Molecular detection and genetic identification of *Borrelia garinii* and *Borrelia afzelii* from patients presenting with a rare skin manifestation of prurigo pigmentosa in Taiwan

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A R T I C L E   I N F O

S U M M A R Y

Objectives: To determine the genetic identity of Borrelia spirochetes isolated from patients with an unusual skin lesion of prurigo pigmentosa (PP) in Taiwan. The causative agents responsible for human borreliosis were clarified.

Methods: Serum samples and skin specimens were collected from 14 patients with suspected PP and five controls. Serological testing by Western immunoblot analysis and isolation of Borrelia spirochetes from skin specimens were used to verify the Borrelia infection. Genetic identities of isolated spirochetes were determined by analyzing the gene sequences amplified by PCR assay based on the 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon gene of *Borrelia burgdorferi* sensu lato.

Results: Borrelia spirochetes were isolated from skin biopsies of three patients. Serological evidence of Borrelia infection in these patients was also confirmed by elevated IgG and IgM antibodies against the major protein antigens of *B. burgdorferi*. Phylogenetic analysis revealed that these detected spirochetes are genetically affiliated to the genospecies of *Borrelia garinii* and *Borrelia afzelii* with high sequence homology within the genospecies of *B. garinii* (91.0–98.7%) and *B. afzelii* (97%).

Conclusions: This study provides the first evidence of *B. garinii* and *B. afzelii* isolated and identified in patients with PP. Whether this unusual skin lesion is a new manifestation of Lyme disease needs to be studied further.

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1. Introduction

Lyme disease (LD) is an emerging tick-borne spirochetal infection that can cause multisystem human illness with varying degrees of clinical symptoms among infected persons, ranging from a relatively benign skin lesion to severe arthritic, neurologic, and cardiac manifestations.[1] Based on the clinical manifestations in patients and the pathogenicity to humans, the causative agent of Lyme disease can be classified into four major genospecies: *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii*, and *Borrelia spielmanii*.[2–4] In previous investigations by our group, Borrelia spirochetes were isolated from rodent hosts and detected in *Ixodes granulatus* ticks in Taiwan.[5,6] Although Borrelia spirochetes have been isolated and identified in patient skin biopsies,[7] isolation from patients with dermal manifestations of prurigo pigmentosa (PP) has never been reported.

PP is a rare dermatosis with an unknown etiology and has been reported most commonly in Japanese patients since its first description in 1971.[8] It is recognized as an inflammatory disorder of the skin characterized clinically by severe pruritic red papules and erythematous urticarial papules that are symmetrically localized on the trunk and nape, accompanied by reticulate pigmentation.[8–10] As PP has been reported increasingly in different races and countries,[11–15] investigations on the causative factors responsible for the etiology and pathogenesis of PP indicate the correlation with various environmental and biological causes, including seasonal variation, contact allergens, primary biliary cirrhosis, *Helicobacter pylori* infection, atopic disease, and anorexia nervosa.[16–20] The chronic and recurrent nature of PP is quite similar to the skin manifestations of LD infection,[21] and together with the seasonal prevalence and effective treatment of both of these disorders with doxycycline, this may imply a possible correlation between PP and LD infection.

Genomic analysis among Borrelia isolates by sequence similarity of a specific target gene has proven useful for the species identification and genomic typing of Borrelia spirochetes

Figure 1. Photograph of the clinical feature of prurigo pigmentosa on the back of a 25-year-old Taiwanese woman. The lesion presented as intensely pruritic erythematous red papules and reticular hyperpigmentation.

isolated from different biological and geographical sources.[22–24] The 5S (rrf)–23S (rrl) intergenic spacer amplicon gene is unique and highly conserved in B. burgdorferi sensu lato.[25,26] The diversity of this gene is useful for distinguishing the genetic heterogeneity among different Borrelia isolates.[27–29] Indeed, the genetic identity of Borrelia spirochetes can be clarified by analyzing the sequence homology of the 5S (rrf)–23S (rrl) intergenic spacer amplicon genes of B. burgdorferi sensu lato isolated from various biological sources,[30,31] including skin specimens from patients.[32,33]

Thus, the objective of this study was to identify the genospecies of Borrelia spirochetes isolated from the skin biopsies of patients characterized with dermal manifestations of PP, by analyzing the sequence similarity of the PCR-amplified 5S (rrf)–23S (rrl) intergenic spacer amplicon genes. In addition, the phylogenetic relationships of these detected spirochetes were compared with other Borrelia species documented in GenBank.

2. Materials and methods

2.1. Specimen collection

We conducted an investigation for LD infection among patients with an unusual clinical manifestation of PP suggestive of LD. A total of 19 patients were identified in the dermatology clinic of Chung Shan Hospital, Taipei; there were 14 cases of suspected PP and five healthy control patients. All of these patients had pruritic red-colored papules with gross reticular pigmentation on the chest, back, and neck. Among these suspected cases, three native Taiwanese patients visited the dermal clinic with the chief complaint of long-lasting skin lesions observed on their body. These skin lesions had been sustained for 3 months to 4 years with an unknown etiology. The patients had no history of travel to any other country during the past 10 years. Based on the clinical features of intensely pruritic erythematous red papules and reticular hyperpigmentation (Figure 1), these skin lesions were diagnosed as cutaneous manifestations of PP. Skin biopsies were collected for culturing of Borrelia spirochetes. In the meantime, serum specimens from the same patient were subsequently collected and tested for serological evidence of B. burgdorferi infection.

2.2. Isolation and purification of Borrelia spirochetes

For the isolation of spirochetes, skin biopsy specimens (measuring 2–4 mm in diameter) from the red papular lesions of patients were cleaned with 75% ethanol solution for 1–2 min and then washed twice in sterile distilled water. Afterwards, individual skin specimens were dissected into pieces and inoculated into a culture tube containing BSK-H medium (B3528; Sigma Co., St. Louis, MO, USA) supplemented with 6% rabbit serum (R7136; Sigma), as described previously.[34] After incubation at 34 °C in a humidified incubator (Sanyo Electric Biomedical Co., Ltd, Osaka, Japan) with 5% CO_2, skin cultures were examined weekly for 8 weeks for evidence of spirochetes by dark-field microscopy (BX-60, Olympus Co., Tokyo, Japan). For the purification of cultured spirochetes, spirochete-positive cultures with other contamination were transferred to new culture tubes by serial dilution and were further filtered with a 0.45-μm pore size syringe filter (Sartorius, Gottingen, Germany), as described previously.[5,34] In addition, all the spirochete-positive cultures were also stored in a deep freezer (−80 °C) until further investigation.

2.3. Serological analysis for LD infection

Serodiagnosis for LD infection was performed with a screening test by indirect immunofluorescence assay (IFA) followed by Western immunoblot (WB) analysis using paired serum samples collected from the same patient, as recommended by the US Centers for Disease Control and Prevention (CDC).[35] Briefly, five strains of Borrelia spirochetes (strains B31, TWKM1, TWKM5, K48, and VS461) from pure cultures were used as antigens for both IFA and WB analysis, as described previously.[5,34] Electrophoresed Borrelia proteins were transferred from the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels to nitrocellulose blotting membranes using a semi-dry electroblotter (PhastTransfer electrode cassette; Pharmacia Biotech). The transferred membranes were blocked for 2 h with 3% gelatin in Tris-buffered saline (TBS, pH 7.5) containing 20 mM Tris and 500 mM NaCl, and then incubated for 2 h at room temperature in a 1:20 dilution of serum specimens. After being washed with buffer solution, the membranes were further immersed for 2 h in alkaline phosphatase (AP)-conjugated goat anti-human IgG (catalog No. 109-055-008; Jackson ImmunoResearch Inc., USA) and IgM (catalog No. 109-055-043; Jackson ImmunoResearch Inc.) diluted 1:5000 with 0.05% Tween 20 in TBS (T-TBS), as described previously.[5,34] The reacted membranes were then washed twice with T-TBS, and a substrate solution (10 ml of nitroblue tetrazolium, catalog No. B-5655; Sigma) was added to develop the color for 1 min; the reacted membranes were then washed with distilled water and air-dried for analysis of spirochetal protein bands.

2.4. DNA extraction from skin cultures

Total genomic DNA was extracted from individual spirochete-positive cultures with the DNeasy Tissue Kit (catalog No. 69506; Qiagen, Taipei, Taiwan) and used as a template for PCR amplification. Briefly, individual positive-culture medium together with dissected skin was placed in a microcentrifuge tube and centrifuged for 20 min at 12 000 × g to pellet the spirochetes. After removing the supernatant, the microcentrifuge tube was filled with 180 μl lysing buffer solution and was further processed with the DNeasy Tissue Kit, as per the manufacturer's instructions. After

filtration, the eluate was collected and the DNA concentration was determined spectrophotometrically with a DNA calculator (Gene-Quant II; Pharmacia Biotech, Uppsala, Sweden).

2.5. DNA amplification by Borrelia-specific PCR

A nested PCR was performed with primers designed to amplify the variable spacer region between two conserved duplicate structures. A primer set corresponding to the 3′ end of the 5S rRNA (rrf) (5′-CGACCTTCTTCGCCTTAAAGC-3′) and the 5′ end of the 23S rRNA (rrl) (5′-TAAGCTGACTAATACTAATTACCC-3′) was designed and applied for the primary amplification, as described previously.[26] In the nested PCR, a primer set of primer 1 (5′-CTGCGAGTTCGCGGGAGA-3′) and primer 2 (5′-TCCTAGGCATT-CACCATA-3′) was used and expected to yield a 210–260-bp fragment depending on the Borrelia strain, as described previously.[36] All PCR reagents and Taq polymerase were obtained and used as recommended by the manufacturer (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. PCR amplification was performed with a Perkin-Elmer Cetus Thermocycler (GeneAmp System 9700; Taipei, Taiwan), and the primary amplification included 2 min denaturation at 96 °C followed by 30 cycles of the following conditions: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 40 s. Nested amplification was performed under the same conditions except the annealing at 59 °C for 30 s. Afterwards, PCR-amplified DNA products were electrophoresed on 2% agarose gels in Tris–borate–EDTA (TBE) buffer and were visualized under ultraviolet (UV) light after staining with ethidium bromide. A DNA ladder (1-kb plus, catalog No. 10787-018; Invitrogen, Taipei, Taiwan) was used as the standard marker for comparison. A negative control of distilled water was included in parallel with each amplification.

2.6. Sequence alignment and phylogenetic analysis

After purification (QIAquick PCR Purification Kit, catalog No. 28104), sequencing reactions were performed by dye-deoxy terminator reaction method using the Big Dye Terminator Cycle Sequencing Kit in an ABI Prism 377–96 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The resulting sequences were initially aligned with the CLUSTAL W software[37] and further analyzed by comparing with other Borrelia sequences available in GenBank. Neighbor-joining (NJ) compared with maximum parsimony (MP) methods were used to estimate the phylogeny of the entire alignment using MEGA 4.0 software package.[38] A similarity matrix was also constructed using the DNASTAR program (Lasergene, version 8.0). All phylogenetic trees were constructed and performed with 1000 bootstrap replications to evaluate the reliability of the constructions, as described previously.[39]

2.7. Nucleotide sequence accession numbers

The nucleotide sequences of PCR-amplified 5S (rrf)–23S (rrl) intergenic spacer amplicon genes from Borrelia spirochetes determined in this study have been registered and assigned the following GenBank accession numbers: **JX649205** (strain Bg-PP-TW1), **JX649206** (strain Bg-PP-TW2), and **JX649207** (strain Ba-PP-TW1). In the phylogenetic analysis, the nucleotide sequences of the 5S (rrf)–23S (rrl) intergenic spacer amplicon gene from various Borrelia species were included for comparison; their GenBank accession numbers are shown in Table 1.

Table 1
Genospecies and strains of Borrelia spirochetes used for phylogenetic analysis in this study

Genospecies and strain	Origin of Borrelia strain		5S (rrf)–23S (rrl) gene accession number[a]
	Biological	Geographic	
Taiwan strain			
Bg-PP-TW1	Human skin	Taiwan	**JX649205**
Bg-PP-TW2	Human skin	Taiwan	**JX649206**
Ba-PP-TW1	Human skin	Taiwan	**JX649207**
B. burgdorferi sensu stricto			
B31	Ixodes scapularis	USA	**L30127**
IP1	Human CSF	France	**AF090977**
TWKM5	Rattus norvegicus	Taiwan	**AY032908**
Bbss-MP-TW	Human skin	Taiwan	**GQ499848**
B. garinii			
20047	Ixodes ricinus	France	**L30119**
NP81	Ixodes persulcatus	Japan	**D84406**
Mp-TW1	Human skin	Taiwan	**FJ686175**
B. afzelii			
VS461	Ixodes ricinus	Switzerland	**L30135**
BO23	Human skin	Sweden	**Z77174**
Ba-MP-TW	Human skin	Taiwan	**GQ499849**
B. bissettii			
DN127	Ixodes pacificus	USA	**L30126**
CA376	Neotoma fuscipes	USA	**AY177634**
B. spielmanii			
A14S	Human skin	Netherlands	**U76616**
PC-Eq17N5	Ixodes ricinus	France	**DQ133526**
B. valaisiana			
VS116	Ixodes ricinus	Switzerland	**L30134**
CKA2a	Apodemus agrarius	China	**AB022124**
B. sinica			
CMN3	Niviventer sp	China	**AB022131**
CWO1	Ixodes ovatus	China	**AB022130**

CSF, cerebral spinal fluid.
[a] GenBank accession numbers **JX649205** to **JX649207** were submitted by this study.

3. Results

3.1. Culture of spirochetes

Cultures of skin biopsies from patients with skin lesions of PP demonstrated *B. burgdorferi*-like spirochetes in the skin cultures of only three patients, at 10 to 20 days after initial inoculation (Table 2). Purification of cultivable spirochetes was performed by serial dilution of filtrated cultures, and pure isolates of *B. burgdorferi*-like spirochetes were also observed in culture medium at 3 to 7 days after filtrated passage. To obtain sufficient quantities of cultivable spirochetes, all positive cultures were allowed to grow in BSK-H medium for another 2 weeks.

3.2. Serodiagnosis of LD infection

Serodiagnosis of LD infection in our patients was made with the IFA test, with a positive reactivity (>1:40) against Borrelia spirochetes, and confirmed by WB analysis, with the presence of several major antigenic proteins that are known to be correlated with *B. burgdorferi* infection. As shown in Table 2, WB analysis using paired serum samples collected from the same patient revealed elevated IgG and IgM antibodies against the major protein antigens of *B. burgdorferi*. In two patients, significant IgG reactivity with the 41-, 58-, 66-, and 72-kDa protein antigens of *B. burgdorferi* was observed, with significant IgM reactivity against the 41-, 66-, 72-, and 76-kDa protein bands of *B. burgdorferi* (Table 2). Another patient with long-lasting PP for 4 years showed significant IgG and IgM reactivities with the 41-, 66-, and 72-kDa protein antigens of *B. burgdorferi*. In contrast, all healthy control patients showed no seroreactivity against the major protein antigens of *B. burgdorferi* and only two out of 11 culture-negative patients showed weak seroreactivity with the 41-kDa protein antigen of *B. burgdorferi*.

3.3. DNA amplification and genetic analysis

To clarify the genetic identity of Borrelia spirochetes, we employed a nested PCR assay to achieve a high sensitivity and specificity for Borrelia detection. In this study, nested PCR assays revealed amplified DNA fragments of approximately 260 bp from skin cultures of infected patients (Figure 2). Based on comparison of the 5S–23S intergenic spacer amplicon gene sequences among 21 Borrelia spirochetes, the genetic identity of strains Bg-PP-TW1 and Bg-PP-TW2 isolated from patient skin was verified with a high sequence homology within the genospecies of *B. garinii* (91.0–98.7%), and the strain Ba-PP-TW1 was verified with a high sequence homology within the genospecies of *B. afzelii* (97%) (Table 3). Phylogenetic analysis also revealed that these detected spirochetes represent two monophyletic groups closely affiliated to the genospecies of *B. garinii* and *B. afzelii*, and constitute two separate clades distinguished clearly from other *Borrelia* genospecies by both neighbor-joining and maximum parsimony methods (Figure 3).
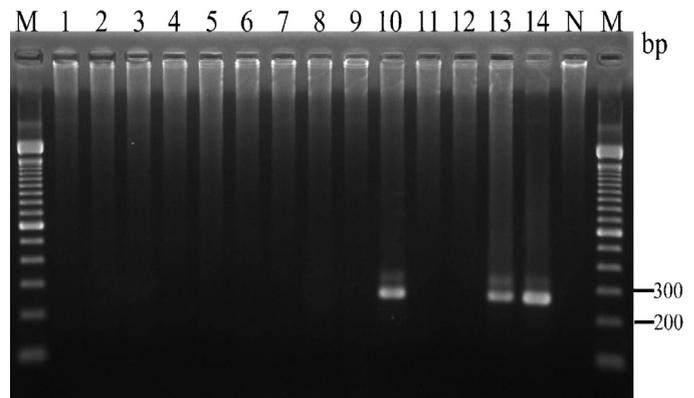

Figure 2. Amplification of Borrelia DNA from skin cultures of patients by nested PCR assay based on the 5S (*rrf*)–23S (*rrl*) rRNA gene. Lanes: M, molecular marker; 1 to 14, patients with prurigo pigmentosa-like skin lesions; N, negative control.

4. Discussion

Our report provides the first convincing evidence of *B. garinii* and *B. afzelii* spirochetes isolated from patients presenting with rare PP skin lesions in Taiwan. In our previous investigations, LD spirochetes were isolated from rodent hosts captured at various locations in Taiwan[5], and the *Ixodes granulatus* tick was recognized as the principle vector tick for enzootic transmission of Borrelia spirochetes in Taiwan.[40] In addition to the discovery of human borreliosis in Taiwan,[7,41–43] the isolation and identification of the causative agents responsible for LD infection in patients presenting with various clinical manifestations are required to verify the genetic polymorphism of Borrelia spirochetes. Indeed, the results from the present study confirm the heterogeneity of Borrelia spirochetes existing in the skin biopsies of humans and demonstrate that these detected spirochetes are genetically affiliated to the genospecies of *B. garinii* and *B. afzelii*. Further study focusing on the detection of Borrelia spirochetes among patients with varying degrees of clinical symptoms would help to elucidate the pathogenesis of Borrelia spirochetes in humans in Taiwan.

Although all these PP patients were characterized by pruritic red-colored papules with gross reticular pigmentation on the trunk, the etiological role of Borrelia spirochetes in the pathogenesis of PP remains unclear. It is interesting that the skin lesions of PP share some clinical and histopathological characteristics with acrodermatitis chronica atrophicans (ACA), which is a late cutaneous manifestation associated with LD infection.[44,45] Indeed, late LD may develop among some patients months to a few years after a tick-transmitted infection, and ACA appears to be the most common manifestation of late LD in Europe.[45,46] The possible association of Borrelia spirochetes in the pathogenesis of ACA has been assumed by the isolation of Borrelia spirochetes from patients with ACA.[47] In the present study, Borrelia spirochetes were isolated from skin specimens of patients with PP skin lesions and *Borrelia* species was also verified by a nested PCR targeting the 5S–23S

Table 2
Clinical features and laboratory analysis of *Borrelia burgdorferi* infection in three patients

Patient sex/age (years)	Clinical features	Reactivity bands by Western immunoblot test	Borrelia culture detected	*Borrelia* species identified[a]
Female/25	PP skin lesion (3 months)	IgG: 41, 58, 66, 72 kDa IgM: 41, 66, 72, 76 kDa	Positive at 10 days	*B. afzelii*
Male/33	PP skin lesion (2 years)	IgG: 41, 58, 66, 72 kDa IgM: 41, 66, 72, 76 kDa	Positive at 20 days	*B. garinii*
Male/63	PP skin lesion (4 years)	IgG: 41, 66, 72 kDa IgM: 41, 66, 72 kDa	Positive at 20 days	*B. garinii*

PP, prurigo pigmentosa.
[a] Genospecies identification based on 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon gene of *B. burgdorferi* sensu lato.

Table 3
Sequence similarity between 5S (*rrf*)–23S (*rrl*) gene sequences from Taiwan strains isolated from patients with prurigo pigmentosa and strains of other *Borrelia* genospecies

Genospecies and strain[a]	B31	Bbss-MP-TW	20047	Mp-TW1	Bg-PP-TW1	Bg-PP-TW2	VS461	Ba-MP-TW	Ba-PP-TW1	DN127	A14S	VS116	CMN3
B31	–	97.0	91.4	90.6	84.5	88.4	85.8	85.8	83.3	91.8	77.3	89.3	80.3
Bbss-MP-TW		–	91.0	90.1	84.1	88.0	86.3	86.3	83.7	89.7	76.8	88.4	79.8
20047			–	98.7	91.4	94.8	89.3	89.3	86.7	89.3	78.5	91.0	80.3
Mp-TW1				–	91.0	95.3	88.8	88.8	86.3	88.4	77.7	90.6	79.4
Bg-PP-TW1					–	91.0	83.3	83.3	82.0	82.0	72.1	84.1	73.4
Bg-PP-TW2						–	87.6	87.6	88.8	85.8	76.0	88.4	77.3
VS461							–	100.0	97.0	85.0	74.7	88.0	81.1
Ba-MP-TW								–	97.0	85.0	74.7	88.0	81.1
Ba-PP-TW1									–	82.4	72.5	85.4	78.5
DN127										–	76.8	89.3	79.4
A14S											–	79.0	83.3
VS116												–	81.5
CMN3													–

[a] Strains: B31 and Bbss-MP-TW, *B. burgdorferi* sensu stricto; 20047 and Mp-TW1, *B. garinii*; VS461 and Ba-MP-TW, *B. afzelii*; DN127, *B. bissettii*; A14S, *B. spielmanii*; VS116, *B. valaisiana*; CMN3, *B. sinica*.

intergenic spacer amplicon gene of *B. burgdorferi* sensu lato. These results suggest that Borrelia spirochetes may play a role in the pathogenesis of PP in Taiwan.

Because the recurrent urticarial- or eczema-like skin lesions on the trunk were observed in the majority of patients with suspected PP. It is possible that seasonal variation and contact allergens may have contributed to the cause of PP in our Borrelia culture-negative patients. Indeed, previous investigations have also indicated that the causative factors responsible for the etiology and pathogenesis of PP include seasonal variation, contact allergens, mechanical stimuli (friction from clothing), primary biliary cirrhosis, *Helicobacter pylori* infection, atopic disease, and anorexia nervosa.[16–20] Thus, it is highly recommended that PP is differentiated from other common dermatological diseases in patients with recurrent urticarial- or eczema-like skin lesions in the clinical diagnosis.

The existence of two tandem duplicated copies of the *rrl* (23S) and *rrf* (5S) genes in LD spirochetes is unique and has not been found in other members of the genus *Borrelia* or in other eubacteria.[25,26] Taking advantage of this unique genomic characteristic, the genetic identity of Borrelia spirochetes can be distinguished by their differential reactivities with genospecies-specific PCR primers targeting the 5S (*rrf*)–23S (*rrl*) intergenic spacer amplicon gene. Indeed, genetic heterogeneity can be further classified among Borrelia isolates that have previously been identified as the same genospecies, or atypical strains of Borrelia spirochetes.[28,29] Results from the present study also clarify that the genetic identity of Borrelia spirochetes isolated from skin biopsy specimens of Taiwanese patients is highly homogeneous within the genospecies of *B. garinii* and *B. afzelii*, and is clearly distinguished from other genospecies of Borrelia spirochetes
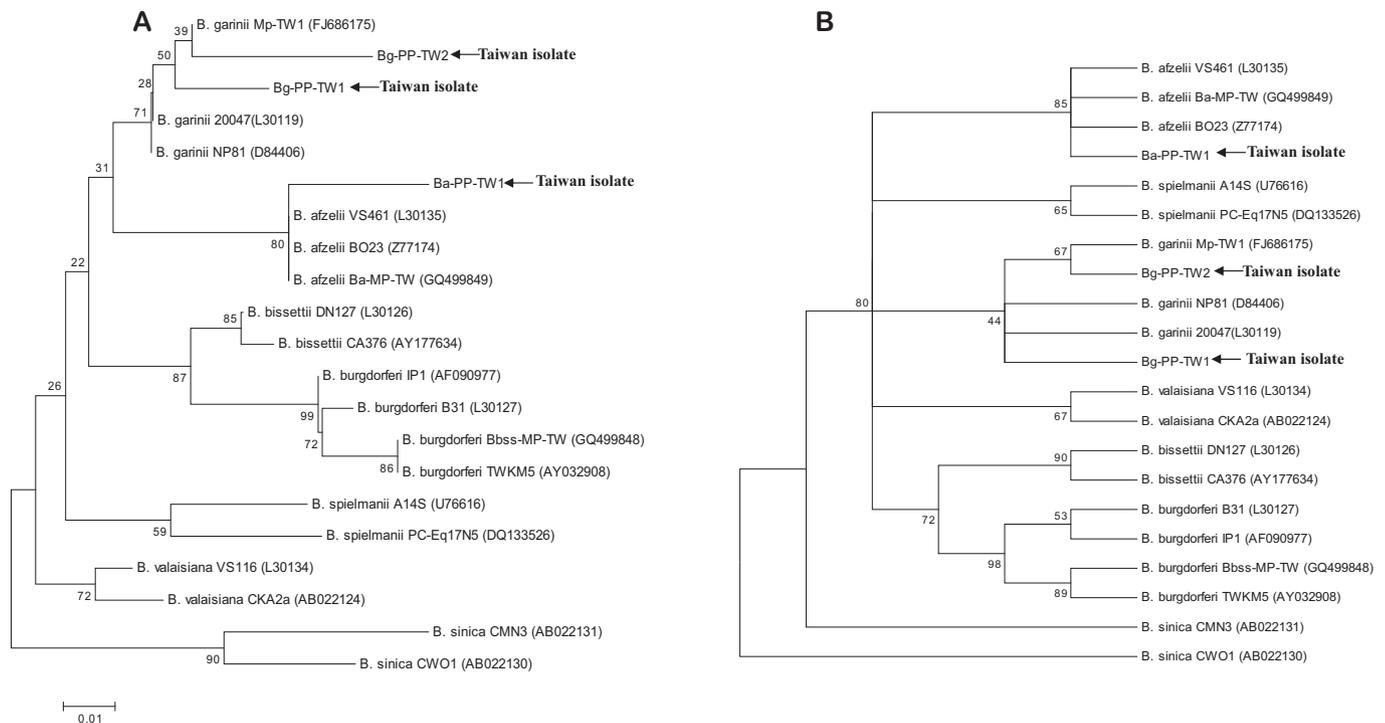

Figure 3. Phylogenetic relationships based on a comparison of the 5S (*rrf*)–23S (*rrl*) rRNA gene sequences between three Borrelia spirochetes (Bg-PP-TW1, Bg-PP-TW2, and Ba-PP-TW1) isolated from human skin in Taiwan and 18 other strains of Borrelia spirochetes. The trees were constructed and analyzed by neighbor-joining (A) and maximum parsimony (B) methods using 1000 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.

(Table 3). Further application of genospecies-specific PCR primers based on the *rrf–rrl* genes to analyze the Borrelia spirochetes detected in various vector ticks may help to clarify the natural transmission cycle of Borrelia spirochetes in Taiwan.

A spirochetal infection can be verified by the presence of cultivable spirochetes in skin cultures. Although the in vitro isolation of Borrelia spirochetes from the infected skin is insensitive, culture of Borrelia spirochetes undoubtedly provides the best confirmation of active infection. Indeed, culture of skin biopsy specimens gives the highest success rates for clinical samples,[48,49] and the successful recovery of Borrelia spirochetes has been reported from patients characterized with late cutaneous manifestations more than 10 years after the onset of the skin lesions.[47] Results from the present cases also showed the positive culture of Borrelia spirochetes from skin specimens of infected patients with long-lasting skin lesions at 3 months to 4 years after onset. Indeed, *B. burgdorferi*-like spirochetes were observed in skin cultures at 10 to 20 days after initial inoculation (Table 2). These observations reveal the feasibility of culturing Borrelia spirochetes from skin specimens of patients with a chronic skin lesion of PP presenting as a late cutaneous manifestation of LD.

Regardless of the two-tier testing approach recommended by the CDC,[35] a positive diagnosis as suggested by the European Union Concerted Action on Lyme Borreliosis (EUCALB) requires only two seroreactive bands among 10 major Borrelia protein antigens for IgG WB, and only one seroreactive band among three major Borrelia protein antigens (17, 23, and 39 kDa) or a strong 41-kDa band for IgM WB.[50] Thus, our patients meet the defined requirement for positive seroconversion associated with human LD according to the diagnostic criteria recommended by EUCALB.

The differential WB banding in infected patients may depend on the duration of infection and the manifestations of LD. In early LD, IgG and IgM antibody reactivities to the OspC (21–23 kDa) and flagellar (41 kDa) antigens are frequently observed.[51–53] However, a previous study on the frequency of WB reactivity to specific Borrelia protein antigens has also revealed that seroreactivity of IgG and IgM antibodies against the higher molecular weight mass bands (41 kDa and above) is most frequently detected in infected patients.[54] Results from the present cases also revealed significant seroreactivities of IgG and IgM antibodies against the higher molecular weight mass bands of Borrelia protein antigens (Table 2). Moreover, the present cases also showed the persistence of elevated anti-*B. burgdorferi* IgG and IgM antibody titers during the prolonged infection course. These results demonstrate the significance of persistent seroreactivity of individual patients against Borrelia protein antigens. Further investigations focused on the variant seroreactivities of WB banding in culture-positive patients classified by various clinical manifestations would be beneficial to the establishment of standardized criteria for the serodiagnosis of LD infection in Taiwan.

In conclusion, our findings provide the first convincing evidence regarding the identification of *B. garinii* and *B. afzelii* from patients presenting with PP, an unusual dermal manifestation of LD in Taiwan. Further investigations focusing on the detection of Borrelia spirochetes among patients with various clinical manifestations may enhance our understanding of the significance of genetic diversity of Borrelia spirochetes in relation to the pathogenicity of LD infection in Taiwan.

Acknowledgements

Ethics: All examinations and interventions were part of routine clinical practice. Informed consent was obtained from all patients. The study was approved by the Expert Committee of the Institutional Review Board.

Conflict of interest: No conflict of interest to declare.

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