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Molecular characterization of *Trypanosoma evansi* isolates from water buffaloes (*Bubalus bubalis*) in the Philippines

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Abstract

Trypanosoma evansi infection in the Philippines is frequently reported to affect the country's livestock, particularly, the buffaloes. To assess the prevalence and intraspecific diversity of *T. evansi* in the country, blood samples from water buffaloes in different geographical regions were collected during an outbreak. *T. evansi* was detected in all 79 animals tested using PCR targeting the *RoTat 1.2 VSG* gene. Sequencing of the rDNA complete internal transcribed spacer (ITS) region including the 5.8S subunit showed high similarity (99–100%) between Philippine isolates and known *T. evansi* isolates in Genbank. Tree construction based on the same region confirmed the close relationship between Philippine and reported Thai isolates as compared to Egyptian isolates separated by relatively small genetic distances, 47 polymorphisms, despite the clustering in four branches. Overall, the results of this study prove genetic diversity within *T. evansi* species despite previous reports on limited heterogeneity among isolates worldwide.

Keywords

Trypanosoma evansi, ITS region, DNA sequencing, phylogeny, water buffaloes, Philippines

Introduction

In the Philippines, *Trypanosoma evansi* infection, commonly known as *surra*, is regarded as one of the most economically important animal parasitic diseases affecting livestock. Large epidemics of the disease have occurred predominantly in the islands of Visayas and Mindanao during the past two decades wherein high morbidity and mortality rates were reported in most domestic animals, particularly in buffaloes (Manuel 1998, Reid 2002, Dargantes *et al*. 2009). Buffaloes are considered as cryptic hosts of *T. evansi* usually harboring asymptomatic infections (Luckins 1988, Reid 2002, Dobson *et al*. 2009). However, abortions and stillbirths were also implicated in *T. evansi* infection among buffaloes (Dargantes *et al*. 2009). The disease, therefore, causes significant impact on buffalo's productivity consequently resulting in financial losses and affecting country's agricultural industry.

Correlation between the variation at the nucleotide sequence level and the changes in pathophysiological properties were reported in other trypanosome species (Majiwa and Webster 1987, Aymerich and Goldenberg 1989). As previously reported, *T. evansi* exhibits a widespread geographic distribution, a variety of host species infected, and an array of clinical symptoms and pathogenicity, hence suggesting genetic diversity within *T. evansi* species (Luckins 1988, Uche *et al*. 1992). In some studies, variations in the virulence pattern and pathogenesis of *T. evansi* independent to its origin were observed although the genetic basis for these variations is yet to be determined (Uche *et al*. 1992, Carmona *et al*. 2006, Antoine-Moussiaux *et al*. 2008, Perrone *et al*. 2009).

The advent of sequencing technology has provided more information on genetic variations in most organisms. In particular, DNA sequencing has been very useful in classifying organisms and among the common targets are the ribosomal DNA (rDNA) internal transcribed spacer (ITS) regions flanking the 5.8S subunit, which have been used in investigating genetic diversities in a number of protozoans including some trypanosome species (Homan *et al*. 1997, Santos *et al*. 2002, Cacciò *et al*. 2010, Lollis *et al*. 2011). In fact, this marker has been very useful in molecular analysis to establish relationships among and within trypanosome species such as *T. b. brucei*, *T. b. gambiense*, *T. cruzi*, *T. rangeli*, and *T. evansi* (Agbo *et al*. 2001, Santos *et al*. 2002, Beltrame-Botelho *et al*. 2005, Khuchareontaworn *et al*. 2007, Areekit *et al*. 2008, Tian *et al*.

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2011). Previous study based on ITS regions of *T. brucei* has established genotypic discrimination between its two subspecies (Agbo *et al*. 2001). In *T. evansi* species, recent investigations on ITS sequences provided evidence on the intraspecific differentiation of *T. evansi* within a single host and from several hosts (Khuchareontaworn *et al*. 2007, Areekit *et al*. 2008, Amer *et al*. 2011). This information on the genetic diversity of *T. evansi* can be used for epidemiological studies specifically in assessing geographical distribution, transmission dynamics, and virulence pattern of the parasite for the prevention and/or management of the disease that it causes particularly during outbreaks.

Currently, limited data on genetic variation among *T. evansi* isolates from the Philippines has been reported. For that reason, DNA sequencing and phylogenetic analysis based on the complete ITS region including the 5.8S subunit of *T. evansi* isolated from water buffaloes in the country were done in this study. In addition, the prevalence of *T. evansi* infection was also assessed via targeted PCR assay using *RoTat 1.2 VSG* gene.

Materials and Methods

Blood sample collection

Blood samples used in this study were kindly provided by the Philippine Carabao Center (PCC) in Muñoz, Nueva Ecija and the College of Veterinary Medicine, Central Mindanao University (CMU) in Musuan, Bukidnon. To check for the disease prevalence, a total of 79 samples were collected from water buffaloes (*Bubalus bubalis*) located in distinct geographical areas, specifically from Nueva Ecija and Bohol. On the other hand, 10 samples were obtained from Balb/c mice used to propagate *T. evansi* isolates collected from infected water buffaloes in the year 2006 to 2009. Table I shows the host location and date of collection of these isolates. These isolates were inoculated to another mouse via intraperitoneal injection of 0.1 ml infected murine blood to increase parasitemia level. All samples were transported to the Molecular Protozoology Laboratory of the Natural Sciences Research Institute, University of the Philippines Diliman for further processing.

Parasite isolation and DNA extraction

Daily examination of murine blood by wet blood film preparation was done for at least two weeks to confirm infection and to check for the parasitemia level. At the peak of parasitemia, about 10^8 cells/ml, murine blood was extracted via cardiac puncture and was stored at ≤ 4ºC. Parasite cells were concentrated by centrifugation at 10,000 rpm for 10–15 min at room temperature. Resulting pellets were used for DNA extraction, which was carried out using Chelex 100 (Bio-Rad Laboratories, Inc.) in accordance to the methods used by Herrera *et al*. (2005) with slight modification wherein 5% instead of 1% Chelex 100 was added into the pellets as recommended by Walsh *et al*. 1991.

Polymerase chain reaction (*PCR*) *assay*

PCR amplification was carried out using *i-Taq*™ DNA polymerase (Intron Biotechnology, Inc.) and TC-412 thermal cycler (Techne® Inc.). Specific primers used for the detection of *T. evansi* were TeRoTat920F and TeRoTat1070R, which amplify a 151 bp gene fragment of the *RoTat 1.2 VSG* gene (Konnai *et al*. 2009). PCR was performed under the following cycling conditions: an initial denaturation at 94ºC for 5 min, followed by 40 cycles of denaturation at 94ºC for 30 sec, annealing at 64ºC for 30 sec, and extension at 72ºC for 30 sec. Final extension was done at 72ºC for 7 min (Mekata *et al*. 2009). Amplification of *T. evansi* rDNA complete ITS region was done using the primers ITS/F and ITS/R, which generate amplicons of ~1,300 bp (Khuchareontaworn *et al*. 2007, Areekit *et al*. 2008). The amplification conditions followed were as described by Khuchareontaworn *et al*. 2007, which were set to give an initial denaturation at 94ºC for 2 min, followed by 30 cycles each of denaturation at 94ºC for 1 min,

Table I. Source location and date of collection of *T. evansi* isolates for genetic diversity studies

Control no.	Source Location		Date of Collection
	Region	Barangay/Municipality/Province	
B01	Visayas	Lomangog, Ubay, Bohol	Aug. 22, 2009
B02	Visayas	Lomangog, Ubay, Bohol	Aug. 25, 2009
B03	Luzon	Muñoz, Nueva Ecija	Aug. 14, 2009
B06	Visayas	Lomangog, Ubay, Bohol	Aug. 25, 2009
B10	Mindanao	Surigao	Aug. 25, 2008
B13	Luzon	Los Baños, Laguna	Sept. 28, 2006
B14	Mindanao	Surigao	Aug. 24, 2008
B18	Luzon	Urdaneta, Pangasinan	Nov. 24, 2009
B19	Luzon	Urdaneta, Pangasinan	Nov. 24, 2009
B22	Luzon	Calabalabaan, Muñoz, Nueva Ecija	July 24, 2009

Table II. Reference *Trypanosoma* spp. ITS region sequences obtained from GenBank

Accession Number	Species/Isolate	Host	Location	Reference
DQ472685.1	*Trypanosoma evansi evansi* clone CL8	Buffalo	Bangkok, Thailand	Khuchareontaworn *et al*. 2007
DQ472705.1	*Trypanosoma evansi evansi* clone CL36	Buffalo	Bangkok, Thailand	Khuchareontaworn *et al*. 2007
DQ472708.1	*Trypanosoma evansi evansi* clone CL40	Buffalo	Bangkok, Thailand	Khuchareontaworn *et al*. 2007
DQ472696.1	*Trypanosoma evansi evansi* clone CL20	Buffalo	Bangkok, Thailand	Khuchareontaworn *et al*. 2007
DQ472688.1	*Trypanosoma evansi evansi* clone CL12	Buffalo	Bangkok, Thailand	Khuchareontaworn *et al*. 2007
DQ472704.1	*Trypanosoma evansi evansi* clone CL34	Buffalo	Bangkok, Thailand	Khuchareontaworn *et al*. 2007
DQ472700.1	*Trypanosoma evansi evansi* clone CL30	Buffalo	Bangkok, Thailand	Khuchareontaworn *et al*. 2007
DQ472703.1	*Trypanosoma evansi evansi* clone CL33	Buffalo	Bangkok, Thailand	Khuchareontaworn *et al*. 2007
DQ472710.1	*Trypanosoma evansi evansi* clone CL49	Buffalo	Bangkok, Thailand	Khuchareontaworn *et al*. 2007
AY912271.1	*Trypanosoma evansi evansi* strain B15.2	Buffalo	Bangkok, Thailand	Unknown
AY912273.1	*Trypanosoma evansi evansi* strain B17.2	Buffalo	Bangkok, Thailand	Unknown
AY912276.1	*Trypanosoma evansi evansi* strain KAI.1	Cattle	Sing Buri, Thailand	Unknown
AY912277.1	*Trypanosoma evansi evansi* strain KAI.2	Cattle	Sing Buri, Thailand	Unknown
AB551919.1	*Trypanosoma evansi evansi* Egy.1.	Camel	Cairo, Egypt	Amer *et al*. 2011
AB551921.1	*Trypanosoma evansi evansi* Egy.1.	Camel	Cairo, Egypt	Amer *et al*. 2011
AB551922.1	*Trypanosoma evansi evansi* Egy.1.	Camel	Cairo, Egypt	Amer *et al*. 2011
AF306770.1	*Trypanosoma brucei* H3	Lion	Luangwa Valley, Zambia	Agbo *et al*. 2001

annealing at 60ºC for 1 min, and extension at 72ºC for 1 min. Final extension was at 72ºC for 5 min. Amplicons were checked by electrophoresis in 1.5% agarose gel and visualized by ethidium bromide staining and UV illumination. Sizes of the bands were estimated using Quick-load® 100 bp DNA ladder (New England Biotech).

PCR product purification, *DNA sequencing and phylogenetic tree construction*

Amplicons of the ITS regions were subsequently purified using QIAquick® Gel Extraction Kit (QIAGEN) following the manufacturer's instructions. Purified PCR products were sent to Macrogen, Inc. (Seoul, South Korea) for DNA sequencing and data obtained were checked in BioEdit v. 7.0.9.0 sequence alignment program (Hall 1999). Using BLAST in GenBank (http://www.ncbi.nlm.nih.gov/BLAST), nucleotide sequences of isolates were compared to reported trypanosome ITS sequences, 17 of these sequences were used as reference in tree construction, specifically 16 from *T. evansi* and one from *Trypanosoma brucei* (Table II). Alignment of sequences was carried out using the ClustalW application of BioEdit followed by visual inspection for manual editing of sequences. The optimal model used to account for DNA sequence evolution was estimated by comparing the log likelihood scores of 9 different models, the HKY (Hasegawa *et al*. 1985), TrN (Tamura and Nei 1993), and GTR (Lanave *et al*. 1984) models with additional parameters such as gamma distributed rates (Γ) and proportion of invariant sites (I) incorporated into each models. Log likelihood scores were generated using PAUP* v. 4.0b10 program (Swofford 2002). Using the parameters of the optimal model, phylogenetic tree based on the complete ITS region was constructed using neighbor-joining (NJ) and maximum parsimony (MP) methods in the PAUP* program wherein bootstrapping was done 1000 times. Maximum likelihood (ML)-based tree was constructed in the Phyml 3.0 website with bootstrap values replicated 100 times (http://atgc.lirmm.fr/phyml/) (Guindon and Gascuel 2003). Trees were viewed using TreeView v. 1.6.6 (Page 1996).

Nucleotide sequence accession numbers

DNA sequences generated from this study were deposited in GenBank and are available through accession numbers HQ593637-HQ593646.

Results and Discussion

The present study specifically aimed to assess the disease prevalence and to investigate variability among Philippine *T. evansi* isolates from water buffaloes. As reported previously, importation of animals from countries where *surra* outbreaks were accounted lead to the transmission of the disease in Philippines. Introduction of the disease subsequently resulted to severe outbreaks in several provinces which caused thousands of deaths in affected animals, hence affecting country's livestock industry (Yutuc 1935, Venus and Dumag 1967, Manuel 1998, Reid 2002). Despite the prevention and control measures implemented by the government to eradicate *surra*, in the present study, PCR-based detection of *T. evansi* using the *RoTat 1.2 VSG* gene as molecular target showed positive infection in all buffaloes tested. This is in contrast to the results

of the initial screening done at the Philippine Carabao Center by mice inoculation test (MIT), in which 25 out of the 79 buffaloes tested were found negative for *T. evansi*-infection (unpublished data). Regardless of the difference in the sensitivity of the tests, wherein PCR-based detection had 100% sensitivity while MIT had 68% sensitivity, the results still confirm the persistence, high incidence, and widespread distribution of the organism in the country.

Evaluating the diversity of Philippine *T. evansi* isolates from water buffaloes was done through amplification and direct sequencing of the rDNA complete ITS region including 5.8S subunit. PCR amplification of ITS regions of *T. evansi* produced amplicons of approximately 1,200 bp in size. Nucleotide sequence length of the isolates ranged from 1168 bp (B03) to 1,218 bp (B19 and B22). BLAST search in GenBank showed relatively high sequence similarity (99–100%) between Philippine *T. evansi* isolates and other known *T. evansi* strains reported to date. In particular, the highest percentage similarities (100%) were observed among the ITS sequences of B13, B14 and that of *T. evansi* isolates from a buffalo in Thailand. This supports previous studies which suggested that *T. evansi* displays high degree of homogeneity

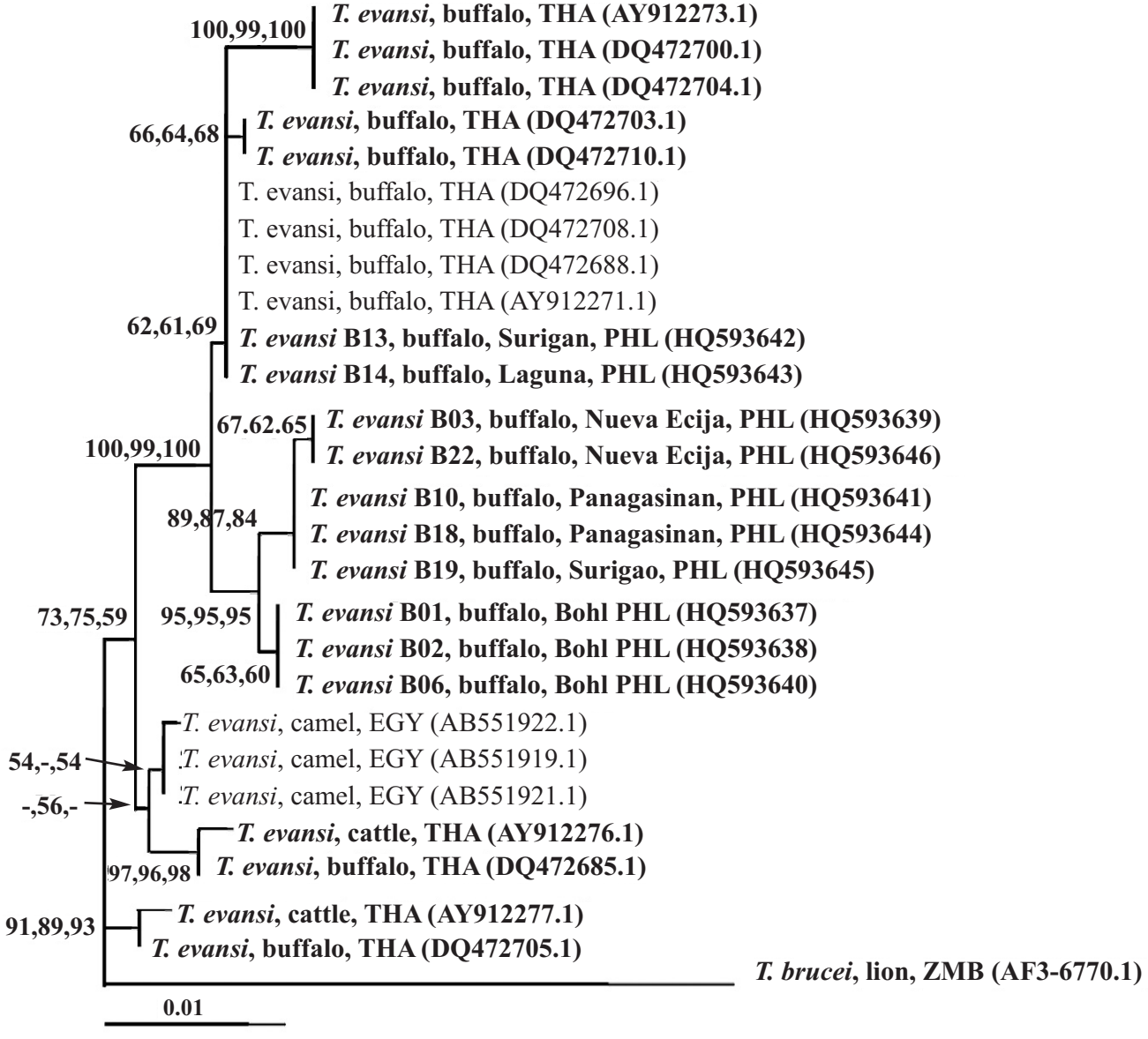

Fig. 1. Consensus tree constructed based on the nucleotide sequence data of the rDNA ITS1, 5.8S, and ITS2 regions (1,097 bp) of *T. evansi* isolates from Philippine water buffaloes and reference GenBank sequences. The tree was rooted with the outgroup, *T.brucei*. Bootstrap support values inferred from NJ, MP, and ML methods are shown in each node, respectively. Values less than 50% are not shown. Scale bar represents substitutions per 100 nucleotides

regardless of wide geographic distribution and host range (Lun *et al*. 2004, Masiga *et al*. 2006, Nijru *et al.* 2007, Perrone *et al.* 2009).

Despite the high sequence similarity, multiple sequence alignment of the complete ITS region of the 10 Philippine isolates and 16 *T. evansi* reference sequences revealed 47 variable sites consisting of single nucleotide polymorphisms (SNPs) characterized by substitutions and indels. In reference to this nucleotide sequence data, the consensus tree constructed using NJ, MP, and ML methods produced four groups supported by strong bootstrap values, greater than 50% (Fig. 1). Two Philippine isolates (B13 and B14) grouped together with 9 isolates from a Thai buffalo with bootstrap support of 61–69% hence suggesting a close relationship between Philippine and Thai isolates. The other 8 isolates (B01, B02, B03, B06, B10, B18, B19, and B22) grouped together with bootstrap support of 95%. This group is further separated into two subgroups, one of which consists of four Luzon and one Mindanao isolates while the other includes all Visayas isolates. The clustering of Visayas isolates implies the predominance of this strain during the disease outbreak, which was reported to cause several abortions in buffaloes within the area. The grouping of Luzon and Mindanao isolates, regardless of the distance of its host location and time of isolation proves the persistence of this particular strain in these regions. The other two groups generally consist of known Thai and Egyptian isolates from various hosts with considerable bootstrap support. In particular, one group consists of Thai and Egyptian isolates from a cow, a buffalo, and camels while the other group consists of Thai isolates from both cow and buffalo. Overall, the ITS tree thus confirmed diversity among *T. evansi* isolates independent to its host and geographical origin.

Characterization of *T. evansi* at the molecular level has been done previously for better species classification and assessment of evolutionary relationships with other organisms (Gibson 2002, Masiga *et al*. 2006, Amer *et al*. 2011). The present study has employed sequencing and phylogenetic tree construction based on the rDNA complete ITS region to evaluate intraspecific diversity of *T. evansi* from water buffaloes in the Philippines. This region has been generally used in assessing genetic variation in a number of protozoans including *T. evansi* (Khuchareontaworn *et al*. 2007, Tian *et al*. 2011, Areekit *et al*. 2008, Amer *et al*. 2011). Other DNA targets that were used to distinguish *T. evansi* interspecifically and intraspecifically to date include 18S rDNA, kinetoplast DNA, microsatellite sequences, and the expression-site-associated genes (ESAGs) (Biteau *et al*. 2000, Witola *et al*. 2005, Nijru *et al*. 2007, Mekata *et al*. 2009). Nevertheless, only ESAG6 and rDNA complete ITS region were proven to be useful genetic markers for classification within this species (Witola *et al*. 2005, Khuchareontaworn *et al*. 2007, Areekit *et al*. 2008, Mekata *et al*. 2009). In the study by Mekata *et al*. 2009, phylogenetic analysis of *T. evansi* ESAG6 yielded 10 clades, 6 of which contain Philippine isolates (not the same isolates used in the present study). The data obtained from the present study showed that Philippine *T. evansi* isolates are separated into 2 groups only, one of which is further divided into 2 subgroups. Despite the clear demonstration of intraspecific diversity within the species by ITS regions, it is obvious that ESAG6 represent a better molecular marker to assess variability in Philippine isolates. Still, data obtained can be used for epidemiological studies such as the investigations on pathogenesis, host range, geographic distribution, and virulence pattern of *T. evansi* in addition to subtyping which can subsequently provide useful information for disease prevention and management in the country.

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