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A survey of xerophilic Aspergillus from indoor environment, including descriptions of two new section Aspergillus species producing eurotium-like sexual states

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Abstract
Xerophilic fungi grow at low water activity or low equilibrium relative humidity and are an important part of the indoor fungal community, of which Aspergillus is one of the dominant genera. A survey of xerophilic fungi isolated from Canadian and Hawaiian house dust resulted in the isolation of 1039 strains; 296 strains belong to Aspergillus and represented 37 species. Reference sequences were generated for all species and deposited in GenBank. Aspergillus sect. Aspergillus (formerly called Eurotium) was one of the most predominant groups from house dust with nine species identified. Additional cultures deposited as Eurotium were received from the Canadian Collection of Fungal Cultures and were also re-identified during this study. Among all strains, two species were found to be new and are introduced here as A. mallochii and A. megasporus. Phylogenetic comparisons with other species of section Aspergillus were made using sequences of ITS, β-tubulin, calmodulin and RNA polymerase II second largest subunit. Morphological observations were made from cultures grown under standardized conditions. Aspergillus mallochii does not grow at 37 °C and produces roughened ascospores with incomplete equatorial furrows. Aspergillus megasporus produces large conidia (up to 12 µm diam) and roughened ascospores with equatorial furrows. Echinulin, quinolactacin A, & A2, preechinulin and neoecinulins A & B were detected as major extrolites of A. megasporus, while neoecinulins A & B and isoechinulin A, B & C were the major extrolites from A. mallochii.
Introduction

Species of *Aspergillus* section *Aspergillus*, the “A. glaucus” group of Thom and Raper (1941) and Raper and Fennell (1965), typically produce yellow cleistothecia (white in *A. leucocarpus*) with lenticular ascospores and the section includes species that were traditionally classified in the genus *Eurotium*. Species of section *Aspergillus* have a broad distribution in nature, but their xerophilic physiology makes them significant for the built environment and the food industry. In the built environment, species of section *Aspergillus* are among the primary colonizers of building materials (Flannigan and Miller 2011). Modern heating systems are designed to remove humidity from buildings, creating opportunities for xerophiles to dominate indoor fungal communities. Also of concern is the growth of these fungi in museums or libraries on historic artefacts such as books, carpets or paintings. They also commonly grow on/in leather, dust, softwood, a variety of textiles and even dried specimens in herbaria (Cavka et al. 2010; Micheluz et al. 2015; Pinar et al. 2013; Pinar et al. 2015; Pitt and Hocking 2009; Raper and Fennell 1965; Samson et al. 2010). For the food industry, these species have an economic impact because they can grow on stored grain, cereals or preserved foods with high sugar (i.e. jams, maple syrup) or salt content (i.e. biltong, dried fish) (Pitt and Hocking 2009; Samson et al. 2010).

Xerophily is a common physiological property of many *Aspergillus* species from several subgenera and sections, enabling those species to grow at low water activity (*a_w*) or equilibrium relative humidity (ERH) (Flannigan and Miller 2011; Pitt 1975). Water activity is a measure of available water in liquid or solid substrates that has a significant effect on which organisms can grow on foods or other matrices, including building materials (Scott 1957). Reducing *a_w* is widely used in the food industry to reduce spoilage (Pitt and Hocking 2009). For the built environment, however, it is very difficult and often impractical to measure *a_w* and as a result relative humidity (RH) is often used as a proxy. Because RH measures moisture in air rather than available water in a substrate, it is not considered a reliable indication of whether growth will actually occur on surfaces in the built environment (Flannigan and Miller 2011). A better measure is ERH because it is more representative of available water and is numerically proportional to *a_w* (Flannigan and Miller 2011; Pitt and Hocking 2009).

Species of section *Aspergillus* produce many extrolites exhibiting a wide range of biological activities (Frisvad and Larsen 2015a; Gomes et al. 2012; Kanokmedhakul et al. 2011; Li et al. 2008a; Li et al. 2008b; Slack et al. 2009; Smetanina et al. 2007). Most notably, compounds from *A. chevalieri* were shown to be active against *Plasmodium falciparum* (malaria), *Mycobacterium tuberculosis* and cancer cell lines (Kanokmedhakul et al. 2011), an antitumor compound was reported from *A. cristatus*
A survey of xerophilic Aspergillus from indoor environment...

(Almeida et al. 2010), while many compounds are known to be antioxidants. They also produce mycotoxins, especially echinulin, flavoglaucin and physcion, which are toxic to animals (Ali et al. 1989; Bachmann et al. 1979; Cole and Cox 1981; Greco et al. 2015; Nazar et al. 1984; Rabie et al. 1964; Semeniuk et al. 1971; Slack et al. 2009; Veesonder et al. 1988), but toxicity has not been reported in humans. These species are not considered significant human pathogens, because most infections are superficial, with few cases of invasive infections known (de Hoog et al. 2014). Species commonly grow as saprobes on clinical specimens, such as skin and nails (Hubka et al. 2012). The biggest concern to humans, or nuisance, is the growth of these species inside homes, where exposure to spores and fragments, which contains β-(1, 3)-D-glucan, and other metabolites, cause allergies (Green et al. 2006; Slack et al. 2009).

Xerophilic fungi are well studied from a morphological point of view, but much work remains to develop reference sequence data for them. In this paper, we report on the diversity of Aspergillus isolated from house dust using media with low a\textsubscript{w} that select for the growth of xerophiles. Reference sequences are released for all species, including those received as Eurotium from the Canadian Collection of Fungal Cultures and re-identified here. Furthermore, we describe two new species and report on their extrolite production.

**Materials and methods**

**Strains/sampling and isolations**

House dust samples were received from various areas in North America. A modified dilution-to-extinction method (Collado et al. 2007) was used to isolate cultures, as described in Visagie et al. (2014a). Modifications included the use of 48-well titre plates rather than 96-well microtube plates and the use of Dichloran 18% Glycerol agar (DG18; Hocking and Pitt 1980), Malt extract yeast extract 10% glucose 12% NaCl agar (MY10-12) and Malt extract yeast extract 50% glucose agar (MY50G) (Samson et al. 2010) isolation media to select for xerophilic fungi.

In addition to newly obtained house dust isolates, several strains, including unidentified isolates and some reference or ex-type cultures, of Aspergillus sect. Aspergillus were obtained from the Canadian Collection of Fungal Cultures, Canada (DAOMC) and the CBS-KNAW Fungal Biodiversity Centre, the Netherlands (CBS).

**Morphology**

Colony characters were recorded from cultures grown for 7 d on various media, including CYA (Czapek yeast autolysate agar), MEA (Blakeslee’s malt extract agar), CREA (Creatine sucrose agar), CY20S (CYA with 20% sucrose agar), MEA20S (MEA with
20% sucrose agar), DG18, YES (yeast extract sucrose agar), M40Y (Harrold’s agar; 2%
malt, 0.5% yeast extract, 40% sucrose), MY50G and MY10-12 (Harrold 1950; Pitt
and Hocking 2009; Samson et al. 2014). Plates were incubated upside down in the
dark at 25 °C and left unwrapped. Additional CY20S, DG18 and MEA20S plates were
wrapped and incubated at 37 °C. Colour names and codes in descriptions are from
Kornerup and Wanscher (1967). Microscopic preparations were made from colonies
growing on DG18 and observations made using an Olympus SZX12 dissecting and
Olympus BX50 compound microscopes equipped with Infinity3 and InfinityX cameras
using Infinity Analyze v. 6.5.1 software (Lumenera Corp., Ottawa, Canada). Variation
of conidia and ascospores was evaluated by measuring at least 50 structures and pre-
sented as mean +/- standard deviation. Photographic plates were prepared in Pixelma-
tor iOS v. 2.3 (http://www.pixelmator.com/ios), with photomicrographs modified for
aesthetic purposes using the repair tool, without altering scientifically significant areas.

**DNA extraction, sequencing and phylogenetic analysis**

DNA was extracted from 8–10 d old colonies grown on DG18 using the UltracleanTM
Microbial DNA isolation Kit (MoBio Laboratories Inc., Solana Beach, USA). Loci chosen
for amplification included ITS barcodes (internal transcribed spacer rDNA region, in-
cluding ITS1-5.8S-ITS2) (Schoch et al. 2012), BenA (partial β-tubulin), CaM (partial
calmodulin) and RPB2 (RNA polymerase II second largest subunit). Thermocycler pro-
grams used for amplification followed Samson et al. (2014) and employed primer pairs
V9G & LS266 (ITS; (de Hoog and Gerrits van den Ende 1998; Masclaux et al. 1995),
Br2a & Br2b (BenA; (Glass and Donaldson 1995)), CF1 & CF4 or sometimes CMD5
& CMD6 (CaM; (Hong et al. 2006; Peterson et al. 2005)) and 5F & 7CR (RPB2;
(Liu et al. 1999)). Sequencing was done as described in Visagie et al. (2016). Contigs
were assembled in Geneious v. 8.1.8 (Biomatters Ltd, New Zealand) and newly generat-
ed sequences submitted to GenBank.

As a preliminary step in identification, CaM sequences derived from the newly
isolated cultures were compared to an ex-type reference sequence database published
by Samson et al. (2014). Then, gene sequences of the two presumed to be new sect.
*Aspergillus* species were compared to reference datasets obtained from Peterson (2008),
Hubka et al. (2013) and Visagie et al. (2014a). All datasets were aligned in MAFFT v.
7.221 (Katoh and Standley 2013) using the L-INS-i option for ITS and G-INS-i op-
tion for the other genes. All alignments were trimmed in Geneious and then analysed
as single and concatenated datasets using Maximum Parsimony (MP) and Bayesian In-
ference of phylogenetic trees (BI). For concatenated phylogenies, a partitioned dataset
of ITS, BenA, CaM and RPB2 regions was used.

MP analyses were run in PAUP* v. 4.0b10 (Swofford 2002) using heuristic searches
with 100 random taxon additions and gaps treated as missing data. Support in nodes was
calculated using a bootstrap analysis with the heuristic search option and 1000 replicates.
A survey of xerophilic *Aspergillus* from indoor environment...

BI analyses were run in MrBayes v. 3.2.5 (Ronquist et al. 2012). Model selections for BI were made for each gene based on the lowest Akaike Information Criterion (AIC) value, calculated in MrModeltest v. 2.3 (Nylander 2004). Analyses were run with two sets of four chains and stopped at a split frequency of 0.01. The sample frequency was set at 100 and 25 percent of trees removed as burnin. Trees were visualized in FigTree v. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and prepared for publication in Adobe® Illustrator® CS6. Aligned datasets, command blocks and trees were uploaded to TreeBase (www.treebase.org) under submission number 19771.

**Extrolite analysis**

For extrolite analysis, all strains were grown on 9 cm polystyrene Petri dishes on MEA supplemented with 7.5% NaCl at 25 °C for 14 d. Six agar plugs from each fungal isolate were removed with a sterilized 7 mm cork borer and placed into a 13 mL polypropylene tube. Ethyl acetate (2 mL) was added to the tubes and vortexed for 30 s, followed by 1 h of sonication at 30 °C and vortexed again for 30 s. The supernatants were transferred into clean polypropylene tubes and dried on a centrifugal vacuum concentrator at 35°C. Extracts were reconstituted in 1 mL of 8:2 methanol:water and filtered into 2 mL amber glass HPLC vials using a 0.45 µm PVDF syringe filter. Extracts were immediately stored at -20 °C until LC-MS analysis. Extracts were analyzed on a Q-Exactive orbitrap coupled to a 1290 Agilent HPLC in both positive and negative polarities. Chemical formula of observed extrolites were determined with Xcalibur® software using accurate mass measurements and manually verified by isotopic pattern. Chemical formulae were searched against AntiBase2013 and Scifinder and putatively confirmed by comparing product ions observed with those published in the literature or through manual interpretation. The fungi were also analysed using the HPLC-DAD method described by Frisvad and Thrane (1987) as modified by Nielsen et al. (2011), by taking two agar plugs from each of the following media: DG18, CYA20S and YES agar, and extracting the combined 6 agar plugs of the colonies of *Aspergillus* with ethylacetate / isopropanol (3:1, vol./vol.) with 1% (vol.) formic acid added to that mixture. The retention indices and UV spectra were compared to those given in the supplementary material of the Nielsen et al. (2011) paper.

**Results**

**Sampling, isolations and identification**

Isolations from house dust collected in Canada and Hawaii resulted in 1039 isolates of xerophilic/xerotolerant fungi. 296 isolates were identified as *Aspergillus*, of which members from sections *Aspergillus*, *Nidulantes* (*A. versicolor* clade) and *Restricti* were most
abundant. Strains were identified to species using CaM sequences and identities confirmed by morphological examination. They include *A. chevalieri*, *A. cibarius*, *A. montevidensis*, *A. proliferans*, *A. pseudoglaucus*, *A. ruber* and *A. tonophilus* from sect. *Aspergillus*. In section *Nidulantes* (*A. versicolor* clade), *A. jensenii* and *A. sydowii* were isolated most frequently, while *A. creber*, *A. fructus*, *A. protuberus*, *A. tennesseensis* and *A. versicolor* were also recovered. A large degree of sequence diversity was observed in sect. *Restricti* and will be presented in a separate study. Other *Aspergillus* species identified include *A. aureolatus*, *A. candidus*, *A. calidoustus*, *A. flavus*, *A. japonicus*, *A. lentulus*, *A. luchuensis*, *A. micronesiensis*, *A. niger*, *A. pragensis*, *A. tamarii*, *A. terreus*, *A. tubingensis*, *A. welwitschiae* and *A. westerdijkiae*. Reference sequences, mostly *CaM*, obtained for these species were uploaded to GenBank under accession numbers KX894565–KX894666 and KY351765–KY351785, and are included in Suppl. material 1: Table 1 to assist with future identifications. This table also include additional information with regard to strains’ location and growth medium used for their isolations. During this survey, two sect. *Aspergillus* species with eurotium-like sexual states could not be identified as known species and are described below as new species, based on growth characters on a wide range of culture media. The new species are compared with their close relatives and notes are provided on their diagnostic phenotypic characters, including extrolite production.

**Phylogeny**

To demonstrate genealogical concordance for the two new species, phylogenies for all known species of sect. *Aspergillus* were prepared (Table 1) using alignments of ITS, *BenA*, *CaM*, and *RPB2* (Fig. 1) and overall phylogenetic relationships considered as a concatenated dataset (Fig. 2).

The ITS alignment was 535 bp long and contained 68 variable characters, of which 27 were parsimony informative. MP analysis resulted in two equally parsimonious trees (length 79 steps, CI = 0.987, RI = 0.992). HKY+I was found to be the most suitable model for BI analysis. ITS is highly conserved in sect. *Aspergillus*, as demonstrated in the phylogenetic analysis, making it uninformative as an identification barcode in section *Aspergillus*. Of the 22 species, including the two new species described here, only *A. cumulatus*, *A. leucocarpus*, *A. osmophilus* and *A. xerophilus* have unique ITS barcodes. The alignments for the *BenA*, *CaM* and *RPB2* datasets were respectively 389 (151 variable, 136 parsimony informative), 556 (221 variable, 177 parsimony informative) and 871 bp (202 variable, 162 parsimony informative) long. MP analyses resulted in 84 (length 287 steps, CI = 0.728, RI = 0.923), 12 (length 275 steps, CI = 0.7, RI = 0.904), 28 (length 364 steps, CI = 0.648, RI = 0.911) and 24 (length 798 steps, CI = 0.692, RI = 0.907) equally parsimonious trees for *BenA*, *CaM*, *RPB2* and concatenated dataset. K80+G (*BenA*), SYM+G (*CaM*) and SYM+I+G (*RPB2*) were the most suitable models for BI.

Tree topologies did not differ for respective genes between MP and BI; therefore, MP trees were used to present results. Some species are consistently resolved as sister
Table 1. Strains used for phylogenetic analyses.

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<th>Origin</th>
<th>GenBank accession numbers</th>
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<td>NRRL 114; ATCC 10076; IMI 211808</td>
<td>Unknown</td>
<td>EF652051, EF651987, EF651886, EF651933</td>
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<tr>
<td>Aspergillus proliferans</td>
<td>NRRL 62482; CCF 4096</td>
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<td>Aspergillus proliferans</td>
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<td>Aspergillus proliferans</td>
<td>NRRL 62497; CCF 4115</td>
<td>Toenail, Czech Republic</td>
<td>FR851850, HE578090, FR851855, HE578107</td>
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</table>
A survey of xerophilic Aspergillus from indoor environment...

<table>
<thead>
<tr>
<th>Species</th>
<th>Strains</th>
<th>Origin</th>
<th>GenBank accession numbers</th>
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</thead>
<tbody>
<tr>
<td><em>Aspergillus proliferans</em></td>
<td>NRRL 71</td>
<td>Leafhoppers, USA</td>
<td>EF652047 EF651986 EF651885 EF651932</td>
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<td><em>Aspergillus pseudoglaucus</em></td>
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<td>Unknown</td>
<td>EF652050 EF652007 EF651917 EF651952</td>
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<tr>
<td><em>Aspergillus pseudoglaucus</em></td>
<td>CBS 379.75; IMI 278373; ETH 8218; DSM 1370</td>
<td>Leaf from <em>Vaccinium myrtillus</em>, Switzerland</td>
<td>HE615131 HE801322 HE801336 HE801311</td>
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<tr>
<td><em>Aspergillus pseudoglaucus</em></td>
<td>CBS 529.65; NRRL13; NRRL 24; ATCC 9294; IMI 016114; IMI 016114ii; MUCL 15649</td>
<td>Prunus domestica, France</td>
<td>EF652048 EF652005 EF651915 EF651950</td>
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<td><em>Aspergillus pseudoglaucus</em></td>
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<td>Animal dung, China</td>
<td>HE615130 HE801321 HE801335 HE801310</td>
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<td><em>Aspergillus ruber</em></td>
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<td>EF652049 EF652006 EF651916 EF651951</td>
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<td><em>Aspergillus ruber</em></td>
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<td>Coffee beans, United Kingdom</td>
<td>EF652080 EF652010 EF651922 EF651949</td>
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<td><em>Aspergillus ruber</em></td>
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<td>Unknown</td>
<td>EF652066 EF652009 EF651920 EF651947</td>
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<td><em>Aspergillus sloanii</em></td>
<td>CBS 138179; DTO 245-A9</td>
<td>House dust, United Kingdom</td>
<td>KJ775543 KJ775314 KJ775077 KX450901</td>
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<td><em>Aspergillus sloanii</em></td>
<td>CBS 138231; DTO 245-A6</td>
<td>House dust, United Kingdom</td>
<td>KJ775541 KJ775311 KJ775075 KX450899</td>
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<tr>
<td><em>Aspergillus tonophilus</em></td>
<td>CBS 405.65T; NRRL 5124; ATCC 14567; ATCC 16440; ATCC 36504; DSM 3462; IFO 6529; IMI 108299; IMI 108299ii</td>
<td>Binocular lens, Japan</td>
<td>EF652081 EF652000 EF651919 EF651969</td>
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<td><em>Aspergillus xerophilus</em></td>
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<td>Desert soil, Egypt</td>
<td>EF652085 EF651983 EF651923 EF651970</td>
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<td><em>Aspergillus xerophilus</em></td>
<td>NRRL 6132</td>
<td>Desert soil, Egypt</td>
<td>EF652086 EF651984 EF651924 EF651971</td>
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</table>
**Figure 1.** One of the most parsimonious trees of *Aspergillus* sect. *Aspergillus* based on ITS, CaM, BenA and RPB2. Trees were rooted to *A. xerophilus*, *A. leucocarpus* and *A. osmophilus*. Support in nodes higher than 80% bootstrap values and 0.95 posterior probabilities are shown above thickened branches. New species are shown in bold and colour, while ex-type strains are followed by $^T$. 

![Tree Diagram](image-url)
A survey of xerophilic *Aspergillus* from indoor environment...

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**Figure 1.** Continued.
Figure 1. Continued.
A survey of xerophilic *Aspergillus* from indoor environment...

**Figure 1.** Continued.
Figure 2. One of the most parsimonious trees of *Aspergillus* sect. *Aspergillus* based on a combined dataset of ITS, BenA, CaM and RPB2. The tree was rooted to *A. xerophilus*, *A. leucocarpus* and *A. osmophilus*. Support in nodes higher than 80% bootstrap values and 0.95 posterior probabilities are shown above thickened branches. New species are shown in bold and colour, while ex-type strains are followed by T.
A survey of xerophilic *Aspergillus* from indoor environment...

15 species such as *A. proliferans* and *A. glaucus*, *A. brunneus* and *A. niveoglaucus*, *A. montevidensis* and *A. intermedius*, and *A. osmophilus* and *A. xerophilus*. On a deeper level, however, the backbones in all gene trees were generally poorly supported, resulting in inconsistent clades among different gene trees. The addition of more newly discovered species of section *Aspergillus* in future may result in better backbone support. With regards to the new species, *A. mallochii* was sister to *A. appendiculatus*, although RPB2 placed it on a unique branch. *Aspergillus megasporus* resolves in different positions depending on gene analyzed, but based on the concatenated phylogeny belongs in a clade with *A. brunneus*, *A. niveoglaucus*, *A. neocarnoyi*, *A. glaucus* and *A. proliferans*. For species identifications, it is clear that all three of these genes are superior to ITS and distinguish between all 22 accepted species in sect. *Aspergillus*.

**Extrolites**

*Aspergillus mallochii* and *A. megasporus* produced several related tryptophan derived alkaloids including, echinulins, neoechinulins and isoechochinulins, but in varying amounts (Table 2). *Aspergillus mallochii* (DAOMC 146054 = KAS 7618) *Aspergillus megasporus* (DAOMC 250799 = KAS 6176). Both species show some production of echinulin class of alkaloids to varying amounts. Quinolactacin A1, A2 and B were not detected in *A. mallochii*.

![Figure 3](image-url) Base peak chromatograms observed in positive ionization mode. **a** *Aspergillus mallochii* (DAOMC 146054 = KAS 7618) **b** *Aspergillus megasporus* (DAOMC 250799 = KAS 6176). Both species show some production of echinulin class of alkaloids to varying amounts. Quinolactacin A1, A2 and B were not detected in *A. mallochii*.
**Table 2.** Overview of the major extrolites detected and product ions.

<table>
<thead>
<tr>
<th>Extrolite</th>
<th>m/z</th>
<th>Formula</th>
<th>RT (min)</th>
<th>Product ions m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>echinulin</td>
<td>462,311</td>
<td>C_{29}H_{39}N_{3}O_{2}</td>
<td>4.16</td>
<td>338,186 266,190 198,128 210,128 270,124</td>
</tr>
<tr>
<td>preechinulin</td>
<td>392,233</td>
<td>C_{24}H_{29}N_{3}O_{2}</td>
<td>3.63</td>
<td>268,108 336,170 256,108 69,071 –</td>
</tr>
<tr>
<td>isoechinulin A</td>
<td>390,217</td>
<td>C_{24}H_{27}O_{2}N_{3}</td>
<td>3.75</td>
<td>266,092 322,155 334,155 254,092 306,123</td>
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<tr>
<td>isoechinulin B</td>
<td>406,212</td>
<td>C_{24}H_{27}O_{3}N_{3}</td>
<td>3.37</td>
<td>334,155 266,092 237,138 338,150 –</td>
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<tr>
<td>isoechinulin C</td>
<td>324,171</td>
<td>C_{19}H_{21}O_{2}N_{3}</td>
<td>3.14</td>
<td>256,108 268,108 185,071 69,071 –</td>
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<td>neoechinulin A</td>
<td>322,155</td>
<td>C_{19}H_{21}O_{2}N_{3}</td>
<td>3.26</td>
<td>254,092 266,095 69,071 226,097 –</td>
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<td>preechinulin</td>
<td>326,186</td>
<td>C_{19}H_{21}O_{2}N_{3}</td>
<td>2.99</td>
<td>130,065 198,128 270,123 258,124 –</td>
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<tr>
<td>quinolactacin A1</td>
<td>271,144</td>
<td>C_{16}H_{18}N_{2}O_{2}</td>
<td>2.66</td>
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<td>quinolactacin A2</td>
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<td>C_{16}H_{18}N_{2}O_{2}</td>
<td>2.72</td>
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<td>quinolactacin B</td>
<td>257,129</td>
<td>C_{16}H_{18}N_{2}O_{2}</td>
<td>2.54</td>
<td>214,073 – – – –</td>
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<td>questin*</td>
<td>283,061</td>
<td>C_{16}H_{18}O_{3}</td>
<td>3.39</td>
<td>268,038 240,042 – – –</td>
</tr>
</tbody>
</table>

* observed in negative ionization mode

**Figure 4.** Chemical structure of major compounds produced by *A. mallochii* and *A. megasporus.*

**Taxonomy**

*Aspergillus mallochii* Visagie, Yilmaz & Seifert, sp. nov.
MycoBank MB 819025
Fig. 5

**Etymology.** Latin, *mallochii,* named after Prof. David Malloch, a Canadian specialist in 'Plectomycetes' who first collected this species in the 1960’s.

**Typus.** USA, California, San Mateo, pack rat dung, added to DAOMC in 1969, collected by David Malloch, Holotype DAOM 740296, culture ex-type DAOMC 146054 = CBS 141928 = DTO 357-A5 = KAS 7618.
A survey of xerophilic Aspergillus from indoor environment...

Additional material examined. The Netherlands, ‘chocolat miroir’ icing for a cake, unknown date and collector, culture CBS 141776 = DTO 343-G3.

ITS barcode. KX450907. Alternative identification markers: BenA = KX540889, CaM = KX450902, RPB2 = KX450894.

Colony diam, 7 d (in mm), 25 °C. CYA 6–8; CY20S 14–17; MEA 3–4; MEA20S 29–31; DG18 48–50; YES 9–10; M40Y 48–50; MY50G 35–40; MY10-12 29–30; CY20S, DG18, MEA20S at 37 °C no growth; CREA no growth.

Colony characters. CYA: Colonies with restricted growth; conidiophores sparse; cleistothecia absent. CY20S: Colonies grow faster than on CYA; sporulation sparse to moderately dense, greyish to dark green (30E5–F5); cleistothecia dark yellow, abundant at colony centre. MEA: Colonies with restricted growth; conidiophores and cleistothecia absent. MEA20S: Colonies grow faster than on MEA; sporulation sparse, greyish to dark green (30E5–F5); cleistothecia yellow to orange, abundant. DG18: Colonies very fluffy with aerial mycelia giving rise to conidiophores; sporulation sparse to moderately dense, greyish to dark green (30E5–F5); cleistothecia abundant at colony centre, yellow to orange. Homothallic.

Micromorphology on DG18. Cleistothecia eurotiun-like, wall consisting of one layer of flattened cells, yellow to orange, turning deep brown with age, globose, 95–250 µm diam. Ascii eight-spored, globose, ellipsoidal to pyriform, 10–15 µm diam, maturing after 7–14 d. Ascospores lenticular, equatorial crest present but incomplete, convex surface roughened, 4.5–6 × 3.5–4.5 µm (5.1±0.3 × 3.9±0.3), n = 52. Conidiophores radiate and columnar, uniseriate; stipes smooth, 200–1000 × 7.5–17(–19) µm; vesicle globose, (25–)40–65 µm diam; phialides ampulliform, covering 80–100% of vesicle, 7–11 × 3–5 µm; conidia roughened to spiny, ellipsoidal, connectives easily visible, 4.5–6.5 × 4.5–5.5 µm (5.4±0.4 × 4.5±0.3), average width/length = 0.83, n = 68.

Extrolites. Isoechinulin A, B & C; neoechinulin A & B; unknowns C_{19}H_{32}O_{18}N_{9}, C_{19}H_{21}O_{3}N_{2}, C_{19}H_{30}O_{3}N_{3}, C_{24}H_{30}O_{3}N_{3}, C_{24}H_{21}O_{3}N_{3}, C_{24}H_{21}O_{3}N_{3}, C_{39}H_{43}O_{6}N_{5}. Additionally, echinulin, erythroglaucin, auroglaucin, flavoglaucin, dihydroauroglaucin, tetrahydroauroglaucin were found in CBS 141776. Some extrolites tentatively identified as tetracyclic compounds were detected in CBS 141776.

Notes. Aspergillus mallochii is phylogenetically and morphologically most similar to A. appendiculatus. Both are unable to grow at 37 °C and both have ascospores with incomplete equatorial furrows. Ascospores of the new species, however, are generally smaller and at least finely roughened compared to the smoother ascospores of A. appendiculatus.

Aspergillus megasporus Visagie, Yilmaz & Seifert, sp. nov.
MycoBank MB 819028
Fig. 6

Etymology. Latin, megasporus, in reference to the large conidia produced by this species.
Figure 5. *Aspergillus mallochii* (DAOMC 146054). a Colonies on MEA, MEA20S, MY10-12 (top row, from left to right), DG18, CY20S, MY50G (bottom row, from left to right) b Texture on DG18 c Asci d Ascospores e Cleistothecium f, g Conidiophores h Conidia. Scale bars: e = 50 µm, c, d, f–h = 10 µm.
A survey of xerophilic *Aspergillus* from indoor environment...

**Figure 6.** *Aspergillus megasporus* (DAOMC 250799). **a** Colonies on MEA, MEA20S, MY10-12 (top row, from left to right), DG18, CY20S, MY50G (bottom row, from left to right) **b** Texture on DG18 **c** Asci **d** Ascospores **e** Cleistothecium **f, g** Conidiophores **h** Conidia. Scale bars: **e** = 50 µm, **c, d, f–h** = 10 µm.


ITS barcode. KX540910. Alternative identification markers: BenA = KX450892, CaM = KX450905, RPB2 = KX450897.

Colony diam, 7 d (in mm), 25 °C. CYA 3–8; CY20S 30–35; MEA 3–5; MEA20S 24–35; DG18 47–50; YES 15–16; M40Y 45–47; MY50G 35–40; MY10-12 40–44; CY20S, DG18, MEA20S at 37 °C no growth, CREA no growth.

Colony characters. CYA: Colonies with restricted growth; conidiophores and cleistothecia absent. CY20S: Colonies grow faster than on CYA; sporulation moderately dense, greyish to dark green (30E5–F5); cleistothecia yellow, sparse. MEA: Colonies with restricted growth; conidiophores and cleistothecia absent. MEA20S: Colonies grow faster than on MEA; sporulation moderately dense, greyish to dark green (30E5–F5); cleistothecia yellow, moderately abundant. DG18: Colonies very fluffy with abundant aerial mycelia giving rise to conidiophores; sporulation moderately dense, dull to dark green (28E3–F3); cleistothecia abundant, dark yellow to orange. Homothallic.

Micromorphology on DG18. Cleistothecia eurutium-like, wall consisting of one layer of flattened cells, yellow to orange, globose, 115–205 µm diam. Asci eight-spored, globose, ellipsoidal to pyriform, 14–19.5 µm diam. Ascospores lenticular, equatorial crest roughened, convex surface smooth, 5–8 × 3.5–6 µm (6.4±0.6 × 4.9±0.5), n = 51. Conidiophores radiate and columnar, uniseriate; stipes smooth, (30–)60–1000 × (9–)13–20 µm; vesicle globose, (8.5–)20–60 µm diam; phialides ampulliform, covering 70–100% of vesicle, (9–)11–15 × 5–7 µm; conidia roughened to spiny, ellipsoidal, connectives often visible, 7–12 × 6–8.5 µm (9.5±1.0 × 6.9±0.5), average width/length = 0.72, n = 85.

Extrolites. Echinulin; neoechinulin A & B; preechinulin; quinolactacin A₁ & A₂; unknowns C₁₅H₂₀O₂, C₂₁H₃₇N, C₂₄H₃₀O₆, C₂₉H₃₇O₂N₃, C₂₁H₄₄O₂. In addition, asperflavin, emodin, erythroglauca, physcion and bisanthron were found in CBS 141772. Some additional extrolites, tentatively identified as tetracyclic compounds, were detected in CBS 141772.

Notes. The concatenated phylogeny of BenA, CaM and RPB2 resolves A. megasporus in a clade with A. brunneus, A. niveoglaucus, A. neocarnoyi, A. glaucus and A. proliferans. None of these species are able to grow on CY20S at 37 °C. Aspergillus niveoglaucus and A. megasporus can be distinguished from other species by their large conidia, which are up to 11 and 12 µm in the longest axis respectively. Aspergillus megasporus colonies grow faster than A. niveoglaucus on DG18.
Discussion

Species of *Aspergillus* section *Aspergillus* are xerophilic and widespread in nature. Indoor environments, including homes and public buildings, are designed to be as dry as possible, especially in temperate countries, and these conditions select for these xerophiles to thrive. This partially explains the dominance of *Aspergillus*, *Penicillium*, *Cladosporium* and *Wallemia* in indoor fungal communities (Amend et al. 2010; Flannigan and Miller 2011; Samson et al. 2010; Visagie et al. 2014a). In our isolations of xerophiles occurring in Canadian and Hawaiian house dust, these genera were also found to be dominant. Xerophily is spread broadly across *Aspergillus*. Thirty *Aspergillus* species were isolated in our survey, excluding the many section *Restricti* species that will be addressed in another study. All *Aspergillus* are capable of growth on DG18, but MY10-12 and MY50G have much lower water activities. Species isolated from these selective media included species of sections *Aspergillus*, *Candidi*, *Flavipes*, *Nidulantes*, *Nigri*, *Restricti* and *Versicolores* (summarized in Suppl. material 1: Table 1). One new species was discovered from our house dust samples, described as *A. megasporus*. We also re-identified all cultures from DAOMC deposited as *Eurotium*. Among these, we discovered an additional species that we described as *A. mallochii* using morphology, extrolite and phylogenetic analyses.

*Aspergillus megasporus* was isolated from Canadian house dust collected in Wolfville, Nova Scotia and Little Lepreau, New Brunswick, and was also isolated from chocolate butter in the Netherlands. Phylogenetically, the position of this species varies depending on which gene is analysed; *CaM* resolves it in its own distinct clade, *BenA* in a clade with a poorly supported branch with *A. glaucus* and *A. proliferans*, and *RPB2* closest to *A. niveoglaucus*. The multigene phylogeny places it in a large clade, including *A. brunneus*, *A. niveoglaucus*, *A. neocarnoyi*, *A. glaucus* and *A. proliferans*. Both *A. niveoglaucus* and *A. megasporus* produces conidia respectively reaching 11 and 12 µm, easily distinguishing them from other species of section *Aspergillus*. *Aspergillus megasporus* can be distinguished from *A. niveoglaucus* based on its faster growth on DG18. *Aspergillus megasporus* produces extrolites commonly detected in species of section *Aspergillus*, including echinulin, neoechinulin and preechinulin. However, we also detected quinolactacin, a first report for the group. In an independent study using different methods and media, compounds detected from CBS 141772 include asperflavin, auroglaucin, bisanthron, dihydroauroglaucin, echinulin, emodin, erythroglauacin, flavoglaucin, isoechinulins, neoechochinulins, preechinulin, physcion, quinolactacin, tetracyclic compounds, and tetrahydroauroglaucin (Frisvad, personal communication).

*Aspergillus mallochii* was isolated from pack rat dung collected from San Mateo, California, USA. An additional strain was recently isolated from ‘*Chocolat miroir*’ icing for a cake in the Netherlands. Phylogenetically, it has *A. appendiculatus* as sister species, originally described by Blaser (1975) from German smoked sausages. These two species share identical ITS sequences that are distinct from all others in the section (Fig. 2). All other genes, especially *RPB2*, easily distinguish the two. Morphologically
they are also similar, but the roughened ascospores of *A. mallochii* are distinct from the smoother ascospores of *A. appendiculatus*. In an independent study using different methods and media, compounds detected from CBS 141776 included auroglaucin, dihydroauroglaucin, echinulins, erythroglaucin, flavoglaucin, isoechinulins, neoechinulins, tetracyclic compounds and tetrahydroauroglaucin. Comparisons revealed that *A. appendiculatus* produced several compounds not observed in *A. mallochii*, such as asperflavin, asperentins, bisanthrons, 5-farnesyl-5,7-dihydroxy-4-methylphthalide, mycophenolic acid, physcion and questin (Nielsen et al. 2011). None of the extrolites identified in *A. mallochii* are unique to the species.

Quinolactacin A1, A2 & B were the major compounds produced by *A. megasporus*, the only species of section *Aspergillus* that produces these. These quinolone structures with a γ-lactam ring were first characterized from fermentations of an unknown *Penicillium* species (Kakinuma et al. 2000; Takahashi et al. 2000) and further characterized by Kim et al. (2001) in *Penicillium citrinum*, where they were demonstrated to be acetylcholinesterase inhibitors. Quinolactacins have since been reported from multiple *Penicillium* species from sections *Citrina* (Houbraken et al. 2011), *Brevicompacta* (Frisvad et al. 2013; Perrone et al. 2015) and *Robsamsonia* (Houbraken et al. 2016); *Aspergillus quadricinctus*, *A. stramenius* (section *Fumigati*) (Frisvad and Larsen 2015b; Samson et al. 2007), *A. karnatakaensis* (section *Aenei*) (Varga et al. 2010); and from the distantly related marine derived *Xylariaceae* (Nong et al. 2014). Based on current knowledge, the echinulins (including echinulin, neoechinulin and isoechinulin) detected in both *A. megasporus* and *A. mallochii* seem specific to *Aspergillus* sections *Aspergillus* and *Restricti* (Frisvad and Larsen 2015a). Echinulin was first discovered in *A. brunneus* (= *E. echinulatum*) by Quilico and Panizzi (1943). It was subsequently detected in many more section *Aspergillus* species (Ali et al. 1989; Almeida et al. 2010; Greco et al. 2015; Li et al. 2008b; Slack et al. 2009; Smetanina et al. 2007; Vesonder et al. 1988) and shown to be toxic to animal cells (Ali et al. 1989; Umeda et al. 1974), while swine and mice respectively refused feed and water containing echinulin (Vesonder et al. 1988). The presence of echinulin in the environment is not well documented however. In contrast to the negative effects of echinulin, neoechinulin has anti-oxidant properties (Yagi and Doi 1999) and protected PC12 cell lines, used in neurological research, against cell death by peroxynitrite (Kimoto et al. 2007; Maruyama et al. 2004).

Visagie et al. (2014a) emphasized that despite the existence of comprehensive ITS barcode reference databases, this marker is insufficient for identifying most *Aspergillus*, *Penicillium* and *Talaromyces* to species level in culture-independent surveys such as those of Amend et al. (2010) and Adams et al. (2013a; 2013b). The reference sets include sequences for multiple genes obtained from ex-type cultures for all accepted species in these genera (Samson et al. 2014; Visagie et al. 2014b; Yilmaz et al. 2014) and are invaluable as anchoring points for species. Curating databases is laborious and has many complications, but both UNITE and NCBI have ongoing curation projects involving ITS barcodes (Kõljalg et al. 2013; Nilsson et al. 2015; Schoch et al. 2014). ITS will always have limited resolution for species identification. In *Aspergillus* and *Penicillium*, ITS is highly conserved in many sections, as is observed in our phylogeny.
A survey of xerophilic Aspergillus from indoor environment...

Barcode-based metagenomic studies commonly use Last Common Ancestor (LCA) analyses for assigning OTU’s to GenBank taxonomic nodes. In LCA, when the analysis cannot identify an operational taxonomic unit (OTU) at a taxonomic rank, it will move up one level until it can make a confident assignment. As now implemented, most species of sect. Aspergillus species would be identified only to the generic level. For species-rich genera such as Aspergillus and Penicillium, this is problematic. Different ecologies, functions, extrolites etc. are often associated with specific groups (i.e. true xerophily in at least three sections of Aspergillus), and much potentially important information is lost because of this imprecision. To circumvent this problem, a few recent studies have used alternative genes combined with next generation sequencing (NGS) for making “mass” identifications in Aspergillus. Lee and Yamamoto (2015) assessed the accuracy of high-throughput amplicon sequencing using ITS, BenA and CaM, and identified OTU’s using the ex-type sequences published by Samson et al. (2014). Results were promising with both BenA and CaM, which are obviously more accurate than ITS. Unfortunately, amplifications of these alternative barcodes were sometimes problematic, perhaps because they are single copy genes or undocumented sequence variation, especially considering comparisons to only ex-type sequences. Similar results were obtained in a subsequent study by Lee et al. (2016). Even though these types of studies are promising, considerable optimisation is required to amplify and sequence low copy markers from a complex matrix, and shotgun sequencing may be more effective. No matter what the experimental approach used by ecologists, taxonomists need to make identifications as easy as possible, not only in the traditional morphological sense, but also by generating reference data that will enhance the robustness of analyses of data generated using new technologies such as NGS. Surveys such as ours are thus important not only for discovering previously unnamed species, but for providing more reference sequences in public databases that capture infraspecies sequence variation for multiple barcodes.

Recently, the International Code of Nomenclature for algae fungi and plants (ICN, Melbourne Code; (McNeill et al. 2012)) adopted single name nomenclature for pleomorphic fungi, meaning decisions are needed to choose either the teleomorph (sexual morph) or anamorphic (asexual morph) name to represent the genus. In anticipation of this change, Houbraken and Samson (2011) reviewed the taxonomy and phylogeny Trichocomaceae, of which Penicillium and Aspergillus are the largest groups, using a four gene combined analysis. The situation with the generic concept and name for Aspergillus is complicated and controversial, partly because of conflicting interpretations of phylogenies, and partly because of differing opinions on how much taxonomic weight to apply to sexual states in generic concepts (Houbraken and Samson 2011; Pitt and Taylor 2014; Taylor et al. 2016). In this paper, we have followed the traditional broad concept of Aspergillus advocated by the International Commission of Penicillium and Aspergillus (ICPA), which includes species formerly classified in the sexual genera Eurotium, Emericella, Neosartorya and Petromyces in Aspergillus. The section of Aspergillus that is the focus of our paper includes the type species of both Aspergillus (A. glaucus) and Eurotium (E. herbariorum). With the community decision of section Aspergillus (Fig. 2).
to respect the priority of *Aspergillus* in both the nomenclatural and practical sense, the new species described here would be described in *Aspergillus* whether a broad or narrow generic concept is applied. The recent proposal by Taylor et al. (2016) to conserve *Aspergillus* with the type species changed to *A. niger* is still being discussed, but at this time seems unlikely to be accepted. If the proposal is implemented, along with the narrower generic concept endorsed by these authors, approximately 180 *Aspergillus* species would be renamed, including those described in this paper.

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**References**

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A survey of xerophilic Aspergillus from indoor environment...


**Supplementary material I**

**Species isolated from house dust using selective xerophilic media**

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Data type: Occurrence, GenBank info

Explanation note: Species isolated from house dust using selective xerophilic media, their occurrence and GenBank numbers for sequences generated for these strains.

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