International Journal of Tropical Insect Science Vol. 28, No. 1, pp. 3–11, 2008 DOI: 10.1017/S1742758408901351

Cytotaxonomy of *Simulium soderense* sp. nov. and a redescription of the 'Kulfo' form (Dunbar, 1969) of *Simulium damnosum* s.l. (Diptera: Simuliidae) as *Simulium kulfoense* sp. nov. in Ethiopia

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(Accepted 22 November 2007)

Abstract. The exact taxonomic identity of members of the *Simulium damnosum* Theobald complex in Ethiopia was not known until recently. A cytotaxonomic analysis of larval chromosomes from the Kulfo River area in southern Ethiopia, from where the 'Kulfo' form was reported, and from the Awash River at Sodere in central Ethiopia has revealed the existence of two newly recognized species, namely *Simulium kulfoense* and *Simulium soderense*. Although three fixed and 11 floating inversions in the chromosomes of the 'Kulfo' form were described in an idiogram, in the present study four fixed and 17 floating inversions were observed, out of which only seven floating inversions were the same as Dunbar's, indicating that the remaining 10 inversions were either missed or misinterpreted. Though both species share many inversions, *S. kulfoense* sp. nov. has a diagnostic inversion on chromosome II, IIL-E6, with no sex-linked chromosomal arrangement, while *S. soderense* sp. nov. has a sex-linked inversion, IIS-3, which warrants it as a distinct species. Both of these new species also share many inversions with the recently described new species from south-western Ethiopia, *Simulium kaffaense*, including the fixed inversions IIL-E1 and E3. Since both the study sites are known to be free of onchocerciasis, and the flies are not anthropophilic, both the species are not presumed to be vectors of human onchocerciasis. The presence of IS-1 and a complex inversion possibly involving IL-3 strongly indicates that the two species belong to the 'Nile' phylogenetic group of *S. damnosum* s.l.

Key words: Diptera, Simuliidae, *Simulium damnosum* complex, onchocerciasis, *Simulium kulfoense*, *Simulium soderense*

Introduction

Since the description of *Simulium damnosum* Theobald (Diptera: Simuliidae) as a species complex (Dunbar, 1966), around 60 cytoforms, some with formal scientific names, have been reported (Crosskey and Howard, 2004; Krüger *et al.*, 2004; Mustapha *et al.*, 2004; Hadis *et al.*, 2005; Krüger

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et al., 2006b). The number of cytospecies is expected to rise as new areas are prospected. Probably, as a result of heterogeneity of topography, there is a preponderance of segregates in eastern and Central Africa, about twice, compared with West Africa (Raybould and White, 1979; Vajime and Gregory, 1990; Crosskey and Howard, 2004). However, the number of vectors in West Africa is much higher than in East Africa: while all the nine species of *S. damnosum* s.l. in West Africa are vectors of *Onchocerca volvulus* Bickel (Nematoda: Filarioidea) (WHO, 1995), only three are vectors in East Africa (Krüger *et al.*, 1999; Vajime *et al.*, 2000; Hadis *et al.*, 2005).

Compared with other African countries with endemic occurrence of onchocerciasis, information on the *S. damnosum* complex in Ethiopia is scanty, except for the recent work by Hadis *et al.* (2005), which described the vector populations in south-western Ethiopia. Dunbar (1969, 1976) reported the existence of the 'Jimma' form in an area where onchocerciasis is endemic and the 'Kulfo' and 'Kisiwani' forms in other onchocerciasis-free areas. While the 'Kisiwani' form is reported in Tanzania and Kenya, the 'Kulfo' form is so far reported only in Ethiopia (Crosskey and Howard, 2004). However, Dunbar (1969, 1976) failed to provide chromosomal maps of the respective cytoforms; instead, described the 'Kulfo' form using idiograms.

From 12 specimens of *S. damnosum* s.l. from the Kulfo River in southern Ethiopia, Dunbar (1969) described three fixed inversions, namely IS-1, IL-3 (characteristic inversions for the 'Nile' group) and IIL-3, and 11 floating inversions for the 'Kulfo' form. Since no further cytotaxonomic study was conducted on this population, the breakpoints of the inversions are not known making it difficult to identify this form especially if found in sympatry with other members of *S. damnosum* s.l. Two inversions, IIL-13 and IIIL-8, of the 'Kulfo' form have been assumed to be the same with that of *Simulium kaffaense* Hadis indicating some level of similarity between the two cytospecies (Hadis *et al.*, 2005). Krüger *et al.* (2005) have also emphasized the similarity of the 'Kulfo' form with *S. kaffaense* based on an ITS-1 amplicon that has the same size for the two cytospecies.

Although the *S. damnosum* s.l. along the Awash River had been reported to be anthropophilic (Raybould and White, 1979) in an onchocerciasis-free area, no attempt has been made to know the identity of the population. The objective of this study, therefore, is to describe the cytotaxonomy of the two populations and provide chromosomal maps for future reference.

Materials and methods

Study sites

Sodere is a resort site 125 km east of Addis Ababa along the Awash River, in an arid area, 930 m above sea level (masl). The Awash River is one of the biggest rivers in Ethiopia. No onchocerciasis is known to exist east of Addis Ababa.

Arba Minch is a provincial town 550 km south of Addis Ababa, at 1243 masl. It is a mountainous area with woodland vegetation. No onchocerciasis is known to occur around Arba Minch. Descriptions of the study sites are summarized in Table 1.

Collection of samples

Simulium damnosum s.l. larvae were picked off from vegetation substrates and transferred into a freshly prepared Carnoy's solution (containing three parts of absolute ethanol and one part of glacial acetic acid) in universal sample bottles. The specimens were transported in iceboxes under cold conditions to the laboratory where they were kept at 4 °C until needed.

Identification of S. damnosum *s.l. larvae*

Simulium damnosum s.l. larvae were examined under a dissection microscope and identified according to the method described by Crosskey (1973).

Preparation and analysis of chromosomes

Chromosomes of *S. damnosum* were prepared and examined as described by Hadis *et al.* (2005). The photographs of the chromosomes were compared with similar photographs of other *Simulium*

Table 1. Collection data also describing the vegetation and altitude of the study sites in Ethiopia

Locality	Vegetation and landscape	Altitude (metres above sea level)	Co-ordinates	Date of collection
Awash River, Sodere	Riverine vegetation, next to arid area with acacia trees	930	08°23N, 039°23E	4–6 December 2002
Kulfo River, Arba Minch	Riverine vegetation, next to savannah woodland, mountainous area	1243	06°02N, 037°32E	28 November 2002–3 December 2002

chromosomes: firstly, with those of the standard 'Nyamagasani' form (Dunbar, 1969; Dunbar and Vajime, 1981) to determine the inversion breakpoints and then, secondly, with those of other relevant species or cytoforms (Vajime and Dunbar, 1975; Post, 1986; Boakye, 1993; Maegga and Cupp, 1993; Procunier and Muro, 1993; Hadis *et al.*, 2005; Krüger *et al.*, 2006a) to examine whether any of the inversions observed had been described previously.

Statistical analysis

Sex linkage of inversions in the respective populations was examined using χ^2 tests. The analyses were carried out using SPSS version 10 (SPSS, Inc., USA).

Results

Cytotaxonomy of Simulium kulfoense *sp. nov*

Synonym: 'Kulfo' form Dunbar (1969).
Etymology: This species is named *S. kulfoense* after the cytoform's name 'Kulfo' given by Dunbar (1969), which is also the name of the river from where the species was collected.

Description: Examination of 40 karyotypes of *S. kulfoense* revealed the presence of four fixed and 17 floating inversions. Chromosome IL has a new fixed inversion designated as IL-ZE. It is a three-step complex inversion involving segments 29–33 (Fig. 1). A floating inversion presumed to be IL-5 reported by Dunbar (1969) was found in 52.5% of the specimens. It runs from part of segment 25 to part of segment 28 (Figs 1 and 2). Another, also new, floating inversion, IL-37 (Fig. 2), occurred at relatively lower frequency (17.5%) (Table 2). It includes segments 35 and 36. IL-9, an inversion overlapping the fixed inversion IL-ZE, also reported by Dunbar (1969), occurred only as a heterozygote inversion involving segments 34, 31, 30, and 29 (Fig. 3). Inversions, IL-1E and IL-CE, that were also reported in *S. kaffaense* (Hadis *et al.*, 2005) are also common in this population. One rare inversion, IL-J, appeared on one specimen as a heterozygous inversion and it involves segments 34–38.

Inversion IS-1 has a 100% frequency with the majority existing as homozygous. IS-BE (Hadis *et al.*, 2005) was also found to be common (Table 2).

Chromosome IIL has three fixed inversions, E1, E3 (which were also reported in *S. kaffaense*; Hadis *et al.*, 2005), and E6 (a new inversion involving segment 67 to part of segment 70; Fig. 4). One specimen was heterozygous for IIL-E6 (2.5%) of the 40 specimens identified. The floating inversions include IIL-E4, IIL-E5 and IIL-13, which are inversions reported earlier (Dunbar, 1969; Hadis *et al.*, 2005) (Table 3).

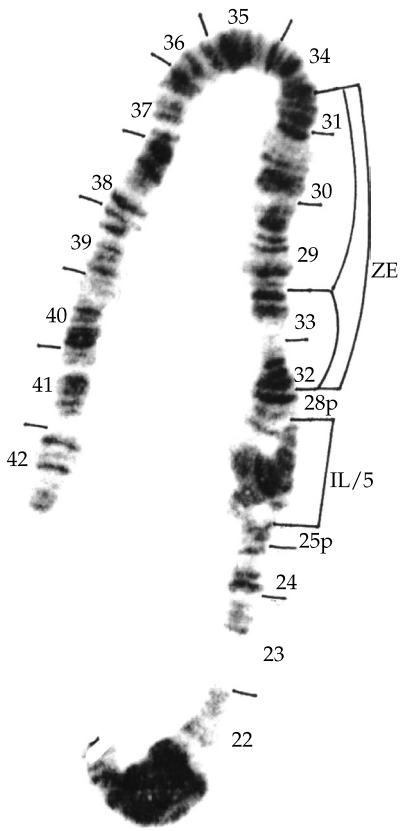

Fig. 1. Long arm of chromosome I of *Simulium kulfoense* (a male collected from Kulfo River at Arba Minch, Ethiopia) showing the fixed inversion IL-ZE and floating inversion IL/5

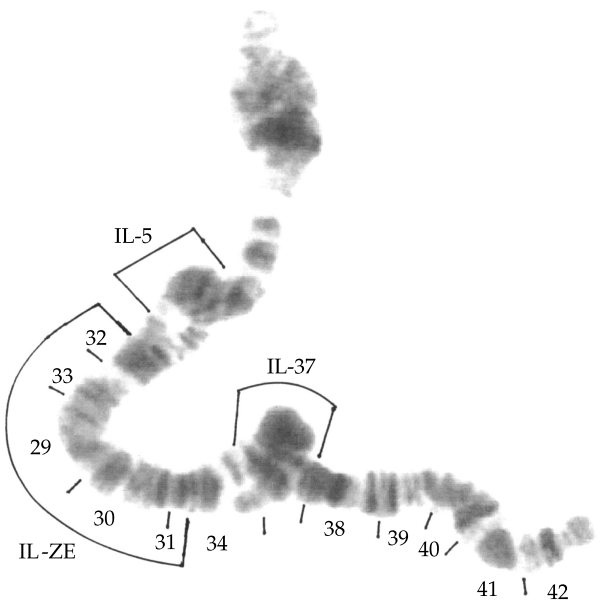

Fig. 2. Long arm of chromosome I of *Simulium kulfoense* (a female collected from Kulfo River at Arba Minch, Ethiopia) showing floating inversions IL-37 and IL-5 and fixed inversion IL-ZE

Table 2. Inversion frequencies in *Simulium kulfoense* and *Simulium soderense* collected from Kulfo and Awash rivers, respectively, in Ethiopia

	No. and (%)	
Karyotype	Kulfo	Awash
Chromosome I		
IL-ZE	40 (100)	47 (100)
IL-5	6 (15.0)	44 (93.6)
IL/5	15 (37.5)	2 (4.3)
IL-37	1 (2.5)	0 (0)
IL/37	6 (15.0)	9 (19.1)
1L/1E	11 (27.5)	7 (14.9)
IL/9	22 (55.0)	1 (2.1)
IL-CE	9 (22.5)	0
IL/CE	4 (10.0)	0
IL/J	1 (2.5)	0
IL/SE	0 (0)	4 (8.5)
IS-1	25 (62.5)	43 (91.5)
IS/1	15 (37.5)	4 (8.5)
IS-BE	8 (20.0)	1 (2.1)
IS/BE	0 (0)	2 (4.3)
IS/UE	0 (0)	4 (8.5)
Chromosome II		
IIL-E1.E3	40 (100)	47 (100)
IIL-E6	39 (97.5)	0 (0)
IIL/E6	1 (2.5)	3 (6.4)
IIL-E4	27 (67.5)	0 (0)
IIL/E4	12 (30.0)	0 (0)
IIL/E5	4 (10.0)	0 (0)
IIL-13	2 (5.0)	0 (0)
IIL/13	5 (12.5)	2 (4.3)
IIS-2	7 (17.5)	47 (100)
IIS/2	26 (65.0)	0 (0)
IIS-3	0 (0)	21 (44.7)
IIS/3	3 (7.5)	26 (55.3)
Chromosome III		
IIIS-A	19 (47.5)	0 (0)
IIIS/A	17 (42.5)	0 (0)
IIIL-8	8 (20.0)	0 (0)
IIIL/8	17 (42.5)	6 (12.8)
IIIL-F	7 (17.5)	0 (0)
IIIL/F	15 (37.5)	0 (0)
IIIL/DA	20 (50.0)	1 (2.1)

Chromosome IIS has two dependent floating inversions, IIS-2 and IIS-3, which were also reported by Dunbar (1969). IIS-2 involves segments from 48 to part of 53, while IIS-3 exists within IIS-2 running from segment 48 to part of 51. IIS-3 existed only as a heterozygous inversion and at a lower frequency (Table 3). Figure 4 shows the heterozygous inversion for IIS/2.

Four floating inversions, IIIS-A, IIIL-8, IIIL-D and IIIL-F (Fig. 5), were recorded on chromosome III, and all were previously reported (Dunbar, 1969; Hadis *et al.*, 2005).

No inversion was found to be associated differentially with sex in this population. Hence, it was not possible to determine which chromosome is involved in sex determination.

Cytotaxonomy of S. soderense *sp. nov*

Synonym: 'Sodere' form Hadis (2006)
Etymology: This cytoform is named *S. soderense* after the collection site Sodere, along the Awash River.
Description: It has four fixed inversions, namely IL-ZE, IIL-E1, E3 and IIS-2. IIS-3 and IS-1 have been found in all the specimens examined but were polymorphic (Figs 6–8). IIS-3 is sex linked: 91.7% of the males and 5.9% of females (percentage calculated from 41 larvae whose sex was determined) are heterozygous, and the remainder are homozygous for IIS-3 ($P < 0.0001$) (Table 4). *Simulium soderense*, therefore, has two X and Y chromosomes: X_S (3%) and X_3 (97%), and Y_S (91.4%) and Y_3 (8.6%). IIS-3 in *S. soderense* is, therefore, the main X chromosome while IIS-standard is the Y chromosome.

IL-5 is the commonest floating inversion in this population at 97.9% frequency. The shared inversions

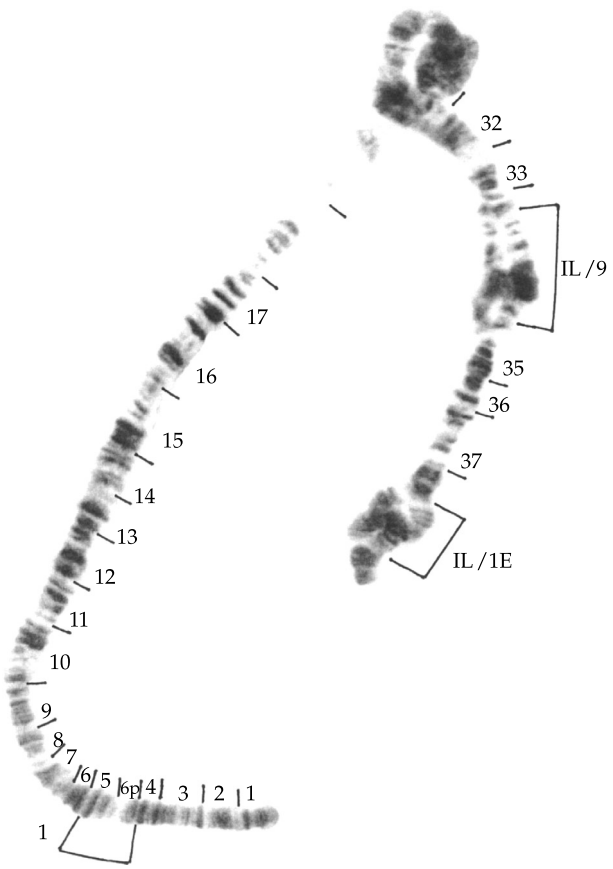

Fig. 3. Chromosome I of *Simulium kulfoense* (a male collected from Kulfo River at Arba Minch, Ethiopia) showing the floating inversions IL-9, IL-IE and IS-1 (the fixed inversion IL-ZE is not shown)

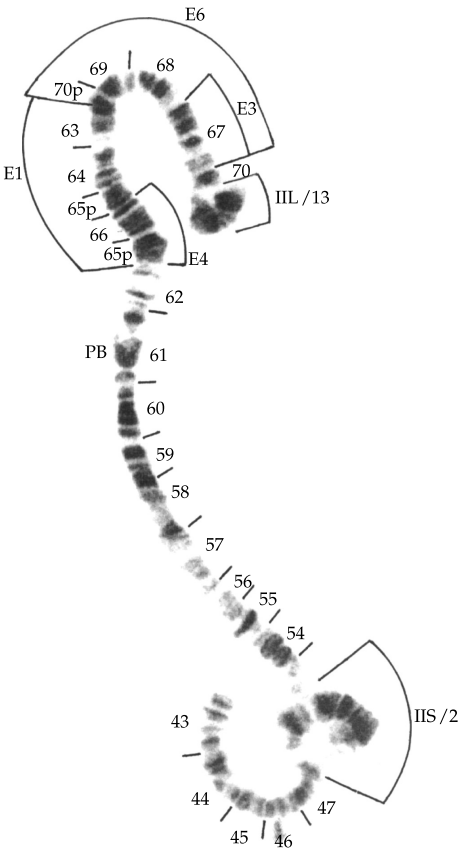

Fig. 4. Chromosome II of *Simulium kulfoense* (a male collected from Kulfo River at Arba Minch, Ethiopia) showing fixed inversions IIL-E1, E3, and E6, and floating inversions IIL-13 and IIS-2

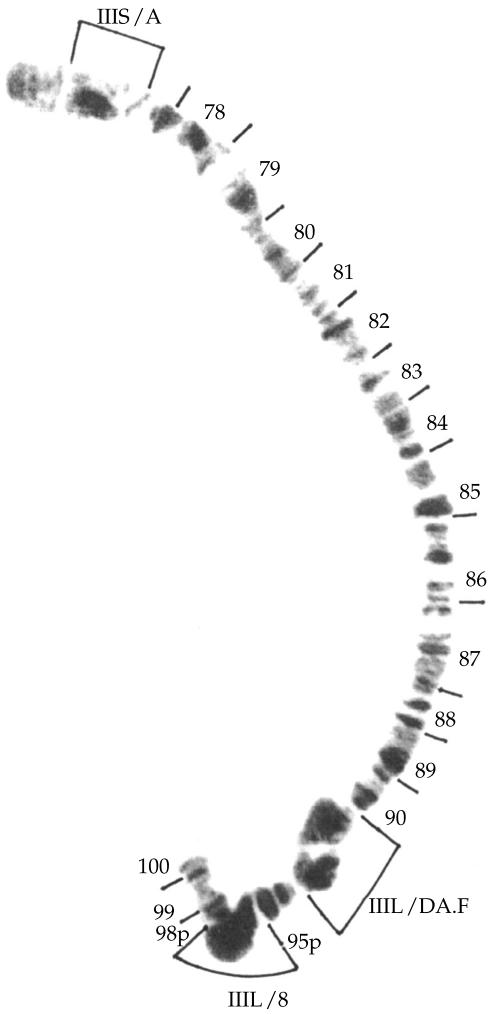

Fig. 5. Chromosome III of *Simulium kulfoense* (a female collected from the Kulfo River at Arba Minch, Ethiopia) showing floating inversions IIIL-8, IIIL-F, IIIL-D and IIIS-A

like IS-BE, IL-1E and IIIL-8 have low frequencies. Unique inversions in *S. soderense* include IL-SE and IS-UE. IL-SE overlaps both IL-ZE and IL-5, and IS-UE includes segments 1–3.

Chromosome III is fairly homogenous: more than 85% of *S. soderense* have standard arrangement. Only IIIL-8 (12.8%) and IIIL-D (2.1%) were recorded.

Table 3. Inversions reported in the 'Kulfo' form of *Simulium damnosum* s.l. by Dunbar (1969) and the present study

	Dunbar (1969)		Present study	
Chromosome arm	Fixed	Floating	Fixed	Floating
IS	1	8, 7, 6	–	1, 6
IL	3	5, 9, 4	ZE	5, 9, 56, 1E, CE, J
IIS	–	2, 3	–	2, 3
IIL	3	12, 13	E1, E3, E6	13, E4, E5
IIIS	–	–	–	A
IIIL	–	8	–	8, F, D

Discussion

From 12 specimens of *S. damnosum* s.l. from the Kulfo River in southern Ethiopia, Dunbar (1969) described three fixed inversions, namely IS-1, IL-3 (characteristic inversions for the Nile group), and IIL-3, and 11 floating inversions. In the present study, four fixed and 17 floating inversions are reported (Table 3). Since Dunbar (1969) did not provide chromosomal maps, direct comparison of the inversions is very difficult except for some of them. Out of the three fixed inversions Dunbar (1969) has mentioned, only IS-1 is reported by the present investigation. However, this inversion was found to be polymorphic as in *S. kaffaense* (Hadis *et al.*, 2005) and not fixed as Dunbar (1969) claimed. Instead of IL-3, a three-step complex inversion (IL-ZE) was found on chromosome IL, possibly involving IL-3 (Fig. 1).

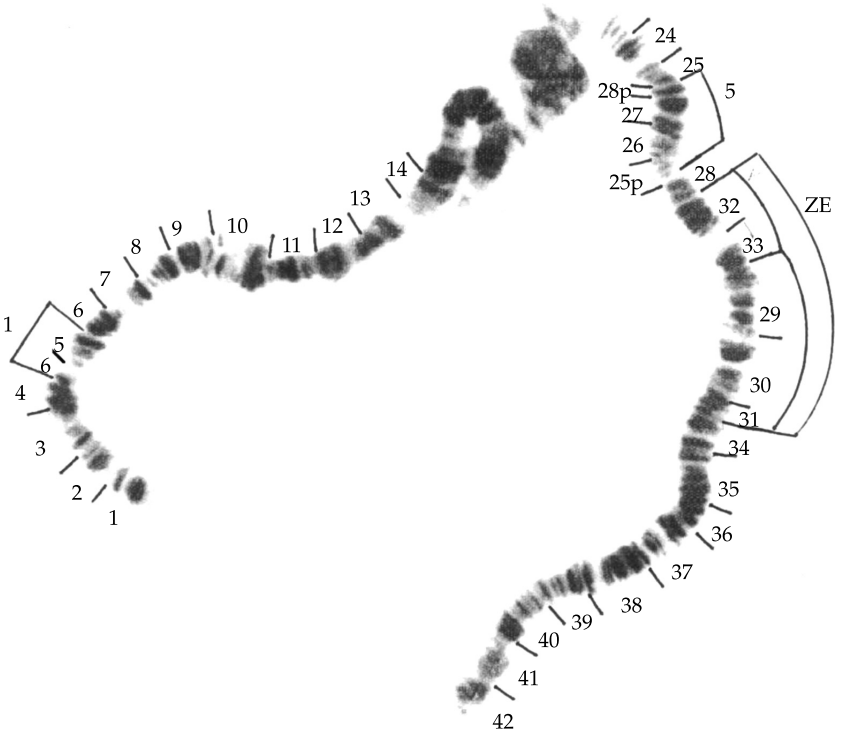

Fig. 6. Chromosome I of *Simulium soderense* (from a larva of undetermined sex collected from Awash River at Sodere) showing fixed inversion IL-ZE and floating inversions IL-5 and IS-1

IL-ZE is very similar to IL-NE of *S. kaffaense*; the difference being that segment 34 is involved in IL-ZE as a part of the floating inversion IL-9 while it is part of the fixed inversion in IL-NE.

On chromosome IIL, three fixed inversions E1, E3 and E6 were found instead of only one fixed simple inversion IIL-3, as reported by Dunbar (1969). However, it is possible that Dunbar (1969) might have seen the three interdependent inversions as a single simple inversion as they are fixed. The floating inversion, IIL-13 (Dunbar, 1969), has been confirmed in the present study; however, the presence of a rather large inversion, IIL-12, could not be confirmed. Apparently, Dunbar (1969) missed the floating inversions IIL-E4 and IIL-E5. One larva was found to be heterozygote for IIL-E6, which means only three larvae would be expected to be homozygote for IIL-St/St for IIL-E6 out of 10,000 larvae. Hence, this inversion can be considered fixed and diagnostic.

All the inversions found on chromosome IIL of *S. kaffaense* (Hadis *et al.*, 2005), except IIL-E2, do also occur in *S. kulfoense*, but only IIL-E1, IIL-E3 and IIL-13 were found in *S. soderense* showing the closeness of *S. kulfoense* and *S. kaffaense*.

The common inversions IIS-2 and IIS-3 reported in this study were mistakenly reported by Hadis *et al.* (2005) as IIS-B and IIS-G, respectively, in *S. kaffaense* due to the confusion by the fixed inversion IIS-DA that overlaps them. IIS-2 was found to be fixed in *S. soderense* but floating in *S. kaffaense* and *S. kulfoense*.

IIIL-8 was the only inversion reported by Dunbar (1969) on chromosome III for the 'Kulfo' form. IIIS-A, IIIL-F and IIIL-D were all missed. All the inversions on chromosome III of *S. kulfoense* were reported in *S. kaffaense*, but IIIS-A and IIIL-F were fixed in the latter. Only IIIL-8 (12.8%) and IIIL-D (2.1%) were recorded in *S. soderense*, showing its isolation compared with the other two species.

On chromosome I, there are four floating inversions reported in the 'Kulfo' form according to Dunbar (1969): IS-8, IS-7, IS-6 and IL-4. We could not observe them in the present study. The inversions are either very rare, which is very unlikely since with 40 specimens a considerably greater number have been examined in the present study compared with the 12 specimens used by Dunbar (1969); hence we believe that a misinterpretation is more likely.

In addition to the inversions shown on the idiogram of 'Kulfo' by Dunbar (1969), IIL-5 has also been mentioned in the text as one of the inversions for 'Kulfo'. Since the breakpoints of IIL-5 are known (Maegga and Cupp, 1993), the inversions are obvious and we would have noticed them. However, this inversion was not detectable. Another inversion, IIL-10, was also associated with 'Kulfo' and 'Kagera' forms in a phylogenetic tree produced by Dunbar and Vajime (1981). However, it was not presented in

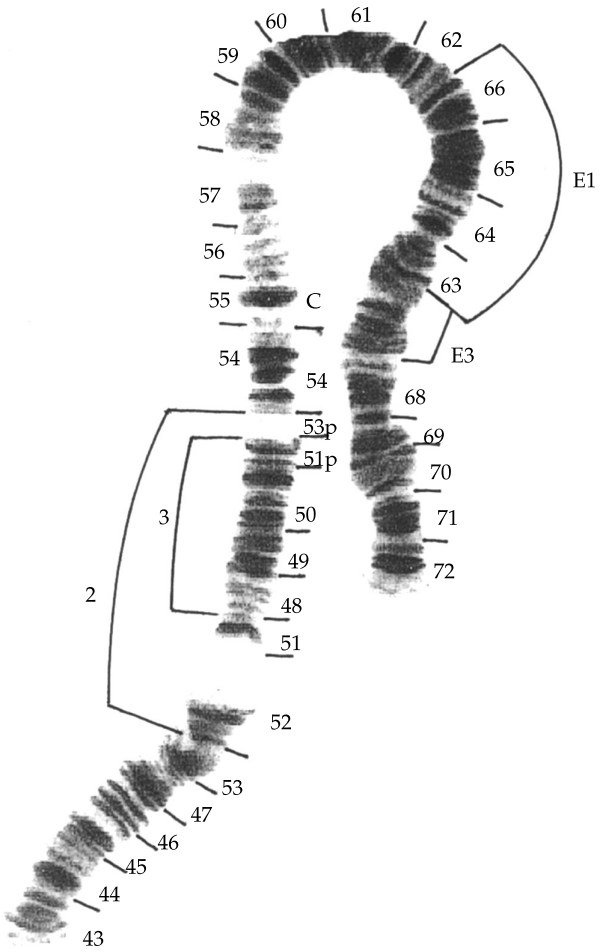

Fig. 7. Chromosome II of *Simulium soderense* (a female collected from Awash River at Sodere, Ethiopia) showing fixed inversions IIS-2, IIL-E1 and IIL-E3 and floating inversion IIS-3

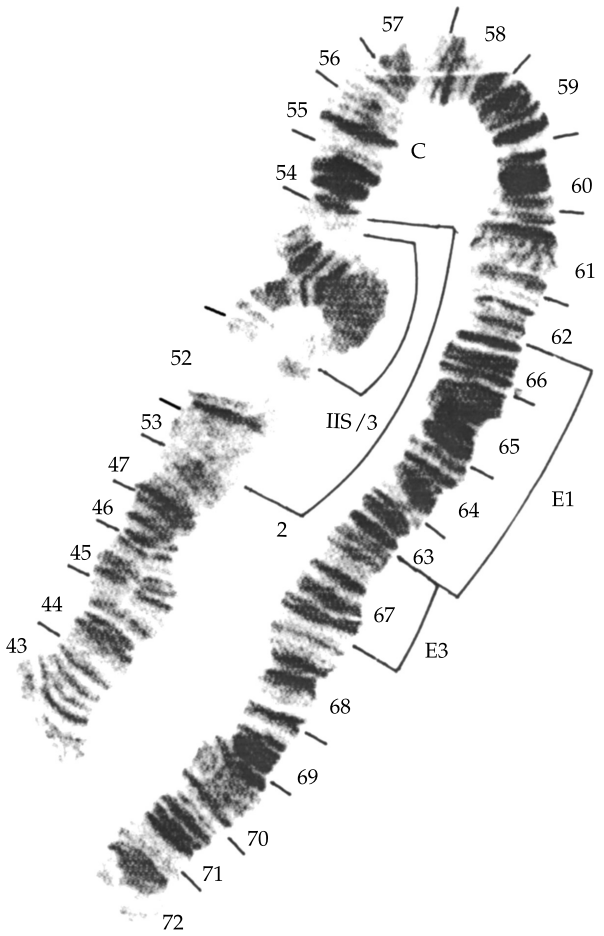

Fig. 8. Chromosome II of *Simulium soderense* (a male collected from Awash River at Sodere, Ethiopia) showing the heterozygote inversion IIS/3 and the fixed inversions IIL-E3, IIL-E1 and IIS-2

the idiogram of both 'Kagera' and 'Kulfo' (Dunbar, 1969), therefore, making it difficult to ascertain its presence or absence.

Out of the 17 floating inversions reported in the present study (Table 3), 7 of them are assumed to be the same as IS-1, IL-5, IL-9, IIL-13, IIS-2, IIS-3 and IIIL-8 reported by Dunbar (1969). The remaining 10 are likely to have been missed or misinterpreted by Dunbar (1969).

The presence of the diagnostic fixed inversion, IIL-E6, in *S. kulfoense* and its absence (or rarity) in *S. soderense* coupled with the sex linkage of IIS-3 in *S. soderense* justifies their distinctive species status.

The 'Kulfo' form was assigned to the 'Nile' group based on the fixed inversions, IS-1 and IL-3, and to the *squamosum* subgroup due to the fixed inversion IIL-10 (Dunbar and Vajime, 1981). Though IS-1 was found in all specimens examined in *S. kulfoense*, it was polymorphic and the sections involved in the inversion IL-3 were found to be part of a complex inversion IL-ZE hinting that IL-3 could be part of the complex inversion. The presence of IS-1 and the possible involvement of IL-3 in IL-ZE, therefore, strongly indicate that both *S. kulfoense* and *S. soderense* belong to the 'Nile' group.

Although Raybould and White (1979) had reported that *S. damnosum* s.l. along the Awash River is anthropophilic, repeated attempts in the present study failed to catch flies on human bait, at

Table 4. Sex linkage of IIS-3 in *Simulium soderense* from central Ethiopia. Only gonadally sexed larvae are used ($P < 0.0001$)

	Sex and no. (%)	
IIS-3 Inversions	Male	Female
IIS-St/St	0 (0)	0 (0)
IIS/3	22 (91.7)	1 (5.9)
IIS-3/3	2 (8.3)	16 (94.1)
Total	24 (100)	17 (100)

least at the collection site in Sodere. However, it is possible that the *S. soderense* itself can be anthropophilic down- or upstream of the Awash River as has been the case for *Simulium yahense* (Vajime and Dunbar) (Cheke, 1998) and *Simulium konkourense* (Boakye) (Post and Boakye, 1992), which exhibit either anthropophily or zoophily in different geographical areas.

Conclusion

Based on the criteria for distinguishing species using cytotaxonomy, the presence of fixed inversion and/or sex-linked inversion, among others, two new members of the *S. damnosum* species complex, *S. soderense* and *S. kulfoense*, are reported from southern and central Ethiopia in this study. The *S. damnosum* species complex so far described in Ethiopia, therefore, comprises four cytospecies: three formally named, *S. kaffaense*, *S. kulfoense*, and *S. soderense*, and one informally named 'Kisiwani' form.

Acknowledgements

The authors thank Mr Samson Otoo, Mr Tony Tetteh-Kumah, and Mr Jonas Asigbe for their help both in the laboratory and field work during the first phase of MH's training. We also thank Mr Charles Brown for his help in editing the pictures. We are grateful to Messers Teffera Asfaw, Dejene Tilahun, Alemayoh Abate, and Berhane Asfaw for their assistance with the field collections. We are also indebted to the former Director of Noguchi Memorial Institute for Medical Research, Prof. David Ofori-Adjei and former Directors of the Ethiopian Health and Nutrition Research Institute, Dr Abera Geyid, and the late Mr Tilahun W/Michael for their help throughout the study. We also thank the current Director of the Ethiopian Health and Nutrition Research Institute, Dr Tsehanesh Messele, for her encouragement and help. This study was funded by UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) and Training Grant to MH (No. M8/181/4/H.221).

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