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Molecular and morphological characterization of fungi isolated from nutmeg (*Myristica fragrans*) in North Sulawesi, Indonesia

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Abstract. *Arifah F, Aini LQ, Muhibuddin A. 2023. Molecular and morphological characterization of fungi isolated from nutmeg (*Myristica fragrans*) in North Sulawesi, Indonesia. Biodiversitas 24: 441-453.* Nutmeg (*Myristica fragrans* Houtt) is one of the predominant commodities from Indonesia exported to the EU market. However, it is frequently rejected due to mycotoxin (especially aflatoxin) content exceeding the aflatoxin standard set by the EU. The high levels of aflatoxin were hypothesized to be produced by a toxigenic fungus infecting nutmeg during and after harvest. The study aims to determine and identify fungal contaminants in nutmeg kernels and provide information on the types of mycotoxins produced. Ten fungal morphotypes were isolated from nutmeg kernels collected from North Minahasa, North Sulawesi, Indonesia, and were grouped into four genera (*Aspergillus*, *Lasiodiplodia*, *Rhizopus*, and *Penicillium*) based on morphological characters. Ten fungal morphotypes were identified, i.e., *Aspergillus flavus* (Groups I and II), *A. tamarii* (Groups I and II), *A. niger*, *A. aculeatus*, *A. ochraceus*, *Lasiodiplodia* sp., *Rhizopus delemar*, and *Penicillium* sp. Isolates of *A. flavus* and *A. tamarii* were divided into two groups based on slight differences in their colony appearances. Molecular identification using homology of 18S rDNA sequences and ITS regions supports morphological identification except for isolates of *A. flavus* (Groups I and II), *Lasiodiplodia* sp., and *Penicillium sp.* These isolates were identified as *A. nomiae* and *A. aflatoxiformans* (*A. autswikcii)*, *L. theobromae*, and *P. citrinum*. The maximum likelihood phylogenetic tree showed that isolates from the same species were grouped in the same clade. The *L. theobromae* was the most dominant type of fungus in the nutmeg kernels (55.61%), followed by *A. niger* (23.8%) and *A. tamarii* (10.53%). It is the first report of *A. aflatoxiformans* or *A. autswikcii* in nutmeg kernels, and no incidence of *L. theobromae* in nutmeg kernels from Indonesia previously.

Keywords: *Aspergillus*, fungi, ITS, morphology, *Myristica fragrans*, phylogenetic analysis

INTRODUCTION

The nutmeg (*Myristica fragrans* Houtt) cultivated in Indonesia is exported mainly to the European Union (EU) market. Vietnam, the United States, the Netherlands, Germany, and Italy are the main export markets for Indonesian nutmeg (in terms of volume). Indonesia is the world's largest exporter of nutmeg, covering 75% of the global market. In 2018, the total amount of nutmeg exported was 20,202 tons, with a value of USD 111.69 million, equivalent to Rp 1.62 trillion (USD 1 = Rp 14,500 at the current exchange rate) (Directorate General of Estate Crops 2019; World Bank 2018). North Sulawesi is one of the centers of nutmeg producers in Indonesia, with a total yield of 18% (5.201 tons) of the total national nutmeg production (Directorate General of Estate Crops 2019).

North Sulawesi has a tropical climate with a humidity range of 77-90%, with temperatures ranging from 21.3°C to 25.8°C throughout the year (Statistic of Minahasa District 2021), physical variables (e.g., environment and climate), pathogen virulence, and improper agricultural practices determine the intensity of fungi contaminate food and food products (Hedayati et al. 2007; Muhibuddin, 2008; Muhibuddin et al. 2011; Nurtjahja et al. 2018). Fungal contamination of nutmeg kernels can occur during storage, transportation, and marketing throughout the supply chain (Dharmaputra et al. 2015). Fungal contamination by *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. has become a global concern, mainly since nutmeg kernels are imported and exported worldwide (Eskola et al. 2020). Fungal contamination of nutmeg reduces the quality and nutritional value of the nutmeg kernels and contributes to mycotoxin contamination. Mycotoxin interferes with human and animal health because mycotoxin can prevail in the food chain (Ráduly et al. 2020; Schrenk et al. 2020).

Mycotoxin (namely, aflatoxin) contamination is one of the significant issues in nutmeg exports from Indonesia. The maximum limit for aflatoxin B1 in nutmeg is 0.5 µg/kg, whereas the total aflatoxin (B1, B2, G1, and G2) is 10 µg/kg (EU 2016). During 2016-2019, there were 39 rejection cases of Indonesian nutmeg by the EU, which were published through the RASFF (Rapid Alert System for Food and Feed) and Notifications of Non-Compliance (NNC) submitted to the Indonesian Agricultural Quarantine Agency (IAQA). The nutmeg was rejected because its aflatoxin content exceeded the EU aflatoxins standard (IAQA 2019-2020). The high levels of aflatoxin were hypothesized due to toxigenic fungi infecting nutmeg during and post-harvest. As a result, accurate species identification and characterization of the contaminating

fungus from nutmeg kernels are critical because they will provide information on the types of mycotoxin production.

Studies on fungal identification that infects *M. fragrans* kernels are still limited. Recently there has been a report on fungi in stored nutmeg kernels. Fungal identification could be performed using conventional approaches (Dharmaputra et al. 2015) based mainly on morphological characteristics such as conidiophores' shape and each species' conidial dimension. However, morphological characters have limitations, making the classification difficult, and may not always perform well. Furthermore, the morphological characteristics have limitations and could not be utilized as the only feature for classification or identification for lower-level (species) classifications (El Khoury et al. 2011; de Hoog et al. 2017; Larena et al. 2018). Moreover, fungal identification based on morphology alone can be challenging, especially when non-experts are dealing with fungal cultures since there are a limited number of morphological characters that can be used for identification up to the species level (Raja et al. 2017).

Species-level identification was carried out using DNA barcoding the Internal Transcribed Spacer (ITS) region method as the official barcode for fungi by the mycologist consortium (Schoch et al. 2012). In addition, DNA barcoding compares unknown sequences to sequence databases, such as International Sequence Database in GenBank has been employed to identify species based on sequence similarity (Raja et al. 2017; Adeniyi et al. 2018). Therefore, fungal identification at the species level could be carried out by morphological and molecular identification beneficial for various fungal identification (Ezeonuegbu et al. 2022). Therefore, the current study used several methods to identify seed-borne fungi associated with nutmeg kernels, including micro- and macromorphology assays, molecular detection using ITS, homology of 18S rDNA sequences, and phylogenetic analysis.

MATERIALS AND METHODS

Sample collection

Ten kilograms of ripe nutmeg fruits from North Minahasa District, North Sulawesi Province, Indonesia, were collected in January 2022. The seeds were stored in sterile, tightly sealed plastic bags. The samples will be delivered to Surabaya for three days under moist conditions, and the kernels will be encased in their seed coats and mace. The seeds were sun-dried for ten days until their moisture content was 10%. Then, the kernels were manually peeled using a pestle. This experiment only used whole seeds. The nutmeg kernels were randomly collected to obtain 80 incremental samples.

Isolation and morphological identification of fungi

The modified direct plating method was used to isolate fungi on four different media (Pitt and Hocking, 2022). The media, including Water Agar (WA), V8 agar at 5% (V8), potato dextrose agar at 1/2 strength (PDA), and filter paper (FP), were used for fungal isolation from nutmeg kernels. The direct plating method was performed by placing sterilized nutmeg seeds on the media. The growth media (WA, 20gr/L dH_2O, V8 50gr/L dH_2O, PDA, 19,5 g/L dH_2O) were prepared following the manufacturer's instructions (WA and PDA; Merck, Germany, V8; Himedia, India), supplemented with 0,1 mg chloramphenicol to prevent bacterial contamination, and autoclaved (Hirayama, Japan) at 121°C and 15 psi for 15 min. The sterilized media were allowed to cool for 20 minutes before being transferred to Petri dishes Ø 90 mm. The Petri dishes were stored at four °C until inoculation. The direct plating method is a semi-quantitative method to determine the percentage of fungal-infected seeds. Each fungus's frequency of occurrence (F) was determined using the formula F = (NF/NT) x 100. F is the frequency of occurrence (%) of a fungus, NF is the total number of samples from which a specific fungus was isolated, and NT is the total number of samples from which isolations were performed (Iqbal and Saeed 2012).

Fungal isolation

Eighty nutmeg kernels were surface sterilized for 10 minutes with a 1% sodium hypochlorite solution and then washed three times with sterile distilled water. The kernels were sliced aseptically into 1.5-cm^2 pieces and placed on each sterilized FP, WA, PDA, and V8 medium (eight cut kernels per Petri dish). The uninoculated agar plate served as the control. The uninoculated and inoculated plates were incubated at 28 °C for seven days. The fungal growth and spore development were observed daily. After seven days of incubation, the fungal colonies from each growth medium were subcultured on newly produced PDA and incubated for an additional seven days to isolate and purify the fungi. This method follows Okayo et al. (2020) with some modifications. Fungal colonies were observed, counted, and identified using fungi identification keys based on morphological characteristics, such as mycelium structure and spore morphology (Barnett and Hunter 1998; Zheng et al. 2007; Baquião et al. 2013; Samson et al. 2014; Visagie et al. 2014; Frisvad et al. 2019). The modified slide culture technique was performed for microscopic examinations (Prakash and Bhargava 2016).

Molecular characterization

Fungal identification was verified using a molecular method. The Zymo Quick-DNA Fungal/Bacterial MiniPrep Kit (Zymo Research, D6005) was used to extract genomic DNA from fungus in line with the manufacturer's instructions. The universal primers for fungus species, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), were employed (Manter and Vivanco 2007). A 1% agarose gel (w/v) and a NanoDrop Spectrophotometer were used to assess DNA quality and quantity. The genomic DNA was adjusted to a final concentration of 20 ng/L and kept at -22°C for PCR amplification. PCR amplification using MyTaq HS Red Mix (Bioline) was done in a 25 µL reaction volume containing 2x MyTaq Red Mix, ten pmol of each primer, and 40 ng of genomic DNA. The amplification conditions were as follows: initial denaturation at 95°C for 3 min,

followed by 35 cycles of denaturation at 95°C for 10 sec, annealing at 52°C for 30 sec, extension at 72°C for 45 sec, and final extension at 72°C for 10 min. Several amplicons were submitted for Sanger sequencing by 1st BASE (Malaysia). The data was examined using BioEdit version 7.0.5.2 for manual evaluation (Tippmann 2004), BLAST, and MEGA 11. The acquired sequences were searched in the NCBI-BLAST database (http://www.ncbi.nlm.nih.gov/BLAST) (Raja et al. 2017). The programs of Molecular Evolutionary Genetics Analysis (MEGA) version 11 (Stecher et al. 2020; Tamura et al. 2021); the Maximum Likelihood (ML) method; and the Kimura 2-parameter model with bootstraps (BS) of 1000 replicates were used to determine the phylogenetic relationships between fungal species.

RESULTS AND DISCUSSION

Morphological identification

Morphological identification is accomplished through the observation of macroscopic and microscopic characteristics. The culture and growth characteristics of the 437 isolates are summarized in Table 1. These isolates were grouped into ten morphotypes based on the similarity of their cultural characters (Lacap et al. 2003; Samson et al. 2014; Visagie et al. 2014a,b; Vesth et al. 2018; Frisvad et al. 2019; Kowalski and Cramer 2020).

Among the 437 isolates, morphotype 4 was the most abundant species (243 isolates), representing 55.61% of the collected isolates (Table 2). It was followed by morphotypes 5, 3, 9,7, and 6. Other isolates were obtained with a percentage of less than 2%. In this study, 437 fungal isolates were collected from *M. fragrans* kernels, which were grouped into ten morphotypes of 4 genera: *Aspergillus*, *Lasiodiplodia*, *Rhizopus*, and *Penicillium*.

Aspergillus

Aspergillus is fungal species typically detected in *M. fragrans* kernels (Dharmaputra et al. 2015; Nurtjahja et al. 2018, 2019; Fendiyanto et al. 2021). There were seven morphotypes identified as *Aspergillus* with varying microscopic properties. The micromorphological structures of seven different morphotypes are shown in Table 3. The cultural characteristics of morphotypes 1, 2, 3, and 8 were similar to those of microfungal *Aspergillus* section *Flavi*. Among these isolates, morphotypes 1 and 2 were identified as *A. flavus*. Characteristics of the flavus group are yellow-green conidia, globose to subglobose vesicles, and uniseriate or biseriate seriations. The flavus group has relatively rapid growth.

Table 1. Cultural characteristics of the mycelia of the isolates (10 morphotypes on PDA)

Morphotypes	Size (mm) of colony[a]		Shape	Colony	Mycelium	Edge	Elevation	Texture
	3 days	7 days						
1	38,80	80,38	Circular	Yellow-green	White	Entire	Convex	Floccose
2	36,07	76,27	Circular	Yellow-green	White	Entire	Flat	Floccose
3	34,62	70,32	Concentric	Dark olive green	White	Undulate	Flat	Floccose
4	>C	>C	Filamentous	White	White-black	Entire	Flat	Wolly
5	39.94	>C	Round with raised margin	Black	White	Entire	Raised	Floccose
6	>C	>C	Rhizoid	Brownish-grey	White	Entire	Convex	Cottony
7	57,72	88.76	Circular	Blackish brown	White	Lobate	Raised	Floccose
8	33,83	67.40	Concentric	Yellow-green	White	Curled	Flat	Floccose
9	8,04	13,84	Irregular	Greenish grey	White	Undulate	Crateriform	Wrinkled
10	27,95	60,78	Concentric	Yellow-orange	Colorless-white	Entire	Raised	Floccose

Note: >C Completely covering plate. [a]An average of 3 colonies, in mm; PDA

Table 2. Morphotypes of isolated fungi from *M. fragrans* kernels from several growing media

Morphotypes	Number of isolates in media[a]				∑	Percentage (%)
	FP	PDA	WA	V8		
1	-	-	-	2 (1.25)	2	0.46
2	-	3 (1.88)	2 (1.25)	2 (1.25)	7	1.60
3	3 (1.88)	13 (8.13)	14 (8.75)	9 (5.63)	39	8.92
4	61 (38.13)	45 (28.13)	57 (35.63)	80 (50.00)	243	55.61
5	33 (20.63)	6 (3.75)	4 (2.50)	61 (38.13)	104	23.80
6	1 (0.63)	2 (1.25)	5 (3.13)	2 (1.25)	10	2.29
7	4 (2.50)	2 (1.25)	5 (3.13)	-	11	2.52
8	-	-	-	7 (4.38)	7	1.60
9	4 (2.50)	2 (1.25)	3 (1.88)	3 (1.88)	12	2.75
10	-	2 (1.25)	-	-	2	0.46
Total isolates	106	75	90	166	437	100

Notes: [a]Numbers in parenthesis showed the percentage of isolates

The diameter colony of the flavus group reaches about 76-80 mm in seven days with velvety colonies and floccose tufts, greenish-yellow conidia, with uniseriate or biseriate seriation (Figure 2). In general, the characteristics of *A. flavus* were similar to those of Klich (2002) and Samson et al. (2010). However, the *A. flavus* isolates were separated into two groups as the colony appearances were slightly different. Group I isolate grew optimally on the plate, with less compact yellow-green, floccose tufts and velvety colonies. Microscopically, vesicles are globose to subglobose with hyaline conidiophores. Group II isolates produced more compact yellowish-green colonies than group I, subglobose to pyriform vesicles, and hyaline rough conidiophores. Morphotypes 3 and 8 were identified as *A. tamarii* by morphological analyses. Morphotypes 3 and 8 have light green to olive green color colonies and appear floccose. Morphotype 3 has less floccose, more compact, and olive green to shade brown colonies than morphotype 8. Morphotype 3 grew slightly slower than morphotype 8, with a colony diameter reaching about 67 mm and 70 mm in seven days. Therefore, morphotype 3 was identified as *A. tamarii* group I. Morphotype 8 was recognized as *A. tamarii* group II with slightly different properties. Those morphotypes are difficult to discern due to their overlapping microscopical properties. The conidiophores vary in length and morphology (Table 3).

Morphotypes 5 and 7 were identified as *Aspergillus* section *Nigri*. Morphologically, morphotype 5 was identified as *A. niger* (F6_VE1.2) due to the presence of tints of black and brown in the colony. The colony expanded maximally and uniformly on the PDA after seven days of incubation. *A. niger* had colorless to slightly brown conidiophores, biseriate conidial heads, and compact conidia (2.57-4.22 µm (Figure 1). It was congruent with the descriptions of *A. niger* by Samson et al. (2010) and Varga et al. (2011). Morphotype 7 was identified as *A. aculeatus* (F8_BT4.2). The isolates were morphological characteristics with chocolate brown colonies and thick white mycelia underneath the colonies. *A. aculeatus* exhibited relatively strong development, displaying 88-90 mm on the PDA at seven days. Microscopically, *A. aculeatus* isolates have enormous uniseriate conidial heads but small conidiophore.

The colony morphology of morphotype 11 was related to the yellow *Aspergilli* (*Aspergillus* section *Circumdati*). Therefore, the isolate was identified as *A. ochraceus* (F11_PD14.2) (Figure 3). It is characterized by densely sporulating colonies and the formation of orange-brown sclerotia on PDA. The colony diameter after seven days of incubation on PDA at 27°C was 50-60 mm. The colonies were yellow-orange or caramel with colorless to white mycelia and lacked exudates and soluble pigmentation. The opposite color was yellow.

Lasiodiplodia

Morphotype 4 was identified as *Lasiodiplodia* sp. The isolate showed a fast growth rate on PDA. Within three days of isolation, the fungi consumed the entire PDA media. Initially, the colony's initial development produced white colonies, but they turned gray to black after 3 to 7 days. The fast expansion of aerial mycelium is followed by dense mycelium (Figure 4A). The fungus has a hyaline initial hypha that turns dark after ten days of inoculation. The chlamydospore was formed intercalarily (Figure 4B-D).

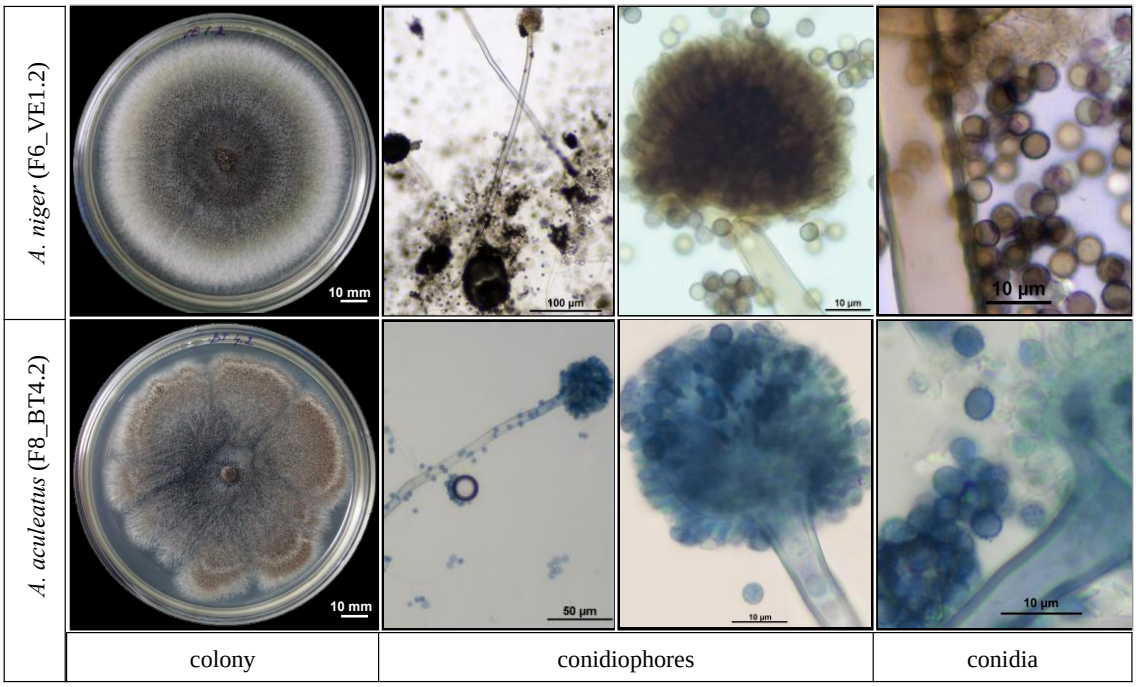

Figure 1. Cultures and microscopic examinations of *Aspergillus* section *Nigri* on PDA after seven days at 27°C. *A. niger* has smooth globose conidia and vesicles that biseriate. *A. aculeatus* was uniseriate with rough conidia and large vesicles

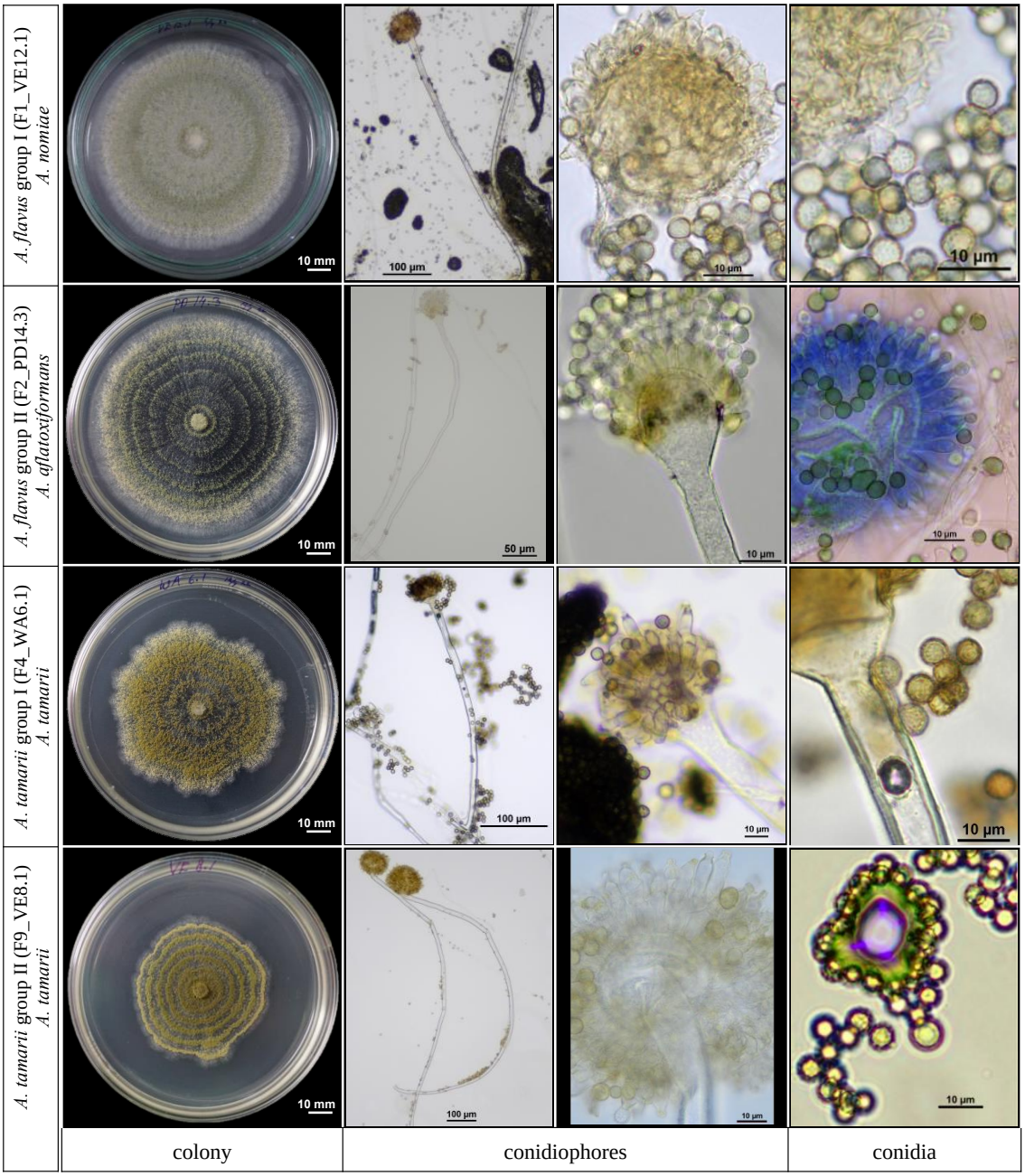

Figure 2. Cultures and microscopic examinations of *Aspergillus* section *Flavi* isolates on PDA after seven days at 27°C. The blue color indicates the presence of methylene blue staining

Table 3. Morphological characterization of isolates from genera *Aspergillus*

Morphotypes	Vesicles[a]		Conidia[b]			Conidiophore[c]		Identification result
	Shape	Diam. (µm)	Seriation	Shape	Diam. (µm)	Color	Dimension	
1	g-sg	29.94 - 32.57	u	g, r	3.69 - 4.50	h	318.29 - 541.93	*A. flavus* group I
2	sg-p	20.65 - 35.78	u	sg, s	2.91 - 4.48	h, r	212.11 - 557.37	*A. flavus* group II
3	g-sg	23.03 - 46.55	b	g-sg, r	3.93 - 5.88	h, r	503.42 - 808.75	*A. tamarii* group I
5	g	35.57 - 58.41	b	g, s-r	2.57 - 4.22	h-bw	721,26 - 844.89	*A. niger*
7	g	47.75 - 51.81	u	g, r	2.97 - 3.68	bw	460.69 - 481.78	*A. aculeatus*
8	g-sg	23.0 - 39.15	b	g, r	4.28 - 5.69	h	437.23 - 1035.50	*A. tamarii* group II
10	g	22.29 - 23.76	b	g, s-r	3.18 - 3.71	h-bw	379.31 - 621.70	*A. ochraceus*

Notes: [a] g: globose, sg: subglobose, p: pyriform. [b] u: uniseriate, b: biseriate, g: globose, sg: subglobose, r: rough, s: smooth. [c] h: hyaline, bw: brown, r: rough

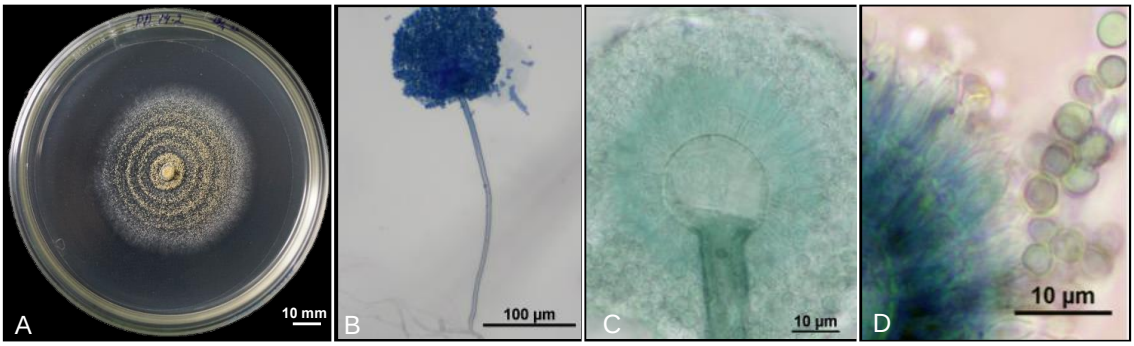

Figure 3. Macro- and micromorphology (after methylene blue staining) of *Aspergillus ochraceus* grown on potato dextrose agar plates for seven days of incubation at 27°C. (A) The colony as from the top view; (B) conidiophore; (C) vesicle, metula, and phialide; (D) conidia

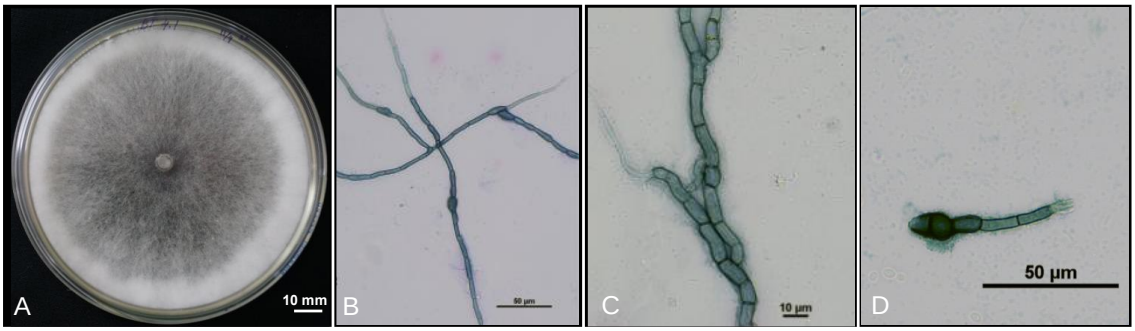

Figure 4. Characteristics of the *Lasiodiplodia* sp. (F5_BT4.1) grown on potato dextrose agar plates after three days of incubation at 27°C. (A) Colony from above view; (B) micromorphology of hyphae; (C-D) swollen hyphae after methylene blue staining (blue) and after Shear's solution (brown-black)

Rhizopus

Morphotype 6 has specific traits that match those of *Rhizopus delemar*. Generally, the phenotypic morphology was the same as that of Zheng et al. (2007). The colonies on PDA reached 90 mm in diameter within three days at 27°C. The initial colony is white and quickly turns deep gray to black at the upper portion or the entire portion. The appearance of the colony ranged from white to gray and created a blackish gray at maturity with a wooly texture (Figure 5A).

Under the microscope, stolons are well developed without septa, brown in color, and sometimes undulating with small protuberances. Chlamydospores were globose on mycelia, which were present in mass intercalary. The rhizoid's finger-like structure is grayish-brown to brown. Sporangiophores appeared to emerge solitary or in groups of two or three from stolons with opposite rhizoids or from mycelia without rhizoids (finger-like shaped) (Figure 5B). Sporangiophores are yellowish brown to brown, 325.64-853.28 µm in size, and aseptate. Sporangiophores were smooth, evenly distributed, slightly increasing at the apical section, often bulging, and typically behind the apophysis. Sporangiospores varied in morphology and size, ranging between 3.11 and 7.38 µm.

Penicillium

Penicillium has the following characteristics: moderately slow growth on PDA, colony diameter reaching about 13 mm in seven days, and white and greenish-grey colonies (Figure 6A). The colonies grew irregularly and were velvety in texture. The micromorphology of the isolates showed the presence of hyalin hyphae and septate. Conidiophores that were smooth-walled, 96.52-300 µm in length, and biverticillate were attached to the septate. Metulae were discovered in whorls of 3-5 divergent structures, 12.77-15.09 µm in size. Phialides were ampuliform and 6.72-9.51 µm in length. Conidia produced relatively lengthy chains, globose to subglobose in shape (1.98-2.64 µm dia.), and smooth (Figure 4B-D). Isolates with these traits were identified as *Penicillium* sp. (F10_BT7.3).

Molecular identification

Molecular identification was carried out to validate the morphologically identified fungus (Schoch et al. 2012; Raja et al. 2017). All fungal morphotypes were determined based on their 18S rDNA-ITS region sequence. Results of sequence analysis showed four genera of *Aspergillus*, *Penicillium*, *Lasiodiplodia*, and *Rhizopus* at the species level. An oligonucleotide segment of ITS-rDNA sequences was compared to their reference sequences. Based on nucleotide database pairing in GeneBank, there are several

recommendations on identification results, as represented by the list of species sorted by their scores, query covers, and maximum identities. Sequence analysis of the ITS regions showed a significant alignment of 99-100% with the previously isolated fungi. Table 4 shows the molecularly identified fungal species recovered from the *M. fragrans* kernels.

Diversities of fungal species in *M. fragrans* kernels were classified based on the rDNA-ITS region tree generated from ML phylogenetic tree analysis (Figure 7). Isolates of the same species were grouped into the same clade or sub-clade. For identification, the combined 34 taxa of the ITS sequences were used. According to phylogenetic analysis, F4 WA6.1 and F9 VE8.1 were in concordance with *A. tamarii* NRRL 20818 (RefSeq). The monophyletic clades of *A. tamarii*, *A. austwickii*, and *A. aflatoxiformans* were further separated at 78% bootstrap value, making both species in different clades. The phylogram successfully assigned isolate F1_VE12.1 to the same clade of *A. nomia*e containing strains NRRL 13137 (RefSeq) and SK1. This clade formed a monophyletic clade with high BS (96%). Isolate F11_PD14.2, F6_VE1.2, F8 BT4.2, F10_BT7.3, and F5_BT4.1 were confirmed as *A. ochraceus*, *A. niger*, *A. aculeatus*, *P. citrinum*, and *L. theobromae*, which formed monophyletic clades with BS 100%, respectively. The sequence obtained 100% matching with those sequences in the database, as indicated by the strong BS values. Therefore, the examined fungus presumably belonged to its respective genus and was firmly aligned with its species, as demonstrated by the strong sequence similarities with the said species. Isolate F7_VE4.1 was classified to the same clade as the two kinds of species, *R. arrhizus* ATCC 11145AF543519.1 and *R. delemar* UICC 9 LC514300.1. It is an intriguing occurrence that should be investigated further for taxonomic studies using morphological characters and a multigene approach to validate the identification of these isolates.

Table 4. Fungal species isolated from *M. fragrans* kernels with accession numbers of ITS sequences based on BLASTN homology searches

Morphotypes	Code	Species	Max % identity	Length	Score	Origin	Gen bank accession no.
1	F1_VE12.1	*A. nomiae*	99.49	618	1077	Indonesia	LC638671.1
2	F2_PD18.1	*A. austwickii*	100	982	1103	Nigeria	NR_171607.1
		A. aflatoxiformans	100	982	1103	Nigeria	NR_171606.1
3	F4_WA6.1	*A. tamarii*	99.83	603	1103	Netherlands	MH279435.1
4	F5_BT4.1	*Lasiodiplodia theobromae*	100	790	981	Malaysia	MK530016.1
5	F6_VE1.2	*A. niger*	99.83	638	1109	China	ON194328.1
6	F7_VE4.1	*Rhizopus arrhizus*	100	665	1166	Japan	AB109754.1
		R. delemar	100	665	1166	Indonesia	LC514331.1
7	F8_BT4.2	*A. aculeatus*	100	579	1068	China	MN088378.1
8	F9_VE8.1	*A. tamarii*	100	601	1109	India	OP526911.1
9	F10_BT7.3	*Penicillium citrinum*	99.46	568	1014	China	MN826202.1
10	F11_PD14.2	*A. ochraceus*	99.49	592	1077	China	OP237390.1

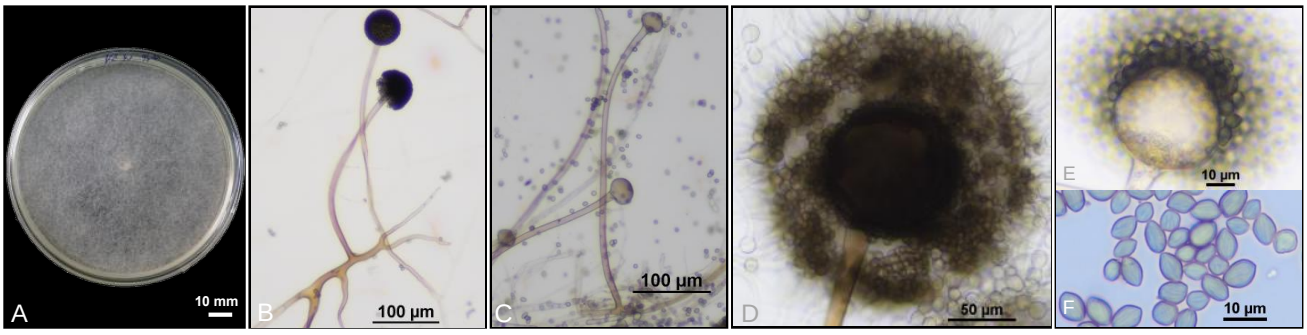

Figure 5. Macro- and micromorphology of *Rhizopus* sp. grown on potato dextrose agar plates after three days of incubation at 27°C. (A) The colony as from the top view; (B) a finger-like shaped rhizoid generates sporangiophores; (C) columella with distinct apophyses; (D-E) columella with apophyses; (F) irregular-shaped sporangiospores

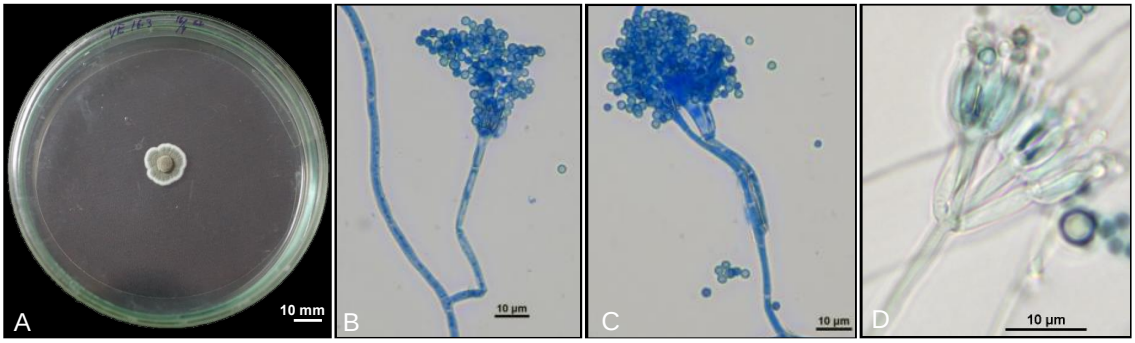

Figure 6. Macro- and micromorphology (after methylene blue staining) of *Penicillium* sp. grown on potato dextrose agar plates after seven days of incubation at 27°C. (A) Colony as from top view; (B-C) conidiophores were joined to the septate with conidia globose to subglobose; (D) biverticillate conidiophores

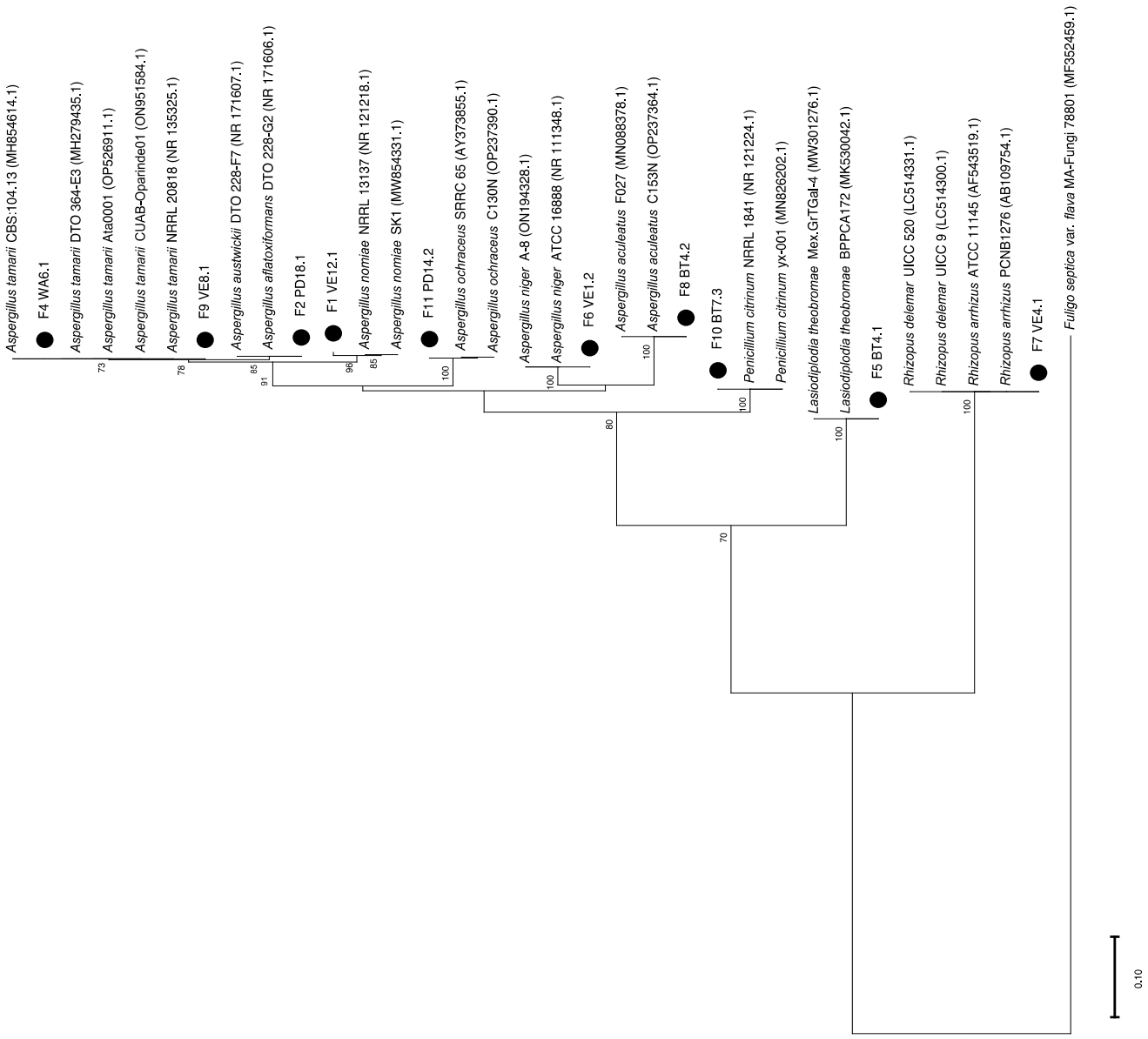

Figure 7. Phylogram of fungal isolates from nutmeg kernels and related species. The phylogram was derived from the ITS sequences' maximum likelihood analysis of 34 taxa. *Fuligo septica* var. *flava* MA-Fungi 78801 was used as the outgroup. The numbers above branches are bootstrap values. The branch lengths are measured in the number of substitutions per site. Bootstrap values ≥70% are displayed. The scale bar represents the expected number of nucleotide substitutions per site. The sequence of the fungal species obtained in this study is in the black circle

Discussion

Growth media including Malt Extract Agar (MEA), Sabouraud Dextrose Agar (SDA), Dichloran Rose Bengal Chloramphenicol Agar (DRBC), and Czapek Dox Agar (CZA) (Guchi et al. 2014; Thathana et al. 2017) and PDA (Diba et al. 2007; Thathana et al. 2017) have been previously applied. These media may provide an appropriate environment for fungal colony formation that supports the development of macroscopic and microscopic characteristics sufficient for the appraisal (Diba et al. 2007; Thathana et al. 2017; Khan et al. 2020). Morphological identification was performed in the current study using PDA as growth media. Isolates identified at the species level were *A. niger, A. aculeatus, A. ochraceus*, and *R. delemar*. *Aspergillus* section *Flavi* and *Penicillium* cannot be distinguished using morphological characteristics. The *Penicillium* and *Aspergillus* section *Flavi* isolates in nutmeg kernels had different characters, but the character discrimination was relatively difficult to recognize because of interspecific similarities. Current findings support those of Demirel et al. (2013), Samson et al. (2014), and Vesagie et al. (2014a), who reported that phenotypic identification (micro- and macromorphological traits) are subjective. Doubtful findings impede precise identification or misleading results. According to Frisvad and Samson (2004), the colonies in the species of the subgenus *Penicillium* had various phenotypic patterns. These patterns are consistent in freshly isolated cultures, but the typical features may be lost after transferring and maintaining the cultures regularly. Classifications and identifications based on any of those traits alone have been unsatisfactory, among others, because *Penicillium* consists of several taxa. The present results showed that the morphological investigations suggested that the isolated fungus belonged to the *Penicillium* genus and had green colonies of cotton texture. Although these conventional approaches for detecting *Penicillium* in the laboratory were time-consuming, morphological traits are still frequently employed. According to this research, a single medium PDA was only beneficial for identifying *Penicillium* at the genus level. This finding was corroborated by the research conducted by Bandh et al. (2011). Moreover, this approach is essential to classify the *Aspergillus* isolates according to groups or sections, which enables further identification by other methods.

A. flavus Grup I isolates were molecularly identified as *A. nomiae*. In this current work, phylogenetic analysis of ITS sequences demonstrated that isolates of *A. flavus* Group I exhibited 99.49% similarity with *A. nomiae* LC638671.1. In phylogenetic analysis, all the isolates were classified in the same clade. Only slight differences in colony appearance were observed between *A. flavus* and *A. nomiae*, of which *A. nomiae* (morphologically identified as *A. flavus* Group I) produced uniformly less compact, yellow-green, floccose tufts, and velvety colonies. According to Horn et al. (2011) and Tam et al. (2014), *A. nomiae* and *A. flavus* were macroscopically similar. Therefore, they might be misidentified as *A. flavus*, and several fungal isolates identified as *A. flavus* could be *A. nomiae.* Both isolates can be differentiated based on synnemata or synnemata-like production. However, synnemata were not observed in the present study. *A. flavus* isolates could produce synnemata or synnemata-like structures when grown on Czapek-Dox media but not for *A. nomiae* (Danmek et al. 2011).

The comparison of the nucleotide sequence of the ITS region between *A. flavus* Grup II isolates and the ITS dataset demonstrated similarities with *A. austwickii* and *A. aflatoxiformans*. In phylogenetic analysis, all the isolates were placed in the same clade as the reference strains. In this instance, comparing the nucleotide sequence of the ITS region between different fungi didn't produce a good differentiation because there was a relatively low degree of ITS variability. Schoch et al. (2012) discovered that while the ITS is the official DNA barcode for fungi, it does not always contain enough variation to distinguish between all *Aspergillus* species. Several studies have demonstrated that the ITS region alone cannot resolve species-level linkages, but it does suggest the likely number of species (Maharachchikumbura et al. 2021). Therefore, an *Aspergillus* ITS phylogeny requires a secondary barcode or identification marker to establish the absence of variation in particular clades to reliably identify an *Aspergillus* at the species level (Lücking et al. 2020). Frisvad et al. (2019) stated that phylogenetic analysis based on combined data sets of loci calmodulin (*CaM*), β-tubulin (*BenA*), or the RNA polymerase II second largest subunit (*RPB2*) could differentiate *Aspergillus* section *Flavi* species, such as *A. flavus, A. aflatoxiformans*, and *A. austwickii*.

A. austwickii and *A. aflatoxiformans* were two of the five most recently described *Flavi* species in 2019 by Frisvad et al. (2019). All three produced yellow-green and floccose-textured colonies. According to Frisvad et al. (2019), *A. flavus* and *A. aflatoxiformans* or *A. austwickii* might be classified depending on sclerotia formation and growth in extract sucrose agar (YES) culture. Most *A. flavus* strains exhibit L-type sclerotia, whereas S-type *A. flavus* strains emerge. The *A. aflatoxiformans* and *A. austwickii* generate S-type sclerotia solely. *A. aflatoxiformans* is closely related to *A. austwickii*, while *A. austwickii* grows slower on YES. Nonetheless, growth in YES was not observed in the present research.

The *A. aflatoxiformans* (described as a synonym for *A. parvisclerotigenus*) is a species that generates mycotoxins detected in various foods. In 2005, *A. aflatoxiformans* was reported for the first time as an isolate producing B and G aflatoxins in peanuts (Frisvad et al. 2005). In 2014, Perrone et al. (2014) reported acquiring *A. parvisclerotigenus* for the first time at low frequencies (1 out of 135 strains of collected *Aspergillus* Section *Flavi*) in the maize of Nigeria and Ghana. Ezekiel et al. (2013) detected *A. parvisclerotigenus* in dried edible mushrooms from Nigeria. In addition, the species has been obtained in sesame from Nigeria (Ezekiel et al. 2014), while *A. austwickii* has been present in stored rice grains and sesame kernels in Nigeria (Frisvad et al. 2019). In Indonesia, *A. aflatoxiformans* was obtained for the first time from rubberwood (Salman et al. 2020). Neither the identification of *A. aflatoxiformans* nor *A. austwickii* from nutmeg kernels in Indonesia has been previously performed. So, it

is the first report. More detailed phenotypic (growth in YES) and molecular studies (using *BenA*, *CaM*, and *RPB2* markers) of the isolates are still needed to determine differences in phenotypic or molecular that have not been assigned to a species in this study.

A. tamarii groups I and II were identified as *A. tamarii* by molecular analyses. Using the standard NCBI BLASTn tool, the F4_WA6.1 and F9_VE8.1 ITS sequences were identical to the sequences from the *A. tamarii* NRRL 20818 reference sequence. All the isolates were sorted into the same clade in the phylogenetic tree. The two colonies were slightly different in terms of color. Group I produced colonies that were uniformly dark olive green to shade brown, whereas Group II produced colonies that were light to olive green. Group II created dark green-brown conidia at maturity with floccose tufts and displayed phenotypic morphology that was the same as that of the description by Klich (2002).

The molecular identification of *A. ochraceus* isolates was validated by morphological identification. In addition, the *A. ochraceus* isolates exhibited 99.49% similarity with *A. ochraceus* OP237390.1, and in phylogenetic analysis, all the isolates were classified in the same clade. The colony grew from white mycelium to a light yellow pigment ranging from dull to dark yellow. The *A.ochraceus* had biseriate sterigmata and yellow or ochraceus conidial heads with tiny, thin-walled, and smooth. It was consistent with Visagie et al. (2014b). Similar traits were reported by Diba et al. (2007) and Nyongesa et al. (2015). The phylogenetic relationships of *Rhizopus* strains isolated from nutmeg kernels were examined using the DNA sequences of the rDNA ITS. The isolates had the same DNA sequence as ITS and were grouped into the same clade in the ML tree (Figure 7). The monophyletic clades with *R. delemar* and *R. arrhizus* (synonym *R. oryzae*) had 100% bootstrap value, so the relationship between molecular phylogeny and morphological taxonomy is quite complicated. The isolates *R. delemar* F7_VE4.1 shared precisely the same sequence with *R. delemar* and *R. arrhizus* in this study, although they are divided at the species level in the current taxonomy. The presence of that clade could be due to ancestral polymorphism or homoplasy rather than ongoing gene flow. Two cryptic species matching groups, *R. arrhizus* and *R. delemar*, may be detected (Abe et al. 2007).

Morphological observations (Zheng et al. 2007; Hartanti et al. 2015, 2020) and organic acid production (Abe et al. 2007) could differentiate *R. delemar* and *R. arrhizus*. *R. delemar* had sporangiospores that varied in shape and size, and the sporangiophores at the apical were swollen. *R. arrhizus* has sporangiospores that are uniform in shape and size (Zheng et al. 2007). The *R. delemar* was a fumaric-malic acid producer, while *R. arrhizus* was a lactic acid producer (Abe et al. 2007). However, organic acid production was not observed in the present study. Morphological analysis validated the *Rhizopus* isolates recovered from nutmeg kernels as *R. delemar* (Figure 7). In this study, colonies of *R. delemar* F7_VE4.1 on PDA had white to deep gray. The rhizoids underwent evolution into finger-like structures. *R. delemar* had sporangiophores emerge from rhizoids (finger-like structures), either individually or in clusters of two or three. These properties were typical of the descriptive taxonomic keys by Zheng et al. (2007) and the characteristics of *R. delemar* previously recorded by Hartanti et al. (2015, 2020).

Due to interspecific similarities, the morphological difference is inadequate to differentiate many *Penicillium* species (Frisvad and Samson 2004). The current study showed that identification to a certain level was complicated since the species share many features. In the present study, *Penicillium* sp. isolates were molecularly identified as *P. citrinum*. Molecular data indicated that the isolates had 99.46% similarity with *P. citrinum* MN826202.1. The phylogenetic analysis of ITS sequences showed that *Penicillium* sp. isolates from nutmeg kernels formed monophyletic clades with 100% BS value using *P. citrinum* NRRL 1841 (RefSeq) and strain yx-001. Therefore, ITS sequences can differentiate *P. citrinum*, corroborating with morphological identification.

Penicillium is well known for its ability to produce various chemical compounds that exhibit numerous biological properties. For example, a study by Firdausi et al. (2021) showed that *P. citrinum* inhibits 60.04% of the growth of *Alternaria porri* due to antifungal metabolites, i.e., citrinin and emodin. Citrinin mycotoxins impede the development of diseases caused by the genus *Alternaria*. Emodin antifungal causes damage to cell membranes and changes in the structure of hyphal cells, including additional lysis, swelling, deformation, loss of hyphal color, and disruption of membrane permeability.

Molecular studies of the sequences of ITS clarified the species identification of *Lasiodiplodia* sp. isolated from *M. fragrans* kernels. BLAST studies confirmed that the isolates had 100% sequence homology to *L. theobromae* MK530016.1. Based on the phylogenetic tree, the sequences of the isolates from the current investigation were aligned with reference *L. theobromae* strains BPPCA172 and Mex. GrTGal-4 formed a monophyletic clade with a high BS (100%). In this study, *L. theobromae* was the most dominant fungal species isolated from the nutmeg kernels, accounting for 55.61% of the total isolates. In Indonesia, *L. theobromae* was reported for the first time as causing agent of gummosis disease in *Citrus* spp. (Henuk et al. 2017). The fungus was related to stem cancer in rubber plants (Febbiyanti et al. 2018; Salman et al. 2020). *L. theobromae* has been isolated and identified from citrus, cocoa, rubber, banana, and mangosteen plants (Sandra et al. 2021). Another study reported that *L. theobromae* in the nutmeg tree is associated with dieback disease in South Aceh (Susanna et al. 2020). Still, it has not yet been reported to be present in nutmeg kernels. As a result, *L. theobromae* would set a new record for house range distribution in Indonesia.

Aspergillus section *Nigri* was the most abundant among *Aspergilli*. The sections were found in nutmeg kernels, namely *A. niger* (23.80%) and *A. aculeus* (2.59%). A previous study by Fendiyanto et al. (2021) reported that *A. niger* is the most common fungal population that emerged from postharvest nutmeg seeds. According to Dharmaputra et al. (2015), all nutmeg kernels obtained from farmers and collectors in all sample locations in North Sulawesi

contained *A. niger*. In a previous study, Patty et al. (2022) identified *A. niger* as the predominant species in nutmeg kernels from Ambon. *A. flavus, Penicillium* sp., *R. stolinifer,* and *Trichoderma* sp., among others, were also isolated in the research by Patty et al. (2022).

The *Flavi* sections in this study included three species: *A. nomiae*, *A. aflatoxiformans (A. autswikcii)*, and *A. tamarii*. Among these species, *A. tamarii* had the highest population of fungi (10.53%) isolated from *M. fragrans* kernels, followed by *A. aflatoxiformans (A. autswikcii)* (1.60%) and *A. nomiae* (0.46%). In addition, *A. ochraceus* belonging to the section *Circumdati* had an occurrence of 0.46%. Previous studies have shown that members of *Flavi* and *Cicumdati* sections are common fungi that colonized nutmeg kernels (Dharmaputra et al. 2015; Nurtjahja et al. 2018; Patty et al. 2022).

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