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Cytotaxonomy, morphology and molecular systematics of the Bioko form of *Simulium yahense* (Diptera: Simuliidae)

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Abstract

Cytotaxonomic analysis of the polytene chromosomes from larvae of the *Simulium damnosum* Theobald complex from the island of Bioko in Equatorial Guinea is reported, and a new endemic cytoform is described. Chromosomally this cytoform is close to both *S. squamosum* (Enderlein) and *S. yahense* Vajime & Dunbar, but is not identical to either. However, it is morphologically and enzymatically identical to *S. yahense*. The Bioko form was also found to differ from other cytoforms of the *S. damnosum* complex in West Africa in the copy number or RFLP pattern of several different repetitive DNA sequences. It is clear that the Bioko form is genetically distinct from other populations of the *S. damnosum* complex, and whilst it is closest to *S. yahense*, it shows features that suggest a high degree of geographical and genetic isolation. Such isolation is an important consideration in the assessment of the potential for onchocerciasis vector eradication on Bioko.

Introduction

Human onchocerciasis is a severely debilitating and blinding disease caused by infection with the parasitic nematode *Onchocerca volvulus* (Leuckart) (Nematoda:

Filarioidea). In West Africa onchocerciasis is transmitted exclusively by sibling species of the *Simulium damnosum* Theobald complex (Diptera: Simuliidae), with blindness rates reaching as high as 15% in the savanna with up to 100% of adults infected. Infection rates can be similarly high in the forest bioclimatic zone, and although the ocular manifestation is usually very much less than in the savanna (with blindness rates typically around 0.5%, see Duke, 1990)

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onchocercal skin disease can be a significant socio-economic problem (Remme, 1995). The major patterns of epidemiological variation are correlates of the taxonomy of both the parasite and the vector (Post & Boakye, 1992), and so not all sibling species are equally important.

The island of Bioko (formally Fernando Po) is part of the Republic of Equatorial Guinea, and is situated 40 km off the coast of Cameroon. An epidemiological survey of the disease on Bioko (Mas *et al.*, 1990, 1995) found an overall prevalence of 75% with a mean microfilarial density of 32 per skin biopsy. Blindness, due to all causes was registered at 0.8%, which is similar to that seen in the forest endemic areas of West Africa and Cameroon (WHO, 1987), and 29% of the total population had onchocercal skin disease. Thus onchocerciasis was shown to be hyperendemic and the whole population of 62,000 was estimated to be at risk. There have been almost no systematic studies of the biology of the vector and the level of transmission on the island, but *S. damnosum* s.l. is known to breed in the numerous small (sometimes seasonal) streams (Calvo Picó, 1962; McCall *et al.*, 1998). It seems to be chromosomally distinctive (Post *et al.*, 1995), but is morphologically similar to *S. yahense* Vajime & Dunbar (Simuliidae) (Wilson *et al.*, 1994) and is undoubtedly the vector (Cheke *et al.*, 1997). Duke *et al.* (1966) included flies from Bioko in their classic study which defined the existence of separate forest and savanna *Onchocerca-Simulium* complexes, and concluded that Bioko fell into the forest group. Furthermore Lewis & Duke (1966) described a sample of 20 flies as being 'dark' and belonging to the forest group. McCall *et al.* (1997) used some flies from Bioko in their analysis of oviposition aggregation pheromone and concluded that the composition of the pheromone was similar throughout the *S. damnosum* complex.

Until recently, onchocerciasis control in West Africa was effected solely by vector control because no suitable drugs were available for community treatment. The prospects for vector control over the whole island of Bioko are at first sight not good, because of the very large number of streams in which *S. damnosum* s.l. breeds. However, actual eradication of local vectors in isolated situations has been achieved elsewhere in the past using larvicides (McMahon, 1967; Davies, 1994), including the eradication of the Djodji form of *S. sanctipauli* Vajime & Dunbar (Simuliidae) in West Africa (Walsh, 1990a; Fiasorgbor *et al.*, 1992). The success of local elimination has depended upon the accessibility of breeding sites to control operations, and their concomitant isolation from vector immigration (Garms *et al.*, 1989; Walsh, 1990b). The distribution of breeding sites on Bioko has been described by McCall *et al.* (1998) with special reference to their accessibility to possible vector control operations, but very little is known about the potential geographical isolation of the Bioko population of vectors (Post *et al.*, 1995).

In 1990 an onchocerciasis chemotherapy programme was established as a joint initiative by the governments of Equatorial Guinea (Ministry of Health) and Spain (Spanish International Cooperation Agency), and the microfilaricidal drug ivermectin has been distributed once a year to the entire population of Bioko since 1995. The clinical effects have been monitored (Mas *et al.*, 1995), but the parasitological effects and effects on transmission have not been assessed. There are undoubted clinical benefits to ivermectin treatment although it is generally not clear whether ivermectin on its own can interrupt transmission of the

parasite (Boatin *et al.*, 1998). Hence, the World Health Organization African Programme for Onchocerciasis Control (APOC) has a strategy not only to support ivermectin distribution in onchocerciasis-endemic countries, but also to effect focal vector eradication where appropriate in isolated areas (Remme, 1995). However, any chance of blocking transmission on Bioko by vector eradication will be affected by the possibility of recolonization by flies from the mainland, and similarly any potential effect of ivermectin distribution on transmission will be moderated by the possibility of immigrant infective flies.

Bioko is the only island with endemic onchocerciasis transmission and, as such, represents a special case of isolation (fig. 1). The Atlantic ocean is to the west of Bioko, and to the south lie the islands of Príncipe (which is the nearest, at 210 km SSW), São Tomé and Annobón, in that order. *Simulium damnosum* s.l. has not been found on any of these islands (respectively, R.J. Post unpublished data; dos Santos Gracio, 1998; J. Mas, unpublished data), and hence the only potential sources of reinvading flies are on the mainland near Mount Cameroon (40 km NNE) and the other coastal parts of Cameroon 80–130 km to the east. There are almost no published records of the distribution of the various cytospecies of the *S. damnosum* complex in southern and eastern Cameroon, including the Mount Cameroon area. However, Traore-Lamizana & Lemasson (1987) and Mafuyai *et al.* (1996) review the general situation for northern Cameroon and Nigeria respectively, and Traore-Lamizana *et al.* (2001) have reviewed the cytotoxicology of the *S. squamosum* subcomplex for both countries.

In this paper we will describe the chromosomal, morphological and molecular (DNA and isoenzymes) characteristics of *S. damnosum* s.l. from Bioko in order to determine its taxonomic status and its distinctiveness from continental populations.

Materials and methods

Collection of materials

Larvae and pupae were collected from riverine breeding sites on Bioko in May and November 1989, April–May and July 1996 (table 1). Larvae were fixed in Carnoy's solution at 4°C for cytotoxicological studies, and pupae were kept in a dark humid cage at room temperature until adults emerged. Neonate adults were fixed in 100% ethanol and kept at 4°C for DNA analysis (Post *et al.*, 1993) or frozen at –40°C or –70°C for isoenzyme analysis. For comparison, additional collections of larvae and neonate adults were used from Cameroon (table 2) and other parts of mainland West Africa, including; *S. squamosum* (Enderlein) (Simuliidae) from R. Amou at Amou Oblo Togo (07°24'N/00°53'E) collected by R.A. Cheke on 22.iii.90; *S. yahense* from R. Mpebo at Akakro Côte d'Ivoire (05°32'N/04°28'W) collected by M.D. Wilson on 05.x.90, and from Kenema waterfall Sierra Leone (07°54'N/11°14'W) collected by R.J. Post on 20.vii.85; *S. sanctipauli* from R. Sassandra at Koperagui Côte d'Ivoire (05°38'N/06°38'W) collected by M.D. Wilson on 02.x.90; *S. leonense* Boakye, Post & Mosha (Simuliidae) and/or *S. soubrense* Vajime & Dunbar (Simuliidae) from R. Rokel near Makpankaw Sierra Leone (08°44'N/11°56'W) collected by R.J. Post on 05.xii.88; *S. sirbanum* Vajime & Dunbar (Simuliidae) from R. Niger at Tienfala Mali (12°43'N/07°44'W) collected by M.D. Wilson on 15.xi.88;

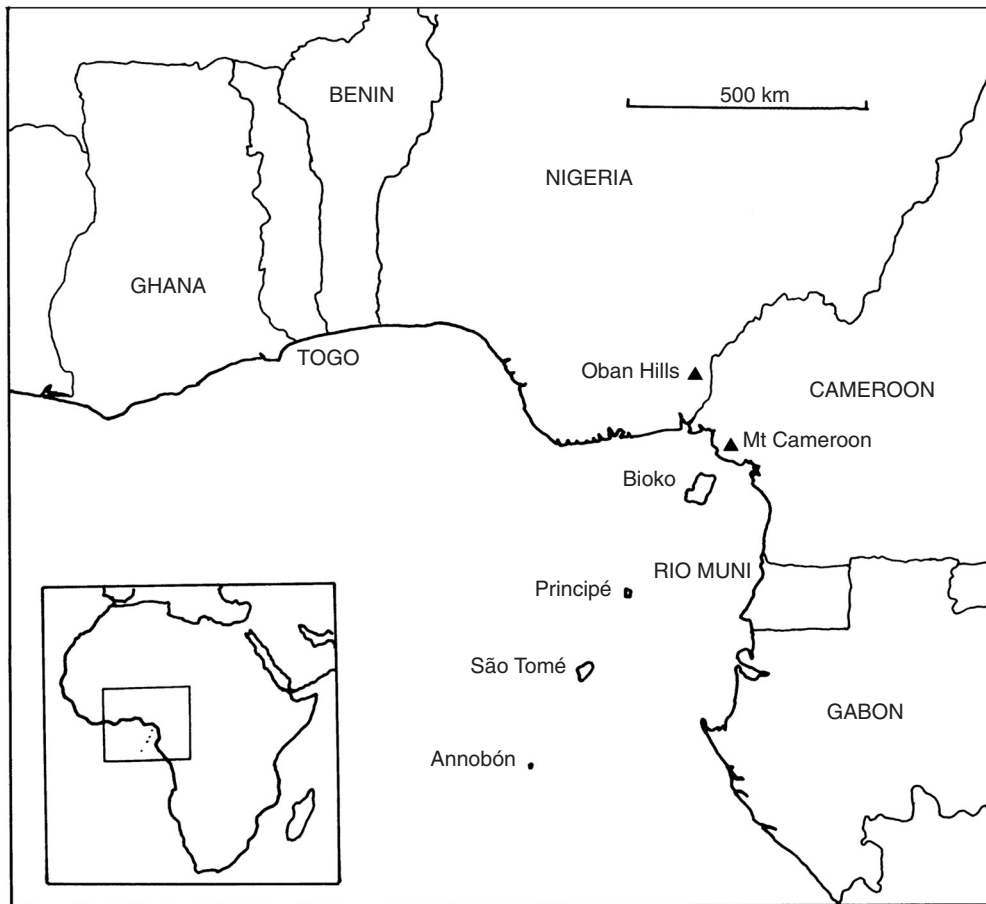


Fig. 1. Map showing the location of the island of Bioko in the Gulf of Guinea. The Republic of Equatorial Guinea consists of Rio Muni and the islands of Bioko and Annobón.

and cytotaxonomically uncharacterized material from R. Pra at Hemang Ghana (05°11'N / 01°36'W) and from R. Tano at Abesim waterworks Ghana (07°15'N / 02°15'W) collected respectively on 01.ii.97 and 29.i.97 by A.K. Tetteh and M.D. Wilson.

Cytotaxonomic analysis of larvae

Larval silk glands were dissected out and the polytene chromosomes prepared according to standard methodologies (Boakye *et al.*, 1993). Sibling species were identified and intraspecific variation was assessed according to the pattern of fixed and polymorphic inversions, which were scored with reference to the nomenclature and standards published by Vajime & Dunbar (1975), taking into account later modifications summarized by Boakye (1993) and Traore-Lamizana *et al.* (2001).

Zymotaxonomy

Flies were either collected at human bait by J. Mas at Sampaca on 28.viii.96, or reared from pupae collected by P.J. McCall and R.A. Cheke on 17/18.v.96 from a number of rivers off the main road, along the west of the island between R. Tiburones and R. Bisoco. Frozen preserved flies were later individually homogenized in buffer to extract

water soluble enzymes. The extract was subjected to cellulose acetate electrophoresis and stained for enzyme activity according to the methods of Thomson *et al.* (1989). Flies were characterized for phosphoglucosmutase and trehalase, which together allow the differentiation of *S. squamosum* and *S. yahense* from each other and from all other West African sibling species (Meredith & Townson, 1981).

Morphotaxonomy

Adult flies of both sexes that had been reared from pupae or females collected at human bait (table 4) were preserved in either 100% or 80% ethanol. The lengths of the antennae and thoraces were measured according to Garms (1978). The colour of the fore coxa was scored as either pale or dark and the arculus was scored as pale, intermediate or dark, as described by Wilson *et al.* (1993). The stem vein setae (wing tufts) were scored on a scale A–E (A, all pale; B, up to five dark hairs; C, mixed; D, up to five pale hairs; E, all dark; and O, character missing) according to Kurtak *et al.* (1981), and the scutellar hairs, ninth abdominal tergite setae, basicostal setae, and postcranial hairs were scored in a similar way but considering all mixtures together (A, all pale; C, mixed; E, all dark; and O, character missing), as described by Wilson *et al.* (1993).

Table 1. Karyotype analysis of *Simulium* larval samples from Bioko.

Collection date	Site ¹	Bioko form 2L-18 karyotype data ^{2,3}						No. of larvae heterozygous inversion 3L-I
		Females		Males		Sex unknown		
		st/st	st/18	st/st	st/18	st/st	st/18	
12.v.89	R. Rupe	3			2			
13.v.89	R. Ebá					5	7	
13.v.89	R. Matogi	10			7			1
13.v.89	R. Lada	3			4			
15.v.89	R. Malaho	1			6			2
16.v.89	R. Tiburones	10			4			1
17.v.89	R. Togecha				1			
18.v.89	R. Biala	8		2	5			
18.v.89	R. Apu	8	2		4	4	5	2
23.v.89	R. Ruma	13	3		4			3
08.xi.89	R. Biala	5			4	3	1	
08.xi.89	R. Apu	11		1	8			2
09.xi.89	R. Bioco				2			
09.xi.89	unnamed	8			3			2
11.xi.89	unnamed	1						
11.xi.89	R. Ebá	9	2		11	4	5	
11.xi.89	unnamed	4			5			1
14.xi.89	R. Ruma	6			6	2	2	
15.xi.89	R. Bosao	1			8			1
15.xi.89	unnamed	8			10			1
16.xi.89	R. Apu	11			9	10	4	
18.xi.89	R. Ko	3			1			
13.iv.96	R. Matogi/Lada	3			6	2	1	
20.iv.96	R. Rupe	1			3	6		
23.iv.96	R. Ruma	10			3	4	1	
23.iv.96	R. Grande	13			5	1	1	1
24.iv.96	R. Boolo				1	6		
24.iv.96	Boolo canal	8			4	2		
24.iv.96	R. Boolo/canal	5			3	6	2	1
24.iv.96	R. Musola dam	1			1	1	1	
29.iv.96	R. Muedede	5			8			
30.iv.96	R. Iladyi	5		1	6	6		
02.v.96	R. Leke					1		
03.v.96	R. Ole				1			
08.v.96	R. Malaho	3			1	1	2	2
08.v.96	R. Rupe	2			2	1		
08.v.96	R. Eneca	2		1	2	3		
08.v.96	R. Ope	2				1		
08.v.96	R. Biala					1		
08.v.96	R. Apu				2			
09.v.96	R. Cíbitá					2	2	
10.v.96	R. Bisoco				1	1		
10.v.96	R. Matogi	10			3	3	3	1
16.vii.96	R. Rupe	4			4	3	1	
16.vii.96	R. Apu	4			4			1
16.vii.96	R. Biala	1			2		1	
16.vii.96	R. Tiburones				1			
17.vii.96	R. Matogi				1			
17.vii.96	R. Musola	2			1		1	
19.vii.96	R. Ruma/Grande	1			1			1
Total		205	7	5	170	79	40	23

¹Details of locations can be found in McCall *et al.* (1998).²st indicates standard (i.e. uninverted) sequence as defined by Vajime & Dunbar (1975).³2L-18/18 homozygotes were never found.

Table 2. Karyotype analysis of *Simulium* larval samples from Mount Cameroon area.

Collection date	Site	Coordinates Lat./Long.	Cytospecies id ¹			<i>S. squamosum</i> 2L-18 karyotype data ²						Other polymorphic inversions
			<i>dam</i>	<i>meng</i>	<i>squ</i>	females		males		unknown		
						st/st	st/18	st/st	st/18	st/st	st/18	
North of Mount Cameroon												
05.v.89	Bikili dam	04°37'/09°21'	6		7	4		3				3L-7
06.viii.90	Bikili dam	04°37'/09°21'	5		3	2	1					1S-22, 1L-15 & 3L-7
05.viii.90	R. Menge	04°45'/09°29'			8	4		4				
07.v.01	R. Menge	04°45'/09°29'			3					3		
07.v.01	Bolo Moboka	04°52'/09°28'			3	2		1				
08.v.01	R. Kake	04°39'/09°25'	6									
08.v.01	R. Bile	04°35'/09°21'			5	3		1			1	
09.v.01	Trib. of Njanje	04°24'/09°19'		1								
10.v.01	R. Kumba	04°32'/09°28'			6	1		4			1	
Southwest of Mount Cameroon												
08.vii.96	R. Sanje	04°14'/09°00'			4							
08.vii.96	R. Mossingili	04°15'/08°59'			7							
11.v.01	R. Oonge	04°16'/08°57'			5							
13.v.01	R. Sanje	04°14'/09°00'			11							
Southeast of Mount Cameroon												
26.ii.91	R. Ombe	04°05'/09°17'			21	12	1	5	1	1	1	
03.iii.91	R. Yoke	04°18'/09°26'			2	1		1				
31.i.94	R. Limbe	04°01'/09°12'			11	6		4	1			
07.vii.96	R. Limbe	04°02'/09°12'			18	4		5			9	
07.vii.96	R. Benoe	04°05'/09°19'			21	9		5			3	
07.vii.96	R. Likomba	04°05'/09°20'			4	1		2			1	
07.vii.96	R. Ombe	04°05'/09°17'			2			2				
07.vii.96	R. Yoke	04°18'/09°26'			16	7		6			3	
06.v.01	R. Benoe	04°07'/09°18'			7	3		4				
06.v.01	R. Ndongo	04°05'/09°21'			2			2				
10.v.01	R. Yoke	04°18'/09°26'			2	2						
12.v.01	R. Limbe	04°01'/09°12'			15	6		4		5		3L-I
13.v.01	R. Essuke	04°05'/09°18'			1			1				
13.v.01	R. Moliwe	04°04'/09°15'			5	2		3				
13.v.01	R. Ombe	04°05'/09°17'			12	6		6				
Total			17	28	174	75	2	63	2	27	1	1S-22, 1L-15, 3L-I & 3L-7

¹*dam*, *S. damnosum* s.str.; *meng*, *S. mengense*; *squ*, *S. squamosum* (no other cytospecies were identified).

²2L-18/18 homozygotes were never found.

DNA extraction and restriction digestion

DNA was extracted from individual flies using the protocol of Flook *et al.* (1992) and was suitable for EcoRI restriction enzyme digestion using manufacturer's instructions. To avoid star activity of enzymes, a low enzyme concentration was used (0.5 U μl^{-1}) overnight at 37°C. This DNA from individual flies was used in Southern blot analysis with hybridization to probe pSO11 (see below). To prepare high quality genomic DNA from 10–100 flies for the construction of a genomic library or melting curve analysis (see below), the method of Bingham *et al.* (1981) was used. Both methods yielded approximately 1 μg DNA per fly.

Agarose gel electrophoresis and Southern blotting

Agarose gels (0.9% FMC SeaKem) were prepared using 1 \times TAE buffer and run overnight at 2 V cm^{-1} . Post-electrophoresis gels were prepared by denaturation and neutralisation (Southern, 1975; Sambrook *et al.*, 1989).

Depurination in HCl was used initially as a preliminary to denaturation, but later limited exposure to ultraviolet light on a transilluminator was used instead. Three methods were used to transfer DNA to nitrocellulose filters (Biotrace NT), including capillary transfer, vacuum blotting and pressure blotting. Of these, capillary blotting was used preferentially because of superior band resolution in autoradiography.

DNA–DNA hybridization

Insert DNA from EcoRI digested cloned probes pSO11 (for Southern blots), pSY29 and pBS22 (both for melting curve analysis) (see below) was recovered from agarose gels and radiolabelled with [^{-32}P]-dCTP using random-primed labelling. Filters were prehybridized (5 \times SSC, 50% deionized formamide, 5 \times Denhardt's, 0.05 M phosphate buffer, 50 mg ml^{-1} denatured herring sperm DNA) at 42°C for 24 h, and the buffer was changed (5 \times SSC, 50% deionized formamide, 5 \times Denhardt's, 0.02 M phosphate

buffer, 100 mg ml⁻¹ denatured herring sperm DNA) immediately before hybridization. Labelled probes were denatured at 100°C and added at a concentration of 1×10^7 – 1×10^8 cpm cm⁻², and hybridization continued overnight (Southern blots) or for 24 h (melting curve analysis, to ensure saturation of all binding sites) at 42°C. Filters were washed in various concentrations of SSC, 0.1% SDS at different temperatures and exposed to Fuji RX film at –45°C or –70°C.

Melting curve analysis

Genomic DNA (100 ng) was resuspended in $6 \times$ SSC and denatured at 100°C for 10 min before application to a nitrocellulose filter using a dot-blot manifold (Kafatos *et al.*, 1979). Filters were dried and hybridized to radiolabelled pSY29 or pBS22 insert DNA (see above) and subsequently washed nine times in $0.1 \times$ SSC + 0.1% SDS at increasing temperatures (steps of 5°C), and the amount of DNA–DNA hybridization after each wash was compared by Cerenkov counting for 1 min (Kafatos *et al.*, 1979; Sambrook *et al.*, 1989). Two replicates of each sample were used. Analysis of the hybrid dissociation data was performed on the combined replicate data using Maximum Likelihood Programme (Numerical Algorithms Group, MLP user manual). The data best fitted the generalized logistic equation, $y = A + C/(1 + \exp(-B(x - M)))$, where the parameter A is the lower asymptote, A + C is the upper asymptote, X = M is the point of inflection, and B is the slope parameter. Statistical analysis of fitted curves was performed by parallel curve analysis. Using this approach the statistical significance of differences between linear parameters (A and C, corresponding to copy number) and non-linear parameters (M and B, corresponding to melting temperatures of sequences) could be examined.

Source and characterization of DNA probes

The DNA probe pSO11 was originally isolated from *S. leonense* for its potential use in sibling species identification (Post & Flook, 1992), because it is repetitive and shows copy number variation between sibling species. Sequencing revealed a composite pattern of repetition (Post *et al.*, 1992), resembling the organization found in certain transposable elements (Flook & Post, 1997), and EcoRI Southern blot analysis of genomic DNA revealed a hypervariable banding profile. To isolate additional DNA probes, a series of genomic libraries was prepared using genomic DNA from three sources; *S. squamosum* and *S. damnosum* s.str. from Cameroon (collected simultaneously from Bikili Dam, 05 May 1989, and identified morphologically as adults – Wilson *et al.*, 1994), and *S. damnosum* complex from Bioko (collected Rio Apu, 18 May 1989), see table 1. Libraries were prepared as described by Flook (1992). Over 100 recombinants were selected after primary screenings by differential hybridization, arranged in ordered arrays and rescreened. Plasmid DNA was prepared from recombinants that showed potentially species specific hybridization patterns. These DNA preparations were radiolabelled and hybridized to genomic DNA dot-blot and Southern blot filters containing different sibling species. In particular, differences in hybridization between specimens from Bioko and other *S. squamosum* subcomplex DNA samples were investigated.

Results

Cytotaxonomy

A total of 506 larvae from 50 samples from Bioko was examined chromosomally (table 1). No new inversions were found. All populations were found to be similar to each other, and closely related to *S. squamosum* and *S. yahense*. Full karyotype analysis did not reveal any fixed inversion differences, but the Bioko populations were found to be cytologically distinct from both *S. squamosum* and *S. yahense* in terms of sex-linkage of inversion IIL-18. Throughout West Africa, IIL-18 is normally absent from *S. squamosum* and strongly X-linked in *S. yahense* (Boakye, 1993), but when inversion IIL-18 is found in *S. squamosum* it is never sex-linked. This is the pattern that was found in larvae of *S. squamosum* from sites sampled near the coast of Cameroon, around Mount Cameroon (table 2). *Simulium yahense* was not identified from sites visited in Cameroon, but it has been reported by Mafuyai *et al.* (1996) from southern Nigeria on the border with Cameroon, where it shows normal X-linkage of IIL-18. The difference is that on Bioko, IIL-18 was clearly Y-linked in all populations (table 1) with an X frequency of 1.7% and a Y frequency of 97.1%. This means that using normal cytogenetic criteria (Boakye, 1993), females from Bioko would be identified as *S. squamosum* and males as *S. yahense*. Hence it is not possible on chromosomal evidence alone to determine whether the Bioko populations are an island race of *S. squamosum*, *S. yahense* or a new species. Besides IIL-18, the only polymorphic inversion found on Bioko was IIII-I (see Post, 1986), although this was rare (4.5% heterozygotes) and never observed homozygously (table 1). This polymorphism was also observed in a single larva of *S. squamosum* from Cameroon, but this observation probably has no taxonomic significance because this inversion, whilst rare, has been found in most of the West African cytospecies (Vajime & Dunbar, 1975; Post, 1986; Boakye, 1993).

Zymotaxonomy

Of 91 flies collected from human bait during August 1996, none stained for phosphoglucosyltransferase and only 67 stained for trehalase. All 67 trehalase-positive flies were identified as being either *S. squamosum* or *S. yahense* (which cannot be distinguished by trehalase). The results for flies collected during May 1996 (and experimental control flies) are shown in table 3. The 14 experimental control flies from the rivers Pra and Tano in southern Ghana were shown to be a mixture of *S. yahense* and some other cytospecies (but not *S. squamosum*). This is broadly consistent with previous cytogenetic identifications from these rivers (R.J. Post, unpublished). *Simulium yahense* is the most common species in the upper Tano, followed by *S. squamosum*, *S. sirbanum* and *S. damnosum* s.str. The most common species on the Lower Pra is *S. sanctipauli*, followed by *S. damnosum*, *S. soubrense*, *S. squamosum* and *S. yahense*. All 25 flies from Bioko which stained for both phosphoglucosyltransferase and trehalase could be identified as *S. yahense* according to the criteria defined by Meredith & Townson (1981).

Morphotaxonomy

Totals of 58 male and 606 female flies were examined from 20 samples. Not all flies could be scored for all

Table 3. Cellulose acetate electrophoresis identification of neonate flies from Bioko and control sites.

PGM/TRE isozyme ¹ Species identity	C/B <i>sanctipauli</i> ²	B1/A <i>yahense</i>	?/A ³ <i>squamosum</i> or <i>yahense</i>	Total
Source				
Ghana				
R. Pra at Hemang	7	1	0	8
R. Tano at Abesim	2	4	0	6
Bioko	0	25	9	34

¹C, B1, B and A are phosphoglucomutase (PGM) or trehalase (TRE) isozyme genotypes which were distinguished by their electrophoretic mobility according to Meredith & Townson (1981). No other genotypes were obtained.

²Or some other cytospecies, not *Simulium yahense* and not *S. squamosum*.

³? = indistinct PGM band.

characters due to missing body parts. Two female flies had a few pale hairs in their wing tufts (score D), and one had lost its hairs, but otherwise all female flies examined had entirely dark wing tufts ($n = 603$), entirely dark basicostal setae ($n = 524$), entirely dark postcranial hairs ($n = 606$), a dark arculus ($n = 383$) and dark fore coxae ($n = 606$). In most specimens the colour of the setae of the scutellum ($n = 593$) and ninth abdominal tergite ($n = 415$) was all dark, but 13 and 7 specimens had mixed setae respectively (table 4). The values of the thorax length:antenna length ratios for females ranged from 1.77 to 2.07, but most were in the range 1.90–2.00, consistent with other populations of *S. yahense* (Garms & Zillman, 1984). The antennae of all females lacked compression, consistent with membership of the forest group of flies (Garms & Cheke, 1985), and were uniformly dark, except for the basal two segments and one third to one half of the third segment.

According to the setae of the ninth abdominal tergite, 415 female specimens would be identified as *S. yahense* and seven specimens as some other species (Garms & Zillman, 1984; Fryauff & Trpis, 1986; Davies *et al.*, 1988). According to the qualitative characters (colour of ninth abdominal tergite setae, colour of arculus and colour of scutellar setae) described in the 'hierarchical' classification method of Wilson *et al.* (1993 – table 4) all female specimens that could be scored for all three characters ($n = 258$ – table 5) were identified as *S. yahense* except for five which had mixed abdominal setae, dark arculus and mixed scutellar setae. However, all five of these flies had a thorax length:antenna length ratio of less than 2.05, which is considered to be the lower cut-off point for practical identification of *S. squamosum* (Garms & Cheke, 1985), and a mean of 1.92 which is closer to the mode for *S. yahense* (1.98 – Garms & Zillman, 1984) than for *S. squamosum* (2.175 – Garms &

Table 4. Morphological variation in adult females of the Bioko form of *Simulium yahense*.

Location	Date	Collector ¹	Status ²	Sample size	Thorax length ³ mean (SD)	Antenna length ³ mean (SD)	Scutellar setae ⁴				Abdominal setae ⁴				
							A	C	E	O	A	C	E	O	
R. Musola	22.iv.96	RAC	Neonates	1	1.02 (-)	0.56 (-)			1				1		
R. Boolo, irrigation canal	22.iv.96	RAC	Neonates	6	1.11 (0.02)	0.57 (0.02)			6				6		
R. Grande	23.iv.96	RAC	Neonates	13	1.09 (0.03)	0.57 (0.02)			13				13		
R. Ruma	23.iv.96	RAC	Neonates	50	1.09 (0.04)	0.57 (0.03)			50				50		
R. Sampaca	10.v.96	RAC	At host	12	1.11 (0.08)	0.56 (0.04)			12				7	5	
R. Apu at Izaguire	16.vii.96	MDW	Neonates	82	1.01(0.09)	0.53 (0.05)	2	80			1		81		
R. Musola at Musola	17.vii.96	MDW	Neonates	17	1.11 (0.07)	0.57 (0.03)	1	16					17		
R. Musola at Musola	26.iv.99	RAC	Neonates	29	1.08 (0.07)	0.57 (0.03)			29				12	17	
Malabo airport pub	24.vii.96	MDW	At host	8	1.07 (0.05)	0.55(0.02)			8				8		
R. Timbabe by airport rd	22.vii.96	MDW	Neonates	13	1.10 (0.05)	0.57 (0.01)			13				13		
R. Timbabe by airport rd	22.vii.96	MDW	At host	65	1.05 (0.07)	0.53 (0.03)	5	60					65		
R. Ruma at Balancha de Riaba	19.vii.96	MDW	At host	5	1.06 (0.04)	0.55 (0.01)	1	4					5		
R. Ruma/Grande at power-station	19.vii.96	MDW	At host	34	1.06 (0.06)	0.54 (0.02)	2	32				2	32		
R. Matogi	17.vii.96	MDW	At host	36	1.08 (0.05)	0.55 (0.02)	2	34				4	32		
R. Machuchumvano	19.vii.96	MDW	At host	1	1.04 (-)	0.55 (-)		1					1		
R. Apu	22.iv.96	RAC	At host	16	1.14 (0.05)	0.58 (0.02)		16					11	5	
R. Apu	26.iv.99	RAC	At host	107	1.13 (0.06)	0.57 (0.03)		107					40	67	
R. Apu	27.iv.99	RAC	At host	40	1.10 (0.07)	0.57 (0.02)		40					17	23	
R. Ruma at Balacha de Riaba	26.iv.99	RAC	At host	35	1.03 (0.05)	0.54 (0.03)		35					1	34	
R. Sampaca	26.iv.99	RAC	At host	36	1.13 (0.07)	0.60 (0.03)		36					3	33	
Total				606			13	593				7	415	184	

¹Collectors were M.D. Wilson and R.A. Cheke.

²Specimens were either reared from pupae (neonates) or collected at human bait (at host).

³Lengths are in mm.

⁴Setae were scored as described in the text.

Table 5. List of all female flies exhibiting mixed colour characters.

Site ¹	Specimen	Arculus ²	Scutellar setae ²	Ninth tergite setae ²	Thorax:antenna ratio
R. Apu (16.vii.96)	1	Dark	C	C	2.02
	2	Dark	C	E	2.18
R. Timbabe (at host)	1	Dark	C	E	1.93
	2	Dark	C	E	2.00
	3	Dark	C	E	1.94
	4	Dark	C	E	2.03
R. Ruma/Grande	1	Dark	C	C	1.80
	2	Dark	C	C	1.80
	3	Dark	C	C	1.96
	4	Dark	E	C	1.98
	5	Dark	C	C	2.00
	6	Dark	E	C	1.95
R. Musola (17.vii.96)	1	Dark	C	E	2.20

¹See table 1 for details.

²See text for details of classification.

Cheke, 1985). Rare variation in these colour characters has already been described as potentially resulting in low levels (approximately 1%) of misidentifications of some *S. yahense* populations elsewhere (Garms & Zillman, 1984; Wilson *et al.*, 1993). Therefore, in view of the thorax length:antenna length ratios, it is not considered that these few flies provide evidence for species other than *S. yahense* on Bioko.

All the 58 males examined were collected on 22 and 23 April 1996 from the first four sites listed in table 4. Forty-eight had scutal patterns of type IIIh and 10 of type IIIg, using the terminology described by Meredith *et al.* (1983), consistent with *S. yahense* (R.A. Cheke & R. Garms, unpublished data), and distinguishing them from members of the *S. sanctipauli* subcomplex which usually have type I or type II scutal patterns (Meredith *et al.*, 1983), or, in the case of the Beffa form of *S. soubrense*, type IV patterns. However, other cytospecies including *S. squamosum* and *S. damnosum* s. str., may have type III patterns. The 58 flies had a mean thorax length of 0.96 (range 0.86–1.08, SD = 0.04) and a mean antenna length of 0.52 (range 0.46–0.58, SD = 0.03). The antennae were also typical of those of forest flies, being robust, broadening towards the tip and mostly dark, with thorax: antenna ratios ranging from 1.70 to 2.09. The arculus and scutellar hairs on these flies were consistently dark.

Southern blot analysis with pSO11

Totals of 334 flies from 24 Bioko samples and 141 flies from the mainland (including 75 *S. squamosum* from Cameroon) were examined for Southern blot variation using

EcoRI digested genomic DNA from individual flies hybridized to radiolabelled probe pSO11. The banding profiles were hypervariable, but the Bioko populations were found to have a unique 3.27 kb band (fig. 2) at a frequency of 90.4% of flies (table 6). Table 6 also includes estimates of relative copy number of pSO11 from Flook & Post (1997). These show an obvious similarity between the Bioko specimens and *S. squamosum* in comparison with those other members of the complex which were tested.

Melting curve analysis with pSY29 and pBS22

Plasmid DNA was prepared from 23 clones, but in subsequent hybridization analyses only three clones showed any useful species-specific variation with respect to the Bioko flies. In all three cases clones pSY2, pSY29 and pBS22 showed stronger hybridization to Bioko flies than to either *S. squamosum* from Cameroon or *S. yahense* from Sierra Leone. In Southern blot analyses, hybridization smears were observed in hybridizations with these probes against all species, and no differential banding patterns were observed against EcoRI digested DNA, even after high stringency washes.

Two potentially taxa-specific sequences (pSY29 and pBS22) were investigated further by melting curve analysis. The melting curves are shown in fig. 3, and the estimated parameters from which these curves were constructed are shown in table 7. Statistical significance of these parameters was estimated by parallel curve analysis, and for both probes significant differences were detected between the

Table 6. pSO11-profile analysis.

Population	Cytospecies	No. specimens	Relative copy no. ²	Frequency 3.27kb band
Bioko	Bioko form	334	1.0	0.904
Mt Cameroon ¹	<i>S. squamosum</i>	75	1.5	0
Côte d'Ivoire	<i>S. sanctipauli</i>	18	4.22	0
Sierra Leone	<i>S. leonense</i> + <i>S. soubrense</i>	48	6.00	0

¹Mt Cameroon samples are from Bikili Dam, and river Menge. The *Simulium squamosum* was separated from *S. damnosum* by morphological examination (Wilson *et al.*, 1994).

²Data from Flook & Post (1997).

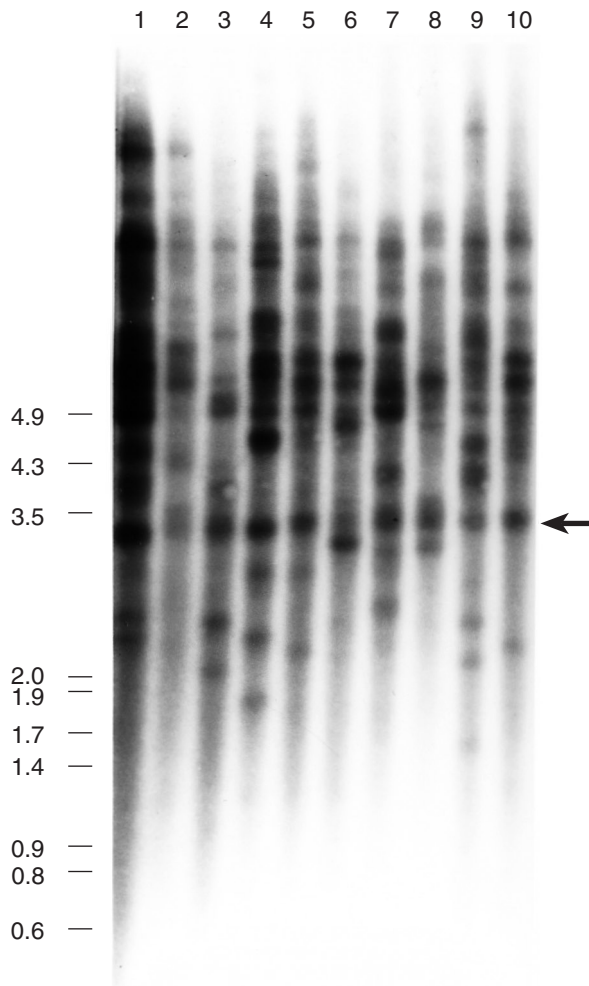


Fig. 2. Autoradiograph of Southern blot hybridization with [α - P^{32}] labelled pSO11 probe. Lanes 1–10 are genomic DNA extracted from individual adult females (caught at human bait near a tributary of the Rio Rupe, Bioko, 11 November 1989) digested with EcoRI. Arrow indicates the position of the high frequency 3.27 kb band.

common linear parameters. These significant differences indicate that the copy number of sequences hybridizing to the probes is significantly higher in the Bioko form of *S. yahense* than in the other members of the *S. squamosum* subcomplex examined. In contrast, no significant differences were detected between the non-linear parameters, indicating that there was little sequence divergence between repeats in the different species.

Discussion

Chromosomal evidence (supported by the relative copy number of pSO11) clearly indicates that the Bioko form belongs to the *S. squamosum* sub-complex although it was found to be genetically distinct from other members on the basis of the pattern of sex-linkage of inversion IIL-18. The evidence from the enzyme and morphological analyses clearly indicates a greater similarity to *S. yahense* than to *S. squamosum* (or any other West African cytospecies), which

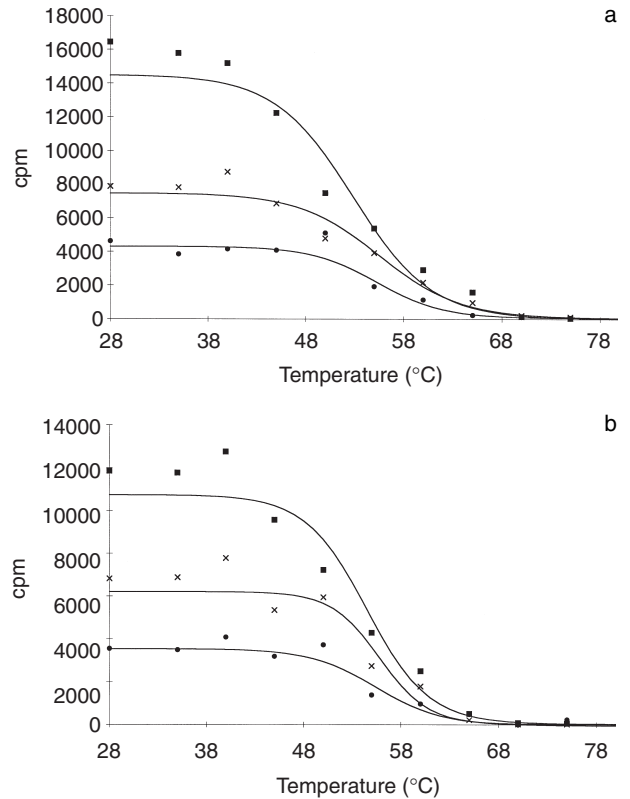


Fig. 3. Melting curves for pBS22 (a) and pSY29 (b). ■, *Simulium yahense* from Bioko; ×, *S. squamosum* from Cameroon (Bikili dam); ●, *S. yahense* from Sierra Leone (Kenema waterfall).

supports an earlier morphotaxonomic analysis of a single sample from the river Apu (Wilson *et al.*, 1994). However, differences in copy number of two interspersed repetitive sequences (pBS22 and pSY29), the presence of the 3.27 kb pSO11 band at high frequency and the unique sex-chromosome system, indicate that the Bioko populations are genetically distinctive. Together these differences indicate a high degree of genetic isolation of the Bioko form, but it cannot be concluded that this isolation is complete because none of these differences is fixed. In view of the evidence for a close relationship with *S. yahense*, in combination with some significant genetic differences from *S. yahense* and the uncertainty surrounding the exact degree of reproductive isolation (see below), it is proposed that these populations be referred to as 'the Bioko form of *S. yahense*'.

The genetic distinctiveness of the Bioko form could be the result of either geographical or reproductive isolation, or a combination of both, but because of the self-evident geographical separation, it is very difficult to determine the level of reproductive isolation of the Bioko form of *S. yahense* from mainland populations (i.e. the extent to which they have the potential to mate successfully and produce viable, fertile offspring). Whatever the extent of reproductive isolation, the evidence suggests that the level of geographical isolation must be very high.

It is impossible that flies could migrate to Bioko from the west (because there is only ocean), or from the islands to the south, not just because of the great distance, but primarily because they do not seem to occur on those islands.

Table 7. Melting curve analysis of pSY29 and pBS22.

Logistic curve Parameter DNA Probe and species		A	B	C	M	TM50
pBS22	Bioko form	-1.575	-0.2504	14497	52.842	53
	<i>S. squamosum</i>	45.36	-0.2438	7418.2	55.520	56
	<i>S. yahense</i>	50.66	-0.2972	4243.5	55.264	55
pSY29	Bioko form	34.838	-0.3162	10696	54.495	54
	<i>S. squamosum</i>	19.216	-0.4038	6180.4	55.811	56
	<i>S. yahense</i>	-47.7	-0.2955	3575.5	55.436	55

The parameters A, B, C and M refer to the equation for the logistic curve (see Materials and methods), of which the melting temperature (TM50°C) is M. The different species are: Bioko = *Simulium damnosum* s.l. from Bioko; *S. squamosum* from Cameroon (Bikili dam); and *S. yahense* from Sierra Leone (Kenema waterfall).

Meteorological factors reinforce Bioko's isolation, and severely limit the possibility of movement of flies from the mainland to the island. For almost the entire year the prevailing winds blow from the south or southwest across the island towards the mainland, and even at the height of the dry season the intertropical convergence zone rarely lies south of the island (Teran, 1962). Nosti (1942) illustrated average wind direction data for Malabo in 1940, which showed that northerly or easterly winds were generally uncommon, and this is a consistent pattern as confirmed by records from recent years. Between 1991 and 1995 the mean wind direction at Malabo for all months came from the southwest, except May 1991, September and November 1992 and April 1994 when it came from either the north or east (unpublished data from Servicio de Meteorología – ASECNA, Malabo). It is, therefore, unlikely that flies could easily migrate to Bioko from the north or east, because this is contrary to the usual prevailing winds. The northerly winds that do blow are usually sea-breezes (Nosti, 1947; Capuz, 1953), and hence do not blow all the way from the mainland. Occasional easterly winds are usually associated with line-squalls. In other parts of West Africa, the available evidence suggests that line-squalls do not carry migrating *S. damnosum* s.l. (Garms *et al.*, 1982), which usually move with the prevailing winds (Garms *et al.*, 1979, 1982, Baker *et al.*, 1990). In Benin, line-squalls are usually followed by a decrease in fly numbers (Cheke & Garms, 1983). It is of course true that Bioko must have been colonized at some time by *S. damnosum* s.l., but during the Pleistocene, Bioko was connected to Mount Cameroon by a land bridge (Jones, 1994) and hence there is no necessity to postulate a recent transoceanic colonization event.

Simulium yahense has not been recorded from continental sources (near the coast of Cameroon) of potentially migrant flies, and in any case immigrant females and hybrid males on Bioko would be IIL-18 homozygotes, which were not found. The nearest known mainland breeding site is in southern Nigeria, approximately 230 km north of Bioko, on the other side of the Ndian/Cross river watershed which runs through the Oban Hills (Mafuyai *et al.*, 1996). There is no chromosomal evidence for other cytospecies breeding on the island. *Simulium damnosum* s.str. and *S. mengense* Vajime & Dunbar (Simuliidae) (both of which are known around the southern edges of Mount Cameroon) would be immediately obvious, and were not found on Bioko. *Simulium squamosum* from Cameroon east of Bioko would be easily recognized if it was rare but indigenous to Bioko because 60–70% of its males would show a sex-linked non-pairing section near the

centromere of the first chromosome (Traore-Lamizana *et al.*, 2001) and this was never found. On the other hand, rare indigenous or immigrant *S. squamosum* from the southern side of Mount Cameroon would often be chromosomally indistinguishable from the endemic Bioko form of *S. yahense* (compare tables 1 and 2), but the morphology of the adult females did not indicate the presence of *S. squamosum*.

It is probable that the taxonomic differentiation of the Bioko form of *S. yahense* results from genetical and ecological isolation caused by the position of the island (see above). There is some variation in taxonomic characters within the Bioko form, but very little evidence that any of this is subdivided geographically around the island. The sex chromosome variation (table 1) is too rare to show a definite pattern, but is apparently scattered around the island. Similarly, inversion 3L-I shows no discernible pattern, as illustrated by the frequency of heterozygotes clockwise around the island from Rio Ruma (considering only samples of ten or more larvae: 0.07, 0.00, 0.00, 0.03, 0.00, 0.00, 0.05, 0.29, 0.00, 0.18, 0.00 and 0.06), which show no significant clustering (one-sample runs test $P > 0.05$). The occurrence of mixed scutellar and abdominal setae is too rare to draw a meaningful pattern from, although there might be some clustering at the southern end of the sampling distribution (R. Ruma and R. Matogi). Size of adult *S. damnosum* s.l. (as indicated by lengths of the thorax) is known to be strongly influenced by the environment (Cheke & Harris, 1980), and there is again little indication of geographical structure on Bioko because nearly all of the standard deviations overlap the various sample means (table 4), and the four samples from the R. Apu include both the largest and smallest flies (where $n > 1$). However, it is interesting that irrespective of the time of year all three samples from the R. Ruma are amongst the smallest (although this is not statistically significant). Flook & Post (1997) compared RFLP profiles for pSO11 DNA (such as fig. 2) for different Bioko populations and also obtained some weak evidence for the R. Ruma population being different from those on the western side of the island, and some suggestion of differences between north and south. In summary, there is no clear evidence for geographical structuring of the Bioko form of *S. yahense*, but a number of independent characters show some possible differences between the R. Ruma and the western samples.

The high level of geographic isolation implied by the results have important implications for the potential for vector eradication from the island. There is no evidence for vector immigration, and the genetic distinctiveness of the Bioko populations actually suggests that if immigration does

occur it must be uncommon. Therefore, if all vector breeding sites on the island can be accessed and treated with insecticide it is likely that the vector will not return for some time at least. Furthermore, and probably more importantly, if vector populations do not disappear from Bioko during the course of an eradication programme (or if they reappear thereafter), it will be possible to examine their taxonomic characteristics and to determine whether the flies are immigrants from the mainland, or whether they are the Bioko form of *S. yahense* (which must have continued breeding locally due to some sort of treatment failure). The first case would indicate an impossible obstacle to eradication, but in the second case it may be possible to locate the breeding sites and treat them to overcome the problem. Unfortunately, the taxonomic characteristics of the surviving populations will not help to locate their breeding sites, because there was very little evidence for population subdivision around the island.

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