

KAVAKA 39: 23—26, 2011

## Occurrence of the halophilic black yeast *Hortaea werneckii* from brackish waters of mangroves of Goa-India

Sarita Nazareth\*, Shweta Nayak and Valerie Gonsalves  
 Department of Microbiology, Goa University, Taleigao Plateau, Goa-403206, India  
 (Submitted on 14 December 2011; Accepted on 21 February 2012)

### ABSTRACT

The black yeast *Hortaea werneckii* was isolated from hypersaline solar salterns, considered as its primary ecological niche, as well as from brackish waters of mangroves, which has not been reported thus far. None of the isolates showed an obligate requirement of salt for growth, but grew optimally with 5-10% salt, with maximal tolerance of 20% salt, being facultative halophiles.

**Key Words:** *Hortaea*; mangroves; salterns; brackish, hypersaline.

### INTRODUCTION

The black yeast *Hortaea werneckii* was described by Nishimura and Miyaji (1984). It has been found in tropical and sub-tropical climates. Hypersaline waters have been considered as its primary ecological niche and it has been isolated from a number of salterns at Slovenia-Adriatic (Gunde-Cimerman *et al.*, 2000; Butinar *et al.*, 2005; Kogej *et al.*, 2005; Gunde-Cimerman and Plemenitas, 2006) and at Cabo-Rojo, Puerto Rico (Diaz-Munoz and Montalvo-Rodriguez, 2005; Cantrell *et al.*, 2006).

*Hortaea* has also been found in diverse saline habitats, such as sea-water, beach-soil, evaporite ponds, saltern microbial mats, marine fish and salt-preserved foods, arid inorganic and organic surfaces (Gunde-Cimerman and Plemenitas, 2006; Plemenitas *et al.*, 2008), existing at 0.5-5.2M NaCl of salterns (Plemenitas *et al.*, 2008)

The present work reports for the first time the occurrence of *H. werneckii* in the non-hypersaline, brackish waters of mangrove as well as in hypersaline salterns of Goa, India. Its absence from estuarine waters is indicated.

### MATERIALS AND METHODS

Samples of water/(w) and sediment/(s) were collected during the pre-monsoon summer month of May, from mangroves/(M) and solar salterns/(S), one site at Ribander/(R) alongside the Mandovi estuary, Goa, leading into the Arabian Sea, one interiorly at Santa Cruz/(C), along an offshoot of the estuary. The waters from the mangroves are channeled into the salterns. Sampling at the mangroves was carried out at

low tide, at a distance of 1m from the shore, from five areas 30cm apart. Water samples were collected at a depth of 0.5m and pooled together, while the sediment was scooped from the surface and likewise pooled together. Similarly, sampling of water and sediment at the solar salterns was carried out at five different salt pans and pooled. Mandovi estuary (EM) top water, bottom water and sediment were sampled using Niskin and Grab samplers, at ten stations beginning from the mouth, moving hinterland, between 73° 46.65 to 74° 2.5 longitude.

The pH and salinity of the water samples were determined using a Cyberscan pH meter and Atago hand-held refractometer. The sediment sample was suspended in deionised water (1:5, w/v), centrifuged at low speed to remove particulate matter. The pH was then measured as above.

The five samples from each of the locations were pooled together and processed as recorded by Nazareth *et al.*, (2011). They were plated on Czapek Dox Agar+20% solar salt (20%S-CzA) having 0.5g/L streptomycin and incubated at 30°C. Purified isolates were identified by colony and morphology characteristics, and by inhibition of growth at 37°C (McGinnis and Schell, 1985; <http://www.doctorfungus.org/thefungi/hortaea.php>). The identification was confirmed by analysis of the ITS1, 5.8S rRNA gene and ITS2, complete sequence (Merck-GeNei Services). The following primers were used:

ITS1-F: CTT GGT CAT TTA GAG GAA GTA A  
 ITS4 R: TCC TCC GCT TAT TGA TAT GC

The PCR conditions were one cycle of denaturation at 94°C (5 min), followed by 35 cycles of denaturation at 94°C (1min), annealing at 55°C (45 sec) and extension at 72°C (90 sec) and holding at

\*Corresponding author.

E-mail: saritanazareth@yahoo.com

72°C (10 min). The sequence was deposited at the GenBank with the accession number HQ711621. The entire sequence obtained was used to acquire sequence similarities using NCBI BLAST (Altschul *et al.*, 1997) and the nucleic acid databases, and the phylogenetic tree was constructed in Clustal X version 2 and the NJ distance method (Saluja and Prasad, 2007).

Halotolerance was studied as described earlier (Nazareth *et al.*, 2011), by spot inoculation in triplicates, of cell suspensions on CzA containing different concentrations of 0-30% solar salt; growth was measured in terms of colony diameter after 7d incubation, or 15d for those showing delayed growth.

## RESULTS

The pH of the mangroves and estuarine samples, saltern brine and sediment was  $7.0 \pm 0.16$ ,  $7.1 \pm 0.6$ ,  $7.8 \pm 0.2$  and  $6.1 \pm 0.5$  respectively. The salinity of mangrove waters was  $28 \pm 3\%$ . Salinity of the estuary waters varied from  $30 \pm 5\%$  for seven stations, to  $13 \pm 3\%$  at the last three stations hinterland. The saltern brine had a salinity of  $280 \pm 10\%$ .

A total of six black yeasts were obtained, three from the Mangrove water sample at Santa Cruz (MCw), two from the Saltern water sample at Santa Cruz (SCw) and one from the Saltern sediment sample

at Riband (SRs). No black yeast was found in the twenty samples of top and bottom waters and ten sediment samples obtained from ten stations spread over  $73^{\circ} 46.65'$  to  $74^{\circ} 2.5'$ , which indicated their absence from this estuarine habitat.

All the isolates had the same characteristics. They showed slow growth, forming moist, shiny colonies, turning black and covered with a thin layer of mycelium. They were unable to grow at  $37^{\circ}\text{C}$ . Mounts showed yeast-like conidia and annelloconidia; thick-walled mycelial forms were also observed as the colony aged. On the basis of these cultural and morphological characteristics, they were identified as *Hortaea werneckii*. The ITS sequence analysis of MCw215 confirmed it to be *H. werneckii*. The percent similarity with sequences of other isolates is shown in Table 1 and Fig. 1.

Isolates had a wide range of halotolerance (Fig.2), able to grow in the absence of added salt, with optimal growth of MCw215, MCw217, SCw250 and SCw251 at 5% salt, SRs238 at 5% – 10% salt and MCw214 at 10% salt; all grew at concentrations of 25% salt. Two isolates showed a delayed growth, with no colony formation in the first seven days and were recorded as 'no growth'; the colony diameter obtained after a total of 2 weeks, is shown by an unconnected

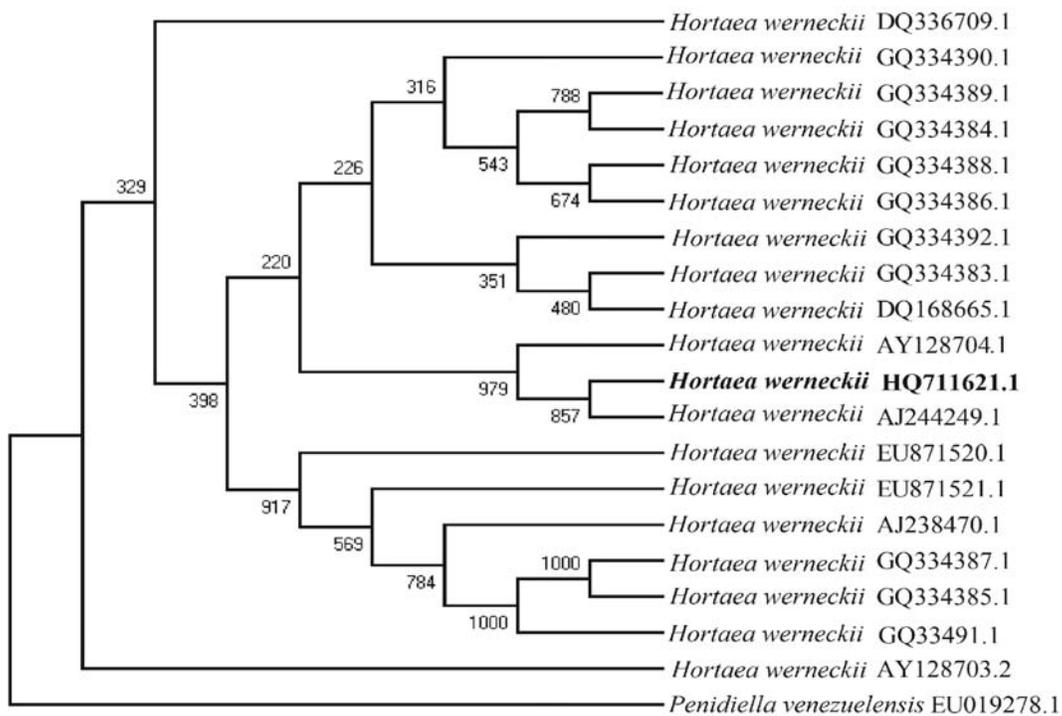


Fig. 1. Phylogenetic tree obtained from the alignment of ITS region of rDNA of *Hortaea werneckii* species; the isolate obtained from the present study is indicated in bold, along with the GenBank accession number.

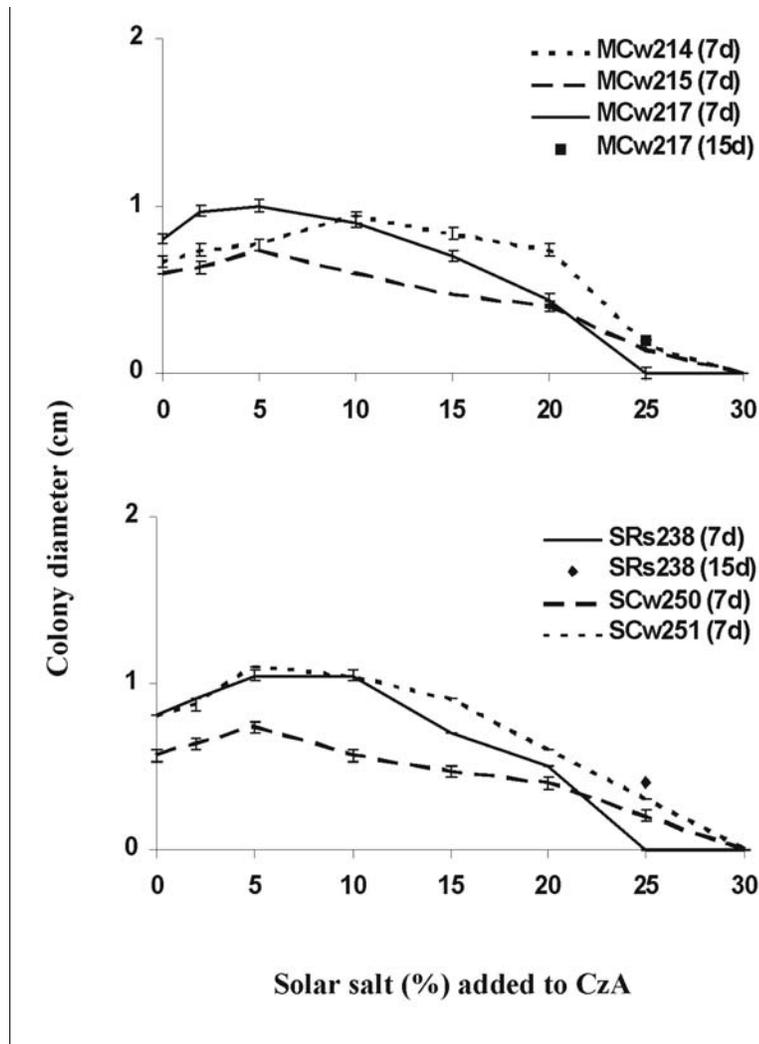


Fig. 2. Salt tolerance of isolates recorded after 7 days incubation; unconnected points indicate delayed growth at the respective salt concentration, recorded after 15 d incubation.

symbol in the figure, to indicate the difference in the incubation period.

**Table 1.** Percent similarity of *Hortaea werneckii* MCw215 (HQ711621) with other isolates of *H. werneckii*

Accession Number	Percent Similarity
FJ755827	100
AY213656	100
GQ334392	99
GQ334390	99
GQ334389	99
GQ334384	99
GQ334383	99
GQ334388	98
GQ334387	98
GQ334386	98
GQ334385	98
DQ168665	97

## DISCUSSION

*Hortaea werneckii* was amongst the first mycobiota isolated from hypersaline environments (Gunde-Cimerman *et al.*, 2000), their halophilic behaviour and complex polymorphic life cycle ensuring their dominance over other fungi (Gunde-Cimerman and Plemenitas, 2006). However, it has not been found in all hypersaline waters. Although isolated from eutrophic thalassohaline waters, Puerto Rico, they have not been found in oligotrophic salterns of Eilat, at Red Sea, Israel, nor in athalassohaline waters of Salt Lake, Utah, or that of Dead Sea (Gunde-Cimerman and Plemenitas, 2006; Nazareth *et al.*, 2011).

The absence of *H. werneckii* in estuarine waters in this study, corroborates findings that melanized fungi predominate the mycobiota at higher salinities (Gunde-Cimerman and Plemenitas, 2006); its isolation from solar salterns, Goa, a first such record from the

Indian sub-continent, confirms the observation that they are present globally in such environments (Butinar *et al.*, 2005). Furthermore in this study, *H. werneckii* was also isolated from mangroves, hitherto unreported. The mangrove ecosystem, which has a salinity of 2.5–3.0‰ or 0.5M, is rich in detritus, which lends itself to sustaining *H. werneckii* even outside hypersaline environments. The presence of *Hortaea* in soil and decaying vegetation is known [<http://www.doctorfungus.org/thefungi/hortaea.php>].

It is noteworthy that although isolation of *H. werneckii* poses difficulties because of its slow-growing nature and low competitive ability (Gunde-Cimerman *et al.*, 2000), despite its co-existence with a diverse group of microorganisms, it was successfully isolated from mangroves, supported by the choice of CzA+salt isolation medium, in preference to a rich, more easily assimilated carbon medium with salt, the latter encouraging growth of faster-growing, halotolerant fungi, that could outgrow the black yeast.

The slow growth of isolates and a wide salt tolerance range of 0-25% added salt, with optimal growth at 5-10% salt, is in keeping with earlier findings (Gunde-Cimerman *et al.*, 2000; Kogej *et al.*, 2005; Diaz-Munoz and Montalvo-Rodriguez, 2005). *H. werneckii* has been termed as halophilic because of its ability to grow at near saturation salt concentrations of 32%, with a broad growth optimum of 6-14% salt (Gunde-Cimerman *et al.*, 2000; Marbaniang and Nazareth, 2006). However, because of its ability to grow even in absence of salt, it has also been categorized as halotolerant (Plemenitas *et al.*, 2008). They have been termed here as facultative halophiles, on the basis that the isolates showed no obligate requirement for salt, yet showed halophilic nature, with optimal growth in presence of 5–10% salt, growing at concentrations of even 25% salt.

Hypersaline waters have been named as an ecological niche for *Hortaea* (Gunde-Cimerman *et al.*, 2000); the data presented here, records for the first time its existence in solar salterns of Goa and of India. It is suggested that salt might be a decisive factor in its ecology (Gunde-Cimerman and Plemenitas, 2006). However, this black yeast has now been isolated from the brackish waters of mangroves, this being the first report for its occurrence in mangroves world-wide.

## REFERENCES

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. 1997. Gapped

BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389-3402.

Butinar, L., Santos, S., Spencer-Martins, I., Oren, A. and Gunde-Cimerman, N. 2005. Yeast diversity in hypersaline habitats. *FEMS Microbiol. Lett.* 244: 229-234.

Cantrell, S.A., Casillas-Martínez, L. and Molina, M. 2006. Characterization of fungi from hypersaline environments of solar salterns using morphological and molecular techniques. *Mycol. Res.* 110: 962-970.

Diaz-Munoz, G. and Montalvo-Rodriguez, R. 2005. Halophilic black yeasts in the Cabo Rojo solar salterns: its first record for this extreme environment in Puerto Rico. *Caribb. J. Sci.* 41: 360-365.

Gunde-Cimerman, N. and Plemenitas, A. 2006. Ecology and molecular adaptation of the halophilic black yeast *Hortaea werneckii*. *Rev. Environ. Sci. Biotechnol.* 5: 323-331.

Gunde-Cimerman, N., Zalar, P., de Hoog, G.S. and Plemenitas, A. 2000. Hypersaline water in salterns – natural ecological niches for halophilic black yeasts. *FEMS Microbiol. Ecol.* 32: 235-240.

Kogej, T., Ramos, J., Plemenitas, A. and Gunde-Cimerman, N. 2005. The halophilic fungus *Hortaea werneckii* and the halotolerant fungus *Aureobasidium pullulans* maintain low intracellular cation concentrations in hypersaline environments. *Appl. Environ. Microbiol.* 71: 6600-6605.

Marbaniang, T. and Nazareth, S. 2006. Isolation of halotolerant *Penicillium* species from mangroves and salterns and their resistance to heavy metals. *Curr. Sci.* 92: 895-896.

McGinnis, M.R., Schell, W.A. and Carson, J. 1985. Phaeoannellomyces and the Phaeococcomycetaceae, new dematiaceous blastomycete taxa. *Sabouraudia* 23: 179-88.

Nazareth, S., Gonsalves, V. and Nayak, S. 2011. A first record of obligate halophilic aspergilli from the Dead Sea. *Ind. J. Microbiol.* 52: 22-27.

Nishimura, K. and Miyaji, M. 1984. *Hortaea*, a new genus to accommodate *Cladosporium werneckii*. *Jpn. J. Med. Mycol.* 26(2): 139-146.

Plemenitas, A., Vaupotic, T., Lenassi, M., Kogej, T. and Gunde-Cimerman, N. 2008. Adaptation of extremely halotolerant black yeast *Hortaea werneckii* to increased osmolarity: a molecular perspective at a glance. *Stud. Mycol.* 61: 67-75.

Saluja, P., and Prasad, G.S. 2007. *Cryptococcus rajasthanensis* sp. nov., an anamorphic yeast species related to *Cryptococcus laurentii*, isolated from Rajasthan, India. *Int. J. Syst. Evol. Micr.* 57: 414-418.