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RAPID ASSAY TO IDENTIFY THE TWO GENETIC FORMS OF *CULEX (CULEX) PIPIENS* L. (DIPTERA: CULICIDAE) AND HYBRID POPULATIONS

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Abstract. A previously developed method to identify members of the *Culex pipiens* complex exploiting polymorphisms in a nuclear intron (acetylcholinesterase [ACE] based-assay) cannot differentiate the two forms of *Cx. pipiens*: form pipiens and form molestus. Notably, the two forms seem to differ extensively in behavior and physiology and likely have very different epidemiologic importance. Because they are morphologically indistinguishable, molecular methods are critical for the evaluation of their relative importance. Although the two forms of *Cx. pipiens* have been distinguished using a panel of microsatellite loci, such a protocol is laborious and expensive. We developed a rapid assay based on polymorphisms in the flanking region of a microsatellite locus. Used in conjunction with the ACE-assay, this new assay allows the identification of pure and hybrid populations of the two *Cx. pipiens* forms as well as those including *Cx. quinquefasciatus*. We discuss the usefulness of the method as well as limitations to its application.

INTRODUCTION

Understanding vector population dynamics is fundamental to the development of disease control strategies. Complexes of sibling species present unique challenges because of the often-large differences in vectorial capacity between taxa that are morphologically indistinguishable.[1] DNA-based rapid assays have emerged as tools to overcome the challenges of sibling species identification,[2,3] but the *Culex pipiens* complex, which includes important disease vectors,[4,5] has proven particularly difficult. This difficulty likely stems from the very recent divergence of behaviorally and physiologically distinct sub-groups, possibly associated with domestication. The nominal species of the complex, *Culex (Culex) pipiens* L., a temperate species, has two distinct forms: form pipiens and form molestus.[6] Whereas *Cx. pipiens* f. pipiens diapauses, requires a blood meal to lay eggs (anautogeny), and is unable to mate in confined spaces, *Cx. pipiens* f. molestus does not diapause, is able to lay its first batch of eggs without a blood meal (autogeny), and mates in confined spaces (stenogamy).[7] The combination of stenogamy and autogeny seems to allow *Cx. pipiens* f. molestus to occur in underground areas in urban settings.[8] Although conclusive evidence is still lacking, the two forms are thought to have different blood host preferences (pipiens biting mainly birds and molestus mainly mammals, especially humans) and therefore very different vectorial capacities.[9] The two forms have been shown to be genetically isolated in Northern Europe; but there is clear evidence of hybridization in North America.[10] Further complicating the epidemiologic landscape in North America, *Culex (Culex) quinquefasciatus* Say, a tropical and sub-tropical species, hybridizes extensively with the temperate forms (D. M. Fonseca and others, unpublished data).[10,11] *Cx. quinquefasciatus* is non-diapausing, anautogenous, and stenogamous. Also, as a species, it has no distinct preference for birds or mammals as blood sources and can be a vicious human biter.[12,13] Although the shape of the male phallosome (genitalia) is diagnostic for *Cx. pipiens* and *Cx. quinquefasciatus,* the male phallosome is indistinguishable between the two forms of *Cx. pipiens.*[6]

With the objective of studying the phylogenetic relationships between members of the *Cx. pipiens* complex, we sequenced multiple alleles of several of the microsatellite loci currently available for the complex.[14] During that process, we realized that locus CQ11 was diagnostic for the two forms of *Cx. pipiens* and decided to develop it as a rapid assay. The DNA-based rapid assay designed by Smith and Fonseca[15] to identify most of the members of the *Cx. pipiens* complex, as well as other morphologically similar species, does not distinguish between the two forms of *Cx. pipiens*, indicating the need for an additional molecular assay. Although there has been a recent report that several transitions in the mitochondrial cytochrome oxidase subunit I allow the identification of the two forms,[16] that assertion has since been refuted by the authors.[17] Here, we describe a rapid assay that can be used to identify *Cx. pipiens* f. pipiens and *Cx. pipiens* f. molestus based on indels in the flanking region of a microsatellite locus. We also expand this assay to recognize the occurrence of populations that include hybrids of the two genetic forms as well as those that include *Cx. quinquefasciatus*.

MATERIALS AND METHODS

To prevent the need for cloning, we only identified specimens for sequencing that were homozygous at alleles representative of the range of allele sizes of CQ11 in *Culex pipiens*.[14] We amplified using primer pairs CQ11F2/R3[145] or CQ11F/R[18] for *Cx. pipiens* and *Cx. quinquefasciatus*, respectively, using the conditions in Smith and others.[14] To identify and discard Taq polymerase errors, two separate independent amplifications from each individual were sequenced. Cycle sequencing was performed using BigDye Terminator v.3.1 (Applied Biosystems [ABI], Foster City, CA). Sequences were analyzed using a capillary automated sequencer (ABI 3730) and aligned using Sequencher 4.2 (GeneCodes, Ann Arbor, MI). Based on observed diagnostic sequence differences between the two forms, we designed form-specific reverse primers using Primer3.[19] We calculated the number of base pair differences between sequences using Arlequin 3.0.[20] We excluded the actual microsatellite region contained in these sequences from this analysis and express the differences as average percent differences between taxa corrected for intra-taxa variation.[20]

We tested this assay in up to 14 specimens from four populations of *Cx. pipiens* f. pipiens, eight populations of *Cx. pipiens* f. molestus, and four populations of *Cx. quinquefasciatus*

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