Bulletin of Entomological Research (2003) **93**, 169–171 DOI: 10.1079/BER2002219

SHORT COMMUNICATION

Comparison of polymerase chain reaction assay and cytotaxonomy for identification of sibling species of *Anopheles fluviatilis* (Diptera: Culicidae)

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Introduction

Anopheles fluviatilis s.s. James (Diptera: Culicidae) is one of the major malaria vectors in India, Pakistan and Nepal. It is widely distributed and found in hills, foothills and plains up to an altitude of 2500 m above mean sea level. Subbarao *et al*. (1994) have shown that *A. fluviatilis* is a complex of three species, identified by species-specific diagnostic inversions seen in the banding pattern of the polytene chromosomes. In the districts of Orissa State in India, which are highly endemic for malaria, *A. fluviatilis* has been incriminated as the main vector (Jambulingam *et al*., 1991) and the population comprises three sibling species S, T and U (Subbarao, 1998). In Koraput and Malkangiri districts, only two sibling species namely S and T have been reported based on cytotaxonomical identification (Nanda *et al*., personal communication). Subsequently, from the specimens collected from these two districts, a polymerase chain reaction (PCR) assay was developed from the rDNA region and two species were identified and provisionally designated as X and Y (Manonmani *et al*., 2001).

In order to correlate the results of the two identification techniques, field-collected specimens of *A. fluviatilis* were simultaneously examined cytotaxonomically and by PCR assay and the results compared.

Materials and methods

Samples were obtained from daytime indoor and outdoor resting mosquito catches, all night man landing collections and light trap catches made in selected villages (table 1) of Koraput (600 m above msl) and Malkangiri (150m above msl) districts of Orissa over one year period (10.III.98–13.IV.99). Daytime resting mosquitoes were collected in the morning hours (0600–0800 h) from human dwellings, cattle sheds and pit shelters using an aspirator and flashlight. All night man landing collections were done between 1800 h and 0600 h. Modified CDC miniature light traps (Sudia & Chamberlain, 1962; Gunasekaran *et al*., 1994), were suspended approximately 60 cm above ground level in cattle sheds and above 150 cm in human dwellings between 1800 h and 0600 h. The next morning, the mosquito-collecting cages were removed and the adults collected. *Anopheles fluviatilis* samples from all the catches were identified morphologically and used for identification of sibling species by the two techniques.

Ovaries were removed from semigravid females and placed in modified Carnoy's fixative (1:3 acetic acid/methanol). Ovaries were processed in 50% propionic acid and stained in 2% lacto-aceto-orcein following the method of Green & Hunt (1980) for making polytene chromosome preparations. The specimens were identified to sibling species by examining the inversion genotypes of chromosome arm 2, i.e. $2+q^1+r^1$ and $2q^1+r^1$ diagnostic for species S and T respectively (Subbarao *et al*., 1994). The specimens in polymorphic state q1, +q1/q1, and +q1 have been identified based on resting and feeding behaviour, viz. those collected from outdoor resting places and with bovine blood were classified as species T while those resting indoors and with human blood as species S. Blood from the gut of the same mosquito was smeared on to a Whatman No. 1 filter paper and tested by countercurrent immunoelectrophoresis (Bray *et al*., 1984) for blood meal assay. The remaining portions of the mosquito sample were dried at 90°C overnight for PCR assay. The blood smear, ovaries and the tissue for PCR assay of each individual mosquito were allocated the same number for tracking purposes.

Parts of individual mosquitoes were taken in separate microfuge tubes and ground to a fine powder with

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Table 1. Details of *Anopheles fluviatilis* samples used in the study.

Area	Village	Habitat	Type of collection	Samples collected	Blood meal analysis			Cytotaxonomy		PCR assay	
					Hu	Bo	Hu+Bo	S	T	X	Y
Jeypore	Champapadar	HD	Hand catch	49	46	3	0	46	3	41	8
		CS	Hand catch	6	0	6	0	0	6	3	3
		PS	Hand catch	34	20	11	3	22	12	23	11
		ANMLC	Hand catch	18	18	0	0	18	0	18	0
		HD	Light trap	1	0	1	0	0	1	0	1
		CS	Light trap	3	0	3	0	0	3	2	1
	Chapper	HD	Hand catch	12	6	5	1	4	8	3	9
		CS	Hand catch	5	0	5	0	0	5	0	5
		PS	Hand catch	7	0	7	0	0	7	1	6
		ANMLC	Hand catch	4	2	1	1	3	1	4	0
		CS	Light trap	24	0	24	0	0	24	1	23
	K. Maliguda	HD	Hand catch	2	2	0	0	2	0	2	0
	Lariguda	CS	Light trap	73	0	73	0	0	73	0	73
Malkangiri	Balalguda	HD	Hand catch	66	62	2	2	66	0	63	3
		CS	Hand catch	1	1	0	0	1	0	1	0
		ANMLC	Hand catch	7	7	0	0	7	0	7	0
		CS	Light trap	1	0	1	0	0	1	1	0
	Batriantal	HD	Hand catch	8	8	0	0	8	0	8	0
	Kandhaguda	HD	Hand catch	42	41	0	1	42	0	40	2
		PS	Hand catch	1	1	0	0	1	0	1	0
		ANMLC	Hand catch	37	37	0	0	37	0	37	0
Total				401	251	142	8	257	144	256	145

ANMLC, all night man landing collection; Bo, bovine; CS, cattle shed; HD, human dwelling; Hu, human; PS, pit shelter. S, T, X and Y refer to the sibling species of *A. fluviatilis* identified cytologically and by PCR assay.

microfuge pestle grinders. They were then overlaid with 100 ml Tris-EDTA ($T_{10}E_1$) buffer, vortexed briefly and incubated at 90^0C for 10 min. After centrifugation at 13,000 rpm, the supernatant containing the DNA was transferred to a fresh tube and stored at 4^0C. The polymerase chain reactions were carried out as described by Manonmani *et al*. (2001). The species diagnostic bands were resolved by electrophoresing the PCR products through a 1.5% agarose gel. The bands were visualized on a UV light trans-illuminator after staining the gels in ethidium bromide. Each gel contained a DNA size standard, a negative control and a positive control using DNA from both sibling species of *A. fluviatilis*. The DNA of the two sibling species of *A. fluviatilis* produced fragments of two distinct sizes, 350 bp for species X and 450 bp for species Y, respectively.

Results

A total of 401 specimens of *A. fluviatilis* was subjected to cytological identification and rDNA-ITS2 PCR assay (table 2). The majority (91%) of the indoor resting mosquitoes collected by aspirators from human dwellings and all night man landing collections were found to be species X, while the majority (96%) of the light trap samples from cattle sheds were identified as species Y. Species Y was found in greater proportion in the aspirator collections from cattle sheds (67%) while species X was represented in greater numbers in pit shelters (60%). In Koraput district, the occurrence of species T/Y was almost double that of species S/X. In Malkangiri district, species S/X was predominant with very few individuals of species T/Y observed in field samples.

Out of 257 specimens identified as 'S' cytologically, 98% had fed on human blood (table 1) while out of 144 specimens identified as 'T' cytologically 99% had fed on bovine blood.

Table 2. *Anopheles fluviatilis* mosquitoes identified by polymerase chain reaction (PCR) assay and cytotaxonomy.

Cyto-taxonomy	PCR assay		Total	% concordance
	X	Y		
S	242	15	257	94.1
T	14	130	144	90.2

The results from 242 specimens (out of 401 tested) confirmed that species S and X were the same, since about 94% of the specimens identified as species S cytologically were identified as species X by PCR assay (table 2). Similarly, about 90% of the specimens identified as T cytologically were identified as Y by PCR, indicating that species T and Y were the same. However, results from 29 mosquitoes (7.2%) were contradictory. Fifteen mosquitoes that were identified as S cytologically were identified as Y by PCR, while 14 mosquitoes identified as T cytologically were identified as X by PCR. Samples which gave contradictory results were from all types of collections.

Discussion

Both the techniques demonstrated that in Malkangiri, one sibling species (S/X) was predominant as observed by Jambulingam *et al*. (personal communication). However, in Koraput, both sibling species existed sympatrically (X:Y::2:3) their within habitat preferences providing evidence of their separate species status. The study showed that the sibling species showed distinct behavioural characteristics, S/X being predominantly endophilic (resting in human dwellings) and anthropophagic and T/Y exophilic (resting

https://doi.org/10.1079/BER2002219 Published online by Cambridge University Press

in cattle sheds) and zoophagic. An earlier study showed that the human blood index (HBI) for mosquitoes of the *A. fluviatilis* complex was 0.83 in Malkangiri district compared with 0.26 for mosquitoes of the same complex in Koraput zone/district (Gunasekaran *et al.*, 1994). The higher values recorded in Malkangiri were due to the predominance of sibling species S/X in contrast to Koraput district where both sibling species were present. In other areas, the sibling species S/X was found to exhibit a high degree of anthropophagy (Nanda *et al.*, 1996) and in the areas of its occurrence, malaria was found to be hyper-endemic with a prevalence of *Plasmodium falciparum* (Welch) (Plasmodidae) leading to deaths.

The small proportion (7.2%) of disagreement observed in this study between cytotaxonomy and the PCR assay might be due to the existence of polymorphism in *A. fluviatilis* in this area. As stated earlier, the q1 inversion has been reported to exist in a polymorphic state in the population of *A. fluviatilis*. In areas where such polymorphism is observed, biological characteristics of the population such as resting and feeding behaviour have been used, in addition to inversion genotypes, to classify the population as species S or T (Subbarao, 1998). However, an earlier study on host-feeding patterns of the same species has shown that although species S is predominantly anthropophilic, a small proportion may feed on animals depending on the man:cattle ratio in the immediate vicinity (Nanda *et al.*, 1996). Similarly, a small proportion of species T, which is mainly zoophilic may feed on man as also found in the present study. Such a phenomenon has also been observed with one of the members of the *A. funestus* Giles group namely *A. vaneedeni* Gillies & Coetzee (Diptera: Culicidae). This species though described as predominantly exophilic and zoophilic has been found resting indoors and feeding on humans in the absence of suitable animal hosts (Brooke *et al.*, 2001). Therefore, using resting and feeding behaviours as the main identification characters may result in some degree of error in the identification.

Paskewitz *et al*. (1993) found absolute concordance in the identification of *A. gambiae* Giles and *A. arabiensis* Patton (Diptera: Culicidae) by cytotaxonomy and polymerase chain reaction assay. However, Kampen *et al*. (1995) found a discrepancy of about 5.3% between the two techniques which they traced back to mistakes in the cytotaxonomic identification procedure.

Except for the small discrepancy in the identification results, both the techniques revealed the presence of only two species of the *A. fluviatilis* complex in these areas as has been observed earlier (Manonmani *et al.*, 2001). The study shows that the species identified as X and Y by PCR assay are the sibling species S and T identified cytologically.

Acknowledgements

The authors wish to thank Dr P.K. Das, Director, Vector Control Research Centre, Pondicherry for his keen interest in this study and for providing the required facilities. They also thank Dr C. Sadanandane, for reviewing the manuscript and for his valuable suggestions. The technical assistance rendered by Mr S. Murugaraj and the staff of the Cytogenetics Division, Malaria Research Centre, Delhi and VCRC Field Station, Malkangiri is gratefully acknowledged.

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(Accepted 24 October 2002)

Tsetse Biology and Ecology: Their Role in the Epidemiology and Control of Trypanosomosis

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ISBN 0 85199 300 1
December 1998 592 pages
Hardback £70.00 (US$120.00)

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Medical and veterinary entomologists, parasitologists and epidemiologists.

Description

Domestic livestock in Africa are of importance not only as a source of milk and meat but also as a source of animal traction enabling farmers to cultivate larger areas, with crops providing the staple foods. Trypanosomosis, a parasitic disease transmitted cyclically by the tsetse fly (*Glossina* spp.), is arguably still the main constraint to livestock production on the continent, preventing full use of the land to feed the rapidly increasing human population. Sleeping sickness, the disease caused in humans by species of *Trypanosoma*, is an important and neglected disease posing a threat to millions of people in tsetse-infested areas. Often wrongly thought of as a disease of the past, the prevalence of human sleeping sickness is increasing in many areas.

https://doi.org/10.1079/BER2002219 Published online by Cambridge University Press