotroph *Spongospora subterranean* (Qu, 2006).The phylogenetic data supported the morphological analysis to a noticeable extent. Ac6 and Ac7 were the fungal isolates from the soil sample A, isolated using feathers as bait. Both of them were white in colour and brown reverse colony characteristics, but Ac6 had a puffy texture while Ac7 had velvety texture. Ac6 had elongated doughnut shaped, thin walled spores with size (9-14) x (5-7) µm, while Ac7 had oval spores of size (9-10) x (2-3) µm. This indicates that these may be different species of a same genus. Ac3, 5 and 9 were isolated from the soil sample 'B' using feather baits. They had white colonies with cottony texture and reverse colony characteristics were yellowish brown. Ac5 had pyriform rough walled spores, racquet hyphae and abundant arthrospores were also present. The spore size was calculated as (7-10) x (4-8) µm. It was closely related to Ac4 which was isolated from the soil sample A using feather baits. It too had pyriform spores and spore size was (9-14) x (7-9) µm. Ac8 emerges as an outgroup and remained aloof from the other isolates as revealed from the dendrogram. It was isolated from soil sample A using hair bait. It was white in colour and had cottony mycelia. Spore size could not be calculated due to absence of sporulation.

The other phylogentic cluster contained Ac1 and Ac2 indicates their close relatedness and identical origin. These were isolated from the soil sample B using hair bait. The morphological colony characteristics were similar with slight differences in the textures. Ac1 had a velvety texture while Ac 2 was cottony in appearance. The spore shape was oval in both of them but spore sizes differed to a small extent.

Conclusion

With the inventions on the newer techniques of isolation, the studies on the keratinophilic fungi are in progression. Soil is the main reservoir of this group of fungi, where they are involved in the degradation of the keratinous substances like hair, nails, feathers *etc*. A marked effect of the habitat on the biological properties of the microbes is well known. We isolated these fungi from high altitude regions. Morphologically all the selected fungal isolates had white colonies, so their differentiation was difficult. But at the molecular levels the variations were revealed using RAPD based approach. Furthermore, their phylogenetic relatedness was a better indicator for the identification of these fungal isolates. Though RAPD has some limitations like non specificity, presence of repetitive sequences but still it serves as an informative molecular tool. Further biochemical screening and protein characterization of the isolated fungal isolates may offer them as a boon for industries.

References

Aho, R.,1988. Mycological studies on zoophilic dermatophyte isolates of Finnish and Swedish origin. ***Mycose,*** 31: 295-302.

Benedek, T., 1962. Fragmenta mycological: Some historical remarks on the development of 'hairbaiting' of Toma-KarlingVanbreuseghem (The Tokava-hair baiting method). ***Mycopathol Mycol Appl,*** 16: 104-106.

Bockle, B. Galunsky, B. and Muller , R., 1995. Characterization of a keratinolytic serine proteinase from *Streptomyces pactum* DMS-40530. ***Appl Environ Microbiol,*** 61: 3705-3710.

Buchta, V. and Hejtmanek, M., 1985. Keratinolytic activity and its use in the identification of dermatophytes. ***Acta Univ Polackianae Olomucensis,*** 109: 53-61.

Chang, H. C. , Leaw, S. N. Huang, A. H., Wu, T. L. and Chang, T. C. , 2001. Rapid identification of positive blood cultures by a multiplex PCR method. ***J. Clin. Microbiol***, 39: 3466-3471.

Deshmukh, S., 2002. Incidence of dermatophytes and other keratinophilic fungi in the glacier bank soils of the Kashmir valley, India. ***Mycologist,*** 4: 165-167.

Gupta, R. and Ramnani, P., 2006. Microbial keratinases and their prospective applications: an overview. ***Appl Microbiol Biotechnol,*** 70: 21-33.

Iwen, P. C. Friefeld, T. A. and Hinrich, S. H., 2002. Use of ITS regions as molecular targets to detect and identify human fungal pathogens. ***Med Mycol***, 40: 87-109.

Kanbe, T. Yamaki, K. and Kikuchi, A., 2002. Identification of pathogenic *Aspergillus* species by nested PCR using a mixture of specific primers to DNA topoisomerase II gene. ***Microbiol Immunol***, 46: 841-848.

Kaul, S. and Sumbali, G., 1997. Keratinolysis by poultry farm soil fungi. ***Mycopathologia***, 139: 137-140.

Kaul, S. and Sumbali, G., 1999. Impact of some ecological factors on the occurrence of poultry soil-inhabiting keratinophiles. ***Mycopathologia,*** 143: 155-9.

Lin, W., Kelemen, D. W., Miller, E. S. and Shih, J. C. H., 1995. Nucleotide sequence and expression of KerA, the gene encoding a keratinolytic protease of *Bacillus licheniformis PWD-1*. ***Appl Environ Microbiol,*** 61: 1469-1474.

Marchisto, V. F. , Fusconi, A. and Rigo, S., 1994. Keratinolysis and its morphological expression in hair digestion by airborne fungi. ***Mycopathologia***, 127: 103-115

Maroof, S., Soliman, K. M., Jorgenson, A. R. and Allard, R. W. ,1984. Ribosomal DNA spacer length polymorphism in barley: Mandelian inheritance, chromosomal location and population dynamics. ***PNAS,*** 81:8014-8018.

Mercantini, R., Marsella, R., Caprilli, F. and Dovgiallo, G. 1980. Isolation of dermatophytes and correlated species from the soil of public gardens and parks in Rome. ***Sabouradia,*** 18: 123-8.

Okoroma, E., Purchase, D. Garelick, H. and Abiola, O. 2009. *Bacterial keratinase: prospects for prion degradation*. Society for Gen Microbiol, Spring Meeting.

Powell, B. C. , Arthur, J. and Nesci, A., 1995. Characterization of a gene encoding a cysteine-rich keratin associated protein synthesized late in rabbit hair follicle differentiation. ***Differentiation*** 58: 227–232.

Qu, X. and Christ, B. J., 2006. Single *Cystosorus* Isolate Production and Restriction Fragment Length Polymorphism Characterization of the Obligate Biotroph *Spongospora subterranea* f. sp. *subterranea*. ***Phytopathol***, 96:1157-63.

Velasquez, V. B., Carcamo, M. B., Merino, C. R., Iglesias , A. F. and Duran, J. F., 2007. Intraspecific differentiation of Chilean isolates of the entomopathogenic fungi *Metarhizium anisopliae* var. *anisopliae* as revealed by RAPD, SSR and ITS markers. ***Genet Mol Biol***, 30: 89-99.

Yokoyama, K., Wang, L., Miyaji, M. and Nishimura, K., 2001. Identification, classification and phylogeny of *Aspergillus* section Nigri inferred from mitochondria cytochrome b gene. ***FEMS Microbiol***, 200: 241-246.