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Molecular Approach to the Systematics of European Tortricini (Lepidoptera: Tortricidae)

Józef RAZOWSKI, Sebastian TARCZ and Magdalena GRECZEK-STACHURA

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Tortricini is a cosmopolitan tribe of the subfamily Tortricinae of the lepidopteran family Tortricidae. The most recent systematic of Tortricini are based on the external morphology of imagines and the structure of their genital organs. The present paper is the first comparative molecular study of the representatives of this tribe. We examined DNA variation in a 606 bp fragment of COI mtDNA obtained from 23 species of Tortricini and two representatives of other tribes (*Archips podanus* of Archipini and *Aethes hartmanniana* of Cochylini). The position of *Spatalistis*, *Tortrix*, *Aleimma* and *Acleris*, and some groupings of species within *Acleris* were confirmed by molecular data, including the synonymization of *Croesia* and *Phylacophora* with *Acleris*. The positions of a few groupings of the *Acleris* species remain unresolved.

Key words: Tortricini, Tortricidae, Europe, molecular, systematics.

Józef RAZOWSKI, Sebastian TARCZ, Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Sławkowska 17, 31-016 Kraków, Poland.
E-mail: razowski@isez.pan.krakow.pl
E-mail: starcz@isez.pan.krakow.pl
Magdalena GRECZEK-STACHURA, Institute of Biology, Pedagogical University, Podbrzezie 3 31-054 Kraków, Poland.
E-mail: magresta@wp.pl

Tortricini is a cosmopolitan tribe of the subfamily Tortricinae of the lepidopteran family Tortricidae. It consists of over 300 species and 40 genera. In Europe there are four genera constituting 50% of the Palaearctic (and Holarctic) fauna and as much as 43 species (in the Palaearctic there are 164 species). The remaining taxa are distributed mainly in the Afrotropical and Oriental regions.

The most recent systematics (RAZOWSKI 1987, 2002, 2008) of Tortricini are based on the external morphology of imagines and the structure of their genital organs.

The present paper is the first comparative molecular study of the representatives of this tribe. Until now, only four representatives of the genus *Acleris* (*A. variegana, A. holmiana, A. forsskaleana, A. comariana*) were studied (DE WAARD *et al*. 2009) and have not been compared with other species of this tribe. The molecular literature on other groups of this family is rather sparse. A mitochondrial DNA fragment containing the genes COI, and COII (2300 bp) was used to determine the phylogenetic relationships among *Argyrotaenia francisca* (LANDRY *et al*. 1999). A similar analysis using the COI segment was carried out by comparing the sequences obtained from closely related species of the genus *Archips* (KRUSE & SPERLING 2001, 2002). COI mtDNA fragment was successfully applied for species identification in *Adoxophyes honmai* species complex (LEE *et al*. 2005). Three mtDNA fragments consisting of COI, CR and ND5 genes were used in extensive research on molecular systematic and population structure of *Cydia pomonella* (MERANGER *et al*. 2008). A fragment of the mitochondrial COI gene was proposed as a universal DNA barcode and used for butterfly species identification in *Astraptes fulgerator* (HEBERT *et al*. 2004). Attempts to use the DNA barcode were also applied in Tortricidae (HULCR *et al.* 2007, LANGHOFF *et al.* 2009).

Trends in molecular studies of advanced moths and butterflies are reviewed by REGIER *et al*. (2009) which we take under consideration in our reconstruction of the evolution of Tortricidae.

The aim of this study was the molecular examination of all European genera and possibly a high

number of species. We compare the current system of genera and species based on morphology with the results from the molecular study. This comparison allowed us to correct or confirm some previous interpretations. It was also possible to assess some morphological characters. The results for particular genera are described in the discussion.

Material

The molecular study is based on 23 species of Tortricini collected mainly in Poland. Only one species, *A. kochiella*, occurring throughout the Palearctic region, comes from Iran. Extraction of DNA from other material outside of Central Europe did not give the expected results despite the circumstance that specimens were not more than five years old. The material used is deposited in the collection of the Institute of Systematics and Evolution of Animals PAS, Kraków. The moths were collected and donated by Jarosław BUSZKO (Toruń), Marek KOPEĆ, Witold ZAJDA (Kraków) and Rosław LEWANDOWSKI (Poznań).

A list of the examined taxa of Tortricini arranged alphabetically is presented below. The accession numbers of the NCBI GenBank are added after each studied species.

Acleris abietana (Hübner, 1822), (GU989462)

Acleris aspersana (Hübner, 1817), (GU989450)

Acleris bergmanniana (Linnaeus, 1758), (GU989460)

Acleris cristana (Denis & Schiffermüller, 1775), (GU989464)

Acleris emargana (Fabricius, 1775), (GU989448)

Acleris ferrugana (Denis & Schiffermüller, 1775), (GU989446)

Acleris forsskaleana (Linnaeus, 1758), (GU989453)

Acleris holmiana (Linnaeus, 1758), (GU989454)

Acleris kochiella (Goeze, 1763), (GU989469)

Acleris laterana (Fabricius, 1794), (GU989447)

Acleris lipsiana (Denis & Schiffermüller, 1775), (GU989466)

Acleris lorquiniana (Duponchel, 1835), (GU989451)

Acleris logiana (Clerck, 1759), (GU989463)

Acleris notana (Donovan, 1806), (GU989452)

Acleris rhombana (Denis & Schiffermüller, 1775), (GU989467)

Acleris scabrana (Denis & Schiffermüller, 1775), (GU989468)

Acleris shepherdana (Stephens, 1852), (GU989455)

Acleris sparsana (Denis & Schiffermüller, 1775), (GU989449)

Acleris umbrana (Hübner, 1799), (GU989465)

Acleris variegana (Denis & Schiffermüller, 1775), (GU989445)

Aleimma loeflingianum (Linnaeus, 1758), (GU989456)

Spatalistis bifasciana (Hübner, 1787), (GU989461)

Tortrix viridana Linnaeus (1758), (GU989457)

Other genera and species mentioned in this paper

Acleris hastiana (Linnaeus, 1758)

Croesia Hübner, 1825

Phylacophora Filipjev, 1931

Aethes hartmanniana (Billberg, 1820), (GU989459)

Archips podanus (Hübner, 1825), (GU989458)

Methods

DNA was extracted usually from two hind legs of dry specimens. We could not completely destroy the museum material using other parts of the body (e.g. the entire tagmata). On the other hand the collection of new, rare species was not possible. The best results were obtained from 1-3 year old individuals. In a few cases we had a chance to examine fresh specimens (e.g. *Tortrix viridana*, *Aleimma loeflingianum*) which we treated in a similar way as the collection examples and the results confirmed by an examination of entire moths.

For the outgroups the representatives of two other tribes of Tortricinae were selected, viz., *Archips podanus* of Archipini and *Aethes hartmanniana* of Cochylini.

Genomic DNA was isolated without protocol modification using the NucleoSpin Tissue Kit (Macherey-Nagel, Germany). To elute purified DNA we applied on the silica membrane 100 μl of Elution Buffer (EB). To amplify a fragment of the COI mtDNA gene (650bp) the following primer pair designed for Lepidoptera was used: LEP-F1, 5'-ATTCAACCAATCATAAAGATAT-3'; and LEP-R1, 5'-TAAACTTCTGGATGTCCAAAAA-3'. They are universal primers used for species identification in DNA barcoding (HEBERT *et al.* 2004). PCR amplification was carried out in a final volume of 40 μl containing: 4μl of template, 1.5 U Taq-Polymerase (Qiagen, Germany), 0.6 μl 10 mM of each primer, 10x PCR buffer, 0.6 μl of 10 mM dNTPs in a Mastercycler ep (Eppendorf, Germany). The amplification protocol was the same as in (HEBERT *et al.* 2004). To check amplification, 10 μl of each PCR product was electrophoresed in 1% agarose gels for 45 min at 85 V with a DNA molecular weight marker (Mass Ruler Low Range DNA Ladder, Fermentas, Lithuania). For purifying

PCR reactions we used NucleoSpin Extract II (Macherey-Nagel, Germany). In 30% of PCR reactions apart from the main band additional subbands were obtained. In these cases 30 μl of each PCR product was separated on a 1.8 % agarose gel (100V/60min). Then, the band representing the examined fragment was cut out and purified.

Cycle sequencing was done in both directions applying the BigDye Terminator v3.1 chemistry (Applied Biosystems, USA). The primers LEP-F1 and LEP-R1 were used for sequencing. Sequencing reaction was carried out in a final volume of 10 μl containing: 3 μl of template, 1 μl of BigDye (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 at the NCBI GenBank database (for accession numbers see list of examined taxa).

Sequences were checked by eye using Chromas Lite (Technelysium, Australia) to evaluate and correct chromatograms. Alignment and consensus of the studied sequences were performed using Clustal W (THOMPSON *et al*. 1994) in the BioEdit program (HALL 1999). Trees were constructed for the studied fragments in Mega version 4.1 (TAMURA *et al*. 2007), using the Neighbor-Joining method (NJ) (SAITOU & NEI 1987). The NJ analysis was performed using a correction model (KIMURA 1980) and Jukes-Cantor method (JUKES & CANTOR 1969) by bootstrapping with 1000 replicates (FELSENSTEIN 1985). The haplotype diversity value, nucleotide diversity and analysis of variable nucleotide positions were carried out using DnaSP v. 5.10.01 (LIBRADO *et al.* 2009). The nucleotide frequencies and transition/transversion rate ratios were calculated with Mega version 4.1 (TAMURA *et al*. 2004, 2007).

Results and Discussion

Both on morphological and molecular grounds the Tortricini appeared as the most generalized European tribe. The four examined genera were rather well separated from each other. The positions of the outgroup species fits well (Fig. 1) with the

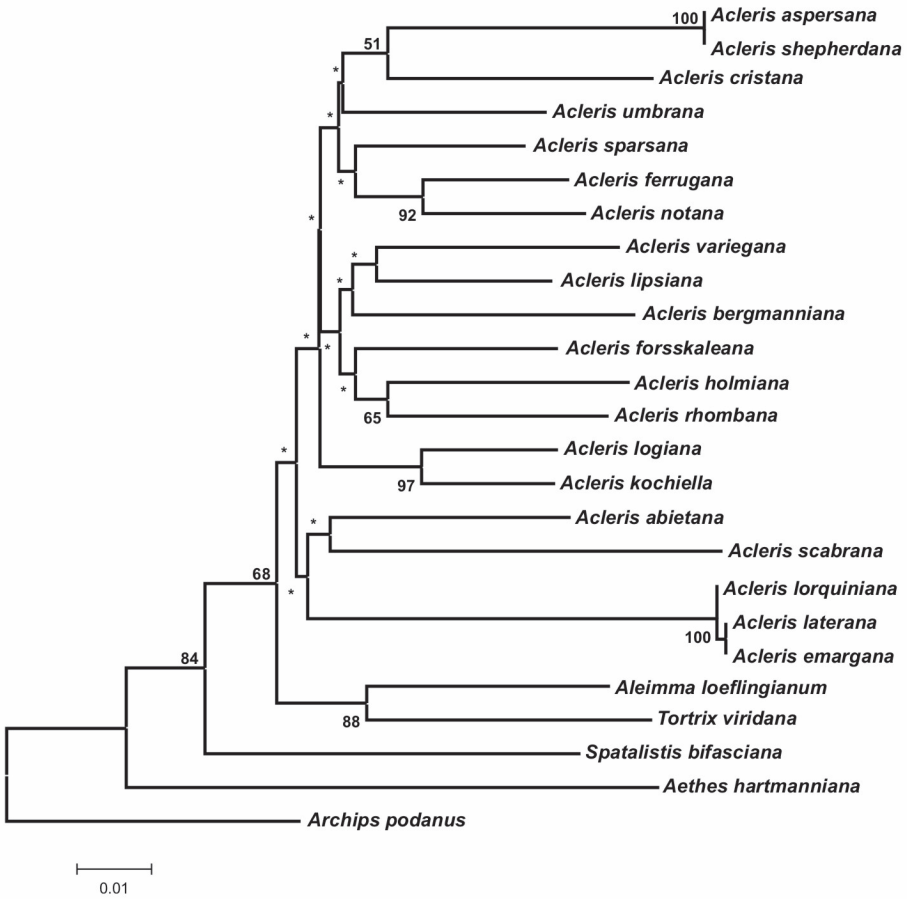

Fig. 1. Phylogenetic tree constructed for 25 species of *Tortricidae (Archips podanus* as outgroup), based on a comparison of sequences from COI mtDNA (606bp) fragment using the NJ (neighbor joining) method (with the application of with the application of the Kimura two-parameter and Jukes-Cantor correction model). Bootstrap values are presented as percentages for 1000 replicates. An asterisk appears in the case of bootstrap values less than 50. Phylogenetic analyses were conducted in MEGA 4.1.

commonly accepted relationships (e.g. RAZOWSKI 2008).

We obtained a portion of the COI mtDNA sequence (606bp) from 25 species of Tortricidae. Twenty three haplotypes among the studies species were found. Identical haplotypes occurred in species pairs : *A. laterana – A. emargana*, and *A. aspersana – A. shepherdana.* The haplotype diversity value was Hd=0.993 and nucleotide diversity was đ=0.08482. G+C content for 606 analyzed nucleotide positions was 0.312. The nucleotide frequencies were A=0.314, T=0.374, C=0.156 and G=0.156. The transition/transversion rate ratios were k_1=5.306 (purines) and k_2=6.705 (pyrimidines). The overall transition/transversion bias was *R*=1.995 (TAMURA *et al*. 2004). In the analyzed mtDNA fragment there were 194 variable positions (139 parsimony informative) (for details see Table 1). Divergence (using JC and K2P models) over all sequence pairs was 0.091 (varied from 0.000 to 0.153). The mean divergence of the 23 studied mtDNA fragments obtained from tribe Tortricini was 0.084 (varied from 0.000 to 0.126) and genus *Acleris* 0.08 (varied from 0.000 to 0.114). In the studied fragment only four polymorphic sites (7, 253, 284, 484) were nonsynonymous. All of them had two nucleotide variants and three of them (7, 253, 284) appeared only in one sequence in the studied dataset (Table 1).

The phylogeny of the studied species was reconstructed using the Neighbour-Joining (NJ) method (Fig. 1). Twenty three species of the tribe Tortricini are discriminated from two representatives of other tribes (*Archips podanus*, *Aethes hartmaniana*). *Acleris* forms the main group on the tree. A second group is formed by *Aleimma loeflingianum* and *Tortrix viridiana*. *Spatalistis bifasciana* is separated from other species of the tribe Tortricini.

Arrangement of the genera

Genus *Spatalistis* is represented by four Palaearctic and only one European species (*S. bifasciana*); apart from this region 17 species are known from the Oriental region.

The *Spatalistis* clade is well separated from the clades of the remaining examined genera and its position is supported by a bootstrap value of 84 (Fig. 1).

According to RAZOWSKI (1966, 1987, 2002), *Spatalistis* is regarded as the most generalized genus. Morphologically this is supported mainly by the presence of the uncus in the male genitalia. In earlier arrangements the wing venation was suggested to be more important (veins M3 – CuA1 in both pairs of wings are stalked – this is also a progressive character). In a recent monograph (RAZOWSKI 2008) *Spatalistis* is placed before *Tortrix* and *Aleimma*, close to *Acleris*. The present molecular study and the phylogenetic tree obtained by the Neighbour-Joining method confirm the above interpretation by RAZOWSKI (1966, 1987, 2002).

Table 1

Polymorphic nucleotide positions obtained by comparing COI mtDNA sequences (606nt) obtained from 25 Tortricidae species. Nonsynonymous polymorphic sites are marked in bold

Type of variable sites	Number of variable sites	Nucleotide positions of studied COI mtDNA fragment
Singleton variable sites (two variants)	45	**7** 30 36 58 69 84 90 93 102 114 126 138 156 172 186 195 201 204 219 231 249 **253** 267 **284** 300 315 339 375 378 411 417 444 474 477 486 504 511 529 549 553 582 585 592 600 606
Parsimony informative sites (two variants)	92	1 6 9 10 15 18 22 27 28 31 39 42 45 46 51 54 57 63 75 78 87 96 108 132 135 162 165 169 171 178 189 198 207 213 222 235 237 238 259 264 270 276 279 285 306 312 321 354 357 358 366 369 373 390 396 399 400 408 414 420 426 429 432 435 438 447 450 454 457 462 472 478 **484** 489 495 502 505 508 514 519 520 522 525 537 543 550 561 567 573 579 588 597
Singleton variable sites (three variants)	10	3 99 309 327 360 459 510 516 564 603
Parsimony informative sites (three variants)	34	12 60 105 111 141 177 192 228 246 256 258 324 342 345 348 351 363 372 384 387 393 405 423 441 456 492 501 513 528 540 546 558 576 594
Singleton variable sites (four variants)	0	---
Parsimony informative sites (four variants)	13	72 153 174 210 240 243 318 336 455 471 480 534 591

The present molecular study and the phylogenetic tree confirm the position of *Spatalistis* as more generalized than the three above mentioned genera.

Genera *Tortrix* and *Aleimma*

Tortrix is a Palaearctic genus consisting of two very closely related species; *Aleimma* is monotypic West Palaearctic. The clade containing these two genera is opposite to the clade of *Acleris* similarly as in the systematics by RAZOWSKI (2002, 2008) and *Tortrix* is regarded as more generalized than *Aleimma*. The position of this clade in the Neighbour-Joinig tree is supported by a bootstrap value of 68 (Fig. 1). The latter interpretation has never been presented in the systematics. The separation of *Tortrix* and *Aleimma* was usually based on the plesiomorphic position of all veins in both pairs of wings in contrast to *Acleris* in which the two last veins of the forewing are connate. *Spatalistis* was described on the basis of stalked veins in both pairs of wings. The separation of forewing veins R4-R5 is, however, not constant in *Acleris*. On the other hand, the membranization of transtilla (most probably an apomorphic character but possibly convergent) may separate *Tortrix* from *Aleimma*. Strongly shortened brachiola and elongated, pocked-shaped signum are the autapomorphies for *Aleimma*. The floricomous ovipositor characteristic of the two genera is convergent and directly depends on the mode of oviposition. The mentioned characters of *Aleimma* are certainly more progressive than in *Tortrix* and confirm the more advanced position of *Aleimma.* The reciprocal close systematic positions of *Tortrix* and *Aleimma* are confirmed by a bootstrap value of 88.

Genus *Acleris* is distributed in all zoogeographic regions and consists of over 340 species (Palaearctic – 136, Nearctic – 60, Oriental – more than 40, Afrotropical – 7, Neotropical – 9). The European fauna is represented by 40 species of which we examined 20, mainly Central European ones. The Neighbour-Joining tree presents *Acleris* as a rather compact grouping supported by a bootstrap value 68 (Fig. 1). It consists of four clades.

The first clade includes *A. laterana*, the type-species of the already synonymized genus *Phylacophora* in which several Holarctic species occur. The close position of *A. emargana* and *A. laterana* is acceptable despite the more elongate aedeagus in the former. It is probable that the elongate socius with median attachment to the tegumen is a more important character than the length of the aedeagus. It is thus a distinguishing character of the discussed clade. Many Asian species morphologically close to *A. abietana* can be thus included here. However, two species (*A. lorquiniana*, *A. scabrana*) included in this clade differ in the genitalia and require further study.

The next clade includes two morphologically close species (*A. kochiella* and *A. logiana*) and certainly several other Asian species which may fulfill the gap between them.

Three species (*A. holmiana*, *A. forsskaleana*, *A. Bergmanniana*) previously representing the distinct genus *Croesia* are included in the third clade, however, *A. holmiana* is placed near *A. rhombana* and regarded as less related with *A. forsskaleana*. On the other hand *A. bergmanniana* is opposite to the clade of the subgroup *A. lipsiana* + *A. variegana*. The systematic positions of both *A. holmiana* and *A. rhombana* were always obscure but their females have some common characters except for the presence of parabursa in the former. This character may be, however, species specific. The position of *A. bergmanniana* which is close to *A. lipsiana* + *A. variegana* and that of the two latter species cannot be explained but certainly all of them belong to different groupings as their morphology shows. However, the most important conclusion is that the mentioned above *Croesia* was correctly synonymized with *Acleris* (RAZOWSKI 1987).

The following fourth clade demonstrates a very close relationship between *A. notana* and *A. ferrugana*, completely in congruence with the morphological study. Previously, *A. sparsana* was regarded as close to the clade of *A. lorquiniana*, *A. laterana* and *A. emargana* but this was not strongly supported on the basis of morphology. Based on the molecular study these last two species are identical, but this relationship is at odds with the traditional point of view based on both morphology of adults, larvae, and biology. They strongly differ from one another and certainly represent different species. We cannot explain this discrepancy. The last clade demonstrates no molecular difference between *A. aspersana* and *A. shepherdana.* Morphologically these two species are very closely related, show very slight genital differences and have similar facies. However, they were always treated as two distinct species.

Conclusions

The systematic arrangement of the European genera of Tortricini based on morphology is essentially confirmed by the obtained molecular data. In the phylogenetic tree *Spatalistis* is, however, treated as the most primitive European genus and not directly related to *Acleris* which together with *Tortrix* and *Aleimma* forms its sister group. The reciprocal systematic position of *Aleimma* and

Tortrix and the former synonymization of *Croesia* and *Phylacophora* with *Acleris* based on the morphological characters are confirmed by the present study. Based on the positions of some species of *Acleris*, it can be hypothesized that the shape of the socii and their attachment to the tegumen may be interpreted as more important characters than the length of the aedeagus. The lack of molecular differences in two morphologically distinct species, *A. emargana* and *A. laterana* needs to be studied using other DNA fragments.

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