Mitochondrial genome nucleotide substitution pattern between domesticated silkmoth, *Bombyx mori*, and its wild ancestors, Chinese *Bombyx mandarina* and Japanese *Bombyx mandarina*

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Abstract

*Bombyx mori* and *Bombyx mandarina* are morphologically and physiologically similar. In this study, we compared the nucleotide variations in the complete mitochondrial (mt) genomes between the domesticated silkmoth, *B. mori*, and its wild ancestors, Chinese *B. mandarina* (ChBm) and Japanese *B. mandarina* (JaBm). The sequence divergence and transition mutation ratio between *B. mori* and ChBm are significantly smaller than those observed between *B. mori* and JaBm. The preference of transition by DNA strands between *B. mori* and ChBm is consistent with that between *B. mori* and JaBm, however, the regional variation in nucleotide substitution rate shows a different feature. These results suggest that the ChBm mt genome is not undergoing the same evolutionary process as JaBm, providing evidence for selection on mtDNA. Moreover, investigation of the nucleotide sequence divergence in the A+T-rich region of *Bombyx* mt genomes also provides evidence for the assumption that the A+T-rich region might not be the fastest evolving region of the mtDNA of insects.

Key words: nucleotide substitution pattern, mitochondrial genome, *Bombyx mori*, Chinese *Bombyx mandarina*, Japanese *Bombyx mandarina*.

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between *B. mori* and *ChBm*. The purpose of this study was to determine whether the nucleotide variation pattern between *B. mori* and its wild ancestor, *ChBm*, is consistent with that between *B. mori* and its close relative, *JaBm*.

The mt genome sequences of *B. mori* C108 (AB070264), Backokjam (NC_002355), Aojuku (AB083339), Xiafang (AY048187), and *JaBm* (AB070263) were downloaded from GenBank, aligned by Clustal X (Thompson et al., 1997), and the alignment was analyzed by MEGA version 4.1 (Tamura et al., 2007), including the variable nucleotide sites and the substitution sites.

The four mt genome sequences of *B. mori* C108 (15,656 bp), Backokjam (15,643 bp), Aojuku (15,635 bp), and Xiafang (15,664 bp) were aligned to produce a 15,685 nt consensus alignment, of which only 89 (0.57%) nucleotide sites are variable, including insertions-deletions (indels) (data not shown). Within the four *B. mori* strains, only 33 substitutions were detected, including 24 transition (ts) and 9 transversion (tv) mutations. The variable nucleotide sites ranged from 63 to 74 nt when each two of the four *B. mori* mt genome sequences were compared, showing no significant differences in variable nucleotide rate ($\chi^2 = 1.25$, d.f. = 5, $p > 0.05$).

However, the mt genome sequences of *ChBm* (15,682 bp) and *JaBm* (15,928 bp) were aligned to produce a 15,970 nt consensus alignment, of which 639 (4.00%) nucleotide sites are variable including indels, and 325 substitutions were detected. Compared to these data observed within the four *B. mori* strains, the sequence divergence within *B. mandarina* is significantly greater (4.00% vs 0.57%; $\chi^2 = 415.26$, d.f. = 1, $p < 0.001$). Moreover, we identified 484 variable nucleotide sites including indels (3.08% sequence divergence) from a 15,722 nt consensus alignment between the mt genomes of *B. mori* C108 and *ChBm*, whereas 855 variable nucleotide sites including indels (5.35% sequence divergence) were identified from a 15,970 nt consensus alignment between *B. mori* C108 and *JaBm*. Compared to the sequence divergence observed between *B. mori* C108 and *JaBm*, that between *B. mori* and *ChBm* was significantly smaller (3.08% vs 5.35%; $\chi^2 = 101.36$, d.f. = 1, $p < 0.001$). These results led us to further compare the nucleotide substitution pattern between *B. mori* C108 and *ChBm* with that between *B. mori* C108 and *JaBm*.

A total of 381 substitutions were detected between the mt genomes of *B. mori* C108 and *ChBm*: 230 transition and 151 transversion mutations (ts:tv = $\kappa \approx 230/151 = 1.52$). This value is significantly deviatory from neutral expectation (1:2, $\chi^2 = 125.30$, d.f. = 1, $p < 0.001$), indicating that evolutionary pressures are acting upon the two mt genomes. However, between *B. mori* C108 and *JaBm*, 514 substitutions were detected: 414 transition and 100 transversion mutations ($\kappa \approx 4.14$), also significantly deviated from neutral expectation (1:2, $\chi^2 = 517.47$, d.f. = 1, $p < 0.001$). Excess transition mutation was also reported in *Drosophila* ($\kappa = 761/180 \approx 4.23$) and *Ostrinia* ($\kappa = 134/48 \approx 2.88$) mt genomes, and attributed to non-neutral evolutionary forces or population effects (Ballard, 2000; Coates et al., 2005). Compared to the data observed between *B. mori* and *JaBm*, the transition mutation ratio between *B. mori* and *ChBm* was significantly smaller (1.52 vs 4.14) ($\chi^2 = 44.14$, d.f. = 1, $p < 0.001$), suggesting that their nucleotide substitution pattern is different.

The nucleotide differences between *B. mori* and *ChBm* mt genes except for tRNA genes are shown in Table 1. The numbers and rates of nucleotide difference between the mt genes of *B. mori* and *ChBm* were found to be decreased compared to those seen between *B. mori* and *JaBm*, except for the srRNA gene and A+T-rich region, in which the numbers and rates of nucleotide difference were increased. The full srRNA4 sequences of *B. mori* C108 and *ChBm* were aligned to produce a 785 nt consensus alignment, of which 12 substitutions were detected, showing a higher nucleotide sequence divergence than the lrRNA gene. Comparing *B. mori* C108 and *ChBm*, the NADH dehydrogenase subunit encoding nad6 gene was the most conserved among genes or regions, followed by the ATP synthase F0 subunit encoding atp6 gene. The cytochrome b (cob) gene was the most variable, followed by the cytochrome c oxidase subunit encoding cox3 gene. The nucleotide sequence divergence in the A+T-rich region was moderate among these genes or regions. However, comparing the mt genes of *B. mori* and *JaBm*, the srRNA gene was the most conserved among genes or regions, and nucleotide sequence divergences in the lrRNA gene and the A+T-rich region were also low, compared to those of protein-coding genes.

In this study, we found no significant differences in transition rates for the protein-coding genes encoded by the major strand (22 A-G and 101 C-T transitions in 6,909 bp) between the mt genomes of *B. mori* C108 and *ChBm*, compared to those encoded by the minor strand (48 A-G and 13 C-T transitions in 4296 bp) ($\chi^2 = 2.13$, d.f. = 1, $p > 0.05$). However, on the major strand there were significantly more changes for T-C transitions in protein-coding genes than observed on the minor strand ($\chi^2 = 35.35$, d.f. = 1, $p < 0.001$), while on the minor strand there were significantly more changes for A-G transitions than observed on the major strand ($\chi^2 = 27.23$, d.f. = 1, $p < 0.001$). These data are consistent with those previously reported (Ballard 2000; Yukihiro et al., 2002).

The rates of nucleotide substitutions between the mt genomes of *B. mori* C108 and *JaBm* vary by genomic region: the five genes surrounding the A+T-rich region and two rRNA genes (Class 1: nad2, cox1, cox2, atp6, and nad1) are more conserved than the remaining genes (Class 2: cox3, nad4, nad5, and cob), whereas no significant dif-
Table 1 - Nucleotide differences between mitochondrial genes of B. mori and Chinese B. mandarina, except for tRNA genes.

| Genes or regions | Base pairs | Transitional changes | Transversional changes | Sum I | Sum II |
|------------------|------------|----------------------|------------------------|-------|--------|
|                  |            | A-G | T-C | Total | T-A | C-A | T-G | C-G | Total |               |               |               |               |               |               |               |               |               |               |               |               |               |               |               |
| nad2             | 1,023      | 2   | 13  | 15    | 2   | 3   | 0   | 0   | 5    | 20 (0.0196) | 32 (0.0313) |
| cob              | 1,152 (1,158) | 9   | 26  | 35    | 7   | 3   | 4   | 1   | 15   | 50 (0.0434) | 55 (0.0477) |
| nad5             | 1,719      | 25  | 4   | 29    | 5   | 0   | 4   | 1   | 10   | 39 (0.0227) | 71 (0.0413) |
| nad4             | 1,341      | 13  | 7   | 20    | 7   | 0   | 4   | 1   | 12   | 32 (0.0238) | 51 (0.0380) |
| nad4L            | 291        | 3   | 0   | 3     | 3   | 0   | 3   | 0   | 6    | 9 (0.0309)  | 12 (0.0412) |
| nad1             | 945        | 7   | 2   | 9     | 1   | 0   | 3   | 3   | 7    | 16 (0.0169) | 24 (0.0257) |
| lrRNA            | 1,378 (1,350) | 12  | 1   | 13    | 4   | 0   | 1   | 0   | 5    | 18 (0.0133) | 26 (0.0189) |
| srRNA            | 783 (784)  | 3   | 1   | 4     | 7   | 1   | 0   | 0   | 8    | 12 (0.0153) | 7 (0.0089)  |
| A+T-rich region  | 494 (484)  | 7   | 0   | 7     | 5   | 1   | 0   | 0   | 6    | 13 (0.0269) | 8 (0.0162)  |

Note: The numbers in parentheses in the second column indicate the gene size of Chinese B. mandarina, when gene sizes vary between Chinese B. mandarina and B. mori C108.

Protein coding genes nad2, cob, nad1, nad3, nad6 and cob are encoded by the major strand, whereas nad5, nad4, nad4L and nad1 are encoded by the minor strand.

Genes lrRNA and srRNA are encoded by the minor strand.

Data in the Sum I column resulted from the comparison between mitochondrial genes of B. mori C108 and Chinese B. mandarina; data in the Sum II column resulted from the comparison between B. mori C108 and Japanese B. mandarina (Yukuhiro et al., 2002).

The A+T-rich region is the only major non-coding region in the mt genomes of animals. Based on the assumption that the A+T-rich region is the fastest evolving region of the mtDNA (Zhang and Hewitt, 1997; Giuffra et al., 2000), it becomes popular as a molecular marker for population genetic and phylogeographic studies of animals, including insects. However, according to Zhang and Hewitt (1997), in terms of nucleotide substitution, the A+T-rich region might not be the fastest evolving region of the mtDNA of insects. Between the mt genes of B. mori and JaBm, the nucleotide sequence divergence in the A+T-rich region is low, higher only than that of the srRNA gene, whereas between B. mori and ChBm, the A+T-rich region presents a moderate sequence divergence, lower only than those of the three protein-coding genes cob, nad3, and nad4L. These observations of nucleotide sequence divergence in the A+T-rich region showed that this is not the fastest evolving gene or region in Bombyx mt genomes, providing direct evidence for the above mentioned assumption of Zhang and Hewitt (1997).

The primary finding of this study was that the nucleotide substitution pattern between the mt genomes of B. mori and ChBm is different from that between B. mori and JaBm. Although the preference of transition by DNA strands between B. mori and ChBm was consistent with that between B. mori and JaBm, the regional variation in nucleotide substitution rate showed a different feature. Comparative mt genomics revealed a lower level of transition mutation (ts:tv = 1.52) between B. mori and ChBm, but a significantly higher level of transition mutation (ts:tv = 4.14) between B. mori and JaBm. It has been suggested that the A+T richness in the mt genome would cause an apparently lower transition bias ratio in closely related species (Tamura, 1992; Yu et al., 1999; Liao and Lu, 2000; Arunkumar et al., 2006). However, the present investigation of the A+T content of the mt genome from the three samples showed that
they were almost identical (from 81.36% in *B. mori* to 81.68% in *Ch*Bm), indicating that the heavy bias towards A+T is not suitable to explain this case. Therefore, the distinct nucleotide substitution pattern suggests that the mt genomes of *Ch*Bm and *Ja*Bm are the result of different evolutionary forces. The mechanism responsible for that difference remains unclear. A possible explanation is that the environmental selection effect could lead to different mutational biases. In line with previous reports (Messier and Stewart, 1997; Wise et al., 1998; Creevey and McInerney, 2002; Jansa et al., 2003), this study presents evidence for mtDNA selection.

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