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# Genotypic Identification of Rickettsiae and Estimation of Intraspecies Sequence Divergence for Portions of Two Rickettsial Genes

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**DNA sequences from specific genes, amplified by the polymerase chain reaction technique, were used as substrata for nonisotopic restriction endonuclease fragment length polymorphism differentiation of rickettsial species and genotypes. The products amplified using a single pair of oligonucleotide primers (derived from a rickettsial citrate synthase gene sequence) and cleaved with restriction endonucleases were used to differentiate almost all recognized species of rickettsiae. A second set of primers was used for differentiation of all recognized species of closely related spotted fever group rickettsiae. The procedure circumvents many technical obstacles previously associated with identification of rickettsial species. Multiple amplified DNA digest patterns were used to estimate the intraspecies nucleotide sequence divergence for the genes coding for rickettsial citrate synthase and a large antigen-coding gene of the spotted fever group rickettsiae. The estimated relationships deduced from these genotypic data correlate reasonably well with established rickettsial taxonomic schemes.**

Multiple species of spotted fever group (SFG) and typhus group rickettsiae are recognized (41). Although different species within each group may share certain common features of ecologic interest (e.g., geographic distribution, common arthropod vectors) and share considerable serologic cross-reactivity, these species differ in ability to cause human disease. To deduce correctly the natural histories of these specialized bacteria, as well as to determine accurately their prevalence, it is necessary to identify naturally occurring rickettsiae reliably. Such identification is required, for example, when studying the relation between isolates obtained from humans and vectors of rickettsial disease.

However, methods currently available for identification of rickettsial species have technical limitations. Serologic identification of rickettsial isolates is complicated by substantial cross-reactivity between recognized species. Specially prepared mouse polyclonal antisera and mouse monoclonal antibodies have been used to differentiate selected species (4, 9, 17, 28), and immunologic relationships among SFG rickettsial species have been determined by serologic neutralization of toxic effects of rickettsial suspensions (8). However, the serologic approach to rickettsial identification has not proven to be entirely practical or satisfactory because of the scarcity of specially prepared, representative antisera and antigens that are required for complete analysis of any one rickettsial isolate. Species-specific monoclonal antibodies are available only for a limited number of rickettsial species (19). Polypeptide patterns from dissociated rickettsiae and immunologically labeled specific epitopes have been studied for the purpose of species differentiation (2, 13, 26); however, as an approach to unambiguous identification of closely related rickettsiae, electrophoretic polypeptide analysis is also complicated by several factors. Rickettsiae must be rigorously purified away from contaminating host cell proteins without the loss or modification of important rickettsial proteins and epitopes. The large number of structural and nonstructural rickettsial proteins naturally occur in differing molar concentrations and presumably include inducible polypeptide gene products.

Complete nucleotide analysis of specific genes provides the best basis for determining genetic relationships among specific genes from various species (6); however, complete gene sequencing cannot currently be considered a reasonable approach to routine identification of multiple isolates.

Analysis of restriction endonuclease digests of DNA from purified rickettsiae has been used on a limited basis for discrimination of genotypes of rickettsiae that can be grown to relatively high titers (18, 29, 31). Isotope-labeled, cloned DNA probes have also been used experimentally for discrimination of rickettsial genomes, digested with restriction endonucleases, which contained a relatively uniform, high percentage of DNA homology to the cloned probe (15, 30, 32).

Analysis of ribosomal gene sequences has proven to be useful for identifying genotypic relationships between major groups of rickettsia-like organisms (39); however, the extreme sequence conservation observed among ribosomal genes may preclude using these genes for accurate identification of multiple genotypic markers for closely related organisms.

Cross-hybridization of genomic DNA purified from different rickettsial species has been used to estimate rickettsial relatedness (22, 23). Rickettsial genome DNA hybridization studies are constrained by the necessity to make relatively large amounts of genome DNA, free of any contaminating sources of DNA (e.g., mitochondrial or host cell nuclear DNA). Experimental deviations for hybridization experiments between closely related organisms can approach the estimates of genuine genotypic differences.

The polymerase chain reaction (PCR) procedure (33) has been used to detect the presence of rickettsiae in clinical specimens (37). We describe here a method that involves the use of defined rickettsial sequences, amplified by the PCR

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technique, as substrata for nonisotopic restriction endonuclease fragment length polymorphism (RFLP) differentiation of rickettsial species and genotypes as well as for estimating genetic divergence among selected genes. These applications of the PCR/RFLP techniques should greatly facilitate routine recognition of isolates of rickettsial species and genotypes.

## MATERIALS AND METHODS

**Rickettsial isolates.** Whenever possible, rickettsial isolates were obtained directly from the American Type Culture Collection (ATCC), Rockville, Md. Specific information about these isolates is referenced in the ATCC catalog (1). The ATCC isolates include *R. akari* (ATCC VR-148) strain Kaplan; *R. canada* (ATCC VR-610); *R. conorii* (ATCC VR-613) strain #7; *R. conorii* (ATCC VR-141) strain Moroccan; *R. montana* (ATCC VR-611); *R. typhi* (ATCC VR-144) strain Wilmington; *R. prowazekii* (ATCC VR-143) strain Breinl; *R. prowazekii* (ATCC VR-233) strain Madrid E; *R. rickettsii* (ATCC VR-149) strain Smith; *R. rickettsii* (ATCC VR-891) strain "R"; *R. sibirica* (ATCC VR-151); *Rochalimaea quintana* (ATCC VR-152) strain Fuller; and Thai tick typhus (ATCC VR-599).

Several isolates of *R. rickettsii* were made from human patient tissue inoculated into guinea pigs at the Centers for Disease Control, ca. 1980. These isolates came from widely separated geographic locations and represent diverse examples of clinical severity. They include isolate AF, clinically mild, Georgia; isolate RC, clinically severe, Georgia; isolate RL, fatal, Georgia; isolate JW, fatal, North Carolina; isolate JC, fatal, Nebraska; and isolate DR, fatal, Oklahoma.

Various other rickettsial isolates were generously shared with the Centers for Disease Control laboratory by other rickettsial laboratories. These included an *R. rickettsii* isolate, courtesy of Luis Fuentes, designated CCM (not the CR John isolate previously referenced [14]), made from a fatal human infection in Costa Rica, ca. 1980. The HLP SFG isolate was obtained from Robert L. Anacker, Rocky Mountain Laboratory, Hamilton, Mont. *R. bellii* and *R. australis* were obtained from Willy Burgdorfer, Rocky Mountain Laboratory. *R. rhipicephali* was obtained from the Rocky Mountain Laboratory via the Ohio State Health Laboratory (courtesy of Chip Pretzman). *R. slovaka* (#246) was obtained from Mort Peacock, Rocky Mountain Laboratory, and was originally isolated in Slovakia (11). An SFG isolate recovered from a tick removed from a hedgehog in Israel, and identified as T-487, was kindly supplied by Robert Goldwasser. References to the original isolation of most SFG rickettsiae used in this study are summarized by Philip et al. (28).

*R. prowazekii* isolates, including flying squirrel isolates and human isolates from North Africa and Europe, are the same as previously referenced (10, 29, 32).

**Rickettsial cultivation.** Passage histories for the various rickettsiae used in this study varied considerably. *Rochalimaea quintana* was grown in liquid medium (40). Otherwise, all other rickettsial isolates were routinely cultivated in Vero cell (E-6 clone) monolayers. Vero cell monolayers were infected with rickettsiae and maintained at either 32°C (SFG rickettsiae) or 35°C (typhus group rickettsiae). Cell culture medium (minimal essential medium) was supplemented with 2% fetal bovine sera, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer, nonessential amino acids, L-glutamine, and sodium bicarbonate.

Infected cell cultures were monitored for the presence of rickettsiae. When infections were judged to be maximal (e.g., 3 to 10 days), the supernatant was discarded, and 5 ml of buffer (Tris, pH 7.6; NaCl, 150 mM; EDTA, 2 mM) was added per 150-$cm^2$ cell culture flask. The infected cells were removed from the tissue culture flasks with glass beads and transferred to a 15-ml screw-cap centrifuge tube. Cytoplasmic extracts from infected cells were made in a similar manner to that described previously by addition of Triton X-100 (10%, vol/vol) to a final concentration of 0.25% (30). The suspension was allowed to stand at room temperature, with occasional gentle rocking of the tube in a nearly horizontal mode. As the host cell cytoplasmic membranes dissolved, the nuclei formed flocculent aggregates which, after rocking of the tube was terminated, settled into a single gelatinous mass. The supernatant was carefully removed and passed through a large-pore disposable Uniflo prefilter (Schleicher & Schuell, Inc., Keene, N.H.) which retained residual nuclear material. The above procedure was reproducible for making cytoplasmic extracts from Vero cells.

**DNA extraction.** Rickettsial DNA-protein complexes were dissociated by addition of sodium dodecyl sulfate (final concentration, 0.5%) and proteinase K (Sigma Chemical Co., St. Louis, Mo.), 0.5 mg/ml, to the cytoplasmic fraction followed by incubation at 50°C for 2 h (after which the rickettsiae were considered inactivated). DNA was partitioned by extractions in buffer-saturated phenol (once), phenol-chloroform (once) and chloroform (twice) and precipitated by the addition of 2 volumes of ethanol at 4°C (34). The ethanol-treated DNA was gently pelleted (e.g., 3,000 rpm) and the pellet was washed with 70%, and subsequently 100%, ethanol. After removal of the final ethanol wash, the DNA was allowed to dry at 37°C. DNA was resuspended overnight at 4°C in a small volume (e.g., 50 μl per original 150-$cm^2$ infected cell culture monolayer) of 10 mM Tris, pH 8.0. DNA extracted in this fashion has been stored at 4°C for several years and remains a suitable substrate for PCR amplification.

**PCR amplification.** PCR amplification was accomplished in 100-μl volumes, using the protocols supplied with the GeneAmp DNA amplification reagent kit (Perkin-Elmer Cetus, Norwalk, Conn.). Typically, 1 μl of undiluted, cytoplasmic extract DNA was used as PCR template. DNA amplification was done in a Perkin-Elmer Cetus DNA Thermal Cycler, using 35 cycles of denaturation (20 s at 95°C), annealing (30 s at 48°C), and extension (2 min at 60°C).

**DNA digestion and electrophoresis.** PCR amplification of DNA was verified by rapid agarose electrophoresis of a small amount of PCR product. Restriction endonuclease digestion was done with 20 μl of PCR reaction mixture, following standard techniques (34), and incubations were at 37°C. All restriction endonucleases were obtained from New England BioLabs, Beverly, Mass. After addition of dye-Ficoll loading mixture (34), the digested reactions were loaded on 1.5 mm thick, 8% polyacrylamide vertical gels (Bio-Rad Laboratories, Richmond, Calif.) made by standard procedures (34). Gels were run at 80 V for 4 h in simple vertical electrophoresis chambers (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, Md.). The gels were then stained with ethidium bromide prior to illumination on a UV light source (365 nm; Spectronic Corp., Westbury, N.Y.) and photographed with Polaroid type 655 P/N film (Polaroid Corp., Cambridge, Mass.).

**Estimation of DNA fragment size.** ϕX174 double-stranded, replicative form DNA (New England BioLabs), cleaved with *Hin*fI restriction endonuclease, was used for DNA fragment size standards (35); each rickettsial DNA PCR digest prod-

TABLE 1. Oligonucleotide primers

| Primer | Species[a] | Gene | Nucleotide sequence (5'-3') | Produce size (bp) |
|---|---|---|---|---|
| *Rp*CS.877p | *R. prowazekii* | Citrate synthase | GGGGGCCTGCTCACGGCGG | 381 |
| *Rp*CS.1258n | *R. prowazekii* | Citrate synthase | ATTGCAAAAAGTACAGTGAACA | |
| *Rr*190.70p | *R. rickettsii* | 190-kDa antigen | ATGGCGAATATTTCTCCAAAA | 532 |
| *Rr*190.602n | *R. rickettsii* | 190-kDa antigen | AGTGCAGCATTCGCTCCCCCT | |
| *Rr*190.4442p | *R. rickettsii* | 190-kDa antigen | CGATAGCCGGAGGCAAGACGT | 1,222 |
| *Rr*190.5664n | *R. rickettsii* | 190-kDa antigen | TGCAAGCGGTCCACCCGGTGC | |

[a] Oligonucleotide primer sequences were derived from either *R. prowazekii* or *R. rickettsii* genes.

uct was run adjacent to a size standard for direct reference and to minimize any possible effects of gel distortion on obtaining accurate size estimates. Photographic negatives of the electrophoretograms were enlarged, and the resulting prints were used for analysis of DNA fragment sizes. The DNA fragment sizes were estimated by entering digitized migration data with an electronic stylus (Houston Instruments, Austin, Tex.) directly to a desk top computer which contained a best-fit spline curve algorithm for estimating DNA fragment migration relative to known size standards.

**Oligonucleotide primers.** Oligonucleotide primers used for priming PCRs are shown in Table 1. Generic rickettsial oligonucleotide primers for PCR were derived from two areas of the citrate synthase gene which have significant homologies for both *R. prowazekii* and *Escherichia coli* analogous sequences (24, 43); the exact nucleotide sequences of both primers were derived from the nucleotide sequence of the *R. prowazekii* gene. It was initially posited that sequences conserved between diverse bacterial genera would most likely also be conserved within either of the genera from which the sequences were initially derived.

Oligonucleotide primers for PCR amplification of regions of the 190-kDa SFG antigen gene were derived from the *R. rickettsii* (R strain) sequence without specific prior information regarding possible genus or species genetic conservation (5).

Primer pairs were referred to by the initials of the genus and species from which the nucleotide sequence was derived, combined with a simple alphanumeric identifier of the gene amplified, and followed by the position of the first nucleotide of the "positive" strand of the primer (using conventional 5'-3' presentation of sequence data) and then the position of the last nucleotide of the "negative" strand of the corresponding primer from the complementary strand of DNA. This system allowed for an unambiguous primer pair designation that also displayed the position and extent of the PCR-amplified product from the organism from which the original sequence data was obtained. For example, *Rp*CS.877p and *Rp*CS.1258n refer to the *R. prowazekii* citrate synthase primer pair for a region between nucleotides 877 and 1258. Likewise, *Rr*190.70p and *Rr*190.602n refer to primers derived from the *R. rickettsii* (R strain) 190-kDa antigen gene sequence and which prime an amplified product between nucleotides 70 and 602 of the homologous DNA sequence.

Specific primer sequences used in this study are included in Table 1. Nucleotide primers were produced by the Division of Viral and Rickettsial Diseases, Biotechnology Resource Facility, Centers for Disease Control, and these primers are available from the Centers for Disease Control.

**Numerical analysis.** To quantify the differences and similarities found between PCR/RFLP patterns, digests from various rickettsial isolates were analyzed to determine the number of comigrating DNA fragments. Multiple digests of PCR-amplified sequences were used for this analysis so that individual restriction site differences would not be overemphasized (see legends to Fig. 5 and 7). The fraction of comigrating fragments for two digests was defined as the number of bands from two digests which have the same migration characteristics (or two times the number of comigrating fragment pairs), divided by the total number of fragments from both digests (38).

DNA sequence divergence between the portions of rickettsial genomes amplified by the PCR reactions was estimated by using the fraction of comigrating fragments, restriction endonuclease recognition sequence size, and mathematical formulations derived by Upholt (38) and subsequently used by others to estimate sequence divergence between closely related bacteria (12, 21). Inferred percent sequence divergence values for rickettsial gene segments from various isolates were displayed as dendrograms, using the unweighted-pair group methodology with arithmetic averages and CLUSTAN 1C commercial computer programming (36, 42).

## RESULTS

**PCR amplification of DNA with *R. prowazekii* citrate synthase primers.** Nucleotide primers designated *Rp*CS.877p and *Rp*CS.1258n, derived from near the amino terminus area of the *R. prowazekii* citrate synthase gene, primed the synthesis of DNA products from all species of rickettsiae tested with the exception of members of the *R. tsutsugamushi* complex, organisms whose relative taxonomic status is uncertain (41). Additional primers have been used to distinguish among *R. tsutsugamushi* isolates (36a). The citrate synthase primer pair described above also primed a product from *Rochalimaea quintana*, a potentially free-living but apparently related bacterium (39). The *R. prowazekii*-derived primers did not prime products from either *E. coli* or *Mycobacterium* species; other bacterial species have not been tested.

**DNA amplification with *R. rickettsii* 190-kDa antigen primers.** Restriction endonuclease digests of the PCR-amplified rickettsial citrate synthase products from several species of SFG rickettsiae were indistinguishable because of the appar-

ent conservation of nucleotide sequences for this relatively well-conserved gene (43). For this reason, additional PCR primer pairs were investigated for differentiation of closely related species of SFG organisms. Complete nucleotide sequence information is available for a large, 190-kDa protein antigen of *R. rickettsii* (5). This antigen, often referred to in the literature as the 155-kDa antigen, is thought to be exposed on the surface of rickettsiae (20) and therefore may be subjected to increased evolutionary pressures, which would be manifested as increased variability in the nucleotide sequences from various species and isolates. Four sets of nucleotide primer pairs were investigated for priming PCR products from four different regions of the gene. Two of these PCR primer pairs were considered most useful by virtue of their ability to prime products of appropriate lengths and produce a minimum of nonspecific products. These two primer pairs (designated *Rr*190.70p and *Rr*190.602n, and *Rr*190.4442p and *Rr*190.5664n) primed PCR product formation from a region beginning at the amino terminus of the gene and another area in the center of the gene.

**Electrophoretic analysis.** Preliminary electrophoretic analysis of uncut PCR products suggested that all rickettsial citrate synthase PCR-amplified products were similar in overall size (data not shown). The sizes of the PCR products, estimated by summing the fragment sizes from restriction endonuclease digestions, also suggested that the products formed from the different isolates and species were approximately the same size (see Fig. 1 and 4). The PCR-amplified product from the one representative of the genus *Rochalimaea* was exceptional in that the PCR-amplified product was approximately 70 bp larger than the PCR-amplified products from the species of the genus *Rickettsia* (Fig. 1). Sets of PCR reactions always included a control reaction containing no template DNA and another control containing material extracted from uninfected Vero cells. Both sets of negative controls yielded minimal amounts of products when the primary PCR products were assayed. However, the controls were valuable for interpretation of occasional faint bands seen in some restriction endonuclease digests of PCR-amplified products after separation on electrophoretic gels. Minimal amounts of partial digest products were sometimes present after exhaustive digestion (e.g., see Fig. 3). Preliminary results indicated that increased PCR temperatures did not appear to either increase the quantity of rickettsial products produced or decrease the minimal amounts of presumably nonspecific amplified DNA (data not shown).

Small DNA fragments differing in only two to six nucleotides formed discrete banding patterns (Fig. 1 and 2). In contrast, estimation of DNA fragment sizes larger than approximately 330 bp often led to modest overestimation of fragment size (e.g., compare summed weights of digest products in Fig. 4A with the different digests of the same amplified products in Fig. 4B, some of which are derived from fragment sizes larger than 330 bp). When DNA digests were subjected to electrophoresis on two occasions, the estimated sizes of DNA fragments varied by <2% (data not shown). Accuracy and consistency of DNA fragment size estimates can be further evaluated by summing the DNA fragment sizes from digests for which DNA sequences are available and comparing the gel fragment size estimates with the size of the fragment deduced from the sequencing data. For example, the citrate synthase, *R. prowazekii*-derived PCR product has a deduced theoretical size of 381 bp. Four different digests (different enzymes) and the resulting four separate gels yielded additive size estimates of 388, 380, 381, and 380 bp (average difference <2% from the theoretical size).

For numerical comparison of digests from different species we conservatively adopted a criterium of 4% or less estimated size difference as constituting "homologous" migration for fragments of <330 bp. Exceptions were occasionally made for well-resolved, small fragments when the 4% window would have included two bands, with clearly different migration patterns, from the same digest.

Although accurate size estimation of DNA fragments, by whatever means, is an important prerequisite for making genotypic comparisons with isolates referenced in the literature, side-by-side visual comparisons of PCR/RFLP patterns are suitable for determining pattern homology, or lack of pattern homology, for a prototype strain and a novel isolate.

**Differentiation of rickettsial species.** PCR/RFLP patterns, for members of what are recognized as the same rickettsial species, were extremely well conserved. All PCR/RFLP patterns (PCR amplification of DNA with primers *Rp*CS.877p and *Rp*CS.1258n) derived from isolates of *R. prowazekii*, whether obtained from humans or flying squirrels or from Europe, North America, or Africa, were identical.

Isolates of *R. rickettsii* from different areas of North and Central America were compared with these techniques. Nine different isolates of *R. rickettsii* made from various human sources were analyzed; the clinical outcomes of the diseases varied from mild to rapidly fatal. By the PCR/RFLP criteria used in this study, all of the *R. rickettsii* isolates from human sources were essentially identical with two minor exceptions; the two isolates from Montana (and often regarded as prototypes for the species) had PCR/RFLP patterns very slightly different from other human isolates when comparing citrate synthase gene sequences (Fig. 1 and 2). This difference was the equivalent of a 2- to 4-bp deletion (relative to other isolates) in one of the two smallest fragments of the *Alu*I digest pattern. Hence, the two Montana isolates investigated displayed two small distinct bands instead of a single "doublet" band (the presence of single and double bands was confirmed by scanning densitometry). RFLP analysis of the 190-kDa antigen primer-amplified DNA did not disclose additional differences between the two Montana isolates and other *R. rickettsii* isolates (Fig. 3).

The HLP strain of *R. rickettsii*, recovered from various species of ticks and demonstrated to have reduced virulence for guinea pigs (7), has subtle antigenic differences when compared with other isolates of *R. rickettsii* (3, 28). The HLP isolate analyzed by PCR/RFLP was identical to other *R. rickettsii* isolates in both *Pst*I and *Rsa*I digests of the portion of the 190-kDa antigen gene amplified in the presence of the *Rr*190.70p-*Rr*190.602 primer pair (Fig. 3). However, the *Alu*I digest of the same amplified material produced a unique pattern, apparently lacking one *Alu*I recognition site (data not shown). In addition, DNA extracted from the HLP isolate failed to serve as template for PCR amplification in the presence of the *Rr*190.4442p-*Rr*190.5664n primer pair. This suggests that, unlike all other SFG isolates studied, a difference occurs within an HLP sequence homologous to at least one of the primers which was critical for PCR amplification. Due to this absence of comparable amplification and resultant RFLP data, the HLP isolate could not be included in the statistical analysis of estimated sequence divergence within the 190-kDa antigen gene sequences studied. However, it is clear that differences occur within the sequences which code for the 190-kDa antigen of the HLP isolate that

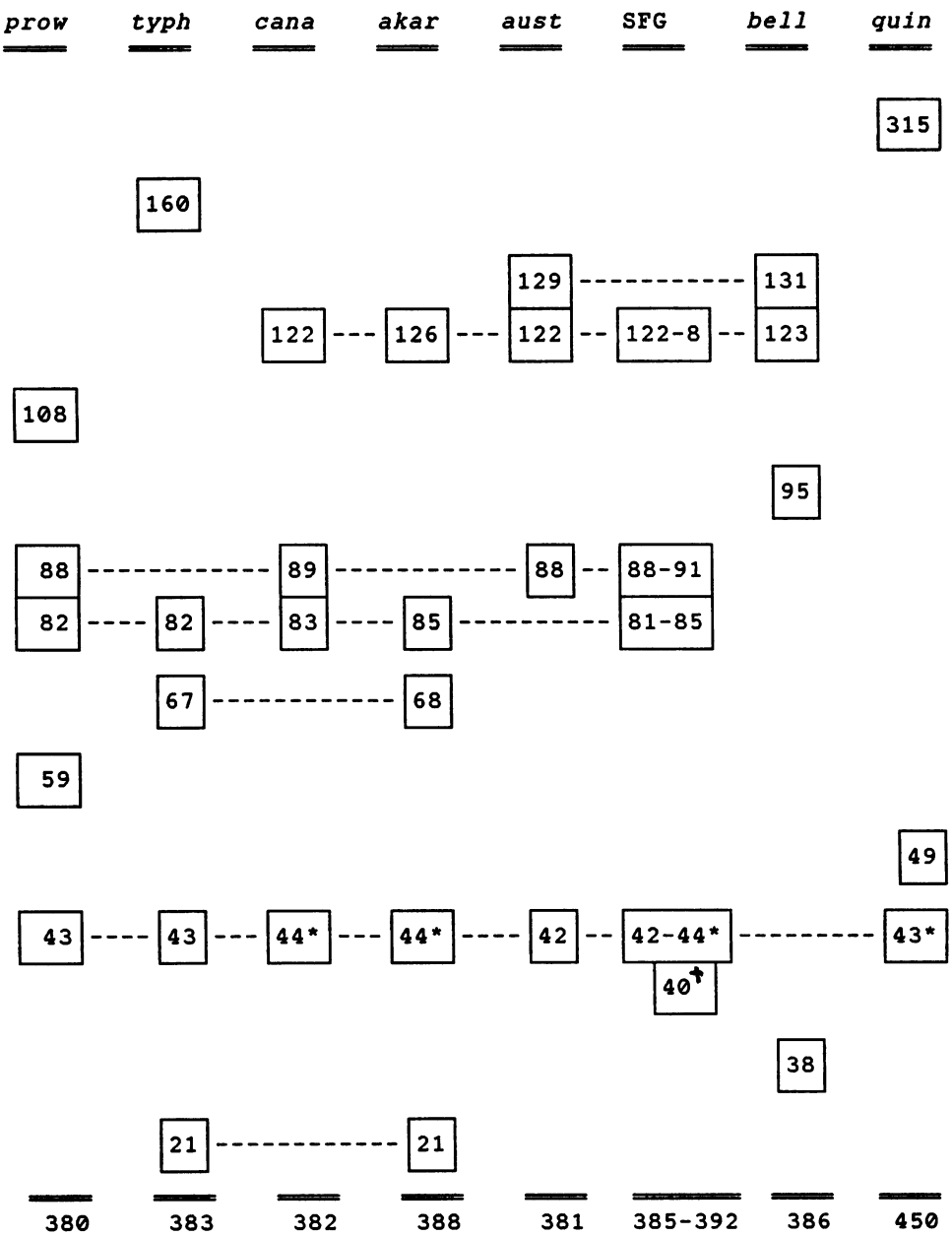


FIG. 1. Composite representation of diagnostic *Alu*I digest patterns of PCR-amplified DNA derived from a portion of citrate synthase genes from various rickettsial species, schematically displayed as if fragment migration ran from top to bottom (see Fig. 2). Sizes for each fragment are displayed as number of nucleotide base pairs. Comigrating fragments from different species are connected by dashed lines. Doublet bands are indicated with an asterisk (*). The *R. prowazekii* (*prow*) digest pattern represents all *R. prowazekii* isolates from various sources described in Materials and Methods. Two *R. rickettsii* isolates ("R" and Smith) produced patterns with a discrete 40-bp fragment (indicated with a †) as well as a single band at 42 to 44 bp. All other SFG organisms had doublet bands at 42 to 44 bp. Sizes for all fragments for each species are totaled near the bottom. The expected size of the total PCR product from *R. prowazekii*, based on sequence information (43), is 381 bp. Several SFG rickettsiae produced essentially identical fragment patterns (grouped together under SFG): *R. rickettsii* isolates R, AF, and HLP; *R. conorii* strain Morocco (Mor); *R. sibirica* (*sib*); *R. slovaka* (*slo*); *R. rhipicephali* (*rhp*); and *R. montana* (*mon*). Note that *R. akari* (*akar*) and *R. australis* (*aust*) have patterns distinct from other SFG rickettsiae. Additional abbreviated identifiers include *R. prowazekii* (*prow*), *R. typhi* (*typh*), *R. canada* (*cana*), *R. bellii* (*bell*), and *Rochalimaea quintana* (*quin*).

are not found in other *R. rickettsii* isolates or isolates of several other SFG species. PCR/RFLP analysis of citrate synthase primed DNA from the HLP isolate was indistinguishable from other closely related SFG organisms.

Multiple recent human isolates of *R. conorii* from France and Spain appear to be nearly identical by the PCR/RFLP analysis (13a). A subtle difference (approximately 10 bp) between the sizes of *Rr*190.70p-*Rr*190.602n-primed products from the ATCC Moroccan strain of *R. conorii* and several other closely related SFG isolates was consistently noted (Fig. 3A and 4A).

While the conservation of PCR/RFLP patterns was well maintained at the species level, differences between recognized species were readily observed (Fig. 1 to 4). Diagnostic

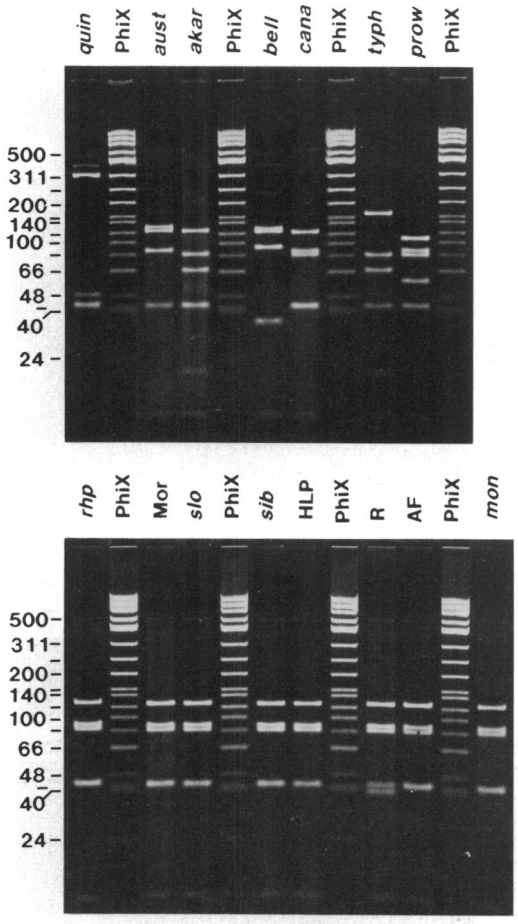


FIG. 2. Ethidium bromide-stained, polyacrylamide gel electrophoretograms of *Alu*I restriction endonuclease-digested, PCR-amplified rickettsial DNA derived from the citrate synthase gene, using primers *Rp*CS.877p and *Rp*CS.1258n. φX174 replicative form DNA (PhiX) cleaved with *Hin*fI was used as a size standard adjacent to each rickettsial sample. Size estimates of individual DNA fragments are given in Fig. 1.

PCR/RFLP patterns for nearly all recognized species were obtained with, at most, three digests of two different amplified products (Fig. 1 and 4). *R. prowazekii*, *R. typhi*, *R. akari*, *R. australis*, *R. bellii*, and *Rochalimaea quintana* all had distinctive PCR/RFLP patterns when the single citrate synthase-primed products from each species were digested with *Alu*I restriction endonuclease (Fig. 1 and 2). The ambiguity between the *Alu*I digests of *Rp*CS.877p-*Rp*CS.1258n-amplified products of *R. canada* and certain SFG members could be clarified by a *Sau*3a digest (which cleaved the products of the SFG one time but which did not cleave the product of *R. canada*), or a double digest with *Sca*I and *Rsa*I (which showed an additional restriction endonuclease site with SFG PCR products). In addition, no PCR products for *R. canada* was primed by any of the primers derived from the 190-kDa antigen gene used to subsequently differentiate SFG rickettsiae.

**Differentiation of SFG rickettsiae by PCR/RFLP analysis.** Whereas digests of the PCR products derived from the citrate synthase gene were especially valuable for identifying relatively diverse groups of rickettsia, the PCR products derived from the *R. rickettsii* 190-kDa antigen gene were useful for differentiating the members of the closely related SFG rickettsial complex. These SFG species (e.g., *R. rickettsii*, *R. conorii*, *R. slovaka*, *R. sibirica*, *R. rhipicephali*, and *R. montana*) all had distinctive patterns readily identifiable from two digests of DNA from a single set of primers (Fig. 3 and 4). SFG isolates that as yet are not recognized as distinct species also had unique PCR/RFLP patterns (e.g., Israel tick typhus and Thai tick typhus).

The Israel tick typhus isolate and the *R. conorii* #7 had identical PCR/RFLP patterns when digested with the restriction endonucleases used to estimate numerical conservation of DNA fragment patterns (all enzymes used recognized sequences 4 nucleotides in length [Fig. 5 to 10]). However, when the enzyme *Pst*I (which has a recognition/cleavage site 6 nucleotides long and therefore could not be included in the same numerical calculations) was used to produce digests of Israel tick typhus and *R. conorii* #7, a different pattern was noted (Fig. 3A and 4A).

*R. akari* and *R. australis*, considered members of a larger SFG of rickettsiae and which were readily differentiated by analysis of citrate synthase-primed PCR products (Fig. 1 and 2), were not primed with 190-kDa antigen primer pairs used for this study.

## DISCUSSION

Using the PCR/RFLP techniques described above, it was possible to identify genotypes characteristic of all rickettsial species tested with the exception of isolates of *R. tsutsugamushi*. *Coxiella burnetii* has not been tested because of lack of available DNA. Isolates of *R. helvitica* and *R. parkeri* were not available for analysis. The PCR primers derived from the relatively well-conserved citrate synthase gene were most useful for differentiation among the relatively distinct rickettsial species. The PCR products from the citrate synthase primer pair cannot be used to differentiate among certain closely related SFG species or genotypes (e.g., *R. rickettsii*, *R. sibirica*, *R. conorii*, and *R. montana*). However, DNA from these same closely related SFG organisms, after PCR amplification with primers derived from the 190-kDa antigen gene of *R. rickettsii*, could be readily differentiated by RFLP analysis.

Stylized representations of selected diagnostic rickettsial PCR/RFLP patterns were prepared as references to assist in future recognition of rickettsial genotypes (Fig. 1 and 4). DNA fragment sizes for each band are indicated in each diagram. By using the same primer pairs used in this study for PCR amplification and appropriate restriction endonuclease digestion, it should be possible to relatively rapidly identify (non-*R. tsutsugamushi*) rickettsial genotypes from unidentified rickettsial isolates. Such future unidentified isolates will fall into one of probably three categories: (i) genotypes essentially identical to those described in this communication; (ii) genotypes with obvious similarities to those rickettsial genotypes described herein (estimates of genotypic similarities can be readily calculated); (iii) novel genotypes from previously unrecognized rickettsiae or closely related bacteria (reasonable estimates of genotypic similarities depends on the number of comigrating DNA fragments). Examples of all three categories of genotyping by PCR/RFLP analysis, as well as identification of *R. tsutsugamushi*, will be the subject of forthcoming communications (13a, 13b, 36a).

The extent of DNA base substitutions within the relevant portions of rickettsial genomes can be estimated from the fractions of comigrating DNA fragments derived from the

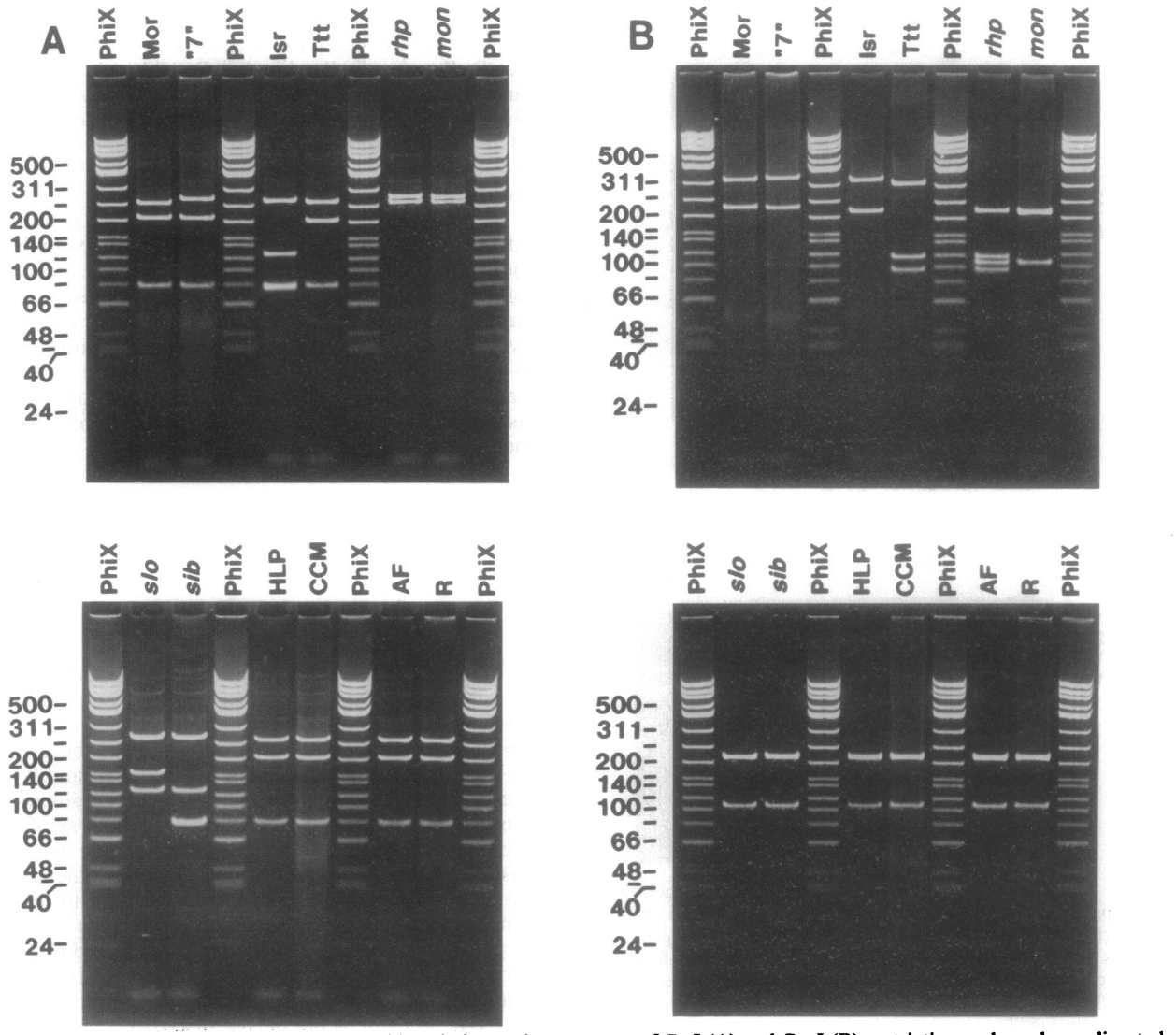


FIG. 3. Ethidium bromide-stained, polyacrylamide gel electrophoretograms of *Pst*I (A) and *Rsa*I (B) restriction endonuclease-digested, PCR-amplified SFG rickettsial DNA derived from the 190-kDa antigen gene, using primers *Rr*190.70p and *Rr*190.602n. *R. rickettsii* is represented by the virulent R, CCM, and AF isolates and the nonpathogenic HLP isolate. Old World SFG rickettsiae are represented by *R. conorii* #7 and Morocco (Mor), Israel tick typhus (Isr), Thailand tick typhus (Ttt), *R. sibirica* (*sib*), and *R. slovaka* (*slo*). *R. rhipicephali* (*rhp*) and *R. montana* (*mon*) are thought to be nonpathogenic for humans and are isolates made only from ticks.

various PCR/RFLP digest patterns (Fig. 5 to 8). Such estimates of sequence divergence apply only to limited portions of the rickettsial genomes. These data suggest, as previously suspected, that the 190-kDa antigen gene has greater intraspecies genetic variability than the citrate synthase gene. Sequence divergence within most species of pathogenic SFG rickettsiae, as determined by analysis of the citrate synthase-derived primers, is almost lacking. However, there is measurable PCR/RFLP divergence observed among species of SFG when the 190-kDa antigen-derived PCR primers are used.

The divergence estimates among various rickettsial species, derived from the citrate synthase sequences studied by PCR/RFLP analysis, are conservative when compared with estimates of divergence previously derived from entire genome hybridization experiments. For example, compare the 2.1% PCR/RFLP estimate of divergence between *R. prowazekii* and *R. typhi* citrate synthase gene sequences with the 30% divergence for the same rickettsiae as measured by DNA homology (22). This may be construed as evidence that the citrate synthase gene is relatively well conserved, perhaps by at least a factor of 10, relative to the rickettsial genome in general.

One can also compare the PCR/RFLP estimates of gene sequence divergence for the citrate synthase and 190-kDa antigen genes with the sequence divergence data derived from the well-conserved rickettsial 17-kDa antigen gene previously analyzed by Anderson and Tzianabos (6). From the sequence data provided in these authors' report, it can be calculated that *R. prowazekii* and *R. typhi* are 5.8% divergent for the 17-kDa antigen gene as compared with the 2.1% divergence estimate obtained from the PCR/RFLP citrate synthase analysis. Likewise, Anderson and Tzianabos have demonstrated that actual sequence divergence between the 17-kDa antigen gene of SFG rickettsiae and the typhus group rickettsiae is approximately 12%, whereas we estimate that

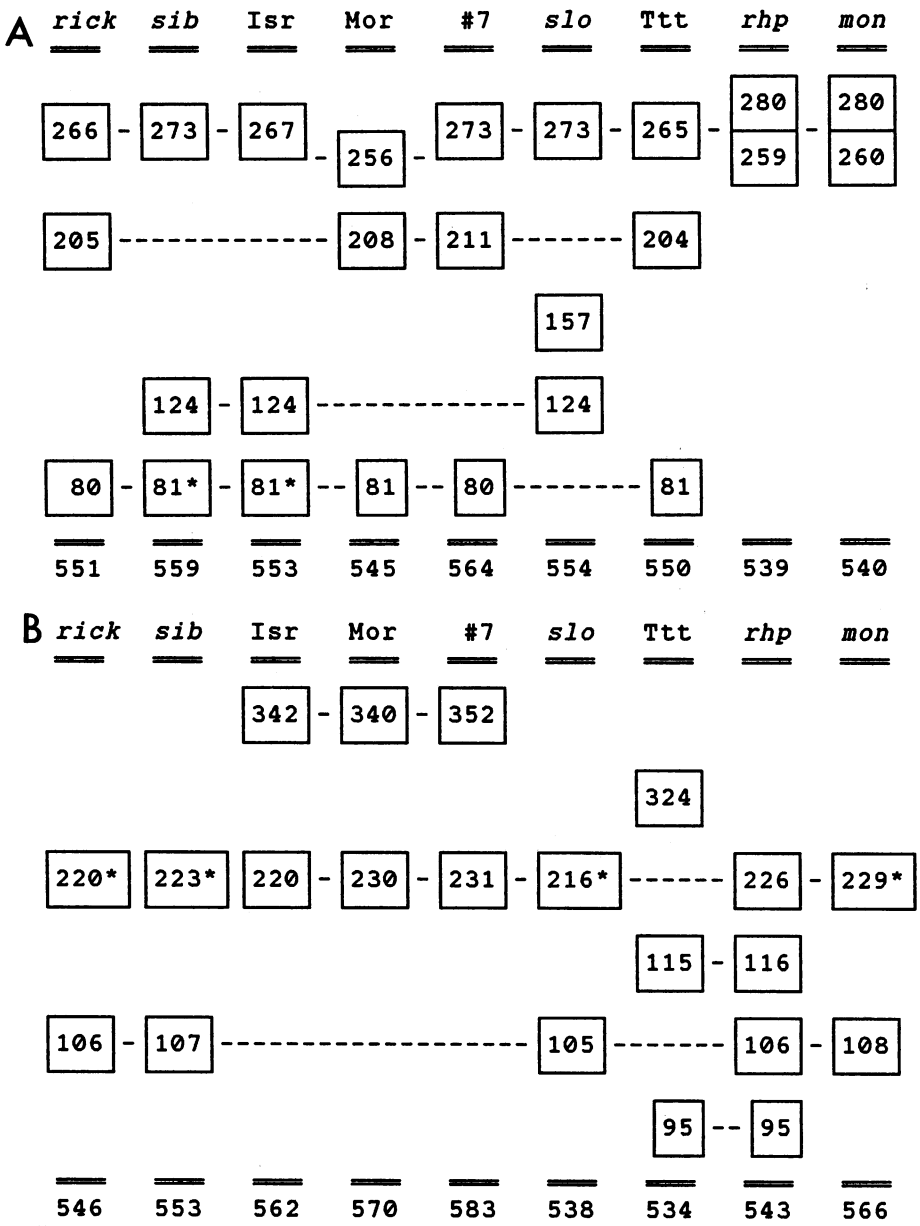


FIG. 4. Schematic, diagnostic composite of PCR-amplified DNA, derived from the 190-kDa antigen gene (PCR primers *Rr*190.70p and *Rr*190.602n) of various species and isolates of SFG rickettsiae, digested with either *Pst*I (A) or *Rsa*I (B) and displayed as if electrophoretic migration of DNA fragments ran from top to bottom (see Fig. 3). All *R. rickettsii* (*rick*) isolates described in Materials and Methods produced essentially identical fragment patterns. Abbreviated identifiers are the same as in the legend to Fig. 3. Doublet bands are indicated with an asterisk.

the sequence divergence between the citrate synthase sequences of the SFG and the typhus group is only approximately 4% (Fig. 6). Anderson and Tzianabos compared the 17-kDa antigen sequences for *R. rickettsii* and *R. conorii* and found only a single base pair difference (0.2% difference); the PCR/RFLP estimate of sequence divergence for the 190-kDa antigen gene for these two species is approximately 1.7 to 2.4%, depending on the *R. conorii* strain (Fig. 8). By comparing the estimated divergence data from the PCR/RFLP analysis for the citrate synthase and the 190-kDa antigen genes with the sequence data from the 17-kDa antigen gene, it can be tentatively deduced that the citrate synthase is the most stringently conserved of these three rickettsial genes, followed by the 17-kDa antigen gene and finally the 190-kDa antigen gene.

When multiple isolates from within recognized rickettsial species were analyzed, the PCR/RFLP patterns were almost totally free of intraspecies genetic variation. This virtual absence of significant observed sequence divergence among isolates of a given species is noteworthy and is one reason why the PCR/RFLP technique works well for identification of rickettsial species. The estimates of genetic divergence derived by the PCR/RFLP techniques appear to agree with estimates of genetic conservation within the SFG, and within individual member species of the SFG, estimated by analysis of genomic digests by using undefined, isotopically labeled

| | *prow* | *typh* | *cana* | *akar* | *aust* | SFG | R,SS | *rhp* | *bell* | *quin* |
|---|---|---|---|---|---|---|---|---|---|---|
| *prow* | (14) | 22/28 | 16/26 | 16/29 | 16/27 | 18/28 | 18/28 | 16/27 | 0/22 | 6/32 |
| *typh* | | (14) | 14/26 | 20/29 | 14/27 | 16/28 | 16/28 | 14/27 | 0/26 | 6/32 |
| *cana* | | | (12) | 18/27 | 16/25 | 20/26 | 18/26 | 18/25 | 2/20 | 8/30 |
| *akar* | | | | (15) | 22/28 | 26/29 | 24/29 | 22/28 | 2/23 | 12/33 |
| *aust* | | | | | (13) | 24/27 | 24/27 | 20/26 | 4/21 | 8/31 |
| SFG | | | | | | (14) | 26/28 | 24/27 | 2/22 | 12/32 |
| R,SS | | | | | | | (14) | 22/27 | 2/22 | 10/32 |
| *rhp* | | | | | | | | (13) | 2/21 | 12/31 |
| *bell* | | | | | | | | | (8) | 0/26 |
| *quin* | | | | | | | | | | (18) |

FIG. 5. Conserved citrate synthase sequence-primed, PCR-amplified, RFLP fragments among rickettsial species. Numbers in parentheses indicate total number of fragments used from each species' analysis. PCR-amplified DNA from each species was digested individually by, or with combinations of, *Alu*I, *Dde*I, *Rsa*I, *Sau*3a, *Sca*I, and *Ssp*I. Fractions indicate the number of comigrating bands for each pair of species/isolates divided by the number of fragments present for both species/isolates. *R. rickettsii* isolates "R" and Smith are indicated together as R, SS; otherwise, abbreviated identifiers are the same as in the legends to Fig. 1 and 3.

probes, has recently been reported to agree with the estimates of genetic divergence derived by the PCR/RFLP techniques (15, 28a).

Stringent genetic conservation within a bacterial species is also found within the genus *Mycobacterium*, especially within the species *Mycobacterium leprae* (12, 21). It may be significant that *M. leprae* is also a naturally occurring obligate intracellular parasitic bacterium.

Analysis of *R. tsutsugamushi* isolates was accomplished by using the PCR/RFLP technique with primers derived from the heat shock-like protein gene (25, 36a), which did not prime synthesis of PCR products from rickettsial species

| | *prow* | *typh* | *cana* | *akar* | *aust* | SFG | R,SS | *rhp* | *bell* | *quin* |
|---|---|---|---|---|---|---|---|---|---|---|
| *prow* | 1/0 | 0.79 | 0.62 | 0.55 | 0.59 | 0.64 | 0.64 | 0.59 | 0 | 0.19 |
| *typh* | 2.1 | 1/0 | 0.54 | 0.69 | 0.52 | 0.57 | 0.57 | 0.52 | 0 | 0.19 |
| *cana* | 4.1 | 5.3 | 1/0 | 0.67 | 0.64 | 0.77 | 0.69 | 0.72 | 0.1 | 0.27 |
| *akar* | 5.2 | 3.2 | 3.4 | 1/0 | 0.79 | 0.90 | 0.83 | 0.79 | 0.09 | 0.36 |
| *aust* | 4.5 | 5.7 | 3.8 | 2.0 | 1/0 | 0.89 | 0.89 | 0.77 | 0.19 | 0.26 |
| SFG | 3.8 | 4.8 | 2.2 | 0.9 | 1.0 | 1/0 | 0.93 | 0.89 | 0.09 | 0.38 |
| R,SS | 3.8 | 4.8 | 3.2 | 1.6 | 1.0 | 0.6 | 1/0 | 0.81 | 0.09 | 0.31 |
| *rhp* | 4.5 | 5.7 | 2.8 | 2.0 | 2.2 | 1.0 | 1.8 | 1/0 | 0.1 | 0.39 |
| *bell* | 100 | 100 | 20.5 | 21.4 | 14.7 | 21.4 | 21.4 | 20.5 | 1/0 | 0 |
| *quin* | 14.7 | 14.7 | 11.6 | 8.9 | 11.9 | 8.5 | 10.3 | 8.28 | 100 | 1/0 |

FIG. 6. Estimates of both citrate synthase sequence divergence and fraction of comigrating PCR-amplified, RFLP DNA fragments for various rickettsial species. Diagonal series indicates fractions of homologous "comigrating" bands (1) over the amount of homologous divergence (0). Values in the upper right sector are the fractions of comigrating bands, and those in the lower left sector are the percent sequence divergences inferred from analysis of comigrating DNA fragments. Isolate and species abbreviated identifiers are the same as in the legend to Fig. 1 and 3.

| | *rick* | *slo* | *sib* | #7 | Isr | Mor | Ttt | *rhp* | *mon* |
|---|---|---|---|---|---|---|---|---|---|
| *rick* | (28) | 48/56 | 48/58 | 46/56 | 46/56 | 42/56 | 46/56 | 40/55 | 46/56 |
| *slo* | | (28) | 50/58 | 46/56 | 46/56 | 44/57 | 46/56 | 42/55 | 48/56 |
| *sib* | | | (30) | 52/58 | 52/58 | 48/57 | 46/58 | 40/57 | 46/58 |
| #7 | | | | (28) | 56/56 | 52/56 | 48/56 | 40/55 | 44/56 |
| Isr | | | | | (28) | 52/56 | 48/56 | 40/55 | 44/56 |
| Mor | | | | | | (28) | 44/56 | 38/55 | 46/56 |
| Ttt | | | | | | | (28) | 44/55 | 44/56 |
| *rhp* | | | | | | | | (27) | 44/55 |
| *mon* | | | | | | | | | (28) |

FIG. 7. Conserved RFLP fragments among various SFG isolates primed for PCR amplification with 190-kDa antigen gene primers. Numbers in parentheses indicate the total number of fragments used for the analysis of each species/isolate. Fractions display the total number of comigrating bands for each pair of species/isolates divided by the total number of fragments present for both species/isolates. Amplified DNA beginning from the amino terminus of the gene (primed with *Rr*190.70p–602n) were digested with *Alu*I and *Rsa*I restriction endonucleases. Amplified DNA from nearer the center of the gene (primed with *Rr*190.4442p–5664n) was digested with the same enzymes and with *Msp*I. One apparently homologous DNA band, found in *Alu*I digests of all isolates primed with *Rr*190.4442p–5664n, as well as two sets of apparently homologous DNA fragment bands found in all *Msp*I digests from the same PCR-amplified DNA, were excluded from this analysis due to their relatively large sizes (>400 bp) which made precise estimation of fragment size difficult to estimate. *R. rickettsii* (*rick*) isolates are represented by isolates R, CCM, and AF; otherwise abbreviated species and isolate identifiers are the same as in the legend to Fig. 1 and 3.

other than *R. tsutsugamushi*. Unlike other rickettsia-like organisms, *R. tsutsugamuchi* isolates appear to exhibit modest intraspecies genotypic variability which appears to correlate with the presence of multiple serovars noted previously for this species.

Few useful taxonomic schemes or techniques for microbial isolate identification rely on accounting for all genetic variability. Total genomic sequencing not only remains impractical for identifying multiple microbial isolates, but still may not reveal which sequences are critical for observed

| | *rick* | *slo* | *sib* | #7 | Isr | Mor | Ttt | *rhp* | *mon* |
|---|---|---|---|---|---|---|---|---|---|
| *rick* | 1/0 | 0.86 | 0.83 | 0.82 | 0.82 | 0.75 | 0.82 | 0.73 | 0.82 |
| *slo* | 1.3 | 1/0 | 0.86 | 0.82 | 0.82 | 0.77 | 0.82 | 0.76 | 0.86 |
| *sib* | 1.6 | 1.3 | 1/0 | 0.90 | 0.90 | 0.84 | 0.79 | 0.70 | 0.79 |
| #7 | 1.7 | 1.7 | 0.9 | 1/0 | 1.00 | 0.93 | 0.86 | 0.73 | 0.79 |
| Isr | 1.7 | 1.7 | 0.9 | 0 | 1/0 | 0.93 | 0.86 | 0.73 | 0.79 |
| Mor | 2.4 | 2.2 | 1.5 | 0.6 | 0.6 | 1/0 | 0.79 | 0.69 | 0.82 |
| Ttt | 1.7 | 1.7 | 2.0 | 1.3 | 1.3 | 2.0 | 1/0 | 0.80 | 0.79 |
| *rhp* | 2.7 | 2.3 | 3.0 | 2.7 | 2.7 | 3.2 | 1.9 | 1/0 | 0.80 |
| *mon* | 1.7 | 1.3 | 2.0 | 2.0 | 2.0 | 1.7 | 2.0 | 1.9 | 1/0 |

FIG. 8. Estimates of 190-kDa antigen gene sequence divergence and the fraction of comigrating PCR-amplified RFLP DNA fragments for various SFG species/isolates. Diagonal series indicates fractions of homologous comigrating fragments (1) over the amount of homologous divergence (0). Values in the upper right sector are the fractions of comigrating fragments, and those in the lower left sector are the percent sequence divergence inferred from analysis of comigrating DNA fragments. Restriction endonucleases used in this analysis are indicated in the legend to Fig. 7. All enzymes used for sequence divergence estimation identify the same number of nucleotide recognition sequences (four nucleotides in this example) (38). Data from *Pst*I digests (six nucleotide recognition site), although useful for diagnostic identification, were therefore not included in this analysis of sequence divergence. Abbreviated species and isolate identifiers are the same as in the legends to Fig. 1 and 3.

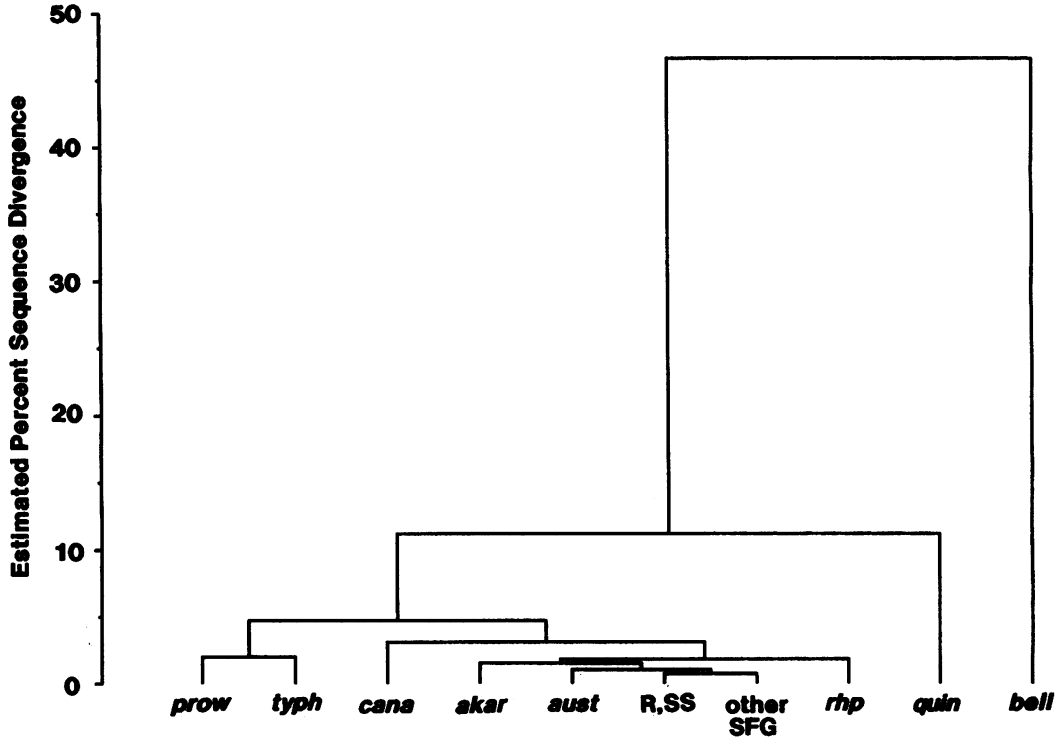


FIG. 9. Dendogram of genotypic relationships of rickettsial citrate synthase sequences derived by PCR/RFLP analysis and estimation of percentage of base substitution. SFG species and isolates, with the exceptions of *R. rickettsii* R and Smith (SS) isolates, but including all other *R. rickettsii* isolates as well as *R. conorii*, *R. slovaka*, *R. sibirica*, and *R. montana* are represented as other SFG. All other abbreviated species identifiers are the same as in the legends to Fig. 1 and 3.

differences in gene function. Whole genomic DNA cross-hybridization experiments can be used to account for similarities between entire genomes (22, 23). However, as noted earlier, practical considerations limit the usefulness of genomic hybridization as a technique for comparing potentially large numbers of closely related rickettsial isolates. In lieu of the practical problems associated with measuring total genome relatedness, rapid characterization and identification of rickettsial genotypes must rely on techniques that are reflections of total genome relatedness.

How well do the PCR/RFLP data reflect accepted concepts of rickettsial taxonomy? The data from citrate synthase genotypic numerical analysis support previous phenotypic and genetic findings that *R. bellii* is not closely related to either SFG or typhus group rickettsiae (Fig. 6 and 9) (27). The genotypic relationship of *R. bellii* to other members of the genus, derived from the numerical analysis of the citrate synthase sequences, suggests that this organism may be more distantly related to other members of the genus *Rickettsia* than is *Rochalimaea quintana*, which belongs to another genus. However, one must exercise caution when interpreting such numerical data from more distantly related species when the number of comigrating fragments are inherently few (38).

The citrate synthase genes of *R. australis* and *R. akari* appear to be distinct from each other and both species are obviously closely aligned with the SFG rickettsiae. However, neither *R. australis* nor *R. akari* was primed by the 190-kDa antigen primers which successfully primed all other recognized SFG rickettsiae; therefore, it was not possible to include these two distinct SFG species with other SFG agents when making estimates of the genetic divergence within the 190-kDa antigen gene. *R. canada* citrate synthase sequences seem to be intermediate between the well-defined SFG and the typhus group; however, the *R. canada* sequences appear to be more closely related to the SFG (with which this species shares several properties, including ticks as vectors and passage through nuclear membranes). DNA hybridization studies support the status of *R. canada* as distinct from either the SFG or typhus group (23). Analysis of additional genes should permit increasingly accurate estimates of overall relatedness of rickettsial species and isolates. Thus, the citrate synthase-based PCR/RFLP genotypic analysis correlates well with other accepted taxonomic schemes for the rickettsiae.

Among SFG members, <2.3% sequence divergence was estimated for citrate synthase gene sequence and <3.2% sequence divergence was noted for the SFG 190-kDa antigen sequences studied (Fig. 6 and 8) (*R. akari* and *R. australis* were not included in 190-kDa antigen sequence analysis). Unweighted-pair analysis may overemphasize the relationship of the 190-kDa sequences of *R. rickettsii* and *R. slovaka* (Fig. 10). The estimated genotypic relationship between the sequences of these two isolates is no closer than the relationship between several other combinations of SFG species (Fig. 8 and 10). However, the degrees of estimated divergences between *R. rickettsii* and *R. slovaka* and most other SFG isolates studied are approximately equivalent, thereby contributing to the apparent statistical link between the DNA sequences of the two agents.

Although both of the *R. rickettsii* isolates from Montana had distinctive PCR/RFLP citrate synthase sequence and *Alu*I digest patterns (comparable to a 2- to 4-bp deletion) and are regarded as virulent isolates, other isolates from clini-

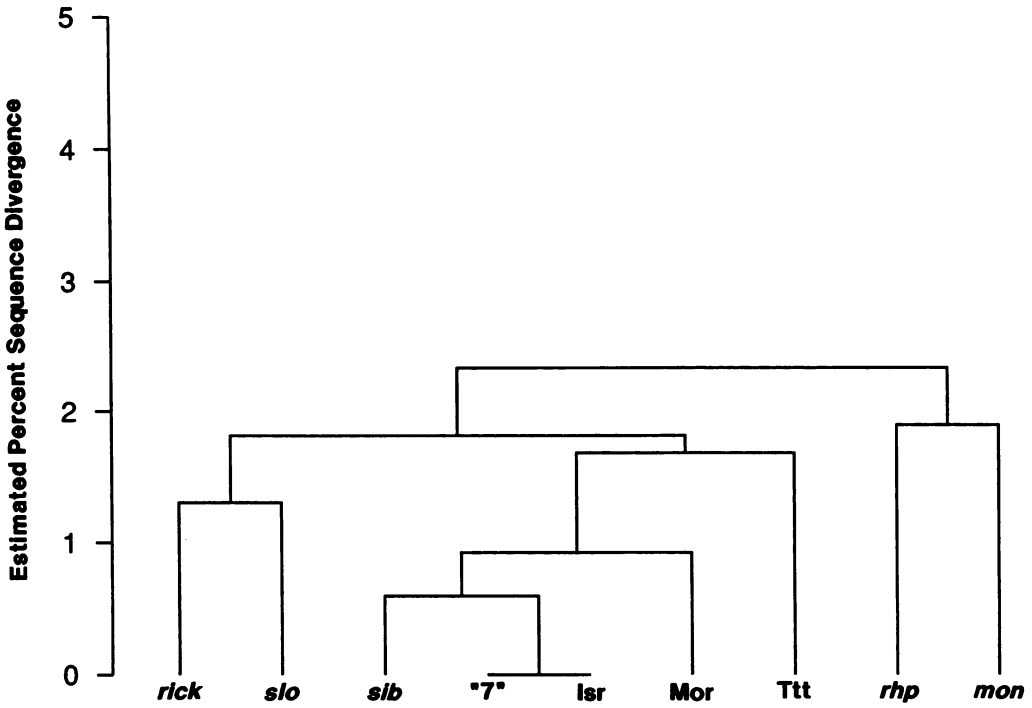


FIG. 10. Dendogram of genotypic relationships of SFG sequences coding for a large antigen protein (190-kDa antigen) derived by PCR/RFLP analysis and estimation of percentage of base substitution. Abbreviated species and isolate identifiers are the same as in the legend to Fig. 3.

cally severe cases of Rocky Mountain spotted fever did not display this variation in PCR/RFLP pattern. This observation does not support the possibility that this subtle PCR/RFLP difference could be directly correlated with a difference in pathogenic genotypes. Additional isolates from the Rocky Mountain region, as well as additional genetic sequences, must be analyzed before the possible significance of a region-specific *R. rickettsii* genotype can be considered.

Genotypic differences within the gene coding for the 190-kDa antigen, noted between the HLP and other isolates of *R. rickettsii*, appear to correlate well with differences noted previously for the HLP isolate regarding the reactivity of monoclonal antibodies reactive for the same protein (3). Further evaluation of the relative similarities between additional genes of the HLP isolate and the genes of other SFG agents is needed to clarify the subtle, but apparently distinct, genotypic or phenotypic character of this isolate.

The genotypic differences noted between the Moroccan isolate, #7 isolate, and Israel tick typhus (Fig. 3, 4, 8, and 10) appear to mirror the subtle serologic nonidentity reported to occur among the same Mediterranean SFG group rickettsial isolates (16).

*R. montana* and *R. rhipicephali*, two rickettsial species recovered only from ticks and not believed to cause human disease, are closely allied with the recognized pathogenic SFG rickettsiae by genotypic analysis. The citrate synthase PCR/RFLP patterns of *R. montana* are indistinguishable from other SFG isolates; the isolate of *R. rhipicephali* had a single restriction site difference noted for digests of the same sequence (a *Dde*I-*Sca*I double digest). The estimated sequence divergence for the 190-kDa antigen gene sequences was ≤3% for both of these isolates when compared with other SFG agents. Nevertheless, digests of PCR-amplified 190-kDa antigen sequences were distinctive for both species.

Our findings show that estimates of genetic similarities and differences among rickettsial species, obtained by comparing citrate synthase gene PCR/RFLP patterns, correlate well with other accepted concepts concerning relative relatedness of rickettsial species; the differences noted between digest patterns from the 190-kDa antigen gene of closely related SFG rickettsiae are at least diagnostic. Intraspecies PCR/RFLP patterns appear to be stringently conserved. Data derived from the PCR/RFLP analysis of discrete genetic sequences can be used as meaningful taxonomic markers for identification of both recognized and novel rickettsial species.

The PCR/RFLP technique for rickettsial characterization is relatively simple, free of most subjective interpretation, and free from many sources of experimental "deviation" experienced, for example, with DNA hybridization experiments. Specific sequence information is not required before a novel isolate can be characterized, and purification of rickettsiae is also not required. The PCR/RFLP methods bypass the requirements for isotope-labeled probes or Southern blots. After a small amount of an unknown isolate becomes available, identification should be readily possible and, if desired, the DNA from various isolates can be analyzed simultaneously. Due to the reproducibility of the system, and with the inclusion of appropriate DNA size standards, it should be readily possible for laboratories equipped to do PCR and gel electrophoresis to identify directly rickettsial species-specific DNA fragment size patterns by comparison with established reference patterns. Other communications will provide examples of genotypic identification of a cluster of human pathogenic rickettsiae and recognition of a rickettsial genotype from an unexpected geographical region (13a, 13b).

Ultimately, if sufficient PCR signal amplification sensitiv-

ity can be demonstrated, it should be possible to identify rickettsiae directly from field specimens and tissue samples accurately without any cultivation and traditional rickettsial isolation. Until then, these PCR/RFLP methods offer a convenient alternative to serologic testing or other nucleic acid methods for identification of rickettsial isolates. The increased ease of accurate isolate identification should encourage further ecologic and epidemiologic studies that may have been frustrated in the past, in part, by the inability to identify multiple rickettsial isolates easily and reliably.

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