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## Halophilic Black Yeast *Hortaea werneckii* in the Cabo Rojo Solar Salterns: Its First Record for this Extreme Environment in Puerto Rico

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**ABSTRACT.**—*Hortaea werneckii* is a black yeast-like hyphomycete associated with the human superficial infection tinea nigra. This fungus has been reported from several environments around the world and it has shown a preference to hypersaline habitats. Since 2001, we have been studying the fungal diversity present at the solar salterns of Cabo Rojo, Puerto Rico. Morphological analyses revealed that several of the isolated strains can be classified as members of the genus *Hortaea*. These strains had the highest frequency of isolation and grew at the maximum NaCl concentration (25%) tested (optimum at 10%) and had an optimal growth temperature of 29°C. Restriction pattern length polymorphism analysis of a PCR amplicon containing the 5.8S rDNA gene and the internal transcribed spacer regions 1 and 2 showed a consensus pattern for the 12 *Hortaea* isolates. Therefore, strains SC1 and SC29 were selected randomly as representatives of this group for further analyses. Phylogenetic analysis of the ITS rDNA sequenced molecule for these representatives places them in the *Hortaea werneckii* monophyletic cluster. This report constitutes the first record of this fungus from a hypersaline environment in Puerto Rico.

**KEYWORDS.**—*Hortaea werneckii*, hypersaline, Caribbean, mycobiota, molecular taxonomy

*Hortaea werneckii* is a melanized yeast-like fungus capable of causing tinea nigra in humans (de Cock 1994). This infection is characterized by an asymptomatic brown or black macule on the hands and feet and

is common to tropical habitats (de Hoog and Gerrits van den Ende 1992). Even though *H. werneckii* has been recovered from several environments, its ecological niche is hypersaline water habitats (Gunde-Cimerman et al. 2000). Reports on the diversity and role of fungi at these environments are scarce (Buchalo et al. 1998; Gunde-Cimerman et al. 2000). Solar salterns are originated from the evaporation of sea water and, therefore, have an ion composition dominated mainly by sodium and chloride making it an extremely halophilic niche. The NaCl concentration of these man-made pools can range from 3% to saturation (35%). Other properties of this environment are: low oxygen concentration, high light intensity, ample nutrient availability, and neutral pH (Brock 1979). Even though the microbial diversity present at this hostile habitat is low compared to mesophilic environments, representatives can be found from the three domains of life: Archaea, Bacteria and Eukarya (Benlloch et al. 2000; Litchfield and Gillevet 2002; Gunde-Cimerman et al. 2000).

Like other salterns, the Cabo Rojo system consists of a series of interconnected ponds in which the concentration of salt increases as sea water evaporates by solar radiation (Davis 1978). We have been studying the prokaryotic diversity of these salterns for the past years (Montalvo-Rodríguez et al. 1997, 1998, 2000). To our knowledge, there are no reports about the occurrence of halophilic and extremely halotolerant fungi from extreme environments in the Caribbean, especially in Puerto Rico. Here we report the isolation and characterization of *Hortaea werneckii* which constitutes the first record of this fungus from a hypersaline environment in Puerto Rico and the Caribbean. This study represents the first attempt to determine the mycobiota present at an extreme environment in Puerto Rico.

In sterile plastic bags, we collected 36 samples of 250 ml of saltern water from each corner of three different ponds at the solar salterns of Cabo Rojo, Puerto Rico. The average values for temperature and NaCl concentration were 35.5°C and above 28%, respectively. From each sample, 50 mL were filtered through 0.45 µm nitrocel-

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lulose membranes and transferred onto three different agar media: Sehgal and Gibbons medium with 15% NaCl (Sehgal and Gibbons 1960), Malt extract medium with 15% NaCl and Potato dextrose medium with 15% NaCl, each at a final pH of 5. Solidification of each medium was achieved by adding 20 g/L agar. Plates were incubated at 30°C. Pure colonies were obtained by repeated transferring of separate colonies on agar plates.

After colony growth, samples were prepared for bright-field microscope and Scanning Electron Microscope (SEM) analyses. For SEM analysis, a piece of membrane of approximately 1 cm<sup>2</sup> (containing cell mass) was removed and fixed using 2% glutaraldehyde-2.5% paraformaldehyde in a 0.1 M potassium phosphate buffer solution. Cells were fixed for 24 hours. The samples were then rinsed in phosphate buffer 3 times. Samples were dehydrated in an ethyl alcohol gradient using the following concentration (in %): 10, 25, 35, 45, 55, 65, 75, 85, 95 (30 min each). The sample was then treated three times (30 min each) with 100% ethyl alcohol. The preparations were dried using the critical point drying technique and all samples were placed on an aluminum stub to be coated with gold in Argon gas. The preparations were analyzed in a JEOL JSM-541 OL SEM microscope at 15 kv.

Optimal growth was measured in SG medium containing NaCl at 0, 5, 10, 15, 20 and 25% (wt/v) and at temperatures of 25, 29, 35 and 40°C each. Comparison and reproducibility of results was achieved by always inoculating 10<sup>6</sup> spores. The spore suspension was prepared as described by Stretch et al. 2001. Data were recorded after 7 days of incubation.

Genomic DNA extraction was performed using Q-Biogene Fast DNA Kit® according to manufacture's protocol. ITS rDNA regions were selected to perform the molecular taxonomy on the isolates and a set of primers described by White et al. (1990) was used for amplification (Fig. 2). These primers are ITS1 5'-TCC GTA GGT GAA CCT GCG G-3' and ITS4 5'-TCC TCC GCT TAT TGA TAT G-3' and they amplify a fragment of approximately 550 bp containing the ITS1, 5.8S rDNA gene and ITS2 re-



FIG. 1. Location of the Solar Salterns of Cabo Rojo, Puerto Rico.

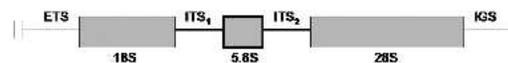


FIG. 2. Schematic representation of ribosomal genes organization present in most fungi. Target areas used in the amplification of ITS and 5.8S rDNA regions are shown in bold.

gions. PCR reactions (small and large scale volume) were performed using a protocol modified by Giovanni López (pers. comm.). The reaction mixture consisted of  $\approx 10$  ng of template, 1X PCR buffer, 300  $\mu$ M dNTP's, 3 mM MgCl<sub>2</sub>, 0.66 pmol $\mu$ l<sup>-1</sup> of each primer and 0.025 U $\mu$ l<sup>-1</sup> of AccuTaq LA DNA Polymerase. After initial denaturation of DNA at 95°C for 5 minutes, thirty cycles of amplification were performed. Each cycle consisted of a denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension step at 72°C for 1 minute and a final extension at 72°C for 10 minutes. Following amplification, PCR products were cleaned using MinElute Gel Extraction Kit (USA QIAGEN Inc.) according to manufacture's protocol. Digestion of the amplified

PCR products for RFLP analysis was performed with restriction enzymes *BsaHI* and *HaeIII* for an hour (twice) at 37°C in a final volume of 10 µl. Selected PCR products were sent to the University of Iowa's DNA sequencing facility. The DNA sequences obtained (GeneBank accession numbers AY820141 and AY820140 for strains SC1 and SC29 respectively) were used for *in silico* similarity analysis using the National Center for Bioethnology Information BLAST program

(<http://www.ncbi.nlm.nih.gov/BLAST/>). Selected sequences were used to determine the isolates phylogenetic position with respect to similar strains. ITS rDNA sequence similarity values were calculated by pairwise comparison of the sequences within the alignment. Seqboot was used to generate 100 bootstrapped data sets. Distance matrices were calculated with dnadist. One hundred trees were inferred by using neighbor joining analyses. Any bias introduced by the order of sequence addition was minimized by randomizing the input order. Consense was used to determine the most frequent branching order. The final tree was drawn using treeview program (Page 1996).

After 4 days of incubation the inoculated membranes, containing water samples from the solar salterns of Cabo Rojo, Puerto Rico, showed several fungal colonies. Among the 59 isolates obtained on the 36 samples, several strains were morphologically classified as *Aspergillus*, *Cladosporium*, *Alternaria*, *Penicillium*, and *Stemphylium*. Under bright field microscopy and SEM several isolates (SC1, SC8, SC9, SC10, SC12, SC13, SC14, SC15, SC16, SC18, SC19 and SC29) showed dark septate mycelia and an-ellidic budding (Fig.3). This group had the highest frequency of isolation among all the fungi obtained from the solar salterns. The morphological characteristics observed on these strains corresponded to those described in the literature for *Hortaea* (de Hoog and Gerrits van den Ende 1992). Restriction fragment length polymorphism analysis of a PCR amplicon containing the 5.8S rDNA and ITS1-ITS2 regions was used to molecularly classify the strains into groups. The restriction pattern obtained

with enzymes *BsaHI* and *HaeIII* revealed that the isolates could not be divided in different groups and might represent the same *Hortaea* species. Therefore, strains SC1 and SC29 were selected randomly as representatives of this group for further analyses. Physiological experiments on strains SC1 and SC29 revealed that even though growth was recorded at 0% NaCl, they can be classified as halophilic by their ability to grow at 25% NaCl. Growth at NaCl saturation conditions was previously reported for *Hortaea werneckii* isolated from a solar saltern in Slovenia (Gunde-Cimerman et al. 2000). The optimum salt concentration for both strains was around 10% NaCl and the optimal temperature was around 29°C: this combination showed the highest colony diameter (10 mm) of conditions tested. The least growth occurred in all salinities tested at 35 and 40°C (average colony diameters of 3.5 mm). Even though it has been reported that *Hortaea werneckii* isolated from extreme environments grow optimally at temperatures between 20-25°C (Zalar et al. 1999a; Gunde-Cimerman et al. 2000), our results indicated that the isolated strains in this study might prefer higher temperatures (29°C). This might show an adaptation of the genus to the usual high temperatures of tropical environments. De Hoog and van den Ende (1992) reported that their cultures of *Hortaea werneckii* produced more hyphae and became more melanized at 30°C.

A PCR amplicon consisting of the ITS1-5.8SrDNA-ITS2 for each isolate was submitted for sequencing to determine the taxonomical position of these strains molecularly. *In silico* analysis of these sequences revealed similarities to strains of *Hortaea werneckii*. This information was used to construct a phylogenetic tree using the neighbour-joining method (Fig. 4). The consensus distance tree places these isolates in the *H. werneckii* monophyletic cluster. All the results presented here strongly suggest that isolates SC1 and SC29, as well as the group they represent, are strains of *H. werneckii*.

There have been several reports on the existence of fungi on hypersaline environments. *H. werneckii* was among these iso-

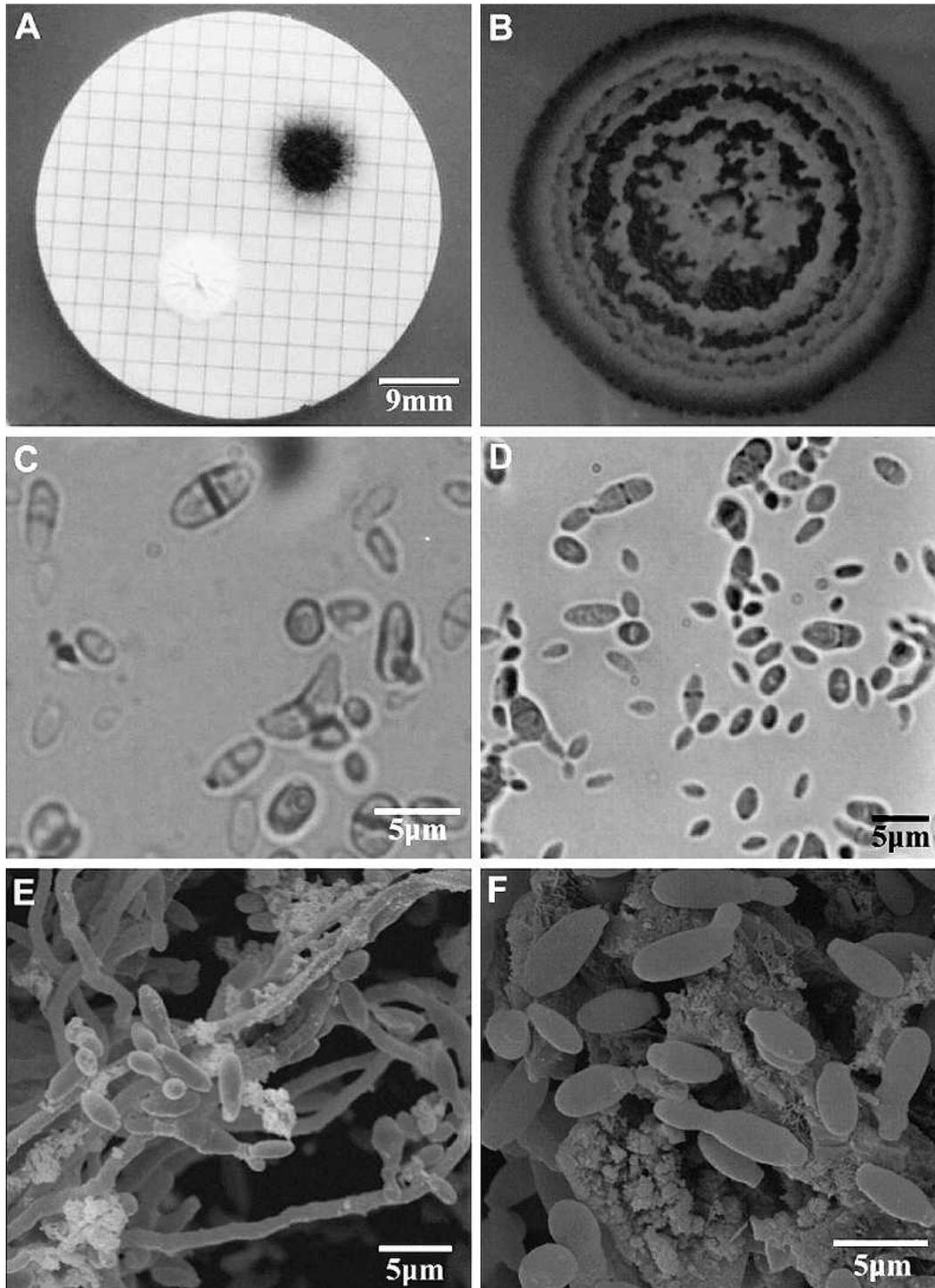


FIG. 3. (A) Strain SC1 (represented by the dark colony) growing on Sehgal and Gibbons (15% NaCl) after 4 days of incubation at 30°C; (B) Colony appearance for strain SC29 on Sehgal and Gibbons (10% NaCl); Bright Field micrographs for Strain SC1 (C-D) and SEM for strain SC29 (E-F) showing different types of propagation: multilateral budding and annelidic budding (unipolar or bipolar).

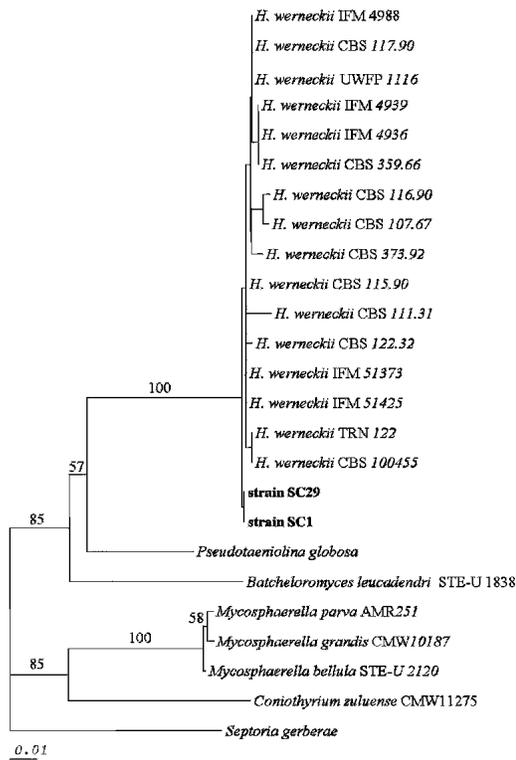


FIG. 4. Neighbour-joining distance tree of partial ITS1-5.8S rDNA gene-ITS2 sequences for strains SC1 and SC29 (accession numbers AY820141 and AY820140 respectively). Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 50% are shown. *Septoria gerberae* was used as the outgroup.

lates and it has been determined that the natural ecological niche is this type of habitat. In the solar saltern ponds from Slovenia, several black yeast-like fungi species from the genera *Phaeotheca* and *Trimmatostroma* and *Aureobasidium pullulans* were also found (Gunde-Cimerman et al. 2000). The halophilic yeast *Trimmatostroma salinum* was reported before as new species from the same Slovenian solar saltern (Zalar et al. 1999b). The isolation of strains of *Hortaea werneckii*, a halophilic yeast-like melanized fungus, from the solar salterns of Cabo Rojo, Puerto Rico constitutes the first report of this organism from a hypersaline niche in the Caribbean.

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