RESEARCH NOTE

REVIEWED Mitochondrial genomes of *Anopheles arabiensis*, *An. gambiae* and *An. coluzzii* show no clear species division

[version 2; peer review: 2 approved]

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Abstract

Here we report the complete mitochondrial sequences of 70 individual field collected mosquito specimens from throughout Sub-Saharan Africa. We generated this dataset to identify species specific markers for the following *Anopheles* species and chromosomal forms: *An. arabiensis*, *An. coluzzii* (The *Forest* and *Mopti* chromosomal forms) and *An. gambiae* (The *Bamako* and *Savannah* chromosomal forms). The raw Illumina sequencing reads were mapped to the NC_002084 reference mitogenome sequence. A total of 783 single nucleotide polymorphisms (SNPs) were detected on the mitochondrial genome, of which 460 are singletons (58.7%). None of these SNPs are suitable as molecular markers to distinguish among *An. arabiensis*, *An. coluzzii* and *An. gambiae* or any of the chromosomal forms. The lack of species or chromosomal form specific markers is also reflected in the constructed phylogenetic tree, which shows no clear division among the operational taxonomic units considered here.

Keywords

Mitogenome, species identification, Africa, malaria vector, mosquitoes, *Anopheles*, single nucleotide polymorphisms, phylogenomics

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Approval Status

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REVISED Amendments from Version 1

The main difference between this version and the previous one is the analysis we performed to construct the phylogenetic tree. The newly created tree is shown in [Figure 1](#). This approach is more in line of what previous studies that looked at mitogenomes in *Anopheles* specimens have done. This did not change the conclusion of the paper. We also added a new table ([Table 1](#)) where we list the chromosomal inversion of each specimen, as was suggested by one of the reviewers. Furthermore, we added [Supplementary Table S1](#) with all the detected SNPs on the mitogenome for the different *Anopheles* species and chromosomal forms. We also addressed most of the comments the reviewers had and clarified where needed.

See referee reports

Introduction

Historically, mtDNA sequence has been used in taxonomy as a source of species diagnostic markers ([Cronin et al. \(1991\)](#); [De Barba et al. \(2014\)](#); [Pegg et al. \(2006\)](#)) or in population genetics and evolutionary studies ([Fu et al. \(2013\)](#); [Harrison \(1989\)](#); [Llamas et al. \(2016\)](#)). One advantage of using mitochondrial over nuclear DNA for such studies is that the mutation rate of mtDNA is about 10 times faster than nuclear DNA ([Brown et al. \(1979\)](#); [Haag-Liautard et al. \(2008\)](#)), hence amplifying the evolutionary trajectory of populations and species. In addition, mtDNA is easy to amplify, because there are more copies of mitochondrial DNA relative to nuclear DNA. Also, universal primers can be applied to a wide range of species. Widely used universal primers target the cytochrome b and cytochrome oxidase 1 genes ([Tahir et al. \(2016\)](#)), because both have conserved and highly variable regions. In addition to these, other genes as described in [De Mandal et al. \(2014\)](#), can also be used as markers. However, phylogenetic trees based on mtDNA can deviate from the ones that are derived from nuclear DNA ([Phillips et al. \(2013\)](#); [Shaw \(2002\)](#); [Sota & Vogler, 2001](#)).

The *Anopheles gambiae* species complex consists of eight morphologically identical species that can only be distinguished with molecular markers ([Scott et al. \(1993\)](#); [Coetzee et al., 2013](#)) or, for some of the species, by cytological examination of polytene chromosomes ([Green, 1972](#); [Pombi et al., 2008](#)). The currently used molecular markers to distinguish between *An. coluzzii* and *An. gambiae* ([Lee et al., 2014](#)) are located within genomic islands of divergence located proximal to the centromeres ([Turner et al. \(2005\)](#)). Monitoring additional species-specific markers on mitochondrial DNA (mtDNA) could increase the ease of application and accuracy of species detection assays. In addition, mtDNA markers could enhance our understanding of divergence times among taxa within the complex.

Previous studies showed that there is a high amount of interspecific gene flow in mtDNA between *An. coluzzii*, *An. gambiae* and *An. arabiensis* specimens ([Besansky et al., 2003](#); [Besansky et al., 1997](#); [Donnelly et al., 2004](#)). Although these data suggested no evidence for clear species division among the various species, the studies only focused on the ND5 loci ([Besansky et al., 2003](#); [Donnelly et al., 2004](#)) or included also cytochrome b and *ND1* loci ([Besansky et al., 1997](#)). In our study we use the

complete mitogenome for comparison, which would make the analysis more robust. In addition, we specifically included the different chromosomal forms in our analysis. These chromosomal forms are genetically diverged from each other and display strong assortative mating in the *An. gambiae* chromosomal forms ([Touré et al., 1998](#)). The *An. coluzzii* chromosomal forms differ from each other in their ecology: *An. coluzzii*-Mopti is found in dry areas whereas the *An. coluzzii*-Forest restrict themselves to a wet climate ([Lee et al., 2009](#)).

In this study we wished to identify species-specific markers within the mtDNA for *Anopheles arabiensis*, *An. coluzzii* and *An. gambiae*, including among the chromosomal forms currently subsumed under the designations *An. gambiae* and *An. coluzzii*, with the goal of adding these to our existing *Anopheles* species detection assay ([Lee et al. \(2014\)](#)). We sequenced the whole mitogenomes of 70 individual mosquito specimens collected throughout Sub-Saharan Africa. The raw Illumina sequencing reads were mapped to the AgamP4 reference sequence, which included both nuclear and mitochondrial sequences. We explore the relationship among *An. arabiensis*, *An. coluzzii*, *An. gambiae* and four of the sub-specific chromosomal form mitogenome sequences.

Methods

Sample collection

Anopheles arabiensis raw Illumina sequencing reads were obtained from our previous study ([Marsden et al. \(2014\)](#)). These included 20 female *An. arabiensis* mosquitoes which were collected indoors in houses using mouth aspirators from three villages in Tanzania in 2012 (Lupiro ((-8.38000°N, 36.66912°W), Sagamaganga (-8.06781°N, 36.80207°W), and Minepa (-8.25700°N, 36.68163°W) in the Kilombero Valley) and 4 samples from Cameroon collected in 2005 (9.09957°N, 13.72292°W). The DNA was extracted from the head and thorax of each mosquito species and *An. arabiensis* mosquitoes were identified using Scott primers ([Scott et al., 1993](#)). The adult *An. gambiae* and *An. coluzzii* samples were collected indoors using mouth aspirators in Kela, Mali (11.88683°N, -8.44744°W) in 2012 and Mutengene, Cameroon (4.0994°N, 9.3081°W) in 2011. We subdivided the *An. coluzzii* specimen into the *Forest* and *Mopti* chromosomal forms. Similarly, we did this for the *An. gambiae* *Savannah* and *Bamako* chromosomal forms. We examined the polytene chromosome to characterize the chromosomal forms as in [Lanzaro & Lee, 2013](#) and used the same definitions. The results of chromosome determination are listed in [Table 1](#). The *An. quadriannulatus* mosquito, used as an outgroup for the phylogenetic analysis, was collected as larvae in the Shingwidzi area (23.1160°S 31.3752°E) in South Africa in 2015 and was reared to adult.

Genome sequencing

Sequencing methods for *An. arabiensis* samples are as described in [Marsden et al. \(2014\)](#). In short, individually barcoded Illumina paired-end sequencing libraries, with insert sizes of 320-400 basepairs (bp) using NEXTflex Sequencing kits (NOVA-5144) and barcodes (NOVA-514102)(Bio Scientific, Austin, TX, USA), were sequenced on an Illumina HiSeq2000 (Illumina, San Diego, CA, USA) with 100-bp paired-end

Table 1. List of detected chromosomal inversions to detect chromosomal forms of *An. coluzzii* and *An. gambiae* according Toure and co-workers (Touré *et al.*, 1998). '2' represents homozygous for the inversion, '1' heterozygous for the inversion and '-' for homozygous for the standard arrangement.

Banked ID	Chromosomal Form	2La	2Rb	2Rc	2Rd	2Rj	2Ru
11MUTE470	<i>An. coluzzii</i> -Forest	-	-	-	-	-	-
11MUTE472	<i>An. coluzzii</i> -Forest	-	-	-	-	-	-
11MUTE476	<i>An. coluzzii</i> -Forest	-	-	-	-	-	-
11MUTE477	<i>An. coluzzii</i> -Forest	-	-	-	-	-	-
11MUTE479	<i>An. coluzzii</i> -Forest	-	-	-	-	-	-
11MUTE480	<i>An. coluzzii</i> -Forest	-	-	-	-	-	-
11MUTE483	<i>An. coluzzii</i> -Forest	-	-	-	-	-	-
11MUTE487	<i>An. coluzzii</i> -Forest	-	-	-	-	-	-
11MUTE490	<i>An. coluzzii</i> -Forest	-	-	-	-	-	-
11MUTE491	<i>An. coluzzii</i> -Forest	-	-	-	-	-	-
11MUTE493	<i>An. coluzzii</i> -Forest	-	-	-	-	-	-
2012KELA022	<i>An. coluzzii</i> -Mopti	1	1	1	-	-	-
2012KELA024	<i>An. coluzzii</i> -Mopti	2	1	1	-	-	-
2012KELA046	<i>An. coluzzii</i> -Mopti	2	1	1	-	-	-
2012KELA085	<i>An. coluzzii</i> -Mopti	2	2	2	-	-	-
2012KELA087	<i>An. coluzzii</i> -Mopti	1	2	2	-	-	-
2012KELA088	<i>An. coluzzii</i> -Mopti	2	-	-	-	-	1
2012KELA099	<i>An. coluzzii</i> -Mopti	2	-	-	-	-	1
2012KELA112	<i>An. coluzzii</i> -Mopti	2	2	2	-	-	-
2012KELA161	<i>An. coluzzii</i> -Mopti	2	-	-	-	-	1
2012KELA210	<i>An. gambiae</i> -Savannah	2	2	-	-	-	-
2012KELA214	<i>An. gambiae</i> -Bamako	2	-	2	-	2	2
2012KELA219	<i>An. gambiae</i> -Bamako	2	-	2	-	2	2
2012KELA228	<i>An. gambiae</i> -Savannah	2	2	-	-	-	-
2012KELA233	<i>An. gambiae</i> -Savannah	2	2	-	-	-	-
2012KELA234	<i>An. gambiae</i> -Savannah	1	2	-	-	-	-
2012KELA239	<i>An. gambiae</i> -Bamako	2	1	2	-	2	2
2012KELA240	<i>An. gambiae</i> -Bamako	2	1	2	-	2	2
2012KELA244	<i>An. gambiae</i> -Bamako	2	-	2	-	2	2
2012KELA285	<i>An. gambiae</i> -Savannah	2	2	-	-	-	-
2012KELA321	<i>An. gambiae</i> -Savannah	2	2	-	-	-	-
2012KELA334	<i>An. gambiae</i> -Savannah	2	2	-	-	-	-
2012KELA348	<i>An. gambiae</i> -Savannah	2	2	-	-	-	-
2012KELA367	<i>An. gambiae</i> -Bamako	2	1	2	-	2	2
2012KELA400	<i>An. coluzzii</i> -Mopti	2	-	-	-	-	2
2012KELA406	<i>An. gambiae</i> -Bamako	2	-	2	-	2	2
2012KELA409	<i>An. gambiae</i> -Savannah	2	2	-	-	-	-
2012KELA420	<i>An. coluzzii</i> -Mopti	2	-	-	-	-	2
2012KELA423	<i>An. coluzzii</i> -Mopti	2	2	2	-	-	-
2012KELA443	<i>An. gambiae</i> -Bamako	2	1	2	-	2	2
2012KELA457	<i>An. gambiae</i> -Bamako	2	-	2	-	2	2
2012KELA458	<i>An. coluzzii</i> -Mopti	2	-	-	-	-	2
2012KELA467	<i>An. gambiae</i> -Bamako	2	-	2	-	2	2
2012KELA468	<i>An. gambiae</i> -Savannah	2	1	-	-	-	-
2012KELA481	<i>An. gambiae</i> -Bamako	2	2	2	-	2	2
2012KELA496	<i>An. coluzzii</i> -Mopti	2	1	-	-	-	-
2012KELA651	<i>An. gambiae</i> -Bamako	2	2	2	-	2	2
2012KELA812	<i>An. gambiae</i> -Savannah	2	1	-	-	-	-

reads using twelve samples per lane. For the *An. coluzzii* and *An. gambiae* samples we used the same methods as described in Norris *et al.* (2015) and Main *et al.* (2015). For the latter species, libraries were created using the Nextera DNA Sample Preparation Kit (FC-121-1031) and TruSeq dual indexing barcodes (FC-121-103)(Illumina) and the samples were sequenced on an Illumina HiSeq2500 with 100-bp paired end reads. We sequenced the whole genome, but only mapped the raw sequences to the NC_002084 reference mitogenome sequence.

Data analysis

De-multiplexed raw reads were trimmed using Trimmomatic (Bolger *et al.* (2014)) version 0.36 and mapped to the mitogenome reference sequence of *An. gambiae* (Genbank accession number = NC_002084 (Beard *et al.* (1993))). Freebayes (v1.0.1)

(Garrison & Marth, 2013) was used for mitochondrial variant calling assuming single ploidy and without population prior. Mapping statistics were calculated using qualimap version 2.2 (Okonechnikov *et al.* (2016)) and the data is represented in Table 2. Following the recommendation of Crawford and Lazzaro (Crawford & Lazzaro, 2012), we used a minimum depth of 8 to call variants for each individual. Between positions 1-13,470bp of the mitogenome, we obtained consistently high quality reads for all samples, which were used for further analysis. An AT-rich region located between 13,471 and 15,388 suffers from low or zero coverage for sequences generated with the Nextera library preparation kit. Therefore, we excluded these regions from further analysis. The Vcf2fasta program (Danecek *et al.* (2011)) was used to extract mitogenome sequences from vcf file to fasta format. Geneious version 10.1.3 was used for mitogenome

Table 2. List of samples that are used for the study. Mapped reads indicates the reads that are mapped to the reference genome. Mean coverage indicates the average depth of reads on the mitochondrial DNA and standard deviation indicates the coverage deviation across the mitochondrial DNA.

Species	Banked_id	Year	Country	Village	Mapped bases	Mean coverage	Standard deviation
<i>An. coluzzii</i> -Forest	11MUTE470	2011	Cameroon	Mutengene	4265836	277.7	144.5
<i>An. coluzzii</i> -Forest	11MUTE472	2011	Cameroon	Mutengene	1862892	121.3	23
<i>An. coluzzii</i> -Forest	11MUTE476	2011	Cameroon	Mutengene	2130531	138.7	50.5
<i>An. coluzzii</i> -Forest	11MUTE477	2011	Cameroon	Mutengene	806611	52.5	16.7
<i>An. coluzzii</i> -Forest	11MUTE480	2011	Cameroon	Mutengene	804015	52.3	21
<i>An. coluzzii</i> -Forest	11MUTE483	2011	Cameroon	Mutengene	1702247	110.8	42.9
<i>An. coluzzii</i> -Forest	11MUTE487	2011	Cameroon	Mutengene	812839	52.9	21.2
<i>An. coluzzii</i> -Forest	11MUTE490	2011	Cameroon	Mutengene	1882088	122.5	52.4
<i>An. coluzzii</i> -Forest	11MUTE491	2011	Cameroon	Mutengene	1422997	92.6	46.6
<i>An. coluzzii</i> -Forest	11MUTE493	2011	Cameroon	Mutengene	627590	40.9	17.3
<i>An. coluzzii</i> -Mopti	12KELA022	2012	Mali	Kela	3695920	240.6	64.4
<i>An. coluzzii</i> -Mopti	12KELA024	2012	Mali	Kela	574282	37.4	30.8
<i>An. coluzzii</i> -Mopti	12KELA046	2012	Mali	Kela	4152520	270.3	87.2
<i>An. coluzzii</i> -Mopti	12KELA085	2012	Mali	Kela	10883282	708.4	345
<i>An. coluzzii</i> -Mopti	12KELA087	2012	Mali	Kela	3351158	218.1	79.8
<i>An. coluzzii</i> -Mopti	12KELA088	2012	Mali	Kela	1704283	110.9	91.3
<i>An. coluzzii</i> -Mopti	12KELA099	2012	Mali	Kela	349531	22.8	11
<i>An. coluzzii</i> -Mopti	12KELA112	2012	Mali	Kela	8550102	556.5	198.2
<i>An. coluzzii</i> -Mopti	12KELA161	2012	Mali	Kela	33794208	2199.7	629.3
<i>An. gambiae</i> -Savannah	12KELA210	2012	Mali	Kela	3007375	195.8	53.3
<i>An. gambiae</i> -Bamako	12KELA214	2012	Mali	Kela	26441050	1721.1	566.4
<i>An. gambiae</i> -Bamako	12KELA219	2012	Mali	Kela	3617355	235.5	130.2
<i>An. gambiae</i> -Savannah	12KELA228	2012	Mali	Kela	7783776	506.7	262.8
<i>An. gambiae</i> -Savannah	12KELA233	2012	Mali	Kela	7827363	509.5	138.6
<i>An. gambiae</i> -Savannah	12KELA234	2012	Mali	Kela	6721204	437.5	205.9
<i>An. gambiae</i> -Bamako	12KELA239	2012	Mali	Kela	6683521	435	126.4

Species	Banked_id	Year	Country	Village	Mapped bases	Mean coverage	Standard deviation
<i>An. gambiae-Bamako</i>	12KELA240	2012	Mali	Kela	15131480	984.9	270.8
<i>An. gambiae-Bamako</i>	12KELA244	2012	Mali	Kela	12851754	836.5	306.5
<i>An. gambiae-Savannah</i>	12KELA285	2012	Mali	Kela	407888	26.6	119.8
<i>An. gambiae-Savannah</i>	12KELA321	2012	Mali	Kela	1034014	67.3	43.8
<i>An. gambiae-Savannah</i>	12KELA334	2012	Mali	Kela	20949015	1363.6	400.4
<i>An. gambiae-Savannah</i>	12KELA348	2012	Mali	Kela	12053890	784.6	280.9
<i>An. gambiae-Bamako</i>	12KELA367	2012	Mali	Kela	12109235	788.2	240.1
<i>An. coluzzii-Mopti</i>	12KELA400	2012	Mali	Kela	13707820	892.3	398.2
<i>An. gambiae-Bamako</i>	12KELA406	2012	Mali	Kela	17605437	1146	463.2
<i>An. gambiae-Savannah</i>	12KELA409	2012	Mali	Kela	10526480	685.2	259.1
<i>An. coluzzii-Mopti</i>	12KELA420	2012	Mali	Kela	31785953	2069	845.5
<i>An. gambiae-Bamako</i>	12KELA443	2012	Mali	Kela	25740781	1675.5	669.1
<i>An. gambiae-Bamako</i>	12KELA457	2012	Mali	Kela	1360654	88.6	36.6
<i>An. coluzzii-Mopti</i>	12KELA458	2012	Mali	Kela	153686	10	10.4
<i>An. gambiae-Bamako</i>	12KELA467	2012	Mali	Kela	10499093	683.4	249.1
<i>An. gambiae-Savannah</i>	12KELA468	2012	Mali	Kela	10315033	671.4	197.1
<i>An. gambiae-Bamako</i>	12KELA481	2012	Mali	Kela	20308589	1321.9	307.6
<i>An. coluzzii-Mopti</i>	12KELA496	2012	Mali	Kela	2975297	193.7	162.9
<i>An. gambiae-Bamako</i>	12KELA651	2012	Mali	Kela	376689	24.5	11.3
<i>An. gambiae-Savannah</i>	12KELA812	2012	Mali	Kela	799071	52	29.3
<i>An. arabiensis</i>	12LUPI001	2012	Tanzania	Lupiro	2843317	185.1	34.9
<i>An. arabiensis</i>	12LUPI007	2012	Tanzania	Lupiro	6288802	409.3	40
<i>An. arabiensis</i>	12LUPI024	2012	Tanzania	Lupiro	6328898	412	78.5
<i>An. arabiensis</i>	12LUPI056	2012	Tanzania	Lupiro	5440256	354.1	39.2
<i>An. arabiensis</i>	12LUPI059	2012	Tanzania	Lupiro	39721262	2585.5	801.8
<i>An. arabiensis</i>	12LUPI071	2012	Tanzania	Lupiro	3433158	223.5	59.2
<i>An. arabiensis</i>	12LUPI074	2012	Tanzania	Lupiro	10096062	657.2	100.5
<i>An. arabiensis</i>	12LUPI082	2012	Tanzania	Lupiro	5732773	373.2	69.6
<i>An. arabiensis</i>	12MINE001	2012	Tanzania	Minepa	7768923	505.7	66.9
<i>An. arabiensis</i>	12MINE040	2012	Tanzania	Minepa	2784428	181.2	54.9
<i>An. arabiensis</i>	12MINE100	2012	Tanzania	Minepa	10753877	700	93.9
<i>An. arabiensis</i>	12MINE101	2012	Tanzania	Minepa	5684230	370	41.9
<i>An. arabiensis</i>	12MINE105	2012	Tanzania	Minepa	1526829	99.4	32.8
<i>An. arabiensis</i>	12MINE111	2012	Tanzania	Minepa	5578562	363.1	76.3
<i>An. arabiensis</i>	12SAGA066	2012	Tanzania	Sagamaganga	12745079	829.6	142.3
<i>An. arabiensis</i>	12SAGA107	2012	Tanzania	Sagamaganga	14460217	941.2	259.2
<i>An. arabiensis</i>	12SAGA131	2012	Tanzania	Sagamaganga	15333239	998.1	282.9
<i>An. arabiensis</i>	12SAGA133	2012	Tanzania	Sagamaganga	3792945	246.9	62.5
<i>An. arabiensis</i>	12SAGA134	2012	Tanzania	Sagamaganga	2439101	158.8	34.5
<i>An. arabiensis</i>	12SAGA141	2012	Tanzania	Sagamaganga	3130504	203.8	33.3
<i>An. arabiensis</i>	05OKJ017	2005	Cameroon	Ourodougoudje	9041052	588.5	78.8
<i>An. arabiensis</i>	05OKJ042	2005	Cameroon	Ourodougoudje	148752684	9682.5	785.7
<i>An. arabiensis</i>	05OKJ045	2005	Cameroon	Ourodougoudje	35514980	2311.7	262.8
<i>An. arabiensis</i>	05OKJ070	2005	Cameroon	Ourodougoudje	22847478	1487.2	400.5

alignments. The phylogenetic tree was generated using PhyloBayes MPI (Lartillot *et al.*, 2013) using the CAT-GTR model on the genomic sequences, which is shown to give similar results compared to amino acid sequences (Foster *et al.*, 2017). We ran the program twice for over 30000 iterations. Max difference between the two runs was 0.045 and minimum effective size was > 100 and created a consensus tree that we visualized in Geneious version 10.1.3. We used *scikit-allele* (v1.1.9), a software package for Python (Miles & Harding (2017)), to identify species specific markers.

Dataset 1. Aligned FASTA file of mitogenome samples

<http://dx.doi.org/10.5256/f1000research.13807.d192892>

Results and Discussion

We identified a total of 783 single nucleotide polymorphisms (SNPs) over the entire mitogenome. The majority of these (58.7%) were singletons (found on one of the 70 mitogenomes). We did not identify any SNPs unique to the species or chromosomal forms (Supplementary Table S1) and therefore conclude that mtDNA is not suitable for *Anopheles gambiae* complex species identification.

The lack of species-specific markers is also reflected in the phylogenetic tree (Figure 1). *An. arabiensis*, *An. coluzzii* and *An. gambiae* did not cluster separately, which is consistent with previous reports that compared mitochondrial genome sequence data from specimens originating from Kenya, Senegal

Figure 1. Phylogenetic tree inferred from mtDNA genome sequence data. The phylogenetic tree fails to reveal a clear division of the operational taxonomic units included in this analysis. Colors indicate the species or chromosomal form and numbers at the branches indicate the accuracy of the inferred branches on a scale of 0–1, where 1 represents the highest confidence. The three *An. arabiensis* lineages are previously reported by Maliti and co-workers (Maliti *et al.*, 2016).

and South Africa (Besansky *et al.* (1997)) and Burkina Faso, Cameroon, Kenya, Mali, South Africa, Tanzania and Zimbabwe (Fontaine *et al.* (2015), Supplemental material).

Our data may indicate that there is no divergent selection in mitogenome among *An. gambiae* complex. Since mitochondrial genomes have a higher (1–10 times) substitution rate than nuclear genomes (Havird & Sloan, 2016; Lynch & Walsh, 2007), one might expect some level of divergence in the mitogenome in the absence of selection if the taxa have been separated by reproductive barrier even if they are in sympatry just as people have observed in nuclear genome. Therefore, our data showing lack of any species-specific markers on the mitogenome may due to the results of episodic hybridizations occurred between two species. Of note, 36 of the samples that we used in our study originated from Kela (Mali). Kela is located near the village of Selinkenyi, where previous studies have shown a history of hybridization and introgression between *An. gambiae* and *An. coluzzii* (Lee *et al.* (2013); Main *et al.* (2015); Norris *et al.* (2015)), which may have resulted in shared polymorphisms in their mitochondrial genomes. Shared polymorphisms in their mitochondrial genomes, where history has not been reported, also appeared to have occurred in Mutengene (Cameroon), where both *An. gambiae* and *An. coluzzii* occur sympatrically. Hybridization between either *An. coluzzii* or *An. gambiae* with *An. arabiensis* yields sterile males (Slotman *et al.* (2004)), but phylogenomic analysis of these species show patterns of introgression between all of them (Fontaine *et al.* (2015)), which could be the reason that we do not find any species-specific

markers on the mitogenome. Our mitochondrial genome study does not provide conclusive evidence for hybridization and introgression among the taxa under study. However, our data suggest that this is a possibility and it would be consistent with results reported by (Fontaine *et al.*, 2015) and (Besansky *et al.*, 1997). Future modeling work may illuminate the likely contribution of different evolutionary forces that shapes mitogenome and nuclear genome evolution.

Data availability

Aligned sequences were submitted to the National Center for Biotechnology Information (NCBI) Accession number: MG930826 - MG930896

Dataset 1. Aligned FASTA file of mitogenome samples 10.5256/f1000research.13807.d192892 (Hanemaaijer *et al.*, 2018)

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Acknowledgments

We thank Michelle Sanford for her assistance in the field collection in Cameroon in 2011. We thank Clare Marsden for providing the raw data of *An. arabiensis* samples.

Supplementary material

Supplementary Table S1. List of SNP variants in the different *Anopheles* species and chromosomal forms.

[Click here to access the data](#)

References

- Beard CB, Hamm DM, Collins FH: **The mitochondrial genome of the mosquito *Anopheles gambiae*: DNA sequence, genome organization, and comparisons with mitochondrial sequences of other insects.** *Insect Mol Biol.* 1993; 2(2): 103–124.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Besansky N, Krzywinski J, Lehmann T, *et al.*: **Semipermeable species boundaries between *Anopheles gambiae* and *Anopheles arabiensis*: evidence from multilocus DNA sequence variation.** *Proc Natl Acad Sci U S A.* 2003; 100(19): 10818–10823.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Besansky NJ, Lehmann T, Fahey GT, *et al.*: **Patterns of mitochondrial variation within and between African malaria vectors, *Anopheles gambiae* and *An. arabiensis*, suggest extensive gene flow.** *Genetics.* 1997; 147(4): 1817–1828.
[PubMed Abstract](#) | [Free Full Text](#)
- Besansky NJ, Lehmann T, Fahey GT, *et al.*: **Patterns of mitochondrial variation within and between African malaria vectors, *Anopheles gambiae* and *An. arabiensis*, suggest extensive gene flow.** *Genetics.* 1997; 147(4): 1817–1828.
[PubMed Abstract](#) | [Free Full Text](#)
- Bolger AM, Loshe M, Usadel B: **Trimmomatic: a flexible trimmer for Illumina sequence data.** *Bioinformatics.* 2014; 30(15): 2114–2120.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Brown WM, George M Jr, Wilson AC: **Rapid evolution of animal mitochondrial DNA.** *Proc Natl Acad Sci U S A.* 1979; 76(4): 1967–1971.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Coetzee M, Hunt RH, Wilkerson R, *et al.*: ***Anopheles coluzzii* and *Anopheles amharicus*, new members of the *Anopheles gambiae* complex.** *Zootaxa.* 2013; 3619(3): 246–274.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Crawford JE, Lazzaro BP: **Assessing the accuracy and power of population genetic inference from low-pass next-generation sequencing data.** *Front Genet.* 2012; 3: 66.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Cronin MA, Palmisciano DA, Vyse ER, *et al.*: **Mitochondrial DNA in wildlife forensic science: species identification of tissues.** *Wildlife Soc B (1973-2006).* 1991; 19(1): 94–105.
[Reference Source](#)

- Danecek P, Auton A, Abecasis G, *et al.*: **The variant call format and VCFtools.** *Bioinformatics.* 2011; **27**(15): 2156–58.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- De Barba M, Adams JR, Goldberg CS, *et al.*: **Molecular species identification for multiple carnivores.** *Conserv Genet Resour.* 2014; **6**(4): 821–824.
[Publisher Full Text](#)
- De Mandal S, Chhakchhuak L, Gurusubramanian G, *et al.*: **Mitochondrial markers for identification and phylogenetic studies in insects – A Review.** *DNA Barcodes.* 2014; **2**(1).
[Publisher Full Text](#)
- Donnelly M, Pinto J, Girod R, *et al.*: **Revisiting the role of introgression vs shared ancestral polymorphisms as key processes shaping genetic diversity in the recently separated sibling species of the *Anopheles gambiae* complex.** *Heredity (Edinb).* 2004; **92**(2): 61–8.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Fontaine MC, Pease JB, Steele A, *et al.*: **Mosquito genomics. Extensive introgression in a malaria vector species complex revealed by phylogenomics.** *Science.* 2015; **347**(6217): 1258524.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Fontaine MC, Pease JB, Steele A, *et al.*: **Mosquito genomics. Extensive introgression in a malaria vector species complex revealed by phylogenomics.** *Science.* 2015; **347**(6217): 1258524.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Foster PG, de Oliveira TMP, Bergo ES, *et al.*: **Phylogeny of Anophelinae using mitochondrial protein coding genes.** *R Soc Open Sci.* 2017; **4**(11): 170758.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Fu Q, Mitnik A, Johnson PLF, *et al.*: **A revised timescale for human evolution based on ancient mitochondrial genomes.** *Curr Biol.* 2013; **23**(7): 553–559.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Garrison E, Marth G: **Haplotype-based variant detection from short-read sequencing.** *arXiv: 1207.3907.* Cornell University Library; 2013.
[Reference Source](#)
- Green CA: **Cytological maps for the practical identification of females of the three freshwater species of the *Anopheles gambiae* complex.** *Ann Trop Med Parasitol.* 1972; **66**(1): 143–7.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Haag-Liautard C, Coffey N, Houle D, *et al.*: **Direct estimation of the mitochondrial DNA mutation rate in *Drosophila melanogaster*.** *PLoS Biol.* 2008; **6**(8): e204.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Hanemaaijer MJ, Houston P, Collier TC, *et al.*: **Dataset 1: Mitochondrial genomes of *Anopheles arabiensis*, *An. gambiae* and *An. coluzzii* show no clear species division.** *F1000Research.* 2018.
<http://dx.doi.org/10.5256/f1000research.13807.d192892>
- Harrison RG: **Animal mitochondrial DNA as a genetic marker in population and evolutionary biology.** *Trends Ecol Evol.* 1989; **4**(1): 6–11.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Havird JC, Sloan DB: **The Roles of Mutation, Selection, and Expression in Determining Relative Rates of Evolution in Mitochondrial versus Nuclear Genomes.** *Mol Biol Evol.* 2016; **33**(12): 3042–3053.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Lanzaro GC, Lee Y: **Speciation in *Anopheles gambiae* —The distribution of genetic polymorphism and patterns of reproductive isolation among natural populations.** *Anopheles mosquitoes-New insights into malaria vectors.* InTech, 2013.
[Publisher Full Text](#)
- Lartillot N, Rodrigue N, Stubbs D, *et al.*: **PhyloBayes MPI: phylogenetic reconstruction with infinite mixtures of profiles in a parallel environment.** *Syst Biol.* 2013; **62**(4): 611–615.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Lee Y, Cornel AJ, Meneses CR, *et al.*: **Ecological and genetic relationships of the Forest-M form among chromosomal and molecular forms of the malaria vector *Anopheles gambiae sensu stricto*.** *Malar J.* 2009; **8**: 75.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Lee Y, Marsden CD, Nieman C, *et al.*: **A new multiplex SNP genotyping assay for detecting hybridization and introgression between the M and S molecular forms of *Anopheles gambiae*.** *Mol Ecol Resour.* 2014; **14**(2): 297–305.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Lee Y, Marsden CD, Nieman C, *et al.*: **A new multiplex SNP genotyping assay for detecting hybridization and introgression between the M and S molecular forms of *Anopheles gambiae*.** *Mol Ecol Resour.* 2014; **14**(2): 297–305.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Lee Y, Marsden CD, Norris LC, *et al.*: **Spatiotemporal dynamics of gene flow and hybrid fitness between the M and S forms of the malaria mosquito, *Anopheles gambiae*.** *Proc Natl Acad Sci U S A.* 2013; **110**(49): 19854–9.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Llamas B, Fehren-Schmitz L, Valverde G, *et al.*: **Ancient mitochondrial DNA provides high-resolution time scale of the peopling of the Americas.** *Sci Adv.* 2016; **2**(4): e1501385.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Lynch M, Walsh B: **The origins of genome architecture.** Sinauer Associates; Sunderland, MA. 2007.
[Reference Source](#)
- Main BJ, Lee Y, Collier TC, *et al.*: **Complex genome evolution in *Anopheles coluzzii* associated with increased insecticide usage in Mali.** *Mol Ecol.* 2015; **24**(20): 5145–5157.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Maliti DV, Marsden C, Main B, *et al.*: **Investigating associations between biting time in the malaria vector *Anopheles arabiensis* Patton and single nucleotide polymorphisms in circadian clock genes: support for sub-structure among *An. arabiensis* in the Kilombero valley of Tanzania.** *Parasit Vectors.* 2016; **9**: 109.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Marsden CD, Lee Y, Kreppel K, *et al.*: **Diversity, differentiation, and linkage disequilibrium: prospects for association mapping in the malaria vector *Anopheles arabiensis*.** *G3 (Bethesda).* 2014; **4**(1): 121–131.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Miles A, Harding N: **cggh/scikit-allel: v1.1.8 (Version v1.1.8).** *Zenodo.* 2017.
[Publisher Full Text](#)
- Norris LC, Main BJ, Lee Y, *et al.*: **Adaptive introgression in an African malaria mosquito coincident with the increased usage of insecticide-treated bed nets.** *Proc Natl Acad Sci U S A.* 2015; **112**(3): 815–820.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Okonechnikov K, Conesa A, Garcia-Alcalde F: **Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data.** *Bioinformatics.* 2016; **32**(2): 292–294.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Pegg GG, Sinclair B, Briskey L, *et al.*: **MtDNA barcode identification of fish larvae in the southern Great Barrier Reef–Australia.** *Sci Mar.* 2006; **70**(S2): 7–12.
[Reference Source](#)
- Phillips MJ, Haouchar D, Pratt RC, *et al.*: **Inferring kangaroo phylogeny from incongruent nuclear and mitochondrial genes.** *PLoS One.* 2013; **8**(2): e57745.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Pombi M, Caputo B, Simard F, *et al.*: **Chromosomal plasticity and evolutionary potential in the malaria vector *Anopheles gambiae sensu stricto*: insights from three decades of rare paracentric inversions.** *BMC Evol Biol.* 2008; **8**(1): 309.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Scott JA, Brogdon WG, Collins FH: **Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction.** *Am J Trop Med Hyg.* 1993; **49**(4): 520–529.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Scott JA, Brogdon WG, Collins FH: **Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction.** *Am J Trop Med Hyg.* 1993; **49**(4): 520–529.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Shaw KL: **Conflict between nuclear and mitochondrial DNA phylogenies of a recent species radiation: what mtDNA reveals and conceals about modes of speciation in Hawaiian crickets.** *Proc Natl Acad Sci U S A.* 2002; **99**(25): 16122–16127.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Slotman M, Della Torre A, Powell JR: **The genetics of inviability and male sterility in hybrids between *Anopheles gambiae* and *An. arabiensis*.** *Genetics.* 2004; **167**(1): 275–287.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Sota T, Vogler AP: **Incongruence of mitochondrial and nuclear gene trees in the Carabid beetles *Ohomopterus*.** *Syst Biol.* 2001; **50**(1): 39–59.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Tahir HM, Mehwish, Kanwal N, *et al.*: **Genetic diversity in cytochrome c oxidase I gene of *Anopheles* mosquitoes.** *Mitochondrial DNA A DNA Mapp Seq Anal.* 2016; **27**(6): 4298–4301.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Touré Y, Petrarca V, Traore S, *et al.*: **The distribution and inversion polymorphism of chromosomally recognized taxa of the *Anopheles gambiae* complex in Mali, West Africa.** *Parassitologia.* 1998; **40**(4): 477–511.
[PubMed Abstract](#)
- Turner TL, Hahn MW, Nuzhdin SV: **Genomic islands of speciation in *Anopheles gambiae*.** *PLoS Biol.* 2005; **3**(9): e285.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

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Maria Anice Mureb Sallum

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The revised version is suitable for publication and previously mentioned concerns have been clarified. The mitogenome annotation was well done, and the phylogenetic analyses were adequate. The writing is clear and correct. This work is an important contribution to our knowledge of the mitochondrial genome of the Anophelinae species complexes.

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.

Version 1

Reviewer Report 14 May 2018

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General comment

Phylogenetic analysis need to be improved, and the choice for NJ methods and JC model, justified

in the article. There are several programs that have been largely employed for phylogenetic analysis, including for mitogenome data. The paper authored by Foster et al.¹ contains useful information about analyses that have been carried out for inferring phylogenetic relationships within Anophelinae mosquitoes. I strongly suggest authors to verify how analyses were done.

Sample collection

Authors - "The *An. gambiae* and *An. coluzzii* samples were collected as resting adults using mouth aspirators in Kela, Mali (11.88683°N, -8.44744°W) in 2012 and Mutengene, Cameroon (4.0994°N, 9.3081°W) in 2011."

Comment - Can you please give more details the micro environment where your specimens of *An. gambiae* and *An. coluzzii* were resting?

Authors - "Similarly, we did this for the *An. gambiae* Savannah and Bamako chromosomal forms. We used the same definitions and methods to characterize the chromosomal forms as in Lanzaro & Lee, 2013."

Comment - It is not clear to me if you examined the polytene chromosome of each specimen you identified as the Savannah, Bamako, Forest and Mopti forms. Please clarify.

Genome sequencing

Authors - "For the *An. coluzzii* and *An. gambiae* samples we used the same methods as described in Norris et al. (2015) and Main et al. (2015). For the latter species, libraries were created using the Nextera DNA Sample Preparation Kit (FC-121-1031) and TruSeq dual indexing barcodes (FC-121-103) (Illumina) and the samples were sequenced on an Illumina HiSeq2500 with 100-bp paired end reads."

Comment - Please add a short sentence to clarify if you sequenced the whole genome and from the full sequence data you obtained the positions 1-13,470 of the mitogenome.

Data analysis

Authors - "The phylogenetic tree was generated using the Jukes-Cantor genetic distance model and Neighbor-Joining tree methods available in Geneious version 10.1.3."

Comment - Authors should clarify their choice for sequence analysis. The Geneious software has been developed for editing and aligning DNA / amino acid sequences. There are several softwares, which have been largely used to infer phylogenetic relationships. I suggest authors to refining and improving the phylogenetic analysis using appropriate programs and models that have been chosen for the mitogenome data you have at hand.

References

1. Foster PG, de Oliveira TMP, Bergo ES, Conn JE, et al.: Phylogeny of Anophelinae using mitochondrial protein coding genes. *R Soc Open Sci.* 2017; **4** (11): 170758 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

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, however I have significant reservations, as outlined above.

Reviewer Report 30 April 2018

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General comments

The present research note entitled: "Mitochondrial genomes of *Anopheles arabiensis*, *An. gambiae* and *An. coluzzii* show no clear species division" is well analysed, reported and written. As already reported in previous study the submitted manuscript suggested the absence of any species-specific differences in the mitogenome of the three species examined. Although the manuscript is not innovative and the research is not based on any previous evidence, the present note confirms previous suggestions by examining the whole mitogenome of 70 specimens from field specimens and find the lack of species or chromosomal form specific markers.

Title and abstract

Title and abstract are appropriate and summarize well the content of the article.

Introduction

The introduction gives a good description of the aims of the present study, although I would have added some references to previous studies performed on mtDNA of the examined species (for example Besansky 1997) and why you expected to obtain different results compared to previous studies.

Please revise also:

“morphologically identical species that can only be distinguished with molecular markers” (Scott *et al.*, 1993; Coetzee *et al.*, 2013)

The currently used molecular markers are located within genomic islands of divergence located proximal to the centromeres (Lee *et al.* (2014); Turner *et al.* (2005)) please rephrase the citation and refer it only to detect genomic differences between *An.gambiae* e and *An.coluzzii*.

Please insert a sentence about chromosomal forms of *An.gambiae*.

Methods

Please specified the method for collecting *An. arabiensis* as you already described for *An.gambiae* (e.g. indoor specimens, mouth aspirators, PSC collections).

Please insert a table with inversion polymorphism of chromosomal forms analyzed.

Please add the source of the *An. quadriannulatus* specimens you included in the phylogenetic analysis.

Results

Study design is well explained and results are given concisely.

Please add in Table 2 also the number of specimens you included for each species in the analysis.

Please add in Figure two an explanation of what “lineage” means for *An. arabiensis* specimens.

Please give results (also without table or figure) for each country separately.

Discussion

Discussion is very concise but deals with most major points of interest. We would just suggest to explain better the conclusion on possible introgression (the more plausible hypothesis) between taxa and to evaluate other possible explanations for the absence of fixed differences between species (e.g. absence for divergent selection, or evolutionary characteristic of mitogenomes).

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

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.

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