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Preliminary studies on the usefulness of DNA mini-barcodes for determining phylogenetic relationships within shieldbugs (Hemiptera: Heteroptera: Pentatomidae)

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Abstract. The paper presents results of preliminary studies on the usefulness of DNA mini-barcodes for the phylogenetic inference within Pentatomidae. Mini-barcodes for six Polish shieldbug species representing three pentatomid subfamilies (Asopinae, Pentatominae, Podopinae) have been obtained from the museum specimens (collected between 1994-2004). These mini-barcodes were then analysed for generating phylogenetic trees, using the neighbor joining (NJ), minimum evolution (ME), maximum parsimony MP), and UPGMS methods, as well as the Bayesian inference (BI). A squashbug, *Coreus marginatus* (Coreidae) was treated as an outgroup species in all analyses. Our findings suggest that DNA mini-barcodes can be employed not only for species identification, but may also be used for the phylogenetic inferences.

Key words: Hemiptera, Heteroptera, Pentatomidae, mtDNA, mini-barcodes, molecular phylogeny, Poland.

Introduction

DNA barcoding, widely used to identify and distinguish between animal species (e.g., Hebert *et al.* 2003a, 2003b; Rach *et al.* 2008; Jinbo *et al.* 2011), has recently become a promising means also for identifying true bug species (Jung *et al.* 2011; Park *et al.* 2011).

However, all studies in which sequences of the COI were fully recovered, have been based on freshly collected or alcohol-preserved specimens (Jung *et al.* 2011; Park *et al.* 2011).

Because freshly preserved samples are often unavailable for such analyses, and it is difficult to recover full barcode sequences from museum specimens, an universal DNA mini-barcode (a short sequence ~100 bp) was proposed for identification of animal specimen whose DNA is degraded (Hajibabaei *et al.* 2006; Meusnier *et al.* 2008).

Such mini-barcodes for insects were obtained from museum specimens of Lepidoptera, Hymenoptera, Diptera, Ephemeroptera, Plecoptera, and Trichoptera (Hajibabaei *et al.* 2006; Meusnier *et al.* 2008), but never from true bugs (Heteroptera).

Because recently, it was shown that it is possible to recover amplifiable mitochondrial DNA (*i.e.*, 12S and 16S subunits of rDNA) from old, dried museum specimens of pentatomoid bugs (Lis *et al.* 2011a), we have decided to find out whether the same is promising for DNA mini-barcodes from the museum samples of Pentatomidae.

Moreover, we have tried to find out if the mini-barcode could be used as a marker for phylogenetic analyses in Pentatomidae, the same way as the full-length barcode (cytochrome oxidase I) (Jung *et al.* 2011; Park *et al.* 2011).

Material and methods

Material. Eleven dried museum specimens (collected between 1994-2004) of six species representing three subfamilies of shield bugs (Asopinae, Pentatominae, Podopinae) have been studied; additionally, *Coreus marginatus* (Coreidae), treated as an outgroup, was also utylized (Table 1 & 2). All specimens come from the heteropteran collection of the Department of Biosystematics (Opole University, Poland).

Table 1. A list of species used in the study.

Family	Subfamily	Species [numer of specimens]
Coreidae	Coreinae	*Coreus marginatus* (L.) [2]
Pentatomidae	Pentatominae	*Carpocoris fuscispinus* (Boh.) [1]
Pentatomidae	Pentatominae	*Carpocoris purpureipennis* (DeG.) [2]
Pentatomidae	Pentatominae	*Aelia acuminata* (L.) [2]

Pentatomidae	Pentatominae	*Dolycoris baccarum* (L.) [2]
Pentatomidae	Pentatominae	*Piezodorus lituratus* (F.) [1]
Pentatomidae	Asopinae	*Picromerus bidens* (L.) [1]
Pentatomidae	Podopinae	*Graphosoma lineatum* (L.) [2]

Table 2. Details of museum specimens used in the study.

Family	**Species**	**Locality**	**Collection date**
Coreidae	*Coreus marginatus*	Poland: East Sudetes: Opawskie Mountains, Jarnołtówek	3.8.2001
Coreidae	*Coreus marginatus*	Poland: Upper Silesia, Łężczok reserve	30.9.2001
Pentatomidae	*Dolycoris baccarum*	Poland: East Sudetes: Opawskie Mountains, Pokrzywna	13.8.1997
Pentatomidae	*Dolycoris baccarum*	Poland: Upper Silesia, St. Anna Mount	21.8.1998
Pentatomidae	*Carpocoris purpureipennis*	Poland: Upper Silesia, Kędzierzyn-Koźle	8.9.2001
Pentatomidae	*Carpocoris purpureipennis*	Poland: East Sudetes: Opawskie Mountains, Pokrzywna	8.8.2001
Pentatomidae	*Aelia acuminata*	Poland: Lower Silesia, vicinity of Polska Nowa Wieś	8.8.2004
Pentatomidae	*Aelia acuminata*	Poland: Podlasie, Biebrza Valley, Czerwone Bagno reserve	10.7.1994
Pentatomidae	*Eurydema oleraceum*	Poland: Upper Silesia: Łężczok reserve	12.5.2001
Pentatomidae	*Eurydema oleraceum*	Poland: Upper Silesia: Kluczbork	12.8.2001
Pentatomidae	*Carpocoris fuscispinus*	Poland: Bieszczady Mts., Brzegi Górne	4.6.2000
Pentatomidae	*Carpocoris fuscispinus*	Poland: Małopolska Upland, Murawy Dobromierskie reserve	14.8.2001
Pentatomidae	*Piezodorus lituratus*	Poland: East Sudetes: Opawskie Mountains, Jarnołtówek	6.05.2000
Pentatomidae	*Picromerus bidens*	Poland: West Beskides, Babia Góra Mt.	20.8.1999
Pentatomidae	*Picromerus bidens*	Poland: Beskid Żywiecki Mts., Sopotnia	20.9.2003
Pentatomidae	*Graphosoma lineatum*	Poland: Upper Silesia, Strzelce Opolskie	27.7.2004
Pentatomidae	*Graphosoma lineatum*	Poland: West Sudetes, Karkonosze National Park	9.8.2002

Methods. An universal DNA mini-barcode (a short sequence of about 100 bp) for each specimen was obtained according to the procedure presented in Hajibabaei *et al.* (2006) and Meusnier *et al.* (2008); the primer sequences used for PCR amplification are provided in Table 3.

DNA extraction and PCR amplification were done according to the techniques described in Lis *et al.* (2011a). The vouchers were inserted in tubes with 96% ethanol and lodged in deep-freezer at the Center for Biodiversity Studies (Department of Biosystematics, Opole University, Poland). Sequencing was conducted at the Health Care Center GENOMED (Warsaw, Poland).

Table 3. Primers used in the study.

Primer name	Primer sequence
Uni-Minibar (R)	GAA AAT CAT AAT GAA GGC ATG AGC
Uni-Minibar (F1)	TCC ACT AAT CAC AAR GAT ATT GGT AC

Analyses. Electropherograms were edited by the Trace Data File Editor (Figs 1-12); their accompanied sequences were sent to the Web Browser for conducting BLAST searches (with use of *blastn* on NCBI) (as was done for 12S and 16S subunits of mtDNA of museum specimens by Lis *et al.* 2011a). Sequence alignments were made with Clustal X (using default parameters) in MEGA 4.0.2 software (Tamura et al. 2007, Kumar et al. 2008).

Phylogenetic analyses using neighbor-joining (NJ), minimum evolution (ME), maximum parsimony (MP), and UPGMA (unweighted pair-group method with arithmetic means) approaches were performed using MEGA 4.0.2 software (Tamura et al. 2007, Kumar et al. 2008).

The phylogenetic tree was also constructed in MrBayes v. 3.2 software (Ronquist et al. 2012) using the Bayesian inference (BI) method, with the parameters described by Lis *et al.* (2011b).

All trees for NJ, ME, MP, and UPGMA, were edited using MEGA 4.0.2 (op. cit.); the BI tree was edited using FigTree 1.3.1 (Rambaut 2009).

Results and discussion

PCR reactions success. The DNA mini-barcode sequences were recovered from ten of the eleven pin-mounted museum specimens exam-

ined; the mtDNA of *Piezodorus lituratus* appeared to be a DNA of the blowfly *Chrysomya putoria* (Wied.) (Diptera: Calliphoridae), and were excluded from the further studies. All other sequences showed high similarities to sequences (obtained from GenBank) of other pentatomomorphan species (for details see the Table 4).

Table 4. Similarities of the obtained sequences to those available from GenBank

Species/specimen	Highest similarity to species from GenBank
Coreus marginatus/ 1st	*Physopelta gutta* (Pentatomomorpha; Pyrrhocoroidea; Largidae)
Coreus marginatus/2nd	*Physopelta gutta* (Pentatomomorpha; Pyrrhocoroidea; Largidae)
Dolycoris baccarum/1st	*Nezara viridula* (Pentatomomorpha; Pentatomoidea; Pentatomidae)
Dolycoris baccarum/2nd	*Nezara viridula* (Pentatomomorpha; Pentatomoidea; Pentatomidae)
Carpocoris purpureipennis/1st	*Aeschyntelus notatus* (Pentatomomorpha; Coreoidea; Rhopalidae)
Carpocoris purpureipennis/2nd	*Aeschyntelus notatus* (Pentatomomorpha; Coreoidea; Rhopalidae)
Aelia acuminata/1st	*Nezara viridula* (Pentatomomorpha; Pentatomoidea; Pentatomidae)
Aelia acuminata/2nd	*Nezara viridula* (Pentatomomorpha; Pentatomoidea; Pentatomidae)
Carpocoris fuscispinus	*Riptortus pedestris* (Pentatomomorpha; Coreoidea; Alydidae)
Picromerus bidens	*Physopelta gutta* (Pentatomomorpha; Pyrrhocoroidea; Largidae)
Graphosoma lineatum/1st	*Riptortus pedestris* (Pentatomomorpha; Coreoidea; Alydidae)
Graphosoma lineatum/2nd	*Riptortus pedestris* (Pentatomomorpha; Coreoidea; Alydidae)

The oldest PCR amplifiable mtDNA sample was extracted from a specimen of *Eurygaster maura* (Scutelleridae) which was collected in June, 1994; the specimen of *Aelia acuminata* was another one collected the same year (July, 1994).

Electropherograms. All obtained sequences are characterized by high quality of DNA; noises in electropherograms were absent or almost indistinct (Figs 1–12).

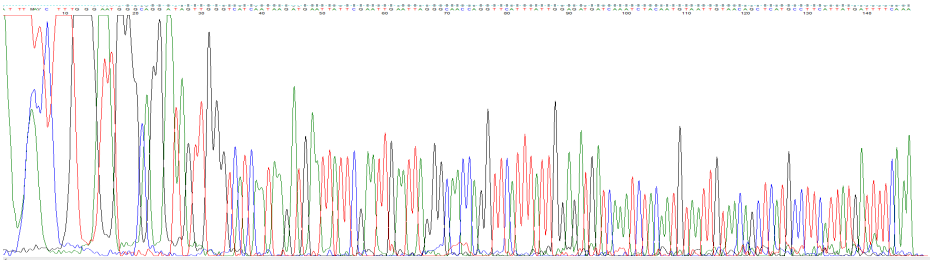

Figure 1. Trace file (electropherogram) of *Coreus marginatus* (1st specimen).

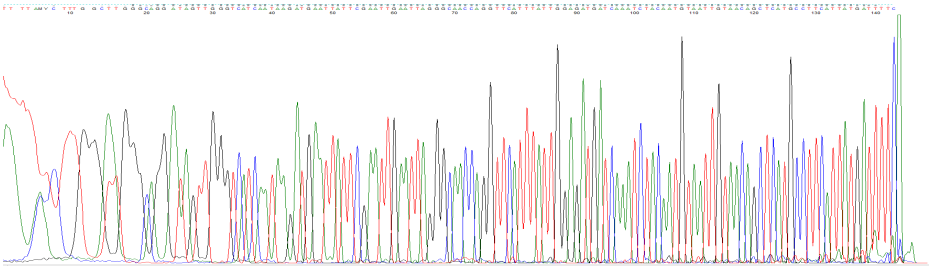

Figure 2. Trace file (electropherogram) of *Coreus marginatus* (2nd specimen).

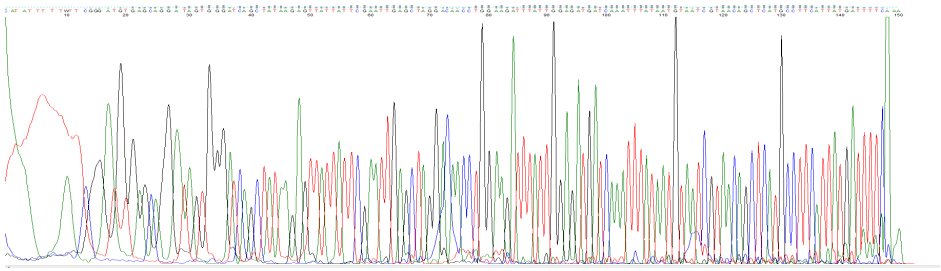

Figure 3. Trace file (electropherogram) of *Dolycoris baccarum* (1st specimen).

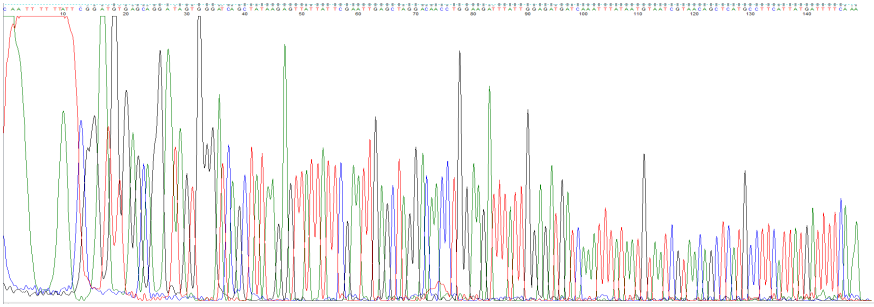

Figure 4. Trace file (electropherogram) of *Dolycoris baccarum* (2nd specimen).

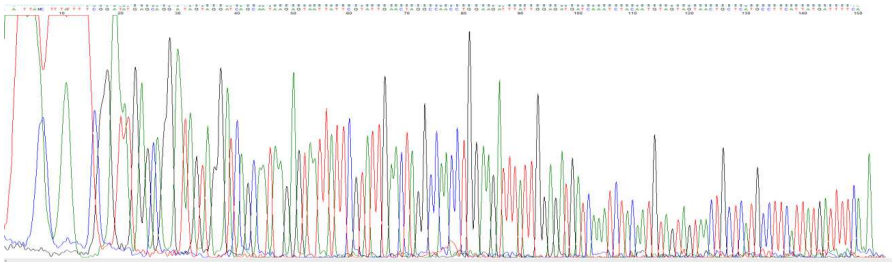

Figure 5. Trace file (electropherogram) of *Carpocoris purpureipennis* (1st specimen).

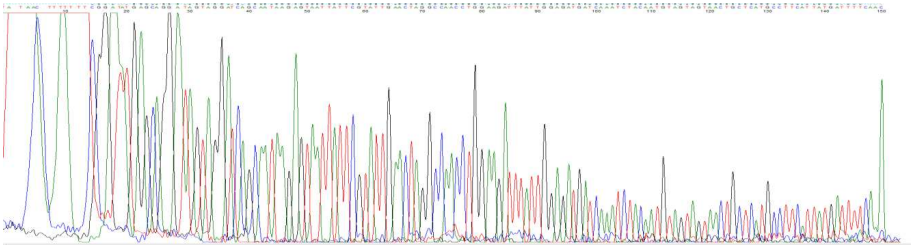

Figure 6. Trace file (electropherogram) of *Carpocoris purpureipennis* (2nd specimen).

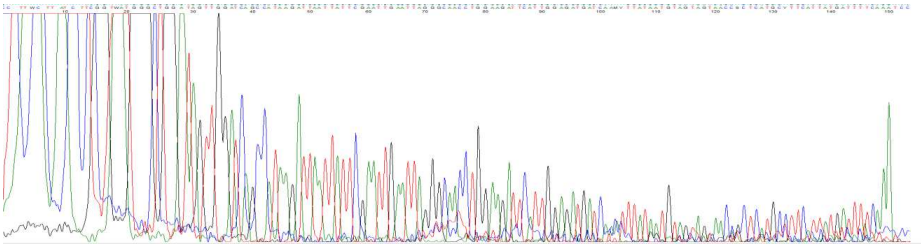

Figure 7. Trace file (electropherogram) of *Aelia acuminata* (1st specimen).

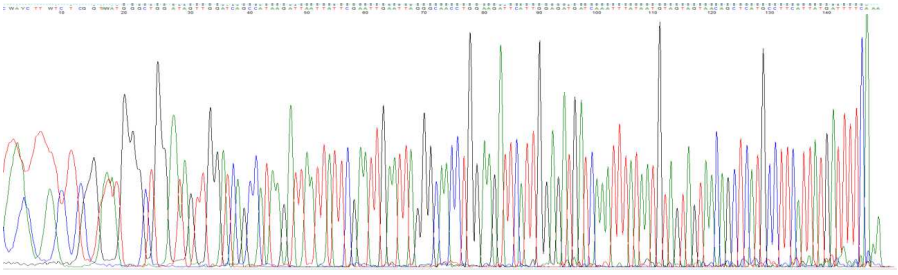

Figure 8. Trace file (electropherogram) of *Aelia acuminata* (2nd specimen).

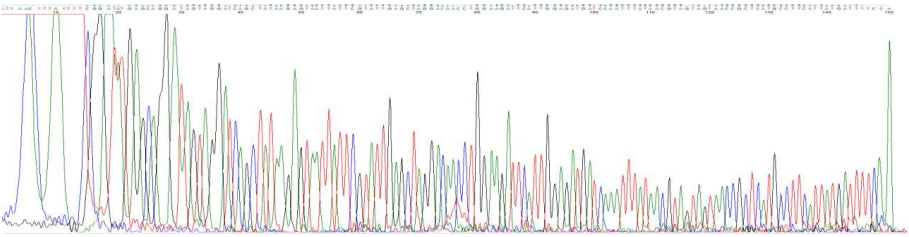

Figure 9. Trace file (electropherogram) of *Carpocoris fuscispinus*.

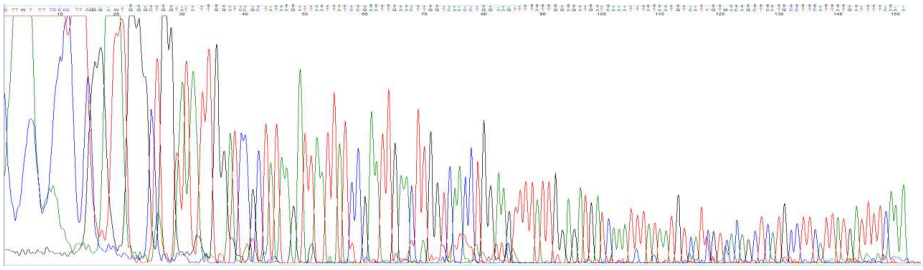

Figure 10. Trace file (electropherogram) of *Picromerus bidens*.

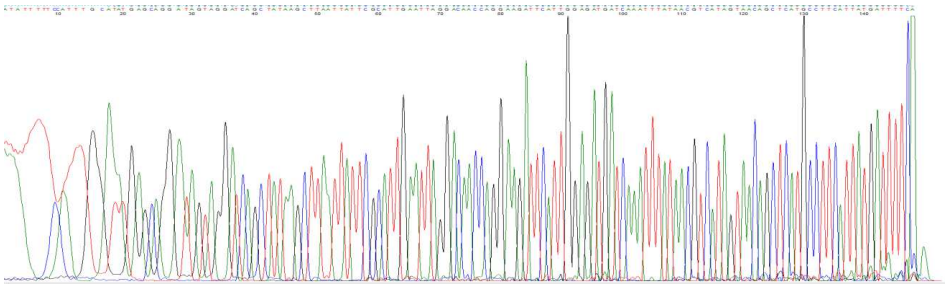

Figure 11. Trace file (electropherogram) of *Graphosoma lineatum* (1st specimen).

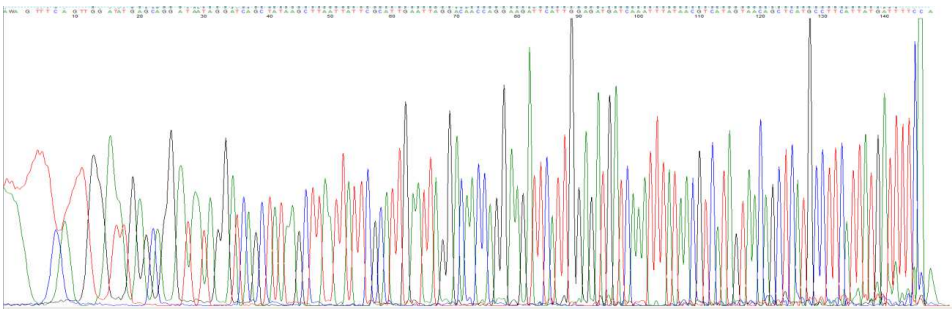

Figure 12. Trace file (electropherogram) of *Graphosoma lineatum* (2nd specimen).

Phylogenetic analyses. All analyses resulted in well resolved phylogenetic trees (Figs 13-17). All trees, except for the one (that derived from the analysis using the MP method – Fig. 15), identified the *Carpocoris*-group (sensu Gapud 1991, *i.e.*, *Carpocoris fuscispinus*, *C. purpureipennis*, *Dolycoris baccarum*) as a monophylum. Moreover, they always showed *Graphosoma lineatum* (Podopinae) as a sister taxon of the *Carpocoris*-group (Pentatominae).

The fact, that *Aelia acuminata* (Pentatominae) and *Picromerus bidens* (Asopinae) are permanently outside the monophylum consisting of *Graphosoma lineatum* (Podopinae) and the *Carpocoris*-group (Pentatominae), is also worth to mention.

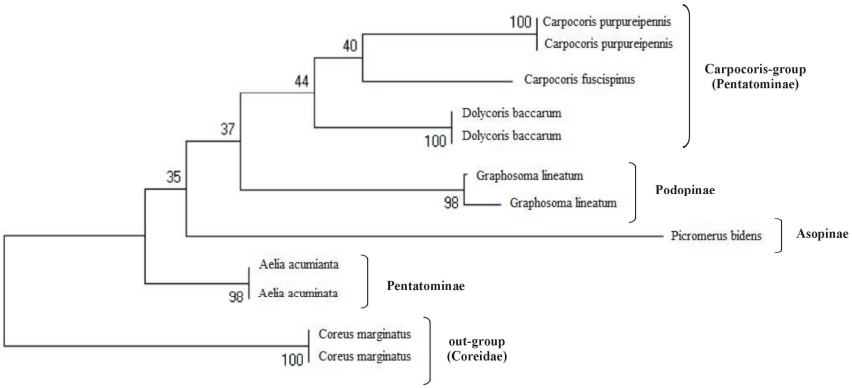

Figure 13. Phylogenetic tree generated by the Neighbor-Joining (NJ) analysis.

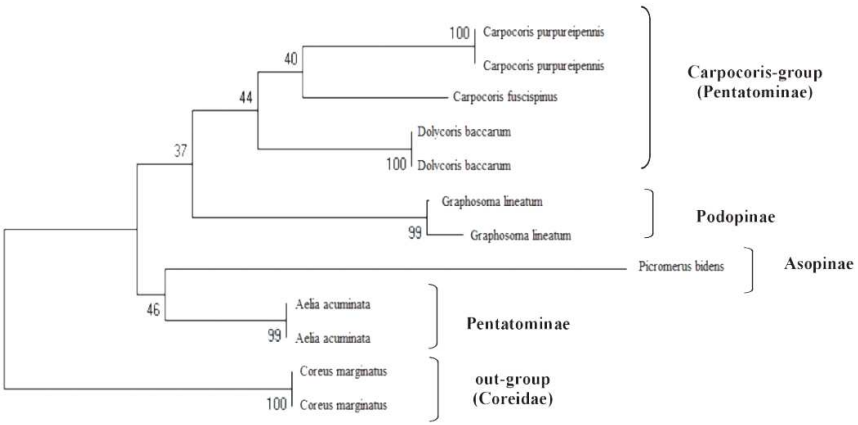

Figure 14. Phylogenetic tree generated by the Minimum Evolution (ME) analysis.

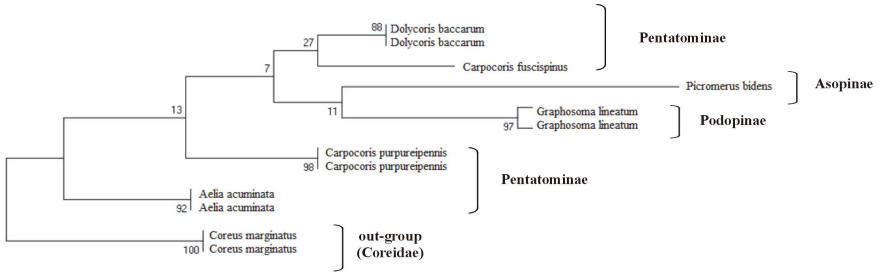

Figure 15. Phylogenetic tree generated by the Maximum Parsimony (MP) analysis.

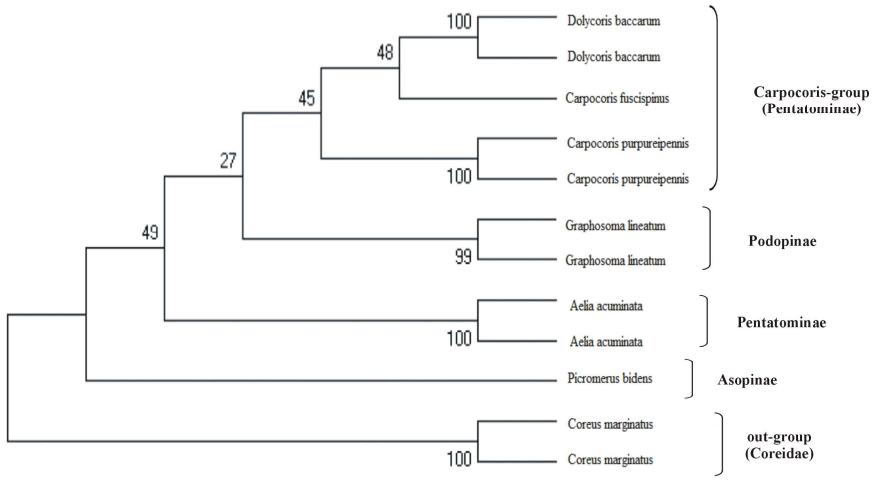

Figure 16. Phylogenetic tree generated by the UPGMA analysis.

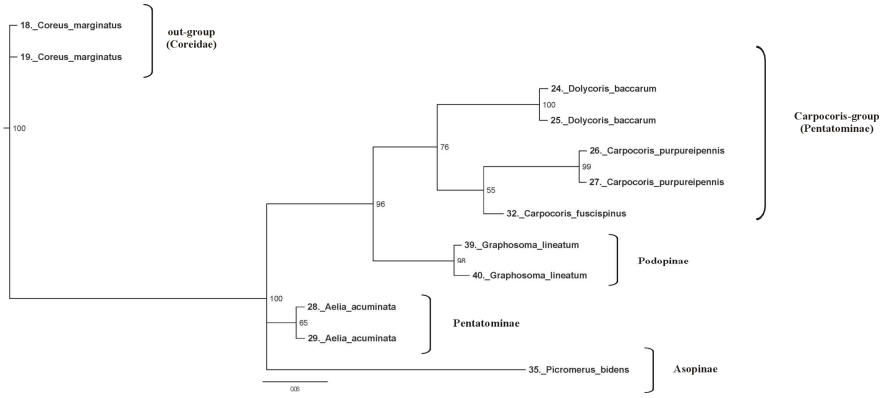

Figure 17. Phylogenetic tree obtained from the Bayesian inference analysis.

Conclusions

This was the first molecular study to examine the usefulness of DNA mini-barcodes for the phylogenetic inference within the family Pentatomidae.

The results of this preliminary studies show that DNA mini-barcodes can be employed not only for species identification, but may also be used for the phylogenetic inferences.

It is especially important when the freshly collected specimens are unavailable, and only museum material can be used for investigation.

However, our results are inconsistent with the present classification of the family Pentatomidae when the relationships among three studies subfamilies are considered. All the phylogenetic trees refuted a monophyly of the subfamily Pentatominae, but such a result may be caused by a limited number of species utilized in the study.

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