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## *Spiroplasma eriocheiris* sp. nov., associated with mortality in the Chinese mitten crab, *Eriocheir sinensis*

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A motile bacterium, designated strain TDA-040725-5<sup>T</sup>, was isolated from the haemolymph of a Chinese mitten crab, *Eriocheir sinensis*, with tremor disease. Based on 16S rRNA gene sequence analysis, the strain was phylogenetically distinct from other spiroplasmas but was closely related to *Spiroplasma mirum* ATCC 29335<sup>T</sup>. Cells of strain TDA-040725-5<sup>T</sup> were variable in length and shape, helical and motile, as determined by phase-contrast light microscopy. Examination by electron microscopy revealed wall-less cells delimited by a single membrane. The strain grew in M1D or R-2 liquid media at 20–40 °C, with optimum growth at 30 °C. Doubling time at the optimal temperature was 24 h. The strain catabolized glucose and hydrolysed arginine but did not hydrolyse urea. The DNA G + C content was 29.7 ± 1 mol%. The genome size was ~1.4–1.6 Mbp. Serological analysis, performed using the deformation test, did not reveal any reciprocal titres ≥ 320, indicating that strain TDA-040725-5<sup>T</sup> had minimal cross-reactivity to strains of recognized species of the genus *Spiroplasma*. Based on this evidence, strain TDA-040725-5<sup>T</sup> (=CCTCC M 207170<sup>T</sup> =DSM 21848<sup>T</sup>) represents a novel species of the genus *Spiroplasma*, for which the name *Spiroplasma eriocheiris* sp. nov. is proposed, belonging to the novel *Spiroplasma* serological group XLIII.

With the development of aquaculture in China, many diseases affecting farmed aquatic organisms have emerged in recent years, one of which is tremor disease (TD). This disease affects Chinese mitten crabs, *Eriocheir sinensis*, and has had disastrous effects on aquaculture in China (Wei, 1999). Based on morphological and pathological studies (Wang & Gu, 2002; Wang *et al.*, 2002; Zhang *et al.*, 2002), the TD agent (TDA) was previously thought to be a rickettsia-like organism. Analysis using universal primers for bacterial 16S rRNA gene sequences showed that the agent was not a rickettsia, but a spiroplasma, showing 98 % 16S rRNA gene sequence similarity to *Spiroplasma mirum*

(Wang *et al.*, 2003, 2004b). This was supported by the fact that the TDA had typical characteristics of spiroplasmas; very small (passing through a 220 nm membrane filter), mobile, helical, lacking a cell wall and culturable in M1D (Whitcomb *et al.*, 1982) or R2 (Moulder *et al.*, 2002) culture medium. This agent was the first spiroplasma isolated from a freshwater crustacean, challenging our understanding of the range of hosts affected by spiroplasmas (Regassa & Gasparich, 2006). In studies based on 16S rRNA gene sequence analysis, two species of marine shrimp, Alvinocarid shrimp (*Rimicaris exoculata*) (Zbinden & Cambon-Bonavita, 2003) and Pacific shrimp (*Penaeus vannamei*) (Nunan *et al.*, 2004), have also been reported as being hosts for spiroplasmas. The strain of spiroplasma isolated from *P. vannamei* showed 99 % 16S rRNA gene sequence similarity to *Spiroplasma insolitum* (GenBank accession no. AY771927); it was identified as a novel species, *Spiroplasma penaei* sp. nov. (Nunan *et al.*,

**Abbreviations:** CCU, colour-change unit; DF, deformation; MP, maximum-parsimony; ML, maximum-likelihood; TD, tremor disease; TDA, tremor disease agent; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of strain TDA-040725-5<sup>T</sup> is DQ917753.

2005), and was designated the reference strain for subgroup I-9 of the genus *Spiroplasma*.

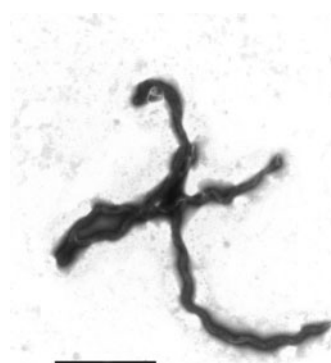
In this paper, the results of a taxonomic study are presented on the causative organism of the TD epizootic in *Eriocheir sinensis* in China. Using a polyphasic approach, analyses demonstrated that the isolate can be clearly distinguished from its closest phylogenetic neighbour *S. mirum*. Based on the guidelines recommended by Brown *et al.* (2007) for the taxonomic classification of novel species of spiroplasma, strain TDA-040725-5<sup>T</sup> is considered to represent a novel species of the genus *Spiroplasma* that constitutes a new serogroup of spiroplasma, group XLIII.

Strain TDA-040725-5<sup>T</sup> was isolated as previously described by Wang *et al.* (2005). Cell filtrate was used for *in vitro* culture in M1D or R2 broth. A pure culture was obtained by serial dilution, determining one colour-change unit (CCU) (Tully, 1983), which represents the number of cells contained in the highest dilution of a cell suspension that will produce a colour change in broth medium. Tubes containing 1 ml medium were inoculated with 0.05 ml liquid culture, incubated at 4, 16, 20, 22, 25, 28, 30, 32, 35, 37, 40, 42 and 60 °C, and observed daily. Any colour change was noted to determine whether growth was established in each of the tubes. Upon acidification of the culture medium, electron microscopy with negative staining was used, as described previously (Wang *et al.*, 2004a), to examine the media for the presence of cells and to determine their morphology. *Spiroplasma mirum* ATCC 29335<sup>T</sup>, the suckling mouse cataract agent (Tully *et al.*, 1976, 1977), originally isolated from rabbit ticks (*Haemaphysalis leporispalustis*) (Tully *et al.*, 1982), was purchased from ATCC and cultured using the same method but incubated at 37 °C.

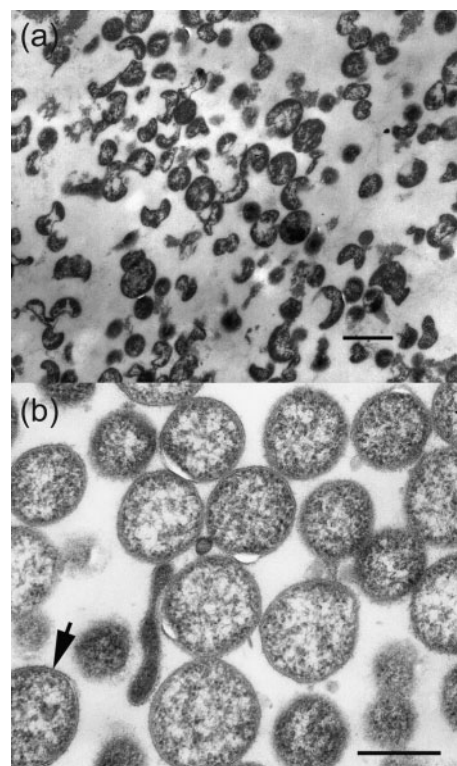
Cell morphology was determined by phase-contrast microscopy and transmission electron microscopy (TEM) after negative staining. Cell-membrane structure was also determined with TEM using standard ultrathin sectioning

procedures (Williamson, 1983). Briefly, cells were fixed for 2 h in 4 % glutaraldehyde, which was added directly to the culture, centrifuged at 8000 r.p.m., post-fixed with 1 % phosphate-buffered osmium tetroxide for 2 h, dehydrated in acetone and embedded in Epon 812 medium. The embedded pellet was cut in to 75–90 nm-thick sections using a Richard Jung Ultracut-E microtome, stained with 2 % aqueous uranyl acetate and lead citrate and viewed with an H-600-2A electron microscope.

To test for sterol requirement, strain TDA-040725-5<sup>T</sup> was transferred from R2 medium into a basal medium containing 8 % sucrose and 2.5 % heart infusion broth (HIB) and supplemented with 0, 5, 10, 15 or 20 % bovine serum. Tests were also carried out in basal medium supplemented with albumin, palmitic acid, Tween 80 and 0, 5, 10, 15 or 20 µg cholesterol ml<sup>-1</sup> solubilized in 95 % ethanol. Each test medium was replicated in three tubes. Colour changes in the media were noted to determine whether growth was established. Phase-contrast microscopy was used to examine the media for the presence of cells and to determine cell motility. Negative staining followed by electron microscopy was used to examine cell morphology. Growth was assessed by monitoring the number of CCU ml<sup>-1</sup> every 3 days (Tully *et al.*, 1983).



**Fig. 1.** Electron micrograph of negatively stained spiroplasma from exponential-phase cultures of strain TDA-040725-5<sup>T</sup>. Bar, 2.0 µm.



**Fig. 2.** Electron micrographs of a sectioned and stained pellet from strain TDA-040725-5<sup>T</sup>. The sections were stained with lead citrate and uranyl acetate and observed at both low magnification (bar, 0.5 µm) (a) and high magnification (bar, 0.1 µm) (b). Arrow indicates the single cytoplasmic membrane.

**Table 1.** Growth response of strain TDA-040725-5<sup>T</sup> to cholesterol (sterol)

HIB, Heart infusion broth; NG, no growth.

Basal medium	Bovine serum or cholesterol supplement	Spiroplasma titres (CCU ml <sup>-1</sup> ) after incubation at 30 °C		
		Day 3	Day 6	Day 9
8 % Sucrose, 2.5 % HIB + bovine serum	20 %	10 <sup>9</sup>	10 <sup>9</sup>	10 <sup>9</sup>
	15 %	10 <sup>9</sup>	10 <sup>9</sup>	10 <sup>9</sup>
	10 %	10 <sup>7</sup>	10 <sup>8</sup>	10 <sup>8</sup>
	5 %	NG	NG	NG
8 % Sucrose, 2.5 % HIB, 1 % albumin, 20 µg palmitic acid ml <sup>-1</sup> , 0.01 % Tween 80 + cholesterol	20 µg ml <sup>-1</sup>	10 <sup>9</sup>	10 <sup>9</sup>	10 <sup>9</sup>
	15 µg ml <sup>-1</sup>	10 <sup>9</sup>	10 <sup>9</sup>	10 <sup>9</sup>
	10 µg ml <sup>-1</sup>	10 <sup>8</sup>	10 <sup>9</sup>	10 <sup>9</sup>
	5 µg ml <sup>-1</sup>	10 <sup>7</sup>	10 <sup>8</sup>	10 <sup>8</sup>
	0 µg ml <sup>-1</sup>	NG	NG	NG

Procedures for determining carbohydrate fermentation and arginine and urea hydrolysis were performed as described by Aluotto *et al.* (1970). The ability of cells to pass through a 220 nm filter membrane and the requirement of serum for growth were determined using R2 broth, following procedures previously described by Tully (1983). All tests were repeated in triplicate.

Utilization of D-glucose, D-fructose, xylose, rhamnose, semiose and D-sorbitol was determined by inoculating strain TDA-040725-5<sup>T</sup> into basal supply medium (BSM: 2.5 % HIB; 0.2 % NaCl; 15 % bovine serum fraction) supplemented with 5 % of one of the carbohydrates listed above. Cells were inoculated into R2 broth and BSM as positive and negative controls (Aluotto *et al.*, 1970; Ye & Chen, 1991). Colour changes in the media were noted to determine whether growth was established. Phase-contrast microscopy and electron microscopy with negative staining were used to examine cell motility and morphology.

For serological tests, antiserum to strain TDA-040725-5<sup>T</sup> was produced in a rabbit as described by Wang *et al.* (2009). The *Spiroplasma* deformation (DF) test was used as described by Williamson *et al.* (1978) to compare strain TDA-040725-5<sup>T</sup>, serologically, with strains of species of the genus *Spiroplasma* with which it shared >0.94 % 16S rRNA gene sequence similarity. These included strains of the following taxa: *Spiroplasma citri* (I-1), *S. melliferum* (I-2), *S. kunkelii* (I-3), *Spiroplasma* sp. 277F (I-4), *Spiroplasma* sp. LB-12 (I-5), *S. insolitum* (I-6), *Spiroplasma* sp. N525 (I-7), *S. phoeniceum* (I-8), *S. penaei* (I-9), *S. poulsonii* (II), *S. mirum* (V), *S. syrphidicola* (VIII-1), *S. chrysopicola* (VIII-2) and *Spiroplasma* sp. TAAS-1 (VIII-3). Antisera for these strains were produced following the protocols of Williamson *et al.* (1978) and Wang *et al.* (2009) at the time of taxonomic classification. A complete set of antisera for all strains is maintained at Towson University (Gasparich laboratory), USA. Reciprocal reactions were performed when any positive reactions were observed in the one-way reactions.

Genomic analysis was performed as described previously (Bi *et al.*, 2008) using DNA extracted from cells of strain TDA-040725-5<sup>T</sup> grown in R2 medium. This process was repeated three times using *Escherichia coli* DNA (diluted in the same buffer) as a control. 16S rRNA gene sequences used in this study and their GenBank accession numbers are provided in Fig. 3. Sequences were aligned by using CLUSTAL\_X version 1.8 (Thompson *et al.*, 1997). Phylogenetic analyses were performed using maximum-parsimony (MP) and maximum-likelihood (ML) methods with PAUP version 4.0b10 (Swofford, 2002). Bootstrap values, based on 1000 replications, were obtained by the random stepwise-addition of taxa, repeated 10 times. The model of nucleotide sequence evolution and ML parameters were estimated using Modeltest 3.06 software (Posada & Crandall, 1998).

Cells of strain TDA-040725-5<sup>T</sup> grown to mid-exponential phase in M1D or R2 medium were long, helical, motile filaments, 3–20 µm long and 0.1–0.2 µm in diameter (Fig. 1). One end of the cells was usually more pointed than the other. Electron microscopy of ultrathin sections of pellets obtained from cultures of strain TDA-040725-5<sup>T</sup> (Fig. 2a) showed cells with a single cytoplasmic membrane and no evidence of a cell wall (Fig. 2b).

Strain TDA-040725-5<sup>T</sup> grew normally in M1D or R2 with 15–20 % bovine serum fraction but did not grow in media with <5 % bovine serum fraction. In media with 10 % bovine serum fraction, the strain did multiply but its morphology was abnormal, showing swollen cells (Table 1). Cells could recover from this abnormality back to a helical filamentous form when the bacterium was recultivated in M1D or R2 medium with 15–20 % serum. The strain was maintained through serial passage in media with a serum supplement and could not be maintained through two consecutive 10-fold serial passages in medium without serum supplement, indicating that serum is a requirement of growth.

Strain TDA-040725-5<sup>T</sup> fermented glucose and hydrolysed arginine but did not hydrolyse urea. Filtration studies indicated that a 48 h R2 broth culture of TDA-040725-5<sup>T</sup>

**Table 2.** Serological relationship between strain TDA-040725-5<sup>T</sup> and *S. mirum* by the deformation test

Strains: 1, TDA-040725-5<sup>T</sup>; 2, *S. mirum* ATCC 29335<sup>T</sup>. Titres are given as reciprocals of the final dilution of antiserum in which one-half of the spiroplasmas were deformed.

Antiserum	Antigen	
	1	2
1	2048	160
2	80	1024

contained  $\sim 10^9$  CCU ml<sup>-1</sup>. Cultures passed through 0.45 and 0.22  $\mu$ m membrane filters contained  $\sim 10^9$  and  $\sim 10^8$  CCU ml<sup>-1</sup>, respectively, after filtration. Strain TDA-040725-5<sup>T</sup> grew at 20–40 °C, with optimal growth at 30 °C.

The *Spiroplasma* DF test using antisera (Wang *et al.*, 2009) from strain TDA-040725-5<sup>T</sup> showed no reaction with nine group I, one group II or three group VIII subgroup reference strains. Reciprocal cross-reactivity was observed using *S. mirum* as an antigen and antiserum as indicated in Table 2. Strain resolution to group placement requires a reciprocal DF titre  $\geq 320$ . This was not observed in any of the DF tests performed in this study according to the minimal standards for taxonomic characterization of novel species of the class *Mollicutes* (Brown *et al.*, 2007).

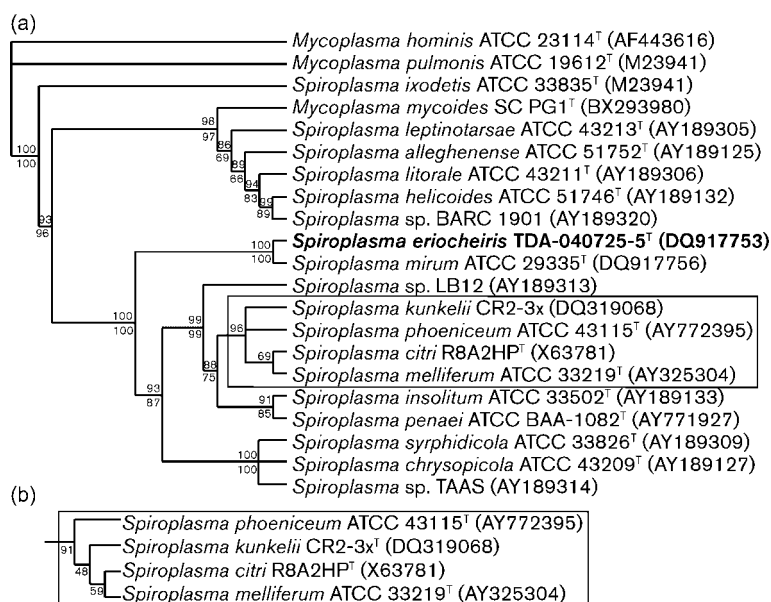
The genome size of strain TDA-040725-5<sup>T</sup> was 1.4–1.6 Mbp as determined by PFGE (Bi *et al.*, 2008). The genome size of *S. mirum* SMCA<sup>T</sup> was previously determined as 1.2–1.3 Mbp (Tully *et al.*, 1976; Carle *et al.*, 1995). The DNA G + C content of strain TDA-040725-5<sup>T</sup> was determined, giving results of 29.56, 29.80 and 29.85 mol% (mean  $29.7 \pm 1$  mol%).

Phylogenetic trees (Fig. 3) constructed by using MP and ML methods grouped strain TDA-040725-5<sup>T</sup> consistently with the group V spiroplasma cluster and it was most closely associated with *S. mirum*, the only member of the group V spiroplasmas (Gasparich *et al.*, 2004).

Phylogenetic analysis of strain TDA-040725-5<sup>T</sup> has been described in detail by Bi *et al.* (2008) with analysis of partial nucleotide sequences of 16S rRNA, *gyrB* and *rpoB* genes, as well as complete 23S rRNA gene sequences and 16S–23S rRNA intergenetic spacer regions. For comparison, sequences from closely related species or strains of the genus *Spiroplasma* were obtained from the GenBank database (Regassa *et al.*, 2004). These sequences clearly showed that strain TDA-040725-5<sup>T</sup> belonged to the *Mirum* clade and showed a close relationship with *S. mirum*.

Strain TDA-040725-5<sup>T</sup> was isolated from the haemolymph of a Chinese mitten crab, *Eriocheir sinensis*, with tremor disease. The pathogen exhibited a predilection for muscle, nerve and connective tissues and was transported to various tissues and organs by haemocytes. The infected cells had inclusions that stained purple with Giemsa stain. The pathology seen in experimentally infected crabs was similar to that seen in naturally occurring TD (Wang & Gu, 2002).

The properties described here for strain TDA-040725-5<sup>T</sup> fulfil the proposed criteria and revised minimal standards for description of new species of the class *Mollicutes* as described in Brown *et al.* (2007). Serological comparisons of strain TDA-040725-5<sup>T</sup> with type strains representing all groups and subgroups of the genus *Spiroplasma* with which it showed >94% 16S rRNA gene sequence similarity demonstrated the uniqueness of the strain. It is therefore proposed that this organism be designated as the reference strain of a novel serological group of the genus *Spiroplasma*,



**Fig. 3.** Phylogenetic tree showing the position of strain TDA-040725-5<sup>T</sup> among representatives of the genera *Spiroplasma* and *Mycoplasma*. (a) MP and ML trees derived from the 16S rRNA gene partial sequences. *Mycoplasma hominis* and *Mycoplasma pulmonis* were used as outgroups. The dataset was resampled 1000 times and bootstrap percentage values are indicated for each branch. Numbers above and below branches represent the bootstrap values of the MP and ML analyses, respectively. (b) A branch of the ML tree that differed to the corresponding branch in the MP tree.

designated group XLIII. The phenotypic and morphological characteristics of this organism, such as its helical shape, motility and inability to hydrolyse urea, together with its phylogenetic position based on 16S rRNA gene sequence analyses indicate that strain TDA-040725-5<sup>T</sup> represents a novel species of the genus *Spiroplasma*, for which the name *Spiroplasma eriocheiris* sp. nov. is proposed.

### Description of *Spiroplasma eriocheiris* sp. nov.

*Spiroplasma eriocheiris* (e.ri.o.cheir'is. N.L. n. *Eriocheir* a genus of crab; N.L. gen. n. *eriocheiris* of *Eriocheir sinensis*, the species of crab from which the organism was isolated).

Cells are helical, motile filaments with a mean diameter of 190 nm and lack true cell walls. Acid is produced from glucose and arginine is hydrolysed. Grows at 20–40 °C (optimum 30 °C) in M1D or R2 broth. Genome size is 1.4–1.6 Mbp. The DNA G+C content of the type strain is 29.7 ± 1 mol%.

The type strain, TDA-040725-5<sup>T</sup> (=CCTCC M 207170<sup>T</sup> =DSM 21848<sup>T</sup>), was isolated from haemolymph of the Chinese mitten crab, *Eriocheir sinensis*, to which the organism is pathogenic, and is serologically distinct from closely related species of the genus *Spiroplasma*.

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