
A psychrophilic and halotolerant strain of *Thelebolus microsporus* from Pangong Lake, Himalaya

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Over recent decades, the Ladhak regions of the Himalaya have been investigated mainly for the presence and exploitation of psychrophilic bacteria and Archaea, but mycological studies in the region have been infrequent. Here we report the occurrence of *Thelebolus microsporus* for the first time from the Himalayan region. The strain, which was isolated from Pangong Lake, is a psychrophile, with an optimum growth temperature of 4°C and halotolerance of up to 6% NaCl. Microscopic studies found that the strain does not produce conidia, and that asci are produced in cleistohymenial ascomata, with each ascus containing eight ascospores. Molecular phylogenetic analysis of the internal transcribed spacer region of the ribosomal RNA gene of the strain showed 97–98% similarity with strains of *Thelebolus microsporus* from Antarctica.

Key words – Himalaya – Pangong Lake – phylogeny – psychrophilic fungi – *Thelebolus microsporus*

Article

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Introduction

Psychrophilic fungi, which grow at optimum temperatures of 15°C or lower (Robinson 2001), are distributed from the deep seas to mountainous and polar regions. The physiological and ecological mechanisms needed for the survival and growth of this group of fungi at low temperatures are still largely unknown. However, cold adapted fungi have successfully evolved both genotypic and phenotypic features to survive at low temperatures.

These fungi have several ways to protect themselves against extreme conditions, *viz.*, the synthesis of melanin to protect the thallus and spores, and the production of mycosporine, antifreeze proteins, antioxidants, glycogens, exopolysaccharides, trehalose, sterols and fatty acids (Ruisi et al. 2007). Psychrophilic fungi

are known to be important for their biotechnological and pharmaceutical applications. Some of the secondary metabolites produced by these fungi are unique to cold ecosystems (Rosa et al. 2008), and enzymes obtained from psychrophilic micro-organisms are economically important, as these enzymes are active at low and moderate temperatures (Georlette et al. 2003).

Previous studies on psychrophilic and psychrotolerant micro-organisms have investigated the mechanisms that they adopt to survive in polar regions (Singh et al. 2006, Arenz et al. 2006). However, the Himalayan region, containing some of the highest mountain ranges on earth, has been much less explored for novel psychrophilic microbes (Petrovic et al. 2000). There are reports indicating the presence in the Himalaya of the novel sulphate

reducing psychrotolerant bacterium *Desulfovibrio psychrotolerans*, the novel yeast *Rhodotorula himalayensis* and a new terricolous species, *Aspicilia tibetica* (Jyothsna et al. 2008, Shivaji et al. 2008, Sohrabi et al. 2010), but mycological studies in the region have been infrequent. Pangong Lake, a salt lake situated at an altitude of 4,350 m above sea level in the Himalaya, has yet to be explored for psychrophilic halotolerant fungi. The aim of the present study was to isolate and identify filamentous fungi from the lake, as well as to determine the morphological and microscopic features of selected fungi and their growth characteristics under a range of temperature and salinity conditions.

Materials and methods

Sampling site

Pangong Lake (otherwise known as Pangong Tso) is 134 km in length and 5 km wide at its broadest point, and extends from India to Tibet. Two thirds of the lake falls in the People's Republic of China. Five 200–250 mL samples of water were collected in sterile 500 mL plastic bottles in January 2009 from beneath the ice crust that forms on the lake during winter. The position of the sampling site was at 33°57'40.51''N, 78°25'34.69''E. The samples were transferred to the laboratory in ice bags and stored at 4°C for further processing.

Isolation

The water samples were serially diluted (10^{-1} – 10^{-10}) with sterile distilled water. Dilutions (1 mL) were spread on yeast-malt agar medium (YM; HiMEDIA, India, containing peptone, 5 g; yeast extract, 3 g; malt extract, 3 g; dextrose, 10 g; agar, 15 g L⁻¹) supplemented with 100 mg streptomycin L⁻¹, to eliminate bacterial contamination, in 90 mm diam. Petri dishes. The dishes were incubated at 4°C for up to one month.

Morphological and microscopic characterization

The morphological characteristics of mature colonies such as colour, growth pattern and spore formation were observed at regular intervals. Fresh material was also mounted in

lactophenol cotton blue and observed under bright field microscopy. Several hundred spore heaps from monospore isolates were counted, as described by Wicklow & Malloch (1971). For the enumeration of ascospores, a clean cover glass was mounted over sporulating ascospores and was pressed carefully in a drop of lactophenol on a slide, so that all of the spores were separated in a single layer.

For scanning electron microscopy, material was placed on cover slips and exposed to osmium tetroxide (2%) for 24 h at 20°C and transferred to copper stubs over double adhesive tape coated with gold in Polaron AU/ PD sputter coater. The material was scanned (SEM model JSM 5600, JEOL, Japan) and photographed.

Molecular characterization

Isolation of genomic DNA

Genomic DNA was isolated using a modified Lysis buffer method (Lee & Taylor 1992). Mycelial mat was collected from a fully grown culture kept at –20°C and was crushed in liquid nitrogen to powder form. Lysis buffer (200 µL; 50 mM Tris HCl, 50 mM EDTA, 3% SDS, 1% β mercaptoethanol) was added and the mixture incubated at 65°C for 60 min. Water-saturated phenol (500 µL) was added and the mixture was centrifuged at 12,000 rpm for 12 min at 4°C. The supernatant was retained and water-saturated phenol: chloroform: isopropanol was added in the ratio of 25: 24: 1 before mixing. The mixture was centrifuged at 12,000 rpm for 12 min and, after adding 0.1 × chloroform and 0.7 × isopropanol to the total volume of supernatant, was centrifuged at 12,000 rpm for 10 min. DNA was precipitated by adding 70% alcohol and incubating at 4°C. The supernatant was removed after centrifugation at 12,000 rpm for 12 min. TE buffer (50 µL) was added to the pellet, which was mixed well and stored at 4°C.

Amplification of ITS rDNA and sequencing

Internal transcribed spacer (ITS) region rDNA was amplified using the primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990) in a thermo cycler using the following profile: 94°C for 5 min, then 35 cycles of 94°C

for 1 min, 52°C for 30 sec and 72°C for 1 min, followed by a final extension step of 72°C for 5 min. Amplicons were visualised on 1.2% agarose gels. Amplicons were purified using a PCR product clean up system (Genei). Sequencing was performed using an automated DNA sequencer (Applied Biosystem 3130XL) using ITS1 forward and ITS4 reverse primers. The ensuing sequence data were analysed by Chromas software and assembled into a consensus sequence. The FASTA sequence was compared to data in the GenBank sequence database using BLASTn (Altschul et al. 1990, Zhang et al. 2000) and the best match was recorded and analysed. Sequence data have been deposited in the National Centre for Biotechnological Information (NCBI) database under accession no. GQ483644.

Phylogenetic analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al. 2007). Sequences were aligned using clustal software (Thompson et al. 1997). A neighbour joining phylogenetic tree (Saitou & Nei 1987) was constructed using Mega 4 based on the Kimura 2 parameter model (Kimura 1980) with bootstrap values derived from 1000 replications (Felsenstein 1985).

Ecophysiological characterisation

Growth was studied in yeast-malt broth (YMB; HiMEDIA, India, containing peptone, 5 g; yeast extract, 3 g; malt extract, 3 g; dextrose, 10 g L⁻¹) inoculated with colonised 5 mm diameter blocks of YM agar medium. Mycelial mat was harvested by filtration through Whatman filter paper and dried at 45°C overnight. The dry weight of the mycelium was then recorded. Growth at 4°C, 15°C, 20°C and 25°C was measured in this way at 7, 14, 21 and 28 d after inoculation. Growth at 4°C and at 2%, 4%, 6% and 8% NaCl concentrations was also measured in this way after 28 d.

Results

The total culturable fungal population in the five water samples from Pangong Lake ranged between 6×10^4 and 8×10^4 CFU mL⁻¹. Amongst 25 isolated morphotypes, strain L-22 was selected for further study based on its low temperature tolerance (data not shown). The

strain was initially white in colour, but turned yellowish after 30 d of incubation at 4°C, when spores were formed.

Microscopic characterization

Light microscopic observations

Hyphal branching was observed after 7 d of incubation at 4°C. Hyphae were hyaline and highly septate. Dense globules were observed in hyphae after 15 d of incubation. Hyphae started to coil after 7 d of incubation, and developed into mature oval shaped ascomata at 15–20 d (Fig. 1a). Cylindrical asci developed about 14 d after the ascomata had formed. Eight ascospores were present in each ascus (Fig. 1b, c). Ascospores were oval in shape, and were double layered with a thick, dark outer layer (Fig. 1d). The mean number of spores per ascus was 210 (range 128–224).

Electron microscopic observations

Cleistohymenial ascomata opened in the meso- or telohymenial phase, and were at first sub globular, often becoming apothecioid, forming a palisade of asci and paraphyses (hymenium), usually with forcible discharge of ascospores through an irregular split above a subapical ring at the top of the ascus (Fig 2a–d). Conidia were absent.

Molecular characterization

The ITS region of the 28 S ribosomal RNA gene of strain L-22 showed 97–98% similarity with three *Thelebolus microsporus* isolates from the Antarctic (Table 1). However, the ITS sequence of strain L-22 had only 40% similarity with another named *T. microsporus* sequence, and 86% similarity with a *Thelebolus* sequence, both derived from soils of the Taylor Valley in the Antarctic. The ITS region of the strain bore 96–97% similarity to sequences from *T. caninus*, *Thelebolus* sp. and a member of the *Thelebolaceae*, all from the Antarctic and the latter two from the Ross Sea region. Lower similarities (58–87%) were recorded with two sequences from *T. caninus* and one from *T. stercoreus* (Table 1).

The affiliation between strain L-22 and the above fungi was further confirmed by a neighbour joining phylogenetic tree. Based on this analysis, the ITS region sequence of strain

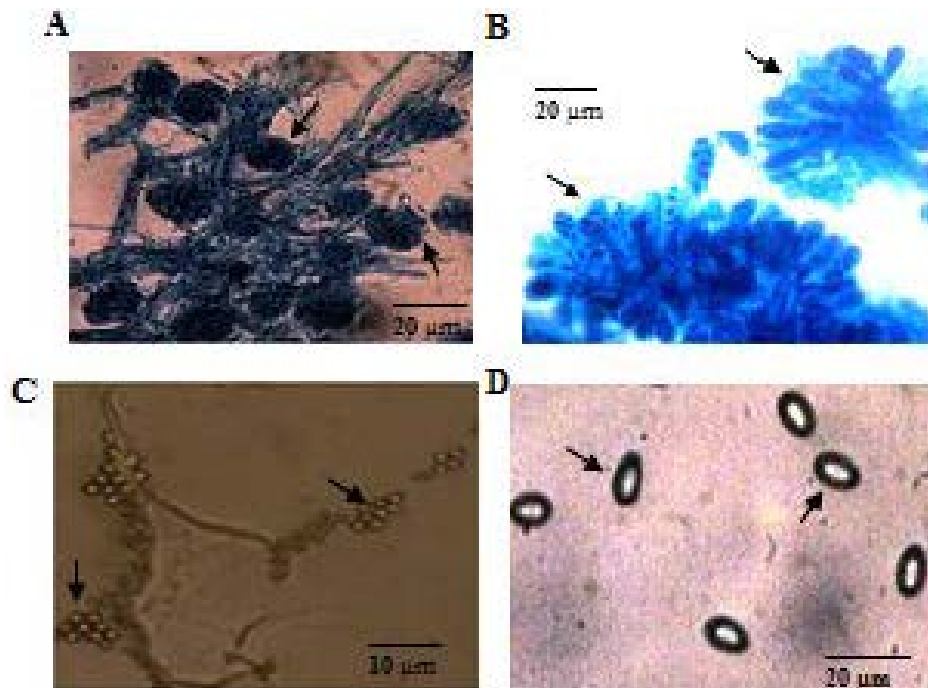


Fig. 1(a–d) – **a** Mature ascomata of strain L-22 (arrows), **b** between 10 and 80 asci per ascomata containing eight ascospores per ascus (arrows), **c** ascomata with reduced receptacle and naked shooting asci (arrows) and **d** oval ascospores with thick, double layered walls (arrows).

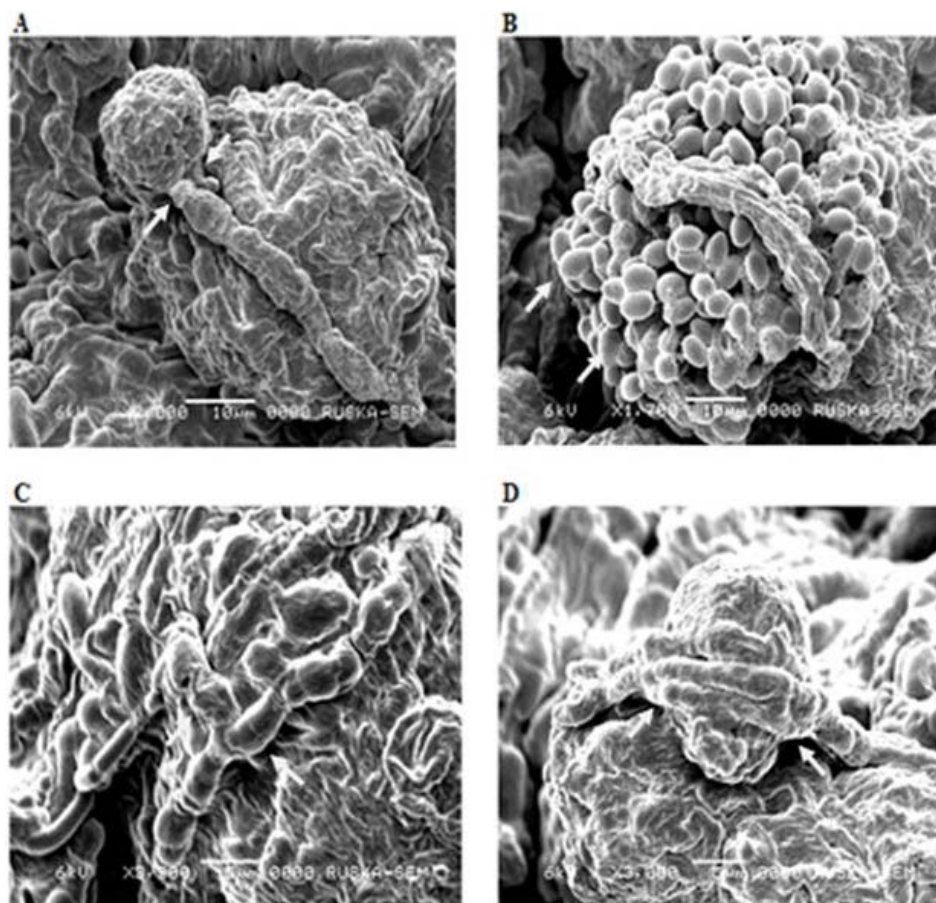


Fig. 2 – Scanning electron microscope images of **a** mature ascomata (arrows), **b** mature ascospores (arrows), **c** mature asci (arrow) with separating rigid thick outer layer and **d** ascomata with strongly reduced receptacles and naked shooting asci (arrows).

Table 1 Best matches in GenBank database to the ITS rDNA region of strain L-22.

Accession no.	Best BLAST match	% similarity ^a	e-value	Overlap ^b	% ID ^c	Location ^d
DQ317351	<i>Thelebolaceae</i> BC 18	97	0.0	512/514	99	RS
DQ317350	<i>Thelebolus</i> sp. BC 17	96	0.0	512/522	98	RS
DQ028268	<i>Thelebolus microspores</i> CBS 113940	97s	0.0	472/476	99	Antarctica
AY957552	<i>Thelebolus</i> sp.	98	0.0	464/468	99	Antarctica
AY957551	<i>Thelebolus microsporus</i> CBS 109909	98	0.0	464/468	99	Antarctica
AY957554	<i>Thelebolus microspores</i> CBS 113940	98	0.0	463/367	99	Antarctica
DQ402532	<i>Thelebolus</i> sp. LC-03-068	86	0.0	463/472	98	TV
AY957549	<i>Thelebolus caninus</i> CBS 709.69	97	0.0	461/469	98	Antarctica
AY957550	<i>Thelebolus caninus</i> CBS 113937	87	0.0	463/473	97	Antarctica
DQ402525	<i>Thelebolus microsporus</i>	40	9e-82	182/185	93	TV
AY957553	<i>Thelebolus caninus</i> CBS 109908	58	3e-76	164/173	94	Antarctica
AY957548	<i>Thelebolus stereoreus</i> CBS 717.69	77	2e-112	361/429	84	Antarctica

^a % similarity: similarity to best match (Clustal W), ^b Overlap: overlap of ITS alignment with best BLAST match (base pairs), ^c % ID: Similarity to best match (BLAST), ^d Location: RSR; Ross Sea region of Antarctica, TV; Taylor Valley, Antarctica.

L-22 showed 61% bootstrap support with a clade including *Thelebolus microsporus*, *T. caninus* and a species of *Thelebolus* (Fig. 3). However, a separate branch of the tree did not differentiate strain L-22 from other species of *Thelebolus*.

Growth at different temperatures and salt concentrations

The optimum growth temperature for strain L-22 was 4°C, followed by 15°C and 20°C (Fig. 4). It failed to grow at 25°C (Fig. 4). At 4°C, strain L-22 grew at NaCl concentrations of up to 6%. Growth did not occur at a salt concentration of 8% (Fig. 5).

Discussion

It is apparent from the observations reported here that strain L-22 is a psychrophile, with the ability to grow well at 4°C, and an inability to grow at temperatures exceeding 20°C (Russell et al. 2006). It thus appears to be adapted to the low temperatures encountered in Himalayan water bodies such as Pangong Lake. The strain was also capable of growing at salinities of up to 6%, which is similar to the 2–4% concentrations of salt measured in Pangong Lake at the time of sampling. Other studies have noted the ability of fungi to live at low temperatures in hypersaline solutions, with

the diversity of fungi isolated from hypersaline biotopes being relatively high. For example, Grishkan et al. (2003) investigated a psychrophilic isolate of *Geomyces* obtained from cryopegs. The isolate was capable of growth at up to 10% NaCl concentrations under low positive and negative temperatures. In addition, Kochkina et al. (2007) reported spore germination of *Geomyces pannorum* (strains FW-224 and FW-2238) isolated from Arctic cryopegs after incubation at –2°C for 60 d.

The morphological features of strain L-22 indicated that it was most probably a species of *Thelebolus*; as in previous studies, radial extension was slow (40–50 mm in 3 wk at 4°C) and the isolate was pale in colour on YM agar medium. Similarly, Montemartini et al. (1993) reported that *T. microsporus* grew slowly (30–50 mm in 2 wk at 15°C) on carrot agar medium and was white in colour. Since the number of specific characters in *Thelebolus* is very low, the number of spores present in an ascus has played an important role in the distinction of taxa within the genus. Although some authors have had their doubts concerning the value of this character (Karsten 1866), microscopic observations suggested that strain L-22 was an isolate of *T. microsporus*. The strain produced cylindrical asci and oval-shaped, thick-walled and double layered ascospores, with eight

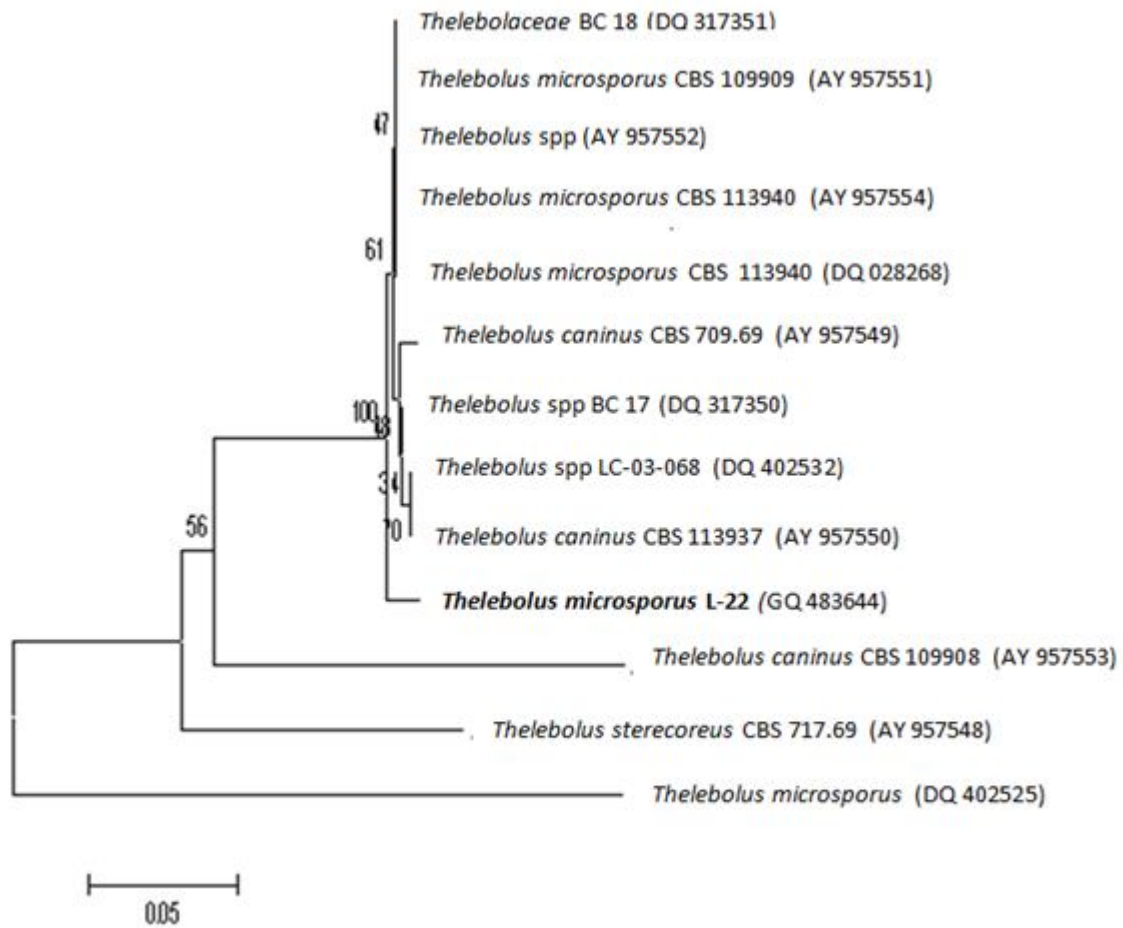


Fig. 3 – Phylogenetic tree showing the relationship of strain L-22 (accession no. GQ483644) with other strains of *Thelebolus*. Reference sequences, the accession numbers of which are indicated in the tree, were retrieved from GenBank.

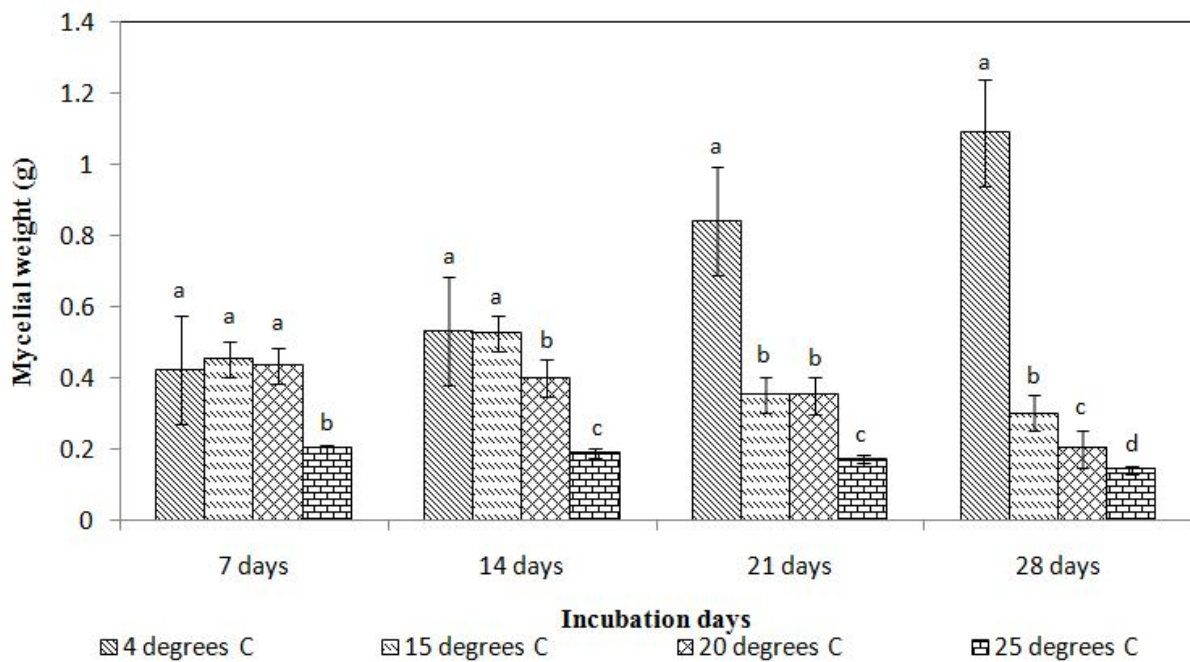


Fig. 4 – Mean dry weight of strain L-22 grown at different temperatures for 7, 14, 21 and 28 d. 0.2 g of mycelia were inoculated into media. Bars represent the standard error of the mean. The same letter above bars indicates that means were not significantly different ($p \leq 0.05$) after Duncan's Multiple Range test.

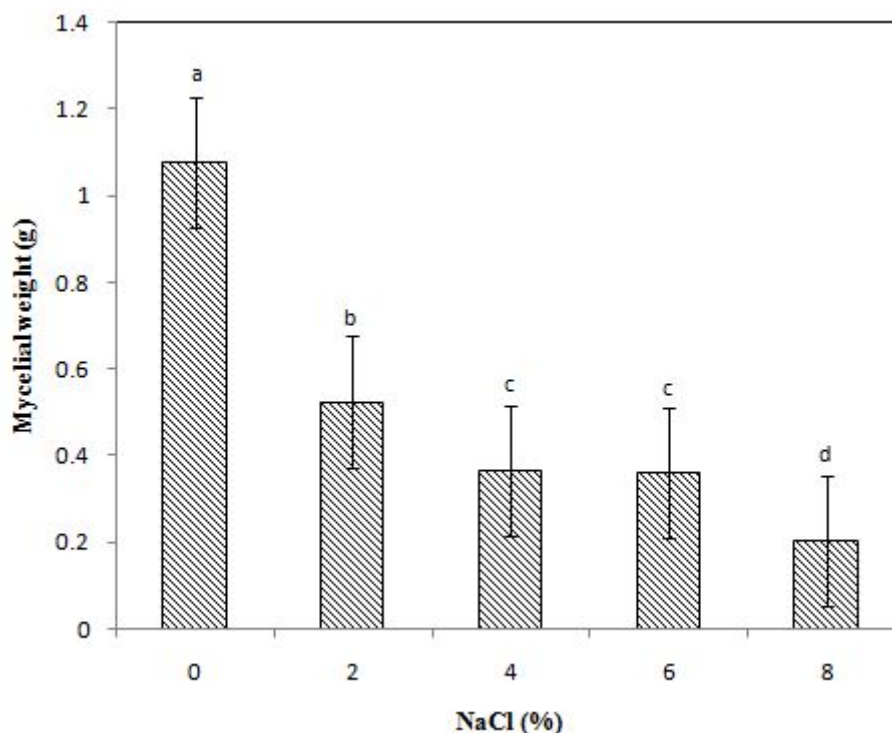


Fig. 5 – Mean dry weight of strain L-22 grown at different salt concentrations for 28 d at 4°C. 0.2 g of mycelia were inoculated into media.

ascospores being present in each ascus, features that distinguish *Thelebolus microsporus* from the other species of *Thelebolus* (Hoog et al. 2005, Kimbrough et al. 1972, Cannon et al. 1985, Raitviir & Prokhorov 1988, Prokhorov 1998).

The morphological observations reported here were supported by phylogenetic analyses, showing the ITS region sequence of strain L-22 to be 97–98 % similar to that of *Thelebolus microsporus*. Strikingly, all of the best matches with strain L-22 in the GenBank database were to *Thelebolus* sequences from the Antarctic, with matches to *T. microsporus* isolated from soil in the Ross Sea region of Antarctica (Arenz et al. 2006) and *T. microsporus* from soil in the Taylor Valley, in the Dry Valleys of the Antarctic (Connell et al. 2006). As for Pangong Lake, the Ross Sea region and Dry Valleys are exposed to subzero temperatures for prolonged periods of each year, which most probably selects for psychrophilic fungi such as *T. microsporus*. This species is apparently one

of the predominant fungi in Antarctica and has also been reported from colder regions of Europe and North America (Russell 1990, Robinson 2001, Hoog et al. 2005, Arenz et al. 2006, Singh et al. 2006, Ruisi et al. 2007). The occurrence of *T. microsporus* in an Himalayan lake is hence not surprising.

The results of our research emphasize the importance of using a combination of molecular techniques, traditional culturing methods and ecophysiological measurements in order to obtain an holistic view of the ecology of fungi in the natural environment. Although there are reports of psychrophilic yeasts from the Himalayan region (Shivaji et al. 2008), the work reported here indicates for the first time the presence of a psychrophilic species of *Thelebolus* in this region of India.

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References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990 – Basic local alignment search tool. *Journal of Molecular Biology* 215, 403–410.
- Arenz BE, Held BW, Jurgens JA, Farrell RL, Blanchette RA. 2006 – Fungal diversity in soils and historic wood from the Ross Sea Region of Antarctica. *Soil Biology and Biochemistry* 38, 3057–3064.
- Cannon PF, Hawksworth DL, Sherwood-Pike MA. 1985 – The British Ascomycotina: an annotated checklist. CMI, Slough, U.K.
- Connell LB, Redman R, Craig SD, Rodriguez R. 2006 – Distribution and abundance of fungi in the soils of Taylor Valley, Antarctica. *Soil Biology and Biochemistry* 38, 3083–3094.
- Felsenstein J. 1985 – Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791.
- Georlette D, Damiens B, Blaise V, Depiereux E, Uversky NV, Gerda C, Feller G. 2003 – Structural and functional adaptations to extreme temperatures in psychrophilic, mesophilic and thermophilic DNA ligases. *The Journal of Biological Chemistry* 278, 37015–37023.
- Grishkan I, Nero E, Wasser SP. 2003 – Soil micromycetes in the hypersaline dead sea coastal area, Israel. *Mycological Progress* 2, 19–28.
- Hoog de GS, Gottlich E, Platas G, Genilloud O, Leotta G, Brummelen V. 2005 – Evolution, taxonomy and ecology of the genus *Thelebolus* in Antarctica. *Studies in Mycology* 51, 33–76.
- Jyothsna TS, Sasikala CH, Ramana V CH. 2008 – *Desulfovibrio psychrotolerans* sp. nov., a psychrotolerant and moderately alkaliphilic sulfate-reducing delta proteobacterium from the Himalayas. *International Journal of Systematic and Evolutionary Microbiology* 58, 821–825.
- Karsten PA. 1866 – Fungi Fenniae exsiccati, Sammling of Finska svampar, 301–600.
- Kimbrough JW, Luck-Allen ER, Cain RF. 1972 – North American species of *Coprotus* (*Thelebolaceae*: *Pezizales*). *Canadian Journal of Botany* 50, 957–971.
- Kimura M. 1980 – A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16, 111–120.
- Kochkina GA, Ivanushkina NE, Akimov VN, Gilichinskii DA, Ozerskaya SM. 2007 – Halo- and psychrotolerant *Geomyces* fungi from Arctic cryopegs and Marine deposits. *Microbiology* 76, 31–38.
- Lee SW, Taylor JW. 1992 – Isolation of DNA from fungal mycelia and single spores. In: *A guide to Methods and Applications: PCR protocols*. (eds MA Innis, DH Gelland, JJ Sninsky, TJ White). San Diego: Academic Press. 282–287.
- Montemartini A, Caretta G, Del Frate G. 1993 – Notes on *Thelebolus microsporus* isolated in Antarctica. *Mycotaxon* 48, 343–358.
- Petrovic U, Gunde-cimerman N, Zalar P. 2000 – Xerotolerant mycobiota from high altitude Anapurna soil, Nepal. *FEMS Microbiology Letters* 182, 339–342.
- Prokhorov VP. 1998 – The genera *Coprotus*, *Ascozonus*, *Thelebolus* and *Trichobolus* (*Pezizales*): the keys to the identification of species. *Mikologia i Fitopatologia* 32, 40–43.
- Raitviir A, Prokhorov V. 1988 – Discomycetes of Middle Asia IV. New data on *Asco-bolaceae* and *Thelebolaceae*. *Proceedings of the Academy of Sciences of the Estonian SSR. Biology* 37, 215–222.
- Robinson CH. 2001 – Cold adaptation in Arctic and Antarctic fungi. *New Phytologist* 151, 341–353.
- Rosa M, Franz S, Jean-Claude M, Charles G. 2008 – Psychrophiles from Biodiversity to Biotechnology In: *Cold-adapted fungi as a source for valuable metabolites* (Ed. CF Jens). Springer, 381–384.
- Ruisi S, Barreca D, Selbmann L, Zucconi L, Onofri S. 2007 – Fungi in Antarctica.

- Reviews in Environmental Science and Biotechnology 6, 127–141.
- Russell NJ. 1990 – Cold adaptation of microorganisms. *Philosophical Transactions of the Royal Society of London (Series B), Biological Sciences* 326, 595–611.
- Russell N, Grishkan I, Nero E, Wasser SP. 2006 – Soil micromycetes in the hypersaline dead seas coastal area, Israel. *Mycological progress* 2, 19–28.
- Saitou N, Nei M. 1987 – The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406–425.
- Shivaji S, Bhadra B, Sreenivas Rao R, Pradhan S. 2008 – *Rhodotorula himalayensis* sp. nov., a novel psychrophilic yeast isolated from Roopkund Lake of the Himalayan mountain ranges, India. *Extremophiles* 12, 375–381.
- Singh SM, Puja G, Bhat DJ. 2006 – Psychrophilic fungi from Schirmacher Oasis, East Antarctica. *Current Science* 90, 1388–1392.
- Sohrabi M, Owe-Larsson B, Nordin A, Obermayer W. 2010 – *Aspicilia tibetica*, a new terricolous species of the Himalayas and adjacent regions. *Mycological Progress* 9, 491–499.
- Tamura K, Dudley J, Nei M, Kumar S. 2007 – MEGA4: Molecular Evolutionary Genetics Analysis software version 4.0. *Molecular Biology and Evolution* 24, 1596–1599.
- Thompson JD, Toby JG, Plewniak F, Jeanmougin F, Desmond GH. 1997 – The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25, 4876–4882.
- White TJ, Bruns T, Lee S, Taylor JW. 1990 – Amplification and direct sequencing of fungal ribosomal RNA genes for Phylogenetics. In: *PCR Protocols: a Guide to methods and applications*. (eds MA Innis, DH Gelland, JJ Sninsky, TJ White). Academic Press, San Diego, 315–322.
- Wicklow D, Malloch D. 1971 – Studies in the genus *Thelebolus*: temperature: optima for growth and ascocarp development. *Mycologia* 63, 118–131.
- Zhang Z, Schwartz S, Wagner L, Miller W. 2000 – A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology* 7, 203–214.