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Genotypic evaluation of rickettsial isolates recovered from various species of ticks in Portugal

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SUMMARY

Twelve rickettsial isolates, from *Rhipicephalus sanguineus*, *R. turanicus*, *Dermacentor marginatus* and *Hyalomma marginatus*, were subjected to genotypic analysis. Amplification of specific DNA sequences, restriction endonuclease digestion of amplified DNA products, and gel electrophoresis were used to identify specific DNA fragment-banding patterns. Five patterns were resolved. Four were homologous with those of previously described rickettsial genotypes, *R. conorii*, *R. slovaca*, *R. rhipicephali* and *R. massiliae*. The fifth pattern differed by only a single altered restriction endonuclease cleavage site. For the first time in Portugal a widely distributed spectrum of spotted fever group rickettsia was found among potential vector species stressing the need to determine their potential for human and domestic animals infection.

INTRODUCTION

Until relatively recently *Rickettsia conorii* was thought to be the only spotted fever group rickettsia (SFGR) of significant public health importance in most of western Europe and the Mediterranean region. *R. sibirica* occurs in more north-easterly areas of the former Soviet Union. *R. slovaca* and *R. helvetica* had been described as isolates from ticks collected within limited geographic regions and these organisms ability to infect humans was not well established [1–3]. Antigenic and pathogenic differences had been noted from isolates of *R. conorii* made in Israel [4, 5].

In Portugal, boutonneuse fever, presumably caused by *R. conorii*, is relatively common: over 1000 human cases are reported per year [6]. Self-prescribed antibiotic treatment is common and it is suspected that a large proportion of

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human infections go unreported [7, 8]. SFGR isolates have been made in Portugal in the past [9], however no recent reports have combined isolation with definitive identification of the etiologic agent(s). Some attempts have been made to establish the prevalence and distribution of rickettsia-like organisms (RLO) in potential tick vector species. Several species of ticks exist in relatively large numbers in close proximity to rural human populations. Domestic mammals including cattle, sheep, goats, and dogs, are common in the rural environment and in spring and summer these animals may become highly parasitized with ticks; domestic animals not only amplify the numbers of ticks but help to bring the ticks into close proximity to humans [10–12].

Spotted fever group rickettsiae are morphologically similar. They are also known to have significant cross-reactive antigenic determinants, and human convalescent antisera are well known for serological cross-reactivity among members of the SFGR. For these reasons, neither traditional serology nor morphologic analysis of rickettsiae from ticks can be relied upon to differentiate closely related SFGR. Estimation of the prevalence and distribution of human pathogenic SFGR, as judged by serological response and morphological evaluation of tick hemolymph, may be influenced by the existence of other, perhaps non-pathogenic, rickettsiae that may occur within the ecosystem(s).

To better understand the role of various *Ixodidae* tick species as potential vectors of human spotted fever, a programme for collection and evaluation of ticks for presence of rickettsial agents was initiated at the National Health Institute (CEVDI), Águas de Moura, Portugal. Recently described methods were used to isolate rickettsiae from ticks [13] as well as polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis (RFLP) of conserved rickettsial genetic material for differentiation of rickettsial isolates [14]. This report summarizes the results from this study and contrasts them with those from other regions.

MATERIALS AND METHODS

Tick collection

Ticks were collected from various animal hosts and vegetation in ecologically distinct areas throughout the year. Adult male and female non-engorged ticks were collected by flagging the vegetation adjacent to paths frequented by cattle, goats and sheep. Flagging was continued on a weekly basis over a 2-year period [12]. Ticks were also collected directly from domestic (cattle, horses, goats, sheeps, dogs) and wild (foxes, wild pig) mammals during vaccination programmes and hunting seasons [11].

Rickettsial isolation

Hemolymph samples from individual ticks were stained by the Giménez method [15, 16]. Only ticks with rickettsial-like organisms (RLO) were processed for isolation using a slightly modified shell-vial technique [13].

Briefly, each tick was disinfected by immersion in iodated alcohol for 5 min, then washed in sterile water, and dried on sterile filter paper under a laminar flow hood. A drop of hemolymph was obtained by sectioning the distal portion of the first leg, and mixed with 250 ml of Eagle's minimum essential medium with 2·2 g

of $NaHCO_2$ per litre, 2 mM glutamine and 8% bovine serum without antibiotics (this medium will be referred to as complete MEM). This material was transferred to a 48 h-old Vero E6 cell monolayer growing on the flat bottom of a 4 ml glass vial. The vials were centrifuged at 5000 rpm for 45 min at 20 °C. The supernatant was discarded and 3 ml of complete MEM added. The vials were incubated at 32 °C for at least 5 days. After this primary incubation, the cells were scraped from the vial and the cell suspension placed on a 25 cm^3 flask containing a second Vero E6 monolayer. Complete MEM (5 ml) was added, and the cell cultures reincubated for 7 days. Rickettsial growth was evaluated by removing some cells from the flask. These were stained by the Giménez method or tested against a human positive control serum to *R. conorii* in an indirect fluorescent-antibody test. Isolates were further passaged 2 or 3 times in Vero cells, and then stored frozen at −80 °C. During the isolation procedure cultures were tested for bacterial and fungal sterility and any contaminated isolate discarded.

Rickettsiae type strains

R. conorii (ATCC VR-613) strain # 7, *R. conorii* (ATCC VR-141) strain Moroccan, *R. slovaca* and *R. rhipicephali* (both a gift from the Centers for Disease Control, Atlanta, Georgia, U.S.A.) were used as standard strains.

DNA extraction, amplification and restriction-endonuclease digestion

The procedure for cultivation of rickettsia (isolates and standards strains) to extract DNA, the PCR amplification and the DNA digestion and electrophoresis were done as previously described [14]. PCR–RFLP was performed by using the following oligonucleotides primers pairs (available from CDC) and restriction endonucleases (New England Biolabs, Beverly, Mass): RpCS.877p and RpCS.1258n (encoding a 381 bp-sequence) digested with *Alu*I and Rr190.70p and Rr190.602n (532 bp-sequence) digested with *Alu*I, *Rsa*I and *Pst*I.

Restriction-endonuclease fragment length polymorphism analysis

Digested DNA fragments were separated by electrophoresis on pre-cast, 6% polyacrylamide gels (Novex, San Diego, Calif) [17]. The gels were stained with ethidium bromide and photographed by transillumination with ultraviolet light. Easily discernible DNA bands were recorded; their migration in gels and the size of amplified DNA bands calculated [17].

RESULTS

A total of 3328 ticks, representing 12 tick species were collected in Portugal during 1991–2 (Table 1). Ninety-eight ticks, positive in the hemolymph test for RLO, were processed for isolation (Table 2). Thirty isolates of RLO were obtained; 12 isolates, representing isolates from three genera of ticks obtained from different mammalian hosts on different dates and from different collection sites (Table 3; Fig. 1), were characterized in detail by PCR/RFLP genotypic analysis.

All 12 isolates yielded PCR products of an appropriate size for rickettsial organisms when primed with oligonucleotides originally derived from the citrate synthase gene of *R. prowazekii* (Fig. 2) [14]. When digested with restriction-

Table 1. *Ticks collected in Portugal during 1991/2*

Species	Female	Male	Total
Boophilus annulatus	32	4	36
Dermacentor marginatus	241	305	546
Haemaphysalis punctata	32	110	142
Hyalomma marginatum	48	159	207
H. lusitanicum	31	50	81
Ixodes ricinus	17	16	33
I. simplex	6	0	6
I. vespertilionis	8	6	14
Rhipicephalus sanguineus	659	828	1487
R. bursa	150	237	387
R. turanicus	95	133	228
R. pusillus	95	66	161
Total	1414	1914	3328

Table 2. *Ticks with positive hemolymph test and total of rickettsial isolates*

Species	Positive ticks	Isolates
Dermacentor marginatus	35	18
Haemaphysalis punctata	3	0
Hyalomma marginatum	26	1
Rhipicephalus sanguineus	29	8
R. turanicus	5	3
Total	98	30

Table 3. *Data about the ticks and isolates characterized by PCR/RFLP*

Isolate code	Tick species	Sex	Collection site*	Collection date	Host
PoTiR10	*Dermacentor marginatus*	Female	Marateca(1)	21.04.91	Vegetation
PoTiR4	*D. marginatus*	Male	Pinheiro(2)	17.10.91	Vegetation
PoTiR5	*D. marginatus*	Male	Pinheiro	13.11.91	Vegetation
PoTiR6	*D. marginatus*	Male	Pinheiro	11.10.91	Vegetation
PoTiR14	*D. marginatus*	Male	Pinheiro	21.10.91	Vegetation
PoTiR15	*D. marginatus*	Male	Pegões(3)	26.01.92	Horse
PoTiR8	*Hyalomma marginatum*	Female	São Domingos(4)	02.04.92	Cow
PoTiR3	*Rhipicephalus sanguineus*	Female	Palhota(5)	13.08.91	Vegetation
PoTiR1	*R. sanguineus*	Female	Setúbal(6)	15.04.92	Dog
PoTiR12	*R. sanguineus*	Male	Padeiras(7)	30.04.92	Dog
PoTiR11	*R. turanicus*	Male	Grândola(8)	03.04.92	Cow
PoTiR9	*R. turanicus*	Male	Grândola	03.04.92	Cow

* See Fig. 1.

endonuclease *Alu*1 and subjected to electrophoresis on polyacrylamide gels, the resultant RFLP patterns were readily separable into two categories. Eight (PoTiR4, PoTiR5, PoTiR6, PoTiR8, PoTiR10, PoTiR12, PoTiR14 and PoTiR15) of the 12 isolates RFLP patterns were identical to one another and likewise identical to the RFLP patterns derived from reference isolates of *R. conorii* and other closely related SFGR (Fig. 2) [14]. Four (PoTiR1, PoTiR3, PoTiR9 and PoTiR11) of the RFLP patterns from isolates made from *Rhipicephalus* sp. were unlike the other eight *Alu*l digest patterns (Fig. 2).

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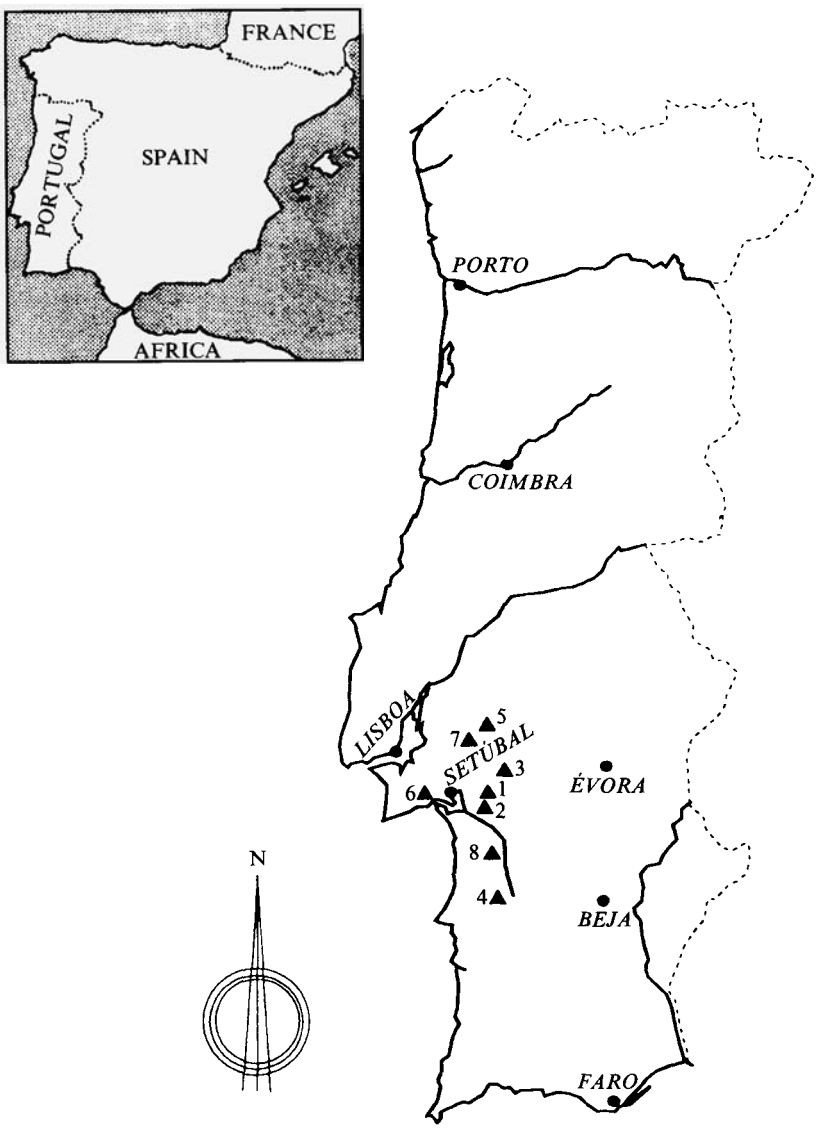

Fig. 1. Map showing areas (▲) where ticks were collected.

RLO isolates were analysed further using PCR and oligonucleotide primers derived from a DNA sequence of *R. rickettsii* known to code for a large 190 kiloDalton (kDa) protein antigen [14, 18]. These PCR products were digested individually with three different restriction-endonucleases (*Rsa*I, *Alu*I, and *Pst*I) and the resulting DNA fragments resolved on polyacrylamide gels (Figs. 3–5).

Isolate PoTiR12, made from *Rhipicephalus sanguineus*, had PCR/RFLP patterns for each amplification/digestion that were indistinguishable from the # 7 type strain of *R. conorii* (Figs. 2–5). The same, approximately 10 bp, difference between the sizes of Rr190.70p-Rr190.602n-primed products from the *R. conorii* Moroccan strain and other closely related SFG isolates were observed [14].

Isolates PoTiR4, PoTiR5, PoTiR6, PoTiR10, PoTiR14, and PoTiR15, were

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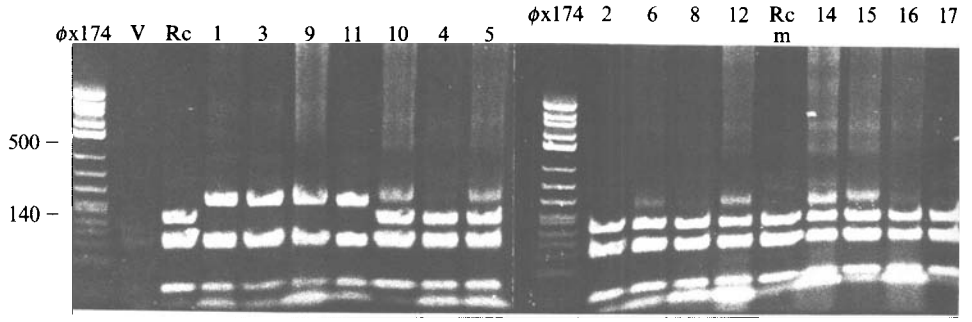

Fig. 2. Ethidium bromide-stained polyacrylamide gel of *Alu*I restriction endonuclease-digests of rickettsial DNA amplified by using *Rp*.CS.877p-CS.1258n primer pairs. φx174, DNA molecular size marker cleaved with *Hinf*I. V, Vero E cells DNA. Rc, *Rickettsia conorii* # 7. Rcm, *R. conorii* Morroccan strain. Lanes 1, 3, 12, Portuguese ticks (PoTi) isolates from *R. sanguineus*. Lanes 9, 11, PoTi isolates from *R. turanicus*. Lanes 4, 5, 6, 10, 14, 15, PoTi isolates from *D. marginatus*. Lane 8, PoTi isolate from *H. marginatum*. PCR/RFLP DNA prepared at different time from the same isolates can be seen as lane 2, 8 and 16 from *H. marginatum* and lane 17 is *R. conorii* # 7.

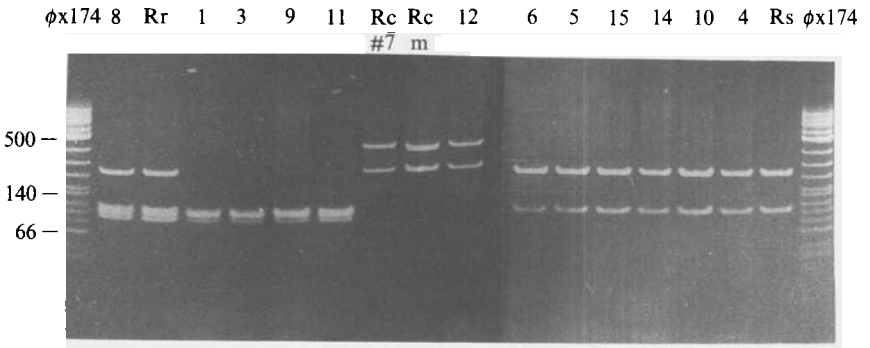

Fig. 3. Ethidium bromide-stained polyacrylamide gel of *Rsa*I restriction endonuclease-digests of rickettsial DNA amplified by using *R.r.*, 90.70p-190.602n. Rs, *R. slovaca*; Rr, *R. rhipicephali* (Other abbreviated identifiers are the same as in the legend of Fig. 2).

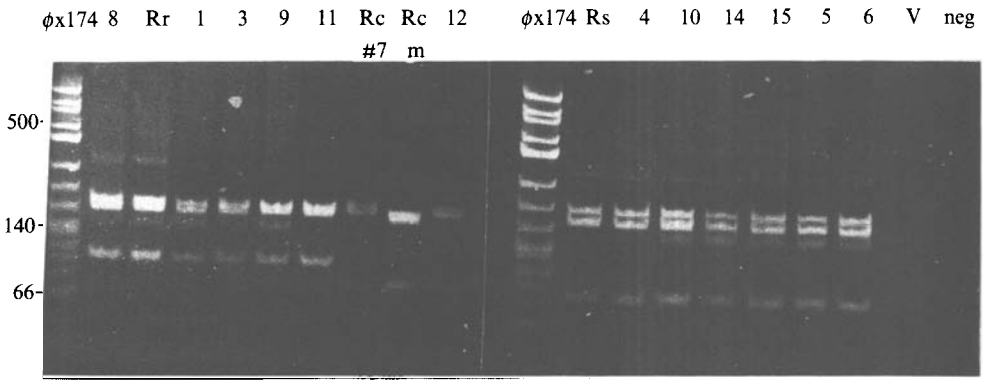

Fig. 4. Ethidium bromide-stained polyacrylamide gel of *Alu*I restriction endonuclease-digests of rickettsial DNA amplified by using *R.r.*190.70p-190.602n. neg, negative control (Abbreviated identifiers are the same as in the legend of Fig. 3).

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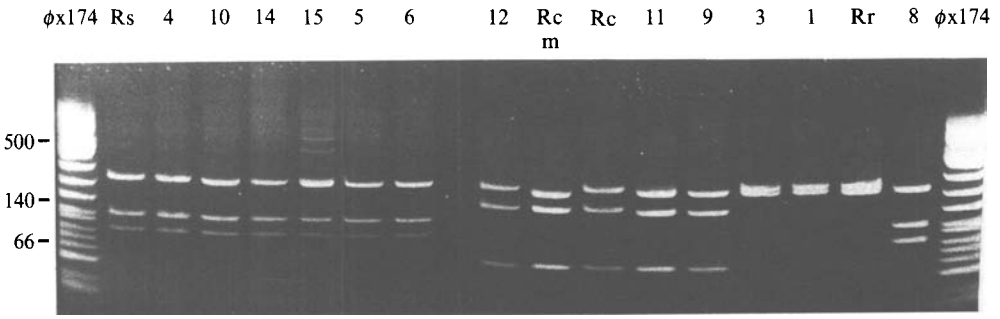

Fig. 5. Ethidium bromide-stained polyacrylamide gel of *Pst*I restriction endonuclease-digests of rickettsial DNA amplified by using *R.r*.190.70p-190.602n. (Abbreviated identifiers are the same as in the legend of Fig. 3).

made from *Dermacentor marginatus*, and had PCR/RFLP patterns identical with the comparable digests made from the type strain of *R. slovaca* (Figs. 2–5).

Isolate PoTiR8, made from a *Hyalomma marginatum* tick, had PCR/RFLP patterns similar to, but not identical, to those described for *R. rhipicephali* [14]. The PCR/RFLP digestion pattern which distinguishes this European isolate from the isolate of *R. rhipicephali* described from North America is visualized in the *Pst*I digest of amplified DNA from the 190 kDa antigen gene (Fig. 5). Three fragments 262, 152 and 118 bp instead of the expected 290 and 262bp yielded by *R. rhipicephali*.

Isolates PoTiR9 and PoTiR11 were indistinguishable from the french Mtu1 recently named *R. massiliae* [19], when *Alu*1 digests of the amplified DNA from the citrate synthase gene (Fig. 2) were considered, together with *Rsa*I and *Pst*I digests of amplified 190 kDa gene DNA (Fig. 3, 5). *Alu*I enzyme digest of amplified 190 kDa gene DNA was not used in the description of the French isolates (Fig. 4).

Additional Portuguese isolates, PoTiR1 and PoTiR3, had *Alu*I digests of the amplified citrate synthase sequence and RsaI and AluI digests of the amplified 190 kDa antigen gene similar to PoTiR9 and PoTiR11 (Fig. 2). However, isolates PoTiR1 and PoTiR3 had *Pst*I digestion patterns unlike the other isolates. *Pst*I digests of the DNA from these Portuguese isolates apparently lack a restriction endonuclease recognition site found in similar isolates made in both Portugal and France (Fig. 5).

DISCUSSION

This analysis demonstrates several important aspects for the understanding of SFGRs in Portugal and perhaps elsewhere in Europe.

PCR/RFLP patterns from a single rickettsial-like isolate, PoTiR12 (recovered from the tick *R. sanguineus*), were identical to that ascribed to the prototype isolate of the Mediterranean spotted fever, *R. conorii*, type # 7. Human isolates of this genotype, made from persons diagnosed with Mediterranean spotted fever in southern France, have been identified by using very similar genotypic methods [20; Drancourt, personal communication]. The results of this study demonstrate that this apparently pathogenic genotype is not the predominant SFGR isolate present in *Ixodidae* ticks in Portugal. The genotypic differences noted between the intraspecies of *R. conorii*-group only mirror the subtle serological and pathogenic

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non-identity reported to occur among the Mediterranean SFG rickettsial isolates [4, 5, 14].

The six rickettsial isolates made from *D. marginatus* (PoTiR4, PoTiR5, PoTiR6, PoTiR10, PoTiR14, and PoTiR15), were all of the *R. slovaca* species genotype [14]. The original isolates of this species were described in Slovakia and, recently, in ticks in southern France [21]. *D. marginatus* will feed on humans in Portugal [22] although the potential for this agent to induce disease in humans remains unknown [2]. We have found that *R. slovaca* is prevalent in *D. marginatus* in Portugal, thus extending the recognized range of this SFGR species. Further evaluation of human isolates of SFGRs from Portugal and elsewhere is warranted since the results may shed additional light on the prospect of human infections with *R. slovaca* and acquired SFGR immunity.

Isolate PoTiR8, made from *H. marginatum*, had PCR/RFLP patterns apparently identical in three of four restriction endonuclease digests to that described for *R. rhipicephali*, a SFGR originally isolated in North America from *R. sanguineus* [14, 23]. Isolates with potentially identical genotypic markers have been described from *R. sanguineus* ticks in southern France [20]. To date, no isolates conforming to *R. rhipicephali*-like genotypic characteristics have been recognized among human SFGR isolates in North America or Europe. The natural history of this organism deserves further study to confirm their identity to *R. rhipicephali*.

Isolates PoTiR9 and PoTiR11, made from *Rhipicephalus turanicus*, appear identical to two examples of *Rickettsia massiliae* isolated in southern France (Mtu1 and Mtu11) [19, 24].

PoTiR1 and PoTiR3 isolates, made from *R. sanguineus*, possessed PCR/RFLP patterns similar to other, previously described European SFGR (e.g., the French Mtul and Mtu11 isolates) as well as the Portuguese isolates PoTiR9 and PoTiR11. However, digestion of amplified 190 kDa antigen gene DNA from PoTiR1 and PoTiR3 yielded patterns apparently lacking a restriction–endonuclease site found in the most closely related digestion patterns (e.g., isolates PoTiR9 and PoTiR11, Fig. 5). Although isolates PoTiR1 and PoTiR3 displayed the same combination of genotypic markers, they were obtained from ticks at different collection sites and on different dates (Table 3). This suggests that the subtle PCR/RFLP differences observed between this genotype and other genotypes were not the result of a isolated mutational event (for example, perhaps during cultivation in the laboratory) and that this genotype may be stable and genetically distinct from closely related organisms.

In the original description of PCR/RFLP methods as applied to rickettsial species, each species could be readily distinguished by only 2 different PCR amplifications and at most 3 or 4 different restriction endonuclease digestions [14]. In this same study, different human isolates of *R. rickettsii* were shown to possess monotypic PCR/RFLP patterns regardless of geographic origin and severity of human disease. However, the resurgence in interest in rickettsial isolates from ticks in Europe [19, 21, 24], the Middle East [5, 25], and Africa [26] has demonstrated that considerable genotypic variability exists in RLO isolates from ticks. As noted in the example given above most of SFGR genotypes have been isolated independently in different laboratories which support the concept that

the genotypes represent stable genetic entities. The taxonomic and epidemiological significance of this genetic variability has yet to be elucidated and is hampered by lack of standardized criteria for the designation of rickettsial species. Hopefully matters will become clear as the new laboratory techniques are developed.

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