Agriculture and Natural Resources 50 (2016) 427–431

Contents lists available at ScienceDirect

Agriculture and Natural Resources

journal homepage: http://www.journals.elsevier.com/agriculture-and-natural-resources/

Original Article

Phylogenetic variation of the green muscadine fungus, *Metarhizium anisopliae* (Metchnikoff) Sorokin, and its virulence to larvae of the sugarcane longhorn stem borer, *Dorysthenes buqueti* Guerin (Coleoptera: Cerambycidae)

Nichanun Kernasa,[a, *] Sopon Uraichuen,[a, b, 1] Naoto Kamata[a, c, 1]

[a] Department of Entomology, Faculty of Agriculture, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand
[b] National Biological Control Research Center, Central Regional Center, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, 73140, Thailand
[c] The University of Tokyo Hokkaido Forest, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Furano 079-1563, Japan

ARTICLE INFO

Article history:
Received 6 February 2016
Accepted 17 July 2016
Available online 11 January 2017

Keywords:
Biological control
Conidial suspension
Cryptic species
DNA sequence
Molecular variation

ABSTRACT

The sugarcane longhorn stem borer (SLSB), *Dorysthenes buqueti* Guerin (Coleoptera: Cerambycidae) has recently become a serious insect pest of sugarcane in Thailand and effective biological control agent must be evaluated. The green muscadine fungus (GMF), *Metarhizium anisopliae* (Metchnikoff) Sorokin is a species complex of entomopathogenic fungi, which includes many cryptic subspecies and species. It has been reported that GMF infects and kills the sugarcane longhorn stem borer (SLSB), *D. buqueti* Guerin, so that GMF is a possible biological control agent of SLSB. Molecular analyses were conducted to gain a better understanding of the taxonomic position of GMF Thai strains. Virulence bioassays were carried out on four isolates of GMF to 5th–9th instars of SLSB. This study revealed that an isolate from Khon Kaen (KK) showed the highest virulence to 5th–9th instars of SLSB. In biological control, an aqueous suspension containing 1×10^8 conidia/mL of KK isolate was best from the viewpoint of a tradeoff between the economic cost/benefit of the mass production cost and the consequent mortality after application. Comparing suspensions containing 1×10^8 conidia/mL with those containing 1×10^{13} conidia/mL, 100,000 times as much quantity of suspension can be obtained from the same quantity of conidia, though the difference in the *D. buqueti* mortality was relatively small. Six isolates of GMF from SLSB in Thailand were likely a cryptic species, although further molecular analysis using factor 1-alpha sequences is needed.

Introduction

The sugarcane longhorn stem borer (SLSB), *Dorysthenes buqueti* Guerin (Coleoptera: Cerambycidae), has recently become a serious insect pest of sugarcane in Thailand and it has been reported that severe injury by SLSB has extended to sugarcane growing areas, especially in eastern and northeastern Thailand. In 2004–2006, MitrPhol Sugar Group reported the outbreak of *D. buqueti* in the Northeastern region of Thailand, and caused yield losses of 13–43%, and sugar losses of 11–46% in harvested plant cane (Pliansinchai et al., 2007). Damage by SLSB is caused by its larval stage boring into a ratoon or the base of a stalk and feeding on the sugarcane tissue inside, which results in brown discoloration and mortality of the whole plant (Pitaksa, 1993).

Metarhizium spp. are always designated as soil saprophytes that are observed in associations with plant roots in the rhizosphere stage and survive well in that environment over a long period (Shelton, 2014). The green muscadine fungus (GMF), *Metarhizium anisopliae* (Metchnikoff) Sorokin, is an entomopathogenic fungus that is distributed worldwide from the arctic to the tropics and has been recorded in both agricultural and forest soils (Zimmermann, 2007). Approximately 200 species of insects including the orders Symphyla, Orthoptera, Dermaptera, Isoptera, Homoptera, Heteroptera, Diptera, Hymenoptera, Siphonaptera and Lepidoptera and other arthropods are known as hosts of GMF (Bidochka and Small,

* Corresponding author.
E-mail addresses: agropk@ku.ac.th (N. Kernasa), agrspu@ku.ac.th (S. Uraichuen), kamatan@uf.a.u-tokyo.ac.jp (N. Kamata).
[1] Co-first authors.

http://dx.doi.org/10.1016/j.anres.2016.07.001

2005). However, it was also reported that each isolate of GMF is found specific to an insect species as its host (Zimmermann, 2007). Nishi et al. (2011) proved that GMF was a species complex that was separated into many subspecies and different species according to morphology and phylogenetic trees using internal transcribed spacer (ITS) and elongation factor 1-alpha (EF1-α) sequences. This study used the term GMF for the *M. anisopliae* species complex.

Marannini et al. (2006) revealed that *M. anisopliae* in an aqueous suspension containing 1×10^8 conidia/mL showed pathogenicity for neonate larvae of *Capnodis. tenebrionis* with a variation in mortality from 23.5% to 100%. A similar result was shown by Cherry et al. (2005), who revealed that an aqueous suspension containing 1×10^8 conidia/mL showed high virulence to *Callosobruchus maculatus* in stored cowpea. Mishra et al. (2011) also reported that a suspension containing 4.1×10^8 conidia/mL acted as an effective larvicide to *Musca domestica*. Benserradj and Mihoubi (2014) reported that a suspension containing 1×10^9 conidia/mL showed the highest mortality to the 4th instar of *Culex pipiens*. Various strains of GMF were also recorded in Thailand (Tangthirasunun et al., 2010) but the GMF found from *D. buqeuti* cadavers have not been identified exactly taxonomically. Suasa-ard et al. (2008) reported that *M. anisopliae* is frequently found infecting *D. buqueti* in sugarcane fields. Their isolates of *M. anisopliae* were collected from the cadavers of *D. buqueti* in Chonburi, Suphanburi, Kanchanaburi and Khon Kaen provinces, Thailand. All isolates exhibited virulence to *D. buqueti* larvae under laboratory conditions.

The objectives of the present study were to obtain a virulent strain of GMF isolates collected from *D. buqeuti* cadavers found in sugarcane plantations in Thailand by bioassays with *D. buqeuti* larvae and to also get a better understanding of the taxonomic position of the GMF Thai strains.

Materials and methods

Collecting green muscadine fungus and molecular analysis

The GMF was isolated from cadavers of SLSB larvae that were collected from six sugarcane plantations in central, eastern, northeastern and southern Thailand (Table 1). Fungal bodies including conidia and mycelium were scraped from the surface of an SLSB cadaver and streaked on a potato dextrose agar culture media. After incubation, subcultures were transplanted to obtain pure cultures. The GMF isolates were used for virulence bioassay and molecular analysis.

All molecular analyses were conducted at the Biotechnology Research and Development Office, Department of Agriculture, Ministry of Agriculture and Cooperatives, Pathum Thani, Thailand according to Curran et al. (1994) and Nishi et al. (2011).

DNA extraction, amplification and sequencing

Fifty mg of mycelia from each isolate were homogenized in DNA extraction buffer composed of 4 mM Tris–HCl, 250 mM NaCl, 25 mM EDTA, and 1% sodium dodecyl sulfate. The isolates were extracted with an equal volume of phenol/choloform/isoamyl alcohol (25:24:1) and were combined using a vortex mixer for 10 min. The solutions were centrifuged at 17,000 $\times$ *g* to separate the phases. The upper aqueous solution was transferred to a 1.5 mL tube and 0.8 volume of iso-propanol was added for DNA precipitation and then washed with 70% ethanol followed by drying under a vacuum, after which, it was put back into suspension in RNase solution (10 μM pH 8 Tris–HCl, 1 μM pH 8 EDTA, 1% RNase A).

Using aliquot parts of extracted DNA, the region of the ribosomal repeat from the 3′ end of 18S rDNA across the internal transcribed spacer 1 (ITS1), 5.8S rDNA and internal transcribed spacer 2 (ITS2) and the 5′ end of the 28S rDNA were amplified using polymerase chain reaction. The used primer sequences were 5′ –TCCGTAGGTGAACCTGCGG- 3′ (ITS1) and 5′ –TCCTCCGCTTATTGATATGC- 3′ (ITS4).

Phylogenetic analysis

Six sequences of GMF were aligned and analyzed together with the sequences obtained from the GenBank database (NCBI) such as *M. anisopliae* KJ921602, FJ5455318, FJ545310, JX912940 and JN133852, *M. anisopliae* var. *anisopliae* AF136376, AF135210 and AF136375, *Metarhizium majus* AY387580, *Metarhizium lepidiotae* AB524442, AB524437 and AB524438, *M. flavoviride* var. *flavoviride* AF138267 and *Nomuraea rileyi* KJ728727 that were used as an outgroup because these strains were used as a sister to *Metarhizium* monophyly in the phylogenetic analysis according to Nishi et al. (2011) and Sung et al. (2007). The variation among different nucleotide sites was analyzed by constructing neighbor-joining method and maximum parsimony (MP) trees. Branch support was estimated using bootstrap analysis based on 1000 bootstrap replicates. The MP tree was obtained using the subtree-pruning-regrafting algorithm with a search level of 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis elaborated 20 nucleotide sequences. All positions having less than 95% site coverage were excluded. Thus, less than 5% alignment gaps, missing data and ambiguous bases were allowed at any position. There were 452 positions in the final dataset. Phylogenetic reconstruction and evolutionary analyses were conducted using the software package MEGA6, version 6.0.6 (Felsenstein, 1985; Saitou and Nei, 1987; Nei and Kumar, 2000; Tamura et al, 2013).

The sequences were deposited in the Genbank Database (DDBJ) (accession nos. LC062590-LC062595) (Table 1).

Virulence bioassays

The bioassay was carried out at the National Biological Control Research Center (NBCRC), Central Regional Center (CRC), Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand.

The larvae of SLSB that were collected from sugarcane fields in Lao Khwan district, Kanchanaburi and Kamphaeng Saen district, Nakhon Pathom were reared individually in plastic cups with an artificial diet for the sugarcane borer, *Diatraea saccharalis*,

Table 1
Six isolates of *Metarhizium anisopliae* collected from different locations in Thailand and their accession numbers in Genbank.

Isolate identification	Location	Latitude E	Longitude N	Accession number
KK	Khon Kaen (NBCRC CRC[a] collection)	–	–	LC062592
LKKB	Nong Nok Kaeo, Lao Khwan district, Kanchanaburi	14 29 29.7	99 40 58.1	LC062595
BBCB	Nong Phai Kaeo, Ban Bueng district, Chon Buri	13 12 9.5	101 13 25.6	LC062591
DMTKB	Tha Yae, Dan Makham Tia district, Kanchanaburi	13 49 11.3	99 23 42.5	LC062594
PBPKK	Khao Noi, Pran Buri district, Prachuap Khiri Khan	12 24 4.4	99 50 25.7	LC062593
KPSNP	Thung Luk Nok, Kamphaeng Saen district, Nakhon Pathom	14 3 32.2	99 54 56.3	LC062590

[a] National Biological Control Research Center (NBCRC), Central Regional Center (CRC), Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand.

(Southland Product Inc; Lake Village, AR, USA) for 30 d to ensure they had not been parasitized by any parasitoids. The healthy larvae were used for the virulence bioassays. The 5th–9th instars (L5–L9) of SLSB were evaluated against four fungal isolates (KK, LKKB, BBCB and DMTK as detailed in Table 1) from among the six. Fifty microliters of a conidial suspension of three concentration levels—1×10^3 conidia/mL, 1×10^8 conidia/mL and 1×10^{13} conidia/mL (conidia mixed with sterile distilled H_2O 80 mL and Triton X 0.05%)—were dropped on larvae cuticle. Larvae treated with 50 μL of distilled water mixed with Triton X 0.05% were equally used as the control. The larvae were discretely placed in a plastic box, 23 cm diameter by 8.5 cm height, with five pieces of sugarcane stalk each 5 cm long as food. The sugarcane pieces were replaced with new ones every 2 wk. Five replications were conducted with five larvae per replication for each isolate. The treated SLSB larvae were incubated at 25 ± 2 °C and 43 ± 2% relative humidity and were checked daily with the naked eye for mortality based on white conidia appearing on the cuticle of the larvae.

The effects of collection site (KK, LKKB, BBCB, and DMTKB), instar (5th–9th), and concentration of conidial suspension (1×10^3 conidia/mL, 1×10^8 conidia/mL and 1×10^{13} conidia/mL) on the larval mortality were evaluated using a generalized linear model, with a binomial error structure and a logit link function (a logistic regression) in the R software package, version 3.1.0 (R Developer Core Team, 2014). Of the three explanatory variables, the collection site was categorical with the KK isolate as a base model, while instar (5, 6, …, 9) and concentration (3, 8, 13) were numeric. A response variable was the grouped data of numbers of dead and alive insects in each plastic box that contained five individuals of SLSB. To determine if an effect of each variable was significant, the Akaike information criterion (AIC) was compared between the full model and a model that contained two explanatory variables out of the three.

Results and discussion

Phylogenetic analysis

Fig. 1 shows the neighbor-joining tree and the maximum parsimony tree (Fig. 2) of GMF isolates. The similarity coefficient among the six sequences of GMF was more than 98% (Fig. 1). The maximum parsimony tree presented the five most parsimonious trees (length = 166). The consistency index (CI) was 0.83, the retention index (RI) was 0.87 and the composite index was 0.77 (Fig. 2). These indicated that the evolutionary history of all these isolates originated from the same ancestor. The results of clustering the six Thai isolates seemed reasonable when compared with their geographic distance: The two isolates from Kanchanaburi (LKKB and DMTKB) were close to each other and originated from the same ancestor as an isolate from Kamphaeng Saen (KPSNP). Isolates from Khon Kaen (KK), Chon Buri (BBCB) and Prachuap Khiri Khan (PBPKK) also originated from one ancestor. However, the result of neighbor-joining clustering also indicated the genetic variability of different isolates of GMF obtained from *D. buqueti* larvae in different locations in Thailand (Fig. 1). Two isolates from Kanchanaburi (LKKB and DMTKB) were highly similar to each other. However, the phylogenetic relationship of the other isolates did not match with their geographic distances. The isolates from Khon Kaen (KK), Chon Buri (BBCB) and Prachuap Khiri Khan (PBPKK) showed 95% of bootstrap values that shared a more recent common ancestor than others although these appeared in different locations. The isolate from Kamphaeng Saen (KPSNP), which was geographically closer to Kanchanaburi, was most distant from the others. The neighbor-joining clustering (Fig. 1) showed that the six sequences of GMF were in the same group with the sequence of *M. anisopliae* KJ921602, FJ5455318, FJ545310, JX912940 and JN133852, *M. anisopliae* var. *anisopliae* AF136376, AF135210 and AF136375, *M. majus* AY387580, *M. lepidiotae* AB524442, AB524437 and AB524438 with a highly supported bootstrap value (100%) and separated from *Metarhizium flavoviride* var. *flavoviride* AF138267 and *N. rileyi* KJ728727. These results indicated that the six isolates of GMF in the present study could be included in the complex of *M. anisopliae* var. *anisopliae* and *M. anisopliae* var. *majus* by Nishi et al. (2011). To clarify the phylogenetic relationships within the complex, Nishi et al. (2011) analyzed the EF-1α region and divided the complex into six monophyletic groups corresponding to the six phylogenetic species defined by Bischoff et al. (2009): *M. anisopliae*, *Metarhizium brunneum*, *Metarhizium guizhouense*, *M. majus*, *Metarhizium pingshaense*, and *Metarhizium robertsii*. Because the six GMF isolates in

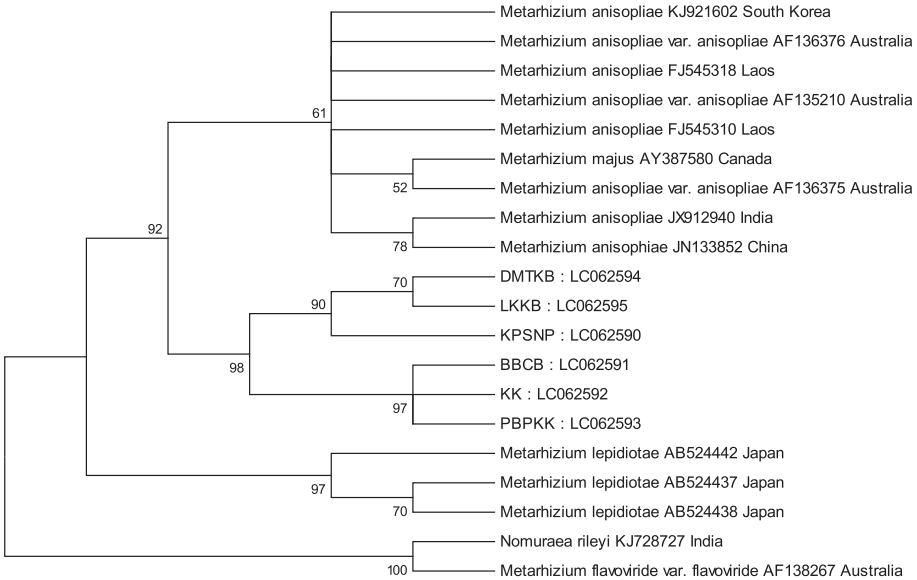

Fig. 1. *Metarhizium anisopliae* relationships derived from neighbor-joining analysis (MEGA 6, Version 6.0.6) of internal transcribed spacer genes. Numbers under branches indicate bootstrap support based on 1000 pseudoreplicates.

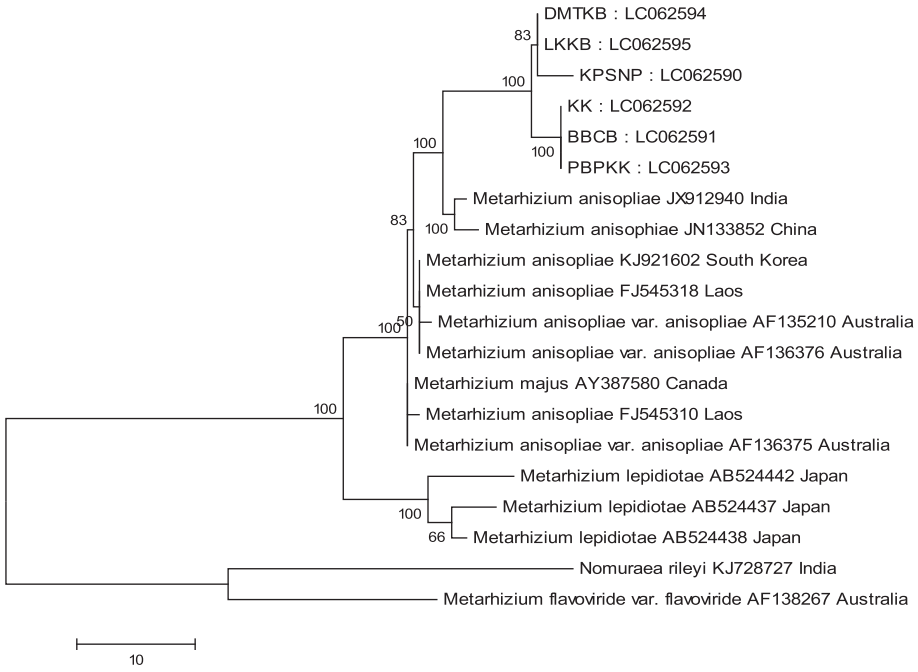

Fig. 2. *Metarhizium anisopliae* relationships derived from parsimony analysis (MEGA 6, Version 6.0.6) of internal transcribed spacer genes. Strict consensus based on five trees of length 600 steps, consistency index = 0.83, retention index = 0.87. Numbers under branches indicate bootstrap support based on 1000 pseudoreplicates.

this study were classified into one clade, these isolates are likely a cryptic species though the same molecular analysis as Nishi et al. (2011) is needed using the EF-1α region.

Virulence bioassays

From the inoculation experiment, mortality tended to increase with concentration of conidial suspension. No mortality was observed in the control (data not shown) or at 1×10^3 conidia/mL. The difference between AIC values between the full model and a model with two explanatory variables among the three was greater than 2.57 for all three explanatory variables indicating that the effects of the three factors were all significant ($p < 0.01$) as shown in Table 2. In the full model result, the coefficient of concentration was positive and significantly different from zero ($p < 0.01$) supporting the tendency for mortality to increase with concentration (Fig. 2). The coefficient of instar was negative and significantly different from zero ($p < 0.01$) indicating that the mortality decreased with instar. Indeed, the mortality of the 9th instar was smaller than the other instars at concentrations of 1×10^8 conidia/mL and 1×10^{13} conidia/mL. The result also indicated that the effects of the KK isolate were greater than the other three isolates because all the coefficients of other three isolates were negative. The difference was significant for the LKKB isolate ($p < 0.01$) and for the DMTKB isolate ($p < 0.05$). Comparing the suspensions containing 1×10^8 conidia/mL with those containing 1×10^{13} conidia/mL, 100,000 times as much quantity of suspension would be obtained from the same quantity of conidia though the difference in the *D. buqueti* mortality would be relatively small. Sommartya et al. (2007) found that the mortality of larvae was 100% at 14 d after inoculation with a conidial suspension of 1×10^8 conidia/mL in the greenhouse. This supports the conclusion from the current study that GMF containing 1×10^8 conidia/mL was the most suitable concentration to control *D. buqueti* larvae.

Table 2
Results of generalized linear model evaluating effects of green muscadine fungus isolates, concentration of the conidial suspension and *Dorysthenes buqueti* instar on mortality by the fungus with a binominal error structure and a logit link function, using the KK isolate as a base model.

Model	AIC†	ΔAIC†
Full model	666.5	0
- Isolate	677.5	11.0
- Instar	844.7	178.2
- Concentration	1557	890.5

	Estimate	SE	*z* value	*p*-value‡
Intercept	0.44292	0.41803	1.060	0.2893
Isolate (BBCB)	−0.37403	0.22111	−1.692	0.0907
Isolate (DMTKB)	−0.44678	0.22139	−2.018	0.0436*
Isolate (LKKB)	−0.90906	0.22429	−4.053	5.06e−05***
Instar	−0.76833	0.06453	−11.907	<2e−16***
Concentration	0.58340	0.02879	20.267	<2e−16***

†AIC = Akaike information criterion, ΔAIC = Difference between AIC value for the model compared to the model with the lowest AIC value.
‡significance codes: *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

This study revealed that the six isolates of the GMF used in this study were likely a cryptic species although further molecular analysis using EF-1α is needed. The KK isolate which was collected from Khon Kaen showed the highest virulence to L5–L9 of *D. buqueti*. The results support a recommendation that an aqueous suspension containing 1×10^8 conidia/mL of KK isolates is best to utilize as a biological control agent from the viewpoint of an economical cost/benefit trade-off between the mass production cost for an optimum concentration of GMF and the subsequent mortality after application, as this suspension has a similar effect to an aqueous suspension containing 1×10^{13} conidia/mL.

Conflict of interest

None.

Acknowledgements

This study was funded by the Ministry of Science and Technology and a grant from the Thailand Research Fund. Thanks are recorded to the sugar mill companies: Rajburi Sugar Company Limited, Cholburi Sugar & Trading Corporation Limited, Burirum Sugar Public Company Limited, Saharuang Company Limited, The Kumphawapi Sugar Company Limited, Mitrhphol Sugar Corporation Limited (PhuViang), Wangkanai Sugar Company Limited and Erawan Sugar Company Limited and to the sugarcane growers who allowed us to collect the infected larvae in the field. The authors also sincerely thank Assistant Professor Anuchit Chinajariyawong, Institute of Agricultural Technology, Walailak University, Nakhon Si Thammarat, Thailand for assistance with editing the manuscript.

References

Benserradj, O., Mihoubi, I., 2014. Larvicidal activity of entomopathogenic fungus *Metarhizium anisopliae* against mosquito larvae in Algeia. Int. J. Curr. Microbiol. App. Sci. 3, 54–62.

Bidochka, M.J., Small, C.L., 2005. Phylogeography of Metarhizium, an insect pathogenic fungus. In: Vega, F.E., Blackwell, M. (Eds.), Insect-fungal Associations. Ecology and Evolution. Oxford University Press, Oxford, UK, pp. 28–50.

Bischoff, J.F., Rehner, S.A., Humber, R.A., 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. Mycologia 101, 512–530.

Cherry, A.J., Abalo, P., Hell, K., 2005. A laboratory assessment of the potential of different strains of the entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vullemin and *Metarhizium anisopliae* (Metschnihoff) to control *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) in stored cowpea. J. Stored Prod. Res. 41, 295–309.

Curran, J., Driver, F., Ballard, J.W.O., Milner, R.J., 1994. Phylogeny of *Metarhizium*: analysis of ribosomal DNA sequence data. Mycol. Res. 98, 547–552.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Marannini, P., Santiago-Álvarez, C., de Lillo, E., Quesada-Morago, E., 2006. A new bioassay method reveals pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* against early stages of *Capnodis tenebrionis* (Coleoptera: Buprestidae). J. Invertebr. Pathol. 93, 210–213.

Mishra, S., Kumar, P., Malik, A., Satya, S., 2011. Adulticidal and larvicidal activity of *Beauveria bassiana* and *Metarhizium anisopliae* against housefly, *Musca domestica* (Diptera: Muscidae), in laboratory and stimulated field bioassays. Parasitol. Res. 108, 1483–1492.

Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York, NY, USA.

Nishi, O., Hasegawa, K., Iiyam, K., Yasunaga-Aoki, C., Shimizu, S., 2011. Phylogenetic analysis of *Metarhizium* spp. Isolated from soil in Japan. Appl. Entomol. Zool. 46, 301–309.

Pitaksa, C., 1993. Ecological Studies, Crop Loss Assessment and Potential Control of Sugarcane Stem Boring Grub, *Dorysthenes buqueti* Guerin (Coleoptera: Cerambycidae). Ph.D. Thesis. Kasetsart University, Bangkok, Thailand.

Pliansinchai, U., Jarnkoon, V., Siengsri, S., Kaenkong, C., Pangma, S., Weerathaworn, P., 2007. Ecology and destructive behavior of cane boring grub (*Dorysthenes buqueti* Guerin) in north eastern Thailand. In: Proceedings of the XXVI Congress of the International Society of Sugar Cane Technologists, 29 July-2 August 2007. Durban, South Africa, pp. 863–867.

R Developer Core Team, 2014. A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Saitou, N., Nei, M., 1987. The neighbor-joining method - a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Shelton, A., 3 March 2014. Biological Control: a Guide to Natural Enemies in North America. Department of Entomology, College of Agriculture and Life Science, Cornell University. http://www.biocontrol.entomology.cornell.edu/pathogens/Metarhizium.html.

Sommartya, P., Suasa-ard, W., Puntongcum, A., 2007. Natural enemies of sugarcane longhorn stem borer, *Dorysthenes buqueti* Guerin (Coleoptera: Cerambycidae), in Thailand. In: XXVI Congress, International Society of Sugar Cane Technologists, ISSCT. Durban, South Africa, 29 July–2 August 2007, pp. 858–862.

Suasa-ard, W., Sommartya, P., Buchatian, P., Puntongcum, A., Chiangsin, R., 2008. Effect of *Metarhizium anisopliae* on infestation of sugarcane stems borer, *Dorysthenes buqueti* Guerin (Coleoptera: Cerambycidae) in laboratory. In: Proceedings of the 46th Kasetsart University Annual Conference (Plants). 29 January–1 February 2008, Kasetsart University, Bangkok, pp. 155–160.

Sung, G., Hywel-Jones, N.L., Sung, J., Luangsa-ard, J.J., Shrestha, B., Spatafora, J.W., 2007. Phylogenetic classification of Cordyceps and the clavicipitaceous fungi. Stud. Mycol. 57, 5–59.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.

Tangthirasunun, N., Poeaim, S., Soytong, K., Sommartya, P., Popoonsak, S., 2010. Variation in morphology and ribosomal DNA among isolates of *Metarhizium anisopliae* from Thailand. J. Agri. Technol. 6, 317–329.

Zimmermann, G., 2007. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. Biocontrol. Sci. Tech. 17, 879–920.