

Supplementary Data file

**Extensive New *Anopheles* Cryptic Species Involved in Human Malaria Transmission
in Western Kenya**

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Supplementary Table S1 Pairwise comparison of ITS2 sequence similarities of the 21 *Anopheles* species identified in western Kenya

Species	AR	CF	CH	CO	FUN	GA	LEE	MA	PH	PR	RU	An.sp.1	An.sp.6	An.sp.7	An.sp.9	An.sp.11	An.sp.14	An.sp.15	An.sp.17	An.sp.18	An.sp.19
AR	-																				
CF	0.46	-																			
CH	0.74	0.48	-																		
CO	0.34	0.33	0.34	-																	
FUN	0.31	0.38	0.30	0.32	-																
GA	0.99	0.45	0.75	0.33	0.31	-															
LEE	0.46	0.66	0.47	0.32	0.36	0.46	-														
MA	0.53	0.45	0.54	0.30	0.29	0.53	0.44	-													
PH	0.23	0.20	0.22	0.22	0.25	0.24	0.20	0.19	-												
PR	0.53	0.46	0.54	0.31	0.28	0.53	0.44	0.84	0.20	-											
RU	0.52	0.47	0.52	0.29	0.30	0.52	0.45	0.77	0.19	0.74	-										
An.sp.1	0.46	0.56	0.46	0.31	0.38	0.45	0.51	0.47	0.20	0.48	0.45	-									
An.sp.6	0.46	0.59	0.48	0.33	0.37	0.46	0.56	0.47	0.20	0.48	0.50	0.72	-								
An.sp.7	0.46	0.57	0.45	0.34	0.37	0.46	0.55	0.47	0.20	0.47	0.49	0.71	0.88	-							
An.sp.9	0.47	0.58	0.46	0.35	0.37	0.47	0.57	0.48	0.21	0.49	0.50	0.71	0.87	0.89	-						
An.sp.11	0.20	0.18	0.19	0.18	0.22	0.20	0.17	0.19	0.49	0.19	0.18	0.17	0.19	0.19	0.18	-					
An.sp.14	0.46	0.58	0.46	0.34	0.38	0.45	0.57	0.48	0.20	0.48	0.49	0.71	0.84	0.81	0.80	0.20	-				
An.sp.15	0.33	0.32	0.34	0.90	0.33	0.33	0.30	0.29	0.21	0.30	0.29	0.30	0.33	0.33	0.34	0.18	0.33	-			
An.sp.17	0.46	0.59	0.46	0.31	0.39	0.46	0.58	0.48	0.19	0.47	0.49	0.68	0.76	0.75	0.77	0.19	0.72	0.31	-		
An.sp.18	0.29	0.27	0.29	0.85	0.27	0.29	0.28	0.27	0.21	0.27	0.26	0.26	0.29	0.30	0.31	0.17	0.30	0.81	0.27	-	
An.sp.19	0.40	0.48	0.39	0.27	0.32	0.40	0.45	0.40	0.20	0.41	0.39	0.78	0.64	0.63	0.63	0.19	0.64	0.26	0.59	0.29	

AR, *An.arabiensis*; CF, *An.cf.rivulorum*; CH, *An.christyi*; CO, *An.coustani*; FUN, *An.funestus*; GA, *An.gambiae*; LEE, *An.leesonii*; MA, *An.maculipalpis*; PH, *An.pharoensis*; PR, *An.pretoriensis*; RU, *An.rufipes*.

Supplementary Table S2. GenBank accession numbers of ITS2 sequences identified in the 21 *Anopheles* species in western Kenya

Sequence_ID	Species	Isolate	GenBank accession
Seq1	<i>An. gambiae</i>	KH01	MT408564
Seq2	<i>An. arabiensis</i>	KH02	MT408565
Seq3	<i>An. funestus</i>	KH03	MT408566
Seq4	<i>An. cf.rivulorum</i>	KH04	MT408567
Seq5	<i>An. christyi</i>	KH05	MT408568
Seq6	<i>An. coustani</i>	KH06	MT408569
Seq7	<i>An. leesonii</i>	KH07	MT408570
Seq8	<i>An. maculipalpis</i>	KH08	MT408571
Seq9	<i>An. pharoensis</i>	KH09	MT408572
Seq10	<i>An. pretoriensis</i>	KH10	MT408573
Seq11	<i>An. rufipes</i>	KH11	MT408574
Seq12	<i>An. sp.1</i>	KH12	MT408575
Seq13	<i>An. sp.6</i>	KH13	MT408576
Seq14	<i>An. sp.7</i>	KH14	MT408577
Seq15	<i>An. sp.9</i>	KH15	MT408578
Seq16	<i>An. sp.11</i>	KH16	MT408579
Seq17	<i>An. sp.14</i>	KH17	MT408580
Seq18	<i>An. sp.15</i>	KH18	MT408581
Seq19	<i>An. sp.17</i>	KH19	MT408582
Seq20	<i>An. sp.18</i>	KH20	MT408583
Seq21	<i>An. sp.19</i>	KH21	MT408584

Supplementary Table S3. GenBank accession numbers of COX1 haplotype sequences identified in *Anopheles* species of western Kenya

Sequence_ID	Species	Haplotype	GenBank accession
>Seq1	<i>An. sp.17</i>	H01	MT375202
>Seq2	<i>An. sp.17</i>	H02	MT375203
>Seq3	<i>An. sp.17</i>	H03	MT375204
>Seq4	<i>An. sp.17</i>	H04	MT375205
>Seq5	<i>An. sp.17</i>	H05	MT375206
>Seq6	<i>An. sp.17</i>	H06	MT375207
>Seq7	<i>An. sp.17</i>	H07	MT375208
>Seq8	<i>An. sp.17</i>	H08	MT375209
>Seq9	<i>An. sp.17</i>	H09	MT375210
>Seq10	<i>An. sp.17</i>	H10	MT375211
>Seq11	<i>An. sp.17</i>	H11	MT375212
>Seq12	<i>An. sp.17</i>	H12	MT375213
>Seq13	<i>An. sp.17</i>	H13	MT375214
>Seq14	<i>An. funestus</i>	H01	MT375215
>Seq15	<i>An. funestus</i>	H02	MT375216
>Seq16	<i>An. funestus</i>	H03	MT375217
>Seq17	<i>An. funestus</i>	H04	MT375218
>Seq18	<i>An. funestus</i>	H05	MT375219
>Seq19	<i>An. funestus</i>	H06	MT375220
>Seq20	<i>An. gambiae</i>	H01	MT375221
>Seq21	<i>An. gambiae</i>	H02	MT375222
>Seq22	<i>An. gambiae</i>	H03	MT375223
>Seq23	<i>An. gambiae</i>	H04	MT375224
>Seq24	<i>An. sp.6</i>	H01	MT375225
>Seq25	<i>An. sp.9</i>	H01	MT375226
>Seq26	<i>An. cf.rivulorum</i>	H01	MT375227
>Seq27	<i>An. maculipalpis</i>	H01	MT375228
>Seq28	<i>An. maculipalpis</i>	H02	MT375229

Supplementary Table S4 Number of *Plasmodium* positive mosquitoes detected indoor and outdoor in western Kenya highlands.

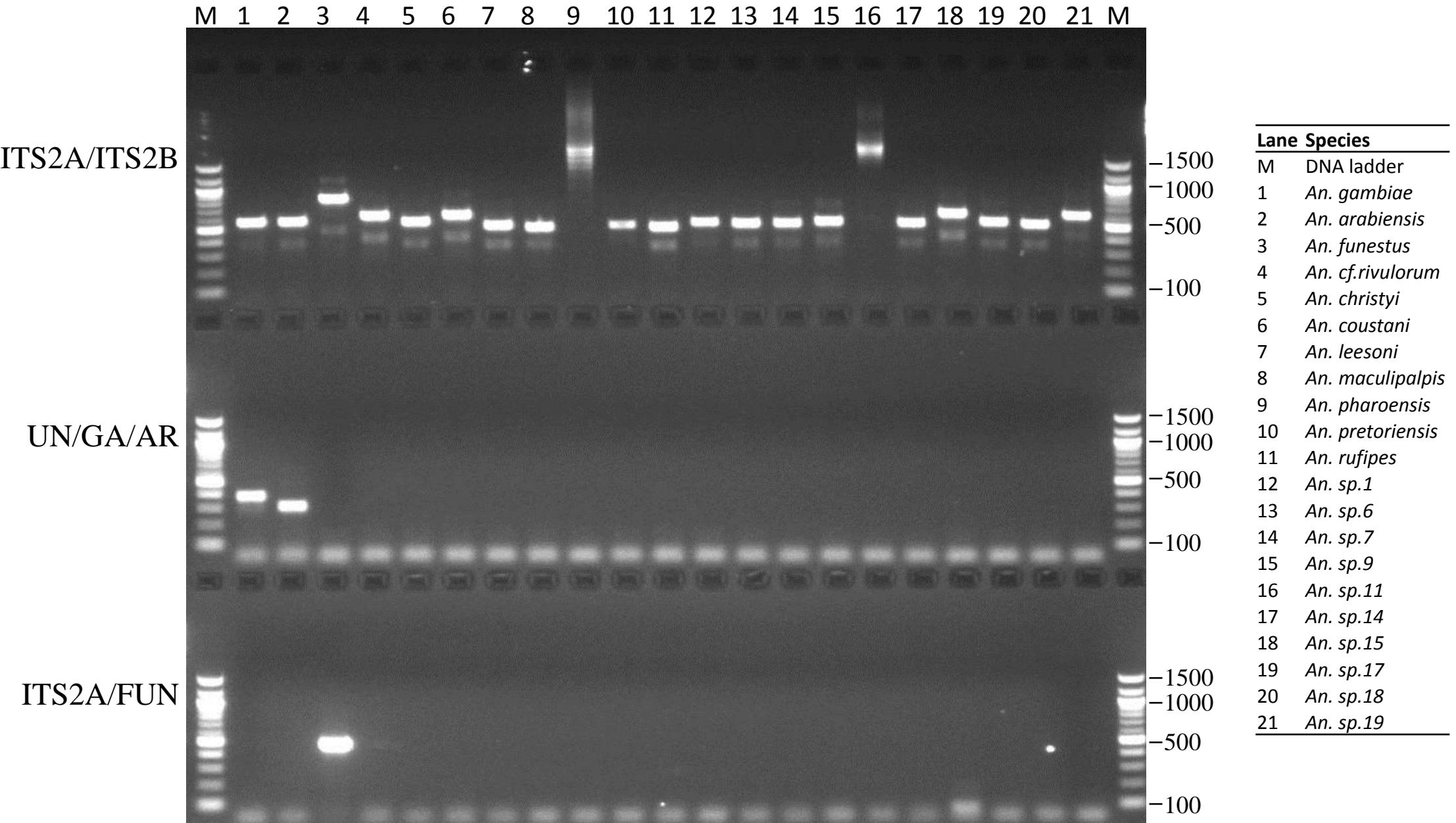
Species	Iguhu (2016)		Emutete (2017)	
	Indoor	Outdoor	Indoor	Outdoor
<i>An. gambiae</i>	10(153)*	7(119)	2(32)	1(14)
<i>An. arabiensis</i>	0(11)	0(2)		0(2)
<i>An. funestus</i>	9(210)	0(14)	5(40)	1(5)
<i>An. cf.rivulorum</i>	0(1)			
<i>An. leesonii</i>	0(1)			
<i>An. maculipalpis</i>				0(1)
<i>An. rufipes</i>	0(2)			0(2)
<i>An. sp.1</i>		0(1)		
<i>An. sp.6</i>	0(3)	0(3)		
<i>An. sp.7</i>	0(6)	0(1)	0(4)	0(4)
<i>An. sp.9</i>	0(5)	1(5)		
<i>An. sp.11</i>		1(1)		
<i>An. sp.14</i>	0(1)	0(1)		
<i>An. sp.15</i>	0(2)	1(2)		0(1)
<i>An. sp.17</i>			2(15)	1(7)
Total	19(395)	10(149)	9(91)	3(36)

* The number in parenthesis represents the total number of mosquitoes tested.

Supplementary Table S5 Species component and proportion of *Plasmodium* infections detected in *Anopheles* mosquitoes in western Kenya.

Species	<i>pf</i>	<i>pm</i>	<i>po</i>	<i>pf+pm</i>	<i>pf+po</i>	<i>pm+po</i>	<i>pf+pm+po</i>
<i>An. gambiae</i>	129(90.2)	1(0.7)	8(5.6)	3(2.1)	2(1.4)		
<i>An. arabiensis</i>	26(86.7)		3(10.0)	1(3.3)			
<i>An. funestus</i>	138(84.7)	3(1.8)	8(4.9)	5(3.1)	7(4.3)	1(0.6)	1(0.6)
<i>An. coustani</i>	1(100)						
<i>An. rufipes</i>	1(100)						
<i>An. sp.1</i>	2(100)						
<i>An. sp.6</i>	2(100)						
<i>An. sp.9</i>	1(100)						
<i>An. sp.11</i>	1(100)						
<i>An. sp.15</i>	1(100)						
<i>An. sp.17</i>	3(100)						

* The number in parenthesis represents the percentage of individuals within the mosquito species. *pf*, *P. falciparum*; *pm*, *P. malariae*; *po*, *P. ovale*.



Supplementary Fig. S1 Agarose gel electrophoresis of PCR products amplified with the 21 *Anopheles* species in western Kenya. Upper panel, PCR amplification with ITS2A and ITS2B primers for sequencing; Middle panel, PCR amplification with multiplex PCR method with UN/GA/AR primers for identification of *An. gambiae* and *An. arabiensis*; Lower panel, PCR amplification with ITS2A and FUN primer for identification of *An. funestus*. Lane M, 100 bp molecular ladder; Lines 1-21, PCR results of the 21 *Anopheles* species.

Supplementary Fig. S2 shows some examples of multiple sequence alignment comparison for the similarity. (a) ITS2 sequence alignment of *An. funestus*; (b) ITS2 sequence alignment of *An. gambiae*; (c) ITS2 sequence alignment of *An. sp.17*; (d) COX1 sequence alignment of *An. funestus*; (e) COX1 sequence alignment of *An. gambiae*; (f) COX1 sequence alignment of *An. sp.17*. Multiple sequence alignments were performed using BioEdit software (<https://bioedit.software.informer.com>). The aligned sequences were reformatted by MView web-based tool (<http://wavis.img.cas.cz/>).

(a)

Reference sequence (1): JN994135_An.funestus

Identities normalised by aligned length.

Colored by: identity

		cov	pid	1	[80
1	JN994135_An.funestus	100.0%	100.0%	TG	GAAC	TGAGGACACAT	
2	MT408566	100.0%	99.6%	TG	GAAC	TGAGGACACAT	
		cov	pid	81	.	.	160
1	JN994135_An.funestus	100.0%	100.0%	TT	C	TTGAGTGCTTA	
2	MT408566	100.0%	99.6%	TT	C	TTGAGTGCTTA	
		cov	pid	161	.	.	240
1	JN994135_An.funestus	100.0%	100.0%	AGCAGCCCG	T	CTAGTGTCGT	
2	MT408566	100.0%	99.6%	AGCAGCCCG	T	CTAGTGTCGT	
		cov	pid	241	.	.	320
1	JN994135_An.funestus	100.0%	100.0%	AGGGCGCT	T	GAAAGTAAAGGGT	
2	MT408566	100.0%	99.6%	AGGGCGCT	T	GAAAGTAAAGGGT	
		cov	pid	321	.	.	400
1	JN994135_An.funestus	100.0%	100.0%	CGTAGGATA	CCGC	TAAAGAGTACGTTG	
2	MT408566	100.0%	99.6%	CGTAGGATA	CCGC	TAAAGAGTACGTTG	
		cov	pid	401	.	.	480
1	JN994135_An.funestus	100.0%	100.0%	CCGATTCG	T	CGTAATACTGGATCAAC	
2	MT408566	100.0%	99.6%	CCGATTCG	T	CGTAATACTGGATCAAC	
		cov	pid	481	5	.	560
1	JN994135_An.funestus	100.0%	100.0%	AUCGATGCCG	GAGGGAACAT	GTGTTCCAATACAA	
2	MT408566	100.0%	99.6%	AUCGATGCCG	GAGGGAACAT	GTGTTCCAATACAA	
		cov	pid	561]	570	
1	JN994135_An.funestus	100.0%	100.0%	TCCAAGT	T	CGA	
2	MT408566	100.0%	99.6%	TCCAAGT	T	CGA	

(b)

Reference sequence (1): EU104646_An.gambiae

Identities normalised by aligned length.

Colored by: identity

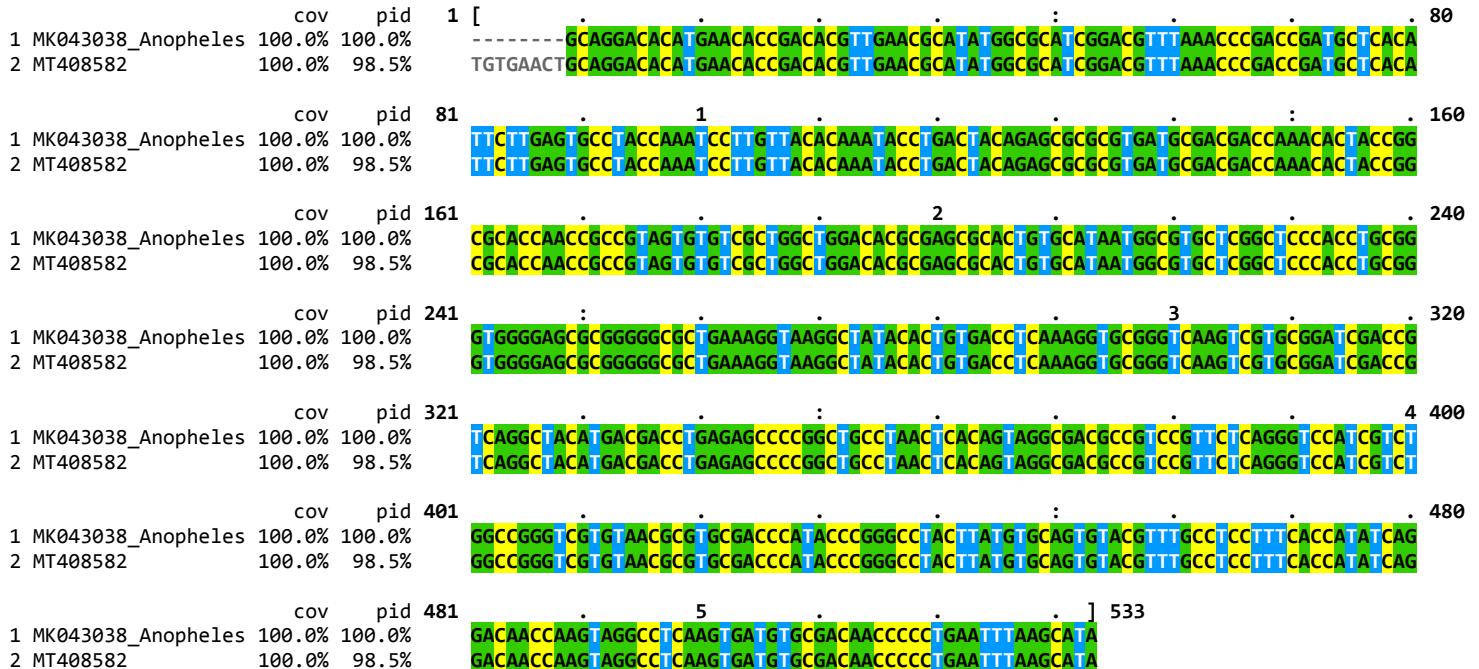
		cov	pid	1			80	
1	EU104646_An.gambiae	100.0%	100.0%	TGTGAAC	TGCAGGACACA	TGAACATTGATAACTTGAACCCATA	TGGCGCATCGGACGTTAACTCCGACCA	
2	MT408564	100.0%	99.8%	TGTGAAC	TGCAGGACACA	TGAACATTGATAACTTGAACCCATA	TGGCGCATCGGACGTTAACTCCGACCA	
		cov	pid	81			160	
1	EU104646_An.gambiae	100.0%	100.0%	TTCCTTGAGT	GCCACTAA	TTACCAAAAGTC	TCAAGTGGCCGTCGCCGAAGGTTCCCCGGTCATCCGAC	
2	MT408564	100.0%	99.8%	TTCCTTGAGT	GCCACTAA	TTACCAAAAGTC	TCAAGTGGCCGTCGCCGAAGGTTCCCCGGTCATCCGAC	
		cov	pid	161			240	
1	EU104646_An.gambiae	100.0%	100.0%	GCAC	TGGCGGTCGCT	GTCATAA	TGACGTGGCTTGGCCCCGTC	TGCGGGTCCTCGGGCTTGAAGTGGACACTCICGA
2	MT408564	100.0%	99.8%	GCAC	TGGCGGTCGCT	GTCATAA	TGACGTGGCTTGGCCCCGTC	TGCGGGTCCTCGGGCTTGAAGTGGACACTCICGA
		cov	pid	241			320	
1	EU104646_An.gambiae	100.0%	100.0%	GCGTATGTTGGATCG	CGTTCTGTTGG	GTTGGTGG	GTTGGATGCGTAGGGCTTGGTGTGTC	TCAGGCCCAGGGTTCGGTGTGTC
2	MT408564	100.0%	99.8%	GCGTATGTTGGATCG	CGTTCTGTTGG	GTTGGTGG	GTTGGATGCGTAGGGCTTGGTGTGTC	TCAGGCCCAGGGTTCGGTGTGTC
		cov	pid	321			400	
1	EU104646_An.gambiae	100.0%	100.0%	AATGCTACGTCG	TCCCCGATGGCC	ACCGGCAGTCTAC	TCTCCAGGCTAAAGTCGGCTCGTCTAGGGATT	CGGAAAGGTAA
2	MT408564	100.0%	99.8%	AATGCTACGTCG	TCCCCGATGGCC	ACCGGCAGTCTAC	TCTCCAGGCTAAAGTCGGCTCGTCTAGGGATT	CGGAAAGGTAA
		cov	pid	401			480	
1	EU104646_An.gambiae	100.0%	100.0%	GTCGCTGTAAC	TCATGTGGGCCCA	TACACGGCGTTGCCTTACCAACGCTAAGTTAGCCCTACATAYACAAGCATCAACCCA		
2	MT408564	100.0%	99.8%	GTCGCTGTAAC	TCATGTGGGCCCA	TACACGGCGTTGCCTTACCAACGCTAAGTTAGCCCTACATAYACAAGCATCAACCCA		
		cov	pid	481			560	
1	EU104646_An.gambiae	100.0%	100.0%	CGGCACGGCG	TAGCTGAAATAC	TTACGTC	CGGTTAACCACGTAAGGTGACTACCCCTAAATT	
2	MT408564	100.0%	99.8%	CGGCACGGCG	TAGCTGAAATAC	TTACGTC	CGGTTAACCACGTAAGGTGACTACCCCTAAATT	
		cov	pid	561			566	
1	EU104646_An.gambiae	100.0%	100.0%	AAGCAT				
2	MT408564	100.0%	99.8%	AAGCAT				

(c)

Reference sequence (1): MK043038_Anopheles

Identities normalised by aligned length.

Colored by: identity

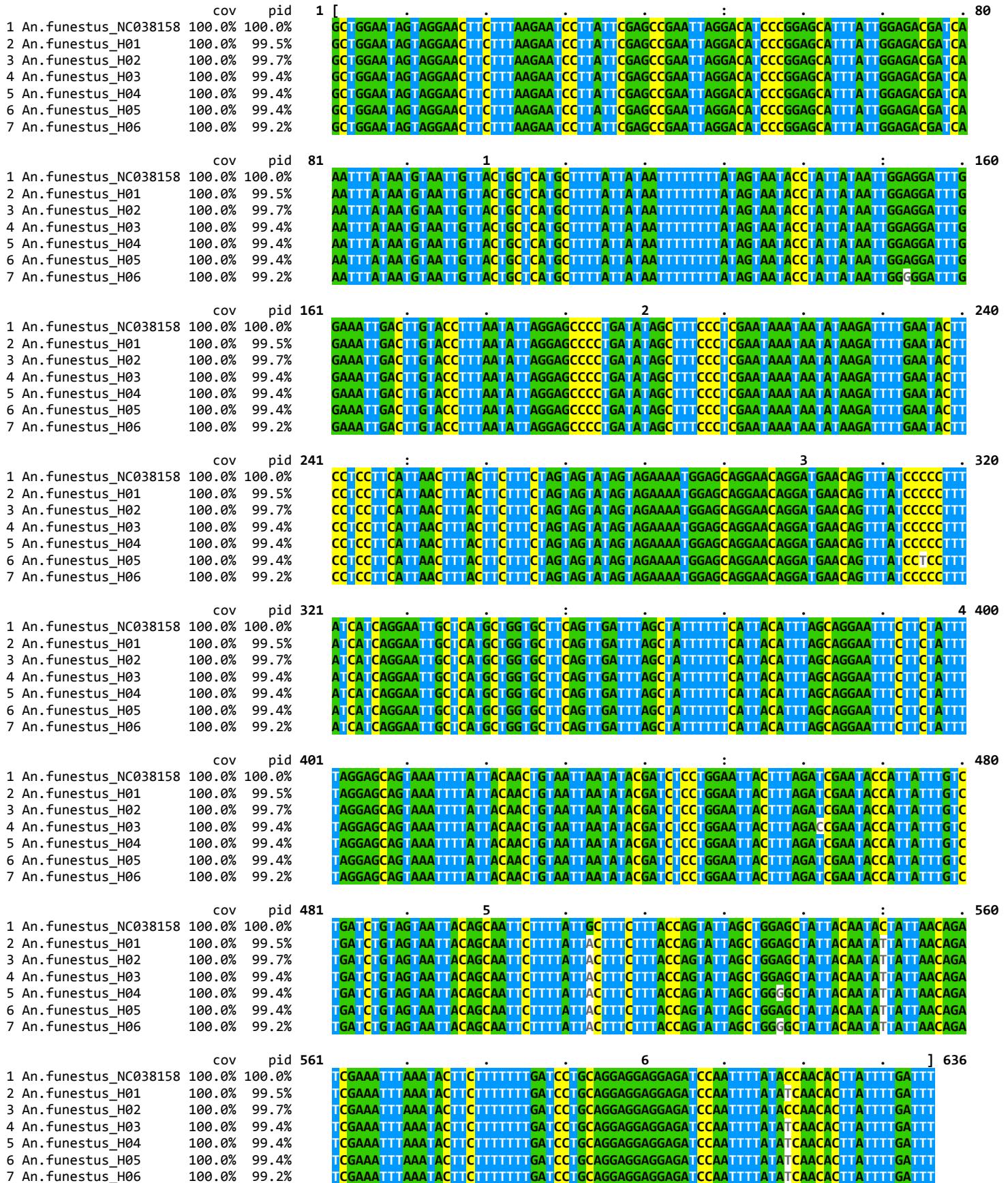


(d)

Reference sequence (1): An.funestus_NC038158

Identities normalised by aligned length.

Colored by: identity

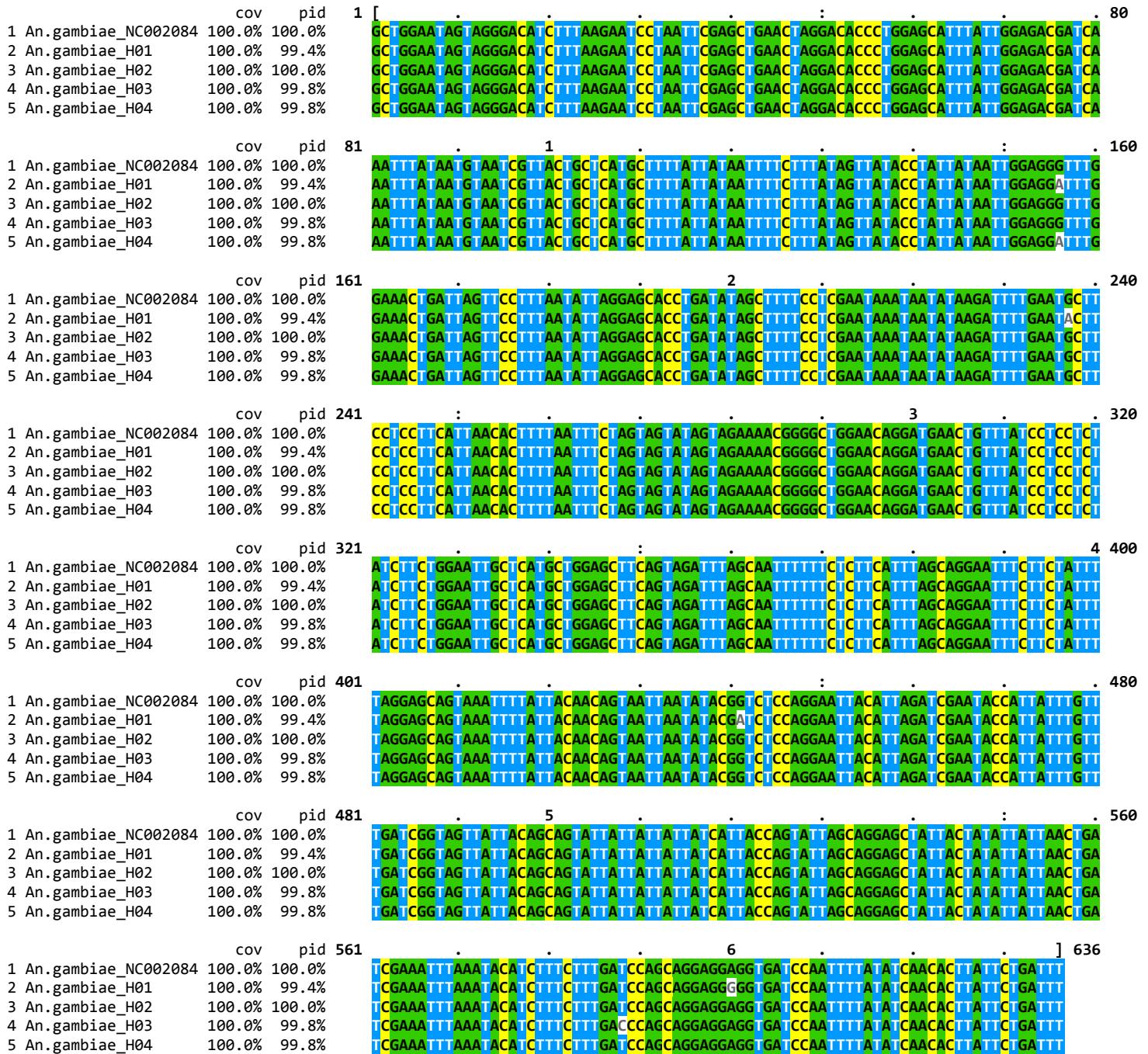


(e)

Reference sequence (1): An.gambiae_NC002084

Identities normalised by aligned length.

Colored by: identity

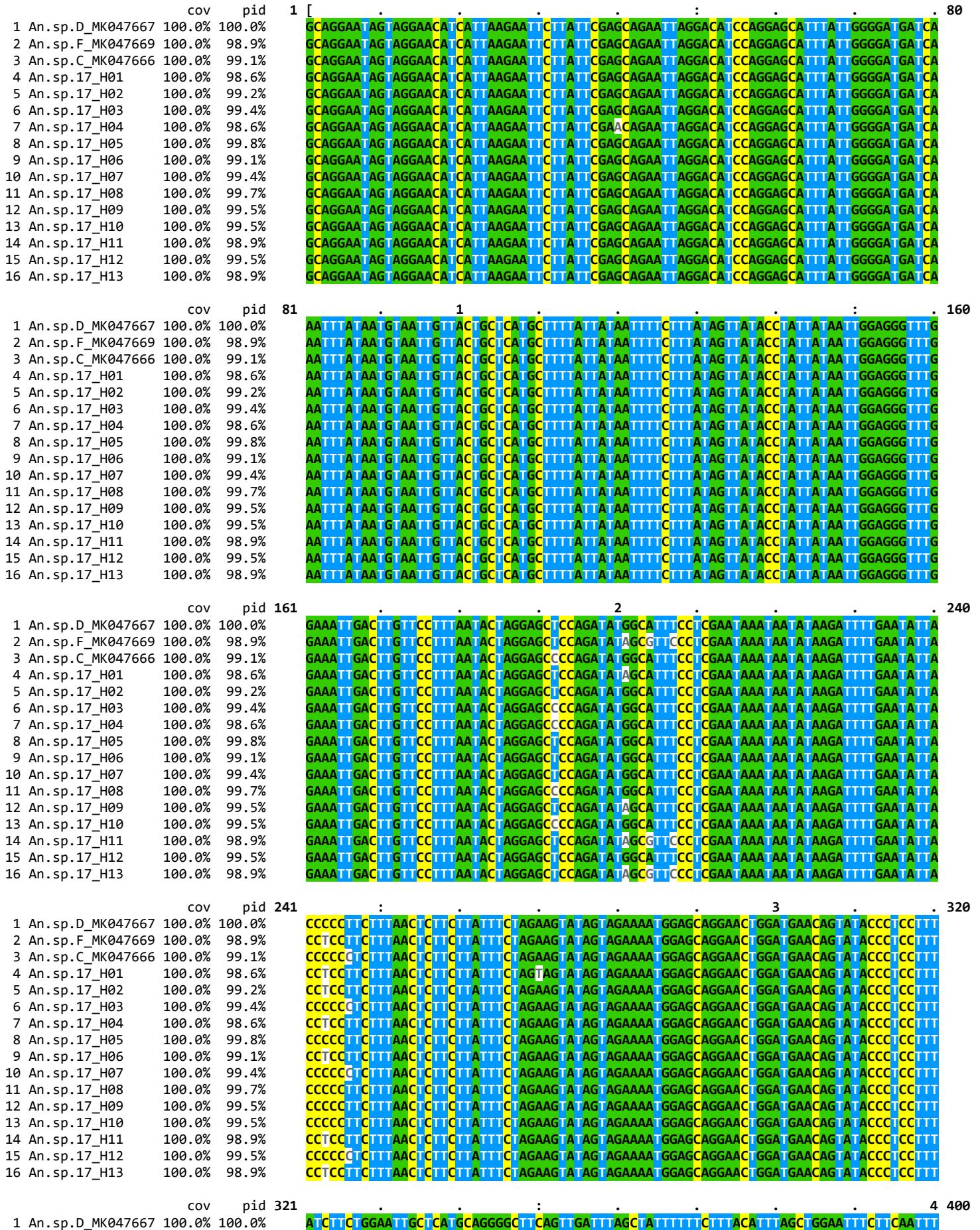


(f)

Reference sequence (1): An.sp.D_MK047667

Identities normalised by aligned length.

Colored by: identity



2	An.sp.F_MK047669	100.0%	98.9%
3	An.sp.C_MK047666	100.0%	99.1%
4	An.sp.17_H01	100.0%	98.6%
5	An.sp.17_H02	100.0%	99.2%
6	An.sp.17_H03	100.0%	99.4%
7	An.sp.17_H04	100.0%	98.6%
8	An.sp.17_H05	100.0%	99.8%
9	An.sp.17_H06	100.0%	99.1%
10	An.sp.17_H07	100.0%	99.4%
11	An.sp.17_H08	100.0%	99.7%
12	An.sp.17_H09	100.0%	99.5%
13	An.sp.17_H10	100.0%	99.5%
14	An.sp.17_H11	100.0%	98.9%
15	An.sp.17_H12	100.0%	99.5%
16	An.sp.17_H13	100.0%	98.9%

Sequence alignment across positions 401 to 480. The sequence is highly conserved, with most sites showing identical or very similar nucleotides across all samples. The sequence starts with TAGGGG and ends with GTT.

	cov	pid	401	480
1	An.sp.D_MK047667	100.0%	100.0%	
2	An.sp.F_MK047669	100.0%	98.9%	
3	An.sp.C_MK047666	100.0%	99.1%	
4	An.sp.17_H01	100.0%	98.6%	
5	An.sp.17_H02	100.0%	99.2%	
6	An.sp.17_H03	100.0%	99.4%	
7	An.sp.17_H04	100.0%	98.6%	
8	An.sp.17_H05	100.0%	99.8%	
9	An.sp.17_H06	100.0%	99.1%	
10	An.sp.17_H07	100.0%	99.4%	
11	An.sp.17_H08	100.0%	99.7%	
12	An.sp.17_H09	100.0%	99.5%	
13	An.sp.17_H10	100.0%	99.5%	
14	An.sp.17_H11	100.0%	98.9%	
15	An.sp.17_H12	100.0%	99.5%	
16	An.sp.17_H13	100.0%	98.9%	

Sequence alignment across positions 481 to 560. The sequence is highly conserved, with most sites showing identical or very similar nucleotides across all samples. The sequence starts with TGATCAGT and ends with AACAGA.

	cov	pid	481	560
1	An.sp.D_MK047667	100.0%	100.0%	
2	An.sp.F_MK047669	100.0%	98.9%	
3	An.sp.C_MK047666	100.0%	99.1%	
4	An.sp.17_H01	100.0%	98.6%	
5	An.sp.17_H02	100.0%	99.2%	
6	An.sp.17_H03	100.0%	99.4%	
7	An.sp.17_H04	100.0%	98.6%	
8	An.sp.17_H05	100.0%	99.8%	
9	An.sp.17_H06	100.0%	99.1%	
10	An.sp.17_H07	100.0%	99.4%	
11	An.sp.17_H08	100.0%	99.7%	
12	An.sp.17_H09	100.0%	99.5%	
13	An.sp.17_H10	100.0%	99.5%	
14	An.sp.17_H11	100.0%	98.9%	
15	An.sp.17_H12	100.0%	99.5%	
16	An.sp.17_H13	100.0%	98.9%	

Sequence alignment across positions 561 to 636. The sequence is highly conserved, with most sites showing identical or very similar nucleotides across all samples. The sequence starts with TCGAAA and ends with GATTT.

	cov	pid	561	636
1	An.sp.D_MK047667	100.0%	100.0%	
2	An.sp.F_MK047669	100.0%	98.9%	
3	An.sp.C_MK047666	100.0%	99.1%	
4	An.sp.17_H01	100.0%	98.6%	
5	An.sp.17_H02	100.0%	99.2%	
6	An.sp.17_H03	100.0%	99.4%	
7	An.sp.17_H04	100.0%	98.6%	
8	An.sp.17_H05	100.0%	99.8%	
9	An.sp.17_H06	100.0%	99.1%	
10	An.sp.17_H07	100.0%	99.4%	
11	An.sp.17_H08	100.0%	99.7%	
12	An.sp.17_H09	100.0%	99.5%	
13	An.sp.17_H10	100.0%	99.5%	
14	An.sp.17_H11	100.0%	98.9%	
15	An.sp.17_H12	100.0%	99.5%	
16	An.sp.17_H13	100.0%	98.9%	

Sequence alignment across positions 561 to 636. The sequence is highly conserved, with most sites showing identical or very similar nucleotides across all samples. The sequence starts with TCGAAA and ends with GATTT.