Infection, Genetics and Evolution 9 (2009) 271–277

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Multiplex PCR assay and phylogenetic analysis of sequences derived from D2 domain of 28S rDNA distinguished members of the *Anopheles culicifacies* complex into two groups, A/D and B/C/E

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ARTICLE INFO

Article history:
Received 31 October 2008
Received in revised form 8 December 2008
Accepted 9 December 2008
Available online 24 December 2008

Keywords:
Anopheles culicifacies
Sibling species
28S rDNA
D2 variable region
Phylogeny
PCR assay

ABSTRACT

A multiplex PCR assay was developed using the sequences of the D2 region of 28S ribosomal DNA (rDNA) to discriminate the five members of the *Anopheles culicifacies* complex provisionally designated as species A, B, C, D and E. Two minus strand primers derived from sequence differences in the D2 variable region and a universal plus strand primer derived from the conserved 28S (rDNA) has delimited five members into species A and D (group 1) and species B, C and E (group 2) in a PCR diagnostic assay. The complete 28S rDNA-D2 region sequence of *A. culicifacies* sibling species is reported for the first time. Inter-specific sequence divergence was greater than the intra-specific divergence. The phylogenetic relationships inferred from maximum likelihood, maximum parsimony and the neighbor joining analysis confirmed the presence of two unambiguous monophyly clades one consisting of species A and D and the other of species B, C and E and that the *A. culicifacies* sibling species diverged relatively recently in evolutionary terms despite their considerable differences in bionomics.

1. Introduction

Malaria is one of the most important infectious diseases of humans globally with more than 300 million reported clinical cases and causes more than one million deaths per year (Centers for Disease Control and Prevention, 2007). *Anopheles culicifacies* sensu lato are major vectors of malaria in India and in the rest of south East Asia. Members of this species complex have a broad geographic distribution, from India eastward into Nepal, Bangladesh, Thailand, Myanmar, China, Vietnam, and Cambodia, westward into Afghanistan, Pakistan, Iran, Yemen, and Ethiopia, and southward into Sri Lanka (Harrison, 1980; Rao, 1984; Van Bortel et al., 2002).

A. culicifacies has three pairs of chromosomes, including two autosomes and X/Y sex chromosomes. In some tissues the chromosomes appear as large polytene chromosomes with distinctive patterns of bands associated with chromosomal inversions (WHO, 2007). Earlier studies (Subbarao et al., 1983; Vasantha et al., 1991) found evidence of restricted gene flow among populations of *A. cuilicifacies* on the basis of polytene chromosome inversion frequencies. This medically important species otherwise recognizing as single taxa, which in fact is a complex of five sibling species.

A. culicifacies thus far been recognized as a complex of five sibling species that are 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). Until the 1990s polytene chromosome examination and Y-chromosome karyotyping were the only methods available to differentiate all members of this complex. A Lactate dehydrogenase (*Ldh*) electrophoresis assay described by Adak et al. (1994) showed some promise by separating group 1 (A and D) from group 2 (B, C and E). Polymerase Chain Reaction (PCR) assays based on sequence differences within the ITS2 ribosomal DNA region (rDNA) (Manonmani et al., 2007), 28S-D3 rDNA domains (Singh et al., 2004) and a PCR–RFLP assay based on ITS2 amplicon (Goswami et al., 2005) met similar fates of only being able to separate group 1 from group 2 species; while, COII amplicon based PCR–RFLP assay (Goswami et al., 2005) could successfully able to distinguish sp. B from sp. E. More recently, a two-step multiplex PCR assay based on sequence differences within the COII region that distinguishes all five sibling species was

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doi:10.1016/j.meegid.2008.12.007

developed (Goswami et al., 2006). However, Surendran et al. in 2006 reported non-usability of COII PCR assay to distinguish the species B from species E in Sri Lanka.

In an attempt to design a less expensive and more efficient multiplex PCR assay to distinguish *A. culicifacies s. l.* we examined sequence variations among members of the complex within the variable D2 domain of the 28S rDNA subunit. These sequences were used to study the phylogenetic relationship among the reported five sibling species of the culicifacies complex. Sequences derived from the D2 variable domain within the 28S rDNA subunit have proven useful in phylogenetic studies of *anopheline* mosquitoes (Porter and Collins, 1996). The designed PCR assay was evaluated using DNA from specimens of the *A. culicifacies* complex from different areas of India that had been previously identified by examination of ovarian nurse cell polytene chromosomes and mitotic karyotypes.

2. Materials and methods

2.1. Mosquitoes and collection sites

Indoor resting *A. culicifacies s. l.* were collected in the morning (from 6 to 8 am) from cattle sheds and human dwellings from villages across India as listed in Table 1. Ghaziabad (Longitude 77° 28E′ and Latitude 28° 40′N) in Uttar Pradesh State (U.P.); Sonepat (Longitude 76° 38′E and Latitude 28° 54′N) and Gurgaon (Longitude 77° 04′N and latitude 28° 37′E) in Haryana State in Northern India; district Alwar (Longitude 76° 38′E and latitude 27° 34′N) in Rajasthan State in Northwest India; district Jabalpur (Longitude 79° 59′E and Latitude 23° 10′N) in Madhya Pradesh State in central India; district Kheda (Longitude 22° 45′E and Latitude 72° 45′N) in Gujarat State in East India; Rourkela (Longitude 84° 54′E and Latitude 22° 12′N) in Orissa State East India; and district Ramanathapuram (longitude 79° 22′E and Latitude 9° 17′N) in Tamil Nadu State in southern India (Table 1). Specimens were also used from established colonies of *A. culicifacies s. l.* originating from the districts Sundargarh (Orissa State), Ghaziabad (Uttar Pradesh State), Jabalpur (Madhya Pradesh State) and from periurban areas of Delhi State. Colonies were established from the progeny of cytologically identified females.

2.2. Sibling species identification

In the morning collections of *A. culicifacies s. l.*, half-gravid females generally constitute 20–30% of the total females collected.

2.2.1. Cytological method

Ovaries were removed from half-gravid *A. culicifacies* and stored in vials containing modified Carnoy's fixative (3 parts methanol to 1 part glacial acetic acid) and the remaining carcass was stored in 50–100 μl of isopropanol for DNA isolation. Matched ovaries and carcasses were given the same collection number. From ovaries, polytene chromosomes were prepared following the method of Green and Hunt (1980). Sibling species identifications were based on the diagnostic paracentric inversion scheme described below (Subbarao et al., 1988d):

Species A X+a+b, $2+g^1+h^1$, $2i^1/+i^1$
Species B Xab, $2g^1+h^1$
Species C Xab, $2+g^1$ h^1
Species D X+a+b, $2i^1+h^1$
Species E Xab, $2g^1+h^1$, mitotic metaphase Y-chromosome sub-metacentric (Kar et al., 1999).

Specimens (larvae, pupae, and adults) from pooled and iso-female laboratory colonies established from chromosomally identified females were processed directly for DNA isolation. Y-chromosome karyotyping was performed on F_1 male larvae reared from individual females.

Table 1
Cytogenetic and COII-PCR identifications of *Anopheles culicifacies* sibling species in India.

State and district	Chromosome karyotype identification							PCR identification	
	Sp. A			Sp. B	Sp. C	Sp. D	Sp. E[a]	Sp. A/D	Sp. B/C/E
	$+i^1$	$+i^1/i^1$	i^1						
Rajasthan									
Alwar	10	25	25	8	1	–	–	60	9
Haryana									
Sonepat	4[b]	–	–	7[b]	–	–	–	4	7
	6	4	–	1	–	–	–	10	1
Gurgaon	19[b]	5[b]	5[b]	17[b]	–	–	–	29	17
Uttar Pradesh									
Ghaziabad	30	–	–	–	–	–	–	30	0
Madhya Pradesh									
Jabalpur	–	–	–	7	11	42	–	42	18
Tamil Nadu									
Ramanathapuram	–	–	–	–	–	–	2[a,c] + 2[a]	0	4
Orissa									
Sundargarh	–	–	–	–	4	–	–	–	4
Gujarat									
Surat	–	–	–	1	–	–	3[d]	–	4
Kheda	–	–	–	2	–	–	–	–	2
Lab colonies									
Pooled cyclic	2	–	–	2	–	–	–	2	2
Iso-female lines	3[b]	–	–	8[b]	5[b]	–	–	3	13

[a] F_1 females identified by polytrene chromosomes and F_1 males identified by mitotic Y chromosome karyotype.
[b] DNA was extracted by trituration in sterile water.
[c] Two iso-female lines.
[d] Field collected females identified by COII PCR assay.