Identification of resistant carboxylesterase alleles in *Culex pipiens* complex via PCR-RFLP

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Carboxylesterase overproduction is a frequently observed resistance mechanism of insects to organophosphate insecticides. As a major transmitter of human diseases, mosquitoes in the *Culex pipiens* complex have evolved 13 carboxylesterase alleles (Ester) that confer organophosphate resistance. Six alleles, Ester²¹, Ester², Ester⁸, Ester⁹, Ester¹⁰, and Ester¹¹, have been observed in field populations in China, sometimes co-existing in one population. To differentiate the carboxylesterase alleles found in these field populations, PCR-RFLP was designed for use in resistance monitoring.

Based on the DNA sequences of resistant and nonresistant carboxylesterase alleles, Ester B alleles were first amplified with PCR-specific primers and then digested with the restriction enzyme DraI. In this step, Ester² and Ester¹¹ were differentiated from the other Ester alleles. When the other Ester B alleles were digested with the restriction enzyme XbaI, Ester²¹ and the susceptible *C. p. pallens* Ester were screened out. Ester⁸ and Ester⁹ were differentiated from Ester¹⁰ and the susceptible *C. p. quinquefasciatus* esterase allele, respectively, by amplifying and digesting the Ester A alleles with the restriction enzyme ApaLI. The effectiveness of the custom-designed PCR-RFLP was verified in two field mosquito populations.

**Conclusions:** A PCR-RFLP based approach was developed to differentiate carboxylesterase alleles in *Culex pipiens* complex mosquitoes. These processes may be useful in monitoring the evolutionary dynamics of known carboxylesterase alleles as well as in the identification of new alleles in field populations.

**Background**

Insecticides are vital to agricultural production and public health, particularly in countries with huge human populations, such as China. In China, large quantities of chemical insecticides have been used to control mosquitoes since the mid-1950s. Consequently, resistance has developed in vector mosquitoes, which makes their control increasingly difficult [1]. Mosquitoes in the *Culex pipiens* complex (Diptera: Culicidae) are common in temperate and tropical countries. These insects have been subjected to insecticide control in many places around the world [2]. Four subspecies comprise the complex: *C. p. quinquefasciatus*, *C. p. pallens*, *C. p. pipiens*, and *C. p. molestus*. *C. p. quinquefasciatus* and *C. p. pallens* are prevalent in South China and North China, respectively [3].

Global surveys of insecticide resistance have indicated that one of the major mechanisms of resistance is the increased detoxification in resistant individuals [4]. Three primary detoxifying enzymes involved in insecticide resistance are carboxylesterase (or esterases), glutathione-S-transferases, and P450 monooxygenases, which are qualitatively or quantitatively changed to confer resistance [5]. Esterase overproduction is a common resistance mechanism of *C. pipiens* complex mosquitoes to organophosphate (OP) insecticides. This process is achieved mainly by gene amplification or occasionally by gene up regulation [6,7]. Some studies indicated that gene duplication or amplification may be a more common adaptive evolutionary mechanism in arthropods, and that certain genomic loci may be “hot spots” for gene duplication, as evidenced by parallel evolution in several arthropod species [8].

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In the *C. pipiens* complex, two carboxylesterase loci, *Est-3* (encoding esterase A) and *Est-2* (encoding esterase B), are amplified in the genome and subsequently confer resistance to insecticides [9,10]. *Est-3* and *Est-2* are usually in complete linkage disequilibrium when amplified and are thus referred to as the *Ester* superloci [11]. To date, 13 alleles that confer insecticide resistance have been identified at the *Ester* superloci in the *C. pipiens* complex. These alleles (with the corresponding overpro-

| Alleles | GenBank ID | PCR (bp) | Digestion (bp) | In gel | XbaI digestion (bp) |
|---------|------------|----------|----------------|--------|---------------------|
| B1      | M32328     | 945      | 512, 369, 64   | 512, 369 | 945                 |
| B2      | Z86069     | 941      | 677, 244       | 677, 244 | 941                 |
| B3      | EF174325   | 2443     | 951, 888, 424, 89, 69, 22 | 951, 424, 180 | 2443             |
| B9      | JQ866911   | 936      | 402, 292, 242  | 402, 292, 242 | 936               |
| B11     | EF174328   | 942, 1165| 519, 423, 742, 423 | 742, 519, 423 | 942, 1165         |
| B10     | EF174326   | 2442     | 951, 888, 424, 89, 68, 22 | 951, 424, 180 | 2442             |
| Sp-B1   | JQ812615   | 942      | 519, 375, 48   | 519, 375 | 353, 589           |
| Sq-B1   | JQ341053   | 936      | 402, 292, 242  | 402, 292, 242 | 936               |

*Susceptible Ester B allele of *C. p. pallens.*

*Susceptible Ester B allele of *C. p. quinquefasciatus.*

beads. The most applied method in studies of *Culex* *Ester* alleles is the starch gel electrophoresis, which only reveals the phenotype of *Ester* alleles instead of the genotype. In this paper, we designed specific primers and selected the appropriate restriction endonucleases for use in PCR-RFLP to differentiate between resistant and nonresistant *Ester* alleles in *C. pipiens* complex mosquitoes. The effectiveness of the PCR-RFLP system was verified in field-collected mosquitoes.

**Methods**

**Mosquitoes**

Eight standard *C. pipiens* complex strains were used. These strains are S-LAB, which is an OP-susceptible *C. p. quinquefasciatus* strain without increased esterase activity [20]; BJSU, an insecticide-susceptible *C. p. pallens* strain collected from Beijing in the 1970s and laboratory-reared for over 40 years without insecticide exposure; and SB1, SA2, MAO2, LING, KARA2, and WU, which are homozygous *C. p. quinquefasciatus* for *Ester* A1, *Ester* B2, *Ester* B4, *Ester* B5, and *Ester* B11, respectively [13,18,21]. Two field populations were also obtained: R-SG, which was *C. p. quinquefasciatus* and collected from Foshan in Guangdong Province (South China) in July 2007, and TAA, which was *C. p. pallens* and collected from Tai’an in Shandong Province (North China) in July 2010 [22].

**DNA isolation and PCR-RFLP**

Genomic DNA from intact single adult mosquitoes or from their head-thoraces was extracted using the cetyl trimethyl ammonium bromide (CTAB) method, as described by Roger and Bendich [23]. Based on the GenBank *Ester* allele sequences (Tables 1 and 2), the *EsterB* allele fragments were amplified using the Bdir (5’AGCTTAAACCGTTACAGCA3’) and Brev (5’CAGTCCAACGTTTCGGTCCA3’) primers, whereas the *EsterA* alleles were amplified using the Adir (5’CTTTATGAGAGATCTAGTGG3’) and Arev (5’CAAGCTTCACGACAC
ATCTC3 primers. The 50 μl PCR mixture contained 1 μl of genomic DNA (50 ng/μl to 100 ng/μl), 0.25 μM of each primer, 0.2 mM of each dNTP, and 2.5 units of LA Taq polymerase (Takara, Otsu, Shiga, Japan) in a 1× reaction buffer. PCR was performed on a thermocycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany) at a denaturing step of 94°C for 4 min, followed by 35 cycles of 94°C for 45 s, 58°C for 45 s, and 72°C for 2 min and 40 s for the Ester B gene or 1 min and 10 s for the EsterA gene. A final extension of 10 min at 72°C followed. After verification via 1% agarose gel electrophoresis, the PCR products were digested with the restriction enzymes DraI or XbaI for EsterB and ApaLI for EsterA at 37°C for 2 h in accordance with the manufacturer’s instructions (BioLabs, Ipswich, MA, USA). The digested products were electrophoresed in 2% agarose gel and photographed under ultraviolet light illumination using a gel imaging system (Gene Company, Hong Kong, China).

Starch gel electrophoresis
Single adult mosquitoes from the R-SG and TAA field populations were cut into two parts: the head-thorax and the abdomen. The abdomen of each mosquito was homogenized in 10 μl distilled water using a pestle. The homogenate was spread onto a Whatman Grade No. 3 filter paper (3 mm × 8 mm) and analyzed via starch gel electrophoresis to identify the carboxylesterase phenotypes [24]. The head-thorax was used in PCR-RFLP for carboxylesterase genotype identification. At least 10 individuals from each population were analyzed.

Results and discussion
Discrimination of carboxylesterase alleles in standard strains via PCR-RFLP
To discriminate the carboxylesterase alleles, the Ester B allele fragments were first amplified by PCR. Most PCR products of the Ester B alleles were approximately 900 bp long, whereas those of Ester B8 and Ester B10 were approximately 2400 bp in length (Figure 1a and Table 1). Meanwhile, the PCR product of Ester B11 showed two predominant bands that correspond to 942 and 1165 bp fragments. Sequencing data show that the 942 bp band is Ester B11, whereas the 1165 bp band had a 223 bp insertion in one Ester B11 intron. This insertion may have originated from gene recombination or transposition in the genome. Whether this insertion affects the insecticide resistance in mosquitoes remains to be determined.

After the PCR products were digested by the restriction enzyme DraI, the Ester B alleles showed various digestion patterns. Given the resolution limit of the 2% agarose gel electrophoresis, some Ester B allele digestion patterns in the agarose gel electrophoresis differed from the predicted theoretical patterns (Table 1). In the electrophoresis graph (Figure 2a), the Ester B2 (lane 2) and Ester B11 (lane 5) patterns were unique, thereby allowing the differentiation of Ester2 and Ester11 from the other alleles. The 223 bp insertion in the 1165 bp Ester B11 PCR product resulted in the appearance of a 742 bp excess band during digestion. Based on DraI digestion, three pairs of Ester B alleles were distinguished by differences in their migration patterns (Figure 2a): Ester B1 (lane 1) and the susceptible C. p. pallens Ester B (lane 7); Ester B8 (lane 3) and Ester B10 (lane 6); Ester B9 (lane 4) and the susceptible C. p. quinquefasciatus Ester B (lane 8).

Table 2 PCR and RFLP products of esterase A alleles

| Alleles | GenBank ID | PCR (bp) | ApaLI digestion (bp) |
|---------|------------|----------|----------------------|
| B1-A    | JQ780068   | 714      | 321, 393             |
| A2      | Z86069     | 713      | 321, 392             |
| A8      | AJ302089   | 713      | 98, 223, 392         |
| A9      | AJ302090   | 713      | 98, 615              |
| A11     | EF174327   | 713      | 98, 223, 409         |
| B10-A   | AJ302090   | 713      | 98, 615              |
| Sp-A1   | JQ812614   | 713      | 321, 392             |
| Sq-A2   | JQ812613   | 713      | 321, 392             |

1Susceptible Ester A allele of C. p. pallens.
2Susceptible Ester A allele of C. p. quinquefasciatus.
The PCR products of the three pairs of Ester B alleles were digested by the restriction enzyme XbaI (Figure 2b) to allow differentiation among members of the same pair. The pair consisting of Ester B1 (lane 1) and the susceptible C. p. pallens Ester B (lane 7) displayed different digestion profiles, which allowed the differentiation of EsterB1 from the susceptible C. p. pallens.

However, the XbaI enzyme could not discriminate EsterB8 (lane 3) from Ester B10 (lane 6), or Ester B9 (lane 4) from the susceptible C. p. quinquefasciatus Ester B (lane 8). At this step, four Ester alleles had already been distinguished from one another: Ester2, Ester11, EsterB1, and the susceptible C. p. pallens.

Ester8 and Ester9 were differentiated from EsterB10 and the susceptible C. p. quinquefasciatus, respectively, by amplifying an approximately 700 bp fragment of their Ester A via PCR using specific primers (Figure 1b and Table 2) and then digesting the fragment with the restriction enzyme ApaLI. Ester A8 (lane 3) displayed digestion profiles that differ from that of the EsterB10 Ester A (lane 6) (Figure 2c). In addition, Ester A9 (lane 4) displayed profiles that differ from those of the susceptible C. p. quinquefasciatus Ester A (lane 8) (Figure 2c). Thus, the six resistant Ester alleles EsterB10, EsterB1, Ester2, Ester8, Ester9, and Ester11, as well as the susceptible alleles, were correctly identified (Figure 3).

EsterA4, EsterB5, and EsterB6, EsterB7, EsterA12, and EsterA13 are found in other places in the world. However, given that no sequence information on the association of EsterB6, EsterB7, and Ester B to EsterA1 and EsterA13 is available, and that only a partial sequence of EsterA12 is currently known, the designed PCR-RFLP cannot be used to differentiate these alleles. The predicted PCR product sizes for Ester B4 and Ester B5 are 1289 and 1337 bp according to the Ester4 and Ester5 sequences, respectively; these values deviate from those of other Ester B alleles (Table 1). However, the DraI digestion profiles of Ester B4 and Ester B5 (764 and 467 bp for Ester B4, and 770 and 518 bp for Ester B5) may be difficult to distinguish in 2% agarose gel electrophoresis. No XbaI cut site exists in Ester B4 and Ester B5. The predicted PCR products of Ester A4 and Ester A5 are 714 and 713 bp, respectively. Only Ester A5 can be cut by ApaLI to produce 321 and 392 bp bands. Thus, the designed PCR-RFLP can identify eight resistant Ester alleles (EsterB1, Ester2, Ester4, Ester5, Ester8, Ester9, EsterB10, and Ester11) and two susceptible alleles.

Identification of carboxylesterase alleles in mosquitoes from field populations

The phenotype and genotype of carboxylesterase alleles in mosquitoes from two field populations, R-SG (C. p. quinquefasciatus) and TAA (C. p. pallens), were identified through starch gel electrophoresis and PCR-RFLP. Of the eight mosquitoes from the R-SG population, Nos. 4, 5, 7, and 9 showed the A8-B8 phenotype in starch gel electrophoresis (Figure 4a). PCR-RFLP results confirm that these mosquitoes were homozygous with...
Esterase (Figure 5 and Figure 6). Starch gel electrophoresis results suggest that the Nos. 3 and 8 mosquitoes are heterozygous to A2-B2 and A9-B9 or A9-B10 and A9-B9 because of the similar mobilities of A2-B2 and A9-B10 [14]. However, PCR-RFLP results indicate that these mosquitoes are actually heterozygous to A9-B10 and B2 (Figure 5 and Figure 6). Three esterase Bs and two esterase As were found in the starch gel electrophoresis profile of Nos. 2 and 10 mosquitoes (Figure 4a). The whole set of PCR-RFLP shows that the No. 2 mosquito expressed A8-B8, A9-B9, and B2, whereas the No. 10 mosquito expressed A8-B8, A9-B10, and B2 (Figure 5 and Figure 6). The fewer than expected DraI digestion bands of esterase B alleles in mosquito No. 2 (Figure 6A, lane 2) could be due to the higher gene copy number of B2 in the genome over B8 and B9. Without the PCR-RFLP results, the differentiation of the phenotypes of the two mosquito types would have been difficult.

Most of the eight mosquito types from the TAA population (Figure 4b) overproduced esterase B1 (No. 2, 4, 6, 7, 8, and 9 mosquitoes). The No. 3 mosquito was heterozygous to B1 and A2-B2. These phenotypes were confirmed by PCR-RFLP (Figure 7 and Figure 8). The No. 5 individual did not appear to have overproduced esterases based on the light-colored band in the starch gel electrophoresis. However, PCR-RFLP results indicate the presence of A2-B2 and A9 (Figure 7 and Figure 8). The results for the two field populations show that the A2-B2 and A9-B9 linkages are not always established. This result deviates from the usual observations and theories [7]. By now the recombination event leading to the existence of three esterase B alleles in one individual
mosquito has only been observed in field populations of China [13 and this study]. The dislinkage between A2 and B2 or A9 and B9 probably results from the rare recombination event among these esterase alleles.

Conclusions

The custom-designed PCR-RFLP method can be used to differentiate the insecticide-resistant esterase alleles existing in China. The method can help monitor the evolutionary dynamics of these esterase alleles and identify new esterase alleles in field populations of C. pipiens complex.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

HZ performed the study, analyzed the data and drafted the manuscript. FM supplied the BJSU strain and helped draft the manuscript. CQ supervised the study and helped draft the manuscript. FC conceived, designed the study, and finalized the manuscript. All authors read and approved the final version of the manuscript.

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References

1. Cui F, Raymond M, Qiao CL: Insecticide resistance in vector mosquitoes in China. Pest Manag Sci 2006, 62:1013–1022.
2. Ben Cheikh R, Berticat C, Berthomieu A, Pasteur N, Ben Cheikh H, Weill M: Characterization of a Novel High-Activity Esterase in Tunisian Populations of the Mosquito Culex pipiens. J Econ Entomol 2008, 101:484–491.
3. Zhao TY, Lu BL: Biosystematics of Culex pipiens complex in China. Insect Sci 1995, 2:1–8.
4. Yan SG, Wu ZH, Cui F, Zhao Q, Qiao CL: Dynamics of esterase alleles in Culex pipiens complex mosquitoes in Beijing. J Econ Entomol 2008, 101:1897–1902.
5. Oppenoorth FJ: Biochemistry and genetics of insecticide resistance. In Comprehensive Insect Physiology, Biochemistry and Pharmacology. Edited by Kerlut GA, Gilbert Li. New York: Pergamon; 1985:731–773. Insect Control, vol.12.
6. Roques S, Guillemaud T, Berge J, Pasteur N, Raymond M: Coamplification of esterase A and B genes as a single unit in Culex pipiens mosquitoes. Heredity 1996, 77:555–561.
7. Raymond M, Chevillon C, Guillemaud T, Lenormand T, Pasteur N: An overview of the evolution of overproduced esterases in the mosquito Culex pipiens. Phil Trans R Soc Lond B Biol Sci 1998, 353:1707–1711.
8. Bass C, Field LM: Gene amplification and insecticide resistance. Pest Manag Sci 2011, 67:886–890.
9. Guillemaud T, Makate N, Raymond N, Hirsh B, Callaghan A: Esterase gene amplification in Culex pipiens. Insect Mol Biol 1997, 6:319–327.
10. Guillemaud T, Lenormand T, Bourgue D, Chevillon C, Pasteur N, Raymond M: Evolution of resistance in Culex pipiens: allele replacement and changing environment. Evolution 1998, 52:443–453.
11. Lenormand T, Guillemaud T, Bourgue D, Raymond M: Evaluating gene flow using selected markers: a case study. Genetics 1998, 149:1383–1392.
12. Raymond M, Berticat C, Weill M, Pasteur N, Chevillon C: Insecticide resistance in the mosquito Culex pipiens: what have we learned about adaptation? Genetica 2001, 112–113:287–296.
13. Cui F, Lin LY, Qiao CL, Xu Y, Marquine M, Weill M, Raymond M: Insecticide resistance in Chinese populations of the Culex pipiens complex through esterase overproduction. *Entomol Exp Appl* 2006, 120:211–220.

14. Cui F, Weill M, Berthomieu A, Raymond M, Qiao CL: Characterization of novel esterases in insecticide-resistant mosquitoes. *Insect Biochem Mol Biol* 2007, 37:1131–1137.

15. Ben Cheikh R, Berticat C, Berthomieu A, Pasteur N, Ben Cheikh H, Weill M: Genes conferring resistance to organophosphorus insecticides in *Culex pipiens* (Diptera: Culicidae) from Tunisia. *J Med Entomol* 2009, 46:523–530.

16. Raymond M, Callaghan A, Fort P, Pasteur N: Worldwide migration of amplified insecticide resistance genes in mosquitoes. *Nature* 1991, 350:151–153.

17. Qiao CL, Marquine M, Pasteur N, Raymond M: A new esterase gene amplification involved in OP resistance in *Culex pipiens* mosquitoes from China. *Biochem Genet* 1998, 36:417–425.

18. Weill M, Marquine M, Berthomieu A, Dubois MP, Bernard C, Qiao CL, Raymond M: Identification and characterization of novel organophosphate detoxifying esterase alleles in the Guangzhou area of China. *J Am Mosq Control Assoc* 2001, 17:238–244.

19. Cui F, Tan Y, Qiao CL: Filariasis vector in China: insecticide resistance and population structure of mosquito *Culex pipiens* complex. *Parasit Vectors* 2011, 4:236–242.

20. Roger SO, Bendich AJ: Extraction of DNA from plant tissues. In *Plant Molecular Biology Manual*. Edited by Gelvin SB, Schilperoort RA. Boston: Kluwer Academic Publishers; 1988:1–10.

21. Liu YY, Zhang HY, Qiao CL, Lu XP, Cui F: Correlation between carboxylesterase alleles and insecticide resistance in *Culex pipiens* complex from China. *Parasit Vectors* 2011, 4:236–242.

22. Pasteur NP, Bonhomme G, Catalan F, Britton-Davidian J: Practical isozyme genetics. Chichester: Ellis Horwood Ltd; 1988.215.