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Multi-gene Phylogenetic Analysis and Genetic Diversity of Discrete Elytral Color Phenotypes in *Menochilus sexmaculatus* (Coleoptera: Coccinellidae)

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XC managed the project and led the writing of the manuscript. WH and XC conceived and designed the experiments. XW and XC collected the fresh samples. WH, XL and XX conducted laboratory experiments and data analyses. WH and XC contributed to the writing of the manuscript.

Key words
Phylogenetic analyses, Genetic diversity, Elytral color polymorphism, *Menochilus sexmaculatus*, Coccinellidae

ABSTRACT

The phenotype variations of elytral color patterns are common in ladybird beetles. However, phylogenetic relationships and genetic diversity of discrete color patterns in coccinellids is still poorly known. Here, we present a comprehensive genetic diversity analyses and phylogenetic relationships within seventeen different phenotypes of elytral color patterns in *Menochilus sexmaculatus* based on two mitochondrial genes, cytochrome oxidase subunit I (COI) and II (COII), and two nuclear genes, carbamoyl phosphate synthetase (CAD) and histone subunit 3 (H3). Results indicated the average genetic distance was 0.005 among the different elytral forms of *M. sexmaculatus* based on combined dataset, which shows very close genetic relationships among them. Results also showed a high level of haplotype diversity (H_d = 0.902) and the low level of nucleotide diversity (P_i = 0.004). In addition, the number of haplotypes was 17 and the same elytral color patterns of *M. sexmaculatus* didn't share the same haplotype. Furthermore, phylogenetic analyses showed all *M. sexmaculatus* samples formed a single clade, but the identical elytral pattern individuals do not cluster together as no special relationships among different phenotypes individuals. Our systematic analyses illustrated the same elytral forms of *M. sexmaculatus* do not possess closely-related phylogenetic relationships. However, these clear photographs of different elytral color patterns of *M. sexmaculatus* and the results of our analyses may prevent our incorrect identification for this species.

INTRODUCTION

Insect body coloration often shows genetically different forms even within a population, which may be important for intra- and interspecific communication and adaption to the local environment (Noriyuki and Osawa, 2015). Color polymorphisms provide some of the best characterized examples of functionally and ecologically important polymorphism. Insect melanism describes the occurrence of varied pigment patterns both within and between closely linked species making them polymorphic (True, 2003). During the past two decades, the genetics underlying variation in melanism have been unraveled for several species of insects (Lommen *et al*., 2012). These variations result either from genetic polymorphism or phenotypic plasticity (Schilthuizen and Kellermann, 2014).

The family Coccinellidae (ladybird beetles) belonging to the superfamily Coccinelloidea within the suborder Polyphaga of the order Coleoptera (Hunt *et al*., 2007; Robertson *et al*., 2015), are well known biological control agents. Ladybird beetles have long been studied by geneticists and evolutionary biologists to investigate the origin and maintenance of discrete color pattern forms in natural population (Majerus, 1994). Variations of elytral color patterns are widespread within different coccinellid species, such as *Harmonia axyridis* (Pallas, 1773), *Phrynocaria unicolor* (Fabricius, 1792), *Calvia quatuordecimguttata* (Linnaeus, 1758), *Propylea japonica* (Thunberg, 1781), *Menochilus sexmaculatus* (Fabricius,

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1781) and others (Yu, 2008). In particular, the harlequin ladybird *H. axyridis* is an emblematic species of elytral color pattern polymorphism, with more than 200 distinct color patterns described around the world (Ando *et al*., 2018; Gautier *et al*., 2018), and have brought difficulties in their identification or nomenclature. Intraspecific variation in color patterns of ladybird beetles has been studied for many decades (Millar *et al*., 1999). Recently, the phylogenetic relationships of different color patterns within *H. axyridis* was investigated based on 12S rRNA and 16S rRNA genes (Yao *et al*., 2011). The color polymorphism of this species was controlled by the transcription factor *pannier* (Gautier *et al*., 2018).

Menochilus sexmaculatus is a polymorphic aphidophagous ladybird with wide prey range and distribution (Kawakami *et al*., 2013). However, the broad distribution and multiple color forms in *M. sexmaculatus* were usually confused for their identification (Fig. 1). This species is known to have 20 phenotypes of elytral polymorphs according to the ratios of elytral red and black areas (Kawakami *et al*., 2013, 2018). Meanwhile, the phylogenetic relationships and genetic diversity of different color patterns within *M. sexmaculatus* is still poorly known.

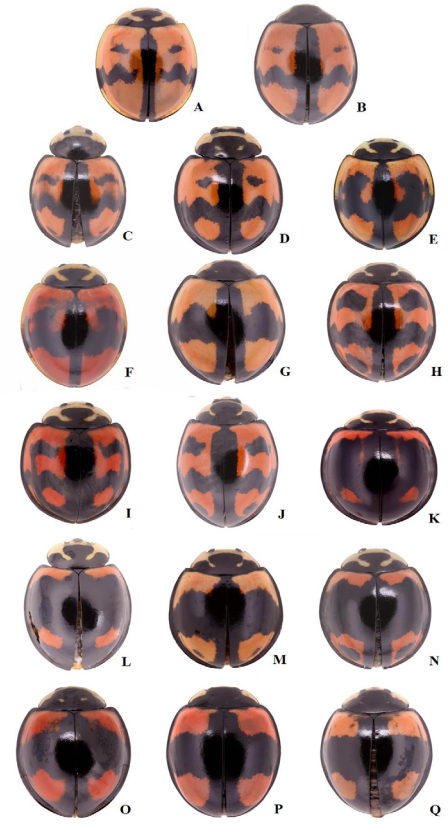

Fig. 1. The different elytral forms in *M. sexmaculatus*.

Recently, polymorphism has become the focus of attention in population ecology as well as evolutionary biology as it can contribute to population productivity, stability and persistence (Wennersten and Forsman, 2012; Forsman, 2013; Takahashi *et al*., 2014). Here we investigate the molecular phylogenetic analyses and genetic diversity of different elytral color forms in *M. sexmaculatus* based on two mitochondrial genes (cytochrome oxidase subunit I (COI) and II (COII)) and two nuclear genes (carbamoyl phosphate synthetase (CAD) and histone subunit 3 (H3)). The aim of this study is testing the different color patterns of *M. sexmaculatus* whether possess phylogenetically specific relationships.

MATERIALS AND METHODS

Sampling

Multiple elytral color forms in *M. sexmaculatus* (Fig. 1) were collected from Guangdong Province, China during September 2017. Each phenotype was sampled by two individuals. After collection, the specimens were preserved in absolute ethanol. Detailed sample information is shown in Table I.

DNA extraction and PCR

Total genomic DNA was extracted using the TIANGEN DNA extracting kit (TianGen Biochemistry, Beijing, China) following the manufacturer's instructions. Four gene regions (two mitochondrial genes COI, COII, and two nuclear genes CAD, H3) were amplified and sequenced. The primers information was listed in Table II. Polymerase chain reactions (PCR) were performed in 25 μL volumes containing 12 μL 2 × EasyTaq PCR SuperMix (TransGen Biotech, Beijing, China), 10 μL ultrapure water, 1 μL of each primer and 1 μL DNA template. PCR cycling conditions consisted of initial denaturation at 94℃ for 3 min, 35 cycles of 94℃ for 30 s, 50℃ for 30 s, 72℃ for 1 min, and ending with a final extension at 72℃ for 5 min. For COII and CAD, we used a hemi-nested approach. We performed an initial PCR reaction using following primer pairs: CD806F3/CD1098R2 for CAD, COIIF-leu/COIIR-lys for COII. One microliter of product from initial reaction was used as template for the hemi-nested reaction using primers CD821F/CD1098R2 for CAD, COIIF-leu/COIIR-9b for COII. Successful amplification was assessed using gel electrophoresis on 1% agarose gels by adding 5 μL PCR product. All obtained sequences were compared using BLAST against GenBank to ensure that the target sequences were amplified.

Alignment and sequence analyses

Target sequences were manually cleared, trimmed and

Table I. All samples number and code information.

Number	Code	Specimen	Haplotype	Number	Code	Specimen	Haplotype
1	A1	*M. sexmaculatus*	H1	18	I2	*M. sexmaculatus*	H4
2	A2	*M. sexmaculatus*	H2	19	J1	*M. sexmaculatus*	H4
3	B1	*M. sexmaculatus*	H3	20	J2	*M. sexmaculatus*	H13
4	B2	*M. sexmaculatus*	H4	21	K1	*M. sexmaculatus*	H14
5	C1	*M. sexmaculatus*	H5	22	K2	*M. sexmaculatus*	H7
6	C2	*M. sexmaculatus*	H6	23	L1	*M. sexmaculatus*	H15
7	D1	*M. sexmaculatus*	H4	24	L2	*M. sexmaculatus*	H16
8	D2	*M. sexmaculatus*	H7	25	M1	*M. sexmaculatus*	H11
9	E1	*M. sexmaculatus*	H4	26	M2	*M. sexmaculatus*	H4
10	E2	*M. sexmaculatus*	H8	27	N1	*M. sexmaculatus*	H7
11	F1	*M. sexmaculatus*	H7	28	N2	*M. sexmaculatus*	H7
12	F2	*M. sexmaculatus*	H9	29	O1	*M. sexmaculatus*	H11
13	G1	*M. sexmaculatus*	H4	30	O2	*M. sexmaculatus*	H13
14	G2	*M. sexmaculatus*	H10	31	P1	*M. sexmaculatus*	H4
15	H1	*M. sexmaculatus*	H11	32	P2	*M. sexmaculatus*	H7
16	H2	*M. sexmaculatus*	H12	33	Q1	*M. sexmaculatus*	H13
17	I1	*M. sexmaculatus*	H7	34	Q2	*M. sexmaculatus*	H17

Table II. Information on the primer sequences and corresponding genes information.

Marker	Primer name	Primer sequence (5'-3')	Reference
COI	Jerry F	CAACATTTATTTTGATTTTTT	Timmermans *et al*., 2010
	Spat R	GCACTAWTCTGCCATATTAGA	
COII	COIIF-leu	TCTAATATGGCAGATTAGTGC	Robertson *et al*., 2013
	COIIR-lys	GAGACCAGTACTTGCTTTCAGTCATC	
	COIIR-9b	GTACTTGCTTTCAGTCATCTWATG	
CAD	CD806F3	TTAYTGYGTTGTNAARATWCCNMGNTGGGA	Wild and Maddison, 2008
	CD821F	AGCACGAAAATHGGNAGYTCNATGAARAG	
	CD1098R2	GCTATGTTGTTNGGNAGYTGDCCNCCCAT	
H3	H3F	ATGGCTCGTACCAAGCAGACVGC	Robertson *et al*., 2013
	H3R	ATATCCTTRGGCATRATRGTGAC	

aligned using Geneious 9.1.5 (Kearse *et al*., 2012), the four gene alignments were then concatenated to obtain a supermatrix using the program Sequence Matrix 1.7.8 (Vaidya *et al*., 2011). MEGA 7.0 (Kumar *et al*., 2016) was used to analyze nucleotides composition and pairwise genetic distance based on Kimura-2-parameter (K2P) (Kimura, 1980) models using combined dataset. DnaSP 5.1 (Librado and Rozas, 2009) was performed to calculate the number of polymorphic sites, haplotype diversity (H_d) and nucleotide diversity (P_i) used single gene and combined dataset.

Phylogenetic analyses

Both methods of maximus likelihood (ML) and Bayesian inference (BI) were employed to explore the phylogenetic relationships within different elytra color forms in *M. sexmaculatus*. Three datasets were assembled for phylogenetic analyses: (1) the P1 matrix, including only mtDNA; (2) the P2 matrix, including only nuclear genes; (3) the P3 matrix, including both mtDNA and nuclear genes. *Psyllobora vigintiduopunctata* (Linnaeus, 1758) and *Halyzia straminea* (Hope, 1831) were chosen as outgroups. Together with *M. sexmaculatus* they belong

to the tribe Coccinellini according to previous studies (Escalona *et al*., 2017).

Partition Finder 1.1.1 (Lanfear *et al*., 2012) was used to infer the optimal partition schemes and models of molecular evolution for the concatenated data sets, applying an all search approach with branch lengths unlinked across partitions and the Bayesian information criterion (BIC). The ML analyses were conducted with the program RAxML 8.0 (Stamatakis, 2006). Since it is not currently possible to specify different models of substitution for different partitions in RAxML, we used GAMMA model for each ML analysis. Branch support was estimated with 500 replicates using a rapid bootstrapping algorithm (Stamatakis *et al*., 2008). The BI analyses were calculated in MrBayes 3.2 (Ronquist *et al*., 2012). Two Markov Chain Monte Carlo (MCMC) runs were performed with one cold and three heated chains for 30 million generations and sampled every 1000 generations. The consensus tree was estimated after a burn-in of 25% of the sampled trees. The chain stationarity was visualized by plotting likelihoods against the generation number using the program Tracer 1.6 (Rambaut *et al*., 2014).

RESULTS

Genetic diversity and structure

To gain insight into the genetic diversity of the different elytral forms of *M. sexmaculatus*, we analyzed the genetic variation of 34 individuals standing for 17 phenotypes of elytral forms, based on 759 bp for CAD, 829 bp for COI, 722 bp for COII and 328 bp for H3, 2638 bp in total. These sequences were deposited in GenBank under the accession number MH589128-MH589261 (Supplementary Table S1). The average nucleotide contents of A, T, G and C were 35.1%, 25.9%, 22.4% and 16.6% for CAD, 33.7%, 37.9%, 13.8% and 14.6% for COI, 35.3%, 38.9%, 11.2% and 14.6% for COII, 27.7%, 20.8%, 23.8% and 27.7% for H3, and 33.8%, 32.6%, 16.8% and 16.8% for combined dataset, respectively. The nucleotide compositions of CAD, COI and COII were similar, having high A + T content, but H3 gene G + C content were slightly higher than A + T content. In four gene regions, there were 15 variable sites for CAD gene sequences, 9 of which were parsimony informative, 17 variable sites for COI gene sequences, 11 of which were parsimony informative, 11 variable sites for COII gene sequences, 9 of which were parsimony informative, 2 variable sites for H3 gene sequences, 2 of which were parsimony informative, whilst 45 variable sites for combined dataset sequences, 31 of which were parsimony informative.

Based on single gene, the genetic structure of 17 different phenotypes of color forms in *M. sexmaculatus* was also analyzed. The number of CAD haplotypes was higher, with 13 haplotypes, whilst the number of haplotypes for H3 was least, only 3 haplotypes were found. The haplotype diversity (*h*) was remarkably high (0.731) for CAD, and the nucleotide diversity (π) of COI was highest (0.007) (Table III). Besides, the combined dataset was used to analyse the genetic structure of the different phenotypes of elytral forms in *M. sexmaculatus*. Results show that the number of haplotypes was 17, the haplotype diversity was 0.902 and the nucleotide diversity was 0.004. For the haplotypes of the combined dataset, eight individuals shared the H4 haplotype and seven individuals shared the H7 haplotype, H1-H3, H5-H6, H8-H10, H12, H14-H17 were represented by only one individual (Table I). However, the same elytral color patterns in *M. sexmaculatus* did not shared the same haplotype.

The estimated intra-specific genetic distance based on combined dataset among the different elytral forms in *M. sexmaculatus* ranged from 0 to 0.011 (Supplementary Table S2). The average genetic distance was 0.005, and the maximum genetic distance between *M. sexmaculatus*-C2 and *M. sexmaculatus*-H1 was 0.011.

Table III. Genetic structure of different elytral forms in *M. sexmaculatus* been revealed by CAD, COI, COII and H3 gene.

Gene	Nh	H_d	P_i
CAD	13	0.731	0.003
COI	6	0.693	0.007
COII	5	0.624	0.005
H3	3	0.324	0.002
Combined	17	0.902	0.004

Nh, number of haplotypes; H_d, haplotype diversity; P_i, nucleotide diversity

Table IV. Partitions and evolutionary substitution models of different datasets.

Dataset	Composition	Partition scheme	Evolutionary substitution models
P1	mtDNA gene	COI + COII	TIM + I
P2	Nuclear gene	CAD + H3	TRN + G
P3	mtDNA and nuclear gene	COI + COII + CAD + H3	GTR + G

Phylogenetic analyses

The best-fit partition scheme with corresponding substitution models for each dataset was shown in Table IV. Based on P1 and P3 datasets, the ML and BI topologies respectively resulting from RAxML and MrBayes analyses

were largely congruent except for several specimens showing the different placement (Figs. 2 and 4). We recover the thirty-four specimens of *M. sexmaculatus* forming monophyletic clade with strong support in both ML and BI analyses (P1: BS/PP = 100/1; P3: BS/PP = 88/1). Two major clade groups were recognized one clade includes 16 individuals, the second clade includes 18 individuals. However, when P2 dataset was used the resulted topology was different that the tree topologies inferred from P1 and P3 datasets (Fig. 3). This difference is mainly manifested in incongruent basal branching.

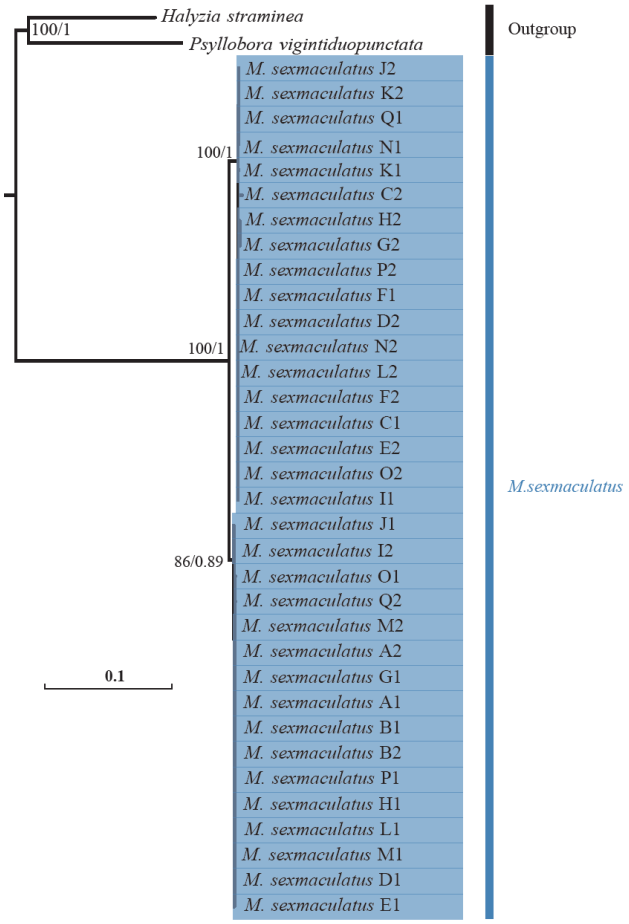

Fig. 2. Phylogenetic tree of the different elytral forms of *Menochilus sexmaculatus* based on mitochondrial genes (P1: COI and COII) obtained from RAxML and MrBayes. All *M. sexmaculatus* samples are labeled by steel-blue, the codes following sample name in this figure correspond to the code in Table I. Bootstrap support and posterior probabilities shown near the nodes.

DISCUSSION

Morphological polymorphisms are well studied and provide evidence of natural variation and micro evolutionary processes occurring in nature (Ford, 1964). Ladybird beetles are one of the classical groups in studying the mechanisms that determine local and temporal trends in color polymorphism. Hence among the frequently studied polymorphic taxa ladybird beetles occupy the main position, especially the results of studies on the polymorphism in the pattern and color of their head (Rogers *et al*., 1971), pronotum (Blehman, 2007) and elytra (Yao *et al*., 2011).

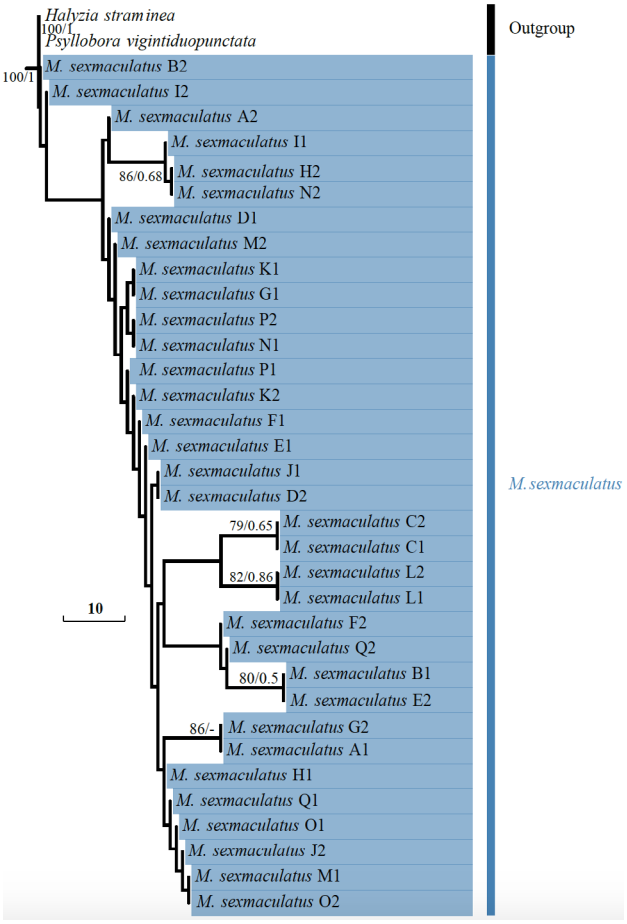

Fig. 3. Phylogenetic tree of the different elytral forms of *Menochilus sexmaculatus* based on nuclear genes (P2: CAD and H3) obtained from RAxML and MrBayes. All *M. sexmaculatus* samples are labeled by steel-blue, the codes following sample name in this figure correspond to the code in Table I. Bootstrap support and posterior probabilities are shown near the nodes.

It is important to derive information from molecular research, because they provide direct evidence of interaction between environmental factors and animal characteristics at the organismic level (Lowe *et al*., 2004). Based on two

mitochondrial (COI and COII) and two nuclear (CAD and H3) genes, we analyzed the phylogenetic relationships and genetic diversity of seventeen different phenotypes of elytral forms in *M. sexmaculatus*. Base composition analyses indicated the *M. sexmaculatus* of mitochondria genes COI and COII with A + T content apparently higher than G + C content, according with the feature of A + T high content in insect mitochondrial genes (Simon *et al*., 1994) and the average genetic distance based on combined dataset was 0.005 and shows their close relationships. Either single gene or combined data sets consistently revealed the lower levels of nucleotide diversity in different phenotypes in *M. sexmaculatus*. In phylogenetic analyses of molecular sequence data, choosing an appropriate partitioning scheme is an important step in most analyses due to it can affect the accuracy of phylogenetic reconstruction (Lanfear *et al*., 2012). Based different datasets resulted in molecular phylogenies analyses showed thirty-four individuals of 17 different elytral forms in *M. sexmaculatus* formed a clade with high support value, and the two individuals of the same elytral forms do not cluster together indicated the different color patterns of *M. sexmculatus* do not possess specific relationship phylogenetically. Similar research has been reported by Yao *et al*. (2011) who conducted phylogenetic analyses based on mitochondrial genes among different elytral forms of *H. axyridis*. In most cases the occurrence of morphs appears to be associated with climatic factors such as temperature, visual predation (Brakefield, 1985) and industrial pollution (Zakharov, 2003). Dubey *et al*. (2016) used different temperature regimes to assess the mate choice, reproductive success and offspring coloration of typical and melanic morphs of the *M. sexmaculatus*. Their findings on offspring phenotype variation indicated that the degree of melanism in morphs is a result of environmentally regulated expression of the parental genotype.

In addition to these factors have mentioned above, understanding the genetic mechanisms generating and maintaining such phenotypic variation within species is essential to comprehending morphological diversity. Indeed the genetic and ecological mechanism for the maintenance of elytral color polymorphism in ladybirds is not fully understood (Noriyuki and Osawa, 2015). However, Gautier *et al*. (2018) combined whole-genome sequencing, population genomic, gene expression and functional analyses, showed that the gene *pannier* controls melanic pattern polymorphism in *H. axyridis*. They also pointed out that *pannier*, which encodes an evolutionary conserved transcription factor, is necessary for the formation of melanic elements on the elytra. Allelic variation in *pannier* leads to protein expression in distinct domains on the elytra, and thus determines the distinct color patterns in *H. axyridis*. Meanwhile, Ando *et al*. (2018) through loss-of-function analyses, genetic association studies, de novo genome assemblies, and gene expression data revealed that repeated inversions within a *pannier* intron drive diversification of intraspecific color patterns of *H. axyridis*. These findings provide a reference for our understanding the genetic mechanisms of the elytral color polymorphism in *M. sexmaculatus*. Therefore, in order to definitely unravel specific causes result in phenotype diversity for *M. sexmaculatus*, combine genetic data and environmental data is needed in further research.

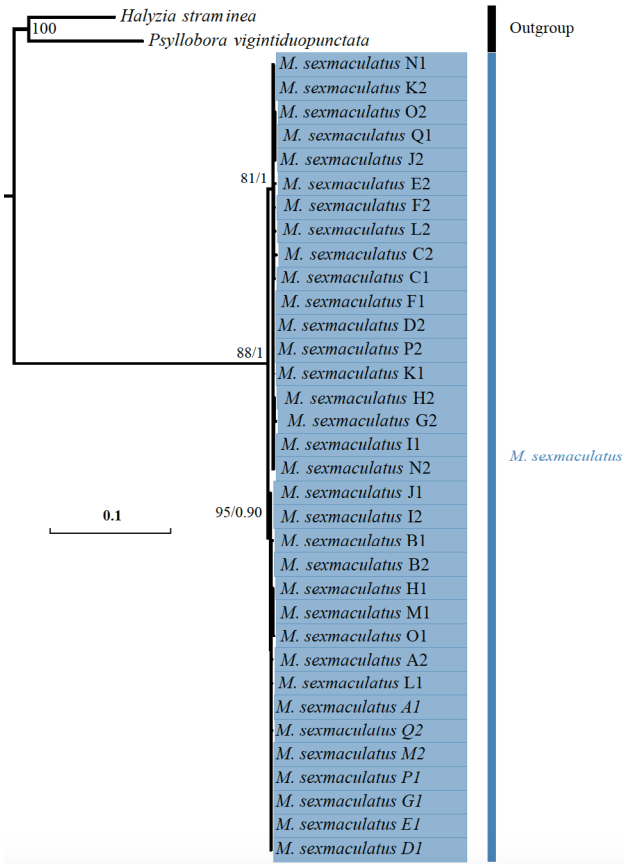

Fig. 4. Phylogenetic tree of the different elytral forms of *Menochilus sexmaculatus* based on mitochondrial genes (COI and COII) and nuclear genes (CAD and H3) obtained from RAxML and MrBayes. All *M. sexmaculatus* samples are labeled by steel-blue, the codes following sample name in this figure correspond to the code in Table I. Bootstrap support and posterior probabilities are shown near the nodes.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20210128120158

Statement of conflict of interest

The authors declare no conflict of interest.

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