NOTE *Parasitology*

Epidemiology of bovine hemoprotozoa parasites in cattle and water buffalo in Vietnam

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ABSTRACT. A PCR-based survey of hemoprotozoa parasites detected *Babesia bigemina*, *Theileria orientalis* and *Trypanosoma theileri* among cattle and water buffalo in Vietnam, and a new *Babesia* sp. closely related to *Babesia ovata* was detected in cattle only. In addition, *Theileria annulata* and *Trypanosoma evansi* were not detected in both cattle and water buffalo. Phylogenetic analysis detected *T. orientalis MPSP* genotypes 3, 5, 7 and N3 in cattle and 5, 7, N1 and N2 in water buffalo. Additionally, water buffalo-derived *T. theileri CATL* sequences clustered together with a previously reported cattle-derived sequence from Vietnam. This is the first report of a new *Babesia* sp. in cattle, and *T. orientalis MPSP* genotype 7 and *T. theileri* in water buffalo in Vietnam.
KEY WORDS: cattle, epidemiology, hemoprotozoa, vietnam, water buffalo

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Bovine hemoprotozoa parasites, including species of *Babesia*, *Theileria* and *Trypanosoma*, infect cattle populations worldwide, causing significant economic damage to the livestock industry. Among the *Babesia* parasites infecting cattle, *Babesia bovis* and *Babesia bigemina* are virulent species reportedly causing infections in tropical and sub-tropical regions of the world [6]. Whereas, *Babesia ovata*, a less virulent species of *Babesia*, is known to be associated with clinical anemia in immunocompromised or *Theileria orientalis*-infected cattle [9, 31]. *Theileria parva* and *Theileria annulata*, lymphoproliferative *Theileria* parasites, severely compromise the health status of infected cattle [5]. In addition, *T. orientalis*, a non-lymphoproliferative *Theileria* species that has a worldwide distribution, occasionally causes severe anemia in infected cattle [28]. Although *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei*, which are endemic in Africa, are highly pathogenic, *Trypanosoma evansi* and *Trypanosoma theileri* are also sometimes reported to be involved in clinical diseases [7, 19, 37]. In general, although most of the bovine hemoprotozoa parasites are known to cause asymptomatic infections in buffalo, control strategies should focus on the elimination of these parasites among buffalo as well, as these animals can act as potential reservoirs [1, 15, 20, 23].

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Recent studies conducted in Vietnam demonstrated that cattle populations were infected with *B. bovis*, *B. bigemina*, *T. orientalis* and *T. theileri*, and that water buffalo were infected with *B. bovis*, *T. orientalis* and *T. evansi* [12–14, 16, 30, 36, 39]. Previously, 96 cattle and 43 water buffalo reared in Thua Thien Hue province of Vietnam were analyzed for *T. orientalis* major piroplasm surface protein (*MPSP*) genotypes [16]. The *MPSP* genotypes 1, 3, 5, 7 and N3 were detected in cattle, whereas in water buffalo, genotypes 5, N1 and N2 were detected. By contrast, in Thailand, a country neighboring Vietnam, several other genotypes were detected in water buffalo [1]. Therefore, the possible presence of other *MPSP* genotypes in Vietnamese water buffalo cannot be ruled out. Extensive studies analyzing the genetic diversity of *T. theileri* revealed pronounced host specificity of the parasite genotypes that infect different host species, including cattle, water buffalo and deer [8, 10, 11, 24, 25]. By contrast, in a recent investigation in Sri Lanka, some of the *T. theileri* cathepsin-L like protein gene (*CATL*) fragments derived from cattle and water buffalo clustered together phylogenetically [38]. However, the host specificity of *T. theileri* genotypes is still unclear in Vietnam, as the parasite has not yet been detected in Vietnamese water buffalo [30]. In the case of *B. ovata*, despite being detected in cattle populations in a number of Asian countries, including Japan [21], China [4], Korea [32], Thailand [40] and Mongolia [40], this parasite has not been surveyed in Vietnam. Therefore, in the present study, several species of *Babesia*, *Theileria* and *Trypanosoma* were surveyed in Vietnamese cattle and water buffalo.

Archived blood DNA samples sourced from cattle (n=258) and water buffalo (n=49) reared in Thua Thien Hue province of Vietnam, which had been previously used to detect and

Table 1. Diagnostic PCR primers used in the present study

Parasite	Target gene	Primer sequence (5′–3′)	Amplicon size (bp)	Annealing temp. (°C)	Reference
B. bigemina	*Apical membrane antigen - 1*	F: TACTGTGACGAGGACGGATC R: CCTCAAAAGCAGATTCGAGT	211	62	[27]
B. ovata	*Apical membrane antigen - 1*	F: GATACGAGGCTGTCGGTAGC R: AGTATAGGTGAGCATCAGTG	504	56	[31]
T. annulata	*Merozoite-piroplasm surface antigen*	F: ATGCTGCAAATGAGGAT R: GGACTGATGAGAAGACGATGAG	768	52	[18]
T. orientalis	*Major piroplasm surface protein*	F: CTTTGCCTAGGATACTTCCT R: ACGGCAAGTGGTGAGAACT	776	58	[22]
T. evansi	*Minicircle DNA*	F: CAACGACAAAGAGTCAGT R: ACGTGTTTTGTGTATGGT	373	55	[3]
T. theileri	*Cathepsin L-like protein*	F: CGTCTCTGGCTCCGGTCAAAC R: TTAAAGCTTCCACGAGTTCTTGATGATCCAGTA	289	55	[24]

F, forward primer; R, reverse primer.

Table 2. The findings of the PCR assays targeting *Babesia*, *Theileria* and *Trypanosoma* species among Vietnamese cattle and water buffalo

Animal type[a)]	Sample No.	*B. bovis*[b)]		*B. bigemina*		*B. ovata*[c)]		*T. orientalis*		*T. theileri*	
		Positive No. (%)	CI[d)]	Positive No. (%)	CI	Positive No. (%)	CI	Positive No. (%)	CI	Positive No. (%)	CI
Cattle	258	23 (8.9)	6.0–13.0	28 (10.9)	7.6–15.2	3 (1.2)	0.4–3.4	182 (70.5)	64.7–75.8	88 (34.1)	28.6–40.1
Buffalo	49	16 (32.7)	21.2–46.6	2 (4.1)	1.1–13.7	0		22 (44.9)	31.8–58.7	16 (32.7)	21.2–46.6

a) *T. annulata* and *T. evansi* were not detected in both cattle and water buffalo. b) *B. bovis* infection data were obtained from a previous study that analyzed the same DNA samples [39]. c) Although these samples were positive for *B. ovata* by PCR, sequencing and phylogenetic analyses demonstrated that they were infected with a *Babesia* sp. closely related to *B. ovata*. d) 95% confidence interval.

genetically characterize *B. bovis* [39], were screened for *B. bigemina*, *B. ovata*, *T. annulata*, *T. orientalis*, *T. evansi* and *T. theileri* using previously described parasite-specific PCR assays [3, 18, 22, 24, 27, 31]. The PCR reaction mixtures and cycling conditions were the same as those previously reported [29, 30, 40]. Briefly, 10 μl PCR reactions for *B. bigemina*, *B. ovata*, *T. annulata*, *T. evansi* and *T. theileri* contained 1 μl of 10× PCR reaction buffer, 200 μM dNTPs (Applied Biosystems, Branchburg, NJ, U.S.A.), 0.5 μM of forward and reverse primers (Table 1), 0.5 units of Taq polymerase (Applied Biosystems), 5.9 μl of double distilled water (DDW) and 1 μl of DNA sample. For *T. orientalis*, 10 μl reaction mixture contained 5 μl of 2× Ampdirect plus (Shimadzu Biotech., Kyoto, Japan), 0.1 μM of forward and reverse primers (Table 1), 0.1 μl of Extaq DNA polymerase (Takara, Tokyo, Japan), 3.7 μl of DDW and 1 μl of DNA sample. After an enzyme activation step at 95°C for 5 min, PCR reaction mixtures were subjected to 35 (*T. orientalis*) or 45 (*B. bigemina*, *B. ovata*, *T. annulata*, *T. evansi* and *T. theileri*) cycles, each consisting of a denaturing step at 95°C for 30 sec, an annealing step at the appropriate temperature (Table 1) for 1 min and an extension step at 72°C for 1 min. After a final elongation step at 72°C for 7 min, PCR products were analyzed by agarose gel electrophoresis and then visualized under UV light. The findings demonstrated that both cattle and water buffalo were infected with *B. bigemina*, *T. orientalis* and *T. theileri*, as summarized in Table 2. In addition, three DNA samples from cattle tested positive in the PCR assay targeting *B. ovata*. However, none of the surveyed samples tested positive for *T. annulata* or *T. evansi*. Positive rates of the parasite species were analyzed by OpenEpi software (http://www.openepi.com/Proportion/Proportion.htm) and a Chi-squared test (https://www.medcalc.org/calc/comparison_of_proportions.php) to determine the 95% confidence intervals and to calculate the *P* values, respectively. *P* values <0.05 were considered to indicate statistically significance. The findings demonstrated that the positive rate of *T. orientalis* in cattle was significantly higher than that of other parasite species detected in the present study. Similarly, *T. theileri*-positive rate in cattle was significantly higher than the positive rate of *B. bovis* and *B. bigemina*. *B. bovis* and *B. bigemina* are usually transmitted by one-host ticks [6], whereas *T. orientalis* is transmitted by three-host tick species [5]. Therefore, in theory, a tick infected with *T. orientalis* may transmit the parasite to more number of host animals as compared to *B. bovis*- or *B. bigemina*-infected tick. This could be a reason for the higher positive rate of *T. orientalis* as compared with that of other parasite species. Additionally, the differences in the densities of specific vectors that can transmit different species *Babesia*, *Theileria* and *Trypanosoma* could also explain the difference between the positive rates of these parasite species. On the other hand, although *B. bigemina*-positive rate was significantly lower than that of *B. bovis*, *T. orientalis* and *T. theileri* in water buffalo, the small sample size may not allow us to make fair comparisons.

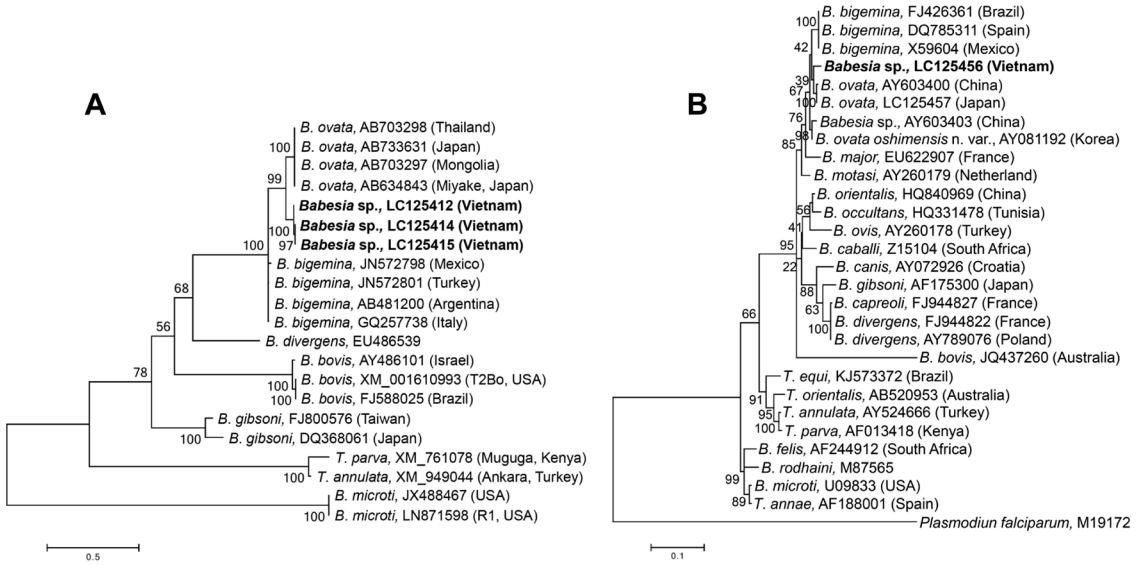

Fig. 1. Phylogenetic trees of the *AMA-1* and 18S rRNA gene sequences. The *AMA-1* (panel A) and 18S rRNA (panel B) gene sequences of *Babesia* and *Theileria* parasites, together with the sequences isolated from the Vietnamese DNA samples that tested positive in the *B. ovata*-specific PCR assay, were used to construct phylogenetic trees. The Vietnamese sequences are highlighted in boldface type letters. Bootstrap values are provided at the beginning of each branch. The scale bars in panels A and B represent 0.5 and 0.1 substitutions per site, respectively. Note that the Vietnamese sequences from *Babesia* sp. formed sister clades to *B. ovata* in both of the phylogenetic trees.

Table 3. Multiple infections of *Babesia, Theileria* and *Trypanosoma* in the surveyed DNA samples

Combination[a)]	Cattle		Water buffalo	
	Positive no. (%)[b)]	CI[c)]	Positive no. (%)	CI
4 parasites				
B. bovis + *B. bigemina* + *T. orientalis* + *T. theileri*	4 (4.3)	1.7–10.7	1 (6.3)	1.1–28.3
3 parasites				
B. bovis + *B. bigemina* + *T. orientalis*	2 (2.2)	0.6–7.6	0	
B. bovis + *B. bigemina* + *T. theileri*	2 (2.2)	0.6–7.6	0	
B. bovis + *T. orientalis* + *T. theileri*	1 (1.1)	0.2–5.9	1 (6.3)	1.1–28.3
B. bigemina + *T. orientalis* + *T. theileri*	7 (7.6)	3.7–14.9	0	
2 parasites				
B. bovis + *T. orientalis*	7 (7.6)	3.7–14.9	5 (31.3)	14.2–55.6
B. bovis + *T. theileri*	1 (1.1)	0.2–5.9	3 (18.8)	6.6–43.0
B. bigemina + *T. orientalis*	8 (8.7)	4.5–16.2	0	
B. bigemina + *T. theileri*	4 (4.3)	1.7–10.7	1 (6.3)	1.1–28.3
Babesia sp. + *T. orientalis*	2 (2.2)	0.6–7.6	0	
Babesia sp. + *T. theileri*	1 (1.1)	0.2–5.9	0	
T. orientalis + *T. theileri*	53 (57.6)	47.4–67.2	5 (31.3)	14.2–55.6
Total	92		16	

a) *B. bovis* infection data were obtained from a previous study that analyzed the same DNA samples [39]. b) Expressed as a percentage of the total number of co-infected cattle (n=92) or water buffalo (n=16). c) 95% confidence interval.

The previous studies that analyzed 96 cattle DNA samples from Thua Thien Hue province in Vietnam found positive rates lower than those determined in the present investigation for *B. bigemina*, *T. orientalis* and *T. theileri*, whereas *B. bovis*-positive rate was lower in cattle surveyed in the present work [16, 30]. The variations in the distribution of specific transmission vectors in different sampling localities within Hue province and the difference between the sample numbers might explain these discrepancies. Of 208 cattle and 38 water buffalo DNA samples that tested positive for at least one parasite species, 92 and 16, respectively, were infected with multiple parasite species (Table 3). However, the co-infection rates for any two parasite species were not significantly higher than the expected values. Although *T.*

theileri infections are generally considered to be benign, the parasite may be associated with clinical disease when co-infected with other parasite species [19, 37]. As 73 of 88 *T. theileri*-positive cattle DNA samples were co-infected with *Babesia* and *Theileria* parasites, future studies in Vietnam should focus on the clinical significance of *T. theileri* in co-infected animals.

Five (3 cattle and 2 water buffalo), three, eighteen (10 cattle and 8 water buffalo) and eight (5 cattle and 3 water buffalo) PCR amplicons from *B. bigemina*-, *B. ovata*-, *T. orientalis*- and *T. theileri*-specific PCR assays, respectively, were cloned, and 2 clones per PCR amplicon were sequenced, as previously described [27]. As the sequences were different between the two clones for 1 *B. bigemina* apical membrane antigen-1 (*AMA-1*) (cattle), 12 *T. orientalis MPSP* (6 cattle and 6 water buffalo) and 2 *T. theileri CATL* (cattle) gene fragments, a total of 6, 30 and 10 of these gene sequences, respectively, together with 3 *AMA-1* gene sequences amplified by the PCR assay targeting *B. ovata*, were registered in GenBank. Six *B. bigemina AMA-1* gene sequences, including four (LC125406–LC125409) from cattle and two (LC125410 and LC125411) from water buffalo, shared high identity scores (98.6–100%) with a *B. bigemina AMA-1* gene sequence (AB845438) isolated in Sri Lanka, confirming the findings of the PCR assay. The *AMA-1* gene sequences (LC125412, LC125414 and LC125415) amplified by the *B. ovata*-specific PCR assay shared only 93.5–93.7% identity scores with known *B. ovata* sequences (AB634843, AB703297, AB703298 and AB733631). In a maximum likelihood phylogenetic tree constructed based on the Kimura 2-parameter model [17] using the MEGA software version 6.06 [35], these *AMA-1* gene sequences clustered and formed a sister clade to the *B. ovata* clade (Fig. 1A). These findings suggested that the *AMA-1* gene sequences amplified by the *B. ovata*-specific PCR could have been derived from a *Babesia* species that is closely related to *B. ovata*. To test this hypothesis, a fragment of 18S rRNA was amplified from the DNA samples that tested positive in the *B. ovata*-specific PCR, using a pair of forward (SSBab18SF1, 5′-CATTACAACAGTTATAGTTTCTTTGG-3′) and reverse (SSBab18SR1, 5′-GTTAAATACGAATGCCCCCAACC-3′) primers. The sequences of the 18S rRNA gene fragments (694 bp) isolated from these DNA samples were identical to each other. These newly determined sequences shared identity scores of 97.3% and 97.1% with known *B. ovata* (AY603400 and LC125457) and *B. bigemina* (X59604 and DQ785311) sequences, respectively. On phylogenetic analysis based on the maximum likelihood method and Tamura-Nei model [34], the Vietnamese 18S rRNA sequence formed a sister clade to *B. ovata* (Fig. 1B), confirming the hypothesis that the *AMA-1* gene fragments amplified by the *B. ovata*-specific PCR were derived from a *Babesia* sp. that is closely related to *B. ovata* but has not been described previously.

The genotypic diversity of *T. orientalis* was analyzed using 16 (LC125416–LC125431) cattle-derived and 14 (LC125432–LC125445) water buffalo-derived *MPSP* gene sequences, respectively. A maximum likelihood phylogeny constructed based on the Tamura 3-parameter model [33]

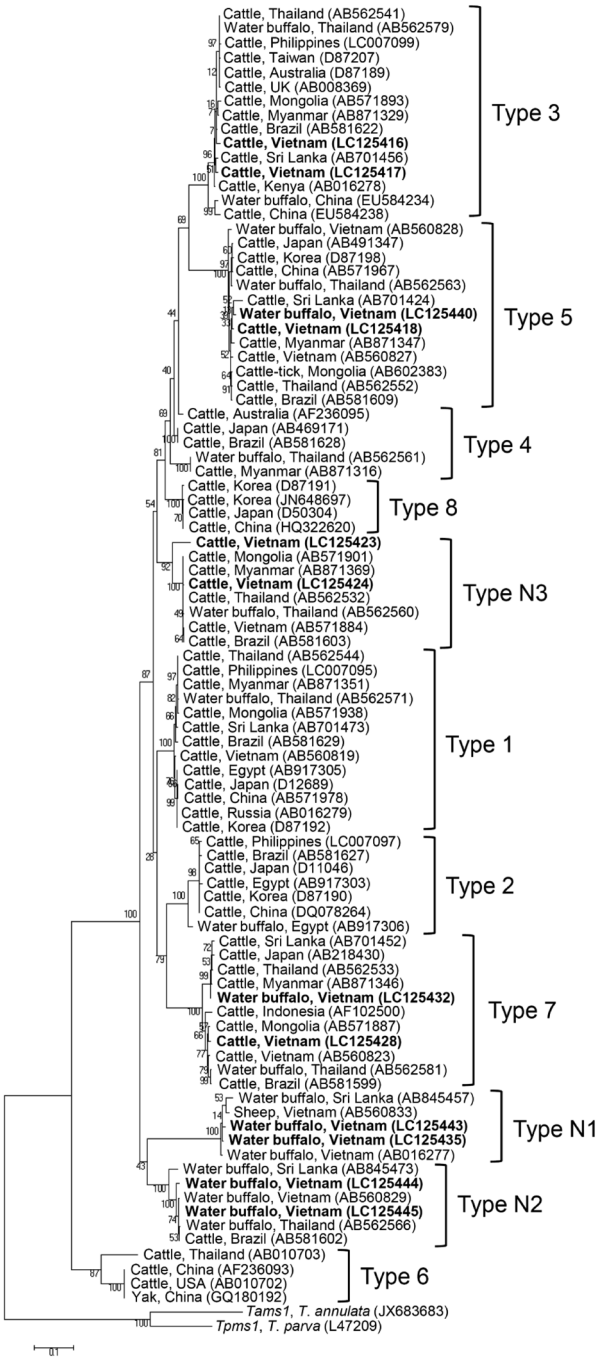

Fig. 2. Phylogenetic tree of *T. orientalis MPSP*. The sequences isolated in the present investigation are indicated by boldface type letters. Bootstrap values are provided at the beginning of each branch. The scale bar represents 0.1 substitutions per site. Note that the newly generated cattle-derived sequences were found within *MPSP* genotypes 3, 5, 7 and N3, while those derived from water buffalo occurred within genotypes 5, 7, N1 and N2.

placed the cattle-derived *MPSP* gene sequences within genotypes 3 (n=3), 5 (n=9), 7 (n=2) and N3 (n=2), and the previ-

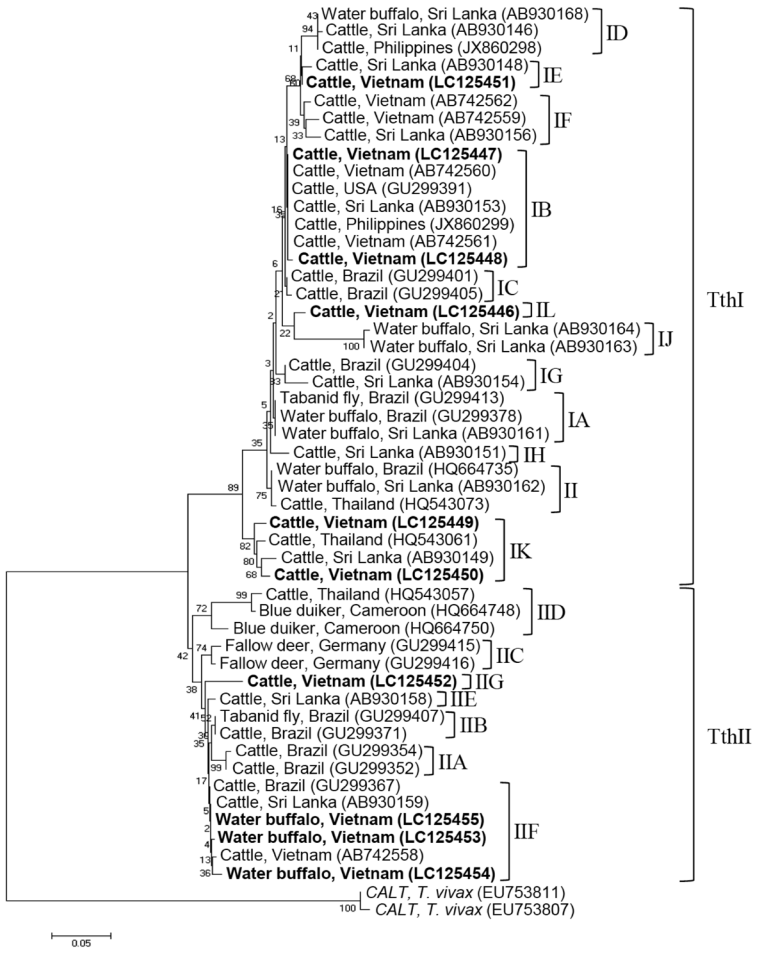

Fig. 3. Phylogenetic tree of *T. theileri CATL* gene sequences. The sequences determined in the present study are shown in boldface type letters. Bootstrap values are provided at the beginning of each branch. The scale bar represents 0.05 substitutions per site. Note that the cattle-derived sequences from Vietnam belonged to genotypes IB, IE, IK, IL and IIG and that the water buffalo-derived sequences clustered together with the previously reported cattle-derived sequences from Vietnam, Brazil and Sri Lanka to form genotype IIF.

ously reported genotype 1 was not detected [16] (Fig. 2). The *MPSP* gene sequences of water buffalo origin clustered with genotypes 5 (n=3), 7 (n=2), N1 (n=7) and N2 (n=2). In Vietnamese water buffalo, this is the first report of genotype 7, which has been implicated in several clinical cases of oriental theileriosis among cattle in India [2].

A neighbor-joining phylogenetic tree [26] was constructed using *T. theileri CATL* gene sequences, based on the Tamura 3-parameter model [33]. Seven *T. theileri CATL* gene sequences (LC125446–LC125452) isolated from cattle DNA samples in the present study belonged to five different clades (IB, IE, IK, IL and IIG), two of which (IB and IE) had been previously identified in Vietnam [30] (Fig. 3). Clades IL and IIG were formed by two individual *CATL* gene sequences determined in the present investigation. The three water buffalo-derived sequences (LC125453–LC125455) clustered with the previously determined cattle-derived sequences isolated in Vietnam, Brazil and Sri Lanka to form clade IIF. In previous investigations, the genotypes of *T. theileri* have been determined based on several marker genes, including small subunit rRNA (*ssrRNA*), internal transcribed spacer 1 (*ITS1*), cytochrome b (*Cyt b*), spliced leader (*SL*), glycosomal glyceraldehyde 3-phosphate dehydrogenase (*gGAPDH*) and *CATL*, which were found to be host specific in cattle, water buffalo and deer [8, 10, 11, 24, 25]. By contrast, similar to the findings of the present study, some of the *CATL* genotypes were shared between cattle and water buffalo in Sri Lanka, questioning the host specificity of *T. theileri CATL* genotypes [38]. Further studies, such as experimental infections and complete sequence analysis from cultured (cloned) parasites, are now needed to clarify the host specificity of these parasites.

In summary, our survey of bovine hemoprotozoa parasites among Vietnamese cattle and water buffalo is the first to report a new *Babesia* sp. in cattle, and *T. theileri* and *T. orientalis MPSP* genotype 7 in water buffalo in Vietnam. These findings provide valuable insight into the epidemiology of bovine hemoprotozoa parasites infecting livestock in Vietnam.

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