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ORIGINAL ARTICLE

Molecular test shows the color pattern is not so reliable in diagnostic of genus *Dysphaea* Selys (Odonata: Euphaeidae)

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Abstract A molecular study based on COI, 16S and 28S genes reveals that a batch of specimens (7 males and 4 females) of *Dysphaea* Selys, 1853 collected from central Vietnam, which include different color patterns of wings and body, and were originally identified as three different species, are all the same species. This study implies that, in some group of Odonata, identification only depending on color pattern may be unreliable, no matter what huge variations there are.

Key words Odonata, dragonfly, wing color pattern variation, molecular phylogeny.

1 Introduction

Nowadays, more and more researches have demonstrated that only use one type of evidence (usually morphological characters) in taxonomy can be problematic, which is especially true when color polymorphism or cryptic species present (Sánchez-Herrera *et al.*, 2010, 2015). New concepts and methods, like integrative taxonomy (Dayrat, 2005) and taxonomic circle concept (Damm *et al.*, 2010) have been suggested to address these problems. Thus, as important independent evidence, molecular identification has become indispensable in current taxonomy.

The genus *Dysphaea* Selys, 1853 is an oriental euphaeid genus with nine recognized and named species occurring from southwestern India in the west to Java and Borneo in the east, and to Yunnan, Guangxi and Guizhou of China in the north (Hämäläinen *et al*., 2015; Schorr & Paulson, 2018). Species in this group usually have diversed wing color pattern but similar structures on genital ligula and caudal appendages. Hämäläinen *et al*. (2015), using both morphologic and molecular data, provided an extensive revision on *Dysphaea* species from the Sundaland region. This work also provided a good DNA sequences database for future studies on *Dysphaea*. Recently, Phan *et al*. (2018) reported three *Dysphaea* species occurring in Vietnam, namely *D. basitincta* Martin, 1904, *D. gloriosa* Fraser, 1938, and *D. haomiao* Hämäläinen, 2012. In Vietnam, *D. basitincta* is limited to the northern area while *D. gloriosa* has its distribution scope almost cover the whole central and southern area. *D. haomiao*, in turn, was reported to distribute both in the northernmost and in central area; but this seems incorrect (see the Discussion below). During a field work in Vietnam in May 2015, the first author and Dr. Quoc Toan Phan both found groups of *Dysphaea* individuals from Quang Nam and Quang Binh Provinces which superficially looked like representing all these three species known from Vietnam. In some cases, these ‘species’ even occurred in the same river. These groups of *Dysphaea* individuals show a distinct variation on the wing color patterns (Fig. 1). To make a further identification and explore the diversity of the collected batch of *Dysphaea* specimens from Quang Nam and Quang Binh, a molecular study was conducted following the protocol in Hämäläinen *et al*. (2015).

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2 Materials and methods

2.1 Samples

Twelve specimens of *Dysphaea* were selected (originally thought to represent *D. basitincta*, *D. gloriosa* and *D. haomiao*) for molecular work (Table 1). All of them were collected from Quang Nam and Quang Binh provinces in central Vietnam in 2015 except one (*D. haomiao*) from Mulun, Hechi, Guangxi, China in 2015 (only 33 km away from the type locality of *D. haomiao*, i.e. Xiaoqikong, Libo, Guizhou Province, China). This specimen has wholly dark color wings and it was identified as *D. haomiao* (Fig. 1), based on the description and illustrations by Hämäläinen (2012). We added the unique specimen of *D. haomiao* since by now no molecular study has included it. All these specimens were obtained by hand nets in the field and were took off one leg each to do DNA extracting. Therefore, our samples in both morphological and molecular works can be linked directly to each other. Specimens were deposited in the collections of the College of Life Sciences, Chongqing Normal University, Chongqing. Furthermore, DNA sequences data of all the 25 specimens used for combined COI+16S+28S phylogenic analysis in Hämäläinen *et al.* (2015) were also downloaded from the databases NCBI and added into our dataset

Figure 1. Habitual photos of all the newly sequenced specimens in this study. The ID numbers are in line with those on the phylogenetic tree. All of them appear to be the same species, except DyHXml01 is a *D. haomiao*.

for molecular phylogenetic analysis, including *D. basitincta*, *D. dimidiata* Selys, 1853, *D. gloriosa*, *D. ulu* Hämäläinen, Dow & Stokvis, 2015, *D. vanida* Hämäläinen, Dow & Stokvis, 2015 and outgroups (*Anisopleura furcata* Selys, 1891, *Euphaea decorata* Hagen in Selys, 1853, *E. superba* Kimmins, 1936, *Cryptophaea vietnamensis* (van Tol & Rozendaal, 1995) and *Lestes praemorsus decipiens* Kirby, 1894).

2.2 Morphological examining

All the specimens were examined and dissected under a Zeiss V8 stereomicroscope. Character photos were taken by a digital camera (Nikon D750, Thailand). Photos were rendered and arranged to form plates using Photoshop cc®.

2.3 DNA extraction and sequencing

Total genomic DNA was isolated from muscle of one leg of each dry specimen using UniversalGen DNA Kit (Beijing ComWin Biotech), following the manufactures protocols. Voucher specimens were preserved in the collections of the College of Life Sciences, Chongqing Normal University, Chongqing. The PCR procedures for the nuclear 28S rRNA, mitochondrial 16S rRNA and COI genes were conducted using primers designed by Dijkstra *et al*. (2014) as below. Two pairs of primers were used to amplify 28S. One was ODO_28S_f2_2 (5'-CCCGGCCGGGTCCCCGACGGT-3') and ODO_28S_r2_p3 (5'-TTACACACTCCTTAGCGGATTC-3'), another was ODO_28S_f3 (5'-ACCATGAAAGGTGTTGG TTG-3') and ODO_28S_r3_p3 (5'-ATCTCCCTGCGAGAGGATTC-3'). ODO_12852F (5'-AGAAACCGACCTGGCTT AAA-3') and ODO_13393R (5'-CGCCTGTTTATCAAAAACAT-3') were used to amplify the 16S, whereas ODO_ LCO1490d (5'-TTTCTACWAACCAYAAAGATATTGG-3') and ODO_HCO2198d (5'-TAAACTTCWGGRTGTCCAAA RAATCA-3') were used to amplify the COI gene. PCR amplification were performed in a 40μL volume including 20μL Mix, 15.5μL or 14.5μL ddH_2O, 1.5μL of each primer and 1.5μL or 2.5μL genomic DNA. The PCR cycling procedure was 2min at 94℃, followed by 30–35 cycles of 30s at 94℃, annealing temparature at 48℃–51℃ (28S) or 50℃ (COI) for 1min, 45℃ (16S) for 30s, and at 72℃ for 1min ending with a final extension at 72℃ for 8min. All PCR products were visualized via 1% agarose gel electrophoresis and amplifications were purified using a gel extraction kit (Sangon Biotech), then sent to commercial companies (BGI TechSolutions or GENEWIZ) for sequencing based on Sanger's chain termination method, the target PCR product were sequenced in both directions.

2.4 Sequence analysis

Sequence were edited and assembled in BioEdit v7.2.0 (Hall, 1999). Alignments of protein coding genes were translated to amino acids using MEGA v6.06 (Tamura *et al*., 2013) to detect frameshift mutations and premature stop codons, which may indicate the presence of pseudogenes. Sequence alignment was performed in ClustalX version 2.1 program with default settings, and corrected manually in terms of the sequence chromatogram to ensure each mutation loci was credible.

2.5 Phylogenetic analysis

To assess the phylogenetic signal contained in each dataset, the individual datasets 16S, 28S and COI and combined datasets 16S+28S+COI were used for Phylogenetic analyses. Neighbor-joining (NJ), maximum-likelihood (ML) and the Bayesian inference (BI) analyses were performed on the basis of all the datasets above. The NJ trees were derived using MEGA v6.06 (Tamura *et al*., 2013) based on the Kimura two-parameter model. ML analyses were performed using RAxML v8.0.0 (Stamatakis, 2014) under the GTR+I+G model estimated by ModelTest 3.7 (Crandall & Posada, 1998) with partitioned model. The node support values were assessed by bootstrap resampling calculated using 1000 replicates. BI analyses were performed using MrBayes 3.2.1 (Huelsenbeck & Ronquist, 2003) with the best-fit model GTR+G for 16S, GTR+I+G for 28S, and HKY+I+G for COI respectively estimated by MrModeltest v2.3 (Nylander, 2004), each fragment was treated as a separate partition. The BI trees were set to 1000000 generations and for every 1000 generations the chain was sampled. The Markov chain Monte Carlo (MCMC) process was run over four parallel chains, one cold and three incrementally heated. It could not be stopped until the average standard deviation of split frequencies was down to < 0.01. Convergence diagnostic was determined with Tracer 1.5 (Rambaut & Drummond, 2007). Trees sampled after burn-in of the first 25% of each run from the four runs were combined and used to construct a 50% majority rule consensus tree. Trees were displayed with Fig Tree v1.4.0 (Rambuat, 2012).

Table 1. Specimens with their sequences data used in the present study.

Specise	Date sources	ID	Location	Date	Collector	SN of Locus (COI, 16S, 28S)
Dysphaea sp.	this study	DyGVm01	Vietnam, Quang Nam, Tay Giang, Bhalee	20150527	X. Yu	
Dysphaea sp.	this study	DyGVm02	Vietnam, Quang Nam, Tay Giang, Bhalee	20150527	X. Yu	
Dysphaea sp.	this study	DyGVm03	Vietnam, Quang Nam, Tay Giang, Bhalee	20150527	X. Yu	
Dysphaea sp.	this study	DyGVm04	Vietnam, Quang Nam, Tay Giang, Bhalee	20150527	X. Yu	
Dysphaea sp.	this study	DyBVm01	Vietnam,Quang Binh, Minh Hoa district, Tan Hoa	20150529	X. Yu	
Dysphaea sp.	this study	DyBVm02	Vietnam,Quang Binh, Minh Hoa district, Tan Hoa	20150529	X. Yu	
Dysphaea sp.	this study	DyBVm03	Vietnam,Quang Binh, Minh Hoa district, Tan Hoa	20150529	X. Yu	
Dysphaea sp.	this study	DyBVm04	Vietnam,Quang Binh, Minh Hoa district, Tan Hoa	20150529	X. Yu	
Dysphaea sp.	this study	DyBVm05	Vietnam,Quang Binh, Minh Hoa district, Tan Hoa	20150529	X. Yu	
Dysphaea sp.	this study	DyBVm06	Vietnam,Quang Binh, Minh Hoa district, Tan Hoa	20150529	X. Yu	
Dysphaea sp.	this study	DyBVm07	Vietnam,Quang Binh, Minh Hoa district, Tan Hoa	20150529	X. Yu	
Dysphaea haomiao	this study	DyHXml01	China, Guangxi, Hechi, Huanjiang, Mulun	20150721	X. Yu	
Dysphaea basitincta	NCBI	*Dysphaea basitincta* 1	China, Hainan, Baisha, Yinggeling	20110502	H. Zhang	KP979480, KP979512, KP979561
Dysphaea basitincta	NCBI	*Dysphaea basitincta* 2	China, Hainan, Baisha, Yinggeling	20110502	H. Zhang	KP979481, KP979513, KP979562
Dysphaea dimidiata	NCBI	*Dysphaea dimidiata* 1	Indonesia, Riau, Riau Regency, Rama Rama	20140218	R. A. Dow	KP979484, KP979514, KP979563
Dysphaea dimidiata	NCBI	*Dysphaea dimidiata* 2	Indonesia, Riau, Riau Regency,Rama Rama	20140218	R. A. Dow	KP979485, KP979515, KP979564
Dysphaea dimidiata	NCBI	*Dysphaea dimidiata* 3	Malaysia, Sarawak, Bintulu division, Kakus	20101112	S. Stone	KP979486, KP979516, KP979565
Dysphaea dimidiata	NCBI	*Dysphaea dimidiata* 4	Thailand, Narathiwat, Sungai Ko-Lok	20030605	A. Pinratana	KP979488, KP979517, KP979566
Dysphaea dimidiata	NCBI	*Dysphaea dimidiata* 5	Indonesia, Kalimantan Timur, Paser district	20051114	J. van Tol	KP979489, KP979518, KP979567
Dysphaea dimidiata	NCBI	*Dysphaea dimidiata* 6	Malaysia, Sarawak, Kapit division, Between Kapit	20130618	R.A. Dow	KP979494, KP979519, KP979568
Dysphaea dimidiata	NCBI	*Dysphaea dimidiata* 7	Malaysia, Johor, Gunung Belumut	20120802	R.A. Dow	KP979495, KP979520, KP979569
Dysphaea dimidiata	NCBI	*Dysphaea dimidiata* 8	Malaysia, Terengganu, Sekayu Recreational	20110819	R.A. Dow	KP979496, KP979521, KP979570
Dysphaea dimidiata	NCBI	*Dysphaea dimidiata* 9	Malaysia, Sarawak, Miri division, Upper Baram	20100719	L. Southwell	KP979497, KP979522, KP979571
Dysphaea dimidiata	NCBI	*Dysphaea dimidiata* 10	Malaysia, Pahang, Kuala Tahan	20101210	R.A. Dow	KP979499, KP979523, KP979572
Dysphaea dimidiata	NCBI	*Dysphaea dimidiate* 11	Brunei, Temburong district	20040101	K.D.B. Dijkstra & V.J. Kalkman	KF369377, KF369707, KF370106

Table 1 (continued)

Specise	Date sources	ID	Location	Date	Collector	SN of Locus (COI, 16S, 28S)
Dysphaea gloriosa	NCBI	*Dysphaea gloriosa*	China, Hainan, Baisha, Yinggeling Reserve	20110502	H. Zhang	KP979500, KP979524, KP979573
Dysphaea ulu	NCBI	*Dysphaea ulu* 1	Malaysia, Sarawak, Kapit division, Lanjak	20130822	R.A. Dow	KP979501, KP979525, KP979574
Dysphaea ulu	NCBI	*Dysphaea ulu* 2	Malaysia, Sarawak, Miri division, Usun Apau	20120428	G.T. Reels	KP979506, KP979526, KP979576
Dysphaea vanida	NCBI	*Dysphaea vanida* 1	Thailand, Kanchanaburi, Lam Klong Ngu	20030525	A. Pinratana	KP979507, KP979527, KP979577
Dysphaea vanida	NCBI	*Dysphaea vanida* 2	Thailand, Ranong	20010325	M. Hämäläinen	KP979508, KP979528, KP979578
Dysphaea vanida	NCBI	*Dysphaea vanida* 3	Thailand, Ranong, Khlong Nakha, Khlong Bang Man	20020502	M. Hämäläinen	KP979509, KP979529, KP979579
Dysphaea vanida	NCBI	*Dysphaea vanida* 4	Thailand, Kanchanaburi, Lam Klong Ngu	20030523	M. Hämäläinen	KP979510, KP979530, KP979580
Anisopleura furcata	NCBI	*Anisopleura furcata*	Thailand, Chiang Mai	20020101	M. Hämäläinen	KF369297, KF369617, KF370015
Euphaea decorata	NCBI	*Euphaea decorata*	China, Guangdong, Nankunshan		K.D.B. Dijkstra	KP979511, KP979531, KP979581
Euphaea superba	NCBI	*Euphaea superba*	China, Guangxi	20050101	V.J. Kalkman	KF369389, KF369722, KF370121
Cryptophaea vietnamensis	NCBI	*Cryptophaea vietnamensis*	Vietnam, Northern Vietnam		M. Hämäläinen	KF369354, KF369682, KF370080
Lestes praemorsus	NCBI	*Lestes praemorsus*	Malaysia, Sarawak	20090101	R.A. Dow	KF369423, KF369759, KF370158

3 Results

Habitus photos of all samples used in this study were provided to dominate the variation of color pattern of wing and body (Fig. 1). Each sample can be linked to the OTU on the phylogenetic tree (Fig. 3) in term of its ID number. To make the wing color patterns easy to compare, right wings of five representatives were split off and put together with the photo of a syntype of *D. basitincta* (Fig. 2). According to the wing color pattern, specimens can be divided into at least three groups: dark-winged (DyBVm01, DyBVm02, DyBVm03, and DyBVm07), orange-winged (DyGVm01, DyGVm03, and DyGVm04), and wholly black winged (DyHXml01), *c.f. D. basitincta, D. gloriosa* and *D. haomiao* respectively.

The target genes of all samples were amplified and sequenced successfully. The final datasets of each individual gene and combined gene (COI 578 bp, 16S 426 bp, and 28S 1390 bp) consisted of 37 sequences respectively (Table 1). Results of phylogenetic analysis using NJ, BI and ML methods on the basis of individual gene and combined gene datasets are consistent (Fig. 3). *Dysphaea* is strongly supported as a monophyletic group (Bayesian posterior probability, BPP = 1; ML bootstrap value, MLB = 100). All *Dysphaea* sp. samples from central Vietnam, no matter variations on wing color or different species (i.e. originally identified by us as *D. basitincta* or *D. gloriosa*), together with the Hainan samples of *D. basitincta* from Hämäläinen *et al*. (2015) formed one distinct clade with strong support (BPP = 0.95; MLB = 100). Specimen from Guizhou (i.e. identified as *D. haomiao*) is closely related to the sample of *D. gloriosa* from Hämäläinen *et al*. (2015) with strong support (BPP = 1; MLB = 99). Furthermore, the two clades, i.e. (central Vietnam samples + *D. basitincta*) and (*D. haomiao* + *D. gloriosa*), are strongly (BPP = 1; MLB = 99) supported as sister groups (Fig. 3).

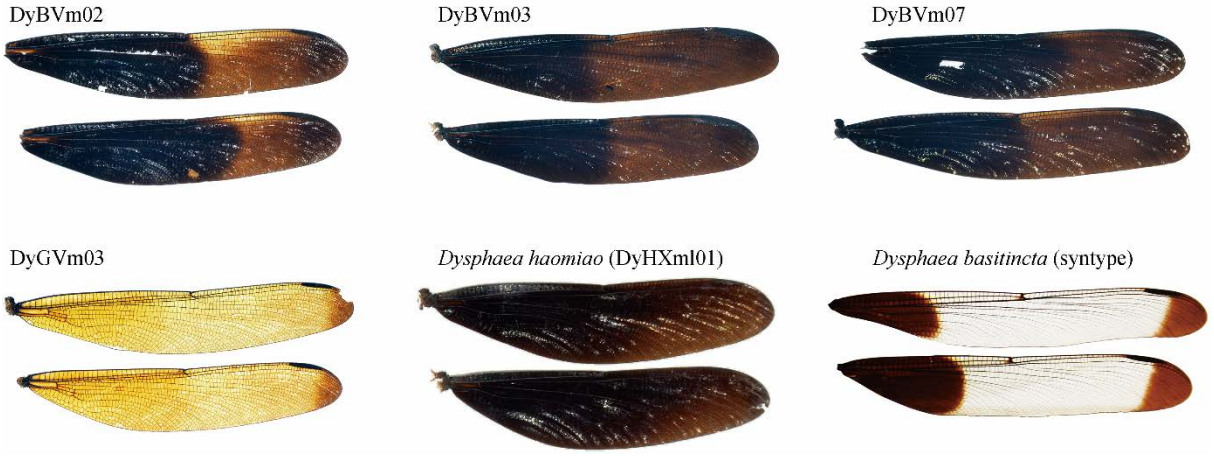

Figure 2. Photos of the right pair of wings of some male specimens showing the variations of wing color patterns. The wings of the syntype of *D. basitincta* (at MNHN, Paris) was kindly provided by Matti Hämäläinen.

4 Discussion

The topology of the phylogenetic tree implies an inconceivable result that all the specimens from central Vietnam, no matter dark-winged (DyBVm01, DyBVm02, DyBVm03, and DyBVm07 in Fig. 1) or orange-winged (DyGVm01, DyGVm03, and DyGVm04 in Fig. 1), together with the two Hainanese specimens identified as *D. basitincta* in Hämäläinen *et al*. (2015), are totally mixed up with strong support, which indicated that all of them belong to one single clade. Therefore, in molecular terms all these specimens should be *D. basitincta*, if the Hainanese specimens really are conspecific with the topotypical *D. basitincta* from northern Vietnam (type locality in Lang Son Province). However, so far, no topotypical north Vietnamese specimens of *D. basitincta* have been analyzed molecularly.

Unexpectedly, the wholly black-winged *D. haomiao* (Fig. 2) rather than those orange-winged central Vietnamese specimens is closely related to *D. gloriosa* which usually has orange color wings. However, it remains to be studied whether the Hainanese specimens identified as *D. gloriosa* in Hämäläinen *et al*. (2015) are conspecific with the topotypical *D. gloriosa* from southwestern Thailand (type locality in Prachuap Khiri Khan Province). Since only one sample each for both *D. haomiao* and *D. gloriosa* involved in our analysis, it is hard to draw a further conclusion presently.

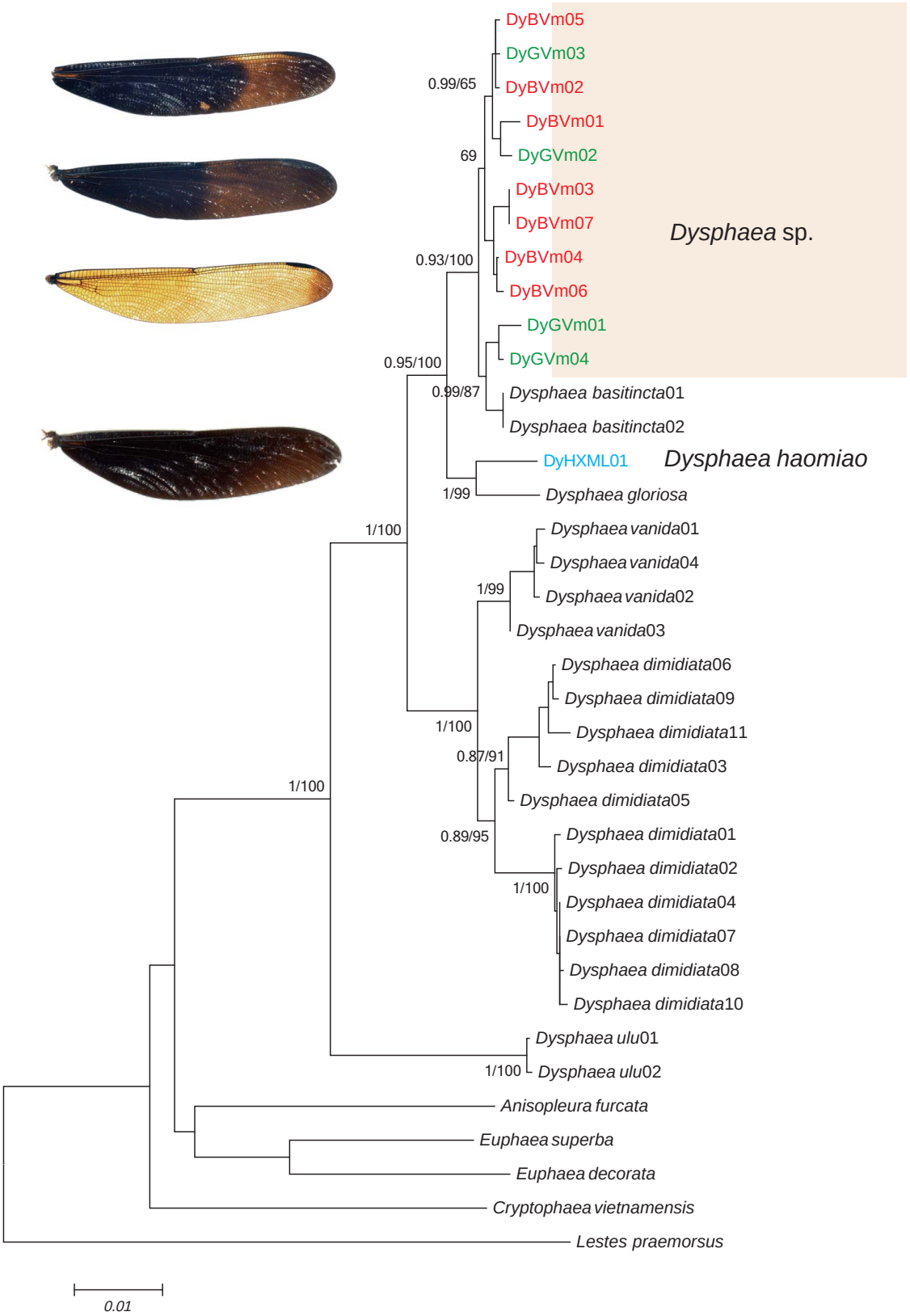

Figure 3. Phylogenetic reconstruction of 37 samples based on combined gene dataset (COI+16S+28S, 2394 bp). Bayesian posterior probabilities (left) and ML bootstrap value (right) are indicated at nodes. Among the central Vietnamese specimens of *Dysphaea* sp. the ID numbers in red color indicate those specimens with dark colour which were originally identified by us as *D. basitincta*. Those with ID in green indicate specimens originally identified by us as *D. gloriosa*. The blue ID refers to *D. haomiao*.

The color pattern on the wings is an important diagnostic characters for species of *Dysphaea* (Hämäläinen *et al*., 2015). Male specimens from central Vietnam showed large variation on the wing color pattern when the wings folding at rest just like our specimen photos demonstrate (Fig. 1). In fact, in the field, this variation is even larger than our limited samples have showed. That is why we first thought that there are three species in central Vietnam. The real reason of this variation is still unknown. However, we speculate that it is not the similar case in *Mnais* (Tsubaki, 2003) or any possible reasons mentioned in (van Gossum *et al*., 2008). It really deserves a further study on this topic.

We believe that the field observations of *D. haomiao* from Quang Binh by Tom Kompier (in Phan *et al*., 2018) represent the same species to ours, similarly as the record of *D. haomiao* from Quang Binh in Hämäläinen (2012), which was based on field photographs by Philip Steinhoff. When the wings are spread like Figure 2 showing, we can tell that all our male specimens from Vietnam have one common character, i.e. the relative dense color at wing base always extends to the level about node and sometimes even a little further (including those orange-winged samples like DyGVm03 (Fig. 2), of which the relative basal opaque area still can be recognized). However, in all other known *D. basitincta* populations in Vietnam and China, the wing pattern is quite uniform on that the basal opaque area is never extend to the node but more short, usually half the distance, and also the wings look proportionally narrower (Fig. 2), which seems different from our specimens (Hämäläinen *pers. comm*).

The present study, although it will not provide any final definite taxonomic conclusions, has detected a meaningful fact that the specimens from central Vietnam obviously belong to one species with different color patterns. They are very close to the expected *D. basitincta* from Hainan, if not the same species. However, in order to decide whether the specimens from central Vietnam represent an already known species, a new species, or even a possible hybrid of *D. basitincta* and *D. gloriosa*, more samples must be included in the further work, both for molecular and morphological. The future samples should include enough representatives of *D. basitincta*, *D. gloriosa* and *D. haomiao*, from their whole ranges. Also more samples from the Hainan populations are needed (all the three specimens of *D. basitincta* and *D. gloriosa* used in Hämäläinen *et al*. (2015) and in the present work come from Hainan).

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