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Original Research Article

Geophylogenetic analysis of *Teinopalpus aureus* Mell based on re-sequencing of the whole mitochondrial genome

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Ying Huang[1], Shanyi Zhou*[1], Chaobin Huang[1] and Juping Zeng[2]

[1]Key Laboratory of Ecology of Rare and Endangered Species and Environmental Protection, Ministry of Education, Guangxi Normal University, Guilin, 541004, China; [2]College of Forestry, Jiangxi Agricultural University, Nanchang, 330045, China

*Corresponding Author
Email: syzhou@mailbox.gxnu.edu.cn
Tel: (+086)-773-5845952 (O), 18078337092 (Mobile)
Fax: (+086)-773-5806680

The complete mitochondrial genomes of three geographic populations of *Teinopalpus aureus* Mell, i. e. the populations of Fujian Meihuashan (MHS), Jiangxi Jiulianshan (JLS), and Jiangxi Pingshan (PS), were sequenced and compared with that of the population of Guangxi Dayaoshan (DYS). The entire length of the *T. aureus* mitochondrial genome is among 15232–15237 bp. Sequence variation was found in different geographic populations and occurred in genes tRNA^{Ala}, tRNA^{Glu}, ND5, 16SrRNA, and spacer regions. Among protein-coding genes, stop codons of ND5 gene were the same in all geographic populations. The same gene had the same start codon, but the stop codons sometimes varied among different geographic populations. Genetic relationships among them were analyzed. Sequences of a close species *Papilio machaon* from Papilionidae and a distant species *Antheraea pernyi* from Saturniidae downloaded from GenBank were used as outgroups. Maximum parsimony trees were constructed using 12S rRNA gene, COI gene, Cytb gene, and ND3 gene, respectively. Two lineages were found among the four geographic populations. One lineage was clustered by DYS and JLS, and the other lineage by MHS and PS. Relationship was close between DYS and JLS, and between MHS and PS.

Key words: *Teinopalpus aureus*, geographic populations, mitochondrial genome, genetic relationship

INTRODUCTION

Teinopalpus aureus belongs to the family Papilioninae, order Lepidoptera (Chou 2000). This butterfly was listed as the national first (I) vulnerable species in the *List of the China Wildlife Conservation* in 1998, and as a data-deficient species in the *IUCN Red List of Threatened Species* in 2004. Many Chinese entomologists offered contributions in the national butterfly nomination of *T. aureus* at the fourth symposium sponsored by the Butterfly Branch of Entomological Society of China in 2002. After the species was nominated by Mell (1923), *T. aureus* was found distributing in Fujian, Guangxi, Hainan, Zhejiang (Tong et al., 1995), Yunnan (Wu 1999); Jiangxi (Song et al., 2007), Vietnam, and Laos (Morita 1998; Masui and Uehara 2000).

Previous studies on *T. aureus* in China mainly focus on host plant (Zeng et al. 2005, 2014; Chen et al. 2009; He and Jia 2012), population sizes (Zhou et al., 2013), biological traits (Zhou and Jiang, 1996; Zeng et al., 2004, 2008, 2012), and life history (Zeng et al., 2007). Qin et al. (2011) and Lin et al. (2011) discussed relationships of *T. aureus* with other butterflies in the phylogeny of Papilionoid species. Qin et al. (2012) sequenced the complete mitochondrial genomes of *T. aureus guangxiensis*. There have no studies of the genetic analysis of *T. aureus* in a wider geographical scale based on the complete mitochondrial genome.

The genetic relationships of *T. aureus* bewteeen four geographical populations were analyzed in this study through re-sequencing of the complete mitochondrial genome. The results would provide contributions to

Table 1. Samples of *Teinopalpus aureus* used for re-sequencing of the mitochodrinal genome

Serial number	Samples	Geographic area	Accession No.	Tissues collected
1	DYS1	Dayaoshan of Guangxi	KP_941013	Adult, male
2	DYS2	Dayaoshan of Guangxi	KP_941014	Adult, female
3	JLS	Jiulianshan of Jiangxi	KP_941015	Adult, male
4	MHS	Meihuashan of Fujian	KP_941016	Adult, male
5	PS1	Pingshan of Jiangxi	KP_941017	1st-instar larva
6	PS2	Pingshan of Jiangxi	KP_941018	2nd-instar larva
7	PS3	Pingshan of Jiangxi	KP_941019	2nd-instar larva
8	*Papilio machaon*	outgroup	NC_018047	Sequences obtained from GenBank
9	*Antheraea pernyi*	outgroup	NC_004622	Sequences obtained from GenBank

systematics of *T. aureus* among different geographic populations.

MATERIALS AND METHODS

Materials

T. aureus specimens characterizing different geographic populations used were three larvae from Pingshan of Jiangxi (PS1, PS2, and PS3), and one adult from each Jiulianshan of Jiangxi (JLS) and Meihuashan of Fujian (MHS). Specimens were deposited in absolute ethyl alcohol at 4°C or indoor temperature. Absolute ethyl alcohol was periodically replaced, the specimens were preserved. Specimen collection was approved by the China's State Forestry Administration (No. [2012] 1707).

Sequences of the population of Daoyaoshan (*T. aureus guangxiensis*, No. DYS) were compared with those of the three populations; sequences of a close species *Papilio machaon* from Papilionidae and a distant species *Antheraea pernyi* from Saturniidae were also obtained from GenBank and used as outgroups for analysis of genetic relationships among four geographic populations of *T. aureus* (Table 1).

Methods

Genome DNA extraction

Genome DNA was extracted using a standard phenol/chloroform isolation buffer (Miller et al., 1988) with several modifications.

Primer design

The primers for re-sequencing of the whole mtDNA genome were designed according to Qin et al. (2012), The primers for re-sequencing COI gene sequence fragment were designed according to Folmer (1994). Other primers were designed by our group using Primer 5 and Oligo7 software (Rachlik, 2007). Twenty pairs of primers were used in total (Table 2).

PCR product re-sequencing

PCR amplification used a 50 µL reaction system that comprised 25 µL of 2× Taq Master Mix, 1 µL of template DNA, 0.4 or 0.8 µmol/L start primer, 0.4 or 0.8 µmol/L final primer concentration and up to 50 µL of double distilled water.

Two reactions were performed. The first reaction was as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, 38–54.5 °C for 45 s, 70 °C for 90 s and a final extension step of 72 °C for 10 min. The PCR products were then stored at 10 °C. The second reaction was as follows: initial denaturation at 94 °C for 5 min, followed by 15 cycles at 94 °C for 50 s, 38–54.5 °C for 50 s and 68 °C for 3 min and then followed by 15 cycles at 94 °C for 55 s, 38–54.5 °C for 55 s, 68 °C for 185 s and a final extension step of 68 °C for 10 min. The PCR products were then stored at 10 °C. PCR products were visualised by electrophoresis on a 1.0% agarose gel.

Re–sequencing from both forward and reverse primers for all PCR products was performed on an ABI 3730 sequencer by Shanghai Bioengineering Technological Service Co. Ltd., or Shanghai Life Sequencing Co. (Shanghai, China).

Data processing

Sequences alignment and annotating

The sequences were edited using SeqMAN in DNAStar software packages, aligned using Clustal X 1.83 (Thompson et al., 1997), then mapped to the reference genome according to Qin et al. (2012). All aligned fragments were also undergone blasting with the published sequences in NCBI for further guarantee. Location and secondary structure of tRNA was determined using tRNA Scan-SE 1.21(Lowe and Eddy, 1997). Each gene was interpreted, and the relative synonymous codon usage (RSCU) of *T. aureus* was counted using MEGA 4.1 (Tamura et al., 2007). Repetitive sequences of the A+T–rich region were estimated using an online tool on http://tandem.bu.edu/trf//trf.

Phylogenetic tree construction

Sequences of 12S rRNA, three protein-coding genes of

Table 2. The PCR primers for re-sequencing of *Teinopalpus aureus* mitochondrial genome

Primer no.	Primer sequence	Starting site	Ending site	Length (bp)	Tm/℃
TA1	CTTTTGGGCTCATACCTC TATGATAATAAAATTATGGGG	22	673	652	49.3
TA2	ATCTTTTATCATCAGAAGCAGC GTTCCAATATCTTTATGGTTTG	400	1498	1099	52.8
TA3	TTCGAATTATTTATTCCTCT GTAATAAAATTAATGGCACCT	1111	1945	835	41.0
AT4	GGTCAACTCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	1474	2197	724	48.0
TA5	AGGTGGAGCCATTACGATAC AATAGCTGGGATAACGGTTC	2054	3268	1215	54.5
TA6	TTTAGAAATGGCAACTTGA AAAATAAATTGTCGGTTAGGAA	3049	4163	1115	46.8
TA7	TATTCCTCAAATAATACCAA AGGAGGTCATATAATTCCA	3874	5060	1187	39.0
TA8	ACGAGATATTTGCCGAGA AAATTAGGGCAATTTCAACA	4880	5780	901	43.0
TA9	GGCAGCTTGATATTGACATT TACCAAATTTATCGACTTATGGA	5417	6551	1135	43.0
TA10	TATCTTCAATATCATGCTCT ATTGACAATGTTTATGGCTG	6277	7294	1018	43.0
TA11	AATTCCACATAAAGACAAA TTTTATACAGGTATTTCTCG	6991	8227	1237	35.0
TA12	AAAAGAAATAATTTCCCACTC ATTATTTGCTTCTTTACCGAT	7924	9026	1103	49.3
TA13	TTAGGTAATCATAAATGAACAA ATATTCCTGATAAAAGGCAAG	8850	10023	1174	42.0
TA14	TCTCTCTATCAATAATCTCC GGTAATTACTGTAGCACCT	9841	10879	1039	38.0
TA15	ATTCACATATTGGACGAGGA TTTATTTGAGTTACGGGGAC	10722	11786	1065	45.2
TA16	TGAGCGTGTTCAAGCGTTTG TGAGCCAGGTGAGTTTCCATCT	11580	12787	1208	46.0
TA17	AATCATTACATCTTTCCTGCC ATTGTATCCTGTGTATCCGAGT	12634	13843	1210	39.0
TA18	ATTTATTTTAAAGCTTATCCCT ACAATTGATAATCCACGAA	13581	14405	825	40.0
TA19	TTCAATTTATATATGAAAGCG TTTTACATATAAATTTTAGTGTT	14114	14805	692	47.4
TA20	TTAAATTATTATTTGTATAACCG	14642	199	800	48.0

interest, and all 13 protein-coding genes were separately used for phylogenetic analysis. Nucleotide content, single nucletide polymorphism (SNP), and ratio between transition and trasversion were calculated using MEGA 4 software. Relative genetic distance was calculated based on Kimura-2-parameter model (Kimura, 1980). Maximum parsimony (MP) tree was constructed using PAUP*4.0b10 (Swofford 2002) with 1000 bootstrap replicates.

RESULTS

Characteristics in the complete mitochondrial genomes

Sequence features of the complete mitochondrial genome in different populations

The entire length of the *T. aureus* mitochondrial genome is among 15232–15237 bp (GenBank accession numbers: KP_941015 to KP_941019). In all, 37 genes were annotated. Among them, 13 genes were protein-coding genes (PCG), 2 ribosomal RNAs (12S rRNA and 16S rRNA), 22 transfer RNAs, and a noncoding control region (A+T–rich region) (Table 3). A total of 12 spacer regions and 11 overlapping regions were found. Spacers were 1- 47 bp long, with the longest between $tRNA^{Gln}$ and ND2, the overlapping sequences were 1-8 bp long, with the longest between $tRNA^{Trp}$ and $tRNA^{Cys}$. Sequence variation of genes $tRNA^{Ala}$, $tRNA^{Glu}$, ND5, 16SrRNA, and spacer regions ($tRNA^{Asn}$, $tRNA^{Ser}$(AGN) , $tRNA^{Phe}$, and ND5 was found in different geographic populations , with variation among 1 bp to 2 bp (Table 3).

For all the protein-coding genes, the stop codon of ND5 gene was the same in all individuals of different geographic populations of *T. aureus*.

Table 3. Gene alignment in the *Teinopalpus aureus* mitochondrial genome

Genes	Direction	Locus	Length(bp)	Spacer or overlaping	Start codon	Stop codon
$tRNA^{Met}$	F	1–68	68	0		
$tRNA^{Ile}$	F	69–132	64	–3		
$tRNA^{Gln}$	R	130–198	69	47		
ND2	F	246–1259	1014	–2	ATT	TAA
$tRNA^{Trp}$	F	1258–1322	65	–8		
$tRNA^{Cys}$	R	1315–1380	66	8		
$tRNA^{Tyr}$	R	1389–1455	67	2		
COI	F	1458–5988	1530	0	CGA	T- $tRNA^{Leu}$
$tRNA^{Leu}$(UUR)	F	2989–3055	67	0		
COII	F	3056–3737	682	0	ATG	T-$tRNA^{Lys}$
$tRNA^{Lys}$	F	3738–3808	71	–1		
$tRNA^{Asp}$	F	3808–3874	67	0		
ATP8	F	3875–4033	159	–7	ATT	TAA
ATP6	F	4027-4704	678	–1	ATG	TAA
COIII	F	4704–5492	789	–1	ATG	TAA
$tRNA^{Gly}$	F	5492–5557	66	0		
ND3	F	5558–5909	352	0	ATT	T-$tRNA^{Ala}$
$tRNA^{Ala}$	F	5910–5974	65*	-1		
$tRNA^{Arg}$	F	5974–6037	64	0		
$tRNA^{Asn}$	F	6038–6305	68	5		
$tRNA^{Ser}$(AGN)	F	6111–6170	60	13		
$tRNA^{Glu}$	F	6184–6249	66*	–2		
$tRNA^{Phe}$	R	6248–6313	66	2		
ND5	R	6316–8054	1728*	0	ATT	TAA[3]
$tRNA^{His}$	R	8056–8120	65	0		
ND4	R	8121–9459	1339	2	ATG	T-$tRNA^{His}$
ND4L	R	9462–9752	291	2	ATG	TAG
$tRNA^{Thr}$	F	9755–9819	65	0		
$tRNA^{Pro}$	R	9820–9884	65	2		
ND6	F	9887–10419	532	–1	ATT	TAA
Cytb	F	10420–11568	1149	–2	ATG	TAA
$tRNA^{Ser}$(UCN)	F	11567–11632	65	16		
ND1	R	11648–12586	939	1	ATG	TAG
$tRNA^{Leu}$(CUN)	R	12588–12655	68	2		
16SrRNA	R	12658–13991	1334*	0		
$tRNA^{Val}$	R	13992–14057	66	0		
12SrRNA	R	14058–14833	776	0		
A+T rich region		14834–15234	401			

Note: 1) Data from one MHS sample is presented in this table. Variations are present in the initiation and termination loci of each gene in different populations.
2) A positive number is indicative of a spacer, and a negative number for the overlapping length.
3) The symbol "*" is indicative of nucleotide variation among different populations.

Four nucleotides, T, C, A, and G, in the mitochondrial genome of *T. aureus* were 40% ± 0.005%, 12.5% ± 0.005%, 39.7% ± 0.009% and 7.7% ± 0.10% respectively. It is obviously biased toward A+T nucleotides (Table 4). A+T content in the complete genome was 79.8% ± 0.01%, among which PCG contained 78.2% ± 0.02%; 16SrRNA gene contained 82.1% ± 0.01%; 12S rRNA gene contained 85.8% ± 0.01%; control region (A+T-rich region) contained 93.6% ± 0.06%, which had the highest A+T content.

AT–skew and GC–skew in the *T. aureus* mitochondrial genome was –0.004 ± 0.00009 and –0.239 ± 0.00053 respectively, indicating that T and C were more than A and G. A total of 12 spacer regions were found in the genome (121 to 124 bp long, covering 0.8% of the entire genome). The longest region was between $tRNA^{Gln}$ gene and ND2 genes (47 bp long). Spacer length between the neighbour genes was the same with no obvious variation found among different geographic populations of *T. aureus*.

Eleven overlapping regions were found in the *T. aureus* mitochondrial genome (26 bp long, occupying 0.2% of the entire genome). Each overlapping region was 1-8 bp long. The length of the overlapping region between the neighbour genes was also the same with no obvious variation found among different geographic populations.

Protein-coding genes

The mitochondrial genome of *T. aureus* contained 13 protein-coding genes, 11 187 bp long in total, with 3 719 AA codons (excluding stop codon).

Table 4. Characteristics of the *Teinopalpus aureus* mitochondrial genome

Samples	Size（bp）	A+T content（%）	No. codons	PCGA+T content(%)	16S rRNA		12S rRNA		Control region	
					Size（bp）	A+T(%)	Size（bp）	A+T(%)	Size（bp）	A+T(%)
DYS1	15234	79.8	3719	78.3	1334	82.1	776	86.0	401	93.8
DYS2	15233	79.9	3719	78.3	1334	82.1	776	86.0	401	93.8
MHS	15234	79.8	3719	78.3	1334	82.0	776	85.8	401	93.3
JLS	15234	79.8	3719	78.2	1334	82.1	776	85.8	401	93.8
PS1	15234	79.8	3719	78.3	1334	82.0	776	85.8	401	93.5
PS2	15235	79.8	3719	78.3	1335	82.0	776	85.8	401	93.3
PS3	15234	79.8	3719	78.3	1334	82.0	776	85.8	401	93.3

Note: Codon calculation excluding stop codon

The same gene from different geographic populations had the same start codon. Genes of ATP6, COII, COIII, Cyt b, ND1, ND4, and ND4L used 'ATG'; ATP8, ND2, ND3, ND5, and ND6 genes used 'ATT'; COI used the nonstandard codon 'ATN' as the start codon.

But the stop codon sometimes varied among different geographic populations of *T. aureus*. ATP6, ATP8, COIII, Cytb, ND2, and ND6 genes used TAA; ND1 and ND4L genes used TAG as the stop codon, whereas COI, COII, ND3, and ND4 genes ended with residue T.

In protein-coding genes, four nuclueotides (T, C, A, and G) were 45.1%±0.011%, 10.8%±0.012%, 33.1%±0.010%, and 11.0%±0.009% respectively, A+T content was 78.2%, with an obvious AT bias. A+T content in the third codon was 90.6%, much higher than that in the first (73.9%) and second (70.2%) codons. GC skews in the first, second and third codons were 0.204, −0.105 and −0.162 respectively, showing that G was more than C in the first codon, and C was more than G in the second and third codons.

The relative synonymous codon usage (RSCU) of *T. aureus* was counted. Results showed that codon usage of protein-encoding genes in *T. aureus* was strongly biased. RSCUs of NNU and NNA were generally greater than 1, indicating that the codon with A or U at the third position was more frequently used than the other codons. AT was biased at the third position of the codons. Five codons with a high frequency were TTA, ATT, TTT, ATA, and AAT, indicating that AT was biased in protein-coding genes (Tables 5 and 6).

tRNA and rRNA genes

tRNA genes in *T. aureus* were 60–71 bp long. $tRNA^{Ser}$ and $tRNA^{Leu}$ were expressed by two tRNA genes (($tRNA^{Ser}$(UCN), $tRNA^{Ser}$(AGN) and $tRNA^{Leu}$(UUR), $tRNA^{Leu}$(CUN)) respectively. And the remaining 18 tRNAs were expressed by a single tRNA gene. A total of 14 J-chain encoding tRNA genes and eight N-chain encoding tRNA genes were found. DHU arm was absent in $tRNA^{Ser}$(AGN) in stead of a loop containing five A and one U to form an incomplete cloverleaf structure. The remaining 21 tRNAs formed a typical cloverleaf structure. Base mismatch was found in Lepidoptera (Aubert et al., 1999). And mismatches of 19 bp were also observed in the *T. aureus* mitochondrial tRNA.

Among them, eight pairs were mismatched on the acceptor arms, three pairs on the DHU arm, four pairs on the anticodon arm, and four pairs on the TΨC arm. Base-pairs of mismatch were always U.U, G.U, and U.G. Among them U.U pairs had 4, G.U pairs had 7, and U.G pairs had 8 mismatches. Lengths of $tRNA^{Ala}$ and $tRNA^{Glu}$ differed among the geographic populations of *T. aureus*. Lengths of $tRNA^{Ala}$ and $tRNA^{Glu}$ were 65 bp and 66 bp in the populations of Guangxi Dayaoshan and Jiangxi Jiulianshan; and were 64 bp and 67 bp in the populations of Jiangxi Pingshan and Fujian Meihuashan, respectively.

A+T-rich region

A+T-rich regions in the *T. aureus* mitochondrial genome were found between 12S rRNA and tRNAMet (396 bp long; A+T content=93.6%±0.06%).

An "ATAGA" was found at the 27 bp position of the 3' prime, followed by an 18 bp poly-T structure, and a 6 bp poly-A structure at the 5' prime (near to $tRNA^{Met}$). A similar microsatellite structure $(AT)_6$ was found at the positions of 45 bp, and $(AT)_8G(TA)_5$ was found at the position of 317 bp from the 3' prime. A 5 bp conservative sequences "ATTTA" were found at the positions of 228 bp and 276 bp of 3' prime, e.g. between $(AT)_6$ and $(AT)_8G(TA)_5$, or in a repetitive sequences "AAATTAATAAATTA" (Figure 1).

Table 5. Average usage codon of the PCGs in *Teinopalpus aureus*

Codon	Usage	Codon	Usage	Codon	Usage	Codon	Usage
UUU(F)	**328.9(1.76)**	UCU(S)	113.4(2.70)	UAU(Y)	171.8(1.82)	UGU(C)	30.0(1.82)
UUC(F)	45.3(0.24)	UCC(S)	17.8(0.42)	UAC(Y)	17.2(0.18)	UGC(C)	3.0(0.18)
UUA(L)	**438.7(4.89)**	UCA(S)	82.6(1.97)	UAA(*)	0.0(0.00)	UGA(W)	89.1(1.88)
UUG(L)	18.8(0.21)	UCG(S)	5.4(0.13)	UAG(*)	0.0(0.00)	UGG(W)	5.9(0.12)
CUU(L)	39.6(0.44)	CCU(P)	71.9(2.38)	CAU(H)	60.7(1.79)	CGU(R)	15.9(1.20)
CUC(L)	8.0(0.09)	CCC(P)	17.1(0.56)	CAC(H)	7.3(0.21)	CGC(R)	1.0(0.08)
CUA(L)	32.1(0.36)	CCA(P)	30.1(0.99)	CAA(Q)	57.9(1.81)	CGA(R)	34.1(2.58)
CUG(L)	0.9(0.01)	CCG(P)	1.9(0.06)	CAG(Q)	6.0(0.19)	CGG(R)	2.0(0.15)
AUU(I)	**428.7(1.87)**	ACU(T)	86.5(2.26)	AAU(N)	**236.6(1.87)**	AGU(S)	30.2(0.72)
AUC(I)	30.2(0.13)	ACC(T)	5.0(0.13)	AAC(N)	16.1(0.13)	AGC(S)	3.2(0.08)
AUA(M)	**258.7(1.73)**	ACA(T)	57.6(1.51)	AAA(K)	83.4(1.72)	AGA(S)	83.2(1.98)
AUG(M)	40.1(0.27)	ACG(T)	3.8(0.10)	AAG(K)	13.6(0.28)	AGG(S)	0.0(0.00)
GUU(V)	63.8(1.95)	GCU(A)	70.0(2.37)	GAU(D)	58.2(1.79)	GGU(G)	50.9(1.03)
GUC(V)	8.0(0.24)	GCC(A)	15.9(0.54)	GAC(D)	6.7(0.21)	GGC(G)	2.1(0.04)
GUA(V)	52.7(1.61)	GCA(A)	26.9(0.91)	GAA(E)	65.8(1.80)	GGA(G)	115.0(2.33)
GUG(V)	6.2(0.19)	GCG(A)	5.2(0.18)	GAG(E)	7.4(0.20)	GGG(G)	29.8(0.60)

Table 6. The composition of protein-coding genes in *Teinopalpus aureus*

Samples	PCGs					The first codon					The second codon					The third codon				
	T	C	A	G	Base number	T	C	A	G	Base number	T	C	A	G	Base number	T	C	A	G	Base number
DYS1	45.1	10.7	33.2	11.0	11157	37	10.4	37.1	15.7	3719	48	16.4	21.8	13.3	3719	50	5.4	40.6	4.0	3719
DYS2	45.1	10.7	33.2	11.0	11157	37	10.4	37.1	15.7	3719	48	16.4	21.8	13.3	3719	50	5.4	40.6	3.9	3719
MHS	45.1	10.7	33.1	11.0	11157	37	10.4	37.1	15.7	3719	48	16.4	21.8	13.3	3719	50	5.4	40.6	3.9	3719
JLS	45.1	10.8	33.1	11.0	11157	37	10.4	37.1	15.7	3719	48	16.4	21.7	13.3	3719	50	5.5	40.6	4.0	3719
PS1	45.1	10.7	33.2	11.0	11157	37	10.4	37.1	15.7	3719	48	16.4	21.8	13.3	3719	50	5.4	40.6	3.9	3719
PS2	45.1	10.7	33.1	11.0	11157	37	10.4	37.1	15.7	3719	48	16.4	21.8	13.3	3719	50	5.4	40.6	3.9	3719
PS3	45.1	10.7	33.2	11.0	11157	37	10.4	37.1	15.7	3719	48	16.4	21.8	13.3	3719	50	5.4	40.6	3.9	3719
Avg.	45.1	10.8	33.1	11.0	11157	37	10.4	37.1	15.7	3719	48	16.4	21.8	13.3	3719	50	5.5	40.6	4.0	3719

Note: Composition of PCGs sequence excluding termination codon.

Sequences of the A+T-rich region were very consistent among the different populations, with few differences observed (Table 7), suggesting that the A+T-rich region was a highly conservative property in the *T. aureus* mitochondrial genome.

Geophylogenetic analysis

Geophylogenetic tree based on 12SrRNA gene data

A maximum parsimony tree based on 12S rRNA gene data was constructed (Figure 2) with a structure of geophylogenetic (MHS + (PS + (JLS + DYS))).

Geophylogenetic tree based on 13 PCG data

A maximum parsimony tree based on 13

Figure 1: Structure of A+T-rich region in *Teinopalpus aureus*

Table 7. Base substitutions and distribution in A+T-rich regions in *Teinopalpus aureus*

No.	Position of base substitutions/nt									
	39	41	66	72	158	168	177	216	328	330
DYS1	.	.	.	A		.	T	.	.	.
DYS2	.	.	.	A		.	T	.	.	.
JLS	.	.	.	.		.	T	T	.	.
MHS	.	A	A	.		C	T	.	.	.
PS1	.	.	.	.		.	T	.	.	.
PS2	.	.	.	.		.	.	.	.	.
PS3	.	.	.	.		.	.	.	.	.

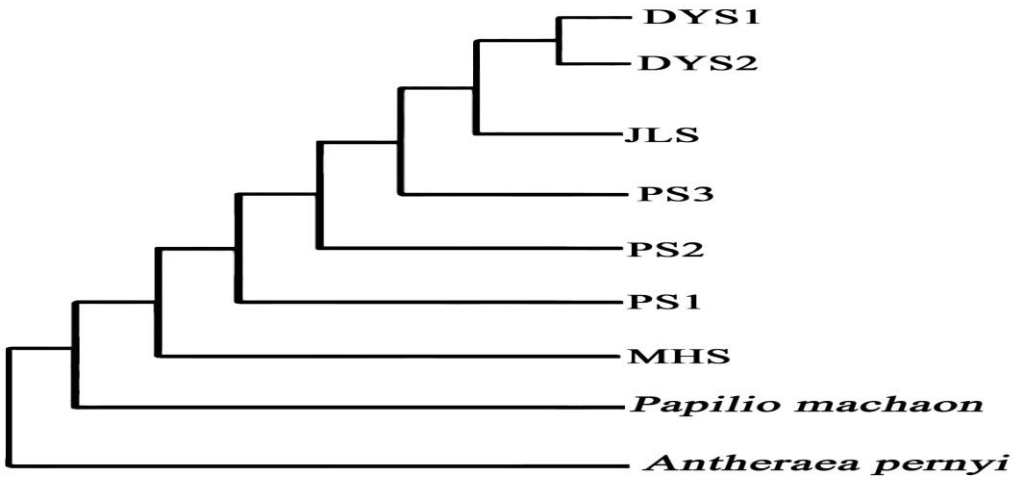


Figure 2: Maximum parsimony tree based on 12SrRNA gene data

PCG data was similar to that on 12S rRNA, with a geophylogenetic structure of (DYS + JLS) + (MHS + PS) (Figure 3).

Geophylogenetic tree based on COI, Cytb, and ND3 gene data

All topological structures in maximum parsimony trees based on COI gene, Cytb gene, and ND3 gene data were similar to the tree based on 13 PCG data, with a geophylogenetic structure of (DYS + JLS) + (MHS + PS) (Figure 4A–C).

DISCUSSION

We collected five individuals from three geographic populations of *T. aureus* (Nos. MHS, JLS, PS1, PS2, and PS3. Specimen collection was approved by the China's State Forestry Administration (No. [2012] 1707)). Maximum

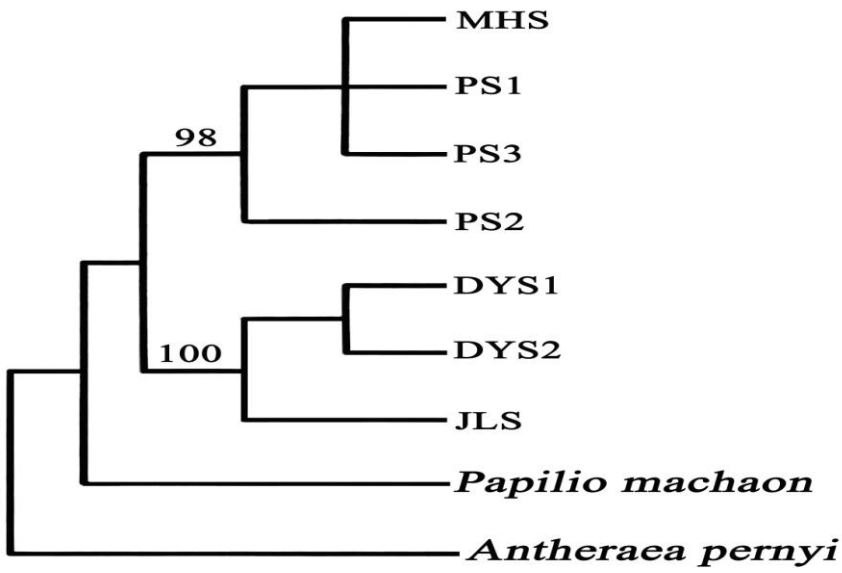
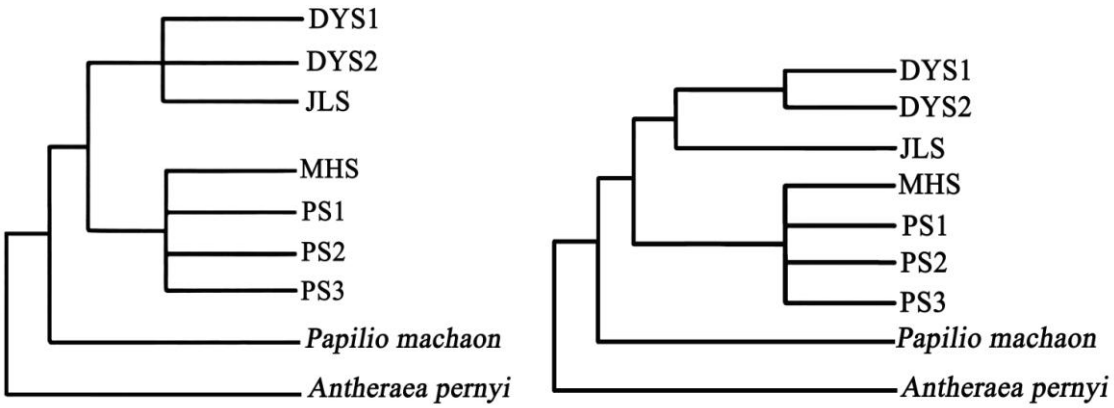


Figure 3: Maximum parsimony tree based on 13 PCG data
（Numbers on nodes correspond to percentage bootstrap values for 1,000 replicates）



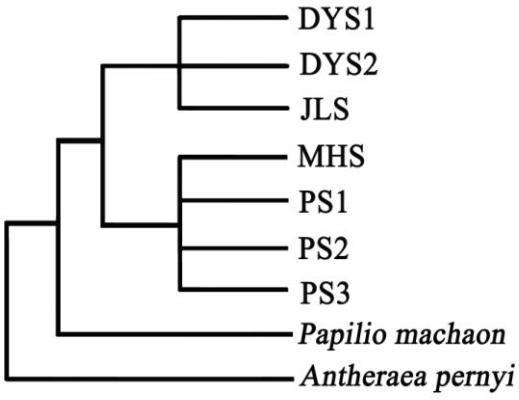


Figure 4: Maximum parsimony tree based on COI gene (A), Cyt b gene (B), and ND3 gene (C) of four geographic populations of *Teinopalpus aureus*

parsimony trees based on 12SrRNA gene, 13 protein-encoding genes, COI gene, Cytb gene, and ND3 gene data strongly supported genetic relationships among the four geographic populations: JLS was close to DYS, and MHS was close to PS. From a geographic point of view, the population in Jiulianshan of Jiangxi is close to that in Nanling Mountains of Guangdong, and the later is close to the population from Guangxi. We speculated that JLS and DYS may be derived from the same population. Pingshan of Jiangxi near Meihuashan of Fujian both belonged to Wuyi Mountains. We also speculated that both PS and MHS may be derived from the same ancestor.

T. aureus is ranked as the national protection species, thus the sampling was limited for collection and studies. Therefore, only one individual from both Meihuashan of Fujian and from Jiulianshan of Jiangxi were availabe in this study. Relationships among different populations need more samples for confirmation.

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REFERENCES

Aubert J, Legal L, Descmon H (1999). Molecular phylogeny of swallowtail butterflies of the tribe Papilioni (Papilionidae, Lepidoptera). Mol. Phylog. Evol., 12 (2): 156–167.

Chen RL, Cai YS, Gong YN, Gu MB (2009). Two new hosts of *Teinopalpus aureus* Mell Found in the Nanling National Nature Reserve. Guangdong For. Sci. Technol., 25(6): 119–120.

Chou I (2000). Monograph of Chinese butterflies. Zhengzhou, Henan Sci. Technol. Publ. H., China: 852 pp.

Folmer O, Black MB, Hoch HW, Lutz R, Vrijenhoek R. (1994). DNA primers for amplification of mitochonodrial cytochrome c oxidase subunti I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol., 3(5): 294–299.

He GQ, Jia FH (2012). Research on population quantity and host plants of *Teinoplpus aureus* Mell in Jinggang Mountain. J. Nanchang Inst. Techol., 31(4): 68–70.

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.

Lin XD, Liang Z, Hao Y, Ma EB, Zhang M (2011) Phylogenetic analysis of some species in Papilioninae (Papilionidae, Lepidoptera) based on mitochondial cytochrome oxidase I gene. Acta Zoot. Sin., 36(3): 639–647.

Lowe TM, Eddy SR (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucl. Aci. Res., 25: 955–964.

Masui A, Uehara J (2000). Butterflies resently collected from Laos P. D. R. (7). Gekkan-Mushi, 352: 2–3.

Mell R (1923). Noch unbeschriebene Lepidopteren aus Süchina, II. Deuts. Entomol. Zeits.: 153–160.

Miller SA , Dykes DD, Polesky HF (1988). A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16(3):1215.

Morita S (1998) A new subspcies of *Teinopalpus aureus* Mell, 1923 from Vietnam (Lepidoptera: Papilionidae). Wallace 4(2):14–15. pls.13–14.

Qin F, Fu WB, Zhou SY (2011). Molecular systematics of six genera of Papilionidae (Lepidoptera) based on mitochondrial cytochrome oxidase Iand Cyt b gene sequences. Acta Entomol. Sin., 54(3): 339–351.

Qin F, Jiang GF, Zhou SY (2012). Complete mitochondrial genome of the *Teinopalpus aureus guangxiensis* (Lepidoptera: Papilionidae) and related phylogenetic analyses. Mitochondrial DNA, 23(2): 123–125.

Rachlik W (2007). PCR primer design. Meth. Mol. Biol. 402: 35-59.

Song YZ, Zeng GB, Chen CQ, Zuo CX, Cheng Y (2007). *Teinopalpus aureus* found in Jinggangshan of Jiangxi. Jiangxi Sci., 25(4): 481–482.

Swofford DL (2002). PAUP*: Phylogenetic Analysis Using Parsimony(*and other methods), ver. 4.0b10. Sinauer Associates, Sunderland, MA.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol. Biol. Evol., 24(8):1596–1599.

Thompson JD, Gibson TJ, Plewnisk F. (1997). The Clustal X windows interface; strao for multiple sequences alignment aided by quality analysis tools. Nucl. Aci. Res., 24: 4876–4882.

Tong XS, Qian ZQ, Kubo K (1995). First record of *Teinopalpus aureus* Mell (Rhopalocera: Papilionidae) from Zhejiang Province, China. Lepidop. Soc. Jap., 162: 2.

Wu Y (1999). The world famous butterfly appreciation. Kunming, Yunnan Educ. Press, China: 117 pp.

Zeng JP, Lin BZ, Zhu XF, Liu LY (2014). A Host Plant "*Michelia maudiae*" Widespread - distributed in South China for the Endangered Butterfly of *Teinopalpus aureus.* Acta Agric. Univ. Jiangxiensis, 36 (3): 550–555.

Zeng JP, Zhou SY, Ding J, Luo BT, Qin K (2012). Behavior characteristics and habitat adaptabilities of the endangered butterfly *Teinopalpus aureus* in Mount Dayao. Acta Ecol. Sin., 32 (20): 6527–6534.

Zeng JP, Zhou SY, Li CC, Wu JS, Qin K (2005). The pupa of *Teinopalpus aureus guangxiensis* and discovery of its host plant. Chin. Bull. Ent., 42(1): 71–73.

Zeng JP, Zhou SY, Liang YL, Li CC, Qin K (2004). Ecological study on pupas of *Teinopalpus aureus* Mell. In: LI DM (ed.) Studis on contemporary entomology.

Agri. Sci. Tech. Publ. H. Beijing, China: pp. 701–703.
Zeng JP, Zhou SY, Luo BT, Qin K, Liang YL (2008). Morphology and bionomics of the endangered butterfly golden kaiserihind, *Teinopalpus aureus*, in Dayaoshan of Guangxi. Chin. Bull. Ent., 45(3): 457–464.
Zeng JP, Zhou SY, Luo BT, Qin K, Wu JS (2007). Life history of *Teinopalpus aureus guangxiensis* Chou et Zhou (Lepidoptera: Palilionidae). Guangxi Sci., 14(3): 323–326.
Zhou CL, Jiang GF. (1996). Characteristics and conservation of *Teinopalpus aureus* Mel1 in Guangxi. J. Guangxi Acad. Sci. 12(1): 24–26.
Zhou SY, Huang Y, Zeng JP, Chen ZL, Qin K, Mo NB (2013). A preliminary investigation on the population quantity of Teinoplpus aureus guangxiensis Chou et Zhou. In: Liang SC, MA JM. (ed.) Ecological studies on animals and plants of Guangxi. Chin Forest. Pub. H., Beijing: pp.101–103.