

Full Length Research Paper

Halophilic fungi in a polyhaline estuarine habitat

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Halophilic mycobiota was isolated from Mandovi estuary and it was dominated by *Aspergillus* and *Penicillium* species. *Cladosporium* and *Eurotium* were found in lesser numbers while obligate halophiles were found only amongst the aspergilla and they were all identified as *Aspergillus penicillioides*. Some aspergilli and all the isolates of *Penicillium*, *Cladosporium* and *Eurotium* were facultative halophiles. There were significant differences in growth of each isolate at different salt concentrations. Most of the isolates were euryhaline, having a wide range of salt tolerance; a few were stenohaline, with a narrow range of halotolerance. The isolates were mainly moderate halophiles, with a very few slight halophiles. Isolation of obligate halophilic fungi from polyhaline environment of an estuary is hereby reported for the first time.

Key words: *Aspergillus penicillioides*, obligate halophiles, facultative halophiles.

INTRODUCTION

An estuary is under the influence of marine conditions such as tides, waves, influx of saline water, as well as the flow of fresh water and sediment of the river (Manoharachary et al., 2005). Studies on the mycobiota in estuarine ecosystems have focused on the diversity or metabolic activities of fungi from estuarine waters, sediments, wood and litter, marshes and mangroves (Borut and Johnson, 1962; Shearer, 1972; Cooke and Lacourse, 1975; Rai and Chowdhery, 1978; D'souza et al., 1979; Maria and Sridhar, 2002; da Silva et al., 2003; Tsui and Hyde, 2004; Anita et al., 2009; Karamchand et al., 2009; Nambiar and Raveendran, 2009; Rani and Panneerselvam, 2009; Pearman et al., 2010; Mohamed and Martiny, 2011). The halophilic nature of these fungi has not been described.

Hypersaline environments have been a focus of study for halophilic organisms that are able to survive in these environments. *Gymnascella marismortui* was the first halophilic fungi to be isolated from the Dead Sea (Buchalo et al., 1998). The black yeasts and closely related dematiaceous *Cladosporium* were among the first halophilic fungi to have been isolated from salterns in Secovlje, Slovenia-Adriatic (Gunde-Cimerman et al.,

2000; Butinar et al., 2005a) and subsequently from Cabo Rojo, Puerto Rico (Diaz-Munoz and Montalvo-Rodriguez, 2005). The isolation of other filamentous fungi from salterns (Cantrell et al., 2006; Nayak et al., 2012), as well as from the Dead Sea (Kis-Papo et al., 2003a, b; Wasser et al., 2003; Nazareth et al., 2012), Mono Lake, California (Steiman et al., 2004), coastal environments of Arctics (Gunde-Cimerman et al., 2005) and from saline soils of Soos, Czech Republic (Hujsova et al., 2010) has followed.

Microbes that inhabit high-salt environments may be of halotolerant or halophilic nature, being adapted to high levels of ions, as well as to low a_w (Grant, 2004). Halophiles have been further classified as facultative or obligate (Kushner, 1978; Nazareth et al., 2012).

The occurrence of halophilic filamentous fungi, particularly that of obligate halophilic aspergilli, in a polyhaline estuarine environment, is recorded herein for the first time.

METHODOLOGY

Samples

Sampling was done along the estuary of the Mandovi (EM) which flows into the Arabian Sea, on the West Coast of the Indian Peninsula. Samples of top and bottom water (wt) and (wb), and of sediment(s) were obtained using the Niskin and Grab samplers,

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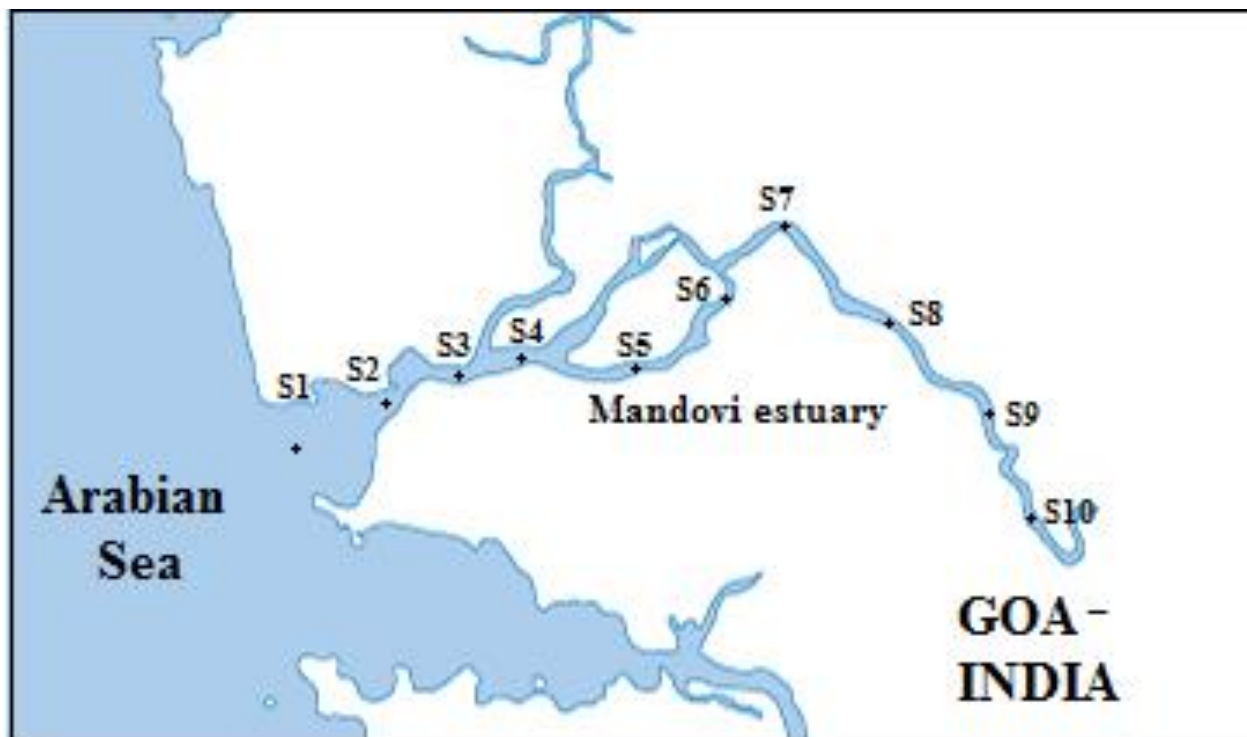


Figure 1. Map indicating the stations of sampling along the Mandovi estuary.

respectively, from 10 Stations, S1 to S10 (Figure 1), beginning at the mouth and moving hinterland between 73°46.65' to 74°2.5' longitude (courtesy G. N. Nayak, Goa University), as shown in Figure 1.

The salinity and pH were measured as described by Nazareth et al. (2012).

Isolation and identification of fungi

The samples were processed under sterile conditions. The water samples and suspensions of sediment were each plated on to Czapek-Dox Agar + 20% solar salt (20% S-CzA) for isolation of halophilic fungi, which were then incubated at room temperature of about 30°C, up to 30 days.

The isolates were picked based on dissimilarity in the colony characteristics, purified and numbered according to the station and the sample of top or bottom water and of sediment. Purified isolates were maintained on 10% S-CzA. Fungal identification was done on the basis of colony and micro morphology characteristics with reference to identification keys (Raper and Fennell, 1965; Ellis, 1971; Domsch et al., 1980).

The identification of an obligate halophilic isolate was confirmed by ITS rDNA sequence analysis (Merck-GeNei Services), using consensus primers for 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA gene fragment. A GenBank accession number was obtained. Sequence similarities were obtained using NCBI BLAST. Alignment and phylogenetic tree were constructed in Clustal X version 2 and the NJ distance method.

Halophilic nature of the isolates

Salt tolerance of the isolates was checked by inoculating the cultures in triplicate on CzA amended with salt up to concentrations

of 30% w/v (Nazareth et al., 2012). Growth was recorded after 7 days incubation in terms of colony diameter; plates that did not show growth in 7 days were further incubated till 15 days to check for delayed growth and then sub-cultured onto corresponding media to confirm the salt tolerance level. Salt tolerance curves were obtained by plotting the arithmetic mean of colony diameter with the standard error.

Statistically significant difference ($P < 0.05$) in the effect of different salt concentrations on growth of all isolates, as well as in growth within the species, was analyzed by two-way ANOVA.

RESULTS

Sample Salinity and pH

The salinity of the surface water was comparable to that of the bottom water at all stations. The salinity at S1 at the mouth of the estuary was 37‰, close to that of sea water, with a gradual decrease to approximately 30 to 31‰ at S5; it decreased drastically to approximately 24 to 25‰ at S6 and S7 and further there was a sequential decrease to approximately 10 to 16‰ till the last station. The sediment had a salinity of 5‰ at S6 and 10‰ at the rest of the stations, which was comparatively much lower than that of the water column at all stations.

The pH of the samples was around neutral.

Fungal isolates

The isolates obtained belonged to the genera of

Table 1. Species identification of isolates from different stations.

Species	Isolate number
<i>A. flavus</i>	EM2s111, EM7w _b 140
<i>A. fumigatus</i>	EM2s112, EM4w _t 119, EM6w _b 135
<i>A. nidulans</i>	EM5w _t 129
<i>A. penicillioides</i>	EM2w _b 107, EM4w _t 118, EM4w _t 120, EM4w _b 121, EM4w _b 123, EM4w _b 125, EM5w _t 130, EM6w _t 133, EM6s137, EM7w _t 138, EM7w _t 139, EM7w _b 141, EM7w _b 142, EM7w _b 143, EM7s144, EM7s145, EM8w _t 146, EM8w _t 147, EM8w _t 148, EM8w _b 149, EM9w _b 155, EM8s153, EM9s156
<i>A. sydowii</i>	EM8s 152
<i>A. versicolor</i>	EM2w _t 104, EM2w _t 105, EM2w _t 106, EM2w _b 108, EM2s110, EM6w _b 136
<i>P. asymetrica</i> sub-section <i>fasciculata</i>	EM5w _b 131, EM8s151
<i>P. canescens</i>	EM1w _b 101
<i>P. chrysogenum</i>	EM1s103, EM6wt134
<i>P. corylophilum</i>	EM2s109, EM3w _b 114, EM5s132, EM8s150
<i>P. steckii</i>	EM1w _t 102, EM3w _t 113, EM3s115, EM4w _t 117
<i>E. amstelodami</i>	EM4w _b 124
<i>E. repens</i>	EM3s116, EM4s126, EM4s127
<i>C. carpophilum</i>	EM9w _b 154
<i>C. cladosporioides</i>	EM4w _b 122, EM4s128

Aspergillus, *Penicillium*, *Cladosporium* and *Eurotium* (Table 1). Culturally dissimilar isolates from each sample were picked and identified as *Aspergillus*: *A. flavus*, *A. fumigatus*, *A. nidulans*, *A. penicillioides*, *A. sydowii*, *A. versicolor*; *Penicillium*: *P. asymetrica* sub-section *fasciculata*, *P. canescens*, *P. chrysogenum*, *P. corylophilum*, *P. steckii*; *Cladosporium*: *C. cladosporioides* and *C. carpophilum* and *Eurotium*: *E. amstelodami* and *E. repens*.

Halophilic nature of the isolates

The isolates that did not grow in absence of salt were characterized as obligate halophiles and those that grew in absence of added salt, but showed enhanced growth at increased levels of salt were termed as facultative halophiles. The halotolerance curves of the isolates are presented according to their obligate or facultative halophilic nature as shown in Figures 2a and b, respectively.

Obligate halophiles

Twenty-three obligate halophiles were obtained, all identified as *A. penicillioides*. These required a minimum of 2, 5 or 10% salt for growth (Figure 2a). Most were moderate halophiles, with optimal growth at 10% salt, and two at 5% salt. Most showed a euryhaline nature, having a capacity to grow on a wide range of salt concentrations up to 20% or even 30% salt; three

showed a stenohaline characteristic, with a narrow range of salt tolerance of 5 to 15%. Significant difference ($P < 0.05$) was obtained in the growth at different salt concentrations, as well as in the halotolerance curves of the isolates.

The isolates were obtained mainly from samples of top or bottom water, or sediment of S4 – S9. *A. penicillioides* had a very different appearance in colony characteristics from the other aspergilli, forming comparatively small colonies with a compact, tough felt growth and furrowed appearance; this also ensured that such isolates were picked from each of the samples in which they were found. Identification of a representative isolate, EM6s137, was confirmed by gene sequence analysis as *A. penicillioides*, the GenBank accession number given as JQ240645. The phylogenetic relationship of this isolate is shown in Figure 3.

Facultative halophiles

Thirteen isolates of aspergilli and all isolates of *Penicillium*, *Cladosporium* and *Eurotium* were facultative halophiles, randomly distributed throughout the stations; *Cladosporium* was isolated only from bottom water and sediment samples.

The isolates of *A. sydowii* and *A. penicillioides* showed maximal growth at 10% salt and could grow at salt concentrations up to 25 and 30%, respectively; *A. fumigatus* and *A. nidulans* isolates grew maximally at salt concentration of 5% and *A. versicolor* isolates showed

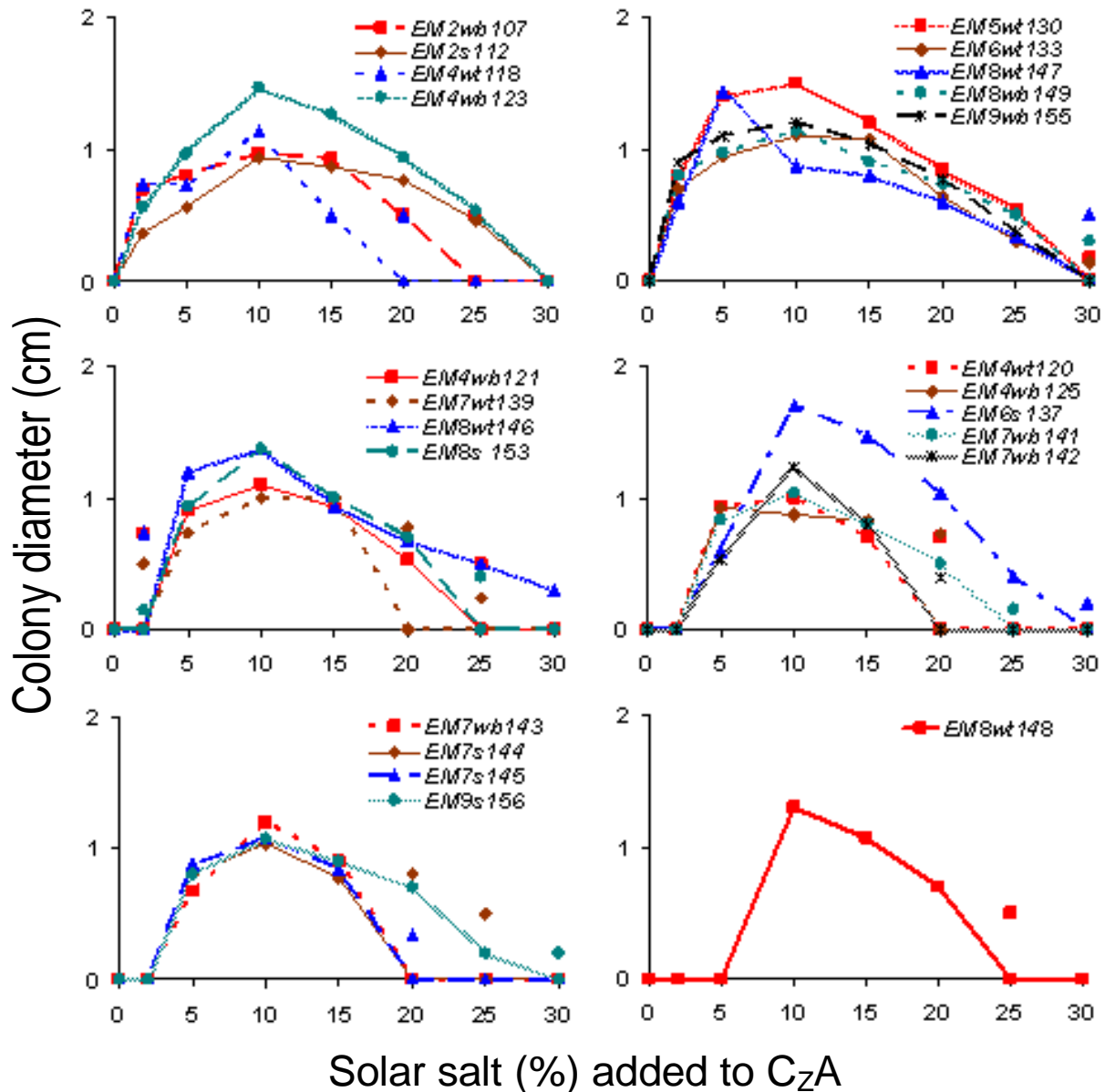


Figure 2a. Salt tolerance curves of obligate halophilic *A. penicillioides* as recorded after 7d incubation; unconnected symbols indicate delayed growth at respective salt concentrations, after 15d incubation.

optimum growth at 2 to 10% salt, with a tolerance level of 20 to 25% salt; *A. flavus* isolates had optimal growth in presence of 2 and 10% salt and tolerated a concentration of 15 and 25% salt, respectively.

The isolates of *P. asymetrica* subsection *fasciculata*, *P. canescens* and *P. chrysogenum* grew optimally at 5% salt and exhibited a tolerance level of 20 to 30% salt; *P. corylophilum* showed optimal growth with 2 or 5% salt, and *P. steckii* with 5 or 10% salt, all able to grow in presence of 20 to 25% salt.

Salt concentrations of 2 and 5% supported optimal growth of *C. cladosporioides* and *C. carpophilum*, respectively; these species tolerated a maximum of 20%

salt. Isolates of *Eurotium*: *E. amstelodami* and *E. repens* showed maximum growth in presence of 2 or 5% salt, and tolerance to 20 to 25%.

The isolates were euryhaline in nature, most of which were moderate halophiles; some grew optimally with 2% salt and were termed as slight halophiles. Significant difference ($P < 0.05$) was obtained in the growth of a given species at different salt concentrations, as well as between the strains of species of *A. versicolor*, *P. corylophilum* and *P. steckii*; however, similarity was found in the growth of strains of *P. asymetrica* subsection *fasciculata*, of *P. chrysogenum*, of *E. repens* and of *C. cladosporioides*.

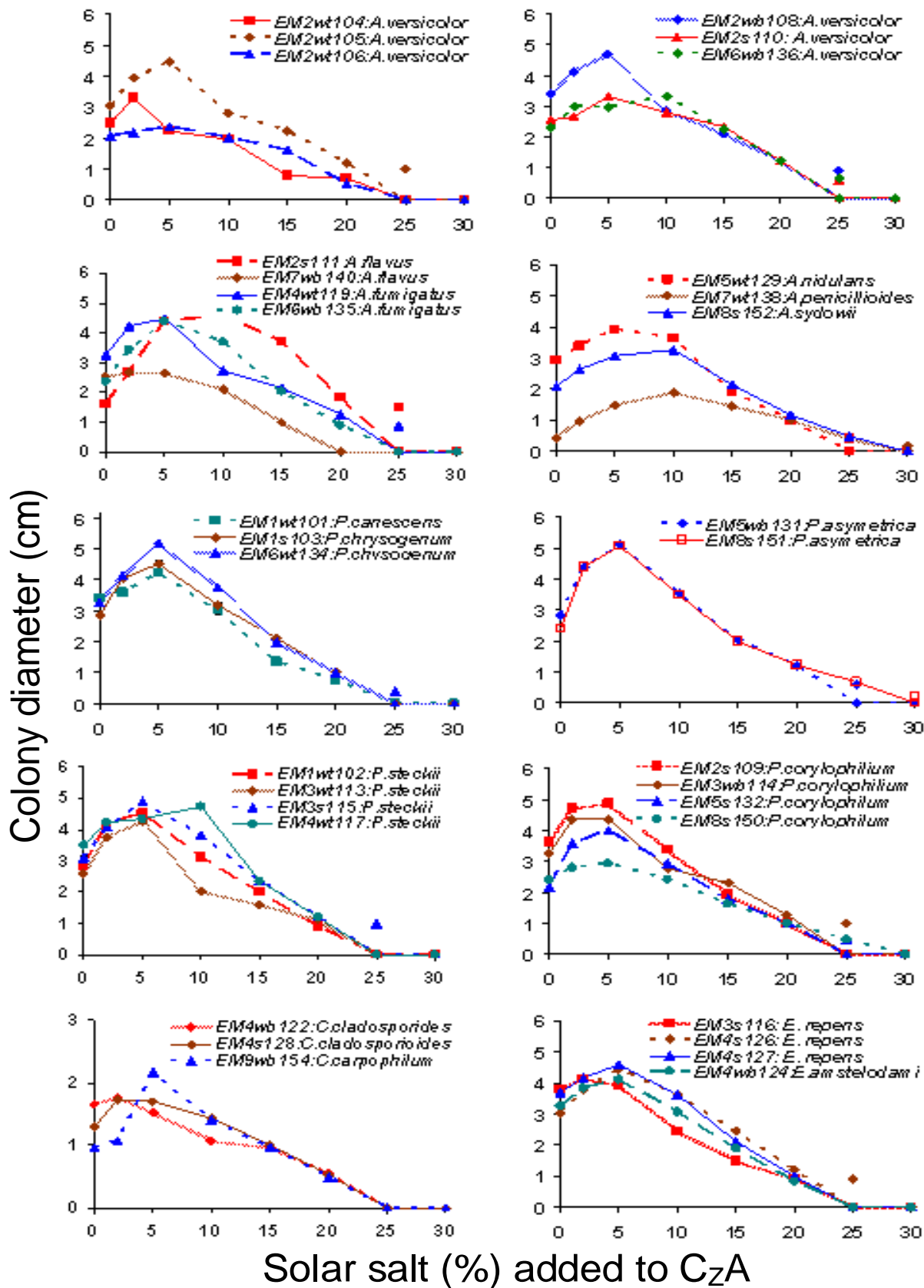


Figure 2b. Salt tolerance curves of facultative halophilic *Aspergillus*, *Penicillium*, *Cladosporium* and *Eurotium* as recorded after 7d incubation; unconnected symbols indicate delayed growth at respective salt concentrations, after 15d incubation.

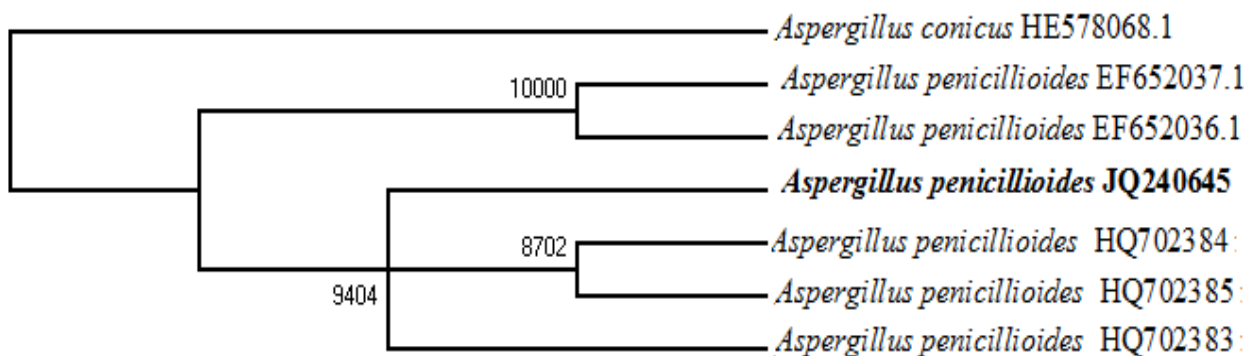


Figure 3. Phylogenetic tree obtained from the alignment of ITS region of rDNA of *A. penicillioides* species; the isolate *A. penicillioides* JQ240645 was obtained during the present study.

DISCUSSION

The work demonstrates the presence of moderate halophiles, in particular, the obligate halophile *A. penicillioides*, as well as other species belonging to the genera *Aspergillus*, *Penicillium*, *Eurotium* and *Cladosporium*, in the polyhaline environment of the estuary. The high concentration of salt used in the media for isolation, helped in the selective isolation of halophilic fungi over that of the non-halophiles.

Aspergillus and *Penicillium* were the dominant halophilic genera; *Eurotium* and *Cladosporium* were isolated in lesser numbers. While the genus *Aspergillus* had both obligate and facultative halophiles, *Penicillium*, *Cladosporium* and *Eurotium* species were exclusively facultative halophiles. These results corroborate earlier findings (Nazareth et al., 2012; Nayak et al., 2012). The obligate halophiles, by means of their absolute requirement for salt, are truly of marine origin. The facultative halophiles could be from terrestrial or fresh water environment, which have adapted so as to grow and sporulate in marine environment.

It was observed that the obligate halophilic *A. penicillioides* were found in greater numbers at the stations hinterland, although the salinity was lower, as compared to that at the mouth, where the salinity was nearly equal to that of sea water. Similar results were reported by Borut and Johnson (1962) wherein marine fungi were isolated from fresh and brackish sediments of an estuary, the irregular pattern of water currents giving rise to changes in environmental factors as well as determining the direction and extent of spore transport.

The presence of obligate halophiles upstream could be due to their ability to synthesize or take up compatible solutes from the estuarine environment, which is abundantly lined with mangroves. Tolerance of salinity has been viewed as a partial function of nutrient level (Borut and Johnson, 1962). It has been reported that fungi associated with standing litter of macrophytes could adapt to daily fluctuations of water availability through adjustment of their intracellular solute concentrations by

degradation of organic matter (Kuehn et al., 1998). It is known that in estuarine ecosystems, the detritus and marsh vegetation constitute a major part of the organic content (Manoharachary et al., 2005). The mangroves bordering the estuary, are more abundant upstream, where there is also a decrease in the cross-sectional area, and would serve as a nutrient source for the obligate halophiles. Earlier results have in deed shown the presence of the obligate halophile *A. penicillioides* in mangroves bordering the Mandovi estuary (Nayak et al., 2012). Tidal movement which extends to a distance of about 50 km (Sundar and Shetye, 2005), a point just beyond station 10, would aid the availability of the nutrient, the receding tide bringing particulate material from upstream of the estuary, and the rising tides carrying it back, thereby forming a nutrient reservoir (Verma and Agarwal, 2007). Interestingly, no halophile was obtained from the samples from station 10 hinterland where there were no mangroves.

Earlier reports have focused on isolation of halophiles only from hypersaline econiches (Buchalo et al., 1998; Gunde-Cimerman et al., 2000; Kis Papo et al., 2003a, b; Wasser et al., 2003; Steiman et al., 2004; Butinar et al., 2005a,b; Diaz-Munoz and Montalvo-Rodriguez, 2005; Gunde-Cimerman et al., 2005; Cantrell et al., 2006; Gunde-Cimerman et al., 2009; Hujsova et al., 2010; Nazareth et al., 2012; Nayak et al., 2012), which are expected to support the growth of such organisms. However, recent work records the finding of obligate halophiles from brackish waters of mangroves, with a salinity of 32‰ (Nayak et al., 2012). In this work, obligate halophiles were found in the polyhaline waters of the estuary, at a salinity range of 12 to 33‰.

This work demonstrates the occurrence of obligate moderately halophilic aspergilli in estuarine environment having salinity lower than that of the sea, thus indicating that hypersaline environments are not the sole econiches for true halophiles.

This is a first report on the isolation and description of an obligate halophilic *A. penicillioides* species from a polyhaline environment of an estuary.

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