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Sauvik Dasgupta
Vector Control Research Centre, Indian Council of Medical Research, Medical Complex, Indira Nagar, Puducherry, Tamil Nadu, India

Smrutidhara Dash
Vector Control Research Centre, Indian Council of Medical Research, Medical Complex, Indira Nagar, Puducherry, Tamil Nadu, India

Thankachy Sonia
Vector Control Research Centre, Indian Council of Medical Research, Medical Complex, Indira Nagar, Puducherry, Tamil Nadu, India

Ashokkumar Mathivanan
Vector Control Research Centre, Indian Council of Medical Research, Medical Complex, Indira Nagar, Puducherry, Tamil Nadu, India

Dr. Sudhansu Sekhar Sahu
Vector Control Research Centre, Indian Council of Medical Research, Medical Complex, Indira Nagar, Puducherry, Tamil Nadu, India

Correspondence
Dr. Sudhansu Sekhar Sahu
Vector Control Research Centre, Indian Council of Medical Research, Medical Complex, Indira Nagar, Puducherry, Tamil Nadu, India

Preferential breeding habitats of sibling species complexes of *Anopheles fluviatilis* in east-central India

Sauvik Dasgupta, Smrutidhara Dash, Thankachy Sonia, Ashokkumar Mathivanan and Dr. Sudhansu Sekhar Sahu

Abstract
In this article, we examine the preferential breeding habitats of *Anopheles fluviatilis* at sibling species level in malaria endemic district of Koraput, Odisha State, East-Central India. The study included the collection and rearing of the larva from different breeding habitats in three terrains and identification of the emerged adults at morphological as well molecular level. Out of 150 *An. fluviatilis* emerged, 149 was sibling species T and only one was sibling species S. Sibling species T was found to be breeding mostly in streams (66.4%) followed by paddy-fields (28.9%). Ponds, mud-puddles and pits together contributed only 4.7% of sibling species T. Since, sibling species S has been incriminated as the main human malaria vector in the area; it is recommended that a longitudinal immature survey covering three seasons over a larger temporal and geographical range would be useful to find out the preferential breeding habitats of *An. fluviatilis* species S.

Keywords: *Anopheles fluviatilis*, breeding habitats, koraput, Odisha, sibling species

1. Introduction
Malaria is a major health problem in rural/tribal areas of the central, eastern and north-eastern States, particularly in 16 States including seven north eastern States and nine States of central India [1]. Odisha State on the east central India has been afflicted with a high incidence of malaria for many decades [2]. Malaria is highly complex in Odisha because of the State's vast tracts of forest with tribal settlements. The dynamics of malaria in the State vary from place to place. Odisha State having 3.8% of the total population of India accounts for 58.2% of *Plasmodium falciparum* cases and 33.7 % of all reported deaths due to malaria in the country during 2016 [1]. *Anopheles fluviatilis* James is considered to be the primary malaria vector in the malarious districts of Odisha State [3, 4]. It is essentially a species of hills and foot hills and is a vector of malaria in all areas wherever it is prevalent [4]. The ecological and geographical conditions of the State favour the persistence of *falciparum* malaria transmitted by *An. fluviatilis.* Among the present hard core malarious areas in Odisha, the hilly tracts of Koraput district contribute a significant number of *P. falciparum* cases and *An. fluviatilis* is mainly involved in transmission [5]. The presence of *An. fluviatilis* complexes S and T has been reported from the Koraput district and species S has been incriminated as the main human malaria vector [4].
Vector control continues to be the main stay of national malaria control programme, and the regions for its setback in the programme are too many [3]. Two rounds of indoor residual spraying with DDT since last six and half decades and distribution of LLINs from 2009 by the national programme have not produced the adequate level of *An. fluviatilis* control in Odisha State [3]. In such a situation, bioenvironmental method of vector control could be an alternative or supplementary option, wherever feasible [6]. Therefore, detailed information on behavior and bionomics of this vector is crucial for disease-threat analysis and for the development and implementation of vector control strategies [4]. Important among these is the breeding behaviour. *An. fluviatilis* had been extensively studied for its breeding habits and habitats [7]. This species was known to breed in habitats with perceptible flow of waters [6]. However, in spite of the well-known vectorial status of sibling species S of *An. fluviatilis*, no information on the preferential breeding habitats of its sibling species complexes was available in India. Hence,

this study was undertaken to find out the preferential breeding habitats of *An. fluviatilis* sibling species complexes in Koraput district of Odisha State where both the sibling species S and T were reported earlier.

2. Materials and Methods

2.1 Study area

Koraput district has a mottled topography encompassing varied terrains in it. This was a cross sectional study. The study was carried out in the tribal area of Laxmipur and Dasamantapur Community Health Centres (CHCs) of Koraput district, Odisha State (Fig. 1). These two CHCs were selected on the basis of random formula in MS-Excel to avoid bias. The villages selected were categorized into 3 types on the basis of the terrain, i.e., hill top, foot hill and plain. Three villages each from all the three terrains were selected randomly for immature survey at weekly intervals from April 2015 to May 2015. The details of the selected villages from different terrains were depicted in Table 1.

Table 1: Details of terrain and villages surveyed

Sl. No.	Terrain	No. of Villages Surveyed	Village Name	Location	Altitude
1.	Hill top	3	Barigaon	19.84501 N, 83.10541 E	2353 ft.
			Piskadanga	19.14993 N, 83.21694 E	2814 ft.
			Chuchukona	19.14658 N, 83.22251 E	2146 ft.
2.	Foothill	3	Kutuli	19.51190 N, 83.43831 E	2565 ft.
			Khalkona	19.32522 N, 83.81208 E	2653 ft.
			Talakutinga	19.08114 N, 83.17943 E	2293 ft.
3.	Plain	3	Bamansuku	18.87821 N, 82.85829 E	2864 ft.
			Bhejapadar	18.82770 N, 82.88729 E	2985 ft.
			L. Maliguda	18.84165 N, 82.82878 E	2798 ft.

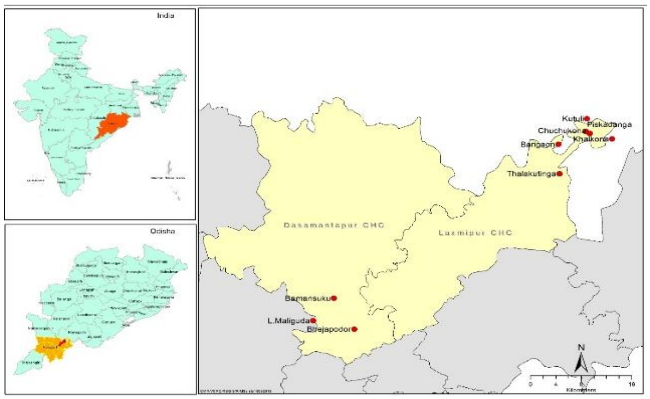

Fig 1: Map showing study area in Koraput district of Odisha State

2.2 Immature survey

The breeding habitats available in all the three terrains ranging from small mud puddles, pits, paddy fields, ponds, wells and streams were surveyed. Sequential sampling was done with the help of standard enamel larval dipper (10 cm diameter and 300 ml capacity) in all the habitats except well where bucket was used for the survey [8]. One dip was taken at every two meter intervals in streams and ponds along the edges and at one meter intervals in paddy fields along the fringes. Five dips were taken from the pit, while three bucket samples (2.5 lit capacities) were taken from the wells. One dip was taken from the mud puddles depending on their size. The number and types of breeding habitats surveyed in each village, total dips taken and the number of positive dips were also recorded. The number of habitats surveyed in different terrains is presented in Table 2. Since, this was a short term preliminary study, the first and second instars immature were not collected as these stages of immature would take long time for emergence. Only the third, fourth instars and pupae collected were brought to Vector Control Research Centre, Field Station laboratory located at Koraput and reared to adults and identified using the keys of Christophers [9].

The emerged *An. fluviatilis* were transferred to 1.5 ml eppendorf tubes with a hole punched on the lid for air circulation and were kept in a dry-bath for 2 hours and at 90 ^{0}C and were labeled. Their breeding habitats were also noted simultaneously. They were then kept in sealable plastic bags with a little silica gel to prevent fungal infections and kept for molecular identification of the sibling complex. Two legs were separated from the individual mosquito for DNA extraction and subsequent identification of sibling species was done using the molecular methods described by Manonmani *et al*. [10, 11].

Table 2: No. of habitats surveyed in different terrains

Terrain	Stream	Paddy field	Pond	Pit	Well	Mud-puddles
Hill top	3	6	1	0	0	10
Foothill	3	9	2	2	1	20
Plain	2	15	4	5	0	0

3. Results

3.1 Species composition

A total of 3562 adult emergence was recorded which belonged to two genera, *Anopheles* and *Culex.* Out of 3562, 2864 (80.4%) were anophelines and 698 (19.6%) were culicines. Among the anophelines, a total of 12 anopheline species were encountered breeding in different habitats. This included *An. fluviatilis* and *An. culicifacies*, the recognized vectors of malaria and *An. annularis, An. jeyporiensis* and *An. maculatus*, the known vectors of secondary importance in India. Among the anophelines, the most abundant one was *An. jeyporiensis* (29.4%) followed by *An. splendidus* (11.3%), *An. theobaldi* (11.1%), *An. culicifacies* (10.0%). *An. fluviatilis* constituted 5.2% and the remaining seven anopheline species *An. maculatus, An. vagus, An. annularis, An. subpictus, An. barbirostris, An. nigerrimus* and *An. tessellatus* constituted 33.0% of the total anophelines.

There was a variation in the availability of breeding habitats in all the study villages with respect to the terrain. In hill top villages, only four available breeding habitats namely streams,

paddy-fields, ponds and mud puddles were surveyed. Stream and paddy fields were found to be the preferential breeding habitats of *An. fluviatilis* in this terrain. A total of 2700 dips were taken from the streams and 429 anopheline immature were brought to the laboratory for emergence. Out of these, the number of *An. fluviatilis* emerged was 17 (Table 3). Molecular assay of *An. fluviatilis* showed that all the 17 *An. fluviatilis* belonged to sibling species, T. A total of 354 anopheline immature were collected from 1350 dips taken from the paddy fields and out of these, 13 *An. fluviatilis* were emerged and all the 13 *An. fluviatilis* belonged to sibling species, T (Table 3). In ponds, out of 450 dips, 175 anopheline immature were collected. Three *An. fluviatilis* were emerged which belonged to sibling species, T. In mud puddles, out of 76 immature collected from 90 dips, only one *An. fluviatilis* emerged, which belonged to sibling species, T. Thus, in hill top villages, a total of 34 *An. fluviatilis* emerged from 1034 anopheline immatures collected from 4590 dips. All the 34 *An. fluviatilis* were belonged to sibling species, T. No sibling species S was recorded from hill top terrain.

In foot hill villages, all the six breeding habitats were surveyed. Stream and paddy fields were found to be the preferential breeding habitats of *An. fluviatilis* in the foot hill villages also. A total of 2700 dips were taken from the streams and 464 anopheline immature were collected (Table 3). Out of these, the number of *An. fluviatilis* emerged was 80. Sibling species identification showed that, out of 80, only one was sibling species, S and the rest 79 were sibling species, T (Table 3). A total of 296 anopheline immature were collected from 2025 dips taken from the paddy fields and out of these, 29 *An. fluviatilis* were emerged. All the 29 *An. fluviatilis* belonged to sibling species, T. From pits, a total of 121 anopheline immature were collected from 90 dips and only one *An. fluviatilis* was emerged which belonged to sibling species, T. A total of 241, 157 and 199 anopheline immature were collected from 900, 180 and 27 dips, respectively from ponds, mud puddles and wells (Table 3). No *An. fluviatilis* emergence was observed from these three habitats. Thus, in foot hill villages, a total of 110 *An. fluviatilis* emerged from 1478 anopheline immature collected from 5922 dips. Out of 110 *An. fluviatilis*, one was sibling species S and all the rest 109 were sibling species T (Table 3).

In plain villages, streams, paddy fields, ponds and pits were surveyed. The other two breeding habitats were not found in plain terrain. Only few emergences were recorded from these breeding habitats. A total of 1800 dips were taken from the streams and 210 anopheline immature were collected. Out of these, the number of *An. fluviatilis* emerged was 3. Sibling species identification showed that, all the 3 were sibling species T. A total of 43 anopheline immature were collected from 1800 dips taken from ponds and out of these, 2 *An. fluviatilis* were emerged which belonged to sibling species, T. From paddy fields, a total of 90 anopheline immature were collected from 3375 dips and only one *An. fluviatilis* was emerged which belonged to sibling species T. A total of 9 anopheline immature were collected from 225 dips from the pits and no emergence was observed (Table 3). Thus, in plain villages, a total of 6 *An. fluviatilis* emerged from 352 anopheline immature collected from 7200 dips. All the six *An. fluviatilis* emerged were sibling species T.

Among the three terrains, maximum number of emergence was recorded from the foot hills. In all the terrains, streams were found to be the preferential breeding habitats of *An. fluviatilis.* Out of total 150 emergence, 100 (66.7%) emergence were from the streams and 43 (28.7%) were from paddy fields (Fig. 2).

3.2 Sibling species composition of *An. fluviatilis*

A total of 150 samples from all the terrain were subjected to the ITS2-PCR assay for determining the sibling species complex. The results revealed a composition of two sibling species complexes of *An. fluviatilis* i.e., S and T. Out of 150, only one *An. fluviatilis* collected from the foot hill stream was S and all other 149 were T. Combining the results of three terrains, it was observed that species T was 66.4% from the streams, 28.9% from the paddy fields, 3.3% from ponds and 0.7% each from mud-puddles and pits (Fig. 3). This showed that sibling species T is prevalent in the study area and its preferential breeding habitat is slow running streams followed by paddy fields.

Table 3: Emergence of sibling species of *An. fluviatilis* from different breeding habitats

Breeding Habitats	Terrain	No. of dips taken	Total immature	No. of *An. Fluviatilis* emerged	Sibling species	
					S	T
Streams	Hill top	2700	429	17	0	17
	Foot hill	2700	464	80	1	79
	Plain	1800	210	3	0	3
Paddy fields	Hill top	1350	354	13	0	13
	Foot hill	2025	296	29	0	29
	Plain	3375	90	1	0	1
Ponds	Hill top	450	175	3	0	3
	Foot hill	900	241	0	0	0
	Plain	1800	43	2	0	2
Pits	Hill top	NA	NA	NA		
	Foot hill	90	121	1	0	1
	Plain	225	9	0	0	0
Mud puddles	Hill top	90	76	1	0	1
	Foot hill	180	157	0	0	0
	Plain	NA	NA	NA		
Wells	Hill top	NA	NA	NA		
	Foot hill	27	199	0	0	0
	Plain	NA	NA	NA		
Total	Hill top	4590	1034	34	0	34
	Foot hill	5922	1478	110	1	109
	Plain	7200	352	6	0	6

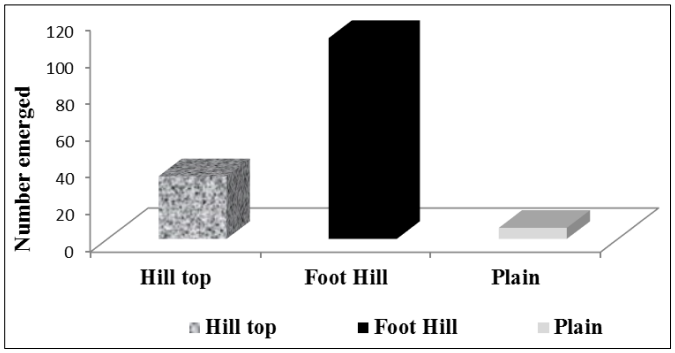

Fig 2: Emergence of *An. fluviatilis* sibling species T from all terrains

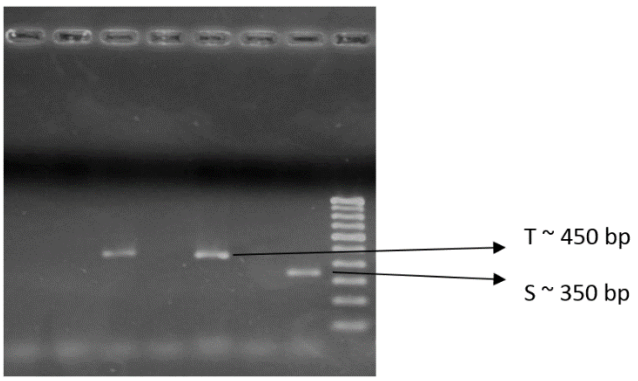

Fig 3: PCR gel plate showing sibling complexes (S & T) of *An. fluviatilis*

4. Discussion

An. fluviatilis is one of the primary malaria vectors in the hilly-forested regions of Southeast Asia [12, 13]. Despite a large number of studies over its range of distribution, it is difficult to have an overall view of its ecological and bionomical characteristics [5]. In the study area, *An. fluviatilis* was the incriminated vector of malaria [5]. The present study was undertaken in a hilly and forested area, which has been hyper-endemic for *falciparum* malaria since many decades [5]. The study villages are situated at an altitude range between 2000 ft to 3000 ft mean sea level and are of different ecotypes. Therefore, variations in density of the vector species occurred between the study villages [4]. In the current study area, streams, rivers, ponds and wells were the perennial breeding habitats and paddy fields, pits and mud puddles were seasonal breeding habitats. However, some paddy fields located in the low lying area and adjacent to the slow running streams behaves as perennial breeding habitats of the mosquitoes. The results of the current study showed that though *An. fluviatilis* breeds in all the habitats, the intensity of breeding was higher in streams followed by paddy fields. This agrees with earlier observation of Ramchandra Rao (1945) in North Kanara District [14]. There are controversial reports on the breeding of *An. fluviatilis* in paddy fields. While some reports showed the breeding of *An. fluviatilis* in paddy fields [14, 15], Covell and Harbhagwan (1939) stated that *An. fluviatilis* larvae were not found in paddy fields in Wynaad area [16]. Present study revealed that breeding of *An. fluviatilis* occurred in paddy fields in all the three terrains. In the study area, paddy cultivation is done in terraced stream beds, especially in hill top and foot hill ecotype and this association is the reason for maintaining perceptible flow of water which is the factor that has been reported to favour the breeding of *An. fluviatilis*.

The breeding potentials of all the available breeding habitats in three terrains were analysed and calculated. There was a variation in the availability of breeding habitats in all the study villages with respect to the terrain. As understood, ponds and pits were less in frequency with respect to hill top and foot hill terrains due to the undulation. The slope of the hill top villages surveyed were more with an average of 35^0 gradient, which was indeed very steep for any stagnant water sources to persist. The foothill villages were found to be having a wide array of breeding habitats starting from streams, paddy fields, wells and ponds to mud puddles. The number of paddy fields surveyed was less than compared to the plains. The form of agriculture is seasonal in the Koraput district [5, 7]. As they practice terrace farming, the fields only closest to the streams get regular supply of water than that of the other terraced fields which are only rain fed. Whereas, mud puddles were found to be absent in plain areas as due to the low-lying areas were flooded with the rain water during rainy season.

The emergence of *An. fluviatilis* was more from the foot hill villages (73.3%) compared to hill top (22.7%) and plain villages (4%), respectively. That is why; probably the adult density was more in hilly villages compared to plain villages as reported elsewhere [4]. The emergence of adults of *An. fluviatilis* from different habitats also signifies their individual variations in their productive capacity. The streams and paddy fields have been the two most productive habitats for this vector species, whereas the other habitats such as pond, mud puddles and wells also have a minimal contribution for their breeding.

An. fluviatilis ranks second to *An. culicifacies* in contributing to the total number of malaria cases in India [5, 13]. Its role in malaria transmission varies from place to place due to its existence as a complex of three sibling species named S, T and U [12], of which only species S is an efficient vector and highly anthropophagic whereas the other two species are zoophagic and considered as non-vectors [17]. Species S is considered as a principal vector of malaria in hilly and foothill regions of India because of its high density, high survival capacity and high human mosquito contact [12, 13, 18]. However in the current study, sibling species S was found to be very negligible (0.7%) among the emerged *An. fluviatilis*, hence the preferential breeding habitat of this sibling could not be identified. This could be due to the mass distribution of long lasting insecticide nets (LLINs) in the district during 2012 [19], as a result of which the pyrethroid susceptible population of *An. fluviatilis* S [20] which prefers to rest in human dwellings reduced to a great extent. As a result of which, out of 150 *An. fluviatilis* emergence, 149 (99.3%) were sibling species T (non vector), which prefers to rest in cattle sheds and does not come in contact with LLINs and predominantly zoophagic [12, 17]. This fact is reflected by the low malaria incidence prevailed in the study area during this period of the year [18]. The most preferential breeding habitats of the sibling species T was found to be streams followed by paddy fields.

There are several limitations in this study. The study was not covered all the three seasons. Study was conducted in summer season, which was not the favorable season for the target species. There was low abundance of water bodies as a result of which less number of *An. fluviatilis* immature was collected. This was a preliminary observation. Because of these limitations, the findings of this study should be interpreted cautiously. However, the strength of the manuscript is all the three terrains available in the district were covered and three villages in each terrain were surveyed, thereby all the terrains were well represented. The study was

conducted in a hard core malarious area in east-central India. Results obtained in the study clearly indicate the status of the sibling species composition of *An. fluviatilis* in summer season in the area. This is the first attempt in the country to study the breeding behavior of sibling species complexes of *An. fluviatilis.*

5. Conclusion

There are two important findings observed in the current study. *An. fluviatilis* sibling species T was predominant in summer season during the study period and the preferential breeding sites of species T is streams followed by paddy fields. However, the study could not establish the preferential breeding habitat of sibling species S which is the major malaria vector in the study area. With the results of this study, one would not come to a conclusion that species S is not available in this area because, it was a short term survey conducted in summer season. Currently, indoor residual spraying using DDT or synthetic pyrethroids (deltamethrin and alphacypermethrin) is the major vector control intervention measure being implemented in the districts of Odisha State. In addition, LLINs have been distributed in these districts. In spite of implementing these intervention measures, the population of *An. fluviatilis* in these areas is not controlled totally. Hence, it is pertinent to know the preferential breeding habitats of *An. fluviatilis* sibling species S, which plays major role in malaria transmission, in order to plan any larval control if required. The results of the study could be used to lead for further investigation to characterize the preferential breeding habitats of *An. fluviatilis* species S. Given the diversity and heterogeneity of larval habitats in forest environment available in the current study area, longitudinal investigations covering three seasons and involving systematic entomological surveys over a larger temporal and geographical range would be useful to establish the preferential breeding habitats of *An. fluviatilis* species S.

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