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Molecular Variability of the *COI* fragment Supports the Systematic Position of Enarmoniini within the Subfamily Olethreutinae (Lepidoptera: Tortricidae)

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The Tortricidae, a globally distributed family of Lepidoptera, consists of approximately 10000 described species, of which a large number do not have clearly defined taxonomic positions. In the present paper the systematics of Enarmoniini based on molecular data is compared to systematics based on morphology. Two genera of Enarmoniini were used for analysis: the type-genus *Enarmonia* (one species examined) and *Ancylis* (7 species examined). A comparison of a 606 bp homologous fragment of the *COI* mitochondrial gene revealed that Enarmoniini form a cluster distinct from Olethreutini (3 genera and 7 species examined), Eucosmini (2 genera, 4 species) and Grapholitini (4 genera, 9 species). In our opinion the molecular studies combined with previously obtained morphological data should facilitate a more natural classification system of this relatively poorly explored family of Microlepidoptera. Altogether, 30 species of Tortricidae were examined.

Key words: Enarmoniini, Olethreutinae, Tortricidae, Lepidoptera, molecular variability, mitochondrial *COI*.

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The classification of species using genomic information is particularly significant in the case of described taxa in which morphological characteristics do not provide an unambiguous answer. An example of this is the family Tortricidae, Lepidoptera (BROWN 2005), a group consisting of about 10000 described species. A taxonomic system based on morphological characters of members of this family has been improved for more than 150 years, but the inclusion of many genera to tribes is doubtful. Therefore, a comparative analysis of genome fragments provides an opportunity for taxonomic progress and determination of the relationships among various taxa.

Currently, the most popular molecular marker is a part of the mitochondrial *COI* gene which was proposed as a universal DNA barcode (HEBERT *et al*. 2003) and used successfully in butterfly species identification in the *Astraptes fulgerator* species complex (HEBERT *et al*. 2004). Recent studies based on a comparison of the *COI* fragment relate mainly to systematics within the genera of the Tortricidae, such as species relationships (KRUSE & SPERLING 2002; LANDRY *et al*. 1999) or the population structure of Tortricid pests (SCHROEDER & DEGEN 2008; TIMM *et al*. 2010). A preliminary study based on comparative analysis of the *COI* gene fragment resolved uncertain relationships between the tribes Bactrini and Endotheniini (RAZOWSKI & TARCZ 2012) as well as reassessed the systematic position of the Neotropical genus *Orthocomotis* (RAZOWSKI *et al*. 2013).

Enarmoniini is a rather small tribe of Tortricidae in the subfamily Olethreutinae. There was no consensus as to its systematic and phylogenetic position. The present molecular data confirm the recent suppositions by HORAK (2006) and RAZOWSKI (1976a, 2003) that Enarmoniini are more closely related to Olethreutini than to Eucosmini or other tribes of Olethreutinae.

Two genera were examined: *Enarmonia* consists of four Palaearctic species whilst *Ancylis* has a worldwide distribution with approximately 130 representatives. Moreover 20 other genera and about 60 species are known from Australia

(HORAK 2006). Until now, there was no conclusive evidence based on morphological characteristics that allowed classifying Enarmoniini to any of these tribes, or to demonstrate that it is a separate tribe. Although a multilocus attempt at Tortricidae classification has been made (REGIER *et al*. 2012), only one species of the tribe Enarmoniini was analyzed. Furthermore, DNA sequence variability within the studied tribe has not been characterized. The present survey provides molecular evidence of the separate position of Enarmoniini within the subfamily Olethreutinae as well as a preliminary analysis of intra-tribe relationships.

Material and Methods

Material

The specimens were preserved dry. Due to problems associated with obtaining good quality DNA suitable for molecular analysis, *COI* sequences of other species were taken from GenBank. Two representatives of the tribe Polyorthini were used as the outgroup. A list of examined taxa arranged alphabetically is presented in Table 1. No permits were required for collection of these specimens, and no endangered species were used.

Molecular methods

DNA was extracted from two hind legs of dry specimens because we could not completely destroy the museum material using other parts of the bodies (e.g. the entire tagmata). The examined specimens were not older than 10 years and were first identified by comparison of the genitalia. Specimens older than ten years usually gave insufficient results. The best results were obtained from 1-3 year old individuals.

Genomic DNA was isolated without protocol modification using the NucleoSpin Tissue Kit (Macherey-Nagel, Germany). To elute purified DNA we applied 100 μl of elution buffer onto the silica membrane. To amplify a fragment of the mitochondrial *COI* gene (650bp) the following primer pair designed for Lepidoptera was used: LEP-F1, 5'-ATTCAACCAATCATAAAGATAT-3'; and LEP-R1, 5'-TAAACTTCTGGATGTCCAAAAA-3'. These are universal primers used for species identification in DNA barcoding (HEBERT *et al*. 2004). PCR amplification for all analyzed DNA fragments was carried out in a final volume of 40 μl containing 30 ng of DNA, 1.5 U Taq-Polymerase (EURx, Poland), 0.8 μl of 20 μM of each primer, 10 × PCR buffer, and 0.8 μl of 10 mM dNTPs in a Mastercycler ep (Eppendorf, Germany). The amplification protocol was the same as in (HEBERT *et al*. 2004).

In order to assess the quality of the amplification, PCR products were electrophoresed in a 1% agarose gel for 45 min at 85 V with a DNA molecular weight marker (Mass Ruler Low Range DNA Ladder, Thermo Scientific, USA). NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany) was used for purifying PCR products. In some PCR products, additional sub-bands were obtained apart from the main band. In these cases, 30 μl of each PCR product was separated on 1.8% agarose gel (100 V/60 min) with a DNA molecular weight marker (Mass Ruler Low Range DNA Ladder, Live Technologies, USA). Then the band representing the examined fragment was cut out and purified.

Cycle sequencing was done in both directions with the application of BigDye Terminator v3.1 chemistry (Life Technologics, USA). Primers LEP-F1 and LEP-R1 were used for sequencing. Each sequencing reaction was carried out in a final volume of 10 μl containing: 3 μl of template, 1 μl of BigDye Master Mix (1/4 of standard reaction), 1 μl of sequencing buffer, 1 μl of 5 mM primer. Sequencing products were precipitated using Ex Terminator (A&A Biotechnology, Poland) and separated on an ABI PRISM 377 DNA Sequencer (Applied Biosystems, USA). Sequences are available in the GenBank database (for accession numbers see Table 1). As a positive control of PCR and sequencing, a successfully amplified and sequenced *COI* fragment from *Olethreutes subtiliana* (Table 1) from previous analyses was used.

Data analysis

Sequences were examined using Chromas Lite (Technelysium, Australia) to evaluate and correct chromatograms. The alignment of the studied sequences was performed using ClustalW (THOMPSON *et al*. 1994) within the BioEdit software (HALL 1999). Phylograms were constructed for the studied fragments with Mega v5.2 (TAMURA *et al*. 2011) using neighbor-joining – NJ, (SAITOU & NEI 1987), maximum parsimony – MP, (NEI & KUMAR 2000), and maximum likelihood – ML, (FELSENSTEIN 1981). NJ analysis was performed using the Kimura 2-parameter correction model (KIMURA 1980) by bootstrapping with 1000 replicates (FELSENSTEIN 1985). MP analysis was evaluated with the minmini heuristic parameter (at level 2) and bootstrapping with 1000 replicates. Bayesian inference (BI) was performed with MrBayes 3.1.2 (RONQUIST & HUELSENBECK 2003); the analysis was run for 5,000,000 generations and trees were sampled every 100 generations. All trees were constructed with TreeView 1.6.6 (PAGE 1996). Analysis of

Table 1

Species of Tortricidae used in the present study. Two species of tribe Polyorthini were used as an outgroup. For better orientation, specimens for which *COI* sequences were obtained in the present study are highlighted in gray

No.	DNA Voucher	Tribe	Genus	Species	Origin	*COI* acc
1.	TLMF Lep 02146	Enarmoniini	*Ancylis* HÜBNER, 1825	*apicella* DENIS & SCHFFERMÜLLER, 1775	Italy, South Tyrol, Etschtal	JF859730
2.	TLMF Lep 02145	Enarmoniini	*Ancylis*	*laetana* FABRICIUS, 1775	Italy, South Tyrol, Etschtal	JF859729
3.	TORT083	Enarmoniini	*Ancylis*	*mitterbacheriana* DENIS & SCHFFERMÜLLER, 1775	Poland	KF493842
4.	TLMF Lep 02051	Enarmoniini	*Ancylis*	*myrtillana* TREITSCH, 1830	Italy, South Tyrol, Etschtal	JF859643
5.	TORT081	Enarmoniini	*Ancylis*	*uncella* DENIS & SCHFFERMÜLLER, 1775	Poland	KF493843
6.	TORT082	Enarmoniini	*Ancylis*	*unculana* HAVORTH, 1811	Poland	KF493844
7.	TLMF Lep 02053	Enarmoniini	*Ancylis*	*unguicella* LINNAEUS, 1758	Italy, South Tyrol, Etschtal	JF859645
8.	DA04	Enarmoniini	*Enarmonia* HÜBNER, 1825	*formosana* SCOPPOLI, 1763	Poland	KF493845
9.	TORT115	Eucosmini	*Blastesthia* OBRASCOV, 1960	*mughiana* ZETTERSCHEDT, 1868	Poland	KF493846
10.	TORT071	Eucosmini	*Blastesthia*	*turionella* LINNAEUS, 1758	Poland	KF493847
11.	TLMF Lep 02358	Eucosmini	*Epinotia* HÜBNER, 1825	*granitana* HERRICH-SCHÄFFER, 1851	Italy, South Tyrol, Etschtal	JF859912
12.	TORT079	Eucosmini	*Epinotia*	*tetraquetrana* HAVORTH, 1811	Poland	KF493848
13.	TORT098	Grapholitini	*Cydia* HÜBNER, 1825	*nigricana* FABRICIUS, 1794	Poland	KF493849
14.	04HBL006698	Grapholitini	*Cydia*	*pomonella* LINNAEUS, 1758	Canada, Ontario, Puslinch	GU093187
15.	TLMF Lep 02100	Grapholitini	*Cydia*	*strobilella* LINNAEUS, 1758	Italy, South Tyrol, Etschtal	JF859687
16.	TORT092	Grapholitini	*Grapholita* TREITSCH, 1829	*caecana* SCHLÄGER, 1848	Poland	KF493850
17.	Jflandry1667	Grapholitini	*Grapholita*	*molesta* BUSCK, 1916	Canada, Ontario, Vineland	GU096466
18.	10-JDWBC-2183	Grapholitini	*Hystrichophora* WALSINGHAM, 1879	*asphodelana* KEARFOTT, 1907	Canada, British Columbia	HM863928
19.	TLMF Lep 02164	Grapholitini	*Pammene* HÜBNER, 1825	*albuginana* GUENÉE, 1845	Italy, South Tyrol, Etschtal	JF859748
20.	TLMF Lep 02374	Grapholitini	*Pammene*	*argyrana* HÜBNER, 1796-99	Italy, South Tyrol, Etschtal	JF859925
21.	TLMF Lep 02160	Grapholitini	*Pammene*	*fasciana* LINNAEUS, 1761	Italy, South Tyrol, Etschtal	JF859744
22.	TORT131	Olethreutini	*Apotomis* HÜBNER, 1825	*inundana* DENIS & SCHFFERMÜLLER, 1775	Poland, Hajnówka	JF730060
23.	TORT132	Olethreutini	*Apotomis*	*sauciana* FRÖLICH, 1828	Poland, Kojszówka	JF730061
24.	TORT127	Olethreutini	*Apotomis*	*sororculana* ZETTERSCHEDT, 1839	Poland, Brzoskwina	JF730062
25.	TORT103	Olethreutini	*Celypha* HÜBNER, 1825	*cespitana* HÜBNER, 1814-17	Poland, Brzoskwina	JF730064
26.	TORT109	Olethreutini	*Celypha*	*rufana* SCOPPOLI, 1763	Poland, Kraków-Nowa Huta	JF730065
27.	TORT104	Olethreutini	*Celypha*	*striana* DENIS & SCHFFERMÜLLER, 1775	Poland, Brzoskwina	JF730066
28.	TORT102	Olethreutini	*Olethreutes* HÜBNER, 1822	*subtiliana* FALKOVITSH, 1958	Poland	JF730067
29.	TORT039	Polyorthini	*Pseudatteria* WALSINGHAM, 1913	*heliocausta* DOGNIN, 1912	Ecuador	JX144973
30.	TORT040	Polyorthini	*Pseudatteria*	*chrysanthema* MEYRICK, 1912	Ecuador	JX144974

haplotype diversity, nucleotide diversity and variable nucleotide positions was done with DnaSP v5.10.01 (LIBRADO & ROZAS 2009). Analysis of nucleotide frequencies, p-distance estimation and identification of substitution model (GTR+G+I for *COI* mtDNA fragments) for ML analysis were done with Mega v5.2 (TAMURA *et al*. 2004, 2011).

Results and Discussion

Besides a fragment of the *COI* gene, several nuclear markers (ITS2 rDNA, *EF1a*, *wingless* and *CAD* genes) were tested on the presently studied museum material. However, we failed to obtain good quality sequences or even amplicons of these genome fragments, probably because of the poor quality of DNA isolated from dry legs of specimens.

Therefore our current analysis, based on the *COI* fragment only, shows the possibility of applying this part of the genome as a molecular marker not only for Tortricidae species but also for initial tribe delineation. In some cases we obtained unresolved phylogenies. This may be connected with a fast rate of evolution of the studied *COI* fragment and also may be caused by the close relationship and lack of monophyly of some of the genera (for example within *Cydia* and *Grapholita*). A total of 30 sequences of the gene encoding cytochrome oxidase subunit I (606 bp) from species of Enarmoniini, Eucosmini, Grapholitini, Olethreutini and Polyorthini (outgroup) were used in this study (Table 1).

The interspecific haplotype diversity value was Hd=1, indicating substantial variability of the studied DNA fragment. Nucleotide diversity amounted to π=0.11106. The nucleotide frequencies were A=0.308, T=0.374, C=0.163 G=0.155 and revealed a high proportion of A-T pairs which corresponds to typical characteristics of insect mitochondrial DNA. Mean divergence over all studied Tortricidae (N=30) sequence pairs was p=0.111/SE=0.008 (p-distance/standard error). The mean divergence over all studied Enarmoniini (N=8) sequence pairs was p=0.097/SE=0.008; Eucosmini (N=4) (p=0.073/SE=0.008); Grapholitini (N=9) (p=0.096/SE=0.007) and Olethreutini (N=7) (p=0.092/SE=0.007). In the analyzed dataset we found 211 variable positions across all studied species in the *COI* fragment, 180 of which were parsimony informative (98 with two variants, 61 – three variants, 21 – four variants). A total of 30 haplotypes were found among the studied species.

All constructed trees (NJ, MP, ML, BI) showed the existence of four well separated clusters which represent particular studied tribes (Enarmoniini, Eucosmini, Grapholitini and Olethreutini), so we decided to present only one tree reconstructed by Bayesian Inference (Fig. 1). The Enarmoniini cluster is well separated and is composed of a monophyletic group containing seven studied *Ancylis* species and a distinct branch represented by *Enarmonia formosana*. On the *COI* tree, the Enarmoniini cluster appears between a group composed by two tribes: Eucosmini and Grapholitini and the more generalized Olethreutini. Although all tribes are well defined on the tree, it is difficult to hypothesize about their reciprocal relationships because there was low or even no bootstrap support for basal nodes of the studied trees (only posterior probabilities are acceptable – see Figure 1). This could be connected with the *COI* gene which is a fast evolving genome fragment, however in the present analysis we were not able to obtain DNA sequence for other, more slowly evolving molecular markers.

The Enarmoniini DIAKONOFF, 1953 (synonyms: Ancylisidii PIERCE & METCALFE, 1922, Anchyloperidae STAINTON, 1859) was given a variable position within the Olethreutinae and their monophyly was also controversial. The most recent and important interpretations of their systematics and phylogeny are as follows.

KUZNETZOV and STEKOLNIKOV (1973) based on the musculature of the male genitalia placed Enarmoniini (under the name Ancylidini) in the supertribe Eucosmidii as a sister group of Eucosmini + Laspeyresiini (= Grapholitini); similarly, RAZOWSKI (1976a) divided Eucosmini into Enarmoniina and Eucosmina + Grapholitina; KUZNETZOV and STEKOLNIKOV (1977) divided Eucosmidii into Enarmoniii and Grapholitini and the latter into Grapholitae and Dichroramphae; the same authors (KUZNETZOV & STEKOLNIKOV 1984) used only a different nomenclature of Eucosmidii in an analogical phylogenetic tree: Enarmoniini, Eucosmini, Laspeyresiina and Lipoptychina; and SAFONKIN (2007) in his phylogeny based on pheromones divided Olethreutinae into Gatesclarkeanidii consisting of Gatesclarkeanini and Endotheniini and Olethreutidii in which he placed three tribes, viz., Microcorsini, Bactrini and Olethreutini. The sister group of Olethreutidii is Grapholitidii composed with Enarmoniini, Eucosmni and Grapholitini tribes. In all the mentioned publications (except for RAZOWSKI 1976a and SAFONKIN (2007) Enarmoniini are included in Eucosmini or Grapholitini and essentially followed the earlier proposals by HEINRICH (1923) and HORAK and BROWN (1991). In the most recent molecular approach (REGIER *et al*. 2012) Enarmonnini was represented by a single genus *Ancylis*, which formed a clade separated both from Eucosmini and Grapholitini but was closer to them than to Olethreutini + Endotheniini + Bactrini. These authors suggested that their data

"do strongly support removal of Enarmoniini from Eucosmini".

At present the systematics of the Enarmoniini is not yet resolved. The only recent approach concerning the Australian fauna was revealed by HORAK (2006) because in RAZOWSKI (1976b) the diagnoses of the genera are not supported by a phylogenetic discussion. In this paper two Palaearctic genera with their type-species were studied (only one in REGIER *et al*. 2012). One representative of *Enarmonia*, which is the type genus of the tribe, seems to be regarded as a more generalized genus than *Ancylis*. The phylogenetic relationships of its seven species, based on sequences of the *COI* mtDNA fragment, differ from that based on morphology, chiefly genital characters. In conclusion, future projects using comparative analysis of DNA fragments should be verified by an analysis of several genital characters as well as a phylogeny based on pheromones.

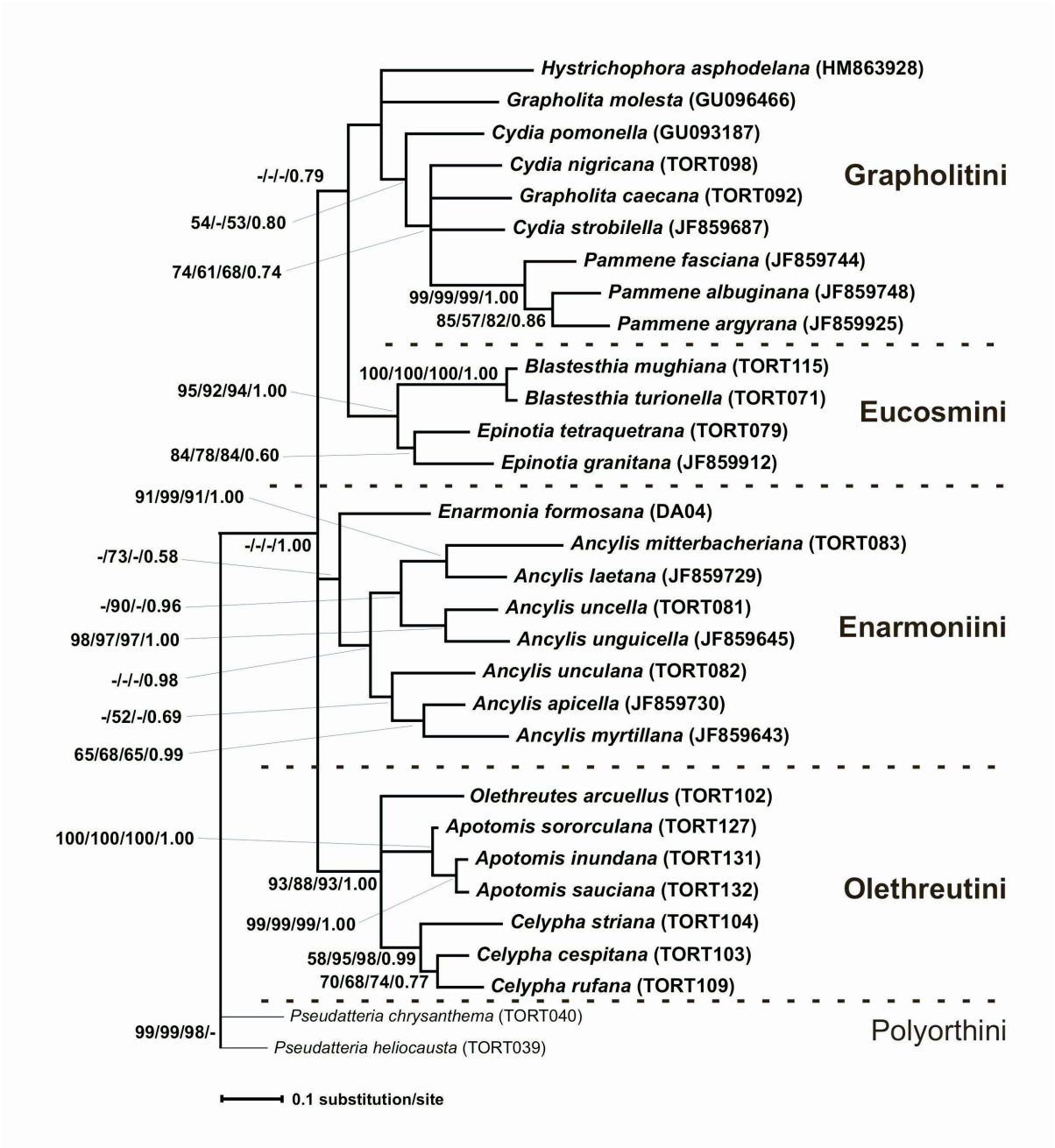

Fig. 1. Phylogenetic tree constructed for 8 species of Enarmoniini, 4 species of Eucosmini, 9 species of Grapholitini and 7 species of Olethreutini (two species of Polyorthini were used as an outgroup).
The tree was constructed on the basis of a comparison of sequences from mitochondrial *COI* fragment using the Bayesian inference method. Bootstrap values for neighbor joining, maximum parsimony analysis, maximum likelihood, and posterior probabilities for Bayesian inference are presented. Bootstrap values smaller than 50% (posterior probabilities <0.50) are not shown. Dashes represent no bootstrap or posterior value at a given node. All positions containing gaps and missing data were eliminated. Phylogenetic analyses were conducted using MEGA 5.2 (NJ/MP/ML) and MrBayes 3.1.2 (BI). The analysis involved 30 nucleotide sequences. There were a total of 606 positions in the final dataset.

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