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Short Report: Identification of a Novel Uncultured *Rickettsia* Species Strain (*Rickettsia* Species Strain Tselenti) in Cyprus

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Abstract. In a previous study conducted in Cyprus, various spotted fever group *Rickettsia* species were detected and identified in ticks by molecular analysis. Among them, a partially characterized *Rickettsia* species was detected in *Hyalomma anatolicum excavatum* and *Rhipicephalus turanicus* ticks. We report characterization of this rickettsial strain by using polymerase chain reaction sequencing analysis of partial citrate synthase A, outer membrane protein A, outer membrane protein B, and 17-kD protein genes. We propose a provisional name *Rickettsia* sp. strain Tselenti for this strain until it is isolated and further characterized.

INTRODUCTION

Rickettsia spp. are obligate intracellular arthropod-borne bacteria that cause mild to severe diseases in humans and other vertebrate animals worldwide.[1–3] Several controversial theories have been suggested for the taxonomic classification of rickettsiae. A widely accepted taxonomic scheme for classification of rickettsiae at the genus and species levels is based on molecular methods; sequencing of genes for 16S ribosomal RNA (*rrs*), citrate synthase A (*gltA*), outer membrane protein A (*ompA*), *ompB*, surface cell antigen 4 (*sca4*), and the 17-kD common antigen.[4–6] However, other methods have been proposed.

One aspect of taxonomic classification was reported by Gillespie and others in examining rickettsial genes belonging to the core genome of rickettsial genomes.[7,8] They proposed the transitional group of rickettsiae as an intermediate evolutionary stage between the ancestral group and the typhus group and the spotted fever group (SFG). Perlman and others[9] and Weinert and others[10] reported the influence of hosts, types of transmission, and habitat transitional events in the rickettsial evolutionary pathway, and created a robust phylogenetic tree of the genus.

Except for well-known rickettsial diseases (epidemic typhus caused by *R. prowazekii*, Rocky Mountain spotted fever caused by *R. rickettsii*, Mediterranean spotted fever caused by *R. conorii*, and murine typhus caused by *R. typhi*), at least 10 other rickettsial diseases have been described and recognized in the past 20 years, many of which have been attributed to *Rickettsia* species within the SFG.[1,3] The number of pathogenic rickettsiae within the SFG has increased in recent decades and they are recognized as emerging pathogens worldwide. Seventeen distinct species within this group are currently recognized as human pathogens, causing the so-called tick-borne rickettsioses, which show clinical features such as fever, headache, rash, lymphadenitis, and occasional eschar formation at the site of the tick bite.[3] Some species have been isolated from ticks, a large number of species have been recognized or are partially characterized as *Rickettsia* spp., and most species have been detected in arthropods, but not yet associated with disease.[1–3]

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In Cyprus, three rickettsial species have been detected: *R. conorii*, *R. typhi*, and *R. felis*.[11–15] In addition, SFG *Rickettsia* spp. have been detected by molecular methods in ticks and their animal hosts.[16,17] In a recent study, five rickettsial species (*R. aeschlimannii*, *R. massiliae*, *R. sibirica mongolotimonae*, *R. hoogstraalii*, and "*Candidatus* R. barbariae") were detected and genetically characterized by using polymerase chain reaction (PCR) sequencing analysis in naturally infected hard ticks (n = 3,950) collected from domestic and wild animals.[18]

Some *Rickettsia* species PCR-amplified DNA sequences have been compared with those of published *Rickettsia* spp.[18] We detected a *Rickettsia* species in *Rhiphicephalus* ticks (11 of 805, infection rate = 1.3%), collected from goats, hares and, sheep and in *Hyalomma anatolicum excavatum* ticks (5 of 301, infection rate = 1.6%) collected from sheep.[18] In the current study, we report characterization of this rickettsial strain by PCR sequencing analysis of portions of the *gltA*, *ompA*, *ompB*, and 17-kD genes.

Tick DNA extracts that were obtained during our previous study were stored at –20°C for further processing.[18] The Rr19070p-Rr190602n, targeting a 532-basepair portion of the *ompA* gene; BG1-21/BG2-20, targeting a 650-basepair portion of the *ompB* gene; and 17kdf/17kdr, targeting a 434-basepair portion of the 17kDa protein gene.[19–22] The four sets of primers were used in each of the 16 samples of the unrecognized *Rickettsia* sp. (total = 64 amplicons). Amplicons were purified using by the QIAquick PCR Purification Spin Kit (QIAGEN, Hilden, Germany).

Sequencing was performed twice by using the described primers in a CEQ 8000 Beckman Coulter Sequencer (Bioanalytica Genotype, Athens, Greece). The BLAST algorithm (National Center for Biotechnology Information, Bethesda, MD) (http://www.ncbi.nlm.nih.gov/BLAST) was used to test homology against known *Rickettsia* species. Product sequences were aligned by using ClustalW (http://www.clustal.org/) IUB DNA weight matrix with default pairwise and multiple parameters. Accession numbers were obtained from GenBank. Phylogenetic analysis was performed using the DAMBE version software package for data analysis in molecular biology and evolution.[3] Tree-building parameters were analyzed by using the neighbor-joining together method and a bootstrap test of 1,000 replicates.[23]

All *gltA* amplicons (358 basepairs) showed 99.72% (357 of 358 basepairs) sequence homology with *Rickettsia* species RR01 (GU056205), *R. rhipicephali* strain HJ5 (DQ865206), "*Candidatus* R. Kulagini" strain Kertch (DQ365806), and

R. rhipicephali 3-7-6 (U59721). The *gltA* amplicon was altered by a single amino acid (H282Y–DQ365806 numbering) when compared with *Rickettsia* species RR01 (GU056205). All partial *ompA* amplified sequences (510b basepairs) showed 99.21% (506 of 510 basepairs) homology with *Rickettsia* species ZJ43/2007 (EU258735). In this case, there was a nucleotide alteration, 14C > T, and a triplet insertion, 240^241insGAT, giving an amino acid alteration, S5F, and insertion D80^G81insD, respectively (EU258735 numbering). The *ompB* gene PCR products (616 basepairs) showed 99.83% (615 of 616 basepairs) sequence homology with *Rickettsia* species TwKM01 (EF219464).

The difference between the two sequences was made only on the basis of nucleotides because the single base mutation (1248 G > A) is a silent mutation (A416A) (EF219464 numbering). The 399-basepair nucleotide sequence of the 17-kD gene showed 99.24% (396 of 399 basepair) similarity with *R. massiliae* strain MTU5 (CP000683) and 99.24% (395 of 398 basepair) similarity with *R. rhipicephali* strain HJ5 (DQ865207). Nucleotide sequences compared with those of *R. massiliae* strain MTU5 differed at three nucleotide positions (225 A > G, 256 G > A, and 458 G > A), resulting in three amino acid mutations (I75M, A86T, and G153E, respectively).

Phylogenetic analysis placed each gene differently in clusters of related phylogenetic trees, making it difficult to determine accurately the species of *Rickettsia* detected. On the basis of the *gltA* and *ompB* amplicon sequences, the new species was placed within the branch of the *R. rhipicephali* group. Conversely, on the basis of the *ompA* and 17-kD amplicons, this *Rickettsia* species seems to be closely related the *R. rhipicephali–R. massiliae* group, but it forms a separate evolutionary branch. Thus, for a more accurate classification of the organism, the four gene sequences were concatenated for the part of the sequences corresponding to the amplicons length. Phylogenetic analysis placed the new strain in a branch next to *Rickettsia* species TwKM01 and was supported by a strong bootstrap value (Figure 1).

According to genotypic criteria and *Rickettsia* species definition,[5] the species analyzed in this study was difficult to assign to one of the valid *Rickettsia* species. Under these conditions and until isolates are obtained and clearly characterized, we suggest that the species detected in Cyprus be provisionally named *Rickettsia* species strain Tselenti.

This new *Rickettsia* strain seems to belong to the SFG, which constitute a phylogenetically distinct clade of rickettsiae composed of more than 30 recognized and validated rickettsial species. Seveteen sequences of *Candidatus* species are in the process of validation (National Center for Biotechnology Information). In addition, more than 150 non-characterized *Rickettsia* species, most of whose pathogenicity to humans or animals is unknown, have been deposited and await further identification and description.

Current and past studies[18] had detected other SFG rickettsiae in Cyprus, in addition to those previously identified (*R. conorii*, *R. typhi*, and *R. felis*).[12] We report a new strain of *Rickettsia* species that has been characterized and classified as belonging to the SFG. Further investigations are warranted to determine if this organism causes disease to humans. The disease ecology of SFG in Cyprus is complex and needs further research.

GenBank accession numbers of genes sequenced in this study are EU448155, EU448156, EU448157, EU448158, EU448159, EU448160, JF803898, JF803899, GU353188, and GU353185.

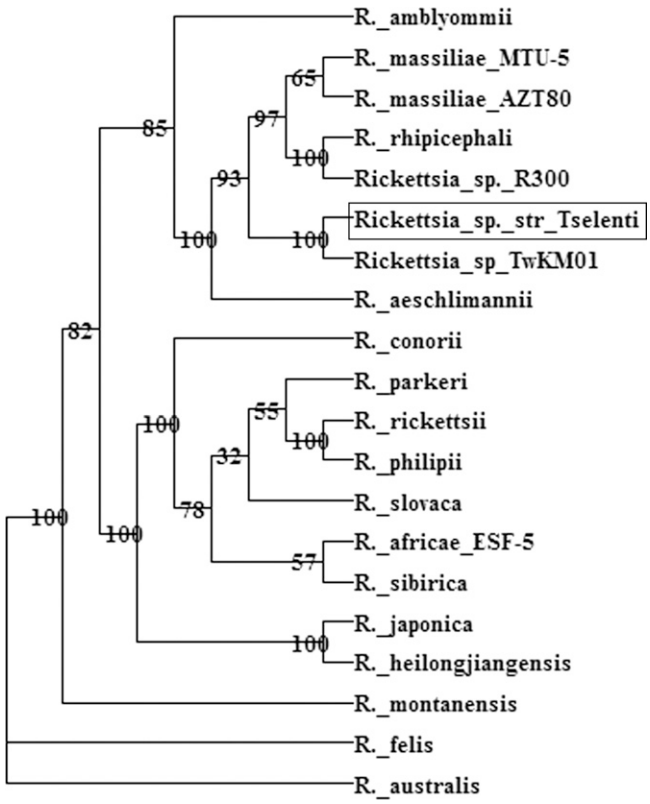

FIGURE 1. Evolutionary relationships of four concatenated genes for citrate synthase A, outer membrane protein A, outer membrane protein B, and 17-kD protein of *Rickettsia* species strain Tselenti. Box indicates sequences of the new *Rickettsia* species. Evolutionary history was inferred by using the neighbor-joining method with bootstrap testing (1,000 replicates) with DAMBE version 5.2.68 software.[23]

Six additional (three for *ompB* and three for 17-kD) accession numbers are still pending.

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