Journal of General Virology (2003), **84**, 465–473 DOI 10.1099/vir.0.18765-0

Phylogenetic relationships among members of the genus *Phlebovirus* (*Bunyaviridae*) based on partial M segment sequence analyses

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Viruses in the *Phlebovirus* genus of the family *Bunyaviridae* cause clinical syndromes ranging from a short, self-limiting febrile illness to fatal haemorrhagic fever. The genus currently consists of 68 antigenically distinct virus serotypes, most of which have not been genetically characterized. RT-PCR with four 'cocktail' primers was performed to amplify a region of the M segment of the genome of 24 phleboviruses included in the sandfly fever Naples, sandfly fever Sicilian and Punta Toro serocomplexes. Partial M segment sequences were successfully obtained and phylogenetic analysis was performed. The three resultant genotypic lineages were consistent with serological data. The sequence divergences were 27·6 % (nucleotide) and 25·7 % (amino acid) within the Sicilian serocomplex, 33·7 % (nucleotide) and 34·4 % (amino acid) within the Naples serocomplex and 35·6 % (nucleotide) and 37·5 % (amino acid) within the Punta Toro serocomplex. Overall, the diversities among viruses of Sicilian, Naples and Punta Toro serocomplexes were 48·2 % and 57·6 % at the nucleotide and amino acid levels, respectively. This high genetic divergence may explain the difficulties in designing a consensus primer pair for the amplification of all the phleboviruses using RT-PCR. It also suggests that infection with one genotype may not completely immunize against infection with all other genotypes in a given serocomplex. These findings have implications for potential vaccine development and may help explain clinical reports of multiple episodes of sandfly fever in the same individual.

Received 9 August 2002
Accepted 15 October 2002

INTRODUCTION

Viruses in the genus *Phlebovirus* (*Bunyaviridae*) are of considerable public health importance, as they can cause a variety of clinical syndromes ranging from a brief, self-limiting febrile illness to retinitis, encephalitis, meningoencephalitis and fatal haemorrhagic fever (Bartelloni & Tesh 1976; Laughlin *et al.*, 1979; Meegan *et al.*, 1979; Peters & Slone, 1982; Tesh, 1988; Nicoletti *et al.*, 1991; Braito *et al.*, 1998). Rift Valley fever (RVF) virus is the type species for the genus. Phleboviruses have been isolated in southern Europe, Africa, Central Asia and the Americas. Eight phleboviruses (Alenquer, Candiru, Chagres, sandfly fever Naples, Punta Toro, Rift Valley fever, sandfly fever Sicilian and Toscana) have been linked to diseases in humans. These viruses contain a negative-sense, single-stranded RNA genome, consisting of three segments, designated large (L), medium (M) and small (S) (Elliott, 1990). The S segment exhibits an ambisense coding strategy and encodes the N and NSs proteins from mRNAs of opposite polarities (Giorgi *et al.*, 1991). The M segment of viruses in the phlebotomus fever group, but not viruses in the Uukuniemi group, has a pre-glycoprotein coding region that encodes a non-structural protein (NSm) (Elliott *et al.*, 2000). The L segment encodes a polypeptide in the viral complementary sense, which is a putative RNA-dependent RNA polymerase (Elliott *et al.*, 1992; Accardi *et al.*, 1993; Muller *et al.*, 1994).

The *Phlebovirus* genus currently consists of 68 distinct virus serotypes, most of which have not been genetically characterized. These viruses are antigenically unrelated to members of other genera within the family *Bunyaviridae*, but they show various degrees of cross-reactivity among themselves in serological tests, such as haemagglutinin-inhibition (HI) tests and complement-fixation (CF) tests (Tesh *et al.*, 1976, 1982; Travassos da Rosa *et al.*, 1983). The 68 known phleboviruses are divided into two major antigenic groups: the phlebotomus fever (or sandfly fever) group with 55 members and the Uukuniemi group with

The sequences obtained in this study have been deposited in GenBank under the accession numbers AY129732–AY129752.

13 members. The phlebotomous fever group is further divided into 13 serocomplexes and 12 members not assigned to any serocomplex. These include the eight serocomplexes listed in the *Seventh Report of the International Committee on Taxonomy of Viruses* (Elliott *et al.*, 2000) and five additional serocomplexes recently defined in our laboratory, namely Aguacate, Chargres, Arboledas, Arumowot and Tapara (A. P. A. Travassos da Rosa, unpublished data). The phlebotomus fever viruses are transmitted by sandflies, mosquitoes or ceratopogonids of the genus *Culicoides*, whereas the Uukuniemi group viruses are transmitted by ticks (Elliott *et al.*, 2000).

Because of the paucity of genetic information about the phleboviruses, viruses within this genus are presently defined by their serological relationships. Antigenic relationships between the viruses as determined by HI tests are used for genus placement, relationships by CF tests for serogroup and complex assignment, and plaque reduction neutralization tests (PRNTs) for serotype and subtype differentiation (Tesh *et al.*, 1982; Travassos da Rosa *et al.*, 1983). The current antigenic (serological) classification of the phleboviruses is unsatisfactory for the following reasons: (i) to date, a total of 68 phlebovirus species have been discovered and undoubtedly many more exist in nature; (ii) based on their genetic structure, abundance and complex patterns of antigenic cross-reactivity, it is probable that natural reassortment occurs among some of these viruses, confounding their antigenic classification and identification; (iii) some of the phleboviruses do not produce readable plaques under agarose and do not readily infect or kill common laboratory animals, making reagent production and antigenic comparisons difficult; and (iv) relatively few research laboratories are still capable of performing the 'classical' serological tests necessary for characterization of phleboviruses. Therefore, other techniques are needed to study and characterize fully this large and diverse genus.

In the present study, we have searched for relatively homologous regions in the M segment sequence of selected viruses of the phlebovirus genus and designed primers to amplify cDNA products using RT-PCR. The products were sequenced and phylogenetic analyses were carried out based on the nucleotide sequences of the RT-PCR products. The cluster pattern of the viruses studied matched the grouping by serological methods; thus, we believe that the technique can serve as a framework for taxonomic placement of other uncharacterized or yet to be discovered phleboviruses.

METHODS

Viruses. The 26 phleboviruses that were included in this study are listed in Table 1 along with their host source, geographical origin and year of isolation. These viruses were obtained from the World Arbovirus Reference Center at the University of Texas Medical Branch, Galveston, Texas, USA. The sandfly fever Naples prototype (Sabin) strain was propagated in BHK cells; all other viruses were propagated in Vero E6 cells. The viruses were harvested when the culture demonstrated a cytopathic effect of 3 to 4+.

RNA extraction. Total RNA was extracted from the infected cell monolayer with Trizol reagent (Invitrogen) following the manufacturer's instructions. After phase separation with Trizol/chloroform, RNA was precipitated with isoproponol, washed with 75 % ethanol and briefly air-dried. The RNA pellet was dissolved in RNase-free water and stored at −80 °C.

DNase I treatment and reverse transcription. RNA was treated with Amplification Grade RNase-free DNase I (Invitrogen) before reverse transcription to eliminate DNA contamination. The reaction included 1 μg total RNA, 1 unit Amplification Grade RNase-free DNase I, 20 mM Tris/HCl, pH 8·3, 50 mM KCl and 2 mM $MgCl_2$, in a total volume of 10 μl. After incubation at room temperature for 15 min, the reaction was terminated by adding 1 μl 25 mM EDTA, heated for 10 min at 65 °C and then chilled on ice. The reaction mixture was used directly for reverse transcription. dNTPs (0·01 μmol; Sigma) and 0·1 μg random hexamers (Promega) were added into the 11 μl terminated DNase I reaction mixture, followed by heating at 65°C for 5 min and quick chilling on ice. Four μl 5 × buffer (250 mM Tris/HCl, pH 8·3, 375 mM KCl, 15 mM $MgCl_2$), 0·2 μmol DTT, 40 units RNaseOUT recombined ribonuclease inhibitor (Invitrogen) and 50 units SuperScript II RNase H^- Reverse Transcriptase (Invitrogen)] were added; the final volume was 20 μl. The reaction was incubated at 25 °C for 10 min followed by 42 °C for 60 min and terminated at 70 °C for 15 min.

Primer design. The amino acid sequences deduced from the available nucleic acid sequences of the M segment of Punta Toro (PT) virus, Rift Valley fever (RVF) virus, Toscana (TOS) virus and sandfly fever Sicilian (SFS) virus (see Table 1 for GenBank accession numbers) were aligned with the CLUSTAL W program (Fig. 1). Four conserved regions (designated 1–4) were selected for primer location. Initially, degenerate primers were synthesized according to the most conserved bases from these regions, but these primers failed to amplify most of the viruses. Subsequently, a different approach was used. Individual oligonucleotides specific for each of the sequences were synthesized from these same regions. These specific primers from one region were pooled as a 'cocktail' primer for RT-PCR. The sequences and position of primers are listed in Table 2. As shown, the forward primer Ph-M-2FM 'cocktail' consisted of Ph-M-2F-PT, Ph-M-2F-RVF, Ph-M-2F-SFS and Ph-M-2F-TOS, which corresponded to sequences of PT, RVF, SFS and TOS at the conserved region 2, respectively. The reverse primer Ph-M-3RM mix was initially composed of Ph-M-3R-PT, Ph-M-3R-RVF, Ph-M-3R-SFS and Ph-M-3R-TOS, which corresponded to sequences of PT, RVF, SFS and TOS viruses at the conserved region 3, respectively. Later, as more sequences were obtained during the study, two additional oligonucleotides were made and added to this 'cocktail' as new components, namely, Ph-M-3R-ELB and Ph-M-3R-27. Another reverse primer, Ph-M-4R2I, corresponding to the conserved region 4, was synthesized using inosine to reduce the degeneracy of the primer. In addition, another forward primer mix, Ph-M-1FM, was designed for conserved region 1. It consisted of Ph-M-1TF and Ph-M-1SF, specific for Toscana and Sicilian viruses, respectively. This primer mix (Ph-M-1FM) was paired with Ph-M-3RM to amplify the Naples prototype (Sabin) virus.

Specific primers were designed according to the newly obtained sequences of the Naples complex (except for the Naples prototype virus) and Sicilian complex viruses (Table 3) as the project progressed. Viruses with similar sequences shared the same primers. These specific primers were used to reamplify the individual viruses, to ensure specificity and authenticity of the original RT-PCR.

Table 1. Source of isolation, geographical origin, year of isolation and GenBank accession numbers of the phleboviruses used in this study

Virus type (strain)	Source	Geographic origin	Year	GenBank accession no.
(a) Sandfly fever group				
Sicilian serocomplex				
Sicilian sandfly fever (Sabin)	Human	Sicily, Italy	1943	U30500
Sicilian-like (I-91025 B)	*Phlebotomus* species	Isfahan, Iran	1975	
Sicilian-like (91045 I)	*Phlebotomus* species	Isfahan, Iran	1975	
Sicilian-like (RM-09)	Human	Cyprus	1985	
Sicilian-like (R-18)	*Phlebotomus papatasi*	Cyprus	1985	
Sicilian-like (I-701735)	*Phlebotomus* species	Aurangabad, India	1970–1971	
Corfou (Pa Ar 814)	*Phlebotomus major*	Corfou, Greece	1981	
Naples serocomplex				
Sandfly fever Naples (Sabin)	Human	Naples, Italy	1944	
Naples-like (POONA 7101795)	Human	Aurangabad, India	1970	
Naples-like (YU 8-76)	*Phlebotomus perfiliewi*	Yugoslavia	1976	
Naples-like (R-3)	Human	Cyprus	1985	
Tehran (I-47)	*Phlebotomus papatasi*	Tehran, Iran	1950	
Toscana (ISS PHL 3)	*Phlebotomus perniciosus*	Toscany, Italy	1971	X89628
Toscana (ELB)	Human	Portugal	198?	
Naples-like (NAMRU 840055)	Human	Egypt	1985?	
Punta Toro serocomplex				
Punta Toro (Balliet)	Human	Panama (western)	1966	M11156
Punta Toro (Adames)	Human	Panama (eastern)	1972	
Buenaventura (Co Ar 3319)	*Lutzomyia* species	Colombia	1964	
Co Ar 171616	*Lutzomyia* species	Colombia	1987	
Co Ar 170255	*Lutzomyia* species	Colombia	1984	
Pa Ar 2381	*Lutzomyia sanguinaria*	Panama	1975	
GML 902878	Sentinel hamster	Panama	1976	
VP-334K	*Lutzomyia* species	Panama	1970	
VP-366G	*Lutzomyia* species	Panama	1970	
Rift Valley fever serocomplex				
Rift Valley fever (ZH-501)*	Human	Egypt	1977	M11157
(b) Uukuniemi group				
Uukuniemi (S23)*	*Ixodes ricinus*	Finland	1960	M17417

*Rift Valley fever (ZH-501) and Uukuniemi (S23) viruses were not examined by RT-PCR, but sequences were included as outgroups in the phylogenetic analysis.

RT-PCR and sequencing of amplicons. For first-strand cDNA synthesis, random hexamers, instead of the forward primer, were used. The PCR reaction started with a non-stringent annealing temperature of 40 °C for five cycles, followed by 35 cycles with the annealing temperature at 50 °C. The total volume of each PCR reaction was 50 μl and contained 2 μl cDNA, 40 pmol each of the forward and reverse primers, 400 μM of each dNTP (Sigma), 2·5 units *Taq* DNA polymerase (Sigma), 10 mM Tris/HCl, pH 8·3, 50 mM KCl and 2·5 mM $MgCl_2$, overlaid with 50 μl mineral oil. The PCR reaction was carried out as follows: initial denaturing was at 94 °C for 5 min; the first five cycles were 94 °C for 1 min, 40 °C for 1 min and 72 °C for 2 min; the next 35 cycles were 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min; and finally 72 °C for 7 min after the last cycle. Amplified products were detected by staining with ethidium bromide following electrophoresis in an agarose gel. The PCR products were purified from the gel with a QIAquick Gel Purification Kit (Qiagen), following the manufacturer's instructions, and were sequenced with PE Biosystem's 373XL automated DNA sequencer. Both strands of each PCR product were sequenced. The same procedures (from RNA extraction to sequencing) were repeated for some of the samples to eliminate possible cross-contamination. In doing these confirmations, specific primers were used for amplification; the RT-PCR conditions were similar to those with 'cocktail' primers, except that 10 pmole of each of the forward and reverse primer were used. The cycles for these latter reactions were: initial denaturing at 94 °C for 5 min; 35 cycles at 94 °C for 1 min; 50 °C for 1 min; 72 °C for 1 min; and finally, 72 °C for 7 min after the last cycle. Products amplified using these specific primers were again detected and sequenced as described above.

Sequence and phylogenetic analyses. The sequences were examined with BLASTX in the MacVector 7.0 program (Accelrys) to search for the homologous sequences in GenBank. All nucleotide sequences of the amplified viruses and available sequences from GenBank were aligned with CLUSTAL W in the MacVector 7.0 program.

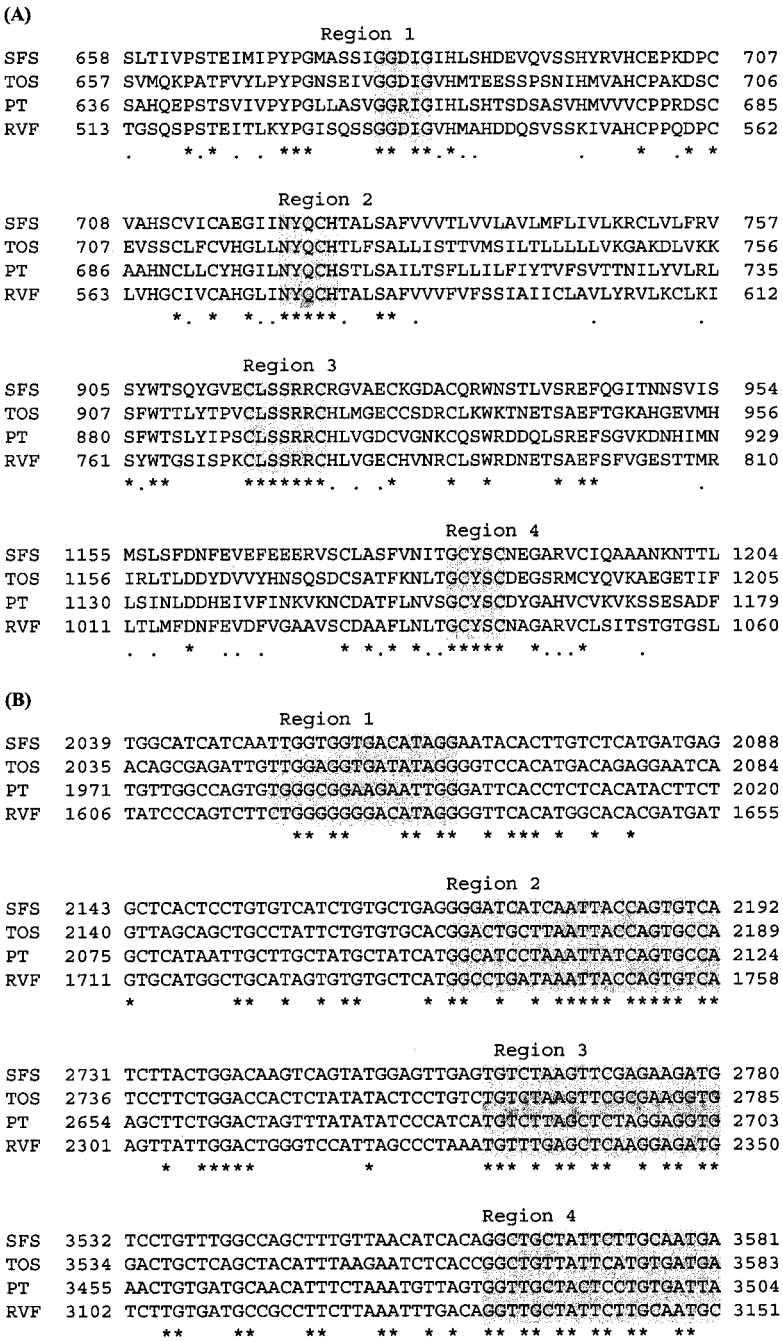

Fig. 1. (A) Partial alignment of M segment amino acid sequences of the Sabin strain of sandfly fever Sicilian (SFS) virus, the ISS PHL3 strain of Toscana (TOS) virus, the Balliet strain of Punta Toro virus (PT) and the ZH-501 strain of Rift Valley fever (RVF) virus. Multiple alignment parameters: open gap penalty = 10·0, extend gap penalty = 0·0; delay divergent = 40 %; gap distance = 8; similarity matrix: blosum. (B) Partial alignment of M segment nucleotide sequences of SFS, TOS, PT and RVF viruses. Multiple alignment parameters: open gap penalty = 10·0, extend gap penalty = 5·0; delay divergent = 40 %; transitions: weighted. The conserved regions 1–4 correspond to regions in the amino acid sequence alignment shown in (A).

Phylogenetic analyses were carried out with PAUP 4.0 Beta version (Swofford, 2002). A bootstrap consensus tree was generated by the neighbour-joining method. The number of bootstrap replicates was 1000. The M segment sequences of Uukuniemi virus and RFV virus were used as outgroups (see Table 1 for GenBank accession numbers).

RESULTS

PCR amplification of the partial M segment genome

For primer design, the M segment sequences of four phleboviruses (PT, RVF, TOS and SFS) were retrieved from GenBank as described above; four regions of high homology (conserved regions) were identified by both amino acid sequence and nucleic acid sequence alignment. Initially, consensus primers were designed, based on the regions with high sequence homology and either by choosing the most plausible bases for the non-conserved positions or by using a 'degenerate' base at these positions. RT-PCR reactions were subsequently carried out, but amplification only occurred with a few of the viruses. After several unsuccessful attempts, we used another approach, based on the assumption that most phleboviruses belong to one of the 13 serocomplexes and that viruses belonging to a given serocomplex most likely share very

Table 2. 'Cocktail' primers (bold) and their components used for RT-PCR amplification of a partial M segment of the phleboviruses

Name	Sequence (5′→3′)	Position (nt)
Ph-M-1FM	WRTTGGWGGTGAYATAGG	
Ph-M-1TF	AATTGGAGGTGATATAGG	TOS 2049–2066
Ph-M-1SF	TGTTGGTGGTGACATAGG	SFS 2053–2070
Ph-M-2FM	GGVMTSMTHAATTAYCAGTGYCA	
Ph-M-2F-PT	GGCATCCTAAATTATCAGTGCCA	PT 2102–2124
Ph-M-2F-RVF	GGCCTGATAAATTACCAGTGTCA	RVF 1737–1759
Ph-M-2F-SFS	GGGATCATCAATTACCAGTGTCA	SFS 2170–2192
Ph-M-2F-TOS	GGACTGCTTAATTACCAGTGCCA	TOS 2166–2188
Ph-M-3RM	CAYCTYCKNGARCTNARRCA	
Ph-M-3R-PT	CACCTCCTAGAGCTAAGACA	PT 2703–2684
Ph-M-3R-RVF	CATCTCCTTGAGCTCAAACA	RVF 2350–2331
Ph-M-3R-SFS	CATCTTCTCGAACTTAGACA	SFS 2780–2761
Ph-M-3R-TOS	CACCTTCGCGAACTTAGACA	TOS 2785–2766
Ph-M-3R-ELB	CATCTTCTTGAGCTTAAGCA	
Ph-M-3R-27	CACCTTCTGGAACTGAGGCA	
Ph-M-4R2I	TCATCGCAAGARTARCAICC	PT 3504–3485
		RVF 3151–3132
		SFS 3581–3562
		TOS 3583–3564

Table 3. Specific primers for Naples and Sicilian serocomplex viruses

Virus	Primer pair	Primer sequence (5′→3′)
Sicilian-like (I-91025 B), Sicilian-like (I-91054)	Ph-M-22-F, Ph-M-22-R	TGCATTTGTAGTCGTGAC, TGAGTGGTCCAGTAAGA
Sicilian-like (RM-09), Sicilian-like (R-18)	Ph-M-24-F, Ph-M-24-R	CGCATTCGTTGTTGTAAC, CTGACTTGTCCAATAAGA
Sicilian-like (I-701735)	Ph-M-27-F, Ph-M-27-R	CGCATTTGTTGTTGTAAC, TTGACTTGTCCAGTAAGA
Corfou (PA AR 814)	Ph-M-Corfou-F, Ph-M-Corfou-R	TGCTTTTGTGGTCTGCAC, TTGTGAAGTCCAGTAGGA
Tehran (I-47), Naples-like (YU 8-76)	Ph-M-Naples-Yu-F, Ph-M-Naples-Yu-R	GCTCTGCTGGTCTCTACTGT, AAACAAGACTGGAGAGTATAG
Naples-like (NAMRU-840055), Naples-like (POONA 7101795) Naples-like (R-3)	Ph-M-Naples-R3-F, Ph-M-Naples-R3-R	GCGCTCCTTCTGTCTACTAT, AGACAGACTGGAGTGTATAA
Toscana (ELB)	Ph-M-TosELB-F, Ph-M-TosELB-R	GCATTGCTCATATCAACTAC, AAGCAGATGGGTGTGTATAG

high sequence homology in these conserved regions. We assumed that primers designed for the conserved regions of a given serocomplex should be able to amplify most viruses within that serocomplex. Furthermore, it was assumed that specific primers for PT, RVF, TOS and SFS viruses, if mixed as a 'cocktail,' would amplify the majority of phlebovirus isolates regardless of their serocomplex. The two primer constructs, Ph-M-2FM (forward) and Ph-M-3RM (reverse) (Table 2), amplified 22 of the 24 viruses studied, with an expected product size of 600 bp. For the remaining two viruses, primers Ph-M-2FM and Ph-M-4R2I were used to amplify the Co Ar 170255 virus (product length 1400 bp), and primers Ph-M-1FM and Ph-M-3RM were used to amplify the Naples prototype (Sabin) virus (product length 730 bp).

To ensure the fidelity of the PCR products, we designed a specific primer pair for each of the Naples and the Sicilian

complex viruses, based on the sequences obtained for each virus; these specific primer pairs (Table 3) were used to amplify the individual viruses again. All of these reactions generated products with sequences that matched those obtained with the 'cocktail' primers. Therefore, the 'cocktail' approach of designing primers to amplify these phleboviruses appears to be reliable.

Sequence analysis

All sequences obtained from the PCR-amplified products were deposited in GenBank. A BLASTX search confirmed that these sequences were homologous to M segment sequences of phleboviruses previously available from GenBank.

Phylogenetic analyses based on the partial M segment nucleotide sequences

Since all of the PCR products encompassed a common 600 bp region of the M segment, the sequences of this region (between primers Ph-M-2FM and Ph-M-3RM) from these viruses were utilized for alignment, using the CLUSTAL W program of the MacVector software. Phylogenetic analysis was carried out by the neighbour-joining procedure, using the PAUP 4.0 program (Beta version 8). The resultant phylogenetic tree is shown in Fig. 2. Phylogenic analysis of these viruses resulted in three distinct genotypic lineages, corresponding to the Sicilian, Naples and Punta Toro complexes, which are shown as lineages I, II and III, respectively. The percentage differences of amino acid and nucleic acid sequences among the different groups of phleboviruses are listed in Table 4.

DISCUSSION

The M segment of the phlebotomus fever group viruses encodes two glycoproteins: G1 and G2, and a non-structural protein, NSm. As with other members of the *Bunyaviridae*, the phlebovirus envelope glycoproteins are important for viral infection, pathogenesis and immunity; they serve as neutralizing and haemagglutinin-inhibiting antibody targets (Keegan & Collett, 1986; Battles & Dalrymple, 1988; Pifat *et al.*, 1988; Besselaar & Blackburn, 1991). In general, neutralizing epitopes depend on the tertiary structure of proteins and even a single amino acid substitution may be important for biological activity (Schmaljohn *et al.*, 1990; Horling & Lundkvist, 1997). Glycoproteins are present on the surface of virions and thus are exposed to the selective pressures of the host. It is probable that these proteins vary noticeably from one strain to another, particularly for those epitopes that are important for reaction with antibodies. Consequently, genetic analyses based on the M segment of phleboviruses may provide a more sensitive approach to classification than would analyses based on the S segment. Also, since the most sensitive serological method of differentiating phleboviruses is the PRNT (results of antibody reactions with the surface glycoproteins), it seems probable that M segment analyses should provide the best correlation with results obtained by PRNT. Nevertheless, a combined approach based on serological and genetic data is still the best one for classification of these viruses.

In order to characterize genetically the many phleboviruses in our reference collection, we wanted to develop a method to amplify any phlebovirus by RT-PCR. We first attempted to find a 'universal' primer pair, as was done in the original phylogenetic study for hantaviruses (Xiao *et al.*, 1992, 1994). However, this approach did not work, due to the high sequence divergence among the phleboviruses as later discovered in this study (Table 4). Nevertheless, the second approach, which used 'cocktail' primers (Table 2), allowed us to amplify successfully the vast majority of the viruses examined.

As shown in Fig. 2, the phylogenetic clustering of individual viruses studied corresponded well with their serological groupings. Lineage I consisted of the Sicilian-like viruses and Corfou virus. Viruses isolated from the same localities and in the same year were closely related, but not identical. By CF test, Corfou cross-reacted with sandfly fever Sicilian virus (unpublished data). It was compatible with the position of Corfou in this lineage.

Lineage II consisted of viruses in the sandfly fever Naples serocomplex, which were distributed into three branches: Toscana prototype (ISS PHL3) and Toscana (ELB); Tehran and Yu 8-76; and Naples prototype (Sabin), Poona 7101795, R-3 and NAMRU-840055. No geographical clustering could be inferred from this group.

Lineage III consisted of Punta Toro serocomplex viruses. All members of this group were isolated from South America. In one branch, Punta Toro virus strains Adames, Pa Ar 2381, GML 902878 and Balliet, all from Panama, were grouped together. The two viruses, Pa Ar 2381 and GML 902878, isolated from Bayano, Panama, in 1975 and 1976, respectively, were very similar; the difference between their nucleotide sequences was 0·2 %, while their amino acid sequences were identical. Balliet virus, an isolate from western Panama, is less virulent in hamsters than Adames, an isolate from eastern Panama (Anderson *et al.*, 1990); the percentage differences between their partial M segment nucleotide and amino acid sequences were 11 % and 6 %, respectively. Another PT branch contained three viruses from Colombia and two from Panama.

As shown in Fig. 2 and Table 4, the sequence diversities within a serogroup and among the different serogroups were variable and larger than those of many other viruses. For example, the sequence difference between Corfou and the other members in the Sicilian serogroup was up to 42 %, yet by both CF test and phylogenetic analysis, they fall into the same serogroup or lineage. Similar diversities were seen in all the other serogroups.

In summary, phleboviruses from both the Old and New Worlds group into different lineages. The positions of

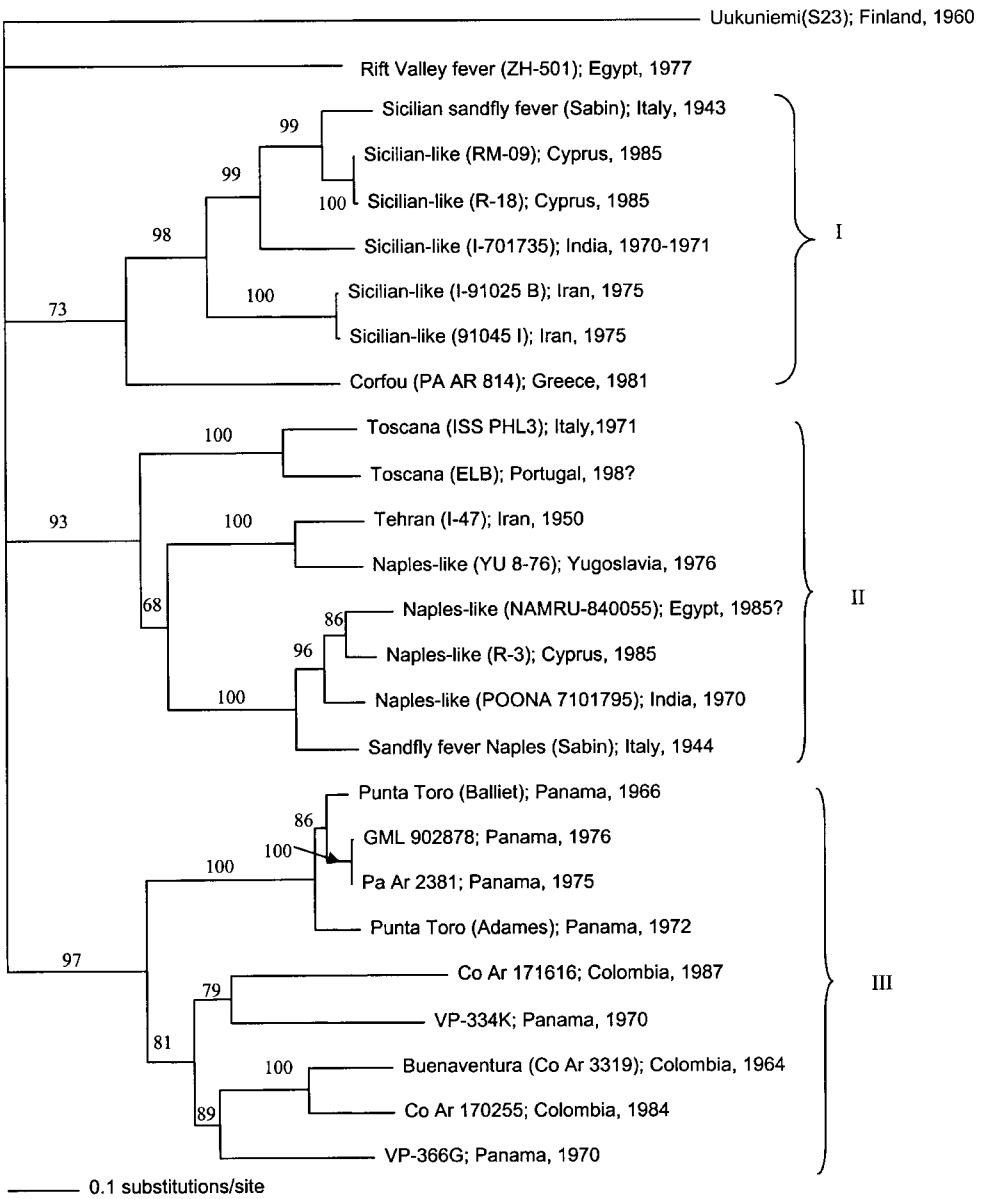

Fig. 2. Phylogeny by the neighbour-joining method, based on nucleotide sequences. Uukuniemi and Rift Valley fever viruses were set as outgroups. Ties were broken randomly. Distance measure = HKY 85. Rates for variable sites were equal. 'Missing data' sites (including gaps) and ambiguous codes were distributed proportionally to unambiguous changes. All substitutions were estimated or counted. Objective function was minimum evolution. Negative branch length was allowed and set to zero when calculating tree scores. Bootstrap method with neighbour-joining search was carried out with the same options. Number of bootstrap replicates = 1000. Number of characters resampled in each replicate = 558. Groups with frequency > 65 % were retained. The numbers attached to branches are bootstrap values. A value of 0·1 substitutions per site is equivalent to 10 % changes.

phleboviruses in the phylogenetic tree are not necessarily related to their geographical distribution. Furthermore, the geographical distributions of some phleboviruses overlap (Tesh *et al.*, 1976). Consequently, viruses present in the same area could theoretically co-infect a single host and produced reassortants.

The approach of using a mixture or 'cocktail' of specific primers, representing different groups of viruses, appears to be an effective alternative to the previously used 'consensus' primer; the method is particularly useful for virus genera that have high genetic diversity, such as the phleboviruses. We are currently using the same approach

Table 4. Sequence differences observed among phleboviruses based on partial M segment sequences

Comparison	Nucleotide difference (%)*			Amino acid difference (%)*		
	Range	Mean	SD	Range	Mean	SD
Within Sicilian serocomplex	0·6–42·0	27·6	13·0	0·6–46·4	25·7	14·8
Within Naples serocomplex	10·0–44·7	33·7	12·2	5·0–48·9	34·4	17·1
Within Punta Toro serocomplex	0·2–46·2	35·6	13·6	0–50·3	37·5	17·1
Between Sicilian and Naples serocomplexes	50·8–57·7	54·5	1·5	64·9–70·4	67·3	1·2
Between Sicilian and Punta Toro serocomplexes	51·2–59·7	54·8	2·1	64·4–74·4	68·4	2·8
Between Naples and Punta Toro serocomplexes	52·7–60·0	55·7	1·5	64·6–74·0	68·9	2·0
Among Sicilian, Naples and Punta Toro serocomplexes	0·2–60·0	48·2	12·6	0–74·4	57·6	18·7
Sicilian, Naples and Punta Toro serocomplexes to RVFV	50·5–57·3	54·4	1·5	60·0–69·3	64·6	3·1
Sicilian, Naples and Punta Toro serocomplexes and RVFV to UUK	59·5–66·4	63·7	1·8	74·5–83·1	79·4	2·3

*Mean character differences (adjusted for missing data) multiplied by 100.

for amplification of the S and L segment sequence of these viruses.

ACKNOWLEDGEMENTS

The authors are grateful to Drs Scott C. Weaver, Xue-Jie Yu, and Juliet E. Bryant for helpful discussions on the phylogenetic analyses, and Dora Salinas for assistance in preparing the manuscript.

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