NOTE *Parasitology*

Molecular Epidemiological Survey of *Theileria orientalis* in Thua Thien Hue Province, Vietnam

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ABSTRACT. *Theileria orientalis* is a benign bovine protozoan parasite that occasionally causes serious economic loss in the livestock industry. We report the findings of a molecular epidemiological survey of *T. orientalis* in 94 Vietnamese yellow cattle, 43 water buffaloes, 21 sheep, 21 goats and 85 blood-sucking ticks of cattle in the Thua Thien Hue province of Vietnam. The major piroplasm surface protein (*MPSP*) gene of *T. orientalis* was detected using polymerase chain reaction from 13 cattle (13.8%), 11 water buffaloes (25.6%), 1 sheep (4.8%) and 9 ticks (10.6%). Phylogenetic analysis using *MPSP* gene sequences showed the presence of seven genotypes, four previously categorized genotypes (Types 1, 3, 5 and 7) and three new genotypes (Types N-1, N-2 and N-3).
KEY WORDS: cattle, genotype, MPSP, *Theileria orientalis*, Vietnam.

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Theileria orientalis is a tick-transmitted, intraerythrocytic protozoa belonging to the phylum Apicomplexa, and a member of the benign *Theileria* group (*Theileria sergenti/buffeli/orientalis*) [19]. *T. orientalis* or related species are widely distributed in many Asia-Pacific countries, including Japan [11, 20], Korea [7, 13], China [14], Taiwan [12], Thailand [8, 16], Cambodia [4], Vietnam [9], Indonesia [2] and Australia [6]. Generally, the pathogenicity of *T. orientalis* is lower than that for *T. parva* and *T. annulata*, however can occasionally cause symptoms including fever, anemia, and anorexia in infected cattle [18]. Serious economic losses in the livestock industry can subsequently arise.

Major piroplasm surface protein (MPSP) of *T. orientalis* is expressed as an immunodominant protein on the parasite surface during the intraerythrocytic stage (piroplasm) [5, 10]. This coding gene exhibits significant sequence diversity among field isolates of *T. orientalis* [3, 8, 23]. Currently, eight genotypes of *T. orientalis* are known worldwide based on registered *MPSP* gene sequences [15]. Our recent survey carried out in Hokkaido, Kumamoto, and Okinawa prefectures of Japan revealed at least five genotypes of *T. orientalis* (Types 1, 2, 3, 4 and 5) in Japan [15, 22]. Thus, the *MPSP* gene may be a reliable molecular marker for studies on the epidemiology of *T. orientalis*.

Although a worldwide distribution of this parasite has been reported, there is insufficient data regarding the sequence identification and genotyping of *T. orientalis*, especially using the *MPSP* gene. In the present study, we conducted a molecular epidemiological survey of *T. orientalis* in domestic animals of the Thua Thien Hue province of Vietnam during 2010 (Fig. 1A). Vietnam is located within Southeast Asia and exhibits high temperatures and humidity throughout the year. The present field survey was carried out in February after a long rainy season (Temperature: 22–27°C). Blood samples from Vietnamese yellow cattle (*Bos taurus*; Fig. 1B), water buffaloes (*Bubalus arnee*; Fig. 1C), sheep (*Ovis aries*) and goats (*Capra aegagrus hircus*) were randomly collected from 13, 6, 1 and 1 farm/s, respectively, as shown in Table 1. Furthermore, half-engorged ticks (nymphs or adults) were collected from the skin of randomly selected Vietnamese yellow cattle (1–3 ticks per animal) and then fixed in acetone. All of the collected ticks were morphologically identified as *Rhipicephalus* (*Boophilus*) *microplus* (data not shown). Individual families typically managed each dairy farm, whereby farmers owned 10–15 domestic animals and had occasionally exchanged animals with neighboring farms. Although field veterinarians in the province had often detected *Theileria* infection of domestic animals through classical blood microscopic observation, species identification of the parasites had not been performed.

Genomic DNA was extracted from 100 μl of whole blood using a QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) and from acetone-fixed ticks using a commercial kit (Isohair, Nippon Gene Co., Tokyo, Japan), according to the manufacturer's instructions. The resulting 200 μl eluted DNA samples (approximately 6 $\mu g/\mu l$) were stored at –30°C for subsequent PCR analyses. PCR amplification, DNA sequencing and phylogenetic analysis were based on the *MPSP* gene of *T. orientalis* according to previous reports [15, 22]. Pairwise comparisons of the *MPSP* gene sequences were conducted using the EMBOSS needle program (European Molecular Biology Laboratory) to deter-

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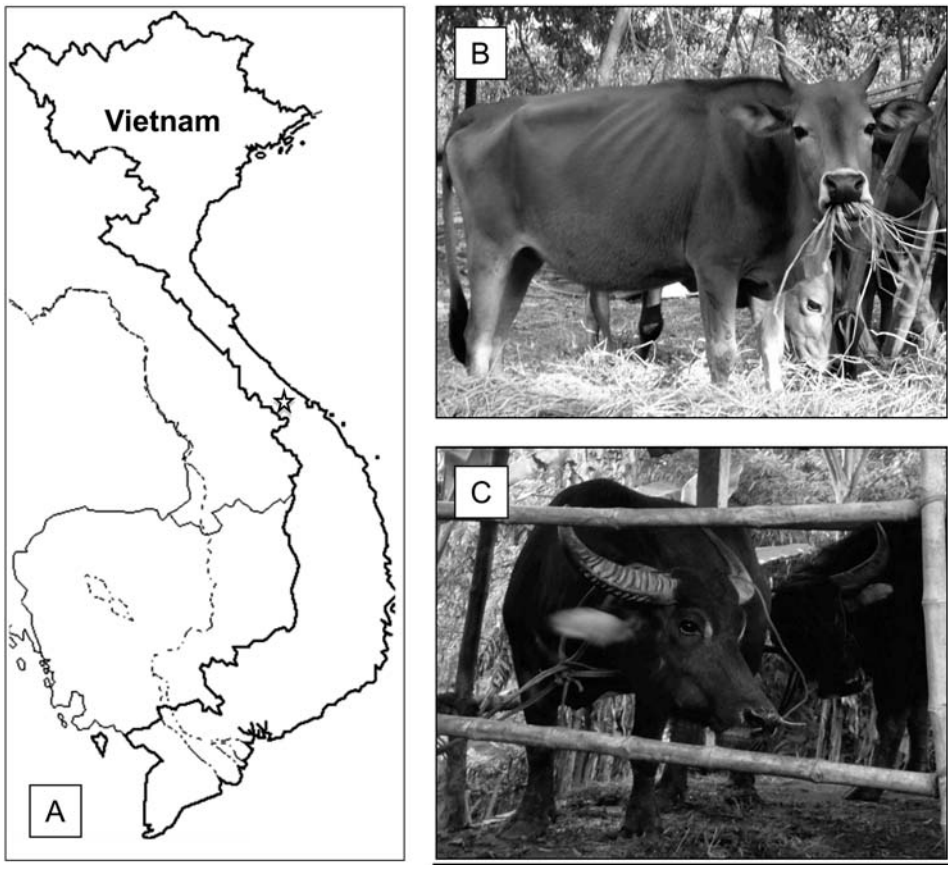

Fig. 1. Geographical map of Vietnam (**A**) and images of Vietnamese yellow cattle (**B**) and water buffalo (**C**). Star indicates the location of Thua Thien Hue Province, where blood and tick samples were collected.

Table 1. MPSP-PCR diagnosis and genotyping of *T. orientalis* from animal and tick samples collected in the Thua Thien Hue province of Vietnam, February 2010

Species	Total number of collected samples	Number of positive samples (%)	Isolated Genotypes (Number)[a)]
Cattle	94	13 (13.8)	1(1), 3(5), 5(5), 7(1), N-3(1)[b)]
Water buffalo	43	11 (25.6)	5(1), N-2[b)](4)
Sheep	21	1 (4.8)	N-1[b)](1)
Goat	21	0 (0.0)	
Tick[c)]	85	9 (10.6)	5(5), 7(1)

a) Refers to Fig. 3.
b) New genotypes detected in the present study.
c) Collected from cattle.

mine percentage homology among all *T. orientalis* genotypes.

Using the diagnostic MPSP-PCR assay, the *MPSP* gene was positively detected in 13 (13.8%) cattle, 11 (25.6%) water buffaloes, 1 (4.8%) sheep and 9 (10.6%) ticks (Table 1). Some farms were positive and others were negative in the diagnosis. Specific DNA amplification in the ticks was possibly derived from the blood meals consumed from infected cattle. Although *Rhipicephalus* (*Boophilus*) *microplus* is not reported as a vector for *T. orientalis*, none of the common vectors, *Haemaphysalis* spp., *Amblyomma* spp. and *Dermacentor* spp. [17, 19, 21], were detected in the present study. Although our study did not detect the parasite in goats (Table 1), there have been previous reports on *T. orientalis* infection in other ruminants, such as wild deer and antelopes [19]. Therefore, a large-scale epidemiological survey would be especially beneficial for sheep and goat populations.

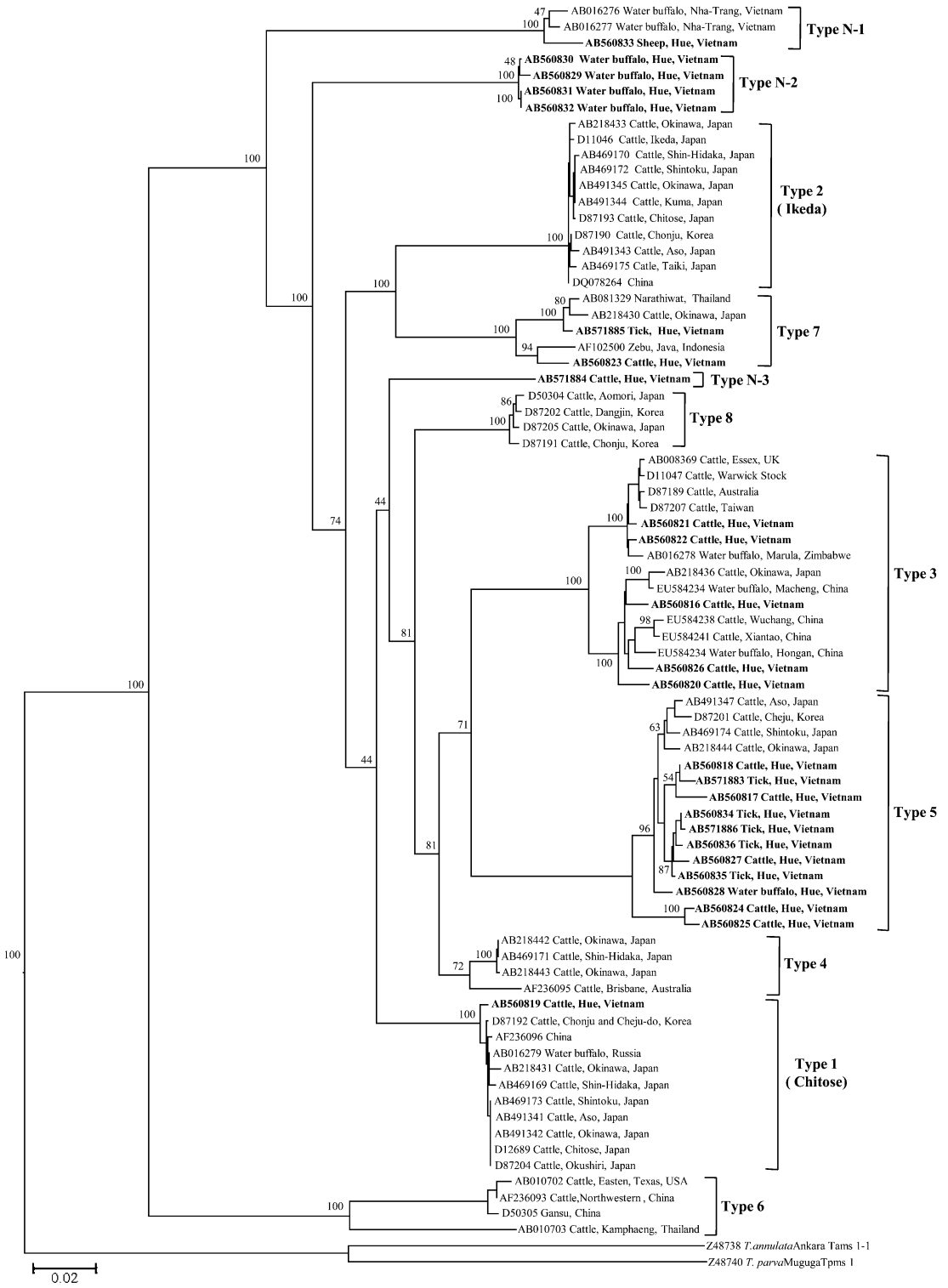

Fig. 2. Phylogenetic tree of the *MPSP* gene sequences derived from the blood and tick samples collected from domestic animals in the Thua Thien Hue province of Vietnam, together with previously registered sequences from the GenBank database. *MPSP* gene sequences determined in the present study are shown in bold-faced type and refer to the GenBank accession numbers as indicated at the end of each branch. Numbers shown at branch nodes indicate bootstrap values. Recently, a group of type 6 was classified as *T. sinensis* [14].

Group	Ikeda group			Chitose group									Buffalo 1		Buffalo 2		*T. sinensis*	*T. annulata*
Type	2	7	7	3	3	5	5	4	8	N-3	1	1	N-2	N-2	N-1	N-1	6	Ta
Acc.	D11046	AB571885	AB560823	AB560821	AB560826	AB560818	AB560824	AB469171	D50304	AB571884	AB560819	D12689	AB560830	AB560831	AB016276	AB560833	D50305	Z48738
D11046	100.0	88.8	88.6	66.0	65.5	64.1	64.3	68.7	84.0	68.8	69.0	87.5	66.5	66.6	80.2	63.3	67.2	57.2
AB571885		100.0	96.6	83.9	84.0	82.3	82.6	87.0	82.6	85.1	88.5	88.3	83.5	83.6	81.1	81.4	74.6	61.2
AB560823			100.0	84.5	83.1	82.6	82.7	86.9	82.9	84.3	88.3	88.2	83.3	83.4	81.1	81.5	74.6	61.9
AB560821				100.0	96.3	86.8	87.1	92.1	85.6	87.0	87.8	87.6	83.7	83.5	79.5	78.9	60.3	60.3
AB560826					100.0	86.7	87.4	91.6	84.7	86.0	87.3	87.0	83.2	83.1	79.1	77.7	72.2	60.0
AB560818						100.0	96.8	88.7	84.5	84.8	86.6	86.5	82.6	82.4	77.7	77.0	74.8	62.0
AB560824							100.0	88.1	83.8	84.3	85.7	85.4	81.6	81.4	77.9	77.4	74.6	60.3
AB469171								100.0	90.5	91.9	92.4	92.4	88.7	88.5	81.1	80.9	74.4	61.0
D50304									100.0	87.9	88.5	88.5	82.9	82.9	77.4	77.0	72.4	60.0
AB571884										100.0	91.4	91.1	86.7	86.9	80.1	80.4	73.8	61.5
AB560819											100.0	99.4	87.2	87.4	82.5	82.4	74.9	63.5
D12689												100.0	87.2	87.4	82.4	82.3	74.7	63.0
AB560830													100.0	99.9	81.8	82.0	74.8	63.0
AB560831														100.0	81.8	82.0	74.9	62.9
AB016276															100.0	98.2	72.6	62.5
AB560833																100.0	72.3	62.1
D50305																	100.0	60.8
Z48738																		100.0

Fig. 3. Pairwise comparisons of the representative *MPSP* gene sequences categorized in each genotype. Comparisons were conducted using the EMBOSS needle program to determine the percentage homology between all *T. orientalis* genotypes. Acc. indicates GenBank accession numbers, and a *T. annulata* sequence was used as an outgroup species.

MPSP genes from field isolates were successfully cloned into a TA-cloning plasmid vector using 13, 5, 1 and 6 PCR fragment/s derived from the cattle, water buffaloes, sheep and tick samples, respectively. Inserts were sequenced and used for subsequent phylogenetic and pairwise comparative analyses (Figs. 2 and 3, respectively). Only two DNA sequences derived from the water buffaloes were identical (GenBank accession numbers, AB560831 and AB560832), while intra-sequence variation was observed among the remaining DNA sequences. Phylogenetic analysis using *MPSP* gene sequences revealed four genotypes (Types 1, 3, 5 and 7), which had been previously categorized, from cattle, water buffalo or tick samples (Table 1 and Fig. 2). Additionally, three new genotypes (Types N-1, N-2 and N-3) were found in the sheep, water buffalo and cattle samples, respectively. While type N-2 detected in the water buffaloes was obviously separated on the phylogenetic tree (Fig. 2), type N-1 detected in the sheep formed a unique cluster with *T. buffeli* gene sequences previously isolated from the water buffaloes in Vietnam [3, 9]. N-1 and N-2 types are potentially genotypes specific to water buffalo in Vietnam. Five genotypes (Types 1, 3, 5, 7 and N-3) of *T. orientalis* were identified in the cattle, whereas only two genotypes (Types 5 and N-2) were detected from the water buffaloes (Table 1). Additionally, two genotypes (Types 5 and 7) of *T. orientalis* were detected in *Rhipicephalus* tick samples (Table 1). Types 2, 4 and 8 of *T. orientalis* were not detected in Vietnam. The finding in which the two genotypes from individual tick sample matched genotypes from the host cattle supports the assumption that PCR amplification in the tick samples may be due to the blood meal as described above.

Based on the phylogenetic findings, it is concluded that *T. orientalis* population in the cattle can be divided into the "Ikeda group" consisting of types 2 (Ikeda type) and 7 (88.6–96.6% homology in Fig. 3), and the "Chitose group" consisting of types 1 (Chitose type), 3, 4, 5, 8 and N-3 (83.8–99.4% homology). Certain genotypes (at least types 1, 3 and 5) in the Chitose group are potentially transmitted between cattle and water buffaloes. Types N-1 and N-2 may form separate groups ("Buffalo groups") consisting of *Theileria* populations distributed in water buffaloes and other ruminant(s) [1]. Types N-1 and N-2 showed low genetic homology to each other (81.8–82.0%), and to the other genotypes (66.5–88.5%) (Fig. 3). Further study is required to determine whether the parasites of Buffalo groups are also detected in cattle. Type 6 was previously found in the cattle and yaks, and recently classified as *Theileria sinensis* [14]. This genotype was distinguishable from the other *T. orientalis* groups in our study (60.3–74.9% homology, Fig. 3). Further elucidation is necessary on the relationship of *T. orientalis* which was first identified in sheep in our study to that in the water buffaloes.

Our results indicate that at least seven genotypes of *T. orientalis* currently exist in Vietnam. Further large-scale epidemiology would be useful in better understanding the geographical distributions, host specificities and clinical pathologies of the different genotypes of *T. orientalis,* and their relationships with the tick populations.

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