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Cytochrome Subunit b and NADH Oxido-reductase Subunit I Genes of Mitochondrial Genome from the Eri Silkworm, *Samia cynthia ricini* and Phylogenetic Analysis

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Abstract **Mitochondrial DNA of eri silkworm, *Samia cynthia ricini* was prepared from newly-hatched larvae with the method described previously. After mtDNA and pBluescript SK(+) (Amp^r) vector were doubly digested by *Eco*R I and *Xba* I respectively, a 3.3-kb restriction fragment was cloned and screened out. It contains partial 16S-rRNA gene, complete genes of tRNA^{Leu} (UUA), NADH oxido-reductase subunit I (nd-1), tRNA^{Ser} (AGU), cytochrome subunit b (*cytb*) and partial NADH oxido-reductase subunit VI (*nd-6*) gene orderly. The sequences of *cytb* and *nd-1* of *S. c. ricini* were compared with those of nine published species of *Insecta* and a phylogenetic tree was constructed based on their sequences. The results indicated that *S. c. ricini* has closest evolutionary relationship with *Bombyx mori* and *B. mandarina*. Meanwhile the possible secondary structures of tRNA^{Leu} (UUA) and tRNA^{Ser} (AGU) of *S. c. ricini* were inferred from their sequences.**

Key words : cytochrome subunit b, NADH oxido-reductase subunit I, mitochondrial DNA, eri silkworm, *Samia cynthia ricini*

Introduction

Mitochondrial genomes of animal contain thirteen polypeptide-encoding genes including cytochrome subunit b gene (*cytb*), three cytochrome oxidase subunit genes (*cox-1*, *cox-2* and *cox-3*), seven NADH oxido-reductase subunit genes (*nd-1*, *nd-2*, *nd-3*, *nd-4*, *nd-4L*, *nd-5* and *nd-6*) and ATP synthase subunit VI and VIII genes (*atp-6* and *atp-8*). Their expression products are components of oxidative phosphorylation system on mitochondrial inner membranes and join in the process of energy metabolism. They are, therefore, significant to cells and organisms (Dey *et al*, 2000). In these thirteen genes, *cytb*, *cox-1*, *cox-2* and *cox-3* are the most conservative ones with about 80% of nucleotide sequence similarity among different animal species. They are useful for studying molecular evolution and classification of species (Garcia-Machodo *et al*., 1999; Gaunt *et al*., 2002). Studies of molecular evolution have been carried out on the basis of nucleotide sequences of these

conservative genes (Lo Galbo *et al.*, 2002; Prychitko *et al.*, 2000; Howell, 1989; Bernasconi *et al.*, 2000; Koulianos *et al.*, 2000; Miya *et al.*, 2000; Wei *et al.*, 2002).

The eri silkworm, *S. c. ricini*, is one of important industrial insects living mainly on leaves of castor-oil plant, *Ricinus comunis* and economically significant as *Antheraea pernyi*. Many researches have been performed on domestication and improvement of breeds. The *S. c. ricini* has relatively close relationship with *B. mori*, so molecular studies on mitochondrial genome of *S. c. ricini* are expected to provide us insights on evolution of both *B. mori* and *S. c ricini*. Recently, a primary restriction endonuclease map has been drawn (Ling *et al.*, 1993). Further, the *cox3*, 12S-rRNA and a few other genes were published or registered (Genbank accession No.: AF288145, AY037829). In this research, as the first time we reported the sequences of *cytb* of *S. c. ricini* and from which the evolutionary relationship with other insect species was discussed.

Materials and Methods

Eri silkworm and reagents

The eri silkworms for mtDNA preparation is named as Hualan preserved in our laboratory. The cloning vector pBluescript SK(+) (Amp^r) and host bacterial *E. coli* JM109 are also housekeeping. Restriction endonucleases and T4 DNA ligase were purchased from Gibco-BRL Ltd. The sequences of mitochondria genes of eri silkworm *S. c. ricini* were determined from our research and registered into Genbank (Accession No.: AF527774). The other data for phylogenetic research were retrieved from the GenBank.

Cloning and screening

MtDNA was extracted from newly-hatched larvae of eri silkworm with the method described previously (Tamura *et al.*, 1988; Shen *et al.*, 2000). Before cloning, both mtDNA and cloning vector were doubly digested with *EcoR* I and *Xba* I, respectively. And the large fragment of vector was obtained by agarose gel electrophoresis segregation. Then they were ligated by T4 DNA ligase at 14~16 ℃ and the resultant product was transformed into *E. coli* JM109 (Sambrook *et al.*, 1989). Bacterial colonies were cultured in LB medium with ampicillin and screened with the method as described previously (Beuken *et al.*, 1998).

Sequencing and analysis

Primer-walking method was employed in sequencing of the recombinant nucleotide. By comparison and online retrieve of Genbank, the order of genes in the fragment of *S. c. ricini* mtDNA have been determined. The gene structure and phylogenetic analyses were carried out with Dnastar software.

Results and Discussion

Identification of 3.3 kb fragment of mtDNA of eri silkworm

By analyzing plasmid DNAs with endonulease digestion and electrophoresis, finally one recombinant DNA was found. When the recombinant DNA digested by *EcoR* I or *Xba* I, respectively, only one band about 6.3 kb was observed. But, when it doubly digested by *EcoR* I and *Xba* I, there were two bands, about 3 kb of the vector DNA and about 3.3 kb of an insert DNA. Sequence analysis confirmed that the 3.3 kb insert was a fragment of eri silkworm mtDNA.

Gene structure analysis

The cloned fragment was 3296 bp long (Fig. 1). By comparison with genes of mitochondrial genome of other species of Insecta such as *B. mori* and *Drosophila*, we deduced genes and their order in the sequence as follows: ① From the 1st to 723rd nt is one-second sequence of 16S-rRNA (the 3' end part) ending by AGA; ②From the 726th to 798th nt is $tRNA^{Leu}$ (UUA); ③From the 789th to 1718th nt is NADH oxsidoreductase subunit 1 (*Scrnd-1*); ④From the 1719th to 1784th nt is the complementary sequence of $tRNA^{Ser}$ (AGU); ⑤From the 1814th to 2962nd nt is the complementary sequence of cytochrome oxidease b (*Scrcytb*); ⑥From the 2969th to 3296th nt is the complementary sequence of the 3' end part of *nd-6* (*Scrnd-6*). The $tRNA^{Leu}$ and *Scrnd-1* are overlapped for 9 nts, while short intervening sequences exist

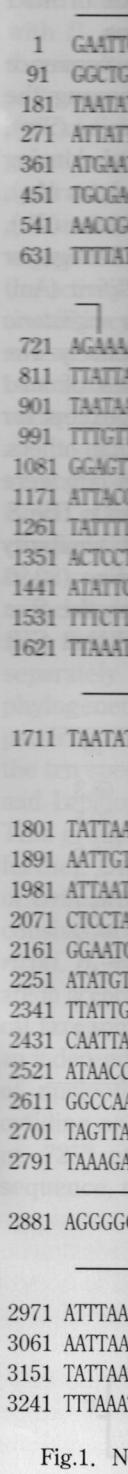


Fig.1. N

Fig.1. Nucleotide sequence of 3.3 kb fragment from *EcoR* I - *Xba* I doubly digested mtDNA of *S. c. ricini*

between other adjoining genes in this fragment.

Scrnd-1 is 930 nts encoding 309 amino acids, which is 15 nts shorter than that of *B. mori* (*Bmnd-1*). Its initiation and stop codon are ATA and TAA, respectively, the same as that of *B. mori*. The percentage of A+T is 76.56 similar to those of other insect species listed in Table 1.

Scrcytb is 1149 nts encoding 382 amino acids, which is 3 nts shorter than that of B. mori (*Bmcytb*). Both *Scrcytb* and *Bmcytb* are initiated with ATA and stopped with TAA.

There are two tRNA genes at the flanking of *nd-1* in the cloned fragment. One is $tRNA^{Leu}$ (UUA) and the other is $tRNA^{Ser}$ (AGU). They are 72 nts and 66 nts respectively. Their secondary structures were deduced according to their nucleotide sequences (Fig. 2a, 2b). The diagrams showed that $tRNA^{Leu}$ (UUA) is stable. The $tRNA^{Ser}$ (AGU) base pairs are however highly mis-paired especially in the D-loop indicated its unstable structure.

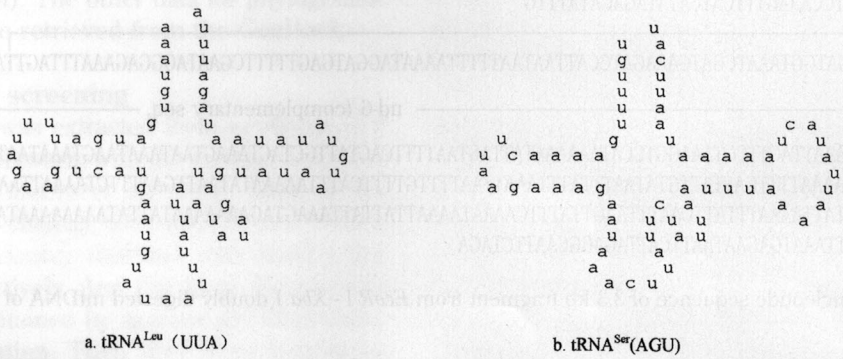
Comparison of nucleotide sequence of *nd-1* and *cytb* and phylogenetic analyses

The comparison of nucleotide sequence of *nd-1* and *cytb* were carried out among the following 10 insects species: *S. c. ricini* (Scr), *B. mandarina* (Bma) and *B. mori* (Bm) belonging to Lepidoptera, *Anopheles quadrimaculatus* (Aq), *A. gambiae* (Ag), *Cochilomyia hominivorax* (Ch), *Drosophila yakuba* (Dy) and *D. melenogaster* (Dm) belonging to Diptera, *Apis mellifera* (Am) belonging to Hymenoptera and *Locusta migratoria* (Lm) belonging to Orthoptera, respectively. The results indicated that the similarities of *nd-1* and *cytb* ranged from 66.9% to 97.5% except for *A. mellifera*, which was much lower than others. The average similarities of *nd-1* and *cytb* were 71.8% and 71.3%, respectively (Table 1). The *S. c. ricini nd-1* showed the highest similarity with that of *B. mori* and *B. mandarina* (80.9% and 80.7%, respectively), and so does the *S. c. ricini cytb* (77.2%, both). It indicated that *S. c.*

Table 1. Comparison of homologies of *nd-1* and *cytb* from mtDNAs of 10 insect species

%	1	2	3	4	5	6	7	8	9	10
1. Scr	/	71.1	57.5	68.9	80.7	80.9	71.9	70.7	71.6	68.3
2. Ag	71.9	/	57.3	89.2	69.7	68.6	72.7	73.9	74.8	70.5
3. Am	55.1	57.4	/	57.3	60.1	59.2	54.1	57.4	57.7	56.6
4. Aq	72.8	90.4	57.1	/	71.0	69.7	75.5	75.8	77.7	71.2
5. Bma	77.2	74.2	58.0	74.9	/	95.0	70.5	72.0	73.5	72.1
6. Bm	77.2	75.0	58.2	75.1	97.5	/	70.3	71.2	72.9	71.4
7. Ch	71.7	77.1	54.8	78.7	70.8	70.7	/	81.4	82.9	74.1
8. Dm	71.9	80.2	56.6	79.3	72.9	72.9	80.0	/	91.0	73.3
9. Dy	72.8	79.8	57.2	79.7	71.9	71.7	81.5	93.2	/	74.1
10. Lm	65.7	72.1	53.5	72.7	69.0	70.0	69.0	71.2	71.0	/

Note: Above the diagonal are homologies of *nd-1* genes and below are homologies of *cytb* genes.



a. $tRNA^{Leu}$ (UUA)　　b. $tRNA^{Ser}$(AGU)

Fig. 2 The deduced secondary structures of $tRNA^{Leu}$ and $tRNA^{Ser}$ of *S. c. ricini* mtDNA

ricini has a close evolutionary relationship with *B. mori* and *B. mandarina*. According to the results of sequences alignment by Dnastar software, we recognized that differences of *cytb* and *nd-1* among these species increased greatly at both ends of their sequences. In contrast, in the middle parts, about 1100 nts in *cytb* and 880 nts in *nd-1*, were conservative. It suggested that the middle parts are related to the activity domain of their expression products.

The *cytb*, *cox-1*, *cox-2* and *cox-3* are the most conservative genes in mitochondrial genomes of animals and followed by *nd-1*, *atp-6*, *atp-8*, control region and so on. So *cytb* and *cox* genes are useful for studying molecular evolution among species (Howell 1989; Zardoya 1996; Lo Galbo *et al*, 2002; Gaunt *et al*., 2002). Here, a comparative phylogenetic tree is constructed from combined sequences of *cytb* and *nd-1* in mitochondrial genomes from ten insect species (Fig. 3). When we used *cytb* and *nd-1* sequences separately and combined for construction of phylogenetic trees, they showed the similar patterns (data not shown). In this tree, nine of the ten species belonging to Orthopetra, Diptera and Lepidoptera are distributed into one group. This group can be divided into two sub-groups further. One sub-group consists of six species of *Orthopetra* and *Diptera*. The other sub-group consists of three Lepidopteran species, i. e. *S. c. ricini*, *B. mori* and *B. mandarina*. These results are in accordance with that of classical morphological taxonomy. Exceptionally, *A. mellifera* formed an independent clade in this tree. The comparison of nucleotide sequences showed that single cytidine or adenosine was inserted at the 479th nt, 725th nt and 754th nt of *A. mellifera nd-1* sequence, respectively.

Moreover, at the 104th nt, 110th nt, 114th nt, 502nd nt, 773rd nt and 848th nt sites, the absence of short sequences resulted in the movement of codon and led to lower similarities with other species. It implicated that these mutated sites are not the activity domain of *nd-1*. Similarly, at the 726th nt of *A. mellifera cytb* a single adenosine was inserted. The results indicated that the base replacements had happened much frequently on *A. mellifera* than those on the other species because of its specific reproductive style (David *et al*., 1994). A swarm of *A. mellifera* usually only has one queen who bears the task of producing offsprings. Once a genetic variation happens, it is easy to be fixed in the swarm and strictly follow the maternal inheritance. Their base replacement is about five to ten times as that of nuclear genome, i.e. their evolution rates are five to ten times as that of nuclear genome.

Mitochondrial genomes are an important model system for studying molecular evolution of the eukaryote (Clary *et al*., 1985; David *et al*., 1994). Up to the date, many phylogenetic trees have been constructed based on nucleotide sequences or deduced amino acid sequences of mitochondrial genes. However, the results of these molecular evolutionary analyses varied when different genes were analyzed, and often conflict to that of classical morphological taxonomy (Boore *et al*., 1998; Wilson *et al*., 2000). Thus, more comprehensive studies should be done to understand molecular evolution of the insect mtDNA.

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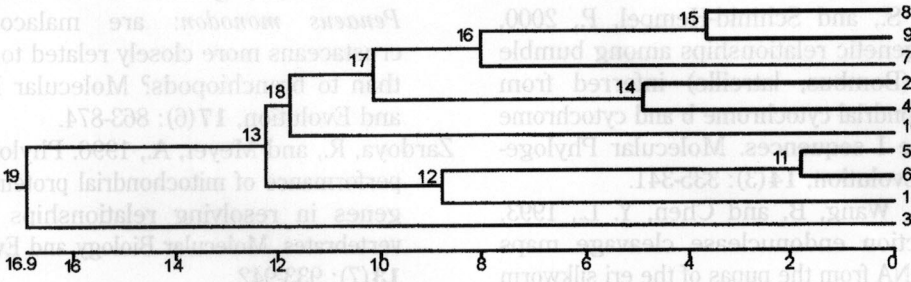

Fig. 3 Phylogenetic tree based on combined sequences of mitochondrial *nd-1* and *cytb* from ten insect species

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