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Jianming Tang
University of Alabama, Birmingham, jtang@uab.edu

Kenneth Pruess
University of Nebraska-Lincoln

Eddie W. Cupp
Auburn University

Thomas R. Unnasch
University of Alabama, Birmingham, tunnasch@health.usf.edu

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Molecular Phylogeny and Typing of Blackflies (Diptera: Simuliidae) That Serve as Vectors of Human or Bovine Onchocerciasis

Jianming Tang,[1] Kenneth Pruess,[2] Eddie W. Cupp,[3]

and Thomas R. Unnasch[1]

1. Division of Geographic Medicine, University of Alabama at Birmingham, Birmingham, Alabama, USA
2. Department of Entomology, University of Nebraska–Lincoln, Lincoln, Nebraska, USA
3. Department of Entomology, Auburn University, Auburn, Alabama, USA

Corresponding author – Dr. T. R. Unnasch, Division of Geographic Medicine, BBRB Box 7, University of Alabama at Birmingham, Birmingham, AL 35294, USA

Abstract

A subregion of the mitochondrial large subunit (16s) rRNA gene was amplified by polymerase chain reaction (PCR) from nine species of blackflies (Diptera: Simuliidae) which serve as natural or experimental vectors of human or bovine *Onchocerca* parasites. PCR products from each species of blackfly were tested by directed heteroduplex analysis (DHDA), and their genotypes established according to diagnostic banding patterns of the heteroduplex products. Three alleles of mitochondrial 16s rRNA were found to exist in members of the *Simulium* (*Edwardsellum*) *damnosum sensu lato* complex from West Africa, and two alleles were found in the Neotropical *Simulium* (*Psilopelmia*) *ochraceum* Walker complex and the *Simulium* (*Simulium*) *metallicum* Bellardi complex. Different single alleles were detected in *Austrosimulium bancrofti*, in English *S.*(*S.*)*noelleri* and in two North American laboratory vectors: *Simulium* (*Psilozia*) *vittatum* Zetterstedt and *S.*(*S.*)*decorum* Walker. Phylogenetic analysis of 16s sequences indicated that blackflies from West Africa and the Americas formed distinct clades. Neotropical onchocerciasis vectors were found to be more closely related to Nearctic and Palaearctic nonvector *Simulium* species than to the African vectors of onchocerciasis.

Keywords: Simuliidae, onchocerciasis vectors, directed heteroduplex analysis, 16s rRNA, molecular phylogenetics

Introduction

Filarial nematodes of the genus *Onchocerca* Nematoda: Onchocercidae are a widespread group of tissue-dwelling parasites responsible for animal and human onchocerciasis (Bain, 1981). In all Faunal Regions except Australasia, blackflies (Diptera: Simuliidae) serve as the vectors for these parasites. For example, the North American *Simulium* (*Simulium*) *jenningsi* Malloch *sensu lato* (*s.l.*) transmits the bovine parasite *Onchocerca lienalis* Stiles (Lok et al., 1983). In Africa, members of the *Simulium* (*Edwardsellum*) *damnosum* Theobold complex transmit the human parasite *Onchocerca volvulus* Leuckart and bovine parasites such as *Onchocerca ochengi* Bwangamoi (Wahl, 1996). *O. volvulus* is also found in South and Central America where its primary vectors are *Simulium* (*Psilopelmia*) *ochraceum* Walker *s.l.*, *S.*(*S.*)*metallicum* Bellardi *s.l.* and *S.*(*Notolepria*)*exiguum* Roubaud *s.l.* In Australia, bovine onchocerciasis is caused by *Onchocerca gibsoni* Cleland and Johnston transmitted by *Forcipomyia townsvillensis* (Diptera: Ceratopogonidae) instead of endemic blackflies such as *Austrosimulium* (*Novaustrosimulium*) *bancrofti* Taylor (Ottley & Moorhouse, 1980).

Human onchocerciasis in the Americas is restricted to the Neotropics, despite the fact that *Simulium* blackflies are abundant in North America, and some of these species are vectors of bovine onchocerciasis. Recent studies have shown that *O. volvulus* was probably introduced to South and Central America through the African slave trade (Zimmerman et al., 1994). As the majority of slaves from *O. volvulus* endemic regions of West Africa were imported into North America, the reasons why human onchocerciasis did not become established in the USA may have included the vectorial incompetence of Nearctic blackflies.

We have used directed heteroduplex analysis (DHDA) to distinguish alleles of the mitochondrial 16s rRNA gene of diverse *Simulium* species that are capable of transmitting human or bovine *Onchocerca* parasites under natural or laboratory conditions. The nucleotide sequence in 16s rRNA alleles from each of the major onchocerciasis vectors was determined. Phylogenetic analyses of these sequence data revealed no correlation with vectorial capacity of the different blackfly species.

Materials and Methods

Collection of Simuliidae

Blackfly larvae and adults were collected from various locations around the world (Table 1). Specimens were identified from conventional morphometric and cytological criteria (Laird, 1981; Kim & Merritt, 1987) and preserved in ethanol.

Table 1. Simuliidae used in this study. The underlined genotypes identified by DHDA were sequenced. *n* = number of individuals examined.

Species/cytotype	Genotype	*n*	Collection site*	Date of collection
Au. bancrofti	ab1	4	Cotter River, New South Wales, Australia	27/02/1993
S. meridionale	sm1	1	Niobrara River, Knox Co., NE, USA	15/05/1989
S. bivittatum	sb1	1	Calamus River, Loup Co., NE, USA	12/10/1990
S. vittatum				
/IIIL	sv1	1	Pedernales River, Gillespie Co., TX, USA	16/12/1989
	svl	1	North Platte River, Scottsbluff Co., NE, USA	26/03/1990
	sv1	1	Bellamy River, Strafford Co., NH, USA	29/05/1990
	sv1	1	Ashford Scout Camp, Thurston Co., NE, USA	16/05/1993
	sv1	1	Trout Creek, Teller Co., CO, USA	03/08/1993
/IS-7	sv1	1	Colebrook, Coos Co., NH, USA	17/06/1993
	sv1	1	Deer Lodge, Powell Co., MT, USA	01/06/1993
	sv1	1	Trout Creek, Teller Co., CO, USA	03/08/1993
/?	sv1	1	River Laxa, Iceland	31/08/1990
	sv1	1	Broad Cove River, St. Johns, Newfoundland, Canada	06/05/1993
S. decorum	se1	2	Red Bank Creek, Saluda Co., SC, USA	09/05/1990
	se1	3	Buckskin Hills, Dixon Co., NE, USA	15/05/1993
S. noelleri	sn1	4	South Teigne River, Dartmoor, Devonshire, England, UK	06/05/1989
S. ochraceum	sol	2	Guatemala	Nov., 1988
	so2	1	Guatemala	Nov., 1988
S. metallicum	st1	2	Guatemala	28/08/1990
	st2	1	Guatemala	28/8/1990
S. damnosum complex				
squamosum/ yahense	sd1	59	Togo, Liberia, Ghana, Côte d'Ivoire	1987–94
sirbanum/ damnosum s.s.	sd2	93	Mali, Togo, Côte d'Ivoire, Ghana	1987–94
	sd3	3	Fétékro, Côte d'Ivoire	1994

* Abbreviations: CA, California; CO, Colorado; MO, Montana; NE, Nebraska; NH, New Hampshire; SC, South Carolina; TX, Texas; UK, United Kingdom of Great Britain and Northern Ireland; USA, United States of America

Preparation of DNA from individual blackflies

Individual blackflies were homogenized in 500 μl of lysis buffer (10 mM Tris-HCl, (pH 8.0), 2 mM EDTA, 10 mM NaCl, 1% SDS, 50 mM DTT, 0.4 mg ml^{-1} proteinase K) and incubated for 2 h at 55°C. RNAse A was added to a final concentration of 10 μg ml^{-1}, and the mixture incubated for 30 min at 37°C. The lysate was extracted with a 1/1 mixture of phenol and chloroform, and the genomic DNA precipitated with ethanol. The purified DNA was re-suspended in 100 μl TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and stored at –70°C.

Polymerase chain reaction (PCR), cloning and sequencing of amplified DNA fragments

PCR conditions and primers used to amplify a 556 bp fragment of the 16s rRNA gene (16s/556) were as described by Xiong & Kocher (1991). PCR products were separated by electrophoresis through 1.2% agarose gel in 40 mM Tris-acetate (pH 8.0), 1 mM EDTA. The DNA bands were visualized by UV transillumination after staining in 2 μg ml^{-1} ethidium bromide solution. The 16s/556 bands were cut out of the gel and purified by adsorption to a glass slurry (Geneclean, Bio 101, San Diego, California). The purified DNA fragments were cloned into a T-tailed PCR cloning plasmid vector (Invitrogen, La Jolla, California). The nucleotide sequence of the cloned PCR products was determined using T4 DNA Sequenase™ Version 2.0 (US Biochemical Corp., Cleveland, Ohio), according to the procedures outlined by the manufacturer. Multiple clones from each individual were compared to the parental uncloned PCR products using DHDA (as described below) to ensure that the cloned 16s/556 PCR products selected for sequencing did not contain PCR induced mutations (Tang & Unnasch, 1995).

***Directed heteroduplex analysis* (*DHDA*)**

DHDA was performed as described elsewhere (Tang et al., 1995b). Oligonucleotide primers were end-labeled with [ψ^{32}P]ATP and polynucleotide kinase (US Biochemical, Cleveland, Ohio), following standard methods (Sambrook et al., 1989). PCR amplification of cloned 16s/556 from *Simulium bivittatum* was done with labeled 3′ primer and nonlabeled 5′ primer to generate antisense strand probe. Following heteroduplex formation between the probe and target PCR reactions, homoduplex and heteroduplex products were separated by electrophoresis on a 5% acrylamide gel (acry1amide:bis, 19:l) prepared in a buffer consisting of 90 mM Tris-borate (pH 8.3). 2 mM EDTA and 2.7 M urea. Heteroduplex patterns were detected by autoradiography. Electrophoretic retardation (ER) for heteroduplex molecules was defined as:

$$ER = \left(\frac{(D_{hm} - D_{hd})}{D_{hm}}\right) \times 100$$

where D_{hm} = distance the homoduplex band migrated and D_{hd} = distance the heteroduplex band migrated.

Phylogenetic analyses

Sequence data were analyzed using the maximum parsimony based methods encoded in the PAUP program package (Swofford, 1991). In the analysis, gaps introduced in the alignment process were treated as missing data, topological constraints were not enforced, and branches of zero length were collapsed to yield polytomies. *Austrosimulium bancrofti* was designated as an outgroup in the analysis. Sequence data were further analyzed using the method of Fitch & Margoliash (1967) provided in the PHYLIP program package (Felsenstein, 1993). In this case the distance matrix was calculated using the two-parameter method developed by Kimura (1980), with gaps treated as missing data, with *Au. bancrofti*

again designated as the outgroup. The robustness of the phylogeny produced by both parsimony and distance methods was examined by analysis of 1000 bootstrap replicate datasets.

Results

Blackflies collected from the sites given in Table 1 were initially analyzed by DHDA in order to assess the degree of intraspecific polymorphism present in the amplified portion of 16s rRNA gene. Different single alleles were detected in most species, with the exceptions of *S. metallicum s.l.*, *S. ochraceum s.l.*, and *S. damnosum s.l.* (Table 1, Fig. 1). Two 16s/556 alleles were found in *S. metallicum s.l.* (Fig. 1, lanes e and f; ER = 21.7% and 17.8%, respectively). Similarly, two alleles were detected in *S. ochraceum s.l.* (Fig. 1, lanes g and h, ER = 7.3% and 4.5%, respectively), and three alleles were found in the *S. damnosum s.l.* complex (Fig. 1, lanes i–p; ER = 52.2%, 10.2%, and 15.9%, respectively).

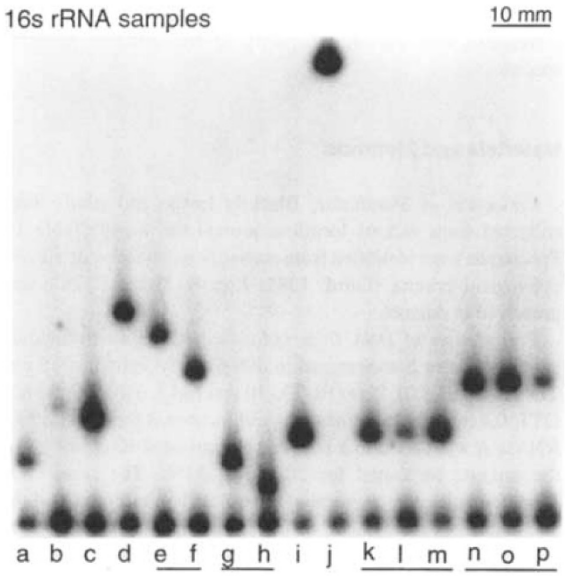

Figure 1. DNA typing of onchocerciasis vectors by DHDA based on 16s rRNA sequences; using the antisense strand of a cloned 16s PCR product derived from *Simulium bivitrafuum* as the probe. Samples were separated at 24 mA for 6 h and homoduplex band migrated 157 mm. Lane a = *S. noelleri*; b = *S. decorum*; c = *S. vittatum*; d = *Au. bancrofti*; e and f = *S. metallicum s.l.*; g and h = *S. ochraecum s.l.*; i = *S. damnosum* (sd2); j = *S. damnosum* (sdl); k–m = *S. damnosum* (sd2); and n–p = *S. damnosum* (sd3).

PCR products generated from representative individuals containing each of the alleles detected by DHDA were cloned, and the identity of the clones confirmed by DHDA. The DNA sequences of all alleles were aligned using the Clustal V algorithm (Higgins et al.,

1992), followed by manual alignment. The aligned sequences are shown in Figure 2. Differences among the sequences were not evenly distributed, with a large proportion of the changes concentrated in the 50 bp domain spanning positions 256–305 in Figure 2.

The DNA sequence data shown in Figure 2 were analyzed, together with a number of previously published sequences from other species of *Simulium* (Tang et al., 1995b), in order to explore the relationship between those species which are *Onchocerca* vectors and those which are not. Branch and bound analysis of the dataset revealed six most parsimonious trees which differed only in the placement of *Simulium* (*Byssodon*) *meridionale* Riley and *S. metallicum s.l.* relative to the other North American species, and the arrangement of the three alleles of *S. damnosum s.l.* relative to one another. The data were then reanalyzed using the heuristic method, which resulted in the identification of the same six most parsimonious trees. The data were reweighted according to the rescaled consistency index of the six most parsimonious trees. Further analysis of the reweighted dataset identified three most parsimonious trees, which differed only in the arrangement of the *S. damnosum s.l.* alleles relative to one another. This result remained stable through subsequent iterations of reweighting and reanalysis of the data. Reanalysis of twenty randomly ordered replicate datasets also resulted in the identification of the same three most parsimonious trees. A strict consensus of the three most parsimonious trees showed several groupings supported by bootstrap analysis (Fig. 3). The three alleles of *S. damnosum s.l.* formed a single clade, which was distinct from the clade containing all other members of the genus *Simulium* collected in the Americas and in England. Similarly, the two alleles found in *S. ochraceum s.l.* formed a clade, as did the two alleles present in *S. metallicum s.l.* Additional clades supported by the analysis included one grouping *S. decorum* and *S.*(*S.*)*noelleri* Friederichs, and another clade grouping *S. bivittatum* and *S. ochraceum s.l.*

Figure 2. Alignment of 16s/556 sequences of seven species of blackflies from five continents. Dots indicate positions where the given sequence is identical to *Austrosimulium bancrofti*, and gaps (–) are introduced to achieve maximum sequence alignment. The primer sequences (20 bp at the 5′ and 3′ sides, respectively) are not shown.

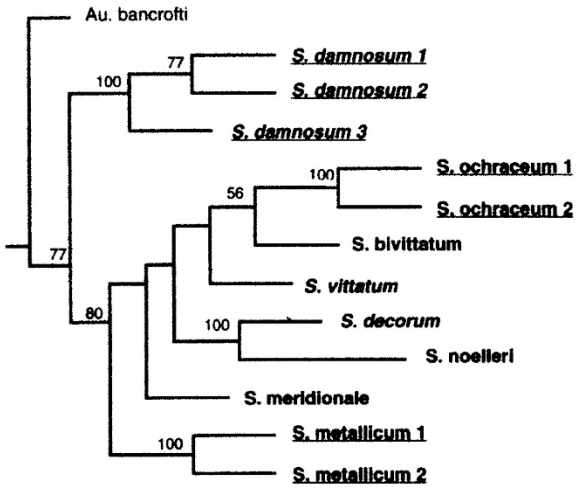

Figure 3. Maximum parsimony analysis of the phylogenetic relationships of nine *Simulium* species. Branch lengths shown are proportional to the estimated genetic distances between taxa. The number at each node indicates the percentage of times given groups to the right of the node were grouped together in an analysis of 1000 bootstrap replicate datasets. Only nodes supported by bootstrap frequencies of greater than 50% are labeled. Underlined species are known vectors of human onchocerciasis, italicized species are vectors of various bovine *Onchocerca* spp. Sequences for *S. bivittatum*, *S. meridionale*, and two alleles of *S. damnosum s.l.* (sdl and sd2) have been reported elsewhere (Tang et al., 1995b). The three trees used to construct the strict consensus tree shown were all identical in overall length and the following statistics: Consistency index = 0.889; retention index = 0.855; rescaled consistency index = 0.760.

The phylogeny produced by distance analysis was almost identical to that produced by parsimony, differing only in its placement of *S. metallicum s.l.* and *S. meridionale* relative to the remaining species from North America (Fig. 4). Once again, the sister alleles present in *S. damnosum s.l.*, *S. metallicum s.l.*, and *S. ochraceum s.l.* all formed separate clades supported by the bootstrap analysis. The analysis also supported clades grouping *S. ochraceum s.l.* with *S. bivittatum*, and *S. decorum* with *S. noelleri*. In addition, the West African *S. damnosum s.l.* complex formed a clade which was distinct from other American and the one English *Simulium* species examined.

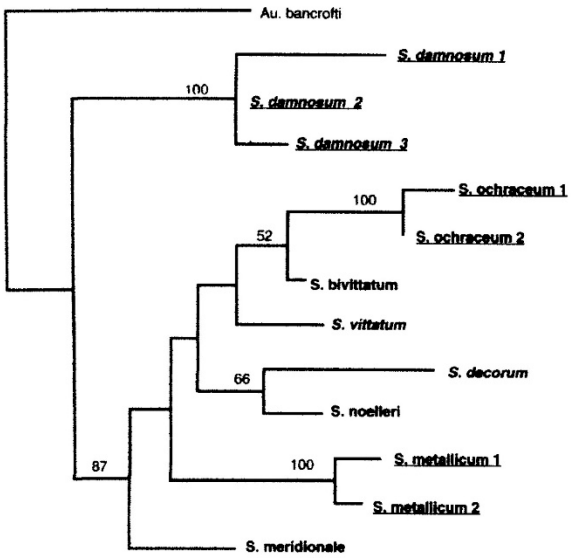

Figure 4. Neighbor-joining analysis of phylogenetic relationships of selected *Simulium* species. Branch lengths drawn to scale; other features of labeling as in Figure 3. Sum of squares = 0.475; average percent standard deviation = 5.55.

Discussion

One of the problems inherent in using DNA sequences for phylogenetic analyses is that, due to intraspecific heterogeneity, data from a particular individual may contain substitutions that are not representative of the species as a whole. To overcome this, we have determined the DNA sequence of PCR products derived from a number of individuals. That approach is time-consuming and expensive, and limits the number of individuals that may be examined from each taxon. In this study, DHDA was applied to detect intraspecific heterogeneity within blackfly species prior to DNA sequence analysis. This method allows a large number of individuals to be characterized quickly. It is also sensitive enough to detect a single nucleotide difference between two PCR fragments of the size used in the current study (Tang et al., 1995a). Thus, DHDA can be used to survey comprehensively the degree of intraspecific variability within a given locus prior to detailed sequencing analysis. The individuals of a given species which carry distinct alleles at the locus in question can be easily identified, and their representatives chosen for more detailed analyses. A further advantage of DHDA is that it may be used to compare cloned PCR products with the uncloned parental DNA, thus proving a useful tool to eliminate clones with either PCR-induced mutations, or those representing minor heteroplasmic variants.

The DHDA results obtained in our study suggested that intraspecific polymorphism of 16s/556 is quite limited within the genus *Simulium*. Furthermore, the intraspecific polymorphisms noted within *S. damnosum s.l.*, *S. metallicum s.l.*, and *S. ochraceum s.l.* are consistent with the fact that these are actually species complexes containing a number of

sibling species (Crosskey, 1987). Region 16s/556 appeared to be an informative marker at the species complex level, at least for the species compared here.

The phylogenies developed from the sequence data supported several groupings. First, in those species where intraspecific polymorphisms were identified by DHDA, the polymorphic alleles all formed distinct clades. Therefore the degree of intraspecific variation within the 16s/556 of *Simulium* is less than for interspecific variation. Moreover, the data supported several additional clades, all of which were generally consistent with the conventional morphological classification of the different *Simulium* species (Fig. 5). For example, the sequence-based phylogenies identified a clade consisting of *S. decorum* and *S. noelleri*. These species have been considered sister species belonging to the *S. noelleri* species group (Fig. 5). Similarly, *S. ochraceum s.l.* and *S. bivittatum* were found to group together. These species are classified together in the subgenus *Psilopelmia* (Fig. 5). In contrast, the sequence-based phylogenies were not capable of resolving the relationship of *S. metallicum s.l.* to *S. noelleri* and *S. decorum*, all of which are classified as members of the subgenus *Simulium sensu stricto*, according to morphological criteria (Crosskey, 1987).

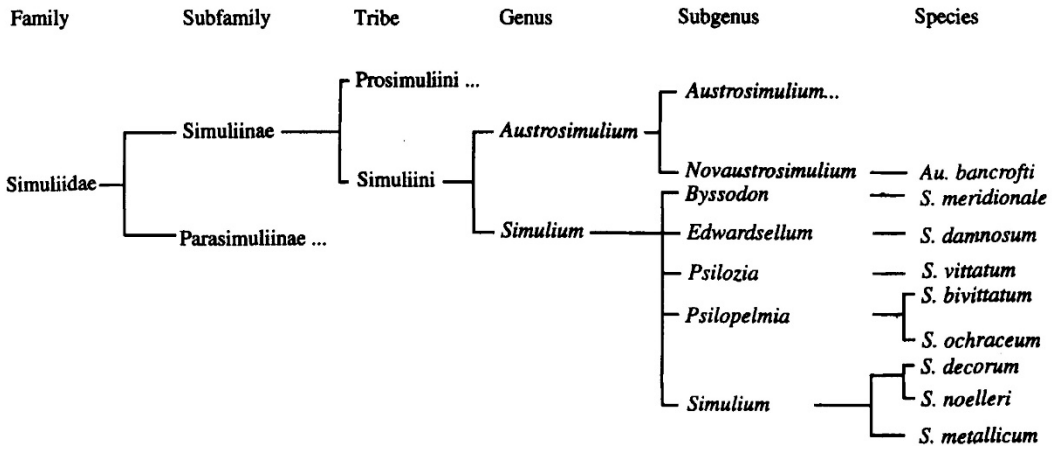

Figure 5. Morphological classification (Crosskey, 1987) of selected blackfly species.

The phylogenetic analyses presented above agree with the conventional taxonomic view that *S. damnosum s.l.* is not closely related to Central American Simuliidae, despite the fact that we compared *Simulium* species that are all capable of transmitting *O. volvulus*. Indeed from their 16s/566 sequences, the Neotropical vector species *S. ochraceum s.l.* and *S. metallicum s.l.* appear to be more closely related. to the English blackfly *S. noelleri* than they are to the Afrotropical *S. damnosum s.l.* Even within the clade containing all the American and English species, no evidence was obtained for a classification grouping the American vectors together. Therefore, the vector competence or incompetence of blackflies for human and bovine onchocerciasis does not correlate with the phylogenetic relationships derived from the 16s/556 sequence data. This notion is supported by the morphological classification scheme (Fig. 5). It is more likely, therefore, that some combination of shared physiological, behavioral, and/or anatomical characteristics determine the vectorial role of

a particular *Simulium* species for *Onchocerca*, and that the presence or absence of these characteristics is not correlated with the formal taxonomic relationships of these species. For example, positive associations have been demonstrated between the levels of blackfly salivary factors, such as erythema activity (Cupp et al., 1994) and apyrase activity (Cupp et al., 1995). and the vector competence for *Onchocerca*. Furthermore, comparisons of taxonomically closely related vector and nonvector species have suggested that saliva composition is associated with host selection for blood-feeding (i.e., anthropophily versus zoophily), critical factors in determining vector potential.

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References

Bain, O. (1981) Le genre *Onchocena:* hypotheses sur son evolution et cle dichotomique des especes. *Annales de Parasitologie.* 56, 503–526.

Crosskey, R.W. (1987) An annotated checklist of the world black flies (Diptera: Simuliidae). *Black Flies* (ed. by K.C. Kim and R.W. Merritt), pp. 425–521. Pennsylvania State University Press.

Cupp, M.S., Cupp, E.W., Ochoa-A, J.O. & Moulton, J.K. (1995) Salivary apyrase in New World black flies (Diptera: Simuliidae) and its relationship to onchocerciasis vector status. *Medical and Veterinary Entomology*, 9, 325–330.

Cupp, M.S., Ribeiro, J.M.C. & Cupp, E.W. (1994) Vasodilative activity in black fly salivary glands. *American Journal of Tropical Medicine and Hygiene,* 50, 235–240.

Felsenstein, J. (1993) *PHYLIP* (*phylogeny Inference Package*), version 3.5c. Department of Genetics, University of Washington, Seattle, Washington.

Fitch, W.M. & Margoliash, E. (1967) Construction of phylogenetic trees. *Science,* 155, 279–284.

Higgins, D.G., B1easby, A.J. & Fuchs, R. (1992) CLUSTAL V: improved software for multiple sequence alignment. *Computer Applications in the Biosciences, 8*, 189–191.

Kim, K.C. & Merritt, R.W. (eds.) (1987) *Blackflies: Ecology, Population Management, and Annotated World List*. Pennsylvania State University Press.

Kimura, M. (1980) A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution,* 16, 111–120.

Laird, M. (ed.) (1981) *Blackflies: The Future for Biological Methods in Integrated Control.* Academic Press, London.

Lok, J.B., Cupp, E.W. & Bernardo, M.J. (1983) *Simulium jenningsi* Mallock (Diptera: Simuliidae): a vector of *Onchocerca lienalis* Stiles (Nematoda: Filarioidea) in New York. *American Journal of Veterinary Research,* 44, 2355–2358.

Ottley, M.L. & Moorhouse, D.E. (1980) Laboratory transmission of *Onchocerca gibsoni* by *Forcipomyia* (*Lasiohelea*) *townsvillensis*. *Australian Veterinary Journal,* 56, 559.

Sambrook, I., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: Laboratory Manual*. Cold Spring Harbor Laboratory, New York.

Swofford, D.L. (1991) *PAUP: Phylogenetic Analysis Using Parsimony*, version 3.0. Illinois Natural History Survey, Champaign, Illinois.

Tang, J., Toe, L., Back, C. & Unnasch, T.R. (1995a) Mitochondrial alleles of *Simulium damnosum sensu lato* infected with *Onchocerca volvulus*. *International Journal for Parasitology,* 25, 1251–1254.

Tang, J., Toe, L., Back, C., Zimmerman, P.A., Pruess, K. & Unnasch, T.R. (1995b) The *Simulium damnosum* species complex: phylogenetic analysis and molecular identification based upon mitochondrially encoded gene sequences. *Insect Molecular Biology,* 4, 79–88.

Tang, J. & Unnasch, T.R. (1995) Discriminating PCR artifacts using DHDA (directed heteroduplex analysis). *BioTechniques,* 19, 902–905.

Wahl, G. (1996) Vector competence of the *Simulium damnosum* complex in Cameroon for *Onchocerca* spp. from cattle. *Medical and Veterinary Entomology,* 10, 200–202.

Xiong, B. & Kocher, T.D. (1991) Comparison of mitochondrial DNA sequences of seven morphospecies of black flies (Diptera: Simuliidae). *Genome,* 34, 306–311.

Zimmerman, P.A., Katholi, C.R., Wooten, M.C., Lang-Unnasch, N. & Unnasch, T.R. (1994) Recent evolutionary history of American *Onchocerca volvulus,* based on analysis of a tandemly repeated DNA sequence family. *Molecular Biology and Evolution,* 11, 384–392.