

Phylogenetic relationships of phlebotomine sandflies inferred from small subunit nuclear ribosomal DNA

A. M. Aransay, E. Scoulica, Y. Tselentis and
P. D. Ready¹

Laboratory of Clinical Bacteriology, Parasitology,
Zoonoses & Geographical Medicine, Faculty of Medicine,
University of Crete, Heraklion, Greece, and ¹Molecular
Systematics Laboratory, Department of Entomology, The
Natural History Museum, London, UK

Abstract

Relationships among seventy specimens, fifteen species and three genera of phlebotomines were inferred from the phylogenetic analysis of small subunit nuclear rDNA, obtained by the PCR amplification and cloning of almost full-length genes. Outgroups included fifteen dipterans, and single representatives of four other insect orders. The more distant the taxa compared, the larger were the regions of ambiguous sequence alignment that needed to be deleted in order to avoid circularity in performing parsimony analyses. Phlebotomine sequences formed a monophyletic clade within the suborder Nematocera, with the progressively more basal sister groups of Diptera being Culicomorpha, Tipulomorpha and the suborder Brachycera. Within Phlebotominae, subgeneric relationships were resolved and the genus *Phlebotomus* was shown to be monophyletic, but markers for intraspecific geographical populations were not found and intergeneric relationships were not resolved.

Keywords: SSU rDNA, phlebotomines, Diptera, phylogeny.

Introduction

Phlebotomine sandflies (Diptera: Psychodidae, Phlebotominae) are often studied in relation to their role as the natural vectors of parasitic protozoa of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae). However, there have been few phylogenetic analyses of relationships among

sandfly taxa or between sandflies and other insects. For many specialists the subfamily Phlebotominae comprises six genera: *Phlebotomus*, *Sergentomyia* and *Chinius* in the Old World (OW) and *Lutzomyia*, *Brumptomyia* and *Warileya* in the New World (NW) (Lewis *et al.*, 1977), and all the *Leishmania* vectors are usually placed in *Phlebotomus* and *Lutzomyia* (Killick-Kendrick, 1990) (Authorities for all higher taxa and OW species are given by Seccombe *et al.* (1993)). However, a recent phylogenetic analysis of morphological characters led to the conclusion that the OW species should be classified in seven genera (*Chinius*, *Phlebotomus*, *Australophlebotomus*, *Idiophlebotomus*, *Spelaeophlebotomus*, *Sergentomyia* and *Spelaeomyia*), although the monophyly of *Sergentomyia* was questioned (Rispail & Léger, 1998a,b).

Concerning higher taxonomic levels, the family Psychodidae is classified in the rather heterogeneous infraorder Psychodomorpha within the suborder Nematocera, the other dipteran suborder being Brachycera (Hennig, 1981; Wood & Borkent, 1989). The Diptera and other orders of holometabolous insects are easily distinguished morphologically, but phylogenetic relationships among them have not been fully resolved (Kristensen, 1991; Oosterbroek & Courtney, 1995).

Comparative analyses of nuclear ribosomal (r) RNA genes have been used to infer phylogenetic histories across a broad spectrum of taxa, from the basal lineages of life to relationships among closely related species and populations (Hillis *et al.*, 1996). The small subunit (SSU) nuclear gene (16–18S rRNA) has been most studied, partly because it contains some of the slowest evolving sequences in living organisms and therefore has proven useful for examining ancient evolutionary events (Hillis & Dixon, 1991). In addition, the slow rate of change of some of these sequences permits the design of universal primers which, along with the gene's high copy number, facilitate polymerase chain reaction (PCR) amplification of SSU rDNA fragments from groups of organisms not previously investigated (Hillis *et al.*, 1996). Several phylogenetic studies of the Holometabola have been based on SSU rDNA (Carmean *et al.*, 1992; Miller *et al.*, 1997; Whiting *et al.*, 1997; Carreno & Barta, 1998).

Although there are often several hundred copies of SSU rDNA per haploid genome in Diptera, the pool of coding

Received 7 May 1999; accepted 27 September 1999. Correspondence:
Ana M. Aransay, Molecular Systematics Laboratory, Department of Entomology, The Natural History Museum, Cromwell Road, London SW7 5BD, UK. E-mail: a.aransay@nhm.ac.uk

sequences is thought to remain homogeneous because of concerted evolution resulting from unequal crossing over or gene conversion (Arnheim *et al.*, 1980; Dover, 1982; Arnheim, 1983; Coen & Dover, 1983). Some intraspecific sampling is still advisable (Williams *et al.*, 1988) and, in this connection, it was reassuring that many restriction sites were diagnostic for phlebotomine species regardless of geographical origins (Aransay *et al.*, 1999).

The present report on the SSU rDNA sequences of sandflies progresses beyond PCR-RFLP diagnostics (Aransay *et al.*, 1999) to analyse phylogenetic relationships. The specific aims were to determine whether or not there is concordance between the species' relationships inferred from SSU rDNA and morphological characters, to discover whether full sequence information might provide markers for geographical populations, and to relate sandflies to other holometabolous insects. Our study concentrated on taxa found in Greece and Cyprus, and for this ten species of the genus *Phlebotomus* and three of *Sergentomyia* were analysed. The only sandfly SSU rDNA sequence reported until now was isolated from *Lutzomyia (Psathyromyia) shannoni* (Dyar) (Miller *et al.*, 1997), and this was added to our SSU rDNA data set along with newly characterized sequences from *Lutzomyia (Lutzomyia) longipalpis* (Lutz & Neiva), the type species of *Lutzomyia*.

Results and discussion

Consensus sequences of specimens and species

PCR with primers F1 and R1 amplified a fragment of about 2000 base pairs (bp) from each of the seventy samples tested (Table 1). All the PCR products were successfully cloned, and complete SSU rDNAs were amplified by PCR from single bacterial colonies before being sequenced on each strand (Fig. 1). For each specimen, complete sequence assembly (from 'contigs') was unambiguous, and consensus sequences ranged from 2024 bp (*L. longipalpis*) to 2120 bp (*P. tobii*) (Table 1).

Consensus sequences for each sandfly species were constructed manually and labelled P(Eu)arg (*P. argentipes*), P(La)tob (*P. tobii*), P(La)perf (*P. perfiliewi*), P(La)pern (*P. perniciosus*), P(La)neg (*P. neglectus*), P(La)syr (*P. syriacus*), P(Ad)sim (*P. simici*), P(Pa)alex (*P. alexandri*), P(Pa)serg (*P. sergenti*), P(Ph)pap (*P. papatasi*), Smin (*S. minuta*), Sdent (*S. dentata*), Sfal (*S. fallax cypriotica*) and Llong (*L. longipalpis*).

Intraspecifically, there were only a few base differences, or none at all between some specimens of *P. simici* (SIM4, SIM5), *P. neglectus* (NEG1, NEG2), *P. alexandri* (ALEX3, ALEX7), *P. sergenti* (SERG1, SERG2, and SERG3, SERG4), *P. papatasi* (PAP1, PAP-b) and *S. minuta* (MIN1, MIN2). PCR error could account for some of the intraspecific polymorphism, but other explanations include stochastic or selective amplification and cloning of

different copies of the SSU rRNA gene (at different loci). However, concerted evolution is expected to homogenize the copies (see Introduction). In order to test the homogeneity of the amplified SSU rRNA genes, PCR products of four samples (Table 1) were directly sequenced. Unique sequences were obtained for each sample, identical to those obtained from the cloned PCR products.

When distant taxa were compared, large ambiguously aligned regions were found in positions occupied by 'expansion segments' in the secondary structure model of *Drosophila melanogaster* (Hancock *et al.*, 1988). However, we are unaware of an algorithm that provides a single secondary structure for any expansion segment within higher taxa of insects. Certainly, based on the predictions of the MFOLD algorithm (GCG 1994), the secondary structure of the expansion segment V4 is variable among tiger beetles (family Cicindelidae) (Vogler *et al.*, 1997) and *Lutzomyia* species (P. D. Ready and J. C. Day, unpublished observations). One solution to this problem is to use an algorithm such as MALIGN (Wheeler & Gladstein, 1994) to optimize sequence alignments. However, we rejected this option for two reasons. Firstly, such algorithms attempt to optimize for the number of changes on a cladogram during sequence alignment and therefore later inferences from parsimony analysis can suffer from circular reasoning. Secondly, phylogenies inferred from expansion segments such as V4 have not been found to be consistent with those from other molecular data because, it was concluded, nonhomologous regions were being aligned (Vogler *et al.*, 1997). For these reasons, we decided to exclude from our comparative sequence analyses regions that could not be unambiguously aligned.

Relationships with other holometabolous insects

The fifteen phlebotomine consensus sequences were aligned with those of fifteen non-psychodid dipterans, one mecopteran, one siphonapteran, one trichopteran and one lepidopteran (Fig. 1; alignment 1, EMBL accession number ds39837). Neither the beginning (nucleotides (nt) 1–40) nor the end (nt 2200–2220) of the SSU rDNA alignment was included in our analyses, because they were not available for all taxa.

A total of 826 bp from nine regions of ambiguously aligned sequences (labelled A to I) was excluded from the phylogenetic analysis (Fig. 1). Using the codes of 'expansion segments' (V1–7) in the secondary-structure model of *Drosophila melanogaster* 18S rRNA (Hancock *et al.*, 1988), our region A (nt 130–137) corresponded to a single-stranded part of V2, B (nt 179–334) to three stems in V2 (E9–1, E9–2, 10), C (nt 719–965), D (nt 1002–1028) and E (nt 1066–1115) to parts of V4, F (nt 1320–1342) and G (nt 1362–1377) to parts of V5, H (nt 1612–1784) to a part of V6, and I (nt 2095–2149) to a part of V7. Regions A–I were delimited by sequences homologous

Table 1. List of the phlebotomine taxa used in this study. (*) Samples whose SSU rDNAs have been directly sequenced from the PCR products, as well as from the cloned PCR products

Specimen code	Species (Sex: 'M' or 'F', or larva 'L ₄ ')	Collection place and date	SSU rDNA GenBank/EMBL	
			Length	Accession no.
ARG1	<i>Phlebotomus (Euphlebotomus) argentipes</i> (F)	India, established colony	2084	AF145133/AJ244359
ARG2	<i>Phlebotomus (Euphlebotomus) argentipes</i> (M)	India, established colony	2082	AF145134/AJ244360
SIM1	<i>Phlebotomus (Adlerius) simici</i> (F)	Athens, 1996	2088	AF145135/AJ244361
SIM2	<i>Phlebotomus (Adlerius) simici</i> (F)	Athens, 1996	2087	AF145136/AS244363
SIM3	<i>Phlebotomus (Adlerius) simici</i> (F)	Athens, 1996	2087	AF145137/AJ244364
SIM4	<i>Phlebotomus (Adlerius) simici</i> (F)	Crete, 1996	2088	AF145138/AJ244365
SIM5	<i>Phlebotomus (Adlerius) simici</i> (F)	Crete, 1996	2088	AF145139/AJ244366
NEG1 (*)	<i>Phlebotomus (Larroussius) neglectus</i> (F)	Athens, 1996	2099	AF145140/AJ244367
NEG2	<i>Phlebotomus (Larroussius) neglectus</i> (F)	Athens, 1996	2099	AF145141/AJ244368
NEG3	<i>Phlebotomus (Larroussius) neglectus</i> (F)	Cefalonia, 1996	2097	AF145142/AJ244369
NEG4	<i>Phlebotomus (Larroussius) neglectus</i> (F)	Cefalonia, 1996	2098	AF145143/AJ244370
NEG5	<i>Phlebotomus (Larroussius) neglectus</i> (F)	Cefalonia, 1996	2098	AF145144/AJ244371
NEG6	<i>Phlebotomus (Larroussius) neglectus</i> (F)	Cefalonia, 1996	2097	AF145145/AJ244372
NEG7	<i>Phlebotomus (Larroussius) neglectus</i> (F)	Crete, 1996	2099	AF145146/AJ244373
NEG8	<i>Phlebotomus (Larroussius) neglectus</i> (F)	Crete, 1996	2098	AF145147/AJ244374
SYR1	<i>Phlebotomus (Larroussius) syriacus</i> (M)	Lebanon, established colony	2098	AF145148/AJ244375
SYR2	<i>Phlebotomus (Larroussius) syriacus</i> (M)	Lebanon, established colony	2098	AF145149/AJ244376
TOB1 (*)	<i>Phlebotomus (Larroussius) tobii</i> (F)	Cyprus, 1996	2096	AF145150/AJ244377
TOB2	<i>Phlebotomus (Larroussius) tobii</i> (F)	Cyprus, 1996	2098	AF145151/AJ244378
TOB3	<i>Phlebotomus (Larroussius) tobii</i> (F)	Cyprus, 1996	2097	AF145152/AJ244379
TOB4	<i>Phlebotomus (Larroussius) tobii</i> (F)	Cyprus, 1996	2096	AF145153/AJ244380
TOB5	<i>Phlebotomus (Larroussius) tobii</i> (F)	Cyprus, 1996	2095	AF145154/AJ244381
TOB6	<i>Phlebotomus (Larroussius) tobii</i> (F)	Cyprus, 1996	2117	AF145155/AJ244382
TOB7	<i>Phlebotomus (Larroussius) tobii</i> (F)	Cyprus, 1996	2120	AF145156/AJ244383
TOB8	<i>Phlebotomus (Larroussius) tobii</i> (F)	Cefalonia, 1996	2098	AF145157/AJ244384
TOB9	<i>Phlebotomus (Larroussius) tobii</i> (F)	Cefalonia, 1996	2094	AF145158/AJ244385
PERF1 (*)	<i>Phlebotomus (Larroussius) perfiliewi</i> (F)	Cyprus, 1996	2100	AF145159/AJ244386
PERF2	<i>Phlebotomus (Larroussius) perfiliewi</i> (F)	Cyprus, 1996	2100	AF145160/AJ244387
PERF3	<i>Phlebotomus (Larroussius) perfiliewi</i> (F)	Cyprus, 1996	2099	AF145161/AJ244388
PERF4	<i>Phlebotomus (Larroussius) perfiliewi</i> (F)	Cyprus, 1996	2101	AF145162/AJ244389
PERF5	<i>Phlebotomus (Larroussius) perfiliewi</i> (F)	Cyprus, 1996	2113	AF145163/AJ244390
PERF6	<i>Phlebotomus (Larroussius) perfiliewi</i> (F)	Cefalonia, 1996	2102	AF145164/AJ244391
PERF7	<i>Phlebotomus (Larroussius) perfiliewi</i> (F)	Cefalonia, 1997	2102	AF145165/AJ244392
PERN1	<i>Phlebotomus (Larroussius) perniciosus</i> (M)	Spain, established colony	2094	AF145166/AJ244393
PERN2	<i>Phlebotomus (Larroussius) perniciosus</i> (M)	Spain, established colony	2096	AF145167/AJ244394
ALEX1	<i>Phlebotomus (Paraphlebotomus) alexandri</i> (F)	Athens, 1996	2095	AF145168/AS244395
ALEX2	<i>Phlebotomus (Paraphlebotomus) alexandri</i> (M)	Athens, 1996	2095	AF145169/AJ244396
ALEX3	<i>Phlebotomus (Paraphlebotomus) alexandri</i> (F)	Cyprus, 1996	2095	AF145170/AJ244397
ALEX4	<i>Phlebotomus (Paraphlebotomus) alexandri</i> (F)	Cyprus, 1996	2092	AF145171/AJ244398
ALEX5	<i>Phlebotomus (Paraphlebotomus) alexandri</i> (F)	Cyprus, 1996	2095	AF145172/AJ244399
ALEX6	<i>Phlebotomus (Paraphlebotomus) alexandri</i> (F)	Crete, 1996	2094	AF145173/AJ244400
ALEX7	<i>Phlebotomus (Paraphlebotomus) alexandri</i> (F)	Crete, 1996	2095	AF145174/AJ244401
SERG1	<i>Phlebotomus (Paraphlebotomus) sergenti</i> (F)	Cyprus, 1996	2097	AF145175/AJ244402
SERG2	<i>Phlebotomus (Paraphlebotomus) sergenti</i> (L4)	Cyprus, 1996	2097	AF145176/AJ244403
SERG3	<i>Phlebotomus (Paraphlebotomus) sergenti</i> (F)	Crete, 1996	2096	AF145177/AJ244404
SERG4	<i>Phlebotomus (Paraphlebotomus) sergenti</i> (F)	Crete, 1996	2096	AF145178/AJ244405
PAP1	<i>Phlebotomus (Phlebotomus) papatasi</i> (F)	Athens, 1996	2095	AF145179/AJ244406
PAP2	<i>Phlebotomus (Phlebotomus) papatasi</i> (F)	Athens, 1996	2095	AF145180/AJ244407
PAP3	<i>Phlebotomus (Phlebotomus) papatasi</i> (F)	Athens, 1996	2096	AF145181/AJ244408
PAP4	<i>Phlebotomus (Phlebotomus) papatasi</i> (F)	Cyprus, 1996	2096	AF145182/AJ244409
PAP5	<i>Phlebotomus (Phlebotomus) papatasi</i> (F)	Cyprus, 1996	2095	AF145183/AJ244410
PAP6	<i>Phlebotomus (Phlebotomus) papatasi</i> (F)	Crete, 1996	2094	AF145184/AJ244411
PAP7	<i>Phlebotomus (Phlebotomus) papatasi</i> (F)	Crete, 1996	2096	AF145185/AJ244412
PAP-a	<i>Phlebotomus (Phlebotomus) papatasi</i> (F)	Israel, established colony	2095	AF145186/AJ244413
PAP-b	<i>Phlebotomus (Phlebotomus) papatasi</i> (M)	Israel, established colony	2095	AF145187/AJ244414
MIN1	<i>Sergentomyia minuta</i> (M)	Athens, 1996	2059	AF145188/AJ244415
MIN2	<i>Sergentomyia minuta</i> (F)	Athens, 1996	2059	AF145189/AJ244416
MIN3	<i>Sergentomyia minuta</i> (F)	Cyprus, 1996	2065	AF145190/AJ244417
MIN4	<i>Sergentomyia minuta</i> (F)	Cyprus, 1996	2057	AF145191/AJ244418
MIN5	<i>Sergentomyia minuta</i> (F)	Cefalonia, 1996	2058	AF145192/AJ244419
MIN7	<i>Sergentomyia minuta</i> (F)	Crete, 1996	2058	AF145193/AJ244420

Table 1. (continued)

Specimen code	Species (Sex: 'M' or 'F', or larva 'L ₄ ')	Collection place and date	SSU rDNA GenBank/EMBL	
			Length	Accession no.
MIN8	<i>Sergentomyia minuta</i> (F)	Crete, 1996	2058	AF145194/AJ244421
DENT1 (*)	<i>Sergentomyia dentata</i> (F)	Cyprus, 1996	2053	AF145195/AJ244422
DENT2	<i>Sergentomyia dentata</i> (F)	Cyprus, 1996	2057	AF145196/AJ244423
DENT3	<i>Sergentomyia dentata</i> (F)	Cefalonia, 1996	2057	AF145197/AJ244424
DENT4	<i>Sergentomyia dentata</i> (F)	Cefalonia, 1996	2057	AF145198/AJ244425
FAL1	<i>Sergentomyia fallax cypriota</i> (F)	Cyprus, 1996	2057	AF145199/AJ244426
FAL2	<i>Sergentomyia fallax cypriota</i> (F)	Cyprus, 1996	2057	AF145200/AJ244427
LONG1	<i>Lutzomyia longipalpis</i> (Lutz & Neiva) (F)	Mexico, established colony	2024	AF145201/AJ244428
LONG2	<i>Lutzomyia longipalpis</i> (Lutz & Nerva) (F)	Mexico, established colony	2025	AF145202/AJ244429

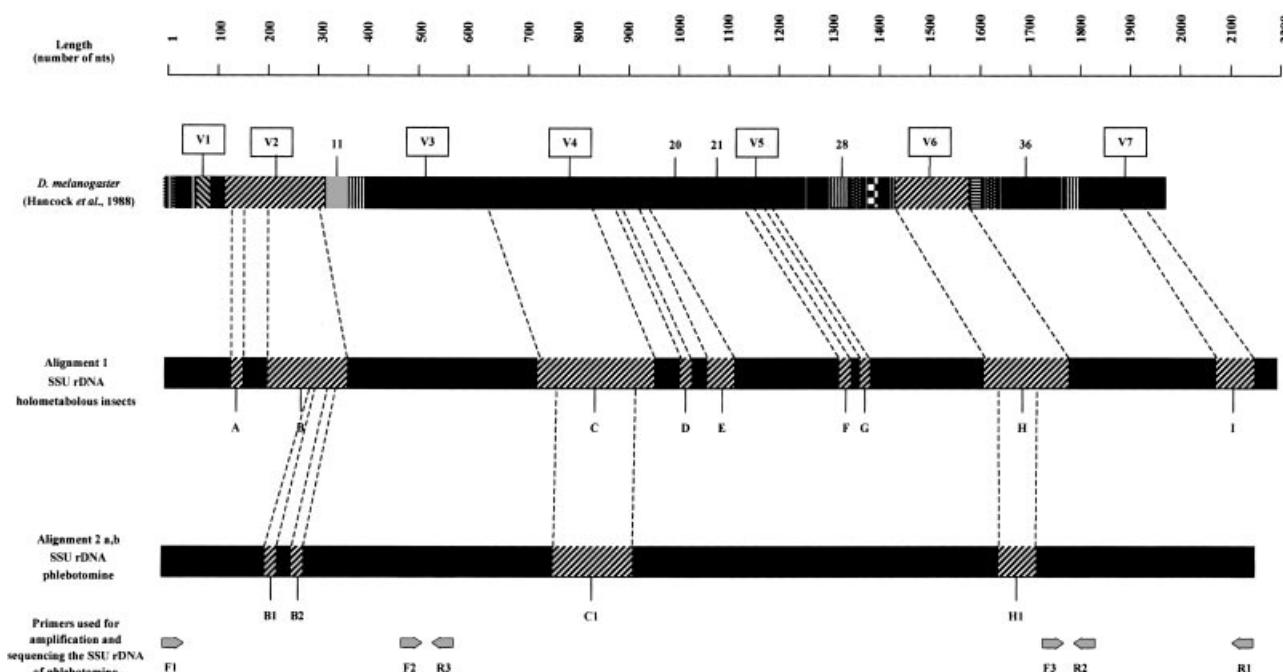


Figure 1. Positions of the variable regions in SSU rDNA alignments. In alignments 1 and 2 (a, b), black areas represent conserved regions where nucleotide sequences were unambiguously aligned between taxa, and other areas represent variable regions. Dashed lines relate the variable regions to those in the model for *D. melanogaster* (Hancock *et al.*, 1988). Primers used for amplification (F1 and R1) and sequencing (F2, F3, R2 and R3) are located within the alignment.

to conserved double-stranded rRNA stems in *D. melanogaster* (Hancock *et al.*, 1988). Excluding regions A–I, values of absolute genetic distances varied from 0.1% (*P. tobii* vs. *P. perniciosus* or *P. argentipes*; *P. neglectus* vs. *P. argentipes*) to 21.1% (*Hydropsyche* sp. (Trichoptera) vs. *Eucorethra underwoodi* (Diptera: Chaoboridae); *Galleria mellonella* (Lepidoptera) vs. *Dixella cornuta* (Diptera: Dixidae)). The average transition/transversion ratio was 1.22 : 1. The lowest ratio (0.45 : 1) was between the culicid dipterans *Aedes albopictus* and *Aedes punctor*, and the highest (4 : 1) was also between culicids (*Culex tritaeniorhynchus* and *Ae. punctor* or *Toxorhynchites amboinensis*). This is not consistent with a higher transition/transversion ratio for more closely related taxa, and

the same is true for our other alignments. Out of 1394 characters analysed phylogenetically, 818 (58.6%) were constant, 252 (18.0%) were variable but parsimony-uninformative, and 324 (23.4%) were variable and parsimony-informative as defined by PAUP 4.0 (Swofford, 1998). When two or more sequences were identical, only one was used in the parsimony analysis (the consensus sequences P(L)syr, P(L)perf and P(L)tob were identical, as were the consensus sequences P(P)alex, P(P)serg and P(P)pap). Maximum parsimony trees were sought using the branch-and-bound option of PAUP 4.0 (Swofford, 1998). The analysis resulted in eighteen equally parsimonious trees when all characters had equal weighting and *Hydropsyche* sp. and *Galleria mellonella* were designated

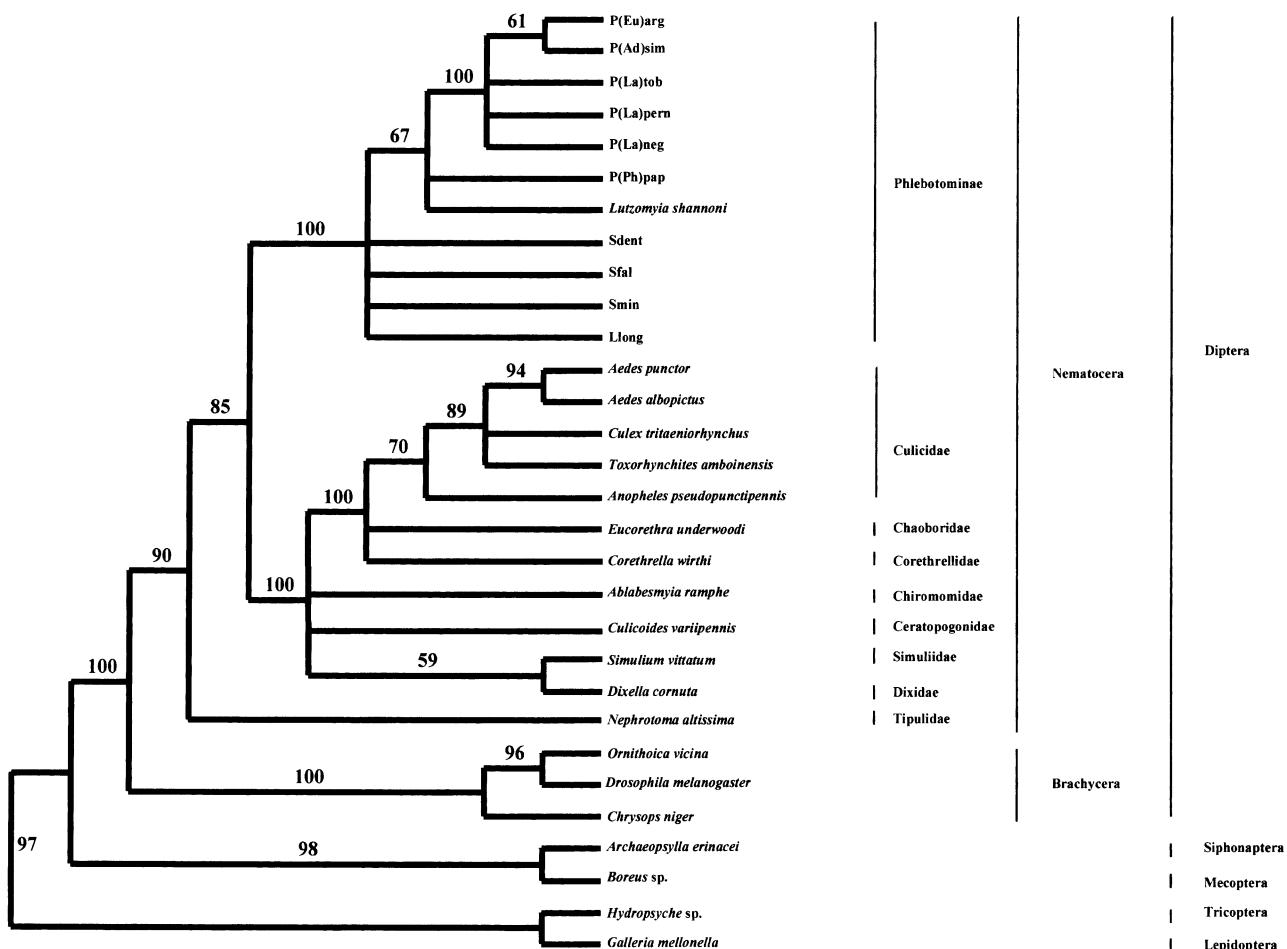


Figure 2. The strict consensus tree (maximum parsimony) for SSU rDNA sequences of holometabolous insects, based on branch-and-bound searches of alignment 1 (EMBL accession number ds39837). Bootstrap values (for those branches shared with 50% majority-rule consensus tree) were calculated with the heuristic search option (100 replicates, each with ten searches using random addition and TBR branch swapping).

as the outgroup (tree length = 1084 steps; retention index (RI) = 0.81, rescaled consistency index (RC) = 0.52). After each of three rounds of reweighting characters by the maximum RC value, the same thirteen equally parsimonious trees were obtained (tree length = 570; RI = 0.92 and RC = 0.78), and the strict consensus is shown in Fig. 2, along with bootstrap values for the branches shared with the 50% majority-rule consensus tree. Most differences were in the relationships among the three sequences of *Phlebotomus* (*Larroussius*) – P(La)tob, P(La)pern and P(La)neg.

Archaeopsylla erinacei (Siphonaptera) and *Boreus* sp. (Mecoptera) formed the sister group to Diptera and this agrees with the phylogeny of Whiting *et al.* (1997), who combined morphology with SSU and large subunit (LSU) rDNA sequence data. Monophyly of the dipterans was strongly supported (bootstrap: 100%), as there were fifty-three diagnostic characters out of 103 apomorphies. Within Diptera, the suborder Brachycera (represented

by two muscomorphans, *D. melanogaster* and *Ornitohica vicina*, and the tabanid *Chrysops niger*) was the sister clade to the suborder Nematocera, in which the phlebotomines formed a monophyletic group (infraorder Psychodomorpha). The nematoceran clade included the crane fly *Nephrotoma altissima* (infraorder Tipulomorpha), as outgroup of Psychodomorpha and its sister infraorder Culicomorpha. This supports previous morphological studies (Hennig, 1981; Wood & Borkent, 1989), unlike a molecular phylogeny based on LSU rDNA sequences that failed to resolve relationships among four strongly supported dipteran clades (Tipulomorpha, Bibionomorpha, Culicomorpha and Brachycera) or the position of Psychodidae, which was represented only by *Psychoda cinerea* (Friedrich & Tautz, 1997).

All dipterans of the infraorder Culicomorpha formed one clade, characterized by sixty apomorphies, out of which eighteen characters were diagnostic (158, 161, 561, 584, 584, 613, 660, 697, 970, 1498, 1535, 1539, 1544, 1793,

1818, 1843, 1949, 2169). Relationships within the clade agreed with the parsimony consensus tree based on SSU and 5.8S rDNA reported by Miller *et al.* (1997). As reported by these authors, the position of *Dixella cornuta* (Dixidae) in the molecular trees conflicts with comparative morphology, which places the family as a sister group to the Chaoboridae-Culicidae clade in the superfamily Culicoidea (Wood & Borkent, 1989).

The phlebotomine clade was well-supported (bootstrap: 100%) and there were twelve diagnostic characters out of thirty-one apomorphies (characters: 116, 124, 164, 351, 551, 606, 1161, 1185, 1817, 1896, 1933, 2076). The transition: transversion ratio within Phlebotominae ranged from 0 : 1 (*P(La)tob* vs. *P(Eu)arg* or *P(La)pern*) to 3 : 1 (*P(La)pern* vs. *P(La)neg*). The phylogenetic relationships among phlebotomine species were not robustly resolved: absolute genetic distances between their SSU rDNA sequences were low following the exclusion of regions that did not align well between insect families, e.g. the I-region of V7 (nt 2095–2149) that contains many of the diagnostic apomorphies within Phlebotominae. Nevertheless, all species of the same genus usually clustered together, the exception being the grouping of *L. shannoni* with *Phlebotomus* species rather than with *L. longipalpis* (*Llong*). However, the branch containing *Phlebotomus* and *L. shannoni* was only supported by two non-diagnostic apomorphies (characters 106 and 1196, with a consistency index (CI) of 0.25 and 0.50, respectively).

Phylogenetic relationships among phlebotomines

A first parsimony analysis was carried out with consensus sequences of each species and the major variants found in some specimens, namely: *P(Eu)arg*, *P(La)tob*, *TOB2*, *TOB7*, *TOB8*, *P(La)perf*, *PERF5*, *P(La)pern*, *P(La)neg*, *P(La)syr*, *P(Ad)sim*, *P(Pa)alex*, *P(Pa)serg*, *P(Ph)pap*, *Smin*, *MIN3*, *Sdent*, *DENT1*, *Sfal*, *Llong* and *L. shannoni* (Fig. 1; alignment 2a, EMBL accession number ds39838). Four ambiguously aligned regions were omitted from the maximum parsimony analysis: B1 (nt 192–211), B2 (nt 246–272), C1 (nt 751–911) and H1 (nt 1630–1696) were within three of the eight variable regions that created alignment problems among families (Fig. 1). The transition/transversion ratio varied from 0.67 : 1 (*TOB8* vs. *P(La)syr* or *P(La)tob*) to 7 : 1 (*MIN3* vs. *Sfal*).

Of the total of 1855 characters considered for a branch-and-bound search in PAUP 4.0, 1669 were constant, ninety-two were variable but parsimony-uninformative, and ninety-four were variable and parsimony-informative. The analysis gave twenty equally parsimonious trees (tree length = 235; RI = 0.91; RC = 0.77) when characters were unweighted and *Lutzomyia* sequences (*Llong*, *L. shannoni*) were selected as outgroup. Outgroup selection was justified by the basal position of *Llong* in the previous analyses. After each of three rounds of reweighting charac-

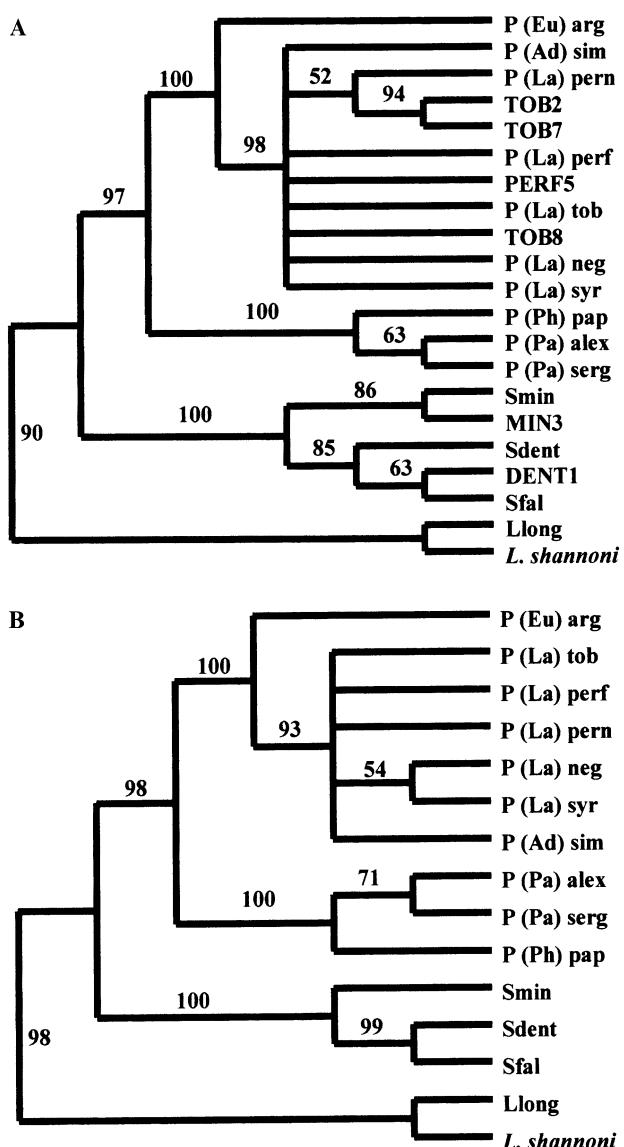


Figure 3. The strict consensus tree (maximum parsimony) for SSU rDNA sequences of phlebotomines, based on branch-and-bound searches of alignment 2a. Bootstrap values (for those branches shared with the 50% majority-rule consensus tree) were calculated with the heuristic search option (1000 replicates, each with 100 searches using random addition and TBR branch swapping). (A) All consensus sequences and major variants for each species (B) only consensus sequences for each species.

ters by the maximum RC value, a branch-and-bound search gave the same twenty equally parsimonious trees (tree length = 186; RI = 0.97; RC = 0.91), and the strict consensus is shown in Fig. 3(A) together with the bootstrap support for the branches shared with the 50% majority-rule consensus tree (with heuristic searches). This phylogeny is concordant with the results of Rispail & Léger (1998a,b), based on morphological characters, except for the position of *Euphlebotomus* subgenus, which appears to be the basal clade to all samples of

Larroussius and *Adlerius* subgenera; and it gives strong bootstrap support for the monophyletic clades that include all *Phlebotomus* species and all *Sergentomyia* species.

The same procedure was followed to analyse only the species consensus sequences, i.e. excluding the major intraspecific variants that might not be orthologous (Fig. 1; alignment 2b, EMBL accession number ds39839). Four ambiguously aligned sequence regions were omitted from the analysis. Regions B1 (nt 193–203), B2 (nt 245–274), C1 (nt 776–911) and H1 (nt 1639–1696) differed little from those excluded in the previous analysis, except that they were usually shorter. The transition/transversion ratio varied from 0.50 : 1 (Sdent vs. Sfal) to 5 : 1 (P(Pa)alex vs. P(Pa)serg). A total of 1901 characters was used for a branch-and-bound search in PAUP 4.0. Of these characters, 1626 were constant, 158 were variable but parsimony-uninformative and 117 both variable and parsimony-informative. When characters were unweighted and *Lutzomyia* sequences (*Llong*, *L. shannoni*) were selected as outgroup, the analysis gave four equally parsimonious trees (tree length = 237; RI = 0.86; RC = 0.68). After each of three rounds of reweighting characters by the maximum RC value, a branch-and-bound search retained a single most parsimonious tree (Fig. 3B; tree length = 163; RI = 0.96; RC = 0.90). Using an heuristic search and 1000 replicates, bootstrap analysis produced a 50% majority-rule consensus tree (bootstrap percentages plotted on branches in Fig. 3B) that was concordant with the results of Rispail & Léger (1998a,b) based on morphological characters, except for the position of *Euphlebotomus* subgenus that is, as in the previous analysis, the basal clade to all samples of *Larroussius* and *Adlerius* subgenera. The relationships among *Phlebotomus* species in the subgenera *Larroussius* and *Adlerius* were not resolved, except for the clade that united P(La)neg and P(La)syr. Within each subgenus, the pair-wise sequence divergence was 0.1–5%.

Further analyses were then carried out to resolve the relationships among species and their geographical populations within each of the three clades of OW phlebotomines identified above, namely: (A) the clade containing the subgenera *Euphlebotomus*, *Adlerius* and *Larroussius* (EMBL accession number ds39841); (B) the clade comprising the subgenera *Paraphlebotomus* and *Phlebotomus* (EMBL accession number ds38648); and (C) the clade of species in the genus *Sergentomyia*, with *Lutzomyia* sequences as outgroup (EMBL accession number ds39840). The results of the branch-and-bound searches for the most parsimonious trees are given in Table 2 and Fig. 4. There were no ambiguously aligned regions of SSU rDNA sequence when comparing taxa from closely related subgenera (sets A or B), in contrast to comparisons of *Sergentomyia* and *Lutzomyia* sequences (set C).

The strict consensus of the 198 equally parsimonious

trees obtained by a branch-and-bound search of the alignment of *Euphlebotomus*, *Adlerius* and *Larroussius* sequences is concordant with species classifications based on morphology (Fig. 4A). Branches defining each subgenus were well supported: the *Euphlebotomus* clade (ARG1, ARG2), chosen as the outgroup based on previous analyses, was supported by forty-seven apomorphic characters, out of which thirty-seven were diagnostic (bootstrap support ingroup: 100%); the *Adlerius* clade (SIM 1–5) was supported by fourteen diagnostic characters out of twenty-two apomorphies (bootstrap: 100%); and the *Larroussius* clade (the rest) was supported by eleven apomorphies (eight diagnostic characters: 197, 378, 739, 753, 760, 844, 1614, 1616; bootstrap: 90%). However, internal branches within *Larroussius* were not well supported, except for the clade that united the former subspecies *P. neglectus* and *P. syriacus* of the *Phlebotomus major* group (Léger & Pesson, 1987), characterized by four diagnostic characters (734, 749, 834, 1633) out of six apomorphies (bootstrap: 95%). Interspecific absolute genetic distances varied from 0.43% (NEG4 with SYR2) to 4.24% (ARG2 with TOB8), and intraspecific distances varied from 0.05% (ARG1 with ARG2) to 1.53% (TOB2 with TOB8). *Phlebotomus neglectus* and its sibling *P. syriacus* formed the basal clade of *Larroussius*, in which *P. perniciosus* and *P. tobii* appeared as sister species. This conforms with the findings of Esseghir *et al.* (1997, 2000) based on mitochondrial (cytochrome b) and nuclear (elongation factor alpha) DNA.

The other two parsimony analyses also grouped sequences (bootstrap ranged from 98 to 100%) according to the subgeneric or species classification based on morphological similarities except for *S. dentata* (Fig. 4B,C). There was no obvious geographical grouping of the SSU rDNA sequences for any of the species, which suggests either a very low rate of sequence divergence or an absence of geographical isolation of populations. Looking in detail at the three alignments of the sequences, we found that the most similar sequences do not have the same origin. For example, in the alignment ds39841, samples collected within the same area show many differences (e.g. SIM1 compared with SIM2; NEG4 compared with NEG6 or NEG7), and samples from different populations show very similar sequences (e.g. SIM1 compared with SIM3, SIM4 or SIM5; NEG1 compared with NEG2, NEG3, NEG5 or NEG8).

General conclusions

The SSU rRNA gene has been shown to be useful for diagnostic PCR-RFLP of sandflies (Aransay *et al.*, 1999), and we now confirm that it is also useful for inferring phylogenetic relationships within the subfamily Phlebotominae. We show for the first time that analyses of SSU rDNA sequences can be used to infer the relationships among sandflies at subgenus and species levels,

Table 2. Species used for each phylogenetic analysis of the SSU rDNA sequences with branch-and-bound searches for the most parsimonious trees. (*) Outgroup taxa in each study. (**) Regions excluded because of ambiguous alignment problems. CI = consistency index; HI = homoplasy index; RI = retention index and RC = rescaled consistency index.

Genus (subgenus)	Species	Ambiguous regions (**secondary structure)	Total characters in input matrix (PAUP)	Constant characters	Variable characters, parsimony- uninformative	Variable characters, parsimony- informative	No. of retained trees- unweighted characters	Tree length	CI	HI	RI	RC	Alignment EMBL accession number
<i>P. (Larroussius)</i>	<i>P. neglectus</i> <i>P. syriacus</i> <i>P. tobbi</i> <i>P. perfilliewi</i> <i>P. perniciosus</i>	None	2149	1907	111	131	198	309	0.8382	0.1618	0.9006	0.7549	ds39841
<i>P. (Adlerius)</i>	<i>P. simici</i>												
<i>P. (Euphlebotomus)</i>	<i>P. argentipes</i> (*)												
<i>P. (Phlebotomus)</i>	<i>P. papatasi</i> (*)												
<i>P. (Paraphlebotomus)</i>	<i>P. alexandri</i> <i>P. sergenti</i>	None	2102	2039	26	37	1	69	0.9855	0.0145	0.9941	0.9797	ds38648
<i>Sergentomyia</i>	<i>S. minuta</i> <i>S. dentata</i> <i>S. fallax cypriotica</i>	Primer F1 (bp1 to 35) C2 ('V4')	1955	1822	45	88	90	144	0.9514	0.0486	0.9600	0.9133	ds39840
<i>Lutzomyia</i>	<i>L. longipalpis</i> (*) <i>L. shanoni</i> (*)	(bp808 to 843) H2 ('V6') (bp1564 to 1623)											

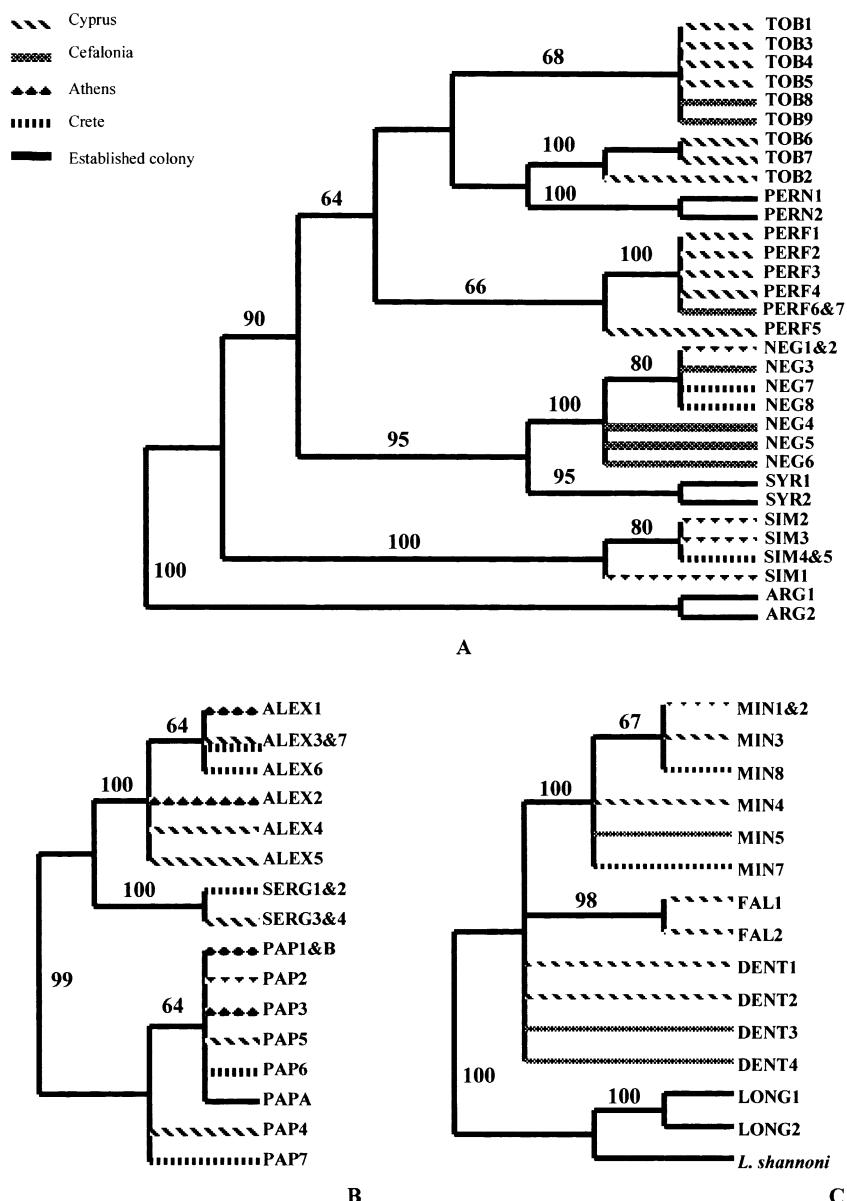


Figure 4. The strict consensus tree (maximum parsimony) for SSU rDNA sequences of phlebotomines, based on branch-and-bound searches of alignments: (A) *Larroussius*, *Adlerius* and *Euphlebotomus* (EMBL accession number ds39841); (B) *Phlebotomus* and *Paraphlebotomus* (EMBL accession number ds 38648) and (C) *Sergentomyia* and *Lutzomyia* taxa (EMBL accession number ds 39840). Bootstrap values (for those branches shared with the 50% majority-rule) on branches were calculated with the heuristic search option (100 replicates, each with ten searches using random addition and TBR branch swapping).

although there are problems deriving unambiguous sequence alignments.

The more distant the taxa compared, the larger were the regions of ambiguous sequence alignment. The highly variable regions were located in positions occupied by 'expansion segments' in the secondary structure model of *D. melanogaster* (Hancock *et al.*, 1988). For the present study, we decided to exclude regions that could not be unambiguously aligned and, unsurprisingly, the number and length of these increased with genetic distance. Consequently, separate phylogenetic analyses had to be undertaken for each alignment, and this reduced the number of characters available for analysing relationships among phlebotomine genera. Generic relationships were

not resolved, but this problem is probably better addressed when more subgenera have been sampled.

Based on our alignment of sequences from different insect orders, Tipulidae was shown to form the basal branch in Nematocera, as selected by Miller *et al.* (1997) on the basis of morphological studies (Hennig, 1973; Wood & Borkent, 1989). However, our result disagrees with the relationships among dipteran sub- and infra-orders inferred by Carreno & Barta (1998) in their analysis of SSU rDNAs. This discrepancy could be due to differences in alignments (including the number of characters used) or to the selection and number of the compared samples.

The five *Phlebotomus* subgenera studied (*Phlebotomus*, *Paraphlebotomus*, *Euphlebotomus*, *Adlerius* and *Larroussius*)

Table 3. rDNA sequences of holometabolous insects from GenBank

Species	Order/family	GenBank no.	Reference
<i>Hydropsyche</i> sp.	Trichoptera	X89483	Chalwatzis & Zimmermann, 1995 (Unpublished)
<i>Galleria mellonella</i>	Lepidoptera	X89491	Chalwatzis & Zimmermann, 1995 (Unpublished)
<i>Boreus</i> sp.	Mecoptera	X89487	Chalwatzis & Zimmermann, 1995 (Unpublished)
<i>Archaeopsylla erinacei</i>	Siphonaptera	X89486	Chalwatzis & Zimmermann, 1995 (Unpublished)
<i>Ornithoica vicina</i>	Diptera: Brachycera		
	Hippoboscidae		
<i>Chrysops niger</i>	Diptera: Brachycera	AF073888	Carreno & Barta, 1997
	Tabanidae	AF073889	Carreno & Barta, 1997
<i>Drosophila melanogaster</i>	Diptera: Brachycera		
	Drosophilidae	M21017	Tautz <i>et al.</i> , 1987
<i>Nephrotoma altissima</i>	Diptera: Nematocera		
	Tipulidae	U48379	Miller <i>et al.</i> , 1996
<i>Lutzomyia shannoni</i>	Diptera: Nematocera		
	Psychodidae	U48382	Miller <i>et al.</i> , 1996
<i>Culex tritaeniorhynchus</i>	Diptera: Nematocera		
	Culicidae	U48385	Miller <i>et al.</i> , 1996
<i>Aedes punctor</i>	Diptera: Nematocera		
	Culicidae	U48378	Miller <i>et al.</i> , 1996
<i>Aedes albopictus</i>	Diptera: Nematocera		
	Culicidae	X57172	Baldridge, 1991 (Unpublished)
<i>Toxorhynchites amboinensis</i>	Diptera: Nematocera		
	Culicidae	U48372	Miller <i>et al.</i> , 1996
<i>Anopheles pseudopunctipennis</i>	Diptera: Nematocera		
	Culicidae	U49735	Miller <i>et al.</i> , 1996
<i>Eucorethra underwoodi</i>	Diptera: Nematocera		
	Chaoboridae	U07981	Miller <i>et al.</i> , 1996
<i>Corethrella wirthi</i>	Diptera: Nematocera		
	Corethrellidae	U49736	Miller <i>et al.</i> , 1996
<i>Ablabesmyia ramphe</i>	Diptera: Nematocera		
	Chironomidae	U48384	Miller <i>et al.</i> , 1996
<i>Simulium vittatum</i>	Diptera: Nematocera		
	Simuliidae	U48383	Miller <i>et al.</i> , 1996
<i>Culicoides variipennis</i>	Diptera: Nematocera		
	Ceratopogonidae	U48380	Miller <i>et al.</i> , 1996
<i>Dixella cornuta</i>	Diptera: Nematocera		
	Dixidae	U48381	Miller <i>et al.</i> , 1996

were clustered in a monophyletic group supported by several synapomorphies (thirteen diagnostic characters out of seventeen apomorphies, 100% bootstrap value), and this contrasted with the paraphyly of the genus *Phlebotomus* inferred from the analysis of the D2 domain of LSU rDNA of a smaller number of sandfly species (Depaquit *et al.*, 1998). The position of the subgenus *Euphlebotomus*, as basal to *Larroussius* and *Adlerius* subgenera, contradicts the results of Rispail & Léger (1998a,b) based on morphological characters. Interestingly, these three subgenera contain most of the OW vectors of the *Leishmania donovani* complex.

Experimental procedures

Specimens

Phlebotomine characterized in this study were from field collections or were obtained from established colonies (Table 1). Specimens were stored in liquid nitrogen, 70% ethanol or dried over silica-gel, until they were processed. Sandflies were dissected

in order to make slide mounts of heads and last abdominal segments for morphological identification, based on the keys described by Adler (1946) and Léger *et al.* (1986).

PCR and sequencing

Genomic DNA from individual sandflies was extracted by a potassium acetate protein precipitation method as described by Aransay *et al.* (1999). The SSU rRNA genes were amplified by the polymerase chain reaction (PCR) (Aransay *et al.*, 1999) using degenerate primers complementary to conserved sequences at the 5' end of the gene (forward (F1), 5'-GCGGTTGATYCTRC CAGT-3') and the 3' end (reverse (R1), 5'-CYGCAGGTTCAC CTACRG-3') (F1: from bp 4–21 and R1: from 1984 to 1966 of *Drosophila melanogaster* 18S rDNA sequence; Tautz *et al.*, 1988).

One-fifth of the PCR products was resolved in 1.2% agarose gels and visualized under UV transillumination. The remaining PCR reaction was column-purified (GFX™ PCR DNA and Gel Band Purification kit, Pharmacia-Biotech). About 100 ng of each amplified product were ligated into either pGEM-T Easy vector (Promega Corp., Madison, WI) or pMOSblue T-vector (Amersham Life Science Ltd, UK). XL-2 competent cells were transformed with the recombinant plasmids. Recombinant bacterial colonies were

the target for reamplifying the SSU rRNA genes with primers –47 (5'-CGCCAGGGTTTCCCAGTCACGAC-3') and RP-OPTI (Reverse primer, 5'-GGAATTGTGAGCGGATAACA-3'), which are in the poly linker of both plasmids used, and products were purified by precipitation with a polyethylene glycol (PEG) buffer (15% PEG-8000, 1 M NaCl and 15 mM EDTA pH 8.0).

Sequencing of 200 ng of each PCR product was carried out by cycle sequencing (Sequitherm Excell II Long-Read DNA Seq. Kit) using M13-Universal and M13-Reverse primers as well as internal primers F2 (5'-GGCGCGTAAATTACCAATC-3'), F3 (5'-GCTTCTTAAATGGACAAAAT-3'), R2 (5'-ACCTGTTATT GCTCAATCTC-3') and R3 (5'-CTCGGATGTGAGTCCTGTAT-3') (Fig. 1). Sequences were resolved in a LI-COR 4200 automated sequencer.

Database sequences

Published SSU rDNA sequences belonging to Diptera, Mecoptera, Siphonaptera and Lepidoptera were retrieved from GenBank (Table 3).

Sequence analyses

Partial sequences were aligned in DNAMAN for windows, version 2.6 (Woffelman, 1994–96), so that sequences of complete genes were determined on both strands. Sequences were assembled by SeqPUP (Gilbert, 1995). Multiple sequence alignments were achieved using CLUSTAL W Multiple Sequence Alignment Program, version 1.7 (Higgins *et al.*, 1997), applying default settings (Gap opening penalty: 10.00; Gap extension penalty: 0.05; delay divergent sequences: 40%; and DNA transitions weight: 0.50). The resulting alignments were compared and divergent (or ambiguously aligned) regions were excluded, based on the secondary-structure model described for *Drosophila melanogaster* 18S rRNA (Hancock *et al.*, 1988). Maximum parsimony analyses were accomplished using the branch-and-bound option or, for bootstrap analyses, the heuristic search option in PAUP 4.0 (Swofford, 1998). Absolute genetic distance and transition: transversion ratio values were determined using subroutines of PAUP 4.0 (Swofford, 1998).

Acknowledgements

A.M.A. was a recipient of a predoctoral fellowship from The Basque Government. We are grateful to numerous colleagues for specimens: Dr Ricardo Molina (Instituto de Salud Carlos III, Majadahonda, Spain) and Prof. Nicole Léger (Faculte de Pharmacie, Reims, France).

References

- Adler, O.B.E. (1946) The sandflies of Cyprus (Diptera). *Bull Entomol Res* **36**: 497–511.
- Aransay, A.M., Scoulica, E., Chaniotis, B. and Tselentis, Y. (1999) Typing of sandflies from Greece and Cyprus by DNA polymorphism of 18S rRNA gene. *Insect Mol Biol* **8**(2): 1–6.
- Arnheim, N. (1983) Concerted evolution of multigene families. In: *Evolution of Genes and Proteins* (Nei, M. and Koehn, K., eds), pp. 38–61. Sinauer Associates Inc., Sunderland, Massachusetts, USA.
- Arnheim, N., Krystal, M., Schmickel, R., Wilson, G., Ryder, O. and Zimmer, E. (1980) Molecular evidence for genetic exchanges among ribosomal genes on nonhomologous chromosomes in man and apes. *Proc Natl Acad Sci USA* **77**(12): 7323–7327.
- Carmean, D., Kimsey, L.S. and Berbee, M.L. (1992) 18S rDNA sequences and the holometabolous insects. *Mol Phylogenetic Evol* **1**(4): 270–278.
- Carreno, R.A. and Barta, J.R. (1998) Small subunit ribosomal RNA genes of Tabanids and Hippoboscids (Diptera: Brachycera): evolutionary relationships and comparison with other Diptera. *J Med Entomol* **35**(6): 1002–1006.
- Coen, E.S. and Dover, G.A. (1983) Unequal exchanges and coevolution of X and Y rDNA arrays in *Drosophila melanogaster*. *Cell* **33**: 849–855.
- Depaquit, J., Perrotey, S., Lecointre, G., Tillier, A., Tillier, S., Ferte, H., Kaltenbach, M. and Léger, N. (1998) Systématique moléculaire des Phlebotominae: étude pilote. Paraphylie du genre *Phlebotomus*. *C R Acad Sci Paris, Sci la Vie* **321**: 849–855.
- Dover, G. (1982) A molecular drive through evolution. *Bio-science* **32**(6): 526–533.
- Esseghir, S., Ready, P.D. and Ben-Ismail, R. (2000) Speciation of *Phlebotomus* sandflies of the subgenus *Larroussius* coincided with the late Miocene-Pliocene aridification of the Mediterranean subregion. *Biol J Linn Soc* in press.
- Esseghir, S., Ready, P.D., Killick-Kendrick, R. and Ben-Ismail, R. (1997) Mitochondrial haplotypes and phylogeography of *Phlebotomus* vectors of *Leishmania major*. *Insect Mol Biol* **6**(3): 211–225.
- Friedrich, M. and Tautz, D. (1997) Evolution and phylogeny of the Diptera: a molecular phylogenetic analysis using 28S rDNA sequences. *Syst Biol* **46**(4): 674–698.
- Gilbert, D.G. (1995) SeqPUP a biosequence editor and analysis application program. Biology department, Indiana University, New Albany, USA. WWW address: <<http://ftp.sunet.se/pub/molbio/seqpup/c++/optional/SeqPup-help/>>.
- Hancock, J.M., Tautz, D. and Dover, A.G. (1988) Evolution of the secondary structures and compensatory mutations of the ribosomal RNAs of *Drosophila melanogaster*. *Mol Biol Evol* **5**(4): 393–414.
- Hennig, W. (1973) Diptera (Zweiflugler). *Handb Zool Berl* **4**: 1–337.
- Hennig, W. (1981) *Insect Phylogeny*. John Wiley & Sons, Chichester, New York.
- Higgins, D.G., Thomson, J.D. and Gibson, T.J. (1997) CLUSTAL W Multiple Sequence Alignment Program, Version 1.7. WWW address: <<http://www.sgi.com/chembio/resources/clustalw/>>.
- Hillis, D.M. and Dixon, M. (1991) Ribosomal DNA: molecular evolution and phylogenetic inference. *Q Rev Biol* **66**: 411–453.
- Hillis, D.M., Moritz, C. and Mable, B.K. (1996) *Molecular Systematics*, 2nd edn. Sinauer Associates Inc., Sunderland, Massachusetts, USA.
- Killick-Kendrick, R. (1990) Phlebotomine vectors of the leishmaniasis: a review. *Med Vet Entomol* **4**: 1–4.
- Kristensen, N.P. (1991) Phylogeny of extant Hexapods. In: *The Insects of Australia* (CSIRO, eds), 2nd edn, pp. 125–140. Melbourne University of Press, Melbourne.
- Léger, N. and Pesson, B. (1987) Sur la taxonomie et la répartition géographique de *Phlebotomus (Adlerius) chinensis* s. l. et de *P. (Larroussius) major* s. l. (Psychodidae-Diptera). Status des espèces présentes en Grèce. *Bull Société Pathologie Exotique* **80**: 252–260.
- Léger, P.N., Pesson, B. and Madulo-Leblond, G. (1986) Les Phlébotomes de Grèce. *Biologia Gallo-Hellenica* **11**(2): 165–192.

- Lewis, D.J., Young, D., Faichild, G.B. and Minter, D.M. (1977) Proposals for a stable classification of Phlebotomine sandflies (Diptera: Psychodidae). *Syst Ent* **2**: 319–332.
- Miller, B.R., Crabtree, M.B. and Savage, H.M. (1997) Phylogenetic relationships of the Culicomorpha inferred from 18S and 5.8S ribosomal DNA sequences (Diptera: Nematocera). *Insect Mol Biol* **6**(2): 105–114.
- Oosterbroek, P.F.L.S. and Courtney, G. (1995) Phylogeny of nematocerous families of Diptera (Insecta). *Zool J Linn Soc* **115**: 267–311.
- Rispail, P. and Léger, N. (1998a) Numerical taxonomy of Old World Phlebotominae (Diptera: Psychodidae) 1. Considerations of morphological characters in the genus *Phlebotomus* Rondani & Berté 1840. *Mem Inst Oswaldo Cruz, Rio Janeiro* **93**(6): 773–785.
- Rispail, P. and Léger, N. (1998b) Numerical taxonomy of Old World Phlebotominae (Diptera: Psychodidae) 2. Restatement of classification upon subgeneric morphological characters. *Mem Inst Oswaldo Cruz, Rio de Janeiro* **93**(6): 787–793.
- Seccombe, A.K., Ready, P.D. and Huddleston, L.M. (1993) A catalogue of Old World phlebotomine sandflies (Diptera: Psychodidae, Phlebotominae). *Occ Pap Syst Ent* **8**: 1–57.
- Swofford, D.L. (1998) Phylogenetic Analysis using Parsimony (PAUP), Versions PAUP* 4.0d (5b–5B). Illinois Natural History Survey, Urbana, Illinois.
- Tautz, D., Hancock, J.M., Webb, D.A., Tautz, C. and Dover, G.A. (1988) Complete sequences of the rRNA genes of *Drosophila melanogaster*. *Mol Biol Evol* **5**(4): 366–376.
- Vogler, A.P., Welsh, A. and Hancock, J.M. (1997) Phylogenetic analysis of slippage-like sequence variation in the V4 rRNA expansion segment in tiger beetles (Cicindelidae). *Mol Biol Evol* **14**: 6–19.
- Wheeler, W.C. and Gladstein, D.S. (1994) MALIGN: a multiple sequence alignment program. *J Hered* **85**: 417–418.
- Whiting, F.M., Carpenter, J.C., Wheeler, Q.D. and Wheeler, W.C. (1997) The Strepsiptera problem: phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. *Syst Biol* **46**(1): 1–68.
- Williams, S.M., DeBry, R.W. and Feder, J.L. (1988) A commentary on the use of ribosomal DNA in systematic studies. *Syst Zool* **37**(1): 60–62.
- Woffelman, C. (1994–96) DNAMAN for Windows, Version 2.6. Lyndon Biosoft, Institute of Molecular Plant Sciences, Leiden University, the Netherlands.
- Wood, D.M. and Borkent, A. (1989) Phylogeny and classification of the Nematocera. In: *Manual of Nearctic Diptera*, 3 (McAlpine, J.F. and Wood, D.M., eds), pp. 1333–1370. Agriculture Canada, Ottawa.