Genetic and electron-microscopic characterization of *Rickettsiella* bacteria from the manuka beetle, *Pyronota setosa* (Coleoptera: Scarabaeidae)

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**Abstract**

Larvae of manuka beetles, *Pyronota spp.* (Coleoptera: Scarabaeidae) cause pasture damage in New Zealand by feeding on the roots of grasses. Surveys for potential biocontrol agents revealed a putative disease, expressed as whitened larvae of one of the outbreak species, *Pyronota setosa*. Microbial diagnosis indicated an intracoelomic, intracellular infection, and intracellular bacteria have been identified with subcellular structures characteristic of infection by *Rickettsiella*-like microorganisms. These bacteria were rod-shaped, often slightly bent with a mean of 628 nm in length and 220 nm in width. Numerous associated protein crystals of variable size and shape occurred within round to oval shaped “giant bodies” either singly or as clusters of smaller crystals. Molecular phylogenetic analysis based on 16S ribosomal RNA and signal recognition particle receptor (FtsY) encoding sequences demonstrates that the manuka beetle pathogen belongs to the taxonomic genus *Rickettsiella*. Therefore, the pathotype designation ‘*Rickettsiella pyronotae*’ is proposed to refer to this organism. Moreover, genetic analysis makes it likely that – on the basis of the currently accepted organization of the genus *Rickettsiella* – this new pathotype should be considered a synonym of the nomenclatural type species, *Rickettsiella popilliae*.

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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 are frequently associated with protein crystals. The current taxonomy of these bacteria (Fournier and Raoult, 2005) is based on a pathotype designation that is partially superposed by the morpho- and serologically based differentiation of three recognized species; these are named according to the pathotype of the respective type strain, i.e. the nomenclatural type species *Rickettsiella popilliae* (Dutky and Goo-den) as well as *Rickettsiella grylli* (Vago and Martoja) and *Rickettsiella chironomi* (Weiser).

Due to their early perception as “rickettsiae of insects” (Wille and Martignoni, 1952), *Rickettsiella* bacteria had originally been assigned to the alpha-proteobacterial order *Rickettsiales* (Weiss et al., 1984) in contrast to an alternative classification in the order *Chlamydiales* that had been considered as well (Federici, 1980). However, based on 16S rRNA sequencing results from a strain of *Rickettsiella grylli* (Roux et al., 1997), the genus *Rickettsiella* has recently been reassigned to the gamma-proteobacterial order *Legionellales* (Garrity et al., 2005). On a genomic basis, this reorganization has been largely confirmed for a different *R. grylli* strain (Leclerque, 2008a) and receives additional support from the determination of 16S rRNA-encoding sequences from further *Rickettsiella* pathotypes, e.g. from ticks (Kurtti et al., 2002), collombola (Czarnecki and Tebbe, 2004), crustaceans (Cordaux et al., 2007), scarabaeids (Leclerque and Kleespies, 2008a), dipteran insects (Leclerque and Kleespies, 2008b). However, further arthropod-associated bacteria originally described as likely *Rickettsiella* pathotypes (Drobne et al., 1999; Radek, 2000) were removed from this taxon and reorganized instead in the candidate genus ‘*Rhabdochlamydia*’ of the order *Chlamydiales* after the respective 16S
rRNA-encoding sequences had been determined (Kostanjsek et al., 2004; Corsaro et al., 2007). The significance of these findings for the monophyly of the genus *Rickettsiella* has been critically discussed (Cordaux et al., 2007; Leclerque, 2008b).

Within the taxonomic family *Coxiellaceae*, several protein-encoding genes have been investigated as possible markers for phylogenetic studies beyond the 16S rRNA gene level, often with limited success (Sekeyová et al., 1999; Leclerque and Kleespies, 2008a,c). The *ftsY* gene, which encodes the bacterial homolog of the eukaryotic signal recognition particle receptor subunit alpha involved in protein translocation, has previously been identified as the most appropriate single gene marker for the estimation of the G + C content in prokaryotic genomes (Fourrier et al., 2006) and as one of the most phylogeny informative and reliable protein-encoding marker genes for phylogenetic studies of the *Legionellales* (Leclerque, 2008a).

Manuka beetles, *Pyronota* spp. Bois, (Coleoptera: Scarabaeidae) are widespread throughout New Zealand where the bright green, diurnal, flying beetles are often seen associated with the flowering manuka shrub (*Leptospermum scoparium*) in summer. Several species are found within the genus, with larvae from several species being reported to cause pasture damage (Thomson et al., 1978; Stewart, 1987). In 2009, a serious outbreak of combined populations have been monitored for the presence of diseases. Some larvae infected by fungi and protozoa have been recovered (Townsend and Jackson, 2008), but recently whitened larvae indicative of the presence of disease were encountered. Preliminary microscopic diagnosis indicated an intracoelomic, intracellular infection of the fat body characteristic of infections by *Rickettsiella*-like bacteria. Here we report on light and electron microscopic studies and the genetic characterization of bacteria present in fat body tissue of diseased *P. setosa* grubs as a new pathotype belonging to the genus *Rickettsiella*, and propose to refer to it as ‘*Rickettsiella pyronotae’.*

2. Materials and methods

In whitened larvae of the manuka beetle, *Pyronota setosa*, derived from new grasslands in New Zealand, infections with *Rickettsiella* bacteria were detected by light and electron microscopy of fat body tissues. For electron microscope studies of ultrathin sections, larval fat-body tissues were fixed in 2.0% osmium tetroxide in veronal buffer (pH 7.2) for 17 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 902 electron microscope (Zeiss, Oberkochen, Germany). To determine the average size of *Rickettsiella* bacteria, negatively stained preparations were examined. A suspension of these bacteria was applied on Formvar-covered grids. After drying, grids were covered with a drop of 2.0% sodium phosphotungstate in *aquad bidest* (pH 7.4) which immediately was soaked off with filter paper. For measurements of the associated crystals, ultrathin sections have been used. Micrographs taken with a CCD camera Txs sharp:eye (Tromede, Moorenwies, Germany) served for measurements by using “Software ImageSP” of the same company.

Genomic DNA of bacterial pathogens was extracted from two individual *Pyronota setosa* third instar larvae (from the West Coast region of the South Island, New Zealand), by a DNeasy Kit protocol (Qiagen). PCR amplification of almost complete 16S rRNA encoding sequences was performed from the extract with primers fD1 and rP2 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 containing an appropriately sized product as determined by agarose gel electrophoresis were purified by passage over a Qiagquick PCR purification column (Qiagen) and sequenced on both strands using the fluorescence-labeled dideoxynucleotide technology with primers fD1, rP2, 522f and 760r (Weisburg et al., 1991). PCR amplification and determination of the complete *ftsY* gene sequence with degenerate primers ftsYF (5′-AGTTTNNCCNCCNCCAYTGNCCNCC) and ftsYR (5′-AGRTCRAANCNCCNCCRAACCAACC) differed from the above protocol by the annealing temperature being 52 °C and the elongation time 90sec. Raw sequence data were analyzed and combined into consensus sequences using the DNA Strider 1.3 program. Orthologous sequences available in the GenBank1 database were identified with the BlastN or BlastX software tools (Altschul et al., 1997) in searches over both single sequence entries and the *Rickettsiella grylli* genome project. Identified sequences assignable to a described pathotype or recognized species were retained for phylogenetic analysis together with the orthologous genes from further taxonomically related or arthropod-associated bacteria.

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 or a Gontet protein weight matrix, respectively. The TREE-Puzzle 5.2 software (Schmidt et al., 2002) was used to estimate data set specific parameters. The most appropriate models of DNA sequence evolution were chosen according to the rationale outlined by Posada and Crandall (1998). From nucleotide sequence alignments, organism 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); protein-encoding nucleotide data were filtered by systematic suppression of third codon positions. Additional Neighbor Joining (NJ) and Minimum Evolution (ME) phylogenies were reconstructed in MEGA 4 from unfiltered nucleotide sequence data under, respectively, the MCL (Tamura et al., 2004) and the K2P (Kimura, 1980) model of nucleotide substitution. With both the NJ and ME models of phylogenetic reconstruction, sites of synonymous substitutions were filtered out from protein-encoding DNA sequence alignments applying the Jukes–Cantor corrected modified Nei–Gojobori method (Nei and Gojobori, 1986).

From amino acid sequence data, organism phylogenies were reconstructed using the JTT (Jones et al., 1992) model of substitution with the ML, NJ, and ME methods. In all cases, a F− distribution based model of rate heterogeneity (Yang, 1993) allowing for eight rate categories was assumed. Tree topology confidence limits were explored in non-parametric bootstrap analyses over 1000 pseudo-replicates. Consensus tree topologies were generated by means of the Consense module of the *Phylib* 3.6 software package (Felsenstein, 2004). A pairwise p-distance matrix for the aligned 16S rRNA genes was constructed in the MEGA 4 program under pairwise deletion of alignment gaps and missing data; the distance values obtained for sequences AM490937-9 that show only 68% sequence coverage with the remaining 16S sequences, were corrected accordingly.

3. Results and discussion

Microscopic examination of the infected *P. setosa* larvae revealed no apparent abnormalities in the alimentary tract and an

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1 GenBank accession numbers for new sequences reported here are HM017957 (16S rRNA gene) and HQ589037 (ftsY).

were replaced by masses of bacteria (Figs. 2 and 3). Most striking
areas of relatively dense stroma had been formed which finally
istic features of
were destroyed. As shown in Fig. 2, thin sections display character-
ment. By disruption of infected cells, large areas of fat body lobes
ically showed the tiny bacteria dancing in rapid Brownian move-
phase contrast, squash preparations of infected fat body cells typ-
and with myriads of pathogens floating in the hemolymph. In
of bacteria and associated crystals in hypertrophied fat body cells
vae were in a late state of infection with massive accumulations
Ultrathin section of infected fat body of
Fig. 1.
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Fig. 1. Electron micrograph of Rickettsiella bacteria (R) isolated from fat body tissue of
P. setosa, negatively stained with sodium phosphotungstate. Note: Bacteria are rod-shaped, with more than half of them being slightly bent (arrows).
Fig. 2. Ultrathin section of infected fat body of
P. setosa in a progressing stage of bacterial multiplication. Rickettsiella bacteria (R) are often observed in vesicles (V) and finally are also replacing areas of relatively dense stroma material (S). Giant bodies (G) transforming into associated crystals (C) are also shown.

in ultrathin sections were round to oval shaped “giant bodies”,
most of them carrying either one large compact crystal or a cluster
of smaller crystals (Figs. 2 and 3) Such associated crystals are
known from other diseases caused by pathogens of the Rickettsiella
group.

Rickettsiella bacteria were rod-shaped, with more than half of
them being slightly bent (Fig. 1). In measurements of negatively
stained preparations (n = 100), their length ranged from 535 to
834 nm with a mean of 628 nm (SD = 55.6 nm), and their width
from 173 to 277 nm with a mean of 220 nm (SD = 19.7 nm). Assoc-
ciated crystals were of variable size and shape ranging from 978 to
2477 nm in length with a mean of 1611 nm (SD = 339.0 nm), and
from 427 to 2071 nm in width with a mean of 1093 nm (SD = 256.3 nm) (n = 50).

Taken together, the histopathological, cytopathological, and
morphological features revealed by microscopic investigation of
diseased manuka beetle larvae are consistent with and indicative
of infection by Rickettsiella-like bacteria.

The 1357 nucleotides comprising the 16S rRNA gene sequences
determined for the bacteria from each of the infected manuka bee-
tle larvae were found to be 100% identical. When this consensus
sequence was used as the BlastN query, 16S rRNA gene sequences
from the Rickettsiella pathotypes ‘R. melolonthae’ and ‘R. tipulae’
(GenBank accession numbers EF408231 and EU180598, respect-
ively) were identified as the two best hits (99% sequence identity
covering 100% of the query sequence). Moreover, a number of 16S
rRNA encoding sequences from further Rickettsiella-like bacteria
displayed identity values in the range from 99% to 94%. Most of
these sequences have been identified from biodiversity studies and
are associated with a diverse range of invertebrates, including:
the collembola, Folsomia candida (AF327558); the antlion, Myrme-
leon bore (AB291637); the earthworm, Eisenia fetida (FJ542982);
the ground beetle, Poecilus chalcites (EF608533); the cricket, Gryllus
bimaculatus (U97547); different isopods (AY447041, AM490937-
39); ixodes ticks (AF383621, GQ857549); and the psyllid, Cec-
idiotrioza sozanica (AF286124).

Consistently, the Maximum Likelihood phylogeny (Fig. 4)
reconstructed from the alignment of 16S rRNA genes from 23 se-
lected bacterial genomes firmly places the Pyronota setosa
associated bacterium among the described gamma-proteobacterial
Rickettsiella pathotypes. With the respective Rickettsiella clade
receiving optimal bootstrap support, the new bacterial specimen
was clearly distinct from other groups of arthropod-associated prok-
yarotes such as (i) gamma-proteobacterial endosymbiotic Buch-
nera and Wigglesworthia, (ii) alpha-proteobacterial pathogenic
Rickettsia and endosymbiotic Wolbachia and (iii) chlamydial
arthropod pathogens now described as ‘Candidatus Rhabdoclamy-
dia’. The exact overall topology of the ML tree as well as the 100%
bootstrap support value for the root of the Rickettsiella clade
are reproduced in both the Minimum Evolution and Neighbor-Joining
phylogenies (data not shown). We therefore propose to refer to
the new specimen as pathotype ‘Rickettsiella pyronotae’.

In order to determine more exactly the taxonomic position of ‘R.
pyronotae’ within the genus Rickettsiella, phylogenetic reconstruc-
tion was performed on the basis of the 16S rRNA encoding se-
quences from Rickettsiella-like organisms identified from GenBank.
The tree topologies obtained using ML (Fig. 5) as well as
ME and NJ (not shown) coincide in grouping ‘R. pyronotae’ to-
gether with two synonyms of the species R. popilliae, namely the
pathotypes ‘R. melolonthae’ and ‘R. tipulae’, as well as with Rickett-
siella bacteria associated with the antlion, Myrmeleon bore, and
the ground beetle, Poecilus chalcites. However, with any of the methods
of phylogenetic reconstruction employed, the bootstrap support
value for this clustering (ML: 86%; ME: 65%; NJ: 77%) is unconvinc-
ing, and the considerable divergence in the overall ML, ME, and NJ
tree topologies underlines that species delineation within the
genus *Rickettsiella* remains problematic on the basis of currently available sequence data. In view of the inapplicability of the classical species concept to asexual organisms, the degree of similarity of orthologous nucleotide sequences from different organisms is widely used as an indicator of taxonomic relationships. Whereas Stackebrandt and Goebel (1994) have proposed a 16S rRNA gene sequence identity of 97% as a general threshold value for prokaryotic species delineation, diverging values are accepted for different groups of prokaryotes. Within the order *Chlamydiales*, for instance, 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). In the present study, the relatedness of the 16S rDNA gene sequence from *R. pyronotae* to orthologous sequences from other *Rickettsiella*-like bacteria has been estimated from a pairwise *p*-distance matrix calculated from the sequence alignment underlying the phylogenetic tree presented in Fig. 5 together with the respective sequence identity percentages. The pathotype ‘*R. pyronotae*’ appears with >99% sequence identity most closely related to a group of *Rickettsiella* bacteria including two synonyms of the species *R. popilliae*. With <98% sequence identity, ‘*R. pyronotae*’ is found to be less clo-

Fig. 3. Ultrathin section of fat body of *P. setosa* in a late stage of infection, stuffed with masses of *Rickettsiella* bacteria (R) together with numerous giant bodies (G) that either contain a single crystal (C) or clusters of smaller crystals (C₁, C₂, C₃, C₄).

Fig. 4. Phylogenetic tree reconstructed from bacterial 16S rRNA encoding sequences by the Maximum Likelihood (ML) method. Terminal branches are labeled by either taxonomic genus and species or pathotype designations together with strain identifiers and GenBank accession numbers (with numbers reading “NC” referring to whole genome sequences). Numbers on branches indicate bootstrap support values superior to 50%; an asterisk (*) denotes optimal (100%) bootstrap support. The tree has been outgroup rooted by the ‘*Candidatus Rhabdochlamydia*’ branch. The bar indicates a 5% relative sequence divergence.

spon of the pathotype ‘Rickettsiella armadillidi’; the latter has been proposed to represent an additional Rickettsiella species (Cordaux et al., 2007; Leclerque and Kleespies, 2008b), but has not been recognized as such to date. This situation suggests a synonymization of the pathotype ‘R. pyronota’ to the species R. popilliae.

Amplification of the complete ftsY marker from ‘R. melolon-thae’, ‘R. tipulae’, and ‘R. pyronota’ generated a 996 bp comprising nucleotide sequence for each of these pathotypes. An ftsY gene sequence of 990 nucleotides has been identified in the R. grylll genome, whereas the orthologous genes from Coxella burnetii and Legionella pneumophila comprise 975 bp and 1068 bp, respectively. Estimation of the genomic G + C content following the approach of Fournier et al. (2006) gives a calculated G + C content of 37.3% for ‘R. pyronota’ that is very similar to the corresponding values for ‘R. melolon-thae’ (37.3%) and ‘R. tipulae’ (37.2%) and lower than the 39.2% G + C content calculated from the ftsY gene of Rickettsiella grylll; however, the real G + C content obtained from the R. grylll genome sequence has been found to be 37%. All these values are well within the range of genomic G + C contents (36–41%) predicted for Rickettsiella bacteria on the basis of DNA hybridization studies (Frutos et al., 1994). In contrast, the ftsY gene sequences of C. burnetii and L. pneumophila give rise to a considerably higher calculated G + C content of 42.5% for both organisms.

The complete peptide (331aa) deduced from the ‘R. pyronota’ ftsY gene displays highest amino acid sequence identity to the deduced FtsY peptides from ‘R. melolon-thae’ (95.5%) and ‘R. tipulae’ (95.2%) and considerably lower degrees of sequence identity to the complete FtsY peptide sequences from R. grylll (72.4%), C. burnetii (56.9%), and L. pneumophila (49.5%). Consistently, the ML phylogeny reconstructed from the central 230aa of these FtsY proteins, i.e. mainly after removal of little conserved N-terminal signal peptides, is characterized by both (i) an optimally bootstrap supported Rickettsiella clade and (ii) an equally well supported and rather divergent subclade that comprises the new pathotype ‘R. pyronota’ together with the recognized R. popilliae synonyms, ‘R. melolon-thae’ and ‘R. tipulae’, but excludes the R. grylll genome project strain (Fig. 6A). These two topological structures – a Rickettsiella clade and a presumed R. popilliae subclade comprising ‘R. pyronota’ – are present in all nine tree topologies that have been obtained from FtsY peptide as well as unfiltered and filtered ftsY gene sequence alignments by the ML, NJ, and ME methods of phylogenetic reconstruction from FtsY peptide as well as unfiltered and filtered ftsY gene sequence alignments; ftsY nucleotide sequences were analyzed both unfiltered and under synonymous substitution filtering. Ratios on branches indicate in how many of the nine original trees the respective branch has been conserved.
phylogenetic reconstruction and that are presented in Fig. 6B in the aggregated form of a Majority Rule consensus tree topology.

Thus, despite the limited comparative sequence data available, the results obtained with the fitsY gene marker clearly and fully support the conclusions drawn from the analysis of 16S rRNA gene sequences, namely (i) assignment of the new bacterial pathogen from Pyronota festiva to the bacterial genus Rickettsiella and (ii) its co-assignment with the pathotypes ‘R. melolonthae’ and ‘R. tipulatue’ that have in turn been placed in synonymy with the species R. popilliae.

4. Conclusions

In conclusion, the results of the present study identify a new bacterial pathogen associated with grubs of Pyronota setosa, which belongs to the taxonomic genus Rickettsiella (Gammaproteobacteria, Legionellales) and therefore represents the new pathotype, ‘R. pyrnonata’. Moreover, keeping in mind the caveat related to the problematic current status of species delineation within the genus Rickettsiella, the results presented motivate the classification of ‘R. pyrnonata’ as a synonym of the nomenclatural type species, Rickettsiella popilliae.

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