

***Culicinae* mosquito Tra-2 RNAi, a method to genetically produce maleness populations.**

Field of the invention

The present invention relates to the field of molecular biology to develop a novel method for controlling mosquito populations. *Culicinae* mosquitoes carrying one or more loci of transformant Tra-2 RNAi constructs which target to mosquito Transformer-2 locus in respective or none respective *Culicinae* mosquitoes. Tra-2 sequences used to assemble Tra-2 RNAi recombinant constructs are Tra-2 gene sequences of *Culicinae* mosquitoes and can be derived from endogenous or exogenous sequences. The Tra-2 RNAi expression is conditional, wherein the expression causing a knockdown effect into the endogenous Tra-2 gene results in mortality of X (m) chromosome bearing sperms and produces maleness mosquito population in the nature environmental of the species.

Background of the invention.

Nowadays, there are many biological control techniques have been invented for controlling insects and plants (Smith and Borstel, 1972. *Science*. 178. 1164-1174). A method invented to control of insect populations is named the "sterile insect technique" SIT. This method is also known with a different name as "sterile insect release method" SIRM. A model of the SIRM based on population parameters obtained from a large scale experiment to eradicate melon fly was first developed by Ito (1977. *Appl. Ent. Zool.* 12:310-312; 1979. *Res. Popul. Ecol.* 20. 216-226). The SIT method creates sterile male insects and releases them into natural habitats where the males would look for natural females to mate. These females would be sterile or to produce offspring that cannot develop up to the harmful stages. When a huge number of sterile males released in a chronic time, it can cause a collapse of the natural insect populations or even extinction. However, the insect created by the SIT method need to be undergone a sexing step to remove females. The reason is that in many insect species, even sterile females, if being released, they would look for blood meals and may transmit diseases (in mosquitoes) or damage fruits (in med fly). As such, release of the female insects must be avoided.

Currently, there are different methods to separate male insects from a total based on the differences of sexual traits as body sizes or eclosion time. However, systematic errors of those are high and more or less depending on each species, for instance, our data indicated that 8-15% females of *Aedes aegypti* mosquitoes can be misidentified as males (K. P. Hoang, unpublished data). Alternatively, insects can be frozen down on ice to separate females and males. However, this method is too high labor costs and can damage small insects as male mosquitoes.

In another approach, X and gamma rays are used to translocate a chromosome fragment which carries genes encoding for different colors of silk worm male and female eggs (Strunnikov, 1979. *Theor. Appl. Genet.* 55, 17-21; Strunnikov. 1983. *Control of silkworm Reproduction, Development and Sex.* MIR Publishers. Moscow). However, the mutant strains created by radiation are usually accompanied with a significant decline in male mating competitiveness in comparison with its wild type males. This can result in failures in vector control strategies if applying for the release of insect males. Besides, irradiation method is not specific to the certain target, the radiation not only causes big mutations in chromosome systems of the target organisms but can also be dangerous for producers. This is also an expensive method with plenty of limitations.

Asburner et al., (1998) disclose a method by introduction of an exogenous DNA fragment into the insect genetic system to create insect transgenic species (*Insect Molecular Biology*, 7 (3), 201-213). This approach was lately improved by a patent of Handler (2006. PN : US 7005296B1).

DeVault disclose to use a female specific promoter to be ligated into a lethal gene. The gene is only activated in females and therefore males are uniquely remained in the selection. These males are irradiated for sterilization before releasing into nature (DeVault et al., 1996; *Biotechnology*, Vol 14; 46-49; DeVault et al., 1996. *Genome Res.* 6: 571-579). This achievement gains a big progress in the genetic sexing experiments, however the use of radiation can severely damage for small insects with its consequences of decreasing male mating competitiveness ability.

To avoid the radiation damages, a new method named RIDL (Release of Insects carrying a dominant lethal) has been disclosed in a patent (Alphey, 1999. PN : WO 01/39599 A2; Alphey, 2007. *Area-Wide Control of Insect Pests: From Research to Field*

Implementation, Springer, Dordrecht, The Netherlands). The RIDL offers a solution to many of the drawbacks of traditional SIT that have limited its application in mosquitoes as mentioned above. RIDL differs from conventional SIT in that the released insects are not sterilized by irradiation but its sterility is resulted from a homozygote for a dominant lethal gene. Highly efficient repressible RIDL systems were first demonstrated in *Drosophila* models and recently in the Mediterranean fruit fly (Thomas et al., 2000. *Science*, 287: 2474-2476.; Gong et al., 2000. *Nature Biotech.*, 23: 453-456.). This system exploits a tetracycline-repressible transactivator (tTA) to control expression of the dominant lethal (Gossen and Bujard, 1992. *Proc Natl Acad Sci USA* 1992, 89(12):5547-5551). The tetracycline (Tet) that to be mixed in larval rearing medium or food can bind to tTA and preventing it binding to tetO sequences and driving the effector gene. The tetO sequences plays a role of an operator would be free to suppress the lethal gene. In the absence of Tet, tTA protein binds to the operator sequence and the effector gene would be free to express. In natural environment where Tet is absent, released transgenic males mates with wild type females and their offspring would be killed by the effector gene activation. In *Aedes* mosquitoes, RIDL has been proved to be efficient, of which the males created haven't been declined their fitness when competing with wild type males. (Phuc et al., 2007; *BMC Biology* <http://www.biomedcentral.com/1741-7007/5/11>). However, the RIDL method has a serious shortcoming that it still produces offspring in both sexes. It therefore needs an addition step of sexing to remove females before releasing.

Fu et al., (2010. *PNAS*, Vol. 107, No. 10, 4550-4554) discloses a method in which a fusion between RIDL system and a female sex-specific regulation based on an endogenous Actin-4 promoter that derived from *Aedes aegypti* females. The effector gene is specifically activated only in the direct-flight muscle of female mosquitoes and this expression makes females to be flightless. These females after eclosion would be stuck on the water surface and to be dead eventually. By this method, only 50% of offspring becomes males which can continuously pass the transformant genetic systems into next generation. However, this method in practice has a shortcoming. This happens when plenty of the flightless females staying on the water surface, their bodies and leg movements can prevent other eclosion males to come up with the water which may eventually drown males. The higher rearing density is the higher "collateral damage" for males, but in industrial insectary, rearing at high density is the only option.

Transformer-2 gene has been seen as a key factor in combination with Tra for sex determination in different eukaryotes although it may involve differently in different taxa depending on evolutionary divergence. Fortier and Belote (2000. *Genesis* 26(4): 240--244), Salvemini et al (2009. *Int. J.Dev. Biol.* 53: 109–120) and Sarno et al (2010. <http://www.biomedcentral.com/1471-2148/10/140>) used the RNAi method to knock down the Tra-2 genes in *Drosophila*, *Ceratitis Capitata* and *Anastrepha*, respectively. The knockdown effect can convert females of these species into pseudo males carrying XX chromosomes. In their studies, the RNA interference method is performed by injection of Tra-2 double stranded RNA (dsRNA) into embryos after an invitro synthesized step.

No tra-2 orthologue has been identified in *Anopheles* and the Tra-2 orthologue in *Aedes aegypti* mosquitoes seems to involve in a different genetic mode. A full length mRNA transcription of Tra-2 gene in *Aedes aegypti* is not necessarily required for its downstream gene cascade, doublesex (dsx) to be spliced. One female specific Dsx can be default spliced to be females (Salvemini et al., 2011. *BMC Evolutionary Biology* 2011, 11:41 <http://www.biomedcentral.com/1471-2148/11/41>). It, therefore, suggests that the wish to create all maleness offspring, including 50% pseudo [XX (mm)] males by a conversion from females is impossible if targeting Tra-2 in *Culicinae*. In fact, in our experiments, Tra-2 dsRNA injection into *Culicinae* mosquito eggs hasn't caused a significant bias in sex ratio.

The present invention sets out to overcome all the shortcoming of the previous methods by using the common principles of the RIDL method (Alphey, 1999. *PN : WO 01/39599 A2*; Alphey, 2007. *Area-Wide Control of Insect Pests: From Research to Field Implementation*, Springer, Dordrecht, The Netherlands) in combination with a discovery of X (m) bearing sperm killing effect due to Tra-2 RNAi genetic system. These transgenic *Culicinae* mosquitoes are therefore to produce more than 90% genetic maleness offspring.

Summary and technique principles of the invention

We discovered that is not like the Tra-2 in *Drosophila*, *Ceratitis Capitata* and *Anastrepha*, the Tra-2 gene in *Culicinae* mosquitoes is involved in male specific, spermatogenesis processes and the knockdown of Tra-2 gene hasn't resulted in a conversion from females to males as occurred in the other Dipteran insects. Because, a transient effect of dsRNA cannot last from eggs to adults to consistently cause a knockdown effect into spermatogenesis stages and following consequences in next generation, therefore all attempts to use dsRNA injections to obtain transient effects would be invalid.

To successfully repress the Tra-2 gene in mosquitoes, it is necessary to create permanent transgenic lines with Tra-2 RNAi constructs by which the interference effect will be stably expressed during the spermatogenesis stages. We found that Tra-2 gene knockdown in *Culicinae* mosquitoes can cause lethality of X (m) chromosome bearing sperms and therefore only Y-chromosome (M) bearing sperms are survival and maleness offspring would be produced. These males are genetic males which carry a Y chromosome. Males created by this method are not sterile but they produce healthy Y chromosome bearing sperms only. Theoretically, maleness offspring would continuously pass the Tra-2 RNAi constructs into natural populations until the populations gone extinct.

This invention discloses all of the methods to create Tra-2 RNAi DNA constructs, to transform it into mosquitoes and to observe its expression.

The invention uses putative Transformer-2 encoding gene sequences from *Aedes albopictus*, *Aedes polynesiensis*, *Culex quinquefasciatus* or the other *Culicinae* mosquitoes as materials to assemble Tra-2 RNAi genetic constructs by using DNA recombination techniques. In Examples of this invention, parts or whole RRM (RNA recognition motif) sequences which are belonging to putative Transformer-2 encoding sequences from *Aedes albopictus*, *Aedes polynesiensis* and *Culex quinquefasciatus* are used.

The Tra-2 of *Aedes aegypti* is identified by blasting against the *Aedes aegypti* Genbank database with an input is the *Drosophila* Tra-2A amino acid sequence. The outcome was AAEL004293-RA protein belonging to supercontig 1.113 (*Aedes aegypti*-

Vectorbase). *Aedes aegypti* are closely related species with *Aedes albopictus* and *Aedes polynesiensis*, therefore primers derived from the AAEL004293-RA sequence can be used to amplify Tra-2 sequences of *Aedes albopictus* and *Aedes polynesiensis* mosquitoes. The regions with highest similarity among the orthologous Tra-2 genes are RRM s (RNA recognition motives) with a length of 240bp. For many other *Aedes spp* these primers were tested and can successfully amplify these 240bp regions. We found two RRM s loci (or allele), each exists in both *Aedes albopictus* and *Aedes polynesiensis*. They are different 10% amino acid from each other and named as SEQ ID: No 1 (RRM1) and SEQ ID: No 2(RRM2) (Figure 5). To knock down these two loci (allele), it may be required to transform two respective RNAi constructs into each species to repress the respective RRM locus (allele).

A putative Tra-2 gene of *Culex quinquefasciatus* is identified by blasting *Culex quinquefasciatus* database with the RRM1 and RRM2 sequences. The name of the outcome is CPIJ016646; supercontig 3.780:5008-5247. The RRM s of *Culex* orthologous Tra-2 gene is named as SEQ ID: No 3 (RRM3) (Figure 5). For many other *Culex spp*, primers derived from the first and the end of the RRM3 region have been tested and can successfully amplify these 240bp regions.

In order to knock down the Tra-2 genes in *Aedes albopictus*, *Aedes polynesiensis*, *Culex quinquefasciatus* and the other *Culicinae* mosquitoes by the RNAi technique, there are three solutions to be disclosed in the invention.

- The first solution is to use SEQ ID: No 1 (RRM1) as materials to invitro create an RNAi kernel sequence 1. This is a recombinant DNA fragment combining two identical sequences of RRM1 but in opposite directions. A connection between the two RRM1 repeats is a straight intron or linker DNA sequence. The RNAi kernel sequence 1 is then ligated with transactivator and regulatory elements and a fluorescent marker within a *PiggyBack* plasmid. This plasmid would then be available for transforming into both *Aedes albopictus* and *Aedes polynesiensis* to knock down their RRM1 locus.

The regulatory element for the kernel sequence is a minimal promoter associated with operator sequences (tetOx7). The minimal promoter plus tetOx7 can be conditional by using the commercial transactivator regulation systems (Clontech). Gene expression is activated as a result of binding of the Tet-Off protein (tTA) to tetracycline response elements (TREs) located within the minimal promoter.

For the best of expression, the invention suggests to use insect spermatogenesis specific promoters to control tTA protein gene. Besides, we also prefer to derive a minimal promoter from an insect spermatogenesis specific promoter for controlling the RNAi kernel sequence 1 which helps to eliminate all leakiness effect. This solution may be applied for any other *Aedes spp* which has a highly similar sequence of its RRM in comparison with the RMM1 or after obtaining its own Tra-2 RRM sequences by the same pair of primers.

- The second solution is to use SEQ ID: No 2 (RRM2) as materials to invitro create an RNAi kernel sequence 2. This is a recombinant DNA fragment combining two identical sequences of RRM2 but in opposite directions. A connection between the two RRM2 repeats is a straight intron or linker DNA sequence. The RNAi kernel sequence 2 is then ligated with regulatory elements and a fluorescent marker within a *piggyBack* plasmid. This plasmid would then be available for transforming into both *Aedes albopictus* and *Aedes polynesiensis* to knock down their RRM2 locus.

In the details, the regulatory elements in the second solution are identical as those described in the first solution.

This solution may be applied for any other *Aedes spp* which has a highly similar sequence of its RRM in comparison with the RMM2 or after obtaining its own Tra-2 RRM sequences by the same pair of primers.

- The third solution is to use SEQ ID: No 3 (RRM3) as materials to invitro create a RNAi kernel sequence 3. This is a recombinant DNA fragment combining two identical sequences of RRM3 but in opposite directions. A connection between the two RRM3 repeats is a straight intron or linker DNA sequence. The RNAi kernel sequence 3 is then ligated with regulatory elements and a fluorescent marker within a *piggyBack* plasmid. This plasmid would then be available for transforming into *Culex quinquefasciatus*.

In the details, the regulatory elements in the third solution are identical as those described in the first and second solutions. This solution can be applied for any other *Culex spp* which has a highly similar sequence of its RRM in comparison with the RMM3 or after obtaining its own Tra-2 RRM sequences by a pair of primers to be mentioned in Examples.

The connective intron or linker between two Tra-2 RRM inverted repeats can be any eukaryote intron sequence. However, introns from the respective species are preferred. The length of connective linker or intron can be from few to few hundred nucleotides. We prefer that two nucleotides of GT and AG need to be inserted at the beginning and at the end of intron or linker, respectively. These are the strengthening signals for spliceosomes to recognize and to splice out the connective introns or linkers.

Three DNA Tra-2 RNAi RRM-1, Tra-2 RNAi RRM-2, Tra-2 RNAi RRM-3 kernel sequences are disclosed here as examples of using specifically Tra-2 DNA sequences to produce an interference effect into the respective species. The transcription of the DNA kernel structure containing two identically inverted repeats would produce single strands of mRNAs, which exposes two complementary sequences at its two ends. The complementary sequences would bind together forming a double strand hairpin mRNA structure (dsRNAi), in which the looping part is formed by the intron or linker. Mosquito cells recognize the abnormal structure and react by dicing the dsRNA interference in a defend mechanism activity which is followed by destroying intact single strand Tra-2 mRNAs from itself (Wang et al., 2006. *Cell* 127, 803–815; Hammond et al., 2001. *Nat Rev Genet.* 2(2):110-9). The Tra-2 gene is, therefore, knocked down.

Illustrations and its explanations.

Figure 1. The RNAi kernel sequences 1.

Tra-2 RRM RNAi- 1

Forward sequence	Connection	Reverse sequence
5' AGTAAGTGCCTCGGTGTGTTTCGGC CTAAGCAGCTACACCAACGAAACCAG CCTGATGGACGTTTTTCGCACCGTACG GAACCATTGACAAGGCGATGATTGTC TACGATGCCAAGACGAAGGTTTCCCG NGGGTTTCGGATTTCGTGTACTTCCAGG AGCAGAGTGCGGCCACCGAAGCCAAA ATGCAGTG ^Y AATGG ^S NATGATGCTGCA TGAGCGC ^A CGATTAGAGTGGATTATT CGGTGACC-3'	GT-Intron-AG (or linker)	5' GGTCACCGAATAATCCACTCTAA TCGTGCGCTCATGCAGCATCAT ^N CC ATT ^Y CACTGCATTTTGGCTTCGGTG GCCGCACTCTGCTCCTGGAAGTACA CGAATCCGAACCCNC ^S GGGAAACCTT CGTCTTGGCATCGTAGACAATCATC GCCTTGTCAATGGTTCCGTACGGTG CGAAAACGTCCATCAGGCTGGTTTC GTTGGTGTAGCTGCTTAGGCCGAAC ACACCGAGGCACTTACT-3'

The respective amino acid sequence

1 -S--K--C--L--G--V--F--G--L--S--S--Y--T--N--E--T--S--L--M--D--
 21 -V--F--A--P--Y--G--T--I--D--K--A--M--I--V--Y--D--A--K--T--K--
 41 -V--S--R--G--F--G--F--V--Y--F--Q--E--Q--S--A--A--T--E--A--K--
 61 -M--Q--C--N--G--M--M--L--H--E--R--T--I--R--V--D--Y--S--V--T--

Note : 3' end of the forward sequence is connected with an intron or linker sequence by GT. 5' end of reverse sequence is connected with the intron or linker sequence by AG. The underlined characters indicate substitutions. Y, S, N, K etc show different possibilities of changed nucleotides at the same position. The substitutions may or may not change amino acid code.

Figure 2. The RNAi kernel sequences 2.**Tra-2 RRM RNAi- 2**

Forward sequence	Connection	Reverse sequence
5' AGTAAGTGCCTCGGTGTGTTCCGGC CTNAGYAGCTAYACCA ^C MC ^A GAARCCAR CCTGATGGAYGTNTTCKCNCCGTWCG GNACCATHGACAAGGCNATGATTGTC TACGATGCCAAGACGAAGGYNTCCCG NGGGTTYGGNTTCGTGTAYTTCCAGG AGCAGAGTKCGGCCACNGARGCCAAA MTGCAGTGYAAYGGAATGRWRCTGCA YGAGCGNACGATTAGAGTGGATTATT CGGTGACC-3'	GT-Intron- AG (or linker)	5' GGTCACCGAATAATCCACTCTAATCG TNCGCTCYTGCAGRWCATTCCYTTYCA CTGCAMTTTGGCRTCNGTGGCCGKACTC TGCTCCTGGAAYTACACGAANCCYAACC CNCGGGAN ^C YCCTTCGTCTTGGCATCGTA GACAATCATNGCCTTGTCHATGGTNCCG WACGGNGKGAANACYTCCATCAGGR ^T TGG RTTCGMTGGTYTAGCTYCTNAGGCCGAA CACACCGAGGCACTTACT-3'

The respective amino acid sequence

1 -S--K--C--L--G--V--F--G--L--S--S--Y--T--T--E--T--N--L--M--D--
 21 -V--F--S--P--F--G--T--I--D--K--A--M--I--V--Y--D--A--K--T--K--
 41 -A--S--R--G--F--G--F--V--Y--F--Q--E--Q--S--S--A--T--E--A--K--
 61 -L--Q--C--N--G--M--E--L--H--E--R--T--I--R--V--D--Y--S--V--T--

Note : 3' end of the forward sequence is connected with an intron or linker sequence by GT. 5' end of reverse sequence is connected with the intron or linker sequence by AG. The underlined characters indicate substitutions. Y, S, N, K etc show different possibilities of changed nucleotides at the same position. The substitutions may or may not change amino acid code.

Figure 3. The RNAi kernel sequences 3.**Tra-2 RRM RNAi- 3**

Forward sequence	Connection	Reverse sequence
5' CGTAACGGAATAGTCCACCCGGA TGGTTCGCTCGTGCATTACCATTCC GTTGCACTGCACCTTGGCTGCGGAA GCGTCCTCCAGGTTGACAAAGTACA CGAATCCGAACCCGCGGGACGCCTT CGTCTTGGCATCGTACACGATCTGC ACCTTCTCGATCAATCCGAACCGGC CAAACACGGTCCTCAGGTCCGCCTC CTGGGTGTAATTGCTGAGGCCAAAC ACGCCGAGGCAGGTCGA-3'	GT-Intron-AG (or linker)	5' TCGACCTGCCTCGGCGTGTTTGGC CTCAGCAATTACACCCAGGAGGCGGA CCTGAGGACCGTGTTTGGCCGGTTCG GATTGATCGAGAAGGTGCAGATCGTG TACGATGCCAAGACGAAGGCGTCCCG CGGGTTCGGATTCTGTGTACTTTGTCA ACCTGGAGGACGCTTCCGCAGCCAAG GTGCAGTGCAACGGAATGGTAATGCA CGAGCGAACCATCCGGGTGGACTATT CCGTTACG-3'

The respective amino acid sequence

1 -S--T--C--L--G--V--F--G--L--S--N--Y--T--Q--E--A--D--L--R--T--
 21 -V--F--G--R--F--G--L--I--E--K--V--Q--I--V--Y--D--A--K--T--K--
 41 -A--S--R--G--F--G--F--V--Y--F--V--N--L--E--D--A--S--A--A--K--
 61 -V--Q--C--N--G--M--V--M--H--E--R--T--I--R--V--D--Y--S--V--T--

Note : 3' end of the forward sequence is connected with an intron or linker sequence by GT. 5' end of reverse sequence is connected with the intron or linker sequence by AG. The red characters indicate substitutions. Y, S, N, K etc show different possibilities of changed nucleotides at the same position. The substitutions may or may not change amino acid code.

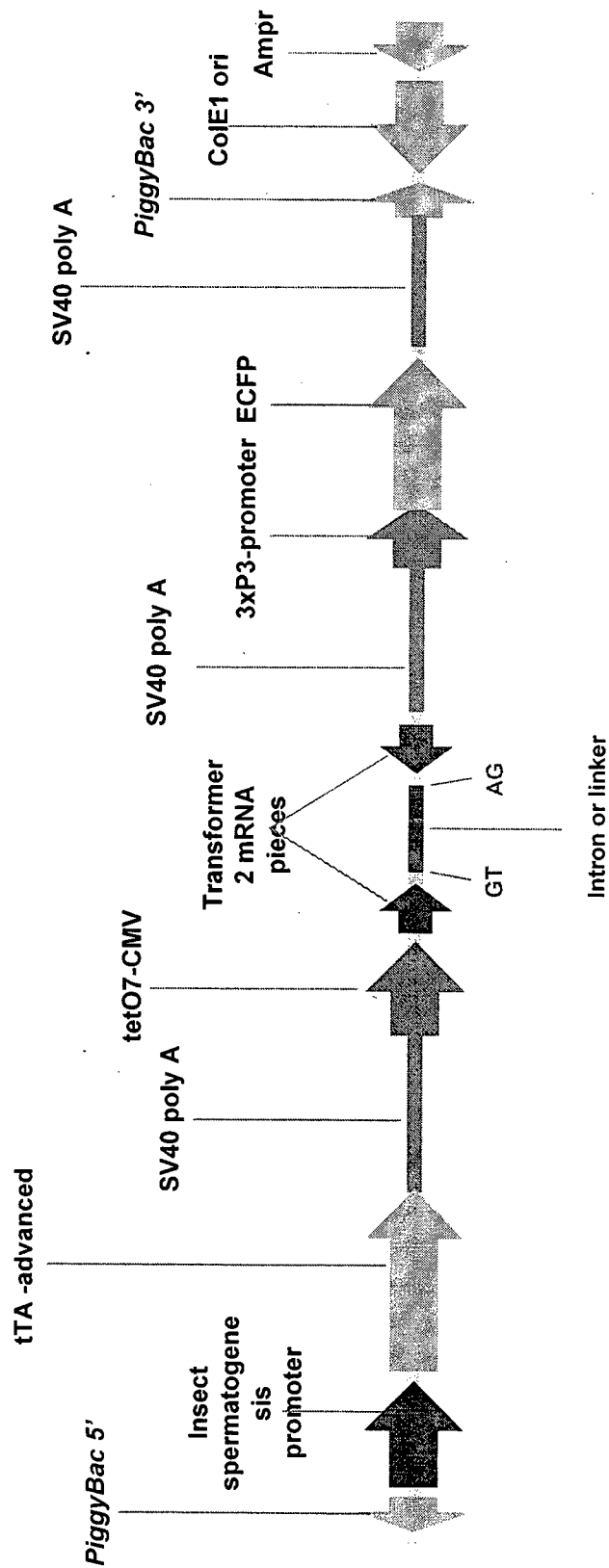


Figure 4. The RNAi kernel sequences is under controlled by a regulatory element in a transactivator system within a *PiggyBac* plasmid.

Diagrammatic representation of a completed Tra2-RNAi system, linearized at the 3' end of the piggyBac transposon. There are three functional segments within the ends of the transposon: the marker (3xP3 promoter-ECFP-SV40 poly A3') to allow detection of transgenic individuals by fluorescence. tTA protein is under the control of an insect spermatogenesis promoter (*Drosophila* $\beta 2$) and terminated by a SV40 poly A 3' sequence. The third segment is the Tra-2 Rnai cassette (tetO7-CMV minimal promoter – two Tra2 mRNA inverted repeats joined by a fragment of a linker or intron sequence. This is also terminated by a SV40 poly A 3' sequence. The Tra-2 Rnai cassette acts by a tTA protein binding into the tetO7 in the absence of Tetra cycline to free CMV minimal promoter to drive.

Figure 5. Sequence ID number.**SEQ ID: No 1 RRM1**

5'AGTAAGTGCCTCGGTGTGTTTCGGCCTAAGCAGCTACACCAACGAAACCAGCCTGATGG
ACGTTTTTCGCACCGTACGGAACCATTGACAAGGCGATGATTGTCTACGATGCCAAGACGA
AGGTTTCCCGNGGGTTCGGATTCGTGTACTTCCAGGAGCAGAGTGCGGCCACCGAAGCC
AAAATGCAGTGAAATGGNATGATGCTGCATGAGCGCACGATTAGAGTGGATTATTCGGTG
ACC-3'

SEQ ID: No 2 RRM2

5'AGTAAGTGCCTCGGTGTGTTTCGGCCTNAGYAGCTAYACCAMCGAARCCARCCTGATGG
AYGTNTTCKCNCCGTWCGGNACCATHGACAAGGCNATGATTGTCTACGATGCCAAGACG
AAGGYNTCCCGNGGGTTYGGNTTCGTGTAYTTCCAGGAGCAGAGTKCGGCCACNGARGC
CAAAMTGCAGTGAAAYGGAATGRWRCTGCAYGAGCGNACGATTAGAGTGGATTATTCGG
TGACC-3'

SEQ ID: No 3 RRM3

CGTAACGGAATAGTCCACCCGGATGGTTCGCTCGTGCATTACCATTCGGTTGCACTGCAC
CTTGGCTGCGGAAGCGTCCTCCAGGTTGACAAAGTACACGAATCCGAACCCGCGGGACG
CCTTCGTCTTGGCATCGTACACGATCTGCACCTTCTCGATCAATCCGAACCGGCCAAACA
CGGTCCTCAGGTCCGCCTCCTGGGTGTAATTGCTGAGGCCAAACACGCCGAGGCAGGTC
GA.

Detailed description of the invention.

The Tra-2 RNAi system in the present invention may be any part of Tra-2 encoding sequences (mRNA) of Tra-2 genes originated from *Aedes albopictus*, *Aedes polynesiensis*, *Culex quinquefasciatus* or the other *Culicinae* mosquitoes which are capable of producing a knockdown (interference) effect to the Tra-2 gene of the respective species. We not rule out the possibilities that a Tra-2 RNAi system containing Tra-2 recombinant sequences from a certain *Culicinae* mosquito species can also cause a knockdown (interference) effect to the other closely related mosquito species within *Culicinae*.

Definitions :

- *Culicinae* mosquitoes refer to mosquito species which have a pair of chromosome (chromosome I) that are similar in size but are distinguishable in many species by the presence in the X (m) or absence in the Y(M) of C-banding intercalary heterochromatin (Knudson et al., 1996. 175-214. *The Biology of Disease vectors. University Press of Colorado*).
- Tra-2 gene sequences from *Culicinae* mosquitoes refer to mRNA coding sequences only (Latchman, 1998. *Gene regulation. A eukaryotic perspective. 3rd edition. Stanley Thornes Publishers*).
- The RNAi kernel sequences refer to any recombinant DNA sequence which includes two inverted repeats (IR) in conjunction by a linker or intron sequence. Sequence of the IR is derived from any part of *Culicinae* Tra-2 mRNA sequences.
- We definite that Tra-2 RNAi kernel sequences is an RNAi encoding sequence, its expression is under the control of a repressible transactivator protein system.

As mentioned above, we look for an existence of the Tra-2 genes in *Aedes albopictus*, *Aedes polynesiensis* and *Culex quinquefasciatus* mosquitoes which may contain a highly conserved region with a length of 240bp (80 amino acids). This region has been identified in *Drosophila*, *Ceratitis Capitata* as Tra-2 RRM specific region (RNA recognition motif) <http://www.expasy.org/cgi-bin/prosite-search-ac?PDOC00030>.

Using a sequence from 1221182 to 1220943 of *Aedes aegypti* Tra-2 gene (GenBank accession number : AAEL004293-RA), two primers have been designed. A forward is CLF, 26bp at beginning of the RRM region (**AGTAAGTGCCTCGGTGTGTTCCGGCCT**) and a reverse is CLR, 23bp at the end of the RRM region (**CCGGTCACCGAATAATCCACTCAA**). PCR products amplified on DNA templates from *Aedes albopictus* and *Aedes polynesiensis* were sequenced. It revealed that in each species there are two different RRM sequences, RRM1 and RRM2. RT-PCR has shown expression from both of those RRMs. RRM1 is identical with the Tra-2 RRM in *Aedes aegypti* (AAEL004293-RA) but RRM2 has 10% amino acid differences. These pair of primers can be used to amplify ortholog Tra-2 RRM 240bp regions from the other *Aedes spp*, even the distance species as *Aedes niveus*, *Aedes annandalei* or *Aedes pseudoalbopictus*. The amplification condition is similar.

A Tra-2 sequence of *Culex quinquefasciatus* is available from Genbank (GenBank accession number: CPIJ016646) when using the RRM1 and RRM2 sequence as inputs to blast. Its RRM sequence belongs to the supercontig 3.780, from 5008 to 5247. We named it as RRM3. 24bp at beginning and 22bp at the end of RRM3 are used to create a pair of primers to amplify it from *Culex quinquefasciatus* DNA. These pair of primers can be used to amplify ortholog Tra-2 RRM 240bp regions of the other *Culex spp*, even the distance species as *Culex visnue*, *Culex pipiens*. The amplification condition is similar.

For convenience in designing primers whole or a part of the RRM regions (RRM1, RRM2 and RRM3) can be used to assemble the Tra-2 RNAi constructs by DNA recombinant techniques. However, in this invention, it doesn't limit to use other Tra-2 encoding parts outside the RRM region of these mosquitoes to build other Tra-2 RNAi constructs.

The elements that regulate the RNAi kernel sequences should be located on the same chromosome as the RNAi kernel sequences. In figure 4 shows the RNAi kernel sequences and Tetracycline (Tet) transactivator system. An insect spermatogenesis promoter, for instance *Drosophila* $\beta 2$, controls the tTA protein gene. In the presence of Tet in larva rearing medium, tTA protein binds to Tet and the operator sequence (tetO7) would bind to the minimal promoter which regulates the transcription of the kernel structure. The promoter is inactivated and no RNAi product is transcribed. In the absence of Tet, this artificial protein binds to operator tetOx7 in the absence of

Tetracycline (Tet) and the minimal promoter would be free to transcribe the RNAi strand. In the same plasmid, a reporter gene as ECFP, Dsred2 or EGFP can be ligated to a 3xP3 or Actin5C promoter. We can trace the plasmid by following this fluorescent marker. The entire packet is ligated into a *PiggyBac* plasmid. This complex can be transformed into mosquito genetic background in one or more loci which can be in the same or different chromosomes.

We suggest that a single locus of the transgene in a transgenic line can be used as a background for another transformation. A second transformant locus which occurs in the same chromosome with the first one, would be particularly preferred. Transformants occurred in the same chromosome would prevent them to be segregated in the next generations and especially in the case where the two Tra-2 loci (or allele) exist in same species as *Aedes albopictus* and *Aedes polynesiensis*, in which two respective RNAi transformants are necessary to repress two loci (or allele).

The expression of the RNAi kernel sequences would knock down the Tra-2 gene in the transgenic species. The knockdown effect results in lethal X chromosome bearing sperms and only male offspring is outcome.

Examples

Components : 1/RRM Tra-2 sequences : In this examples, we used three types of RRM (RRM1, RRM2 and RRM3) to create Tra-2 RNAi kernel constructs. These sequences are obtained from sequencing the target species or blasting from (<http://www.vectorbase.org/>). It doesn't limit to use a different part of the Tra-2 gene sequences which are belonging to *Aedes albopictus*, *Aedes polynesiensis*, *Culex quinquefasciatus* or the other *Culicinae* mosquitoes in the invention. All the other components of plasmids are identical. 2/ *Drosophila* $\beta 2$ tubulin promoter (or other insect spermatogenesis promoter) : PCR from *Drosophila* DNA. 3/ Transactivator component (tTA) : Clontech. 4/Regulator element (tetOx7) : Clontech. 5/Reporter gene: <http://piggybac.bio.nd.edu/>. 6/ Piggybac plasmids : <http://piggybac.bio.nd.edu/>. 7/Helper plasmid : <http://piggybac.bio.nd.edu/>.

I. RRM from *Aedes albopictus* and *Aedes polynesiensis*.

These examples show how the RRM sequences of Tra-2 were identified from *Aedes albopictus* and *Aedes polynesiensis*. It also shows the way to create the RNAi kernel

sequence by using the RRM sequences from *Aedes albopictus* and *Aedes polynesiensis*.

As mentioned above, RRM regions of *Aedes albopictus* and *Aedes polynesiensis* have been amplified by a PCR used a pair of primers of 26bp at beginning and 23 bp at the ending of *Aedes aegypti* supercontig 1.113 (1221182-1220943).

CLF **AGTAAGTGCCTCGGTGTGTTCGGCCT**

CLR **CCGGTCACCGAATAATCCACTCAA**

DNA from *Aedes albopictus* and *Aedes polynesiensis* are extracted using a QIAGEN kit. PCR is carried out in 25µl reaction in a condition of 2.5µl PCR buffer; 1.5µl MgCL (25mM); 0.5 µl dNTPs (10mM); each primer 0.5µl (10pmol/µl); 0.15µl Taq DNA polymerase (5U/µl); 10-40ng DNA template. Thermal profile of PCR is [94⁰C/4; (94⁰C/30"; 59⁰C/30"; 72⁰C/45")x3; (94⁰C/30"; 57⁰C/30"; 72⁰C/45")x3; (94⁰C/30"; 54⁰C/30"; 72⁰C/45")x35; 72⁰C/10"]. PCR products are then purified and sequenced with the same primers. Two 240bp sequences of RRM are obtained bellow.

RRM1 DNA sequence

5'AGTAAGTGCCTCGGTGTGTTCGGCCTAAGCAGCTACACCAACGAAACCAGCCTGATGG
ACGTTTTCGCACCGTACGGAACCATGACAAGGCGATGATTGTCTACGATGCCAAGACGA
AGGTTTCCCGNGGGTTCGGATTCGTGTACTTCCAGGAGCAGAGTGCGGCCACCGAAGCC
AAAATGCAGTGAAATGGNATGATGCTGCATGAGCGCACGATTAGAGTGGATTATTCGGTG
ACC-3'

RRM2 DNA sequence

5'AGTAAGTGCCTCGGTGTGTTCGGCCTNAGYAGCTAYACCAMCGAARCCARCCTGATGG
AYGTNTTCKNCCTGTCGGNACCATHGACAAGGCNATGATTGTCTACGATGCCAAGACG
AAGGYNTCCCGNGGGTTCGGTTCGTGTAYTTCCAGGAGCAGAGTKCGGCCACNGARGC
CAAAMTGCAGTGAAAYGGAATGRWRCTGCAYGAGCGNACGATTAGAGTGGATTATTCGG
TGACC-3'

RRM1 amino acid sequence

1 -S--K--C--L--G--V--F--G--L--S--S--Y--T--N--E--T--S--L--M--D-
21 -V--F--A--P--Y--G--T--I--D--K--A--M--I--V--Y--D--A--K--T--K-
41 -V--S--R--G--F--G--F--V--Y--F--Q--E--Q--S--A--A--T--E--A--K-
61 -M--Q--C--N--G--M--M--L--H--E--R--T--I--R--V--D--Y--S--V--T-

RRM2 amino acid sequence

1 -S--K--C--L--G--V--F--G--L--S--S--Y--T--T--E--T--N--L--M--D-
21 -V--F--**S**--P--F--G--T--I--D--K--A--M--I--V--Y--D--A--K--T--K-
41 -**A**--S--R--G--F--G--F--V--Y--F--Q--E--Q--S--**S**--A--T--E--A--K-
61 -L--Q--C--N--G--M--**E**--L--H--E--R--T--I--R--V--D--Y--S--V--T-

(Underlines indicate the region selected for primers. The bold characters indicated nucleotide and amino acid substitutions).

Beside, these pair of primers can be used to amplify this Tra-2 RRM 240bp region of the other *Aedes spp*, even from the distance species as *Aedes niveus*, *Aedes annandalei* or *Aedes pseudoalbopictus*. Using the same PCR condition, an exact 240bp band would be amplified among other bands. An agarose gel extraction step is performed for the 240bp band by Qiagen columns. The DNA elution is diluted between 10-20 times in water and 1µl to be used as template for the same PCR. A 240bp specific band would be amplified and can be used to assemble Tra-2 RNAi constructs for the respective species.

Two fragments of 135bp from the bottom parts of these RRM1 and RRM2 regions are used to assemble Tra-2 RNAi constructs. Because the sequences of RRM1 and RRM2 are only different in some parts, therefore the primers derived outside of those parts can be used for amplifying both RRMs. PCR is carried out in 25µl reaction in a condition of 2.5µl PCR buffer; 1.5µl MgCL (25mM); 0.5 µl dNTPs (10mM); each primer 0.5µl (10pmol/µl); 0.15µl Taq DNA polymerase (5U/µl); 10-40ng DNA template. Thermal profile of PCR is [94⁰C/4; (94⁰ C/30"; 59⁰ C/30"; 72⁰ C/45")x3; (94⁰ C/30"; 57⁰ C/30"; 72⁰ C/45")x3; (94⁰ C/30"; 54⁰ C/30"; 72⁰ C/45")x35; 72⁰ C/10']

1-(BA-EX1F) 5'CGATCTCGGATCCATGCCAAGACGAAGGTTTCCCGAG 3'
 2-(X-Ex1R) 5'CGGCAATGACCTCGAGACCGGTCACCGAATAATCCACTCAA 3'
 3-(SAL-EX1F) 5'GGCGTCAATGTCGACATGCCAAGACGAAGGTTTCCCGAG 3'
 4-(ECORI-EX1R) 5'CGGACGTTGGAATTCGACGGTCACCGAATAATCCACTCAA 3'

Primers 1 &3 or 2&4 are similar forward and reverse primers. A combination between 1&2 would produce the same PCR product as that of 3&4. The differences in those PCR products are endonuclease restriction enzyme sequences inserted in the front parts of the primers (underline). This allows the PCR products can be ligated to an intron or linker that contains the same restriction sites in a desired direction. If a connection between the two inverted repeats is a linker about 10bp, PCRs to amplify these fragments can use the same reverse primer (2 or 4) and therefore products would contain the same restriction sites at 3' end (XhoI or EcoRI). Two PCR products would be easily inversely connected after an XhoI or EcoRI enzyme treatments. However, if we want to insert an intron between the two inverted repeats, it needs to use both inverse primers. Two PCR products would then have different sticky ends at 3' (XhoI and EcoRI) and can be easily ligated with an intron that ends by XhoI and EcoRI restriction sites. In this invention, any linker or intron sequence from other

insects can be used in conjunction with the two inverted repeats, provided that two nucleotides GT and AG would be inserted at the first and the end of those sequences, respectively. These are recognition signals for intron splicing sites.

After two identical DNA fragments are reversely connected via an intron or linker, these RNAi kernel sequences (1&2) can be easily ligated into the transactivator system in a desired direction provided that the transactivator plasmids contain the same restriction sites.

II. RRM from *Culex quinquefasciatus*

Genomic sequences of *Culex quinquefasciatus* are available in the Vectorbase.org website (<http://www.vectorbase.org/>). We used two RRM s (RRM1 and RRM2) as queries to blast against the database. Outcome is a 240bp sequence which is highly similar with RRM1 and RRM2 in its helix structure as well as phylogenetic relationship. RRM3 contains up to 69% and 73% amino acid similarity with RRM1 and RRM2, respectively. The annotation of Tra-2 *Culex quinquefasciatus* is CPIJ016646; supercont3.780:5008-5247.(RRM3)

RRM3 DNA sequence

CGTAACGGAATAGTCCACCCGGATGGTTCGCTCGTGCATTACCATTCCGTTGCACTGCAC
CTTGCTGCGGAAGCGTCCTCCAGGTTGACAAAGTACACGAATCCGAACCCGCGGGACG
CCTTCGTCTTGGCATCGTACACGATCTGCACCTTCTCGATCAATCCGAACCGGCCAAACA
CGGTCCTCAGGTCCGCCTCCTGGGTGTAATTGCTGAGGCCAAACACGCCGAGGCAGGTC
GA

RRM3 amino acid sequence

1 -S--T--C--L--G--V--F--G--L--S--N--Y--T--Q--E--A--D--L--R--T--
 21 -V--F--G--R--F--G--L--I--E--K--V--Q--I--V--Y--D--A--K--T--K--
 41 -A--S--R--G--F--G--F--V--Y--F--V--N--L--E--D--A--S--A--A--K--
 61 -V--Q--C--N--G--M--V--M--H--E--R--T--I--R--V--D--Y--S--V--T--
 (Underlines indicate the region selected for primers).

In *Culex quinquefasciatus* whole RRM3 sequence can be used to create an RNAi kernel sequence as its nucleotide sequences at beginning and at the end are suitable to design good primers. 24bp at beginning and 22bp at the end of RRM3 (underline) are used to create a pair of primers. These pair of primers can be used to amplify this Tra-2 RRM 240bp region of the other *Culex spp*, even the distance species as *Culex vishnue*, *Culex pipiens* or *Culex tritaeniorhynchus* by a similar condition. Using the same PCR condition, an exact 240bp band would be amplified among other bands. A gel extraction step is performed for the 240bp band by Qiagen columns. The DNA

elution is diluted between 10-20 times in water and 1µl to be used as template for the same PCR. A 240bp specific band would be amplified and can be used to assemble Tra-2 RNAi constructs for the respective species.

7-(BA-EX1F) 5' CGATCTCGGATCCCGTAACGGAATAGTCCACCCGGAT 3'
 8-(X-Ex1R) 5' CGGCAATGACCTCGAGACTCGACCTGCCTCGGCGTGTGTTG 3'
 9-(SAL-EX1F) 5' GCGGTCAATGTCGACCGTAACGGAATAGTCCACCCGGAT 3'
 10-(ECORI-EX1R) 5' CGGACGTTGGAATTCGATCGACCTGCCTCGGCGTGTGTTG 3'

PCR is carried out in 25µl reaction in a condition of 2.5µl PCR buffer; 1.5µl MgCL (25mM); 0.5 µl dNTPs (10mM); each primer 0.5µl (10pmol/µl); 0.15µl Taq DNA polymerase (5U/µl); 10-40ng DNA template. Thermal profile of PCR is [94° C/4; (94° C/30"; 59° C/30"; 72° C/45")x3; (94° C/30"; 57° C/30"; 72° C/45")x3; (94° C/30"; 54° C/30"; 72° C/45")x35; 72° C/10']. Afterward, these PCR products are also performed in the same manner with those have been done in *Aedes albopictus* and *Aedes polynesiensis*. Whatever, these fragments are connected by a linker or intron, after this RNAi kernel sequence (3) is constructed, it would be available to ligate into the transactivator plasmids to transform *Culex quinquesfasciatus* embryos.

III. Connection of the RNAi kernel structures with Tre repressor.

The pTre-tight plasmid (Cat. No. 631059) from Clontech is mixed with the RNAi kernel sequence (1 or 2 or 3) in 1:3 molar ratio in a 30µl reaction in the presence of BamHI and Sall restriction enzymes. After digestion, ligation is performed by adding T4 ligation into the denatured restriction enzyme mixture. The circle plasmid is transformed into competent cells (DH5α™ derivative, New England Biolabs), isolated and cultured overnight to harvest a larger amount of plasmid DNA from each clone. The size of new plasmid would be 2.6kb plus the size of the RNAi kernel sequences. In the case of RRM1 and RRM2 from *Aedes albopictus* and *Aedes polynesiensis*, only 135 bp of each RRM are used, the plasmid size would be about 3070bp if using an intron of 200bp. If a linker of 10bp is used, the plasmid is about 2870bp. In the case of *Culex quinquesfasciatus*, whole 240bp is used, if it is accompanied with 200bp intron, the fragment size would be 3280bp. If a linker of 10bp is used, the plasmid is about 3090bp.

A fragment includes the Tre operator and the RNAi kernel sequence (tetOx7+PminCMV+RNAi kernel sequence +SV40 polyA) can now be amplified by two

primers which contain HindIII and Acc65I restriction sites. These pending sites are available for ligation with *Piggybac* plasmid and the other parts of the construct.

(Tre-HindIII)	CGATCTAAGCTTCTCGAGTTTACTCCCTATCAGTGA
(Tre-Acc65I)	CGATCTGGTACCAGTCAGTGAGCGAGGAAGCTCGAG

PCR is carried out in 25µl reaction in a condition of 2.5µl PCR buffer; 1.5µl MgCL (25mM); 0.5 µl dNTPs (10mM); each primer 0.5µl (10pmol/µl); 0.15µl Taq DNA polymerase (5U/µl); 10-40ng DNA template. Thermal profile of PCR is [94°C/4; (94°C/90"; 54°C/60"; 72°C/ 2 min 30")x35; 72°C/10']. The PCR products amplified from the RRM of *Aedes albopictus* and *Aedes polynesiensis* with a 10 bp linker would be 875bp, meanwhile a 200bp intron would produced 1065bp products. The PCR products amplified from the RRM of *Culex quinquesfasciatus* would be 1085bp and 1275bp, which are respectively to a 10bp linker or 200bp intron. The PCR products are digested by Acc65I and HindIII and purified by Qiagen columns. The product is available for a final ligation.

IV. Connection of the *Drosophila* β2 tubulin promoter with a transactivator sequence.

Drosophila β2 tubulin promoter sequence is obtained from GenBank or <http://flybase.org/reports/FBgn0003889.html>. Two primers which contain EcoRI and Apa I are designed from the sequence. These primers amplify 230bp of 5'UTR of β2 tubulin gene from *Drosophila* genomic DNA. Thermal profile of PCR is [94°C/4; (94°C/30"; 55°C/30"; 72°C/45")x35; 72°C/10'].

β2-ApaI-F	CGATCTGGGCCCCGGAAATCGTAGTAGCCTATTTGTGA
β2-EcoRI-R	CGGACGTTGGAATTCCTGAATGTGTACAATTTACGCAT

The pTet-Off-Advanced plasmid (Clontech, Catalog Nos. 630934) is digested by two restriction enzymes EcoRI and HindIII producing a band of 1222bp. This DNA fragment is then ligated to the β2 tubulin promoter sequence via the EcoRI restriction site to produce a fragment of 1458bp. tTA protein is now controlled by β2 tubulin promoter. The ligation product is digested by ApaI and purified by Qiagen columns. The product is available for a final ligation.

V. Whole plasmid assemblies.

pXL-BacII-ECFP plasmid from <http://piggybac.bio.nd.edu/> is used to assemble all the above fragments into completed Tra-2 RNAi constructs. The pXL-BacII-ECFP plasmid

carries a 3xP3 promoter which drives ECEP reporter gene. This reporter gene would be tissue specific expressed under the promoter. When mosquitoes are transformed with this marker, mosquito eyes would be fluorescently cyan color.

The pXL-BacII-ECFP plasmid is digested by *Apal* and *Acc65I* and purified by Qiagen columns. The linear plasmid is 5390bp. The plasmid is then mixed with *Tre* fragments (III), $\beta 2$ +tTA fragment (IV) in 1:3:3 molar ratio. T4 ligation is added into a 30 μ l reaction. Ligation product is used to transform into competent cells. Ligation products are expected in a range of different sizes as follow :

For *Aedes albopictus* and *Aedes polynesiensis*, two plasmid containing 10bp linker or 200bp intron are 7723bp and 7913bp, respectively. Meanwhile, plasmids of *Culex quinquesfasciatus* would be 7933bp and 8123bp for 10bp linker and 200bp intron, respectively.

VI. Plasmid injection and transformant selection.

The Tra-2 RNAi plasmids is mixed with a pBSII-IE1-orf (<http://piggybac.bio.nd.edu/>) helper plasmid. The helper produces transposase enzyme which helps *Piggybac* in the Tra-2 RNAi plasmids jumping into mosquito genome. A good concentration of the injection mixture would be 600ng of the Tra-2 RNAi plasmid plus 400ng of the helper per micro liter (μ l) of 1x phosphate buffer. Mosquito eggs would be injected within 2hrs after oviposition into egg posterior ends. After 4 days post injection, the eggs are submerged into tetracycline solution (0.008g per liter). Go survivors would be kept to cross with wild type males or females. G1 larvae are screened under a stereo fluorescent microscope. Any fluorescent larva found that would be the transformant one and to be crossed to build transformant lines. These lines would be tested in Tet-on and Tet-off conditions to check sex ratio. Any line having maleness skew over 80% in Te-off condition would be kept for further analysis and for vector control applications.

Invention effects

The method exposed in this invention would help to produce one sex (maleness) in *Culicinae* mosquitoes. Males created by this invention would pass on the Tra-2 RNAi genetic system into natural population when being released. If the number of released males is big enough, it can result in a collapse of natural vector population, even gone extinct of whole population in a certain time.

Claims

1. A homomorphic sex chromosome (*Culicinae*) mosquito carrying one or more heterozygous or homozygous Tra-2 RNAi loci, to knockdown the Transformer-2 locus, the interference effect is specifically expressed to males or male tissue and is conditional, wherein the knockdown effect caused by the Tra-2 RNAi occurs in the natural population of the target species.
2. An organism according to claim 1 wherein the interference effect of the Tra-2 RNAi system is expressed in male spermatogenesis and interrupt the development of X(m) bearing sperms and results in a severe male bias in the progeny.
3. An organism according to claim 1 and 2, wherein any sequence to create an RNAi genetic recombinant construct used for transgenic of the organism is derived from its own or from other *Culicinae* mosquito Tra-2 gene sequences.
4. An organism according to any preceding claim, wherein the Tra-2 RNAi effect is located on any chromosome.
5. A method of biological control according to any preceding claim, wherein the Tra-2 RNAi effect results in producing of more than 90% males in the progeny of the target population when matings between released males and wild females.
6. An RNAi recombinant construct according to any preceding claim, is used to transform *Culicinae* mosquitoes wherein contains parts or whole sequence from the DNA recombinant sequence (Tra-2 RRM RNAi 1. Fig.1) encoding hairpin mRNA sequences.
7. An RNAi recombinant construct according to any preceding claim, is used to transform *Culicinae* mosquitoes wherein contains parts or whole sequence from the DNA recombinant sequence (Tra-2 RRM RNAi 2. Fig.2) encoding hairpin mRNA sequences.
8. An RNAi recombinant construct according to any preceding claim, is used to transform *Culicinae* mosquitoes wherein contains parts or whole sequence from the DNA recombinant sequence (Tra-2 RRM RNAi 3. Fig.3) encoding hairpin mRNA sequences.

9. An RNAi recombinant construct according to any preceding claim, is used to transform *Culicinae* mosquitoes wherein contains an insect spermatogenesis specific promoter which regulates the conditional transactivator (tTA gene), for instance *Drosophila* $\beta 2$.
10. An RNAi recombinant construct according to any preceding claim, is used to transform *Culicinae* mosquitoes wherein contains an insect spermatogenesis specific minimal promoter fused with a tetOx7 (n) operator sequence which regulates a *Culicinae* Tra-2 RNAi sequence.