NOTE *Parasitology*

Genetic Diversity of Benign *Theileria* Parasites of Cattle in the Okinawa Prefecture

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ABSTRACT. Benign *Theileria* parasites of cattle distributed in the Okinawa prefecture were characterized by allele-specific polymerase chain reaction (PCR) and DNA sequence analysis of the major piroplasm surface protein (MPSP) gene. Using universal or allele-specific primer sets, parasite DNA was amplified in 31 out of 48 blood samples obtained from beef cattle. Among the positive cases, mixed infections involving various combinations of I-, C-, and B-type parasites were detected in 24 (77.4%) samples. Phylogenetic analysis based on the *MPSP* gene sequences revealed that parasites with the MPSP types 1–5 and 7, exist within the Okinawa prefecture.
KEY WORDS: genomic diversity, Okinawa, *Theileria*.

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Benign *Theileria* species of cattle transmitted by ticks, which occasionally cause anemia and icterus, are distributed worldwide [1, 8, 13, 18]. The taxonomy and nomenclature of this group of *Theileria* parasites remain to be assigned [3]. In the literature, parasites identified in Japan, Korea, Russia and Australia are described as *Theileria sergenti* or *Theileria buffeli*, which are synonyms *Theileria orientalis* [16]. Kubota *et al.* [14] have developed allele-specific PCR for the major piroplasm surface protein (MPSP) gene, using specific primers to differentiate parasite populations. With this method, molecular epidemiological studies on *Theileria* parasites of the three main islands in Japan (Hokkaido, Honshu and Kyushu) were conducted. The data show that the major populations include parasites with Chitose (C) and Ikeda (I) types of *MPSP* gene, and cattle usually harbor mixed parasite populations involving both-types [14].

The Okinawa prefecture, consisting of several islands, is located in the subtropical region in Japan where cattle are pastured all year round. Asian buffalos (*Bos bubalis*) distributed around a number of these islands are often infected with apathogenic *Theileria* species closely related to *T. orientalis*, and sometimes referred to as *T. buffeli*. Serious babesiosis and anaplasmosis cases were frequent before eradication of the vector tick species, *Rhipicephalus* (*Boophilus) microplus*, in 1999. Benign *Theileria* parasites morphologically indistinguishable from *T. orientalis* observed in the main islands of Japan are additionally prevalent, as reported from microscopic and direct fluorescent antibody (FA) experiments [5, 6]. However, detailed molecular biology analyses of the parasite strains are yet to be performed. In this study, we characterize field isolates of *Theileria* parasites from cattle in Okinawa using allele-specific PCR and DNA sequence analysis of the *MPSP* gene.

Blood samples of 48 Japanese Black calves (<10 months of age) were collected from 4 islands (Ishigaki, Iriomote, Kuroshima and Yonaguni) located in the southern part of Okinawa in 2002. Cattle in the majority of farms on these islands are pastured, and vector tick species, *Haemaphysalis longicornis*, are active all year round. The inspected calves were born and reared in individual farms, and clinically healthy at the time of sampling. Giemsa-stained blood samples were examined under a microscope. The packed cell volume (PCV) was measured with an Auto Cell Counter (Nihon Denko Co, Japan). Parasite DNA was extracted from whole blood samples using the SepaGene kit (Viogene, U.S.A.), according to the manufacturer's protocol. The primers used for amplification of parasite DNA are specified below. The first set, comprising 5'-CACGCTATGTTGTCCAAGAG-3'(Ts-U) and 5'-TGTGAGACTCAATGCGCCTA-3' (Ts-R), was used to amplify the gene encoding MPSP p32 of *T. orientalis* [18]. The second set, 5'-TATGTTGTCCAAGAGATCGT-3' and 5'-TGAGACTCAGTGTCGCCTAGA-3', was specific for the gene encoding MPSP p33/34 of *T. orientalis* and *T. buffeli* [10]. A combination of Ts-U and Thai 3'–510 (5'-CGACGAAGTCATAGAGGCAC-3') was employed for the *MPSP* gene of Thai-type parasites, which was not obtained with Ts-U and Ts-R primers [8]. Three other sets of primers were used in allele-specific PCR to distinguish between parasite populations within the *T. orientalis* group. For allele-specific PCR, three sense primers, Ts-I: 5;'AAGGATCCGTCTCTGCTACCGCCGC-3', Ts-C: GCGGATCCTCATCGTCTCTGCAACT-3', or Ts-B: 5'-GCGGATCCG CTCTGCAACCGCAGAG-3', together with the anti-sense primer, Ts-R, were employed to amplify the *MPSP* genes of two *T. orientalis* stocks (I and C), and Warwick stocks of *T. buffeli* (B), respectively [14, 15]. PCR was performed with 1 μl of DNA template in 50 μl of reaction mixture containing 10 mM Tris-HCl, pH8.3; 50 mM KCl; 2 mM $MgCl_2$; 200 μM dNTPs; 0.5 μM of each oligonucleotide primer; and 1.25 units of *Taq* DNA polymerase (Takara, Japan). All PCRs were performed in an automatic DNA thermal cycler (Model TP600 Takara). The following conditions were employed: denaturation for 1 min at 94°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C for 30 cycles, with an additional 4 min at 72°C. Next, 5–10 μl aliquots of

Table 1. Analysis of *Theileria* parasite population in Okinawa by allele-specific PCR

Region (Island)	Farm	No. of samples	Microscopic examination	Result with primer set for					
				p33/34[a)]	p32[b)]	Thai[c)]	I[d)]	c[d)]	B[e)]
Ishigaki	K	8	+	+	+	–	+	+	+
		1	+	+	+	–	+	+	–
		1	+	+	–	–	+	+	+
	T	1	+	+	+	–	+	+	–
		1	+	+	+	–	–	+	–
		2	–	+	–	–	–	–	–
		1	+	–	–	–	–	–	–
		5	–	–	–	–	–	–	–
Iriomote	Y	8	+	+	+	–	+	+	+
		1	+	+	+	–	+	+	+
		1	+	–	+	–	+	+	+
Kuroshima	E	10	–	–	–	–	–	–	–
Yonaguni	H	1	+	+	+	–	–	+	+
		1	+	+	+	–	–	+	+
		1	+	+	+	–	+	+	–
		2	+	+	+	–	+	–	–
		1	+	+	+	–	–	–	+
		1	+	+	–	–	–	–	–
		1	–	–	–	–	–	–	–
Total (%)		48	30 (62.5)	30 (62.5)	27 (56.3)	0 (0.0)	24 (50.0)	25 (52.1)	22 (45.8)

a) Kawazu *et al.* [10], b) Tanaka *et al.* [18], c) Kakuda *et al.* [8], d) Kubota *et al.* [14], e) Kubota *et al.* [15].

samples were subjected to 1.2% agarose gel electrophoresis. Amplified *MPSP* gene products were cloned into pGEM T Easy vector (Promega, U.S.A.), and the complete nucleotide sequences determined from both strands using ABI PRISM 3100 or 3700 Genetic Analyzer (Applied Biosystem, U.S.A.) with the BigDys Teriminator Cycle sequencing kit (Applied Biosystem). Nucleotide sequences were applied to a Basic Local Alignment Search Tool (BLAST) on the DNA Data Bank of Japan (DDBJ) database for homology analysis with other *MPSP* gene types. Phylogenetic analyses of *MPSP* genes and deduced amino acid sequences were performed by neighbor-joining [17] using CLUSTAL W [20], and the bootstrap probabilities of each node were calculated with 1,000 replications.

Among 48 blood samples collected from five farms on four islands in Okinawa, *Theileria* parasites were identified in 31 from four farms on three islands by PCR using primer sets specific for the p33/34 or p32 genes. However, 30 samples were microscopically positive (Table 1). No products were observed upon PCR with the primer set for Thai-type MPSP in all cases. Allele-specific PCR disclosed that 24, 25, and 22 field isolates contained I-, C-, and B-type MPSP, respectively (Table 1). Moreover, 77.4% of the infected cows displayed mixed infections comprising these three types. Interestingly, the majority of parasites identified in other regions of Japan involve the I- and C-types, while B-type is specifically detected in cattle imported from Australia [14]. In contrast, high rates of infection by the B-type parasite are observed in Okinawa. Previously, one Okinawa isolate exhibited an SDS-PAGE protein profile similar to that of *T. buffeli* (Zakimi *et al.*, unpublished data). According to epidemiological studies, I-type, rather than C- and B-type parasites, tends to be associated with clinical theileriosis [9, 21]. However, no obvious correlations between MPSP types and clinical signs as measured by PCV were evident in this study, (data not shown). In fact, few clinical cases of theileriosis have been reported in Okinawa, possibly due to natural resistance against the parasite. The Japanese Black cattle variety is reportedly more resistant than Friesian cattle [19].

Products amplified using either allele-specific or p33/34 primer sets were cloned. In total, 18 clones were sequenced. The *MPSP* sequence homology among the clones ranged from 81.2% to 100% at the nucleotide level, and 81.6% to 100% at the amino acid level, respectively. Figure 1 depicts a phylogenetic tree for the *MPSP* sequences of 7 typical clones obtained from Okinawa isolates and reference stocks or isolates. Kim *et al.* [13] originally proposed that benign *Theileria* parasites in East Asia can be divided into six types (types 1–6), depending on the *MPSP* gene. Recently, an additional type, 7 was identified in Japan [12]. In the present study, we detect the MPSP types 1–5 and 7, in the Okinawa prefecture (Fig. 1). In particular, clones Okinawa C14-4 (C1-3) and C-9-3 are clustered in types 3 and 5 respectively, which has not been reported in cattle from Japan to date.

In conclusion, parasites in Okinawa islands display higher level of *MPSP* gene diversity, compared to isolates from the main islands of Japan. This genetic complexity may be due to the geographical, agricultural and metrologi-

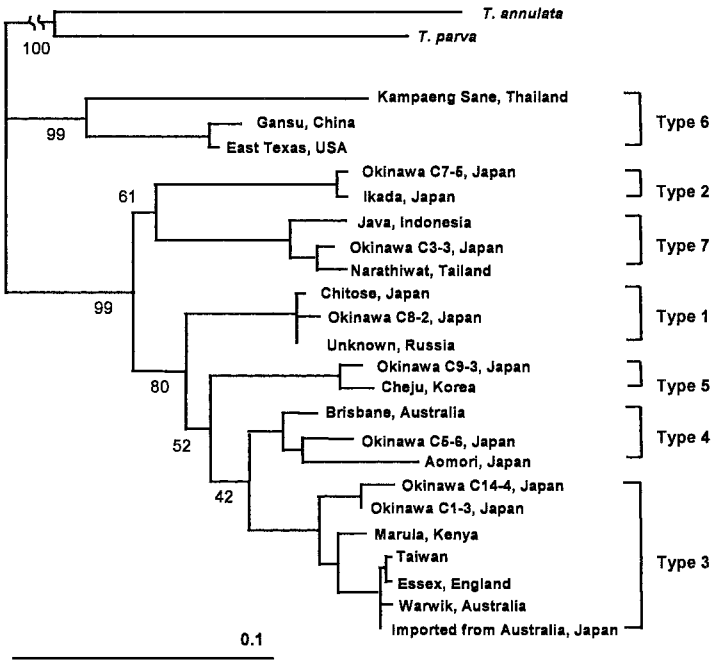

Fig. 1. The phylogenetic tree was constructed by the neighbor-joining method using the predicted sequence data of *MPSP* of *Theileria* parasites from the DNA bank, and seven representative Okinawa isolates. *T. annulata* and *T. parva* were employed as outgroups in the tree. The scale bar indicates a 10% amino acid difference. Bootstrup values are shown at branch nodes classified into each type. GenBank accession numbers of the MPSP sequences are: *T. annulata* (Z48738), *T. parva* (Z48740), Kampaeng Sene, Thailand (AB010703), Gansu, China (D50305), East Texas, U.S.A. (AB010702), Ikeda, Japan (D11046), Java, Indonesia (AF102500), Narathiwat, Thailand (AB081329), Chitose, Japan (D12689), unknown, Russia (AB016279), Cheju, Korea (D87198), Brisbane, Australia (AF236095), Aomori, Japan (D50304), Marula, Kenya (AB016278), Taiwan(D87207), Essex, England (AB008369), Warwick, Australia (D11047) imported from Australia, Japan (D87189), Okinawa C7–5, Japan (AB218433), C3–3 (AB218430), C8–2 (AB218431), C9–3 (AB218444), C5–6 (AB218442), C14–4 (AB218436), and C1–3 (AB218438).

cal uniqueness of the Okinawa islands. Firstly, Asian water buffalo (*Bubalus arnee*) used for drafting in Okinawa is a carrier of apathogenic *Theileria parasites*, which coexist with *Theileria* orientalis in cattle. Gubbels *et al*. proposed that all known *T. buffli*-like isolates originate in a disperse group of buffalo-derived parasites that have adapted to cattle [4]. The subtropical climate also allows the flourishing of a variety of tick species that do not inhabit the main islands of Japan. Among these, *Haemaphysalis mageshimaensis*, an effective vector for *T. orientalis* [2], may function in transmitting the benign *Theileria* parasite, and maintaining genetic diversity.

The population structure of pathogens is important in relation to vaccine development, and higher diversity makes this process more difficult. Antigenic differences among MPSP may also cause problems in serological diagnosis [7, 11]. Therefore, a survey on how such a mixed population is maintained under natural infection cycles between tick vectors and cattle, and changes in population structure during a period of persistent infection is currently in progress.

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