MOLD DIVERSITY OF WREATHED HORNBILL (*Rhyticeros undulatus*) NEST IN MOUNT UNGARAN

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ABSTRACT

Wreathed Hornbill (*Rhyticeros undulates*) is known to build nests in three cavities where they managed to live and breed. This edifice is predicted to contain various molds needed to maintain micro-environmental steadiness. This study was aimed to identify molds diversity in the Wreathed Hornbill's nest, using samples collected from empty structure with no bird activity. The samples were obtained from the Kalisidi and Nglimut observation stations on two occasions, i.e., in 2016 and 2017. Furthermore, the samples comprised cover soil, wood and inner material, which were collected aseptically and placed in sterile ziplock plastic bags. These samples were then diluted in sterilized distilled water to attain 10^{-3} mg/mL, and subsequently inoculated on Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Czapek Dox Agar (CDA). The inoculants were incubated at 37 °C, followed by the observation of mold colony after the 11th day. The results identified seven and nine species of molds in the Kalisidi and Nglimut observation stations, respectively. The most abundant species was *Penicillium* sp. which was found in composted nest materials for the whole observation periods.

Keywords: mold diversity, Mount Ungaran, nest, *Rhyticeros undulates*, Wreathed Hornbill

INTRODUCTION

Wreathed Hornbill or *Rhyticeros undulauts* Shaw 1811 is a protected bird, which is categorized into Apendix II, according to the *Convention on International Trade of Endangered Spesies of Wild Fauna and Flora* (CITES). Appendix II lists "all species which although not necessarily now threatened with extinction may become so, unless trade in specimens of such species is subject to strict regulation". This is an indication that the species is tradable under certain conditions, e.g., for scientific research purposes (Rahayuningsih & Kartijono 2013). In addition, breeding and nesting occur during fruiting season. The Wreathed Hornbill selects nest location based on the availability of fruiting trees and a conducive environment (Rahman *et al.* 2019). The nest selection process is followed by the nest building process, which involves the use of existing cavities from other birds and cracked branches (Rahayuningsih *et al. 2017)*. Generally, the nest selection and building process are initiated in dry season, characterized by low humidity, which is suitable for breeding and protecting the eggs from parasites. (Supa-Amornkul *et al.* 2011). However, the *R. undulatus* in Khao Yai, Thailand is capable of completing the breeding process before the heavy rains.

The created nests provide a suitable physical environment, alongside microorganisms needed for the development of eggs and chicks (Rahayuningsih *et al.* 2017; Utoyo *et al.* 2017). In addition, the strength of the physical structure is ensured by building the inner and outer parts of the nest using different soil sources, while the outer cover comprises high mircroorganism diversity, including mold. There have been minimal studies on mold diversity in *R. undulatus* nests present on Mount Ungaran, although Supa-Amornkul *et al.* (2011) reported the importance of micro-fungus in breaking down wood and organic materials. This phenomenon is exploited in nest cavity expansion; hence the study aimed to determine the diversity of molds in *R. undulatus* nests on Mount Ungaran.

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MATERIALS AND METHODS

This research was an observational exploratory study on the diversity of mold present in the nest of *R. undulatus*. The identification process was conducted in the Microbiology Laboratory, Department of Biology, Universitas Negeri Semarang (UNNES). Samples were obtained from the Kalisidi observation station located in the Kalisidi Village, West Ungaran District, Semarang, and Nglimut observation station located in Nglimut Village, Gonoharjo District, Kendal District, Central Java. The first observation period was conducted in 2016, and the last observation period was conducted in 2017. Samples were taken at each observation period.

Nest Sampling

The nest sampling process was performed at the end of the nesting season, marked by the absence of female and juvenile birds. Samples were collected aseptically, using sterile pinset and spatula, and then placed in ziplock plastic bags. The collected samples included nest cover, internal wood and nest materials of each nest in the two observation periods. All of the prepared samples were then stored in a freezer at -20 °C, prior to further analysis. Three samples were collected at each observation period. Thus, our study collected six samples altogether for the two observation period.

Each of the six samples were mashed up aseptically, followed by taking 10 g of each sample and dissolving the 10 g of each sample by using 100 mL of distilled water. This mixture was then homogenized and kept as a sample solution, from which 10% was diluted to attain 10^{-3}. Subsequently, the inoculation step was performed by spreading 1 mL of the sample solution onto culture media, i.e., Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Czapek Dox Agar (CDA). The isolates were then incubated at room temperature for 3-7 days in an incubator. A microscope was used for identification purposes, followed by the observation of wide mold colony growth.

Sterilization and Medium Preparation

The culture media used were Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Czapek Dox Agar (CDA). Each of the culture media was measured for 250 mL and dissolved with distilled water. As much as 0.05 g of chloramphenicol was added into the culture media as antibacterial agent, followed by sterilization using an autoclave at 121 °C and 2 atm, for 15 min. Subsequently, the culture media were poured into different Petri dishes under aseptic conditions, stored inside the Biological Safety Cabinet (BSC), and allowed to solidify, before being placed in a media cooler.

Calculating and Identification of Mold Colony

The grown mold colonies were stained using lactophenol, prior to observation with a microscope at magnifications of 100x - 1,000x. Subsequently, identification was carried out based on the guidelines of mold morphological structure developed by Samson *et al.* (2019) and Robinson (2011) to determine the genus and species of the samples.

RESULTS AND DISCUSSION

Environmental conditions of the Kalisidi and Nglimut observation stations are presented in Table 1.

Table 1 Enviromental condition of the observation stations

Enviromental conditions	Kalisidi station	Nglimut station
Light intensity (C)	613-1,740	656-1,480
Soil pH	5.2-6.2	3.7-4.5
Soil humidity (%)	25-55	70-90
Air humidity (%)	71-75	75
Air temperature °C	29.4-29.9	28.9
Tree type	Dead tree (unidentified)	*Weinmannia fraxenia* (nuts)

Despite the similarities, there was a variation in light intensity, with Nglimut station amounting 656 C to 1,480 C, due to the presence of tighter and thicker canopies. High and low light intensities impacted the air temperature around the nest, which was relatively higher in Kalisidi station. Kalisidi station featured higher soil pH and lesser soil humidity (Table 1).

After reaching a diameter of 1.5 cm, the mold colonies were transferred into the subsequent culture medium, followed by staining and observation by a microscope for identification purpose. Mold colonies having a diameter less than 1 cm were re-incubated to ensure further growth for easier identification. Table 2 shows the relatively higher density of samples obtained in 2017, compared to those obtained in 2016.

Within the incubated conditions, the MEA culture medium was colonized by various molds, where four and seven species were observed in samples obtained in 2016 and 2017, respectively. On the other hand, two and seven mold species grew in the PDA culture medium, while two and four mold species were recognized in CDA culture medium.

Aspergillus was successfully identified as the only genera present in all medium, across both sampling years. Furthermore, *Aspergillus niger* occurred less frequently than *A. terreus*, despite the fact that *A. niger* is commonly found in general environment. Meanwhile, *Acremonium* sp. and *P. variabile* were both identified in the MEA medium of 2016, while *Rhizopus* sp. was only seen in the CDA medium of 2017. Based on the collection period, there was a possibility that the mold species abundance was affected by the growth medium applied (Mahadevan & Shanmugasundaram 2018).

These mold species generally grow better in the MEA culture medium, made from wheat extract, maltosa, long chain carbohydrate and glycerol. The next culture medium preferred by mold to grow is PDA and followed by CDA. In addition, the composition of MEA provides sugar and nutrients as a source of energy for molds and yeast (Aziz *et al.* 2018; Cvetkovic & Markov 2002). The pepton content in MEA functions as a nitrogen source, presents in higher amounts compared to other media. Pepton is important for amino acid synthesis, which is required in the production of various functional protein, cell structure and hyphae conformation (Wang *et al.* 2010) and is responsible for the relatively higher amount of essential amino acid in MEA, including triptophan and tyrosin. These are important components in the conformation of metabolic protein, and also during cell communication.

PDA media includes the semi synthetic variety, resulting from the natural and synthetic material (*dextrose* and agar) components. In addition, complex carbohydrate molecules have been identified as the main source of carbon in potato. Carbon is the base material for PDA production, needed for the growth of molds and yeast. PDA is used because of the vitamin and mineral contents required to support fungi growth. Also, the dextrose component in PDA provides additional sugar as an energy source.

Table 2 Types of mold grown in the three culture media

Species	2016			2017		
	PDA	MEA	CDA	PDA	MEA	CDA
Aspergillus sp.	√	√	√	√	√	√
Aspergillus niger	-	√	-	-	-	-
Aspergillus terreus	√	-	-	√	√	-
Acremonium sp.	-	√	-	-	-	-
Curvularia sp.	-	-	-	√	√	-
Fusarium sp.	-	-	-	-	√	√
Geotrichum sp.	-	-	-	√	√	-
Neosartorya fischeri	-	-	-	√	√	-
Penicillium sp.	-	-	√	√	√	-
Penicillium variabile	-	√	-	-	-	-
Rhizopus sp.	-	-	-	-	-	√
Trichoderma sp.	-	-	-	√	-	√
Total spesies	2	4	2	7	7	4

Notes: PDA = Potato Dextrose Agar; MEA = Malt Extract Agar; CDA = Czapek Dox Agar; 2016 and 2017 = sampling years.

On the other hand, CDA medium consists of various nutrient molecules, of which sucrose has been identified as the main energy source, while nitrogen is obtained from the natrium nitrate component. Furthermore, other constituents, including dipotassium phosphate is known to serve as a buffer solution, while magnesium sulfate and iron sulfate are essential ions. However, both MEA and PDA used in this study are manufactured medium, ready to use, while CDA was created using a formula according to the manufacture's protocol (HiMedia Laboratories, Mumbai), hence the tendency for unclear and unstandardized composition accuracies and capabilities.

The molds obtained in 2016 at the Kalisidi station were successfully identified, and three species were observed in the first collection (P1), with two in the second collection (P2). Two species were accumulated in P1, and four species in P2 for samples collected in 2017. However, the Nglimut station portrayed a relatively higher diversity (Table 3).

Aspergillus sp. was the only species present five times in the *R. undulatus* nest, i.e., two times in Kalisidi station and three times in Nglimut station, while *Penicillium* sp. were observed two times in each station. In addition, *A. terreus* was identified on three instances, i.e., one and two times for the respective stations. On the other hand, *Curvularia* sp., *Fusarium* sp., *Geotrichum* sp., *N. fischeri* and *Trichoderma* sp. were only identified twice in both stations, while *A. niger, Acremonium* sp., *P. variabile*, and *Rhizopus* sp. were rarely present (Table 4).

Table 3 Types of molds in the cover Wreathed Hornbill (*Rhyticeros* undulatus) obtained in the 2016 and 2017 sampling period

Species	Kalisidi station				Nglimut station			
	2016		2017		2016		2017	
	P1	P2	P1	P2	P1	P2	P1	P2
Aspergillus sp.	√	√	-	-	√	-	√	√
Aspergillus niger	-	-	-	-	√	-	-	-
Aspergillus terreus	-	√	-	-	-	-	√	√
Acremonium sp.	√	-	-	-	-	-	-	-
Curvularia sp.	-	-	-	-	-	-	√	√
Fusarium sp.	-	-	-	-	-	-	√	√
Geotrichum sp.	-	-	√	√	-	-	-	-
Neosartorya fischeri	-	-	-	√	-	-	-	√
Penicillium sp.	√	-	-	√	-	-	√	√
Penicillium variabile	-	-	-	-	√	-	-	-
Rhizopus sp.	-	-	-	-	-	-	-	√
Trichoderma sp.	-	-	√	√	-	-	-	-
Total spesies	3	2	2	4	3	-	5	7

Notes: P1 = first collection; P2 = second collection.

Table 4 Types of molds in *R. undulatus* nest during sampling in 2017

Types of molds	Kalisidi station						Nglimut station					
	P1			P2			P1			P2		
	CN	WM	CM	CN	WM	CM	CN	WM	CM	CN	WM	CM
Aspergillus sp.	-	-	-	-	-	-	√	√	√	√	√	√
Aspergillus niger	-	-	-	-	-	-	√	-	-	√	-	-
Aspergillus terreus	-	-	-	-	-	-	-	-	√	-	-	√
Acremonium sp.	-	-	-	-	-	-	-	-	√	-	-	-
Curvularia sp.	-	-	-	-	-	-	-	-	√	-	-	√
Fusarium sp.	-	-	-	-	-	-	√	√	-	√	√	√
Geotrichum sp.	√	-	-	-	-	√	-	-	-	-	-	-
Neosartorya fischeri	-	-	-	-	√	√	-	-	-	-	-	√
Penicillium sp.	-	-	-	-	-	√	√	√	√	√	√	√
Penicillium variabile	-	-	-	-	√	-	-	-	-	-	-	-
Rhizopus sp.	-	-	-	-	-	-	-	-	-	√	-	-
Trichoderma sp.	-	√	-	√	√	-	-	-	-	-	-	-
Total spesies	1	1	-	1	3	2	4	3	5	5	3	6

Notes: P1 = first collection; P2 = second collection; CN = cover nest; WM = wood material; CM = compost material.

Samples obtained from the nests were divided into three parts, i.e., cover, internal compost and wood material. The most abundant molds identified at the Kalisidi station included *Geotrichum* sp., *N. fischeri* and *Trichoderma* sp. The *Trichoderma* sp. Was observed in the first and second collection periods, while the wood material containing *N. fischeri* and *P. variabile* was observed in the second collection period. This result indicated that *N. fischeri* is the most widespread mold species.

The high diversity shown in Nglimut station included four mold species in the nest cover, with three in the wood material and four species from the compost. In addition, *Aspergilus* sp. and *Penicillium* sp. were identified as the most common molds in all parts, during both collection periods, while *A. niger* was only found in the nest cover, with *A. terreus* in the nest inner material for both collection periods.

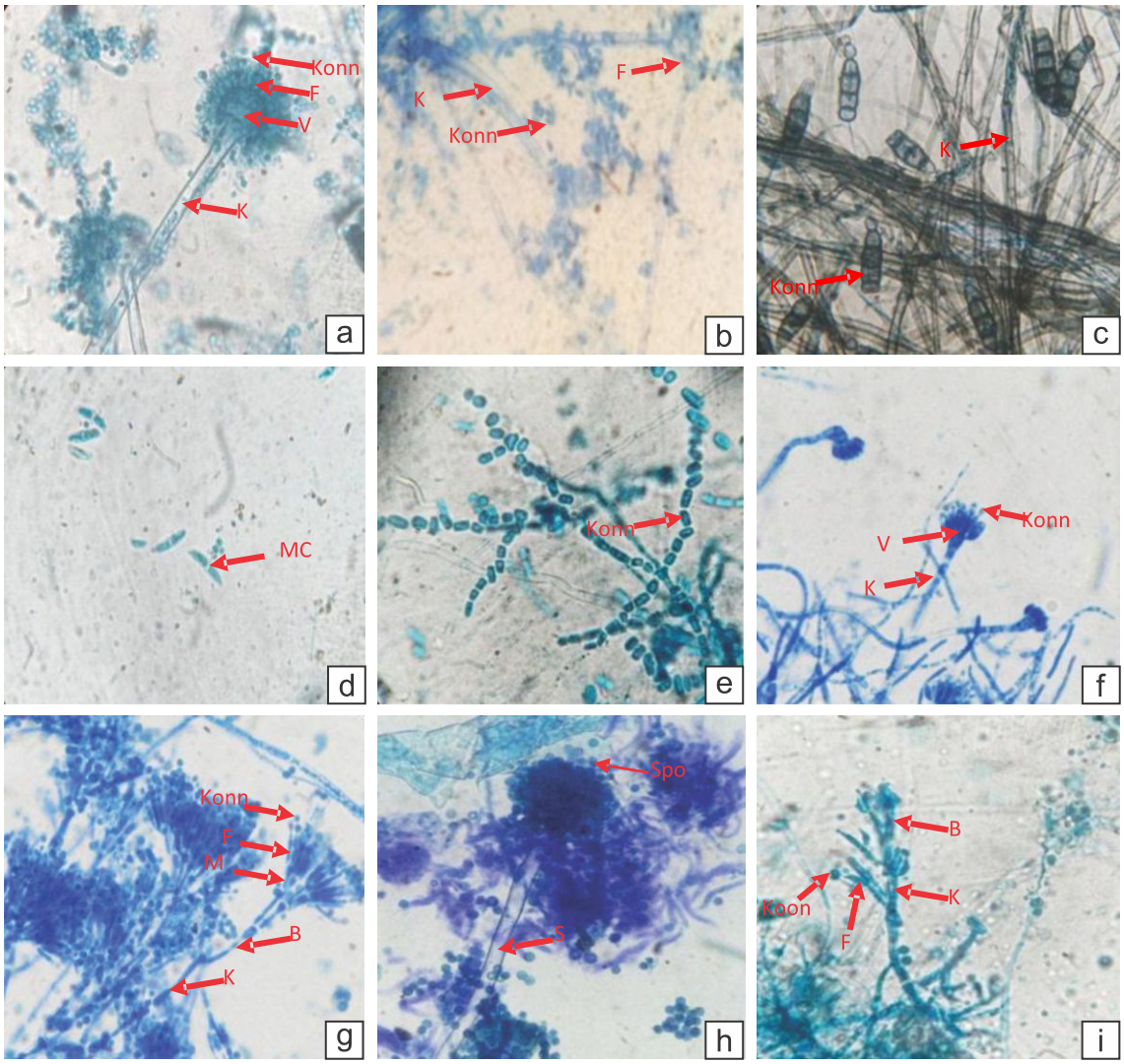

Figure 1 Molds types identified in *R. undulatus* nest
Notes: a = *Aspergillus* sp.; b = *Acremonium* sp.; c = *Curvularia* sp.; d = *Fusarium* sp.; e = *Geotrichum* sp.; f = *Neosartorya fischeri*; g = *Penicillium* sp.; h = *Rhizopus* sp.; i = *Trichoderma* sp. (B = Branch; F = Phialid; K = Conidiophore; Kon = Conidia; M = Metula; MC = Microconidia; S = Sporangiophore; Spo = Spora; V = Vesicle). Microscope magnification: 1,000x.

The morphologycal identification process was based on two main characteristics, including: 1) the colony formation and color and 2) the morphological structure. Based on these two main characteristics, the molds were identified into genus and species (Table 5).

Table 5 Description of molds types identified in the nest of *R. undulatus*

Mold	Description
Aspergillus sp.	The fruit body consisted of *Aspergillus* formed conidiophores (non-septate), vesicles, metula, phialid, stolone (vegetative hyphae) and conidia. The identified *Aspergillus* sp. possessed radiate- and biseriate-conidial heads (sideways/ deviate), with phialid organs that grow in the metula, as seen in Figure 1.a. Hence, molds with similar criteria were designated as *Aspergillus* (Diba *et al.* 2007)
Acremonium sp.	The typical organs present included a cluster of aerial hyphae, conidiophores, phialids and ellipse extended conidia. Furthermore, the phialid grew directly on aerial hyphae, and was tapered in the form of a needle, while the conidiophores were single-celled, erected and unbranched condia (Fig. 1.b) (Samson *et al.* 1984). The microscopic size range of these components were 17.5-37.5 (-50.4) × 3.2-4 μm, and 6-8.5 (-9.3) × 2.1-2.8 (-4) μm, respectively (Gräfenhan *et al.* 2011; Hill *et al.* 1990).
Curvularia sp.	The conidiophores were branched, brown, and tightly arranged in groups. In addition, the conidia were elliptical with 3-4 bulkheads in each, with brownish white coloration, and comprising of 4-5 cells. The colonies were dark black in color and round, with cotton texture (Fig. 1.c). This was in accordance with the description by Kusai (2015) and Hosokawa (2003), except with the addition of velvet pigmentation. The conidiophores appeared singly or in groups, simple or branched, straight or crouched, with pale brown or young cones, while the conidia had 3-4 septa. The specimen was thin-walled, measuring 20-30 x 9-15 μm (Hosokawa *et al.* 2003; Kusai *et al.* 2016).
Fusarium sp.	The *Fusarium* genera was identified using the method by (Bashyal *et al.* 2016; Gräfenhan *et al.* 2011). The microconidia appeared as fusiform and ovoid form, with 0-1 septate, while the conidiophorous structure present was insulated. In addition, phialid and macroconidia were not seen under microscopic observations, although microconidia were recognized (Fig. 1.d).
Geotrichum sp.	Conidia were cylindrical, oval, and tubular (barrel) in shape, with green-blue coloration, and also a chain-like and clustered arrangement. In addition, the upper part of this mold was formed from broken fertile hyphae (Fig. 1.e), with conidia diameter of 3.7-4.8-12.5 (-13.8) x (1.7-) 2.4-5 μm, and no conidiophores. Also, the fertile hyphae present was branched off dichotomous and insulate.
Neosartorya fischeri	The morphological structure of *Neosartorya fischeri* was similar with *Aspergillus*, characterized by vesicles, phialid and conidia, with seemingly insulated hyphen, alongside blue densely arranged conidiophores and hyphae. In addition, the vesicles were slightly elongated in shape, with conidial columnar head, which was also uniseriate (direct phialid growth in vesicles) (Fig. 1.f). The colony was white in color, with cotton-like texture, and ± 0.2-2 cm in diameter. The conidia of *Neosartorya fischeri* was round in shape, half round and elliptical, with slightly coarse wall, at ± 2-3 μm diameter, while the conidiophores ranged from 300-500 μm, with characteristic smooth walls (Udagawa *et al.* 1996).
Penicillium sp.	The morphological structures possessed insulated vegetative hyphae, alongside conidiophores, branches, metula, phialid and conidia. The conidiophores were of the two-stage branched (biverticillate-asymmetrical) type, while the conidia appeared round (Fig. 1.g). In addition, the colonies were grayish and light green-old, with ± 0.2–2 cm diameter. Also, *Penicillium* is included as a Deuteromycota, characterized by fast growing colonies, which is green in appearance and sometimes white. The conidiophores had several branching pattern forms, including one to three-stages and more-stage branched (Visagie *et al.* 2014).
Rhizopus sp.	The morphological structure had sporangiofor, sporangium, featuring the release of spores (sporangiospor). In addition, the mold contains the non-septate stolone (vegetative hyphae), alongside the rhizoid, although only the columella covered by sporangium. The Sporangiofor stands tall, with a round shape (Fig. 1.h) (Hartanti *et al.* 2015).
Trichoderma sp.	Morphological structures are similar with *Trichoderma*, featuring vegetative hyphae (aerial hypha), conidiophores with side branches, slim and elongated phialids, and also round conidia, with white and dark green colonies (Fig. 1.i). According to Gusnawaty *et al.* (2014), *Trichoderma* sp. has branched conidiophores resembling pyramids, with more to the end, and the branching becomes shorter. Also, the conidia are smooth walled and semi-round to oval in shape. This species have green colonies that were initially white (Supa-Amornkul *et al.* 2011).

The nest cover collected in this study consisted of soil and wood. Molds identified from the nest cover were of various species, although the more abundant molds were observed in the organic material of the nest's inner part. This was possibly caused by composted organic materials, including feces, fermented fruit, e.g., *Ficus*, insects and decayed wood. Particularly, the *Ficus* fruit or fig (*Ficus carica*) contains 8.98% protein, 6.57% fat, 10.26% moisture content, 18.23% ash content, 20.31% crude fiber, 0.0395% calcium, 0.002% phosphor, 25.48 mg/100 g and 1.64 mg/100 g of vitamin C and E, respectively. In addition, fig also contains various minerals needed by *R. undulates,* including N, P, K, Ca, Mg and others (Mendoza-Castillo 2019). Aside from fruits, the fecal matter was high in N for mold protein synthesis, while the soil and wood were characterized by water, fat, carbohydrate and protein. This results were confirmed with the results of previous studies on the nest of *R. undulatus* containing 53.30 mg/mL of water, 39.02 mg/mL of fat, 35.03 mg/mL of carbohydrates, 5.82 mg/mL of ash and 20.12 mg/mL of protein. In addition, the humidity and the warm and dark conditions of the inner part of the nest form an appropriate and suitable environment for mold growth.

CONCLUSION

Mold species obtained from *R. undulatus* nests consisting of cover, composted material and wood material in Kalisidi and Nglimut stations during the sampling in 2016 comprised 6 mold species including *Aspergillus niger, Aspergillus terreus, Aspergillus* sp., *Penicillium* sp., *Penicillium variabile* and *Acremonium* sp. On the other hand, 9 mold species were reported during the sampling in 2017, including *Aspergillus terreus, Aspergillus* sp., *Curvularia* sp., *Fusarium* sp., *Penicillium* sp., *Rhizopus* sp., *Geotrichum* sp., *Trichoderma* sp., and *Neosartorya fischeri.*

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