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MOLECULAR DIVERGENCE OF THE MITOCHONDRIAL CYTOCHROME OXIDASE II GENE IN THREE MOSQUITOES

WANG JINFU AND HUANG CHAOHUI

College of Life Sciences, Zhejiang University, Hangzhou, Zhejiang 310012, People's Republic of China

ABSTRACT. The cytochrome oxidase II (COII) genes in the mitochondrial DNA of 3 mosquito species (*Anopheles sinensis*, *Aedes albopictus*, and *Culex quinquefasciatus*) were amplified and sequenced. Both the gene order and direction of transcription were identical to those of other species of *Anopheles*, *Aedes*, and *Culex*. The polymerase chain reaction–amplified fragments in these mosquitoes were approximately 700 base pairs and the nucleotide sequences exhibited more than 82% similarity, whereas amino acids were more than 85% similar. The frequency of transitions was less than that of transversions. Four highly conserved segments of COII proteins are similar to those in other insects. These segments contain the major amino acid residues of cytochrome *c* oxidase involved in electron transport and ligand binding.

KEY WORDS Amino acid sequence, *Aedes*, *Culex*, *Anopheles*

INTRODUCTION

Cytochrome *c* oxidase is an important respiratory enzyme involved in the terminal oxidation steps of the mitochondrial election transport chain. Cytochrome *c* oxidase includes 3 large subunits and is encoded by mitochondrial DNA (mtDNA) (Capaldi et al. 1983; Clary and Wolstenholme 1983, 1985). Among these subunits, cytochrome *c* oxidase subunit II (COII) has been studied extensively in vertebrates (Busse et al. 1978, Bisson et al. 1980, Brown and Simpson 1982, Millett et al. 1983, Lee et al. 1989, Pan et al. 1993) and insects (Liu and Beckenbach 1992, Ho et al. 1995).

Cytochrome *c* oxidase subunit II has a high affinity binding site for cytochrome *c* and contains ligands for copper. Moreover, this subunit evolves at different rates for human, rat, mouse, and cow (Cann et al. 1984). Insects are divergent in both COII amino acid and nucleotide sequences (Liu and Beckenbach 1992). The COII gene is located between 2 transfer RNA genes, $tRNA^{Leu}$ and $tRNA^{Lys}$, in mosquitoes (Cockburn et al. 1990, Beard et al. 1993, Mitchell et al. 1993, Ho et al. 1995). We isolated the template DNA from 3 species of mosquitoes (*Anopheles sinensis* Wiedemann, *Aedes albopictus* (Skuse), and *Culex quinquefasciatus* Say) and amplified the COII gene with the polymerase chain reaction (PCR) by using primers located within these 2 tRNAs. The COII gene was analyzed by sequencing PCR products that had been cloned into plasmid DNA. The amino acid sequence of COII was deduced by using the insect mitochondrial code and was compared with the sequences of other insect species. The conserved segments of the physiologically important segments of the COII gene in these 3 mosquitoes were analyzed.

MATERIALS AND METHODS

Mosquitoes: Adult *Cx. quinquefasciatus* and *Ae. albopictus* were collected in Hangzhou, and *An. sinensis* was collected in Jinhua, Zhejiang Province, China. The mosquitoes were kept in screened cages (29 × 22 × 23 cm) and provided with 10% sucrose solution. Eggs of *Cx. quinquefasciatus* and *Ae. albopictus* were laid in a 50-ml beaker containing water. Eggs of *An. sinensis* were laid on moist silk fabric in a culture plate. Larvae were reared in enamel bowls containing 200 ml of water (Wang et al. 1996). The insectary was maintained at 80% relative humidity, 27°C, and a photoperiod of 14:10 h light : dark. The emerging adults were used for extracting total DNA.

Extraction of total DNA: Total DNA was extracted from each adult (Cockburn and Seawright 1988). Fifty adults of each species were frozen at −70°C and then ground in a cooled mortar and pestle. Powdered mosquitoes were homogenized and the DNA was extracted by phenol : chloroform from the proteinase K digested homogenate. The crude DNA solution was obtained by centrifugation and was then dialyzed against 10 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid (pH 8.0) at 4°C for 16 h. The dialysate was concentrated 5-fold with PEG 20,000 (polyethylene glycol; SANGON, Ltd., Shanghai, China) and stored at 4°C. The DNA concentration was determined by absorbancy at 260 nm.

Oligonucleotide primers: The oligonucleotide primers used in this study were designed according to Ho et al. (1995). The sequence of the 5′ forward primer was 5′-AGATTTTATCTTTTGTTAGAA-3′ located in the $tRNA^{Leu}$ gene and the 3′ reverse primer was 5′-TTGCTTTCAGTCATCTAATG-3′ situated at the beginning of the $tRNA^{Lys}$ gene.

Polymerase chain reaction conditions: Amplification reactions were modified from Kocher et al. (1989). The total volume of reaction mixture was 50 μl. The reaction mixture contained 0.2 mM each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate; 300 ng of each primer; 2.5 units of *Taq* DNA (SANGON Ltd.); and 1 μg of the mosquito DNA. This mixture was amplified

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          tRNA^Leu←------|     |-----→COII

AEALB  AGATTTTATCTTTTGTTAGAA....AATGGCAACATGAATAAATCTAGGACTTCAAAATA  56
CXQUI  ---------------------TACT---------C---GC----T--T--T-A-------  60
ANSIN  ----------------------....----------GC----T----TT-A---G---  56

AEALB  GTACTTCTCCTTTAATAGAACAATTAAATTTTTTTCATGATCATACTTTATTAATTTTAA 116
CXQUI  --T-------A-----------------------------------AG-T----------  120
ANSIN  --T----A-------------------------------------C-TC-T-------  116

AEALB  TTACAATTACTATTATAATTGCATATATTATATTTATATTATTTTTTAATAAATTTACAA 176
CXQUI  ---T------AG-A------A-T---G-A---GG-----------C-------------  180
ANSIN  CA-T------A---T--G---G----------AGA---C--A-A------C----C--T-  176

AEALB  ATCGATATTTACTTCACGGACAAACAATTGAAATTATTTGAACTATTCTTCCTGCAATTA 236
CXQUI  -----------T-A--T--------T--------C--------A----------------  240
ANSIN  ----T------T-A--T---------------------------G--T-A----------  236

AEALB  TTTTAATATTTATTGCCTTTCCTTCTTTACGACTTTTATACTTAATAGATGAAATTAATT 296
CXQUI  ----------------T-----A--AC-T--GT-A-----T---T---------------  300
ANSIN  ----------C-----A--------------TT-A-----T--------C---------A 296

AEALB  CTCCTTTAATTACTTTAAAAGTTATTGGCCATCAATGATATTGAAGTTATGAATATTCTA 356
CXQUI  -------------------G-C------A----------C-------------------  360
ANSIN  -A----C------A-----GTCGG----T-----------------C------------G 356

AEALB  ATTTTTTAAATTTAGAATTTGATTCTTACATAATTCCAACTAATGAATTAGATATTAATG 416
CXQUI  -----A-------------------A--T----------A------------T-A----  420
ANSIN  -------------------------A--T--------T--A------C----A-CA----  416

AEALB  GATTTCGTTTATTAGATGTTGATAATCGAGTTATTCTTCCAATAAATAATCAAATTCGAA 476
CXQUI  ----C--A-C-------------------A-----T-A---T------------------  480
ANSIN  -------AC-T------------------A--G--T-A--T------------------  476

AEALB  TTTTAGTAACTGCTACTGATGTAATTCATTCTTGAACAGTTCCCTCTATAGGAATAAAAA 536
CXQUI  ----------------------TC----C--A----------T---T-----G------  540
ANSIN  -------T--A------------T-A-----A--------A--A---T-----G----GG 536

AEALB  TTGATGCTACTCCCGGACGTTTAAATCAAACTAATTTTTTAATAAATCAACCTGGATTAT 596
CXQUI  -------------A--C--A------------------C----T------T----TC-T-  600
ANSIN  -A--------A--A-----A----------T-----------C----G----------  596
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Fig. 1. Nucleotide sequences of the cytochrome oxidase II gene and the 5′ and 3′ flanking regions in $tRNA^{Leu}$ and $tRNA^{Lys}$ of *Anopheles sinensis* (ANSIN), *Aedes albopictus* (AEALB), and *Culex quinquefasciatus* (CXQUI). Dots and asterisk represent inserted nucleotide and termination, respectively. Dashes indicate the identical nucleotides among 3 species. For the best alignment, 4 gaps (.) were inserted in AEALB and ANSIN.

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AEALB TTTATGGACAATGCTCAGAAATTTGTGGAGCAAATCATAGTTTCATACCAATTGTTATTG 656
CXQUI ---T---------T--T-----C--------T-----------T-----T---------- 660
ANSIN ---T---T-----T-----------------------------T-----------A---- 656

AEALB AGAGAATCCCAATAAATTATTTTATTAAATGAATTTCTTCTCAAATAAATTCATTAGATG 716
CXQUI -A-----T------------------------G-----------T--------------- 720
ANSIN -A-----T--------C-----------G------A--AA-ATG-CT------------- 716
                                      *|---→tRNA^Lys

AEALB ACTGAAAGCAA                                                  727
CXQUI -----------                                                 731
ANSIN -----------                                                 727
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Fig. 1. Continued.

in a RoboCycler GRADIENT-40 thermal cycler (Stratagene, La Jolla, CA). The PCR profile consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min. After 30 cycles, extension occurred at 72°C for 8 min. The amplified product was analyzed and purified by electrophoresis in ethidium bromide–stained 1% agarose gel at 100 V for 30 min in Tris/borate electrophoresis (TBE) buffer.

Cloning and sequence analysis of COII gene: Amplified fragments of approximately 0.7 kilobases were extracted by phenol : chloroform and precipitated by ethanol (Sambrook et al. 1989), then ligated to the pUC-T Vector (SANGON Ltd.). *Escherichia coli* strain DH5α was transformed with the ligated plasmid DNA. The transformants were selected by a LB agar (Sigma, St. Louis, MO) plate containing ampicillin (50 μg/ml), isopropylthio-β-D-galactoside (200 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (20 μg/ml).

Nucleotide sequences of 3 or 4 clones inserts for each mosquito species were determined with an automatic DNA sequencer and genetic analysis systems (LI-COR Ltd., Lincoln, NE) (Sanger et al. 1977). Sequencing reactions were performed on double-stranded DNA by using modified T7 DNA polymerase (Sequenase version 2.0, USB, Cleveland, OH). The mitochondrial COII gene coding regions were confirmed by comparison with *Drosophila yakuba* (Clary and Wolstenholme 1983, 1985), *Anopheles quadrimaculatus* Say (Cockburn et al. 1990), and *Cx. quinquefasciatus* (Ho et al. 1995).

Data analysis: Nucleotide sequences were aligned by using ClustalW (Thompson et al. 1994) and translated into amino acid sequences by using Mega 1.02 (Kumar et al. 1993). *Anopheles gambiae* Giles (Beard et al. 1993), *An. quadrimaculatus* (Cockburn et al. 1990), *Aedes australis* (Erichson) (Brust et al. 1998), and *D. yakuba* Burla (Clary and Wolstenholme 1983, 1985) were used to compare nucleotide and amino acid sequences.

RESULTS

The amplified fragment was 727 base pairs (bp) for *An. sinensis* and *Ae. albopictus*, and 731 bp for *Cx. quinquefasciatus*. Figure 1 shows the DNA sequences for the COII gene and flanking regions on $tRNA^{Leu}$ and $tRNA^{Lys}$. The nucleotide sequences of $tRNA^{Lys}$ are identical in these 3 species. The nucleotide sequences of $tRNA^{Leu}$ are also identical between *An. sinensis* and *Ae. albopictus*, but an addition of 3 nucleotides was found in *Cx. quinquefasciatus*. Five intergenic nucleotides occur between $tRNA^{Leu}$ and the COII gene of *Cx. quinquefasciatus*, but only 1 is found in *An. sinensis* and *Ae. albopictus*. No nucleotides separating COII and $tRNA^{Lys}$ were found in these species. The COII genes of these species were 685 nucleotides long (sequences can be found in GenBank AF324898 [*Ae. albopictus*], AF325715 [*An. sinensis*], and AF325716 [*Cx. quinquefasciatus*]). An ATG initiation site and a presumptive terminating T at the 3′ end were found.

Figure 1 shows the locations of nucleotide substitutions. Table 1 shows the substitutions classified according to the type of base change between these 3 species. The COII gene is 84% similar between *An. sinensis* and *Ae. albopictus* and between *An. sinensis* and *Cx. quinquefasciatus*, but is 87% similar between *Ae. albopictus* and *Cx. quinquefasciatus*. The transition frequency was less than the transversion frequency. The frequency of C+G is lower (24.9% for *An. sinensis*, 23.2% for *Ae. albopictus*, and 23.8% for *Cx. quinquefasciatus*). This is similar to other insects.

For a comparison of COII, amino acid sequences were deduced by using the insect mitochondrial code. Two hundred twenty-eight amino acid residues are present and similarity is shown in Figure

Table 1. Nucleotide changes in the cytochrome oxidase II gene among *Anopheles sinensis, Aedes albopictus,* and *Culex quinquefasciatus.*

	An. sinensis–Cx. quinquefasciatus			*An. sinensis–Ae. albopictus*			*Ae. albopictus–Cx. quinquefasciatus*		
	Synonymous	Nonsynonymous	Total	Synonymous	Nonsynonymous	Total	Synonymous	Nonsynonymous	Total
Codon position									
1st	8	29	37	11	22	33	8	19	27
2nd	0	10	10	1	13	14	0	6	6
3rd	48	14	62	47	12	59	44	6	50
Total	56	53	109	59	47	106	52	31	83
Transition									
G↔A	2	14	16	4	12	16	3	7	10
T↔C	20	8	28	23	8	31	22	5	27
Total	22	22	44	27	20	47	25	12	37
Transversion									
G↔T	1	3	4	0	3	3	0	4	4
G↔C	0	2	2	0	1	1	0	0	0
A↔T	31	23	54	28	20	48	23	14	37
A↔C	2	3	5	3	4	7	4	1	5
Total	34	31	65	31	28	59	27	19	46

2. The greatest similarity was found at the residues 50–86 (region I), 101–128 (region II), 144–167 (region III), and 194–212 (region IV). To examine the divergence of COII among additional mosquito species, nucleotide and amino acid sequences of 7 additional insects were compared (Table 2). The nucleotide divergences between mosquitoes is less than 16%, and the amino acid divergence is less than 15%.

DISCUSSION

The COII subunit has 5 conserved regions in vertebrates (Lee et al. 1989, Pan et al. 1993) and 2 conserved regions in insects (Clary and Wolstenholme 1983, 1985; de Bruijn 1983; Haucke and Gellissen 1988; Liu and Beckenbach 1992; Beard et al. 1993; Crozier and Crozier 1993; Mitchell et al. 1993; Ho et al. 1995). These regions contain the functional domains of cytochrome *c* oxidase. These amino acids are located in 4 identical regions in *Ae. albopictus, An. sinensis,* and *Cx. quinquefasciatus* (Fig. 2). Highly conserved Glu-18 is located in region I. Also in this region, both *Ae. albopictus* and *Cx. quinquefasciatus* have a substitution of Asn for Asp-11. Region II has aromatic amino acids (Trp-104, Trp-106, Tyr-105, Tyr-108, and Tyr-110) that are involved in electron transport (Capaldi et al. 1983). A similar substitution of Asn for Asp-112 in this region occurs in mosquitoes and has been identified in cytochrome *c* binding (Capaldi et al. 1983). The His-161 in region III is a copper-binding ligand (Capaldi et al. 1983). The residues Cys-196, Cyt-200, His-204, and Met-207 in region IV are the ligands for copper binding (Capaldi et al. 1983, Covello and Gray 1990). The Glu-198 in this region also binds cytochrome *c* (Millett et al. 1983). In addition to these conserved amino acids, Asn-158, Asn-173, and Glu-212 also are conserved in insects. These indicate that the functional domains of cytochrome *c* oxidase are preserved perfectly in the COII subunit of mosquitoes.

The number of transitions was less than that of transversions (44 vs. 65 for *An. sinensis* and *Cx. quinquefasciatus,* 47 vs. 59 for *An. sinensis* and *Ae. albopictus,* and 37 vs. 46 for *Ae. albopictus* and *Cx. quinquefasciatus*). In all transversions, the frequency of change between A and T was greater (83.1% for *An. sinensis* and *Cx. quinquefasciatus,* 81.4% for *An. sinensis* and *Ae. albopictus,* and 80.4% for *Ae. albopictus* and *Cx. quinquefasciatus*). In addition, if only the substitution at the 3rd position is considered, the transitions also were less than the transversions (16 vs. 46 for *An. sinensis* and *Cx. quinquefasciatus,* 18 vs. 41 for *An. sinensis* and *Ae. albopictus,* and 17 vs. 33 for *Ae. albopictus* and *Cx. quinquefasciatus*). This is different from comparisons of mtDNA between closely related mammalian species in which transitions occur more frequently than transversions (Brown 1985).

The number of synonymous substitutions was

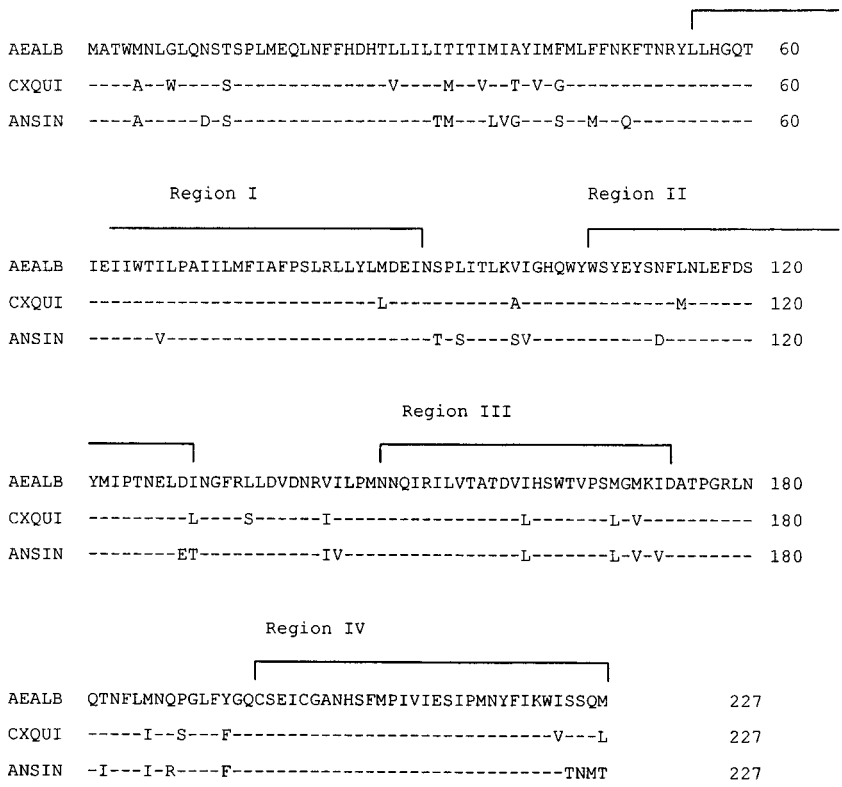

Fig. 2. Comparison of mitochondrial cytochrome oxidase II amino acid sequences of *Anopheles sinensis* (ANSIN), *Aedes albopictus* (AEALB), and *Culex quinquefasciatus* (CXQUI). Four regions with highly conserved amino acid sequences are marked. Regions I–IV contain residues 50–86, 101–128, 144–167, and 194–212, respectively.

Table 2. Interspecies divergences in cytochrome oxidase II gene nucleotides (above the diagonal) and deduced amino acid sequences (below the diagonal) in 7 insect species.

Species	1	2	3	4	5	6	7
1. *Drosophila yakuba*		20.0	19.1	19.5	19.2	19.4	18.9
2. *Anopheles quadrimaculatus*	19.3		9.1	11.7	16.7	18.1	17.1
3. *An. sinensis*	19.7	2.2		9.8	15.5	15.6	15.9
4. *An. gambiae*	18.4	3.5	3.9		16.5	16.8	16.5
5. *Aedes albopictus*	20.6	14.9	14.5	13.6		9.4	12.1
6. *Ae. australis*	21.9	14.5	13.2	13.2	8.3		12.1
7. *Culex quinquefasciatus*	22.4	15.4	14.9	14.5	10.5	9.2	

greater than that of nonsynonymous substitutions (51.4% for *An. sinensis* and *Cx. quinquefasciatus,* 54.7% for *An. sinensis* and *Ae. albopictus,* and 62.7% for *Ae. albopictus* and *Cx. quinquefasciatus*). Furthermore, the frequencies of replacement generated by transversions were higher than those generated by transitions (58.5% or 31 vs. 22 for *An. sinensis* and *Cx. quinquefasciatus,* 58.3% or 28 vs. 20 for *An. sinensis* and *Ae. albopictus,* and 61.3% or 19 vs. 12 for *Ae. albopictus* and *Cx. quinquefasciatus*). A difference of 3 nucleotides and 2 amino acids was found between 2 geographic populations of *Cx. quinquefasciatus* from the Chinese mainland and Taiwan (Ho et al. 1995)

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REFERENCES CITED

Beard CB, Hamm DM, Collins FH. 1993. The mitochondrial genome of the mosquito *Anopheles gambiae*: DNA sequence, genome organization, and comparisons

with mitochondrial sequences of other insects. *Insect Mol Biol* 2:103–124.

Bisson R, Jacobs B, Capaldi RA. 1980. Binding of arylazidocytochrome *c* derivatives to beef heart cytochrome *c* oxidase: cross-linking in the high and low affinity binding site. *Biochemistry* 19:4173–4178.

Brown GG, Simpson MV. 1982. Novel features of animal mtDNA evolution as shown by sequence of the rat cytochrome oxidase subunit II genes. *Proc Natl Acad Sci USA* 79:3246–3250.

Brown WM. 1985. The mitochondrial genome of animals. In MacIntyre RJ, ed. *Molecular evolution genetics* New York: Plenum. p 95–130.

Brust RA, Ballard JWO, Driver F, Hartley DM, Galway NJ, Curran J. 1998. Molecular systematics, morphological analysis, and hybrid crossing identify a third taxon, *Aedes (Halaedes) wardangensis* sp.nov., of the *Aedes (Halaedes) australis* species-group (Diptera: Culicidae). *Can J Zool* 76:1236–1246.

Busse G, Steffens GJ, Steffens GCM. 1978. Studies on cytochrome *c* oxidase. III. Relationship of cytochrome oxidase subunits to electron carriers of photophosphorylation. *Hoppe-Seyler's Z Physiol Chem* 359:1011–1013.

Cann RL, Brown WM, Wilson AC. 1984. Polymorphic sites and the mechanism of evolution in human mitochondrial DNA. *Genetics* 106:479–499.

Capaldi RA, Malatesta F, Darley-Usmar VM. 1983. Structure of cytochrome *c* oxidase. *Biochim Biophys Acta* 726:135–148.

Clary DO, Wolstenhome DR. 1983. Nucleotide sequence of q segment of *Drosophila* mitochondrial DNA that contains the genes for cytochrome *c* oxidase subunits II and III and ATPase subunit 6. *Nucleic Acids Res* 11: 4211–4227.

Clary DO, Wolstenhome DR. 1985. The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. *J Mol Evol* 22:252–271.

Cockburn AF, Mitchell SE, Seawright JA. 1990. Cloning of the mitochondrial genome of *Anopheles quadrimaculatus*. *Arch Insect Biochem Physiol* 14:31–36.

Cockburn AF, Seawright JA. 1988. Techniques for mitochondrial and ribosomal DNA analysis of anopheline mosquitoes. *J Am Mosq Control Assoc* 4:261–265.

Covello PS, Gray MW. 1990. Sequence and the nature of the Cu_A-binding site in cytochrome *c* oxidase. *FEBS Lett* 268:5–7.

Crozier RH, Crozier YC. 1993. The mitochondrial genome of the honeybee *Apis mellifera*: complete sequence and genome organization. *Genetics* 133:97–117.

de Bruijn MHL. 1983. *Drosophila melanogaster* mitochondrial DNA, a novel organization and genetic code. *Nature (Lond)* 304:234–241.

Haucke HR, Gellissen G. 1988. Different mitochondrial gene orders among insects: exchanged tRNA gene positions in the COII/COIII region between and orthopteran and a dipteran species. *Curr Genet* 14:471–476.

Ho CM, Liu YM, Wei YH, Hu ST. 1995. Gene for cytochrome *c* oxidase subunit II in the mitochondrial DNA of *Culex quinquefasciatus* and *Aedes aegypti*. *J Med Entomol* 32:174–180.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Vilablance FX, Wilson AC. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc Natl Acad Sci USA* 86:6196–6200.

Kumar S, Tamura K, Jakobson I, Nei M. 1993. *MEGA: molecular evolutionary genetics analysis* Version 1.02. University Park, PA: Pennsylvania State University.

Lee YFW, Liaw LL, Tsai TY, Wei YH, Lo SJ. 1989. Chicken mitochondrial cytochrome *c* oxidase subunit II: comparative analysis among the vertebrates. *Biochem Int* 19:889–898.

Liu H, Beckenbach A. 1992. Evolution of the mitochondrial cytochrome oxidase II gene among 10 orders of insects. *Mol Phylogenet Evol* 1:41–52.

Millett F, de Jong K, Paulson L, Capaldi RA. 1983. Identification of specific carboxylate groups on cytochrome *c* oxidase that is involved in binding cytochrome *c*. *Biochemistry* 22:546–552.

Mitchell SE, Cockburn AF, Seawright JA. 1993. The mitochondrial genome of *Anopheles quadrimaculatus* species A: complete nucleotide sequence and gene organization. *Genome* 36:1058–1073.

Pan YF, Lee YHW, Wei YH, Chiang AN. 1993. A gene for cytochrome *c* oxidase subunit II in duck mitochondrial DNA: structural features and sequence evolution. *Biochem Mol Biol Int* 30:479–489.

Sambrook J, Fritsch EF, Maniatis TM. 1989. *Molecular cloning: a laboratory manual* 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanger F, Nicklen S, Coulsin AR. 1977. DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* 74:5463–5467.

Thompson JD, Higgins DG, Gibson DJ. 1994. CLUSTALw: improving the sensitivity of progressive multiple sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680.

Wang J, Lu S, Shen H, Chen J. 1996. Analysis on the probability of high esterase activity and the tendency of organophosphate resistance in *Culex pipiens* populations. *Acta Parasitol Med Entomol Sin* 3:223–230.