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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).