RESEARCH NOTE

REVISED 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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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 restrtict 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	*An. coluzzii*-Forest	-	-	-	-	-	-
11MUTE472	*An. coluzzii*-Forest	-	-	-	-	-	-
11MUTE476	*An. coluzzii*-Forest	-	-	-	-	-	-
11MUTE477	*An. coluzzii*-Forest	-	-	-	-	-	-
11MUTE479	*An. coluzzii*-Forest	-	-	-	-	-	-
11MUTE480	*An. coluzzii*-Forest	-	-	-	-	-	-
11MUTE483	*An. coluzzii*-Forest	-	-	-	-	-	-
11MUTE487	*An. coluzzii*-Forest	-	-	-	-	-	-
11MUTE490	*An. coluzzii*-Forest	-	-	-	-	-	-
11MUTE491	*An. coluzzii*-Forest	-	-	-	-	-	-
11MUTE493	*An. coluzzii*-Forest	-	-	-	-	-	-
2012KELA022	*An. coluzzii*-Mopti	1	1	1	-	-	-
2012KELA024	*An. coluzzii*-Mopti	2	1	1	-	-	-
2012KELA046	*An. coluzzii*-Mopti	2	1	1	-	-	-
2012KELA085	*An. coluzzii*-Mopti	2	2	2	-	-	-
2012KELA087	*An. coluzzii*-Mopti	1	2	2	-	-	-
2012KELA088	*An. coluzzii*-Mopti	2	-	-	-	-	1
2012KELA099	*An. coluzzii*-Mopti	2	-	-	-	-	1
2012KELA112	*An. coluzzii*-Mopti	2	2	2	-	-	-
2012KELA161	*An. coluzzii*-Mopti	2	-	-	-	-	1
2012KELA210	*An. gambiae*-Savannah	2	2	-	-	-	-
2012KELA214	*An. gambiae*-Bamako	2	-	2	-	2	2
2012KELA219	*An. gambiae*-Bamako	2	-	2	-	2	2
2012KELA228	*An. gambiae*-Savannah	2	2	-	-	-	-
2012KELA233	*An. gambiae*-Savannah	2	2	-	-	-	-
2012KELA234	*An. gambiae*-Savannah	1	2	-	-	-	-
2012KELA239	*An. gambiae*-Bamako	2	1	2	-	2	2
2012KELA240	*An. gambiae*-Bamako	2	1	2	-	2	2
2012KELA244	*An. gambiae*-Bamako	2	-	2	-	2	2
2012KELA285	*An. gambiae*-Savannah	2	2	-	-	-	-
2012KELA321	*An. gambiae*-Savannah	2	2	-	-	-	-
2012KELA334	*An. gambiae*-Savannah	2	2	-	-	-	-
2012KELA348	*An. gambiae*-Savannah	2	2	-	-	-	-
2012KELA367	*An. gambiae*-Bamako	2	1	2	-	2	2
2012KELA400	*An. coluzzii*-Mopti	2	-	-	-	-	2
2012KELA406	*An. gambiae*-Bamako	2	-	2	-	2	2
2012KELA409	*An. gambiae*-Savannah	2	2	-	-	-	-
2012KELA420	*An. coluzzii*-Mopti	2	-	-	-	-	2
2012KELA423	*An. coluzzii*-Mopti	2	2	2	-	-	-
2012KELA443	*An. gambiae*-Bamako	2	1	2	-	2	2
2012KELA457	*An. gambiae*-Bamako	2	-	2	-	2	2
2012KELA458	*An. coluzzii*-Mopti	2	-	-	-	-	2
2012KELA467	*An. gambiae*-Bamako	2	-	2	-	2	2
2012KELA468	*An. gambiae*-Savannah	2	1	-	-	-	-
2012KELA481	*An. gambiae*-Bamako	2	2	2	-	2	2
2012KELA496	*An. coluzzii*-Mopti	2	1	-	-	-	-
2012KELA651	*An. gambiae*-Bamako	2	2	2	-	2	2
2012KELA812	*An. gambiae*-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 Lazarro (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
An. coluzzii-Forest	11MUTE470	2011	Cameroon	Mutengene	4265836	277.7	144.5
An. coluzzii-Forest	11MUTE472	2011	Cameroon	Mutengene	1862892	121.3	23
An. coluzzii-Forest	11MUTE476	2011	Cameroon	Mutengene	2130531	138.7	50.5
An. coluzzii-Forest	11MUTE477	2011	Cameroon	Mutengene	806611	52.5	16.7
An. coluzzii-Forest	11MUTE480	2011	Cameroon	Mutengene	804015	52.3	21
An. coluzzii-Forest	11MUTE483	2011	Cameroon	Mutengene	1702247	110.8	42.9
An. coluzzii-Forest	11MUTE487	2011	Cameroon	Mutengene	812839	52.9	21.2
An. coluzzii-Forest	11MUTE490	2011	Cameroon	Mutengene	1882088	122.5	52.4
An. coluzzii-Forest	11MUTE491	2011	Cameroon	Mutengene	1422997	92.6	46.6
An. coluzzii-Forest	11MUTE493	2011	Cameroon	Mutengene	627590	40.9	17.3
An. coluzzii-Mopti	12KELA022	2012	Mali	Kela	3695920	240.6	64.4
An. coluzzii-Mopti	12KELA024	2012	Mali	Kela	574282	37.4	30.8
An. coluzzii-Mopti	12KELA046	2012	Mali	Kela	4152520	270.3	87.2
An. coluzzii-Mopti	12KELA085	2012	Mali	Kela	10883282	708.4	345
An. coluzzii-Mopti	12KELA087	2012	Mali	Kela	3351158	218.1	79.8
An. coluzzii-Mopti	12KELA088	2012	Mali	Kela	1704283	110.9	91.3
An. coluzzii-Mopti	12KELA099	2012	Mali	Kela	349531	22.8	11
An. coluzzii-Mopti	12KELA112	2012	Mali	Kela	8550102	556.5	198.2
An. coluzzii-Mopti	12KELA161	2012	Mali	Kela	33794208	2199.7	629.3
An. gambiae-Savannah	12KELA210	2012	Mali	Kela	3007375	195.8	53.3
An. gambiae-Bamako	12KELA214	2012	Mali	Kela	26441050	1721.1	566.4
An. gambiae-Bamako	12KELA219	2012	Mali	Kela	3617355	235.5	130.2
An. gambiae-Savannah	12KELA228	2012	Mali	Kela	7783776	506.7	262.8
An. gambiae-Savannah	12KELA233	2012	Mali	Kela	7827363	509.5	138.6
An. gambiae-Savannah	12KELA234	2012	Mali	Kela	6721204	437.5	205.9
An. gambiae-Bamako	12KELA239	2012	Mali	Kela	6683521	435	126.4

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

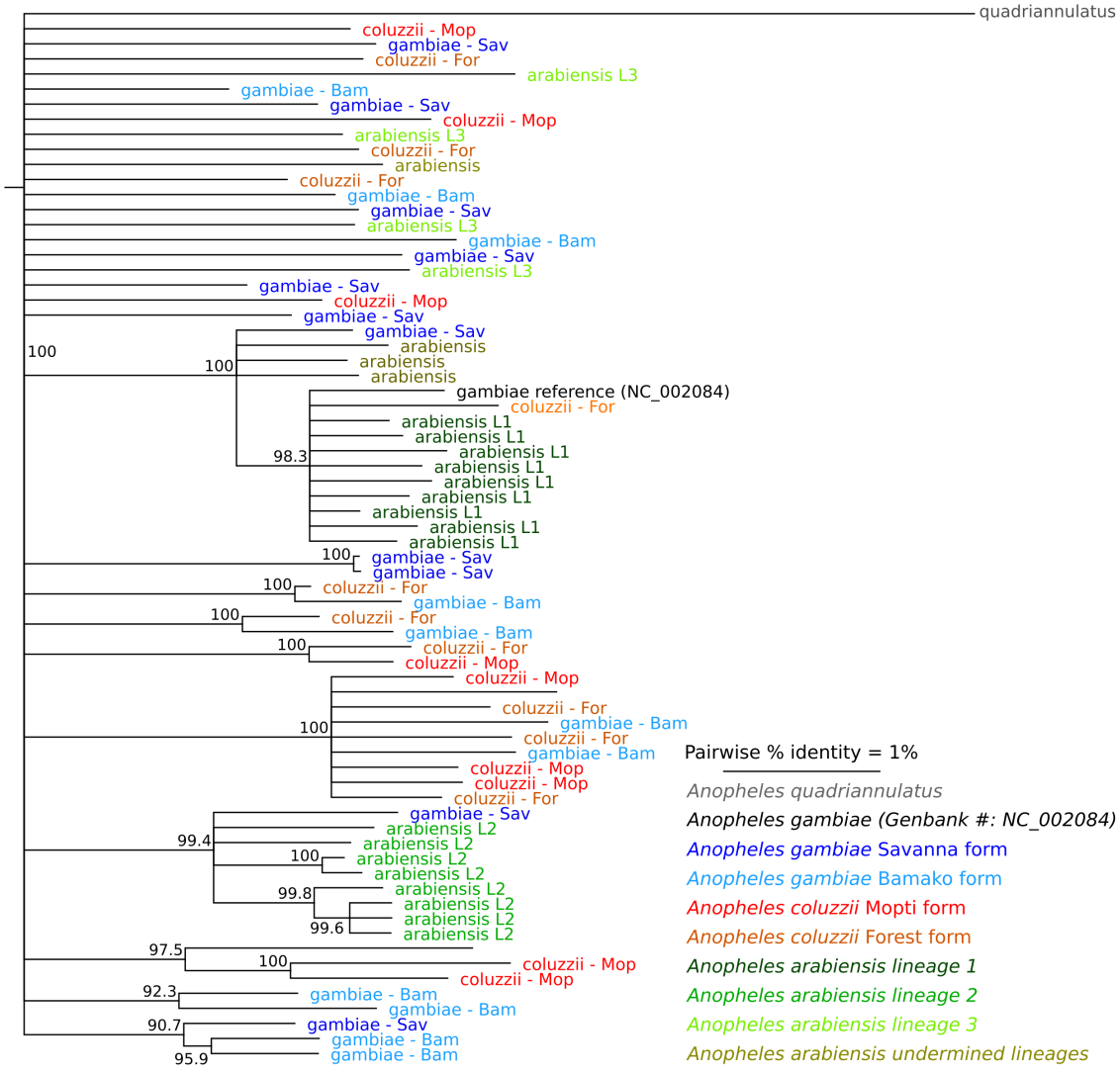

Figure 1. Phylogenetic tree inferred from mtDNA genome sequence data. The phylogenetic tree fails to reveal a clear division of the operational taxonomic units included in this analysis. Colors indicate the species or chromosomal form and numbers at the branches indicate the accuracy of the inferred 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 evoluationary 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)

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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

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Current Referee Status: ✔ ✔

Version 2

Referee Report 29 April 2019

https://doi.org/10.5256/f1000research.20094.r45747

✔ **Maria Anice Mureb Sallum**
Department of Epidemiology, Faculty of Public Health, University of São Paulo, São Paulo, Brazil

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

Version 1

Referee Report 14 May 2018

https://doi.org/10.5256/f1000research.15009.r33371

? **Maria Anice Mureb Sallum**
Department of Epidemiology, Faculty of Public Health, University of São Paulo, São Paulo, Brazil

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.

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

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

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

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

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

Are the conclusions drawn adequately supported by the results?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

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, however I have significant reservations, as outlined above.

Referee Report 30 April 2018

https://doi.org/10.5256/f1000research.15009.r32266

Beniamino Caputo [1], **Verena Pichler** [2]
[1] Department of Public Health and Infectious Diseases, Sapienza University of Rome, Rome, Italy
[2] Department of Public Health & Infectious Diseases, Sapienza University of Rome, Rome, Italy

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 characherestic of mitogenomes).

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

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

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

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

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

Are the conclusions drawn adequately supported by the results?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

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