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Anopheles subpictus B and its role in transmission of malaria in Odisha, India

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Abstract. Odisha state is highly endemic for malaria and among several Anopheline species found in the state, *Anopheles subpictus* is known to be one of the most prevalent species. *An. subpictus* complex consists of four sibling species i,e A,B,C and D. However, no work has been conducted on prevalence of sibling species of *An. subpictus* in Odisha. Hence attempt was made to study the prevalence of sibling species of *An. subpictus* and their role in transmission of malaria in four districts i,e Angul, Khurda, Cuttack and Puri of Odisha using Mitochondrial Gene, Cytochrome C Oxidase subunit I (COI) known as DNA barcode. The sibling species B of *An. subpictus* with sporozoite were reported for the first time in Odisha.

INTRODUCTION

Anopheles subpictus Grassi belongs to the subgenus *Cellia* and the Pyretophorous Series (Harbach, 2004). It is a ubiquitous mosquito widely distributed in South East Asian Countries. In India it is found throughout the mainland and in Lakshdweep islands but not in Andaman and Nicobar Islands (Rao, 1984). This species has been reported as malaria vector in Sri Lanka (Amerasingha *et al.*, 1991; Premasiri *et al.*, 2005; Kusumawathie *et al.*, 2006). *Anopheles subpictus* s.l has been reported as vector of local importance in some parts of India (Paniker *et al.*, 1981; Kulkarni 1987; Chatterjee & Chandra, 2000; Kumari *et al.*, 2009). Based on the morphological differences in the egg of *An. subpictus* s.l (Reid., 1966) suggested that this might be a species complex while this taxon was found to be a complex of four sibling species A, B, C and D by examining polytene chromosomes from the ovaries of adult females (Suguna *et al.*, 1994). These sibling species are morphologically indistinguishable and could be identified only cytotaxonomically using polytene chromosomes specific to certain tissue in particular developmental stages. Hence identification tools that could identify mosquito species even from a small piece of tissue from any developmental stages would be important for the taxonomy of mosquitoes (Kumar *et al.*, 2007). The mitochondrial genome, Cytochrome C Oxidase subunit I (COI) called as DNA Barcode was selected for this approach owing to its advantages such as maternal lineage, lack of recombination, lack of "indels" and higher mutation rates (Saccone *et al.*, 1999). The Cytochrome C oxidase subunit I (COI) of mitochondrial gene is the most conserved gene in the amino acid sequences and hence has distinct advantage for studies (Knowlton & Weight, 1998). *Anopheles subpictus* s.l.was found to be the most abundant species among anophelines in several parts of India (Paniker, 1981; Kulkarni *et al.*, 1987). It was also a predominant species in Odisha (Dash *et al.*, 2000). According to Subbarao (1998) the

distribution of sibling species of *An. subpictus* s.l. should be mapped in all areas wherever it was found prevalent. As this species is among the most prevalent species in Orissa, study was carried out to find out the prevalence of sibling species of *An. subpictus* and their role in malaria transmission in Odisha.

MATERIALS AND METHODS

Sample Collection

Indoor resting mosquitoes were collected from human dwellings of high malaria endemic villages of four districts i,e Angul, Khurda, Cuttack and Puri of Odisha. *Anopheles subpictus* was identified using taxonomic keys of Christophers (1933). The head and thorax part were preserved in isopropyl alcohol for molecular analysis i,e sporozoite detection and sibling species identification using DNA barcodes (Cytochrome C oxidase I of mtDNA) as described by Kumar *et al.* (2007), while the abdomen part was kept in carnoy's fixative (Hunt & Coetzee., 1986) for cytotaxonomic study. Ovaries from individual semi-gravid females were pulled out and stored in modified Carnoy's fixative (1:3 glacial acetic acid and methanol) and ovaries were processed for the preparation of polytene chromosomes and inversions of polytene X-chromosome are used as diagnostic characters in the identification of sibling species. After pulling out the ovary, rest of the body parts were processed for identification of sibling species by bar coding method

DNA extraction

Total DNA from individual mosquitoes was extracted following a modified method proposed by (Collins *et al.*, 1987). The DNA was pelleted, dissolved in water and subjected to a Phenol chloroform isoamyl extraction followed by chloroform. Then DNA was precipitated using ethanol and dissolved in 50µl of deionised water.

Nested PCR for sporozoite detection

Amplification of genus and species specific *Plasmodium* was done by using the primers: rPLU5(5'CCTGTTGTTGCCTTAAACTTC3'), PLU6(5'TTAAAATTGTTGCAGTTAAAACG3'), rFAL1(5'TTAAACTGGTTTGGGAAAACCAA ATATATT3'), rFAL2(5'ACACAATGAACTCA ATCATGACTACCCGTC3'), rVIV1(5'CGCTT CTAGCTTAATCCACATAACTGATAC3') & rVIV2(5'ACTTCCAAGCCGAAGCAAAGAAA GTCCTTA-3') as described by Snounou *et al.* (1993a,b) and Mahapatra *et al.* (2006). Each 20 µl reaction mixture for nest-1 amplifications contained 12 µl of template DNA, 250 nM of each primer (rPLU5 & rPLU6), 4 mM $MgCL_2$, PCR buffer (50 mMKCl, 10mMTris-HCl), 200 µM of each dNTPs and 0.4 units of Taq DNA Polymerase. The PCR conditions (nest-1) were as follows: step-1: 94°C for 4 min; 94°C for 30 sec, 55°C for one min; extension at 72°C for one min; 35 cycles and final extension at 72°C for 4 min. About 8 µl of the nest-1-amplification products served as the DNA template for each of the 20 µl of second PCR (nest 2) amplification. The concentration of the nest 2 primers and other constituents were identical to nest-1 amplification, except that 0.3 unit of Taq DNA Polymerase was used. The second PCR (nest-2) amplification conditions were identical to those of first PCR (nest-1) except that the annealing temperature was 58°C for the species-specific primer. The PCR products were analyzed after electrophoresed in 1.5% agarose gel and stained with ethidium bromide.

Polymerase Chain Reaction (PCR) to amplify COI region of mitochondrial DNA

The DNA of eight samples of *An. subpictus* found positive for sporozoite were coded BF1 to BF8 and used to study the sibling species using barcode region (COI region of mitochondrial DNA). PCR was performed to amplify the 5' COI region of mitochondrial DNA was done by using the primers: Forward Primer: 5'-GGA TTT GGA AAT TGA TTA GTT CCT T-3' and Reverse Primer: 5'-AAA AAT TTT AAT TCC AGT TGG AAC AGC 3' as per

the protocol proposed by (Kumar *et al.*, 2007). Each 50 µl reaction mixture included 1.5 U of Taq DNA Polymerase , 5 µl of 10 X PCR buffer, 2.5 mM $MgCL_{2,}$ 2.5 µl of Q solution and 0.5 µl of 10 pmol each of forward and reverse primers along with the DNA of the mosquito samples. The PCR conditions were as follows: initial denaturation: 95°C for 5 min; followed by 35 cycles of 94°C for 40 sec (denaturation), 45°C for one min (annealing); and 72°C for one min (extention); and final extension at 72°C for 4 min. The PCR products were analyzed after electrophoresed in 1.5% agarose gel and stained with ethidium bromide.

DNA Sequencing

After performing PCR, the PCR products were electrophoresed in 1.5% agarose gel and stained with ethidium bromide to obtain amplified fragments. The PCR products were purified by Chromous RKT 34 purification kit and the purified products were eluted to 30 µl of deionized water and used for obtaining DNA sequences using automated DNA sequencer.

Bioinformatics Analysis

The annotated sequences reported from literature review (Kumar *et al.*, 2007) (DQ310145, DQ310146, DQ310147, DQ310149, DQ267688, AY729970, AY917203) were obtained from The European Bioinformatics Institute (EBI) (Sagliano *et al.*, 1998) with Sequence Retrieval System (SRS) (Etzold *et al.*, 1996) in FASTA (Pearson, 1990) format. As an initial approach for genetic characterization, the raw nucleotide sequences (BF1-BF8) and annotated sequences were threaded in CLUSTALW (Higgins *et al.*, 1996) application embedded in BioEdit (Hall *et al.*, 1999) for multiple alignment with default gap opening and gap extension parameters. Finally the alignment results were studied with Jalview 2.4 (Clamp *et al.*, 2004).

RESULTS AND DISCUSSION

A total of 844 *An. subpictus* were collected from Angul (294), Khurda (208), Cuttack (160) and Puri (182) districts of Odisha (Table 1). A total of 8 samples of *An. subpictus* were found positive for sporozoite (Table 1). In all the four districts *Plasmodium vivax* was detected while in three districts except Cuttack, *Plasmodium falciparum* was detected in samples of *An.subpictus* (Table 1). Out of 844 samples only 25 numbers were found in the semi gravid condition and were subjected to Cytotaxonomic method for identification of sibling species of which 19 samples were found to be species B and rest six could not be identified. Out of the 19 samples identified as sibling speices B, five samples were found positive for sporozoite and out of six unidentified three were positive for sporozoite. Hence all the eight samples of *An. subpictus* found positive for sporozoite were subjected to DNA isolation. The eight DNA samples were coded as BF1, BF2, BF3,

Table 1. Results of sporozoite detection in *An. subpictus* collected from study areas

District	No. of samples Tested	No. of positive samples detected	Parasite species detected		Sporozoite rate (%)
			Pf	Pv	
Angul	294	3	1	2	1.02
Khurda	208	2	1	1	0.96
Cuttack	160	1	0	1	0.62
Puri	182	2	1	1	1.09
Total	844	8	3	5	

Pf – *Plasmodium falciparum*
Pv – *Plasmodium vivax*

BF4, BF5, BF6, BF7 & BF8 (Table 2) which were then subjected to PCR for amplification of Cytochrome C Oxidase subunit I (COI) of mitochondrial DNA. The amplification of fragments was obtained at 720 bp (Fig. 1). The amplified products were used for sequence analysis of COI region of mitochondrial DNA of *An. subpictus* to study their sibling species status. The study showed that DNA sample BF1 collected from Khurda, BF3 and BF4 collected from Angul, BF5 and BF6 collected from Cuttack showed sequence similarities with accession number DQ310147 and DQ310149 which were reported as sibling species B by Kumar *et al.* (2007). Whereas the remaining sample of *An. subpictus* BF2 collected from Angul showed sequence similarities with accession number AY729970 while samples BF7 collected from Khurda and BF8 collected from Puri showed sequence similarities with accession number DQ267688 (Fig. 2). The accession number AY729970 and DQ267688 were reported to be *An. subpictus* s.l. by Kumar *et al.* (2007) (Fig. 2). Five out of eight samples (BF1, BF3, BF4, BF5 and BF6) of *An. subpictus* collected from Khurda, Angul and Cuttack were sibling species B, while the sibling species status of remaining three samples (BF2, BF7 & BF8) could not be detected. The inversion arrangements of samples BF1, BF3, BF4, BF5 and BF6 were observed as Xa,b i.e. species B. Hence the present study showed the prevalence of sibling species B of *An. subpictus* in the four districts selected for the study. Moreover samples BF1- BF8 were also found positive for sporozoites (Table 1 and 2). Hence it can be assumed that *An.subpictus* sibling species B found in

Table 2. Coding of sporozoite positive DNA samples of *An. subpictus*

Sl.no	Coding of DNA samples of *An. subpictus* found Positive for sporozoite	Area/ District
1.	BF1	Khurda
2.	BF2	Angul
3.	BF3	Angul
4.	BF4	Angul
5.	BF5	Cuttack
6.	BF6	Cuttack
7.	BF7	Khurda
8.	BF8	Puri

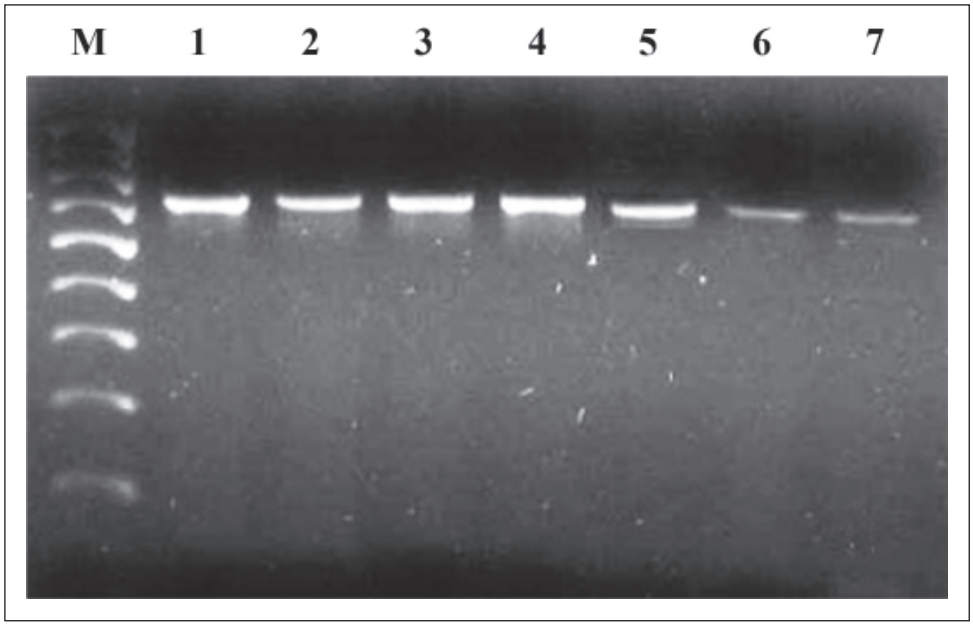

Figure 1. PCR Lane M: 100bp ladder, lane 1– 7: showing an amplification product of 720 bp for COI region of *An. subpictus*

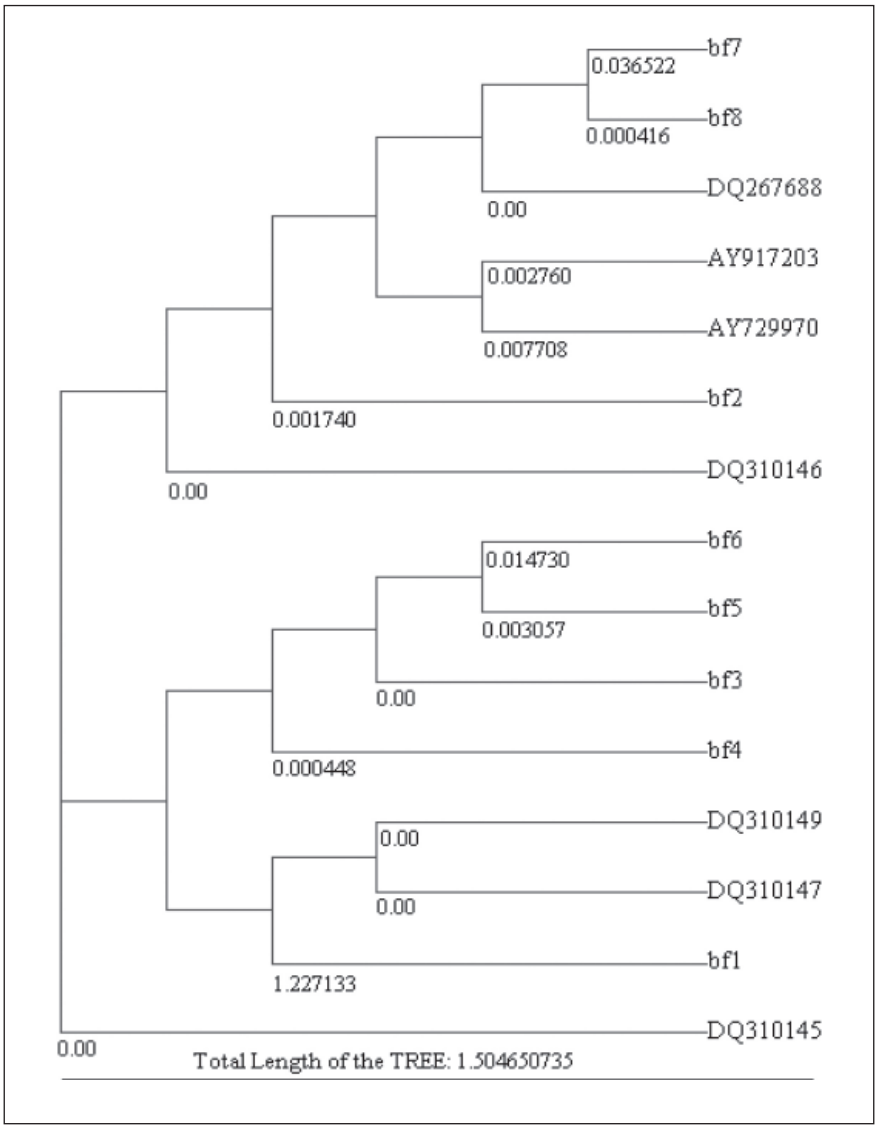

Figure 2. Rectangular Cladogram obtained from Jalview 2.4 by Neighbour Joining methods using % identity of aligned sequences and distances shown in the unit of 0.1

different regions of Orissa might be playing role in malaria transmission. Kumar *et al.* (2007) in their study used seven samples of *An. subpictus* which included four *sensu lato* specimens, two specimens as species B and one specimen identified as species A based on egg ridge number. The nucleotide diversity between the specimens identified as species A and species B was 11.3%, indicating them to be very distinct from each other. The *sensu lato* specimens matched with the clade of species A and hence could be the same species. Thus, study evinced that the DNA barcode approach could distinguish members of sibling species complexes in insects as reported by Harbach *et al.* (2004). Sibling species B of *An. subpictus* was predominant on the east coast of the Jaffna district and west coast of the Chilaw districts in the north western province of Sri Lanka (Surendran & Ramasamy, 2010). Species C was predominant in inland localities of Jaffna and Chilaw districts (Abhayawardana *et al.*, 1996, 1999a). The density of sibling species

A and D in the coastal areas was found to be low in comparison with the inland areas (Abhayawardana *et al.*, 1999a and b). Study carried out by Kannathasan *et al*. (2008) showed the predominance of sibling species B (67%) out of 409 samples of *An. subpictus.* Sibling species A, C, and D occur in freshwater habitats of inland areas, while the salt water species B occurs sympatrically with the others in coastal villages of Pondicherry, Southeast India (Suguna *et al.*, 1994). In our study samples identified to be sibling species B were found to be positive for sporozoites. Transmission of human malaria by sibling species B of *An. subpictus* was also reported by Paniker *et al.* (1981). Thus development of an appropriate molecular tool for distinguishing sibling species of *An. subpictus* will permit more detailed studies on entomological parameters such as longevity, fecundity, vectorial capacity, breeding rhythm, feeding and resting preferences, breeding habitats, susceptibility to insecticides etc. The findings from such studies will in turn yield a better understanding of the dynamics of malaria transmission in endemic localities (Surendran & Ramasamy, 2010).

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REFERENCES

Abhayawardana, T.A., Wickramasinghe, M.B. & Amerasinghe, F.P. (1999a). Sibling species of *Anopheles subpictus* and seasonal abundance in Chilaw area. *Proceedings of the Sri Lanka Association of Advance Science* **14**: 55.

Abhayawardana, T.A., Wickramasinghe, M.B. & Amerasinghe, F.P. (1999b). Indoor resting behavior of different sibling species of *Anopheles subpictus*. *Proceedings of the Sri Lanka Association of Advance Science* **55**: 17.

Abhayawardana, T.A., Wijesuria, S.R.E. & Dilrukshi, R.R.K.C. (1996). *Anopheles subpictus* complex: distribution of sibling species in Sri Lanka. *Indian Journal of Malariology* **33**: 53-60.

Amerasinghe, F.P., Amerasingha, P.H., Peiris, J.M.S. & Witz, R.A. (1991). Anopheline ecology and malaria infection during the irrigation development of an area of Mahawali project, Sri Lanka. *American Journal of Tropical Medicine and Hygiene* **45**: 226-35.

Chatterjee, S. & Chandra, G. (2000). Role of *Anopheles subpictus* as primary vector of malaria in an area in India. *Japanese Journal of Tropical Medicine and Hygiene* **28**(3): 177-181.

Christophers, S.R. (1933). *The Fauna of British India* – including Ceylon and Burma, Diptera.. Vol. 4 – Family Culicidae, Tribe Anopheline. Taylor and Francis, Landon. pp.271.

Clamp, M., Cuff, J., Searle, S.M. & Barton, G.J. (2004). The Jalview Java Alignment Editor, *Bioinformatics* **20**: 426-427.

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

Dash, A.P., Hazra, R.K., Mahapatra, N. & Tripathy, H.K. (2000). Disappearance of malaria vector *Anopheles sundaicus* from Chilika lake area of Odisha state in India. *Medical and Veterinary Entomology* **14**: 1-5.

Etzold, T., Ulyanov, A. & Argos, P. (1996). SRS: information retrieval system for molecular biology data base. *Methods in Enzymology* **266**: 114-128.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.

Harbach, R.E. (2004). The classification of the genus *Anopheles* (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. *Bulletin of Entomological Research* **94**: 537-553.

Higgins, D.G., Thompson, J.D. & Gibson, T.J. (1996). Using CLUSTAL for multiple sequence alignment. *Methods in Enzymology* **266**: 383-402.

Hunt, R.H. & Coetzee, M. (1986). Field sampling of anopheles mosquitoes for correlated cytogenetic and morphological studies. *Bulletin of the World Health Organisation* **64**: 896-900.

Kannathasan, S., Antonyrajan, A., Srikrishnaraj, K.A., Karunaratne, S.H.P.P., Karunaweera, N.D. & Surendran, S.N. (2008). Studies on prevalence of anopheline species and community perception of malaria in Jaffna district, Sri Lanka. *Journal of Vector Borne Diseases* **45**: 231-239.

Knowlton, N. & Weight, L.A. (1998). New dates and new rates for divergence across the Isthmus of Panama. *Proceedings of the Royal Society of London B Biological Sciences* **265**: 2257-2263.

Kulkarni, S.M. (1987). Feeding behavior of Anopheline mosquitoes in area endemic for malaria in Bastar District, Madhya Pradesh. *Indian Journal of Malariology* **24**: 163-171.

Kusumawathie, P.H.D., Wickremasinghe, A.R., Karunaweera, N.D., Wijeyaratne, M.J.S. & Yapabandara, A.M.G.M. (2006). Anopheline breeding in river bed pools below major dams in Sri Lanka. *Acta Tropica* 30- 33.

Kumar, N.P., Rajaval, A.R., Natarajan, R. & Jambulingam, P. (2007). DNA barcode can distinguish species of Indian mosquitoes (Diptera: Culicidae). *Journal of Medical Entmol*ogy **44**(1): 1-7.

Kumari, S., Parida, S.K., Marai, N., Tripathy, A., Hazra, R.K., Kar, S.K. & Mahapatra, N. (2009). Vectorial role of *Anopheles subpictus* and *Anopheles culicifacies* in Angul district of Orissa, India. *Southeast Asian Journal of Tropical Medicine and Public Health* **40**(4): 713-719.

Mahapatra, N., Marai,N.S., Ranjit, M.R. & Hazra, R.K. (2006). Detection of *Plasmodium falciparum* in Anopheles mosquitoes from Angul district, Orissa, India. *Journal of Vector Borne Diseases* **43**: 191-194.

Paniker, K.N., Geetha Bai, M., Bheema Rao, U.S., Viswam, K. & Suryanarayana, M.U. (1981). *Anopheles subpictus* vector of malaria in coastal villages of South East India. *Current Science* **50**: 694.

Pearson, W.R. (1990). Rapid and sensitive sequence comparison with FASTP and FASTA. *Methods in Enzymology* **183**: 63-98.

Premasiri, D.A., Wickremasinghe, A.R., Premasiri, D.S. & Karunaweera, N. (2005). Malarial vectors in an irrigated rice cultivation area in southern Sri Lanka. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **99**(2): 106-114.

Rao, T.R. (1984). *The Anophelines of India*. Malaria Research Centre, Indian Council of Medical Research, Delhi.

Reid, J.A. (1966). A note on *Anopheles subpictus* Grassi and *An. indefinitus* Ludlow (Diptera: Culicidae). *Journal of Medical Entomology* **3**: 327-331.

Saccone, C., Carla, G.D., Gissi, C., Pesole, G. & Renys, A. (1999). Evolutionary genomics in metazoa: the mitochondrial model DNA as a model system. *Gene* **238**: 195-210.

Sagliano, A., Volpicella, M., Gallerani, R. & Geci, L.R. (1998). *Nucleic Acids Research* **26**: 154-155.

Snounou, G., Pinheiro, L., Goncalves, A., Fonseca, L., Dias, F. & Brown, K.N. (1993a). The importance of sensitive detection of malaria parasites in the human and insect hosts in epidemiological studies, as shown by the analysis of field Sample from Guinea Bissau. Transaction of the *Royal Society of Tropical Medicine and Hygiene* **87**: 649-653.

Snounou, G., Vriyakosol, S., Jarra, W., Thaithong, S. & Brown, K.N. (1993b). Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Molecular Biochemical Parasitology* **58**: 283-292.

Subbarao, S.K. (1998). Anopheline species complexes in South-East Asia. World Health Organization, Technical Publication number 18, SEARO.

Suguna, S.G., Rathinam, K.G., Rajavel, A.R. & Dhanda, V. (1994). Morphological and chromosomal descriptions of new species in the *Anopheles subpictus* complex. *Medical and Veterinary Entomology* **8**: 88-94.

Surendran, S.N. & Ramasamy, R. (2010). The *Anophelese culicifacies* and *An. subpictus* species complexes in Sri Lanka and their implications for malaria for malaria control in the country. *Tropical Medicine and Health* **38**(1): 1-11.