FULL PAPER *Parasitology*

First Molecular Characterization of *Anaplasma marginale* in Cattle and *Rhipicephalus* (*Boophilus*) *microplus* Ticks in Cebu, Philippines

Adrian Patalinghug YBAÑEZ[1,2], Thillaiampalam SIVAKUMAR[3], Rochelle Haidee Daclan YBAÑEZ[3], Jowarren Catingan RATILLA[4], Zandro Obligado PEREZ[5], Shirleny Reyes GABOTERO[5], Hassan HAKIMI[2,3], Shin-ichiro KAWAZU[3], Kotaro MATSUMOTO[1], Naoaki YOKOYAMA[2,3] and Hisashi INOKUMA[2,3]*

[1]*Department of Veterinary Clinical Sciences, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan*
[2]*United Graduate School of Veterinary Sciences, Gifu University, Gifu 501–1193, Japan*
[3]*National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan*
[4]*National Dairy Authority, Cebu City 6000, Philippines*
[5]*GPY Veterinare Animale - Group of Veterinary Clinics, Cebu City 6000, Philippines*

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ABSTRACT. *Anaplasma marginale* has been detected in the Philippines only by peripheral blood smear examination and serological methods. This study generally aimed to molecularly detect and characterize *A. marginale* in cattle and ticks in Cebu, Philippines. A total of 12 bovine blood samples and 60 *Rhipicephalus (Boophilus) microplus* ticks were collected on the Cebu Island in 2011. 16S rRNA-based screening-PCR and DNA sequencing revealed 8 cattle (66.7%) and 8 ticks (13.3%) to be positive for *A. marginale*, and 1 tick (1.7%) to be positive for *A. centrale*. Selected positive DNA samples were further characterized based on 16S rRNA (longer sequence), *Msp5, Msp1α*, *gltA* and *groEL* genes for phylogenetic analyses. Sequence identities of partial DNA fragments of *A. marginale* from the Philippines revealed 99.1–100% (16S rRNA, *gltA*, *groEL* and *Msp5*) and 94.3–97.6% (*Msp1α*) identities to the closest isolates from other countries. Moreover, sequence analysis of the *Msp1α*, gene showed 3 variants, including a case of co-infection with 2 variants. Phylogenetic analyses based on *Msp1α* and *Msp5* genes revealed that Philippine *A. marginale* isolates formed a monophyletic lineage, and were phylogenetically related to Brazilian and Chinese isolates. On the other hand, a highly specific and sensitive nested PCR based on *groEL*, with a detection limit of 2 copies/PCR, was developed to detect *A. marginale* in the Philippines. This study reported the first molecular detection and characterization of *A. marginale* in cattle and *R. microplus* in Cebu, Philippines.
KEY WORDS: *Anaplasma marginale,* cattle, PCR, Philippines, *Rhipicephalus (Boophilus) microplus.*

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Anaplasma marginale is a rickettsial Gram-negative intra-erythrocytic pathogen which causes bovine anaplasmosis characterized by a mild to severe hemolytic disease [15]. *A. marginale* has a worldwide occurrence [1], and is considered as one of the most prevalent pathogens causing costly tick-borne diseases in cattle [25]. It is transmitted by the tick *Rhipicephalus (Boophilus) microplus* [15]. To date, only limited information about *A. marginale* infection in cattle in the Philippines can be accessed. The Australian Centre for International Agricultural Research (ACIAR) and the Bureau of Animal Industry of the Philippines had a collaborative project (ID: AS2/2000/098) from 2001–2004 which partly dealt with bovine anaplasmosis. However, data on the national prevalence of the disease through the project are still not made readily available (personal communication). Moreover, the project mainly utilized ELISA-based technologies and peripheral blood smear examination technique for the detection of the pathogen. Published reports detailing *A. marginale* in the Philippines were mainly on water buffaloes in the Luzon area [17]. Hitherto, no specific molecular detection method of *A. marginale* in the Philippines has been developed yet.

Molecular methods (e.g. PCR) based on the heat-shock operon (*groEL*) gene are commonly used for the detection of *Anaplasma* spp. organisms. The *groEL*, which belongs to the chaperonin family, can be found in numerous bacteria [33]. It has the advantage of having more variations between species than the 16S rRNA gene [12, 31], while supplementing most of the results of the 16S rRNA phylogenetic trees [10, 12, 31]. Therefore, highly specific PCR primers based on *groEL* can be designed from the conserved regions of *A. marginale*. This is the advantage of *groEL* over the widely used 16S rRNA gene that has very close homology among closely related species [32].

The island of Cebu, which hosts the 2nd largest metropolitan city (Cebu City) of the country, is found in the Visayas region. To the author's knowledge, there have been no published reports about tick-borne diseases in cattle in the province. Hence, confirmation on the presence of *A. marginale* in Cebu will be beneficial. In the present study, *A. marginale* was detected in cattle and *R. microplus* ticks,

*CORRESPONDENCE TO: INOKUMA, H., Department of Veterinary Clinical Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan.
e-mail: inokuma@obihiro.ac.jp

and was characterized based on 16S rRNA, *Msp5, Msp1α*, *groEL* and *gltA* genes for comparison and phylogenetic analyses. Furthermore, a new specific and sensitive PCR method based on *groEL* was developed for the detection of *A. marginale* in the Philippines.

MATERIALS AND METHODS

DNA samples: A total of 12 cattle and 60 *R. microplus* ticks collected in 2011 from Mandaue City and Consolacion Municipality, Cebu, Philippines were sampled (Fig. 1). All cattle showed varying degrees of emaciation. Five milliliter of blood sample from each cattle was collected using BD K_3EDTA Vacutainer® tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.), while ticks (5 per cattle) were manually picked and stored at 70% ethyl alcohol. DNAs from blood and individual ticks were extracted using QIAmp DNA blood Mini kit (Qiagen, Hilden, Germany), eluted with 200 *µl* of the buffer provided in the kit, and stored at −30°C until further use. DNAs prepared from infected cattle from Mongolia (9 samples) [2] and Japan (1 sample) [19] were used for the evaluation of a newly designed specific nested PCR method based on *groEL*, which will be described later. All these samples were *A. marginale* DNA-positive by 16S rRNA PCR. In addition, DNA samples extracted from the following species were used as negative controls: *Ehrlichia canis* (supplied by Dr. S. Harrus, Israel), *E. muris* [26], *Ehrlichia* sp. from *Ixodes ovatus* (supplied by Dr. H. Fujita, Japan), *A. bovis* [14], *A. centrale* [13], *A. platys* [12], *A. phagocytophilum* from human (formerly human granulocytic ehrlichia; supplied by Dr. P. Brouqui, France), horse (formerly *E. equi;* supplied by Dr. P. Brouqui, France) and cattle (supplied by Dr. G. Jouncour, France), *Anaplasma* sp. closely related to *A. phagocytophilum* [31], *Theileria orientalis*, *Babesia bovis* and *B. ovata* [24].

16S rRNA screening PCR and purification methods: DNA samples were initially screened using a nested PCR (nPCR) based on the 16S rRNA gene. The oligonucleotide sequences of the primers used in the present study are presented in Table 1. The initial amplification utilized the universal outer primers fD1 and Rp2 [20]. The subsequent amplification utilized the primer pairs EHR16SD/EHR16SR [20], which amplifies a partial 345-bp fragment in all *Anaplasma* and *Ehrlichia* species, and the fD1/GA1UR [31], which amplify a partial 426 bp-fragment containing the divergent region of the 16S rRNA gene. DNA sequencing was performed on all positive amplicons for confirmation. The blank and positive controls used were double distilled water (DDW) and *A. platys* DNA, respectively. The amplification products were visualized in 1.5% agarose gel after migration, and subsequently purified using either QIAquick® PCR Purification Kit (Qiagen) or QIAquick® Gel Extraction Kit (Qiagen).

16S rRNA (longer sequence), Msp5, Msp1α, groEL and gltA amplification: Four *A. marginale* DNA-positive samples and an *A. centrale*-positive sample were further amplified using the 16S rRNA, *Msp5*, *Msp1α*, *groEL* and *gltA* genes. For longer sequences of the 16S rRNA gene, primer pairs fD1/EHR16SR and EHR16SD/Rp2 in the second round PCR were used to amplify an approximately 1,400-bp fragment from the selected positive tick DNA samples. Moreover, inner primers pairs AM-87F/AM-963F were used to amplify an 875-bp fragment from the selected positive bovine DNA samples. For the *Msp5* PCR, primers AM-49F1 and AM-595R1 were used to amplify a 576-bp fragment. For the *Msp1α*PCR, the assay was performed according to a published method [16]. For the non-specific *groEL* PCR, partial fragments (513 bp) were amplified using AMgroES-111F1/AMgroEL1557R1 (outer primers) and AMgroES-67F2/AMgroELGr513R2 (inner primers), with starting and final annealing temperatures of 71 to 61°C for the first round PCR, and 68 to 58°C for the second round PCR. For the *gltA* PCR, partial fragments (422 bp) were amplified using a published method [31].

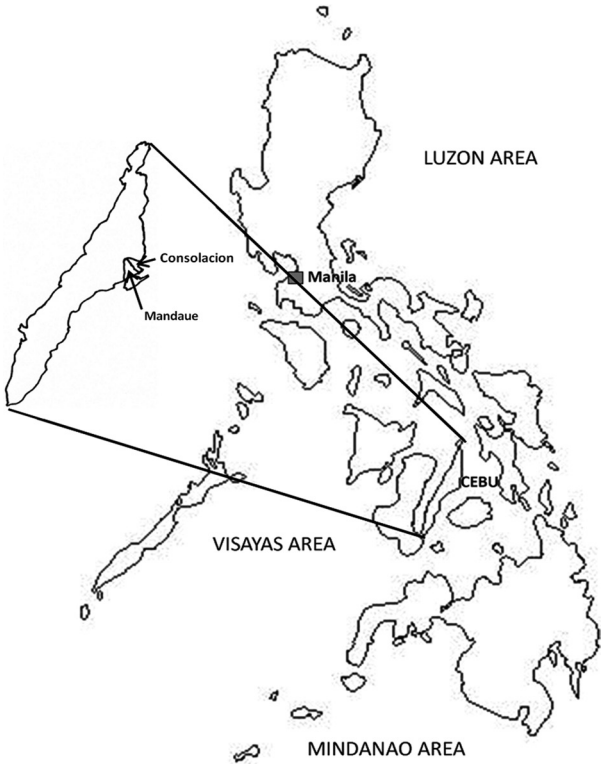

Fig. 1. Map of the sampling area.

Development of the specific groEL PCR: A new method, herein referred to as specific *groEL* PCR, was designed based on the multiple alignment of several *A. marginale* sequences from GenBank. Primers were designed to target a final 866-bp product. Oligonucleotide sequences of the newly designed primers are listed in Table 1. In the first round PCR, a final volume of 10 *µl* was set. It was composed of 4.9 *µl* of DDW, 1 *µl* of dNTPs (2 mM each),1 *µl* of 10× PCR buffer, 1 *µl* of each primer (10 pmol/reaction), 0.1 *µl* of 5 units AmpliTaq Gold polymerase (Roche Molecular Systems, Inc., Pleasanton, CA, U.S.A.) and 1 *µl* of DNA template. The step-down cycling conditions were the following: initial denaturation at 95°C for 9 min, followed by 35 cycles of

Table 1. Oligonucleotide sequence of primers

Primer	Sequence	Reference
16S rRNA		
fD1	AGAGTTTGATCCTGGCTCAG	Parola *et al*., 2000 [20]
Rp2	ACGGCTACCTTGTTACGACTT	Parola *et al*., 2000 [20]
EHR16SD	GGTACCY*ACAGAAGAAGTCC	Parola *et al*., 2000 [20]
EHR16SD	TAGCACTCATCGTTTACAGC	Parola *et al*., 2000 [20]
GA1UR	GAGTTTGCCGGGACTTCTTCT	Warner and Dawson, 1996 [30]
AM-87F	TACGCAGCTTGCTGCGTGTATG	This study
AM-963R	GCCCTTCTGTTAAGAAGGATCTAG	This study
Msp5		
AM-211F2	AAGCACATGTTGGTAATATTCGGCTTCTCA	This study
AM-376R2	AATTCTCGCATCAAAAGACTTGTGGTACTC	This study
AM-49F1	GTGTTCCTGGGGTACTCCTATGTGAACAAG	This study
AM-595R1	AAGCATGTGACCGCTGACAAACTTAAACAG	This study
MSP1a		
MSPa733F1	TGTGCTTATGGCAGACATTTCC	Lew *et al*., 2002 [16]
MSPa2957R2	AAACCTTGTAGCCCCAACTTATCC	Lew *et al*., 2002 [16]
MSPa3134R1	TCACGGTCAAAACCTTTGCTTACC	Lew *et al*., 2002 [16]
gltA		
CS7F2	ATGR*TAGAAAAW*GCTGTTTT	Ybañez *et al*., 2012 [31]
CSF1b	GATCATGAR*CAR*AATGCTTC	Inokuma *et al*., 2002 [12]
HG1085R	ACTATACCK*GAGTAAAAGTC	Inokuma *et al*., 2002 [12]
groEL for *A. marginale* and *A. centrale*		
AMgroES-111F1	AGAGCTCGAAGGAAAGAAGTTCATAGT	This study
AMgroEL1557R1	CATGAATACAGCTGCR*AGTGACACAGCCA	This study
AMgroES-67F2	TAATCGCTAAGGAGGCGTAGTC	This study
AMgroEL513-R2	GTCTTTGCCAACTTCCCTTACGCACTGTG	This study
Specific *groEL* for *A. marginale*		
AM265F1	GACTACCACATGCTCCATACTGACTG	This study
AMA424F2	GTCTGAAGATGAGATTGCACAGGTTG	This study
AM1574R1	GACGTCCACAACTACTGCATTCAAG	This study
AM1289R2	CCTTTGATGCCGTCCAGAGATGCA	This study

*Degenerate primers: Y=C or T, R= A or G, W=A or T, K=G or T.

95°C for 30 sec, 74°C (with 2°C incremental decrease until reaching a final annealing temperature at 65°C) for 30 sec and 72°C for 1.5 min, and final extension at 72°C for 5 min. In the 2nd phase of the amplification, a final volume of 25 *μl* was set. It was composed of 16.35 *μl* of DDW, 2.5 *μl* of dNTPs (2 mM each), 2.5 *μl* of 10× PCR buffer, 1.25 *μl* of each primer (10 pmol/reaction), 0.15 *μl* of 5 units AmpliTaq Gold polymerase (Roche Molecular Systems, Inc.), and 1 *μl* of the 1st round PCR amplicon. Similar cycling conditions as the 1st round PCR were employed, except for the final annealing step which used 68°C.

Evaluation of the specific groEL PCR: The specific *groEL* PCR was initially evaluated using DNA from 20 *A. marginale*-positive samples (10 from the present study, 1 prepared from bovine blood in Japan, and 9 prepared from bovine blood in Mongolia), and from the *A. centrale*-positive sample detected in the present study. The positivity of the DNA samples from Japan and Mongolia was validated using a 16S rRNA PCR (using fD1/GA1UR), and subsequent direct sequencing. To further evaluate the specificity of the specific *groEL* PCR, DNA samples prepared from several other *Ehrlichia*, *Anaplasma*, *Babesia* and *Theileria* species that were mentioned earlier were tested. To evaluate its sensitivity, serial dilution PCR assays were performed. From an *A.marginale*-positive DNA sample, a longer amplicon was produced by a standard PCR using *groEL* outer primers AM/AC265F1 and AM1574R1, which was ligated into a pCR 2.1 plasmid (PCR 2.1-TOPO, Invitrogen, Carlsbad, CA, U.S.A.), and subsequently cloned into One Shot® Top10 chemically competent *Escherichia coli* using the TA Cloning kit (Invitrogen). The transformed *E. coli* was grown overnight in a shaker at 37°C in Luria Bertani broth (Invitrogen) supplemented with 1 *μl*/m*l* of 50 mg/m*l* ampicillin (Wako Chemical Laboratories, Ltd., Osaka, Japan). The plasmid was extracted and purified from the pelleted *E. coli* using QIAprep® Spin Miniprep Kit (Qiagen), and eluted with 50 *μl* of DDW. The initial concentration of the plasmid DNA (210.1 *ng*/*μl*) was adjusted to a starting concentration of 1 *ng*/*μl* (approximately 2×10^8 copies/*μl*), and was subsequently 10-fold serially diluted using DDW until 10^{-8} for use as templates for the standard PCR (using only the inner primers) and the nPCR assays. The same cycling conditions were performed as described previously for the specific *groEL* nPCR.

Sequencing and phylogenetic analyses: Direct sequencing method was performed using the same nPCR inner primers. In case, if the sequence result was of low quality, amplicons were cloned into a vector using TOPO TA cloning (Invitrogen), and sequenced using the primers provided in the kit. Nucleotide sequence results were initially checked using BLAST search for comparison with other known sequences. Percent identities were computed without considering the gaps. The multiple sequence alignment was performed using the MUSCLE program [11]. Phylogenetic analyses were performed by neighbor-joining (maximum composite likelihood model) and maximum likelihood methods utilizing the MEGA software version 5.05 [27]. The tree stability was estimated by bootstrap analysis for 1,000 replications. Separate analyses using translated amino acid sequence characters were performed for protein-encoding genes for comparison.

Nucleotide accession numbers: The accession numbers of nucleotide sequences used for comparison are indicated beside the organism's name, as shown in Figs. 2 and 3. The nucleotide sequences obtained in the current study were registered at GenBank and DNA Database of Japan with the following accession numbers: *A. marginale* 16S rRNA (*R. microplus*: JQ839011, JQ839012; *Bos taurus*: JQ839008; JQ839009), *A. marginale Msp5* (*B. taurus*: AB704328), *A. marginale groEL* (*R. microplus*: JQ838999, JQ839015, JQ839003; *B. taurus*: JQ839001, JQ839002, JQ839013, JQ839014), *A. marginale Msp1α* (*B. taurus*:JQ839005, JQ839006, JQ839007), *A. marginale gltA* (*R. microplus, B. taurus*: JQ839004), *A. centrale* 16S rRNA (*R. microplus*: JQ839010), *A. centrale groEL* (*R. microplus*: JQ839000) and *Bartonella* sp. 16S rRNA (*B. taurus*: JQ839016).

RESULTS

Examination of Giemsa-stained blood smears revealed 3 cattle (25%) showing *A. marginale* inclusion bodies. However, 16S rRNA screening PCR and subsequent sequencing revealed 8 cattle (66.7%) to be positive for *A. marginale*. On the other hand, 8 ticks (13.3%) were found positive for *A. marginale*, and 1 tick (1.7%) positive for *A. centrale*. The *A. marginale*-positive ticks were obtained from 3 *A. marginale*-positive cattle, while the *A. centrale*-positive tick was obtained from a cattle found negative with any *Anaplasma* sp. Shorter partial 16S rRNA sequences (345–426 bp) from the detected *Anaplasma* spp. isolates revealed 99.7–100% identities with several registered sequences from U.S.A., China, Japan, Zimbabwe, Israel, South Africa and Australia. Longer representative 16S rRNA sequences of *A. marginale* from cattle (JQ839008, 668 bp; JQ839009, 612 bp) still revealed the same identities with the isolates from the same countries mentioned earlier. On the other hand, longer representative 16S rRNA sequences of *A. marginale* from ticks (JQ839011, 1,326 bp; JQ839012, 1,354 bp) showed 99.8–99.9% identities to isolates from U.S.A., China, Australia, Uruguay, South Africa and Israel, while that of *A. centrale* (JQ839010, 1,402 bp) showed 99.9% identities to the isolates from Israel. On the other hand, DNA fragments (JQ839016; 450 bp) of a *Bartonella* sp. were detected in 1 cattle, which was found 99.1% identical to *B. schoenbuchensis*.

For the *Msp5* PCR, only the *A. marginale* positive samples were amplified. Representative sequence (AB704328, 576 bp) revealed 100% identity with a Chinese isolate, and 95–99.8% identities with other registered *A. marginale* sequences. On the other hand, only 4 *A. marginale*-positive DNA samples (out of 8) were amplified using the *Msp1α* nPCR. Three variants of different lengths (JQ839005, 698 bp; JQ839006, 780 bp; JQ839007, 960 bp) were identified, which shared 96.1–99.3% identities. One variant, JQ839005, was found closest to a U.S.A. isolate (M32870), with 94.3% identity. Two other variants, JQ839006 and JQ839007, were found closest to Brazilian isolates (AY998121, AY998120), with 97.6 and 95.9% identities, respectively. One cattle was found co-infected with 2 variants (JQ839005 and JQ839006).

Partial *groEL* fragments of *A. marginale*-DNA positive samples shared 99.1–100% identities. Those that were detected from *R. microplus* ticks, JQ838999 (573 bp) and JQ839003 (764 bp), were found to be 100 and 99.1% identical to U.S.A. (CP001079, AF165812) and Japanese (FJ226455) isolates, respectively. Those that were detected from cattle (JQ839001, JQ839002) were found to be 100% identical to isolates from Japan (FJ226455), Israel (AF414861) and Australia (AF414860). On the other hand, partial *groEL* sequence of *A. centrale* (JQ839000) from a *R. microplus* tick (1,402 bp) was found 99.6% identical to isolates from Israel (CP100759) and Australia (AF414867). Meanwhile, the partial *gltA* sequences of *A. marginale* from cattle and ticks indicated 100% identities with each other, and 99.1% identities with U.S.A. strains (CPOO1079, AF304140 and AF304139). DNA fragments of *A. centrale* were not amplified in the *gltA* PCR assay.

Phylogenetic tree analyses based on 16S rRNA (Fig. 2A), *Msp5* (Fig. 2B), *Msp1α* (figure not shown) and *groEL* (Fig. 3A) genes demonstrated that the Philippine *A. marginale* isolates generally clustered with those from other countries. It was also observed that the Philippine *A. marginale* isolates were positioned closer to the Chinese isolates for the 16S rRNA gene, and to isolates from U.S.A., Japan, Australia and Israel for *groEL*. In contrast, the *Msp1α* phylogenetic tree results (using more than 100 registered sequences) showed closer positions of the Philippine *A. marginale* isolates to Brazilian isolates, while *Msp5* phylogenetic tree results showed closer positions to Brazilian and Chinese isolates. In the *gltA* phylogenetic tree, the Philippine *A. marginale* isolates was positioned in a separate branch (under the same clade) from all other registered isolates (only U.S.A.) supported by a high bootstrap value (Fig. 3B). On the other hand, the Philippine *A. centrale* isolate formed a cluster with *A. centrale* isolates from other countries in the 16S rRNA and *groEL* phylogenetic trees. Similar trees for *gltA, Msp5, Msp1α* and *groEL* phylogenetic analyses were obtained using deduced amino acid sequences (figures not shown).

In the evaluation of the specific *groEL* PCR, all *A. marginale*-positive samples were found positive using the nPCR method. However, the *A. centrale*-positive DNA sample was also found positive using the nPCR method (figure not

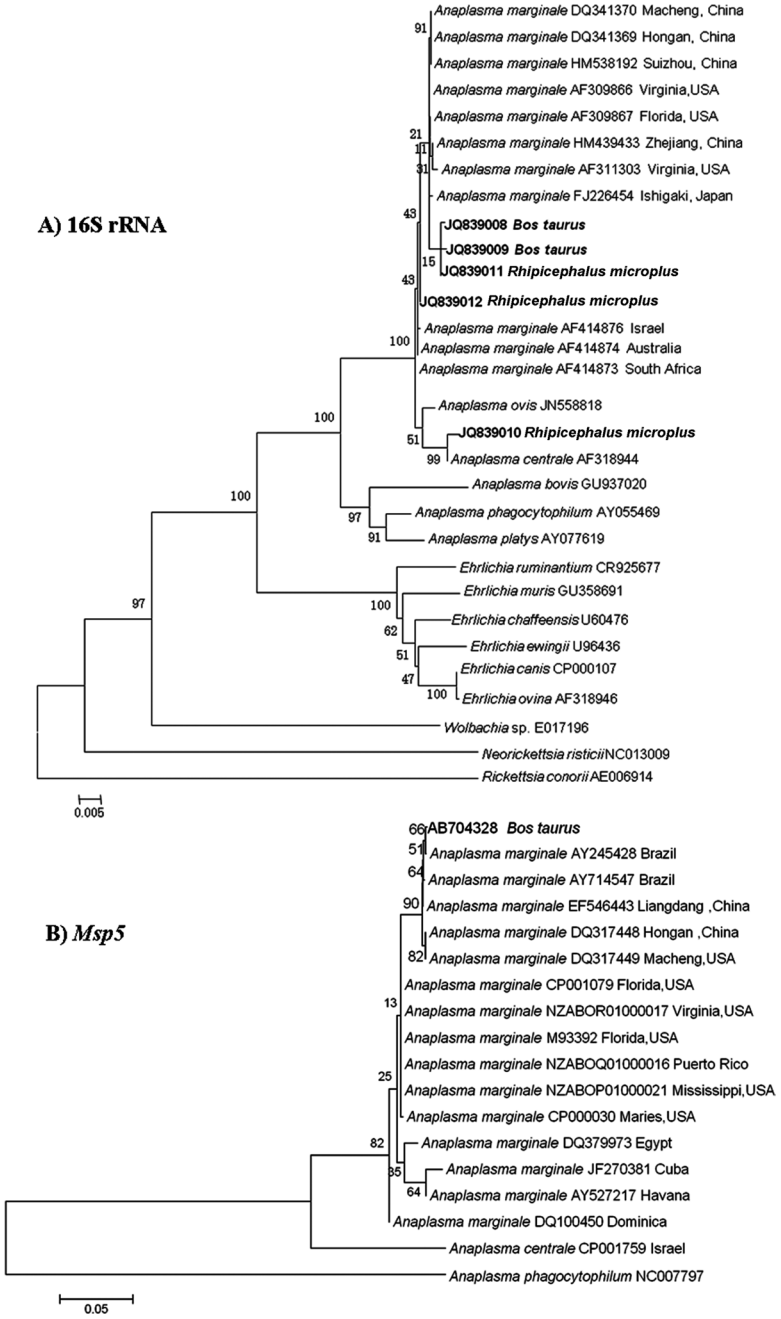

Fig. 2. Phylogenetic relationships of *Anaplasma* spp. detected from cattle and *R. microplus* ticks in Cebu, Philippines based on A) 16S rRNA and B) *Msp5* genes. The trees were analyzed using nucleotide sequences by neighbor-joining method (maximum composite likelihood model; pairwise deletion), and were supported by 1,000 bootstrap replications. *Rickettsia conorii* and *Anaplasma phagocytophilum* were used as outgroups for A) and B), respectively. The *Anaplasma* spp. studied are set in bold.

shown). Direct sequencing of the amplicon (JQ839015; 826 bp) revealed 99.8% identities to the closest *A. marginale* isolates from Australia (AF414860), Japan (FJ226455) and Israel (AF414860). Meanwhile, DNAs from other *Anaplasma, Ehrlichia, Theileria* and *Babesia* species mentioned earlier were found negative using the nPCR method (Fig. 4). Serial dilution assays yielded sensitivity limits of 2×10^2 and 2 copies/PCR for the standard PCR (using the inner primers) and specific *groEL* nPCR assays, respectively (Fig. 5).

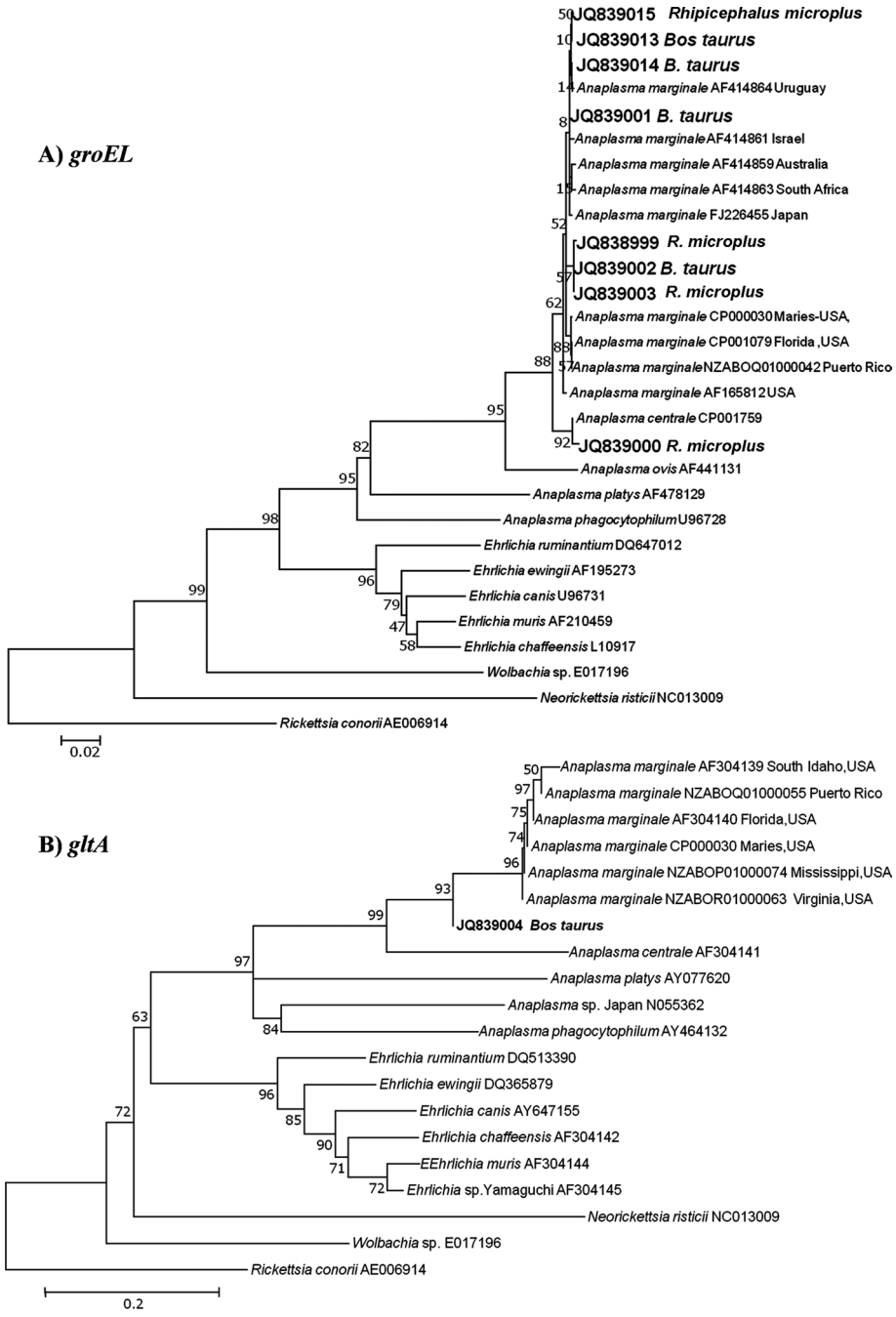

Fig. 3. Phylogenetic relationships of *Anaplasma* spp. detected from cattle and *R. microplus* ticks in Cebu, Philippines based on A) *groEL* and B) *gltA* genes. The trees were analyzed using nucleotide sequences by neighbor-joining method (maximum composite likelihood model; pairwise deletion), and were supported by 1,000 bootstrap replications. *Rickettsia conorii* was used as the outgroup for both trees. The *Anaplasma* spp. studied are set in bold.

DISCUSSION

The number of *A. marginale*-positive animals that were detected using the peripheral blood smear examination (PBSE) method was lower than the PCR assay. The PCR assay is considered to be more sensitive than the PBSE method in the detection of *A. marginale,* because of the higher possibility of false-negative results of the latter [26]. On the other hand, the *A. marginale*-positive ticks came from 3 (out of 8) infected cattle. The PCR positive results of the ticks and their

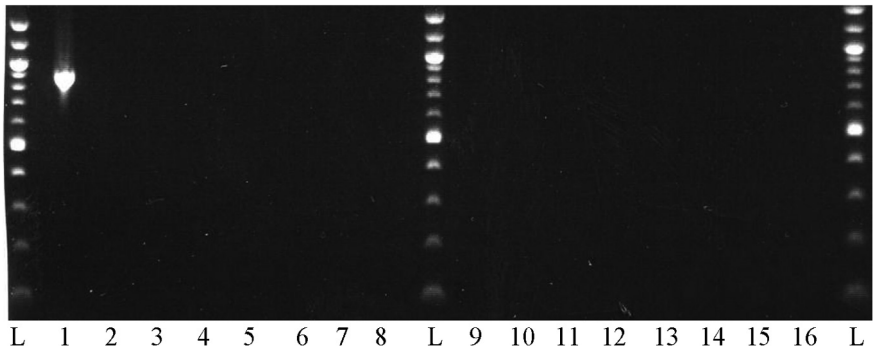

Fig. 4. The specificity of the specific *groEL* nPCR assay. The following are represented in the respective lanes: 1) *A. marginale* from Philippine cattle, 2) *Anaplasma phagocytophilum*-equine strain, 3) *A. phagocytophilum*-human strain, 4) *A. phagocytophilum*-bovine strain, 5) *Anaplasma* sp. closely related to *A. phagocytophilum* of Japan, 6) *A. bovis*, 7) *A. centrale* from Japanese cattle, 8) *A. centrale* from *R. microplus* in Philippines, 9) *A. platys*, 10) *Ehrlichia canis*, 11) *E. muris*, 12) *Ehrlichia* sp. from *Ixodes ovatus*, 13) *Theileria orientalis*, 14) *Babesia bovis*, 15) *B. ovata*, and 16) blank control (double distilled water). L represents the 100-bp DNA ladder marker.

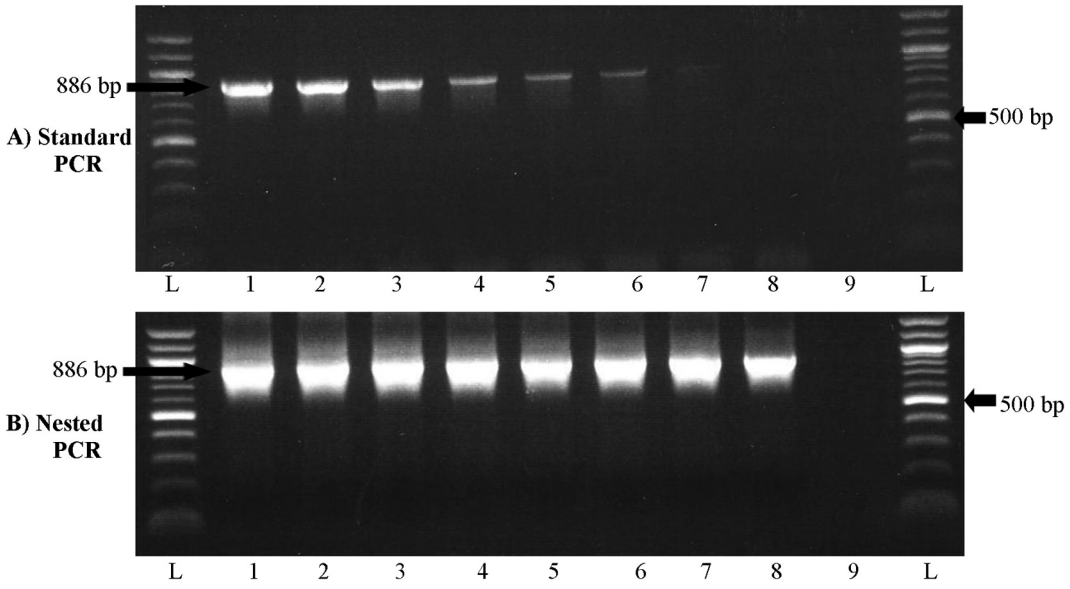

Fig. 5. Sensitivity of the specific *groEL*-based PCR assay by 10-fold serial dilution of DNA using A) standard PCR (using internal primers) and B) nPCR assays. Lanes 1–8 represent assays of the same template dilutions of 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7} and 1×10^{-8} for each panel, respectively. Lane 9 and L represent the blank control (double distilled water) and 100-bp DNA ladder marker, respectively.

cattle sources indicate the carrier status of the latter with *A. marginale*. Xenodiagnosis, which uses ticks in the detection of a pathogen, is employed together with the PCR assay to confirm the carrier status of an animal with a pathogen [23].

For the *A. centrale*-positive tick, its cattle source was found negative with the pathogen. It is possible that the levels of bacteremia were low, and thus was undetectable using PCR [22]. The presence of *A. centrale* in the Philippines has not been documented before. The potential role of *R. microplus* in the transmission of *A. centrale* would warrant further studies. *R. microplus*, a known vector of *A. marginale*, was first reported in the capital, Manila [3]. This vector is prevalent in cattle all throughout the Philippines [5].

The shorter partial 16S rRNA sequences of the *Anaplasma* species detected in the present study showed 100% identities from several registered sequences, while the longer representative 16S rRNA sequences showed variation (0.1–0.2%) from the closest registered sequences. Results indicated that longer DNA sequences should be desired, if the 16S rRNA gene is to be used for sequence comparison. Moreover, the close identities of the *Anaplasma* species detected in the present study make it difficult to come up with stronger assumptions about species diversity, if only the 16S rRNA gene is used as the basis. The 16S rRNA gene is less variable

compared to other genes, because it shares higher similarities among closely related species [18, 31].

Msp5 sequence identity of the Philippine *A. marginale* isolate was found closest to the Chinese and Brazilian isolates. The *Msp5* PCR did not amplify the Philippine *A. centrale* isolate from the tick, which connotes that the *Msp5* PCR used in the present study may be highly specific for *A. marginale*. *Msp5* is a good tool for molecular detection due to its high conservation in *A. marginale* species. The *Msp5* is a highly conserved 19-kDa protein, which is encoded by a single-copy 633-bp gene on the genome of *A. marginale*. It has been used in several detection studies utilizing different primer sets [28, 29].

Partial *gltA* sequences of *A. marginale* from cattle and ticks were found identical to each other, which implied that the isolates from both sources are highly related. The registered partial *gltA* sequence from the Philippine samples represented the only *gltA* sequence from an Asian isolate, since other registered *gltA* sequences of *A. marginale* came from U.S.A. territories. Moreover, the 99.1% identities of the Philippine isolates with the registered sequences can be considered low, since other sequences shared 99.7% to 100% identities, which have a maximum of 4 bp differences in the whole span of *A. marginale gltA* (data not shown). This indicates that the *gltA* may be a good genetic indicator in studying intra-species variation and diversity. The *gltA* is known to exhibit low sequence identities within and between *Ehrlichia* and *Anaplasma* species [13].

Comparison of partial *groEL* fragments from Philippine *A. marginale* isolates was found to have close identities with U.S.A., Japan, Israel and Australia, while the Philippine *A. centrale* isolate was found closer to Israel and Australian isolates. Australia remains as one of the major cattle importers to the Philippines, and cattle movement from Australia can lead to the possibility of introducing pathogens like *A. marginale* and *A. centrale* in the Philippine area. However, it is also possible that these pathogens are endemic in the Philippines, and that they were already present in the area even before the introduction of cattle imports.

Although found DNA positive using PCR based on the other 4 genes, only a few of the Philippine *A. marginale*-positive DNA samples were DNA positive using the *Msp1α* PCR method. Although it was proven that the method was highly specific and sensitive for detecting *A. marginale* in Australia [16], results suggested that this PCR method may differ in its sensitivity if used with the Philippines isolates. Moreover, it appears that the method is apparently more suitable for Australian isolates, in which only 1 genotype or variant is known to exist [6], than for the Philippine isolates, in which at least 3 variants were identified in the present study. *Msp1α* has been used to analyze distribution and genetic diversity of *A. marginale* strains within and between countries due to its variable tandem repeats [6, 16]. Due to the low detection sensitivity of the *Msp1α* PCR method used in the present study, alternative genes or methods can be sought to analyze phylogeographic distribution of *A. marginale.*

In the phylogenetic analyses, results demonstrated that the Philippine *A. marginale* isolates were not divergent from the isolates of other countries. In the present study, 4 other genes were sought other than the 16S rRNA gene due to the apparent limitations of the latter gene in resolving relationships between closely related species [31]. Based on results, the Philippine *A. marginale* isolates appeared phylogenetically closer to Chinese, Brazilian and U.S.A. strains. However, the absence of representative DNA sequences of nearby Asian countries from the 5 different genes used in the present study precludes making further assumptions, because the inclusion of the desired sequences in the analyses will probably change the phylogenetic tree results. This is particularly observed in the *gltA* phylogenetic analyses of the present study, in which other than the Philippine *A. marginale* isolate, all other sequences came from U.S.A. isolates. The distinction of the Philippine *A. marginale* isolate from U.S.A. strains may be a good indication that the *gltA* gene may be used as an alternative gene for studying the phylogeographic distribution of *A. marginale* strains. A study analyzing *A. marginale* strains using the *Msp1α* gene found out that the *Msp1α* did not provide phylogeographic information [7].

The newly developed specific *groEL* PCR was proven to be highly specific and sensitive. Since the *groEL* exhibits more variation than the 16S rRNA gene [5], this gene was chosen for the development of an nPCR method. In another related study, a duplex real-time PCR method included probe/primers which were designed based on *groEL* for the specific detection of *A. centrale.* It was recognized that the *groEL* contains regions, which were variable between *A. marginale* and *A. centrale* [8].

DNA fragments of a *Bartonella* sp. closely related to *B. schoenbuchensis* in cattle were amplified using primers fD1 and GA1UR. The reverse primer GA1UR has been used for the detection of *Ehrlichia* and *Anaplasma* spp. [12, 13, 26, 30]. Results imply that when GA1UR is paired with the universal primer fD1, non-specific amplifications may occur. The present molecular detection of the *Bartonella* sp. closely related to *B. schoenbuchensis* in cattle is the first report in the Philippines. *B. schoenbuchensis* is known to infect cattle and deer [9, 21]. In the Philippines, the only reported *Bartonella* sp. infections were in domestic cats, which were caused by *B. henselae* and *B. clarridgeiae* [4].

This study detected and characterized *A. marginale* isolates from cattle and *R. microplus* ticks in Cebu, Philippines based on 5 different genes: 16S rRNA, *Msp5, Msp1α, gltA* and *groEL*. In addition, *A. centrale* was detected and characterized (using 16S rRNA and *groEL*) in a *R. microplus* tick for the first time in the Philippines. *Msp1α* characterization revealed at least 3 variants that are found in Cebu, Philippines. Meanwhile, a new *groEL*-based nPCR method that can detect *A. marginale* in cattle and ticks was developed. Conducting an epidemiological study on anaplasmosis using the new method in large ruminants in Cebu will be beneficial in identifying specific areas that are affected by the pathogen, and in establishing the prevalence of *A. marginale* in the area. In this way, relevant animal health program scheme can be designed, which will ultimately benefit the local farmers. It is also desired that its national prevalence should be determined for the surveillance of the disease. Nevertheless,

local animal health officers and large animal veterinarians should be made aware about the presence of *A. marginale* in the Philippines.

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