Fungal diversity inside caves of Southern India

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Thirty-five species of sporulating mesophilous fungi and seven types of non-sporulating fungi were isolated from the soil samples collected at the entrance, twilight and dark zones of six caves. Of these, 27 species belong to Deuteromycetes, 8 species belong to Zygomycetes and one species belongs to Ascomycetes. The genus *Aspergillus* and *Penicillium* were present in large numbers in all the three zones. Fungi like *Aspergillus flavus, Penicillium cyclopium* and *Chaetomium* sp. were predominantly available from all the samples in all the six caves. Ten species of fungi such as *A. chevalieri, A. versicolor, A. sydowi, Aspergillus* sp., *Curvularia brachyspora, Fusarium* sp., *Geotrichum candidum, P. fellutanum, Mucor* sp. and *Rhizopus stolonifer* were isolated from light available zones. The number of species of fungi and the number of propagules/gram of soil obtained from the entrance zone soil sample were significantly more compared to that of the twilight and dark zone soil samples of the caves. The fungi, *Cunninghamella echinata* was isolated only from the dark zone of caves 2 and 5, which were occupied exclusively by a colony of carnivorous bat *Megaderma lyra*. Insectivorous bats such as *Hipposideros speoris, H. fulvus* and *Rhinopoma hardwickei* occupied other areas. *Syncephalis* sp., a rare fungus, has been isolated only from the dark zone of a cave.

CAVES *are divided into different zones based on the* prevailing light and temperature[1]. According to Poulson and White[2], each cave has three zones: (i) twilight zone, which is located at the entrance area; (ii) middle zone, in which relative darkness prevails with fluctuating temperature; and (iii) dark zone, in which total darkness and constant temperature prevails[3].

Many sets of organisms live inside caves; generally cave life is divided into three groups[2,4]. The organisms that live inside the cave but come out periodically everyday for feeding are known as *trogloxenes* (e.g. bats). The second group of animals live their entire lives inside the cave but their conspecifics found outside the *cave are also named as troglophiles* (e.g. cockroaches). The third group is the peculiar and most specialized for the cave environment, especially to the dark zone and are not found elsewhere. They are known as *troglobites* (e.g. cave millipede, *Glyphiulus cavernicolus*). The constancy of temperature, humidity and darkness[5] might be the favourable factors for cave life. Another interesting feature of the cave environment is the absence of green plants deep inside caves[3], due to lack of sunlight.

Fungi are remarkable for their antiquity, diversity, ubiquitous distribution and longevity[6]. Fungi are known to occur in almost all environments including soils[7–9], among seeds[10], marshy plants[11–13], plants in lakes[14], sewage and polluted waters[15], peat soils[16] and also in caves[17,18].

Ingold[19] has stated that the majority of fungi known to develop in culture are mesophiles growing between 5° and 37°C. Apparently no report is available on fungi from caves in India. An attempt was made to isolate and culture the mesophilic fungi from soil samples collected at different zones from six caves in South India.

We collected soil samples from six different caves: (1) Samanar cave, (2) Pannian cave, (3) KKB cave I, (4) KKB cave II, (5) Ramanathapuram cave and (6) Veerasihamani cave. The Samanar cave is towards the south-east, whereas Pannian cave, KKB cave I and KKB cave II are towards the north-west and all are at a distance of about 10 km from the Madurai Kamaraj University campus (9°58'N, 78°10'E). The Ramanathapuram cave and Veerasihamani cave are located at a distance of about 140 km from Tirunelveli (8°44'N, 77°42'E) towards north. The temperature and humidity were recorded inside the first four caves continuously for a period of one year[5,20,21]. The physical factors such as temperature, relative humidity and light intensity fluctuate at the entrance and twilight zones of the caves. At the dark region, the temperature and humidity showed relatively constant values at 27°C and 95%, respectively[3]. We arbitrarily divided the cave into three zones in terms of the prevailing light, temperature and humidity: *the entrance zone near the cave mouth into which* the environmental light penetrates with varying temperature and humidity, the twilight zone in which diffused light is available and the dark zone, characterized by constant temperature and humidity with mysterious total darkness[3]. Cave 3 does not have a dark zone since the environmental light can be perceived even from the deeper area. The cave soil samples (bat guano) were collected from the entrance, twilight and dark zones of caves between September 1997 and August 1998. They were screened for fungi using soil dilution plating method[22]. *Czapek-Dox* agar medium (pH 4.5) was used for isolating mesophilous fungi. Six replicates were prepared for each sample. A total of 102 plates were prepared. Petri dishes were incubated at 25 ± 1°C for one

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Table 1. Quantitative estimation of the number of propagules/gram of dry soil ($\times 10^3$) of entrance, twilight and dark zones in six caves

Fungi	Entrance zone						Twilight zone						Dark zone				
	C_1	C_2	C_3	C_4	C_5	C_6	C_1	C_2	C_3	C_4	C_5	C_6	C_1	C_2	C_4	C_5	C_6
Deuteromycetes																	
Aspergillus japanicus	3	2	1	7	–	4	3	1	2	–	2	–	2	3	3	–	2
A. flavus	19	34	3	5	2	21	8	27	2	4	4	19	1	2	3	–	5
A. versicolor	–	11	–	–	5	–	–	7	–	–	3	–	–	–	–	–	–
A. tamari	2	7	2	6	–	11	3	5	–	–	–	6	1	2	3	–	5
A. sydowii	7	9	–	–	–	10	2	5	–	–	–	3	–	–	–	–	–
A. chevalieri	4	–	–	–	–	7	–	–	–	–	–	–	–	–	–	–	–
A. ochraceous	1	–	–	4	–	–	2	–	–	–	–	–	–	–	–	–	2
A. niger	11	22	–	18	–	6	8	16	3	10	–	6	5	14	8	–	2
A. parasiticus	2	21	3	5	–	9	6	10	1	2	–	–	4	4	1	–	5
A. fumigatus	21	9	1	9	–	14	16	4	1	2	–	6	16	14	1	–	6
A. terreus	5	2	–	–	–	–	5	3	–	–	–	–	2	2	–	–	–
A. wenti	–	–	–	–	–	–	–	–	–	–	–	–	4	–	–	–	–
Aspergillus sp.	14	–	–	–	–	–	–	3	–	–	2	2	–	–	–	–	–
Chrysosporium sp.	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	1	1
Curvularia brachyspora	7	9	–	11	5	–	7	11	6	–	5	–	–	–	–	–	–
Dactylella sp.	6	9	–	4	–	11	–	–	–	–	–	–	–	–	–	–	4
Fusarium sp.	4	2	1	7	–	14	12	22	–	–	–	–	6	3	4	–	10
Geotrichum candidum	–	2	–	–	6	–	–	–	–	–	4	–	–	–	–	–	–
Humicola sp.	2	3	–	5	–	–	–	–	–	–	–	–	–	–	–	7	5
Mycogone sp.	1	2	6	9	–	14	2	2	7	11	–	16	3	4	9	–	19
Penicillium cyclopium	19	11	2	8	9	10	15	8	2	7	5	9	3	5	2	3	4
P. fellutanum	–	4	–	–	6	7	–	–	–	–	7	–	–	–	–	–	–
P. citreonigrum	12	7	4	2	–	6	–	5	–	–	–	–	1	4	2	–	4
Penicillium sp.	5	14	–	1	5	11	4	–	–	–	4	9	–	–	–	–	4
Sepedonium sp.	6	–	–	–	7	–	–	–	–	–	15	–	–	–	–	–	6
Trichoderma viride	–	–	–	–	–	6	–	–	–	–	–	–	–	1	–	–	–
Trichophyton sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	1
Zygomycetes																	
Absidia corymbifera	–	–	19	6	–	–	–	–	16	–	–	–	–	–	14	–	–
Absidia sp.	–	–	–	–	–	–	–	–	–	12	–	–	14	–	–	–	–
Cunninghamella echinata	–	–	–	–	–	–	–	–	–	–	–	–	–	6	–	4	–
Mucor sp.	–	–	–	9	–	–	–	–	14	–	–	–	–	–	–	–	–
Rhizopus stolonifer	12	15	–	–	–	6	14	19	6	–	4	3	–	–	–	–	–
Rhizopus sp.	7	–	–	–	12	–	10	–	–	–	–	–	16	–	–	–	–
Syncephalis sp.	–	–	–	–	–	–	–	–	–	–	–	–	–	12	–	–	–
Ascomycetes																	
Chaetomium sp.	5	16	5	9	14	18	8	21	8	9	5	19	12	29	10	14	21
Non-sporulating fungus																	
A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	5	11
B	–	–	–	–	–	–	–	–	–	–	–	–	14	–	23	2	–
C	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	29
D	–	–	–	–	–	–	–	–	–	–	–	–	–	25	–	22	7
E	–	–	–	–	–	–	–	–	–	–	–	–	6	2	16	9	–
F	–	–	–	–	–	–	–	–	–	–	–	–	3	–	27	9	–
G	–	–	–	–	–	–	–	–	–	–	–	–	4	17	–	–	–

– Indicates absence of fungi; C_1 represents cave 1; C_2 represents cave 2; and so on.

week and the mycoflora were observed under microscope. The data were quantified to obtain the relative numbers of colony forming units (cfu) of different species of sporulating and non-sporulating fungi per gram of dry cave soil from various zones. The number of propagules of fungi was calculated by the following formula[23]

$$\frac{\text{Average number of colonies per plate}}{\text{Weight of the soil}} \times \text{Dilution fact}$$

A total of 35 species of sporulating fungi belonging t 18 genera and seven species of non-sporulating fun was isolated from the soil samples of six caves. Table shows the distribution of all the species in differe

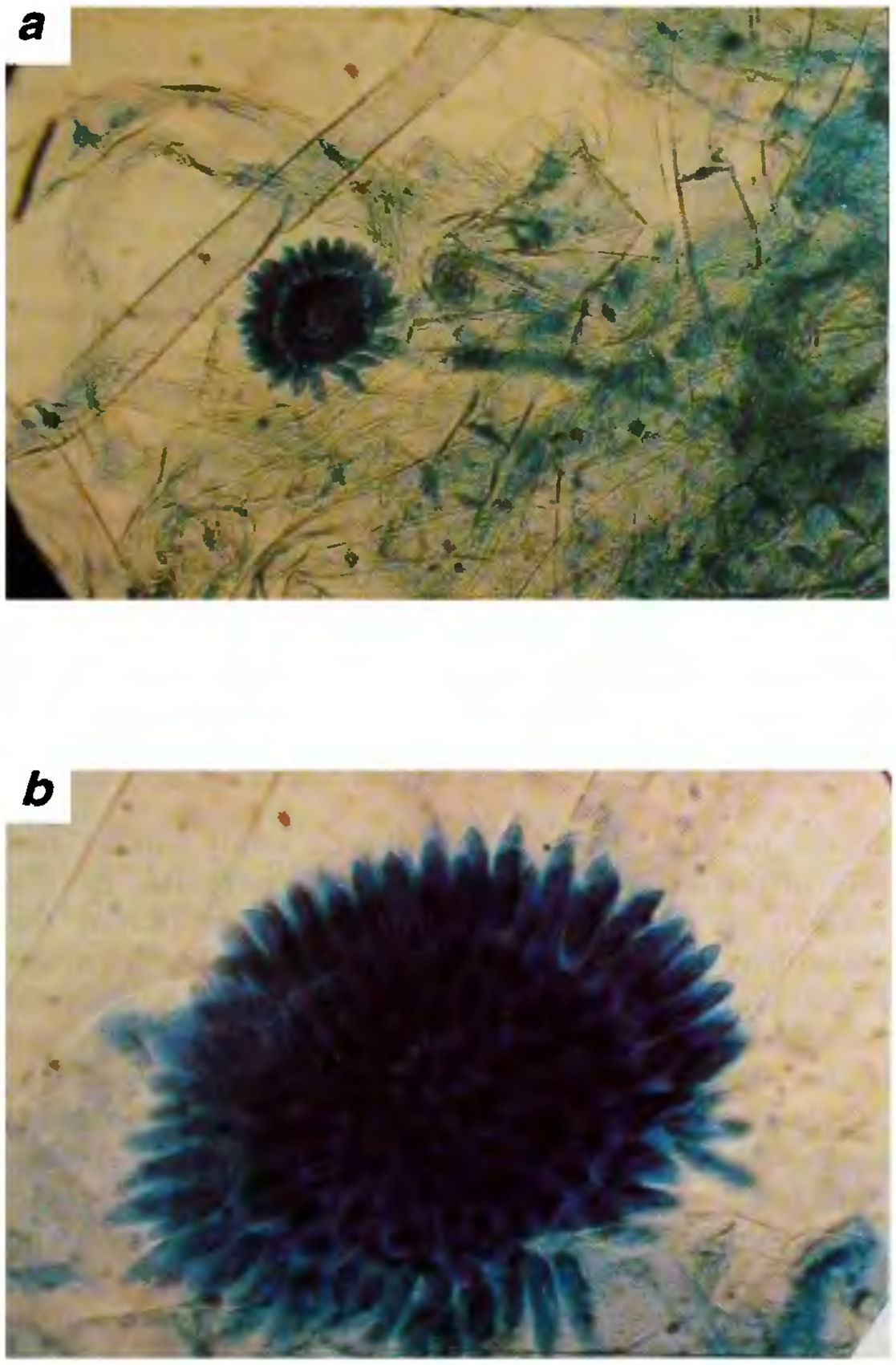

Figure 1. Light photomicrographs of a sporulating fungus *Syncephalis* sp. at *a*, × 40; *b*, × 100.

zones of the caves as indicated cfu. Among them, the fungi belonging to Deuteromycetes were more than those belonging to Zygomycetes and Ascomycetes. The genus *Aspergillus* was represented by 13 species, *Penicillium* represented by 4 species, *Absidia* and *Rhizopus* represented each by 2 species and *Chrysosporium, Curvularia, Dactylella, Fusarium, Geotrichum, Humicola, Mycogone, Sepedonium, Trichoderma, Trichophyton, Cunninghamella, Mucor, Syncephalis* and *Chaetomium* were represented each with a single species. The fungus *A. chevalieri, A. versicolor, A. sydowi, Aspergillus* sp., *C. brachyspora, Fusarium* sp., *G. candidum, P. cyclopium, Mucor* sp. and *R. stolonifer* were isolated only from light available areas (entrance and twilight zones). Four species of fungi, *A. wenti, Chrysosporium, Trichophyton* sp. and *Syncephalis* sp. (Figure 1 *a* and *b*) were isolated only from dark zone samples. The fungi like *A. flavus, P. cyclopium* and *Chaetomium* sp. were isolated invariably from all the samples in all the six caves.

The distribution of number of colonies of species of the predominantly occurring genera *Aspergillus* and *Penicillium* decreased from the entrance towards the interior, whereas, there was an increase in the number of colonies *Mycogone, Sepedonium, Absidia, Rhizopus, Mucor* and *Chaetomium* towards the interior. One way completely randomized ANOVA shows that the number of species found in the entrance zone of all the caves was significantly higher than the twilight and dark zones ($F_{2,645} = 6.18$, $P < 0.005$). The fungus *Cunninghamella echinata* was isolated in the dark zone of caves 2 and 5, occupied by a colony of carnivorous bat *Megaderma lyra*. Insectivorous bats, *Hipposideros speoris, H. fulvus* and *Rhinopoma hardwickei* occupied other areas. All seven different types of non-sporulated fungi were isolated only from dark zone samples of the caves.

The results of the present study provide the comparative analysis of the diversity of fungal species as well as propagules available at the entrance, twilight and dark zones of different caves. Natural agents such as flood and air introduce fungal spores and mycelium into the cave environment[24]. The spores might also enter into the cave through organic substances such as plant and animal remains which are carried into the cave by trogloxenes[4,25,26]. The spores that enter into a cave begin to grow on suitable substrate like remnants of insects[17] and bat guano[27]. The total number of colonies of fungi/gram cave soil at the entrance zone was presumably higher when compared to twilight and dark zones. The entrance and twilight zones accommodated more number of fungal species. This may be due to the higher temperature, lower relative humidity and the availability of light. In addition to physical parameters, the guano of carnivorous and insectivorous bats also plays an important role in the occurrence of specific fungal species at different zones of the caves. It was clearly pointed out that many of the fungi recorded from caves would eventually be found in the epigean domain also because their substrates may not restrict them to the hypogean domain[28]. Fungi form a component of the food chain that prevails in the cave ecosystem. They depend on the trogloxenes and troglophiles for food and are in turn eaten by other organisms[29]. Some invertebrates like springtails[30] and glow worm larvae feed largely on fungus[4]. Seven species of non-sporulated fungi that were isolated from the samples collected only at the dark zone substantiate the essential role of light on sporulation. The constancy of temperature and humidity in addition to darkness might presumably be conducive for the growth of fungi.

Five species of *Syncephalis* are reported from different habitats in India[31–34]. In the present study *Syncephalis* sp. was isolated for the first time from the dark area of a cave. According to an earlier report, the

growth from spores was disrupted at less than 90% relative humidity and germination occurred readily in total darkness than under normal conditions[35]. The reason for the isolation of well-documented, highly sporulating species in the present study may be due to the isolation technique used, viz. the dilution plate technique. There is a possibility that many slow-growing fungi belonging to Basidiomycetes might have been eliminated by using this technique.

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Evaluation of two *in vitro* test systems employing *Brugia malayi* parasite for prescreening of potential antifilarials

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In the present study, we employed adult worms and microfilariae of the human filarial parasite, *Brugia malayi*, in two *in vitro* systems and evaluated the suitability of the systems as prescreens for identifying potential antifilarials. A total of 38 new synthetic compounds and 19 plant products that were found active or inactive in *Acanthocheilonema viteae–Mastomys coucha* (rodent filariid in rodent host) and/or *B. malayi–M. coucha* (human filariid in rodent host) models, were tested in the *in vitro* systems using inhibition of worm motility (motility assay) and inhibition of MTT reduction potential (MTT assay) of the parasite as test parameters. Two known antifilarials, ivermectin and diethylcarbamazine, were included as standards. All (100%) the synthetic and plant products that were active in *B. malayi–M. coucha* model were also found active in the *in vitro* systems: About 82% and 20% of synthetic and plant products respectively that were active in *A. viteae–M. coucha* system were positive and 87.5% of the synthetic products and 9% of the plant products found inactive in *A. viteae–M. coucha* system were also positive in the *in vitro* systems. The results show that plant products showing LC_{100} in the range of 31.25 μg/ml to 62.5 μg/ml in the *in vitro* systems can be considered as potential antifilarials and followed-up in *in vivo* assay systems. It is concluded that the motility and MTT assays using both the life forms of *B. malayi* are reliable prescreens with high predictive value; both the assays are necessary for screening synthetic compounds whereas the motility assay using adult worms alone appears sufficient for screening plant products.

IN our antifilarial drug development programme, animal models of rodent and human filarial parasites (*Acanthocheilonema viteae* in *Mastomys coucha* and

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