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# DIFFERENTIATION OF MEMBERS OF THE *ANOPHELES FLUVIATILIS* SPECIES COMPLEX BY AN ALLELE-SPECIFIC POLYMERASE CHAIN REACTION BASED ON 28S RIBOSOMAL DNA SEQUENCES

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*Abstract.* *Anopheles fluviatilis,* one of the major vectors of malaria in India, is a complex of at least three cryptic species provisionally designated as species S, T, and U. Identification of the cryptic species of *An. fluviatilis* complex is of paramount importance in disease control program due to contrasting differences in their vectorial efficiency, preference for feeding on humans, and resting behavior. Species S, T, and U are morphologically indistinguishable at any stage of their life cycle and can be identified only by the examination of species-specific fixed inversions in the polytene chromosomes. We report an allele-specific polymerase chain reaction assay for the differentiation of members of *An. fluviatilis* complex, which is based on differences in nucleotide sequences in D3 domain of 28S ribosomal DNA. The assay was evaluated against chromosomally examined individuals from different localities with different sympatric associations and was found to differentiate unambiguously all the members of the complex.

## INTRODUCTION

Vector control is an essential component of any malaria control program, the success of which relies on knowledge of vector species present in area and their bionomics. Most of the malaria vectors found in southeast Asia are known to be complexes of cryptic species.[1] Members of these complexes may differ in biologic characteristics that have direct relevance to epidemiology and control of malaria such as vectorial efficiency,[2–4] insecticide resistance,[5–7] feeding and resting preference.[8,9] Therefore, mapping their distribution of cryptic species and understanding the bionomics and vectorial efficiency are essential for planning vector control strategies.

*Anopheles fluviatilis* James is a vector of primary importance in hilly and foothill regions of India and ranks second in contributing to total malaria cases in India.[10] It is considered a vector in India, Pakistan, and Nepal, although it is found widely distributed in eastern Asia (Pakistan, Afghanistan, India, Nepal, Bangladesh, Myanmar, Thailand, and southern China) and in parts of western Asia (Iran, Iraq, eastern and southern Saudi Arabia, Oman, Bahrain, and Russia).[11] Due to marked differences in density, host preference, and role in malaria transmission among different *An. fluviatilis* populations, the existence of two distinct biologic forms of *An. fluviatilis* was suggested by several investigators.[11] Cytotaxonomic studies subsequently revealed that *An. fluviatilis* is a complex of at least three reproductively isolated cryptic species, designated as species S, T, and U, which are distinguishable on the basis of fixed inversion genotypes detectable in polytene chromosome arm 2.[12] The three cryptic species of *An. fluviatilis* are found to vary in their distribution pattern,[1,12] feeding preferences[9] and their role in disease transmission.[1,13,14] Species S is found mainly in hilly and forested regions, is predominantly anthropophagic[9,13] (~90%), and is highly efficient vector of malaria. *An. fluviatilis* s.l. has been found to have high sporozoite rate in areas of India where species S is predominant (Malkangiri and Koraput Districts in Orissa State).[15] Species T and U are almost totally zoophagic (~99%)[9] and regarded as poor or non-vectors. In fact, only species S has been incriminated as a vector.[1]

The identification of cryptic species is of great concern for success of vector control programs, especially when they differ significantly in vectorial potential, host preference, and/or response to insecticides. Until now, there was no method of differentiating all the cryptic species of *An. fluviatilis* apart from the cytologic method, which is based on examination of polytene chromosomes from ovarian nurse cells in ovaries at Christopher's stage late III. However, the cytologic method can only differentiate semi-gravid females that constitute a small portion of the wild mosquito population and it requires highly skilled personnel. Therefore, an alternative technique that can identify all stages and sexes is needed.

DNA-based methods for the identification of cryptic species or closely related species have successfully been used with a number of anophelines. These methods are based mainly on probe hybridization and the polymerase chain reaction (PCR).[16,17] However, the hybridization assay can be unreliable due to its sensitivity to unequal amounts of target DNA loaded and variation in copy number across the species range.[16] The ribosomal DNA cistron, one of the multigene families frequently distributed in genome in arrays of tandem repeats, is the preferred candidate region for PCR-based species-diagnostic assays because of useful feature of concerted evolution acting on the rDNA array that maintains sequence homogeneity in a species.[18] The rDNA genes in eukaryotes occur in hundreds or thousands copies arranged in tandem arrays; therefore, small amounts of insect bodies are sufficient for the PCR assay. For taxonomic purposes, the non-coding spacers of rDNA i.e., internal transcribed spacers (ITS 1 and 2) and intergenic spacers, that frequently vary interspecifically both in length and sequence are most studied regions. The variable regions within 28S rDNA have also been studied, although less frequently for such purposes. We hereby report an allele-specific PCR method for the identification of all three known members of the *An. fluviatilis* complex, which is based on the differences in nucleotide sequences in D3 domain of 28S ribosomal DNA.

Recently Manomonai and others[19] designed a PCR-based diagnostic assay for differentiation of two genotypes, which have been designated as putative species X and Y, based on differences in the ITS 2 of rDNA. Subsequent correlation with cytotaxonomy revealed that 94% of species X were spe-

cies S and 90% of species Y were species T.[20] The present paper describes PCR-based diagnostic method for the differentiation of all three members of *An. fluviatilis* complex, which have been established to be reproductively isolated, as shown by total absence of heterozygotes between three inversion genotypes.[12]

## MATERIALS AND METHODS

**Mosquitoes.** *Anopheles fluviatilis* collected from different parts of India having different sympatric associations of the three cryptic species were used for this study. The details of the localities from where mosquitoes were used in this study are shown in Figure 1 and Table 1. In addition to field-collected mosquitoes, laboratory-reared species T that originated in Haldwani were also used in the study.

Adult female mosquitoes were collected during from 6:00 AM to 8:00 AM from indoor resting habitats (human dwellings and cattle sheds). Fully fed females were kept in paper cups or cloth cages at a temperature of approximately 27 ± 2°C and a high relative humidity of 70–80% for optimal ovarian development. Ovaries were removed from individual semi-gravid *An. fluviatilis* mosquitoes (in Christopher's stage late III) and preserved in modified Carnoy's fixative (1:3 acetic acid: methanol). The rest of the body was preserved in isopropanol for DNA isolation. Both the ovaries and the rest of the body of individual mosquitoes were labeled with identical numbers. Mosquitoes not suitable for cytotaxonomy were preserved in isopropanol and transported to laboratory at Delhi.

**Mosquito processing.** The ovaries of individual *An. fluviatilis* were used for polytene chromosome preparation following the procedure of Green and Hunt[21] and identified at cryptic species level by examining species-specific paracentric inversions present in chromosome arm 2.[12] The diagnostic

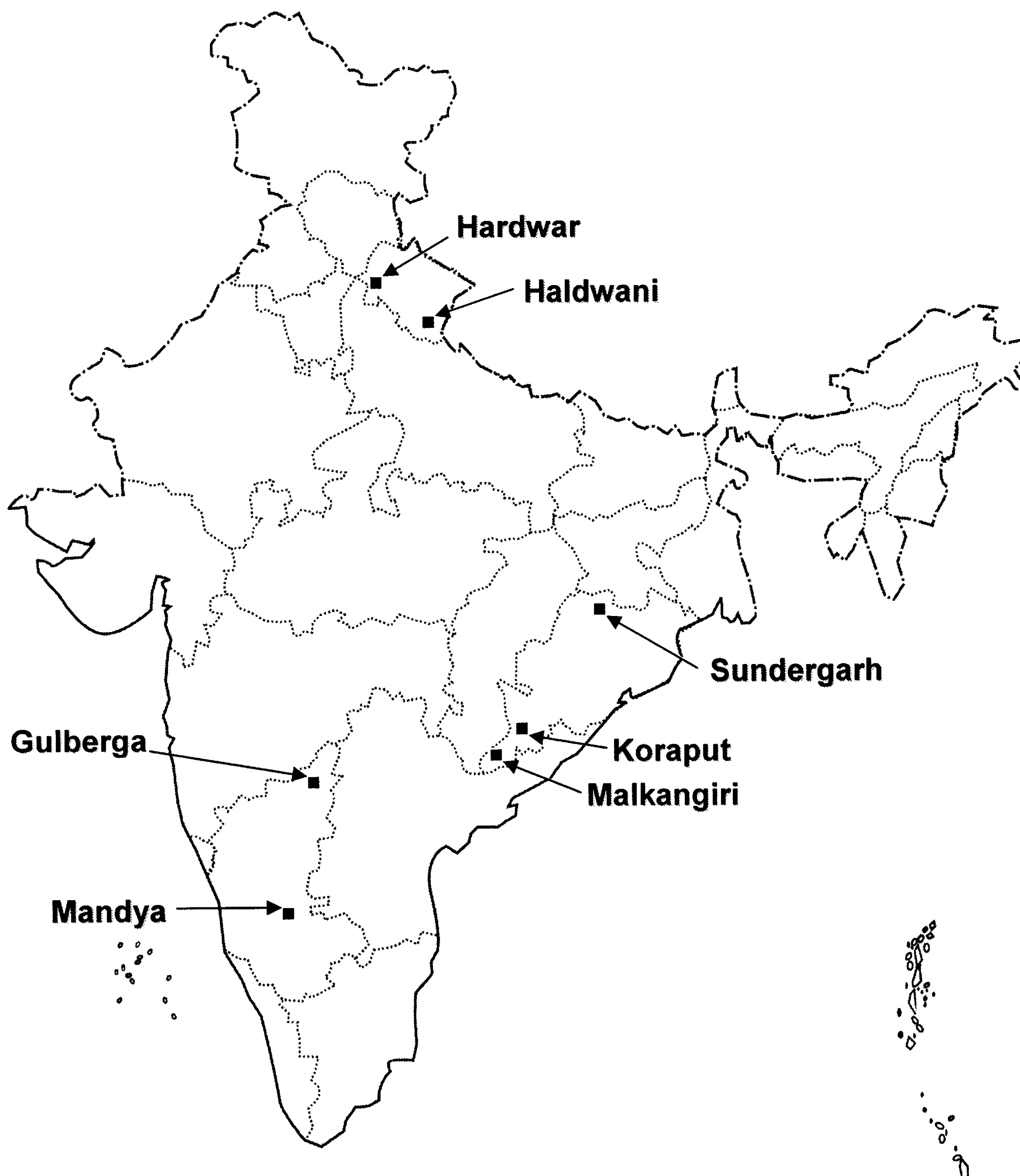


FIGURE 1. Localities in India from which *Anopheles fluviatilis* used in this study were obtained.

TABLE 1

Result of a polymerase chain reaction (PCR) assay of *Anopheles fluviatilis* and correlation with chromosomal inversion genotypes

| | | Inversion genotypes | | | | |
|---|---|---|---|---|---|---|
| Locality | Result of the allele-specific PCR | $+^{q^1}+^{r^1}$ (S) | $q^1+^{r^1}$ (T) | $+^{q^1}r^1$ (U) | $+^{q^1}/q^1$ heterozygote | Unidentified |
| Haldwani, Uttaranchal (29.2°N, 79.5°E) | T | | 18 | | | 9 |
| Hardwar, Uttaranchal (30.0°N, 78.2°E) | T | | 34 | 1 | | 93 |
| | U | | | 47 | | 116 |
| Sundergarh, Orissa (22.1°N, 84.0°E) | S | 34 | | | | 261 |
| | T | | | | | 1 |
| Koraput, Orissa (18.8°N, 82.7°E) | S | 12 | | | | 7 |
| Malkangiri, Orissa (18.4°N, 81.9°E) | S | | | | | 9 |
| Mandya, Karnataka* (12.6°N, 76.9°E) | T | 13 | 1 | | 7 | 16 |
| Gulberga, Karnataka* (17.3°N, 76.8°E) | T | | 3 | | 1 | |
| Laboratory colony (Origin Haldwani) | T | | 10 | | | |

* Area where *An. fluviatilis* is polymorphic for the $q^1$ inversion and no chromosomal marker is available for species identification.

paracentric inversion genotypes are $+^{q1}+^{r1}$ for species S, $q^1+^{r1}$ for species T, and $+^{q1}r^1$ for species U. Genomic DNA was isolated using the method of Coen and others,[22] resuspended in 200 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and stored at 4°C.

**Sequencing of DNA.** The D3 domain of 28S rDNA region was amplified by a 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 by Litvaitis and others[23] for platyhelminths and later used by Sharpe and others[24] for *An. minimus*. The PCR mixture contained 20 μM of each dNTP, 1× buffer, 1.5 mM $MgCl_2$, 1.0 units of *Taq* polymerase/25-μL reaction volume, and 0.2 μM of each primer. The concentrations of dNTPs and primers were kept at a minimum so that they would nearly consumed during PCR and that the product can be directly sequenced. The PCR conditions were an initial denaturation at 95°C for five minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 48°C for 30 seconds, and extension at 72°C for 60 seconds, and a final extension at 72°C for seven minutes. The PCR product was directly subjected to a cycle sequencing reaction using the BigDye Terminator Ready Reaction Kit (Applied Biosystems, Foster City, CA). Sequences were analyzed on the ABI Prism 310 Genetic Analyzer (Applied Biosystems) following the manufacture's instructions. Sequencing was done in both directions. Five specimens of species S from Sundergarh, Orissa, three specimens of species T each from Hardwar and Haldwani, Uttaranchal, and three specimens of species U from Hardwar, Uttaranchal were sequenced in both directions. Alignment of sequences and designing of primers was done with using DNASTAR software (DNASTAR, Inc., Madison, WI). The sequence data have been submitted to GenBank (accession numbers AF 437880–AF 437882).

## RESULTS

**Sequence alignment and primer design.** Analysis of the sequences of products amplified with universal primers D3A and D3B corresponding to the D3 domain of 28S rDNA showed that the amplified products were 375 basepairs in all three species of *An. fluviatilis*. Alignment of sequences with the database of the National Center for Biotechnology Information (Bethesda, MD) using a BLAST search confirmed that the sequences are from D3 domain of 28S ribosomal RNA. Alignment of sequences of three cryptic species is shown in Figure 2. The sequences of all three species are closely similar (> 99%). Species S differs from species T and U by two nucleotide bases, at base position 76-77 (Figure 2), and all three cryptic species differ by one base from each other at base position 92. These differences in nucleotide sequences among the three members were used for designing allele-specific primers. During the designing of these primers, the mismatching base(s) were kept at the extreme 3′ ends of the primers. Different primers were evaluated for species specificity.

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Species S  CCAAGAAGTC TATCTTGCGC GCAAGCCAAT GGGTAAATGG   40
Species T  ---------- ---------- ---------- ----------   40
Species U  ---------- ---------- ---------- ----------   40

Species S  TGCGGTACGC CGCCCATGAC TGGAAACCCA CAGGCACAGA   80
Species T  ---------- ---------- ---------- -----GA---   80
Species U  ---------- ---------- ---------- -----GA---   80

Species S  CAAATCGAGT GTTGCGGGAT TACGGGTACG GCCGATGGCG  120
Species T  ---------- -G-------- ---------- ----------  120
Species U  ---------- -A-------- ---------- ----------  120

Species S  CAAGCCTTCG TCGGACCCCT CCATCCCAGG GTGTCCCGTT  160
Species T  ---------- ---------- ---------- ----------  160
Species U  ---------- ---------- ---------- ----------  160

Species S  CGGGTGCTTG CACCCAGCGG ACATCCCCGG AGTGCGTAGG  200
Species T  ---------- ---------- ---------- ----------  200
Species U  ---------- ---------- ---------- ----------  200

Species S  ATGTGACCCG AAAGATGGTG AACTATGCCT GATCAGGTTG  240
Species T  ---------- ---------- ---------- ----------  240
Species U  ---------- ---------- ---------- ----------  240

Species S  AAGTCAGGGG AAACCCTGAT GGAGGACCGA AGCAATTCTG  280
Species T  ---------- ---------- ---------- ----------  280
Species U  ---------- ---------- ---------- ----------  280

Species S  ACGTGCAAAT CGATTGTCAG AGTTGGGCAT AGGGGCGAAA  320
Species T  ---------- ---------- ---------- ----------  320
Species U  ---------- ---------- ---------- ----------  320

Species S  GACCAATCGA ACCAT  335
Species T  ---------- -----  335
Species U  ---------- -----  335
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FIGURE 2. Sequence alignment of the D3 domain of 28S ribosomal DNA of members of the *Anopheles fluviatilis* complex (species S, T, and U). - = identity with species S. Genbank accessions numbers are AF 437880 for species S, AF 437881 for species T, and AF 437882 for species U. Sequences corresponding to the primers used for polymerase chain reaction amplification have been removed.

**Development of the diagnostic PCR.** The schematic representation of the PCR strategy is shown in Figure 3. Four primers were used, i.e., the universal primers D3A (forward) and D3B (reverse) and two allele-specific primers: AFS (forward, 5′-TGG AAA CCC ACA GGC AC-3′) and AFT (reverse, 5′-TAC CCG TAA TCC CGC AC-3′), which are specific for species S and T, respectively. The S species-specific primer (AFS) forms a 295-basepair product with D3B, and the T species-specific primer (AFT) forms a 128-basepair product with D3A. Additionally, the external primers D3A and D3B form a 375-basepair product in all cases, which serves as a positive control. The absence of the 375-basepair product indicates the failure of the reaction. Species U can be differentiated from species S and T by the absence of species S- or T-specific products and the presence of a positive control product (Figure 4). The optimized PCR conditions were as follows: one cycle of heating at 95°C for 10 minutes (hot-start method) with *Taq* polymerase AmpliTaq Gold (Applied Biosystems), followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, and a final extension at 72°C for seven minutes. The optimum primer concentrations were 1.5 μM for D3A, 1.6 μM for D3B, 1.5 μM for AFS, and 1.4 μM for AFT. The reaction mixture contained 1× buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, and 0.375 units of Taq polymerase (AmpliTaq Gold)/15-μL reaction volume. In rare instances in which a hot-start method is not used and an excess of primer is used, mispriming of species T-specific primer with species S was noticed and resulted in a faint band of 128 basepairs, but such mispriming was never observed with species U. Thus, the 295-basepair product identified species S, the 128-basepair product and the absence of the 295-basepair product identified species T, and absence of both the 295- and 128-basepair products identified species U. The 375-basepair product (control) was observed in all species. The amplified products were observed after electrophoresis on a 2.0% agarose gel containing ethidium bromide under ultraviolet illumination.

**Evaluation of the allele-specific PCR.** A total of 683 field collected specimens originating from different localities and sympatric associations were assayed with this diagnostic PCR, of which 323 were identified as species S, 197 as species T, and 163 as species U. Among these, 171 mosquitoes were also identified chromosomally. Additionally, 10 laboratory-colonized specimens of species T were also assayed with the PCR. The details of the localities from where mosquito populations were examined and the result of their diagnosis with the allele-specific PCR and the cytotaxonomic method are

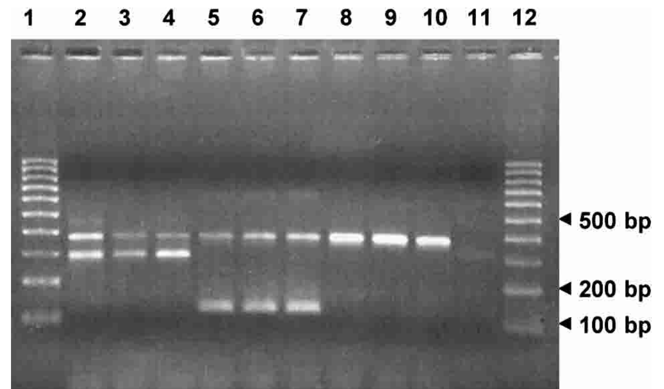


FIGURE 4. Polymerase chain reaction (PCR) assay for differentiation of members of the *Anopheles fluviatilis* complex. The PCR products were subjected to electrophoresis on a 2% agarose gel containing ethidium bromide and visualized under ultraviolet illumination. Lanes 1 and 12, 100-basepair (bp) ladder; lanes 2–4, species S; lanes 5–7, species T; lanes 8–10, species U; lane 11, negative control without DNA.

shown in Table 1. The allele-specific PCR results were in agreement with the cytotaxonomic results for all specimens collected from regions where the three diagnostic inversion genotypes ($+^{q1}+^{r1}$ for species S, $q^1+^{r1}$ for species T, and $+^{q1}r^1$ for species U) in chromosome 2 have been found to be fixed, except for one sample (1 of 82) from Hardwar, which was identified as species T by the allele-specific PCR and as species U by chromosomal analysis. This may be due to incorrect labeling at various stages during processing of the mosquito in the field and laboratory. In the Mandya and Gulberga districts of Karnataka, where the *An. fluviatilis* population is polymorphic for the $q^1$ inversion, the three inversion genotypes ($+^{q1/}+^{q1}$, $+^{q1/q1}$, and $q^{1/q^1}$) were found in Hardy-Weinberg equilibrium ($P = 0.998$ for the Mandya population), and all were identified as species T by the allele-specific PCR.

The allele-specific PCR was also tested with *An. culicifacies,* which is sympatric with *An. fluviatilis* in most of the areas. All five members of the *An. culicifacies* complex did not react with species S or T-specific primers. However, they reacted with the universal primers, giving an amplicon of ~380 basepairs.

## DISCUSSION

The allele-specific PCR assay reported in this study was able to differentiate unambiguously the three known isomor-

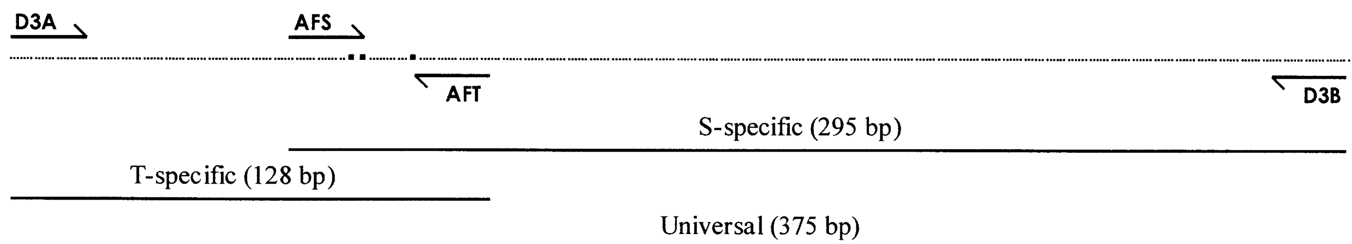


FIGURE 3. Schematic representation of the polymerase chain reaction (PCR) strategy for differentiation of members of the *Anopheles fluviatilis* complex. The dotted line represents the DNA template, the arrows represent primers, and the solid lines represent the expected PCR product. bp = basepairs.

phic members of the *An. fluviatilis* complex. In all localities studied where there was no polymorphism for chromosomal inversion, the results of the allele-specific PCR were in agreement with chromosomal identifications of the three species. In the Mandya and Gulberga districts in Karnataka State where the heterozygotes for $q^1$ inversion ($+^{q1/q^1}$) were found, the question arises whether these specimens belong to species S, T, or to both. The fact that the three genotypes (i.e., $+^{q^1/}+^{q^1}$, $+^{q^1/q1}$, and $^{q1/q1}$) were in Hardy-Weinberg equilibrium suggests the presence of only one species. The allele-specific PCR identified all these genotypes as species T. In the absence of a chromosomal marker for this population, this population was regarded as species T on the basis of its zoophagic nature,[11] similar to species T, and unlike species S, which is predominantly anthropophagic. However, definite conclusions can be drawn after careful genetic analysis of this chromosomally polymorphic population.

Although the cytotaxonomic analysis of *An. fluviatilis* is highly reliable method (except for *An. fluviatilis* population where the $q^1$ inversion is polymorphic), the PCR has advantage of being easy to perform, sensitive, applicable to all stages and either sexes of mosquito, and not requiring highly skilled personnel. Since the rDNA cistron belongs to a multiple gene family having hundreds/thousands of copies, a small amount of DNA is sufficient to perform this diagnostic PCR. Even a small piece of insect's appendages is sufficient for such an assay, making it possible to identify living specimen that can be used for species-specific experimental purposes. In field studies, a single mosquito can be used for sporozoite/oocyst dissection, parity, blood meal analysis, and sibling species identification by the PCR. The specimens can be stored in isopropanol, eliminating the need for a refrigerator or sophisticated laboratory conditions. Mosquitoes stored in a wide variety of conditions may be used for the PCR assay. We have also used part of the ovary of a mosquito, which was preserved in Carnoy's fixative for cytotaxonomic analysis, for successful species identification by the PCR. The cost concern of the PCR limits its use to research laboratories only. However, the cost of the PCR is being reduced as the cost of reagents decreases. Furthermore, the cost of the PCR can be substantially reduced by reducing the volume of reaction mixture to 10–15 μL instead of 25–50 μL.

Since species U is identified in this assay on the basis of the absence of S- or T-specific amplicons, it may not be possible to differentiate other anophelines from this species. It is expected that most anophelines will produce universal amplicons (produced by the conserved primers D3A and D3B). This has been shown in present study with *An. culicifacies*, which is widely distributed in India and is sympatric with *An. fluviatilis* in most of the areas studied. Since this test is not designed to differentiate *An. fluviatilis* from other anophelines, care must be taken to identify mosquito morphologically before testing with the PCR assay.

Although this method has been tested in many different populations, care must be taken to confirm the specificity of this method in a particular population by correlating its results with cytologic identification because variation in nucleotide sequences may be found in geographically distant populations. Once the results of this method are found to be in agreement with those of a cytologic method in a specific population, it can be applied for identification of any stage and either sex of mosquitoes.

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