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Characterization of two xerophilic *Aspergillus* spp. from peanuts (*Arachis hypogaea*)

Nur Amalina Kamarudin and Latiffah Zakaria*
School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia.
Email: lfah@usm.my

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ABSTRACT

Aims: Xerophilic *Aspergillus* spp. promote the growth of toxigenic species. Since mycotoxins are toxic to human and animal, identification of these species is important.
Methodology and results: Two xerophilic species isolated from peanuts (*Arachis hypogaea*) were identified based on morphological characteristics, molecular identification, and phylogenetic analysis using internal transcribed spacer region, β-tubulin, and calmodulin sequences.
Conclusion, significance and impact of study: The occurrence of *A. chevalieri* and *A. amstelodami* on peanuts provides favorable growth conditions for less xerophilic *Aspergillus* as well as other spoilage-related fungal genera, particularly mycotoxin-producing species that could lead to mycotoxin contamination. The occurrence of *A. chevalieri* and *A. amstelodami* on peanuts might also reduce shelf life and affect the quality of the kernels. To our knowledge, this is the first report of the occurrence of *A. chevalieri* and *A. amstelodami* on a food product in Malaysia, and the finding of this study contributes to the repertoire of *Aspergillus* species that are associated with food products.

Keywords: Aspergillus amstelodami, Aspergillus chevalieri, peanuts

INTRODUCTION

Peanut (*Arachis hypogaea*) is generally used as an ingredient in local dishes or are consumed as snacks in Malaysia. This legume is often imported and thus contamination with fungi can easily occur under favorable conditions during shipment, storage, and marketing. Among fungal contaminants, *Aspergillus* is the most common genus found on peanuts, especially those belonging to the sections *Nigri* and *Flavi* (Palencia *et al*., 2010; Passone *et al*., 2008; Pitt and Hocking, 2009; Zorzete *et al*., 2013). Several species from these sections are well-known mycotoxin producers, including *A. niger*, *A. flavus*, and *A. parasiticus*. Thus, contamination of food products with these toxigenic *Aspergillus* spp. can also lead to contamination with mycotoxins secondary metabolites toxic to humans and animals.

Growth of the toxigenic *Aspergillus* spp. on food products can be facilitated by xerophilic *Aspergillus* spp., which are classified under the section *Aspergillus.* Xerophilic *Aspergillus* spp. can grow on substrates with low water activity, which can cause deterioration of stored food products including peanuts (Hubka *et al*., 2013). During growth, as a result of metabolic activities, xerophilic *Aspergillus* species release water in substrates with low water activity; this creates favorable conditions for the growth of less xerophilic fungi such as toxigenic *Aspergillus* species (Hubka *et al*., 2013). Therefore, identification of xerophilic aspergilli is as important as that of toxigenic aspergilli.

Both morphological and molecular methods should be employed to identify and characterize *Aspergillus* isolates because morphological features of some *Aspergillus* species are very similar, especially in closely related species, leading to inaccuracy in identification. Furthermore, medium composition and incubation conditions can affect morphological features (Okuda *et al*., 2000) used as identification keys; thus, taxonomical expertise is essential for morphological identification of *Aspergillus* isolates. Hence, for species confirmation, molecular methods are typically used. For identification of *Aspergillus*, sequence-based approaches using the internal transcribed spacer regions (ITS), β-tubulin, and calmodulin genes is recommended in addition to phylogenetic analysis (Samson *et al*., 2014). The aim of this study was to identify and characterize two xerophilic *Aspergillus* species isolated from peanuts using morphological and molecular methods for accurate species determination.

MATERIALS AND METHODS

Peanuts were purchased from sundry shops and supermarkets in seven Malaysian states: Kuala Lumpur,

*Corresponding author

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Pulau Pinang, Sarawak, Kedah, Johor, Perak, and Terengganu. These food items were stored in gunny sacks or storage bins at room temperature. Peanuts stored in the gunny sacks or storage bins were apparently in good condition. Hundred grams of peanut was randomly scooped from the top surface of each gunny sack or storage bin and then purchased.

Aspergillus isolates were obtained using direct plating and surface sterilization techniques. For direct plating, five to seven peanuts were directly plated onto potato dextrose agar (PDA). The peanuts were surfaced sterilized with 10% sodium hypochlorite for 3 min, soaked in 70% alcohol solution for 3 min, soaked in sterile distilled water for 1 min, and dried using filter paper. The surface sterilized peanuts were plated on PDA, equidistant from each other. After direct plating and surface sterilization, plates were incubated at 27 ± 1 °C for 5 to 7 days or until the growth of fungal mycelia was observed. The mycelia were then plated on new PDA plates. PDA was used because it is general purpose medium for the cultivation of fungi, and our goal was to obtain as many different types of fungi as possible. In this study, 20 isolates of *Aspergillus* were examined and were maintained in the Plant Pathology Lab, School of Biological Sciences, Universiti Sains Malaysia.

Fungal isolates were identified macroscopically using differential media as described by Samson *et al*. (2010), specifically, Malt Extract Agar (MEA), Czapek Yeast Agar 25 (CYA25), Czapek Yeast Agar 37 (CYA37), and Creatine Sucrose Agar (CREA) were used. The isolates were plated in three-point inoculation and incubated in the dark at 25 °C for 7 days. For observation of microscopic characteristics, cultures grown on MEA were used. The microscopic and macroscopic characteristics were compared to the descriptions of Klich (2002) and the description of the lab manual by Samson *et al*. (2010).

Molecular identification was performed using the internal transcribed spacer regions (ITS), β-tubulin, and calmodulin genes. For DNA extraction, mycelia were grown in malt extract broth at 25 °C. Mycelia were harvested after 2 days and freeze dried for 48 h, after which the mycelia were ground to a fine powder using liquid nitrogen. DNA was extracted using Invisorb Spin Plant Mini Kit (Stratec Molecular GmbH, Berlin, Germany) according to the protocols of the manufacturer.

PCR amplification was conducted using the following primers: ITS, ITS1 (TCCGTAGGTGAACCTGCG G) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al*., 1990); β-tubulin, Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (ACCCTCAGT GTAGTGACCCTTGGC) (Glass and Donaldson, 1995) and calmodulin, CMD5 (CCGAGTACAAGGARGCCTTC) and CMD6 (CCGATRGAGGTCATRACGTGG) (Hong *et al*., 2005). PCR amplification of ITS, β-tubulin, and calmodulin was performed in a total volume of 25 µL using a PTC-100 Peltier Thermal Cycler (MJ Research Inc., Waltham, MA, USA). The PCR reaction mixture contained 5 µL 5× green buffer, 4 µL 25 mM $MgCl_2$, 0.5 µL dNTP mix (Promega, Madison, WI, USA), 0.15 µL containing 5 U *Taq* polymerase (Promega), 4 µL of each primer (at 5 mM), and 0.5 µL DNA template. PCR was performed using the following conditions: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 sec, annealing for ITS at 58 °C for 30 sec and for β-tubulin and calmodulin at 56 °C for 30 sec, and extension at 72 °C for 1 min; a final extension at 72 °C for 5 min. The PCR products were sent to a service provider for DNA sequencing.

The consensus sequences of ITS, β-tubulin, and calmodulin were compared with sequences in GenBank using Basic Local Alignment Search Tool (BLAST). The DNA sequences were then analyzed for phylogenetic relatedness using Molecular Evolution Genetic Analysis (MEGA5) software (Tamura *et al*., 2011). The individual and combined sequences of ITS, β-tubulin, and calmodulin were used to generate a phylogenetic tree using the maximum-likelihood (ML) method. Bootstrap analysis was performed with 1,000 replicates to determine the support for each clade. Two *Aspergillus* species, *A. sydowii* and *A. ustus*, served as the outgroup.

RESULTS AND DISCUSSION

After isolation, we identified 20 isolates of slow growing *Aspergillus* on CYA and MEA that were not commonly found during our routine survey of *Aspergillus* spp. on different substrates. The observed microscopic and macroscopic characteristics suggested that 10 isolates were similar to *A. chevalieri*, as described by Klich (2002). For the other 10 isolates, it was difficult to identify microscopic and macroscopic similarities based on the species descriptions of Klich (2002) or the description in the lab manual by Samson *et al.* (2010); thus, these isolates were referred to as *Aspergillus* sp. Table 1 shows the macroscopic and microscopic characteristics of morphologically identified *A. chevalieri* and *Aspergillus* sp. The colony appearance of *Aspergillus* sp. on MEA and CYA, as well as the shape of the vesicle, conidiophore, and conidia are shown in Figure 1 (A, B, C, D). *A. chevalieri* isolates were identified based on its slow growth on MEA and CYA, small vesicles, and rough walled conidia (Figure 1E, F, G, H).

The ITS region, β-tubulin and calmodulin genes were successfully amplified and a single band of approximately 600 bp was produced by the isolates. A BLAST search of ITS, β-tubulin, and calmodulin sequences of morphologically identified isolates of *Aspergillus* sp. indicated 98–100% similarity with *A. amstelodami*. Therefore, morphologically-identified isolates of *Aspergillus* sp. were molecularly identified as *A. amstelodami*.

A BLAST search of β-tubulin and calmodulin sequences from isolates morphologically identified as *A. chevalieri* revealed 98–100% similarity with *A. chevalieri*. However, the ITS sequences of morphologically-identified *A. chevalieri* isolates were 100% similar to those of *A. amstelodami*. The ITS, β-tubulin, and calmodulin sequences of *A. amstelodami* and *A. chevalieri* isolates were deposited in GenBank and the accession numbers

Table 1: Macroscopic and microscopic characteristics of *Aspergillus* sp. and *A. chevalieri* isolated from peanuts.

	Aspergillus sp.	*A. chevalieri*
MEA	Velutinous mycelia, yellow color at the center surrounded by green rings and white at the margin, pale green pigments	Floccose and brown color in the center with yellow at the margin and yellow pigments
CYA25	Velutinous mycelia, yellow color at the center surrounded by green rings and white at the margin, pale green to brown pigments	Floccose and brown color in the center with yellow at the margin and yellow pigments
CYA37	White colonies and cream pigments	White colonies and cream pigments
CREA	Grew poorly with limited acid production	Poor growth with limited acid production
Diameter	MEA: 13.0–23 mm CYA25: 13.0–20 mm CYA37: 2.0–4.0 mm	MEA: 14.0–24.0 mm CYA25: 15.0–23.0 mm CYA37: 2.0–4.0 mm
Conidiophore	Uniseriate, radiate and columnar conidial head, globose and subclavate vesicle Size of vesicle: 10.0–24.6 µm	Subglobose vesicle and radiate conidial head Size of vesicle: 8.4–24.3 µm
Conidia	Globose to subglobose and smooth walled 3.1–5.4 × 4.0–6.0 µm	Ellipsoidal to cylindrical 2.0–5.4 µm × 3.0–5.8 µm

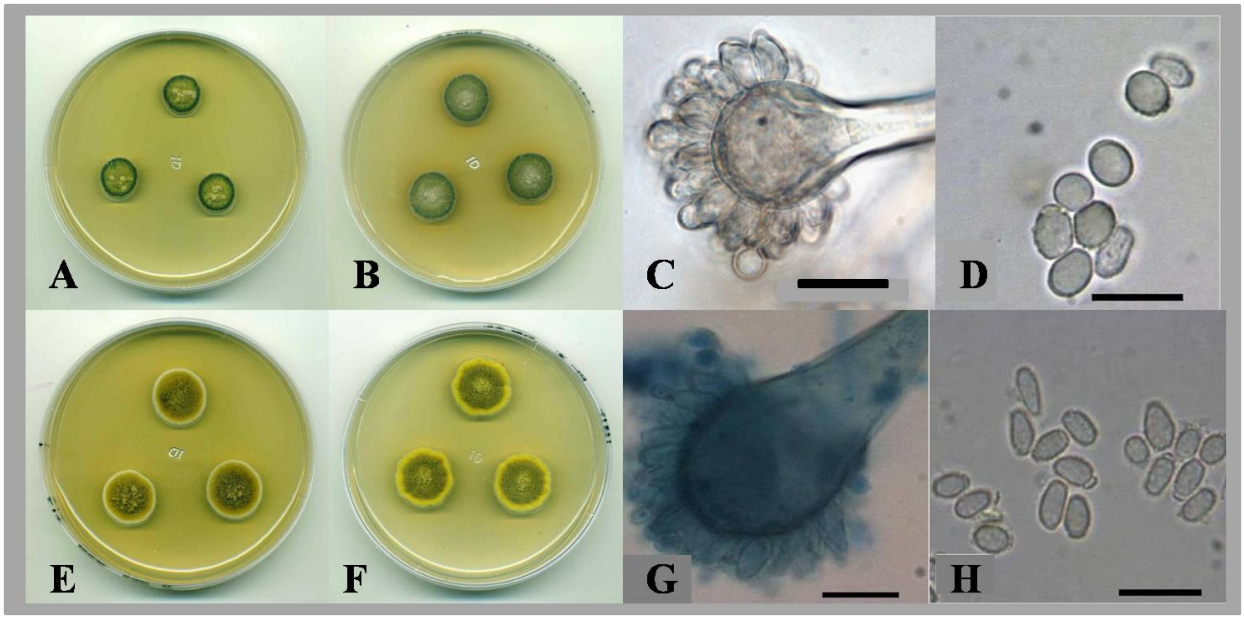

Figure 1: Macroscopic and microscopic characteristics of *Aspergillus* sp. (A–D) and *A. chevalieri* (E–H) isolated from peanuts. (A) Colonies of *Aspergillus* sp. on MEA, (B) CYA25, as well as appearance of (C) conidiophores and (D) conidia. (E) Colonies of *A. chevalieri* on MEA, and (F) CYA25, as well as appearance of (G) conidiophores, (H) conidia.

are shown in Tables 2 and 3.

A maximum likelihood tree based on combined ITS, β-tubulin, and calmodulin sequences was similar to the ML tree based on individual β-tubulin and calmodulin sequences (data not shown). In the combined tree, isolates of *A. amstelodami* from peanuts grouped together in the same clade as *A. amstelodami* type strains CBS 651.74 and NRRL 35697 and isolates of *A. chevalieri* also grouped with *A. chevalieri* type strains CBS 121704, KP330049, and NRRL 4755 (Figure 2).

In this study, two xerophilic *Aspergillus* species, *A. chevalieri* and *A. amstelodami,* were isolated from peanuts obtained from sundry shops and supermarkets in seven states of Malaysia. The species identification was based on morphological characteristics, molecular identification, and phylogenetic analysis using sequences of ITS, β-tubulin, and calmodulin.

Of the 20 isolates of slow growing *Aspergillus* isolated from the peanut samples*,* 10 fungal isolates were identified as *A. chevalieri,* a xerophilic species from the *Aspergillus* section. The description of *A. chevalieri* by Klich (2002) was based on the teleomorph species *Eurotium chevalieri*. In the lab manual by Samson *et al*. (2010), all species in the section *Aspergillus* were also described as *Eurotium*. With current nomenclature based on one fungus, one name, the name *Aspergillus* has taken over *Eurotium* (Hubka *et al*., 2013). Thus, teleomorph genera including *Eurotium* are synonymous with *Aspergillus* (Samson *et al*., 2014). According to Hubka *et al.* (2013), the concept of naming teleomorph genera *Aspergillus* has been applied to other *Aspergillus* sections including *Usti, Terrei, Aspergillus*, and *Fumigati.*

The 10 isolates, the species of which could not be identified morphologically, were molecularly identified as *A. amstelodami*. Notably, ITS sequences could not be used to distinguish *A. chevalieri* and *A. amstelodami* isolates. Phylogenetic analysis solely based on ITS also could not distinguish *A. chevalieri* and *A. amstelodami* isolates of which the isolates of both species were grouped together. According to Samson *et al.* (2014), ITS sequences lack variation in some *Aspergillus* clades or sections and therefore cannot be used to accurately identify certain species of this genus. Moreover, *A. chevalieri* and *A. amstelodami* are morphologically similar and is considered closely related species (Klich, 2002; Samson *et al*., 2010). Schoch *et al*. (2012) also stated that although ITS sequences represent the official DNA barcode for fungi, sometimes this region does not contain enough variation for species differentiation. Distinction of *A. chevalieri* and *A. amstelodami* isolates was achieved using β-tubulin and calmodulin sequences, which are recommended as secondary markers for identification of *Aspergillus* species (Samson *et al*., 2014). Phylogenetic analysis of combined sequences of ITS, β-tubulin and calmodulin strengthen the distinction of *A. chevalieri* and *A. amstelodami.* Several studies have also shown multiple loci are necessary for accurate species determination of species within section *Aspergillus* (Hubka *et al*., 2012; Jurjevic *et al*., 2012; Peterson, 2012).

Aspergillus chevalieri and *A. amstelodami* are xerophilic fungi and thus have the ability to grow on substrates with low water activity; these species are widely distributed in the environment (Hubka *et al*., 2013). Both *A. chevalieri* and *A. amstelodami* are physiologically similar and reside in similar ecological niches (Hocking, 2001; Pettersson and Leong, 2011). Commonly, *A. chevalieri* and *A. amstelodami* grow at a minimum of 0.71 a_w and at 33 °C with optimum growth at 30–35 °C and maximum growth at 40–43 °C (Hocking, 2001). These conditions, present in the Malaysian tropical climate, are suitable for growth of *A. chevalieri* and *A. amstelodami*.

Aspergillus chevalieri has been found in cereals and cereal products as well as in processed and dried peanuts, pecan nuts, hazelnuts, and walnuts in Southeast Asian countries (Pitt and Hocking, 2009). However, Pitt and Hocking (2009) reported that *A. amstelodami* was less common than *A. chevalieri*. Nevertheless, *A. amstelodami* has been isolated from maize, peanuts, soybeans, cashews, copra, paddy, mung beans, sorghum, and peppercorns (Pitt *et al*., 1993; Pitt *et al*., 1994; Pitt and Hocking, 2009). *Aspergillus chevalieri* and *A. amstelodami* have also been found in mixed feed (Accensi *et al*. 2004), indoor air (Slack *et al*., 2009) and hypersaline water (Butinar *et al*., 2011). However, these species had not yet been isolated from food and feed in Malaysia.

The occurrence of *A. chevalieri* and *A. amstelodami* on peanuts could provide favorable growth conditions for less xerophilic *Aspergillus* species, particularly *A. niger, A. flavus*, and *A. fumigatus*, which are well-known mycotoxin producers. Xing *et al*. (2016) reported that during the initial stages of peanut storage, *Eurotium* (teleomorph species of *Aspergillus* section *Aspergillus*) grows rapidly and can release water as a metabolic byproduct, which in turn increases the water activity, creating favorable conditions for the growth of various species of *Aspergillus* including *A. niger* and *A. flavus*. In their study, Xing *et al*. (2016) found that when peanuts are stored for 30 to 90 days, the relative abundance of other *Aspergillus* species increases, and that of xerophilic genera of *Rhizopus*, *Eurotium*, and *Wallemia* decreases. This finding corroborated with Hocking (2008) who showed that *Eurotium* species are usually the first colonizers of stored commodities that were improperly dried; in addition, growth of these strains was found to raise the water activity of the substrate. This allowed other potentially toxigenic *Aspergillus* and *Penicillium* species to grow on the substrate. *A. chevalieri* and *A. amstelodami* possibly facilitate the growth of mycotoxin producers in a similar manner in peanuts.

Bukelskienė *et al*. (2006) reported that *A. amstelodami* produces patulin, ochratoxin A, and sterigmatocystin. *Aspergillus chevalieri* has been reported to produce echinulin and neoechinulin, resulting in feed refusal in swine (Hocking, 2008, Pettersson and Leong, 2011). Other toxic compounds produced by *A. chevalieri* include epiheveadride and flavoglaucin, which are also produced

Table 2: *Aspegillus amstelodami* isolates from peanuts with GenBank accession number and percentage of similarity.

Isolates	GenBank accession number			Percentage of similarity (%)		
	ITS regions	β-tubulin	Calmodulin	ITS regions	β-tubulin	Calmodulin
KL1	KU569230	KU569223	KU872148	*A. amstelodami* (100%)	*A. amstelodami* (99%)	*A. amstelodami* (99%)
KL5a	KU569231	KU569224	KU872149	*A. amstelodami* (100%)	*A. amstelodami* (99%)	*A. amstelodami* (98%)
KL8	KU569232	KU569225	KU872150	*A. amstelodami* (100%)	*A. amstelodami* (99%)	*A. amstelodami* (99%)
KL10	KU872153	KU872154	KU886694	*A. amstelodami* (100%)	*A. amstelodami* (99%)	*A. amstelodami* (99%)
KL12a	KU872158	KU872155	KU886695	*A. amstelodami* (100%)	*A. amstelodami* (99%)	*A. amstelodami* (99%)
KL15	KU872159	KU872156	KU886696	*A. amstelodami* (100%)	*A. amstelodami* (99%)	*A. amstelodami* (99%)
KL21	KU872160	KU872157	KU886697	*A. amstelodami* (100%)	*A. amstelodami* (99%)	*A. amstelodami* (99%)
SRW6a	KU569233	KU569226	KU872151	*A. amstelodami* (100%)	*A. amstelodami* (99%)	*A. amstelodami* (99%)
SRW8b	KU569234	KU569227	KU872152	*A. amstelodami* (100%)	*A. amstelodami* (99%)	*A. amstelodami* (99%)
KDH15	KU569228	KU569229	KU872147	*A. amstelodami* (100%)	*A. amstelodami* (99%)	*A. amstelodami* (99%)

Table 3: *Aspergillus chevalieri* isolates from peanuts with GenBank accession number and percentage of similarity.

Isolates	GenBank accession number			Percentage of similarity (%)		
	ITS regions	β-tubulin	Calmodulin	ITS regions	β-tubulin	Calmodulin
KDH11	KU872161	KU872170	KU872162	*A. amstelodami* (99%)	*A. chevalieri* (100%)	*A. chevalieri* (99%)
KL6	KU872179	KU872171	KU872163	*A. amstelodami* (100%)	*A. chevalieri* (99%)	*A. chevalieri* (99%)
KL7	KU872180	KU872172	KU872164	*A. amstelodami* (100%)	*A. chevalieri* (100%)	*A. chevalieri* (100%)
PRK6a	KU872181	KU872173	KU872165	*A. amstelodami* (100%)	*A. chevalieri* (100%)	*A. chevalieri* (98%)
PRK9a	KU872183	KU872182	KU872184	*A. amstelodami* (100%)	*A. chevalieri* (100%)	*A. chevalieri* (99%)
SRW3b	KU872186	KU872174	KU872166	*A. amstelodami* (100%)	*A. chevalieri* (100%)	*A. chevalieri* (99%)
SRW8a	KU872187	KU872175	KU872167	*A. amstelodami* (99%)	*A. chevalieri* (100%)	*A. chevalieri* (99%)
SRW9	KU872188	KU872176	KU872168	*A. amstelodami* (100%)	*A. chevalieri* (99%)	*A. chevalieri* (100%)
TE6	KU872189	KU872177	KU872169	*A. amstelodami* (100%)	*A. chevalieri* (100%)	*A. chevalieri* (100%)
TGN9	KU872190	KU872178	KU872185	*A. amstelodami* (100%)	*A. chevalieri* (100%)	*A. chevalieri* (99%)

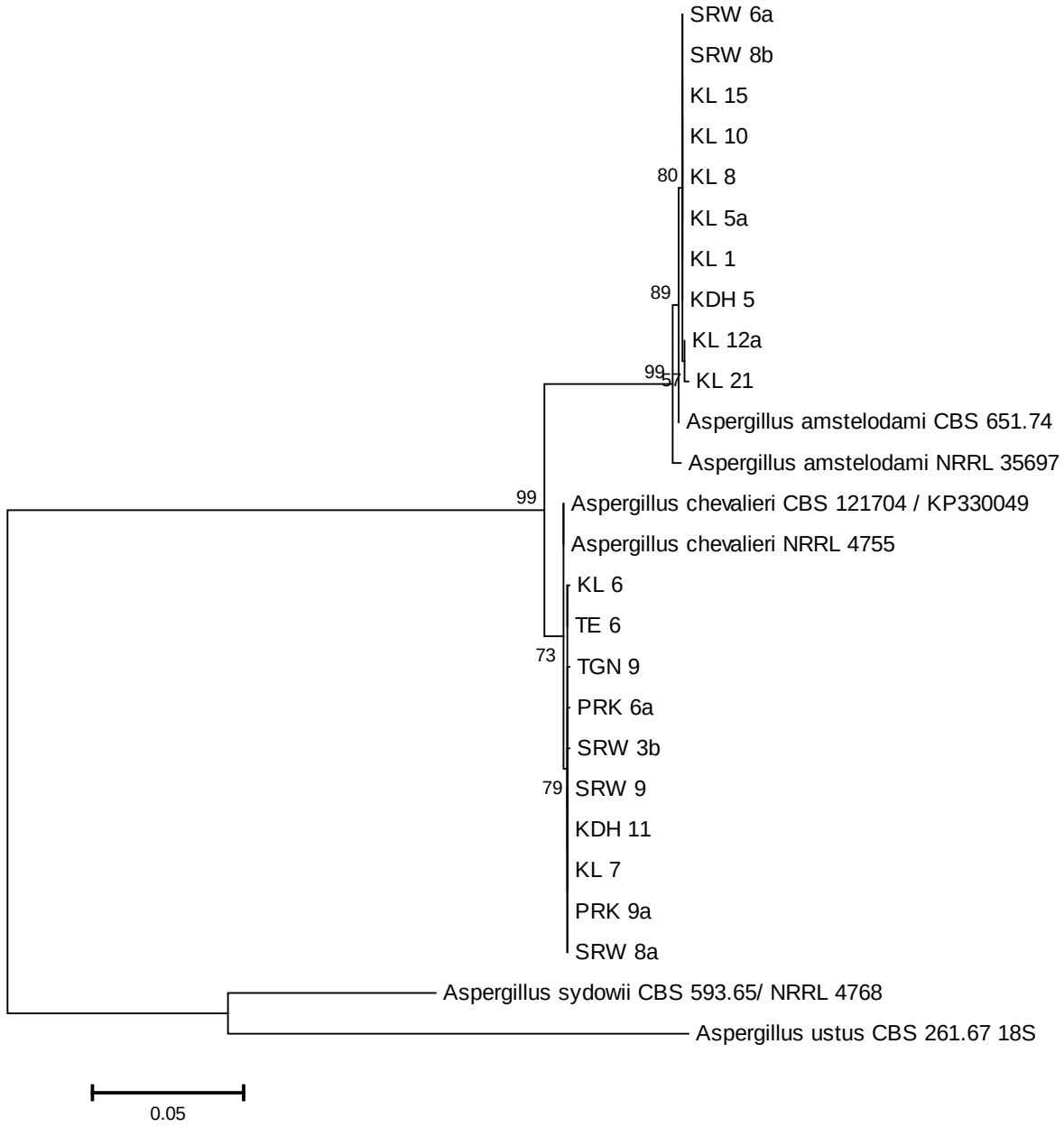

Figure 2: Maximum likelihood tree based on combined sequences (ITS, β-tubulin and calmodulin) of *A. amstelodami* and *A. chevalieri* with 100 bootstrap replicates. *Aspergillus sydowii* and *A. ustus* are the outgroup.

by *A. amstelodami* (Klich, 2002). According to Frisvad *et al.* (2002; 2007) reports of mycotoxin production in species of the *Aspergillus* section should be interpreted with caution as such isolates have not been verified using taxonomically well-characterized strains. This is an agreement with a study by Hocking (2008) showing that *Eurotium* species (species in section *Aspergillus*) do not produce important mycotoxins. However, these strains can produce secondary metabolites and cause oxidative rancidity in grains and nuts. Unlike other toxigenic *Aspergillus* spp. such as *A. flavus* and *A. fumigatus*, which can also cause diseases in humans and animals, *A. chevalieri* and *A. amstelodami* are not considered important human pathogens (Samson *et al*., 2014) however, occasional cases of infection have been reported (de Hoog, 2009; Naidu and Singh, 1994).

The occurrence of *A. chevalieri* and *A. amstelodami* on peanuts can possibly reduce their shelf life and the quality of the kernels. There is also the potential of mycotoxin contamination from the growth of mycotoxin-producing

Aspergillus. In addition, identifying these species on peanuts contributes to the understanding of *Aspergillus* species diversity in food products in Malaysia. To our knowledge, this is the first report of *A. chevalieri* and *A. amstelodam* on peanuts occurrence in Malaysia.

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