THE HABITAT SUITABILITY OF JAVAN LANGUR (*Trachypithecus auratus* E. Geoffroy Saint-Hilaire, 1812) IN KUCUR RESORT AT ALAS PURWO NATIONAL PARK

THESIS

by

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FACULTY OF MATHEMATICS AND NATURAL SCIENCES
UNIVERSITAS JEMBER
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submitted to complete the final project and fulfill one of the requirements
to complete the Biology Study Program (S1)
and achieve a Bachelor of Science degree

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DEDICATION

With praise and gratitude to the presence of God Almighty, for all the graces and gifts, this thesis is dedicated to:

1. My mom Nike Sulistyorini and my beloved dad Edi Hendrowardaja H., thank you for the love, prayers, materials, sacrifices, as well as support and patience that never stopped in educating me since childhood;
2. My beloved sister Haidar Yahya Maulahila, Naila Aulia Maulahila, and Muhammad Haibat Maulahila who always give joy, enthusiasm and support;
3. Teachers at Rahmatullah Kindergarten Banyuwangi, SDU Habibullah Banyuwangi, MTs Acceleration Amanatul Ummah, MTsN 2 Jember, and MAN 2 Probolinggo City, as well as all lecturers at the Biology Study Program, FMIPA Universitas Jember who have guided, educated, and taught their knowledge;
4. Contribute for Universitas Jember.

MOTO

"*Let men visit the Orang utan in domestication……see its intelligence. Man in his arrogance think himself a great work……more humble and I believe true to consider him created from animals*."*)

*) Charles Darwin dalam Aydon, C. 2008. *A Brief Guide to Charles Darwin: His Life and Times*. London: Robinson.

STATEMENT

I, the undersigned below:

Name : Haikal Idris Maulahila

NIM : 171810401031

I solemnly declare that the scientific work entitled "The Habitat Suitability Of Javan Langur (*Trachypithecus auratus* e. Geoffroy saint-hilaire, 1812) in Kucur Resort Alas Purwo National Park" is truly the result of his own scientific work, unless the source is mentioned in the citation of the substance and has never been submitted by any institution, and is not the result of plagiarism. This research was fully funded by the Tropical Biodiversity Conservation Research Group. I am responsible for the validity and correctness of the contents in accordance with a scientific attitude that must be upheld.

Thus, I make this statement in truth, without any pressure and coercion from any party and am willing to receive academic sanctions if it turns out that in the future this statement is not true.

Jember, July 2021

That state

Haikal Idris Maulahila

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VALIDITY

Thesis entitled "The Habitat Suitability of Javan Langur (*Trachypithecus auratus* e. Geoffroy saint-hilaire, 1812) in Kucur Resort Kucur at Alas Purwo National Park" by Haikal Idris Maulahila has been evaluated and approved on:

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SUMMARY

The Habitat Suitability of Javan Langur (*Trachypithecus auratus* E. Geoffroy Saint-Hilaire, 1812) in Kucur Resort Alas Purwo National Park; Haikal Idris Maulahila, 171810401031; 2021; 32 page; Department of Biology Faculty of Mathematics and Natural Science.

Javan langur (*Trachypithecus auratus* E. Geoffroy Saint-Hilaire, 1812) is an Indonesian primate whose population continues to decline due to land conversion, illegal logging, and poaching in protected areas. Therefore, the current status of the Javan langur has been categorized as a vulnerable by the IUCN and Appendix II by CITES. Efforts to monitor and manage wildlife need to be carried out comprehensively. One of the protected areas where the Javan langur is still found is Resort Kucur Alas Purwo national Park (APNP), which until now has no information about the number and condition of its habitat. This study aims to determine the use and level of habitat suitability for the Javan langur so that it can be followed up for its conservation efforts.

This research was conducted in January-June 2021 at the Kucur Resort, APNP. This study uses two types of data, namely primary data and secondary data. Primary data in the form of encounter points for Javan langurs was obtained by collecting data on five transect lines along 0.5-2.5 km using two-way sampling. Meanwhile, secondary data collection in the form of habitat variables includes Normalized Difference Vegetation Index (NDVI), elevation, river distance, settlement distance, and tourist distance downloaded from the website providing geographic information database. All the data that has been obtained were analyzed using Microsoft Excel 2019 and ArcGIS 10.1 to determine the number of encounter points and the use of Javan langur habitat. Habitat suitability analysis of the Javan langur, the data were analyzed by scoring overlay using ArcGIS 10.1.

Based on the results of the analysis, it can be seen that the Javan langur prefers habitats with medium density and vegetation cover (NDVI 0.1 -0.5) because the vegetation has provided a source of food and protection for its survival. These primates also prefer to use habitats at an altitude of about 0-100 meters above sea

level which has a higher plant diversity and physical environmental factors that are suitable for Javan langurs in their activities. In addition, the habitat that the Javan langur prefers is quite far from the river, which is >300 m because the primate's water needs can already be met from its feed. Based on the results of this study, Javan langurs also choose habitats that are far away (>1000 m) from settlements and tourism to avoid anthropogenic disturbances. The results of the analysis also show that Resort Kucur has a very high level of habitat suitability (1456.25 Ha) and high (898.89 Ha) for Javan langurs. This shows that Kucur Resort is an optimal habitat for Javan langurs because it is able to provide the needs of Javan langurs to continue to survive and breed.

PREFACE

Praise and gratitude to the presence of God Almighty for all His graces and gifts so that the author can complete the thesis entitled "The Habitat Suitability of Javan Langur (*Trachypithecus auratus* e. Geoffroy saint-hilaire, 1812) in Kucur Resort Alas Purwo National Park". This thesis was prepared to fulfill one of the requirements in completing the bachelor degree at the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Jember (UNEJ).

The preparation of thesis could not be separated from the help and assistance of various parties, therefore the author would like to express his gratitude to:

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The author also accepts all criticism and suggestions from various parties for the perfection of this thesis. Finally, the author hopes that this thesis can be useful for goodness.

Jember, July 2021

Author

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BAB I. INTRODUCTION

1.1 Background

Javan langur (*Trachypithecus auratus* E Geoffroy Saint-Hilaire 1812) is one of the Indonesia endemic primates that have conservation status as vulnerable species according to the International Union for Conservation of Nature and Natural Resources (IUCN) (Nijman & Supriatna, 2008). The conservation status of Appendix II has also been granted by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (UNEP-WCMC, 2014). In addition, the javan langur has been protected by the Indonesian Government through the decree of the Minister of Forestry and Plantation No. 733/Kpts-II/1999 due to the declining population. As an endemic animal, this primate can be found in the Kucur Resort of Alas Purwo National Park (APNP).

Javan langurs at this resort use habitat which are entirely lowland forests with quite varied vegetation structures such as teak forests and jungle forests. The existence of the Javan langur depends on the preferences and conditions of its habitat [3], it can affect the habitat uses and habitat suitability of these primates. The identification of these habitat conditions can be done by using some habitat-forming factors include food sources, cover, water, and topographic conditions (McComb, 2007; Rode *et al,* 2013).

The availability of food sources can be represented through the condition of the vegetation in the Javan langur habitat because this primate exploits young leaves, seeds, flowers, and fruit a source of feed (National Research Council, 2003). In addition, the condition of the vegetation also can represent a protective availability for the Javan langur to get away from predators and anthropogenic disturbances (Leca *et al.,* 2013). Water sources can provide a guarantee for the existence of Javan langur as instrumental to the metabolism. These primates are also found to occupy habitats with varying altitudes from the lowland forest to the highland forest (Nijman, 2013).

All of these factors can be analyzed using a Geographic Information System (GIS) approach through mapping (Store & Jokimaki, 2003) based on the

Normalizes Difference Vegetation Index (NDVI), altitude, distance to water sources, settlements, and tourism parameters. Analysis of vegetation conditions as a representative of food sources and protection can be done using NDVI parameters. This data analysis can detect the density and area of vegetation by utilizing Landsat satellite imagery (Muhsoni, 2015). Especially for the distance between the Javan langur habitat and water sources, settlements, and tourism, it can be analyzed using Euclidean distance which is a technique that can measure the distance of an object with other objects in vector mapping (Reddy, 2008).

Based on these descriptions, the available information related to the use and suitability of habitat for Javan langur is based on these parameters is important to know for sure. This is because the information can be used as the basis for the management of Javan langur conservation for the APNP manager planning development around the Javan langur habitat. Therefore, this research was conducted to obtain information concerning the use and level of suitability habitat for Javan langur in Resort Kucur APNP, so the results can be used to minimize threats to the Javan langur population as a species endemic.

1.2 The Statement of the Problems

1. How is the habitat used of Javan langur in Kucur Resort based on NDVI, altitude, river distance, settlement distance, and tourist distance?
2. How is the level of habitat suitability of Javan langurs in Kucur Resort based on NDVI variables, altitude, river distance, residential distance, and tourist distance?

1.3 Objective

Adapun tujuan dilakukannya penelitian ini yaitu:

1. Determination the habitat used of Javan langur in Kucur Resort based on NDVI, altitude, river distance, settlement distance, and tourist distance.
2. Determination the level of habitat suitability of Javan langurs in Kucur Resort based on NDVI variables, altitude, river distance, residential distance, and tourist distance.

1.4 Benefit

1. Provide scientific information regarding the habitat suitability of the Javan langur in the lowland forest at Kucur Resort.
2. Provide the information to Alas Purwo National Park regarding the distribution and level of habitat suitability of Javan langur at Kucur Resort so that conservation management in the area can be carried out more optimally.
3. Provide information to the surrounding community about the importance of the Javan langur and their habitat to be conserved so that the community can play a role in its management.

BAB 2. LITERATURE REVIEW

2.1 Biology of Javan Langur (*Trachypithecus auratus*)

2.1.1 Taxonomy

Trachypithecus classification has undergone quite a lot of development and changes to finally be distinguished as a separate genus and separate from *Presbytis* and *Semnopithecus* (Brandon-Jones 1995; Zhang & Ryder, 1998; Groves, 2005; Parelman *et al.,* 2011; Wang *et al.,* 2012). The presence of *Trachypithecus* in Indonesia was initially considered to be a conspesific (one species) with *T. cristatus,* but analysis of cranial morphometry and genetic markers of the cytochrome-b gene showed that *T. auratus* is a different species and is known as Javan langur. (Weitzel & Groves, 1985; Rosenblum *et al.,* 1997; Ingicco *et al.,* 2011).

Javan langur can be divided into two subspecies, i.e *T. a. auratus* and *T. a. mauritius*. The division of this subspecies is based on the comparison of body morphometry and analysis of cytochrome-b and D-loop genetic markers (Brandon-Jones, 1995; Groves, 2005; Rahmawati *et al.,* 2015; Riskyani *et al.,* 2015). Roos *et al.* (2008) and Roos *et al.* (2014) then used the genetic marker cytochrome-b to determine the differences between these two subspecies of Javan langur and show that the two subspecies are different species. The classification of Javan langurs according to Roos et al, (2014) is as follows:

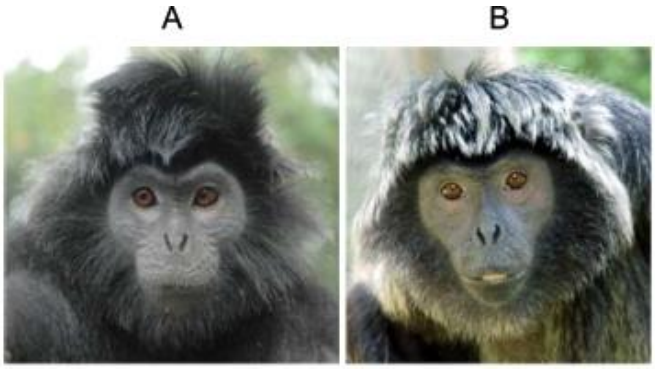
Kingdom : Animalia
Phylum : Chordata
Class : Mamalia
Ordo : Primata
Family : Cercopithecidae
Genus : *Trachypithecus*
Species : *T. auratus* E. Geoffroy Saint-Hilaire, 1812

2.1.2 Morphology

Javan langurs generally have a body length of about 42-60 cm, a tail length of 61-82 cm, and a weight of 6.3 - 7.1 kg. The body of the Javan langur is covered by black hair with protruding cheek tassels except for the face which only has dark skin. In contrast, baby Javan langurs have a different color from their mother, namely orange (Figure 2.1) (Rowe, 1996; Supriatna & Wahyono, 2000; Kurniawan et al., 2016). The pelvic part of the female Javan langur has pale hair with a slight black tinge, while the male Javan langur only has black hair without any markings. The difference in color and pattern of the pelvis in the Javan langur is one of the distinguishing characteristics between individual females (Tsuji et al., 2013).

(A) Male Javan langur; (B) Female Javan langur and child
Gambar 2.1 Characteristic of Javan langur (Source: Wedana et al., 2013; Leca et al., 2016)

Differences in hair color can also be found between the two Javan langur species. Body hair of *T. auratus* is black with a combination of silver at each end and hair around the face that falls or forms bangs (Maryanto et al., 1997; Brandon-Jones, 1995; Groves, 2005). Meanwhile, its relative, *T. mauritius*, has completely jet black body hair without any color combinations (Figure 2.2) (Roos et al., 2014; Nijman, 2000). Another difference that was found was the presence of adult T. auratus which had orange hair. The unique phenomenon that occurs in T. auratus is thought to be caused by mutations in the MC1R gene (Abdillah et al., 2016).



(A) *Trachypithecus mauritius*; (B) *Trachypithecus auratus*
Figure 2.2 Differences of *T. Mauritius* and *T. auratus* (Source: Rahmawati, 2015)

2.1.3 Distribution Range

Trachypithecus is one of the genera of the Cercopithecidae which has a fairly wide distribution area which includes India, Sri Lanka, China, and Southeast Asia (Groves, 2005). According to Roos et al., (2008) in Indonesia, only three species can be found, i.e *T. crisatus, T. mauritius*, and *T. auratus* with different distributions. *Trachypithecus cristatus* inhabits Sumatra and Kalimantan, *T. mauritius* is found in West Java, while *T. auratus* is found in Central-East Java, Bali and Lombok (Figure 2.3). According to Nijman (2000) Nijman & Supriatna (2008) the distribution of Javan langurs in Lombok is thought to be due to the introduction process.

The distribution of *T. auratus* in East Java was detected in 12 areas, including Meru Betiri National Park (MBNP), Baluran National Park (BNP), Alas Purwo National Park (APNP), and others. Especially in the APNP area, *T. auratus* is found in Pancur, Kucur, and Trianggulasi (Nijman, 2000). These animals are also found in various types of ecosystems, such as tropical forests, highland forests, lowland forests, mangrove forests, and even savannas (Supriatna, 2019; Hansen 2021).

Figure 2.3 Map of Distribution Range of *T. cristatus*, *T. mauritius* dan *T. auratus* (Source: Roos et al., 2008)

2.1.4 Behaviour

Javan langurs are primates that live in groups of 6-33 individuals, this group consists of one male and many females, so it is called a unigroup. These animals are active during the day (diurnal) and move on trees (arboreal) to find food, move places, or other social activities (Rowe, 1996; Utami, 2010; Rahmawati, 2017; Praditya, 2018).

The average Javan langur activity starts in the morning until the afternoon which begins with eating or moving places and then begins to increase by doing social activities such as playing and grooming. During the day, the activity of the Javan langur begins to decline which is indicated by resting activities (Geovana, 2015). Foraging and moving are the activities with the highest intensity. Javan langurs carry out these activities in a roaming area of 7-13 ha with a daily roaming area of up toi 540-740 m^2 (Vogt, 2003 dalam Leca *et al.,* 2013; Geovana, 2015; Santono *et al.,* 2016).

2.2 Habitat

2.2.1 The Component and Habitat Characteristics

Habitat is one of the basic needs for wildlife including primates in providing useful resources as a place to live to survive and breed (Bissonate & Storch, 2002).

According to the Texas Parks and Wildlife Department (2010), these habitat resources can include three main components:

a. Food Resource

Feed is a primary resource needed by animals because in quantity or quality it can affect the success of reproduction, development, and survival of wild animals (Smith, 1980). Sources of food for each wild animal can certainly be different, but for herbivores, the presence, abundance, and composition of vegetation is the most important thing. Therefore, the Javan langur, which is a folivorous primate, is very dependent on the presence of trees because it uses leaves as its main food source. In addition, this primate known as ebony leaf langur also uses fruits, seeds, flowers, and several types of insects as secondary food sources (Kool, 1993).

b. Water

One of the most fundamental needs for wildlife in supporting their life is water. The factors that make up this habitat have a crucial role in the body's metabolism of wild animals. Water sources for wild animals can be obtained from three main sources, namely (1) free water, water that comes from water sources such as rivers or lakes; (2) preformed water, water contained in food; and (3) metabolic water, water produced by the body during metabolic processes (Sinclair et al., 2006).

In general, wildlife tends to use preformed water. This is because using water directly from rivers or lakes can pose a high risk for the survival of these wildlife such as threats from predators or pathogenic microbes. In addition, the water content in the feed is also sufficient to meet the water needs of some wild animals. Generally, the water content in the feed varies from 30% -80% of the total mass (Barboza et al., 2009).

Water not only plays a direct role in meeting the metabolic needs of wild animals, but can also play an indirect role by influencing habitat conditions such as the physical structure of the environment. One example of the significant role of water is by influencing the presence and pattern of vegetation. Some plant species are able to adapt to high water content and vice versa so that the vegetation that composes can be different (McComb, 2007).

c. Cover

The cover is part of the habitat used by wild animals to nest or escape from various disturbances (McComb, 2007). The existence of this factor for wildlife is something that cannot be separated from food and water sources. This is because every wildlife, including primates, depends on the existence of protection from various threats such as predators, weather, or anthropogenic (Texas Parks and Wildlife Department, 2010). Some of these protectors are represented by the shape of the nest, the character of the trees used, or the distance of the habitat from anthropogenic disturbances. (McComb, 2007; Febriyanti, 2008; Karanth et al., 2010).

Javan langurs generally choose trees with the characteristic architectural forms of cook's (umbrella-shaped branching), leeuwenberg's (narrowing upward branching), and schoute's (widening branching) as shelter according to their needs (Febriyanti, 2008). In addition, these primates will tend to choose habitats with low human intensity because of their shy and sensitive characteristics to protect themselves from anthropogenic disturbances. However, in some cases Javan langurs are also found in transitional areas between forest and plantations with a fairly high intensity of human activity. This is thought to be related to the efforts of the Javan langur in meeting the needs of food sources (Sulistiyadi et al, 2013).

In addition to these three resource components, environmental physical conditions such as topography are also important habitat components. Smith (1980) states that altitude is one of the abiotic factors that can affect environmental conditions such as the presence of vegetation, temperature, atmospheric pressure, rain precipitation, and ultraviolet (UV) radiation. These factors influence primates in choosing a suitable habitat (Grow et al., 2014). In general, primates prefer lowland habitats, but some primates can also be found up to 3000 meters above sea level (FAO, 1996). Especially for Javan langurs, their presence can be found in lowland forests (<800 masl) to highland forests (1600-2400 masl) (Nijman, 2013).

2.2.2 Habitat Suitability Model

Habitat Suitability Model (HSM) is an approach that relates the distribution of wild animals with their environmental conditions to obtain an overview of

suitable habitats for wildlife survival (Thuiller & Munkemuller, 2010). The HSM approach can be constructed using the Habitat Suitability Index (HSI), a numerical index that represents the level of habitat suitability for wildlife. This index has a minimum value of 0.0 which means the habitat is not suitable and a maximum value of 1.0 which indicates the suitability of the habitat is very optimal (U.S. Fish and Wildlife Service, 1981).

2.3 Geography information System

Geographic Information System (GIS) is a computer-based system capable of storing, analyzing and displaying data related to the earth's surface. In general, GIS is composed of five main interrelated components, namely people, applications, data, software, and hardware (figure 2.4). People are the components that run GIS, applications and data are the techniques and information needed to run a GIS. Meanwhile, software is the software used in conducting the analysis and hardware is the device that supports the software so that it can be used for GIS (Harmon & Anderson, 2003).

This system is also able to process geographic data simultaneously with physical or biological components in a database which is then displayed into a spatial component in the form of visual data (Darmawan, 2006; Sinclair et al., 2006). This system can also be combined with multivariate analysis so that in practice it can be used in multidisciplinary fields (Darmawan, 2006; Prayogo, 2014). In the last few decades, GIS has been widely used in the analysis of the distribution, occupancy, preference, and habitat suitability of a wildlife, one of which is also used in the analysis of distribution maps for endangered or protected primates (Jaya, 2002; Jorgensen, 2009;). This capability allows GIS to also be used in analysis and providing accurate data for wildlife conservation efforts (Guisan & Zimmerman, 2000; Calenge et al. 2008).

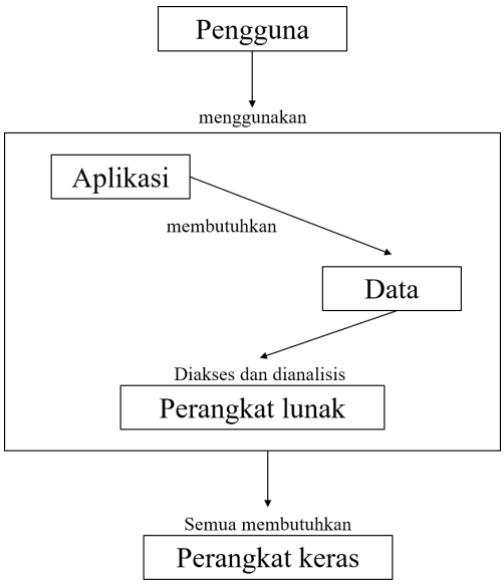


Figure 2.4 Component of GIS (Siurce: Harmon & Anderson, 2003)

The GIS approach is also often used in vegetation analysis or better known as the vegetation index. This index is an algorithm that is able to show vegetation conditions such as density, chlorophyll concentration, biomass, and even plant stress levels. However, to be able to perform the analysis, there are several types of indexes with different functions. One type of index that can be used to see the density of a vegetation is NDVI (Muhsoni, 2015).

The Normalized Different Vegetation Index (NDVI) is an index developed by the National Oceanic and Atmospheric Administration (NOAA) by utilizing weather satellite images that also pay attention to the global phenomenon of vegetation (Muhsoni, 2015). This index describes the greenness of a plant by using comparisons and subtractions from the reflection of satellite imagery of the red band (Red) and the Near Infrared (NIR) band (Lillesand et al., 2004). According to Jaya (2014), the NDVI vegetation index has a value range of (-1) to 1. The range of values <0 is non-vegetated areas such as vacant land, rocks, clouds, or bodies of water. Meanwhile, areas with an NDVI value range of > 0.1 are areas that have vegetation cover

2.4 Kucur Resort

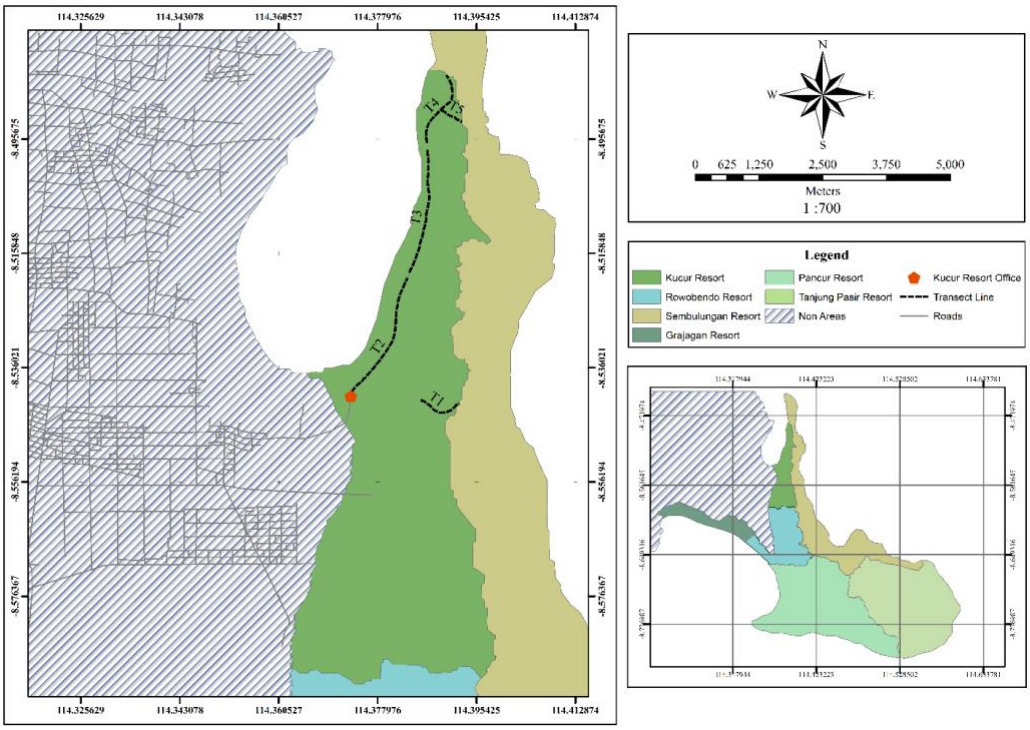
Kucur Resort is one of the resorts of APNP which is administratively included in the working area of SPTN II Muncar. This resort is directly adjacent to two other resorts, namely Sembulungan Resort in the east and Rowobendo Resort in the south. Meanwhile, the northern area is directly adjacent to Pang-Pang Bay and directly adjacent to the Perum Perhutan production forest in the west (Awang et al., 2011). The resort has been divided into three zones, namely the jungle zone, utilization zone, and rehabilitation zone (TNAP, 2017).

Kucur Resort also has varied contours with an altitude of 0-322 meters above sea level so that with this altitude this resort can be classified as lowland forest. (Awang et al., 2011). The lowland forests in TNAP are generally dominated by kepuh (Streculia foetida), bendo (Artocarpus elastica), Kedawung (Parkia roxburghii), candlenut (Aleurites moluccana), banyan (Ficus benjaminica), and forest kedondong (Spondias pinnata) (Ariyanto et al. al., 2011). But apart from that, teak (Tectona grandis) is also quite dominant as a remnant of the transition from Perum Perhutani production forest (Awang et al., 2011)

BAB 3 RESEARCH METHODS

3.1 Research Area

The study was conducted in January-June 2021 with data collection at the Kucur Resort APNP (Figure 3.1). The data analysis process was carried out at the Ecology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Jember.



(T1) Transect line 1; (T2) Transect line 2; (T3) Transect line 3; (T4) Transect line 4; (T5) Transect line 5

Figure 3.1 Research location in Kucur Resort, Alas Purwo National Park

3.2 Methods Procedure

2.7.1 Priliminary Survey

a. Identification and Verification of Javan langur (*Trachypithecus auratus*)

The survey began by conducting interviews with APNP officers and the surrounding community who are active in the Kucur Resort area regarding the presence of Javan langurs. This process was then continued by observing the Javan langur in the field of observation and recording the physical characteristics of the

Javan langur using a 10x50 mm Bushnell binocular and documenting it using a Canon 60d DSLR camera and a 300 mm telephoto lens. The Javan langur information obtained was then identified using books from Supriatna (2019) and KLHK (2019). Furthermore, this species is protected by assistance from the supervisor through a statement letter by the supervisor (Appendix 1).

b. Transect Survey Location

The determination of the location, size, and distance of the transect line is based on the results of interviews with TNAP officers, surrounding communities who are active in the TNAP area, and field observations. The location of the transect line that has been determined is recorded with the name and coordinates using the Garmin 64s Global Positioning System (GPS). Based on the survey, the five transect lines used include: the T1 transect line is on the trail to Pasir Putih Beach with teak forest and jungle ecosystem types, the T2-T4 transect line is on the trail to Sembulungan with the ecosystem type in the form of teak forest, and the T5 transect which is on the path to Payaman Beach with ecosystem types in the form of teak forests and jungle forests (Picture 3.1).

The habituation (getting used to) is carried out on the transect line that has been made. The habituation has also been carried out with the appropriate range of view to ensure the accuracy of the observations. This process is carried out so that the observation process can be carried out more clearly, efficiently, and easily.

3.2.2 Data Collecting

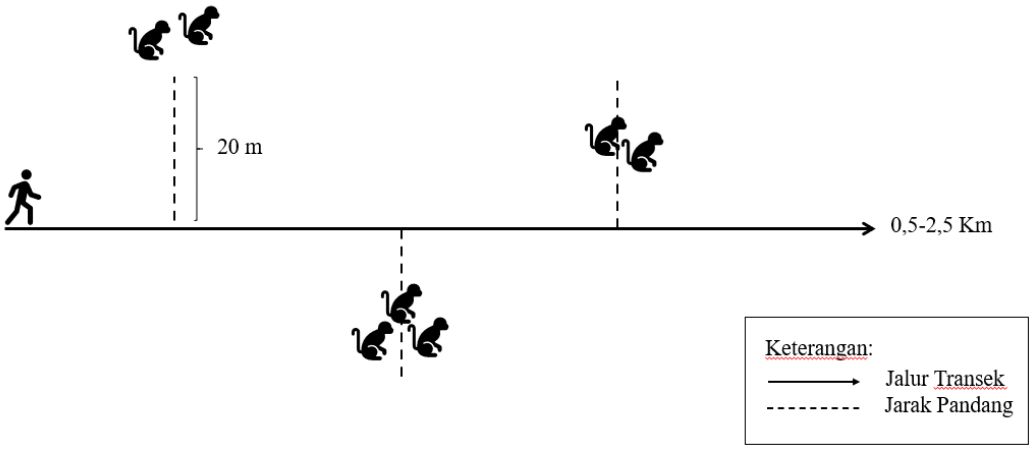
a. Data Type

The data consisted of primary and secondary data. The primary data in this study is data on encounters with Javan langurs obtained from the field. Secondary data includes Normalized Difference Vegetation Index (NDVI), river distances, settlement distances, and tourist distances in the form of satellite images and shapefile maps (shp) obtained from websites providing geographic information databases.

b. Encounter of Javan langurs

The encounter of Javan langur was carried out using a transect from the trail (Peres, 1999; Buckland *et al*., 2010; Hillario *et al*., 2011) at 07.00-16.30 WIB

according to the daily behavior of the Javan langur. The research team walked using the two-way sampling method (round-trip) on a transect line with a distance of about 0.5-2.5 km and when they met a Javan langur they would stop for about 30-45 minutes to make observations..



Picture 3.3 Design of transect line for encounters Javan langurs.

This was done with visibility as far as 20 m from the transect line using a 10x50 mm Bushnell binocular to make the observation process clearer (Figure 3.3). The observation process was also assisted by a Canon 60d DSLR camera and a 300 mm telephoto lens to document the group of Javan langurs encountered. The number of individuals, age ratio, and species that were observed were then recorded. Next, the coordinates of the location of the Javan langur are recorded using a Garmin 64s GPS (Leca *et al*, 2013).

c. Habitat Variable

The variable habitat is a determination based on the use of habitat classification variables by Guisan & Zimmerman (2000), which include using four main factors, that is vegetation, topography, water resources, and anthropogenic disturbances. The five factors are represented into five variables: (1) NDVI (Normalized Vegetation Index Difference), (2) altitude, (3) river, (4) settlement areas, and (5) tourist areas. Altitude variable data at Kucur Resort was obtained from the Earth Explorer website (https://earthdata.nasa.gov/) in the form of a Landsat 8 image map obtained on August 25, 2020. Meanwhile, river and

settlement variable data were obtained from the Indonesian Earth Map (RBI) (https http://portal.ina-sdi.or.id/), while the tourism variable was obtained from Google Earth Pro.

3.3 Data Analysis

3.3.1 Penggunaan Habitat Lutung Jawa

Primary and secondary data have been analyzed spatially into maps using ArcGIS 10.1 and grouped in Microsoft Excel 2019. The analysis was performed with the following stages:

a. NDVI Map

NDVI map of Resort Kucur APNP made with data that has been obtained from EarthData and then processed along with the data digitization Javan langur encounter using ArcGIS 10.1. The equation used to determine NDVI values are based on Landsat 8 bands (NIR= Near-Infrared; Red= Infrared) as in Equation 3.1 below (Jaya, 2014).

$$NDVI = \frac{NIR - RED}{NIR + RED} \quad \text{.....(3.1)}$$

Note:
NIR = Near Infrared
RED = Infrared

b. Altitude Map

The altitude map of Resort Kucur APNP was created using data obtained from EarthData and then processed together with digitized data on encounters with Javan langurs using ArcGIS 10. So the altitude used by javan langurs can be determined.

c. River Distance Map

Map distance of the river created using data obtained from https://portal.ina-sdi.or.id/ and then processed along with the data digitization encounter Javan langur using spatial analysis tools in ArcGIS 10.1 so that the distance between the Javan langur river can be determined.

d. Settlements Distance Map

The settlement distance map was made using data obtained from the Indonesian Earth Map (RBI) and then processed together with digitized data on encounters of Javan langurs using the spatial analysis tool in ArcGIS 10.1 and then the distance between Javan langurs so that the distance between Javan langurs and settlements can be determined.

e. Tourism Distance Map

The distance map of the tourist area was made using data obtained from Google Earth and then processed together with digitized data on encounters of Javan langurs using spatial analysis tools in ArcGIS 10.1 so that the distance between Javan langurs and tourism can be determined.

3.3.2 Habitat Suitability Level

The five maps that have been made based on the habitat variable were then analyzed simultaneously with the scoring overlay method using ArcGIS 10.1. Each habitat variable was classified and given a score based on the Javan langur habitat preferences. The condition of the habitat variable that is most favored by the Javan langur is given the highest score of 4, while the condition of the habitat variable that is least favored will be given the lowest score of 1 [Table 1]. (U.S. Fish and Wildlife Service, 1981).

$$\text{HSI} = \frac{Output\ Rank\ (skor) for\ the\ Area\ of\ Interest}{20} \quad \text{.....(3.2)}$$

Tabel 3.1 *Ranking classification of Java langur habitat uses and suitability* (Abdillah, 2014; Jaya, 2014)

No	Habitat Variable	Classification	*Rank*
1.	NDVI	< -0,5	1
		-0,5 – 0,0	2
		0,0 – 0,5	3
		>0,5	4
2.	Altitude (masl)	0-100	4
		100-200	3
		200-300	2
		>300	1
3.	River Distance (m)	0-100	4
		100-200	3
		200-300	2
		>300	1

4.	Settlement Distance (m)	0-250	1
		250-500	2
		500-750	3
		>750	4
5.	Tourism Distance (m)	0-250	1
		250-500	2
		500-750	3
		>750	4

The scores obtained are then totaled and converted into a habitat index using equation (2) [17]. This was done to determine the overall level of suitability of the Javan langur habitat at Kucur Resort APNP. The area level is based on four classifications, namely Excellent, High, average, and below-average (Table 3.2) (U.S. Fish and Wildlife Service, 1981).

Tabel 3.2 level classification of Javan langur habitat suitability

Habitat Suitability Level	Habitat Suitability Index (HSI)
Excellent	0,76-1,0
High	0,51-0,75
Average	0,26-0,50
Below-average	0,0-0,25

BAB 4. RESULTS AND DISCUSSION

4.1 Javan Langur Habitat Use

The observation shows that the javan langurs can only be found on three transects, that is a T1 line, T2 line, and T3 line with the total encounter points obtained were seven points and from that, three different groups of javan langurs were identified (Figure 4.1). The difference in the number of those encounters (Table 4.1) is assumed to have relation with the habitat uses of the javan langur on the five variables in this study.

Table 4.1 The number of encounter points of Javan langurs

Group	Transect Line	Number of Encounter Points
I	T1	2
II	T2	3
III	T3	2

(A) Group I, (B) Group II, (C) Group III
Picture 4.1 The javan langur groups were found at T1, T2, and T3.

Based on the spatial analysis using ArcGIS 10.1 the habitat use by the Javan langur is explained as follows:

a. NDVI

The results of spatial analysis on the NDVI variable show that Kucur Resort tends to be dominated by areas with NDVI values in the range of 0.0 - 0.5 (Table 4) and can be categorized as vegetation with medium density. The results of this analysis show that most of the Kucur resort areas can reflect the Nir-infrared band satellite imagery, which means that the vegetation at Kucur Resort is in fairly

healthy condition. The condition indicates that the vegetation in this resort has a fairly dense canopy as described by Zaitunah et al. (2018) As a result of this NDVI value, it is not surprising that all Javan langur encounter points were found to dominate in areas with medium vegetation density (Figure 4.2).

Tabel 4.2 Titik perjumpaan lutung jawa berdasarkan variabel NDVI

No	NDVI	Luas (Ha)	Jumlat Titik Perjumpaan
1.	< -0,5	0	0
2.	-0,5 – 0,0	316,60	0
3.	0,0 – 0,5	2053,08	7
4.	>0,5	0	0

The number of encounter points that dominate in habitats with medium vegetation density can occur because Javan langurs are very dependent on the presence of trees. This arboreal primate uses the canopy and tree branches to carry out all its activities such as resting, mobilizing, socializing, and sheltering from predators. The condition of the canopy and dense tree branches at this resort will certainly provide convenience for the Javan langur in carrying out these activities. This was confirmed by Subarkah et al., (2013) and Tsuji et al., (2016) which states that the Javan langur vegetation utilizes as much as 20-40% in the move. (Figure 4.2).

Therefore, the area with a medium NDVI value can attract many Javan langurs to use this habitat. In addition, this resort is also dominated by forage plants that are often used by Javan langurs. Plant species such as kepuh (Sterculia foetida), bendo (Artocarpus elastica), kedawung (Parkia roxburghii), and kedodong (Spondias pinnata) are forage plants that are quite often used by Javan langurs (Tsuji, 2019).

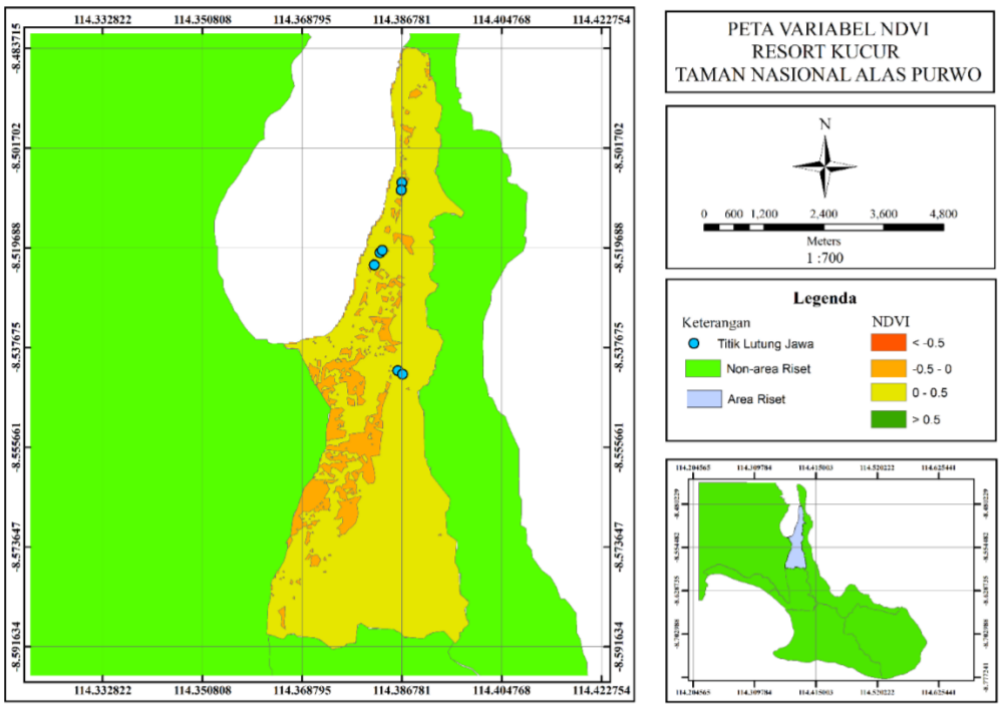


Figure 4.2 Map of Habitat Use of Javan Langur Based on NDVI

b. Altitude

The results of the spatial analysis on the altitude variable show that Kucur Resort has an altitude ranging from 0-300 masl (Figure 4.3). The meeting point is the javan langur gained as much as 6 points are located at an altitude of 0-100 meters above sea level and 1 point at an altitude of 100-200 meters above sea level (Table 4.3). The lowest altitude found in javan langur encounters was at 13 meters above sea level, while the highest altitude was at 120 meters above sea level.

Javan langurs are primates that can utilize habitats with very varied altitudes, even reaching a height of 3000 masl (Nijman, 2013). However, based on the results of this analysis, javan langurs are more dominant using habitats located at an altitude of <100 masl. The dominance of habitat use showed that the javan langur prefers to use habitat in the lowlands compared to the habitat in the highlands. It is thought to be related to two reasons, namely the structure of vegetation and physical factors of the environment.

Table 4.3 Javan langur encounter points based on the altitude variable

No	Altitude (masl)	Area (Ha)	Number of Encounter Points

1.	0-100	1402.02	6
2.	100-200	507.43	1
3.	200-300	463.22	0
4.	>300	0	0

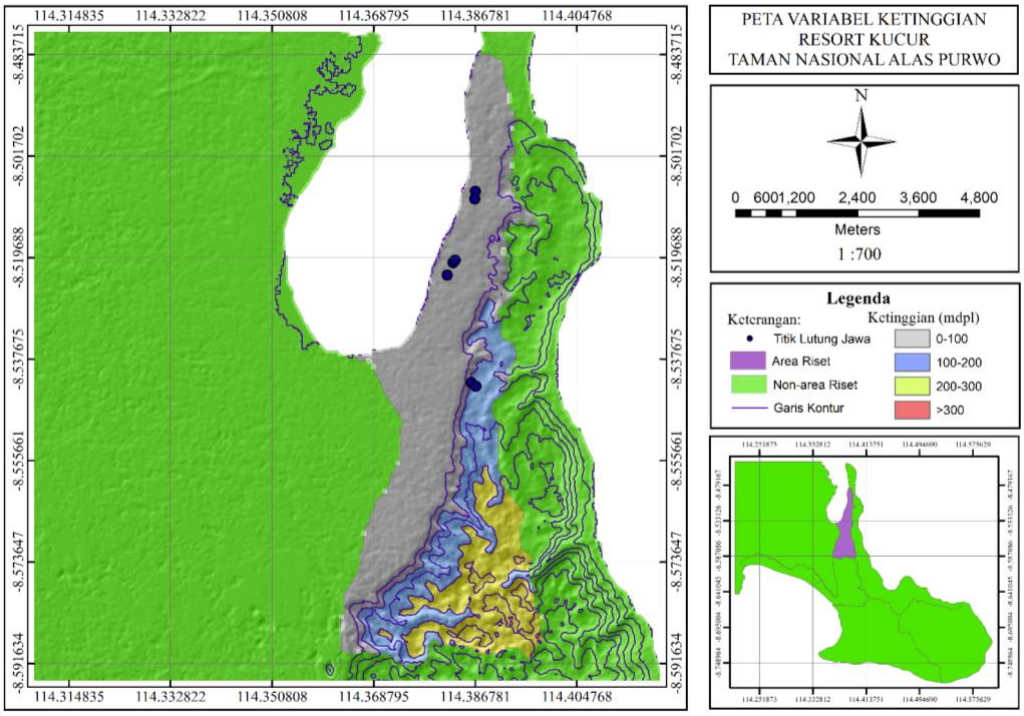


Figure 4.3 Map of Habitat Use of Javan Langur Based on altitude

Lowlands in general have a more diverse vegetation structure compared to the highland. High tend plant diversity allows the javan langur to be able to have more variant feed sources. This factor is according to Lomolino (2000) and Monge-Gonzalez (2019) which state that plant diversity will tend to increase as altitude decreases. In addition, the vegetation in the highlands also tends to be dominated by coniferous vegetation with a small number and form of branches and not too wide in size so that these arboreal primates are unable to use these branches optimally in carrying out their activities.

Altitude is also capable of affecting physical factors in an environment such as temperature and humidity. Regions that are in the lowland will have warm temperatures and humid air conditions, while the highland tends to be cold and dry. The warm and humid conditions are very suitable for the javan langur in carrying

out various activities such as feeding, grooming, and other social activities. Eliana et al., (2017) and Aryanti & Azizah (2019) state that the ideal habitat conditions for javan langur activity are temperatures in the range of 22-33 oC and humidity of around 79-89%.

Javan langur also uses habitats with low altitudes to avoid other physical factors as exposure to ultraviolet radiation (UV). Excessive exposure to UV rays from solar radiation can affect the quality of javan langur feed. According to Sayers (2014), UV can influence the susceptibility of parasites and reduce the productivity of the plant so that the feed quality of wildlife can be decreased.

c. River Distance

The results of the spatial analysis of river distance variables showed that all meeting points of javan langurs obtained at Kucur Resort were at a distance of >300 m from the river (Table 4.4). The closest encounter point to the river is at a distance of 469 m and the furthest distance is 2227 m. These data indicate that the javan langur prefers to use habitat away from the river (Figure 4.4). It is quite in contrast to some previous studies (Abdillah, 2014; Astriani, 2015). However, this contradiction shows that the Javan langur is not entirely dependent on the existence of river water to meet its needs, but other factors also play a role in influencing it. Based on this analysis, three factors are assumed to influence the Javan langur to stay away from the river and do not use the water directly. These factors include (1) the use of preformed water, (2) the avoidance of predation, and (3) the arboreal behavior of the Javan langur.

Table 4.4 Javan langur encounter points based on the river distance variable

No	River Distance (m)	Areas (Ha)	Number of Encounter Points
1.	0-100	291.18	0
2.	100-200	261.71	0
3.	200-300	254.73	0
4.	> 300	1565.82	7

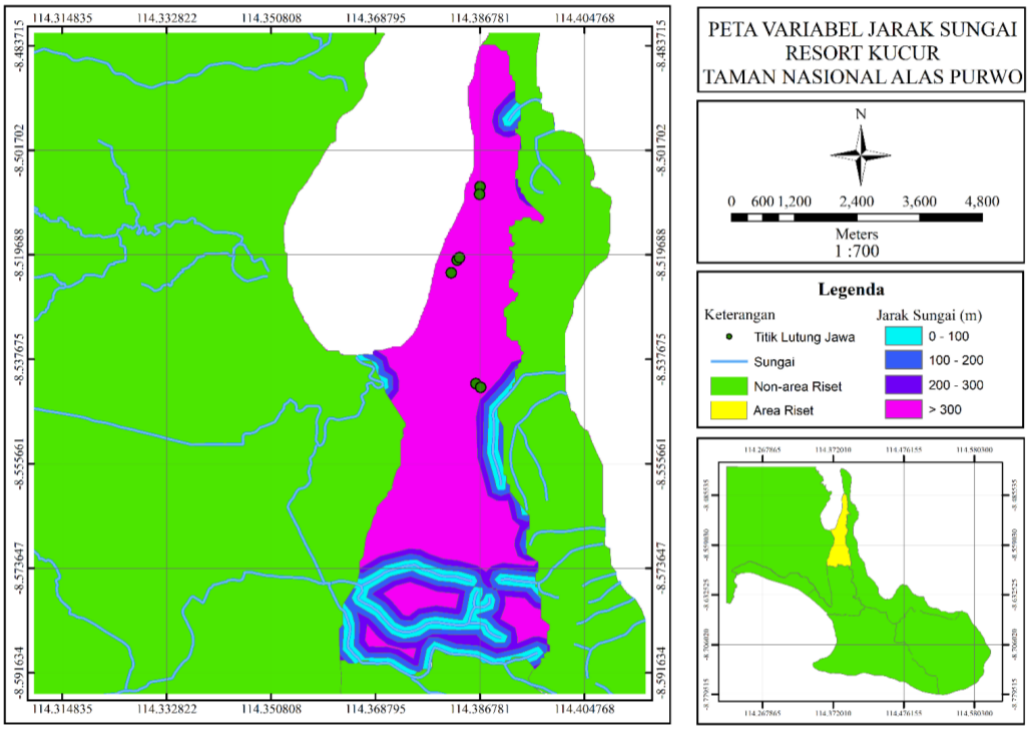


Figure 4.4 Map of Habitat Use of Javan Langur Based on River Distance

Primates are included in the subfamily Colobines known to utilize the presence of preformed water by their feed to meet water needs. These primates, including the javan langur, can utilize their feed such as young leaves, flowers, and fruit to meet the needs of the water. This is because the water in the feed has been able to meet the body's water needs by 60% (Kullik, 2010; Suarez 2013) of what it needs. In addition, there is also another possibility that these primates use water sources that come from hollows in trees or dew on leaves.

Javan langurs in these results prefer habitats away from rivers even though the river used in the analysis is a year-round river. This is thought to be related to the second assumption, namely the efforts of the javan langur to increase survival from the threat of predation. Generally, the river is an open area that allows all wildlife to come to the area to drink, including the javan langur predator, the leopard (Panthera pardus melas). If the javan langur down to the forest floor and use the river water, indirectly increases the risk of predation.

The arboreal behavior of the javan langur is also another factor that plays a role in influencing the Javan langur to be near the river. This is because these

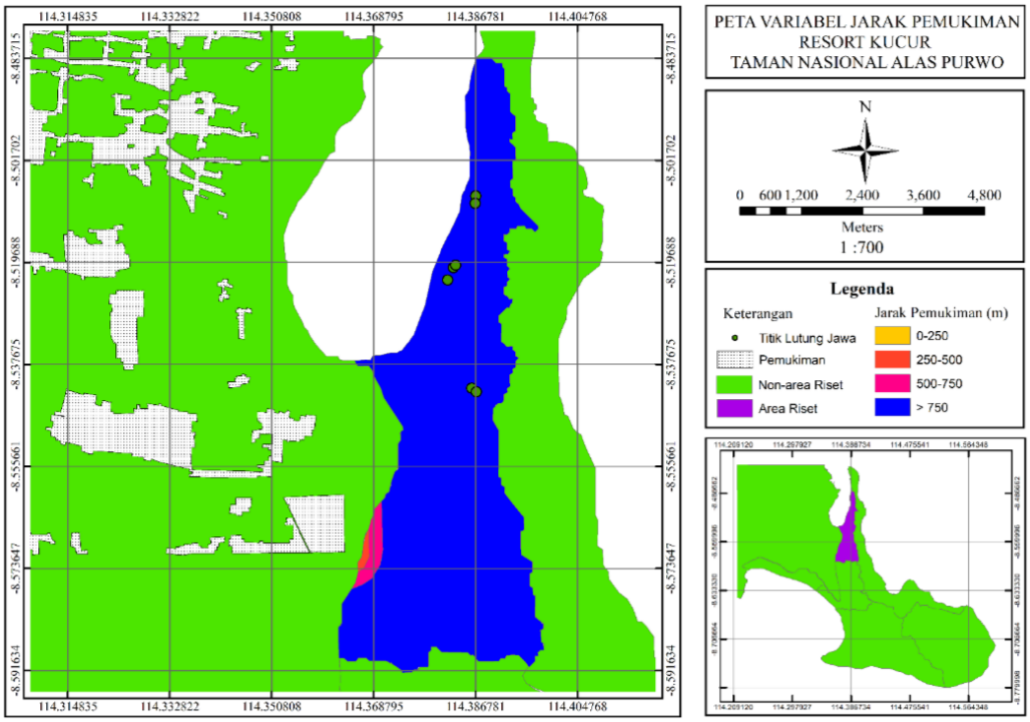
primates spend more of their time up in the trees to eat, move, groom, or rest rather than go down to the ground to carry out these activities. Meanwhile, to descend to the forest floor only in certain cases these primates do it, such as picking up fallen food and also geophagy. This was confirmed by Qamariah (2015) and Santono *et al*., (2016) which states at least the intensity of the javan langur to descend to the forest floor.

d. Settlements Distance

The results of the spatial analysis of the settlement variables around the Kucur Resort show that all javan langur encounter points are found at a distance of >750 m from the settlement (Table 4.5). Javan langur was found using the nearest habitats with human settlements at a distance of 3,107 m. Meanwhile, the farthest habitat used is at a distance of 4,130 m. Based on these results it can be seen that the javan langur prefers to use habitat away from settlements (Figure 4.5). Activities and human high-intensity residential areas are the reason that causes this primate to away from the area. This is because the habitat close to the settlement is not able to protect the javan langur against anthropogenic disturbance.

Tabel 4.5 Javan langur encounter points based on the settlements distance.

No	Settlement Distance (m)	Areas (Ha)	Number of Encounter Points
1.	0-250	0.00	0
2.	250-500	8.85	0
3.	500-750	38.72	0
4.	>750	2325.12	7



Picture 4.5 Map of Habitat Use of Javan Langur Based on Settlement Distance

In addition, the close distance between Kucur Resort and settlements in Tegaldlimo District, Banyuwangi makes access to this resort easier. This is proven by a sufficient number of community activities around the entrance into this resort area for fishing or search for clams. This increased human activity can influence the javan langur to choose a habitat, such as the opinion of Karanth et al. (2010) which states that the presence of settlements in a national park area can influence the existence of primates.

e. Tourism Distance

Analysis of the variables showed that the entire travel distance javan langur habitat encountered using a distance> 750 m from the tourist areas (Table 4.6). As for the point of closest encounter javan langur is located at a distance of 1371 m from the tourist areas and the encounters point farthest Javan langur is at a distance of 3900 m. The tendency of Javan langurs to use habitats away from the tourist area is assumed to be related to the high intensity of human presence around the mangrove jungle track tourism area.

Table 4.6 Javan langur encounter points based on the tourism distance variable

No	Tourism Distance (m)	Areas (Ha)	Number of Encounter Points
1.	0-250	27.52	0
2.	250-500	57.07	0
3.	500-750	60.57	0
4.	> 750	2228.83	7

Kucur Resort is one of the resorts in APNP which is also used as a mangrove jungle track tourism area. The attraction thas located in Pang-Pang Bay has been built since 2018 and is still actively operating to receive tourists. The presence of these tourists can cause the intensity of human activity in the Kucur Resort area to increase and is expected to affect the habitat use of the Javan langur.

Javan langurs can also be known to use mangrove forests as one of their habitats (Nowak, 2013). However, during observations, there were no encounters with Javan langurs that approached the mangroves around the tourist area. The activity of javan langurs approaching the mangrove was found between the T2 transect and the T3 transect. It can be caused by the influence of human presence can indirectly cause stress in primates (Figure 4.6). According to Russon & Wallis (2014), tourism can also cause stress, even in severe conditions it can change behavior to reduce the mortality of primate chicks. Therefore, the javan langur chooses to stay away from tourist areas to still be able to meet their needs in the mangrove forest.

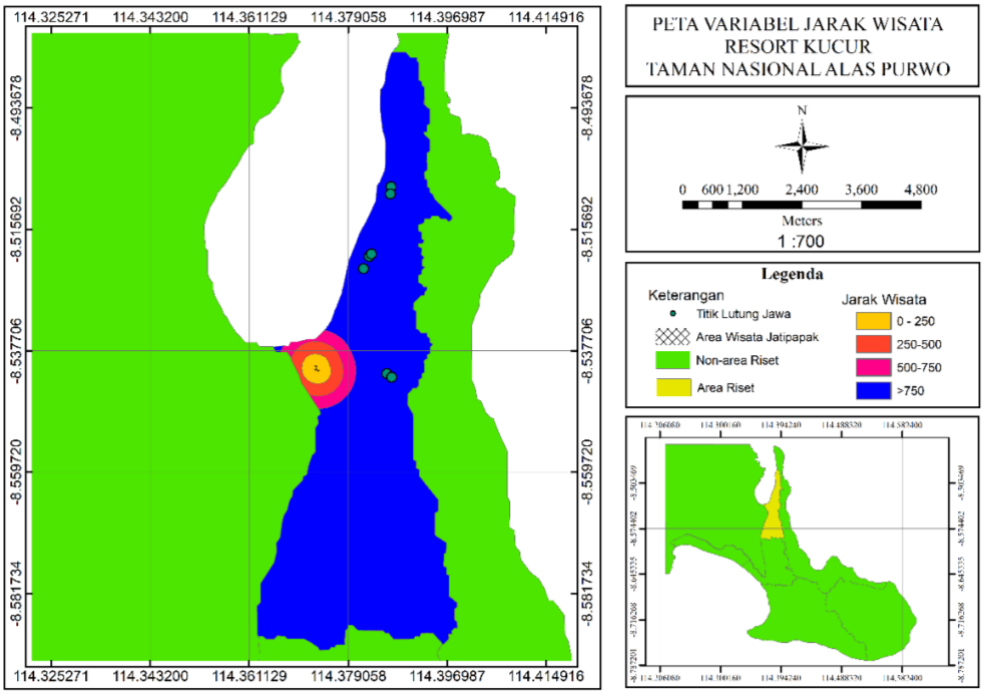


Figure 4.6 Map of Habitat Use of Javan Langur Based on Tourism Distance

4.2 Habitat Suitability Level

Spatial analysis of the habitat suitability level showed that Resort Kucur has an HSI value in the range of 0.60 to 0.95 (Appendix 4). Based on these results, the Javan langur habitat at Kucur Resort can be classified as a habitat with an excellent and high level of suitability. Habitat with an excellent level of suitability at this resort has an HSI value range of 0.80-0.95 with an area of 1456.5 Ha. Meanwhile, habitats with a high level of suitability have a range of HSI values in the range of 0.6-0.75 with an area of 898.89 Ha (Table 4.7). The analysis showed that habitats that have an excellent level of suitability are the dominant habitats at Kucur Resort. These results are assumed to be due to the influence of several variables used in determining the level of habitat suitability, including NDVI, altitude, and Settlement distance.

Table 4.7 Habitat suitability level of javan langur

No	Habitat Suitability Level	Habiat Suitability Index (HSI)	Areas (Ha)	Total Meeting Point
1.	Sangat Tinggi	0,76-1,0	1456,25	6

2.	Tinggi	0,51-0,75	898,89	1
3.	Sedang	0,26-0,50	0	0
4.	Rendah	0,0-0,25	0	0

The NDVI value can affect this level because most of the Kucur Resort areas have NDVI values which indicate medium density and vegetation cover. Based on the altitude variable, this resort has a height that is widely used by javan langurs, which is at an altitude of <100 meters above sea level. Meanwhile, the habitat of the javan langur is still quite away from community settlements so that anthropogenic disturbances are quite rare. These results are consistent with Dong et al., (2019) which states that altitude and community settlement are important factors in determining habitat suitability.

The results of this analysis also show that six encounter points were found at an excellent level (Figure 4.7). Javan langur groups II and III were found in habitats with an HSI value of 0.8. While the Javan langurs from group I were found to be at two levels of habitat suitability with HSI values of 0.8 and 0.75. The high number of encounter points in habitats with an excellent level of suitability can be caused by the carrying capacity of these habitats in providing the needs of javan langurs to survive. The availability of vegetation as a source of food and shelter, suitable altitude, and minimal anthropogenic disturbance will help javan langurs to use these habitats. This is following Yumarni (2012) which states that a wide and good quality habitat will certainly attract the presence of wild animals to inhabit the habitat.

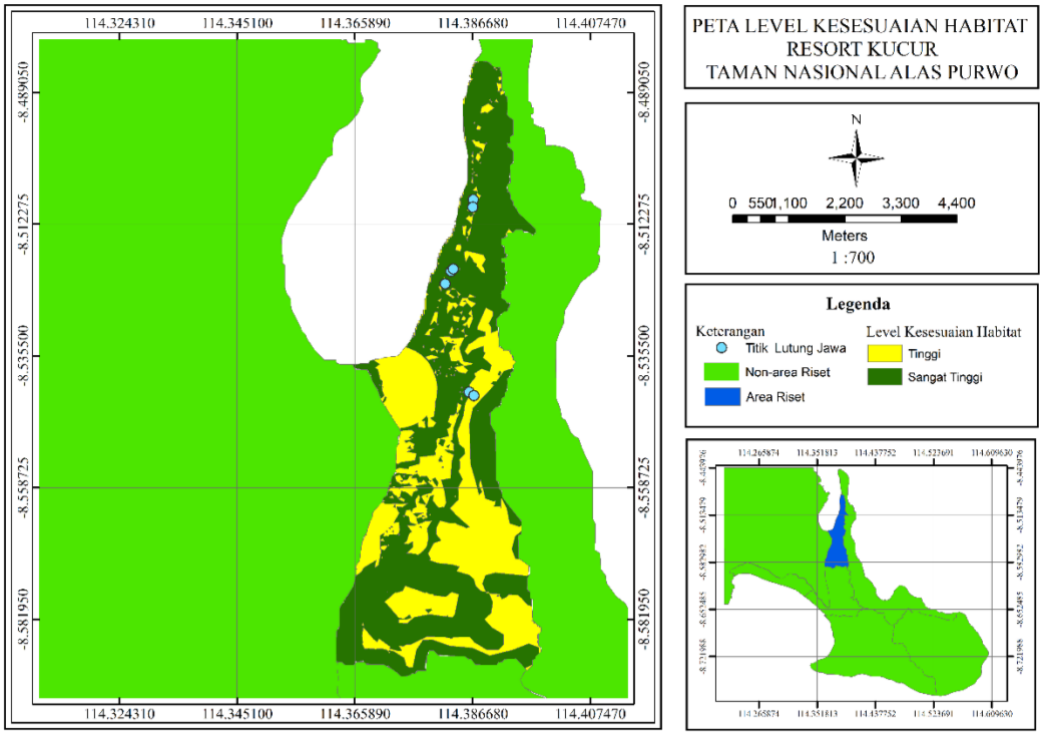


Figure 4.7 Map of Habitat Suitability of Javan Langur in Kucur Resort APNP

Based on these results, it can be seen that Kucur Resort is quite suitable for the survival of the javan langur. This is because the Kucur resort can meet the needs of the javan langur. Wedana et al., (2013) even categorize the entire APNP area as one of the good habitats for jvan langurs because it has a fairly low level of disturbance. However, the APNP, especially the Kucur Resort, still needs to improve its efforts to improve habitat quality for the javan langur. One of these efforts is to increase the density and quality of vegetation cover in the Kucur Resort area so that it becomes higher so that it can provide more abundant availability of food trees or sleeping trees for javan langurs.

BAB 5. CONCLUSION AND SUGGESTION

5.1 Conclusion

1. Javan langurs in Kucur Resort used habitats that have an NDVI category with medium vegetation density, lowlands, and a considerable distance from rivers, settlements, and tourism.
2. The level of suitability of the Javan langur habitat at Kucur Resort can be classified into two levels, namely very high (1456.25 Ha) and high (898.89 Ha).

5.2 Suggestion

1. Data collection on Javan langur encounters can be carried out using non-pathway transects to increase the number of Javan langur encounter points.

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APPENDIX

Appendix 1. Verification Letter

KEMENTERIAN PENDIDIKAN DAN KEBUDAYAAN
UNIVERSITAS JEMBER
FAKULTAS MATEMATIKA DAN ILMU PENGETAHUAN ALAM
JURUSAN BIOLOGI
Jalan Kalimantan 37 – Kampus Tegal Boto Kotak Pos 159 Jember 68121
MIPA (0331) 337818, Fax. (0331) 337818

SURAT KETERANGAN VERIFIKASI

Berdasarkan pengamatan morfologi hasil dokumentasi spesimen lutung jawa yang dilakukan di Taman Nasional Alas Purwo dan Laboratorium Ekologi, Jurusan Biologi, FMIPA Universitas Jember oleh:

Nama	: Haikal Idris Maulahila
Tempat/Tgl.Lahir	: Banyuwangi, 03 September 2000
Status	: Mahasiswa S1
Nim	: 171810401031
Jurusan	: Biologi

Hasil identifikasi yaitu lutung jawa (*Trachypithecus auratus* Geoffroy, 1812) yang termasuk dalam Famili Cercopithecidae. Verifikasi tersebut didampingi oleh Arif Mohamad Siddiq, S.Si., M.Si dengan mecermati referensi:

1. Supriatna, J. 2019. *Field Guide to the Indonesia Primates*. Jakarta: Yayasan Pustaka Buku Obor.
2. Kementerian Lingkungan Hidup dan Kehutanan. 2019. *Panduan Identifikasi Jenis Satwa Liar Dilindungi*. Jakarta: Kemeterian Kementerian Lingkungan Hidup dan Kehutanan & Lembaga Ilmu Pengetahuan Indonesia.

Demikian surat keterangan ini dibuat untuk dapat digunakan sebagaimana mestinya.

Jember, 19 Maret 2021

Pendamping

Arif Mohammad Siddiq, S.Si., M.Si.
NIP. 760018007

Lampiran 2. Kelompok Lutung Jawa

Kode Koordinat	Jalur Transek	Jarak transek (km)	Kelompok	Jumlah Individu (ekor)	Jenis Kelamin		Struktur Umur			Jumlah titik perjumpaan
					Jantan	Betina	Anakan	Remaja	Dewasa	
2851-LT1; 2851-LT2	T1	1	I	10		3	3		7	2
2850-LT1; 2850-LT2; 2850-LT5.2	T2	5	II	11	1	1	1	3	7	3
2850-LT4; 2850-LT1	T3	5	III	9		2		1	3	2

Appendix 3. Habitat use of Javan langur based on NDVI, altitude, river distance, river distance, settlement distance, and tourism distance

No	Code	NDVI	Altitude (masl)	River_Dstnc (m)	Stlment_Dstnc (m)	Tourism_Dstnc (m)
1.	2850-LT1	0.21	13	960.469	4108.36	3903.33
2.	2850-LT2	0.24	13	2206.17	3712.86	2183.3
3.	2850-LT3	0.26	14	1973.85	3969.65	2447.3
4.	2850-LT4	0.26	18	1001.3	4130.21	3770
5.	2850-LT5.2	0.31	16	1909.66	4012.04	2509.54
6.	2851-LT1	0.34	94	553.173	3107.28	1370.58
7.	2851-LT2	0.27	120	445.982	3186.36	1464.14

Appendix 4. Value of HSI Level of Habitat Suitability Javan Langur.

FID	NDVI	Altitude	River	Settlement	Tourism	HSI	Level
148	2	4	1	4	1	0.6	Tinggi
151	2	4	1	4	1	0.6	Tinggi
153	2	4	1	4	1	0.6	Tinggi
163	2	4	1	4	1	0.6	Tinggi
165	2	4	1	4	1	0.6	Tinggi
196	2	4	1	4	1	0.6	Tinggi
122	2	4	1	4	2	0.65	Tinggi
124	2	4	1	4	2	0.65	Tinggi
126	2	4	1	4	2	0.65	Tinggi
131	2	4	1	4	2	0.65	Tinggi
134	2	4	1	4	2	0.65	Tinggi
149	2	4	1	4	2	0.65	Tinggi
152	2	4	2	4	1	0.65	Tinggi
154	3	4	1	4	1	0.65	Tinggi
156	2	4	2	4	1	0.65	Tinggi
171	2	4	2	4	1	0.65	Tinggi
188	2	4	1	4	2	0.65	Tinggi
197	2	4	1	4	2	0.65	Tinggi
200	2	4	2	4	1	0.65	Tinggi
252	2	4	1	2	4	0.65	Tinggi
262	2	4	1	2	4	0.65	Tinggi
351	3	4	1	4	1	0.65	Tinggi
118	2	4	1	4	3	0.7	Tinggi
119	2	4	1	4	3	0.7	Tinggi
120	2	4	1	4	3	0.7	Tinggi
125	2	4	1	4	3	0.7	Tinggi
127	2	4	1	4	3	0.7	Tinggi
137	2	4	1	4	3	0.7	Tinggi
150	2	4	1	4	3	0.7	Tinggi
155	3	4	1	4	2	0.7	Tinggi
157	2	4	3	4	1	0.7	Tinggi
159	3	4	1	4	2	0.7	Tinggi
160	3	4	1	4	2	0.7	Tinggi
166	3	4	1	4	2	0.7	Tinggi
167	3	4	1	4	2	0.7	Tinggi
172	2	4	2	4	2	0.7	Tinggi
173	2	4	3	4	1	0.7	Tinggi
177	2	4	2	4	2	0.7	Tinggi
182	3	4	1	4	2	0.7	Tinggi
186	3	4	1	4	2	0.7	Tinggi
189	2	4	1	4	3	0.7	Tinggi

FID	NDVI	Elevasi	Sungai	Pemukiman	Wisata	HSI	Level
190	2	4	1	4	3	0.7	Tinggi
192	2	4	1	4	3	0.7	Tinggi
194	2	4	1	4	3	0.7	Tinggi
198	2	4	1	4	3	0.7	Tinggi
201	2	4	2	4	2	0.7	Tinggi
235	2	4	1	3	4	0.7	Tinggi
236	2	4	1	3	4	0.7	Tinggi
238	2	4	1	3	4	0.7	Tinggi
239	2	4	1	3	4	0.7	Tinggi
241	2	4	1	3	4	0.7	Tinggi
243	2	4	1	3	4	0.7	Tinggi
245	3	4	1	2	4	0.7	Tinggi
251	2	2	2	4	4	0.7	Tinggi
253	2	4	1	3	4	0.7	Tinggi
256	3	4	1	2	4	0.7	Tinggi
257	2	4	1	3	4	0.7	Tinggi
263	2	4	2	2	4	0.7	Tinggi
274	2	3	1	4	4	0.7	Tinggi
302	3	2	1	4	4	0.7	Tinggi
317	3	2	1	4	4	0.7	Tinggi
322	3	2	1	4	4	0.7	Tinggi
325	3	2	1	4	4	0.7	Tinggi
326	3	2	1	4	4	0.7	Tinggi
349	3	4	1	2	4	0.7	Tinggi
352	3	4	1	4	2	0.7	Tinggi
357	3	4	2	4	1	0.7	Tinggi
0	2	4	1	4	4	0.75	Tinggi
1	2	4	1	4	4	0.75	Tinggi
2	2	4	1	4	4	0.75	Tinggi
3	2	4	1	4	4	0.75	Tinggi
4	2	4	1	4	4	0.75	Tinggi
5	2	4	1	4	4	0.75	Tinggi
6	2	4	1	4	4	0.75	Tinggi
7	2	4	1	4	4	0.75	Tinggi
8	2	4	1	4	4	0.75	Tinggi
9	2	4	1	4	4	0.75	Tinggi
10	2	4	1	4	4	0.75	Tinggi
11	2	4	1	4	4	0.75	Tinggi
13	2	4	1	4	4	0.75	Tinggi
14	2	4	1	4	4	0.75	Tinggi
15	2	4	1	4	4	0.75	Tinggi
16	2	4	1	4	4	0.75	Tinggi
17	2	4	1	4	4	0.75	Tinggi

FID	NDVI	Elevasi	Sungai	Pemukiman	Wisata	HSI	Level
18	2	4	1	4	4	0.75	Tinggi
19	2	4	1	4	4	0.75	Tinggi
20	2	4	1	4	4	0.75	Tinggi
21	2	4	1	4	4	0.75	Tinggi
22	2	4	1	4	4	0.75	Tinggi
23	2	4	1	4	4	0.75	Tinggi
24	2	4	1	4	4	0.75	Tinggi
26	2	4	1	4	4	0.75	Tinggi
27	2	4	1	4	4	0.75	Tinggi
28	2	4	1	4	4	0.75	Tinggi
29	2	4	1	4	4	0.75	Tinggi
30	2	4	1	4	4	0.75	Tinggi
31	2	4	1	4	4	0.75	Tinggi
32	2	4	1	4	4	0.75	Tinggi
33	2	4	1	4	4	0.75	Tinggi
34	2	4	1	4	4	0.75	Tinggi
35	2	4	1	4	4	0.75	Tinggi
36	2	4	1	4	4	0.75	Tinggi
37	2	4	1	4	4	0.75	Tinggi
38	2	4	1	4	4	0.75	Tinggi
39	2	4	1	4	4	0.75	Tinggi
40	2	4	1	4	4	0.75	Tinggi
41	2	4	1	4	4	0.75	Tinggi
42	2	4	1	4	4	0.75	Tinggi
43	2	4	1	4	4	0.75	Tinggi
44	2	4	1	4	4	0.75	Tinggi
45	2	4	1	4	4	0.75	Tinggi
46	2	4	1	4	4	0.75	Tinggi
47	2	4	1	4	4	0.75	Tinggi
48	2	4	1	4	4	0.75	Tinggi
49	2	4	1	4	4	0.75	Tinggi
50	2	4	1	4	4	0.75	Tinggi
51	2	4	1	4	4	0.75	Tinggi
52	2	4	1	4	4	0.75	Tinggi
53	2	4	1	4	4	0.75	Tinggi
54	2	4	1	4	4	0.75	Tinggi
55	2	4	1	4	4	0.75	Tinggi
56	2	4	1	4	4	0.75	Tinggi
57	2	4	1	4	4	0.75	Tinggi
58	2	4	1	4	4	0.75	Tinggi
59	2	4	1	4	4	0.75	Tinggi
60	2	4	1	4	4	0.75	Tinggi
61	2	4	1	4	4	0.75	Tinggi

FID	NDVI	Elevasi	Sungai	Pemukiman	Wisata	HSI	Level
62	2	4	1	4	4	0.75	Tinggi
63	2	4	1	4	4	0.75	Tinggi
64	2	4	1	4	4	0.75	Tinggi
65	2	4	1	4	4	0.75	Tinggi
66	2	4	1	4	4	0.75	Tinggi
67	2	4	1	4	4	0.75	Tinggi
68	2	4	1	4	4	0.75	Tinggi
69	2	4	1	4	4	0.75	Tinggi
70	2	4	1	4	4	0.75	Tinggi
71	2	4	1	4	4	0.75	Tinggi
72	2	4	1	4	4	0.75	Tinggi
73	2	4	1	4	4	0.75	Tinggi
74	2	4	1	4	4	0.75	Tinggi
75	2	4	1	4	4	0.75	Tinggi
76	2	4	1	4	4	0.75	Tinggi
77	2	4	1	4	4	0.75	Tinggi
78	2	4	1	4	4	0.75	Tinggi
79	2	4	1	4	4	0.75	Tinggi
80	2	4	1	4	4	0.75	Tinggi
81	2	4	1	4	4	0.75	Tinggi
82	2	4	1	4	4	0.75	Tinggi
83	2	4	1	4	4	0.75	Tinggi
84	2	4	1	4	4	0.75	Tinggi
86	2	4	1	4	4	0.75	Tinggi
87	2	4	1	4	4	0.75	Tinggi
88	2	4	1	4	4	0.75	Tinggi
89	2	4	1	4	4	0.75	Tinggi
90	2	4	1	4	4	0.75	Tinggi
91	2	4	1	4	4	0.75	Tinggi
92	2	4	1	4	4	0.75	Tinggi
93	2	4	1	4	4	0.75	Tinggi
94	2	4	1	4	4	0.75	Tinggi
95	2	4	1	4	4	0.75	Tinggi
96	2	4	1	4	4	0.75	Tinggi
97	2	4	1	4	4	0.75	Tinggi
98	2	4	1	4	4	0.75	Tinggi
99	2	4	1	4	4	0.75	Tinggi
100	2	4	1	4	4	0.75	Tinggi
101	2	4	1	4	4	0.75	Tinggi
102	2	4	1	4	4	0.75	Tinggi
103	2	4	1	4	4	0.75	Tinggi
104	2	4	1	4	4	0.75	Tinggi
105	2	4	1	4	4	0.75	Tinggi

FID	NDVI	Elevasi	Sungai	Pemukiman	Wisata	HSI	Level
106	2	4	1	4	4	0.75	Tinggi
107	2	4	1	4	4	0.75	Tinggi
108	2	4	1	4	4	0.75	Tinggi
109	2	4	1	4	4	0.75	Tinggi
112	2	4	1	4	4	0.75	Tinggi
113	2	4	1	4	4	0.75	Tinggi
115	2	4	1	4	4	0.75	Tinggi
116	2	4	1	4	4	0.75	Tinggi
117	2	4	1	4	4	0.75	Tinggi
121	2	4	1	4	4	0.75	Tinggi
123	2	4	1	4	4	0.75	Tinggi
132	2	4	1	4	4	0.75	Tinggi
135	2	4	1	4	4	0.75	Tinggi
136	2	4	1	4	4	0.75	Tinggi
138	2	4	1	4	4	0.75	Tinggi
139	2	4	1	4	4	0.75	Tinggi
140	2	4	1	4	4	0.75	Tinggi
141	2	4	1	4	4	0.75	Tinggi
142	2	4	1	4	4	0.75	Tinggi
143	2	4	1	4	4	0.75	Tinggi
144	2	4	1	4	4	0.75	Tinggi
145	2	4	1	4	4	0.75	Tinggi
146	2	4	1	4	4	0.75	Tinggi
147	2	4	1	4	4	0.75	Tinggi
158	2	4	1	4	4	0.75	Tinggi
161	2	4	1	4	4	0.75	Tinggi
162	2	4	1	4	4	0.75	Tinggi
168	2	4	1	4	4	0.75	Tinggi
169	3	4	2	4	2	0.75	Tinggi
174	2	4	3	4	2	0.75	Tinggi
175	2	4	4	4	1	0.75	Tinggi
178	3	4	2	4	2	0.75	Tinggi
179	3	4	1	4	3	0.75	Tinggi
181	2	3	2	4	4	0.75	Tinggi
183	3	4	1	4	3	0.75	Tinggi
184	2	4	1	4	4	0.75	Tinggi
187	3	4	1	4	3	0.75	Tinggi
191	3	4	1	4	3	0.75	Tinggi
193	2	4	1	4	4	0.75	Tinggi
195	2	4	1	4	4	0.75	Tinggi
199	2	4	1	4	4	0.75	Tinggi
202	2	4	1	4	4	0.75	Tinggi
208	2	4	1	4	4	0.75	Tinggi

FID	NDVI	Elevasi	Sungai	Pemukiman	Wisata	HSI	Level
211	2	4	1	4	4	0.75	Tinggi
214	2	4	1	4	4	0.75	Tinggi
218	2	4	1	4	4	0.75	Tinggi
219	2	4	1	4	4	0.75	Tinggi
221	2	4	1	4	4	0.75	Tinggi
222	2	4	1	4	4	0.75	Tinggi
224	2	4	1	4	4	0.75	Tinggi
225	2	4	1	4	4	0.75	Tinggi
226	2	4	1	4	4	0.75	Tinggi
234	2	4	1	4	4	0.75	Tinggi
237	2	4	1	4	4	0.75	Tinggi
240	2	4	1	4	4	0.75	Tinggi
242	2	4	1	4	4	0.75	Tinggi
244	2	4	1	4	4	0.75	Tinggi
246	3	4	1	3	4	0.75	Tinggi
247	2	4	1	4	4	0.75	Tinggi
248	2	4	1	4	4	0.75	Tinggi
254	2	4	1	4	4	0.75	Tinggi
258	2	4	2	3	4	0.75	Tinggi
269	2	4	1	4	4	0.75	Tinggi
287	2	3	2	4	4	0.75	Tinggi
292	3	3	1	4	4	0.75	Tinggi
293	3	3	1	4	4	0.75	Tinggi
294	3	3	1	4	4	0.75	Tinggi
303	3	2	2	4	4	0.75	Tinggi
304	3	2	2	4	4	0.75	Tinggi
305	3	3	1	4	4	0.75	Tinggi
318	3	2	2	4	4	0.75	Tinggi
319	3	2	2	4	4	0.75	Tinggi
323	3	2	2	4	4	0.75	Tinggi
327	3	2	2	4	4	0.75	Tinggi
333	3	3	1	4	4	0.75	Tinggi
337	3	3	1	4	4	0.75	Tinggi
340	3	3	1	4	4	0.75	Tinggi
350	3	4	1	3	4	0.75	Tinggi
353	3	4	1	4	3	0.75	Tinggi
355	3	4	2	2	4	0.75	Tinggi
358	3	4	2	4	2	0.75	Tinggi
368	3	4	3	4	1	0.75	Tinggi
12	3	4	1	4	4	0.8	Sangat Tinggi
25	2	4	2	4	4	0.8	Sangat Tinggi
85	3	4	1	4	4	0.8	Sangat Tinggi
110	3	4	1	4	4	0.8	Sangat Tinggi

FID	NDVI	Elevasi	Sungai	Pemukiman	Wisata	HSI	Level
111	3	4	1	4	4	0.8	Sangat Tinggi
114	3	4	1	4	4	0.8	Sangat Tinggi
128	2	4	3	4	3	0.8	Sangat Tinggi
170	3	4	3	4	2	0.8	Sangat Tinggi
176	2	4	4	4	2	0.8	Sangat Tinggi
180	2	3	3	4	4	0.8	Sangat Tinggi
185	3	4	1	4	4	0.8	Sangat Tinggi
203	3	4	1	4	4	0.8	Sangat Tinggi
204	3	4	1	4	4	0.8	Sangat Tinggi
205	3	4	1	4	4	0.8	Sangat Tinggi
206	3	4	1	4	4	0.8	Sangat Tinggi
207	3	4	1	4	4	0.8	Sangat Tinggi
209	3	4	1	4	4	0.8	Sangat Tinggi
210	3	4	1	4	4	0.8	Sangat Tinggi
212	3	4	1	4	4	0.8	Sangat Tinggi
213	3	4	1	4	4	0.8	Sangat Tinggi
215	3	4	1	4	4	0.8	Sangat Tinggi
216	3	4	1	4	4	0.8	Sangat Tinggi
217	3	4	1	4	4	0.8	Sangat Tinggi
220	3	4	1	4	4	0.8	Sangat Tinggi
223	3	4	1	4	4	0.8	Sangat Tinggi
227	3	4	1	4	4	0.8	Sangat Tinggi
228	3	4	1	4	4	0.8	Sangat Tinggi
229	3	4	1	4	4	0.8	Sangat Tinggi
230	3	4	1	4	4	0.8	Sangat Tinggi
231	3	4	1	4	4	0.8	Sangat Tinggi
232	3	4	1	4	4	0.8	Sangat Tinggi
233	3	4	1	4	4	0.8	Sangat Tinggi
249	2	4	2	4	4	0.8	Sangat Tinggi
259	2	4	2	4	4	0.8	Sangat Tinggi
260	2	4	3	3	4	0.8	Sangat Tinggi
265	2	4	2	4	4	0.8	Sangat Tinggi
267	2	4	2	4	4	0.8	Sangat Tinggi
270	2	4	2	4	4	0.8	Sangat Tinggi
290	3	4	1	4	4	0.8	Sangat Tinggi
295	3	3	2	4	4	0.8	Sangat Tinggi
298	3	3	2	4	4	0.8	Sangat Tinggi
315	3	2	3	4	4	0.8	Sangat Tinggi
321	3	2	3	4	4	0.8	Sangat Tinggi
324	3	2	3	4	4	0.8	Sangat Tinggi
328	3	2	3	4	4	0.8	Sangat Tinggi
334	3	3	2	4	4	0.8	Sangat Tinggi
338	3	3	2	4	4	0.8	Sangat Tinggi

FID	NDVI	Elevasi	Sungai	Pemukiman	Wisata	HSI	Level
341	3	3	2	4	4	0.8	Sangat Tinggi
354	3	4	1	4	4	0.8	Sangat Tinggi
356	3	4	2	3	4	0.8	Sangat Tinggi
359	3	4	2	4	3	0.8	Sangat Tinggi
361	3	4	1	4	4	0.8	Sangat Tinggi
364	3	4	1	4	4	0.8	Sangat Tinggi
369	3	4	3	4	2	0.8	Sangat Tinggi
373	3	4	4	4	1	0.8	Sangat Tinggi
129	2	4	3	4	4	0.85	Sangat Tinggi
164	3	4	4	4	2	0.85	Sangat Tinggi
250	2	4	3	4	4	0.85	Sangat Tinggi
255	2	4	3	4	4	0.85	Sangat Tinggi
261	2	4	3	4	4	0.85	Sangat Tinggi
266	2	4	3	4	4	0.85	Sangat Tinggi
268	2	4	3	4	4	0.85	Sangat Tinggi
271	2	4	3	4	4	0.85	Sangat Tinggi
275	2	4	3	4	4	0.85	Sangat Tinggi
276	2	4	3	4	4	0.85	Sangat Tinggi
278	2	4	3	4	4	0.85	Sangat Tinggi
280	2	4	3	4	4	0.85	Sangat Tinggi
288	2	4	3	4	4	0.85	Sangat Tinggi
299	3	3	3	4	4	0.85	Sangat Tinggi
306	3	3	3	4	4	0.85	Sangat Tinggi
308	3	3	3	4	4	0.85	Sangat Tinggi
313	3	2	4	4	4	0.85	Sangat Tinggi
314	3	2	4	4	4	0.85	Sangat Tinggi
316	3	2	4	4	4	0.85	Sangat Tinggi
320	3	2	4	4	4	0.85	Sangat Tinggi
329	3	2	4	4	4	0.85	Sangat Tinggi
330	3	3	3	4	4	0.85	Sangat Tinggi
332	3	3	3	4	4	0.85	Sangat Tinggi
336	3	3	3	4	4	0.85	Sangat Tinggi
339	3	3	3	4	4	0.85	Sangat Tinggi
342	3	3	3	4	4	0.85	Sangat Tinggi
346	3	4	2	4	4	0.85	Sangat Tinggi
360	3	4	2	4	4	0.85	Sangat Tinggi
362	3	4	2	4	4	0.85	Sangat Tinggi
365	3	4	2	4	4	0.85	Sangat Tinggi
366	3	4	2	4	4	0.85	Sangat Tinggi
367	3	4	3	3	4	0.85	Sangat Tinggi
370	3	4	3	4	3	0.85	Sangat Tinggi
374	3	4	4	4	2	0.85	Sangat Tinggi
130	2	4	4	4	4	0.9	Sangat Tinggi

FID	NDVI	Elevasi	Sungai	Pemukiman	Wisata	HSI	Level
133	2	4	4	4	4	0.9	Sangat Tinggi
264	2	4	4	4	4	0.9	Sangat Tinggi
272	2	4	4	4	4	0.9	Sangat Tinggi
273	2	4	4	4	4	0.9	Sangat Tinggi
277	2	4	4	4	4	0.9	Sangat Tinggi
279	2	4	4	4	4	0.9	Sangat Tinggi
281	2	4	4	4	4	0.9	Sangat Tinggi
282	2	4	4	4	4	0.9	Sangat Tinggi
283	2	4	4	4	4	0.9	Sangat Tinggi
284	2	4	4	4	4	0.9	Sangat Tinggi
285	2	4	4	4	4	0.9	Sangat Tinggi
286	2	4	4	4	4	0.9	Sangat Tinggi
289	2	4	4	4	4	0.9	Sangat Tinggi
296	3	3	4	4	4	0.9	Sangat Tinggi
297	3	3	4	4	4	0.9	Sangat Tinggi
300	3	3	4	4	4	0.9	Sangat Tinggi
301	3	3	4	4	4	0.9	Sangat Tinggi
307	3	3	4	4	4	0.9	Sangat Tinggi
309	3	3	4	4	4	0.9	Sangat Tinggi
310	3	3	4	4	4	0.9	Sangat Tinggi
311	3	3	4	4	4	0.9	Sangat Tinggi
312	3	3	4	4	4	0.9	Sangat Tinggi
331	3	3	4	4	4	0.9	Sangat Tinggi
335	3	3	4	4	4	0.9	Sangat Tinggi
343	3	3	4	4	4	0.9	Sangat Tinggi
345	3	4	3	4	4	0.9	Sangat Tinggi
348	3	4	3	4	4	0.9	Sangat Tinggi
363	3	4	3	4	4	0.9	Sangat Tinggi
371	3	4	3	4	4	0.9	Sangat Tinggi
372	3	4	4	3	4	0.9	Sangat Tinggi
375	3	4	4	4	3	0.9	Sangat Tinggi
291	3	4	4	4	4	0.95	Sangat Tinggi
344	3	4	4	4	4	0.95	Sangat Tinggi
347	3	4	4	4	4	0.95	Sangat Tinggi
376	3	4	4	4	4	0.95	Sangat Tinggi

INVENTORY OF PARASITOID INSECTS ORDER HYMENOPTERA ON POMEGRANATE (*Punica granatum* L.) CROPS IN MERAK VILLAGE, SITUBONDO

THESIS

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CHAPTER 1 INTRODUCTION

1.1 Background

Merak Village, Sidomulyo Hamlet, Sumberwaru Village, Banyuputih District, Situbondo which is located in the utilization zone of Baluran National Park is an area with a pomegranate commodity, Punica granatum Linnaeus (Myrtales: Lythraceae), in Indonesia. A total of 350 trees were planted by the people of Kampung Merak in 2018. Over a period of two years (2018 – 2020) the people's fields planted with corn and chilies were replaced with pomegranates. This led to a significant increase in the number of pomegranates planted, bringing them to 7,250 trees by 2020.

Pomegranates in Kampung Merak can be harvested all year round with a variety of fruit colors from red to white with a sweet taste. However, the pomegranates in Kampung Merak have poor skin texture and color and are not large in size when compared to imported pomegranates. This is partly due to pest attacks on pomegranate plants in the area. The texture and color of the skin that is not good and the size of the fruit is not large enough to cause the price of Kampung Merak pomegranate to decrease, from the price of Rp. 50,000.00 per kilogram to Rp. 25,000.00 – 30,000.00 per kilogram.

According toHartley and Jones (2003), the diversity of insects in a system depends on the diversity of the dominant vegetation. Changes in the structure of the vegetation in the community's fields, which were originally corn and chili fields, turned into pomegranates, may cause changes in the structure of the insect community in the ecosystem. Likewise with the existing parasitoid insects.

The immature insects that parasitize Arthropods or other insects are called parasitoids(Gauld and Bolton, 1988). Adult parasitoids lay their eggs on arthropods or other insects that become hosts in various phases (eggs, larvae, adults, and larval eggs). Eggs laid by adult parasitoids will grow and develop in the body of the Arthropod or insect host by absorbing its nutrients. This causes the Arthropods or host insects to be unable to provide nutrients for themselves and

die(Gauld and Bolton, 1988). This parasitoid ability makes it an ecosystem controlling agent(Meilin and Nasamsir, 2016).

The role of parasitoids as ecosystem control agents is commonly used in agriculture as a biological control for plant pests. Pomegranate plantations in various exporting countries use a lot of parasitoids to control pest attacks. The main pest of pomegranate in the region of Iran which is the country of origin of this plant is Ectomyelois ceratoniae Zeller (Lepidoptera: Pyralidae). The attack of E. ceratoniae causes the pomegranate to become infected by the saprophytic fungus that enters through the hole punctured by the pest, causing the fruit to rot. This causes the commodity of pomegranates to decline by 30-80%.(Sobhani et al., 2015). Control of E. ceratoniae using biological control by releasing parasitoids is considered more effective and efficient than chemical insecticides(Kishani-Farahani et al., 2012; Nobakht et al., 2015; Sobhani et al., 2015)due to the endophytic behavior of E. ceratoniae and the position of the pomegranate on the hanging tree(Ksentini et al., 2010).

According toLewis et al. (1998), the presence of parasitoids is indicated by the presence of food sources and hosts. The increasing number of pomegranate trees planted in Merak Village provides opportunities for parasitoids to obtain food and protection for their continued reproduction. Therefore, a study was conducted on the inventory of parasitoid insects on pomegranate plantations in Merak Village, Sidomulyo Hamlet, Sumberwaru Village, Banyuputih District, Situbondo. Through this research, it is hoped that parasitoid insects found in pomegranate plantations can be identified.

1.2 Formulation of the problem

The problem formulation of this research is which parasitoid insects from the Order Hymenoptera are found in pomegranate plantations (P. granatum) in Merak Village, Sidomulyo Hamlet, Sumberwaru Village, Banyuputih District, Situbondo?

1.3 Destination

The purpose of this study was to determine what parasitoid insects from the Order Hymenoptera were found in pomegranate plantations (P. granatum) in Merak Village, Sidomulyo Hamlet, Sumberwaru Village, Banyuputih District, Situbondo.

1.4 Benefit

The expected benefit of this research is that it can provide information about parasitoid insects from the Order Hymenoptera which are found in pomegranate plantations (P. granatum) in Merak Village, Sidomulyo Hamlet, Sumberwaru Village, Banyuputih District, Situbondo.

CHAPTER 2. LITERATURE REVIEW

2.1 Parasitoid Insects Order Hymenoptera

Parasitoids are immature insects that live as parasites on Arthropods or other insects. Parasitoids parasitize other insects in various phases (eggs, larvae, adults, and larval eggs) both endoparasitoids and ectoparasitoids. Endoparasitoids are parasites by inserting their eggs into the host's body, while ectoparasitoids are outside the host's body.(Gauld and Bolton, 1988).

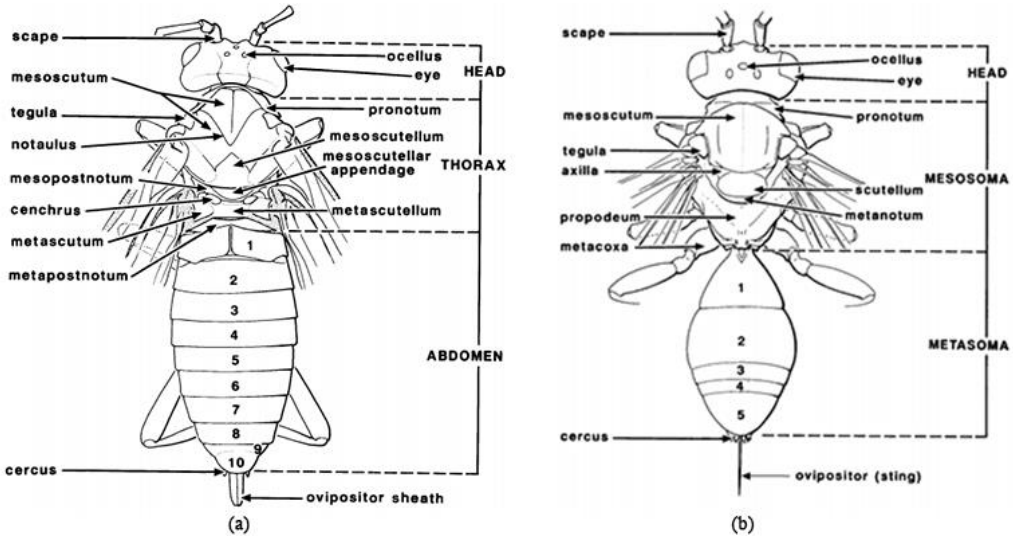
Parasitization is carried out by parasitoids to provide nutrition for larval development by absorbing host nutrients. The strategy used by adult parasitoids against the host so that nutrients for the larvae are met is done by preventing the growth of the host (idiobiont) or allowing the host to continue to grow (koinobiont). Most idiobiont insects are ectoparasitoids. They use a chemical (venom) released at the time of laying their eggs to make the host die, be permanently paralyzed, or stop developing. This is different from that of the conoobiont insects, which are generally endoparasitoids. Endoparasitoid coinobiont insects temporarily paralyze the host to be able to lay their eggs in the host's body and adjust their body physiology to suit the host,(Gauld and Bolton, 1988).

Most insects that act as parasitoids according toBorror et al. (1989), belongs to the order Hymenoptera. Hymenoptera is one of the orders in the Insect Class which consists of social insect groups such as bees, wasps, and ants. This order has a fairly high diversity and makes it one of the four largest orders in the Insect Class(Goulet and Huber, 1993).

The high diversity of Hymenoptera makes the existence of Hymenoptera in nature quite easy to find. Hymenoptera also have distinctive morphological characteristics, making them easy to identify(Gauld and Bolton, 1988). Characteristics that distinguish Hymenoptera from other orders include having a mandibulate (biter) type mouth apparatus, thin wings with hamuli (small hooks) on the anterior edge of the hind wings, and an ovipositor (modified abdominal

segment that functions to lay eggs) in the female parent. mature(Goulet and Huber, 1993).

Traditionally (based on morphology) Hymenoptera are grouped into two sub-orders, namely Symphyta and Apocrita (Figure 2.1). Each sub-order is divided into several super-families. The sub-order Symphyta consists of six superfamilies, namely Cephoidea, Megalogontoidea, Orussoidea, Siricoidea, Tenthredinoidea, and Xyeloidea. The sub-order Apocrita consists of 14 superfamilies, namely Apoidea, Chrysidoidea, Vespoidea, Ceraphronoidea, Chalcidoidea, Cynipoidea, Evanioidea, Ichneumonoidea, Megalyroidea, Mymarommatoidea, Platygastroidea, Proctotrupoidea, Trigonalyoidea, and(Goulet and Huber, 1993).



(a) Sub-Order Symphyta; (b) Sub-Order Apocrita

Figure 2.1 The main morphological structure of the sub-orders Symphyta and Apocrita (Goulet and Huber, 1993)

2.1.1 Sub-Order Symphyta

Sub-order Symphyta has a small number and consists of the ancestors of Hymenoptera. Like the ancestor of Hymenoptera, Symphyta generally contain p insects

eat plants. Some of its super-families are plant-parasitic insects(Goulet and Huber, 1993). Plant parasites of the sub-order Symphyta lay their eggs in plant tissues by making holes in plant stems.(Gauld and Bolton, 1988).

Symphytes have morphological characteristics in the form of wings with many venations, unmodified abdomen(Goulet and Huber, 1993), and primitive ovipositor(Gauld and Bolton, 1988). Symphyta wings have a characteristic as the primitive Hymenoptera group, namely the number of venation (wing skeleton)(Goulet and Huber, 1993)with at least three closed cells at the base of the hind wings(Triplehorn and Johnson, 2005). Symphytes have a dilated abdomen that connects to the thorax (Fig. 2.1)(Triplehorn and Johnson, 2005). Ovipositor in Symphyta is a primitive ovipositor that maintains its function to lay eggs properly (Figure 2.1)(Gauld and Bolton, 1988).

a. Super-Family Cephoidea

The morphological feature of this superfamily is that it does not have senkri. Senkri is a round or oval shaped structure on the sub-lateral part of the metascutum which amounts to a pair. Another characteristic is that there is a slight narrowing between the first and second segments of the abdomen(Goulet and Huber, 1993).

Super-Family Cephoidea consists of one family, namely Cephidae (Figure 2.2)(Goulet and Huber, 1993). The Cephidae family has a thin black or dark brown integument. The abdomen has a yellow ribbon-shaped pattern(Slave et al., 2011). The larvae of this group feed on the stems of herbaceous or woody plants. Therefore, this group of insects is commonly referred to as stem sawflies.(Goulet and Huber, 1993).

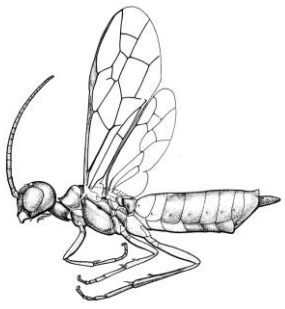


Figure 2.2 Morphology of the Cephidae Family (Goulet and Huber, 1993)

b. Super-Family Megalogontoidea

A distinctive feature of this group is the size of its very large and prominent head. Klipeus on the head is wide. Superfamily Megalogontoidea has a separate mandibular foramen(Gauld and Bolton, 1988; Goulet and Huber, 1993).

Super-Family Megalogontoidea consists of two families, namely Megalodontidae and Pamphiliidae (Figure 2.3). The difference between the Megalodontidae and Pamphiliidae families is the shape of the antennae and the presence of longitudinal folds on the second to fifth abdominal terga segments that lie beneath the spiracles. The shape of the antennae of the Pamphiliidae family resembles a thread (filiform), while the Megalodontidae family has an antenna that resembles a saw or a comb. The Pamphiliidae family has longitudinal folds on the second to fifth abdominal terga segments located below the spiracles, while the Megalodontidae family does not.(Gauld and Bolton, 1988; Goulet and Huber, 1993). The larvae of this group feed on the leaves of some herbs. However, some larval species of the Pamphiliidae family also feed on fruit on some woody and coniferous trees(Goulet and Huber, 1993).

(a) (b)

(a) Family Pamphiliidae; (b) Family Megalodontidae

Figure 2.3 Morphology of Families Pamphiliidae and Megalodontidae (Goulet and Huber, 1993)

c. Super-Family Orussoidea

A distinctive feature of the Orussoidea superfamily is that it has spines between its pair of ocelli(Goulet and Huber, 1993). The larvae of this group are characterized by having chemosensitive and mechanosensitive organs. Chemosensitive organs are present in one of the larval antennae of this group, while one of the other antennae has mechanosensitive seta.(Gauld and Bolton, 1988).

Super-Family Orussoidea consists of one family, namely Orussidae (Figure 2.4). The morphological feature of this group is the antennae which are located below the ventral line of the eye. The antenna consists of 10-11 segments. Super-Family Orussoidea has a body measuring 9-15 mm. Adult female insects have long and thin ovipositors. The venation on the wings is reduced. This group contains the parasitoids of the order Coleoptera borer and several larvae of other Order Hymenoptera. The adult female parent will lay her eggs outside the host's body. The eggs will be laid between the flight muscles in the center of the host's thorax(Gauld and Bolton, 1988; Goulet and Huber, 1993).

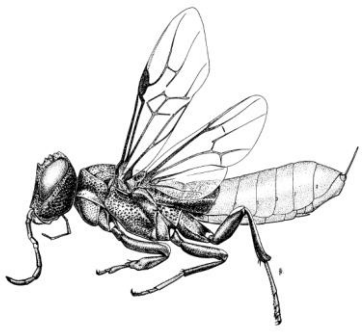


Figure 2.4 Morphology of the Orussidae family (Goulet and Huber, 1993)

d. Super-Family Siricoidea

Super-Family Siricoidea is a member of the order Hymenoptera wood borer. The hallmark of this group is to have senkri on the sub-lateral metascutum. Super-

Family Siricoidea's abdominal shape is cylindrical and not slender(Gauld and Bolton, 1988).

Super-Family Siricoidea consists of one family, namely Siricidae (Figure 2.5). This group has a light colored body. Its body size is more than 14 mm. Antenna family Siricidae long and flat with 14-30 segments. The ovipositor in adult female insects is clearly visible past the tip of the abdomen. Adult female insects will lay their eggs on tree trunks that are dead or still alive. When the adult female insect lays her eggs, she secretes mucus which causes an increase in fungal growth. The growth of this fungus causes water transport in the xylem to be disrupted. This results in the physiology of living trees being disturbed, thus causing death(Gauld and Bolton, 1988; Goulet and Huber, 1993).

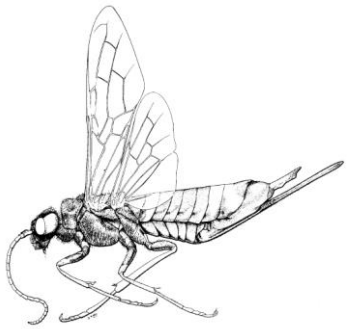


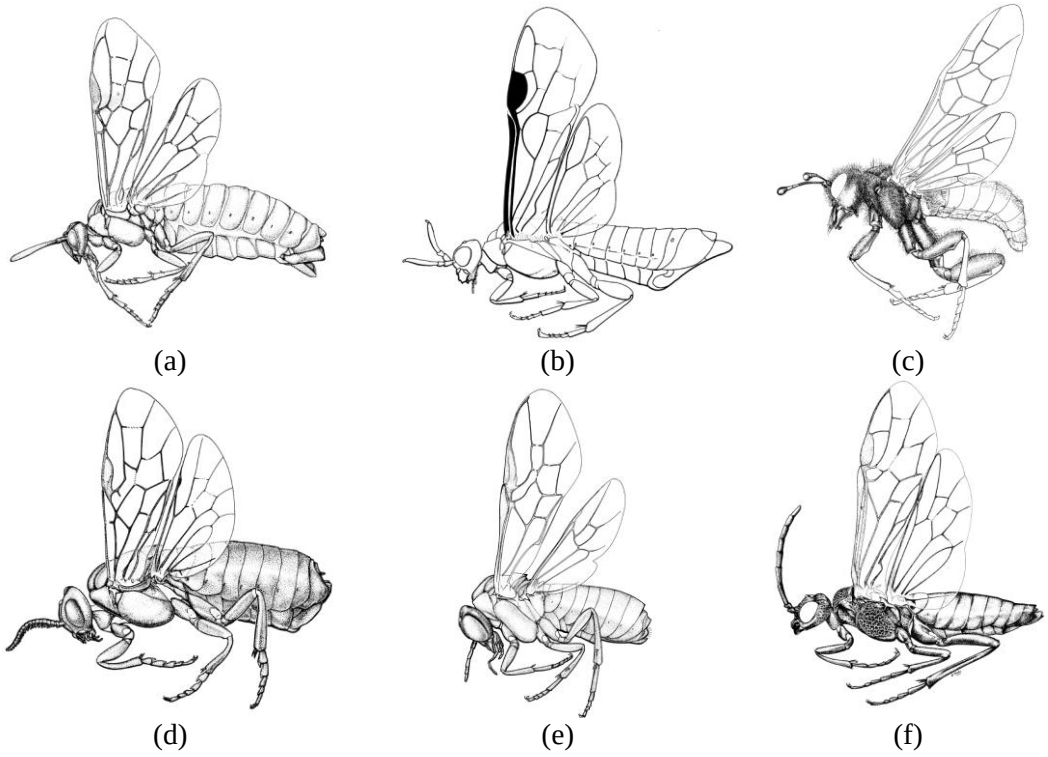
Figure 2.5 Morphology of the Siricidae Family (Goulet and Huber, 1993)

e. Super-Family Tenthredinoidea

A distinctive feature of this group is the curved back edge of the pronotum when viewed from the dorsal side. The labrum of the super-Family Tenthredinoidea is clearly visible. The middle and hind tibia have no spines (preapical spurs). The antenna has 3 – 32 segments(Gauld and Bolton, 1988).

The Super-Family Tenthredinoidea consists of six families, namely Argidae, Blasticotomidae, Cimbicidae, Diprionidae, Peridae, and Tenthredinidae (Figure 2.6). The family Argidae is characterized by antennae with one flagelomer. The family Blasticotomidae is characterized by one or two flagelomers on its

antennae. The Cimbicidae family has an antenna shaped like a club. The family Diprionidae has comb-like antennae in males and saw-like in females, with 20 flagelomers. The Perigidae family is characterized by antennae with more than four flagelomers. The family Tenthredinidae has antennae with five to nine flagelomers. The larvae of this group are all plant eaters. They eat the tissue on the leaves(Gauld and Bolton, 1988; Goulet and Huber, 1993).



(a) Family Argidae; (b) Family Blasticotomidae; (c) Family Cimbicidae; (d) Family Diprionidae; (e) the Perigidae family; (f) Family Tenthredinidae

Figure 2.6 Morphology of the superfamily Tenthredinoidea (Goulet and Huber, 1993)

f. Super-Family Xyeloidea

The distinctive feature of this group is that it has modified antennae in the form of three long and sturdy segments at the base. These segments are followed by filaments totaling nine or more segments. Another feature is the venation of Rs (Radial Sector) on the forewing forewings(Gauld and Bolton, 1988).

Super-Family Xyeloidea consists of one family, namely Xyelidae (Figure 2.7). The Xyelidae family has a body length of less than 5 mm. The head lacks a

hypostomal bridge (sclerotization of the posterior part of the head that separates the foramen magnum from the oral cavity). The Xyelidae family has a long pronotum. The ovipositor in adult female insects is long, clearly visible from the tip of the abdomen. Larvae of this group are commonly found in pine trees. They eat the pollen-producing part of the pine flower and some also eat the leaf buds(Gauld and Bolton, 1988; Goulet and Huber, 1993).

Figure 2.7 Morphology of superfamily Xyeloidea (Goulet and Huber, 1993)

2.1.2 Sub-Order Apocrita

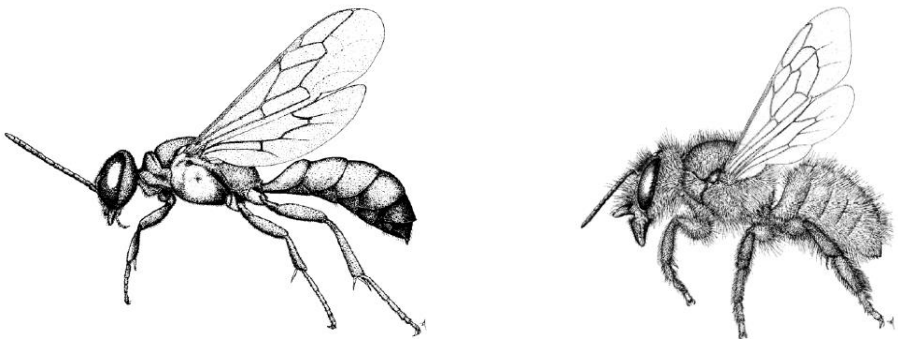
The sub-order Apocrita is the majority group of the Order Hymenoptera. The sub-order Apocrita is a modern group of the Order Hymenoptera that evolved from the sub-order Symphyta(Gauld and Bolton, 1988). The evolutionary development of this sub-order resulted in the Hymenoptera group consisting of carnivorous insects. As many as 75% of the species act as parasitoids(Goulet and Huber, 1993).

The sub-order Apocrita has morphological characteristics in the form of wings with little venation and a modified abdomen and ovipositor. Sub-Order Apocrita is a group of the Order Hymenoptera with more advanced development. Wing venation is few, there are no closed cells at the base of the wing, even some superfamilies do not have venation. The abdomen of the sub-order Apocrita is narrowed in the 1st and 2nd segments (Fig. 2.1), the first segment of the abdomen is associated with the metathorax.(Gauld and Bolton, 1988). The ovipositor in this group is thin and cylindrical (Goulet and Huber, 1993) with a function as a tool for laying eggs and immobilizing prey (Figure 2.1)(Gauld and Bolton, 1988).

The sub-order Apocrita is morphologically divided into two groups, namely Parasitica and Aculeata(Gauld and Bolton, 1988). This difference is based on the number of trochanteric segments, the presence of a jugum lobe (jugal lobe) on the hind wings, and modification of the ovipositor into a stinger.(Carpenter, 1986). The Parasitica group has an ovipositor that functions to lay eggs and immobilize the host. The Aculeata group has an ovipositor that functions as a weapon in hunting or defense(Gauld and Bolton, 1988). Although this grouping based on morphology is inconsistent because in fact the stinger in Aculeata still functions as an ovipositor in several families.(Carpenter, 1986).

a. Super-Family Apoidea

Super-Family Apoidea (Figure 2.8) is characterized by antennae with 12 segments on female insects and 13 on male insects. The pronotum is short and does not touch the sugar. The forewings and hind wings are filled with venation and closed cells. The wings of the superfamily Apoidea have jugum lobes and claval lobes. Some members of the super-family Apoidea have bodies covered with fine hairs, but some do not(Gauld and Bolton, 1988).



(a) (b)

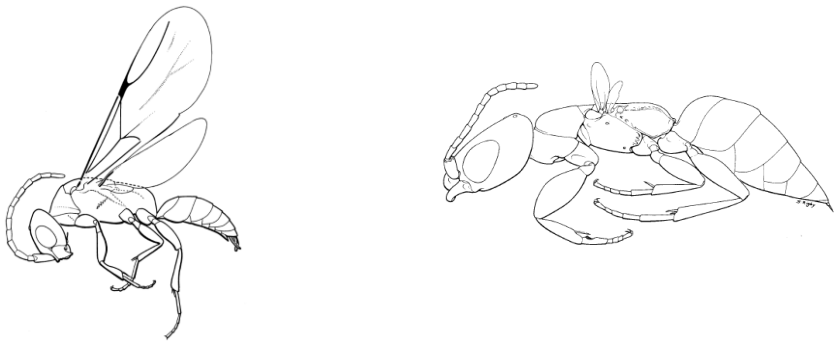
(a) Family Sphecidae; (b) Family Apidae

Figure 2.8 Morphology of superfamily Apoidea (Gauld and Bolton, 1988)

Super-Family Apoidea consists of two families, namely Sphecidae and Apidae (Figure 2.8). Adult families Sphecidae and Apidae feed on nectar on flowers. This is different from the larvae of the Sphecidae and Apidae families. The larvae of the Sphecidae family grow and develop by preying on other insects, while the larvae of the Apidae family are plant eaters.(Gauld and Bolton, 1988).

b. Super-Family Chrysidoidea

Super-Family Chrysidoidea (Fig. 2.9) is characterized by flagelomers on its antennae which are eight or eleven segments. The number of flagelomeric segments in both female and male adult insects is the same. This group has a pronotum with a posterolateral tip that touches the sugar. The metaposnotum is short, transverse, and fused with the propodeum. Wing venation reduces. The hind wings do not have jugum lobes. Adult female insects of the superfamily Chrysidoidea have an ovipositor that will be hidden when resting and is modified into a stinging organ.(Goulet and Huber, 1993).



(a) (b)

(a) Family Bethylidae male; (b) Family Bethylidae female

Figure 2.9 Morphology of superfamily Chrysidoidea (Goulet and Huber, 1993)

Super-Family Chrysidoidea consists of three major families and four minor families. The large family Chrysidoidea consists of Bethylidae (Figure 2.9), Chrysididae, and Dryinidae. The small family Chrysidoidea consists of Embolemidae, Plumariidae, Sclerogibbidae, and Scolebythidae. Chrysidoidea consists of parasitoid insects on the eggs of the Order Cheleutoptera, outside the body of the Order Homoptera, nymphs of the Order Embioptera, larvae of the Order Coleoptera, and the Order Lepidoptera.(Goulet and Huber, 1993).

c. Super-Family Vespoidea

Super-Family Vespoidea (Fig. 2.10) is characterized by antennae with ten flagelomer in female insects and eleven in male insects. The pronotum has a posterolateral tip that touches the sugar. The metaposnotum is short, transverse,

and fused with the propodeum. The venation of the wings is well developed. The hind wings of the Vespoidea superfamily usually have jugum lobes. The sterna of the metasoma of the first and second segments are sometimes separated by a streamline. Adult female insects have an ovipositor that will be hidden at rest and modified into a stinging organ.

Figure 2.10 Morphology of super-Family Vespoidea, Family Rhopalosomatidae (Goulet and Huber, 1993)

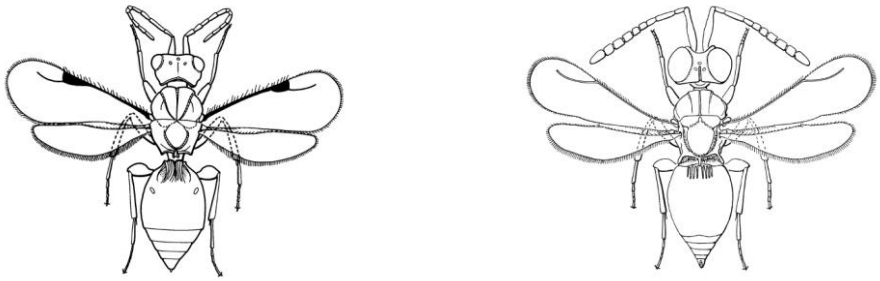
Super-Family Vespoidea consists of ten families. The families of Vespoidea are Bradynobaenidae, Formicidae, Mutillidae, Pompilidae, Rhopalosomatidae (Figure 2.10), Sapygidae, Scoliidae, Sierolomorphidae, Tiphiidae, and Vespidae. Some members of the superfamily Vespoidea are predatory insects and parasitoids. Parasitoids in this group usually parasitize the larvae of the Order Coleoptera(Goulet and Huber, 1993).

d. Super Family Ceraphronoidea

Super-Family Ceraphronoidea (Fig. 2.11) has a body with a length of 0.3 – 3.5 mm. The hallmark of this superfamily is that it has spikes at the ends of its protibia. This superfamily has antennae with nine to eleven segments. The forewings of this group have fused costal (C) and radial (R) venations. The second metasoma segment is very large, appears to be connected to the propodeum(Gauld and Bolton, 1988; Goulet and Huber, 1993).

Super-Family Ceraphronoidea consists of two families, namely Megaspilidae and Ceraphronidae (Figure 2.11). Several species of this group are parasitoids. The super-family Ceraphronoidea parasitoids attack the Order Homoptera,

Neuroptera, and pupae of various types of the Order Diptera or Homoptera (Goulet and Huber, 1993).



(a) (b)

(a) Family Megaspilidae; (b) Family Ceraphronidae

Figure 2.11 Morphology of superfamily Ceraphronoidea (Goulet and Huber, 1993)

e. Super-Family Chalcidoidea

Super-Family Chalcidoidea (Figure 2.12) has a body size of 3 – 5 mm. This group is characterized by reduced venation of the forewings. The most complete wing venation in this group consists of sub-marginal, marginal, stigmal, and posmarginal venation. Another characteristic is that it has separate sclerites called prepectus. The pronotum in the superfamily Chalcidoidea is shaped like a saddle or a horseshoe(Goulet and Huber, 1993).

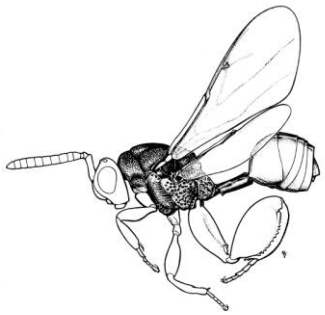


Figure 2.12 Morphology of super-Family Chalcidoidea, Family Chalcididae (Goulet and Huber, 1993)

Super-Family Chalcidoidea consists of 20 families. The families are Agaonidae, Aphelinidae, Chalcididae (Figure 2.12), Elasmidae, Encyrtidae, Eucharitidae, Eulophidae, Eupelmidae, Eurytomidae, Leucospidae, Mymaridae,

Ormyridae, Perilampidae, Pteromalidae, Rotoitidae, Signiphoridae, Tanaostigmatidae, and Trigramidae. Super-Family Chalcidoidea is a group of Order Hymenoptera with high parasitoid diversity as well as super-Family Ichneumonoidea. Super-family Chalcidoidea parasitoids generally attack their hosts in the egg to pupa stage by endoparasitoids or ectoparasitoids.(Gauld and Bolton, 1988; Goulet and Huber, 1993).

f. Super-Family Cynipoidea

The superfamily Cynipoidea (Fig. 2.13) has a body length of less than 30 mm. A distinctive feature of this group is its unbent antennae. The antennae have eleven flagelomers in female insects and 12-13 in male insects. The pronotum reaches the sugar cane. Tarsi in the superfamily Cynipoidea have five segments(Goulet and Huber, 1993).

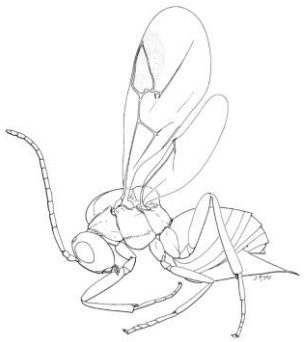


Figure 2.13 Morphology of super-Family Cynipoidea, Family Cynipidae (Goulet and Huber, 1993)

The superfamily Cynipoidea has six families. The families that fall into the Superfamily Cynipoidea are Charipidae, Cynipidae (Figure 2.13), Eucoilidae, Figitidae, Ibaliidae, Liopteridae. Super-Family Cynipoidea has members that are predominantly endoparasitoids in holometabolous insect groups (complete metamorphosis)(Gauld and Bolton, 1988; Goulet and Huber, 1993).

g. Super-Family Evanioidea

Super-Family Evanioidea (Fig. 2.14) is characterized by a higher metasoma than the propodeum. Most of the adult female super-Family Evanioidea

ovipositors are conspicuous and elongated in size. Male insects generally have antennae with 11 flagelomeric segments, while females have 12 segments(Goulet and Huber, 1993).

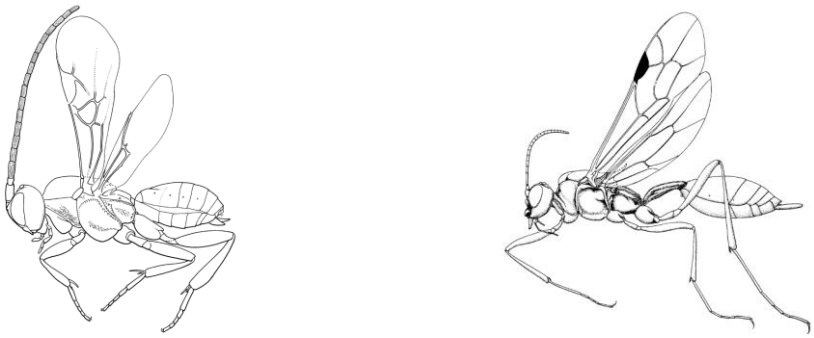
Figure 2.14 Morphology of super-Family Evanioidea, Family Aulacidae (Goulet and Huber, 1993)

Super-Family Evanioidea consists of three families. These families are Aulacidae (Figure 2.14), Evaniidae, and Gasteruptiidae. Members of this group are generally predators and parasitoids. The family Evanioidae parasitoids usually parasitize the larvae of the Order Coleoptera and some sawflies. A member of the Evanioidae family is a parasitoid on cockroach eggs (Dictuoptera: Blattodea)(Goulet and Huber, 1993).

h. Super Family Ichneumonoidea

Super-Family Ichneumonoidea (Figure 2.15) is a super-family of the Order Hymenoptera with a characteristic feature of fused costal and radial venation on the forewings. The antennae in this group are not bent. The number of flagelomers in the antennae of the superfamily Ichneumonoidea is always more than eleven segments in almost all of its members. Adult female insects have a long ovipositor(Goulet and Huber, 1993).

Super-Family Ichneumonoidea has two families, namely Braconidae and Ichneumonidae (Figure 2.15). This superfamily has the greatest diversity of parasitoids among other groups of the Order Hymenoptera. Super-Family Ichneumonoidea generally parasitize the larvae and pupae of insect groups with complete metamorphosis (holometabola) except for the Order Megaloptera and Siphonaptera.(Goulet and Huber, 1993)



(a) (b)

(a) Braconidae; (b) Ichneumonidae

Figure 2.15 Morphology of superfamily Ichneumonoidea (Goulet and Huber, 1993)

i. Super Family Megalyroidea

Super-Family Megalyroidea (Figure 2.16) is characterized by a cylindrical body. This superfamily has very large, deep, oval-shaped gene structures. The antennae of this group have 12 flagelomeric segments. Another characteristic is that it has a flat mesoscutum(Goulet and Huber, 1993).

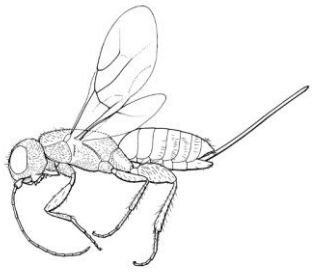


Figure 2.16 Morphology of super-Family Megalyroidea, Family Megalyridae (Goulet and Huber, 1993)

Super-Family Megalyroidea consists of one family, namely Megalyridae (Figure 2.16). Members of the superfamily Megalyroidea are generally parasitoids of the larvae of the Order Coleoptera. The larvae of the Order Coleoptera parasitized by the super-Family Megalyroidea usually live under the bark(Goulet and Huber, 1993).

j. Superfamily Mymarommatoidea

A distinctive feature of the superfamily Mymarommatoidea (Fig. 2.17) that distinguishes it from other Hymenoptera groups is the petiole on its antennae which has two segments. In addition to the number of petiole segments, the shape of the head is quite interesting, namely with a shape that is similar to a pubic ring. Super-Family Mymarommatidea wings are also quite interesting because they have a spoon-like shape(Goulet and Huber, 1993).

Super-Family Mymarommatoidea consists of one family, namely Mymarommatidae (Figure 2.17). The host of this group is not widely known. Most species of Mymarommatidae are found in bracket fungi.(Goulet and Huber, 1993).

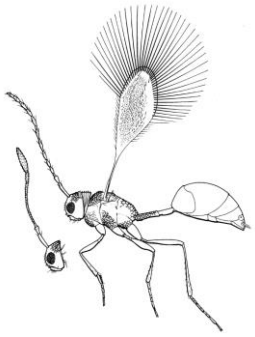
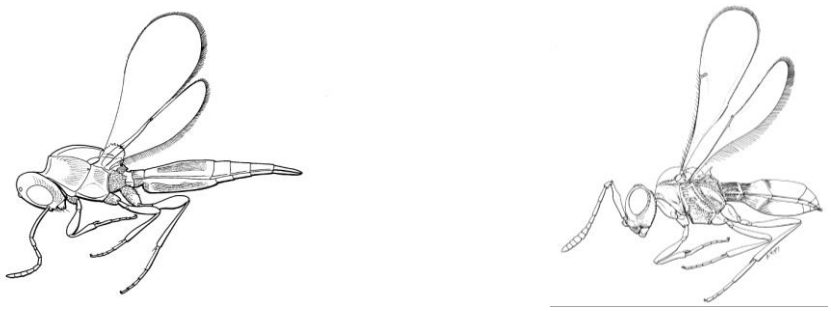


Figure 2.17 Morphology of super-Family Mymarommatidea, Family Mymarommatidae (Goulet and Huber, 1993)

k. Super-Family Platygastroidea

The superfamily Platygastroidea (Fig. 2.18) has metasoma with six or seven terga visible. Terga in the second or third segment metasoma has a larger size than the other segment metasoma. Has large eyes. Adult female insects have ovipositor that is not strongly sclerotic(Goulet and Huber, 1993).



(a) (b)

(a) Family Platygastridae; (b) Family Scelionidae

Figure 2.18 Morphology of superfamily Platygastroidea (Goulet and Huber, 1993)

Super-Family Platygastroidea has two families within it, namely Platygastridae and Scelionidae (Figure 2.18). The family Platygastridae are generally parasitoids of various eggs of the Order Coleoptera and Homoptera. The family Scelionidae is an endoparasitoid in the eggs of other insects and the spider group (Araneae)(Goulet and Huber, 1993).

l. Super-Family Proctotrupoidea

Super-Family Proctotrupoidea (Fig. 2.19) have antennae with flagelomer number 9 – 13 segment and some sometimes have flagelomer less than seven. The size of the terga in the second segment of metasoma is longer than that of the other segments. The terga in the second segment of the metasoma of the Proctotrupoidea superfamily is also wider than the first segment(Goulet and Huber, 1993).

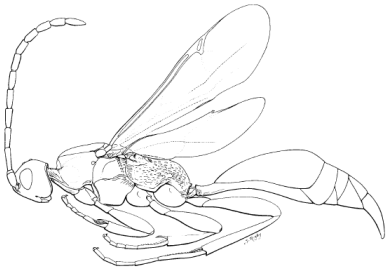


Figure 2.19 Morphology of super-Family Proctotrupoidea, Family Proctotrupidae (Goulet and Huber, 1993)

Super-Family Proctotrupoidea has nine families. These families are Austroniidae, Diapriidae, Heloridae, Monomachidae, Pelecinidae, Peradeniidae, Proctotrupidae (Figure 2.19), Roproniidae, and Vanhorniidae. Members of the superfamily Proctotrupoidea are parasitoids in various groups of the Order Coleoptera, several Suborders Symphyta, and the Order Neuroptera.(Goulet and Huber, 1993).

m. Super-Family Stephanoidea

Super-Family Stephanoidea (Figure 2.20) is characterized by a long and slender body. This is because the pronotum has a shape similar to a neck. The antennae have flagelomers with more than 20 segments. The forewings have no costal venation. The metafemur is very large and is serrated on the ventral side. The adult female insect has a long ovipositor(Goulet and Huber, 1993).

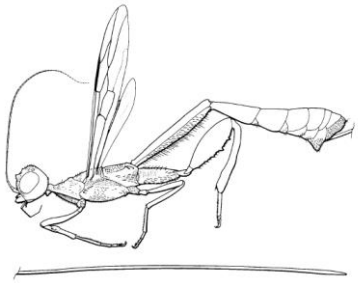


Figure 2.20 Morphology of super-Family Stephanoidea, Family Stephanidae (Goulet and Huber, 1993)

Super-Family Stephanoidea has only one family. The family of Stephanoidea is Stephanidae (Figure 2.20). Generally, members of Stephanoidea are parasitoids of various types of the order Coleoptera wood borer(Goulet and Huber, 1993).

n. Super Family Trigonalyoidea

Super-Family Trigonalyoidea (Fig. 2.21) has morphological characteristics in the form of antennae inserted in the frons. The antennae have a flagellum with 20 segments. The forewings have ten closed cells, while the hind wings have two closed cells. Super-Family Trigonalyoidea has one to four tarsomers. The ovipositor in adult female insects of this group is reduced(Goulet and Huber, 1993).

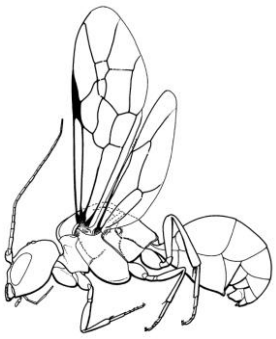


Figure 2.21 Morphology of super-Family Trigonalyoidea, Family Trigonalyidae (Goulet and Huber, 1993)

Super-Family Trigonalyoidea has one family, namely Trigonalyidae (Figure 2.21). The adult female insects of the Super Family Trigonalyoidea lay their eggs on the leaf bones. The eggs of the superfamily Trigonalyoidea have a very thick shell. These eggs will be eaten by larvae of various types of insects except for the sub-Order Symphyta and Order Lepidoptera. The eggs that enter the larva's digestion will hatch due to exposure to the digestive enzymes of the larva's body. Then the eggs that hatch will grow and develop in the larva's body as a parasite(Goulet and Huber, 1993).

2.2 Parasitoids of the Order Hymenoptera Pomegranate (*Punica granatum* L.)

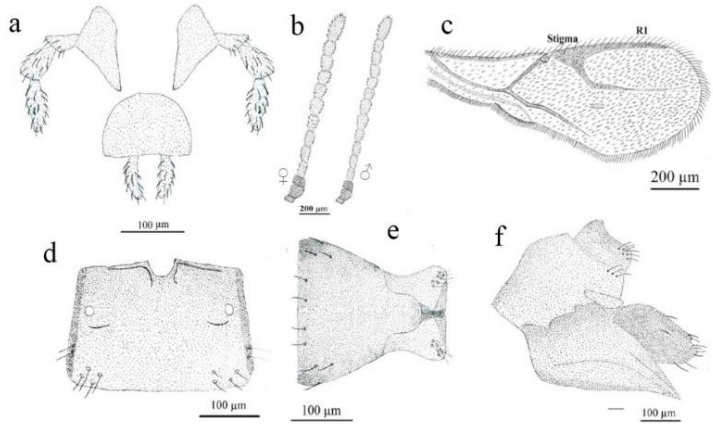
The role of parasitoids in agriculture is as a biological agent that can control the number of insect pests in the ecosystem (Meilin and Nasamsir, 2016). Parasitization carried out by parasitoids on insects by making them a host causes the insect to be unable to continue its development. The inability of the host insect to grow and develop further is because it is unable to provide nutrients for itself, so that population growth can be suppressed.(Gauld and Bolton, 1988).

The diversity of parasitoids of the Order Hymenoptera in pomegranate is quite high. Parasitization is carried out at various stages in the life cycle of the host insect, starting from the egg, larva, pupa, and imago. Some types of parasitoids in pomegranates include the following:

a. *Lysiphlebus fabarum*Marshall

*Lysiphlebus fabarum*Marshall is a species of the Order Hymenoptera that belongs to the super-family Ichneumonoidea, family Braconidae, and sub-family Aphidiinae. This species is the main parasitoid that attacks aphids (Aphids) in an endoparasitoid manner(Barahoei et al., 2011; Farrokhzadeh et al., 2014). One of the hosts of L. fabarum is Aphis punicae Passerini which is a pest on pomegranate plants(Farrokhzadeh et al., 2014).

Morphologically, L. fabarum is characterized by a black body with a dark brown head. The body length is 1.4 – 1.6 mm. This species has maxillary palps with three palpomeres, whereas the labial palps have one palpomer (Fig. 2.22). The antennae on female insects have 13 segments, while the male insects have 13-15 segments (Figure 2.22). The petiole has a triangular shape (Fig. 2.22). The venation of the forewings of L. fabarum is incomplete (Fig. 2.22). The ovipositor of the adult female insect is weak and curved when viewed from the dorsal side (Fig. 2.22).(Farrokhzadeh et al., 2014).



(a) Mouth Tool; (b) Antenna; (c) Front Wing; (d) Propodeum; (e) Petiola; (f) Ovipositor View Lateral

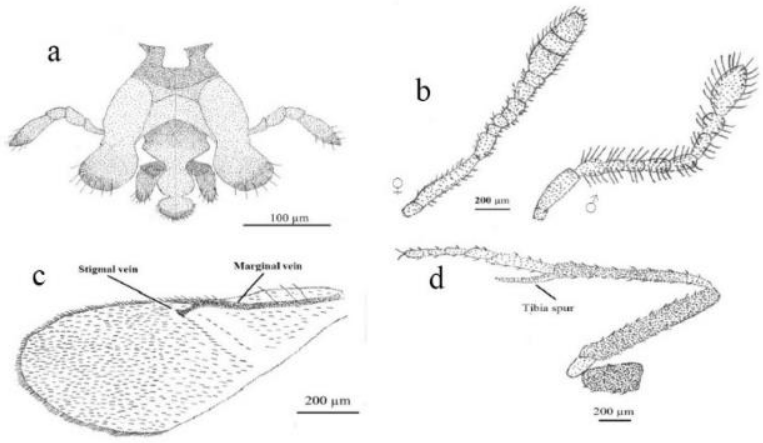
Figure 2.22 Morphological characters of L. fabarum (Farrokhzadeh et al., 2014)

b. *Syrphophagus aphidivorus*Mayr

*Syrphophagus aphidivorus*Mayr is a species of the Order Hymenoptera that belongs to the super-Family Chalcidoidea and Family Encyrtidae.(Martínez-Chávez et al., 2019). This species is a hyperparasitoid that parasitizes several

other parasitoid species. One of the hosts*S. aphidivorus*is A. punicae(Farrokhzadeh et al., 2014).

The morphological feature of S. aphidivorus (Figure 2.23) is that it has a black body with a dark brown head. Body length 1.1 - 1.7 mm. This species has maxillary palps with four segments and labial palps with two segments (Fig. 2.23). Both female and male insects have antennae with nine flagellar segments (Fig. 2.23). The forewings are longer than the body. The wings of S. aphidivorus have very short marginal and postmarginal venations (Fig. 2.23). The tibia on the forelimbs has spikes (Fig. 2.23)(Farrokhzadeh et al., 2014).



(a) Oral apparatus; (b) Antenna; (c) Front Wing; (d) Forelimb

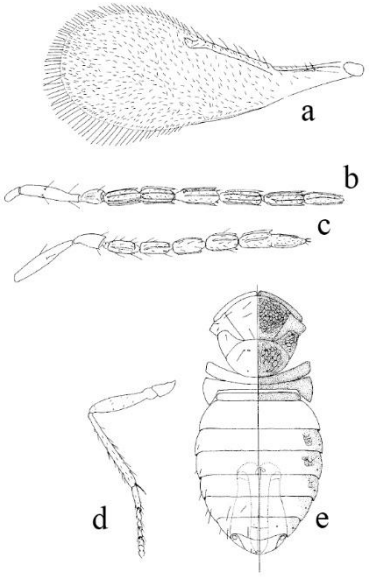
Figure 2.23 Morphological characters of S. aphidivorus (Farrokhzadeh et al., 2014)

c. *Encarsia inaron*Walker

*Encarsia inaron*Walker is a species of the Order Hymenoptera which is grouped into the super-Family Chalcidoidea, Family Aphelinidae.(Abd-Rabou and Simmons, 2010). This species belongs to the genus Encarsia, which is an endoparasitoid in the whiteflies group. This species is one of the parasitoids on Siphoninus phillyreae which is a species of whitefly that mostly attacks pomegranate plants.(Abd-Rabou and Simmons, 2010; Heraty et al., 2008).

The morphological feature of E. inaron is that it has four-segmented flagelomers on its antennae (Fig. 2.24). The wings of this species have reduced venation (Fig. 2.24). The marginal venation is very short. There are many seta

covering the wings. This species has a tarsus with the formula 5-5-5 (Fig. 2.24). Adult female insects have an ovipositor that is shorter than the middle tibia plus the basitarsus. The male E. inaron species has a dark brown head and mesosomes, while the female does not (Figure 2.24).(Hernández- Suárez et al., 2003).



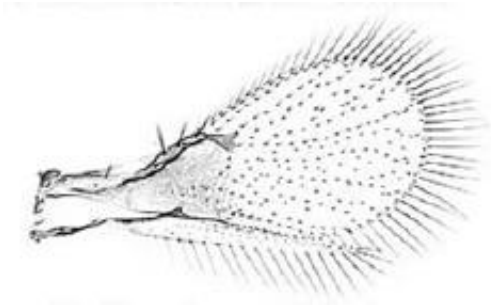
(a) Wings; (b) Male Insect Antenna; (c) Female Insect Antenna; (d) Middle Limb; (e) Mesosoma and Metasoma

Figure 2.24 Morphological characters of E. inaron(Hernández- Suárez et al., 2003)

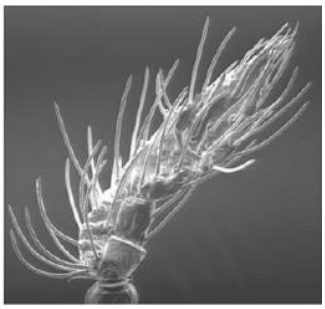
d. *Trichogramma evanescens*Westwood

*Trichogramma evanescens*Westwood is a species of the Order Hymenoptera which is grouped into the super-Family Chalcidoidea and Family Trichogrammatidae. This species is an egg parasitoid. One of the hosts for this species is the eggs of Apomyelois ceratoniae, which is a pest on pomegranate plants(Nobakht et al., 2015).

The morphological feature of the genus Trichogramma is that it has antennae covered by setae (flagelliform setae and unsocketed setae) with two funicular segments (Figure 2.25). The front and hind wings of this genus are covered by seta. Venation on the wings of Genus Trichogramma is reduced, the forewings only have sigmoid venation and 1Rs (First Radial Sector) setal track (Figure 2.25)(Querino et al., 2009).



(a)



(b)

(c)

(a) Front and Rear Wings; (b) The female genus Trichogramma murmur; (c) Antenna of the genus Trichogramma male

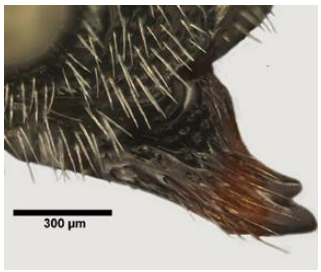
Figure 2.25 Morphological characters of the genus Trichogramma (Querino et al., 2009)

e. *Brachymeria minuta*Linnaeus

*Brachymeria minuta*Linnaeus is a species of the Order Hymenoptera which is grouped into the super-Family Chalcidoidea and Family Chalcididae. This insect is an endoparasitoid coinobiont in the pupa. One of the hosts of B. minuta is A. ceratoniae which is a pest on pomegranate plants(Nobakht et al., 2015).

The morphological feature of B. minuta is that it has a prominent head (Figure 2.26). Each pair of mandibles had two blunt-ended teeth (Fig. 2.26). The setae on the posterior pretarsus are slender and less curved (Fig. 2.26).(Delvare and Huchet, 2017).

(a)



(b)

(c)

(a) Head View Front (Frontal); (b) Right Mandible; (c) Rear Pretarsus

Figure 2.26 Morphological characters of B. minuta (Querino et al., 2009)

2.3 Biological Characteristics of Pomegranate (Punica granatum L.)

The word pomegranate in English is called pomegranate. The word pomegranate comes from the Latin pomum which means apple and granatus which means full of seeds. Pomegranate is a plant that comes from the Balkans to the Himalayas(Melgarejo et al., 2020). Since ancient times, pomegranate has been grown throughout the Mediterranean region including Asia, Africa, and Europe(Morton, 1987).

According toNCBI (2021b), pomegranate is classified into:

Kingdom :Viridiplantae

phylum :Streptophyta

Class : Magnoliopsida

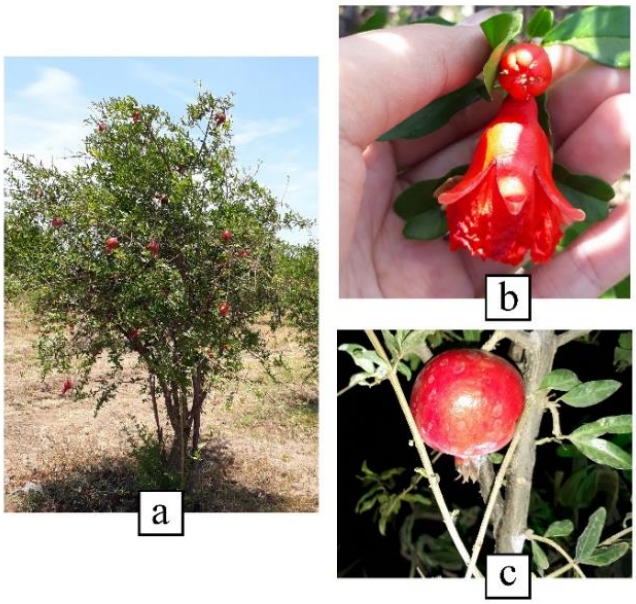
Order : Myrtales

Family :Lythraceae

Genus : Punica

Species : Punica granatum Linnaeus

Pomegranate is a plant with a tree habitus (Figure 2.27). However, most pomegranate farmers often prune their pomegranate trees, so that their shape becomes like a bush(Haque et al., 2015). Morphologically, this plant has roots that grow spread, touch the ground, tuberous, strong, and reddish in color. Pomegranates have stems with alternate branches and open with thorns at the ends. Pomegranate leaves have an elongated and lanceolate shape (lanceolate or oblanceolate), without stipules, short petioles, and the leaves sit opposite(Melgarejo et al., 2020; Rajaei and Yazdanpanah, 2015).



(a) Pomegranate Tree; (b) Pomegranate Flowers; (c) Pomegranate

Figure 2.27 Pomegranate plant and its parts

The pomegranate has a single bright red flower with five corolla and nine petals (Figure 2.27). The pistil and stamen of the pomegranate flower are in one flower (hermaphrodite) (Melgarejo et al., 2020), so that pollination occurs in one individual (self-pollination). Pollination in pomegranate can also occur cross-pollinated with the help of insects and wind(Morton, 1987).

Pomegranate is often called the pulpy berry because it has a fruit filled with prism-shaped granules. These grains are called seeds (Figure 2.28), have parts called testa and tegmen. Testa has a watery texture, while tegmen has a woody texture. The average diameter of a pomegranate is 6 – 12 cm. The shape of the

pomegranate is round like an apple with a crown-shaped structure (persistent calyx) formed from flower petals. The skin of the pomegranate has a hard structure with colors ranging from bright red to green with a few red parts(Melgarejo et al., 2020).

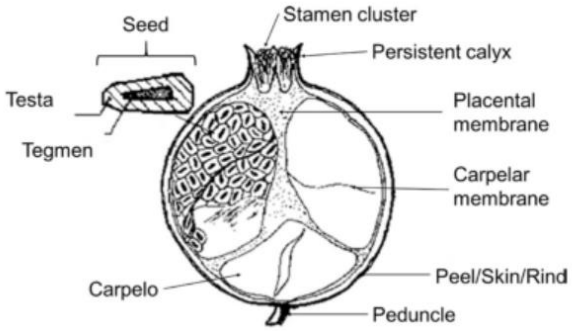


Figure 2.28 The parts of a pomegranate (Melgarejo et al., 2020)

Pomegranate is the main commodity of countries in the Balkans to the Himalayas, such as India. Pomegranate production in India is the highest in the world(Bulbule and Kadam, 2020)with a total production of 2,845,000 tons from a pomegranate garden covering an area of 234,000 hectares in one year(Holticulture Statistics Division, 2018). However, pomegranate production can decrease to30-80% due to disease, pests, or other natural factors that attack the roots, stems, leaves, and pomegranates(Sobhani et al., 2015).

2.4 Merak Village, Situbondo

Kampung Merak is located inSidomulyo Hamlet, Sumberwaru Village, Banyuputih District, Situbondo Regency, East Java Province, Indonesia. Kampung Merak is a residential area within the Baluran National Park area since before Baluran was designated a National Park. Based on the Minister of Forestry Regulation No. P.56/Menhut-II/2006 Kampung Merak is designated as a special zone by the Baluran National Park Office(Ramadhanita and Satiawan, 2019).

The position of Kampung Merak is between forests, mountains, and the ocean. Soil characteristics in this area are dry and sandy. These soil characteristics cause only plants that are able to adapt to grow. Therefore, the people of

Kampung Merak who make a living as field farmers only plant crops that can survive dry and sandy soil conditions. One of the plants that are able to adapt to dry and sandy soil characteristics is pomegranate (Figure 2.29).(Ramadhanita and Satiawan, 2019).

Figure 2.29 Pomegranate garden in Merak Village

CHAPTER 3. RESEARCH METHODS

3.1 Place and Time of Research

The study consisted of sample collection and identification. The sample collection was carried out in Merak Village, Sidomulyo Hamlet, Sumberwaru Village, Banyuputih District, Situbondo (Figure 3.1). Insect samples of the Order Hymenoptera were obtained through passive collection and active collection. This insect sample collection was carried out in pomegranate gardens scattered in the area. The collection is carried out four times, namely on 18-20 December 2020, 2-4 January 2021, 30 January - 1 February 2021, and 13-15 February 2021 at 08.00 - 12.00 WIB according to the active time of insects in their activities.

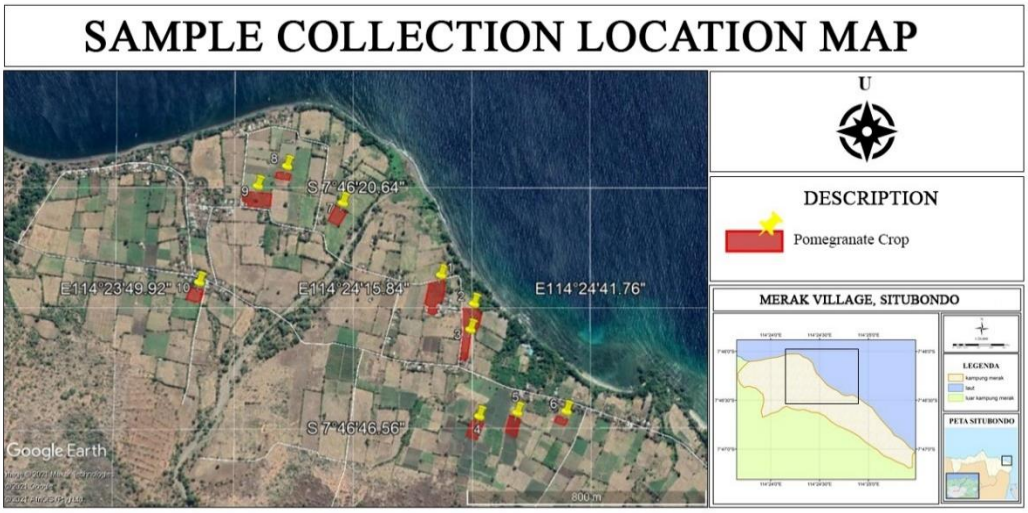


Figure 3.1 Distribution map of pomegranate gardens used in sampling in Merak Village, Situbondo

The identification and validation process of the collected insects of the Order Hymenoptera was carried out at the Zoology Laboratory, Faculty of Mathematics and Natural Sciences (FMIPA), Jember University. The identification and validation were carried out from February to April 2021. The validation was carried out under the guidance of Purwatiningsih, S.Si., M.Sc., Ph.D.

3.2 Library Tools, Materials, and Reference

The tools used in this study were containers, funnels, small glass bottles, insect nets, brushes, insect needles, petri dishes, stereo microscopes (Nikon SMZ 445), pipettes, tweezers, glass bottles with wide mouths, and cameras. The materials used in this study were small triangular paper, 70% alcohol, black plastic, tissue, cork, and insect glue. The references used in the identification are Goulet and Huber's (1993) identification book, Hymenoptera of the World: an Identification Guide to Families.

3.3 Research Design

This research is a qualitative research with descriptive method. The research was conducted by passively and actively collecting insects of the Order Hymenoptera. Insects of the Order Hymenoptera collected were then identified for their morphological characters by using the identification guide book Hymenoptera of the World: an Identification Guide to Families written by Goulet and Huber (1993). The identification results were then described and literature tests were carried out regarding its role as a parasitoid.

3.4 Research Procedure

3.4.1 Sampling

Insect sampling of the Order Hymenoptera was carried out passively and actively. Passive collection was carried out to collect insects of the Order Hymenoptera in pomegranate fruit, while active collection was carried out to collect insects of the Order Hymenoptera in pomegranate plantation areas. The collection was carried out randomly in ten pomegranate gardens spread out in Merak Village, Sidomulyo Hamlet, Sumberwaru Village, Banyuputih District, Situbondo with the highest pest attack rate. The high level of pest attack can be seen from the damage to the fruit produced.

Passive collection begins with collecting pomegranates that are indicated to be attacked by pests. Indications of pest attack on pomegranates are punctures and burrs on the surface of the pomegranate skin (Figure 3.2). The collection of

pomegranates indicated by pests was carried out purposively as much as possible in each selected garden.

Figure 3.2 Pomegranate indicated by pests

The collected fruits are then placed in an emergency container (Figure 3.3). The dark container (emergence container) consists of a dark plastic container, a funnel, and a small glass bottle (collection bottle). The collection bottle is placed on the top of the container to attract insects. This allows insects that are thought to be inside the fruit to be attracted to the light source coming from the collecting bottle and trapped inside. This dark container is placed in the laboratory by conditioning the room at a temperature of 27 – 30°C, humidity 65 – 75%, and the ratio of dark and light 12:12 hours. The emergence of insects of the Order Hymenoptera in bCollecting bottles were observed every day between 12.00 – 14.00 WIB. Insects of the Order Hymenoptera that appeared in the collection bottle were then transferred into a glass bottle with a wide mouth containing 70% alcohol using a brush.

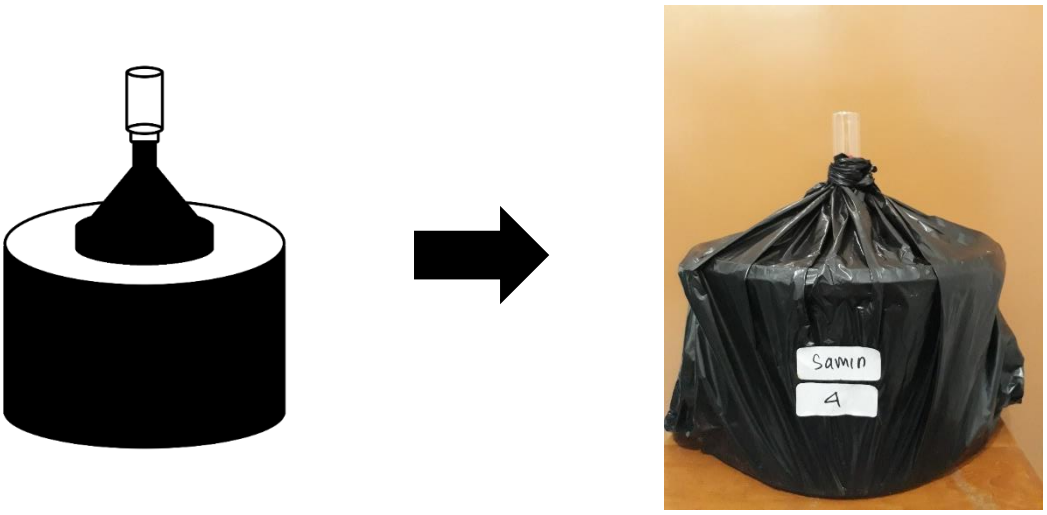


Figure 3.3 Emergency container

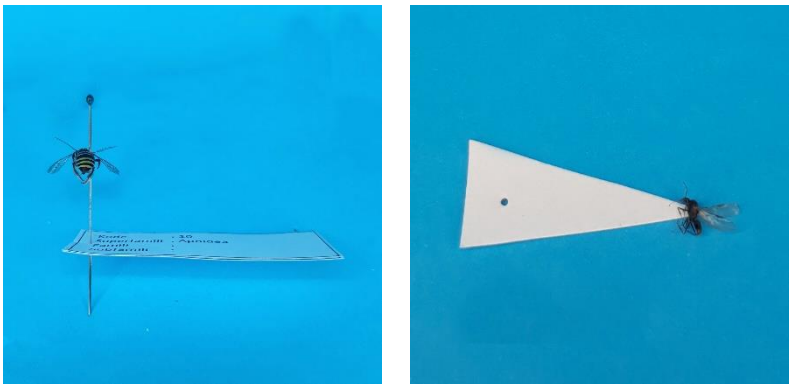
Active collection using insect nets (Figure 3.4) was carried out by tracing each lane in the pomegranate garden. The insect net was swung several times near the pomegranate plant. Insects of the Order Hymenoptera trapped in insect nets will then be put into wide-mouthed glass bottles containing 70% alcohol.

Figure 3.4 Active collection using insect nets

3.4.2 Mounting Insects of the Order Hymenoptera

Insects of the Order Hymenoptera obtained and stored in wide-mouthed glass bottles containing 70% alcohol were sorted according to size. Mounting is done in two ways according to the size of the collected insects. After sorting according to the size of the specimen, the specimen was softened using 70% alcohol to arrange the appendages. Appendages such as wings, legs, and antennae are arranged in

such a way with the help of a brush and needle under a stereo microscope. Large insects can be mounted directly by inserting an insect needle into the metathorax (Figure 3.5 a), while small insects can be mounted using a small triangular paper (Figure 3.5 b).



(a) (b)

(a) Puncture of the Mesothorax; (b) Use of Small Triangle Paper

Figure 3.5 Mounting insects of the Order Hyemenoptera

*Mounting*using a small triangle of paper is done by sticking the side of the paper base using an insect needle at a height of 2 – 2.5 cm from the tip of the needle. The small triangular paper is dripped with a small amount of insect glue on the opposite end of the needle. The thoracic part of the specimen that has been arranged in such a way is touched on insect glue that has been dripped on small triangular paper. The body parts of the specimen are arranged in such a way that there are no overlapping parts.

3.4.3 Specimen Identification

The identification process is carried out by observing the morphology of the collected insects. The observed morphology of each collection is matched with a key of determination that refers to the identification bookGoulet and Huber (1993), Hymenoptera of the world : an identification guide to families. Morphological observations were carried out using a stereo microscope. The identification results are validated inZoology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), University of Jember.

3.4.4 Labeling

Labeling was carried out on all specimens collected using small paper. The data listed on the label contains the location where the specimen was found, the date it was collected, the name of the collector, classification, and other important information. The label is placed approximately 1 cm from the tip of the needle, so that its position is under the insect (Figure 3.6). The position of the label faces up, making it easier to read without having to change the position of the specimen.

3.5 Data Analysis

Data in the form of an inventory of insects of the Order Hymenoptera which were collected and identified and validated, were analyzed descriptively. The description is carried out based on the observed morphological characteristics and refers to the identification book Hymenoptera of the world: an identification guide to families(Goulet and Huber, 1993)used. Literature tests were also carried out on the inventoried specimens regarding their role as parasitoids.

CHAPTER 4. RESULTS AND DISCUSSION

The results of the Insect Inventory of the Order Hymenoptera were obtained from passive collection in collecting bottles and actively using insect nets. The passive collection on the collecting bottle failed to find any insects of the Order Hymenoptera that appeared. This is different from direct collection using insect nets, which collected 41 individuals of the Order Hymenoptera from four different superfamilies (Table 4.1).

4.1 Passive Collection

Passive collection using dark containers on pomegranates which are indicated to be attacked by pests, is a collection carried out by attracting the attention of insects using light entering from a collection bottle in the form of a small clear glass bottle. Based on daily observations (for 30 days) on collecting bottles from ten containers containing pomegranate fruit which were indicated to be attacked by pests, no insects of the Order Hymenoptera appeared. However, some insects and other arthropods were found in the collection bottles. These insects and other arthropods are a group of Coleoptera, mites, and spiders.

Adults of the Order Coleoptera were collected from collection bottles numbered 1 – 6 on the fifth day the containers were placed in the laboratory. Mites (mites) were found on the 17th day in collection bottles from the same container. The spider was found on the seventh day in the collection bottle from container number ten.

4.1.1 Carpophilus

The adults of the Order Coleoptera found have a body size of less than 5 mm. The body is dark brown tend to black. The antennae have eleven segments with three segments distally forming a club or club. This insect has a tarsus consisting of five segments and a short elitra, showing its pygidium. This insect is grouped into the Genus Carpophilus(Larson, 2013).

Figure 4.1 Carpophilus

According toNCBI (2021a), Carpophilus classified into the following classifications.

Kingdom : Animalia

Phylum : Arthropods

Class :Insects

Order : Coleoptera

Family :Nitidulidae

Genus :*Carphophilus*

*Carpophilus*or commonly known as sap beetles (sap beetles) are polyphagous insects that are generally attracted by the odor released from fermenting or rotting fruit. The presence of wounds on the fruit due to attack by other insects causes the inside of the fruit to be exposed to the outside environment. Exposure of the inside of the pomegranate to the external environment accelerates the spoilage of the fruit. It is the odor released by the rotting fruit that attracts Carpophilus to it. The arrival of Carpophilus to the injured pomegranate makes Carpophilus a vector for the spread of yeast and bacterial cells, thereby accelerating the occurrence of fruit spoilage.(Emekci and Moore, 2015).

According toEmekci and Moore (2015), adult Carpophilus enters the pomegranate, through the tip of the calyx or through the hole formed by a wound in the fruit, eats the flesh of the pomegranate, and lays its eggs in the fruit. These eggs will hatch within 2-5 days and pass through the larval stage in 3 instars for 14 days. The hatched larva will eat the flesh of the fruit where it develops, so the

life cycle occurs inside the fruit. Based on this statement, it is estimated that the pomegranates collected are pomegranates that have begun to rot and are attacked by Carpophilus. When these collected fruits are placed in dark containers, spoilage in the fruit causes Carpophilus' food sources to deplete, prompting it to go outside and look for other food sources. Therefore, Carpophilus was found in the collecting bottle.

*Carpophilus*according toEmekci and Moore (2015), host several parasitoids of the Order Hymenoptera such as Cerchysiella utilis, Microctonus nitidulidis, and Brachyserphus abruptus. However, when the pomegranates that serve as shelter and food for Carpophilus are placed in dark containers to spawn parasitoids that attack it, collection is unsuccessful. This is possible because Carpophilus found in pomegranates is not a suitable host for the parasitoids present in the pomegranate plantation in Kampung Merak.

4.1.2 Tetranychidae

Mites or mites (Figure 2.1) collected in the collection bottle, have a size of less than 0.3 mm. The body has no differentiation between the head and body. It has a greenish-white body with four pairs of legs. According toTriplehorn and Johnson (2005), this arthropod is a group of Tetranychidae.

According toTriplehorn and Johnson (2005), Tetranychidae are classified into the following classifications.

Kingdom : Animalia
Phylum : Arthropods
Class : Arachnida
Order : Acari
Family : Tetranychidae

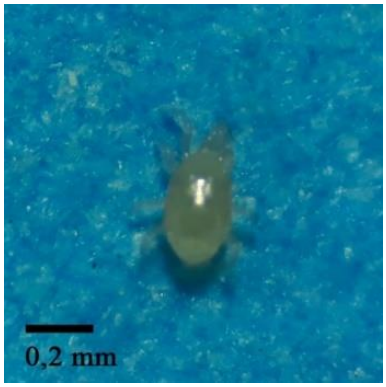


Figure 4.2 Tetranychidae

The Tetranychidae group or commonly referred to as Spider Mites are plant-eating mites. These mites eat the leaves or fruit of various types of plants, and even become pests. According toCocuzza et al. (2016), the Tetranychidae group found in pomegranate plantations attacks the leaves of the pomegranate, causing the leaves to turn yellow and then dry out. This occurs because the Tetranychidae eat the mesophyll tissue in the leaves, so the leaves cannot photosynthesize, turn yellow, and then dry out.(Attia et al., 2013; Elango et al., 2021). In addition to attacking pomegranate leaves, Tetranychidae also attacks the fruit, namely the skin. Attacks on the skin of the pomegranate cause a brown discoloration and cracks(Ebeling and Pence, 1949). The appearance of Tetranychidae in collecting bottles was possible due to the presence of Tetranychidae on the skins of the collected pomegranates.

4.1.3 Araneae

The spider that appeared in the collection bottle (Figure 4.3), was less than 4 mm in size. Has a dominant body brownish white. The body consists of a cephalothorax and an opistoma (abdomen). The abdomen is segmented and attached to the cephalothorax via a slender stalk. This spider has four pairs of legs attached to the cephalothorax. Several eyes are also present in the cephalothorax which is visible from the dorsal side. According toTriplehorn and Johnson (2005)This arthropod belongs to the Araneae group.

Figure 4.3 Araneae

According toTriplehorn and Johnson (2005), these spiders are classified into the following classifications.

Kingdom : Animalia

phylum : Arthropods

Class : Arachnida

Order : Araneae

Spiders are Arthropods that are widely distributed in the world. Spiders are carnivores that eat insects or other arthropods by building snares when hunting for prey(Ahmed, 2017). As natural predators, spiders can play a role in reducing the density of pests that attack plantation or agricultural crops(Bianchi et al., 2006). This can be seen from their behavior in the collecting bottle where on observation on February 8, 2021, along with their appearance on the collecting bottle, they began to build a snare (Figure 4.4 a). The appearance of Carpophilus in the collection bottle a few days later, causing the beetle to be trapped in a constructed noose (Figure 4.4 b). The spider that acted as a predator then preyed on the entangled Carpophilus (Figure 4.4 c).

The discovery of spiders in the collection bottles was possible because there were adult female spiders on the collected pomegranates, so that the spiders appeared in the collection bottles when the pomegranates were placed in a dark container. According toHosseini et al. (2017), generally predators such as Araneae take refuge in the crown of the pomegranate. The female spider that may have hidden in the crown of the collected pomegranate is thought to have also carried

her egg sac, so that in the collection bottle many smaller spiders also appeared. This small spider is thought to be his offspring. This is supported by some literature which states that the adult female spider lays her eggs in a bag which she then carries in her mouth until they hatch.(Ahmed, 2018; Zaed, 2019).

(a) Araneae appearance on the collecting bottle; (b) Carpophilus Trapped in a Constructed Snare; (c) Carpophilus exoskeleton remains

Figure 4.4 The behavior of Araneae preying on Carpophilus

This behavior of spiders as predators may be the reason for the absence of the order Hymenoptera insects in the collection bottles. Spiders in dark containers allegedly prey on insects in containers as they prey on Carpophilus (Figure 4.4). This may also happen to other insects, including insects of the Order Hymenoptera, so that insects of the Order of Hymenoptera were not found in the collection bottles.

4.2 Active Collection

Based on the active collection of the Order Hymenoptera, 41 individuals were obtained from four different superfamilies (Table 4.1). Active collection is different from passive collection which aims to collect Hymenoptera insects found in pomegranates. Insects of the Order Hymenoptera are actively collected from all areas of the pomegranate garden and its surroundings.

The most active collections of the Order Hymenoptera insects come from the Vespoidea superfamily. The next most abundant superfamily in the active

collection was Apoidea. Proctotrupoidea and Ichneumonoidea are the least collected superfamilies.

Table 4.1 Identification of active collection insects using insect nets

Order	Super-Family	Family	Number found
Hymenoptera	Vespoidea	Scoliidae	15
		Vespidae	5
		Tiphiidae	1
	Proctotrupoidea		6
	Ichneumonoidea		1
	Apoidea		13
		Amount	41

4.2.1 Super-Family Vespoidea

The identification key used in insects of the Order Hymenoptera in this group, according toGoulet and Huber (1993), is as follows.

The key to the super-family:

1(a) The forewings when stretched backwards extend far past the ends of the mesosomes……...…….2

2(aa) Body with marked constriction between the first and second abdominal segments that line the mesosoma and metasoma, when viewed from the dorsal and lateral sides (Apocrita).

12(aa) The head is not globular (round) and there is no circle of teeth around the middle ocelli.

(bb) Body shapes and sizes vary, often fat or small; The ovipositor is often short or hidden.

(cc) Partthe metacoxal base is usually narrow, the two articulations at the base are close to each other (difficult to see) and arranged obliquely; the long axis of the metacoxes forms an angle away from the metasoma…….13

13(aa) Distancethe shortest point between the base of the mandible and the rim of the compound eye (malar space) without an indentation.

(bb) The mesoscutum is rare with a large axilla and medial groove, but when with both, the body length is usually less than 3 mm and the number of flagelomeric segments is not 12.

(cc) Sparse antennae with 12 flagellomeric segments…….……14

14(a) Forewings with 3 or more closed cells formed by conspicuous tubular venation.

(b) Longbody usually more than 5 mm, but sometimes less than 2 mm…….…….……15

15(a) Front wing without stigma……...……16

15(aa) Front wing with stigma……...…….18

16(a) Sforelimb with at least 5 closed cells formed by tubular venation……...17

17(aa) The profemur is smaller than the metafemur.

(bb) Antenna with 10 or more flagelomer segments.

(cc) Torulus is closer to the center of the head and not below the transverse ledge..……..18

18(a) Forewings with C (Costal) and R (Radial) venation touching or fused, eliminating costal cells.

18(aa) Forewings with separate C and R venations; there is a small long costal cell……...21

19(aa) The abdominal sterna is sclerotized as strongly as the terga.

(bb) anterior sterna (usually sternal metasoma 1 – 3) are undivided and are generally convex ventrally…..…….20

20(a) Antenna with 10 or 11 flagellomer……...24

21(aa) Antenna with 10 or more flagelomeric segments…….22

22(aa) The forewings with Cu (Cubital) venation are deflected abruptly (30-90o angle or greater) posteriorly, gradually, at the base of the 1M-Cu (1Medial-Cubital) venation.

(bb) Forewings with 2Cu-A (2Cubital-Anal) venation are usually less than half the length of 1Cu-A (1Cubital-Anal) venation.

(cc) Sforewing with 2R-M (2Radial-Medial) and 2M-Cu (2Medial-Cubital) venation……...23

23(aa) Antenna with 10 or 11 flagelomeric segments.

(bb) Tarsomeres without a protrusion or rarely with small flat lobes……24

24(aa) SUtra on the thorax around the tegula are visible.

(bb) KThe density and structure of the hairs around the body vary, usually sparse or short.

(cc) The hind limbs with tarsomer 1 are usually cylindrical in shape, not 3 times larger than the tarsomer.

25(aa) Shind wings with 1 – 3 closed cells formed by tubular venation……..28

28(aa) The tegula touches the pronotum when the wings fold, so that the mesoscutum appears to be separated from the mesopleura……..30

30(aa) Rthe hairs around the body are thin and fine, very rarely with short or long branches.

(bb) Thind limbs with tarsomer 1 are cylindrical, not more than 3 times the length of tarsomer 2…..……31

31(aa) Forewings with 3 (very rarely 4) venation approaching or touching the apical edge beyond the stigma.

(bb) STerna metasoma 1 and 2 articulate with each other, their junctions form straight curves…….....32

32(aa) Pronotum on the lateral side with the medial cross section fused with the mesonotum, not raised on the lobes like a transverse roll; the posterior edge of the pronotum is often lobeless, the lobes are defined by concave angles above and below; lobe protrusion is sometimes present but occurs completely beneath the sugar; the posterior border of the pronotum on the dorsal side forms a relatively uniform curve that continues back to the tegula or to the overlying pointed lobe.

(bb) The tegula touches the pronotum margin, but the point of contact is over the protruding lobe which may be on the posterior border.... Vespoidea

Based on the identification results, three different families were obtained from the super-Family Vespoidea, namely the Scoliidae, Vespidae, and Tiphiidae families. The results of the collection from the Scoliidae family found five different insects that both belong to this family. The number of insects found in the Vespidae family were two different insects, while from the Tiphiidae family, one was found.

a. Family Scoliidae

The identification key used in the insects of the superfamily Vespoidea of this group according toGoulet and Huber (1993), is as follows.

Key to family:

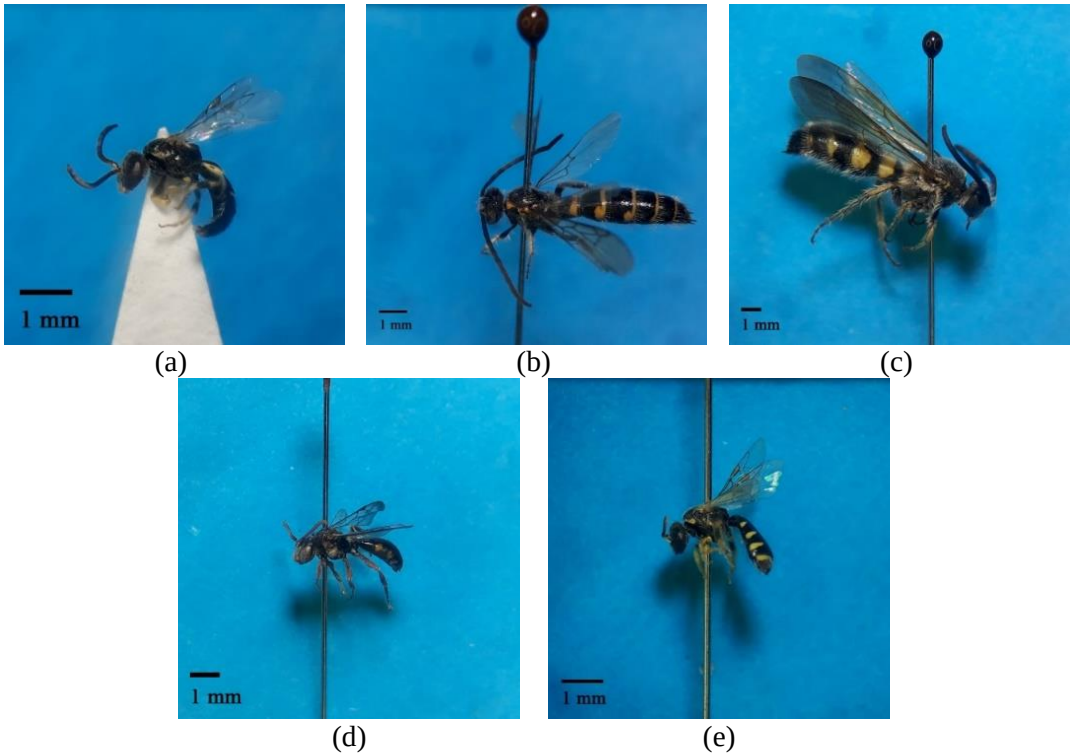
1(a) Wings are fully developed (forewings have a length of approximately reaching the mesosoma)……..……2

2(a) The metacoxes are separated by a flattened metasternum that extends to the mesosternum.

(b) The wing membrane has fine wrinkles longitudinally in an apical direction.

(c) Propodeumdivided into 3 by 2 longitudinal grooves connecting the two plates and ramps……..……..Scoliidae

The collected family Scoliidae has a dominant black body with a yellow band-shaped pattern on the metasoma (Figure 4.5). The body size of the Scoliidae family obtained varied from 4 mm to more than 1 cm. Scoliidae 1 and 5 have a body measuring 4 mm, Scoliidae 4 measuring 6 mm, Scoliidae 2 measuring 8 mm, and Scoliidae 3 measuring 12 mm.



(a) (b) (c)

(d) (e)

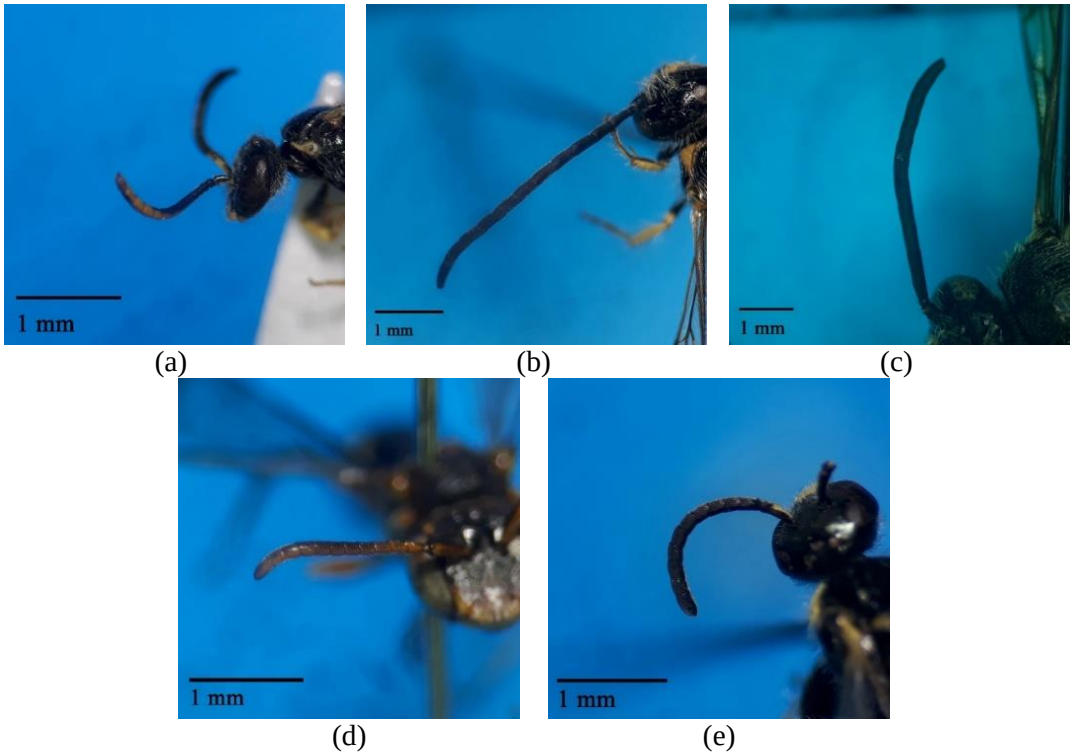
(a) Scoliidae 1; (b) Scoliidae 2; (c) Scoliidae 3; (d) Scoliidae 4; (e) Scoliidae 5

Figure 4.5 Family Scoliidae

According toGoulet and Huber (1993), this group is classified into the following classification.

Kingdom : Animalia
Phylum : Arthropods
Class :Insects
Order : Hymenoptera
Super-Family : Vespoidea
Family :Scoliidae

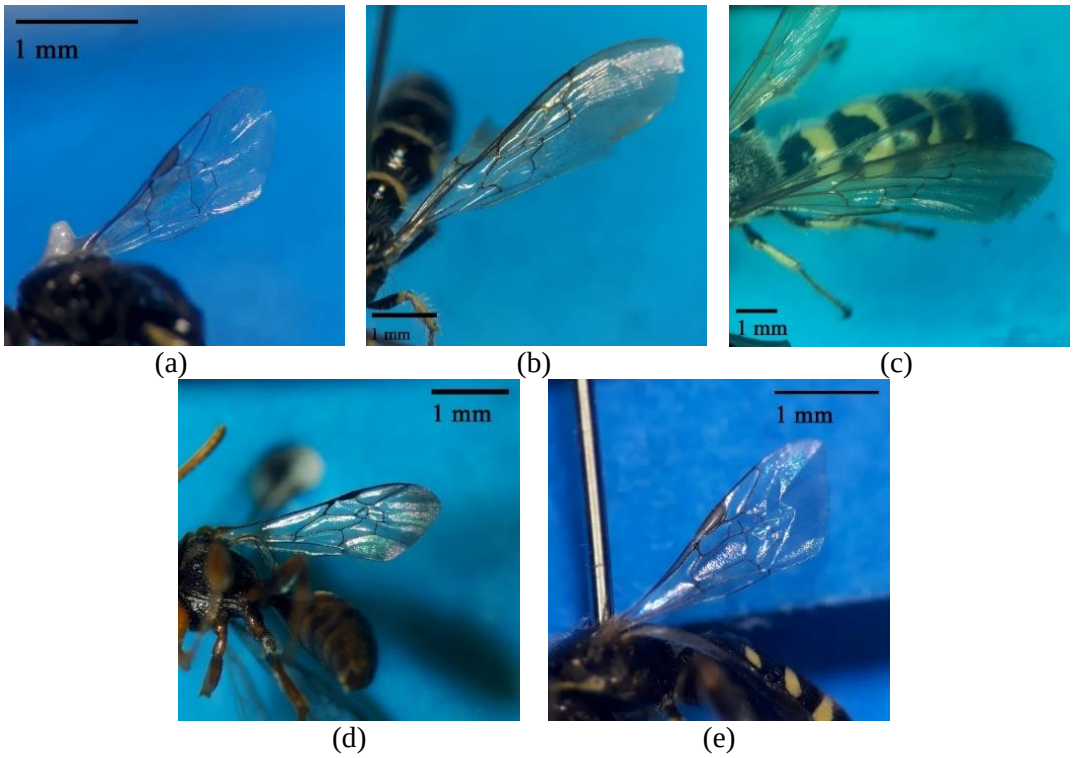
Based on the identification results, Scoliidae have antennae that are not angled. The number of flagelomernya 11 to 12 segments. Scoliidae 1 and 3 have 12 flagelomeric segments (Figures 4.6 a and c), while Scoliidae 2, 4, and 5 have 11 flagelomeric segments on their antennae (Figures 4.6 b, d, and e).



(a) Scoliidae 1; (b) Scoliidae 2; (c) Scoliidae 3; (d) Scoliidae 4; (e) Scoliidae 5

Figure 4.6 Antenna morphology in the Scoliidae family

This insect has full-sized wings with well-developed venation. Scoliidae have nine to eleven closed cells on the forewings and two on the hindwings, which are formed by tubular venation. Scoliidae 2 and 3 have nine closed cells on their forewings (Figure 4.7 b and c), Scoliidae 4 have ten closed cells on their forewings (Figure 4.7 d), while Scoliidae 1 and 5 have eleven closed cells on their forewings (Figure 4.7 a and e). The hind wings of Scoliidae 1–5 have two closed cells (Fig. 4.8).



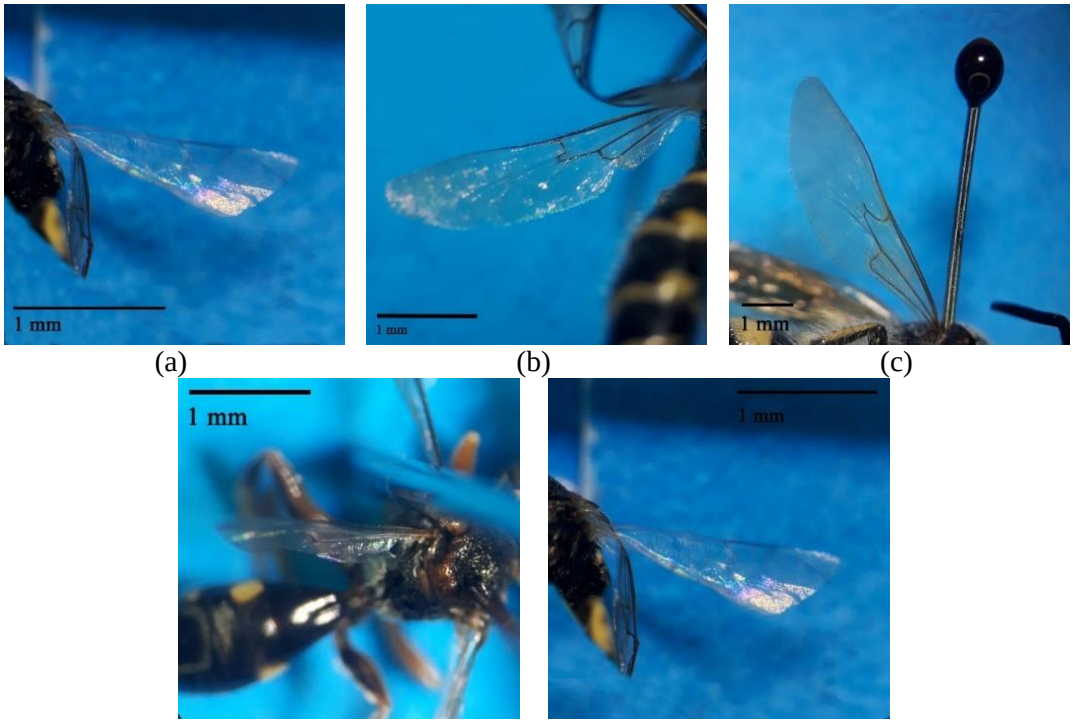
(a) (b) (c)

(d) (e)

(a) Scoliidae 1; (b) Scoliidae 2; (c) Scoliidae 3; (d) Scoliidae 4; (e) Scoliidae 5

Figure 4.7 Morphology of the forewings in the Family Scoliidae

The forewings of Scoliidae have a stigma with separate C and R venations, but some do not have a stigma. Scoliidae 1, 2, 4, and 5 have stigmas on their forewings with separate C and R venations (Fig. 4.7 a, b, d, and e), except for Scoliidae 4 (Fig. 4.7 d). It has hind wings equipped with a very well-defined jugular lobe in Scoliidae 1 – 5 (Fig. 4.8). The forewings of Scoliidae also have fine wrinkles longitudinally pointing apically.



(a) (b) (c)

(d) (e)

(a) Scoliidae 1; (b) Scoliidae 2; (c) Scoliidae 3; (d) Scoliidae 4; (e) Scoliidae 5

Figure 4.8 Morphology of hindwings in the Family Scoliidae

The tip or corner of the pronotum is attached to or close to the tegula when viewed from the dorsal or lateral side. Has a flattened meso- and metasternal. The metasoma is attached to the underside of the propodeum, so that it appears close to the coxa. The limbs have similarly sized tarsomes that are cylindrical in shape. Compound eyes have edges that curve inward.

The Scoliidae family is a cosmopolitan wasp group that predominantly lives in the tropics. Larvae of the family Scoliidae are ectoparasitoids of Coleoptera larvae. Coleoptera larvae which usually become hosts are from the Scarabaeoidea group and not infrequently from the Curculionoidea group.(Goulet and Huber, 1993). Adult Scoliidae insects will detect the presence of their hosts living in the soil by following the trail of kairomone or attractant substances released by insects, residual substances from the cuticle, or feces left by potential hosts.(Inoue and Endo, 2008).

b. Family Vespidae

The identification key used in insects of the superfamily Vespoidea of this group, according toGoulet and Huber (1993), is as follows.

Key to Family:

1(a) Wings are fully developed (forewings have a length of approximately reaching the mesosoma)……2

2(aa) Mthe etacoxes are close together; the metasternum is not dilated and is usually not in the same stratum as the mesosternum; metacox is not covered by lamina.

(bb) Wing membrane without fine wrinkles longitudinally but sometimes with an irregular surface.

(cc) Propodeumwithout longitudinal grooves connecting the two plates and ramps…… …3

3(aa) MThe esophagus does not extend posteromedially beyond the mesocosa, although sometimes adjacent to the margin.

(bb) The hind wings are sometimes without a jgal lobe, but usually with a jgal lobe…….....5

5(aa) Mnode-like segmentless athasoma. Tergum 1 on lateral appearance does not narrow markedly or abruptly apically, although occasionally it swells slightly in the middle and/or tergum 2 occasionally narrows anteriorly.

(bb) The second and third segments of the metasoma without narrowing between them, but if there is narrowing then a similar narrowing is present between the following segments.....12

12(aa) 2nd tergum and 2nd sternum metasomawithout thick lines and without longitudinal indentations on the lateral sides; If there is an indentation, it extends from the anterior edge to almost the length of the sclerite.

(bb) Sthe ternum of the last metasoma is usually simple, never with 3 apical spines.

(cc) Sforewings with marginal and/or sub-marginal cells surrounded by tubular venation…….……..14

14(aa) Metasomathe latter is usually simple in appearance, never forming an upward curving medial hook.

(bb) Metasomararely cylindrical, usually less than twice the length of the mesosomes and the width of the mesosomes 15

15(a) Pronotumwith the posterolateral end having a clear angle or rectangular shape, extending slightly above and beyond the edge of the sugar.

(b) Forewings with 1st discal cells (1D) which are the same length as the sub-basal cells (SB), but rarely shorter.

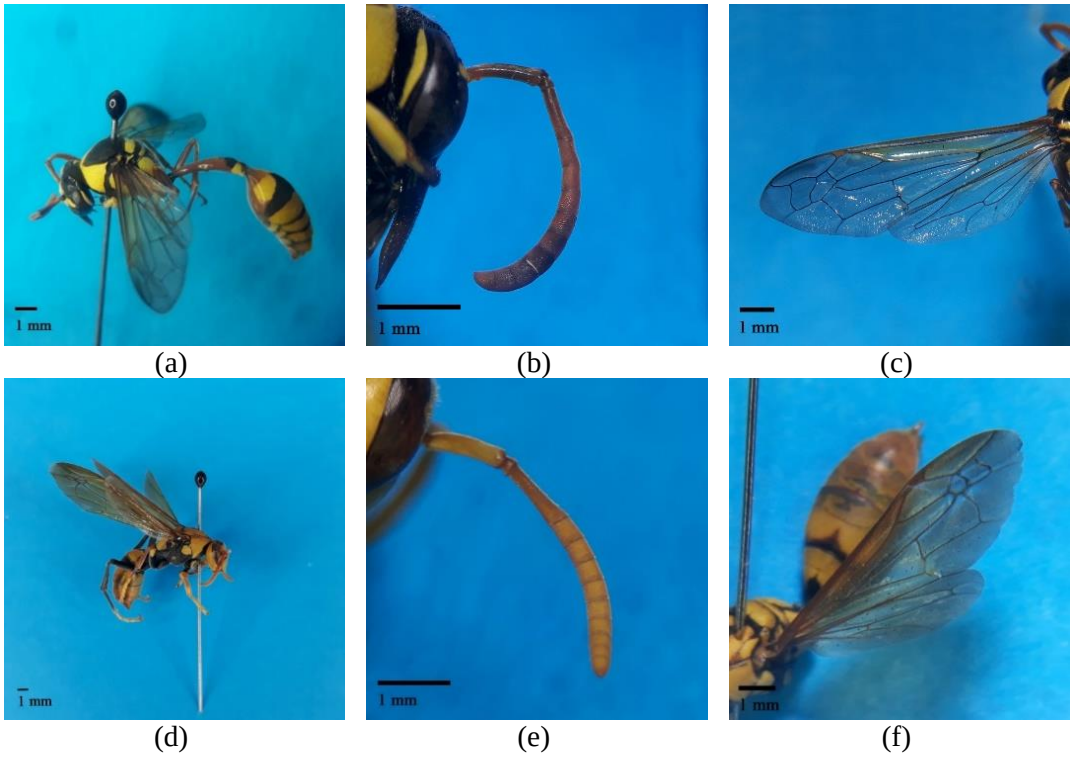
(c) The forewings are usually folded longitudinally, but sometimes flatten.

(d) Mcompound ata with well-margined inner edges…….Vespidae

From the Vespidae family, two different insects were found, namely Vespidae 1 (Figure 4.9 a) and Vespidae 2 (Figure 4.9 d). Both have a dominant yellow body with black and brown patterns. Both body size is the same, which is less than 2 cm.

According toGoulet and Huber (1993), this group is classified into the following classification.

Kingdom : Animalia
phylum : Arthropods
Class :Insects
Order : Hymenoptera
Super-Family : Vespoidea
Family : Vespidae



(a) (b) (c)

(d) (e) (f)

(a) Vespidae 1; (b) Vespidae 1 antenna; (c) Vespidae 1 wing; (d) Vespidae 2; (e) Vespidae 2 antennae; (f) Wings of Vespidae 2

Figure 4.9 Morphological characters of the Family Vespidae

Based on the identification results, Vespidae 1 and 2 have ten flagelomeric segments on their antennae (Figure 4.9 b and e). The Vespidae are a group of social wasps with full-sized, longitudinally folded wings and well-developed venation (Figs. 4.9 c and f). Vespidae 1 has nine closed cells on the forewings and two on the hind wings, while Vespidae 2 has 10 closed cells on the forewings and 2 closed cells on the hind wings formed by tubular venation. The forewings of Vespidae 1 have a stigma with separate C and R venations, whereas Vespidae 2 is fused. Both Vespidae 1 and 2 have hind wings equipped with a jugular lobe smaller than the sub-basal cells.

The tip or corner of the pronotum is attached to or close to the tegula when viewed from the dorsal or lateral side. The metasoma is attached to the underside

of the propodeum, so that it appears close to the coxa. The limbs have similarly sized tarsomes that are cylindrical in shape. The pair of compound eyes have edges that curve inward.

The Vespidae family is a family of groups of wasps with different behaviors. The behavior of these wasps ranges from solitary species to social groups which are generally opportunistic. Wasps in the Vespidae family usually return to places with large food supplies in order to optimize their search for food and reduce search efforts. This is related to the behavior in order to survive carried out by this group(Oliveira et al., 2017).

The Vespidae family utilizes the resources in the surrounding environment by preying on other insects, utilizing plant fibers, and eating nectar. The social species of the adult Vespidae prey on other insects to feed their larvae, while the larvae of the solitary species prey on other insects(Goulet and Huber, 1993). Therefore this group acts as a natural predator. Plant fibers are used by Vespidae to build nests which are places for their larvae to grow and develop.(Oliveira et al., 2017).

c. Family Tiphiidae

The identification key used in insects of the superfamily Vespoidea of this group, according toGoulet and Huber (1993), is as follows.

Key to Family:

1(a) Wings are fully developed (forewings have a length of approximately reaching the mesosoma)…….……2

2(aa) Mthe etacoxes are close together; the metasternum is not dilated and is usually not in the same stratum as the mesosternum; metacox is not covered by lamina.

(bb) Wing membrane without fine wrinkles longitudinally but sometimes with an irregular surface.

(cc) Propodeumwithout longitudinal grooves connecting the two plates and ramps…… 3

3(aa) MThe esophagus does not extend posteromedially beyond the mesocosa, although sometimes adjacent to the margin.

(bb) The hind wings are sometimes without a jgal lobe, but usually with a jgal lobe…….…… 5

5(a) The 1st segment of the metasoma forms like a node, on the lateral view the terga widens to the middle and forms a sudden constriction.

(b) The 2nd segment of the metasoma sometimes is node-like or with marked constriction on dorsal and ventral appearances between the 2nd and 3rd segments and usually without constriction between the following segments……...…… ……6

6(aa) Terga and 2nd sterna metasomawithout thick lines and deep longitudinal grooves on lateral sides…….……8

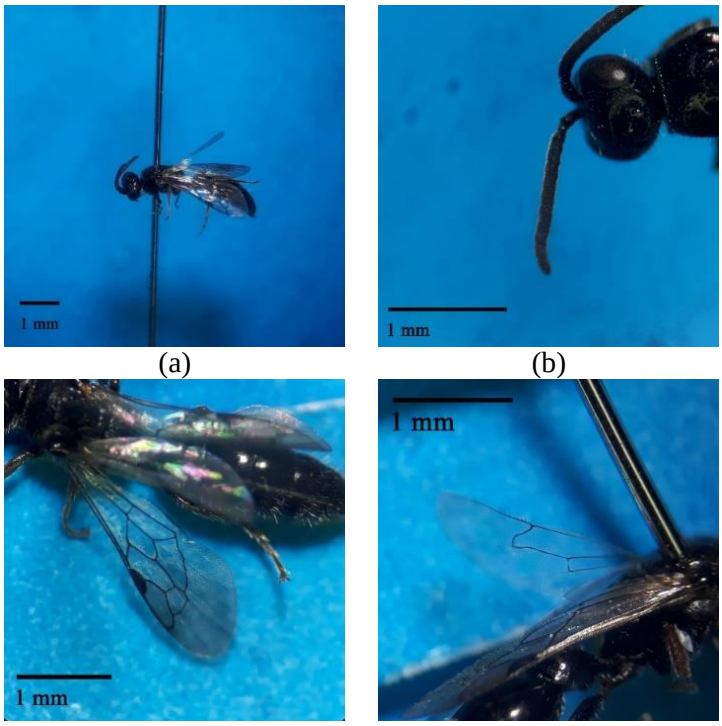
8(a) Sternathe last segment of the metasoma forms a hook that curves upward.

(b) Metasomacylindrical in shape, at least twice as long and narrower than the mesosoma……..…….Tiphiidae

Based on the results of the collection, it was found that one insect was included in the Tiphiidae family (Figure 4.10 a). This insect has a metallic black body overall. Its body size is less than 5 mm.

According to(Goulet and Huber, 1993), this group is classified into the following classification.

Kingdom : Animalia
phylum : Arthropods
Class :Insects
Order : Hymenoptera
Super-Family : Vespoidea
Family :Tiphiidae



(a) (b)

(c) (d)

(a) Tiphiidae; (b) Tiphiid antennae; (c) Tiphiidae Forewings; (d) Tiphiidae hind wings

Figure 4.10 Morphological characters of the Family Tiphiidae

The collected family Tiphiidae has 12 flagelomeric segments on its antennae (Fig. 4.10 b). It has full-sized wings with well-developed venation. The forewings have ten closed cells and a stigma with separate C and R venations (Fig. 4.10c). The hindwings have two closed cells with well-defined jugular and claval lobes (Fig. 4.10 d).

The tip or corner of the pronotum is attached to or close to the tegula when viewed from the dorsal or lateral side.The sternal segment of the last metasoma forms a hook that curves upward. The metasoma is cylindrical, at least twice as long and narrower than the mesosoma. The eyes have convex or straight edges that don't curve inward.

The family Tiphiidae is a group of wasps whose species are all solitary insects. Larvae of this group are generally ectoparasitoids in the larvae of ground

beetles (Soil-dwelling Coleoptera)(Goulet and Huber, 1993)The adult female Tiphiidae, which will lay their eggs, look for the right host by digging the ground and following the smell of ground beetles in the soil. The egg will be laid between the last thoracic segment and the first abdominal segment of the ground beetle after temporarily immobilizing it. Tiphiidae eggs that hatch and become larvae will later eat the outer shell and internal fluids by piercing the cuticle of the ground beetle(Shanovich et al., 2019).

4.2.2 Super-Family Proctotrupoidea

The identification key used in insects of the Order Hymenoptera in this group, according toGoulet and Huber (1993), is as follows.

The key to the super-family:

1(a) The forewings when stretched backwards extend far past the ends of the mesosomes……...…….2

2(aa) Body with marked constriction between the first and second abdominal segments that line the mesosoma and metasoma, when viewed from the dorsal and lateral sides (Apocrita).

12(aa) The head is not globular (round) and there is no circle of teeth around the middle ocelli.

(bb) Body shapes and sizes vary, often fat or small; The ovipositor is often short or hidden.

(cc) Partthe metacoxal base is usually narrow, the two articulations at the base are close to each other (difficult to see) and arranged obliquely; the long axis of the metacoxes forms an angle away from the metasoma……..……13

13(aa) Distancethe shortest point between the base of the mandible and the rim of the compound eye (malar space) without an indentation.

(bb) The mesoscutum is rare with a large axilla and medial groove, but when with both, the body length is usually less than 3 mm and the number of flagelomeric segments is not 12.

(cc) Sparse antennae with 12 flagellomeric segments……14

14(a) Forewings with 3 or more closed cells formed by conspicuous tubular venation.

(b) Longbody usually more than 5 mm, but sometimes less than 2 mm….15

15(aa) Front wing with stigma……18

18(aa) Forewings with separate C and R venations; there is a small long costal cell……...21

19(aa) The abdominal sterna is sclerotized as strongly as the terga.

(bb) anterior sterna (usually sternal metasoma 1 – 3) are undivided and are generally convex ventrally..…….20

20(a) Antenna with 10 or 11 flagellomer……...24

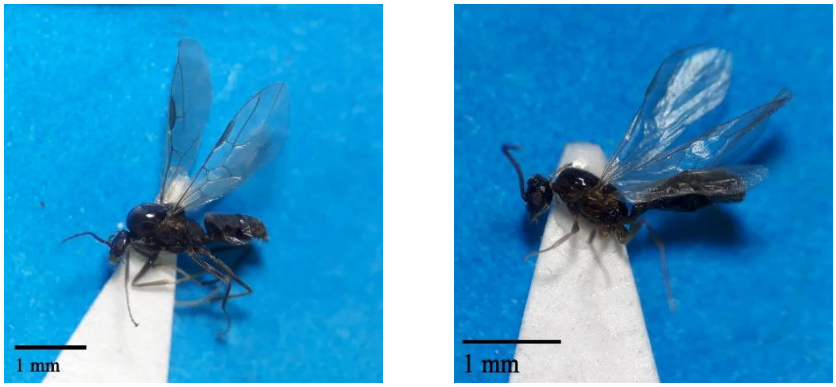
21(aa) Antenna with 10 or more flagelomeric segments ……22

22(a) The forewings with Cu venation extend straight to the edge of the wing.

(b) Forewings with 2Cu-A venation equal to or more in length than 1Cu-A venation.

(c) Forewings without 2R-M and 2M-Cu venation.…….Proctotrupoidea

Based on the results of the collection, two species were obtained from the superfamily Proctotrupoidea (Figure 4.11). Based on the identification results, this insect has a black non-metallic body. The body size is less than 3 mm.



(a) (b)

(a) Proctotrupoidea 1; (b) Proctotrupoidea 2

Figure 4.11 Super-Family Proctotrupoidea

According to(Goulet and Huber, 1993), this group is classified into the following classification.

Kingdom : Animalia

phylum : Arthropods

Class :Insects

Order : Hymenoptera

Super-Family :Proctotrupoidea

Based on the identification results, Proctotrupoidea 1 has 12 flagelomeric segments, while Proctotrupoidea 2 has 14 flagelomeric segments on its antennae. Proctotrupoidea have full-sized wings with well-developed venation. The forewings have eight closed cells and have a stigma with separate C and R venations. The hind wings have two closed cells without any jugular lobes.

The forewings of Proctotrupoidea 1 and 2 have Cu venations that extend straight toward the wing margins. The forewings also have a 2Cu-A venation which is longer than the 1Cu-A venation. The identified Proctotrupoidea 1 and 2 also lacked 2R-M and 2M-Cu venation on their forewings.

The pronotum of the collected Proctotrupoidea is prominent. The second metasoma segment has the largest size when compared to other metasoma segments. The back end of the pronotum is attached to the sugar. The anterior tibia is equipped with a single spur.

Super-Family Proctotrupoidea is a group of entomophagous insects. Proctotrupoidea are egg or larval parasitoids of various types of insects and other arthropods. Some of the host insects for Proctotrupoidea include the Orders Orthoptera, Heteroptera, Coleoptera, Lepidoptera, Hymenoptera, and Diptera. Proctotrupoidea also parasitize spider eggs(Goulet and Huber, 1993; Mani and Sharma, 1982).

4.2.3 Super-Family Ichneumonoidea

The identification key used in insects of the Order Hymenoptera in this group, according toGoulet and Huber (1993), is as follows.

The key to the super-family:

1(a) The forewings when stretched backwards extend far past the ends of the mesosomes……...…….2

2(aa) Body with marked constriction between the first and second abdominal segments that line the mesosoma and metasoma, when viewed from the dorsal and lateral sides (Apocrita) ……..…….……12

12(aa) The head is not globular (round) and there is no circle of teeth around the middle ocelli.

(bb) Body shapes and sizes vary, often fat or small; The ovipositor is often short or hidden.

(cc) Partthe metacoxal base is usually narrow, the two articulations at the base are close to each other (difficult to see) and arranged obliquely; the long axis of the metacoxes forms a far angle from the metasoma ..13

13(aa) Distancethe shortest point between the base of the mandible and the rim of the compound eye (malar space) without an indentation.

(bb) The mesoscutum is rare with a large axilla and medial groove, but when with both, the body length is usually less than 3 mm and the number of flagelomeric segments is not 12.

(cc) Sparse antennae with 12 flagellomeric segments……14

14(a) Forewings with 3 or more closed cells formed by conspicuous tubular venation.

(b) Longbody usually more than 5 mm, but sometimes less than 2 mm ……..……..…….15

15(aa) Front wing with stigma……18

18(a) Forewings with C and R venation touching or fused, eliminating costal cells……..19

19(a) sterna on abdomenless strongly sclerotized when compared to terga.

(b) anterior sterna (usually sternal metasoma 1 – 3) each divided into several sclerites……..……..…Ichneumonoidea

Based on the results of the collection, obtained one insect from the superfamily Ichneumonoidea (Figure 4.12). This insect has a light brown body overall. Has a body size of less than 2 mm.

Figure 4.12 Super-Family Ichneumonoidea

According toGoulet and Huber (1993), this group is classified into the following classifications:

Kingdom : Animalia

phylum : Arthropods

Class :Insects

Order : Hymenoptera

Super-Family :Ichneumonoidea

Based on the identification results, the collected Ichneumonoidea have antennae that are not angled. There are 16 flagelomeric segments. The antennae emerge from the center between a pair of compound eyes.

The wings on the collected Ichneumonoidea are full-sized with well-developed venation. The forewings have seven closed cells. The forewings also have a stigma with fused C and R venations. The hind wings have two closed cells without any jugular lobes.

The sterna of the collected Ichneumonoide abdomen was less strongly sclerotized when compared to the terga. The ovipositor is very long. The collected ichneumonoidea also have trochantelli (basal end of the femur) on their legs.

Super-Family Ichneumonoidea contains egg and larval parasitoids. Ichneumonoidea become parasites by endoparasitoid and ectoparasitoid. All holometabolous insects, except Megaloptera and Siphonaptera, host Ichneumonoidea. Endoparasitoids from Ichneumonoidea generally become parasites by means of a coinobiont parasitizing the eggs or early larval stages of the host. Ectoparasitoids Icneumonoidea generally become parasites in an idiobion way in parasitizing the mature larval stage, prepupa stage, or pupae of the host.(Goulet and Huber, 1993).

4.2.4 Super-Family Apoidea

The identification key used in insects of the Order Hymenoptera in this group, according toGoulet and Huber (1993), is as follows.

The key to the super-family:

1(a) The forewings when stretched backwards extend far past the ends of the mesosomes……...…….2

2(aa) Body with marked constriction between the first and second abdominal segments that line the mesosoma and metasoma, when

viewed from the dorsal and lateral sides (Apocrita) ……..……..12

12(aa) The head is not globular (round) and there is no circle of teeth around the middle ocelli.

(bb) Body shapes and sizes vary, often fat or small; The ovipositor is often short or hidden.

(cc) Partthe metacoxal base is usually narrow, the two articulations at the base are close to each other (difficult to see) and arranged obliquely; the long axis of the metacoxes forms a far angle from the metasoma ……..…13

13(aa) Distancethe shortest point between the base of the mandible and the rim of the compound eye (malar space) without an indentation.

(bb) The mesoscutum is rare with a large axilla and medial groove, but when with both, the body length is usually less than 3 mm and the number of flagelomeric segments is not 12.

(cc) Sparse antennae with 12 flagellomeric segments…….14

14(a) Forewings with 3 or more closed cells formed by conspicuous tubular venation.

(b) Longbody usually more than 5 mm, but sometimes less than 2 mm ……..…..15

15(a) Front wing without stigma……...16

15(aa) Front wing with stigma……18

16(a) Sforelimb with at least 5 closed cells formed by tubular venation…….…..17

17(aa) The profemur is smaller than the metafemur.

(bb) Antenna with 10 or more flagelomer segments.

(cc) Torulus is closer to the center of the head and not below the transverse ledge……18

18(a) Forewings with C and R venation touching or fused, eliminating costal cells……..19

18(aa) Forewings with separate C and R venations; there is a small long costal cell……...21

19(aa) The abdominal sterna is sclerotized as strongly as the terga.

(bb) anterior sterna (usually sternal metasoma 1 – 3) are undivided and are generally convex ventrally..……20

20(a) Antenna with 10 or 11 flagellomer……...24

21(aa) Antenna with 10 or more phlegelomeric segments ……..…….22

22(aa) The forewings with Cu venation are deflected abruptly (30-90o angle or greater) posteriorly, gradually, at the base of the 1m-cu venation.

(bb) Forewings with 2Cu-A venation are usually less than half the length of 1Cu-A venation.

(cc) Sfront wing with 2R-M and 2M-Cu venation…….……..…23

23(aa) Antenna with 10 or 11 flagelomeric segments.

(bb) Tarsomeres without a protrusion, or rarely with small flat lobes …….24

24(a) Sutures on the thorax in the area around the tegula are covered by long thick hair, so that the surrounding sutures are not visible.

(b) The hairs on the body, especially in the propodeum are not fine, but with short to long lateral branches.

(c) hind limbs with a flattened 1st segment tarsomer, about four times the length of the 2nd segment tarsomer and twice the width…… Apoidea

24(aa) SUtra on the thorax around the tegula are visible.

(bb) KThe density and structure of the hairs around the body vary, usually sparse or short.

(cc) The hind limbs with tarsomere 1 are usually cylindrical in shape with a size not 3 times larger than tarsomere 2……25

25(aa) Shind wings with 1 – 3 closed cells formed by tubular venation……..28

28(a) Tegulaseparated from the pronotum, so that the mesoscutum touches the mesopleura……...……..29

28(aa) The tegula touches the pronotum when the wings fold, so that the mesoscutum separates from the mesopleura……30

29(a) Pronotum onlateral appearance with prominent posterior border, forming rounded lobes with prominent angles; the edge of the lobe on the dorsal view forms a 30-90o angle concave with the pronotum margin……..……..Apoidea

30(aa) Rthe hairs around the body are thin and fine, very rarely with short or long branches.

(bb) Thind limb with tarsomere 1 cylindrical, not more than 3 times long tarsomere 2……..31

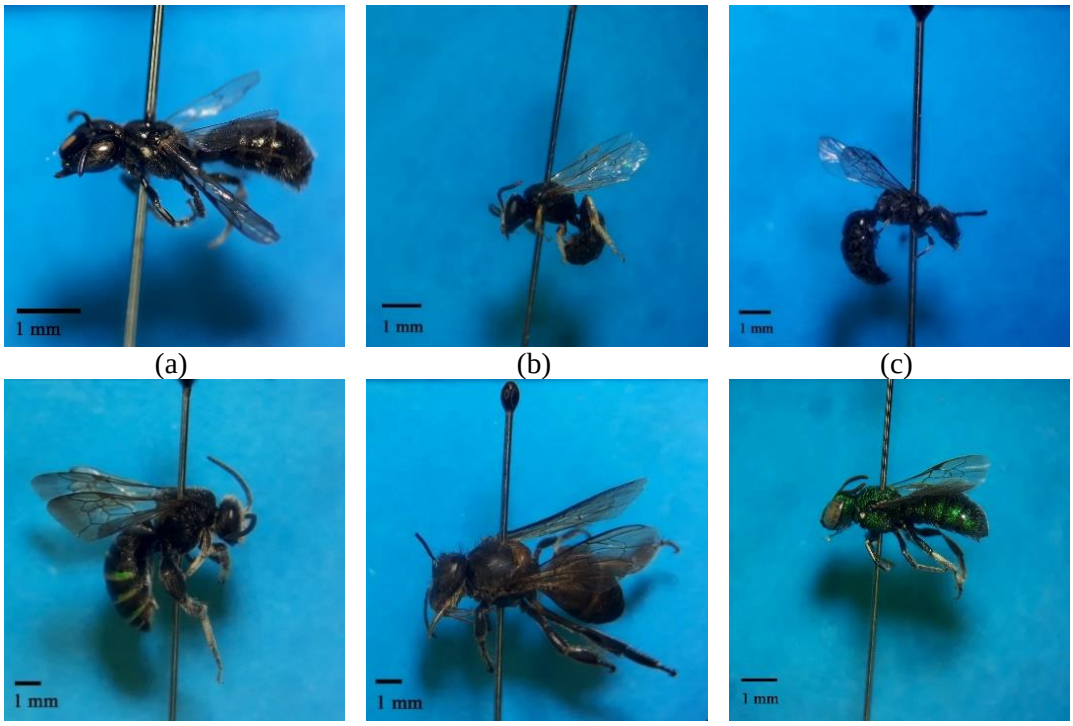
31(aa) Forewings with 3 (very rarely 4) venation approaching or touching the apical edge beyond the stigma.

(bb) STerna metasoma 1 and 2 articulate with each other, their junctions form a straight curve……32

32(a) Pronotum onthe lateral view forms a sharp angle at the mid-joint part of the mesoscutum or forms a deep indentation; The posteromedial portion of the pronotum margin on the dorsal view is straighter and meets the upper edge of the posterior lobe sharply, forming an approximately right angle.

(b) Tthe sugar touches the lobe that protrudes at the posterior edge of the pronotum……Apoidea

Based on the results of the collection, from the Super-Family Apoidea (Figure 4.13), six different species were found. The bodies of the collected Apoidea, five of which are predominantly black and one has a metallic green body. Body size ranging from 4-10 mm.



(a) (b) (c)

(d) (e) (f)

(a) Apoidea 1; (b) Apoidea 2; (c) Apoidea 3; (d) Apoidea 4; (e) Apoidea 5; (f) Apoidea 6

Figure 4.13 Super-Family Apoidea

According to Goulet and Huber (1993), this group is grouped into the following classification.

Kingdom : Animalia

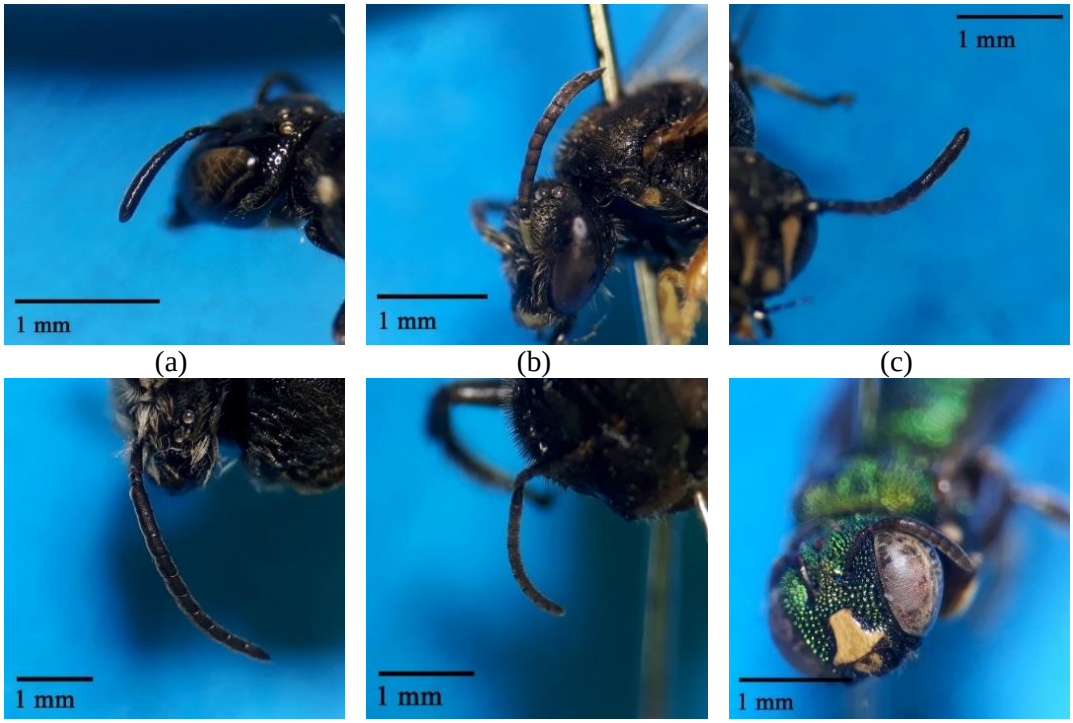
phylum : Arthropods

Class :Insects

Order : Hymenoptera

Super-Family : Apoidea

Based on the identification results, Apoidea has antennae with 10 to 11 flagelomeric segments. Apoidea 1 and 5 have 10 flagelomeric segments (Figs. 4.14 a and e), whereas Apoidea 2, 3, 4, and 6 have 11 flagelomeric segments (Figs. 4.14 b, c, d, and f).



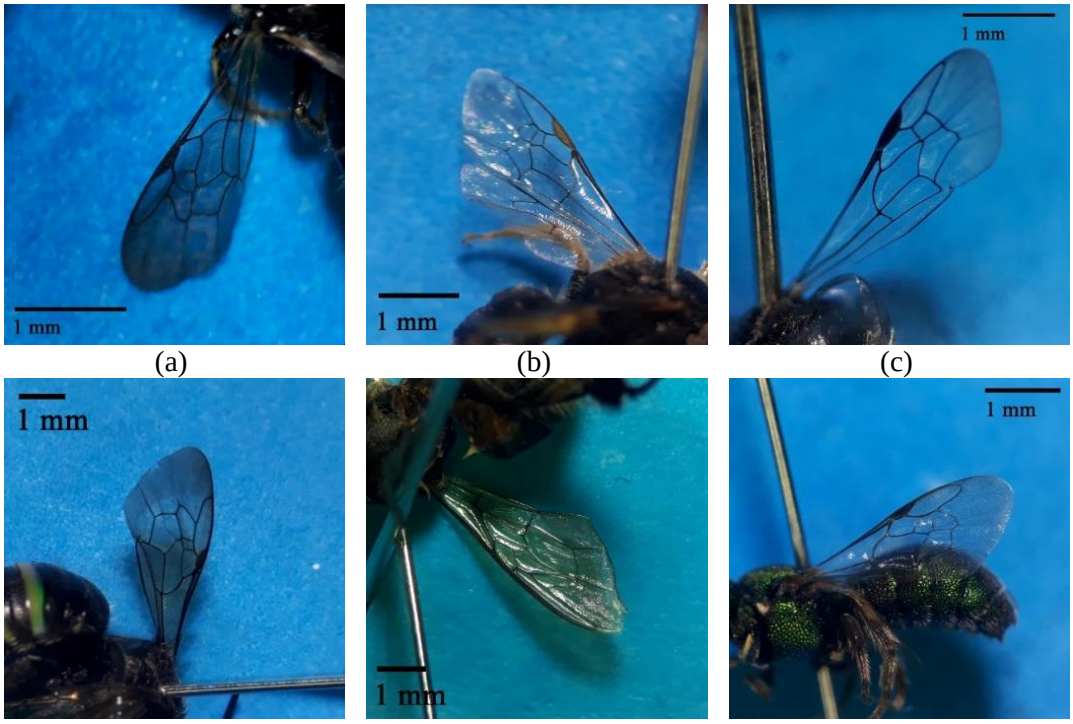
(a) (b) (c)

(d) (e) (f)

(a) Apoidea 1; (b) Apoidea 2; (c) Apoidea 3; (d) Apoidea 4; (e) Apoidea 5; (f) Apoidea 6

Figure 4.14 Antenna morphology of super-Family Apoidea

This insect has full-sized wings with well-developed venation. Based on the identification results, the collected Apoideans had nine to eleven closed cells on the forewings and two on the hind wings which were formed from tubular venation. Apoidea 3 and 5 have nine closed cells on their forewings (Figs. 4.15 c and e), Apoidea 1 has ten closed cells on their forewings (Fig. 4.15 a), while Apoidea 2, 4, and 6 have eleven closed cells on their forewings (Fig. Figure 4.15 b, d, and f). All Apoidean insects collected had two closed cells on their hind wings (Fig. 4.16).



(a) (b) (c)

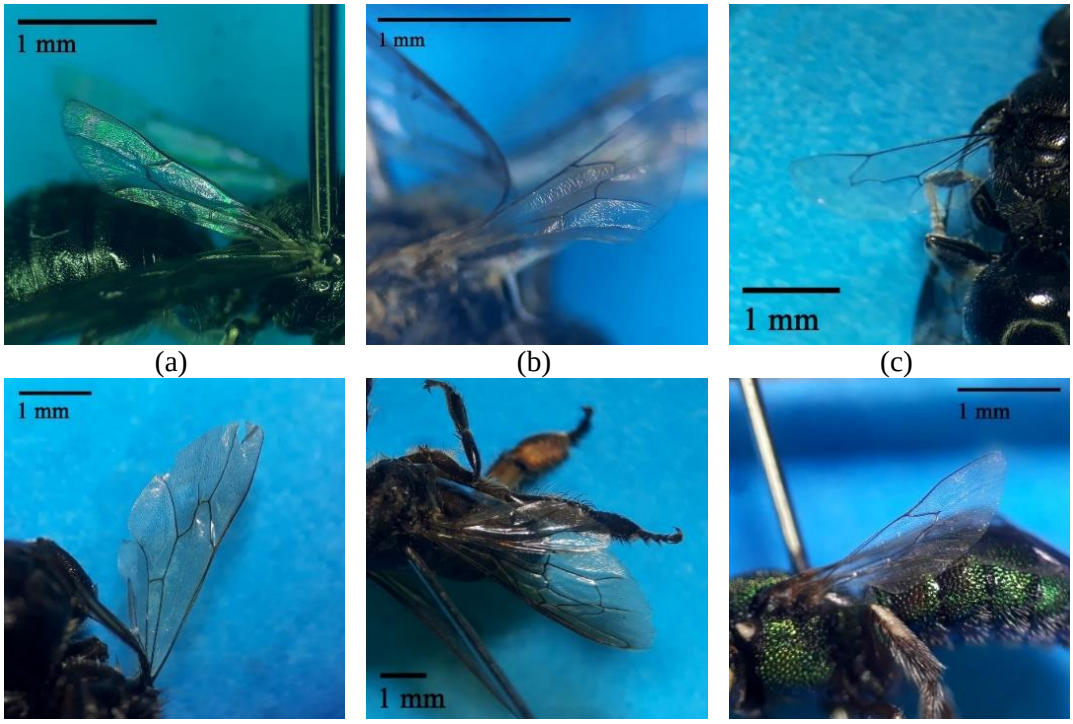
(d) (e) (f)

(a) Apoidea 1; (b) Apoidea 2; (c) Apoidea 3; (d) Apoidea 4; (e) Apoidea 5; (f) Apoidea 6

Figure 4.15 Morphology of the superfamily Apoidea depan forewings

The forewings of Apoidea 1, 2, 3, 4, and 6 have stigmas with separate C and R venations in Apoidea 2, 4, and 6 (Figure 4.15 b, d, and f), whereas those in Apoidea 1 and 3 are fused (Figure 4.15). a and c). Apoidea 5 has no stigma on its forewings (Fig. 4.15 e). All Apoidea species collected have hind wings equipped with well-definedjugal lobes and claval lobes (Fig. 4.16).

Superfamily Apoidea based on the identification results have a short pronotum. The tip or corner of the pronotum is far apart from the tegula when viewed from the dorsal or lateral side. Most of its body is covered by fine hairs.



(a) (b) (c)

(d) (e) (f)

(a) Apoidea 1; (b) Apoidea 2; (c) Apoidea 3; (d) Apoidea 4; (e) Apoidea 5; (f) Apoidea 6

Figure 4.16 Morphology of hind wings super-Family Apoidea

Super-Family Apoidea is a super-family of the Order Hymenoptera which consists of bees. Most species of Apoidea are solitary insects. Apoidea visit flowers to utilize carbohydrates produced by nectar and pollen produced by flowers. Adult female Apoidea are able to build nest-forming spaces to supply nectar needs, lay eggs in these spaces, close them, and then build another nest. This is different from the behavior of other Hymenoptera groups which feed their larvae by preying on other insects, while nectar as a food source is only for adults. Apoidea behavior when visiting flowers to take advantage of the nectar and pollen produced, helps to spread pollen with the help of hairs which generally in the Apoidea group grow in all parts of the body. Therefore, the Apoidea group is a group of insects that are useful in helping pollination(Triplehorn and Johnson, 2005).

Parasitoids of the Order Hymenoptera that have been collected using active collections are from the Family Scoliidae, Family Tiphiidae, super-Family Proctotrupoidea, and super-Family Ichneumonoidea. According toLewis et al. (1998), the behavior of parasitoids in the selection of hosts is not only used for their reproductive purposes, but also to find the location of food sources to meet their nutritional needs. The behavior of parasitoids in looking for hosts and food sources takes into account efficiency. The landscape of pomegranate orchards in Merak Village, Situbondo (Figure 4.17) supports parasitoids to streamline their time and energy in hunting for food and hosts.

Figure 4.17 Pomegranate garden in Merak Village, Situbondo used for collecting insects of the Order Hymenoptera

The pomegranate plantation landscape used in the collection is of high complexity. According toLizmah et al. (2018), high-complexity landscapes where the presence of non-agricultural plants is dominant such as trees, shrubs, and weeds. The high complexity of the pomegranate plantation landscape in Merak Village can be observed from the characteristics of the plantations there. Pomegranate gardens used in the collection are polyculture plantations. Polyculture plantations are plantations in which more than one type of plant is planted in one area(Lizmah et al., 2018). Plants that are also planted in pomegranate gardens in Merak Village include dragon fruit, chili, peanuts,

watermelon, elephant grass, and others. The location of these pomegranate gardens is also in between orchards of citrus, chili, dragon fruit, and others. Conditions around pomegranate gardens are overgrown with trees, shrubs, and weeds. These pomegranate gardens are also very rarely even sprayed with insecticides in an effort to reduce pest attacks.

The landscape condition of pomegranate plantations with such high complexity allows for the availability of hosts and food sources with good accessibility in one location. This will increase the efficiency of the parasitoid, thus affecting its abundance in an area(Lewis et al., 1998). Therefore, parasitoid insects of the Order Hymenoptera were successfully collected through active collection in pomegranate plantations in Kampung Merak.

In addition to parasitoid insects, the active collection also found predatory wasps from the Vespidae family and pollinators from the Apoidea superfamily which were successfully inventoried. This is because the pomegranate plantations in Kampung Merak, with their high complexity, maintain a semi-natural habitat. Semi-natural habitats are ecosystems that maintain their naturalness despite human intervention. Habitats like this support ecosystem services for insects, both parasitoids, predators, and pollinators. This causes the density and diversity in an area to increase (Blaauw and Isaacs, 2012; Thomson and Hoffmann, 2013; Holland et al., 2017).

CHAPTER 5. CONCLUSIONS AND SUGGESTIONS

5.1 Conclusion

The conclusion obtained from the research that has been carried out is that no parasitoid insects from the Order Hymenoptera were found in pomegranate (P. granatum) in Merak Village, Sidomulyo Hamlet, Sumberwaru Village, Banyuputih District, Situbondo. However, we found insects of the order Hymenoptera, family Scoliidae, family Tiphiidae, super-family Ichneumonoidea, and super-family Proctotrupoidea which act as parasitoids in several other insects. This group of insects of the Order Hymenoptera which acts as a parasitoid was found in a pomegranate garden in Merak Village, Situbondo.

5.2 Suggestions

Suggestions that can be given based on an inventory of parasitoid insects of the Order Hymenoptera on a pomegranate plantation (Punica granatum L.) in Merak Village, Situbondo is the need for further identification to the species level of the insects that have been inventoried. This cannot be done in this study because there are obstacles in the institution that validates it. The Indonesian Institute of Sciences (LIPI) as a validation institution in the identification process cannot validate until an indefinite period due to the COVID-19 pandemic. Therefore, in this study, it was only possible to identify up to the super-family and familial levels with independent validation at the Faculty of Mathematics and Natural Sciences, University of Jember.

Suggestions that can be given for further research is the need for a re-examination of the methods used, especially in passive collection. Pomegranate collection in passive collection needs to be done more selectively. This aims to minimize the presence of predators on the collected pomegranates, thereby minimizing the occurrence of predation in dark containers. In addition, to minimize the collection of fruits that are attacked by pests due to starting to rot. This is because fruit rot that occurs very quickly becomes a major obstacle in using dark containers to remove insects in pomegranates.

In addition to conducting a review in the passive collection method, suggestions that can be given for further research are the need to collect in the dry season. Fruit rot in the dry season is not as fast as during the rainy season. Therefore, it is more possible to passively collect insects of the Order Hymenoptera.

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THE ALKALOIDS AND FLAVONOIDS CONTENT IN THREE SPECIES OF POTENTIALLY MEDICINAL ORCHID FROM THE GUMITIR MOUNTAIN AREA OF JEMBER REGENCY

THESIS

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UNIVERSITY OF JEMBER
2021

THE ALKALOIDS AND FLAVONOIDS CONTENT IN THREE SPECIES OF POTENTIALLY MEDICINAL ORCHID FROM THE GUMITIR MOUNTAIN AREA OF JEMBER REGENCY

THESIS

Submitted to complete the final project and fulfill one of the requirements to complete the Biology Study Program (S1) and achieve a Bachelor of Science degree

By

Desy Lutfianasari
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DEPARTMENT OF BIOLOGY
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2021

DEDICATION

In the name of Allah, the Most Gracious, the Most Merciful. This work is the result of a journey through the Biology Study Program (S1) and the intention to worship Allah SWT with the hope that the knowledge obtained can be useful for society and myself, I dedicate this scientific work to: My

1. parents, Sri Kartini's mother and Jaka Wahyono's father beloved, thank you for all your prayers, love, sacrifices both material and support that has been given, as well as patience in educating until now;
2. My beloved big family, who have given me motivation and support;
3. Ladies and gentlemen, teachers from kindergarten to university who have educated and imparted knowledge with great patience;
4. Alma mater Department of Biology, Faculty of Mathematics and Natural Sciences, Jember University

MOTTO

"Everything is a plan from Him, we just run"

"and I give them respite. Indeed, My plan is very strong"
(translation of QS Al-Qalam verse 45)*)

*) Ministry of Religion of the Republic of Indonesia. 2009. Al-Quran and its Translation. Bandung. Bandung: PT Syaamil Cipta

STATEMENT

I, the undersigned:

Name : Desy Lutfianasari

Nim : 161810401009

I solemnly declare that the scientific paper entitled "The Alkaloids and Flavonoids in Three Species of Orchid Potential Medicine from Mount Gumitir, Jember Regency" is completely the result of the scientific work itself, unless the source is stated in the citation of the substance and has never been submitted to any institution, and is not a plagiarized scientific work. This research was fully funded by the KERIS SYMPLAST Project. I am responsible for the validity and correctness of the contents in accordance with a scientific attitude that must be upheld.

Thus, I make this statement truthfully, without any pressure and coercion from any party and am willing to receive academic sanctions if it turns out that in the future this statement is not true.

Jember, 04 February 2021

Who stated

Desy Lutfianasari

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THESIS

THE ALKALOIDS AND FLAVONOIDS CONTENT IN THREE SPECIES OF POTENTIALLY MEDICINAL ORCHID FROM THE GUMITIR MOUNTAIN AREA OF JEMBER REGENCY

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APPROVAL

Thesis entitled "The Alkaloids and Flavonoid Content in Three Species of Orchid Potential Medicine from Mount Gumitir Area, Jember Regency", was tested and ratified on:

Day, date :
Place : Faculty of Mathematics and Natural Sciences, University of Jember

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SUMMARY

The Alkaloids and Flavonoids Content in Three Species of Orchid Potential Medicine from Mount Gumitir Area, Jember Regency; Desy Lutfianasari, 161810401009; 2021; 48 Pages; Department of Biology, Faculty of Mathematics and Natural Sciences.

Plants used as medicine are known to contain certain secondary metabolites with great therapeutic value. One of the plants that are efficacious as medicine is orchids. Orchids have secondary metabolites such as alkaloids and flavonoids. Groups of orchids that have medicinal properties include the genera *Dendrobium, Vanda,* and *Bulbophyllum.* In the same type with different plant organs produce different secondary metabolite content. Likewise in different types in the same organ. One of the orchid habitats in East Java is in the area of Mount Gumitir, Jember Regency which has not been studied until now, until this research was carried out. The purpose of this study was to determine the content of alkaloids and flavonoids in *Bulbophyllum odoratum* Lindl., *Dendrobium linearifolium* Teijsm.& Binn., and *Vanda tricolor* Lindl. orchids.

Orchid sample *Bulbophyllum odoratum* Lindl. *Dendrobium linearifolium* Teijsm & Binn., and *Vanda tricolor* Lindl. collected from the Mount Gumitir coffee plantation area, Jember Regency. Sample preparation was carried out by separating each orchid organ which included pseudobulbs, stems and leaves, then washed and dried. The sample was then blended to obtain simplicia. The simplicia was then macerated with methanol solvent in the ratio (1:100) or 1 g in 100 ml of methanol for 3x24. Then it is evaporated to get *crude extract*. *The crude extract* was then used for quantitative assay by spectrophotometric method at a wavelength of 346 nm for alkaloids and flavonoids 435 nm.

The content of alkaloids and flavonoids in the three species of orchids has different medicinal potential, as well as in the organs studied. Content The highest levels of alkaloids and flavonoids are found in the leaves. The highest alkaloid content was found in the leaves of *Dendrobium linearifolium* Teijsm & Binn (43.84 mgBE/gr), followed by pseudobulb *Bulbophyllum odoratum* Lindl. (21.91 mgBE/gr), and leaves of *Bulbophyllum odoratum* Lindl. (18.01 mgBE/gr).

Meanwhile, the highest flavonoid content was found in the leaves of *Dendrobium linearifolium* Teijsm.&Binn (219.59 mgQE/gr), then leaves of *Bulbophyllum odoratum* Lindl., (132.21 mgQE/gr) and leaves of *Vanda tricolor* Lindl. (122.69 mgQE/gr). Based on these data the distribution of secondary metabolites in the organs of each type of orchid is uneven, the content of secondary metabolites in leaves is higher than pseudobulbs and stems, this is because the distribution of secondary metabolites is influenced by the function of these secondary metabolites for plants as a defense against the environment. In the three orchid species studied, the highest content of alkaloids and flavonoids was found in the leaves. This is because the leaf organ is an organ that is susceptible to pests and diseases compared to stems and pseudobulbs, so that secondary metabolites in leaves are higher.

Based on the research, it can be concluded that different types of orchid plants and organs contain different alkaloids and flavonoids. The content of alkaloids and flavonoids of the orchid *Bulbophyllum odoratum* (Blume) Lindl. on pseudobulb were (21.91 mgBE/gr) and (57.45 mgQE/gr), on leaves (18.01 mgBE/gr) and (132.21 mgQE/gr).orchid *Dendrobium linearifolium* Teijsm& Bin. on pseudobulbs (16.37 mgBE/gr) and (66.97 mgQE/gr), stems (10.06 mgBE/gr) and (56.26 mgQE/gr), leaves (43.84 mgBE/gr) and (219.59 mgQE/gr). While the *Vanda tricolor* Lindl orchids on the leaves were (9.35 mgBE/gr) and (122.59 mgQE/gr).

PREFACE

Praise the presence of Allah SWT for all His grace and guidance, so that the author can complete the thesis entitled "Content of Alkaloids and Flavonoids in Three Species of Orchid Potential Medicine from Mount Gumitir, Jember Regency"

. Therefore, the writer would like to express many thanks to:

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The author realizes that there are still many shortcomings in writing this thesis. The author accepts criticism and suggestions from all parties, for the perfection of this thesis. Hopefully this thesis can be useful for those who read it.

Jember, 04 February 2021

Author

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CHAPTER I. INTRODUCTION

1.1 Background

Indonesia is a tropical country that is rich in biological resources. There are about 30,000 species of plants found in Indonesia (Rahayu and Arista, 2019). These plants are widely used by the community for economic purposes (Rahayu and Arista, 2019) including as food and medicine (Hasairin, 2010; Setiawan *et al.*, 2019). Knowledge about plants that have the potential as drugs is a cultural heritage from generation to generation (Evizal *et al.*, 2013). Plants that have the potential as drugs in Indonesia are known to number 940 species (Rahayu and Arista, 2019), and many of these plants are found growing wild in the forest (Mais *et al*., 2018).

Medicinal plants are natural products that have properties to maintain, prevent and treat a disease or pain complaint (Christomo *et al.*, 2018). Plants used as drugs are known to contain certain chemicals (phytochemicals or secondary metabolites) with great therapeutic value (Pandey *et al.*, 2012). Therapeutic value is a value that encourages to improve disease healing (Siti *et al.*, 2016).

One of the plants that are efficacious as medicine is the orchid plant (Gutierrez, 2010). Orchid plants are generally used as ornamental plants because they have high aesthetic value with beautiful flowers and various shapes and colors that vary (Sabran *et al.*, 2003). In its development, the use of orchid plants is not only as an ornamental plant, but also as a medicine (Silalahi and Nisyawati, 2015). Groups of orchid plants that are efficacious as drugs include the genera *Dendrobium, Vanda,* and *Bulbophyllum* (Silalahi and Nisyawati, 2015). The genus *Dendrobium* as many as 20 species are known to be useful as anti- *cancer*, as a tonic to maintain stomach health, increase body fluids, reduce fever (Wahyudiningsih *et al.*, 2017), and cure ear diseases (Sujarwo and Lestari, 2019). The potential of orchids as medicine is due to the presence of secondary metabolites. Orchids have bioactive compounds or secondary metabolites including alkaloids, flavonoids, carotenoids, anthocyanins (*Maridass et al.*, 2008) and sterols (Hossain, 2011).

Secondary metabolites contained in Dendrobium orchids are alkaloids, phenanthren, bibenzil, fluorenone and sesquiterpenes (Lo *et al.*, 2004).orchids *Bulbophyluum* contain secondary metabolites such as flavonoids, terpenoids, saponins, tannins, phlobatannins, steroids, glycosides and antroquinones (Akter *et al.*, 2018), while the genus Vanda contains flavonoids, tannins, and terpenoids (Maridass *et al.,* 2008). Different genera contain different secondary metabolites (Sahoo *et al.,* 2010). Orchid plants are often found in tropical rain forests (Mardiyana *et al.,* 2019), one of the orchid habitats in East Java is on Mount Gumitir.

Mount Gumitir is an area that connects the city of Jember with Banyuwangi. The Gumitir mountain area is also a large coffee plantation. Coffee plantations in the Gumitir mountain area have an area of 1,165,730 ha. The plantation is managed by PT Perkebunan Nusantara XII (Persero) (Perhutani, 2008). Based on previous research belonging to (Susilowati, 2018) there were 16 species of orchids found in the Mount Gumitir area, including *Arundina graminifolia* (D. Don) Hocker, *Bulbophyllum odoratum* (Blume) Lindl. , *Cymbidium bicolor* Lindl., *Dendrobium linearifolium* Teijsm. & Bin. *Pomatocalpa spicata* Breda, *Trixspermum arachnites* (Blume.) Rehb. and *Vanda tricolor* Lindl.. Based on the survey results at Mount Gumitir, of the 16 orchid species, three species were *Bulbophyllum odoratum* (Blume) Lindl., *Dendrobium linearifolium* Teijsm. & Binn, and *Vanda tricolor* Lindl. The population is abundant and is known to be efficacious as medicine. Based on this background, a research entitled. The content of alkaloids and flavonoids in three species of orchids with medicinal potential from the Gumitir mountain area, Jember Regency.

1.2 **Problem Formulation**

What is the content of alkaloids and flavonoids in three species of orchids with medicinal potential from the Gunung Gumitir area, Jember Regency?

1.3 **Research Objectives**

To determine the content of alkaloids and flavonoids in three species of orchids with medicinal potential from the Gunung Gumitir area, Jember Regency.

1.4 **Limitation of the Problem The**

limitations of the problem in this study were:

1. The organs used for secondary metabolite analysis were leaves and *pseudobulbs* (*Bulbophyllum odoratum* (Blume) Lindl), leaves, stems, and *pseudobulbs* (*Dendrobium linearifolium* Teijsm. &Binn) and leaves. (*Vanda tricolor* Lindl.)
2. Analysis of secondary metabolite content of alkaloids and flavonoids was carried out quantitatively by spectrophotometric method.

1.5 **Research**

Benefits The benefits obtained in this research are:

1. For science, this research can provide information about the content of secondary metabolites of alkaloids and flavonoids found in the orchids *Bulbophyllum odoratum* (Blume) Lindl., *Dendrobium linearifolium* Teijsm. & Binn., and *Vanda tricolor* Lindl. from the Gumitir mountain area, Jember district.
2. Provide information to the public regarding the content of secondary metabolites of alkaloids and flavonoids found in the orchids *Bulbophyllum odoratum* (Blume) Lindl., *Dendrobium linearifolium* Teijsm.& Binn., and *Vanda tricolor* Lindl. from the Gumitir mountain area, Jember district.
3. Government or related agencies, the content of secondary metabolites of alkaloids and flavonoids found in the orchids *Bulbophyllum odoratum* (Blume) Lindl., *Dendrobium linearifolium* Teijsm.& Binn., and *Vanda tricolor* Lindl. potential as a drug and is expected to be further developed as a useful herbal medicine to treat disease.

CHAPTER II. LITERATURE REVIEW

2.1 Orchid Plants Orchids

are flowering plants belonging to the *Orchidaceae family* (Maridass *et al.*, 2008). In Indonesia, there are approximately 5000 species of orchids (Dwiyani *et al.*, 2012), including those of the genus *Bulbophyllum, Vanda, Dendrobium, Eria, Cerathostylis* (Nusantara *et al., 2017).* Orchids are plants that have a high beauty appeal with beautiful flowers with various flower colors. This makes orchids widely used as ornamental plants (Rahmatia and Pipit, 2009).

Orchid cultivation is also widely carried out to meet the needs of the community related to ornamental plants and the preservation of orchids (Rahmatia and Pipit, 2009). In addition, orchids can also be used as medicine (Gutierrez, 2010) as in (Table 2.1). The cultivation also provides economic benefits to the community. Orchids are found in all parts of the world except in Antarctica and the desert areas of Eurasia. Orchid plants are mostly found in tropical forest areas such as Indonesia (Rahmatia and Pipit, 2009).

Orchid plants have the characteristics of their flowers growing on a stalk called a pedicel (Rahmatia and Pipit, 2009). The flower consists of three beautiful colored sepals, three petals with two strands forming an angle of 120º facing each other and one strand modified as a labellum. The labellum serves to attract insects to land. Orchids have leaf shapes that vary with the shape of the leaf bones that are parallel to the leaf blade, the leaves are thin to thick (succulent) (Gunawan, 2006). There are two types of orchid stem growth, namely monopodial and sympodial. Monopodial is a type of growth with stems that extends upward and only has one stem, while the sympodial type is a type of growth with stems to the side and in one plant there is more than one stem (Hartati and Linayanti, 2015). Orchids also have another uniqueness, namely most orchids have *pseudobulbs* as a place to store water (Rahmatia and Pipit, 2009). Sympodial orchid roots come out of the base of the *pseudobulp* or along the rhizome, while monopodial orchid roots come out of the stem segments (Gunawan, 2006).

Table 2.1 Orchid Potential Drug

No	Orchid	Potential Drug	Reference
1	*Dendrobium linawianum Reichb. f.*	Digestion, increases body fluid production,	Nalawade *et al., 2003.*
2	*Bulbophyllum Kwangtungense* Schlecht	Healthy lungs, produces body fluids, fever,	Wu *et al.,* 2006.
3	*Bulbophyllum odoratissimum*,	Leokimia, stomach cancer, tuberculosis, chronic inflammation and fractures	Chen *et al.,* 2007.
4	*Dendrobium chrysanthum* Wall.	Antipyretic (fever lowering, anti-pain), eye, and immunomodulator	Yang *et al.,* 2006.
5	*Dendrobium linearifolium* Teijsm & Binn.	Ear Disease	Sujarwo and Lestari, 2019
6	*Dendrobium aurantiacum*	Antibiotic	Yang *et al., 2007*
7	*Dendrobium salaccense* Lindl.	Stomach*ache*Nisyawati	, 2015

Based on their habitat, orchids are divided into three, namely terrestrial, epiphytic and lithophytic (Agustini *et al.,* 2015). Terrestrial orchids are orchids that grow on the ground. Epiphytic orchids are orchids that live attached to living or dead tree hosts on stems, branches and twigs. (Demena *et al.,* 2020), while lithophytic orchids are orchids that live among rocks (Pasaribu *et al.,* 2015). Orchid growth in some of these habitats is influenced by the environment in the form of biotic factors such as microorganisms, animals, humans and competition between plants, and abiotic factors such as light intensity, temperature, and humidity (Tirta, 2004).

2.2 Orchid Botany

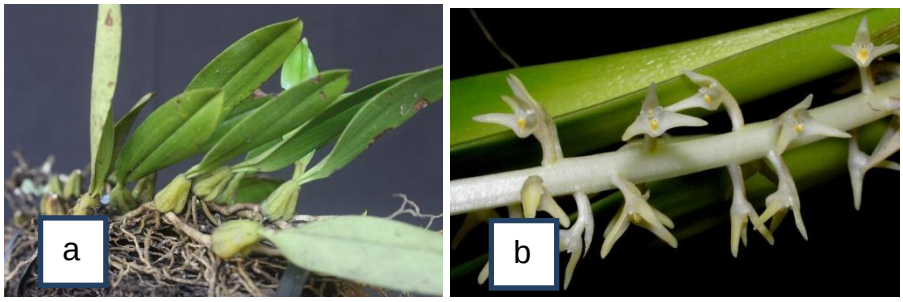
2.2.1 *Bulbophyllum odoratum* (Blume) Lindl.

Classification of orchid plants *Bulbophyllum odoratum* (Blume) Lindl. are as follows:

Kingdom : Plantae
Division : Tracheophyta
Class : Liliopsida
Order : Asparagales
Family : Orchidaceae
Genus : *Bulbophyllum*
Species : *Bulbophyllum odoratum* (Blume) Lindl. (Tropicos, 2020).

Bulbophyllum odoratum (Blume) Lindl. generally grows in forests with an altitude of 700-1800 meters above sea level, including epiphytic orchids that grow in clusters (Lungrayasa and Deden, 2000). These orchids have *pseudobulbs* , not flat, with a size of 0.2 - 0.5 x 0.4 - 1 cm, elliptical leaves measuring 21-29cm x 3-7 cm, thick, 35-125 mm long. . Inflorescence (*Inflorescence)* has a length of 28-68 cm including the stem. Flowers are white, cream, yellowish or greenish, with sepal tips and yellowish petals, labellum with or without yellow to *orange*in the center, dorsal sepals measuring 2.2-7.5 x 1-1.5 mm, rounded tip, lateral *sepals* measuring 2.2-9 x 1.2-2 mm, petals measuring 0.7-1.2 x 0.3-0.8mm, thick *lip recurve* (labellum), elliptical to ovoid in size. 0.8-1.5 x 0.4-0.7 mm (Seidenfaden and Jeffrey, 1992) with flowers can reach 100 buds on each stalk (Lungrayasa and Deden, 2000). Morphology *of Bulbophyllum odoratum* (Blume) Lindl. can be seen in (Figure 2.1).

Distribution of *Bulbophyllum odoratum* (Blume) Lindl. in Sumatra, Java, Lesser Sunda Islands, Borneo, Sulawesi, Maluku, Pahang Malaysia, Selangor, Kelantan and Perak (Seidenfaden and Jeffrey, 1992). *Bulbophyllum odoratum* (Blume) Lindl has other names (synonyms) for this species: *Diphyes odorata*, *Phalorchis odorata* (Lungrayasa and Deden, 2000).



(a) Habitus, (b) Interest

Figure 2.1. Morphology *of Bulbophyllum odoratum* (Blume) Lindl. (Susilowati, 2018 ; https://orchid.unibas.ch/phpMyHerbarium/documents/55/185255m.jpg)

2.2.2 *Dendrobium linearifolium* Teijsm. & Bin.

Classification of orchid plants *Dendrobium linearifolium* Teijsm. & Bin. They are as follows:

Kingdom : Plantae
Division : Tracheophyta
Class : Liliopsida
Order : Asparagales
Family : Orchidaceae
Genus : *Dendrobium*
Species : *Dendrobium linearifolium* Teijsm. & Bin. (Tropicos, 2020).

Dendrobium linearifolium Teijsm. & Bin. is an epiphytic orchid, with roots appearing at the base of the *pseudobulb,* oval-shaped pseudobulb (Darmawati *et al*, 2018), green to brown, fusiform (enlarged middle part, pointed tip and base), 4-5 cm long (Rahadi and Luchman, 2007). 2018) with a diameter of 1-3 cm (Teoh, 2016) which serves to store nutrients. The stem is oval-shaped like a needle, emerging from the *pseudobulb* (Rahadi and Luchman, 2018) with a branched length of up to 70 cm (Teoh, 2016). The leaves are light green to dark green (Rahadi and Luchman, 2018) in a linear shape with a size of 6 x 0.6 cm along the stem. The flowers are white with dark red stripes with a size of 2.5 x 1.7

cm. The labellum is shaped like a kite (Teoh, 2016). Flowers grow along the stem (Hidayati *et al*, 2016). Morphology *of Dendrobium linearifoliumis* Teijsm. & Bin. can be seen in (Figure 2.2). Distribution of *Dendrobium linearifoliumis* Teijsm. & Bin. found in Southeast Asia, Myanmar, Thailand, and Indonesia (Rahadi and Luchman, 2018) such as Bali, Java and Sumatra (Teoh, 2016).

(a) Habitus, (b) Flowers

Figure 2.2 Morphology *of Dendrobium linearifolium* Teijsm. & Binn (Dewi *et al.*, 2020; https://www.orchidsforum.com/threads/dendrobium-linearifolium.13880/)

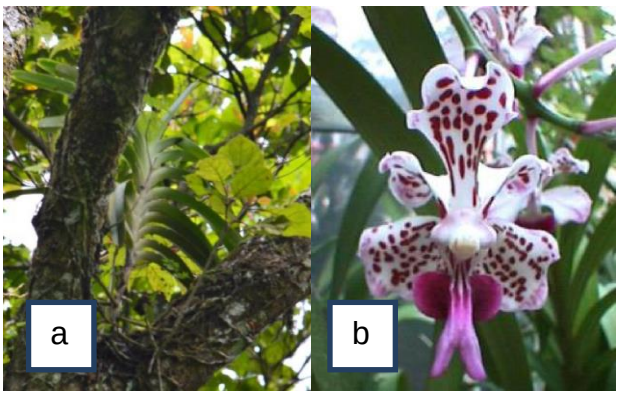
2.2.3 *Vanda tricolor* Lindl.

Classification of the orchid plant *Vanda tricolor* Lindl. are as follows:

Kingdom : Plantae
Division : Tracheophyta
Class : Liliopsida
Order : Asparagales
Family : Orchidaceae
Genus : *Vanda*
Species : *Vanda tricolor* Lindl. (ITIS.gov, 2020).

Geographically the ancestors of the *Vanda tricolor* Lindl. originated from Sulawesi and the Philippines, and then spread to West Java, Central Java, before going to Bali and East Java (Gardiner, 2007). *Vanda tricolor* Lindl is known as an orchid native to Java and Bali. *Vanda tricolor* Lindl has a monopodial stem (Purwantoro *et al.*, 2000) which is thick, cylindrical, 1-2 m long. The leaves are

45 x 5 cm, oval in shape. Inflorescence erect with the number of 8-15 flowers, flower diameter 6-7.5 cm, fragrant and surface. The shape of the sepals and petals is ovoid, and blooms together (Kusumastianto *et al*., 2013). Curved inflorescences about 4 cm high. Petals and sepals twist backwards. The color of the flowers varies widely, from white to *cream* or pale purple (*mauve*), with many brown spots arranged in longitudinal rows. The distribution *Vanda tricolor* extends from Java to northern Australia (Soon, 2005). Morphology *of Vanda tricolor* Lindl. found in (Figure 2.3)



(a) Habitus (b). Flower

Figure 2.3 Morphology *of Vanda tricolor* Lindl.

(Dewi *et al.*, 2018; Dewi *et al.*, 2020)

2.3 **Secondary**

Metabolites Metabolites are classified into primary metabolites and secondary metabolites. Primary metabolites are the main types of metabolites that are important in the growth and life processes of living things, while secondary metabolites are compounds that are not needed or not used in a growth process (Nofiani, 2008). Secondary metabolites are compounds produced from detoxification products from toxic metabolites that cannot be removed by an organism (Kristanti *et al*., 2008). These compounds are considered as end products of non-functioning metabolism or as metabolic wastes (Anulika *et al.*, 2016). Secondary metabolite compounds contained in plants are more than those produced by animals. This is because animals have a better system so they are better able to process the waste products of these toxic metabolites (Kristanti *et*

al., 2008). These secondary metabolites are formed or derived from primary metabolites under certain conditions (Nofiani, 2008).

These secondary metabolites are not directly involved in the growth, development and reproduction of plants (Kusbiantoro and Purwaningrum, 2018). Secondary metabolites are not required by cells (organisms) to live, but secondary metabolites play a role in the interaction of cells (organisms) with their environment (Parage *et al.,* 2015). If a plant does not have or does not contain secondary metabolites, then the plant will not experience death directly, only in the long term it will experience disturbances from decreased survival (ability to survive with other organisms) or organism aesthetics (Bizzo *et al.* , 2012; Anulika *et al.,* 2016). These compounds can be used in plant reproduction and defense, because in general these secondary metabolites are toxic to animals (Kusbiantoro and Purwaningrum, 2018), so that secondary metabolites in plants also affect the color and aroma of the plant (Cowan, 1999).

Secondary metabolites found in plants can be found in plant parts such as roots, stems, leaves, and tree bark, depending on the type of secondary metabolites produced. Secondary metabolites found in plants can be classified into three groups, namely: Terpenes, phenolic compounds, and nitrogen-containing compounds. Derivatives belonging to the terpene group include saponins, steroids, triterpenes, glycosides, and carotenoids. Derivatives belonging to phenolic compounds include simple phenyl propanoids, benzoic acid derivatives, anthocyanins, isoflavones, tannins, lignins, and flavonoids. While those included in the group of nitrogen-containing compounds are alkaloids, cyanogen glucosides, and glucosine. These secondary metabolites have been widely used by scientists to make drugs such as increasing immunity, protecting the body from free radicals, killing pathogenic germs and much more (Anulika *et al.,* 2016). For humans, secondary metabolites are useful as drugs, dyes, fragrances, insecticides and flavors (Leslie *et al.,* 2000).

2.3.1 Factors Affecting Secondary Metabolite Content

Factors influencing secondary metabolite production include media nutrition, light intensity, osmotic pressure. Media/soil nutrition, secondary

metabolite production depends on the availability of nutrients in the growth medium, especially nitrogen and carbon. Because nitrogen and carbon are used to increase the production of biomass associated with the production of secondary metabolites. The addition of nutrients can be controlled by adding fertilizer to plants, but it is different from plant species that are genetically adapted to media that exist in nature (Bernays, 2019).

Light intensity, plants exposed to high light intensity were able to respond by producing more secondary metabolites compared to species in a shady environment. Osmotic pressure, (dryness and salinity), drought conditions of a plant can cause an increase (phenotypic) in several types of secondary metabolites such as glucosinoates, cyanogenic glycosides, terpenoids, alkaloids, but this depends on the level of stress and the time during which it occurs in the plant itself. Short term effects can lead to increased production compared to long term. Salinity is considered a stress factor proportional to drought. An increase in salinity affects the production of secondary metabolites, including an increase in simple phenolic acids, alkaloids and a decrease in terpenes (Bernays, 2019).

2.3.2 Biosynthesis of Secondary Metabolites

Secondary metabolites are produced through 3 main pathways, namely: the shikimate pathway, the mevalonate and methylerythritol phosphate (MEP) pathways, and the malonate pathway (Figure 2.4) (Anggraito et al., 2018). Secondary metabolites are produced through pathways outside the biosynthesis of carbohydrates and proteins. The shikimic acid pathway can synthesize cinnamic acid, phenol, benzoic acid and quinone. The mevalonate pathway is capable of synthesizing essential oils, squalents, monoterpenoids, menthol, corrosives, steroids, terpenoids, sapogenins, geraniol, ABA, and GA3. While the malonic acid pathway is capable of synthesizing fatty acids (including lauric, myristic, palmitic, stearic, oleic, linoleic, linolenic), glycerides, polyacetylene, phospholipids, and glycolipids (Tando, 2018).

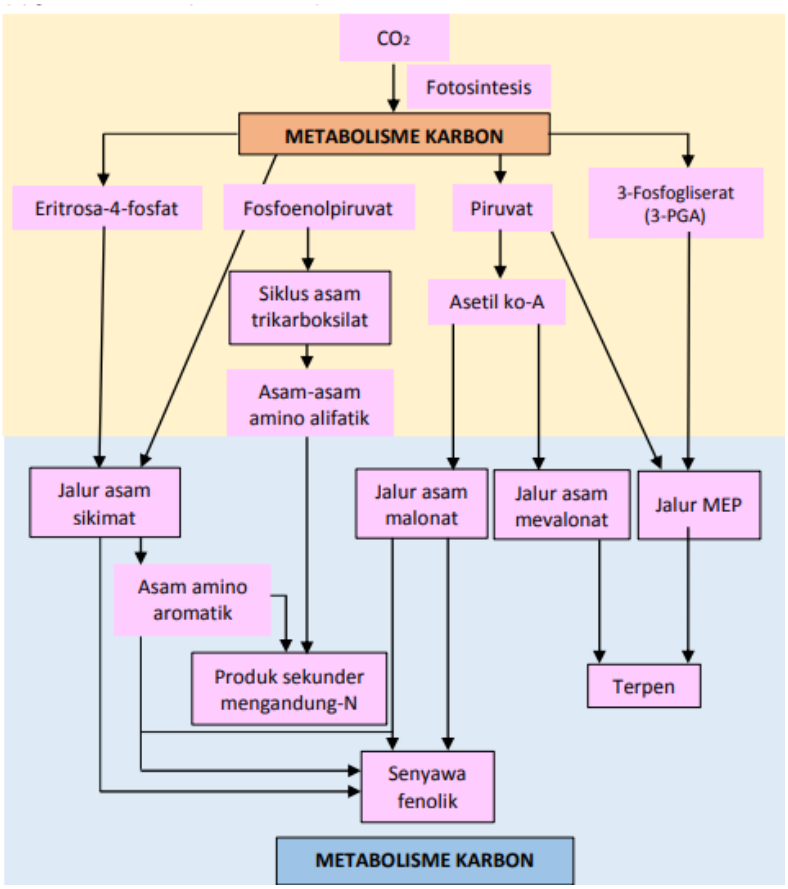


Figure 2.4 Main pathways of secondary metabolite biosynthesis (Anggraito *et al*., 2018)

2.3.3 Alkaloids

Alkaloids are basic compounds synthesized by living organisms containing one or more heterocyclic nitrogen atoms, which are amino acid derivatives (Bizzo et al., 2012) except steroidal alkaloids (Schäfer and Michael, 2009) and are pharmacologically active as antibiotics. Alkaloids are a very large group of secondary metabolites, and more than 12,000 have been isolated. Almost all alkaloids are basic compounds (alkali). Many alkaloids are toxic and even deadly even in small amounts. Some also have antibiotic activity (Bizzo et al., 2012). Alkaloids are a group of compounds that are among the most abundant in nature. Alkaloid compounds are characterized by having at least one basic N atom and are part of the heterocyclic ring of the heterocyclic ring (Figure 2.5). One example of a well-known group of alkaloid compounds is Dendrobine (Figure 2.6).

diisolasi dalan bentuk garamnya dengan HCl atau H_2SO_4. Garam ini atau alkaloid bebasnya berbentuk padat membentuk kristal yang tidak berwarna. Banyak alkaloid yang bersifat optis aktif dan biasanya hanya satu isomer optik yang dijumpai di alam, meskipun dikenal juga campuran rasemat alkaloid.

Senyawa golongan alkaloid diklasifikasikan menurut jenis cincin heterosiklik nitrogen yang merupakan bagian dari struktur molekul. Cincin heterosiklik nitrogen tersebut adalah:

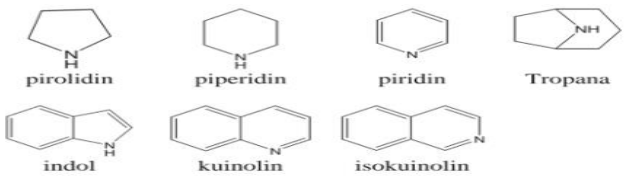
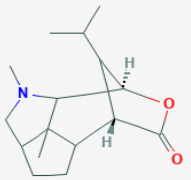


Figure 2.5 Heterocyclic nitrogen ring part of the structure of the alkaloid molecule (Kristianti *et al*., 2008)

Alkaloids are classified into 3 groups, namely, true alkaloids, combined alkaloids and pseudo alkaloids. True alkaloids are compounds that have a heterocyclic nitrogen ring, are basic and are derived from amino acids. Combined alkaloids are amino acid derivatives, the nitrogen atom is not in the form of a heterocyclic ring. The combined alkaloids are basic in nature, derived from the biosynthesis of the amino acids themselves. For example mescalina. Pseudo-alkaloids are plant bases that contain heterocyclic nitrogen, have activity and have no biosynthetic relationship with amino acids. Pseudo-alkaloids are derived from terpenoid compounds derived from acetic acid and polyketonic acid (Illing et al., 2017).



Dendrobine

Figure 2.6. Chemical structure of Dendrobine (One of the alkaloid compounds) (https://pubchem.ncbi.nlm.nih.gov/compound/Dendrobine#section=2D-Structure)

Alkaloids can be found in various parts of the plant although less than 1%. The content of alkaloids in some orchids (Table 2.2).

Table 2.2 Alkaloid Content in Several Orchid Species

Species	Plant organs	Alkaloids Total	Reference
Dendrobium nobile (di Meghalaya) (etanol)	Stem	51.31 ± 0.19 (mg ATP/gr)	Bhattacharyya *et al.*, 2015
Dendrobium crepidatum Maghalaya) (aseton)	Stem	32.22 ± 0.2b	Bhattacharyya *et al*., 2016
	Leaf	56.33 ± 0.5a	
	Root	10.22 ± 0.2d	
Dendrobium fimbriatum (metanol)	PLB (Protocrom Like- Bodies)	49.44 ± 0.01l	Paul dan Suman, 2020

Based on the nature of the base alkaloids can be separated from some other plant components. These compounds are generally or often isolated in the form of HCL or H2SO4 (Kristanti et al., 2008). Alkaloid compounds are known to be contained in several species of orchids such as; Dendrobium nobile, namely dendrobine compounds (Wahyudiningsih et al., 2017), Habendaia loerzingi, Habendaia undulata, (Garvita and Hary, 2020) Phalaenopsis lueddemanniana, Paphiopedilum javanicum, Liparis parviflora (Kong *et al*., 2003).

2.3.4 Flavonoids

Flavonoids are the largest phenolic compounds found in nature. This is due to the varying degrees of hydroxylation, alkoxylation or glycosylation of the structure. Flavonoids can function as PGR, regulators of the photosynthesis process, antimicrobial, antiviral, and anti-insecticide substances (Kristanti et al., 2008). Flavonoids in plants as well as pigmentation and plant defense. Flavonoids have a carbon skeleton consisting of 15 carbon atoms arranged in 2 aromatic rings

connected by three carbon bridges (Anulika et al., 2016; Terahara, 2014). The general structure of flavonoids can be seen in (Figure 2.7).

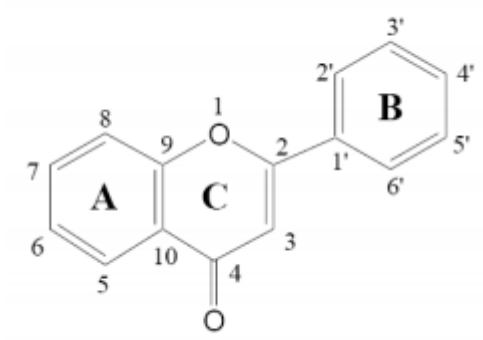


Figure 2.7 General structure of flavonoids

(Pambudi *et al*., 2014)

Flavonoids have a C6-C3-C6 arrangement with 3 structural products, including 1,3-diarylpropane or flavonoids, 1,2-diarylpropane or isoflavonoids, and 1,1-diarylpropane or neoflavonoids (Figure 2.8) (Kristanti et al., 2008). Flavonoid compounds are classified into 10, namely, anthocyanins, proantocyanins, flavonols, flavones, glycoflavones, biflavonils, chalcones, aurons, flavanones and isoflavones (Harborne, 1973). These flavonoid compounds are known to be found in several species of orchids such as; Acampe praemorsa (Roxb.), Bulbophyllum aureum, Calanthe masuca (D. Don) Lindl., Coelogyne nervosa, Dendrobium heyneanum Lindl., Vanda tessellate (Roxb.) Hook. (Maridass et al., 2008). Such as the flavonoid content in several species of orchids contained in (Table 2.3). There are many kinds of flavonoid compounds that can be found in several plants such as quercetin, rutin, kaemperol, isoquercetin, taxifolin, diadzein, apigenin hesperidin and many more (Arifin and sanusi, 2018; Brodowska, 2017; Terahara, 2014).

Table 2.3 Content of flavonoids in several species of orchids

Species	Plant organs	Flavonoids Total (mg QE/grDW)	Reference
Dendrobium nobile (di Meghalaya) (etanol)	Stem	3.11 ± 0.11	Bhattacharyya *et al.*, 2015
Dendrobium crepidatum (Maghalaya) (aseton)	Stem	8.56 ± 0.2b	Bhattacharyya *et al*., 2016
	Leaf	10.32 ± 0.2a	
	Root	2.23 ± 0 .2e	
Dendrobium fimbriatum (metanol)	(PLB) Protocrom Like-Bodies	0.62 ± 0.01k	Paul dan Suman, 2020

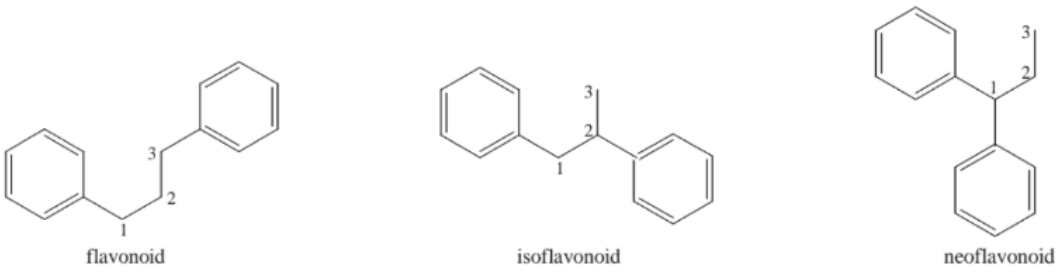


Figure 2.8 Chemical structure of flavonoids, isoflavonoids, and neoflavonoids (Kristanti et al., 2008).

2.4 Extraction and Maseration Method

Extraction is a technique for separating one or more compounds from an existing sample using a suitable solvent. The principle of separation by extraction is a separation based on the dissolving ability of the components contained in the mixture (Leba, 2017). There are two kinds of extraction, namely solid-liquid extraction (Leaching) and liquid extraction. Solid-liquid extraction is a process of separating solutes from insoluble solids which are called inerts. The solvent to be used in this extraction process must have the condition that it can dissolve the

solute contained in the inert (Perina et al., 2007). Based on the method of solid-liquid extraction, it can be divided into maceration, perlocation, and soxhletation (Leba, 2017).

Maceration method is a method by immersing the sample using a solvent at room temperature and humidity. Soaking plant samples with solvents will be able to break cell walls and membranes due to pressure between inside and outside the cell so that the secondary metabolite compounds contained in the plant cytoplasm will be dissolved in the solvent (Prayoga et al., 2015). This maceration method is included in the simplest method. The advantage of using this method is that the tool and method are very simple, it can be used for compounds that are resistant to heating and compounds that are not resistant to heating. While the weakness is to use a lot of solvents (Leba, 2017). Several types of solvents that can be used for maceration include methanol, ethanol, ethyl acetate, water, chloroform, and N-hexane.

Methanol is a type of solvent that has universal properties, with these universal properties making methanol able to dissolve polar and non-polar analytes (Astarina et al., 2013). Methanol is also the most widely used solvent for the isolation of organic compounds from natural materials (Susanti et al., 2012). The advantage of using methanol as a solvent is that methanol has a low boiling point. The low boiling point is easily evaporated at lower temperatures, but the methanol is toxic (Atun, 2014).

Ethanol is a solvent that has relatively non-toxic properties, but ethanol has a high boiling point, which makes it more difficult to evaporate (Atun, 2014). In addition, ethanol has a high solubility and is inert, so it will not react with other components (Susanti et al., 2012). Ethanol is also an organic solvent capable of dissolving almost all secondary metabolites (Lela et al., 2010). Ethyl acetate is a semipolar solvent. Ethyl acetate can attract both polar and non-polar analytes (Artini et al., 2013). The advantage of using this solvent is that it is low in toxicity, besides that ethyl acetate is also easy to evaporate (Putri et al., 2013).

Water is an excellent solvent for ionic compounds and is a polar solvent capable of extracting other polar and non-polar components. Water is a liquid

filter that can be obtained easily because it is cheap, besides that water has stable, flammable, non-volatile, non-toxic properties, but the extract with water is easily overgrown with mold (Sa'adah and Henny, 2015). Chloroform is a semi-polar solvent (Saputro, 2020). Chloroform is able to attract polar and non-polar compounds (Iffah et al., 2018). N-hexane is a good solvent for non-polar compounds. The advantages of n-hexane are stable, volatile, selective. N-hexane is able to extract more bioactive components with low polarity (Budilaksono et al., 2014). Based on the advantages of the above solvent, methanol is a solvent that is widely used for the extraction of natural materials. In addition, methanol is also easy to evaporate. Research by Maridass et al., 2008 and Sohag et al., 2008 used methanol as the solvent.

CHAPTER III. RESEARCH METHODS

3.1 Research Implementation

This research was conducted from July to December 2020 at the Botanical Laboratory and Biotechnology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Jember.

3.2 Research Tools and Materials

3.2.1 Research Tools

The tools used in this research are winnowing, tray, knife, scissors, scales, blender, analytical balance, brush, glass bottle, Erlenmeyer, test tube, measuring cup, micropipette, beaker, dropper pipette, spatula, rotary evaporator, spectrophotometer , sieve, GPS (Global Positioning System) GARMIN GPSMAP 64s, oven, Thermohygrometer UNI-T UT333, lux meter LUTRON LX-107 Digital

3.2.2 Research Materials

The material used in this research is the orchid plant Bulbophyllum odoratum (Blume) Lindl. Dendrobium linearifolium Teijs & Binn., and Vanda tricolor Lindl. obtained from the Gumitir mountain area, water, methanol, hydrochloric acid, berberine, phosphate buffer pH 4.7; BCG (Bromocresol Green), chloroform, quercetin, 10% AlCl3, 1 M potassium acetate, and aquades.

3.3 Research Procedure

3.3.1 Sampling

Sampling of orchids *Bulbophyllum odoratum* (Blume) Lindl., *Dendrobium linearifolium* Teijs & Binn., and *Vanda tricolor* Lindl was carried out at a coffee plantation in the Gumitir mountain area in Jember Regency.

3.3.2 Sample Preparation

The orchid plant samples that had been obtained were then washed with water and cleaned of adhering dirt. The orchid plant samples were then separated between the stems, leaves and pesudobulbs, then each was thinly sliced and weighed as the wet weight of the sample. The samples that have been weighed are then air-dried. Each dried sample was blended until smooth and then sieved to

obtain simplicia powder. The simplicia powder was then weighed and stored in a glass bottle. The simplicia powder is ready to be used for secondary metabolite testing.

3.3.3 Maceration of the Orchid Bulbophyllum odoratum (Blume) Lindl., *Dendrobium linearifolium* Teijs & Binn., and *Vanda tricolor* Lindl

Simplicia from each organ of the orchid plant Bulbophyllum odoratum (Blume) Lindl., Dendrobium linearifolium Teijs & Binn., and Vanda tricolor Lindl, soaked in methanol in the ratio of simplicia: methanol (1:100) or 1 gram in 100 ml of methanol for 3x24 hours with stirring every 24 hours until homogeneous. The soaked solution was then filtered using filter paper, then evaporated with a rotary evaporator for 20 minutes. The result of evaporation is in the form of crude extract which will then be used for quantitative testing of secondary metabolites of alkaloids and flavonoids.

3.3.4 Quantitative Analysis of Secondary Metabolic Compounds

3.3.4.1 Alkaloids

1. Determination of the maximum wavelength (λ max) of berberine

Determination of the maximum wavelength was carried out with 25 g of berberine dissolved in 5 ml of phosphate buffer pH 4.7 and 5 ml of BCG solution. Then the solution was extracted with 5 ml of chloroform and then the chloroform phase was taken. The extract was then added 5 ml of chloroform. The absorbance of the solution was measured at a wavelength of 200-400 nm. The results of the running show the maximum standard wavelength of berberine (Salamah et al., 2017).

2. Berberine standard curve Pembuatan

The compound used as a standard for determining alkaloids is berberine chloride. Berberine chloride is used to identify the total alkaloids present in plant extracts (Qoriati, 2018). The standard curve was made with 10 mg of berberine dissolved in 10 ml of methanol until the concentration obtained was 1000 ppm. The next solution is taken 1 ml and added methanol to reduce the concentration to 100 ppm, then made with a series of concentrations to 10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm, 60 ppm, 70 ppm, 80 ppm, 90 ppm, and 100 ppm. The standard solution of berberine of each concentration was taken 1 ml and added with 5 ml of

phosphate buffer pH 4.7 and 5 ml of BCG. Each was then extracted with 5 ml of chloroform and the chloroform phase was taken, then the chloroform phase was added 5 ml of chloroform again. The resulting solution was then measured absorbance at the maximum wavelength (Salamah et al., 2017).

3. Determination of the total alkaloid content of orchid extract *Dendrobium linearifolium* Teijsm.& Binn., *Bulbophyllum odoratum* (Blume) Lindl., and Vanda tricolor Lindl.

Determination of the total alkaloid content in each orchid was carried out by dissolving 50 mg of orchid extract with 3 ml of 2 N HCl. The solution was pipetted 1 ml then added 5 ml of phosphate buffer pH 4.7 and 5 ml BCG. Then extracted by adding 5 ml of chloroform and the chloroform phase was taken, then added 5 ml of chloroform again. The resulting solution was then measured absorbance at the maximum wavelength (Salamah et al., 2017).

3.3.4.2 Flavonoids

1. Determination of the maximum wavelength (λ max) of quercetin

Determination of the maximum wavelength (λ max) of quercetin can be done by running the quercetin solution in the wavelength range of 400-450 nm. The results of the running will then determine the maximum wavelength used to measure the absorption (Aminah *et al*., 2017) from the extracts of the orchid extracts of *Bulbophyllum odoratum* (Blume) Lindl., *Dendrobium linearifolium* Teijsm. & Binn., and Vanda tricolor Lindl.

2. Preparation of quercetin standard curve

Determination of the amount of flavonoid extract of orchids, Bulbophyllum odoratum (Blume) Lindl., Dendrobium linearifolium Teijs & Binn., and Vanda tricolor Lindl. The compound used as a standard for determining flavonoid levels is quercetin. Quercetin is used because it is categorized as a flavonoid from the flavonol group. Quercetin has a keto group on the C-4 atom and a hydroxyl group on the C-3 and C-5 atoms which are neighbors to the flavones and flavonol groups (Azizah *et al*., 2014).

The determination of the amount of flavonoid was carried out by complementary colorimetry – AlCl3. This complementary colorimerty has the

principle of measuring by color formation. The principle of this method is to form a complex between AlCl3 with C-3, C-4, and C-5 groups (Desmiaty et al., 2009). This method is used to determine the number of flavonoids and flavonol groups (Cahyanta, 2016). The quercetin standard curve was made by making a stock solution. 10 mg of quercetin was dissolved into 10 ml of methanol to obtain a concentration of 1000 ppm. The stock solution was then reduced in concentration by taking 1 ml of stock solution and dissolved in 10 ml of methanol until the concentration obtained was 100 ppm, then a series of concentrations were made, namely 10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm, 60 ppm, 70 ppm. , 80 ppm, 90 ppm, and 100 ppm (Aminah et al., 2017). The standard solutions of quercetin with different concentration series were each taken 0.5 ml and added with 0.1 ml 10% aluminum chloride (AlCl3); 0.1 1 M potassium acetate and 2.8 ml of distilled water. Each sample solution was incubated for 30 minutes at room temperature. The absorbance was determined by the UV-Vis spectrophotometer method at the maximum wavelength (Desmiaty et al., 2009; Aminah *et al*., 2017).

3. Determination of the total flavonoid content of *Dendrobium linearifolium* Teijs & Binn orchid extract. , *Bulbophyllum odoratum* (Blume) Lindl. , and *Vanda tricolor* Lindl.

The determination of the total flavonoid content in the orchid extract was carried out the same as the determination of the quercetin standard curve. The extract from each orchid was taken 10 mg dissolved in 10 ml of methanol until the concentration obtained was 1000 ppm. The solution is pipetted 0.5 ml; added 1.5 methanol; 0.1 ml (AlCl3) 10% and 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. The absorbance was determined by the UV-Vis spectrophotometer method at the maximum wavelength. Samples were made in three replications for each analysis to obtain the average absorbance (Desmiaty *et al*., 2009; Aminah *et al*., 2017).

3.3.5 Measurement of Abiotic Factors

Measurement of abiotic factors at the sampling site in the Gunung Gumitir area, Jember Regency was carried out by measuring altitude, temperature,

humidity, and light intensity. Each measurement of abiotic factors was repeated three times to take the average measurement. Measurement of the altitude of the sampling location is carried out using the Global Positioning System (GPS) tool, by pressing the on button to turn on the GPS, then pressing the menu button and selecting "trip computer", it will display the size of the altitude of the place. Measurement of air temperature and humidity using a thermohygrometer, how to put the thermohygrometer in the place to be measured, then install the sensor on the tool and wait 3-5 minutes, the number at the top will show the temperature, while the bottom number shows the humidity. Measurement of light intensity is carried out using a lux meter which is then calibrated, by pressing the on button and directing the sensor at the light source where to be measured, then pressing "hold" so that the light intensity number shown does not change.

3.4 Data Analysis

Data from secondary metabolite test results, namely quantitative data in the form of absorbance values from each orchid sample, were used to determine the total content of alkaloids and flavonoids calculated using the formula:

Content mg(BE/QE)/gr extract

$$= \frac{Konsentrasi\ alkaloid\ atau\ flavonoid\ dalam\ volume\ ekstrak\ sampel\ (mg)}{massa\ ekstrak\ (gr)}$$

$$\frac{Konsentrasi\ alkaloid\ atau\ flavonoid\ dalam\ volume\ ekstrak\ sampel\ (mg)}{massa\ ekstrak\ (gr)}$$

(Setyati *et al.*, 2020)

as well as measurement data of abiotic factors in the form of average data arranged in tabular form. The data is then analyzed descriptively which describes the environmental conditions at the time of the study.

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CHAPTER IV. RESULTS AND DISCUSSION

4.1 Standard curve measurement

The maximum wavelength absorption measurement results show that the maximum wavelength of the standard standard berberine is at a wavelength of 346 nm while quarcetine is at a wavelength of 435 nm. This is in accordance with the research of Setyati et al., 2020 which states that the maximum wavelength of berberine is 346 nm and quercetin is 435 nm. Standard absorbance measurements were used to obtain standard calibration curves for berberine and quercetin. Berberine standard curve obtained a calibration curve with the absorbance regression equation y = 0.0031x-0.0138, the results of the standard solvent calibration curve for berberine obtained a linear relationship between absorbance and concentration indicated by the value of the correlation coefficient (R2) 0.9902 (Appendix). Meanwhile, for the standard curve of Quercetin, the absorbance equation y = 0.0042x-0.0033, with a correlation coefficient (R2) of 0.9847 (Appendix) is very strong. The correlation coefficient value is stated to be very strong if the value (R2) obtained is above 0.9 but less than 1.0 (Padmaningrum and Siti., 2015).

4.2 Alkaloid content of methanol extract of orchid plant *Bulbophyllum odoratum* (Blume) Lindl., *Dendrobium linearifolium* Teijsm. & Binn., and *Vanda tricolor* Lindl.

Based on table 4.1 the average alkaloid content between species and between organs of orchid plants Bulbophyllum odoratum (Blume) Lindl., Dendrobium linearifolium Teijsm. & Binn., and Vanda tricolor Lindl. different. This difference indicates that each plant organ has different amounts of alkaloids from each other. In accordance with the statement by Bribi, 2018 which states that certain plants have different/varied concentrations of alkaloids, as well as in each plant organ. The difference in the content of secondary metabolites (alkaloids) between plant organs is related to the important role of plant organs for defense and reproduction. This is in accordance with the statement of Wink et al., 2010 that the mechanism of secondary metabolite accumulation depends on the place and time of storage. Furthermore, according to Wink et al. (2010) the content of alkaloids (Quinolizidine alkaloids/QA) of Lupinus plants in

leaves <4% QA, stems (epidermis 6%QA), flowers 4%QA, fruit 3.9% and seeds 4-8%. This shows the uneven distribution of secondary metabolites (alkaloids) in each plant organ.

Table 4.1 Average alkaloid content of three species of orchids with medicinal potential from the Gunung Gumitir area, Jember Regency

Species	Plant of part	Total alkaloid content (mgBE/gr)
Bulbophyllum odoratum (Blume) Lindl.	Pseudobulb	21,91 ± 3,01
	Leaf	18,01 ± 0,91
Dendrobium linearifolium Teijsm. & Binn.	Pseudobulb	16,37 ± 0,89
	Stem	10,06 ± 0,24
	Leaf	43,84 ± 4,81
Vanda tricolor Lindl.	Leaf	9,35 ± 0,216

Based on the results table (4.1), the highest average alkaloid content was found in the leaves (*Dendrobium linearifolium* Teijsm & Binn. (43.84 mgBE/gr), followed by pseudobulb *Bulbophyllum odoratum* (Blume) Lindl. (21.91 mgBE/gr) and leaves of *Bulbophyllum odoratum* (Blume) Lindl. (18.01 mgBE/gr), while the lowest alkaloid content was found in leaves of Vanda tricolor (9.35 mgBE/gr).This difference in alkaloid content was also influenced by the function of these secondary metabolites for plants. et al., 2012 stated that plants have acquired various mechanisms to deal with their environment and modify growth and development as needed. Each plant has different constitutive defense mechanisms in response to it. Plants experience biotic stress such as disturbances from other organisms, then the plant then will activate its defense system by increasing the production of the required secondary metabolites.Wink., 2008 stated that the alkaloid compounds jw.org en plants serve as a chemical defense against herbivores or other predators. Many alkaloid compounds are toxic to animals that eat them so that they can cause the animal to die, this is a protection for plants from other animals, besides that alkaloid compounds are also used for insecticides (Bribi, 2018).

The distribution of alkaloid compounds in plants is also influenced by the age of the plant. Alkaloid compounds in plants in the first year of growth are

distributed in various plant organs, but when the plant gets older, the distribution of alkaloids is only found in some parts of the plant, according to the needs of the plant (Djoronga et al., 2014). Alkaloid compounds have mutagenic or carcinogenic, antibacterial, antifungal, antioxidant, anti-inflammatory and antiviral activities (Bribi, 2018).

The content of alkaloids in each species and organ of orchid is different. Leaves of *Dendrobium linearifolium* Teijsm & Binn. from the Gumitir mountain area contained alkaloids (43.84 mgBE/gr) (Table 4.1). The alkaloid content is lower than the alkaloid content in the leaves of Dendrobium crepidatum (56.33 mgATP/gr) using acetone as a solvent (Bhattacharyya et al., 2016) and in the stems of *Dendrobium nobile* (51.31 mgATP/gr) with ethanol as solvent (Bhattacharyya). et al., 2015). The difference in the alkaloid content was caused by the different types of samples used and the solvent used during extraction. Based on research by Hartanti et al., 2019 the different types of solvents used for the extraction of secondary metabolites affect the yield of secondary metabolites obtained. Different types of solvents produce different contents. This is because secondary metabolites in plants depend on the solubility of secondary metabolites in the solvent.

4.3. Flavonoid content of methanol extract of orchid plant *Bulbophyllum odoratum* (Blume) Lindl., *Dendrobium linearifolium* Teijsm. & Binn., and *Vanda tricolor* Lindl.

Quantitative test of determination of flavonoid content was carried out to determine how much flavonoid compound contained in the extract of the orchid plant *Bulbophyllum odoratum* (Blume) Lindl., *Dendrobium linearifolium* Teijsm. & Binn., and *Vanda tricolor* Lindl. obtained from the Gumitir mountain area, Jember district. The results of the quantitative test of the flavonoid compounds of these orchids can be seen in (Table 4.2).

Based on (Table 4.2) the average flavonoid content was different between orchid species and the organs observed. This shows that the difference in flavonoid content in each organ of the orchid is due to the uneven distribution of

secondary metabolites in plants. The study of Manurung *et al*., 2017 which examined the flavonoid content and antioxidant activity of the Barito tabat plant (*Ficus deltoidea* Jack.) on differences in plant organs and age showed that at the age of 6 months after planting the flavonoid content of stems was 138.67 gCE/mg, fresh leaves 206.67 gCE/mg and fruit 328.21 gCE/mg. In accordance with the statement of Hermawan, 2008 which states that the difference in flavonoid content in each organ of orchids is due to the uneven distribution of secondary metabolites in plants.

Table 4.2. The average flavonoid content of three species of orchids with medicinal potential from the Mount Gumitir area, Jember Regency

Species	Plant of Part	Total flavonoid content mgQE/gr
Bulbophyllum odoratum (Blume) Lindl.	Pseudobulb	57,45 ± 9,64
	Leaf	132,21 ± 6,20
Dendrobium linearifolium Teijsm. & Binn.	Pseudobulb	66,97 ± 1,71
	Stem	56,26 ± 0,36
	Leaf	219,59 ± 10,47
Vanda tricolor Lindl.	Leaf	122,69 ± 3,69

The highest average total flavonoid content was found in the leaves of *Dendrobium linearifolium* Teijsm. & Binn (219.59 mgQE/gr), followed by *Bulbophyllum odoratum* (Blume) Lindl (132.21 mgQE/gr) and *Vanda tricolor* Lindl leaves. (122.69mgQE/gr). The difference in the flavonoid content of leaves, stems, and pseudobulbs is due to the location of the accumulation of these secondary metabolites influenced by the function of these secondary metabolites for plants (Hermawan., 2008). Leaves are organs that are more susceptible to pests and diseases than stems or pseudobulbs, therefore the growth of flavonoid compounds is higher in the leaves than in other plant parts. Flavonoids in plants function to defend plants against their environment. The function of flavonoids is to protect plants from various abiotic stresses such as UV radiation which can cause cell damage and biotic stresses such as pests, microbes and insects (Samanta et al., 2011). The high content of total flavonoids in the leaves of *Dendrobium linearifolium* Teijsm. & Bin. intended for plant self-defense considering that the

leaf is an important organ that functions as a place for photosynthesis, transpiration and respiration for the plant concerned. Photosynthesis, transpiration, and respiration are important processes in producing secondary metabolites (Syukriah and Liuvita, 2016). The high content of total flavonoids in the leaves of *Dendrobium linearifolium* Teijsm. & Bin. also influenced by plant morphology. As stated by Felicia et al., (2016) that differences in flavonoid content are influenced by the morphology and increasing age of each plant organ. Leaves of *Dendrobium linearifolium* Teijsm. & Bin. has thinner leaves than those of *Bulbophyllum odoratum* (Blume) Lindl. and leaves of Vanda tricolor Lindl., to leaves of *Dendrobium linearifolium* Teijsm. & Bin. more susceptible to attacks by caterpillars and other insects that prey on them, this makes the leaves of Dendrobium linearifolium Teijsm. & Bin. produce more flavonoids than the other two orchid species.

The total flavonoid content in each orchid species has a different amount. One of the differences in flavonoid content is influenced by the solvent used during extraction. This is in accordance with the statement of Suryani et al., 2015 that the solvent is one of the factors that affect the high content of flavonoids in plants. This is because the ability and nature of the solvent in dissolving different compounds, depending on the level of polarity of the solvent and the extracted compounds. Leaves of *Dendrobium linearifolium* Teijsm & Binn. has a higher total flavonoid content (219.59 mgQE/gr) than *Dendrobium nobile* using ethanol as solvent (Bhattacharyya et al., 2015), *Dendrobium crepidatum* with acetone solvent (Bhattacharyya et al., 2016), and *Dendrobium fimbriatum* with solvent methanol (Paul and Suman, 2020). This proves that the type of solvent affects the total flavonoid content in plants. As with methanol, it is suitable to be used as a solvent for the determination of the total flavonoid content. If the total flavonoid content is high, the solvent used is able to dissolve the compound (flavonoid). This happened in the leaves of *Dendrobium linearifolium* Teijs & Binn. which used methanol as a solvent resulted in a higher total flavonoid content than other species using different solvents. This is in accordance with the

statement of Ghasemzadeh et al., 2011 which states that methanol as a solvent is better used for the extraction of secondary metabolic compounds (flavonoids).

4.4 Measurement of abiotic factors at the orchid sampling site

The content of secondary metabolites in plants can be influenced by several factors, such as internal factors such as genes and external factors such as altitude, temperature, light intensity, humidity, pH, and nutrient content (Katuuk et al., 2019). Based on the results of measurements of abiotic factors in (Table 4.3), the average value of the altitude on Mount Gumitir (755.63 masl) is obtained. The humidity and temperature values at Mount Gumitir are (52.93% rh) and 32.85 C, respectively, while the light intensity value is (654.3 lux). The environmental conditions of Mount Gumitir from the measurement results of the abiotic factors are in accordance with the habitat needed by the orchids *Bulbophyllum odoratum* (Blume) Lindl., *Dendrobium linearifolium* Teijs & Binn and Vanda tricolor Lindl. According to a statement by Fitriany *et al*., 2019 which states that from the aspect of temperature, orchids are divided into three types, namely: (1) cold temperature orchids that grow in mountainous areas at an altitude of 2000 – 4000 m above sea level. This type of orchid grows well at temperatures of 15 – 21 C during the day and 10 – 13 C at night, (2) Medium temperature orchids that grow at an altitude of 750 – 2000 m above sea level. This type of orchid grows well at temperatures of 21 – 32 C during the day and 13 – 18 C at night. (3) Hot temperature orchids that grow in the lowlands at an altitude between 0 – 750 m above sea level. This type of orchid grows well at temperatures of 26 – 35 C during the day and 18 – 24 C at night, so it can be said that based on the measurement of abiotic factors the three species of orchids are included in moderate temperature orchids, with normal conditions for the environment in which they live.

Table 4.3 Average measurements of abiotic factors in the Gumitir mountain area, Jember regency

No	Abiotic Factors	Averagea
1	Altitude (mdpl)	755,63 ± 22,89
2	Humidity (%rh)	52,93 ± 7,25
3	Temperature(ºC)	32,85 ± 4,11
4	Light Intensity (lux)	654,3 ± 5,68

Environmental conditions greatly affect the growth of orchids, according to Tirta., 2004 which states that orchids cannot live in poor environmental conditions, because the intensity of sunlight, humidity and nutrients that are not in accordance with the needs of each type of orchid, orchids cannot grow. and thrive in their habitat. Air humidity is certainly influenced by altitude, the higher the place, the higher the humidity (Tanamal *et al*., 2017). Other factors such as temperature also affect plant growth and development, because temperature affects physiological processes such as photosynthesis, respiration and plant transpiration (Pratama *et al*., 2017). If the environmental conditions in which they live are suitable, the orchid can carry out the photosynthesis process well, so that the products produced from the photosynthesis process also increase, which of course will increase the content of secondary metabolites such as alkaloids and flavonoids, because secondary metabolites such as alkaloids and flavonoids are indirectly a product of photosynthesis process.

CHAPTER V. CONCLUSIONS AND SUGGESTIONS

5.1 Conclusions

Based on the research, it can be concluded that different types of orchid plants and organs contain different alkaloids and flavonoids. The content of alkaloids and flavonoids of the orchid *Bulbophyllum odoratum* (Blume) Lindl. on pseudobulb were (21.91 mgBE/gr) and (57.45 mgQE/gr), on leaves (18.01 mgBE/gr) and (132.21 mgQE/gr). *Dendrobium linearifolium* Teijsm orchid. & Bin. on pseudobulbs (16.37 mgBE/gr) and (66.97 mgQE/gr), stems (10.06 mgBE/gr) and (56.26 mgQE/gr), leaves (43.84 mgBE/gr) and (219.59 mgQE/gr). While the *Vanda tricolor* Lindl orchids on the leaves were (9.35 mgBE/gr) and (122.59 mgQE/gr).

5.2 Suggestion

Improvements for further research are:

1. Determination of the content of alkaloids and flavonoids in this study using the spectrophotometric method, in future studies other methods can be used, for example using the gavimetric method, so that the results can be compared between the two methods.
2. This study used methanol as a solvent, in future studies it is better to use a different solvent, such as ethyl acetate, ethanol, n-hexane or others, so that the results of the alkaloid and flavonoid content can be compared using different solvents.

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GROWTH CURVE PATTERN AND DEGRADATION ABILITY OF CAFFEINE DEGRADING BACTERIA CONSORTIUM

THESIS

By

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NIM 171810401051

BIOLOGY PROGRAM

FACULTY OF MATHEMATICS AND NATURAL SCIENCES

JEMBER UNIVERSITY

2021

GROWTH CURVE PATTERN AND DEGRADATION ABILITY OF CAFFEINE DEGRADING BACTERIA CONSORTIUM

THESIS

submitted to complete the final project and fulfill one of the requirements
to complete the Biology Study Program (S1)
and achieve a Bachelor of Science degree

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DEDICATION

This thesis is dedicated to:

My mother Sri Wahyuni and my father Dadang Dwi Karianto for all the support, advice, love, and prayers that are always offered.

MOTTO

Maka bersabarlah kamu, sesungguhnya janji Allah itu benar, dan mohonlah ampun untuk dosamu dan bertasbihlah seraya memuji Tuhanmu pada waktu petang dan pagi
(Terjemahan Surat Ghafir ayat 55)[*)]

Dia memberikan hikmah kepada siapa yang Dia kehendaki. Barangsiapa diberi hikmah, sesungguhnya dia telah diberi kebaikan yang banyak. Dan tidak ada yang dapat mengambil pelajaran kecuali orang-orang yang mempunyai akal sehat.
(Terjemahan Surat Al-Baqarah ayat 269)[*)]

"*Don't limit yourself. Many people limit themselves to what they think they can do. You can go as far as your mind lets you. What you believe, remember, you can achieve*."
(Mary Kay Ash)[**)]

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PRONOUNCEMENT

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I hereby sincerely state that the thesis titled "Growth Curve Pattern and Degradation Ability of Caffeine Degrading Bacteria Consortium" is the real masterpiece. The things out of my masterpiece in the thesis are signed by citation and referred to in my bibliography. This research was funded by Dr. Sattya Arimurti, S.P., M.Si. I am responsible for the validity and correctness of the contents following a highly upheld scientific attitude.

Thus, I make this statement truthfully, without any pressure or coercion from any party, and am willing to take the academic sanctions if it turns out that this statement is not true in the future.

Jember, July 2021

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THESIS

GROWTH CURVE PATTERN AND DEGRADATION ABILITY OF CAFFEINE DEGRADING BACTERIA CONSORTIUM

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PENGESAHAN

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SUMMARY

Growth Curve Pattern and Degradation Ability of Caffeine Degrading Bacteria Consortium; Nadhea Ayu Sukma; 171810401051; 2021; 45 pages; Biology Faculty of Mathematics and Natural Sciences Jember University.

Caffeine is a methylxanthine group that is naturally found in plants including berries, chocolate, tea, and coffee. Caffeine in organic waste from the coffee industry is toxic to bacteria, so the presence of caffeine in coffee waste will affect the growth of bacteria in the soil which plays an important role in the decomposition process of organic matter. Therefore, the caffeine content in organic waste needs to be reduced by utilizing caffeine-degrading bacteria. Caffeine-degrading bacteria *Paracoccus denitrificans* KAFS 16, *Pseudomonas plecoglossicida* KAFS 34, and *Acinetobacter gerneri* KAFS 47 showed caffeine degradation of 100%, respectively; 98.15%; and 100% on a minimum medium of M9 + 2.5 g/L caffeine within 72 hours of incubation. The three bacteria have the potential to be used as a consortium of caffeine degradation agents in organic waste. The conditions for bacteria to be able to be in a consortium must have a positive association relationship between isolates and the viability of the consortium. Viability analysis of the consortium bacterial isolates can be carried out by testing for resistance to antibiotics because the resistance profile of each bacterium to antibiotics is specific. This study aimed to analyze the potential of a consortium of caffeine-degrading bacteria *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47 based on growth patterns and caffeine degradation.

This research was conducted from January to April 2021 at the Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Jember. The research method used was an association test between isolates of caffeine-degrading bacteria; a test for antibiotic resistance of caffeine-degrading bacteria; making a pattern for the growth of a consortium of caffeine-degrading bacteria; analysis of the viability of single isolates in a consortium of bacteria based on antibiotic resistance markers; patterning of caffeine degradation by a consortium of caffeine-degrading bacteria; and correlation analysis (Pearson) of bacterial growth pattern with caffeine degradation pattern.

The results showed that the association test between caffeine-degrading bacteria using a cell-free supernatant showed a positive association, so it could potentially be used as a consortium of caffeine-degrading bacteria. *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47 were resistant to the antibiotic lincomycin (50 ppm); sanprima (50 ppm); erythromycin (50 ppm), cefixime (100 ppm); and metronidazole (50 ppm). Cefadroxil antibiotics (75 ppm) as markers of *P. plecoglossicida* KAFS 34. Antibiotics Ampicillin (75 ppm) as markers of *P. plecoglossicida* KAFS 34 and *A. gerneri* KAFS 47, and colsancetine (20 ppm) as markers of *P. denitrificans* KAFS 16 and *P. plecoglossicida* KAFS 34.

The growth of the bacterial consortium (54.779 CFU/mL) was lower than that of *P. denitrificans* KAFS 16 (84.940 CFU/mL) and *A. gerneri* KAFS 47 (93.481 CFU/mL) at 4 days of incubation. However, the growth of the bacterial consortium

was higher than that of *P. plecoglossicida* KAFS 34 (49,277 CFU/mL) at the same time. The results of the bacterial consortium based on the viability of bacteria to antibiotics showed that all bacteria could still grow. The degradation of caffeine by a consortium of bacteria and *P. plecoglossicida* KAFS 34 was 24 hours faster than that of *P. denitrificans* KAFS 16 and *A. gerneri* KAFS 47. The consortium of bacteria and *P. plecoglossicida* KAFS 34 was able to degrade 100% of caffeine on M9 + 2.5 g/L caffeine medium within 3 days of incubation. The bacterial consortium has the same caffeine degradation potential as a single isolate (*P. plecoglossicida* KAFS 34), therefore it is necessary to consider the use of the bacterial consortium as a caffeine degradation agent in organic waste.

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Jember, July 2021

The writer

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CHAPTER 1. INTRODUCTION

1.1 Background of Study

Caffeine (1,3,7-trimethylxanthine) is a methylxanthine in addition to theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine) (Hermawati, 2015; Misfadhila *et al*., 2017). Caffeine is naturally contained in coffee waste (Win *et al.,* 2019). Caffeine is toxic to bacteria, so the presence of caffeine in coffee waste will affect the growth of bacteria in the soil, which play an important role in the process decomposition of organic matter (Iswanto *et al*., 2019: Ramanaviciene *et al.,* 2003; Dash and Gummadi, 2008; Al-Janabi, 2011). Therefore, the caffeine content in coffee waste needs to be reduced.

Caffeine in a coffee waste can be reduced by utilizing caffeine-degrading bacteria. These bacteria can degrade caffeine and use it as a source of carbon and nitrogen for growth (Summers *et al.,* 2012). These bacteria can be used as caffeine degrading agents in organic waste. Several genera of bacteria reported having the ability to degrade caffeine are *Pseudomonas, Serratia, Rhodococcus, Klebsiella, Acinetobacter, Alcaligenes, and Acetobacter* (Summers *et al.,* 2015). Caffeine-degrading bacteria *Paracoccus denitrificans* KAFS 16, *Pseudomonas plecoglossicida* KAFS 34, and *Acinetobacter gerneri* KAFS 47 were individually able to degrade caffeine by 100%, respectively; 98.15%; and 100% (Irsyadah, 2020; Malasari, 2020; Sindiana, 2020). These three bacteria have the potential to be used as a consortium of caffeine degrading agents in organic waste.

A bacterial consortium is a collection of bacterial populations that live in symbiosis (Kumar and Jagadesh, 2016). The symbiosis between the bacterial isolates in the consortium must show a positive association which is indicated by not inhibiting each other's growth. Several research results show that the use of bacterial consortium has higher effectiveness than single isolates. The use of a consortium of endophytic bacteria (*Bacillus* sp. SJI and *S. marcescen* isolate JB1E3) to inhibit the pathogenic bacterium *Rasltonia solanacearum* showed an inhibition zone of 9.25 cm compared to single isolates unable to suppress pathogenic bacteria (Resti *et al.,* 2018). Research by Poszytek *et al.* (2016) showed

that a consortium of cellulolytic bacteria was able to efficiently hydrolyze corn silage and increase biogas production by 38% compared to the single isolate (16%). The consortium of oil-degrading bacteria consisting of *Pseudomonas* sp., *Micrococcus* sp., *Bacillus* sp., *Flavobacterium* sp., and *Corynebacterium* sp. showed degradation activity on oil substrates up to 78% compared to single isolates whose degradation activity reached 41-66% (Hamzah *et al.*, 2013).

Analysis of the viability of each bacterial isolate in the bacterial consortium can be carried out with markers of resistance to certain antibiotics (Langden *et al.*, 2017). Antibiotics have different mechanisms for inhibiting bacterial growth including inhibition of bacterial cell wall synthesis, inhibition of protein synthesis, inhibition of the translation process, and destruction of cell membranes (Fatiqin, 2015; Soleha 2015). Markers of resistance of each bacterium to antibiotics are specific. Gram-positive bacteria are resistant to penicillin antibiotics while Gram-negative bacteria are not resistant (Febiana *et al.*, 2012).

The use of a consortium of caffeine-degrading bacteria for caffeine degradation has not been reported. It is necessary to analyze the potential of a consortium of caffeine-degrading bacteria *Paracoccus denitrificans* KAFS 16, *Pseudomonas plecoglossicida* KAFS 34, and *Acinetobacter gerneri* KAFS 47 based on growth patterns and degradation patterns on caffeine media. This supports the development of caffeine-degrading biological agents in organic waste.

1.2 Identification of Problem

Identification of the problem of this research based on the above background is:

1. What are the growth and degradation patterns of the consortium of caffeine-degrading bacteria *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47?
2. How is the correlation between bacterial growth pattern and caffeine degradation pattern by a consortium of caffeine-degrading bacteria *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47?

1.3 Limitation of the Problem

The problem limitations of this research are:

1. The Association test between isolates of caffeine-degrading bacteria was determined based on the formation of a cell-free supernatant inhibition zone of one bacterial isolate against other bacterial isolates in the consortium.
2. The growth ability test in the consortium was carried out using several antibiotics.

1.4 Purpose of the Study

The aims of this research are:

1. Analyzing the growth and degradation patterns of a consortium of caffeine-degrading bacteria *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47;
2. Analyzing the correlation of bacterial growth patterns with caffeine degradation patterns by a consortium of caffeine-degrading bacteria *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47.

1.5 Significance of the Research

1. The benefits of research for Science, Technology, and the Arts (IPTEKS) can increase knowledge about the potential of a consortium of caffeine-degrading bacteria in degrading caffeine.
2. The benefits of research for the government can be a consideration for the use of biological agents in organic waste management.
3. The benefits of research for the community can take advantage of the potential of this bacterial consortium as a caffeine degradation agent in organic waste.

CHAPTER 2. LITERATURE REVIEW

2.1 Caffeine

Caffeine (1,3,7-trimethylxanthine) is a methylxanthine in addition to theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine) (Hermawati, 2015; Misfadhila *et al*., 2017). Caffeine is a purine alkaloid compound with the chemical formula $C_8H_{10}N_4O_2$ (Nonthakaew *et al.,* 2015). Pure caffeine is in the form of a white powder, odorless, and weakly alkaline, so it has a slightly bitter taste (Yonata and Saragih, 2016). Caffeine is composed of carbon, hydrogen, nitrogen, and oxygen components (Nonthakaew *et al.,* 2015). The molecular structure of caffeine is presented in Figure 2.1.

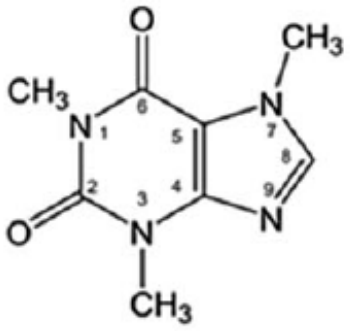


Figure 2.1 Caffeine molecular structure (Source: Summers *et al.,* 2015)

Caffeine is naturally contained in more than 60 plant species spread around the world, such as berries, chocolate, tea, and coffee (Win *et al.*, 2019). The coffee and tea industry produces solid waste in the form of tea, husks, and coffee grounds which produce organic waste containing caffeine (Nonthakaew *et al*., 2015). Industrial waste can contaminate soil and water because it is toxic to the environment (Gummadi *et al*., 2012; Ibrahim *et al*., 2016). Caffeine has antibacterial properties in several genera of bacteria that are unable to utilize caffeine such as *Bacillus subtilis, Listeria monocytogenes, Escherichia coli, Salmonella typhimurium, Enterobacter aerogenes*, and *Staphylococcus aureus* (Ramanaviciene *et al*., 2003; Dash and Gummadi, 2008; Al Janabi, 2011). The presence of caffeine in coffee waste will also affect the growth of bacteria in the soil which play an important role in the decomposition of organic matter. Besides

being toxic to bacteria, caffeine can also inhibit the growth of onion cells (Pincheira *et al.,* 2003), lettuce (Gomes *et al*., 2013), and sunflowers (Khursheed *et al.,* 2019). Therefore, caffeine in coffee waste needs to be degraded.

Conventional decaffeination techniques can be carried out using supercritical solutions and solvent extraction. This method uses a solvent that is not specific to caffeine, is expensive, and has an impact on the environment because it uses a toxic solvent (Gummadi *et al.,* 2012). The use of microorganisms as caffeine biodegradation agents is more environmentally friendly, specific, sustainable, and requires less cost than conventional extraction techniques (Win *et al*., 2019). Therefore, the potential for caffeine degradation by microorganisms, especially bacteria, is still being studied.

2.2 Caffeine Degrading Bacteria

2.2.1 Caffeine as a Source of Carbon and Nitrogen for Caffeine Degrading Bacteria

Caffeine-degrading bacteria are bacteria that can grow on media containing caffeine by utilizing it as a carbon and nitrogen source (Summers *et al*., 2015; Iswanto *et al.,* 2019). Utilization of carbon sources as the main energy source that will be used for the growth and metabolism of bacterial cells. Nitrogen sources are used for protein synthesis, and nucleic acids, and can increase cell production (Subagyo *et al.,* 2015). Caffeine degradation using several bacterial species can be used for decaffeination (Win *et al*., 2019).

Bacterial metabolism in the caffeine degradation process goes through two pathways, namely N-demethylation and C-8 oxidation. Both of these metabolic pathways will convert caffeine into carbon dioxide and ammonia (Summers *et al*., 2015). Caffeine in the N-demethylation pathway will be degraded by the demethylase enzyme to theobromine and paraxanthine (Dash and Gummadi, 2006). Theobromine and paraxanthine will be converted into an intermediate product, namely 7-methylxanthine, and then into xanthine. The next step is xanthine will be degraded into carbon dioxide and ammonia through purine catabolism (Figure 2.2) (Gummadi *et al*., 2012; Summers *et al*., 2015; Kim *et al*., 2019). The bacterial genus that uses the N-demethylation pathway to degrade caffeine is *Pseudomonas* (Summers *et al*., 2015).

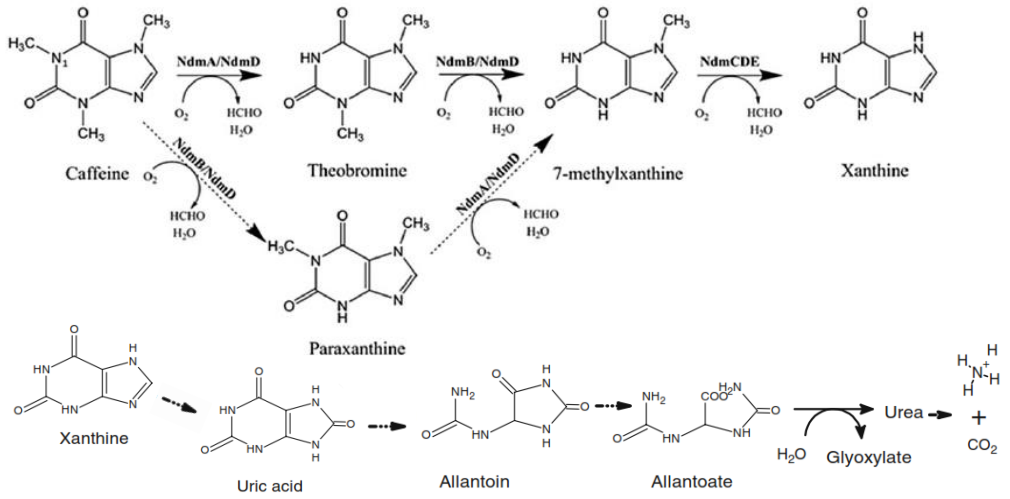


Figure 2.2 N-demethylase pathway caffeine degradation (Source: Summers *et al*., 2015)

Caffeine degradation through C-8 oxidation occurs in *Klebsiella*, *Rhodococcus*, and *Alcaligenes* bacteria (Summers *et al.*, 2015). The C-8 oxidase pathway will convert caffeine into 1,3,7-trimethyluric acid (TMU) with the enzyme caffeine oxidase as a catalyst. Trimethyluric acid (TMU) will be converted into 3,6,8-trimethylallantoin (TMA) and will then be converted into allantoin (Summers *et al*., 2015). Allantoin will be degraded through purine catabolism pathways to produce carbon dioxide and ammonia (Figure 2.3) (Gummadi *et al.*, 2012).

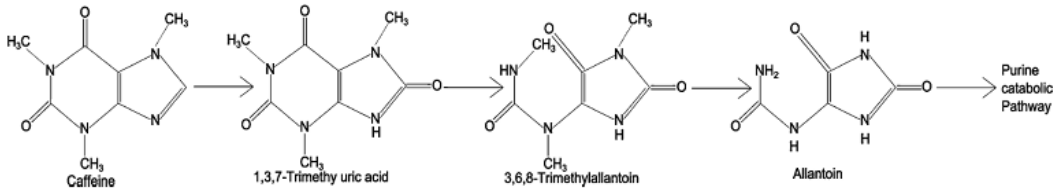


Figure 2.3 C-8 oxidase pathway caffeine degradation (Source: Gummadi *et al.*, 2012)

2.2.2 Types of Caffeine Degrading Bacteria

Summers *et al*. (2015) have reported several bacteria capable of degrading caffeine, namely *Pseudomonas, Serratia*, *Rhodococcus, Klebsiella, Acinetobacter, Alcaligenes, Acetobacter, Coryneform, Flavobacterium*, and *Moraxella*. Research conducted by Arimurti at the people's coffee plantation *Coffea arabica* Sempol, Bondowoso, found three isolates of caffeine-degrading bacteria that could degrade caffeine above 95%. The bacterial isolates were able to grow on a minimal medium

of M9 + 1000 ppm caffeine. The three bacterial isolates were *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47 (Arimurti *et al.,* 2018).

P. denitrificans KAFS 16 is a Gram-negative bacterium, coccoid in shape, non-motile, and has potential for bioremediation (Yoshida *et al.,* 2017). These bacteria can also grow both under aerobic conditions by utilizing oxygen and under anaerobic conditions by utilizing nitrogen as the final electron acceptor in the respiration process (Cheng *et al*., 2016). The results of Irsyadah's research (2020) showed that *P. denitrificans* KAFS 16 was able to degrade 100% of the caffeine in M9 + 2.5 g/L caffeine media with an incubation time of 72 hours. *P. plecoglossicida* KAFS 34 is a Gram-negative and rod-shaped bacterium. These bacteria are found in environments that have aerobic metabolism (Lugito *et al*., 2015). *P. plecoglossicida* KAFS 34 was able to degrade 98.15% caffeine for 72 hours on M9 + 2.5 g/L caffeine media (Malasari, 2020). *A. gerneri* KAFS 47 is a Gram-negative bacterium, cococobacilli form, non-motile, and aerobic (Alvarez-Perez *et al*., 2013). The ability of *A. gerneri* KAFS 47 in degrading caffeine was shown in the Sindiana study (2020) of 100% grown in M9 + 2.5 g/L caffeine medium for 72 hours.

2.2.3 Caffeine Degrading Bacteria Growth Pattern

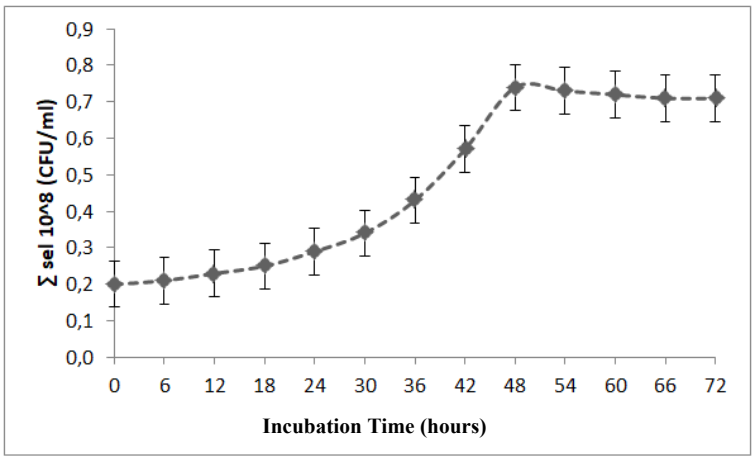
Bacterial growth is an increase in the number of cells, cell size, cell mass, and cell volume. The growth pattern of bacteria generally forms a sigmoid curve. Bacterial growth consists of four phases, namely the lag phase, the exponential growth phase, the stationary phase, and the death phase. The lag or adaptation phase is the initial growth phase and adjustment to the culture medium, increasing the number of cells to a small number. The length of this phase indicates the time required for cells to adjust to the culture medium. This phase is characterized by a horizontal curve. The exponential growth phase or log phase is a phase of rapid growth by cells due to significant metabolic activity with their ability to utilize nutrients in the media. This phase is characterized by the shape of an exponential curve. The stationary phase is a phase of constant cell growth, the number of dead and living cells is the same. In this phase, some cells continue to grow, but some cells die due to decreased nutrient content in the media, and the formation of

secondary metabolites that will inhibit cell growth. This phase is characterized by a horizontal curve. The death phase is a phase of decreasing the number of cells because the number of dead cells is more than the number of living cells. This happens because the nutrients needed for growth have been depleted. This phase is characterized by a downward curve (Madigan *et al*., 2015).

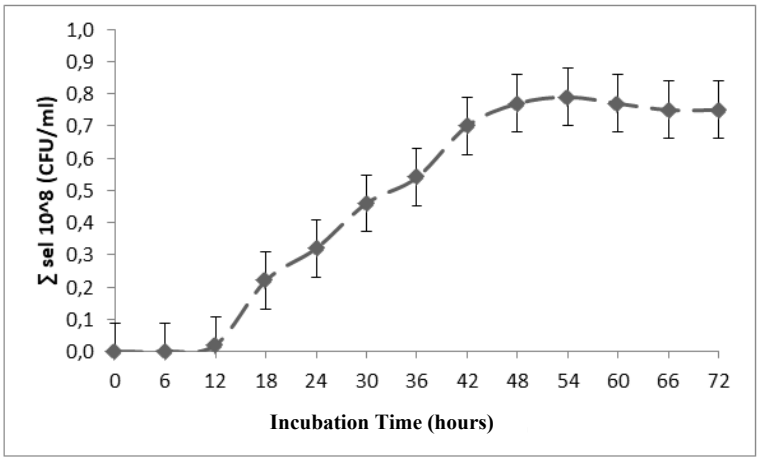
Caffeine-degrading bacteria utilize carbon and nitrogen sources from the caffeine degradation process and the degradation products are used for growth (Summers *et al*., 2015). The growth pattern of caffeine-degrading bacteria depends on the type of media used. This is related to the utilization of carbon and nitrogen sources resulting from the degradation of caffeine contained in the media. The growth patterns of *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47 on M9 + 2.5 g/L caffeine media are presented in Figure 2.4.

The growth pattern of caffeine-degrading bacteria at 0 to 48 hours has experienced an exponential phase for KAFS 16 isolate (Figure 2.4a). The exponential phase of the growth pattern of caffeine-degrading bacteria isolates KAFS 34 was shown at 12 to 48 hours (Figure 2.4b). The growth pattern of KAFS 47 isolates reached an exponential phase at 24 to 66 hours (Figure 2.4c).

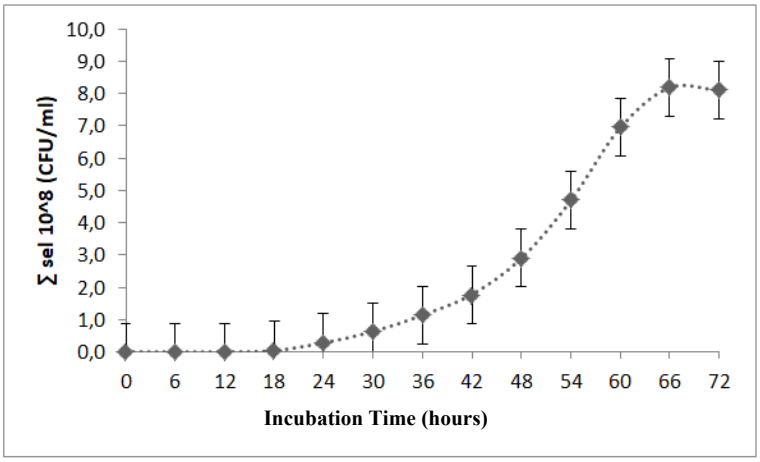
a)



b)



c)



(a) *Paracoccus denitrificans* KAFS 16; (b), *Pseudomonas plecoglossicida* KAFS 34 (c); *Acinetobacter gerneri* KAFS 47

Figure 2. 4 Growth pattern of caffeine-degrading bacteria on a minimum medium of M9 + 2.5 g/L caffeine for 72 hours (Source: Irsyadah, 2020; Malasari, 2020; Sindiana, 2020)

2.2.4 Caffeine Degradation Patterns Caffeine Degrading Bacteria

The pattern of caffeine degradation shows the utilization of caffeine as a carbon and nitrogen source used for the bacterial growth process (Arimurti *et al.*, 2018). The carbon and nitrogen sources are obtained from carbon dioxide and ammonia as the end products of caffeine degradation. The first step of caffeine degradation results in N-demethylation to produce theobromine (Summers *et al.*, 2015; Kim *et al.*, 2019). The research results of Ogawa *et al*. (2012) based on LC-MS/MS analysis showed that caffeine-degrading bacteria produced metabolites in the form of theophylline and theobromine which had a molecular weight of 181 g/mol.

The pattern of caffeine degradation of the isolates of the caffeine-degrading bacteria *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47 on a minimum medium of M9 + 2.5 g/L caffeine is shown in Figure 2.5. The fastest decrease in caffeine concentration was by KAFS 16 and KAFS 34, followed by KAFS 47. The graph shows the decrease in caffeine concentration of the three isolates due to their degradation activity.

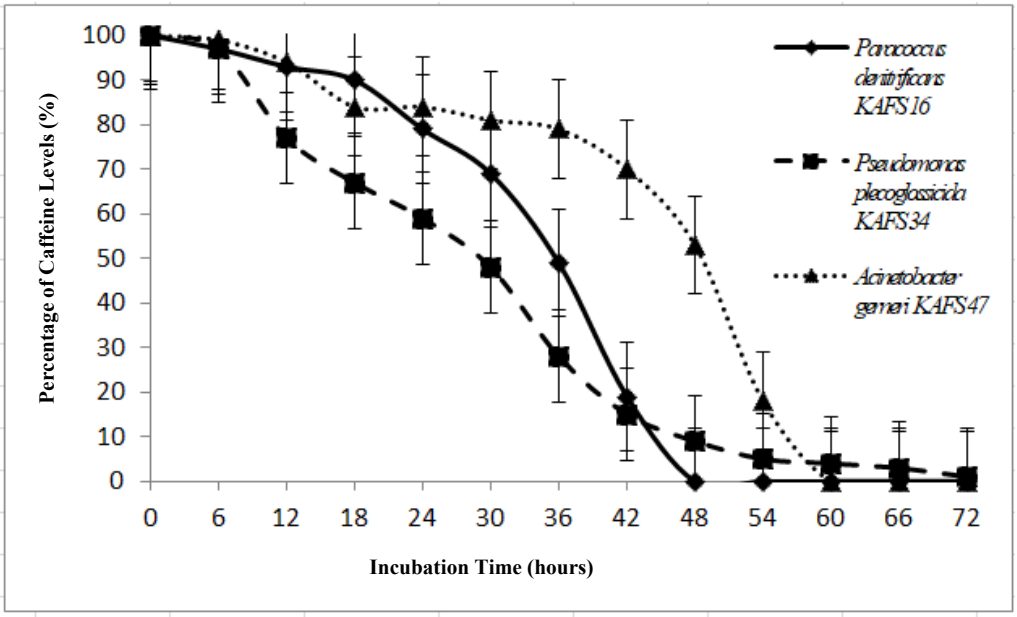


Figure 2.5 Pattern of degradation of caffeine-degrading bacteria *Paracoccus denitrificans* KAFS 16, *Pseudomonas plecoglossicida* KAFS 34, and *Acinetobacter gerneri* KAFS 47 on a minimum medium of M9 + 2.5 g/L caffeine for 72 hours (Source: Irsyadah, 2020; Malasari, 2020; Sindiana, 2020)

2.3 Bacteria Consortium

A bacterial consortium is a mixture of two or more different bacterial isolates on the same medium (Amanah *et al.*, 2017). Bacterial consortia can form cooperative, commensal, and mutualistic interactions either naturally or artificially or on a laboratory scale. According to research by Sahlan *et al*. (2014), bacterial consortia can complement each other's needs in the same ecosystem, both naturally and artificially formed consortia. Bacterial activities that inhibit the growth of other bacteria due to competition for nutrients, space, and even produce secondary metabolites are called antagonistic bacteria while the opposite is called synergistic. Synergistic bacteria are bacteria whose activities do not interfere with each other (Rifai *et al*., 2020).

Synergism is an interaction that occurs in bacteria that does not show an inhibitory mechanism between one type of bacteria and another. The activity of these bacteria can interact, share nutrients, and can behave cooperatively so that they support each other in the growth process of each bacterium (Rifai *et al*., 2020). Positive interactions between consortium bacteria that live together using the same nutrients can be determined by conducting a synergism test. The synergistic form of the consortium bacteria is indicated by the absence of a clear zone, which is an indication of the inhibition mechanism (Safitri *et al*., 2018). Factors that support synergistic interactions include one of the bacteria being able to provide nutrients that cannot be synthesized by other bacteria, bacteria that cannot degrade organic matter will depend on bacteria capable of degrading organic matter, and one of the bacteria will protect other bacteria sensitive to organic matter by reducing the concentration of organic matter and producing protective compounds (Asri and Zulaika, 2016).

The use of consortium bacteria has better potential than single isolates (Amanah *et al*., 2017). According to Resti *et al*. (2018), a consortium of endophytic bacteria was able to suppress the development of the pathogen *R. solanacearum*. This is indicated by the presence of an inhibition zone of 6-9.25 cm and when compared with the use of a single isolate, it does not show an inhibition zone (Resti *et al.,* 2018). According to Istifadah in Resti *et al*. (2018), a consortium of

antagonistic microbes with biological fertilizers can suppress seedling fall disease (*Rhizoctonia solani*) by 67.4-91.8% and can increase growth in chili plants. According to Simamarta and Sukiman in Resti *et al*., (2018) the microbial consortium can increase the percentage of seed weight in soybean plants by 47.9%. The bacterial consortium consisting of *Bacillus* sp. SJI and *Serratia marcescens* JB1E3 can increase the number of leaves on chili plants by 70% (Resti *et al.*, 2018). According to research by Marsandi *et al*. (2016), the association of the bacterial consortium *Micrococcus luteus* and *Pseudomonas pseudoalcaligenes* has a good interaction form with lamtoro to increase petroleum bioremediation. The consortium interaction was able to reduce the TPH (Total Petroleum Hydrocarbon) value of petroleum by 2.85%. The bacterial consortium consisting of *Pseudomonas* sp., *Micrococcus* sp., *Bacillus* sp., *Flavobacterium* sp., and *Corynebacterium* sp. showed degradation activity on oil substrates up to 78% compared to single isolates whose degradation activity reached 41-66% (Hamzah *et al.*, 2013).

2.4 Test for Bacterial Resistance to Antibiotics

Antibiotics are metabolites that are produced naturally by organisms (actinomycetes, bacteria, or molds) or synthetically that have the effect of inhibiting and suppressing biochemical processes in bacteria (Fatiqin, 2015; Soleha 2015). The resistance of microorganisms to antibiotics is a specific characteristic of these microorganisms because they can survive and adapt to their environment containing antibiotics (Haryanto *et al*., 2016).

Various types of antibiotics are classified based on the chemical structure of the antibiotic, selective toxicity, and the mechanism of action of the antibiotic. Antibiotics based on their selective toxicity are divided into antibiotics that are bacteriostatic (inhibits bacterial growth) and bactericidal (kill bacteria) (Febiana *et al.*, 2012). Antibiotics based on the mechanism of action of antibiotics are grouped into antibiotics that inhibit bacterial cell wall synthesis, inhibit bacterial protein synthesis, inhibit folate synthesis, change cell membrane permeability, and interfere with nucleic acid synthesis (Febiana *et al*., 2012; Wilarsih, 2019). Types of antibiotics based on the mechanism of action of antibiotics are presented in Figure 2.6. Antibiotics are selective, which means they have components that are specific

to their targets, such as the cell membrane, cell wall, and ribosomes. The inhibitory activity of the antibiotic test can be seen from the formation of a clear zone found in the culture media (Fatiqin, 2015).

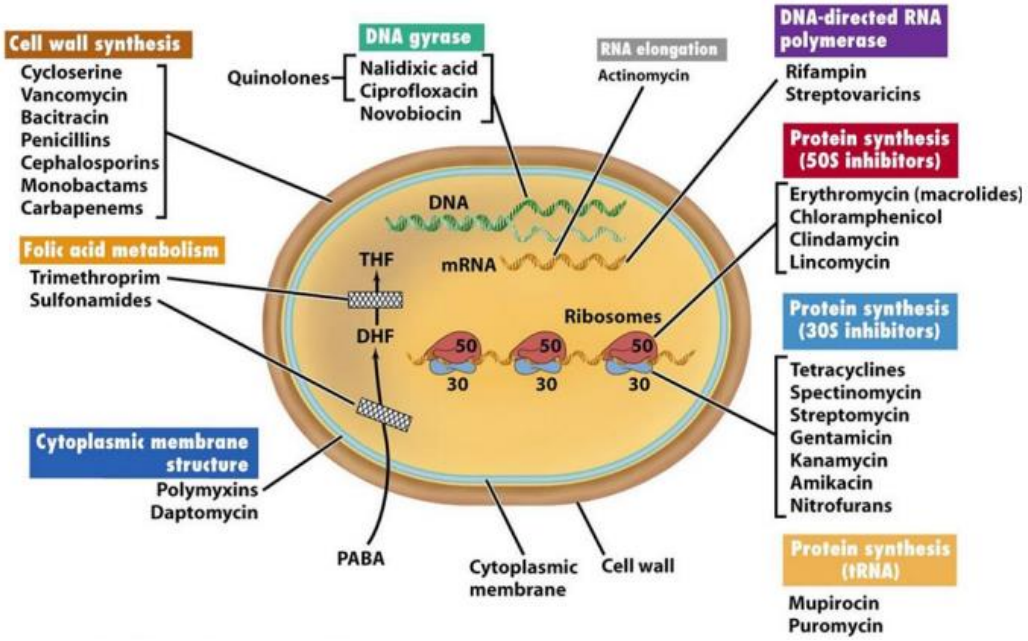


Figure 2.6 Antibiotics based on the mechanism of antibiotic inhibition (Source: Etebu Ebimieowei and Ibemologi Arikekpar, 2016

CHAPTER 3. RESEARCH METHODS

3.1 Research Time and Place

The research was conducted from January to April 2021. The research was conducted at the Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Jember.

3.2 Tools and materials

3.2.1 Tools

The tools used in this study include glassware, non-glass tools, and electric tools. The glassware used were test tubes, Beaker glasses, measuring cups, Schott bottles, Erlenmeyer flasks, Petri dishes, and Bunsen lamps. Non-glass tools used include ose needle, microtube, cork borer, microtip, and micropipette. The electrical equipment used includes an incubator, hot plate, centrifuge (Hermle Z 180 M), vortex (Turbo Mixer), shaker (Thermoline eyebrow), spectrophotometer (UV/VIS Spectrophotometer), autoclave, and laminar airflow.

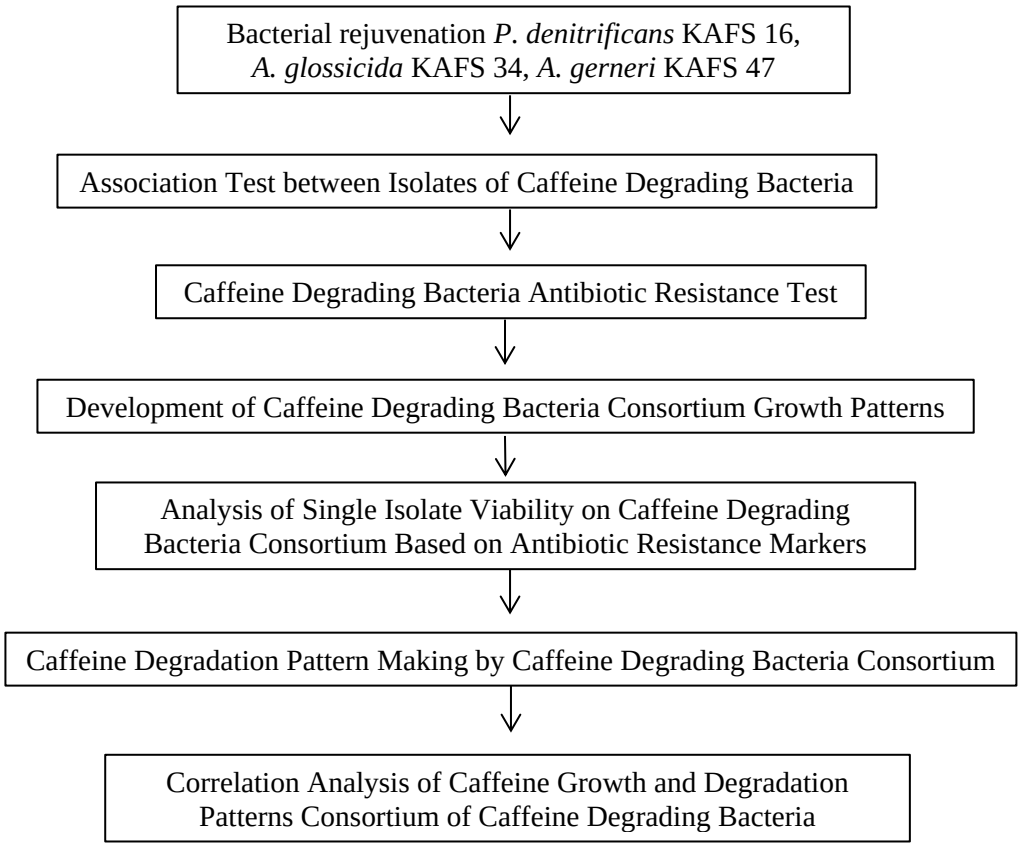
3.2.2 Materials

The bacterial isolates used in this study were *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47. The media used included: media M9 (Appendix 3.1); 0.25% Sigma-Aldrich caffeine; nutrient agar media; nutrient broth medium; aquades. The solutions used include antibiotics (ampicillin, cefadroxil, tetracycline, lincomycin, sanprima, rifampicin, colsancetine, erythromycin, cefixime, ofloxacin, and metronidazole), 70% alcohol, and physiological saline solution (0.85% NaCl) (Appendix 3.2).

3.3 Research Design

This study uses a descriptive research design. This research is used to systematically explain data obtained through research in the laboratory (Payuyasa, 2017; Suardi *et al*., 2019). The study was carried out with the stages of bacterial rejuvenation, association test between bacterial isolates, growth patterns of a consortium of caffeine-degrading bacteria, analysis of the viability of single isolates in a consortium of caffeine-degrading bacteria based on antibiotic resistance

markers, patterns of caffeine degradation by a consortium of caffeine-degrading bacteria, and correlation analysis of bacterial growth patterns with caffeine degradation pattern consortium of caffeine-degrading bacteria (Figure 3.1).



Gambar 3.1 Skema rancangan penelitian

3.4 Research procedure

3.4.1 Caffeine Degradation and Growth Patterns Consortium of Caffeine Degrading Bacteria

a. Caffeine Degrading Bacteria Growth Pattern

1) Bacterial Isolate Rejuvenation

Rejuvenation of bacterial isolates of *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47 was initiated by the culture purification stage. Culture purification was carried out by inoculating each bacterium on M9 + 2.5 g/L caffeine media using the quadrant streak method and incubating for 3-4 days at 30 C. The single colonies that emerged were rejuvenated

on M9 + 2.5 g/L caffeine oblique media and incubated for 3-4 days at 30 C. The rejuvenation of the bacteria that grows is then used for further research.

2) Association Test between Caffeine Degrading Bacterial Isolates

a) Culture Preparation

The association test between bacterial isolates was carried out based on the ability to grow one bacterial isolate on the cell-free supernatant of the other bacterial isolate that the consortium would carry out. This test was carried out with three repetitions. One cycle of bacterial isolate culture on NA media was inoculated on 10 mL of NB media and incubated on a shaker at 125 rpm at room temperature for 24 hours. The inoculum for the association test was obtained from the cell density of each isolate with an OD of 0.4 on measurements using a spectrophotometer with a wavelength of 600 nm (Arimurti, 2018). Culture dilution as a source of inoculum was carried out using sterile distilled water. A total of 100 L of inoculum for each bacterium was inoculated on 15 mL of NA medium using the pour plate method. The use of the pour cup method was carried out to grow the isolates evenly on all media under both aerobic and anaerobic conditions.

b) Preparation of Cell-Free Supernatant

Cell-free supernatants were obtained by filtration using a millipore from the culture of each isolate on NB media. A total of 20 L of cell-free supernatant from other bacteria for the association test was placed in the holes that had been made in the test medium.

c) Association Test between Isolates of Consortium Candidates

The media containing the isolated culture and solidified were perforated using a sterile cork borer with a diameter of 5 mm and as many as 4 holes. Each treatment was repeated three times. The parameters of the association test were observed based on the inhibitory activity of the cell-free supernatant of bacterial isolates on the growth of dispersed bacteria. A positive association test was indicated by the absence of an inhibition zone around the colony which meant that the consortium candidate bacteria did not inhibit the growth of other bacteria. The positive control used NB media and the negative control used ofloxacin (50 ppm) antibiotic solution

(Arimurti, 2018). These results are used as the basis for determining the bacterial consortium that will be carried out for further research (Appendix 4.3).

3) Antibiotic Resistance Test of Caffeine Degrading Bacteria

An antibiotic resistance test by bacteria was carried out to test the growth ability of bacterial isolates on each added antibiotic as confirmation of bacterial growth during the consortium test. Each bacterial isolate was taken one ose and inoculated in 10 mL of NB media then incubated using a shaker at 125 rpm at room temperature for 24 hours. Culture incubation was carried out until the number of cells reached an OD of 0.4 (λ=600 nm) and it was necessary to dilute using NB media if the OD was more than 0.4. Each bacterial isolate was taken 100 L and inoculated on 15 mL of NA medium using the pour plate method. Seven holes were made for each medium using a sterile cork borer (5 mm diameter). Each hole was filled with 20 L of different antibiotics. The antibiotics used were ampicillin (20 ppm, 50 ppm, 75 ppm, 100 ppm); cefadroxil (20 ppm, 50 ppm, 75 ppm, 100 ppm); tetracycline (20 ppm); lincomycin (20 ppm, 50 ppm); sanprima (20 ppm, 50 ppm); rifampicin (20 ppm); colsancetine (20 ppm); erythromycin (20 ppm, 50 ppm); cefixime (50 ppm, 75 ppm, 100 ppm); ofloxacin (50 ppm); and metronidazole (50 ppm). Parameters of antibiotic resistance test based on the sensitivity of the bacteria indicated by the presence or absence of an inhibition zone. Negative control using sterile distilled water.

4) Development of Caffeine Degrading Bacteria Consortium Growth Patterns

a) Development of a Consortium Growth Standard Curve

The standard curve for the consortium growth was initiated by inoculating each bacterial isolate in 50 mL of M9 liquid medium + 2.5 g/L caffeine, then incubating the shaker at a speed of 125 rpm at room temperature. Incubation was carried out until each bacterium reached an OD of 0.4 at a wavelength of 600 nm. 1.67 mL of each culture was taken and then inoculated into 100 mL of M9 liquid medium + 2.5 g/L caffeine so that a consortium of bacteria was obtained. The bacterial consortium was then incubated in a shaker at a speed of 125 rpm at room temperature for 3-4 days. The inoculum was then made in a series of 10-1-10-8 dilutions using 0.85%

sterile NaCl. Cell density was determined by the absorbance value of 3 mL of culture and the calculation of the number of colonies by inoculation of 1 mL of culture in M9 + 2.5 g/L caffeine medium from each dilution series. Measurement of absorbance using a wavelength of 600 nm was carried out directly, while the calculation of colonies was carried out using the drop plate method which was incubated at 30°C for 3-4 days. The range of the number of colonies that can be counted is 3-30 colonies. The number of colonies was then converted in equation (1) so that the number of cells (CFU/mL) was obtained. The absorbance value (OD) and the number of cells (CFU/mL) were then made into a linear regression curve of the relationship between the number of cells (CFU/mL) on the x-axis and the absorbance value on the y-axis (Appendix 3.3.1).

$$\text{Cell count}\left(\frac{\text{CFU}}{\text{mL}}\right)=\Sigma\ \text{Bacterial colony x}\ \frac{1000\ \mu\text{L}}{10\ \mu\text{L}}\ \text{x}\ \frac{1}{\text{dillutin factor}} \qquad (1)$$

b) Single Isolate Starter manufacture

Culture stock of *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47 was inoculated as much as 1 ose in 50 mL of M9 liquid medium + 2.5 g/L caffeine. The culture was incubated on a shaker at 125 rpm at room temperature. Incubation was carried out until the bacterial cells had reached OD 0.4 by measuring the absorbance value using a spectrophotometer at a wavelength of 600 nm.

c) Development of Bacterial Consortium Growth Patterns

A single bacterial isolate starter that had reached an OD of 0.4 at a wavelength of 600 nm was then inoculated into the growth medium, namely 100 mL of M9 liquid medium + 2.5 g/L caffeine. A single bacterial isolate starter was taken 1.67 mL each and inoculated into the growth medium so that a consortium of bacteria was obtained. Bacterial consortium cultures and three single isolates were made three times the growth medium repeated. The four types of culture were incubated with a shaker at room temperature at a speed of 125 rpm during the measurement of the number of cells.

Measurement of the number of cells based on their absorbance at a wavelength of 600 nm was carried out every day (24 hours) for 7 days. Measurement of bacterial cell growth was carried out by converting the standard curve equation to the number of cells (CFU/mL). Bacterial generation time is calculated using equations (2 and 3) to calculate the speed of bacterial division (Septiani, 2016).

$$\text{Generation time (jam)} = \frac{\text{unit of time}}{\text{number of generations}} \quad (2)$$

$$\text{Number of generations} = \frac{\log \text{final cell count} - \log \text{ initial cell count}}{\log 2} \quad (3)$$

5) Analysis of Single Isolate Viability in Caffeine Degrading Bacteria Consortium Based on Antibiotic Resistance Markers

Analysis of the viability of single isolates in a consortium of caffeine-degrading bacteria was carried out by growing the consortium which was being tested for growth and degradation patterns of caffeine aged 1-7 days. This analysis was carried out by growing a consortium of bacteria on media that had been labeled with antibiotics according to the results in step 3.4.3. The bacterial consortium inoculation method used the drop plate method (10 L). Observations were made on the ability to grow colonies on the media.

b. Caffeine Degradation Pattern by Caffeine Degrading Bacteria Consortium

1) Creation of Caffeine Level Standard Curve

The standard curve for caffeine concentration was made with a caffeine concentration of 0.02 mM; 0.04 mM; 0.08 mM; 0.1 mM; 0.12 mM; 0.016 mM. Absorbance measurement using a spectrophotometer at a wavelength of 272.5 nm with distilled water blank. The absorbance value obtained was converted into a linear regression curve of the relationship between the caffeine concentration on the x-axis and the absorbance value on the y-axis (Appendix 3.3.2).

2) Caffeine Degradation Pattern Making by Caffeine Degrading Bacteria Consortium

Caffeine degradation was measured at 24-hour time intervals for 7 days. Extraction of residual caffeine was obtained by centrifuging the bacterial culture every hour interval at 5000 rpm at room temperature for 5 minutes. The supernatant obtained is a residual extract of caffeine. The residual caffeine content was determined based on the measurement of the absorbance value of the sample using a spectrophotometer with a wavelength of 272.5 nm. The caffeine content was determined based on the conversion of the absorbance value of the sample to the standard curve. The absorbance value was then converted into the caffeine standard curve equation. The percentage of caffeine degradation (%) is determined in equation (4).

$$\text{Caffeine degradation}(\%)=\frac{[\,]\text{initial caffeine (mM)}-[\,]\text{final caffeine (mM)}}{[\,]\text{initial caffeine (mM)}}\times 100\% \quad (4)$$

3.4.2 Correlation Analysis of Bacterial Growth Patterns with Caffeine Degradation Patterns

Data analysis was carried out descriptively and statistically. Association test, bacterial resistance test to antibiotics, bacterial consortium growth pattern, and caffeine degradation are presented descriptively in the form of figures and tables. The correlation between the growth and degradation of caffeine was statistically analyzed using the SPSS program (IMB SPSS Statistics 21). The statistical analysis begins with a normality test using Kolmogorov-Smirnov with a significance value of 0.05. The next statistical analysis is correlation analysis to determine the strength of the relationship between one variable and another. Correlation test using Pearson with a significance level of 0.05. This correlation test is carried out if the data is normally distributed in the normality test (Windarto, 2020).

CHAPTER 4. RESULTS AND DISCUSSION

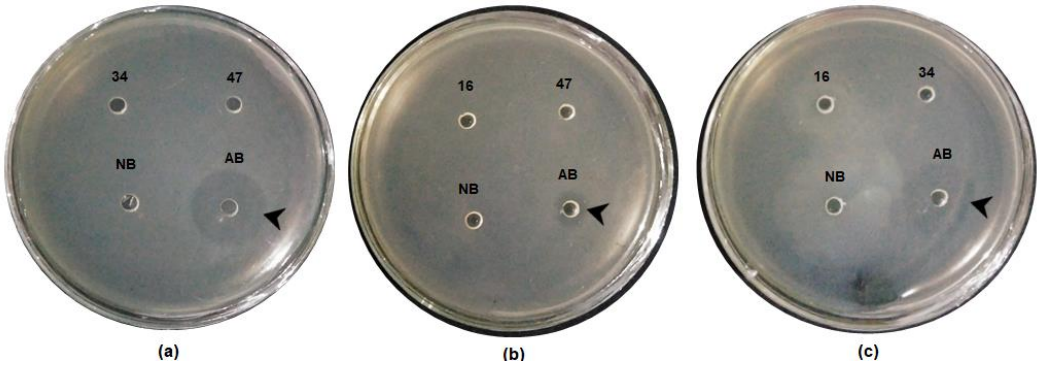
4.1 Association between Caffeine Degrading Bacterial Isolates

The bacteria *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47 were bacteria as a single isolate was able to degrade caffeine. These three bacteria have the potential to be made into a consortium to increase the degradation activity of caffeine in organic waste. The main standard for making a consortium is a positive association between these bacterial isolates.

The bacterial association assay between *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47 was carried out using a cell-free supernatant inhibitory test against other bacteria using NB media as a positive control and the antibiotic ofloxacin (50 ppm) as a negative control. NB medium was used as a positive control and showed no zone of inhibition against the tested bacteria. The cause is NB media is a bacterial culture medium that contains a protein, nitrogen, and mineral salt, so as not to inhibit bacterial growth (Mulyadi *et al.*, 2017). An inhibition zone was formed in the media with the addition of ofloxacin (50 ppm) as a negative control. Ofloxacin is a synthetic antibiotic compound belonging to the quinolone group. An antibiotic type of quinolone has activity a broad spectrum and is bactericidal which works actively on Gram-negative bacteria. Based on their mechanism of action, quinolones include antibiotics that inhibit nucleic acid synthesis by inhibiting DNA gyrase (which plays a role in opening and closing DNA coils) (Triono and Purwoko, 2012). Therefore, ofloxacin can inhibit the growth of bacteria. The inhibition zone or clear area (arrow in Figure 4.1) indicates the sensitivity of the three bacteria to the antibiotic ofloxacin (50 ppm) (Appendix 4.3).

The results of the bacterial association assay between isolates of P. *denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47 showed that the cell-free supernatant of each bacteria did not have inhibition activity against other bacteria, which was indicated by the absence of an inhibition zone (Fig. 4.1). Cell-free supernatants contain metabolites that can inhibit the growth of other bacteria (Afriani *et al.,* 2017). The results of treatment the addition

of a cell-free supernatant in the association test showed that the three isolates were positively associated so that they could potentially be used as candidates for a consortium of caffeine-degrading bacteria. According to Rifai *et al*. (2020), the synergistic relationship in the bacterial consortium shows cooperative behavior between bacteria because bacteria can support each other's growth.



(a) *P. denitrificans* KAFS 16; (b) *P. plecoglossicida* KAFS 34; (c) *A. gerneri* KAFS 47

Figure 4.1 Bacterial association assay between isolates of caffeine-degrading bacteria on the measurement of the inhibition of cell-free supernatants. NB: positive control using NB media; AB: negative control using antibiotics (ofloxacin 50 ppm). Inhibition is indicated by the presence of an inhibition zone (arrows in the picture)

4.2 Resistance of Caffeine Degrading Bacteria to Antibiotics

Antibiotics are compounds that can inhibit the growth of bacteria with different inhibitory mechanisms. The inhibition of bacterial growth was indicated by an inhibition zone around the antibiotic in the media containing the bacterial suspension. According to Tanauma *et al.* (2016), the inhibition zone marked as a clear zone indicates the sensitivity of bacteria to a compound that can affect the growth of these bacteria. The resistance of each bacterium to growth inhibition by antibiotics is specific and can be used as a marker in the manufacture of bacterial consortia.

The results of the antibiotic resistance test showed that *P. denitrificans* KAFS 16 was not resistant to the antibiotic ampicillin (50 ppm, 75 ppm, 100 ppm); cefadroxil (50 ppm, 75 ppm, 100 ppm); tetracycline (20 ppm), rifampicin (20 ppm), and ofloxacin (50 ppm). *P. plecoglossicida* KAFS 34 was not resistant to tetracycline (20 ppm) and ofloxacin (50 ppm), and *A. gerneri* KAFS 47 was not

resistant to the antibiotics cefadroxil (50 ppm, 75 ppm, 100 ppm), tetracycline (20 ppm), colsancetine (20 ppm), and ofloxacin (50 ppm) because their growth can be inhibited. The three bacteria were not resistant to tetracycline and ofloxacin (Table 4.1).

Table 4.1 Resistance of caffeine-degrading bacteria to antibiotics

Types of Antibiotics	**[ ] AB (ppm)**	**Antibiotic Inhibitory**		
		***Paracoccus denitrificans* KAFS 16**	***Pseudomonas plecoglossicida* KAFS 34**	***Acinetobacter gerneri* KAFS 47**
Ampicillin	20	R	R	R
	50	1,03 ± 0,03 mm	R	R
	75	1,54 ± 0,25 mm	R	R
	100	2,06 ± 0,04 mm	R	R
Cefadroxil	20	R	R	R
	50	1,07 ± 0,05 mm	R	1,08 ± 0,07 mm
	75	1,60 ± 0,03 mm	R	1,62 ± 0,03 mm
	100	2,14 ± 0,06 mm	R	2,16 ± 0,02 mm
Tetrasiklin	20	2,13 ± 0,02 mm	0,82 ± 0,01 mm	2,34 ± 0,05 mm
Lincomisin	20	R	R	R
	50	R	R	R
Sanprima	20	R	R	R
	50	R	R	R
Rifampicin	20	0,88 ± 0,02 mm	R	R
Colsancetine	20	R	R	0,95 ± 0,20 mm
Eritromisin	20	R	R	R
	50	R	R	R
Cefixime	50	R	R	R
	75	R	R	R
	100	R	R	R
Ofloxacin	50	2,06 ± 0,03 mm	1,12 ± 0,04 mm	1,89 ±0,05 mm
Metronidazole	50	R	R	R

(R) : resistant to antibiotics

Tetracyclin based on their activity includes antibiotics that inhibit protein synthesis. This antibiotic is bacteriostatic, has a broad spectrum, and has inhibition of both Gram-positive and Gram-negative bacteria (Wasitaningrum, 2009; Wilarsih, 2019). Ofloxacin as in the previous discussion (association between bacterial isolates) inhibits the nucleic acid synthesis and works actively on Gram-negative bacteria (Triono and Purwoko, 2012). This shows that the growth of the

three caffeine-degrading bacteria can be inhibited by antibiotics with the activity of inhibition of bacterial protein and nucleic acid synthesis.

Cefadroxil belongs to the cephalosporin group; has the activity of inhibiting the synthesis of cell walls; is bactericidal; has a broad spectrum and has moderate activity against Gram-negative bacteria and is effective against Gram-positive bacteria (Wasitaningrum, 2009; Wilarsih, 2019). *P. denitrificans* KAFS 16 and *A. gerneri* KAFS 47 are Gram-negative bacteria (Alvarez-Perez *et al.*, 2013; Yoshida *et al*., 2017), so they are not resistant to the antibiotic cefadroxil (50 ppm, 75 ppm, 100 ppm). ppm). Based on this, cefadroxil can be used as a growth marker for *P. plecoglossicida* KAFS 34 because only this bacterium is resistant when given cefadroxil and is indicated to have uninhibited growth.

Ampicillin belongs to the penicillin class of antibiotics with its mechanism of action by inhibiting bacterial cell wall synthesis, has a broad spectrum, is bactericidal, and is active against Gram-positive and Gram-negative bacteria (Yunensa, 2016; Wilarsih, 2019). *P. denitrificans* KAFS 16 was not resistant to the antibiotic ampicillin (50 ppm, 75 ppm, 100 ppm), while the other two bacteria (*P. plecoglossicida* KAFS 34 and *A. gerneri* KAFS 47) were resistant to the antibiotic because they did not show any inhibition. Therefore, ampicillin can be used as a growth marker for *P. plecoglossicida* KAFS 34 and *A. gerneri* KAFS 47 when bacteria consort.

A. gerneri KAFS 47 is not resistant to the antibiotic colsacentine (20 ppm). This antibiotic belongs to the chloramphenicol class of antibiotics. Antibiotics of this class are bacteriostatic (Wasitaningrum, 2009), have a broad spectrum with their mechanism of action inhibiting protein synthesis, and actively inhibiting Gram-positive and Gram-negative bacteria (Wilarsih, 2019). Colsacentine can be used as a growth marker of *P. denitrificans* KAFS 16 and *P. plecoglossicida* KAFS 34 when all three bacteria consort. That was because *P. denitrificans* KAFS 16 and *P. plecoglossicida* KAFS 34 were resistant to the antibiotic colsacentine (20 ppm).

4.3 Caffeine Degrading Bacteria Consortium Growth Pattern

Growing bacteria need nutrients contained in the media. Bacterial growth media must contain a source of carbon, nitrogen, and mineral salts (Mulyadi *et al.*,

2017). Caffeine-degrading bacteria utilize caffeine as a carbon and nitrogen source for bacterial cell growth. The use of caffeine as a carbon and nitrogen source shows the growth pattern of a consortium of bacteria and a single isolate that degrades caffeine which can be seen in Figure 4.2.

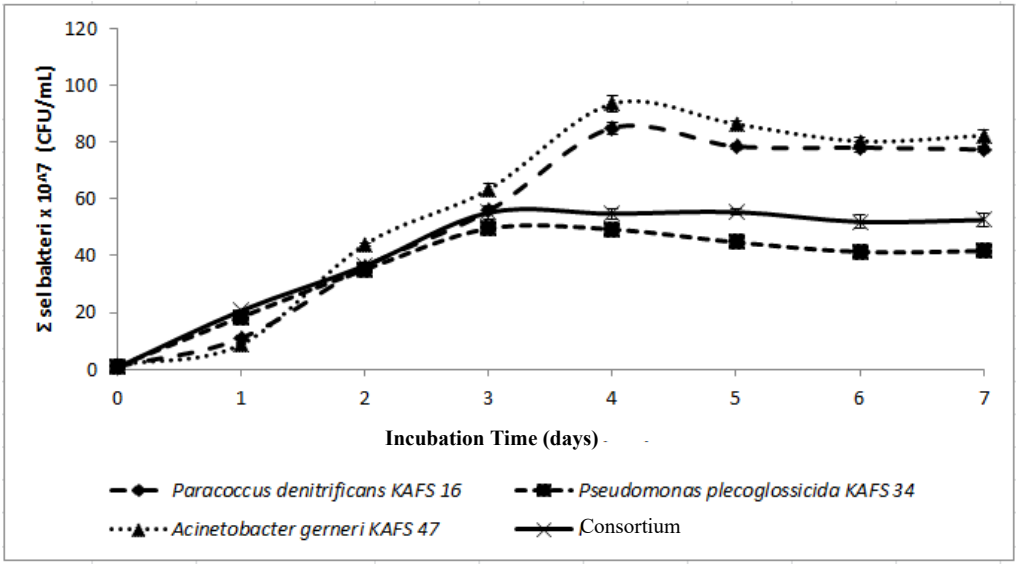


Figure 4.2 Growth pattern of a consortium of bacteria and a single isolate that degrades caffeine

The results showed that *A. gerneri* KAFS 47 (93.481 x 10^7 ± 2,981 CFU/mL) had better growth than *P. denitrificans* KAFS 16 (84.940 x 10^7 ± 2,118 CFU/mL); bacterial consortium (54.779 x 10^7 ± 1.739 CFU/mL); and *P. plecoglossicida* KAFS 34 (49.277 x 10^7 ± 0.733 CFU/mL) on M9 + 2.5 g/L caffeine medium. The growth pattern of the bacterial consortium was almost the same as that of *P. plecoglossicida* KAFS 34, while the growth pattern of *P. denitrificans* KAFS 16 and *A. gerneri* KAFS 47 was almost the same. The growth of the bacterial consortium showed an exponential phase at the beginning of inoculation until day 3 (55,181 x 10^7 ± 2,539 CFU/mL) with a generation time of 40 hours, followed by a stationary phase (Appendix 4.3). *P. denitrificans* KAFS 16 reached its final exponential phase on incubation day 4 (84,940 x 10^7 ± 2,118 CFU/mL) with a generation time of 40 hours. Irsyadah's research (2020) showed the number of *P. denitrificans* KAFS 16 cells in the final exponential phase at an incubation time of 48 hours (7.41 x 10^7 CFU/mL) with a generation time of 5 hours. The exponential phase of *P.*

plecoglossicida KAFS 34 on the 2nd day of incubation (35,141 x 10^7 ± 0.387 CFU/mL) with the fastest generation time of 26 hours. Malasari's study (2020) stated that *P. plecoglossicida* KAFS 34 experienced an exponential phase at 12 to 54 hours (0.79 x 10^8 CFU/mL). *A. gerneri* KAFS 47 experienced an exponential phase starting on day 1 and reaching a peak exponential phase on incubation day 3 (55,181 x 10^7 ± 2,539 CFU/mL) with the longest generation time of 43 hours. The Sindiana study (2020) stated that the final exponential phase of *A. gerneri* KAFS 47 was at the 66th hour incubation time with a cell number of 8.19 x 10^8 CFU/mL with a generation time of 7 hours.

Based on Figure 4.2, it is suspected that *P. plecoglossicida* KAFS 34 plays a more important role in the growth pattern of the bacterial consortium. This is supported by the growth pattern and caffeine degradation pattern in *P. plecoglossicida* KAFS 34 are not much different from the consortium. However, based on the results of the association test between bacterial isolates, showed there was no inhibition, which was carried out based on the inhibitory power of the cell-free supernatant. This cell-free supernatant contains metabolites that can inhibit the growth of other bacteria (Afriani *et al.,* 2017). However, based on the growth pattern when the bacteria were grown as a consortium, it showed that two bacteria (*P. denitrificans* KAFS 16 and *A. gerneri* KAFS 47) had better growth. Competition for nutrients may occur when bacteria are grown as a consortium on the same medium that has the same carbon source. According to Rifai *et al*. (2020) competition between bacteria can occur when grown in the same medium. The competition will compete with each other regarding nutrition, place, air, and water.

The results of the single isolate viability test on a consortium of caffeine-degrading bacteria based on antibiotic resistance markers can be seen in Table 4.2. This test was carried out based on the antibiotic markers of each bacteria. Based on the viability of the media containing the antibiotic, it showed that all the bacterial isolates consorted by each bacteria were still able to grow. It suspected that the growth of a single bacterium was inhibited, but it was still able to grow with a lower number of bacterial consortium cells compared to the single isolate.

Table 4.2 Viability of single isolates in a consortium of bacteria on media containing antibiotic markers

Incubation Time (days)	**Cefadroxil 75 ppm** (*P. plecoglossicida* KAFS 34)	**Ampisilin 75 ppm** (*P. plecoglossicida* KAFS 34 and *A. gerneri* KAFS 47)	**Colsancetine 20 ppm** (*P. denitrificans* KAFS 16 and *P. plecoglossicida* KAFS 34)
0	+	+	+
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	+	+
7	+	+	+

(+) : bacteria grow

4.4 Caffeine Degradation Pattern by Caffeine Degrading Bacteria Consortium

The results of caffeine degradation by caffeine-degrading bacteria carried out as a single isolate or as a consortium are shown in Figure 4.3. and Appendix 4.4. The results showed the growth of the bacterial consortium followed by a decrease in the concentration of caffeine in the media. This shows that the bacterial consortium can utilize caffeine in the media for its growth, which is supported by the number of cells of the bacterial consortium reaching 20,683 x 10^7 CFU/mL ± 0.347 in 1-day incubation. The percentage of caffeine degradation by the bacterial consortium and *P. plecoglossicida* KAFS 34 increased to 100% within 3 days of incubation, an increase in the number of cells of the bacterial consortium (55,181 x 10^7 CFU/mL ± 2,539). The research of Win *et al*. (2019) states that the use of carbon and nitrogen sources by caffeine-degrading bacteria use for bacterial growth can reduce the concentration of caffeine in the media.

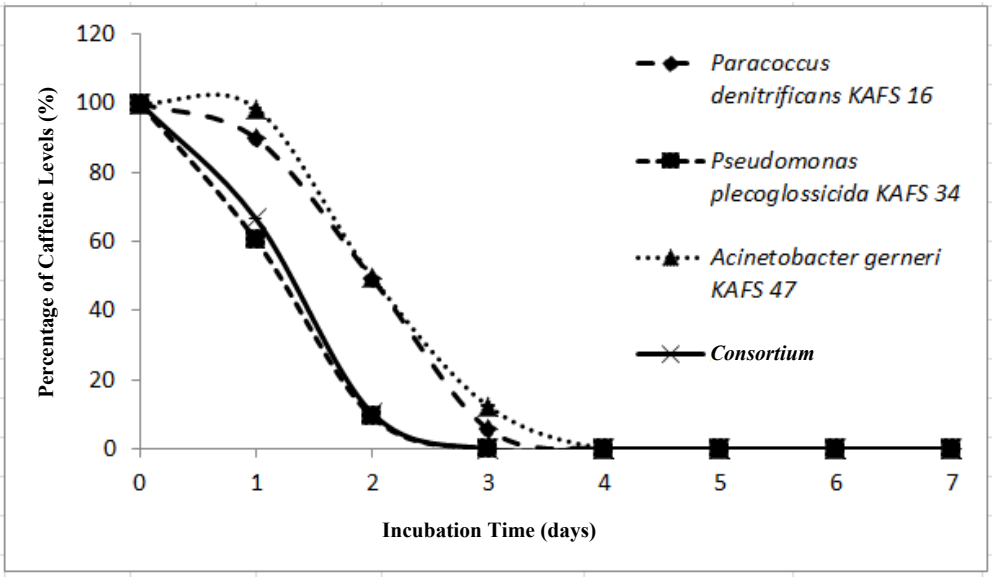


Figure 4.3 Caffeine degradation pattern by a consortium of bacteria and a single isolate of caffeine-degrading bacteria

The pattern of caffeine degradation by a consortium of bacteria and *P. plecoglossicida* KAFS 34 based on Figure 4.3 shows a pattern that is not much different. *P. plecoglossicida* KAFS 34 was able to degrade caffeine up to 100% at an incubation time of 3 days with a cell count of 49.598 x 10^7 ± 0.487 CFU/mL. According to Malasari's research (2020), *P. plecoglossicida* KAFS 34 can degrade caffeine up to 98.15% within 3 days of incubation. *P. denitrificans* KAFS 16 (84,940 x 10^7 ± 2,118 CFU/mL) and *A. gerneri* KAFS 47 (93.481 x 10^7 ± 2,981 CFU/mL) had a caffeine degradation pattern that was almost the same with an incubation time of 4 days able to degrade caffeine 100%. According to Irsyadah (2020), the degradation of caffeine by *P. denitrificans* KAFS 16 reached 100% at an incubation time of 2 days. Sindiana's research (2020) reported that *A. gerneri* KAFS 47 was able to degrade 100% of the caffeine in an incubation time of 2.5 days.

The growth of the bacterial consortium (54.779 x 10^7 ± 1.739 CFU/mL) and *P. plecoglossicida* KAFS 34 (49.277 x 10^7 ± 0.733 CFU/mL) at an incubation time of 4 days was lower than that of *P. denitrificans* KAFS 16 (84.940 x 10^7 ± 2,118 CFU) /mL) and *A. gerneri* KAFS 47 (93.481 x 10^7 ± 2.981 CFU/mL). However, the degradation activity of caffeine by a consortium of bacteria and *P.*

plecoglossicida KAFS 34 was higher, which is 24 hours faster than that of *P. denitrificans* KAFS 16 and *A. gerneri* KAFS 47. Based on these results, the pattern of growth and degradation of caffeine by a consortium of bacteria and *P. plecoglossicida* KAFS 34 is almost the same. Therefore, it is necessary to consider the use of a consortium of bacteria as a caffeine degrader because it has a caffeine degradation potential that is not much different from a single isolate (*P. plecoglossicida* KAFS 34).

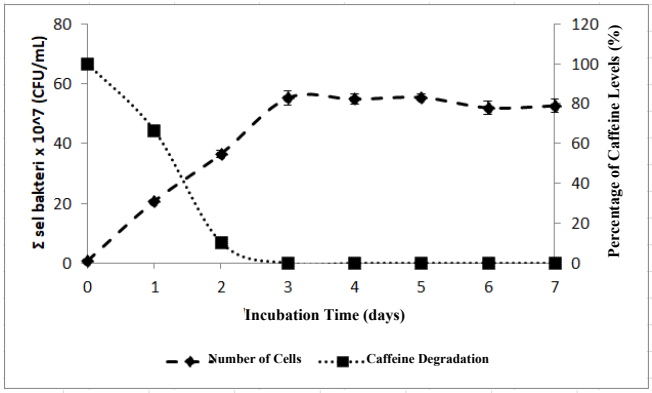
4.5 Correlation of Growth Patterns and Caffeine Degradation Patterns Caffeine Degrading Bacteria

The data were normally distributed based on the results of the Kolmogorov-Smirnov normality test (> 0.05). This is indicated by a significance value of 0.566 (*P. denitrificans* KAFS16); 0,960 (*P. plecoglossicida* KAFS 34; 0,998 (*A. gerneri* KAFS 47) and 0,481 (bacteria consortium). The Pearson correlation test was conducted to determine the relationship between bacterial growth and caffeine degradation in media.

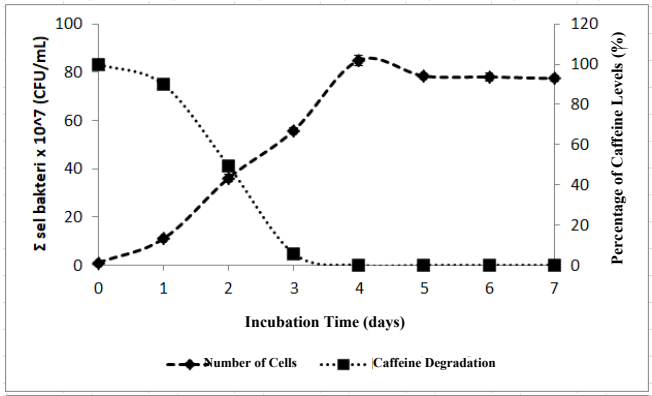
The significance value for *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, *A. gerneri* KAFS 47, and the bacterial consortium was 0.000 (p>0.05). That means there is a significant correlation between the number of bacterial cells and the percentage of caffeine degradation (Figure 4.4). The correlation value of the bacterial consortium (Appendix 4.6), *P. denitrificans* KAFS 16 (Appendix 4.7), *P. plecoglossicida* KAFS 34 (Appendix 4.8), and *A. gerneri* KAFS 47 (Appendix 4.9) was >0.950. According to Rahmatih *et al.* (2020), the correlation criterion value of 0.81-1.00 indicates a perfect correlation.

The results of the Pearson correlation test showed a negative correlation between the bacterial consortium (Appendix 4.6), *P. denitrificans* KAFS 16 (Appendix 4.7), *P. plecoglossicida* KAFS 34 (Appendix 4.8), and *A. gerneri* KAFS 47 (Appendix 4.9). This is indicated by a negative sign on the Pearson correlation test. The negative correlation showed that with the increasing growth of bacteria, the concentration of caffeine in the media decreased. That shows that bacterial growth utilizes media containing caffeine so that bacteria can degrade 100% of caffeine (Subagyo *et al.,* 2015).

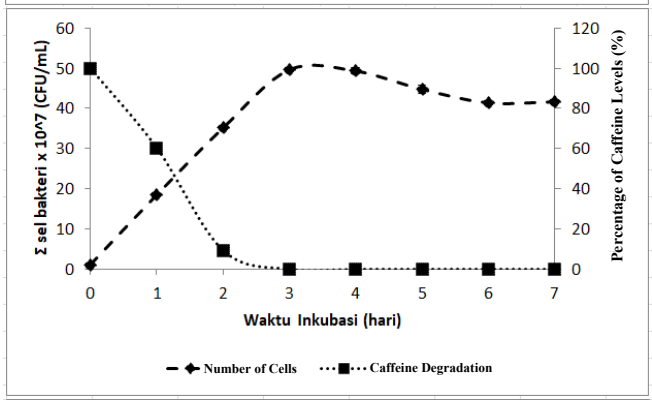
(a)



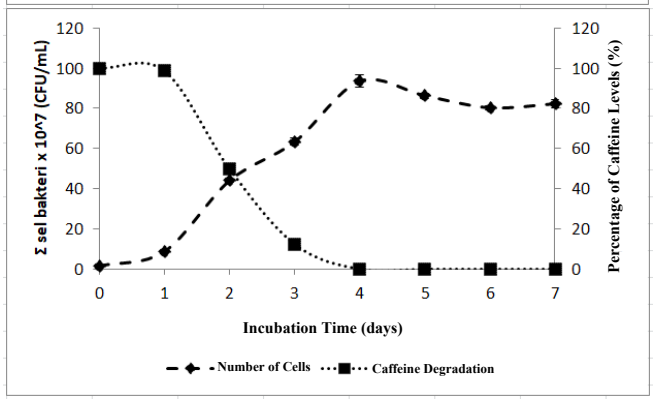
(b)



(c)



(d)



(a) bacterial consortium; (b) *P. denitrificans* KAFS 16;)c) *P. plecoglossicida* KAFS 34; (d) *A. gerneri* KAFS 47

Figure 4.4 The relationship between the number of bacterial cells and the percentage of caffeine content

CHAPTER 5. CONCLUSION AND SUGGESTION

5.1 Conclusion

The conclusion of this study is

1. The bacteria *P. denitrificans* KAFS 16, *P. plecoglossicida* KAFS 34, and *A. gerneri* KAFS 47 have positive associations, so they can be used as a consortium of caffeine-degrading bacteria. The growth of the bacterial consortium had a lower cell count than *P. denitrificans* KAFS 16 and *A. gerneri* KAFS 47, but higher than *P. plecoglossicida* KAFS 34 in the exponential phase on caffeine media. The three isolates showed growth in the consortium based on the viability test of resistance to antibiotics. The bacterial consortium had a caffeine degradation ability of 100% and was similar to *P. plecoglossicida* KAFS 34, namely and higher than *P. denitrificans* KAFS 16 and *A. gerneri* KAFS 47.
2. The correlation between bacterial growth and caffeine degradation in the bacterial consortium showed a perfect correlation value (>0.950) and was negative.

5.2 Suggestion

1. Based on the research conducted, other markers that are specific for every single isolate are needed.
2. Analysis of caffeine degradation was carried out using organic waste media containing caffeine.
3. Analysis of caffeine degradation was carried out using the LCMS method.

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APPENDIX

Appendix 3.1 Material Composition

Table 3.1 Material composition

No.	Medium	Materials	Composition
1.	M9	$Na_2HPO_4.7H2O$	6 gram
		KH_2PO_4	3 gram
		NaCl	0,5 gram
		$MgSO_4$	0,25 gram
		NH_4Cl	1 gram
		Aquades	1.000 mL
2.	Medium 2.5 g/L caffeine (liquid)	M9	1.000 mL
		Caffeine	2,5 gram
3.	Medium 2.5 g/L caffeine (solid)	M9	1.000 mL
		Caffeine	2,5 gram
		Bacto agar	15 gram
4.	Nutrient Agar Medium	*Nutrient Broth*	13 gram
		Bacto agar	15 gram
		Aquades	1.000 mL

Appendix 3.2 Solution Composition

Table 3.2 Solution Composition

Solution	Materials	Composition	Description
Physiological salt	NaCl	2,55 gram	0,85%
	Aquades	1.000 mL	

Appendix 3.3 Standard Curve

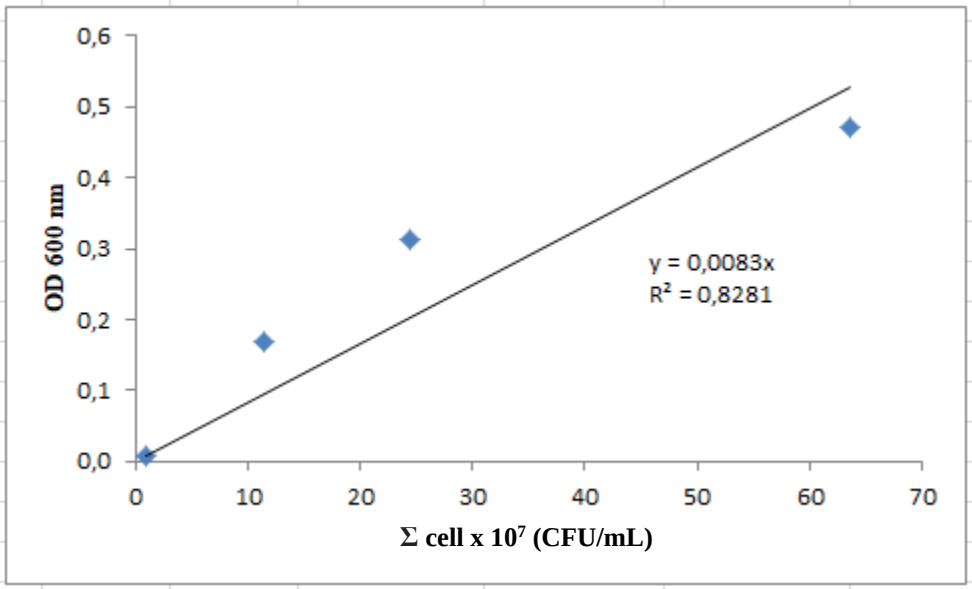

Figure 3.3.1 Caffeine-degrading bacteria consortium growth standard curve

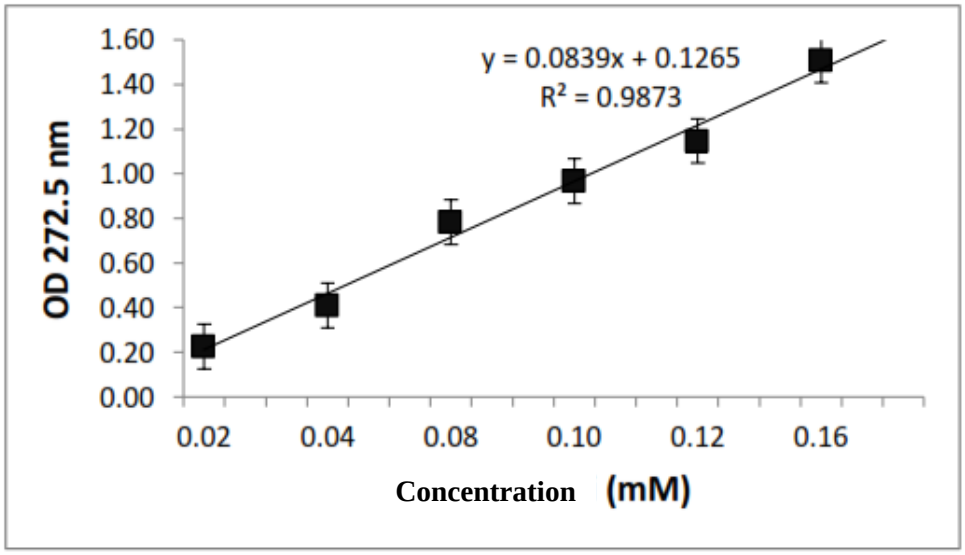

Figure 3.3.2 Caffeine standard curve

Appendix 4.3 Association Test between Bacterial Isolates

Table 4. 3 Association Test between Caffeine Degrading Bacterial Isolates

Isolation Code	Cell-Free Supernatant			Control + (Media NB)	Control - (Ofloxacin 50 ppm)
	***Paracoccus denitrificans* KAFS 16**	***Pseudomonas plecoglossicida* KAFS 34**	***Acinetobacter gerneri* KAFS 47**		
KAFS 16		-	-	-	2,14 mm ± 0,13
KAFS 34	-		-	-	1,02 mm ± 0,02
KAFS 47	-	-		-	2,17 mm ± 0,03

(blue block) : no addition of the same isolate cell-free supernatant
(-) : does not indicate any inhibition zone

Appendix 4.4 Caffeine Degrading Bacteria Growth

Table 4. 4 Caffeine-degrading bacteria growth

Incubation Time (days)	Σ Sel x 10^7 (CFU/mL)			
	***Paracoccus denitrificans* KAFS 16**	***Pseudomonas plecoglossicida* KAFS 34**	***Acinetobacter gerneri* KAFS 47**	**Consortium**
0	1,044 ± 0,070	1,124 ± 0,278	1,593 ± 0,273	0,776 ± 0,046
1	11,004 ± 0,368	18,474 ± 0,387	8,835 ± 0,290	20,683 ± 0,357
2	36,145 ± 1,446	35,141 ± 0,387	44,003 ± 0,615	36,506 ± 1,109
3	55,823 ± 1,025	49,598 ± 0,487	63,333 ± 1,829	55,181 ± 2,539
4	84,940 ± 2,118	49,277 ± 0,733	93,481 ± 2,981	54,779 ± 1,739
5	78,434 ± 0,835	44,739 ± 0,936	86,332 ± 1,314	55,382 ± 1,025
6	78,072 ± 1,783	41,365 ± 0,387	80,295 ± 1,075	51,928 ± 2,312
7	77,390 ± 1,145	41,647 ± 0,618	82,289 ± 2,192	52,597 ± 2,245

Appendix 4.5 Caffeine Degradation by Caffeine Degrading Bacteria

Table 4. 5 Caffeine degradation by caffeine-degrading bacteria on caffeine medium M9 + 2.5 g/L caffein

Incubation Time (days)	Caffeine Degradation Percentage (%)			
	***Paracoccus denitrificans* KAFS 16**	***Pseudomonas plecoglossicida* KAFS 34**	***Acinetobacter gerneri* KAFS 47**	**Consortium**
0	100,0 ± 0,067	100,0 ± 0,093	100,0 ± 0,126	100,0 ± 0,068
1	89,8 ± 0,121	60,4 ± 0,024	98,6 ± 0,022	66,6 ± 0,141
2	49,4 ± 0,121	9,4 ± 0,094	49,8 ± 0,056	10,4 ± 0,044
3	5,8 ± 0,062	0,0 ± 0,035	12,4 ± 0,130	0,0 ± 0,045

4	0,0 ± 0,099	0,0 ± 0,008	0,0 ± 0,158	0,0 ± 0,064
5	0,0 ± 0,014	0,0 ± 0,004	0,0 ± 0,020	0,0 ± 0,010
6	0,0 ± 0,036	0,0 ± 0,004	0,0 ± 0,030	0,0 ± 0,044
7	0,0 ± 0,056	0,0 ± 0,032	0,0 ± 0,019	0,0 ± 0,022

Appendix 4.6 Normality and Pearson Correlation Test Bacterial Cell Number and Caffeine Degradation in a bacterial consortium

Table 4.6.1 Normality test (One-Sample Kolmogorov-Smirnov Test) caffeine-degrading bacteria consortium

One-Sample Kolmogorov-Smirnov Test

		Unstandardized Residual
N		8
Normal Parameters[a,b]	Mean	,0000000
	Std. Deviation	8,55701400
Most Extreme Differences	Absolute	,297
	Positive	,221
	Negative	-,297
Kolmogorov-Smirnov Z		,840
Asymp. Sig. (2-tailed)		,481

a. Test distribution is Normal.

b. Calculated from data.

Table 4.6.2 Correlation test (Pearson) cell number and percentage of caffeine degradation consortium of caffeine-degrading bacteria on M9 + 2.5 g/L caffeine media

Correlations

		Jumlah Sel Konsorsium	Degradasi Kafein Konsorsium
Jumlah Sel Konsorsium	Pearson Correlation	1	-,976**
	Sig. (2-tailed)		,000
	N	8	8
Degradasi Kafein Konsorsium	Pearson Correlation	-,976**	1
	Sig. (2-tailed)	,000	
	N	8	8

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix 4.7 Normality and Pearson Correlation Test Bacterial Cell Number and Caffeine Degradation in a *P. denitrificans* KAFS 16

Table 4.7.1 Normality test (One-Sample Kolmogorov-Smirnov Test) *P. denitrificans* KAFS 16

One-Sample Kolmogorov-Smirnov Test

		Unstandardized Residual
N		8
Normal Parameters[a,b]	Mean	,0000000
	Std. Deviation	9,30737353
Most Extreme Differences	Absolute	,278
	Positive	,141
	Negative	-,278
Kolmogorov-Smirnov Z		,786
Asymp. Sig. (2-tailed)		,566

a. Test distribution is Normal.

b. Calculated from data.

Table 4.7.2 Correlation test (Pearson) cell number and percentage of caffeine degradation *P. denitrificans* KAFS 16 on M9 + 2.5 g/L caffeine media

Correlations

		Jumlah Sel KAFS 16	Degradasi Kafein KAFS 16
Jumlah Sel KAFS 16	Pearson Correlation	1	-,976**
	Sig. (2-tailed)		,000
	N	8	8
Degradasi Kafein KAFS 16	Pearson Correlation	-,976**	1
	Sig. (2-tailed)	,000	
	N	8	8

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix 4.8 Normality and Pearson Correlation Test Bacterial Cell Number and Caffeine Degradation in a *P. plecoglossicida* KAFS 34

Table 4.8.1 Normality test (One-Sample Kolmogorov-Smirnov Test) *P. plecoglossicida* KAFS 34

One-Sample Kolmogorov-Smirnov Test

		Unstandardized Residual
N		8
Normal Parameters[a,b]	Mean	,0000000
	Std. Deviation	8,23988667
Most Extreme Differences	Absolute	,179
	Positive	,179
	Negative	-,129
Kolmogorov-Smirnov Z		,506
Asymp. Sig. (2-tailed)		,960

a. Test distribution is Normal.

b. Calculated from data.

Table 4.8.2 Correlation test (Pearson) cell number and percentage of caffeine degradation *P. plecoglossicida* KAFS 34 on M9 media + 2.5 g/L caffeine

Correlations

		Jumlah Sel KAFS 34	Degradasi Kafein KAFS 34
Jumlah Sel KAFS 34	Pearson Correlation	1	-,976**
	Sig. (2-tailed)		,000
	N	8	8
Degradasi Kafein KAFS 34	Pearson Correlation	-,976**	1
	Sig. (2-tailed)	,000	
	N	8	8

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix 4.9 Normality and Pearson Correlation Test Bacterial Cell Number and Caffeine Degradation in an *A. gerneri* KAFS 47

Table 4.9.1 Normality test (One-Sample Kolmogorov-Smirnov Test) *A. gerneri* KAFS 47

One-Sample Kolmogorov-Smirnov Test

		Unstandardized Residual
N		8
Normal Parameters[a,b]	Mean	,0000000
	Std. Deviation	7,35598418
Most Extreme Differences	Absolute	,137
	Positive	,106
	Negative	-,137
Kolmogorov-Smirnov Z		,388
Asymp. Sig. (2-tailed)		,998

a. Test distribution is Normal.

b. Calculated from data.

Table 4.9.2 Correlation test (Pearson) cell number and percentage of caffeine degradation of *A. gerneri* KAFS 47 on M9 + 2.5 g/L caffeine media

Correlations

		Jumlah Sel KAFS 47	Degradasi Kafein KAFS 47
Jumlah Sel KAFS 47	Pearson Correlation	1	-,986**
	Sig. (2-tailed)		,000
	N	8	8
Degradasi Kafein KAFS 47	Pearson Correlation	-,986**	1
	Sig. (2-tailed)	,000	
	N	8	8

**. Correlation is significant at the 0.01 level (2-tailed).

Screening of Associated Bacteria from the Midgut of Main Dengue Mosquito's Vector, *Aedes aegypti*

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Abstract

The female *Ae. aegypti* mosquito is the primary vector of the dengue virus that causes Dengue Hemorrhagic Fever (DHF). The success of the transmission process is influenced by the ability of the dengue virus to infect several mosquito organs, one of which is the midgut organ. The midgut organ serves as the site for the incubation phase for viral replication. The incubation phase of the dengue virus can be delayed or even fail due to the bacteria associated with the midgut organ. Therefore, this research aimed to determine the bacteria associated with the midgut organ of *Ae. aegypti*. The object of this research is the morphological characteristics and 16S rDNA sequences of bacteria. The research is divided into two main stages, namely in silico analysis and experimental works. The experimental works included sampling and morphological identification of mosquito, and midgut isolation and identification of association bacteria based on morphological and molecular approach. Based on the results, in silico analysis is relevant with experimental results. Five bacterial isolates have been isolated, MG1 and MG2 had high similarity of their 16S rDNA sequences with *Delftia lacustris* and *Pseudomonas aeruginosa*, respectively. From morphological characteristics of colonies, isolates MG3 and MG5 were possibly belongs to bacteria from the genus *Serratia.*

Keywords: associated bacteria, mosquito's midgut, dengue vector, and 16S rDNA.

Introduction

Dengue Hemorrhagic Fever (DHF) is one of the infectious diseases caused by dengue virus infection (DENV), which often occurs every year in tropical and subtropical regions. Dengue virus transmission mediated by its primary vector, *Ae. aegypti* female [1]. The success of dengue virus transmission to the human body influenced by the ability of the dengue virus to infect several mosquito organs that play an essential role in the transmission cycle, one of them is midgut. The midgut organ functions as a site for the incubation phase for self-propagation or replication of the dengue virus [2]. The transmission cycle can be hampered or even fail due to the association bacteria that live in the midgut organ [3].

Association bacteria can activate several molecular defense pathways in the midgut if there is an infection with a pathogen such as the dengue virus [2]. In addition, association bacteria in

the midgut organ can secrete several secondary metabolites that are virucidal. Association bacteria can also accelerate the death of mosquitoes by degrading the Peritrophic Matrix (PM) layer or increasing uncontrolled Reactive Oxygen Species (ROS) [4].

Based on the role of association bacteria for inhibit dengue virus infection, many studies were carried out to characterize and analyze the associated bacteria species in the midgut of *Ae. aegypti*. Bacterial characterization can be conducted using a molecular approach because it has high accuracy in determining the genus or species of bacteria [5]. The DNA sequence often used for bacterial characterization is the 16S rDNA sequence because it is universal, conservative, and has an identification accuracy of 95% at the genus level and 97% at the species level [6]. Analysis of the bacteria associated species with the midgut of *Ae. aegypti* can be conducted by in silico analysis to identify the mosquito-associated microbial community taxa through Operational Taxonomic Units (OTU) clustering [7].

Results

In silico analysis

In silico analysis was used to predict the common order of bacteria from database which have been found on mosquito's midgut before, especially *Ae. aegypti.* The results are used as a reference when the results from wet laboratory have been obtained. In silico analysis result from the 100 sequences that we have been collected, show that the most abundance order from both of the groups are Enterobacterales which has 37% abundance from both cultured and uncultured groups. The other order that also present are Pseudomonadales, Burkhoideriales, Aeromonadales, Flavobacteriales, and Bacillales that has different percentage in each group. From the cultured groups, Bacillales, Psudomonadales, Flavobacteriales, and Burkhoideriales has 23%, 11%, 9%, and 5% respectively. From uncultured groups, Pseudomonadales, Burkhoideriales, and Aeromonadales has 30%, 16% and 4% respectively. Detail of this krona result percentage can be seen in **Figure 1**. This result should indicate the expectation of what bacteria groups possbily could be found in the midgut of *Ae. aegypti* with laboratory experimental work.

Samples collection and morphological identification of mosquito

From samples collection, the 137 female and 109 male *Ae. aegypti* mosquitoes have been collected. Based on the morphological characteristics, *Ae. aegypti* mosquitoes have separated mesepimeron in lateral thorax, white submedian-longitudinal and lyre-shape markings in mesonotum, and white longitudinal line in anterior midfemur [8]. The mosquitoes that we used are female, so it can be distinguished by the antenna, palps, and proboscis. Antenna's female *Ae. aegypti* has shorter hair than male *Ae. aegypti.* The female *Ae. aegypti* also has palps that shorter than proboscis, while male *Ae. aegypti* has the same length [8]. The results of morphological characteristic and sexing of *Ae. aegypti* observation can be seen in **Figure 2**.

Isolation of bacterial associated with midgut organ

The midgut organ consists of two parts, there are anterior midgut and posterior midgut. The two parts have different function, the anterior midgut is for nectar digestion that conducted by male and female mosquito, while the posterior midgut is for blood meal digestion that conducted by only female mosquito [9]. The virus can be ingested in blood meal and interacts

first with the posterior midgut, which the midgut can represent a barrier to successful virus infection and transmission [10].

In this research, the posterior midgut organ isolated from mosquitoes after blood feeding for 24 hours. The posterior midgut after blood feeding 24 hours contained blood bolus that caused the size is swollen and change the color to dark red-drown [11]. We collected about 10 posterior midguts that already formed into blood bolus. Posterior midgut isolated after blood feeding 24 hours because several associated bacteria will proliferate up to 24 times faster after the blood meal [4]. The posterior midgut after blood feeding 24 hours can be seen in **Figure 3**.

Bacterial morphological identification

The results showed that the colonies produced from the spread plate process contains serial dilutions suspension of 10 midguts after incubated for 24h at 37°C were very large, so only five colonies were taken because it had different characteristics. The colonies named MG1, MG2, MG3, MG4, and MG5. The colonies were identified macroscopically by observing the single colonies and microscopically by Gram staining that can be seen in **Table 1** and **Figure 4**. Based on the colony morphology, the five bacteria had different colony appearances, especially in their color, while on the results of Gram staining, all bacteria were Gram negative bacteria with bacillus or coccobacillary cell shapes.

Bacterial molecular identification

First, genomic DNA was isolated by freeze and thaw method which had heat shock treatment to separate the genome from the cells. The results of visualization of the bacterial genome showed that the length of the bacterial genome DNA of isolates MG1 and MG2 has above 10.000 bp. In addition, amplification of 16S rDNA sequences and purification of PCR products have same sequences length about 1500 bp. All of the DNA visualization results can be seen in **Figure 5**.

The amplified sequence is then carried out in the sequencing stage and followed by bioinformatics analysis. Based on bioinformatics analysis, from BLAST result, the MG1 isolate has a high similarity with *D. lacustris,* and *D. tsuruhatensis* with 100.00% of percent identity. Furthermore, the MG2 isolate has a high similarity with *P. aeruginosa* with 99.93% of percent identity that can be seen more clearly in **Table 2**. The results of further bioinformatics analysis, namely the relationship of bacterial species based on the reconstruction of the phylogenetic tree in **Figure 6** showed that the isolate MG1 had a close relationship with *D. lacustris* and *D. tsuruhatensis* with bootstrap value 93%, while the isolate MG2 had a close relationship with *P. aeruginosa* with bootstrap value 100%.

Discussion

Therefore, it could be concluded that the use of self-designed primers showed reproducible results. This study focused on observing the associated bacterial species living in the midgut *Ae. aegypti* female by comparing the results of in silico analysis and molecular identification using 16S rDNA sequences. Based on the results of the in silico analysis shown in **Figure 1**, Enterobacterales is the most abundant order from database based on the result earlier. We hypothesize that this order being the most probably abundant because Enterobacterales is a

bacteria order that can be found in many environments, such as animal gut [12], including mosquito. Based on metagenomic result from various publications also found genera belonging to Enterobacterales such as *Enterobacter, Serratia, Erwinia* [13,14,15]. This result should also support the expectation of what bacteria groups possbily could be found in the midgut of *Ae. aegypti* with laboratory experimental work along with in silico analysis result. Some of its species play roles in mosquito midgut such as *Serratia*, *Escherichia* and *Shigella* genera.

Escherichia have roles in *Ae. aegypti* larvae development [16], *Escherichia* and *Shigella* can inhibit dengue virus infection by reducing virus dissemination [17]. Some species of *Serratia* genus also have direct roles in mosquito immune system such as *S. marcescens and S. odorifera. S. marcescens* is known capable to reduce mosquito lifespan by producing SmEnhancin, a secondary metabolite protein which can break mucin bound on Peritrophic matrix layer of mosquito resulting in DENV susceptibility to mosquito [4], whereas *S. odorifera* can increase DENV-2 susceptibility due to the prohibitin molecules blockade by the polypeptide of S. *odorifera* [18].

Based on molecular identification, the MG1 isolate has a high similarity with *D. lacustris,* and *D. tsuruhatensis* with 100.00% of percent identity. Furthermore, the MG2 isolate has a high similarity with *P. aeruginosa* with 99.93% of percent identity that can be seen in **Table 2**. These results are valid because sample sequences can be categorized into one genus or species using the GenBank database if the percent identity value is close to 100% [19]. Specifically for isolate MG1, there is ambiguity about the alignment results because it refers to two different species. *D. lacustris,* and *D. tsuruhatensis* indeed have high similarity with value 99,9% based on 16S rDNA sequence. The two bacteria divided into different species based on the activity test of D-mannitol and D-malic acid which can only be utilized by *D. lacustris* as a carbon source [20].

Delftia genus is a Gram-negative aerobic bacteria included Burkholderiales order. These bacteria have also been found in the midgut organ of *Ae. aegypti* after blood feeding at 2019 [15]. While *Pseudomonas* genus is Gram-negative aerobic facultative bacteria included Pseudomonadales order. The discovery of *P. aeruginosa* in the midgut of *Ae. aegypti* is not the first time, this bacteria was also found in the midgut organ of *Ae. aegypti* in 2013 [21]. These results are relevant with the in-silico analysis, the orders Burkholderiales and Pseudomonadales are one of the orders of bacteria associated with the midgut organ of the *Ae. aegypti* mosquito with a percentage of 5% and 11% based on the result of cultured bacteria pie chart in **Figure 1**.

Based on the results of the phylogenetic tree reconstruction (**Figure 6**), it showed that isolate MG1 had a close relationship with *D. lacustris* and *D. tsuruhatensis*, while isolate MG2 had a close relationship with *P. aeruginosa*. This is evidenced by the position and short branch formed in the phylogenetic tree. A sequence that has a high relationship value will form short and close branches [22]. Further analysis, the formed branch can be trusted for the validity of the data due to the high value of the bootstrap formed. Formed branch in isolates MG1 with *D. lacustris* and *D. tsuruhatensis* had a bootstrap value of 93%, while isolates MG2 with *P. aeruginosa* had a bootstrap value of 100%. The minimum bootstrap value that can be trusted for its validity on a phylogenetic tree is >70% [23].

The results of a literature review about the function of bacteria for inhibit the dengue virus infection in mosquitoes show that *P. aeruginosa* is symbiont bacteria in the midgut organ that can shorten the mosquito's lifespan, about 72 hours if infected the female wild types of *Ae. aegypti* [24]. This species can produce several metabolites that can inhibit the pathogenic infection include prodigiosin, cytotoxic metalloproteases, hemolysins, antibiotics, and haemagglutinins. Prodigiosin is a secondary metabolite which has the ability to antimicrobial, immunosuppressive, and antimalarial [25]. *P. aeruginosa* also stimulated the formation of AMPs and melanozation by secreting cytotoxic metalloproteases [26]. In addition, the hemolysins secreted can inhibit the infection from *Plasmodium* because hemolysins can lyse the red blood cells [27]. While *D. lacustris* has never had any research discussing its function against dengue virus infection in mosquitoes.

In this research, the experimental results of bacterial screening from the midgut of *Ae. aegypti* were relevant to in silico analysis. From five bacterial isolates, MG1 and MG2 had high similarity of their 16S rDNA sequences with *D. lacustris* and *P. aeruginosa*, respectively. From morphological characteristics of colonies, isolates MG3 and MG5 were possibly belongs to bacteria from the genus *Serratia.*

Material and Methods

General Laboratory Equipment

1. Computer
2. Tray
3. Autoclave
4. Freezer -20°C and 4°C
5. Mosquito aspirator
6. Laminar Air Flow
7. Stereo microscope
8. Compound microscope
9. Sterile glass object
10. Pipette
11. Micropipette
12. Sterile petridish
13. Sterile microtube
14. Micropistil
15. Vortex
16. Hot plate
17. Centrifuge
18. Glass reaction
19. Glass beaker
20. PCR machine
21. Electrophoresis machine

22. Sterile cutter
23. UV-Transilluminator

Material

1. Fish concentrate
2. Sucrose
3. Wistar rats
4. Ethanol 75%
5. Sterile PBS pH 7.4
6. Sterile 0,5% NaCl
7. Nutrient Agar medium
8. Nutrient Broth medium
9. Ketoconazole
10. Gram staining step (crystal violet, iodine, ethyl alcohol, safranin)
11. Sterile ddH_2O
12. 16S-rDNA marker
13. Agarose
14. TAE buffer
15. PCR master mix solution
16. DNA template
17. Wizard SV Gel and PCR Clean-Up System kit (Promega, USA)

In silico analysis

In silico analysis is carried out to make important data about the possibilities of what bacterial taxa in the midgut of *Ae. aegypti*. The sequences collected from two groups, cultured and uncultured bacteria groups, 100 sequences each from the NCBI database which are submitted as bacteria of *Ae. aegypti* midgut related publication title (https://www.ncbi.nlm.nih.gov/). The sequences collected were selected using by sequence length filter to acquire the longest 100 sequences approximately to 1500 base pairs [28]. Visualization was carried out using the PATRIC bioinformatics website (Pathosystems Resource Integration Center), from (https://patricbrc.org/). Taxonomic classification was used to obtain the results of in silico analysis are in the form of a pie chart, Krona. Krona can be used to visualize the results of the microbial composition of metagenomic analysis [29]. Krona contains the composition of bacterial taxa based on sequence data inputted from the database with the percentage of the number of bacterial taxa present in the midgut of Ae. aegypti based on NCBI database.

Sampling and morphological identification of mosquito

The sample taken is *Ae. aegypti,* which is still in the larval stage from densely populated settlements around the University of Jember, Sumbersari District, Jember Regency. Larvae of *Ae. aegypti* were collected from water containers around residential areas such as buckets, jugs, and baths for rearing to become adult mosquitoes. Larvae from the landing collection were placed on a tray filled with water and given adequate feed in the form of fish concentrate. The larvae that have turned into pupae are then transferred to the pupae cup in the mosquito cage to become imago. The imago was fed with 10% sucrose and Wistar rats for the blood-feeding

needs of female imago.

Morphological identification of *Ae. aegypti* was carried out using a stereo microscope based on its morphological characteristics. The part that can be used as an identification key for *Ae. aegypti*, namely the dorsal part of the head, mesepimeron, and anterior midfemur. These characteristics are based on a journal entitled Pictorial Keys for the Identification of Mosquitoes (Diptera: Culicidae) associated with Dengue Virus Transmission [8].

Midgut isolation and bacterial culture

Ten mosquitoes female *Ae. aegypti* 24 hours after performing blood-feeding soaked in sterile 75% ethanol solution for 10 seconds. The midgut isolation process was carried out using a sterile stereo microscope. Mosquitoes isolated from the midgut were first soaked with PBS solution (pH 7.4) 2 times. The next step is to add sterile 0.5% NaCl to the glass object every time the midgut organ is isolated. The part of the midgut organ that will be isolated is the posterior midgut. The posterior part of the midgut can be seen in **Figure 7**.

The isolated posterior midgut was then inserted into a microtube containing 100µl of sterile PBS for further grinding using a micropistil and homogenized by vortex process. The homogeneous suspension was then diluted using the serial dilution method at 900µl PBS 5 times. The suspension resulting from the 3rd, 4th, and 5th serial dilutions was then taken 100 µl to be inoculated on NA medium by the spreading method and incubated at 37°C for 48 hours. Ketoconazole has been added to NA medium as an antifungal. To obtain single colonies, the grown bacteria were purified on NA medium by streak quadrant technique and incubated at 37°C for 48 hours.

Morphological identification of bacteria

Morphology identification of the midgut symbiont of *Ae. aegypti* will be conducted by observing their colony morphology macroscopically and bacterial morphology microscopically. Colony shape, colony color, colony edge, and colony elevation surface will be identified in this study. The reference book in the identification of macroscopic morphology is Bergey's Manual of Determinative Bacteriology 9ed. Bacterial cell morphological observations will be carried out by observing cell shape and Gram staining of bacteria.

Molecular identification of bacteria

Genome isolation

Molecular identification started with the genome isolation used is the freeze and thaw method. Bacterial isolates aged ± 24 hours, resulting from incubation at a temperature of ± 37°C in NB medium, were taken 1000µl and transferred to a microtube for further centrifugation at 10,000 rpm at 4°C for 5 minutes. The centrifuged pellet was then taken and then washed with 1000µl of PBS solution (pH 7.4). The washed pellets were then centrifuged again at 10,000 rpm and 4°C for 5 minutes. The pellets obtained from the previous stage were then incubated at -20°C for 24 hours. The genomic DNA extraction phase begins with boiling the results of the previous incubation for 4 minutes. Next, the sample was dissolved in 50µl of sterile distilled water and resuspended by micropipetting and vortexing. The suspension was then centrifuged at a speed of 10,000 rpm and a temperature of 4°C for 5 minutes. The centrifuged supernatant was then visualized using an electric current on 1% agarose gel with 500 mA and a voltage of 100 volts for 40 minutes.

16S-rDNA sequence amplification

The amplification stage was carried out by mixing 25µl of 2x PCR master solution, 15µl of sterile ddH_2O, 2µl of DNA template, 1.25µl of forward and reverse primers on a PCR machine with conditions as shown in **Table 2**. The amplification of the 16S-rRNA coding gene sequence was carried out by using primers 16S-rDNA forward 27F (5' AGA GTT TGA TCM TGG CTG AG 3'), and reverse 1492 R (5' GGT TAC CTT ACG AT T 3') [30]. This pair of primers can produce PCR products with a length of ± 1500 bp [31]. The amplified 16S-rDNA sequence was then visualized using an electric current on 1.5% agarose gel with an electric current of 500 mA and 100 volts for 40 minutes.

Purification of PCR results

The amplified 16S-rDNA sequence was purified by cutting the agarose gel containing DNA bands using a sterile cutter on top of a UV-transilluminator. The cut agarose gel was then purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega, USA).

Sequencing and bioinformatics analysis

The sequencing stage was carried out at 1st BASE using the Sanger method. The data from the sequences were then edited with the chromatogram using the BioEdit application to make a consensus. The edited consensus is then aligned with the existing database in GenBank using the BLAST feature. The next step is to take 3 sequences from GenBank, which are highly similar to the sample sequences. After the BLAST stage, analysis was carried out using the ClustalX2 application to determine the nucleotide differences between sequences by aligning the sample sequences with 3 sequences from GenBank. Finally, reconstruct the phylogenetic tree using the neighbor-joining method with 1000 bootstrap and calculate the similarity of the 16S-rDNA sequences of bacterial isolates with the GenBank database using pairwise distance MEGAX application.

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Conflict of Interest

The authors declare no competing interests.

Figure Legends

Figure 1: In silico analysis result. (A) Krona of cultured group. (B) Krona of uncultured group.

Figure 2: Morphological characteristic and sexing of *Ae. aegypti*. (A) Mesepimeron of Ae. aegypti have a V sign that separated in lateral thorax. (B) Mesonotum have white submedian-longitudinal (sl) and lyre-shape markings (lm). (C) Anterior midfemur have white longitudinal line (gl). (D) Female mosquito has thin hair in the antenna (an) and the palps (pl) shorter than proboscis (pr). (E) Male mosquito has thick hair in the antenna (an) and the palps (pl) have same length with proboscis (pr).

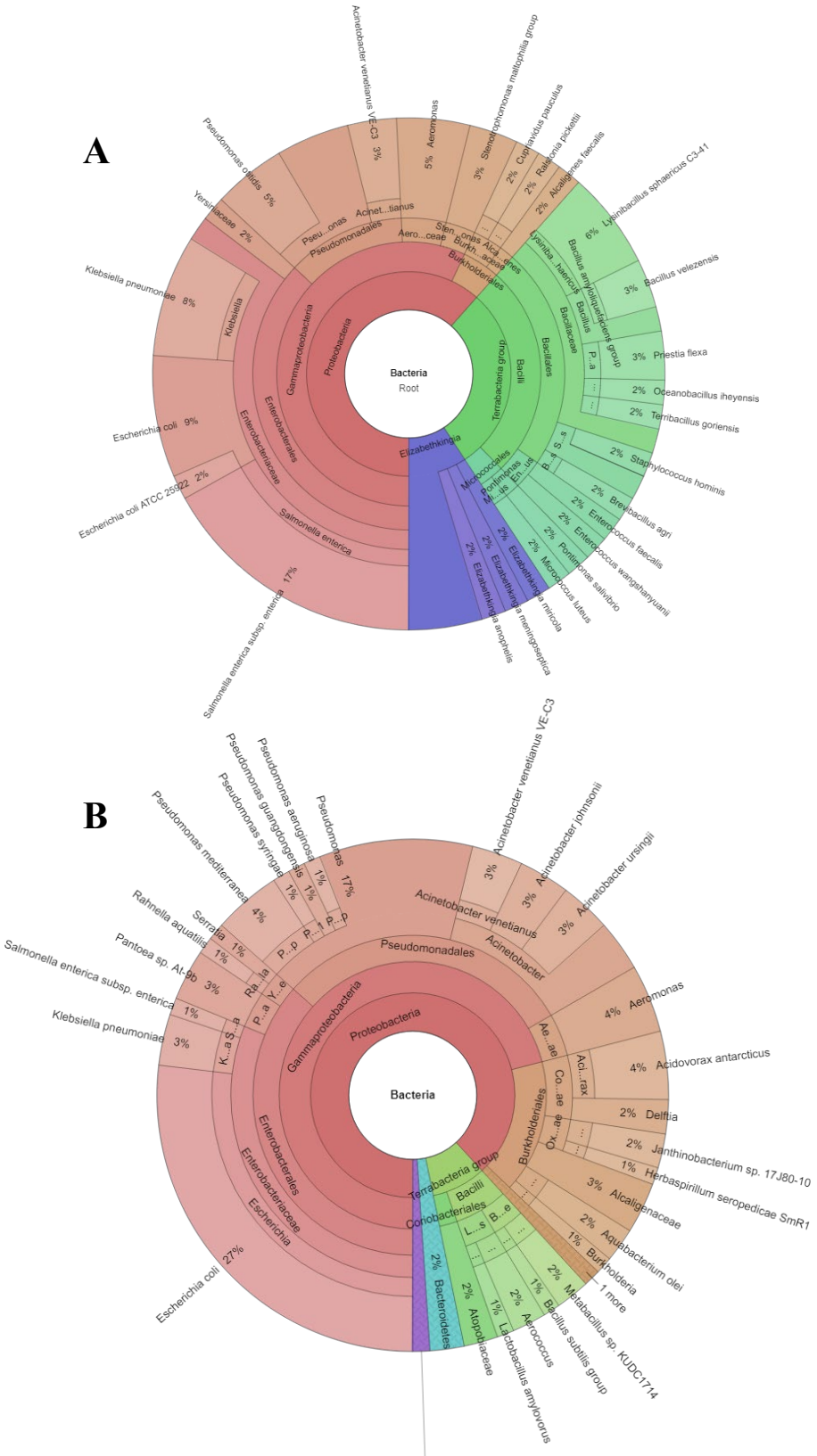
Figure 3: Midgut organ after 24h blood feeding. Description: anterior midgut (amg); posterior midgut (pmg); ileum (il); Malpighian tubules (mt); rectum (rc).

Figure 4: Bacterial morphological identification

Figure 5: DNA visualization. (A) Genome extraction. (B) Amplification of 16S rDNA sequences. (C) Purification of PCR products.

Figure 6: The result of phylogenetic tree reconstruction of MG1 and MG2 isolates

Figures



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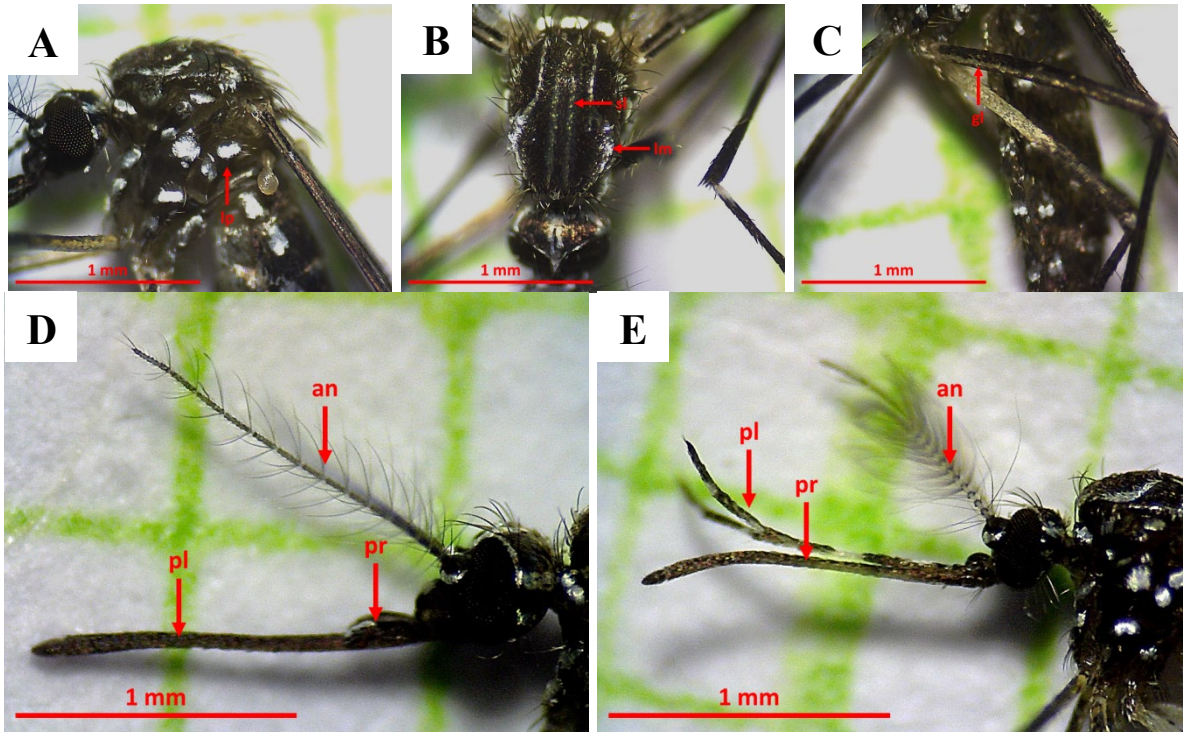

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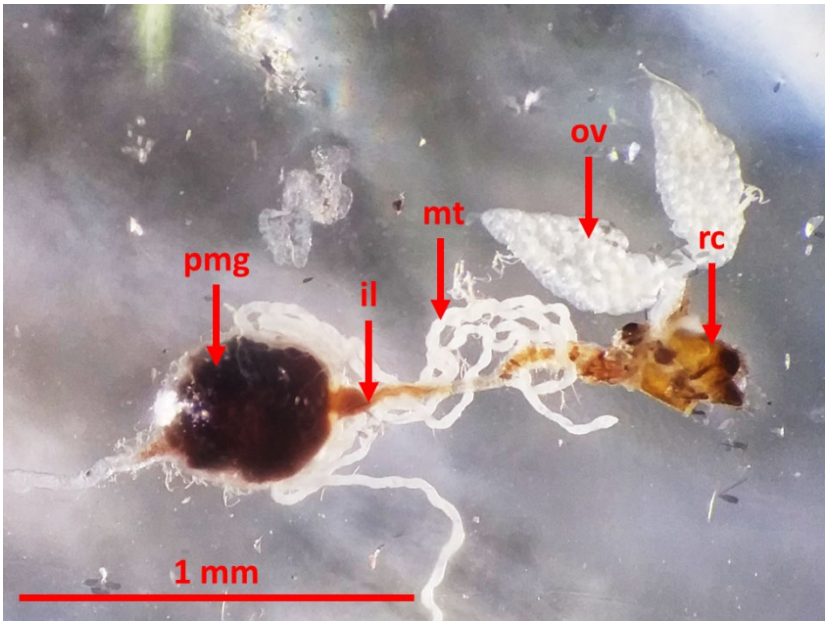

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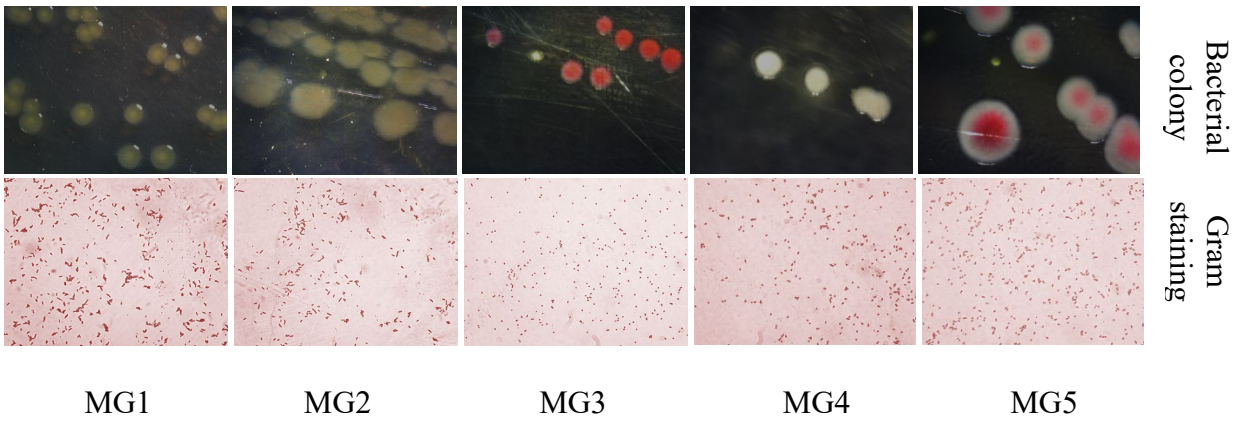

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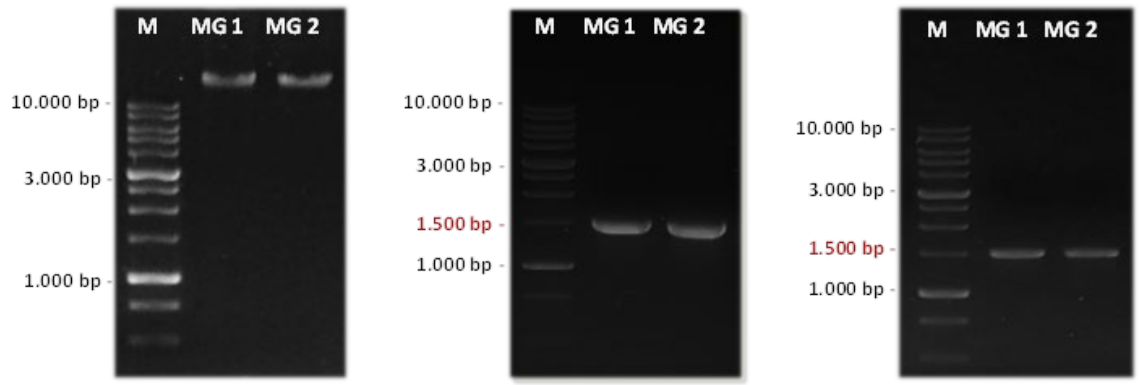

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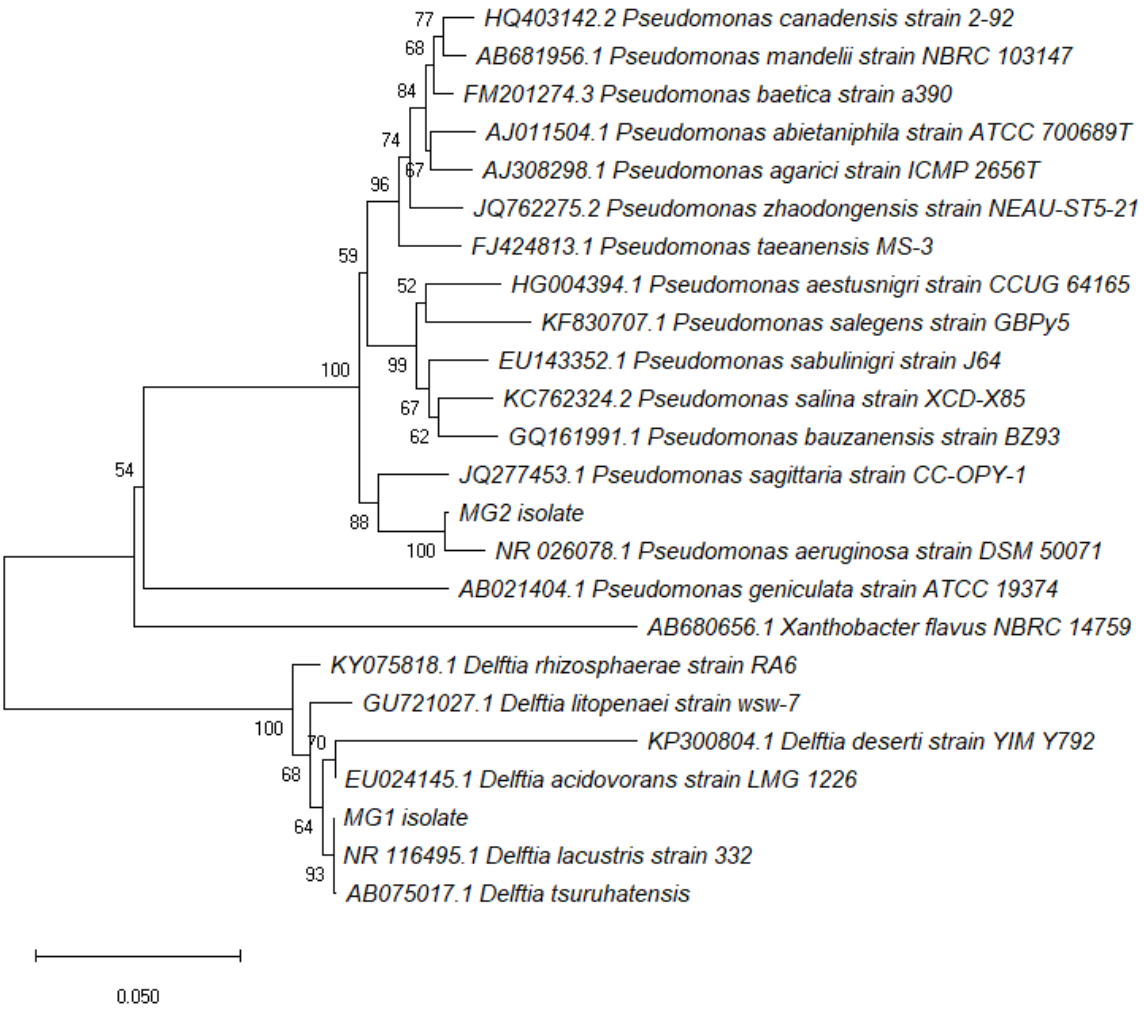

Figure 6. The result of phylogenetic tree reconstruction of MG1 and MG2 isolates

Table legends

Tables

Table 1. Bacterial morphological identification

characteristics	**Isolates**				
	MG1	MG2	MG3	MG4	MG5
Form	Circular	Circular	Circular	Circular	Circular
Margin	Entire	Filamentous	Entire	Entire	Entire
Color	Yellow	Yellow	Red	White	Combination red and white
Elevation	Convex	Raised	Convex	Convex	Convex
Gram type	Negative	Negative	Negative	Negative	Negative
Cell shape	Bacilli	Bacilli	Bacilli	Cocobacilli	Cocobacilli

Table 2. BLAST result of MG1 and MG2 isolates

Isolat	**Data *GenBank***						
	Scientific Name	***Max Score***	***Total Score***	***Query Cover***	***E Value***	***Per. Ident***	***Accession***
A1	*Delftia lacustris strain* NG12	2584	2584	100%	0.0	100.00%	MT266922.1
	Delftia tsuruhatensis strain OsEnb_HZB_E2	2584	2584	100%	0.0	100.00%	MN889373.1
	Delftia sp. *strain* MS2As2	2584	2584	100%	0.0	100.00%	MT226500.1
A2	*Pseudomonas aeruginosa strain* D3	2595	2595	100%	0.0	99.93%	JN995664.1
	Pseudomonas aeruginosa strain SRDchr3	2595	2595	100%	0.0	99.93%	EU714901.1
	Pseudomonas aeruginosa strain 172A	2593	2593	99%	0.0	99.93%	KF254528.1

Table 3: Condition of PCR machine during amplification

Step	Temperature (°C)	Time	Description
Initial denaturation	94	5 minutes	-
Denaturation	94	1 minute	
Annealing	56	1 minute	9 cycles
Extention	72	1 minute	
Denaturation	94	1 minute	
Annealing	53	1 minute	24 cycles
Extention	72	1 minute	
Final extention	72	10 minutes	-