**Aureobasidium thailandense** sp. nov. isolated from leaves and wooden surfaces

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**Aureobasidium thailandense** sp. nov. is described from cultures of material collected on leaves and wooden surfaces in Thailand and the type isolate is NRRL 58539. Phylogenetically it is distinct from other species of the genus *Aureobasidium*. Phenotypically it is distinguished by its cardinal growth temperatures, salt tolerance and production of reddish brown hyphal pigmentation in PDA cultures, but micro-morphologically it is not clearly distinguishable from *Aureobasidium pullulans*. Unlike *A. pullulans*, *A. thailandense* sp. nov. produces a non-pullulan extracellular polysaccharide whose characteristics are unknown. The two known isolates of *A. thailandense* sp. nov. possess an approx. 500 bp type I intron in the 18S rRNA gene that is present in ITS amplifications using primers ITS4 and ITS5. *A. pullulans* isolates uniformly lack this intron.

**Aureobasidium pullulans** is a yeast-like fungus in the Ascomycota class Dothideomycetes (Schoch *et al.*, 2006) that is related to leaf-spot fungi from grasses, shrubs and trees (Hermanides-Nijhof, 1977; de Hoog & Yurolva, 1994; Nichols *et al.*, 2010). It has been isolated from the phylloplanes of diverse plants, maize silks, brackish water, mangrove swamps, glacial ice in Norway and wooden or painted structures (Arfi *et al.*, 2012; Manitchotpisit *et al.*, 2009; Santo *et al.*, 2012; Zalar *et al.*, 2008). *Aureobasidium proteae* was reported to cause chronic fungal meningitis (Kutleša *et al.*, 2012) as well as a leaf spot disease of Proteaceae (Crous *et al.*, 2011). Some isolates of *A. pullulans* produce semiochemicals (Davis *et al.*, 2012) that influence wasp behaviour in nature and that may be involved in dispersal of members of the genus *Aureobasidium*, while other isolates produce large amounts of the extracellular polysaccharide (EPS) pullulan (Manitchotpisit *et al.*, 2009), a polysaccharide with many valuable industrial and medical uses (Leathers, 2002; Cheng *et al.*, 2011). Still other isolates biosynthesize products that have commercial value such as laccase (Rich *et al.*, 2011), xylanase (Manitchotpisit *et al.*, 2009) or poly β-L-malic acid (Manitchotpisit *et al.*, 2012).

The taxonomy of the genus *Aureobasidium* as viewed by different authors has consisted of its division into phenotypic varieties of *A. pullulans* (Hermanides-Nijhof, 1977; Zalar *et al.*, 2008), descriptions of monophyletic clades in *A. pullulans* without specific Linnaean ranking (Manitchotpisit *et al.*, 2009) or descriptions of distinct species (Crous *et al.*, 2011). The broad clade containing the genus *Aureobasidium* (Schoch *et al.*, 2006; Bills *et al.*, 2012) includes other genera, such as *Kabatiella* and *Selenophoma* that express *Aureobasidium* and other anamorphic states under different growth conditions (synanamorphosis).

Manitchotpisit *et al.* (2009) isolated two putative *Aureobasidium pullulans* strains whose phylogenetic position was not fully resolved. These two isolates contain an 18S rRNA intron not found in other *A. pullulans* isolates and produce an uncharacterized EPS. We conducted phylogenetic analysis of these isolates to resolve their relationship to *A. pullulans* and, as they form a monophyletic clade distinct from other species, we provide a description and name for these two isolates.

**Culture growth and examination**

Cultures used in this study and their provenance are listed in Table 1 and are available from the Agricultural Research Service (NRRL, Peoria, IL, USA; http://nrrl.ncaur.usda.gov/). Numerous DNA sequences were obtained from GenBank and their GenBank and culture...
collection accession numbers are listed in the phylogenetic tree.

Cultures were initiated from lyophilized preparations and maintained on potato dextrose agar (PDA; Difco) during the course of this study. Malt extract agar (MEA), Hay infusion agar, Harold’s M40Y and M60Y media were prepared as described (Raper & Fennell, 1965). MEA media containing salt were prepared prior to sterilization. Cultures were incubated at 25 °C unless otherwise noted. The mature culture appearance was recorded and culture plates were photographed with a Kodak 14n digital camera.

Microscopy was conducted on cellular material mounted in 0.1 % Triton X-100 on a microscope slide. The Zeiss axioskop was fitted with a Kodak 14n camera and had phase-contrast, DIF contrast and plain light illumination. Photographs of culture plates and micromorphology were sized and fitted into composite illustrations using Photoshop Elements 10 (Adobe).

DNA sequencing

Each isolate was grown in 2 ml YM broth (Difco) overnight, and cells were collected by centrifugation and resuspended in 1 ml TE buffer, pH 8.0. A 0.5 ml volume of glass beads (0.5 mm Zirconia/Silica beads, BioSpec Products, Inc.) was added and cells were then disrupted using a FastPrep instrument (Qbiogene), set at number 4, for 30 s. Total DNA was isolated and purified using the QIAquick PCR purification kit (Qiagen).

The ITS and LSU-rRNA gene were amplified as previously described (Zalar et al., 2008; Manitchotpisit et al., 2009). The amplified fragments were sequenced using the amplification primers and BigDye v3 Terminator Cycle Sequencing kit (Applied Biosystems) and an ABI 3730 (capillary) DNA Analyzer (Applied Biosystems).

Phylogenetic analyses

All sequences were determined from bidirectional sequencing, and errors were viewed and corrected using Sequencher (http://www.genecodes.com). Corrected sequences were aligned with CLUSTAL W (Thompson et al., 1994) and trimmed to equal length. Phylograms and bootstrap analysis were generated using PAUP* (Swofford, 2003) version 4.0 b10 with parsimony criterion. Maximum-parsimony analysis was conducted with the dataset initially subjected to a heuristic search with random sequence addition (500 repetitions), NNI (nearest neighbour interchange) branch swapping, and maximum trees set to 5000. Subsequently, the random addition trees were used as the starting point for a heuristic search using ‘as-is’ sequence addition, TBR (tree bisection-reconnection) branch swapping, and maximum trees set at 5000. Bootstrap analysis was performed in PAUP* using the parsimony criterion, TBR and 1000 repetitions with maximum trees set to 100. Tree diagrams were viewed using TreeView (Page, 1996) and redrawn using CorelDraw X3.

Results

Growth characteristics of A. pullulans and A. thailandense sp. nov. isolates on media with varying sugar or salt concentrations and at different temperatures are shown in Table 2. Amplification and sequencing of the ITS region (primers ITS5, ITS4) of A. thailandense sp. nov. demonstrated an approx. 500 bp insert in the 3’ end of the 18S rRNA gene from isolates NRRL 58539T and NRRL 58543, but sequencing of numerous A. pullulans isolates revealed no introns (Manitchotpisit et al., 2009). In a BLAST search of the GenBank database, this rRNA insert segment demonstrated 100 % similarity to the comparable region from Neoscytalidium dimidiatum over 68 % of the segment length and probably represents a group I intron in the nuclear SSU-rRNA gene (Bhattacharya et al., 2005).

The nuclear LSU-rRNA gene alignment included 529 nt positions. Four base positions were indels and these positions were eliminated from further analysis. Out of the 525 remaining positions analysed, 467 were constant and 61 were variable with 41 of those positions being phylogenetically informative. Parsimony analysis produced four equally most parsimonious trees of 77 steps. The consistency index was 0.8701 and the rescaled consistency index was 0.7951. One of those trees is shown in Fig. 1. Bootstrap values above 70 % are listed on the tree diagram.

Table 1. Provenance of Aureobasidium thailandense sp. nov. and A. pullulans isolates

<table>
<thead>
<tr>
<th>NRRL accession number</th>
<th>Provenance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aureobasidium pullulans MB 508998</td>
<td>France, Beaujolais, isol. ex Vitis vinifera, 1974, E. J. Hermanides-Nijhof, ex neotype</td>
</tr>
<tr>
<td>58012T</td>
<td>USA, isol. ex corn silks, 1974, R. Bothast</td>
</tr>
<tr>
<td>Y-7703</td>
<td>Bahama, isol. ex seaweed, 1985, T.D. Leathers</td>
</tr>
<tr>
<td>Y-12974</td>
<td></td>
</tr>
<tr>
<td>Aureobasidium thailandense MB 801148</td>
<td>Thailand, Nakhonratchasima, isol. ex leaf of Cerbera odollum, 2006, P. Manitchotpisit, type</td>
</tr>
<tr>
<td>58539T=CBS 133856T</td>
<td>Thailand, Prachuapkhirikhan, isol. ex wood surface, 2006, P. Manitchotpisit</td>
</tr>
<tr>
<td>58543=CBS 133857</td>
<td></td>
</tr>
</tbody>
</table>
formed a distinct clade with a 92 % bootstrap value. Aureobasidium thailandense isolates formed a sister clade to the trichotomy of Selenophoma mahoniae, A. leucospermi and A. pullulans varieties; and A. pullulans, A. proteae, Discosphaeria fagi and Kabatiella microsticta. Sydowia polydora was chosen as the outgroup species based on prior studies (Schoch et al., 2006; Crous et al., 2011; Bills et al., 2012) showing that the genus Sydowia is representative of a strongly supported clade sister to the Aureobasidium clade.

**Description of Aureobasidium thailandense sp. nov. S. W. Peterson, Manitchotpisit & Leathers Figs 2, 3**

**Mycobank number:** MB 801148.

**Holotype:** NRRL 58539<sup>T</sup> was isolated from leaves of Cerbera odollum Gaertn. 2006, Nakhonratchasima, Thailand by Pennapa Manitchotpisit, and is kept metabolically inactive as a lyophilized culture (article 8.4) in the Agricultural Research Service Culture Collection, Peoria, IL.

**Diagnosis:** Aureobasidium thailandense sp. nov. differs from A. pullulans by displaying reddish brown melanized hyphae after 4–5 days growth on PDA medium, while A. pullulans remains white to pinkish for 2–3 weeks and its varieties darken in different colours.

**Description:** Aureobasidium thailandense sp. nov. colonies on PDA (Fig. 2a, b) attain 24–25 mm diam after 7 d growth at 25 °C, are reddish brown centrally, appearing slinky from spor and EPS production, margins irregular, reverse white peripherally to pale brown centrally, produces non-pullulan EPS. Microscopically (Fig. 3a, b, c, d) composed of short broad hyphae, 7–10 × 20–40 μm producing blastoconidia terminally or laterally, conidia subglobose, ovoid, or pyriform 3–10 × 5–12 μm, smooth walled, individual cells proliferating by polar budding.

### Table 2. Diameter (mm) of A. pullulans and A. thailandense sp. nov. colonies under different conditions and on different media

<table>
<thead>
<tr>
<th>Condition</th>
<th>A. pullulans</th>
<th>A. thailandense sp. nov.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Y-12974</td>
<td>NRRL 58012&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td>PDA</td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>Hay infusion</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>M40Y</td>
<td>30</td>
<td>28</td>
</tr>
<tr>
<td>M60Y</td>
<td>26</td>
<td>23</td>
</tr>
<tr>
<td>MEA + 5% NaCl</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>MEA + 10% NaCl</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>MEA + 15% NaCl</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>MEA + 20% NaCl</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MEA at 4 °C</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>MEA at 15 °C</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>MEA at 25 °C</td>
<td>38</td>
<td>27</td>
</tr>
<tr>
<td>MEA at 28 °C</td>
<td>36</td>
<td>30</td>
</tr>
<tr>
<td>MEA at 30 °C</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>MEA at 37 °C</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Growth temperature was 25 °C unless noted otherwise and incubation was for 7 d.

**Phenotypic recognition**

A. thailandense sp. nov. resembles A. pullulans in producing initially pale yeast-like colonies with glistening moist surfaces where spores and EPS are produced. After 7 d growth, most of the A. thailandense sp. nov. colony is reddish brown coloured (Fig. 2a). A. pullulans colonies on PDA remain white to pink coloured for at least a week, usually 2–3 weeks. A. pullulans var. melanogenum isolates become melanized early in colonial growth appearing greenish black. A. pullulans var. subglaciale isolates remain pinkish at 7 d but are melanized by 14 d. A. pullulans var. namibiae isolates become melanized in the first week producing an olive–brown colour.

A. thailandense sp. nov. has different cardinal growth temperatures from A. pullulans (Table 2), failing to grow at 4 °C while some growth is seen in A. pullulans at that temperature. A. pullulans grows on MEA supplemented with 15 % (w/v) NaCl (Table 2, Zalar et al., 2008), but A. thailandense sp. nov. can only tolerate concentrations of up to 10 % NaCl. The optimum growth temperature for both species is in the 25–28 °C range. There was little difference in growth of the two species on very weak media (hay infusion agar), on moderate sugar level media (PDA) or media with high sugar levels (M40Y, M60Y, Table 2).

Micromorphologically A. thailandense sp. nov. is difficult to distinguish from A. pullulans because of overlapping spore sizes and shapes, and similar types of conidium production.

**Genotypic recognition**

The A. thailandense sp. nov. DNA sequences from β-tubulin, calmodulin, ITS, LSU-rRNA and RPB2 are distinct from those of A. pullulans and all other sequences available either in our laboratory or in the GenBank databases.
Discussion

The genera *Aureobasidium* (de Bary) G. Arnaud (1891) and *Kabatiella* Bubak (1907) were based on the different anamorphic states seen in each. Hermanides-Nijhof (1977) treated the different anamorphic states as representative of different form-genera, a situation no longer accepted under recent changes to the nomenclatural code (Norvell, 2011; Hawksworth, 2012). We have subsequently learned that species from genera such as *Kabatiella* (Nichols et al., 2010) and *Selenophoma* (Ramaley, 1992) may display several anamorphic states, including *Aureobasidium* states, under different growth conditions. *Aureobasidium thailandense* sp. nov. was isolated as part of a larger survey of pullulan production in *A. pullulans* and initially was thought to be a ‘colour variant’ isolate of *A. pullulans* (Manitchotpisit et al., 2009). The species was never studied *in situ* so we do not know if synanamorphs in addition to the *Aureobasidium* state might be found in the leaf habitat, a common phenomenon (Nichols et al., 2010; Ramaley 1992; Crous et al., 2011).

DNA studies (Manitchotpisit et al., 2009) demonstrated that *A. thailandense* sp. nov. could not be accommodated in a phylogenetically defined *A. pullulans sensu Zalar et al.* (2008) except by broadening the concept of an already variable species (Loncaric et al., 2009). Bills et al. (2012) showed that *Aureobasidium thailandense* sp. nov. (labelled *Aureobasidium* sp. in that study) occurs in the statistically supported *Aureobasidium* clade rather than the sister *Sydowia* clade (Schoch et al., 2006; Crous et al., 2011).

Upon discovering the sibling relationship of *K. proteae* and *A. pullulans*, Crous et al. (2011) placed *Kabatiella proteae* in the genus *Aureobasidium* as *A. proteae* using the priority concept. Other genera and species in *Kabatiella, Selenophoma* or *Discosphaeria* also produce several anamorphic states and careful study may show that they should be transferred to the genus *Aureobasidium*.

We found a type I intron in both isolates of *A. thailandense* sp. nov., while in *A. pullulans* type I introns were not found in any isolates examined. Côté et al. (2004) reported that in *Monilinia fructicola* isolates only some isolates of the species contained a SSU-rRNA type I intron. For identification of *A. thailandense* sp. nov., we place more importance on the sequences from the

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**Fig. 1.** Parsimony analysis of *A. thailandense* sp. nov., *A. pullulans* and related taxa based on partial LSU-rRNA sequences. The *A. thailandense* sp. nov. isolates form a distinct clade separate from *A. pullulans*. *A. pullulans* isolates occur on an unresolved trichotomy that includes *Aureobasidium* species and species of the genera *Discosphaeria, Kabatiella* and *Selenophoma*. *Sydowia polyspora* is the outgroup species (Schoch et al., 2006; Crous et al., 2011). Bootstrap support >70% is indicated on the branches. NRRL accession numbers refer to the Agricultural Research Service Culture Collection; CBS refers to the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CPC refers to the culture collection of Pedro Crous housed at the CBS. Bar, 1 substitution per nucleotide position.

**Fig. 2.** Obverse and reverse views of 7 d PDA cultures of: (a, b) *Aureobasidium thailandense* sp. nov. NRRL 58539T and (c, d) *Aureobasidium pullulans* NRRL 58012T.
different loci than on the presence of the SSU-rRNA intron as this may be a variable character. Bhattacharya et al. (2005) reported that group I introns are absent from the family Dothideomycetidae and this may represent a first report in this taxonomic group.

The morphological diversity of synanamorphs found in the *Aureobasidium* clade species has been misleading about phylogenetic relationships in the past. Current studies employing phylogenetic analysis of DNA sequence data has clarified the relationships among species of these genera. The broad use of nuclear ITS barcode sequences (Schoch et al., 2012) or sequences from other loci in the identification process will allow improved identification of isolates not previously possible.

**Acknowledgements**

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**References**


