

MOLECULAR MARKER-BASED DETECTION OF PRIMARY BACTERIAL ENDOSYMBIONT ASSOCIATED WITH THE COCONUT SCALE INSECTS, *Aspidiotus destructor* Signoret AND *Aspidiotus rigidus* Reyne (HEMIPTERA: DIASPIDIDAE)¹

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ABSTRACT

The primary endosymbionts associated with coconut scale insects (CSI), *Aspidiotus rigidus* and *A. destructor*, were detected using the 16S *rRNA*. Sequences of this gene were sourced from *A. destructor* and *A. rigidus* populations from different geographical locations namely, Orani, Bataan; Cagbalate 1 and Cagbalate 2, Mauban, Quezon; Sampaloc, Quezon; and Tranca, Bay, Laguna. Results revealed variation between the primary endosymbiont associated with the *Aspidiotus* species, but not among the primary endosymbionts associated within each CSI species. GenBank mining using BLAST revealed 98% to 99% similarity of the endosymbionts of *A. destructor* and *A. rigidus*, respectively, with *Candidatus Uzinura diaspidicola* (GenBank Acc. No. DQ868800.1), - the identified primary bacterial endosymbiont of armored scale insects. Maximum likelihood analysis, via the endosymbiont 16S *rRNA* gene comparison, inferred that the *U. diaspidicola* of the two CSI species were entirely different and thus, could serve as a target in designing sound management strategies against these economically important pests. With the various vital roles of endosymbionts in their host ecology and evolution, more in-depth studies on *U. diaspidicola* could definitely help understand the mechanism of relative higher invasiveness of *A. rigidus* over *A. destructor* in coconut. Moreover, our results suggest that, with the difficulties encountered through conventional techniques of species identification, molecular-based approach such as utilization of 16S *rRNA*

gene of an endosymbiont could be an efficient additional tool for a more accurate and faster identification of scale insect species.

Key words: *16S rRNA*, *Aspidiotus destructor*, *Aspidiotus rigidus*, *Candidatus Uzinura diaspidicola*, coconut scale insects, primary endosymbiont

INTRODUCTION

The earliest record of coconut scale insect (CSI) infestation that posed serious economic and ecological impacts on the Philippine coconut industry was reported in 2010 in Barangay Balele, Tanauan, Batangas (Villareal, 2014). Contributing to the crisis was the difficulty of identifying the CSI species causing the severe damage, since *Aspidiotus destructor* Signoret and *A. rigidus* Reyné co-existed in the outbreak populations (Caoili et al., 2014; 2015). *A. rigidus* was found to cause more severe infestation to more than 1.2 million coconut trees in Batangas province alone, consequently threatening the coconut industry and livelihood of no less than 3.5 million local Filipino coconut farmers (PCA, 2010). Infestations by *A. rigidus* (Hemiptera: Diaspididae) then spread to the rest of the provinces of Cavite, Laguna, Batangas, and Quezon (CALABARZON). By 2015, more than 2.6 million nut-bearing coconut trees were affected due to the coconut scale insect outbreak in CALABARZON (PCA, 2015). Serious infestations in Bataan as well as Basilan provinces were also reported (Caoili et al., 2015). The Department of Agriculture has informed that these coconut scale insects have spread and infested coconut trees in mainland Sulu, Zamboanga Peninsula, Caraga Region (TDMM, 2016), and most recently, in Romblon (Tablas Island).

Armored scale insects have been documented to harbor primary and secondary endosymbionts, which belong to the phylum Bacterioidetes, and genus *Cardinium*, similar to *Wolbachia*, respectively. The vital roles of endosymbionts in the ecology and evolution of their hosts are widely recognized (Zchori-Fein & Perlman, 2004), and therefore, are potential targets for insect pest management. Bacterial endosymbionts have shown an array of fitness advantages for the insect host such as, nutritional supplementation (Hossokawa et al., 2010), tolerance to stress factors (Bian et al., 2010), enhanced immune response against microbial pathogens (Rances et al., 2012), parasitoid resistance (Kaltenpoth et al., 2005; Oliver et al., 2005; Teixeira et al., 2008) as well as insecticidal resistance (Kikuchi et al., 2012; Werren, 2012; Pan et al., 2013; de Almeida et al., 2017). Moreover, in a scale insect species, *A. nerii* Bouche, it was observed that endosymbionts played an important role in inducing parthenogenesis (Gruwell et al., 2007).

Aside from an evolutionary point of view, the identification of associated bacteria in armored scale insect is necessary to provide basic information to design efficient pest management strategies (Douglas, 2007; Sreerag et al., 2014). As critical determinants for the survival of the scale insects, these endosymbionts can be potential targets for the development of biotechnological control tactics such as, incompatible insect technique through symbiosis

disruption (Berasategui et al., 2016). Recently, identification of endosymbionts in armored scale insects was accomplished with sequence analysis of the small ribosomal subunit, *16S rRNA* (Gruwell, 2008; Andersen et al., 2010). In the Philippines, however, there has been no study on the identification of *A. destructor* and *A. rigidus* bacterial endosymbiont. The mechanism of the endosymbiont relationship could give us a clue on the vast growth of *A. rigidus* populations in the outbreak areas, given the potential influence of these microorganisms in inducing reproductive phenotypes, host fecundity, and fertility (Dedeine et al., 2005; Werren & Clark, 2008). Hence, accurate identification of the endosymbiont is crucial in designing efficient management strategy against *A. rigidus* devastating coconuts in various localities and provinces in the country through targeted disruption of host-endosymbiont association. With the uncultivable nature of most insect endosymbionts using laboratory media, molecular techniques, such as use of DNA barcode (Hebert et al., 2003), offer a more accurate and faster detection method (Kolbert & Persing, 1999; Gruwell et al., 2008). This paper, therefore, aimed to detect the primary endosymbiont of the CSI species based on the *16S rRNA* gene sequence.

MATERIALS AND METHODS

Field collection of coconut scale insects

The test insects were collected from infested coconut trees in different geographic locations. Populations of *A. destructor* were obtained from Orani, Bataan; Cagbalete 1, Mauban, Quezon; and Sampaloc, Quezon. On the other hand, *A. rigidus* samples were collected from Tranca, Bay, Laguna; Cagbalete 2, Mauban, Quezon; and Sampaloc Quezon. The CSI species was confirmed, in another study, using 28S nucleotide sequence (Caoili et al., 2014).

Amplification of *16S rRNA* gene from CSI bacterial endosymbionts

Nucleic acid isolation was done following the standard protocol of the Animal and Fungi DNA Preparation Kit (Jena Bioscience GmbH, Jena, Germany, www.jenabioscience.com) with some modifications. Quantification of DNA samples was done using a spectrophotometer, BioDrop 125 (www.biodrop.co.uk). The small ribosomal subunit, *16S rRNA*, was specifically used for the identification of endosymbiotic bacteria, *Candidatus Uzinura diaspidicola* Gruwell et al., associated with the scale insect. Amplification of the *16S rRNA* gene was based on Gruwell et al. (2007) and Andersen et al. (2010), using the *U. diaspidicola*-specific primer pair (s688DIASP: 5'-GGAATGTATGGTGTAGCGGTGAAAT-3') and a1271DIASP: 5'-CATTGTAGCACGTGGGTAFCCCAAG-3'), which targets a 583 bp region of the gene, with some modifications. The polymerase chain reaction (PCR) was carried out using 25 µl reaction volume containing 12.5 µl 2x Taq Master Mix (Vivantis, Malaysia), 20 pmol of each primer and 3 µl genomic DNA, with the following thermal cycler protocol: initial denaturation temperature of 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 52°C for 80 sec, and 72°C for 2 min and

then finishing with a final extension at 72°C for 10 min. Amplicons were resolved in 1.0% agarose gel via electrophoresis stained with GelRed™ (Biotium, Inc. Fremont, California, USA).

Nucleotide sequence analysis for endosymbiont identification

The amplified DNA fragments were sent to 1st BASE DNA Sequencing Services (Axil Scientific Pte Ltd, Singapore Science Park II, Singapore, www.base-asia.com) through ASIAGEL CORPORATON (Quezon City, Philippines, www.asiagel.com) for sequencing. Moreover, the nucleotide sequences were checked and the less informative sites were trimmed using BioEdit 7 (Hall, 1999). The high-quality nucleotide sequences were compared with the existing data in the GenBank using Basic Local Alignment Search Tool (BLAST) (NCBI). Multiple sequence alignment was carried out using BioEdit7 built-in ClustalW function, while phylogenetic tree construction was done through MEGA 7 (Tamura et al., 2016).

RESULTS AND DISCUSSION

Amplification of 16S rRNA genes from genomic DNA of *A. destructor* and *A. rigidus*

Partial sequences of the 16S rRNA gene were deduced from the resulting PCR products using total genomic DNA from CSI samples representing the Philippine populations of *A. destructor* and *A. rigidus*. The 16S rRNA gene amplicons from the representative *A. destructor* and *A. rigidus* samples (Figure 1) had a molecular size of approximately 583 base pairs (bp). The resulting length of the amplicons was consistent with the reported expected size of the PCR product as deduced from the *Uzinura diaspidicola*-specific primer set used by Gruwell et al. (2007).

Analysis of 16S rRNA gene of *A. destructor* and *A. rigidus* bacterial endosymbiont

The 16S rRNA sequences were obtained from the amplicons generated from *A. destructor* populations from Cagbalete 1 (GenBank Acc. No. MH923496-506), Bataan (GenBank Acc. No. MH923486-495), and Sampaloc (GenBank Acc. No. MH923507). On the other hand, 36 16S rRNA sequences from Cagbalete 2 (GenBank Acc. No. MH923448-458), Tranca (GenBank Acc. No. MH923474-475, MH923477-478, MG923480-485) and Sampaloc (GenBank Acc. No. MH923459-473) represented the *A. rigidus* populations. All partial nucleotide sequences subjected to BLASTn for confirmation showed 98% to 99% similarity with the Flavobacteriales endosymbiont of *A. nerii* (GenBank Acc. No. DQ868800.1), belonging to phylum Bacterioidetes (Gruwell et al., 2007) (Table 1).

Armored scale insects are known to host bacterial endosymbionts that are vertically transmitted. Given that the *A. destructor* and *A. rigidus* samples were

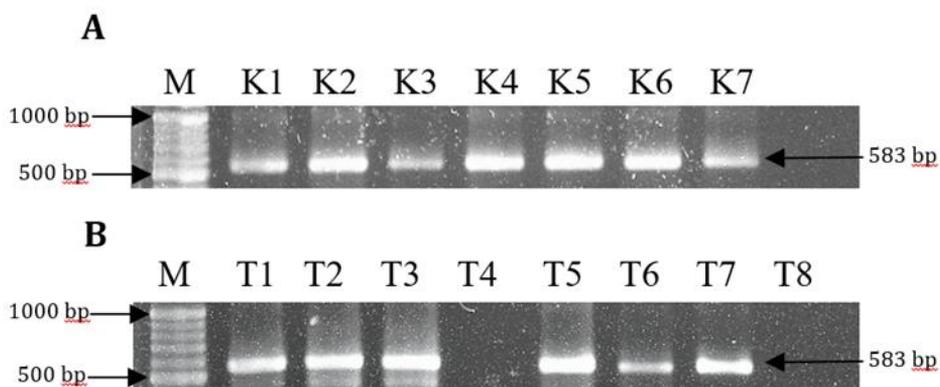


Figure 1. Representative amplicons of the *16s ribosomal RNA* gene of the primary symbiont of *Aspidiotus destructor* and *A. rigidus* visualized in 1% agarose gel stained with GelRed™ (Biotium, Inc. Fremont, California, USA). (A) *A. destructor* samples were collected from Orani, Bataan (K1-K7), while *A. rigidus* samples were from Tranca, Bay, Laguna (T1-T8). The VC 100 bp Plus DNA Ladder (Vivantis, Malaysia) was used as standard (M) to estimate the molecular size of the amplicons.

Table 1. Significant alignment of the *16S ribosomal RNA* gene of the primary endosymbionts associated with *Aspidiotus destructor* Signoret and *A. rigidus* Reyne with the *16S rRNA* gene sequence in the GenBank using Basic Local Alignment Search Tool (BLAST). The Most Significant BLAST Hit for all samples/sequences is Flavobacteriales endosymbiont of *Aspidiotus cf. nerii* with GenBank Accession No. DQ868800.1 and Reference - Gruwell et al. (2007).

CSI Species	Collection Site	No. of Samples	Identity (%)
<i>Aspidiotus destructor</i>	Orani, Bataan	10	98
	Cagbalete 1, Mauban, Quezon	11	98
	Sampaloc, Quezon	1	98
<i>Aspidiotus rigidus</i>	Tranca, Bay, Laguna	10	99
	Cagbalete 2, Mauban, Quezon	11	99
	Sampaloc, Quezon	15	99

collected from different geographical locations, analyses of the *16S rRNA* genes amplified from *Aspidiotus* spp.-extracted DNA showed close affinity to Flavobacteriales endosymbiont of *A. nerii*. Gruwell et al. (2007) proposed the name *Candidatus Uzinura diaspidicola* for this armored scale insect primary bacterial endosymbiont. This bacterium was found to show a distinct monophyletic clade among other scale insects. Just like other primary endosymbionts, which are critical for host development, survival and reproduction (Gibson & Hunter, 2009), *U. diaspidicola* plays vital roles in the ecology and evolution of its scale insect hosts (Zchori-Fein & Perlman, 2004). A recent study on whole genome sequence analysis of *U. diaspidicola* of *A. nerii*

(Sabree et al., 2013) showed that this endosymbiont supplies its insect host with essential amino acids that are scarce in *A. nerii* feeding site - the parenchyma cells.

Multiple alignment of consensus *16S rRNA* gene sequences deduced from the endosymbionts of both *Aspidiotus* spp. revealed variations between the primary bacterial endosymbiont of *A. destructor* and *A. rigidus* (Figure 2). Several polymorphic sites exhibiting both transition and transversion are found across the aligned sequences. The same was observed when the endosymbionts of *A. destructor* and *A. rigidus* were compared with the reference *U. diaspidicola* associated with *A. nerii* (Figure 3). These intraspecific variations can be targeted for marker development for the rapid species delimitation of the *Aspidiotus* hosts such as in the case of Rounds et al. (2012) where characterization of heritable endosymbiotic bacteria of ticks led to the development of a PCR/electrospray ionization-mass spectrometry-based assay for tick species identification.

In line with this, the constructed phylogenetic tree based on the *16S rRNA* sequences showed three distinct clusters with reference to insect host species. (Figure 4). High bootstrap values of 89% and 99% obtained for *U. diaspidicola* within the *A. rigidus* and *A. destructor* clades, respectively, supports the groupings formed. This could imply that *U. diaspidicola* strains show host

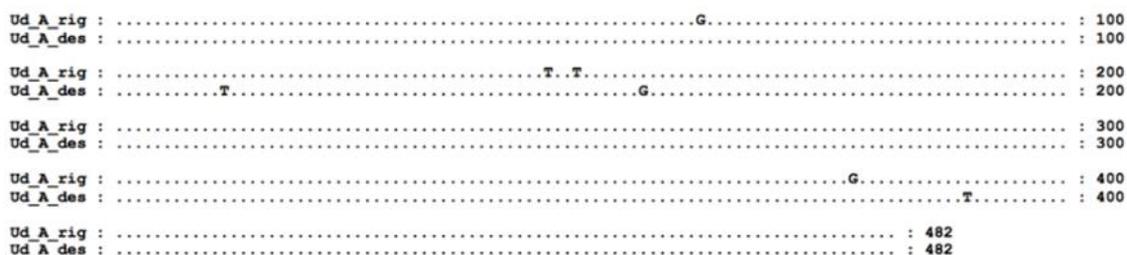


Figure 2. Nucleotide sequence alignment of the consensus *16S ribosomal RNA* gene of primary bacterial endosymbiont, *Ca. Uzinura diaspidicola* associated with *Aspidiotus rigidus* (Ud_A_rig) and *A. destructor* (Ud_A_des).

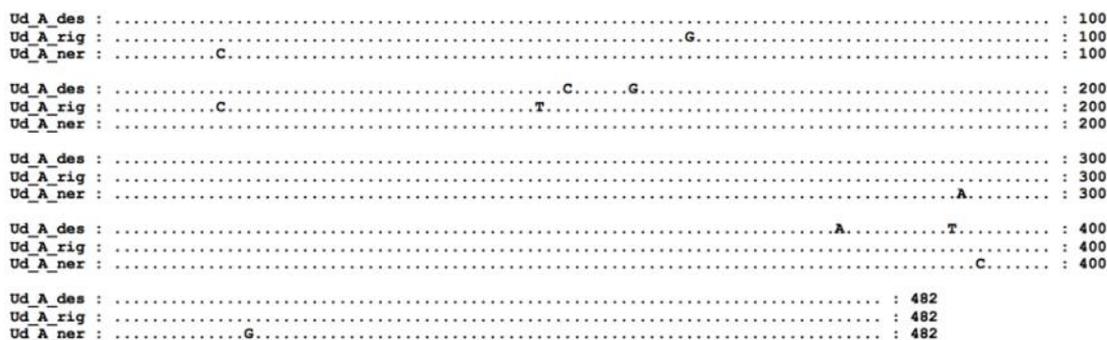


Figure 3. Nucleotide sequence alignment of the consensus *16S ribosomal RNA* gene of primary bacterial endosymbiont, *Ca. Uzinura diaspidicola* associated with *Aspidiotus destructor* (Ud_A_des) and *A. rigidus* (Ud_A_rig) compared with reference *U. diaspidicola* associated with *A. nerii* (Ud_A_ner).

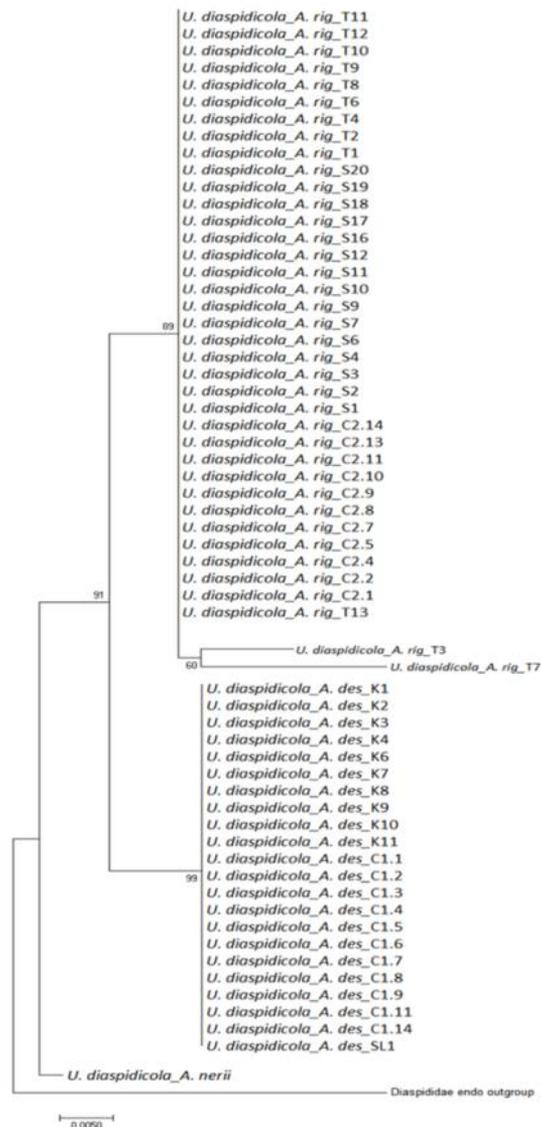


Figure 4. Maximum likelihood tree constructed based on Tamura-Nei model with 1000 bootstrap replications in MEGA 7 (Tamura et al., 2016) using partial *16s ribosomal RNA* gene nucleotide sequences showing distinct clades of *Uzinura diaspidicola* strains based on host association (*A. des*: *Aspidiotus destructor*, *A. rig*: *A. rigidus*);). The strains were named after the collection sites namely, C1 (GenBank Acc. No. MH923496-506): Cagbalete 1, Mauban, Quezon; C2 (GenBank Acc. No. MH923448-458): Cagbalete 2, Mauban, Quezon; K (GenBank Acc. No. MH923486-495): Orani, Bataan; S (GenBank Acc. No. MH923459-473) & SL (GenBank Acc. No. MH923507): Sampaloc, Quezon; T (GenBank Acc. No. MH923474-75, MH923477-78, MH923480-85): Tranca, Bay, Laguna). A diaspidid endosymbiont (Diaspididae endo outgroup) (GenBank Acc. No. DQ868827.1) was included as the outgroup.

specificity and suggests a possible separate evolution of these strains despite the sympatry of *A. destructor* and *A. rigidus*. Although evolutionary records show that bacterial genes are highly conserved among species (Patel, 2001), the different lifestyles and biology of *A. rigidus* (Reyne, 1948) and *A. destructor* (Reyne, 1947) might have contributed to a likely co-evolution of these armored scale insects with specific strains of *U. diaspidicola*. (Wernegreen, 2002; Sabree et al., 2013).

The study presents the first report on the detection of the armored scale insects' primary endosymbiont, *U. diaspidicola*, based on the *16S rRNA* marker from both *A. destructor* and *A. rigidus* populations. Based on the *U. diaspidicola*-based marker used in this study, a distinct association between the primary endosymbiont and its scale insect host was observed, and thus, can be a reliable technique to resolve phylogenetic inconsistencies encountered on morphology-based or conventional methods. However, there is an observable narrow degree of variability on the locus examined as shown by limited number of polymorphic sites. Hence, other markers based on the full sequence of the *16S rRNA* region and other fast-evolving gene regions can also be utilized for the development of a more robust marker-based diagnostic assay for coconut scale insect species without nucleotide sequencing.

Studies on the phylogenetic congruence of *Aspidiotus* spp. and their corresponding primary symbionts can also be explored to further prove host-symbiont co-evolution. This phenomenon perhaps could explain the relative higher invasiveness of *A. rigidus* in coconut as compared with *A. destructor*. Further researches on more potential roles of *U. diaspidicola* on its insect host would be highly recommendable, since a lot of symbiont-dependent processes still remain a mystery. Moreover, the knowledge on the bacterial endosymbionts shall serve as a foundation for future targets for the control of armored scale insects they associate with.

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