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# Molecular identification and phylogeny of Myzomyia and Neocellia series of *Anopheles* subgenus Cellia (Diptera: Culicidae)

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ABSTRACT

Any biological study is only meaningful if the concerned organism is accurately identified; this is particularly important in vector-borne disease studies where correct and precise identification of the target species has medical and practical implications, such as in vector control. The Myzomyia series is divided into four groups including the Funestus group, which consists of five subgroups, i.e. Aconitus, Culicifacies, Funestus, Minimus, Rivulorum, and the Neocellia series, which is divided into three groups Annularis, Jamesii and Maculatus. Members of the Funestus group of Myzomyia and the Annularis group of the Neocellia series are difficult to identify because of the morphological overlap that exists within the groups. Therefore a multiplex polymerase chain reaction (PCR) assay was developed based on the sequence of the D3 region of 28S rDNA to distinguish between four members (*An. fluviatilis*, *An. culicifacies*, *An. varuna* and *An. aconitus*) of three subgroups (Minimus, Aconitus, Culicifacies) of the Funestus group of Myzomyia and three members (*An. annularis*, *An. pallidus* and *An. philippinensis*) of the Annularis group of the Neocellia series of the Anopheles subgenus Cellia, prevalent in Orissa, India. Polymorphism present on the D3 region of rDNA allowed the development of a species-specific primer that when combined with two universal primers lead to a simple and sensitive multiplex allele-specific polymerase chain reaction (AS-PCR) assay. This assay can be applied as an unbiased confirmatory method for the identification of morphological variants, imperfectly preserved specimens and life stages for which taxonomic keys do not allow a definitive species determination. Finally, phylogenetic relationships between the members of the two series were determined using D3 sequence data. The phylogenetic relationships inferred from maximum parsimony and the neighbour joining analysis separated two distinct monophyletic clades, one consisting of species of Myzomyia and other of species of the Neocellia series. The molecular phylogeny obtained in this work matches with that of the classical morphological taxonomy reasonably well, with proper species arrangements.



## 1. Introduction

*Anopheles* mosquitoes are responsible for human malaria transmission. Most of the important malaria vectors are members of species complexes or species groups, which are often difficult to distinguish morphologically from one another. Their accurate identification is the most basic requisite for understanding vector biology, malaria risk factors and epidemiology, and for designing and adequately measuring the impact of disease control interventions. Studies on mosquito susceptibility to *Plasmodium* infection, behaviour and geographical distribution are all dependent on the correct species designation of acquired samples. Therefore, proper identification of malaria vectors is of the utmost importance for the success of vector control programmes.

The subgenus Cellia, of the genus *Anopheles*, includes 217 formally recognized species and a growing number of unnamed members of sibling species complexes which are divided between six series comprised of groups and subgroups believed to represent phylogenetically related assemblages, based principally on morphological similarities (Harbach, 2004). The species of the Myzomyia and Neocellia series comprise those species, which are morphologically very similar. The Myzomyia series of the subgenus Cellia, of the genus *Anopheles*, consists of 69 species found in Afrotropical, Mediterranean and Oriental regions. The series is divided into four groups, including the Funestus group, which consists of five subgroups: Aconitus, Culicifacies, Funestus,

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Minimus and Rivulorum. The Neocellia series consists of 33 species found in Oriental and Afrotropical regions, which are divided into three groups (Harbach, 2004). In India, the Myzomyia and Neocellia series consists of 7 and 12 species, respectively (Rao, 1984). The 7 species of Myzomyia are *An. culicifacies*, *An. fluviatilis*, *An. minimus*, *An. varuna*, *An. aconitus*, *An. jeyporiensis* and *An. majidi*, and the 12 species of Neocellia are *An. moghulensis*, *An. stephensi*, *An. maculatus*, *An. theobaldi*, *An. karwari*, *An. jamesii*, *An. ramsayi*, *An. splendidus*, *An. annularis*, *An. philippinensis*, *An. pallidus* and *An. pulcherrimus*. Among these, *An. fluviatilis*, *An. culicifacies*, *An. varuna* and *An. aconitus*, of Myzomyia and *An. annularis*, *An. pallidus* and *An. philippinensis* of the Neocellia series are mainly prevalent in Orissa, India. *An. culicifacies s.l.* is a major vector of malaria in India, contributing to ~60–70% of malaria cases in the country. *An. fluviatilis James* is a primary vector in the hilly and foothill regions of India and ranks second in contributing to the total malarial cases of the country. *An. fluviatilis* is the most anthropophilic species; additionally, *An. fluviatilis* and *An. culicifacies* are endophilic in nature and are the most efficient vectors of malaria (Sharma, 1998). In India, the role of *An. annularis* in malarial transmission has been established in Orissa, Assam, West Bengal and Andhra Pradesh (Ghosh et al., 1985; Prakash et al., 2004; Mahapatra et al., 2006). The other members of these two series are mainly zoophilic and feed on human beings depending upon the availability of the host, and are regarded as secondary vectors of malaria in India. Vectorial and behavioural variations among these species and groups constitute the major factors in an accurate and precise identification.

The identification of anophelines in Orissa is complicated by the biodiversity, which characterizes the region. Some of the members of the two series are the major vectors of malaria in Orissa. The major problem in working with these series is the difficulty in identifying one species from another within the series. Morphological identification is dependent on various characteristics found in the different life stages of a species. The adults of the species are morphologically very similar and are often difficult to distinguish between, especially *An. philippinensis* and *An. pallidus* of the Annularis group of the Neocellia series, which cannot always be identified reliably unless accompanied by larval and pupal exuviae (Reid, 1968). Using immature characteristics, *An. varuna* and *An. aconitus* can be identified by both egg and larval morphologies (Rao, 1984). Examination of these characteristics entails capturing blood-fed females from the wild variety for egg laying and rearing of larvae to fourth instars, which is by no means simple as larvae are difficult to maintain under laboratory conditions. Mortality is high during the rearing process, and also time consuming. Cytogenetic studies showed that *An. fluviatilis*, *An. varuna* and *An. aconitus* of Myzomyia and *An. annularis* of the Neocellia series each posse unique chromosomal inversion rearrangements that can be used to identify them (Subbarao et al., 1994; Baimai et al., 1996; Atrie et al., 1999). Polytene chromosome identification is much faster than using morphology but there are disadvantages in routinely using cytogenetic tool for the identification of the half gravid females which are required for this purpose. These difficulties stimulated the development of molecular tools for precise and reliable species identification. Recent developments in the field of DNA-based techniques, such as allele-specific PCR, PCR direct sequencing, PCR restriction fragment length polymorphism and single-strand conformational polymorphism assay (Collins and Paskewitz, 1996; Proft et al., 1999; Wilkerson et al., 2004; Li and Wilkerson, 2005) have proven to be potential tools for the differentiation of numerous *Anopheles* species (Beebe and Saul, 1995; Van Bortel et al., 2001).

One of the most widely used regions of the genome to infer genetic variations and phylogenetic relationships is the ribosomal DNA (rDNA) cluster, a tandemly repeated multigene family. The tandemly arrayed rDNA is a common target of such methods because of useful features in its sequence organization and evolution. Concerted evolution acting on rDNA arrays maintains sequence homogeneity within species as it drives differentiation between species, a pattern which explains the utility of rDNA for species diagnostic assays (Collins and Paskewitz, 1996). This locus has many advantages, such as the fact that it is represented in multiple copies leads to high amplification signals, and it also contains variable regions that facilitate the selection of primer binding sites for each species to generate specific amplification products of different sizes. As such, the rDNA cluster has become an increasingly popular tool in molecular entomology (Collins and Paskewitz, 1996), in particular as a means for developing diagnostic tests to differentiate between anopheline species. Besides being useful for species identification, molecular sequences also provide phylogenetic information.

The ability to efficiently and unequivocally identify these species is a priority for obtaining a clear understanding of malarial transmission in the region. We structured a single step multiplex PCR assay to improve the accuracy of identification of a number of key vectors which display significant morphological overlap within the two series. The assay is useful because it relies on a single PCR to produce fragments which can be easily fractionated by gel-electrophoresis and which can clearly differentiate between taxa that are often misidentified or unsuitable for morphological taxonomy. Finally, the phylogenetic relationships between the members of the Myzomyia and Neocellia series were estimated using the D3 sequences.

## 2. Materials and methods

### 2.1. Sample collection and identification

The samples used in the study originated from various localities of Orissa. The samples were collected from Keonjhar, Mayurbhanj, Rayagada and Gajapati (Table 1 and Fig. 1). Adult female anopheline mosquitoes were collected with the help of a mechanical aspirator and light trap. The mosquitoes were collected from human dwellings, cattle sheds and mixed dwelling. After field capture, all mosquitoes were first identified on the basis of their morphology (Christophers, 1933; Nagpal et al., 2005). After identification the blood-fed females were transported to the laboratory in individual tubes for egg laying. The egg morphology was examined before the eggs were put into bowls with aerated water. The larvae were fed on a high protein diet consisting of yeast tablets. Fourth instar larvae and pupae characteristics were studied and compared with those of the adults for species verification. Morphological identification was carried out on all reared species according to standard taxonomic keys (Christophers, 1933; Nagpal et al., 2005).

### 2.2. DNA extraction, PCR amplification and sequencing of the D3 region of Anopheles mosquitoes

Genomic DNA was extracted from single individual adult mosquitoes using the method described by Coen et al. (1982). The D3 forward 5′-GACCCGTCTTGAAACACGGA-3′ and D3 reverse 5′-TCGGAAGGAACCAGCTACTA-3′ primers were used to amplify the D3 region of 28S rDNA (Litvaitis et al., 1994) from four members of the Funestus group of Myzomyia and three members of the Annularis group of the Neocellia series. The PCR conditions were 1× PCR buffer (Genei, Bangalore, India), 2 mM $MgCl_2$, 500 μM dNTP, 1 μM of each primer and 1unit of Taq DNA polymerase (Genei, Bangalore, India) per 30 μl reaction. A 2 μl volume of DNA sample was used per 30 μl PCR reaction mix. The samples were heated at 94 °C for 5 min before 35 cycles of amplification at 94 °C