



Molecular taxonomy of mosquitoes by end-point PCR.

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This protocol describes the PCR components and conditions required for molecular taxonomical identification of mosquitoes. This PCR-SSP technique allows for the identification of *Anopheles*, *Aedes* and *Culex* genera, as well as species identification for *Aedes aegypti* and *Aedes albopictus* through a single end-point multiplex PCR reaction per mosquito using standard agarose gel electrophoresis.

Oligonucleotide primer features

Name	Specificity	Sequence*	bp	%GC	Tm	Hair	HmD	Ref
Mosq-F	<i>Anopheles</i>	5'-TGTGAACTGCAGGACACAT-3'	19	47.4	53.9	-1.75	-10.24	1
Mosq -R2	<i>Aedes</i> <i>Culex</i>	5'-TTTAGGGGGTAGTCACACAT-3'	20	45	52.6	-0.02	-1.47	2
Mosq -RAE2	<i>Aedes</i>	5'-GAAAACRTCACBMYGCATACG-3'	21	48.4	54.4	0.97	-14.23	2
Mosq -RCX	<i>Culex</i>	5'-GTCTTGAATGTTTTGCCAGC-3'	20	45	52.8	1.04	-3.14	2

Name	Heterodimer (HtD) ΔG analysis			
	Mosq-F	Mosq-RAE2	Mosq-RCx	Mosq-R2
Mosq -F	—	-11.71	-5.09	-6.82
Mosq -R2	-6.82	-9.46	-4.77	—
Mosq -RAE2	-11.79	—	-11.37	-8.02
Mosq -RCX	-5.09	-11.37	—	-4.77

* Reverse oligonucleotide primer sequences given in this table are the reverse-complement of the sequence present in the alignments and as they should be ordered for synthesis. Hairpin (Hair), homodimer (HmD) and heterodimer (HtD) ΔG 's are given in kcal/mol.





Oligonucleotide primer specificity

Genus	Species	TotSeq	Mosq-F (generic)			Mosq-RAE2 (Aedes)			Mosq-RCX (Culex)			Mosq-R2 (generic)		
			Mewara A, 2018			Jurado-Sánchez N, 2022			Jurado-Sánchez N, 2022			Jurado-Sánchez N, 2022		
			BC ¹	Seq ²	Exten ⁴	BC	n/%	Exten ⁴	BC	n/%	Exten ⁴	BC	n/%	Exten ⁴
Aedes	abserratus	2		Unknown ³		All bp	All seq	Yes	None	All seq	No	All bp	All seq	Yes
Aedes	aegypti	19	All bp	Some seq ³	Yes	All bp	All seq	Yes	None	All seq	No	All bp	All seq ³	Yes
Aedes	albopictus	166	Mostl bp ³	All seq ³	Yes	All bp	All seq	Yes	None	All seq	No	Most bp ³	All seq ³	Yes
Aedes	atlanticus	2	Mostl bp ³	All seq ³	Yes	All bp	All seq	Yes	None	All seq	No	All bp	All seq	Yes
Aedes	atropalpus	1		Unknown ³		All bp	All seq	Yes	None	All seq	No			Unknown ³
Aedes	atropos	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	aurifer	1		Unknown ³		All bp	All seq	Yes	None	All seq	No	All bp	All seq	Yes
Aedes	campestris	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	campitorhynchus	8	Mostl bp ³	All seq ³	Yes	All bp	All seq	Yes	None	All seq	No	All bp	All seq	Yes
Aedes	canadensis	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	cantator	1		Unknown ³		All bp	All seq	Yes	None	All seq	No			Unknown ³
Aedes	cataphylla	3		Unknown ³		All bp	All seq	Yes	None	All seq	No	Some bp ³	All seq ³	Likely ³
Aedes	cinereus	4	All bp	Some seq ³	Yes	All bp	All seq	Yes	None	All seq	No	All bp	All seq ³	Yes
Aedes	dorsalis	1		Unknown ³		All bp	All seq	Yes	None	All seq	No			Unknown ³
Aedes	earlei	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	excrucians	1		Unknown ³		All bp	All seq	Yes	None	All seq	No			Unknown ³
Aedes	flavescens	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	fulvus	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	grossbecki	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	hensilli	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	hespernotius	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	infirmatus	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	intrudens	1		Unknown ³		All bp	All seq	Yes	None	All seq	No			Unknown ³
Aedes	japonicus	5		Unknown ³		All bp	All seq	Yes	None	All seq	No	Most bp ³	Most seq ³	Likely ³
Aedes	kochi	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	melanimon	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	mitchellae	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	nigromaculis	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	notoscriptus	25	All bp	All seq ³	Yes	All bp	All seq	Yes	None	All seq	No	All bp	All seq	Yes
Aedes	polynesiensis	2	All bp	Most seq ³	Yes	All bp	All seq	Yes	None	All seq	No	Some bp ³	All seq ³	Likely ³
Aedes	solicitans	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	squamiger	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	sticticus	3		Unknown ³		All bp	All seq	Yes	None	All seq	No			Unknown ³
Aedes	taeniorhynchus	1		Unknown ³		All bp	All seq	Yes	None	All seq	No			Unknown ³
Aedes	territans	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	triseriatus	2	All bp	Most seq ³	Yes	All bp	All seq	Yes	None	All seq	No	All bp	Most seq ³	Yes
Aedes	trivittatus	0	-	-	-	-	-	-	-	-	-	-	-	-
Aedes	vexans	5	Mostl bp ³	Most seq ³	Yes	All bp	All seq	Yes	None	All seq	No	All bp ³	Most seq ³	Yes
Aedes	vigilax	0	-	-	-	-	-	-	-	-	-	-	-	-
Anopheles	albimanus	8	Mostl bp ³	Most seq ³	Yes	None	All seq	No	None	All seq	No	All bp	All seq ³	Yes
Anopheles	apicimacula	10	Mostl bp ³	All seq ³	Yes	None	All seq	No	None	All seq	No	All bp	All seq ³	Yes
Anopheles	crucians	3	All bp	All seq	Yes	None	All seq	No	None	All seq	No	All bp	All seq ³	Yes
Anopheles	darlingi	26	Mostl bp ³	Most seq ³	Yes	None	All seq	No	None	All seq	No	All bp	All seq ³	Yes
Anopheles	gambiae	14	All bp	All seq ³	Yes	Most bp	All seq	Likely	None	All seq	No	All bp	All seq ³	Yes
Anopheles	pseudopunctipennis	2	All bp	Most seq ³	Yes	None	All seq	No	None	All seq	No	All bp	All seq ³	Yes
Anopheles	punctimacula	11	All bp	All seq	Yes	None	All seq	No	None	All seq	No	All bp	All seq ³	Yes
Anopheles	quadriraculatus	5	All bp	All seq	Yes	None	All seq	No	None	All seq	No	All bp	All seq	Yes
Coquillettidia	perturbans	0	-	-	-	-	-	-	-	-	-	-	-	-
Culex	annulirostris	11	All bp	All seq	Yes	None	All seq	No	Most bp	All seq	Yes	All bp	All seq	Yes
Culex	erraticus	69	All bp	Some seq ³	Yes	All bp	All seq	Yes	Some bp	All seq	Unlikely	All bp	All seq	Yes
Culex	erythrorhox	2	All bp	All seq	Yes	None	All seq	No	All bp	All seq	Yes	All bp	All seq	Yes
Culex	nigripalpus	6		Unknown ³		None	All seq	No	Most bp	All seq	Yes	All bp	All seq	Yes
Culex	pipiens	23	All bp	Most seq ³	Yes	None	All seq	No	All bp	All seq	Yes	All bp	All seq	Yes
Culex	quinquefasciatus	59	All bp	Most seq ³	Yes	None	All seq	No	All bp	All seq	Yes	All bp	All seq	Yes
Culex	restuans	6	All bp	All seq	Yes	None	All seq	No	Most bp	All seq	Yes	All bp	All seq	Yes
Culex	salinarius	6	All bp	All seq	Yes	None	All seq	No	All bp	All seq	Yes	All bp	All seq	Yes
Culex	tarsalis	4	All bp	All seq	Yes	None	All seq	No	Most bp	All seq	Yes	All bp	All seq	Yes
Culiseta	inornata	0	-	-	-	-	-	-	-	-	-	-	-	-
Culiseta	melanura	0	-	-	-	-	-	-	-	-	-	-	-	-
Culiseta	morsitans	0	-	-	-	-	-	-	-	-	-	-	-	-
Deinocerites	cancer	0	-	-	-	-	-	-	-	-	-	-	-	-
Deinocerites	mathesoni	0	-	-	-	-	-	-	-	-	-	-	-	-
Deinocerites	pseudes	0	-	-	-	-	-	-	-	-	-	-	-	-
Mansonia	dyari	0	-	-	-	-	-	-	-	-	-	-	-	-
Mansonia	signipennis	0	-	-	-	-	-	-	-	-	-	-	-	-
Mansonia	titillans	0	-	-	-	-	-	-	-	-	-	-	-	-
Psorophora	ciliata	0	-	-	-	-	-	-	-	-	-	-	-	-
Psorophora	ferox	1		Unknown ³		All bp	All seq	Yes	None	All seq	No	Most bp ³	All seq	Yes
Uranotaenia	lowii	0	-	-	-	-	-	-	-	-	-	-	-	-
Uranotaenia	sapphirina	0	-	-	-	-	-	-	-	-	-	-	-	-
Wyeomyia	mitchellae	0	-	-	-	-	-	-	-	-	-	-	-	-
Wyeomyia	smithii	0	-	-	-	-	-	-	-	-	-	-	-	-
Wyeomyia	vanduzeei	0	-	-	-	-	-	-	-	-	-	-	-	-





Notes to oligonucleotide primer specificity table:

1. Base complementarity, number of oligonucleotide primer bases annealing to mosquito sequences (All if $\geq 90\%$ bp, Most if between 75%-90% bp, Some if between 50%-75% bp, Few if between 25%-50%, None if $< 25\%$)
2. Number of mosquito sequences (Seq) annealing with described BC to oligonucleotide sequences (All if $\geq 90\%$ seq, Most if between 75%-90% seq, Some if between 50%-75% seq, Few if between 25%-50% seq, None if $< 25\%$ seq)
3. Nucleotide sequence present in NCBI's GenBank database is not informative of oligonucleotide annealing site.
4. Likelihood of oligonucleotide primer being extended (Yes, No, Likely, Unlikely, Probably)
5. *Culex erraticus* will paradoxically amplify with Mosq-RAE2 but not with Mosq-RCX.

SSP-PCR amplicon lengths

Genus	Species	Amplicon size (bp)		
		Mosq-F + Mosq-R2	Mosq-F + Mosq-RAE2	Mosq-F + Mosq-RCX
Aedes	abserratus	~ 354	~ 178	–
	aegypti	328	146	–
	albopictus	529	161	–
	atlanticus	391	178	–
	atropalpus	* Unknown	~ 179	–
	aurifer	* Unknown	~ 178	–
	camptorhynchus	341	167	–
	cantator	* Unknown	~ 178	–
	cataphylla	~ 384	178	–
	cinereus	347	155	–
	dorsalis	* Unknown	~ 178	–
	excrucians	* Unknown	~ 178	–
	intrudens	* Unknown	~ 178	–
	japonicus	~ 411	~ 221	–
	notoscriptus	416	191 / 342	–
	polynesiensis	434	143	–
	sticticus	* Unknown	~ 178	–
taeniorhynchus	* Unknown	~ 210	–	
triseriatus	375	179	–	
vexans	378	168	–	
Anopheles	albimanus	502	392	–
	apicimacula	473	–	–
	crucians	431	–	–
	darlingi	536	–	–
	gambiae	557	262	–
	pseudopunctipennis	502	–	–
	punctimacula	386	–	–
quadrimaculatus	466	–	–	
Culex	annulirostris	401	–	189
	erraticus	359	155	186
	erythrothorax	411	–	180
	nigripalpus	399	–	175
	pipiens	462	–	168
	quinquefasciatus	460	–	169
	restuans	409	–	185
	salinarius	417	–	194
tarsalis	415	–	191	
Psorophora	ferox	362	~ 192	–





Notes to SSP-PCR amplicon length table:

1. ~ Amplicon length inferred from alignments to other mosquito sequences of the same genus provided for sequences not spanning oligonucleotide binding sites.
2. ⇐ No amplification possible given oligonucleotide specificity.
3. * Amplicon length estimation not possible.
4. *Aedes notoscriptus* has two complementary sites for the extensible 3' end (last 7 bases) of Mosq-RAE2

PCR components and conditions

Taq DNA polymerase (Vivantis Cat No. PL1202)

dH ₂ O	cf	16.613 μL
10x Buffer	1 x	2.5 μL
50 mM MgCl ₂	1.5 mM	0.75 μL
10 mM dNTPs	200 μM	0.5 μL
10 μM MOSQ-F	300 nM	0.75 μL
10 μM MOSQ-RAE2	75 nM	0.187 μL
10 μM MOSQ-RCX	300 nM	0.75 μL
10 μM MOSQ-R2	300 nM	0.75 μL
Taq DNA polymerase	0.04 IU	0.2 μL
Template	~100 ng	2 μL
		vf: 25 μL



Run Mosq Taxonomy (see note 1)



Total time: 1:34 hrs		
94 °C	2 min	30 cycles
94 °C	30 sec	
47 °C	30 sec	
72 °C	45 sec	
72 °C	5 min	

Electrophoresis conditions

Add 5 μL of orange loading buffer to each PCR reaction corresponding to individual mosquitos and load 25 μL into a 3% agarose gel and run at 90 VDC (3.6 V/cm) for 90 minutes.



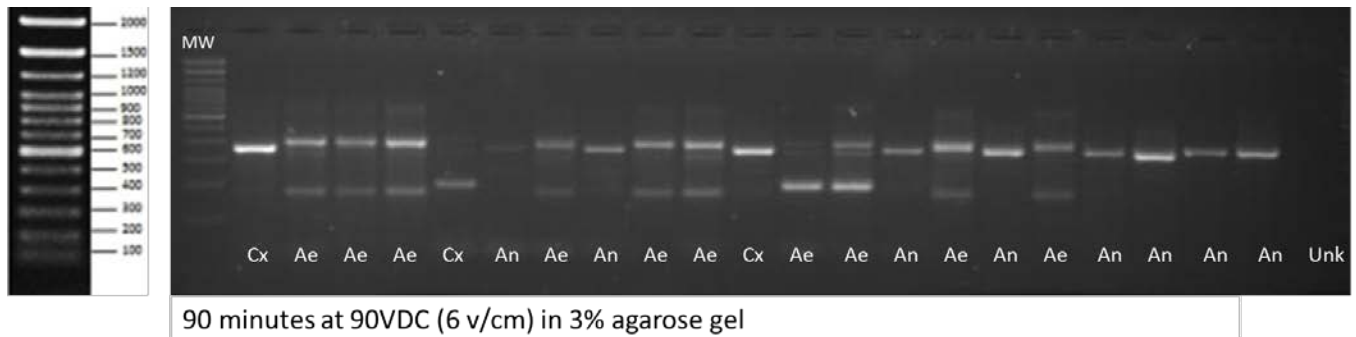


Figure 1. Typical multiplex SSP-PCR banding patterns for Aedes (Ae), Anopheles (An) and Culex (Cx) mosquitos. Unk: Mosquito of unknown taxonomical classification.

Notes

1. Thermocycling tried and tested on Axygen Maxygene and Thermo Scientific Mini Amp Plus thermalcyclers (1 through 4).
2. Mosquito DNA extracted with phenol-chloroform-isoamyl alcohol (PCI) method (http://www.genomica.uaslp.mx/Protocolos/Mol_Mosquito_DNA_PCI.pdf).
3. Clean workbench with 0.1% NaOCl and 70% Ethanol before and after setting up the PCR.
4. Preparation of mastermix should only be performed in the pre-PCR workbench.
5. Addition of sample DNA should only be performed in the pre-PCR workbench.
6. Prepare PCR reactions on ice to prevent evaporation of small liquid volumes.
7. Vortex all reagents (except DNA and Taq DNA polymerase) before dispensing into master mix.
8. Vortex master mix after adding all required reagents then dispense into each tube.
9. Vortex all PCR reactions after adding reagents and DNA. Spin briefly and load into cyclers.

References

1. Mewara Abhishek, Sharma Megha, Kaura Taruna, Zaman Kamran, Yadav Rakesh, Sehgal Rakesh. Rapid identification of medically important mosquitoes by matrix-assisted laser



desorption/ionization time-of-flight mass spectrometry. *Parasites & Vectors*. 2018 11:281.

2. N Jurado-Sánchez, SE Guerra-Palomares, CA García-Sepúlveda. 2022.

Revision history

- 1.0 Original document.
- 2.0 Updated protocol.
- 3.0 Update protocol for 25 uL vf PCR's and Thermo Scientific MiniAmp Plus thermalcyclers.
- 4.0 Protocol updated for new oligonucleotide primers (MOSQ-RAE2 & MOSQ-R2).

