ISHAM INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2016, 54, 567–575
doi: 10.1093/mmy/myw014
Advance Access Publication Date: 26 April 2016
Original Article

Original Article

The environmental source of emerging *Apophysomyces variabilis* infection in India

Hariprasath Prakash, Anup Kumar Ghosh, Shivaprakash Mandya Rudramurthy, Raees Ahmad Paul, Sunita Gupta, Vishwanand Negi and Arunaloke Chakrabarti*

Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

*To whom correspondence should be addressed: Dr. Arunaloke Chakrabarti, Professor and Head, Department of Medical Microbiology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India. Tel: +91 172 2747990; Fax: +91 172 2744401; E-mail: arunaloke@hotmail.com

Received 3 November 2015; Revised 2 February 2016; Accepted 15 February 2016

Abstract

The rare mucoraceous fungus, *Apophysomyces* species complex ranks second after *Rhizopus arrhizus* causing mucormycosis in India. The source of this agent in the environment is not clearly known. We conducted an environmental study to find its presence in Indian soil. The soil samples from different geographical locations were analyzed for isolation of *Mucorales*. *Rhizopus arrhizus* (24.6%) was most commonly isolated from soil, followed by *Lichtheimia* spp. (23.2%), *Cunninghamella* spp. (21.7%), *Rhizopus microsporus* (14%) and *Apophysomyces* spp. (4.5%). The isolation of *Apophysomyces* species complex was significantly associated with low nitrogen content of the soil. Based on sequencing of internal transcribed spacer (ITS) and 28S (D1/D2) regions of ribosomal DNA, the *Apophysomyces* isolates were identified as *Apophysomyces variabilis* with 98 to 100% similarity to type strain *A. variabilis* (CBS658.93). The analysis of amplified fragment length polymorphism (AFLP) fingerprinting data demonstrated genomic diversity of *A. variabilis* isolates with multiple clades (similarity 40–90%). The minimum inhibitory concentrations (MIC), MIC_{50} and MIC_{90} for *A. variabilis* isolates were 1 and 4 μg/ml for amphotericin B, 0.25 and 0.5 μg/ml for itraconazole, 0.125 and 0.25 μg/ml for posaconazole, 0.06 and 0.12 μg/ml for terbinafine, respectively. The present study revealed abundant presence of *A. variabilis* in Indian soil with low nitrogen content, its genetic heterogeneity and relatively high MICs for amphotericin B.

Key words: *Mucorales*, *Apophysomyces variabilis*, ecology, molecular typing, AFLP, antifungal susceptibility testing, amphotericin B.

Introduction

Fungi under the order *Mucorales* cause serious angio invasive infections with high morbidity and mortality. *Rhizopus*, *Lichtheimia*, and *Mucor* species are commonly isolated from patients with mucormycosis across the world. Whereas, isolation of *Cunninghamella*, *Syncephalastrum*,

Apophysomyces, *Saksenaea* species are relatively rare.[1,2] In India, the prevalence of mucormycosis appears to be disproportionately high in patients with uncontrolled diabetes compared to Europe and United States.[1,3,4] Contrary to other studies, *Apophysomyces* species complex is the second most common agent causing mucormycosis in India and accounts for nearly 60% of the reported cases worldwide.[2,5,6] The reason of this high isolation rate and the specific environmental source of *Apophysomyces* species complex in India are not known. In 1979, *Apophysomyces elegans* was isolated from soil of a mango orchard in India.[7] Following that, there is no further report on the environmental isolation of *Apophysomyces* species complex from India. *Apophysomyces elegans* had been isolated once from the soil in Australia.[8] The fungus was recently isolated from the environment of semiarid regions of Brazil.[9] Other than these occasional reports, the ecological niche of this fungus is not clearly delineated.

The taxonomy of the genus *Apophysomyces* is also evolving. Presently, the fungi under this genus are considered as *Apophysomyces elegans* species complex comprising *A. elegans*, *A. variabilis, A. ossiformis*, *A. trapeziformis*, *and A. mexicanus*.[7,10,11] These species are differentiated on the basis of variation in the morphological characters, biochemical properties and molecular characterisation. DNA sequences of ITS, 28S, and histone 3 genes help in the differentiation of various species described. It is also believed that majority of human cases in India is due to *A. variabilis*.[5,12] The present study was conducted to isolate *Apophysomyces* species complex from Indian soil and to perform the molecular characterisation of those isolates. In addition, we performed antifungal susceptibility testing of the isolates, as our earlier study reported high minimum inhibitory concentration (MICs) for clinical isolates to amphotericin B.[13]

Materials and methods

Environmental isolation of *Apophysomyces* species

A total of 2188 soil samples were collected from four provinces: Haryana, Punjab, Himachal Pradesh (North India) and Tamilnadu (South India). Soil samples were collected during rainy (200 samples), winter (147 samples) and summer (1841 samples) seasons. The soil samples from both agricultural and non-agricultural lands were collected by scooping soil from the surface into sterile zipper bags. For selective isolation of *Mucorales,* around 5 g of soil was mixed with 20 ml of dichloran rose-bengal chloramphenicol (DRBC) agar (Himedia, Mumbai, India) supplemented with benomyl to a final concentration of 10 μg/ml.[10,14,15] Plates were incubated at 37°C for 16–24 hours. Any cottony growth was sub-cultured on potato dextrose agar (PDA) to obtain pure culture. The isolates were presumptively identified on the basis of macroscopic and microscopic morphology.[16]

Phenotypic Characterisation of *Apophysomyces* species

For phenotypic characterisation of *Apophysomyces* species complex, isolates were sub cultured on DRBC agar with benomyl, water agar, and PDA plates and incubated at 37°C for 2 to 7 days to induce sporulation.[10,16] The isolates were also evaluated for growth at different temperature (25, 37 and 42°C), pH (4 to 9) and salt concentration (0.5%, 1%, and 1.5%). An attempt was made to induce sexual stage of *Apophysomyces* species by mating different combination of isolates for 45 days.[10]

Molecular identification of *Apophysomyces* species

The morphologically identified *Apophysomyces* isolates were subjected to molecular identification by DNA sequencing of internal transcribed spacer region (ITS) and large ribosomal subunit DNA (28S) genes.[17–19] The DNA was extracted by phenol: chloroform: isoamyl alcohol method.[20] The ITS and 28S genes were amplified by polymerase chain reaction (PCR) using the primers; NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGT CCGTGTTTCAAGACGG-3′) for 28S rDNA, ITS-5(5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC 3′) for ITS region. The PCR was performed using ~5 to 10 ng/μl genomic DNA template in 20 μl reactions. Taq DNA polymerase (0.5 unit), 0.2 mM deoxynucleotide triphosphates, and 0.2 mM of each primer were added for each reaction. The amplification protocol included initial denaturation at 94°C for 7 mins followed by 35 cycles at 94°C for 1min, 52°C for 1 min and 72°C for 2 min. Sequencing PCR was carried out using the both forward and reverse primers separately, the amplified products were analysed using ABI Prism 3130 automated DNA analyser (Applied Biosystems, California, USA). The consensus sequences were obtained using BIONUMERICS v6.6 software (Applied Maths, Ghent, Belgium). To identify the fungus, the sequences were analysed using the NCBI BLAST search (http://blast.ncbi.nlm.nih.gov/blast).

Phylogenetic analysis of *Apophysomyces variabilis*

The 28S rDNA and ITS sequences of different *Apophysomyces* species were retrieved from NCBI nucleotide database (Table S1). The DNA sequences of our

Table 1. The isolation of different species of *Mucorales* from soil.

Organism	Number	Percentage	Cochran's Q	*P* value
Rhizopus arrhizus	538	24.6	2282.759	<.0001
Lichtheimia spp.	507	23.2		
Cunninghamella spp.	474	21.7		
Rhizopus microsporus	307	14		
Apophysomyces variabilis	99	4.5		
Rhizomucor spp.	32	1.5		
Mucor spp.	33	1.5		
Rhizopus homothallicus	24	1.1		
Other *Rhizopus* spp.	21	1		
Syncephalastrum spp.	7	0.3		

'*p*' valve < 0.05 were considered statistically significant.

isolates and those retrieved from GenBank were aligned using multiple sequence alignment mode in ClustalX2 software. The sequence alignments were exported to Molecular Evolutionary Genetics Analysis software version 6 (MEGA 6). The phylogenetic analysis was performed using the neighbor-joining method with Kimura 2 parameter model. The phylogenetic tree was constructed by comparing 1000 bootstrap replications.[10,18,19] *R. oryzae* was used as an outgroup isolate. The constructed phylogenetic tree was further analysed using maximum parsimony analysis to determine the consistency, retention, and composite index.[10]

Amplified fragment length polymorphism analysis of *Apophysomyces variabilis*

Molecular typing of *A. variabilis* isolates was performed by AFLP as previously described with modifications.[13,17] For standardization, duplicate samples of ten randomly selected isolates were used to check the reproducibility of this technique. Though the number and the size of the amplified bands varied, the clustering patterns were similar. The protocol in brief, ~50 ng of genomic DNA was subjected to combined restriction-ligation procedure containing 5 U each of EcoRI and HindIII (New England Biolabs), 50 pmol each of EcoRI and HindIII adapter (Sigma-Aldrich) and 1 U of T4 DNA ligase (Fermentas) in 20 μl of reaction mixture and incubated at 37°C for 2 hr. The pre-selective amplification was performed by using pre-selective primers of 10 μM EcoRI primer (5′- GACTGCGTACCAATTC-3′), 10 μM HindIII primer (5′- GACTGCGTACCA GCTT -3′). For the selective amplification, primers labelled with 6-carboxyfluroscein (6-FAM), 10 μM HindIII primer with one selective residue (5′-GACTGCGTACCAGCTTT-3′) and 10 μM EcoRI primer with two selective residues were used (5′-GACTGCGTACCAATTCAC -3′). The fingerprint curves were converted into bands and correct band of each lane was assigned using the band position of the reference dye (LIZ500). The fingerprint data were analyzed in BioNumerics v6.6 software (Applied Maths, Ghent, Belgium) using Pearson correlation coefficient and clustering by UPGMA (Unweighted Pair Group Method with Arithmetic Mean).

Antifungal Susceptibility testing of *Apophysomyces variabilis*

The *in vitro* antifungal susceptibility testing was performed for 99 *A. variabilis* isolates against four antifungal agents including amphotericin B, itraconazole, posaconazole and terbinafine by micro broth dilution method of CLSI.[21] Briefly, inoculum was prepared by harvesting spores in 0.85% sterile normal saline and the spore concentrations were adjusted spectrophotometrically at a 530-nm to the optical density of 0.15–0.17, equivalent to spore count of 0.2×10^6 to 2.5×10^6 cfu/ml. The drug dilutions were prepared from stock concentration of 16 mg/l. The MIC was defined as complete inhibition in the range of 0.03 μg/ml to 16 μg/ml after 24 hours of incubation in comparison to the growth control. *Candida krusei* (ATCC 6258) and *Aspergillus flavus* (ATCC 204304) were used as quality control strains. The MIC_{50} and MIC_{90} were determined by selecting the median and 90th percentile, respectively.[13,17]

Soil chemistry

The data on soil chemistry including pH, organic carbon, nitrogen, phosphorus, potassium, and micronutrients like zinc, copper, iron, and manganese of the study areas were tested at Indian Institute of Soil Sciences, Bhopal. The already available data from the areas where we collected soil

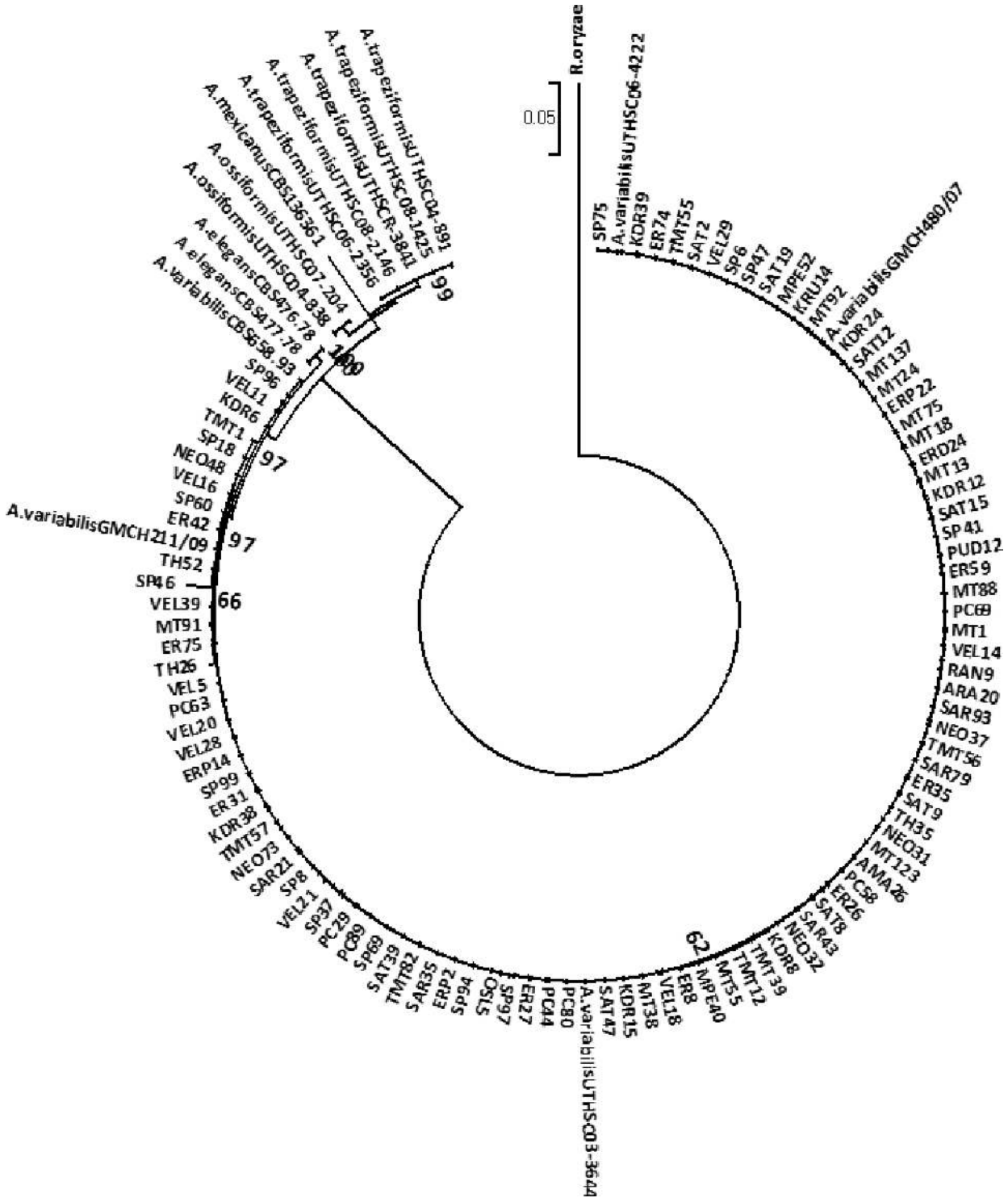

Figure 1. Neighbor-joining (NJ) phylogenetic tree (Kimura-2 parameter method) generated from combined data of 28S rDNA and ITS region. Bootstrap values were expressed as percentages of 1000 replications. The scale represents the number of substitutions per site.

samples, were also extracted from National bureau of Soil Survey and Land Use Planning, Government of India, Nagpur, India.[22,23] The association of each parameter favouring growth of *Apophysomyces* species complex in soil, was analysed.

Statistical analysis

The data were entered into Microsoft Excel and analysed using SPSS V16.0. Cochran's Q test was used to test the significant difference among the proportions of *Mucorales* isolated from the soil. Chi square test was used to test the

association between the different study variables and the isolation of *Apophysomyces* species complex from soil. Logistic regression was used to evaluate the confounding factors significantly associated with isolation of *Apophysomyces* species complex from soil.

Results

Isolation of *Mucorales* from soil

A total of 2188 soil samples processed, and 2042 isolates of *Mucorales* were grown from 1949 (89.1%) samples. Multiple colonies appearing similar on macroscopic and microscopic morphology on any culture plate were considered as single isolate. Of the 2042 *Mucorales* isolated, *R. arrhizus* (538, 24.6%) was the most common agent followed by *Lichtheimia* spp. (507, 23.2%), *Cunninghamella* spp. (474, 21.7%), *R. microsporus* (307, 14%), *A. variabilis* (99, 4.5%), *Rhizopus homothallicus* (24, 1.1%), *Rhizomucor* spp. (32, 1.5%), and *Mucor* spp. (33, 1.5%). The difference in isolation of different species of *Mucorales* was statistically significant. (Cochran's Q = 2282.759, $P < .0001$) (Table 1). *A. variabilis* was isolated from all the places sampled irrespective of geographical region and season.

Phenotypic and genotypic characterisation of *Apophysomyces* species

Based on DNA sequences of ITS and 28S rDNA regions *Apophysomyces* isolates were identified as *A. variabilis* with 98–100% match with the type strain *A. variabilis* CBS 658.93. The isolates could grow at 25, 37, and 42°C, and the sporulation was better in water agar and DRBC agar with benomyl compared to PDA. The isolates grew in the presence of salt (0.5 to 1.5%) and pH (4 to 9). Zygospore formation was not successful in the mating experiments.

Phylogenetic analysis of *Apophysomyces variabilis*

The combined analysis of both ITS and 28S demonstrated close degree of similarity among *A. variabilis* isolates, with minor degree of strain variations. Maximum parsimony analysis of *A. variabilis* by combined analysis of ITS and 28S region showed consistency, retention, and composite index of 0.948546, 0.906504, and 0.859861, respectively (Fig. 1). The phylogenetic tree constructed using 28S rDNA showed the clustering of *A. variabilis* isolates with clear separation from other *Apophysomyces* species (Fig. S1). The ITS sequences also revealed close similarity among *A. variabilis* isolates with minor strain variation and clear

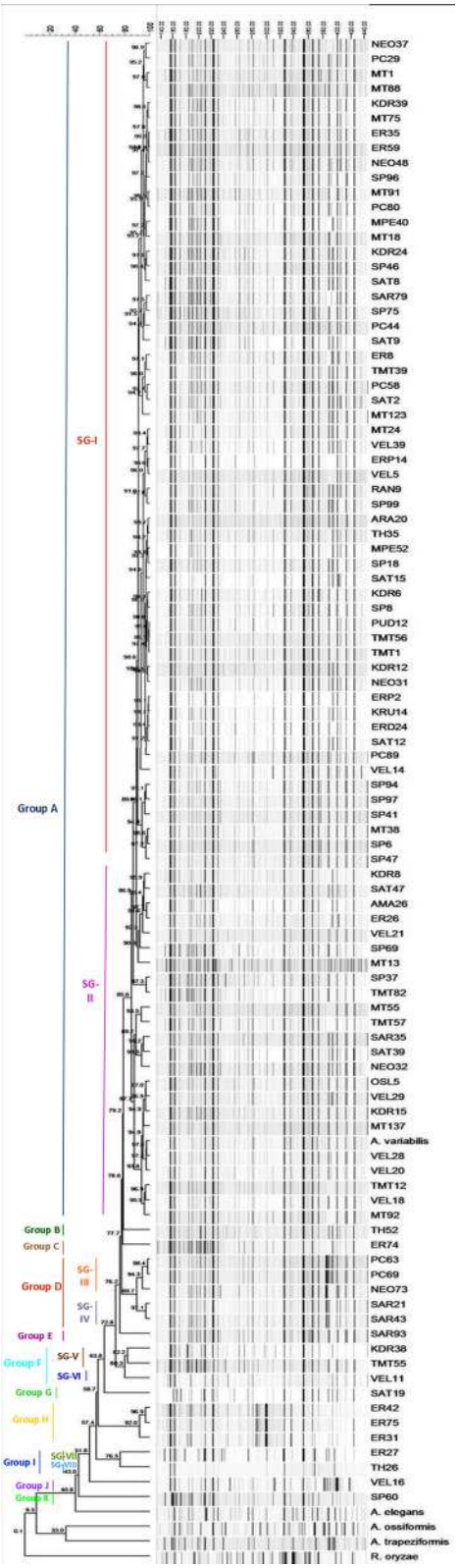

Figure 2. AFLP of 98 environmental *Apophysomyces* isolates along with 4 control isolates of *Apophysomyces* species. *R. oryzae* was included as out group isolate. Bands in the range of 150 to 450 base pairs are shown. The scale bar indicates the percentages of similarity between the isolates.

Table 2. *In vitro* antifungal susceptibility pattern for *Apophysomyces variabilis* isolates against four antifungal agents.

					Cumulative percentage of 99 *A. variabilis* isolates inhibited at MIC μg/ml									
Antifungals	Range*	GM*	50%*	90%*	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
Amphotericin B	0.125–4	1.07	1	4	0	0	10.1	15.2	24.2	56.6	83.8	100	100	100
Itraconazole	0.03–1	0.24	0.25	0.50	2.02	6.1	32.3	70.7	96.9	100	100	100	100	100
Posaconazole	0.03–1	0.13	0.125	0.25	2.02	21.2	73.7	93.9	98.9	100	100	100	100	100
Terbinafine	0.03–0.5	0.08	0.06	0.12	12.1	65.7	90.9	98.9	100	100	100	100	100	100

*All values in μg/ml, GM-Geometric Mean, MIC-Minimum inhibitory concentration.

distinction from other *Apophysomyces* species. The type strain of *A. variabilis* formed a sub-cluster with few of our environmental isolates (Fig. S2).

Amplified fragment length polymorphism analysis of *Apophysomyces variabilis*

The AFLP results of the isolates grouped *A. variabilis* into 11 clades (Group A to K). The isolates with percentage similarity >80% were grouped into single clade (Group A). Group A was further divided into two subgroups (SGI and SGII). SGI isolates had >90% similarity indicating genetic homogeneity among the isolates; SGII isolates had 80–90% similarity. The isolates in Group B to K had similarity ranging between 45% to 79%. Though all the isolates were identified as *A. variabilis* by sequencing, AFLP pattern of Group F to K isolates had similarity percentage of 43% to 60% indicating genetic heterogeneity within the species. *A. elegans* type strain had 40% similarity with *A. variabilis* isolates. *A. ossiformis* (CBS 125533) and *A. trapeziformis* (CBS125534) showed <35% similarity to *A. variabilis* (Fig. 2). The isolates from different geographical regions had no significant difference in AFLP types.

Antifungal susceptibility testing of *Apophysomyces variabilis*

No significant difference in susceptibility to antifungal drugs was observed among the isolates from different geographical locations. Majority (56.6%) of the *A. variabilis* isolates had MIC more than 1 μg/ml to amphotericin B (Table 2).

Isolation of *Apophysomyces* and its association with soil parameters

The parameters of the soils tested during the study were similar to the available data from National Bureau of Soil Survey and Land Use Planning, India. The rate of isolation of *A. variabilis* was significantly higher (15.6%; $P < .0001$) from *Manilkara zapota* (chickoo) orchards compared to other soil sources. No significant difference in isolation of different species under *Mucorales* was observed with the variation of seasons or the environmental temperature. The low nitrogen, medium phosphorous, marginal zinc, adequate copper content, and acidic pH were significantly associated with the isolation of *A. variabilis* (Table 3). However, on logistic regression analysis only low nitrogen content was significantly associated with *A. variabilis* isolation (adjusted OR = 0.132, $P < .0001$).

Discussion

The isolation of fungal agents from soil is generally influenced by soil characteristics. *Mucorales* are found in soil rich in composite vegetation, decaying vegetables and fruits.[24] Humans acquire the infection through skin trauma from contaminated soil or by inhalation of sporangiospores that have been dispersed from the soil. Mucormycosis cases are relatively common in tropical and subtropical countries, where the climatic conditions favour the survival and growth of *Mucorales* in nature.

Apophysomyces species complex usually produces cutaneous and subcutaneous mucormycosis.[4–6] As the acquisition of the agent is commonly associated with trauma, it is assumed that the incriminating organism comes from soil or other sources of environment.[5,6,8] A cluster of necrotizing cutaneous mucormycosis cases due to *A. trapeziformis* was reported after a Tornado in Joplin, Missuori.[25] In Australia, a burn wound patient acquired cutaneous mucormycosis due to *Apophysomyces elegans* after he rolled on the ground to extinguish fire and the agent was isolated from the soil at the same site.[8]

The present study is the first systematic environmental survey to determine the possible source of *Apophysomyces* species complex in Indian soils. Low nitrogen content in soil was significantly associated with *A. variabilis* isolation. The different fungal species are known to inhabit in various nutrient conditions, like *Cryptococcus* species survive in the soil rich in nitrogen, low organic content, and acidic pH; *Coccidioides immitis* in sandy, alkaline soil.[26,27] *Mucorales* are known to produce battery of enzymes to degrade complex nutrients in environment.[28]

Table 3. Association of the environmental parameters with the isolation of *Apophysomyces variabilis*

Factor	*A. variabilis* N (%)	Chi square	*P* value
Season			
Summer	85 (5.2)	0.593	.743
Winter	7 (5.4)		
Monsoon	7 (3.9)		
Soil Source			
Chickoo orchards	14 (15.6)	24.929	<.0001*
Grass sites	40 (4.2)		
Mango orchards	19 (4.8)		
Vegetative fields	26 (5.7)		
Nitrogen			
Low	73 (6)	5.722	.017*
Medium	26 (3.5)		
Phosphorus			
Low	26 (3.4)	7.161	.007*
Medium	73 (6.1)		
Potassium			
High	94 (5.1)	0.025	.874
Medium	5 (5.4)		
Organic Carbon			
High	4 (7.8)	0.830	.362
Low	95 (5)		
pH			
5.6–6.8	4 (7.8)	8.364	.039*
6.8– 7.3	21 (3.1)		
7–7.5	69 (6.1)		
8.1–8.9	5 (5.4)		
Zinc			
Marginal	43 (8.7)	18.289	<.0001*
Adequate	26 (3.9)		
Low	30 (3.8)		
Copper			
High	26 (3.4)	7.161	.007*
Adequate	73 (6.1)		
Iron			
Marginal	69 (5.6)	2.457	.293
Adequate	25 (4)		
Low	5 (5.4)		
Manganese			
Adequate	56 (3.6)	32.724	<.0001*
Marginal	43 (10.6)		

*P valve < .05 were considered statistically significant.

Unlike other *Mucorales*, *Apophysomyces* species complex produces sterile hyphae on primary isolation media. The induction of sporulation in nutrient deficient media is a standard practice to identify the agent phenotypically.[29] As *Mucorales* produce aseptate hyphae, which are fragile structures, the prolific sporulation helps the fungi to survive hostile environment. The same logic may not hold true for *Apophysomyces* species complex, as it does not produce spores easily. However, the present study showed that *A. variabilis* survives in wide range of temperatures (25–42°C), pH (4–9), salt content (0.5–1.5%), low organic carbon, and nitrogen content. Humans may acquire the infection from soil contaminated with hyphae rather than spores.

The taxonomy of *Apophysomyces* is evolving. The molecular phylogenetic analysis indicated multiple cryptic species under *Apophysomyces*, and the majority of human infection is due to *A. variabilis*.[5,12] Our soil isolates were also identified as *A. variabilis*. Phylogenetic analysis of 28S rDNA and ITS region showed clustering of our isolates in one group, with clear species delineation from other described *Apophysomyces* species.[10] Both the ITS and 28S sequences showed minimal nucleotide variation among *A. variabilis* isolates. The 18S and actin gene sequences also did not show any variation within *A. variabilis*.

Various molecular methods have been used to assess the phylogenetic structure of *Apophysomyces* species complex.[10,13,30,31] AFLP is one of the economical and highly discriminatory technique used widely to differentiate closely related species and to determine the strain variation within the species. In the present study, AFLP typing was used to estimate the extent of genetic diversity among *A. variabilis* isolates. The isolates formed multiple clusters with similarity percentage ranging from 40 to 90%; which strongly suggests the genetic heterogeneity, multiple genotypes and possibility of cryptic species.[13] However, other *Apophysomyces* species could be clearly differentiated from *A. variabilis* with the similarity percentage of 30–40%. The earlier Indian environmental *A. elegans* (CBS 477.78) isolate did not cluster with *A. variabilis* isolates, indicating genetically different species. A similar observation was made in our earlier study with clinical isolates.[13] The phylogenetic analysis by whole genome sequencing reported genetic homoplasy among *Apophysomyces* species complex, with the possibility of recombination by lateral gene transfer.[30] Our AFLP data also indicate the existence of multiple species in the genus *Apophysomyces* and the possibility of further cryptic species under *A. variabilis*. The AFLP typing of clinical isolates of *Apophysomyces* species complex also indicated greater genetic diversity and possibility of cryptic species within the genus.[13] However, the limitation of the AFLP data is that occasionally it may generate erroneous genotyping results.[32] The whole genome sequence (WGS) analysis of divergent isolates of *A. variabilis* may clarify the genetic structure of this species.

The data on antifungal susceptibility pattern of *Apophysomyces* species complex are sparse, and practically no data are available on the environmental isolates. The available studies on clinical isolates revealed varying degree of susceptibility for amphotericin B in the range of 0.125 to 4 μg/ml.[13,33,34] The environmental *A. variabilis* isolates in the present study also exhibited high MIC for amphotericin

B with MIC_{50} and MIC_{90} at 1 and 4 μg/ml respectively. Itraconazole had better *in vitro* activity against environmental *A. variabilis* isolates with MICs ranging between 0.03 to 1 μg/ml compared to earlier reports (ranged from 0.03 to 8 μg/ml).[13,33,34] Posaconazole also showed good *in vitro* activity in the present study with MIC_{50} and MIC_{90} at 0.125 μg/ml and 0.25 μg/ml, respectively. The *in vitro* activity of terbinafine is less studied against *Apophysomyces* species complex. The present study showed good *in vitro* susceptibility of *A. variabilis* isolates to terbinafine, with MIC_{50} and MIC_{90} at 0.06 μg/ ml and 0.125 μg/ml, respectively.

In conclusion, the study identified the natural habitat of *A. variabilis* in low nitrogen containing soil in India. The AFLP pattern suggests high genetic heterogeneity among the *A. variabilis* isolates. We propose to study the genetic relatedness between clinical and environmental isolates in future. *In vitro* antifungal susceptibility showed high MIC for amphotericin B, but better susceptibility to posaconazole, itraconazole, and terbinafine.

The GenBank accession numbers of 28S ribosomal RNA and internal transcribed spacer (ITS) gene sequences are **KT387372 - KT387470** and **KT387471 - KT387569,** respectively.

Acknowledgements

We deeply acknowledge the financial support of Department of Biotechnology, Ministry of science and technology, New Delhi, India.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Supplementary material

Supplementary material is available at Medical Mycology online (http://www.mmy.oxfordjournals.org/).

References

1. Roden MM, Zaoutis TE, Buchanan WL et al. Epidemiology and outcome of zygomycosis: a review of 929 reported cases. *Clin Infect Dis* 2005; **41**: 634–653.
2. Gomes MZ, Lewis RE, Kontoyiannis DP. Mucormycosis caused by unusual mucormycetes, non-*Rhizopus*, -*Mucor*, and *Lichtheimia* species. *Clin Microbiol Rev* 2011; **24**: 411–445.
3. Chakrabarti A, Singh R. The emerging epidemiology of mould infections in developing countries. *Curr Opin Infect Dis* 2011; **24**: 521–526.
4. Chakrabarti A, Singh R. Mucormycosis in India: unique features. *Mycoses* 2014; **57**(Suppl 3): 85–90.
5. Chander J, Stchigel AM, Alastruey-Izquierdo A et al. Fungal necrotizing fasciitis, an emerging infectious disease caused by *Apophysomyces* (*Mucorales*). *Rev Iberoam Micol* 2015; **32**: 93–98.
6. Meis JF, Chakrabarti A. Changing epidemiology of an emerging infection: zygomycosis. *Clin Microbiol Infect* 2009; **15**(Suppl 5): 10–14.
7. Misra PC, Srivastava KJ, Lata K. *Apophysomyces, a new genus of the Mucorales*. *Mycotaxon* 1979; **8**: 377–382.
8. Cooter RD, Lim IS, Ellis DH et al. Burn wound zygomycosis caused by *Apophysomyces elegans*. *J Clin Microbiol* 1990; **28**: 2151–2153.
9. de Azevedo Santiago AL, Dos Santos PJ, Maia LC. *Mucorales* from the semiarid of Pernambuco, Brazil. *Braz J Microbiol* 2013; **44**: 299–305.
10. Alvarez E, Stchigel AM, Cano J et al. Molecular phylogenetic diversity of the emerging mucoralean fungus *Apophysomyces*: Proposal of three new species. *Rev Iberoam Micol* 2010; **27**: 80–89.
11. Bonifaz A, Stchigel AM, Guarro J et al. Primary cutaneous mucormycosis produced by the new species *Apophysomyce mexicanus*. *J Clin Microbiol*.2014; **52**: 4428–4431.
12. Guarro J, Chander J, Alvarez E et al. *Apophysomyces variabilis* infections in humans. *Emerg Infect Dis* 2011; **17**: 134–135.
13. Chakrabarti A, Shivaprakash MR, Curfs-Breuker I et al. *Apophysomyces elegans*: epidemiology, amplified fragment length polymorphism typing, and *in vitro* antifungal susceptibility pattern. *J Clin Microbiol* 2010; **48**: 4580–4585.
14. Summerbell RC. The benomyl test as a fundamental diagnostic method for medical mycology. *J Clin Microbiol* 1993; **31**: 572–577.
15. Benny GL. Methods used by Dr. R. K. Benjamin, and other mycologists, to isolate zygomycetes. *Aliso* 2008; **26**: 37–61.
16. de Hoog GS, Guarro J, Gene J et al. *Atlas of Clinical Fungi*, 2nd edn. Utrecht, Reus: Centraalbureau voor Schimmelcultures, Universitat Rovira i Virgili. 2000.
17. Chakrabarti A, Rudramurthy SM, Kale P et al. Epidemiological study of a large cluster of fungaemia cases due to *Kodamaea ohmeri* in Indian tertiary care centre. *Clin Microbiol Infect* 2014; **20**: O83–O89.
18. Babu AG, Kim SW, Yadhav DR et al. A new record of *Pseudallescheria boydii* isolates from crop field soil in Korea. *Mycobiology* 2014; **42**: 397–400.
19. Klang A, Loncaric I, Spergser J et al. Disseminated histoplasmosis in a domestic cat imported from the USA to Austria. *Med Mycol Case Rep* 2013; **2**: 108–112.
20. Lee SB, Taylor JW. Isolation of DNA from fungal mycelia and single spores. In: Innis MA, Gelfand DH, Shinsky J, White TJ Eds. *PCR Protocols: A Guide to Methods and Applications*. Orlando, Florida:Academic Press, 1990: 282–287.
21. Clinical and Laboratory Standards Institute. *Reference method for broth dilution antifungal susceptibility testing of yeasts; filamentous fungi; approved standard CLSI document M38-A2*. Wayne, Pa: CLSI; 2008.
22. National bureau of soil survey and land use planning. Soils of India. NBSS Publication. 1994.
23. Singh MV. Micronutrient fertility mapping for Indian Soil, Technical Bulletin. *AICRP Micronutrients*, IISS, Bhopal. 2008; **7**: 1–60.

24. Richardson M. The ecology of the zygomycetes and its impact on environmental exposure. *Clin Microbiol Infect* 2009; **15**(Suppl 5): 2–9.
25. Neblett Fanfair R, Benedict K, Bos J et al. Necrotizing cutaneous mucormycosis after a tornado in Jolpin, Missouri, in 2011. *N Engl J Med* 2012; **367**: 2214–2225.
26. Kidd SE, Chow Y, Mak S et al. Characterization of environmental sources of the human and animal pathogen *Cryptococcus gattii* in British Columbia, Canada, and the Pacific Northwest of the United States. *Appl Environ Microbiol* 2007; **73**: 1433–1443.
27. Lacy GH, Swatek FE. Soil ecology of *Coccidioides immitis* at Amerindian middens in California. *Appl Microbiol* 1974; **27**: 379–388.
28. Ko HS, Taguchi H, Takizawa K et al. The enzymatic approach of zygomycosis-causing *Mucorales*. *Kor J Med Mycol* 2007; **12**: 9–17.
29. Padhye AA, Ajello L. Simple method of inducing sporulation by *Apophysomyces elegans* and *Saksenaea vasiformis*. *J Clin Microbiol* 1988; **26**: 1861–1863.
30. Etienne KA, Gillece J, Hilsabeck R et al. Whole genome sequence typing to investigate the *Apophysomyces* outbreak following a tornado in Joplin, Missouri, 2011. *PLos One* 2012; **7**: e49989.
31. Chakrabarti A, Ghosh A, Prasad GS et al. *Apophysomyces elegans*: an emerging zygomycete in India. *J Clin Microbiol* 2003; **41**: 783–788.
32. Crawford LA, Koscinski, Keyghobadi N. A call for more transparent reporting of error rates: the quality of AFLP data in ecological and evolutionary research. *Mol Ecol* 2012; **21**: 5911–5917.
33. Almyroudis NG, Sutton DA, Fothergill AW et al. *In vitro* susceptibilities of 217 clinical isolates of zygomycetes to conventional and new antifungal agents. *Antimicrob Agents Chemother* 2007; **51**: 2587–2590.
34. Sun QN, Fothergill AW, McCarthy DI et al. *In vitro* activities of posaconazole, itraconazole, voriconazole, amphotericin B, and fluconazole against 37 clinical isolates of zygomycetes. *Antimicrob Agents Chemother* 2002; **46**: 1581–1582.