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Morphological characterization and analysis of molecular mediated genetic variations in *Anopheles subpictus s.l.* (Diptera: Culicidae) in Sri Lanka

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Declaration by the candidate

"The work described in this thesis was carried out by me under the supervision of Prof. B. G. D. N. K. De Silva and Dr. N. V. Chandrasekharan and a report on this has not been submitted in whole or in part to any university or any other institution for another Degree/Diploma".

D. P. W. Jayatunga-Katuwawalage

We certify that the above declaration made by the candidate is true and accurate.

Prof. B.G.D.N.K. De Silva.

Dr. N.V. Chandrasekharan.

Certification of supervisors

We certify that the candidate has incorporated all corrections, additions and amendments recommended by the examiners.

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Adenine	- A
ABC	-ATP-Binding Cassette
AFLP	- Amplified Fragment Length Polymorphism
AMC	- Anti-Malaria Campaign
BLAST	- Basic Local Alignment Search Tool
BoLD	- Barcode of Life Database
BSC	- Biological Species Concept
bp	- base pair
C	- Cytosine
°C	- Centigrade
CBCs	- Compensatory Base Changes
CBHC	- Cattle-Baited Hut Collection
CBNC	- Cattle-Baited Net Collection
CHC	- Cuticular Hydrocarbons
COI	- Cytochrome *c* Oxidase I
CR	- Control region
Cyt-b	- Cytochrome *b*
DDT	-Dichlorodiphenyltrichloroethane
DNA	- Deoxyribo Nucleic Acid
dNTP	- deoxynucleotides

EtBr	- Ethidium Bromide
G	-Guanine
GC-MS	- Gas Chromatography-Mass Spectrometry
HMM	- Hidden Markov Models
HMS	-Hybrid Male Sterility
ITS1	- Internal Transcribed Spacer 1
ITS2	- Internal Transcribed Spacer 2
kb	-kilo base pair
Mb	- Mega base pair
MCMC	- Markov Chain Monte Carlo
ML	- Maximum Likelihood
mM	-millimoles per liter
mtDNA	- mitochondrial DNA
NCBI	- National Center for Biotechnology Information
ng	- nanogram
NJ	- Neighbor-Joining
NTS	- Non-Transcribed Spacer
PCR	-Polymerase Chain Reaction
RAPD	- Random Amplified Polymorphic DNA
RFLP	- Restriction Fragment Length Polymorphism
RNA	- Ribonucleic acid
rpm	- resolutions per minute
scnDNA	- single-copy nuclear DNA

SNP	- Single Nucleotide Polymorphism
SRF	- Spectral Repeat Finder
SSCP	- Single Strand Conformation Polymorphism
STR	- Simple Tandem Repeats
T	-Thymine
TEDHA	-Tropical and Environmental Disease & Health Associates
TROL	- Tandem Repeat Occurrence Locator
ts	- transitions
tv	- transversions
U	- Uracil
U	- Units
UPGMA	- Unweighted Pair Group Method with Arithmetic Mean
V	- Volt
WHO	- World Health Organization
µg	- microgram
µl	- microliter

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by

Dona Pamoda Wajirapanie Jayatunga-Katuwawalage

ABSTRACT

Anopheles subpictus sensu lato is an important secondary vector of malaria in Sri Lanka. Literature based on morphology and cytology evidence *An. subpictus* to be a species complex consisting sibling species A, B, C and D. Recent molecular based studies carried out in Sri Lanka using Internal Transcribed Spacer 2 (ITS2) and Cytochrome *c* Oxidase subunit-I (*COI*) gene have identified two sibling species, *An. subpictus* A and B. However, characterization of sibling species of *An. subpictus* using several molecular markers is yet to be established. Thus, objective of the present study was to determine validity of standard taxonomic keys based on mophomertry and identification of genetic variations in selected nuclear DNA markers and mitochondrial DNA markers among different populations of *An. subpictus s.l.* in Sri Lanka. As the first step of the study morphometric features of larvae, pupae and adults of morphologically confirmed *An. subpictus* specimens which had been collected from five localities in the country were observed through laboratory rearing. In identification of molecular variations, five DNA loci (3 nuclear markers ITS2, D3, *white* gene and 2 mitochondrial DNA markers; *COI* and *Cyt-b*) were subjected to PCR amplification and DNA sequencing. The resulted DNA sequences were analyzed using

modern bioinformatics tools and phylogenies of UPGMA, NJ, ML and Bayesian inference for each loci. Further, for ITS2, secondary structures were constructed and analyzed for the presence of Compensatory Base Changes.

Significant morphological variations were detected in standard morphometric features including larval mesothoracic seta 4 and adult palpi. Phylogenetic analyses of each DNA marker, together with two distinct ITS2 secondary structures confirmed the presence of two sibling species that corresponded to sibling species A and B. The two ITS2 secondary structures were found to have two CBCs each, denoting their reproductive isolation. Most importantly, in addition to *COI* and *Cyt-b* which inherent high mutation rates, low to moderate levels of polymorphism were detected in ITS2 and single-copy nuclear gene, *white*, in terms of many haplotypes. Present investigations highlighted that morphological characterization of *An. subpictus* using currently available standard morphometric characters was erroneous and hence could no longer be recommended as valid for *An. subpictus* species identifications in Sri Lanka. Further, findings of current study confirmed the presence of two distinct sibling species A and B based on molecular mediated genetic variations of *An. subpictus* populations in Sri Lanka. Also, extent of polymorphism in ITS2 and *white* gene indicated the potential occurrence of more yet unexplained sibling species in *An. subpictus* species complex in Sri Lanka. Therefore, more intensive molecular characterization of the species from across the island should be undertaken to verify unknown members and also to determine present vector status of *An. subpictus* complex in Sri Lanka.