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Detection and molecular characterization of outdoor fungi from the coastal city of Visakhapatnam, India

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ABSTRACT

Visakhapatnam is a coastal city situated on the east coast of India. It is one of the cities included under the Smart Cities mission of Government of India. Recent studies have reported around 35 fungal taxa from the outdoor air of the city. There have been, however, few studies that has employed modern taxonomic tools such as gene sequence analysis to understand the fungal diversity. The present study was, therefore, initiated to characterize the phylogenetic diversity and clarify the taxonomic positions of the fungi isolated from aerosol samples collected from urban Visakhapatnam. In total, 22 fungi were isolated, of which 9 isolates were subjected to ITS-based phylogenetic analysis. In the phylogenetic tree, the newly generated nine ITS sequences clustered within *Alternaria alternata*, *Aspergillus elegans*, *A. micronesiensis, Penicillium citrinum, P. oxalicum* and *P. steckii* clades. Further studies employing a polyphasic approach, including mycotoxin analysis, will be required to accurately identify these fungi to species level and assess their possible effects on human health and coastal ecology of Visakhapatnam.

Keywords: Air-borne fungi, Colony morphology, ITS, Phylogeny, Taxonomy

INTRODUCTION

It is well-known that the atmospheric air contains microorganisms (sizes range 50 nm to 10 μm) known as bioaerosols, including pollen, fungi, microalgae, bacteria and viruses. Fungi are the well-studied aerosol component, considering their implications in air quality and human health. Fungi are primarily present in aerosols as spores and occasionally as fungal mycelia. Fungal spores, including those of ascomycetes and basidiomycetes, are reportedly dominant in the outdoor coarse particle aerosol (1-10 μm) (Singh and Kumar, 2002; Singh and Dahiya, 2008). It is estimated that fungi constitute about 35% of the bioaerosols in tropical rainforests (Elbert *et al.,* 2007).

Fungal contamination of indoor and outdoor air environments comes from a variety of sources such as sewage treatment plants, stock farming, fermentation processes, industrial and agricultural activities (Abdel Hameed, 1996). Common air-borne fungi are *Penicillium, Aspergillus, Acremonium, Paecilomyces, Mucor* and *Cladosporium* (Lugauskas, 2004). They act as aeroallergens and also produce mycotoxins. Mycotoxins are lipid soluble and readily absorbed by the intestinal lining, airways and skin, and are capable of causing adverse health effects such as inflammatory reactions (Etzel *et al.,* 1998). Volatile products of fungal metabolism are capable of inducing sensory irritation to eyes and upper respiratory tract (Korpi *et al*., 1999).

Recently, Yadav *et al*., (2016) investigated the composition of aerosols over the city of Visakhapatnam and its impact on phytoplankton biomass. It was suggested that 52-89% of Visakhapatnam's aerosol-borne nitrogen deposited over waters within 10 km from the coastline. It is also suggested that atmospheric deposition of nutrients enhances phytoplankton biomass in waters along the central east coast of India during the winter monsoon period. It is important to improve our understanding of possible roles of air-borne fungi on the coastal productivity. Such studies may provide interesting perspectives on fate of fungal biomass, especially of terrestrial origins, present in aerosols on encountering sea water.

There are limited reports on gene sequence-based taxonomy of airborne fungal diversity in the Visakhapatnam (**Table 1**). Thus this study was initiated with a focus on taxonomy of fungi isolated from outdoor aerosols in the coastal city of Visakhapatnam. Visakhapatnam, situated on the East Coast of India, is one of the designated cities under Smart Cities Mission of the Government of India. The present study had two objectives:

1) To collect outdoor aerosol samples from Visakhapatnam and detect aerosol-associated fungi using culturing method
2) To characterize the phylogenetic diversity of the aerosol-associated fungi based on ITS sequence analysis.

MATERIALS AND METHODS

Study area

Visakhapatnam is a port city and industrial center in the Indian state of Andhra Pradesh, on the East Coast of India (**Fig. 1**). It is the second largest city in the state with an area of $550 km^2$ and is primarily an industrial city (https://en.wikipedia.org/wiki/Visakhapatnam). The city has many heavy industries and steel plant. Pollution from industries is found very high in Visakhapatnam (http://timesofindia.indiatimes.com/city/visakhapatnam/Ranked-5th-clean-city-Vizag-finds-air-pollution-on-the-rise/articleshow/51737679.cms). The anthropogenic sources to aerosols in and around this city mainly include ore transport through an open belt system, burning of coal by National Thermal Power Corporation (NTPC), fossil fuel burning by industries mainly involving automobiles and fertilizers usage in agricultural activities contributing organic and inorganic nitrogen. The city has pollution index (PI) 79.31, exponential pollution index (EPI) 151.82 and stands 6^{th} as the most polluted cities of India (www.hoparoundindia.com, 2013).

Table 1: Outdoor airborne fungi reported from Visakhapatnam, India

Sl. no.	Taxon/ Clade	References
1	***Alternaria alternata***	Reddy *et al*., 2011, 2012, 2015; Reddy and Srinivas, 2012; **This study.**
2	*A. solani*	Reddy *et al.,* 2011, 2012, 2015; Reddy and Srinivas, 2012.
3	*Alternaria* spp.	Bomala *et al*., 2016.
4	*A. candidus*	Reddy *et al*., 2011, 2012, 2015;Reddy and Srinivas, 2012.
5	***A. elegans***	**This study.**
6	*A. flavus*	Reddy *et al.,* 2011, 2012, 2015;Reddy and Srinivas, 2012.
7	*A. fumigatus*	Reddy *et al*., 2011, 2015; Reddy and Srinivas, 2012.
8	***A. micronensis***	**This study.**
9	*A. niger*	Reddy *et al*., 2012, 2015; Reddy and Srinivas, 2012.
10	*A. parasiticus*	Reddy *et al*., 2011, 2012, 2015;Reddy and Srinivas, 2012.
11	*Aspergillus* spp.	Bomala *et al*., 2016.
12	*A. terreus*	Reddy *et al*., 2015.
13	*A. versicolor*	Reddy *et al*., 2011, 2015.
14	*Botrytis* spp.	Reddy *et al*., 2011, 2012, 2015; Reddy and Srinivas, 2012.
15	*Cephalosporium* spp.	Reddy *et al*., 2012, 2015.
16	*Cercospora* spp.	Reddy *et al*., 2011, 2012, 2015.
17	*Cladosporium cladosporioides*	Reddy *et al*., 2011, 2012;Reddy and Srinivas, 2012.
18	*Cladosporium* spp.	Reddy *et al*., 2015; Bomala *et al*., 2016.
19	*Colletotrichum* sp.	Reddy et al., 2015.
20	*Curvularia affinis*	Reddy *et* al., 2011, 2012, 2015;, Reddy and Srinivas, 2012.
21	*C. lunata*	Reddy *et al*., 2011, 2015; Reddy and Srinivas, 2012.
22	*Fusarium moniliforme*	Reddy *et al*., 2011, 2012, 2015; Reddy and Srinivas, 2012.
23	*F. oxysporum*	Reddy *et al*., 2012, 2015
24	*F. solani*	Reddy *et al*., 2011, 2015;, Reddy and Srinivas, 2012.
25	*Fusarium* spp.	Bomala *et al*., 2016
26	*Helminthosporium* spp.	Reddy *et al*., 2011, 2012, 2015.
27	*Mortierella zonata*	Reddy *et al*., 2011, 2012, 2015.
28	*Mucor microsporus*	Reddy *et al*., 2011, 2012, 2015;Reddy and Srinivas, 2012.
29	*M. racemosus*	Reddy *et al*., 2011, 2015.
30	*Mucor* spp.	Bomala *et al*., 2016
31	***Penicillium citrinum***	**This study.**
32	***P. oxalicum***	**This study.**
33	*Penicillium* spp.	Reddy *et al*., 2011, 2012, 2015; Reddy and Srinivas, 2012; Bomala *et al*., 2016.
34	***P. steckii***	**This study.**
35	*Rhizopus oryzae*	Reddy *et al*., 2011, 2012, 2015; Reddy and Srinivas, 2012.
36	*Rhizopus* spp.	Bomala *et al*., 2016
37	*R. stolonifer*	Reddy *et al*., 2011, 2015;Reddy and Srinivas, 2012.
38	*Stachybotrys* spp.	Reddy and Srinivas, 2012; Reddy *et al*., 2011, 2012, 2015.
39	*Trichoderma* spp.	Reddy *et al*., 2011, 2012, 2015; Reddy and Srinivas, 2012; Bomala *et al*., 2016.
40	*Trichothecium* spp.	Reddy *et al*., 2011, 2015; Reddy and Srinivas, 2012.
41	*Verticillium* spp.	Reddy *et al*., 2011, 2012, 2015.
42	Yeast cells	Reddy *et al*., 2012, 2015.

Sample collection

The aerosol sampling was done in Visakhapatnam city at the top (at a height of 20 m above the mean sea level) of NIO laboratory building (17.69° N: 83.22° E) located at about 200 m from the coastline. Atmospheric aerosol samples were collected on pre-combusted quartz filters (395.5 cm^2, PALLFLEX) using respirable dust sampler (Environtec APM 460NL) during the period of January to March (20.01.2016, 23.02.2016, and 16.03.2016). The automatic weather-station data for the sampling days provided by Dr. Prakash Mehra from CSIR-NIO, Goa are presented in **Table 2**.

Fig. 1 Visakhapatnam map (Source: Google)

Table 2: Automatic weather station data for the sampling days

Date	Time	Speed km/h	Direction (º)	Temp. (°C)
20.01.2016	10am-4pm	2.0627	282.362	26.47
23.02.2016	10am-4pm	4.6513	273	27.78
16.03.2016	10am-4pm	3.1702	262.905	29.03

Sample processing for isolation of fungi

The collected aerosol filter papers were carefully folded and transferred to the lab in a zip-lock bag and stored at 4 °C. A 47 mm punch hole was used to take aliquots from the filter paper (A4 size). Each aliquot was cut into small pieces and transferred into 10 ml Tarson centrifuge tube containing 5 ml of sterile distilled water. A separate set of 10 ml Tarson centrifuge tube containing 5 ml of sterile sea water was prepared. The centrifuge tubes were vortexed at maximum speed for two minutes. 100 µl sample from each aliquot was directly plated on PDA medium amended with chloramphenicol (100 mg/ l). Fungal growth on PDA medium was monitored and growing fungal mycelia were transferred to fresh PDA plates to obtain pure cultures (**Table 3**). The fungal cultures were maintained at 28 °C in incubator till further processing.

Sequencing and phylogenetic analysis of the ITS region

Nine fungal cultures were processed to extract DNA and PCR amplification of the ITS region, following the methods detailed in Khandavilli *et al*., (2016) and White *et al*., (1991) . The sequencing of ITS gene region was done using Genetic Analyzer 3130*xl* (ABI) based on the Big Dye terminator v3.1 (Chain terminator) chemistry at Biological Oceanography Division, CSIR-National Institute of Oceanography, Goa. The forward and reverse sequences obtained from each primer were aligned in MEGA v 7.0 (Kumar *et al*., 2015) to generate a consensus sequence. A multiple sequence alignment was prepared in MEGA using the newly-generated ITS sequences and the reference sequences retrieved from NCBI-GenBank (**Fig. 2**). The evolutionary tree was inferred in MEGA using Maximum-Likelihood method.

RESULTS

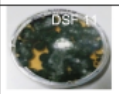
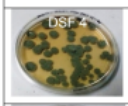
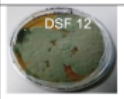
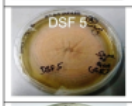
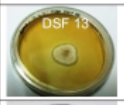
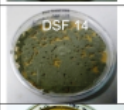
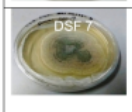
Isolation of fungal cultures from aerosol samples

Totally, 22 fungi were isolated from the aerosol samples. The culture morphology of select 15 fungal isolates is presented in **Table 3**.

ITS-based phylogenetic tree

The ML tree shown in **Fig. 2** represents the evolutionary relationships of the 9 fungal isolates. Tree details obtained from MEGA is reproduced here, with some minor modifications: "The evolutionary history was inferred by

Table 3: Colony morphology of fungal cultures

Image of culture	Colony morphology	Image of culture	Colony morphology
	Colony on PDA+MW at 27-29 °C. White, edge of the colony Entire. Reverse White yellowish.		Colony on PDA+DW at 27-29 °C. Brown with White outline, edge of the colony entire. Reverse Black.
	Colony on PDA+DW at 27-29 °C. Apricots with white outline, edge of the colony undulate. Reverse brownish yellow.		Colony on PDA+MW at 27-29 °C. White, edge of the colony undulate. Reverse yellow.
	Colony on PDA+MW at 27-29 °C. Brown with white outline, edge of the colony undulate. Reverse Brown with yellow.		Colony on PDA+DW at 27-29 °C. White and Black, edge of the colony undulate. Reverse yellow.
	Colony on PDA+DW at 27-29 °C. Green, edge of the colony entire. Reverse cream.		Colony on PDA+MW at 27-29 °C. Forest green, edge of the colony undulate. Reverse yellow.
	Colony on PDA+DW at 27-29 °C. Peach, edge of the colony entire. Reverse yellow.		Colony on PDA+DW at 27-29 °C. White, edge of the colony undulate. Reverse Brown.
	Colony on PDA+MW at 27-29 °C. White, edge of the colony undulate. Reverse yellow.		Colony on PDA+MW at 27-29 °C. Olive greens, edge of the colony undulate. Reverse yellow.
	Colony on PDA+DW at 27-29 °C. Black and White with yellow outline, edge of the colony undulate. Reverse contains yellowish circle and white outline.		Colony on PDA+MW at 27-29 °C. Green, edge of the colony undulate. Reverse yellow.
	Colony on PDA+MW at 27-29 °C. Green, edge of the colony undulate. Reverse yellow.		

MW: Marine water; DW: Distilled water

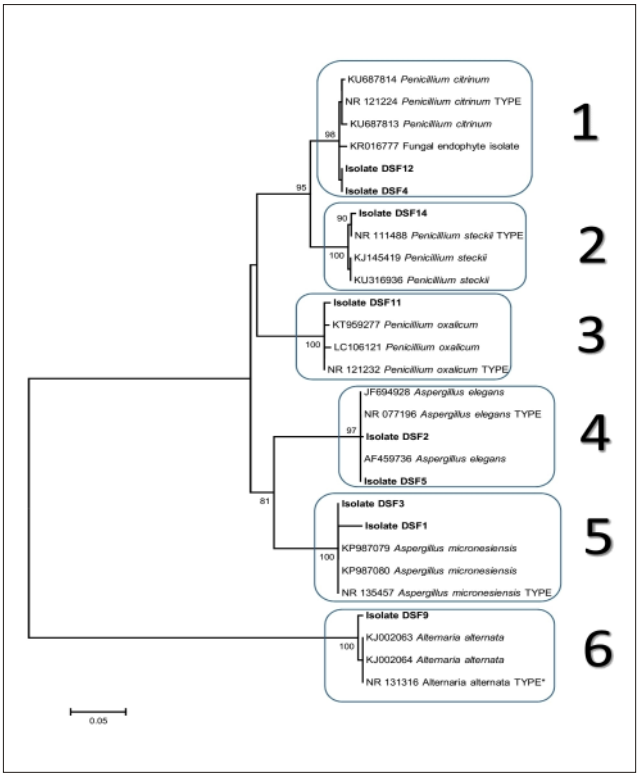


Fig. 2 Maximum Likelihood tree depicting the evolutionary relationships of 9 fungi isolated from aerosol samples in Visakhapatnam. The tree was constructed based on the ITS sequence-data. Captions: Clade 1: *Penicillium citrinum,* Clade 2: *P. steckii,* Clade 3: *P. oxalicum,* Clade 4: *Aspergillus elegans,* Clade 5: *A. micronesiensis* and Clade 6: *Alternaria alternata*

using the Maximum Likelihood method based on the General Time Reversible model. The tree with the highest log likelihood (-2414.4527) is shown in **Fig. 2**. The percentage of trees in which the associated taxa are clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.6952)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 32.6895% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 28 nucleotide sequences. There were a total of 673 positions in the final dataset". In the ML tree (**Fig. 2**), isolates DSF12 and 14 clustered within the *Penicillium citrinum* clade (Clade 1), while DSF14 and DSF11 clustered within *P. steckii* clade (Clade 2) and *P. oxalicum* clade (Clade 3), respectively. Isolates DSF2 and 5 clustered with *Aspergillus elegans* clade (Clade 4), while isolates DSF1 and 3 clustered with *A. micronesiensis* clade (Clade 5). Isolate DSF9 is a part of *Alternaria alternata* clade (Clade 6).

DISCUSSION

This study reveals that 9 aerosol-associated fungi included in the phylogenetic analysis belong to 3 fungal genera and 6 species (**Fig. 2**). To the best of our knowledge, this is the first study employing DNA based phylogenetic analysis to investigate fungal diversity present in aerosol samples of Visakhapatnam (**Table 1**). *Aspergillus micromesiensis* (DSF1), *A. elegans* (DSF5), *Penicillium oxalicum* (DSF11) and *P. citrinum* (DSF12) were recovered from aerosol samples collected on 16.03.2016, while *Aspergillus micromesiensis* (DSF3) and *Penicillium steckii* (DSF14) were recovered from aerosol samples collected on 20.01.2016. Fungal isolates (*Aspergillus elegans*) DSF2 and DSF 4 (*P. citrinum*) were recovered from the aerosol samples collected on 23.02.2016. *Aspergillus micromesiensis* and *P. citrinum* were recovered from aerosol samples collected on two different dates.

Aspergillus micronensis, A. elegans, Penicillium citrinum, P. oxalicum and *P. steckii* are apparently new records for outdoor aerosol-associated fungi from Visakhapatnam. Further studies employing a polyphasic approach and enhanced taxon sampling are required to get better insights into diversity of fungi in aerosols from Visakhapatnam and their ability to produce harmful mycotoxins. Additional studies, including

mesocosm experiments, are required to understand the fate of fungal biomass present in outdoor aerosols on encountering sea water and their possible roles in coastal productivity along the East Coast of India.

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