Preliminary study of black aspergilli inhabiting *Piper* rhizosphere from Eka Karya Botanical Garden, Bedugul Bali

M Ilyas1,2*, D A Nurcahyanto1, Y Yuliani1, W Mangunwardoyo2, and I Hidayat3

1Microbiology Division, Research Center for Biology - Indonesian Institute of Sciences (LIPI) Jl. Raya Jakarta-Bogor Km. 46, Cibinong, West Java, 16911- Indonesia
2Department of Biology, Faculty of Mathematics and Natural Sciences, University of Indonesia, Kampus UI-Depok, West Java, 16424 – Indonesia
3Research Center for Biomaterials - Indonesian Institute of Sciences (LIPI) Jl. Raya Jakarta-Bogor Km. 46, Cibinong, West Java, 16911- Indonesia

*Corresponding email: ilyasmould@yahoo.com

Abstract. The black aspergilli (*Aspergillus* section *Nigri*) occupy a broad spectrum of habitat, and they are an essential group of species in food mycology, medical mycology, and biotechnology. This study aimed to isolate and identify the black aspergilli inhabiting five samples of *Piper* rhizosphere collected from Eka Karya Botanical Garden, Bedugul Bali. In this study, 71 strains of black aspergilli were isolated, and 20 strains of them have selected and characterized using morphological, molecular, and phylogenetic analysis. Morphological data results are useful for preliminary identification, but they did not have been totally effective in describing and elucidating 20 selected strains into species level. Further molecular identification and phylogenetic analysis based on ITS rDNA showed that from 20 selected strains, four strains were identified as *Aspergillus aculeatus* sensu stricto, three strains were identified as *Aspergillus japonicus* sensu stricto, four strains were identified as *Aspergillus niger* sensu lato, and nine strains were identified as *Aspergillus tubingensis* sensu stricto. Preliminary exploration of fungi through isolation and identification was the first step to discover the potential strains that able to produce the promising metabolites and bioactive materials. The study results are expected to be the initial microbial resources and information for screening and utilizing the black aspergilli through bioprospecting.

Keywords: *Aspergillus* section *Nigri*, black aspergilli, identification, isolation, ITS rDNA

1. Introduction

The black aspergilli or *Aspergillus* section *Nigri* sensu Gams et al (1985) is a subgroup of *Aspergillus* with black-spored aspergilli. The black aspergilli’s main habitat is in the soil, although many of them have been isolated from various substrates [1-3]. The black aspergilli have a significant impact on the modern human socio-economy, especially in biotechnology, food mycology, and medical mycology [4-6]. Through the fermentation industry, the black aspergilli were used to produce an assemblage of commercially utilized enzymes such as amylase and lipase and other secondary metabolites such as citric acid and gluconic acid [7-9]. Despite their economic importance, the black aspergilli are also responsible for some the food spoilage and biodeterioration. Several black aspergilli strains are known...
to produce mycotoxin, i.e. ochratoxin A (OTA), which often contaminate agricultural products and wine [10-12].

The black aspergilli are one of the more challenging groups regarding classification and identification since they have many variations in morphological and physiological characteristics [5,13,14]. The morphological characteristics such as color, shape, size, and conidial ornamentation are still useful for describing the strains [1,15]. However, this approach are only useful as an initial identification to grouping the black aspergilli into two major groups, i.e., uniseriate and biseriate black aspergilli. Moreover, identification based solely on morphological characters cannot be applied in the cryptic and or complex species of black aspergilli [8,16]. Fungal identification with another approaches such as molecular and phylogenetic analysis is necessary for a better results in determining the identity of the black aspergilli [5,13,14].

An assemblage of molecular identification approaches were used to identify and classify the black aspergilli. These methods included the analysis of restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), polymerase chain reaction-RFLP (PCR-RFLP), and DNA sequencing at specific loci [5,17]. Molecular identification based on the DNA sequencing analysis is the most widely used because of its accuracy and effectiveness in identify and classify the black aspergilli. Sequencing analysis for identifies the black aspergilli generally conducted by multilocus analysis with the primary amplification target ITS DNA, ß-tubulin, and calmodulin loci [8,14,17,18].

More than 50% of the black aspergilli species are found in the tropics, but most of the described species are from subtropics [19]. The genus Piper are plants that have major distribution in the tropics. Piper plants have a high diversity with more than 1000 species [20-21]. There are many microorganisms that have closely related and associations with the plant rhizosphere. Fungal rhizosphere such as Aspergillus, Fusarium, Penicillium, and Trichoderma are frequently reported as plant growth-promoting fungi (PGPF) because the interactions between fungal rhizosphere and the plant will induce the plant resistance to the diseases and improving the plant growth [22]. The previous study of fungal isolation from rhizosphere samples of black pepper (Piper nigrum L.) reported that 14 fungal taxa were isolated, and the fungi were dominated by the genus Aspergillus [23]. This study was conducted with the objective to isolate and identify the black aspergilli inhabiting five Piper rhizosphere samples collected from Eka Karya Botanical Garden, Bedugul Bali. The fungal identification will be carried out through the morphological, molecular, and phylogenetic analysis.

2. Materials and Methods

2.1. Sample materials

The sample material consists of soil and rhizosphere samples of Piper plant were collected from Eka Karya Botanical Garden, Bedugul Regency, Bali Province, Indonesia. Sampling technique was conducted based on the purposive random sampling method. The soil and rhizosphere samples were collected from 15-20 cm depth around the Piper plantation using a sterilized hand shovel. Total of five soil and rhizosphere samples were collected from five different Piper species (Table 1). All the soil and rhizosphere samples were packed carefully in the 1kg size black polyethylene bag, marked, and then transferred to the laboratory for isolation purposes within less than 72 hrs.

2.2. Fungal isolation

The fungal isolation was carried out based on the serial dilution method with slight modification [24]. The fungal isolation was carried out with the following steps; 10 g of rhizosphere samples were diluted into 90 ml of distilled water, shake the sample suspension by vortexing (Preparation I, 10^{-1} dilution). Using the micropipette, put 1 ml of preparation I into 9 ml of distilled water, then shake by vortexing (Preparation II, 10^{-2} dilution). Repeat the next dilution process with the same steps until the sample dilution at 10^{-3} (Preparation III), 10^{-4} (Preparation IV), and 10^{-5} (Preparation V). Starting with the weakest dilution (10^{-5}), aseptically pipette 200 µL sample suspension, poured and then spread using Drigalski spatula on rose bengal chloramphenicol agar (RBCA) plate. Repeat this step until 10^{-3}, and
each dilution was made in three replications. Seal the plates, and then incubated cultures in the incubator with the temperature set at 27°C for 3-7 days. Observe the culture after 48 hours, record the number of fungal colonies/ plate/dilution, and then calculated the average of colony-forming unit (CFU)/ ml in each sample. The selected fungal strain then purified by picked up and transferred on PDA slant for working culture. For the back-up and long-term preservation, the selected strain was preserved in freezing and L-drying preservation method [25].

2.3. Morphology identification
Morphological identification was made by observing both macroscopic and microscopic phenotypic characters of the black aspergilli were grown on malt extract agar (MEA) and czapek yeast extract agar (CYA) after seven days incubation at 25°C. Macroscopic characterization includes observation of color, colony shape, surface, texture, exudates drop, and reverse color. For microscopic observation, fungal mycelia were mounted in one drop of 1% lactophenol blue solution. Microscopic characterization was conducted under a light microscope by observing hyphae, hyphae pigmentation, septate, vesicle, conidial color, shape, size, and ornamentation.

2.4. Molecular identification
The molecular identification by sequence analysis was based on the DNA sequence of an internal transcribed spacer (ITS1 and ITS2) of rDNA regions, includes the 5.8S rRNA. The fungal genomic were isolated using Nucleon PhytoPure (GE Healthcare) according to the manufacturer’s instruction. DNA amplification of the ITS rDNA region was performed by polymerase chain reaction (PCR). PCR amplification performed in 25-µl reaction mixtures containing 10 µl distilled water, 12.5 µl GoTaq Green Master Mix (Promega), 0.5 µl DMSO, 0.5 µl each primer (10 pmol), and 1 µl (5 to 10 ng) extracted genomic DNA as a template. The primer set of ITS4 (5`-GGAAGTAAAAGTCGTAACAAGG-3`) and ITS5 (5`-GGAAGTAAAAGTCGTAACAAGG-3`) was used to amplified approximately 550 nucleotides from ITS1 and ITS 2, including 5.8S rDNA [26]. Amplification performed in a TaKaRa PCR Thermal Cycler P650 (TAKARA BIO Inc.), programmed under the following conditions: initial denaturation at 95°C for 3 min, 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The PCR products then subjected to the purification and sequence analysis.

2.5. Phylogenetic analysis
The raw sequence data edited using ChromasPro version 1.5 program (http://www.technelysium.com.au/ChromasPro.html). The assembled sequences aligned with those downloaded from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) using the Muscle (http://www.ebi.ac.uk/Tools/msa/muscle). The phylogenetic analyses of sequence data conducted based on the neighbor-joining (NJ) method [27] using Molecular Evolutionary Genetics Analysis (MEGA) version 5.05 program [28]. The reliability of each branch was evaluated by bootstrapping with 1000 resampling in NJ [29].

3. Results and discussion
The black aspergilli and other fungal colonies were starting growth on rose bengal chloramphenicol agar (RBCA) plates after three days incubation at 27°C (Figure 1A). The average of colony-forming unit (CFU) of fungal colonies in each soil and Piper rhizosphere samples are ranged between 15–190 x10^3 CFU/ml. The highest fungal CFU/ml sample was obtained from P. betle L. rhizosphere, while the lowest fungal CFU/ml sample was obtained from P. bantamense Blumei rhizosphere sample. The average CFU/ml of fungal colonies on each sample was shown in Table 1.

**Table 1.** The average of fungal colony-forming unit (CFU)/ml in each rhizosphere sample
| No. | Sample code | Plant rhizosphere | Sample description | Sample pH | GPS coordinate | Altitude (m.alt) | Fungal colony-forming unit (CFU/ml x 10^3) |
|-----|-------------|-------------------|--------------------|-----------|----------------|-----------------|-----------------------------------------|
| 1   | P1Rh1       | Piper bantamense   |                    | 6.8       | S 08°16’490”  | 1378            | 15                                      |
|     |             | Blumeti           |                    |           | E 115°09’140”|                 |                                         |
| 2   | P2Rh2       | Piper nigrum L.   |                    | 5.5       | S 08°16’490”  | 1378            | 96,5                                    |
|     |             |                   |                    |           | E 115°09’140”|                 |                                         |
| 3   | P3Rh3       | Piper betle L.    |                    | 6.8       | S 08°16’490”  | 1371            | 190                                    |
|     |             |                   |                    |           | E 115°09’135”|                 |                                         |
| 4   | P4Rh4       | Piper sarmentosum |                    | 6.5       | S 08°16’472”  | 1374            | 145                                    |
|     |             | Roxb.             |                    |           | E 115°09’136”|                 |                                         |
| 5   | P5Rh5       | Piper macropiper  |                    | 6.8       | S 08°16’475”  | 1374            | 75                                     |
|     |             | Pennant           |                    |           | E 115°09’145”|                 |                                         |

**Figure 1.** Black aspergilli and other fungal colonies growth on RBCA isolation media (A); Sclerotia (arrow mark) formed on strain P18 were grown on MEA (B1), and strain P14 were grown on CYA (B2); Soluble pigment (orange color) formed around the fungal colony of strain P15 (C1) and P19 (C2) were grown on CYA.

The macroscopic and microscopic morphological characteristics were carried out on the black aspergilli were grown on malt extract agar (MEA) and czapex yeast extract agar (CYA) after seven days incubation at 25°C. Based on the macroscopic observation, among the 20 selected black aspergilli strains, the sclerotia only found in strain P18 were grown on MEA (Figure 1 B1), meanwhile, in the CYA, sclerotia were formed in black aspergilli strain P14 (Figure 1 B2), strain P15, P16, P18, and P20. In general, the sclerotia morphology are a globular, whitish or cream color, and diameter size 0.4-0.9 mm. Several black aspergilli species have been reported to produce sclerotia, such as; *Aspergillus aculeatus, A. ellipticus, A. carbonarius, A. costaricaensis, A. piperis, A. sclerotioniger, A. aculetianus* and *A. sclerotici carbonarius*. The black aspergilli *A. sclerotici carbonarius* was easily recognized by its yellowish-orange color sclerotia. The sclerotia in *A. tubingensis* was found only in a few strains, whereas in *A. niger*, the sclerotia were almost never found on well-known growth media [5,9,30]. The soluble pigment-containing metabolites indicated by the orange color was formed around the black aspergilli colony. The soluble pigments found in the black aspergilli strain P15, P16, and P19 were grown on CYA (Figure 1 C1, C2). Several fungal pigments containing metabolites were already used in the market, such as yellow pigment of citrinin produced by *Monascus purpureus*, the orange pigment of β-carotene, and lycopene produced by *Blakeslea trispora*, and `arpink red` a commercial colorant produced by *Penicillium oxalicum*. Water-soluble pigments produced by fungi are more suitable in the industry because easily extracted in mass-producing, cost-efficient, and environmentally friendly [31].
Figure 2. Representatif microscopic view of black aspergilli with the uniseriate (Strain P03) and biseriate conidial heads (Strain P16). Notes: A.ur-c = uniseriate radiate collumnar, A.br-c = biseriate radiate collumnar, Ana.s = anamorphic structures, St = stipe of conidiophores, Vs = vesicle, Mt = metulae, P = phialide, Cd = conidia, Bar (Scale bar = 10 µm).

The microscopic observation results of 20 representatives black aspergilli strains growing on MEA, seven strains of them have the uniseriate conidial heads, i.e., strain P03, P08, P09, P10, P12, P13, P14. Meanwhile, the rest of the 13 strains have the biseriate conidial heads, i.e. strain P01, P02, P04, P05, P06, P07, P11, P15, P16, P17, P18, P19, and P20. Representative strains with uniseriate and biseriate conidial heads were depicted in Figure 2. Microscopically, the black aspergilli fungi have dark-brown to black conidia, uniseriate or biseriate conidial heads, hyaline, or pigmented conidiophores, and vesicle structures which are generally spherical in shape [9,32]. The anamorphic structures of uniseriate strains have the ellipsoidal-shaped conidia, 4-5 µm in diameter, and echinulate ornamentation. The tip of conidiophores or vesicle size varies between 20-60 µm in diameter. The anamorphic structures of biseriate strains have the globular, subglobular, and or ellipsoidal conidia. The conidia size is 3-5 µm in diameter, fine spines (warty or verrucose) ornamentation, and vesicles size 30-60 µm in diameter. According to the conidial ornamentation, the black aspergilli can be distinguished into two main groups. The black aspergilli with echinulate conidia consist of *Aspergillus atrovioaceus*, *A. carbonarius*, *A. ellipticus*, *A. heteromorphus*, *A. helicothrix*, and *A. japonicus*, while the black aspergilli with warty or verrucose conidia consist of *A. fonsecaeus*, *A. acida*, *A. niger var. niger*, *A. niger var. phoenicis*, *A. niger var. ficuum*, *A. niger var. tubingensis*, *A. niger var. purvulurentus*, *A. niger var. awamori*, *A. citricus* (*A. foetidus*) and *A. citricus* var. *pallidus* [1,5]. However, in practice, many black aspergilli species, based on the morphological characteristics, are indistinguishable one and another, such as the morphology of *A. niger* vs. *A. awamori* [16], the morphology of *A. niger* vs. *A. tubingensis*, *A. foetidus*, and *A. brasiliensis* which are known as 'aggregate', of *A. niger* species [13,33]. Another analysis such as molecular identification is absolutely necessary for valid results in determining the identity of the black aspergilli.

The query lengths of ITS nucleotides aligned online at NCBI (http://www.ncbi.nlm.nih.gov/) ranged from 550-580 base pairs. According to the BLAST result black aspergilli strain P01, P02, P11, and P17 have the highest similarity with *A. tubingensis* A1KA (AM 745112) with the homology range from 99-100%. Other black aspergilli strain P04, P05, P06, and P07 have the highest similarity with *A. niger* MACN2 (AM 745113) with 100% homology. The black aspergilli strain P03 and P14 have the highest similarity with *A. japonicus* A1.1 (EU 833207) with 100% homology. Meanwhile, the black aspergilli strain P08, P09, P12, P13, and P14 have the highest similarity with *A. aculeatus* A1.9 (EU 833205) with 99-100% homology, while the P15, P16, P18, and P20 strains have the highest similarity with *Aspergillus* sp. r308 (HQ 649946) with 99-100% homology. The BLAST results showed that the 20 representative strains have high homology with the previously identified black aspergilli taxa with the 99-100% ITS rDNA homology. The ITS rDNA nucleotide differentiation were 1-2 bases with the gap
0-1 base pair. This high homology results did not fully indicate fungal species low independence since the ITS rDNA sequences on black aspergilli were conserved and many black aspergilli species have an identical ITS rDNA sequences. For example, the ITS rDNA sequence of *A. niger* was idenitic with *A. lacticoffeatus*. The ITS rDNA sequence of *A. tubingensis* was idenitic with *A. foetidus*, *A. vadensis*, and *A. piperis*, while the ITS sequence of *A. carbonarius* was idenitic with *A. sclerotioniger*, *A. japonicus*, *A. aculeatus*, and *A. uvarum* [5,14].

The phylogenetic tree of neighbor-joining (NJ) was constructed according to the ITS sequence data entry with the nucleotide length 550-580 base pairs. In total, 30 representative black aspergilli type strains and neotype strains were used as in-group and one strain, i.e., *A. flavus* CBS 100927T was used as the out-group. The phylogenetic analysis showed that the biseriate strain of black aspergilli i.e., P01, P02, P11, P15, P16, P17, P18, P19, and P20 (nine strains) are in the *A. tubingensis* sensu lato clade with a bootstrap value of 87%. Other biseriate strains, i.e., P04, P05, P06, and P07 (four strains), are in the *A. niger* 'aggregate' clade with the bootstrap value 98%. Meanwhile, the uniseriate black aspergilli strain i.e., P08, P09, P12, and P13 (four strains), were in the *A. aculeatus* sensu stricto clade with the bootstrap value 88%. Other uniseriate black aspergilli strain, i.e., P03, P10, and P14 (three strains) in the *A. japonicus* sensu stricto clade with the bootstrap value 65% (Figure 3).

![Phylogenetic tree of 20 representative strains of black aspergilli based on ITS rDNA sequence using the neighbor-joining (NJ) method and *A. flavus* as out-group. Phylogeny test with a bootstrap value of 1000 resampling and only bootstrap values> 50% were listed.](image)

According to Figure 3, the NJ analysis resulted in the phylogenetic tree with the two main clades, i.e., the biseriate clade of *A. niger* sensu lato and uniseriate clade of *A. aculeatus* sensu lato. The biseriate clade of *A. niger* sensu lato consists of two main subclades, i.e., *A. tubingensis* subclade and *A. niger* 'aggregate' subclade. The subclade of *A. tubingensis* consists of several black aspergilli species with the *A. tubingensis* identical ITS sequence such as *A. foetidus*, *A. vadensis*, and *A. piperis* [5,14]. Meanwhile, in the subclade *A. niger* 'aggregate’, the black aspergilli species, *A. awamori* was also found in the clade.
According [16], A. niger and A. awamori are known as cryptic species. The uniseriate clade of *A. aculeatus* sensu lato showed the two slight branches, i.e., *A. japonicus* and *A. aculeatus* subclades. The slight and discrete branching of the two subclades is possible since the *A. japonicus*, *A. aculeatus*, and *A. awarum* have an identical ITS sequences [5,14].

4. Conclusion

In total, 71 strains of black aspergilli (*Aspergillus* section *Nigri*) were isolated from five samples of *Piper* rhizosphere growing at Eka Karya Botanical Garden, Bedugul Bali. Twenty representative strains have been identified based on morphological, molecular, and phylogenetic analysis. Morphological observation on MEA and CYA have been identified *Piper* strains into variations.

However, the morphological identification results cannot identify the 20 representative strains into species level. Further molecular identification approach based on ITS rDNA sequence and phylogenetic analysis using the neighbor-joining (NJ) method showed that from 20 selected strains; four strains of them (P08, P09, P12, and P13) were identified as *Aspergillus aculeatus* sensu stricto, three strains (P03, P10, and P14) were identified as *Aspergillus japonicus* sensu stricto, four strains (P04, P05, P06, and P07) were identified as *Aspergillus niger* sensu lato and nine strains (P01, P01, P02, P11, P15, P16, P17, P18, P19, and P20) were identified as *Aspergillus tubingensis* sensu stricto.

Acknowledgments

This study was partially funded by The Indonesian Ministry of Research and Technology and DIPA project of Research Center for Biology, Indonesian Institute of Sciences (LIPI).

5. References

[1] Kozakiewicz Z 1989 *Myc. Papers* 161 1-188
[2] Abarca M L, Accensi F, Cano J and Cabanes F J 2004 *Antonie van Leeuwenhoek* 86 33-49
[3] Samson R A, Houbraken J A M P, Kuijpers A F A, Frank J M and Frisvad J C 2004 *SMYCA2* 50 45-61
[4] Bennet J W and Klich M A 1992 *Aspergillus: Biology and Industrial Application* (Boston: Butterworth-Heinemann)
[5] Samson R A, Noonim P, Meijer M, Houbraken J, Frisvad J C and Varga J 2007 *SMYCA2* 59 129-145
[6] Varga J, Frisvad J C, Koscube S, Brankovics B, Toth B, Szigeti G and Samson R A 2011 *SMYCA2* 69 1-17
[7] de Vries R P, Burgers K, van de Vondervoort P J I, Frisvad J C, Samson R A and Visser J 2004 *Appl. Environ. Microbiol.* 70 3954-3959
[8] Howard S J, Harrison E, Bowyer P, Varga J and Denning D W 2011 *Antimicrob. Agents Chemother* 55 4802-4809
[9] Silva D M, Batista L R, Rezende E F, Fungaro M H P, Sartori D and Alves E 2011 *Braz. J. Microbiol* 42 761-773
[10] Varga J, Kevei E, Rinyu E, Teren J and Z. Kozakiewicz Z 1996 *Appl. Environ. Microbiol.* 62 4461-4464
[11] Cabanes F J, Accensi F, Bragulat M R, Abarca M L, Castela G, Minguéz S and Pons A 2002 *Int. J. Food Microbiol.* 79 213-215
[12] Perrone G, Varga J, Susca A, Frisvad J C, Stea G, Koscube S, Toth B, Kozakiewicz Z and Samson R A 2008 *Int. J. Syst. Evol.* 58 1032-1039
[13] Parenicova L, Skouboe P, Frisvad J, Samsons R A, Rossen L, Hoor-Suykerbuyk M and Visser J 2001 *Appl. Environ. Microbiol.* 67 521-527
[14] Varga J, Koscube S, Toth B, Frisvad J C, Perrone G, Susca A, Meijer M and Samson R A 2007 *Int. J. Syst. Evol.* 57 1925-1932
[15] Samson R A, Houbraken J A M P, Kuijpers A F A, Frank J M and Frisvad J C 2004 *SMYCA2* 50
45-61

[16] Perrone G, Stea G, Epifani F, Varga J, Frisvad J C and Samson R A 2011 *Fungal Biol*. 115 1138-1150

[17] Geiser D M, Klich M A, Frisvad J C, Peterson S W, Varga J and Samson R A 2007 *SMYCA2* 59 1-10

[18] Peterson S W 2008 *Mycologia* 100 205-226

[19] Domisch K H, Gams W and Anderson T H 1980 *Compendium of Soil Fungi* (London: Academic Press)

[20] Mabberley D J 1987 *The Plant Book* (Cambridge: Cambridge University Press)

[21] van Steenis C G G J 1988 *Flora* 5th ed (Jakarta: PT Pradnya Paramita)

[22] Hyakumachi M and Kubota M 2003 Fungi as plant growth promoter and disease suppressor. *Fungal Biotechnology in Agricultural, Food and Environmental Application* ed Arora D K (New York: Marcel Dekker) pp 101-110

[23] Noveriza R and Quimio T H 2004 *Indones. J. Agric. Sci.* 5 1-10

[24] Ilyas M, Rahmansyah M and Kanti A 2006 *Seri Panduan: Teknik Isolasi Fungi* (Jakarta: LIPI-Press)

[25] Kanti A, Ilyas M, Nurkanto A, Sulistiyani T R and Meliah S 2018 *Panduan Pengelolaan Koleksi Mikroorganisme InaCC* (Jakarta: LIPI-Press)

[26] White T J, Bruns T D, Lee S B and Taylor J W 1990 Amplification and direct sequencing of fungal RNA genes for phylogenetics. *PCR protocols* ed M A Innis, D H Gelfand, J J Sninsky and T J White (New York: Academic Press) pp 315-322

[27] Saito N and Nei M 1987 *Mol. Biol. Evol.* 4 406-425

[28] Kumar S, Nei M, Dudley J, and Tamura K 2008 *Brief. Bioinform.* 9 299-306

[29] Felsenstein J 1985 *Evolution* 39 783-791

[30] Frisvad J C, Petersen L M, Lyhne E K and Larsen T O 2014 *PLOS One* 9 e94857

[31] Heo Y M, Kim K, Lwhe E K and Larsen T O 2014 *PLOS One* 9 e94857

[32] Klich M A 2002 *Identification of Common Aspergillus Species* (Utrecht: Centraalbureau voor Schimmecultures)

[33] Ferracin L M, Frisvad J C, Taniwaki M H, Imanaka B T, Sartori D, Schapovaloff M E and Fungaro M H P 2009 *Braz Arch Biol Technol* 52 241-248