Rapid and high throughput molecular identification of diverse mosquito species by high resolution melting analysis [version 1; referees: 2 approved]

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Abstract
Mosquitoes are a diverse group of invertebrates, with members that are among the most important vectors of diseases. The correct identification of mosquitoes is paramount to the control of the diseases that they transmit. However, morphological techniques depend on the quality of the specimen and often unavailable taxonomic expertise, which may still not be able to distinguish mosquitoes among species complexes (sibling and cryptic species). High resolution melting (HRM) analyses, a closed-tube, post-polymerase chain reaction (PCR) method used to identify variations in nucleic acid sequences, has been used to differentiate species within the Anopheles gambiae and Culex pipiens complexes. We validated the use of PCR-HRM analyses to differentiate species within Anopheles and within each of six genera of culicine mosquitoes, comparing primers targeting cytochrome b (cyt b), NADH dehydrogenase subunit 1 (ND1), intergenic spacer region (IGS) and cytochrome c oxidase subunit 1 (COI) gene regions. HRM analyses of COI amplicons from all the six primer pairs successfully differentiated two or more mosquito species within one or more genera (Aedes (Ae. vittatus from Ae. metallicus), Culex (Cx. tenagius from Cx. antennatus, Cx. neavei from Cx. duttoni, cryptic Cx. pipiens species), Anopheles (An. gambiae s.s. from An. arabiensis) and Mansonia (Ma. africana from Ma. uniformis)) based on their HRM profiles. However, PCR-HRM could not distinguish between species within Adoeomyia (Ad. africana and Ad. furfurea), Mimomyia (Mi. hispida and Mi. splendens) and Coquillettidia (Cq. aurites, Cq. chrysosoma, Cq. fuscopennata, Cq. metallica, Cq. microannulatus, Cq. pseudoconopas and Cq. versicolor) genera using any of the primers. The IGS and COI barcode region primers gave the best and most definitive separation of mosquito species among anopheline and culicine mosquito genera, respectively, while the other markers may serve to confirm identifications of closely related sub-species. This approach can be employed for rapid identification of mosquitoes.
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Introduction
Mosquitoes are among the most important disease vectors, known to transmit and maintain the circulation of pathogens that cause both global and neglected tropical diseases in humans and animals. The correct identification of different field-collected mosquito species, endemic to distinct ecologies, with high parasite and arthropod-borne virus (arbovirus) diversities is crucial to the planning of targeted vector control strategies to mitigate disease transmission. The last and most comprehensive Afrotropical mosquito identification keys were published in 1941 for culicides and in 1987 for anophelines. Molecular approaches that efficiently differentiate conspecific mosquitoes such as the barcode region improve identification accuracy considerably, but are time consuming, expensive in terms of post-polymerase chain reaction (post-PCR) processing and depend heavily on DNA sequencing.

Recent approaches have taken advantage of the unique melting profiles generated by homologous PCR products with small sequence differences during high resolution melting (HRM) analysis. Indeed, PCR-HRM has been used to differentiate mosquito transmitted arboviruses and malaria Plasmodium, vertebrate blood meals of mosquitoes, between two members of the Anopheles gambiae complex and amongst three members of the Culex pipiens complex. HRM analysis has proven to offer a higher resolution of PCR product based species identification on sequence variants than electrophoretic methods by revealing even higher resolution of PCR product based species identification on the vertebrate blood meals of mosquitoes and medium plants.

Although HRM has been successfully used to differentiate between specific Anopheles and Culex mosquitoes, the approach’s broader applicability and most suitable markers have not been evaluated. Previously, only the ribosomal DNA was targeted for An. gambiae sensu lato (s.l.) and only the acetylcholinesterase gene was used in distinguishing the Cx. pipiens complex. This study aimed at validating the use of HRM analysis for high throughput molecular culicine and anopheline mosquito identification and differentiation, comparing the utility of one ribosomal IGS (previously used to differentiate An. gambiae s.l.) and three mitochondrial (COI, ND1, cyt b) gene markers.

Table 1. Number (N) of mosquito species (GenBank accessions) used for HRM analyses from Baringo County, Kenya.

| Mosquito species | N | Logumgum 0.455 N, 36.078 E | Sirata 0.462 N, 36.097 E | K Ampi ya Samaki 0.620 N, 36.028 E | Nosuguro 0.605 N, 36.126 E |
|------------------|---|--------------------------|--------------------------|-------------------------------|---------------------------|
| Ad. africana     | 4 | 4 (KU186980, KU186981, KU186982, KU186985) | | | |
| Ad. furfurea     | 4 | 4 (KU186979, KU186983, KU186984, KU186986) | | | |
| An. funestus     | 3 | 3 (KU187102, KU187103, KU187105) | | | |
| An. gambiae s.l. | 3 | 1 | 2 | | |
| Cq. aurites      | 2 | 2 (KU187114, KU187117) | | | |
| Cq. chrysosoma   | 1 | 1 (KU187115) | | | |
| Cq. fuscocephala | 1 | | | | | (KU187116) |
| Cq. metallica    | 2 | 2 (KU187112, KU187113) | | | |
| Cx. antennatus   | 2 | 1 (KU187050) | | | |
| Cx. perexiguus   | 4 | 4 (KU380423, KU380348, KU380476, KU380382) | | | |
| Cx. tenagi       | 1 | 1 (KU187054) | | | |
| Ma. africana     | 4 | 2 (KU187124, KU187130) | 2 (KU187127, KU187129) | | |
| Ma. uniformis    | 3 | 2 (KU187170, KU187171) | 1 (KU187164) | | |
| Total            | 34 | 8 | 16 | 6 | 4 |

GenBank accessions are provided only for samples with confirmed identity and from which the COI/DNA sequences were obtained during a previously published mosquito diversity study.
Table 2. Number (N) of mosquito species (GenBank accessions) used for HRM analyses from Homa Bay County, Kenya.

| Mosquito species | N | Mbita 0.432 S, 34.210 E | Luanda Nyamasare 0.478 S, 34.287 E | Ngodhe 0.505 S, 34.363 E | Ungoye 0.612 S, 34.098 E | Mfangano Island 0.462 S, 33.999 E | Rusinga Island 0.399 S, 34.193 E | Chamaunga Island 0.431 S, 34.228 E | Takawiri Island 0.472 S, 34.091 E |
|------------------|---|-------------------------|-----------------------------------|--------------------------|---------------------------|-------------------------------|-----------------------------|-------------------------------|-------------------------------|
| Ae. metallicus   | 3 | 1 (KU187014)            | 1                                 |                          |                           |                               |                             |                               |                               |
| Ae. vittatus     | 8 |                          | 6 (KU187004, KU187006, KU187008-KU187011) |                          |                           |                               |                             | 2 (KU187005, KU187007)       |                               |
| An. coustani s.l.| 10| 3 (KU187098, KU187100, KU187101) | 2 (KU187095, KU187096)          |                          |                           |                               |                             | 2 (KU187097, KU187099)       | 2                              |
| An. funestus     | 2 | 1 (KU187104)            |                                   |                          |                           |                               |                             |                               | 1                              |
| An. gambiae s.l. | 2 |                          |                                   |                          |                           |                               | 2 (KU187108, KU187109)       |                               |                               |
| Cq. aurites      | 1 | 1 (KU187121)            |                                   |                          |                           |                               |                             |                               |                               |
| Cq. microannulatus | 2 |                          |                                   |                          |                           | 1                             |                             |                               | 1 (KU187118)                  |
| Cq. pseudoconopas | 1 |                          |                                   |                          |                           |                               |                             |                               | 1                              |
| Cq. versicolor   | 2 |                          |                                   |                          |                           |                               |                             | 2 (KU187119, KU187120)       |                               |
| Cx. antennatus   | 4 |                          |                                   |                          |                           |                               |                             |                               |                               |
| Cx. duttoni      | 6 | 1 (KU187075)            |                                   |                          |                           | 5 (KU187067, KU187068, KU187070-KU187072) | 1                             | 1                             | 1                             |
| Cx. neavei       | 3 |                          |                                   |                          | 3 (KU187032, KU187040, KU187046) |                               |                             |                               |                               |
| Cx. perexiguus   | 1 | 1 (KU380445)            |                                   |                          |                           |                               |                             |                               |                               |
| Cx. pipiens      | 6 |                          | 1 (KU187083)                       |                           |                           |                               | 5 (KU187077, KU380366, KU380372, KU380431, KU380444) |                               |                               |
| Culex sp. GPA    | 5 | 3 (KU380352, KU380455, KU380394) | 2 (KU380377, KU380413)          |                          |                           |                               |                             |                               |                               |
| Cx. univittatus  | 3 | 3 (KU187056, KU187059, KU187060) |                               |                          |                           |                               |                             |                               |                               |
| Cx. watti        | 5 | 2 (KU187063, KU187064)  |                                   |                          |                           |                               |                             | 2                              | 1                              |
| Ma. africana     | 2 |                          | 1                                 |                           |                           |                               |                             | 1 (KU187153)                 |                               |
| Ma. uniformis    | 3 | 1 (KU380460)            |                                   |                           |                           |                               |                             |                               | 1 (KU187175)                 |
| Mi. hispida      | 2 |                          |                                   |                           |                           |                               |                             |                               |                               |
| Mi. splendens    | 3 | 3 (KU187093, KU187094)  |                                   |                           |                           |                               |                             |                               |                               |
| **Total**        | 75| 21                      | 5                                 | 5                        | 1                         | 22                            | 12                           | 6                             | 3                              |

GenBank accessions are provided only for samples with confirmed identity and from which the COI DNA sequences were obtained during a previously published mosquito diversity study.6
Baringo County (Table 1) and Lake Victoria in Homa Bay County (Table 2) in Kenya. Before sampling, we obtained ethical clearance for the study from the Kenya Medical Research Institute (KEMRI) ethics review committee (Approval Ref: Non-SSC Protocol #310). These mosquitoes were morphologically identified during a previous study, Baringo County is a known hotspot for arbovirus outbreaks, while Homa Bay County is endemic to malaria and is located in a region with a history of arbovirus activity. One sample each of Anopheles gambiae sensu stricto (s.s.) and An. arabiensis, Aedes aegypti and Culex pipiens from laboratory colonies maintained in the Insectary of the International Centre of Insect Physiology and Ecology (icipe), Nairobi, Kenya, were used as controls. Also, specimens with confirmed identity that have been previously sequenced and submitted to GenBank (Table 1 and Table 2) were used as both controls and samples.

DNA extraction

From each mosquito, we extracted DNA according to the hot sodium hydroxide and Tris (HotSHOT) DNA extraction protocol from a single mosquito leg that was detached from the rest of the body using a pair of forceps and dissecting pin. Without crushing, the mosquito leg was put in a 0.2 ml microcentrifuge tube containing 30 µl of Alkaline Lysis buffer (25 mM NaOH (Thermo Fisher Scientific), 0.2 mM disodium EDTA (Thermo Fisher Scientific), pH 8.0) and incubated in a thermocycler at 95°C for 30 minutes and cooled at 4°C for 5 minutes. Then, 30 µl neutralising solution (40 mM Tris-HCl (Thermo Fisher Scientific)) was added. The resulting DNA was stored at -20°C until required as templates for PCR assays.

Table 3. Primers used for the amplification of gene fragments.

| Target gene                      | Primer name       | Primer Sequence (5’ to 3’)                  | Reference genome | Primer coordinates | Amplicon size (bp) |
|----------------------------------|-------------------|---------------------------------------------|------------------|--------------------|-------------------|
| Mitochondrial COI (within barcode region) | COI-AnophF       | GCAGGAATTCTCTATTTTAGG                       | L20934           | 1,874–1,896        | 275               |
|                                  | HCO2198R          | TAAACTCAGGGTGACAAAAAATCA                    | L20934           | 2,148–2,123        |                   |
| Mitochondrial COI                | Uni-Minibar-JVF   | ACAATCTAAGATATGGAAAC                      | L20934           | 1,445–1,467        | 173               |
|                                  | Uni-Minibar-JVR   | AAAATTATAAAAWGCATGAGC                     | L20934           | 1,617–1,55         |                   |
| Mitochondrial COI                | Mos-Co1-JVF       | ATAGTWATACCATYATAATGGG                    | L20934           | 1,622–1,644        | 299               |
|                                  | Mos-Co1-JVR       | ACWGTAAGTATAAATTTACTGCG                  | L20934           | 1,920–1,898        |                   |
| Mitochondrial ND1                | Mos-ND1F          | TATGCTCTGAAAACATAAGAAG                     | L20934           | 11,569–11,591      | 275               |
|                                  | Mos-ND1R          | CGDTATGATAAATTTATGTAG                     | L20934           | 11,717–11,741      |                   |
| Mitochondrial cyt b              | CYT BF            | GGACAAATACATTTGAGGAGCGAAGAC              | L20934           | 10,821–10,848      | 470               |
|                                  | CYT BR            | ATTACTCTCTTCAGCTTTAGGAATTT               | L20934           | 11,290–11,263      |                   |
| Ribosomal DNA IGS                | AgamUni F         | GTGAAGCTTTGCGTTC                      | KT284724         | 126–174            | 169               |
|                                  | AgamUni R         | GCAGGAGGACAGCTCA                           | KT284724         | 319–303            |                   |

F is forward primer direction; R is reverse primer direction.

Primer design, PCR and HRM analyses

Based on multiple alignments using Geneious software version 8.1.4 of mitochondrial genomes of mosquitoes (GenBank accessions NC_015079, NC_028616, NC_028223, KR068634, NC_010241, NC_014574, EU352212, NC_008070, KT358413, KT382816, KU494979, JX040513, AY729979, KU494979), we designed four sets of primers from two mitochondrial gene regions: COI (COI-AnophF/HCO2108R; Uni-Minibar-JVF/Uni-Minibar-JVR; Mos-CO1-JVF/Mos-CO1-JVR) and ND1 (Mos-ND1F/Mos-ND1R) genes (Table 3). The COI AnophF primer was initially designed specifically for Anopheles mosquitoes to be used with the HCO2108R primer, but tested on other species as well. Using samples of morphologically and molecularly identified Culex, Aedes, and Anopheles mosquito species (Table 1 and Table 2), we amplified different gene regions of their genomes using six pairs of primers (Table 3) in three replicate runs of single-plex PCRs in a RotorGene Q HRM real time PCR thermocycler (QIAGEN, Hannover, Germany). PCR grade water was used as negative control while mosquito species from Ae. aegypti, An. gambiae s.s., An. arabiensis, and Cx. Pipiens quinquefasciatus colonies maintained in the International Centre of Insect Physiology and Ecology (icipe) Insectary Unit were used as positive controls. The PCR mix contained 5 µl of 5X Hot Firepol EvaGreen HRM Mix (Solis BioDyne, Tartu, Estonia), 0.5 µM of each primer, 1 µl of DNA template and distilled water in a final volume of 10 µl. The thermal cycling conditions involved an initial denaturation for 1 minute at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 20 seconds, and extension at 72°C for 30 seconds, and a
final extension at 72°C for 7 minutes. Without stopping the reaction, the PCR amplicons were denatured at 95°C for 1 minute, held for another minute at 40°C and melted by gradually raising the temperature from 70°C to 95°C by 0.1°C in 2 second steps, waiting for 90 seconds of pre-melt conditioning on first step and 2 seconds in subsequent steps. The outcome was automatically plotted on a connected computer and visually observed and analysed using the Rotor-Gene Q Series software v2.1. Representative samples of differentiated mosquito species that had similar HRM curves were purified with ExoSAP-IT (USB Corporation, Cleveland, OH) and submitted for DNA sequencing at Macrogen (South Korea). To confirm the identity of PCR-HRM differentiated mosquitoes, DNA sequences were edited with Geneious version 8.1.4 and queried against the GenBank nr database (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST N) version 2.3.0.

Results

We differentiated 12 mosquito species in the Aedes (two), Anopheles (two), Culex (six), and Mansonia (two) genera by HRM analyses (Table 4). The COI sequences of some of the mosquito samples analyzed and differentiated were obtained during a previously published mosquito diversity study and their respective GenBank Accession numbers are listed in Table 2. Despite the fact that the COI-AnophF/HCO2198R primers were originally designed based on Anopheles mitochondria genome alignments, they were most efficient in differentiating among Mansonia (Ma. africana and Ma. uniformis (Figure 1A)), Culex (Cx. neavei and Cx. duttoni, Cx. tenagius and Cx. antennatus, and two genetic variants of Cx. pipiens (Figure 2A)), and Aedes (Ae. vittatus and Ae. metallicus (Figure 3)) mosquitoes (Table 4). Indeed, the DNA sequences flanked by the COI-AnophF/HCO2198R primers included multiple polymorphic sites in species within these genera (Figure 4). Although there are SNPs within species DNA that resulted to the slight changes observed in their HRM profiles, the SNPs across species were enough to distinguish between them.

Mansonia africana and Ma. uniformis could also be differentiated by Mos-COI-JV (Figure 1B) and CYT B (Figure 1C) PCR-HRM analysis. Some Culex species were similarly differentiated by HRM based on their CYT B, Uni-Minibar-JV and Mos-ND1 (Figure 2B–D) primer pair PCR products. The morphologically indistinguishable Cx. tenagius and Cx. antennatus were distinguishable based on their CYT B, Uni-Minibar-JV and Mos-ND1 HRM profiles (Figure 2B–D). Similarly, HRM analysis of only two of the COI (COI-AnophF/HCO2198R and Uni-Minibar-JV) and the ND1 primer pairs grouped morphologically identical and difficult to differentiate Cx. pipiens into two distinct clades; one with Cx. pipiens voucher sequences from GenBank (KF919189) and those with a sequence that we identified as Culex sp. GPA (GenBank accessions KU380352, KU380455, KU380394) (Figure 2A, C and D; Table 4). However, unlike the COI HRM profiles (Figure 2A, B), the ND1 HRM profiles (Figure 2D) of Cx. pipiens amplicons showed a melting temperature shift of to the right (higher temperature) compared to the Culex sp. GPA amplicons, possibly due to greater GC

Table 4. Differentiation of mosquito species using the six primer pairs amplifying four loci.

| Mosquito genera | COI | cyt b | ND1 | IGS |
|-----------------|-----|-------|-----|-----|
|                 | COI-AnophF/HCO2198R | Mos-COI-JV | Uni-Minibar-JV | CYT B | Mos-ND1 | AgamUni |
| Anopheles       | DNS | DNS   | DNS | DNS | DNS | Separated An. gambiae from An. arabiensis |
| Mansonia        | Separated Ma. africana from Ma. uniformis | Separated Ma. africana from Ma. uniformis | DNS | Separated Ma. africana from Ma. uniformis | DNS | DNS |
| Aedes           | Separated Ae. vittatus from Ae. metallicus | DNS | DNS | DNS | DNS |_dns |
| Culex           | Separated Cx. tenagius from Cx. antennatus, Separated Cx. pipiens from Culex sp. GPA, Separated Cx. neavei from Cx. duttoni | DNS | Separated Cx. pipiens from Culex sp. GPA, Separated Cx. tenagius from Cx. antennatus | DNS | Separated Cx. pipiens from Cx. antennatus | DNS | DNS |
| Aedeomyia       | DNS | DNS   | DNS | DNS | DNS | DNS |
| Mimomyia        | DNS | DNS   | DNS | DNS | DNS | DNS |
| Coquillettidia  | DNS | DNS   | DNS | DNS | DNS | DNS |
Figure 1. HRM profiles of two *Mansonia* species. *Mansonia uniformis* and *Ma. africana* mosquitoes were differentiated by PCR-HRM using the (A) COI-AnophF/HCO2198R, (B) MOS-CO1 and (C) CYT B primer pairs.

richness of *Cx. pipiens* at this locus\(^8\). Similarly, the IGS primers (AgamUni) differentiated *Anopheles gambiae* s.s. from *An. arabiensis* (Figure 5). In addition, the COI-AnophF/HCO2198R primers were also used to separate *Cx. neavei* from *Cx. duttoni* (Figure 2A), which belong to the same subgenus of *Culex* mosquitoes.

HRM analysis of all the six primer pairs could not differentiate *Aedeomyia* (Ad. africana and Ad. furfurea), *Mimomyia* (Mi. hispida and Mi. splendens) and *Coquillettidia* (*Cq. aurites*, *Cq. chrysosoma*, *Cq. fuscopennata*, *Cq. metallica*, *Cq. microannulatus*, *Cq. pseudoconopas* and *Cq. versicolor*) species (Table 4) or among *An. funestus* and *An. coustani* species complexes.

**Discussion**

We compared six pairs of primers for their potential to differentiate at least two morphologically similar mosquito species within each of seven mosquito genera by PCR-HRM analysis and identified suitable markers for differentiating species within *Anopheles*, *Aedes*, *Culex* and *Mansonia* mosquitoes. However, none of the markers were suitable for HRM analysis to distinguish among species of *Aedeomyia*, *Mimomyia* or *Coquillettidia* genera mosquitoes. Also, *Cx. watti*, which can be misidentified morphologically as *Cx. duttoni* or *Cx. pipiens*, could not be differentiated by PCR-HRM analyses. Nonetheless, we were able to distinguish *Ma. africana* from *Ma. uniformis*, *An. gambiae* s.s. from *An. arabiensis* (sibling species of *An. gambiae* s.l.), *Ae. vittatus* from *Ae. metallicus,
HRM profiles of *Aedes* mosquitoes. *Aedes vittatus* and *Ae. metallicus* were differentiated by PCR-HRM using the COI-AnophF/HCO2198R primer pair.

**Figure 2.** HRM profiles of *Culex* species. *Culex* species were differentiated by PCR-HRM using the (A) COI-AnophF/HCO2198R, (B) CYT B, (C) Uni-Minibar-JV, and (D) Mos-ND1 primer pairs.

Surprisingly, HRM analysis of PCR products from the COI-AnophF/HCO2198R primers, which were designed for *Anopheles*, could not distinguish between these sibling species, yet were most effective in discriminating species within the *Mansonia*, *Aedes* and *Culex* genera, including between the cryptic *Culex pipiens* species. *Anopheles gambiae* and *An. arabiensis* were only distinguished using the IGS gene, which was also designed for *An. gambiae* and is routinely used for distinguishing these sibling species by conventional PCR and HRM analysis. In contrast, species complexes of *An. coustani* and *An. funestus* were not

**Figure 3.** HRM profiles of *Aedes* mosquitoes. *Aedes vittatus* and *Ae. metallicus* were differentiated by PCR-HRM using the COI-AnophF/HCO2198R primer pair.

as well as *Cx. neavei* from *Cx. duttoni*, *Cx. tenagius* from *Cx. antennatus* and two cryptic sympatric species of morphologically identical *Cx. pipiens*. Most notably, the two *Cx. pipiens* species with distinct COI barcode sequences were indeed first identified by HRM analysis of numerous samples. Thus, the relative economy of HRM analysis compared to sequencing facilitates the rapid identification of cryptic species.
differentiated with any of the primers. The data suggest that COI\textsuperscript{10},
cyt \textit{b} and ND1 loci may be unsuitable for distinguishing among
\textit{Anopheles} sibling species. Similarly, the \textit{Aedes} species could only
be differentiated by the COI-AnophF/HCO2108R primers. This
could be as a result of more recent speciation, insufficient to allow
for sibling species resolution at these markers. Such scenarios have
been observed for recent or rapidly evolving groups, such as the
Cichlid fishes of eastern Africa, where mitochondrial divergence is
not concordant with morphological variations\textsuperscript{11}.

In contrast, \textit{Ma. africana} and \textit{Ma. uniformis} were separated by the
COI and cyt \textit{b} loci, but not by the ND1 and IGS gene primers and
\textit{Culex} species were variably distinguishable by all markers,
extcept IGS. For both \textit{Mansonia} and \textit{Culex}, as with \textit{Aedes}, the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Single nucleotide polymorphisms (SNP) between mosquito species separated by the COI-AnophF/HCO2108R primer pair amplicons. Polymorphic sites vary more between than within species.}
\end{figure}
COI-AnophF/HCO2198R primers were most sensitive in discriminating morphologically indistinct species. This highlights the power of the COI barcode region for identifying diverse cryptic species\(^3\). The SNPs present in the COI genes of the ten mosquito species confirms that the COI gene is conserved and polymorphic enough to differentiate these species even in cases of morphological misidentification. The polymorphisms between species were enough to robustly separate them based on their HRM profiles, while sequence polymorphisms within species were too few to significantly alter their HRM profiles.

We, therefore, recommend the initial use of the COI-AnophF/HCO2198R primers Bar-HRM to differentiate Mansonia, Culex and Aedes mosquito species and the IGS primers for anopheline mosquito identification\(^2,13,33\) by HRM. The inability of all the six primer pairs to differentiate many mosquito species among all seven genera tested is an indication that the genetic diversity of many mosquito species is complicated and still poorly understood. Also, the number (sample size) of many of the analyzed mosquito species was small (<3) because these species were scarcely present in the study areas. More samples (≥3) should be used and more study areas should be sampled in subsequent studies to test genetic differentiation of mosquito species\(^3\). Additional polymorphic DNA loci should also be identified, tested and used in combination with existing ones for the identification of mosquito species, especially among species complexes and across genera.

Conclusions
This study shows that specific PCR markers can be used to distinguish closely related species of mosquitoes using HRM analysis. We distinguished two sibling species of *An. gambiae s.l.*, two species each of *Mansonia* and *Aedes*, and six species, including cryptic species, of *Culex* using six pairs of primers targeting the mitochondrial and ribosomal genes. HRM is a low cost (<$1 per reaction), effective tool that enhances culicine and anopheline mosquito identification and may also reveal population differences in conserved mitochondrial sequences. This approach can improve vector surveillance associated with *Plasmodium* (malaria) or arbovirus transmission and inform targeted vector control strategies.

Data availability
All sequence data associated with this manuscript are freely available in GenBank. All relevant accession numbers are listed in Table 1 and Table 2.

**Author contributions**
YUA, DM, JV, and AM conceived of, designed and coordinated the study. YUA, DO and TOO did sample collection and identification. YUA and EM carried out the molecular genetic studies. YUA and JV carried out the sequence analyses and drafted the manuscript. DM, JV and YUA contributed materials used for the study. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

**Competing interests**
No competing interests were disclosed.

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Overall, this is a well-written article on the development of HRM for identification of different species of mosquitoes. The authors also mentioned about the limitations of the study. However, the six pairs of primers used in this study did not seem to have clear directions or purpose. Did the authors want to come out with a pair of universal primers to differentiate all the clinically important mosquito species mentioned in the article? Or one specific primer pair for each genus of mosquitoes? Or there is other intention? Although the authors showed the ability of primer pairs to discern some mosquito species, for instance, COI-AnophF/HCO2198R could distinguish *Mansonina* spp., *Aedes* spp. and *Culex* spp., how about the species of the same genera that could not be discriminated? It would be more focused and directional if the primer pairs are genus-specific.

Methods:
1. It would be great if the clinical importance of or disease transmitted by each mosquito genus/species is listed in Table 1 and Table 2 so that the readers understand the significance of discriminating the mosquito species.

2. Please mention the $T_m$ for each primer in Table 3.

3. Please elaborate more on the primer design: on what basis the authors design the primers and for what reason they want to amplify those regions with amplicon sizes mentioned in Table 3.

4. What was the amount of DNA template used for HRM analyses?

Results:
1. Please show the limitation of detection for each assay in the detection of each mosquito species.

2. Was auto-calling mode used for clustering? What was the confidence interval? Please mention the mean $T_m$ for each species with standard deviation.

3. Figure 2A, B, D: There was only one sample of *C. tenagius* included in the analysis. It is difficult to conclude the melting profile of this species if there was only one sample available.

4. In Table 1, COI-AnophF/HCO2198R has been shown to be able to separate *C. tenagius* from *C. antennatus*, *C. pipiens* from *Culex sp. GPA*, and *C. neavei* from *C. duttoni*. Since I am not an...
expert in mosquitoes, is there any specific reason the authors wanted to report the separation between two species of Culex rather than reporting it as a separation between all species?

5. In Figure 2A, the melt curves of Cx. antennatus, Cx. neavei, Culex sp. GPA and Cx. pipiens looks closely apart and hard to differentiate. Was the clustering auto- or manually called? What was the confidence interval if it was auto-called? What is the possibility of all these species being present or analysed at the same time? It would be hard to differentiate them if all of them are present in a same run of HRM analysis.

6. How do the melt curves of the unseparated species appear as compared to those in the same genus which could be differentiated? For instance, Cx. perexiguus vs. Cx. tenagius, Cx. antennatus, Cx. pipiens, Culex sp. GPA, Cx. neavei and Cx. duttoni. And also melting curves of Aedeomyia (Ad. africana and Ad. furfurea), Mimomyia (Mi. hispida and Mi. splendens) and Coquillettidia (Cq. aurites, Cq. chrysosoma, Cq. fuscopennata, Cq. metallica, Cq. microannulatus, Cq. pseudoconopas and Cq. versicolor) species (Table 4) or among An. funestus and An. coustani species complexes, which could not be differentiated in this study.

7. In Figure 4, intraspecies variation in the target sequence is seen, for instance, in the four strains of Ma. africana. Although the authors did mention on Page 6 “Although there are SNPs within species DNA that resulted to the slight changes observed in their HRM profiles, the SNPs across species were enough to distinguish between them”. I am not sure how confident it is to employ these assays in the presence of intraspecies variations, plus the sample size may be too small to validate the assays.

8. Figure 5: please revise the figure legend.

Discussion:

1. On page 8, 2nd paragraph, the authors mentioned “Surprisingly, HRM analysis of PCR products from the COIAnophF/HCO2198R primers, which were designed for Anopheles, could not distinguish between these sibling species...” Can the authors explain the possible reason for this?

2. Again, since I am not a mosquito expert, are the mosquitoes of the same genus morphologically identical? If the assays are successfully developed, are the mosquitoes going to be morphologically identified prior to subjecting them to HRM analysis? How are the authors going to decide which primer pairs to use later in the mosquito identification?

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

**Competing Interests:** No competing interests were disclosed.
Ajamma have an article on the use of the technique high resolution melt analysis towards the identification of morphologically indistinct species of mosquito. The specific goal is to expand the current set of primers in the research literature in order to identify more species from multiple genera of mosquitoes.

The methodology is clear with sufficient details for it to be reproduced by listing all appropriate reagents, DNA primer sequences and real-time PCR instrumentation. I do recommend adding the criteria by which the melt curves were deemed to be sufficiently different to allow identification of the species as compared to the "Did Not Separate" state as reported in Table 4.

Figures 1 - 3 and 5 are clear and support the results summarized in table 4. I appreciate the authors efforts to repeat and report data from the previously published primers ("AgamUni") as a point of comparison. Appropriate controls were used with (1) water as a negative control for amplification and (2) samples from defined colonies and samples previously sequenced as positive controls. The most significant limitation is the number of replicates, and the diversity of sample collection points for each species. The authors clearly acknowledge these limitations in the conclusion and clearly state the need for additional samples to assess the intra-specific variation which is critically important information to make this method highly useful.

In summary, the paper is clearly and concisely written with 1 minor recommendations for additional information on the method. The goals of the research are clearly stated, and the results as well as the conclusions support the goals. The researchers have achieved the goals by identifying and confirming at least one primer pair for each of 4 genera that identify various species that are difficult to identify by morphology alone.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

**Competing Interests:** No competing interests were disclosed.