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Cytotaxonomic and Molecular Analysis of *Simulium (Edwardsellum) damnosum* sensu lato (Diptera: Simuliidae) from Abu Hamed, Sudan

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ABSTRACT The northernmost focus for *Onchocerca volvulus* Leuckhart (Nematoda: Onchocercidae), the causative agent of human onchocerciasis, is found along the Nile near the town of Abu Hamed in Sudan. The vector for *O. volvulus* at this focus is a single monomorphic population of *Simulium (Edwardsellum) damnosum* Theobald. This black fly population is limited to a small area between the fourth and fifth cataracts of the Nile River that is isolated geographically from all other populations of *S. damnosum* sensu lato. Phylogenies produced from cytological analyses and sequence data derived from the NADH dehydrogenase subunit 4 and 16S rRNA genes indicate that Abu Hamed black flies are similar to, but distinct from, the savanna-dwelling sibling species of *S. damnosum* s.l., *Simulium (Edwardsellum) damnosum* sensu strictu Theobald, and *S. (Edwardsellum) sirbanum* Vajime & Dunbar. The DNA sequence and the cytological data support the hypothesis that the black fly population present in Abu Hamed may represent a new sibling species of *S. damnosum* s.l. We propose that this population be informally designated as the hamedense form of the *Simulium damnosum* complex.

KEY WORDS *Simulium damnosum*, *Onchocerca volvulus*, Sudan, heteroduplex analysis, phylogeny, mitochondrial genes

Onchocerca volvulus LEUCKHART (Nematoda: Onchocercidae), the causative agent of onchocerciasis or river blindness, is a filarial parasite that is endemic throughout most of sub-Saharan Africa. Sudan is the northernmost country in the world endemic for onchocerciasis (Baker and Abdelnur 1986). Onchocerciasis in Sudan is endemic at three foci, the largest of which is found in the southwestern part of the country. At this focus, blinding onchocerciasis is prevalent and infection rates are often >90%. A second focus of onchocerciasis, located in the eastern portion of the country, has not been studied in detail. Finally, a focus of onchocerciasis exists in the north of the country, along the Nile River at Abu Hamed between the fourth and fifth cataracts (Baker and Abdelnur 1986). This focus is unique in many respects, including its geographic isolation and in the arid nature of the surrounding countryside. Previous work has documented that although the intensity of infection in the Abu Hamed focus is relatively low, the prevalence and severity of onchodermatitis is high (Williams et al. 1985).

In the major *O. volvulus* endemic areas of West Africa, the vectors are black flies in the *Simulium (Edwardsellum) damnosum* Theobald species complex (World Health Organization 1995). *Simulium damnosum* s.l. consists of at least nine sibling species and numerous geographically distinct cytotypes. Six of these cytospecies serve as major vectors for *O. volvulus* in West Africa (World Health Organization 1995). Previous studies have implicated the savanna-dwelling species of *S. damnosum* s.l., *S. damnosum* sensu strictu Theobald and *S. sirbanum* Vajime & Dunbar, as the vectors for *O. volvulus* in the southern focus of onchocerciasis in Sudan (Baker and Abdelnur 1986). In the Abu Hamed focus, the status of the vector population remains unclear. The black flies present at this focus represent a single monomorphic population. The larval habitat for these flies is limited in size and is isolated geographically from all other *S. damnosum* s.l. populations in Africa by the surrounding Nubian desert. The black flies at the Abu Hamed breeding site provisionally were identified in previous studies as *S. sirbanum.* However, the flies at this focus exhibited some cytologic characteristics that distinguished them from the typical West African populations of *S. sirbanum* (Procunier 1989).

Recently, the different sibling species of *S. damnosum* s.l. endemic to West Africa have been distin-

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guished based on sequence differences encoded in the mitochondrial genome (Tang et al. 1995a, 1995b). Portions of the 16S rRNA and NADH dehydrogenase subunit 4 (ND4) mitochondrial genes have proven particularly useful in studying the phylogenetic relationships among different species of black flies of the genus *Simulium*, both in Africa and in South America (Tang et al. 1995a, 1995b, 1996).

The objective of the current study was to apply cytotaxonomic and the recently developed DNA sequence-based methods to explore the relationship of the black flies found at Abu Hamed to the *S. damnosum* s.l. sibling species found throughout West Africa. Results of this study support the hypothesis that the black flies from Abu Hamed may represent a new sibling species of *S. damnosum* s.l., which we propose should be designated as the hamedense form of the *Simulium damnosum* complex.

Materials and Methods

Collection of Black Flies. Specimens were collected from the Nile River near the town of Abu Hamed (33° 20′ E, 19° 30′ N). Larvae were collected from rocks and vegetation, and adults were collected using human bait following standard methods (Walsh 1983). Informed consent was obtained from all individuals involved in collection of adult flies. Collections were carried out in March 1997 and February 1999. Adults were preserved in isopropanol for DNA isolation, whereas larvae were preserved in Carnoy's solution (three parts ethanol: one part acetic acid) for cytotaxonomic identification. Voucher specimens of adults and larvae have been deposited in the Natural History Museum, London.

Cytology. Chromosome preparations were made as previously described (Boakye et al. 1993). The preparations were examined and photographed at 400× magnification. Chromosomes were mapped and inversions identified based upon previously published criteria (Vajime and Dunbar 1975, Garms et al. 1988, Vajime 1989, Boakye 1993, Boakye et al. 1993).

Molecular Techniques. DNA was prepared from individual adults preserved in isopropanol following previously published procedures (Tang et al. 1996) and used as a template in the polymerase chain reaction (PCR) to amplify portions of the 16S rRNA and ND4 genes (Tang et al. 1995a). The 16S rRNA fragment was amplified simultaneously from DNA of well-characterized standards of the West African members of *S. damnosum* s.l. for use in DNA heteroduplex analysis (HDA). The HDA was performed as previously described (Tang et al. 1995a), using a PCR product generated from the 16S rRNA gene of *Simulium (Psilopelmia) bivittatum* Malloch as the heteroduplex driver.

PCR products of the ND4 and 16s rRNA fragments were purified by electrophoresis through 1.5% agarose gel followed by adsorption to a glass slurry (Bio 101, Geneclean, San Diego CA). The purified DNA fragments were cloned into a T-tailed PCR cloning vector (Invitrogen, La Jolla, CA). The nucleotide sequence of plasmid clones containing the ND4 insert were determined using T4 DNA Sequenase, version 2.0 (U.S. Biochemical, Cleveland, OH), according to the procedures outlined by the manufacturer. The DNA sequences of multiple-cloned PCR products were examined to confirm that differences noted were not caused by PCR induced mutations. The ND4 and 16S rRNA sequences derived from the Abu Hamed flies are available from the Genbank sequence database under accession numbers AF213336 and AF213337, respectively.

Phylogenetic Analysis. Phylogenies were constructed from the cytotaxonomic data using the parsimony-based program package PAUP version 4.0 (Swofford 1998). For each of the sibling species of the *S. damnosum s.l.* complex and the Abu Hamed specimens, each diagnostic inversion was scored as absent (0), present but not fixed (1), or present and fixed (2). In total, 22 inversions were scored in this fashion, of which 13 represented informative character states. The data were treated as unordered in the analysis, and phylogenetic analysis was completed using the branch and bound algorithm. The phylogeny produced from the cytotaxonomic data were not rooted. Statistical support for the phylogeny was evaluated by bootstrap reanalysis of 1,000 replicate datasets.

DNA sequence data were analyzed using both the parsimony-based algorithms found in the PAUP program package (Swofford 1998), and the numerical methods found in the PHYLIP program package (Felsenstein 1993). Apart from the DNA sequences derived from the Abu Hamed black flies described in the current work, the DNA sequences of the North American and African species of *Simulium* have been reported previously (Tang et al. 1995a, 1995b, 1996) and are available from Genbank (accession numbers U17727-U17741, U18231, U18232, U27114, and U27115). All of the ND4 sequences were the same length, making it unnecessary to introduce any gaps into the sequences to maximize the sequence alignments. The 16S rRNA sequences were aligned using the Clustal V algorithm (Higgins et al. 1992) and the introduced gaps treated as character states in the analysis. Parsimony analysis used the branch and bound algorithm of the PAUP program package. Genetic distances were calculated from the sequence data using the two-parameter method of Kimura (1980) and phylogenies constructed from distance data using the neighbor joining method (Saitou and Nei 1987). Phylogenies derived from the DNA sequence data were rooted using the corresponding sequences derived from *Austrosimulium (Novaustrosimulium) bancrofti* Taylor and *Simulium (Psilozia) vittatum* Zetterstedt.

Results

As a first step in comparing *S. damnosum* s.l. from Abu Hamed to those of the rest of West Africa, 25 larvae (13 males and 12 females) were subjected to cytotaxonomic analysis. The diagnostic inversions found are listed in Table 1. Inversions IS-1 and IL-3 (Fig. 1, panels A and B) were found in all individuals.

Table 1. Comparison of diagnostic inversions in Abu Hamed black flies with the West African sibling species of *S. damnosum* s.l.

Inversion	Species of *Simulium*						
	S. hamedense	*S. sirbanum*	*S. damnosum*	*S. santipauli*	*S. squamosum*	*S. yahense*	*S. leonense*
IS-2	2	2	1	0	1	0	0
IS-A	0	0	0	1	0	0	1
IS-3	2	2	1	0	0	0	0
IL-A	0	0	0	0	0	0	2
1L-1	2	2	2	0	0	0	0
1L-B	0	0	0	2	0	0	0
1L-2	0	1	1	0	0	0	0
IIS-7	0	0	0	0	0	0	2
IIL-C	2	2	2	0	0	0	0
IIL-8	2	2	1	0	0	0	0
IIL-3	0	1	0	0	0	0	0
IIL-4	0	0	0	2	0	0	2
IIL-6	0	0	0	2	0	0	2
IIL-7	0	0	0	1	0	0	1
IIL-A	0	0	0	2	0	0	0
IIL-18	0	0	0	0	0	2	0
IIIL-6	2	1	1	0	0	0	0
IIIL-7	0	1	1	0	0	0	0
IIIL-2	2	2	2	2	0	0	2
IIIL-17	0	0	0	2	0	0	1
IIIL-4	0	0	0	2	0	0	1
IIIL-B	0	0	0	1	0	0	0

0, absent; 1, floating; 2, fixed.

These diagnostic inversions occur in *Simulium damnosum* s.l. populations across West Africa and support the hypothesis that the specimens from Abu Hamed were related closely to the West African sibling species in this complex. Furthermore, the chromosomal rearrangement IL-1 (Fig. 1, panel A) was present, indicating that the Abu Hamed flies could be grouped in the *S. damnosum* s.s./*S. sirbanum* subcomplex (Boakye 1993). Other inversions recorded were IS-2+3 (Fig. 1, panel B), IIL-C.8 (Fig. 1, panel C), and IIIL-2.6 (Fig. 1, panel D). No sex linkage was observed among these different inversions. The chromosomes were highly monomorphic, with the seven diagnostic inversions present in 100% of the individuals examined. Heterozygous rearrangements were not observed for any of the inversions except for a micromorphological feature of the centromere. Centromere dimorphism (Fig. 1, panels A and C) was observed for all three chromosomes at 25% frequency.

Cytotaxonomic data were subjected to phylogenetic analysis together with previously described cytotaxonomic data for the six major West African vector species of *S. damnosum* s.l. A single most parsimonious tree resulted from this analysis (Fig. 2). The Abu Hamed specimens grouped in a clade that included *S. damnosum* s.s. and *S. sirbanum*, consistent with the observations described above. However, the phylogeny indicated that the Abu Hamed isolates were related more distantly to *S. damnosum* s.s. and *S. sirbanum* than these two savanna-dwelling species were to each other.

Adult flies collected from Abu Hamed also were examined by comparing their morphological characteristics with the West African species of *Simulium damnosum* s.l. The flies had broad and swollen fore basitarsi with tufts of setae on the dorsal side. The hind tarsi had a narrow black base, and the middle region was bright white and had a broad black base. These characters place the flies in the *S. damnosum* complex. Abu Hamed adults also possessed pale wing tufts (01 category) (Kurtak et al. 1981) and arculus. The antennae (i.e., at least the four basal segments) were pale. The fore coxa and ninth abdominal tergal hairs were also pale in color. These features indicated that the Abu Hamed flies were related more closely to the savanna species of the *S. damnosum* complex in West Africa (*S. sirbanum* and *S. damnosum* s.s.) than to the forest- and transition zone-dwelling sibling species.

To further explore the relationship of the Abu Hamed specimens to the major sibling species of *S. damnosum* s.l. from West Africa, a 556-bp fragment of the 16S rRNA gene previously found to be phylogenetically informative was amplified from 12 individual Abu Hamed flies. Heteroduplex analysis of the 16s rRNA fragment was used to compare the specimens obtained from Abu Hamed with the *S. damnosum* s.l. species endemic to West Africa. As previously described (Tang et al. 1996), three different heteroduplex variants are found in the 16S rRNA gene in the six sibling species of *S. damnosum* s.l. from West Africa. One of these is found in the forest dwelling sibling species *S. yahense* Theobald and *S. squamosum* Theobald, whereas the other two represent alleles shared among the remaining four sibling species endemic to the savanna and transition bioclimes (*S. damnosum* s.s., *S. sirbanum*, *S. leonense* Theobald, and *S. sanctipauli* Theobald). The Abu Hamed flies produced a heteroduplex pattern that was distinct from the three-sequence variants found in the West African sibling species (Fig. 3). No intraspecific variation was seen in the heteroduplex pattern among the 12 individuals examined (data not shown). Because previous studies have demonstrated that the heteroduplex assay used here was capable of detecting a single nu-

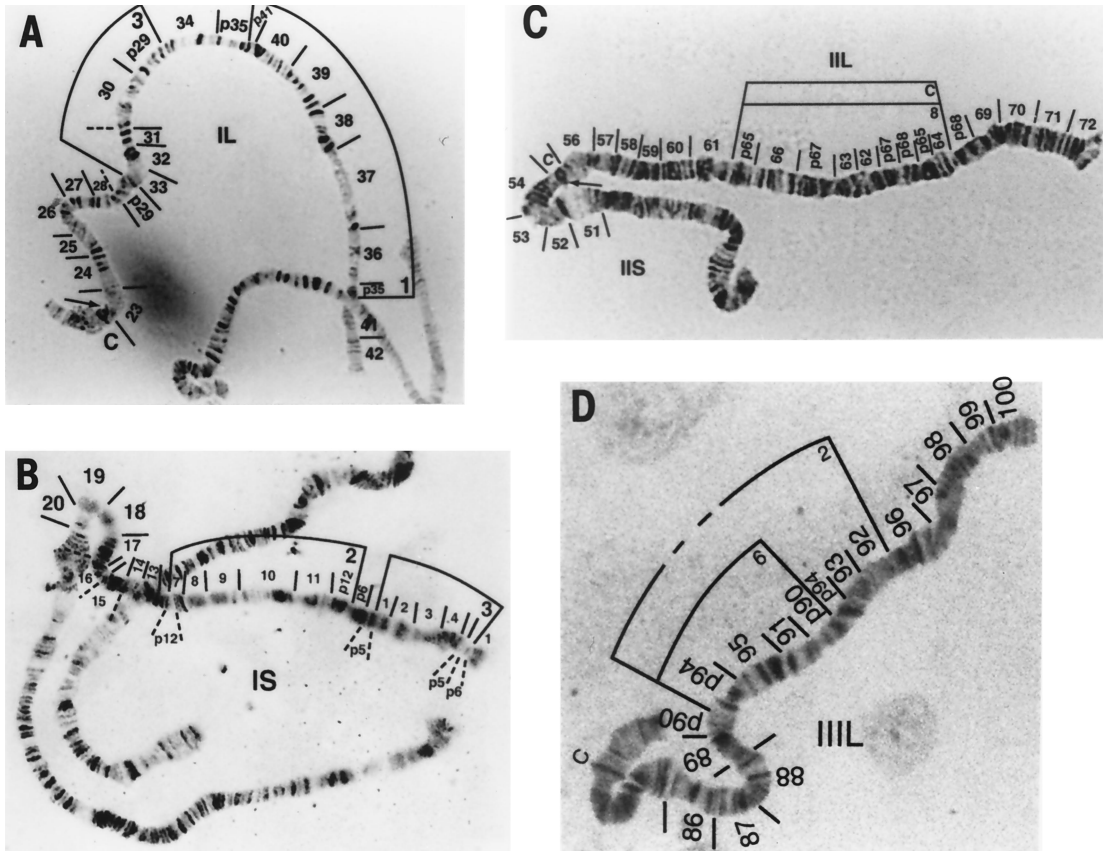


Fig. 1. Cytotaxonomy of Abu Hamed black flies. Numbers in each panel indicate the division of the chromosome into standard cytotaxonomic units as described in *Materials and Methods.* (A) Chromosome IL: Inversions IL-1 and IL-3 are indicated by brackets. The centromere dimorphism is indicated by the arrow. (B) Chromosome IS: Inversions IS-2 and IS-3 are indicated by brackets. Inversion IS-1 is located at the tip of chromosome IS and is included within inversion IS-3. The boundaries of inversion IS-1 are indicated by the three dotted lines labeled p5 and p6 at the tip of chromosome IS. (C) Chromosome IIL: The double inversion consisting of inversions IIL*C* and IIL-8 is indicated by the bracket, and the centromere dimorphism by the arrow. (D) Chromosome IIIL. Inversions IIIL-2 and IIIL-6 are indicated by brackets. The dotted portion of the bracket highlights the fact that inversion IIIL-6 is contained within inversion IIIL-2. In all panels, C is centromere and *P* is partial unit divided by inversion breakpoints.

cleotide difference in the 556-bp 16S rRNA PCR product (Tang and Unnasch 1995), these results indicated that the DNA sequences of the 16S fragment were identical in all of the individuals examined. To confirm this and to further explore the relationship of the Abu Hamed specimens to the West African sibling species of *S. damnosum* s.l., the DNA sequence of the 16S rRNA gene fragment from two randomly chosen individuals collected from Abu Hamed was determined. As expected, both sequences were identical. The DNA sequence data then were used in phylogenetic studies comparing the Abu Hamed flies with the West African sibling species of *S. damnosum* s.l. and to several other *Simulium* species from around the world. These phylogenies were rooted by comparison to the sequence derived from *Austrosimulium (Novaustrosimulium) bancrofti* Taylor, a black fly species known to be related closely to flies of the genus *Simulium* (Crosskey 1987). Phylogenies were derived using both parsimony and numerical methods, as described in the *Materials and Methods.* Both methods produced identical phylogenies (Fig. 4). As expected, the isolates from Abu Hamed were grouped with the three 16S rRNA sequences found in the *S. damnosum* s.l. sibling species. Together, the four African black flies formed a clade that was distinct from the American [*S. (Psilopelmia) ochraceum* Walker, *S. (Psilopelmia) bivittatum* Malloch, *S. (Psilozia) vittatum* Zetterstedt, *S. decorum* Walker, and *S. (Byssodon) meridionale* Riley] and European (*S. noelleri* Friederichs) *Simulium* species, consistent with previously published results (Tang et al. 1996). Within the African clade, the Abu Hamed sequence separated from the clade representing the remaining sibling species of *S. damnosum* s.l., although bootstrap support for the *S. damnosum* s.l. clade was not strong (Fig. 4).

Previous studies suggested that a 252-bp fragment of the ND4 gene was capable of resolving relationships within the *S. damnosum* s.l. complex (Tang et al. 1995a, b). Therefore, this informative fragment was amplified

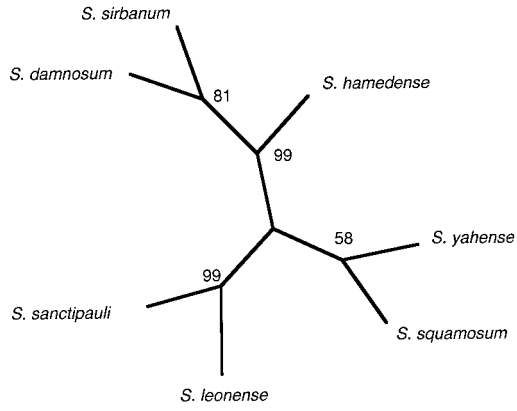
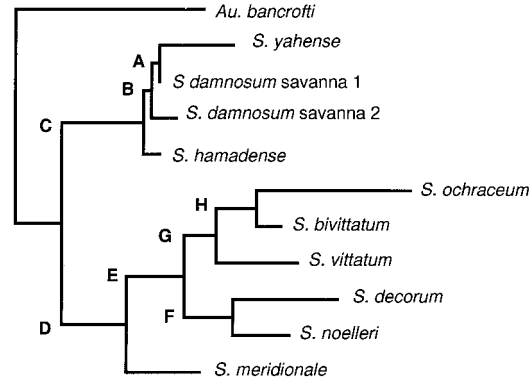


Fig. 2. Phylogenetic analysis of *Simulium damnosum* sibling species, based upon cytotaxonomic data. An unrooted phylogeny was developed from the cytotaxonomic data as described in *Materials and Methods.* A single most parsimonious tree was identified in the analysis shown here. Length and fit measures for this tree were as follows: length 29 (minimum length 28; maximum length 44); consistency index 0.996; rescaled consistency index 0.905. Numbers at the nodes indicate the percentage of times the group distal to the node were grouped in a bootstrap analysis of 1,000 replicate datasets.



Node	Parsimony	Numerical
A	<50	64
B	66	53
C	100	100
D	89	91
E	84	63
F	57	85
G	63	51
H	63	63

Fig. 4. Phylogenetic analysis of 16S rRNA gene sequences from Abu Hamed black flies. Phylogenies were constructed using both parsimony and numerical algorithms, as described in *Materials and Methods.* In the parsimony analysis, 90 characters were identified as variable, of which 40 were informative. Numbers in the table below the figure refer to the percentage of times a group distal to the labeled node were found to occur in a bootstrap analysis of 1,000 replicate datasets.

from two flies from the Abu Hamed focus. Both individuals produced identical sequences. These data were then subjected to phylogenetic analysis as described in *Materials and Methods.* The phylogenies derived from the ND4 data were rooted by comparison with the DNA sequence derived from the North American black fly species *S. vittatum.* Both parsimony and numerical phylogenetic analysis of these data produced phylogenies with identical topologies (Fig. 5). In this phylogeny, the flies from the Abu Hamed focus clustered with the savanna dwelling species *S. damnosum* s.s. and *S. sirbanum.* Within the *S. damnosum* s.l. complex, two other clades were recognized, consisting of the transition zone-dwelling sibling species (*S. leonense–S. sanctipauli*) and the forest-dwelling species (*S. yahense–S. squamosum*). Consistent with the phylogeny developed from the 16S rRNA gene fragment, *S. damnosum* s.s. and *S. sirbanum* form a clade separate from the flies collected from Abu Hamed. However, as in the case of the 16S dataset, bootstrap support for the *S. damnosum* s.s.–*S. sirbanum* clade was not strong.

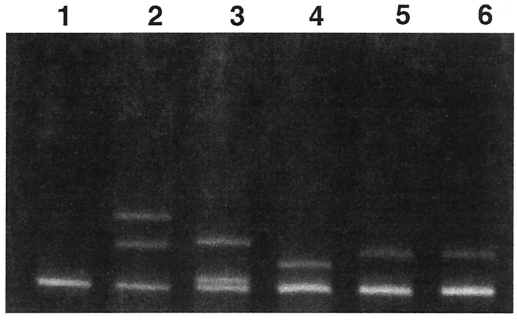
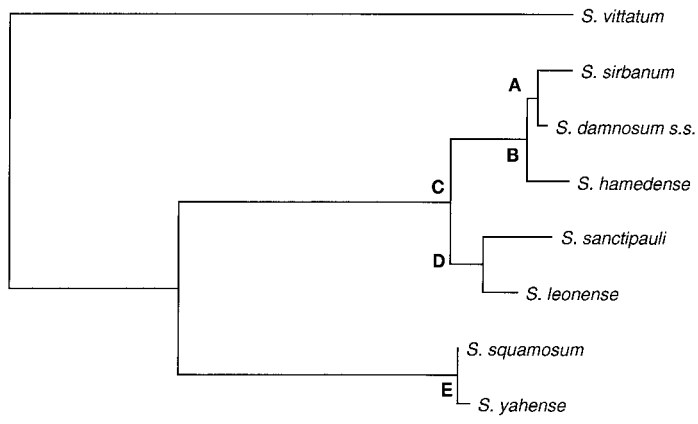


Fig. 3. Heteroduplex analysis of the 16S rRNA gene PCR product from flies from the Abu Hamed focus. HDA was carried out using the 16S PCR product from *S. bivittatum* as the driver DNA. Lane 1, *S. bivittatum* driver alone (homoduplex); lane 2, *S. yahense* target DNA; lane 3, *Simulium damnosum* s.l. savanna allele one-target DNA; lane 4, *Simulium damnosum* s.l. savanna allele two-target DNA; lanes 5 and 6, PCR product from Abu Hamed black flies target DNA.

Discussion

Previous reports suggested that the *Simulium* population endemic to the Abu Hamed focus was a monomorphic form of *S. sirbanum* (Procunier 1989). Consistent with this conclusion, the cytological data placed Abu Hamed cytotype within the *S. damnosum* s.s. subcomplex. However, the data indicated that these flies were distinct from the West African species of *S. damnosum* s.l., including *S. sirbanum.* First, unlike *S. sirbanum,* there was a complete absence of any sex-relatedness to the inversion IS-3. Sex chromosome differentiation is considered to be important in population substructuring leading to speciation in *Simulium* (Procunier 1982). Closely related members differ by only sex chromosomes, but distant taxa differ by both fixed inversions and sex chromosomes (Procunier 1989). Second, the Abu Hamed samples were notable for the highly monomorphic nature of their chromosomes. The floating inversions IL-2, IIL-3, and



Node	Parsimony	Numerical
A	49	56
B	100	83
C	100	97
D	74	80
E	100	100

Fig. 5. Phylogenetic analysis of ND4 gene sequences from Abu Hamed black flies. Phylogenies were constructed from the ND4 sequence data using both parsimony and numerical algorithms as described in *Materials and Methods.* In the parsimony analysis, 46 variable characters were identified, of which 22 were informative. Numbers in the table refer to the percentage of times a given group was found to exist in a bootstrap analysis of 1,000 replicate datasets.

IIIL-7, which commonly are found in *S. sirbanum* populations (Boakye 1993) were absent in these individuals. Together, these findings indicated that, although the flies from Abu Hamed were related closely to *S. sirbanum,* they did not appear to be simply an isolated population of the latter. This conclusion is supported by the phylogenetic analysis carried out on the cytotaxonomic data. If the Abu Hamed flies were indeed an isolated population of *S. sirbanum,* one would predict that they would have formed a clade with the West African *S. sirbanum* populations, excluding *S. damnosum* s.s. However, the Abu Hamed isolates did not group with *S. sirbanum.* In fact, the data provide some support for the conclusion that the West African *S. sirbanum* and *S. damnosum* s.s. are related more closely to one another than they are to the Abu Hamed population.

The DNA sequence data also supported the hypothesis that the Abu Hamed flies are related closely to, but distinct from, the savanna dwelling species *S. sirbanum* and *S. damnosum* s.s. First, the Abu Hamed population encoded a new variant of the 16S rRNA sequence which was distinct from the West African sibling species. Similarly, analysis of the ND4 gene sequence from the Abu Hamed flies demonstrated that it differed from the sequences found in the West African sibling species of *S. damnosum* s.l. Both parsimony and numerical methods grouped this sequence with the savanna-dwelling West African species. However, the Abu Hamed sequence did not form a clade with *S. sirbanum.* Therefore, the DNA sequence data and the cytological data together support the hypothesis that the black fly population present in Abu Hamed is related closely to, but distinct from, *S. sirbanum,* and may represent a new sibling cytospecies of *S. damnosum* s.l. We propose that this be named the hamedense form of the *Simulium damnosum* complex. In accordance with standard usage in simuliid taxonomy, this is an informal name outside the regulation of the International Code for Zoological Nomenclature. Voucher specimens of the hamedense form have been deposited in the Natural History Museum, London. Although distinct from the West African members of the *S. damnosum* complex, both the cytotaxonomic and DNA sequence data indicate that the hamedense form is related most closely to the savanna-dwelling species *S. damnosum* s.s. and *S. sirbanum.*

Onchocerciasis in Eastern and northern Sudan may be amenable to vector control because of the focal nature of the infection at these foci (Baker and Abdelnur 1986). However, this will depend to a large extent on whether there is vector dispersal among the various foci, and in particular between the large focus in the South and those in the East and North. The finding that the black fly population of Abu Hamed represents a unique population, together with its extreme geographic isolation, indicates that migration may not be a significant confounding factor if vector control is used as a control measure in this area. Further studies of the eastern focus will be needed to determine if this also will be the case at this focus.

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