African Journal of Biotechnology Vol. 8 (12), pp. 2707-2712, 17 June, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Mitochondrial DNA analysis reveals a low nucleotide diversity of *Caligula japonica* in China

Yu-Ping Li[1γ], Bao-Shan Yang[1,2γ], Huan Wang[1], Rui-Xi Xia[1], Lin Wang[3], Zhi-Hong Zhang[1], Li Qin[1], Yan-Qun Liu[1]*

[1]College of Bioscience and Biotechnology, Shenyang Agricultural University, Shenyang 110161, China.
[2]School of City Development, University of Jinan, Shandong, Jinan 250002, China.
[3]Technical Center for Inspection and Quarantine, Beijing Entry-Exit Inspection and Quarantine Bureau, Beijing 100026, China.

Accepted 14 April, 2009

***Caligula japonica* (Lepidoptera: Saturniidae), an endemic species in Eastern Asia, is not only an economically important pest to be controlled, but also a producer of expensive silk. We have demonstrated previously the presence of a higher genetic diversity and a certain degree of genetic differentiation related to geographical distribution in *C. japonica* based on RAPD and ISSR markers. In this paper, we determined the 574 bp segment of the mitochondrial cytochrome oxidase subunit I (COI) gene sequences of *C. japonica* in China to assess nucleotide sequence diversity (GenBank accession no. FJ358506-FJ358517). The pairwise COI sequence identity between these samples reached 99 - 100%, showing a low nucleotide sequence diversity (Pi = 0.0035). In NJ tree, these samples were divided well into Northern and Southern group, which was consistent with that previously reported based on RAPD and ISSR markers and suggested the genetic differentiation between them might have been occurred. Further work is needed to define the genetic structure of different populations of *C. japonica*.**

Key words: *Caligula japonica*, mitochondrial DNA, COI, genetic diversity, China.

INTRODUCTION

Caligula japonica (Lepidoptera: Saturniidae) is an endemic species in Eastern Asia including China, Korea, Japan and Russia. This species is also a wide-distributed species in China. It enters diapause in the stage of egg for living through the winter and enters dormancy in the stage of pupa through the summer (Zhang et al., 2005). The heterogametic females of *C. japonica* are XO type and the present chromosome X might be fused by the progenitor chromosome X and Y (Song et al., 1996). The omnivorous larva can survive by feeding the economic forest leaves form more than 38 species including *Ginkgo biloba*, *Populus tremula*, *Betula*, *Diospyros*, *Juglans*, *Malus*, *Pyrus*, *Quercus*, *Rhus*, which belong to more than 30 genus of 20 families (Ding et al., 2006). The moths of this species have not the ability of long distance migration, so they were often introduced into a new place of residence accompanying with the introduction of the seedling of plant.

C. japonica is one of the important forestry and medicinal plant pest to be monitored and controlled in China (Wei, 2006). In recent years, it occurred frequently in some areas of China and became the outbreak of the disaster. In 2006, it out-breaked in Longnan area of Gansu Province and more than 140 thousands of walnut trees were eaten up, resulting in a direct economic loss of 150 million RMB for walnut production (Xin and Du, 2006).

C. japonica is also considered as a precious wild silkmoth to be reared for silk production. It produces a long oval-shaped, chestnut brown, mesh-like cocoon. The silk of *C. japonica* has unique flashes of fluorescence and exhibits distinct flashes in natural light: the reflectance of fluorescence is higher than that of oak silkworm, *Antheraea pernyi* and mulberry silkworm, *Bombyx mori* (Huang et al., 2006). The price of *C. japonica* silk is about ten times higher than that of the *A. pernyi* silk. This natural

*Corresponding author. E-mail: liuyqlyp@yahoo.com.cn. Fax: 86-24-88411127.

γThese authors contributed equally to this work.

Figure 1. Map of China with locations from which *Caligula japonica* was sampled indicated a black dot (see Table 1 for descriptions).

fiber has been used as raw material silk to spin the silk yarn, which is used as the decorative goods to suit the expensive fabric and the identification to mark the high-security goods (Dong et al., 2006). The domestication study of *C. japonica* has been carried out by Sericulture Institute of Liaoning Province since 1995. Recently, *C. japonica* has been reared successfully using *Juglans Mandshurica* and *Pterocarya stenoptera* as feed (Zhang et al., 2005).

Clarifying the genetic diversity of *C. japonica* will provide a scientific basis for the regional control of forestry pest or rational utilization and conservation of wild silk moth resources. In our laboratory, we have showed a higher genetic diversity of *C. japonica* sampled from different areas by using RAPD and ISSR technical (Cao et al., 2007; Yang et al., 2008). The polymorphism of *C. japonica* on RAPD and ISSR markers ranged 60 - 91% with genetic distance and 73 - 95%, respectively. Cluster analysis showed the individuals from the same areas were well clustered into one group and the samples from different areas were well divided into Northern group and Southern group.

In this study, genetic diversity based on mitochondrial cytochrome oxidase subunit I (COI) gene sequences of 12 *C. japonica* samples from different areas in China, along with one Japanese sample were investigated. The COI gene sequences have been extensively used to investigate the genetic diversity within a species for their rapid evolution rates (Schery et al., 2005; Froufe et al., 2008; Xiao YS et al., 2008).

MATERIALS AND METHODS

Samples

Cocoons (pupae) of *C. japonica* from distinct geographic locations (Figure 1) representing eight provinces in China were sampled from July 2007 to August 2007 (Table 1). Five samples collected from Liaoning province were used. Upon collection, pupae were kept alive until they reached the laboratory where they were stored at -20℃ Single pupa was selected randomly as a representative for each population. The voucher, numbered from LC 001 to 012 respectively, was deposited at College of Bioscience and Biotechnology, Shenyang Agricultural University.

DNA isolation, polymerase chain reaction and DNA sequencing

Total genomic DNA was extracted from partial abdomen of pupa according to the method previously published (Zhao et al., 2000). The COI gene was PCR amplified (polymerase chain reaction) and sequenced using universal primers from Simon et al. (1994). The

Table 1. Samples collected in this study.

Population source	Code	Latitude/ longitude	Host plant	Voucher	Accession no.	Haplotype
Mudanjiang, Heilongjiang	HLJ	44°35′/ 129°34′	*Juglans Mandshurica*	LC001	FJ358509	H3
Jiaohe, Jilin	JL	43°75′/ 127°33′	*J. Mandshurica*	LC002	FJ358510	H5
Kuandian, Liaoning	LNK	40°80′/ 124°80′	*J. Mandshurica*	LC003	FJ358517	H3
Fengcheng, Liaoning	LNF	40°46′/ 124°05′	*J. Mandshurica*	LC004	FJ358513	H3
Xiuyan, Liaoning	LNX	40°30′/ 123°30′	*J. Mandshurica*	LC005	FJ358514	H4
Donggang, Liaoning	LND	39°90′/ 124°10′	*J. Mandshurica*	LC006	FJ358516	H3
Zhuanghe, Liaoning	LNZ	39°70′/ 122°96′	*J. Mandshurica*	LC007	FJ358511	H3
Longnan, Gansu	GS	33°70′/ 105°70′	*J. Mandshurica*	LC008	FJ358506	H1
Enshi, Hubei	HB	30°20′/ 109°40′	*Ginkgo biloba*	LC009	FJ358515	H2
Wuxi, Chongqing	CQ	31°42′/ 109°60′	*J. Mandshurica*	LC010	FJ358512	H2
Guiyang, Guizhou	GZ	26°56′/ 106°72′	*J. Mandshurica*	LC011	FJ358507	H1
Quanzhou, Guangxi	GX	25°96′/ 111°06′	*Pterocarya stenoptera*	LC012	FJ358508	H1
Japan	Japan	Unknowm	Unknowm	-	AB015869	H6

two primers were LYQ3 (5`-CCTGG ATCTT TAATT GGAGA-3`) and LYQ4 (5`-GGTAA AATTA AAATA TAAAC TTC-3`) respectively. PCRs were performed in a 50 µL volume with 0.5 U Taq (TIANGEN), 1 µL (about 20 ng) of Total DNA, 10 × PCR buffer, 4 µL of $MgCl_2$ (25 mmol/L), 4 µL of dNTPs (2.5 mmol/L for each), 10 pmol each primer and water. PCR products were detected by 1% agarose gels. The PCR fragments were directly sequenced in both directions after purification with PCR product purification kit (TIANGEN, China). DNA sequencing was performed on an ABI 3730 Genetic Analyzer (Applied Biosystems, USA). The sequence data have been deposited in GenBank under accession no. FJ358506 - FJ358517.

Sequence analysis

DNA sequences generated in both directions were assembled and edited to obtain a consensus sequence for each sample using Staden Package. The COI gene sequence of Japanese *C. japonica* population (AB015869) was downloaded from GenBank database. The sequence alignment was carried out using ClustalX version 1.8 (Thompson et al., 1997). MEGA version 4 (Tamura et al., 2007) was used to calculate the number of variable sites and nucleotide composition.

Sequence divergences were calculated by using the Kimura-2-Parameter (K2P) distance model of base substitution (Kimura, 1980) and phylogenetic tree was constructed using Neighbour-Joining (NJ) method (Saitou and Nei, 1987) with bootstrap test of 1000 replications. The K2P model was well suited to produce the distance matrix when the genetic distance between species analyzed was small (Nei and Kumar, 2000). DnaSP version 3.51 (Rozas and Rozas, 1999) was used to define the haplotype and estimate the population genetic parameters (*Pi*).

RESULTS AND DISCUSSION

Only a single specific PCR product was amplified from genomic DNAs of 12 *C. japonica* samples with the primer pair LYQ3 and LYQ4, although three PCR amplifications were separately carried out. The PCR products were sequenced and assembled to have a consensus sequence of 574 bp for each sample excluding two primer sequences. We carried out a BLAST search on NCBI with the sequence of LNF sample (Accession no. FJ358513) as query sequence. BLAST analysis showed that the determined sequence of LNF sample has 99% sequence identity with that of the COI gene of Japanese *C. japonica* (GenBank accession no. AB015869). No stop codons, insertions or deletions were found in the COI sequences. Translation analysis indicated that a total of 191 amino acids were encoded by the 574 bp fragment. These results affirmed that the determined sequence should be amplified from mitochondrial COI gene fragment, rather than nuclear gene fragment (Bensasson et al., 2000). The base compositions of the COI sequence of LNF sample of *C. japonica* varied among the individuals as follows: T 40.42%, C 17.07%, A 28.40% and G 14.11%, with a strong AT bias (68.82%), as usually found in insect mitochondrial genomes. As for the codon in the use of the base frequency, the COI sequence of *C. japonica* showed almost unanimous bias with that of the other known

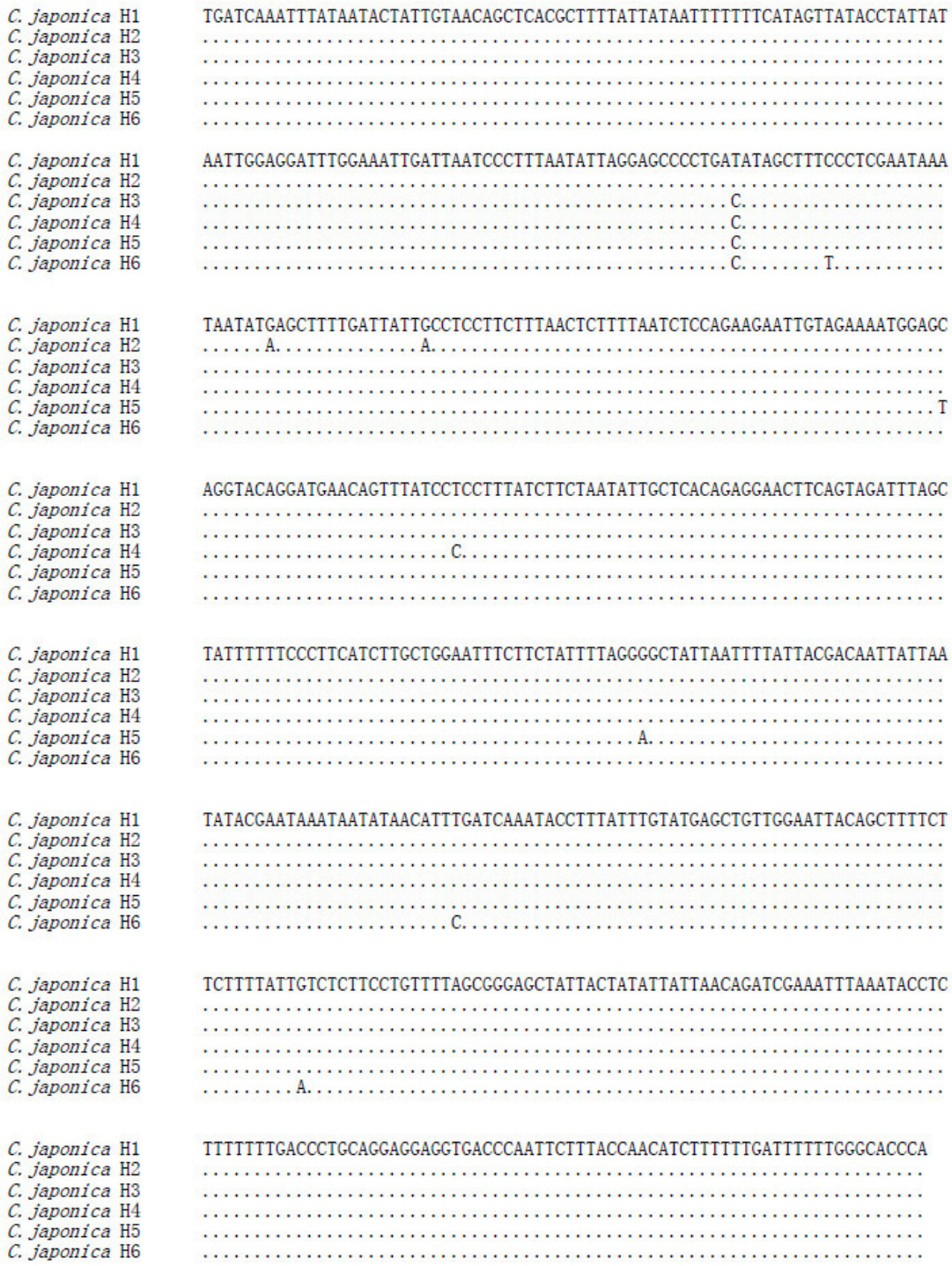

Figure 2. COI sequence alignment of different haplotypes of *Caligula japonica*. Only nine variable sites were detected from the alignments with a total of 574 bp in length. *C. japonica* H1-H6 refer to the six haplotypes of *Caligula japonica* as shown in Table 1 (. = Identical).

Saturniidae insects (Pu et al., 2009).

Analysis of the COI sequences of 13 *C. japonica* samples including Japanese sample, produced a 574 bp sequence alignment. Figure 2 shows COI sequence alignment result of different haplotypes of *C. japonica*. Only nine variable nucleotide sites were identified within these sequences, accounting for only 1.6% and of which were the transitions between T and C. Only five haplotypes were identified in 12 sequences from Chinese samples and three haplotypes shared. We designated

Table 2. Genetic distance of different haplotypes of *Caligula japonica*

	H1	H2	H3	H4	H5
H1					
H2	0.003				
H3	0.002	0.005			
H4	0.003	0.007	0.002		
H5	0.005	0.009	0.003	0.005	
H6	0.007	0.011	0.005	0.007	0.009

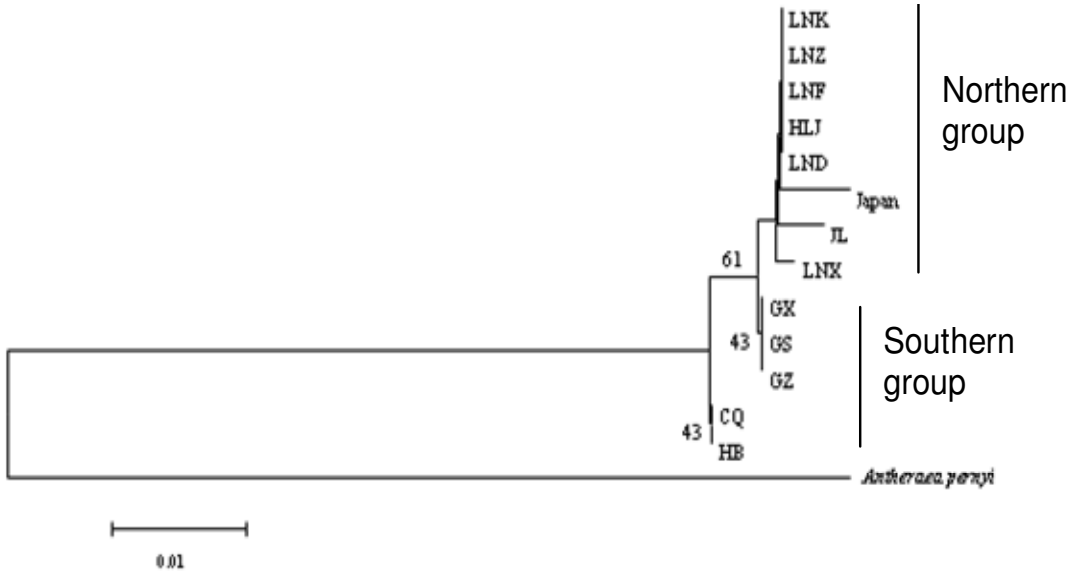

Figure 3. NJ tree based on the COI gene sequence of *Caligula japonica* samples. *Antheraea pernyi* was the outgroup. Bootstrap values from 1 000 replications are indicated above branches. The population codes of *C. japonica* were shown in Table 1.

them as haplotype H1 - 5 (Table 1). The shared haplotype H1 come from GS, GZ and GX samples; H2 from HB and CQ samples; H3 from LNF, LND, LNZ, LNK and HLJ samples with the highest frequency. Two haplotypes, H3 and H4, were identified in five samples that were collected from Liaoning province. We identified the COI sequence of Japanese sample as haplotype H6. Sequence analysis indicated that the pairwise sequence identity between *C. japonica* samples reached 99 - 100%, with a nucleotide sequence diversity (*Pi*) of 0.0035. This value was well within the previously observed data in the range from 0.0015 to 0.0047 which meant lower genetic diversity (Lan and Shi, 1993). The nucleotide sequence diversity of *Caligula japonica* in China observed in this study was also lower when compared to that in *Thanasimus dubius* (Coleoptera: Cleridae) with the range from 0.010 to 0.014 (Schery et al., 2005).

Genetic distance of different haplotypes of *C. japonica* was calculated based on the K2P model (Table 2). Among the five haplotypes in Chinese samples, the lowest pair wise genetic distance was 0.002 between H1 and H3 and between H3 and H4; the largest pair wise genetic distance was 0.009 between H2 and H5. Mean genetic distance of all samples was only 0.006, which was similar with that of *A. pernyi* geographic populations (range 0.002 - 0.006) (Zhu et al., 2008).

Phylogeny analysis showed *C. japonica* samples used in this study were well divided into Northern and Southern group according to the geographical position (Figure 3), although the bootstrap value on the evolution branches was not strong. The result was consistent with that previously reported based on RAPD and ISSR markers. The samples including LNK, LNF, LND, LNX, LNZ, HLJ, JL and Japan were grouped into Northern group. The samples including GS, CQ, HB, GZ and GX were clustered into Southern group. The lower bootstrap values resulted from the smaller genetic differences between COI sequences of *C. japonica* samples. There was no shared haplotype between Northern and Southern group, whereas there were shared haplotypes within groups. The result showed that genetic differences might have been occurred between the Northern and Southern group. Due

to the few variable sites, analysis of molecular variance was not carried out.

In analysis of the COI gene sequence, 13 *C. japonica* samples showed low nucleotide diversity, although a higher genetic diversity of these samples had been revealed by RAPD and ISSR markers with the maximal genetic distance of 0.49 between HLJ and GZ samples (Cao et al., 2007; Yang et al., 2008). These results were consistent with that found in Chinese oak silkworm, *A. pernyi* (Lepidoptera: Saturniidae) (Liu et al., 2006; Zhu et al., 2008). The semi-domestic *A. pernyi* was originated in Shandong province of China and its spread history was estimated to be only four hundred years (Gu, 1995; Zhang, 1982). Therefore, *C. japonica* is likely to be a recent spread species like *A. pernyi*.

The result of this study provides a preliminary molecular biology clue for further studies on population genetics of *C. japonica*. In order to fully grasp the internal relations of geographic populations of *C. japonica*, the number of samples, collection ranges and sequence length of molecular markers selected should be increased. It is helpful to produce more genetic information of *C. japonica* for the regional control of forestry pest or rational utilization and conservation of wild silk moth resources.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the National Natural Science Foundation of China (No. 30800803), the Scientific Research Project for Common weal Industry of Agricultural Ministry (No. nyhyzx 07-020-17), the Scientific Research Project for High University of the Educational Department of Liaoning Province (No. 2008643) and the Young Scholar Foundation of Shenyang Agricultural University (No. 20070112) respectively.

REFERENCES

Bensasson D, Zhang D, Hartl DL, Hewitt GM (2000). Mitochondrial pseudogenes: evolution's misplaced witnesses. Trends Ecol. Evol. 16: 314-321.

Cao LJ, Yang BS, Li J, Li M, Wang Z, Yang RS, Qin L (2007). RAPD analysis of *Dictyoploca japonica* from different areas. Acta Sericologica Sinica, 33: 293-296.

Ding DS, Zhong LY, Chen H, Chen CY, Wang L (2006). Biology and No-general Pollution Control of *Dictyoploca japonica*. Jiangxi Forest. Sci. Technol. 4: 21-22.

Dong FC, Pan ZJ, Jia YT (2006). Structure and performance analysis of wildmoth silks. Silk, 3: 18-20.

Froufe E, Sousa P, Alves PC, Harris DJ (2008) Genetic diversity within *Scorpio maurus* (Scorpiones: Scorpionidae) from morocco: Preliminary evidence based on COI mitochondrial DNA sequences. Biologia, 63: 1157-1160.

Gu KB (1995). Review of phylogeny on tussah sericulture. Agric. Archaeol. 3: 206-214.

Huang XM, Liu FY, Ding SX (2006). Preliminary analysis of fluorescence reflectivity of hand spun silk yarn of Camphor silkworm. Acta Sericologica Sinica, 32: 285-288.

Kimura M (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111-120.

Lan H, Shi L (1993). The origin and genetic differentiation of native breeds of pigs in South west China: An approach from mitochondrial DNA polymorphism. Biochem. Genet. 31: 51-60.

Liu YQ, Lu C, Qin L, Xiang ZH (2006). Genetic relationships of *Antheraea pernyi* cultivars based on RAPD markers. Scientia Agricultura Sinica, 39: 2608-2614.

Nei M, Kumar S (2000). Molecular evolution and phylogenetics. Oxford University Press, New York.

Pu JM, Wu S, Huo XM, Wang H, Xia RX, Li XS, Wang ZD, Luo CB, Liu YQ (2009). The DNA Barcode and primary phylogenetic analysis of the moon silkworm, *Actias selene*. Acta Sericologica Sinica, 35: 155-160.

Rozas J, Rozas R (1999). DnaSp version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics, 15: 174-175.

Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Schery MM, Reeve JD, Anderson FE (2005). Mitochondrial DNA analysis of the bark beetle predator *Thanasimus dubius* F. (Coleoptera: Cleridae) reveals genetic differentiation. Mol. Ecol. 14: 3317-3324.

Simon C, Frait F, Bechenback A, Crespi B, Liu H, Flook PK (1994). Evolution, weighting and phylogentic utility of mitochondrial gene sequence and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87: 651-701.

Song FZ, Xiang ZH, Liu SL, Yutaka B, Yutaka K, Katsumi K (1996). Studies on the sex chromosome of the silkworms *Philosamia cynthia ricini* and *Dictyoploca japonica*. Acta Sericologica Sinica, 22: 170-174.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596-1599.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 25: 4876-4882.

Wei JF (2006). Fifteen pests endanger the safety of forest resources in Shennongjia area. Hubei Forest. Technol. 3: p. 9.

Xiao YS, Zhang Y, Gao TX, Yabe M, Sakurai Y (2008). Phylogenetic relationships of the lancelets of the genus *Branchiostoma* in China inferred from mitochondrial genome analysis. Afr. J. Biotechnol. 7(21): 3845-3852.

Xin G, Du WY (2006). The prevention and control of *Dictyoploca japonica* on the walnut tree. Northwest Hortic. 12: p. 21.

Yang BS, Cao LJ, Li J, Li YR, Wang Z, Qin L (2008). Genetic diversity assessment of *Dictyoploca japonica* from different areas. Entomol. Knowledge, 45: 418-421.

Zhang CH, Liu FY, Qi RH, Zuo SC (2005). The biological characteristics of *Dictyoploca japonica*. China Sericult. 26(2): 50-51.

Zhang K (1982). Origin and Radiation of oak silkworm in China. Acta Sericologica Sinica, l8: 112-116.

Zhao QL, Zhang ZF, He JL (2000). Rapid preparation method of the genomic DNA from *Bombyx mori* pupa. Acta Sericologica Sinica, 26: 63-64.

Zhu XW, Liu YQ, Li XS, Huo XM, Jiang YR, Qin L (2008) Taxonomy status of wild oak silkworm in Yunnan revealed by DNA barcoding. Acta Sericologica Sinica, 34: 424-428.