16S rRNA-, GroEL- and MucZ-based assessment of the taxonomic position of '*Rickettsiella melolonthae*' and its implications for the organization of the genus *Rickettsiella*

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'*Rickettsiella melolonthae*' is an intracellularly multiplying bacterial pathogen of European cockchafer, *Melolontha melolontha* (Linnaeus, 1758) and *Melolontha hippocastani* (Fabricius, 1801) (Coleoptera: Scarabaeidae). We report the first determination of nucleotide sequences from this organism, i.e. the 16S rRNA encoding *rrs* gene, the chaperonin encoding *groEL* gene and the *mucZ* gene encoding the orthologue of a capsule synthesis-inducing factor of *Coxiella burnetii*. Within the genus *Rickettsiella*, the pathotype '*Rickettsiella melolonthae*' is currently classified as a synonym of the nomenclatural type species *Rickettsiella popilliae*. Previous sequencing of a 16S rRNA gene from a different species, *Rickettsiella grylli*, has motivated the transfer of the entire genus from the alphaproteobacterial order *Rickettsiales* to the gammaproteobacterial order *Legionellales*, family *Coxiellaceae*. We investigated the validity of this taxonomic reorganization beyond the species *Rickettsiella grylli* by reconstructing the organismal phylogeny from comparisons of 16S rRNA gene and GroEL and MucZ protein sequences from a selected set of alpha- and gammaproteobacteria as well as bacterial pathogens from the order *Chlamydiales*. Our analysis strongly supported the transfer of the genus *Rickettsiella* to the order *Legionellales*, but not its classification in one of the recognized families present in this order. Furthermore, our results substantiated inconsistencies in the internal organization of the genus. In particular, the currently accepted delineation of *Rickettsiella* species and the claimed synonymy of '*Rickettsiella melolonthae*' with *Rickettsiella popilliae* are not simultaneously consistent with our findings.

The genus *Rickettsiella* (Philip, 1956) comprises intracellularly multiplying bacterial pathogens of insects, crustaceans and arachnids that were originally perceived as 'rickettsiae of insects' (Dutky & Gooden, 1952; Krieg, 1955) and classified in the order *Rickettsiales*. An alternative assignment of these organisms to the order *Chlamydiales* has been judged less convincing (Weiss & Moulder, 1984). Within the genus *Rickettsiella*, there are currently four species with validly published names: the type species *Rickettsiella popilliae* (Dutky & Gooden, 1952), *Rickettsiella grylli* (Vago & Martoja, 1963), *Rickettsiella chironomi* (Weiser, 1963) and *Rickettsiella stethorae* (Hall & Badgley, 1957). However, according to Fournier & Raoult (2005), there is not sufficient evidence available to warrant the consideration of *Rickettsiella stethorae* as a distinct species.

Abbreviations: ME, minimum evolution; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the *rrs*, *groEL* and *mucZ* gene sequences of '*Rickettsiella melolonthae*' are EF408231, EF635858 and EF635859, respectively.

Numerous further pathotypes studied by electron microscopy have been suggested to represent synonyms of these species, while others still await conclusive species assignment. The pathotype '*Rickettsiella melolonthae*' was discovered and first described as the causative agent of the 'Lorsch Erkrankung' (Lorsch disease) of white grubs of the European cockchafer species *Melolontha melolontha* (Linnaeus, 1758) and *Melolontha hippocastani* (Fabricius, 1801) (Coleoptera: Scarabaeidae) from near the town Lorsch, State of Hessen, Germany (Wille & Martignoni, 1952). Originally named '*Rickettsia melolonthae*' (Krieg, 1955), more detailed morphological and serological examinations (Krieg, 1958a, b, 1960; Huger & Krieg, 1967) have led to its assignment as a synonym of the species *Rickettsiella popilliae*.

Strains of the genus *Rickettsiella* are generally considerably heterogeneous in terms of the cellular stages of the life cycle, the hosts and host tissues infected, the presence of protein crystals and the serological properties (for a review see Tanada & Kaya, 1993 and Garrity *et al.*, 2005). Detailed

descriptions of developmental cycles point to a relatively close relationship between *Rickettsiella popilliae* and *Rickettsiella grylli* as compared with *Rickettsiella chironomi* (Götz, 1972) and reveal considerable similarities between the latter species and members of the genus *Chlamydia* (Federici, 1980). Nevertheless, a more recent study including *Rickettsiella* strains assigned to three of the species concluded from the results of DNA–DNA hybridization experiments, DNA G+C content ratios and DNA fine melting profiles that the genera *Rickettsiella* and *Chlamydia* are ‘appropriately placed in different orders’ (Frutos *et al.*, 1994).

Sequencing of the 16S rRNA encoding *rrs* gene of a strain of *Rickettsiella grylli* revealed that it possessed the highest sequence similarity with the corresponding gene of *Coxiella burnetii* (Roux *et al.*, 1997), i.e. a bacterial species that due to earlier 16S rRNA gene sequence analyses had been transferred from the alphaproteobacterial order *Rickettsiales* to the distantly related gamma subclass of the proteobacteria (Weisburg *et al.*, 1989). As this finding calls into question the taxonomic classification of at least *Rickettsiella grylli* and reflects the original description of *Rickettsiella popilliae* as *Coxiella popilliae*, the entire genus *Rickettsiella* has recently been ‘provisionally removed from the order *Rickettsiales*’ and instead has been assigned to the gammaproteobacterial order *Legionellales*, family *Coxiellaceae* (Garrity *et al.*, 2005). While this taxonomic reorganization is confirmed for *Rickettsiella grylli* by the recently published genome draft sequence (GenBank accession number NZ_AAQJ00000000) from one strain (strain TIGR) and partial 16S rRNA gene sequences (Cordaux *et al.*, 2007) from three additional strains assigned to this species, the taxonomic positions of *Rickettsiella popilliae* and *Rickettsiella chironomi* remain uncertain due to the complete lack of sequence data from these species or from synonymous pathotypes such as ‘*Rickettsiella melolonthae*’.

The determination of 16S rRNA gene sequences from three *Rickettsiella* pathotypes that, on the basis of electron microscopic studies, had not been assigned to any of the recognized species has produced conflicting results. On one hand, the *rrs* gene sequence from a bacterial endosymbiont of the tick *Ixodes woodi* (strain GSU) was found to display the highest similarity with that of *Rickettsiella grylli* (Kurtti *et al.*, 2002). On the other hand, a presumed unnamed *Rickettsiella* strain infecting the terrestrial isopod *Porcellio scaber* that shows ‘a high degree of morphological similarity with *Rickettsiella grylli*’ (Drobne *et al.*, 1999) and a putative rickettsiellal pathogen of the cockroach *Blatta orientalis* (‘*Rickettsiella blattae*’, Huger, 1963; ‘*Rickettsiella crassificans*’, Radek, 2000) were transferred to the newly created candidate genus ‘*Rhabdochlamydia*’ within the order *Chlamydiales* after the respective 16S rRNA gene sequences had been determined (Kostanjsek *et al.*, 2004; Corsaro *et al.*, 2006). Irrespective of these conflicting results, monophyly has recently been claimed for the genus *Rickettsiella* (Cordaux *et al.*, 2007).

The highly conserved Hsp60 chaperonin GroEL is distributed ubiquitously throughout the eubacterial lineage, appears functionally involved in bacterial endosymbiosis (Fares *et al.*, 2004) and pathogenicity (Garduño *et al.*, 1998) and has been used in molecular phylogenetic studies of proteobacterial endosymbionts of insects (Viale *et al.*, 1994; Casiraghi *et al.*, 2005). The *mucZ* gene product from *C. burnetii* induces capsule synthesis (mucoidy) when expressed in *Escherichia coli* (Zuber *et al.*, 1995). MucZ proteins contain a C-terminal domain sharing significant similarity with the J domain of the bacterial Hsp40 co-chaperone DnaJ, but are otherwise highly variable. Orthologues of the *C. burnetii* protein are identifiable to date only in a subset of the class *Gammaproteobacteria* including *Legionella pneumophila*. The *mucZ* gene has been used as a molecular marker to investigate the phylogeny of the genus *Coxiella* (Sekeyová *et al.*, 1999). In a preliminary study using *C. burnetii* gene sequences as a query, we identified two identical *rrs* genes, as well as single copies of the *mucZ* and the *groEL* genes in the genome of *Rickettsiella grylli*. The finding of only a single copy of the *groEL* gene was in contrast to the situation usually found in chlamydial genomes where duplication of *groEL* genes is the rule (McNally & Fares, 2007).

In this study, using the 16S rRNA gene and the GroEL and MucZ proteins as taxonomic markers, we report the first molecular sequence data for a *Rickettsiella* strain belonging to the type species *Rickettsiella popilliae*. We investigated whether these data: (i) corroborate the previously merely assumed assignment of *Rickettsiella popilliae* to the class *Gammaproteobacteria*, (ii) support the current family level classification of the genus *Rickettsiella* within the order *Legionellales*, and (iii) help to elucidate the internal taxonomic structure of the genus *Rickettsiella*, with particular emphasis on the validity of current species delineations and species–pathotype synonymies.

Genomic DNA of ‘*Rickettsiella melolonthae*’ strain BBA1806 was extracted from infected fat body tissue of diseased *Melolontha hippocastani* grubs collected in the Lorsch area, Germany, by using a standard protocol (Walsh *et al.*, 1991) based on Chelex 100 resin (Bio-Rad). PCR amplification of an almost complete 16S rRNA gene 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 29 cycles of 15 s at 94 °C, 30 s at 50 °C and 2 min at 72 °C. With identical PCR settings, the following pairs of degenerate oligonucleotides (MWG Biotech) were used to amplify internal fragments of the *groEL* (*groEL*F1: 5'-GANGCNGGNGAYGGNACNACN-AC; *groEL*R1: 5'-ACYTTNATNACNGCNACNCCNCC) and the *mucZ* (*mucZ*F1: 5'-GATGGACGTGTTTCTGAG-GCAGARAT; *mucZ*R1: 5'-CCTTTAGAGAGTAATTTA-TCNGGRTG) genes from the same template. For each gene, 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. The six raw sequences were analysed, combined into a single consensus sequence and translated using the DNA Strider 1.3 program.

Similar nucleotide and amino acid sequence entries in the GenBank database were identified with the BLASTN and BLASTP software tools, respectively (Altschul *et al.*, 1997). Sequence alignments were performed by means of the CLUSTAL W function (Thompson *et al.*, 1994) of the MEGA 3.1 program (Kumar *et al.*, 2004) using an International Union of Biochemistry (IUB) DNA and a Gonnet protein weight matrix. The TREE-PUZZLE 5.2 program (Schmidt *et al.*, 2002) was used to estimate dataset-specific parameters. The most appropriate model of DNA sequence evolution was chosen according to the rationale outlined by Posada & Crandall (1998), while amino acid sequence evolution models were directly chosen with the analysis tool available in TREE-PUZZLE. Phylogenies were reconstructed with the maximum-likelihood (ML) method as implemented in the PHYL software tool (Guindon & Gascuel, 2003), using the Hasegawa–Kishino–Yano (HKY) model of nucleotide substitution (Hasegawa *et al.*, 1985) for the analysis of the 16S rRNA gene and the JTT model (Jones *et al.*, 1992) for the GroEL and MucZ amino acid sequence alignments. Additional minimum-evolution (ME) and neighbour-joining (NJ) 16S rRNA gene phylogenies were reconstructed in the MEGA 3.1 program under a Kimura two-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. A maximum-parsimony (MP) tree was constructed in MEGA 3.1 from the 16S rRNA gene sequence alignment on the basis of a max-mini branch-and-bound algorithm. Tree topology confidence limits were explored in non-parametric bootstrap analyses over 1000 replicates. Trees were outgroup rooted by the respective sequence from the epsilonproteobacterium *Myxococcus xanthus* and arranged for more convenient comparison.

From each of the three sets of raw data generated from PCR products, reliable consensus sequences could be generated as the pairwise sequence similarities within a set were in no case <99.5%. Both protein coding consensus sequences were translated into partial sequences of 282 amino acids for the GroEL and 157 amino acids for the MucZ protein of '*Rickettsiella melolonthae*'. When used as the query sequence of a BLASTP search for similar database entries, the respective orthologue from *Rickettsiella grylli* was identified as the best hit for both sequences. The 16S rRNA consensus gene sequence derived from our data comprised 1462 nucleotides or approximately 95% of the *rrs* gene of '*Rickettsiella melolonthae*' and, when used as BLASTN query, the best hits of >95% sequence similarity at close to 100% query coverage were identified as the two available full-length 16S rRNA gene sequences from *Rickettsiella grylli*, together with the 16S rRNA gene sequences from the above-mentioned *Ixodes woodi* endosymbiont (strain GSU) and a number of uncultured arthropod-associated bacteria.

The maximum-likelihood tree constructed from the alignment of bacterial *rrs* sequences (Fig. 1) displayed a clear distinction of two maximally supported branches comprising, respectively, members of the orders *Chlamydiales*, including the candidate genus '*Rhabdochlamydia*', and *Rickettsiales*, including insect-associated *Wolbachia*, from a gammaproteobacterial branch. The latter, in turn, contained one major consistently structured sub-branch comprising, among others, the insect endosymbiotic genera *Buchnera* and *Wigglesworthia* in a sister position to the enterobacterial clade. These topological features were clearly supported in phylogenetic trees based on ME, MP and NJ analysis of 16S rRNA gene sequences and the GroEL protein tree (data not shown). As *mucZ* genes are absent from most of these genera, the MucZ protein tree was non-informative at this level. The gammaproteobacterial branch was made up of several maximally supported clades representing single genera. Within this structure, the 16S rRNA gene sequence from '*Rickettsiella melolonthae*' clustered firmly with the sequences from the two *Rickettsiella grylli* strains (Roux and TIGR) and the tick endosymbiont (strain GSU), thereby forming a presumed *Rickettsiella* clade located next to equally well supported *Coxiella* and *Legionella* clades. When included in the analysis, the three additional partial sequences from the isopod-pathogenic *Rickettsiella grylli* strains formed an additional branch within this clade (data not shown). With a similarly positioned *Rickettsiella* clade receiving near optimal bootstrap support in all of the further phylogenetic trees presented in Fig. 2, both of the considered alternative assignments of '*Rickettsiella melolonthae*', i.e. as either a member of the candidate genus '*Rhabdochlamydia*' of the order *Chlamydiales* or as according to its original classification in the order *Rickettsiales*, can be ruled out, while the co-assignment of '*Rickettsiella melolonthae*' and *Rickettsiella grylli* is positively corroborated. This result is consistent with the monophyly hypothesis for the genus *Rickettsiella*. However, as long as sequence data are still lacking for the third, and in this respect, probably most critical species, *Rickettsiella chironomi*, such a claim must seem premature. Our view is that, under the assumption of the claimed synonymy of '*Rickettsiella melolonthae*' with *Rickettsiella popilliae*, the transfer of the rickettsiellae to the gammaproteobacterial order *Legionellales* appears justified for two of the three *Rickettsiella* species.

There are, however, further features of the phylogenetic trees presented that are not in congruence with the current classification of the genus *Rickettsiella*. Firstly, the ML tree based on 16S rRNA gene sequences places the *Rickettsiella* clade in a sister position to the *Coxiella* clade, whereas the trees in Fig. 2 instead show a sister relationship of the *Rickettsiella* and *Legionella* clades. Clearly, this finding does not lend support to the current recognized classification of the genus *Rickettsiella* in the family *Coxiellaceae* and with neither of the two alternative topologies receiving a convincing bootstrap support, it might thus be more parsimonious to renounce a hierarchy building family assignment.

Fig. 1. Bacterial phylogeny reconstructed from 16S rRNA gene sequences by the ML method. Terminal branches are labelled by genus, species, and, where appropriate, the strain designation of the organism as well as the GenBank accession number of the respective sequence. Designations *Rickettsiella grylli* (Roux) and *Rickettsiella 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. Bar, 10% relative sequence difference.

In addition, there is also incongruence with respect to the internal structure of the *Rickettsiella* clade as displayed in the 16S rRNA gene trees presented. Irrespective of the method of phylogeny reconstruction applied, one of the two strains of *Rickettsiella grylli* is more closely related to '*Rickettsiella melolonthae*' than to the second *Rickettsiella grylli* isolate, but under different reconstruction methods this is true for a different *Rickettsiella grylli* strain. The *Rickettsiella* clade thus lacks any topological structure reflecting the assumed species delineation. With pairwise sequence similarities of the respective *rrs* genes of 95–97 %, this is clearly not due to a lack of relevant phylogenetic information. On the contrary, the 16S rRNA gene sequences of the genus *Rickettsiella* appear rather heterogeneous if compared, for instance, with the >99 % sequence similarity found without exception for different isolates of *C. burnetii* (Stein *et al.*, 1993) or to the ≥ 90 %, ≥ 95 % and ≥ 98.5 % sequence similarity thresholds employed, respectively, as criteria for the family, genus and species assignment of chlamydial strains (Everett *et al.*, 1999). Application of these cut-off values would place each

of the four *Rickettsiella* isolates (*Rickettsiella grylli* strains Roux and TIGR, '*Rickettsiella melolonthae*' strain BBA1806 and *Ixodes woodi* endosymbiont strain GSU) in a different species, with the species concept thereby undercutting the pathotype concept, and, moreover, it would also remove the genus *Rickettsiella* from the family *Coxiellaceae* (data not shown). Due to their relatively short length, the further available partial 16S rRNA gene sequences from three additional strains of *Rickettsiella grylli* do not contribute any further conclusive data. The topological structure of the *Rickettsiella* clade resulting from our reconstructions of the 16S rRNA gene sequence phylogeny is, therefore, inconsistent with the currently accepted internal organization of this genus. The two studied strains of *Rickettsiella grylli*, Roux and TIGR, can be either placed in different species, with the consequence that the generally questionable pathotype-based system of species delineation has to be relinquished, or alternatively, if they are kept together in the same species, our results demand the inclusion of the strain BBA1806 of '*Rickettsiella melolonthae*' (as well as the *Ixodes woodi* endosymbiont strain GSU) in this species. As

Fig. 2. Partial phylogenetic trees constructed from 16S rRNA gene sequences by the ME (a), MP (b) and NJ (c) methods, as well as from GroEL (d) and MucZ (e) protein sequences by the ML method. The labels for the trees in a–c are given at the right hand side of the figures. Bars: 5% relative sequence difference (a and c), 50 nucleotide substitutions per site (b), 20% relative sequence difference (d) and 50% relative sequence difference (e).

'*Rickettsiella melolonthae*' is a synonym for *Rickettsiella popilliae*, this means that the current delineation of the species *Rickettsiella popilliae* and *Rickettsiella grylli* would disappear. This last consequence could, in turn, be avoided by giving up the assumption of the synonymy of the species *Rickettsiella popilliae* and '*Rickettsiella melolonthae*'.

In conclusion, our study lends firm support to the previous reorganization of the genus *Rickettsiella* as part of the gammaproteobacterial order *Legionellales* by furnishing the first molecular genetic evidence for the taxonomic position of a *Rickettsiella* pathotype classified in the species *Rickettsiella popilliae*. At the same time, our study emphasizes the weaknesses in the family level classification and internal organization of the genus. On one hand, the assignment of the genus *Rickettsiella* to the family *Coxiellaceae* is not supported by our findings and we feel that a hierarchically neutral assignment at the family level might be more appropriate. On the other hand, the currently accepted delineation of *Rickettsiella* species and the assumption of synonymy of '*Rickettsiella melolonthae*' with *Rickettsiella popilliae* are not simultaneously consistent with our findings. Sequencing of the 16S rRNA gene from the *Rickettsiella popilliae* type strain will be the most straightforward way to properly decide which of these concepts will finally have to be abandoned. Meanwhile, the systematically most conservative, even if phylogenetically little convincing approach, consistent with our results would be to give up the morphologically and serologically comparatively well-founded claim of synonymy of *Rickettsiella popilliae* and '*Rickettsiella melolonthae*'. Having secured the taxonomic position of the genus *Rickettsiella*, our study emphasizes the need for a reliable molecular taxonomic criterion for its internal organization.

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