Aspergillus aeneus rediscovered in India from an extreme habitat

Skarma Nonzom* and Geeta Sumbali

Department of Botany, University of Jammu, B.R. Ambedkar Road, Jammu 180 006, India

During a survey of psychrotrophic soil fungi from a high-altitude cold desert (4000 masl), nine species of the genus *Aspergillus* were recovered. One of the species was identified as *Aspergillus aeneus* based on its morphological and molecular characterization. This species has been rediscovered from India from an extreme habitat as a psychrotolerant after 56 years of its first report from tropical soil in Madhya Pradesh. Globally, this fungus is a rare species with limited occurrence, being reported so far only from India, Somalia and Costa Rica. It is a novel representative of desert fungi that showed cellulase and protease activity, but lack lipase activity.

Keywords: *Aspergillus aeneus*, cold desert isolate, phenotypic and genotypic characterization, rediscovered.

Aspergillus aeneus has been recently placed in a novel section Aeni to accommodate a new *Aspergillus* species *A. karnatkaensis* along with some other related species[1]. This species had been earlier placed in the nidulans group by Raper and Fennell[2], with many of its perfect states assigned to the genus *Emericella*. However, this group was later assigned the formal subgenus 'Nidulantes' by Gams *et al.*[3] *Aspergillus* subgenus Nidulantes encompasses species of *Aspergillus* characterized by the production of biseriate condiophores, with pale to pigmented stipes; ascomata (if present) embedded in masses of hulle cells[3–5].

This species has been recovered from a high-altitude pass, Sapi La (La means pass), at 4000 masl in Kargil district, Ladakh (34.5539°N lat, 76.1349°E long), India. This high-altitude pass experiences strong wind currents round the year with an extremely dry and cool climate from April to October. However, from November to March, the whole region is covered with huge layers of snow and ice leading to chilling cold winters with temperature recorded as low as –35°C. This region along with the others parts of Ladakh remains cut-off from rest of the country for half of the year due to heavy snowfall during winter. The recovered isolate when assessed for its ability to produce extracellular enzyme, was found to produce a potential amount of protease and a lesser extent of cellulase and complete absence of lipase.

*For correspondence. (e-mail: skarmanunzom@yahoo.com)

Materials and methods

Sampling and isolation

Soil samples were collected aseptically from Sapi La pass in July (18°C) by scraping the superficial layer, not exceeding 3–5 cm in depth, with the help of properly sterilized spatula. They were brought to the laboratory in pre-sterilized polythene bags. For isolation, dilution pour plate method was adopted using modified Czapek Dox agar (CDA) supplemented with streptomycin sulphate (50 mg/1000 ml) and Rose Bengal (0.1 mg/100 ml).

Phenotypic characterization

Microscopic observations were made in lactophenol cotton blue and microscopic line drawings were made with the help of camera lucida (Erma, Japan) at 400× and 1000× magnification. Dimensions were determined for Hulle cells, hyphae, conidiophores, metulae (primary sterigmata), phialides (secondary sterigmata) and conidia with the help of an ocular micrometer. Microphotography of the lactophenol cotton blue mounted fungal cultures was done using a camera (Sony N50, Japan) attached to a binocular microscope (Olympus CH 20i, Japan). The recovered fungal isolate was identified by studying its cultural and morphological characters. For the purpose of identification, the recovered fungal species was grown and made to sporulate on different culture media, such as potato dextrose agar medium (PDA) and malt extract agar medium (MEA). The relevant literature and various keys used for identification of fungal species are those given in Raper and Fennell[2], Sappa[6] and Rai *et al.*[7].

Genotypic characterization

PCR amplification and sequencing of ITS region: The *Aspergillus* isolate identified on the basis of its cultural and morphological characters was further confirmed by molecular characterization (ITS sequencing), which was carried out at the sequencing facility of the National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune, India. Genomic DNA was isolated by the standard phenol/chloroform extraction method given by Sambrook *et al.*[8], followed by PCR amplification of

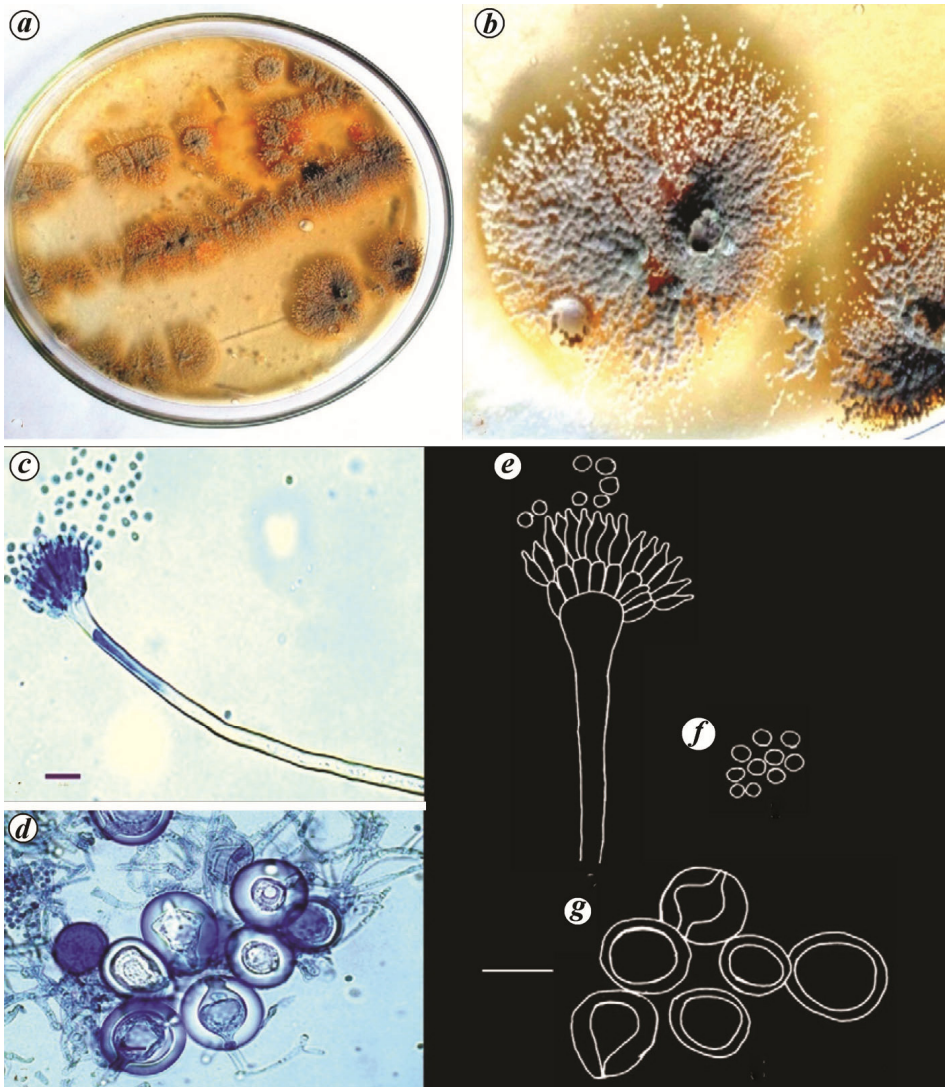

Figure 1. *Aspergillus aeneus*. ***a***, Colonies growing on malt extract agar. ***b***, Closer view of colony showing crustose appearance and fimbriate margins. ***c–d***, photomicrographs: ***c***, Conidial head with conidia. ***d***, Hulle cells (bar = 10 μm). ***e–g***, Camera lucida drawings of conidial head, conidia and Hulle cells (bar = 14 μm).

the ITS regions using universal primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). The amplified ITS PCR product was purified by PEG-NaCl precipitation and directly sequenced on an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., USA, CA) according to the manufacturer's instructions. Sequencing was carried out from both ends so that each position was read at least twice. Further assembly was carried out using Lasergene package followed by NCBI BLAST against sequences from type material for tentative identification[9].

The phylogenetic tree was constructed using maximum-likelihood method and Tamura–Nei model[10] implemented in the program MEGA X[11] with 1000 bootstrap replicates (Figure 2). Sequences were retrieved from GenBank based on their closest related species showing maximum identity. The consensus sequences were deposited at the National Centre for Biotechnology Information, GenBank under the accession number MW002556.

Enzymatic potential

Cellulase activity: Qualitative estimation of cellulase activity of *A. aeneus* was done following the method of Pointing[12], whereas quantitative estimation was done following the method of Miller *et al.*[13].

Protease activity: Qualitative estimation of protease activity of *A. aeneus* was done following the method of Hankin and Anagnostakis[14], whereas quantitative estimation was done following the method of Kembhavi *et al.*[15].

Lipase activity: Qualitative estimation of lipase activity of *A. aeneus* was done following the method of Cardenas *et al.*[16].

Results

Taxonomy

Aspergillus aeneus Sappa, Allionia 2: 84. 1954
Holotype MB292832
Habitat: Soil, forest soil, leaf litter of forest.
Distribution: Rare, only reported from Somalia (1954), India (1964) and Costa Rica (2000).
Current isolate examined: India, trans-Himalaya, Kargil district, Sapi mountains, isolated from a high altitude cold desert soil, July 2017.
Description: Based on Raper and Fennel (1965).
Description of cold desert isolate: Asexual form; sexual form–not observed.

Culture characteristics

Colonies on MEA grew rapidly attaining 25–35 mm diameter in 7 days at 28° ± 2°C, raised and radially wrinkled at the centre, becoming plane at margins, consisting of a dense crusty layer of Hulle cells and their supportive hyphae, zonate from the centre outward but more pronounced in marginal and sub-marginal areas with irregularly lobed and fimbriate margins; the entire colony surface later assuming a crustose appearance and consistency from the formation of abundant Hulle cells and becoming olive to deep olive buff (Figure 1 *a*, *b*, *d* and *g*).

Micromorphology

Conidial heads: Conidial heads borne directly from the substrate between masses of Hulle cells mostly 14–25 µm, radiate when young or sparsely produced but columnar in age or in areas of heavy conidial development; vesicles hemispherical, thin, fragile, fertile over the upper two-thirds, measuring 8.4–12.6 µm in diameter (Figure 1 *c* and *e*).

Sterigmata: In two series, crowded; metulae measuring 4.9–5.6 × 1.8–2.1 µm; phialides measuring 4.2–8.4 × 1.5–1.6 µm (Figure 1 *c* and *e*).

Conidia: Globose, deep yellow–green, conspicuously roughened, 2.5–2.8 µm in diameter (Figure 1 *c* and *e*).

Molecular identification

Blast analysis of the ITS region (700 bp) showed its closest similarity to the type material *A. aeneus*, accession no. MW002556 (GenBank: NR_13537.1; E-value 0; identity: 99.62% and coverage: 100%). Simultaneously, results were confirmed using UNITE and CBS databases. Phylogenetic analysis of the sequences of isolate SN56 based on combined sequences of 15 selected isolates of closest type strains confirmed that our isolate forms a strongly supported clade (98% bootstrap value) with *A. aeneus* (Figure 2). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 16 nucleotide sequences, including the present *A. aeneus* (Figure 2).

Substrate degradation

Aspergillus aeneus SN56 was tested for its ability to produce extracellular enzymes, viz. cellulase, protease and lipase. Among the three enzymatic activity tested, the cold desert isolate of *A. aeneus* showed the ability to degrade protein and slightly cellulose, but was unable to degrade lipid. As depicted in Figure 3, on qualitative estimation, this isolate showed more protease activity

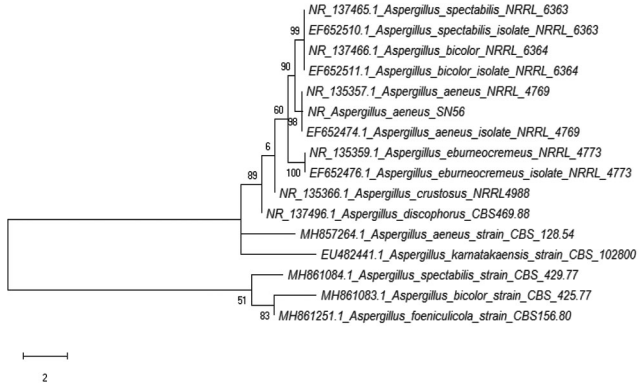

Figure 2. Phylogenetic tree based on maximum likelihood analysis of ITS data for *A. aeneus* (SN56) related to the isolate obtained in this study. Bootstrap values of >60% are indicated at the nodes. Scale bar is represents the number of substitutions per site.

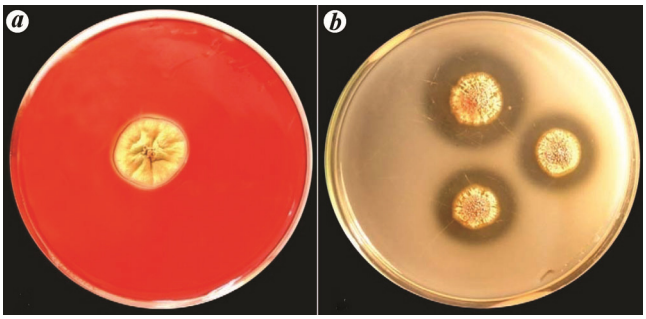

Figure 3. ***a***, Cellulase activity of *A. aeneus* grown on cellulose basal agar medium plated petri plates supplemented with carboxymethyl cellulose, hydrolysis of which resulted in a clear zone against red colour background of Congo red stain. ***b***, Protease activity on skimmed milk agar medium, clear zone around the colony indicates skimmed milk hydrolysis.

Table 1. Morphological characteristics of the Indian isolates of *Aspergillus aeneus* from tropical soil (Bansa, Madhya Pradesh) and cold desert soil (Kargil, Jammu & Kashmir)

	Phenotypic characters					
Habitat	Altitude (m msl)	Colonies on malt extract agar	Vesicle	Primary sterigmata	Secondary sterigmata	Conidia
Soil of river beds and leaf litter	306	Raised, radially wrinkled at the centre, crustose with dense sea-foam yellow crusty layer of Hulle cells zonate, irregularly lobed and fimbriate margins	Hemispherical, dome-like, upper half or two-thirdth fertile, 6.4–14.4 μm in diameter	4.8–6.4 × 2.4 μm	4.8–8.0 × 1.6 μm	Subglobose to slightly elliptical, smooth, 2.4–3.2 μm
Cold desert soil	4000	Circular, deeply radially wrinkled, crateriform, with abundant sea-foam yellow clusters of Hulle cells superficially resembling cleistothecia	Hemispherical, fertile over the upper two-thirds, 8.4–12.6 μm in diameter	4.9–5.6 × 1.8–2.1 μm	4.2–8.4 × 1.5–1.6 μm	Globose, slightly roughened, 2.5–2.8 μm

with a clearance zone of 8 mm in diameter and relatively less cellulase activity with a clearance zone of only 1 mm. However, it showed negative response for lipid substrate. Quantitative estimation of protease and cellulase revealed protease activity of 1.263 ± 0.43 μmol/ml/min and cellulase activity of 0.037 ± 0.21 μmol/ml/min respectively.

Discussion

During the present study, 39 fungal isolates were recovered from a barren, high-altitude pass while exploring the diversity of psychrotolerant fungi of some unexplored regions of Kargil district. One of the isolates which was identified and described on the basis of morphology combined with molecular data, represents a microfungi of rare occurrence and distribution. This species of *Aspergillus* was originally isolated and described by Sappa[6] in 1954 from forest soils of Somalia. The same species was later reported after a decade by Rai *et al.*[7] in 1964 from India, which was isolated from leaf litter and soil samples of Machrar river bed in Bansa, Madhya Pradesh. Since then, there were no reports on the occurrence of this species until 2000, when Polishook *et al.*[17] reported the occurrence of *A. aeneus* on soil and litter fragments of forest soil. No reports on its occurrence are available thereafter. However, in 2010, a novel *Aspergillus* species, *A. karnatakaensis* was reported from a tropical soil. This species was found to be conspecific and closely related to *A. aeneus*[1], and both these species along with some other species were placed in a new section Aeni.

Incidentally, Costa Rica and Sapi La (the present study area) shared another rare fungus *Geosmithiar rufescens*, although from two markedly contrasting habitats, i.e. ambrosia beetles[18] and high altitude cold desert soil[19]. Nevertheless, no conclusion can be drawn from this fact and in the present study also this species shows occurrence in two different habitats, but common region.

From the reports of its incidence, it is unambiguous that this fungal species apparently prefers tropical soils with ample moisture and nutrients, as in two of the cases it is associated with river beds and with leaf litter in all the cases. However, in contrast and surprisingly, this isolate of *A. aeneus* has been reported from an extreme oligotrophic, high-altitude, cold habitat indicating its ability to survive not only in favourable habitats, but also in those that are considered to be detrimental for the survival of numerous microfungi. Globally, there are no reports of this fungal isolate from an extreme environment and therefore it is a novel addition to extreme desert fungi.

As discussed earlier, from India *A. aeneus* has been reported as early as in 1964 inhabiting tropical soils and leaf litter, in contrast to the current cold desert isolate. It has been rediscovered in the present study after a huge gap of 56 years. This vast gap recommends the need for extensive studies and exploration of diverse unexplored habitats, as many factors which are considered hostile for the existence of living forms are not actually constraints for the survival of microfungi, as observed during the present study. The extensive efforts that have been made worldwide to unravel the diversity and distribution of microbes from extreme environments have revealed that what we are aware of today is mere a tip of the iceberg. More efficient attempts and extensive studies are needed to explore the range of unexplored extreme habitats to reveal more rare and novel fungal species of importance.

On comparing the morphological features with the other reported isolates, the isolate SN56 showed more similarities with the Indian isolate; for example, colony texture, colour, microscopic features such as dimensions of vesicle, primary and secondary sterigmata and conidia (Table 1).

The capability of producing cellulase and protease might be one of the strategies to surmount the various extreme conditions prevailing in the area. Although these enzymes were not produced in considerable concentrations compared to their mesophilic counterparts, it is evident that it might be the conditions in the area that are curbing the various metabolic processes as the soil usually remains covered under snow for maximum time, and even when the snow melts during summer, the temperature of the soil remains low. The simultaneous limiting factors include constant strong wind currents, aridity, oligotropic conditions, and so on. These factors might have led to prolonged dormancy of the described *Aspergillus* species, which could have facilitated their survival in such extreme environments for several years. Since the study area is completely barren, the greater protease activity could be attributed to soil-harbouring protein-associated substrate than cellulose or lipid. For successful surmounting of the negative effects, the diverse microfungi thriving in such extreme conditions might have evolved a range of structural and functional adaptations. One such adaptive survival strategy could be the secretion of these cold active enzymes (although in small amounts) that might have permitted their prevalence and endurance in such extreme environments.

Conflicts of interest: The authors declare that they have no conflicts of interest.

1. Varga, J., Frisvad, J. C. and Samson, R. A., *Aspergillus* sect. Aenei sect. nov., a new section of the genus for *A. karnatakaensis* sp. nov. and some allied fungi. *IMA Fungus*, 2010, **1**, 197–205.
2. Raper, K. B. and Fennell, D. I., *The Genus Aspergillus*, The Williams and Wilkins Co, Baltimore, USA, 1965, p. 686.
3. Gams, W., Christensen, M., Onions, A. H. S., Pitt, J. I. and Samson, R. A., Infrageneric taxa of *Aspergillus*. In *Advances in Penicillium and Aspergillus systematics* (eds Samson, R. A. and Pitt, J. I.), NATO ASI Series A: Life Sciences, Plenum Press, New York, 1985, vol. 102, pp. 55–62.
4. Frisvad, J. C. and Samson, R. A., *Emericella venezuelensis*, a new species with stellate ascospores producing sterigmatocystin and aflatoxin B1. *Syst. Appl. Microbiol*., 2004, **27**, 672–680.
5. Chen, A. J., Frisvad, J. C. and Sun, B. D., *Aspergillus* section Nidulantes (formerly *Emericella*): polyphasic taxonomy, chemistry and biology. *Stud. Mycol*., 2016, **84**, 1–118.
6. Sappa, F., Nuove specie di *Aspergillus* deiterreni forestall Somali, Allionia, Bouettino dell' Istituto ed Orto Botanieo dell' UniversitS di Torino, 1954, **2**, 79–95.
7. Rai, J. N., Tewari, J. P. and Murekji, K. G., Cultural and taxonomic studies on two rare species of *Aspergillus – A. paradoxus* and *A. aeneus*, and an interesting strain of *A. variecolor* from Indian soils. *Mycopathol. Mycol. Appl*., 1964, **24**, 369–376.
8. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning a Laboratory Manual*, Cold Spring Harbor Laboratory Press, NY, USA, 1989, pp. 1–1626.
9. Boratyn, G. M. *et al.*, BLAST: a more efficient report with usability improvements. *Nucleic Acids Res*., 2013, **41**, 29–33.
10. Tamura, K. and Nei, M., Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol*., 1993, **10**, 512–526.
11. Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K., MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol*., 2018, **35**, 1547–1549.
12. Pointing, S. B., Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi. *Fungal Divers*., 1999, **2**, 17–33.
13. Miller, G. L., Blum, R., Glennon, W. E. and Burton, A. L., Measurement of carboxymethyl cellulase activity. *Anal. Biochem*., 1960, **2**, 127–132.
14. Hankin, L. and Anagnostakis, S. L., The use of solid media for detection of enzyme production by fungi. *Mycologia*, 1975, **67**, 597–607.
15. Kembhavi, A. A., Kulharni, A. and Pant, A., Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM No 64. *Appl. Biochem*., 1993, **38**, 83–92.
16. Cardenas, J. *et al.*, Screening and catalytic activity in organic synthesis of novel fungal and yeast lipase. *J. Mol. Catal B*, 2001, **14**, 111–123.
17. Polishook, J. D., Pelaez, F., Platas, G., Asensio, F. J. and Bills, G. F., Observations on *Aspergilli* in Santa Rosa National Park, Costa Rica. *Fungal Divers*., 2000, **4**, 81–100.
18. Kolarik, M. and Kirkendall, L. R., Evidence for a new lineage of primary ambrosia fungi in *Geosmithia* Pitt (Ascomycota: Hypocreales). *Fungal Biol*., 2010, **114**, 676–689.
19. Nonzom, S. and Sumbali, G., New record of *Geosmithiar rufescens* from a high altitude pass in the trans-Himalayan region. *Österr. Z. Pilzk.*, 2018, **27**, 1–4.

ACKNOWLEDGEMENTS. The first author thanks the University Grants Commission (UGC), New Delhi for financial assistance through the Rajiv Gandhi National Fellowship, which facilitated the study. We also thank UGC special assistance programme DRS-II for providing laboratory facilities.

Received 9 September 2019; revised accepted 1 October 2020

doi: 10.18520/cs/v120/i3/514-518