The Comparative Molecular Study between Bombycidae and Saturniidae Based on mtDNA RFLP and Cytochrome Oxidase I Gene Sequences: Implication for Molecular Evolution

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The phylogenetic relationships between *Bombyx mori* and *Bombyx mandarina* species of Bombycidae, and *Antheraea yamamai* and *Antheraea pernyi* species of Saturniidae were investigated based on mtDNA RFLP and cytochrome oxidase I gene. The sizes of the mtDNA of all the species were estimated at approximately 16 kbp ± 500 bp by total length of all the restricted fragments and no variation in size was recognized. Of the fourteen different restriction endonucleases used, *Bam*HI, *Hin*dIII, *Pst*I, *Eco*RI and *Xba*I showed RFLP. Among these, only *Hin*dIII showed RFLP between *B. mori* and *B. mandarina.* A comparative analysis of sequences was also conducted with the mitochondrial cytochrome oxidase I genes of each species. The results indicated that *B. mori* shared a 97%, 85% and 87% sequence identity with *B. mandarina*, *A. yamamai* and *A. pernyi*, respectively. *B. mandarina* shared a 87% and 88% sequence identity with *A. yamamai* and *A. penyi*, respectively. *A. yamamai* shared 92% sequence identity with *A. pernyi*. The results of the phylogenetic analysis exhibited monophyly and confidence limits of more than 99% in all trees for both Bombycidae and Saturniidae.

Introduction

The analysis of mitochondrial DNA (mtDNA) sequence is a powerful tool for understanding insect population genetics and phylogenetics, since the small size of the mtDNA, the relatively rapid rate of its evolutionary change, and maternal inheritance all make it suitable for examining population background and evolution among closely related taxa (Moritz *et al.,* 1987; Gray, 1989; Lansman *et al.,* 1981). Recently, RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), mtDNA sequence and the sequence variation of target genes have been used in the molecular phylogenetics of insects.

Among insects, complete mtDNA sequences only exist for four insects: *Drosophila yakuba* (Clary and Wolstenholme, 1985), *Apis mellifera* (Crozier and Crozier, 1993), *Anopheles quadrimaculatus* (Mitchell *et al.,* 1993), *Anopheles gambiae* (Beard *et al.,* 1993). It is generally recognized known that insect mtDNA consists of two ribosomal RNA genes, twenty-two transfer RNA genes and thirteen protein coding genes of the mitochondrial inner membrane respitory complexes (Clary and Wolstenholme, 1985; Crozier and Crozier, 1993; Beard *et al.,* 1993). Further studies on mtDNA variation have also enhanced our understanding of population and evolutionary biology at both the intraspecific and interspecific levels. The PCR revolution and discovery of universal primers for mtDNA that work in a number of different species (Simon *et al.,* 1994; Lunt *et al.,* 1996; Kambhampati and Smith, 1995; Roehrdanz, 1995) have greatly increased the speed at which data can be obtained.

Silkmoths are holometabolous insects belonging to either the family Bombycidae or Saturniidae, both of which belong to the superfamily Bombycoidea. Bombycidae, *Bombix mori* and *Bombix mandarina,* live on mulberry leaves and make

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higher quality silk fiber than Saturniidae, *Antheraea yamamai* and *Antheraea pernyin*, which live on leaves of oak tree. *B. mori* and *B. mandarina* can be crossed, and the resulting hybrid progeny shows normal fertility (Kawaguchi, 1928; Aratake *et al.*, 1973). The chromosome numbers are quite different between *A. yamamai* (31) and *A. pernyin* (49), but they can be closed to produce F1 progeny (Kawaguchi, 1934; Shimada *et al.*, 1988) which, however, are sterile due to incomplete oogenesis and other abnormalities (Shimada and Kobayashi, 1992; Kirimura, 1962). It is prerequisited to clarifying the phylogentic relationships between Bombycidae or Saturniidae, with respect to understand physiological differences under molecular level and to create genetically transformed silkworms which may produce high quality amount of silk fiber using molecular breeding techniques. With in this mind, here, we have investigated the construction of mtDNA RFLP and sequenced the mitochondrial cytochrome oxidase I gene in *B. mori, B. mandarina, A. yamamai* and *A. pernyi* to understand the phylogenetic relationship between these species of Bombycidae and Saturniidae.

Materials and Methods

The preparation of mtDNA from silkgland was purified as described by Tamura and Aotsuka (1988). One gram of silkgland was homogenized in a 10 ml chilled homogenizing buffer [0.25 M sucrose, 10 mM EDTA, 30 mM Tris-HCl, (pH 7.5)], and centrifuged at 1,000×*g* for 1 min at 4 C°. The supernatant was recentrifuged at 12,000×*g* for 10 min at 4 C°. The resulting pellet of mitochondria was suspended in a 500 μl of 10 mM Tris-EDTA buffer (pH 8.0) containing 0.15 M NaCl, 0.18 N NaOH and 1% SDS. After phenol-chloroform (1:1) and ethanol extraction, the mtDNA was collected at 12,000×*g* for 15 min at 4 C°. The resulting mtDNA was rinsed twice with 70% cold ethanol and digested with 20 μg/ml of DNase-free RNase in a 20 μl of 10 mM Tris-EDTA buffer (pH 8.0) at 37 C° for 30 min. The mtDNAs were digested with 14 kind of restriction enzymes at 37 C° for 2 h; *Bam*HI, *Hin*dIII, *Xho*I, *Sal*I, *Sma*I, *Pst*I, *Eco*RI, *Xba*I, *Eco*RV, *Dra*I, *Kpn*I, *Sca*I, *Bgl*I, *Bgl*II. Digested DNA fragments were separated on the 1% agarose gels in TBE buffer, the gels were stained with ethidium bromide solution and were photographed using Polaroid type 667 films on a UV transilluminator.

A PCR was set up in a 50 μl volume as described by Kambhampati *et al.* (1992) in a GeneAmp PCR system 9700 (Perkin-Elmer cetus Co). Ten ng of each mtDNA, *B. mori, B. mandarina, A. yamamai* and *A. pernyin*, was used as a PCR template DNA. The primers for cytochrome oxidase I gene (Cy1–1,632; 5′-TGATCAAATTTATAAT-3′ and Cy2–2,191; 5′-GGTAAAATTAAAATATAAACTTC-3′) were derived from the already known mtDNA sequences of *D. yakuba* (Clary and Wolstenholme, 1985), *A. mellifera* (Crozier and Crozier, 1993), and *A. quadrimacutatus* (Mitchell *et al.*, 1993) and used 5 pmol in this experiments. The temperature profile for the amplification was 95 C° for 30 sec, 50 C° for 1 min, then 72 C° for 1 min for 35 cycles, and a final extension step of 72 C° for 5 min was added. The PCR products were separated on a 1% agarose gel, and the bands of the expected size were recovered using a Gene clean II Kit (Bio 101, USA). The eluted DNA fragments were directly ligated with a pGemT Easy vector (Promega, USA) and transformed into *E. coli* JM 109. The recombinant plasmids were isolated according to procedures prescribed by the Wizard SV plasmid purification Kit (Promega, USA).

Fluorescent cycle sequencing was performed with 300–500 ng of this double stranded DNA in a 20 μl sequencing reaction mixture and prepared using an ABI PRISM Dye terminator Cycle Sequencing Ready Reaction Kit. Three clones, both orientations, were examined for each sequence.

The genetic similarity coefficient was calculated from $F = 2n_{xy}/(n_x+n_y)$ in which n_x and n_y are the number of fragments in species X and Y, respectively, where n_{xy} is the number of fragments shared by the two species (Nei and Li, 1979). Computer analyses of mtDNA sequences were carried out using the CLUSTAL W program (Thompson *et al.*, 1994). Phylogenetic trees for the data set were inferred by using the neighbor-joining program of MEGA (Saitou and Nei, 1987; Kumar *et al.*, 1993). The stability of the relationships was assessed by performing bootstrap analyses of the neighbor-joining data based on 1,000 resampling.

Results and Discussion

Silkworms, in addition to being important targets for improvement by genetic engineering, provide chances to research unique biological aspects, including developmental pattern formation and tissue specific expression. To investigate these phenomena and to genetically engineer silkworms showing better characteristics, comparison of silkworms both dominant, Bombycidae (*Bombix mori* and *Bombix mandarina)* and wild type, Saturniidae (*Antheraea yamamai* and *Antheraea pernyin)* at a molecular level is prerequisited, since little information is still available on the base of molecular level.

As described above, it is important to establish the phylogentic relationships between Bombycidae or Saturniidae on the molecular level to understand a silkworm physiology and to create a transgenic silkworm may produce a lot of useable biomatters including silk and drugs. With in this mind, here, we have established the molecular relationship of *B. mori*, *B. mandarina* and *A. yamamai*, *A. pernyi* based on those mtDNA RFLP data and mitochondrial cytochrome oxidase I gene sequences partially amplificated by PCR.

Phylogenetic relationships among the *B. mori* and *B. mandarina* species of Bombycidae and *A. yamamai* and the *A. pernyi* species of Saturniidae were investigated based on mtDNA RFLP and the cytochrome oxidase I gene. The mtDNA sizes of all the species tested were estimated at approximately 16 kb ± 500 bp in length, in which the nonvariation of sizes and restriction enzyme sites were observed as shown in Figs 1A and 1B. The resulting genomic sizes are similar to those previously

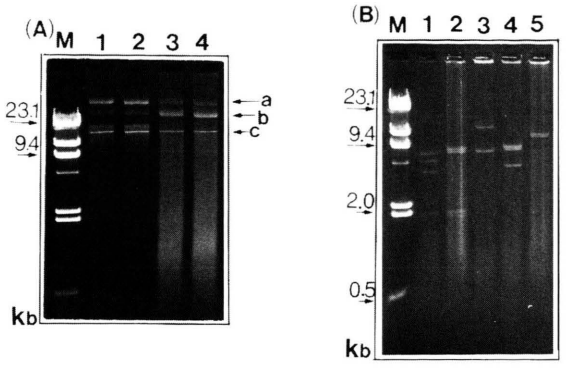

Fig. 1. Determination of mitochondrial genome sizes of silkmoth.
(A) Uncut form of mtDNAs purified from silkmoths, separated on a 1% agarose gel. M: molecular marker of λ-*Hin*d III, Lane 1: *Bombyx mori*, Lane 2: *Bombyx mandarina, Lane 3: Antheraea yamamai, Lane 4: Antheraea pernyi.* Arrows a: mtDNA nicked circular form, b: super coiled form, c: linear form.
(B) Electrophoretic patterns of *B. mori* mtDNA digested with five restriction endonucleases, separated on a 1% agarose gel. M: molecular marker of λ-*Hin*d III. Lane 1: *Hin*d III, 2: *Eco*R I, 3: *Bam*H I, 4: *Xba* I, 5: *Pst* I.

Table I. Numbers of restriction sites on the mtDNAs of the major silkmoths.

	BamHI*	*Hin*dIII*	*Xho*I	*Sal*I	*Sma*I	*Pst*I*	*Eco*RI*	*Xba*I*	*Eco*RV	*Dra*I	*Kpn*I	*Sca*I	*Bgl*I	*Bgl*II
B. mori(Jam305)	2	4	-	1	1	2	4	3	1	7	-	-	-	2
B. mori(Jam306)	2	4	-	1	1	2	4	3	1	7	-	-	-	2
B. madarina	2	2	-	1	1	2	4	3	1	7	-	-	-	2
A. yamamai	1	4	-	1	1	2	3	1	1	7	-	-	-	2
A. pernyi	1	4	-	1	1	3	3	3	1	7	-	-	-	2

-: No restriction sites. *: Restriction endonuclease showing RFLPs among the five silkmoth. Jam305: Japonic type. Jam306: Chinese type.

Table II. Genetic similarity values among silkmoths, based on the RFLP markers of mtDNA.

	B. mori(Jam305)	*B. mori*(Jam306)	*B. mandarina*	*A. yamamai*	*A. periny*
B. mori(Jam305)	1.000				
B. mori(Jam306)	1.000	1.000			
B. mandarina	0.848	0.848	1.000		
A. yamamai	0.303	0.302	0.394	1.000	
A. periny	0.227	0.228	0.348	0.500	1.000

Jam305: Japonic type; Jam306: Chinese type.

reported for *A. gambiae* (15,363 bp), *A. quadrimaculatus* (15,455 bp), and *D. yakuba* (16,019 bp), however, they are slightly smaller than that for *D. melanogaster* (19,517 bp). Of the fourteen different restriction endonucleases used, *BamH*I, *Hin*dIII, *Pst*I, *Eco*RI and *Xba*I showed RFLP (Table I and Fig. 1B). A variation in the enzyme-digested fragments between *B. mori* strains, Japanese (Jam305) and Chinese (Jam306), was not detected (Table I). A matrix for genetic similarity was constructed based on sixty eight mtDNA RFLP markers obtained from the two families di-

Fig. 2. Alignment of PCR amplified nucleotide sequences of a part (ca. 600 bp) of the cytochrome oxidase I gene. The origins of the amplified insects are indicated to the left. Asterisks (*) indicate nucleotides common to all species. Bmocy: *Bombyx mori*, Bmacy: *Bombyx mandarina*, Aycy: *Antheraea yamamai*, Apcy: *Antheraea pernyi*.

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Bmocy   TGATCAAATTTATAATACTATTGTAACAGCACATGCTTTTATTATAATTTTTTTTATAGT 60
Bmacy   TGATCAAATTTATAATACTATTGTAACAGCACATGCTTTTATTATAATTTTTTTTATAGT 60
Aycy    TGATCAAATTTATAATACTATTGTAACAGCTCATGCTTTTATTATAATTTTTTTTATAGT 60
Apcy    TGATCAAATTTATAATACTATTGTAACAGCTCATGCTTTTATTATAATTTTTTTCATAGT 60
        ****************************** ********************** ***** 60

Bmocy   TATACCTATTATAATTGGAGGATTTGGAAATTGATTAGTTCCTCTTATACTAGGAGCACC 120
Bmacy   TATACCTATTATAATTGGAGGATTTGGAAATTGATTAGTTCCTCTTATACTAGGAGCACC 120
Aycy    TATACCTATTATAATTGGAGGATTTGGAAATTGACTAATTCCGTTAATATTAGGAGCTCC 120
Apcy    TATACCTATCATAATTGGAGGATTTGGAAATTGATTAATTCCATTAATATTAGGAGCCCC 120
        ********  ************************ ** ****  * ***  ******* ** 120

Bmocy   AGATATAGCATTCCCACGAATAAATAATATAAGATTTTGACTCCTACCCCCCTCCCTTAT 180
Bmacy   AGATATAGCATTCCCACGAATAAGTAATATAAGATTTTGACTCCTACCCCCCTCCCTTAT 180
Aycy    TGATATAGCTTTCCCACGAATAAATAATATAANTTTTTGATTGCTCCCCCCTTCTTTAAC 180
Apcy    TGATATAGCTTTCCCACGAATAAATAATATAAGTTTTGGACTATTACCCCCCTCTTTAAC 180
        ******** ************* ********  *** ** *  * ***** **  * * 180

Bmocy   ATTATTAATTTCAAGAAGAATTGTAGAAAATGGTGCAGGAACAGGATGAACAGTTTACCC 240
Bmacy   ATTATTAATTTCAAGAAGAATTGTAGAAAATGGTGCAGGAACAGGATGAACAGTTTACCC 240
Aycy    TTTATTAATTTCTAGAAGAATTGTAGAAAATGGAGCTGGAACTGGATGAACAGTTTATCC 240
Apcy    CCTATTAATCTCCAGAAGAATTGTAGAAAATGGAGCTGGAACTGGATGAACAGTTTACCC 240
        ******* ** ********************** **  ***** **************  ** 240

Bmocy   CCCACTTTCATCTAATATCGCACATAGAGGAAGATCCGTAGATCTTGCTATTTTTTCACT 300
Bmacy   CCCACTTTCATCTAATATTGCACATAGAGGAAGATCCGTAGATCTTGCTATTTTTTCACT 300
Aycy    CCCCCTTTCTTCAAATATTGCTCATGGAGGCTCTTCAGTAAATCTTGCTATTTTTTCATT 300
Apcy    CCCTCTCTCTTCAAATATTGCTCATGGAGGATCTTCAGTAGATCTTGCTATTTTTTCCCT 300
        *** ** ** ** ***** ** *** ****     ** *** ***************  * 300
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Bmocy   ACATTTAGCAGGTATTTCATCAATTATAGGAGCAATTAATTTTATTACAACAATAATTAA 360
Bmacy   ACATTTAGCAGGTATTTCATCAATTATAGGAGCAATTAATTTTATTACAACAATAATTAA 360
Aycy    ACATCTTGCAGGTATTTCTTCAATTTTAGGAGCAATTAATTTTATTACTACAATTATTAA 360
Apcy    TCATCTTGCAGGTATTTCGTCAATTTTAGGAGCAATTAATTTTATTACTACAATTATTAA 360
        *** * ********** ****** ********************** * ** ** **** 360

Bmocy   TATACGATTAAATAATATATCATTTGATCAATTACCCTTATTTGTATGAGCTGTATCGGA 420
Bmacy   TATGCGATTAAATAATATATCATTTGATCAATTACCCTTATTTGTATGAGCTGTAG-GAA 420
Aycy    TATACAAATAAATAATTTATCATTTGATCAAATACCTTTATTTGTTTGAGCTGTCG-GAA 420
Apcy    TATACGAATAAATAATTTATCATTTGATCAAATACCTTTATTTGTCTGAGCTGTTG-GAA 420
        *** * * ******** ************** **** ******** ********  * * 420

Bmocy   TTACAGCATTTT-ATTATTATTATCACTACCCGTTTTAGCTGGAGCTATTACAATATTAT 480
Bmacy   TTACAGCATTTTTATTATTATTATCACTACCTGTTT-AGCTGGAGCTATTACAATATTAT 480
Aycy    TTACAGCCTTTTTACTTCTTTTATCATTACCTGCTTTAGCTGGAGCTATTACAATATTAC 480
Apcy    TTACAGCTTTCTTACTTCTTTTATCATTACCTGTTTTAGCTGGAGCTATTACTATACTTT 480
        ******* ** * * *  * ****** **** * ** ********** *** *  480

Bmocy   TAACAGATCGAGACTTAAATACATCATTTTTTGATCCTGCTGGAGGAGGAGACCCAATTT 540
Bmacy   TAACAGATCGAAACTTAAATACATCATTTTTTGATCCTGCTGGAGGAGGAGACCCAATTT 540
Aycy    TAACAGATCGAAATTTAAATACTTCTTTCTTTGATCCTGCTGGAGGGGGAGATCCTATTC 540
Apcy    TAACAGATCGAAACTTAAATACTTCTTTTTTTGATCCTGCTGGTGGAGGAGATCCAAATT 540
        *********** * ******** ** ** ************** ** ***** ** * *  540

Bmocy   TATATCAACATTTATTTTGAATTTTTGGACATCCTGAAGTTTATATTTTAATTTTACC 598
Bmacy   TATATCAAC-TTTATTTTGATTTTTTGGGCATCCTGAAGTTTATATTTTAATTTTACC 598
Aycy    TTTATCAACATTTATTTTGATTTTTTGGTCACCCTGAAGTTTATATTTTAATTTTACC 598
Apcy    TATATCAACATTTATTTTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTTTACC 598
        * ******* ********** ******* ** ** ********************** 598
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gested with the fourteen restriction endonucleases by using the equation of Nei and Li (1979). The genetic similarity coefficients varied from 0.227 to 1.000 with an average of 0.498. *B. mori* (Jam305) shared a 0.848, 0.303 and 0.227 genetic similarity coefficient with *B. mandarina*, *A. yamamai*, *A. pernyi*, respectively. *B. manadarina* shared a 0.394 and 0.348 genetic similarity coefficient with *A. yamamai* and *A. pernyi*, respectively, and the genetic similarity coefficient between *A. yamamai* and *A. pernyi* was 0.500 (Table II). As a result, the genetic similarity coefficient between *B. mori* and *B. mandarina* from the four silkmoths was determined at 0.848 which indicates a highly homogeneous genetic background.

The nucleotide sequence homology of a mitochondrial cytochrome oxidase I gene from the four silkmoths was investigated using PCR. The resulting PCR products of the cytochrome oxidase I gene were about 600 bp corresponding to the position of 1,617~2,213 in the cytochrome oxidase I gene of the *D. yakuba* (Clary and Wolstenholme, 1985). The sequences of the four amplified fragments were aligned using the CLUSTAL W program as shown in Fig. 2. As a result, *B. mori* shared a 97%, 85% and 87% identity with *B. mandarina*, *A. yamamai* and *A. pernyi*, respectively. *B. mandarina* shared a 87% and 88% identity with both *A. yamamai* and *A. penyi*, and *A. yamamai* shared a 92% identity with *A. pernyi*. These sequences were compared with four insect species previously reported on (Table III). These sequences were also used for a phylogenetic analysis. All the trees showed monophyly for both Bombycidae and Saturniidae (Fig. 3). The confidence limits for the monophyly of these trees were estimated using bootstrapping tests. The confidence limits for both Bombycidae and Saturniidae were more than 99% for all trees.

Until now, many studies have examined the relationships between Bombycidae and Saturniidae. *B. mori* and *B. mandarina* can be crossed, and the resulting hybrid progeny shows normal fertility (Kawaguchi, 1928; Aratake *et al.*, 1973). Studies of the fibroin gene (Kusuda *et al.*, 1986), ribosome gene (Makawa *et al.*, 1988), the immunological properties of arylphorin (Shimada *et al.*, 1992) and the arylphorin gene (Shimada *et al.*, 1995), all suggest close genetic relationships between *B. mori* and *B. mandarina*. Although their chromosome numbers are quite different (*yamamai*=31, *pernyi*=

Table III. Levels of similarity based on cytochrome oxidase I gene sequences in silkmoths and other insects.

Organisms	% Cytochrome oxidase I gene sequence similarity							
	1	2	3	4	5	6	7	8
Bombyx mori	100							
Bombyx mandarina	97	100						
Antheraea yamamai	85	87	100					
Antheraea pernyi	87	88	92	100				
Drosophila yakuba	80	81	82	82	100			
Drosophila melanogaster	82	83	82	82	91	100		
Anopheles gambiae	82	82	82	82	86	87	100	
Apis mellifera	76	78	75	75	75	75	76	100

1. *Bombyx mori*, 2. *Bombyx mandarina*, 3. *Antheraea yamamai*, 4. *Antheraea pernyi*, 5. *Drosophila yakuba*, 6. *Drosophila melanogaster*, 7. *Anopheles gambiae*, 8. *Apis mellifera*.

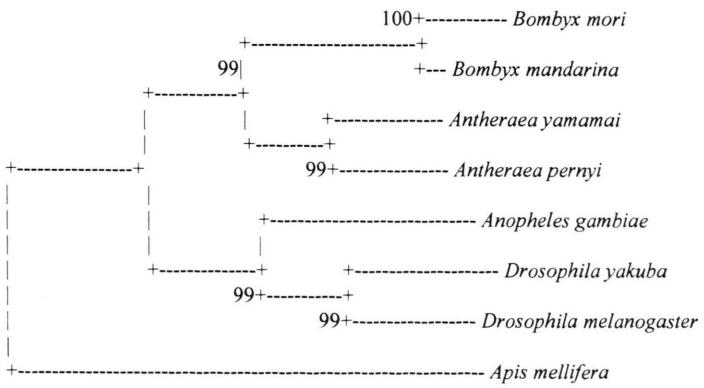

Fig. 3. Dendrogram of the phylogenetic relationships in silkmoths and other insects based on mtDNA cytochrome oxidase I sequence data.

49), *A. yamamai* and *A. pernyi* can be closed to produce F1 progeny (Kawaguchi, 1934; Shimada *et al.,* 1988). The progeny, however, are sterile due to incomplete oogenesis and other abnomalities (Shimada and Kobayashi, 1992). Kirimura (1962) showed that the fibroin of *A. yamamai* and *A. pernyi* are clearly similar. It is generally known that a mitochondria genome in a higher eukaryote is higher than a somatic genome on the evolutional change-speed. In addition, Kimura (1968) reported in his paper on 'Evolutionary rate at the molecular level' that evolution on the molecular level does not depend on Darwinian selection but rather on the result of a spontaneous change of genetic matters in the unimportant genetic region. In other words, natural selection on the molecular level also chooses optimal best adaptability in response to ever changing environments in the evolutional process. Therefore, genetic variation requires a minimum of change in the less important regions of genes. Here, we investigated the phylogenetic relationships between the *B. mori* and *B. mandarina* species of the Bombycidae and the *A. yamamai* and *A. pernyi* species of the Saturniidae based on mtDNA RFLP and the cytochrome oxidase I gene. From the results of the mtDNA RFLP and the cytochrome oxidase I sequence homology, we can assume a closer genetic relationship between the *B. mori* and *B. mandarina* species than between *A. yamamai* and *A. pernyi*. Although this report is not exhaustive to prove that wild silkworm, *B. mandarina* is a possible ancestor of the domesticated *B. mori,* it does provide data to understand the mtDNA relationship of *B. mori* and *B. mandarina*. In addition, this study indicates that the mtDNA gene can serve as a good basis for the phylogenetic analysis of silkmoths. At least mtDNA RFLP and cytochrome oxidase I gene sequences can provide evidence on the phylogenetic relationships among genus and families.

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