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Ultrastructural characterization and multilocus sequence analysis (MLSA) of '*Candidatus* Rickettsiella isopodorum', a new lineage of intracellular bacteria infecting woodlice (Crustacea: Isopoda)☆

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ABSTRACT

The taxonomic genus *Rickettsiella* (*Gammaproteobacteria*; *Legionellales*) comprises intracellular bacteria associated with a wide range of arthropods including insects, arachnids and crustaceans. The present study provides ultrastructural together with genetic evidence for a *Rickettsiella* bacterium in the common rough woodlouse, *Porcellio scaber* (Isopoda, Porcellionidae), occurring in Germany, and shows that this bacterium is very closely related to one of the same genus occurring in California that infects the pill bug, *Armadillidium vulgare* (Isopoda, Armadillidiidae). Both bacterial isolates displayed the ultrastructural features described previously for crustacean-associated bacteria of the genus *Rickettsiella*, including the absence of well-defined associated protein crystals; occurrence of the latter is a typical characteristic of infection by this type of bacteria in insects, but has not been reported in crustaceans. A molecular systematic approach combining multilocus sequence analysis (MLSA) with likelihood-based significance testing demonstrated that despite their distant geographic origins, both bacteria form a tight sub-clade within the genus *Rickettsiella*. In the 16S rRNA gene trees, this sub-clade includes other bacterial sequences from woodlice. Moreover, the bacterial specimens from *P. scaber* and *A. vulgare* are found genetically or morphologically different from each of the four currently recognized *Rickettsiella* species. Therefore, the designation '*Candidatus* Rickettsiella isopodorum' is introduced for this new lineage of isopod-associated *Rickettsiella* bacteria.

Introduction

The bacterial genus *Rickettsiella* (Philip) comprises intracellular bacteria associated with a wide range of arthropods. While mostly described as pathogens, at least in one case a mutualistic relationship to the host – the pea aphid, *Acyrthosiphon pisum* – has been reported [36,49]. Bacteria of this genus typically multiply in cytoplasmic vesicles within fat body cells or hemocytes, and multiplication frequently yields protein crystals within these vesicles. Infections by members of this genus typically proceed slowly, often lasting for many weeks, and are characterized by a complex histopathology and cytopathology that includes polymorphic bacterial stages of development along with the release of infective cells after host cell lysis [22].

Originally perceived as "*Rickettsia*-like bacteria" (RLBs) or "rickettsiae of insects", *Rickettsiella* bacteria have been organized in the order *Legionellales* (*Gammaproteobacteria*) based on 16S rRNA gene sequence analyses. Currently, four species are recognized within the genus: the type species *Rickettsiella popilliae* (Dutky and

Abbreviations: RLB, *Rickettsiella*-like bacterium; MLSA, multilocus sequence analysis; *ftsY*, gene encoding signal recognition particle-receptor subunit alpha; *gidA*, gene encoding glucose inhibited cell division protein A; *rpsA*, gene encoding 30S ribosomal protein S1; *sucB*, gene encoding dihydrolipoamide succinyltransferase; ML, maximum likelihood method of phylogenetic reconstruction; ME, minimum evolution method of phylogenetic reconstruction; NJ, neighbor joining method of phylogenetic reconstruction; HKY, Hasegawa–Kishino–Yano model of nucleotide substitution; MCL, maximum composite likelihood model of nucleotide substitution; K2P, Kimura 2-parameter model of nucleotide substitution; JTT, Jones–Taylor–Thornton model of amino acid substitution; 1sKH/2sKH, one-/two-sided Kishino–Hasegawa significance test.

☆ *Note*: Nucleotide sequence data reported are available in the GenBank database under the accession numbers HQ660943–44, JQ679309–11, JX406180–84.

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Gooden); *Rickettsiella grylli* (Vago and Martoja); *Rickettsiella chironomi* (Weiser); and *Rickettsiella stethorae* (Hall and Badgley). At present, no genetic data are available for the last two species, and conclusive species assignments are lacking for most isolates of *Rickettsiella*. Instead, these are described at the level of pathotypes that in turn are sometimes claimed subjective synonyms of a species. Since their discovery in 1952 [8,56], RLBs from numerous insects and arachnids have been described morpho- and histopathologically [14, and references therein], and intracellular bacteria from crickets [43], ticks [27,32], collembola [6], aphids [36,49] as well as coleopteran [34,45], or dipteran insects [31] have been demonstrated to belong to the gamma-proteobacterial genus *Rickettsiella* by 16S rRNA gene sequencing.

In addition, RLBs have been reported in terrestrial, freshwater, and marine crustaceans as, for instance, several species of woodlice including the pill bug, *Armadillidium vulgare* [4,50], and the common rough woodlouse, *Porcellio scaber* [1,7], freshwater amphipods [13,29], crayfish [9,40], crabs [2] and shrimps [26,39]. Infections by RLBs of hemocytes and midgut glands (hepatopancreas) appear to be the rule in crustaceans, as is the absence of well-defined protein crystals [13,40] that are a prominent histological feature in *Rickettsiella* infections of insects. Molecular analyses of 16S rRNA sequences have resulted in the assignment of several isopod pathogens to the genus *Rickettsiella* (*Gammaproteobacteria*) [4,52], whereas a crayfish pathogenic RLB has been assigned to the neighboring genus *Coxiella*, giving rise to the introduction of a new species, *Coxiella cheraxi* [3,48]. However, RLBs from further isopods have genetically been identified as members of the taxonomic orders *Rickettsiales* (*Alphaproteobacteria*) [51] or *Chlamydiales*; in particular, the to date only genetic analysis of a supposed *Rickettsiella* pathotype associated with *P. scaber* [7] proved its affiliation to the new chlamydial lineage, '*Candidatus* Rhabdochlamydia' [25].

For the first RLB described in an isopod, i.e. the pill bug, *Armadillidium vulgare* (Latreille, 1804) (Isopoda, Armadillidiidae), the species name *Rickettsiella armadillidii* has been proposed [50], but not formally recognized. Instead, due to the finding of serological cross-reactions between the cricket and woodlouse pathogens [5,35], the pathotype '*R. armadillidii*' has been considered a subjective synonym of the recognized species *R. grylli* [55]. Accordingly, a full genome sequence determined for a RLB from *A. vulgare* has been termed a representation of the *R. grylli* genome. However, partial sequencing of the 16S rRNA genes from RLBs from three different species of woodlice has substantiated the earlier hypothesis that '*R. armadillidii*' and *R. grylli* should rather be considered members of distinct species [4]. Moreover, DNA–DNA hybridization studies [17] revealed a more distant relationship of '*R. armadillidii*' to *R. grylli* in comparison to '*Rickettsiella melolonthae*', a recognized synonym of the type species, *R. popilliae*, resulting in the current assignment of the pathotype '*R. armadillidii*' to this latter taxon [14].

16S rRNA encoding sequences alone have been found to be of limited resolving power for phylogenetic studies at or below the species level [41]. Recently, a comparative genomics approach has identified multilocus sequence analysis (MLSA) schemes for numerous bacterial genera and species, but without considering members of the taxonomic order *Legionellales* [28]. For the genus *Rickettsiella*, a systematic evaluation of possible phylogenetic markers that operate reasonably well at the infra-generic level has revealed a set of MLSA markers consisting of the *gidA* gene encoding glucose-inhibited cell division protein A, the *rpsA* gene encoding the 30S ribosomal protein S1, the *sucB* gene encoding dihydrolipoamide succinyltransferase, and the *ftsY* gene encoding the bacterial homolog of the eukaryotic signal recognition particle receptor subunit alpha involved in protein translocation [30,33]. The *ftsY* gene had previously been identified as the most appropriate marker for the estimation of the G+C content in prokaryotic genomes [16]. This MLSA scheme has recently been employed with *Rickettsiella* pathotypes associated with insects [34,45] and arachnids [32].

In the present study, we used this MLSA approach combined with electron-microscopy, along with 16S rRNA phylogenetic reconstruction and likelihood-based significance testing to elucidate the taxonomic position and status of *Rickettsiella*–like bacteria that infect terrestrial crustaceans, namely the pill bug, *Armadillidium vulgare* (Latreille, 1804) (Isopoda, Armadillidiidae), and the common rough woodlouse, *Porcellio scaber* (Latreille, 1804) (Isopoda, Porcellionidae). For simplicity, these bacteria are henceforth referred to as the pathotypes '*Rickettsiella armadillidii*' and '*Rickettsiella porcellionis*', respectively. The data presented comprise the first ultrastructural together with genetic evidence for *Rickettsiella* bacteria from woodlice.

Materials and methods

Specimens of *Armadillidium vulgare* originated from a bed of ivy on the campus of the University of California at Riverside, CA, USA (sample JKI A174), and those of the common rough woodlouse, *Porcellio scaber*, from a garden at Maintal, Frankfurt/Main region, Germany (sample JKI D244), where alive and dead specimens were found under stones and wooden boards distributed over an area of about 500 m^2 in March, 2012. The bacterial isolates from these woodlice were provisionally named '*Rickettsiella armadillidii*' JKI A174 and '*Rickettsiella porcellionis*' JKI D244, respectively. In dead hypertrophied larvae of both isopod species, infections with *Rickettsiella* bacteria were detected by light and electron microscopy as indicated in Kleespies et al. [24].

Extraction of genomic DNA, PCR amplification of bacterial genetic markers using the oligonucleotide primers and annealing temperatures described in Table 1, and DNA sequence determination and comparison as well as phylogentic reconstruction were performed as described by Schuster et al. [45].

For the reconstruction of a 16S rRNA gene phylogeny, GenBank database entries identified using the BlastN or BlastX software tools were ranked by decreasing maximal identity with the query sequence from '*Rickettsiella armadillidii*' and '*Rickettsiella porcellionis*', with a value of <90% sequence similarity being applied as a cut-off criterion.

Woodlouse 18S rRNA marker sequences were amplified from the same extracted DNA samples using primers F02 and R01 described in Mattern and Schlegel [37] applying the reaction conditions specified therein.

Likelihood-based significance testing of tree topologies was performed by the pairwise one-sided Kishino–Hasegawa (1sKH) test that has been shown to be superior to the original two-sided Kishino–Hasegawa (2sKH) test [23] if evaluated tree topology sets are permutatively incomplete [19]. Candidate tree topologies (Table S1) for significance testing were generated manually in Newick format according to the rationale outlined in Fig. S1. The 1sKH test was performed as implemented in the Tree-Puzzle 5.2 software package applying a 10% significance threshold. For protein-encoding markers, candidate topologies were evaluated against hypervariability filtered nucleotide sequence alignments.

Results

Comparison of amplified 18S rRNA sequences with the orthologs from a set of further isopods unequivocally identified the woodlice from samples A174 and D244 investigated for bacterial infections as members of the species *Armadillidium vulgare* and *Porcellio scaber*, respectively (Fig. S2).

In wet-mount preparations of infected tissues of both woodlice, tiny elementary bodies of the rickettsiellae were observed with