Detection of Borrelia garinii, Borrelia tanukii and Borrelia sp. closely related to Borrelia valaisiana in Ixodes ticks removed from dogs and cats in Japan.

Veterinary Parasitology Revised 15 June, 2005

Short Communication

Detection of *Borrelia garinii, Borrelia tanukii* and *Borrelia* sp. closely related to *Borrelia valaisiana* in *Ixodes* ticks removed from dogs and cats in Japan

Hiroko Hiraoka [a], Yojiro Shimada [b], Yoshimi Sakata [c], Malaika Watanabe [a], Kazuhito Itamoto [a], Masaru Okuda [a], Toshiyuki Masuzawa [d], Hisashi Inokuma [e, *]

[a] *Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515*

[b] *Nippon Zenyaku Kogyo Co., Ltd, Koriyama, Fukushima 963-0196*

[c] *Merial Japan Ltd., Tokyo 100-0014*

[d] *Department of Microbiology, School of Pharmaceutic Science, University of Shizuoka, and COE program in the 21st Century, Shizuoka, 422-8526*

[e] *Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555 Japan.*

* Corresponding author: H.INOKUMA

Laboratory of Veterinary Internal Medicine,

Obihiro University of Agriculture and Veterinary Medicine

Inada, Obihiro, 080-8555 Japan

TEL/FAX +81-155-49-5370, e-mail: inokuma@obihiro.ac.jp

Abstract

Ticks removed from 1136 dogs and 134 cats all over Japan were examined for *Borrelia* infection by PCR and sequencing. The 5S-23S rDNA intergenic spacer of *Borrelia* was detected from 2 *Ixodes persulcatus* ticks from 2 dogs and 2 unidentified *Ixodes* spp. from another 2 dogs in Hokkaido, and 2 *Ixodes granulatus* ticks from 2 cats in Okinawa. Additional 2 I. granulatus from the same cats also showed positive. Sequence analysis of the PCR products revealed that the one from Hokkaido was similar to *B. garinii,* the three from Hokkaido to *B. tanukii*, and the four from Okinawa to a novel *Borrelia* sp. closely related to *B. valaisiana.* The data was confirmed by analysis of the flagellin gene sequence. Infected ticks carried by companion animals can be introduced into the human environment.

1. Introduction

Ticks can transmit various pathogens, including bacteria and protozoa. Recently, interest in ticks of domestic animals has increased, because of emerging and re-emerging tick-borne diseases and their zoonotic nature. *Borrelia* is one such tick-borne bacteria that causes Lyme disease in both man and animals. Many *Borrelia* species are known to exist in the world. *Borrelia burgdorferi sensu stricto* is the most well known causative agent of Lyme disease and has been isolated in North America and European countries (Baranton et al., 1992). *Borrelia garinii* and *Borrelia afzelii* are known to be pathogenic to human and animals (Baraton et al. 1992), and they are transmitted by *Ixodes persulcatus* in Japan (Masuzawa et al., 1991; Miyamoto and Masuzawa, 2002; Nakao et al., 1994). *Borrelia japonica, Borrelia tanukii* and *Borrelia turdi* have also been isolated in Japan from *Ixodes ovatus, Ixodes tanuki*, and *Ixodes turdus*, respectively (Fukunaga et al., 1996a, b; Kawabata et al., 1993). More recently, a new *Borrelia* species closely related to *Borrelia valaisiana* has been isolated from ticks and rodents in China, Taiwan, Korea and Okinawa, the southern most islands of Japan (Takada et al., 2001; Masuzawa et al., 2004), though the pathogenesities are still unknown.

Dogs and cats are often exposed to a large number of tick species, depending upon the distribution of these arthropod vectors in the environment (Shimada et al., 2003a, b). As dogs and cats are in close contact with human beings, they are possible carriers of tick vectors to the human environment. However, there have been few reports of tick-borne *Borrelia* in ticks recovered from dogs and cats (Beichel et al. 1996). Because blood sucking vectors contain infected host blood and the pathogen itself, they are reliable tools with which to demonstrate the existence of pathogens in a specific area. Thus ticks have been used for the epidemiological studies of tick-borne pathogens (Spragam et al., 1999). Molecular techniques including the polymerase chain reaction (PCR) and sequence analysis have been used effectively for the epidemiological studies and phylogenetic analyses of tick-borne pathogens (Inokuma et al., 2003, 2004). Thus in the present study, detection and analysis of *Borrelia* species from ticks recovered from dogs and cats in Japan

were attempted using molecular methods including screening PCR and sequence analysis.

2. Materials and Methods

The methods for tick collection and extraction of DNA from tick samples have already been reported in our previous paper (Shimada et al. 2003a, 2003b, Inokuma et al., 2003). In brief, using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), DNA was successfully extracted from 1136 and 133 ticks recovered from 1136 dogs and 133 cats, respectively, from all over Japan. One tick was selected from each animal. Most of the ticks selected were semi- or fully-engorged adult females or nymphs. The DNA samples were examined with nested PCR. The first PCR was performed with a set of primers, RIS1 and RIS2 (Postic et al. 1994), and the second PCR with primers, rrf2 (CGA GTT CGC GGG AGA GTA) and rrl2 (TAG GCA TTC ACC ATA GAC TC). This nested PCR was designed to amplify a 250-bp fragment of the 5S-23S rDNA intergenic spacer of *Borrelia*. When the samples revealed appropriate amplicons in the screening PCR, we carried out another PCR based on the flagellin gene with the primer set, flaC and flaL4 (Frankel et al., 2002), to further establish the phylogenetic position of the organisms.

The nucleotide sequencing of positive PCR products were determined by the direct sequencing method as described previously (Inokuma et al., 2003). The GenBank accession numbers of *Borrelia* 5S-23S rDNA intergenic spacer sequences to analyze the percent identities and construct a phylogenetic tree are as follows; *B. burgdorferi* B31, L30127; *B. garinii* , L30130; *B. garinii* 20047, L30119; *B. afzellii* , L30131; *B. japonica*, L30125; *B. lusitaniae* PotiB2, L30131; *B. turdi* Ya501, D84407; *B. tanukii* , D84404; *B. valaisiana* VS116 , L30134; *B. valaisiana* Am501, D84402; *Borrelia* sp. closely related to *B. valaisiana* OG1/01, AB091441; OM43, AB091442; OS49, AB091443. The GenBank accession numbers of the flagellin sequences used in this study are as follows; *B. burgdorferi* B31, X15561; *B. hermsii*, M86838; *B. garinii*, D82846; *B. afzellii* , D63366; *B. japonica* , D82852; *B. lusitaniae* PotiB2, D82856; *B. turdi* Ya501, D82849; *B. tanukii* , D82847; *B. valaisiana* VS116 , D82854; *B. valaisiana* Am501, D82855; *Borrelia* sp. closely related

to *B. valaisiana* OG1/01, AB091701; OM43, AB091703; OS49, AB091714. Multiple alignment analysis and construction of a phylogenetic tree were also performed as described previously (Inokuma et al. 2003). The gene sequences of *Borrelia* detected from ticks determined in this study have been deposited in the GenBank database under the following accession numbers as follows; (a) 5S-23S rRNA gene intergenic spacer, HK-Ip4, AY854023; HK-Ip5, AY854024; HK-I564, AY854025; HK-I575, AY854026; OK-Ig132, AY854027; OK-Ig132-2, AY854028; OK-Ig133, AY854029 and OK-Ig133-2, AY854030, (b) flagellin gene, HK-I564, AY854031; HK-I575, AY854032; OK-Ig132, AY854033.

3. Results and Discussion

Two females of *Ixodes persulcatus* (HK-Ip4 and HK-Ip5) from 2 dogs and two females of unidentified *Ixodes* sp. (HK-I564 and HK-I575) from another 2 dogs in Hokkaido, the northern part of Japan (Fig.1), were positive following screening PCR based on the 5S-23S rRNA gene intergenic spacer. Two *Ixodes granulatus* (OK-Ig132 and OK-Ig133) ticks from 2 cats in Okinawa, the southern part of Japan, were also positive (Fig.1). As the two cats in Okinawa were infested with other *I. granulatus* females, one more tick from each cat (OK-Ig132-2 and OK-Ig-133-2) was submitted for additional analysis and were found to be positive.

By analyzing the intergenic spacer sequences, HK-I575 was the most similar to *B. garinii* (Table 1, Fig.2). The greatest homology with *B. garinii* was also found in the flagellin gene analysis with 97.9% percent identity (Table 1, Fig.2). The results suggest that HK-I575 is probably *B. garinii*. *B. garinii* is one of the etiological agents of Lyme borrelisosis in both man and animals (Baranton et al., 1992), and is known to be transmitted by *I. persulcatus* in Japan (Masuzawa et al., 1991). As the dog did not show any clinical symptoms at the time of presentation and serological examination for *Borrelia* infection was not performed, it is impossible to determined whether the dog infected with *B. garinii* or not.

HK-Ip4, HK-Ip5 and HK-I564 were most similar to *B. tanukii* with percent identities of

96.8, 95.3 and 97.0 % on 5S-23S rDNA intergenic spacer sequences, respectively. Randomly selected HK-I564 among these three ticks also showed higher percent identity (97.0%) with the flagellin gene of *B. tanukii* and belongs to the same cluster as *B. tanukii* in a phylogenetic tree based on the flagellin sequence (Table 1, Fig.2). The data for the three ticks from Hokkaido, HK-Ip4, HK-Ip5 and HK-I564, suggest that these 3 samples are either *B. tanukii* or a *Borrelia* sp. closely related to *B. tanukii*. *I. tanuki* is one of the known vectors of *B. tanukii* (Fukunaga et al., 1996 a & b); however it is not a dominant tick species of companion animals in Japan (Yamaguchi et al., 1971, Shimada et al. 2003a and b). The pathogenesis of *B. tanukii* to man and animals is unknown, but the dogs in this study did not show any clinical signs at the time of presentation.

There have been only two dogs diagnosed with Lyme borreliosis in Japan (Azuma et al., 1993). Neurological abnormalities were recorded in both cases; however, the causative agent was not identified in either case. All the 4 dogs that were infested with positive ticks in this study were toy breeds and kept mainly indoors. They were supposed to be infested with the ticks at the time of bush walking with their owners. Clinical veterinarians should be aware that tick infested dogs may carry potentially pathogenic *Borrelia* species. Infected ticks carried by companion animals can be introduced into the human environment.

Four *Borrelia* intergenic spacer sequences detected from *I. granulatus* in Okinawa were identical to each other and showed 99.5% identities with a novel *Borrelia* sp. closely related with *B. valaisiana* found in Okinawa Prefecture (Masuzawa et al., 2004; Takada et al., 2001) (Table 1). A phylogenetic tree based on the intergenic spacer sequences also showed that OK-Ig132, OK-Ig132-2, OK-Ig133 and OK-Ig133-2 belong to the same cluster as the *Borrelia* sp. (Fig.2). The flagellin gene sequence of OK-Ig132 also showed the highest identity of 98.3 % with the novel *Borrelia* species closely related with *B. valaisiana.* In the phylogenetic tree based on the flagellin gene sequences, OK-Ig132 and the *Borrelia* sp. closely related with *B. valaisiana* make one cluster (Fig.2). The *Borrelia* found in *I. granulatus* removed from cats in this study may be the novel *Borrelia* sp. closely related to *B. valaisiana* found in Okinawa recently. These 2 cats were free

roaming and presented to a veterinarian for tick infestation without any clinical signs, although the pathogenesis of this newly found *Borrelia* to man and animals is still unknown. *I. granulatus* is a suspected vector of this *Borrelia* (Masuzawa et al 2004), and the main host of this tick is rodents (Yamaguchi et al. 1971). Attention should be paid to the fact that *I. granulatus* ticks infected with *Borrelia* have been recovered from domestic cats.

ACKNOWLEDGMENTS. The authors would like to acknowledge the technical expertise of the DNA Core facility of the Center for Gene Research, Yamaguchi University, supported by a Grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan. They would also like to thank Nippon Zenyaku Kogyo Co., Ltd. for collecting and sending the tick samples. This work was supported in part by Merial Japan Ltd. and a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (Grant No. 14360190).

References

Azuma, Y., Kawamura, K., Isogai, H., Isogai, E., 1993. Neurologic abnormalities in two dogs suspected Lyme Disease. Microbiol. Immunol. 37, 325-329.

Baranton, G., Postic, D., Saint Girons, I., Boerlin, P., Piffaretti, J. C., Assous, M., Grimont, P. A., 1992. Delination of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. Nov., and group VS461 associated with Lyme borreliosis. Int. J. Syst. Bacteriol. 42, 378-383.

Beichel E, Petney TN, Hassler D, Bruckner M, Maiwald M. 1996. Tick infestation patterns and prevalence of *Borrelia burgdorferi* in ticks collected at a veterinary clinic in Germany. Vet Parasitol. 65, 147-155.

Fraenkel, C. J., Garpmo, U., Berglund, J., 2002. Determination of Novel *Borrelia* genospecies in Swedish *Ixodes ricinus* ticks. J. Clin. Microbiol. 40, 3308-3312.

Fukunaga, M., Hamase, A., Okada, K., Inoue, H., Tsuruta, Y., Miyamoto, K., Nakao, M., 1996a. Characterization of spirochetes isolated from ticks (*Ixodes tanuki*, *Ixodes turdus*, and *Ixodes columnae*) and comparison of the sequences with those of *Borrelia burgdorferi* sensu lato strains. Appl. Environ. Microbiol. 62, 2338-2344.

Fukunaga, M., Hamase, A., Okada, K., Nakao, M. 1996b., *Borrelia tanukii* sp. nov. and *Borrelia turdae* sp. nov. found from ixodid ticks in Japan: rapid species identification by 16S rRNA gene-targeted PCR analysis.

Microbiol. Immunol. 40, 877-881.

Inokuma, H., Yoshizaki, Y., Shimada, Y., Sakata, Y., Okuda, M., Onishi, T. 2003. Epidemiological survey of *Babesia* species in Japan performed with specimens from ticks collected from dogs and detection of new *Babesia* DNA closely related to *Babesia odocoilei* and *Babesia divergens* DNA. J. Clin. Microbiol. 41, 3494-3498.

Inokuma, H., Beppu, T., Okuda, M., Shimada, Y., Sakata, Y., 2004. Detection of ehrlichial DNA in *Haemaphysalis* ticks recovered from dogs in Japan that is closely related to a novel *Ehrlichia* sp. found in cattle ticks from Tibet, Thailand and Africa. J. Clin. Microbiol. 42, 1353-1355.

Kawabata, H., Masuzawa, T., Yanagihara, Y., 1993. Genomic analysis of *Borrelia japonica* sp. nov. isolated from *Ixodes ovatus* in Japan. Microbiol. Immunol. 37, 843–848.

Lissman, B. A., Bosler, E. M., Camay, H., Ormiston, B. G., Benach, J. L. 1984. Spirochete- associated arthritis (Lyme disease) in a dog. J. Am. Vet. Med. Assos. 185, 219-220.

Masuzawa, T., Okada, Y., Yanagihara, Y., Sato, N., 1991. Antigenic properties of *Borrelia burgdorferi* isolated from *Ixodes ovatus* and *Ixodes persulcatus* in Hokkaido, Japan. J. Clin. Microbiol. 29, 1568-1573.

Masuzawa, T., Hashimoto, N., Kudeken, M., Kadosaka, T., Nakamura, M., Kawabata, H., Koizumi, N., Imai, Y. 2004. New genomospecies related to *Borrelia valaisiana*, isolated from mammals in Okinawa archipelago, Japan. J. Med. Microbiol. 53, 421-426.

Miyamoto, K., Masuzawa, T., 2002. Ecology of *Borrelia burgdorferi* sensu lato in Japan and East Asia. In: Gray, J. S., Kahl, O., Lane, R. S., Stanek, G. (Eds.), Lyme Borreliosis: Biology, Epidemiology and Control. CABI Publishing, Walilingford, UK, pp. 201-222.

Nakao, M., Miyamoto, K., Fukunaga, M., Hashimoto, Y., Takahashi, H., 1994. Comparative studies on *Borrelia afzelli* isolated from a patient of Lyme disease, *Ixodes persulcatus* ticks, and *Apodemus speciosus* rodents in Japan. Microbiol. Immunol. 38, 413-420.

Postic, D., Assous, V. M., Grimont, P. A. D., Baranton, G., 1994. Diversity of *Borrelia burgdorferi* sensu lato evidenced by restriction fragment length polymorphism of *rrf*(5S)-*rrl*(23S) intergenic spacer amplicons. Int. J. Syst. Bacteriol. 44, 743-752.

Scotarzak, B., 2002. Canine borreliosis – epidemiology and diagnosis. *Ann. Agric. Environ.* 9, 137-140.

Shimada, Y., Beppu, T., Inokuma, H., Okuda, M., Onishi, T., 2003a. Ixodid tick species recovered from domestic dogs in Japan. Med. Vet. Entomol. 17, 38-45.

Shimada, Y., Inokuma, H., Beppu, T., Okuda, M., Onishi, T., 2003b. Survey of ixodid tick species on domestic cat in Japan. Vet. Parasitol. 111, 231-239.

Sparagano, O. A. E., Allsopp, M. T. E. P., Mank, R. A., Rijpkema, S. G. T., Figueroa, J. V., Jongejan, F. 1999. Molecular detection of pathogen DNA in ticks (Acari: *Ixodidae*): A review. Exp. Appl. Acarol. 23: 929-960.

Takada, N., Fujita, H., Yano, Y., Ishiguro, F., Iwasaki, H., Masuzawa, T., 2001a. First records of tick-bone pathogens, *Borrelia*, and spotted fever group rickettsiae in Okinawajima Island, Japan. Microbiol. Immunol. 45, 163-165.

Yamaguchi, N., Tipton, V.J., Keegan, H.L., Toshioka, S., 1971. Ticks of Japan, Korea, and the Ryukyu Islands. Brigham Young Univ. Sci. Bull. Biol. Ser. XV: 1-226.

Figure legends

Fig.1. A map of Japan. Ticks were recovered from dogs and cats in all over Japan (small dots). *Borrelia* DNA were detected from ticks in Hokkaido and Okinawa Prefectures (circles)

Fig.2. A) Phylogenetic relationship of various *Borrelia* spp. based on the nucleotide sequences of the 5S-23S rDNA intergenic spacer. The numbers at nodes are the proportions of 100 bootstrap resamplings that support the topology shown. The scale bar represents 1 % divergence. The bacteria detected in this work are highlighted. B) Phylogenetic relationship of various *Borrelia* spp. based on the nucleotide sequences of the flagellin gene. The numbers at nodes are the proportions of 100 bootstrap resamplings that support the topology shown. The scale bar represents 1 % divergence. The bacteria detected in this work are highlighted.

Table 1. Percent identities of partial sequences of the 5S-23S rDNA intergenic spacer and flagellin gene of *Borrelia* detected in ticks recovered from dogs and cats, with *Borrelia* species registered in GenBank.

Species	Accession No.	HK-I575	HK-I564	OK-Ig132
5S-23S rDNA intergenic spacer gene				
B. garinii	L30130	96.1 %	90.2 %	91.0 %
B. tanukii	D84404	90.0 %	97.0 %	90.6 %
*B. valaisiana*VS116	L30134	90.5 %	92.0 %	94.7 %
*B. valaisiana*Am501	D84402	91.3 %	89.5 %	93.4 %
B. afzelli	L30135	90.1 %	88.5 %	91.7 %
B. japonica	L30125	88.2 %	86.4 %	77.7 %
B. turdi	D84407	90.1 %	82.0 %	89.6 %
Borrelia sp.OG1/01	AB091441	91.0 %	90.0 %	99.5 %
Borrelia sp.OM43	AB091441	91.0 %	90.0 %	99.5 %
Borrelia sp.OS49	AB091441	91.0 %	90.0 %	99.5 %
Flagellin gene				
B. garinii	D82846	97.9 %	91.3 %	92.4 %
B. tanukii	D82847	93.1 %	97.0 %	93.6 %
*B. valaisiana*VS116	D82854	92.4 %	94.8 %	94.5 %
*B. valaisiana*Am501	D82855	92.4 %	93.9 %	94.5 %
B. afzelli	D63366	90.7 %	91.7 %	91.9 %
B. japonica	D82852	91.7 %	90.9 %	91.9 %
B. turdi	D82849	92.7 %	93.0 %	94.5 %
Borrelia sp.OG1/01	AB091701	92.0 %	93.9 %	98.3 %
Borrelia sp.OM43	AB091703	92.0 %	93.9 %	98.3 %
Borrelia sp.OS49	AB091714	91.7 %	93.9 %	95.8 %

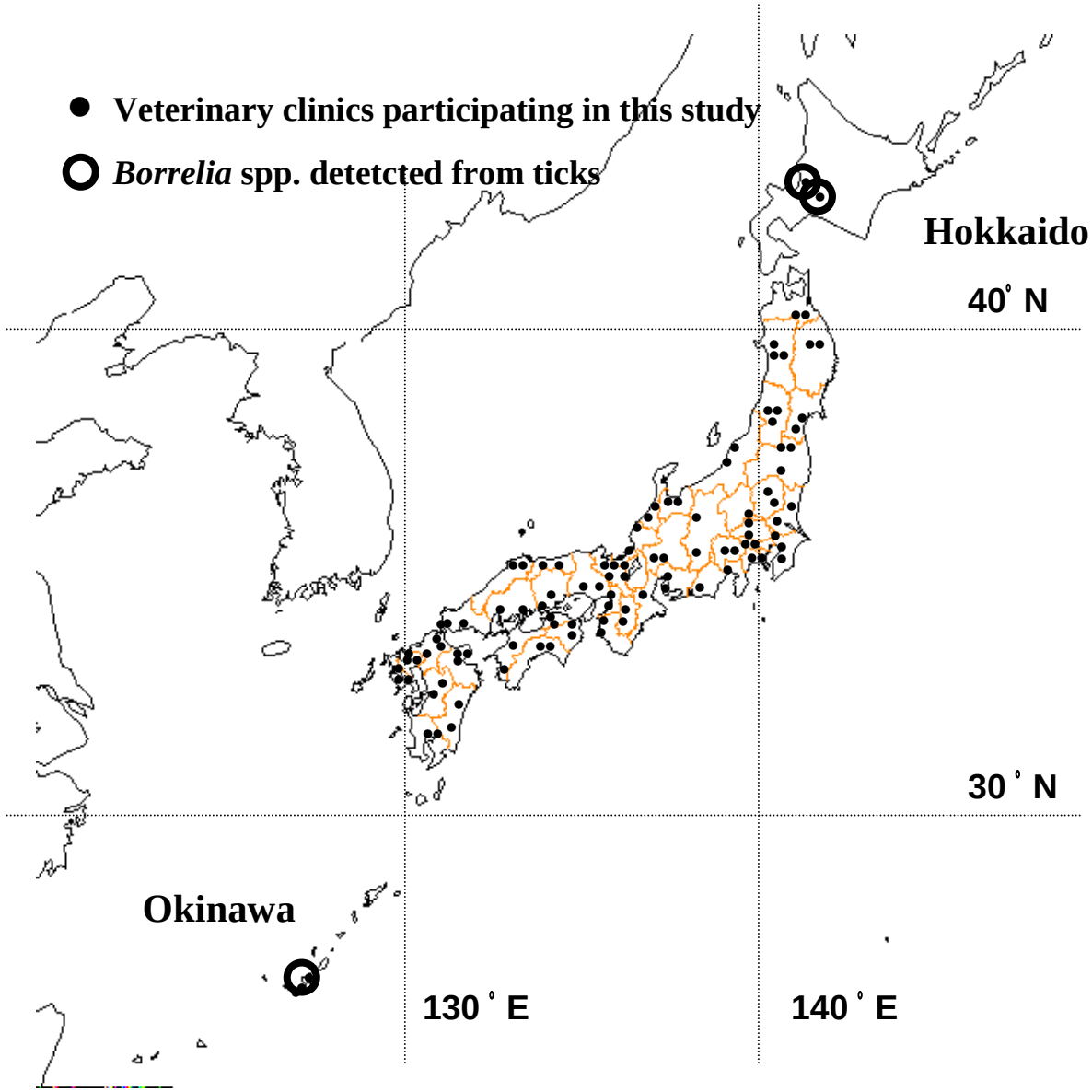
Veterinary clinics participating in this study
Borrelia spp. detetcted from ticks
Hokkaido
40˚ N
30 ˚ N
Okinawa
130 ˚ E
140 ˚ E

Fig.1

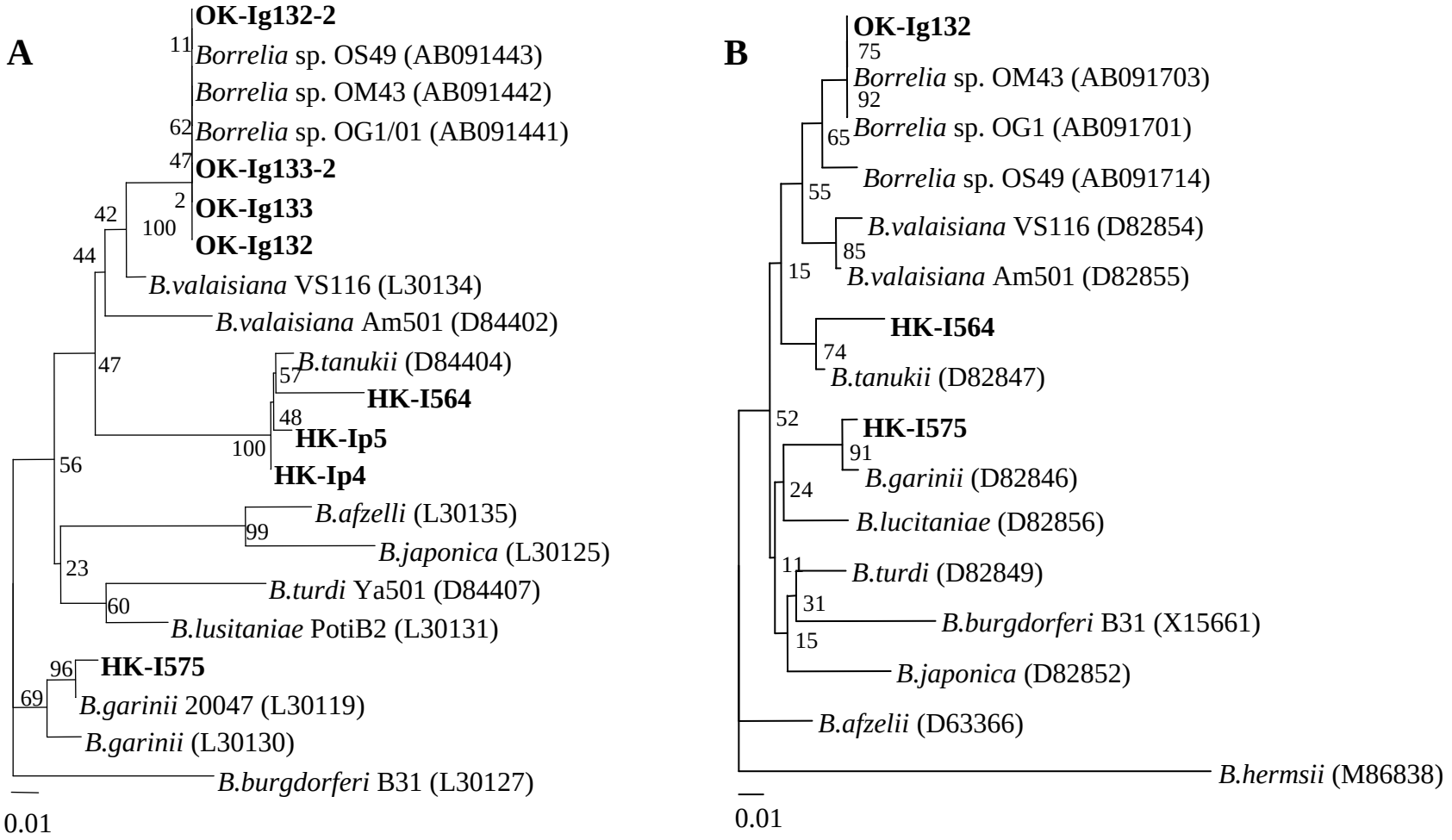
A
OK-Ig132-2
11
Borrelia sp. OS49 (AB091443)
Borrelia sp. OM43 (AB091442)
62
Borrelia sp. OG1/01 (AB091441)
47
OK-Ig133-2
2
OK-Ig133
42
100
OK-Ig132
44
B.valaisiana VS116 (L30134)
B.valaisiana Am501 (D84402)
47
B.tanukii (D84404)
57
HK-I564
48
HK-Ip5
100
HK-Ip4
56
B.afzelli (L30135)
99
B.japonica (L30125)
23
B.turdi Ya501 (D84407)
60
B.lusitaniae PotiB2 (L30131)
96
HK-I575
69
B.garinii 20047 (L30119)
B.garinii (L30130)
B.burgdorferi B31 (L30127)
0.01
B
OK-Ig132
75
Borrelia sp. OM43 (AB091703)
92
65
Borrelia sp. OG1 (AB091701)
Borrelia sp. OS49 (AB091714)
55
B.valaisiana VS116 (D82854)
85
15
B.valaisiana Am501 (D82855)
HK-I564
74
B.tanukii (D82847)
52
HK-I575
91
B.garinii (D82846)
24
B.lucitaniae (D82856)
11
B.turdi (D82849)
31
B.burgdorferi B31 (X15661)
15
B.japonica (D82852)
B.afzelii (D63366)
B.hermsii (M86838)
0.01

Fig.2