Electrophoretic and DNA identification of *Anopheles bwambae* and *A. gambiae* (Diptera: Culicidae) in western Uganda

M. Charalambous[1], H. Townson[2], R.E. Harbach[1]* and L.G. Mukwaya[3]

[1] Department of Entomology, The Natural History Museum, Cromwell Road, London, SW7 5BD, UK: [2] Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA, UK: [3] Department of Entomology, Uganda Virus Research Institute, Entebbe,Uganda

Abstract

Collections of mosquitoes of the *Anopheles gambiae* Giles complex were made from the geothermal springs and surrounding area in the Semliki Valley, Bwamba County, Uganda, which is the only known locality of *A. bwambae* White. Specimens were analysed in one of three ways: rDNA-PCR for unequivocal species identification, allozyme electrophoresis to determine superoxide dismutase (*Sod*) and octanol dehydrogenase (*Odh*) genotypes, or both methods. Ribosomal DNA-PCR identification revealed the presence of *A. bwambae* and *A. gambiae*. Allozyme electrophoresis of 181 individuals showed that *A. bwambae* possessed the Sod^{105} and Sod^{100} alleles and was not monomorphic for Sod^{105} as reported previously. In adults reared from collections made in the vicinity of the geothermal springs, the frequency of Sod^{105} was found to be 0.614. *Anopheles gambiae* was fixed for Sod^{100}. The majority of individuals homozygous for the Sod^{100} allele could be identified to species using *Odh*. Odh^{95} was found to be common in *A. bwambae* (frequency = 0.988) while *A. gambiae* appeared to be fixed for Odh^{100}. Since Odh^{100} occurred at a frequency of 1.2% in *A. bwambae* (concomitant with *Sod* genotypes of *105/105*, *100/105* or *100/100*), individuals homozygous for Sod^{100} and Odh^{100} could be either species. Among 25 *A. bwambae* specimens homozygous for Sod^{100}, one (4%) was also homozygous for Odh^{100}. At present, this subset of the *A. bwambae* population can only be correctly identified to species using rDNA-PCR analysis.

Introduction

The mosquito *Anopheles bwambae* White (Diptera: Culicidae) is the most restricted and poorly known member of the *A. gambiae* Giles complex of malaria vectors in Africa. It is only known to occur within a 10 km radius of the geothermal springs located within the Semuliki (previously, Semliki) National Park of Bwamba County, Uganda where it is believed to transmit human malaria and filariasis (White, 1973). *Anopheles bwambae*, originally regarded as an abnormal population of *A. gambiae* (Haddow, 1945; Haddow *et al*., 1951), was afforded species status (initially as *A. gambiae* species D) based on the results of crossmating experiments and polytene chromosome analysis (Davidson & White, 1972; Davidson & Hunt, 1973; White, 1973). Within the complex, *A. bwambae* appears to be most closely related to *A. melas* Theobald, which occurs in brackish water habitats along the western coast of Africa. These species share the fixed paracentric chromosome inversion 3La (Coluzzi *et al*., 1979) and have a high level of homology in the control region of mitochondrial DNA (Caccone *et al*., 1996).

* Author for correspondence.
E-mail: reh@nhm.ac.uk
Fax: +44 (0)171 938 8937

https://doi.org/10.1017/S0007485399000188 Published online by Cambridge University Press

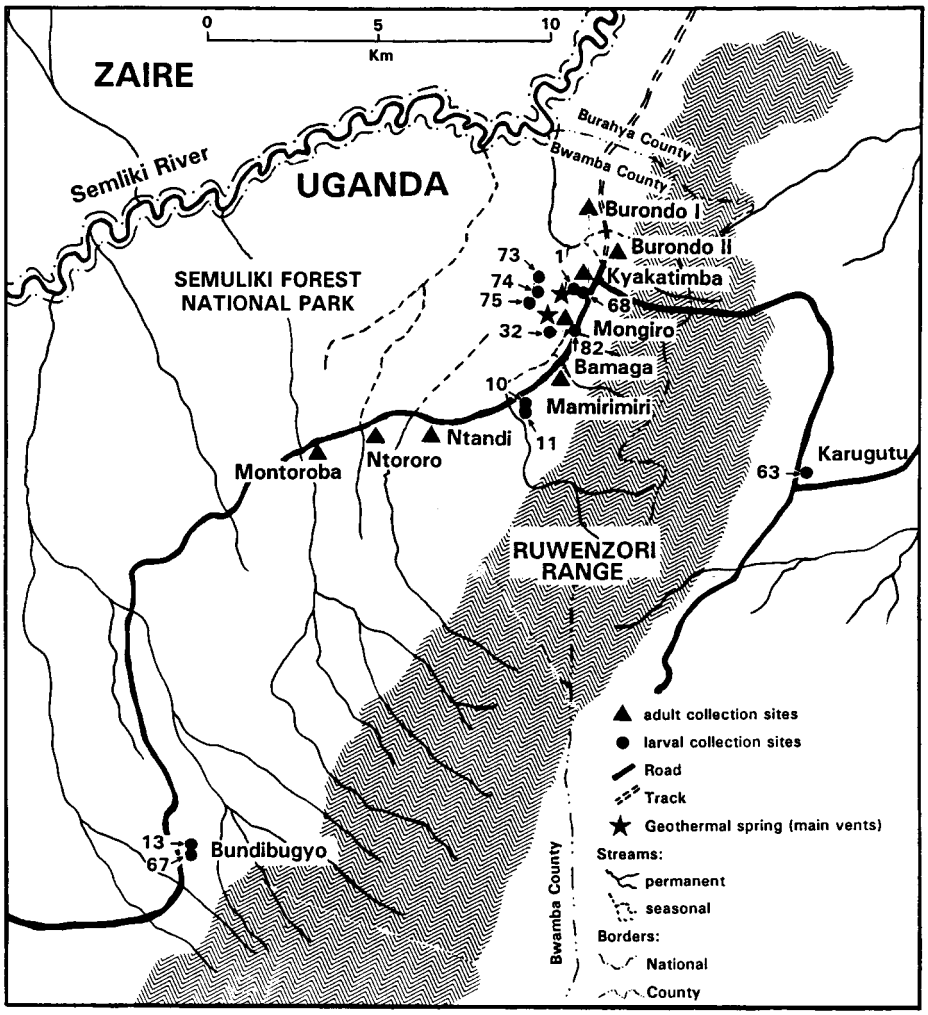

Fig. 1. Map of the study area and collection sites of *Anopheles bwambae* and *A. gambiae* in Bwamba County, Uganda. Larval collections are numbered (see table 1).

Miles (1978, 1979) produced a biochemical key based on starch gel electrophoresis of specific enzymes which were believed to distinguish all species of the *A. gambiae* complex (with some very small degree of error). In his key, *A. bwambae* (as *A. gambiae* species D) was identified as being homozygous for the *105* allele of superoxide dismutase (Sod^{105}). Other members of the complex were homozygous for Sod^{100}, except *A. melas* which was shown to be polymorphic for the *100* and *95* alleles. *Anopheles gambiae* and *A. arabiensis* Patton are the only other members of the complex which occur in Bwamba County (White, 1973). In eastern Africa there is a higher probability of correctly identifying these two species using octanol dehydrogenase (*Odh*) than the esterase genotypes used by Mahon *et al.* (1976). *Anopheles gambiae* is homozygous for Odh^{100}, while the majority of *A. arabiensis* individuals possess either the Odh^{95} or Odh^{90} allele. However, *A. arabiensis* individuals homozygous for Odh^{100} are expected to occur at a frequency of 0.3% (in Hardy-Weinberg equilibrium) (Miles, 1979).

Miles (1979) stressed that the identification of *A. bwambae* using *Sod* required confirmation because he only examined eight individuals. This paper reports the results of enzyme studies (allozyme electrophoresis) and some ribsomal DNA-PCR identifications of a much larger sample and addresses the usefulness of the biochemical key for identifying members of the *A. gambiae* complex in western Uganda.

Materials and methods

Collections of larval and adult mosquitoes were made at various sites in Bwamba County, Bundibugyo District, Uganda during June 1995. Specimens analysed in this study were obtained from 11 larval habitats and seven adult resting sites (houses and trees in forest) primarily along the road between Montoroba (located about 9 km southwest of the geothermal springs) and Burondo I (located about 3 km north of the springs) (fig. 1). Larvae were individually reared, in water obtained from their collection sites, to obtain adults with associated larval and pupal exuviae. Approximately equal numbers of the reared adults from each collection were pinned for morphological study and to serve as voucher specimens, frozen in liquid nitrogen for

https://doi.org/10.1017/S0007485399000188 Published online by Cambridge University Press

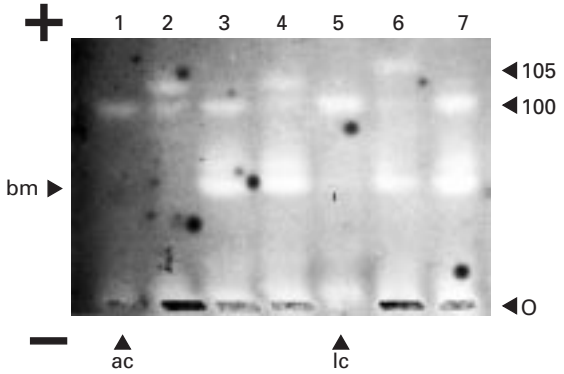

Fig. 2. *Sod* alleles of females of the *Anopheles gambiae* complex captured resting in houses at Kyakatimba, Bwamba County, Uganda, some of which had a blood meal (bm). An adult and larva of the R70 strain of *A. gambiae* were run as controls (ac and lc, respectively). The alleles were loaded at the origin (o) and migrated anodally.

allozyme electrophoresis, and dried in gelatin capsules over silica gel for molecular genetic studies and identification using species-specific rDNA-PCR primers. Resting adult mosquitoes were collected with aspirators from houses (six sites) and in the Semliki Forest at Mongiro near the geothermal springs (the type locality of *A. bwambae*). Mosquitoes collected in the same house on different days were treated as a single collection. Approximately half of these specimens were frozen for allozyme electrophoresis; the remainder were either pinned or dried for other studies. The following analyses were performed without knowledge of where the specimens were collected.

Cellulose acetate gel electrophoresis of superoxide dismutase (*Sod*, E.C. 1.15.1.1) and octanol dehydrogenase (*Odh*, E.C. 1.1.1.73) generally followed the method of Herbert & Beaton (1989). Whole mosquitoes were homogenized in 10–20 µl of autoclaved, double-distilled water in a cold grinding block, 3.5 µl of each homogenate was loaded into a well of a 12-sample plate, and the remaining homogenate was stored at −70°C. The abdomens of blood-fed females from the adult resting collections were removed because haemoglobin was found to have a very similar mobility to the slowest *Odh* band (marked bm in fig. 2). An homogenate from a R70 strain of *A. gambiae* from Tanzania (maintained at the London School of Hygiene and Tropical Medicine), known to be homozygous for both Sod^{100} and Odh^{100} was used as a control on each gel plate. Individuals of the KGB strain of *A. arabiensis* from Zimbabwe (maintained at the London School of Hygiene and Tropical Medicine) were used to check the assignation of allele mobilities.

Electrophoresis of the two enzyme systems required different tank and soaking buffers: 0.1M Tris–0.3M boric acid–10% sucrose, pH 7.6 (Stevens *et al*., 1989) for *Sod* and 0.025M tris-glycine, pH 8.5 (Richardson *et al*., 1986) for *Odh*. Electrophoresis was conducted at 200V for 40 min at room temperature. The staining solution for *Sod* was modified from Le Blanq *et al*. (1986) and Stevens *et al*. (1989) and consisted of 2 ml 0.0032% riboflavin in 0.1M sodium phosphate at pH 8.0, 0.1 ml 3.5 mg ml^{-1} EDTA, and 0.1 ml 7.5 mg ml^{-1} *p*-iodonitrotetrazolium (INT). The staining solution for *Odh* was based on Herbert & Beaton (1989), with slight modification of the quantities of reagents used. After completing each run, 7.8 ml of 1.6% molten agar was mixed with the appropriate staining solution. The staining solution/agar mixture (10 ml in total) was then poured immediately onto the plate and left in the light at room temperature for 2 min until the overlay had solidified. *Sod* plates were placed in the dark at 37°C for 10 min and then on a light box for 10 min; *Odh* plates were incubated in the dark at 37°C for about 20 min. The *Sod* bands appeared white on a pink background; *Odh* bands were dark on a light background.

The homogenates of several specimens of each electrophoretic genotype were identified by PCR, to give both an enzyme and DNA identification for each specimen. This PCR-based method relies on sequence differences in the intergenic spacer region of the ribosomal RNA gene cluster (Paskewitz & Collins, 1990). A number of dried specimens from each collection were also identified by the same PCR technique. DNA was extracted from homogenates by the method of Collins *et al*. (1987) and PCR carried out using methods described by Scott *et al*. (1993) with their species-specific primer for *A. gambiae* and *A. arabiensis*, and an additional species-specific primer for *A. bwambae* (Townson, Besansky & Collins, unpublished). For dried mosquitoes we found that a phenol extraction step improved the recovery of amplifiable DNA (Townson *et al*., 1999).

Results

A total of 181 mosquitoes from Bwamba County (including specimens from Mongiro, the only site sampled by Miles, 1978) were scored for both *Sod* and *Odh*. Both the Sod^{100} and Sod^{105} alleles were present. As well as the expected homozygotes, heterozygotes were also found ($Sod^{100/105}$; table 1). *Sod* allozymes produced heterozygotes asymmetrical for relative intensity (see fig. 8.8 in Richardson *et al*., 1986), so that the majority appeared two-banded instead of the usual three-banded phenotype expected for a dimeric enzyme (Hunt, 1987). The uppermost band, equivalent in mobility to the Sod^{105} allele (see lane 6 in fig. 2), was missing. However, the relative intensity of the two remaining bands was that expected for a dimer (see lanes 2 and 4 in fig. 2). Increasing the run-time of electrophoresis, altering the buffer pH and the quantities of staining reagents did not aid resolution. Since the quaternary structure of the enzyme is known, it was not difficult to score the heterozygotes and homozygotes.

Odh^{95} and Odh^{100} were also found in the samples, giving rise to four main combinations of *Sod* and *Odh* genotypes. A fifth genotype, $Sod^{100/100}$ + $Odh^{95/100}$, was found only once (table 1).

A number of individuals of each genotype combination were identified by rDNA-PCR to give an unambiguous species determination for each specimen (table 2). The results indicated that *A. bwambae* and *A. gambiae* were present in the study area, but that *A. arabiensis* was absent. Furthermore, *A. bwambae* was seen to be polymorphic for *Sod*, possessing both the *100* and *105* alleles, while *A. gambiae* was fixed for Sod^{100} (see also DNA results of individuals from Bundibugyo and Karugutu in table 1). Therefore, *Sod* cannot be used alone to identify all individuals of *A. bwambae* since those homozygous for Sod^{100} might be misidentified as *A. gambiae*. Fortunately, the *Odh* locus, originally intended to separate *A. gambiae* and *A. arabiensis*,

https://doi.org/10.1017/S0007485399000188 Published online by Cambridge University Press

Table 1. Biochemical analysis of specimens of the *Anopheles gambiae* complex collected during June 1995 in Bwamba County, Uganda.

			Allozyme genotypes					rDNA-PCR identification	
Location (collection no.)	Latitude/ longtitude†	Total no.	$Sod^{105/105}$ + $Odh^{95/95}$	$Sod^{100/105}$ + $Odh^{95/95}$	$Sod^{100/100}$ + $Odh^{95/95}$	$Sod^{100/100}$ + $Odh^{95/100}$	$Sod^{100/100}$ + $Odh^{100/100}$	*A. bwambae*	*A. gambiae*
Adults reared from larval collections									
North Spring (1)	0°50′42.3″N/ 30°09′36.5″E	6	1	2	3			10	
North Spring (68)	0°50′42.3″N/ 30°09′36.5″E	4	1	3				5	
South Spring (32)	0°49′51.0″N/ 30°09′50.5″E	2	1		1			3	
Spring savanna (73)	0°50′08.4″N/ 30°09′41.2″E	3	2				1*	6	
Spring savanna (74)	0°50′06.4″N/ 30°09′41.2″E	2	2					5	
Spring savanna (75)	0°50′05.4″N/ 30°09′43.2″E	5	3	2				6	1
Mamirimiri (10)	0°48′54.2″N/ 30°09′12.5″E	2					2		2
Mamirimiri (11)	0°48′54.2″N/ 30°09′12.5″E	3					3		4
Karugutu‡ (63)	0°47′27.4″N 30°14′21.0″E	23					23		9
Bundibugyo (13)	0°42′37.7″N/ 30°03′44.6″E	6					6		5
Bundibugyo (67)	0°42′06.3″N/ 30°03′49.6″E	21				1**	20		10
Sub-total		77	10	7	4	1	55	35	31
Adult resting collections									
Burondo I	0°51′58.8″N/ 30°10′24.4″E	7	2	3	2			11	
Burondo II	0°51′04.9″N/ 30°10′28.1″E	23	5	16	2			25	
Kyakatimba	0°50′16.4″N/ 30°10′03.7″E	40	13	14	13			70	1
Mongiro	0°49′48.8″N/ 30°09′57.0″E	8	3	5				31	
Bamaga	0°49′21.1″N/ 30°09′36.7″E	5		4	1			10	
Ntandi	0°48′38.3″N/ 30°08′43.0″E	18	6	11			1	30	
Ntororo	0°48′22.9″N/ 30°07′28.8″E	3		2	1			3	7
Sub-total		104	29	55	19		1	180	8
Total		181	39	62	23	1	56	215	39

The majority of specimens from each locality were subjected to either electrophoretic analysis or identified using rDNA-polymerase chain reaction analysis. A few specimens of each electrophoretic genotype (including those marked * and **) were later identifed using rDNA–PCR (see table 2). †GPS coordinates (accuracy 30–100 m). ‡Burahya County.

can be used to distinguish most specimens of *A. gambiae* and *A. bwambae* in the vicinity of the geothermal springs where *A. arabiensis* was absent. According to Miles (1978), *A. bwambae* is fixed for Odh^{95}. However, we identified (using rDNA-PCR) an individual of this species from the geothermal springs drainage area that was homozygous for Odh^{100} (from collection 73) and another individual from Bundibugyo that was $Odh^{95/100}$ (from collection 67; tables 1 and 2). Including these two individuals plus the 124 $Odh^{95/95}$ specimens (in table 1), the frequency of Odh^{95} and Odh^{100} in *A. bwambae* was 0.988 and 0.012, respectively. Consequently, as *A. bwambae* possesses both *Odh* alleles and *A. gambiae* is fixed for Odh^{100}, Odh^{95} can be used to positively identify specimens of *A. bwambae*. However, this will only be valid in

https://doi.org/10.1017/S0007485399000188 Published online by Cambridge University Press

Table 2. rDNA-PCR identification of allozyme-typed mosquitoes from Bwamba County, Uganda.

Allozyme genotype		rDNA-PCR identification	
Sod	*Odh*	*A. bwambae*	*A. gambiae*
105/105	95/95	6	
100/105	95/95	5	
100/100	95/95	3	
100/100	95/100	1**	
100/100	100/100	1*	2

*Indicates an individual from the springwater savanna (collection 73); ** indicates an individual from Bundibugyo (collection 67; table 1).

regions, such as the study area, where *A. arabiensis* does not occur as it also possesses Odh^{95} (Miles, 1978, 1979).

Using both *Sod* and *Odh* loci to identify specimens, a proportion of individuals of *A. bwambae* which are homozygous for Sod^{100} and Odh^{100} might still be incorrectly identified as *A. gambiae* using the biochemical key (Miles, 1979). In our study, 20% of the total population of *A. bwambae* were $Sod^{100/100}$ (n = 25/126, 23 in table 1 which are also $Odh^{95/95}$ plus the $Odh^{95/100}$ and $Odh^{100/100}$ individuals identified by rDNA-PCR in table 2). Only one of these individuals (i.e. 0.8% of the total population) was homozygous for both Sod^{100} and Odh^{100}. As only 16 *A. bwambae* specimens of known enzyme genotype were identified by rDNA-PCR (table 2), our data are insufficient to estimate the frequency of misclassification. However, it does seem that the frequency of *A. bwambae* homozygotes for Sod^{100} and Odh^{100} would be low since the expected frequency of $Odh^{100/100}$ individuals is 0.00014 (frequency of Odh^{100} = 0.012 assuming Hardy-Weinberg equilibrium).

The allozyme and DNA identifications of adult mosquitoes reared from the same larval sites were generally in complete agreement in terms of the species present. The *Sod* genotypes of adults reared from larvae collected in water derived from the geothermal springs (collections 1, 68, 32, 73–75) were in Hardy-Weinberg proportions ($\chi^2 = 2.38$, df = 2, $P > 0.5$, frequency (*105*) = 0.614, f (*100*) = 0.386) (table 3), suggesting that the population may be randomly mating (panmictic) and could therefore represent mainly one species, *A. bwambae*. This was confirmed by rDNA-PCR identification of additional specimens from these collections, which showed all but one specimen to be *A. bwambae*. This lone specimen of *A. gambiae* was found in association with *A. bwambae* in a site located at the margin of the springwater drainage system (collection 75). The larval collections made away from the geothermal springs runoff, or outside of the known distribution of *A. bwambae*, contained only *A. gambiae* according to rDNA-PCR identification – except for an *A. bwambae* specimen from Bundibugyo (collection 67) which was of suspect origin (see Discussion) – and possessed the $Sod^{100/100}$ + $Odh^{100/100}$ genotype expected for this species.

The adult resting collections were made within the known range of *A. bwambae* and consisted mainly of this species based on allozyme analysis and rDNA-PCR identification (97%, n = 292; table 1). The *Sod* genotype frequencies of adult females caught resting in houses in the vicinity of the springs (table 3) were in Hardy-Weinberg proportions ($\chi^2 = 0.2$, df = 2, $P > 0.9$, frequency (*105*) =0.53, f (*100*) = 0.47), again suggesting the presence of a single panmictic population.

Discussion

Chromosomal studies of the *A. gambiae* complex in Bwamba County by White (1973) revealed the presence of three species, *A. gambiae*, *A. arabiensis*, and *A. bwambae*. These three species may also be distinguished using allozyme electrophoresis of superdioxide dismutase and octanol dehydrogenase (Miles, 1978, 1979). However, Miles (1979) urged caution in ascribing diagnostic value to Sod^{105} for the identification of *A. bwambae* until additional samples had been examined from Bwamba County. His study of eight individuals from Mongiro was clearly not enough to reveal the extent of variation in the *Sod* locus (Miles, 1978). Our analysis of 181 individuals has shown that *A. bwambae* is polymorphic at this locus, possessing both Sod^{105} and Sod^{100} at frequencies of 0.614 and 0.386, respectively, in adults reared from larvae collected in water derived from the geothermal springs. We examined eight individuals collected from a resting population in the Semliki forest at Mongiro and detected five heterozygous. Consequently, it is striking that Miles (1978) did not report the occurrence of Sod^{100} in *A. bwambae*, especially as we found the *Sod* locus to be in Hardy-Weinberg equilibrium. We found no evidence of hybridization between *A. bwambae* and *A. gambiae*, which would be a potential source of Sod^{100}. If effective population sizes are very small, it is possible that Sod^{100} has arisen and spread through the population by random genetic drift. However, we believe that the anomaly is more likely to be due to sampling error.

As *A. gambiae* and *A. arabiensis* also occur in Bwamba County (White, 1973), and possess Sod^{100}, only those individuals which are homozygous or heterozygous for the *105* allele can be identified as *A. bwambae*. Odh^{95} in combination with *Sod* can be used to positively identify $Sod^{100/100}$ individuals of *A. bwambae* because rDNA-PCR analysis of the same individuals indicates that *A. gambiae* is

Table 3. *Sod* genotypes of mosquitoes collected in the vicinity of the geothermal springs in Bwamba County, Uganda. The Mongiro collection is omitted as it contained males and females collected in the Semliki Forest not in houses.

	Allozyme genotype			
	$Sod^{105/105}$	$Sod^{100/105}$	$Sod^{100/100}$	Total
Adults reared from larval collections	10	7	5	22
Females resting in houses	26	50	20	96

https://doi.org/10.1017/S0007485399000188 Published online by Cambridge University Press

fixed for Odh^{100}. Positive identification by allozyme electrophoresis will not be possible in areas where *A. arabiensis* also occurs because this species contains the Odh^{95} allele at a high frequency (Miles, 1978, 1979). We did not find *A. arabiensis* in the study area in 1995. *A. bwambae* possessed the Odh^{100} allele at a very low frequency of 1.2% and only 4% of $Sod^{100/100}$ individuals were also $Odh^{100/100}$. These individuals, representing 0.8% (1/126) of the total population, would be misidentified as *A. gambiae* using the biochemical key (Miles, 1979). Since only three individuals homozygous for Sod^{100} and Odh^{100} were subjected to both DNA and allozyme analysis, it is possible that the real frequency of Odh^{100} is higher in *A. bwambae* than the 1.2% reported here. As a case in point, a single adult from Ntandi homozygous for Sod^{100} and Odh^{100}, and thus *A. gambiae* according to the biochemical key, could be *A. bwambae* as indicated by the DNA identification of 30 other individuals captured at this location (table 1). If this specimen was *A. bwambae*, then the occurrence of Odh^{100} in *A. bwambae* would rise to 2%.

Our results suggest that the both *A. bwambae and A. gambiae* might have a wider distribution in Bwamba County than previously thought. For example, an adult reared from a larval collection from Bundibugyo (collection 67) located about 8 km southwest of the known distribution of *A. bwambae,* was confirmed to be *A. bwambae* and had an enzyme genotype of $Sod^{100/100}$ + $Odh^{95/100}$. However, as other specimens reared from this collection were identified as *A. gambiae* by rDNA-PCR (table 1), it is possible that this individual came from another collection and was mislabelled during the process of rearing and preserving specimens in the field. A specimen of *A. gambiae* was identified using rDNA-PCR from the margins of the springwater drainage system (collection 75), which is a typical *A. bwambae* site. This indicates that the larvae of these species may occur together where water derived from the geothermal springs interfaces with surrounding grasslands and forests (Harbach *et al*., 1997) and is supported by DNA identification of specimens collected in 1996 (N. Thewell, personal communication).

The reported occurrence of all three species in the study area in 1996 (N. Thelwell, personal communication) suggests that the *Odh* locus might not be as useful for identification as we had hoped. Consequently, only $Sod^{105/105}$ and $Sod^{100/105}$ individuals can be positively identified as *A. bwambae*, i.e. only 56% (101/181) of the known population. Ribosomal DNA-PCR analysis, therefore, remains the only unequivocal method of distinguishing all members of the *A. gambiae* complex in Bwamba County.

Miles (1978, 1979) indicated that *Sod* was monomorphic and diagnostic for *A. bwambae*, and this information was included in the formal description of the species (White, 1985). However, Sod^{105} has been found in *A. arabiensis* in northeastern Tanzania (Marchand & Mnzava, 1985) and in the Yaka-Yaka population of *A. gambiae* in the People's Republic of the Congo (Hunt & Coetzee, 1989). Both of these species were thought to be monomorphic for Sod^{100}. Similarly, we have shown that *Sod* is not monomorphic in *A. bwambae*, both the *100* and *105* alleles occur in this species. It is apparent, therefore, that the biochemical key of Miles (1979) should be regarded as an evolving entity which will change as more populations, with differing gene frequencies, are sampled from wider geographic areas.

Acknowledgements

We thank Chris Curtis and Barbara Sawyer at the London School of Hygiene and Tropical Medicine for providing the R70 and KGB strains, Anoushka Smits formerly of the Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium for discussions on *Sod* staining recipes, Abraham Mnzava for a helpful e-mail discussion, Theresa Howard at The Natural History Museum for preparing fig. 1, and Rory Post and Martin Hall for their comments on the manuscript. Financial support from the National Geographic Society, Washington, DC (Grant. No. 5351–94) and The Natural History Museum is gratefully acknowledged.

References

Caccone, A. Garcia, B.A. & Powell, J.R. (1996) Evolution of the mitochondrial DNA control region in the *Anopheles gambiae* complex. *Insect Molecular Biology* **5**, 51–59.

Collins, F.H., Mendez, M.A., Rasmussen, M.O., Mehaffey, P.C., Besansky, N.J. & Finnerty, V. (1987) A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. *American Journal of Tropical Medicine and Hygiene* **37**, 37–41.

Coluzzi, M. Sabatini, A., Petrarca, V. & Di Deco, M.A. (1979) Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **73**, 483–497.

Davidson, G. & Hunt, R.H. (1973) The crossing and chromosome characteristics of a new, sixth species in the *Anopheles gambiae* complex. *Parassitologia* **15**, 121–128.

Davidson, G. & White, G.B. (1972) The crossing characteristics of a new, sixth species in the *Anopheles gambiae* complex. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **66**, 531–532.

Haddow, A.J. (1945) On the mosquitoes of Bwamba County, Uganda. I. Description of Bwamba with special reference to mosquito ecology. *Proceedings of the Zoological Society of London* **115**, 1–13.

Haddow, A.J., Van Someren, E.C.C., Lumsden, W.H.R., Harper, J.O. & Gillet, J.D. (1951) The mosquitoes of Bwamba County, Uganda. VIII. Records of occurrence, behaviour and habitat. *Bulletin of Entomological Research* **42**, 207–238.

Harbach, R.E., Townson, H. & Mukwaya, L.G. (1997) Observations on the bionomics and ecological distribution of *Anopheles bwambae* and *Anopheles gambiae* in sympatry (Diptera: Culicidae). *Medical and Veterinary Entomology* **11**, 329–334.

Herbert, P.D.N. & Beaton, M.J. (1989) *Methodologies for allozyme analysis using cellulose acetate electrophoresis - a practical handbook*. Ontario, Helena Laboratories.

Hunt, R.H. (1987) Location of genes on the chromosome arms in the *Anopheles gambiae* group of species and their correlation to linkage data for other anopheline mosquitoes. *Medical and Veterinary Entomology* **1**, 81–88.

Hunt, R.H., & Coetzee, M. (1989) A parasitological, cytogenetic, and biochemical study of *Anopheles gambiae* (Diptera: Culicidae) from the People's Republic of Congo. *South African Medical Journal* **76**, 362–364.

Le Blanq, S.M., Schnur, L.F. & Peters, W. (1986) *Leishmania* in the Old World. I. The geographical and hostal distribution of *L. major* zymodemes. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **80**, 99–112.

Mahon, R.J., Green, C.A. & Hunt, R.H. (1976) Diagnostic allozymes for routine identification of adults of the *Anopheles gambiae* complex (Diptera, Culicidae). *Bulletin of Entomological Research* **66**, 25–31.

Marchand, R.P. & Mnzava, A.E.P. (1985) A field test of a biochemical key to identify members of the *Anopheles gambiae* group of species in north-east Tanzania. *Journal of Tropical Medicine and Hygiene* **88**, 205–210.

Miles, S.J. (1978) Enzyme variation in the *Anopheles gambiae* Giles group of species (Diptera: Culicidae). *Bulletin of Entomological Research* **68**, 85–96.

Miles, S.J. (1979) A biochemical key to adult members of the *Anopheles gambiae* group of species (Diptera: Culicidae). *Journal of Medical Entomology* **15**, 297–299.

Paskewitz, S.M. & Collins, F.H. (1990) Use of the polymerase chain reaction to identify mosquito species of the *Anopheles gambiae* complex. *Medical and Veterinary Entmology* **4**, 367–373.

Richardson, B.J., Baverstock, P.R. & Adams, M. (1986) *Allozyme electrophoresis*. San Diego, Academic Press.

Scott, J.A., Brogdon, W.G. & Collins, F.H. (1993) Identification of a single specimen of the *Anopheles gambiae* complex by the polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene* **49**, 520–529.

Stevens, J.R., Nunes, V.L.B., Lanham, S.M. & Oshiro, E.T. (1989) Isoenzyme characterization of *Trypanosoma evansi* isolated from capybaras and dogs in Brazil. *Acta Tropica* **46**, 213–222.

Townson, H., Harbach, R.E. & Callan, T.A. (1999) DNA identification of musuem specimens of the *Anophles gambiae* complex: an evaluation of PCR as a tool for resolving the formal taxonomy of sibling species complexes. *Systematic Entomology* **24**, 95–100.

White, G.B. (1973) Comparative studies on sibling species of the *Anopheles gambiae* Giles complex (Dipt., Culicidae). III. The distribution, ecology, behaviour and vectorial importance of species D in Bwamba County, Uganda, with an analysis of biological, morphological and cytogenetical relationships of Ugandan species D. *Bulletin of Entomological Research* **63**, 65–97.

White, G.B. (1985) *Anopheles bwambae* sp., a malaria vector in the Semliki Valley, Uganda, and its relationships with other sibling species of the *An. gambiae* complex (Diptera: Culicidae). *Systematic Entomology* **10**, 501–522.

(Accepted 1 December 1998)

https://doi.org/10.1017/S0007485399000188 Published online by Cambridge University Press