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MICROBIAL COMMUNITY ASSOCIATED WITH AMBROSIA BEETLE, *Euplatypus parallelus* ON SONOKEMBANG, *Pterocarpus indicus* IN MALANG

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ABSTRACT

Recently, most of sonokembang, *Pterocarpus indicus* trees are dying in Malang. In 2012, the death rate of trees reached ca. 11%. In addition, death of trees spread to other regencies in East Java. *Euplatypus parallelus* is a specific species of ambrosia beetles that were the causal agents to the dying and wilting of sonokembang trees in Malang. Wilting is caused mainly by the pathogenic fungi carried by ambrosia beetles. To confirm the microbial communities related to *E. parallelus* that attack sonokembang, *E. parallelus* and some attacked trees were collected in Malang city. Isolation and identification of these species were conducted at the Laboratory of Mycology, Faculty of Agriculture, University of Brawijaya and Laboratory of Molecular Biology, Islamic State University, Malang. Results showed that there were nine microbes including five genera of fungi, two genera of yeasts and one genus of bacterium were identified. The microbial communities that were found namely *Aspergillus* spp., *Penicillium* spp., *Trichoderma* spp., *Fusarium* spp., *Acremonium* spp., *Gliocladium* spp. (fungi), *Streptomyces* spp. (bacteria), *Saccharomyces* spp., and *Candida* spp. (yeast).

Keywords: ambrosia beetle; *Euplatypus parallelus*; microbial community; *Pterocarpus indicus*

INTRODUCTION

Recently, most of sonokembang trees are dying in Malang. Tarno, Suprapto, & Himawan, (2014) reported that a death rate of trees reached ca. 11%. In addition, such occurrence had spread to the other regencies in East Java. Wilting is commonly caused by pathogenic fungi carried by *Euplatypus parallelus*, an ambrosia beetle that was responsible to wilting and dying of sonokembang in Malang (Tarno, Suprapto, & Himawan, 2014).

Ambrosia beetle has mycangia located in the thorax of adult female (Kuroda & Yamada, 1996). Mycangia has specific character and be used to carry symbiotic microbes such as fungi, yeast or bacteria (Endoh, Suzuki, Benno, & Futai, 2008). However, the ambrosia beetle (*Platypus quercivorus*) could carry both pathogenic and non-pathogenic microbes.

Some microbes are advantageous to ambrosia beetles (Harris et al., 2009) as they can serve as food for the ambrosia beetles and aid in their growth and development (Moon, Park, Oh, & Kim, 2008). Several ambrosia beetles feed on yeasts; however, some of these microbes are classified as pathogens that can reduce plant resistance and eventually leads to wilting and dying of trees (Henriques, Inácio, & Sousa, 2009).

Infected trees by ambrosia fungi showed general symptoms such as fallen leaves, wilting and dying, including discoloration of sapwood (Kubono & Ito, 2002). In this study, the microbial communities related to *E. parallelus* that attack sonokembang were investigated.

MATERIALS AND METHODS

Research was conducted from early December 2013 to the end of June 2014 in Malang City. Ambrosia beetle, wood and frass samples were collected randomly from five points of attacked coordinate sites. Stratified Random Sampling as sampling method was used in this research (Singarimbun & Effendi, 2005). Each collected sample was identified and then compared between three groups of samples such as insect body, gallery in the wood and frass. Identification

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was conducted at Laboratory of Mycology, Faculty of Agriculture, University of Brawijaya and Laboratory of Molecular Biology, Islamic State University, Malang.

Microbial Isolation, Collected from Insect Body, Gallery and Frass

Thorax of female beetles was incised to get microbes from the mycangia. For the gallery, wood samples were cut in small parts (ca. 1 cm). In the case of frass, two types of frass such as fibrous and powdery frass were collected from the mouth of tree tunnels. All of the samples were isolated according to the method of Larran, Rollán, Ángeles, Alippi, & Urrutia (2002).

Purification

All microbial colonies were purified on Potato Dextrose Agar (PDA) medium. Nutrient Agar (NA) medium was used for bacteria and Yeast Mannitol Agar (YMA) medium was used for yeast. Each microbial colony characterizing a different color and form based on macroscopic morphology was purified. Specific microbes were separated from each other, taken by oose, and then cultured on Petridish on their respective medium.

Microbial Preparates

Microbial preparates were made on object glass. Fungal spores were taken by oose and placed on small PDA media on object glass, then covered by cover glass. Preparates were placed into tray with sterile paper and then incubated for 2–3 days.

Identification

Macroscopic and microscopic observations were used during the identification process. Color, form (concentric or non-concentric), texture and growth (cm day^{-1}) of colonies were used to distinguish each colony. Macroscopic observation was conducted every day after period of inoculation until microbe covered all of space on Petri dish (ϕ ca. 9 cm). Microscopic observation was conducted within 5–7 days by microscope. Types of hyphae, growth of hyphae, color of hyphae, color of conidia, form of conidia, form of mycelia, size of conidia, conidiophores and form of spores were used to identify each microbe as microscopic variables. In addition, form, color, growth and development of colonies were used to identify as macroscopic variables.

Data Analysis

Based on the data of each variable, some ecological indices, such as Simpson Index (D), Shannon-Wiener Indices (H`), Species Evenness Indices, and Simpson's dominance index were calculated:

1. Simpson Index (D) and species diversity indices, Shannon-Wiener Indices (H`) are described as (Davari, Jouri, & Ariapour, 2011; Krebs, 2014, Pawhestri, Hidayat, & Putro, 2015):

$$\mathbf{D} = \sum_{i=1}^{s} P_i^2 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

$$\mathbf{H'} = -\sum_{i=1}^{s} (P_i)(\log_2 P_i) \quad \ldots\ldots\ldots(2)$$

Remarks: *D, H’, P_i, S* and *log* are Simpson index, index of species diversity, proportion of individuals of species *i* in the community, number of species in the sample, and logarithm respectively (Table 1).

Table 1. Value and description of Shannon-Wiener Indices (H')

Index values	Description
< 1	low level of diversity, low individual distribution of each species
1-3	middle level of diversity, middle level of individual distribution for each species
>3	high level of diversity, high level of individual distribution for each species

2. Species Evenness Indices (E) (Table 2) is formulated as (Pawhestri, Hidayat, & Putro, 2015):

$$\mathbf{E} = \frac{H'}{\ln(S)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(3)$$

Remarks: *H’*, *ln* and *S* are index of species diversity, exponential logarithm, and proportion of individuals of species.

Table 2. Value and description of index for species evenness indices (E)

Index values	Description
0.00<E<0.50	Evenness is low, Community is underpressure
0.50<E<0.75	Evenness is medium level, Community is unstable
0.75<E<1.00	Evenness is high, Community is stable

3. Simpson's dominance index (C) (Table 3) is used to measure the dominance of microbes in the community. Formula of Simpson's dominance index is described as (Davari, Jouri, & Ariapour, 2011):

$$\mathbf{C} = \sum_{i=1}^{n} \left[\frac{ni}{N}\right]^2 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(4)$$

Remarks: *C*, n_i and *N* are Simpson's dominance index, population of i-species, and population total of all species, respectively.

Table 3. Value and description of dominance index

Index values	Description
0.00<C<0.50	Low dominance of species
0.50<C<0.75	Middle dominance of species
0.75<C<1.00	High dominance of species

RESULTS AND DISCUSSION

Symptom and Mortality of Sonokembang Trees in Malang City Caused by Ambrosia Beetle, *E. parallelus*

Sonokembang that attacked by *E. parallelus* showed distinguished characteristics. High amounts of fallen leaves indicated wilting and dying of trees. In addition, a high number of frass as a special sign was also produced during Ambrosia beetle attacks. Tarno et al. (2010) reported that there are two types of frass produced by ambrosia beetles, namely: fibrous and powdery frass. Fibrous and powdery frass are caused by adults and larva, respectively (Tarno et al., 2010). Powdery frass was much higher produced by Ambrosia beetle than fibrous frass. Both of frass are expelled by male adult to keep the gallery clean (Tarno, Qi, Yamasaki, Kobayashi, & Futai, 2016).

Ambrosia beetle made hole on the stem and then construct longer and complicated tunnels inside the wood as known as gallery. Tarno, Suprapto, & Himawan (2014) explained that the diameter of entrance holes is ca. 1.90 ± 0.21 mm. During constructing tunnels, ambrosia beetle ejects frass from their tunnels (Tarno et al., 2010; Tarno, Qi, Yamasaki, Kobayashi, & Futai, 2016). Figure 1 describes the signs and symptoms of sonokembang that were attacked by the Ambrosia beetle, *E. parallelus*.

In case of an attacked tree, a cross-section of the wood will show a vivid discoloration of the xylem as shown in Figure 2A (Kuroda & Yamada, 1996), which indicate that the xylem tissue already died. Pattern of gallery is easily observed if the wood were incubated. Likewise, the growing fungi can also be seen during incubation. In this study, the conditions of wood before and after incubation are shown in Figure 2B and 2C, respectively.

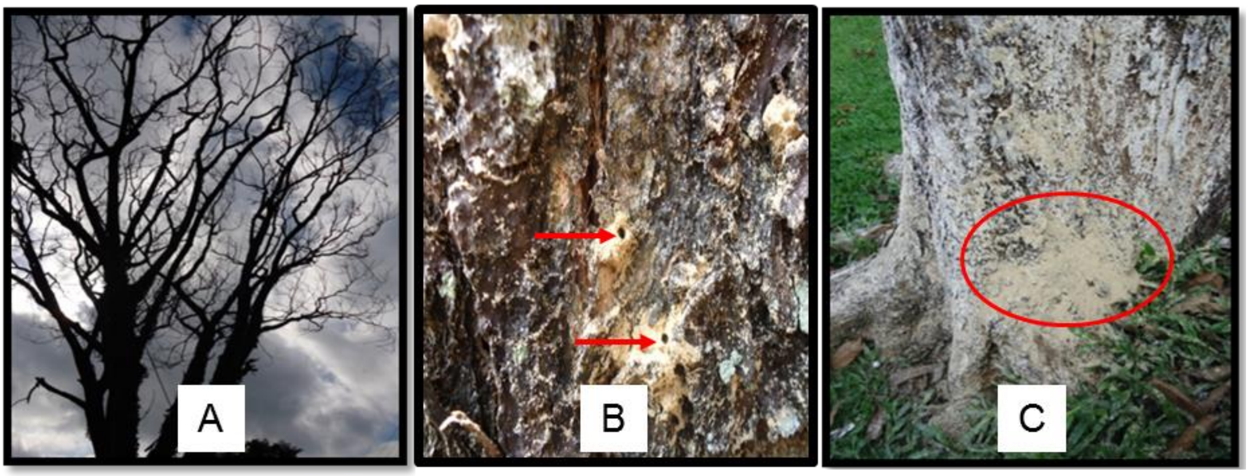

Figure 1. Sign and symptoms of microbial disease on sonokembang: (A) dying tree (B) holes on stem of tree (C) *frass* on stem of tree

Figure 2. Morphological characteristics in the stem of tree before and after incubation. (A) Discoloration on xylem (Kuroda & Yamada, 1996) (B) condition of wood before incubation and (C) condition of wood after incubation.

Diversity of Microbial Community on Insect Body, Gallery and Frass

Based on the identified isolates, there were 48 isolates of microbes which were associated with insect body of ambrosia beetle, gallery of tunnels and frass. Genus of isolates are described in Table 4.

Nine genera of microbes were identified from the insect body, gallery of tunnel and frass such as *Trichoderma, Aspergillus, Acremonium, Fusarium, Gliocladium, Streptomyces, Saccharomyces* and *Candida*.

There were 15 species of microbes that were identified from the insect body of ambrosia beetle, *E. parallelus*. Five species were isolated from mycangia such as *Acremonium* spp., *Gliocladium* spp. (fungi), *Streptomyces* spp. (bacteria), *Saccharomyces* spp. and *Candida* spp. (yeasts). However, *Fusarium* was not found in the insect body of *E. parallelus*.

In the tunnel gallery, there were 19 identified species of microbes. Nine and ten species were identified from the entry holes and the inner part of galleries. One species of *Trichoderma*, two species of *Aspergilus*, two species of *Penicilium*, one species of *Acremonium*, one species of *Gliocladium*, and one species of *Candida* were found in the entry holes of galleries. One species of *Trichoderma*, two species of *Aspergilus*, one species of *Penicilium*, one species of *Acremonium*, two species of *Fusarium*, one species of *Gliocladium*, one species of *Saccharomyces* and one species of *Candida* were found in the inner part of galleries.

In the case of frass, 14 species of microbes were identified. Six and eight species were identified in powdery and fibrous frass, respectively. Only *Fusarium* was not found on both powdery and fibrous frass. Two species of yeasts were not found on the powdery frass and three species of yeasts were found in the fibrous frass.

Based on the composition of microbes in the three different samples (insect body, gallery and frass), species of microbes in gallery were the highest. Frass was lowest in terms of number of microbes. *Fusarium* species were found only in the gallery. In addition, Simpson diversity index (D), Shannon (H'), Evenness (E) and Dominance index (C) of microbial communities were described in Table 5.

As shown in Table 5, diversity, evenness and dominance indices of microbial communities in gallery are highest according to the number of colonies and species, with 29 colonies and 19 species.

Table 4. Genera of microbial community isolated from insect body, gallery and frass

Genera	Insect body			Gallery		Frass		∑ Species of microbes
	Intestine	Exoscleleton	Mycangia	Entry hole of gallery	Inner part of gallery	Powdery Frass	Fibrous Frass	
Trichoderma	1	1	0	1	1	1	2	8
Aspergillus	2	1	0	2	2	1	1	9
Penicillium	2	1	0	2	1	1	1	8
Acremonium	0	0	1	1	1	1	1	5
Fusarium	0	0	0	1	2	0	0	3
Gliocladium	0	0	1	1	1	1	0	4
Streptomyces	0	1	1	0	0	1	0	2
Saccharomyces	0	0	1	0	1	0	2	4
Candida	1	0	1	1	1	0	1	5
∑ microbial colonies/part	6	4	5	9	10	6	8	
∑ microbial colonies/group	15			19		14		48
∑ microbial genus/group	8			8		8		

Table 5. Simpson's diversity index (D), Diversity index of Shannon (H'), Evenness (E) and dominance index (C) of microbial communities between insect body, gallery and frass

Sources of isolates	Index values				∑ genus	∑ species	∑ colony
	D	H'	E	C			
Insect body	0.085	1.991	0.957	0.062	8	15	23
Gallery	0.090	2.013	0.969	0.065	8	19	27
Frass	0.076	2.007	0.968	0.063	8	14	22
Total	0.251	6.011	2.895	0.190	24	48	72
Average	0.084	2.004	0.965	0.063	8	16	24

Macroscopic and Microscopic Characteristic of Microbial Isolates on Insect Body, Gallery and Frass

Based on the macroscopic and microscopic characteristic of microbial isolates, description of each genus is explained in Table 6, Table 7 and Figure 3. Most of the ambrosia fungi belong to four mitosporic genera: *Ambrosiella*, *Raffaelea*, *Monacrosporium* and *Phialophoropsis*. However, more genera have been reported to be involved with ambrosia beetles including *Fusarium*, *Acremonium*, *Candida* and *Graphium* (Batra, 1963; 1967; Baker & Norris, 1968).

Fusarium spp. as plant pathogenic fungi such as *F. oxysporum* and *F. solani* were isolated from all samples, except the insect body. It was confirmed that *F. oxysporum* was the causal agent for the death of *P. indicus* in Malaysia due to wilt disease (Philip, 1999). Similarly, the dying *P. indicus* infested by *E. parallelus* in this study showed wilt symptom. *Fusarium oxysporum* may be introduced into the tunnels by beetles and may be the causal agent of death of *P. indicus* in the south of Thailand (Bumrungsri, Beaver, Phongpaichit, & Sittichaya, 2008).

Table 6. Description of each genus of microbial isolates that associated with ambrosia beetle, *E. parallelus* in Malang city based on macroscopic characteristics

Genera	Upper surface				Lower surface
	Textures	Densities	Color of colonies	Patterns of growth	
Aspergillus	Smooth, powdery	high	Green or black	Concentric	Similar to upper face, orange medium
Penicillium	Smooth, thick, powdery	high	Green to brown	Symmetry	Similar to upper face, Yellowish medium
Trichoderma	Rough, thick, powdery	high	Early white to dark green	Concentric	Similar to upper face
Fusarium	Smooth and thick like cotton	high	White	Concentric, end portion of wavy	purple/red medium
Acremonium	Smooth, feathered	high	White	Concentric	Similar to upper face
Gliocladium	Feathered	high	Yellowish with white	Concentric & wavy	Yellowish medium
Streptomyces	Slick, powdery	high	grey	Radial	Yellowish medium
Saccharomyces	Slick shiny, thick.	high	White to grey	Radial	Yellowish medium
Candida	Slick not shiny	high	White to grey	Radial	Yellowish medium

Table 7. Description of each genus of microbial isolates associated with the ambrosia beetle, *E. parallelus* in Malang city based on microscopic characteristics

Genera	Microscopic characteristics of microbes					Observations
	Mycelia		Spores / Conidia			
	Shape	Color	Shape	Color	ϕ (µm)	
Aspergillus	Elongated, unbranched	Hyaline	Spherical serrated of conidia	Blackish brown	4.7 – 5.2	Tightly clustered conidia
Penicillium	Mycelium branched	Hyaline	Oval conidia	Hyaline	3.3 – 3.6	Tip of conidia clustered phialide
Trichoderma	Sectional has many branches	Hyaline	Elliptical clustered conidia	Hyaline	3.7 – 4.2	Tip of conidia clustered phialide
Fusarium	Sectional, branched	Hyaline	Elongated oval conidia	Hyaline	3.7 - 4.3	Conidia taper at both ends
Acremonium	Branched has septa	Hyaline	Elliptical cylindrical conidia	Hyaline	3.3 – 7.0	Conidia groups such as ball
Gliocladium	Branched has septa	Hyaline	Ovoid conidia	Blackish brown	4.6 – 5.2	Conidiophores branches such as brush
Streptomyces	Branched	Hyaline	Chain spherical spore	Blackish brown	0.5 – 2.0	Spore chain consists of three or more
Saccharomyces	Pseudohyphae	Hyaline	Conidiogenous cells poliblastic, cylindric & thick	Hyaline	4.5 – 5.0	Did not form hyphae
Candida	Pseudohyphae	Hyaline	Conidiogenous cells poliblastic, oval	Hyaline	4.8 – 5.2	Cell form varies

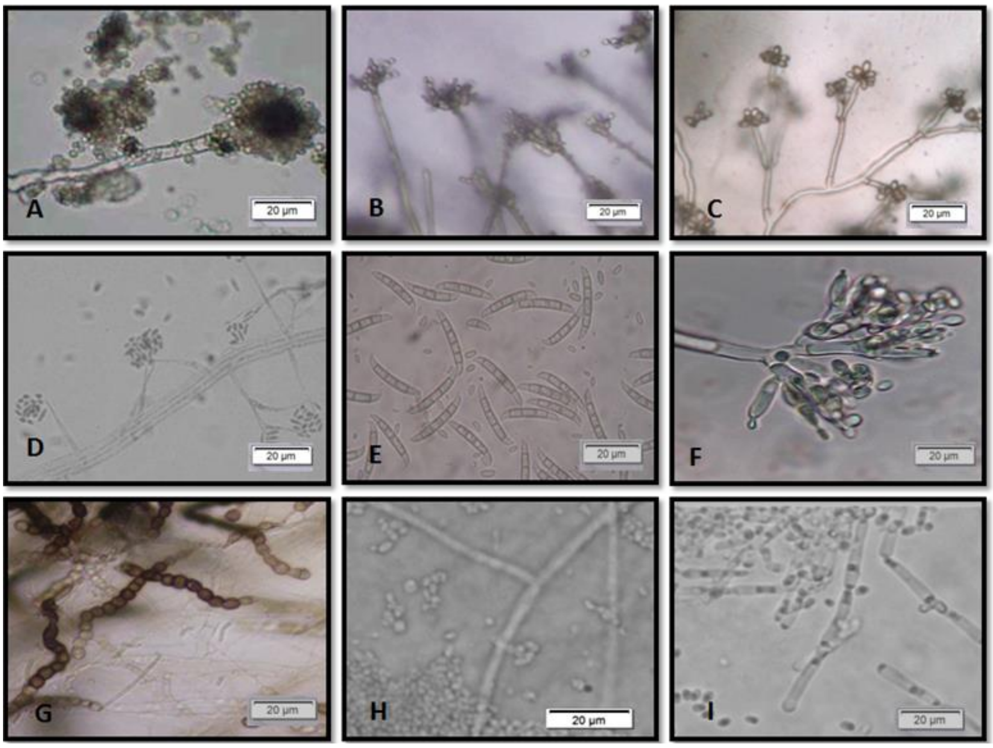

Figure 3. Microscopic characteristics of microbial communities' associated with the ambrosia beetle, *E. parallelus* on sonokembang in Malang. (A) *Aspergilus* (B) *Penicillium* (C) *Trichoderma* (D) *Acremonium* (E) *Fusarium* (F) *Gliocladium* (G) *Streptomyces* (H) *Saccharomyces* and (I) *Candida.*

In Singapore, Sanderson, Fong, Yik, Ong, & Anuar (1997) considered that *P. indicus* attract *E. parallelus* when they became stressed by lightning strike. If the beetles were carrying spores of *F. oxysporum*, infection by the fungus is likely to follow, and result in the death of the trees (Sanderson, Fong, Yik, Ong, & Anuar, 1997).

Fusarium and *Acremonium* are plant pathogenic fungi. Kiffer & Morelet (1997) stated that *Acremonium* is plant pathogen for woody plants. In case of *Fusarium*, it commonly attacks on xylem and phloem of plants (Kiffer & Morelet, 1997). Streptomyces is classified as actionbacteria that have gained high commercial interest for the production of a variety of metabolites acting as potential insecticides (Ruiu, 2015). In addition, *Penicilium* and *Gliocladium* are potential antagonistic fungi (Gouli, V., Gouli, S., Marcelino, Skinner, & Parker, 2013; Vázquez-Martínez, Cirerol-Cruz, Torres-Estrada, & López, 2014).

CONCLUSION

From nine identified microbes, there were five genera of fungi, two genera of yeasts and one genus of bacterium. The microbial communities that were associated with *E. parallelus* namely *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp., *Fusarium* sp., *Acremonium* sp., *Gliocladium* sp. (fungi), *Streptomyces* sp. (bacteria), *Saccharomyces* sp., and *Candida* sp. (yeast).

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