An allele-specific polymerase chain reaction assay for the differentiation of members of the *Anopheles culicifacies* complex

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Anopheles culicifacies, the principal vector of malaria in India, is a complex of five cryptic species which are morphologically indistinguishable at any stage of life. In view of the practical difficulties associated with classical cytotaxonomic method for the identification of members of the complex, an allele-specific polymerase chain reaction (ASPCR) assay targeted to the D3 domain of 28S ribosomal DNA was developed. The assay discriminates *An. culicifacies* species A and D from species B, C and E. The assay was validated using chromosomally-identified specimens of *An. culicifacies* from different geographical regions of India representing different sympatric associations. The assay correctly differentiates species A and D from species B, C and E. The possible use of this diagnostic assay in disease vector control programmes is discussed.

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1. Introduction

Anopheles culicifacies is one of the major vectors of malaria in India responsible for approximately 65% of total malaria cases (Sharma 1998). The *An. culicifacies* complex comprises five cryptic species, provisionally designated as species A, B (Green and Miles 1980), C (Subbarao *et al* 1983), D (Vasantha *et al* 1991) and E (Kar *et al* 1999). Variations in the biological characteristics exist among members of *An. culicifacies* complex, most importantly in malaria transmission efficiency (Subbarao *et al* 1988a 1992), susceptibility to plasmodial infections (Adak *et al* 1999, Kaur *et al* 2000) and insecticide resistance (Subbarao *et al* 1988b, Raghavendra *et al* 1991, 1992) that have important bearing in planning effective vector-control strategies. Among the members of the complex, species B is the poor or non-vector (Subbarao *et al* 1988a, 1992). This is primarily due to inherent poor susceptibility of species B to malaria sporogony as demonstrated in laboratory feeding experiments on three members of *An. culicifacies* complex, species A, B and C (Adak *et al* 1999; Kaur *et al* 2000). Therefore it is imperative to identify *An. culicifacies* at cryptic species level to discriminate vector species from non-vector in any of the vector control programmes.

The members of *An. culicifacies* are classically identified on the basis of fixed paracentric inversions present in chromosome-X and chromosome arm-2 detectable in polytene chromosome complement (Subbarao 1998). However, species B and E being homosequencial for polytene chromosomes, are distinguished on the basis of Y-chromosome karyotype of F_1 male (larvae) progeny (Kar *et al* 1999). It is difficult to carry out cytological identification of members of *An. culicifacies* complex in disease control programme due to the requirement of highly skilled technical personnel and applicability of this technique to half-gravid female mosquitoes only. Among the alternative methods available, isozyme analysis of lactate dehydrogenage (*ldh*) differentiates species A and D from species B and C (Adak *et al* 1994) with 94·6% specificity. DNA probe hybridization assay – based on highly repetitive DNA sequences – (Gunasekara *et al* 1995) was

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able to distinguish species A from species B/C when single mosquito-extract was diluted to 1/200. However such hybridization assay, which is based on difference in copy number across species, is sensitive to variation in amount of template DNA loaded and can be unreliable (Krzywinski and Besansky 2003). Identification by cuticular hydrocarbon analysis (Mulligan *et al* 1986) was not promising due to intraspecific variability in field samples (Subbarao 1988).

Polymerase chain reaction (PCR) and PCR-based assays have emerged as reliable and sensitive method for the differentiation of closely related species or cryptic members of the species complex. The ribosomal DNA (rDNA) regions are usually used for such PCR-based species-diagnostic assays (Collins and Paskewitz 1996). In the present study we report an ASPCR assay targeting D3 domain of 28S-rDNA which differentiates species A and D from species B, C and E.

2. Experimental procedures

2.1 *Mosquito collection and processing*

Adult female *An. culicifacies* were collected from different parts of India with different sympatric associations with respect to sibling species (table 1). In addition to field collected mosquitoes, laboratory reared species A, B and C were also used. The method of collection of mosquitoes and their processing were as described by Singh *et al* (2004), except for mosquitoes collected from district Ramanathapuram, where individual mosquitoes were allowed to lay eggs separately and isofemale larval progeny was obtained (Kar *et al* 1999). Following oviposition, the mosquitoes were re-fed on rabbit, allowed to attain semi-gravid condition, and subsequently ovaries were removed. The ovaries of mosquitoes from all localities were processed for polytene chromosome plates

Table 1. Result of ASPCR assay of field-collected and laboratory-reared *An. culicifacies* and their correlation with result of cytotaxonomy.

Locality (longitude and latitude)	Result of ASPCR	Result of cytotaxonomy				
		A	D	B	C	E
Udaipur, Rajasthan (24°N, 73°E)	A/D	6				
	B/C/E			6		
Hardwar, Uttaranchal (30°N, 78°E)	A/D					
	B/C/E			10		
Sonepat, Haryana (28°N, 77°E)	A/D	3				
	B/C/E			2		
Nadiad, Gujarat (22°N, 72°E)	A/D	14				
	B/C/E			5		
Allahabad, Uttar Pradesh (25°N, 81°E)	A/D	21				
	B/C/E			18		
Jabalpur, Madhya Pradesh (23°N, 79°E)	A/D		25			
	B/C/E				8	
Ranchi, Jharkhand (23° N, 85°E)	A/D					
	B/C/E			14		
Gulberga, Karnataka (17°N, 76°E)	A/D	10				
	B/C/E			8		
Ramanathapuram, Tamil Nadu (09°N, 79°E)	A/D					
	B/C/E			5		23
Laboratory cyclic colonies	A/D	10				
	B/C/E			10	10	
Total	A/D	64	25			
	B/C/E			78	18	23

(Green and Hunt 1980) and identified to cryptic species by species-specific paracentric inversions present in X-chromosome and chromosome arm-2 (Subbarao 1998). For differentiation of species B and E in Ramanthapuram population, metaphase karyotype of Y chromosome from neurogonial cells of F_1 male larvae (IV instar) was examined (Kar *et al* 1999). Genomic DNA was extracted from rest of the body (Coen *et al* 1982) and stored at 4°C. DNA was also isolated from other anophelines – *An. fluviatilis* species S, T and U; *An. minimus* species A; *An. stephensi*; and *An. subpictus*.

2.2 *PCR amplification and DNA-sequencing*

The targeted region, D3 domain of 28S rDNA, was amplified by PCR using universal primers, D3A (5′-GAC CCG TCT TGA AAC ACG GA-3′) and D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) designed for platyhelminth (Litvaitis *et al* 1994) and later used for *An. minimus* (Sharpe *et al* 1999) and *An. fluviatilis* (Singh *et al* 2004). For PCR amplification and sequencing of D3 domain of 28S rDNA, Singh *et al* (2004) method was followed. Both strands of DNA were sequenced using at least three specimens of each member of the complex. The sequences were deposited at GenBank (accession numbers for species A, B, C, D and E are AY531531, AY531532, AY531533, AY531534 and AY531535 respectively). Alignment of sequences and designing of primers was done with software DNASTAR.

3. Results

3.1 *Sequence analysis and designing of allele-specific primers*

Alignment of sequences of D3 domain of 28S rDNA of five members of *An. culicifacies* complex is shown in figure 1. The size of amplified products was 382 base pair in species A and D and 385 base pair in species B, C and E. The sequences of species A and D, and of species B, C and E are identical. The differences in nucleotide sequences between species A/D and species B/C/E were used for designing allele-specific primers – with the mismatching bases kept at extreme 3′ of primers.

3.2 *Development of allele-specific PCR strategy*

To develop ASPCR assay, four primers were selected including two universal primers D3A and D3B used for amplification of D3 domain of 28S rDNA. Two allele-specific primers ACA (forward, sequence 5′-GCC GTC CCC ATA CAC TG-3′) and ACB (reverse, sequence 5′-CCG TAA TCC CGT GAT AAC TT-3′), which are specific to species A/D and species B/C/E respectively, were selected for design of multiplex ASPCR. While the A/D-specific primer (ACA) in conjunction with D3B produces 313 bp amplification product, B/C/E-specific primer (ACB) forms 133 bp product with D3A. Additionally, the external primers D3A and D3B form common product in all the cases (382 bp in species A and D; and 385 bp in species B/C/E) serving as positive control (figure 2). The optimized PCR conditions were: one cycle of denaturation at 95°C for 5 min (10 min with 'AmpliTaq Gold'), followed by 35 cycles of each of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 60 s, and final extension at 72°C for 7 min. The optimum primer concentrations were 1.5 μM for each primer. When 'AmpliTaq Gold' Taq polymerse (Applied Biosystems) was used, the concentration of primers ACA and ACB was reduced to 1·25 μM. The reaction mixture contained 1X buffer, 1·5 mM of $MgCl_2$, 200 μM of each dNTP and 0·375 unit of Taq Polymerase/15 μl reaction.

3.3 *Validation of allele-specific PCR assay*

To validate the ASPCR assay, mosquitoes which were successfully identified chromosomally to cryptic species were assayed. A total of 208 such chromosomally-identified specimens of *An. culicifacies* originating from various parts of India and representing different sympatric associations and ecological regions, were assayed with ASPCR assay (table 1). All the specimens were correctly grouped into species A/D or species B/C/E by ASPCR assay.

3.4 *Cross-reactivity with other mosquitoes*

Five specimens of each of other anophelines i.e., species S, T and U of *An. fluviatilis* complex, *An. minimus* species A, *An. stephensi* and *An. subpictus* were also tested with this ASPCR to examine cross-reactivity with unrelated anophelines. With all these anophelines, a single amplicon of ~ 380 bp (produced by universal primers D3A and D3B) was amplified (figure 3) and no amplicon matching 313 or 133 base pair specific to *An. culicifacies* species A/D and B/C/E, respectively, was amplified indicating that both the culicifacies-specific primers (ACA and ACB) do not react with other anophelines tested in this study.

4. Discussion

The identification of members of *An. culicifacies* complex or vector species from non-vector species is a

serious concern in the malaria control programme. The classical technique of sibling species identification, i.e., cytotaxonomy is difficult and has limited use since this technique requires on the one hand semi-gravid females only, which constitute small proportion of any population and on the other hand requires highly skilled personnel. The PCR assay on the other hand has advantage of being simple and sensitive, and is applicable to all stages and either sexes of mosquito. Much vital information about biology of cryptic members can be generated using samples belonging to any sex or stages of life cycle. Since this ASPCR is targeted to rDNA, a multigene family,

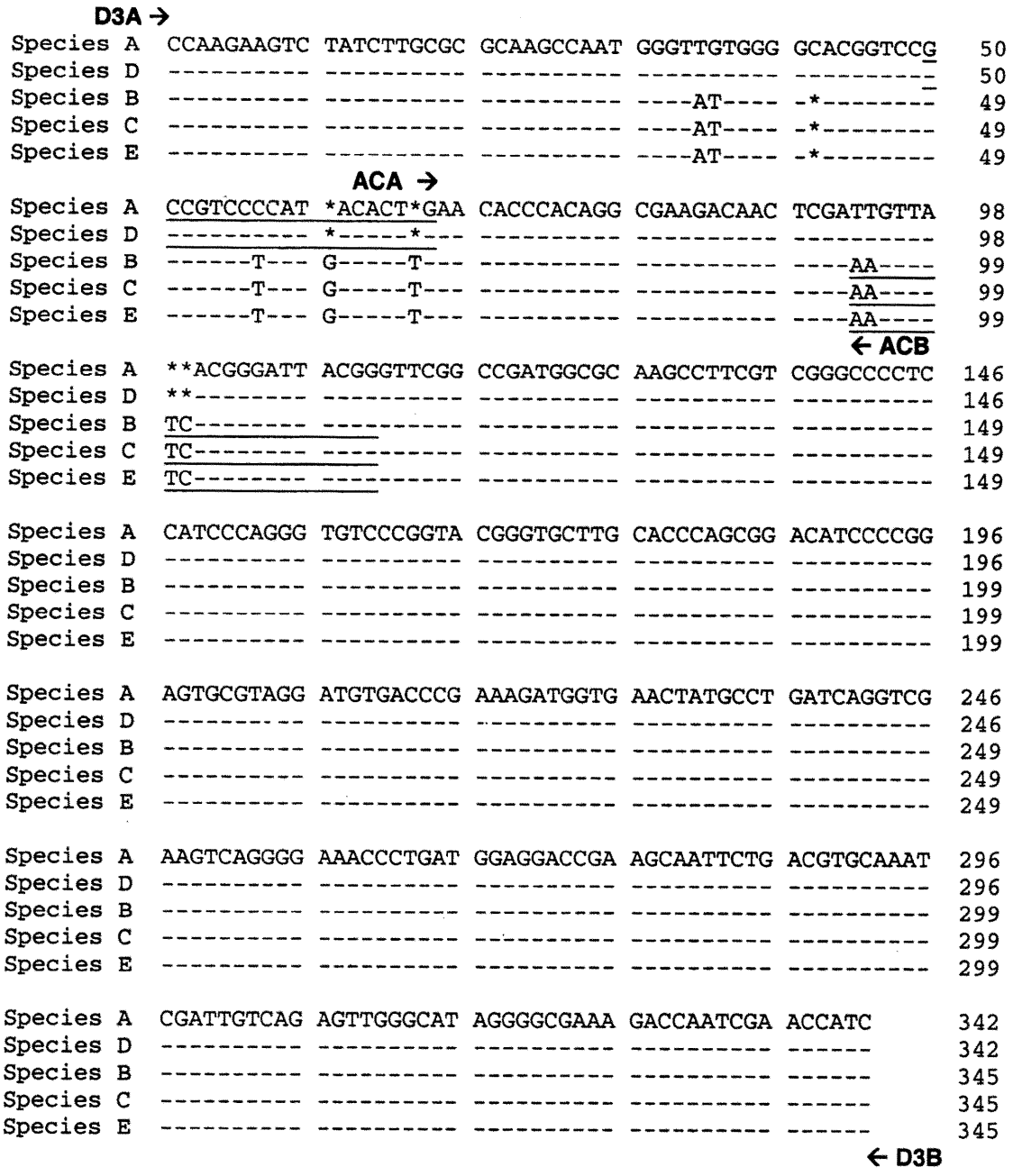

Figure 1. Alignment of 28S-D3 rDNA nucleotide sequences of five members of *Anopheles culicifacies* complex. Dashes represent identity with species A and asterisks missing nucleotide base. Underlined areas correspond to allele-specific primers designed. The regions corresponding to primers D3A and D3B (used for amplification) are not shown.

small amount of DNA is sufficient for PCR, making it possible to identify live mosquito by taking out a small piece of appendages like leg, antennae, palpi, etc.

Though this PCR assay is not able to differentiate all five members of the *An. culicifacies* complex and classifies the complex correctly into 2 groups, this method is highly useful in discriminating vector from non-vector in areas with certain sympatric association of sibling species especially where species C and E (latter is reported in Rameshwaram Island and Sri Lanka only) are not present. For example, in most of the northern India species A and B are sympatric with restricted presence of species D in some areas (Subbarao 1998). In such areas this assay is an excellent tool for discrimination of vector species (species A/D) from non-vector species B.

Since both the allele-specific primers (ACA and ACB) do not react to some common species (members of *An. fluviatilis* complex, *An. minimus*, *An. stephensi* and *An. subpictus*), this assay also has a check upon misdiagnosis of other anophelines as *An. culicifacies*. However more anopheline species – particularly of Myzomia series – which are morphologically closely related, are yet to be tested to arrive at final conclusion. Among Myzomia, series, only members of *An. fluviatilis* complex (species S, T and U) and *An. minimus* (species A) have been tested for cross-reactivity under this study.

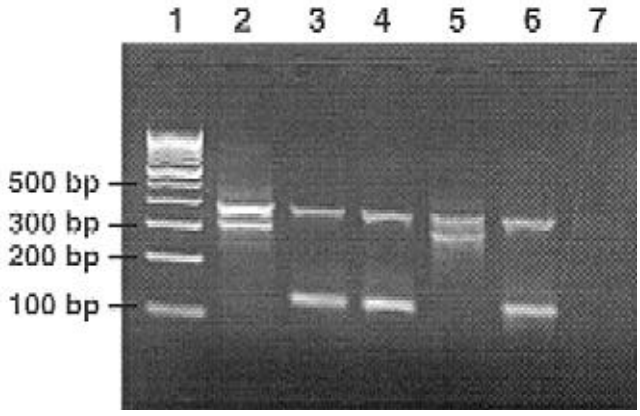

Figure 2. Allele-specific PCR assay of different members of *An. culicifacies* complex. PCR product as seen on 2·25% agarose gel under UV illumination. Lane 1, 100 bp DNA ladder; lane 2, species A; lane 3, species B; lane 4, species C; lane 5, species D; lane 6, species E; and lane 7, negative control, without DNA.

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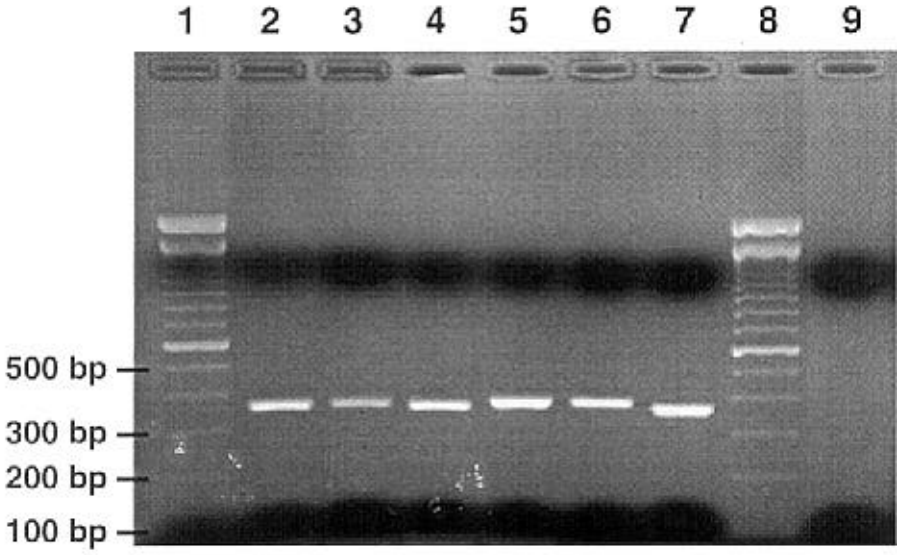

Figure 3. Reactivity of allele-specific PCR assay with other anophelines. PCR products were run on 2% agarose gel and visualized under UV illumination. Lanes 1 and 8, 100 bp DNA ladder; lane 2, *An. fluviatilis* species S; lane 3, *An. fluviatilis* species T; lane 4, *An. fluviatilis* species U; lane 5, *An. subpictus*; lane 6, *An. stephensi*; lane 7, *An. minimus* species A; lane 9, negative control, without DNA. None of the *An. culicifacies*-specific primers (ACA or ACB) reacted with these species.

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