Molecular Identification of Bacterial Pathogen Infecting Coconut Leaf Beetle
*Brontispa longissima* (Coleoptera:Chrysomelidae)

**ABSTRACT**

Many species of microorganisms can cause diseases and mortality of insect pests. Accurate detection and identification of the entomopathogens are essential for development of biological control agent to the pest. *Brontispa longissima*, a serious and invasive pest of coconut, was infected by bacterium causing mortality of the larvae and pupae in coconut field. Objective of the research was to identify bacterium as a causal agent of the field-infected *B. longissima* using molecular technique. Research was conducted between April and August 2011. Molecular identification using polymerase chain reaction (PCR) amplification of 16s ribosomal RNA of the infected larvae and sequencing of the gene showed that *Serratia marcescens* is the causal agent of the disease.

**Keywords**: *Brontispa longissima*, coconut, 16s rRNA, *Serratia marcescens*.

**INTRODUCTION**

The plant-protecting role of bacterial entomopathogens in controlling insect pest population has been well known. Entomopathogens attack insect pests through a number of mechanisms including antibiosis (Gerc *et al*., 2012) and production of enzymes that disrupt proteins used by the insect in response to the insect’s attack (Flyg *et al*., 1983). Most of the researches using microbial bacteria to control insect pests in several past years have been dominated by *Bacillus thuringiensis* (Bt) (Ruu *et al*., 2015). However, adaptation of some herbivorous insects to this bacterium has lead to the need of finding a novel bacterial species with new mode of action.

*Brontispa longissima*, a serious and invasive pest of coconut has been found in the field to be infected by entomopathogen putatively identified as *Serratia* sp. based on the red pigmentation of the infected larvae (Alouw *et al*., 2008a,b). However, *Serratia* sp. exists in nature as either pigmented or unpigmented bacteria (Grimont *et al*., 1978, 2006). Therefore, identification of the causal agent based on the pigmentation on the insect is still needed further investigation. The toxic effects of *Serratia* spp. are responsible for naturally death of about 70 insect spesies (Grimont *et al*., 1978; Petersen *et al*., 2012; Lauzon *et al*., 2003) including *B. longissima* (Alouw *et al*., 1998). *Serratia* sp. is a bacterial entomopathogen that exist in various environments including soil, water, air, plants and insects (Grimont *et al*., 1978). Pigment produced by...
Serratia is called prodiogolin. Prodiogolin and prodiogolin-like pigment are also produced by other species such as Vibrio psychrothermus, Pseudomonas magnesibora, Alteromonas rubra, Streptomyces spp., and Nocardia (Actinomadura spp.) (Grimont et al., 1978).

B. longissima was firstly reported in 1885, more than a century ago from Aru Island (Waterhouse et al., 1987, Singh and Rethinam, 2005). Larvae and adult of the beetle feed on tissues of unopened fronds of coconut palm causing browning of the leaves. Light attack results in only minor leaf injury. Fruit production is significantly reduced if eight or more leaves are destroyed (Waterhouse, 1987). Under prolonged outbreak condition, fruit-shedding might occurs, newly-formed leaves remain small, and the trees appear ragged (Alouw et al., 2008a, b; Singh and Rethinam, 2005). Successive severe defoliation can lead to death of the palm. Seventeen species of palm trees including oil palm, nipa palm and many ornamental palms are host of the pest, and among them coconut palm is the most preferred host. The beetle was believed accidentally introduced from Indonesia into several other countries in the Pacific in the 20th century. During 1919-1934 B. longissima had been recognized as a pest of coconut in only few provinces of Indonesia and since 2004 it has been reported from almost every province in Indonesia (Singh and Rethinam, 2005; Hosang et al., 2004; Alouw et al., 2008a, b). The pest was not reported from continental Southeast Asian countries until the late 1990’s when it was found in Mekong Delta of Vietnam and the Maldives (Nakamura et al., 2006). It is suspected that shipment of ornamentals has contributed to the introduction of this pest into both countries (Nakamura et al., 2006). The pest has been expanding in areas around Southeast Asian countries, and it was found in Myanmar in 2004, followed by the Philippines in 2005 (Nakamura et al., 2006, Singh and Rethinam, 2005). Since there are a large number of coconut industries in coconut pro-ducing countries, the pest incursion would be catastrophic to those countries.

Bacterial entomopathogen can exist as heterogeneous species complexes (Grimont and Grimont, 1978). Distinct molecular genotypes can also exist within bacterial entomopathogen species and may have different pathogenic or virulence levels to the insect host. Therefore, rapid and accurate detection of bacteria is needed to differentiate them from others according to their role and to potentially use them as biological control. It has been known that identification through molecular is more sensitive than the culture method (Rhoads et al., 2012).

The purposes of this study were to identify Brontispa-infecting bacterium using conventional polymerase chain reaction (PCR) amplification of 16s ribosomal RNA, sequencing the gene and comparing the sequences with genebank database. 16S rRNA gene sequences can be found in almost all bacteria species and have been the most common housekeeping genetic marker for studying bacterial phylogeny and identification (Janda and Abbott, 2007).

MATERIAL AND METHOD

Insect samples

Bacterium-infected larvae were collected from coconut leaves attacked by B. longissima in Kaiwatu experimental garden. The reddish larva, which is the typical sign of bacterial infection, was individually separated from the healthy one for further investigation.

Isolation, purification and postulat Koch of field-infected Brontispa longissima larvae

Larvae infected by red-pigmenting bacterium in coconut field attacked by B. longissima were isolated and purified on nutrient agar. The pure culture was then infected to the healthy larvae to ensure similar symptom of infection. Laboratory-infected larvae were isolated and purified again on nutrient agar and Luria bertani (LB). The symptom was observed every day under laboratory condition.

Extraction of DNA of red pigmening bacteria

Field-infected larvae were isolated and cultured in LB agar. A culture bacterium in 5 ml of liquid LB media was shaken for 18 hours in an orbital shaker (75 rpm) under room temperature. The culture was centrifuged at 10,000 rpm for 5 min to obtain bacterial cells. Cells was suspended with 150 μl solution buffer I, and was added with the same amount of buffer II. The suspension was mixed well and incubated for 5-20 min under room temperature. 250 μl solution III was added and mixed well. The suspension was centrifuged at 10,000 rpm for 5 min. The supernatant was placed in new tube and was added with 100 μl phenol and chloroform isoamyl and was centrifuged at 10,000 rpm for 10 min. 600 μl supernatant was added with 600 μl iso-propanol, and was centrifuged again. The supernatant was removed, precipitated and was added with 20 μl purified water.
Amplification of 16S ribosomal RNA

Identification of the red bacteria was done based on 16S ribosomal RNA nucleotide sequences. Ribosomal DNA provides the genetic coding from which rRNA molecules are constructed. Amplification of DNA fragment was done with a pair of primer 63F (C A G G C C T AACACATGCAAGTC) and 1387R (G G G C G G A/T G T G T A C A G G C) using PCR machine. Composition 20 µl reaction of PCR consist of 2 µl 10X buffer PCR, 1.5 µl 50 mM MgCl₂, 0.5 µl 4 mM dNTP, 1 µl 20 mM primer forward, 1 µl 20 mM primer reverse, 0.5 µl 5U Taq Polimerase, and 13.5 µl distilled sterilized water. PCR reaction was placed in 200 µl microcentrifugation tubes. DNA template for PCR used single bacteria colony from 18 hours culture on LB agar in petridish. PCR was performed with an initial of 94°C for 10 minutes, followed by 35 cycles of 94°C denaturation for 1 min, 50°C annealing for 45 seconds and 72°C extension for 1 minute 30 second. The quality of PCR product was confirmed by agarose gel electrophoresis at 90 volt for 30 min in buffer TAE.

Analysis of nucleotide sequences

The PCR product was subjected to DNA sequencing. Analysis of the sequence was performed using software Blastn of National Center for Biotechnology Information (NCBI) to know the close relationship with bacteria in database GeneBank (NCBI). 16S DNA ribosomal sequence in GeneBank having close relationship with 16S DNA ribosomal sequence of red bacteria was analyzed using phylogenetic tree.

Sequencing of DNA fragment

The PCR product was subjected to DNA sequencing by ABI PRISM 3070 DNA Sequencer. Sequencing reaction was performed by ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, USA). Sequencing reaction contain 200 ng DNA, 3.2 pmol primer, 1 µl big dye terminator ready reaction mix and destilled water that was added to a final volume of 10 µl. Sequencing was performed by PCR thermal cycler (Biometra, USA) with program of 96°C for 30 second, 50°C for 5 second and 60°C for 1 min. PCR product was purified using ethanol precipitation method 1 µl 125 mM EDTA, 1 µl 3 M sodium acetate pH 4, and 25 µl 100% ethanol were added to the sequencing reaction. The mixed solution was centrifuged at 12,000 rpm for 20 minute at 4°C after incubating it for 15 min under room temperature. Pellet was washed with 70% ethanol and was dried at 65°C for 10 min.

Phylogenetic analysis was performed by PHYLIP program version 3.6 (University of Washington) after the nucleotide sequences of the chosen isolates were formatted by ClustalX 1.83. The matrix of the genetics distance was calculated with parameter matrix in DNAML computer program. Bootstrap analysis with 100 replications was performed by using SEQBOOT and the consensus of the phylogenetic tree was built with CONSENSE program. Phylogenetic tree was drew with MEGA4 program in PHYLIP program.

RESULT AND DISCUSSION

Symptom of B. longissima larvae infected by red-pigmenting bacterium (Figure 1a) in the field was similar to those infected by pure culture of the bacterium (Figure 1b) under laboratory condition. The result confirmed that the isolated bacterium infecting larvae of B. longissima in the field can be isolated on culture medium.
Molecular identification of red bacteria-infected *Brontispa longissima*

The 16s rRNA gene is also designated as 16s rDNA. The terms have been used interchangeably (Claridge, 2004). Using 16s rDNA primers, a predicted band for red bacteria DNA template was obtained (Figure 2). As the DNA double helix unwinds, RNA molecules read the template that is provided from this DNA sequence and an rRNA molecule is formed. Since these DNA segments do not provide code for specific proteins, the rRNA products produced from these DNA genes are considered their end products. Region of 16s rRNA from red bacteria is 1.3 kb. 16s rRNA genes have often been used as markers for identification and phylogenetic differences of inter- and intra-species. For example, utilization of 16s rDNA showed the usefulness of the genetic markers to identify a number of bacteria including *Klebsiella pneumonia* (Fatimawali, 2013).

![Figure 2. Elektroforesis of PCR product of 16S rRNA red-pigmenting bacterium isolated from *B. longissima*. M: marker (1 kb), 1: red-pigmenting bacterium.](image)

**Gambar 2. Elekroforesis dari produk PCR 16S rRNA bakteri berpigmen merah yang diisolasi dari *Brontispa longissima*. M: marker (1 kb), 1: bakteri berpigmen merah.**

The 16s rDNA gene sequences of red bacteria sample from infected *B. longissima* had a degree of identity of 99% to *S. marcescens* with 0.0 E value. Zero E-value means that these sequences are essentially similar to the query. The result confirmed that *S. marcescens* is a causal agent of the disease causing the death of *B. longissima, after* being reddish and sluggish.

*Serratia* spp., is a gram-negative bacterium (Enterobacteriaceae) containing insect pathogenic strains (Tambong et al., 2014) against several insect species including locusts (Tao et al., 2006, 2007). There are more than 70 species of insects are susceptible to *Serratia* infections including insects from order orthoptera, isothen, coleoptera, lepidoptera, hymenoptera and diptera (Grimont et al., 1978). There are a number of mechanisms used by *S. marcescens* in infecting insects. Extracellular proteins such as nuclease, phospholipase, protease, and hemolysin, have been reported to be involved in the pathogenesis of locusts (Tao et al., 2006, 2007). Extracellular metalloproteases are mostly associated with pathogenic bacteria (Tao et al., 2006, 2007; Chaston et al., 2011). Serralysin metalloprotease contained in *S. marcescens* suppresses immune system of silkworms (*Bombyx mori*) by inhibiting immune cell adhesion or degrading adhesion molecules (Ishii et al., 2014a), and also by inhibiting wound healing, which leads to a massive loss of hemolymph in silkworm larvae (Ishii et al., 2014b). Cecropins are used by insect as the main defence against gram-negative bacteria. Three different proteases exist in *S. marcescens* can destroy cecropins (Fly et al., 1983). It has been proposed that outer membrane vesicles (OMVs) produced by bacteria might act as long-range toxin delivery vectors, and the result from laboratory test showed that OMVs of *S. marcescens* are optimally produced at 22°C to 30°C (McMahon et al., 2012).

In addition to its role as a biological control agent, *Serratia* spp. are considered as opportunistic human pathogens (Kurz et al., 2003). However, among several species of the genus *Serratia* (Holt et al., 1994), some of them produce prodigiosin, a nondiffusible, water-insoluble red pigment. Prodigiosin is also known as a secondary metabolic producing red-pigment antibiotic (Coulthurst et al., 2006). Most of the strains isolated from humans are nonpigmented, while those isolated from insects are red-pigmented (Sikorowski et al., 2001). It seems that *Serratia* species infecting human is different from those that infect insects. However, further studies are needed to confirm the effects of this bacterium to human health and other important organisms. Metalloproteases produced by *S. marcescens* are potential to be developed for biological control of important insect pests (Tambong et al., 2014) including *B. longissima*, that is naturally infected by this entomopathogen in the field.
CONCLUSION

*Serratia marcescens* was confirmed as a causal agent of *B. longissima* disease. This present study based on 16s rRNA genetic markers provided a mean for rapid and accurate identification of entomopathogenic bacterium infecting *B. longissima*.

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