Molecular Confirmation of Novel Morphological Characters to Identify Two Medically Important Mosquito (Diptera: Culicidae) Species That Were Previously Indistinguishable as Adult Females

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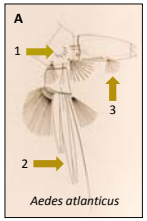
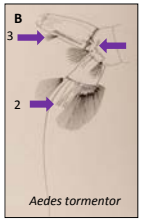
Current Problem

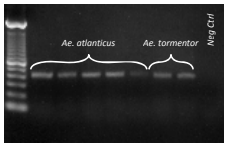


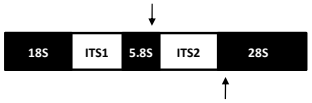


Distinct morphological characters exist to identify *Ae. atlanticus* **[A]** and *Ae. tormentor* **[B]** larvae (left) and adult males (not shown). Differences in the comb scales [1], anal papilli [2], and setae 1-S [3] support the current taxonomic assessment that the two mosquitoes are distinct species.

To date, there is not an effective way to morphologically distinguish both species as adult females. The Centers for Disease Control DVBID website **[Image Right]** does not distinguish the adult females and they are simply referred to as *Aedes atlanticus/tormentor*. An effective assay to distinguish both species would allow for proper epidemiological data to be collected for both species.

ITS2 PCR





The above agarose gel demonstrates successful amplification of the rDNA ITS2 for *Ae. atlanticus* and *Ae. tormentor* by PCR. Amplification was achieved with conserved primers that anneal to the highly conserved 5.8S and 28S regions of the rDNA cistron. The arrows depict the annealing sites of the CP-1A/CP-1B primer pair (Wesson et al., 1992).

TOPO-TA Cloning

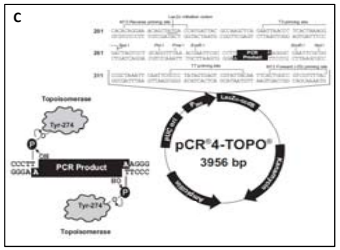
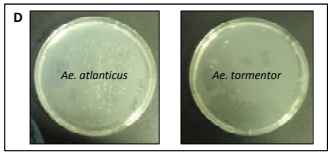
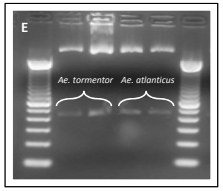


Figure **[C]** shows a map of the pCR4-TOPO cloning vector that was used to clone the ITS2 PCR amplicons for both species. Topoisomerase cleaves the phosphodiester backbone after 5'-CCCTT with energy being conserved by a covalent bond between the 3' phosphate and tyrosol residue (Tyr-274). The bond can be reversed between DNA and enzyme when attacked by the 5' hydroxyl of an original cleaved strand. This can be exploited by PCR products because *Taq* polymerase leaves a nontemplate dependent deoxyadenosine (A) to the 3' end of PCR products.

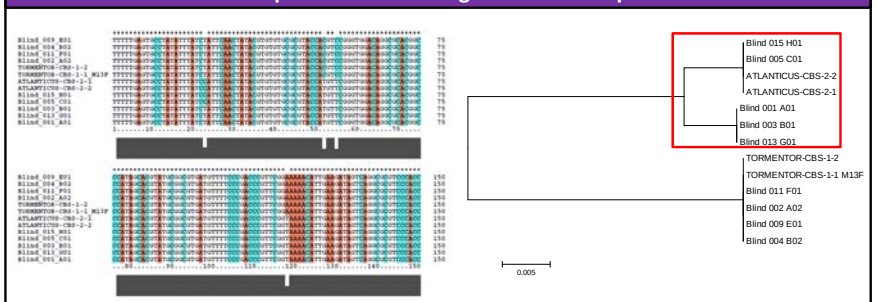
Competent TOP10 *E. coli* cells were transformed **[D]** with the cloning vector containing the ITS2 amplicon and an ampicillin resistance gene. Successful insertion of the amplicon in the plasmid was confirmed using an *Eco*R1 restriction digest **[E]**.





ABSTRACT: *Aedes atlanticus* and *Ae. tormentor* are well known as nuisance mosquitoes and potential vectors of Eastern Equine Encephalomyelitis, Keystone, and West Nile viruses. Unfortunately, these mosquitoes are not readily distinguishable as adult females. Because the adult female is the medically important life stage, a method to distinguish the species should improve surveillance efforts and provide a better understanding of the epidemiological importance of the two species. Molecular methods were employed to investigate the rDNA second internal transcribed spacer (ITS2) as a useful gene target to distinguish the two species. Briefly, DNA was extracted from known specimens and then PCR amplified using conserved ITS2 primers. The resulting amplicons were cloned using the pCR4-TOPO vector and *E. coli* (TOP10 strain). Analyses of the resulting sequences demonstrated minimal size difference but useful sequence heterogeneity (94% sequence similarity). There is a 12 base pair (bp) difference between the ITS2 of both species: 415 bp (*Ae. atlanticus*) and 403 bp (*Ae. tormentor*). Direct sequences of blinded specimens were then obtained and used to successfully validate a novel morphological character that now may be used to distinguish the two species. This study solved a significant problem in mosquito ecology/taxonomy that has existed for more than sixty years.

ITS2 Sequence Data Distinguish the Two Species



Complete ITS2 sequences, partial 5.8S and partial 28S sequences for both species were initially obtained from the cloned PCR amplicons. The sequences were verified as ITS2 after evaluating the results of a BLAST query, secondary structure analysis, and the identification of specific sequence motifs known to exist on the ITS2 of all mosquitoes. Direct sequences (~150 bp) of blinded specimens were then obtained and the sequence heterogeneity was used to successfully identify the species. Furthermore, sequence data was used to validate a novel morphological character that now may be used to distinguish the two species. A multiple sequence alignment (above) was used to identify useful sequence differences between the two species. A phylogenetic approach also proved useful and supported the use of the sequences for species identification. Every blinded specimen was correctly identified using the partial ITS2 sequences obtained from direct sequencing.

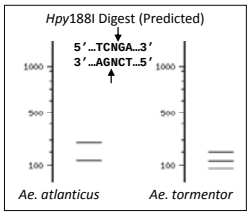
Validation of Novel Morphological Characters

Aedes atlanticus

Aedes tormentor

Novel morphological characters to distinguish the two species were validated using the ITS2 sequence data. The above images demonstrate that on *Ae. tormentor* (right) brown scales located on the occiput do not extend and touch the compound eye. However, the brown scales on *Ae. atlanticus* (left) do extend and touch the compound eye.

Diagnostic Restriction Enzyme Digest



Using sequence data, we have designed (*in silico*) a restriction enzyme assay that should distinguish the two species. The *Hpy*188I enzyme is predicted to cut the PCR amplified ITS2 of *Ae. atlanticus* once and *Ae. tormentor* twice (see underlined sequences above), thus providing a diagnostic assay to distinguish the two species without sequencing. This work is still on-going.

Selected References

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Acknowledgements

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