

## Short Communication

# Detection of Ehrlichial DNA in Small Rodents Captured in a Woodland Area of Hokkaido, the Northernmost Island of Japan, Where Lyme Disease Is Endemic

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**SUMMARY:** The ehrlichial gene was detected in small rodents trapped in a Lyme disease-endemic area in Hokkaido, the northernmost island of Japan. Primer pairs of 16S rDNA targeting the genus *Ehrlichia* and other regions of the 16S rDNA specific for *E. chaffeensis* and *E. muris* were used for identification. The DNA fragment specific for 16S rDNA of *Ehrlichia* spp. was detected in 4 of 94 *Apodemus speciosus* mice (positive rate: 4.3%) and 5 of 73 *Clethrionomys rufocanus bedfordiae* mice (positive rate: 6.8%). The nucleotide sequence of the amplified 16S rDNA fragment was most similar to those of *E. muris*-like *Ehrlichia*, *Ehrlichia* spp. HF565 and Shizuoka-36, originating in the northern and central parts of Japan. In phylogenetic analysis based on 16S rDNA sequences, the northern, central and western groups of *E. muris*-like *Ehrlichia* from a cluster with microorganisms of the *E. muris* group. These results suggest that there are a group of *E. muris* microorganisms and a group of *E. muris*-like microorganisms in Japan.

*Ehrlichia muris* strains have been isolated from wild mice (*Eothenomys kageus*) in Aichi Prefecture and from other wild mice, *Apodemus speciosus* and *A. argenteus*, in Tokyo in Japan (1,2). Also, *E. muris* and *E. muris*-like microorganisms have been isolated and/or genetically detected from *Haemaphysalis flava* and *Ixodes ovatus* ticks in several areas of Japan (2-4). These facts suggest that *Ehrlichia* spp., including *E. muris*, are broadly distributed among ticks and small wild rodents in Japan. Several studies have shown a correlation between *Anaplasma* spp. microorganisms (formerly known as granulocytic *Ehrlichia*) and *Borrelia burgdorferi*, the causative agent of Lyme disease in humans, small rodents and ticks (5-7). Co-infection with *B. burgdorferi* and *E. chaffeensis* in *Ixodes* ticks has also been reported (8). The purpose of the present study was to detect ehrlichial DNA in small rodents captured in an area in which Lyme disease is endemic and to analyze its sequence.

A total of 167 wild mice (94 *A. speciosus* and 73 *Clethrionomys rufocanus bedfordiae*) were captured in two woodland areas of Hokkaido (latitude: 42°48'N, longitude: 142°08'E and latitude: 42°49'N, longitude: 142°08'E) between October 1997 and September 1998. The woodland is located in an area in which Lyme disease is endemic (9) (personal communication with Dr. E. Isogai, School of Dentistry, Health Sciences University of Hokkaido). The mice were anesthetized with chloroform and then euthanized by cardiac puncture to aseptically remove their spleens. The livers were stored at -80°C until use. Genomic DNA was extracted from each spleen by the SDS-proteinase K-phenol extraction method (10). The extracted DNA samples were used as a template for amplification of ehrlichial DNA by PCR methods. PCR assays using an ECC/ECB primer pair were performed for the screening of *Ehrlichia* spp. (11).

Subsequently, DNA samples positive for ECC/ECB-PCR were subjected to other PCR assays for amplification of *E. chaffeensis* or *E. muris* 16S rDNA using primers ECC1 (5'-TAATACTGTATAATCCCTGC-3') and ECBR (5'-GTTTGC CGGGACAACCTTCTA-3'); these assays were performed according to advice given by Dr. M. Kawahara, Nagoya City Public Health Research Institute, Aichi, Japan. The ECC/ECB-PCR amplicon was sequenced and analyzed with the 36 reference sequences listed in Table 1.

The DNA fragment specific for 16S rDNA of *Ehrlichia* spp. was detected in 4 of 94 *A. speciosus* mice (positive rate: 4.3%) and 5 of 73 *C. rufocanus bedfordiae* mice (positive rate: 6.8%). All DNA samples positive for ECC/ECB-PCR were also positive for ECC1/ECBR-PCR. The difference between the positivity of *A. speciosus* mice and that of *C. rufocanus bedfordiae* mice was not statistically significant. Of the 9 positive samples, 2 samples obtained from *C. rufocanus bedfordiae* mice were sequenced, and the 481-bp sequences of the fragments of *Ehrlichia* spp. were found to be identical (Nos. 184 and 192). One of the DNA fragments was provisionally named HoCr184. The sequence of HoCr184 was most similar to that of *Ehrlichia* sp. HF565 and that of *Ehrlichia* sp. Shizuoka-36 originating in Fukushima and Shizuoka Prefectures in the northern and central areas of Japan (99.57 to 99.56% similarity, Figs. 1 and 2). The sequence of HoCr184 also showed high similarity to *Ehrlichia* sp. Anan and *Ehrlichia* sp. HI-2000 originating in Tokushima and Yamaguchi Prefectures in the western area of Japan (99.17 and 99.34% similarity, Figs. 1 and 2). On the other hand, similarity between the sequences of HoCr184 and *Anaplasma* spp. ranged from 91.84 to 93.14% (Fig. 1). In a phylogenetic tree, HoCr184 formed a cluster with *Ehrlichia* spp. HF565, Anan, HI-2000 and Shizuoka-36, as well as with four strains of *E. muris* (Fig. 1). In the cluster, HoCr184 was categorized into a subgroup consisting of *Ehrlichia* spp. HF565, Anan, HI-2000 and Shizuoka-36, and branched with another subgroup containing the four strains of *E. muris* (55.0% bootstrap value; Fig. 1). The cluster including HoCr184 branched

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Table 1. *Ehrlichia* spp., *Anaplasma* spp. and *Neorickettsia* microorganisms used for homology and phylogenetic analyses

Microorganisms	Geographical origin (Prefecture)	GenBank accession no.
<i>Ehrlichia</i> sp. Anan	Japan (Tokushima)	AB028319
<i>E. sp.</i> HI-2000	Japan (Yamaguchi)	AF260591
<i>E. sp.</i> Shizuoka-36	Japan (Shizuoka)	AB178793
<i>E. sp.</i> HF565	Japan (Fukushima)	AB024928
<i>E. sp.</i> EH1087	Japan (Miyazaki)	AY309971
<i>E. sp.</i> Ehf669	Japan (Saitama)	AY309969
<i>E. sp.</i> EH727	Japan (Shizuoka)	AY309970
<i>E. sp.</i> TS37	unpublished	AB074459
<i>E. sp.</i> Eht224	Niger	AF311968
<i>E. sp.</i> EBm52	Thailand	AF497581
<i>E. sp.</i> Tibet	China	AF414399
<i>E. sp.</i> Germishuys	South Africa	U54805
<i>E. muris</i>	Russia	AF312907
<i>E. muris</i> NA1	Japan (Aichi)	AB013009
<i>E. muris</i> AS145	Japan (Aichi)	U15527
<i>E. muris</i> I268	Japan (Tokyo)	AB013008
<i>E. canis</i> Kagoshima1	Japan (Kagoshima)	AF536827
<i>E. canis</i> Gzh982	China	AF162860
<i>E. canis</i> Oklahoma	U.S.A.	M73221
<i>E. chaffeensis</i> St. Vincent	U.S.A.	U86665
<i>E. chaffeensis</i> Arkansas	U.S.A.	AF416764
<i>E. chaffeensis</i> 91HE17	U.S.A.	U23503
<i>E. ruminantium</i> Omatjenne	Namibia	U03776
<i>E. ruminantium</i> Crystal Springs	Zimbabwe	X61659
<i>E. ruminantium</i> Welgevonden	South Africa	CR767821
<i>E. ruminantium</i> Gardel	Guadeloupe	CR925677
<i>E. ruminantium</i> Senegal	Senegal	X62432
<i>E. ewingii</i>	U.S.A.	M73227
<i>E. ewingii</i> 95E-9-TS	U.S.A.	U96436
<i>Anaplasma platys</i> Okinawa1	Japan (Okinawa)	AY077619
<i>A. phagocytophilum</i> group HGE agent	U.S.A.	AF189153
<i>A. phagocytophilum</i> group HGE agent PL59	U.S.A.	U02521
<i>A. phagocytophilum</i> group <i>A. equi</i>	U.S.A.	M73223
<i>A. phagocytophilum</i> group LGE agent	U.S.A.	AF241532
<i>A. phagocytophilum</i> group USG3	U.S.A.	AY055469
Candidatus <i>Neorickettsia mikurensis</i>	Japan (Tokyo)	AB074460

with other clusters consisting of other *Ehrlichia* spp. (70.5% bootstrap value; Fig. 1). *Anaplasma* spp. formed another cluster and branched with all of the *Ehrlichia* spp. used in the present study (86.6% bootstrap value; Fig. 1).

We detected the ehrlichial gene not only in *Apodemus* sp. but also in *Clethrionomys* sp. mice captured in the endemic area of Lyme disease in Hokkaido. In a previous study, high seropositivity to Lyme *Borrelia* (16-18%) was shown in a high-risk group of people (forestry workers) in this area (9) (personal communication with Dr. Isogai). To the best of our knowledge, this is the first report of detection of the ehrlichial gene from *Clethrionomys* mice in Japan. Phylogenetic analysis provided evidence that *E. muris*-like microorganisms in Japan can be genetically separated into two groups: a group of *E. muris* strains and another group of *E. muris*-like strains (Fig. 1). However, the sequence obtained in this study does not correspond to the sequences of any strains of *E. muris*-like microorganisms (Fig. 1). *C. rufocanus bedfordiae* is an inhabitant of Hokkaido but is not found in any other parts of Japan, as shown by the zoogeographical border known as Blakiston's line (Fig. 2). The uniqueness of this sequence is probably due to the ecological limitations of this rodent

species in Japan.

*Clethrionomys* spp. mice have been a reservoir of *Anaplasma* spp. in addition to *Peromyscus leucopus* (white-footed mouse), which is a major reservoir of *Anaplasma* spp. microorganisms (12,13). On the other hand, *Apodemus* spp. mice have acted as a reservoir of *E. muris* (2). It remains an enigma whether or not *Apodemus* sp. mice captured in the study area harbor *Ehrlichia* spp. microorganisms, because sequence data were not obtained from this rodent species.

*Clethrionomys* spp. mice are competent hosts of *Ixodes* ticks (12,13), and co-infection with *B. burgdorferi* and *E. chaffeensis*, the etiological agent of human monocytic ehrlichiosis, has been reported in *Ixodes* ticks (8). *E. muris* is closely related to *E. chaffeensis* (14). There have been several reports on the existence of *Anaplasma* spp. microorganisms in areas in which Lyme disease is endemic (7,15). Co-infection with *Anaplasma* spp. and *B. burgdorferi* in *Clethrionomys* spp. mice has also been reported (12). In the present study, we demonstrated that a *Clethrionomys* sp. mouse is an important reservoir of *Ehrlichia* spp. microorganisms as well as *Anaplasma* spp. microorganisms. However, we did not detect any ehrlichial DNA closely related

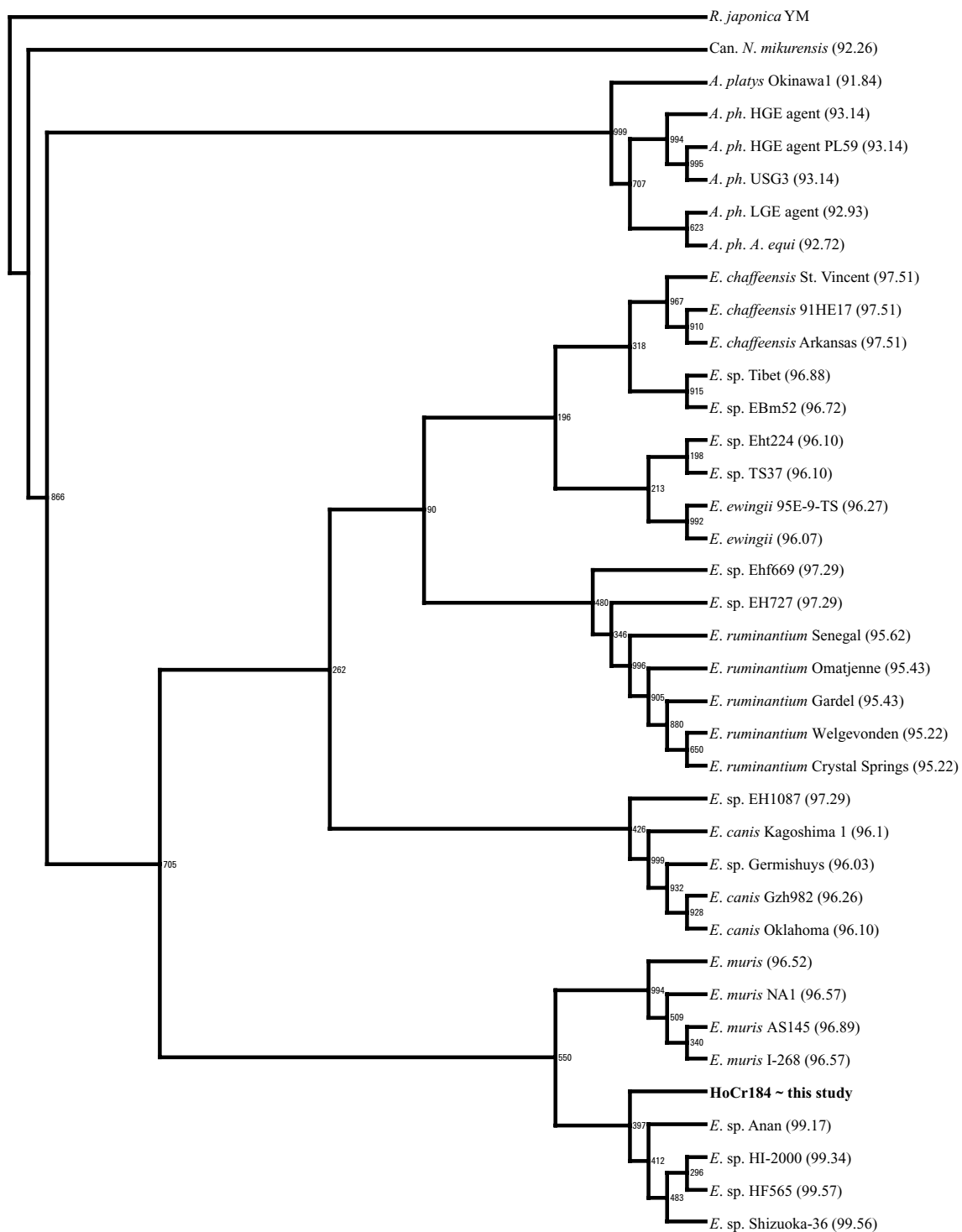


Fig. 1. 16S rDNA-based phylogenetic tree of *Ehrlichia* spp., *Neorickettsia* sp. and *Anaplasma* spp. The percentages of similarity were determined by using the FASTA program, and phylogenetic analysis was performed with the Clustal W program in the DNA Data Bank of Japan (DDBJ; Mishima, Japan). *Rickettsia japonica* YM strain (GenBank accession no. L36213) was used as an outgroup of the neighbor-joining phylogenetic tree. Each numerical value in parenthesis indicates the percentage of homology to the ehrlichial DNA obtained in this study.

to *Anaplasma* spp. microorganisms. The small number of positive samples might have affected the results of this study.

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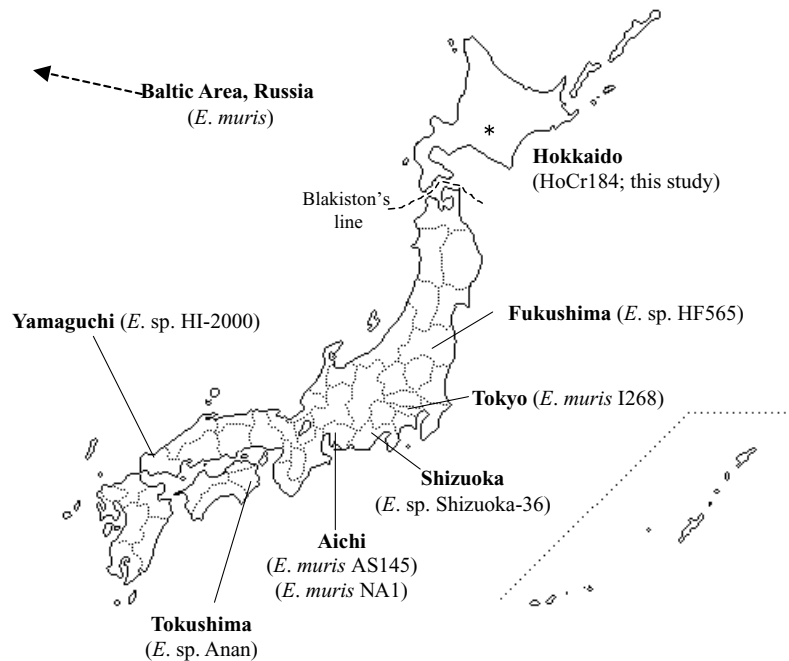


Fig. 2. Geographical origins of *Ehrlichia* spp. closely related to the ehrlichial DNA detected in this study. Letters in boldface indicate the geographical origins of *Ehrlichia* spp. microorganisms. Letters in parentheses indicate the names of *Ehrlichia* spp. microorganisms. The asterisk indicates the sampling site in this study.

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