Genetic Classification of “Rickettsia heilongjiangii” and “Rickettsia hulinii,” Two Chinese Spotted Fever Group Rickettsiae

J. Z. ZHANG,1,2 M. Y. FAN,3 Y. M. WU,3 P. E. FOURNIER,1 V. ROUX,1 AND D. RAOUULT1*

Unité des Rickettsies, Faculté de Médecine, CNRS UPRES-A6020, 13385 Marseille Cedex 05, France,1 and Department of Rickettsiology, Institute of Epidemiology & Microbiology, Chinese Academy of Preventive Medicine, Beijing 102206,2 and Department of Microbiology, Institute of Military Medicine, Shenyang Military Command, Shenyang 110034,3 Peoples Republic of China

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To determine the phylogenetic position of two new rickettsial strains isolated from ticks in China, 16S ribosomal DNA, gltA, and ompA (apart from the tandem repeat units) genes were amplified by PCR and sequenced. The phylogenetic relationships between these strains and other rickettsiae were inferred from the comparison of sequences of the three genes by the parsimony, neighbor-joining, and maximum-parsimony methods. The results demonstrated that the 054 strain, a rickettsia pathogenic in humans, and the HL-93 strain were related and clustered together with Rickettsia japonica. Significant statistical bootstrap values (100 and 92%) supported the nodes in this cluster. Based on previous genotypic and antigenic data and the phylogenetic analysis presented here, the 054 and HL-93 strains should be considered as new species, and we formally propose that they be named “Rickettsia heilongjiangii” and “Rickettsia hulinii,” respectively.

The advent of new culture techniques, such as the shell vial-centrifugation technique (16), and the detection of rickettsial species detected (20). Before 1984, only six spotted fever group (SFG) rickettsioses were recognized in the world (20): Rocky Mountain spotted fever caused by Rickettsia rickettsii, Mediterranean spotted fever caused by Rickettsia conorii, Siberian tick typhus caused by Rickettsia tsutsugamushi, Queensland tick typhus caused by Rickettsia australis, rickettsialpox caused by Rickettsia akari, and Israeli spotted fever caused by Israeli tick typhus rickettsia. Over the last 12 years, eight new rickettsioses have been reported, including Japanese spotted fever caused by Rickettsia japonica, Flinders Island spotted fever caused by Rickettsia honei, Astrakhan fever caused by Astrakhan fever rickettsia, African tick bite fever caused by Rickettsia africana, California flea typhus caused by the ELB agent, and three unnamed spotted fevers caused by “Rickettsia slovaca,” Rickettsia helvetica, and Rickettsia hulinii. In China, 20 strains of tick-associated rickettsiae, belonging to at least five serotypes, have been isolated over the last few years, including R. sibirica; the BJ-90 isolate (42); the Heilongjiang isolate (strain 054, also called “Rickettsia heilongjiangii”), the Inner Mongolian isolate HA-91, or “R. mongolotimonae,” also found in France; and the Hulin isolate (strain HL-93, also called “Rickettsia hulinii”) (9, 19, 33). Three of these rickettsiae, R. sibirica, “R. mongolotimonae,” and “R. heilongjiangii,” are human pathogens. “R. heilongjiangii” was first isolated in 1982 from Dermacentor silvarum ticks collected in SuiFenhe, a city of Heilongjiang Province in China (Fig. 1). Between May and July 1988, 12 patients in Chunhua, Huichun County, Jilin Province, were reported to be infected by this strain on the basis of serological results. All patients had a history of fever, headache, rash, eschar, regional lymphadenopathy, and conjunctivitis following a tick bite, with fourfold rises in complement fixation antibodies in convalescent-phase sera (14). Between May and June 1996, seven patients with clinical manifestations of SFG rickettsial infections were found in SuiFenhe and Dongning, in Heilongjiang Province. Isolates from these patients were found to be identical to the 054 strain by microimmunofluorescence and PCR plus restriction fragment length polymorphism (30). The “R. hulinii” strain was first isolated in 1993 from Haemaphysalis concinna ticks collected in Hulin County, Heilongjiang Province (Fig. 1), and was considered to be a unique SFG rickettsia (38). To date, the pathogenicity of this strain has been experimentally demonstrated in animals (38), but not in humans. In order to estimate the phylogenetic classification of strains 054 and HL-93, we sequenced their 16S rRNA (16S ribosomal DNA [rDNA])- citrate synthase (gltA), and rOmpA (ompA)-encoding genes and compared these sequences with those of other SFG rickettsiae.

Strains 054 and HL-93 were cultivated and purified as previously described (12), and their DNA was extracted from purified organisms with the QIAmp tissue kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. PCR amplification and sequencing reactions were performed with the oligonucleotide primers mentioned below. All primers were purchased from Gibco BRL (Custom Primers, Life Technologies Sarl. B.P., Cergy Pontoise Cedex, France), and amplifications were carried out in a PTC-200 DNA thermal cycler (MJ Research, Inc., San Francisco, Calif.) under conditions previously described (11, 22–24). R. japonica and distilled water were used as a positive and negative control, respectively. Each amplicon obtained was purified for sequencing with a QIAquick Spin PCR purification kit; Perkin-Elmer) as described by the manufacturer’s instructions. Sequencing reactions were carried out with the Dye Terminator kit (d-rhodamine terminator cycle DNA sequencing kit; Perkin-Elmer) as described by the manufacturer. Sequencing reaction products were resolved by electrophoresis with an ABI Prism 377 Sequencer (Perkin-Elmer). The results obtained were processed into sequence data by using the Se-
sequence Navigator and AutoAssembler software. Each base position was established at least three times in both the forward and reverse directions. Sequences of the 16S rDNA, \( \text{gltA} \), and \( \text{ompA} \) genes were aligned by using the multisequence alignment program CLUSTAL, within the BISANCE environment (7). Phylogenetic relationships between strains 054 and HL-93 and other SFG rickettsiae were inferred by using version 3.4 of the PHYLIP software package (10). The distance matrices generated by DNADIST were determined under the assumptions of Kimura (13) and were used to infer dendrograms by the neighbor-joining method (25). Two other dendrograms were constructed by data processing with the maximum-likelihood and parsimony programs in PHYLIP. A bootstrap analysis based on 100 randomly generated trees by using SEQBOOT and CONSENSE in PHYLIP was performed to estimate the node reliability of the trees obtained by the three phylogenetic methods (4).

The primers fD1 and rP2 amplified 1,463- and 1,461-bp fragments of the 16S rDNA gene from strains 054 and HL-93, respectively. The \( \text{gltA} \) gene was amplified in two fragments. Overall, sequences of 1,238 and 1,237 bp were obtained from the O54 and HL-93 strains, respectively. PCR amplification and sequencing of the \( \text{ompA} \) gene excluded the central tandemly repeat region. The 5' end fragment of the gene was 611 bp for both the 054 and HL-93 strains. The 3' part of the gene was amplified in four fragments. Overall, sequences of 3,187 and 3,181 bp were obtained from strains 054 and HL-93, respectively. The sequenced fragments of the rOmpA gene were analyzed from base positions 91 to 680 and 3608 to 6789 with respect to the sequence published for \( R. \text{rickettsii} \) (2) and were therefore submitted to GenBank as two separate fragments. Strains 054 and HL-93 clustered together with \( R. \text{japonica} \) into a well-defined, strongly supported monophyletic group within the SFG rickettsiae, with bootstrap values of 91 and 100% for the node where strains and HL-93 branched together in the \( \text{gltA} \) and \( \text{ompA} \) trees, respectively, and values of 92 and 100% for the node where both strains clustered with \( R. \text{japonica} \). Similar phylogenetic organizations were inferred from the analysis of all three genes by using the three phylogenetic analysis methods (Fig. 2).

Historically, the classification and identification of rickettsiae have been based on differences in their epidemiology, serology, and intracellular growth characteristics. Because different species may have common ecological features (e.g., geographic distribution, arthropod vectors, etc.) and due to serological cross-reactivity, it is difficult to distinguish between rickettsiae. Moreover, DNA-DNA hybridization, a criterion used for the definition of species in the family \( \text{Enterobacteriaceae} \), is not applicable to SFG rickettsiae (8). Other recognized tools for the taxonomic classification of bacteria, such as the study of protein profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, monoclonal antibodies, Western blotting, and hybridization of labeled cloned DNA probes (1, 3, 17, 21, 31, 32, 34), are not widely applicable and lack interlaboratory reproducibility. Therefore, for over 10 years, the reference method for the identification of members of SFG rickettsiae has been the indirect microimmunofluorescence serologic typing test with mouse sera (18). Recently, phylogenetic analyses and the determination of taxonomic relationships based on comparisons of sequences of a combination of various genes have greatly facilitated the study of rickettsial taxonomy. The 16S rDNA (23, 26, 28), \( \text{gltA} \) (24), and \( \text{ompA} \) (11, 22) genes appear to be the most useful sequences. In our study, the 054 and HL-93 strains demonstrated \( \text{ompA} \) sequence homologies of 98.1% with each other and 96.6 and 95.6% with \( R. \text{japonica} \), respectively, which are higher than...
ettsia are the first showing isolates clustering with grouped into a well-supported cluster in the phylogenetic trees species (Fig. 2). Strains 054 and HL-93 and from our study show that strains 054 and HL-93 are distinct (94 and 90%, respectively). Although closely related, the data GenBank accession numbers are AF172942 and AF178037 for *Rickettsia hulinii*, respectively. *Rickettsia* strains should be strongly considered as members of new spe-
vious genotypic and antigenic studies and on the phylogenetic alternative genomic tools. However, based on data from pre-
on serotyping (11), and, to date, there is no consensus on Japan and in China. The current rickettsial taxonomy is based main islands. The island is located between 132° to 135° East

those observed for strain HL-93 with *R. rickettsii* or *R. conorii* (94 and 90%, respectively). Although closely related, the data from our study show that strains 054 and HL-93 are distinct species (Fig. 2). Strains 054 and HL-93 and *R. japonica* were grouped into a well-supported cluster in the phylogenetic trees developed by the three analysis methods (Fig. 2). These results are the first showing isolates clustering with *R. japonica* and are in concordance with previous phenotypic and genotypic studies (5, 6, 15, 29, 30, 35–41; L. Chen, J. Z. Zhang, and D. Z. Bi, Program Abstr. EUWOG-ASR Joint Meeting, p. 12, 1999). Tick-associated rickettsiae are characterized by limited, specific geographic distribution areas (11). *R. japonica* was first isolated on Shikoku Island (27), the smallest of Japan’s four main islands. The island is located between 132° to 135° East and 33° to 35° North. To the north is the Seto Inland Sea, while to the south is the Pacific Ocean. Heilongjiang Province of China, where the 054 and HL-93 strains were isolated, is located between about 130° to 140° East and 45° to 50° North. This suggests that an SFG rickettsia may have evolved separa-
ritely in this area of the world and diverged more recently into Japan and in China. The current rickettsial taxonomy is based on serotyping (11), and, to date, there is no consensus on alternative genomic tools. However, based on data from previous genotypic and antigenic studies and on the phylogenetic analysis presented here, we believe that the 054 and HL-93 strains should be strongly considered as members of new spe-
pecies, and we formally propose that these be named “*Rickettsia heilongianguai*” and “*Rickettsia hulinii***,” respectively.

**Nucleotide sequence accession numbers.** The 16S rDNA GenBank accession numbers are AF172942 and AF178037 for 054 and HL-93, respectively. The *gltA* accession numbers are AF178034 and AF172943 for 054 and HL-93, respectively. The *ompA* accession numbers are AF179362 and AF179364 for the 5’ fragments of 054 and HL-93, respectively, and AF179363 and AF179366 for the 5’ fragments of 054 and HL-93, respectively.

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