RESEARCH ARTICLE

Molecular phylogeny of silk-producing insects based on 16S ribosomal RNA and cytochrome oxidase subunit I genes

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Abstract

We have examined the molecular-phylogenetic relationships between nonmulberry and mulberry silkworm species that belong to the families Saturniidae, Bombycidae and Lasiocampidae using 16S ribosomal RNA (16S rRNA) and cytochrome oxidase subunit I (*coxI*) gene sequences. Aligned nucleotide sequences of 16S rRNA and *coxI* from 14 silk-producing species were used for construction of phylogenetic trees by maximum likelihood and maximum parsimony methods. The tree topology on the basis of 16S rRNA supports monophyly for members of Saturniidae and Bombycidae. Weighted parsimony analysis weighted towards transversions relative to transitions (ts, tv4) for *coxI* resulted in more robust bootstrap support over unweighted parsimony and favours the 16S rRNA tree topology. Combined analysis reflected clear biogeographic pattern, and agrees with morphological and cytological data.

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Introduction

Silk-producing insects of Lepidoptera can be divided into two major groups, namely mulberry and nonmulberry silkworms. Mulberry silk is mainly contributed by *Bombyx mori* of the family Bombycidae. It is domesticated, wide in distribution, and is a major part of the sericulture industry. Unlike mulberry silks, nonmulberry silks are more heterogeneous and have apparently originated in the Indo-Australian region of the Gondwana belt, or may have even wider distribution (Jolly *et al*. 1974). The nonmulberry silks are tasar, muga, eri and fagaria, which are mainly produced by species of the Antheraea and Attacini tribes of the family Saturniidae. Most of the species in this family are wild and polyphagous in nature, and the silks produced by them are specific to a particular geographical zone: temperate tasar is produced by *Antheraea pernyi*, *A. roylei* and *A. proylei*, tropical tasar by *A. mylitta*, muga silk by *A. assama*, eri silk by *Philosamia ricini*, fagaria by *Attacus atlas*, and shashe silk by *Gonometa postica*. The silk-producing insects have been classified on the basis of morphological clues such as follicular imprints on the chorine egg, the arrangement of tubercular setae on the larvae, and karyotyping data (Jolly *et al*. 1969, 1970a; Sinha *et al*. 1994). Classification and phylogenetic analysis of species on the basis of morphological attributes may be riddled with problems because morphological features may be variable with environment (Shouche and Patole 2000). Molecular features provide an alternative. Different molecular markers are available for characterization of genetic diversity of *Bombyx mori* (Reddy *et al*. 1999; Nagaraju and Goldsmith 2002; Goldsmith *et al*. 2005), whereas very little attention has been focussed on the genetic diversity of nonmulberry silkworms. It is essential to develop a DNA marker system to study genetic diversity among all varieties of silkworms (Prasad *et al*. 2002; Chatterjee and Tanushree 2004).

Selection of a gene for phylogenetic analysis plays a pivotal role (Whitfield and Cameron 1998). Ribosomal RNA and cytochrome oxidase genes encoded by the mitochondrial genome are commonly used in molecular-phylogenetic studies because they have conserved sequences whose evolutionary rate is appropriate to resolve phylogenetic relationships between small taxonomic units like genera or families (Brower 1994; Canapa *et al*. 2000; Han 2000; Niehuis and Wagele 2004). There are several reports on the utility

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of mitochondrial genes for inferring relationships among closely related species and species groups in butterflies (Davies and Bermingham 2002; Blum *et al*. 2003). The phylogenetic relationships among members of Saturniidae and Bombycidae have earlier been studied on the basis of nuclear genes (elongation factor 1α, DOPA decarboxylase and arylphorin) and mitochondrial DNA (16S rRNA and RFLP of mtDNA) (Shimada *et al*. 1995; Friedlander *et al*. 1998; Hwang *et al*. 1999a,b). The previous analysis was limited to *Antheraea pernyi*, *A. yamamai* and *Attacus atlas* of Saturniidae, and *Bombyx mori* and *B. mandarina* of Bombycidae.

Here we present a phylogenetic analysis of 14 silkworm species based on sequences of 16S ribosomal RNA (rRNA) and cytochrome oxidase subunit I (*coxI*) genes.

Materials and methods

Species collection

Fresh live cocoons were obtained from different locations in India and other countries. The cocoons were stored frozen at −80°C. The geographical distribution and host plants of 10 collected silk-producing insects are given in table 1.

Genomic DNA isolation

Total genomic DNA was isolated from either fresh or frozen pupae by the standard laboratory protocol (Datta *et al*. 2001). Prior to DNA extraction, the digestive tract was taken out from the pupal body by dissection. About one gram of fat body tissue was ground in liquid nitrogen and incubated overnight at 50°C in 10 ml digestion buffer (0.01 M NaCl, 0.1 M Tris-HCl, 0.25 M EDTA and 0.5% SDS with 100 μg/ml proteinase K). The digested samples were extracted twice with an equal volume of Tris-HCl, saturated phenol (pH 8.0), and then centrifuged at 5000 *g* for 15 min to remove protein contaminants and debris. The supernatant was transferred into a fresh tube and treated with RNAase at 37°C for 30 min, followed by chloroform extraction and centrifugation at 5000 *g* for 15 min. Finally, the aqueous phase from each tube was transferred separately to clean centrifuge tubes and mixed with 0.1 volume of 3 M sodium acetate, pH 5.2. Genomic DNA was precipitated with two volumes of cold ethanol, spooled, washed twice with 70% ethanol, dried, and suspended in 10 mM Tris-Cl (pH 8.0).

Amplification and sequencing of 16S rRNA and coxI genes

A 383-bp region of 16S rRNA gene and a 597-bp region of *coxI* gene were amplified by polymerase chain reaction (PCR). The primers used for amplification of the 383-bp fragment of 16S rRNA gene were forward 5′-GTGCAAAGGTAGCATAATCA-3′ and reverse 5′-TGTCCTGATCCAACATCGAG-3′. The primers for amplification of the 597-bp fragment of *coxI* were forward 5′-TGATCAAATTTATAATAC-3′ and reverse 5′-GTAAAATTAAAATATAAAC-3′ (Hwang *et al*. 1999a,b). PCR was carried out in 50 μl using 1 U of *Taq* polymerase (Roche) in a Perkin Elmer 2400 thermal cycler. The PCR reaction conditions for 16S rRNA were 94°C for 30 sec (denaturation), 55°C for 1 min (annealing) and 72°C for 1 min for 35 cycles. The PCR conditions for *coxI* were identical to those for 16S rRNA except that the annealing temperature was 50°C. The PCR products were

Table 1. Collected silk-producing species.

Species (Host plants)	Place of collection	Distribution	Chromosome number (2*n*)
Antheraea mylitta (*Terminalia arjuna, T. tomentosa, Shorea robusta*)	West Bengal, India	North and Central India	62
Antheraea frithi (*Lithocarpus dealbata, Quercus dealbata*)	Imphal, India	Eastern India	62
Antheraea assama (*Machilus bombycina*)	Assam, India	Eastern India	30
Antheraea proylei (*Quercus serrata, Q. incana*)	Imphal, India	Himalayan belt of India	98
Antheraea roylei (*Quercus* spp.)	Imphal, India	Himalayan belt of India	60, 62, 64, 68
Antheraea polyphemus (*Quercus alba, Q. nigra, Q. rubra*)	Edward Island, Canada	North America	60
Philosamia ricini (*Ricinus communis, Manihot utilissima*)	Assam, India	India	28
Attacus atlas (*Ailantus altissima, Ligustrum, Syringa*)	Yogyakarta, Indonesia	Southeast Indonesia	–
Gonometa postica (*Colophospermum mopane*)	Kalahari desert	Southeast Namibia	–
Hyalophora cecropia (*Acer saccharinum, Prunus* spp, *Quercus* spp.)	Edward Island, Canada	North America	62

(Modified from Jolly et al., 1974).

gel-purified using Qiagen gel extraction kit (Qiagen, Hilden, Germany). The purified DNA fragments were cloned into a pCR2.1 TOPO TA cloning vector (Invitrogen, USA). The ligated products were used to transform *E. coli* DH5α. The plasmids were isolated, and the inserts were verified according to Sambrook and Russell (2001) and sequenced using our automated cycle sequencing facility (ABI Prism 3770). The sequencing was performed using 400 ng of plasmid DNA as a template and 2.0 pmol of primer (M13 forward or reverse). The Sequencher (Gene Codes Corp.) was used for sequence assembly and evaluation. The sequences of 16S rRNA and *coxI* genes were analysed using BLAST (Altschul *et al*. 1990). The obtained sequences were aligned with their orthologues from *B. mori* (AB070264 for 16S rRNA and *coxI*), *B. mandarina* (AB070263 for 16S rRNA and *coxI*), *A. yamamai* (AF027952 for 16S rRNA and AF029067 for *coxI*), *A. pernyi* (AY242996 for 16S rRNA and *coxI*), *Hyalophora cecropia* (AY165720 for *coxI*) and *Spodoptera frugiperda* (M76713 for 16S rRNA and U72976 for *coxI*) using ClustalW (Thompson *et al*. 1994). Sequence statistics were obtained using MEGA 2.1 (Kumar *et al*. 2001). The sequences of 16S rRNA and *coxI* genes have been submitted to GenBank (accession numbers AY598830 (*A. mylitta* 16S rRNA gene), AY604239 (*H. cecropia* 16S rRNA gene), AY604240 (*Genometa postica* 16S rRNA gene), AY601275 (*A. frithi* 16S rRNA gene), AY601276 (*A. assama* 16S rRNA gene), AY601277 (*A. proylei* 16S rRNA gene), AY601278 (*A. polyphemus* 16S rRNA gene), AY601279 (*Attacus atlas* 16S rRNA gene), AY601280 (*Philosomia ricini* 16S rRNA gene), AY960275 (*A. roylei* 16S rRNA gene), AY605248 (*A. frithi coxI* gene), AY605249 (*A. assama coxI* gene), AY605250 (*A. proylei coxI* gene), AY605251 (*A. polyphemus coxI* gene), AY605252 (*Attacus atlas coxI* gene), AY605253 (*P. ricini coxI* gene), AY605254 (*G. postica coxI* gene), AY605255 (*A. mylitta coxI* gene), AY960274 (*A. roylei coxI* gene)). The saturation analysis and filtering of data in relation to the type of substitution and codon position were performed for all three codon positions for *coxI* (Griffiths 1997).

Phylogenetic analysis

The two genes (16S rRNA and *coxI*) were initially analysed separately, because different genes may experience different evolutionary pathways. The phylogenetic analyses were performed with PAUP* 4.01 version beta 10 (Swofford 2001) using maximum parsimony (MP) and maximum likelihood (ML) approaches. MP trees were produced using heuristic searches with 50 repetitions using random stepwise addition of taxa and gaps were also treated as missing characters. Tree-bisection-reconnection (TBR) branch swapping, Multrees option, and accelerated transformation (ACCTRAN) character-state optimizations were in effect. Multiple-state taxa were interpreted as uncertain and the branches were collapsed if the maximum length was zero. For all analyses, tree length (L), consistency indices (CI; Kluge and Farris 1969), and retention indices (RI; Farris 1989) were reported. The internal stability of the inferred MP tree was measured by bootstrapping using 1000 replications (Felsenstein 1985). Further, the phylogenetic analysis was conducted on a combined data set (16S rRNA and *coxI*). To examine the effect of transition-to-transversion weighting schemes on tree topology, further analyses were run with transitions down-weighted relative to transversions by a factor of four to compensate for the observed transition bias. The transition–transversion ratio was calculated using the program MEGA 1.01 (Kumar *et al*. 2001).

The most appropriate substitution model for ML analysis was determined by using Modeltest version 3.06 (Posada and Crandall 1998) for data on each gene and for the combined data. Sequence divergence values were also calculated from the selected model. Rate of heterogeneity was expressed using gamma distribution with the shape parameter. In both the approaches insertions, deletions and missing data were completely excluded and the sequences were analysed in favour of the outgroup selection. *Spodoptera frugiperda*, a Noctuidae species, was considered as outgroup. One hundred replicates were performed in ML to find the phylogenetic confidence at each node of the tree. Minimum evolutionary trees were constructed for combined data analysis.

Results and discussion

Molecular genetics and biology of the domesticated silkworm *Bombyx mori* are better understood than those of wild nonmulberry silkworms. Mitochondrial genes have been used to study the relationship between wild and domesticated species (Hwang *et al*. 1999a,b; Yukuhiro *et al*. 2002), but those studies considered only a few species. In this study emphasis has been given to the phylogenetic relationships of more economically important wild species on the basis of 16S rRNA and *coxI* gene sequences. BLAST analysis of amplified and cloned 383-bp region of 16S rRNA and 597-bp region of *coxI* from 10 species of silkworms (table 1) showed significant homology with *A. pernyi* and *A. yamamai* (Saturniidae), and with *B. mori* and *B. mandarina* (Bombycidae). Both sequence regions showed AT-rich nucleotide composition typical of mitochondrial genes. The average percentage nucleotide composition among silk-producing taxa for 16S rRNA is A 39.2, T 40.4, G 6.9, C 13.5, and for *coxI* A 30.0, T 39.3, G 13.8, C 16.8. In comparison with *coxI*, 16S rRNA showed high AT bias and also possessed higher variability. Multiple sequence alignment of 16S rRNA and *coxI* sequences from 14 silkworm taxa was performed using ClustalW (Thompson *et al*. 1994). No stop codons, insertions or deletions were found in the *coxI* sequence.

Phylogenetic analysis

Earlier studies on the basis of morphological clues such as follicular imprints over the chorion of the egg, tubercular

setae of the larvae, eyespots on wings, karyotype, and silk gland (Jolly *et al*. 1969, 1970a,b; Gupta 1985) inferred that *A. mylitta, A. frithi, A. pernyi, A. roylei, A. proylei, A. assama* and *A. yamamai* are closely related. *A. proylei* is a hybrid between *A. pernyi* of China ($2n = 98$) and *A. roylei* of the Himalayan belt of India ($2n = 60$) (Nagaraju and Jolly 1985). A controversy arose during the 1970s, since one group assigned the hybrid a status of species, calling it *A. proylei*. Some taxonomists considered *A. proylei* as just a hybrid. Despite these facts, *A. proylei* parents produce fertile *A. proylei* offspring, and *A. proylei* is considered as a species in eastern India. However, Bhagirath *et al*. (1988) have observed that the chromosome number of the present-day *A. proylei* is $2n = 98$. Our study examines the phylogenetic relationships among some of these silk-producing insects on the basis of 16S rRNA and *coxI*.

Phylogenetic analysis on the basis of 16S rRNA

Phylogenetic analysis of the 14 silk-producing species was carried out using PAUP* 4.01 (Swofford 2001) software. Besides the small differences in the bootstrap values, heuristic maximum parsimony (MP) and maximum likelihood (ML) methods yielded similar trees (figure 1). These trees support monophyly for the major silk-producing families, Saturniidae and Bombycidae. Within Saturniidae two distinct clades are observed with significant bootstrap. One includes *A. proylei, A. pernyi* and *A. roylei*, and the second includes *A. mylitta* and *A. frithi*, followed by *A. yamamai*. Moreover, the bootstrap analysis showed low confidence values between *Antheraea* and *Attacus*. This analysis does not support monophyly for members of Antheraea and Attacini. The North American large silkmoth *Hyalophora cecropia* clusters with *Philosamia ricini* (the only semidomesticated species included in our study) and the two form a clade with *Attacus atlas*. In this MP tree the relationship of *A. assama* and *A. polyphemus* with other *Antheraea* species is not clear. *Gonometa postica* (Lasiocampidae) clusters with members of Bombycidae.

Phylogenetic analysis on the basis of coxI

Unweighted MP analysis resulting in a single most-parsimonious tree with low bootstrap value at each node did not resolve the position of *A. yamamai, A. assama* and *A. polyphemus* and displayed polytomy (data not shown). It might be due to effect of transition/transversion ratio at the third codon position in *coxI*. Therefore, we examined the effects of saturation in phylogenetic analysis (figure 2).

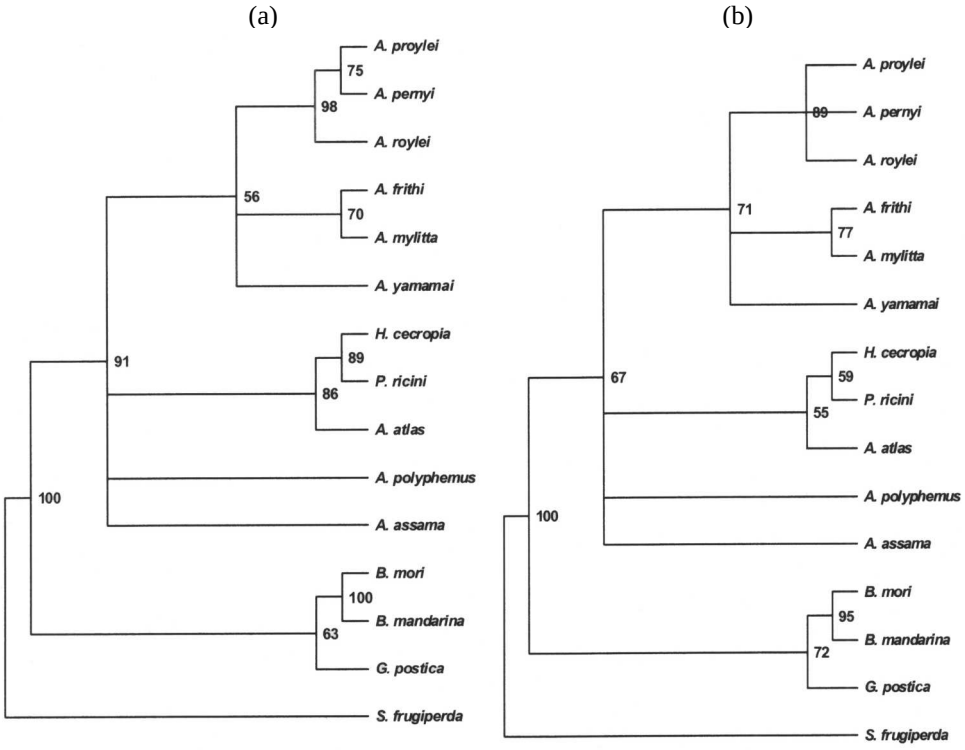

Figure 1. Maximum parsimony (a) and maximum lilkelihood (b) trees for 16S rRNA. Maximum parsimony analysis yielded a single tree with a length of 202, CI = 0.58 and RI = 0.59. The selected model for maximum likelihood analysis is GTR+G+I ($-\ln L = 857.55221$). Incorporating this model, the base frequencies were unequal: 0.394 (A), 0.079 (C), 0.061 (G), 0.465 (T). The estimated shape parameter for the gamma distribution was $\alpha = 0.326$. The tree was rooted using *S. frugiperda* as outgroup. Numbers inside the branches are bootstrap values for 1000 and 100 replications.

The figure shows that substitutions at the first codon position can be easily fitted to a straight line, indicating that they are unsaturated (Griffiths 1997; Guryev *et al*. 2001). On the other hand, substitutions at the second and third codon positions are in mutation pressure. In the case of transversions two clouds in the third-codon-position plot indicate that one group has no transversions and another has several. The more distantly related species might have lower third-codon-position divergences than closer relatives. This illustrates the preferential accumulation of further substitutions at the first and second positions and saturation starts at the third codon position. The data were also evaluated excluding the third position, and also considering the third position alone, but we could not resolve the deeper nodes and also the question of monophyly between Saturniidae and Bombycidae. To understand the necessity and usefulness of differential weighting we estimated the relative frequencies of transitions and transversions using Kimura two-parameter distance method

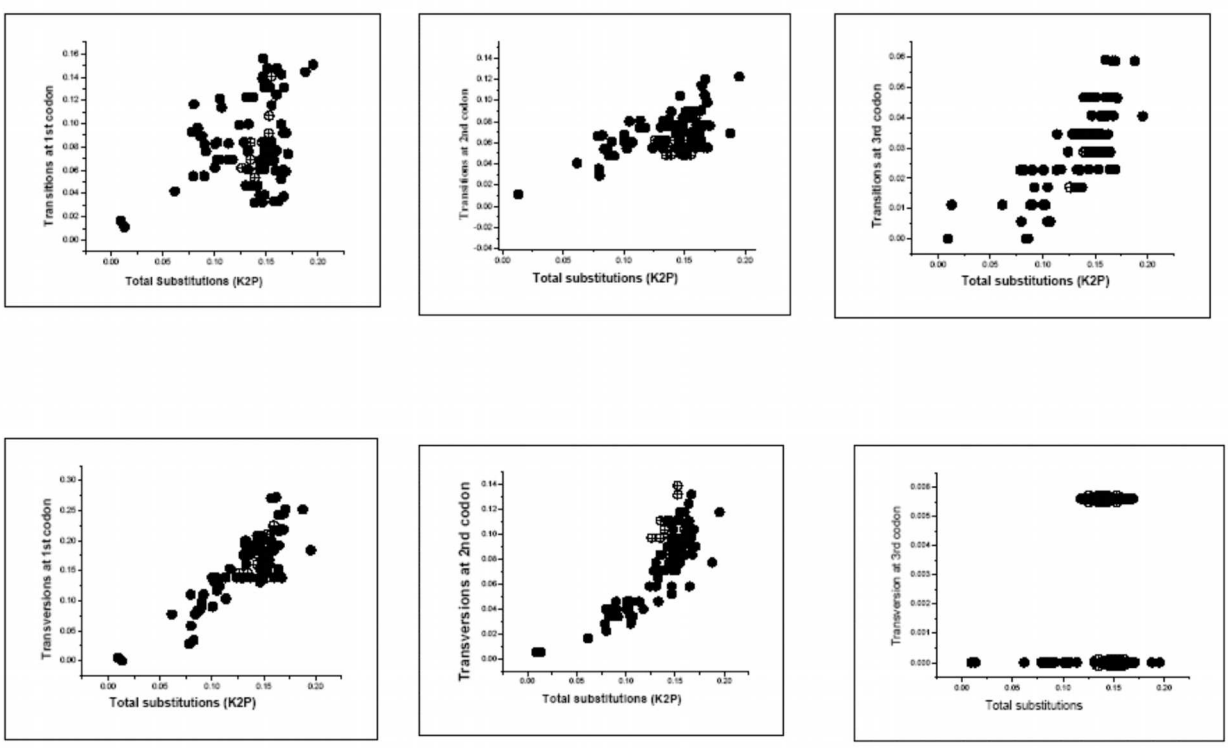

Figure 2. Plots illustrating saturation assessments for three data sets of *coxI*. Pairwise sequence differences within each partition are plotted against total sequence divergence. The dark and square symbols used in all saturation plots indicate comparison of substitution rate between silk-producing insects with outgroup.

Table 2. Transition/transversion ratio for *coxI* for all species studied.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *A. mylitta*														
2. *A. frithi*	1.02													
3. *A. assama*	0.85	0.64												
4. *A. pernyi*	1.03	1.13	1.12											
5. *A. roylei*	0.98	0.93	1.12	2.03										
6. *A. yamamai*	1.35	1.03	0.99	2.64	2.09									
7. *A. proylei*	1.18	1.31	1.26	1.50	3.74	3.17								
8. *A. polyphemus*	1.27	0.77	0.92	0.75	0.77	0.93	0.88							
9. *P. ricini*	0.98	0.87	0.91	0.53	0.50	0.81	0.62	0.86						
10. *A. atlas*	1.12	0.94	0.97	1.02	0.99	1.28	1.12	0.90	1.08					
11. *H. cecropia*	1.03	1.15	1.22	0.91	0.89	1.15	1.02	0.82	1.94	1.02				
12. *B. mori*	0.55	0.55	0.71	0.60	0.74	0.73	0.63	0.50	0.42	0.67	0.67			
13. *B. mandarina*	0.54	0.54	0.67	0.56	0.70	0.70	0.59	0.51	0.41	0.67	0.68	6.06		
14. *G. postica*	0.51	0.53	0.69	0.59	0.51	0.59	0.62	0.67	0.64	1.05	0.86	0.67	0.66	
15. *S. frugiperda*	0.48	0.46	0.52	0.64	0.53	0.58	0.72	0.62	0.61	0.64	0.86	0.64	0.63	0.56

(K2P) and reanalysed the data giving transversions four times more weight than transitions (table 2). The resultant weighted tree topology (figure 3, left) differs from the unweighted MP tree and supports monophyly of Saturniidae and Bombycidae. The relationship of *A. yamamai*, *A. assama and A. polyphemus* clade with other species in the same genera supports monophyletic origin for both Antheraea and Attacini tribes. *A. assama* and *A. polyphemus* resolved at the root of the *Antheraea* cluster. For ML analysis the justified model GTR+G+I was selected using Modeltest version 3.06 (Posada and Crandall 1998). In this model, the base frequencies were unequal: 0.302 (A), 0.143 (C), 0.145 (G), 0.408 (T). In comparison with the weighted MP tree this tree could not resolve the position of *A. mylitta*, *A. frithi*, *A. assama* and *A. polyphemus*, and displayed polytomy. This analysis reveals that sequence evolution is under different constraints in these species (Dobler and Muller 2000).

Combined analysis

There is considerable disagreement over whether data sets should be combined or considered separately in phylogenetic analysis (Kluge and Farris 1969). The partition-homogeneity test did not indicate a significant conflict between the molecular data sets ($P = 0.93$), and thus the 16S rRNA and *coxI* data were combined for total evidence analysis. The topologies obtained under the optimality criteria of MP (figure 4a) and ML (figure 4b) were almost similar to the weighted MP tree for *coxI*. Like in ML analysis of 16S rRNA and *coxI*, GTR+G+I was found to be the best model by Modeltest for combined data also, with likelihood ratio $-\ln L = 3766.4749$ and shape parameter $\alpha = 0.8538$. In this model the resolution of nodes is generally high within and between tribes of Saturniidae. In both MP and ML analysis monophyletic relationships were found between Saturniidae and Bombycidae members. A similar observation was made on the basis of two nuclear genes (Regier *et al*. 2002). Within Saturniidae *Antheraea* and *Attacus* also showed sister group relationship. The different silk-producing species of *Antheraea* fall into distinct clusters according to the type of silk (figure 4). For example the Chinese oak silkmoth *A. pernyi* makes a distinct clade with the Indian oak tasar silkmoth *A. proylei*, and also shows close relationship with another Indian oak tasar silkmoth, *A. roylei*. Two other tasar-silk-producing species, *A. mylitta* and *A. frithi*, make a distinct clade with significant bootstrap value. In this combined analysis also, the status of muga-silk-producing species *A. assama* and *A. polyphemus* is not clear. The representative genera of Attacini (*Philosamia*, *Attacus* and *Hyalophora*) form a monophyletic clade. *B. mori* and *B. mandarina* of the Bombycidae also group together, like in the individual tree

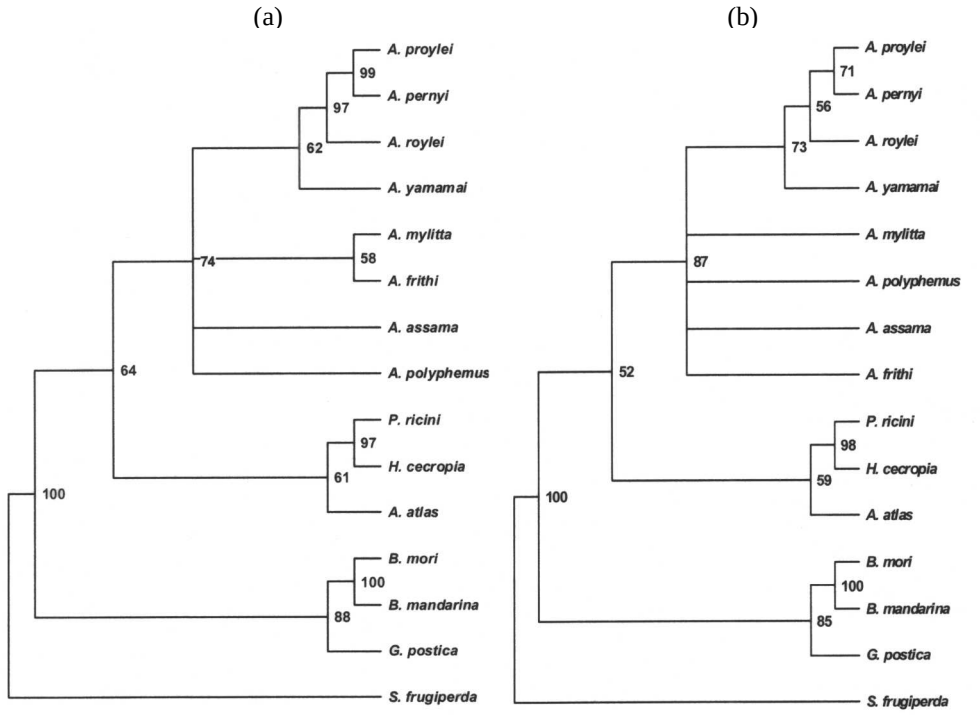

Figure 3. Weighted parsimony (a) and maximum likelihood (b) analysis for *coxI*. The weighted tree topology differs from the unweighted MP tree, and has a length of 1105, with CI = 0.487 and RI = 0.550. The justified model is GTR+G+I ($-\ln L = 1713.338$). The estimated shape parameter for the gamma distribution was $\alpha = 0.968$. The tree was rooted using *S. frugiperda* as outgroup. Numbers inside the branches are bootstrap values for 1000 and 100 replications.

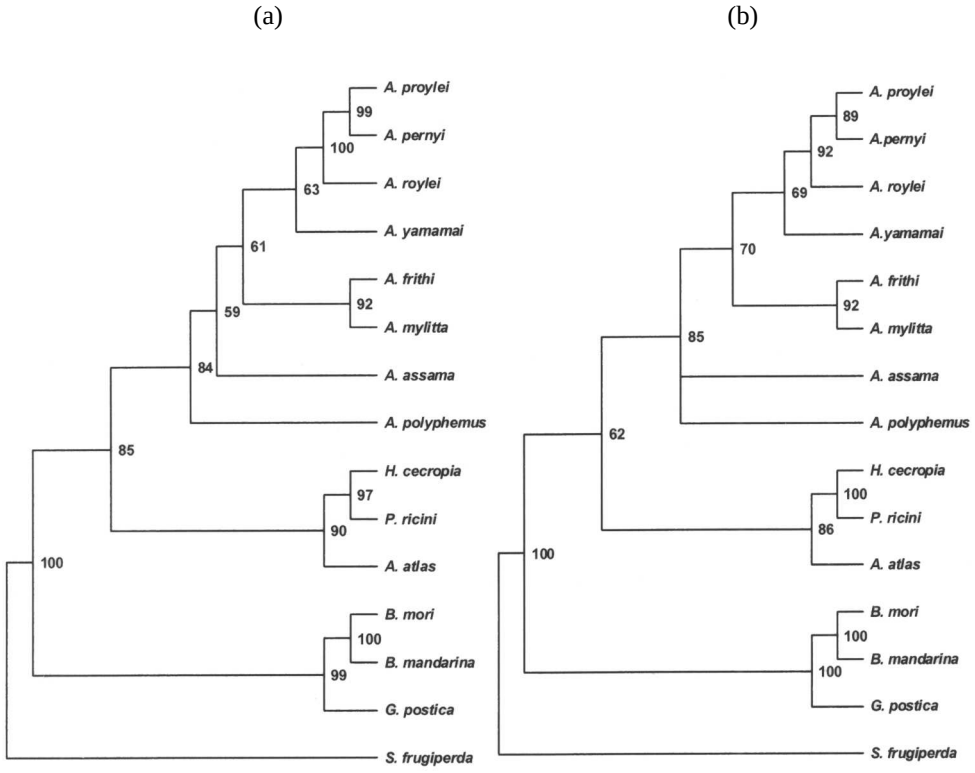

Figure 4. Phylogenetic relationships among species of Saturniidae, Bombycidae and Lasiocampidae based on the combined sequence data from 16S rRNA and *coxI* genes. (a) Combined data set comprises 633 constant characters, and 227 sites are parsimony informative. (b) Maximum likelihood analysis; the tree was rooted using *S. frugiperda* as outgroup. Numbers inside the nodes are bootstrap values.

topologies. The combined data also support the close resemblance of *G. postica* with members of Bombycidae.

The tasar-producing species *A. mylitta* showed resemblance to the geographically neighbouring species *A. frithi*. From karyotype data, *A. assama*, with the lowest chromosome number, $2n = 30$ (Deodikar *et al*. 1962), has been considered as a probable ancestor of all species in the genus *Antheraea*. However, *A. polyphemus* (the only *Antheraea* species available in the Palaearctic region) occupies the basal position within the genus *Antheraea* and is very close to *A. assama*, owing to probable occurrence of rapid speciation in association with colonization of new continents, and continental drift. A similar phenomenon has been reported in *Chironomus* (Diptera) species (Guryev *et al*. 2001). The position of *A. proylei* in MP and ML trees and high transition/transversion ratio suggest that it is the most recently evolved species in the genus *Antheraea*. However, more species in Bombycoidea should be analysed to understand the systematic position of superfamily relationships across Macrolepidoptera.

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References

Altschul S. F., Gish W., Miller W., Myers E. W. and Lipman D. J. 1990 Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Bhagirath T. H., Kundu S. C. and Ibotombi N. 1988 Ultimate karyotype of the hybrid between *Antheraea pernyi* and *A. roylei*. *Sericologia* **28**, 91–94.

Blum M. J., Bermingham E. and Dasmahapatra K. 2003 A molecular phylogeny of the neotropical butterfly genus *Anartia* (Lepidoptera: Nymphalidae). *Mol. Phylogenet. Evol*. **26**, 46–55.

Brower A. V. Z. 1994 Phylogeny of *Heliconius* butterflies inferred from mitochondrial DNA sequences (Lepidoptera: Nymphalidae). *Mol. Phylogenet. Evol*. **3**, 159–174.

Canapa A., Barucca M., Marinelli A. and Olmo E. 2000 Molecular data from the 16S rRNA gene for the phylogeny of Pectinidae (Mollusca: Bivalvia). *J. Mol. Evol*. **50**, 93–97.

Chatterjee S. N. and Tanushree T. 2004 Molecular profiling of silkworm biodiversity in India: an overview. *Genetica* (Russian) **10**, 1–9.

Datta A., Ghosh A. K. and Kundu S. C. 2001 Differential expression of fibroin gene in developmental stages of silkworm *A. mylitta* (Saturniidae). *Comp. Biochem. Physiol*. **B129**, 197–204.

Davies N. and Bermingham E. 2002 The historical biogeography of two Caribbean butterflies (Lepidoptera: Heliconiidae) as inferred from genetic variation at multiple loci. *Evolution* **56**, 573–589.

Deodikar G. B., Chowdhury S. N., Bhuyan B. N. and Kshirsagar K. K. 1962 Cytogenetic studies in Indian silkworms. Chromosome number in muga silkworm, *A. assamensis* WW. *Curr. Sci*. **31**, 147–148.

Dobler S. and Muller J. K. 2000 Resolving phylogeny at the family level by mitochondrial cytochrome oxidase sequences: Phylogeny of carrion beetles (Coleoptera, Silphidae). *Mol. Phylogenet. Evol*. **15**, 390–402.

Farris J. S. 1989 The retention index and homoplasy excess. *Syst. Zool*. **38**, 406–407.

Felsenstein J. 1985 Congruence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783–791.

Friedlander T. P., Horst K. R., Regier J. C., Mitter C., Peigler R. S. and Fang Q. Q. 1998 Two nuclear genes yield concordant relationships within Attacini (Lepidoptera: Saturniidae). *Mol. Phylogenet. Evol*. **9**, 131–140.

Goldsmith M. R., Shimada T. and Abe H. 2005 The genetics and genomics of the silkworm, *Bombyx mori. Annu. Rev. Entomol*. **50**, 71–100.

Griffiths C. 1997 Correlation of functional domains and rates of nucleotide substitution in cytochrome *b. Mol. Phylogenet. Evol*. **7**, 352–365.

Gupta K. S. 1985 Non-mulberry sericulture—its problems and prospects. *Sericologia* **25**, 89–94.

Guryev V., Makarevitch I., Blinov A. and Martin J. 2001 Phylogeny of the genus *Chironomus* (Diptera) inferred from DNA sequences of mitochondrial cytochrome *b* and cytochrome oxidase I. *Mol. Phylogenet. Evol*. **19**, 9–21.

Han H.-Y. 2000 Molecular phylogenetic study of the tribe Trypetini (Diptera: Tephritidae), using mitochondrial 16S ribosomal DNA sequences. *Biochem. Syst. Ecol*. **28**, 501–513.

Hwang J. S., Lee J. S., Goo T. W., Yun E. Y., Sohn H. Y., Kim H. R. and Kwon O. Yu. 1999a Molecular genetic relationships between Bombycidae and Saturniidae based on the mitochondrial DNA encoding of large and small rRNA. *Genet. Anal. Biomol. Eng*. **15**, 223–228.

Hwang J. S., Lee J. S., Goo T. W., Kang H. A., Yun E. Y., Sohn H. Y., Kim H. R. and Kwon O. Yu. 1999b The comparative molecular study between Bombycidae and Saturniidae based on mtDNA RFLP and cytochrome oxidase I gene sequences: Implication for molecular evolution. *Z. Naturforsch*. **C54**, 587–594.

Jolly M. S. and Sen S. K. 1969 Patterns of follicular imprints in egg shell—a species-specific character in *Antheraea. I. Bull. Entomol*. **10**, 32–38.

Jolly M. S., Narasimhanna M. N., Sinha S. S. and Sen S. K. 1969 Interspecific hybridization in *Antheraea. Indian J. Hered*. **1**, 45–48.

Jolly M. S., Narasimhanna M. N. and Wartkar V. B. 1970a Arrangement of tubercular setae in *Antheraea* species (Lepidoptera: Saturniidae). *Entomologist* **103**, 18–23.

Jolly M. S., Sen S. K. and Sinha S. S. 1970b Chromosome number in the Indian oak feeding tasar silkworm *A. proylei* Mr. *Curr. Sci*. **39**, 423–424.

Jolly M. S., Sen S. K. and Ahsan M. M. 1974 *Tasar culture*. Ambika Publishers, Bombay.

Kluge A. G. and Farris J. S. 1969 Quantitative phyletics and the evolution of anurans. *Syst. Zool*. **18**, 1–32.

Kumar S., Tamura K., Jakobsen I. B. and Nei M. 2001 MEGA 2.1, molecular evolutionary genetics analysis software. *Bioinformatics* **17**, 1244–1245.

Nagaraju J. and Goldsmith M. R. 2002 Silkworm genomics—progress and prospects. *Curr. Sci*. **83**, 415–425.

Nagaraju J. and Jolly M. S. 1985 Interspecific hybrids of *Antheraea roylei* and *A. pernyi*—a cytogenetic reassessment. *Theor. Appl. Genet*. **72**, 269–273.

Niehuis O. and Wagele J. W. 2004 Phylogenetic analysis of the mitochondrial genes LSU rRNA and COI suggests early adaptive differentiation of anal teeth in chrysidine cuckoo wasps (Hymenoptera: Chrysidae). *Mol. Phylogenet. Evol*. **30**, 615–622.

Posada D. and Crandall K. A. 1998 MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.

Prasad M. D., Nurminsky D. L. and Nagaraju J. 2002 Characterization and molecular phylogenetic analysis of *mariner* elements from wild and domesticated species of silkworms. *Mol. Phylogenet. Evol*. **25**, 210–217.

Reddy K. D., Abraham E. G. and Nagaraju J. 1999 Genetic characterization of the silkworm, *Bombyx mori*, by inter-simple sequence repeat (ISSR)-anchored PCR. *Heredity* **83**, 681–687.

Regier J. C., Mitter C., Peigler R. S. and Friedlander T. P. 2002 Monophyly, composition, and relationships within Saturniinae (Lepidoptera: Saturniidae): Evidence from two nuclear genes. *Insect Syst. Evol*. **33**, 9–21.

Sambrook J. and Russell D. W. 2001 *Molecular cloning: a laboratory manual*, 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Shimada T., Kurimoto Y. and Kobayashi M. 1995 Phylogenetic relationship of silk moths inferred from sequence data of the arylphorin gene. *Mol. Phylogenet. Evol*. **4**, 223–234.

Shouche Y. S. and Patole M. 2000 Sequence analysis of mitochondrial 16S ribosomal RNA gene fragment from seven mosquito species. *J. Biosci*. **25**, 361–366.

Sinha A. K., Sinha R. K., Goel A. K., Sinha B. R. R. P. and Thangavelu K. 1994 A review on the breeding and genetic aspects of tropical tasar silkworm, *Antheraea mylitta* D. *Proc. Con. Cytol. Genet*. **4**, 7–16.

Swofford D. L. 2001 PAUP* phylogenetic analysis using parsimony (* and other methods), Ver. 4. Sinauer Associates, Sunderland, USA.

Thompson J. D., Higgins D. G. and Gibson T. J. 1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res*. **22**, 221–244.

Whitfield J. B. and Cameron S. A. 1998 Hierarchical analysis of variation in the mitochondrial 16S rRNA gene among Hymenoptera. *Mol. Biol. Evol*. **15**, 1728–1743.

Yukuhiro K., Sezutsu H., Itoh M., Shimizu K. and Banno Y. 2002 Significant levels of sequence divergence and gene rearrangements have occurred between the mitochondrial genomes of the wild mulberry silkmoth, *Bombyx mandarina*, and its close relative, the domesticated silkmoth, *Bombyx mori. Mol. Biol. Evol*. **19**, 1385–1389.

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