C. R. Biologies 336 (2013) 449–456

Contents lists available at SciVerse ScienceDirect

Comptes Rendus Biologies

www.sciencedirect.com

Microbiology: bacteriology, mycology, parasitology, virology/Microbiologie : bactériologie, mycologie, parasitologie, virologie

DNA barcoding for the identification of eight species members of the Thai Hyrcanus Group and investigation of their stenogamous behavior

Adulsak Wijit [a], Atiporn Saeung [b,*], Visut Baimai [c], Yasushi Otsuka [d], Sorawat Thongsahuan [e], Kritsana Taai [b], Wichai Srisuka [f], Siripan Songsawatkiat [b], Sriwatapron Sor-suwan [b], Chayanit Hempolchom [b], Pradya Somboon [b], Wej Choochote [b]

[a] *Office of Disease Prevention and Control, 10th, Department of Disease Control, Ministry of Public Health, Chiang Mai 50100, Thailand*
[b] *Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand*
[c] *Department of Biology and Centre for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*
[d] *Department of Infectious Disease Control, Faculty of Medicine, Oita University, Oita 879–5593, Japan*
[e] *Faculty of Veterinary Science (Establishment Project), Prince of Songkla University, Songkhla 90110, Thailand*
[f] *Entomology Section, Queen Sirikit Botanic Garden, PO Box 7, Chiang Mai 50180, Thailand*

ARTICLE INFO

Article history:
Received 20 May 2013
Accepted after revision 2 August 2013
Available online 4 September 2013

Keywords:
Anopheles
Hyrcanus Group
DNA barcodes
Stenogamy
Eurygamy

ABSTRACT

Eight species members of the Thai Hyrcanus Group were identified based on the intact morphology and molecular analysis (COI barcoding, 658 bp) of F_1-progenies. Five isofemale lines of each species were pooled in order to establish stock colonies. A stenogamous colony of each species was investigated by making 200 and 300 newly emerged adult females and males co-habit in a 30 cm cubic cage for one week. After ovipositon, the spermathecae of females were examined for sperms. The results revealed that *Anopheles argyropus*, *Anopheles crawfordi*, *Anopheles nitidus*, *Anopheles pursati*, *Anopheles sinensis*, *Anopheles nigerrimus*, *Anopheles paraliae* and *Anopheles peditaeniatus* yielded insemination rates of 0%, 0%, 0%, 31%, 33%, 42%, 50% and 77%, respectively. Continuous selection to establish stenogamous colonies indicated that *An. sinensis*, *An. pursati*, *An. nigerrimus*, *An. paraliae* and *An. peditaeniatus* provided insemination rates of 33–34%, 27–31%, 42–58%, 43–57% and 61–86% in 1, 2, 5, 6 and 20 generations of passages, respectively.

1. Introduction

The Hyrcanus Group Reid (Genus *Anopheles*, Subgenus *Anopheles*) is distributed widely from Europe to East and Southeast Asia, including some of the off-lying islands of the Indian and Pacific Oceans, and at least 27 species are reported within it [1]. In Thailand, eight species members of the Hyrcanus Group have been reported so far, i.e., *Anopheles argyropus*, *Anopheles crawfordi*, *Anopheles nigerrimus*, *Anopheles nitidus*, *Anopheles paraliae*, *Anopheles peditaeniatus*, *Anopheles pursati*, and *Anopheles sinensis* [2–4]. Among these, *An. nigerrimus*, *An. peditaeniatus* and *An. sinensis* are considered as suspected vectors of malaria due to *Plasmodium vivax* [5–9], while *An. sinensis* and *An. peditaeniatus* have been incriminated as vectors of *P. vivax* in China and Korea [10,11] and Japanese

* Corresponding author.
E-mail address: atiporn44@yahoo.com (A. Saeung).

1631-0691/$ – see front matter © 2013 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.crvi.2013.08.001

encephalitis virus in China and India [12–14], respectively. Even though *An. peditaeniatus* has been found abundantly and widely distributed throughout Thailand [3,15], its status as a vector of the Japanese encephalitis virus is still a cryptic question, which needs to be investigated more intensively. Recently, *An. sinensis* and *An. nigerrimus* have been incriminated as a main vector and secondary or incidental vector, respectively, of *Wuchereria bancrofti* in Asia [16]. In addition, *An. peditaeniatus*, *An. crawfordi*, *An. nigerrimus, An. argyropus* and *An. pursati* were reported as high potential vectors of nocturnally subperiodic *Brugia malayi* [17]. Likewise, the *Anopheles hyrcanus* group was also considered as an economic pest of cattle because of its vicious biting behavior and ability to transmit cervid filariae of the genus *Setaria* [2,3].

Establishment of anopheline colonies is the backbone of mosquito researches, and the inability to create a healthy colony of difficult-to-rear species is the principal cause behind every failure in research efforts. Very few research experiments concerning the Hyrcanus Group have been documented, during the past two decades, particularly those with a complete multidisciplinary approach (combination of related-aspects of morphology, cytology, molecular investigation, hybridization, susceptibility and refractory to pathogens, etc.), although eight species members of the Hyrcanus Group are found throughout Thailand and/or other Southeast Asian countries [2,3]. This might result from the lack of biological information and/or available laboratory-raised colonies, particularly the adaptive stenogamous colonies that are easy to maintain and mass produce, which reduces time, workload and manpower for artificially mating adult females with males. Hence, this paper reports establishment of a stenogamous colony of *An. peditaeniatus* that has existed for more than 20 successive generations, as well as promising possible stenogamous colonies of *An. paraliae* and *An. nigerrimus*, which are still being established and detailed. Furthermore, the utility of DNA barcoding, which is incorporated with the taxonomic key for exact identification of the eight species members, is present as well.

2. Materials and methods

2.1. Mosquito species and strains

Eight species members of the Hyrcanus Group were collected in five provinces of western and southern Thailand, where malaria and filariasis are endemic due to *Plasmodium falciparum* and *P. vivax*, and *W. bancrofti*, respectively [16,18]. The species and strains were as follows: *An. argyropus* (Nakhon Si Thammarat strain: 08°29′N, 100°0′E), *An. crawfordi* (Trang strain: 07°33′N, 99°38′E), *An. nigerrimus* (Songkhla strain: 07°13′N, 100°37′E), *An. nitidus* (Phang Nga strain: 08°27′N, 98°31′E), *An. paraliae* (Ratchaburi strain: 13°30′N, 99°54′E), *An. peditaeniatus* (Phang Nga strain: 08°27′N, 98°31′E), *An. pursati* (Ratchaburi strain: 13°30′N, 99° 54′E) and *An. sinensis* (Chumphon strain: 10°29′N, 99°11′E). Wild caught, fully engorged females of these species members were collected from cow-baited traps.

2.2. Species identification

Identification of wild caught females followed standard illustrated keys [2–4]. Subsequently, identification using intact morphology of eggs, larvae, pupal skins and adult females were performed intensively in F_1-progenies of iso-female lines.

2.3. Molecular investigation

In order to guarantee the exact morphological species identification, thus, individual F_1-progeny adult female of each iso-female line was performed for DNA extraction and amplification. Genomic DNA was extracted using DNeasy® Blood and Tissue Kit (QIAGEN, Japan). The LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) barcoding primers of Folmer et al. [19] were used to amplify the cytochrome *c* oxidase subunit I (COI) region of mitochondrial DNA (658 bp, excluding primers). Each PCR reaction was carried out in a 20-μL volume containing 0.5 U of *Ex Taq* (Takara, Japan), 1X of *Ex Taq* buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.25 μM of each primer, and 1 μL of the extracted DNA. The amplification profile comprised initial denaturation at 94 °C for 1 min, 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The amplified products were electrophoresed in 1.5% agarose gels and stained with ethidium bromide. Lastly, the PCR products were purified using the QIAquick® PCR Purification Kit (QIAGEN, Japan) and their sequences directly determined using the BigDye® V3.1 Terminator Cycle Sequencing Kit and 3130 genetic analyzer (Applied Biosystems of Life Technologies, Japan). The sequence data obtained have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under the following accession numbers: *An. argyropus* (Nakhon Si Thammarat strain: AB781747-AB781751), *An. crawfordi* (Trang strain: AB781752-AB781756), *An. nigerrimus* (Songkhla strain: AB781757-AB781761), *An. nitidus* (Phang Nga strain: AB781762-AB781766), *An. paraliae* (Ratchaburi strain: AB781767-AB781771), *An. peditaeniatus* (Phang Nga strain: AB781772-AB781776), *An. pursati* (Ratchaburi strain: AB781777-AB781781) and *An. sinensis* (Chumphon strain: AB781782-AB781786). The newly COI sequences were also compared with those available in GenBank using the Basic Local Alignment Search Tool (BLAST) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. *Anopheles gambiae* (accession number NC_002084) and *Anopheles braziliensis* (accession number DQ076238) were used as outgroup taxa [20,21]. Sequences were aligned with BioEdit version 7.0.5.3 [22]. Genetic distance was calculated using the Kimura two-parameter (K2P) model [23]. Using the distances, construction of neighbor-joining trees [24] and bootstrap test with 10,000 replications were performed with the MEGA version 4.0 program [25]. Bayesian analysis was conducted with MrBayes 3.2 [26] by using two replicates of 1 million generations with the nucleotide evolutionary model. The