NOTE *Parasitology*

Prevalence of Tick-Borne *Rickettsia* and *Ehrlichia* in *Ixodes persulcatus* and *Ixodes ovatus* in Tokachi District, Eastern Hokkaido, Japan

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ABSTRACT. DNA from 111 ticks collected by flagging in Tokachi district, Eastern Hokkaido, Japan were examined for infection with *Rickettsia* and *Ehrlichia*, by PCR and sequencing methodology. For *Rickettsia*, analysis of the partial sequence of the citrate synthase gene was successfully performed on 11 DNA samples from *I. persulcatus*, and 7 of them showed 99.8% identical with *Rickettsia helvetica* while the other 4 showed 99.8% identical with '*Candidatus* Rickettsia tarasevichiae'. For *Ehrlichia*, a partial sequence of the 16S rRNA gene detected from *I. persulcatus* was 100% identical with that from *Ehrlichia muris*, and another DNA sample from *I. ovatus* showed 99.8% identical with *Ehrlichia* species detected from *I. ovatus*. The results suggest that the pathogens detected here might be distributed in this area.

KEY WORDS: *Ehrlichia*, *Rickettsia*, ticks.

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Rickettsia, Ehrlichia and *Anaplasma* are major tick-borne rickettsial pathogens that are obligate intracellular, gram-negative bacteria, among which several species cause diseases in humans and other animals, and have a worldwide distribution. In Japan, several *Rickettsia* spp., including *Rickettsia japonica*, *Rickettsia helvetica* and *Rickettsia* sp. AT-1 have been detected from ticks [1, 4, 5]. There have also been several *Ehrlichia* and *Anaplasma* pathogens reported in Japan since 1995 [3, 6, 15, 18]. *Ehrlichia muris*, which is closely related to *E. chaffeensis*, was found in murine hosts, which is thought to be transmitted by *Haemaphysalis flava* and distributed widely in mainland Japan [6, 18]. A new *Ehrlichia* species (EIO) which is closely related to *E. chaffeensis* was isolated from *Ixodes ovatus* ticks in Japan [15]. More recently, *Anaplasma phagocytophilum* was detected in *Ixodes persulcatus* and deer [7, 11]. This wide variety of rickettsial and ehrlichial species in Japan has been examined only in limited localities.

Tokachi district is a rural area located in the southeast part of Hokkaido. It is surrounded by high mountains, with the Taisetsu Mountains to the north and the Hidaka Mountains to the west, and the Pacific Ocean is located to the south. The climate corresponds to the subarctic zone, being very cold in winter. The main tick species found in this area are *Ixodes persulcatus* and *Ixodes ovatus* [19]. However, little information is available about the distribution of rickettsial and ehrlichial pathogens in this area. Thus in this study, the detection and analysis of rickettsial and ehrlichial pathogens (*Rickettsia, Ehrlichia* and *Anaplasma*) from the ticks collected by flagging in Tokachi district, Eastern Hokkaido, Japan was attempted using molecular methods including PCR and sequence analysis.

Seven cattle pasture lands (A, B, C, D, E, F and G) and four woodlands (H, U, J and K) in Shintoku, Shikaoi, Shimizu, Memuro, Otofuke and Ikeda towns, and Obihiro City, in Tokachi district, Eastern Hokkaido, Japan were selected for this study. A total of 111 ticks were collected by flagging from May to August 2006, and identified morphologically. These ticks included 67 *Ixodes persulcatus* (27 males and 40 females), 41 *Ixodes ovatus* (16 males, 22 females and 3 nymphs) and 3 *Haemaphysalis douglasi* (3 nymphs). Total DNA was extracted from each tick with a method described previously [2]. To detect *Rickettsia* gene fragments from the ticks, *gltA* based-PCR with a genus-specific primer set, RpCS.877p and RpCS.1273r, was used [14]. The ehrlichial DNA samples were screened with PCR using primers EHR16SD and EHR16SR, which amplify a 345-bp fragment of the 16S rRNA gene found in the genera *Ehrlichia, Anaplasma, Neorickettsia* and *Wolbachia* of the family *Anaplasmataceae* [12]. The PCR amplification was performed in a 25-μl reaction mixture containing 5 μl of each DNA template. The PCR was carried out under the following conditions: 35 cycles of denaturation (94°C, 60 sec), annealing (54°C for *Rickettsia* and 55°C for *Ehrlichia*, 60 sec) and extension (72°C, 90 sec). PCR products were electrophoresed at 100 V in 2% agarose gels (Wako Chemicals Ind) for 30 min, stained with ethidium bromide, and verified by UV illumination. When the samples revealed appropriate amplicons for *Ehrlichia*, another PCR was also carried out with primer set fD1/EHR16SR, which amplifies a longer sequence of the 16S rRNA gene, including a divergent region near the 3' end, for further phylogenetic studies for *Ehrlichia* [12]. The PCR product with a positive reaction was purified using the QIA PCR purification kit (QIAGEN, Germany). Direct sequencing of the PCR products and analysis of the sequences obtained were performed as described previously [2]. The sequence data of the PCR products was analyzed using the BLAST 2.0 program (National Center for Biotechnology Information) for homology searching. The determined sequences were then analyzed for phylogenetic relationships with the other sequences registered in GenBank. Multiple alignment anal-

ysis, the determination of pair-wise percent identities of the sequences, distance matrix calculations and the construction of phylogenetic trees by neighbour-joining were all performed with the ClustalW program version 1.8 in the DNA data bank of Japan. Tree figures were generated using the Tree View program version 1.6.6. The GenBank accession numbers of the gltA gene sequences of the other species used to analyze the data are as follows: *R. prowazekii*, M17149; *R. japonica*, U59724; *R. akari*, U41752; *R. felis*, U33922; *R. slovaca*, U59725; *R. conorii*, U59730; *R. cadada*, U59713; *R. honei*, AF022817; *R. helvetica*, U59723; *R. australis*, U59718; *R. montana*, U74756; *R. massiliae*, U59719; '*Candidatus* Rickettsia tarasevichiae', AF503167; *Rickettsia* sp. AT1, AB114796; *R. sibirica*, U59734; *Rickettsia* mongolotimonae, U59731. The GenBank accession numbers of the 16S rRNA gene sequences used to construct phylogenetic trees and to analyze percent identities were as follows: *E. muris* AS145, U15527; *E. muris* I268, AB013008; *E. muris* NA-1, AB013009, *E. muris* FN2619, AB196302 and *E. muris* Russia, AY587608; EIO HF565, AB024928; EIO Anan, AB028319; EIO HI-2000, AF260591; EIO Shizuoka-36, AB178793; *E. canis*, M73221; *E. chaffeensis*, M73222; *E. ewingii*, M73227; *E. ruminantium*, U03777; *A. phagocytophilum*, M73220; *A. platys*, M82801; *A. marginale*, AF414871; *Wolbachia pipientis*, AF179630; *N. risticii*, M21290; *R. prowazekii*, M21789.

The results are summarized in Table 1. For *Rickettsia*, 439-bp of the rickettsial *gltA* sequences (excluding the primer region) were successfully isolated from 11 *I. persulcatus* samples collected from one cattle pasture land (B) and 3 woodlands (H, I and J). The partial *gltA* sequences of 7 DNA samples obtained from the ticks, B-IP13f, B-IP17m, H-IP1f, H-IP5f, I-IP7f, I-IP8f and I-IP10f, were identical to each other and showed the highest homology (99.8%) with *R. helvetica*. The sequences from the other 4 ticks (B-IP16m, B-IP18f, H-IP3f and J-IP21f) were also identical to each other and showed the highest homology (99.8%) with '*C.* R. tarasevichiae'. The phylogenetic tree thus revealed that 7 samples belong to the same clade with *R. helvetica*, and the other 4 with '*C.* R. tarasevichiae' (Fig.1).

Formerly *R. helvetica* had been known to exist only in European countries; however, several evidences have been accumulated to support the wide distribution of *R. helvetica* in Japan, from Hokkaido to Kyusyu region [1, 2, 4]. In Hokkaido, *R. helvetica* was previously detected from *I. persulcatus* in the central mountainous area [1]. The present study is the first detection of *R. helvetica* from ticks in eastern Hokkaido. Although *R. helvetica* is thought to be moderately pathogenic in general, the first human case of *R. helvetica* was reported in Fukui Prefecture recently [10]. The present data also suggest that '*C.* R. tarasevichiae' might be established in Tokachi district, which were originally detected from the ticks in Russia [16]. Although the pathogenicity of '*C.* R. tarasevichiae' is not clear at present, notice should be taken regarding a possibility of rickettsial

Table 1. Numbers of ticks collected in this study and the rates of prevalence of *Rickettsia* and *Ehrlichia* spp. by PCR and sequence methodology

Location	*I. persulcatus*			*I. ovatus*		*H. douglasi*
	F	M	N	F	M	N
(Cattle pasture)						
A	0/1	–	–	1/6 (1 EIO)	0/5	0/1
B	2/6 (1 Rh) (1 Rt)	2/3 (1 Rh) (1 Rt)	–	–	–	–
C	–	0/2	–	–	–	–
D	–	0/1	–	0/14	0/11	0/1
E	–	–	–	0/3	–	–
F	–	0/1	–	0/2	–	–
G	–	0/1	–	–	–	–
(Woodlands)						
H	3/5 (2 Rh) (1 Rt)	–	0/1	–	–	–
I	3/10 (3 Rh)	0/12	–	–	–	–
J	2/10 (1Rt) (1 Em)	0/9	–	–	–	–
K	0/2	0/3	–	–	–	–

Data are shown as numbers positive for *Rickettsia* or *Ehrlichia*/numbers of ticks examined. The numbers in parentheses show the numbers of each species identified. .

F: Female, M: Male, N: Nymph, Rh: *R. helvetica*, Rt: '*C.* R. tarasevichiae', Em: *E. muris*, EIO: *Ehrlichia* species detected from *Ixodes ovatus*.

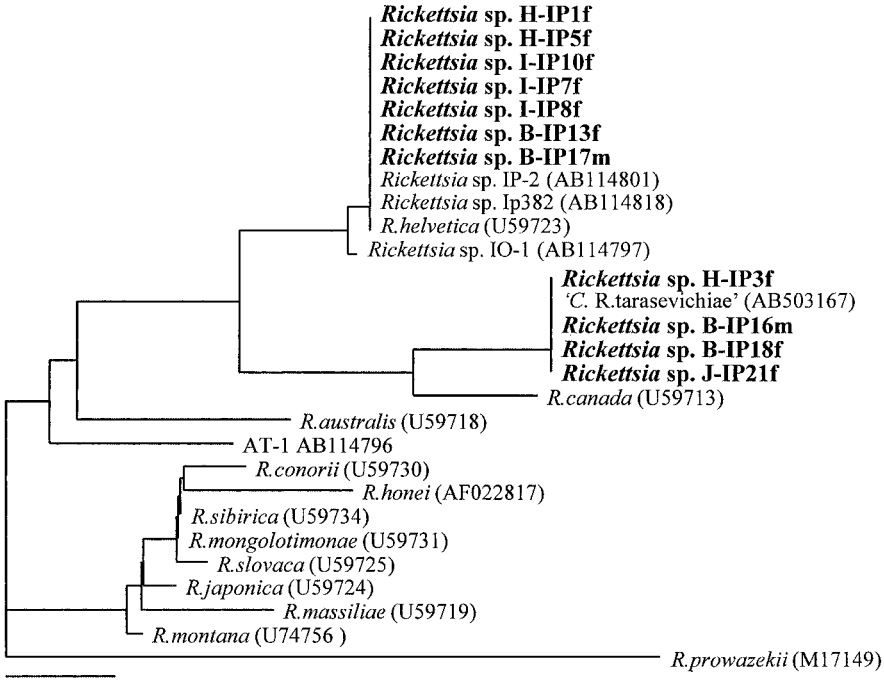

Fig. 1. Phylogenetic relationship of various *Rickettsia* spp. based on the nucleotide sequences of the citrate synthase gene (*gltA*). The scale bar indicates genetic distance (0.01 substitution/site). The *Rickettsia* spp. detected in this work are highlighted.

infection in both human and animals.

An appropriate screening PCR product of 345-bp was obtained from 12 *I. persulcatus* and 2 *I. ovatus*. Homology analysis of the sequences revealed that the sequences from one *I. persulcatus* (J-IP22f) and another *I. ovatus* (A-IO2f) were closely related to those of the genus *Ehrlichia*. However, ehrlichial DNA was not determined in the remaining 12 samples. Subsequent PCR using fD1/EHR16SR, which amplifies a longer sequence of the 16S rRNA gene, including a divergent region near the 3' end, produced a product of approximately 790-bp. Gene analysis of the longer PCR products revealed that the partial 16S rRNA gene sequence detected from the tick J-IP22f was 100% identical with that of *E. muris* AS145. The sequence detected from the tick A-IO2f was similar to that of EIO. This sequence had one nucleotide difference (99.9% identical) from those of EIO strain HF565 from Fukushima Prefecture, EIO strain Shizuoka-36 from Shizuoka Prefecture and EIO strain HI-2000 from Yamaguchi Prefecture, and 2 differences (99.72%) from EIO Anan strain from Tokushima Prefecture. The phylogenetic tree is shown in Fig. 2. *Ehrlichia* spp. J-IP22 and A-IO2 belong to the same clade with *E. muris* and EIO, respectively.

E. muris has also been isolated or detected in Tokyo, Shizuoka and Nagano Prefectures [6, 9]. We have reported here the first detection of *E. muris* DNA in the northern part of Japan. The vector tick of *E. muris* is thought to be *Haemaphysalis flava* in mainland Japan, but *E. muris* DNA was detected from a female of *I. persulcatus* in this study. The present result supports the finding of Naitou *et al*. [9], suggesting that *I. persulcatus* could be an alternative vector of *E. muris*. *E. muris* DNA has also been detected from *I. persulcatus* ticks in Russia [13, 17].

EIO might be related to the *Ehrlichia* species recently isolated from *I. ovatus* [15]. This *Ehrlichia* was isolated from the laboratory mice by intraperitoneal inoculations of homogenates of adult *I. ovatus* collected from vegetation in Aomori, Fukushima and Tokushima Prefectures [15]. The DNA of EIO was also detected from *I. ovatus* in Yamaguchi Prefecture and mice in central Hokkaido [3, 8]. The distribution of both ehrlichial pathogens might be wider than previously known.

The results obtained in our present study suggest that *R. helvetica*, '*C.* Rickettsia tarasevichiae', *E. muris* and EIO are distributed in Tokachi district, eastern Hokkaido. As the epidemiology of these pathogens is not completely understood, more epidemiological studies are required to clarify the reservoir animals, infection rates of ticks and distribution of the pathogens.

Nucleotide sequence accession number. The *gltA* sequences of *Rickettsia* sp. obtained from H-IP1f, H-IP5f, I-IP10f, I-IP7f, I-IP8f, B-IP13f, B-IP17m, H-IP3f, B-IP16m, B-IP18f and J-IP21f have been registered under the accession numbers DQ887269 to DQ887279, respectively. The 16S rRNA gene sequences of *Ehrlichia* sp. detected from ticks A-IO2f and J-IP22f have been deposited in the GenBank database under the accession numbers DQ887267 and DQ 887268, respectively.

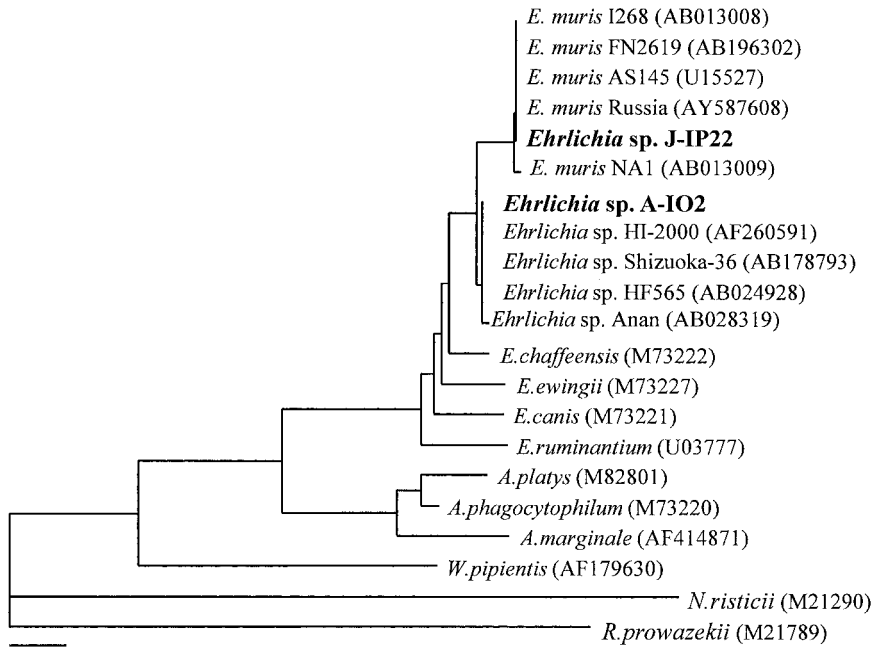

Fig. 2. Phylogenetic relationship of various *Ehrlichia* and *Anaplasma* spp. based on the nucleotide sequences of the 16S rRNA gene. The scale bar indicates genetic distance (0.01 substitution/site). The *Ehrlichia* spp. detected in this work are highlighted.

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