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Usefulness of DNA Barcoding for Identification of Preimaginal Stages Species of the Mecinini: Genus *Miarus* (Schoenherr, 1826) and *Cleopomiarus* (Pierce, 1919) (Coleoptera: Curculionidae)

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Differentiation between the two genera *Miarus* (Schoenherr, 1826) and *Cleopomiarus* (Pierce, 1919) and further identification of species within the genus have still proved to be a problem. The only known method for Polish species is based on detailed analysis of the adult male reproductive organs. Modern taxonomy may support the morphological markings using biological labels. It allows for the generation of DNA barcode that makes possible to identify the species of insects at any stage of development and any part of the body. The aim of the study was to verify the possibility of using the DNA barcoding BOLD database to species identification of the representatives of Mecinini, based on the *MT-CO1* sequence obtained from individuals in the larvae and pupae stages. It was found possible to assign specimens on larvae (L2, L3) and pupae stage to the right genus – *Miarus* or *Cleopomiarus*. The species level identification using *MT-CO1* sequences, especially within the genus *Miarus*, does not allow unambiguous assignment of unknown individuals.

Key words: Mecinini, *Miarus*, *Cleopomiarus*, DNA barcode, preimaginal stages, pupa, larva.

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Miarus (Schoenherr, 1826) and *Cleopomiarus* (Pierce, 1919) are currently considered two very closely related distinct genera (CALDARA 1999, 2001, 2007, 2013; CALDARA & LEGALOV 2016). Previously *Cleopomiarus* – or its synonyms *Miaromimus* (Solari, 1947) and *Hemimiarus* (Franz, 1947) – was considered a subgenus of *Miarus* (HOFFMANN 1958; ROUDIER 1966; SMRECZYŃSKI 1973, 1976; LOHSE & TISCHLER 1983). Open question remains to establish whether some morphospecies correspond to true biological species. It is due to similar features visible in biology of the species, as well as the morphology of the adult specimens (SMRECZYŃSKI 1957, 1973, 1976; DIECKMANN 1962 1978; PETRYSZAK 1979; GOSIK & ŁĘTOWSKI 2008). Sometimes (e.g as regards *Miarus)*, only a detailed analysis of the male reproductive organs (differences in the *aedeagus* anatomy), allows the correct determination of the species. This method made it possible to verify the data considering recording on Polish territory species *Miarus campanulae* (Linnaeus, 1767). After the review, the samples collected from central and south-eastern part of the country showed that they were in fact specimens of *M. ajugae* (Herbst, 1795) and *M. monticola* (Petri, 1912) (GOSIK & ŁĘTOWSKI 2008). One of the aspects of immatures identification might be the fact that the larvae of weevils do much greater damage to crops than imago – the adult form usually only makes holes in different parts of the plant while larva eats biomass and in many cases causes dieback of the whole plant. A study of immatures in these complex groups of weevils might be important and help current taxonomy (based only on adult morphology) by the addition of other useful characters. Unfortunately, previous studies pro-

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vide only fragmentary data on the morphology of the larva stage L3 of species: *Cleopomiarus graminis* (Gyllenhal, 1813), *C. hispidulus* (LeConte, 1876) and *Miarus campanulae* (EMDEN 1938; SCHERF 1964; ANDERSON 1973) and even more modest data about the morphology of the pupal stage of the mentioned species (SCHERF 1964; ANDERSON 1973). The genus *Cleopomiarus* (Pierce, 1919) include 19 Palaearctic species (CALDARA & LEGALOV 2016). This genus is also present in the southern Afrotropical region, whereas *Miarus* is only Palaearctic. In a European region 5 species are represented (including 1 also collected from Poland). In Asia 8 species are noted, 5 are Eurasian (including 2 known from Poland), another 2 are present both in Europe and North Africa, and *Cleopomiarus plantarum* (Germar, 1824) is spread throughout the Palaearctic (CALDARA 2013; CALDARA *et al.* 2014). In Poland occur: *Cleopomiarus distinctus* (Boheman, 1845), *C. graminis* (Gyllenhal, 1813) and *C. micros* (Germar, 1821) (SMRECZYŃSKI 1976; BURAKOWSKI *et al.* 1997; PETRYSZAK 2004; WANAT & MOKRZYCKI 2005; CALDARA & LEGALOV 2016). Genus *Miarus* includes 19 species, 9 of which exist in Europe, one in Asia, 2 in North Africa and 7 inhabits Eurasia (CALDARA 2007; CALDARA 2013; CALDARA *et al.* 2014). In Poland the following were reported so far: *Miarus abnormis* (Solari, 1947), *M. ajugae* and *M. monticola* (SMRECZYŃSKI 1976; BURA- KOWSKI *et al.* 1997; PETRYSZAK 2004; WANAT & MOKRZYCKI 2005; GOSIK & ŁĘTOWSKI 2008). The systematics of the tribe is still reorganized and made completed, an example of which may be a recent verification of the Holarctic species of *Cleopomiarus* (CALDARA & LEGALOV 2016). Modern taxonomy may verify the morphological markings using methods of molecular genetics. DNA barcoding uses a short, standardized sequences of genetic markers, adequately informative for species identification. Selected sequences should have a low variability within the species (approx. 2%) and, at the same time, high variability between species (approx. 10%). Designing PCR primers and subsequent standardization are facilitated by high numbers of repeats in the genome and flanking by the conserved domains. The length of the barcode sequence is also important – short fragments, with a small number of insertions/deletions, greatly accelerate and facilitate identification. The method allows for the generation of DNA barcode that makes it possible to identify the species of insects at any stage of development and all parts of the body. In the animal kingdom, including insects, the DNA barcode is approximately 650 bp mitochondrial sequence of the I subunit of cytochrome c oxidase (*MT-CO1*) (HEBERT *et al.* 2003). In order to accelerate the taxonomic identification, the DNA barcodes are now regarded as an alternative tool for the identification of insects biodiversity. The results of molecular identification including representatives of various orders and families confirmed the utility of DNA barcodes as a crucial tool in the rapid and reliable identification of insects (JALALI *et al.* 2015). It should be noted, however, that the usefulness of DNA barcodes might be a problem, due to the presence of mitochondrial pseudogenes, non-functional copies of mitochondrial DNA genes, present in the nuclear genome that are found in different taxa. When these paralogous sequences are amplified together with mitochondrial DNA, they can stay unnoticed and eventually be analyzed as orthologs, which has also been found in studies in the field of entomology. That is why, it is important to follow the procedures to avoid sequence analysis of pseudogenes (LEITE 2012). DNA barcoding technique refers to the sequencing of DNA barcode from the taxonomically unknown sample and comparison with a library of reference barcodes with known origin, in order to do the identification on the species level. Libraries of DNA barcodes are valuable as reference databases through the relationship between the deposited and marked specimen of the species they come from and other information, especially the names of species in Linnaeus classification, locations of the collections and morphological characteristics in a form of digital images. The first task in DNA barcoding is to link a specific sequence with the name of the species. These sequences supported by conventional morphological methods, are then incorporated into the reference library of DNA barcodes – Barcode of Life Datasystem (BOLD) (WILSON 2012). However, the limited differentiating potential of molecular methods of identification should be noted. And it is strictly associated with the correctness of morphological determination of species. An example might be the studies on *Miarus campanulae* and *Miarus graminis*. On the basis of minor differences in the external anatomy they were divided into several morphospecies. Since separating these new forms proved impossible, molecular data of mitochondrial *MT-CO1* gene and nuclear *ITS2* region were used. The newly isolated morphospecies of the genus *Miarus* proved to be indistinguishable from the traditionally accepted forms on the basis of these sequences. On the other hand, the traditionally recognized species were characterized by a certain number of synapomorphies. Hence, it should not be generalized that molecular evidence reliably reveal the unnoticeable variation of cryptic species (VAHTERA & MUONA 2006). Various studies of the DNA barcodes of insects often provide contrary results and suggest inconsistent efficiency between the orders. Despite the relatively low percentage of type I errors (misidentification of species that are represented in the reference database), the lack of a reference sequence barcode DNA for 98% of the known species of insects means that the identification of this group by DNA barcoding is heavily burden with errors of type II (misidentification of species without the same species in the database) (VIRGILIO *et al.* 2010).

The aim of the study was to verify the possibility of using the DNA barcoding method for species identification of the representatives of the tribe Mecinini, based on the *MT-CO1* sequence obtained from individuals in the larva and pupa stages.

Materials and Methods

The whole procedure was carried out in accordance with the guidelines of insects DNA barcoding (WILSON 2012). All the investigated Mecinini (Gistel, 1848) species were collected from Poland. Preimaginal stages were obtained from the field plants of the genus *Campanula* L. and *Jasione* L and isolated in the laboratory. The adult insects were caught directly from the host plants and isolated from the nets. A more detailed characteristics of the samples and description of the locations are presented in Table 1. After freezing at -70°C, total genomic DNA was extracted from the single specimens in the larva stages of L2, L3, pupa and imago. Before DNA extraction, the weevils were surface sterilized by immersion in 70% ethanol. Beetle remains were deposited in the Department of Zoology, Animal Ecology and Wildlife Management (University of Life Sciences in Lublin). The total genomic DNA was extracted following the QIAamp® DNA Mini Kit procedure (QIAGEN). DNA extraction was performed according to the procedure recommended by the manufacturer and VAHTERA & MUONA (2006), PTASZYŃSKA *et al.* (2012). Purity and concentration of DNA were assessed by spectrophotometry and qualitative assessment was done by electrophoresis in 1% agarose gel. PCR mixtures were prepared using AmpliTaq Gold 360 DNA Polymerase (Thermo Fisher Scientific) in a thermal cycler Labcycler (SensoQuest) in 25 µl volume of sample containing: 2.5 µl (1x) PCR buffer, 2.5 µl (10% v/v) G/C, 0.2 mM dNTP mix, 1.25 U Taq DNA polymerase, 1 µM of each primer, 3 µl of DNA template. For DNA amplification, the following PCR cycling conditions were used: 10 min at 95 °C followed by 30 sec at 95°C, 30 sec at 50.5°C, and 1 min at 72°C, repeated for 30 cycles, and 7 min at 72 °C. A region of about 600 bp of the *MT-CO1* gene was amplified using primers LCO1490-5'GGTCAACAAATCATAAAGATATTGG3' and HCO2198-5'TAAACTTCAGGGTGACCAAA AAATCA3' (FOLMER *et al.* 1994). PCR products were sequenced by a commercial company (Genomed S.A., Poland). Amplified sequences of 28 specimens were supported with *MT-CO1* gene sequences of 9 specimens that were obtained from BOLD v. 4.

The sequences were manually corrected with the DNA Baser v. 3. The amplified 28 *MT-COI* gene sequences of the studied Mecinini species obtained in this study were analyzed with 9 *MT-COI* Mecinini reference sequences downloaded from the BOLD v. 4 database. A total of 37 sequences were aligned using ClustalW and then they were visually checked and corrected. Each sample was individually compared by the 'Identification' tool with the resources of BOLD v.4 database, for the purpose to determine the degree of similarity to known species. Phylogenetic trees were constructed in MEGA 7 using the neighbor joining (NJ) and maximum likelihood (ML) methods. The phylogenetic distances were estimated with Generalised Time-Reversible model (GTR) determined by Model option in MEGA 7 program (KUMAR *et al*. 2016). Robustness of tree branches was determined by bootstrap analysis using 10000 re-samplings. The relationships obtained with distance-based methods were verified using Bayesian algorithm with MrBayes (HUELSENBECK & RONQUIST 2001; RONQUIST & HUELSENBECK 2003). Bayesian analysis with MrBayes used four linked Markov chains per run and two simultaneous runs. Convergence and stationarity of the runs was assessed by checking for plateaus in the time series of parameter values and examining the standard deviation of split frequencies between the two runs. The simulations were carried out for 1×10^6 generations which were logged every 100 generations. The phylogram was presented in the FigTree program (http://tree.bio.ed.ac.uk/software/figtree/). A homologous sequence of *Kyklioacalles anthyllis* (Coleoptera: Curculionidae), (EU286456, ASTRIN & STUEBEN 2008) was used as the outgroup.

Results

The initial indication of the species was made due to the differences in morphological structure and the characteristics of localities and host plant, from which larvae and pupae, as well as adult beetles were collected. It was found that the specimens analyzed in this study, according to the accepted systematics, may belong to a species *Miarus ajugae* and *Cleopomiarus graminis* or *Cleopomiarus distinctus.* Barcodes of 575 bp length, corresponding to the complete DNA barcode sequence (658 bp) in the range 6-580, were successfully obtained from all the analyzed specimens. Comparison of the sequences obtained in our study with the reference sequences deposited in BOLD database made it possible to demonstrate their greatest similarity to two of the possible species. The sequences of specimens identified by the laboratory numbers 11, 12, 13, 15, 26, 30, 31, 32, 33, 36, 47 showed the greatest similarity to *Miarus ajugae*, although it has been found that with equal probability they could be attributed to the species *Miarus monticola*. This species was not included

Table 1

Characteristics of investigated Mecinini specimens and description of localities

No. of sample	Identified species	Stage	Amount in a sample	Locality*
4	*C. graminis*	pupa	3	Łysaków
7	*C. graminis/C. distinctus*	L3	2	Gródek-Czumów
8	*C. graminis/C. distinctus*	L3	3	Gródek
9	*C. graminis/C. distinctus*	L3	3	Gródek
10	*C. graminis/C. distinctus*	L3	3	Gródek
11	*M. ajugae*	L3	3	Jakubowice Murowane
12	*M. ajugae*	L3	3	Łęczna
13	*M. ajugae*	L3	3	Kąty
14	*C. graminis*	L3	3	Kąty
15	*M. ajugae*	L3	2	Bychawa
16	*C. graminis*	L3	1	Jakubowice Murowane
18	*C. graminis/C. distinctus*	pupa	3	Gródek-Czumów
19	*C. graminis/C. distinctus*	pupa	2	Gródek-Czumów
21	*C. graminis/C. distinctus*	pupa	3	Gródek
22	*C. graminis*	pupa	3	Kąty
25	*C. graminis*	pupa	1	Kąty
26	*M. ajugae*	pupa	2	Gródek-Czumów
27	*C. graminis/C. distinctus*	pupa	1	Gródek-Czumów
28	*C. graminis*	pupa	1	Jakubowice Murowane
30	*M. ajugae*	L3	1	Nasutów
31	*M. ajugae*	L2	5	Łęczna
32	*M. ajugae*	L2	4	Jakubowice Murowane
33	*M. ajugae*	L3	1	Niedzieliska
36	*M. ajugae*	L3	2	Jakubowice Murowane
47	*M. ajugae*	imago	2	Kąty
48	*C. graminis*	imago	2	Kąty
49	*C. graminis*	imago	2	Łysaków
50	*C. graminis*	imago	2	Jakubowice Murowane

*Characteristics of localities – alphabetically:

B y c h a w a. Plant communities: *Koelerio-Festucetum rupicolae*, *Inuletum ensifoliae*, *Thalictro-Salvietum pratensis*, *Origano-Brachypodietum*. Host plants species: *Campanula patula* L., *C. persicifolia* L., *C. sibirica* L., *C. trachelium* L.

G r ó d e k. Plant communities: *Molinieteum caeruleae*, *Thalictro-Salvietum pratensis*. Host plants species: *Campanula bononiensis* L., *C. sibirica* L.

G r ó d e k - C z u m ó w. Plant communities: *Festuco psammophilae-Koelerietum glaucae*, *Thalictro-Salvietum pratensis*, *Origano-Brachypodietum*. Host plants species: *Campanula bononiensis* L., *C. glomerata* L., *C. patula* L.

J a k u b o w i c e M u r o w a n e. Plant communities: *Koelerio-Festucetum rupicolae*, *Thalictro-Salvietum pratensis*. Host plants species: *Campanula rapunculoides* L., *C. trachelium* L.

K ą t y. Plant communities: *Inuletum ensifoliae*, *Thalictro-Salvietum pratensis*, *Origano-Brachypodietum*. Host plants species: *Campanula patula* L., *C. sibirica* L.

Ł ę c z n a. Plant communities: *Thalictro-Salvietum pratensis*, *Origano-Brachypodietum*. Host plants species: *Campanula persicifolia* L., *C. rapunculoides* L.

Ł y s a k ó w. Plant communities: *Molinieteum caeruleae*, *Thalictro-Salvietum pratensis*, *Origano-Brachypodietum*, *Prunion fruticosae*. Host plants species: *Campanula patula* L., *C. persicifolia* L., *C. sibirica* L.

N a s u t ó w. Plant communities/Host plants species: *Jasione montana* L., *Campanula patula* L.

N i e d z i e l i s k a. Plant communities: *Inuletum ensifoliae*, *Thalictro-Salvietum pratensis*, *Origano-Brachypodietum*, *Prunion fruticosae*. Host plants species: *Campanula sibirica* L.

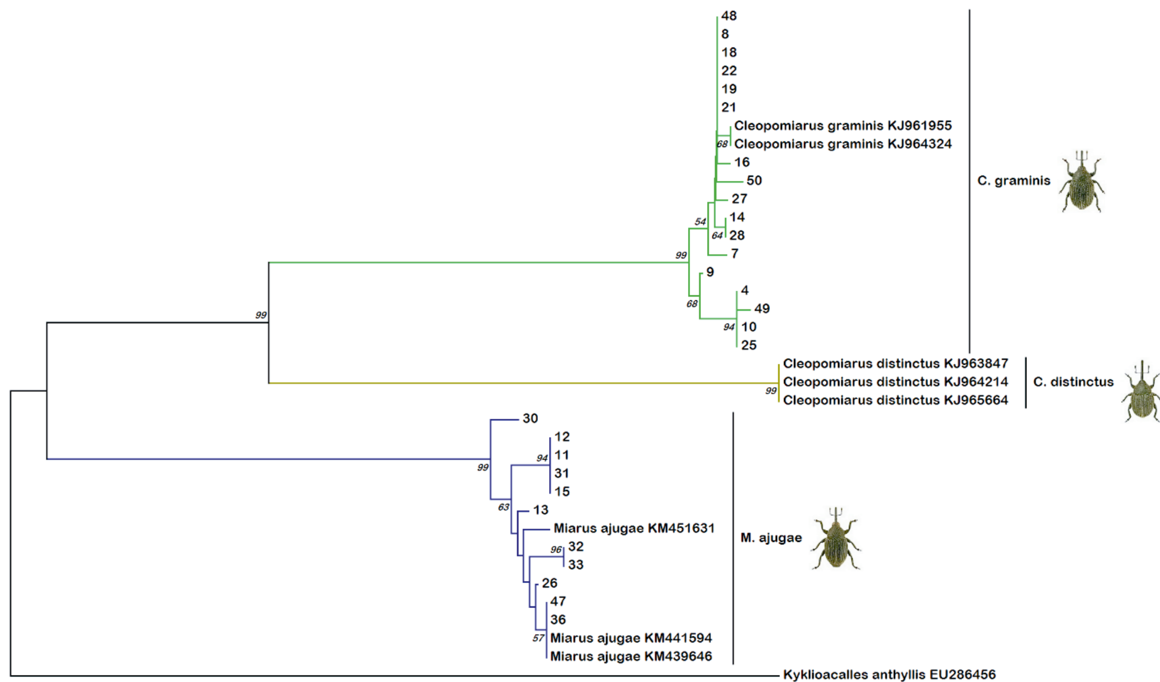

Fig. 1. The phylogenetic tree constructed by NJ method: samples without labels represent laboratory numbers; sequences from the database are described by species name and accession number.

in the tree because *M. monticola* barcode sequence is not available for download. The data of *M. monticola* deposited in BOLD are not public yet and only allow to determine the similarity, but are not capable of processing. At the same time, according to the description of the records in BOLD, the mentioned specimens were each time assigned as *Miarus campanulae*. However, this species was excluded as one of the possible options due to the fact that none Polish *Miarus* specimens proved to be true *M. campanulae*. The degree of similarity to *Miarus ajugae* ranged from 99.1% for the individual no. 30 to 100% for samples 36 and 47. In turn, the sequences derived from samples numbered 4, 7, 8, 9, 10, 14, 16, 18, 19, 21, 22, 25, 27, 28, 48, 49, 50 showed the greatest similarity to the reference sequence of *Cleopomiarus graminis*. The value of the similarity was in the range of 98.6% for the sample no. 49 to 99.8% for sequences belonging to individuals 8, 18, 19, 21, 22, 48. None of the sequences showed the most significant similarity to the reference sequence of *Cleopomiarus distinctus*. It should be noted that in BOLD v.4 there is no deposited sequence of *Cleopomiarus micros*. Own sequences were analyzed together with the sequences from the BOLD database and were used to generate phylogenetic trees that have been constructed by the NJ, ML and Bayesian methods. Tree topologies obtained using all three methods were completely consistent, therefore, only NJ tree is presented as an example (Fig. 1). Two major clades corresponding to the type *Miarus* and *Cleopomiarus* were clearly separated. The additionally use of reference sequences from the BOLD database made it possible to determine the individual species. Within the genus *Cleopomiarus*, a clear division into two species was observed: clade corresponding to *Cleopomiarus distinctus*, focusing exclusively sequences of the species obtained from the database KJ964214, KJ963847, KJ965664 and clade considered as *Cleopomiarus graminis*, within which besides sequences KJ964324 and KJ961955 were also placed samples from own research, characterized by the greatest similarity to the species in accordance with the comparison in BOLD. The level of variation between pairs of sequences within the clade, including reference sequences, was in the range 0-1.6%, with a mean value of 0.6%. In the second main clade specimens which were assigned to the genus *Miarus* were placed, together with the reference sequences of *Miarus ajugae* KM441594 and KM439646. The distance between the pairs of sequences within this group ranged from 0 to 1.4% (mean 0.6%).

Discussion

The values of similarity between the obtained sequences and the references deposited in BOLD database indicate that there are no specimens of *Cleopomiarus distinctus* in the tested samples. Due to the lack of *Cleopomiarus micros* sequence in BOLD or GenBank, it is impossible to certainly

prove the lack of specimens of this species between the investigated samples, which also increases the risk of type II error. It should be noted, however, that the differentiation between pairs of sequences recognized as belonging to *Cleopomiarus graminis*, of up to 1.6% is in the range of intraspecific differentiation accepted for DNA barcodes (approx. 2%). Therefore, there is only a slight probability of erroneous identification of specimens of *C. micros* as *C. graminis*. Moreover, none of the samples were classified by morphology as *C. micros,* which minimizes the risk of error. As regards the genus *Miarus*, all the tested specimens were assigned as *M. ajugae* and the degree of sequence variability within the clade taking a maximum value of 1.4%, is also acceptable as regards the variation within a single species. The order Coleoptera is the most diverse group of insects. According to data from The National Science Museum of Korea (NSMK), obtained from the analysis of 179 specimens and 60 species, it was found that average intra- and interspecies diversity values were 0.70 ± 0.45% and 26.34 ± 6.01% with the variation rates respectively in the range of 0% to 1.45% and 9.83% to 56.23% (JUNG *et al.* 2016). Assignment of the specimens to the species *Miarus monticola* was excluded due to the characteristics of the places, which were the source of samples, and morphology of therein noted adult forms of beetles. However, the comparison of own sequences with the resources of BOLD v.4 database gave less conclusive results of species identification than in the case of *Cleopomiarus*. Obtained similarity values were the same for both *M. ajugae* and *M. monticola*, which sequence, despite the presence in the database, and the possibility of making alignment, is not currently available for download, and therefore could not be included in the prepared phylograms. However, that indicates a very high genetic similarity between the two species, which may hinder or even disable their correct molecular identification from unknown samples based on *MT-CO1* barcode sequence.

Studies of HENDRICH *et al*. (2015) involved 15 948 specimens of beetles from Central Europe and indicated that 92.2% of them can be unambiguously assigned to one known species based on the diversity *MT-CO1* sequence, while 6.8% of the specimens have been assigned to more than one barcode index number (BIN). This is also referred, as in our study, to *Miarus ajugae* whose sequence obtained by these authors was used in this paper as a reference. This requires further testing to determine if this is due to the presence of cryptic species, mitochondrial introgression or regional variability in widespread species.

On the other hand, PENTINSAARI *et al*. (2014) found that as many as 98.3% of the surveyed species had a distinctive sequences of DNA barcodes. Among them there were also specimens belonging to *Cleopomiarus graminis* and *Cleopomiarus distinctus*, whose DNA barcodes deposited in the BOLD database were used as sequences of reference in this research. The observed sequence divergence between the nearest neighbor species were significantly higher than those reported previously for butterflies (11.99% vs 5.80%). The maximum intraspecific divergence was growing and the average divergence between the nearest neighbors was decreasing with increasing number of samples, although these trends rarely hindered the identification based on DNA barcodes because of deep differences in sequence between most species. As in the studies of HENDRICH *et al.* (2015), again 92.1% compatibility was found between the sequences of known species and BIN sequences in BOLD database.

DNA barcodes have proven to be a useful tool for the molecular determination of species in many higher insects taxa, including the beetles. Among others, the effectiveness of DNA barcode to distinguish beetles species of the genus *Bembidion* was tested. Correspondence between molecular identification and traditionally recognized species was 89%. Low maximum interspecies distances of less than 2.2% was found for the three pairs of species, including two pairs of species sharing haplotype. The same time, deep intraspecies sequence divergences have been revealed for two species (RAUPACH *et al*. 2016).

Analyses using the identification of insects species on the basis of *MT-CO1* barcode sequence are undoubtedly highly effective and are a valuable source of information about the biodiversity of this group. However, it should be noted that the difficulties in differentiating species of insects are a common problem, also with the support of the identification by molecular methods. In this study it was found possible to assign specimens on larvae (L2, L3) and pupae stage to the right genus – *Miarus* or *Cleopomiarus*. In contrast, species level identification, especially within the genus *Miarus*, at the current state of knowledge and DNA barcodes databases resources, unfortunately do not allow unambiguous assignment of unknown individuals. It is mainly due to the fact that several species of *Miarus* and *Cleopomiarus*, currently considered as valid taxa, can be distinguished only by a few subtle morphological differences in the external morphology as well as in the genitalia, especially of the male. Therefore, for some of these, it is still impossible to establish whether they are valid, perhaps recently diverged species, or populations of a single species. Moreover, available data show a strong similarity in *MT-CO1* in some species. Following research must be supported and

complemented with an analysis of subsequent mitochondrial and nuclear DNA fragments characterized by high informativeness.

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