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Phylogenetic evaluation of the taxonomic status of *Papilio maackii* and *P. syfanius* (Lepidoptera: Papilionidae)

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Abstract: The taxonomic status of *Papilio maackii* and *P. syfanius* has long been disputed. We conducted a molecular phylogenetic study to evaluate the taxonomic status of *P. maackii* and *P. syfanius*. A total of twenty-four *P. maackii* individuals from six localities and sixteen *P. syfanius* individuals from two localities were analyzed. We sequenced the partial region of the CO-I gene (about 579 bp) and partial CO-II gene sequence (about 655bp) of the two species. The Kimura-2-Parameter distances among *P. maackii* and *P. syfanius* ranged from 0 to 0.6%. Fifteen haplotypes were obtained based on the combined data set. The results strongly supported that all *P. maackii* individuals and all *P. syfanius* individuals formed a large clade, and could not be divided into separated clades. This research indicated that the two species have only very recently undergone speciation.

Key words: *Papilio maackii*; *Papilio syfanius*; CO-I; CO-II; Phylogeny; Taxonomy

基于系统发生探讨绿带翠凤蝶和西番翠凤蝶的分类地位

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摘要：绿带翠凤蝶和西番翠凤蝶的分类问题存在一定的争议。应用分子系统学方法对这一问题进行了研究。对 6 个不同地区的 24 个绿带翠凤蝶、2 个地区的 16 个西番翠凤蝶个体的 COI (579bp)和 COII (655bp)基因测序, 绿带翠凤蝶与西番翠凤蝶的遗传距离为 0 至 0.6%, 共获得了 15 个单倍型。结果显示这些单倍型不能形成各自独立的单系群, 因此认为绿带翠凤蝶和西番翠凤蝶为近期分化的两个种。

关键词：绿带翠凤蝶; 西番翠凤蝶; CO-I; CO-II; 系统发生; 分类
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Butterflies of the *Papilio* genus are one of the best known invertebrate organisms. They are large and colorful, and particularly noticeable in their habitats. About 210 *Papilio* species have been documented worldwide, with some 27 species recorded in China (Wu, 2001). This wide-spread distribution has seen many *Papilio* species used as model organisms for studies in evolutionary biology, ecology, genetics, and conservation biology (Collins & Morris, 1985; Scriber et al, 1995).

The taxonomic status of several *Princeps* species, a subgenus of *Papilio*, has long been disputed. Sometimes *P. maackii, P. dialis* and *P. syfanius* are treated as subspecies of *P. bianor* (Seitz, 1906), yet morphological differences between *P. bianor*, *P. maackii* and *P. syfanius* are often regarded as sufficient to treat them as separate species. These external morphological characteristics of *P. maackii* and *P. syfanius* are as follow: *P. maackii* agrees with *P. bianor* in the character of sexual marks, but differs somewhat in color. Primaries black, thickly powdered with green scales and traversed

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by a submarginal band formed of paler green scales. Secondaries black, costal area suffused with blue, inner and median area; submarginal lunulated band bluish green, and this color is projected along the third median nervule almost to the extremity of the tail, this is a more or less complete reddish ring at anal angle, outer margin sinuate, fringes white as in *P. bianor*. *P. syfanius* is only separable from *P. bianor* by its narrower secondaries, the entire absence of bluish color on the costal area and the presence of a more or less well-defined pale patch on the disc of these wings. This patch is placed at the end of the discoidal cell and is separated into three portions by the discoidal and upper discocellar nervules. These three insects are separated by well-defined and constant characteristics.

The *P. maackii* populations occur in all over China (except in the north-west), Japan, Korea, and Russia, while *P. syfanius* populations occur only in Sichuan, Yunnan, and Xizang. The area inhabited by *P. syfanius* is sympatric to the South China distribution of *P. maackii*. The host plants of *P. maackii* are Rutaceae, but the biology of *P. syfanius* remains unclear (Wu, 2001).

We examined a large number of these two species from different locations in China. We found that the white patch of *P. syfanius* was occasionally indistinct, even absent, and that the bluish green band on the *P. maackii* individuals from Southwest China was also sometimes absent. Wing markings on *P. maackii* and *P. syfanius* were similar in Southwest China, where the two species were sympatric (Wu, 2001).

Despite the extensive use of *Papilio* species in basic research, the phylogeny of *Papilio* remains weak. Recent relevant studies have used mitochondrial DNA (mtDNA) as molecular markers (Sperling & Harrison, 1994; Aubert et al, 1999; Reed & Sperling, 1999; Yagi et al, 1999; Caterino & Sperling, 1999; Caterino et al，2001; Zakharov et al, 2004a, b; Zhu et al, 2007; Silva-Brandão et al, 2008; Wheata & Watt, 2008; Chen et al, 2010), as mtDNA is considered useful in phylogenetic studies due to its rapid evolution. However, *P. maackii* and *P. syfanius* were only distinguished by less pairwise substitutions (0.15%) based on partial mitochondrial CO-I and CO-II gene sequences. The sequences of their CO-I and CO-II gene fragments were the same, although sequence divergences among these two species and other Chinese species of *Princeps* ranged from 3.6% (*P. polyctor*) to 8.28% (*P. demoleus*), and sequence divergences among them and *P. dialis* were 4.33% (Zhu et al, 2007). These results indicated that *P. maackii* and *P. syfanius* could be conspecific.

So, is *P. syfanius* a synonym species of *P. maackii*? Further evidence was required to answer this question. Previous research determined that DNA sequencing of a standard gene region of CO-I gene or "DNA barcoding" (Hebert et al, 2003) might hold the answer. DNA barcoding is helpful in species diagnosis because sequence divergences are ordinarily much lower among individuals of a species than between closely related species (Hebert et al, 2003, 2004a, b; Avise & Walker, 1999). To provide further proof, we sequenced the standard gene region of the CO-I gene or "DNA bar coding" and partial CO-II gene sequence of the two species. We focused on and attempted to evaluate the taxonomic status of *P. maackii* and *P. syfanius*.

1 Materials and Methods

1.1 Insects

We obtained twenty-four adult individuals of *P. maackii* from six localities in China and sixteen adult individuals of *P. syfanius* from two localities in South China, where the two species were sympatric (Fig. 1; Tab. 1). These specimens included both fresh and papered specimens. The sample of *P. bianor* was obtained for use as an out-group. Specimens were either collected by us or donated by colleagues. Sequences from all specimens were available from GenBank (Tab. 1).

1.2 DNA extraction

Tissue samples were comprised of two legs removed from one side of the thorax to preserve both the taxonomic and aesthetic values of the specimens. Voucher labels were attached to all sampled specimens. Excised legs from dried materials were rehydrated in buffer consisting of 5 mmol/L of Tris-HCl, 25 mmol/L of NaCl, pH 8.0, 25 of mmol/L EDTA, and 0.1% SDS (Zimmermann et al, 2000) for at least 48 h. A total of 5 μL μL of 20 mg/mL Proteinase K enzyme digester was added, and samples were incubated at 55°C overnight. Total DNA was extracted sequentially with phenol, phenol/chloroform (1/1), and chloroform and then precipitated with ethanol. The resultant DNA was dissolved in 30 μL ul of double-distilled H_2O.

1.3 Amplification and sequencing of DNA

Total DNAs were used as templates for amplifications

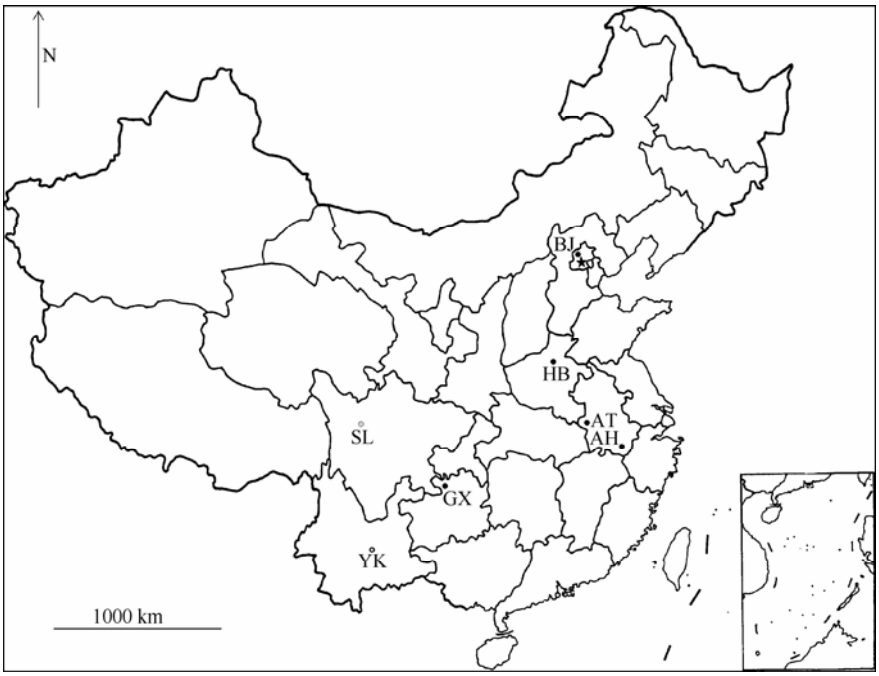

Fig. 1 Distribution of sampling sites in China

The inset shows the collection localities of *Papilio maackii* (black circles), *P. syfanius* (white circles), and both species (gray circles). Letters refer to populations given in Tab. 1.

of the partial CO-I gene (about 579bp) and CO-II gene (about 655bp) by polymerase chain reaction (PCR). These sequences were amplified by the following primer pairs for Lepidoptera: LEP-F1, 5'-ATTCAACCAATCA TAAAATAT-3'; LEP-R1, 5'-TAAACTTCTGATGTCC AAAAA-3' for the COI gene (Hebert et al, 2004a) and PATRICK, 5'-CTAATAT GGCAGATTATATGTATTG GA-3'; EVA, 5'-GAGACCATTACTTGCTTTCAGTCA CT-3'for the COII gene (Caterino & Sperling, 1999).

All PCR mixes had a total volume of 50 μL and contained 10 mmol/L of Tris-HCl (pH 8.3), 50 mmol/L of KCl, 1.5 mmol/L $MgCl_2$, 100 μmol/L of dNTPs, 10−50 ng (1−5μL) of genomic DNA, and 1 U *Taq*DNA polymerase. The thermocycling profile consisted of one cycle of 1 min at 94°C, 6 cycles of 1 min at 94°C, 1 min and 30 sec at 48°C, and 1 min and 15 sec at 72°C, followed by 36 cycles of 1 min at 94°C, 1 min and 30 sec at 52°C, and 1 min and 15 sec at 72°C, and a final step of 5 min at 72°C. The PCR products were electrophoresed in 1.0% TBE agarose gels, stained with ethidium bromide, and visualized under UV light. The PCR products were cleaned using the V-Gene DNA Gel Extraction Kit. The same primers for PCR were used for sequencing. Sequences were analyzed on an ABI 3730 automated sequencer.

1.4 Phylogenetic analysis

The sequence data were aligned with CLUSTALX (Thompson et al, 1997) and by eye. All variable characters, parsimony-informative characters, and the Tis/TVs were estimated from pairwise comparisons of sequences with Mega Version 2.1 based on Kimura-2-Parameter (Kumar et al, 2001). The ML analyses were performed using the equally weighted sequence data with standard settings with PAUP* Version 4.0b10 (Swofford, 1998). The GTR+G+I model was calculated by Modeltest 3.06 (Posada & Crandall, 1998). The statistical confidence for each clade was determined using the bootstrap test based on 100 replicates for ML using PAUP*. MrBayes 3.04b (Ronquist & Huelsenbeck, 2003) was used for the Bayesian estimation of phylogeny. The GTR+I+G model was used for Bayesian inference. An initial run was performed to determine the burn-in value, which was used in further analyses to exclude all trees prior to the stable log likelihood estimate. Searches were conducted with four simultaneous Markov Chains over two million generations, sampling every 100 generations. To estimate the posterior probabilities of recovered branches, the 50% majority rule was applied. To ensure that Bayesian inference was not trapped in local optima, the analysis was performed three times, starting from different random trees. Phylograms were created as average-branch length consensus trees in MrBayes and visualized with Tree-View 1.6.6 (Page, 1996). Bootstrap values were shown above the node. A partition

Tab. 1 Specimens, collecting localities and dates, mtDNA haplotypes and the NCBI accession codes for the *Papilio* mtDNA sequences

Species	Collection location	Collection date	mtDNA haplotypes			GenBank Accession Number	
			COI+COII	COI	COII	COI gene	COII gene
*P. maackii*1	SL(Lushan, Sichuan)	06.15.06	3	A	a	JF281155	JF281195
*P. maackii*2	AT(Tiantangzhai, Anhui)	06.16.04	3	A	a	JF281156	JF281196
*P. maackii*6	AT(Tiantangzhai, Anhui)	06.16.04	3	A	a	JF281157	JF281197
*P. maackii*7	AT(Tiantangzhai, Anhui)	06.16.04	9	A	j	JF281158 JF281155	JF2811988
*P. maackii*10	AT(Tiantangzhai, Anhui)	06.16.04	13	A	i	JF281159	JF281199
*P. maackii*12	BJ(Beijing)	07.21.05	14	F	a	JF281160	JF281200
*P. maackii*15	HB(Balihe, Henan)	07.21.04	1	A	e	JF281161	JF281201
*P. maackii*16	HB(Balihe, Henan)	07.21.04	2	A	b	JF281162	JF281202
*P. maackii*91	GX(Xishui, Guizhou)	08.28.05	5	A	f	JF281163	JF281203
*P. maackii*92	GX(Xishui, Guizhou)	08.28.05	5	A	f	JF281164	JF281204
*P. maackii*102	GX(Xishui, Guizhou)	08.28.05	2	A	b	JF281165	JF281205
*P. maackii*106	GX(Xishui, Guizhou)	08.28.05	1	A	e	JF281166	JF281206 JF281195
*P. maackii*133	SL(Lushan, Sichuan)	05.05.05	1	A	e	JF281167	JF281207
*P. maackii*143	AH(Huangshan, Anhui)	07.05.05	1	A	e	JF281168	JF281208
*P. maackii*146	AH(Huangshan, Anhui)	07.05.05	1	A	e	JF281169	JF281209
*P. maackii*148	AH(Huangshan, Anhui)	07.05.05	1	A	e	JF281170	JF281210
*P. maackii*25	SL(Lushan, Sichuan)	06.15.06	3	A	a	JF281171	JF281211
*P. maackii*26	SL(Lushan, Sichuan)	06.15.06	11	C	a	JF281172	JF281212
*P. maackii*27	SL(Lushan, Sichuan)	06.15.06	10	D	h	JF281173	JF281213
*P. maackii*28	SL(Lushan, Sichuan)	06.15.06	1	A	e	JF281174	JF281214
*P. maackii*29	SL(Lushan, Sichuan)	06.15.06	7	A	d	JF281175	JF281215
*P. maackii*30	SL(Lushan, Sichuan)	06.15.06	7	A	d	JF281176	JF281216
*P. maackii*31	SL(Lushan, Sichuan)	06.15.06	3	A	a	JF281177	JF281217
*P. maackii*32	SL(Lushan, Sichuan)	06.15.06	2	A	b	JF2811788	JF281218
*P. syfanius*1	SL(Lushan, Sichuan)	06.16.06	6	A	c	JF281219	JF281179 JF281179
*P. syfanius*2	SL(Lushan, Sichuan)	06.12.06	6	A	c	JF281220	JF281180
*P. syfanius*3	SL(Lushan, Sichuan)	06.12.06	2	A	b	JF281221	JF281181
*P. syfanius*4	SL(Lushan, Sichuan)	06.16.06	4	B	a	JF281222	JF281182
*P. syfanius*5	SL(Lushan, Sichuan)	06.16.06	4	B	a	JF281223	JF281183 JF281179
*P. syfanius*6	SL(Lushan, Sichuan)	06.16.06	3	A	a	JF281224 JF281219	JF281184
*P. syfanius*7	SL(Lushan, Sichuan)	06.12.06	2	A	b	JF281225	JF281185
*P. syfanius*8	SL(Lushan, Sichuan)	06.12.06	4	B	a	JF281226	JF281186
*P. syfanius*9	SL(Lushan, Sichuan)	06.12.06	12	E	a	JF281227	JF281187
*P. syfanius*10	SL(Lushan, Sichuan)	06.12.06	8	A	g	JF281228	JF281188
*P. syfanius*11	SL(Lushan, Sichuan)	06.14.06	2	A	b	JF281229	JF281189
*P. syfanius*12	SL(Lushan, Sichuan)	06.14.06	2	A	b	JF281230	JF281190
*P. syfanius*13	SL(Lushan, Sichuan)	06.15.06	2	A	b	JF281231	JF281191
*P. syfanius*14	SL(Lushan, Sichuan)	06.15.06	4	B	a	JF281234	JF281192
*P. syfanius*15	SL(Lushan, Sichuan)	06.14.06	4	B	a	JF281232	JF281193
*P. syfanius*16	YK(Kunming, Yunnan)	06.16.04	15	A	k	JF281233	JF281194

homogeneity test was carried out in PAUP* to determine if significantly different signals were being generated by the CO-I and CO-II fragments. To further resolve relationships within major haplotype clusters, a statistical parsimony approach was employed (Templeton et al, 1992), which was carried out using computer program TCS 1.18 (Clement et al, 2000).

2 Results

Sequences used to generate phylogeny represented about 7.7% of the insect mitochondrial molecule. Of the 1234 characters (579bp of COI and 655bp of CO II) in

the data matrix, nineteen (1.54%) were variable and eight (0.65%) were parsimony informative. The partition homogeneity test showed no significant incongruence between phylogenetic signals from CO-I and CO-II fragments (P=0.38). The average transition/transversion ratio was calculated as 4.8. The Kimura-2-Parameter distances among *P. maackii* and *P. syfanius* ranged from 0 to 0.6%, with an overall average of 0.2%.

These sequences produced a combined data set of fifteen haplotypes (Tab. 1). Haplotypes 2 and 3 were shared by the two species. This was particularly true for the haplotypes produced by the CO-I gene and CO-II gene region data (Tab. 1). The CO-I region data included six unique haplotypes, and the CO-II region data included eleven haplotypes. Haplotypes A of the CO-I region and haplotypes A and B of the CO-II region were shared by *P. maackii* and *P. syfanius*. In both the parsimony and Bayesian analyses, *P. bianor* was used as the out-group. The Bayesian majority consensus tree for the combined data set is presented in Fig. 2. The ML tree had a nearly identical topology to the majority consensus tree of the Bayesian inference. Most nodes were supported by both methods. Results showed that all *P. maackii* and *P. syfanius* individuals formed a large clade, and could not be divided into separated clades. These groups corresponded closely to the distribution of the major clades defined by TCS.

The statistical parsimony analysis of the combined data set produced one network, which contained all of the haplotypes (Fig. 3.). The networks provided the relationships of the haplotypes. Haplotype 2 and 3 were central and by far the most numerous in this network (Tab. 1). The network was also unable to separate *P. syfanius* from *P. maackii*.

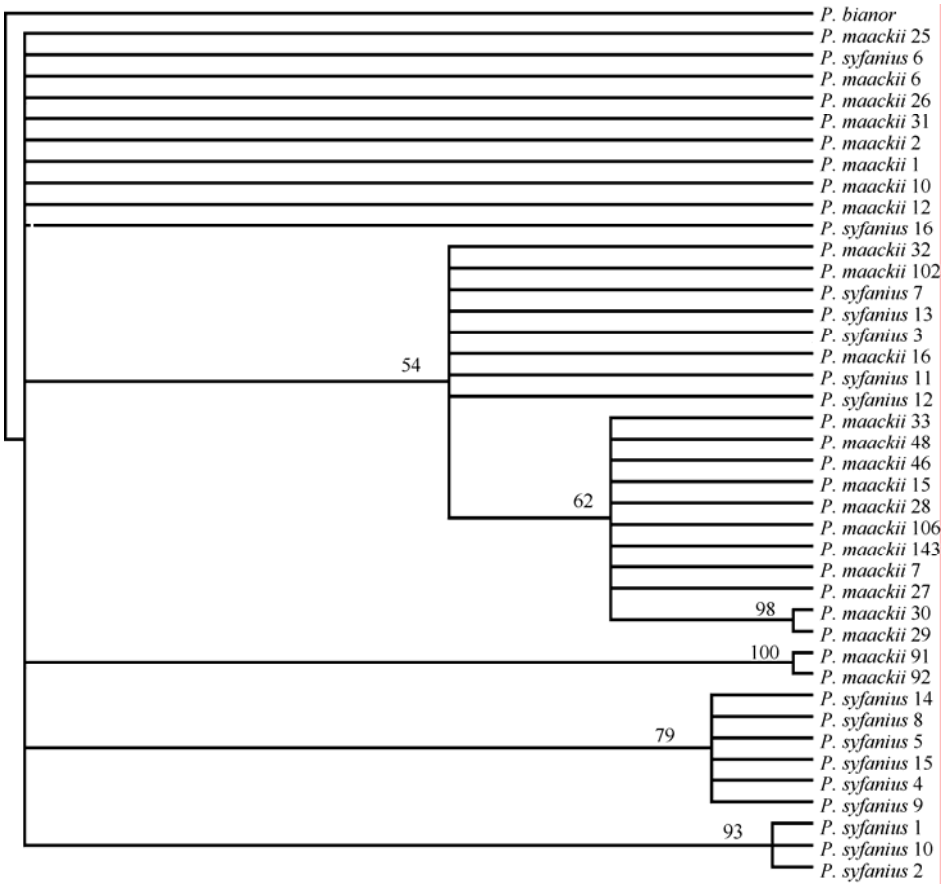

Fig. 2 Bayesian majority consensus tree of *Papilio maackii* and *P. syfanius* based on sequences of CO-I (579 bp) and CO-II (655 bp)
Above the branches are bootstrap values.

3 Discussion

We examined the phylogeny derived from concatenated CO-I and CO-II gene fragments of *P. maackii* and *P. syfanius* from China, and provided an insight into their taxonomic status.

The taxonomic status of *P. maackii* and *P. syfanius* has long been disputed. DNA barcoding can be helpful in species diagnosis because sequence divergences are ordinarily much lower among individuals of a species

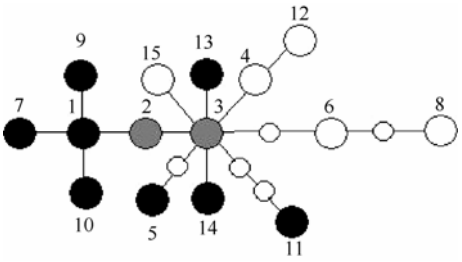

Fig. 3 Statistical parsimony network of haplotypes of *Papilio maackii* (black circles), *P. syfanius* (white circles), and both species (gray circles)

Solid lines connecting each pair of haplotypes represent a single mutational event, regardless of their length. Small circles represent missing or theoretical haplotypes. The codes of haplotypes are presented in accordance to Tab. 1.

than between closely related species (Hebert et al, 2003, 2004a, b; Avise & Walker, 1999). For example, con-generic moth species show an average sequence divergence of 6.5% in the CO-I gene, whereas divergences among con-specific individuals averages only 0.25% (Moore, 1995). Similar values were obtained in birds, with intra-specific divergences at CO-I averaging 0.27%, whereas congener divergences averaged 7.93% (Hebert et al, 2003, 2004a, b; Avise & Walker, 1999). *Princeps* divergences we obtained previously ranged from 3.6% to 8.28% (Zhu et al, 2007). To provide further proof, we sequenced partial CO-II gene sequences of the two species. Based on previous studies of butterflies, con-generic species show an average sequence divergence of 4% in CO-II gene (Sperling et al, 1996; Wang et al, 2004), whereas divergences among con-specific individuals average 1%-2% (Sperling & Hickey, 1994). We sequenced the standard gene region of the CO-I gene (about 579bp), known as "DNA barcoding", and the partial CO-II gene sequence (about 655bp) of the two species.

Following Mayr's (1963) biological species concept, it is generally assumed that species must be monophyletic entities (Harrison, 1998). However, as shown by Pamilo & Nei (1988) and summarized by Wahlberg et al (2003) and Funk & Omland (2003) both polyphyly and paraphyly may arise during the speciation process. Although what constitutes a species is a subject of intense debate, there is a general agreement that species are segments of evolutionary lineages (de Queiroz, 1998).

Our results indicated that sequence divergence between *P. maackii* and *P. syfanius* ranged from 0 to 0.6% across both CO-I and CO-II gene fragments. The phylogenetic analysis could not separate *P. maackii* and *P. syfanius* into different clades, instead supporting a monophyly (Fig.2). The clade included all the *P. maackii* and *P. syfanius* individuals, with neither species able to form separated clades and both sharing the same haplotypes. If we were unfamiliar with *Papilio* butterflies, we could, using the ''classic" 665 bp barcoding segment of CO-I (Hebert et al, 2004a), place the two specimens into their ''correct" major subclades and sometimes into the ''correct" species. They would have genetic distinctness of geographic subgroups of a morphologically conservative assemblage previously called one species. But the phylogeny of many subclades is poorly resolved with this short sequence block, and recent divergences, clear when more diverse signal sources are used, would go undetected. ''Barcoding" is useful for the initial sorting of material, but it is no substitute for phylogenetic studies using more extensive evidence.

Results from the probabilistic modeling approach indicate that accurate species delimitation is possible (Knowles & Carstens, 2007), despite widespread incomplete lineage sorting and discordance among loci, and confirm that it is not necessary to rely on exclusivity criteria. Molecular data may suggest that morphological differentiation proceeded much faster than mtDNA divergence owing to strong selection pressures (Eastwood & Hughes, 2003), since *Acrodipsas* were allopatric and lived in markedly different habitats. This fixed amino acid change may be a "key innovation" of macro-evolutionary consequences, as the derived genotypes carrying it in the warm habitats of the lowland complex, produce much more thermally stable PGI than the basal ones in colder, higher or more northern habitats (Christopher & Ward, 2008). Studies on the intermediate taxa in this phylogeny, in regards to historical relationships and the state of adaptations, may illuminate connections between micro- and macro-evolutionary processes. Our results indicate that the two species have undergone only very recent speciation.

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