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Genetic Homogeneity in the Domestic Silkworm, *Bombyx mori*, and Phylogenetic Relationship Between *B. mori* and the Wild Silkworm, *B. mandarina* Using Mitochondrial COI Gene Sequences

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Genetic variation in the domestic silkworm strains (*Bombyx mori*) and phylogenetic relationships between domestic silkworms and wild silkworms (*B. mandarina*) were investigated by using a portion of mitochondrial COI gene sequences. Ten geographic strains of *B. mori* we sequenced were identical in the 410 bp-section of mitochondrial COI gene. This sequence was also identical to the homologous sequ- ence of the four GenBank-registered strains, but one strain of *B. mori* differed a single nucleotide (0.2%) from others. MtDNA homogeneity in the *B. mori* strains appears to be resulted from fixation into the most frequent mtDNA type during the course of breeding for new strains, in which an extensive indoor rearing and removal of unwanted individuals were accompanied. In the comparisons between domestic and wild silkworms, some wild silkworms were closely related to domestic silkworms (0.2%-1.2% of divergence), but the others were not (2.7%-3.7% of sequence divergence). This result was also reflected in the phylogenetic analyses, showing two independent phylogenetic groups: one including all *B. mandarina* sequences and the other including both *B. mandarina* and *B. mori* sequences. Thus, domestic silkworms may have been derived from the ancestor of *B. mandarina*, which belongs to this group, alth-ough more extensive study will provide better understanding on this issue.

Key words : *Bombyx mori, Bombyx mandarina*, MtDNA, COI gene, Silkworm, Phylogeny

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Introduction

Recently, several researches have attempted to classify the silkworm strains using molecular genetic markers, but this effort was not fully successful (Kang and Seong, 1995; Hwang *et al.*, 1995, 1997, 1998; Seong, 1997). This is probably because domestic silkworms lack general genetic difference among strains and subdivision and/or naming into each strain may have heavily been relied upon a paucity of phenotypic characteristics.

Mitochondrial DNA (mtDNA) is known to have higher evolutionary rate compared to the functional counterpart of nuclear DNA, is inherited maternally, is lacking genetic recombination as in the chromosome, and is easy to handle in the laboratory (Brown *et al.*, 1979; Cantatore and Saccone, 1987; Harrison, 1989; Kim *et al.*, 1998). Because of these characteristics, after and during a full genome of human mtDNA was sequenced (Anderson *et al.*, 1981), mtDNA became popular in the fields of systematics, ecology, population genetics, behavior, biogeography, and conservation, where detection of polymorphism for natural populations is necessary (Avise, 1994). As a result of this, our understanding of the natural population progressed greatly.

On the other hand, researches on genetic variation among domesticated strains and relationships of them to the ancestral species have been attempted in various animals using mtDNA, but they showed quite different pattern according to the process of domestication. For example, the sequence analysis of mitochondrial control region from several domesticated cattle breeds revealed two obvious phylogenetic groups with the sequence divergence of at least 5.01% between them, and this phenomenon was explained by an independent domestication from two pre-existing ancestral subspecies of *Bos primigenius* (Loftus *et al.*, 1994). In case of domesticated

chicken (*Gallus gallus domesticus*), phylogenetic analysis among breeds using control region sequence revealed monophyletic group with relatively low sequence divergence of 0.5%-3%, suggesting a single domestication event within relatively short period of time for the explanation of the current diversified breeds (Fumihito *et al.*, 1994). On the other hand, mitochondrial control region analysis of dog breeds (*Canis familiaris*) and wolves revealed multiple and ancient origin of domesticated dogs (Vilà *et al.*, 1991). Although many domesticated animals were subjected to phylogenetic analysis to illustrate the relationships among domesticated breeds and between domesticated animals and their wild ancestors, no such study is available for silkworms using mtDNA.

It is known that COI gene of mtDNA is highly variable at the DNA level, especially at the silent sites. We selected a portion of COI gene, which includes the membrane-spanning helices M3, M4, and M5, external loops E2 and E3, and internal loops I2 (Lunt *et al.*, 1996). This portion of COI gene has been proved to be useful for the study of intraspecific genetic variation in insects (Simon *et al.*, 1994; Kim *et al.*, 2000a, b; Lee *et al.*, 2000). We sequenced this portion of mtDNA to investigate the genetic divergence among several domestic silkworm strains, which is under maintaining and preserving at the Department of Sericulture & Entomology, National Institute of Agricultural Science & Technology (NIAST), Rural Development Administration (RDA) in Korea. We also obtained homologous sequences of wild silkworms (*B. mandarina*) and other domestic silkworm sequences through GenBank search to form a combined data set for the analysis of genetic divergence among domestic silkworm strains and phylogenetic relationships between domestic and wild silkworms.

Materials and Methods

Insects

Ten domestic silkworm strains used in this study were obtained from the Department of Sericulture & Entomology, NIAST, RDA in Korea and their strain names and origins are listed in Table 1. We also obtained five *B. mori* and 12 *B. mandarina* sequences of the mitochondrial COI gene through GenBank search, and their GenBank accession numbers are listed in Table 1.

MtDNA amplification and sequencing

Total DNA was extracted from the frozen tissue samples by following the standard proteinase K method (Kocher *et al.*, 1989). Primer information and PCR condition for amplification of the partial COI gene (410 base pairs) are in detail described in Kim *et al.* (2000a). To ascertain successful DNA replication, electrophoresis was carried out for 40 min using 1X TBE buffer in 0.7% agarose gel. The PCR product was then purified using PCR purification kit (QIAGEN) by following manufacturers instruction. DNA sequencing was performed using ABI 377 Genetic Analyzer (PE Applied Biosystems). Sequence alignment was performed using IBI MacVector (ver. 6. 5).

Phylogenetic analysis using PAUP, networks, and PHYLIP

PAUP (Phylogenetic Analysis using Parsimony) ver. 3.1 (Swofford, 1990) was used to infer possible phylogenetic relationships among the matralines of the domestic and wild silkworm sequences. Homologous mtDNA sequences of the Japanese oak silkworm, *Antheraea yamamai*, and Chinese oak silkworm, *A. yamamai*, which showed the highest sequence homology to the ingroup sequences were obtained through GenBank search and used as outgroups independently or jointly (GenBank accession numbers AB15864 and AF029985, respectively). PAUP analysis was performed using an equal weighting of transitions and transversions as well as several ratios up to and including 1:20. Heuristic searches were performed, and reliability of the topology was tested by bootstrapping (100 iterations). With intraspecific mtDNA sequence data it often happens that parsimony analysis provide limited resolution because of back and parallel mutations, resulting in polytomies. One solution, which we employed, is to prepare one-step median networks, which can provide insight into probable relationships among closely related lineages (Bandelt *et al.*, 1995). As an alternative to the form of parsimony analysis, we used Neighbor-Joining method in PHYLIP (Phylogeny Inference Package) ver. 3.5c (Felsenstein, 1993). To obtain phylogenetic tree, the data set was first iterated 100 times using the subprogram SEQBOOT. Each iterated data set was run using the subprogram DNADIST to obtain distance matrix between pairs of sequences with the option of Kimura's 2-parameter method (Kimura, 1980). Individual trees from each distance matrix were obtained using the subprogram NEIGHBOR. The two homologous mtDNA sequences of giant silkworm moths (GenBank accession numbers AB 15864 and AF029985) were both included in the analysis to root the trees. Finally, a consensus tree representing reliability at each branch in the tree was obtained using the subprogram CONSENSE.

Results and Discussion

Genetic homegeneity among the strains of *Bombyx mori*

Analysis of a portion of COI gene from 10 strains of *B.*

mori revealed all identical sequences in the 410-bp section, although they were originated from various countries, such as Korea, Japan, China, and Europe (Table 1). These sequences were diposited in the GenBank, and accession numbers are AF248706-AF248715, respectively. Furthermore, among the five GenBank-registered sequences including the strain, Daizo, all were identical except for one strain "W2" (accession number AF167265), which has been registered by National Institute of Sericultural & Entomological Science in Japan. This "W2" differed by one nucleotide from all others (Table 2), and that was a transitional substitution (C⇔T in the nucleotide position 406; Fig. 1), but the point mutation did not substitute amino acid (all aspartic acid). Nucleotide sequences of the strain, Sun3ho, and "W2", are presented in Fig. 1 on the behalf of others. The maximum sequence divergence in *B. mori* thus is 0.2% (one nucleotide), the value of which is the least amount of possible variation. In the comparisons with other natural groups of insect species, which utilized COI gene, this divergence was revealed to be extremely low. For example, the sequence divergences were ~0.4% for spruce budworm species

Table 1. A brief information of the strains of *B. mori* sequenced in this study and GenBank-searched *B. mori* and *B. mandarina* sequences

Species	Type	Strain	Origin	Cocoon color	GenBank accession number
Sequences obtained from this study					
B. mori	1	N12	Japan	Yellow	AF248706
B. mori	1	Shansurian	Europe	Light Yellow	AF248707
B. mori	1	Sun3ho	Korea	Yellow	AF248708
B. mori	1	Sammyunhonghoibak	Korea	Light yellow	AF248709
B. mori	1	C14	China	White	AF248710
B. mori	1	Hansammyun	Korea	Light yellow	AF248711
B. mori	1	N74	Japan	Light green	AF248712
B. mori	1	N24	Japan	Yellow	AF248713
B. mori	1	Bagdad	Europe	Light green	AF248714
B. mori	1	Rhwang	China	Yellow	AF248715
Sequences obtained through GenBank search					
B. mori	1	NA	NA	NA	AB016294
B. mori	1	Daizo	Japan	NA	AF167260
B. mori	1	NA	NA	NA	AF167261
B. mori	1	Aojuku-hakuran	NA	NA	AF167262
B. mori	2	W2	NA	NA	AF167265
B. mandarina	3	Bma282-China	NA	NA	AF167278
B. mandarina	4	Bma281-Korea	NA	NA	AF167277
B. mandarina	5	NA	NA	NA	AF029068
B. mandarina	6	Bma12-Tsushima	NA	NA	AF167276
B. mandarina	7	Bma4-Tsukuba	NA	NA	AF167271
B. mandarina	8	NA	NA	NA	AB016295
B. mandarina	8	Bma9-Fukuoka	NA	NA	AF167266
B. mandarina	8	Bma6-Tsukuba	NA	NA	AF167269
B. mandarina	9	Bma8-Matsumoto	NA	NA	AF167267
B. mandarina	10	Bma1-Tsukuba	NA	NA	AF167274
B. mandarina	11	Bma11-Fukuoka	NA	NA	AF167275
B. mandarina	12	Bma7-Tsukuba	NA	NA	AF167268

NA, Information is not available from GenBank.

Numbers before strain names indicate sequence types and are employed to distinguish different sequences, instead of using similar GenBank numbers in the following Tables and Figures. Thus, identical number indicates identical sequences in the 410-bp section of the mitochondrial COI gene.

Table 2. Pairwise comparisons among mitochondrial COI gene sequences of *B. mori* and *B. mandarina*

Species	Type	GenBank Accession Number	1	2	3	4	5	6	7	8	9	10	11	12
B. mori	1	Sun3ho*	-	0.002	0.002	0.005	0.010	0.029	0.032	0.034	0.034	0.034	0.034	0.037
B. mori	2	AF167265	1	-	0.005	0.007	0.012	0.027	0.029	0.032	0.032	0.032	0.032	0.034
B. mandarina	3	AF167278	1	2	-	0.007	0.012	0.032	0.034	0.037	0.037	0.037	0.037	0.039
B. mandarina	4	AF167277	2	3	3	-	0.005	0.024	0.027	0.029	0.029	0.029	0.029	0.032
B. mandarina	5	AF029068	4	5	5	2	-	0.029	0.032	0.034	0.034	0.034	0.034	0.037
B. mandarina	6	AF167276	12	11	13	10	12	-	0.012	0.010	0.015	0.015	0.015	0.012
B. mandarina	7	AF167271	13	12	14	11	13	5	-	0.002	0.002	0.002	0.002	0.005
B. mandarina	8	AF167266	14	13	15	12	14	4	1	-	0.005	0.005	0.005	0.002
B. mandarina	9	AF167267	14	13	15	12	14	6	1	2	-	0.005	0.005	0.007
B. mandarina	10	AF167274	14	13	15	12	14	6	1	2	2	-	0.005	0.007
B. mandarina	11	AF167275	14	13	15	12	14	6	1	2	2	2	-	0.007
B. mandarina	12	AF167268	15	14	16	13	15	5	2	1	3	3	3	-

*Sun3ho is a strain name as shown in Table 1 and represents four identical sequences of *B. mori* obtained through GenBank search (AB016294, AF167260, AF167261, AF167262) as well as 10 identical COI gene sequences of *B. mori* strains analyzed in this study. Also, *B. mandarina* sequences of AF167266, AB016295, and AF167269, which were found through GenBank search were identical to AF167266 in the 410-bp segment, so only AF167266 was presented. Percent sequence divergence is presented above diagonals and their numbers of nucleotide differences are given below diagonals.

(Sperling and Hickey, 1994), 0.23% and 0.12% for two species of the rice planthoppers (Mun *et al.*, 1999), 0.5% for *Heliconius* butterflies (Brower, 1994), 1.4% for diamondback moth (Kim *et al.*, 2000a), and 1.2% for firefly species (Lee *et al.*, 2000; Kim *et al.*, 2000c). Such a low genetic variation in the domestic silkworms was previously suggested by other studies using different molecules. For example, in the RFLP study of 22 *B. mori* strains, Kang and Seong (1995) only found two polymorphic probes among three and mentioned the possibility of lack of general genetic variation in the *B. mori* strains.

Although further study may provide rather accurate answer for the low genetic divergence in the domestic silkworms in terms of mtDNA (0.2% of maximum sequence divergence), one possible explanation appears to be related to the processes of breeding for better strains. During the course of breeding, cross between strains over generations may result in a few individuals with wanted characteristics, but most with unwanted. These unwanted silkworms will be discarded unlikely in the other domesticated animals, such as dogs. Thus, although the nuclear DNA loci, which encode the wanted phenotypes, are selected, mtDNA genotypes would show the equivalent result of population bottleneck. Thus, theoretically, the selected strains may possess and will be fixed with the mtDNA genotype that was most frequent originally over generation. From 1900 an extensive cross for breeding among once geographically isolated strains and the resultant discard of unwanted individuals may further have eliminated the least mtDNA diversity possesses by geographic strains. The observation that the process of breeding may have caused mtDNA homogeneity was not found in other studies possibly because there is no equivalent, domesticated insect as well as animals, but it seems to be very interesting issue.

On the other hand, several studies, which utilized nuclear markers (*e. g.*, RAPD) proposed that the domesticated silkworm strains possess substantial genetic diversity among them (Hwang *et al.*, 1995, 1996, 1997, 1998). The discrepancy between studies might be explained in terms of difference in the mode of inheritance in each molecule. That is, although a pair of diploid animals can inherit maximum of four different types of nuclear genes (*e. g.*, RAPD), they can only inherit one mtDNA type to the subsequent generation. Therefore, nuclear DNA compared to mtDNA would show less effect in the general background of the subsequent genome during the course of breeding.

Phylogenetic relationships between *B. mori* and *B. mandarina*

Among the 12 mtDNA sequences of *B. mandarina* obtained through GenBank search, pairwise comparisons revealed ten different sequences, but AF167266, AB 016295, and AF167269 were identical in the 410-bp section (Table 1). The 10 sequences diverged from 0.2% (one bp) to 3.9% (16 bp), and these estimates are at least similar or somewhat larger compared with other intraspecific

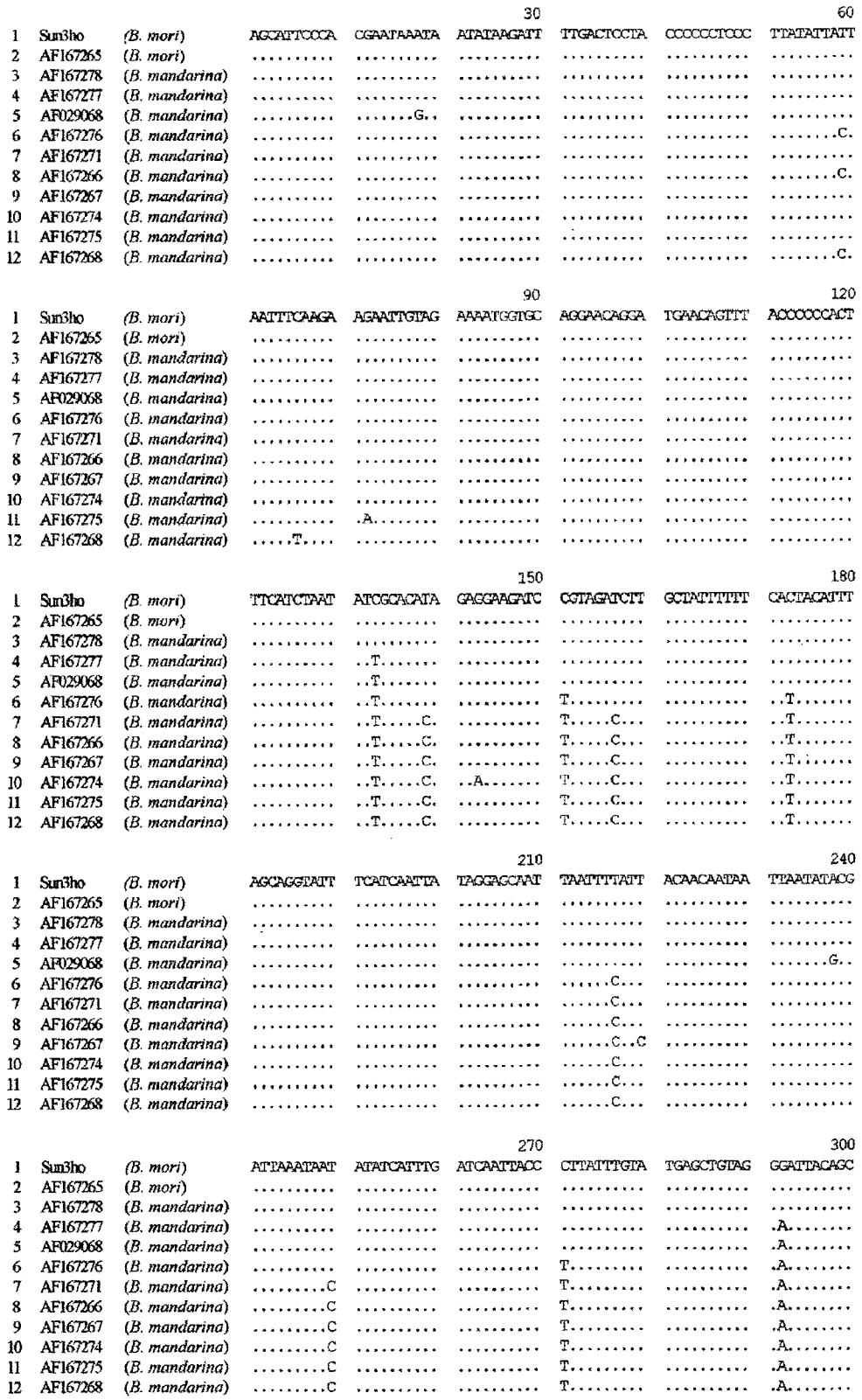

Fig. 1. Alignment of the 410-bp of COI gene sequences from *B. mori* and *B. mandarina*. Only nucleotide positions that differ from Sun3ho (*B. mori*) are indicated. Sun3ho represents 10 identical COI gene sequences of *B. mori* strains analyzed in this study and four identical sequences of *B. mori* obtained through GenBank search (AB016294, AF167260, AF167261, AF167262). Also, *B. mandarina* sequences of AF167266, AB016295, and AF167269, which were found through GenBank search were identical to each other in the 410-bp segment, so only AF167266 was presented.

					330			360
1	Sun3ho	(*B. mori*)	ATTTTTATTA	TTATTATCAC	TACCTGTTTT	AGCTGGAGCT	ATTACAATAT	TATTAACAGA
2	AF167265	(*B. mori*)	....	....	....	....	....	....
3	AF167278	(*B. mandarina*)	....	....	....	....	....	.G........
4	AF167277	(*B. mandarina*)	....	....	....	....	....	....
5	AF029068	(*B. mandarina*)	....	....	....	....	....	....
6	AF167276	(*B. mandarina*)	....	...C.....T	....	....	....	....
7	AF167271	(*B. mandarina*)	....	.........T	....	....	....	....
8	AF167266	(*B. mandarina*)	....	.........T	....	....	....	....
9	AF167267	(*B. mandarina*)	....	.........T	....	....	....	....
10	AF167274	(*B. mandarina*)	....	.........T	....	....	....	....
11	AF167275	(*B. mandarina*)	....	.........T	....	....	....	....
12	AF167268	(*B. mandarina*)	....	.........T	....	....	....	....

					390		410
1	Sun3ho	(*B. mori*)	TCGAAACTTA	AATACATCAT	TTTTTGATCC	TGCTGGAGGA	GGAGACCCAA
2	AF167265	(*B. mori*)	....	....	....	....	.....T....
3	AF167278	(*B. mandarina*)	....	....	....	....	....
4	AF167277	(*B. mandarina*)	....	....	....	....	....
5	AF029068	(*B. mandarina*)	....	....	....	....	....
6	AF167276	(*B. mandarina*)	....	....	.......C..	.........G	.....T....
7	AF167271	(*B. mandarina*)	....	....	.......C..	.........G	.....T....
8	AF167266	(*B. mandarina*)	....	....	.......C..	.........G	.....T....
9	AF167267	(*B. mandarina*)	....	....	.......C..	.........G	.....T....
10	AF167274	(*B. mandarina*)	....	....	.......C..	.........G	.....T....
11	AF167275	(*B. mandarina*)	....	....	.......C..	.........G	.....T....
12	AF167268	(*B. mandarina*)	....	....	.......C..	.........G	.....T....

Fig. 1. Continued.

studies of insect species (see previous section). In the comparisons between *B. mandarina* and *B. mori* sequences, some *B. mandarina* sequences (AF 167278, AF 167277, and AF 029068) diverged only 0.2%-1.2% from *B. mori* sequences (Table 1). These estimates are well within the range of the intraspecific sequences of other insects, in which mitochondrial COI gene sequences were used (see previous section). Furthermore, the maximum sequence divergence within the domestic silkworm strains (3.9%) is larger than that between the two silkworm species (3.7%; AF167268 vs. Sun3ho). These all aspects thus suggest genetic similarity between the two species.

The phylogenetic relationships between *B. mori* and *B. mandarina* are depicted in the PAUP phylogeny in Fig. 2. Analyses run with transition: transversion weightings of several ratios and with two species of giant silkworm moths as outgroups independently or jointly (*A. yamamai* and *A. yamamai* (GenBank accession numbers AB15864 and AF029985, respectively) did not affect the topology of trees. Thus, the result of unordered analysis run with the two species together as outgroups is presented (Fig. 2). The silkworm sequences generally formed two independent groups (named groups A and B) supported by high bootstrapping values (92% and 96%, respectively). Group A included all *B. mori* sequences and also three sequences of *B. mandarina*, which are assumed be collected in China and Korea. Thus, both species are included in this group. On the other hand, group B included several sequences of *B. mandarina*, which are assumed to be collected in the several areas in Japan, but this group did not include *B. mori*. The result of the PHYLIP analysis is shown in Fig. 3. Overall, similar result was obtained compared with

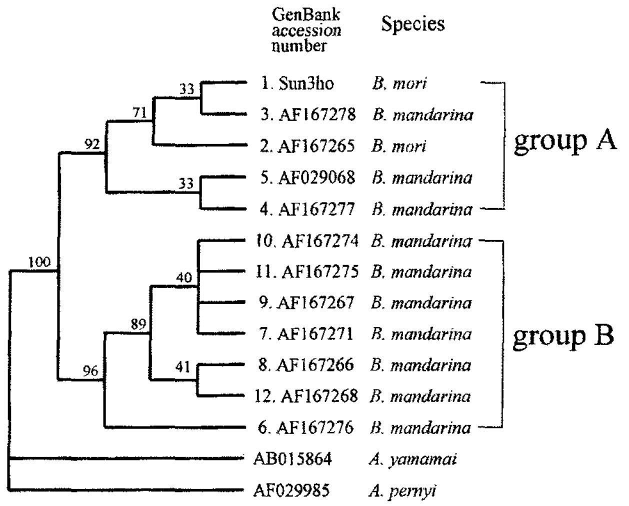

Fig. 2. PAUP analysis of the COI gene sequences of *Bombyx mori* and *B. mandarina* using homologous mtDNA sequences from *Antheraea yamamai* and *A. pernyi* as outgroups. The topology represents the consensus tree from an heuristic search yielding two equally most parsimonious trees, each with 94 steps. The consistency index is 0.957. The retention index is 0.955. The numbers represent bootstrap values for 100 replicates.

PAUP analysis, although more branches were generated in the PHYLIP tree. To further illustrate the genetic relationships among sequences of the two species, we used an unrooted one-step median network, which visualizes a possible evolutionary pathway among them (Fig. 4). As shown in the PAUP and PHYLIP analyses (Fig. 2 and 3) the two groups sustained their own members of sequences. The minimum genetic distance between the two

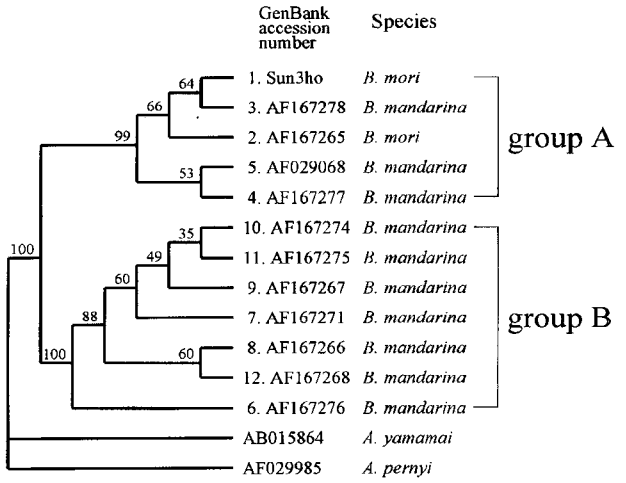

Fig. 3. PHYLIP analysis of the COI gene sequences of *Bombyx mori* and *B. mandarina*. The tree was obtained using the subprogram NEIGHBOR incorporated in PHYLIP with the option of Kimura's 2-parameter method (1980). The tree was rooted using the two species of giant silkworm moths, *Antheraea yamamai* and *A. pernyi*. The numbers shown on branches, which represent bootstrap values for 100 replications, were obtained using the subprogram CONSENSE.

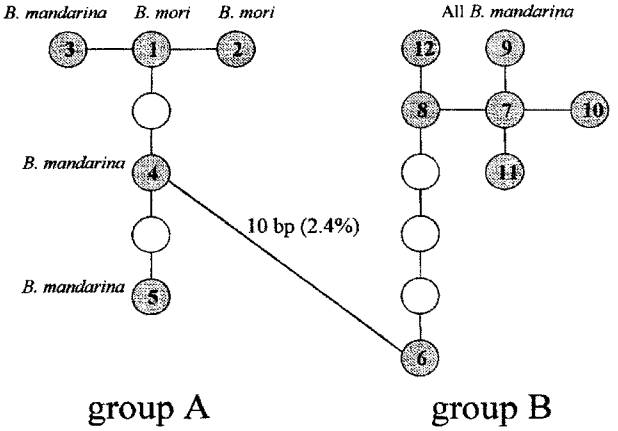

Fig. 4. Parsimonious one-step median networks analysis among the sequences of *B. mori* strains and *B. mandarina*. Numbers in the circle correspond to the type numbers in all Tables and Figures. Each bar between circles indicates one nucleotide difference from the neighboring sequences and white circles without numbers inside indicate the hypothetical sequences, which were not found in this study. To connect the two groups, a minimum of 10 mutational steps (2.4% of sequence divergence) are required.

groups was 2.4% (12 mutational steps), indicating the presence of substantial genetic divergence between the two groups.

Many researches on genetic divergence and phylogenetic relationships among wild silkworms and domestic ones have been studied (Kusuda *et al.*, 1986; Yoshitake, 1966). Generally, there is consensus on the aspect that domestic silkworms were evolved from wild silkworms (Yoshitake, 1968, 1984). However, opinions differ on the magnitude of genetic divergence among the two species, accordingly molecular markers utilized. One is genetic "similarity", based on the fact that the two species can hybridize and, resultantly, have similar characteristics (Kim *et al.*, 1999; Kim and Nho, 1994; Kusuda *et al.*, 1986). The other is genetic "difference" (Hwang *et al.*, 1995, 1998), the notion of which explains an independent evolution of the domestic silkworm in spite of morphological and ecological similarity. If we agree evolutionary neutrality of mtDNA and consider comparatively low mtDNA divergence between the two species (Table 2), it would be a wise conclusion that the general genetic background of the two species is expected to be fairly similar except for the genes, coding for some phenotypes. This low mtDNA divergence between the two species appears to reflect the aspect that evolution of domestic silkworm from wild silkworm happened within relatively limited regions and time period as seen in the example of domesticated chicken (Fumihito *et al.*, 1994, 1996).

There are fairly different opinions about the lineage of the wild silkworm, from which domestic silkworm was originated. One is that the domestic silkworm, which has 2n=56 evolved from the wild silkworm with 2n=54 (Murakami and Imai, 1974). The other is that domestic silkworm was derived from the wild silkworm with 2n=56, because the wild silkworm with 2n=56 are actually found in China and Korea (Astaurov, 1959). Although a conclusive statement is not available because of our limited data, our data at least do provide a ground for a plausible speculation. That is, our mtDNA results clearly suggest that domestic silkworms are genetically close to the members of wild silkworm belonging to group A (Fig. 2, 3, and 4; AF 167278, AF 029068, AF 167277).

Furthermore, this similarity allows us to believe that these wild silkworms are close enough to be considered as an immediate ancestor of domestic silkworm. Thus, we think that this similarity in the mtDNA genome does not necessary require the explanation about chromosome evolution, such as evolution from the wild silkworm with 2n=54 to the domestic silkworm with 2n=56. Therefore, we support the latter hypothesis proposed by Astaurov (1959). As we mentioned earlier, because our data are based on the limited samples, an extensive sampling of *B. mandarina* over a wide geographic area and a variety of *B. mori* strains are required.

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