Black Yeast Diversity on Creosoted Railway Sleepers Changes with Ambient Climatic Conditions

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Abstract The environmental isolation of opportunistic pathogenic black yeasts, which are responsible for a wide spectrum of human infections, is essential to understanding the ecology of clinical fungi. Extreme outdoor environments polluted with aromatic hydrocarbons support the growth of black yeasts in unlikely places, such as railway sleepers. However, there are limited data concerning the diversity of these fungi growing on polluted railway sleepers. In this investigation, we examined 845 railway sleeper samples, obtained from 11 Turkish cities representing altitudes from 25 to 1,893 m, and inoculated the samples onto mycological media for the isolation of black yeasts. Ninety-four samples (11.1 %) yielded positive results for black yeast, with creosoted oak sleepers having a significantly higher number of isolates than concrete sleepers (p<0.05). Identification based on the ribosomal DNA (rDNA) internal transcribed spacer region revealed the highest prevalence of *Exophiala phaeomuriformis*, followed by *Exophiala dermatitidis*, *Exophiala heteromorpha*, *Exophiala xenobiotica*, and *Exophiala crusticola*. This study revealed that railway sleepers harboring black yeasts were predominantly (>75 %) populated with thermophilic species. We observed that altitude might have a significant effect on species diversity. Briefly, *E. phaeomuriformis* exhibited growth over a wide altitude range, from 30 to 1,893 m. In contrast, *E. dermatitidis* had a remarkable aversion to low altitudes and exhibited maximum growth at 1,285 m. In conclusion, we speculate that one can predict what species will be found on railway sleepers and their probability and that species diversity primarily depends on sleeper type and altitude height. We believe that this study can contribute new insights into the ecology of black yeasts on railway sleepers and the railway factors that influence their diversity.

Introduction

*Exophiala* (ascomycete order *Chaetothyriales*) is a genus of black yeasts that contains more than 40 species [1, 2]. To date,
14 of these have been demonstrated to be potential etiological agents of human infections with various clinical presentations, including (sub)cutaneous, systemic or disseminated infections [1, 3]. *Exophiala dermatitidis*, the most common human opportunistic, is prevalent in two very different human-made environments, namely, creosote-treated oak wood railway sleepers [4–8] and hot and moist, low-nutrient environments such as bathrooms [9], dishwashers [10, 11], and steam baths [12]. The natural habitat of this fungus has been speculated to be an association with fruit-eating animals in the tropical rain forest [5].

Ecological data obtained thus far suggest that black yeasts tend to escape from microbial competition by growth in poor or toxic habitats [4–8]. Indeed, studies have shown that (i) several species can be enriched in environments containing aromatic hydrocarbons (e.g., benzene, toluene, and xylene) [13] and (ii) toxic environments such as creosoted railway sleepers containing arsenic and phenolic compounds massively support the growth of black yeasts, although fecal contamination was excluded because its abundance was greater on oak wood than on concrete sleepers [4, 5, 7, 8, 14]. Species composition differs with altitude and climate conditions (tropical, subtropical, and temperate) [4, 5, 7, 8, 14], with temperature being a decisive factor. We hypothesized that the occurrence of *E. dermatitidis* on railway sleepers is limited to warm climatic conditions, i.e., in the (sub)tropics at low altitudes [8].

Expanding the understanding of the ecological factors that influence the growth and reproduction of black yeasts in the environment is necessary to predict the possible routes of infection of these human opportunists. Therefore, we examined the effect of altitude on the species diversity of *Exophiala* on oak wood and concrete railway sleepers, representing 11 cities in Turkey. Railway sleepers compose a habitat that is relatively comparable on a global scale, with the main differences generated by the climatic conditions of humidity and temperature.

**Materials and Methods**

**Sampling Area**

From June to September 2013, we collected samples from 11 different railway stations in 11 different cities in Turkey. These stations were located on five different lines at altitudes ranging from 25 to 1,893 m (Fig. 1). The average temperatures of the sampling area during the study period are also presented in Table 1. The trains running on these lines have open toilets, similar to most other trains in Turkey. The study protocol was reviewed and approved by the Ethics Committee of Gülhane Military Medical Academy, Ankara, Turkey.

**Sampling Method**

A total of 845 railway sleeper samples were collected, including 645 samples from creosoted oak wood and 200 samples from concrete. The oak railway sleepers were treated with arsenic creosote, and the concrete sleepers were stained black with petroleum that leaked from the trains. Fecal contamination was not observed on either type of sleeper; thus, we collected only machine-oil-impacted samples. Cotton swabs moistened with physiological saline were used to collect the samples, which were subsequently transported in sterile tubes and inoculated onto culture plates containing malt extract agar (MEA; Oxoid, Basingstoke, UK) supplemented with 100 μg/ml chloramphenicol (Sigma, Steinheim, Germany). The plates were incubated at 26 °C and monitored daily for 4 weeks to observe the growth of these microorganisms. Slow-growing, olivaceous black colonies were selected and transferred to fresh MEA plates for purification.

**DNA Extraction, PCR, and Molecular Analysis**

DNA extraction and PCR amplification were performed as previously described [15]. Ribosomal DNA (rDNA) sequences spanning the internal transcribed spacer (ITS) 1 region were amplified by Refgen Biotechnology (Ankara, Turkey) using an ABI PRISM 3130XL genetic analyzer and the universal fungal primers ITS1 and ITS4. CAP contig assembly software, included in the BioEdit Sequence Alignment Editor software version 7.0.9.0, was used to edit the sequences [16]. The assembled DNA sequences were examined using the BLAST (nucleotide-nucleotide) software program from the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD, USA).

**Genotyping**

The sequences were aligned using ClustalW [17] and compared through neighbor-joining phylogenetic tree analyses using the Molecular Evolutionary Genetics Analysis (MEGA) version 5.05 software package (www.megasoftware.net). Genotypic indicators of *E. dermatitidis* and *Exophiala phaeomuriformis* were obtained from Zalar et al. [10]. The genotypes were compared using the local CBS research database (www.cbs.knaw.nl) containing approximately 10,000 black yeast sequences for verification.

**Physiology**

The *Exophiala* strains were tested for several growth characteristics. First, thermotolerance tests were performed in 96-well microtiter plates, containing 300 μl of malt extract broth (MEB) inoculated with 10 μl of a cell suspension in each well.
The plates were incubated at 5, 10, 33, 37, 42, or 47 °C. The growth in the media was assessed daily for 2 weeks. Visual and spectrophotometric readings were performed at 450 nm using a Thermo Scientific Multiskan™ GO Microplate Spectrophotometer (Vankaa, Finland). The tolerance at pH 2.5, 4, 10, and 12.5 was assessed in the MEB using appropriate amounts of 0.1 M HCl and 0.1 M NaOH. Halotolerance was assessed in MEB supplemented with 5, 10, or 17 % NaCl (w/v). Tolerance to 100 µg/ml cycloheximide (Sigma) was also tested in MEA [5, 7, 10].

Statistical Analysis

The data were analyzed using both SPSS version 20.0 and SAS version 9.0. In a univariate analysis, two factors (i.e., railway sleeper type and the tendency of these sleepers to harbor thermophilic E. phaeomuriformis or E. dermatitidis strains) were cross-classified, and chi-square analysis was performed to determine the associations between factors. In a multivariate analysis, a version of the analysis of variance on three factors, the so-called log-linear model, was employed for the sleepers to determine which of the main factors — the type of railway sleeper (X), the altitude (Y), and the presence of thermophilic Exophiala species (Z) and their interactions (XY, XZ, YZ, or XYZ)—had effects on the sleepers’ tendency to harbor black yeast. A p value of <0.05 was considered significant.

In the multivariate analysis, the log-linear model (full/saturated) model was defined as

$$\log(m_{ijk}) = \mu + \lambda_X^i + \lambda_Y^j + \lambda_Z^k + \lambda_{XY}^{ij} + \lambda_{XZ}^{ik} + \lambda_{YZ}^{jk} + \lambda_{XYZ}^{ijk}$$

where $\lambda_X^i$, $\lambda_Y^j$, and $\lambda_Z^k$ were parameters for the main effect and $\lambda_{XY}^{ij}$, $\lambda_{XZ}^{ik}$, and $\lambda_{YZ}^{jk}$ represent the two- and three-way interaction effects on the sleepers’ tendency to harbor black yeasts. This model perfectly fit the data at hand but left many parameters unexplained. Thus, we required a model with fewer parameters: a reduced model that fit the data (i.e., X and Y are conditionally independent, given Z).

Results

In the present study, we encountered oily black spots on both types of railway sleepers examined at the 11 stations but did not observe visible fecal contamination. During these experiments, we obtained numerous contaminants on the MEA plates. When the medium was entirely covered with contaminants, the sleeper samples were reinoculated onto another MEA plate and reincubated under the same conditions. Overall, 94 out of 845 samples (11.1 %) yielded positive results for black yeasts, with creosoted oak sleepers having a significantly higher number of isolates than concrete sleepers (13.5 vs. 3.5 %); this difference of 0.10 was significant ($p<0.0001; 95 \% CI 0.06–0.14$).

The fungal isolation rates observed with samples obtained from the railway stations are presented in Table 1. We did not recover any black yeasts in the sleeper samples ($n=62$) obtained from Izmir, representing the first line. The most common black yeast was E. phaeomuriformis (51.1 %), followed by E. dermatitidis (25.5 %), Exophiala heteromorpha (17 %), Exophiala xenobiotica (5.3 %), and Exophiala crusticola (1.1 %). Remarkably, E. phaeomuriformis was observed on railway sleepers located at altitudes ranging from 30 to 1,893 m. In contrast, E. dermatitidis exhibited a predilection for altitudes lower than 1,285 m, with growth predominantly observed at sea level (30 m, Istanbul) or close to sea level (120 m, Osmaniye). In addition, E. heteromorpha was observed independent of the altitude range. Although, E. xenobiotica (at 732 and 1,750 m) and E. crusticola (at 1,750 m) were rarely recovered; sleepers harbored these two
species at high altitudes. The thermophilic species *E. dermatitidis* and *E. phaeomuriformis* grew in 4–8 days, *E. heteromorpha* grew in 4–6 days, *E. xenobiotica* grew in 5–6 days, and *E. crusticola* grew in 5 days.

In the univariate analysis, the odds of predicting positive *E. dermatitidis* were 4.5 times higher among samples from oak sleepers taken from <1,000 m compared with samples taken at altitudes >1,000 m (*p*=0.001; 95 % CI 1.7–12.2) (Table 2). For *E. phaeomuriformis*, no significant differences were identified for either altitude or sleeper type (*p*=0.2). The estimated model indicated that the sleeper type (whether oak or concrete) had significant effects on *E. dermatitidis* growth rate (*p*<0.001) and altitude (*p*=0.04) and that the interaction of altitude with *E. dermatitidis* (*p*=0.003) had significant effects on the *E. dermatitidis* growth rate. Namely, the *E. dermatitidis* rates were significantly different between samples taken from altitudes <1,000 m and samples taken from altitudes >1,000 m. The odds of detecting *E. dermatitidis* were 4.2 times higher among samples from altitudes <1,000 m compared with samples >1,000 m (*p*=0.003). For *E. phaeomuriformis*, a significant difference was identified for sleeper type (*p*<0.01) but not altitude. ITS sequencing indicated that *E. phaeomuriformis* strains were represented by genotypes 1 and 2 (30:18) and *E. dermatitidis* by genotypes A, A2, and B (16:7:1) (Figs. 2 and 3).

Notably, all *E. dermatitidis* isolates tolerated pH 2.5–12.5 and 5–17 % salinity. However, 2/16 of the *E. heteromorpha* genotype A3 and 7/30 of the *E. phaeomuriformis* genotype 1 strains were not viable at 47 °C. In addition, 13/16 of the *E. heteromorpha* isolates grew at 5–47 °C. No *E. xenobiotica* strains were grown at 45–47 °C, and one isolate did not grow at 5–10 °C. Overall, 83/94 isolates were grown on MEA with pH levels ranging from 2.5 to 12.5. Briefly, 2/16 of the *E. heteromorpha* isolates and 9/48 of the *E. phaeomuriformis* strains did not grow at pH 12.5. Furthermore, 4/16 of the *E. heteromorpha* isolates did not grow at 10–17 % salinity, and one strain of *E. phaeomuriformis* genotype 1 did not grow at 17 % salinity. All *Exophiala* isolates tolerated cycloheximide, except for a single *E. xenobiotica* isolate and two *E. phaeomuriformis* genotype 1 strains. Remarkably, a single *E. crusticola* isolate tolerated 5–10 °C, pH 2.5–12.5, and 5–17 % salinity (Table 3).

A selection of the isolates examined in this study was deposited in the reference collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands. In addition, the ITS rDNA sequences of these isolates were deposited in GenBank (Table 4).

### Discussion

In the present study, we examined the effects of climate on the species diversity of the black yeast *Exophiala* spp. growing on
creosoted oak and concrete railway sleepers at temperate and cold climates in Turkey. The isolated black yeasts were subcultured, identified using ITS sequencing, genotyped, and examined for their physiological properties to further characterize the species diversity. Railway sleepers predominantly (76.6%) harbored thermophilic species, namely,
Table 2 Railway sleepers harboring *E. paeomuriformis* and *E. dermatitidis*, according to sleeper type and altitude

<table>
<thead>
<tr>
<th>Railway sleeper</th>
<th>Altitude (m)</th>
<th><em>E. paeomuriformis</em></th>
<th><em>E. dermatitidis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;1,000</td>
<td>16</td>
<td>288</td>
</tr>
<tr>
<td>Oak</td>
<td>&gt;1,000</td>
<td>26</td>
<td>315</td>
</tr>
<tr>
<td>Concrete</td>
<td>&lt;1,000</td>
<td>5</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>&gt;1,000</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td>797</td>
<td>24</td>
</tr>
</tbody>
</table>

+ positive, − negative, m meter

*E. paeomuriformis* and *E. dermatitidis*. Remarkably, *E. paeomuriformis* and *E. dermatitidis* differ in their range of growth temperatures under those climatic conditions: *E. paeomuriformis* has a wide (17.5–28.1 °C) growth range, and *E. dermatitidis* has a narrow (19.3–27.6 °C) growth range. The lowland climate data using Turkish meteorological measurements for the sampling sites in this study are summarized in Table 1. Furthermore, an important limitation of this study is that the data represent only one country: Turkey. A comparable number of samples taken worldwide, representing different altitudes and climatic conditions, will be able to minimize the impact of this limitation.

Overall, in this study, we recovered black yeasts from 11.1 % of the railway sleepers. Recent studies from our group have also revealed consistent results, showing that the presence of black yeasts was 17 % in subtropical regions [7] and 3.6 % in cold and arid areas [8] in Turkey. In accordance with the results obtained in our two previous studies [7, 8], we observed the enhanced growth of black yeasts on creosoted oak (13.5 %) compared with concrete sleepers (3.5 %). *Exophiala* species, including *Exophiala bergeri* [4, 6], *E. crusticola* [8], *E. dermatitidis* [4, 5, 7], *E. heteromorpha* [8], *Exophiala sideris* [14], and *E. xenobiotica* [6, 14], were recovered from railway sleepers. Although there is no realistic health risk, these species significantly differ in pathology and virulence [6, 14] and have been recognized as (i) human opportunists (*E. dermatitidis*, *E. paeomuriformis*, and *E. xenobiotica*) [7, 18], (ii) rare human opportunists (*E. heteromorpha*) [7, 18], and (iii) species with no clinical significance (*E. crusticola* and *E. sideris*) [1, 7, 14, 18]. We suggest that all *Exophiala* species behave the same on railway sleepers and that the massive presence of *E. dermatitidis* indicates that this environment is a preferred habitat for all of these species. *E. dermatitidis* is opportunistic because it is thermostolerant, in addition to its other behaviors, which together enable its invasiveness.

A recent multilocus analysis revealed that the “jeanselmei clade” belonged to two ecologically significant lineages: a subclade around *Exophiala spinifera* (tendency toward human pathogenicity, e.g., *Exophiala jeanselmei sensu stricto* and *Exophiala oligosperma*) and another subclade around *E. bergeri* (often associated with environments rich in toxic hydrocarbons, e.g., *E. sideris* and *E. xenobiotica*) [18]. Currently, the thermophilic *E. spinifera* subclade, growing at 37 °C or higher, has not yet been recovered from railway sleepers, as in the present study. However, Zeng et al. [18] previously described the presence of black yeasts on railway sleepers belonging to a subclade around *E. bergeri* (such as *E. sideris* and *E. xenobiotica*), consistent with previous studies [4, 6, 8, 14] and with the results obtained in the present study.

*E. paeomuriformis* has been detected in bathrooms and hot springs in Europe [12] and is consistently observed in dishwashers worldwide [10, 11]. Our recent findings revealed that moisture slightly affected fungal growth as *E. paeomuriformis* grew well on oak wood but poorly or not at all on concrete sleepers, which were virtually devoid of moisture [7, 8]. In contrast, in the present study, we observed seven *Exophiala* isolates on concrete sleepers, which were all identified as *E. paeomuriformis*. In addition, we also observed that *E. paeomuriformis* grew in both temperate and cold climates, which is also consistent with our two previous reports [7, 8].

The surfaces of fruit (e.g., wild berries) provide a natural niche for the growth of *E. dermatitidis*, with a life cycle involving passage through the intestinal tracts of fruit-eating animals, such as flying foxes and hornbills in Thailand [5], and the presence of this fungus on *Eucalyptus* wood in Brazil [4] suggests a potential origin in the tropical rain forest. Furthermore, Zhao et al. [6] noted the lack of this yeast under temperate climate conditions (e.g., The Netherlands), suggesting that *E. dermatitidis* originated in the tropics. Consistent with these findings, recent studies have revealed that *E. dermatitidis* grows in subtropical areas [7], yet this microbe was not recovered from 658 railway sleepers at altitudes ranging from 1,026 to 1,427 m in Turkey [8]. In contrast, in the present study, we identified five *E. dermatitidis* strains at >1,000 m, including four from Akyonkarahisar (1,013 m) and one from Sivas (1,285 m).

Using univariate analysis, we observed that oak sleepers were 4.5 times more likely to harbor *E. dermatitidis* at altitudes <1,000 m compared with altitudes >1,000 m (p=0.001); no significant differences were detected for *E. paeomuriformis* on either sleeper type (p=0.2; Table 2). In addition, using univariate analysis, both the sleeper type (p<0.001) and altitude (p=0.04) had significant effects on the presence of *E. dermatitidis*, whereas only the sleeper type was noted as having a significant effect on *E. paeomuriformis* (p<0.01). These results suggest that the environmental temperature plays an important role in the life cycle of *E. dermatitidis*. Genomic comparisons are needed, but at present, we can hypothesize that they do not differ greatly in terms of hydrocarbon-related genes but rather differ in terms of temperature-related genes. Sudhadham et al. [5] and Dogen et al. [7] also detected...
E. dermatitidis on concrete sleepers and attributed its presence to machine oil spills and the constant presence of aromatic or aliphatic hydrocarbons. A recent study also revealed the existence of E. dermatitidis in hydrocarbon-polluted soils [19].
E. xenobiotica was also detected in hydrocarbon-rich habitats, such as in polluted soils and gasoline car tanks, or on building façades [4, 6, 20, 21], as observed in the present study. This observation might reflect the growth temperature of this fungus, which grew best at 30 °C, more slowly at 33 °C, and not at all at 37 °C [22]. In addition, E. crusticola grows at 5–30 °C but does not grow at or above 37 °C [23]. This fungus has been detected in biological soil crust in the USA [23] and has been recovered from both creosoted oak and concrete sleepers in cold climates in Turkey [8]. Consistent with these observations, in the present study, we recovered E. crusticola only in Kars (at 1,750 m). Interestingly, to date, Veronaea botryosa, in the ascomycete order Chaetothyriales, has been detected on creosoted oak wood in Brazil [4].

Consistent with a previous study [7], we observed the active growth of E. dermatitidis and E. phaeomuriformis at 5–47 °C, across a wide pH range and up to 17 % NaCl, with tolerance to cycloheximide. In addition, most of the E. heteromorpha isolates shared physiological characteristics with thermophilic species. We recovered E. dermatitidis genotype A more frequently than genotype B but did not identify genotype C, with a ratio of 23:1:0. Remarkably, genotype A is more successful at entering urban environments [7, 10, 11, 24] and is also the most common cause of human infections [25]. E. phaeomuriformis genotype 1 was observed more often than genotype 2, in contrast to our recent findings from both subtropical areas [7] and cold and arid areas in Turkey [8].

In conclusion, black yeasts of the genus Exophiala are enriched by and prevalent (11.1 %) on railway sleepers at altitudes ranging from 30 to 1,893 m and are primarily harbored on creosoted oak wood. The climate has a significant effect on species diversity as railways in cold climates were primarily contaminated with clinically insignificant or rare human opportunists (i.e., E. crusticola and E. xenobiotica), whereas railways in relatively temperate climates and particularly close to sea level had abundant amounts of E. dermatitidis. Conversely, E. phaeomuriformis exhibited growth in both temperate and cold climates. We speculate that the overall implications of these findings may be informative for the ecology of clinical fungi and will require further worldwide sampling.

<table>
<thead>
<tr>
<th>Strains (number)</th>
<th>Temperatures</th>
<th>pH values</th>
<th>Growth on NaCl</th>
<th>Cycloheximide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 °C</td>
<td>10 °C</td>
<td>45 °C</td>
<td>47 °C</td>
</tr>
<tr>
<td>E. crusticola (n=1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. dermatitidis (n=24)</td>
<td>GENOTYPE</td>
<td>GENOTYPE A (n=16)</td>
<td>15/16</td>
<td>15/16</td>
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<td>E. heteromorpha (n=16)</td>
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<td></td>
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<tr>
<td>E. phaeomuriformis (n=48)</td>
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<td>GENOTYPE 1 (n=30)</td>
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<td>23/30</td>
</tr>
<tr>
<td>E. xenobiotica (n=5)</td>
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</tr>
</tbody>
</table>

Table 3 Growth characteristics of Exophiala species over a range of temperatures, pH values, and salt concentrations

Table 4 CBS and GenBank accession numbers (ITS rDNA sequences) for the selected study isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Sampling area</th>
<th>Railway sleeper</th>
<th>GenBank accession no. (ITS rDNA)/CBS no.</th>
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<td>Kars</td>
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<td>E. dermatitidis genotype A</td>
<td>Afyonkarahisar</td>
<td>Oak wood</td>
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<tr>
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<td>Oak wood</td>
<td>KJ522798/CBS 137220</td>
</tr>
<tr>
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<td>Oak wood</td>
<td>KJ522799/CBS 137221</td>
</tr>
<tr>
<td>E. heteromorpha</td>
<td>Sivas</td>
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<td>KJ522800/CBS 137222</td>
</tr>
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<td>KJ522802/CBS 137224</td>
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<td>Oak wood</td>
<td>KJ522804/CBS 137226</td>
</tr>
<tr>
<td>E. xenobiotica</td>
<td>Eskişehir</td>
<td>Oak wood</td>
<td>KJ522805/CBS 137227</td>
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</table>
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Conflict of Interest  The authors report no conflicts of interest. The authors alone are responsible for the content and composition of this manuscript.

References