Table 2
Genogroups of the isolates from Japanese sika deer and sequence similarities between the isolates and ruminant-associated closest *Bartonella* species based on *gltA*, *ftsZ*, *ribC*, and *rpoB*.

Strain	Lineage	GenBank accession numbers/ruminant-associated closest *Bartonella* species (sequence similarity, %)			
		ftsZ (788 bp)	*gltA* (312 bp)	*ribC* (557 bp)	*rpoB* (825 bp)
Honshu-8.1	A	AB703114/cap (99.1)	AB703124/cap (99.4)	AB703132/cho (96.8)	AB703142/B28980 (98.9)
Honshu-18.2	A	AB703115/cap (97.6)	Identical to Honshu-8.1	AB703133/B28980 (97.5)	AB703143/B28980 (98.4)
Yezo-25.1	A	Identical to B28980	Identical to B28980	AB703134/B28980 (99.8)	Identical to B28980
Honshu-9.1	B	AB703116/mel (98.6)	AB703125/cho, mel, sch (93.6)	AB703135/cho (98.6)	AB703144/sch (97.1)
Honshu-9.3	B	AB703117/mel (98.9)	AB703126/bov, cho, mel, sch (94.6)	AB703136/cho (97.8)	AB703145/sch (97.6)
Honshu-11.1	B	AB703118/mel (97.7)	AB703127/bov, cho, mel, sch (94.2)	AB703137/cho (97.5)	Identical to Honshu-9.3
Honshu-12.1	B	AB703119/mel (98.2)	AB703128/bov (94.6)	AB703138/cho (96.8)	AB703146/sch (96.5)
Honshu-18.5	B	AB703120/mel (98.1)	AB703129/bov (94.2)	AB703139/cho (97.7)	AB703147/sch (97.2)
Honshu-53.5	B	AB703121/mel (98.4)	AB703130/cho (94.2)	AB703140/cho (96.8)	AB703148/sch (97.1)
Honshu-58.5	B	AB703122/cap (99.0)	Identical to Honshu-9.1	Identical to Honshu-8.1	Identical to Honshu-9.3
Honshu-16.1	C	AB703123/cap (97.1)	AB703131/bov, cap (93.6)	AB703141/B28980 (94.8)	AB703149/cho (95.3)

Abbreviations: bov, *B. bovis* 91-4^T; cap, *B. capreoli* IBS193^T; cho, *B. chomelii* A828^T; mel, Candidatus *B. melophagi* K-2C; sch, *B. schoenbuchensis* R1^T; B28980, *B. capreoli* B28980 strain isolated from an elk in the USA.

Table 3
Sequence similarities of *gltA* and *rpoB* among the isolates in lineages B and C.

Isolates/lineages	Sequence similarities (%) of *gltA* on the lower left and *rpoB* on the upper right							
	Honsyu-9.1	Honsyu-9.3	Honsyu-11.1	Honsyu-12.1	Honsyu-18.5	Honsyu-53.5	Honsyu-58.5	Honsyu-16.1
Honshu-9.1/B	–	98.1	98.1	97.9	97.9	99.8	98.1	94.5
Honshu-9.3/B	99.0	–	100	97.2	99.9	98.1	100	95.0
Honshu-11.1/B	98.7	99.0	–	97.2	99.9	98.1	100	95.0
Honshu-12.1/B	99.0	99.4	99.7	–	97.1	97.6	97.2	94.7
Honshu-18.5/B	98.7	99.0	99.4	99.7	–	97.9	99.9	94.9
Honshu-53.5/B	98.7	98.4	98.1	98.4	98.1	–	98.1	94.5
Honshu-58.5/B	100	99.0	98.7	99.0	98.7	98.7	–	95.0
Honshu-16.1/C	94.6	94.2	94.6	94.9	94.6	93.9	94.6	–

countries [3,17,20]. In contrast, isolation of *Bartonella* was confirmed only in deer keds infesting wild deer in Germany [3]. Such findings strongly suggest that deer keds may be potential vectors responsible for the transmission of *Bartonella* species among sika deer. Future studies are needed to investigate *Bartonella* prevalence of deer keds collected from sika deer.

The strains in lineage A, identified as *B. capreoli*, were prevalent in both Honshu and Yezo deer, suggesting that this *Bartonella* species is common in wild deer throughout Japan. Bai et al. [8] have suggested that *B. capreoli* may be widely distributed among deer of the genus *Cervus*. Thus, *B. capreoli* may be a common species in *Cervus* deer around the world, including Japan.

La Scola et al. [32] reported that *gltA* and *rpoB* were the most appropriate genes for discriminating species of the genus *Bartonella* and proposed that gene sequence similarities <96.0% in *gltA* and <95.4% in *rpoB* between *Bartonella* species could be used as cut-off values for the designation of a novel *Bartonella* species. The strains in lineage B could be clearly differentiated by the sequence similarities (93.6–94.6%) in the *gltA* gene by the cut-off value. On the other hand, the sequence similarity (96.5–97.6%) in the *rpoB* gene of the lineage was above the cut-off value. A similar phenomenon was observed in designating novel *Bartonella* species isolated from wild rodents [33]. Although most of the strains in lineage B were relatively close to *B. melophagi* for the *ftsZ* gene and *B. chomelii* for the *ribC* gene, the discriminatory power with both of the genes have been reported to be inadequate for identification of *Bartonella* species [8,32]. However, in the phylogenetic analysis based on the concatenated sequences, the strains in lineage B formed a distinct cluster from other known *Bartonella* species, supporting the view that lineage B is a novel *Bartonella* species. Strain Honshu-16.1 in lineage C was confirmed to be a novel *Bartonella* species by the sequence similarities in both *gltA* (93.6%) and *rpoB* (95.3%) genes. Furthermore, the strain was distinct from other known *Bartonella* species based on the phylogenetic analysis of concatenated sequences. Based on the sequence similarities in the *gltA* (93.9–94.6%) and *rpoB* (94.5–95.0%) genes between the strains in lineages B and

Table 4
Prevalence of *Bartonella* lineages in wild Japanese sika deer.

Subspecies of deer	No. of deer examined	No. of positive deer (%)			
		Lineage A	Lineage B	Lineage C	Lineages A and B
Honshu deer	37	12 (32.4)	20 (54.1)	1 (2.7)	8 (21.6)
Yezo deer	18	9 (50.0)	0	0	0
Total	55	21 (38.2)	20 (36.4)	1 (1.8)	8 (14.5)

C, both lineages belonged to different species. From these results, Japanese sika deer harbor three different *Bartonella* species, including *B. capreoli* and two novel species.

Interestingly, the sequences of *ftsZ*, *gltA*, and *rpoB* genes of strain Yezo-25.1 in lineage A were identical to those from *B. capreoli* B28980, which was obtained from an elk in the USA. Japanese sika deer and elk, otherwise known as wapiti or American red deer, were estimated to have diverged into different species 0.57 million years ago [34]. Although this appears to be sufficient time for mutations to occur within the housekeeping genes of *Bartonella* species, no sequence differences were observed in the three genes examined. Since the reason for the accordance between both strains is unclear in our study, more strains derived from Yezo deer and North American elk need to be analyzed to resolve the relationships of *Bartonella* species in deer from Japan and North America.

Honshu deer harbored a higher genetic diversity of *Bartonella* species in comparison with Yezo deer. Japanese sika deer are divided into two genetically distinct groups based on the mitochondrial D-loop region sequences: the Northern and Southern groups. Both deer groups have been estimated to have migrated separately to the Japanese archipelago during the Middle to Late Pleistocene across land bridges from the Asian continent [35]. Nara and Wakayama Prefectures are located at the boundary region of both groups, and some genetic crossing has been suggested to have occurred in this region [36]. Thus, the genetic crossing between Southern and Northern groups might have contributed to the genetic diversity of *Bartonella* species in Honshu deer.

In conclusion, we confirmed that Japanese sika deer harbor *B. capreoli* and two novel *Bartonella* species, with high percentages of infections. The present study also suggested that ectoparasites such as deer ked may be the potential vector of *Bartonella* in Japanese sika deer. Further studies are necessary to explain the role of the ectoparasites in the transmission of *Bartonella* and to determine the potential threat of *Bartonella* species from deer to humans, as reported for *B. melophagi* from sheep to humans [15].

Acknowledgments

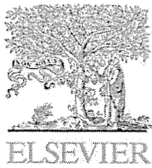
This work was supported in part by a Grant-in-Aid for Scientific Research (No. 23580429), and Strategic Research Base Development Program, International Research on Epidemiology of Zoonoses and Training for Young Researchers by Ministry of Education, Culture, Sports, Science and Technology, Japan. It was also supported in part by a Grant-in-Aid from Ministry of Health, Labour and Welfare, Japan.

References

[1] Chomel BB, Kasten RW, Floyd-Hawkins K, Chi B, Yamamoto K, Roberts-Wilson J, et al. Experimental transmission of *Bartonella henselae* by the cat flea. Journal of Clinical Microbiology 1996;34(8):1952–6.
[2] Chang CC, Chomel BB, Kasten RW, Romano V, Tietze N. Molecular evidence of *Bartonella* spp. in questing adult *Ixodes pacificus* ticks in California. Journal of Clinical Microbiology 2001;39(4):1221–6.
[3] Dehio C, Sauder U, Hiestand R. Isolation of *Bartonella schoenbuchensis* from *Lipoptena cervi*, a blood-sucking arthropod causing deer ked dermatitis. Journal of Clinical Microbiology 2004;42(11):5320–3.
[4] Chomel BB, Boulouis HJ, Maruyama S, Breitschwerdt EB. *Bartonella* spp. in pets and effect on human health. Emerging Infectious Diseases 2006;12(3):389–94.
[5] Chomel BB, Boulouis HJ, Breitschwerdt EB, Kasten RW, Vayssier-Taussat M, Birtles RJ, et al. Ecological fitness and strategies of adaptation of *Bartonella* species to their hosts and vectors. Veterinary Research 2009;40(2):29.
[6] Deng H, Le Rhun DC, Cotte V, Buffet JP, Read A, Birtles RJ, et al. Strategies of exploitation of mammalian reservoirs by *Bartonella* species. Veterinary Research 2012;43(1):15.
[7] Bermond D, Boulouis HJ, Heller R, Van Laere G, Monteil H, Chomel BB, et al. *Bartonella bovis* Bermond et al. sp. nov. and *Bartonella capreoli* sp. nov., isolated from European ruminants. International Journal of Systematic and Evolutionary Microbiology 2002;52(Pt 2): 383–90.
[8] Bai Y, Cross PC, Malania L, Kosoy M. Isolation of *Bartonella capreoli* from elk. Veterinary Microbiology 2010;148(2–4):329–32.
[9] Dehio C, Lanz C, Pohl R, Behrens P, Bermond D, Piemont Y, et al. *Bartonella schoenbuchii* sp. nov., isolated from the blood of wild roe deer. International Journal of Systematic and Evolutionary Microbiology 2001;51(Pt 4):1557–65.
[10] Rolain JM, Rousset E, La Scola B, Duquesnel R, Raoult D. *Bartonella schoenbuchensis* isolated from the blood of a French cow. Annals of the New York Academy of Sciences 2003;990:236–8.
[11] Chang CC, Chomel BB, Kasten RW, Heller RM, Ueno H, Yamamoto K, et al. *Bartonella* spp. isolated from wild and domestic ruminants in North America. Emerging Infectious Diseases 2000;6(3):306–11.
[12] Maillard R, Riegel P, Barrat F, Bouillin C, Thibault D, Gandoin C, et al. *Bartonella chomelii* sp. nov., isolated from French domestic cattle (*Bos taurus*). International Journal of Systematic and Evolutionary Microbiology 2004;54(Pt 1):215–20.
[13] Mediannikov O, Davoust B, Cabre O, Rolain JM, Raoult D. Bartonellae in animals and vectors in New Caledonia. Comparative Immunology, Microbiology and Infectious Diseases 2011;34(6):497–501.
[14] Bemis DA, Kania SA. Isolation of *Bartonella* sp. from sheep blood. Emerging Infectious Diseases 2007;13(10):1565–7.
[15] Maggi RG, Kosoy M, Mintzer M, Breitschwerdt EB. Isolation of Candidatus *Bartonella melophagi* from human blood. Emerging Infectious Diseases 2009;15(1):66–8.
[16] Maillard R, Petit E, Chomel B, Lacroux C, Schelcher F, Vayssier-Taussat M, et al. Endocarditis in cattle caused by *Bartonella bovis*. Emerging Infectious Diseases 2007;13(9):1383–5.
[17] Halos L, Jamal T, Maillard R, Girard B, Guillot J, Chomel B, et al. Role of Hippoboscidae flies as potential vectors of *Bartonella* spp. infecting wild and domestic ruminants. Applied and Environment Microbiology 2004;70(10):6302–5.
[18] Matsumoto K, Berrada ZL, Klinger E, Goethert HK, Telford 3rd SR. Molecular detection of *Bartonella schoenbuchensis* from ectoparasites of deer in Massachusetts. Vector-Borne and Zoonotic Diseases 2008;8(4):549–54.
[19] Reeves WK, Nelder MP, Cobb KD, Dasch GA. *Bartonella* spp. in deer keds, *Lipoptena mazamae* (Diptera: Hippoboscidae), from Georgia and South Carolina, USA. Journal of Wildlife Diseases 2006;42(2):391–6.
[20] Skotarczak B, Adamska M. Detection of *Bartonella* DNA in roe deer (*Capreolus capreolus*) and in ticks removed from deer. European Journal of Wildlife Research 2005;51(4):287–90.
[21] Matsuda H, Kaji K, Uno H, Hirakawa H, Saitoh T. A management policy for sika deer based on sex-specific hunting. Researches on Population Ecology 1999;41(2):139–49.
[22] Tei S, Kitajima N, Takahashi K, Mishiro S. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet 2003;362(9381):371–3.
[23] Wilson PR, Davies PR. Deer diseases and human health: an internationally significant issue. In: Conference proceedings of the 5th international deer biology congress. 2003. p. 27–33.
[24] Maruyama S, Nakamura Y, Kabeya H, Tanaka S, Sakai T, Katsube Y. Prevalence of *Bartonella henselae*, *Bartonella clarridgeiae* and the 16S rRNA gene types of *Bartonella henselae* among pet cats in Japan. Journal of Veterinary Medical Science 2000;62(3):273–9.
[25] Birtles RJ, Raoult D. Comparison of partial citrate synthase gene (*gltA*) sequences for phylogenetic analysis of *Bartonella* species. International Journal of Systematic Bacteriology 1996;46(4):891–7.
[26] Renesto P, Gouvernet J, Drancourt M, Roux V, Raoult D. Use of *rpoB* gene analysis for detection and identification of *Bartonella* species. Journal of Clinical Microbiology 2001;39(2):430–7.
[27] Zeaiter Z, Liang Z, Raoult D. Genetic classification and differentiation of *Bartonella* species based on comparison of partial *ftsZ* gene sequences. Journal of Clinical Microbiology 2002;40(10): 3641–7.

[28] Inoue K, Kabeya H, Kosoy MY, Bai Y, Smirnov G, McColl D, et al. Evolutional and geographical relationships of *Bartonella grahamii* isolates from wild rodents by multi-locus sequencing analysis. Microbial Ecology 2009;57(3):534–41.
[29] Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN, editor. Mammalian protein metabolism. New York: Academic Press; 1969. p. 21–132.
[30] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 2007;24(8):1596–9.
[31] Adamska M. Wild ruminants in the area of the North-Western Poland as potential reservoir hosts of *Bartonella schoenbuchensis* and *B. bovis*. Acta Parasitologica 2008;53(4): 407–10.
[32] La Scola B, Zeaiter Z, Khamis A, Raoult D. Gene-sequence-based criteria for species definition in bacteriology: the *Bartonella* paradigm. Trends in Microbiology 2003;11(7):318–21.
[33] Gundi VA, Taylor C, Raoult D, La Scola B. *Bartonella rattaustraliani* sp. nov., *Bartonella queenslandensis* sp. nov. and *Bartonella coopersplainsensis* sp. nov., identified in Australian rats. International Journal of Systematic and Evolutionary Microbiology 2009;59(Pt 12):2956–61.
[34] Kuwayama R, Ozawa T. Phylogenetic relationships among European red deer, wapiti, and sika deer inferred from mitochondrial DNA sequences. Molecular Phylogenetics and Evolution 2000;15(1):115–23.
[35] Nagata J, Masuda R, Tamate HB, Hamasaki S, Ochiai K, Asada M, et al. Two genetically distinct lineages of the sika deer, *Cervus nippon*, in Japanese islands: comparison of mitochondrial D-loop region sequences. Molecular Phylogenetics and Evolution 1999;13(3):511–9.
[36] Goodman SJ, Tamate HB, Wilson R, Nagata J, Tatsuzawa S, Swanson GM, et al. Bottlenecks, drift and differentiation: the population structure and demographic history of sika deer (*Cervus nippon*) in the Japanese archipelago. Molecular Ecology 2001;10(6):1357–70.

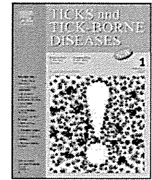
Ticks and Tick-borne Diseases 3 (2012) 259–261



Contents lists available at SciVerse ScienceDirect

Ticks and Tick-borne Diseases

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



Short communication

A novel relapsing fever *Borrelia* sp. infects the salivary glands of the molted hard tick, *Amblyomma geoemydae*

Ai Takano[a,b], Chieko Sugimori[a], Hiromi Fujita[c], Teruki Kadosaka[d], Kyle R. Taylor[e], Toshio Tsubota[e], Satoru Konnai[e], Tomoko Tajima[f], Kozue Sato[a], Haruo Watanabe[a,b], Makoto Ohnishi[a], Hiroki Kawabata[a,b,*]

[a] *Department of Bacteriology-I, National Institute of Infectious Diseases, Tokyo, Japan*
[b] *United Graduate School of Agricultural Science & Veterinary Science, Gifu University, Gifu, Japan*
[c] *Ohara Research Laboratory, Ohara General Hospital, Fukushima, Japan*
[d] *Department of Parasitology, Aichi Medical University, Aichi, Japan*
[e] *Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Hokkaido, Japan*
[f] *Laboratory of Veterinary Microbiology, School of Life and Environmental Science, Osaka Prefecture University, Osaka, Japan*

ARTICLE INFO

Article history:
Received 25 March 2012
Received in revised form 6 June 2012
Accepted 15 June 2012

Keywords:
Hard tick-borne relapsing fever
Amblyomma geoemydae
Transstadial transmission

ABSTRACT

A novel relapsing fever *Borrelia* sp. was found in *Amblyomma geoemydae* in Japan. The novel *Borrelia* sp. was phylogenetically related to the hard (ixodid) tick-borne relapsing fever *Borrelia* spp. *Borrelia miyamotoi* and *B. lonestari*. The novel relapsing fever *Borrelia* sp. was detected in 39 *A. geoemydae* (39/274: 14.2%), of which 14 (14/274: 5.1%) were co-infected with the novel relapsing fever *Borrelia* sp. and *Borrelia* sp. tAG, one of the reptile-associated borreliae. Transstadial transmission of the novel relapsing fever *Borrelia* sp. occurred in the tick midgut and the salivary glands, although *Borrelia* sp. tAG was only detected in the tick midgut. The difference of the borrelial niche in molted ticks might be associated with borrelial characterization.

© 2012 Published by Elsevier GmbH.

Introduction

The spirochete genus *Borrelia* is comprised of arthropod-borne infectious agents classified into 3 major groups by phylogenetic analysis: the Lyme disease (LD) *Borrelia* spp., the reptile-associated (REP) *Borrelia* spp., and the relapsing fever (RF) *Borrelia* spp. (Parola and Raoult, 2001; Takano et al., 2010, 2011). The LD *Borrelia* spp. include 3 LD agents, *B. burgdorferi*, *B. garinii*, and *B. afzelii*, which are transmitted by hard (ixodid) ticks of the genus *Ixodes* (Parola and Raoult, 2001). The REP *Borrelia* spp. have been recently discovered in reptiles and their associated hard ticks, genera *Amblyomma* and *Hyalomma* (Takano et al., 2010). The RF *Borrelia* spp. are transmitted by ticks and human body lice. Most tick-borne RF *Borrelia* spp. are transmitted by soft (argasid) ticks excluding 3 species of RF *Borrelia* spp., *B. miyamotoi*, *B. lonestari*, and *B. theileri*. These *Borrelia* spp. are found in hard ticks: in ticks of the genus *Ixodes*, in *Amblyomma americanum*, and in ticks of the genus *Rhipicephalus*, respectively (Barbour, 2005; Steere et al., 2005). In this study, we found a novel *Borrelia* sp. in *Amblyomma geoemydae* which clusters with hard tick-borne RF *Borrelia* spp. based on phylogenetic analysis, and we investigated the transstadial transmission of the borrelial organism in molted ticks.

Materials and methods

For this study, we examined 274 *A. geoemydae* ticks collected from Ryukyu yellow-margined box turtles (*Cuora flavomarginata evelynae*) and Asian yellow pond turtles (*Mauremys mutica kami*) found on Iriomote-jima Island, Okinawa prefecture, Japan. Of these 274 ticks, 173 ticks (124 larvae, 29 nymphs, 4 males, and 16 females) were analyzed without incubation as blood-feeding ticks. Another 101 ticks (87 larvae, 14 nymphs) were allowed to molt to 87 nymphs, 6 males, and 8 females in our laboratory. Molted ticks were obtained by incubating engorged larvae and nymphs at 24 °C. After molting, the ticks were incubated at 24 °C for an additional month (Takano et al., 2011). All ticks were subjected to DNA preparation of the whole body or in part (salivary glands and midgut) after dissection. The total DNA of ticks was prepared as previously described (Takano et al., 2011). For borrelial DNA detection, conventional PCRs for the flagellin gene (*flaB*) and the glycerophosphoryl diester phosphodiesterase gene (*glpQ*) were performed as previously described (Takano et al., 2011). PCR products were purified by using ExoSAP-IT (GE Healthcare UK Ltd.,

* Corresponding author at: Department of Bacteriology-I, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku, Tokyo, 162-8640, Japan.
Tel.: +81 3 4582 2682; fax: +81 3 5285 1163.
E-mail address: kbata@nih.go.jp (H. Kawabata).

1877-959X/$ – see front matter © 2012 Published by Elsevier GmbH.
http://dx.doi.org/10.1016/j.ttbdis.2012.06.003

Table 1
Number of *Borrelia* spp.-positive ticks.

Stage	Number of ticks examined	*Borrelia* spp.-positive ticks	Infection		
			Borrelia sp. AGRF	Co-infection	*Borrelia* sp. tAG
Blood-feeding					
Larva	124	48	15(12.1%)	10(8.1%)	23(18.5%)
Nymph	29	15	2(6.9%)	3(10.3%)	10(34.5%)
Male	4	3	0 (0%)	1 (25%)	2 (50%)
Female	16	10	3(18.8%)	0 (0%)	7(43.8%)
Subtotal	173	76	20(11.6%)	14 (8%)	42(24.3%)
Molted					
Nymph	87	29	3(3.4%)	0 (0%)	26(29.9%)
Male	6	2	0 (0%)	0 (0%)	2(33.3%)
Female	8	4	2 (25%)	0 (0%)	2 (25%)
Subtotal	101	35	5 (5%)	0 (0%)	30(29.7%)
Total	274	111	25(9.1%)	14(5.1%)	72(26.3%)

UK) and were directly sequenced (ABI Prism 3130xl Genetic Analyzer; Life Technologies Corporation, USA). Sequence data were analyzed using MEGA4 software (http://www.megasoftware.net) (Tamura et al., 2007). The construction of phylogenetic tree and bootstrap tests were carried out according to the protocol previously described (Takano et al., 2011). In this study, 76 feeding ticks (76/173, 43.9%) and 35 molted ticks (35/101, 34.7%) were borrelial DNA-positive by PCR for both genes (Table 1). From sequencing analysis of *flaB* amplicons, these borreliae were classified into 2 phylogenetic groups, REP *Borrelia* spp. and RF *Borrelia* spp. One group was clustered with a REP *Borrelia* sp., *Borrelia* sp. tAG, as previously described (Takano et al., 2011). The other group was an unknown *Borrelia* sp. (preliminarily designated as *Borrelia* sp. AGRF in this study), and it clustered with hard tick-borne RF *Borrelia* spp. (Figs. S1, S2, and S3). To characterize *Borrelia* sp. AGRF, partial *flaB* sequence (397 bp), 16S rRNA gene (*16S rDNA*) (1490 bp), and *glpQ* (1014 bp) were determined according to previously described methods (Takano et al., 2011), and representative sequences of these genes were deposited in GenBank as AB529429, AB529435, and AB529433, respectively.

It was thought that 2 different *Borrelia* spp. were occasionally co-infected within a single tick. To determine mixed infections with these borreliae, we established a *Borrelia* sp. AGRF detection system based on quantitative real-time PCR (qPCR) technology and performed qPCR on ticks that were positive for borrelial DNAs. From sequence analysis of the *flaB* gene of *Borrelia* sp. AGRF, the *Borrelia* sp. was expected to be clonal because nucleic acid substitution rate was less than 0.5%. Primer Express software version 2.0 (Applied Biosystems, Foster City, CA) was used to design oligonucleotide primers and a TaqMan probe, sequence-specific for the *Borrelia* sp. AGRF *flaB*. Forward and reverse primers were MGB_92F (5′-GCTGGAGCACAAGCTTCATG-3′) and MGB_242R (5′-CCTGTTGTGCCCCTTCTTGA-3′), respectively. The dye-labeled probe is MGB_P174 (VIC-CTAATGTTGCAAATCTCTTT-3′) with the 3′ end modified with a minor groove-binding protein (Applied Biosystems). The qPCR was performed using Premix Ex TaqTM (Perfect Real Time) (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions and run on an ABI PRISM 7000 system (Life Technologies Corporation, Gaithersburg, MD). For analysis of qPCR, the threshold line was fixed at 0.2 to avoid detection of nonspecific fluorescence. To evaluate a specificity of the qPCR (designated as *flaB*-qPCR in this study), a total of 37 borrelial strains were used: 32 REP *Borrelia* spp. (8 of *Borrelia* sp. tAG, 9 of *B. turcica*, 9 of *Borrelia* sp. GP, 4 of *Borrelia* sp. BF, *Borrelia* sp. TA2, and *Borrelia* sp. Tick98 M), 5 LD *Borrelia* spp. (*B. burgdorferi* B31, *B. garinii* 20047, *B. afzelii* VS461, *B. valaisiana* VS116, and *B. lusitaniae* PotiB2). As a result, none of the REP *Borrelia* spp. and LD *Borrelia* spp. was detected in this assay. To determine the sensitivity of *flaB*-qPCR, an external standard template containing 10^1 to 10^8 copies of the *Borrelia* sp. AGRF *flaB* was included in a run. For establishing the external standard plasmids, a fragment of the *Borrelia* sp. AGRF *flaB* (397 bp) was amplified by PCR using primer set BflaPAD and BflaPDU and cloned as previously described (Takano et al., 2011). As a result, the limit of detection consistently observed was a minimum of 10 plasmid copies (data not shown). In addition to the *Borrelia* sp. AGRF detection system, we performed qPCR to detect the *Borrelia* sp. tAG phosphotransferase system maltose-specific enzyme IICB component gene (*glvC*) (Takano et al., 2011), for *Borrelia sp. AGRF-positive ticks (Fig. S4)*. As a result, 14 of 274 ticks (10 larvae, 3 nymphs, and 1 male tick) were found to have mixed infections of these borreliae (Table 1 and Fig. S4).

Results and discussion

In a previous study, we found that *Borrelia* sp. tAG colonized the midgut, but not the salivary glands after tick molting (transstadial transmission occurred in the midgut only (Takano et al., 2011)). On the other hand, it was unknown how the transstadial transmission of *Borrelia* sp. AGRF occurred. To elucidate the transstadial transmission niches of *Borrelia* sp. AGRF in ticks, the presence of *Borrelia* sp. AGRF in one salivary gland and the midgut of molted ticks were investigated. Consequently, we detected DNA fragments of *Borrelia* sp. AGRF from both the midgut and the salivary glands of 5 molted ticks. The results were confirmed by immunofluorescence assay of the second and remaining salivary gland. Confocal imaging analysis suggested that the borrelial cells had entered into the salivary gland of molted ticks (Fig. 1).

Prior to this study, a hard tick-borne RF borrelia, *B. miyamotoi*, was suggested to be a co-infection with LD borreliae in a tick (Barbour et al., 2009; Scoles et al., 2001). In addition to these cases, in this study, we found a novel hard tick-borne RF *Borrelia* sp. in *A. geoemydae* and showed evidence of co-infection of *Borrelia* sp. AGRF and *Borrelia* sp. tAG within ticks. In this study, all co-infected ticks were detected among *Borrelia* sp. AGRF-positive ticks (Fig. S4). We used *flaB*-PCR as a first screening. However, the screening may preferentially detect the dominant *Borrelia* species in tick samples. In this study, all co-infected ticks were observed in the tick group which were *Borrelia* sp. AGRF-positive in the first screening (Fig. S4). This result may be due to the borrelial dominance in ticks, similar to that noted for co-infection of *B. miyamotoi* and *B. burgdorferi* in *Ixodes scapularis* nymphs (Barbour et al., 2009). Moreover, we demonstrated the transstadial transmission niches of the hard tick-borne RF *Borrelia* sp. in the vector tick (Fig. 1). Since *Borrelia* sp. AGRF was detected from the salivary gland and the midgut of molted ticks, the transstadial transmission niches of *Borrelia* sp. tAG and *Borrelia* sp. AGRF were somewhat different in the same tick, *A. geoemydae*. According to some reports, it has been shown that transstadial transmission of RF borreliae (e.g. *B. hermsii*) does not occur only in the midgut, but also in the salivary grand of molted

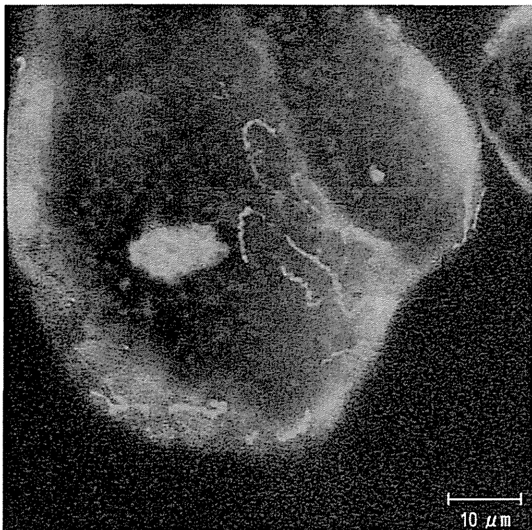


Fig. 1. Borrelial cells (red) in salivary glands of molted *A. geoemydae*. Immunofluorescence assay (IFA) was performed as previously described (Takano et al., 2010). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ticks (Barbour, 2005). This phenotype of RF borreliae was thought to be required for borreliae to be transmitted by fast feeders (soft ticks). This phenotype was not observed, however, in LD borreliae (e.g. *B. burgdorferi*) and REP borreliae (*Borrelia* sp. tAG, our previous study), which are transmitted by hard ticks (Piesman et al., 2001; Takano et al., 2011). In this study, the borrelial phenotype (able to infect the salivary gland of molted ticks) was observed in hard tick-borne RF borreliae (*Borrelia* sp. AGRF in this study), although this phenotype might not be required for *Borrelia* sp. AGRF in *A. geoemydae* (slow feeders). From this result, we speculate that RF borreliae (including hard tick-borne RF borreliae) generally show this phenotype, and the transstadial transmission of *Borrelia* sp. AGRF in the salivary glands may be due to characteristics of the borreliae itself, rather than characteristics (e.g. blood-feeding time) of the vector tick.

In this study, it was suggested that the prevalence of *Borrelia* sp. AGRF was reduced through molting when compared to that of *Borrelia* sp. tAG (Table S1, chi-square test, $P < 0.01$). It was reported that the rate of transstadial transmission from larvae to adult ticks was only 61% in the case of the hard tick-borne RF *Borrelia* sp. in *I. scapularis* ticks (Scoles et al., 2001). In the case of soft tick-borne RF borreliae, a high rate of transstadial transmission has been suggested for *B. hermsii* in *Ornithodoros hermsi* (Lopez et al., 2011). Moreover, the transstadial transmission rate of LD borreliae in vector ticks was estimated at over 90% (Dolan et al., 1998; Piesman, 1995). Although the reason is unclear, the inefficiency in transstadial transmission of hard tick-borne RF borreliae compared to that of soft tick-borne RF borreliae and LD borreliae might be due to difference(s) in characteristics of the borreliae. Furthermore, in the case of hard tick-borne RF *Borrelia* sp., transovarial transmission occurred in *I. scapularis* (Scoles et al., 2001). Thus, we speculate that *Borrelia* sp. AGRF is transovarially transmitted in *A. geoemydae* because *Borrelia* sp. AGRF is phylogenetically related to hard tick-borne RF *Borrelia* spp. However, since we could not collect *Borrelia* sp. AGRF-positive engorged females, transovarial transmission of *Borrelia* sp. AGRF could not be evaluated. To elucidate the mechanism of transstadial transmission of RF borreliae in ticks, further analysis will be required. Our findings may contribute to the elucidation of transstadial transmission mechanisms of borreliae in ticks.

Acknowledgements

This study was funded by the Global Environment Research Fund (F-3 and F-081; Leader: K. Goka) of the Ministry of the Environment AND Japan 2008, and a grant for research on emerging and reemerging infectious diseases from the Japanese Ministry of Health, Labor, and Welfare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2012.06.003.

References

Barbour, A.G., 2005. Relapsing fever. In: Goodman, J.L., Dennis, D.T., Sonenshine, D.E. (Eds.), Tick-borne Diseases of Humans. ASM Press, Washington, DC, pp. 268–291.

Barbour, A.G., Bunikis, J., Travinsky, B., Hoen, A.G., Diuk-Wasser, M.A., Fish, D., Tsao, J.I., 2009. Niche partitioning of *Borrelia burgdorferi* and *Borrelia miyamotoi* in the same tick vector and mammalian reservoir species. Am. J. Trop. Med. Hyg. 81, 1120–1131.

Dolan, M.C., Piesman, J., Mbow, M.L., Maupin, G.O., Péter, O., Brossard, M., Golde, W.T., 1998. Vector competence of *Ixodes scapularis* and *Ixodes ricinus* (Acari: Ixodidae) for three genospecies of *Borrelia burgdorferi*. J. Med. Entomol. 35, 465–470.

Lopez, J.E., Mccoy, B.N., Krajacich, B.J., Schwan, T.G., 2011. Acquisition and subsequent transmission of *Borrelia hermsii* by the soft tick *Ornithodoros hermsi*. J. Med. Entomol. 48, 891–895.

Parola, P., Raoult, D., 2001. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin. Infect. Dis. 32, 897–928.

Piesman, J., 1995. Dispersal of the Lyme disease spirochete *Borrelia burgdorferi* to salivary glands of feeding nymphal *Ixodes scapularis* (Acari: Ixodidae). J. Med. Entomol. 32, 519–521.

Piesman, J., Schneider, B.S., Zeidner, N.S., 2001. Use of quantitative PCR to measure density of *Borrelia burgdorferi* in the midgut and salivary glands of feeding tick vectors. J. Clin. Microbiol. 39, 4145–4148.

Scoles, G.A., Papero, M., Beati, L., Fish, D., 2001. A relapsing fever group spirochete transmitted by *Ixodes scapularis* ticks. Vector Borne Zoonotic. Dis. 1, 21–34.

Steere, A.C., Coburn, J., Glickstein, L., 2005. Lyme borreliosis. In: Goodman, J.L., Dennis, D.T., Sonenshine, D.E. (Eds.), Tick-borne Diseases of Humans. ASM Press, Washington, DC, pp. 176–206.

Takano, A., Goka, K., Une, Y., Shimada, Y., Fujita, H., Shiino, T., Watanabe, H., Kawabata, H., 2010. Isolation and characterization of a novel *Borrelia* group of tick-borne borreliae from imported reptiles and their associated ticks. Environ. Microbiol. 12, 134–146.

Takano, A., Fujita, H., Kadosaka, T., Konnai, S., Tajima, T., Watanabe, H., Ohnishi, M., Kawabata, H., 2011. Characterization of reptile-associated *Borrelia* sp. in the vector tick, *Amblyomma geoemydae*, and its association with Lyme disease and relapsing fever *Borrelia* spp. Environ. Microbiol. Rep. 3, 632–637.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.