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A pilot study on the molecular phylogeny of Drepanoidea (Insecta: Lepidoptera) inferred from the nuclear gene EF-1α and the mitochondrial gene COI

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Abstract

A molecular phylogenetic study of the Drepanoidea based on the EF-1α sequences and combined EF-1α and COI sequences was carried out in order to infer higher classification at and above the subfamily level. The sample contained 14 taxa representing 13 genera recognized in the Drepanoidea. The results revealed that the Drepaninae, Thyatirinae and Cyclidiinae respectively form monophyletic groups. The sister relationship between the Drepaninae and the Thyatirinae was validated. The monophyly of the Cyclidiinae with the Drepaninae + Thyatirinae was supported robustly. *Hypsomadius insignis* and *Oreta vatama* within the traditional definition of the Drepaninae formed an individual clade with robust support (100%) and constitutes a sister relationship to a clade containing the rest of the Drepaninae in all the topologies, which means that the subfamily Oretinae of the Drepanidae should be restored. The family Drepanidae is divided into four subfamilies: Drepaninae, Oretinae, Thyatirinae and Cyclidiinae in this work. The family Epicopeiidae formed a monophyly with high bootstrap values. The result of combined analysis of EF-1α and COI showed that the Epicopeiidae have a closer phylogenetic relationship with the Geometridae than with the Drepanidae and belong to neither the Drepanoidea nor the Geometroidea.

Keywords: Drepanoidea, molecular phylogeny, subfamily

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Introduction

The superfamily Drepanoidea is currently composed of two families, the Epicopeiidae and the Drepanidae (Minet, 1991; Minet & Scoble, 1999). The family Drepanidae is divided into three subfamilies: the Drepaninae, Thyatirinae and Cyclidiinae. The family Epicopeiidae is not divided into subfamilies owing to the relative homogeneity of the limited number of genera. The members of the Drepanoidea were once placed in the Geometroidea in the early years because most of them have abdominal tympanal organs (Imms, 1934). Since that time, different authors have had different viewpoints about the content of the drepanoids. Inoue (1954) recorded them as including the Drepanidae, Thyatiridae and Callidulidae, mainly following McDunnough (1938). Nakamura (1981) considered that the superfamily Drepanoidea consists of the Drepanidae, Thyatiridae, Cyclidiidae and Epicopeiidae. Minet (1983) considered the superfamily as only including the Drepanidae, based on the study of the tympanal organs, and regarded the Thyatiridae and Cyclidiidae as two subfamilies of the Drepanidae. The same author (Minet, 1991) assigned the Epicopeiidae, unplaced by him in 1983, to the Drepanoidea, mainly based on the Epicopeiidae

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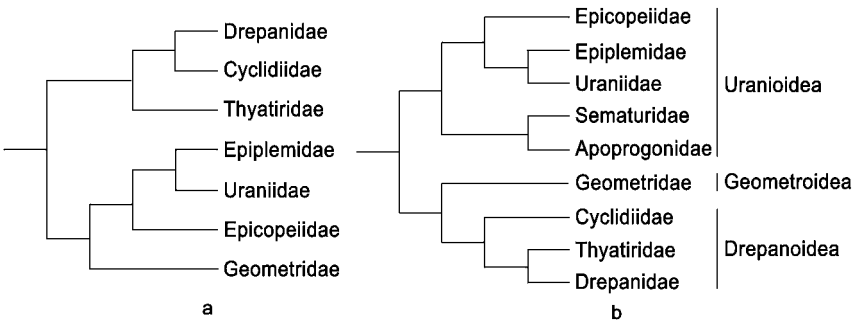

Fig. 1. Phylogeny trees (a) Imms' Geometroidea (Zhu & Wang, 1991); (b) Uranioidea, Geometroidea and Drepanoidea (Kuznetzov & Stekolnikov, 2001).

and Drepanidae sharing four apomorphies: setae of the larval mandible inserted on a large, flat, lateral area delimited ventrally by a projecting line; at least one secondary seta associated with L3 on segments A1-8 of the larva; the femur of the pupal foreleg concealed or very slightly exposed; and adult abdomen with prespiracular sclerite laterally, connecting the first tergite with the first sternite, and modified into tympanal organs in the Drepanidae. Scoble (1992) still doubted the taxonomic position of the Epicopeiidae and tentatively placed them in the Uranioidea. However, he added that there was no similarity with other uraniid groups because the Epicopeiidae lacked tympanal organs; and he concluded that the treatment of Minet (1991), placing the Epicopeiidae in the Drepanoidea, might be more appropriate. Subsequently, the definition of Drepanoidea in Minet (1991) has been widely accepted and used (Holloway, 1998; Minet & Scoble, 1999; Holloway *et al*., 2001; Kristensen *et al*., 2007). But Minet & Scoble (1999) also included some epicopeiid characters which are different from the Drepanidae when they summarized the characteristics of the group, e.g. the abdomen lacking tympanal organs; the tongue being well developed; frenulum and subcostal retinaculum usually present in males, female frenulum absent or strongly reduced; M_2 rarely arising nearer M_3 than M_1 in both forewing and hindwing; $Sc+R_1$ close to Rs in the base of cell and far away beyond cell in hindwing.

Some other researchers did not support the viewpoint of placing the Epicopeiidae in the Drepanoidea. In Imms' family system, the family Epicopeiidae was always a part of the Uraniidae, although he added that, "The Asiatic genus *Epicopeia* has a vestigial frenulum and is often relegated to a separate family—the Epicopeiidae" (Imms, 1934). Inoue (1954) listed the Epicopeiidae under the superfamily Uranioidea. Zhu & Wang (1991) performed a phylogenetic analysis at family level on the Geometroidea based on morphologic characters in the same year in which Minet defined the Drepanoidea. The following characters were used in their analysis: antenna filiform or bipectinate; apex of forewing falcate or not; forewing R_5 or R_{4+5} connected or stalked with R_{2+3} or far apart; hindwing $Sc+R_1$ close to Rs or far apart beyond cell; the base of $Sc+R_1$ in hindwing forked or not; one or two A veins present in hindwing; M_2 located in the middle of M_1 and M_3 or other ways in both wings; frenulum present or not; a pair of hair clusters in the second abdomen segment present or not; abdominal tympanal organs located on the dorsal or ventral side. The analysis results showed (fig. 1a) that the Epicopeiidae formed a sister group with the Epiplemidae + Uraniidae, and the relationship between the Epicopeiidae and the Geometridae was closer than that between the Epicopeiidae and the Thyatiridae + (Drepanidae + Cyclidiidae). Kuznetzov & Stekolnikov (2001) also performed an analysis based on morphological characters and obtained almost the same result (fig. 1b) as those of Zhu & Wang (1991), namely that the Epicopeiidae and Uraniidae + Epiplemidae formed a sister group. The author considered this group as a part of the Uranioidea and, furthermore, that the Uranioidea and Geometroidea + Drepanoidea were a sister group. The result shows that the relationship between the Epicopeiidae and Drepanidae is not closer than that between the Geometridae and Drepanidae. Therefore, it is clear that the monophyly of the Drepanoidea needs to be confirmed and that the taxonomic status of the Epicopeiidae should be reconsidered and further validated.

The phylogenetic analysis results of Zhu & Wang (1991) (fig. 1a) showed that the Drepanidae and Cyclidiidae formed a sister group, and formed a monophyly together with the Thyatiridae. But Minet (2002) considered that the first dichotomy was likely to lie between the Cyclidiinae and the Thyatirinae + Drepaninae within the Drepanidae. His viewpoint is supported by the following three apomorphies: a male frenulum with clubbed apex (e.g. Scoble & Edwards, 1988: fig. 17), a small tympanal chamber provided with a fairly broad dorsal sclerotized wall (e.g. Gohrbandt, 1937: fig. 14), and a large tympanal chamber that is distinctly fused with sternum A2 mesad of the apodemal protrusion (e.g. Minet, 1983: fig. 95). Unlike that of most Thyatirinae and Drepaninae, the small tympanal chamber of the Cyclidiinae has a dorsal sclerotized wall, which varies from extremely narrow to entirely absent.

Different authors divided the Drepaninae into different subgroups based on a set of characters: adult body colour, tongue and frenulum, forewing colour and shape (falcate or not), hind tibial spurs, larval secondary setae and supracoxal vesicle. Many taxonomists considered that the Drepanidae (present Drepaninae) should be divided into two subfamilies: the Drepaninae and Oretinae (Inoue, 1962; Nakajima, 1970; Wilkinson, 1972; Zhu & Wang, 1991; Smetacek, 2002). However, other authors considered that the Drepaninae should be divided into two subgroups rather than subfamilies (e.g. Watson, 1965, 1967; Minet, 1985; Scoble, 1992). Holloway (1998) considered that the subgroups of Drepaninae did not reach the subfamily level and placed them at tribal level: Oretini and Drepanini. Minet & Scoble (1999)

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further divided the Drepaninae into three tribes: Nidarini, Oretini and Drepanini.

Therefore, it is necessary to further investigate the monophyly of each subfamily within the Drepanidae and the relationships between the different subfamilies, especially the taxonomic status of the *Oreta* group.

Currently, many genes are available for phylogenetic analysis. Because the cytochrome oxidase I (COI), 16S rRNA, 18S rRNA and elongation factor-1α (EF-1α) genes have been widely used and are informative across a broad range of divergences. Caterino *et al*. (2000) advocate their use as standards for insect molecular phylogenetics. Since the amino acid sequences of EF-1α are highly conserved, nonsynonymous changes are rare, especially in the Lepidoptera, which have lost all introns and only have a single copy of the gene. These properties render the gene a useful marker for resolving the phylogenetic relationships of the higher classification of insects (Friedlander *et al*., 1992, 1994; Brower & DeSalle, 1994; Cho *et al*., 1995; Belshaw & Quicke, 1997; Mitchell *et al*., 1997; Danforth & Shuqing, 1998). In the Lepidoptera, the EF-1α gene should give phylogenetic information and has been proved useful in reconstructing phylogenies at subfamily or lower levels, such as generic and tribal levels (Cho *et al*., 1995; Mitchell *et al*., 1997; Friedlander *et al*., 1998; Reed & Sperling, 1999; Mitchell *et al*., 2000; Caterino *et al*., 2001; Monteiro & Pierce, 2001; Morinaka *et al*., 2002; Wahlberg & Nylin, 2003; Braby *et al*., 2006).

COI is a widely used mitochondrial protein-encoding gene. In molecular phylogenetic studies on the Lepidoptera, the gene has shown great utility for resolving the phylogenetic relationships within closely related groups (Caterino *et al*., 2000; Sperling, 2003). Different authors have different ideas about whether the saturated third codon positions should be removed when using COI to perform phylogenetic analysis (Gleeson *et al*., 1998; Söller *et al*., 2001; Wares, 2001; Ros & Breeuwer, 2007; Zhang *et al*., 2007; Ketmaier *et al*., 2008).

Because the two genes evolve at different rates, combining both genes will probably increase the reliability of phylogenetic analysis results. Furthermore, it may provide consistent information on nodes (Caterino *et al*., 2000). In the Lepidoptera, several recent studies have demonstrated improved resolution of nodal support at both higher and intermediate systematic categories of divergence in a combined analysis of nuclear and mitochondrial genes (Caterino *et al*., 2001; Monteiro & Pierce, 2001; Wahlberg & Nylin, 2003; Kandul *et al*., 2004; Zakharov *et al*., 2004).

The purpose of this study is to reconstruct the phylogeny of the Drepanoidea based on the analysis of EF-1α sequences and combined EF-1α and COI sequences. It also investigates the taxonomic system of higher categories above the subfamily level and the phylogenetic relationships of different subfamilies. It proved that the Oretinae should be restored, that the sister relationship between the Thyatirinae and Drepaninae + Oretinae was well formed and that the Epicopeiidae did not belong to the Drepanoidea.

Materials and methods

Taxa examined

The collection localities of the material examined in this study and GenBank Accession numbers of all sequences are given in table 1. For the phylogenetic analysis of the Drepanoidea, the EF-1α and COI sequences of 14 taxa belonging to two families, three subfamilies and 13 genera were obtained and used as ingroups. Two sequences were obtained from GenBank based on the published work of Yamamoto & Sota (2007) and Cho *et al*. (2008) (table 1). Two representatives of the Geometridae, which is regarded as the sister group of the Drepanidae based on morphology (Minet, 1983; Zhu & Wang, 1991; Minet & Scoble, 1999; Xue & Zhu, 1999; Young, 2006; Kristensen *et al*., 2007) and molecular phylogenetics (Abraham *et al*., 2001), and two representatives of the Noctuidae, were used as outgroups.

Molecular techniques

The following protocol was adopted to obtain DNA sequences EF-1α and COI.

Specimen preparation

Fresh adult specimens were collected by using light traps and killed in cyanide bottles. Wings were immediately excised and stored in paper envelopes as vouchers for identification, and the bodies or only three legs on the same side were preserved in 100% ethylalcohol. The specimens were stored at −20°C for laboratory use. A few of the specimens were collected and preserved as dried adults. All DNA samples and voucher specimens were deposited in the Zoological Museum, the Institute of Zoology, Chinese Academy of Sciences, Beijing, China.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from single moth thorax or legs by using QIAgen's DNEasy extraction kit according to the manufacturer's protocols and with some slight improvements. Abdomens and wings were conserved in microtubes and paper envelopes, respectively, as vouchers and for confirmation of specimen identification by dissection of the genitalia.

The nuclear gene EF-1α and mitochondrial gene COI were amplified by polymerase chain reaction (PCR) using published primers. The primer sequences were as follows: EF1aF2 (sense) (5′-ACAAATGCGGTGGTATCGACAA-3′) and EF1aR (antisense) (5′-GATTTACCRGWACGACGRTC-3′) (see Yamamoto & Sato, 2007; Kawakita *et al*., 2004) for EF-1α; LCO1490 (sense) (5′-GGTCAACAAATCATAAAG-ATATTGG-3′) and HCO2198 (antisense) (5′-TAAACTTCA-GGGTGACCAAAAAATCA-3′) (see Folmer *et al*., 1994) for COI. PCR reactions were performed in a 50 μl volume, containing 10 μl 5 × PrimeSTAR™ buffer (5 mM of $MgCl_2$); 4 μl dNTP mixture (each of 2.5 mM); 0.5 μl PrimeSTAR™ HS DNA polymerase (2.5 U μl^{-1}), 2 μl of each primer (10 pM); 3 μl DNA template and 28.5 μl distilled water up to 50 μl. The reactions were done on a GeneAmp PCR System 9700 (Applied Biosystem, USA) with the following conditions for EF-1α: 95°C for 2 min, 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min and a final extension period of 72°C for 10 min. The reaction cycle profile of COI PCR amplification was 95°C for 2 min, 30 cycles of 95°C for 30 s, 53°C for 30 s, 72°C for 1 min, and a final extension period of 72°C for 10 min. These two protocols and the two pairs of primers worked well for all examined species. Each PCR product was subsequently gel purified using the AxyPrep™ DNA Gel Extraction Kit (Axygen).

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Table 1. Species information and GenBank accession numbers.

Species and subspecies	Collection locality	Collection date	GenBank accession no: EF-1α/COI
Outgroup			
Geometridae			
Ennominae			
Odontopera bilinearia coryphodes	Yunnan (Baoshan, Bawan)	2007-VIII-8–10	FJ768768/FJ768752
Geometrinae			
Tanaorhinus viridiluteata	Hainan (Lingshui, Diaoluoshan)	2007-V-2	FJ768769/FJ768753
Noctuidae			
Helicoverpa armigera	Beijing	2006-IX-12	FJ768770/EU768941
Catocala fraxini	Beijing (Mentougou, Liyuanling)	2008-IX-27	FJ768771/FJ768754
Ingroup			
Epicopeiidae			
Epicopeia hainesi	Henan (Neixiang, Baotianman)	2008-VIII-12	FJ768755/FJ768738
Psychostrophia nymphidiaria	Hunan (Mangshan, Xiaotiantai)	2008-VII-15	FJ768756/FJ768739
Drepanidae			
Cyclidiinae			
Cyclidia substigmaria	Yunnan (Baoshan, Bawan)	2007-VIII-8–10	FJ768760/FJ768743
Drepaninae			
Callidrepana patrana	Henan (Neixiang, Baotianman)	2008-VIII-10	FJ768767/FJ768751
Ditrigona conflexaria	Henan (Neixiang, Baotianman)	2008-VIII-11	FJ768766/FJ768750
Hypsomadius insignis	Hainan (Lingshui, Diaoluoshan)	2008-III-31	FJ768761/FJ768745
Macrocilix maia	Hainan (Wuzhishan, Shuiman)	2008-IV-1	FJ768765/FJ768749
Macrocilix mysticata	Zhejiang (Lin'an, Tianmushan)	2003-VII-28–29	AB265512/FJ768744
Microblepsis leucosticta	Hainan (Ledong, Jianfengling)	2008-III-25	FJ768764/FJ768748
Oreta vatama	Yunnan (Tengchong, Dahaoping)	2007-VIII-5–7	FJ768762/FJ768746
Tridrepana fulvata	Hainan (Wenchang, Yunlongwan)	2008-III-17	FJ768763/FJ768747
Thyatirinae			
Gaurena fletcheri	Yunnan (Tengchong, Dahaoping)	2007-VIII-5–7	FJ768759/FJ768742
Habrosyne conscripta	Henan (Songxian, Baiyunshan)	2008-VIII-14	FJ768757/FJ768740
Parapsestis lichenea	Tibet (Médog)	2006-VIII-21	FJ768758/FJ768741

Sequencing reactions were performed with the corresponding amplifying primers from both directions and run with ABI 3730 automated sequencer (Applied Biosystem, USA).

Assembling and alignment of sequences

Chromatograms, including sense and antisense, were edited and assembled using DNASTAR 5.0 (DNASTAR, Madison, Wisconsin, USA, Inc.) to obtain single consensus sequences. The nucleotide sequences were translated into amino acid sequences to check for the presence of stop codons that might indicate that pseudogenes had been amplified (Sanders *et al*., 2006). Multiple alignments were done with Clustal version 1.81 (Thompson *et al*., 1997) with default parameter settings and verified by eye. For EF-1α, the consensus sequence of each sample was aligned against the published sequence for *Bombyx mori* (Kamie *et al*., 1993) and primer ends were removed, resulting in 960 bp (corresponding to positions 187–1146). For COI, the consensus sequence of each sample was aligned against the published sequence for *Drosophila yakuba* (Clary & Wolstenholme, 1985) and/or other Lepidoptera sequences on GenBank; the final fragment was 617 bp (corresponding to positions 1556–2172 of *Drosophila*).

Aligned sequence data were imported into MEGA 3.1 (Kumar *et al*., 2004) for analyses of nucleotide composition. Nucleotide saturation was analyzed by plotting the number of transitions and transversions on each codon position against the Tamura & Nei (1993) (TN93) genetic distance using DAMBE (Xia & Xie, 2001). Saturation was considered to have occurred if the scatter of points showed leveling off mutations as sequence divergence increased.

Phylogenetic analysis

Simultaneous analyses of Nuclear EF-1α gene and combined data of EF-1α and mitochondrial COI gene were attempted because phylogenetic resolution from an individual gene was obviously limited. In the search for optimal trees, maximum parsimony (MP), Bayesian and maximum likelihood (ML) analyses for each of the data sets were used. All phylogenetic analyses were performed with PAUP*4.0b10 (Swofford, 2003) and MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003).

A maximum parsimony analysis was carried out first, with all sites weighted equally, using 1000 random additions of sequences and tree bisection reconnection (TBR) branch swapping. The command of 'contree' was used to yield the strict consensus tree. To assess the support for branching events, non-parametric bootstrapping was performed with 1000 pseudo-replicates under the heuristic search strategy and 100 random addition sequences in each pseudo-replicate. A node was interpreted as strongly supported if the bootstrap percentage (BP) was $\geqslant 70\%$ (Hillis & Bull, 1993).

Bayesian analysis was conducted using MrBayes 3.1.2, based on the model selected by ModelTest 3.7 (Posada &

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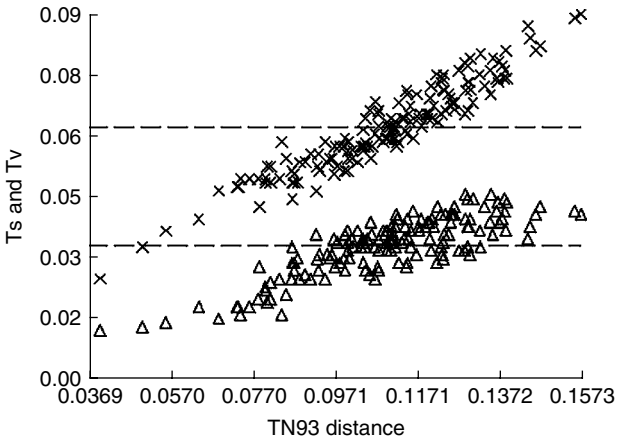

Fig. 2. Saturation plots of Nuclear gene EF-1α. The number of transitions and transversions of each pairwise comparison of taxa are plotted against the TN93 model; corrected distance and the broken lines show the mean value of transition and transversion, respectively (×, Ts; Δ, Tv).

Crandall, 1998). Model parameter values were treated as unknown variables with uniform prior probabilities and were estimated during the analysis. Four chains (three heated and one cold) were run, starting from a random tree and proceeding for 1,000,000 Markov chain Monte Carlo generations, sampling the chains every 100 generations. Two independent runs were conducted to verify the results. For all runs, 1000 trees were discarded as burn-in samples. Remaining trees were used to generate a majority-rule consensus tree, in which the percentage of trees recovering a clade portrayed the clade's posterior probability (PP) (Huelsenbeck *et al*., 2001) or the probability that the clade is correct in the given data and model parameters. The combined data set was treated as two partitions with different models accounted for their heterogeneity. The prior models of sequence evolution employed for both COI and EF-1α data sets were also determined using Modeltest 3.7 (Posada & Crandall, 1998) based on the likelihood ratio tests. The test indicated that GTR+I+G model was the most appropriate model for both of the two data sets. The 'unlink' command was utilized to unlink the following parameters: 'unlink shape=(all) pinvar=(all) statefreq=(all) revmat=(all)'. Probabilities of 95% or more were considered to indicate significant support (Reeder, 2003; Zakharov *et al*., 2004).

The best-fit nucleotide substitution model used in maximum likelihood analysis was selected by using ModelTest 3.7 based on the Akaike Information Criterion (AIC). Maximum likelihood analysis was performed in PAUP* with the selected optional model under the heuristic search strategy with 100 random additions of sequences and TBR branch swapping. Bootstrap analysis was performed under the same model, with 100 pseudo-replicates, ten random additions of sequences per replicate and TBR branch swapping.

Results

Sequences characteristics and saturation analysis

For all the taxa, including outgroups and download sequence directly from GenBank (accession no. AB265512), approximately 960 bp were sequenced for EF-1α. Because transitions and transversions in the nuclear EF-1α were accumulated linearly and showed no saturation patterns at any position (fig. 2), all nucleotide positions were employed in the subsequent analysis. Of a total of 960 characters, 702 sites were conserved, 258 variable and 203 parsimony-informative (717 sites were constant, 243 variable and 185 parsimony-informative for the ingroup only), and average base frequencies were well proportioned with 21.1% T, 28.2% C, 24.7% A and 26.0% G. Nucleotide frequencies average Ti/Tv ratio = 1.9.

As for COI, 14 ingroup taxa were sequenced, and one outgroup sequence of *Helicoverpa armigera* was downloaded directly from GenBank (accession no. EU768941). Because the third codon positions of mitochondrial gene COI exhibited a tendency towards saturation, individual analysis was not performed based on this gene. A sequence of 617 bp for phylogenetic analysis was acquired, with 412 sites conserved, 205 variable and 160 parsimony-informative (for ingroup taxa only, 424 sites conserved, 193 variable and 140 parsimony-informative). These sequences were heavily biased toward A and T nucleotides, as expected from previous studies (Simon *et al*., 1994; Lunt *et al*., 1996). Base-composition averages were 38.6% T, 15.8% C, 31.4% A and 14.2% G. Nucleotide frequencies average Ti/Tv ratio = 0.8.

For combined data of EF-1α and COI sequences, altogether 18 taxa were included. The combined data matrix comprises 1577 characters, and all nucleotide positions were employed in the subsequent analysis. Of the total characters, variable sites accounted for about 29.4%, nucleotide substitution mainly with transition, transition/transversion ratios = 1.3. The average distance was 11.5% in all sequences. Within the ingroup, the minimum distance was 5.8% (between *Hypsomadius insignis* and *Oreta vatama*), and the maximum distance was 14.5% (between *Cyclidia substigmaria* and *Callidrepana patrana*).

Table 2. Parameters for ML and MP analysis.

Data set	EF-1α	EF-1α+COI
Base frequencies (A, C, G, T)	0.2477, 0.2980, 0.2379, 0.2164	0.2776, 0.2364, 0.1941, 0.2920
Best fit model	GTR+I+G	GTR+I+G
I	0.6790	0.6310
G	1.2882	1.0904
−lnL (ML tree fit)	5013.4868	8834.1787
Mp tree length (number of steps)	862	1571
CI	0.426	0.412
RI	0.404	0.344

I, proportion of invariable sites; G, gamma distribution shape parameter; CI, consistency index; RI, retention index.

Phylogenetic analysis

MP, ML and Bayesian analysis were performed on both the EF-1α gene data set and the combined data set of EF-1α and COI using PAUP* and Mrbayes. The MP, ML and Bayesian trees were obtained, but only the Bayesian trees are shown below. The selected optimal models for each data set and the corresponding parameters for ML and MP analysis are listed in table 2. Topologies of these trees are very similar

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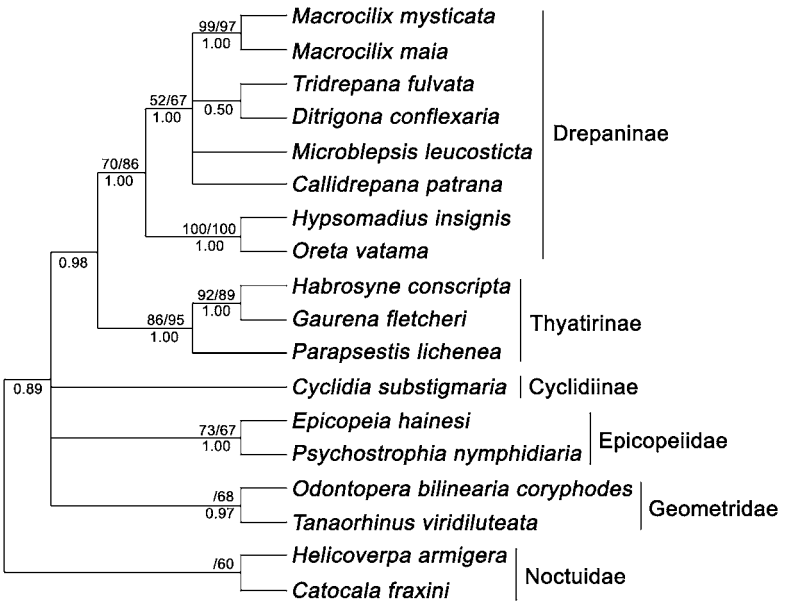

Fig. 3. Bayesian tree of Drepanoidea reconstructed from nuclear EF-1α sequences. Bootstrap percentages of consensus clades with Bayesian tree from maximum parsimony/maximum likelihood (50% and greater) are shown above the branches, and Bayesian posterior probabilities (50% and greater) are shown below the branches.

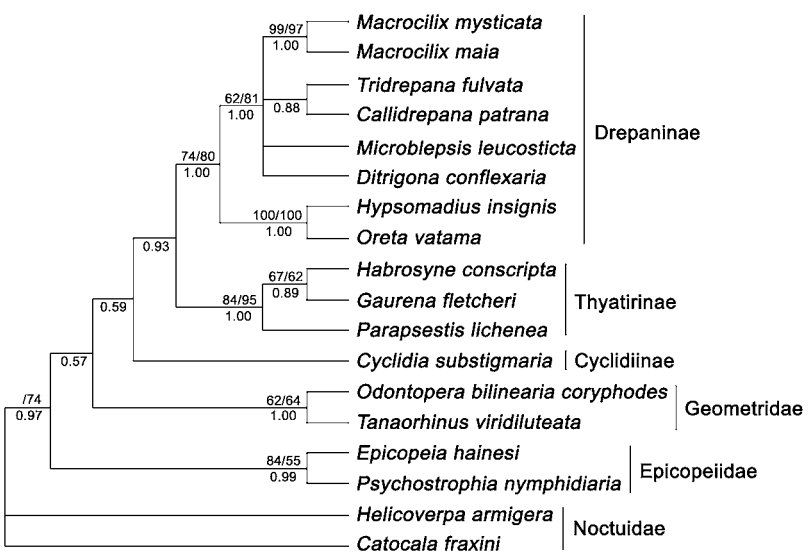

Fig. 4. Bayesian tree of Drepanoidea reconstructed from nuclear EF-1α and mitochondrial COI sequences. Bootstrap percentages of consensus clades with Bayesian tree from maximum parsimony/maximum likelihood (50% and greater) are shown above the branches, and Bayesian posterior probabilities (50% and greater) are shown below the branches.

(figs 3 and 4). In the defined outgroup taxa, only two taxa, *Helicoverpa armigera* and *Catocala fraxini*, in the Noctuidae are at the base of the trees, whereas another two defined outgroup taxa, *Odontopera bilinearia coryphodes* and *Tanaorhinus viridiluteata*, in the Geometridae cluster together with defined ingroup taxa and form a monophyly with robust support (97% support value in combined Bayesian trees, 89% in EF-1α Bayesian trees).

Nuclear EF-1α gene analysis

MP, ML and Bayesian analysis were performed on the EF-1α gene data sets. Topologies of these trees (fig. 3; Bayesian tree, single tree of MP and ML not shown, only the bootstrap percentages of consensus clades shown with Bayesian tree) support the monophyletic groupings of the Drepaninae and Thyatirinae respectively with high bootstrap

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values (70%, 86% support value in MP tree, 86%, 95% support value in ML tree, both clades with 100% support value in Bayesian tree). Drepaninae and Thyatirinae are sister taxa with a high bootstrap value (98%) in the Bayesian tree. The Cyclidiinae also constitute a monophyly. The Epicopeiidae constitute a monophyly with high bootstrap values (73% support value in MP tree, 67% support value in ML tree, 100% support value in Bayesian tree). *Hypsomadius insignis* and *Oreta vatama* in the Drepaninae form a monophyly with robust support (100%) in three topologies. The relationship among Cyclidiinae, Epicopeiidae, Geometridae and Drepaninae + Thyatirinae is not certain in nuclear EF-1α gene analysis.

Combined analysis of EF-1α and COI

The partition-homogeneity test (Farris *et al*., 1995) revealed significant heterogeneity between EF-1α and COI ($P = 0.01$). The combined data of EF-1α and COI was used to perform MP, ML and Bayesian analysis in the following analysis. In MP, ML and Bayesian trees resulting from combined gene data of EF-1α with COI, topologies of these trees (fig. 4; Bayesian tree, single tree of MP and ML not shown, with only bootstrap percentages of consensus clades shown with Bayesian tree) all support both the Drepaninae and Thyatirinae as a monophyletic group respectively with high bootstrap values (74%, 84% support value in MP tree, 80%, 95% support value in ML tree, both clades with 100% support value in Bayesian tree). The Drepaninae and Thyatirinae emerge as sister taxa with high bootstrap value (93%) in the Bayesian tree. The Cyclidiinae with Drepaninae + Thyatirinae form a monophyly with 59% bootstrap value in the Bayesian tree. *Hypsomadius insignis* and *Oreta vatama* in the Drepaninae form a monophyly with robust support (100%) in all the topologies. The Epicopeiidae also form a monophyly with high bootstrap values (84% support value in MP tree, 55% support value in ML tree, 99% support value in Bayesian tree). The relationship between the Epicopeiidae and Geometridae is closer than that between the Epicopeiidae and Cyclidiinae + (Drepaninae + Thyatirinae) from combined analysis of EF-1α and COI genes.

Discussion

Subfamilies of the Drepanidae and their relationship

The Drepanidae have been defined on the basis that the three currently recognized subfamilies (Drepaninae, Thyatirinae and Cyclidiinae) share a distinctive synapomorphy in the adult, namely the possession of abdominal tympanal organs associated with the tergosternal sclerites, which connect tergum 1 with sternum 2 (Minet, 1991; Minet & Scoble, 1999). This study investigated the phylogenetic relationship among the subfamilies of the Drepanidae by using molecular data from EF-1α and COI. The results showed that the monophyly of the Drepaninae, Thyatirinae and Cyclidiinae respectively was well supported (figs 3 and 4), and the sister relationship between the Drepaninae and Thyatirinae of Minet (2002) was validated. It did not support the sister relationship between the Drepanidae (= Drepaninae) and Cyclidiidae (= Cyclidiinae) postulated by Zhu & Wang (1991).

Hypsomadius insignis and *Oreta vatama* in the *Oreta* group in the traditional definition of the Drepaninae form an independent clade with robust support (100%) in all the topologies. Additional evidence from morphology are: both body and wings coloured brown; body stout; tongue undeveloped; labial palpus short, broad and with dense hair, only reaching the underside of face; frenulum undeveloped; hind tibia with only one pair of spurs which have the same length; uncus of male genitalia flat and broad, turtle-head like, socii absent; tergum of metathorax in larva extended and with spinose process; fourth segment of abdomen with one pair of processes. While in other traditional Drepaninae, both body and wings are white or yellow in colour; body is slender; tongue is well developed; labial palpus is slender, reaching the lower edge of the face, the third segment is visible; frenulum is well developed; hind tibia has two pairs of spurs; uncus of male genitalia is stick- or fork-like, socii are present; tergum of the metathorax and fourth segment of the abdomen in larva are without processes. Both morphological characters and the molecular phylogenetic evidence strongly support that the *Oreta* group should be separated from the original Drepaninae and constitutes a sister group with it, and that the Oretinae should be restored as a separate subfamily. We propose a revised higher classification of the Drepanidae. In this classification, the Drepanidae should be divided into four subfamilies (Drepaninae, Oretinae, Thyatirinae and Cyclidiinae). The result of restoring the *Oreta* group to Oretinae perhaps has limitation because the taxa selected were available from China. Therefore, the results in the present analysis should need further studies, which will cover more genera in the Drepanidae and use more molecular markers to test further the strength of support for Oretinae and Drepaninae.

The taxonomic status of the Epicopeiidae

The taxonomic position of the Epicopeiidae has long been disputed. Using the traditional morphology division, it was found to belong to the Geometroidea when Zhu & Wang (1991) performed a phylogenetic analysis on the latter group. Minet (1983) ascribed it to the Uranioidea, but later (Minet, 1991) attributed it to the Drepanoidea based on four apomorphies shared between the Epicopeiidae and Drepanidae. However, Kuznetzov & Stekolnikov (2001) still thought the Epicopeiidae belonged to the Uranioidea. In the present work, all available data analysis (figs 3 and 4) supports the monophyly of the Epicopeiidae, while evidence on the relationship between Epicopeiidae, Drepanidae and Geometridae, derived from the combined analysis between the EF-1α and COI genes, showed that the Epicopeiidae and Geometridae have a closer phylogenetic relationship than that between the Epicopeiidae and Drepanidae (fig. 4). The following additional evidence of these relationships derived from morphology are: the absence in the Epicopeiidae of tympanal organs, which are well developed in the Drepanidae and Geometridae; the fact that in the Epicopeiidae the hindwing vein M_2 is closer to M_1 than to M_3, and in the Geometridae M_2, if present, is never closer to M_3 than to M_1, whereas in the Drepanidae M_2 is close to M_3; the fact that in the Epicopeiidae hindwing $Sc + R_1$ is close to Rs in the base of the cell and distant from it beyond the cell, as in the Geometridae, while in the Drepanidae hindwing $Sc + R_1$ is close to Rs at or beyond the end of the cell (Zhu & Wang, 1991; Minet & Scoble, 1999). Putting together the evidence from the morphology and molecular analysis results in the present work, we think that the family Epicopeiidae could

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not belong within the Drepanoidea. This result differs from the provisional phylogenetic hypothesis based on morphological characters alone (Minet, 1991; Minet & Scoble, 1999). The relationship between the Epicopeiidae and Geometridae is closer than that between the Epicopeiidae and Drepanidae and accords with the phylogenetic analysis on the Geometroidea performed by Zhu & Wang (1991) based on morphological characters. However, our results, especially the combined analysis result from the EF-1α and COI genes, did not support the placing of the Epicopeiidae within the Geometroidea. There are perhaps three possible conclusions. Either the selected gene segments in this paper are insufficient to distinguish or reconstruct the relationship between the Epicopeiidae and other groups; or the two species of Geometridae in this study are an insufficiently representative outgroup and do not reflect the range of the Geometridae sufficiently well to express the relationship between the Geometridae and Epicopeiidae; or, finally, the family Epicopeiidae belongs to neither the Drepanoidea nor the Geometroidea and should be placed in another superfamily. It has been ascribed to the Uranioidea many times in the past, which might be a clue to a realistic phylogenetic relationship (Inoue, 1954; Kuznetzov & Stekolnikov, 2001). However, Minet sunk the Uranioidea in 1991 and placed its members in either the Geometroidea or the Drepanoidea. The phylogenetic relationships of the Geometroidea were outside the scope of this paper. Further work needs to be done as Wahlberg & Wheat (2008) proposed, preferably using the other molecular markers and longer gene sequences, to reconstruct the phylogenetics among the Epicopeiidae, Geometridae and Uraniidae and to verify the results inferred in this paper from EF-1α and the combined EF-1α and COI analysis. On the basis of this, it might be possible to establish whether the Uranioidea should be resurrected to contain the Epicopeiidae and part or all of the Uraniidae or, possibly, whether a separate superfamily, the Epicopeiioidea, should be established.

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