Doctoral Dissertation

Biological Control for Suppressing Human Diseases: a Case Study of *Bacillus thuringiensis* Isolated Indigenously from East Java as a Natural Enemy against *Aedes aegypti*

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We hereby recommend that the dissertation by Ms. ZULFAIDAH PENATA GAMA entitled " Biological Control for Suppressing Human Diseases: a Case Study of *Bacillus thuringiensis* Isolated Indigenously from East Java as a Natural Enemy against *Aedes aegypti* " be accepted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY .

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Appendix A: Abbreviations and acronyms

ABATE	:	O,O,O′,O′-tetramethyl O,O′-thiodi-p-phenylene phosphorothioate
Aedes aegypti L.	:	*Aedes aegypti* Linnaeus
ANOVA	:	Analysis of Variance
ArcGIS	:	Geographic Information System
B2P2VRP	:	Balai Besar Penelitian dan Penembangan Vektor dan Reservoir Penyakit / Central Council of Vector and Disease Reservoir Research and Development
BI	:	Breteau Index
BPS	:	Badan Pusat Statistik (Central Statistics Agency)
Bt	:	*Bacillus thuringiensis*
Bti	:	*Bacillus thuringiensis* var. *israelensis*
CI	:	Container Index
CLAD	:	Cladogram is constructed by calculating nearest distance between each taxon (OTU) and creating one HTU in every bifurcation
cm	:	Centi meter
Cry	:	Crystals
Cyt	:	Cytolysins
DHF	:	Dengue Hemorrhagic Fever
DNA	:	Deoxyribonucleic Acid
EIP	:	Extrinsic Incubation Period
GPI-anchored protein	:	glycosylphosphatidylinositol-anchored proteins
GPS	:	Global Positioning System
Ha	:	a hectare
HI	:	House Index
Hs	:	Hours
HSD	:	Honesty Significance Different
HSP	:	Heat Shock Protein
IPM	:	Integrated Pest Management
IR	:	Incidence Rate
ITB	:	Institute Technology Bandung
IVI	:	Important Value Index
Jumantik	:	Juru Pemantau Jentik
kDa	:	Kilo Dalton
KEGG	:	Kyoto Encyclopedia of Genes and Genomes

LC	:	Lethal Concentration
ml	:	Mili liter
mm	:	Mili meter
MR	:	Methil Red
NA	:	Nutrient Agar
NB	:	Nutrient Broth
nm	:	Nanometer
NOD	:	The nucleotide-binding oligomerization domain receptors
OTU	:	Operational Taxonomic Units
P2PL	:	Pengendalian Penyakit dan Penyehatan Lingkungan
PCR	:	Polymerase Chain Reaction
PFT	:	Pore-forming-toxins
rpm	:	Rotation per minutes
SPSS	:	Statistical Product and Service Solutions
SSM	:	Schaeffer's sporulation medium
TB	:	Total of cell density
TS	:	Total of spores density
UPGMA	:	Unweighted pair group method using arithmetic averages
UV	:	Ultra Violet
VP	:	The Voges-Proskauer test
WHO	:	World Health Organization

Abstract

Biological Control for Suppressing Human Diseases: a Case Study of *Bacillus thuringiensis* Isolated Indigenously from East Java as a Natural Enemy against *Aedes aegypti*

This study aims to investigate safe strategy to control *Aedes aegypti* mosquito using the most potential *Bacillus thuringiensis* isolated indigenous from East Java as a natural enemy, to understand the current condition of biological control in East Java and to formulate some conclusions, which can be taken for future consideration by the local government.

East Java, as one of the provinces in Indonesia, has been growing very fast where the human population and the city are increasing very significantly compared to other regions in Indonesia. East Java is one of the very dense populated areas; unfortunately it still has various health problems. Actually, it is still difficult to be resolved, especially related to dengue hemorrhagic fever (DHF). The case number of DHF is rising every year. The Indonesian government has been reduced the number of DHF patients with various methods. Among others, by sprinkling mosquito larvicides (ABATE) in some containers to kill mosquito larvae at the surface of water, by fogging, and conducted the volunteer person to monitor the number of mosquito larvae / *Jumantik (Juru Pemantau Jentik)*. Unfortunately, all is not yet effective way to control mosquito. Based on this historical background, this study was therefore conducted.

Chapter 1 discusses the general introduction and literature review. Chapter 2 concern on association between climate variability, DHF incidence, and distribution of *Aedes aegypti* as a vector of DHF in East Java. Chapter 3 is the main chapter, which focuses

deeply on indigenous natural enemy selection to control *Aedes aegypti* larvae. Chapter 4 focuses on formulating the safe strategy to control *Aedes aegypti* using the most potential indigenously *Bacillus thuringiensis* isolated from East Java.

The first section of Chapter 2 determines the spatial distribution of *Aedes aegypti* in East Java from 2008 to 2010 and to characterize the temporal patterns of *Aedes aegypti* in the tenth regions of East Java for 3 years (2008, 2009, 2010) and its association with local meteorological variables. For spatial distribution, the studies used the average of DHF incidence rate (IR) data in nine districts in East Java over a monthly period (January - December) from 2008 to 2010. The results of statistical analysis showed there was a significant relationship between the average IR and the number of *Aedes aegypti*. And the mosquitoes were captured by using a bait indoor has the most closely related to the average IR cause *Aedes aegypti* is an *anthropophilic* mosquito, taking its blood meals preferentially from humans. *Aedes aegypti* was more often found in indoor than outdoor. The highest CI and BI were recorded in 36.11 and 70.59 % respectively in Nganjuk District. The highest *Aedes aegypti* house index was 51% in Bangkalan District. In these districts, there are many good habitats for *Aedes aegypti* larvae. All locations of East Java have a higher House Index (HI) than the WHO standard for the high DHF risk area (i.e.10 % HI). The peak season to sucking blood of *Aedes aegypti* is occurring in the morning and in the evening.

The second section of chapter 2 discusses association between climatic factors (maximum and minimum temperature, rainfall, humidity, light duration, wind velocity) associated with DHF incidence in Nganjuk District, East Java Province, Indonesia from 2005 to 2010. The results of this study indicated the climatic variability is clearly associated with the dengue incidence rate (IR). The maximum air temperature, humidity, rainfall and

light duration have played an important role in the transmission of DHF in Nganjuk District. The Spearman correlation analyses showed that in humidity and rainfall have positive correlation with DHF incidence; on the contrary a decreased value of maximum air temperature and light duration would have an impact on increased IR. The result of regression analysis indicated that IR of DHF was affected by the maximum air temperature, minimum air temperature, and rainfall in the rainy season; however, in the dry season, the IR was affected by wind velocity and rainfall.

The third section of chapter 2 investigates the observation which was undertaken to determine the distribution pattern of *Aedes aegypti* and risk factors of DHF between regency and cities in Mojokerto 2012 and the correlation between the elevation of sampling location and distribution of mosquito. Mosquitoes are one of the insects that have an important role as vectors of disease agents. The diseases transmitted by mosquitoes are still public health problems in Indonesia, especially in East Java Province, for example Dengue Hemorrhagic Fever (DHF). DHF is a very alarming disease because the occurrence of this epidemic is no longer just confined to certain geographic locations. At present, Mojokerto is a district in the East Java region known as endemic for DHF in Indonesia. Number of cases in this district tend to rise and expand distributed. The spread pattern of dengue cases in this district is not certainly known. Sampling was conducted in two locations in the Mojokerto district, namely Prajurit Kulon for urban and Dlanggu for rural. We used survey for mosquito larvae by WHO standard and for mosquito eggs by ovitrap. The coordinates of sampling locations recorded using GPS and then identification of mosquitoes is performed at the Laboratory of Ecology and Animal Diversity, University of Brawijaya. Quantitative data were analyzed to determine the abundance, relative abundance, frequency, relative

frequency and IVI (Importance Value Index). Mosquito distribution patterns were analyzed with Morisita index. The research findings indicated that there are five mosquito species consist of *Aedes aegypti*, *Aedes albopictus*, *Aedes laniger*, *Culex bitaeniorchynchus* and *Culex quinquefasciatus*. *Aedes aegypti* is the dominant species in urban areas while *Culex quinquefasciatus* is the dominant species in rural area. Morisita index showed that mosquito dispersal on patterns in Mojokerto district is uniform. The elevation of the area and the density of *Aedes aegypti* has a positive correlation.

The first section of chapter 3 focuses on maintaining the sustainability of the local bacteria which have effectively controlled population of mosquito larvae; it is necessary to observe the toxicity tests for local bacteria from other places around East Java. *B. thuringiensis* is an important insect pathogen that is highly toxic to mosquito larvae and related dipterans. The original *B. thuringiensis* exploration efforts in Indonesia were carried out because the *B. thuringiensis* crystal protein has an arrow host spectrum. Therefore, the ideal effort for killing Indonesian mosquitoes would be using *B. thuringiensis* isolated from Indonesia. This research investigated the toxicity of indigenous *Bacillus thuringiensis* isolates from East Java for controlling *Aedes aegypti* larvae. The result of the study disclosed that *B. thuringiensis* Brht isolates from Surabaya district has the highest percentage of *Aedes aegypti larvae* mortality at 24 hours ($LC_{50\text{-}24h}$ = 1.215x10^8 cells/ml). It also described that these isolate is more effective than the reference *B. thuringiensis* (*B. thuringiensis* var. israelensis HD 567).

The second section of chapter 3 investigates the toxicity of indigenous *Bacillus thuringiensis* isolates from Malang City for controlling *Aedes aegypti* larvae. Soil samples were taken from Purwantoro and Sawojajar sub districts, and bacterial isolation was

performed using *B. thuringiensis* selective media. Phenotypic characteristics of the isolates were obtained with the simple matching method. The growth and prevalence of spores were determined by the Total Plate Count method, and toxicity tests were also performed on the *Aedes aegypti* third instar larval stage. The percentage of larval mortality was analyzed using probit regression. The LC_{50} was analyzed by ANOVA, and the Tukey HSD interval was 95%. Six isolates were obtained among 33 selected bacterial isolates (PWR4-31, PWR4-32, SWJ4-2b, SWJ4-4b, SWJ-4k and SWJ5-1) that have a similar phenotype to reference *B. thuringiensis*. Based on the dendrogram, all of the bacterial isolates were 71% similar. The three isolates that had a higher prevalence of reference *B. thuringiensis* were PWR4-32, SWJ4-4b and SW5-1, of which there was a 52.44%, 23.59%, 34.46% spore prevalence, respectively. These three indigenous isolates from Malang City (PWR4-32, SWJ4-4b, SWJ5-1) successfully killed *Aedes aegypti* larvae. The PWR4-32 isolates were the most effective at killing the larvae. The study concluded that the six indigenous *B. thuringiensis* isolates (PWR4-31, PWR4-32, SWJ4-2b, SWJ4-4b, SWJ4-4k and SWJ5-1) among the 33 bacterial isolates that were found in the Sawojajar and Purwantoro sub districts were toxic to third instar *Aedes aegypti* larvae. The PWR4-32 isolates were identical to the reference *B. thuringiensis* and had 88% phenotype similarity. The PWR4-32 isolates had the highest spore prevalence (52.44%), and the early stationary phase occurred at 36 hs. The PWR4-32 isolates were the most effective at killing *Aedes aegypti* larvae ($LC_{50\text{-}72\ hs}$=2.3x10^8 cells/ml).

The third section of chapter 3 evaluates *Bacillus thuringiensis* isolated from East Java (Lamongan, Bangkalan, Madiun districts and HD 500) was effective bacteria in controlling against *Aedes aegypti* larvae. Mdn I TK2 isolates were the most effective

indigenous *Bacillus thuringiensis* isolated from East Java, as (2,21 x 10^7) cells/ml were required to kill 50% of the third stage *Ae.aegypti* larvae within 48 h. *Trichogaster pectoralis* Regan 1910 was exposed to different concentration (10^6,10^7,10^8,10^9,10^{10},10^{11},10^{12} cells/ml) of *Bacillus thuringiensis* isolated from Lamongan, Bangkalan, Madiun and HD 500 (reference bacteria) suspension no resulted mortality effects. The study resulted that three isolates of *Bacillus thuringiensis* from Bangkalan, Madiun and Lamongan have no direct effect on *trichogaster pectoralis.* Aquatic organisms, such as fresh water fish are generally unaffected (Eder and Iris, 2010). This large safety margin of preparations of *Bacillus thuringiensis* for non-target organisms indicated that their suitability for mosquito control programs in areas where protection of the natural ecosystem is important.

The chapter 4 reviews the safe strategy concept of biological control using indigenous *Bacillus thuringiensis*, the current status, and developmental trends of biological control using *Bacillus thuringiensis,* mode of action. *Bacillus thuringiensis* provides effective alternatives to broad-spectrum larvicides in many situations with little or no environmental impacts. The advantages of microbial control agents' usage are numerous. These include safety for humans and other non-target organisms, reduction of pesticide residues in the aquatic environment, increased activity of most other natural enemies and increased biodiversity in an aquatic ecosystem. This phenomenon indicates that indigenous *Bacillus thuringiensis* from East Java isolates have its potential to become bio control of *Aedes aegypti* larvae.

Chapter 1

Introduction

1.1 Background

Dengue is a mosquito-borne viral infection. The infection causes flu-like illness, and occasionally develops into a potentially lethal complication called severe dengue. The global incidence of dengue has grown dramatically in recent decades. About half of the world's population is now at risk. Over 2.5 billion people – over 40% of the world's population – are now at risk from dengue. WHO currently estimates there may be 50–100 million dengue infections worldwide every year. Before 1970, only nine countries had experienced severe dengue epidemics. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia and the Western Pacific (Figure 1-1). The American, Southeast Asia and the Western Pacific regions are the most seriously affected. Cases across the Americas, Southeast Asia and Western Pacific have exceeded 1.2 million cases in 2008 and over 2.3 million in 2010. Recently the number of reported cases continued to increase. In 2010, 1.6 million cases of dengue were reported in the Americas alone, of which 49 000 cases were severe dengue. Not only is the number of cases increasing as the disease spreads to new areas, but explosive outbreaks are occurring. The threat of a possible outbreak of dengue fever now

exists in Europe and local transmission of dengue was reported for the first time in France and Croatia in 2010 and imported cases were detected in three other European countries. In 2012, an outbreak of dengue on Madeira Islands of Portugal resulted in over 2,000 cases and imported cases were detected in 10 other countries in Europe apart from mainland Portugal. In 2013, cases have occurred in Florida (United States of America) and Yunnan province of China. Dengue also continues to affect several South American countries, notably Honduras, Costa Rica and Mexico. In Asia, Singapore has reported an increase in cases after a lapse of several years and outbreaks have also been reported in Laos. An estimated 500 000 people with severe dengue require hospitalization each year, a large proportion of whom are children. About 2.5% of those affected die (WHO, 2013).

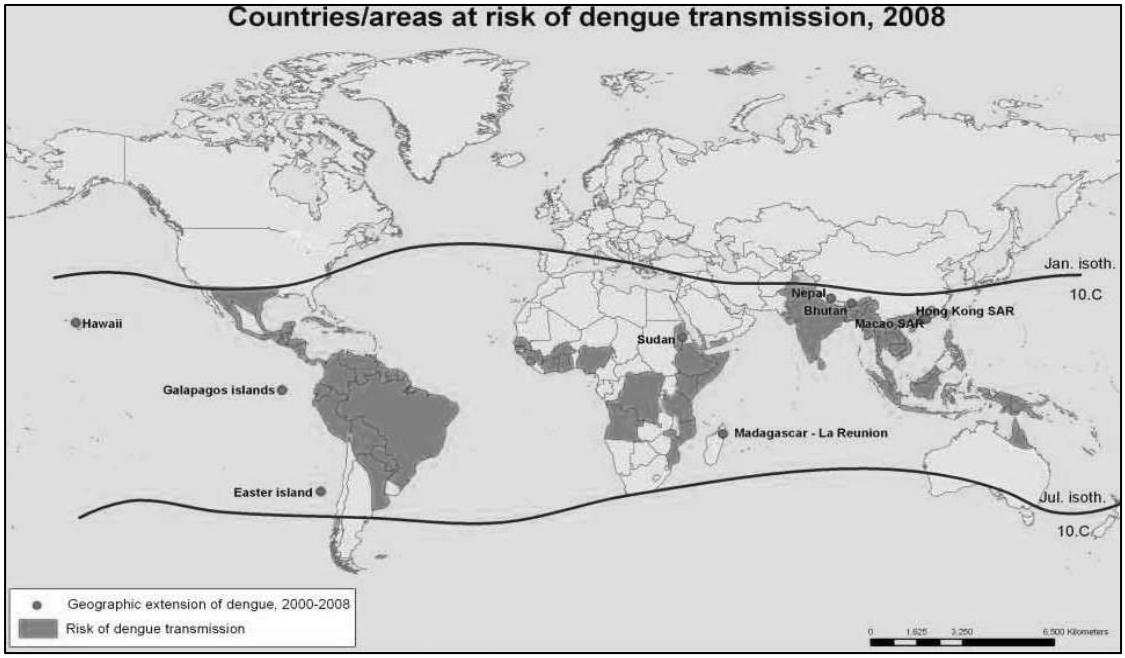

Figure 1-1. Map of Countries/Areas at Risk of DHF Transmission, 2008 (modified from WHO, 2008)

Dengue is currently one of the most relevant human diseases, especially in tropical and subtropical zone, where the proliferation of *Aedes aegypti* is favored by environmental conditions (Huntingford et al. 2007). Dengue is also a significant public health issue in urban and suburban areas (Liaqat et al. 2013). Severe dengue (previously known as Dengue Hemorrhagic Fever) was first recognized in the 1950s during dengue epidemics in the Philippines and Thailand. Today, severe dengue, affects most Asian and Latin American countries and has become a leading cause of hospitalization and death among children in these regions.

In the absence of an effective vaccine, control of *Aedes* mosquito vectors is the only preventive intervention. However, reduction of pesticide residues and the alternative use of insect biological control should be taken into account when environmental benefits including safety for humans and other non-target organism are considered.

Biological control is a bio effector – the method of using natural enemies for controlling insects, mites, weeds and plant diseases. It relies on predation, parasitism, herbivory or other natural mechanisms, but typically also involves an active human management role. Biological control is the use of natural enemies to reduce densities of insect pests. It can be an important component of integrated pest management (IPM) strategy.

The conservation of existing natural enemies in an environment is the third method of biological pest control. Natural enemies are already adapted to the habitat and to the target pest, and their conservation can be simple and cost-effective. Natural enemies of insect pests, also known as biological control agents, include predators, parasitoids, and pathogens.

Bacillus thuringiensis is one of pathogens, which provides effective alternatives to broad-spectrum larvicides in many situations with little or no environmental impacts. The advantages of microbial control agent's usage are numerous. These include safety for humans and other non-target organism, reduction of pesticide residues in the aquatic environment, increased activity of most other natural enemies and increased biodiversity in an aquatic ecosystem.

Many investigations related to biological control used *Bacillus thuringiensis* var. *israelensis* as a natural enemy against mosquito larvae have been conducted around the world. Quesada-Moraga et al. (2004) was performed to study the composition, ecological distributions, and insecticidal activity of isolates of this entomopathogenic bacterium from the Spanish territory. Using a standard isolation method, *B. thuringiensis* was isolated from 115 out of 493 soil samples collected in the Iberian Peninsula and the Canary and Balearic Archipelagos. The percentages of samples with *B. thuringiensis* were 31.7, 27.6 and 18.5 and the *B. thuringiensis* index 0.065, 0.067 and 0.11 for the Iberian Peninsula, Canary and Balearic Archipelagos, respectively. They found variable percentages of isolated active against Coleoptera and Lepidoptera, one isolate highly active against mosquito larvae.

Dengue is also common mosquito-borne diseases endemic to Sri Lanka. *Aedes aegypti*, the major vectors of dengue, were recently shown to undergo pre-imaginal development in brackish water bodies on the island. Therefore, Jude et al. 2012 investigated about the toxicity of *B. thuringiensis israelensis* H-14 larvicidae to *Aedes aegypti* larvae in the tropical coast of Jaffna, Sri Lanka and they focused on the effect of salinity levels naturally tolerated by *Aedes aegypti.* Their research resulted that LC_{50} and

LC_{90} of *B. thuringiensis* toxin for the second instar larvae of *Aedes aegypti* in fresh water were 0.006 ppm and 0.013 ppm, respectively, with corresponding values for brackish water populations of 0.008 and 0.012 ppm respectively. Statistical analysis showed significantly reduced toxicity of *B. thuringiensis* to fresh and brackish water-derived *Aedes aegypti* larvae at high salinities.

Although biological control is a very classical study and abundance publications have been produced in this field, but we believe that this topic is still very actual, especially in developing countries, and many new aspects can still be interesting to deal with. Currently, there are many researches on biological control using *B. thuringiensis* var. *israelensis* as a natural enemy of *Aedes aegypti* larvae, but for using indigenously *B. thuringiensis* isolated from East Java to kill indigenous *Aedes aegypti* itself is also new finding at this time. As Mary Barton of South Australia University (as one of the peer reviewers of my paper in 2013) stated that this study provides a potential solution for controlling the vector of dengue, *Aedes aegypti*, in Indonesia. It finds that the Indonesia indigenous bacterium *B. thuringiensis* can reduce the *Aedes aegypti* larvae, with bright prospect of further promotion. This discovery benefits local residents, what's more, Indonesia as an important tropical part of tourism, commerce and trade, cultural communication, promoting biological control of dengue will reduce the spread of dengue and influence of population outside the island, which means a great significance of public health. The general principal of the methodology had appeared in some publications, but modifications of the methodology seem to be the first only in this study. Some publications still use bacterial isolates from outside the mosquito habitat itself as in Wirth MC (2010), Poopathi S, (2010), and El-kersh TA (2012), whereas this research is the first

study using *B. thuringiensis* isolates from the regions of the mosquito (East Java). The study also used *Aedes aegypti* captured from East Java as the native habitat of the mosquito.

Alternative methods of mosquito management offer adequate levels of pest control and pose fewer hazards. One such alternative is the use of microbial insecticides such as *Bacillus thuringiensis* as biological pathogens, and biological control agents that contain microorganisms or their by-products. *B. thuringiensis* especially valuable because their toxicity to non-target animals and humans is extremely low. Compared to other commonly used insecticides, they are safe for both, the pesticide user and consumers of treated crops. The designing of integrated biological control, it is necessary to know the pattern of spatial and temporal distribution of *Aedes aegypti*. Hence, the spatial and temporal distribution in East Java should be assessed by ecological, climatic, and social factor approach. The climatic factors, including temperature, humidity, rainfall, wind velocity, light duration while social factors concern on demographic condition. Therefore, this research will try to reach and conduct these some aspects of mosquito distribution as an ecological based-approach to develop a role of sustainable biological control.

B. thuringiensis var. *israelensis* is a bacterium that is often used to control larvae of *Aedes aegypti* and locally *B. thuringiensis* was also found in East Java, and these bacteria have the ability to kill mosquito larvae. When the using of chemical insecticides increasingly out of control in East Java and it poses a serious problem, therefore, this research tries to search for potential local bacteria for controlling *Aedes aegypti* larvae.

Before the isolation of *Bacillus thuringiensis* from the soil in the East Java area, previously we needed to study about the distribution and abundance of *Aedes aegypti* at

the number of locations which it is a common outbreak in East Java. The presence and abundance of *Aedes aegypti* are vital to the transmission of DHF. East Java selected as the study site because there were more than 26.059 reported cases of DHF in East Java (Health Department of East Java Province, 2011). Dengue fever remains a serious health problem in East Java. This is due to dengue fever is a disease that causes high mortality every year.

Based on data from BPS (2010) showed that Nganjuk District has a highest Breteau Index (BI) during 3 years from 2008 to 2010, therefore, Nganjuk is selected as one of the study sites in this research. To compare the selected region around East Java, then we selected another location, such as Blitar, Bondowoso, Bangkalan, Ponorogo.Madiun, Tulungagung, Lamongan, Surabaya, Mojokerto and Malang (Figure 1-2).

Screening of *Bacillus thuringiensis* is essential to carry out because we want to find the most potential indigenously *Bacillus thuringiensis* isolated from several regions in East Java to control mosquito. Each isolate have to test against *Aedes aegypti* larvae originating from Madiun district. The mosquito larvae captured during the outbreak in the Madiun district. Then, rearing of mosquito will be carried out in the laboratory. The results of the offspring to be used for toxicity tests using various isolates obtained from several locations in East Java, which is an area endemic of DHF during the last three years. The original *Bacillus thuringiensis* exploration efforts in East Java were carried out because the *Bacillus thuringiensis* crystal protein had an arrow host spectrum. Therefore, the ideal effort in controlling East Java mosquitoes would be using *Bacillus thuringiensis* isolated from East Java Province.

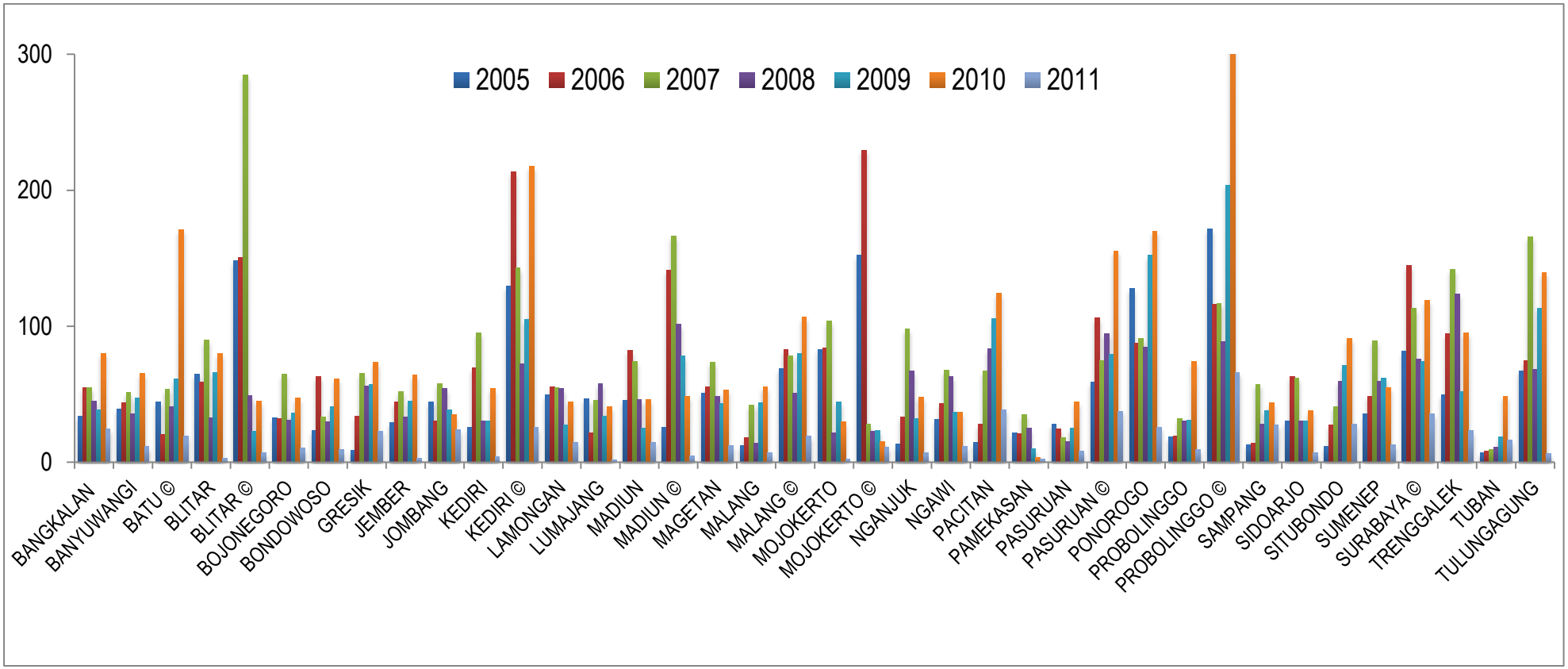

Figure 1-2. The incidence rate (IR/100.000 residents) in East Java Province from 2005 to 2011(produced by Health Department, 2011).

1.2 Research objectives

The main objective of this study is to investigate safe strategy to control *Aedes aegypti* mosquito using the most potential of *Bacillus thuringiensis* isolated from East Java as a natural enemy. In this study case, Nganjuk, Mojokerto, Malang, Madiun were taken as examples of DHF endemic areas. In addition, due to the designing of integrated biological control, the first part of this study is focused on recognizing association between climatic variables, DHF incidence, and distribution of *Aedes aegypti* as a vector of DHF in East Java. Therefore, this study will try to reach and conduct these some aspects of mosquito distribution as an ecological based-approach to develop a role of sustainable biological control.

In order to achieve the above goal, several important works have been conducted. Each chapter investigates about a particular issue and tries to solve its own specific objectives. Therefore, if we break down the main objective of the study into several specific objectives, the list of all objectives in this study can be formulated as follows:

1. To investigate climatic variables (maximum and minimum temperature, rainfall, humidity, light duration, wind velocity) associated with DHF incidence in East Java Province, Indonesia from 2005 to 2010 (a case study of Nganjuk District).
2. To determine the distribution pattern of mosquitoes and its relationship between elevation and abundance of *Aedes aegypti* in East Java (a case study of Mojokerto District) that caused the dengue virus transmission.
3. To investigate the toxicity of indigenous *Bacillus thuringiensis* isolated from East Java for controlling *Aedes aegypti* larvae (study sites: Malang, Surabaya, Lamongan, Madiun, Nganjuk, Tulungagung, Blitar).

4. To investigate the toxicity of indigenous *Bacillus thuringiensis* isolated from East Java on non-target organism *Trichogaster pectoralis* (Class: Actinopterygii).
5. To introduce a safe strategy to control mosquito using the potential of indigenous *Bacillus thuringiensis* isolated from East Java as a natural enemy of mosquitoes (*Aedes aegypti*) and to summarize the current status and developmental trends of biological control based on the published reports.

1.3 Research scopes and limitations

The scopes of this study focus on biological control that using only one type of bacteria. *Bacillus thuringiensis* isolated from soil in East Java compares with *Bacillus thuringiensis* var. israelensis HD-500 and HD-567 was obtained from the Cibinong Science Center (LIPI Bogor) as standard bacteria for identifying all isolates. In addition, this study also covers only a few locations/district in East Java for sampling of soil and mosquito.

It is very important to mention the limitation of this study. Although this study mainly focuses on the biological control using *Bacillus thuringiensis* isolated from East Java, this isn't all isolated can use for toxicity test. *Bacillus thuringiensis* isolates that have a high similarity level with bacterial standards are used for toxicity tests. Some data, such as climate data and DHF cases are secondary data. Therefore, abiotic factor analysis is just based on the availability on the data. For instance, it is good to have the data on temperature, humidity, light duration, wind velocity, and rainfall for each location of sampling, but the data are limited to the year.

1.4 Research framework

As indicated in the previous section, this study is begun with the study on the association between climatic variables, Dengue Hemorrhagic Fever incidence and distribution of *Aedes aegypti* as a vector of DHF in East Java. The next part is focused on biological control using the most potential indigenous *Bacillus thuringiensis* isolated from East Java for suppressing *Aedes aegypti* larvae. Then further study continued to review related to the safety and advantages indigenously *Bacillus thuringiensis* isolated from East Java as an addition. The whole framework of the study is being described as that shown in Figure 1-3.

1.5 Software utilization

We employed software used for data processing and analysis: ArcGIS 10 (ESRI Inc., Redlands, California, USA) and SPSS 16 (SPSS Inc., Chicago, Illinois, USA).

1.6 Dissertation backbone

A doctoral dissertation is a long piece of writing about scientific work. Its contents should not include, but real, facts and scientific findings. All sentences in a dissertation have to be written with full of consideration and responsibility. Ideally, each sentence in a dissertation has to be assessed and reviewed by experts in the field. The only way that all sentences in a scientific work being reviewed is by submitting the work to the journal, so that it can be assessed and criticized by experts in the international society.

It is an important to state here that all parts in this dissertation are based on the published paper, implying that any written sentence in this dissertation has been

scientifically reviewed by experts in the field. At the time of submitting the dissertation, five papers of this dissertation have been published / accepted and two others were still being reviewed the journal. These papers become the backbone for this dissertation; hence any quotation taken from this dissertation should be referred to its corresponding paper.

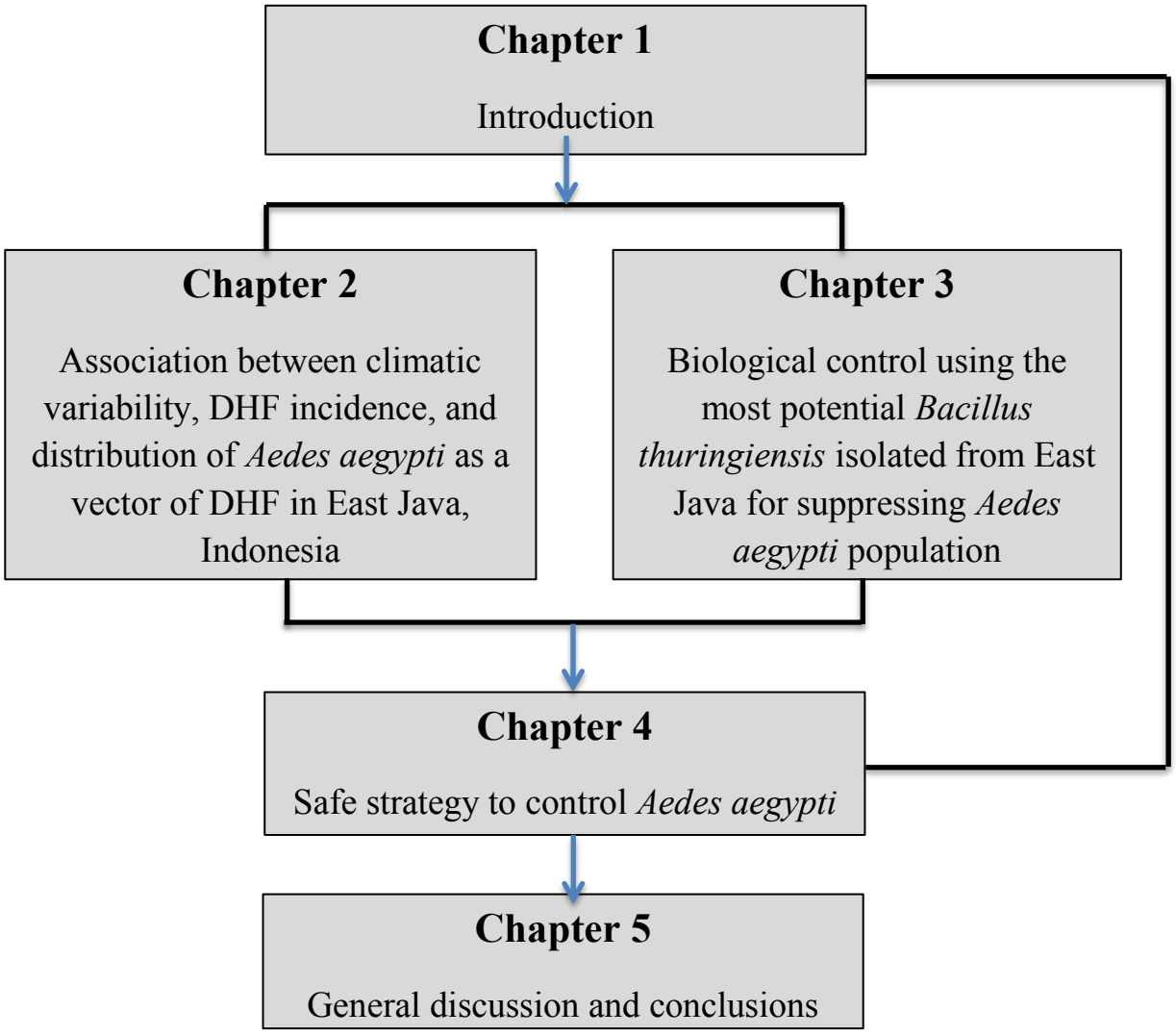

Figure 1- 3 Research framework

Chapter 2

Association between climatic variability, DHF incidence and distribution of *Aedes aegypti* as a vector of DHF in East Java, Indonesia

At the beginning of the study, this chapter had been explained about preliminary research based on secondary data. Secondary data were obtained from the East Java Provincial Health Office. East Java Province has 38 districts that need to be determined which areas is the object of this research. To determine which areas will be used as a place for mosquito sampling and isolation of bacteria from the soil, it is necessary to know the spatial and temporal distribution of *Aedes aegypti*. Based on preliminary research, resulted that information about the location that has the highest dengue incidence rate and the peak season of Dengue Hemorrhagic Fever (DHF) outbreak from 2008 to 2010. Therefore, this preliminary research is very important to support the accuracy of the study site selection before using the data for further research. In addition, Mojokerto is selected as one of districts in East Java to evaluate the distribution and abundance of *Aedes aegypti* due to this location still have a higher breteau index (BI) more than WHO standard.

2.1 A spatial and temporal distribution of *Aedes aegypti* in East Java, Indonesia from 2008 to 2010

2.1.1 Introduction

Global warming causes unstable climate and many serious impacts to physical environment and health. WHO stated that mosquito borne disease, which is Dengue hemorrhagic fever (DHF), is very sensitive to climate change. *Aedes aegypti* (L.) is the main dengue vector worldwide because of its close association with humans in tropical and sub-tropical urbanized areas (Cox *et al.,* 2007). The incidence of dengue has grown dramatically around the world in recent decades. WHO currently estimates there may be 50 million dengue infections worldwide every year. An estimated 500,000 people with severe dengue require hospitalization each year, a large proportion of whom are children. About 2.5% of those affected die. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia and the Western Pacific. Southeast Asia and the Western Pacific are the most seriously affected (WHO, 2012). Indonesia as one of the countries in the tropics, are very susceptible to diseases spread by the *Aedes aegypti* (Suci Astutik *et al.,* 2011). In 2010, there were more than 26.059 reported cases of DHF in East Javas (Health Department of East Java Province, 2011). Therefore, the aim of this study was to determine the spatial distribution of *Aedes aegypti* in East Java from 2008 to 2010.

2.1.2 Material and methods

2.1.2.1 Study site

This study used the average of DHF incidence rate (IR) data in 9 districts in East Java over a monthly period (January - December) from 2008 to 2010. The land area of East Java Province (Figure 2-1) is about 47,130.15 km² and the sea area of 110,764.28 km², located between 111°0 '– 114 °4′ east longitude and 7 °12 '- 8 °48′ latitude (BPS, 2010).

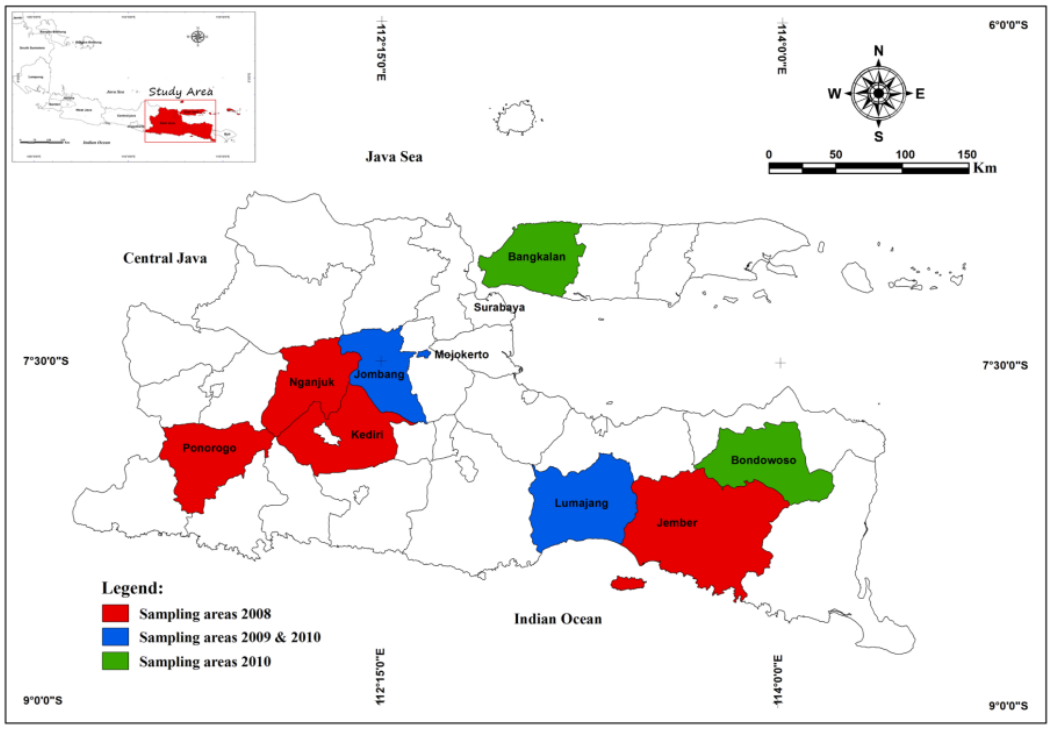

Figure 2- 1 Study site

2.1.2.2 Data Collection

A sample, check survey was carried out in nine locations in East Java. Its purpose was to collect information on the presence or absence of potential habitats of *Aedes aegypti*

in the region. The landing resting of *Aedes aegypti* and total catches of adult mosquitoes by the aspirator was also undertaken.

2.1.2.3 Mosquitoes Capture

In this study we carried out two types for mosquitoes captures. The first is spot check method and the second method is longitudinal methods. Spot check location is selected at many locations, which there have been occurring outbreak of DHF. These locations were Ponorogo, Nganjuk, Kediri, Jember, Jombang, Lumajang, Bondowoso, Bangkalan, Mojokerto. However, for the longitudinal method is only conducted at the special location, and each location necessary monthly checked during the planning time in 2010 (Figure 2-2).

Figure 2- 2 Methods for sampling of mosquitoes

2.1.2.4 Capturing mosquito larvae

Locations were selected for twenty sampling points in each study site based on the previous data in the Health Department in Surabaya Province. Larval sampling method with a dipper from various places such as mosquito breeding places in the

house that shelters consisting of a water bath tub, drinking water containers, jars, bowl of water, and a bucket. Mosquito breeding places outside the home such as drums, cans, bottles, pot scrap, decorative plant pots filled with rainwater. Observation of the presence or absence of mosquito larvae was carried out monthly.

2.1.2.5 Capturing mosquito (adult)

Locations were selected for ten sampling points based on the previous data in the Health Department in Surabaya Province (Figure 2-5). Time for sampling ranged from 07:00 to 17:00. Capturing mosquito used an aspirator by trained technicians both indoor and outdoor locations. The next activity is identifying the captured mosquito by microscope and counting the number of mosquitoes.

2.1.2.6 Data Analysis

The incidence rate (IR) of DHF, House index (HI), Containers index (CI) and Breteau index (BI) were calculated using following formula (Santoso and Anif Budiyanto, 2008):

$$IR = \frac{new\ DHF\ cases}{population} \text{ x } 100.000 \quad (1)$$

$$CI = \frac{number\ of\ containers\ positive\ for\ Ae.aegypti\ larvae}{number\ of\ containers\ surveyed} \text{ x } 100\ \% \quad (2)$$

$$HI = \frac{number\ of\ houses\ positive\ for\ Ae.aegypti\ larvae}{number\ of\ houses\ surveyed} \text{ x } 100\ \% \quad (3)$$

$$BI = \frac{number\ of\ habitats\ positive\ for\ Ae.aegypti\ larvae}{number\ of\ houses\ surveyed} \text{ x } 100\ \% \quad (4)$$

2.1.3 Results

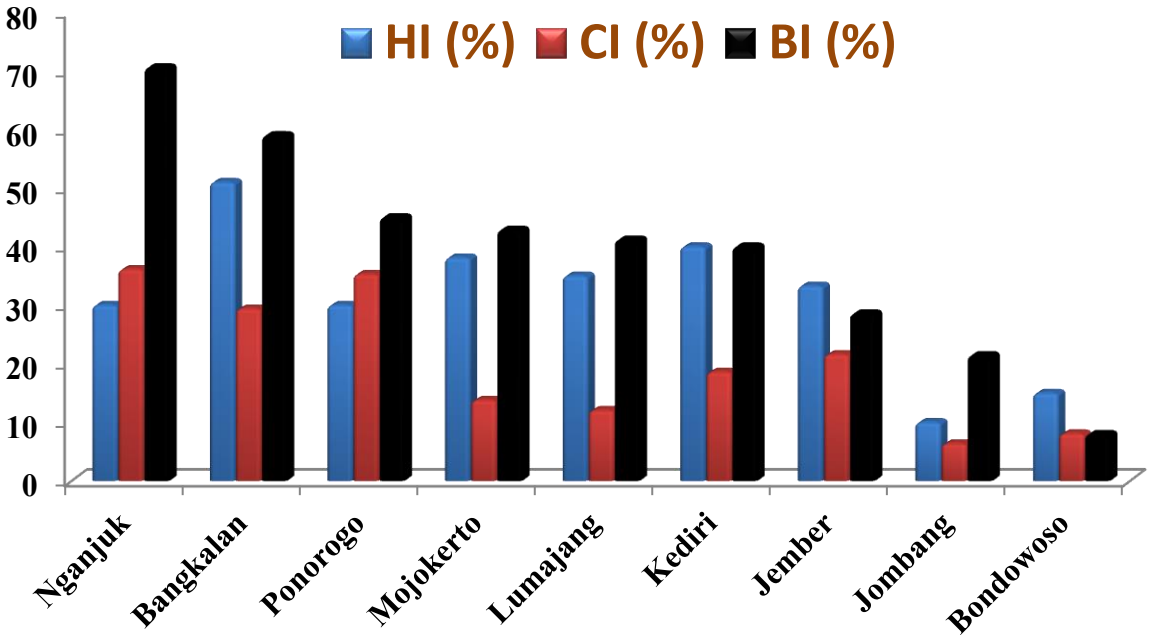

Figure 2- 3 Relationship between HI, CI and BI in 9 districts

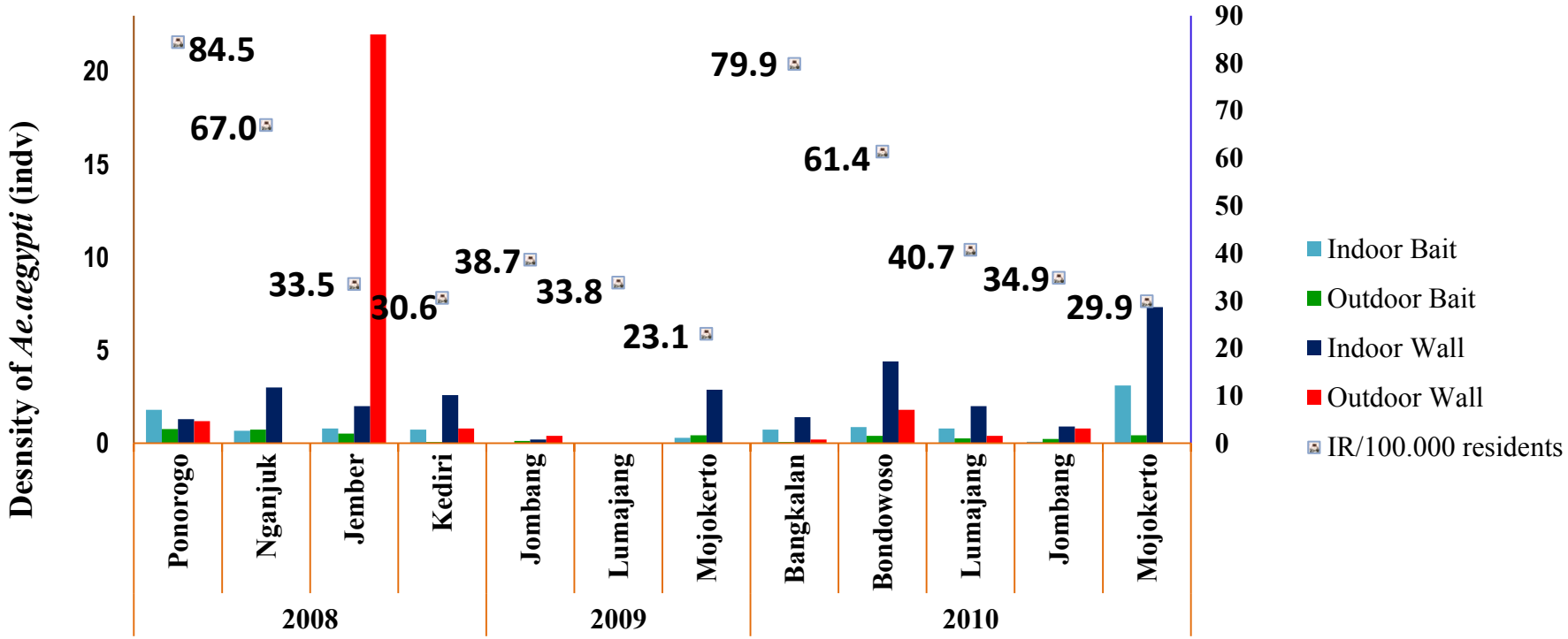

Figure 2- 4 Relationship between density of *Aedes aegypti* (adult) and the average IR of DHF

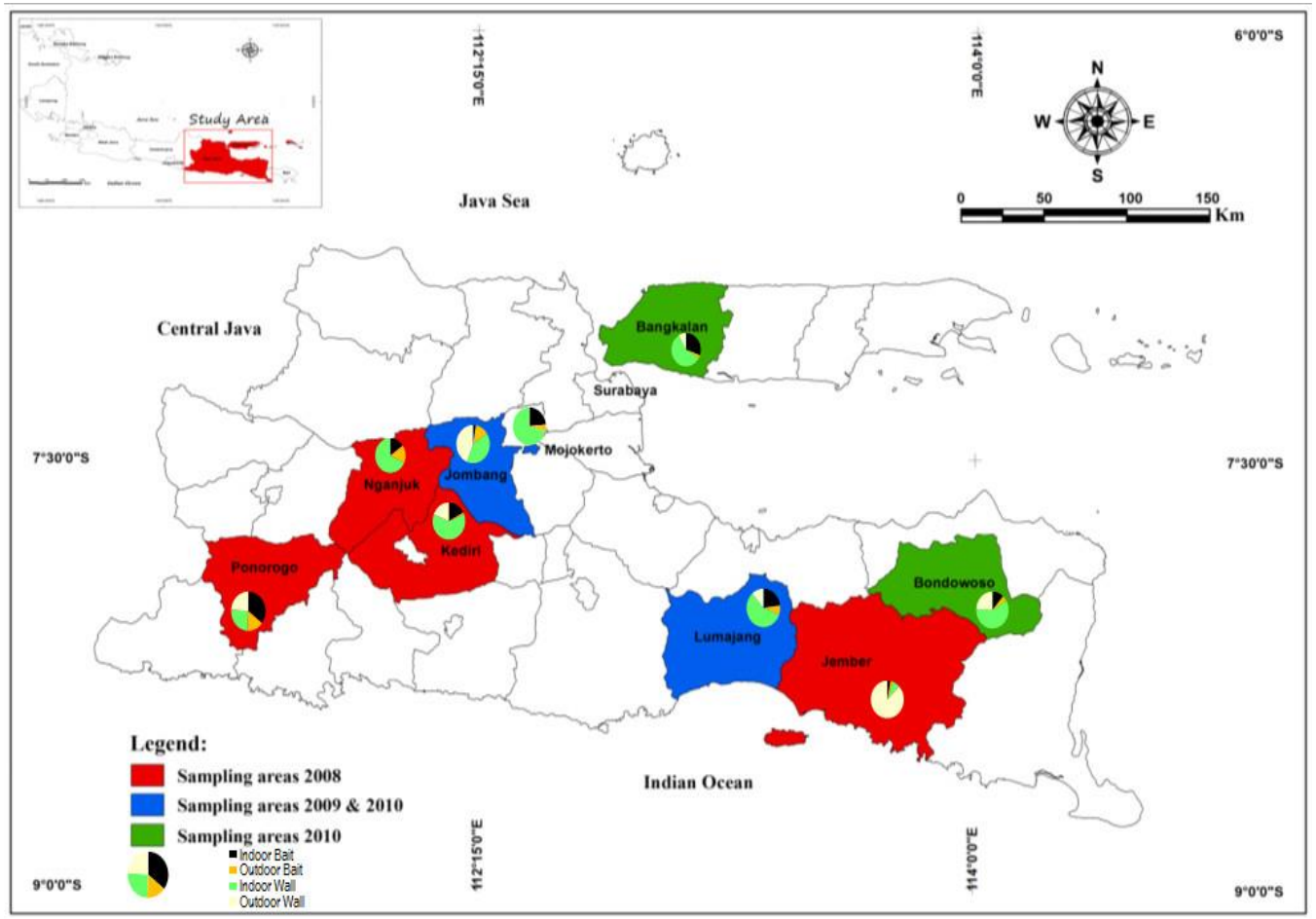

Figure 2- 5. Spatial distribution of *Aedes aegypti* in East Java from 2008 to 2010

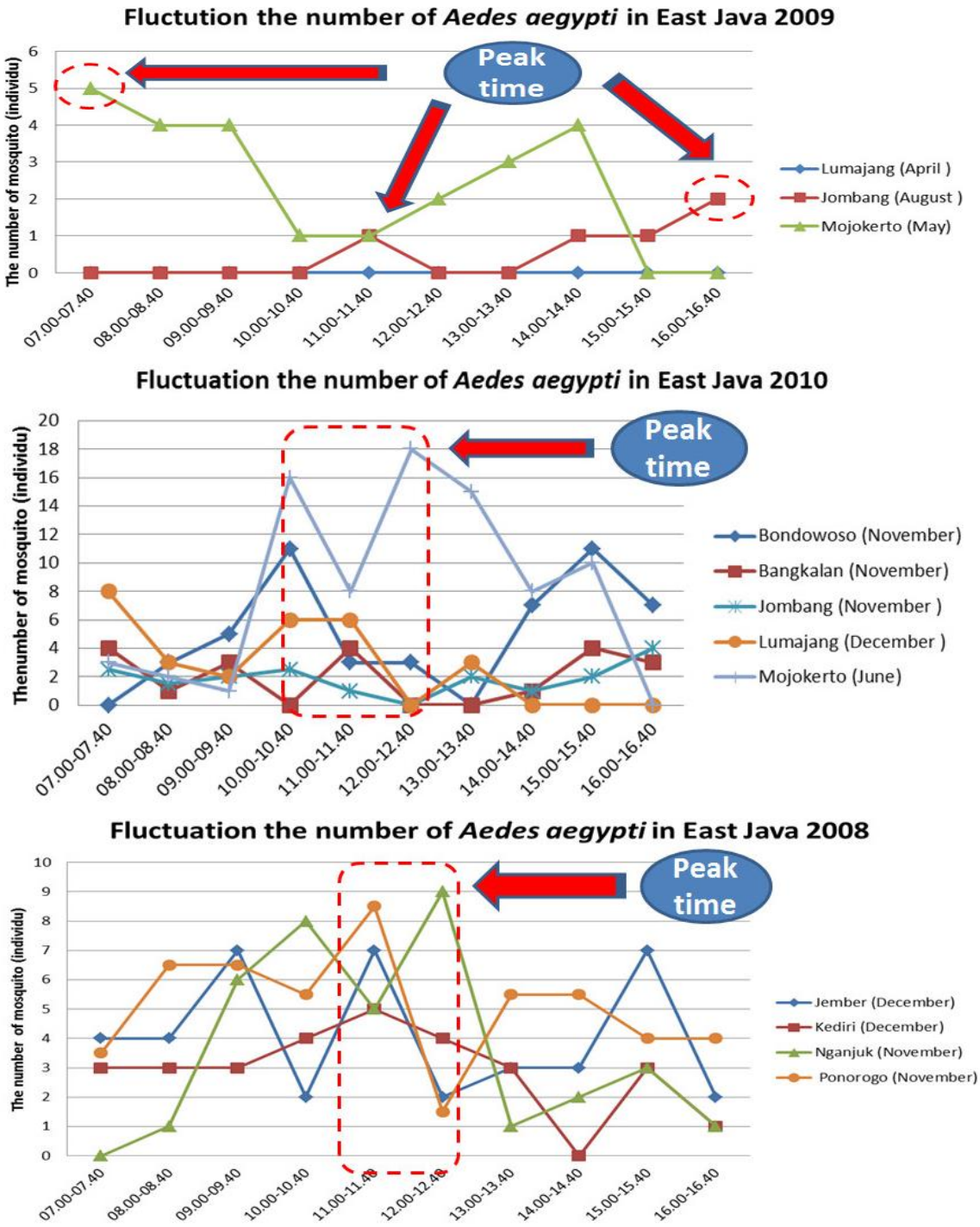

Figure 2- 6. Temporal distribution pattern of *Aedes aegypti* in nine regions for 3 years (2008, 2009, and 2010)

2.1.4 Discussion

The results of statistical analysis showed that there was a significant relationship between the average IR and the number of *Aedes aegypti* (Figure 2-4). And the mosquitoes were captured by using a bait in door has the most closely related to the average IR cause *Aedes aegypti* is an *anthropophilic* mosquito, taking its blood meals preferentially from humans.

The relationship between IR and the number of mosquitoes is shown by the following equation:

Average IR = 1,923 (The density of *Aedes aegypti* in door bait) + *26,791* ($R^2 = 0.92$) (5)

The peak season of *Aedes aegypti* occurred in 2008 and it is ranged at 11:00 to 11.40. The peak time for *Aedes aegypti* in the Mojokerto on 2009 occurs twice in a day. They occurred at 07:00 and 14:00, while in the Jombang area at 11:00 and 16:00. This result indicated (Figure 2-6) that the mosquito *Aedes aegypti* to appear twice a day for sucking human blood. The peak time of *Aedes aegypti* in Bondowoso, Bangkalan, Jombang, Mojokerto Lumajang on 2010 almost simultaneously, these happened during around 2,40 hours between 10:00 to 12:40. *Aedes sp.* living in and around the house, therefore all available food obtained in situ. It may be said that *Aedes aegypti* female love of human blood (antropophylic). Sucking blood habit of mosquitoes, especially in the morning at 08:00 to 12:00 and 15:00 to 17:00 in the evening (Soegiyanto, 2006).

2.1.5 Conclusion

Aedes aegypti was more often found in indoor than outdoor. The highest CI and BI were recorded in 36.11 and 70.59 % respectively in Nganjuk District. The highest *Aedes aegypti* house index was 51% in Bangkalan District. In these districts, there are many good habitats for *Aedes aegypti* larvae. All locations of East Java have a higher House Index (HI) than the WHO standard for the high DHF risk area (i.e.10 percentage HI). The peak season to sucking blood of *Aedes aegypti* is occurring in the morning and in the evening.

2.2 Climatic variability and dengue hemorrhagic fever (DHF) incidence in Nganjuk district, East Java, Indonesia.

2.2.1 Introduction

Dengue Hemorrhagic Fever (DHF) is a vector-based disease that causes many deaths in tropical countries. Indonesia is one of the countries with a serious DHF problem. DHF outbreaks have been recorded in Indonesia since 1986; it firstly occurred in Surabaya area. DHF virus is transmitted to human through the bites of infective female *Aedes* sp. (Diptera: Culicidae) mosquitoes. DHF is especially occuring in over 100 tropical and subtropical countries. It is living in countries located within the equatorial zone where seasonal and geographical distributions are dependent on climate that is conductive to its transmission (Wongkoon et al. 2011). Considerable interest exists in the potential role of the climate in human health issues, especially regarding the effect of climate change on vector-borne diseases such as DHF.

The *Aedes aegypti* mosquito, the principal vector for DHF, which is considered the most pressing vector-borne viral disease in the world, is particularly susceptible to climate variability and climatic change (Hopp et al. 2001). These mosquitoes are well adapted to the urban environment and successfully breed in containers where water is allowed to accumulate, such as discarded can, bottles, plastic containers and tires. The presence and abundance of *Aedes aegypti* is vital to the transmission of DHF. Changes in mean climate conditions and climate variability also can affect human health via indirect pathways,

particularly in the changing of biological and ecological processes that influence infectious disease transmission and food yields (McNeil et al. 1976).

Environmental conditions strongly control the geographic distribution and abundance of *Aedes aegypti*. Breeding habitats for the mosquito consist of any type of water-holding container. In this environment, climatic variables such as temperature, humidity, and rainfall significantly influence mosquito development and survivorship (Suci et al. 2011). Temperature affects the rate of development in the different mosquito life stage, as well as DHF viral development. Based on the background of previous studies, this research investigated climatic variability (maximum and minimum air temperature, rainfall, humidity, light duration, wind velocity) associated with DHF incidence in Nganjuk District, East Java Province, Indonesia from 2005 to 2010.

2.2.2 Materials and method

2.2.2.1 Study area

Nganjuk District is located between 11105’ to 112013′ East longitude and 7020’ to 7059′ South latitude in East Java Province (Figure 2-7). The District shares the boundary with Bojonegoro District in the north, with Kediri Regency and Trenggalek in the south, with Jombang and Kediri regency in the east, and with Ponorogo and Madiun District in the west. Based on BPS (2010) showed that the total area of the Nganjuk District is 122,433 ha consisting of land rice fields = 43,052.5 ha, Dry land = 32,373.6 ha, Timber = 47,007.0 ha. This area was selected as the study site based on the results of previous research that it was the one of major DHF epidemic foci in Indonesia. The DHF incidence was recorded at the district level (lattice data).

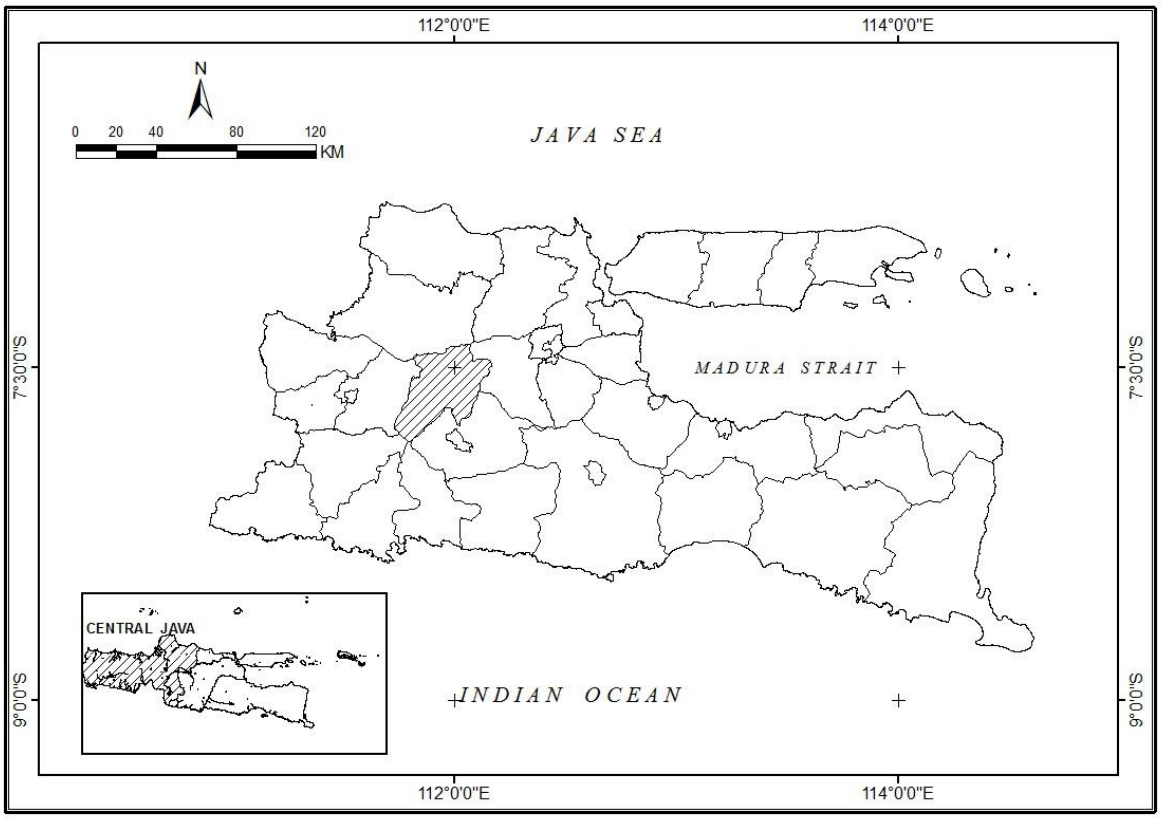

Figure 2- 7 Study area in Nganjuk district

2.2.2.2 Data collection

DHF's incidence rate was obtained from the East Java Provincial Health Office from 2005 to 2010. The data collection procedure during the study period was the same; therefore, this routinely collected data can be used for analyzing factors affecting the occurrence of DHF. Monthly climate data that were obtained from the Meteorology, Climatology and Geophysics Agency in Surabaya city consisted of maximum and minimum air temperature, rainfall, humidity, light duration, and wind velocity components.

2.2.2.3 Statistical analysis

Data analysis was performed to test the correlation between the incidence rate of DHF and climate variability (maximum and minimum temperature, rainfall, humidity, light duration and wind velocity) in Nganjuk District. Bivariate analysis using the Spearman's correlation analysis was carried out to determine the relationship between the monthly climatic variables and the DHF incidence. The DHF incidence was calculated from the number of dengue cases per 100,000 populations in the Nganjuk District over the period of 2005 – 2010 (P2PL of DHF, 2011). For correlation-regression, one year was divided into rainy (October-March) and dry seasons (April-September).

2.2.3 Results and discussion

The results of this study indicated that climatic variability is clearly correlated with the IR (Figure 2-8 to Figure 2-13). The maximum air temperature, humidity, rainfall and light duration played an important role in the transmission of DHF in Nganjuk District. The Spearman correlation analyses indicated that an increase in humidity and rainfall in Nganjuk District from 2005 to 2010 were associated with an increase of DHF incidence; but a decreased value of maximum air temperature and light duration will have an impact on increased IR.

Table 2-1. Spearman correlation between climatic variables and dengue incidence rate (IR) in Nganjuk District from 2005 to 2010

Climatic variables	Spearman correlation	Significant (*two tailed*)
Maximum air temperature (^{o}C)	- 0.445	0.000
Minimum air temperature (^{o}C)	0.166	0.164
Humidity (%)	0.484	0.000
Rainfall (mm)	0.612	0.000
Light duration (%)	- 0.625	0.000
Wind velocity (Knots)	0.166	0.164

Spearman correlation was conducted relating to the monthly incidence rate of DHF to various monthly climatic measures. Table 2-2 showed that monthly humidity and rainfall were a positive associated with monthly-recorded dengue incidence rate in Nganjuk District from 2005 to 2010. Monthly maximum air temperature and light duration were a negative associated with the monthly dengue incidence rate.

Table 2-2. Result of regression analysis between monthly climatic variables and dengue incidence rate

a. In the rainy season model		t	Sig.
	Constant	4.619	0.000
	Max temperature	-2.274	0.029
	Min temperature	-4.001	0.000
	Rainfall	0.274	0.062
b. In the dry season model	Constant	1.967	0.056
	Rainfall	4.454	0.000
	Wind velocity	2.496	0.017

Dependent Variable : IR = %

The relationship between climate variability and the DHF incidence rate was shown in the following figures:

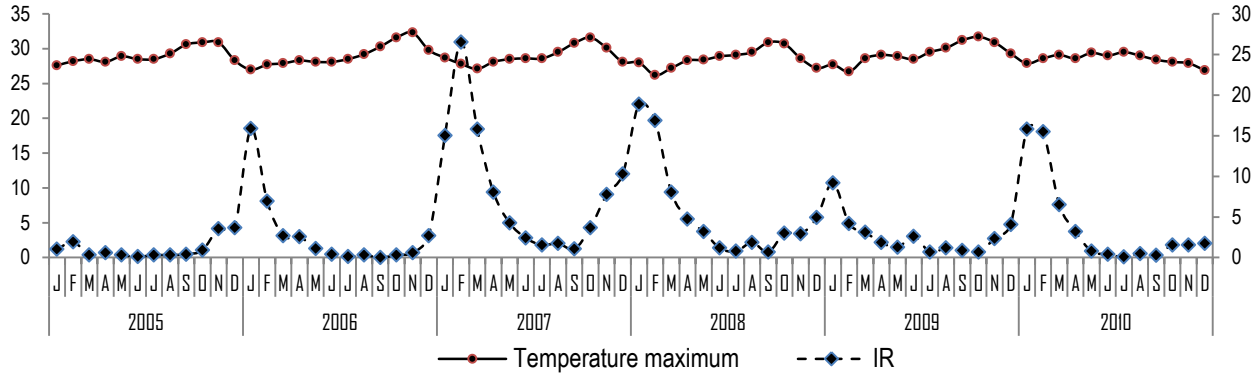

Figure 2- 8 Relationship between the maximum temperature (Celsius) and IR in Nganjuk district

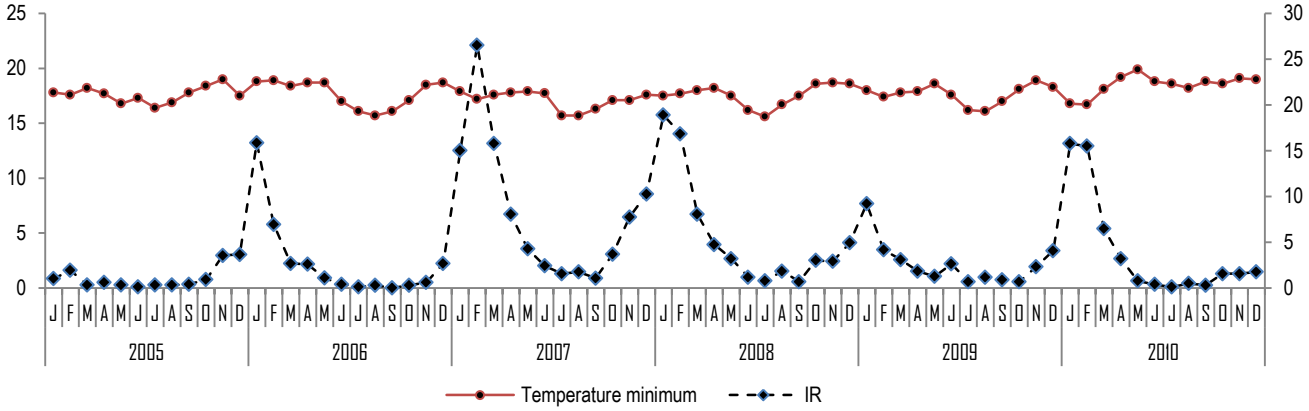

Figure 2- 9 Relationship between the minimum air temperature (Celsius) and IR in Nganjuk district

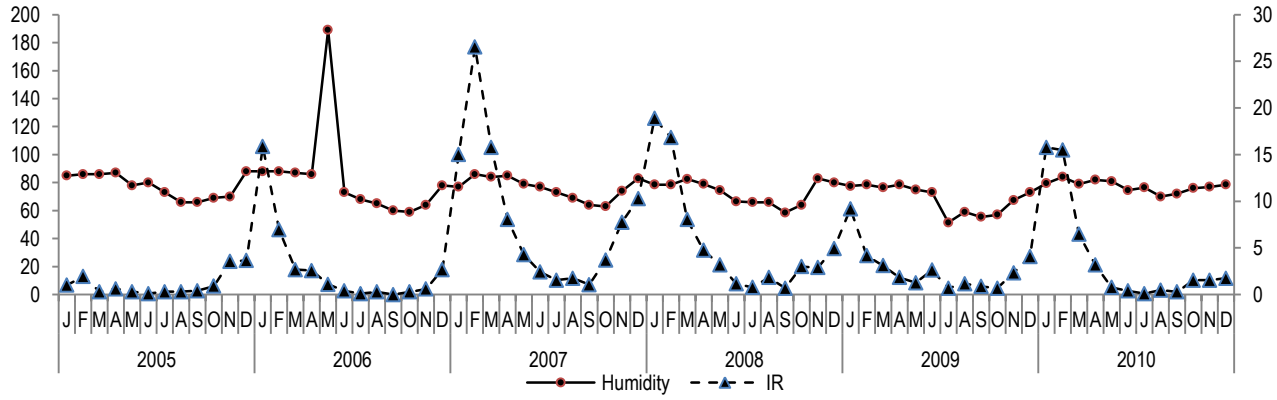

Figure 2- 10 Relationship between the humidity (%) and IR in Nganjuk district

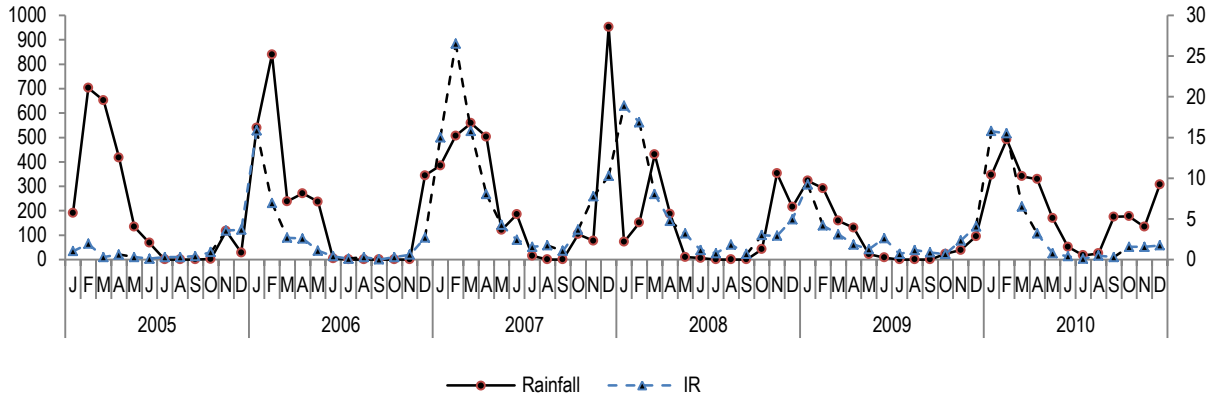

Figure 2-11. Relationship between the rainfall (day) and IR in Nganjuk District

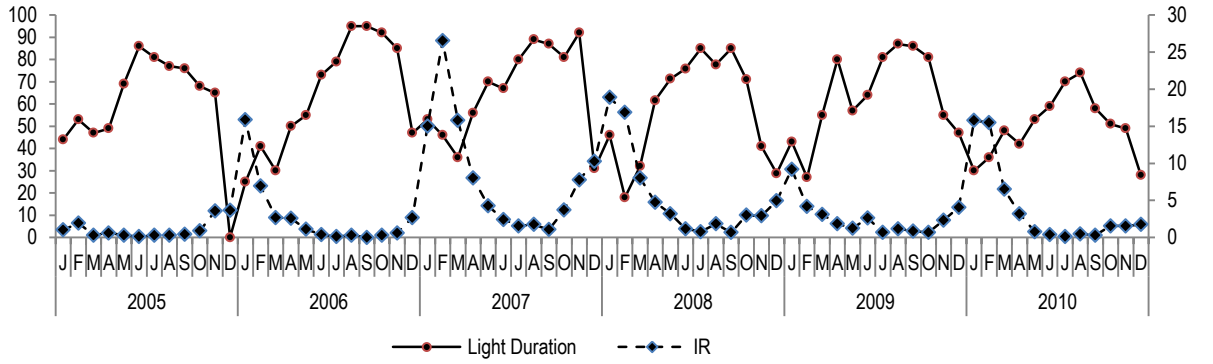

Figure 2-12. Relationship between the light duration (%) and IR in Nganjuk District

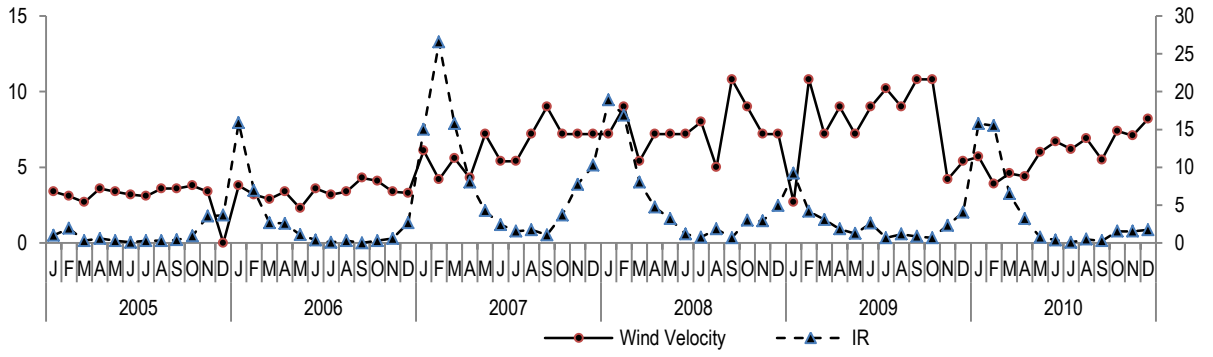

Figure 2-13. Relationship between the wind velocity (knot) and IR in Nganjuk District

Based on Figure 2-8 and Figure 2-9 appear that temperatures ranged from 15.6 to 32.3 degree Celsius in Nganjuk District (2005 – 2010) with the average of 28 degree Celsius. Temperature is an important factor for the development of mosquito larvae (Rowley and Graham 1968). Similar studies have stated that temperature is an important environmental parameter to enhance vector development, gonotropic cycle length, fecundity, time from emergence to first blood meal, biting rates, shortening pathogen incubation period and encouraging adult longevity. In addition, higher temperatures also increase the rate of larval development and therefore the emergence of adult vectors, increase the vector biting rate and reduce the time required for virus replication within the vector, known as the extrinsic incubation period (EIP) of dengue virus in *Aedes aegypti* transmission, meaning vectors are infectious earlier and bite more frequently. Higher temperatures may reduce the vector survival time, which may offset the positive effect on vector abundance to some degree (Thai and Katherine 2011; Wongkoon et al. 2011).

Result of regression between monthly climate variables and DHF incidence rate (Table 2-2) showed that a negative coefficient of the maximum and minimum temperatures during the rainy season. Several studies indicated that the negative coefficient of the maximum and minimum temperatures were caused by the optimum temperature for development of mosquitoes ranged 25 to 27 degree Celsius. When air temperature is at 10 degree Celsius, mosquitoes can survive at low temperature, but if air temperature is lower than 4.5 degree Celsius, then the process of their metabolism is decreased or even stopped. As air temperature above 35 degree Celsius, mosquitoes have limited physiological processes. The air temperature affects the development of virus in the mosquito (Colon-Gonzales et al. 2011; Lambrechts et al. 2011). Humidity is governed by a combination of

rainfall and temperature and influences the lifespan of the mosquito and therefore the potential for transmission of the virus (Thai and Katherine 2011). Additionally, humidity and in general the presence of water is necessary for egg laying and hatching and larval survivorship and relative humidity affects adult mosquito mortality (Herrera-Martinez and Alfonso 2010). This study indicated that monthly rainfall was related to the incidence of the DHF in Nganjuk District both of two seasons, in the rainy season and the dry season (Table 2-2). Rainfall is one of the important elements for the breeding and development of mosquitoes. Many studies have shown that rain plays an important role in the dengue epidemiology. The findings confirmed rainfall is one of the key predictors of dengue transmission. It could be because water not only provides a medium for the aquatic stages of the mosquito's life cycle, but also increases the relative humidity and hence longevity of adult mosquitoes (Bi et al. 2003). They need water to complete their life cycle. They can breed in almost any source of water. Rain may prove beneficial component to mosquito breeding if moderate, but it may destroy existing breeding sites and interrupt the development of mosquito eggs when excessive. Increased rain may increase larval habitat and vector population size by creating a new habitat or increase adult survival. In tropical areas in particular, extensive and continuous rainfall can delay the build up of some mosquito species until late in the season and thus delay transmission (Wongkoon et al. 2011). The same results can also be found in observations of The Thailand (Wongkoon et al. 2011) and it's associated with local rainfall in Puerto Rico (Herrera-Martinez and Alfonso 2010).

Wind velocity will affect a flying range of *Aedes aegypti*. The most extensive coverage of a mosquito, the more opportunity for contact with humans and so increases the

reproductive age and the length of a mosquito. Gubler (1998) suggested that wind indirectly affects the water and air temperature as well as the passive spread of mosquitoes. Wind velocity in Nganjuk District (2005-2010) had a range between 2.30 to 10.80 knots. As the recorded wind velocity is low. The development and spread of mosquitoes are not hindered (Amah et al. 2010). Therefore, the incidence rate is also high during the dry season as mosquitoes can breed and spread widely due to the calm wind conditions.

In natural habitats, the aquatic larvae are usually surrounded by a canopy of vegetation and emergent plants. Dense vegetation usually supports mosquito proliferation. Land cover may also affect larval survivorship and adult productivity as penetration of light and temperature are minimized under such natural conditions (Swain et al. 2008). Various reactions of the larva to stimuli that of negative phototropism is especially characteristic of the larva of *Aegypti aegypti.* When describing the behavior of this larva, notes as especially characteristic, over and above its extreme restlessness, (1) strong negative phototropism, and (2) extreme sensitiveness to vibrations and to light. Christophers (2009) who reveals that *Aedes aegypti* can all develop from the egg to adult in complete darkness. Of these species, *Aedes aegypti* was able in complete darkness to give rise to a second generation. Exposure to light may on the contrary be prejudicial in that this tends to cause the larvae to congregate in one spot in the culture. It is similar to the data analysis results in this study, because it was found that a negative value on the relationship between light duration and incidence rate of DHF. In other words, increasing the light duration will lead to decreasing the incidence of DHF.

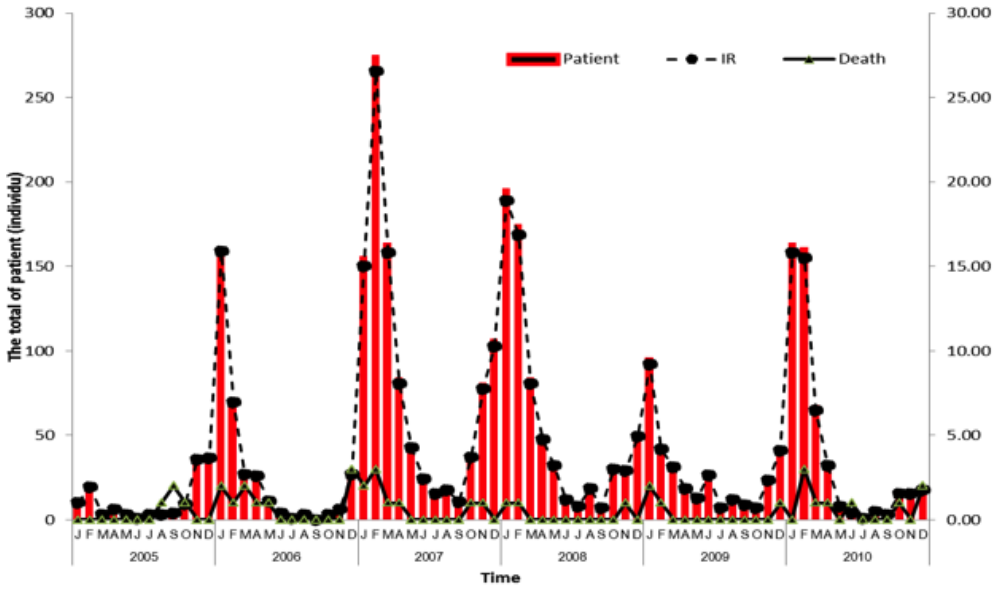

Figure 2-14. Relationship between the total of patients, the total of death and IR in Nganjuk District

The result indicated that an average of DHF incidence in Nganjuk District was 4.05 per 100.000 residents from 2005 to 2010. As Figure 2-14 shown that the highest DHF incidence (26.53 per 100.000 residents) was founded in February 2007 and the lowest DHF incidence was detected on September 2006 (IR = 0). Based on statistical analysis showed that the incidence rate (IR) of DHF was affected by the maximum air temperature, minimum air temperature and rainfall in the rainy season, but in the dry season, the IR was affected by wind velocity and rainfall.

The highest incidence rate occurred in February 2007 (rainy season) when the temperature ranged between 17.20 and 27.80 degree Celsius and rainfall at 507 mm/day (Figure 2-15). When the environment has an optimum air temperature at 25-27^{0}C, mosquitoes have developed optimally; therefore the number of mosquitoes gets to increase.

If the number of mosquitoes increases the likelihood of DHF also increased. Changes in climate may influence the abundance and distribution of vectors. Rainfall events and subsequent floods can lead to outbreaks of DHF mainly by enabling breeding of vector mosquitoes (Hii et al. 2009; Lindsay and Mackenzie 1997).

In many areas of the world, dengue outbreaks occur every year during the rainy season, when conditions are perfect for mosquito breeding. Dengue can pose a particular threat in highly populated regions, because epidemics are more likely where there are larger numbers of people in contact with large numbers of mosquito vectors than in more isolated areas. In countries in the equatorial zone that experience tropical monsoon seasons, such as Indonesia, India, Brazil, Thailand, Sri Lanka, and Myanmar (Angel and Joshi 2008; Libraty et al. 2007). Epidemics of dengue tend to coincide with the rainy season; this is because of significant increases in the mosquito larval populations are seen during the rainy season (Barrera et al. 2011). Furthermore, ambient temperature and relative humidity affect viral propagation in mosquitoes; rates being highest in climates resembling the rainy season (Malavige et al. 2004).

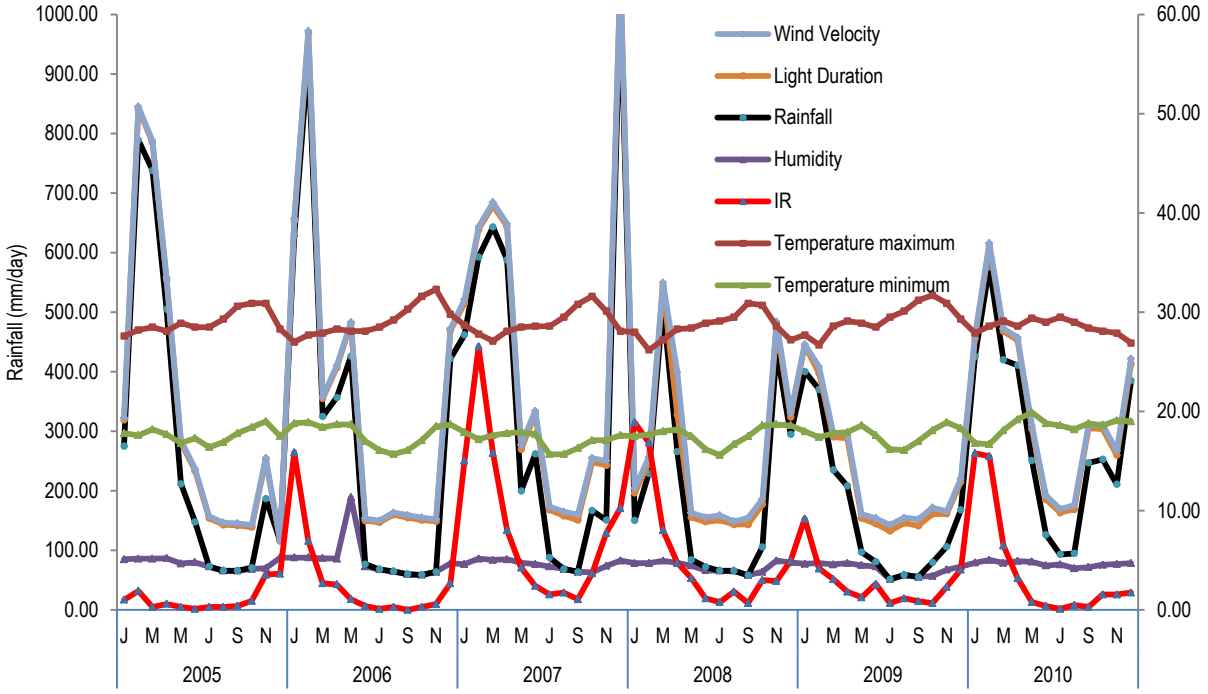

Figure 2-15. Relationship between monthly climate variability and DHF Incidence (IR) in Nganjuk District

Climate can influence the pattern of infectious diseases due to disease vectors (viruses, bacteria, parasites) are sensitive to temperature, humidity and other environmental conditions (Chakravarti and Kumaria, 2005). The WHO (2009) also states that the disease is transmitted by mosquitoes such as DHF is associated with warm weather conditions. Promprou et al. (2005) stated that the transmissions of dengue viruses were climatically sensitive. Air temperature changes affect vector-borne disease transmission and epidemic potential by altering the vector's reproductive rate, biting rate, the extrinsic incubation period of the pathogen, by shifting a vector's geographic range or distribution and

increasing or decreasing vector-pathogen-host interaction and thereby affecting host susceptibility; and there is a distinct seasonal pattern in DHF outbreaks is evident in most places. Rainfall affects the adult female mosquito density, an increase in the amount of rainfall leads to an increase in available breeding sites and the number of mosquitoes, an increase in the number of adult female mosquitoes increases the odds of a mosquito obtaining a pathogen and transmitting it to a second sensitive host.

2.3 Distribution Patterns and Relationship between Elevation and the Abundance of *Aedes aegypti* in Mojokerto City 2012

2.3.1 Introduction

Dengue hemorrhagic fever is one of the most important viral diseases in the world. Dengue viruses are among the most widely distributed and significant arthropod-borne viruses (Arboviruses) affecting humans. DHF potentially affects 2.5 billion people and in more than 100 tropical and sub-tropical regions of the world (Hopp and Jonathan, 2003; Herrera-Martinez and Alfonso, 2010). Dengue is one of the most serious health problems in Indonesia, with *Aedes aegypti* acting as the vector (Abdalmagid and Alhusein, 2008). DHF outbreaks have been recorded in the Surabaya area since 1986. DHF virus is transmitted to human through the bites of infective female *Aedes* sp. (Diptera: Culicidae) mosquitoes (Zulfaidah and Nobukazu, 2013).

Aedes aegypti often breeds in water storage containers used by households without tap water supply and occurs in high numbers even in dense urban areas (Schmidt et al. 2011).

These mosquitoes are well adapted to the urban environment and successfully breed in containers where water is allowed to accumulate, such as discarded can, bottles, plastic containers, and tires. *Aedes aegypti* is particularly susceptible to climate variability and climate change. Changes in mean climate conditions and climate variability also can affect human health via indirect pathways, especially in the changing of biological and ecological processes that influence infectious disease transmission and food yields (Hopp and Jonathan, 2001).

Global emergence and resurgence of dengue can be attributed to multiple factors, including urbanization, transportation and changes in human migration and behavior, resulting in the dengue increase as the second most important vector-borne disease, after malaria, in term of human morbidity and mortality (Herrera-Martinez and Alfonso, 2010). It also occurred in East Java, since it has been discovered in Surabaya, the number of cases tends to rise and expand in distribution. This condition is closely related to the increased mobility of the population in line with the fluent transport links and the spread of dengue virus and mosquitoes in various regions in Indonesia (Health Department of East Java Province, 2005). Environmental circumstance strongly controls the geographic distribution and abundance of *Aedes aegypti.*

Mojokerto is one of the cities in East Java that has endemic DHF. Based on the DHF case incidence data from 2008 to 2010, It was obtained from P2PL of DHF, Health Department of East Java Province showed that Mojokerto district still had House Indices (HI), Containers Indices (CI), and Breteau Indices (BI) quite high at 35%, 27.3% and 42.86% respectively (Morisita, 1959). Therefore, the observation was undertaken to

determine the distribution pattern of *Aedes aegypti* and risk factors of DHF between regency and cities in Mojokerto 2012 and the correlation between the elevation of sampling location and distribution of mosquito.

2.3.2 Materials and methods

2.3.2.1 Study Area

Mojokerto District is located between 112°26′01″ East longitude and 7°27′59″ South latitude in East Java Province. Mojokerto's territory is 872 km², located between 15 m and 3,156 m above sea level (Figure 2-16). It is divided into north and south regions by the Brantas River. The district shares the boundary with Gresik and Lamongan district in the north, with Malang district in the south, with Sidoarjo and Pasuruan in the east, and with Jombang district in the west. South Region has several mountains, which includes Welirang Mountain (3,156 m), Anjasmoro Mountain (2,277 m) and Penanggungan Mountain (1693m). Based on BPS data (2010) showed that the population of the district is about 1,014,785. Many of them earn their living as small farmers and craftsmen (consisting shoemakers, furniture makers, and souvenir makers). This area was selected as the study site based on the results of the previous research that it was the one of major DHF epidemic foci in Indonesia. The DHF incidence was recorded at the district level (lattice data).

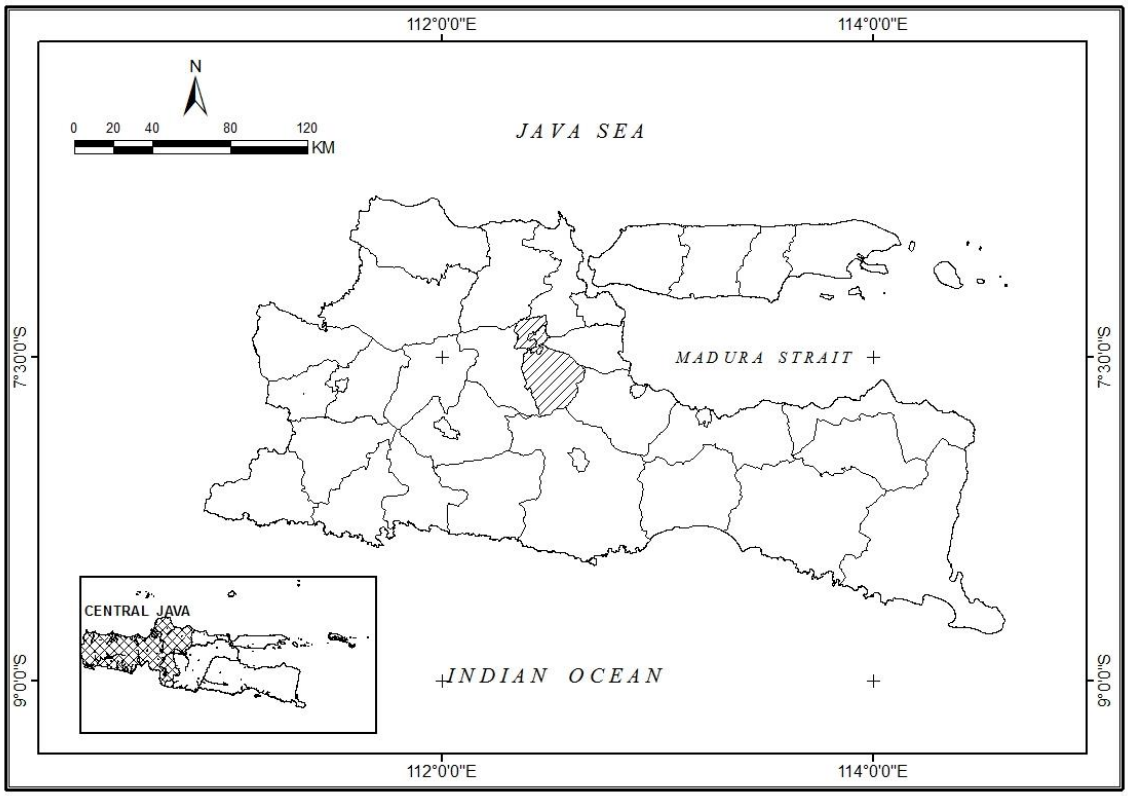

Figure 2-16. Study site

2.3.2.2 Data collection

Sampling was carried out in urban and rural area of Mojokerto District. Selected area of the urban is Prajurit Kulon while the selected rural is Dlanggu. Each area was taken 3 villages, which consists of Surodinawan, Blooto, Kranggan villages for urban area and Mojokarang, Segunung, Kalen for rural area. Each village location in the five-point set was sampled.

2.3.2.3 Capturing larvae of mosquito

Random coordinates were selected for five sampling points in each village site

using a topographical map broken into 1-m^2 grid squares. Coordinates were reselected if they fell in inaccessible areas (e.g., in the middle of ponds), or inside unsecured areas (e.g., on public roads), or were within 30 m of another coordinate (Leisnham et al. 2005). Larval sampling method with a dipper from various places such as mosquito breeding places in the house that shelters consisting of a water bath tub, tubs, toilets, drinking water containers, jars, bowl of water, and a bucket. Mosquito breeding places outside the home such as drums, cans, bottles, pot scrap, decorative plant pots filled with rainwater. Observation of the presence or absence of mosquito larvae was carried out for 4 days.

2.3.2.4 Capturing egg of a mosquito

The capture of the eggs laid by the mosquito is collected in a plastic cup that was given water. The ovitrap was placed in each house is only one piece. Ovitrap discarded and replaced by new water when sampling egg of mosquitoes are conducted at each location. The number of eggs was calculated after they hatched become mosquito larvae.

2.3.2.5 Statistical Analysis

Data analysis was performed to test the correlation between elevations of each sampling location and the number of mosquitoes in Mojokerto District.

2.3.2.6 Determining the Importance Value Index (IVI)

Important Value Index (IVI) is used to establish the dominance of a species to other species or in other words the importance of describing the position of a species in the ecological community. Calculate Frequency, Abundance, Relative Frequency and Relative Abundance, analysis using Microsoft Excel 2007. IVI (Importance Value Index) can be determined by:

IVI = Relative Abundance + Relative Frequency

2.3.2.7 Determination of Population Distribution by Morisita Index

Morisita index is a statistical measure of dispersion of individuals in a population. It is used to compare overlap among samples (Morisita, 1959). This formula is based on the assumption that increasing the size of the samples will increase the diversity because it will include different habitats (i.e. Different faunas). The standardized Morisita index was used to assess the spatial pattern (i.e. Clumped, random or uniform) of the most abundant species. The index was calculated as:

Values of $I_d = 1$ indicate a random dispersion Values of $I_d < 1$ indicate a uniform dispersion

Values of $I_d > 1$ indicate a clumped dispersion

The distribution pattern of population can be determined by using the Morisita index by the following formula:

$$\frac{\sum_{i=1}^{n} X_i^2 - N}{N(N-1)} Id = n \qquad (6)$$

N = total number of individuals in the plot; n = number of plots
Xi2 = the square of the number of individuals in the plot to I (Wolda, 1981)

2.3.3 Results

Mosquitoes were found in Mojokerto region consisting of five species: were *Aedes aegypti, Aedes albopictus, Aedes laniger, Culex bitaeniorchynchus and Culex quinquefasciatus*.

Table 2-3. Abundance of mosquito's larvae in Mojokerto

Species	**Abundance (individual)**	
	City	**Regency**
Aedes aegypti	52	50
Aedes albopictus	49	62
Aedes laniger	-	7
Culex bitaeniorchynchus	-	48
Culex quinquefasciatus	39	104

Table 2-4. Abundance of mosquitoes was captured with ovitrap

Species	Abundance (individual)	
	City	Regency
Aedes aegypti	54	55
Aedes albopictus	20	22
Culex quinquefasciatus	50	99

Table 2-5. Pearson correlation between elevation of each location and the density of *Aedes aegypti* in Mojokerto District

		Elevation	*Aedes aegypti*
Elevation	Pearson correlation	1	0.418*
	Sig. (2-tailed)		0.022
	N	30	30
Aedes aegypti	Pearson correlation	0.418*	1
	Sig. (2-tailed)	0.022	
	N	30	30

* Correlation is significant at the 0.05 level (2-tailed).

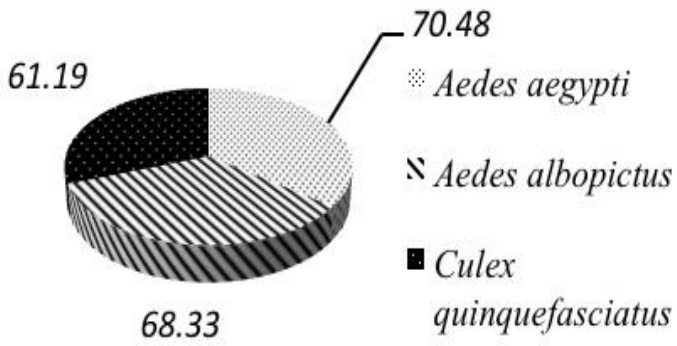

Figure 2-17. Important Value Index (%) of mosquito larvae in Prajurit Kulon Sub district

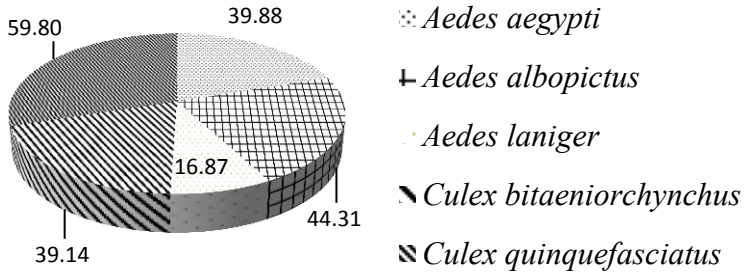

Figure 2-18. Important Value Index (%) of mosquito's larvae in Dlanggu Sub district

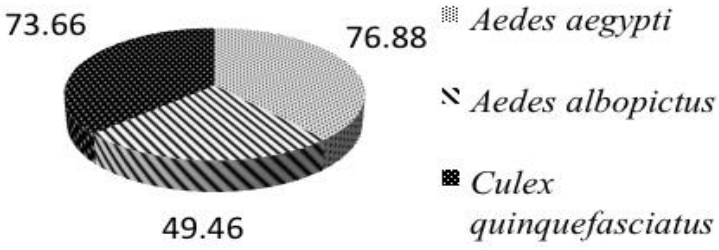

Figure 2-19. Important Value Index (%) of mosquito's larvae in Prajurit Kulon Sub district (captured with ovitrap)

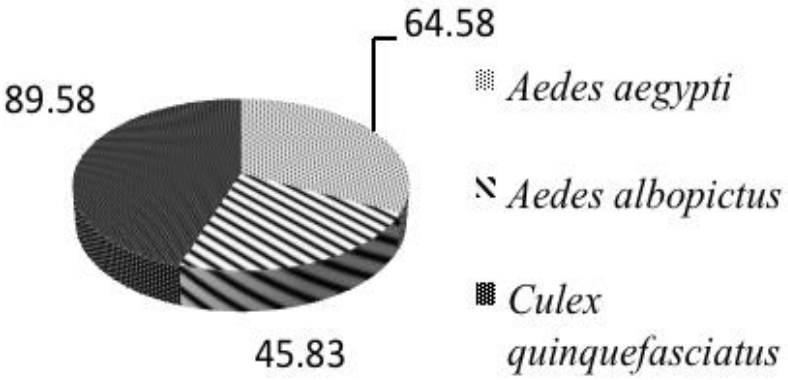

Figure 2-20. Important Value Index (%) of mosquitos' larvae in Dlanggu Subdistrict (captured with ovitrap)

2.3.4 Discussion

Mosquito larvae in Prajurit Kulon sub district (Table 2-3) were encountered in the present study are *Aedes aegypti, Aedes albopictus and Culex quinquefasciatus*. *Aedes aegypti* is the dominant species as the highest IVI value. *Aedes aegypti* with Importance Value Index (IVI) of 70.48%, followed by *Aedes albopictus* (68.33%) and *Culex quinquefasciatus* (61.19%) (Figure 2-17). *Aedes aegypti* is most often found in heavily populated/urban areas (Prajurit Kulon Sub district) than rural area (Dlanggu Sub district). There is also a similar result of the previous studies stated that *Aedes aegypti* is more dominant than *Aedes albopictus* (Budiyanto, 2012).

Species of mosquitoes were found in the Dlanggu sub district (Table 2-3) are *Aedes aegypti, Aedes albopictus, Aedes laniger, Culex bitaeniorynchus, Culex quequinfasciatus. Culex quinquefasciatus* has the highest IVI value (59.8%) than other species, and there is,

followed by *Aedes albopictus* (44.31%), *Aedes aegypti* (39.88%), *Culex bitaeniorynchus* (39.14%), *Aedes laniger* (16.87%) (Figure 2-18).

Data of Health Department of Mojokerto District (2012) showed that the outbreak of DHF was still suffering highly in this region. The occurrence of DHF outbreaks is linked to a number of factors, including the density of mosquito vectors particularly that of *Aedes aegypti.* The precise population density of *Aedes aegypti* that is needed to sustain dengue virus transmission epidemically or endemically has yet to be determined, but experience in Nganjuk during the past 3 years from 2008 to 2010 suggest that house indices as low as 30% are sufficient for the epidemic transmission of dengue in areas where there is a low level of immunity in the human population. In many instances, a small number of actively biting female mosquitoes have infected an entire household. Denser human population increases virus transmission. Urbanization in tropical countries has resulted in both a proliferation of *Aedes aegypti* and an increase in the number of susceptible human hosts (Basiri et al. 2011). In the cities (for example: Prajurit Kulon Sub district), the movement of viraemic persons is a more important means of transporting dengue viruses than the movement of *Aedes aegypti* mosquitoes. A place where people congregate during the day has important sites of dengue virus transmission. Dengue virus may also spread in settings involving large numbers of people such as in hospitals where visitors, patients and staffs may be bitten by infected *Aedes aegypti.*

Some of other factors that also influence DHF outbreaks are the behavior of female mosquitoes to lay their eggs. Various factors that caused a lot of *Aedes* and *Culex* larvae are found in the surrounding area Dlanggu Subdistrict, Mojokerto including residential areas,

shrubs (vegetation) lush, plantation, cattle sheds, gutters, and along the river. Based on the survey in this regency showed that *a Culex quinquefasciatus larva is* the most dominant species. This is in contrast with previous research; it stated that *Culex quinquefasciatus* is a species commonly found in urban residential areas (Figure 2-20).

The capturing egg of mosquitoes using ovitrap in Kulon Prajurit region performed that three types of mosquitoes (Table 2-4). These species are *Aedes aegypti*, *Aedes albopictus* and *Culex quinquefasciatus*. The dominant species is *Aedes aegypti* with Importance Value Index (IVI) of 76.88%, followed by 73.66% for *Culex quinquefasciatus* and *Aedes albopictus* amounted to 49.46%.

Aedes aegypti is the predominant species in urban areas due to the location of housing coincide thus increasing mosquito breeding. Density of eggs / larvae of mosquitoes in the container are influenced by the type, color and ability to absorb water containers. Smooth-walled containers light and does not absorb water, as it is owned by ovitrap glass, relatively less favored by mosquitoes. Smooth surface will make it difficult for mosquito oviposition.

Based on the capturing mosquitoes using ovitrap in Mojokerto regency were found that three species of mosquitoes, including *Aedes aegypti, Aedes albopictus, and Culex quinquefasciatus*. The dominant species are the *Culex quinquefasciatus* with Importance Value Index (IVI) of 89.58%, followed by *Aedes aegypti* (64.58%) and *Aedes albopictus* (45.83%).

Culex quinquefasciatus is the dominant species due to the condition of the house adjacent to the yard there are trees and grass. Around the site, there is also stagnant and dirty water, sewers, which are potential sites for *Culex* mosquito breeding. Based on

observation of my study (2013), there is also some standing water, either rainwater or water reservoirs households that are not covered. Mosquito larvae are generally found in various places such as aquatic ponds, artificial containers, and tree holes in the other pool. Development of mosquito larvae mainly was influenced by physical factors, especially temperature. Optimal temperature in media ranges from breeding places 25-27 ˚C. Based on temperature data obtained from BMKG (BMKG Malang, 2013) showed that in January the average temperature in the area of Mojokerto District reach 27 ˚C, so it can be said immature mosquitoes experiencing normal growth process.

2.3.4.1 The distribution pattern of mosquitoes in Mojokerto District.

Based on calculations using the Morisita index, it was noted that the distribution pattern of mosquitoes in the Mojokerto District is uniform. Mosquitoes have a uniform distribution due to environmental factors such as temperature and humidity at that location is not much different among each site. There are a variety of physical environments that may affect the distribution of mosquitoes, such as home layout, type of container, altitude/elevation and climate. Presence or absence of mosquitoes in residential greatly influenced by the infrastructure of the house itself. The home construction, home wall color and arrangement of goods inside the home are also deeply affected to determine the home being liked or not by mosquitoes. Types of containers, including the location of containers, container material, container shape, container color, the volume of water, cover the container, and the origin of water in the container are also influenced in the selection of female mosquitoes for laying their eggs.

One of the models to determine the distribution pattern of mosquitoes existing in a location is morisita index. It is useful to know the potential areas that are estimated to have high mosquito populations. Regions with high mosquito populations have the potential to contract the disease. Spread of mosquito species in Indonesia came from cities to cities, including the villages, due to transportation venues carrying rainwater as drums, cans, old tires, and other items containing larvae mosquitoes. The spreaders of the mosquito population are also closely related to the development of human settlements due to the establishment of the new homes are equipped with the means of procuring water for daily use.

2.3.4.2 Relationship between elevation factors and density of *Aedes aegypti*.

Pearson correlation was conducted in Mojokerto District, 2012 related to elevation of each area sampling to the density of *Aedes aegypti* measures. The results showed that elevation and the density of mosquitoes were positive association. In general, there were few species per site with increasing altitude. The areas at the lowest elevation produced the greatest number of species, but did not produce a corresponding greater number of specimens. A decrease in the number of mosquito species at the higher elevation has already been reported (Devi and Jauhari, 2004). Other studies argued that the larger number of mosquito species collected at lower elevation may be due to increased human disruption in those areas. There is a similarity between the present findings and those already made in respect of a number of species, which remained relatively constant for the first 800 m from the lowest level followed by a decrease. There are a number of views about the increased diversity at lower altitudes, but the possible explanation could be the availability of

favorable breeding places and preferred host. Another cause may be related to the dispersal of a mosquito, since they have to stop while flying to refuel with blood/nectar. Further, the tropical zone ranging between 300 and 900 m has the maximum temperature between 27.2 and 29.4^{O}C during June whiles the lowest (11.1– 13.3^{O}C) in the month of January. As the optimal range of temperature for the best survival of mosquito is from 22 to 31^{O}C, hence in the present study the diversity is more between 500 and 900 m. A slight variation in the distribution and abundance of most of the mosquito species during the study period could be the result of several interacting climatic factors, which depend on the severity of the amount and duration of rain in the wet season. As these conditions fluctuate season-to-season and place-to-place, henceforth, restriction on elevation distribution is a result of habitat specificity. Another possible explanation is that the elevation may limit niche availability, which results in a differential distribution pattern (Devi and Jauhari, 2004).

2.3.5 Conclusion

The mosquitoes found in Mojokerto region consist of five species. There were *Aedes aegypti , Aedes albopictus, Aedes laniger, Culex bitaeniorchynchus and Culex quinquefasciatus.* Although *Aedes aegypti* is the predominant species found in Mojokerto region, 2012, but this species still plays an important role for the outbreak of DHF. *Aedes aegypti* has an Importance Value Index (IVI) of 70.48%. Based on calculations using the Morisita index, the pattern of spread of mosquitoes in the area of Mojokerto is uniform. The elevation of each sampling area and density of mosquitoes has positive associations.

Chapter 3

Biological control using the most potential *Bacillus thuringiensis* isolated from East Java for suppressing *Aedes aegypti* population

3.1 Toxicity studies for indigenous *Bacillus thuringiensis* isolates from Malang City, East Java on *Aedes aegypti* larvae.

3.1.1 Introduction

Dengue hemorrhagic fever (DHF) is one of the most serious public health problems in Indonesia and many other tropical countries around the world. DHF outbreaks have occurred in Indonesia since 1986; the first case was in Surabaya. In 2010, there were more than 26,059 reported DHF cases in East Java (Provincial Health Office, 2011). Dengue fever remains a serious health problem in both urban and rural areas of East Java. This is because dengue fever is a disease that causes high yearly mortality. Malang City is one of the cities in East Java that has endemic DHF. Sawojajar, Purwantoro, Jatimulyo, Klojen and Sukun are sub-districts of Malang City that became DHF-endemic areas in the last three years (Malang City Health Office, 2008). DHF is transmitted predominantly by

Aedes aegypti mosquitoes that have adapted to living near human-inhabited areas (Mulyatono, et al. 2012).

Aedes aegypti (Linnaeus) is the major urban vector of the dengue virus worldwide (Jansen and Beeb e, 2010). This mosquito species have Cosmo-tropical distribution and is widely dispersed throughout Indonesia. Therefore, an effective environmental management system is necessary to avoid the spread of human disease.

B. thuringiensis is an important insect pathogen that is highly toxic to mosquito larvae and related dipterans (Poopathi and Abidha, 2010). The toxicity is attributed to δ-endotoxin, which is made of proteins that are produced and assembled when the bacteria sporulate (Sanahuja et al. 2011; Haggag and Yousef, 2010)[1]. In Indonesia, some insecticides use active microbial *B. thuringiensis* imported from countries such as Belgium (Bactospeine), the United States (mop) and Switzerland (Thuricide). The original *B. thuringiensis* exploration efforts in Indonesia were carried out because the *B. thuringiensis* crystal protein has an arrow host spectrum. Therefore, the ideal effort in controlling Indonesian mosquitoes would be using *B. thuringiensis* isolated from Indonesia. The objective of this study was to investigate the toxicity of indigenous *B. thuringiensis* isolates from Malang City on *Aedes aegypti* larvae.

3.1.2 Materials and methods

3.1.2.1 Study area

Malang City is the second largest city in the East Java province, Indonesia, and in 2008, it was established as the fourth largest city in Indonesia. It is located approximately 400 meters above sea level, which allows the city to have cooler weather and a temperate climate. Malang City is located between 07°46'48"- 08°46'42" south latitude and

112°31'42"- 112°48'48" east longitude (Malang City Health Office, 2008). The Malang City district borders Mojokerto and Pasuruan to the north, the Lumajang district to the east, the Blitar district to the west, and the Indian Ocean to the south. Soil sample sites were determined based on endemic DHF data from the Malang City health office in a preliminary study using search-sampling methods. Each soil location in the five-point set was sampled (Figure 3-1).

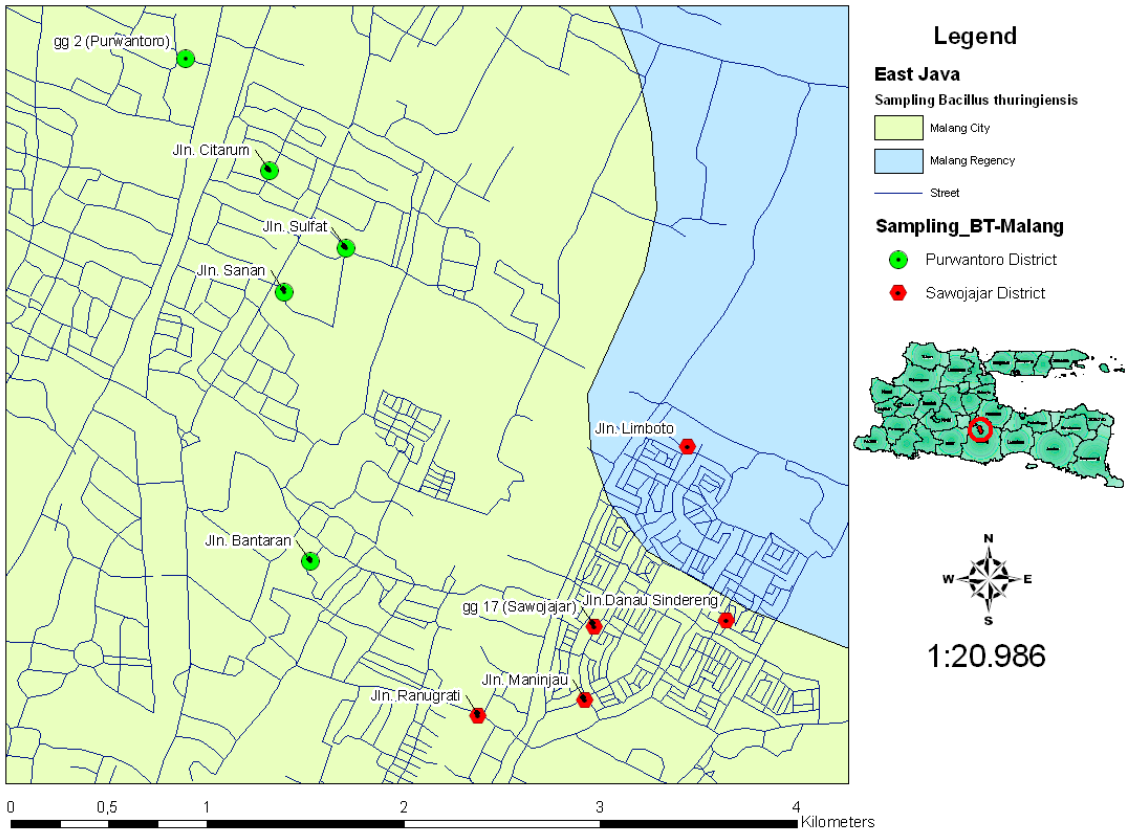

Figure 3-1. Study area in Malang City

3.1.2.2 Isolation of bacteria

Five soil samples were taken from10 cm below the soil surface at each location (Chartterjee et al. 2007). The soil samples were as large as 25 grams and were placed into

225 ml sterile physiological saline solution (0.85% NaCl). Serial 10^{-1}-10^{-6}-fold dilutions of the sample suspensions were made in NaCl. Then, 0.1ml of the suspension was inoculated into a petri dish containing *B. thuringiensis* selective media. Cultures were incubated for 48-72 hs at 30^{o} C. Any isolates that grew were purified on a quadrant streak plate. Single colonies were inoculated into *B. thuringiensis* selective media. Cultures were incubated for 48-72 hs at 30^{o}C. Isolates were stored in NA and *B. thuringiensis* selective media at 4°C and in glycerol at-80°C.

3.1.2.3 Classification of bacteria based on phenotypic attribute similarity

The phenotypes of the bacterial isolates were characterized by morphological observation and biochemical and physiological tests (Zakeel et al. 2009). Morphological observations were performed on single cells or a single colony. Single colonies were observed for color, surface texture, colony structure, gram staining and the presence of endospores. Biochemical tests performed included the catalase test, growth on Simmons citrate agar, urea hydrolysis, methyl red and acid butanediol (Voges Proskauer) fermentation, carbohydrate fermentation, motility, tryptophan hydrolysis, the oxidase test and the nitrate test. Observed physiological test parameters consisted of temperature (30 and 60 °C) and pH (4.0, 7.0 and 9.0). Bacterial density was determined with a spectrophotometer at a 600 nm wavelength (Gong et al. 2012). Phenotypic data with a plus (+) or minus (-) sign were compiled using Microsoft Excel. The data that changed were used to construct a CLAD97 dendrogram reflecting operational taxonomic units (OTU) based on the similarity index value as determined by simple matching methods (SSM). The unweight pair group method using arithmetic averages (UPGMA) algorithm was used.

3.1.2.4 Bacteria and spore number determination

The total number of live bacteria was calculated from stock bacterial suspension cultures that were inoculated into NB and homogenized. In total, 0.1 ml of the stock bacterial suspension from each dilution was inoculated on NA and incubated at 30°C. The number of *B. thuringiensis* was based on the number of colonies that grew after incubating for 12, 24, 36, 48, 72 and 96 hs.

3.1.2.5 Endospore screening

Isolated colonies were inoculated on sporulation media (10 g glucose, 7.5 g peptone, 6.8 g KH_2PO_4, 123 mg $MgSO_4.7H_2O$, 2.33 mg $MnSO_4.4H_2O$, 14 mg $ZnSO_4.7H_2O$, and 320 mg $Fe(SO_4)$ dissolved in 1 N NH_4Cl). The bacterial suspensions were incubated at 30°C and shaken at120 rpm for 24 hs; then, 3.75 ml of the suspension was inoculated into 25 ml of sporulation media and incubated at 30°C, where it was shaken at 120 rpm for 24 hs. Spore prevalence was calculated based on the number of live spores divided by the number of live cells times 100% (Jensen et al. 2002).

3.1.2.6 Testing of Aedes aegypti

Aedes aegypti larvae were provided by the Central Council of Vector and Disease Reservoir Research and Development (B2P2VRP), Salatiga, Indonesia. The larvae were transferred to the laboratory of Ecology and Diversity, Department of Biology, Brawijaya University, where self-perpetuating colonies were established and maintained for the present study.

Toxicity studies were performed on test compounds as described by Wright in 1971(Wright, 1971; El-kersh et al. 2012), with some modifications (Wright, 1971; El-

kersh, et al. 2012). Mortality data were analyzed using log-probit analysis to estimate the probit regression line and calculate LC_{50} (El-maghraby et al. 2012).

3.1.2.7 Evaluation of the potential bacteria on *Aedes aegypti* larvae

Bacillus thuringiensis toxicity against *Aedes aegypti* larvae was evaluated by randomized factorial design. Tests on each isolate were repeated three times. For each *B. thuringiensis* isolate, the effects on larval mortality after 24, 48, and 72 hs were observed. One single and isolated colony was sub-cultured in nutrient broth (NB) media, incubated for 24 hs at 30°C and centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the pellets were added to a 10 ml bacterial culture followed by another centrifugation, which was repeated three times. The subsequent pellets were then added to 1ml 0.85% NaCl and homogenized. The pellets were inserted into a tube, and NaCl was added to the volume reached 10 ml. Suspension test samples were made by adding 10 ml of the suspension pellets to 90 ml of sterile NaCl. Dilutions of bacterial suspension were made based on different bacterial cell densities, such as 1:0, 1:1, 1:3, 1:5, 1:7, 1:10, and 1:20. Each dilution was tested in a tube that contained 20 mosquito larvae, and each isolate was tested three times. Each isolate was exposed for 24, 48 or 72 hs, and it was observed whether there was an effect of *B. thuringiensis* on larval mortality. As a control, 10 mosquito larvae were tested in a tube without *B. thuringiensis*.

3.1.2.8 Statistical analysis

The percentage of larval mortality was analyzed using probit regression. LC_{50} was analyzed by analysis of variance (ANOVA) and it will be continued the TUKEY HSD interval was 95%.

3.1.3 Results

Six of 33 bacterial isolates were selected (PWR4-31, PWR4-32, SWJ4-2b, SWJ4-4b, SWJ-4k and SWJ5-1) that phenocopy reference *B. thuringiensis*. The reference *B. thuringiensis* isolates used in this study was isolated from the swamp in Rancak Upas, Bandung District, and the other bacteria were from the Bandung Institute of Technology (ITB) collection.

3.1.3.1 Phenotypic characteristics and similarity among *B. thuringiensis* isolates

All of the bacterial isolates had similarity values of 71%. Based on the dendrogram (Figure 3-2) and on phenotype similarity values greater than 71%, isolates were classified into two groups based on colony color. The first group, containing PWR4-32, PWR4-31, SWJ5-1, and SWJ4-4b, had a similarity value of 78% compared with *B. thuringiensis*. The second group contained SWJ4-4k and SWJ4-2b and had an 82% similarity value. Subgroup classification was based on endospore location: one subgroup had a sub terminal spore location, and the second subgroup had a central spore location.

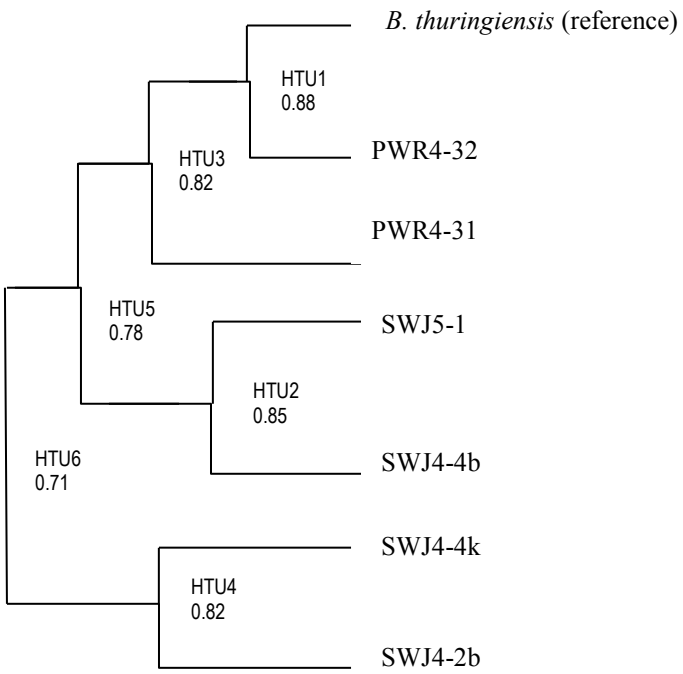

Figure 3-2. Dendrogram showing relationships of Malang City indigenous *B. thuringiensis* isolates and reference *B. thuringiensis*.

The similarity value between *B. thuringiensis* and isolate PWR4-32 was 88% (Figure 3-2). Both of the isolates were in different groups than isolate PWR4-31, which had a similarity value of 82%. A distinguishing characteristic among the three bacteria was the oxidase enzyme. *B. thuringiensis* and isolate PWR4-32 had oxidase, but isolate PWR4-31 did not (Table 3-1). Distinguishing factors between isolate PWR4-32 and *B. thuringiensis* were related to motility and nitrate: PWR4-32 isolates are not motile, and they cannot reduce nitrates. In contrast, *B. thuringiensis* are motile, and they can reduce nitrate. Based on the phenotypic characteristics and literature searches, it was determined that *B. thuringiensis* (reference) has similar characteristics to the *B. thuringiensis subspecies aizawa* (Dulmage et al. 1990).

Table 3-1. Phenotypic characteristics of bacterial isolates

Phenotypic characteristics	PWR4-31	PWR4-32	SWJ4-2b	SWJ4-4b	SWJ4-4k	SWJ5-1	Reference *B. thuringiensis*
colony observation							
* circular	+	+	+	+	+	+	+
* entire	+	+	+	+	+	+	+
elevation							
*effuse	+	+	-	+	-	-	+
*convex	-	-	+	-	+	-	-
*umbonate	-	-	-	-		-	-
*opaque	+	+	+	+	+	+	+
colour							
* dark white	+	+	-	+	-	+	+
* yellow	-	-	+	-	+	-	-
cell observation							
*shape: basil	+	+	+	+	+	+	+
* colour: purple/violet	+	+	+	+	+	+	+
endospore							
* central	-	-	+	+	-	+	-
* terminal	-	-	-	-	+	-	-
* sub terminal	+	+	-	-	-	-	+
catalase	+	+	+	+	+	+	+
oxidase	-	+	-	+	-	+	+
motility							
* rhizoid	-	-	+				+
* arborescent	-	-	-	+	-	-	-
* immobile	+	+	-	-	+	+	-
Simmon's citrate	-	-	-	-	-	+	-
MR	-	-	-	-	-	-	-
VP	-	+	+	-	-	-	+
carbohydrate fermentation							
* glucose	+	+	+	+	+	+	+
* maltose	-	+	+	+	+	+	+
* lactose	+	+	+	+	+	+	+
* sucrose	+	+	+	+	+	+	+
* mannitol	+	+	+	+	+	+	+
urea hydrolysis	-	-	-	+	+	+	-
tryptophan hydrolysis							
nitrate	+	-	+	+	+	+	+
temperature							
* 30^{o}C (mesophyl)	+	+	+	+	+	+	+
* 60^{o} C (thermophile)	+	-	+	-	+	-	-
pH							
* acid (pH 4.0)	+	+	+	+	+	+	+
* neutral (pH 7.0)	+	+	+	+	+	+	+
* alkali (pH 9.0)	+	+	+	+	+	+	+

3.1.3.2 Spore prevalence in *B. thuringiensis* isolates

Determination of the isolates that would be used for testing mosquito larvae toxicity was based on the highest toxic – prevalence among the six isolates. Three isolates, PWR4-32, SWJ4-4b and SW5-1 were more prevalent than *B. thuringiensis* and these isolates had a spore prevalence of 52.44%, 23.59%, and 34.46%, respectively. The results are presented in Table 3-2. Relationship between the cell density and the spore number for reference *B. thuringiensis* is illustrated in Figure 3-3, Figure 3-4, and Figure 3-5, respectively for three isolate, PWR4-32, SWJ4-4b and SW5-1.

Table 3-2. Analysis of spore prevalence in *B. thuringiensis* isolates at 48 hours

Isolate	**Prevalence of spores (%)**
PWR4-31	9.28 ± 11.49 (a)
PWR4-32	52.44 ± 40.09 (a)
SWJ4-2b	0.82 ± 0.55 (a)
SWJ4-4b	23.59 ± 9.91 (a)
SWJ4-4k	4.34 ± 21.38 (a)
SWJ5-1	34.46 ± 12.28 (a)
Reference *B. thuringiensis*	10.02 ± 36.96 (a)

The same letters after the numbers denote no significant difference ($\alpha = 0.05$).

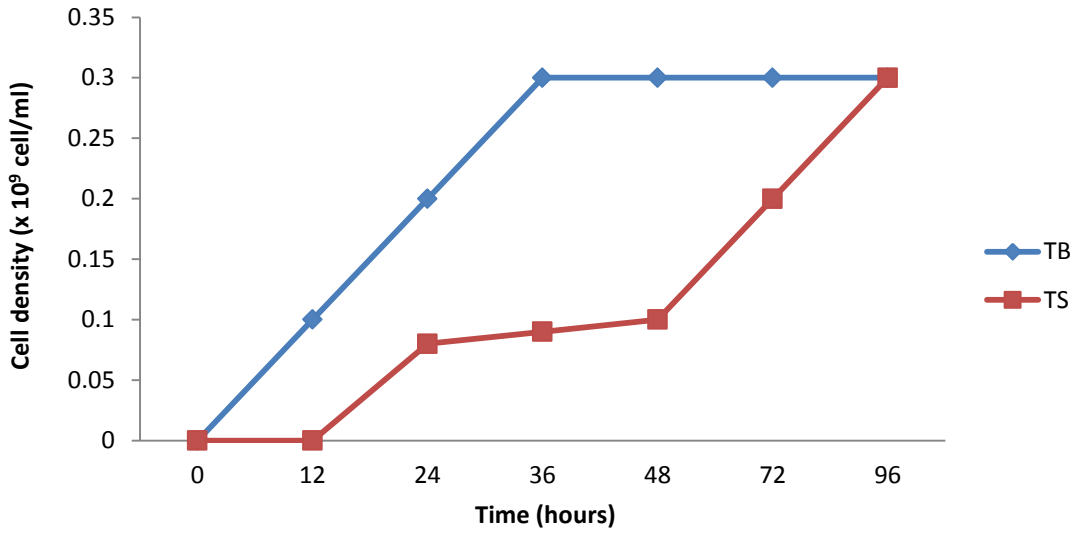

Figure 3-3. Relationship between the cell density (TB) and the spore number (TS) for reference *B. thuringiensis.*

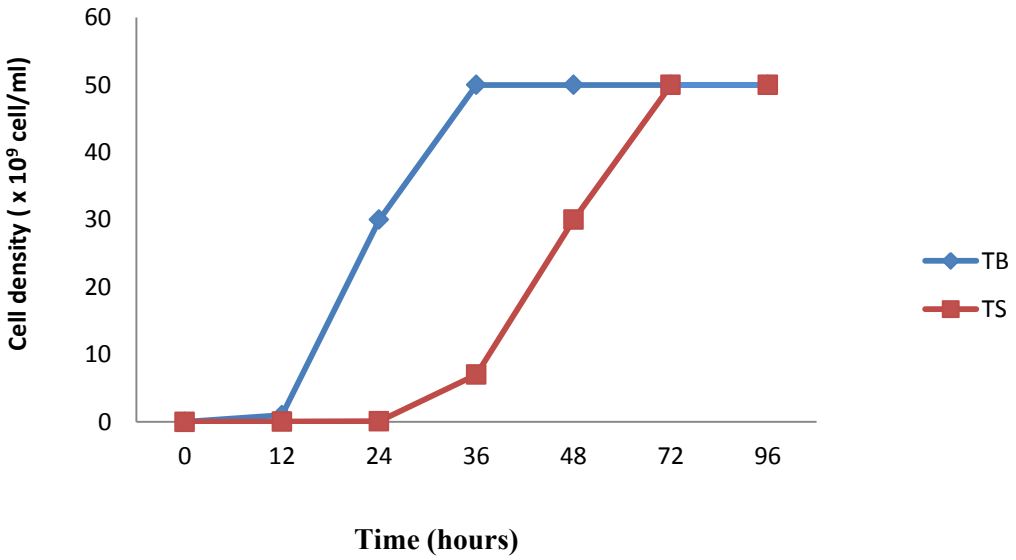

Figure 3-4. Relationship between the cell density (TB) and the spore number (TS) for isolate PWR4-32.

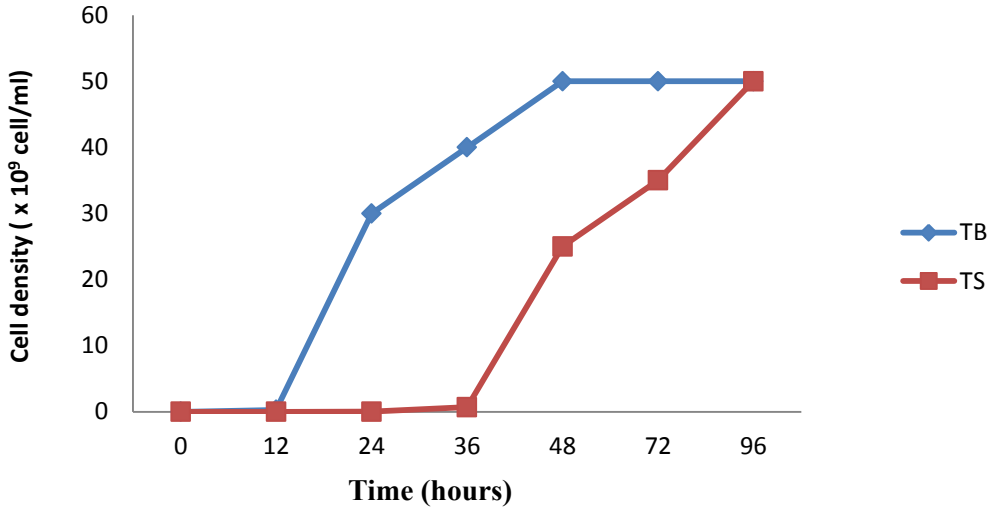

Figure 3-5. Relationship between the cell density (TB) and the spore number (TS) for isolate SWJ4-4b.

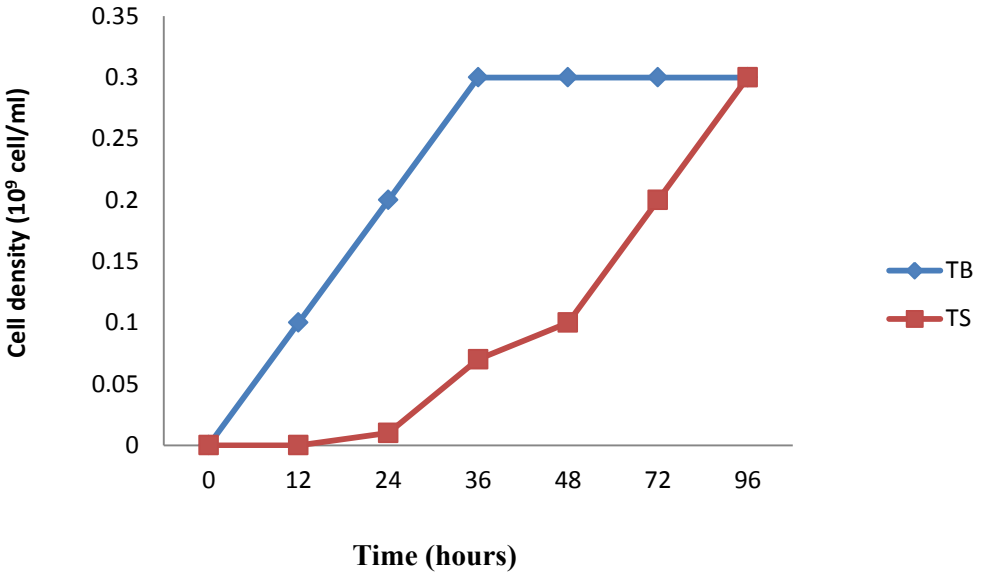

Figure 3-6. Relationship between the cell density (TB) and the spore number (TS) for isolate SWJ5-1.

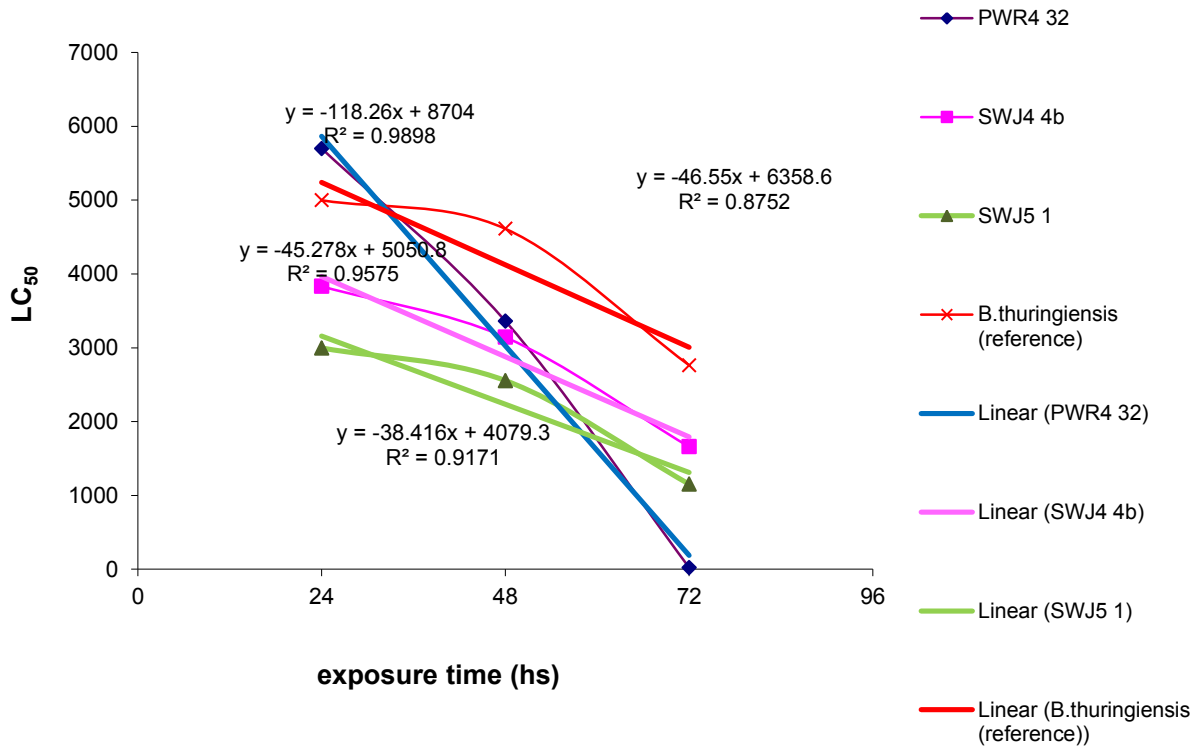

Figure 3-7. Relationship between the LC_{50} and the *B. thuringiensis* exposure time

Increasing the cell density and the spore number for all isolate or increasing the exposure time showed that a proportional increase in mortality rate or decrease in LC50, respectively as Figure 3-7 above.

3.1.3.3 Toxicological evaluation of indigenous *B. thuringiensis* isolates against *Aedes aegypti* larvae

The ANOVA and the LC_{50} (lethal concentration) results indicate a significant effect (p<0.05) among the tested isolates. The three isolates that are indigenous to Malang City (PWR4-32, SWJ 4-4b, SWJ 5-1) killed *Aedes aegypti* larvae. Among those, the PWR4-32 isolates were the most effective, as 22.79 x 10^7 cells/ml were required to kill fifty percent

of the *Aedes aegypti* larvae within 72 hours. The72-hs exposure time was more effective than the 24-hs and 48-hs exposure times.

Table 3-3. ANOVA among the isolates based on LC_{50}

Isolate	Cell density (x 10^9 cells/ml)		
	$LC_{50\text{-}24h}$	$LC_{50\text{-}48h}$	$LC_{50\text{-}72h}$
Control	-	-	-
PWR4-32	56.99 (dA)	33.61 (bB)	0.23 (aC)
SWJ4-4b	38.32 (cA)	31.42 (bA)	16.59 (abB)
SWJ5-1	29.97 (bA)	25.55 (bA)	11.53 (abB)
Reference *B. thuringiensis*	49.98 (dA)	46.11 (cA)	27.63 (bA)

The same letters after the numbers denote no significant difference ($\alpha = 0.05$). The capital letters indicate the time between trials, while the lowercase letters indicate tests among the isolates.

3.1.4 Discussion

3.1.4.1 Phenotypic characteristics and similarity among *B. thuringiensis* isolates

Similar characteristics among the six selected isolates were as follows: they were rod-shaped bacteria, they were gram-positive, they exhibited oval endospore production, they were circular in shape along the entire edge, and they had an opaque inner structure. None of the isolates was able to convert glucose into acid in the methyl red test. The Voges-Proskauer (VP) test revealed that isolates PWR4-32 and SWJ4-2b could convert glucose to acetoin. These results are similar to those of reference *B. thuringiensis*, which had negative MR and positive VP test results. All six of the isolates grew well at 30°C, but three of the isolates (PWR4-32, SWJ4-4b, and SWJ5-1) could not grow at 60°C. All of the isolates grew at pH 4.0, 7.0 or 9.0. In general, neutral (pH 7.0) media are used for

optimum bacterial growth (Das and Dangar, 2008). These data can be used as a basis for bacterial toxicity tests against mosquito larvae. Bacteria can survive for a long time and have high efficacy against mosquito larvae at pH 7.0 (Raina et al. 2009).

3.1.4.2 Spore prevalence in *B. thuringiensis* isolates

If bacterial spore prevalence was increasing, it can be assumed that the amount of toxin produced was also growing. As the number of bacterial toxins increases, one may expect the bacteria to be more effective at killing mosquito larvae. There are differences in spore prevalence that are associated with the individual characteristics of the spore-forming isolates. *B. thuringiensis* has two developmental phases: germination and sporulation (Manonmani et al. 2011). During sporulation, parasporal crystals are released by autolysis. These crystals are toxic and will damage the mosquito larval digestive tract, thus causing larval mortality.

The early stationary phase is marked by vegetative cell death, followed by toxin accumulation, because the cells metabolize the available nutrients, resulting in nutrient shortage and competition. The bacteria will then synthesize secondary metabolites that are used to maintain life. In *B. thuringiensis*, this stationary phase is associated with spore and toxin formation. Toxins from *B. thuringiensis* cells are formed after the cells have formed endospores (Muniady et al. 2011).

3.1.4.3 Toxicological evaluation of indigenous *B. thuringiensis* isolates against *Aedes aegypti* larvae

Toxicity tests were conducted using various dilutions of the bacterial suspension (1:0, 1:1,1:3, 1:5, 1:7, 1:10, 1:15, and 1:20) and exposure times (24, 48, and 72h). The toxin effectiveness of the *B. thuringiensis* isolates was determined. The bacteria form spores and parasporal crystals during the stationary phase, which is a nutritionally deficient state; at that time, the parasporal crystals were toxic and can kill the *Aedes aegypti* larvae (Renganathan et al. 2011). The mosquito third instar larvae were selected because at this stage, the larvae have a complete anatomical structure and the body is divided into three parts (head, thoracic, abdomen); therefore, damage to the larvae can be easily observed within each section. A previous study demonstrated that the numbers of intestinal epithelial cells and peritrophic cells increase in accordance with in creasing larval toxin resistance (Wirth, 2010).

The ANOVA and the LC_{50} (lethal concentration) results indicate a significant effect ($p<0.05$) among the tested isolates. The three isolates that are indigenous to Malang City (PWR4-32, SWJ 4-4b, SWJ 5-1) killed *Aedes aegypti* larvae. Among those, the PWR4-32 isolates were the most effective, as 22.79 x 10^7cells/ml were required to kill fifty percent of the *Aedes aegypti* larvae within 72 hs. The72-hs exposure time was more effective than the 24-hs and 48-hs exposure times. Once the bacterial toxin enters the mosquito larvae digestive tract, the increased time allows the toxin to accumulate, and the toxin's (δ-endotoxin) effects increase in accordance with the exposure. The toxin binds to receptors located on the epithelial midgut cell wall's apical microvillus membrane. After the toxin binds to its receptor, there is a change in the toxin's conformation, allowing toxin insertion

into the membrane. Electrophysiological and biochemical evidence suggest that the toxins generate pores in the cell membrane, which disturbs the osmotic balance; consequently, the cells swell and lyses. The gut becomes paralyzed, and the mosquito stops feeding. Most mosquito larvae generally show reduced activity after two hs and die within six hs of toxin injection (Poopathi and Abidha, 2010; Poopathi, 2010). Mosquito larvae mortality is also influenced by factors such as bacterial concentration, larval age and the bacterial strain used (Ramires-Suero et al. 2011; Poopathi and Tyagi, 2006).

A negative relationship can be observed between the exposure time and the LC_{50} for the *B. thuringiensis* indigenous to Malang City and the reference *B. thuringiensis*. This means that with a longer exposure time, the LC_{50} value will decrease and the larval mortality level will increase (Arrivoli et al. 2011; Valadez-Lira et al. 2011). All of the tested and reference isolates had the highest percentages for larval mortality at the highest cell density and the longest exposure time because the toxins were released by the bacteria and accumulated in the *Aedes aegypti* larvae's digestive tract. Increased mortality was also observed for the mosquito larvae. These combined conditions resulted in a higher mosquito larvae mortality rate.

3.2 Toxicity studies for indigenous *Bacillus thuringiensis* isolated from East Java on *Aedes aegypti* larvae.

3.2.1 Introduction

Dengue hemorrhagic fever (DHF) has become a major International public health concern in recent years; two-fifth of the world's population is now at risk from dengue. It

is estimated that there are 50 million cases of DHF infection worldwide every year (Tolle 2009; WHO 2009, 3-86). DHF is transmitted predominantly by *Aedes aegypti* that has cosmo-tropical distribution throughout Indonesia. DHF is a serious disease that causes high yearly mortality in East Java. DHF outbreaks have occurred since 1986; the first case was in Surabaya. In 2010, there were more than 26,059 reported DHF cases in East Java (Health Department of East Java Province 2011).

Aedes aegypti (L.) is the main dengue vector worldwide because of its close association with humans in tropical and sub-tropical urbanized areas. This mosquito encounters other invasive or native container mosquitoes (mostly tree hole mosquitoes) with similar requirements of aquatic habitats for its immature development (natural and artificial containers) in any part of the world (Cox et al. 2007). The transmission cycle for dengue is human - mosquito – human. Importantly, mosquitoes do not naturally carry the dengue virus, but it must acquire from a dengue-infected person before they can transmit it to another person (Pratt and Chester, 1993).

The incidence of mosquito borne diseases is increasing due to uncontrolled urbanization, creating mosquito genetic conditions for the vector mosquito populations. Therefore, mosquito control forms an essential component in the control of mosquito borne diseases (Poopathi, 2010). Potential approaches to reduce dengue infection include reduction of mosquito abundance, prevention of contact between the vector and humans, genetically manipulated vector mosquito and vaccine (Eisen et al. 2009; Gubler 1988). Currently, the only available control strategies are reducing mosquito abundance, reducing the adult mosquito lifespan, and preventing mosquito-human contact. Therefore, an effective biological control agent for the mosquito vector is a critical component in control of the disease. The ideal environmental management effort in controlling *Aedes aegypti* is

to use *B. thuringiensis* isolated from the local area that has a similar location with the mosquito vector.

Bacillus thuringiensis is an insecticide, gram positive and spore forming bacterium which has capabilities infecting the insect vectors belonging to the order Diptera (mosquitoes) especially dengue causing *Aedes* species (Renganathan et al. 2011). *Bacillus thuringiensis* is well known for its ability to produce parasporal crystalline protein inclusions (usually referred to as crystals), which have attracted worldwide interest in various pest management applications because of their specific pesticide activities (Ali et al. 2010). *B. thuringiensis* is also an important insect pathogen that is highly toxic to mosquito larvae and related dipterans (Poopathi 2010). During the sporulation, this bacterium produces one or more proteinaceous parasporal crystal (Cry), recoqnized as delta-endotoxin. This crystal protein under alkaline condition of midgut of insects, gets solubilized and then activated by intrinsic protease into an active toxin that selectively binds specific receptor in the cell membrane, leading to formation and consequent insect death (Eswarapriya et al. 2010).

The objective of this study was to investigate the toxicity of indigenous *B. thuringiensis* isolates from East Java on *Aedes aegypti* larvae.

3.2.2 Materials and methods

3.2.2.1 Study Area

East Java is a province in the eastern part of Java Island, Indonesia. The capital is Surabaya. East Java has the largest area among the six provinces in Java Island, and has the second largest population in Indonesia after West Java. East Java is bordering the Java Sea in the north, the Strait of Bali in the east, the Indian Ocean in the south, as well as in

the western province of Central Java. East Java also includes the island of Madura, Bawean Island, Kangean Island and a number of small islands in the Java Sea and the Indian Ocean (Sempu Island and Nusa barung). The land area of East Java Province is about 47,130.15 km² and the sea area of 110,764.28 km², located between 111°0 '– 114 °4′ east longitude and 7 °12 ' - 8 °48′ latitude (BPS 2010). Soil sampling sites located in 7 regions were Bondowoso, Blitar, Tulungagung, Nganjuk, Ponorogo, Pamekasan, Surabaya. The determination of these sites was based on altitude. The altitude will affect mosquito populations that exist at each location.

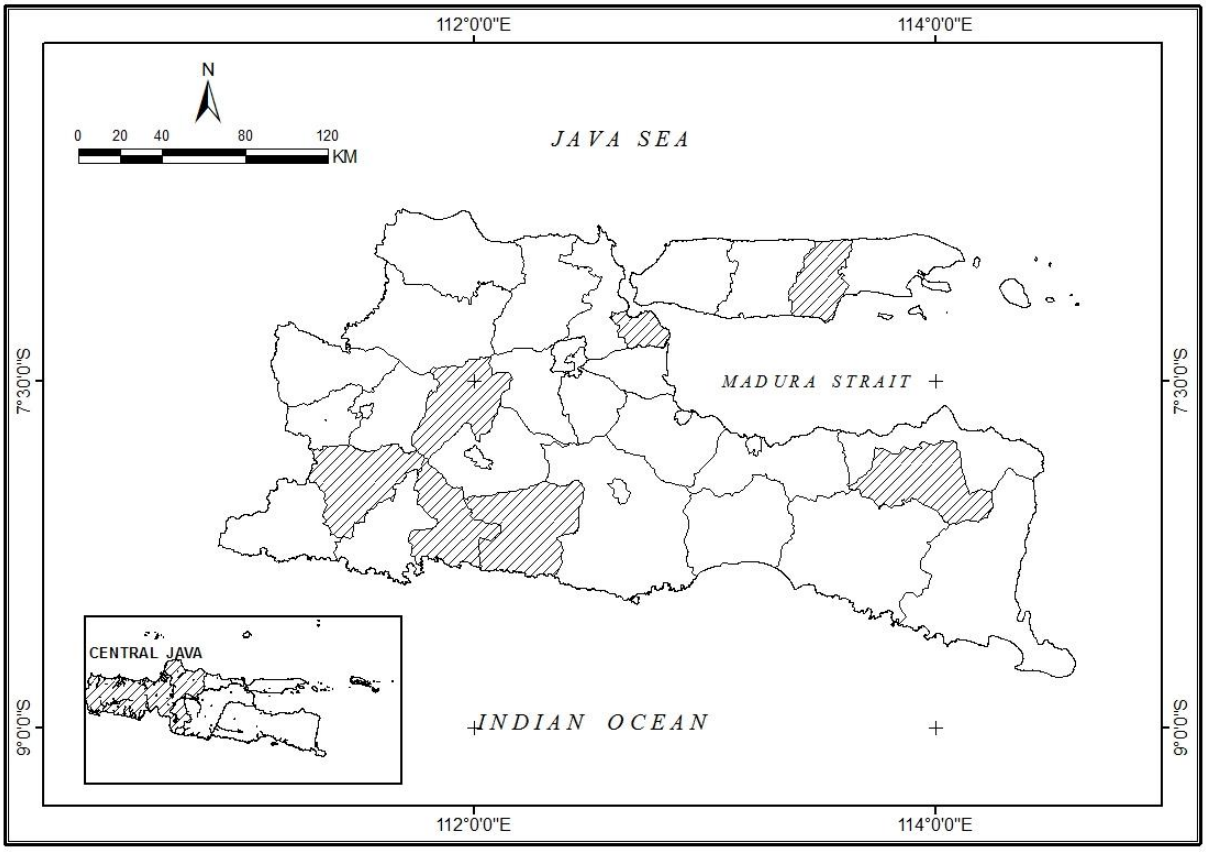

Figure 3-8. Study area

3.2.2.2 Isolation of bacteria

Samples of soil were carried out in seven locations in East Java from August 2011 to September 2012. Three soil samples were taken from 10 cm below the soil surface at each location (Chartterjee et al. 2007). The soil samples were 25 g and were placed into 225 ml sterile physiological saline solution (8.5 g/L NaCl). Serial 10^{-1} to 10^{-6} – fold dilutions of the sample suspensions were made in NaCl. Then, about 0.1 ml of the suspension was inoculated into a petri dish containing *B. thuringiensis* selective media. Cultures were incubated for 48-72h at 30^{o} C. Any isolates that grew were purified on a quadrant streak plate. Single colonies were inoculated into *B. thuringiensis* selective media. Cultures were incubated for 48-72h at 30^{o}C. Isolates were stored on nutrient agar and *B. thuringiensis* selective media at 4^{o}C and in glycerol at -80^{o}C.

3.2.2.3 Classification of bacteria based on phenotypic attribute similarity

The phenotypes of the bacterial isolates were characterized by morphological observation and biochemical and physiological tests (Zakeel et al. 2009). Morphological observations were performed on single cells or a single colony. Single colonies were observed for color, surface texture, colony structure, gram staining and the presence of endospores (Schaeffer-Fulton methods). Biochemical tests performed included the catalase test, growth on Simmons citrate agar, urea hydrolysis, methyl red and acid butanediol (Voges Proskauer) fermentation, carbohydrate fermentation, motility, tryptophan hydrolysis, the oxidase test and the nitrate test. Observed physiological test parameters consisted of temperature (30 and 60 °C) and pH (4.0, 7.0 and 9.0). Bacterial density was determined with a spectrophotometer at a 600 nm wavelength (Gong et al. 2012).

The total number of live bacteria was calculated from stock bacterial suspension cultures that were inoculated into NB and homogenized. In total, 0.1 ml of the stock bacterial suspension from each dilution was inoculated on NA and incubated at 30°C. The number of *B. thuringiensis* was based on the number of colonies that grew after incubating for 12, 24, 36, 48, 72 and 96 hours.

3.2.2.4 Endospore screening

Isolated colonies were inoculated on sporulation media (10 g glucose, 7.5 g peptone, 6.8 g KH_2PO_4, 123 mg $MgSO_4.7H_2O$, 2.33 mg $MnSO_4.4H_2O$, 14 mg $ZnSO_4.7H_2O$, and 320 mg $Fe(SO_4)$ dissolved in 1 N NH_4Cl). The bacterial suspensions were incubated at 30°C and shaken at120 rpm for 24 hs; then, 3.75 ml of the suspension was inoculated into 25 ml of sporulation media and incubated at 30°C, where it was shaken at 120 rpm for 24 hs. Spore prevalence was calculated based on the number of live spores divided by the number of live cells times 100% (Jensen et al. 2002).

3.2.2.5 Testing of *Aedes aegypti*

Aedes aegypti larvae were provided by the Provincial Health Department, Surabaya – East Java, Indonesia. The larvae were transferred to the laboratory of Ecology and Diversity, Department of Biology, Brawijaya University, where self-perpetuating colonies were established and maintained for the present study.

Toxicity studies were performed on test compounds as described by Wright in 1971 (Wright, 1971; El-kersh, 2012) with some modifications. Mortality data were analyzed using log-probit analysis to estimate the probit regression line and calculate LC_{50} (El-Maghraby et al. 2012).

3.2.2.6 Evaluation of the potential bacteria toxicity on *Aedes aegypti* larvae

Bacillus thuringiensis toxicity against *Aedes aegypti* larvae was evaluated by randomized factorial design. Tests on each isolate were repeated three times. For each *B. thuringiensis* isolate, the effects on larval mortality after 24, 48, and 72 hs were observed. Isolates were inoculated with 50 ml of Schaeffer's sporulation medium (SSM). There were incubated at 30^{o} C for 72 hs; and then 5 ml of bacterial suspension in SSM were inoculated on each petri dish containing 45 ml of water. Each dilution was tested in a tube that contained 20 mosquito larvae, and each isolate was tested three times. Each isolate was exposed for 24, 48 or 72 hs, and it was observed whether there was an effect of *B. thuringiensis* on larval mortality. As a control, 20 mosquito larvae were tested in a tube without *B. thuringiensis*.

3.2.3 Results

Of the 24 selected bacterial isolates and all the isolates that had obtained a similar phenotypic to reference *B. thuringiensis*. The reference *B. thuringiensis* isolates used in this study were *B. thuringiensis* var.Israelensis HD 567. This bacteria were from Science Center Cibinong Bogor (LIPI) collection.

3.2.3.1 Spore prevalence in *B. thuringiensis* isolates

Determination of the isolates which would be used for testing mosquito larvae toxicity was based on the high toxic-prevalence among the twenty four isolates. Two isolates that had the highest prevalence and the fastest production of spores were TAT.2.10-2.k1b and TAT.1.10-3.k6b. Both of isolates had a spore prevalence of 100% for

24 hours. Almost all isolates were able to produce 100% of spores within 120 hours. The results are presented in Table 3-4. Percentage of *Aedes aegypti* larvae mortality is presented in Table 3-5.

Table 3-4. Percentage of spore prevalence of *Bacillus thuringiensis*

No	City	Isolates	Percentage of spore prevalence (%)				
			24 hs	48 hs	72 hs	96 hs	120 hs
1	BONDOWOSO	BDWS T2 10-2 K6	0.00	95.47	100.00		
		BDWS T2 10-2 K14	9.28	0.00	89.00	89.00	97.00
		BDWS T1 K1	26.00	0.00	60.90	65.72	91.37
		BDWS T2 K3	33.00	0.00	73.62	75.80	95.50
2	BLITAR	Bltr T1 K6	77.00	0.00	93.56	100.00	
		Bltr T2 k1a	6.40	0.00	76.30	93.40	100.00
		Bltr T2 k1b	3.30	0.00	92.90	100.00	
3	TULUNGAGUNG	TAT.I.K.1a	92.27	100.00			
		TAT.I.k6	5.08	97.00	100.00		
		TA.A.I.k6b 10-1	39.03	69	100.00		
		TA.T II K2	5.97	84	100.00		
		TA.T 2 K1a	70.00	85.21	100.00		
		TAT.2.10-2.k1b	100.00				
		TAT.1.10-3.k6b	100.00				
4	NGANJUK	NGJ I 10-2 K2	16.70	95.16	97.60	100.00	
		NGJ I 10-4 K2	20.97	20.97	95.00	100.00	
		NGJ II 10-3 K1b	94.00	97.90	98.80	99.50	100.00
5	PONOROGO	PNRG TI k2	25.00	50.00	100.00		
		PNRG TII 10-2 k2	51.90	60.00	80.00	100.00	
		PNRG Ti K1a	42.00	75.60	97.38	97.90	100.00
		PNRG TII 10-2 k1a	30.80	66.30	80.00	100.00	
		PNRG A1 K2	23.50	70.00	95.20	95.80	100.00
6	PAMEKASAN	PAMEKASAN	35.19	65.00	100.00		
7	SURABAYA	BrHt	2.36	65.77	88.00	100.00	
8	REFERENCE	HD 567	33.80	45.00	80.00	94.00	100.00

Table 3-5. Percentage of *Aedes aegypti* larvae mortality

No.	City	Altitute of city (m)	Isolate	Percentage of mortality (%)		
				24 hs	48 hs	72 hs
1	Bondowoso	255	BDWS T2 10-2 K6	17.5	20.0	25.0
			BDWS T2 10-2 K14	0.0	2.5	7.5
			BDWS T1 K1	2.5	2.5	5.0
			BDWS T2 K3	0.0	0.0	5.0
2	Blitar	167	Bltr T1 K6	0.0	5.0	10.0
			Bltr T2 k1a	0.0	2.5	3.0
			Bltr T2 k1b	0.0	5.0	5.0
3	Tulungagung	85	TAT.I.K.1a	0.0	0.0	0.0
			TAT.I.k6	5.0	5.0	5.0
			TA.A.I.k6b 10-1	0.0	0.0	0.0
			TA.T II K2	0.0	0.0	0.0
			TA.T 2 K1a	0.0	0.0	0.0
			TAT.2.10-2.k1b	0.0	0.0	0.0
			TAT.1.10-3.k6b	0.0	0.0	0.0
4	Nganjuk	56	NGJ I 10-2 K2	0.0	0.0	0.0
			NGJ I 10-4 K2	2.5	5.0	5.0
			NGJ II 10-3 K1b	0.0	0.0	0.0
5	Ponorogo	49	PNRG TI k2	5.0	15.0	15.0
			PNRG TII 10-2 k2	20.0	25.0	25.0
			PNRG Ti K1a	5.0	35.0	35.0
			PNRG TII 10-2 k1a	0.0	0.0	0.0
			PNRG A1 K2	0.0	2.5	7.5
6	Pamekasan	47	Pamekasan	0.0	0.0	0.0
7	Surabaya	2	BrHt	100.0	100.0	100.0
8	Reference		HD 567	20.0	65.0	80.0
9	Control (without *B. thuringiensis*)		Control	0.0	0.0	5.0

Table 3-6. The relationship between percentage of *Aedes aegypti* larvae mortality and time exposure of *B. thuringiensis* isolate

No	City	Isolates	Model	R square (R^2)
1	Bondowoso	BDWS T2 10-2 K6	y = 0.322x + 4	0.846
		BDWS T2 10-2 K14	y = 0.104x - 1.25	0.833
		BDWS T1 K1	y = 0.062x + 0.25	0.962
		BDWS T2 K3	y = 0.062x - 1	0.6
2	Blitar	Bltr T1 K6	y = 0.145x - 1.5	0.890
		Bltr T2 k1a	y = 0.047x - 0.35	0.860
		Bltr T2 k1b	y = 0.083x - 0.5	0.8
3	Tulungagung	TAT.I.K.1a	y = 0	-
		TAT.I.k6	y = 0.062x + 1.5	0.6
		TA.A.I.k6b 10-1	y = 0	-
		TA.T II K2	y = 0	-
		TA.T 2 K1a	y = 0	-
		TAT.2.10-2.k1b	y = 0	-
		TAT.1.10-3.k6b	y = 0	-
4	Nganjuk	NGJ I 10-2 K2	y = 0	-
		NGJ I 10-4 K2	y = 0.072x + 0.5	0.890
		NGJ II 10-3 K1b	y = 0	-
5	Ponorogo	PNRG TI k2	y = 0.299x + 0.5	0.896
		PNRG TII 10-2 k2	y = 0.33x + 5.5	0.752
		PNRG Ti K1a	y = 0.562x - 1.5	0.852
		PNRG TII 10-2 k1a	y = 0	-
		PNRG A1 K2	y = 0.104x - 1.25	0.833
6	Pamekasan	Pamekasan	y = 0	-
7	Surabaya	BrHt	y = 4.166x	1
8	Reference	HD 567	y = 1.187 x - 1.5	0.962
9	Control (without *B. thuringiensis*)		y = 0.062x - 1	0.60

Increasing the spore number of all isolates or increasing the exposure time showed a proportional increase in *Aedes aegypti* larvae mortality are illustrated in Figure 3-9.

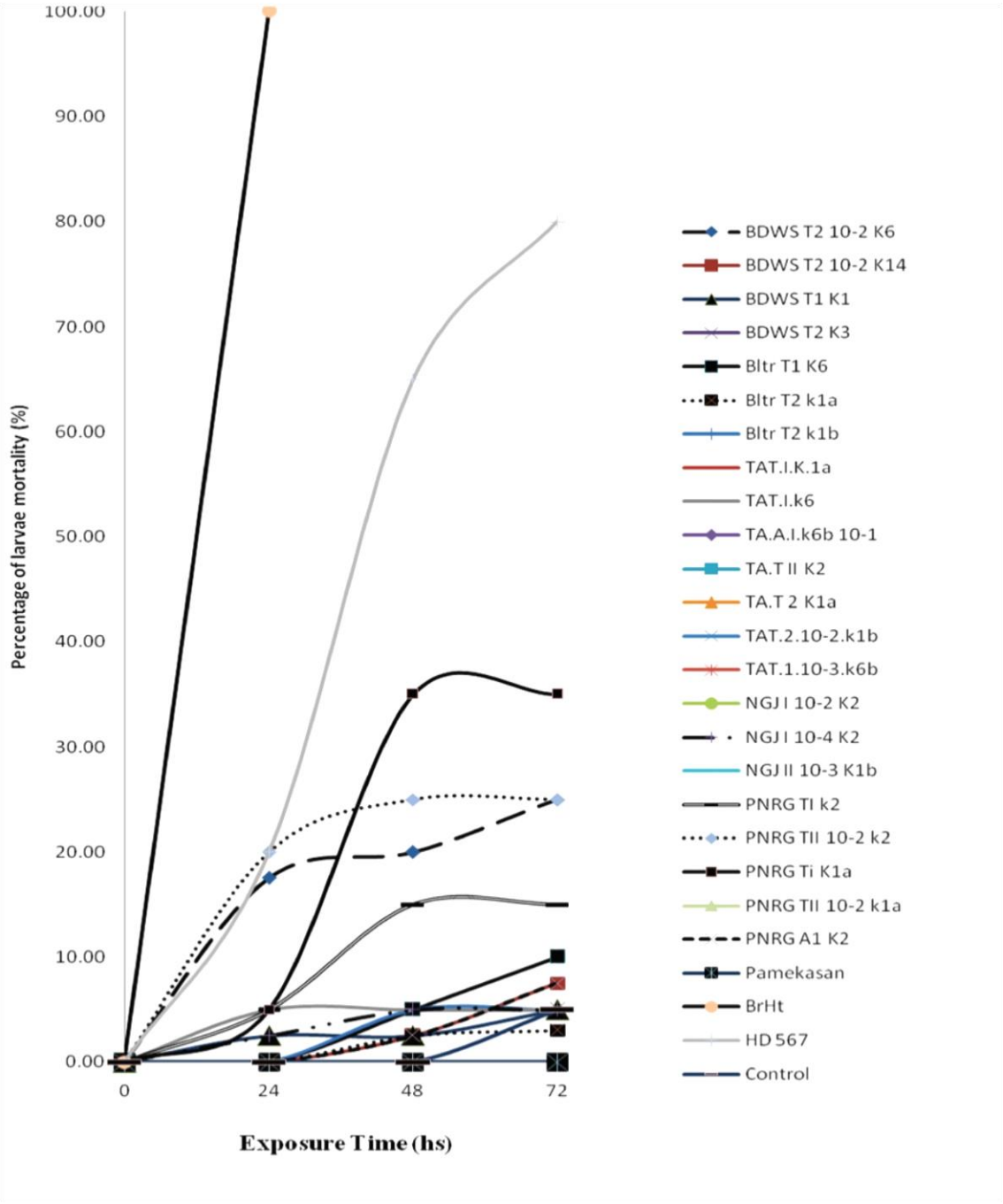

Figure 3-9. Relationship between percentage of *Aedes aegypti* larvae mortality and the *B. thuringiensis* exposure time (hours)

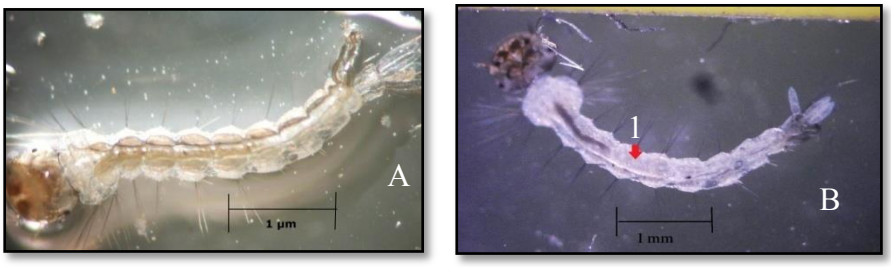

Figure 3-10 Third instar *Aedes aegypti* larvae
(A. Before toxicity test; B. After toxicity test; 1. Digestive tract)

3.2.4 Discussion

In this study, bacterial sampling locations selected by the altitude because of the low altitude lands (0-250 mm above sea level) are usually have the higher mosquito populations. At sites with low latitude location, then it is expected the bacterial density was also great to control mosquito naturally. In contrast to predictions for vector borne parasites, many studies have reported reductions in geographical range size and abundance, and shifts to lower latitudes or high altitudes, in a wide range of organisms that are potential hosts for these parasites (Zouache et al. 2011).

If bacterial spore prevalence was increasing, it can be assumed that the amount of toxin produced was also growing. As the number of bacterial toxins increases, one may expect the bacteria to be more effective at killing mosquito larvae. There are differences in spore prevalence that are associated with the individual characteristics of the spore-forming isolates. *B. thuringiensis* has two developmental phases: germination and sporulation (Manonmani et al. 2011). During sporulation, parasporal crystals are released by autolysis. These crystals are toxic and will damage the mosquito larval digestive tract, thus causing larval mortality.

The early stationary phase is marked by vegetative cell death, followed by toxin accumulation, because the cells metabolize the available nutrients, resulting in nutrient shortage and competition. The bacteria will then synthesize secondary metabolites that are used to maintain life. In *B. thuringiensis*, this stationary phase is associated with spore and toxin formation. Toxins from *B. thuringiensis* cells are formed after the cells have formed endospores (Mudiady et al. 2011).

The result of the study disclosed that *B. thuringiensis* Brht isolate from Surabaya district has the highest percentage of *Aedes aegypti* larvae mortality at 24 hours. It also described that these isolate is more effective than the reference *B. thuringiensis* (*B. thuringiensis* var. Israelensis HD 567). The mosquito third instar larvae were selected because at this stage, the larvae have a complete anatomical structure and the body is divided into three parts(head, thorax, abdomen); therefore, damage to the larvae can be easily observed within each section. A previous study demonstrated that the numbers of intestinal epithelial cells and peritrophic cells increase in accordance with increasing larval toxin resistance (Wirth 2010).

This research reported that only Surabaya isolates (BrHt) was able to kill 100% animal within 24 hours, while reference *B. thuringiensis* isolate was able to kill 80% of mosquito larvae after 72 hours. Therefore, the LC_{50} of the tested bacterial isolates could not be determined because no percentage of mosquito larvae mortality reached 50%. The mortality rates of larvae were under 50%, this result indicated that larvae of *Aedes aegypti* were possible resistance to the toxin of *Bacillus thuringiensis* (Mulyatno et al. 2012).

The LC_{50} (lethal concentration) results indicate a significant effect among the tested isolates. One of all isolates that is indigenous to East Java (BrHt) killed *Aedes aegypti* larvae. This isolate is the most effective, as 1.215×10^8 cells/ml was required to kill fifty

percent of the *Aedes aegypti* larvae within 24 hs. The 72-hs exposure time was more effective than the 24-hs and 48-hs exposure times, especially for the reference *B. thuringiensis* (HD 567 isolate). Once the bacterial toxin enters the mosquito larvae digestive tract, the increased time allows the toxin to accumulate, and the toxin's (δ-endotoxin) effects increase in accordance with the exposure. The toxin binds to receptors located on the epithelial midgut cell wall's apical microvillus membrane. After the toxin binds to its receptor, there is a change in the toxin's conformation, allowing toxin insertion into the membrane. Electrophysiological and biochemical evidence suggest that the toxins generate pores in the cell membrane, which disturbs the osmotic balance; consequently, the cells swell and lyses. The gut becomes paralyzed, and the mosquito stops feeding. Most mosquito larvae generally show reduced activity after two hs and die within six hs of toxin injection (Poopathi and Abidha 2010). Mosquito larvae mortality is also influenced by factors such as bacterial concentration, larval age and the bacterial strain used (Ramirez-Suero et al. 2011; Poopathi and Tyagi 2006). Receptor of larvae midgut can bind to the toxins that were produced by *B. thuringiensis* and it will cause lyses' of the digestive tract mosquito larvae. Digestive tract on mosquito larval damaged can be observed in Figure 3-10.

A negative relationship can be observed between the exposure time and the LC_{50} for the *B. thuringiensis* indigenous to Surabaya and the reference *B. thuringiensis*. This means that with a longer exposure time, the LC_{50} value will decrease and the larval mortality level will increase (Arivoli et al. 2011; Valadez-Lira et al. 2011). All of the tested and reference isolates had the highest percentages for larval mortality at the highest cell density and the longest exposure time because the toxins were released by the bacteria and accumulated in the *Aedes aegypti* larvae's digestive tract. Increased mortality was also

observed for the mosquito larvae. These combined conditions resulted in a higher mosquito larvae mortality rate. The result of this study showed that had a positive relationship between percentage of *Aedes aegypti* larvae mortality and time exposure of *B. thuringiensis* isolate.

3.3 Toxicity Studies for Indigenous *Bacillus thuringiensis* Isolated from East Java on *Aedes aegypti* larvae and the Non-target Species *Trichogaster pectoralis* (Class: Actinopterygii)

3.3.1 Introduction

Control of insect vectors of important human diseases in mainly achieved using chemical insecticides. However, the use of these chemical pesticides has led to several problems, including environmental pollution and increase in human health effects, such as cancer and several immune system disorders. Microbial insecticide has been proposed as substitutes for chemicals their use is limited since most microbes show a narrow spectrum of activity that enables them to kill only certain insect species (Bravo et al. 2011).

The greatest successes in microbial pesticides have come from uses of *Bacillus thuringiensis* var. *israelensis* and *Bacillus thuringiensis kurstaki* which are toxic against Diptera and Lepidoptera, respectively (Obeidat, 2008). *Bacillus thuringiensis* is a naturally occurring bacterial disease of insects. This bacterium is the active ingredients in some insecticides. *Bacillus thuringiensis* is a gram-positive, spore forming, aerobic bacterium that is found in a variety of habitat. During sporulation, *Bacillus thuringiensis* subsp. *israelensis* produces a spherical, parasporal inclusion that contains larvicidal proteins with

activity against Nematoceran Dipterans; primarily mosquitoes and blackflies. Chironomid midges, fungus gnats, and crane flies are also susceptible to a lesser degree. The requisite to control mosquito populations continues, however the insecticides that can be safely used in such efforts are extremely limited. For the reason that, interest has grown in the bacterial insecticides, primarily *B. thuringiensis* subsp. *israelensis*, that produce crystalline proteins that are toxic to mosquitoes and safe for the environment (Wirth, 2010).

The preferences of using biological pesticides over chemical pesticides have been widely accepted in different part of the world for many reasons. The important advantages of the biological pesticides are more safer agents, because they are degradable and have a high level of safety for non-target organisms (humans, animals and fish) in addition to their host specificity, their lower resistance in the target pest population (Obeidat, 2008).

B. thuringiensis is considered safe to people and non-target species. The toxicity is attributed to δ-endotoxin, which is made of proteins that are produced and assembled when the bacteria sporulate (Sanahuja 2011, Hagggag 2010). Research investigation using *B. thuringiensis* isolated from East Java, Indonesia as natural enemies of mosquito's larvae were carried out, because the *Bacillus thuringiensis* crystal protein had an arrow host spectrum (Gama and Nobukazu, 2013), but it needs to be continued with toxicity test on non-target organism before application in the field. The objective of this study was to investigate the toxicity of indigenously *Bacillus thuringiensis* isolated from East Java on *Aedes aegypti* larvae and non-target species *Trichogaster pectoralis* (Class: Actinopterygii).

Trichogaster pectoralis (Snakeskin Gourami) as the fish is known in Asia, is an air-breathing swamp fish and they have a dorsal fin with 6 spines and 10-11 branched rays; origin far behind the pectoral fin base. Snakeskin Gourami or Sepat siam means

freshwater fish with the scientific name of *Trichogaster pectoralis*. Anal fin of this fish with 9-12 spines and 33-38 branched rays. Pelvic fin with a long filamentous ray and 2-3 small rays in the Axil. This fish has a lateral line with 55-63 scales. *T. pectoralis* lives in lakes, ponds and still sluggish waters (Rainboth, 1996). Generally, this species feeds on aquatic plants and they can breathe air directly, as well as absorb oxygen from water through its gills. *T.pectoralis* (synonyms: *Trichopodus* pectoralis), an elongated, moderately compressed fish with a small dorsal fin and they attain about 20 cm for adult, but the common fish is around 12-15 cm. The local name of *T. pectoralis* in Indonesia is sepat siam (Boyd and Tucker, 1998; Froese and Pauly, 2003). These fishes belonged to the Class: Actinopterygii, Ordo: Perciformes, Family: Osphronemidae, Genus: *Trichogaster*, Species: *Trichogaster pectoralis* Regan 1910 (Tampubolon and Rahardjo, 2011).

The previous study on the toxicity of *Bacillus thuringiensis* var. israelensis on European large freshwater branchiopods, with the exceptions of test on adult *Eubranchipus grubii* specimens (Becker and Margalit, 1993) and study indicating that *Bacillus thuringiensis* var. israelensis is well tolerated by adult *Branchipus schaefferi* are already published (Blaustein and Margalit, 1991). Studies on *Triops newberryi, Eulimnadia texana* as non-European large branchiopods and salt-water species (*Artemia salina*) did not show any toxic effects of *Bacillus thuringiensis* var. israelensis (Su and Mulla, 2005).There are only a few published studies on the toxicity of *B. thuringiensis* on non-target organism, especially test on freshwater fish. Therefore an examination of possible toxic effects on *Trichogaster pectoralis* seemed to be indispensable.

3.3.2 Material and methods

3.3.2.1 Study area

East Java is a province in the eastern part of Java Island, Indonesia. The capital is Surabaya. East Java has the largest area among the six provinces in Java Island, and has the second largest population in Indonesia after West Java. East Java is bordering the Java Sea in the north, the Strait of Bali in the east, the Indian Ocean in the south, as well as in the western province of Central Java. East Java also includes the island of Madura, Bawean Island, Kangean Island, and a number of small islands in the Java Sea and the Indian Ocean (Sempu Island and Nusa barung). The land area of East Java Province is about 47,130.15 km² and the sea area of 110,764.28 km², located between 111°0‘– 114 °4′ east longitude and 7 °12‘ - 8 °48′ latitude (BPS 2010). Soil sampling sites located in 7 regions were Blitar, Bondowoso, Ponorogo, Tulungagung, Madiun, Lamongan, Bangkalan, Determinations of these sites were based on altitude. The altitude will affect mosquito populations that exist at each location

3.3.2.2 Soil sampling

Three soil samples were taken from 10 cm below the soil surface at each location (Chartterjee et al. 2007). The determination of the sampling location based on a preliminary study data about an outbreak of dengue cases in East Java (East Java Health Department, 2011) and the altitude of each location of sampling. The highest altitude district was Bondowoso and Blitar, at the middle latitude were Bangkalan, Ponorogo, Madiun and Tulungagung and the lowest altitude were Lamongan.

3.3.2.3 Isolation and Purification of *Bacillus thuringiensis*

Isolation and identification of the *Bt* strain were conducted by the Atlas method (2004); Gama and Marwati (2005); Chatterjee et al. (2007). Approximately 25 g of soil-sediment and 25 ml water samples each put in 225 ml of sterile saline solution (0.85% NaCl) and heated at 80^{o} C for 15 min to eliminate any unsporulated microorganisms. Sample suspension was made serial dilutions 10^{-1} to 10^{-6}. A suspension of 10^{-2}-10^{-6} dilution rate of 0.1 ml was taken and then inoculated into a petri dish and poured with the composition of a selective medium *B. thuringiensis* (glucose 3 g, $(NH_4)_2SO4$ 2 g, *yeast extract* 2 g, $K_2HPO_4.3H_2O$ 0,5 g, $MgSO_4.7H_2O$ 0,2 g, $CaCl_2.2H_2O$ 0,08 g, $MnSO_4.4H_2O$ 0,05 g in 1000 ml of distilled water). Cultures were incubated for ± 48-96 hours in an incubator at 30^{o} C. Bacterial colonies have morphological characteristics that indicate the similarity colonies of *Bacillus thuringiensis*. These are round, raised, opaque, configuration entire and white (Rampersad and Ammons, 2005). The morphology of gram positive cells were rod-shaped, widths between 1.0 and 1.2 µm ; a length of the cell was 3-5 µm (Bravo, 1998) as well as the character of the spores is oval, sub terminal location and size was 1.0 - 1.3 µm. Each isolate were grown and colonies similar to *Bacillus thuringiensis* purified with the quadrant streak plate method on selective media so that the surface of *Bacillus thuringiensis*. Cultures were incubated for 48-72 h ± at 30^{o}C (Chatterjee et al. 2007). Isolates were inoculated in NA medium (*Nutrient Agar*) and selective media of *Bacillus thuringiensis* at 4°C.

3.3.2.4 Biochemical typing

Biochemical tests were carried out to confirm the identity of each isolated which has gram positive bacilli as *Bacillus thuringiensis*, according to Barrow and Feltham, 1993;

Collee *et al*. 1996; Claus and Berkeley, 1986 and Collins *et al*., 1995. These tests were catalase production, *Voges-Proskauer* test, starch hydrolysis, and *Bt* isolates were tested using API 50CHB systems (BioMerieux, Marcyle Etoile, France). *Bt* isolates were divided into biochemical types based on hydrolysis of esculin, urea or lecithin, and acid production from sucrose, or salicin. Then it was incubated at 30°C overnight (Logan and Berkeley, 1984; Martin and Travers, 1989; Lecadet et al. 1999; Keshavarzi, 2008; Aramideh et al. 2010; Martin et al. 2010).

3.3.2.5 Classification of bacteria based on phenotypic attribute similarity

The phenotypes of the bacterial isolates were characterized by morphological observation and biochemical and physiological tests (Zakeel et al. 2009). Morphological observations were performed on single cells or single colonies. Single colonies were observed for colour, surface texture, colony structure, Gram staining and the presence of endospores. Biochemical tests were performed including the catalase test, growth on Simmons citrate agar, urea hydrolysis, methyl red and acid butanediol (Voges Proskauer) fermentation, carbohydrate fermentation, motility, tryptophan hydrolysis, the oxidase test and the nitrate test. Observed physiological test parameters consisted of temperature (30 °C and 60 °C) and pH (4.0, 7.0 and 9.0). Bacterial density was determined with a spectrophotometer at a 600 nm wavelength (Gong et al. 2012). Phenotypic data with a plus (+) or minus (-) sign were compiled using Microsoft Excel. The data that changed were used to construct a CLAD97 dendrogram reflecting operational taxonomic units based on the similarity index value as determined by simple matching methods. The unweighted pair group method of the arithmetic averages algorithm was used.

3.3.2.6 Bioassay *Bacillus thuringiensis* to larvae of *Aedes aegypti*

B. thuringiensis toxicity against *Aedes aegypti* larvae was evaluated by randomized factorial design. Tests on each isolate were repeated three times. For each *B. thuringiensis* isolate, the effects on larval mortality were observed after 24, 48, and 72 h, respectively. One single and isolated colony was sub-cultured on 50 ml nutrient broth media, incubated for 72 h at 30 °C. In every 24 h during 72 h incubation process, 1 ml bacterial suspension was taken to counting the density of cells was 10^8 cells / ml using a hemocytometer. Dilutions of bacterial suspension were made based on different bacterial cell densities, such as 0:50, 5:45, 10:40, 15:35, 20:30, and 25:25. Each dilution was tested in a tube that contained 20 mosquito larvae, and each isolate was tested three times. Each isolate was exposed for 24, 48 or 72 h, and it was observed whether there was an effect of *B. thuringiensis* on larval mortality. As a control, 20 mosquito larvae were tested in a tube without *B. thuringiensis*.

3.3.2.7 Acclimatization of *Trichogaster pectoralis* in the aquaculture

The fish which used in toxicity tests have uniform size, such as the fish length is 4-5 cm. *Trichogaster pectoralis* are acclimatized in a pond measuring 30 X 60 X 30 cubic centimeters for two weeks (14 days) with feeding every morning. Feeding was stopped by 24 hours before toxicity test. *Trichogaster pectoralis* selected as experimental animals because this is a type of fish that often maintained by residents. These fish are often kept in the bathtub or other containers. Further application using indigenously *Bacillus thuringiensis* isolated from East Java will be put in the bathroom or water container, then the fish should be safe from infection of *Bacillus thuringiensis* toxin.

3.3.2.8 Preparation of bacterial culture suspension for toxicity test on *Trichogaster pectoralis*

For toxicity test on non-target species was used only *B. thuringiensis* isolates which had phenotypic characteristics and similarity value was over 82%. Therefore, we used *Bacillus thuringiensis* isolated from Bangkalan district, Lamongan district, and Madiun districts. Each 100 ml culture of the *Bacillus thuringiensis* isolates, and *Bacillus thuringiensis* var. *israelensis* (*Bti*) HD 500 (as Referee bacteria) is inoculated into 250 ml Erlenmeyer of sterile nutrient broth (NB). Then next activity, suspension of bacteria was shaken with 120 rpm at 30 ° C for 24 hours. After 24 hours, a total of 10 ml of inoculum were centrifuged at 10,000 rpm at four degree Celsius for 15 minutes. Supernatant was discarded. Sterile distilled water was added into the desired concentration.

3.3.2.9 Toxicity test on *Trichogaster pectoralis* using *Bacillus thuringiensis* with various concentrations

Bacterial culture suspension inoculated into an aquarium that has been filled up to 400 ml of fresh water. They contain *Bacillus thuringiensis* with various concentrations. These concentrations of suspension are 10^{12}, 10^{11}, 10^{10}, 10^{9}, 10^{8}.10^{7} and 10^{6} cells /ml. Homogenized culture with fresh water. Each aquarium is added with a ten fish and it is given feed every morning and we used aerator for each aquarium. Toxicity test was conducted for 7 days. The calculation of fish mortality carried out every day. The observation of swim agility reflexes, fading, bending the spine, and bleeding conducted every 24 hours. Each treatment was repeated 3 times. Abiotic factors such as pH and temperature are observed during 7 days. As a control, 10 fishes were tested in an aquarium without *B. thuringiensis*. In this study, we used juvenile fishes have average length were 4

to 8 cm and the age of fishes were 30 to 60 days. We decided to use juvenile stages for our tests due to as mosquito larvae and large branchiopods both hatch early after the emergence of temporary pools (Su and Mulla, 2005).

3.3.2.10 Statistical analysis

The percentage of *Aedes aegypti* larvae and fish mortality was analyzed using probit regression. LC_{50} was analyzed by analysis of variance (ANOVA) and it will be continued the TUKEY HSD interval was 95%. The relationship between percentage of *Aedes aegypti* larvae and fish mortality and density of *Bacillus thuringiensis* was analyzed using linear regression and Pearson correlation.

Figure 3-11. Acclimatization process of *Trichogaster pectoralis* in aquaculture (August.1st ,2013)

Figure 3-12. Aerator is used for toxicity test of indigenously *Bacillus thuringiensis* isolated from East Java on *Trichogaster pectoralis* in each aquarium (September 2nd, 2013).

Figure 3-13. *Trichogaster pectoralis* juvenile (September 2nd ,2013)

Figure 3-14. Toxicity test of indigenously *Bacillus thuringiensis* isolated from East Java (Isolates from Bangkalan, Madiun, Lamongan and HD 500) on *Trichogaster pectoralis* in aqua culture (September 3rd, 2013)

3.3.3 Results

Of the 86 selected bacterial isolates, five were obtained (Mdn I TK 2, SK.T, Solokuro TK1, Sekaran TK1, and Bltr TI K6) that had a similar phenocopy to reference *B. thuringiensis* HD 567 (Figure 3-15).

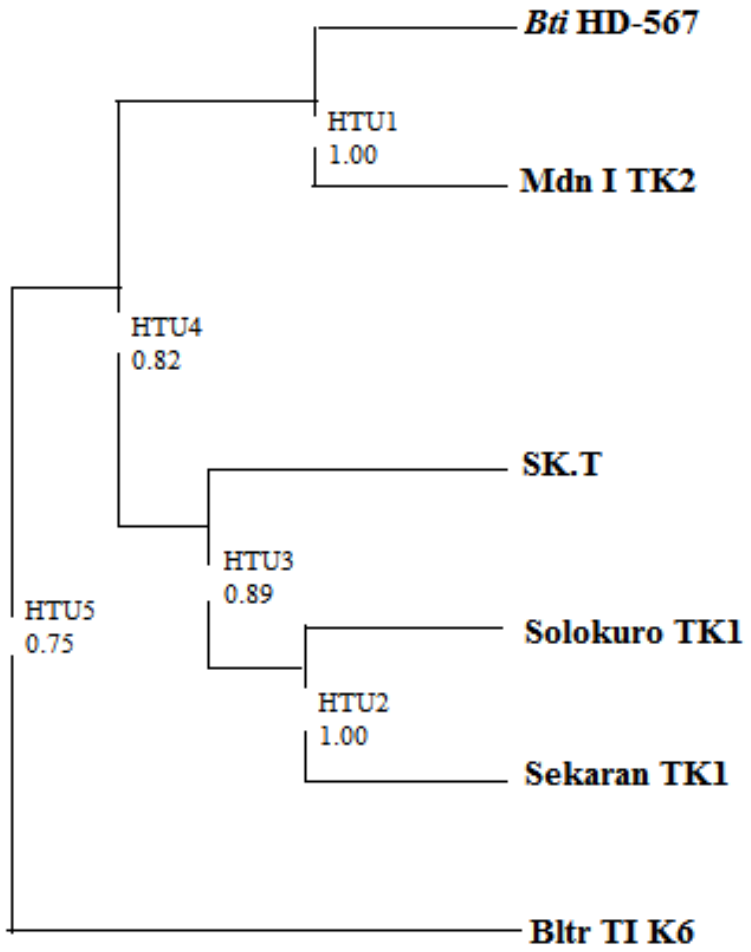

Figure 3-15 Dendrogram of relationships between indigenously *B. thuringiensis* isolates from East Java and reference *B. thuringiensis* HD 567

Table 3-7. Phenotypic characteristics of bacterial isolates similar *B. thuringiensis* by altitude

Area	Districts	Isolated	Gram	Catalase	Strach Hydrolisis	VP test
High altitude	Blitar	Bltr TI k6	+	+	+	+
		Bltr TII K1b	+	+	+	-
		Bltr TII K1a	+	+	+	-
	Bondowoso	Bdws T2 K14	+	+	-	-
		Bdws T2 K6	+	-	-	-
		Bdws T2 K3	+	-	-	-
Middle altitude	Ponorogo	Pono TI K1a	+	+	-	-
		Pono TII K1a	+	+	-	-
		Pono TI K2	+	+	-	-
		Pono TI K1a	+	+	-	-
	Tulungagung	TA.A. K6b	+	+	-	-
		TA.T I K6	+	+	+	-
	Madiun	Mdn I TK2	+	+	+	+
		Mdn JT K1a	+	+	-	-
		Mdn JT K1b	+	+	-	-
		Mdn JT K6b	+	+	-	-
	Bangkalan	Bgklan.T	+	+	-	-
Low altitude	Surabaya	RSBY. T	+	+	-	-
	Lamongan	SK. T	+	+	+	+
		Kltgah TK1	+	+	-	-
		Kltgah TK2	+	+	-	-
		Solokuro TK2	+	+	+	-
		Solokuro TK1	+	+	+	+
		Solokuro TK5	+	+	+	-
		Sekaran Ak1	+	+	+	-
		Sekaran TK1	+	+	+	+
		Kedung TK1	+	+	+	-
		Kedung A 13	+	+	+	-

3.3.3.1 Phenotypic characteristics and similarity among *B. thuringiensis* isolates

All of the bacterial isolates had similarity values of 75%. According to the dendrogram and phenotypic similarity value greater than 75%, isolates were classified into two groups based on the elevation colony (Figure 3-15). The first group, containing Mdn I TK 2, SK.T, Solokuro TK1, Sekaran TK1, had a similarity value of 82% compared with *B. thuringiensis* and the second group, containing only Bltr TI K6. Subgroup classification was based on margins colony: one subgroup had no round margins and the other subgroup had round margins.

The similarity value between *B. thuringiensis* HD 567 (reference) and Mdn I TK2 isolates was 100%. Both of the isolates and SK.T, Solokuro TKI, Sekaran TK1 were in different groups, which had a similarity value of 82%. A distinguishing characteristic among the five bacteria was round with raised margin. *B. thuringiensis* HD 567 and Mdn I TK2 isolate had a round with raised margin, but the other isolates did not (Table 3-8). Distinguishing factors between Solokuro TK1, Sekaran TK1 and SK.T isolates were related to pigmentation. SK.T isolates had white pigmentation and the other isolates had light yellow pigmentation.

Based on figure 3-15, Mdn I TK2 and *B. thuringiensis* HD 567 had a similar strain of bacteria cause they had 100% similarity value. There also had the same strain bacteria between Solokuro TK1 and Sekaran TK1 due to they had 100% similarity value. Mdn I TK2 isolates and SK.T, Solokuro TK1, Sekaran TK1 can be concluded a same genus because they had 82% similarity value. Priest and Austin (1993) stated that bacteria isolates had the same genus when they had 89-98 % phenotypic similarity value, however, it can be concluded to be one species when they had phenotypic similarity value 99%. If

among bacteria isolates had 100% phenotypic similarity index, it can be concluded as one strain.

Table 3-8. Phenotypic characteristics of different bacterial isolates.

Isolate	Circular	Margins				Elevation			Density	Pigmentation	
		Erose	Radially ridged	Round	Round with raised Margin	Flat	Convex	Effuse	Opaque	White	Light yellow
Bltr TI K6	+	-	+	-	-	+	-	-	+	+	-
Bltr TII K1b	+	-	-	+	-	-	-	+	+	+	-
Bltr TII K1a	+	-	-	+	-	-	-	+	+	-	+
Bdws T2 K14	+	+	-	-	-	+	-	-	+	+	-
Bdws T2 K6	+	-	+	-	-	+	-	-	+	+	-
Bdws T2 K3	+	+	-	-	-	-	+	-	-	+	-
Pono TI K1a	+	-	-	+	-	-	-	+	+	-	+
Pono TII K1a	+	-	-	+	-	-	-	+	+	-	+
Pono TI K2	+	-	-	+	-	-	-	+	+	+	-
Pono TIIK2	+	-	-	+	-	-	-	+	+	+	-
TA.T I K6b	+	-	+	-	-	+	-	-	+	+	-
TA.A. K6b	+	-	+	-	-	+	-	-	+	+	-
Mdn I TK2	+	-	-	-	+	-	-	+	+	+	-
Mdn JT K1a	+	-	-	+	-	-	-	+	+	-	+
Mdn JT K1b	+	-	-	+	-	-	-	+	+	+	-
Mdn JT K6b	+	-	+	-	-	+	-	-	+	+	-
Bgklan.T	+	-	-	-	+	-	-	+	+	+	-
RSBY. T	+	-	-	-	+	-	-	+	+	+	-
SK. T	+	-	-	+	-	-	-	+	+	+	-
Kltgah TK1	+	-	-	+	-	-	+	-	+	-	+
Kltgah TK2	+	+	-	-	-	+	-	-	-	+	-
Solokuro TK2	+	-	-	+	-	-	+	-	+	+	-
Solokuro TK1	+	-	-	+	-	-	-	+	+	-	+

3.3.3.2 Toxicological evaluation of indigenous *Bacillus thuringiensis* isolates against *Aedes aegypti* larvae.

Table 3-9. Number of bacterial isolates obtained from soil and water samples by altitude

Origin of Isolates (Location)	Number of Samples		Total of Samples	Number of *Bt* isolates	Number of *Bacillus* spp.	*Bt.* index*
	Soil Sediments	Water				
High Altitude (Biltar and Bondowoso)	2	2	4	6	14	0,42
Middle Altitude (Ponorogo, Tulungagung, and Madiun)	6	4	10	10	33	0,30
Low Altitude (Lamongan)	5	5	10	10	39	0,25
Total	13	11	24	26	86	0,30

Bt index: No. of identified *B. thuringiensis* colonies divided by the total number of *Bacillus*-like colonies examined (Ammouneh et al. 2010).

Based on table 3-10 indicated a significant effect ($P<0.05$) among the tested isolates. The two indigenous isolates of East Java (Mdn I TK2 and SK.T) killed *Ae.aegypti* larvae. Among them, the Mdn I TK2 was the most effective, as (2.21×10^7) cells/ml of which was required to kill 50% of the *Ae.aegypti* larvae within 48 h. The 48-h exposure time was more effective than that of the 24-h and the 72-h exposure time (Figure 3-16).

Table 3-10. The LC_{50} values of *B. thuringiensis* isolates against third stage *Aedes aegypti* larvae (ANOVA test results)

Isolate	Cell Density (x 10^7) cell/ml		
	$LC_{50\ 24hours}$	$LC_{50\ 48hours}$	$LC_{50\ 72hours}$
SK.T	2,49 (a)	2,69 (a)	2,42 (a)
Mdn I TK2	2,37 (ab)	2,21 (ac)	2,17 (ac)
Bti. HD-567	47,08 (d)	46,66 (abc)	19,49 (abc)

The same letters after the numbers denote no significant difference (significant at the 0.05 level).

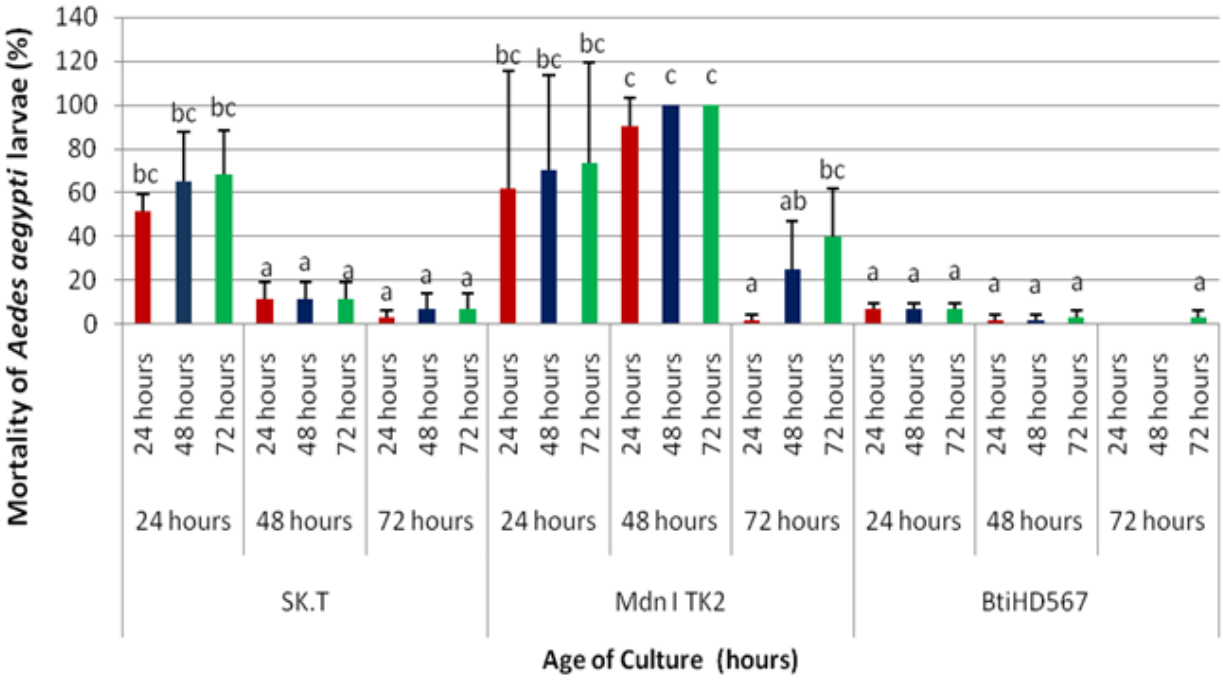

Figure 3-16. The mortality percentage of third stage *Aedes aegypti* larvae by SK.T isolates, Mdn I TK2, and *Bti.* HD-567 was exposed for 24, 48, and 72 hours. The same small letters on the block in the same culture showed no difference.

Table 3-11. Mortality percentage of *Trichogaster pectoralis*

Name of Isolate	Average of microbial density (cell/ml)	Percentage of mortality		
		24 h	48 h	72 h
HD 500	**3.60×10^{6}**	0	0	0
	3.60×10^{7}	0	0	0
	3.60×10^{8}	0	0	0
	3.60×10^{9}	0	0	0
	3.60×11^{10}	0	0	0
	3.60×11^{11}	0	0	0
	3.60×11^{12}	0	0	0
	control	0	0	0
Bangkalan	**3.60×10^{6}**	0	0	0
	3.60×10^{7}	0	0	0
	3.60×10^{8}	0	0	0
	3.60×10^{9}	0	0	0
	3.60×11^{10}	0	0	0
	3.60×11^{11}	0	0	0
	3.60×11^{12}	0	0	0
	control	0	0	0
Lamongan	**3.60×10^{6}**	0	0	0
	3.60×10^{7}	0	0	0
	3.60×10^{8}	0	0	0
	3.60×10^{9}	0	0	0
	3.60×11^{10}	0	0	0
	3.60×11^{11}	0	0	0
	3.60×11^{12}	0	0	0
	control	0	0	0
Madiun	**3.60×10^{6}**	0	0	0
	3.60×10^{7}	0	0	0
	3.60×10^{8}	0	0	0
	3.60×10^{9}	0	0	0
	3.60×11^{10}	0	0	0
	3.60×11^{11}	0	0	0
	3.60×11^{12}	0	0	0
	Control	0	0	0

Based on table 3-11 showed that all isolates of *Bacillus thuringiensis* did not kill *Trichogaster pectoralis* during 7 days. The result indicates that *Bacillus thuringiensis* isolated from Bangkalan, Lamongan and Madiun districts also *Bacillus thuringiensis* var.*israelensis* HD 500 are tolerated by the juvenile of *Trichogaster pectoralis* R.

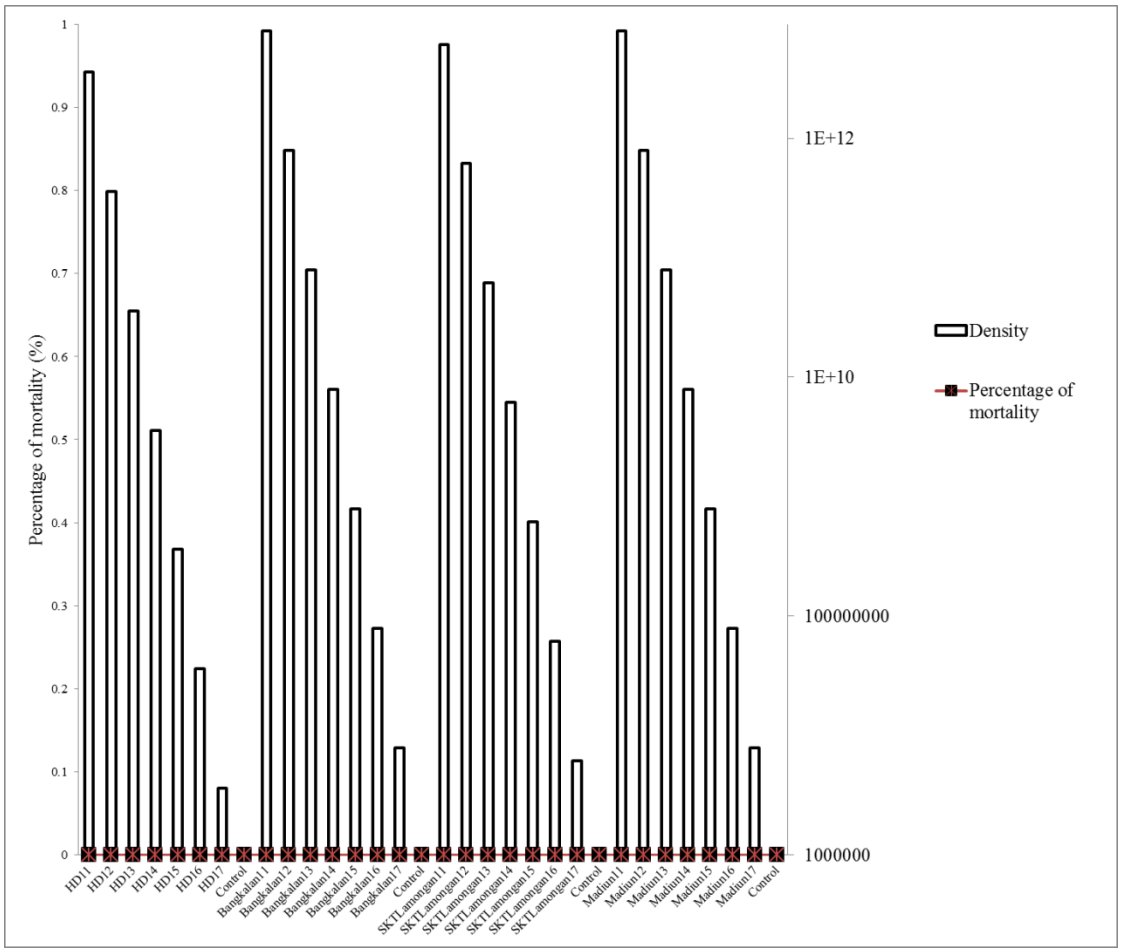

Figure 3-17. The relationship between mortality percentage of *Trichogaster pectoralis* R and density of *Bacillus thuringiensis* isolated from Bangkalan, Lamongan, Madiun districts, and HD 500 (referee *Bacillus thuringiensis*).

Lethal concentration (LC_{50}) for 24, 48, 72, 96, 120, 144, 168 hours could not calculate by statistical analysis using probit analysis because the percentage of fish mortality is zero during the toxicity test. Based on Pearson correlation analysis results between density of *Bacillus thuringiensis* isolated from Lamongan, Bangkalan, Madiun and HD 500 showed that it cannot be computed because at least one of the variables is constant (Figure 3-17).

3.3.4 Discussion

3.3.4.1 Effect of indigenously *Bacillus thuringiensis* isolated from East Java and HD 500 (referee *Bacillus thuringiensis*) on third stage *Aedes aegypti* larvae.

In this study, bacterial isolates selected based on the previous research which they have the most effective *Bacillus thuringiensis* isolated from East Java for killing mosquito larvae and they had a phenotypic similarity value between *B. thuringiensis* and the selected isolates more than 82 %. We had been chosen *Bacillus thuringiensis* isolated from Bangkalan (the previous research isolates), Lamongan, Madiun districts, and *Bacillus thuringiensis* var. *israelensis* HD 500 (referee *Bti*) for toxicity test on non-target species. Similar characteristics among the five selected isolates were: rod-shaped bacteria; gram-positive; subterminal endospores; exhibiting oval endospore production; positive-catalase test results; positive-Strach hydrolysis; positive-Voges-Proskauer test results; opaque-density.

Toxicity tests were conducted using various dilutions of bacterial suspension (0:50, 5:45, 10:40, 15:35, 20:30, and 25:25) and exposure times (24, 48, and 72 h). The toxin effectiveness of *B. thuringiensis* isolates was determined. The bacteria form spores and parasporal crystals during the stationary phase, which is a nutritionally deficient state; at that time, the parasporal crystals would be toxic and could kill the *Ae.aegypti* larvae (Renganathan et al. 2011). The *Aedes aegypti* third stage larvae were selected, because at this level, the larvae have a complete anatomical structure and the body is divided into three parts (head, thorax, abdomen). Therefore, damage to the larvae can be easily observed within each section. According Wirth (2010) the numbers of intestinal epithelial

cells and peritrophic cells were increased in accordance within the increasing larval toxin resistance.

The ANOVA and the LC_{50} results indicate a significant effect ($P<0.05$) among the tested isolates. The two isolates (SK.T and Mdn I TK2), which are indigenous of East Java Province, killed *Ae.aegypti* larvae. Among the isolates, Mdn I TK2 isolates were the most effective, as ($2{,}21 \times 10^7$) cells/ml were required to kill 50% of the third stage *Ae.aegypti* larvae within 48 h. Ramirez-Suero et al. (2011), Poopathi and Tyagi (2006), and Poopathi (2010) stated that once the bacterial toxin enters the mosquito larvae digestive tract, the increased time allows the toxin to accumulate, and the toxin's (δ-endotoxin) effects increase in accordance with the exposure. The toxin binds to receptors located on the epithelial midgut cell wall's apical microvillus membrane. After the toxin binds to its receptor, there is a change in the toxin's conformation, allowing toxin insertion into the membrane. Electrophysiological and biochemical evidence suggest that the toxins generate pores in the cell membrane, which disturbs the osmotic balance; consequently, the cells swell and lyses. The gut becomes paralyzed, and the mosquito stops feeding. Most mosquito larvae generally show reduced activity after two hs and die within six hs of toxin injection. Mosquito larvae mortality is also influenced by factors such as bacterial concentration, larval age and the bacterial strain used.

3.3.4.2 Effect of *Bacillus thuringiensis* isolated from Bangkalan, Lamongan, Madiun districts, and HD 500 (referee *Bacillus thuringiensis*) on non target species *Trichogaster pectoralis*

In this research, we used juvenile fish because the fish was still in its infancy and they were very sensitive to changes in the surrounding environment. Based on Amornsakun *et al.* (2004) showed that the size at sexual maturity of female Siamese gourami was 18.07 ± 1.10 cm (mean ± SD) in average total length and 94.20 ± 13.39 gram in average body weight.

In the aquarium was also did not find any changes included pH and temperature during observation. Average pH and temperature during the exposure with *Bacillus thuringiensis* is 7.8 and 25 degree Celsius, respectively. The present study did not show any toxic effects of examining *Bacillus thuringiensis* isolated from Bangkalan, Lamongan and Madiun even *Bti* HD 500 (as referee *Bti.*) suspension on juvenile specimens of *Trichogaster pectoralis.* Furthermore, no influence on the lifespan could be detected. Charbonneau *et al.,* (1994) and Nayar *et al.,* (1999) argued that temperature is also a major factor affecting *Bti* toxicity. The toxicity of VectoBac WDG and a *Bti* spore/crystal mixture to *C. tepperi* has been shown to display large variations in LC_{50} values at 15^{o}C. According to Cao *et al.,* (2012) that the data with technical materials of *Bti* and *C. kiiensis* larvae showed similar changes at temperatures between 30^{o}C and 15^{o}C. The *Bti* toxins can produce toxicity in the insect midgut after ingestion, and it is likely that an increased larval feeding activity at higher temperatures contributes substantially to the observed increases in *Bti* toxicity (Stevens *et al*., 2004).

In this study, we used aerator for each aquarium due to sufficient oxygen in the ponds during the experiment and better survival rate of fish in aerated ponds. Qayyum *et al.* (2005) argued that fish production in aerated ponds is higher in their studies as compared to the Mayer *et al.* (1973) reported fish production in channel catfish ponds, but lower in un-aerated ponds, which seems to be due to mortality of the whole mori resulting in overall decrease in fish production.

Based on table 3-7 showed that all isolates of *Bacillus thuringiensis* did not kill *Trichogaster pectoralis* during 7 days. The result indicating that *Bacillus thuringiensis* isolated from Bangkalan, Lamongan and Madiun districts are tolerated by the juvenile of *Trichogaster pectoralis* R. In the aquarium was also did not find any changes included pH and temperature during observation. Average pH and temperature during the exposure with *Bacillus thuringiensis* is 7.8 and 25 degree Celsius, respectively. Based on Pearson correlation analysis resulted that it has no significant correlation between density of *Bacillus thuringiensis* isolated from Bangkalan, Lamongan, Madiun districts, *Bti* HD 500 and abiotic factors such as temperature and pH during an experiment.

Lethal concentration (LC_{50}) for 24, 48, 72, 96, 120, 144, 168 hours could not calculate by statistical analysis using probit analysis because percentage of fish mortality is zero during the toxicity test. Based on Pearson correlation analysis results between density of *Bacillus thuringiensis* isolated from Lamongan, Bangkalan, Madiun and HD 500 showed that it cannot be computed because at least one of the variables is constant (Figure 3-15).

Although the toxicity of *Bacillus thuringiensis* isolated from Lamongan, Bangkalan, Madiun and HD 500 on the investigated species seems highly unlikely, the present data

cannot entirely rule out a low toxicity. More important, our result data do not exclude long-term and cumulative effects on the examined species, the species composition of their natural habitats, or effects on the food web. Lacey and Merit (2004) point out that neither the results of repeated *Bacillus thuringiensis* var. *israelensis* application nor long-term influence on the ecosystem are sufficiently known. Boisvert and Boisvert (2000) argued that unpredictable effects on higher tropic levels and on the ecosystem structure should be considered.

3.3.4.3 Effect of *Bacillus thuringiensis* var. *israelensis* (*Bti*) on non-target organism

Bacillus thuringiensis var. *israelensis* showed a weak effect on *Eylais hamata* (Acari: Hydrachnidia) and *Physa marmorata* (Gastropoda: physidae) and this is in accordance with the results of investigation of laboratory studies (Mansouri *et al.*, 2013), that confirmed the safety of the *Bacillus thuringiensis* var. *israelensis* in the presence of non-target species (Garcia *et al.,* 1980; Merrit and Wipfli, 1994). Thus, Merrit and Wipfli (1994) in their research on the density of aquatic invertebrates also resulted that Mollusca, Oligochaeta, Crustacea, Hirudinea, Heteroptera, Ephemeroptera, Odonata, Trichoptera, Coleoptera in different sites in Druskininkai in Lithuania, showed no significant difference. Caquet *et al.* (2011) argued that no adverse effects of the treatments on the abundance of Polychaeta *Nereis diversicolor* and *Corophium volutator* by *Bacillus thuringiensis* var. *israelensis* applications. According to Becker and Margalit (1993), Cnidaria (genus: Hydra) were not affected by *Bacillus thuringiensis* var. *israelensis* in laboratory tests at a concentration of 100 mg/l of *Bacillus thuringiensis* var. *israelensis* and oligochaetes (genus: Tubifex) were not affected by *Bacillus thuringiensis* var. *israelensis* in laboratory tests at a concentration of 180 mg/L. In their 6^{th}-year of survey,

observation on the effects of *Bacillus thuringiensis* var. *israelensis* on non-target invertebrates in Minnesota wetlands, Hershey *et al.,* (1998) did not find any significant difference in the abundance of annelids (including oligochaetes) between control and VectoBac® G-treated areas. Similarly, Barnes and Chapman (1998) found that no effect of VectoBac® 12AS on the abundance of insect larvae crustaceans or mollusk collected in sediment from temperate New South Wales saltmarshes. Other *in situ* studies, found no significant *Bacillus thuringiensis* var. *israelensis* toxicity to chironomids (Charbonneau *et al.,* 1994; Lagadic *et al.,* 2002; Vinnersten *et al.,* 2009; Lundström *et al.,* 2010a; Lundström *et al.,* 2010b). In other studies, Östman *et al*., (2008) resulted that an increase in the taxonomic richness and abundance of protozoans, after *Bacillus thuringiensis* var. *israelensis* application *in situ*. After application of the *Bacillus thuringiensis* var. *israelensis* by spreading air against mosquitoes of the Dalälven River in the Sweden, there was a production of new insects (Vinnersten *et al.,* 2009). Laboratory and field studies showed that *Bacillus thuringiensis* var. *israelensis* can be considered as ineffective and harmless biopescide to the environment due to its selectivity (Mulla *et al.,* 1982; Barnes and Chapman, 1998). According to Caquet *et al.,* (2011), during his study for two years, repeated applications of either VectoBac® 12AS or VectoBac® WG in brackish water pools had no significant negative impact of invertebrate communities. In particular, no adverse effects of the treatments were shown on the abundance of *N. diversicolor , C. volutator* and midge larvae, suggesting that the availability of invertebrate food source for birds was not negatively affected by *Bacillus thuringiensis* var. *israelensis* applications. The World Health Organization declared that *Bti* is safe for use in aquatic environments, including drinking water reservoirs for the control of mosquitoes, black flies, and harmful insect larvae (WHO, 1999).

3.3.5 Conclusions

Bacillus thuringiensis isolated from East Java (Lamongan, Bangkalan, Madiun districts and HD 500) toxicity test was conducted on *Aedes aegypti* larvae, the LC_{50} was applied to non-target species: *Trichogaster pectoralis* (Class: Actinopterigii). These *Bacillus thuringiensis* isolated from East Java has shown a toxic effect on the *Aedes aegypti* larvae. However, they were no resulted mortality effects of different concentration of *Bacillus thuringiensis* suspensions (10^6, 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , 10^{12} cells/ml) were exposed to non-target species.

Chapter 4

Safe strategy to control mosquito using the potential of *Bacillus thuringiensis* isolated indigenously from East Java as a natural agent of *Aedes aegypti*

4.1 Introduction

Mosquitoes are insect vectors responsible for the transmission of many diseases. Mosquitoes cause more human sufferings than any other organism; over one million people worldwide die from mosquito-borne diseases every year. Mosquito borne diseases include malaria, filariasis, yellow fever, Chikungunya and dengue fever cause extensive morbidity and mortality and are a major economic burden within disease-endemic countries (Boutayeb, 2006; Poopathi and Abidha, 2010; Nareshkumar *et al*., 2012). *Aedes aegypty* (L.) is the main dengue vector worldwide because of its close association with humans in tropical and sub-tropical urbanized areas (Jansen and Beebe, 2010; Cox *et al*., 2007). The *Aedes aegypti* mosquito, the principal vector for Dengue Hemorrhagic Fever (DHF), which is considered the most pressing vector-borne viral disease in the world, is particularly susceptible to climate variability and climatic change. These mosquitoes are

well adapted to urban environment and successfully breed in containers where water is allowed to accumulate, such as discarded can, bottles, plastic containers and tires. The presence and abundance of *Aedes aegypti* is vital to the transmission of DHF. Changes in mean climate conditions and climate variability also can affect human health via indirect pathways, particularly in the changing of biological and ecological processes that influence infectious disease transmission and food yields (Barrett *et al*., 1998). As in my study (2013) also mentioned that environmental conditions strongly control the geographic distribution and abundance of *Aedes aegypti*. The result of this study indicated that the climate variability is clearly associated with the DHF incidence rate. The maximum air temperature, humidity, rainfall and light duration have played an important role in the transmission of DHF in Nganjuk district, East Java, Indonesia. Based on statistical analysis in this study, it showed that humidity and rainfall have positive correlation with DHF incidence, on the contrary a decreased value of maximum air temperature and light duration would have an impact on increased IR. Another regression analysis resulted that the maximum and minimum air temperature, rainfall in the rainy season, affected Incidence Rate (IR) of DHF; however, in the dry season, the IR was affected by wind velocity and rainfall.

Dengue hemorrhagic fever is one of the serious arboviral diseases in the tropical America, Asia, and Africa. DHF has been the most significant vector borne disease in increasing distribution and incidence of cases in the recent decades. An estimated 2.5 billion people in the world are at risk of dengue hemorrhagic fever, of which 1.3 billion people live in South East Asian Countries (Sankari *et al*., 2012). The incidences of Dengue Hemorrhagic Fever have been increasing in the last five years, both in rural and urban

areas of the world. The incidence of dengue has grown dramatically in recent decades. WHO currently estimates there may be 50 million dengue infections worldwide every year. An estimated 500,000 people with severe dengue require hospitalization each year; a large proportion of them are children. About 2.5% of those affected die. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia and the Western Pacific regions. Southeast Asia and the Western Pacific are the most seriously affected (WHO, 2010).

Indonesia as one of the countries in the tropics is very susceptible to diseases spread by the *Aedes aegypti* mosquito (Suci *et al*., 2010). DHF outbreaks have occurred in Indonesia since 1986; the first case was in Surabaya city. In 2010, there were more than 26,059 reported DHF cases in East Java (Provincial Health Office, 2011). In the past 50 years, the incidence of dengue has increased 30-fold with a widespread geographic area well into the new state or from urban to rural (WHO, 2009). According to Basic Health Research 2007, in Indonesia dengue cases that were diagnosed by health officers and the existence of the symptoms stated by respondents showed the prevalence of 0.6% (range: 0.3 ‰ - 2.5%) within a period of 12 months (Malang City Health Office, 2008). In addition, the prevalence in East Java is only 0.24%; but monitoring is still needed, considering that East Java is one of the densely populated provinces in Indonesia (Badan Pusat Statistik, 2012). According to the Basic Health Research 2007 of East Java Province, the prevalence of dengue and the prevalence of the dengue fever symptoms in rural areas is smaller than in urban areas (0.23% vs. 0.27%).

Recently, uncontrolled use of chemical insecticides has resulted in irreparable damage to the environment. Continuous use of chemical insecticides has led to the

emergence and spread of resistance in vectors of human diseases and agricultural pest (Georghiou, 1990; El-Kersh *et al*., 2012). A major alternative to chemical control is biological control, which is a crucial part of integrated pest management (Aramideh *et al*., 2010; El-kersh *et al*., 2012). Interestingly, in my study resulted that *Bacillus thuringiensis* is an important insect pathogen that is highly toxic to mosquito larvae and related dipterans (Poopathi, S. and Abidha, S 2010). *Bacillus thuringiensis* is a Gram-positive, facultative anaerobe and spore forming saprophyte soil bacterium that was first isolated from diseased larvae of *Bombyx mori* (an economically important insect, being a primary producer of silk, called the silkworm) in Japan (Ishiwata, 1901; Baig and Samina, 2010). The toxicity is attributed to δ-endotoxin, which is made of proteins that are produced and assembled when the bacteria sporulate (Sanahuja *et al*., 2011; Haggag and Yousef, 2010). *Bacillus thuringiensis* during the sporulation produces one or more proteinaceous parasporal crystals (Cry), recognized as delta-endotoxin. This crystal protein under alkaline condition of midgut of insects, gets solubilized, and then activated by intrinsic protease into an active toxin that selectively binds specific receptor in the cell membrane, leading to pore formation and consequent insect death (Soberon *et al*., 2000; Eswarapriya *et al*., 2010; El-kersh *et al*., 2012). In Indonesia, some insecticides use active microbial *B. thuringiensis* imported from the countries such as Belgium (Bactospeine), the United States (mop) and Switzerland (Thuricide). The original *B. thuringiensis* exploration efforts in Indonesia were carried out because the *B. thuringiensis* crystal protein had an arrow host spectrum. Therefore, we argued that the ideal effort in controlling Indonesian mosquitoes would be using *B. thuringiensis* isolated from Indonesia.

4.2 Concepts of biological control agents

Global usage of insecticides for mosquito vector control in recent decades has caused environmental pollution of aqueous ecosystem and resulted in insecticide resistance in many mosquito species. The last decade has witnessed and increased interest in biological control agents. Biological means to control vectors, based on entomopathogenic bacteria has been studied for more than 20 years. The number of biological control agents were screened for their efficacy, mammalian safety and environmental impact. Many organisms have been investigated as potential agents for vector mosquito control, including viruses, fungi, bacteria, protozoa, nematodes, invertebrate predators and fish. Only a few spores forming bacteria, copepods and fish have reached operational use and are undergoing extensive field trials. The discovery of bacteria like *B. thuringiensis* serovar *israelensis* deBarjac, which are highly toxic to dipteran larvae have opened up the possibility of its use as potential biolarvicides in mosquito eradication programs in the over the world (Poopathi and Tyagi, 2002; Poopathi *et al*., 2002). The larvicidal substances of these preparations are based on endotoxin proteins accumulated as parasporal crystals produced by the bacterial cells during the sporulation growth phase. These biological preparations have some important advantages over conventional insecticides in mosquito control operations, besides being safe to non-target organism including human beings. Also, it is harmless to the environment (Prabakaran and Balaraman, 2006). The *Bacillus thuringiensis* serovar *israelensis* has been used operationally in controlling mosquitoes for over two decades and its formulations are highly effective against *Anopheles, Aedes,* and *Culex* mosquitoes (Mahmood, 1998; Poopathi and Abidha, 2010).

4.3 The potential of local natural agents for suppressing *Aedes aegypti*.

Research using *B. thuringiensis* var. *israelensis* is often done to control mosquitoes, either the formulation of *B. thuringiensis* as commercial products or culturing soil bacteria isolates. However *B. thuringiensis* isolate indigenous from East Java rarely used as a natural enemy. My previous study (2010) showed that the efficacy of *B. thuringiensis* Madura isolates higher than *B. thuringiensis* var. *israelensis* for killing mosquito larvae. The ANOVA results (as Table 4-1) showed that this experiment has significantly different from stage of mosquito larvae and dilution factors in the toxicity test of *the B. thuringiensis* isolated from Madura Island against *Aedes aegypti* larvae, as well as the interaction between the dilution and stadia larvae also have different influences. The results of the study showed that the high density of the bacteria (before dilution) would cause high mortality of mosquito larvae because the amount of protein crystals formed also increased. These results were indicated that the density of bacteria depend on the dilution, which increasing the dilution of bacteria could reduce the density of bacteria.

Based on analysis result in table 4-1, the toxin of *Bacillus thuringiensis* was the most common factors due to larval death. The most susceptible *Aedes aegypti* larvae was the first stage larvae, because of the large percentage of death was 88.89% that had the largest mortality rates. In the second larval stage, the percentage of mortality was 64.44%; then in the third and the fourth larval stage, the mortality percentage was 26.67% and 11.11% respectively.

Table 4-1. ANOVA result of toxicity test of *Bacillus thuringiensis* isolated from Madura on *Aedes aegypti* larvae

Source	DF	SS	MS	F	P
Instar larvae (L)	3	1807.40	602.46	13.56	0.00
Dilution (D)	6	222271.90	3711.90	83.50	0.00
L * D	18	9651.80	536.21	12.07	0.00
Error	57	2488.80	44.44		
Total	84	41511.10			

Table 4-2. The cell density of *Bacillus thuringiensis* isolated from Madura (cells/ml) in each dilution series

Dilution	The cell density of bacteria in each dilution (cells/ml)					
series	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Average	1.51×10^{8}	9×10^{6}	1.27×10^{6}	6.17×10^{5}	4×10^{5}	5×10^{4}

Based on my study also showed that older stage of *Aedes aegypti* larvae were more resistant to *Bacillus thuringiensis* isolated from Madura than the early instar larvae. This was due to the development difference of the number of cells, which it had made up the digestive tract of larvae in each instar. The first and second stage developed digestive tract cells; especially in the middle intestine (midgut) were almost the same compared to the third and fourth stage that have further developed. The fourth stage larvae already have

epithelial tissue and perithrophic membrane in the intestinal tissue. It has been protecting epithelial tissue from the toxin of *Bacillus thuringiensis*, however, it would be broken after toxicity test.

The honesty testing results (see table 4-3) showed that the *Bacillus thuringiensis* isolated from Madura was able to kill 50% of each stage mosquito larvae, which were tested within 24 hours. $LC_{50\text{-}24\ hours}$ values for each instar were relatively similar, although in the first instance was a small compared to the older age. This was because the first stage was more sensitive to the crystal toxin produced by *Bacillus thuringiensis* than the second, third and fourth stage of *Aedes aegypti* larvae.

Table 4-3. Honesty test results in interaction between dilution series of *Bacillus thuringiensis* isolated from Madura and the mortality percentage of *Aedes aegypti* larvae (for each instar larvae)

Dilution series	Percentage of larvae mortality on			
	First instar	Second instar	Third instar	Fourth instar
10^{0}	88.89 d	64.44 c	26.67 b	11.11 ab
10^{-1}	2.22 a	0.00 a	2.22 a	4.44 ab
10^{-2}	2.22 a	2.22 a	4.44 a	0.00 a
10^{-3}	0.00 a	0.00 a	2.22 a	0.00 a
10^{-4}	6.67 ab	0.00 a	0.00 a	0.00 a
10^{-5}	2.22 a	0.00 a	2.22 a	0.00 a

The same letters after the number denote no significant difference (significant at the 0.05 level).

Figure 4-1. Third instar *Aedes aegypti* larvae

Note :

A. Before toxicity test; B. After toxicity test; 1. Digestive tract (lyses).

The same thing also happened in my study that using indigenous *Bacillus thuringiensis* isolates from Malang City, East Java on the third instar *Aedes aegypti* larvae (see Figure 4-1). Based on phenotypic characteristics and literature searches, it was found that indigenous *Bacillus thuringiensis* isolated from Malang had similar characteristics to the *Bacillus thuringiensis* subspecies aizawai. Based on the percentage of spore prevalence in *Bacillus thuringiensis* isolates at 48 hours, it was known that if bacterial spore prevalence was increasing, the amount of toxin produced was also growing. As the number of bacterial toxins increases, one may expect the bacteria to be more effective at killing mosquito larvae. There are differences in spore prevalence, which is associated with individual characteristics of the spore-forming isolates. *Bacillus thuringiensis* has two developmental phases: germination and sporulation (Manonmani *et al*., 2011). During sporulation, parasporal crystals are released by autolysis. These crystals are toxic and will damage the mosquito larval digestive tract, thus causing larval mortality.

The early stationary phase is marked by vegetative cell death, followed by toxin accumulation, because the cell metabolism the available nutrients, resulting in nutrient shortage and competition. The bacteria will then synthesize secondary metabolites that are used to maintain life. In *Bacillus thuringiensis*, this stationary phase is associated with spore and toxin formation. Toxins from *Bacillus thuringiensis* cells are formed after the cells have formed endospores (Muniady *et al*., 2011).

My study showed that this experiment also conducted toxicity tests using various dilutions of the bacterial suspension (1:0, 1:1,1:3, 1:5, 1:7, 1:10, 1:15, and 1:20) and exposure times (24, 48, and 72h). The toxin effectiveness of the *Bacillus thuringiensis* isolates was determined. The bacteria form spores and parasporal crystals during the stationary phase, which is a nutritionally deficient state; at that time, the parasporal crystals were toxic and can kill the *Aedes aegypti* larvae (Renganatan *et al*., 2011). The mosquito third instar larvae are selected because at this stage, the larvae have a complete anatomical structure and the body is divided into three parts (head, thorax, abdomen); therefore, damage to the larvae can be easily observed within each section. A previous study demonstrated the numbers of intestinal epithelial cells and peritrophic cells increase in accordance with increasing larval toxin resistance (Poopathi, 2010).

Based on our results (2013), it was indicated that the statistical analysis of the LC_{50} test (lethal concentration) has a significant effect ($p<0.05$) among the tested isolates. The three isolates that are indigenous to Malang City (PWR4-32, SWJ 4-4b, SWJ 5-1) killed *Aedes aegypti* larvae. Among those, the PWR4-32 isolates were the most effective, as 22.79 x 10^7cells/ml were required to kill fifty percent of the *Aedes aegypti* larvae within 72 hours. The 72-hours exposure time was more effective than the 24-hours and 48-hours

exposure times. Once the bacterial toxin enters the mosquito larvae digestive tract, the increased time allows the toxin to accumulate, and the toxin's (δ-endotoxin) effects increase in accordance with the exposure. The toxin binds to receptors located on the epithelial midgut cell wall's apical microvillus membrane. After the toxin binds to its receptor, there is a change in the toxin's conformation, allowing toxin insertion into the membrane. Electrophysiological and biochemical evidence suggest that the toxins generate pores in the cell membrane, which disturbs the osmotic balance; consequently, the cells swell and lyses. The gut becomes paralyzed, and the mosquito stops feeding. Most mosquito larvae generally show reduced activity after two hours and die within six hours of toxin injection (Poopathi *et al*., 2002; Poopathi, 2010). Mosquito larvae mortality is also influenced by factors such as bacterial concentration, larval age and the bacterial strain used (Ramirez-Suero *et al.*, 2011; Poopathi and Tyagi, 2006).

A negative relationship can be observed between the exposure time and the LC_{50} for the *Bacillus thuringiensis* indigenously from Malang City and the reference *Bacillus thuringiensis*. This means that with a longer exposure time, the LC_{50} value will decrease and the larval mortality level will increase (Arivoli and Tennyson, 2011; Valadez-Lira *et al*., 2011). All of the tested and reference isolates had the highest percentages for larval mortality at the highest cell density and the longest exposure time because the toxins were released by the bacteria and accumulated in the *Aedes aegypti* larvae's digestive tract. Increased mortality was also observed for the mosquito larvae. These combined conditions resulted in a higher mosquito larvae mortality rate. Some researchers also stated that among mosquitoes, different preparations of *Bacillus thuringiensis israelensis* have shown different levels of toxicity to host species. Other factors influencing the susceptibility of

mosquito larva to *Bacillus thuringiensis* var. *israelensis*. For example; the effect of giving dosage of toxin could produce different results depending on whether the lethal dose is administered all at once or in the same doses over a long period (Aly *et al*., 1988; Ramirez-Lepe and Montserrat, 2012).

The same thing is also shown by Abdel-Hameed and Landen (1994) that at moderate concentration, 23 of 40 strains of *Bacillus thuringiensis* isolated from Sweden were toxic to *Aedes aegypti.* Five of the strains were toxic to *Diabrotica undecimpunctata* at high concentrations, two were toxic to *Heliothis virescens* at low concentration and five produced thuringiensin (formerly called β-exotoxin). No strain was toxic towards the beet armyworm *Spodoptera exigua* at low concentration. Twenty-three of the strains produced a *B. cereus*-diarrhoeal-type enterotoxin.

Other research also resulted that the *Bacillus thuringiensis* isolated from the local area have the capability to kill *Aedes aegypti* larvae. As the investigation was conducted by Gonzales *et al.,* (2011), they showed that three soils isolates (A21, A51 and C17) from different regions of the Cuban archipelago were identified, characterized and evaluated against *Aedes aegypti* and *Culex quinquefasciatus*. The new isolates were compared with reference IPS82 strain and two strains isolated from biolarvicides Bactivec and Bactoculicida, respectively. The differentiation was done by morphological, biochemical, bioassays activity, and molecular methods (SDS-PAGE, plasmid profile and random amplified polymorphic analysis). All isolates were identified as *Bacillus thuringiensis*. The A21, A51 and C17 isolate showed higher larvicide activity than Bactivec's isolated reference strain, against both *A. aegypti* and *C. quinquefasciatus*. A21 isolates had a protein profile similar to IPS82 and Bactivec strain. A51 and C17 isolate produced a

characteristic protein pattern. A21 and A51 isolate had plasmid patterns similar to IPS82 standard strain, while C17 isolate had different both plasmid profile and protein bands. All the studied isolates showed a diverse RAPD patterns and were different from the strains previously used in biological control in Cuba.

4.4 The current status and the future perspective of the research

Over the past decades, the trend has been towards abatement in the use of chemical insecticides, progressively replaced by emerging environment-friendly pesticides such as bacteria-insecticides, strongly recommended by the World Health Organization (WHO, 1976). *Bacillus thuringiensis* is one of the most famed spore forming bacterium, which was first isolated in 1901 in Japan by Shigetane Ishiwata as the cause of the sudden ("sotto") death disease of silkworms, larvae of the silkworm moth, *Bombyx mori* (Federici *et al*., 2010). After Ishiwata's discovery, Ernst Berliner, the German bacteriologist, unaware of Ishiwata's publication, described a similar bacterium as the cause of disease in larvae of the flour moth, *Ephestia canola.* The species name "*thuringiensis*" is derived from Thuringia, the German state where the diseased flour moth larvae were found. It was later isolated from Mediterranean flour moths and named *Bacillus thuringiensis* in 1911. It was not until 1958 than *Bacillus thuringiensis* was used commercially in the United States (Jenkins, 1992; Poopathi and Abidha, 2010). Federici *et al*., (2010) argues that *Bacillus thuringiensis* is a species of bacteria that has insecticidal properties affecting a selective range of insect orders. There are at least 34 subspecies of *Bacillus thuringiensis* (also called serotype or varieties) and probably over 800 strains isolates By 1989, *Bacillus thuringiensis* products had captured 90-95 per cent of the biopesticide market. *Bacillus*

thuringiensis products available in the United States are comprised of one of five varieties of *B. thuringiensis; Bacillus thuringiensis* var. Kurstaki and var. Morrisoni which cause disease in moth and butterfly caterpillars; *Bacillus thuringiensis* var.israelensis which causes disease in mosquito and blackfly larvae; *Bacillus thuringiensis* var. Aizawai which causes disease in wax moth caterpillars; and *Bacillus thuringiensis* var. *tenebrionis*, also called var. *San Diego*, which causes disease in beetle larvae. Other strains of *Bacillus thuringiensis* have been discovered that exhibit pesticidal activity against nematodes, mites, flatworms and protozoa (Feitelson *et al*., 1992).

Two general groups of insecticidal crystal proteins made this wide variety of subspecies have been identified by Cyt (cytolysins) and Cry (crystal delta-endotoxins). Höfte and Whiteley (1989), reviewed systematic nomenclature and classified the crystal proteins in five major groups according to their insecticidal and molecular relationship (Cry I, Cry II, Cry III, Cry IV and Cry V, Cyt). As new strains are discovered, a need for a new nomenclature arose. According to new nomenclature which is used today, roman numerals have been exchanged with the Arabic numerals and the strains are named on the basis of their evolutionary divergence. Additionally, beneath the capital letters which were present at the first nomenclature as well, small letters have been brought indicating the minor amino acid differences like the capital letters denoted for the major differences. It is also noted that most *Bt* strains produce more than one type of crystal protein that act in combination. Hastowo *et al*., (1992) have been set up several screening programs that aim to isolate new strains producing novel mosquitocidal crystal proteins that could replace or be used in combination with *Bacillus thuringiensis* subsp. *israelensis. Bacillus thuringiensis* subsp. *entomocidus* INA288 has been isolated from Indonesia soil, and it

produces large cuboidal crystals. Although known mosquitocidal *cry* genes, such as *cry2A, cry4A, cry4B* and *cry cry11A*, were not detected by PCR, this strain showed toxicity comparable to that of *Bacillus thuringiensis* subsp. *israelensis* against second-instar *Aedes aegypti*. This result indicated the presence of a novel mosquitocidal *cry* gene(s). When the structural and sequential similarities are considered, conserved amino acid sequences drew attention among most of the Cry toxins. According to these similarities and insecticidal activities, the properties of the Cry proteins differ and the members of the same group share a number of common features. The types of crystal proteins and the insect orders to which they are active were showed in table below:

Table 4-4. The Cry protein groups and the orders they are pathogenic (Deacon, 2001)

No	The Cry protein groups	The orders for:
1.	cry I [several subgrup:A(a), A(b), A(c), B, C, D, E, F, G]	Lepidopteran
2.	cry II [subgrup A, B,C]	Lepidopteran and Dipteran
3.	cry III [subgrup A, B, C]	Coleopteran
4.	cry IV [subgrup A, B,C, D]	Dipteran
5.	cry V-IX	Lepidopteran and Coleopteran

Bacillus thuringiensis crystals are composed of four major polypeptides with molecular weights of 125, 135, 68 and 28 kDa, now referred to as Cry IVA, Cry IVB, Cry IVD and CytA, respectively. De Maagd *et al*., (2003) stated that insecticidal crystal toxins (Cry) are pore-forming toxins (PFT) produced by this bacterium as crystalline inclusions

during the sporulation phase of growth. Crystal development during sporulation of *Bacillus thuringiensis* strains has been studied extensively. These crystals are predominantly comprised of one or more crystal (Cry) and Cytolytic (Cyt) toxins. Cyt proteins are parasporal inclusion protein from *Bacillus thuringiensis* exhibits hemolytic activity. For example *Bacillus thuringiensis* subsp. *israelensis* produces four protein/polypeptides ranging from 135-27kDa referred to as CryIVA, CryIVB, CryIVD and CytA (Lakxmy *et al*., 2011). These genes, encoding this Cry toxin, are located on 72 kDa resident plasmid and they have been cloned and expressed in various hosts. Chromosomal Cry genes have also been reported in some *Bacillus thuringiensis* strains and the role, structure and molecular organization of genes coding for the parasporal delta endotoxin of *Bacillus thuringiensis* biochemical mechanisms of insects' resistance to *Bacillus thuringiensis* indicates that altered proteolysis processing of *Bacillus thuringiensis* crystal proteins involved in one case of resistance in mosquitoes. The presence of IS240 elements responsible for mosquitocidal action was investigated in sixty-nine *Bacillus thuringiensis* strains. A PCR-based approach for detection of Cry genes in *Bacillus thuringiensis* has been reported. Since the toxins of this bacterium are highly potent for mosquito control, evaluation of the activity of *Bacillus thuringiensis* preparations is currently carried out by bioassay with a target insect and compared to a defined standard (Poopathi and Abidha, 2010).

Federici *et al*., (2010) argued that *Bacillus thuringiensis* produces a variety of insecticidal proteins produced during vegetative growth and sporulation that determines its activity for insect species belonging to different orders, with the most important of these being the Cry protein active against Lepidoptera and Coleopteran pest and a combination

of Cry and Cyt proteins for mosquitoes and blackflies. After intoxication by these proteins, spores typically germinate and invade larvae, contributing to insect mortality. Whereas strains wild type isolates have been commercialized and are now used worldwide, the use of recombinant DNA technique, genetic engineering, has been used over the past decade to recombine the proteins of different *Bacillus thuringiensis* strains with those of *Bacillus sphaericus* to generate recombinant larvicides as much as ten-fold more toxic than the parental strains.

4.5 Mode of action

Bacillus thuringiensis israelensis products contain the spores and parasporal crystals of *Bacillus thuringiensis* var. *israelensis* H-14 serotype that must be ingested by the larval stage of the mosquito to cause mortality. This multi-step toxicity process includes ingestion of the Cry protein by a susceptible insect, solubilization, and processing from a protoxin to an activated toxin core in the insect digestive fluid. The toxin core travels across the peritrophic matrix and binds to specific receptors called cadherins on the brush border membrane of the gut cells. Toxin binding to cadherin proteins results in activation of an oncotic cell death pathway and/or formation of toxic oligomers that bind to GPI-anchored proteins and concentrate on regions of the cell membrane called lipid rafts. Accumulation of toxin oligomers results in toxin insertion in the membrane, pore formation, osmotic cell shock, and ultimately insect death (Poopathi and Abidha, 2010). Cyt genes are active against dipteran and coleopteran pests, and additionally have shown an action against hemipterans (true bugs) and dictyopterans (roaches and termites). Cyt, unlike Cry toxins, do not recognize specific binding sites. *Bacillus thuringiensis* directly

causes mortality in insects and isolates of the toxin from different strains follow similar mode of action. After delta-endotoxin crystals are ingested, they are dissolved in the insect midgut, liberating protections of which they are made. These are proteolytically processed into fragments, one of which binds to cells of the midgut epithelium. The activated protein disrupts the osmotic balance of these cells by forming pores in the cell membrane causing the cell to lyse. The gut becomes paralyzed and the insect stop feeding; and as a result, most insects will die within few hours of ingestion. In order to identify the biological pathways that were activity after toxin ingestion, Cancino-Rezno *et al*., (2012) mapped the differential expressed protein to canonical signaling pathways found in the Kyoto Encyclopedia of Genes and Genomes (KEGG). The KEGG analysis showed the immune system NOD-like receptor pathway since the heat shock protein HSP90 participates in this pathway. In addition, some protein of the pathways of glycolysis, citrate cycle and fatty acid metabolism pathways was also activated by Cry11Aa treatment, suggesting activation of carbohydrate and lipid metabolism after toxin intoxication. The binding affinity of these toxin fragments has been often directly related to the toxicity, though binding does not assure toxicity (Whalon and McGaughey, 1998). *Bacillus thuringiensis* var. *israelensis* treated mosquito larvae generally cease feeding within 1 hour, and then it shows reduced activity by two hours, extreme sluggishness by four hours and general paralysis by six hours after ingestion (Glare and Maureen, 1998).

4.6 Effect of *Bacillus thuringiensis* on non-target organisms

Bacillus thuringiensis has no direct effect on aquatic organisms other than mosquitoes, black flies, lacewing, aphid, Crustacea and chironomids. Other aquatic

organisms, such as shrimps, mites and oysters are generally unaffected (Eder and Iris, 2010). This large safety margin of preparations of *Bacillus thuringiensis* for non-target organisms indicated that their suitability for mosquito control programs in areas where protection of the natural ecosystem is important. Field applications have often been monitored for effects on non-target organisms, but no significant non-target effects have been reported (Ramirez-Lepe and Montserrat, 2012).

4.7 Environmental fate

Bacillus thuringiensis is a biological pest control agent. A living bacteria that occur naturally in many soils. Martin and Travers (1989) argued that *Bacillus thuringiensis* was isolated from 70 % of soil samples taken from around the world, and was most abundant in samples taken in Asia. More than half of these isolates were undescribed varieties of *Bacillus thuringiensis.* Essentially no unexpected toxicities from *Bacillus thuringiensis* sprays have been recorded (Icoz *et al*., 2008) probably because *Bacillus thuringiensis* does not survive or grow well in soil (Petra and Casida, 1985) and its spores are rapidly inactivated by UV radiation (Griego and Spence, 1978). Consequently, there is probably little production of toxins in soil (Vettori, *et al*., 2003) and the persistence of introduced toxins is a function primarily of the: (1) amount added; (2) rate of consumption and inactivation by insect larvae; (3) rate of degradation by microorganisms; and (4) rate of abiotic inactivation *Bacillus thuringiensis* has also been isolated from insect bodies, tree leaves and aquatic environments. It has even been recovered from paper (Vaisanen *et al.,* 1991).

Bacillus thuringiensis generally persist only a short time in soil. The half-life of the insecticidal activity (the time in which half of the insecticidal activity is lost) of the crystal is about 9 days (West and Burges, 1985). However, small amounts can be quite persistent. In one experiment, *Bacillus thuringiensis* spore numbers declined by one order of magnitude after 2 weeks, but then remained constant for 8 months following application (Petra and Casida, 1985). *Bacillus thuringiensis* does not appear to move readily in soil. In one study, two varieties of *Bacillus thuringiensis* were applied in adjacent plots, but did not become cross contaminated, indicating that *Bacillus thuringiensis* does not move laterally in soil (Drobniewski, 1994). Other studies found that *Bacillus thuringiensis* was not recovered past a depth of 6 centimeters after irrigation and that movement beyond the application plot was less than 10 yards (Akiba, 1991).

According to Caquet *et al.,* (2011) *Bacillus thuringiensis* var.*israelensis* (*Bti*) is commonly used for selective control of larval populations of mosquitoes in coastal wetlands. A two year-study was implemented to investigate whether repeated treatments with *Bti* applied either as a liquid (VectoBac® 12AS) or a water-dispersible granule (VectoBac® WG) formulation may affect the abundance and diversity of non-target aquatic invertebrates in saltmarsh pools. The taxonomic composition of the invertebrate communities was typical of brackiswater intermittent ecosystems, with a dominance of annelids, crustaceans, and nematocerans. Caquet *et al.,* (2011) also showed that water temperature and salinity was a significant interaction within the different zones. Many environmental parameters were significantly correlated. Water depth was negatively correlated with salinity, and with chlorophyll *a* and suspended solid concentration, and positively correlated with water pH. An opposite pattern was observed for water

temperature. The correlation between water temperature and depth was not significant. Salinity was positively correlated with suspended solids and chlorophyll *a* concentration, whereas it was negatively correlated with pH and dissolved oxygen concentration. Suspended solids and chlorophyll *a* concentrations were positively correlated. Finally, a significant negative correlation was found between pH and chlorophyll *a* concentration, whereas pH and dissolved oxygen concentration were positively correlated.

4.8 Effective Measures for dengue control

According Liaqat et al. (2013) controlling dengue disease can actually be accomplished in different ways:

1. First available strategy is eliminating the mosquito (*Aedes spp.*) vector by larviciding and adulticiding. Although integrated vector control is appropriate and effective, it may be difficult considering political, geographical and logistical basic, since the resurgence of both the mosquito vector and distinct dengue viruses has been observed in areas where these were assumed to be controlled successfully (Sihuincha et al. 2005; Erlangger et al. 2008).
2. Vaccination, a second effective measure to control disease by administering effective vaccine is currently being tried by both governments supported entities and pharmaceutical companies. The development of vaccines against dengue is an important research area, however; it is somewhat complicated. This is due to the fact that dengue fever is caused by four different serotypes and there is a lack of suitable animal models for monitoring the progression of dengue disease (Sabchareon et al. 2012).

3. Drug therapy, the third method is based on the use and application of suitable drugs and pharmaceutical including natural products that may be effective against dengue fever (Erlanger et al. 2008).

4.9 Methods for dengue vector control.

A variety of methods have been adopted to control *Aedes* mosquitoes depending upon the severity of the dengue vector in different countries. The important factors which determine control strategies are; ecology of vector; availability and practicability of resources, culture of the affected country. According Erlanger et al. 2008; Ahmed et al. 2012). It is important to determine the ecology of vector to apply the most suitable control method, which maybe environmental or biological in nature. Several complimentary intervention strategies including microbial and biological control measure, environmental management and reduction of human-mosquito contact based on community participation may also be applied as integrated dengue vector control.

Chapter 5

General Discussion and Conclusion

5.1 Preamble

Nganjuk, Mojokerto, Malang, Madiun, Blitar, Bondowoso, Ponorogo, Tulungagung, Lamongan, Jember, Probolinggo, Pamekasan, Surabaya, Bangkalan were taken as examples of DHF endemic areas in East Java. This study case focuses on biological control using the most potential indigenously *Bacillus thuringiensis* isolated from East Java for suppressing human disease, especially dengue hemorrhagic fever (DHF) which transmitted by *Aedes aegypti* larvae.

This study also includes investigation of association between climatic variability, DHF incidence, and distribution of *Aedes aegypti* as a vector of DHF in East Java, especially in Nganjuk district. Besides distribution patterns, relationship between elevation and abundance of *Aedes aegypti* in Mojokerto is also investigated in this study. Nganjuk and Mojokerto were chosen as a study site due to these regions have the highest Breateu index (BI) for 3 years (from 2008 to 2010).

Isolation and determination of indigenously *Bacillus thuringiensis* were carried out in some districts of East Java, but not all the *Bacillus thuringiensis* isolates used for toxicity test. We choose only the most potential of indigenously *Bacillus thuringiensis* isolated from East Java based on percentage of spore prevalence and the the phenotypic similarity between these bacteria and referee *Bacillus thuringiensis*. Additionally, toxicity study of indigenously *Bacillus thuringiensis* isolated from East Java on non-target organism (*Trichogaster pectoralis*) is also elaborated in this study. Finally, safe strategy to control mosquito using indigenously *Bacillus thuringiensis* isolated from East Java as a natural enemy of mosquito could help enhance the analysis of this study.

5.2 The problem of biological control using natural agents

Dengue fever remains a serious health problem in both urban and rural areas of East Java. This is because dengue fever is a disease that causes high yearly mortality. DHF is transmitted by *Aedes aegypti* that have adapted to living near human-inhabited areas. *Aedes aegypti* (Linnaeus) is the major urban vector of the dengue virus worldwide. This mosquito species have Cosmo-tropical distribution and is widely dispersed throughout Indonesia. Therefore, an effective environmental management system is necessary to avoid the spread of human disease.

B. thuringiensis is an important insect pathogen that is highly toxic to mosquito larvae and related dipterans. The toxicity is attributed to δ-endotoxin, which is made of proteins that are produced and assembled during the sporulation bacteria. In Indonesia, some insecticides use active microbial *B. thuringiensis* imported from countries such as Belgium (Bactospeine), the United States (mop) and Switzerland (Thuricide). The original

B. thuringiensis exploration efforts in Indonesia were carried out because the *B. thuringiensis* crystal protein has an arrow host spectrum. Therefore, the ideal effort in controlling Indonesian mosquitoes would be using *B. thuringiensis* isolated from Indonesia.

The study using *B. thuringiensis* var. *israelensis* is often done to control mosquitoes, either the formulation of *B. thuringiensis* as commercial products or culturing soil bacteria isolates. However *B. thuringiensis* isolate indigenous from East Java rarely used as a natural enemy. As a necessity in any ecology study and biological control components to have a sustainability, maintenance of the local bacteria, which have effectively controlled population of mosquito larvae, it is necessary to observe the toxicity tests for local bacteria from other places around East Java. *B. thuringiensis* is an important insect pathogen that is highly toxic to mosquito larvae and related dipterans.

The first section of this study (in the second Chapter) analyzed about the LC_{50} (lethal concentration) of local isolated and its results indicated that a significant effect ($p<0.05$) among the tested isolates. The three isolates that are indigenous to Malang City (PWR4-32, SWJ 4-4b, SWJ 5-1) killed *Aedes aegypti* larvae. Among those, the PWR4-32 isolates were the most effective, as 22.79 x 10^7cells/ml were required to kill fifty percent of the *Aedes aegypti* larvae within 72 hs. The72-hs exposure time was more effective than the 24-hs and 48-hs exposure times. Once the bacterial toxin enters a mosquito larvae digestive tract, the increased time allows the toxin to accumulate, and the toxin's (δ-endotoxin) effects increase in accordance with the exposure. The toxin binds to receptors located on the epithelial midgut cell wall's apical microvillus membrane. After the toxin binds to its receptor, there is a change in the toxin's conformation, allowing the toxin

insertion into the membrane. Electrophysiological and biochemical evidence suggest that the toxins generate pores in the cell membrane, which disturbs the osmotic balance; consequently, the cells swell and lyses. The gut becomes paralyzed, and the mosquito stops feeding. Most mosquito larvae generally show reduced activity after two hs and die within six hs of toxin injection. Mosquito larvae mortality is also influenced by factors such as bacterial concentration, larval age and the bacterial strain used.

A negative relationship can be observed between the exposure time and the LC_{50} for the *B. thuringiensis* indigenous to Malang City and the reference *B. thuringiensis*. This means that, with a longer exposure time, the LC_{50} value will decrease, and the larval mortality level will increase. All of the tested and reference isolates had the highest percentages for larval mortality at the highest cell density and the longest exposure time because the toxins were released by the bacteria and accumulated in the *Aedes aegypti* larvae's digestive tract. Increased mortality was also observed for the mosquito larvae. These combined conditions resulted in a higher mosquito larvae mortality rate.

The second section of this study investigated toxicity of local *Bacillus thuringiensis* on *Aedes aegypti* larvae while the bacterial sampling locations selected by the altitude because of the low altitude lands (0-250 m above sea level) are usually have the higher mosquito populations. At sites with low latitude location, then it is expected the bacterial density was also great to control mosquito naturally. In contrast to predictions for vector borne parasites, many studies have reported reductions in geographical range size and abundance, and shifts to lower latitudes or high altitudes, in a wide range of organisms that are potential hosts for these parasites.

If bacterial spore prevalence was increasing, it can be assumed that the amount of toxin produced was also growing. As the number of bacterial toxins increases, one may expect the bacteria to be more effective at killing mosquito larvae. There are differences in spore prevalence that are associated with the individual characteristics of the spore-forming isolates. *B. thuringiensis* has two developmental phases: germination and sporulation. During sporulation, parasporal crystals are released by autolysis. These crystals are toxic and will damage the mosquito larval digestive tract, thus causing larval mortality.

The result of the study disclosed that *B. thuringiensis* Brht isolates from Surabaya district has the highest percentage of *Aedes aegypti* larvae mortality at 24 hours. It also described that this isolate is more effective than the reference *B. thuringiensis* (*B. thuringiensis* var. Israelensis HD 567). The mosquito third instar larvae were selected because at this stage, the larvae have a complete anatomical structure and the body is divided into three parts (head, thorax, abdomen); therefore, damage to the larvae can be easily observed within each section. A previous study demonstrated that the numbers of intestinal epithelial cells and peritrophic cells increase in accordance with increasing larval toxin resistance.

This research reported that only Surabaya isolates (BrHt) was able to kill 100% animal within 24 hours, while reference *B. thuringiensis* isolate was able to kill 80% of mosquito larvae after 72 hours. The LC_{50} (lethal concentration) results indicate a significant effect among the tested isolates. One of all isolates that they have been killed *Aedes aegypti* larvae. This isolate is the most effective, as 1.215×10^8 cells/ml was required to kill fifty percent of the *Aedes aegypti* larvae within 24 hs. The 72-hs exposure time was more

effective than the 24-hs and 48-hs exposure times, especially for the reference *B. thuringiensis* (HD 567 isolate). Once the bacterial toxin enters a mosquito larvae digestive tract, the increased time allows the toxin to accumulate, and the toxin's (δ-endotoxin) effects increase in accordance with the exposure. The toxin binds to receptors located on the epithelial midgut cell wall's apical microvillus membrane. After the toxin binds to its receptor, there is a change in the toxin's conformation, allowing the toxin insertion into the membrane. Electrophysiological and biochemical evidence suggest that the toxins generate pores in the cell membrane, which disturbs the osmotic balance; consequently, the cells swell and lyses. The gut becomes paralyzed, and the mosquito stops feeding. Most mosquito larvae generally show reduced activity after two hs and die within six hs of toxin injection. Receptor of larvae midgut can bind to the toxins that were produced by *B .thuringiensis* and it will cause lyses' of the digestive tract mosquito larvae.

A negative relationship can be observed between the exposure time and the LC_{50} for the *B. thuringiensis* indigenous to Surabaya and a reference *B. thuringiensis*. This means that with a longer exposure time, the LC_{50} value will decrease, and the larval mortality level will increase. All of the tested and reference isolates had the highest percentages for larval mortality at the highest cell density and the longest exposure time because the toxins were released by the bacteria and accumulated in the *Aedes aegypti* larvae's digestive tract. Increased mortality was also observed for the mosquito larvae. These combined conditions resulted in a higher mosquito larvae mortality rate. The result of this study showed that had a positive relationship between the percentage of *Aedes aegypti* larvae mortality and time exposure of *B. thuringiensis* isolate.

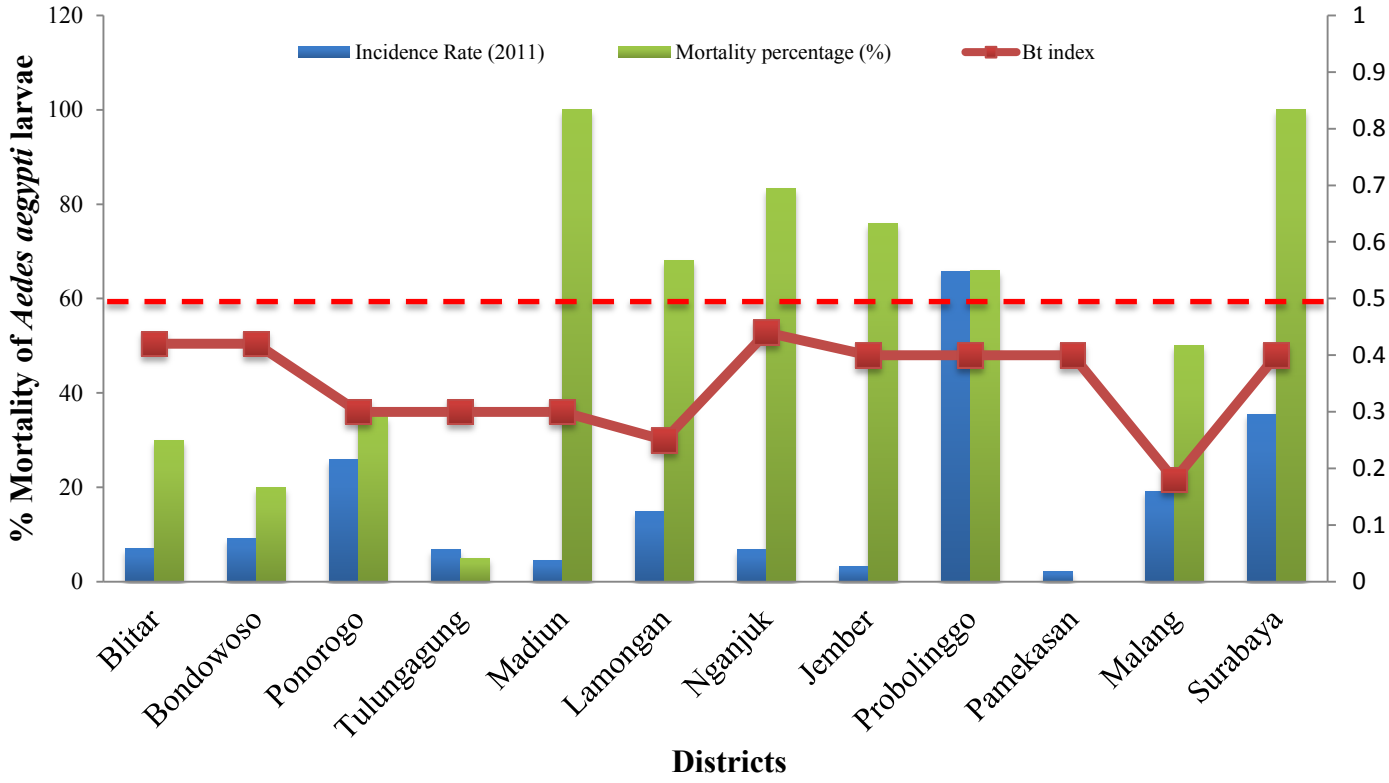

Figure 5-1. Relationship between *Bacillus thuringiensis* index, mortality percentage of *Aedes aegypti* larvae and Incidence Rate (IR) of DHF in East Java 2011

Based on my study results showed that all districts of East Java Province have *Bacillus thuringiensis* index under 0.5. It means that almost all the regions of East Java have low-abundance of *Bacillus thuringiensis* (as Figure 5-1 above). *Bacillus thuringiensis* isolated from Madiun and Surabaya districts have the highest mortality percentage for killing 100 % of *Aedes aegypti* larvae within 48 hours although they only have *a Bacillus thuringiensis* var. *israelensis index* under 0.5. The incidence rate (IR) in Madiun district can be suppressed by presence of *Bacillus thuringiensis* in the same habitat with *Aedes aegypti* larvae. Naturally, bacteria have been killed almost all the mosquito larvae. On the other hand, it can reduce the incidence rate of DHF in the location itself. *Bacillus thuringiensis* isolated from Madiun district is the most potential bacteria for controlling *Aedes aegypti* larvae. *B. thuringiensis* isolated from Madiun is also more effective than referee bacteria (*Bacillus thuringiensis* HD 500 or HD 567) for killing *Aedes aegypti* third larvae in laboratory scale.

Actually, DHF outbreak is still the serious public health problems in East Java. Although *Bacillus thuringiensis* is found in every district of East Java Province, however, their abundance is still low that it would be not able to control the number of mosquito larvae. Therefore, human intervention should be conducted by several methods such as augmentation, conservation of natural enemies as well as an integrated pest management strategy in the environment. The most important thing about the biological control effort is the uses of natural enemies (*B.* thuringiensis) that these came from the same habitat as disease vectors (*Aedes aegypti*). The suitable results of my study have proven that *Bacillus thuringiensis* indigenously isolated from Madiun district has the highest efficacious treatment in killing *Aedes aegypti* third stage larvae are also derived from Madiun district.

The third section of this study-contributed information about all isolates of *Bacillus thuringiensis* did not kill *Trichogaster pectoralis* during the seven day toxicity test. A result indicating that *Bacillus thuringiensis* isolated from Bangkalan, Lamongan and Madiun districts are tolerated by the juvenile of *Trichogaster pectoralis***.** The result of this study showed that in the aquarium was also did not find any changes included pH and temperature during observation.

The present study did not show any toxic effects of examining *Bacillus thuringiensis* isolated from Bangkalan, Lamongan and Madiun even *Bti* HD 500 (as referee *Bti.*) suspension on juvenile specimens of *Trichogaster pectoralis.* Furthermore, no influence on the lifespan could be detected.

Although the toxicity of *Bacillus thuringiensis* isolated from Lamongan, Bangkalan, Madiun and HD 500 on the investigated species seems highly unlikely, the present data cannot entirely rule out low toxicity. More important, our result data do not exclude long-term and cumulative effects on the examined species, the species composition of their natural habitats, or effects on the food web.

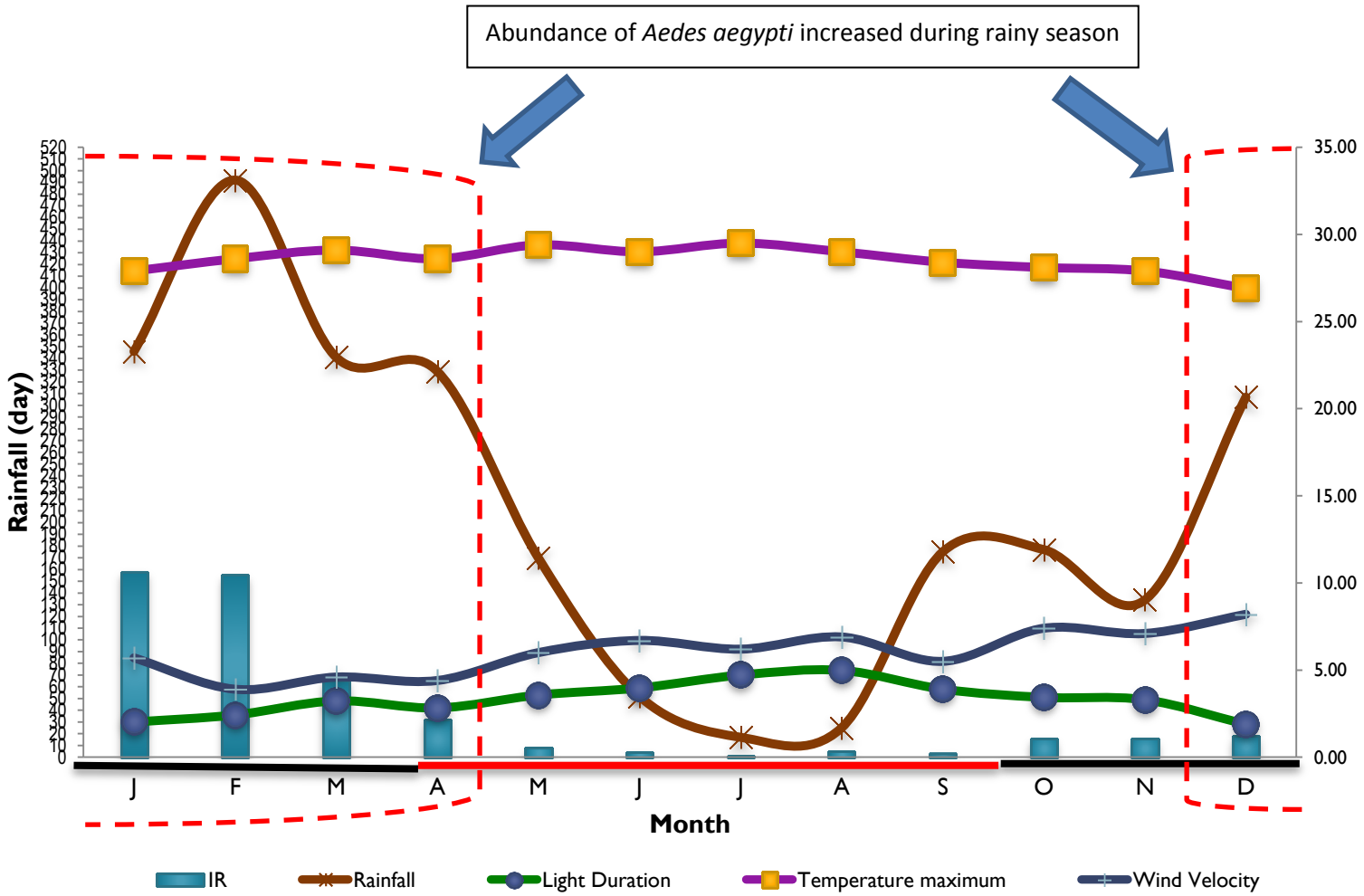

Figure 5-2. Relationship between climatic variability and incidence rate (IR) of DHF in Nganjuk district 2010

Note: ▬ = Rainy season ▬ = Dry season

During the rainy season in the tropical area, rainfall always increased; it means environmental humidity was also increased. According to Buxton and Frazer (1977) bacterial sporulation can be occurred when soil moisture was also increased (soil moisture was over than 70%). However the speed of the sporulation process was also depended on the ambient temperature. When the temperature is over 37 degrees Celsius, then it only takes 8 hours to produce bacterial sporulation. The lower ambient temperature will require a longer time for sporulation of bacteria (if the temperature is 21 degrees Celsius, it will take 24 hours for sporulation. This condition will inhibit bacteria toxin production for killing mosquito larvae (Figure 5-2).

Presence of static surface water in natural or man-made containers is a pre-requisite for *Aedes aegypti* oviposition and larval and pupal development. Despite *Aedes aegypti*'s principal larval habitats being man-made water storage containers (Romero-Vivas and Falconar, 2005) fine-scale temporal relationships between precipitation, vector abundance, and dengue incidence have been established in many locations (Johansson et al. 2009; Li et al. 1985; Hurtado-Diaz et al. 2007). These relationships are not universal, with dengue occurring in dry periods in some locations (Eamchan et al. 1989) and exhibiting varying patterns where two rainy seasons exist (Aiken et al. 1980). In general, however, there is evidence that areas with greater amounts of precipitation are associated with higher dengue infection risk (Wiwanitkit, 2006).

Dengue viruses are established in two habitats: the urban setting where humans and mosquitoes are the only known hosts, and forested areas where mosquito-borne viruses occur in nonhuman primates in a sylvatic cycle, with rare transmission from primates to humans (Simmons et al. 2012). Central to the global emergence of dengue virus has been

the spread of its mosquito vectors. The primary vector of dengue virus, is the highly domesticated, urban-adapted *Aedes aegypti*, found across tropical and subtropical latitudes (Gubler, 1998); however, other secondary vectors including *Aedes albopictus*, *Aedes polynesiensis*, and *Aedes scutellaris* can also transmit the virus. A complex interaction of factors influences the vector efficacy in virus transmission with environmental factors such as precipitation, humidity, and temperature having been most often incorporated into past efforts to model the distribution of dengue transmission (Hales et al. 2002; Barbazan, 2010; Ooi and Gubler, 2009).

At national and international spatial scales, individual human movements drive dengue virus introduction and reintroduction (Adam and Kapan, 2009). Indeed, the global spread of dengue virus in the past sixty years occurred through shipping routes and was characterized by periodic, large, spatial displacements. Globalization has further aided viral transmission by increasing the speed and frequency with which climatically suitable locations for dengue are connected (Hollingsworth et al. 2007; Tatem, 2006). Spread of dengue into new locations requires establishment of a competent local vector population, as the dispersal capabilities of individual mosquitoes are limited (Harrington et al. 2005). Conversely, movement of viremic humans occurs frequently, between a multitude of locations and at varying spatial scales. Therefore, Adams and Kapan (2009) stated that human movement is the key facilitator of the spread of dengue virus at larger spatial scales, particularly in highly accessible, interconnected areas towards which people tend to gravitate. To simultaneously account for accessibility, patterns of human movement, and urban gravitation, then Nelson (2008) used the time required to travel from a given

geographic location to a large city (minimum population 50,000) via land or water-based transportation networks.

5.3 The comparison of LC_{50} between *Bacillus thuringiensis* var.*israelensis* and *Bacillus thuringiensis* indigenously isolated from East Java

Bacillus thuringiensis apparently exists in the environment as spores, which have little or no metabolic processes and can survive periods of low nutrition and desiccation. There is a little multiplication of the vegetative stage in the environment. Bt is also indigenous in many other environments, being found in store products, dust, in deciduous and coniferous plants and in aquatic environments. *Bacillus thuringiensis* var. *israelensis* is generally regarded as specific to larvae of the Diptera (Glare and Maureen, 1998). In the laboratory and occasionally in the field, it has been found to kill other hosts. However, the vast majority of susceptible hosts is recorded in nematocerous Diptera such as *Aedes aegypti.* As shown in table 5-1 below that *Bacillus thuringiensis* var. *israelensis* can kill fifty percent of test animals within 24 hours. When the number of spores is formed, it is assumed that the number of toxins is also produced by *Bacillus thuringiensis* var. *israelensis. Bacillus thuringiensis* var. *israelensis* has a relatively high effectiveness when this bacteria has a number of toxins or cell number slightly, but there was able to kill 50% of mosquito larvae. Based on this table showed that spore production depends on the type of media used for toxicity test. The most appropriate medium which carried out in toxicity testing was milhocina because it is only required in lesser concentration in killing 50% mosquito larvae.

Table 5-1. The LC_{50} of *Bacillus thuringiensis* var. *israelensis* (*Bti*)

No.	Name of isolates	Media tested	$LC_{50\text{-}24hs}$ (mg/l)	Spore (CFU/ml)	References
1	*Bti*	Tryptose	0.0122	9×10^7	Ernandes et al(2013)
		Tryptose+Glucose	0.0084	20×10^7	
		Milhocina	0.0052	20×10^7	
		Milhocina+Glucose	0.0028	30×10^7	
2	*Bti*	Technical powder	0.26	440×10^7	Melo-Santos et al (2009

Based on these findings, it appears that *Bacillus thuringiensis* isolated indigenous from Madiun district (Mdn I K2) has the smallest $LC_{50\text{-}24hours}$. It means that *Bacillus thuringiensis* isolated indigenous from Madiun was the most potential bacteria to control *Aedes aegypti* third larvae because there was only needed 0.47×10^9 cells/ml in killing a half of the test animals (Table 5-2). Related to the number of spores, which there were produced by each bacterial isolates, it appears that *Bacillus thuringiensis* isolated indigenous from Madiun has been reported as the least amount of spores but they have high efficacy compared to the *Bacillus thuringiensis* var.*Israelensis.*

Table 5-2. The LC_{50} of *Bacillus thuringiensis* isolated indigenous from East Java

No.	Name of isolates	Media tested	$LC_{50\text{-}24hs}$ ($\times 10^9$ cells/ml)	Spore (CFU/ml)
1	PWR4-32	sporulation media	0.23	52.44×10^7
2	SWJ4-4b	sporulation media	16.59	23.59×10^7
3	SWJ5-1	sporulation media	11.53	34.46×10^7
4	BrHt	sporulation media	0.122	8.8×10^7
5	SKT	sporulation media	0.025	10×10^7
6	**Mdn I TK2**	**sporulation media**	**0.024**	**3.6×10^7**
7	The reference *Bt*	sporulation media	0.47	10.02×10^7

5.4 Future perspective of the research

Despite recent heightened awareness for dengue prevention, various challenges still exist. These include inadequate funding and resources and the lack of a sound strategy to respond to the increasing problem of dengue outbreaks in a growing number of geographical areas. Rapid urbanization, lack of basic sanitation, increased mobility of populations and international travel has compounded the problem in some countries and areas, and there is no promising solution for sustainable control of dengue vectors. Given the complexity of dengue vector control at national level, a well-organized dengue control program should be established to collaborate with different sectors, ministries, agencies and partners to plan, implement and facilitate these activities.

For vector control, there is a need for increased capacity in outbreak response as a component of a three-pronged strategy. This strategy should include community-based larvae control (using environmental management and other technologies as appropriate), adult mosquito management (including research into novel insecticides and their application) and use of personal protection (including research on repellents, adult reduction devices and their mode of delivery).

Preventive activities should be built into existing health care systems and be well coordinated within primary health care activities rather than within the dengue control program of Ministries of Health. WHO is advocating Integrated Vector Management (IVM) as a further method of dengue vector control. IVM is defined as “a rational decision-making process for the optimal use of resources for vector control.” The important attributes of IVM are advocacy, social mobilization and legislation, collaboration within the health sector and with other sectors, an integrated intervention

method approach, evidence-based decision-making and capacity-building. IVM is suited to dengue management and control programs in dengue endemic countries and should be used when planning dengue prevention in the Region. To improve the efficiency and effectiveness of dengue vector control, increasing the capacity of countries to implement IVM is critical. The basic elements of dengue control under IVM are to adopt evidence-based selection and delivery of different interventions (or combinations of interventions) based on local settings to increase country vector control delivery capacity in all geographical areas and to implement monitoring and evaluation tools.

This biological control study by using toxicity test of indigenous *Bacillus thuringiensis* isolated from East Java against *Aedes aegypti* larvae, is important not only for Malang district and all other districts locating in East Java province, but also for other province to take lessons from what happened in their neighboring condition. It has to be considered that biological control without an integrated pest management strategy (IPM) will create the secondary pest outbreak. In turn, the amount of DHF incidence rate will increase and it will cause the health problems and the environmental degradation are deep impact due to using chemical insecticides to control mosquitoes. Following this study, a managerial and technical study is necessary to overcome the situation, each sampling location.

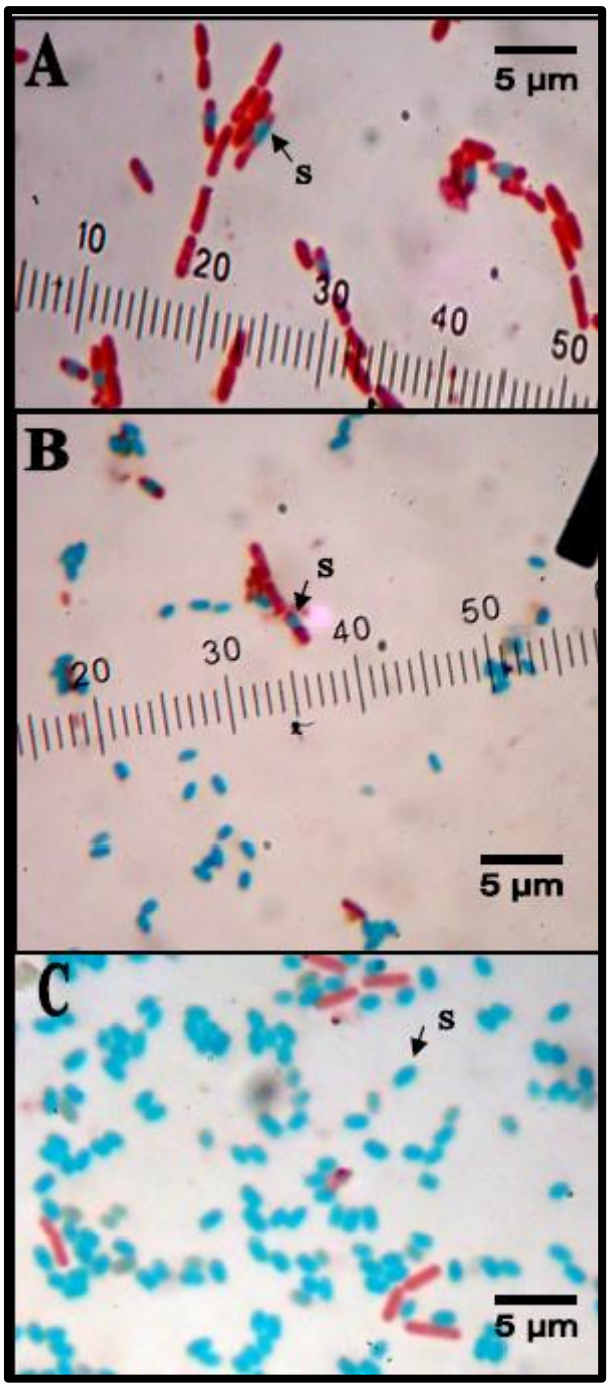

Figure 5-3. Indigenously *Bacillus thuringiensis* isolated from Madiun (A), Lamongan district (B), and *Bacillus thuringiensis* HD 567 (C) (Note : S = Spore)

The usage of indigenous *Bacillus thuringiensis* isolated from East Java for biological control study is also challenging. This study has introduced the utilization *Bacillus thuringiensis* from local soil sampling area to control *Aedes aegypti* from the same area with soil sampling location. The result of biological control study using the same origin for soil sampling and mosquito larvae capture are more effective than using the different source of soil sampling. Although the study was still on a laboratory scale, but the possibility to apply this method in the field is widely open. Before applying to the field, it is necessary to do more experiment at laboratory scale with more comprehensive and more precise measurement. It is fundamental to understand the sensitivity of each target organism (*Aedes* aegypti) is depending on the characteristics of *Bacillus thuringiensis*. Especially for the shape and size of bacteria toxin, density of toxin and abiotic factors and suitability receptors that exist of the mosquito digestive tract have greatly affected the last result.

Biolarvicides based on mosquitocidal toxins of *B. thuringiensis* indigenously isolated from East Java have great potential in controlling the breeding of *Aedes aegypti* mosquito, vectors of dengue hemorrhagic fever in an integrated vector control program, either independently as a larvicide or along with other biological control agents and natural predators of mosquito larvae. *B. thuringiensis* indigenously isolated from East Java as bacterial agents are highly specific in action against *Aedes aegypti* and are safe to other organisms.

Existing *Bti* formulations are, highly effective against *Aedes* mosquitoes. The produce of *B. thuringiensis* indigenously isolated from East Java is also challenging. However, further improvement, particularly to extend their long-term effect and to

enhance control, will accelerate this process further. Tablet and granule formulation of *B. thuringiensis* might be developed which can be used by individuals and community, particularly to control container breeding *Aedes aegypti.* We could be using the simple liquid medium to produce a formulation of *B. thuringiensis* indigenously isolated from East Java such as coconut milk.

B. thuringiensis indigenously isolated from East Java as potent vector control agents are considered as the most potent and successful groups of organism to be utilized. Their specific properties including (i) high efficacy against the target organism (ii) relative ease of mass production of industrial scale (iii) environmental safety (iv) cost-effectiveness (v) minimal development of resistance (vi) simple integration into control programs involving community participation make them potentially the most effective biological control agents.

5.5 Scientific contribution of this study

It is very glad to mention that this study produced several scientific contributions to basic science. As any doctoral dissertation is expected to find something new in its field, this study could deliver several interesting findings, from which the author can consider them as novelties. We believe that some findings are relatively new in its field and some other are kind of confirmation to the previous scientific publication and reports. If these findings are listed from the most important aspect to the least one, the sequence may be as that listed in the following order:

1. **All locations of East Java have a higher House Index (HI) than the WHO standard for the high DHF risk area (i.e.10 % HI).**

The density of *Aedes aegypti* has positive correlation with the average of DHF incidence rate in East Java Province from 2008 to 2010. The results of statistical analysis showed that there was a significant relationship between the average IR and the number of *Aedes aegypti*. And the mosquitoes were captured by using a bait indoor has the most closely related to the average IR cause *Aedes aegypti* is an *anthropophylic* mosquito, taking its blood meals preferentially from humans. *Aedes aegypti* was more often found in indoor than outdoor. The highest *Aedes aegypti* house index was found in Bangkalan District. In these districts, there are many good habitats for *Aedes aegypti* larvae.

2. **The incidence rate (IR) of DHF was affected by the maximum air temperature, minimum air temperature, and rainfall in the rainy season, but in the dry season, the IR was affected by wind velocity and rainfall**.

The average of DHF incidence in Nganjuk District was 4.05 per 100.000 residents from 2005 to 2010. The highest DHF incidence (26.53 per 100.000 residents) was founded in February 2007 and the lowest DHF incidence was detected on September 2006 (IR = 0).

The highest incidence rate occurred in February 2007 (rainy season) when the temperature ranged between 17.20 and 27.80 degree Celsius and rainfall at 507 mm/day.

3. The mosquitoes found in Mojokerto district consist of five species.

There were *Aedes aegypti , Aedes albopictus, Aedes laniger, Culex bitaeniorchynchus and Culex quinquefasciatus.* Although *Aedes aegypti* is the

predominant species found in Mojokerto region, 2012, but this species still plays an important role for the outbreak of DHF. *Aedes aegypti* has an Importance Value Index (IVI) of 70.48%. Based on calculations using the Morisita index, the pattern of spread of mosquitoes in the area of Mojokerto is uniform. The elevation of each sampling area and density of mosquitoes has positive associations.

4. **A negative relationship can be observed between the exposure time and the LC_{50} for the *Bacillus thuringiensis* indigenous to Malang City and the reference *Bacillus thuringiensis***.

This means that with a longer exposure time, the LC_{50} value will decrease and the larval mortality level will increase. All of the tested and reference isolates had the high percentages for larval mortality at the highest cell density and the longest exposure time because the toxins were released by the bacteria and accumulated in the *Aedes aegypti* larvae's digestive tract. Increased mortality was also observed for the mosquito larvae. These combined conditions resulted in a higher mosquito larvae mortality rate.

5. ***Bacillus thuringiensis* isolated from East Java more effective than *Bacillus thuringiensis* var. *israelensis* to control *Aedes aegypti* larvae.**

The result of the study disclosed that *Bacillus thuringiensis* Brht isolates from Surabaya district has the highest percentage of *Aedes aegypti* larvae mortality at 24 hours. This fact indicates that *Bacillus thuringiensis* indigenously isolated from East Java has a high potential natural enemy to control of *Aedes aegypti*.

6. *Bacillus thuringiensis* isolated from East Java has not toxic against *Trichogaster pectoralis.*

Trichogaster pectoralis were exposed to different concentration (10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11} , 10^{12} cells/ml) of *Bacillus thuringiensis* isolated from Lamongan, Bangkalan, Madiun and HD 500 (referee bacteria) suspension no resulted mortality effects.

7. The most important finding about the biological control effort is the usage of natural enemies (*B. thuringiensis*) where they come from the same habitat as disease vectors (*Aedes aegypti*).

B. thuringiensis indigenously isolated from East Java as bacterial agents are highly specific in action against *Aedes aegypti* and are safe to other organisms. My study showed that *Bacillus thuringiensis* indigenously isolated from Madiun district has the highest efficacy in killing *Aedes aegypti* third stage larvae are also derived from Madiun district.

8. All districts of East Java Province have *B. thuringiensis* index under 0.5. It means that almost all the regions of East Java have low-abundance of *Bacillus thuringiensis*.

This causes East Java is still has DHF outbreak, though naturally, bacteria has ability to control the abundance of mosquitoes in the same habitat.

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