

Detection of the New *Ehrlichia* Species Closely Related to *Ehrlichia ewingii* from *Haemaphysalis longicornis* in Yonaguni Island, Okinawa, Japan

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ABSTRACT. We collected a total of 206 *Haemaphysalis longicornis* ticks by flagging in pastures in Yonaguni Island, Okinawa, Japan, in April 2008. Four of the 206 tick DNA samples tested were positive in a polymerase chain reaction (PCR) screening for the 16SrRNA gene of Anaplasmataceae. Partial sequences of 4 PCR products were identical to each other. Longer sequences of the 16SrRNA gene were successfully determined in 2 of the 4 tick samples, and the obtained 1,392 bp and 1,300 bp sequences revealed high similarity to the 16SrRNA gene sequences of the validated *Ehrlichia* species, including *Ehrlichia ewingii*, *E. chaffeensis*, and *E. canis* (98.3–98.6%). We also sequenced 1,304 bp of the *groEL* gene from the 2 tick samples, and found that these had the highest similarity to sequences of *E. ewingii* (94.0–94.4%) in the validated ehrlichial species. Based on the 16SrRNA and *groEL* gene sequences, the ehrlichial agents detected in this study were similar to the *Ehrlichia* species detected in Asia and may compose a new *Ehrlichia* species with other *Ehrlichia* species detected in Asia.

KEY WORDS: 16SrRNA, *Ehrlichia*, *groEL*, *Haemaphysalis longicornis*.

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Ehrlichia and *Anaplasma* species are Gram-negative, obligate intracellular bacteria, and are reported as pathogens of emerging diseases [2, 4]. Bacteria in the genera *Ehrlichia*/*Anaplasma* are transmitted by blood-sucking arthropods, including ticks, fleas, and lice, the reservoirs of which are wild mammals such as rodents and deer [5, 15]. Recently, *Ehrlichia*/*Anaplasma* species have been detected in mammals and ticks in Japan [6, 11]. In Yonaguni Island, which is the westernmost island of Japan and lies between the East China Sea and the Pacific Ocean (Fig. 1), *Anaplasma phagocytophilum* and *A. bovis* were detected in peripheral blood samples of grazing cattle in 2006 [11]. However, no survey of ticks in Yonaguni Island for *Ehrlichia*/*Anaplasma* species has been performed, and the relationship between ticks and *Ehrlichia*/*Anaplasma* species in Yonaguni Island is unclear. The present study aimed to reveal how ticks were involved in the transmission of *Ehrlichia*/*Anaplasma* species by using DNA-based tools to test for the presence of *Ehrlichia*/*Anaplasma* species in ticks in Yonaguni Island.

We used flagging methods to collect a total of 206 ticks from 6 grazing pastures on Yonaguni Island in April 2008. Following tick identification, we extracted DNA from each tick using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Tick DNA samples were stored at –30°C until use.

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Tick DNA samples were screened by PCR for 345 bp of the 16SrRNA gene of Anaplasmataceae using the primer pair EHR16SD and EHR16SR [12]. Sequences of positive PCR products were determined through direct sequencing by BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, U.S.A.). Obtained sequences were compared to known sequences using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Tick DNA samples positive for *Ehrlichia*/*Anaplasma* species were used in PCR and sequencing of nearly the full length of the 16SrRNA gene and part of the *groEL* gene. The 16rRNA gene was amplified with primer pairs fD1 and EHR16SR, as well as EHR16SD and Rp2, which amplify the 16SrRNA gene of Anaplasmataceae bacteria [7]. Nested PCR was used to amplify approximately 1,300 bp of the *groEL* gene of *Ehrlichia* species. Primer pairs HS1a and HS6a as well as HS43 and HSVR were used in the 1st and 2nd PCRs, respectively [10]. Sequencing and BLAST search of PCR products were performed as described above. Phylogenetic trees based on 16SrRNA and *groEL* genes were constructed using the ClustalW software program [14] and MEGA4 software [13] by the neighbor-joining method. The 16SrRNA gene sequences of Yonaguni138 and Yonaguni206 were submitted to GenBank with accession numbers HQ697588 and HQ697589, respectively, and the *groEL* gene sequences of Yonaguni138 and Yonaguni206 were submitted with accession numbers HQ697590 and HQ697591, respectively.

All ticks, comprising 31 females and 175 nymphs, were

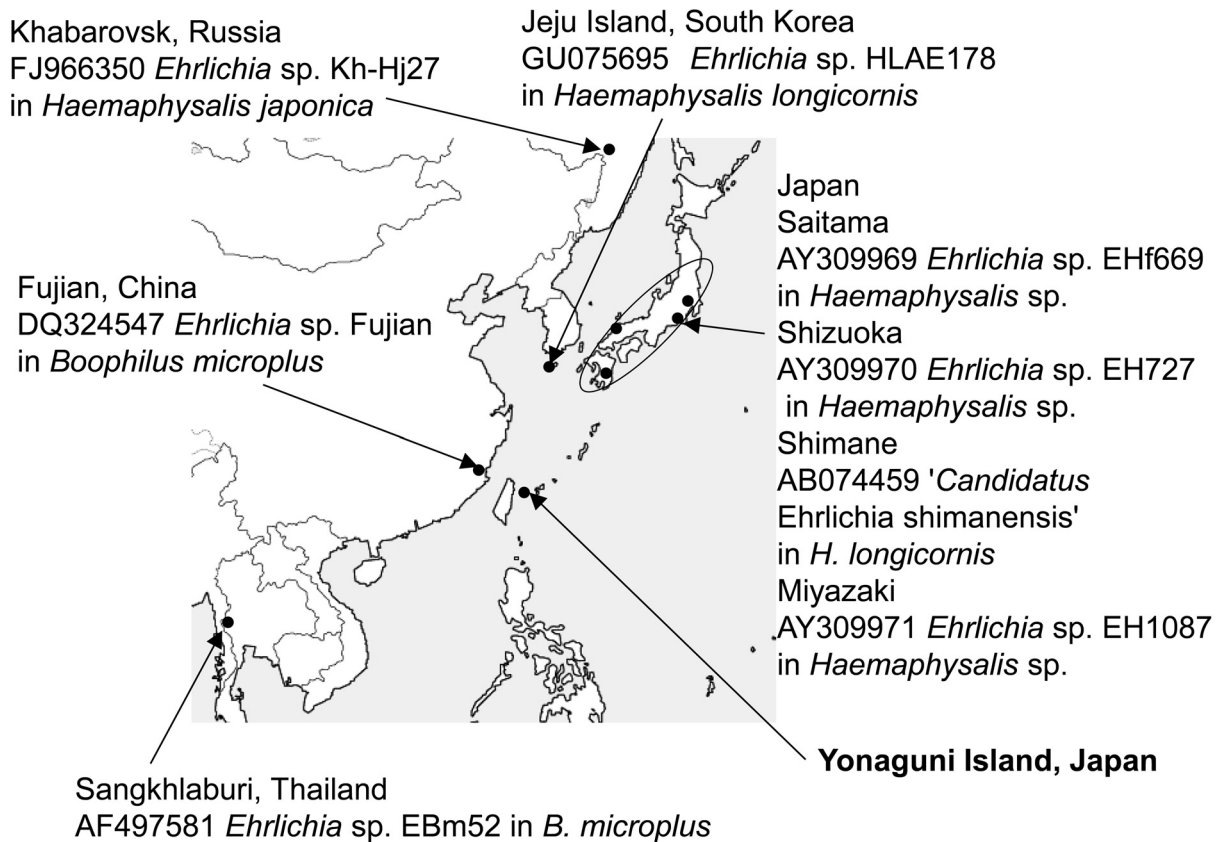


Fig. 1. Geographical relationship of Yonaguni Island with Asian countries. The ehrlichial agents close to *Ehrlichia* species detected in this study, including GenBank accession numbers, tick species and country name, were shown in this figure.

identified as *Haemaphysalis longicornis*, which may be caused by that all ticks were collected from grazing pastures. Two females (numbers 138 and 206) and 2 nymphs of 206 tick DNA samples tested were positive in the PCR screening (1.9%). Sequences (305 bp) of 4 positive PCR products were identical and showed high similarity to the 16SrRNA gene in *Ehrlichia* species. We successfully sequenced the 1,392 bp and 1,300 bp sequences using 16SrRNA primers (numbers 138 and 206, respectively) and 1,304 bp sequences using *groEL* primers in 2 of the female samples. The BLAST search for the obtained sequences using 16SrRNA primers showed the highest similarity (99.8–99.9%) to the 16SrRNA gene of *Ehrlichia* sp. HLAE178 detected from *H. longicornis* in South Korea. The ehrlichial species with the highest similarity to sequences from Japan was *Ehrlichia* sp. EH727 detected in *Haemaphysalis* sp. in Shizuoka Prefecture (99.1%). In the validated ehrlichial species, the 16SrRNA gene sequences of *E. chaffeensis*, *E. canis*, and *E. ewingii* had relatively high similarity to the obtained sequences (98.3–98.6%). The 1,304 bp *groEL* sequences obtained from 2 female ticks in this study showed the highest similarity to that of *Ehrlichia* sp. Kh-Hj27 (94.2–94.4%), which was detected in *Haemaphysalis japonica* in Khabarovsk, Russia. Our obtained

sequences had the highest similarity to the *groEL* genes of *Candidatus E. shimanensis* (91.8%) and *E. ewingii* (94.0–94.4%) in the Japanese ehrlichial agents and the validated species, respectively. *E. ewingii* is a pathogenic agent to humans and dogs, and infection with *E. ewingii* causes fever, headache, thrombocytopenia, and leukopenia [1, 2]. The ehrlichial species detected in *H. longicornis* from pastures on Yonaguni Island may interact with cattle. Further study of the pathogenesis and epidemiology of *Ehrlichia* agents is needed.

Sequences of 16SrRNA and *groEL* genes obtained in this study have relatively low similarity to the validated *Ehrlichia* species and made unique clades with other *Ehrlichia* species, including *Ehrlichia* sp. EBm52, *Ehrlichia* sp. HLAE178, and *Ehrlichia* sp. Kh-Hj27, which were detected in ticks in Thailand, South Korea, and the Russian Far East, respectively (Figs. 1, 2 and 3). These *Ehrlichia* species detected in Asia may represent a new *Ehrlichia* species. Of *Ehrlichia* species shown in figure 1, *Ehrlichia* sp. HLAE178 and 'Candidatus *Ehrlichia shimanensis*' were detected in *H. longicornis*, and the distribution of the ehrlichial species close to Yonaguni 138 and 206 were included in that of *H. longicornis*. Further study including isolation of the ehrlichial agent and more genetic analysis is needed.

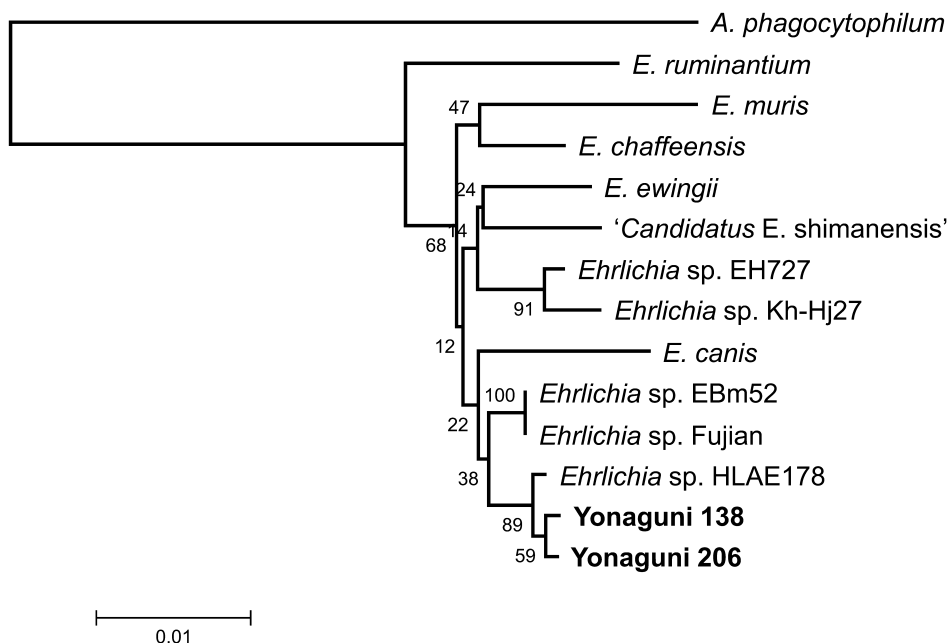


Fig. 2. Phylogenetic tree constructed by the neighbor-joining method based on sequences of the 16S rRNA gene. Yonaguni 138 and Yonaguni 206 were obtained from *H. longicornis* in this study. Numbers at branch nodes indicate bootstrap values. GenBank accession numbers for the sequences used to construct the trees are as follows: *E. canis* (M73221), *E. chaffeensis* (M73222), *E. ewingii* (U96436), *E. muris* (U15527), *E. ruminantium* (U03776), *Candidatus E. shimanensis* (AB074459), *Ehrlichia* sp. EBm52 (AF497581), *Ehrlichia* sp. EH727 (Y309970), *Ehrlichia* sp. Fujian (DQ324547), *Ehrlichia* sp. HLAE178 (GU075695), *Ehrlichia* sp. Kh-Hj27 (FJ966350), and *A. phagocytophilum* (CP000235).

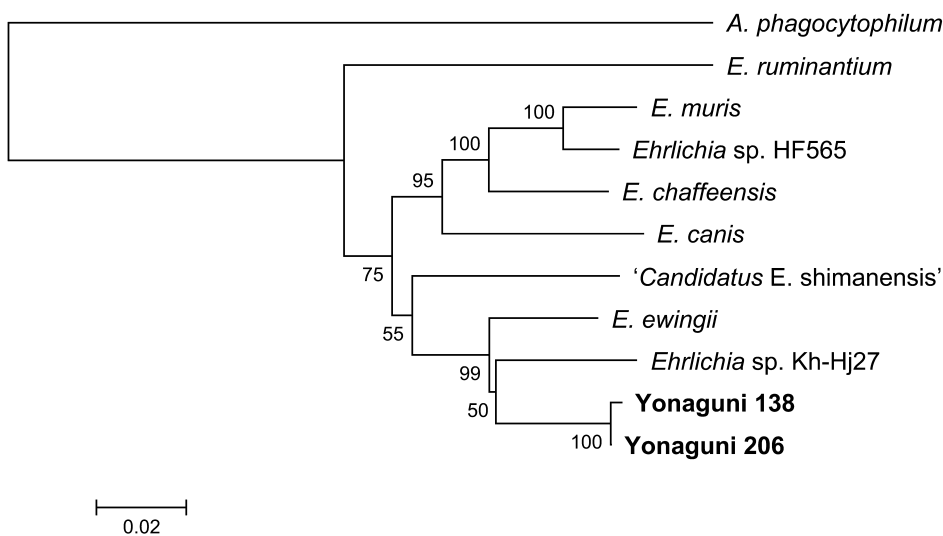


Fig. 3. Neighbor-joining tree based on the sequences of *groEL* gene. Yonaguni 138 and Yonaguni 206 were obtained from *H. longicornis* in the present study. Numbers at branch nodes indicate bootstrap values. GenBank accession numbers for the sequences used to construct the trees are as follows: *E. canis* (U96731), *E. chaffeensis* (L10917), *E. ewingii* (AF195273), *E. muris* (AF210459), *E. ruminantium* (DQ647014), *Candidatus E. shimanensis* (AB074462), *Ehrlichia* sp. HF565 (AB032712), *Ehrlichia* sp. Kh-Hj27 (FJ966349), and *A. phagocytophilum* (CP000235).

An examination of blood samples from cattle on Yonaguni Island in 2006 showed a high prevalence of *A. phagocytophilum* and *A. bovis* [11]. However, *Anaplasma* species were not detected in *H. longicornis* in the present study. The grazing pastures in this study were apart from the farm surveyed previously. In South Korea, *A. phagocytophilum* and *A. bovis* were detected in *H. longicornis* [8], which suggests that *H. longicornis* can harbor these *Anaplasma* species. Moreover, *A. phagocytophilum* was detected in the rodent, *Apodemus agrarius* [3, 9]. The prevalence rate of *A. phagocytophilum* in *H. longicornis* was relatively low (0–2.4%) [3, 7, 9], however, that in *A. agrarius* was high (5.6–23.6%). The distribution of rodents in Yonaguni Island was unclear, and the rodents were not tested in this study. The absence of *A. phagocytophilum* and *A. bovis* in this study may indicate that reservoirs of *A. phagocytophilum* and *A. bovis* were not located in the studied pastures and/or that the number of tested ticks was insufficient to detect *Anaplasma* species.

Prevalence of *Ehrlichia* species of *H. longicornis* in this study was 1.1% (2 of 175) in nymphs and 6.5% (2 of 31) in adults, which may indicate that larvae and/or nymphs of *H. longicornis* become possessed of ehrlichial agents when they take blood from the hosts on the pasture on Yonaguni Island. However, the nature of the reservoir for *Ehrlichia* species on Yonaguni Island is not clear. Further study to reveal infection rates in mammals, including rodents, in pastures on Yonaguni Island is needed.

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