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Phylogenetic analysis of *Theileria orientalis* in cattle bred in Fujian province, China

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ABSTRACT

A molecular epidemiological survey on *Theileria orientalis* was conducted in a cattle population of Fujian province in China. The screening polymerase chain reaction assay showed that 23 (45%) of 51 blood samples were positive for *T. orientalis*. DNA sequencing of the major piroplasm surface antigen-encoding gene indicated the presence of 3 different genotypes in the study area (types 1, 2, and 5) and identified *T. orientalis* types 1 and 2 as the major genotypes in the sample population. Because type 2 (Ikeda type in Japan) has been recognized as a relatively virulent genotype of *T. orientalis*, control and preventive measures to minimize the incidence of infectious diseases among cattle bred in China are necessary.

Keywords: *Theileria orientalis*; cattle; China; epidemiology; genotype

Theileria parasites are broadly classified into 2 groups. The first group consists of *Theileria parva* and *Theileria annulata* is known to cause malignant disease (Onuma *et al.*, 1997), whereas the second group consists of non-lymphoproliferative species (and classification and nomenclature systems have differed between authors) such as *Theileria orientalis* and *Theileria sergenti* (Kim *et al.*, 1998; Minami *et al.*, 1980). Although the second group has been described as having low pathogenicity, occasional outbreaks of infectious disease caused by the members of this group have been reported in many Asian cattle populations (Baek *et al.*, 1990). The clinical picture of *T. orientalis* infection may include anemia and related signs, and asymptomatic infections have been reported (Kawazu *et al.*, 1992).

Recently, the occurrence of different genotypes of *T. orientalis* has been widely reviewed, by using the gene encoding the major piroplasm surface protein (MPSP) of *T. orientalis* as an epidemiological molecular marker (Kakuda *et al.*, 1998; Khukhuu *et al.*, 2010). Previous classification criteria divided the parasite population into 4 genotypes, *i.e.*, I, C, B, and Thai (Kakuda *et al.*, 1998; Sarataphan *et al.*, 1999, 2003). Currently, however, the scheme proposed by Kim *et al.* (1998) has been increasingly adopted for phylogenetic analysis of *T. orientalis* isolates. According to this method, the *T. orientalis* population was initially classified into 6 genotypes (types 1-6) (Kim *et al.*, 1998). Subsequently, 2 more genotypes (types 7 and 8) were identified (Jeong *et al.*, 2010; Kim *et al.*, 1998; Ota *et al.*, 2009), and the recent analysis of the *MPSP* gene sequences of Vietnamese *T. orientalis* revealed 3 additional genotypes (types N1, N2 and N3) in the population (Kawazu *et al.*, 1999; Khukhuu *et al.*, 2010).

Most of these genotypes differ in antigenic structure; therefore, immunity against infection by a particular genotype may not provide protection against subsequent infection by other genotypes (Jeong *et al.*, 2010). The polymorphic nature of *T. orientalis MPSP* genes may hinder the development of a subunit vaccine, although immunization with recombinant MPSP has reduced the level of parasitemia (Onuma *et al.*, 1997). Therefore, regular, widespread epidemiological studies are necessary to identify the current distribution of different *T. orientalis* genotypes in a particular region or country, and to understand the phylogenetic associations between the different genotypes. Consequently, appropriate preventive and control strategies can be formulated against *T. orientalis* infection. Recently, we conducted molecular epidemiological studies on *T. orientalis* found in the circulating blood of domestic animals bred in various

countries (Khukhuu *et al.*, 2010; Ota *et al.*, 2009; Yokoyama *et al.*, 2011). In addition, a recent epidemiological survey indicated the presence of 6 *T. orientalis* genotypes (types 1-5 and 7) in China (Liu *et al.*, 2011). In this study, we investigated a cattle population bred in China and analyzed the *MPSP* gene to identify the molecular epidemiology of *T. orientalis* in the study area, Fujian.

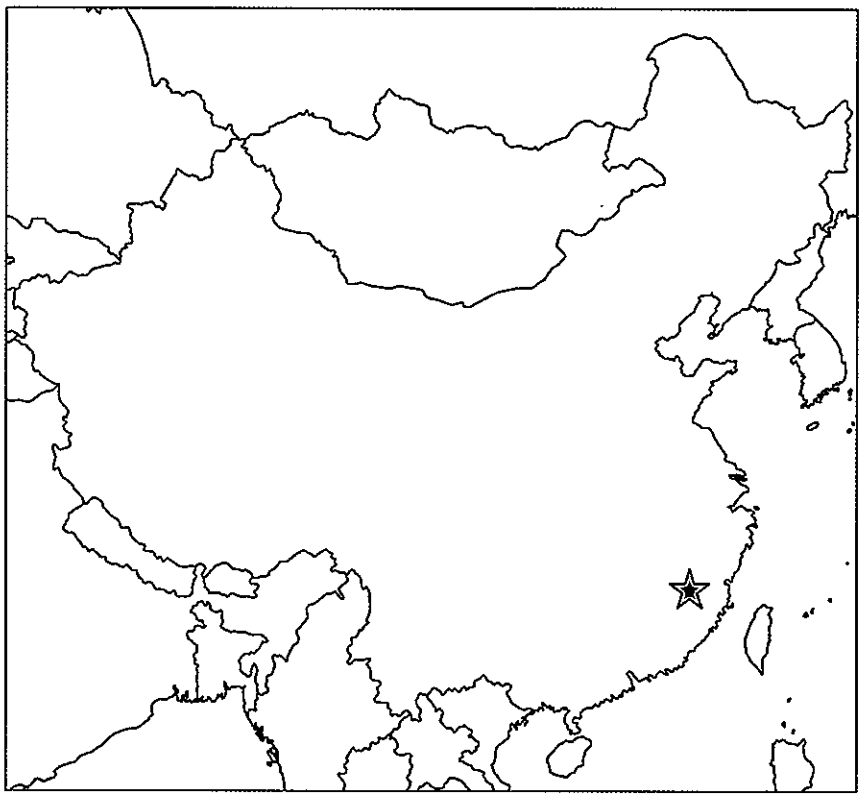

Figure 1: Location of Fujian province in China(★). Blood samples were collected from 51 randomly selected cattle bred in the province.

We randomly collected 51 blood samples from a cattle population bred in Fujian province (Fig. 1), on October 20, 2009, and extracted DNA with a commercial kit (Qiagen Blood Mini Kit, Hilden, Germany). The DNA samples were then screened for the presence of *T. orientalis* by a previously described diagnostic polymerase chain reaction (PCR) assay (Ota *et al.*, 2009). In the PCR assay, a set of forward and reverse primers was used for amplification of the *T. orientalis*-specific *MPSP* gene fragment (Ota *et al.*, 2009). Briefly, 25 μL of the reaction mixture consisted of 1 μL DNA template, 2.5 μL ×10 reaction buffer (Applied Biosystems, Branchburg, NJ, USA), 2.5 μL 2 mM dNTPs (Applied Biosystems), 1 μL 10 μM primers each, 0.1 μL *Taq* DNA polymerase (Applied Biosystems), and 16.9 μL double distilled water. The reaction was amplified under optimized conditions as described previously (Ota *et al.*, 2009). Identification of a 776 bp PCR product on gel electrophoresis indicated the presence of *T. orientalis* in the sample population (Fig. 2), and of 51 DNA samples, 23 samples (45%) were positive in the PCR assay (Table 1). Although this positive percentage was higher than that of previous investigations in China (34%) (Liu *et al.*, 2011), limited sample numbers in this study might account for the observed difference.

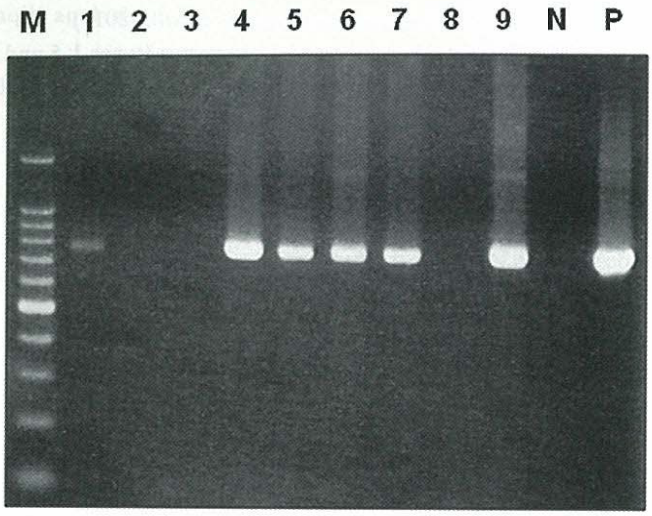

Figure 2: PCR detection of *T. orientalis* genes. M, 100 bp DNA marker; lanes 1–9, field DNA samples extracted from bovine blood; N, negative control; P, positive control.

Table 1. PCR detection and genotyping of *T. orientalis* detected from cattle bred in Fujian province, China.

Total number of collected samples	Number of positive samples(%)	Isolated genotypes (Number)[1)]
51	23(45.0)	1(7), 2(7), 5(4)

[1)] Refers to Figure 3.

Eighteen of the positively amplified PCR products were successfully gel extracted (Alquick Gel Extraction Kit, Qiagen), ligated to a PCR 2.1 plasmid vector (PCR 2.1-TOPO, Invitrogen, Carlsbad, CA, USA), cloned, and then cultured as previously described (Ota *et al.*, 2009). Plasmids were extracted from the positive cultures to obtain the inserted fragment, and the nucleotide sequences of the inserted *MPSP* gene fragments were determined by a genetic analyzer (ABI PRISM 3100 genetic analyzer, Applied Biosystems). The sequences were then analyzed, according to previously documented methods (Ota *et al.*, 2009). Briefly, after multiple alignments with the ClustalW online software program, a phylogenetic tree was constructed, based on a neighbor-joining branching pattern (Perrière *et al.*, 1996). Additionally, a bootstrap test was employed to determine the reliability of the branching pattern of the phylogenetic tree (Felsenstein, 1985).

The phylogenetic analysis identified 3 different genotypes, *i.e.*, types 1, 2 and 5 (Fig. 3), and identified *T. orientalis* types 1 and 2 as the major genotypes in the study area. Type 1 (Chitose type in Japan) has already been reported in China and other countries such as Russia, Japan and Korea (Gubbels *et al.*, 2000), and recently, we indicated the presence of type 1 in Mongolia (Altengerel *et al.*, 2011), Thailand (Altengerel *et al.*, 2011) and Vietnam (Khukhuu *et al.*, 2010). Type 2 (Ikeda type in Japan) has been isolated in China, Japan and Korea (Kakuda *et al.*, 1998; Kim *et al.*, 1998; Ota *et al.*, 2009; Yokoyama *et al.*, 2011). Type 5 was initially reported in Japan, Korea, and very recently in China (Jeong *et al.*, 2010; Kakuda *et al.*, 1998; Kim *et al.*, 1998; Liu *et al.*, 2011; Yokoyama *et al.*, 2011). In addition, type 5 has recently been isolated in Mongolia (Altengerel *et al.*, 2011), Thailand (Altengerel *et al.*, 2011) and Vietnam (Khukhuu *et al.*, 2010).

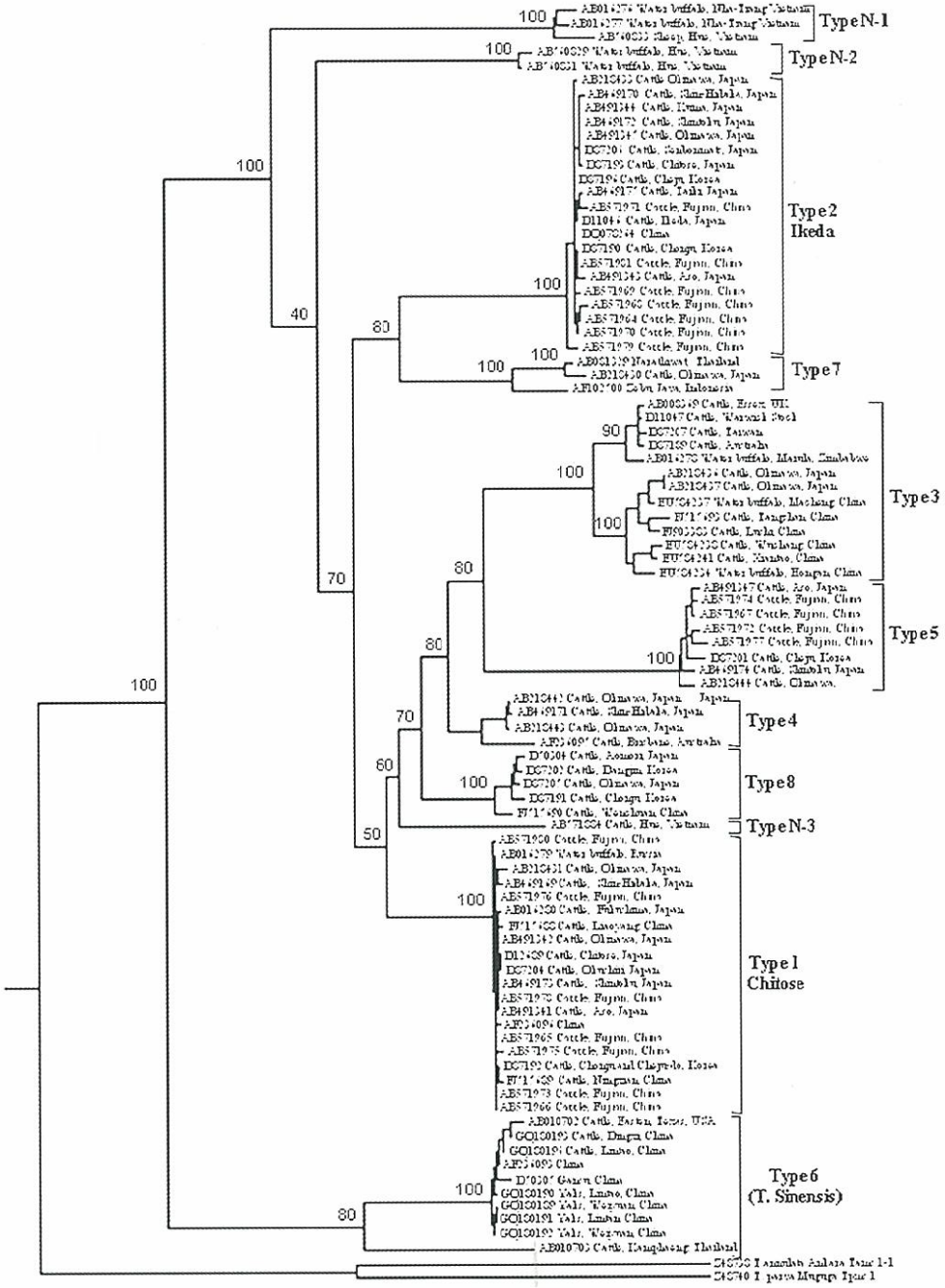

Figure 3: Phylogenetic analysis of *T. orientalis*. Previously reported and currently generated *MPSP* gene sequences were used for the tree construction. The GenBank accession numbers for nucleotide sequences determined in the present study are in bold type.

The results of our present study clearly indicate the presence of 3 different *T. orientalis* genotypes in the sample population, but a large-scale analysis of bovine and/or tick populations is required to generalize the findings of this study. Compared with the other *T. orientalis* genotypes, type 2 (Ikeda type) has been described as a virulent genotype in Japan (Kamau *et al.*, 2011). Because the circulating blood of the bovine population of Fujian province was found to harbor type 2 as one of the major genotypes, the cattle industry in the study area may be susceptible to economical losses due to *T. orientalis* infection. Therefore, an integrated control and preventive strategy must be developed to minimize disease incidence among the cattle bred in Fujian province, China.

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