A preliminary molecular phylogeny of the genus *Scobura*, with a synonym of *Scobura masutaroi* (Lepidoptera, Hesperiidae)

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Abstract

A molecular phylogeny of the genus *Scobura* based on the mitochondrial COI and the nuclear EF-1\(\alpha\) genes using maximum likelihood and Bayesian inference is proposed. The analyses include 19 specimens from nine ingroup species. The monophyly of *Scobura* is not strongly supported, but two strongly supported monophyletic groups within the genus are recognized: the *S. coniata* group and the *S. woolletti* group. Judging from combination of the molecular evidence and morphological features, the former consists of six species, including *S. masutaroi*, while four species belong to the latter. *S. mouchai* Krajcik, 2013 is confirmed to be a syn. n. of *S. masutaroi* Sugiyama, 1996. The key to the species of the genus *Scobura* is modified to reflect these results.

Keywords

COI, EF-1\(\alpha\), *Scobura masutaroi*, *Scobura mouchai*

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Introduction

The skipper genus *Scobura* Elwes & Edwards, 1897 was recently revised by Fan et al. (2010), who recognized 14 species. The genus *Scobura*, however, includes another species, *S. masutaroi*, Sugiyama 1996. Fan et al. (2010) overlooked the existence of this taxon and did not include it in their revisional work, which resulted in Krajcik (2013) proposing a new taxon, *S. mouchai*, from Shaanxi.

Although a comprehensive morphological revision of the genus has been completed, no phylogenetic analysis has been performed to infer relationships within the genus. In the present study, we present a preliminary phylogeny of *Scobura*, based on molecular evidence. By comparing molecular and morphological evidence, we examine whether *S. mouchai* is a synonym of *S. masutaroi*.

Methods

Morphological examination

See Fan et al. (2010) for materials for the morphological study. In order to examine the wing venation, wings were removed from thorax, cleaned with 95% ethanol, and dyed red with acetocarmine (Wang et al. 2011).

Taxon sampling

Twenty-three specimens including nine of the 15 valid species of *Scobura* and four outgroup species were included in the phylogenetic reconstruction. Detailed information on the specimens is provided in Table 1. Specimens used in this study were mainly deposited in the Insect Collection, Department of Entomology, South China Agriculture University (SCAU), except for some specimens in Kyushu University museum (KU) and Mr. Hiroaki Onodera’s private collection.

Laboratory protocols

Genomic DNA was extracted from the thorax of specimens preserved in ethanol, or from legs of dried specimens, using Magen’s Blood/cell/tissue DNA extraction kit. One mitochondrial gene *cytochrome c oxidase I* (COI) and one nuclear gene *elongation factor 1-α* (EF-1α) were used as molecular phylogenetic markers. The following primers were used for amplification and sequencing in this study: for COI – primers LCO1490 and HCO2198 (Folmer et al. 1994); for EF-1α – primers ef44 and efrcM4 (Monteiro and Pierce 2001). Polymerase Chain Reaction (PCR) were performed in 20 μl volumes containing 1 μl
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Table 1. Voucher information and GenBank accession numbers for the specimens in this study.

| Species                                             | Locality          | Latitude | Longitude | Voucher Number | COI     | EF-1x    |
|-----------------------------------------------------|-------------------|----------|-----------|----------------|---------|----------|
| Scobura cephaloides kinka Evans, 1949               | China: Hainan     | 19.02N   | 109.53E   | SCAU He102     | KY049936| KY049958 |
| Scobura cephaloides kinka Evans, 1949               | Laos: Luang Prabang | 19.93N   | 102.07E   | Onodera He553  | KY049937| KY049959 |
| Scobura coniata Hering, 1918                       | China: Guangdong  | 24.91N   | 113.04E   | SCAU He073     | KY049938| KY049960 |
| Scobura coniata Hering, 1918                       | China: Guangdong  | 24.87N   | 113.04E   | SCAU He472     | KY049939| KY049961 |
| Scobura hainana (Gu & Wang, 1997)                  | China: Guangdong  | 24.87N   | 113.04E   | SCAU He471     | KY049940| KY049962 |
| Scobura hainana (Gu & Wang, 1997)                  | China: Guangdong  | 24.87N   | 113.04E   | SCAU He487     | KY049941| KY049963 |
| Scobura hainana (Gu & Wang, 1997)                  | China: Guangdong  | 24.87N   | 113.04E   | SCAU He488     | KY049942| KY049964 |
| Scobura isota (Swinhoe, 1893)                      | Thailand: Kanchanaburi | 14.08N | 99.36E | SCAU He538     | KY049943| KY049965 |
| Scobura isota (Swinhoe, 1893)                      | Thailand: Mae Hong Son | 19.35N | 98.14E | SCAU He468     | KY049944| KY049966 |
| Scobura lyso (Evans, 1939)                         | China: Zhejiang   | 30.15N   | 119.25E   | SCAU He465     | KY049945| —        |
| Scobura lyso (Evans, 1939)                         | China: Zhejiang   | 30.15N   | 119.25E   | SCAU He475     | KY049946| —        |
| Scobura masutaroi Sugiyama, 1996                   | China: Sichuan    | 29.94N   | 102.48E   | SCAU He300     | KY049947| KY049967 |
| Scobura masutaroi Sugiyama, 1996                   | China: Sichuan    | 29.94N   | 102.48E   | SCAU He301     | KY049948| KY049968 |
| Scobura masutaroi Sugiyama, 1996 (=mouchai)       | China: Shaanxi    | 31.91N   | 106.34E   | SCAU He303     | KY049949| KY049969 |
| Scobura parasquilloletti Fan et al., 2010          | China: Hainan     | 19.03N   | 109.53E   | SCAU He116     | KY049950| KY049970 |
| Scobura stellata Fan et al., 2010                  | China: Guangdong  | 24.92N   | 113.01E   | SCAU He036     | KY049951| KY049971 |
| Scobura woolletti (Riley, 1923)                     | Indonesia: Kabundungan | 6.77 S  | 106.60E | KU He535       | KY049952| KY049972 |
| Scobura woolletti (Riley, 1923)                     | Indonesia: Kabundungan | 6.77 S  | 106.60E | KU He536       | KY049953| KY049973 |
| Scobura woolletti (Riley, 1923)                     | Indonesia: Kabundungan | 6.77 S  | 106.60E | KU He537       | KY049954| KY049974 |
| Suastus grenius (Fabricius, 1798)                   | China: Guangdong  | 23.15N   | 113.34E   | SCAU He157     | KY049955| KY049975 |
| Suada sverga (deNicéville, 1884)                   | Thailand: Chiang Mai | 18.80N | 98.92E | SCAU He495     | KY049956| KY049976 |
| Hyarotis quinquepunctatus Fan & Chiba, 2008        | China: Hainan     | 19.03N   | 109.54E   | SCAU He114     | —       | KY049977 |
| Zographetus satva (deNicéville, 1884)              | China: Guangdong  | 24.88N   | 113.03E   | SCAU He442     | KY049957| KY049978 |
template DNA, 2 µl 10× buffer, 1.6 µl dNTPs (containing 2.5 mM of each dNTP), 0.8 µl of each primer (10 uM), 0.2 µl Taq Polymerase (2 U/µl), and 13.6 µl ddH₂O. The PCR Products were amplified using initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 47 °C (COI) for 45 s, 55 °C (EF-1α) for 1 min, elongation at 72 °C for 1.5 min, and final elongation at 72 °C for 5 min.

Amplified DNA products were purified using an Agarose Gel Extraction kit (Magen Biotech), and directly sequenced, or cloned with pMD18-T vector (Takara Inc), and then sequenced. Sequencing was performed using the ABI 3730 automated sequencer. All sequences were submitted to the Genbank database (accession numbers are given in Table 1).

**Phylogenetic analyses**

Alignment of the DNA sequences were performed in Clustal X (Thompson et al. 1997) and edited manually in MEGA 6.0 (Tamura et al. 2013). All base frequencies and molecular character statistics were calculated in MEGA 6.0. Phylogenetic trees were constructed under maximum Likelihood (ML) and Bayesian inference (BI) criteria. For ML analysis, RAxML version 8 (Stamatakis et al. 2014) was used on a concatenated data set of two genes, with 1000 rapid bootstrap replicates using GTR+G substitution model on the CIPRES Science Gateway (Miller et al. 2010). BI was carried out using Markov Chain Monte Carlo (MCMC) randomization in MrBayes v3.2.3 (Ronquist et al. 2012). We used reversible-jump MCMC to allow for sampling across the entire substitution rate models. Four Markov chains (three heated chains, one cold) were run for 500,000 generations, with the first 25% of sampled trees discarded as burn-in. The two independent runs were considered to have converged when the standard deviation of split frequencies value was <0.01. The convergence of the analysis was determined in Tracer v1.6 (Rambaut et al. 2014). Bayesian posterior probabilities (PP) and ML bootstrap values (BP) were used to evaluate branch support.

**Results**

**Sequence data**

From a total of 23 samples, 22 sequences for COI and 21 for EF-1α were obtained. The alignment of the combined sequences consisted of a total of 1724 bp (658 bp of COI and 1066 bp of EF-1α genes, respectively), including 277 variable and 200 informative sites.

The pairwise P2K distances among the sequences were variable between genes. The ranges of sequence divergences for two loci and ingroup taxa are: COI (0–12.4%), EF-1α (0–5.0%). For COI, sequence divergence between conspecific individuals ranged from 0 to 0.6%; inter-specific genetic distances ranged from 3.6% to 12.4% with divergences among species averaging 7.9% (Table 2).
Table 2. Uncorrected pairwise genetic distances (Kimura 2-parameter) for the COI sequences of the genus *Scobura* species.

|     | 1    | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    | 11    | 12    | 13    | 14    | 15    | 16    | 17    |
|-----|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1   | S. cephaloides 102 |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 2   | S. cephaloides 553 | **0.003** |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 3   | S. coniata 73     | 0.096 | 0.098 |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 4   | S. coniata 472    | 0.096 | 0.098 | **0.000** |       |       |       |       |       |       |       |       |       |       |       |       |
| 5   | S. hainana 471    | 0.084 | 0.085 | 0.059 | 0.059 |       |       |       |       |       |       |       |       |       |       |       |
| 6   | S. hainana 487    | 0.084 | 0.085 | 0.059 | 0.059 | **0.000** |       |       |       |       |       |       |       |       |       |       |
| 7   | S. hainana 488    | 0.084 | 0.085 | 0.079 | 0.061 | 0.099 | 0.099 | 0.099 |       |       |       |       |       |       |       |       |
| 8   | S. isota 468      | 0.115 | 0.117 | 0.122 | 0.122 | 0.099 | 0.099 | 0.099 |       |       |       |       |       |       |       |       |
| 9   | S. isota 538      | 0.113 | 0.118 | 0.118 | 0.096 | 0.096 | 0.096 | **0.006** |       |       |       |       |       |       |       |       |
| 10  | S. lyso 465       | 0.087 | 0.089 | 0.061 | 0.061 | 0.039 | 0.039 | 0.105 | 0.101 |       |       |       |       |       |       |       |
| 11  | S. lyso 475       | 0.085 | 0.087 | 0.061 | 0.061 | 0.036 | 0.036 | 0.101 | 0.098 | **0.003** |       |       |       |       |       |       |
| 12  | S. masutaroi 300  | 0.092 | 0.096 | 0.059 | 0.059 | 0.056 | 0.056 | 0.107 | 0.103 | 0.052 | 0.052 |       |       |       |       |       |
| 13  | S. masutaroi 301  | 0.092 | 0.096 | 0.059 | 0.059 | 0.056 | 0.056 | 0.107 | 0.103 | 0.052 | 0.052 | **0.000** |       |       |       |       |
| 14  | S. masutaroi 303  | 0.092 | 0.096 | 0.059 | 0.059 | 0.056 | 0.056 | 0.109 | 0.105 | 0.052 | 0.052 | **0.002** | **0.002** |       |       |       |
| 15  | S. parawoolletti 116 | 0.094 | 0.097 | 0.089 | 0.089 | 0.087 | 0.087 | 0.112 | 0.108 | 0.084 | 0.084 | 0.084 | 0.084 | 0.084 | 0.084 |       |
| 16  | S. stellata 36    | 0.101 | 0.104 | 0.104 | 0.104 | 0.101 | 0.101 | 0.099 | 0.108 | 0.105 | 0.099 | 0.099 | 0.099 | 0.092 | 0.092 | 0.094 | 0.070 |
| 17  | S. woolletti 535  | 0.094 | 0.097 | 0.096 | 0.096 | 0.085 | 0.085 | 0.124 | 0.121 | 0.092 | 0.092 | 0.099 | 0.099 | 0.099 | 0.099 | 0.039 | 0.074 |
| 18  | S. woolletti 536  | 0.092 | 0.096 | 0.094 | 0.094 | 0.084 | 0.084 | 0.123 | 0.119 | 0.090 | 0.090 | 0.097 | 0.097 | 0.098 | 0.038 | 0.072 | **0.002** |
| 19  | S. woolletti 537  | 0.094 | 0.097 | 0.096 | 0.096 | 0.085 | 0.085 | 0.124 | 0.121 | 0.092 | 0.092 | 0.099 | 0.099 | 0.099 | 0.039 | 0.074 | **0.000** | **0.002** |
Phylogenetic analyses

The two model-based analyses (BI and ML) revealed nearly identical topologies, differing mainly in branch support (Fig. 1). In both analyses, the monophyly of the genus *Scobura* is weakly supported (BP = 44, PP = 0.87). Within the genus, although support for the basal clades was low, the *Scobura* species included here are clearly distinguished from each other, and formed four clades: the *S. isota* clade (which only included two representative specimens), Clade A, the *S. cephaloides* clade (only with two representative specimens), and Clade B. Clade A is comprised by *S. stellata* + (*S. parawoolletti* + *S. woolletti*) and receive high bootstrap support and posterior probability (BP = 99, PP = 1.00). We hereafter called the clade *S. woolletti* group.
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Clade B is comprised by *S. masutaroi* and the representatives of *S. coniata* group (Devyatkin 2004): *S. coniata*, *S. lyso* and *S. hainana*, and the latter two are sister species with strong support (BP = 93, PP = 1.00). The monophyly of *S. coniata* group including *S. masutaroi* is strongly supported (BP = 100, PP = 1.00).

In all the analyses, *S. cephaloides* is sister to Clade B, with moderate support (BP = 63, PP = 0.92), whereas the relationships between *S. isota* and the other clades (Clade A, *S. cephaloides* and Clade B) remain unresolved.

**Discussion**

Although our phylogenetic analyses do not strongly support the monophyly of the genus *Scobura*, two strongly supported monophyletic groups within the genus are recognized: the *S. coniata* group and the *S. woolletti* group. The members of the *coniata* group share the following four morphological characters: 1) male band of scent scales on both sides of veins CuA₁ and CuA₂ and above 2A on the forewing (Fig. 2); 2) juxta U-shaped with two spine bearing arms, flat at base; 3) tegumen without socius; and 4) uncus thin and long. *S. masutaroi* is nested within this group. In our present analyses, two individuals (He 300, 301) of *masutaroi* from Nibashan, Sichuan (close to Dujiangyan, Sichuan, the type locality of *S. masutaroi*) and an individual (He303) from Jialingjiang, Fengxian, Shaanxi (the type locality of *S. mouchai*) are clearly grouped together with strong support values (BP = 100, PP = 1.00). Moreover, the pairwise P2K distances in COI between the species in the *S. coniata* group range from 3.3% to 6.1% with divergences between species averaging 4.5%, while divergence between individuals of *S. masutaroi* from Sichuan and Shaanxi province was 0.2%.

Based on the original description, distribution data, and the illustrations provided by Krajcik (2013), as well as our phylogenetic inferences, we conclude that *S. mouchai*...
is identical to *S. masutaroi* and should be considered a junior synonym. The male genitalia are illustrated herein, and the female genitalia are described for the first time. On the basis of morphological study (Devyatkin, 2004), two other species, *S. phuongi* and *S. evani*, which are not included in the present study, likely also belong to this group.

A well-support clade comprised by *S. stellata*, *S. parawoolletti* and *S. woolletti* was recovered in all analyses. These species share the following three characters: 1) hindwing with white spots on underside but not on upperside; 2) socius slender and pointed at tip; and 3) juxta funnel-like, thin and long basally. The generic name *Mimambrix* Riley, 1923 was proposed with *Mimambrix woolletti* as the type species, but later synonymized by Evans (1949). We follow Evans’ treatment and consider this clade as a species group within the genus *Scobura*. Based on morphological characters, the group also includes *S. tytleri* (Evans, 1914).

### Taxonomic account

The key given by Fan et al. (2010) is modified to include *S. masutaroi*. The couplets leading to *S. masutaroi* only are included here. Couplets beyond 11 in the original increase their number by one.

| Couplet | Description |
|---------|-------------|
| 3       | Forewing upper side without spots in spaces M$_2$ or M$_1$ and M$_2$.................4 |
| –       | Forewing upper side with spots in spaces M$_1$, M$_2$ and M$_3$.....................6 |
| 4       | Forewing upper side without spots in spaces M$_1$ and M$_3$. hindwing under side: basal half yellow, distally ferruginous, with five small spots.......*S. cephaloides* |
| –       | Forewing upper side without spot in space M$_3$............................................5 |
| 5       | Hindwing under side with a conspicuous rectangular white spot in space CuA$_2$...............................................................................................*S. cephala* |
| –       | Hindwing upper side without a conspicuous rectangular white spot in space CuA$_2$..........................................................................................*S. isota* |
| 6       | Hindwing upper side without spot in space CuA$_1$, under side with small white spots in spaces Sc+R$_1$, M$_{1,2}$, M$_3$ and cell ......................*S. eximia* |
| –       | Hindwing upper side with the spot in space CuA$_1$...........................................7 |
| 7       | Forewing cell spots conjoined, subequal.......................................................8 |
| –       | Forewing cell spots separated, if conjoined, the lower spot much larger ......9 |
| 8       | Hindwing upper side hyaline spots white.....................................................*S. evansi* |
| –       | Hindwing upper side hyaline spots yellow ..............................................*S. masutaroi* |
| 9       | Forewing upper side the spot in space CuA$_2$ triangular, and with a linear stigma crossing the spots in spaces CuA$_1$ and CuA$_2$..........................*S. coniata* |
| –       | Forewing upper side the spot in space CuA$_2$ not as above ......................10 |
| 10      | Forewing upper side the spot in space CuA$_1$ narrow, hindwing upper side without spot in space.......................................................*S. lyso* |
| –       | Forewing upper side the spot in space CuA$_1$ broad.................................11 |
Hindwing upper side spot in space $M_3$ tiny dot, forewing upper side cell spots cell spots conjoined.......................... $S. hainana$

Hindwing upper side spot in space $M_3$ significant, forewing upper side cell spots cell spots separated.......................... $S. phuongi$

**Scobura masutaroi** Sugiyama, 1996

Fig. 3

*Scobura masutaroi* Sugiyama, 1996: 9 (Type locality: Dujiangyan, Sichuan, China)

*Scobura mouchai* Krajcik, 2013: 2, **syn. n.** (Type locality: Fengxian, Shaanxi, China)

**Material examined.** 1♂, 1♀, Nibashan, Rongjing, Sichuan, 26.VII.2009, Min Wang; 1♂, Jialingjiang, Fengxian, Shaanxi, 15.VII.2010, Min Wang.

**Diagnosis.** Forewing length 17–18 mm. This species is different from other species of *S. coniata* group in the appearance of the wing upper side: forewing with yellow streak in subcosta space basally, a big cell spots solid across cell, the spot in space $CuA_2$ yellow; hindwing with spots in spaces $CuA_1$ and $M_1$-$M_2$ yellow. Wing under side: forewing costal and submarginal spots yellow; hindwing all veins and submarginal spots from spaces $Sc+R_1$ to $CuA_2$ yellow; and all yellow submarginal spots conjoined both forewing and hindwing.

![Figure 3. *Scobura masutaroi* Sugiyama, 1996 (Sichuan): A, B male C, D female; scale bar 10 mm.](image-url)
Figure 4. Male genitalia of *Scobura masutaroi* Sugiyama, 1996. (Sichuan). **A** Genitalia ring, lateral view; **B** aedeagus and juxta. **C** valva, inner view; **D** tegument, dorsal view.

Figure 5. Female genitalia of *Scobura masutaroi* Sugiyama, 1996 (Sichuan)
Description. Male genitalia (Fig. 4): Tegumen without socius, weakly rounded from lateral view; uncus slender and much longer than tegumen; valva with transtilla rounded and sclerotized with small spines, ventro-distal process irregularly shaped with outer edge rounded, inner edge uneven, and distal part rectangular with densely small spines; saccus short and broad; gnathos absent; juxta U-shaped with two arms with densely spines.

Female genitalia (Fig. 5): Papillae anales rectangular, covered with setae; anterior lamella U-shaped with sclerotization; posterior lamella triangular with upper margin arched; ductus bursae membranous and short; copulatrix bursa elongate, membranous.

Distribution. China (Sichuan, Shaanxi).

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