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Molecular phylogeny of silkmoths reveals the origin of domesticated silkmoth, *Bombyx mori* from Chinese *Bombyx mandarina* and paternal inheritance of *Antheraea proylei* mitochondrial DNA

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Abstract

Molecular phylogeny of some of the economically important silkmoths was derived using three mitochondrial genes, *12S rRNA*, *16S rRNA*, and *COI*, and the control region (CR). Maximum likelihood (ML) analyses showed two distinct clades, one consisting of moths from Bombycidae family and the other from Saturniidae family. The mitochondrial CR showed length polymorphisms with indels. The ML analyses for complete mitochondrial genome sequences of *Bombyx mori* (strains Aojuku, C108, Backokjam, and Xiafang), Japanese and Chinese strains of *B. mandarina* (Japanese mandarina and Chinese mandarina) and, *Antheraea pernyi* revealed two distinct clades, one comprising of *B. mori* strains and the other with *B. mandarina*, and *A. pernyi* forming an outgroup. Pairwise distances revealed that all of the strains of *B. mori* studied are closer to Chinese than to Japanese mandarina. Phylogenetic analyses based on whole mitochondrial genome sequences, the finding of a tandem triplication of a 126 bp repeat element only in Japanese mandarina, and chromosome number variation in *B. mandarina* suggest that *B. mori* must have shared its recent common ancestor with Chinese mandarina. Another wild species of the Bombycidae family, *Theophila religiosa*, whose phylogenetic status was not clear, clustered together with the other bombycid moths in the study. Analysis of the interspecific hybrid, *A. proylei* gave evidence for paternal inheritance of mitochondrial DNA.

Keywords: *Bombyx mori*; *Bombyx mandarina*; Saturniidae; Bombycidae; *Theophila religiosa*

1. Introduction

Among lepidopteran insects silkmoths are the best studied. Silkmoths mainly belong to two families, Bombycidae and Saturniidae. The domesticated silkworm, *Bombyx mori*, a member of the family Bombycidae, is a well-studied lepidopteran model system with rich repertoire of genetic information on mutations affecting morphology, development, and behaviour. Recently completed genome sequence of *B. mori* (Mita et al., 2004; Xia et al., 2004) provides much needed molecular genetic resource for studying a broad range of biological problems (Nagaraju and Goldsmith, 2002).

Non-mulberry feeding sericigenous fauna belonging to the family Saturniidae are mostly wild silkmoths. They are diverse and include semi-domesticated species used for silk production spread over mainly India, China, and Japan. Among saturniids the most well-known species are *A. pernyi*, *A. roylei*, *A. proylei*, *A. mylitta*, *A. assama*, *Samia cynthia ricini*, and *A. yamamai*. The wild silk moth, *A. pernyi* originated in southern China found its commercial use during Han and Wei dynasties. *A. roylei* is distributed along the sub-Himalayan belt of India (Jolly et al., 1981). *A. proylei* is a synthetic hybrid derived from the fertile hybrid of the *A. roylei* and *A. pernyi* (Nagaraju and Jolly, 1986). One of the members of the Bombycidae, *Theophila religiosa* is native to northeast India.

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Information on the phylogenetic relationships of these silkmoths is scanty when compared to insects like *Drosophila* species. A few studies employing nuclear genes (Shimada et al., 1995) and mitochondrial DNA sequences (Hwang et al., 1999; Li et al., 2005) carried out with bombycid and saturniid silkmoths, did not provide sufficient evidence for the origin of present day domestic silkmoths.

The wild silkmoth, *B. mandarina*, is believed to be the ancestor of *B. mori*, as these two species can cross-breed and yield fertile hybrid offspring. *B. mandarina* includes significant variation within species (Yukuhiro et al., 2002). For example, *B. mandarina* inhabiting China (Chinese mandarina) has 28 pairs of chromosomes, similar to that of *B. mori*, whereas *B. mandarina* residing in Japan (Japanese mandarina) and in some regions of Korea has 27 pairs of chromosomes (Banno et al., 2004). Although, a few studies have shown *B. mandarina* as the likely close relative of *B. mori* (Hwang et al., 1999; Yukuhiro et al., 2002), the question of whether the apparent progenitor of *B. mori* is the Chinese or Japanese mandarina still remains elusive.

Characterising geographic patterns of genetic variation within and among populations is a necessary prerequisite for understanding the mechanisms of population differentiation and speciation events. Mitochondrial DNA (mtDNA) has been widely employed in phylogenetic studies of animals because it evolves much more rapidly than nuclear DNA, resulting in the accumulation of differences between closely related species (Brown et al., 1979; Mindell et al., 1997; Moore, 1995). In the present study, we investigated the plausible progenitor of the domesticated silkworm, *B. mori*, and inferred the phylogenetic relationships among six economically important saturniid and bombycid silkmoths, based on mitochondrial DNA sequences. We also showed evidence for paternal inheritance of mitochondrial DNA in an interspecific hybrid *A. proylei*.

2. Materials and methods

2.1. Specimens and loci analysed

Six silkmoth species of the superfamily Bombycidae listed in Table 1 were analysed for four mitochondrial loci, *12S rRNA*, *16S rRNA*, *COI*, and the control region (CR). The geographic distribution of these species is shown in Fig. 1. Two strains of *B. mori*, Nistari and NB_4D_2, which have nondiapausing and diapausing characters, respectively, were used. *A. proylei* is the F_{72} generation of an interspecific hybrid of *A. roylei* and *A. pernyi*, where the former was the maternal parent. Of the six species, mitochondrial *12S rRNA*, *16S rRNA* and *COI* genes, and CR from three saturniid moths, two bombycid moths were sequenced. The complete mitochondrial sequences of Japanese mandarina and Chinese mandarina were obtained from GenBank (Accession No. NC_003395 and AY301620).

2.2. DNA extraction, PCR amplification, and sequencing

Genomic DNA was isolated from the silkmoth samples using a standard protocol (Nagaraja and Nagaraju, 1995). The primer sequences for *12S rRNA*, *16S rRNA*, and *COI* genes were taken from Kambhampati and Smith (1995). For the mitochondrial CR, primers were designed based on the complete mitochondrial genome sequence of *B. mori* (Accession No. AB070264). The primer sequences used for CR were CR1: GCAACTGCTGGCACAAAAT and CR2: TGAGGTATGAGCCCAAAAGC.

PCR reactions were carried out in 10 mM Tris–HCl, pH 8.3 (50 mM KCl/1.5–3.0 mM $MgCl_2$/0.01% gelatin/0.01% Triton X-100), 1 mM dNTPs, with 2 pmol of each primer and 0.5 U of *Taq* DNA Polymerase (MBI Fermentas) per reaction. Amplification was carried out in a thermal cycler (PE9700, Applied Biosystems) using the following conditions: initial denaturation of 3 min at 94 °C; 35 cycles of 30 s at 94 °C; 30 s at 40 °C (for *12S* and *16S rRNA* genes), 45 °C (for *COI* gene) and 60 °C (for control region); and 2 min at 72 °C; and final extension of 10 min at 72 °C. PCR products were used directly for sequencing in both forward and reverse orientations. For DNA sequencing, 50 ng of PCR product was used in a sequencing reaction containing 8 μl Ready reaction mix (BDT v 3.0, Applied Biosystems, Foster City, CA) and 5 pmol of primer. The cycling conditions were as follows: 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Samples were precipitated and washed with 70% ethanol and resuspended in Hi-Di™ formamide (Applied Biosystems). Sequencing was carried out on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Table 1
Bombycid and saturniid silkmoths used in the study

Species name	Chromosome number (*n*)	Family	Common name	Geographical distribution
B. mori (Nistari)	28	Bombycidae	Domestic silkmoth	Karnataka, India
B. mori (NB_4D_2)	28	Bombycidae	Domestic silkmoth	Karnataka, India
B. mandarina (Japan)	27	Bombycidae	Wild silkmoth	Tokyo, Japan
B. mandarina (China)	28	Bombycidae	Wild silkmoth	China
T. religiosa		Bombycidae	Wild silkmoth	West Bengal, India
A. roylei	30, 31, 32[a]	Saturniidae	Indian temperate tasar silkmoth	Jammu & Kashmir, India
A. pernyi	49	Saturniidae	Chinese oak silkmoth	China
A. proylei[b]	49	Saturniidae	Synthetic oak silkmoth	Jammu & Kashmir, India

[a] *A. roylei* exhibits chromosome number polymorphism (Jolly et al., 1979).
[b] F_{72} generation of an interspecific hybrid of *A. pernyi* × *A. roylei* (Nagaraju and Jolly, 1986).

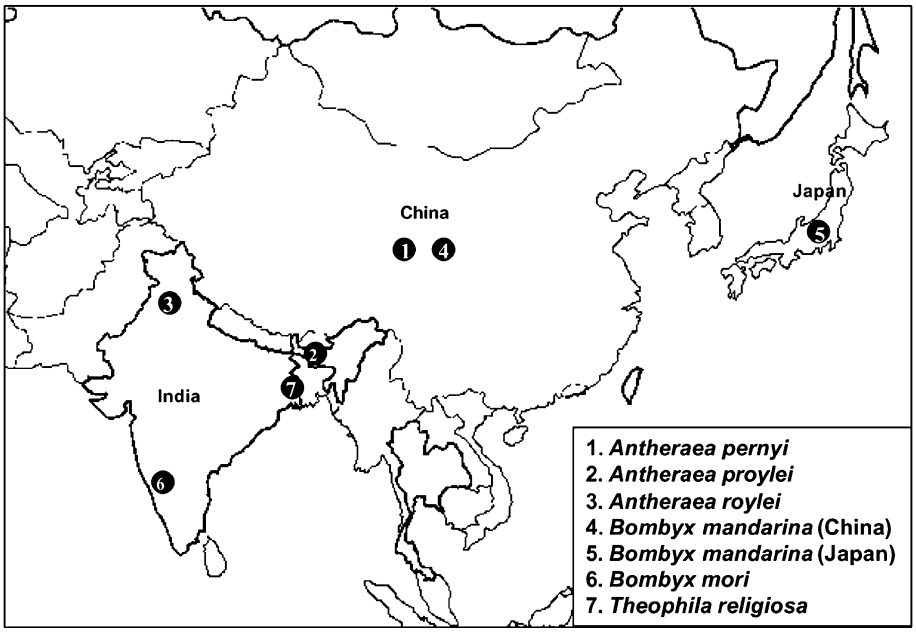

Fig. 1. Geographic distribution of silkmoths used in the study.

2.3. Phylogenetic analysis

In the present study, *Drosophila melanogaster* was used as an outgroup. The complete mitochondrial genome sequences of *B. mori* (strains Aojuku [Japan—AB083339], Backokjam [Korea—AF149768], C108[China—AB0702 64], and Xiafang [China—AY048187]), Chinese and Japanese mandarina, and *A. pernyi* were retrieved from GenBank for analyses.

The sequences were aligned using ClustalX 1.8 (Thompson et al., 1997) and manually edited using GeneDoc Version 2.6.002 (Nicholas et al., 1997). The LogDet transformation distance (Lockhart et al., 1994) and the number of substitutions per synonymous (*Ks*) and non-synonymous sites (*Ka*) were calculated according to Li (1993) as implemented in DAMBE (Data Analysis in Molecular Biology and Evolution) 4.2.13 (Xia and Xie, 2001). Phylogenetic data analyses were executed in PAUP* 4 version beta 10 (Swofford, 2000) using maximum likelihood (ML) optimality criteria. For the ML analysis, the appropriate substitution model was calculated using MODELTEST version 3.06 (Posada and Crandall, 1998). The best-fit maximum likelihood (ML) score was chosen using the Akaike Information Criterion (AIC) (Akaike, 1974), since this reduced the number of unnecessary parameters which contributed little to describing the data by penalizing more complex models (Burnham and Anderson, 2002; Clary and Wolstenholme, 1985; Nylander et al., 2004). Uncorrected sequence divergence values were calculated between samples. Phylogenetic confidence in the nodes recovered from ML was estimated by bootstrapping (Felsenstein, 1985), analyzing 1000 pseudo replicates of data sets. The bootstrap values >50% were regarded as strongly supported.

The transition bias was calculated from the full-length gene sequences of *COI*, *12S rRNA*, and *16S rRNA* to find recently evolved species among three bombycid silkmoths, *B. mori*, Chinese and Japanese mandarina, using DAMBE. The gene sequences were taken from respective complete mitochondrial genome sequences.

3. Results

3.1. Sequence statistics

We sequenced 527 bp of *COI*, 439 bp of *12S rRNA*, 432 bp of *16S rRNA*, and 689 bp of CR (including indels). Since CR primers did not amplify the DNA in *T. religiosa*, which may be attributed to possible mutations in primer binding sites, the CR in this species could not be sequenced. The sequences of *12S rRNA*, *16S* r*RNA*, *COI* and CR were submitted to GenBank (Accession Nos. AY037827–AY037834, DQ415441–DQ415456 and DQ417193). The *16S rRNA* was highly conserved across the species and genera, and only a few informative sites were observed. Since these informative sites were in the form of substitutions only in *B. mori* (strain NB_4D_2) and Chinese mandarina, we ignored this gene for phylogenetic analysis. The remaining three loci (*12S rRNA*, *COI*, and CR) showed a number of informative sites in all the species studied. The sequence polymorphisms and the base composition are given in Table 2. All the loci showed a bias toward high A + T content. Irregular base compositions are a possible general cause of patterns that can mislead tree-reconstruction methods even when high bootstrap values are obtained (Lockhart et al., 1994). To avoid this problem, we measured LogDet transformation distance along with ML distance for *12S rRNA* and *COI* genes. We observed no difference in tree topologies using both measures. The mitochondrial CR showed considerable length polymorphism across the species and genera. Synonymous substitutions outnumbered in

Table 2
Sequence polymorphism details and base composition of the mitochondrial loci analysed in the study

Loci name	Total length (bp)[b]	Variable sites	Parsimony informative sites	A	C	G	T
12S rRNA	439	116	43	0.402	0.087	0.116	0.395
16S rRNA	432	48	30	0.398	0.086	0.134	0.382
COI	527	161	71	0.378	0.144	0.176	0.302
CR	689	183	100	0.453	0.036	0.025	0.486
Complete mitochondrial sequence[a]	16192	613	267	0.431	0.114	0.073	0.383

[a] *A. pernyi* sequence was used as outgroup.
[b] Including indels.

COI compared to the *12S rRNA* gene; further, synonymous substitutions outnumbered non-synonymous substitutions in both the loci.

The transition bias was calculated for the full-length gene sequences of *12S rRNA*, *16S rRNA*, and *COI*. It was found to be low between *B. mori* and Chinese mandarina (1.30), and between Chinese and Japanese mandarina (1.30) compared to the Japanese mandarina and *B. mori* pair (5.0).

3.2. Phylogenetic analysis

The best model for different data sets was selected based on the AIC (Table 3). The ML analyses showed almost similar tree topologies for all the three informative loci in all the species. A 50% bootstrap consensus tree topology was always considered. All of the trees showed two distinct clades, one consisting of silk moths of Bombycidae and the other with Saturniidae (Figs. 2A–C). The LogDet transformation distances also showed similar relationships between species. The *12S rRNA* gene and CR placed Japanese as well as Chinese mandarina at equidistance from both Nistari and NB_4D_2 strains of *B. mori*. However, the *COI* gene data suggested that Chinese mandarina is closer to both strains of *B. mori* than to Japanese mandarina. Saturniid moths formed a separate clade in the phylogenetic tree (Figs. 2A–C). Interestingly the interspecific hybrid *A. proylei* was more close to its male parent, *A. pernyi* than to its female parent, *A. roylei*, in all the three loci studied (Fig. 4).

Table 3
Parameter estimates of substitution rates, proportion of variable sites (I) and the shape parameter (Γ) based on ML analysis

Locus	Model	A–C	A–G	A–T	C–G	C–T	I	Γ
12S rRNA	TVM	0.9712	3.2317	2.6048	0.0001	3.2317	0	0.3381
COI	GTR + I	0.5654	3.9679	1.7638	0.2749	1.1995	0.4959	2.4357
CR	TIM	1.0000	2.6296	0.4538	0.4538	1.3613	0	2.2785
Complete mitochondrial sequence	GTR + I	6.7887	7.4640	7.7657	3.2266	67.3019	0.6750	Infinity

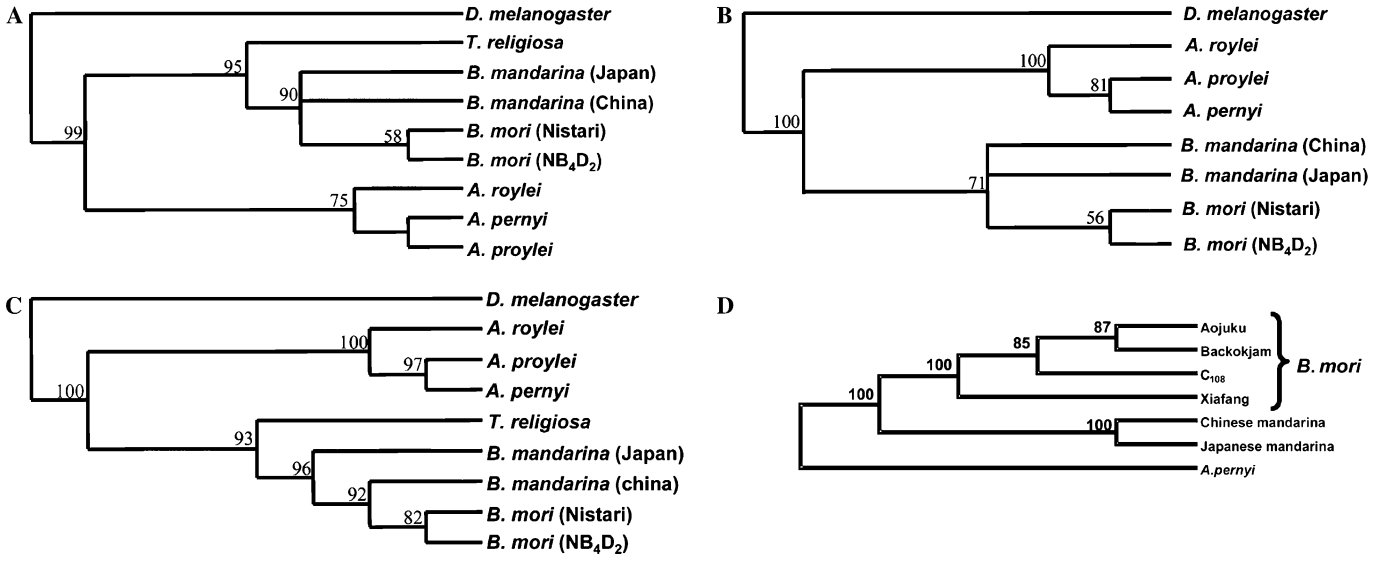

Fig. 2. Maximum Likelihood tree inferred from all informative sites of (A) *12S rRNA*, (B) CR and (C) *COI* for six silkmoth species and, (D) complete mitochondrial genome analysis using four species, *B. mori*, Chinese mandarina, Japanese mandarina, and *A. pernyi*. Percentage bootstrap values based on 1000 replicates are given at the internal branches. The bootstrap values >50% were only considered.

The ML analyses of complete mitochondrial genome sequences of bombycid moths, *B. mori* (strains Aojuku, C108, Backokjam, and Xiafang), Japanese mandarina, Chinese mandarina using *A. pernyi* as outgroup showed two distinct clades, one with strains of *B. mori* and the other with Chinese and Japanese mandarina (Fig. 2D). The ML data unambiguously suggested that all the strains of *B. mori* are closer to Chinese mandarina than to Japanese mandarina. Furthermore, the complete mitochondrial sequence alignment revealed the presence of a~378 bp repetitive region in the CR of Japanese mandarina (Fig. 3). Further analysis showed that this repeat region harboured tandemly triplicated ~126 bp repeat element. The Chinese mandarina and *B. mori* strains, on the other hand, revealed only one 126 bp repeat element. Each of the 126 bp elements is comprised of ~64 bp and ~62 bp repeat units. The 64 bp unit (shown in red in Fig. 3) consists of 44 bp core sequence flanked by 10 bp perfect inverted repeats whereas the 62 bp unit (shown in green in Fig. 3) harboured 50 bp core unit flanked by 6 bp perfect inverted repeats. This repeat region was found to be unique to *Bombyx* species, as it had no apparent homology to any mitochondrial sequence submission in the NCBI database. When the three 126 bp repeat elements of Japanese mandarina were aligned, the first two elements were found to be identical whereas the third element had

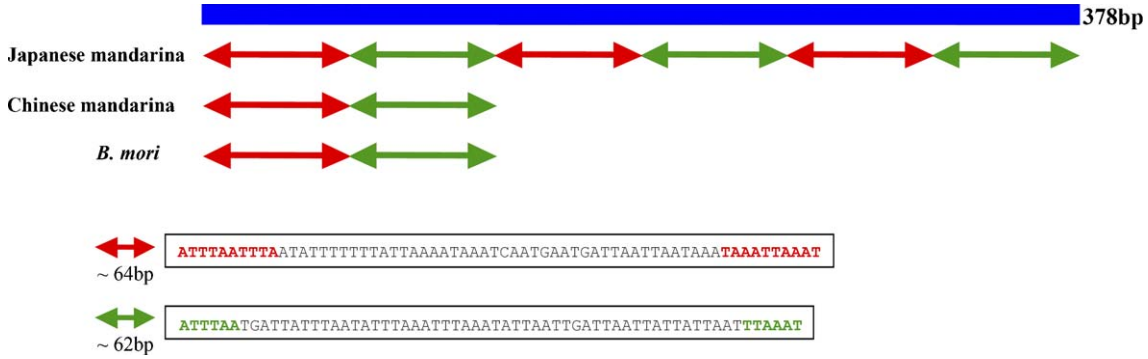

Fig. 3. Comparison of 126 bp repeat element in Japanese mandarina, Chinese mandarina, and *B. mori*. Arrow marks represent inverted repeats and their direction. The 64 bp repeat elements are represented in red and 62 bp elements in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

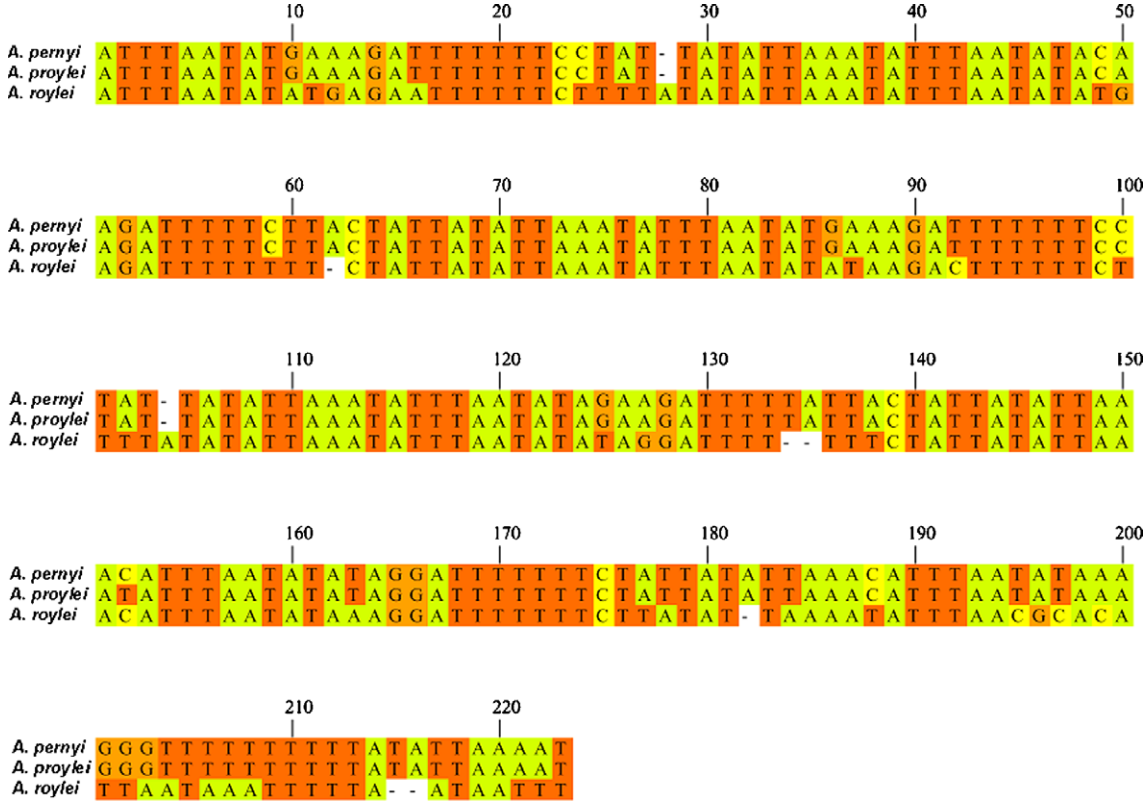

Fig. 4. Multiple sequence alignment of mitochondrial control region (CR) from *A. pernyi*, *A. proylei*, and *A. roylei*.

three substitutions and an AT insertion in the 62 bp unit. However, the 64 bp unit was conserved in all the three 126 bp repeat elements (Supplementary figure 1). Out of the three 126 bp repeat elements of Japanese mandarina, the third repeat unit was closer to Chinese mandarina and *B. mori* strains.

The mitochondrial CR of *A. pernyi*, *A. roylei*, and *A. proylei* showed a characteristic repeat element of 38 bp tandemly repeated six times. This repeat region was unique only to these three species and had no apparent homology to any mitochondrial sequence submission in the NCBI database. Each repeat unit contained ~20 bp core motif flanked by 9 bp perfect inverted repeats (Supplementary figure 2). Phylogenetic analysis using only these repeat regions revealed that *A. proylei* is closer to *A. pernyi* than to *A. roylei*.

We compared the microsatellite repeat motifs in the mitochondrial genome of these moths using a complete mitochondrial genome alignment and observed microsatellite length variation at three loci (Supplementary figure 3). There was a complete absence of microsatellite repeat motifs in *A. pernyi* at both the loci, hinting at a more recent origin of these repeat motifs in bombycid moths after their divergence from a common ancestor. At both the loci, repeat motifs were found to be longer in Chinese and Japanese mandarina than in *B. mori* strains. Further, in Chinese mandarina and Japanese mandarina point mutations were prevalent in the microsatellite motifs rendering them as imperfect repeats.

4. Discussion

In the present study, a molecular phylogeny of six species of silkmoths was derived using four mitochondrial loci. Phylogenetic methods of ML were employed to construct phylogenetic trees. In addition, complete mitochondrial genome sequences were used to determine the phylogenetic relationships among *B. mori*, Chinese mandarina, Japanese mandarina, and *A. pernyi*.

All the three mitochondrial genes and CR in the silkmoths showed strong bias towards higher AT content, as is the case in other insects (Clary and Wolstenholme, 1985; Yu et al., 1999). A lower transition bias ratio between *B. mori* and Chinese mandarina, and between Japanese and Chinese mandarina was observed, and a higher transition bias ratio between *B. mori* and Japanese mandarina. The transition bias was accompanied by A + T richness in the mitochondrial genes of these silkmoths, which causes an apparently lower transition bias ratio in closely related species, as contended by Tamura (1992) and Yu et al. (1999) in *Drosophila*. The lower transition bias between *B. mori* and Chinese mandarina observed in the present study suggested lower nucleotide divergence and closer affinity of the two species. This was also consistent with the lower genetic distances and low rate of nucleotide substitutions observed in the whole mitochondrial genome sequence analysis.

4.1. Evolutionary path of the domesticated silkmoth *B. mori*

The molecular phylogeny of *B. mori* and *B. mandarina* revealed several interesting features about the evolutionary history of the two species. *B. mori* and *B. mandarina* are morphologically and physiologically similar, but differ in flight behaviour, egg laying and a kind of mimicry. The moths of *B. mandarina* fly a good distance for mating, while those of *B. mori* are unable to fly, barely moving over the breeding site. The loss of flight in *B. mori* is considered to have occurred as a consequence of domestication. From the chromosomal study of these species, Astaurov et al. (1959) discovered that Chinese mandarina has 28 pairs of chromosomes (*n*) and in contrast, Japanese mandarina possesses 27 pairs of chromosomes (*n*). Analysis of meiotic chromosomes of the F_1 hybrids of *B. mori* and Japanese mandarina revealed a trivalent, suggesting that one of the *B. mandarina* chromosomes might have split into two during the generation of the *B. mandarina* variant of *B. mori* (Kawaguchi, 1934; Murakami and Imai, 1974). Recent studies on interspecific hybrids of *B. mori* and Japanese mandarina also revealed that the M-chromosome of Japanese mandarina corresponds to two chromosomes of *B. mori* (Banno et al., 2004). Initial speciation of Chinese mandarina must have accomplished by the fragmentation of one of the chromosomes of Japanese mandarina. Analysis of four mitochondrial loci and complete mitochondrial genomes strengthen this speculation. Although, *B. mori* is thought to have originated from *B. mandarina*, large amounts of sequence divergence between the two suggest a significant genetic isolation of the current Japanese population of *B. mandarina* from the population or populations from which *B. mori* was derived (Banno et al., 2004; Yukuhiro et al., 2002). Our studies based on sequence analyses of four mitochondrial loci suggest that Chinese mandarina is a likely progenitor species of *B. mori*.

Additional information from the analysis of the complete mitochondrial genome sequence of four strains of *B. mori*, Chinese mandarina, and Japanese mandarina also strengthens this conclusion. Taken together, these observations allow us to conclude that the present day *B. mori* shares its most recent common ancestor with Chinese mandarina and has subsequently spread to other parts of the world such as Japan, Korea, and India.

Japanese mandarina revealed the presence of tandemly triplicated 126 bp repeat element in the CR region of whereas the Chinese mandarina and all the four strains of *B. mori* have only one such element. Yukuhiro et al. (2002) have also made similar observations for Japanese mandarina and *B. mori* strain C108. We have also confirmed the presence of only one repeat element in all the Indian *B. mori* strains and in Korean mandarina (data not shown). These observations suggest that one of two possible molecular events would have occurred in the course of time: (1) the two repeat elements were probably deleted in Chinese mandarina before the divergence of *B. mori*, after the divergence of Chinese mandarina from Japanese mandarina or

(2) the tandem triplication of the repeat element occurred only in Japanese mandarina after the divergence of Chinese mandarina. Similarly, in an earlier study, Shimada et al. (Shimada et al., 1995) found a retro transposon like sequence insertion in the third intron of the arylphorin gene only in Japanese mandarina.

In previous studies, it has been proposed that *B. mori* probably originated in China from Chinese mandarina about 4600 years ago (Yoshitake, 1968). Moreover, it has been speculated that Japanese mandarina segregated from Chinese mandarina long before the islands of Japan were completely separated from the Asian continent about 0.02 MYA (Minato, 1966; Maekawa et al., 1988). The close relationship of *B. mori* strains with Chinese mandarina than with Japanese mandarina in as revealed by the present study supports the hypothesis that Chinese mandarina diverged from Japanese mandarina before the domestication of *B. mori* (Yamauchi et al., 2000). A recent report on molecular phylogeny of domesticated silkmoth, *B. mori* based on the mitochondrial cytochrome *b* genes also indicated that Chinese mandarina was more close to *B. mori* than to Japanese mandarina (Li et al., 2005).

4.2. Phylogenetic status of T. religiosa

In the present study, we provide phylogenetic status of another bombycid moth *T. religiosa*. This species is the most divergent species in the bombycid clade. Our analysis indicated that this species shared its last common ancestor with the remaining bombycid silkmoths analysed in the present study.

4.3. A. proylei is closer to A. pernyi, its male parent, than to its female parent, A. roylei

The synthetic hybrid, *A. proylei* derived from a backcross of an F_1 hybrid (*A. roylei* female × *A. pernyi* male) females to *A. pernyi* males, showed 98% sequence similarity to *A. pernyi* for all the three mitochondrial loci studied. This is the antithesis to the well-accepted dogma of maternal inheritance of mitochondria and absence of recombination. Evidence for occasional paternal inheritance has been reported in literature including *Homo sapiens* (Schwartz and Vissing, 2002), *Ovis aries* (Zhao et al., 2004), *Bos taurus* (Steinborn et al., 1998), and *Apis mellifera* (Meusel and Moritz, 1993). Based on expected maternal transmission of the mitochondrial genome, *A. roylei* should have contributed the mitochondrial genome to *A. proylei*. Also, no heteroplasmy of mtDNA was observed in *A. proylei*, which excludes the possibility of recombination between maternally and paternally contributed mitochondrial genomes unlike reported in several animal groups (Burzynski et al., 2003; Kraytsberg et al., 2004; Ladoukakis and Zouros, 2001; Lunt and Hyman, 1997; Saville et al., 1998).

Previous reports suggest that paternal mtDNA can enter eggs with sperm, and might be maintained at low level in the fertilized eggs and sperm mitochondria disappear during early embryogenesis by selective destruction, inactivation, or simple dilution by the vast surplus of oocyte mitochondria (Cummins et al., 1998). The studies reveal that paternal inheritance of mtDNA is frequently observed in interspecific hybrids as in the case of mice (Gyllensten et al., 1991; Kaneda et al., 1995), cattle (Sutovsky et al., 1999), and *Drosophila* (Kondo et al., 1990). This tempts us to speculate that because of the failure to identify the mitochondria contributed by the sperm from a different species, the paternally contributed mitochondria might persist in this interspecific hybrid. A comprehensive study encompassing many interspecific hybrids would give better insights into this phenomenon.

The results showed high mitochondrial sequence variability among *A. roylei*, *A. pernyi*, and *A. proylei*. A high mutation rate in interspecific hybrids has been observed in *Drosophila* and other organisms (Gerstel and Burns, 1967; Miller, 1950; Sturtevant, 1939). Human intervention during the interspecific hybridization of *A. roylei* and *A. pernyi* followed by selection for economically important traits for many generations may have hastened the process of genome evolution.

Our studies on 38 bp repeat structure of *A. roylei*, *A. pernyi*, and *A. proylei* also support the paternal inheritance of mitochondria. Interestingly, this diagnostic repeat structure is absent (data not shown) in other saturniids, *A. assama*, *A. mylitta*, and *S. c. ricini*. This repeat region is probably inserted after the divergence of *A. pernyi* and *A. roylei* from other saturniids. The phylogenetic analysis using three mitochondrial genes and CR shows that *A. pernyi* and *A. roylei* appear to have evolved recently. Nagaraju and Jolly (1986) performed cytogenetic assessment of *A. pernyi*, *A. roylei*, and *A. proylei*, and concluded that, in spite of allopatry and karyotypic divergence, a high degree of homology exists between the chromosomal complements of *A. pernyi* and *A. roylei* and *A. pernyi* possibly evolved from *A. roylei* through chromosomal fission. The results on mitochondrial sequence based phylogenetic analysis also suggest close relationship between the two species.

4.4. The control region contains more repeat regions

It has been reported that the lepidopteran CR is prone to indels (Brooks et al., 1997; Sperling, 1991). The CR has been described for only seven species of lycaenids (Taylor et al., 1993) and two moths (McKechnie et al., 1993a; McKechnie et al., 1993b). Thus, data from only nine species of Lepidoptera appear in sequence databases, although complete mitochondrial genomes are given for *B. mori* and *B. mandarina*. Our results showed a length variation in the CR at the interspecies level. The CR has sufficient phylogenetic signal to be informative to distinguish insects at the family level. Nevertheless, when the CR is used for species-level phylogenetic analysis, a clear strategy is necessary while analysing the indels. Overall, the CR showed higher nucleotide diversity than the other three genes studied. Some species tend to show nucleotide variability as indels. Whether

indels have arisen rather than substitutions in those species or past demographic events have caused the differences is not clear. Four species in our study, Japanese mandarina, *A. pernyi*, *A. roylei*, and *A. proylei*, showed the presence of repeat regions in CR with flanking inverted repeats. The inverted repeats could be responsible for the duplication of the regions between them (Ford and Fried, 1986). Further, point mutations within the repeat units suggest the occurrence of duplication events at different time periods. Although, inverted repeats were present in *B. mori* and Chinese mandarina, no duplicated regions were observed. Despite the demonstration that inverted repeats are implicated in gene amplification (Tanaka et al., 2002), in most of the cases inverted repeat harbouring regions are not associated with gene amplification, but they are more likely to be amplified than are random loci (Tanaka et al., 2005). So it may be difficult to decipher the molecular switch associated with amplification of regions containing inverted repeats.

The present study suggests that the mitochondrial *12S*, *COI*, and the CR are suitable for resolving the phylogenetic relationships of the species of the superfamily Bombycidae. The analysis based on chromosome number, transition bias ratio and tandem triplication of a 126-bp repeat element in mitochondrial DNA only in Japanese mandarina, suggest that *B. mori* must have diverged from its wild relative, Chinese mandarina. Another wild species of Bombycidae family, *T. religiosa*, appeared to share a common ancestor along with *B. mori* and *B. mandarina*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2006.02.023.

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