Nucleotide sequence variation in mitochondrial COI gene among 147 silkworm (*Bombyx mori*) strains from Japanese, Chinese, European and moltinism classes

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We characterized the nucleotide sequences of PCR-amplified mitochondrial COI fragments of 147 silkworm (*Bombyx mori*) strains that have been maintained in the National Institute of Agrobiological Sciences. Coding sequences (714 bp) of the 147 COI fragments were classified into eight haplotypes based on nucleotide differences at eight segregating sites. No length variation was identified in this region. The 5'-noncoding region showed different features, wherein changes in the number of Ts in the T-stretch, together with two base substitutions, were observed. As a result, the 147 COI noncoding sequences were classified into six haplotypes. Combining the coding and noncoding regions, we identified 14 haplotypes. One of the 14 haplotypes, Hap1A was exclusively abundant in the Japanese native strain class, while this haplotype was less frequent in the other three native strain classes. This finding suggests that the Japanese strain class underwent significant genetic differentiation from the Chinese, European, and moltinism classes, when the each class is regarded as a population. Comparison of the nucleotide sequences to those of *B. mandarina* (which inhabits Japan) revealed changes that are significantly larger than those within either *B. mori* or *B. mandarina*. Furthermore, we detected no common haplotypes between them, which suggests the concept of suppressed gene flow between the two species.

Key words: *Bombyx mori*, *Bombyx mandarina*, COI haplotype, gene flow

INTRODUCTION

The silkworm *Bombyx mori* is a highly domesticated insect that has been artificially selected to increase silk production and facilitate rearing. Consequently, *B. mori* lacks the ability to find and approach its food source, mulberry leaves, during the larvae stage, and to fly in the adult stage. Commercial strains that have been bred to produce a large amount of silk also tend to have difficulty in mating. Such features prompt us to recognize that *B. mori* cannot live without human care; that is, it cannot survive in the field.

The domestication of *B. mori* is believed to have

occurred in China more than 5,000 years ago. The ancestor of *B. mori* is thought to be the wild mulberry silkworm *B. mandarina*, which inhabits China. These two species share common features such as carrying 28 chromosomes per haploid genome (Astaurov et al., 1959). Recent molecular phylogenetic studies support this hypothesis (Arunkumar et al., 2006).

During the domestication process, many silkworm strains have been yielded. They have differences in physiological traits, such as moltinism and voltinism, as well as phenotypic characteristics, such as differences in cocoon color, egg color, and body size. Such remarkable differences suggest that significant levels of genetic variation exist in *B. mori* genetic resources.

Following completion of the silkworm genome project (International Silkworm Genome Consortium, 2008), the genetic variation in *B. mori* has been carefully observed.

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Abbreviations: COI, cytochrome c oxidase subunit I; CDR, coding region; NCDR, noncoding region.

Chinese research groups demonstrated significant levels of variation between the nucleotide sequence of nuclear and mitochondrial genomes (Xia et al., 2009; Li et al., 2010, respectively). However, their results have not effectively contributed to understanding the population structure in the whole silkworm genetic resource. Because of the small size of samples, their results were not always representatives of whole silkworm genetic resource. In addition, they used quite small number of *B. mandarina* samples derived from limited area of China.

The National Institute of Agrobiological Sciences (NIAS) Japan has maintained hundreds of geographic strains of *B. mori* as genetic resources. These geographic strains are preserved as a collection of native and improved strains (Sorita, 1991; Kosegawa et al., 1997). The four native strain classes (Japanese, Chinese, European, and tropical) were derived from many local areas. In addition, the moltinism strain class, which includes local strains characterized by moltinism mutations, which differ from the standard tetramolter, has traditionally been maintained as the native strain.

To demonstrate the level of genetic variation that has been maintained in the *B. mori* genetic resource at the nucleotide level, we conducted DNA sequencing and subsequent DNA polymorphism analysis of partial mitochondrial COI gene fragments mediated by PCR amplification. The COI gene is a major target gene of the DNA Barcoding Project (Kress et al., 2005). DNA barcoding is a method to identify species of organisms based on matching to short genetic markers. Therefore, a series of sequence results are highly applicable to phylogenetic analysis of the Bombycidae species, particularly the probable ancestor of the silkworm, *B. mandarina*.

We characterized mitochondrial COI nucleotide sequences from 147 native silkworm strains and identified eight segregating nucleotide sites, wherein two different nucleotides were observed in the COI-coding region. Furthermore, length variation, which was caused by variable numbers of thymine (T) stretches, were observed in the 5'-noncoding region. In total, we detected 14 different haplotypes. The 14 COI haplotypes differentially appeared in the four native strain classes, which is indicative of significant genetic differentiation in the Japanese strain class, as compared to others.

We further discuss the relationship between *B. mori* and Japanese *B. mandarina*, which are likely separated by more than a million years from a common ancestor of *B. mori* (Yukuhiro et al., 2002; Pan et al., 2008).

MATERIALS AND METHODS

Genomic DNA samples of native silkworm strains

The DNA samples used in this study were similar to those in Tomita et al. (1997), except we excluded the tropical

strains because of the small number of strains kept in NIAS. We analyzed 48 Japanese, 50 Chinese, 28 European, and 21 moltinism strains. The European strain samples consisted of 26 native and two improved strains, while other three strain classes exclusively contained native strains. The list of strains used in this study is presented in Table 1. The purification method of genomic DNAs was described in Tomita et al. (1997).

PCR amplification of target DNA fragments We used a pair of COI-specific primers TY-J-1460 and Cl-N-2191 (Simon et al., 1994). Nucleotide sequences of the two primers are as follows:

TY-J-1460: 5'-TACAATTTATCGCCTAAACTTCAGCC-3'

Cl-N-2191: 5'-CCCGGTAAAATTAAATATAAACTTC-3'

To read both terminus of amplified fragment, we linked the T7 and M13 reverse primer sequences to TY-J-1460 and Cl-N-2191 primers, respectively, at the 5' terminus.

A reaction mixture contained 1× Ex Taq buffer (TaKaRa, Kyoto, Japan), 0.8 mM dNTP, 0.2 μM each primer, 0.5 unit of Ex Taq (TaKaRa), and approximately 5 ng of genomic DNA, which contained some amount of mtDNA, in a 20 μl reaction mixture. Cycling conditions were as follows: 2-min incubation at 96°C and 35 cycles of 15 s at 96°C, 15 s at 40°C, and 1 min at 68°C, followed by a 5-min incubation at 68°C. We confirmed amplification by agarose gel electrophoresis followed by detection under Gel Red (Biotium, CA, USA) staining.

DNA sequencing Prior to DNA sequencing, we removed the remaining primers and dNTPs by treatment with ExoSap-IT (USB Corp., Cleveland, OH, USA) according to manufacturer's protocol. We used the ABI BigDye Terminator ver. 3.1 (Applied Biosystems, Foster City, CA, USA) for DNA sequencing. The sequencing mixture (10 μl) contained the following components: 1.5 μl 5× buffer (Applied Biosystems), 1 μl Premix (Applied Biosystems), 0.1 μl of 20 μM primer for sequencing, 5.4 μl of dH₂O, and 2.0 μl of PCR purified product. For the initial sequencing, we used M13 reverse primers. Cycling conditions were as follows: 2-min incubation at 96°C followed by 35 cycles at 96°C for 15 s, 50°C for 10 s, and 60°C for 4 min. Upon PCR completion, we purified the sequencing products on a Sephadex column. We used the ABI Prism 3730 (Applied Biosystems) to detect sequencing signals.

When a read did not reach the 5' terminus of the COI fragment, we sequenced it using a T7 primer to read the opposite strand. We also used three additional primers (COI_443_2U, COI_221L, and COI_576L) for sequencing. Nucleotide sequences of the three primers were as follows:

COI_443_2U: 5'-TATTGCACACAGAGGAAGATCTG-3',

COI_221L: 5'-GGRKTWCCTAATTCTGCTCGA-3', and

COI_576L: 5'-AATGCTGTAAATTCCTACAG-3'.

Table 1. List of silkworm strains used in this study

Japanese strains	Chinese strains	European strains	Moltinism strains
Akajuku	Amoi moricaud	Yellow cocoon of Asia	Chojo
Akako	Benishina	Ascoli	Choyo
Aojuku (A)	Segregated white	Yellow cocoon of Ascoli	European No.7 trimolter
Aojuku (Ichinomiya)	Chahan	Bagdad	Trimolter of Kan
Agunisan	C spherical cocoon	91 Bagdad	Trimolter of Korai
Chikuma	Daiento	Pione	Menyo
Chusuotsu	Hekiren	Blanc des C'evennes	Sainan
Dainyorai	Hiko	Ginto	Sanko
Datenishiki	Kaijo	Gubbio	Trimolting white
Himeko	Kanko	Golden pink	Trimolter of Santo
Hinode (Odaka)	Kankoshaken	Black moth	Sen No.3
Kakumata (Aramomi)	Kansen	MaiellaZebra	Golden yellow cocoon of Shisen
Kokin	Golden yellow	Yellow cocoon of Maruke	Trimolter of Shisen
Kinkoshu	Knobbed	No. 500	Kyokuko
Kinshoku (Maebashi)	Kohoku	No. 65	OK39
Koishimaru	Kuroko (Shimane)	Rosa	VC1
(Koishimaru Watari)	Kyugyoku	S No. 9	Chogosan No. 1
Kuniichi	Nanko	Spain	Pentamolting white
Matamukashi	Oha	St. Julien	Pentamolting black
Murasakiko	Renshin	Szegsard	Pentamolter of Kyushu Univ.
Onichijimi	Seikyo	Yellow cocoon of Turkey	N-S
Ryukyu multi-pupal cocoon (Kiaya)	Sekko	Var	
Ryukyu multi-pupal cocoon (Shizuoka)	Shakugen	Y4	
Sagami	Chinese white cocoon	y35	
Sakurahime	Shinshocho	y39	
Seihaku (Shizuoka)	Shokei	Yuhaku	
Sekaiichi	Shuko	European No. 16 (Old)	
Tadamiko	S	European No. 16 (New)	
Tenryuseihaku	Tenmon		
Wako	Tokuishin		
Aojuku (Hanyuda)	Tsunoshina		
Chiyoazuru	Uryu		
Chusu	Y10		
Kinshoku (Matsumoto)	Yoko		
Kojiki	Daiankyo		
Kunitomi	Daikojo		
Kuroko (Takahashi)	Daizo (Nagano)		
Kuroko	Daizo (Matsumura)		
Nihonichi	Hakuyoen		
Daiaojuku	Kinkomaru		
Okusa	Kosetsu		
Onishiki	Striped		
Seihaku (Yamagata)	Shoka		
Shohaku (Maebashi)	Shoko		
Shohaku (Taketoyo)	208AB		
Tamanashi kasuri	209AB		
Tanegashima	Low 209		
Tsunomata (Shizuoka)	210		
	213		
	214		
	216		

Sequence analysis We edited the nucleotide sequences using ATGC (Genetyx Co., Tokyo, Japan) followed by DNA sequence alignment using Clustal W (Thompson et al., 1997) in MEGA (Tamura et al., 2007; Kumar et al., 2008). After aligning the nucleotide sequences, we prepared a fasta-formatted sequence data set. We analyzed this data set using DnaSP version 5.0 (Librado and Rozas, 2009) to detect segregating sites and to identify constitu-

tive haplotypes. We further used this software to estimate the population genetic parameters, S , π , and F_{st} . TCS 1.21 (Clement et al., 2000) was used to construct the maximum-parsimony haplotype network.

Fourteen DNA sequences of *B. mori* COI haplotypes reported here are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB649182, AB649183, AB649184, AB649185, AB649186, AB649187,

AB649188, AB649189, AB649190, AB649191, A1DB649192, AB64918493, AB649194, and AB649195 for BmoHap1A, BmoHap1B, BmoHap1D, BmoHap2A, BmoHap2C, BmoHap2D, BmoHap2E, BmoHap2F, BmoHap3A, BmoHap4D, BmoHap5D, BmoHap6D, BmoHap7A, and BmoHap8D, respectively.

RESULTS AND DISCUSSION

We successfully amplified COI fragments from 147 preserved silkworm strains. The COI fragment can be divided into two regions; the 5'-noncoding region (NCDR) and coding region (CDR). Initially, we separately analyzed the two regions because they showed different features in sequence variation.

Segregation sites in the COI coding region We identified eight segregating sites wherein two different nucleotides were observed at 714 CDR nucleotide sites (Fig. 1A). In the eight segregating sites, we detected eight transitions (T vs. C: 4; A vs. G: 4) and no transversions. Three of eight base changes were nonsynonymous (Fig. 1A). At the 41st CDR site, a C to T transition caused a Thr to Met mutation. G to A transition at site 238 induced a Val to Ile replacement, while a G to A transition

at site 256 yielded an Ala to Thr nonsynonymous change.

We did not identify any indels in the CDR. This is indicative of a non-length polymorphism in the COI CDR, showing significant functional constraint. To assess the magnitude of genetic variation in silkworm COI coding sequences in the 147 preserved strains, we estimated nucleotide diversity as π . The estimated π score was calculated to be 0.00080 ± 0.00008 . Estimated S (the number of segregating sites) and the π scores in four types of the native strains are shown in Table 2.

Haplotypes in the coding region Based on the differences in nucleotide sequence, we identified eight haplotypes. We tentatively termed the eight haplotypes for CDR Hap1 to Hap8 (Fig. 1A). A maximum-parsimony haplotype network generated by TCS 1.21 (Clement et al., 2000) is shown in Fig. 2A. The score of haplotype diversity (Hd) for the 147 COI sequences was calculated to be 0.5001 ± 0.0319 (Table 2). Differences between the individual haplotypes were quite small, which is consistent with the low estimate of π .

The frequency of each haplotype is demonstrated in Table 3, clearly indicating biased occurrences of haplotypes. The most abundant haplotype was Hap2, whose frequency was 0.639 (94/147). The next more frequent

Fig. 1. Segregating nucleotide sites in the *B. mori* COI nucleotide sequences. A. Segregating nucleotide sites in the *B. mori* COI coding region (CDR) and the eight haplotypes detected in the CDR. B. Aligned nucleotide sequences of six haplotypes identified in COI noncoding region (NCDR). Vertical numbers represent nucleotide positions in either the NCDR or CDR using the largest-sized flanking sequence from HapB as a standard. A hyphen means a deleted nucleotide. T-stretch region is marked by an*. We identified six haplotypes, A to F, in the NCDR. C. Segregating nucleotide sites in the *B. mori* COI coding region (CDR) combined with the 5'-noncoding region (NCDR), and 14 haplotypes identified. Vertical numbers represent nucleotide positions in the CDR, wherein a possible start site is defined as 1. An * signifies a nonsynonymous nucleotide difference. A dot indicates an identical nucleotide. A hyphen means a deleted nucleotide.

Table 2. Genetic variation scores of *Bombyx mori* native strains and 11 samples of Japanese *B. mandarina* in the COI coding region (CDR)

	Number of strains or samples	Total number of nucleotide sites	Number of segregating sites (S)	Number of haplotypes	Haplotype diversity (Hd)	Nucleotide diversity (π)
<i>B. mori</i> (Total)	147	714	8	8	0.5001	0.00080
Japanese strains	48	714	2	3	0.4140	0.00059
Chinese strains	50	714	4	5	0.2914	0.00048
European strains	28	714	1	2	0.2540	0.00036
Moltinism strains	21	714	5	5	0.4238	0.00079
<i>B. mandarina</i> * (Japan)	11	714	6	7	0.8730	0.00224

*Accession numbers of the sequences used in this survey are as follows: AF167266, AF167267, AF167268, AF167269, AF167270, AF167271, AF167272, AF167273, AF167274, AF167275, and AF167280.

Table 3. Haplotype classification of *Bombyx mori* native strains

Haplotypes (Total)	Bmo Hap 1A	Bmo Hap 1B	Bmo Hap 1D	Bmo Hap 2A	Bmo Hap 2C	Bmo Hap 2D	Bmo Hap 2E	Bmo Hap 2F	Bmo Hap 3A	Bmo Hap 4D	Bmo Hap 5D	Bmo Hap 6D	Bmo Hap 7A	Bmo Hap 8D
Japanese strain class	29	5	1	4	1	6	1	0	1	0	0	0	0	0
Chinese strain class	4	0	0	10	0	31	0	1	0	2	1	1	0	0
European strain class	3	0	1	18	0	6	0	0	0	0	0	0	0	0
Moltinism strain class	2	0	0	9	0	7	0	0	0	1	0	0	1	1
Total	38	5	2	41	1	50	1	1	1	3	1	1	1	1

Fig. 2. Maximum-parsimony haplotype network of *B. mori* COI sequences. A. CDR. B. NCDR and CDR. A bar signifies a mutation. An open circle means an undetected haplotype. In Fig. 2B, a blue bar signifies a CDR base change, while a red bar indicates a change in the number of T in the NCDR. A green bar represents a base change in the NCDR.

haplotype was Hap1 with a frequency of 0.306 (45/147). The other six haplotypes were less frequent; Hap4 appeared only three times, while the remaining five were singletons.

As shown in Fig. 1A, the nucleotide sequence of Hap1 differed from that Hap2 at site 171, where Hap1 had a G while Hap2 had an A. Hap3 shared an identical sequence to Hap1 except for site 238, where Hap3 carried an A and Hap1 had a G. As described above, this base change was nonsynonymous.

As shown in Fig. 2A, other haplotypes (Hap4, 5, 6, 7, and 8) were derived from Hap2. The nucleotide sequences of Hap4, 6, and 7 differed from that of Hap2 at sites 633, 439, and 538, respectively (Fig. 1A). An additional transition of C to T at site 41 on Hap4 yielded Hap5, and two extra transitions at sites 256 and 633 on Hap2 induced Hap8 (Fig. 1A). Note that Hap3, Hap5, and Hap8, which carried amino acid changes, were singletons.

Differential occurrences of haplotypes among the four native strain classes Heterologous distributions of the eight haplotypes, particularly Hap1 and Hap2, were clearly observed between the four native strain classes (Japanese, Chinese, European and moltinism). As shown in Table 3, the most frequent haplotype was Hap1 in the Japanese strains. In contrast, the other three native strains preferentially carried Hap2.

To assess the magnitude of variation at the nucleotide level within native strain classes, we estimated S , π , and haplotype diversity (H_d) for each of the four classes. Scores are summarized in Table 2. Despite the difference in the number of haplotypes between preserved strain classes, we did not observe remarkable differences in S , π , and H_d scores. This finding infers that Japanese strain class underwent less strong bottleneck effect.

Although Tomita et al. (1997) reported higher levels of genetic variation in Chinese strain classes (compared to the others) by examining the abundance of mariner-like elements mediated by PCR-amplification, our approach did not result in clear differences among the four classes. This discrepancy likely arose from the relatively small number of variable sites in the present study. If a larger fragment were analyzed, clearer differences may be obtained.

To assess the differentiation level between native strain classes, we estimated a series of F_{st} (fixation index). As shown in Table 4A, the results were highly biased; that is, F_{st} scores between the Japanese strain and the others ranged from 0.4 to 0.5, while those among three native strain classes (excluding Japanese) were quite low (approximately 0). These results clearly demonstrate genetic differentiation of the Japanese strain class from others.

Table 4. F_{st} scores

A CDR			
	Japanese	Chinese	European
Chinese	0.5366		
European	0.5149	0.0012	
Moltinism	0.4601	-0.0199	-0.0164
B NCDR + CDR			
	Japanese	Chinese	European
Chinese	0.4863		
European	0.4592	0.0012	
Moltinism	0.4218	-0.0199	-0.0164

Haplotype classification of silkworm COI sequences including the noncoding region We analyzed the 5'-noncoding region of the COI gene. Of the 147 sequences, we identified six different sequence types (HapA to F; Fig. 1B), wherein one transition between C and T (HapA vs. HapF), one transversion between A and T (HapA vs. HapB), and a difference in the number of thymine bases (T) in sites 7 to 15 were observed. The last event was associated with sequence slippage, such as a microsatellite sequence variation.

HapA was the most common at a frequency of 0.551 (81/147), followed by HapD (0.395:58/147). Although HapB appeared in five strains (0.034), the others were singletons.

As shown in Fig. 1C, tight linkage between coding and noncoding haplotypes was not observed. For example, Hap1 in the coding region was associated with HapA, HapB, and HapD, while Hap2 combined with all noncoding haplotypes, except for HapB. Hereafter, we analyzed a combined set of coding and noncoding sequences as a unit (Fig. 1C).

In Fig. 2B, we show a maximum-parsimony haplotype network of combined sequence sets. The differences in the parsimony networks between Fig. 2A and Fig. 2B were derived from a subdivision of Hap1 and Hap2. F_{st} analysis also indicated heterogeneity of the Japanese strains from the others (Table 4B).

Overall, the Chinese, European, and moltinism strains shared a common feature, the mtCOI gene pool, but the Japanese strain possessed a different one. This suggests that the European and moltinism strain classes were derived from the Chinese strains without undergoing severe genetic drift. In contrast, the Japanese strains may have experienced biased sampling of COI haplotypes to change their population structure.

Genetic variation in nuclear gene of silkworm and comparison with that of COI We detected genetic differentiation in mitochondrial COI sequence of *B. mori*. When comparing the result with those of nuclear genes, we can use limited amount of information on genetic variation in nuclear genes. For example, Kim et

al. (2008) analyzed nine independent intronic sequences from 25 *B. mori* strains including four Japanese ones in order to develop genetic markers for use in strain identification. Despite use of only 25 strains, many haplotypes were detected in all of analyses. For example, 15 haplotypes were identified in cytoplasmic action A4 second intronic sequences, wherein 29 nucleotide changes and two events of indels were observed in 533 nucleotide sites. Note that 14 haplotypes were identified in 147 COI sequences of the present study. The excess number of haplotypes relative to that of COI is likely to come from less stringent functional constraint on intronic sequences. Moreover, two out of four Japanese originated strains were classified into a haplotype wherein Chinese, European and Korean strains were observed.

Recently, Yu et al. (2011) demonstrated that Tyrosine hydroxylase (TH) gene had extremely low amount of nucleotide diversity relative to Chinese *B. mandarina*. The TH gene was considered to be one of the target genes for silkworm domestication through multiple approaches of population genetic analyses. Note that despite of analyzing the gene under the artificial selection during domestication process and use of 15 strains, the 13 haplotypes based on nucleotide change was detected. Furthermore, Yu et al. (2011) demonstrated clear difference in haplotype structure between the gene under the artificial selection and neutral genes. Their result showed a common feature to that of Kim et al. (2008), that is, intronic sequences or sequences including introns are relatively abundant in haplotype to that of COI even in genes under the artificial selection.

Similarity of silkworm COI sequences to Japanese *B. mandarina* *B. mori* mates with *B. mandarina* (which inhabits Japan) to generate fertile progenies, although the number of chromosomes differs between them (*B. mori*: $n = 28$; Japanese *B. mandarina*: $n = 27$; Sasaki, 1898; Kawaguchi, 1928). The cross between a *B. mori* male and a *B. mandarina* female is almost impossible without human intervention because the former cannot approach the latter successfully even in capture. Successful mating only occurs between *B. mori* females and *B. mandarina* males. As mitochondrial DNA is maternally inherited, the hybrid progeny between *B. mori* females and *B. mandarina* males carry mitochondrial DNA from *B. mori*. If gene flow had continued to occur between *B. mori* and *B. mandarina*, we could detect mtDNA derived from *B. mori* in the *B. mandarina* natural population.

In this study, we detected eight haplotypes in COI CDR, six in COI NCDR and 14 in the combined data set of 147 *B. mori* preserved strains (Fig. 1, A–C). To confirm the occurrence of gene flow between *B. mori* and *B. mandarina*, we analyzed 11 COI DNA sequences of Japanese *B. mandarina* (available in NCBI). Addition-

ally, we used a Korean *B. mandarina* COI sequence, as well as that of Chinese *B. mandarina*, for the analysis. The 11 Japanese *B. mandarina* COI sequences were classified into seven haplotypes based on seven segregating sites in the coding region (Fig. 3). Although four of the seven segregating sites were silent, three of seven segregating sites underwent nonsynonymous changes: a C to T change caused a Ser to Leu replacement at site 332, a G to A change caused a Ser to Lys replacement at site 338, and a G to A transition caused a nonsynonymous change of Gly to Ser at site 409. The π value was estimated to be 0.00224 ± 0.00046 (Table 2).

Japanese *B. mandarina* sequences had a NCDR, whose organization was different from that of *B. mori* (The data of *B. mori* shown in Fig. 1B). This was caused by the addition of two AC dimers into the T stretch (data not shown). Because the difference in the repetitive structure between *B. mori* and Japanese *B. mandarina* gave us unreliable nucleotide sequence alignment results, we excluded the NCDR from this comparison.

The comparison of CDR nucleotide sequences clearly revealed that none of the seven Japanese *B. mandarina* COI haplotype sequences were identical to the eight *B. mori* sequences (Fig. 3). Eleven base substitutions were detected between *B. mori* and Japanese *B. mandarina* (Fig. 3). Figure 4 demonstrates the separation of Japanese *B. mandarina* and *B. mori* combined with Chinese and Korean *B. mandarina*. Note that the eleven base substitutions are synonymous. It means that *B. mori* and Japanese *B. mandarina* share an identical amino acid sequence for the COI fragments, strongly sug-

[	112222333	344444445	556666667]
[	4573455233	9001238813	581356790]
[	1618306528	9597393667	868346208]
#BmoHap1	CTGGTCGTCG	CTGCTCTTTC	GCACTACTA
#BmoHap2	..A.....		
#BmoHap3	..A.....		
#BmoHap4	..A.....		T..
#BmoHap5	T.A.....		T..
#BmoHap6	..A.....	T....	
#BmoHap7	..A.....		A.....
#BmoHap8	..A...A...		...T....
#BmaC_Hap1	..A..T....		..G.....
#BmaK_Hap1	..A.....	T.....	A.....G
#BmaJ_1	.CA.C..C..	TC.TCTC.CT	AT..CGT..
#BmaJ_2	.CA.C...A	TC.TCTC.CT	AT..CGT..
#BmaJ_3	.CA.C....	TCATCTC.CT	AT..CGT..
#BmaJ_4	.CA.C....	TC.TCTCCCT	AT..CGT..
#BmaJ_5	.CA.C....	TC.TCTC.CT	AT..CGT..
#BmaJ_6	.CA.C..C..	TC.TCTC.CT	AT..CGTC.
#BmaJ_7	.CA.C..CT.	TC.TCTC.CT	AT..CGT..

Fig. 3. Variable nucleotide sites in *B. mori* and the Chinese, Korean, and Japanese *B. mandarina* COI coding region. Vertical numbers represent nucleotide positions. Bmo: *B. mori*; BmaC, BmaK, and BmaJ: Chinese, Korean, and Japanese *B. mandarina*, respectively. A dot represents the same base as that of BmoHap1. Accession numbers of BmaC and BmaK sequences are AF167278 and AF167277, respectively.

Fig. 4. Maximum-parsimony haplotype network of *B. mori* and Chinese, Korean, and Japanese *B. mandarina* COI coding sequences. A bar signifies a mutation. An open circle means an undetected haplotype.

gesting strict functional constraint on COI gene.

The noncoding region was excluded from this comparison because the differences in repetitive structure between *B. mori* and Japanese *B. mandarina* yielded unreliable nucleotide sequence alignment results. Nevertheless, we must emphasize the clear differences in the sequence composition of the NCDR as evidence for genetic differentiation between *B. mori* and Japanese *B. mandarina* (data not shown).

These observations support the lack of gene flow between *B. mori* and Japanese *B. mandarina*, despite the small number of comparisons. To thoroughly analyze this problem, a large-scale COI sequence analysis of Japanese *B. mandarina* populations is currently being

performed.

This study was based on mitochondrial DNA analysis, and the possibility remains that the two species share a common nuclear gene haplotype. Taking genetic differentiation under artificial selection during domestication process into consideration, further studies on the nuclear genes will help to clarify the genetic relationship between *B. mori* and *B. mandarina* in Japan.

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