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Original Article

Morphological characteristics of black aspergilli isolated from clinical wastes

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Abstract

The present study aimed to recognize the microscopic characteristics of black aspergilli species which exhibit similar characteristics on culture media. Forty eight black aspergilli isolates were obtained from clinical wastes and purified using single spore technique on six different culture media. The ultrastructure of fungal conidiophore and spores was detected by using Scanning Electron Microscope (SEM). The fungal isolates were identified within five species included *A. niger*, *A. tubingensis*, *Aspergillus* section Nigri, *A. violaceofuscus*, *A. neoniger*. Besides, two isolates identified as *Aspergillus* sp. strain no. 39, *Aspergillus* sp. strain no. 53 appear as new strains based on the structure of conidiophore and spores. The fungi species have similar culture characteristics. However, SEM observation demonstrated that they have quite different conidiophore and spores morphology. The study revealed that the microstructure of the fungal spores and conidiophores plays an important role in the identification of fungi species based on the phenotypic method.

Keywords: black aspergilli, SEM, ultrastructure, single spore technique

1. Introduction

Many fungi can be found in hospitals and other clinical facilities or laboratories, and form a composite in the wastes, generated from these premises (Efaq *et al*., 2015a). The clinical wastes present an appropriate and fertile environment for the reproduction of fungi due to the right temperature and proper nutrients as well as the adequate moisture and pH of the clinical wastes are favourable for their growth (Efaq *et al*., 2017; United States Environmental Protection Agency [U.S. EPA], 1990). On the other hand, the identification using phenotypic techniques depends on the culture and microscopic characteristics. Fungi have high diversity in their conidiophore and spores, which are useful to identify of fungal to species level (Efaq *et al*., 2016). *Aspergillus* section Negri complex have many common species which are known as the black aspergilli (Balajee *et al*., 2009; Klich, 2009; Silva *et al*., 2011). These species are often misidentified as *A. niger* owing to the culture characteristics such as colony surface and reverse colour are similar (Samson *et al*., 2007). The develop-

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ments for Scanning Electronic Microscopy (SEM), *Fluorescent Microscopy* and *Flow Cytometry* have enhanced the recognition of several details of phenotypic taxonomic significance (Cole & Samson, 1979; Guarro *et al*., 1999). The SEM has the ability to show the slight differences in the spore structure to classify of fungi to the varieties level.

The present work aimed to describe the morphological and culture characteristics of black aspergillli on different culture media included V8A, Potato Dextrose Agar, *Sabouraud dextrose* agar, Czapek-Dox *Agar*, Czapek Yeast Extract A*gar* and Malt Extract Agar. The study focused on the role SEM in the identification of these species. The ultrastructure of conidiophores and spores included shape, texture, spore surface ornamentation which cannot be observed using light microscope were recognized by SEM.

2. Materials and Methods

2.1 Isolation and purification of fungi from clinical waste samples

Clinical waste samples were collected from five divisions of Wellness Centre located within University Science Malaysia (USM). The samples were collected in a biohazards clinical waste bags and then transported to the laboratory inside the polystyrene box containing ice. The fungi isolates were recovered from the clinical waste samples using direct plate method on V8 juice agar medium (V8A) (Yu, 2010) and then purified using single spore technique on PDA medium according to Dr. Nagao technique (Efaq *et al*., 2015b). In brief; the fungal colony grown on V8A was placed under stereo light microscope (4X, Olympus SZ51, Japan). A spot inoculation of the fungal spores (100-500 spores) was transferred onto a new PDA medium by using sterilized scalpel. The inoculum was spread on the surface of the culture medium by using stainless steel spreaders and with 0.1 mL sterilized distilled water (SDW). The plates were placed inside the laminar flow 10 min to dry and then incubated at 28˚C for 16-18 hours. Thereafter, a small piece (0.2×0.2 cm) of agar medium containing a germinated spore was cut out aseptically by using scalpel and under the light microscope to insure the observation of the spore germinated tube and transferred onto PDA medium. The plates were incubated at 28˚C for 7 days and the fungal growth was noted daily.

2.2 Identification of fungal isolates

The identification of fungal isolates was performed according to the culture and microscopic characterization. The following references were used in the identification process; Ellis and Martin (1985); Barnett and Hunter (1998); Watanabe (2002); Samson *et al*. (2007); Robert *et al*. (2011); Silva *et al*. (2011); Campbell *et al*. (2013) and National Mycology Reference Centre (NMRC, 2015).

2.2.1 Cultural characteristics

The culture characteristics for each fungal isolate was investigated on V8A, Potato Dextrose Agar (PDA, Oxoid, UK), Czapek-Dox *Agar* (CZ, R&M Marketing, UK), *Sabouraud dextrose* agar (SDA, Hi Media, India), Malt Extract Agar (MEA, Merck, Germany) and Czapek Yeast Extract A*gar* (CYA, Oxoid, UK). The fungal cultures were separated into groups based on their morphological characteristics including growth diameter of the colonies (mm), surface and reverse colour, texture, zonation on PDA and sporulation as described by Promputtha *et al*. (2005). These characteristics were examined after 7 days of the incubation period at 28˚C.

2.2.2 Microscopic characteristics

The morphological characteristics for each fungal isolate were observed under light microscope (100X, Olympus, BX53F-CCD, Serial No. 1A589796, JAPAN). A small mycelia part from the centre and edge of each colony was placed onto glass slide contained one drop of distilled water and covered by a cover slip. The characteristics of conidiophores and spores shapes and size were described for each fungal isolates. The mean for 25 spores size determination by using Cell Sens Standard (Version 1.4.1) programme was used to give the average of the spore size for each fungus.

2.3 Scanning electron microscope (SEM) analysis

The microstructure of the conidiophore and spore shapes was observed using SEM. Pure culture of each fungal isolate sub-cultured on new PDA medium was sealed with parafilm and incubated at 28˚C for 2 days. Thereafter, the pure culture was sent under aseptic conditions to SEM laboratory at Biological School, USM. A small piece (0.2×0.2 cm) from the edge of grown colony was taken into aluminium petri dish. The sample was dried using liquid nitrogen. The sample was then coated with gold metal and observed using SEM (Zeiss Supra 50 VP, Germany).

3. Results and Discussion

3.1 Recovery of fungal isolates from the clinical waste samples

The first isolation of fungi from clinical waste samples was performed on V8A medium, which was chosen due to the high fungal growth observed on this medium during the preliminary study in comparison to CZ, CYA, PDA, SDA and MEA media. PDA is the most common isolation medium for the fungi from the samples. However, V8A exhibited here more efficiency to improve the *Aspergillus* spp. growth. It has been mentioned that the V8A is superior for the fungi which need a complex medium for their growth and sporulation (Choi *et al*. 1999). This might be due to the high contents of the natural nutrients which enrich the fungal growth. Nonetheless, the reverse colour for the fungal growth on V8A was unclear. Therefore, the morphology of fungal colonies was described on PDA, CZ, CYA, MEA and SDA as presented in Section 3.2.

3.2 Description of culture and morphological characteristics of black aspergilli species

The identification of black aspergilli species in the present study was conducted using phenotypic methods. This technique represent a good method to best understand the similarity and differences between the morphological and culture among the aspergilli species. Moreover, the micro-

structure of the fungal conidiophores and spores were determined using SEM. The culture characteristics and sporulation of fungal species obtained from clinical waste samples were described on V8A, PDA, CZ, CYA, MEA and SDA. It can be observed that the fungal growth and culture characteristics of each fungal isolate rely on the culture medium. Among different culture media, *Aspergillus* spp. produced abundant spores on V8A and MEA than on CZ and PDA media (Figure 1).

Among 48 *Aspergillus* isolates, 73% exhibited high growth on V8A, while 8.1% of fungal species did not differ in thier growth on V8A compared to MEA, CYA and PDA media. However, the reverse colours of fungal colonies were difficult to distinguish on V8A. Fungal species exhibited clear reverse colour on PDA medium. Several researchers reported that PDA medium supported the fungal growth of a wide range of fungi (Maheshwari *et al*., 1999; Saha *et al*., 2008; Xu *et al*., 1984). However, in the present study V8A was the best medium for fungal growth, these finding are consistent with reports for many authors. Lee *et al*. (2007) stated that V8 medium proved optimal growth and biomass yield of *Cladosporium phlei compared to PDA, YEA and CZ media*. Kent *et al*. (2008) revealed that V8A medium is an efficient inducer for sexual development of *C. neoformans.* Moreover, in some fungi such as *M. oryzae, the* sporulation taken a long time (10 days) even in V8A, but this medium still induces sporulation better than PDA medium (Chandrakanth *et al.,* 2014; Nunes *et al.,* 2011).

The differences between culture characteristics on the different culture media were more useful to facility the identification of fungi in this study. Black aspergilli have similar characteristics on one medium but they exhibited different characteristics on the others. Besides that, some of the references used for identification of fungi here described the fungal culture on one medium while others described it on the different media. Therefore, the combination and comparison between these references and the characteristics on different culture media helped to get the accurate name for the fungus isolate.

In the phenotypic methods the mycelia growth, culture characteristics, sporulation and spores colour of fungal isolate are the main keys for the identification. These characteristics rely on the composition of culture medium such as carbon and nitrogen source as well as pH (Emine *et al*., 2010; Germain & Summerbell, 1996; Kuhn & Ghannoum, 2003; Kumara & Rawal, 2008; Northolt & Bullerman, 1982). Thus, it is necessary to subculture of fungal isolates on different culture media to get more characterization of the fungi (Germain & Summerbell, 1996).

The culture characteristics of fungal isolates might give an indication of fungal species, but these are un-useful alone for the identification of fungus to the species level. To achieve correct identification, the microscopic morphology of fungal conidiophore and spores were observed using light microscope. The characteristics of fungal isolates belonged to black aspergilli appeared very similar, which made the identification of the isolates more difficult even under the light microscope. Nevertheless, the sub-culture on several culture media and the ultrastructure of the conidiophore and spore morphology observed using SEM was useful for their identification.

Black aspergilli exhibited very close characteristics on one media. However, they have different growth rate and culture characteristics on the others. The study showed that the growth and culture morphology (colony colour) might also vary on the same medium after being sub-cultured several times and during the incubation period. Moreover, the microscopic morphologies such as conidiophore shape, texture, vesicle shape and phialids as well as spore shape, texture and ornamentation are constant.

In this study, the culture and physiological characteristics were described after 7 days of incubation period, while microscopic morphologies were tested daily during the incubation period. The flowchart used to facility the identification of species within this genus is presented in Figure 2. It was noted that black aspergillus, which included *A. niger, A. tubingensis, Aspergillus* spp. in section Nigri, *Aspergillus* sp. strain no. 39, *Aspergillus* sp. strain no. 53, *A. violaceofuscus* and *A. neoniger* have slight differences in the culture characteristics on the different culture media (Table 1).

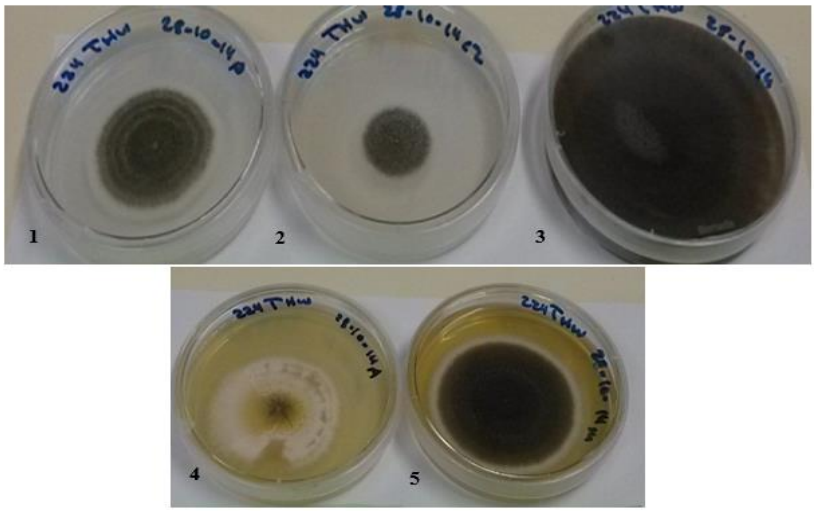

Figure 1. Growth of *A. violaceofuscus* on different culture media after 7 days at 28˚C, 1) PDA; 2) CZ; 3) V8A; 4) CYA; 5) MEA.

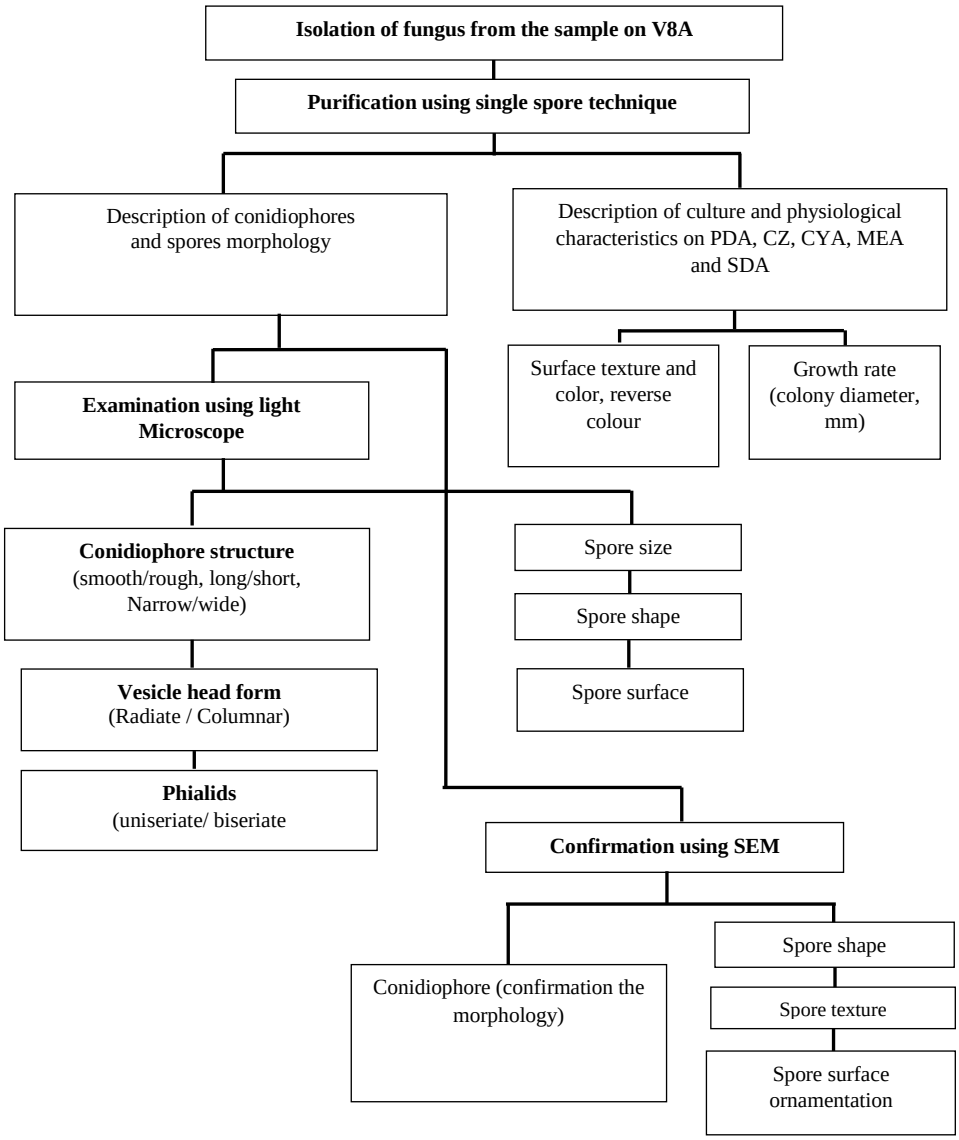

Figure 2. Flowchart of identification of *Aspergillus* spp

On PDA and CZ, *A. niger*, *A. tubingensis*, *Aspergillus* sp. strain no. 39 and *Aspergillus* sp. strain no. 53 grew as black colonies with white edges on both media. *Aspergillus* spp. in section Nigri occurred as black colony mixed with greenish colour centre on PDA, while *A. violaceofuscus* grew as a ring-shaped with slight difference in degree of black colour. *A. neoniger* has black colony on PDA with small zone white edge (Figure 3a). These fungi have different characteristics on V8A. *A. niger*, *A. tubingensis*, *Aspergillus* spp. in section Nigri exhibited black colony, but the different area zone of the white edge. *Aspergillus* sp. strain no 39, *A. violaceofuscus* and *A. neoniger* have black colonies covered the surface area on V8A, while *Aspergillus* sp. strain no. 53, grew as a small white colonies with black centre on this medium.

On CYA, *A. niger* and *Aspergillus* sp. strain no. 53 colonies were light black with white edges. *A. tubingensis* exhibited white colony with light black centre, *Aspergillus* spp. in section Nigri has yellow colony with black centre. *Aspergillus* sp. strain no. 39 grew as a small white colony. *A. violaceofuscus* colony was white colony with black and yellow colour in the centre while *A. neoniger* has large white colony (Figure 3).

Colony of *A. niger* and *A. tubingensis* grown on MEA occurred as black colour in the centre and white edges. *Aspergillus* spp. in section Nigri has black colony with yellow edge. *Aspergillus* sp. strain no. 39 and *Aspergillus* sp. strain no. 53 have similar colonies in the size (moderate size) and colours (Black with white edge). *A. violaceofuscus* and *A. neoniger* exhibited larger colonies than that of others species, but they occurred slight differences in the degree of black colour.

Table 1. Culture characteristics of black aspergilli on different culture media after 7 days of incubation period at 28˚C

Fungus	Media type	Colony Diameter (mm)	Colony character			Zonation (Margin)	Sporulation
			Texture	Surface colour	Reverse colour		
A.niger	V8A	73±5	crisp/ floccose	dark black in centre	not clear	white	high
	CZ	44±9	velvety centre/ floccose centre	black	colour-less	white	moderate
	CYA	38±5	annular velvety	dark brown to black	light yellow	yellow	moderate
	MEA	34±4.5	crisp/ floccose	black	colour-less	white	high
	PDA	45±2.5	velvety/ crisp	dark black	colour-less	white	high
	SDA	55±3.4	velvety	black	black	white	high
A. tubingensis	V8A	78±4.5	crisp/ floccose	black	not clear	white	high
	CZ	49.3±4.5	velvety centre/ floccose centre	black	colourless	white	high
	CYA	66±0.8	velvety/ floccose/ sulcate	light yellow centre	colourless	white	moderate
	MEA	30±3	crisp/ floccose	black	colourless	white	high
	PDA	44±4.3	velvety/ crisp	black	colourless	white	high
	SDA	70±2.5	velvety/ floccose	black	black	white	high
Aspergillus spp. in section Nigri	V8A	77±1.7	velvety	black	not clear	yellow/ white	high
	CZ	25.5±0.5	velvety	black	yellow	white	moderate
	CYA	75±1.5	velvety	yellow/ black centre	yellow	yellow	moderate
	MEA	45±7.5	velvety centre/ sulcate edge	black	brown	yellow	high
	PDA	41±1.8	velvety	black greenish	yellow	white	moderate
	SDA	80 ±0.0	velvety centre/ sulcate edge	black	yellow/ brown	yellow	high
Aspergillus sp. strain no. 39	V8A	80±0.0	velvety	black	not clear	black	high
	CZ	21±4	velvety	black	colourless	white	high
	CYA	19±6.7	creamy growth/ velvety and radially centre	white centre	colourless	beige/ white	less
	MEA	32±4.8	velvety centre/ sulcate edge	black	colourless	white	high
	PDA	34±7.2	velvety/annular	black	light yellow	white	high
	SDA	37.1±2.8	sulcate	black	black	white	High
Aspergillus sp. strain no. 53	V8A	20±3.1	velvety, wrinkled, sulcate	black	colourless	white	low
	CZ	22±1	velvety	black	colourless	white	high
	CYA	70±8.3	velvety, wrinkled/ sulcate	brown centre	colourless	white	moderate
	MEA	48±6.8	velvety/sulcate	black	light brown	white	high
	PDA	29±10	velvety	black	colourless	white	High

Table 1. Continued

Fungus	Media type	Colony Diameter (mm)	Colony character			Zonation (Margin)	Sporulation
			Texture	Surface	Reverse		
A. violaceofuscus	V8A	80±0.0	velvety	black	not clear	black	high
	CZ	24.5±1.5	velvety	light black	colourless	white	moderate
	CYA	46±2.5	floccose/ wrinkled	yellow-ish/ brown	orange	white	low
	MEA	62±5.2	amaranthine	black	colourless	white	high
	PDA	32±7.2	velvety annular	black	light yellow	white	moderate
	SDA	36±0.8	amaranthine	black	colourless	white	high
A. neoniger	V8A	80±0.0	velvety	black	not clear	black	high
	CZ	26±10	velvety centre/ floccose edge	black	colourless	white	high
	CYA	28±8.0	floccose/sulcate	light yellow centre	colourless to light yellow	white	moderate
	MEA	68±2.5	velvety centre / sulcate edge	black	colourless to light yellow	white	high
	PDA	39±1.3	velvety/annular	dark black	yellow	white	high
	SDA	80±0.0	velvety/ sulcate	black	black	white	high

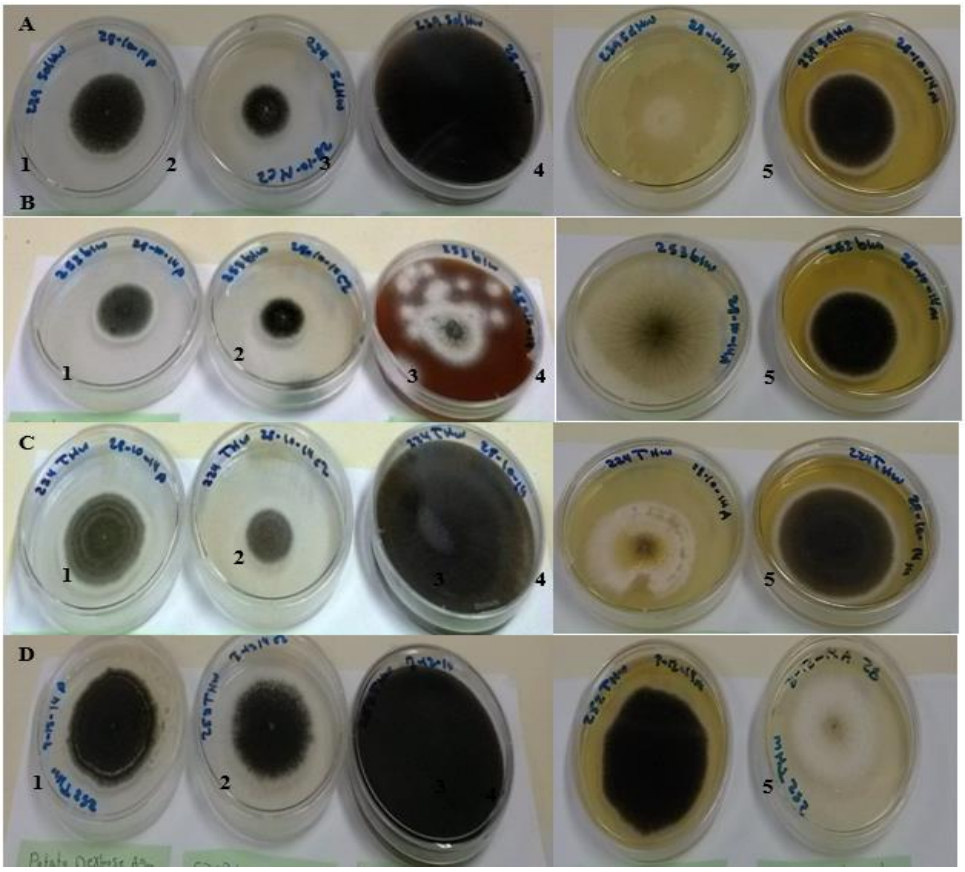

Figure 3. Culture characteristics of *Aspergillus* spp. on different culture media after incubation period for 7 days at 28˚C, A) *Aspergillus* sp. strain no. 39*;* B) *Aspergillus* sp. strain no. 53; C) *A. violaceofuscus*; D) *A. neoniger*; 1) PDA; 2) CZ; 3) V8A; 4) CYA; 5) MEA

The examination using light microscope and SEM confirmed the differences between *A. niger, A. tubingensis, Aspergillus* spp. in section Nigri, *Aspergillus* sp. strain no. 39, *Aspergillus* sp. strain no 53, *A. violaceofuscus* and *A. neoniger* (Table 2, Figure 4 and 5). It was noted that *A. niger* (Figure 4 and 5a), *A. tubingensis* (Figure 4 and 5b), *Aspergillus* spp. strain no. 53 (Figure 4 and 5e) and *A. neoniger* (Figure 4 and 5g) have similar conidiophore (smooth, long and narrow) with round vesicle and radiate head as well as biseriate phialids. *Aspergillus* spp. in section Nigri has smooth, wide and long conidiophore with round vesicle and radiate head but uniseriate phialids (Figure 4 and 5c). *Aspergillus* spp. strain no. 39 has a similar conidiophore shape but it has slightly different vesicle shape and size; it appeared to be round and radiate head as well as biseriate phialids but the phialids did not cover all the surface area of the vesicle (Figure 4 and 5d). *A. violaceofuscus* has large round vesicle and radiate head but uniseriate phialids (Figure 4 and 5f).

The spore sizes of all fungal species ranged from 3 to 5.1 µm with average 3.7, 3.8, 3.9, 4.1, 3.7, 3.9 and 4 µm for *A. niger, A. tubingensis, Aspergillus* spp. in section Nigri, *Aspergillus* spp. strain no. 53, *A. neoniger, Aspergillus* spp. strain no. 39 and *A. violaceofuscus* respectively. The SEM observation for the spores confirmed that these fungi are quite different. *A. niger* has globular spores with spiny texture and echinulate ornamentation (Figure 5a). *A. tubingensis* spores were globular in shape with finely wrinkled texture and warty ornamentation (Figure 5b). In *Aspergillus* spp. in section Nigri the spores were globular and distinctly wrinkled in texture and with warty ornamentation (Figure 5c). *Aspergillus* sp. strain no. 39 spores were globular in shape with spiny texture, connected to each with tube (Figure 5d). *Aspergillus* sp. strain no. 53 spores were globular in shape and wrinkled in texture (Figure 5e). *A. violaceofuscus* spores were elongated in shape with spiny texture (Figure 5f). *A. neoniger* pores were globular shape, concave, spiny to smooth in texture (Figure 5g).

In the present study, SEM analysis was carried out to recognize details of spores and conidiospore of *A. niger, A. tubingensis, Aspergillus* spp. in section Nigri, *Aspergillus* sp. strain no. 53, *A. neoniger, Aspergillus* sp. strain no. 39 and *A. violaceofuscus* for correct identification. Guarro *et al*. (1999) reported that SEM technique facilitated the identification for many fungi to the species level. SEM clearly shows the differences in the spore surface (de Hoog & Guarro, 1995). Freeze fracturing procedure that is used during the imaging process for fungi morphology by SEM reveal fine details of outer wall layers of conidia (Figueras *et al*., 1988; Whalley & Edwards, 1995). Silva *et al*. (2011) identified nine species of *Aspergillus* spp. located within *Aspergillus* section Negri complex which exhibited slight differences in the culture characteristics but they have clear differences in the spore surface texture and ornamentation as observed using SEM analysis; among them were *A. niger* and *A. tubingensis.*

The microscopic and cultural characteristics of fungal species on different media are considered the most important sets for the identification and classification by phenotypic method (Diba *et al*., 2007; Guarro *et al*., 1999). This method is an essential tool, for effective identification of fungal isolates, and gives clear characteristics on culture media and under microscope. Understanding the similarities and differences between *Aspergillus* species is the first step for the correct identification and the confirmation using molecular techniques. According to American society for microbiology, 89% of laboratories identified the fungi based on morphological characteristics (Diba *et al*., 2007).

Table 2. Microscopic morphology of black aspergill

No	Fungal species	Conidiophore			spore size (µm)			Spore shape and surface
		Structure	Vesicle head form	Phialids	mean	max	min	
1	*A.niger*	smooth, long/ narrow	Radiate	biseriate	3.7	4.2	3.2	globular spores with spiny texture and echinulate ornamentation
2	*Aspergillus Nigri Section*	smooth, wide/ long	Radiate	uniseriate	3.9	4.3	3.3	globular and distinctly wrinkled in texture and warty ornamentation
3	*A. tubingensis*	smooth, long and narrow	Radiate	biseriate	3.8	4.2	3.3	globular in shape/ finely wrinkled texture/ warty ornamentation
4	*A. violaceofuscus*	smooth, long and narrow	large round vesicle/ radiate head	uniseriate	4.0	5.1	3	Elongated in shape with spiny texture
5	*Aspergillus* sp. strain no. 53	smooth, long/ wide and swollen	Radiate	biseriate	4.1	5.1	3.5	globular in shape and wrinkled in texture
6	*A. neoniger*	smooth, long and narrow	Radiate	biseriate	3.7	4.3	3.1	globular shape, concave, spiny to smooth in texture
7	*Aspergillus* strain no. 39	smooth, long and narrow	Radiate	biseriate, phialids	3.9	4.3	3.6	globular shape, spiny texture,

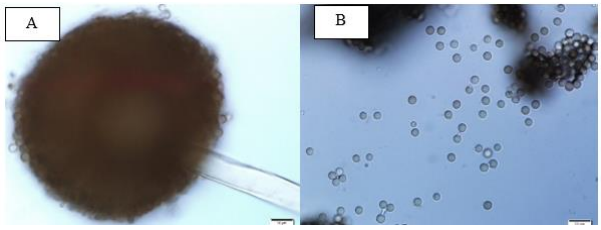

Figure 4a. Light microscope micrographs of *A. niger*, A) conidiophore; B) spores

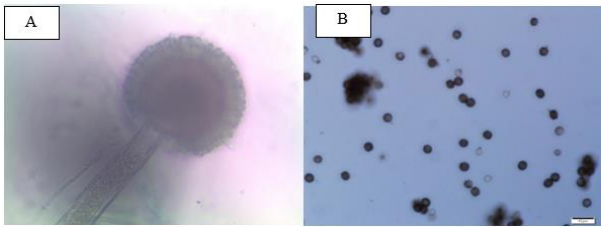

Figure 4b. Light microscope micrographs of *A. tubingensis*, A) conidiophore; B) spores

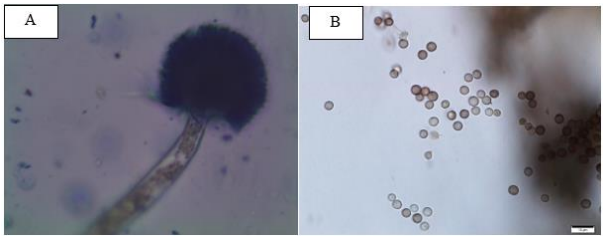

Figure 4c. Light microscope micrographs of *Aspergillus* spp. in section Nigri, A) conidiophore; B) spores

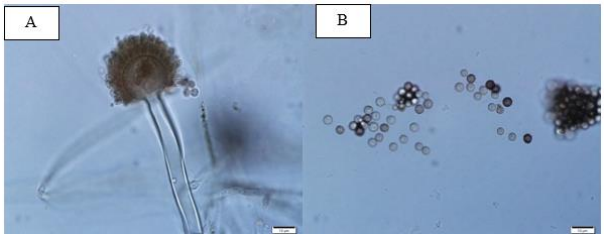

Figure 4d. Light microscope micrographs of *Aspergillus* sp. new strain 39, A) conidiophore; B) spores

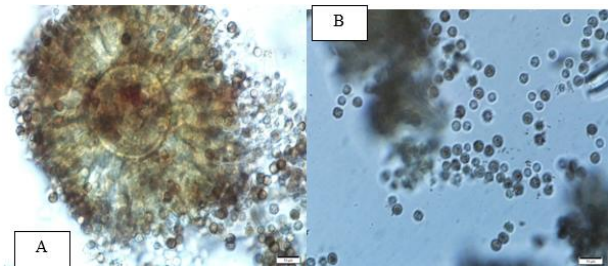

Figure 4e. Light microscope micrographs of *Aspergillus* sp. new strain 53, A) conidiophore; B) spores

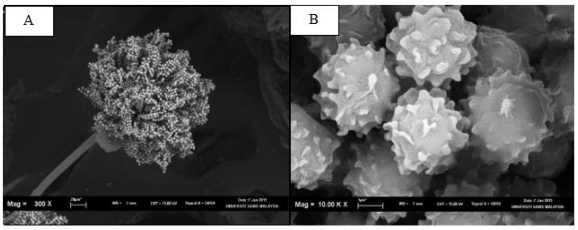

Figure 5a. Scanning electron micrographs of *A. niger* showing the conidiophore (A), which occurs as smooth, long and narrow with round vesicles and radiate head as well as biseriate phialids, and spores (B), which occur as globular spores with spiny texture and echinulate ornamentation. The magnification is 300x for conidiophore and 10.00 kx for the spores

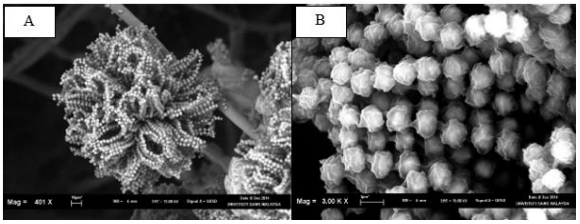

Figure 5b. Scanning electron micrographs of *A. tubingensis* showing the conidiophore (A), which occurs as smooth, long and narrow with round vesicles and radiate head as well as biseriate phialids, and spores (B), which occur globular in shape/ finely wrinkled texture/ warty ornamentation. The magnification is 401x for the conidiophore and 3.00 kx for the spores.

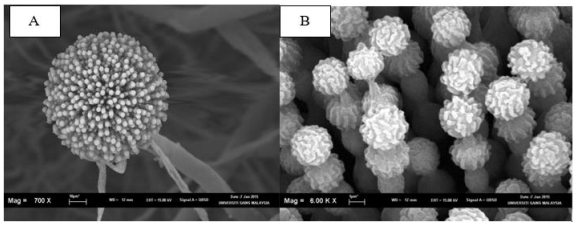

Figure 5c. Scanning electron micrographs of *Aspergillus* spp. in section Nigri showing the conidiophore (A), which occurs as smooth, wide and long conidiophore with round vesicles and radiate head as well as uniseriate phialids, and spores B), which occur as globular and distinctly wrinkled in texture and warty ornamentation. The magnification was 700x for the conidiophore and 6.00 kx for the spores.

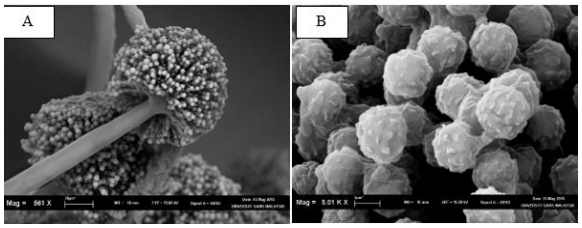

Figure 5d. Scanning electron micrographs of *Aspergillus* sp. strain no. 39 showing the conidiophore (A), which occurs as smooth, wide and long with round and radiate head vesicles as well as biseriate phialids but the phialids have not covered all surface area of the vesicles, and spores B), which occur as globular shape and spiny texture. The magnification is 561x for the conidiophore and 5.01 kx for the spores.

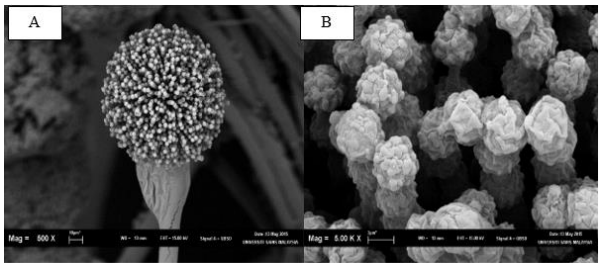

Figure 5e. Scanning electron micrographs of *Aspergillus* sp. strain no. 53 showing the conidiophore (A), which occurs as smooth, long and narrow with round and swollen vesicles and radiate head as well as biseriate phialids, and spores (B), which occur globular in shape and wrinkled in texture. The magnification is 500x for the conidiophore and 5.00 kx for the spores.

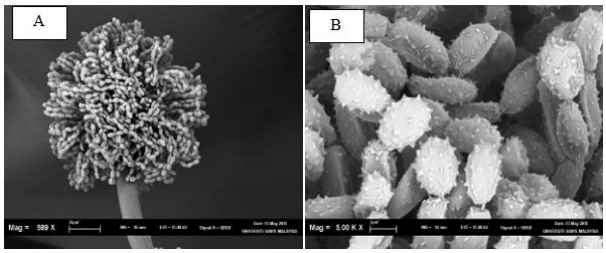

Figure 5f . Scanning electron micrographs of *A. violaceofuscus* showing the conidiophore (A) which occurs as smooth, wide and long with large round vesicles and radiate head but uniseriate phialids, and spores B), which are elongated in shape with spiny texture. The magnification is 589x for the conidiophore and 5.00 kx for the spores.

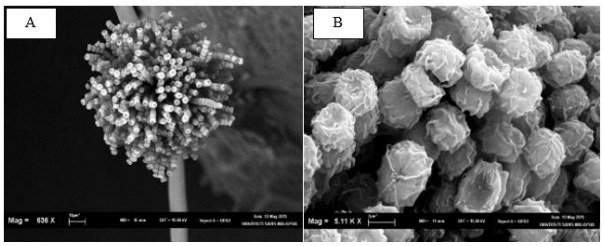

Figure 5g. Scanning electron micrographs of *A. neoniger* showing the conidiophore (A), which occurs as smooth, long and narrow with round vesicle and radiate head as well as biseriate phialids, and spores (B), which occur as globular shape, concave, spiny to smooth in texture. The magnification is 636x for the conidiophore and 5.11 kx for the spores.

The phenotypic method for the identification of fungi is usually easy. However, the culture and microscopic observation using light microscope often create a misconception about its value for the detection some species such as *Aspergillus* spp. especially within black aspergilli as noted in this study. Therefore, SEM observation was used for the determination of the ultrastructure of conidiophore and spores of *Aspergillus* spp. SEM has been previously used as a technique for study and identification of fungi based on the spore structure (Clarke & Griffiths, 1970; Eduard *et al*., 19 85). Gao *et al*. (2007) determined two strains of *A. flavus* (L and S strain) based on the microscopic observation of conidiophores structures. Mares *et al*. (2008) identified 3 new species of *Aspergillus* spp. including *A. quitensis*; E19C, *A. amazonicus*; E19D, and *A. ecuadorensis*; E19F based on SEM analysis of conidiophore and spores, and DNA analysis confirmed that these fungi are quite different. Zhang (2009) described a new species of *Aspergillus* sp. in China based on morphological characteristics using light and SEM observation. Lately, Vestlund *et al*. (2014) used SEM for classification of bioaerosols from composting which included fungal spores. In this study, two new isolates within black aspergilli were identified based on culture and microscopic morphologies as well as ultrastructure determined using SEM. These strains were *Aspergillus* sp. strain no. 39, *Aspergillus* sp. strain no. 53. However, the confirmation for both isolates will be conducted based on molecular analysis in the possible future work.

4. Conclusions

The high diversity in the structure of conidiophore and spores of black aspergilli is more useful for their identification. However, these structures might be difficult to recognize using light microscopy. SEM clearly recognize the slight differences in the spore structure, which includes spore texture and surface ornamentation. Both parameters play an important role in the identification process by phenotypic method.

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References

Balajee, S. A., Kano, R., Baddley, J. W., Moser, S. A., Marr, K. A., Alexander, B. D., . . . Chiller, T. (2009). Molecular Identification of *Aspergillus* Species Collected for the Transplant-Associated Infection Surveillance Network. *Journal of Clinical Microbiology, 47*(10), 3138–3141.

Barnett, H. L., & Hunter, B. B. (1998). *Illustrated genera of imperfect fungi; A comprehensive resource for recognizing, identifying, and learning various aspects of imperfect fungi 4th Edition*. Saint Paul, MN: APS Press.

Campbell, C. K., Johnson, E. M., & Warnock, D. W. (2013). *Identification of pathogenic fungi 2nd Edition*. London, England: A John Wiley & Sons.

Chandrakanth, R., Jagadeesh, D., & Devaki, N. S. (2014). Black light mediated growth and sporulation of magnaporthe oryzae. *International Journal of Agriculture Science and Research, 4*, 25-30.

Choi, Y. W., Hyde, K. D., & Ho, W. H. (1999). Single spore isolation of fungi. *Fungal Diversity, 3*, 29-38.

Clarke, J. H., & Griffiths, D. A. (1970). Ascospores of some common species of *Eurotium (Aspergillus glaucus)* as shown by scanning electron microscopy. *Transactions of the British Mycological Society, 55*, 117–122.

Cole, G. T., & Samson, R. A. (1979). *Patterns of development in conidial fungi*. London, England: Pitman.

de Hoog, G. S., & Guarro, J. (1995). Atlas *of clinical fungi.* Baarn, The Netherlands: Centraalbureau voor Schimmelcultures.

Diba, K., Kordbacheh, P., Mirhendi, S. H., Rezaie, S., & Mahmoudi, M. (2007). Identification of *Aspergillus* species using morphological characteristics. *Pakistan Journal of Medical Science, 23*(6), 867-872.

Eduard, W., Sandven, P., Johansen, B. V., & Bruun, R. (1985). Identification and quantification of mould spores by scanning electron microscopy (SEM): analysis of filter samples collected in Norwegian Saw Mills. *Proceedings of an International Symposium and Workshop on Lung Dosimetry Organised by the British Occupational Hygiene Society in Co-Operation with the Commission of the European Communities*, Cambridge, England. 447–453.

Efaq, A. N., Nik, N. N. A. R., Nagao, H., Al-Gheethi, A. A., Md, S., & Ab. Kadir, M. O. (2015a). Supercritical carbon dioxide as non-thermal alternative technology for safe handling of clinical wastes. *Journal of Environmental Processes, 2*(4), 797–822.

Efaq A. N., Nik, N. N. A. R., Nagao, H., Al-Gheethi, A. A., & Ab. Kadir, M. O. (2017). Inactivation of *Aspergillus* Spores in Clinical Wastes by Supercritical Carbon Dioxide. *Arabian Journal for Science and Engineering, 42*(1), 39-51.

Efaq, A.N., Al-Gheethi, A. A., Nik, N. N. A. R., Nagao, H., & Ab. Kadir, M. O. (2016). Assessment of relevant fungal species in clinical solid wastes. *Environmental Science and Pollution Research*, *23*(19), 19806–19824. doi:10.1007/s11356-016-7161-8

Efaq, A. N., Nagao, H., Nik, N. N. A., Al-Gheethi, A. A., & Ab. Kadir, M. O. (2015b). Survival of opportunistic fungi in clinical wastes. *4th International Conference on Environmental Research and Technology*, Penang, Malaysia.

Ellis, M. B. (1971). *Dematiaceous Hyphomycetes*. London, England: Kew: Commonwealth Mycological Institute.

Emine, S., Kambol, R., & Zainol, N. (2010). Morphological Characterization of Soil Penicillium sp. Strains – Potential Producers of Statin. *Biotechnology Symposium IV*, Universiti Malaysia Sabah, Sabah, Malaysia.

Figueras, M. J., Guarro, J., & Dijk, F. (1988). Rodlet structure on the surface of Chaetomium spores. *Journal of Microbios, 53*, 101–107.

Gao, J., Liu, Z. and Yu, J. 2007. Identification of Aspergillus section Flavi in maize in northeastern China. *Mycopathology Journal, 64*, 91–99.

Germain, S. G., & Summerbell, R. (1996). *Identifying Filamentous Fungi–A Clinical Laboratory Handbook 1st Edition*. Belmont, California: Star Publishing.

Guarro, J., Gene, J., & Stchigel, A. M. (1999). Developments in Fungal Taxonomy. *Clinical Microbiology Review, 12*(3), 454–500.

Kent, C. R., Bermúdez, P. O., Giles, S. S., & Hull, C. M. (2008). Formulation of a Defined V8 Medium for Induction of Sexual Development of *Cryptococcus neoformans. Appled Environmental Microbiology, 74*(20), 6248–6253.

Klich, M. A. (2009). Health effects of *Aspergillus* in food and air. *Toxicology Industrial Health 25*, 657–667.

Kuhn, D. M., & Ghonnoum, M. A. (2003). Indoor mold, toxigenic fungi and Stachybotrys chartarum: Infectious disease perspective. *Clinical Microbiology Review, 16*(1), 144-172.

Kumara, K. L. W., & Rawal, R. D. (2008). Influence of carbon, nitrogen, temperature and pH on the growth and sporulation of some Indian isolates of Colletotrichum gloeosporioides causing anthracnose disease of papaya (Carrica papaya L). *Tropical Agriculture Research Experiment, 11*, 7-12.

Lee, J. K., Kim, B. T., Kim, J. A., Chung, H. J., Park, S. M., Yang, M. S., . . . Kim, D. H. (2007). Cultural characteristics and extraction of the fungal pigment phleichrome from the phytopathogenic fungus *Cladosporium phlei*. *Biotechnology Bioprocess Engineering, 12*(5), 508-515.

Maheshwari, S. K., Singh, D. V., & Sahu, A. K. (1999). Effect of several nutrient media, pH and carbon sources on growth and sporulation of Alternaria alternata. *Journal of Mycopathology Research, 37*, 21-23.

Mares, D., Andreotti, E., Maldonado, M. E., Pedrini, P., Colalongo, C., & Romagnoli, C. (2008). Three New Species of Aspergillus from Amazonian Forest Soil (Ecuador). *Current Microbiology, 57*, 222–229.

National Mycology Reference Centre. (2015, January 2). Mycology Online. National Mycology Reference Centre, the University of Adelaide. Retrieved from http: //www.mycology.adelaide.edu.au

Northolt, M. D., & Bullerman, L. B. (1982). Prevention of mould growth and toxin production through control of environmental condition. *Journal of Food Protection, 6*, 519-526.

Nunes, C. C., Gowda, M., Sailsbery, J., Xue, M., Chen, F., Brown, D. E., . . . Dean, R. A. (2011). Diverse and tissue-enriched small RNAs in the plant pathogenic fungus, *Magnaporthe oryzae*. *BMC Genomics, 12* (288), 2-20.

Promputtha, I., Jeewon, R., Lumyong, S., McKenzie, E. H. C., & Hyde, K. D. (2005). Ribosomal DNA fingerprinting in the identification of non sporulating endophytes from Magnolia liliifera (Magnoliaceae). *Fungal Diversity, 20*, 167-186.

Robert, A. S., János, V., & Christian, F. J. (2011). Taxonomic studies on the genus *Aspergillus*- DTU Orbit. CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands.

Saha, A., Mandal, P., Dasgupta, S., & Saha, D. (2008). Influence of culture media and environmental fac-tors on mycelial growth and sporulation of Lasiodiplodia theobromae (Pat.) Griffon and Maubl. *Journal of Environmental Biology, 29*(3), 407-410.

Samson, R. A., Houbraken, J., Thrane, U., Frisvad, J. C., & Andersen, B. (2010). Food and indoor fungi. CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands.

Silva, D. M., Batista, L. R., Rezende, E. F., Fungaro, M. H. P., Sartori, D., & Alves, E. (2011). Identification of fungi of the genus *Aspergillus* section Nigri using polyphasic taxonomy. *Brazilian Journal of Microbiology, 42*, 761-773.

United States Environmental Protection Agency. (1990). Summary of potential risks from hospital waste incineration: Pathogens in air emission and residues. EACO-R-0238. Retrieved from https://nepis.epa.gov/

Vestlund, A. T., Al-Ashaab, R., Tyrre, S. F, Longhurst, P. J., Pollard, S. J. T., & Drewa, G. H. (2014). Morphological classification of bioaerosols from composting using scanning electron microscopy. *Waste Management, 34*, 1101–1108.

Watanabe, T. (2002). *Pictorial atlas of soil and seed fungi; Morphologies of cultured fungi and key to species 2nd Edition*. Boca Raton, FL: CRC Press.

Whalley, A. J. S., & Edwards, R. L. (1995). Secondary metabolites and systematic arrangement within the Xylariaceae. *Canadian Journal of Botany, 73*, 802–810.

Xu, S. O., Yuan, S. Z., & Chen, X. C. (1984). Studies on pathogenic fungus (Alternaria tennuis Nees) of poplar leaf blight. *Journal of North East Forestry Institute, 12*, 56-64.

Yu, J. (2010). *Identification of fungi and bacteria associated with internally discolored horseradish roots* (Master's thesis, Graduate College, University of Illinois, IL).

Zhang, Z. (2009). A new species of *Aspergillus*. *International Journal of biology, 1*(2), 78-80.