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ORIGINAL ARTICLE

Mitochondrial genomes of two wild silkmoths, *Samia watsoni* and *Samia wangi* (Lepidoptera: Saturniidae), and their phylogenetic implications

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Abstract. The wild silkmoth genus *Samia* Hübner, 1819 (Saturniidae) contains a number of economically important species in industrial silk production. However, the interspecific relationships within the genus remain unclear. We sequence the mitogenomes of *Samia watsoni* Oberthür, 1914 and *Samia wangi* Naumann & Peigler, 2001. Both mitogenomes are annotated and found to be cyclized, with 37 genes (13 PCGs, 2 rRNA genes and 22 tRNA genes). Using maximum likelihood and Bayesian inference methods, we analyze these mitogenomes together with a further 68 downloaded from GenBank (65 Bombycoidea and 5 Lasiocampidae as the outgroup) to investigate the phylogenetic relationships both within the genus and those among the three families of the 'SBS' group: Bombycidae, Saturniidae and Sphingidae. The results show that within *Samia*, *S. ricini* is closely related to *S. canningi*, and not *S. cynthia* of which it has previously been considered to be a subspecies. Although arguments have been proposed to treat *S. ricini* and *S. canningi* as conspecific, we choose to accept the morphological arguments and continue to treat them as two separate species. *Samia watsoni* is corroborated as the sister group of all other *Samia* species, but nevertheless should be included within *Samia* rather than being placed in its own monobasic genus. Our analysis recovers the following relationship among the three families of the 'SBS' group: (Saturniidae + (Bombycidae + Sphingidae)). This agrees with previous studies based on analysis of mitogenomes but continues to contradict the results derived from phylogenomic analysis of nuclear genomes.

INTRODUCTION

The Lepidoptera (butterflies and moths) have unique feeding habits, diverse geographical distributions and multi-directional patterns of species evolution, which makes the group an excellent model for the analysis of the diversity of community systems (De Camargo et al., 2016). Within Lepidoptera, the superfamily Bombycoidea currently comprises ten families (Anthelidae, Apatelodidae, Bombycidae, Brahmaeidae, Carthaeidae, Endromidae, Eupterotidae, Phiditiidae, Saturniidae and Sphingidae), 520 genera and 6092 species (Kitching et al., 2018; Hamilton et al., 2019). However, the phylogenetics of the families within Bombycoidea remains controversial, with a current focus being on the relationship among the three families, Bombycidae, Saturniidae, and Sphingidae (Hamilton et al., 2019).

Early classifications of Bombycoidea were based solely on morphology, but convergent evolution has confused our understanding of their evolution, as exemplified by the two genera *Rotunda* and *Arotros* (Hamilton et al., 2019). Furthermore, although the monophyly of Bombycoidea is supported by six morphological synapomorphies, only two of these are systematically informative (Zwick, 2008). More recently, phylogenetic research has increasingly used the techniques of molecular sequence analysis. In the first such analysis of Bombycoidea, Regier et al. (2008) sequenced

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five nuclear genes from 66 species and found that the families Bombycidae, Sphingidae and Saturniidae, which had been placed on different branches in the superfamily, actually comprised a single monophyletic group, with the relationship: (Sphingidae + (Bombycidae + Saturniidae)). In the same year, based on a study of two nuclear genes (CAD and Ef-1a), Zwick (2008) obtained a different pattern of relationships, namely (Saturniidae + (Bombycidae + Sphingidae)). The following year, a new study by Regier et al. (2009) based on five nuclear genes obtained results consistent with those of Zwick but in the same year, in a study that increased the sampling to 20 genes, Zwick et al. (2009) recovered the original pattern of relationships found by Regier et al. (2008), namely (Sphingidae + (Bombycidae + Saturniidae)). They also introduced the concept of the 'SBS' group for the clade comprising these three families. In one of the first phylogenomic studies of Lepidoptera, Kawahara & Breinholt (2014) analyzed a nuclear gene dataset (combining 33 new transcriptomes with 13 available genes, transcriptomes and expressed sequence tags) for 46 species of butterflies and moths and recovered the third possible topology for the 'SBS' group: (Bombycidae + (Saturniidae + Sphingidae)). Xin et al. (2017) undertook a phylogenetic analysis on 34 complete mitochondrial genomes with the same result and in the same year, Kim et al. (2017) also published an analysis of mitogenomes, concluding the relationship was (Saturniidae + (Bombycidae + Sphingidae)). Wang et al. (2018) then arrived at the same conclusion as Kim et al. (2017), this time based on 39 complete mitogenomes. Most recently, using a newly developed anchored hybrid enrichment probe set sampling 571 genes across 117 species and all major bombycoid lineages, Hamilton et al. (2019) concluded the relationship among the three 'SBS' group families was again (Bombycidae + (Saturniidae + Sphingidae)). Thus, there is still considerable uncertainty over these relationships, with much perhaps depending on the sampling, of both genes and taxa, and the analytical methods employed (Fig. 1).

Within the Saturniidae subfamily Saturniinae, tribe Attacini, the genus *Samia* includes several species that are used as both model organisms in scientific research and in industrial silk production, particularly *S. ricini* W. Jones, 1791. Compared with *Bombyx mori* Linnaeus, 1758, *S. ricini* has the advantages of higher silk yield, greater disease resistance and easier rearing, and so has been regarded as a new model species to replace *Bombyx mori* in molecular and cellular experiments (Meier et al., 2000; Lee et al., 2021). *Samia ricini* has sometimes been treated as a subspecies of *S. cynthia* (Drury, 1773) but molecular phylogenetic analyses have now shown that *S. ricini* is instead very closely related to *S. canningi* (Hutton, 1859) (Lemaire & Peigler, 1982). Indeed, Peigler & Calhoun (2013) confirmed that *S. ricini* is a domesticated species, derived of *S. canningi*, that is not known in the wild and treated the two as conspecific. However, because of their obvious morphological differentiation, many taxonomists continue to regard them as two separate species (Huang et al., 2021).

Another species of *Samia*, *S. watsoni* (Oberthür, 1914), was originally described in the monobasic genus *Desgodinsia* Oberthür, 1914 (Naumann et al., 2014). While some lepidopterists accepted this taxonomy, others, including the great Claude Lemaire (see Lemaire & Peigler, 1982), treated *Desgodinsia* as a synonym of *Samia* and placed the species in that genus. Peigler & Naumann (2003) considered that although *S. watsoni* is the sister taxon to all other *Samia*, this was insufficient to warrant the recognition of a separate genus for it and advocated that *S. watsoni* should be left in *Samia*. In contrast, based on a study of the shapes of the wing eyespots, body size and male genitalia structure, Brechlin (2007) considered that *S. watsoni* did not conform to the diagnostic characteristics that united the other *Samia* species, and described a new genus, *Archaeosamia* Brechlin, 2007, to accommodate the species *watsoni* (this new genus name was required because *Desgodinsia* Oberthür, 1914 is a junior primary homonym of *Desgodinsia* Senna, 1894 in Coleoptera, and is so unavailable for use in the present case). However, Naumann et al. (2014) pointed out that there were probably sufficient genera in Lepidoptera at present, especially monobasic ones, and it was not necessary to recognize a new genus for a single species just because it exhibits some relatively minor differences from the remaining species in its genus. Therefore, they synonymized *Archaeosamia* with *Samia* and returned *watsoni* to the latter genus.

Mitochondria are important organelles in eukaryotic cells, not only providing power for cells but also participating in apoptosis (Wang et al., 2009; Saita et al., 2017). As semi-autonomous organelles, mitochondria contain their own genetic material, comprising two ribosomal RNA genes (rRNAs), 22 transfer RNA genes (tRNAs), one major non-coding sequence (A+T rich region) and 13 protein coding genes (PCGs) (including genes related to autogenesis), and a unique translation system (Singh et al., 2017; Xin et al., 2017; Kim et al., 2018; Wang et al., 2018). The mitochondrial genome is a double stranded circular molecule with a size range of 14–19 kb and has been widely used in animal evolutionary research, including molecular evolution, evolutionary genomics, phylogenetics and population genetics, due to its small size, maternal inheritance, lack of genetic recombination, rapid evolutionary rate, multiple copies within cells and easy amplification (Liu et al., 2012; Chen et al., 2014; Wu et al., 2016).

In this study, we sequenced the complete mitogenomes of *S. watsoni* and *S. wangi* and undertook a phylogenetic analysis based on these new data and sequences from GenBank to explore the internal relationships of *Samia* and the phylogeny of 'SBS' group.

MATERIALS AND METHODS

Sampling and DNA extraction

Adult moths of *S. wangi* and *S. watsoni* were collected in Huangshan city, Anhui Province, China. Species identification was confirmed by examination of the dissected male genitalia. Legs were preserved in absolute ethanol at –20°C before DNA extraction. Total DNA was isolated using a TIANamp Genomic

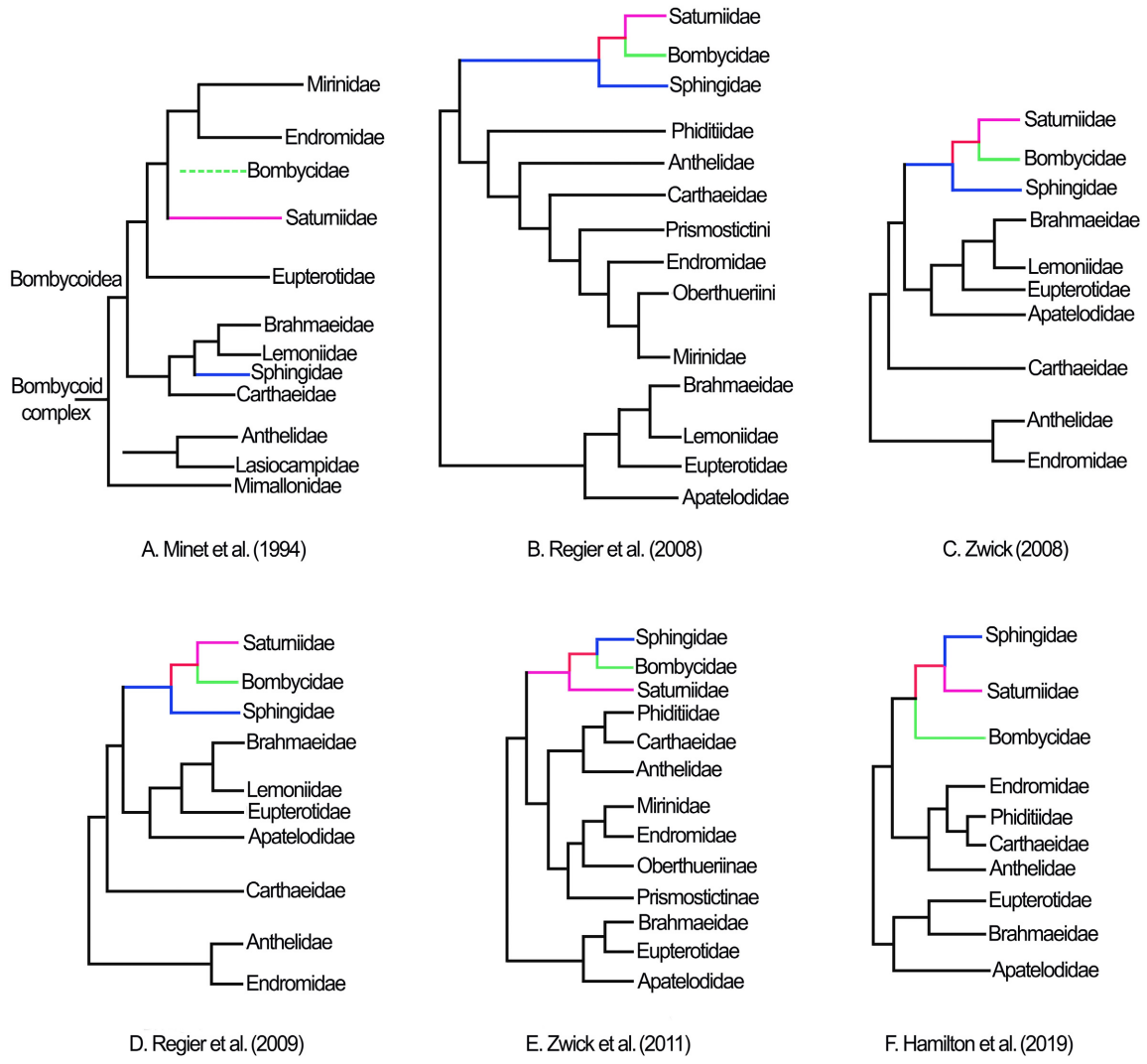

Fig. 1. Phylogenetic relationships of families within the superfamily Bombycoidea based on previous studies.

DNA Kit according to the manufacturer's instructions. The extracted DNA was then used to amplify the complete mitogenomes by PCR following the protocols given by Tyrrell (1997).

Sequencing and assembly

A whole genome shotgun (WGS) strategy was used for sequencing on an Illumina NovaSeq platform. Data quality was checked using FastQC (Andrews, 2020) and mitogenome assembly was undertaken using NOVOPlasty (Dierckxsens et al., 2016).

Mitochondrial genome annotation

MitoZ was used for gene annotation and the MITOS WebSever was used to identify tRNA genes and predict their secondary structure (Bernt et al., 2013; Meng et al., 2019). The parameters were set with Invertebrate Mito genetic code. Each tRNA gene sequence was checked manually. Protein-coding genes (PCGs) were identified as open reading frames corresponding to the 13 PCGs of Saturniidae mitogenomes.

Sequence analysis

MEGA X was used to analyze base composition and relative synonymous codon usage (RSCU) (Kumar et al., 2018). The calculation of AT-skew and GC-skew was based on the formula proposed by Hassanin et al. (2005): AT-skew = (A−T)/(A+T), GC-skew = (G−C)/(G+C). DnaSP was used to compute the numbers of synonymous substitutions per synonymous site (Ks) and nonsynonymous substitutions per nonsynonymous site (Ka) for the 13 PCGs in the mitogenome (Rozas et al., 2003).

Phylogenetic analysis

A total of 68 mitochondrial genomes were downloaded from GenBank (Table S1) and together with the two newly sequenced species were used to construct a phylogenetic tree. Five species of the family Lasiocampidae were used as the outgroup, and the remaining 65 species represent six families of Bombycoidea (Bombycidae, Brahmaeidae Endromidae, Eupterotidae, Saturniidae and Sphingidae). Alignment of PCGs was conducted by MAFFT 7.3.1 using G-INS-I algorithms (Katoh et al., 2016). Two rRNA segments were aligned with MEGA X (Kumar et al., 2018). The alignments were then concatenated into a single matrix using Phylosuite (Zhang et al., 2019). Two data sets were analyzed: (1) PCG, comprising just the 13 protein coding genes; and (2) PCG + rRNA, which comprises the 13 protein coding genes and the two rRNA genes.

To reconstruct the phylogenetic tree, both ML (maximum-likelihood) and BI (Bayesian inference) methods were applied to the concatenated dataset. Maximum likelihood analysis was conducted in W-IQ-TREE (Trifinopoulos et al., 2016) using the best-fit substitution model. An ultrafast bootstrap (UFB) of 1000 replications and the SH-aLRT test were used to assess branch

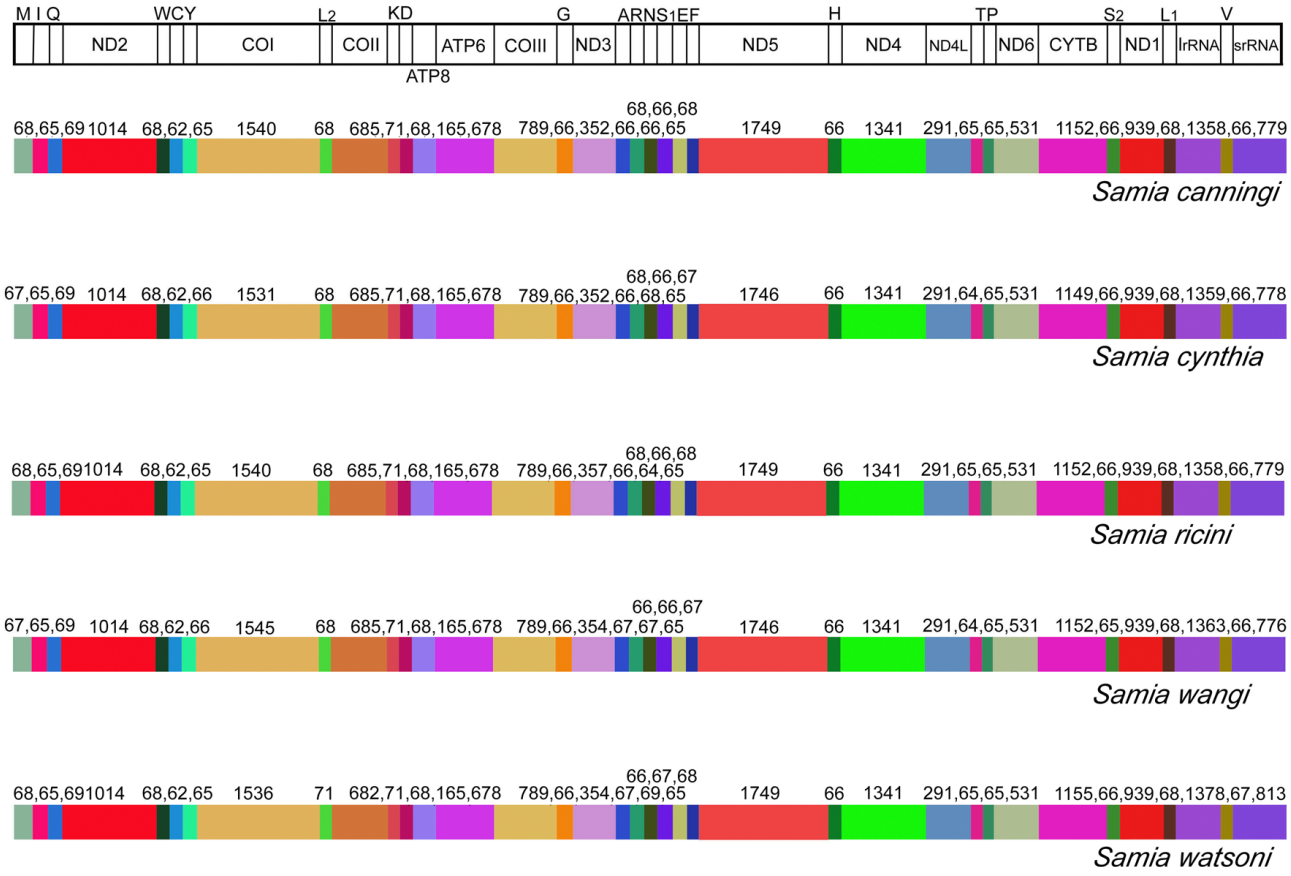

Fig. 2. Mitochondrial genomic characteristics of *Samia canningi*, *Samia cynthia*, *Samia wangi*, *Samia ricini* and *Samia watsoni*.

supports. Bayesian inference analysis was conducted using PhyloBayes (Lartillot et al., 2013). The first 25% of samples were discarded as burn-in and the remaining samples used to generate a 50% majority rule consensus tree. FigTree v.1.4.0 was used to view the resulting trees (Rambaut, 2020).

Comparative mitogenomes analyses within *Samia*

Samia watsoni and *S. wangi* are the newly obtained cyclized sequences and the mitogenomes of *S. canningi*, *S. cynthia* and *S. ricini* were downloaded from GenBank. The genes of these five species were annotated with MITOS WebServer (Bernt et al., 2013) and the secondary structure of their tRNAs analyzed. These tRNAs were mapped with AI (McLean., 2002), and the structural differences of *S. canningi*, *S. cynthia* and *S. ricini* were then compared. Geneious was used to compare the different sites in the mitogenome sequences of *S. canningi*, *S. cynthia* and *S. ricini* (Kearse et al., 2012). MEGA X was used to calculate the pairwise distances among the five species.

RESULTS

Genome structure, organization and composition

The *S. watsoni* and *S. wangi* mitogenomes are 15408 bp and 15334 bp long respectively, and comprise 13 PCGs, 2 rRNA genes and 22 tRNA genes (Fig. 2). Nine PCGs (COX1, ND2, COX2, ATP8, ATP6, COX3, ND3, ND6 and CYTB) and 14 tRNAs (trnM, trnI, trnW, trnL2, trnK, trnD, trnG, trnA, trnR, trnN, trnS1, trnE, trnT and trnS2) are coded on the majority-strand, with the remaining 14 genes encoded by the minority-strand (Fig. S1).

The nucleotide composition of the *S. wangi* mitogenome is A = 6073 (39.6%), T = 6179 (40.3%), C = 1876 (12.2%), G = 1206 (7.9%), and that of the *S. watsoni* mitogenome is A = 6047 (39.2%), T = 6167 (40.0%), C = 1958 (12.7%), G = 1236 (8.0%). The AT and GC skews are both negative in these two mitogenomes, indicating a bias towards the use of T and C. All the mitogenome nucleotide compositions indicate high A+T content, with an average of 79.55%, showing a strong AT bias (Table 1).

Ka/Ks analysis shows this ratio to be less than one in all five species, indicating that these genes are negatively selected. The 13 protein coding genes of *S. canningi* and *S. ricini* are under almost the same selection pressure. The selection pressures of *S. cynthia* and *S. wangi* are also close (Fig. S2).

Protein-coding genes (PCGs)

As in other Lepidoptera, the mitogenomes of *S. wangi* and *S. watsoni* contain three cytochrome c oxidase subunits, seven NADH dehydrogenase subunits, two ATPase subunits and one cytochrome b gene. The total lengths of the 13 PCGs of *S. wangi* and *S. watsoni* are 11227 bp and 11224 bp respectively. Tables S2–S3 list the composition of the mitogenomes of *S. wangi* and *S. watsoni*. The initiation codons of COX1 in *S. wangi* and *S. watsoni* are CGA, and the initiation codons of COX2 in *S. wangi* and *S. watsoni* are GTG (Kim et al., 2009, 2014; Margam et al., 2011; Park et al., 2016). COX2 in *S. wangi* and *S. watsoni* has a single t-termination, and the termination codons of the

Table 1. Nucleotide composition of *Samia wangi* and *Samia watsoni*.

Samia wangi	Size (bp)	A (bp)	T (bp)	G (bp)	C (bp)	A %	T %	G %	C %	AT skew	GC skew
Whole genome	15334	6073	6179	1206	1876	39.6%	40.3%	7.9%	12.2%	–0.00865	–0.21739
Protein-coding genes	11227	4374	4434	965	1454	39.0%	39.5%	8.6%	13.0%	–0.00681	–0.20215
tRNA genes	1462	595	586	118	163	40.7%	40.1%	8.1%	11.1%	0.007621	–0.16014
rRNA genes	2190	896	948	112	234	40.9%	43.3%	5.1%	10.7%	–0.0282	–0.3526
A+T-rich region	328	148	151	12	17	45.1%	46.0%	3.7%	5.2%	–0.01003	–0.17241
Samia watsoni	Size (bp)	A (bp)	T (bp)	G (bp)	C (bp)	A %	T %	G %	C %	AT skew	GC skew
Whole genome	15408	6047	6167	1236	1958	39.2%	40.0%	8.0%	12.7%	–0.00982	–0.22605
Protein-coding genes	11224	4340	4363	995	1526	38.7%	38.9%	8.9%	13.6%	–0.00264	–0.21063
tRNA genes	1472	598	589	120	165	40.6%	40.0%	8.2%	11.2%	0.007582	–0.15789
rRNA genes	2191	889	953	111	238	40.6%	43.5%	5.1%	10.9%	–0.03474	–0.3639
A+T-rich region	322	138	152	11	21	42.9%	47.2%	3.4%	6.5%	–0.04828	–0.3125

other PCGs are complete. The frequencies of A and T in the protein coding genes are significantly higher than those of C and G (Table 1). To further explore the composition of the protein coding genes, we carried out RSCU (relative synonymous codon usage) analysis (Fig. S3). The comparison shows that UUA is the most frequently used codon, and GCG is the least frequently used. The frequency of NNT and NNA is significantly higher than that of NNG and NNC, indicating that there is a strong A and T bias in the third codon position.

Transfer RNA and ribosomal RNA genes

There are 22 tRNAs in each of the two species (Figs S4–S5), and the total lengths are 1462 bp (*S. wangi*) and 1472 bp (*S. watsoni*), accounting for 9.5% and 9.6% of the total mitogenome respectively. A and T are used more frequently than C and G (Table 1). The AT skew is positive and the GC skew is negative. The total lengths of the rRNA gene fragments of these two species are 2190 bp (*S. wangi*) and 2191 bp (*S. watsoni*), accounting for 14.3% (*S. wangi*) and 14.2% (*S. watsoni*) of the total mitogenome respectively.

A+T-rich region

The A+T-rich region of the two species is located between rrnS and trnM, with lengths of 328 bp (*S. wangi*) and 322 bp (*S. watsoni*). A+T accounts for 91.1% of the whole A+T-rich region in both species. A-T skew and G-C skew analysis showed that *S. wangi* and *S. watsoni* have clear T and C usage bias (see Table 1 for further details).

Phylogenetic analysis

The monophyly of *Samia* is highly supported by both datasets (13 PCGs and 13 PCGs+2 rRNA) and both analytical methods (BI and ML) (Fig. 3). *Samia watsoni* is the first species to diverge within the genus (Fig. 3). The remaining four species form a clade within which *S. ricini* + *S. canningi* and *S. cynthia* + *S. wangi* form two reciprocally monophyletic pairs.

The relationships among the families in Bombycoidea are consistent in both the ML and BI analyses based on the two different datasets, with the topology: ((((Bombycidae + Sphingidae) + Saturniidae) + Endromidae) + (Eupterotidae + Brahmaeidae)). Thus, our results agree with those of previous studies that found Bombycidae and Sphingidae form a clade to the exclusion of Saturniidae. All families with more than one representative are recovered as monophyletic and most nodes are highly supported (every node in Endromidae, Bombycidae and Sphingidae). Although Saturniidae was monophyletic, it is only moderately supported. With the exception of some small differences at lower levels, the topological structures of ML and BI trees are the same.

Comparative mitogenomes analyses within *Samia*

The tRNA structures of *S. canningi* and *S. ricini* were almost identical, the only differences being alternative codons at four sites in the sequences of trnM, trnI and trnK. More significant differences are found between the tRNAs of *S. cynthia* and *S. ricini*, in which the TΨC loop of trnR and DHU loop of trnF showed clear structural differences, and trnM, trnI, trnQ, trnY, trnK, trnD, trnA, trnE, trnH, trnT, trnS2 and trnL1 all varied in sequence (Figs S6–S7).

Table S4 shows the conserved and variable sites among *S. ricini*, *S. canningi* and *S. cynthia*. CYTB in *S. ricini* and *S. canningi* has the most variable sites (55/1149). The number of variable sites of *S. canningi* and *S. ricini* are the least among the comparisons. The variation in sites of most genes between *S. canningi* and *S. ricini* is either zero or only a single site, indicating a high degree of sequence similarity.

The pairwise distance analysis shows that *S. wangi* is closest to *S. cynthia*, which is consistent with the results of the phylogenetic analyses. The distance between *S. canningi* and *S. ricini* is only 0.003. *Samia watsoni* is much more divergent from the other four species (Table S5).

DISCUSSION

Previously, *S. ricini* was sometimes treated as a subspecies of *S. cynthia* (Peigler & Calhoun, 2013). However, the results of our study refute this taxonomic treatment for the following reasons. (1) In the Ka/Ks analysis, the selection pressures on *S. ricini* and *S. canningi* are similar, as are those of *S. cynthia* and *S. wangi*, whereas the selection pressures between *S. cynthia* and *S. ricini* are much greater. (2) 14 of the 22 tRNAs of *S. cynthia* and *S. ricini* have a different structure. (3) The number of variable sites in *S. cynthia and S. ricini* is much higher than between *S. canningi* and *S. ricini*, which is particularly evident in the 13 PCGs (Table S4). (4) The results of interspecific genetic distance analysis showed that the genetic distance between *S. cynthia* and *S. ricini* was 0.11 but that between *S. cynthia*

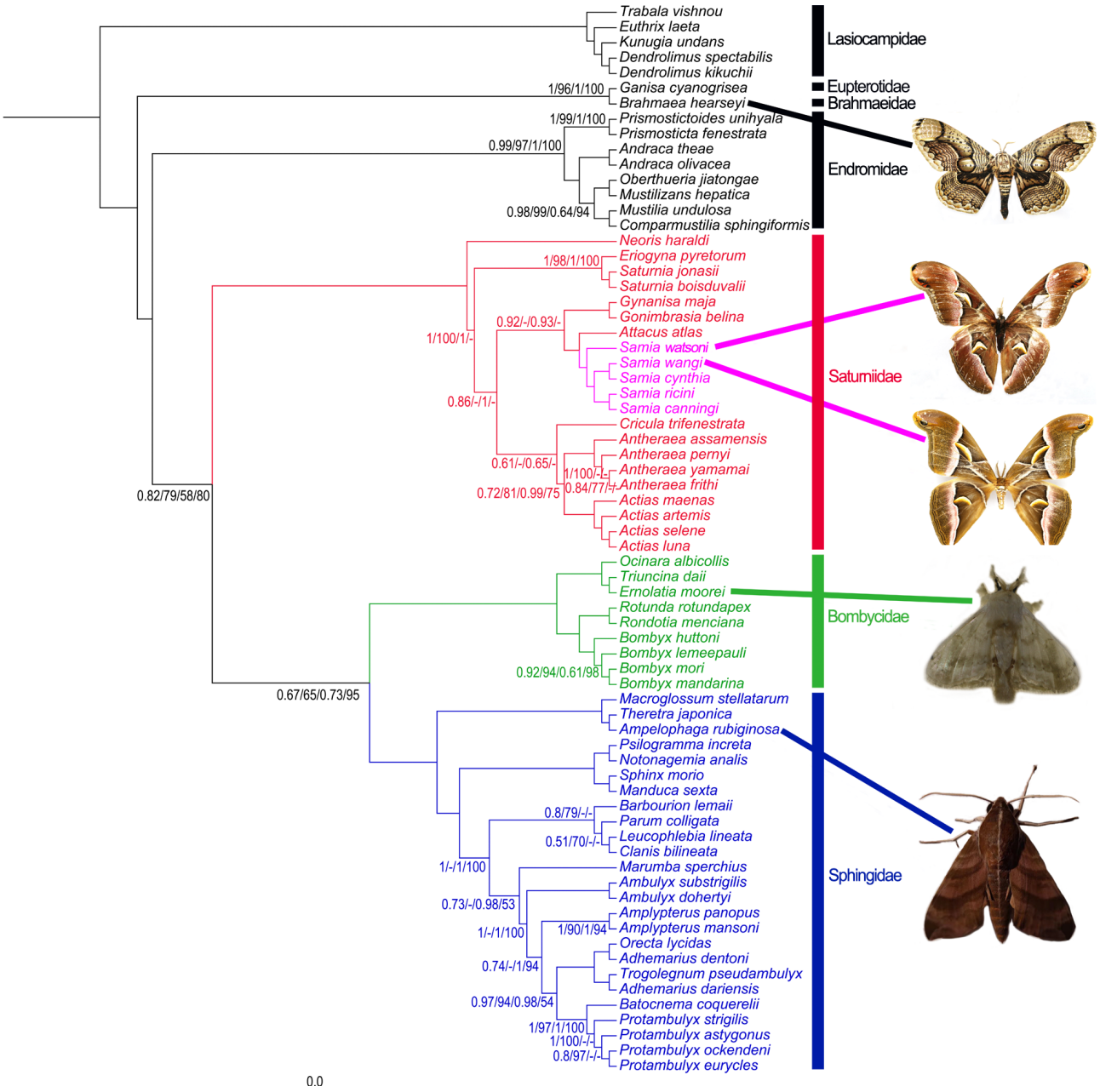

Fig. 3. ML tree and BI tree based on AA (amino acid sequence) and 13 PCGs (protein coding genes) + 2 rRNA data sets. The order is: AA (ML) / AA (BI) / 13 PCGs + 2 rRNA (ML) / 13 PCGs + 2 rRNA (BI). All nodes that do not display support are 1/100/1/100. All images in this figure are provided by Decai Lu.

and *S. wangi* was only 0.005, but *S. cynthia* and *S. wangi* are considered to be two separate species (Table S5). (5) The phylogenetic tree derived from the two data sets and analytical methods showed that *S. cynthia* and *S. wangi* are more closely related each other than either is to *S. ricini*. Therefore, *S. ricini* cannot be a subspecies of *S. cynthia*.

Instead, *S. ricini* is recovered as the sister taxon to *S. canningi*. *Samia ricini* has been considered to be a domesticated species derived from *S. canningi* and as a consequence, the two taxa should be treated as conspecific (Peigler & Calhoun, 2013). To test this conclusion, we carried out a number of analyses. The tRNAs of *S. ricini* and *S. canningi* are very similar, with only four mutations separating them. Evolutionary rates analysis showed that the 13 mitochondrial genes of the two species are under almost the same selection pressure. The codon usage frequencies of *S. ricini* and *S. canningi* are identical and the genetic distance analysis of the COI gene between *S. ricini* and *S. canningi* is zero. All of this evidence supports *S. ricini* and *S. canningi* being one and the same species. Huang et al. (2021) reached similar conclusions using DNA barcoding methods (Huang et al., 2021). They concluded that interspecific genetic distance played an important role in determining species delimitation, a position we adopted here. Moreover, Huang et al. (2021) found that multiple COI genes of *S. ricini* and *S. canningi* nested among each other on their phylogenetic tree, again providing strong evidence that they are the same species. However, another

analysis paints a contradictory picture. Peigler & Naumann (2003) considered *S. ricini* and *S. canningi* to be two different species based on structural differences, as well as behavioural differences. So, given that the names *S. ricini* and *S. canningi* have been used for a long time for two separate species, we consider that, from the perspective of research convenience, it is best to provisionally consider *S. ricini* and *S. canningi* as two different species.

Samia wangi was named by Naumann & Peigler (2001) for those populations of *Samia* in southern Mainland China, Taiwan and northern Vietnam that were once treated as *S. walkeri* (*S. walkeri* is now considered to be a junior synonym of *S. cynthia*). Early scholars clearly failed to distinguish *S. wangi* and *S. cynthia* as separate species, which is perhaps not surprising given how close they are on our phylogenetic tree (Fig. 3). Peigler & Naumann (2003) did note, however, that although the two species look very similar, they occur in very different habitats. *Samia wangi* lives in lowland and lower montane evergreen broad-leaved forest in the south, whereas *S. cynthia* lives in deciduous forests on the northern plain. Thus, their respective ecologies are clearly distinct.

Although Brechlin (2014) proposed a new genus, *Archaeosamia*, to accommodate *Samia watsoni*, this was not supported by Naumann et al. (2014). Our phylogenetic analysis corroborated *S. watsoni* as the sister group of all the other *Samia* studied here and that the genetic distance between it and any other *Samia* is greater than that between any pair of those other four species, However, this genetic distance is still far less than that between *S. watsoni* and *Attacus*, the other genus of the tribe Attacini included in our study. So, we concur that *S. watsoni* should be treated as a member of *Samia* rather than placed in a monobasic genus of its own.

Regarding the relationships among the three families of the 'SBS' group, our mitogenomic analysis yielded the following pattern: (Saturniidae + (Bombycidae + Sphingidae)). Thus, our study corroborates the conclusions of Kim et al. (2017) and Wang et al. (2017) but is based on increased taxon sampling and with greater support values for many clades. However, results from mitogenome analyses continue to disagree with those derived from phylogenomic analyses of nuclear genomes, in which increased taxon and gene sampling now consistently supports a sister group relationship between Saturniidae and Sphingidae to the exclusion of Bombycidae (Kawahara et al., 2014; Xin et al., 2017; Hamilton et al., 2019). Whether this conflict can be resolved by yet further taxon sampling or, if not, what is the underlying explanation for this conflict remains to be determined.

CONCLUSION

The conclusions of our study are as follows: (1) *Samia ricini* is very closely related to *S. canningi*, and more distant from a clade comprising *S. cynthia* and *S. wangi*. (2) Our results are consistent with *S. ricini* being derived from *S. canningi* by a process of domestication, but we regard them as two species, rather than conspecific, based on the morphological evidence provided by previous authors (e.g., Naumann & Peigler, 2014). (3) We concur with Naumann et al. (2014) that *S. watsoni* should be included within the genus *Samia* rather than being placed in its own monobasic genus. (4) Our analysis recovered the following relationship for the three families of the 'SBS' group is: (Saturniidae + (Bombycidae + Sphingidae)). This agrees with previous studies based on analysis of mitogenomes but continues to contradict the results derived from phylogenomic analysis of nuclear genomes.

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DATA AVAILABILITY STATEMENT. The raw data and the assemblies were deposited in the National Center for Biotechnology Information, with the BioProject access number PRJNA818861 (*Samia watsoni*) and PRJNA818466 (*Samia wangi*), with the BioSample access number SAMN26885815 (*Samia watsoni*) and SAMN26863503 (*Samia wangi*), with the SRA access number SRR18441626 (*Samia watsoni*) and SRR18441011 (*Samia wangi*), with the GenBank access number ON059173 (*Samia watsoni*) and ON080860 (*Samia wangi*).

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CONFLICTS OF INTEREST. The authors declare no competing financial interests.

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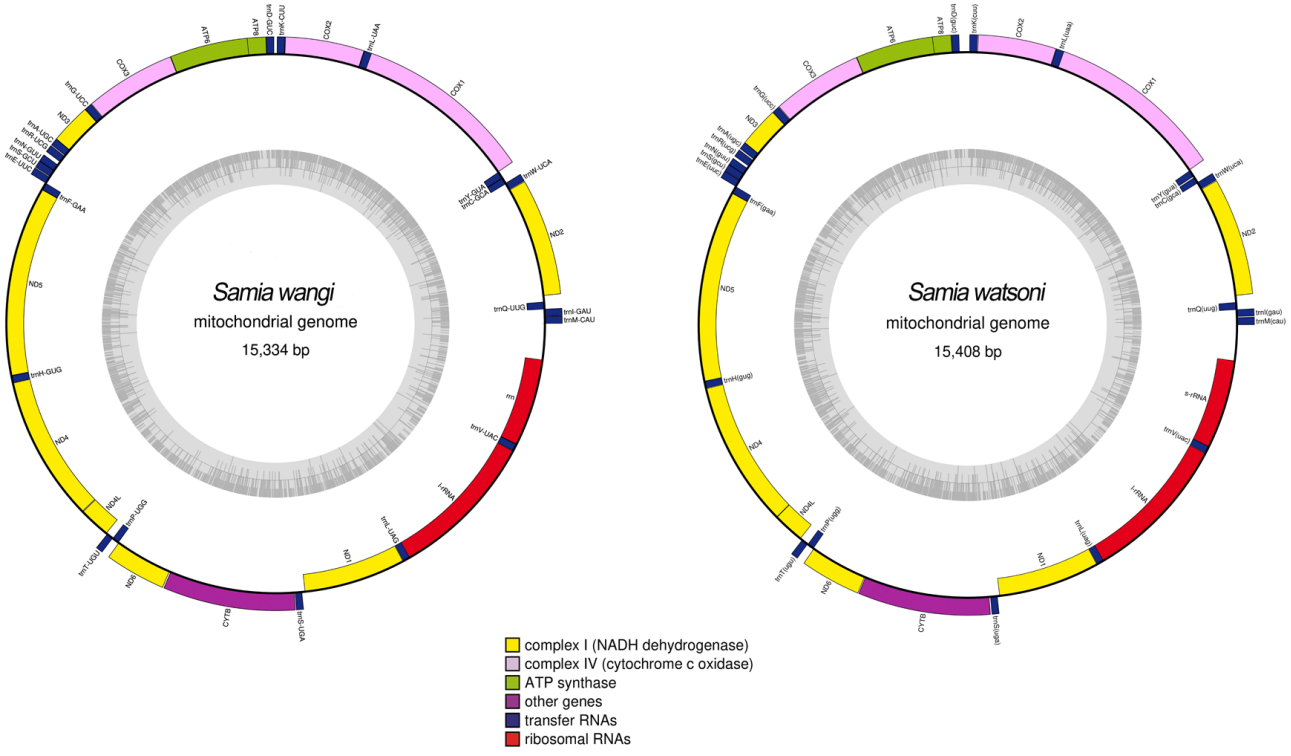

Fig. S1. Circular map of the mitogenomes of *Samia wangi* and *Samia watsoni.*

Table S1. Mitogenomes downloaded from NCBI.

Superfamily	Family	Species	Size(bp)	GenBank
Bombycoidea	Bombycidae	*Bombyx mori* strain Baiyun	15629	KM279431.1
Bombycoidea	Bombycidae	*Bombyx huttoni*	15638	NC_026518.1
Bombycoidea	Bombycidae	*Bombyx lemeepauli*	15801	NC_037149.1
Bombycoidea	Bombycidae	*Bombyx mandarina* from China	15682	GU966631.1
Bombycoidea	Bombycidae	*Ernolatia moorei*	15377	NC_038104.1
Bombycoidea	Bombycidae	*Ocinara albicollis*	15439	MF100144.1
Bombycoidea	Bombycidae	*Rondotia menciana* RM1234	15301	KJ647172.1
Bombycoidea	Bombycidae	*Rotunda rotundapex*	15298	NC_045528.1
Bombycoidea	Bombycidae	*Triuncina daii*	15482	NC_036484.1
Bombycoidea	Brahmaeidae	*Brahmaea hearseyi*	15442	NC_034279.1
Bombycoidea	Endromidae	*Andraca olivacea*	15880	NC_038082.1
Bombycoidea	Endromidae	*Andraca theae*	15737	NC_032694.1
Bombycoidea	Endromidae	*Comparmustilia sphingiformis*	15100	NC_038083.1
Bombycoidea	Endromidae	*Mustilia undulosa*	15720	NC_038085.1
Bombycoidea	Endromidae	*Mustilizans hepatica*	15745	NC_038105.1
Bombycoidea	Endromidae	*Oberthueria jiatongae*	15673	NC_038086.1
Bombycoidea	Endromidae	*Prismosticta fenestrata*	15772	NC_038106.1
Bombycoidea	Endromidae	*Prismostictoides unihyala*	15772	MF100146.1
Bombycoidea	Eupterotidae	*Ganisa cyanogrisea*	15250	NC_038084.1
Bombycoidea	Saturniidae	*Actias artemis aliena*	15243	KF927042
Bombycoidea	Saturniidae	*Actias luna*	15295	NC_045899
Bombycoidea	Saturniidae	*Actias maenas*	15322	MG836834
Bombycoidea	Saturniidae	*Actias selene*	15236	JX186589
Bombycoidea	Saturniidae	*Antheraea assamensis*	15312	NC_030270
Bombycoidea	Saturniidae	*Antheraea frithi*	15338	KJ740437
Bombycoidea	Saturniidae	*Antheraea pernyi*	15566	AY242996
Bombycoidea	Saturniidae	*Antheraea yamamai*	15338	EU726630
Bombycoidea	Saturniidae	*Attacus atlas*	15282	NC_021770
Bombycoidea	Saturniidae	*Cricula trifenestrata*	15425	KY644697
Bombycoidea	Saturniidae	*Eriogyna pyretorum*	15327	NC_012727
Bombycoidea	Saturniidae	*Gonimbrasia belina*	15308	NC_046032
Bombycoidea	Saturniidae	*Gynanisa maja*	15287	MN832541
Bombycoidea	Saturniidae	*Neoris haraldi*	15383	NC_036765
Bombycoidea	Saturniidae	*Samia canningi*	15384	NC_024270
Bombycoidea	Saturniidae	*Samia cynthia*	15345	KC812618
Bombycoidea	Saturniidae	*Samia ricini*	15384	NC_017869.1
Bombycoidea	Saturniidae	*Saturnia boisduvalii*	15360	EF622227
Bombycoidea	Saturniidae	*Saturnia jonasii*	15261	MF346379
Bombycoidea	Sphingidae	*Adhemarius dariensis*	15676	NC_046728.1
Bombycoidea	Sphingidae	*Adhemarius dentoni*	15423	NC_046713.1
Bombycoidea	Sphingidae	*Ambulyx dohertyi*	15304	NC_046714.1
Bombycoidea	Sphingidae	*Ambulyx substrigilis*	15333	NC_046715.1
Bombycoidea	Sphingidae	*Ampelophaga rubiginosa*	15282	NC_035431.1
Bombycoidea	Sphingidae	*Amplypterus mansoni*	15394	MK804152.1
Bombycoidea	Sphingidae	*Amplypterus panopus*	15370	MK804153.1
Bombycoidea	Sphingidae	*Barbourion lemaii*	15366	NC_046718.1
Bombycoidea	Sphingidae	*Batocnema coquerelii*	15361	MK804155.1
Bombycoidea	Sphingidae	*Clanis bilineata*	15426	NC_046720.1
Bombycoidea	Sphingidae	*Leucophlebia lineata*	15454	NC_046721.1
Bombycoidea	Sphingidae	*Macroglossum stellatarum*	15290	NC_037441.1
Bombycoidea	Sphingidae	*Manduca sexta*	15516	EU286785.1
Bombycoidea	Sphingidae	*Marumba sperchius*	15669	MT712138
Bombycoidea	Sphingidae	*Notonagemia analis scribae*	15303	KU934302.1
Bombycoidea	Sphingidae	*Orecta lycidas*	15387	NC_046722.1
Bombycoidea	Sphingidae	*Parum colligata*	15288	NC_039166.1
Bombycoidea	Sphingidae	*Protambulyx astygonus*	15345	NC_046723.1
Bombycoidea	Sphingidae	*Protambulyx eurycles*	15542	NC_046724.1
Bombycoidea	Sphingidae	*Protambulyx ockendeni*	15395	NC_046725.1
Bombycoidea	Sphingidae	*Protambulyx strigilis*	15534	NC_046726.1
Bombycoidea	Sphingidae	*Psilogramma increta*	15252	MF974243.1
Bombycoidea	Sphingidae	*Sphinx morio*	15299	NC_020780.1
Bombycoidea	Sphingidae	*Theretra japonica*	15399	NC_037725.1
Bombycoidea	Sphingidae	*Trogolegnum pseudambulyx*	15387	NC_046727.1
Lasiocampoidea	Lasiocampidae	*Dendrolimus kikuchii*	15382	MF100138.1
Lasiocampoidea	Lasiocampidae	*Dendrolimus spectabilis*	15409	KU558688.1
Lasiocampoidea	Lasiocampidae	*Euthrix laeta*	15368	NC_031507.1
Lasiocampoidea	Lasiocampidae	*Kunugia undans*	15570	KX822016.1
Lasiocampoidea	Lasiocampidae	*Trabala vishnou guttata*	15281	KU884483.1

Table S2. Mitogenome composition of *Samia wangi*.

Gene	Direction	Location	Length	Codon	Start codon	Stop codon	Intergenic nucleotides
trnM	F	1–67	67	ATG	—	—	
trnI	F	69–133	65	ATC	—	—	1
trnQ	R	131–199	69	CAA	—	—	–3
nad2	F	254–1267	1014	—	ATT	TAA	54
trnW	F	1271–1338	68	TGA	—	—	3
trnC	R	1331–1392	62	TGC	—	—	2
trnY	R	1393–1458	66	TAC	—	—	
cox1	F	1459–3003	1545	—	ATT	TAA	
trnL2	F	2999–3066	68	TTA	—	—	–5
cox2	F	3067–3751	685	—	GTG	T	
trnK	F	3752–3822	71	AAG	—	—	
trnD	F	3847–3914	68	GAC	—	—	24
atp8	F	3915–4079	165	—	ATT	TAA	
atp6	F	4073–4750	678	—	ATG	TAA	7
cox3	F	4750–5538	789	—	ATG	TAA	–1
trnG	F	5541–5606	66	GGA	—	—	2
nad3	F	5607–5960	354	—	ATT	TAG	
trnA	F	5959–6025	67	GCA	—	—	–2
trnR	F	6031–6097	67	CGA	—	—	5
trnN	F	6130–6194	65	AAC	—	—	32
trnS1	F	6194–6259	66	AGC	—	—	–1
trnE	F	6264–6329	66	GAA	—	—	4
trnF	R	6328–6394	67	TTC	—	—	–2
nad5	R	6395–8140	1746	—	ATT	TAA	
trnH	R	8141–8206	66	CAC	—	—	
nad4	R	8208–9548	1341	—	ATG	TAA	1
nad4l	R	9552–9842	291	—	ATG	TAA	3
trnT	F	9851–9914	64	ACA	—	—	8
trnP	R	9915–9979	65	CCA	—	—	
nad6	F	9982–10512	531	—	ATA	TAA	2
cytb	F	10519–11670	1152	—	ATG	TAA	6
trnS2	F	11676–11740	65	TCA	—	—	–5
nad1	R	11766–12704	939	—	ATG	TAA	25
trnL1	R	12707–12774	68	CTA	—	—	2
rrnL	R	12775–14137	1363	—	—	—	
trnV	R	14138–14203	66	GTA	—	—	
rrnS	R	14204–14979	776	—	—	—	

Table S3. Mitogenome composition of *Samia watsoni*.

Gene	Direction	Location	Length	Codon	Start codon	Stop codon	Intergenic nucleotides
trnM	F	1–68	68	ATG	—	—	
trnI	F	75–139	65	ATC	—	—	6
trnQ	R	137–205	69	CAA	—	—	–3
nad2	F	264–1277	1014	—	ATT	TAA	58
trnW	F	1281–1348	68	TGA	—	—	3
trnC	R	1341–1402	62	TGC	—	—	–8
trnY	R	1412–1476	65	TAC	—	—	9
cox1	F	1481–3016	1536	—	CGA	TAA	4
trnL2	F	3012–3082	71	TTA	—	—	–5
cox2	F	3083–3764	682	—	GTG	T	
trnK	F	3768–3838	71	AAG	—	—	3
trnD	F	3927–3994	68	GAC	—	—	88
atp8	F	3995–4159	165	—	ATC	TAA	
atp6	F	4153–4830	678	—	ATG	TAA	–7
cox3	F	4830–5618	789	—	ATG	TAA	–1
trnG	F	5621–5686	66	GGA	—	—	2
nad3	F	5687–6040	354	—	ATT	TAG	
trnA	F	6039–6105	67	GCA	—	—	–2
trnR	F	6115–6183	69	CGA	—	—	9
trnN	F	6204–6268	65	AAC	—	—	20
trnS1	F	6268–6333	66	AGC	—	—	–1
trnE	F	6334–6400	67	GAA	—	—	
trnF	R	6399–6466	68	TTC	—	—	–2
nad5	R	6467–8215	1749	—	ATT	TAA	
trnH	R	8213–8278	66	CAC	—	—	–3
nad4	R	8281–9621	1341	—	ATG	TAA	2
nad4l	R	9621–9911	291	—	ATG	TAA	–1
trnT	F	9929–9993	65	ACA	—	—	17
trnP	R	9994–10058	65	CCA	—	—	
nad6	F	10061–10591	531	—	ATA	TAA	2
cytb	F	10597–11751	1155	—	ATG	TAA	5
trnS2	F	11761–11826	66	TCA	—	—	9
nad1	R	11845–12783	939	—	ATG	TAA	18
trnL1	R	12785–12852	68	CTA	—	—	1
rrnL	R	12828–14205	1378	—	—	—	–25
trnV	R	14207–14273	67	GTA	—	—	1
rrnS	R	14274–15086	813	—	—	—	

Table S4. Conserved sites and variable sites between the mitogenomes of *Samia ricini*, *Samia canningi* and *Samia cynthia*.

Gene	*S. canningi* and *S. cynthia*		*S. canningi* and *S. ricini*		*S. cynthia* and *S. ricini*	
	conserved sites	variable sites	conserved sites	variable sites	conserved sites	variable sites
tRNA-Met	67/67	0	66/67	1/67	66/67	1/67
tRNA-Ile	65/65	0	64/65	1/65	64/65	1/65
tRNA-Gln	68/69	1/69	69/69	0	68/69	1/69
ND2	985/1014	29/1014	1012/1014	2/1014	983/1014	31/1014
tRNA-Trp	68/68	0	68/68	0	68/68	0
tRNA-Cys	62/62	0	62/62	0	62/62	0
tRNA-Tyr	63/66	2/66	65/66	0	63/66	2/66
COI	1480/1540	51/1540	1536/1540	4/1540	1478/1540	53/1540
tRNA-Leu	68/68	0	68/68	0	68/68	0
COX2	667/685	18/685	682/685	3/685	666/685	19/685
tRNA-Lys	71/71	0	69/71	2/71	69/71	2/71
tRNA-Asp	67/68	1/68	68/68	0	67/68	1/68
ATP8	164/165	1/165	164/165	1/165	163/165	2/165
ATP6	653/678	25/678	677/678	1/678	652/678	26/678
COX3	765/789	24/789	785/789	4/789	765/789	24/789
tRNA-Gly	66/66	0	66/66	0	66/66	0
tRNA-Ala	65/66	1/66	66/66	0	65/66	1/66
tRNA-Arg	63/70	1/70	64/70	0	63/70	1/70
tRNA-Asn	65/65	0	65/65	0	65/65	0
tRNA-Ser	68/68	0	68/68	0	68/68	0
tRNA-Glu	65/66	1/66	66/66	0	65/66	1/66
tRNA-Phe	66/68	1/68	68/68	0	66/68	1/68
ND5	1694/1749	52/1749	1742/1749	7/1749	1695/1749	51/1749
tRNA-His	63/66	3/66	66/66	0	63/66	3/66
ND4	1307/1341	34/1341	1335/1341	6/1341	1305/1341	36/1341
ND4L	283/291	8/291	290/291	1/291	282/291	9/291
tRNA-Thr	62/65	2/65	65/65	0	62/65	2/65
tRNA-Pro	65/65	0	65/65	0	65/65	0
ND6	505/531	26/531	528/531	3/531	505/531	26/531
CYTB	1096/1149	53/1149	1144/1149	5/1149	1094/1149	55/1149
tRNA-Ser	65/66	1/66	66/66	0	65/66	1/66
ND1	900/939	39/939	930/939	9/939	901/939	38/939
tRNA-Leu	66/68	2/68	68/68	0	66/68	2/68
tRNA-Val	66/66	0	66/66	0	66/66	0
ND3	1325/1356	30/1356	1356/1356	0	1325/1356	30/1356
16S-rRNA	1325/1362	30/1362	1358/1362	0	1325/1362	30/1362
12S-rRNA	761/779	17/779	778/779	1/779	762/779	16/779

Table S5. Pairwise genetic distances between the five species of *Samia*.

	Samia cynthia	*Samia canningi*	*Samia ricini*	*Samia wangi*	*Samia watsoni*
Samia cynthia					
Samia canningi	0.011				
Samia ricini	0.011	0.000			
Samia wangi	0.005	0.009	0.010		
Samia watsoni	0.026	0.025	0.025	0.023	

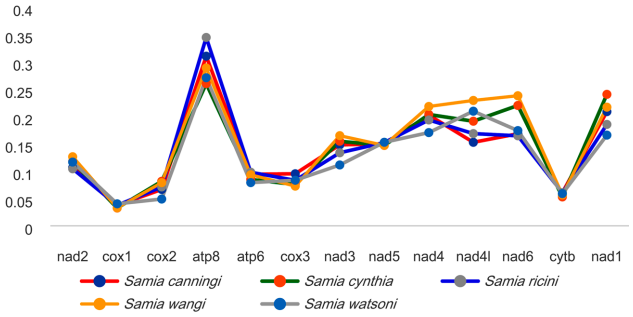

Fig. S2. Evolutionary rates of the mitochondrial genomes of the five species of *Samia*. The ratio of Ka (the number of non-synonymous substitutions per non-synonymous site)/Ks (the number of synonymous substitutions per synonymous site) for every mitochondrial genome are given, using that of *Ambulyx dohertyi* (Sphingidae) as the reference sequence.

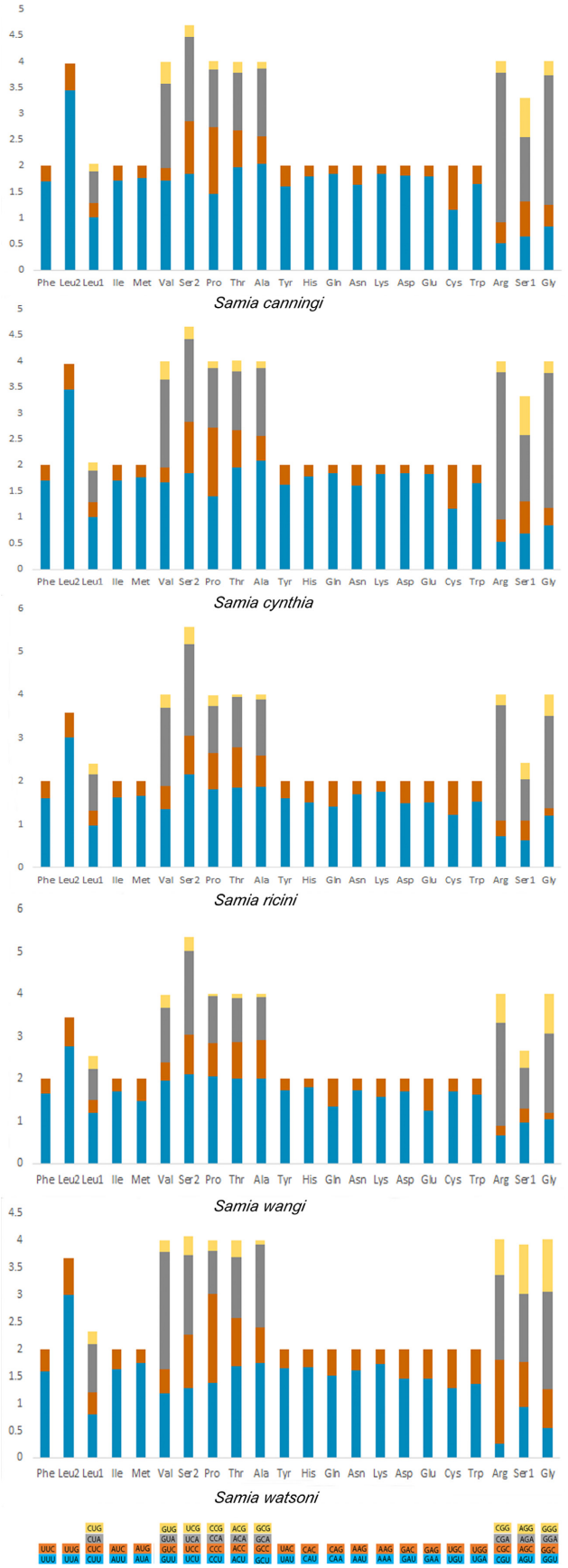

Fig. S3. Relative synonymous codon usage (RSCU) in the mitogenomes of *Samia watsoni* and *Samia wangi*.

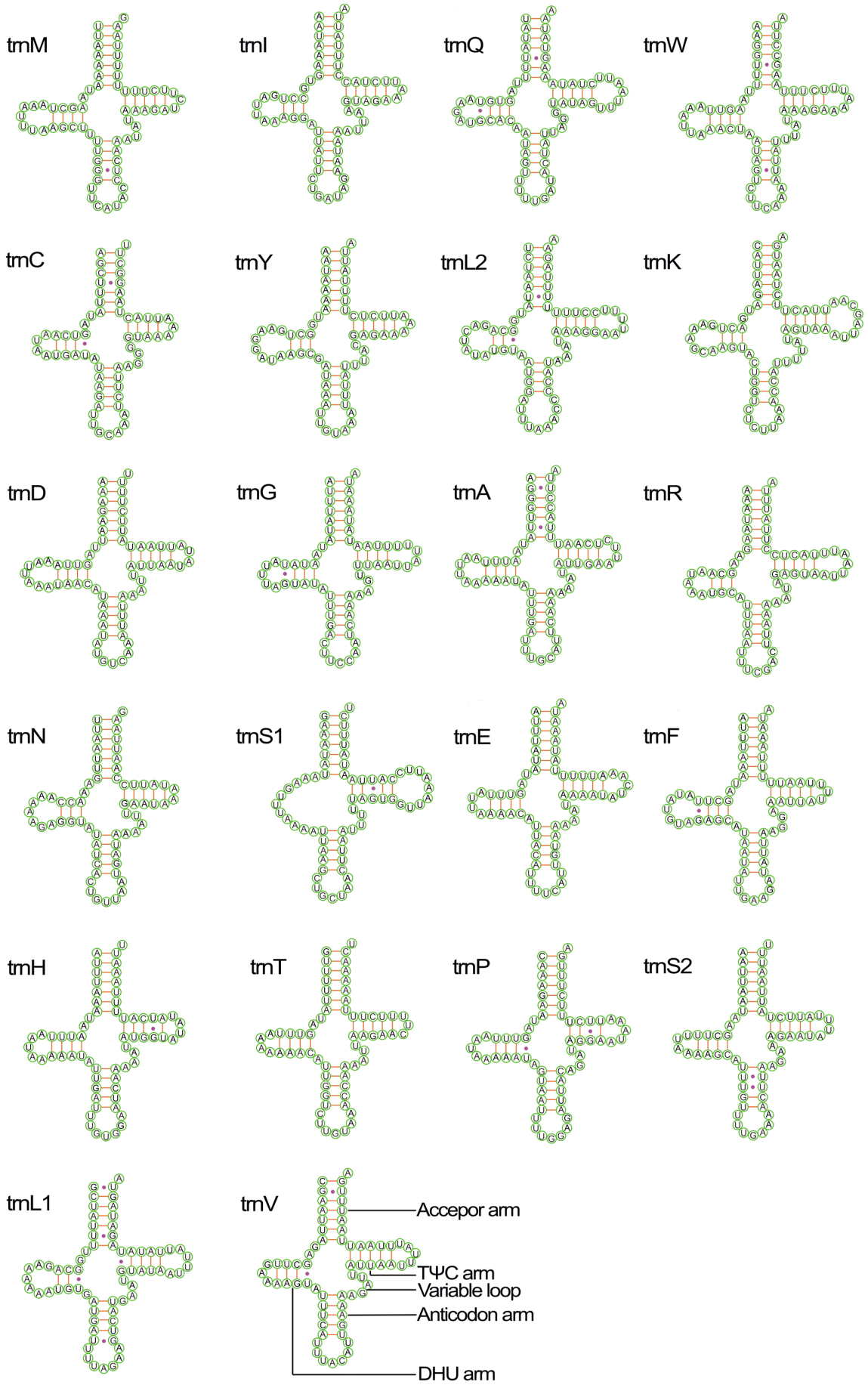

Fig. S4. Putative secondary structures of tRNAs from the *Samia wangi* mitogenome.

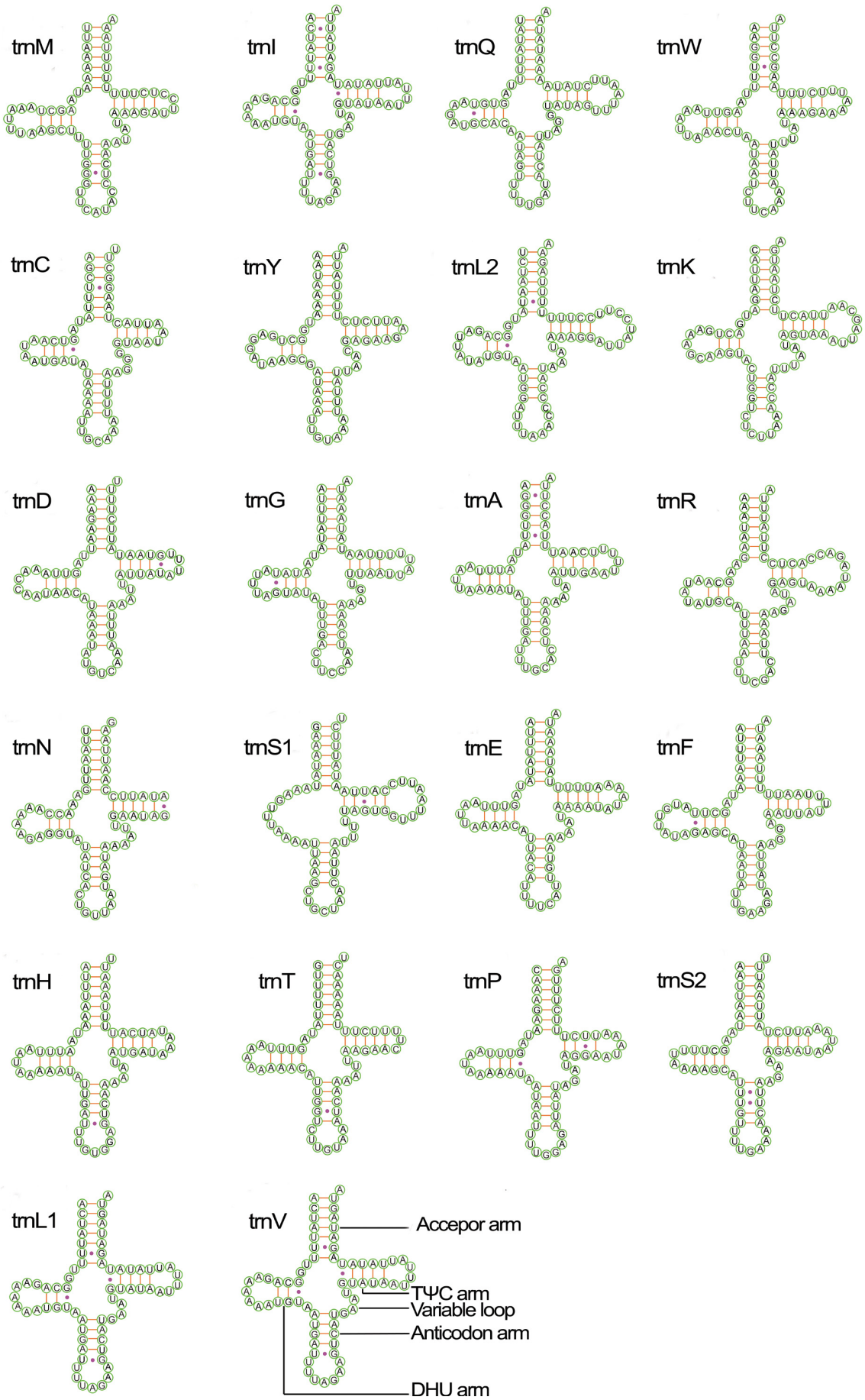

Fig. S5. Putative secondary structures of tRNAs from the *Samia watsoni* mitogenome.

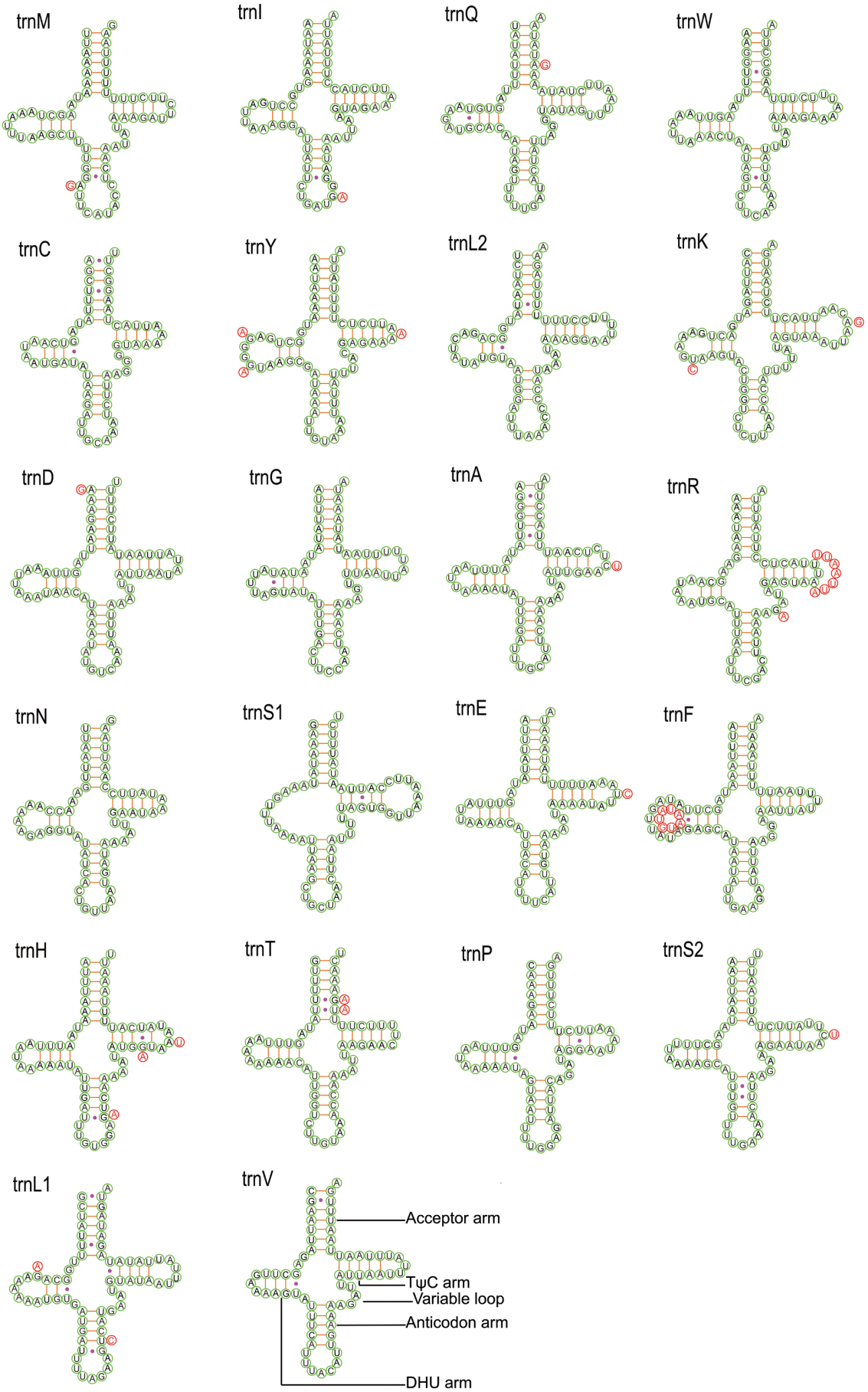

Fig. S6. Inferred secondary structures of 22 tRNAs of *Samia cynthia* and *Samia ricini*. The red sections are the partial tRNA sequences of *Samia cynthia*.

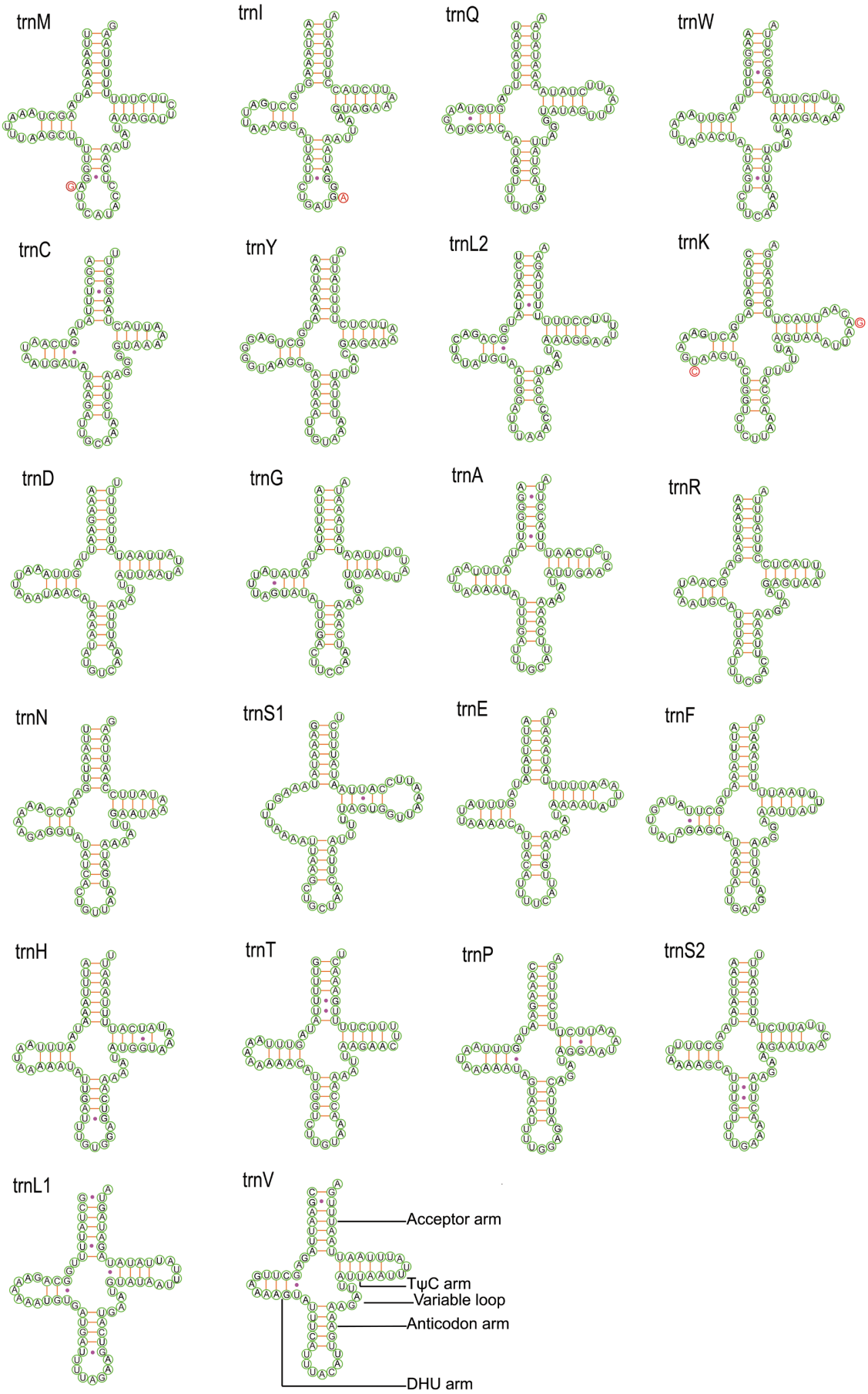

Fig. S7. Inferred secondary structure of 22 tRNAs of *Samia ricini* and *Samia canningi*. The red sections are the partial tRNA sequences of *Samia canningi*.