Ticks and Tick-borne Diseases 10 (2019) 344–351

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Isolation and molecular detection of *Ehrlichia* species from ticks in western, central, and eastern Japan

Masakatsu Taira[a,b,c,*], Shuji Ando[c], Hiroki Kawabata[a,d], Hiromi Fujita[e], Teruki Kadosaka[f], Hiroko Sato[g], Naoto Monma[h], Norio Ohashi[i], Masayuki Saijo[a,c]

[a] United Graduate School of Agricultural Science and Veterinary Science, Gifu University, Gifu, Japan
[b] Division of Virology and Medical Zoology, Chiba Prefectural Institute of Public Health, Chiba, Japan
[c] Department of Virology 1, National Institute of Infectious Diseases, Tokyo, Japan
[d] Department of Bacteriology 1, National Institute of Infectious Diseases, Tokyo, Japan
[e] Mahara Institute of Medical Acarology, Tokushima, Japan
[f] Aichi Medical University, Aichi, Japan
[g] Akita Prefectural Research Center for Public Health and Environment, Akita, Japan
[h] Fukushima Prefectural Institute for Public Health, Fukushima, Japan
[i] Department of Food and Nutritional Sciences, Graduate School of Integrated Pharmaceutical and Nutritional Sciences, University of Shizuoka, Shizuoka, Japan

ARTICLE INFO

Keywords:
Ehrlichiosis
Ixodes ovatus
Ixodes turdus
Japan
Tick-borne diseases

ABSTRACT

Ehrlichiosis is a tick-borne bacterial disease caused by pathogens of the *Ehrlichia* genus. Although human ehrlichiosis has not been reported in Japan, *Ehrlichia* spp., which are closely related to *Ehrlichia chaffeensis*, were detected in several species of ixodid ticks. In this study, the presence of *Ehrlichia* spp. in ticks in Japan was studied by using isolation and molecular detection methods. In total, 1237 ticks were collected from vegetation in western, central, and eastern parts of Japan. The ticks were tested for detection of ehrlichial DNA with a nested polymerase chain reaction and/or isolation by inoculation of mice with the homogenate. Ehrlichial DNA was detected in 29 of these ticks. The ehrlichial DNAs, *groEL* and 16S rRNA genes, detected in *Ixodes turdus* showed a high similarity to those of *E. chaffeensis* with 94.7% and 99.2% identity, respectively. *Ehrlichia* sp. HF and *Candidatus* Neoehrlichia mikurensis were also detected in *I. ovatus*. Furthermore, *Ehrlichia* sp. HF was isolated from laboratory mice that were intraperitoneal inoculated with *I. ovatus* tick homogenate. Some ehrlichial agents detected in *Ixodes* ticks might be a previously unknown *Ehrlichia* species. In this study, *Candidatus* N. mikurensis was detected in *I. ovatus* ticks. Because *I. ovatus* is distributed widely and cases of its tick bite in humans are ubiquitously reported in Japan, there is a potential for ehrlichiosis to be endemic to Japan, necessitating intensive surveillance of this infectious disease.

1. Introduction

Human ehrlichiosis is a tick-borne bacterial diseases caused by obligate intracellular bacteria, *Ehrlichia chaffeensis*, *E. ewingii*, and *E. muris*. Neoehrlichiosis is also a tick-borne bacterial disease caused by *Candidatus* Neoehrlichia mikurensis. These species of bacteria are commonly present in nature in a life cycle between wildlife mammals and some species of ticks (*Ixodes*, *Haemaphysalis*, and *Amblyomma* species), and they may cause asymptomatic infections or mild diseases in wildlife mammals (Naitou et al., 2006; Paddock and Childs, 2003; Tabara et al., 2007; Yabsley et al., 2002).

Ehrlichia chaffeensis, an obligatory intramonocytic bacterium, causes human monocytic ehrlichiosis (HME) (Anderson et al., 1991; Dawson et al., 1991). *Ehrlichia chaffeensis* was discovered for the first time in 1986 in the monocytes of a patient with fever, headache, pharyngitis, nausea, vomiting, and dehydration with multiple tick bites (Anderson et al., 1991; Dawson et al., 1991; Paddock and Childs, 2003). *Ehrlichia chaffeensis* has been detected in *Amblyomma americanum* ticks collected in the field, and the wild white-tailed deer is well known to be its reservoir in the USA (Lockhart et al., 1997; Ijdo et al., 2000; Paddock and Childs, 2003).

Ehrlichia muris-like agent (EMLA), which was detected in febrile patients with tick bites in Minnesota and Wisconsin in the USA, has recently been recognized as a novel pathogenic *Ehrlichia* species (Pritt et al., 2011). EMLA was related genetically to *E. muris*, a species of *Ehrlichia* isolated from *Haemaphysalis* ticks and wild rodents in Japan

* Correspondence author at: D.V.M. Chiba Prefectural Institute of Public Health, 666-2 Nitona-cho, Chuo-ku, Chiba 260-8715, Japan.
E-mail address: m.tir4@pref.chiba.lg.jp (M. Taira).

https://doi.org/10.1016/j.ttbdis.2018.11.010
Received 27 September 2018; Received in revised form 8 November 2018; Accepted 11 November 2018
Available online 13 November 2018

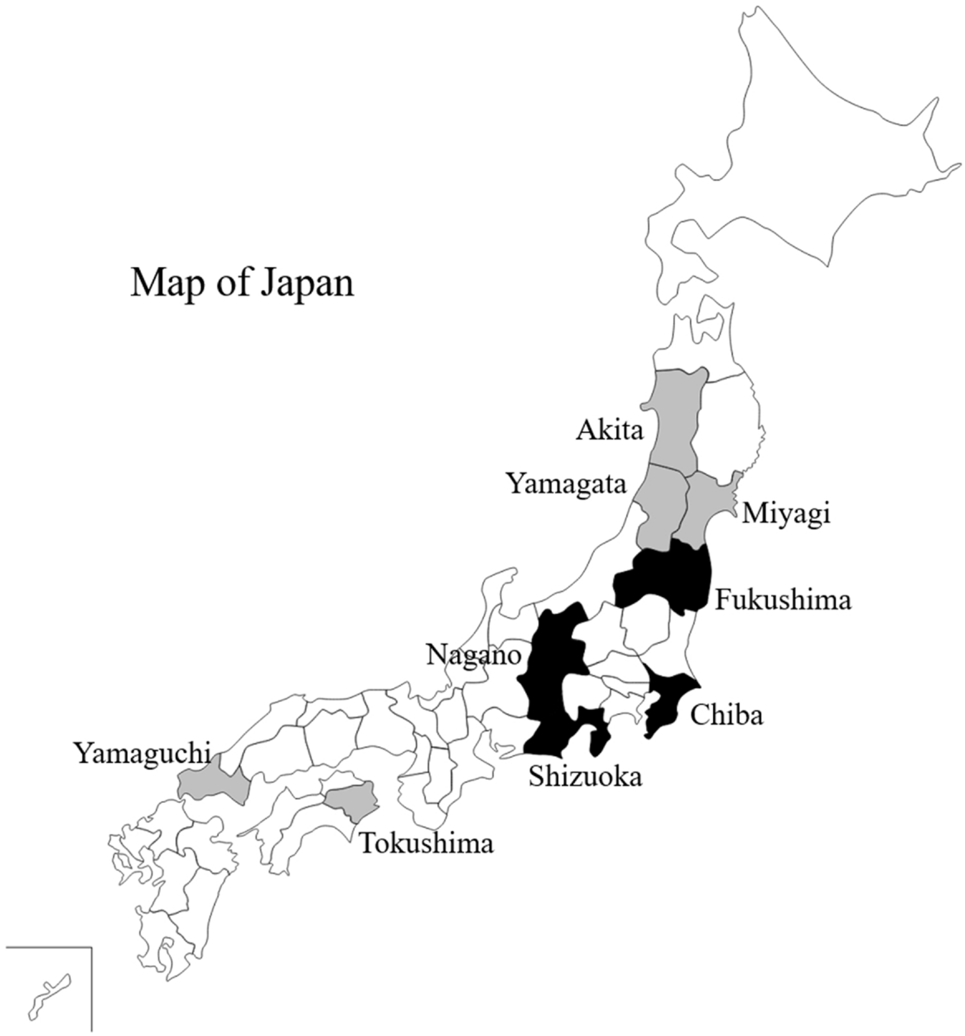

Fig. 1. Geographic areas where ticks were collected from June 2013 to June 2016. The areas, in which the ehrlichial DNA-positive ticks were found are shown in black, and those in which only ehrlichial DNA-negative ticks were found are shown in gray.

Table 1
Primer sets for the amplification of *groEL* and 16S rRNA of the ehrlichial species.

Target gene	PCR step	Length of PCR product	Primer name	Primer sequence 5′-3′	Reference
groEL	1st	687 bp	gro607F	GAAGATGCWGTWGGWTGTACKGC	Tabara et al. (2007)
			gro1294R	AGMGCTTCWCCTTCWACRTCYTC	
	Nest	444 bp	gro677F	ATTACTCAGAGTGCTTCTCARTG	
			gro1121R	TGCATACCRTCAGTYTTTTCAAC	
16S rRNA	2nd	1490 bp	ER5-3	TTGAGAGTTTGATCCTGG	Inayoshi et al. (2004)
			ER-R1	GGAGGTAATCCAGCCGCA	
	Nest	910 bp	E16 s-200F	GATCAGCCACACTGGAACTGAGA	Tabara et al. (2007)
			E16S-1162R	CATTGTAGCACGTGTGTAGCCCA	

Table 2
Nested PCR-positive rate for the ehrlichial *groEL* gene in each tick species.

Tick	Number of samples	Number of PCR-positive ticks	Positive rate (%)
I. turdus	84	9	10.7
I. ovatus	350	16	4.6
H. megaspinosa	99	2	2.0
H. flava	55	1	1.8
H. longicornis	516	1	0.2
H. kitaokai	107	0	0
H. cornigera	15	0	0
A. testudinarium	11	0	0
Total	1237	29	2.3

(Kawahara et al., 1993, 1999). However, despite the use of the term EMLA in several previous reports, it has recently been formally named *E. muris eauclairensis* (Pritt et al., 2017).

Candidatus N. mikurensis was first isolated from wild rats captured in Mikura Island, Izu Archipelago, Japan in 2004 (Kawahara et al., 2004). It is prevalent among ticks and wild rodents in Europe and Asia (Kawahara et al., 2004; Wennerås, 2015). *Candidatus* N. mikurensis is also a novel tick-borne disease agent for neoehrlichiosis. The disease caused by *Candidatus* N. mikurensis was first discovered in an immunocompromised patient with febrile episodes in Sweden in 2010 (Welinder-Olsson et al., 2010). *Candidatus* N. mikurensis infections have been reported not only from Europe but also from Asia (Li et al., 2012). These patients were diagnosed as having neoehrlichiosis by

Table 3
Information on the ticks in which the ehrlichial DNA was detected.

Tick species	Sample ID	Sex (Stage)	Site (prefecture)	Nested PCR		Isolation
				groEL	16S rRNA	
I. turdus	Itp1	Unknown (Nymph)	Chiba	+	+	–
	Itp3	Unknown (Nymph)	Chiba	+[a]	+	–
	It1	Female	Chiba	+[a]	+	–
	It19	Unknown (Nymph)	Chiba	+[a]	+	–
	It20	Unknown (Nymph)	Chiba	+[a]	+[a]	–
	It24	Unknown (Nymph)	Chiba	+[a]	+	–
	It25	Unknown (Nymph)	Chiba	+[a]	+[a]	–
	It40	Unknown (Nymph)	Chiba	+[a]	+[a]	–
	It51	Unknown (Nymph)	Chiba	+	+	–
I. ovatus	Io3	Unknown (Nymph)	Chiba	+[a]	+	–
	Io30	Male	Nagano	+[a]	+[a]	+
	Io67	Female	Fukushima	+[a]	+[a]	+
	Io82	Male	Fukushima	+[a]	+[a]	–
	Io86	Male	Fukushima	+[a]	+[a]	–
	Io106	Female	Nagano	+[a]	+[a]	–
	Io114	Female	Nagano	+[a]	+[a]	–
	Io143	Female	Shizuoka	+[a]	+[a]	–
	Io145	Female	Shizuoka	+[a]	+	–
	Io181	Female	Shizuoka	+[a]	+	–
	Io184	Female	Shizuoka	+[a]	+	–
	Io191	Female	Shizuoka	+[a]	+[a]	+
	Io193	Male	Shizuoka	+[a]	+	–
	Io198	Female	Shizuoka	+[a]	+	–
	Io199	Female	Shizuoka	+[a]	+	–
	Io202	Female	Shizuoka	+[a]	+[a]	+
H. megaspinosa	Hm28	Male	Chiba	+[a]	–	–
	Hm37	Female	Chiba	+[a]	–	–
H. flava	Hf34	Female	Chiba	+[a]	–	–
H. longicornis	Hl9	Female	Chiba	+[a]	–	–

[a] The nucleotide sequence was successfully determined.

detection of *Candidatus* N. mikurensis genes in their blood with the use of polymerase chain reaction (PCR), followed by subsequent sequencing of the DNA amplified (von Loewenich et al., 2010; Welinder-Olsson et al., 2010; Li et al., 2012).

Ticks collected in Japan have already been reported to possess *Ehrlichia* species that are closely related to *E. chaffeensis*, *E. muris*, and *Candidatus* N. mikurensis (Fujita and Watanabe, 1994; Inayoshi et al., 2004; Kawahara et al., 1999, 2004; Naitou et al., 2006; Shibata et al., 2000; Takano et al., 2009).

Both ehrlichiosis and rickettsiosis are endemic to the USA. Although over 200 cases of rickettsiosis are reported annually in Japan, ehrlichiosis has not been reported. The rickettsiosis endemic area may have the potential for other tick-borne infectious diseases. However, neither patients with HME nor those with *Candidatus* N. mikurensis infection have been reported in Japan so far. Ehrlichiosis may have the potential to be endemic to Japan. Because previous investigations surveyed limited areas, further investigation in a wider area is required. The aim of this study was to investigate the prevalence of *Ehrlichia* species in ticks by detection of ehrlichial DNA and/or the isolation of *Ehrlichia* species from ticks collected in multiple regions of Japan to evaluate the potential for human ehrlichiosis in Japan.

2. Material and methods

2.1. Tick collection and preparation

Ticks were collected by use of a flagging method in western, central, and eastern parts of Japan from 2013 to 2016 (Fig. 1), and the tick species were identified morphologically (Yamaguti et al., 1971). The collected ticks were then soaked in 70% ethanol with 0.1% povidone-iodine for 10 min and rinsed with phosphate buffered saline solution (PBS) containing 0.5–1.0% calf serum. The ticks were homogenized in tubes using a pestle with sucrose-phosphate-glutamate (0.0038 M KH_2PO_4, 0.0072 M K_2HPO_4, 0.0049 M L-glutamate, 0.218 M sucrose, pH 7.2) at a volume of 0.3 mL per one tick. Then, 0.2 mL of the homogenized tick samples was applied for the extraction of DNA using a High Pure PCR Preparation kit (Roche Diagnostics, Mannheim, Germany). The remaining 0.1 mL of the homogenized tick sample was stored at -80 °C for further study. DNA extracted from the ticks was also stored at -80 °C until use.

2.2. Detection of ehrlichial DNA

DNA extracted from each tick sample was individually tested for amplification of the ehrlichial genome with PCR targeting a conserved region of the *groEL* and 16S rRNA genes (Table 1) (Inayoshi et al., 2004; Tabara et al., 2007). When the specific DNA was amplified, the nucleotide sequence of the amplified DNA was determined with cycle sequencing using a BigDye Terminator v1.1 cycle sequencing kit and Genetic Analyzer 3130 (Applied Biosystems, Foster City, CA). Alignments were performed using MEGA 6.0 software with bootstrap support (1000 replications), and their phylogenetic relationships were analyzed with the neighbor-joining method (Tamura et al., 2013).

2.3. Isolation of ehrlichial agent in mice

Three 5-week-old male ddY mice (Japan SLC, Shizuoka, Japan) were inoculated intraperitoneally with each tick homogenate, which was ehrlichial *groEL* gene-positive by PCR, per one sample. The mice were observed for 2 weeks or until clinical signs such as ruffled fur appeared or they died. When mice showed the clinical signs, they were sacrificed by excess isoflurane, and spleen and liver were aseptically collected. The organs removed were weighed, and their homogenates were prepared in sucrose-phosphate-glutamate buffer as a 10% w/v concentration. They were applied for the additional propagation of pathogens using ddY mice, in which the ehrlichial *groEL* gene was

Table 4
Information on the ehrlichial species used for the phylogenetic analysis, including the isolate determined in the present study.

Ehrlichia strain	Country	Year	Source	Accession number	
				groEL	16S rRNA
Ehrlichia chaffeensis str. Arkansas	U.S.A.	1991	Human	CP000236	CP000236
Ehrlichia sp. HF565	Japan: Fukushima	1994	Tick (*I. ovatus*)	AB032712	DQ647318
Ehrlichia sp. Anan	Japan: Tokushima	1994	Tick (*I. ovatus*)	AB032711	AB028319
Ehrlichia muris AS145	Japan: Aichi	1983	Wild mouse (*Eothenomys kageus*)	AF210459	NR121714
Ehrlichia canis str. YZ-1	China	2016	Dog	U96731	M73226
Ehrlichia sp. Yonaguni 206	Japan: Yonaguni Island	2012	Tick (*H. longicornis*)	HQ697591	HQ697589
Ehrlichia ewingii	U.S.A.	1999	Human/Dog	AF195273	U96436
Candidatus Ehrlichia shimanensis	Japan: Shimane	1999	Tick (*H. longicornis*)	AB074462	AB074459
Ehrlichia ruminantium str. Welgevonden	South Africa	1985	Tick (*A. hebraeum*)	CR925678	CR925678
Candidatus Neoehrlichia mikurensis str. IS58	Japan: Mikura Island	2000	Tick (*I. ovatus*)	AB074461	AB074460
Anaplasma phagocytophilum str. HZ	U.S.A.	2014	Human	CP000235	CP000235
Present study					
Ehrlichia sp. Itp3 Chiba	Japan: Chiba	2014	Tick (*I. turdus*)	LC385848	-[a]
Ehrlichia sp. It1 Chiba	Japan: Chiba	2016	Tick (*I. turdus*)	LC385847	–
Ehrlichia sp. It19 Chiba	Japan: Chiba	2016	Tick (*I. turdus*)	LC385849	–
Ehrlichia sp. It20 Chiba	Japan: Chiba	2016	Tick (*I. turdus*)	LC385850	LC386012
Ehrlichia sp. It24 Chiba	Japan: Chiba	2016	Tick (*I. turdus*)	LC385851	–
Ehrlichia sp. It25 Chiba	Japan: Chiba	2016	Tick (*I. turdus*)	LC385852	LC386011
Ehrlichia sp. It40 Chiba	Japan: Chiba	2016	Tick (*I. turdus*)	LC385853	LC386013
Ehrlichia sp. Io3 Chiba	Japan: Chiba	2013	Tick (*I. ovatus*)	LC385831	–
Ehrlichia sp. Io30 Nagano	Japan: Nagano	2015	Tick (*I. ovatus*)	LC385832	LC386008
Ehrlichia sp. Io67 Fukushima	Japan: Fukushima	2015	Tick (*I. ovatus*)	LC385833	LC388350
Ehrlichia sp. Io82 Fukushima	Japan: Fukushima	2015	Tick (*I. ovatus*)	LC385834	LC386006
Ehrlichia sp. Io86 Fukushima	Japan: Fukushima	2015	Tick (*I. ovatus*)	LC385835	LC386005
Ehrlichia sp. Io106 Nagano	Japan: Nagano	2015	Tick (*I. ovatus*)	LC385836	LC386010
Ehrlichia sp. Io114 Nagano	Japan: Nagano	2015	Tick (*I. ovatus*)	LC385837	LC386009
Ehrlichia sp. Io143 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385838	LC386007
Ehrlichia sp. Io145 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385839	–
Ehrlichia sp. Io181 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385840	–
Ehrlichia sp. Io184 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385841	–
Ehrlichia sp. Io191 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385842	LC388351
Ehrlichia sp. Io193 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385843	–
Ehrlichia sp. Io198 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385844	–
Ehrlichia sp. Io199 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385845	–
Ehrlichia sp. Io202 Shizuoka	Japan: Shizuoka	2015	Tick (*I. ovatus*)	LC385846	LC388352
Ehrlichia sp. Hm28 Chiba	Japan: Chiba	2013	Tick (*H. megaspinosa*)	LC385855	–
Ehrlichia sp. Hm37 Chiba	Japan: Chiba	2013	Tick (*H. megaspinosa*)	LC385856	–
Ehrlichia sp. Hf34 Chiba	Japan: Chiba	2013	Tick (*H. flava*)	LC385857	–
Ehrlichia sp. Hl9 Chiba	Japan: Chiba	2013	Tick (*H. longicornis*)	LC385854	–

[a] The nucleotide sequence could not be determined.

amplified with PCR for confirmation of isolation. The nucleotide sequence of the PCR product was also determined. *Ehrlichia* sp. HF565 strain was used for ehrlichial isolation as positive controls.

2.4. Ethical considerations

The animal experiments were performed in strict accordance with the Regulation for Animal Experimentation of the Chiba Prefectural Institute of Public Health. The protocol of the experiments in which mice were used was approved by the Institutional Animal Care and Use Committee of the Chiba Prefectural Institute of Public Health (numbers 28-3 and 29-2). All of the mice infected with pathogens were handled in biosafety level 2 animal facilities in accordance with the biosafety guidelines of the Chiba Prefectural Institute of Public Health. The mice were inoculated with tick homogenates while taking all efforts to minimize any potential pain and distress.

3. Results

3.1. Ticks collected

In total, 1237 ticks (84 *I. turdus*, 350 *I. ovatus*, 99 *Haemaphysalis megaspinosa*, 55 *H. flava*, 516 *H. longicornis*, 107 *H. kitaokai*, 15 *H. cornigera*, and 11 *A. testudinarium*) were captured from vegetation in western, central, and eastern parts of Japan (Fig. 1, Table 3).

3.2. Phylogenetic analysis of ehrlichial groEL genes

Ehrlichial *groEL* fragments were detected in 29 tick samples. The ehrlichial *groEL* gene was amplified in the following tick species of *I. turdus*, *I. ovatus*, *H. megaspinosa*, *H. flava*, and *H. longicornis*, but not in *H. kitaokai, H. cornigera*, and *A. testudinarium* (Tables 2 and 3). The ehrlichial *groEL* gene-positive rate in *I. turdus* was the highest among those of the tick species tested. The ehrlichial *groEL* gene fragments were detected in the ticks collected in Chiba, Nagano, Shizuoka, and Fukushima prefectures, but not in those collected in Akita, Yamagata, Miyagi, Tokushima, and Yamaguchi prefectures (Fig. 1, Table 3). The novel sequences of *groEL* genes were deposited in the DNA Data Bank of Japan (DDBJ) (Table 4).

The nucleotide sequences of the *groEL* gene fragments amplified in the 29 ticks were determined, revealing that the ehrlichial *groEL* genes detected in *I. ovatus* were divided into two genotypes (Fig. 2, Table 4). One type including Io30, Io67, Io82, Io86, Io143, Io181, Io184, Io191, Io193, Io198, Io199, and Io202 was positioned in a cluster to which *Ehrlichia* sp. strain HF565 belonged, being that the sequence identities of the *groEL* genes amplified from these *I. ovatus* were 100% identical to that of HF565. The other type of ehrlichial *groEL* genes detected in *I. ovatus* Io3, Io106, Io114, and Io145 was positioned in a cluster to which *Candidatus* N. mikurensis was included (Fig. 2), being that the sequence identities of the *groEL* genes amplified from these *I. ovatus* were 98.6% identical to that of *Candidatus* N. mikurensis (Acc. No. AB074461).

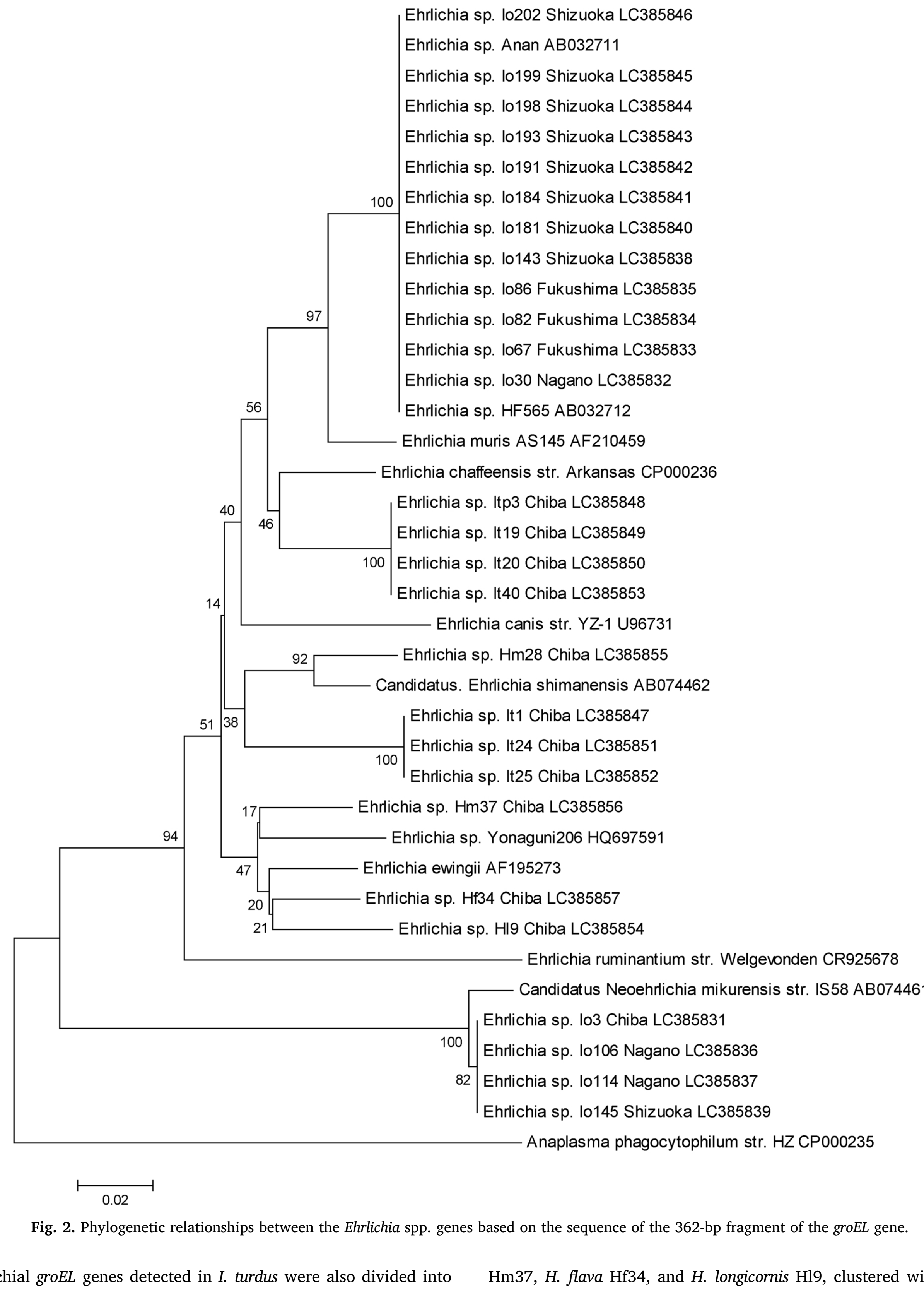

Fig. 2. Phylogenetic relationships between the *Ehrlichia* spp. genes based on the sequence of the 362-bp fragment of the *groEL* gene.

Ehrlichial *groEL* genes detected in *I. turdus* were also divided into two genotypes. The *groEL* genes amplified from *I. turdus* Itp3, It19, It20, and It40 were closely related to that of *E. chaffeensis* (Acc. No. CP000236), whereas *groEL* genes amplified from *I. turdus* Itp1, It1, It24, It25, and *H. megaspinosa* Hm28 were positioned in a cluster with *Candidatus Ehrlichia* shimanensis (Acc. No. AB074462). Novel ehrlichial *groEL* genes sequences, which were amplified from *H. megaspinosa* Hm37, *H. flava* Hf34, and *H. longicornis* Hl9, clustered with those of *Ehrlichia* sp. yonaguni206 (Acc. No. HQ697591) and *E. ewingii* (Acc. No. AF195273).

3.3. Phylogenetic analysis of ehrlichial 16S rRNA genes

The 16S rRNA gene fragments amplified from 12 ticks were

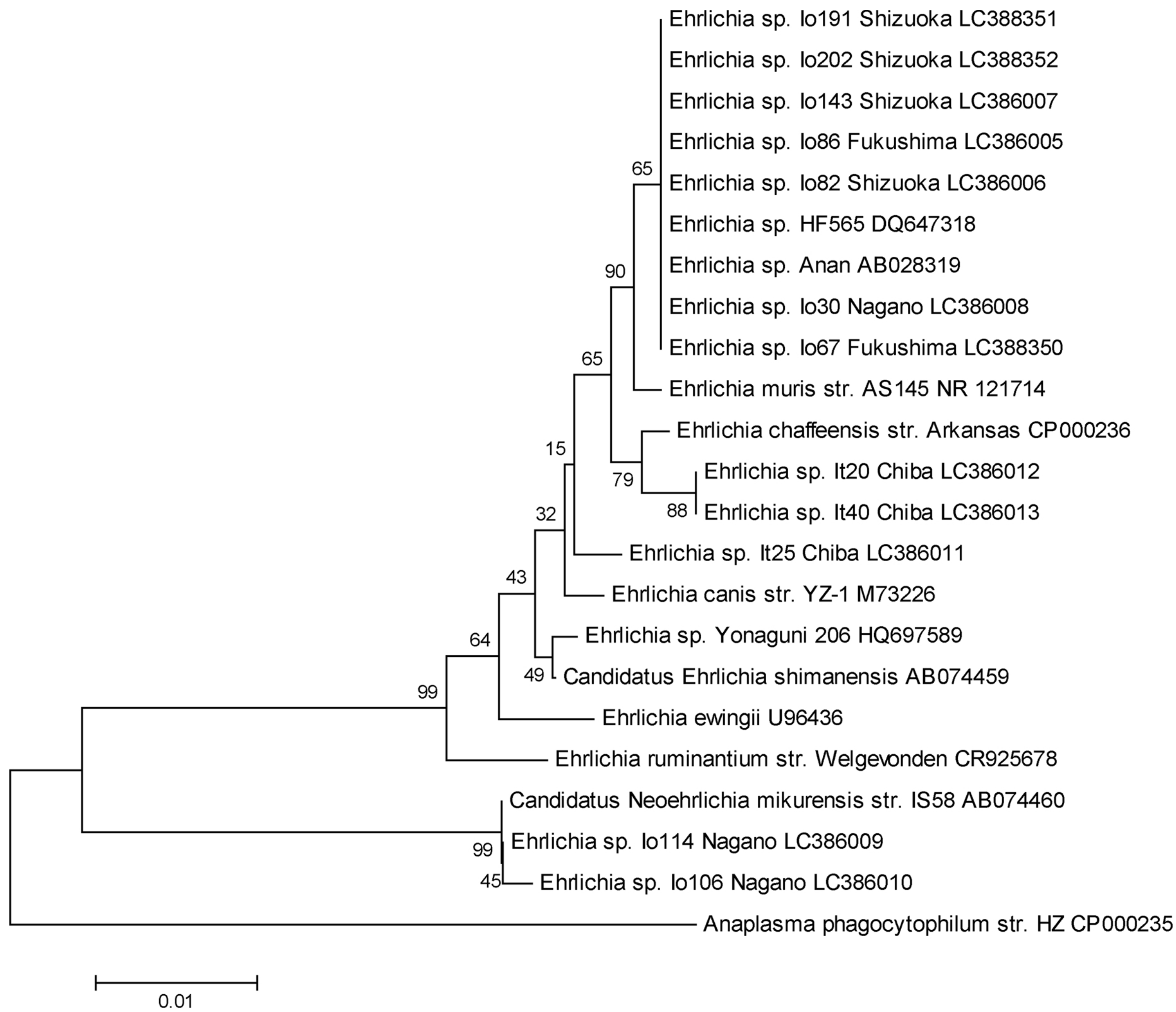

Fig. 3. Phylogenetic relationships between the *Ehrlichia* spp. genes based on the sequence of the 702-bp fragment of the 16 s rRNA gene.

sequenced. The novel sequences of 16S rRNA genes were also deposited in the DDBJ under the accession numbers assigned (Table 4). Ehrlichial 16S rRNA genes detected in *I. ovatus* were divided into two genotypes (Fig. 3). One type including Io30, Io67, Io82, Io86, Io143, Io191, and Io202 was positioned in a cluster to which *Ehrlichia* sp. strain HF565 (Acc. No. DQ647318) belonged, being that the sequence identities of the 16S rRNA genes amplified from these *Ehrlichia* of *I. ovatus* were 100% identical to that of *Ehrlichia* sp. strain HF565. Another type of ehrlichial 16S rRNA genes detected in *Ehrlichia* of *I. ovatus* Io106 and Io114 was positioned in a cluster to which *Candidatus* N. mikurensis (Acc. No. AB074460) was included (Fig. 3), being that the sequence identities of the 16S rRNA genes amplified from these *Ehrlichia* of *I. ovatus* were also 100% identical to that of *Candidatus* N. mikurensis.

Ehrlichial 16S rRNA genes detected in *I. turdus* were also divided into two genotypes. The 16S rRNA genes amplified from It20 and It40 were closely related to that of *E. chaffeensis* (Acc. No. NR074500), whereas 16S rRNA genes amplified from It25 were positioned in a novel cluster.

3.4. Isolation of ehrlichial agent from ticks

Mice were inoculated with each of the homogenates of the 29 ehrlichial DNA-positive samples, composed of 16 *I. ovatus* and 9 *I. turdus* homogenates, for isolation. Ehrlichial organisms were isolated from 4 *I. ovatus* but not from *I. turdus* (Table 3). All of the mice inoculated with each of the Io30, Io67, Io191, and Io202 samples developed clinical signs of ruffled fur and died within 9 to 11 days after inoculation. The organisms isolated were confirmed to be *Ehrlichia* spp. by genetic analysis. *Candidatus* N. mikurensis-like *Ehrlichia* was not isolated from any samples.

4. Discussion

Ehrlichial DNA fragments, which were classified to *E. chaffeensis* or to *Candidatus* N. mikurensis, were detected in multiple tick species collected in central and eastern parts of Japan. The detected ehrlichial DNA fragments had diversities, some of which were closely related to *E. chaffeensis*, which is a causative agent of HME.

Ehrlichia sp. strain HF565-like *Ehrlichia* spp. was isolated from *I. ovatus* ticks collected in Nagano, Shizuoka, and Fukushima prefectures. The *Ehrlichia* sp. strain HF565 isolated was closely related to *E. chaffeensis* isolated in the USA. The present study suggests that *Ehrlichia* sp. strain HF565-like *Ehrlichia* is more widely distributed in Japan than was previously thought.

Candidatus N. mikurensis is a pathogen of neoehrlichiosis first isolated in Japan (Kawahara et al., 1993). However, no patient with neoehrlichiosis has been reported in Japan, whereas the DNA of *Candidatus* N. mikurensis was detected from immunodeficient patients in Europe and China (Li et al., 2012; von Loewenich et al., 2010; Welinder-Olsson et al., 2010; Wennerås, 2015). Because *Candidatus* N. mikurensis was detected in *I. ovatus* collected in Chiba, Nagano, and Shizuoka prefectures, *Candidatus* N. mikurensis is also considered to be distributed more widely in Japan than was previously thought. The *Candidatus* N. mikurensis DNA sequence detected from *I. persulcatus* collected in areas where neoehrlichiosis patients were reported was identical to those amplified from neoehrlichiosis patients in China (Wei et al., 2016). Therefore, *I. persulcatus* is thought to be the vector of *Candidatus* N. mikurensis in China. *I. ovatus* is widely distributed, and cases of its tick bite are ubiquitously reported in Japan (Fukuoka et al., 1989). There is a potential that ehrlichiosis might be endemic to Japan, necessitating intensive surveillance of this infectious disease. To address this assumption, a prospective study is needed.

Ehrlichial DNA detected in *I. turdus* was more closely related to *E. chaffeensis* than to *Ehrlichia* sp. strain HF565. Ehrlichial *groEL* and 16 s RNA DNAs detected in 4 samples of *I. turdus* showed a high similarity to those of *E. chaffeensis* strain Arkansas with 94.8% and 99.6% identity, respectively. Humans are reported to be bitten by *I. turdus* in Japan, suggesting that *I. turdus* may be a potential vector tick for HME in Japan (Woo et al., 1990).

Recently, EMLA and *E. muris eauclairensis* were detected from human ehrlichiosis patients in the USA (Pritt et al., 2011, 2017; Centers for Disease Control and Prevention (CDC), 2018). *E. muris* was not detected in this study, although some species of ticks in Japan, and especially *H. flava*, possess *E. muris* (Kawahara et al., 1999). The total number of *H. flava* ticks collected accounted for only 4% of the total tick collection, and thus the number of samples was limited. Expanding the sampling area and increasing the number of ticks collected are needed to assess the prevalence of *E. muris* accurately.

There are many areas to be addressed in terms of HME and neoehrlichiosis in Japan. *E. chaffeensis*, *E. muris eauclairensis*, and *Candidatus* N. mikurensis have been detected in patients outside Japan (Anderson et al., 1991; Li et al., 2012; Pritt et al., 2011; von Loewenich et al., 2010; Welinder-Olsson et al., 2010). Since 2008, around 1000 cases of *E. chaffeensis* infection have been reported annually in the USA (Centers for Disease Control and Prevention (CDC), 2018). Since the discovery of *E. muris eauclairensis* in the USA, 115 cases of infection by this species have been reported in total (Centers for Disease Control and Prevention (CDC), 2018). In total, 26 cases of *Candidatus* N. mikurensis infection have been reported from several European and Asian countries such as Sweden, Switzerland, Czech Republic, Germany, Poland, and China (Wennerås, 2015).

To evaluate the risk for ehrlichiosis more precisely, further studies are needed. In the present study, ticks were collected in a limited number of areas, and the total number of ticks collected was also limited. Thus, similar studies that undertake an expansion of the sampling areas and an increase in the number of ticks collected will be necessary and important in the future. Infectious *Candidatus* N. mikurensis has not been isolated and cultivated, and its genome sequence has not yet been fully determined. In a previous study, in which the isolation of *Candidatus* N. mikurensis using rats was reported, neoehrlichial DNA was negative up to day 15 after inoculation. However, specimens from days 20–60 after inoculation became positive for *Candidatus* N. mikurensis DNA in PCR (Kawahara et al., 2004). Thus, in the present study, the time period for observation of the mice might have been too short. Furthermore, it seems to be highly likely that mice may be less sensitive than rats in the isolation of *Candidatus* N. mikurensis. The development of an isolation method for infectious *Candidatus* N. mikurensis is needed to enhance the research capacity for sequencing genomes, elucidating host-pathogens interactions, and developing diagnostics and therapeutics.

Japanese Ixodes ticks carry the ehrlichial pathogens, which can cause HME and neoehrlichiosis, and it is suggested that the distribution of Japanese Ixodes ticks with ehrlichial pathogens may be more widely prevalent in Japan than was shown in previous studies (Kawahara et al., 1993, 1999, 2004; Shibata et al., 2000; Inayoshi et al., 2004; Naitou et al., 2006; Tabara et al., 2007). The development of diagnostic systems for ehrlichiosis in patients and a surveillance system should be undertaken.

Acknowledgements

This work was supported financially in part by Gifu University and by grants-in-aid from the Japan Agency for Medical Research and Development (JP17fk0108310, JP18fk0108068). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

Anderson, B.E., Dawson, J.E., Jones, D.C., Wilson, K.H., 1991. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J. Clin. Microbiol. 29, 2838–2842.

Centers for Disease Control and Prevention (CDC), 2018. Ehrlichiosis: Epidemiology and Statistics. (Accessed 31 October 2018). https://www.cdc.gov/ehrlichiosis/stats/index.html.

Dawson, J.E., Anderson, B.E., Fishbein, D.B., Sanchez, J.L., Goldsmith, C.S., Wilson, K.H., Duntley, C.W., 1991. Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. J. Clin. Microbiol. 29, 2741–2745.

Fujita, H., Watanabe, Y., 1994. Ehrlichial organisms isolated from *Ixodes ovatus* ticks and field rodents in Japan. Ann. Rep. Ohara Hosp. 37, 13–17 (in Japanese).

Fukuoka, F., Kawasaki, Y., Dekio, S., Jidoi, J., Sugihara, K., Abe, K., Yamane, Y., 1989. A case of tick-bite from *Ixodes ovatus* Neumann–a report from west Japan. J. Dermatol. 16, 76–78.

Ijdo, J.W., Wu, C., Magnarelli, L.A., Stafford 3rd, K.C., Anderson, J.F., Fikrig, E., 2000. Detection of *Ehrlichia chaffeensis* DNA in *Amblyomma americanum* ticks in Connecticut and Rhode island. J. Clin. Microbiol. 38, 4655–4656.

Inayoshi, M., Naitou, H., Kawamori, F., Masuzawa, T., Ohashi, N., 2004. Characterization of *Ehrlichia* species from *Ixodes ovatus* ticks at the foot of Mt. Fuji, Japan. Microbiol. Immunol. 48, 737–745.

Kawahara, M., Ito, T., Suto, C., Shibata, S., Rikihisa, Y., Hata, K., Hirai, K., 1999. Comparison of *Ehrlichia muris* strains isolated from wild mice and ticks and serologic survey of humans and animals with *E. muris* as antigen. J. Clin. Microbiol. 37, 1123–1129.

Kawahara, M., Rikihisa, Y., Isogai, E., Takahashi, M., Misumi, H., Suto, C., Shibata, S., Zhang, C., Tsuji, M., 2004. Ultrastructure and phylogenetic analysis of' *Candidatus* Neoehrlichia mikurensis' in the family *Anaplasmataceae*, isolated from wild rats and found in *Ixodes ovatus* ticks. Int. J. Syst. Evol. Microbiol. 54, 1837–1843. https://doi.org/10.1099/ijs.0.63260-0.

Kawahara, M., Suto, C., Rikihisa, Y., Yamamoto, S., Tsuboi, Y., 1993. Characterization of ehrlichial organisms isolated from a wild mouse. J. Clin. Microbiol. 31, 89–96.

Li, H., Jiang, J.F., Liu, W., Zheng, Y.C., Huo, Q.B., Tang, K., Zuo, S.Y., Liu, K., Jiang, B.G., Yang, H., Cao, W.C., 2012. Human infection with *Candidatus*Neoehrlichia mikurensis, China. Emerg. Infect. Dis. 18, 1636–1639. https://doi.org/10.3201/eid1810.120594.

Lockhart, J.M., Davidson, W.R., Stallknecht, D.E., Dawson, J.E., Howerth, E.W., 1997. Isolation of Ehrlichia chaffeensis from wild white-tailed deer (Odocoileus virginianus) confirms their role as natural reservoir hosts. J. Clin. Microbiol. 35, 1681–1686.

Naitou, H., Kawaguchi, D., Nishimura, Y., Inayoshi, M., Kawamori, F., Masuzawa, T., Hiroi, M., Kurashige, H., Kawabata, H., Fujita, H., Ohashi, N., 2006. Molecular identification of Ehrlichia species and' *Candidatus* Neoehrlichia mikurensis' from ticks and wild rodents in Shizuoka and Nagano Prefectures, Japan. Microbiol. Immunol. 50, 45–51.

Paddock, C.D., Childs, J.E., 2003. *Ehrlichia chaffeensis*: a prototypical emerging pathogen. Clin. Microbiol. Rev. 16, 37–64.

Pritt, B.S., Allerdice, M.E.J., Sloan, L.M., Paddock, C.D., Munderloh, U.G., Rikihisa, Y., Tajima, T., Paskewitz, S.M., Neitzel, D.F., Hoang Johnson, D.K., Schiffman, E., Davis, J.P., Goldsmith, C.S., Nelson, C.M., Karpathy, S.E., 2017. Proposal to reclassify *Ehrlichia muris* as *Ehrlichia muris* subsp. *Muris* subsp. Nov. And description of *Ehrlichia muris* subsp. *Eauclairensis* subsp. nov., a newly recognized tick-borne pathogen of humans. Int. J. Syst. Evol. Microbiol. 67, 2121–2126. https://doi.org/10.1099/ijsem.0.001896.

Pritt, B.S., Sloan, L.M., Johnson, D.K., Munderloh, U.G., Paskewitz, S.M., McElroy, K.M., McFadden, J.D., Binnicker, M.J., Neitzel, D.F., Liu, G., Nicholson, W.L., Nelson, C.M., Franson, J.J., Martin, S.A., Cunningham, S.A., Steward, C.R., Bogumill, K., Bjorgaard, M.E., Davis, J.P., McQuiston, J.H., Warshauer, D.M., Wilhelm, M.P., Patel, R., Trivedi, V.A., Eremeeva, M.E., 2011. Emergence of a new pathogenic Ehrlichia species, Wisconsin and Minnesota, 2009. N. Engl. J. Med. 365, 422–429. https://doi.org/10.1056/NEJMoa1010493.

Shibata, S., Kawahara, M., Rikihisa, Y., Fujita, H., Watanabe, Y., Suto, C., Ito, T., 2000. New *Ehrlichia* species closely related to *Ehrlichia chaffeensis* isolated from *Ixodes ovatus* ticks in Japan. J. Clin. Microbiol. 38, 1331–1338.

Tabara, K., Arai, S., Kawabuchi, T., Itagaki, A., Ishihara, C., Satoh, H., Okabe, N., Tsuji, M., 2007. Molecular survey of *Babesia microti*, *Ehrlichia* species and *Candidatus* neoehrlichia mikurensis in wild rodents from Shimane Prefecture, Japan. Microbiol. Immunol. 51, 359–367.

Takano, A., Ando, S., Kishimoto, T., Fujita, H., Kadosaka, T., Nitta, Y., Kawabata, H., Watanabe, H., 2009. Presence of a novel *Ehrlichia*sp. in*Ixodes granulatus* found in Okinawa, Japan. Microbiol. Immunol. 53, 101–106. https://doi.org/10.1111/j.1348-0421.2008.00093.x.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729. https://doi.org/10.1093/molbev/mst197.

von Loewenich, F.D., Geissdorfer, W., Disque, C., Matten, J., Schett, G., Sakka, S.G., Bogdan, C., 2010. Detection of "*Candidatus* Neoehrlichia mikurensis" in two patients with severe febrile illnesses: evidence for a European sequence variant. J. Clin. Microbiol. 48, 2630–2635. https://doi.org/10.1128/jcm.00588-10.

Wei, F., Song, M., Liu, H., Wang, B., Wang, S., Wang, Z., Ma, H., Li, Z., Zeng, Z., Qian, J., Liu, Q., 2016. Molecular detection and characterization of zoonotic and veterinary pathogens in ticks from Northeastern China. Front. Microbiol. 7, 1913. https://doi.org/10.3389/fmicb.2016.01913.

Welinder-Olsson, C., Kjellin, E., Vaht, K., Jacobsson, S., Wennerås, C., 2010. First case of human "*Candidatus* Neoehrlichia mikurensis" infection in a febrile patient with chronic lymphocytic leukemia. J. Clin. Microbiol. 48, 1956–1959. https://doi.org/10.1128/jcm.02423-09.

Wennerås, C., 2015. Infections with the tick-borne bacterium *Candidatus* Neoehrlichia mikurensis. Clin. Microbiol. Infect. 21, 621–630. https://doi.org/10.1016/j.cmi.2015.02.030.

Woo, I.C., Baba, S., Suzuki, H., Kawabata, M., 1990. A case of tick bite with *Ixodes turdus* Nakatsuji–a report from Japan. J. Dermatol. 17, 56–58. https://doi.org/10.1111/j.1346-8138.1990.tb01610.x.

Yabsley, M.J., Varela, A.S., Tate, C.M., Dugan, V.G., Stallknecht, D.E., Little, S.E., Davidson, W.R., 2002. *Ehrlichia ewingii* infection in white-tailed deer (*Odocoileus virginianus*). Emerg. Infect. Dis. 8, 668–671. https://doi.org/10.3201/eid0807.020018.

Yamaguti, N., Tipton, V.J., Keegan, H.L., Toshioka, S., 1971. Ticks of Japan, Korea, and the Ryukyu islands. Brigham Young Univ. Sci. Bull. Bio. Ser. 15, 1–226.