Original Article

Evaluation of the mtDNA-COII Region Based Species Specific Assay for Identifying Members of the *Anopheles culicifacies* Species Complex

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Abstract

Background: *Anopheles culicifacies*, a major malarial vector has been recognized as a complex of five sibling species, A, B, C, D and E. These sibling species exhibit varied vectorial capacity, host specificity and susceptibility to malarial parasites/ insecticides. In this study, a PCR assay developed earlier for distinguishing the five individual species was validated on samples of *An. culicifacies* collected from various parts of India.

Methods: The samples were initially screened using the rDNA-ITS2 region based primers which categorised the samples into either A/D group or B/C/E group. A proportion of samples belonging to each group were subjected to the mtDNA-COII PCR assay for identifying individual species.

Results: Among the 615 samples analysed by rDNA-ITS2 PCR assay, 303 were found to belong to A/D group and 299 to B/C/E group while 13 turned negative. Among 163 samples belonging to A/D group, only one sample displayed the profile characteristic of species A and among the 176 samples falling in the B/C/E group, 51 were identified as species B, 14 as species C and 41 as species E respectively by the mtDNA-COII PCR assay. Samples exhibiting products diagnostic of B/C/E, when subjected to PCR-RFLP assay identified 15 samples as species E.

Conclusion: Validation of the mtDNA-COII PCR assay on large number of samples showed that this technique cannot be used universally to distinguish the 5 members of this species complex, as it has been designed based on minor/single base differences observed in the COII region.

Keywords: *Anopheles culicifacies*, COII PCR assay, rDNA-ITS2 PCR assay, Sibling species

Introduction

Anopheles culicifacies, an important mosquito species occurring in the Indian subcontinent, is reported as a complex of five sibling species, A to E. All of them except species B are vectors of malaria (Sunil et al. 2004). Though morphologically similar, members of the complex show ecological and behavioral differences which affect their vectorial capacity (Subbarao 1998). Identification of different members is possible based on the variable banding pattern in the X chromosome and chromosome arm 2, and also structural differences in the male mitotic karyotypes. But these observations require specifically staged specimens and hence cannot be applied to the entire sample collection. Several PCR assays were developed based on the sequences of the D2, D3 and ITS2 region of the ribosomal DNA, which were able to categorize the 5 members into two groups, viz, A/D group and B/C/E group. Recently, a three step PCR assay was developed by Goswami et al. (2006), based on the differences in the COII region of the mitochondrial DNA for identifying the individual members of this species complex. It was later reduced to a two step process by Manonmani et al. (2007), by replacing the ITS2 and RFLP steps by a single ITS2 group diagnostic assay.

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This paper presents the findings of evaluation of this two step PCR assay using field specimens of *An. culicifacies* collected from various parts of India.

Materials and Methods

Mosquito collection

Adult samples of *An. culicifacies* were collected from Malkangiri District, Orissa State, Alwar District, Rajasthan State, Tiruvanamalai and Ramanathapuram districts, Tamil Nadu state (Fig. 1) (Table 1). Daytime resting mosquito collections were done between 6:00 to 8:00AM using aspirators, while all night collections were done between 6:00PM to 6:00AM by using light traps (Gunasekaran et al. 1994). The collections were done from human dwellings and cattle sheds. Samples of *An. culicifacies* from all the catches were identified based on the taxonomic keys of Christophers (1933).

DNA extraction and PCR assays

DNA was extracted from the thoracic region of each individual mosquito sample using Genelute mammalian genomic DNA extraction kit (Sigma, St. Louis, MO, USA). The DNA pellet was dissolved in 50µl of TE (Tris/EDTA) buffer and stored at -20 °C. Two PCR reactions were carried out on each DNA sample. Using the rDNA-ITS2-PCR assay described by Manonmani et al. (2007), samples were initially grouped either as A/D or B/C/E. These samples were then subjected to mtDNA-COII PCR assay reported by Goswami et al. (2006). Samples grouped as A/D were subjected to the AD-PCR assay while those grouped as B/C/E were subjected to the BCE-PCR assay. Details related to the primers used in these PCR assays and the expected products are given in Table 2. The amplified products were electrophoresed on an ethidium bromide stained 1.5% agarose gel, along with DNA molecular weight marker (Genei, Bangalore, India) and visualized under UV light.

Amplification of the COII region

The COII region of the mtDNA for 5 individuals each of species B, C and E, identified cytologically was amplified using a pair of primers, COIIF: 5'-AGAGCTTCTCCTTTAA TGGAACA-3' and COIIR: 5'-CAATTGGTA TAAAACTATGATTTG-3' respectively. The reaction mixture comprised 4.5 mM $MgCl_2$, 200 µM of each dNTP's (GE healthcare UK limited, Buckinghamshire, UK), 10 µM of each primer (Metabion, Martinsried, Germany), 2.5 µl of Taq buffer and 2 units of Taq polymerase (Finnzyme, Espoo, Finland). To this mixture was added 1/100th of the DNA from a whole mosquito and the reaction mixture was made up to 25 µl volume using deionized water. These reactions were amplified in a Bio-Rad cycler PCR machine (Biorad, California, USA). PCR conditions were: denaturation at 94 °C for 4 min. followed by 40 cycles of denaturation at 95 °C for 40 sec, annealing at 50 °C for 1 min, extension at 68 °C for 1 min and a final extension step at 72 °C for 10 min.

After checking a portion of each amplification product by gel electrophoresis, the remainder of the sample was purified using Qiaquick PCR purification kit (Qiagen, Hilden, Germany). The concentration of DNA was quantified and subjected to sequencing in an ABI automatic sequencer (Applied Biosystems, California, USA), using the amplification primers to obtain sequence from both strands.

PCR-RFLP

Samples which exhibited products that are diagnostic of species B, C and E were subjected to COII PCR assay followed by DdeI digestion, which is reported to seperate species E from B and C (Goswami et al. 2005). Each 20µL digest consisted of 17.5µL PCR product, 2µL 10x Buffer and 0.5 U of DdeI enzyme (Sigma, St. Louis, MO, USA). For each sample, paired control reactions were set up that

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contained only PCR product and 10x buffer. Reactions were incubated at 37 °C overnight, followed by heat inactivation at 65 °C for 10 minutes. Digested and undigested PCR products were electrophoresed and observed as mentioned earlier.

Results

The results of the validation of the rDNA-ITS2 PCR assay and mtDNA-COII PCR assay is given in Table 3. Collections from Tiruvanamalai District of Tamil Nadu represented individuals belonging to BCE group in major proportion (86.8%) while those from Alwar District of Rajasthan represented individuals belonging to AD group in major proportion (87.7%) (Table 3). All the *An. culicifacies* collections from Malkangiri district of Orissa and Ramanathapuram District of Tamil Nadu belonged to the BCE group. Thirteen (2.1%) of the samples did not belong to either of the groups. About half the number of samples from each group, representing different types of collections/ habitats/ villages were randomly selected and subjected to the COII-PCR assay developed by Goswami et al. (2006). Among the 163 samples from Tiruvanamalai and Alwar districts, belonging to the AD group, one was found to be species A, 160 were species D and 2 belonged to neither. The single sample belonging to species A originated from Tiruvanamalai District. The samples which belonged to the BCE group when subjected to the species diagnostic mt-DNA PCR assays showed that all the 3 sibling species were present in the 4 districts, though species C was seen in lesser numbers in Tiruvanamalai, Alwar and Malkangiri districts respectively. However, samples exhibiting products diagnostic of all the 3 species were found in sizeable numbers (36.4%) (Fig. 2).

Sequencing of the mtDNA-COII region showed that polymorphism was noticed in the nucleotide positions which were diagnostic. With primer CR designed to amplify species C, it was noticed that the base ’A’ at position 171 which is the only diagnostic point differentiating species C from B was found to be ’G’ in all the species C samples sequenced by us (Fig. 3). With primer ER, the diagnostic point ‘T’ and ‘C’ in species B and E was found to be ‘T’ in one of our samples belonging to species E (Fig. 4). With the primer BCR, the only diagnostic point ’G’ at position 334 differentiating species B and C from E was found to exhibit polymorphism by being either ’G’ or ’A’ (Fig. 5).

Samples which exhibited products diagnostic of species B, C and E were subjected to the PCR-RFLP assay. Amplification of the mtDNA-COII region gave a band of size 573bp. This product, when subjected to digestion with the restriction enzyme, DdeI produced 3 fragments of sizes 300, 156 and 117bp respectively for species E and 2 fragments of sizes 456 and 117bp respectively for species B and C. These fragments were as per the predicted restricted sites of this enzyme. However, only 15 samples out of 64 yielded fragments corresponding to E, 15 produced fragments corresponding to species B and C and the products of 34 samples remained uncut (Table 3).

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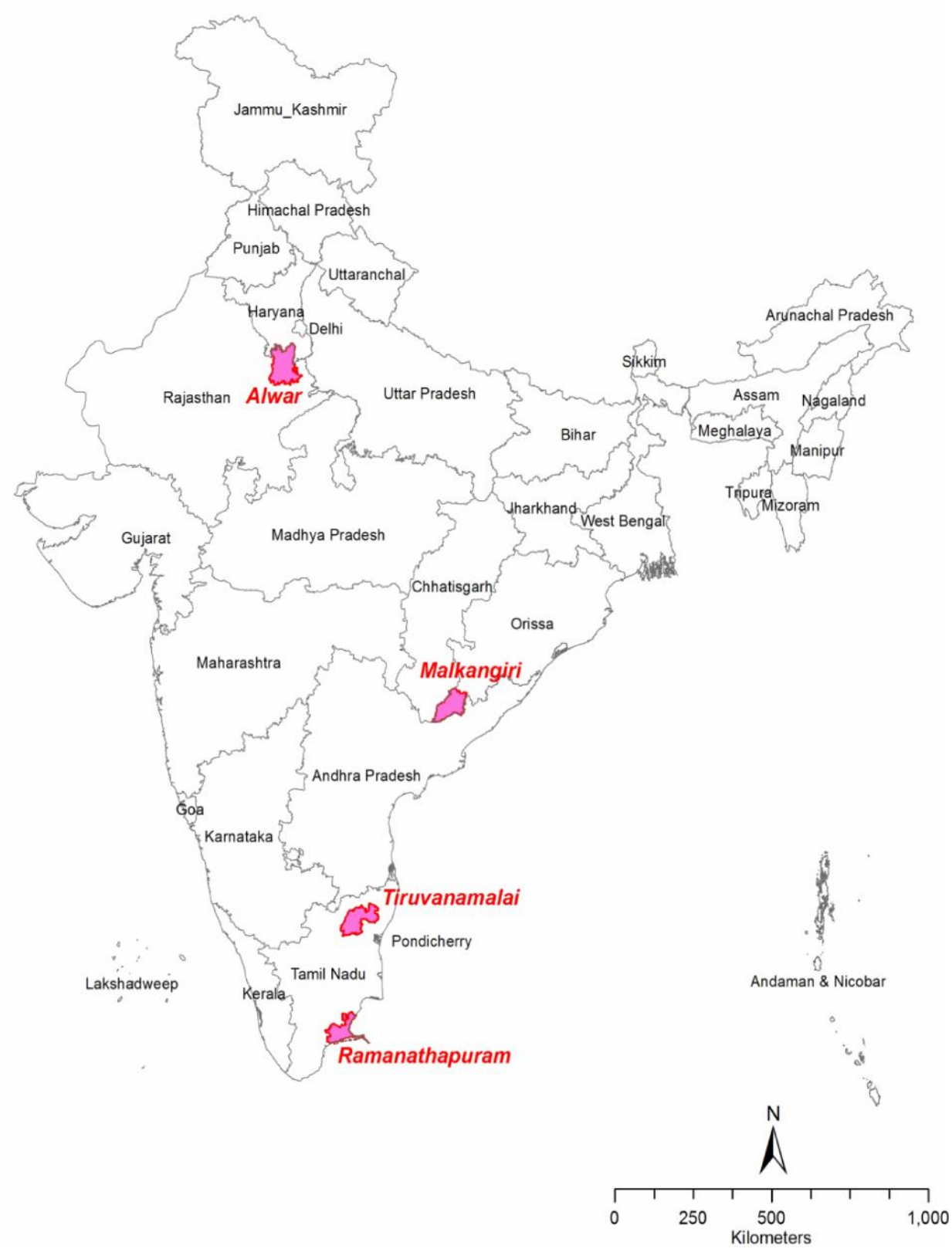

Fig. 1. Map showing the collection sites of *Anopheles culicifacies s.l.*

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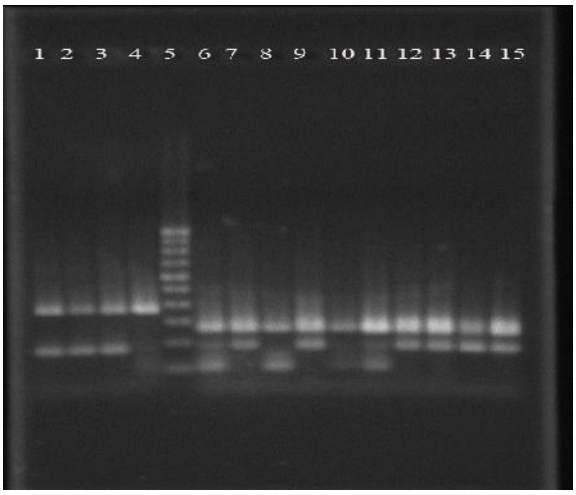

Fig. 2. COII species diagnostic PCR assay for *Anopheles culicifacies*. Lane 1–3: sibling D (359bp+166bp), Lane 4: sibling A (359bp), Lane 5: 100bp ladder, Lane 6, 11: B/C/E (248bp+178bp+95bp), Lane 8: sibling C (248bp+95bp), Lane 10: sibling B (248bp), Lane 7, 9, 12–15: sibling E (248bp+178bp).

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AJ518810 B India TTTAGA ATTTGATTCT TAC 273
HQ377221 B India ...... .......... ... 273
HQ377222 B India ...... .......... ... 273
HQ377223 B India ...... .......... ... 273
COXIIAcSL-B1     C..... .......... ... 273
AJ519493 C India ...... .......... ... 273
HQ377224 C India ...... .......... ..T 273
HQ377225 C India ...... .......... ... 273
AJ534646 E India C..... .......... ... 273
HQ377226 E India ...... .......... ... 273
HQ377227 E India C..... .......... ... 273
HQ377228 E India C..... .......... ... 273
COXIIAcSL-E1     C..... .......... ... 273
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Fig. 4. ER primer site of the mtDNA-COII sequences for species B, C and E of the *Anopheles culicifacies* complex. Accession numbers AJ518810, AJ519493 and AJ534646 (Goswami et al. 2006). Accession numbers COXIIAcSL-B1 and COXIIAcSL-E1 (Surendran et al. 2006). Accesssion numbers HQ377221-HQ377228: mtDNA COII sequences of species B, C and E generated in the current study.

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AJ518810 B India GGACGAAATT AATACTCCTT 190
HQ377221 B India G......... .......... 190
HQ377222 B India G......... .......... 190
HQ377223 B India G...C..... .......... 190
AJ519493 C India A......... .......... 190
HQ377224 C India G......... .......... 190
HQ377225 C India G......... .......... 190
```

Fig. 3. CR primer site of the mtDNA-COII sequences of species B and C of the *Anopheles culicifacies* complex. Accession numbers AJ518810 and AJ519493 Goswami et al. (2006). Accession numbers HQ377221-HQ377225: mtDNA-COII sequences of B and C generated in the current study.

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AJ518810 B India GTTGTTT TACCAATAAA TAA 353
HQ377221 B India ....... .......... ... 353
HQ377222 B India A...... .......... ... 353
HQ377223 B India A...... .......... ... 353
COXIIAcSL-B1     A...... .......... ... 353
AJ519493 C India ....... .......... ... 353
HQ377224 C India A...... .......... ... 353
HQ377225 C India A...... .......... ... 353
AJ534646 E India A...... .......... ... 353
HQ377226 E India A...... .......... ... 353
HQ377227 E India A...... .......... ... 353
HQ377228 E India A...... .......... ... 353
COXIIAcSL-E1     A...... .......... ... 353
```

Fig. 5. BCR primer site of the mtDNA-COII sequences for species B, C and E of the *Anopheles culicifacies* complex. Accession numbers AJ518810, AJ519493andAJ534646 (Goswami et al. 2006). Accession numbers COXIIAcSL-B1 and COXIIAcSL-E1 (Surendran et al. 2006). Accession numbers HQ377221-HQ377228: mtDNA COII sequences of species B, C and E generated in the current study.

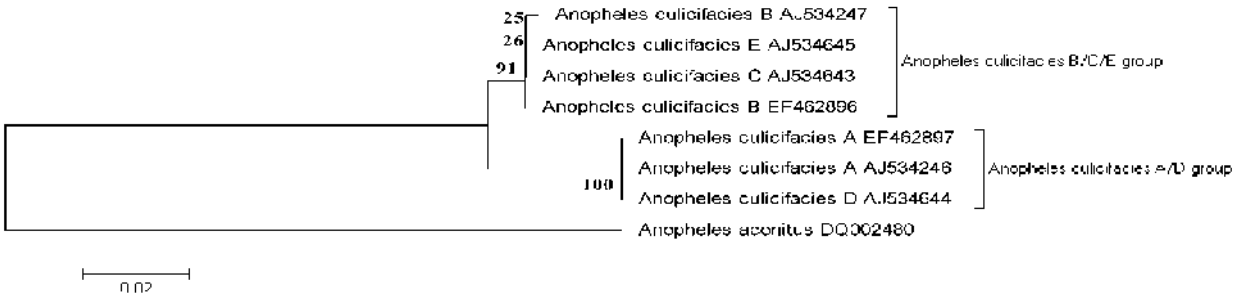

Fig. 6. ITS2 region based phylogenetic tree (Neighbor joining method) for *Anopheles culicifacies* showing two distinct clades of A/D and B/C/E with an out group *Anopheles aconitus*

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Table 1. Details of collected *Anopheles culicifacies* samples

District and State	Geographical co-ordinates	Village	Habitat	Type of collection	No.
Tiruvanamalai Tamilnadu	Lat: 11°55'–13°15'N Long: 78°20'–79°50'E	Kolamanjanur	Cattle shed	Light trap	3
		Kolamanjanur	Cattle shed	Hand catch	141
		Kolamanjanur	Human dwelling	Hand catch	34
		Labour colony	Cattle shed	Hand catch	22
		Labour colony	Human dwelling	Hand catch	5
Ramanathapuram Tamilnadu	Lat: 9°05'–9°50'N Long: 78°10'–79°27'E	Karaiyur	Human dwelling	Hand catch	13
		Rajakoil	Human dwelling	Hand catch	11
		Tharavaithoppu	Human dwelling	Hand catch	36
Alwar Rajasthan	Lat: 27°03'–28°14'N Long: 76°07'–77°13'E	Balana	Cattle shed	Hand catch	20
		Balana	Human dwelling	Hand catch	27
		Lalpura	Cattle shed	Hand catch	109
		Lalpura	Human dwelling	Hand catch	101
		Indok	Cattle shed	Hand catch	2
		Sawar	Cattle shed	Hand catch	40
		Sawar	Human dwelling	Hand catch	25
Malkangiri Orissa	Lat: 17°45'–18°40'N Long: 81°10'–82°00'E	Teakguda	Cattle shed	Hand catch	26
Total samples collected					615

Lat: Latitude, Long: Longitude

Table 2. Details of primers used in PCR assays

S. No	Primer Name/ Sequence (5'-3')	Tm value	GC Content	PCR product size (bp)
1	**5.8S -** ATCACTCGGCTCATGGATCG	60	55	AD group: 409 BCE group: 253
2	**Sp AD -** CAGTGCTGCAAACCACCACTTAT	64	45.8	
3	**Sp BCE -** TGTTAGTAGGCTGCCGGGGTTC	66	59.1	
4	**ADF** - CTAATCGATATTTATTACAC	48	25	Species A: 359 Species D: 359+166
5	**ADR** - TTACTCCTAAAGAAGGC	48	41.2	
6	**DF** - TTAGAGTTTGATTCTTAC	45	27.8	
7	**BCEF** - AAATTATTTGAACAGTATTG	46	20	Species B: 248 Species C: 248+95 Species E: 248+178
8	**BCR** - TTATTTATTGGTAAAACAAC	46	20	
9	**CR** - AAGGAGTATTAATTTCGTCT	49	31.6	
10	**ER** - GTAAGAATCAAATTCTAAG	47	26.3	

1–3: Primers for rDNA-ITS2 group diagnostic PCR assays (Manonmani et al. 2007)
4–10: Primers for mtDNA-COII species diagnostic PCR assays (Goswami et al. 2006)

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Table 3. Evaluation of PCR assays for identification of individual members of *Anopheles culicifacies*

Area	rDNA-ITS2 PCR assay				mtDNA-COII PCR assay											PCR-RFLP	
					A/D			B/C/E									
	Total collected	A/D group	B/C/E group	-ve	Numbers processed	A	D	-ve	Numbers processed	B	C	E	B/C/E	-ve	B/C/E	E	B/C
Tiruvanamalai	205	19	178	8	19	1	17	1	81	25	3	17	32	4	32	7	7
Ramanathapuram	60	0	58	2	0	0	0	0	43	15	9	7	11	1	11	3	1
Alwar	324	284	38	2	144	0	143	1	27	4	1	10	12	0	12	4	1
Malkangiri	26	0	25	1	0	0	0	0	25	7	1	7	9	1	9	1	6
Total	615	303	299	13	163	1	160	2	176	51	14	41	64	6	64	15	15

-ve = negative

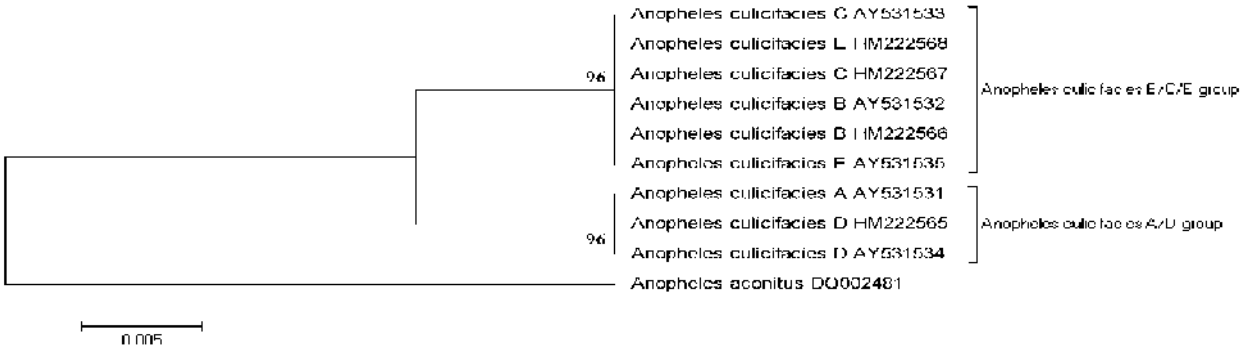

Fig. 7. D3 region based phylogenetic tree (Neighbor joining method) for *Anopheles culicifacies* showing two distinct clades of A/D and B/C/E with an out group *Anopheles aconitus*

Discussion

Accurate identification and knowledge about the distribution of vector and non-vector members of species complexes is required for planning effective control measures. Hence, development of molecular diagnostics for the individual species of the *An. culicifacies* complex was felt necessary as 4 of these species have been incriminated as vectors of malaria in India. Goswami et al. (2006) have developed a three step PCR assay for the identify cation of 5 individual members of this species complex, which involved (1) amplification of the rDNA-ITS2 region, (2) restriction digestion of the ITS2 product and (3) PCR assay based on the COII region of the mtDNA. This three step PCR assay was later reduced by developing a PCR assay based on the rDNA-ITS2 region (Manonmani et al. 2007) which grouped the 5 species into the same 2 categories as obtained after the first two steps of Goswami et al. (2006). In the present study this two step PCR assay was evaluated on

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samples of *An. culicifacies* collected from different regions of India. Of the 615 samples evaluated, 49.3% were categorized as A/D while 48.6% were B/C/E. Selected samples (163) from the A/D group when subjected to mtDNA-COII PCR assay showed that 0.6% were species A and 98.2% were species D. Likewise, when 176 samples belonging to the B/C/E category were analyzed, 29%, 8% and 23.3% exhibited products diagnostic for species B, C and E respectively, while 28% exhibited products diagnostic of all the 3 species.

The specificity of the COII-PCR assay in identifying the individual member within the 2 groups was low. This can be attributed to single base differences in the COII region which has been used for the design of species diagnostic assay. Similar problem was encountered when single base differences of the rDNA-ITS2 region was used for designing PCR assay for species A and C of the *An. minimus* complex (Garros et al. 2004). The major role played by members of the *An. culicifacies* complex in malaria transmission in several regions of India has compelled the authors to use the minor differences in designing these primers. Hence, the basic requirements such as 18–24 nucleotide length, Tm value between 56–62 and GC content between 45–60% could not be met while designing the 7 species diagnostic primers (Table 3). GC content, melting and annealing temperatures being dependent on one another play a crucial role in the efficiency and sensitivity of PCR assays (Rychlik et al. 1990, Dieffenbach et al. 1993, He et al. 1994).

In the case of AD-PCR assay, species A should exhibit a single product of size 359bp while species D should produce 2 products of size 359 and 166 bp respectively. But in this study, all samples produced products characteristic of species D. Goswami et al. (2006), while evaluating this assay found that the AD-PCR results did not agree with the earlier reports on the distribution of these members in Kheda and Sonepat, India. They attributed this to the polymorphic nature of the i^1 inversion in species A in these areas and hence samples collected from these regions, whether homozygous or heterozygous for this inversion, have been identified as species D. This might be the reason for all samples being identified as species D in our study also. The i^1 homozygotes could be species D or polymorphic forms of species A. Hence, the AD-PCR assay will not be useful in areas where the i^1 inversion is polymorphic. Vasantha et al. (1991) using i^1 inversion for detecting species D found that in certain areas, presence of species D becomes evident only due to the deficiency of heterozygotes. Hence, it appears that cytotaxonomy also, cannot be used universally in the identification of species A and D of this species complex.

In our study, sequencing of the mtDNA-COII region for species B, C and E showed polymorphism in the nucleotide positions used for designing of diagnostic primers. Similar observations were made by Surendran et al. (2006) while characterizing species B and E of this species complex from Srilanka. Though they observed acrocentric and submetacentric Y chromosomes in species B and E as reported by Kar et al. (1999), they were not able to correlate these results with those obtained by the PCR-RFLP assay (Goswami et al. 2005). This was because several bases of the COII region were found to exhibit polymorphism. In particular, the base 'C' at position 255 in the COII gene which is the only diagnostic point differentiating species E from B as well as serving as the restriction site for the enzyme, Dde I, itself exhibited polymorphism. This base which was 'T' and 'C' in species B and E respectively, in the samples studied by Goswami et al. (2006) was found to be 'C' in both the species from Srilanka. Hence, the species diagnostic PCR assay and PCR-RFLP cannot be used with 100 % accuracy for distinguishing species B and E.

While using cytotaxonomical methods for identifying species B and C of the *An.*

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culicifacies complex, B/C heterozygotes were seen in forested and deforested villages of Northern Orissa, which represented interspecific hybrids obtained due to breakdown of premating barriers between species B and C (Nanda et al. 2000).

Phylogenetic analysis of the ITS2 and D3 region sequences (Figs. 6, 7) showed that the 5 members fell into 2 clades, one representing species B, C and E and the other representing species A and D. Similar categorization has been made by Dassanayake et al. (2008) and Raghavendra et al. (2009) based on the sequences of the ITS2 and D2 regions of the rDNA respectively. Hence, species A and B of *An. culicifacies* reported by Green and Miles (1980) appears to be the two distinct sibling species of this malaria vector while the others namely species D and species C and E seems to have diverged very recently from species A and species B respectively. Further, the fact that the members within the 2 groups have not yet developed post mating barriers (Raghavendra et al. 2009) shows that the speciation process among the members of the 2 groups is not yet complete.

The present study has shown that the mtDNA-COII PCR assays cannot be used universally to distinguish the members of *An. culicifacies* complex, as has been observed by Surendran et al. (2006) in Srilanka as well.

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