Utilizing Molecular Techniques to Distinguish Mosquito Species and Populations

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ABSTRACT

There are 180 species of mosquitoes established in the United States, many of which are important vectors for a number of diseases affecting human health. The purpose of this project was to distinguish among mosquito species and between mosquito populations using genetic analysis of mitochondrial DNA. Eight individual mosquitoes, encompassing five species, from varying geographical regions were studied for this purpose. Using a primer pair that amplified a region of the NADH dehydrogenase 5 subunit gene, it was possible to distinguish among all five species. On the population level, pairs of three different species were analyzed from differing locations. Two species pairs could not be distinguished, however the third pair resulted in 99% similarity with two base pair differences. These results indicate there is potential to utilize this technique to distinguish between both species and populations of mosquitoes.

Keywords: mosquito barcoding, population genetics, mitochondrial DNA, *Aedes, Ochlerotatus, Anopheles*

INTRODUCTION

Mosquitoes belong to a family of insects, called Culicidae, in the true fly order Diptera. There are over 3,000 species described worldwide, 180 of which are found in North America (Harbach & Kitching 1998, Willott & Ramberg 2007). Female mosquitoes must consume blood in order to obtain the necessary nutrients for laying eggs and readily feed on humans and other

animals (Tanaka et al. 1979). In addition to being considered pests, mosquitoes are important vectors for a number of infectious diseases including malaria, West Nile virus, yellow fever, and LaCrosse encephalitis (CDC 2007). Worldwide, hundreds of millions of people suffer from the effects of mosquito borne illnesses every year and hundreds of thousands of them die (WHO 2013). In addition to spreading disease to humans, mosquitoes are also spread pathogens to domesticated pets and other animals, for example the parasitic roundworm that causes heartworm in dogs and cats (Ledesma & Harrington 2011). The expansion of a number of species northward increases the risk for more disease outbreaks in the future (Rochlin et al. 2013).

For this study, five mosquito species with distributions in the eastern United States were selected for analysis including: *Ochlereotatus triseriatus, Ochlerotatus japonicus, Aedes albopictus, Anopheles punctipennis*, and *Anopheles quadrimaculatus*. *Oc. triseriatus*, a species native to North America, is an important vector for LaCrosse encephalitis (Alto 2011). Populations of this species have declined in conjunction with the introduction of invasives *Oc. japonicus* and *Ae. albopictus* (Hardstone & Andreadis 2012, Alto 2011). These aggressive invaders are capable of carrying a number of types of encephalitis including West Nile virus, and, like *Oc. triseriatus*, are active and biting during the day (Turell et al. 2001). Additionally, *Ae. albopictus* is a competent vector for the emerging infectious disease chikungunya (Vega-Rúa et al. 2014). While not yet established in North America, there was an outbreak of the virus in the Caribbean islands in 2013 and there concerns for potential outbreaks in the United States (Vega-Rúa et al. 2014). *An. punctipennis* and *An. quadrimaculatus* were important vectors for malaria in the eastern U.S. in the mid 1900's (Molaei et al. 2009). They can also carry heartworm and

various forms of encephalitis including eastern equine encephalitis, a rare but potentially very serious disease, and feed primarily at night (Brown et al. 2012, Molaei et al. 2009).

Mitochondrial DNA (mtDNA) has been utilized in mosquito phylogenetic studies for analyzing both interspecific and intraspecific variation (Birungi & Munstermann 2002, Avise 1994). These genes have a population size one-fourth that of nuclear genes and, as a result, mtDNA tends to be more sensitive to differentiation by genetic drift than nuclear DNA (Birky et al. 1982). MtDNA also evolves five to ten times faster due to high mutation rates (Stoneking et al. 1990). The NADH dehydrogenase 5 subunit (ND5) is an especially variable protein-coding gene, and researchers have had success in categorizing the genetic structures of mosquitoes (Besansky et al. 1997, Birungi & Munstermann 2002. The objectives of this study are to distinguish among species and between populations of mosquitoes by using a primer pair that amplifies a region of mtDNA in the ND5 gene.

METHODS

Mosquito Collection

Eight adult mosquitoes constituting five different species were collected for analysis. Specimens included: two individuals of the species *Ae. albopictus*, two individuals of the species *Oc. triseriatus*, two individuals of the species *An. punctipennis*, one individual of the species *An. quadrimaculatus*, and one individual of the species *Oc. japonicus* (Table 1). Paired species samples were collected from different regions for the purpose of population comparison. The two *Ae. albopictus* and two *Oc. triseriatus* specimens were collected from different counties in NY State and the two *An. punctipennis* individuals were collected from NY and TN.

Mosquitoes were caught utilizing one of three battery-operated traps: a Centers for Disease Control and Prevention (CDC) light trap, a CDC gravid trap, or a CDC aspirator (John W. Hock Co., Gainesville, FL). CDC light traps are devices that emit light and carbon dioxide for luring female mosquitoes. Gravid traps utilize water filled containers that are high in organic material for the attracting of gravid, or ovipositing, females. Aspirators consist of a backpack and hose and use suction for collecting of male and female resting mosquitoes.

Table 1. Collected mosquito specimens.

Sample #	Species	County, state	Year	Condition
1	*Ae. albopictus*	Westchester, NY	2013	Frozen
2	*Ae. albopictus*	Rockland, NY	2012	Frozen
3	*Oc. triseriatus*	Westchester, NY	2009	Frozen
4	*Oc. triseriatus*	Sullivan, NY	2006	Frozen
5	*An. punctipennis*	Sullivan, NY	2005	Frozen
6	*An. punctipennis*	Weakley, TN	2012	Dried
7	*An. quadrimaculatus*	Sullivan, NY	2005	Frozen
8	*Oc. japonicus*	Westchester, NY	2003	Frozen

Molecular Techniques

Each sample was homogenized using a FastPrep®-24 instrument (MP Biomedicals, Solon, OH, USA). DNA from the specimens was extracted using the FastDNA® Spin Kit for soil (MP Biomedicals, Solon, OH, USA). Samples were diluted into 5ng/ul solutions and prepared for amplification by polymerase chain reaction (PCR). A primer pair, that amplified a 450 bp region of ND5, was selected from the literature and tagged with T7 and SP6: forward primer 5′-TCCTTAGAATAAAATCCCGC-3,′ reverse primer 5′-GTTTCTGCTTTAGTTCATTCTTC-3′ (Birungi & Munstermann 2002). PCR total reaction volumes were 20 µl including 1µl extracted DNA, 10 µl GoTaq Green 2x (Promega, Madison, WI, USA), 0.5 µl forward primer, 0.5 µl reverse primer, and 8.0 µl dH_2O. Eight samples were

run plus a negative control using the following reaction parameters: 95˚C for 3:00 min for initial denaturation of DNA, followed by 50 cycles of 94˚C at 0:30 min, 58˚C at 0:30 min and 72˚C at 0:30 min, and ending with a final extension at 72˚C for 2:00 min.

PCR products were visualized on a 1% agarose gel. After electrophoresis, samples were purified using the QIquick PCR Purification kit (Qiagen, Venio, Netherlands). Purified PCR products were sent to Genewiz, Inc. (South Plainfield, NJ, USA) for Sanger sequencing.

Sequence Analysis

Sequences were edited using A plasmid Editor (ApE, M. Wayne Davis, University of Utah) and aligned using Clustal Omega (European Bioinformatics Institute, Cambridgeshire, UK). Pairwise comparisons between each of the five species were conducted by inserting the sequences into Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, Bethesda, MD, USA) to determine the percent similarity. This process was repeated between the species pairs to compare within species populations.

RESULTS

Among Species Comparisons

The target region of mtDNA was successfully amplified in the specimens of the five species used in this study (Figure 1). Each band visualized on the 1% agarose gel represented PCR product of 450 bp.

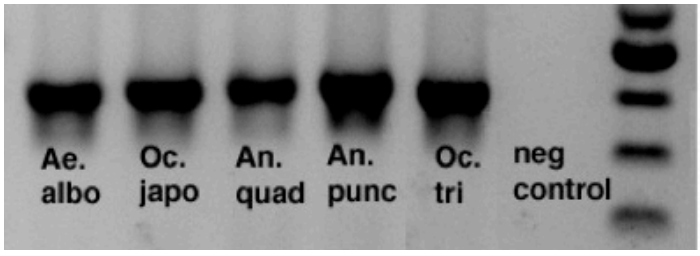

Fig 1. PCR product from 5ng of DNA of five mosquito species.

The sequences for the five species were edited down to 219 bp and aligned in Clustal Omega (Figure 2). There were a number of base pair differences between each species as determined by studying the alignments.

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Pun       CCCTGCACACATAAATAATAAAGCCTTAAAAAGAGCATGAGTTAAAAGATGAAAAAAAGC  60
Quad      CCCAGCACATATAAATAATAACGCCTTAAATAATGCGTGTGTTAAAAGATGAAAAAATGC  60
Jap       CCCTGCACACATAAATAATAAAGCCTTAAATAAAGCATGAGTTAATAAATGAAAAAAAGC  60
Alb       CCCAGCACATATAAATAATAATGCTTTAAATAAAGCATGTGTTAATAAATGAAAAAAAGC  60
Tri       TCCTGCACATATAAATAATAAAGCCTTAAATAAAGCATGAGTTAATAAATGAAAAAAGGC  60
           ** ***** ********** ** ***** *  ** ** ***** * ********* **

Pun       TAATTTATAAAATCCTATAGATAAAATTCTTATTATCAACCCTAATTGACTTAAAGTAGA  120
Quad      TAATTTATAAAACCCTATAGATAAAATTCTTATTATTAACCCTAACTGTCTTAAAGTAGA  120
Jap       TAATTTTAAAAATCCTATTGATAAAATGCTTATTATTAACCCTAATTGACTTAGAGTCGA  120
Alb       TAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAATGTAGA  120
Tri       TAATTTATAAAATCCAATTGATAAAATTCTCATTATTAACCCTAACTGACTTAAAGTAGA  120
          *** **  **** ** ** ******** ** ***** ** ***** ** ****  ** **

Pun       TAAAGCAATAATTTTTTTTAAATCAAATTCAAAATTAGCCCCTAACCCCGCTATAAATAT  180
Quad      CAAAGCAATAATTTTTTTTAAATCAAATTCAAAATTAGCCCCTAACCCAGCTATAAATAT  180
Jap       TAAAGCAATGATTTTTTTTAAATCAAATTCAAAATTTGCCCCTAACCCAGCTATAAATAT  180
Alb       TAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATAT  180
Tri       TAATGCAATAATTTTTTTTAAATCAAATTCAAAATTAGCCCCTAACCCTGCTATAAATAT  180
           ** ***** ** ********************** ** ***** ** ***********

Pun       TGTTAACCCTGAAATTAATAATATAAATTGGCCTATTCA       219
Quad      TGTTAACCCAGAAATTAATAACATAAATTGCCCTATTCA       219
Jap       AGTTAGCCCAGAAACTAGTAACATAAATTGACCCAAAAA       219
Alb       AGTTATTCCGGATATTAATAATAAAAATTGACCTAATGT       219
Tri       TGTTAAACCAGAAATTACTAATAAAAATTGACCTAATAA       219
           ****  ** ** * ** *** * ****** ** *
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Fig 2. Sequence alignment of five mosquito species in Clustal Omega.

Pairwise comparisons of the sequences revealed base pair differences between each of the individual species (Table 2). The greatest homology between sequences was seen in the two Anopheles species at 92% base pair similarity, while the greatest differences were seen between *An. quadrimaculatus* and *Ae. albopictus* and between *An. quadrimaculatus* and *Oc. japonicus* at 86% base pair similarity.

Table 2. Pairwise comparisons between the five mosquito species (% similarity).

	Ae. albo.	*Oc. japo.*	*Oc. tris.*	*An. quad.*	*An. punc.*
Ae. albo.	100				
Oc. japo.	88	100			
Oc. tris.	90	90	100		
An. quad.	86	86	89	100	
An. punc.	87	88	90	92	100

Within Species Comparisons

The target region was also amplified successfully in two specimens from each of three mosquito species (Figure 3)

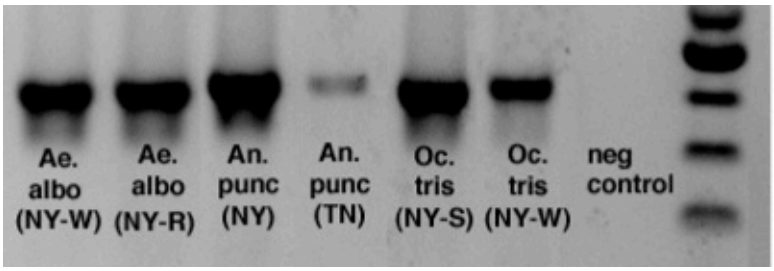

Fig 3. PCR product from 5 ng of DNA of six individual mosquitoes constituting three species.

The sequences for the two *Ae. albopictus* individuals demonstrated 100% similarity, as did the sequences for the two *Oc. triseriatus* specimens. Between the two *An. punctipennis* individuals, however, the sequences showed 99% similarity (Table 3).

Table 3. Pairwise comparisons within species.

Species Pair	% Similarity
Ae. albo. **(NY-W)** ***Ae. albo.*** **(NY-R)**	100
Oc. tris. **(NY-W)** ***Oc. tris.*** **(NY-S)**	100
An. punc. **(NY)** ***An. punc.*** **(TN)**	99

Sequences for the *An. punctipennis* samples differed by two base pairs (Figure 4). To determine if these substitutions altered the amino acid sequence, the Expasy (Swiss Institute of Bioinformatics, Switzerland) translate tool referencing the genetic code of invertebrate mitochondrial DNA was used to translate the nucleotide sequence. It was determined that the amino acid sequence was not altered and the base substitutions were confirmed as synonymous.

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PunNY   1bp...  TTAGCCCCTAACCCCGCTATAAATATTGTTAACCCTGAAATTAATAATATAAATTGGCCT
PunTN           TTAGCTCCTAACCCCGCTATAAATATTGTTAACCCTGAAATTAATAATATAAATTGACCT  ... ≈ 200bp
                ***** ********************************************** ***
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Fig 4. Sequence alignment between the two *An. punctipennis* samples, one from NY and one from TN, in Clustal Omega. Base pair differences are highlighted in red.

DISCUSSION

The ND5 primer pair was able to successfully amplify DNA sequences that were distinguishable among all five species. Closely related mosquitoes of the same genera demonstrated at least 8% base pair differences, indicating this technique may be useful in

developing DNA barcodes for species identification. Some species among certain genera in particular, for example *Culex*, are often difficult to identify based on morphological structures alone. Further analysis may also be useful for phylogenetic studies in determining genetic relatedness among various species.

While the sequences of the two *Ae. albopictus* and the two *Oc. triseriatus* individuals were 100% homologous, the ND5 region sequence allowed me to distinguish between the *An. punctipennis* specimens from NY and TN. These results support the findings of previous studies indicating the potential usefulness of ND5 primer pairs in distinguishing among populations of mosquitoes (Birungi & Munstermann 2002). The greater distance between the NY and TN mosquitoes, compared to the relatively short distances between the other two pairs in NY, may have increased the likelihood of genetic differences. A sample size of one mosquito from each location, however, is not sufficient for a more concrete analysis. There is no way to ascertain whether the mosquitoes were from populations exclusive to the NY or TN area, or if they coexist in both regions. Collecting more individuals of the same species from various locations and repeating these techniques would need to be done in order to better understand the biogeographical relationship of these populations.

In addition to determining variation among populations, interspecific analyses of invasive species can provide clues in regards to location of origin and details surrounding the introduction. Many species of mosquitoes are highly invasive and in order to control for or monitor potential future introductions it is important to understand from where these mosquitoes are being introduced (Kamgang et al. 2013, Fonseca et al. 2010). Additionally, it is not uncommon for there to be multiple introductions, such as is the case of the *Oc. japonicus* populations established in Washington and Oregon compared to the populations established on the east coast

(Fonseca et al. 2010). Understanding these trends and being able to distinguish among populations may have implications for the monitoring of future introductions and for disease transmission (Fonseca et al. 2010).

Future studies with more samples and species will need to be conducted in order to confirm the usefulness of this technique for developing mosquito species barcodes, distinguishing among mosquito populations, and for analyzing the population genetics of invasive species.

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