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Resolving genetic diversity in Australasian *Culex* mosquitoes: Incongruence between the mitochondrial cytochrome *c* oxidase I and nuclear acetylcholine esterase 2

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ABSTRACT

Insects that vector pathogens are under constant surveillance in Australasia although the repertoire of genetic markers to distinguish what are often cryptic mosquito species remains limited. We present a comparative assessment of the second exon–intron region of the acetylcholine esterase 2 gene (ace-2) and the mitochondrial DNA cytochrome *c* oxidase I (COI) using two closely related Australasia mosquitoes *Culex annulirostris* and *Culex palpalis*. The COI revealed eight divergent lineages of which four were confirmed with the ace-2. We dissect out the nuclear chromosomal haplotypes of the ace-2 as well as the exon–intron regions by assessing the protein's tertiary structure to reveal a hypervariable 5'-exon that forms part of an external protein loop and displays a higher polymorphic rate than the intron. We retrace the evolutionary history of these mosquitoes by phylogenetic inference and by testing different evolutionary hypotheses. We conclude that DNA barcoding using COI may overestimate the diversity of *Culex* mosquitoes in Australasia and should be applied cautiously with support from the nuclear DNA such as the ace-2. Together the COI and ace-2 provide robust evidence for distinct cryptic *Culex* lineages—one of which correlates exactly with the southern limit of Japanese encephalitis virus activity in Australasia.

1. Introduction

Despite the importance of mosquitoes as vectors of pathogens that cause significant human and animal diseases, their genetic diversity still requires accurate detailing while an understanding of their phylogeography and dispersal has the potential to resolve longstanding taxonomic and epidemiological questions. *Culex annulirostris* Skuse (Diptera: Culicidae) is an important vector of the Japanese encephalitis virus (JEV) in Papua New Guinea, Indonesia and surrounding islands and territories (Russell and Dwyer, 2000). This morphospecies often occurs in sympatry with its closely related sister species *C. palpalis* and their similar morphology makes studies on these mosquitoes difficult (Beebe et al., 2002; Chapman et al., 2003, 2000). Allozyme studies and observations from a PCR diagnostic developed for these mosquitoes suggested the existence of a complex of cryptic species (Beebe et al., 2002; Chapman et al., 2003, 2000). Mitochondrial DNA cytochrome oxidase I (COI) studies supported these findings by identifying eight separate lineages within the COI sequences (Hemmerter et al., 2007). Importantly, mitochondrial lineages of *C. annulirostris* showed a southern limit matching that of JEV activity in Australasia, leading to the hypothesis that JEV's establishment may be dependent on a specific mtDNA lineage or cryptic taxa (Hemmerter et al., 2007). However, confirmation is needed to determine if the *C. annulirostris* and *C. palpalis* lineages identified are reproductively isolated.

The acetylcholinesterase 2 gene (ace-2) is currently the only protein coding nuclear marker used for the molecular identification of mosquitoes and has been used for discrimination of the *C. pipiens* complex (Bourguet et al., 1998; Kasai et al., 2008; Sanogo et al., 2007; Smith and Fonseca, 2004). The ace-2 codes for a crucial nerve response enzyme acetylcholinesterase (AChE). The function of synaptic AChE is to stop the neurotransmission in the central nervous system of insects by hydrolyzing the neurotransmitter acetylcholine into acetate and choline (Toutant, 1989). Due to AChE's function, it is the target of two major classes of insecticides used for pest management in agriculture and public health (Hemingway et al., 2004).

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The aim of this study was to identify a polymorphic region of ace-2 and test its usefulness as a phylogenetic marker for identification of cryptic taxa within *C. annulirostris* and *C. palpalis* in Australasia. We used the three-dimensional structure of *Drosophila melanogaster* AChE as the proxy to map the nucleotide diversity of the second intron to the flanking exons which code for a hypervariable external loop in *Culex* mosquitoes. The genetic diversity of ace-2 and COI is compared and the evolutionary history of these mosquitoes is retraced in a phylogenetic framework. We conclude that the external loop region of ace-2 is a useful marker to investigate diversity of *Culex* mosquitoes and strongly supports the existence of a novel divergent COI *Culex* lineage matching the southern limit of JEV activity in Australasia.

2. Material and methods

2.1. Specimen collection and identification

Mosquitoes were collected from a wide geographic range that included Australia, PNG, Timor Leste and the Solomon Islands (Bougainville to the north and Guadalcanal to the south) (Fig. 1, Supplementary Table S1). Adult mosquitoes were collected using CO_2-baited encephalitis virus surveillance traps both with and without 1-octen-3-ol (octenol). Specimens were morphologically identified using the available keys (Lee et al., 1989; Marks, 1982). Mosquitoes were then stored in either liquid nitrogen or on dry ice, on silica gel, or in 70–100% ethanol prior to DNA extraction.

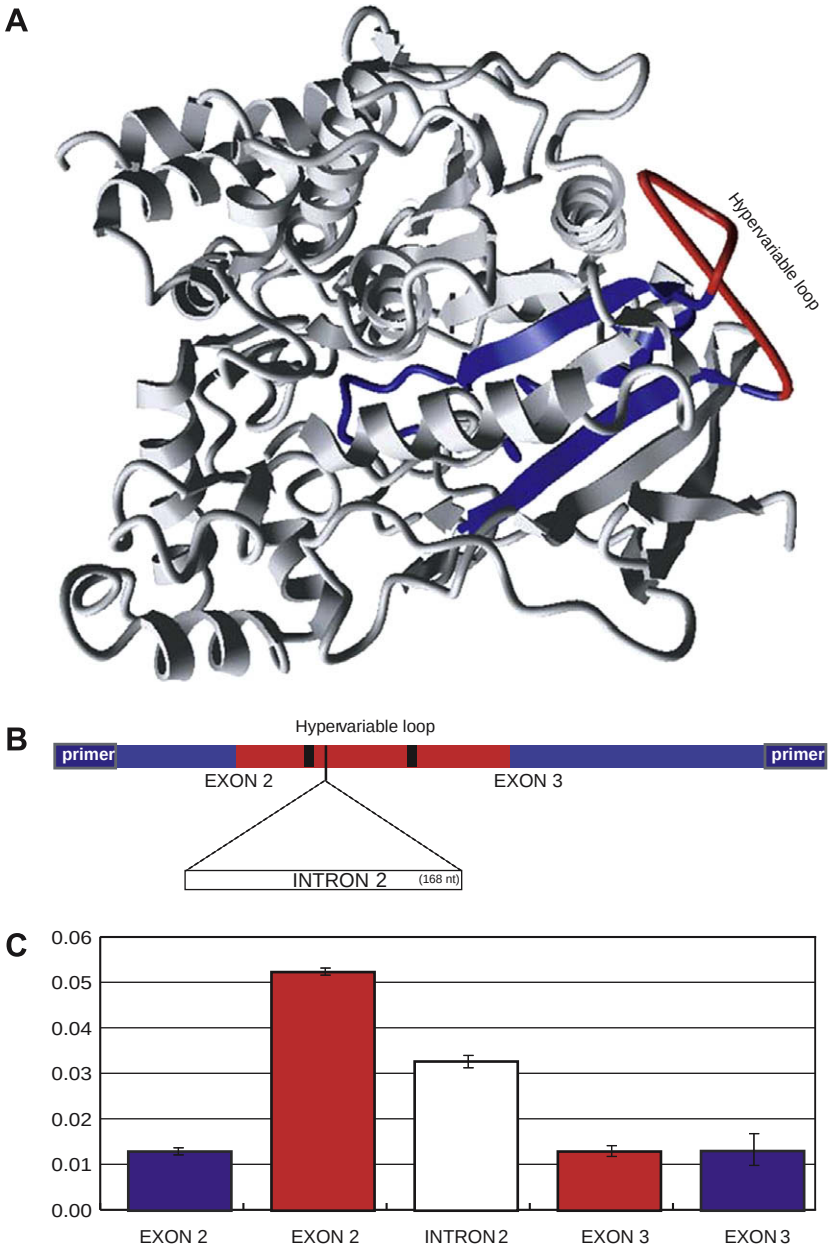

Fig. 1. Three-dimensional structure of the *Drosophila melanogaster* acetylcholinesterase, DmAChE (PDB: 1QO9) with the external hypervariable loop insertion added manually using YASARA (A). This "insertion" is not drawn to scale and is 36 amino acids long for the DmAChE. (B) Graphical representation of the ace-2 intron–exon region amplified in this study which is 392 residues. The vertical bars within the external loop region represent amino acid changes within *C. annulirostris* and *C. palpalis*. (C) Frequency of nucleotide diversity per site of the ace-2 sequence for *C. annulirostris* and *C. palpalis* relative to its position and functionality. The nucleotide diversity per site is showed as a histogram: blue and red = exon; white = intron; red = exon coding for the external loop of the ace-2 protein. Standard deviations are represented for each region.