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Infectivity, transmission and 16S rRNA sequencing of a rickettsia, *Coxiella cheraxi* sp. nov., from the freshwater crayfish *Cherax quadricarinatus*

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ABSTRACT: A rickettsia-like organism isolated from infected, farm-reared *Cherax quadricarinatus* was cultured in the yolk sac of developing chicken eggs, but could not be cultured in 3 continuous cell lines, bluegill fry (BF-2), fathead minnow (FHM), and *Spodoptera frugiperda* (Sf-9). The organism was confirmed by fulfilling Koch's postulates as the aetiological agent of mortalities amongst *C. quadricarinatus*. When *C. quadricarinatus* was inoculated with the organism, mortality was 100% at 28°C and 80% at an ambient temperature of 24°C. Horizontal transmission with food and via the waterborne route was demonstrated, but mortalities were lower at 30 and 10% respectively over a 4 wk period. The 16S rRNA sequence of 1325 base pairs of the Gram-negative, obligate intracellular organism was 95.6% homologous to *Coxiella burnetii*. Of 18 species compared to this rickettsia, the next most closely related bacterium was *Legionella pneumophila* at 86.7%. The suggested classification of this organism is Order Rickettsiales, family Rickettsiaceae, tribe Rickettsieae, within the genus *Coxiella*. We suggest it should be named *Coxiella cheraxi* sp. nov.

KEY WORDS: *Cherax quadricarinatus* · Crayfish · Rickettsia · *Coxiella cheraxi*

INTRODUCTION

The Australian redclaw crayfish *Cherax quadricarinatus* is a tropical freshwater crayfish native to river systems and waterways of northern Australia and southern Papua New Guinea (Jones 1990). It grows rapidly, is non-aggressive and can tolerate a wide range of conditions, thus making it an ideal species for aquaculture (Rouse 1995). Since its introduction into aquaculture, redclaw has generated a vast amount of interest throughout the world (Medley et al. 1994). It is currently cultured in the United States, South Africa, Israel, China, Taiwan, the Caribbean and Latin America (e.g., Ecuador) (Medley et al. 1994).

With the expansion of redclaw aquaculture, diseases have become more evident. To date, diseases have not threatened the industry as a whole, but they have on a local scale (Edgerton 1996). A number of viruses (Anderson & Prior 1992, Edgerton et al. 1994, 1995, Owens & McElnea 2000), bacteria (Ketterer et al. 1992, Owens et al. 1992, Eaves & Ketterer 1994, Webster 1995) and ectoparasites (Herbert 1987, 1988) have been reported in Australia, and the presence of *Cherax* bacilliform virus (Groff et al. 1993) and rickettsiae (Jimenez & Romero 1997) also been reported outside Australia.

During the summer of 1990, heavy losses were reported from growout ponds of commercially farmed redclaw in Australia. Initially, 3 to 4 deaths were seen each day, but after the transfer of redclaw to fresh ponds, mortalities reached up to 300 d^{-1} over 3 to 4 d. By the end of the growing period, mortalities totaled 24 000 (Ketterer et al. 1992). Histological examinations found a rickettsia-like bacteria and ectoparasites, *Lagenophrys* sp., associated with the infected crayfish. Similarly, Edgerton et al. (1995) found the presence of 2 species of rickettsiae, 1 in the cytoplasm of hepatopancreatic cells (Edgerton & Prior 1999) and 1 in the connective tissues throughout moribund crayfish. Rickettsial disease has recently been identified in Ecuador

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with mortalities up to 80% in several farms (Jimenez & Romero 1997). The bacteria exhibited morphology and characteristics similar to those reported by Edgerton et al. (1995).

Rickettsiae are Gram-negative, obligate parasitic bacteria that multiply only within the host cells (Weiss & Moulder 1984). They are well known amongst terrestrial species with insects and arachnids serving as secondary hosts or vectors (Larrson 1982). However, in recent years, rickettsiae have been reported as the aetiological agent of diseases in a number of aquatic organisms including teleosts (Fryer & Lannan 1994) and molluscs (Le Gall et al. 1991). These bacteria may also be the aetiological agent of disease in redclaw and have the potential to devastate the redclaw industry worldwide.

To date, no studies have been conducted beyond microscopic observations to determine the nature of the disease. Accordingly, this study was designed to investigate the pathogenicity of the bacteria, the possible modes of transmission, and the taxonomic placement relative to other species of rickettsia and closely related bacteria.

MATERIALS AND METHODS

Rickettsial isolate. Rickettsiae used in the study were obtained from an infected farm in far northern Queensland which exhibited daily mortalities of redclaw. The isolate of rickettsia from *Cherax quadricarinatus*, named TO-98, was purified according to the procedures adopted by Le Gall & Mialhe (1992) with slight modification. In brief, hepatopancreata, eyestalks, gills and connective tissues were dissected and homogenised in physiological saline (0.9%) and Tween-80 (0.1%) (PST). The homogenate was sieved through a nylon cloth to remove larger cellular material and washed twice by centrifugation (3500 × *g*, 30 min at 6°C). The supernatant was discarded and the pellet resuspended in PST. Several differential centrifugations using discontinuous sucrose gradients (15 and 50%) were performed to concentrate the bacteria. Purification was achieved with an isopycnic centrifugation on a Urografin™ (Scherring) discontinuous density gradient (14 to 21 to 28%) (V/V). The interfaces at 14 to 21% and 21 to 28% were collected, diluted in TN buffer (20 mM Tris-HCl, 400 mM NaCl at pH 7.5), pelleted and resuspended in TN buffer. The purified bacteria were plated onto nutrient agar and identified by Gram stain under light microscopy. Any growth on the nutrient agar was considered due to contaminating heterotrophic bacteria, and the relevant isolate was discarded. Contaminants were removed by filtration through 1.2 and 0.45 µm filter.

Cell culture. Propagation of rickettsia from *Cherax quadricarinatus* was attempted in fathead minnow *Pimephales promelas* (FHM), bluegill *Lepomis macrochirus* fry (BF-2) and *Spodoptera* moth (Sf-9) cell lines. Cells were cultured in 25 cm^2 plastic culture flasks (Corning Glass Works, New York) containing Dulbecco's modified medium (DMEM) supplemented with 5% foetal bovine serum (FBS) for the first two, while the latter was propagated in insect medium TC-100 supplemented with 10% FBS. Cells were incubated at 28°C, and media was changed when the monolayers were confluent. Cells were inoculated with rickettsia directly after their passage.

Inoculation of hens' eggs. Purified rickettsia (100 l^{-1}) suspended in TN buffer were inoculated into the yolk sac of specific pathogen-free (for rickettsia), embryonated hens' eggs at 6 d of age. The eggs were incubated at 28, 32 and 36°C, and harvested after 11 d post-inoculation. Harvested material was examined by Gram stain.

DNA extraction. Infected redclaw were collected from a farm in far northern Queensland and rickettsial isolates were purified as described above. DNA from purified rickettsia was extracted by 100 µg ml^{-1} Proteinase K/1% sodium dodecyl sulfate (SDS) digestion followed by 2 phenol-chloroform and 1 chloroform-isoamyl alcohol extraction. After an absolute-ethanol precipitation, the purified DNA was washed with 70% ethanol and resuspended in 50 µl TE buffer.

Amplification of rRNA genes. The reaction mixes for PCR contained: 5 µl of 10 × PCR buffer, 5 µl dNTPs (2 mM), 100 pmol of each of the 2 primers, 0.1 unit of *Taq* polymerase 10 µl $MgCl_2$, and sterile double distilled water to 50 µl. Light mineral oil was overlaid onto each reaction mix. The primers (5' to 3' orientation) were forward 63for 16SrRNA CAG GCC TAA CAC ATG CAA CTC, and reverse 1387rev 16srRNA GGG CGG WGT GTA CAA GGC, where W = A or T. (See 'Acknowledgements' for primer source.) The thermal program comprised 30 cycles of denaturation at 94°C for 3 min, 95°C for 1 min, hybridization at 55°C for 1 min and extension at 72°C for 90 s. The products were kept at 4°C at the completion of the cycles. Products were run on 1.0% agarose gel made in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) and contained 0.0005% ethidium bromide. The gel was run at 80 V for 60 min. The DNA was extracted and purified from the agarose gel using QIAquick Gel Extraction Kit (QIAGEN, Germany) according to the manufacturer's instructions.

Purified PCR product was sequenced with dRhodamine Terminator Cycle Sequencing Ready Reaction mix (ABI Prism, PE Applied Biosystems). In addition to the 2 primers above, the following rickettsial primers (5' to 3' orientation) PC• (CTA CGG GAG GCA GCA

GTG GG), PC (CTA CGG GAG GCA GTG GG), and PF* (CAT GGC TGT CGT CAG CTC GT) were included to bridge the sequence. (See 'Acknowledgements' for primer source.) The thermal cycling program consisted of 96°C for 30 s, 50°C for 15 s and 60°C for 4 min repeated for 25 cycles.

DNA analysis. A consensus sequence was developed by aligning a minimum of 4 replicates and a maximum of 7 overlapping frames using the program Sequencher. The sequence was then aligned with 18 published sequences of Rickettsiales and closely related bacteria using the multiple aligning software, Clustal X. Once the sequences were aligned, the corrected level of nucleotide divergence of the sequences was calculated using the program PAUP.

Experimental infections. The infectivity trial was conducted in twelve 170 l aquaria containing 5 juvenile redclaw weighing between 10 and 15 g in each tank. Purified TO-98 was centrifuged at 6000 × *g* for 10 min at 6°C and resuspended in DMEM. A total of 100 ml of inoculum was injected into each experimental crayfish in the first abdominal segment. The placebo control was injected with 100 µl of DMEM. Experimentally infected and uninfected control crayfish were maintained separately under similar feeding and water quality conditions. All treatments were in duplicate. All surviving crayfish at the end of the 28 d pathogenicity trial were examined for the presence of rickettsia.

Redclaw heavily infected with rickettsia were macerated and fed to experimental crayfish, which were starved for 24 h prior to feeding. Uneaten flesh was removed 2 h later to avoid water contamination. Purified rickettsia were placed directly into the tank in order to determine the possibility of direct transmission.

Statistical analysis. Testing for significant differences in survival was by a 1-way analysis of variance (ANOVA) and significant variation between the treatment means was by using Tukey HSD analysis within the SPSS statistical package.

RESULTS

Growth of the rickettsia in embryonated hens' eggs

The yolk sac membranes of embryonated hens' eggs inoculated at Day 6 were harvested at Day 11. The eggs were successful for the cultivation of rickettsia at 36°C, but the embryo failed to grow at 28 and 32°C and no rickettsia were recovered (Table 1).

Table 1. Results of inoculation of rickettsiae into embryonic hens' eggs under a number of conditions

Temperature (°C)	Number of eggs tested	Presence of rickettsia	% of infection
28	6	No	0
32	9	No	0
36	12	Yes	75

Cell culture

The current rickettsia of *Cherax quadricarinatus* failed to grow in any of the 3 cell lines, despite an additional re-inoculation with the specific rickettsial isolate (TO-98).

Experimental infections

Rickettsial infections were established in all crayfish injected with TO-98. Grossly, the infected crayfish looked normal but were extremely lethargic shortly before death. Upon death, the crayfish became reddened and putrefied with the eyes of highly infected crayfish totally necrotised and the hepatopancreas liquefied. Mortalities were first detected 2 d post-infection in the waterborne treatment (Fig. 1), but no rickettsia were recovered. The inoculated treatment held at 28°C had significantly higher mortalities with 70% mortalities between 2 and 12 d post-injection, and total mortality by 20 d post-injection. Gram stains of impression smears of the eyestalk revealed masses of Gram-negative bacteria resembling those of rickettsiae. These bacteria failed to grow on nutrient agar.

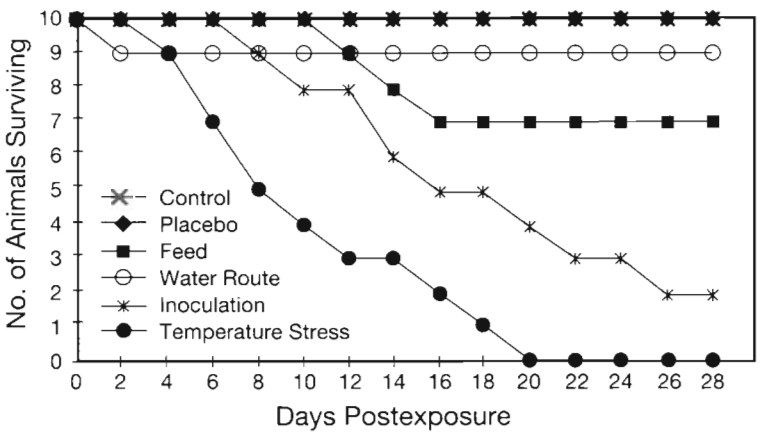

Fig. 1. *Cherax quadricarinatus*. Survival of redclaw over a 28 d pathogenicity trial with rickettsial isolate TO-98 administered by inoculation (24°C), bath, a feeding route, and inoculation without and with a 28°C temperature stress

The inoculated crayfish held at ambient temperatures showed trends similar to those held at the higher temperature and with mortalities at 8 d post-injection. Gradual mortalities were reported throughout the trial period and at the termination of the experiment, survival was 20%. In contrast, feed transmission treatments had a total of 70% survival by Day 28, with mortalities occurring between Days 10 and 16. No crayfish from the control and placebo treatments died during the experimental period.

One-way ANOVA found significant difference (F = 37.3, df = 5,11, $p < 0.01$) between the treatments. Tukey's HSD revealed a significant difference ($p < 0.05$) between both experimentally injected groups and the placebo, control, feed infected and waterborne treatment groups (Table 2). However, no significant difference was found between the rickettsial-inoculated-groups held at ambient and at raised water temperatures.

Re-isolation of rickettsia

Rickettsia were re-isolated and purified from all experimentally infected crayfish groups (Table 3). If the isolates contained small, Gram-negative rods with no growth on nutrient agar, it was presumed that they were rickettsia and that Koch's postulates had been confirmed. In contrast, no rickettsia were present in the placebo and control treatments. Despite the fact that no rickettsia were isolated in the moribund crayfish from the water transmission treatment, low numbers of rickettsia were recovered from 40% of the crayfish in this treatment. Similarly, the oral-infected treatment had a high infection rate at 50%.

Table 3. Recovery rate of rickettsia from experimental *Cherax quadricarinatus*

Treatment	No. of samples tested	No. positive for rickettsia	% positive
Control	10	0	0
Placebo	10	0	0
Waterborne	10	4	40
Oral route	10	5	50
Inoculation	10	10	100
Inoculation + temperature stress	10	10	100

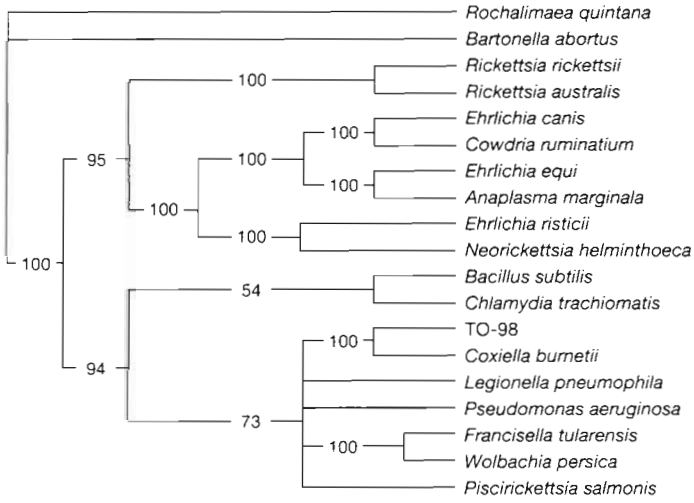

Fig. 2. Phylogenetic tree showing relationships amongst the rickettsiae in this study representing members of the α- and γ-subgroups of Proteobacteria. The bootstrap distances were calculated by the program PAUP

Table 2. Tukey HSDs showing the mean differences in percentage survival (top value) and p-value (bottom value)

Treatment	Control	Placebo	Oral route	Water-borne	Inocu-lation	Inoculation + temperature stress
Control	–					
Placebo	0 1.0	–				
Oral route	10 0.9	10 0.9	–			
Waterborne	30 0.15	30 0.15	20 0.4	–		
Inoculation	80 0.002	80 0.002	70 0.003	50 0.02	–	
Inoculation + temperature stress	100 0.0	100 0.0	90 0.001	70 0.003	20 0.4	–

16S rRNA sequencing

Phylogenetic tree

The partial 16S rRNA sequence characterised 1325 nucleotides of the rickettsia from *Cherax quadricarinatus*. When the sequence was aligned with other rickettsial and closely related bacterial 16S rRNA sequences obtained from Genbank, TO-98 appeared to be a member of the γ-subgroup of the Proteobacteria, with *Coxiella burnetii* its nearest neighbour (Fig. 2). The tree branched with *C. burnetii* at approximately the same point as with *Wolbachia persica* with *Francisella tularensis*, after the splitting off of *Piscirickettsia salmo-*

nis, *Legionella pneumophila*, and *Pseudomonas aeruginosa*. Other genera, *Rickettsia* and *Ehrlichia*, which belong to the α-subgroup, diverged very early from the γ-subgroup. The bootstrap values for the most parsimonious tree were relatively high between 94 and 100% for all nodes except the node connecting *Bacillus subtilis* and *Chlamydia trachiomatis*, and that connection to the γ-subgroup, where the bootstrap values were 54 and 73% respectively.

Evolutionary distances

Pair-wise comparison of TO-98 with other members of Rickettsiaceae and closely related bacteria are summarised in Table 4. TO-98's nearest neighbour, *Coxiella burnetii*, had 55 nucleotides substitutions within the partial sequence of 1325 base pairs or equivalent to 95.6% similarities (Table 4). Of 18 species compared to this rickettsia, the next most closely related bacterium was *Legionella pneumophila* at 86.7%. This rickettsia is not closely related to species of *Ehrlichia*, *Rickettsia* and *Neorickettsia* with similarities between 78.4 and 82.3%, while rickettsial members of the γ-subgroup were approximately 84%.

Primary structure

The aligned primary sequences revealed a number of differences between the α- and γ-subgroups (Fig. 3). The most drastic differences occurred at positions 141 to 157, 347 to 374, and 899 to 915. Despite having 55 nucleotide substitutions between TO-98 and *Coxiella burnetii*, the most significant stretch occurred at position 368 to 379 (Fig. 3). The remaining changes were subtle with only 1 or 2 continuous base pair substitutions along the sequence.

DISCUSSION

The organism isolated from infected farmed redclaw was confirmed by Koch's postulates as the aetiological agent of the disease. Previous publications (Ketterer et al. 1992, Owens et al. 1992) and our findings suggested that the organism is an obligate intracellular pathogen that depends on living host cells for survival, which is very characteristic of the Order Rickettsiale. Owens et al. (1992) suggested that the

Table 4. Pairwise substitution distances and sequence similarities amongst rickettsial and other eubacterial 16S rRNA. Lower triangle: percentage similarity; upper triangle: pairwise substitution distances. *Y*-axis: first letters of the species named in the *x*-axis. GA: GenBank Accession number for the sequence of the species named on the *x*-axis that was used in this comparison

Organism	*Rq*	*Ba*	*Rr*	*Ra*	*Ec*	*Cr*	*Ee*	*Am*	*Er*	*Nh*	*Bs*	**TO-98**	*Cb*	*Lp*	*Pa*	*Ft*	*Wp*	*Ps*	*Ct*	GA
Rocholimeae quintana		80	203	199	249	245	228	238	268	244	280	**213**	246	264	204	277	264	225	700	M11927
Brucella abortus	94.1		212	209	251	248	238	239	256	237	266	**212**	238	265	198	273	274	225	698	AF91354
Rickettsia rickettsii	85.2	84.3		10	224	218	218	219	237	222	299	**228**	249	271	208	293	284	231	720	L36217
Rickettsia australis	85.5	84.6	99.3		229	221	221	223	238	222	295	**228**	256	270	207	290	282	233	719	L36101
Ehrlichia canis	81.7	81.5	83.5	83.1		37	103	106	219	189	307	**241**	283	296	206	312	312	260	711	U26740
Cowdria ruminantium	82.4	81.7	84.1	83.8	97.3		102	103	209	192	301	**239**	269	287	201	307	292	247	713	AF69758
Ehrlichia equi	83.2	82.4	83.9	83.7	92.4	92.5		40	200	187	310	**228**	263	284	189	294	291	243	702	AF36646
Anaplasma marginale	82.9	82.4	84	83.7	92.3	92.7	97.1		211	196	304	**232**	268	279	191	310	305	256	702	M60313
Ehrlichia risticii	80.3	81.1	82.5	82.4	84	84.6	85.3	84.6		49	317	**259**	289	293	208	323	326	260	716	AF36654
Neorickettsia helminthoeca	82.3	82.3	83.4	83.4	86	86.1	86.1	85.8	96.4		305	**255**	268	289	208	317	307	235	722	U12457
Bacillus subtilis	79.8	80.3	78	78.4	77.4	78.1	77.1	78	76.7	77.6		**276**	312	336	246	349	330	299	750	AB16721
TO-98	**82.3**	**82.3**	**81**	**81**	**79.9**	**80.1**	**81**	**80.7**	**78.4**	**78.7**	**77.6**		**55**	**165**	**143**	**199**	**193**	**177**	**663**	
Coxiella burnetii	81.5	82	81.2	80.7	78.8	79.7	80.1	80	78.4	79.6	77.3	**95.6**	87.3	173	144	223	221	192	710	D89792
Legionella pneumophila	80.5	80.3	79.9	80	78.1	78.7	79	79.3	78.2	78.5	75.8	**86.7**	87.3		147	220	217	189	708	M36026
Pseudomonas aeruginosa	80.1	80.6	79.7	79.8	79.8	80.3	81.5	8.3	79.6	79.6	76.8	**86.3**	86.5	86.2		180	172	160	549	AF76039
Francisella tularensis	80	79.7	78.4	78.6	77	77.9	78.2	77.9	76.1	76.8	75.4	**83.8**	83.6	84.1	82.9		29	204	713	Z21932
Wolbachia persica	80.4	79.6	78.8	79	76.9	78.2	78.3	77.4	75.9	76.9	76.2	**84.3**	83.9	84.2	83.8	97.9		184	704	M21292
Piscirickettsia salmonis	80.6	80.4	79.9	79.8	77.6	78.5	78.9	77.9	77.5	79.3	75.1	**84.7**	84	84.1	84.9	82.9	84.7		631	U36915
Chlamydia trachomatis	49.7	49.7	38.9	39	39.4	39.3	40.3	40.3	39	38.6	38.3	**41.7**	41.1	41.5	42.4	40.9	41.8	40.9		U68422

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F. tularensis     GATTAAAGGT GG-----CT- TTCGGCGTGT CGCAGATGGA
W. persica        GATTAAAGGT AG-----CT- TTCGAGCTGT CGCAGATGGA
R. quintana       GGAGAAAGAT T--------- --------TA- CGGAGGTGGA
B. abortus        GGGGAAAGAT T--------- -------TAT CGGCAAATGA
R. rickettsii     GAGGAAAGAT T--------- -------TAT CGCTGATGGA
R. australis      GAGGAAAGAT T--------- -------TAT CGCTGATGGA
E. canis          GGGGAAAGAT T--------- -------TAT CGCTATTAGA
C. ruminatuim     GGGGAAAGAT T--------- -------TAT CGCTATTAGA
E. equi           GGGGAAAGAT T--------- -------TAT CGCTATTAGA
A. marginalla     GGGGAAAGAT T--------- -------TAT CGCTATTAGA
E. risticii       GGGGAAA-AT T--------- -------TAT TGCTATCAGA
N. helminthoeca   GGGGAAAGAT T--------- -------TAT TGCTATCAGA
B. subtilis       GTTCAAACAT AAAAGGTGG- CTTCGGCTAC CACTTACAGA
TO-98             GAGCAAAGCG GGG-GATCT- TCGGACCTCG CGCTAAAAGA
C. burnetii       GAGCAAAGCG NGG-GATCT- TCGGACCTCG TGCTATAAGA
L. pneumorphila   GACGAAAGCT GGG-GACCT- TCGGGCCTGG CGCTTTAAGA
P. aeruginosa     GGAGAAAGTG GGG-GATCT- TCGGACCTCA CGCTATCAGA
P. salmonis       GGTTAAAGAG GGCCTCTATA TATAAGCTCT TGCTAGGAGA
C. trachiomatis   GTTTTACCTT AAG---TCGT TGACTCAACC CGCAAGGGAG
                  131       141       151       161       170

F. tularensis     AGGAAAGCCT CAAGGTTAAT AGCCTTGGGG AG--GACGTT
W. persica        AGGAAAGCCT TGAGGTTAAT NGCCTTTAGG AAT-GACGTT
R. quintana       AAGATAA--- ---------- ---------- ---TGACGTT
B. abortus        AAGATAA--- ---------- ---------- ---TGACGGT
R. rickettsii     AAGATAA--- ---------- ---------- ---TGACGTT
R. australisT     AAGATAA--- ---------- ---------- ---TGACGTT
E. canis          AAGATAA--- ---------- ---------- ---TGACGGT
C. ruminatuim     AAGATAA--- ---------- ---------- ---TGACGGT
E. equi           AAGATAA--- ---------- ---------- ---TGACGGT
A. marginalla     AAGATAA--- ---------- ---------- ---TGACGGT
E. risticii       AAGATAA--- ---------- ---------- ---TGACGGT
N. helminthoeca   AAGATAA--- ---------- ---------- ---TGACGGT
B. subtilis       AAGAACAAGT GCCGTTCAAA TAGGGCGGCT CCTTGACGGT
TO-98             AAGAAATTCT TAAGATTAAT ACTCTTAA-- ---------T
C. burnetii       AAGAAATTCT CAAGGGTAAT ATCCTTGGGC G-TTGACGTT
L. pneumorphila   AGGAGGGTTG ATAGGTTAAG AGCTGATTAA C-TGGACGTT
P. aeruginosa     AGGAAGGGCA GTAAGTTAAT ACCTTGCTGT T-TTGACGTT
P. salmonis       AGGAGAGGTA AGCTAATTAA TACTTGGCTT AATTGACGTT
C. trachiomatis   ATCAAGTATG TTATTGTAAA GAAAT--AA- ---TCATGGT
                  341       351       361       371       380
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Fig. 3. Regions along the aligned 16S rRNA sequences of α- and γ-subgroups of Proteobacteria displaying the variations in nucleotides at the 2 most disparate points. –: a gap or missing nucleotide; N: not known. For full generic names, see Table 4

rickettsia of *Cherax quadricarinatus* might be a member of the genus *Rickettsiella*, based on morphological and pathological evidence. However, the present findings demonstrated that the rickettsia of *C. quadricarinatus* is more closely related to *Coxiella burnetii* in the γ-subgroup of the phylum Proteobacteria. Although this rickettsia may bear some phenotypic resemblance to members of the α-subgroup, genotypic relationships are quite distinct (Weiss & Moulder 1984). Therefore, the common parasitic existence of the rickettsiae must either be very ancient in origin or have undergone convergence evolution, arising independently from 2 lineages (Weisburg et al. 1989).

Coxiella (Philip 1943) Philip 1948 (Skerman et al. 1980) is a monospecific genus with a wide geographical distribution and diverse pathogenic characteristics. Beside the close similarities with the rickettsia of *Cherax quadricarinatus*, both of these bacteria also share a common geographical range in Australia. To date, rickettsial disease has not been detected from any other freshwater crayfish in Australia or worldwide, despite the diverse crayfish fauna (Rouse 1995). The host and specific nature of this rickettsia and its distribution suggests that the divergence from *Coxiella burnetii* could be a recent event. An approximation on the time of divergence may be obtained by applying the molecular clock calibrations proposed for prokary-

otic 16S rRNA genes (Ochman & Wilson 1987). It suggests the divergence of 16S rRNA nucleotide sequence between 2 bacterial taxa occurs at a rate of approximately 1% per 50 million yr (Weisburg et al. 1989). Based on this approximation, the divergence of the rickettsia of *C. quadricarinatus* and *C. burnetii* occurred 200 million yr ago, during which time arthropods were very abundant (Hickman & Roberts 1994). We suggest there is enough evidence of divergence and distinctness to submit that this rickettsia from *Cherax quadricarinatus* is a new species most closely related to *Coxiella burnetii* and that it should be named *Coxiella cheraxi* sp. nov.

The ability of the rickettsiae to grow in yolk sacs of embryonic chicken eggs at 36°C further supports that this rickettsial organism is a member of the genus *Coxiella* in the tribe Rickettsieae of the family Rickettsiaceae. *C. burnetii* is readily cultivated in the yolk sacs of developing chicken embryos, and is highly resistant to adverse physical and chemical agents (Woldehiwet & Aitken 1993). At present, only 2 other published studies, by Harshbarger et al. (1977) and Buchanan (1978), have successfully propagated a rickettsia of aquatic origin in yolk sac embryos of developing chicken eggs. Further work is needed on the culture of this organism in embryonated eggs.

Evidence of the gills providing an effective and fast infection route have been demonstrated for a number of bacteria including *Piscirickettsia salmonis* (Branson & Nieto Diaz Munoz 1991, Almendras et al. 1997), rickettsia-like organisms of *Placopecten magellanicus* (Gulka & Chang 1984), and *Aeromonas salmonicida* (Effendi & Austin 1995). The gills may provide an important site of transmission during the natural infection of rickettsial disease (Gulka & Chang 1984). As the waterborne route seemed to infect crayfish in the present study, the gills are a possible site of infection.

The ability of the rickettsia of *Cherax quadricarinatus* to survive extracellularly (i.e. in the water) for an extended period has also been observed with *Coxiella burnetii* (Weiss & Moulder 1984) and *Piscirickettsia salmonis* (Fryer & Lannan 1994). The time over which *C. burnetii* and this current rickettsia remain infective is uncertain, but Fryer & Lannan (1994) demonstrated that *P. salmonis* could survive and remain infective for up to 15 d. The presence of a vector in most mammalian cases of rickettsial disease suggests that a secondary arthropod host may be involved (Almedras et al. 1997). Perhaps, *C. quadricarinatus* is acting as an alternative arthropod host to some vertebrate.

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