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Molecular detection of *Borrelia valaisiana*-related spirochetes from *Ixodes granulatus* ticks in Taiwan

Li-Lian Chao · Wen-Jer Wu · Chien-Ming Shih

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Abstract *Borrelia valaisiana*-related spirochetes were detected for the first time in *Ixodes granulatus* ticks collected in Taiwan. The genetic identities of these detected spirochetes were determined by analyzing the gene sequences amplified by a genospecies-specific polymerase chain reaction assay based on the outer surface protein A (OspA) gene of *B. burgdorferi* sensu lato. Phylogenetic relationships were analyzed by comparing the sequences of OspA gene obtained from 35 strains of *Borrelia* spirochetes representing six genospecies of *Borrelia*. Eight major clades can be easily distinguished by neighbour-joining analysis and were congruent by maximum-parsimony method. Except one strain (KH-74), all these *Borrelia* spirochetes of Taiwan were genetically affiliated to the same clade with highly homogeneous sequences (97.8–100% similarity), and can be discriminated from other groups of *B. valaisiana* and other genospecies of *Borrelia* spirochetes with a sequence divergence ranging from 3 to 19.6%. Moreover, intraspecific analysis also revealed that three distinct groups are evident between the same species of *B. valaisiana* spirochetes detected in Taiwan. Our results provide the first evidence of *B. valaisiana* spirochetes detected in *I. granulatus* ticks collected in Taiwan and demonstrate that all these *B. valaisiana* spirochetes of Taiwan represent three major groups distinct from the European group of *B. valaisiana* spirochetes.

Keywords *Borrelia valaisiana* · Spirochete · *Ixodes granulatus* · Tick · Taiwan

Introduction

The causative agent for Lyme disease, *Borrelia burgdorferi* sensu lato, was firstly identified within the gut of vector ticks (Burgdorfer et al. 1982) and the spirochete species can

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be classified into at least thirteen genospecies based on their genetic differences (Wang et al. 1999; Masuzawa et al. 2001; Richter et al. 2006; Postic et al. 2007). The tick species of *Ixodes ricinus* complex serve as the main vectors for transmission and perpetuation of *B. burgdorferi* spirochetes through a natural cycle between vector ticks and rodent hosts in North America and Europe (Spielman 1988; Matuschka et al. 1990). Although *I. persulcatus* and *I. ovatus* had been recognized as the principle vector for the transmission of *B. burgdorferi* spirochetes in the Northeast Asia including the northeastern regions of China, Korea, and Japan (Kawabata et al. 1987; Ai et al. 1988; Nakao et al. 1992; Park et al. 1993), the hard ticks of *I. granulatus* and *Haemaphysalis bispinosa* were suggested as the principle vector for the transmission of *B. burgdorferi* spirochetes in the southern regions (adjacent to Taiwan) of China (Zhang et al. 1997; Wan et al. 1998).

The abundance and widespread of *I. granulatus* had been recorded for the first time from various countries in Southeast Asia and Taiwan (Wilson 1970). The medical importance with the recent emergence of human babesiosis (Shih et al. 1997) and Lyme borreliosis (Shih et al. 1998b) in Taiwan raises the focus of research attention on *I. granulatus* tick. In addition, Lyme disease spirochetes (*B. burgdorferi* sensu lato) had also been isolated from six species of rodent hosts parasitized by various stages of *I. granulatus* ticks of Taiwan (Shih and Chao 1998) and all these Taiwan isolates were genetically classified into the genospecies of *B. burgdorferi* sensu stricto, a genospecies firstly identified in Asia (Shih et al. 1998a; Shih and Chao 2002). Although the hard tick of *I. granulatus* was presumed to be the tick vector for the enzoonotic transmission of *Borrelia* spirochetes in Taiwan (Shih and Chao 2004), the genetic diversity of *Borrelia* spirochetes harbored by this tick species remain undefined.

The existence of outer surface protein (Osp) genes in all *Borrelia* isolates belonging to the major genospecies of *B. burgdorferi* sensu lato were verified and described (Bergstrom et al. 1989; Jonsson et al. 1992). Genomic similarities of *Borrelia* spirochetes can be clarified by their differential reactivity with genospecies-specific OspA primer sets and by analyzing the homogeneity of *OspA* sequences (Zumstein et al. 1992; Wilske et al. 1993; Caporale and Kocher 1994; Demaerschalck et al. 1995). In addition, different genospecies of *B. burgdorferi* sensu lato are distributed unevently throughout the world and are associated with distinct ecologic features (Wang et al. 1999). It may be that the *Borrelia* spirochetes exist in *I. granulatus* ticks of Taiwan are genetically distinct from the *Borrelia* spirochetes within common vector ticks (*I. ricinus* complex) in Europe and the United States. The potential of genetic variation in relation to the geographic distribution may also exist among the same *Borrelia* species detected in variant ticks. Thus, the objective of the present study intends to identify *Borrelia* spirochetes in *I. granulatus* ticks by polymerase chain reaction (PCR) assay targeting the OspA gene of *B. burgdorferi* sensu lato and to clarify the genetic identity of detected spirochetes by analyzing phylogenetic relationships with other *Borrelia* species.

Materials and methods

Collection and identification of tick specimens

All specimens of adult ticks were removed from rodents captured at various field sites of Taiwan and all field-collected ticks were subsequently stored in separate mesh-covered vials. Adult ticks of male and female *I. granulatus* collected on Taiwan were identified to species level on the basis of their morphological characteristics, as described and sketched

previously (Teng and Jiang 1991). Ultrastructural observations by scanning electron microscope (SEM) were also used to identify the morphological features of *I. granulatus* ticks of Taiwan, as described previously (Chao et al. 2009).

DNA extraction from tick specimens

Total genomic DNA was extracted from individual tick specimen used in this study. Briefly, tick specimens were cleaned by sonication for 3–5 min in 75% ethanol and then washed twice in sterile distilled water. Afterwards, individual tick specimen was dissected into pieces, placed in a microcentrifuge tube filled with 180-µl lysing buffer solution supplied in the DNeasy Tissue Kit (catalogue no. 69506, Qiagen, Taipei, Taiwan) and then homogenized with a sterile tissue grinder (catalogue no. 358103, Wheaton Scientific Products, Millville, NJ, USA). The homogenate was centrifuged at room temperature and the supernatant fluid was further processed using a DNeasy Tissue Kit, as per manufacturer's instructions. After filtration, the filtrate was collected and the DNA concentration was determined spectrophotometrically with a DNA calculator (GeneQuant II; Pharmacia Biotech, Uppsala, Sweden).

DNA amplification by polymerase chain reaction

DNA samples extracted from the tick specimens were used as template for PCR amplification. A specific primer set, SL-1 (5′-AATAGGTCTAATAATAGCCTTAATAGC-3′) corresponding to the 3′ end of the OspA gene and SL-2 (5′-CTAGTGTTTTGC CATCTTCTTTGAAAA-3′) corresponding to the 5′ end of the OspA gene, were designed to amplify DNA of all major genospecies of *B. burgdorferi* sensu lato, as described previously (Demaerschalck et al. 1995). All PCR reagents and Taq polymerase were obtained and used as recommended by the supplier (Takara Shuzo Co., Ltd., Japan). Briefly, a total of 0.2-µmol of the appropriate primer set and various amounts of template DNA were used in each 50-µl reaction mixture. The PCR amplification was performed with a Perkin-Elmer Cetus thermocycler (GeneAmp system 9700; Applied Biosystems, Taipei, Taiwan) for 40 cycles with denaturation at 93°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Amplified DNA products were electrophoresed in 2% agarose gels in Tris–Borate-EDTA (TBE) buffer and visualized under ultraviolet (UV) light after staining with ethidium bromide. A 1-kb plus DNA ladder (catalogue no. 10787-018, Gibco BRL, Taipei, Taiwan) was used as the standard marker for comparison.

Sequence alignments and phylogenetic analysis

After purification with a QIAquick PCR purification kit (catalogue no. 28104, Qiagen, Taipei, Taiwan), the nucleotide sequences of 13 strains of *Borrelia* spirochetes detected in *I. granulatus* ticks of Taiwan were sequenced using an ABI Prism 377-96 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The resulting sequences were initially aligned with the CLUSTAL W software (Thompson et al. 1994) and further analyzed by neighbour-joining (NJ) compared with maximum parsimony (MP) methods to estimate the phylogeny of the entire alignment using MEGA 4.0 software package (Tamura et al. 2007). A similarity matrix was also constructed using DNASTAR program (Lasergene, version 8.0). The genetic distance values of intra- and inter-specific variations of *Borrelia* spirochetes were also analyzed by the Kimura two-parameter model (Kimura 1980), as

implemented in MEGA 4.0. All phylogenetic trees were constructed and performed with 1,000 bootstrap replications to evaluate the reliability of the constructions, as described previously (Felsenstein 1985).

Nucleotide sequence accession numbers

The nucleotide sequences of PCR-amplified OspA gene of *Borrelia* spirochetes determined in this study have been registered and assigned the following GenBank accession numbers: strains KH-58 (GU002658), KH-71 (GU002659), KS-18 (GU002660), KS-19 (GU002661), KS-42 (GU002662), KS-48 (GU002663), KS-67 (GU002664), KS-68 (GU002665), KC-44-1 (GU002666), KH-62 (GU002667), KH-74 (GU002668), KH-100 (GU002669), and KH-103 (GU002670). For phylogenetic analysis, nucleotide sequences of OspA from 22 strains of *Borrelia* spirochetes downloaded from GenBank were included for comparison and their GenBank accession numbers are shown in Table 1.

Results

Detection of spirochetal infection in *Ixodes granulatus* ticks

To verify the existence of *Borrelia* spirochetes in adult *I. granulatus* ticks removed from rodents of Taiwan. A total of 147 adult ticks (121 female and 26 male) were examined and tested for the evidence of spirochetal infection by PCR using specific primers targeting the OspA gene of *B. burgdorferi* sensu lato. *Borrelia* spirochetes were detected in 23 female and 4 male adult *I. granulatus* ticks with an infection rate of 19.0% (23/121) and 15.4% (4/26), respectively (Table 2). All the positive-infected adult ticks feed on the rodent host of *Rattus losea*.

Sequence alignment and genetic analysis

To clarify the genetic identity of these *Borrelia* spirochetes detected in adult *I. granulatus* ticks of Taiwan, sequences of PCR-amplified OspA fragments of 13 strains of *Borrelia* spirochetes were aligned and compared with the downloaded sequences of 22 strains of *Borrelia* spirochetes (11 *B. valaisiana*, 3 *B. burgdorferi* sensu stricto, 2 *B. garinii*, 2 *B. afzelii*, 2 *B. bissettii*, and 2 *B. califoeniensis*) from GenBank. The lengths of the aligned sequences were measured from 206 to 230 bp, and the nucleotide components indicate that the OspA of these spirochetes is highly AT-rich with average nucleotide frequencies of thymine (T) = 22.2%, cytosine (C) = 13.7%, adenine (A) = 43.7%, and guanine (G) = 20.4%, respectively (Fig. 1). The nucleotide sequences between the 13 *Borrelia* spirochetes of Taiwan were highly conserved with only a few point mutations/substitutions (Fig. 1) and the nucleotide variations within these *Borrelia* spirochetes of Taiwan were measured from 0 to 3.5% (Table 3). In contrast, the nucleotide variations among other genospecies of *Borrelia* compared with the Taiwan strains were measured from 3 to 19.6%. Inter- and intra-specific variations analyzed by the pairwise comparisons of genetic distance values reveal that all these *Borrelia* spirochetes of Taiwan were genetically affiliated with the *B. valaisiana* groups from China and Korea, and can be distinguished from the European group of *B. valaisiana* as well as other genospecies of *B. burgdorferi* sensu lato (Table 4). However, intraspecies analysis of *B. valaisiana* based on the genetic distance

Table 1 Genospecies and strains of *Borrelia* spirochetes analyzed in this study and their GenBank accession numbers[a]

Genospecies and strain	Origin of *Borrelia* strain		OspA gene accession no.
	Biological	Geographic	
Taiwan strains			
KH-58	*Ixodes granulatus*	Taiwan	GU002658
KH-62	*I. granulatus*	Taiwan	GU002667
KH-71	*I. granulatus*	Taiwan	GU002659
KH-74	*I. granulatus*	Taiwan	GU002668
KH-100	*I. granulatus*	Taiwan	GU002669
KH-103	*I. granulatus*	Taiwan	GU002670
KC-44-1	*I. granulatus*	Taiwan	GU002666
KS-18	*I. granulatus*	Taiwan	GU002660
KS-19	*I. granulatus*	Taiwan	GU002661
KS-42	*I. granulatus*	Taiwan	GU002662
KS-48	*I. granulatus*	Taiwan	GU002663
KS-67	*I. granulatus*	Taiwan	GU002664
KS-68	*I. granulatus*	Taiwan	GU002665
B. valaisiana			
VS116	*I. ricinus*	Switzerland	AF095940
UK	*I. ricinus*	England	AF095941
AR-2	*I. ricinus*	The Netherlands	AF095942
M19	*I. ricinus*	The Netherlands	AF095944
M52	*I. ricinus*	The Netherlands	AF095946
Am501	*I. columnae*	Japan	DQ393332
QLZSP1	*I. granulatus*	China	EU325674
QSYSP3	*Haemaphysalis longicornis*	China	EU325676
QTMP2	*I. granulatus*	China	EU325678
5MT	*I. nipponensis*	Korea	AB016977
10MT	*I. nipponensis*	Korea	AB016978
B. burgdorferi sensu stricto			
B31	*I. scapularis*	USA	AE000790
PKa	Human CSF	Germany	X80182
KH-13	*I. granulatus*	Taiwan	EU564838
B. garinii			
PBi	Human CSF	Germany	CP000015
Khab2559	Human skin	Russia	AY260463
B. afzelii			
PKo	Human CSF	Germany	X65599
VS461	*I. ricinus*	Switzerland	Z29087
B. bissettii			
CA128	*I. pacificus*	USA	AF186846
CA389	*I. pacificus*	USA	AF186845
B. californiensis			
CA404	*Dipodomys californicus*	USA	DQ393326
CA443	*D. californicus*	USA	DQ393325

[a] GenBank accession numbers (GU002658 ~ GU002670) were submitted by this study

Table 2 Spirochetal infection detected in adult *Ixodes granulatus* ticks[a] by polymerase chain reaction (PCR) assay targeting the OspA of *B. burgdorferi* sensu lato

Sex of tick	OspA-based PCR assay		% Infected
	No. tested	No. infected	
Female	121	23	19.0
Male	26	4	15.4
Total	147	27	18.4

[a] All unfed adult ticks were removed from the rodent host of *Rattus losea*

values also indicates a lower level of genetic divergence (<0.011) within these *Borrelia* spirochetes of Taiwan and all these Taiwan strains of *Borrelia* spirochetes were genetically more distant to the European group of *B. valaisiana* (>0.024) and other genospecies of *B. burgdorferi* sensu lato (>0.032) (Table 4).

Phylogenetic analysis

Phylogenetic relationships based on the alignment of OspA sequences were performed to analyze the genetic divergence among 35 *Borrelia* spirochetes investigated in this study. Bootstrap analysis was used to analyze the repeatability of the clustering of specimens represented in phylogenetic trees. Phylogenetic trees constructed by both NJ and MP analyses showed congruent basal topologies with eight major branch of distinguished clades (Figs. 2, 3). All *Borrelia* spirochetes detected in adult *I. granulatus* ticks represent three major groups of *Borrelia* spirochetes (groups A–C) which constituted a separate clade that can be easily distinguished from the European group of *Borrelia* spirochetes and other genospecies of *Borrelia* spirochetes. Within the same clade, 13 strains of *Borrelia* spirochetes from Taiwan represent three groups (groups A–C) and can be easily distinguished from the European group of *Borrelia* spirochetes with a bootstrap value of 86 in NJ analysis (Fig. 2). The phylogenetic tree of MP analysis was identical to the NJ tree and strongly support the separation of different lineages between the *Borrelia* spirochetes from Taiwan and Europe with a bootstrap value of 82 (Fig. 3). These results reveal a lower genetic divergence within the same genospecies of *Borrelia* spirochetes from Taiwan, but a higher genetic variations among different genospecies or group of *Borrelia* spirochetes.

Discussion

This report describes the first detection of *B. valaisiana*-related spirochetes in adult *I. granulatus* ticks collected in Taiwan. In our previous investigations, *B. burgdorferi* sensu stricto was isolated from six species of rodent hosts captured at various locations in Taiwan and *I. granulatus* ticks were observed on these highly infected rodent hosts (Shih and Chao 1998). Because of the high prevalence of *Borrelia* infection among captured rodents in Taiwan, the existence of zoonotic transmission of *Borrelia* spirochetes was suggested and the identification of *Borrelia* spirochetes in possible vector ticks is required to clarify the natural transmission cycle in Taiwan. Indeed, results from the present study confirm the existence of *B. valaisiana*-related spirochetes in adult *I. granulatus* ticks and indicate that the *R. losea* serves as the principle reservoir host for perpetuation of *Borrelia* spirochetes in nature. Further studies focused on the seasonal abundance of reservoir hosts and the

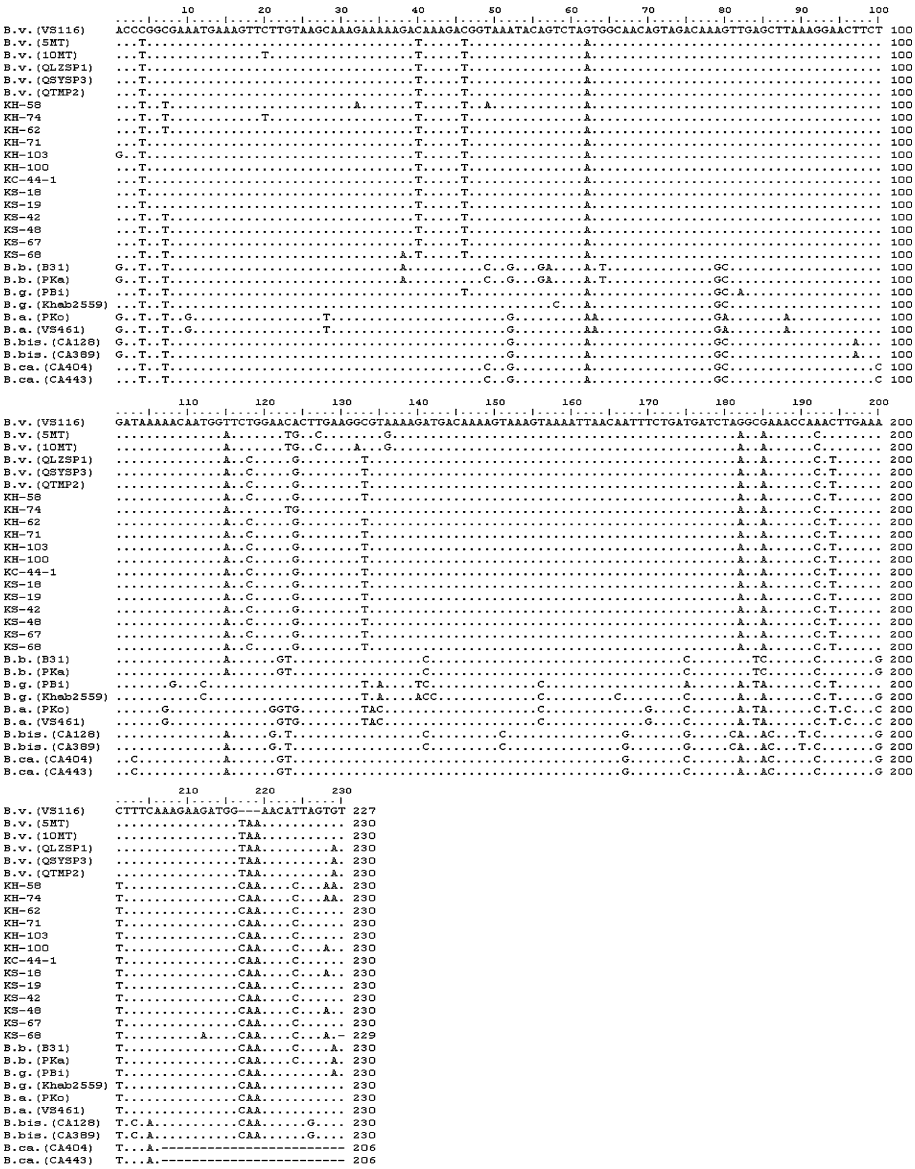

Fig. 1 Nucleotide sequences of the OspA gene of 13 strains of *Borrelia* spirochetes performed by this study were aligned and compared with the downloaded sequences of other 16 strains of *Borrelia* spirochetes from GenBank. The sequence for the *Borrelia* strain named *B. valaisiana* (VS116) is given as reference. *Dots* indicate nucleotides that are identical to the sequence of reference strain. *Dashes* indicate deletions within the sequence

prevalence of spirochetal infection among vector ticks would help to illustrate the ecologic feature regarding the transmission cycle of *Borrelia* spirochetes in Taiwan.

Geographical distribution of *B. valaisiana*-related spirochetes in Asia remains undefined. It is assumed that different genospecies of *B. burgdorferi* sensu lato are associated

Table 3 Sequence similarity between OspA gene sequences from Taiwan strains of *Borrelia* detected in *I. granulatus* and strains of other genospecies of *Borrelia*

Genospecies and strain[a]	VS116	QLZSP1	KH-71	KS-19	KH-62	KS-42	KH-74	5MT	PBi	PKo	B31	CA128	CA404
B. v. VS116	–	93.3	92.6	92.6	92.2	92.2	91.7	93.5	89.1	85.7	88.3	87.4	81.7
B. v. QLZSP1		–	98.3	98.3	97.8	97.8	95.7	97.0	92.2	87.8	88.3	87.8	80.4
KH-71			–	100.0	99.6	99.6	96.5	96.1	92.2	88.7	89.1	88.7	80.9
KS-19				–	99.6	99.6	96.5	96.1	92.2	88.7	89.1	88.7	80.9
KH-62					–	100.0	97.0	95.7	92.6	89.1	89.6	89.1	81.3
KS-42						–	97.0	95.7	92.6	89.1	89.6	89.1	81.3
KH-74							–	96.1	91.3	87.8	90.9	89.6	82.6
B. v. 5MT								–	90.0	87.4	88.7	89.1	81.3
B. g. PBi									–	89.1	89.6	88.3	79.6
B. a. PKo										–	88.3	87.4	78.7
B. b. s.s. B31											–	90.4	83.5
B. bis. CA128												–	83.9
B. ca. CA404													–

[a] Strains: VS116, QLZSP1, and 5MT, *B. valaisiana*; PBi, *B. garinii*; PKo, *B. afzelii*; B31, *B. burgdorferi* sensu stricto; CA128, *B. bissettii*; CA404, *B. californiensis*

with distinct reservoir hosts and vector ticks (Wang et al. 1999). Indeed, *B. valaisiana* has been isolated or detected from *I. ricinus* ticks and avain reservoirs from at least eight European countries (Rijpkema et al. 1996; Postic et al. 1997; Wang et al. 1997; Kirstein et al. 1997; Kurtenbach et al. 1998; Clinco et al. 1998). However, *B. valaisiana*-related spirochetes were isolated mainly from rodent hosts (*R. losea*, *R. norvegicus*, *Mus formosanus*, *Niviventer fulvescens*, and *Apodemus agrarius*) and detected in various hard ticks (*I. nipponensis*, *I. columnae*, *I. granulatus*, and *Haemaphysalis longicornis*) in Northeast Asia and Southwestern China (Masuzawa et al. 1999, 2001, 2004; Chu et al. 2008). Results from this study also verify the existence of *B. valaisiana*-related spirochetes detected in *I. granulatus* ticks removed from the rodent host of *R. losea* in Taiwan. These findings suggested that *B. valaisiana*-related spirochetes may persist in a zoonotic cycle between their rodent reservoir hosts and tick vectors in Eastern Asia.

The genetic identity of *Borrelia* spirochetes can be clarified by their differential reactivities with genospecies-specific PCR primers. In previous investigations, sequence analysis of OspA gene had been used to distinguish closely related *Borrelia* spirochetes and to assess the phylogenetic relationships of diverse *B. burgdorferi* sensu lato spirochetes by comparing their nucleotide variations of the OspA gene (Zumstein et al. 1992; Wilske et al. 1993; Caporale and Kocher 1994; Demaerschalck et al. 1995; Masuzawa et al. 1999; Wang et al. 2000; Shih and Chao 2002; Chu et al. 2008). Results from this study demonstrate that the nucleotide composition of OspA gene derived from these *I. granulatus* ticks of Taiwan is highly A-T rich ($\sim$65.9%) and that is similar to the nucleotide frequency of other *Borrelia* spirochetes either analyzed in this study (Fig. 1) or described in previous investigations (Masuzawa et al. 1999; Chu et al. 2008). This sequence feature observed in this study may imply a recent genetic evolution among these *B. valaisiana*-related spirochetes in Eastern Asia. Furthermore, the genetic divergence of these *B. valaisiana*-related spirochetes of Taiwan can be easily distinguished from the European

Table 4 Inter- and intra-specific analysis of genetic distance values[a] based on the OspA gene sequences between Taiwan strains of *Borrelia* detected in *I. granulatus* and strains of other genospecies of *Borrelia*

Genospecies and strain[b]	VS116	QLZSP1	KH-71	KS-19	KH-62	KS-42	KH-74	5MT	PBi	PKo	B31	CA128	CA404
B. v. VS116	–	0.024	0.026	0.026	0.028	0.028	0.026	0.024	0.046	0.070	0.049	0.058	0.046
B. v. QLZSP1		–	0.002	0.002	0.004	0.004	0.013	0.011	0.036	0.058	0.056	0.056	0.045
KH-71			–	0.000	0.002	0.002	0.011	0.013	0.034	0.056	0.053	0.054	0.042
KS-19				–	0.002	0.002	0.011	0.013	0.034	0.056	0.053	0.054	0.042
KH-62					–	0.000	0.009	0.015	0.032	0.053	0.051	0.051	0.040
KS-42						–	0.009	0.015	0.032	0.053	0.051	0.051	0.040
KH-74							–	0.009	0.038	0.056	0.044	0.044	0.033
B. v. 5MT								–	0.046	0.063	0.051	0.052	0.040
B. g. PBi									–	0.056	0.052	0.057	0.051
B. a. PKo										–	0.058	0.066	0.057
B. b. s.s. B31											–	0.042	0.029
B. bis. CA128												–	0.026
B. ca. CA404													–

[a] The pairwise distance calculation was performed by the method of Kimura 2-parameter, as implemented in MEGA 4 (Tamura et al. 2007)

[b] Strains: VS116, QLZSP1, and 5MT, *B. valaisiana*; PBi, *B. garinii*; PKo, *B. afzelii*; B31, *B. burgdorferi* sensu stricto; CA128, *B. bissettii*; CA404, *B. californiensis*

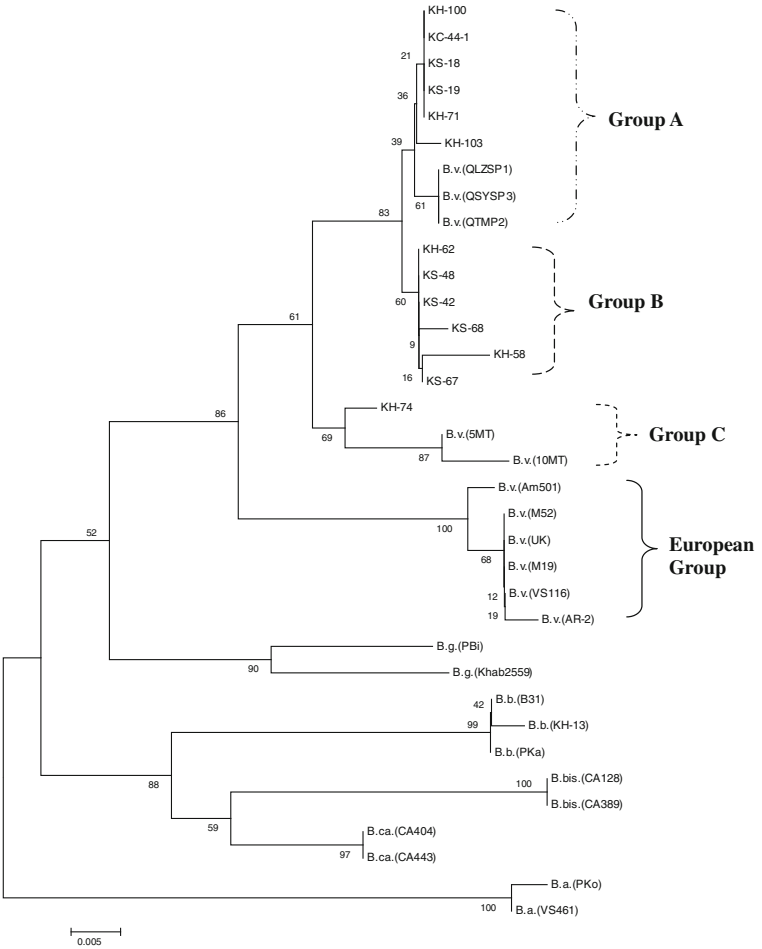

Fig. 2 Phylogenetic relationships of 13 OspA genes of *Borrelia* spirochetes detected in *I. granulatus* were compared with the sequences of six genospecies (i.e., *B. v.*, *B. valaisiana*; *B. g.*, *B. garinii*; *B. a.*, *B. afzelii*; *B. b.*, *B. burgdorferi* sensu stricto; *B. bis.*, *B. bissettii*; *B. ca.*, *B. californiensis*) of *Borrelia* spirochetes. The tree was constructed and analyzed with the neighbour-joining method with 1,000 bootstrap replicates. *Numbers* at the nodes indicate the percentages of reliability of each branch of the tree. Branch lengths are drawn proportional to the estimated sequence divergence

group of *B. valaisiana* spirochetes and other genospecies of *B. burgdorferi* spirochetes by their differential nucleotide variations existed in the OspA gene sequences (Tables 3 and 4). Thus, these observations suggest that the genetic identity of *B. valaisiana*-related spirochetes of Taiwan can be determined either interspecies or intraspecies among *Borrelia* spirochetes by analyzing their genetic divergence of the nucleotide sequences of OspA gene. Although intraspecific variation within these *B. valaisiana*-related spirochetes of Taiwan averaged less than 3.5% sequence variations may not fully represent a new genomospecies, interspecific variation between these *B. valaisiana* of Taiwan and other genospecies of *B. burgdorferi* spirochetes averaged more than 10.9% sequence variations

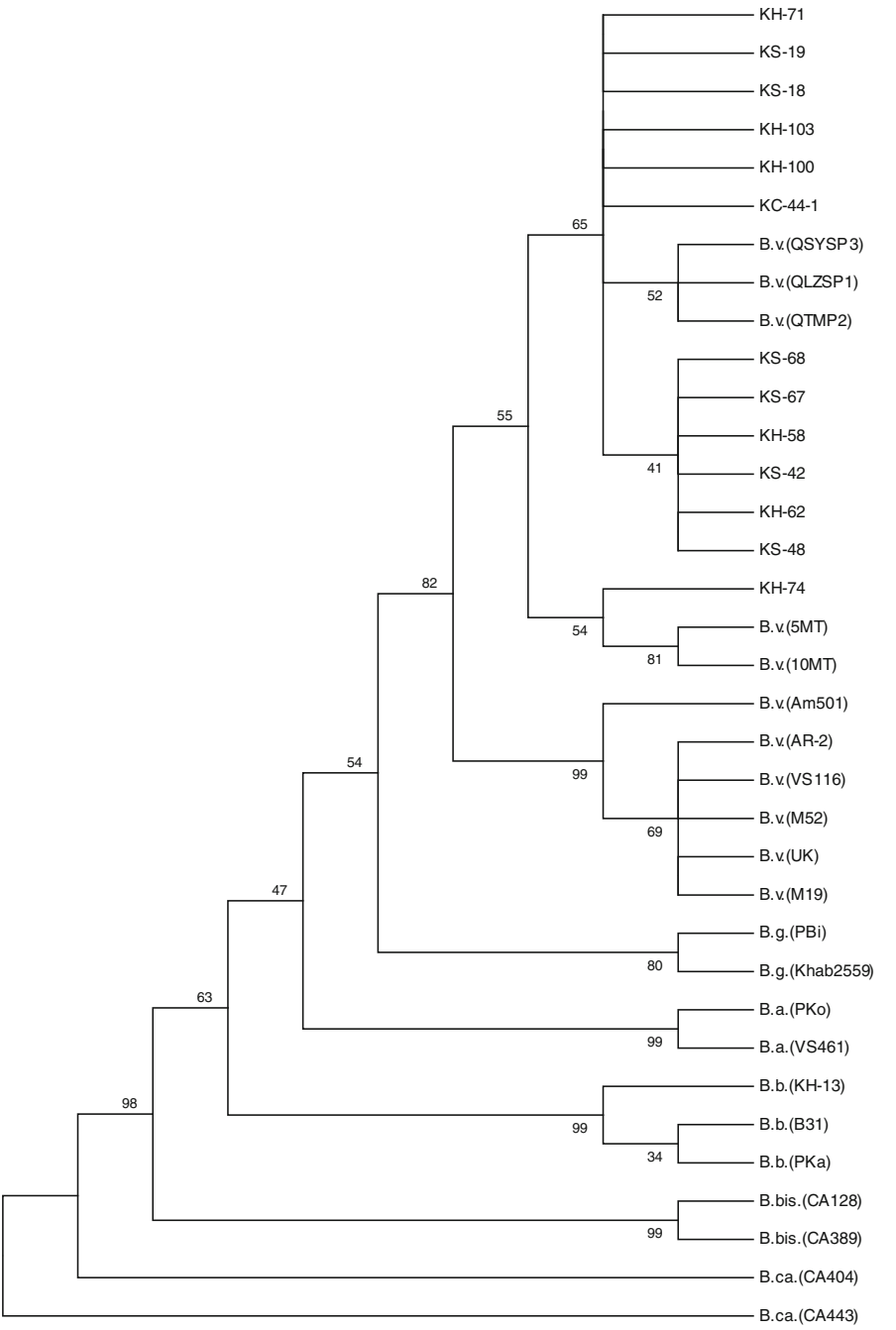

Fig. 3 Phylogenetic relationships of 13 OspA genes of *Borrelia* spirochetes detected in *I. granulatus* were compared with the sequences of six genospecies (i.e., *B. v.*, *B. valaisiana*; *B. g.*, *B. garinii*; *B. a.*, *B. afzelii*; *B. b.*, *B. burgdorferi* sensu stricto; *B. bis.*, *B. bissettii*; *B. ca.*, *B. californiensis*) of *Borrelia* spirochetes. The tree was constructed and analyzed with the maximum parsimony method with 1,000 bootstrap replicates. Bootstrap percentage values from 1,000 replicates are indicated for relevant clades

are much greater than that analyzed by previous studies for distinguishing the sequence variations between the distinct genospecies from different geographical origins (Wang et al. 2000; Masuzawa et al. 1999, 2001, 2004; Chu et al. 2008). Further investigation on the sequence divergence based on various targets of Osp genes of *Borrelia* spirochetes collected from different localities of Taiwan and its adjacent areas would be required to clarify the genetic divergence as well as the evolutionally origin among *Borrelia* spirochetes from Taiwan and its adjacent areas.

Phylogenetic relationships among *Borrelia* spirochetes can be determined by analyzing their sequence heterogeneity of the OspA gene. Indeed, the sequence analysis of OspA gene among various genospecies of *Borrelia* spirochetes had been shown to be useful for evaluating the genetic relatedness of *Borrelia* spirochetes isolated from various biological and geographical origins (Bergstrom et al. 1989; Jonsson et al. 1992; Zumstein et al. 1992; Wilske et al. 1993; Caporale and Kocher 1994; Demaerschalck et al. 1995; Will et al. 1995; Masuzawa et al. 1999; Wang et al. 2000; Shih and Chao 2002; Chu et al. 2008). In previous study, two distinct subgroups of *B. valaisiana* spirochetes are evident by comparing their OspA gene sequences between two closely related *B. valaisiana* isolated from the *I. ricinus* ticks of Europe (Wang et al. 2000). Phylogenetic analysis of *Borrelia* spirochetes related to the members of *B. valaisiana* also revealed intraspecific variation between different biological and geographical origins (Masuzawa et al. 1999, 2001, 2004; Wang et al. 2000; Chu et al. 2008). In this study, phylogenetic analysis based on the OspA gene sequences among various *Borrelia* genospecies demonstrated a high genetic heterogeneity between *B. valaisiana*-related spirochetes and other genospecies of *Borrelia* (Fig. 2). Although a low intraspecific variation was observed among the same genospecies of *B. valaisiana*, all the 13 strains of *B. valaisiana* from Taiwan represented as a separate clade that can be distinguished from the European group of *B. valaisiana* (Figs. 2 and 3). The phylogenetic trees constructed by either NJ or MP analysis strongly support the discrimination recognizing the separation of different lineages between the *B. valaisiana* from Taiwan and Europe. Within the same clade, geographical variation was also observed among a sister group C (strain KH-74) affiliated to the *B. valaisiana* from Korea (strains 5MT and 10MT), group A (strains KH-71, KH-100, KH-103, KS-18, KS-19, and KC-44-1) affiliated to the *B. valaisiana* from Southwestern China (strains QLZSP1, QSYSP3, and QTMP2), and group B (strains KH-58, KH-62, KS-42, KS-48, KS-67, and KS-68) adjacent to the Southeastern China. Accordingly, these observations reveal that all these *B. valaisiana*-related spirochetes detected in *I. granulatus* ticks from Taiwan represent three major groups constructed a unique clade distincted from the genospecies of *B. valaisiana* from Europe.

The pathogenecity of *B. valaisiana*-related spirochetes to humans remains to be determined. Although *B. valaisiana* has been recognized as the predominant *Borrelia* species detected in field-collected *I. ricinus* ticks and the *I. ricinus* ticks attached to human skin (Kirstein et al. 1997; Liebisch et al. 1998), the *B. valaisiana*-related spirochetes has never been isolated from human patients. Indeed, *B. valaisiana* DNA has been detected in cerebrospinal fluid (CSF) of an European patient (Diza et al. 2004) and *B. valaisiana* infection was reported in a Japanese man associated with a suspected bite by an *I. persulcatus* tick in which the DNA of *B. valaisiana* was detected. However, there is no confirmed evidence for the existence of *B. valaisiana* spirochetes in the patient's tissue (Saito et al. 2007). Moreover, the host-associated selection of genetic diversity of *Borrelia* spirochetes was proposed (Kurtenbach et al. 2002) and enzoonotic transmission by tick species that rarely feed on human hosts had also been suggested as the possible factors responsible for the under estimation of human cases (Maupin et al. 1994; Peavey et al.

2000). Indeed, a total of 13 *Borrelia* genospecies within the *B. burgdorferi* sensu lato complex have been described worldwide and only three genospecies (i.e., *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*) are highly pathogenic to humans (Aguero-Rosenfeld et al. 2005). Thus, the *B. valaisiana*-related spirochetes to cause a disease in humans in Asia still ambiguity.

In conclusion, our report provides the first evidence regarding the existence of *B. valaisiana*-related spirochetes within *I. granulatus* ticks collected in Taiwan. The genetic identity of these spirochetes was confirmed by analyzing sequence homology of OspA and indicated that all these spirochetes detected in *I. granulatus* ticks of Taiwan were genetically affiliated to the genospecies of *B. valaisiana* and constituted a separate clade representing three major groups distinguished from the European group of *B. valaisiana* transmitted by the common vector ticks (*I. ricinus* complex) for *B. burgdorferi* sensu lato. Further application of this molecular tool to investigate the genetic variability of OspA and other target genes among *Borrelia* spirochetes detected in different vector ticks and reservoir hosts may help to clarify the genetic diversity of *Borrelia* spirochetes in relation to the epidemiological features as well as their pathogenecity for human infection in Taiwan.

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