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# GENETIC DIVERSITY AMONG THE ISOLATES OF *CORDYCEPS SINENSIS* OF HIGHER HIMALAYAN MEADOWS OF INDIA

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**ABSTRACT**
There is heavy biotic pressure on the Himalayan alpine meadows of Uttarakhand in India for the extraction of *Cordyceps sinensis*, a medicinally important fungus. Molecular characterization of *Cordyceps sinensis* germplasm of Indian origin has not yet been established. In the current study isolates of *C. sinensis* collected from different parts of Uttarakhand have been analyzed by Random Amplified Polymorphic DNA for assessing genetic diversity among the isolates.

**KEY WORDS***: Cordyceps sinensis*, Genetic diversity, phylogeny, 18S rDNA.

## INTRODUCTION

*Cordyceps sinensis* is an ascomycetous, entomophagic fungus widely distributed in alpine regions of south west China including Yannan, Gansu, Qinghai Tibetan plateau, alpine meadows of Uttarakhand in India and Nepal. This fungus infects the larvae of one particular species of month *Hepialus armoricanus*. The name *Cordyceps* comes from the latin words "cord" and "ceps" meaning "club" and "head" respectively (Holliday and Cleaver 2005). *Cordyceps sinensis* is characterized by having perithicioid ascomata that are superficial or embedded in acicular, clavate or capitate-stipitate stromata. The fungus has been shown to produce some potent antibiotics, immune stimulants and anti tumor agents (Hobbs 1986). *Cordyceps sinensis* was recognized as a traditional Chinese medicine 2000 years ago and formerly used as early as 1578 (Li 1975). It has been used for the treatment of many diseases like respiratory diseases, renal dysfunction, hyperlipidemia and hyperglycemia (Zhou *et al*, 1998). The use of *Cordyceps* by athletes came to light in 1993 after the remarkable performance exhibited by the chinese Women's Track and Field team at the chinese National Games. Recent research has confirmed that *Cordyceps* usage increase both the cellular ATP level (Guowei 2001) and the oxygen utilization. Ko and Leung (2007) observed both immunopotentiating and ATP-enhancing activities in *Cordyceps* extracts. Its high price in international market has led to intensive illegal extraction of this species even from the protected biosphere reserves in India. Threat to biodiversity and heavy biotic pressure in such areas compel the need to develop protocol for artificial cultivation and identification of germplasm giving high yield of bioactive molecules such as $N^6$-(2-hydroxyethyl)-Adenosine, Cordycepin (3'deoxyadenosine), Adenosine, Ergosterol etc. Specimens originating from different locations have divergent reputations as medicinal drugs and obtain different market price (Chen *et al*, 2004)

Many workers grouped different fungi according to geographical origin by polymerase chain reaction (Singh *et al*, 2006). In the present study an attempt was made to genetically differentiate *Cordyceps sinensis* isolates collected from different locations in Himalayan alpine meadows of Uttarakhand, India on the basis of random amplified polymorphic DNA (RAPD).

## MATERIALS AND METHODS

### Collection and Isolation:

Sporophores of *Cordyceps sinensis* were collected between May to July from different parts of Uttarakhand. Total 15 isolates were collected from 6 different geographical locations (Table 1). Fruiting bodies were then brushed properly and preserved in dry condition as described by Ainsworth (1971).

Fungal isolation was done using the method described by Sehgal and Sagar (2006). The specimens were first washed with distilled water and then the tissue from the stipe and stroma portion were cut with the help of sterilized blade. The cut pieces of tissue were sterilized in 0.1% Mercuric Chloride for 5-10 seconds and then washed with sterilized distilled water. Tissues were placed on sterilized filter paper to remove the excess moisture and then transferred aseptically on to potato dextrose agar (PDA) medium. Petri plates were then incubated at ambient temperature for 8-10 days for appearance of mycelia. The actively growing mycelial colonies were sub cultured on PDA plates and slants. Slants were then preserved by adding autoclaved mineral oil and stored at $6^0$C.

### Genomic DNA isolation

For isolation of genomic DNA, isolates were grown in 50 ml potato dextrose broth (PDB) for 10 days at $25\pm1^0$C in an environmental shaker. Submerged mycelial mass were then filtered with filter paper, lyophilized and stored at $-20^0$C. For each fungal isolate 5gm of lyophilized mycelium was ground in liquid nitrogen to make a fine powder. Then 25 mg of powdered mycelium was taken for DNA isolation. DNA was extracted by C-TAB protocol (Saghai-Maroof *et al*, 1984) with slight modification. DNA was dissolved in TE-8 buffer and the final concentration of DNA was diluted to 20ng and stored at $-20^0$C until used.

**RAPD analysis**

RAPD-PCR reactions were carried out in a final volume of 25 µl containing 1X taq buffer (2.5µl), 0.25mM of each dNTPs , 2.5mM $MgCl_2$, 0.5mM oligonucleotide primer, 1 unit of amplitaq (Applied biosystems) and 20ng of template DNA. Four RAPD primers *viz.* OPT-9, OPT-14, OPT-19, OPT-20 (Operon technology, USA) were selected after preliminary screening of 40 primers from Operon E and T series. The reaction mixture was microfuged at 10,000g for 1min. Amplification was carried out in gradient thermal cycler (Eppendorf) with initial denaturation at $95^0$C for 2min and 45 cycles at $94^0$C for 1min, $37^0$C for 45 sec, $72^0$C for 1min and a final extension at $72^0$C for 8min. Amplification products were resolved on 1.3% agarose gel containing ethidium bromide (6mg/ml) at 6 µl per 100ml in 1X TAE buffer at 60 V for 3 hours. A high range DNA ladder was loaded for molecular weight comparison. Gels were visualized and photographed through UVP gel documentation system (Fig 1). All RAPD fragments were with no correction for band intensity as suggested by Barry et al. (2002). Isolates were scored for presence (1) or absence (0) and binary matrix was performed by Unweighted Pair Group Method with Arithmetic mean (UPGMA) using Gene Profiler 1-D Phylogenetic analysis and databasing software (Scanalytics, Inc). A combined dendrogram was generated with the help of TREECON software.

**18S nrDNA amplification:**

Partial sequence of 18S nrDNA for all the isolates were amplified using primer1 (5'GTTGGTGGAGTGA TTTGTCTGC3') and primer[2] (5'TAATGATCCTT CCGCAGGTT3') (Ito and Hirona 1997). PCR reaction conditions were same as RAPD except for forward and reverse primers. The primer concentration was 0.2 µM. PCR cycling conditions were as follows: an initial denaturation for 3 min at $96^0$C, followed by 35 cycles of 1 min at $94^0$C, 1 min at $60^0$C and 1 min at $72^0$C and completed by a final elongation step at $72^0$C for 8 min.

**Sequencing**

All the PCR products were sequenced at Genomic lab of Chromous Biotech, Sahakar Nagar, Bangalore, India. The partial 18S nrDNA regions were sequenced directly from the PCR products with primer primer1 and were directly submitted to GenBank (NCBI, USA).

## RESULTS

Thirty isolates of *Cordyceps sinensis* were obtained from Himalayan meadows of Uttarakhand. Identification of isolates were done by BLAST search of partial sequence of 18S ribosomal DNA sequences of 15 isolates and other 15 isolates were compared with the sequenced isolates through morphology and 18S rDNA profiling. RAPD profiles were prepared for 30 isolates using 7 primers. A total of 263 polymorphic bands were scored, with an average of 37.57 bands per primer. Each amplification was repeated twice to conform the reproducibility.

Cluster analysis with UPGMA using genetic distance was performed to generate a dendrogram (Fig 2) illustrating the overall genetic relationship within the species studied. All the 28 isolates were grouped in two main clusters (A and B) and 2 isolates grouped as outlier. The main cluster A consisted 25 isolates where as the main cluster B consisted only 3 isolates. Main cluster B was further divided into five sub clusters (B1, B2, B3, B4 and B5). Sub cluster B1 consisted 8 isolates (1 from Panchachouli, 3 from Patal Nachoni and 4 from Braham), sub cluster B2 consisted 5 isolates (1 of each from Girji and Patal Nachoni and 3 from Chipla Kedar), sub cluster B3 consisted 3 isolates (2 from Kewla Vinayak and 1 from Auli), sub cluster B4 consisted 2 isolates (from Girji) and sub cluster B5 consisted 6 isolates (5 from Girji and 1 from Chipla Kedar). Main cluster A consisted 3 isolates from Chipla Kedar. Isolates ISO-17 and ISO-21 (from Girji and Chipla Kedar) clustered as outlier.

## DISCUSSION

*Cordyceps sinensis* is one of the most studied fungi by researchers for its bioactive molecules. There are few morphological differences between the different species of *Cordyceps* and the characters used to distinguish taxa are unreliable thus imposed identification difficulties. In recent years DNA based identification of fungi has been proved to be a better tool. BLAST search of 18S nrDNA classified the isolates as *Cordyceps* sinensis. Furthermore, as *Cordyceps sinensis* is medicinally very important fungi and in recent years has gained high commercial value but as pointed out by Chen *et al,* (2004) the specimens originating from different locations have divergent reputations, it is wise to screen *Cordyceps sinensis* isolates collected from different geographical regions for their genetic variability. Different authors classified *Cordyceps sinensis* isolates on the basis of geographical origin (Chen *et al,* 1999). From this study we found that RAPD technique is able to differentiate 30 isolates into two main clusters which are further divided into subclusters. Tree from RAPD data showed that the all 30 isolates tend to group with respect to their geographical origin to a greater extent. The low bootstrap value confirmed a high genetic variation found within the isolates of same geographical origin. Two isolates *Viz,* ISO-17 and ISO-21 grouped as outlier showed a possible high genetic diversity among the isolates. As RAPD-PCR scans the full genome and produce consistent polymorphic bands, it can easily be used for genetic differentiation of isolates but may not be useful for phylogenetic studies. It also confirmed that the isolates were geographically diversified but within the geographical region isolates were highly similar. Differentiating isolates on the basis of geographical regions can be useful in studies aiming to characterize and quantify bioactive molecules thus reducing effort by avoiding duplication and conclusively identifying important sites for germplasm conservation and artificial cultivation.

## CONCLUSION

From the present study for the first time the identity of *Cordyceps sinensis* from alpine meadows of Uttarakhand from India has been authenticated based on their molecular characterization by RAPD and BLAST search of 18S rDNA sequence. The genetic variability among isolates with respect to their geographical location was an important observation which may be correlated in further studies with their therapeutic value as their different bioactive principles such as $N^6$-(2-hydroxyethyl)-Adenosine, Cordycepin (3'deoxyadenosine), Adenosine, Ergosterol etc. may either be present or absent in some

isolates or they may have differential production with respect to the isolates or geographical region.

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**FIG 1:** DNA banding patterns from random amplified polymorphism DNA analysis of *Cordyceps sinensis* isolates using primer OPT-19

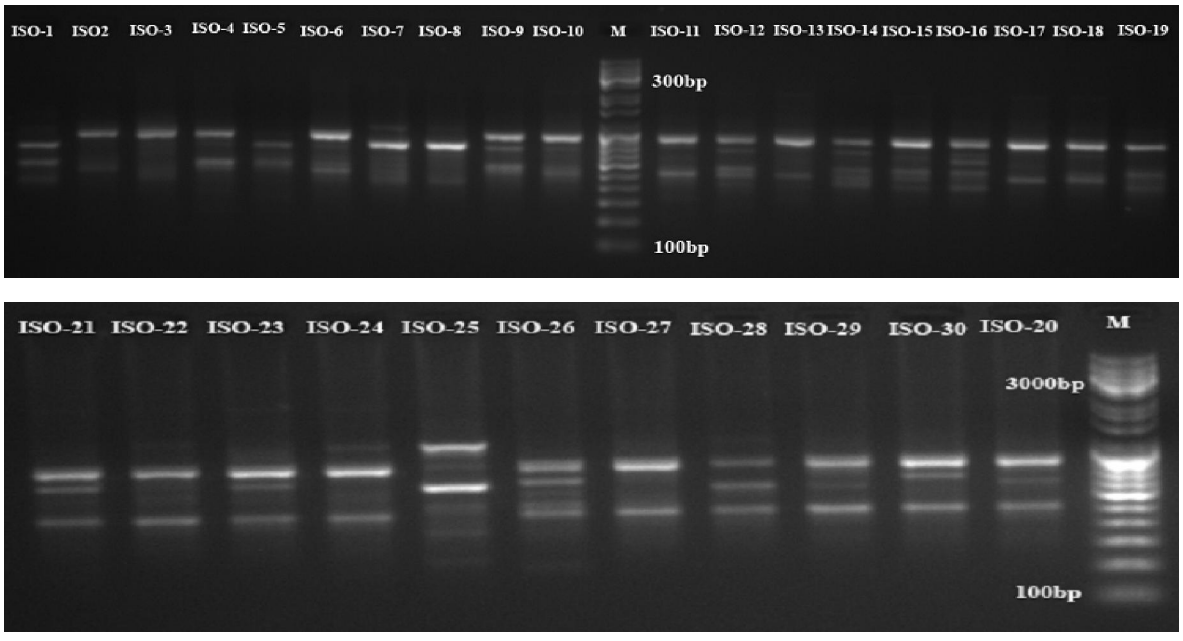

**TABLE 1**: Accession numbers and geographical origin of the isolates used in this study.

| Isolate name | Geographic origin | Accession No(18S rDNA) |
|---|---|---|
| ISO-1 | Panchachauli | GU172150 |
| ISO-2 | Patal Nachoni | GU172151 |
| ISO-3 | Patal Nachoni | GU172152 |
| ISO-4 | Patal Nachoni | GU172153 |
| ISO-5 | Patal Nachoni | GU183546 |
| ISO-6 | Kewla Vinayak | GU183547 |
| ISO-7 | Kewla Vinayak | GU172154 |
| ISO-8 | Auli | GU172155 |
| ISO-9 | Braham | GU172156 |
| ISO-10 | Braham | GU172157 |
| ISO-11 | Braham | GU172158 |
| ISO-12 | Braham | GU172159 |
| ISO-13 | Braham | GU172160 |
| ISO-14 | Girji | GU172161 |
| ISO-15 | Girji | GU172162 |
| ISO-16 | Girji | --- |
| ISO-17 | Girji | --- |
| ISO-18 | Chipla Kedar | --- |
| ISO-19 | Chipla Kedar | --- |
| ISO-20 | Chipla Kedar | --- |
| ISO-21 | Chipla Kedar | --- |
| ISO-22 | Chipla Kedar | --- |
| ISO-23 | Chipla Kedar | --- |
| ISO-24 | Chipla Kedar | --- |
| ISO-25 | Chipla Kedar | --- |
| ISO-26 | Girji | --- |
| ISO-27 | Girji | --- |
| ISO-28 | Girji | --- |
| ISO-29 | Girji | --- |
| ISO-30 | Girji | --- |

**FIG 2:** Combined cluster analysis (UPGMA) of *Cordyceps sinensis* based on distance matrix produced by primers OPT-9, OPT-14, OPT-19, OPT-20. Boot strap values were given next to the branch.