Journal of Invertebrate Pathology 99 (2008) 57–65

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

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

Phylogenetic analysis of *Spiroplasmas* from three freshwater crustaceans (*Eriocheir sinensis*, *Procambarus clarkia* and *Penaeus vannamei*) in China

Keran Bi, Hua Huang, Wei Gu, Junhai Wang, Wen Wang *

Jiangsu Key Laboratory for Biodiversity & Biotechnology, College of Life Sciences, Nanjing Normal University, 1 Wenyuan Road, Nanjing 210046, China

ARTICLE INFO

Article history:
Received 19 December 2007
Accepted 18 June 2008
Available online 24 June 2008

Keywords:
Crustacean
16S rRNA
23S rRNA
16S–23S rRNA intergenetic spacer regions (ISRs)
gyrB
rpoB
Genomic size
Western blot
Spiroplasma
Phylogenetic analysis

ABSTRACT

Disease epizootics in freshwater culture crustaceans (crab, crayfish and shrimp) gained high attention recently in China, due to intensive developments of freshwater aquacultures. *Spiroplasma* was identified as a lethal pathogen of the above three freshwater crustaceans in previous studies. Further characterization of these freshwater crustacean *Spiroplasma* strains were analyzed in the current study. Phylogenetic position was investigated by analysis of partial nucleotide sequences of 16S ribosomal RNA (rRNA), *gyrB* and *rpoB* genes, together with complete sequencing of 23S rRNA gene and 16S–23S rRNA intergenetic spacer regions (ISRs). Phylogenetic analysis of these sequences showed that the above-mentioned three freshwater crustacean *Spiroplasma* strains were identical and had a close relationship with *Spiroplasma mirum*. Furthermore, the genomic size, serological studies and experimental infection characteristics confirmed that three freshwater crustacean *Spiroplasma* strains are a single species other than traditional *S. mirum*. Therefore, these data suggest that a single species of *Spiroplasma* infects all three investigated freshwater crustaceans in China, and is a potential candidate for a new species within the *Spiroplasma* genus. These results provide critical information for the further investigations in fresh aquaculture epizootics related to tremor diseases, caused by this infectious agent.

1. Introduction

The Chinese mitten crab *Eriocheir sinensis*, red swamp crayfish *Procambarus clarkii* and Pacific white shrimp *Penaeus vannamei* are important species in freshwater aquaculture in China. Disease epizootics have occurred concurrently with the development of aquaculture of these crustaceans. One of the most devastating diseases for the aquaculture was tremor disease of the Chinese mitten crab *E. sinensis*. A Spiroplasma was previously identified as a potential causative pathogen of tremor disease in crustaceans (Wang et al., 2003, 2004a, 2004b). Subsequently two other freshwater crustaceans, *P. clarkia* (Wang et al., 2005) and *P. vannamei*, were also found to be infected by Spiroplasmas. These Spiroplasmas caused severe mortalities of *E. sinensis*, *P. clarkia* and *P. vannamei* in southeast China (including Jiangsu, Anhui and Zhejiang provinces).

Spiroplasmas are among the smallest self-replicating prokaryotes, with genomes ranging in size from approximately 780–2220 kbp (Carle et al., 1995; Williamson et al., 1997). They are helical, motile bacteria that apparently evolved via simplification of Gram-positive bacteria, although they lack a cell wall (Woese et al., 1980; Weisburg et al., 1989). As the organisms radiated to occupy multiple habitats, they became able to invade the arthropod gut lumen, either as part of their life cycle or incidentally, to invade other habitats in the hemolymph, ovaries, fat bodies, hypodermis and salivary glands. With much lower frequency, Spiroplasmas have also been found in association with ticks and plants (Tully et al., 1987; Williamson et al., 1989, 1998). Because of their diverse hosts, Spiroplasmas may be one of the most abundant groups of microbes on earth (Hackett and Clark, 1989; Hackett et al., 1992). To date, 36 *Spiroplasma* species have been described and given binomial names. Hundreds of other isolates have been partially described, and some of these undoubtedly represent new *Spiroplasma* species (Regassa and Gasparich, 2006). Based on the 16S rRNA gene sequences analysis, Gasparich et al. (2004) demonstrated the genus *Spiroplasma* including 36 *Spiroplasma* spp. is divided into three major phylogenetic clades, the Apis clade, the Citri-Chrysopicola-Mirum clade and the Ixodetis clade. The Citri-Chrysopicola-Mirum clade is then divided further into the Citri-Poulsonii clade, the Chrysopicola-Syrphidicola-TAAS-1 clade and the Mirum clade. The Mirum clade contains only a single member, *Spiroplasma mirum* that is the only one *Spiroplasma* species from rabbit ticks. Recently, the causative agent of tremor disease in *E. sinensis* has been identified as a member of the genus *Spiroplasma* that is closely related to *S. mirum* based on a limited 16S rDNA (less than 800 bp) phylogenetic analysis (Wang et al., 2004b). Subsequently, the *Spiroplasma* from *P. clarkii* has also been identified in close

* Corresponding author. Fax: +86 858915265.
E-mail address: njnuwang@263.net (W. Wang).

0022-2011/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2008.06.008

relation to *Spiroplasma* sp. strain CRAB and *S. mirum* based on 16S rRNA gene sequences (271 bp) similarity in BLAST (Basic Local Alignment Search Tool) searches (Wang et al., 2005). Therefore Wang et al. (2004b, 2005) have provided a hypothesis that the Mirum clade may contain another strain or species, but the relationship among Spiroplasma strains from three freshwater crustaceans and *S. mirum* have not been clearly explained.

In the phylogenetic analysis of the genus of *Spiroplasma*, several studies showed that 16S rRNA gene is a widely accepted genetic marker for determining Spiroplasma identification and the interspecies or intraspecies evolutionary relationships (Gasparich et al., 2004; Regassa and Gasparich, 2006). However, in some cases the 16S rRNA genetic marker might be insufficient due to the presence of low interspecies polymorphism in closely related species within the genus *Spiroplasma* (Gasparich et al., 2004; Johansson, 2000; Regassa and Gasparich, 2006; Stackebrandt et al., 2002). To circumvent the limitations of that 16S rRNA is too conserved, we performed a phylogenetic study using five phylogenetic markers, 16S, 23S, ISRs, *gyrB* and *rpoB* sequence, to determine the phylogenetic position of the Spiroplasmas from three freshwater crustacean hosts. ISRs sequence was a genetic marker which could successfully determine Spiroplasma identification (Volokhov et al., 2006) and the evolutionary relationships among closely related Spiroplasma strains (Regassa et al., 2004; Von der Schulenburg et al., 2000). The *gyrB* gene was identified as a potential genetic marker for interspecies comparison within the genus *Spiroplasma* (Regassa and Gasparich, 2006). The advantages of the 23S rRNA and *rpoB* genes have been shown in the phylogenetic analysis of other affined bacterial taxa, such as *Mycoplasma* and *Acholeplasma* species (Drancourt and Raoult, 2005; Gasparich et al., 2004; Pitulle et al., 2002; Volokhov et al., 2007).

On the other hand, we also performed genomic size, experimental infection characteristics and serological studies to further elucidate the relationships among Spiroplasmas from three freshwater crustaceans, as well as *S. mirum*.

2. Materials and methods

2.1. Spiroplasmas strains and experimental infection

Spiroplasma mirum was purchased from the American Type Culture Collection (ATCC 29335) and grown in R2 medium (Moulder et al., 2002) at 37 °C. *Spiroplasmas* from *E. sinensis* and *P. clarkii* were cultured in the R2 medium at 30 °C. The *Spiroplasma* from freshwater *P. vannamei* was first isolated from the moribund freshwater cultured *P. vannamei* using the methods described by Wang et al. (2004a). To obtain pure cultures of strains from *E. sinensis*, *P. clarkii* and *P. vannamei*, a dilution cloning technique was used (Whitcomb and Hackett, 1987). Actively growing Spiroplasma cultures were serially diluted in R2 medium containing phenol red indicator. Culture dilutions of 10^{3}–10^{-2} cells/mL were distributed on 96-well microtitre plates with 100 μl per well. As cultures grew and acidified, the media changed from red to yellow. The plates were observed daily for color change. Organisms growing within a single, isolated yellow well at the lowest possible dilution were subcultured after microscopic confirmation of their identity as Spiroplasma. This cloning process was repeated three times. The cloned strains from *Spiroplasma* sp. strain CRAB, *Spiroplasma* sp. strain CRAYFISH, *Spiroplasma* sp. strain SHRIMP and the purchased *S. mirum* were used to infect the healthy *E. sinensis* by experimental infection method described by Wang et al. (2003), respectively, i.e., 60 crabs taken from the markets or aquafarms were used for artificial infection. Healthy crabs ($n = 56$) were chosen for experimental infection and divided into four groups, 10 crabs in each group were inoculated with *S.* sp. strain CRAB, *S.* sp. strain CRAYFISH, *S.* sp. strain SHRIMP and *S. mirum* by injecting them with 0.1 ml (10^{8} cells/mL) each, respectively. The other five crabs were inoculated with 0.1 ml vacant R2 medium as a control. The inoculations were made with a 1 ml syringe through the joint between the pereiopod and the thorax of every crab. Each group of crabs were cultivated in aquaria (0.250 m^3) equipped with a heating system. The water was changed every three days and the temperature was controlled at 28 °C and the O_2 in water was maintained in 7–8 mg/L. Blood smears of the crab were collected every five days and were observed with a Hitachi H-600 TEM for Spiroplasma detection.

2.2. Genomic DNA extraction

Genomic DNA was extracted from three cloned freshwater crustacean Spiroplasma strains and *S. mirum* using the DNeasy Tissue Kit (Qiagen, Chatsworth, California), according to the manufacturer's protocol. Briefly, the density of a *Spiroplasma* cell achieved approximate 10^{8} cells/mL, cells were then harvested by centrifugation at 15,700*g* for 30 min at 4 °C, resuspended in ultrapure water, and lysed with the DNeasy lysis buffer at 56 °C. The lysate was treated with RNase A (Promega Corporation, Madison, Wisconsin) and extracted the genomic DNA. The DNA concentration for each sample was determined by measuring absorbance at 260 nm, and the quality of the DNA sample was verified on the basis of the A260/A280 ratio.

2.3. PCR amplification and sequencing of 16S, 23S, ISRs, gyrB and rpoB sequences

Three pairs of PCR primers, 16F1/16R1, 16F2/23R1 and 23F1/5R, were designed using sequences in the 16S, 23S and 5S rRNA genes conserved for most *Mollicutes*. All PCR products were amplified for four Spiroplasma strains using the PCR primer and corresponding annealing temperature in Table 1. All PCR products were obtained from the four Spiroplasma strains using 50 μl reactions with the following combination of reagents: 100 ng of genomic DNA, 100 pmol of each primer, 1 × *Taq* polymerase Buffer, 0.2 mmol/L deoxynucleotide triphosphate (dNTPs), 2.5 U *Taq* polymerase (Promega), 1.5 mmol/L $MgCl_2$, and ultrapure water to a final volume of 50 μl. Amplification cycles were completed as follows: denaturation at 94 °C for 5 min; 35 cycles at 94 °C for 30 s, annealing temperature in Table 1 for 1 min, 72 °C for 90 s; and 1 cycle at 72 °C for 10 min. PCR products were separated on a 1.5% agarose gel and purified using a BioSpin Gel extraction kit (BioFLUX). Amplified

Table 1
Primers and their anneal temperature

Gene region	Primer type	Primer Sequence 5′–3′	Fragment size (bp)	Anneal temperature
16S				
16F1	PCR and sequencing	CTAATACATGCAAGTCGAACG	16F1/16R1 1300	65 °C
16F2	PCR and sequencing	GGTGCATGGTTGTCGTCAG	16F2/23R1 1000	56 °C
16R1	PCR and sequencing	TTGCTGATTCGCGATTACTAG		
23S				
23F1	PCR and sequencing	GAATGGGGAAACCCGGTGAG	23F1/5R 3000	65 °C
23R1	PCR and sequencing	TTCGCTCGCCGCTACTAAG		
23F2	Sequencing	GTAGACCCGAAACCAGGTGA		
23F3	Sequencing	CCGTGAGGACTGCTGGACTG		
23R2	Sequencing	GGATCACTAAGCCCAGCTTT		
5S:				
5R:	PCR and sequencing	TCGGGATGGGAACGGGTG		