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OCCURRENCE AND MOLECULAR CHARACTERIZATION OF *Aspergillus* SPECIES IN BEACH SAND

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ABSTRACT

A total of 103 *Aspergillus* isolates were obtained from beach sand samples collected along Batu Ferringhi beach, Penang Island. Ten species of *Aspergillus* were identified and the most common species was *A. tubingensis* (33%) followed by *A. aculeatus* (21.4%), *A. flavus* (20.4%), *A. niger* (9.7%), *A. terreus* (6.80%), *A. fumigatus* (2.91%), *A. ibericus* (1.94%), *A. sydowii* (1.94%), *A. carbonarius* (0.98%) and *A. tamarii* (0.98%). Maximum likelihood tree of combined dataset of ITS regions and β-tubulin sequences showed that the same species were grouped in the same clade. The present study indicated that beach sand harbor a variety of *Aspergillus* species and the occurrence of *Aspergillus* in the sand might pose health concern in case of long term exposure as some species such as *A. fumigatus, A. flavus, A. niger* and *A. terreus* are potentially pathogenic especially to immune compromised individual. The present study also contribute to the knowledge on the diversity of *Aspergillus* species in the beach environment as well as contribute knowledge on the taxonomic relationship of *Aspergillus* species in Malaysia.

Key words: *Aspergillus*, beach sand, ITS regions, β-tubulin

INTRODUCTION

The genus *Aspergillus* has high biological diversity which was reflected in the list of species given by Raper and Fennell (1965) and Pitt *et al.* (2000). In addition, *Aspergillus* has significant presence in a variety of ecosystems and different substrates such as the soil, textiles, food and feed (Klich, 2002; Klich, 2009; Klich *et al*., 1992; Perrone *et al.*, 2007; Pitt & Hocking, 2009). A number of *Aspergillus* species are xerophilic and can survive in environments with relatively low moistures (Cantrell *et al.,* 2006).

In Malaysia, sandy beaches are often sought after for recreational purposes and there is significant presence of microorganisms in the beach sand (Velonakis *et al.,* 2014). *Aspergillus* species are one of the common fungi isolated from several sandy beaches worldwide (Larrondo & Calvo, 1989; Oliveira *et al*., 2011). Members of the species are common saprophytes in the beach soil environment, however, they may act as opportunistic pathogens, especially in immune compromised patients (Hoog *et al*., 2000). Moreover, the viable fungal conidia can act as an agent in the transmission of fungal infection in humans (Larrondo & Calvo, 1989). Factors such as the nature of the beach, tidal phenomena, sewage outlets, seasons, the presence of animals and the number of bathers, can encourage the survival and dispersion of pathogens on beach sand (World Health Organization, 2003).

The tropical beaches in Malaysia provide an ideal habitat for a wide diversity of *Aspergillus* species. Considering lack of studies on diversity of *Aspergillus* species in Malaysia, the objective of this study was to isolate and characterize *Aspergillus* species in beach sand using molecular method in which the information can enhance the knowledge on the occurrence and biodiversity of *Aspergillus* in beach ecosystem.

MATERIALS AND METHODS

Beach sand samples were collected along Batu Ferringhi beach areas, Penang Island, Peninsular Malaysia and the sampling was done during a dry season. Twenty four sand samples were taken by scraping off the surface and subsurface to a depth of 10 cm. Approximately 1.5 kg of soils were collected and put in plastic bags and labelled. Three isolation methods, soil dilution plate, debris isolation and direct isolation were used to isolate the *Aspergillus* isolates from the sand samples. The isolation medium used was Malt Extract Agar. From

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soil analysis, the beach sand samples have sand texture and pH ranging from 5.87 to 7.72.

Mycelia for DNA extraction were grown in Universal bottles with Potato Dextrose Broth at 25ºC. Mycelia were harvested by filtration when mycelium was visible with no sporulation, generally after 16-48 h. Mycelia were frozen and lyophilized, and then crushed using liquid nitrogen. Genomic DNA was extracted using Invisorb® Spin Plant Mini Kit (STRATEC Molecular GmbH, Germany) according to the manufacturer's protocol. For amplification of ITS regions, ITS1 and ITS4 primers were used (White *et al*., 1990), while β-tubulin gene were amplified using Bt2a and Bt2b primers (Glass & Donaldson, 1995).

PCR reactions for both ITS regions and β-tubulin were performed in TM Peltier Thermal Cycler Model PTC-100 (MJ-Research, USA). DNA amplifications were performed in a total volume of 25 µl containing 0.5 µl of genomic DNA, 4.0 mM $MgCl_2$, 0.8 mM dNTPs and 0.625 U of *Taq* polymerase (Promega, USA). For amplification of ITS regions, the primer concentration used was 0.5 µM, while the amplification of β-tubulin gene, 0.2 µM. PCR cycles started with an initial denaturation at 95ºC for 5 min, followed by 30 cycles of 30s denaturation at 95ºC, 30s annealing at 58ºC and 1 min extension at 72ºC. Final extension for 5 min at 72ºC was performed after the cycles ended.

PCR products were separated by electrophoresis on 1% agarose gels and checked to ensure that a single DNA band of desired size was produced. PCR products were purified using FavorPrep™ Gel/PCR Purification Kit (Favorgen® Biotech Corp, Taiwan) according to the manufacturer's protocol. Then, the purified products were sent to a service provider for DNA sequencing.

The DNA sequences were analyzed for phylogenetic relationship using Molecular Evolutionary Genetic Analysis (MEGA5) software (Tamura *et al*., 2011). The sequences of *Aspergillus* isolates were compared with sequences in the GenBank by using Basic Local Alignment Search Tool (BLAST). Combined datasets of ITS regions and ß - tubulin sequences were used to generate a phylogenetic tree. Maximum likelihood tree was constructed by using Tamura 3-parameter substitution with discrete Gamma distribution (T92+G) model (Tamura, 1992). Tree was inferred using the ML heuristics search option with nearest-neighbor-interchange (NNI). Bootstrap analysis was performed with 1000 replications in order to determine the support for each clade. The ITS regions and β-tubulin sequences of type specimen for *Aspergillus* culture from Centraalbureau voor Schimmelcultures (CBS) and Genbank are also included for comparison (Table 1). All the sequences were deposited in the Genbank.

RESULTS

A total of 103 *Aspergillus* isolates were obtained from the beach sand samples. The size of the PCR products for the ITS regions was approximately 600 bp and for β-tubulin, 500 bp. The percentage of similarity from BLAST search and accession number of the sequences are listed in Table 2. From BLAST search, ten *Aspergillus* species were identified and the most common species isolated was *A. tubingensis* (33%) followed by *A. aculeatus* (21.4%), *A. flavus* (20.4%), *A. niger* (9.7%), *A. terreus* (6.80%), *A. fumigatus* (2.91%), *A. ibericus* (1.94%), *A. sydowii* (1.94%), *A. carbonarius* and *A. tamarii* (0.98%). All the isolates in this study showed the percentage of similarity ranging from 98% to 100% for ITS regions and 97% to 100% for β-tubulin gene.

Based on ML tree generated using combined datasets of ITS regions and β-tubulin, isolates from the same species including the type specimen were grouped in the same group (Fig. 1). All *A. tubingensis* isolates were clustered in Clade 1, separated from *A. niger* isolates (Clade 2) with 89% bootstrap value. *Aspergillus carbonarius* isolates were grouped in Clade 3 separated from *A. ibericus* isolates which grouped in Clade 4 with 97% bootstrap value. Clade 5 comprised *A. fumigatus* isolates. *Aspergillus tamarii* isolates were grouped in Clade 6, separated from *A. flavus* isolates (Clade 7) with 99% bootstrap support. Clade 8 consisted of *A. terreus* isolates. *Aspergillus aculeatus* isolates were clustered in Clade 9 and *A. sydowii* isolates, in Clade 10.

DISCUSSION

Phylogenetic analysis using ITS regions and β-tubulin gene was useful for studying phylogenetic relationships and to distinguish among closely related *Aspergillus* species (Balajee *et al.*, 2007). Varga *et al*. (2004) were able to clarify the

Table 1. Centraalbureau voor Schimmelcultures (CBS) culture number and GenBank accession numbers of type specimens included in this study

Species	Culture number / Accession number
A. tubingensis	EF661193.1/EF661086.1
A. niger	CBS 554.65
A. carbonarius	CBS 111.26
A. aculeatus	CBS 172.66
A. tamarii	CBS 104.13
A. flavus	CBS 569.65
A. ibericus	EF661200.1/EF661102.1
A. fumigatus	CBS 133.61
A. terreus	CBS 601.65
A. sydowii	CBS 593.65

Table 2. Identity of *Aspergillus* isolates from beach soil based on sequence similarity of ITS regions and β-tubulin gene

Isolates	Percentage of similarity (%)		Accession number (Genbank)	
	ITS regions	β-tubulin	ITS	β-Tubulin
A1S1-D72	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291183	JX463309
A1S4-D31	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (100%)	JX291184	JX463299
A1S5-15	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291185	JX463318
A2S1-7	*Aspergillus tubingensis* (99%)	*Aspergillus tubingensis* (100%)	JX501370	JX545079
A2S1-D59	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291187	JX463303
A2S1-D60	*Aspergillus tubingensis* (99%)	*Aspergillus tubingensis* (99%)	JX291169	JX463330
A2S2-D32	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291194	JX463321
A2S3-2	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (100%)	JX291192	JX463304
A2S3-D5	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291188	JX463326
A2S4-1	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291186	JX463310
A2S5-3	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (97%)	JX501390	JX545080
A2S5-D40	*Aspergillus tubingensis* (99%)	*Aspergillus tubingensis* (99%)	JX291189	JX463331
A2S5-D88	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291190	JX463314
A2S6-7	*Aspergillus tubingensis* (99%)	*Aspergillus tubingensis* (99%)	JX291193	JX463305
A3S1-2	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291171	JX463320
A3S1-D107	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291170	JX392949
A3S1-D50	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX501396	JX545081
A3S2-12	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291172	JX463313
A3S2-D45	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291173	JX463324
A3S2-D94	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291174	JX463307
A3S3-6	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX501398	JX545082
A3S3-D40	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291175	JX463328
A3S5-2	*Aspergillus tubingensis* (99%)	*Aspergillus tubingensis* (99%)	JX501403	JX545083
A3S5-32	*Aspergillus tubingensis* (99%)	*Aspergillus tubingensis* (99%)	JX291195	JX463312
A3S5-D18	*Aspergillus tubingensis* (99%)	*Aspergillus tubingensis* (99%)	JX291196	JX463297
A3S6-2	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX501405	JX545084
A3S6-D9	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291191	JX463316
A4S1-56	*Aspergillus tubingensis* (99%)	*Aspergillus tubingensis* (99%)	JX291176	JX463301
A4S2-16	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291177	JX463322
A4S3-1	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291178	JX463306
A4S4-1	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291179	JX463300
A4S5-21	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX501413	JX545086
A4S5-D2	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291180	JX463325
A4S6-28	*Aspergillus tubingensis* (100%)	*Aspergillus tubingensis* (99%)	JX291181	JX463311
A3S4-D6	*Aspergillus niger* (100%)	*Aspergillus niger* (100%)	JX291197	JX463317
A4S6-D5	*Aspergillus tubingensis* (99%)	*Aspergillus tubingensis* (99%)	JX291182	JX463308
A4S2-1	*Aspergillus niger* (100%)	*Aspergillus niger* (99%)	JX291198	JX463302
A3S6-58	*Aspergillus tubingensis* (99%)	*Aspergillus tubingensis* (99%)	JX501407	JX545085
A2S1-D105	*Aspergillus niger* (99%)	*Aspergillus niger* (99%)	JX501371	JX545077
A2S2-2	*Aspergillus niger* (100%)	*Aspergillus niger* (100%)	JX501376	JX545078
A1S5-D33	*Aspergillus niger* (100%)	*Aspergillus niger* (100%)	JX291199	JX463319
A1S6-13	*Aspergillus niger* (99%)	*Aspergillus niger* (100%)	JX501365	JX545076
A1S6-D20	*Aspergillus niger* (100%)	*Aspergillus niger* (99%)	JX291200	JX463298
A1S3-D97	*Aspergillus niger* (99%)	*Aspergillus niger* (100%)	JX291201	JX463329
A2S6-D3	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX501392	JX545072
A1S2-3	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX291165	JX463296
A1S2-D21	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX291166	JX463323
A1S3-D48	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX501355	JX545059
A1S3-D9	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX501357	JX545060
A1S4-D17	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX501358	JX545061

A1S4-D18	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX501359	JX545062
A1S5-D16	*Aspergillus aculeatus* (99%)	*Aspergillus aculeatus* (99%)	JX501362	JX545063
A1S5-D7	*Aspergillus aculeatus* (99%)	*Aspergillus aculeatus* (99%)	JX501364	JX545064
A1S6-D11	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX501368	JX545065
A2S1-4	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX291167	JX463333
A2S1-D91	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX501374	JX545066
A2S1-D97	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX291168	JX463327
A2S1-D98	*Aspergillus aculeatus* (99%)	*Aspergillus aculeatus* (99%)	JX501375	JX545067
A2S2-D14	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX501377	JX545068
A2S2-D23	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX501378	JX545069
A2S3-D3	*Aspergillus aculeatus* (99%)	*Aspergillus aculeatus* (99%)	JX501381	JX545070
A2S4-D20	*Aspergillus aculeatus* (99%)	*Aspergillus aculeatus* (99%)	JX501384	JX545071
A3S1-16	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX501393	JX545089
A3S1-40	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (98%)	JX501394	JX545073
A3S1-D57	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (99%)	JX501397	JX545074
A4S3-D3	*Aspergillus aculeatus* (100%)	*Aspergillus aculeatus* (97%)	JX501412	JX545075
A1S3-D53	*Aspergillus carbonarius* (100%)	*Aspergillus carbonarius* (99%)	JX291202	JX463315
A2S1-D20	*Aspergillus ibericus* (100%)	*Aspergillus ibericus* (99%)	JX501373	JX489770
A2S2-D4	*Aspergillus ibericus* (98%)	*Aspergillus ibericus* (99%)	JX291203	JX463332
A1S2-D20	*Aspergillus flavus* (100%)	*Aspergillus flavus* (98%)	JX501354	JX545039
A1S3-D84	*Aspergillus flavus* (100%)	*Aspergillus flavus* (99%)	JX501356	JX545040
A1S4-D2	*Aspergillus flavus* (100%)	*Aspergillus flavus* (97%)	JX501360	JX545088
A1S5-D5	*Aspergillus flavus* (100%)	*Aspergillus flavus* (99%)	JX501363	JX545041
A1S6-3	*Aspergillus flavus* (100%)	*Aspergillus flavus* (99%)	JX501366	JX545042
A1S6-8	*Aspergillus flavus* (99%)	*Aspergillus flavus* (100%)	JX501367	JX545043
A2S1-17	*Aspergillus flavus* (100%)	*Aspergillus flavus* (99%)	JX501369	JX545044
A2S1-D104	*Aspergillus flavus* (99%)	*Aspergillus flavus* (99%)	JX501415	JX545045
A2S2-D29	*Aspergillus flavus* (100%)	*Aspergillus flavus* (99%)	JX501379	JX545046
A2S4-12	*Aspergillus flavus* (100%)	*Aspergillus flavus* (99%)	JX501383	JX545047
A2S4-D25	*Aspergillus flavus* (99%)	*Aspergillus flavus* (100%)	JX501385	JX545048
A2S5-D1	*Aspergillus flavus* (99%)	*Aspergillus flavus* (98%)	JX501391	JX545049
A3S1-D101	*Aspergillus flavus* (100%)	*Aspergillus flavus* (99%)	JX501395	JX545050
A3S3-D3	*Aspergillus flavus* (100%)	*Aspergillus flavus* (99%)	JX501399	JX545051
A3S4-88	*Aspergillus flavus* (100%)	*Aspergillus flavus* (99%)	JX501400	JX545052
A3S4-D2	*Aspergillus flavus* (99%)	*Aspergillus flavus* (100%)	JX501401	JX545053
A3S5-58	*Aspergillus flavus* (99%)	*Aspergillus flavus* (100%)	JX501404	JX545054
A3S6-48	*Aspergillus flavus* (99%)	*Aspergillus flavus* (98%)	JX501406	JX545055
A4S3-12	*Aspergillus flavus* (99%)	*Aspergillus flavus* (99%)	JX501409	JX545056
A4S3-13	*Aspergillus flavus* (99%)	*Aspergillus flavus* (99%)	JX501410	JX545057
A4S6-40	*Aspergillus flavus* (100%)	*Aspergillus flavus* (99%)	JX501414	JX545058
A4S3-D1	*Aspergillus tamarii* (99%)	*Aspergillus tamarii* (99%)	JX501411	JX489771
A1S2-D12	*Aspergillus terreus* (99%)	*Aspergillus terreus* (99%)	JX501352	JX501416
A1S2-D18	*Aspergillus terreus* (99%)	*Aspergillus terreus* (99%)	JX501353	JX501417
A1S4-D36	*Aspergillus terreus* (100%)	*Aspergillus terreus* (99%)	JX501361	JX501418
A2S1-D106	*Aspergillus terreus* (99%)	*Aspergillus terreus* (97%)	JX501372	JX501419
A2S4-D7	*Aspergillus terreus* (99%)	*Aspergillus terreus* (98%)	JX501389	JX501421
A2S4-D50	*Aspergillus terreus* (99%)	*Aspergillus terreus* (98%)	JX501387	JX501420
A3S5-1	*Aspergillus terreus* (99%)	*Aspergillus terreus* (97%)	JX501402	JX501422
A2S3-D7	*Aspergillus fumigatus* (100%)	*Aspergillus fumigatus* (100%)	JX501382	JX501424
A2S4-D49	*Aspergillus fumigatus* (99%)	*Aspergillus fumigatus* (100%)	JX501386	JX545087
A2S4-D54	*Aspergillus fumigatus* (99%)	*Aspergillus fumigatus* (100%)	JX501388	JX501423
A2S2-31	*Aspergillus sydowii* (99%)	*Aspergillus sydowii* (99%)	KC795922	KC795920
A2S3-D6	*Aspergillus sydowii* (99%)	*Aspergillus sydowii* (99%)	KC795923	KC795921

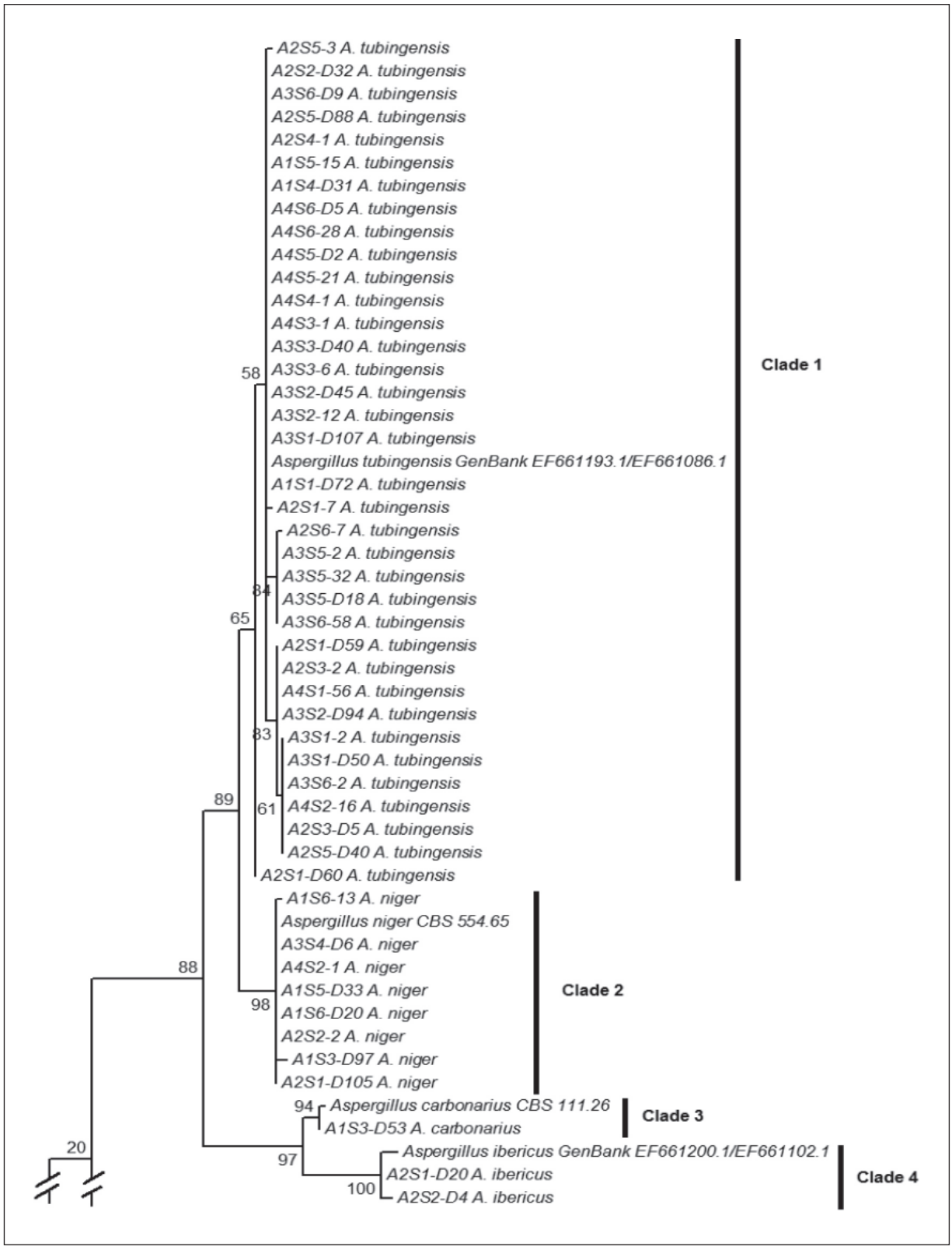

Fig. 1a. Maximum likelihood tree showing phylogenetic relationships among *Aspergillus* species based on the combined dataset of ITS regions and β-tubulin gene using Tamura 3-parameter substitution with discrete Gamma distribution (+G) model and nearest-neighbor-interchange search options with 1000 bootstrap replicates.

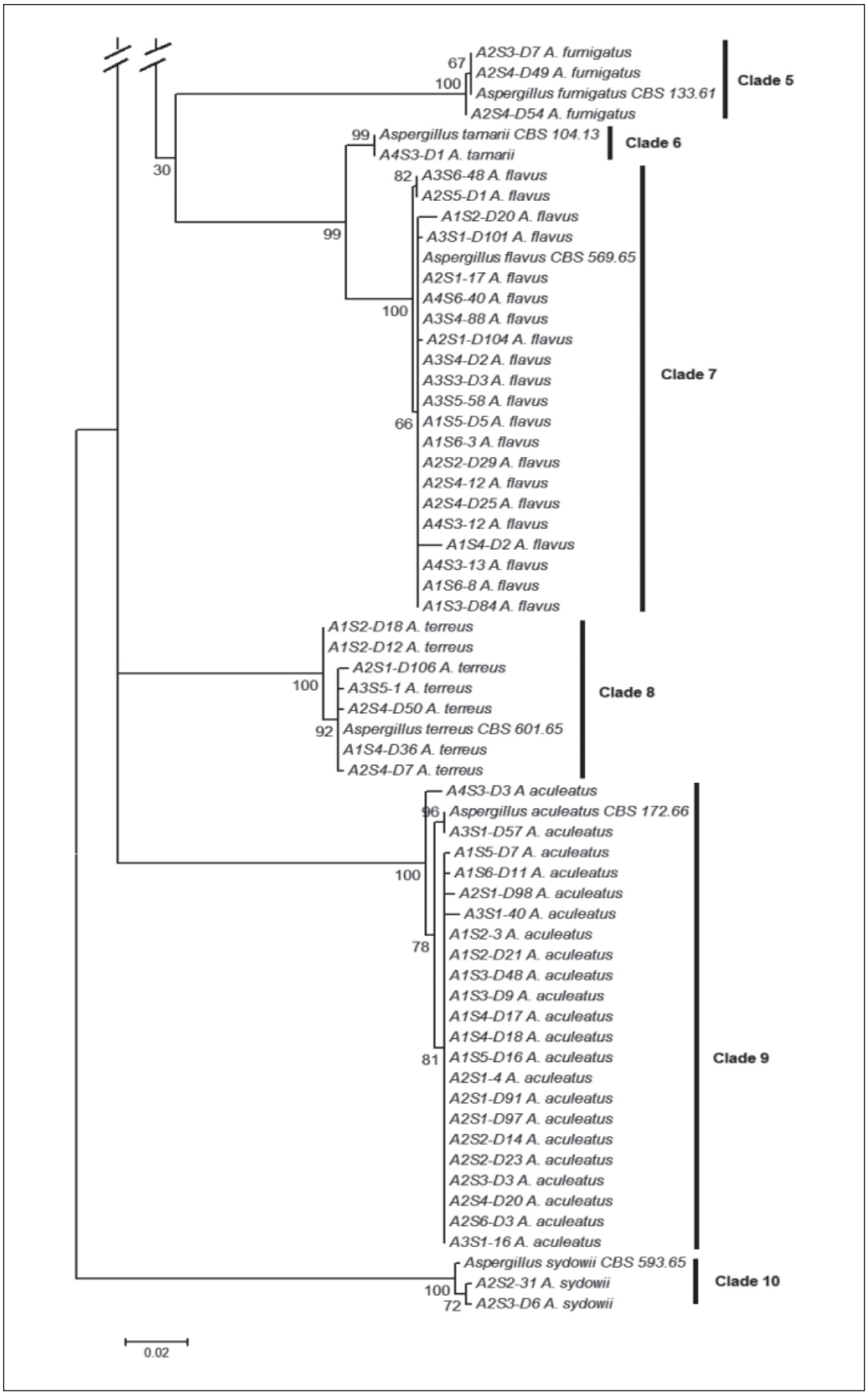

Fig. 1b. Maximum likelihood tree showing phylogenetic relationships among *Aspergillus* species based on the combined dataset of ITS regions and β-tubulin gene using Tamura 3-parameter substitution with discrete Gamma distribution (+G) model and nearest-neighbor-interchange search options with 1000 bootstrap replicates.

taxonomy of the black Aspergilli (*Aspergillus* section Nigri) as well as *A. flavus* and its relatives (*Aspergillus* section Flavi) by using phylogenetic analysis of ITS regions and â-tubulin sequences.

The present study showed that *Aspergillus* species are diverse in beach sand. Sand particles might provide microhabitat under specific conditions for survival of conidia. *Aspergillus* species are regarded as opportunistic pathogens and the sand particle might also play a role as vector of disease infection process (Larrondo & Calvo, 1989). Therefore, beach sand could become a reservoir of *Aspergillus* and pose a health concern for beach users especially immunocompromised individuals which are at higher risk of developing health problems. Infection to human can occur through direct contact with the skin or through inhalation of the conidia and are often asymptomatic (Mancini *et al*., 2005). Many species of *Aspergillus* are well-known mycotoxin producer and one of the fungal genera that are medically important. In clinical setting, *Aspergillus* species caused opportunistic infections such as aspergillosis and is regarded as the most commonly isolated species in invasive infections (Yaguchi *et al.*, 2007). In the present study, several species such as *A. fumigatus, A. niger, A. flavus* and *A. terreus* are commonly associated with aspergillosis.

Aspergillus species have been isolated from beach soils in several parts of the world such as from Ipanema Beach, Rio de Janeiro (Moura Sarquis & Oliveira, 1996), Bairro Novo and Casa Caiad beaches, Olinda, Pernambuco (Gomes *et al*., 2008) Candeias Beach, Pernambuco (Oliveira *et al.,* 2011) in Brazil. In Mexico, González *et al.* (1998) also isolated *Aspergillus* from three coastal beaches located on the coasts of the Caribbean Sea, Gulf of Mexico, and the Pacific Ocean. *Aspergillus* species have even been isolated from hypersaline Dead Sea coastal area (Grishkan *et al.*, 2003) and in sandy soil in Egyptian beaches (Migahed, 2003).

Based on phylogenetic analysis, *A. tubingensis* and *A. niger* isolates were clearly separated into different clades. These two members of black aspergilli are commonly found in soil and litter (Klich, 2002). Both *A. tubingensis* and *A. niger* are known to produce ochratoxin A which is carcinogenic to human (Castegnaro & Wild, 1995; Medina *et al*., 2005; Oliveri *et al*., 2008) and this mycotoxin is receiving increasing attention worldwide as it poses health risk to human and animal (Abarca *et al*., 2004).

Phylogenetic analysis also showed that *A. ibericus* and *A. carbonarius* were separated from the rest of the members of *Aspergillus* section Nigri. These two black aspergilli are commonly isolated from vineyard soils (Klich & Pitt, 1988; Leong *et al.,* 2006a) and *A. carbonarius* has been reported to produce ochratoxin A in grapes (Leong *et al.*, 2006b) and coffee (Taniwaki *et al.*, 2003). Serra *et al.* (2006) reported that *A. ibericus* strains did not produce any ochratoxin A but they produced Naptho-γ-pyrones and pyranonigrin A.

In the present study, *A. aculeatus* isolates and the referral culture *A. aculeatus* CBS 172.66, an isolate which was recovered from tropical soil, formed a well-supported clade separated from the other biseriate black Aspergilli. *Aspergillus aculeatus* is an ubiquitous species and have been isolated from soil (Klich, 2002) as well as dried grapes from Australia (King *et al*., 1981), Egypt (Abdel-Sater & Saber, 1999) and Spain (Abarca *et al*., 2003).

All *A. tamarii* and *A. flavus* isolates were clearly separated from each other by forming separate clades. *Aspergillus flavus* is a saprophyte that degraded dead plant and animal tissues in the soil (Klich, 2002). It is also pathogenic to animals and humans due to its small spores and its ability to grow at 37°C. *Aspergillus flavus* is the most common cause of superficial infection and it is the second leading cause of invasive human aspergillosis (Hedayati *et al*., 2007). *Aspergillus tamarii* has been isolated from acidic tea field soils in Japan (Ito, 1998). In India, *A. tamarii* has been reported to cause keratitis (Kredics *et al.,* 2007), hence resulting it to be regarded as one of the important pathogens in eye infections along with *A. flavus*, *A. terreus*, *A. fumigatus*, and *A. niger*.

Aspergillus fumigatus, *A. sydowii* and *A. terreus* isolates were grouped in individual clades separated from each other. *Aspergillus fumigatus* is predominant agent of invasive pulmonary aspergillosis followed by *A. flavus, A. terreus,* and *A. niger,* but many other species have also been described in human infections. *Aspergillus sydowii* and *A. terreus* are cosmopolitan saprophytic fungi (Klich, 2002). *Aspergillus sydowii* caused aspergillosis not only in human but in invertebrates and bird (Alker *et al*., 2001; Hoog *et al*., 2000), and is also one of the important fungal pathogen of Caribbean sea fan corals (Alker *et al.*, 2001). *Aspergillus terreus* is also another cosmopolitan fungus (Klich, 2002) and is an opportunistic human pathogen, regarded as the third most important cause of human invasive aspergillosis (Balajee *et al*., 2007). *Aspergillus terreus* has been particularly associated with lethal infections (Hachem *et al.,* 2004; Lass-Flörl *et al*., 2000).

The presence of *Aspergillus* isolates in beach soil probably due to presence of discarding organic litter, environmental factors such as suitable temperature and humidity necessary for viability and survival of the fungus. The present study contribute

to the knowledge on the biodiversity of *Aspergillus* species particularly in the beach environment in Malaysia as well as contribute knowledge on the taxonomic relationship of *Aspergillus* species.

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