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Genetic and electron-microscopic characterization of *Rickettsiella tipulae*, an intracellular bacterial pathogen of the crane fly, *Tipula paludosa* ☆

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Abstract

Rickettsiella tipulae is an intracellular bacterial pathogen of larvae of the crane fly, *Tipula paludosa* (Diptera: Tipulidae) and has previously been claimed to represent an independent species within the genus *Rickettsiella*. Recently, this taxon has been reorganized and transferred as a whole from the α-proteobacterial order *Rickettsiales* to the γ-proteobacterial order *Legionellales*. Here we present the electron-microscopic identification of this rickettsial pathogen together with the first DNA sequence information for *R. tipulae*. The results of our 16S rDNA-based phylogenetic analysis demonstrate that the transfer to the order *Legionellales* is justified for *R. tipulae*. However, there is no phylogenetic basis to consider *R. tipulae* an independent species, but instead conclusive evidence substantiating its species level co-assignment with *Rickettsiella melolonthae*. Furthermore, implications of our results for a possible reorganization of the internal structure of the genus *Rickettsiella* are discussed.

Keywords: *Rickettsiella popilliae*; *Rickettsiella tipulae*; *Tipula paludosa*; 16S ribosomal DNA; Giant bodies; Associated proteinaceous crystals; *Rickettsiales*; *Legionellales*

1. Introduction

The genus *Rickettsiella* (Philip) comprises intracellular bacterial pathogens of a wide range of arthropods that typically multiply in vacuolar structures within fat-body cells and form protein crystals. Their taxonomic classification is primarily based on the identity of a strain's original host. At present, this pathotype designation is partly superposed by the morpho- and serologically founded distinction of the nomenclatural type species, *Rickettsiella popilliae* (Dutky and Gooden), and two further recognized species, *Rickettsiella grylli* (Vago and Martoja) and *Rickettsiella chironomi* (Weiser), that are named according to the respective type strain's pathotype. Several *Rickettsiella* pathotypes have been placed in synonymy with one of these species, while others await conclusive species assignment (Garrity et al., 2005). Detailed descriptions of developmental cycles argue in favor of a relatively close phylogenetic relationship of *R. popilliae* and *R. grylli* as compared to *R. chironomi* (Götz, 1972) and reveal considerable similarities of the latter species to chlamydia (Federici, 1980).

Due to their perception as "rickettsiae of insects", *Rickettsiella* had originally been assigned to the α-proteobacterial order *Rickettsiales* while an alternative classification to the order *Chlamydiales* has been considered (Weiss et al., 1984). However, the determination of the 16S rRNA encoding sequence from a *R. grylli* strain revealed highest homology to the ortholog from *Coxiella burnetii* (Roux et al., 1997), i.e. a bacterial species that due to earlier 16S rRNA analyses had been transferred from the order *Rickettsiales* to the distantly related order

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Legionellales of the γ-proteobacteria (Weisburg et al., 1989). As a consequence of this finding, the entire genus *Rickettsiella* has recently been provisionally removed from the order *Rickettsiales* and instead been assigned to the family *Coxiellaceae* within the *Legionellales* (Garrity et al., 2005). Interestingly, *R. popilliae* had originally been described as "*Coxiella popilliae*" (Dutky and Gooden, 1952).

This taxonomic rearrangement has been confirmed for two of the three recognized *Rickettsiella* species by additional 16S rRNA gene sequences available from the recently published genome sequence of a second strain of *R. grylli* (GenBank Accession No. NZ_AAQJ00000000; Leclerque, 2008) as well as from *Rickettsiella* strains of the pathotypes *Rickettsiella armadillidii* (Cordaux et al., 2007) and *Rickettsiella melolonthae* (Leclerque and Kleespies, 2008) that are recognized synonyms of the species *R. popilliae*. Despite the fact that there are yet no sequence data available for the third species, *R. chironomi*, monophyly of the genus *Rickettsiella* has been claimed (Cordaux et al., 2007). However, two further isopod- and cockroach-associated bacteria that had initially been described, respectively, as showing "a high degree of morphological similarity with *R. grylli*" (Drobne et al., 1999) and as "*Rickettsiella crassificans*" (Radek, 2000) were recently transferred to the candidate genus "*Rhabdochlamydia*" of the order *Chlamydiales* (Kostanjsek et al., 2004; Corsaro et al., 2007) after the respective 16S rRNA encoding genes had been sequenced.

Rickettsiella tipulae, a pathogen of the crane fly, *Tipula paludosa* (Diptera: Tipulidae) displays a developmental cycle characteristic for *Rickettsiella*-like bacteria with close similarity to *R. melolonthae* (Huger and Krieg, 1967) and is currently recognized as a pathotype placed in synonymy with the species *R. popilliae* (Garrity et al., 2005). However, results from comparative serological studies have been interpreted in favor of *R. tipulae* being an independent species different from *R. popilliae* and several of its synonyms, including *R. melolonthae* (Müller-Kögler, 1958; Krieg, 1958). Here we use electron microscopy to identify a rickettsial pathogen of *T. paludosa* and provide the first DNA sequence data for *R. tipulae.* Performing a phylogenetic analysis based on the comparison of 16S ribosomal RNA encoding sequences, we aim to assess if *R. tipulae* is correctly placed in the order *Legionellales* and, in case it is, to elucidate its taxonomic status within the genus *Rickettsiella*.

2. Materials and methods

For electron microscope studies of ultrathin sections, larval fat-body tissues were fixed in 1% osmium tetroxide in Veronal buffer (pH 7.2) for 5 h. After dehydration in ascending ethanol series, tissues were embedded in a 7:3 mixture of butyl- and *n*-methylmethacrylate. Thin sections, double-stained with uranyl acetate and lead citrate, were examined in a Zeiss EM 9A electron microscope.

Genomic DNA of *R. tipulae* strain BBA296 was extracted from L3 to L4 larvae of the crane fly, *T. paludosa*, from the area of Burscheid, Germany, by using a standard protocol (Walsh et al., 1991) based on the Chelex 100 resin (BioRad). PCR amplification of an almost complete 16S rRNA encoding sequence was performed from the extract with primers fD1 and rP2 as described by Weisburg et al. (1991) using Phusion High-Fidelity DNA polymerase (Finnzymes) in a reaction running over 25 cycles of 15 s at 94 °C, 30 s at 50 °C, and 2 min at 72 °C. PCR products from three independent amplification reactions containing an appropriately sized product as controlled by agarose gel electrophoresis were purified by passage over a Qiaquick column (Qiagen) and sequenced on both strands using the fluorescence-labeled didesoxynucleotide technology. The six raw sequences were analyzed and combined into a single consensus sequence using the DNA Strider 1.3 program. Orthologous sequences available in the GenBank database were identified with the BlastN software tool in searches over both the whole range of single sequence entries and the *R. grylli* genome project (Altschul et al., 1997).

Sequence alignments were performed by means of the CLUSTAL W function (Thompson et al., 1994) of the MEGA 4 program (Tamura et al., 2007) using an IUB DNA weight matrix. The TREE-PUZZLE 5.2 software (Schmidt et al., 2002) was used to estimate data set specific parameters. The most appropriate model of DNA sequence evolution was chosen according to the rationale outlined by Posada and Crandall (1998). Organismal phylogenies were reconstructed with the Maximum Likelihood (ML) method as implemented in the PhyML software tool (Guindon and Gascuel, 2003) using the HKY model of nucleotide substitution (Hasegawa et al., 1985). Additional Minimum Evolution (ME) and Neighbor Joining (NJ) phylogenies were reconstructed in MEGA 4 under a Kimura 2-parameter model of substitution (Kimura, 1980). In all cases, a Γ-distribution based model of rate heterogeneity (Yang, 1993) allowing for eight rate categories was assumed. Furthermore, a Maximum Parsimony (MP) 16S rDNA tree was constructed in MEGA 4 on the basis of a Max-mini Branch-and-bound algorithm. Tree topology confidence limits were explored in non-parametric bootstrap analyses over 1000 pseudo-replicates. Trees were outgroup rooted by the 16S rRNA gene sequence from Candidatus '*Rhabdochlamydia crassificans*' and arranged for more convenient comparison. A pairwise p-distance matrix for the aligned 16S rDNA sequences was constructed in the MEGA 4 program under pairwise deletion of alignment gaps and missing data.

3. Results and discussion

Electron micrographs of ultrathin sections from the fat-body tissue of infected *T. paludosa* larvae reveal the subcellular structures characteristic for rickettsiosis. Features of rickettsial development are shown in Fig. 1, e.g. intracellular vesicles and vacuolar structures filled with bacterial cells (Fig. 1A). "Giant bodies" can be found in rounded or more

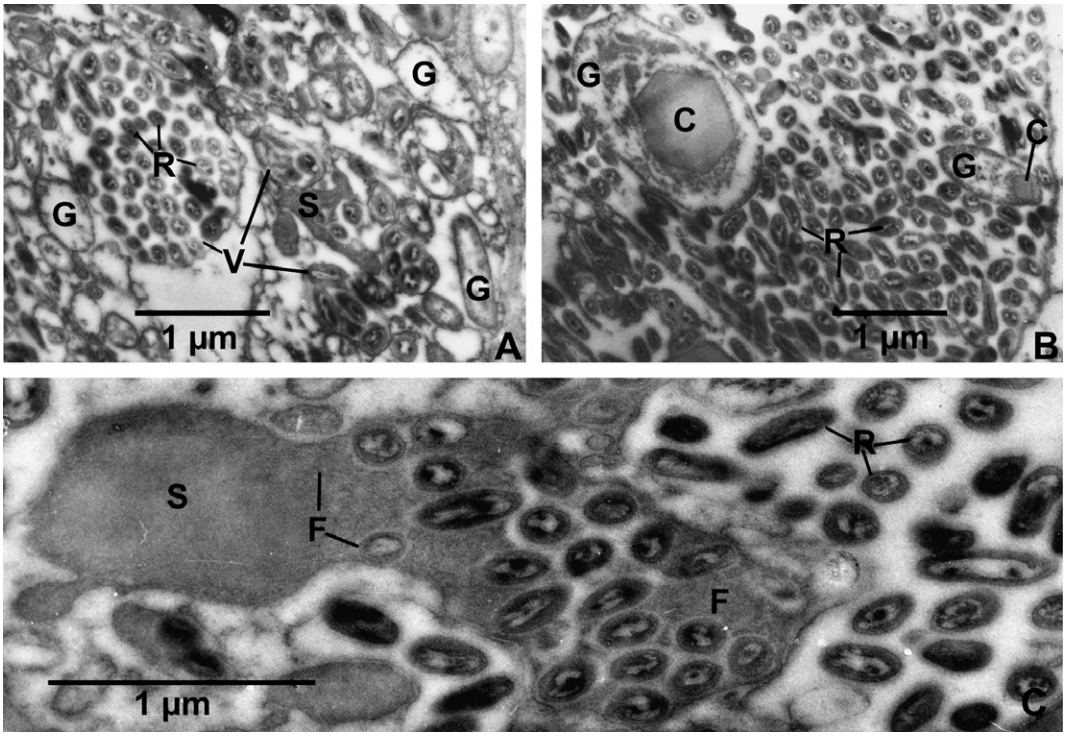

Fig. 1. Electron micrographs from ultrathin sections of fat-body cells of *Tipula paludosa* infected with *Rickettsiella tipulae*. (A) Rickettsiae (R) often assembled in vesicles (V), and "giant bodies" (G). Rickettsiae may also appear within areas of dense stroma (S). (B) Mature rickettsiae (R) and "giant bodies" (G), the latter transforming into associated crystals (C). (C) Formation (F) of rickettsiae (R) within dense stroma (S) (Phot. A.M. Huger, BBA).

or less elongated shape (Fig. 1A and B). Membrane-bounded protein crystals that later on appear naked can be observed in Fig. 1B. The formation of rod- or oval-shaped rickettsiae within dense stroma is shown in Fig. 1C. A detailed ultrastructural description of the development and interconversion of this structure has been given by Huger and Krieg (1967).

The presence of these morphological characteristics of rickettsial infections of insects in our samples demonstrates that the following genetic information and its interpretation refer to the organism of interest.

Pairwise identities between the six raw sequences obtained from our PCR products were in no case inferior to 99.5%. The derived consensus sequence comprises 1357 nucleotides of the 16S rRNA encoding gene of *R. tipulae* BBA296 and, when used as BlastN query, identifies as best hits of >95% sequence identity at close to 100% query coverage the above-mentioned 16S rRNA encoding sequences from *R. grylli* together with the orthologs from *Rickettsiella*-like bacteria associated with different arthropods, e.g. the collembola *Folsomia candida* (Czarnetzki and Tebbe, 2004), the tick *Ixodes woodi* (Kurtti et al., 2002), and the psyllid *Cecidotrioza sozanica* (Spaulding and von Dohlen, 2001), as well as a number of sequences from environmental samples. Furthermore, three shorter sequences from aquatic isopod-associated bacteria belonging to the pathotype *R. armadillidii* (Cordaux et al., 2007) show >95% sequence identity to the *R. tipulae* sequence. Those identified sequences that are currently assigned to a recognized

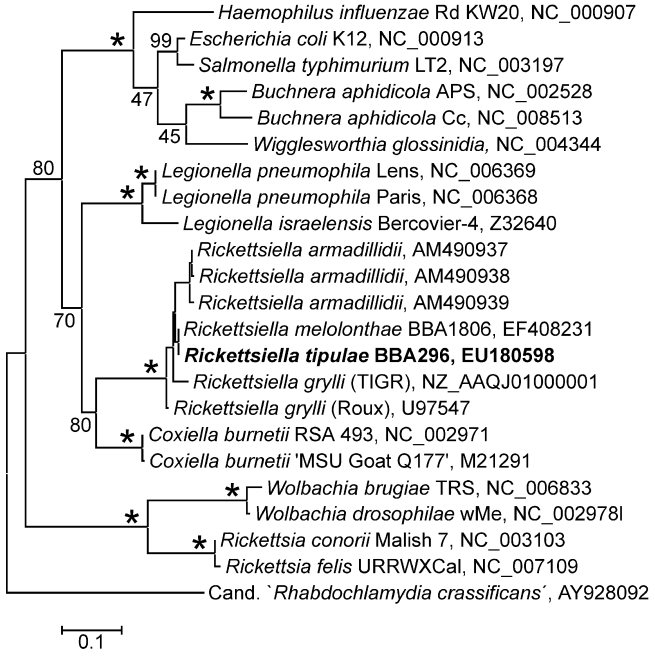

Fig. 2. Bacterial phylogeny reconstructed from 16S rRNA encoding sequences by the Maximum Likelihood (ML) method. Terminal branches are labeled by genus, species or pathotype, and—where appropriate—strain designation of the organism as well as Genbank accession number of the respective sequence. Designations "*R. grylli* (Roux)" and "*R. grylli* (TIGR)" refer to the strain described by Roux et al. (1997) and to the genome project strain, respectively. Numbers on branches indicate bootstrap support values; the asterisk (*) denotes optimal bootstrap support. For confidence limits within and at higher resolution of the *Rickettsiella* clade see Fig. 3.

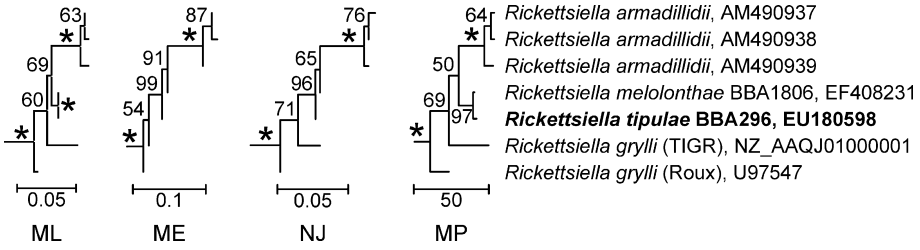

Fig. 3. *Rickettsiella* clades pruned off from 16S rDNA-based phylogenetic trees constructed by the Maximum Likelihood (ML), Minimum Evolution (ME), Neighbor Joining (NJ), and Maximum Parsimony (MP) methods. Terminal branch labels for the four clades are identical and given at the right margin. Further designations are as indicated in the legend to Fig. 2.

pathotype or species were retained for phylogenetic analysis together with orthologs from further taxonomically related or arthropod-associated bacteria. A total of 22 bacterial 16S rDNA sequences were compared to the *R. tipulae* consensus sequence.

The Maximum Likelihood phylogeny (Fig. 2) reconstructed from the resulting alignment expectedly places all supposed *Rickettsiella* sequences among the γ-proteobacteria, in closest proximity to the genera *Coxiella* and *Legionella*, thereby separating them from the α-proteobacterial *Rickettsia* and insect endosymbiotic *Wolbachia* as well as from the γ-proteobacterial insect endosymbionts *Buchnera* and *Wigglesworthia*. These topological features are reproduced in the three additional phylogenetic trees generated by the Minimum Evolution (ME), Maximum Parsimony (MP), and Neighbor Joining (NJ) methods (data not shown). In all these trees, the branch at the root of the *Rickettsiella* clade receives optimal bootstrap support and *R. tipulae* is firmly placed within this clade (Fig. 3). These findings demonstrate that in contrast to the formerly supposed *Rickettsiella*-like organisms now described as *Rhabdochlamydia*, the taxonomic reorganization of the genus *Rickettsiella* in the γ-proteobacterial order *Legionellales* is justified for the pathotype *R. tipulae*.

As the classical species concept is problematic in microbiology, the degree of similarity of orthologous nucleotide sequences from different organisms is widely used as an indicator of taxonomic relationships. Within, for instance, the order *Chlamydiales* 16S rDNA sequence identity thresholds of ⩾90%, ⩾95%, and ⩾98.5% are employed as criterion for the relatedness of specimen at the family, genus, and species level, respectively (Everett et al., 1999). The percentage identity values for the six 16S rDNA gene sequences from *Rickettsiella* used in our alignment were calculated from the corresponding pairwise p-distance matrix (Table 1). Most pairs of *Rickettsiella* sequences are found 95–98% identical, indicating—in terms of the cut-off values applied for chlamydia—that they might belong to different strains of the same genus. Exceptions are the pairwise identity values among the three *R. armadillidii* rRNA genes as well as between the *R. melolonthae* and the *R. tipulae* sequences, all of which are superior to 99%. These values indicate that, firstly, each of these two groups comprises organisms related at the species level. Secondly, the above results strongly suggest that the pathotype *R. armadillidii,* on the one hand, and the pathotypes *R. melolonthae* and *R. tipulae,* on the other hand, might represent different *Rickettsiella* species. All these pathotypes are currently recognized as synonyms of the nomenclatural type species, *R. popilliae*, and yet unavailable sequence information from the pathotype *R. popilliae* will be required to decide about the exact nature of a possible reorganization of the respective species delineations within the genus *Rickettsiella*. As the synonymization to *R. popil-*

Table 1
Pairwise percentage sequence identities calculated from a p-distance matrix of 16S rRNA encoding genes from *Rickettsiella* strains

	Rickettsiella tipulae, BBA296	*Rickettsiella melolonthae*, BBA1806	*Rickettsiella armadillidii*, AM490937	*Rickettsiella armadillidii*, AM490938	*Rickettsiella armadillidii*, AM490939	*Rickettsiella grylli* (Roux)
R. melolonthae BBA1806	**99.9%**					
R. armadillidii, AM490937	96.4%	96.7%				
R. armadillidii, AM490938	97.2%	97.4%	**99.7%**			
R. armadillidii, AM490939	96.7%	96.9%	**99.1%**	**99.3%**		
R. grylli (Roux)	97.6%	97.7%	95.5%	96.1%	95.7%	
R. grylli (TIGR)	97.1%	97.1%	95.0%	95.7%	95.3%	96.3%

Sequence identity values superior to 98.5% are printed in bold.

liae of *R. armadillidii* is on rather tenuous ground (Frutos et al., 1994) as compared to that of *R. melolonthae* (Krieg, 1958), the recognition of *R. armadillidii* as a separate species might seem a likely future development.

Unexpectedly, the two *R. grylli* sequences included in our study diverge strongly, being each more closely related to the *R. melolonthae* and *R. tipulae* orthologs than to each other. As one of both *R. grylli* strains represents the authentic pathotype isolated from the cricket *Gryllus bimaculatus*, this finding sheds considerable doubt on the taxonomic classification of the isopod-derived *R. grylli* genome project strain.

In conclusion, the results of the present study firstly demonstrate that the *Rickettsiella*-like bacterial pathogen associated with larvae of the crane fly, *T. paludosa*, is correctly classified in the γ-proteobacterial genus *Rickettsiella*. However, in contrast to earlier claims, *R. tipulae* should not be considered an independent species, but be co-assigned at least with the pathotype *R. melolonthae*. Secondly, our comparison of 16S rDNA sequence identities is suggestive of a reorganization of the nomenclatural type species, *R. popilliae*, separating the synonymous pathotypes into two different species, one comprising both *R. melolonthae* and *R. tipulae*, the other *R. armadillidii*. More extensive molecular phylogenetic analyses, particularly those including the pathotype *R. popilliae*, will be required to determine the correct species delineation within the genus *Rickettsiella*.

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