During a survey of black yeasts of marine origin, some isolates of *Hortaea werneckii* were recovered from scuba diving equipment, such as silicone masks and snorkel mouthpieces, which had been kept under poor storage conditions. These yeasts were unambiguously identified by phenotypic and genotypic methods. Phylogenetic analysis of both the D1/D2 regions of 26S rRNA gene and ITS-5.8S rRNA gene sequences showed three distinct genetic types. This species is the agent of tinea nigra which is a rarely diagnosed superficial mycosis in Europe. In fact this mycosis is considered an imported fungal infection being much more prevalent in warm, humid parts of the world such as the Central and South Americas, Africa, and Asia. Although *H. werneckii* has been found in hypersaline environments in Europe, this is the first instance of the isolation of this halotolerant species from scuba diving equipment made with silicone rubber which is used in close contact with human skin and mucous membranes. The occurrence of this fungus in Spain is also an unexpected finding because cases of tinea nigra in this country are practically not seen.

**Keywords** *Hortaea werneckii*, polyethylene plastic, scuba diving, silicone rubber, tinea nigra

### Introduction

*Hortaea werneckii* (Horta) Nishim. & Miyaji 1984, formerly classified as *Cladosporium werneckii* Horta, *Exophiala werneckii* (Horta) v. Arx and *Phaeoannellomyces werneckii* (Horta) McGinnis & Schell among other taxa, is the etiologic agent of human tinea nigra, a superficial mycosis affecting palms of hands and sometimes causing similar lesions on the soles of feet [1]. This mycosis was originally observed in 1891 by Alexandre Cerqueira in Salvador (Bahía), Brazil, who named it keratomycosis nigra palmaris [2].

Tinea nigra has generally been considered to be a tropical disease, being rarely diagnosed in Europe. In fact it is considered an imported fungal infection in Europe [3,4] being much more prevalent in warm, humid parts of the world, such as the tropical regions of Central America, South America, Africa, and Asia and infrequently in USA and Australia [5–7]. Predisposing factors associated with tinea nigra are hyperhydrosis and exposure in coastal sea areas or hypersaline environments, where the causative agent may be acquired from the natural habitat [5]. *Hortaea werneckii* is a halophilic saprobe which occurs in natural salt pans [1] being the dominant species in hypersaline evaporation ponds [8].

In searching for the etiologic agent of an *Exophiala* infection of a dolphin in the Mediterranean Sea (unpublished data, F.J. Cabañes & M. Domingo), *H. werneckii* was isolated from moldy scuba diving equipment, such as silicone masks, straps and snorkel mouthpieces, kept in bad storage conditions. In this paper, we describe the unexpected occurrence of this fungus on this special equipment usually used in close contact with human skin and mucous membranes. We have also screened other environmental samples for the presence of *H. werneckii* in close
surrounding areas where the moldy scuba diving equipment was maintained.

**Material and methods**

*Sampling, culture media used and isolation of strains*

Moldy scuba diving equipment was stored in the bilge of a boat in the Sant Feliu de Guixols marina (41°46.6′ N, 3°01.9′ E; Mediterranean Sea coast), Catalonia, Spain. Direct inspection of some silicone rubber and plastic samples showed what appeared to be black fungal microcolonies (Fig. 1). Silicone skirts and straps of three scuba diving masks, mouthpieces of snorkels and a polyethylene plastic bag were sampled by rubbing the surfaces with sterile cotton swabs, pre-moistened in sterile physiological solution. Three plastic dock fenders and wood boards of the dock with visible moldy growth were also sampled using the same technique. Six samples of wet granitic sand in contact with sea water were also aseptically collected from the bay where the boat was moored. Samples were transported to the laboratory within one day of collection where the 32 specimens were screened for the presence of *H. werneckii*. Sampling was conducted during spring and summer 2011.

All samples were inoculated onto Sabouraud glucose agar (SGA; Biolife s.r.l., Milano, Italy) and Sabouraud glucose agar supplemented with 20% of NaCl (SGA20NaCl), both of which contained chloramphenicol (0.05%). The latter medium was prepared and used in this study for the selective isolation of *H. werneckii* since it tolerates NaCl concentrations of up to 27% [9]. Swabs were streaked over the surface of culture media, while granitic sand samples were inoculated onto the same culture media by sprinkling wet sand grains over the surface of the plates. Plates were incubated at 25°C and examined daily for 30 days. When growth of black yeasts was detected, colonies were selected and subcultured on SGA for preservation and storage at −80°C.

*Morphological and physiological characterization*

The identification of the *H. werneckii* isolates was carried out based on macroscopic and microscopic characteristics of the colonies on SGA, potato dextrose agar (PDA; Becton Dickinson) and oatmeal agar (OMA; Becton Dickinson) cultures after incubation at 25°C for 14 days [1]. Other tests, such as the isolates temperature tolerance (20, 25, 30, 35, 37 and 40°C) on SGA for 28 days, were also performed.

**ITS-5.8S rRNA gene and D1/D2 26S rRNA gene sequencing and analysis**

DNA was extracted and purified directly from seven day-old cultures in SGA according to the FastDNA Spin kit protocol with the FastPrep FP-24 instrument (MP Biomedicals, Biolink, Barcelona, Spain). The DNA was kept at −20°C until used as template for PCR amplification.

Following the DNA extraction, internal transcribed spacers (ITS1 and ITS 2) and the 5.8S rRNA gene were amplified with the conserved fungal primer pair ITS5 and ITS4 [10]. The variable D1 and D2 regions of the 26S rRNA gene were amplified by using the conserved fungal primers NL1 and NL4 [11]. The PCR products were purified with MultiScreen filter plates (Millipore, Barcelona, Spain) following the manufacturer’s protocol. The purified product was used as a template for sequencing. The BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and primers ITS5/ITS4 and NL1/NL4 were used for sequencing as specified by the manufacturer. An Applied Biosystems mod. 3730 sequencer was employed to obtain the DNA sequences. Sequence alignments were performed using the software program Clustal X v1.81. The neighbor joining method was performed using Mega 4 package and the algorithm Kimura 2-parameter was used to obtain the distance tree. Support of internal branches was assessed by a search of 1,000 bootstrapped sets of data. Selected
sequences of \textit{H. werneckii} deposited in GenBank were used to determine the isolates phylogenetic position with respect to similar strains. Sequences of \textit{Penidiella venezuelensis} CBS 106.75 and \textit{Pseudotaeniolina globosa} CBS 109889, members of the family Teratosphaeriaceae, were used as outgroup.

The nucleotide sequences of the ITS-5.8S rRNA gene and D1/D2 26S rRNA gene and determined in this study have been deposited in the GenBank and their accession numbers are given in Figure 3.

**Results**

In total, six black yeast isolates were recovered from the samples and all fit the phenotypic characteristics of \textit{H. werneckii} (Fig. 2). Two isolates were recovered from two silicone skirts of scuba diving masks (Hw1 and Hw2), one from a strap of a mask (Hw3), one from a mouthpiece of a snorkel (Hw4) and two from the plastic bag (one from the bag [Hw5] and one from the bag handles [Hw6]). Hw1-Hw6 are the strain numbers assigned to the isolates as part of the Culture Collection of the Veterinary Mycology Group, UAB, Barcelona, Spain. No black yeasts was detected on SGA or SGA20NaCl cultures inoculated with granitic sand, plastic dock fenders and wood boards of the dock.

Growth of \textit{H. werneckii} isolates was detected easily and earlier on SGA20NaCl plates than on SGA plates. A few other molds were found (e.g., \textit{Aspergillus} spp., \textit{Alternaria} spp., \textit{Penicillium} spp.) but they were not consistently recovered on the SGA plates, with the exception of \textit{Cladosporium} spp. High level of salt contained in SGA20NaCl medium inhibited the development of these moulds.

Colonies of \textit{H. werneckii} were slow growing, reaching a diameter of 10–13 mm on SGA, 11–13 mm on OMA, and 13–16 mm on PDA after 14 days at 25°C. Optimal growth was observed at 20°C and 25°C. The isolates grew slowly and formed smaller colonies at 30°C. No growth was observed at 35, 37 and 40°C.

A BLAST search on GenBank of both genes revealed that all strains had an identity of 99–100% to \textit{H. werneckii}. Comparison of the ITS-5.8S rRNA gene sequences of all isolates showed three genetic types. The length of the section of DNA sequenced ranged from 551–553 base pairs. The D1/D2 region of the 26S rRNA gene sequenced included 579 base pairs. Comparison of the sequences of all isolates showed again three genetic types that were at least 99% similar by D1/D2 sequence. Figure 3 shows the molecular phylogenetic trees based on the ITS-5.8S rRNA (Fig. 3a) and the D1 and D2 regions of the 26S rRNA (Fig. 3b) gene sequences constructed by the neighbor-joining method.

**Discussion**

\textit{Hortaea werneckii} is a melanized meristematic fungus included in the Teratosphaeriaceae family which includes mainly saprobes and plant pathogenic species in the Capnodiales, Dothideomycetes [12]. It is an extremely halotolerant species and the dominant black yeast species in hypersaline waters at salinities above 3.0 M NaCl (e.g., hypersaline evaporation ponds) [8,13]. This yeast has been also found in beach soil from the Canary Islands [14] and as a contaminant of an Amazonian salted fish causing food spoilage [15]. In our study, the culture medium containing 20% salt (SGA20NaCl) was a good selective medium for the recovery of \textit{H. werneckii} from natural environments. Growth on this medium was primarily restricted to \textit{H. werneckii} colonies and the medium could be used as a selective isolation medium for this halotolerant species in these kinds of samples.

In our study, isolates of \textit{H. werneckii} were unambiguously identified by phenotypic and genotypic means. Phylogenetic analysis of both the D1/D2 regions of 26S rRNA gene and ITS-5.8S rRNA gene sequences showed three distinct genetic types. Full concordance was observed with clustering of the isolates using the above-mentioned partial genome sequences. These clustered with sequences of \textit{H. werneckii} from different sources (cases of tinea nigra and environment). However, no correlation was observed between sequence type and origin of the isolates. The variability observed in both ITS-5.8S and 26S rRNA genes
Fig. 3  Neighbor-joining trees based on Kimura 2-p corrected nucleotide distances among sequences of the ITS1-5.8S-ITS2 region (a) and of the D1/D2 domains (b) of the rRNA gene from *Hortaea werneckii* strains. Bootstrap replication frequencies over 70% (a) and 50% (b) (1,000 replications) are indicated at the nodes. *Penidiella venezuelensis* (EU19278 and EU19278) and *Pseudotaeniolina globosa* (AY128700 and EU19283) were used as outgroup.
indicated that the species is quite heterogeneous, as reported previously [16].

Although H. werneckii has been reported in Europe from hypersaline water [13], sea water-sprayed marble [9] and a Mediterranean sponge [17], this is, to our knowledge, the first time that it has been recovered from silicone rubber and polyethylene plastic in contact with sea water. Perhaps, the selective common factor to these substrates is a relatively high salt concentration and possibly the synthetic polymers of the polyethylene plastic and silicone rubber. Polyethylene is the most widely used plastic and its use in plastic implants and medical devices has led to a concomitant increase in the incidence of device-related infections, with the most common fungal pathogen being Candida albicans [18]. However, H. werneckii has not been isolated from polyethylene plastic so to our knowledge this is the first time that this species is reported from this material.

Silicone rubber is generally non-reactive, stable, biologically inert and resistant to extreme environments and temperatures and can be found in a wide variety of products such as home sealants, food storage products, sports-wear, footwear, electrical and electronic components, medical devices and implants. Silicone rubber is also used to make the skirts and straps on most modern scuba diving masks. Candida biofilms have been reported in silicone rubber used in medical devices [19–21] or in silicone rubber prostheses [22]. Nevertheless, H. werneckii has been not isolated from silicone rubber in these studies so to our knowledge this is also the first time that this black yeast is reported from this stable material. However, other black yeasts have been isolated from silicone seals in the shower room of a hospital ward [23] or from rubber seals of the doors of dishwashers in private homes [24].

In our study H. werneckii was not isolated from samples of granitic sand in contact with sea water. In fact this species is not reported as a marine yeast [25]. However, it has been found in seawater from Japan [26] and has been recently isolated from a sample of Bathymodiolus azoricus which is a mussel that dominates hydrothermal vent sites along the northern Mid-Atlantic Ridge at 2,300 m depth [27].

Tinea nigra is a superficial mycosis extremely uncommon in Spain [4], as well as being rare in Europe. In fact, only a few cases have been described and all of them are considered imported fungal infections [3,4,28–31]. Cases involving H. werneckii are primarily reported from coastal areas in tropical regions. The fungus is lipophilic, adhering to the stratum corneum and it does not extend below this level [7]. This species is unable to degrade keratin, but shows significant lipolytic activity. It is considered a commensal that shows lipophilic adhesion to human skin and survives by the assimilation of excretion products [32]. However, the isolation of H. werneckii from blood and a splenic abscess of neutropenic patients has been also reported [33].

Although scuba diving equipment should be cleaned and dried at the end of each day’s dive to prevent salt build-up and reduce microbial contamination, it is not unusual to see silicone scuba diving equipment, such as silicone masks, straps and snorkel mouthpieces, with fungal growth. In our study, H. werneckii was isolated from this type equipment which is usually used in close contact with human skin and mucous membranes. As it has been suggested, human skin softened during bathing might be more vulnerable to infection by black yeasts or other fungi in domestic bathrooms [34]. Similarly, fungi that colonize silicone masks, straps and snorkel mouthpieces could gain access by skin and mucous membranes maceration or through the respiratory system during scuba diving activities.

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Hortaea werneckii on scuba diving equipment


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