Absence of kdr resistance alleles in the Union of the Comoros, East Africa [version 1; peer review: 2 approved, 1 approved with reservations]

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Abstract

Knockdown resistance (kdr) and CYP9K1 genotypes were detected by a MOLDI-TOF based SNP genotyping assay (Sequenom iPLEX) in samples of Anopheles gambiae collected at 13 sites throughout the Union of the Comoros and Dar es Salaam, Tanzania during February and March 2011. All A. gambiae specimens collected in the Comoros were homozygous for the susceptible kdr alleles (+/+), while 96% of A. gambiae from Dar es Salaam were homozygous for the East African kdr resistant genotype (E/E). In contrast, all specimens from Dar es Salaam and the Comoros were homozygous for the cyp3 allele (c3/c3) at the CYP9K1 locus; the locus has been implicated in metabolic resistance against pyrethroid insecticides in West Africa. All specimens had typical A. gambiae genotypes for SNPs within the divergence Islands on all three chromosomes. Although further spatial and temporal studies are needed, the distribution of kdr genotypes between the Comoros and Tanzania further supports isolation of the Comoros populations from A. gambiae populations on mainland Africa.

Keywords

DIS, Dar es Salaam, CYP9K1, knockdown resistance, insecticide, resistance
Introduction
A majority of the human population residing in the Union of the Comoros (=94%) live in high malaria transmission zones. Anopheles gambiae (Giles) are the major malaria vectors in the Comoros. Vector control efforts have concentrated on the adult stage using insecticide-treated bednets (ITNs) and indoor residual spraying (IRS) with DDT. ITN distribution was initiated in the Comoros in 2005 and by 2014 roughly 40% of the population has access to ITNs.

Limited insecticide resistance surveillance has been conducted on malaria vectors in Union of Comoros, with to date, published records stemming only from investigations in Mayotte (an island administered by France), where A. gambiae were susceptible to multiple insecticides except for a larvicide, temephos. Insecticide susceptibility studies have been conducted in neighboring East African countries such as in western Kenya (Chen et al. 2008. JME, Mathias et al. 2011. Malaria J, Ochomo et al. 2012 MVE), but little information is available on the coastal regions of Kenya. In Tanzania, information, based on small sample sizes, is available on the kdr allele frequency distribution in coastal districts of Muheza and Hula (Dar es Salaam) where about one third from Dar es Salaam were homozygous for the kdr-East (L1014S) mutation.

Here we present much needed data on kdr allele frequencies and include frequency data for a recently described pyrethroid metabolite resistance gene, CYP9K1. Allele frequencies for Anopheles gambiae collected at 13 sites in the Union of the Comoros, plus Dar es Salaam, Tanzania are presented (Table 1).

Methods
A total of 362 indoor resting adults and larvae were collected from 13 locations from the three islands (Figure 1) making up the Union of the Comoros between February and March, 2011. Larvae were individually rinsed twice in bottled mineral water and placed in 80% ethanol for downstream genomic DNA extraction. A collection of A. gambiae sensu lato from Furvela, Mozambique were collected using light traps inside houses. Mosquitoes from Dar es Salaam were obtained from Dr. Kija Ngh’abi at Ifakara Health Institute.

Samples were transported to the UC Davis Vector Genetics Laboratory for further genetic assay. DNA was extracted using a DNeasy extraction kit (Qiagen, Valencia, CA). Species were determined based on the combination of species diagnostic assays and a divergence island SNP (DIS) genotyping assay.

For DIS, kdr and CYP9K1 genotyping, we used the Sequenom iPLEX Gold Genotyping Reagent Set (Catalog number: Sequenom 10158) on a MassArray (Sequenom) mass spectrometer at the UC Davis Veterinary Genetics Laboratory. This assay was slightly modified from the original DIS assay by adding the kdr and CYP9K1 markers, as described in Supplemental Document S1.

Results & discussion
A. gambiae from Dar es Salaam, Tanzania, had the kdr-East (L1014S) genotype at a frequency of 96%, which is higher than the frequency previously reported from Dar es Salaam by Kabula et al. where respectively, 1/3 and 2/3 of their samples were homozygous and susceptible for kdr-East (L1014S). In contrast, all A. gambiae from

<table>
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<th>Site</th>
<th>Lat</th>
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the Comoros were homozygous for the susceptible kdr alleles. All A. gambiae from both Tanzania and the Comoros were homozygous for the cyp3 allele for the CYP9K1 gene. All specimens from Furvela, Mozambique were A. merus (30/35) or A. arabiensis (5/35) and were excluded from further analysis.

Significant pressure to select for resistance to pyrethroid insecticides in A. gambiae and other indoor biting and resting malaria vectors likely occurs throughout sub-Saharan Africa because of intense IRS and ITN usage. A recent study in Mali noted an adaptive introgression of kdr resistant alleles from A. gambiae stably incorporated into the A. coluzzii genome under high ITN coverage environments. A similar genomic signature of adaptive introgression was also observed in Ghana. A. gambiae populations in the Comoros have had the opportunity, via transport by boat or air, to acquire resistant A. gambiae genotypes from neighboring countries such as Tanzania where high levels of insecticide resistance have been reported. The failure of the Comoros population to acquire insecticide resistance alleles despite long term exposure to insecticide pressure may potentially be due to several factors or combination of factor including: (1) ITN coverage (<25% compared to >60% Mali) is not high enough to drive selection for resistance, (2) these populations are very isolated from mainland populations, requiring them to develop resistance de novo rather than from gene flow from neighboring populations, and/or (3) A. gambiae on the Comoros may be exophilic.

Our study provides much needed information regarding the genetics of insecticide resistance in A. gambiae populations in the Comoros Islands. Although the malaria vectors in Comoros appear to be genetically predisposed to insecticide susceptibility, it is possible that these mosquitoes have developed phenotypic resistance via alternative mechanisms such as metabolic resistance other than CYP9K1 or behavior resistance (e.g. exophily). Further studies are needed to establish levels of phenotypic resistance against insecticides, as well as bionomics of the malaria vectors in this region to understand the impact of insecticide-based malaria control measures in the Comoros.

Author contributions
YL conceived the study, designed experiments, performed data analysis and wrote manuscript. YY, AC, NO conducted experiments. CDM conducted field collections and conducted experiments. AO, GCL and AJC conducted field collections and wrote manuscript. All authors were involved in drafting this manuscript and have agreed to the final content.

Competing interests
No competing interests were disclosed.

Grant information
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I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements
We thank Catelyn C. Nieman for assistance in DNA extraction and species diagnostic assay. We also thank Julia Malvick at the Veterinary Genetics Laboratory of UC Davis School of Veterinary Medicine for assistance in processing iPLEX SNP genotyping assay.
Supplementary materials

Supplemental Document S1.
Modification of the original DIS assay in 7.
Click here to access the data.

References

   Reference Source
   PubMed Abstract | Publisher Full Text
   PubMed Abstract | Publisher Full Text | Free Full Text
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Open Peer Review

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Version 1

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The paper by Lee et al. provides strong evidence for the absence of mutant alleles at the well-characterized kdr locus in populations of the major malaria mosquito Anopheles gambiae from three islands of the Comoros archipelago. The lack of kdr mutants in these populations contrasts with the high frequency of the East-African kdr mutation (L1014S) in a continental population from Dar es Salaam, Tanzania. These findings are consistent with results of work carried out by our research group in the neighbouring island of Mayotte, showing no evidence for phenotypic insecticide resistance in An. gambiae, as well as the absence of target site mutations at the kdr locus on this island. The authors conclude that, altogether, these results suggest restricted gene flow between continental and island populations of An. gambiae in this area.

The paper is concise and clear. The title and abstract are appropriate, and they reflect adequately the content of the paper.

There are, however, a few minor shortcomings to be addressed:

- The number and position of the sampling sites are not consistent in the text, Table and Figure: 13 sampling sites are mentioned in the text and abstract, 15 are shown in the Table and Figure; there are also inconsistencies with the identification codes between the table and the figure, and the caption of Table 1 indicates these codes with the symbol ‘#’ whereas the corresponding column name in the body of the Table is ‘idx’.

- In the first sentence of the ‘Results & discussion’ section, the authors state that “A. gambiae from Dar es Salaam, Tanzania, had the kdr-East (L1014S) genotype at a frequency of 96%,…”. The sentence should either state that 96% of the specimens were homozygous for the kdr allele (as in the abstract) or that the kdr mutation (instead of “genotype”—it is more appropriate as this is a
single nucleotide polymorphism) was found at a frequency of 98% (as shown in Table 1).

- It is mentioned in the Introduction that “population access to ITNs” in 2014 in the Comoros was 40%, whereas it is reported that “ITN coverage” is <25% when discussing the results. Please explain the difference between these figures.

- Table S1 in the Supplementary Materials still uses the non-Linnean nomenclature of molecular forms instead of *An. gambiae* and *An. coluzzii*; we think this should now be superseded by the Linnean taxonomic nomenclature.

- There is little information in the paper about the CYP9K1 locus; for example, does it confer cross-resistance to DDT? Is the cyp3 allele wild-type or ‘resistant’? What is the phenotype of ‘resistant’ alleles? One or more references would be useful in this respect.

- In which year were the mosquitoes sampled in Dar es Salam?

Moreover, the authors should:

- Include the references ‘Chen et al. 2008. JME, Mathias et al. 2011, MalariaJ and Ochomo et al. 2012 MVE’ which are cited in the text (‘Introduction’) in the reference list;

- Specify in the ‘Methods’ (or perhaps in Table 1) which of the 362 specimens were collected as resting adults and which ones were collected as larvae; also, which steps were taken to avoid sampling individuals coming from the same mosquito progeny in larval samples, as this could have an impact on observed genetic diversity.

- Correct the reference number for the Kabula *et al.* paper cited in the ‘Results & discussion’ section.

- Use the abbreviation “An.” rather than “A.” for *Anopheles*, in agreement with taxonomic conventions and community usage.

- Italicize the adverb “sensu lato” given that it is from the Latin.

Finally, it is in our view inappropriate to infer about gene flow between the Comoros and the African continent based on these results. (i) The locus is under selection, which is not ideal for gene flow inference, and, as discussed by the authors, the nature, strength, and geographic distribution of selective pressures may differ between the Comoros and the single continental population that was sampled. (ii) The level of resistance to pyrethroids and other insecticide compounds due to the L1014S mutation is probably low in *An. gambiae* (Ranson *et al.*, 2000), and may differ according to genetic background. (iii) All other genetic markers used in the study, including CYP9K1 argue for gene flow occurring between these populations.

Because there are no phenotypic data for the level of resistance of the *An. gambiae* populations that were included in the study, and because no historical and accurate data on insecticide usage in the Archipelago are available, it is at this point rather speculative to explain the absence of *kdr* mutations in the Comoros populations.

Accordingly, the selective pressures for insecticide resistance operating on these populations need to be assessed before inferring the dynamics of gene exchange between these island and continental
populations. As acknowledged by the authors, vector behaviour (feeding preferences, feeding time, endo-exophagy, endo-exophily) also needs to be investigated before any conclusion can be put forward to explain the differences observed.

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

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

**Frederic Tripet**

Centre for Applied Entomology and Parasitology, School of Life Sciences, Keele University, Staffordshire, UK

This is an interesting short report describing the prevalence of two loci incriminated in metabolic and target-site resistance to pyrethroid pesticides in populations of the malaria mosquito *A. gambiae* s.s. on the Comoros Islands. The cyp3 allele of the CYP9K1 gene was found in all island specimens and also in a single population sampled from mainland Tanzania. In contrast the East African (L1014S) allele was not found on the islands suggesting that these populations are fully geographically isolated from mainland populations, or alternatively that pesticide selection pressures are not very high on the islands due to limited vector control programmes. I just have a few general suggestions that may help make the current report a little bit more informative in key areas and thus make it more relevant to a broader readership.

The current introduction and discussion are very succinct...

Are these 2 loci the only major loci involved in resistance to pyrethroids and why were they chosen in particular? Additional background with references would be useful in the intro.

As noted by the authors, there is limited information on pesticide resistance in general on the Comoros. The authors also state: ‘Our study provides much needed information regarding the genetics of insecticide resistance in *A. gambiae* populations in the Comoros Islands.’ It looks to me that what is first and foremost really needed are detailed bioassay surveys of pesticide resistance for the island populations. These would have provided a better context for the current study and would have made the interpretation of the distribution on the two *kdr* resistance loci easier. Is the cyp3 allele here an ancestral allele or has it swept through due to selection?

Given the above, the main selling point of this study lies, in my view, in the use of the *kdr* locus as a marker for introgression as done previously in studies of West African *A. gambiae* populations. For many years, *kdr* was the only marker suggesting an absence of gene flow between *A. coluzzii* and *A. gambiae* s.s. in Mali. Hence, I would suggest expanding that part of the discussion a little bit. The current section omits some interesting parallels and details.
In the same line of thought, discussing the new findings with those generated using neutral markers in a previous study for the same islands (Marsden et al., 2013) would be useful, again because of the similarities with approaches (neutral versus non-neutral markers) used in past studies of reproductive isolation.

The authors mention possible gene flow from the mainland via boats. It may be worth mentioning that the main boat connections are from Mayotte, Zanzibar and Madagascar whose An. gambiae populations are not included in this and previous study. Why is that? What is the status of those populations? If the Comoros islands were to be used for possible mosquito release programmes, re-colonization from neighbouring islands would possibly also be a concern. On the other hand, gene flow from the Mozambique coast can only be dismissed if additional sampling was made from that region.

This is clearly an area of the world whose vector populations are greatly understudied. Given that these islands represent some of the best locations for testing mosquito release programmes, this study, albeit a small contribution, represents an important step in the right direction.

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

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
Please explain your interest on CYP9K1 gene and add citations.

Add references on insecticide resistance surveillance in Mozambique if any are available.

Add the objective of the study (gene-flow of continental versus island).

Minor comments:
In the 1st paragraph, the last sentence change “has” to “had”.
In the second paragraph substitute citation with numbers.

Methods

Study design and methods are quite well explained.

Please specify how many larvae and adults have been collected from 13 locations from the three islands, and give more details in the results section.

Modify Figure 1 according to Table 1 (Figure 1: image on the right side: you have missed out the number 14 and the number 15 is in a different location to that stated in Table 1. Therefore the number 15 in Figure 1 should be changed to a 14 and number 15 should be added at Dar es Salaam, Tanzania).

In the table you list 15 collection sites but here you cite only 14.

Results

Please specify the statement “malaria vectors in Comoros appear to be genetically predisposed to insecticide susceptibility”and add more details and references.

Correct the citation Kabula et al. 7 to Kabula et al. 4

The samples in Mozambique seem not related to the study since only A. merus or A. arabiensis have been found.

The discussions are balanced and justified based on the results obtained, even if continental sample (Tanzania) is very small.

Competing Interests: No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Reader Comment 22 Jul 2015

Yoosook Lee, UC Davis, USA

Thank you very much for your review.

The kdr we genotyped is also known as L2014F. We will revise our title accordingly in the
The kdr we genotyped is also known as L2014F. We will revise our title accordingly in the upcoming revision.

Clarification on the species *A. gambiae* will be also made.

The formal paper describing how we came across CYP9K1 gene and its evolutionary history among *A. gambiae* and *A. coluzzii* is under review and we should be able to add the proper citation in the next revision.

We have looked for the peer-reviewed articles on insecticide resistance surveillance in Mozambique but we have not found one thus far. We welcome suggestions if you came across such publications.

Other suggestion will be incorporated in the upcoming revision. Once we made revisions, we will post the detailed response to your review.

Thank you very much again for your constructive comments!

**Competing Interests:** none