

**Molecular Identification of Bacterial Pathogen Infecting Coconut Leaf Beetle
Brontispa longissima (Coleoptera:Chrysomelidae)**
**Identifikasi Molekular Bakteri Pathogen yang Menginfeksi Hama Daun Kelapa
Brontispa longissima (Coleoptera:Chrysomelidae)**

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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.

ABSTRAK

Banyak mikroorganisme dapat menimbulkan penyakit pada serangga hama. Deteksi dan identifikasi yang akurat dari pathogen penyebab penyakit pada serangga (entomopathogen) hama merupakan tahap yang penting dalam pengembangan pengendalian biologi untuk hama tersebut. *Brontispa longissima* sebagai hama penting dan bersifat invasif pada tanaman kelapa diinfeksi oleh sejenis bakteri yang menyebabkan kematian larva dan pupa dari serangga tersebut di lapangan. Penelitian ini bertujuan untuk mengidentifikasi organisme penyebab penyakit pada hama *B. longissima* dengan menggunakan teknik molekuler. Penelitian dilaksanakan pada bulan April sampai dengan Agustus 2011. Identifikasi bakteri dilakukan dengan mengamplifikasi 16s ribosomal RNA dari larva yang terinfeksi dengan menggunakan PCR (polymerase chain reaction). Hasil analisis sekuens nukleotida 16s ribosomal RNA dari larva yang terinfeksi menunjukkan bahwa *Serratia marcescens* adalah bakteri penyebab dari penyakit tersebut.

Kata kunci: Brontispa longissima, kelapa, 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) (Ruiu *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 species (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 prodioglosin. Prodioglosin and prodioglosin-like pigment are also produced by other species such as *Vibrio psychoerythrus*, *Pseudomonas magnesiobura*, *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 producing 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 pigmenting 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 isoamil 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 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 distilled 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 formed 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 drawn 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.

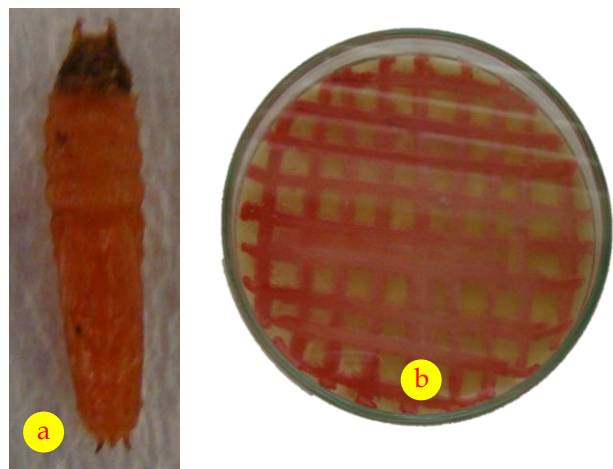


Figure 1. Symptom of *B. longissima* larva infected by red-pigmenting bacterium (a) and pure culture of the red-pigmenting bacterium on Luria bertani agar (b).

Gambar 1. Gejala dari larva *B. longissima* yang diinfeksi oleh bakteri penghasil pigmen merah (a) dan kultur murni dari bakteri berpigmen merah pada media Luria bertani (b).

Molecular identification of red bacteria-infected *Brontispa longissima*

The 16s rRNA gene is also designated as 16s rDNA. The terms have been used interchangeably (Clarridge, 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).

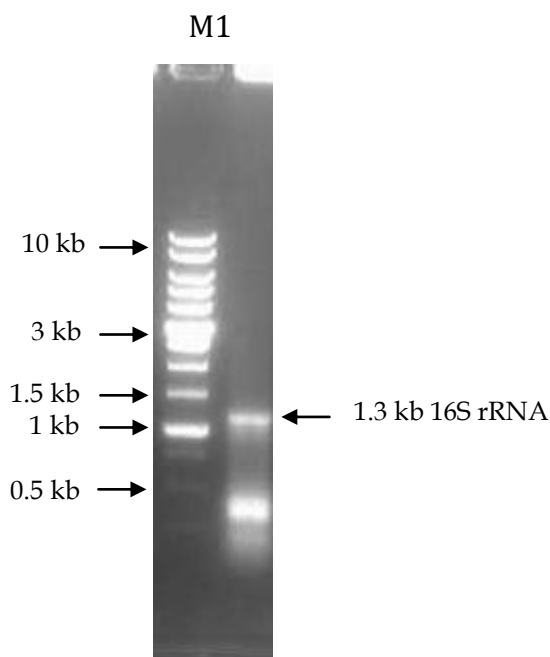


Figure 2. Elektrophoresis of PCR product of 16S rRNA red-pigmenting bacterium isolated from *B. longissima*. M: marker (1 kb), 1: red-pigmenting bacterium.

Gambar 2. Elektrofosis 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, isopteran, 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). Serralyisin 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.

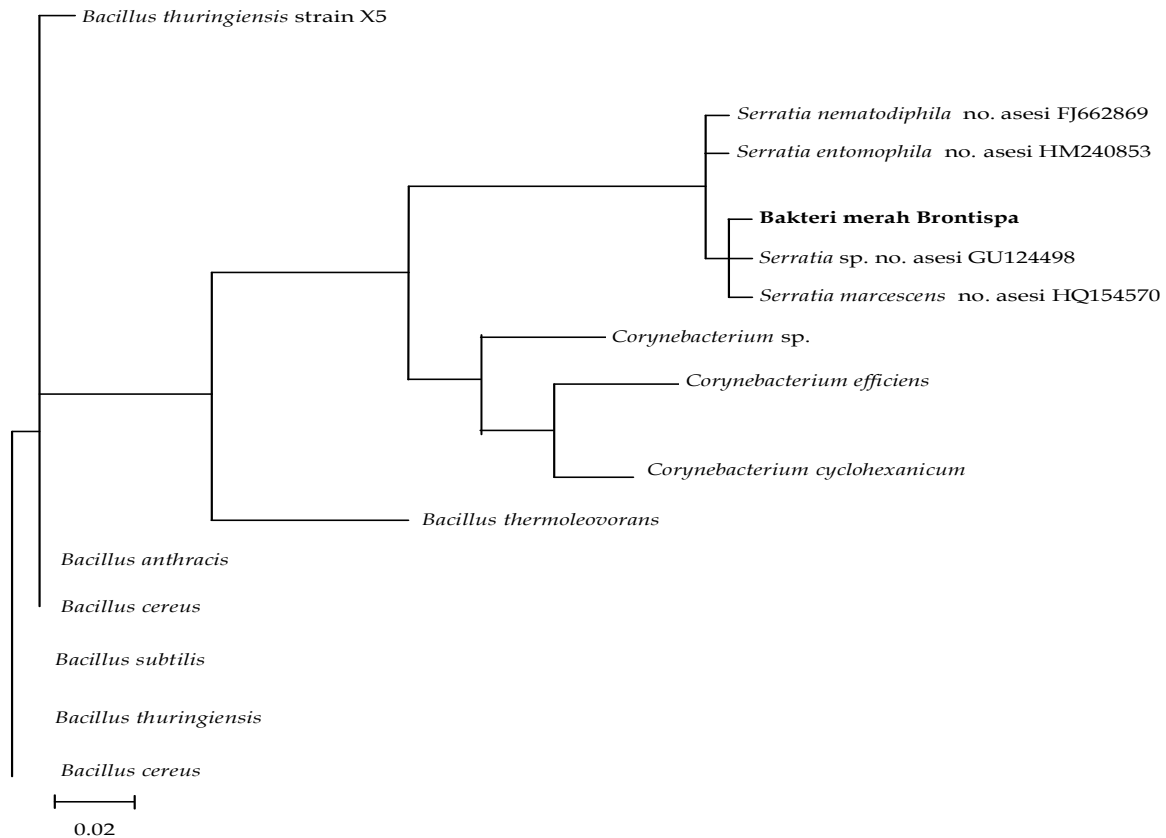


Figure 3. Phylogenetic tree of bacterium infecting *B. longissima* and other bacteria.
 Gambar 3. Pohon filogeni dari bakteri yang menginfeksi *B. longissima* dan bakteri lainnya.

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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REFERENCES

Alouw, J.C. and M.L.A. Hosang. 2008a. Survei hama kumbang kelapa *Brontispa longissima* (Gestro) dan musuh alamnya di Provinsi

sulawesi utara (Survey on coconut hispine beetle *Brontispa longissima* (Gestro) and its natural enemies in north sulawesi province). Buletin Palma (34):9-17.

Alouw, J.C. and M.L.A. Hosang. 2008b. Observasi musuh alami hama *Brontispa longissima* (Gestro) di Propinsi Maluku (Observation of natural enemies of *Brontispa longissima* (Gestro) in Maluku Province. Buletin Palma (35):34-42.

Alouw, J.C. and Diana Novianti. 2010. Status hama *Brontispa longissima* (Gestro) pada pertanaman kelapa di Kabupaten Biak Numfor, Propinsi Papua. Status of *Brontispa longissima* (Gestro) pest on coconut plantation in Biak Numfor District, Papua Province. Buletin palma (39): 154-161.

Chaston, J.M., Suen G., Tucker, S.I., Andersen, A.W., Bhasin, A., Bode, E. 2011. The entomopathogenic bacterial endosymbionts *Xenorhabdis* and *Photorhabdus*: convergent lifestyles from divergent genomes. PLoS One, 6:e27909. Doi:10.1371/journal.pone.0027909. PMID:22125637.

- Clarridge, J.E. 2004. Impact of 16s rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infections diseases. *Clinical Microbiology Reviews* (1): 840-862.
- Coulthurst, S.J., Williamson, N.R., Harris, A.K.P., Spring, D.R., and Salmond, P.C. 2006. Metabolic and regulatory engineering of *Serratia marcescens*: mimicking phage-mediated horizontal acquisition of antibiotic biosynthesis and quorum-sensing capacities. *Microbiology* 152:1899-1911. DOI 10.1099/mic.028803-0.
- Fatimawali. 2013. Identifikasi mikrobiologi dan analisis gen 16SrRNA bakteri resisten merkuri isolat S3.2.2. yang diperoleh dari limbah tambang rakyat. *Pharmakon Jurnal Ilmiah Farmasi-UNSRAT* 2(4) : 2302-2493.
- Flyg C., and Xanthopoulos, K.G. 1983. Insect pathogenic properties of *Serratia marcescens*. Passive and active resistance to insect immunity studied with protease-deficient and phage-resistant mutants. *Journal of general microbiology* (129):453-464.
- Gerc, A.J., Song, L., Chalis, G.L., Stanly-Wall, N. R., and Coulthurst, S.J. 2012. The insect pathogen *Serratia marcescens* Db10 uses a hybrid non-ribosomal peptide synthetase-polyketide synthase to produce the antibiotic althiomycin. *Plos One* 7(9). E44673. Doi:10.1371/journal.pone.0044673.
- Grimont, P.A.D and Grimont, F. 1978. The genus *Serratia*. *Annual reviews Microbiology*, 32: 221-248.
- Hosang, M.L.A, J.C. Alouw and H. Novarianto. 2004. Biological control of *Brontispa longissima* (Gestro) in Indonesia. Report of the expert consultation on coconut beetle outbreak in APPPC member countries. FAO. P 39-52.
- Ishii, K., Adachi, T., hara, T, Hamamoto, H., and Sekimizu, K. 2014a. Identification of *Serratia marcescens* virulence factor that promotes hemolymph bleeding in the silkworm, *Bombyx mori*. *Journal of invertebrate pathology* 117: 61-67.
- Ishii, K., Adachi, T, Hamamoto, H., and Sekimizu, K. 2014b. *Serratia marcescens* suppresses host cellular immunity via the production of an adhesion-inhibitory factor against immunosurveillance cells. *The journal of biological chemistry* 289(9): 5876-5888.
- Janda , J.M., and Abbott, S.L. 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils and pitfalls. *Journal of clinical microbiology* 45(9):2761-2764.
- Kurz, C.L. Chauvet, S., Andres, E., Aurouze, M., Vallet, I., Michel, G.P.F. 2003. Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by in vivo screening. *EMBO Journal* 22: 1451-1460. Doi:10.1093/emboj/cdg159.PMID:12660152.
- Lauzon, C. R., Bussert, T. G., Sjogren, R.E., and Prokopy, R.J. 2003. *Serratia marcescens* as a bacterial pathogen of *Rhagoletis pomonella* flies (Diptera: Tephritidae). *European Journal of Entomology* 100: 87-92.
- Liebrechts, W., and Chapman, K. 2004. Impact and control of the coconut hispine beetle, *Brontispa longissima* Gestro (Coleoptera: Chrysomelidae). Pp. 19-34. In Report of the expert consultation on coconut beetle outbreak in APPC member countries. FAO regional office for Asia and the Pacific, FAO, Bangkok, Thailand.
- Petersen, L.M and L.S. Tisa. 2012. Influence of temperature on the physiology and virulence of the insect pathogen *Serratia* sp. strain SCBI. *Applied and environmental microbiology* 78(24): 8840-8844.
- Ruii, L. 2015. Insect pathogenic bacteria in integrated pest management. *Insects* 6: 352-367. Doi:10.3390/insects6020352.
- McMahon, K.J, Castelli, M.E, Vescovi, E.G. and Feldman, M. 2012. Biogenesis of outer membrane vesicles in *Serratia marcescens* is thermoregulated and can be induced by activation of the Rcs phosphorelay system. *Journals ASM.org*. 194(12): 3241-3249.
- Nakamura, S., Konishi K., and Takasu, K. 2006. Invasion of the coconut hispine beetle, *Brontispa longissima*: current situation and control measures in Southeast Asia. In: Ku, T.Y, Chiang M.Y (eds). Proceedings of an international workshop on development of a database (APASD) for biological invasion, vol 3. Taiwan Agricultural chemicals and toxic substance research institute. Taichung, Taiwan; FFTC (Food and fertilizer technology center) for the Asia and Pacific region, Taipei, Taiwan. Pp 1-9.
- Rhoads, D.D., Wolcott, R.D., Sun, Y and Dowd, S.E. 2012. Comparison of culture and molecular identification of bacteria in chronic wounds. *International journal of molecular sciences* 13: 2535-2550. Doi:10.3390/ijms13032535.
- Sikorowski, P.P. Lawrence, A.M and Inglis, G.D. 2001. Effects of *Serratia marcescens* on rearing of the tobacco budworm (Lepidop-

- tera: Noctuidae). American Entomologist 47(1): 51-60.
- Singh, S.P dan P. Rethinam. 2005. Coconut leaf beetle *Brontispa longissima*. APCC, Jakarta. 35 p.
- Tambong J.T., Xu, R., Sadiku, A., Chen, Q., Badiss and Yu, Q. 2014. Molekular detection and analysis of a novel metalloprotease gene of entomopathogenic *Serratia marcescens* strains in infected *Galleria mellonella*. Canadian Journal of Microbiology 60: 203-209. Dxdoi.org/10.1139/cjm-2013-0864.
- Tao, K., Long, Z., Liu, K., Tao, Y., and Liu, S. 2006. Purification and properties of a novel insecticidal protein from the locust pathogen *Serratia marcescens* HR-3. Curr. Microbiology 52: 45-49. Doi:10.1007/s00284-005-0089-8. PMID:16391997.
- Tao, K., Yu, X., Liu, Y., Shi, G., Liu, S., and Hou, T. 2007. Cloning, expression, and purification of insecticidal protein Pr596 from locust pathogen *Serratia marcescens* HR-3. Curr. Microbiology 55:228-233. Doi:10.1007/s00284-007-0096-z. PMID:17657528.
- Waterhouse, D.F. (1987). *Brontispa longissima* (Gestro). In: Pressley, M (ed). Biological control. Pacific prospects. Inkata Press, Melbourne. Pp 134-141.