Microscopic characteristics as preliminary identification of *Aspergillus* spp. from beach sand

Teh, L.Y.[1] and Latiffah, Z.[1]*

[1]School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia.

Abstract

Aspergillus spp. are prevalent in the environment and commonly isolated from soil. Microscopic characteristics of the genus are mainly defined by the aspergillum-like conidia bearing structure or conidial head. In most cases this structure is essential for rapid and quick identification in the laboratory especially for detection of *Aspergillus* spp. that are known to be clinically important. In the present study, several *Aspergillus* spp. isolated from beach sand were microscopically identified based on the characteristics of the conidial head and conidia of which seven species were identified. Microscopic observation was done using a light microscope and scanning electron microscope. The species were identified as *A. aculeatus*, *A. carbonarius, A. niger, A. flavus, A. tamarii, A. terreus* and *A. sydowii*. The results showed the characteristics of conidial head and the shape of conidia can be used as preliminary identification of *Aspergillus* spp. to sort the species into sections as well as to species levels for certain species.

Keywords*:* *Aspergillus*, scanning electron microscopy, light microscope, beach sand.

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**Corresponding author: Prof. Dr. Latiffah Zakaria*
Tel: +604 599 5262 e-mail: ifah@usm.my
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Introduction

The genus *Aspergillus* is a Hyphomycetes and is characterised by the formation of conidial head or conidiophores with large stipes and swollen apices, known as vesicles [1]. Vesicles are usually roughly sphericalbut are elongated or less conspicuously swollen in a few species [1]. Vesicles bear crowded phialides, or metulae and phialides, which are characteristically all borne simultaneously [1]. These structures are the most important microscopic characters to identify species of *Aspergillus*. Under the microscope, the conidial head has the appearance of aspergilum which is an instrument for sprinkling holy water.

Aspergillus spp. are prevalence in sand beach due to its ability to grow at high temperatures and low water activities [2]. Valero et al. [3] reported that fungal propagules such as conidia and sclerotia of *Aspergillus* were resistant to ultraviolet radiation or sunlight. Horn [4] suggested that sclerotia are the most important survival structure which enable *Aspergillus* species to survive in harsh conditions.

Aspergillus spp. are known to be opportunistic pathogens which cause infections on immunodepressed individuals [5]. Thus, there is a possibility that the occurrence of *Aspergillus* spp. in beach sand might pose health risks to beach visitors. *Aspergillus* species produce large numbers of small, air-borne conidia that could cause allergic reactions to some individuals. Moreover, long-term exposure to high concentration of conidia may cause aspergilloses in humans via inhalation [6]. It was reported that human contact with beach sand through activities like digging in the sand or having one's body buried in the sand was

associated with an elevated health risk [7] as infection can occurred through conidia of which the conidia can enter the host through inhalation, via wound as well as ingestion [8].

Several *Aspergillus* spp. such as *A. fumigatus*, *A. niger* and *A. niger* are regarded as one of the most effective opportunistic fungal pathogens of which the species has the potential to cause medical problems [8], therefore it is vital to identify the species of *Aspergillus* using microscopic characteristics as preliminary identification as this method is relatively quick and easy Thus, the objective of the present study was to identify several species of *Aspergillus* isolated from beach sand using microscopic characteristics.

Materials and methods

Beach sands were collected from beach areas in Batu Ferringhi and Teluk Bahang, Penang. The sands were collected by scraping off the surface to a depth of 10 cm. Approximately 1.5 kg of beach sands were collected and put in plastic bags and labelled. Isolation methods were based on the methods used by Teh & Latiffah [9] of which soil dilution plate, direct plating and debris isolation methods were applied. Malt Extract Agar (MEA) amended with streptomycin and neomycin was used for isolation purposes.

For identification, species descriptions mainly by Klich [10], Pitt & Hocking [1] and Samson et al. [11] were used. Three features commonly used in the classification of *Aspergillus* into species level are the morphological characteristics of the conidial head or conidiophore, the arrangement of metulae or phialides on the vesicle and colony colours [1]. The characteristics of the conidiophore (erect or sub-erect), the arrangement of phialides on the vesicle (uniseriate or biseriate), the shape of the vesicle (globose or clavate) and the shape and texture of conidia produced (smooth or roughed, hyaline to darkly pigmented, globose to ovoid) were observed. The length and width of conidia and stipe, length of phialides and width of vesicle were measured.

The microscopic characteristics were examined under a light microscope (Olympus BX 41) using 400X and 1000X magnifications as well as Scanning Electron Microscope (LEO SUPRA 50VP, (Carl Zeiss, Germany). Microscopic slides were prepared from cultures in MEA using both wet mount and cellophane tape techniques. Lactic acid (60%) without colour dye and lactophenol blue were used as a mounting medium.

Results and discussion

Members of the genus *Aspergillus* were characterized by the conidial head or conidiophore consisting of stipe, vesicle, phialides for uniseriate species or both phialides and metulae for biseriate species bearing dry chains of conidia [10,11]. In this study, *Aspergillus* isolates recovered from beach soil samples were categorized into five sections according to the classification scheme of Gams et al. [12], namely sections Nigri, Flavi, Terrei, Fumigati and Versicolores.

Aspergillus section Nigri

The distinctive characteristics of the isolates that belonged to *Aspergillus* section Nigri were the production of dark-brown to black conidia, with uniseriate or biseriate conidiophores, spherical vesicles and hyaline to sub-hyaline hyphae [1, 13]. Based on these characteristics, three members of *Aspergillus* section Nigri, *A. aculeatus*, *A. carbonarius* and *A. niger* were identified.

Aspergillus niger conidial heads were biseriate and radiated, stipes at length of 450–900 µm, walls were thick, smooth and hyaline, vesicles were globose at 35-58 µm wide (Figure 1A). The conidia were brown, globose to subglobose, 3.2-3.7 µm, finely roughened to rough-walled (Figure 1B). The shapes and sizes of the conidiophores and conidia of *A.*

niger isolates in the present study fit the descriptions of *A. niger* isolates reported by Silva et al. [13] of which the isolates were from different food products and environmental samples.

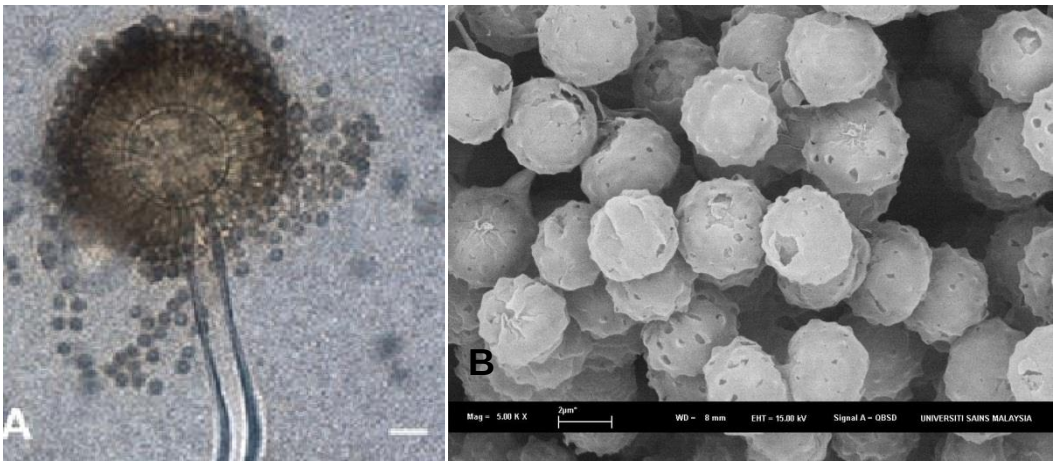

Figure 1: Microscopic characteristics of conidial head and conidia of *A. niger*. (A) Globose vesicle. Scale bar = 10 µm. (B): Rough-walled, globose conidia using SEM.

Aspergillus carbonarius colonies produced biseriate conidiophores with radiated conidial heads, long stipes (640–1350 µm). The vesicles were thick walls, smooth, hyaline and globose measuring about 23-44 µm wide (Figure 2A). The conidia produced were globose, 4.3-7.8 µm, rough-walled to echinulated (Figure 2B). The conidia diameter reported in this study was smaller than the diameter (7-9 µm) reported by Samson et al. [14] which might be due to different substrates and locations the isolates originated.

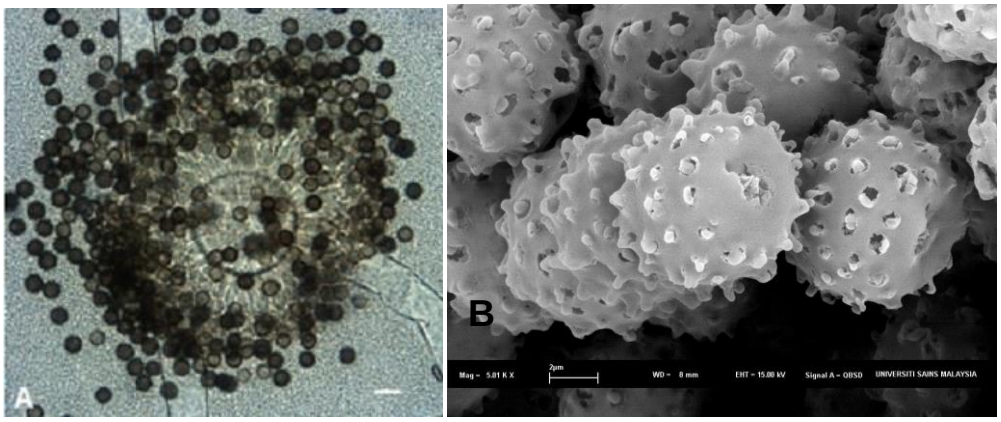

Figure 2: Microscopic characteristics of conidial head and conidia of *A. carbonarius*. (A) Globose vesicle. Scale bar = 10 µm. (B) Echinulated, globose conidia observed using SEM.

Aspergillus aculeatus colonies produced conidiophores with radiated, uniseriate conidial heads and long stipes (600–1600 µm). The vesicles were thick-walled, smooth, hyaline, globose with diameter of 58–68 µm (Figure 3A). The conidia produced were subglobose to ellipsoidal, 3.4-3.8 µm and rough-walled to echinulated (Figure 3B). The diameter of vesicles obtained in this study fits the descriptions of Samson et al. [13] which was in the range of 60 to 80 µm.

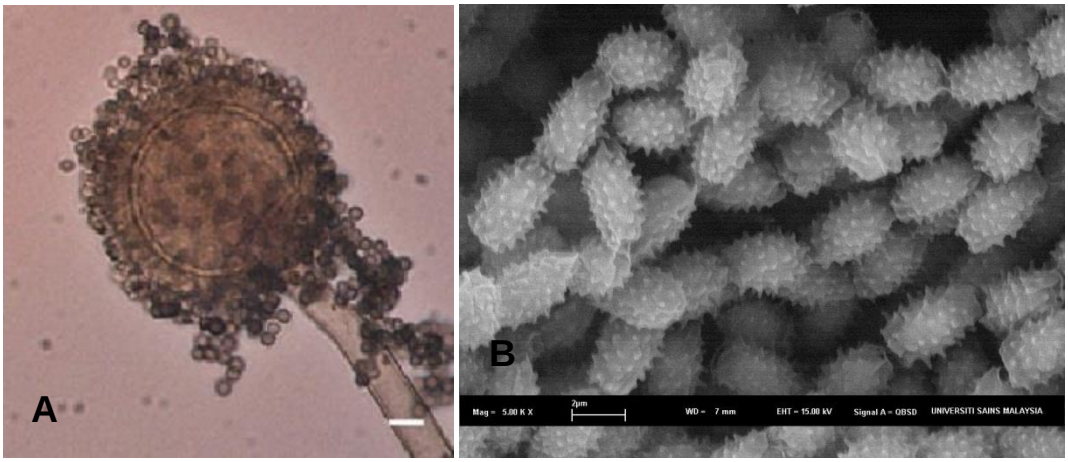

Figure 3: Microscopic characteristics of conidial head and conidia of *A. aculeatus*. (A) Globose vesicle. Scale bar = 10 µm. (B) Echinulated, ellipsoidal conidia observed using SEM.

Aspergillus carbonarius and *A. niger* can be distinguished based on their microscopic characteristics in which *A. carbonarius* produced large, rough-walled conidia with 4.3-7.8 µm in diameter while *A. niger* produced finely roughened to rough-walled conidia with 3.2-3.7 µm in diameter. Similar to this study, Oliveri et al. [14] distinguished *A. niger* and *A. carbonarius* obtained from grapes and environmental samples based on vesicle and conidial size and ornamentation. Silva et al. [13] also distinguished *A. niger* and *A. carbonarius* based on conidial size and ornamentation.

The uniseriate species, A. *aculeatus* can be easily distinguished based on the the size of vesicle and the shape and ornamentation of the conidia. *Aspergillus aculeatus* produced ellipsoidal, echinulated conidia and larger vesicle with the diameter of about 25-60 µm which fits the descriptions by Samson et al. [14]. Perrone et al. [16] also reported that *A. aculeatus* had large conidial heads with vesicles ranging from 22-55 µm in diameter and ellipsoidal conidia.

Aspergillus section Flavi

Two species from section Flavi, namely *A. flavus* and *A. tamarii* were identified. *Aspergillus flavus* produced uniseriate phialides and the conidial head was hyaline, coarsely roughened, 189–892 µm in length. Vesicles were elongated when young, later becoming subglobose or globose, varying from 14 to 50 µm diameter (Figure 4A). Conidia were typically globose to subglobose, varying from 2.8 to 5.1 µm in diameter. The microscopic characteristics of *A. flavus* observed in this study fit the descriptions by Rodrigues et al. [17]. The ornamentation of conidia was smooth-walled when observed under a light microscope but the observations under SEM showed a finely-roughened texture (Figure 4B).

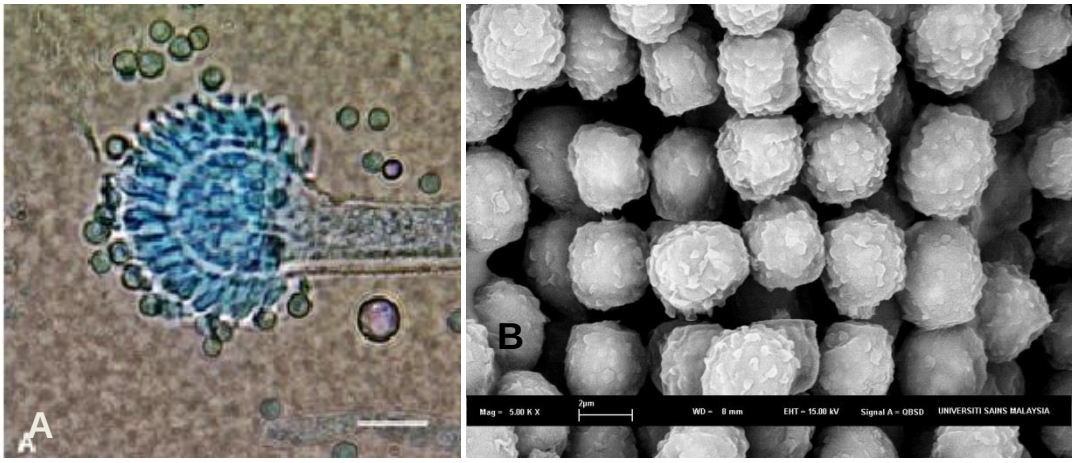

Figure 4: Microscopic characteristics of conidial head conidia of *A. flavus*. (A) Globose vesicle. Scale bar = 10 µm. (B) Rough-walled, sub-globose conidia observed using SEM.

Aspergillus tamarii produced uniseriate phialides and radiated, covering the entire surface of the vesicle (Figure 5A). Vesicles were globose to sub-globose measuring 10–22 µm. The stipes were relatively shorter than *A. flavus* stipes which were 213-500 µm in length. The conidia were rough-walled, globose to sub-globose with 3.2-6.6 µm (Figure 5B). The sizes of the vesicles and conidia of morphologically identified *A. tamarii* isolates observed in this study were smaller than the descriptions in Samson et al. [11] which might be due to different substrates as the isolates described by Samson et al. [11] were from food products.

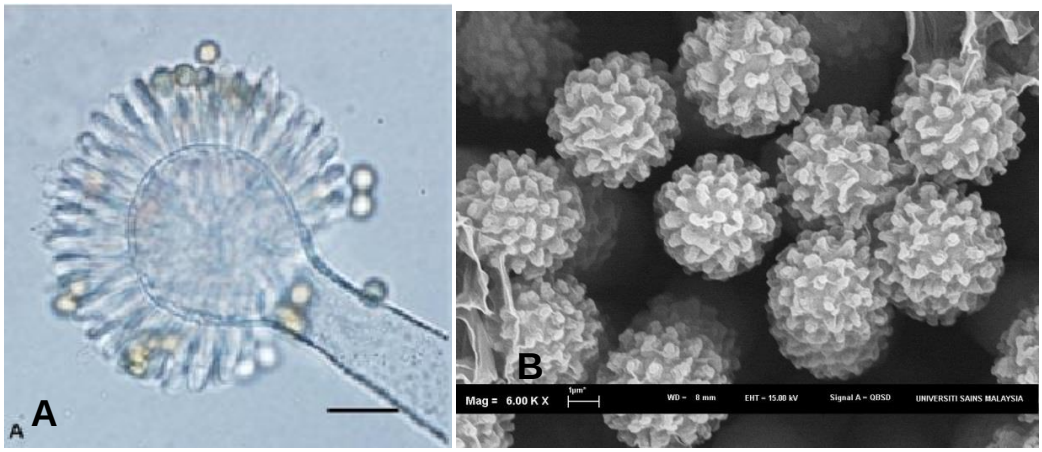

Figure 5: Microscopic characteristics of conidial head and conidia *A. tamarii*. (A) Sub-globose vesicle. Scale bar = 10 µm. (B) Rough-walled, globose conidia observed using SEM.

Other features that can be used to distinguish between *A. flavus* and *A. tamarii* is production of sclerotia. Sclerotia were abundant in *A. flavus* colony grown on Czapek Yeast Autolysate agar (CYA) while *A. tamarii* colony did not produce any sclerotium. Samson et al. [11]. reported that sclerotia were only produced by some strains of *A. tamarii*.

Aspergillus terreus

Aspergillus terreus produced biseriate phialides that were hyaline and smooth with 95–170 µm in length. Vesicles were subglobose or globose, varying from 12 to 16 µm in diameter (Figure 6A). Conidia were typically globose to subglobose, varying from 1.9 to 2.3 µm in diameter (Figure 6B). The size of the conidia was relatively small compared to other species of *Aspergillus* which were usually larger than 3µm. The ornamentation of conidia was smooth-walled when observed under the light microscope but the observations under SEM

showed wrinkled texture (Figure 6B). The range of diameters of conidial heads and conidia recorded in this study were within the size range of *A. terreus* reported by Samson et al. [11].

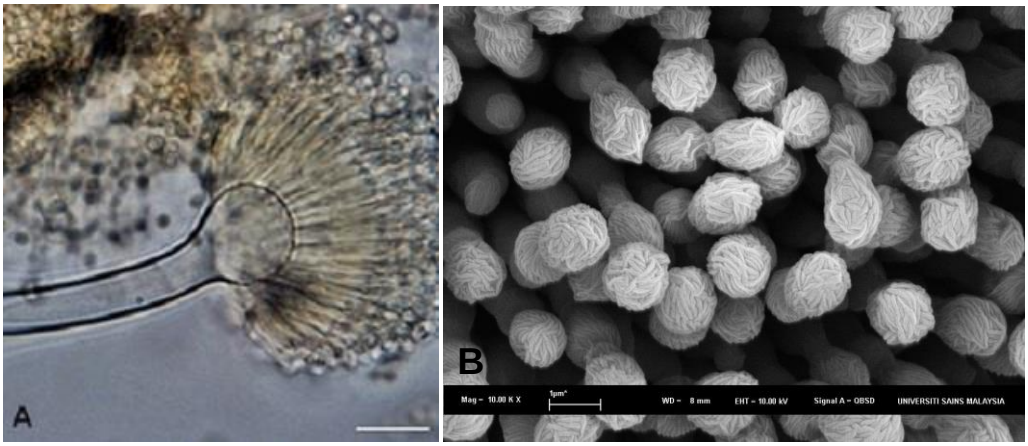

Figure 6: Microscopic characteristics of conidial head and conidia of *A. terreus*. (A) Globose vesicle. Scale bar = 10 µm. (B) Wrinkled, sub-globose conidia observed using SEM.

Aspergillus terreus is members of *Aspergillus* section Terrei, which consisted of species with columnar conidial heads in shades of buff to brown [18]. *Aspergillus terreus* was identified based on the morphological features such as golden-brown colony on the culture media and small conidial size. The colony morphology and microscopic characteristics were commonly used to identify *A. terreus* species [19]. However, the existence of cryptic species made it difficult to distinguish between species using morphological characteristics. Cryptic species of *A. terreus* are morphologically similar but genetically distinct [20].

Aspergillus fumigatus

Microscopic observations of conidial head and conidia of *A. fumigatus* isolates are shown in Figure 7. Uniseriate phialides were hyaline, smooth with 208-250 µm in length. Vesicles were pyriform to subclavate and sometimes subglobose, varying from 18 to 20 in diameter (Figure 7A). Conidia were globose to subglobose, varying from 1.9 to 3.2 mm in diameter in which the size of the conidia was relatively small compared to other species of *Aspergillus*. The ornamentation of conidia was smooth-walled when observed under a light microscope but the observations under SEM showed a finely-roughened texture (Figure 7B). The shape and sizes of conidiophores and conidia recorded in this study fit the descriptions of *A. fumigatus* by Samson et al. [21].

Aspergillus fumigatus is a member of *Aspergillus* section Fumigati which was commonly known as the main causative agent of aspergillosis. Identification of *A. fumigatus* was based on heavily sporulating, dark green colony which showed rapid growth at 37ºC and uniseriate conidiophores with subclavate vesicles. Rinyu et al. [22] studied the variations of morphological features of *A. fumigatus* from different origins such as human sputum, food and forest trees, and concluded that all strains have uniform conidial diameters of 2.5 to 3.5 µm and produced the distinctive subclavate vesicles. These observations were in agreement with observations in the present study. Similar features were observed by Hong et al. [23] and Samson et al. [21] to distinguish *A. fumigatus* from its closely related species within section Fumigati.

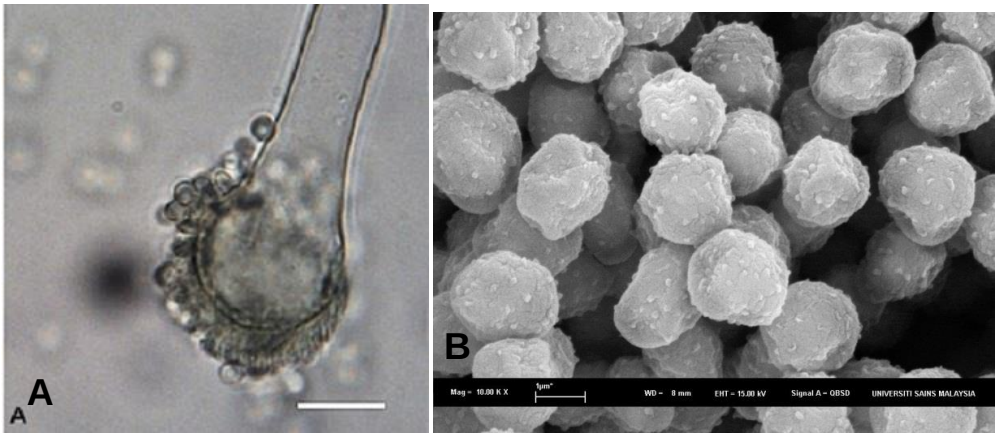

Figure 7. *Aspergillus fumigatus*: Microscopic observations of conidiophore and conidia of *A. fumigatus*. (A) Sub-globose vesicle. Scale bar = 10 µm. (B) Fine-roughened, sub-globose conidia observed using SEM.

Aspergillus sydowii

Aspergillus sydowii isolates were identified based on the shape of the vesicles which were relatively small compared to other *Aspergillus* species. *Aspergillus sydowii* produced biseriate phialides that were hyaline, smooth, 115-192 µm in length (Figure 8A). Vesicles were sub-globose, varying from 8.4 to 10.2 µm in diameter. Conidia were globose and echinulated (Figure 8B) under SEM observations, varying from 2.6 to 3.9 µm in diameter. The shapes and sizes of conidiophores and conidia recorded in this study matched the descriptions given by Jurjevic et al. [24].

Aspergillus sydowii is an important member of *Aspergillus* section Versicolores and this species is the common pathogen of sea fans corals (*Gorgonia* species) [25]. The isolates of *A. sydowii* can be easily mistaken as *Penicillium* species because of the slow growth rate as well as the blue-green colour colonies on MEA. In the present study, two *A. sydowii* isolates were identified based on the small vesicles size (8.4-10.2 µm) and small-sized, echinulated conidia (2.6-3.9 µm). Similar observations were described by Klich [26] and Jurjevic et al. [24] for the morphological identifications of *A. sydowii* isolates.

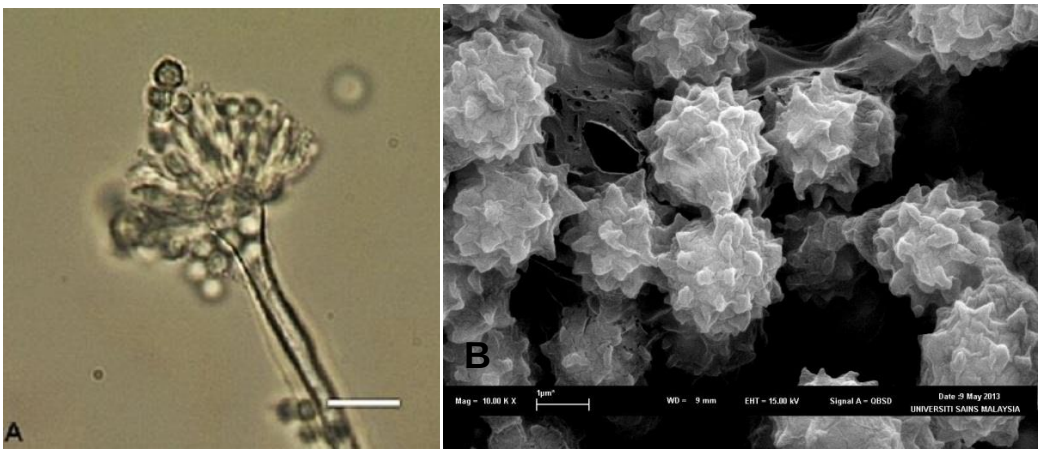

Figure 8: Microscopic characteristics of conidial head and conidia *A. sydowii*.
(A) Sub-globose vesicle. Scale bar = 10 µm. (B) Echinulated, globose conidia observed using SEM.

Conclusion

Preliminary identification of *Aspergillus* spp. is important in clinical settings especially for treatment as differences on antifungal susceptibility were observed between clinical and environmental isolates [26]. Thus, this study provides preliminary identification on the occurrence of *Aspergillus* spp. in beach sand as well as other substrates. Characteristics of conidial heads of different *Aspergillus* spp. can typically be distinguish based on reliable manual and references. Moreover, this method is relatively quick and easy for preliminary identification.

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Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of conflict of interest

The authors have no disclosures to declare.

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