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Isolation of *Bartonella* species from rodents in Taiwan including a strain closely related to '*Bartonella rochalimae*' from *Rattus norvegicus*

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An increasing number of *Bartonella* species originally isolated from small mammals have been identified as emerging human pathogens. During an investigation of *Bartonella* infection in rodent populations carried out in Taiwan in 2006, a total of 58 rodents were tested. It was determined that 10.3 % (6/58) of the animals were *Bartonella* bacteraemic. After PCR/RFLP analysis, four isolates were identified as *Bartonella elizabethae* and one isolate as *Bartonella tribocorum*. However, there was one specific isolate with an unrecognized PCR/RFLP pattern. After further sequence and phylogenetic analyses of the *gltA*, *ftsZ* and *rpoB* genes, and the 16S–23S rRNA intergenic spacer region, the results indicated that this specific isolate from *Rattus norvegicus* was closely related to human pathogenic '*Bartonella rochalimae*'. Further studies need to be conducted to evaluate whether this rodent species could be a reservoir for '*B. rochalimae*'.

INTRODUCTION

Bartonella species are short rod, fastidious, Gram-negative and oxidase-negative bacteria (Boulouis *et al.*, 2005; Breitschwerdt & Kordick, 2000). Since the early 1990s, more than 20 species within the genus *Bartonella* have been recognized (Eremeeva *et al.*, 2007; Zeaiter *et al.*, 2002). These intracellular bacteria are regarded as emerging zoonotic pathogens, which can cause serious diseases in humans worldwide (Boulouis *et al.*, 2005). A new species, '*Bartonella rochalimae*', was recently isolated from a case of splenomegaly in a patient who had travelled to South America (Eremeeva *et al.*, 2007). This event raised the concern that it could be a newly emerged zoonotic pathogen. However, to date, little is known regarding to the reservoirs and vectors for the transmission of '*B. rochalimae*'.

Small mammals are natural hosts for several *Bartonella* species, some of which can cause human diseases (Boulouis *et al.*, 2005; Zeaiter *et al.*, 2002). The order Rodentia has been reported as the reservoir for several *Bartonella* species (Ellis *et al.*, 1999) that are pathogens for humans, such as *Bartonella elizabethae* reported in a case of endocarditis (Daly *et al.*, 1993). *Bartonella grahamii*, isolated primarily from bank voles (*Clethrionomys glareolus*), was identified to be the causative agent of neuroretinitis in humans (Baker, 1946; Kerkhoff *et al.*, 1999). Although the main route of transmission for rodent *Bartonella* species is still unclear, these infections are likely to be vector-borne, mainly by fleas. For instance, *Ctenophthalmus nobilis*, collected on bank voles in the UK, was shown to be a competent vector for *B. grahamii* and *Bartonella taylorii* (Bown *et al.*, 2004; Brenner *et al.*, 1993). In Kabul, Afghanistan, *B. taylorii* was also detected by molecular methods in fleas collected from gerbils (*Meriones libycus*), and *B. elizabethae* and *Bartonella doshiae* were identified in rat fleas (Marié *et al.*, 2006). More recently, *Bartonella clarridgeiae*-like bacteria were detected in rat fleas (*Xenopsylla cheopis*) collected from brown rats (*Rattus norvegicus*) from Egypt (Loftis *et al.*, 2006). Similarly, when studying *Bartonella* infection in flea vectors (*Polygenis gwyni*) parasitizing cotton rats (*Sigmodon hispidus*), some samples were co-infected with a strain phylogenetically related to *B. clarridgeiae* (Abbot *et al.*, 2007).

Abbreviation: ITS, intergenic spacer region.

The GenBank/EMBL/DDBJ accession numbers for the *gltA*, *ftsZ* and *rpoB* genes and the 16S–23S rRNA ITS sequence of *Bartonella* 1-1C strain are EU551154, EU551155, EU551156 and EU551157.

Our laboratory has been collaborating with the Center for Disease Control, Taiwan, to conduct an epidemiological survey of *Bartonella* species in small mammals in central Taiwan. In this study, using molecular methods and sequencing various genes for the obtained isolates, our aim was to determine if a '*B. rochalimae*'-like organism could be isolated from rodents and the prevalence of *Bartonella* infection in rodents in Taiwan.

METHODS

Sample collection. Between March and October 2006, a total of 58 rodents (53 *R. norvegicus*, 2 *Mus musculus* and 3 *Rattus rattus*) were captured in Taichung, a city located in central Taiwan. After being humanely anaesthetized with zoletil 50 (Virbac Laboratories), the animals were bled to death via cardiac puncture by using 3 ml syringes fitted with 22 gauge, 3.8 cm needles. The blood was then collected in a tube containing 10 µl (1.5 mg ml^{-1}) EDTA, and was stored at −80 °C for isolation and molecular identification of *Bartonella* species.

Blood culture for *Bartonella* species. For bacterial culture, 200 µl thawed whole blood sample was plated onto both fresh 10 % sheep blood-enriched Columbia agar and chocolate agar. The plates were incubated at 35 °C in 5 % CO_2 for at least 1 month and checked for growth of *Bartonella* species on a weekly basis (Boulouis *et al.*, 2005). The suspected colonies were subcultured onto a fresh agar plate for further molecular identification of *Bartonella* species by PCR/RFLP and sequence analyses.

DNA extraction and PCR/RFLP procedures for *Bartonella* species. After culturing a single colony pick from the original plate, DNA of the cultured isolates was extracted using a Viogene DNA/RNA extraction kit (Viogene Biotek) following the manufacturer's instructions. The primers used for DNA amplification and sequencing in this study are shown in Table 1. The primers BhCS.781p and BhCS.1137n were used first for amplifying a partial fragment, ranging from 380 to 400 bp, of the *gltA* gene of *Bartonella* species. PCR mixtures were set up as follows: 5 µl DNA template, 0.5 µl 100 µM each primer, 4 µl 2.5 mM dNTPs, 5 µl 10× PCR buffer, 3 µl 25 mM $MgCl_2$, 31.75 µl sterile distilled H_2O and 0.25 µl DNA polymerase (AmpliTaq Gold; Applied Biosystems). The PCR amplification was executed as follows: 1 cycle of 10 min at 95 °C, followed by 45 cycles of 30 s at 95 °C, 1 min at 57 °C and 2 min at 72 °C, and then 1 cycle of 5 min at 72 °C for final extension (Norman *et al.*, 1995). Primers bartsppA and bartsppB amplified a fragment of the 16S–23S rRNA intergenic spacer region (ITS) by single-step PCR (Jensen *et al.*, 2000); the different molecular size of the PCR products allowed for quick differentiation of various *Bartonella* species. The PCR program for the ITS was as follows: 1 cycle of 2 min at 95 °C, followed by 45 cycles of 1 min at 95 °C, 1 min at 52 °C and 30 s at 72 °C, and 1 cycle of 10 min at 72 °C for complete extension (Jensen *et al.*, 2000). The primers for the amplification of the *ftsZ* gene were Bfp1 and Bfp2. A 900 bp fragment was expected from the amplification. The PCR program consisted of: 1 cycle of 4 min at 94 °C, followed by 44 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 68 °C, and then 1 cycle of 10 min at 68 °C for complete extension (Zeaiter *et al.*, 2002). For PCR amplification of the *rpoB* gene, a set of primers 1400F and 2300R was used to amplify an expected 900 bp DNA fragment. The PCR was performed as follows: 1 cycle of 2 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 53 °C and 1 min at 72 °C, and then 1 cycle of 2 min at 72 °C for complete extension (Renesto *et al.*, 2001). RFLP of the *gltA* gene was also used for quick identification of *Bartonella* organisms at the species level with restriction enzyme digestion with *Taq*I (New England BioLabs), *Hha*I (New England BioLabs) and *Mse*I (New England BioLabs) following the manufacturer's instructions. The RFLP banding patterns were then compared to the patterns of *Bartonella* type strains, including *Bartonella alsatica* (IBS382T) CIP 105477, *Bartonella birtlesii* (IBS 325T) CIP 106294, *Bartonella bovis* (91-4T) CIP 106692, *B. clarridgeiae* (Houston-2T) ATCC 51734, *B. doshiae* (R18T) NCTC 12862, *B. elizabethae* (F9251T) ATCC 49927, *B. grahamii* (V2T) NCTC 12860 b, *Bartonella henselae* (Houston-1T) ATCC 49882, *Bartonella koehlerae* (C-29T) ATCC 700693, *Bartonella quintana* (FullerT) ATCC VR-358, *B. taylorii* (M6T) NCTC 12861, *Bartonella tribocorum* (IBS 506T) CIP 104576, *Bartonella. vinsonii* subsp. *arupensis* (OK 94-513T) ATCC 700727, *B. vinsonii* subsp. *berkhoffii* (93-CO1T) ATCC 51672, *B. vinsonii* subsp. *vinsonii* (BakerT) ATCC VR-152.

Sequencing and phylogenetic analyses for *Bartonella* species. The confirmed PCR products were sent for automated sequencing using the primers listed in Table 1 (Mission Biotech). Sequences of several *Bartonella* species were downloaded for comparison from GenBank. The sequences of the *gltA*, *ftsZ* and *rpoB* genes, and the 16S–23S rRNA ITS, of the *Bartonella* strain (1-1C) isolated in this study have been submitted to the GenBank. The accession numbers are EU551154 for the *gltA* gene, EU551155 for the *ftsZ* gene, EU551156 for the *rpoB* gene and EU551157 for the 16S–23S rRNA ITS. Sequences were first aligned by the CLUSTAL W method of the BioEdit program (Tom Hall, Ibis Biosciences, Isis Pharmaceuticals). Phylogenetic analysis was performed on the aligned DNA sequences using maximum-parsimony as implemented in PHYLIP version 3.6 (Joseph Felsenstein, Department of Genome Sciences and Department of Biology, University of Washington, Seattle, WA,

Table 1. Primers used for PCR amplification and sequencing

Primer set	Target gene	Primer sequence	Size of PCR product (bp)	Reference
BhCS.781p	*gltA*	5′–GGGGACCAGCTCATGGTGG–3′	380–400	Norman *et al.* (1995)
BhCS.1137n		5′–AATGCAAAAAGAACAGTAAACA–3′		
Bartsppa	16S–23S rRNA ITS	5′–YCTTCGTTTCTCTTTCTTCA–3′	Molecular size dependent on *Bartonella* species	Jensen *et al.* (2000)
Bartsppb		5′–AACCAACTGAGCTACAAGCC–3′		
Bfp1	*ftsZ*	5′–ATTAATCTGCAYCGGCCAGA–3′	900	Zeaiter *et al.* (2002)
Bfp2		5′–ACVGADACACGAATAACACC–3′		
1400F	*rpoB*	5′–CGCATTGGCTTACTTCGTATG–3′	900	Renesto *et al.* (2001)
2300R		5′–GTAGACTGATTAGAACGCTG–3′		

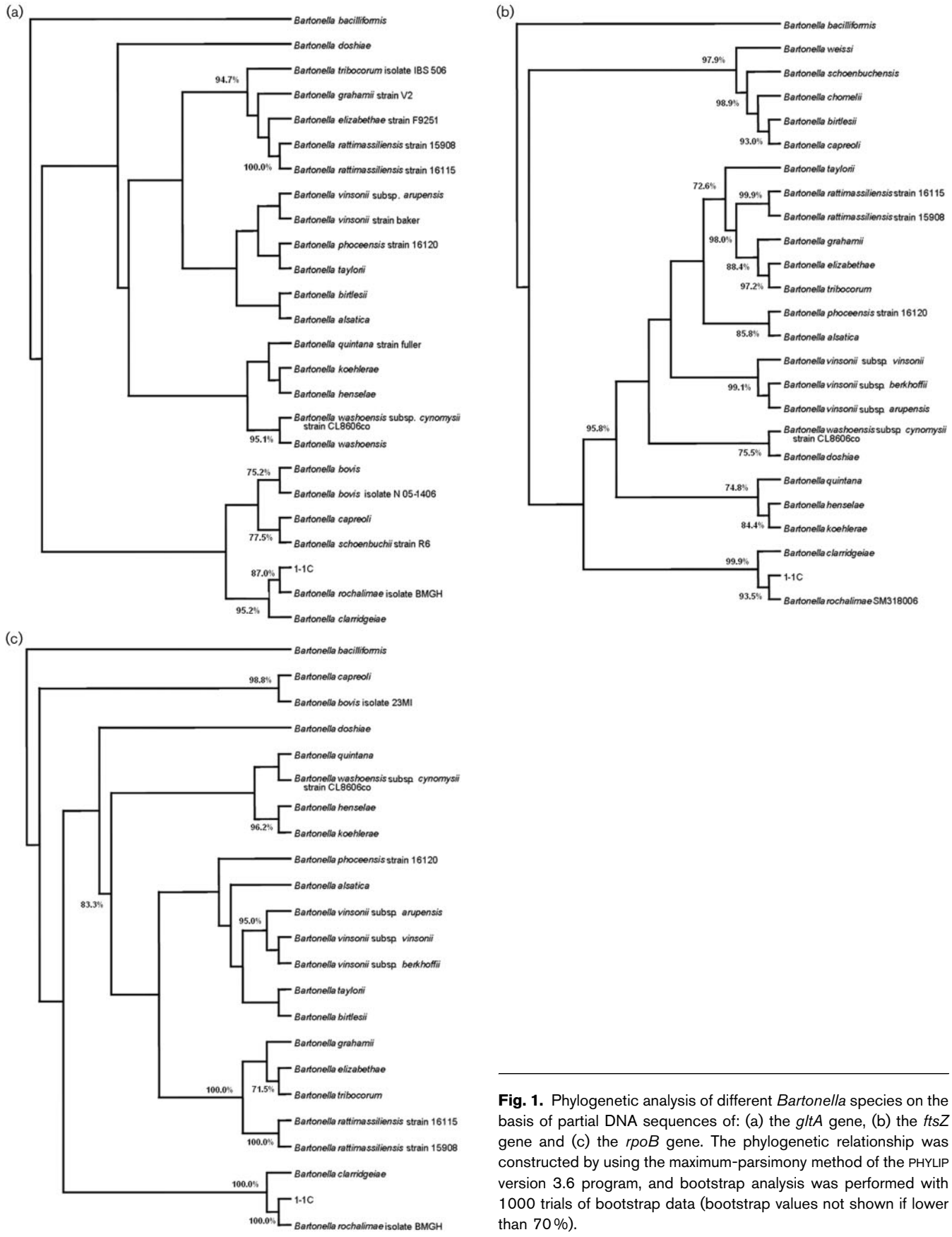

Fig. 1. Phylogenetic analysis of different *Bartonella* species on the basis of partial DNA sequences of: (a) the *gltA* gene, (b) the *ftsZ* gene and (c) the *rpoB* gene. The phylogenetic relationship was constructed by using the maximum-parsimony method of the PHYLIP version 3.6 program, and bootstrap analysis was performed with 1000 trials of bootstrap data (bootstrap values not shown if lower than 70 %).

Table 2. Accession numbers of the reference *Bartonella* species sequences used for phylogenetic analysis in this study

***Bartonella* strain**	**Sequence accession no.**		
	gltA	***ftsZ***	***rpoB***
B. alsatica	AF204273	AF467763	AF165987
B. bacilliformis	AF478357	AF007266	AF165988
B. birtlesii	AF204272	AF467762	AB196425
B. bovis	AF293394	–	–
B. bovis isolate 23MI	–	–	DQ356076
B. bovis isolate N 05-1406N05	EF432056	–	–
B. capreoli	AF293392	AB290192	AB290188
B. chomelii	–	AB290193	–
B. clarridgeiae	U84386	AF141018	AF165990
B. doshiae	AF207827	AF467754	AF165991
B. elizabethae	–	AF467760	AF165992
B. elizabethae strain F9251	Z70009	–	–
B. grahamii	–	AF467753	AF165993
B. grahamii strain V2	Z70016	–	–
B. henselae	L38987	AF061746	AF171071
B. koehlerae	AF176091	AF467755	AY166580
'*B. phoceensis*' strain 16120	AY515126	AY515135	AY515132
B. quintana	–	AF061747	AF165994
B. quintana strain Fuller	Z70014	–	–
'*B. rattimassiliensis*' strain 15908	AY515124	AY515133	AY515130
'*B. rattimassiliensis*' strain16115	AY515125	AY515134	AY515131
'*B. rochalimae*' isolate BMGH	DQ683195	–	DQ683198
'*B. rochalimae*' SM318006	–	DQ676490	–
B. schoenbuchensis	–	AF467765	–
B. schoenbuchii strain R6	AJ278186	–	–
B. taylorii	AF191502	AF467756	AF165995
B. tribocorum	–	AF467759	AF165996
B. tribocorum isolate IBS 506	AJ005494	–	–
B. vinsonii strain Baker	Z70015	–	–
B. vinsonii subsp. *arupensis*	AF214557	AF467758	AY166582
B. vinsonii subsp. *berkhoffii*	–	AF467764	AF165989
B. vinsonii subsp. *vinsonii*	–	AF467757	AF165997
'*B. washoensis*'	AF470616	–	–
B. washoensis subsp. *cynomysii* strain CL8606co	DQ897367	DQ825692	DQ825688
'*B. weissi*'	–	AF467761	–

USA). Bootstrap support was calculated by using 1000 bootstrap data replicates as implemented by SEQBOOT of the PHYLIP program.

RESULTS

Using whole blood culture, 6 (10.3 %) of the 58 animals were *Bartonella* bacteraemic. Among the six bacteraemic animals, five were *R. norvegicus* and one was *R. rattus*. After PCR/RFLP analysis and pattern comparisons of *Bartonella* type strains, it was determined that four of the five isolates from *R. norvegicus* were *B. elizabethae* and the isolate from *R. rattus* was *B. tribocorum*. However, there was one specific isolate with an unrecognized PCR/RFLP pattern, which was one undigested band (380 bp) by *Taq*I, two bands (285 and 94 bp) by *Hha*I and two bands (199 and 132 bp) by *Mse*I digestion. Therefore, this isolate was further characterized by direct DNA sequencing for species determination.

For this specific isolate, three randomly picked colonies from the original plate were subcultured and harvested for DNA extraction after 1 week. There was no *gltA* sequence variation between the isolates, thus a single isolate (designated 1-1C) was used for subsequent sequencing of the other genes. The accession numbers of the *Bartonella* reference strains used for comparison are shown in Table 2. In comparison with partial sequences of the *gltA* gene of *Bartonella* species in GenBank, the DNA similarity value of this specific isolate was highest, 95.6 % with '*B. rochalimae*', followed by *B. clarridgeiae* (95 %), *Bartonella schoenbuchensis* (90.8 %) and '*Bartonella washoensis* subsp. *cynomy-*

sii' strain CL8606co (90.2 %). DNA similarity was less than 90 % to all other *Bartonella* species tested.

After sequence analysis of the *ftsZ* and *rpoB* genes, as well as the 16S–23S rRNA ITS, the isolate 1-1C sequences were still closest to '*B. rochalimae*' (similarity values: 97.1, 97.1 and 96 %, respectively). Similarly, phylogenetic analysis of the *gltA*, *ftsZ* and *rpoB* genes strongly supported that isolate 1-1C and '*B. rochalimae*' belonged to the same clade, as indicated by the high bootstrap value (>90 %) on the basis of 1000 replicates (Fig. 1). This clade also includes *B. clarridgeiae* and is clearly separated from other *Bartonella* species. When we further compared sequence relatedness between the 1-1C strain and *B. clarridgeiae*, we found that the similarity values were 94.3 % for the *ftsZ* gene and 93.2 % for the *rpoB* gene, and the similarity value for the 16S–23S rRNA ITS was only 69.1 %.

DISCUSSION

'*B. rochalimae*' has recently been isolated from a human infection (Eremeeva *et al.*, 2007); however, the reservoir of this new human pathogen remains unknown. We report what is believed to be the first isolation of a *Bartonella* strain closely related to '*B. rochalimae*' from a brown rat captured in central Taiwan. As the data are limited by the small sample size of rats tested and the lack of a further inoculation test in laboratory animals in this study, this unique isolate does not allow confirmation of rats as a possible reservoir of '*B. rochalimae*'. However, several *Bartonella* species have been shown to use various species of small mammals as their natural reservoirs (Baker, 1946; Birtles *et al.*, 1995; Brenner *et al.*, 1993; Heller *et al.*, 1998; Kordick *et al.*, 1996). Of major importance, several rodent *Bartonella* species have been shown to be emerging zoonotic pathogens. The first rodent-associated zoonotic *Bartonella* species was *B. elizabethae* (Daly *et al.*, 1993), and *R. norvegicus* was considered to be its main reservoir (Ellis *et al.*, 1999). '*B. washoensis*' and *B. vinsonii* subsp. *arupensis*, which cause human myocarditis and neurological disorders, respectively, were also suggested to be rodent associated (Kosoy *et al.*, 2003; Welch *et al.*, 1999). The present study clearly showed that rodents in Taiwan can harbour *Bartonella* species, including *B. elizabethae*, *B. tribocorum* and a strain closely related to '*B. rochalimae*'.

B. clarridgeiae is considered to be an agent possibly causing human cat-scratch disease, with cats being the natural reservoir (Boulouis *et al.*, 2005), and recently '*B. rochalimae*', the closest *Bartonella* species to *B. clarridgeiae*, was isolated from a human patient (Eremeeva *et al.*, 2007). In a study conducted in northern California, researchers identified a novel *B. clarridgeiae*-like bacterium from 3 dogs and 22 grey foxes (Henn *et al.*, 2007). These data raise concerns about the existence of other reservoirs and vectors for this emerging infection. For instance, a novel *Bartonella* species close but distinct from *B. clarridgeiae* was identified in a striped field mouse (*Apodemus agrarius*) and a yellow-necked field mouse (*Apodemus flavicollis*) from Slovenia (Knap *et al.*, 2007). *B. clarridgeiae*-like bacteria were also detected in rat fleas (*X. cheopis*) collected on brown rats (*R. norvegicus*) from Egypt (Loftis *et al.*, 2006) and from *P. gwyni* fleas parasitizing cotton rats (*S. hispidus*) in the United States (Abbot *et al.*, 2007). Furthermore, according to a phylogenetic analysis of *Bartonella* species detected in rodent fleas, a genotype designated F15YN, most closely related to *B. clarridgeiae*, was identified in a *Ctenophthalmus lushuiensis* flea in China (Li *et al.*, 2007). Because Taiwan is geographically located close to mainland China, the sequence of the *gltA* gene of the F15YN strain was further compared to the one of our 1-1C strain. After sequence comparison, it showed that a 5 % divergence existed between these two strains. Unfortunately, no sequences for other genes of the F15YN were available for comparison. In this study, we determined that the 1-1C strain was most closely related to '*B. rochalimae*', on the basis of more than 95 % similarity values for the sequences of *gltA*, *ftsZ* and *rpoB* genes, and the 16S–23S rRNA ITS region. Although 95 % similarity for the *gltA* gene was also observed between the 1-1C strain and *B. clarridgeiae*, more divergence was observed by the sequence comparisons of the *ftsZ* and *rpoB* genes, as well as the 16S–23S rRNA ITS (similarity values: 94.3, 93.2 and 69.1 %, respectively). The partial sequence of the 16S–23S rRNA ITS region was shown to be useful to differentiate '*B. rochalimae*' and *B. clarridgeiae*. As the phylogenetic analysis showed a specific clade including '*B. rochalimae*', *B. clarridgeiae* and the 1-1C strain, it will be important to further determine the relationship and biological differences of these *B. clarridgeiae*-like isolates in the future. Although the mode of infection of humans from these rodents is still unclear, ectoparasites might play a role as vectors. Unfortunately, no ectoparasites were collected for detection of *Bartonella* infection on the bacteraemic rats in our study.

In conclusion, the most significant finding of this study is the identification of a '*B. rochalimae*'-like organism from *R. norvegicus*, on the basis of sequence and phylogenetic analyses of *gltA*, *ftsZ* and *rpoB* genes. Due to the small sample size of animals tested, this study cannot conclude that *R. norvegicus* is the reservoir for '*B. rochalimae*', but it certainly warrants further investigation.

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