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RAPID COMMUNICATON

The molecular identification and phylogeny of moths feeding on cereals, belonging to *Cnephasia* species based on cytochrome *c* oxidase subunit I gene

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Abstract

The genus *Cnephasia* is represented by more than 70 species of insects worldwide, including serious pests of agricultural plants, mainly cereals. Since members of this genus are frequently very similar externally, species determination based on morphotaxonomy is time-consuming and difficult, especially for non-taxonomists. Hence, it could possibly be replaced by molecular biology approaches. A short nucleotide sequence of mitochondrial cytochrome oxidase I (*mtCOI*) constitutes a commonly used molecular marker for phylogenetic analyses identification. The aim of this work was molecular species determination of leaf rollers, collected in Poland, that on the basis of external features were hardly distinguishable. We amplified, sequenced and phylogenetically studied the fragment of the *mtCOI* gene for each individual. Comparative analyses showed the highest nucleotide similarity to *C. genitalana*, *C. longana, C. pasiuana, C. asseclana* and *C. stephensiana*, which was also confirmed by phylogeny. Obtained results showed genetic variation of the analyzed fragment of the *mtCOI* gene between analyzed *Cnephasia* spp. found in Poland that can be helpful in proper species determination. This in turn, may be essential for successful biological control and pest monitoring in crop cultivation.

Keywords: cytochrome c oxidase, *Cnephasia*, mitochondrial DNA, species identification

The genus *Cnephasia* Curtis 1826, belongs to the *Tortricidae* family. It currently is comprised of more than 450 species of moths and butterflies, including many pests. Larvae are expansive polyphages, typically leaf rollers, that under favorable conditions cause significant losses in agriculture. In Europe, two species have great economic importance – *C. pumicana* and *C. longana,* reducing mainly cereal crops. In turn, in the USA *C. longana* noticeably reduces strawberry production yield (Powell and Opler 2009). Recently, in Poland, both of these species have increased their range and abundance. However, crop losses do not influence the economy.

The identification of *Cnephasia* species relies mainly on genital morphometry. However, in some cases it is still laborious and problematic (mainly in the case of females). Determination of caterpillars, especially the younger larval stages is also difficult, especially for non-taxonomists. Therefore, to enhance identification molecular and bioinformatic tools become advantageous. Currently, the use of short nucleotide sequences of nuclear, mitochondrial or chloroplast DNA (in plants) is being successfully used in taxonomic studies (Purty and Chatterjee 2016). Among animals, the most useful and satisfactory DNA marker for species identification is mitochondrial cytochrome *c* oxidase I (*mtCOI*) (Dinsdale *et al.* 2010; Purty and Chatterjee 2016). Phylogenetic analyses and molecular species determination based on *mtCOI* have been reported for various insects (Hajibabaei *et al.* 2006; Obrępalska-Stęplowska *et al.* 2008; Nowaczyk *et al.* 2009; Akhilesh and Sebastian 2013; Ghosh *et al.* 2019).

The aim of this study was to characterize the partial sequence of *mtCOI* for 19 specimens of *Cnephasia* found in cereals in Poland followed by phylogenetic analysis. The obtained sequences had similar lengths, however nucleotide variability between species was noticeable and thus it can be useful for species determination.

Imagines and larvae feeding on cereals were collected in Greater Poland (Poznań, Winna Góra – experimental station of Institute of Plant Protection – National Research Institute). They were then bred under laboratory conditions until adulthood. Due to the difficulty in species determination based on external morphology, genitalia preparations were necessary. Therefore, detached abdomens of moths were macerated in 10% KOH. After cleaning and rinsing in distilled water, the prepared genitals were stained with a glycerol solution of chlorazole black. Using an Olympus SZ60 stereo microscope visual identification was performed according to Razowski (2002). Insect samples were stored in 70% ethyl alcohol until molecular tests.

DNA was isolated from the leg with NucleoSpin Tissue kit (Machery-Nagel, Germany). The partial *mtCOI* gene was amplified with specific primers LepF1 5'ATTCAACCAATCATAAAGATATTGG3', LepR1 5'TAAACTTCTGGATGTCCAAAAAATCA3' (Hebert *et al.* 2004). Polymerase chain reaction (PCR) profiles were as follows: denaturation at 95°C for 3 min, 8 cycles at 95°C for 30 s, 45°C for 30 s, 72°C for 40 s, followed by 35 cycles: 95°C for 30 s, 51°C for 30 s, 72°C for 40 s, final elongation at 72°C for 5 min. Electrophoretically analyzed PCR product size was 658 bp. DNA was extracted from the gel and then molecular cloning was performed (Obrępalska-Stęplowska *et al.* 2008). Positively recombined plasmids were sequenced. The obtained sequencing results were compared with sequences deposited in GenBank and analyzed using BLASTn tool. Among 243 of partial *mtCOI* sequences of *Cnephasia* species available in GenBank, about 174 sequences showed similar lengths (658 nts). They were downloaded and ClustalW multiple sequence alignment was created followed by phylogenetic study based on the neighbour-joining (NJ) method (Saitou and Nei 1987) in MEGAX software (Kumar *et al.* 2018).

According to external characters as well as the morphology of genitalia the analyzed individuals were designated as the following *Cnephasia* species: *C. stephensiana* – 2 exx.; *C. genitalana* – 6 exx.; *C. pumicana* – 5 exx.; *C. asseclana* – 3 exx.; *C. l*ongana – 3 exx.

To improve, as well as to verify the species determination in relation to morphological criteria, genetic differentiation analysis of the *mtCOI* gene of Polish representatives was undertaken. The PCR reactions gave a product of 658 bp (Fig. 1).

The obtained nucleotide sequences were analyzed using BLASTn and showed the highest identity of collected specimens to five species: *C. pasiuana*, *C. longana*, *C. stephensiana, C. asseclana* and *C. genitalana*. The percentage of nucleotide identity in relation to the above mentioned species was very high (from 98.9 to 100%). Similarly, the sequences of collected individuals differed from each other showing 89.9% to 100% nucleotide identity. Using MEGAX program, 201 variable sites were indicated (Fig. 2) that might influence species classification. Evolutionary studies were inferred using neighbour-joining criteria. The topology of the generated tree showed distance between

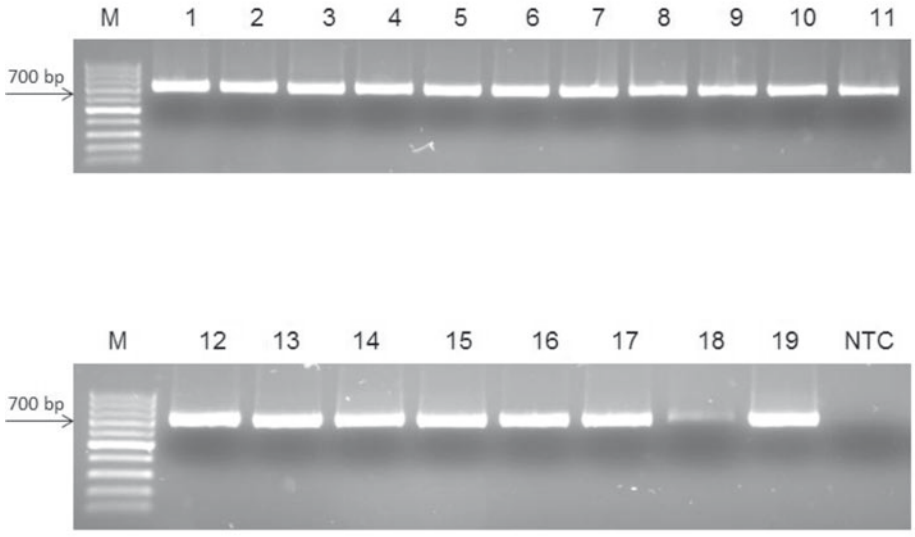

Fig. 1. Electrophoresis of PCR products of *mtCOI***.** From left to right: M – DNA ladder, GeneRuler 100 bp (ThermoFisher, USA), 1–19 specimens collected in Poland, NTC – no template control

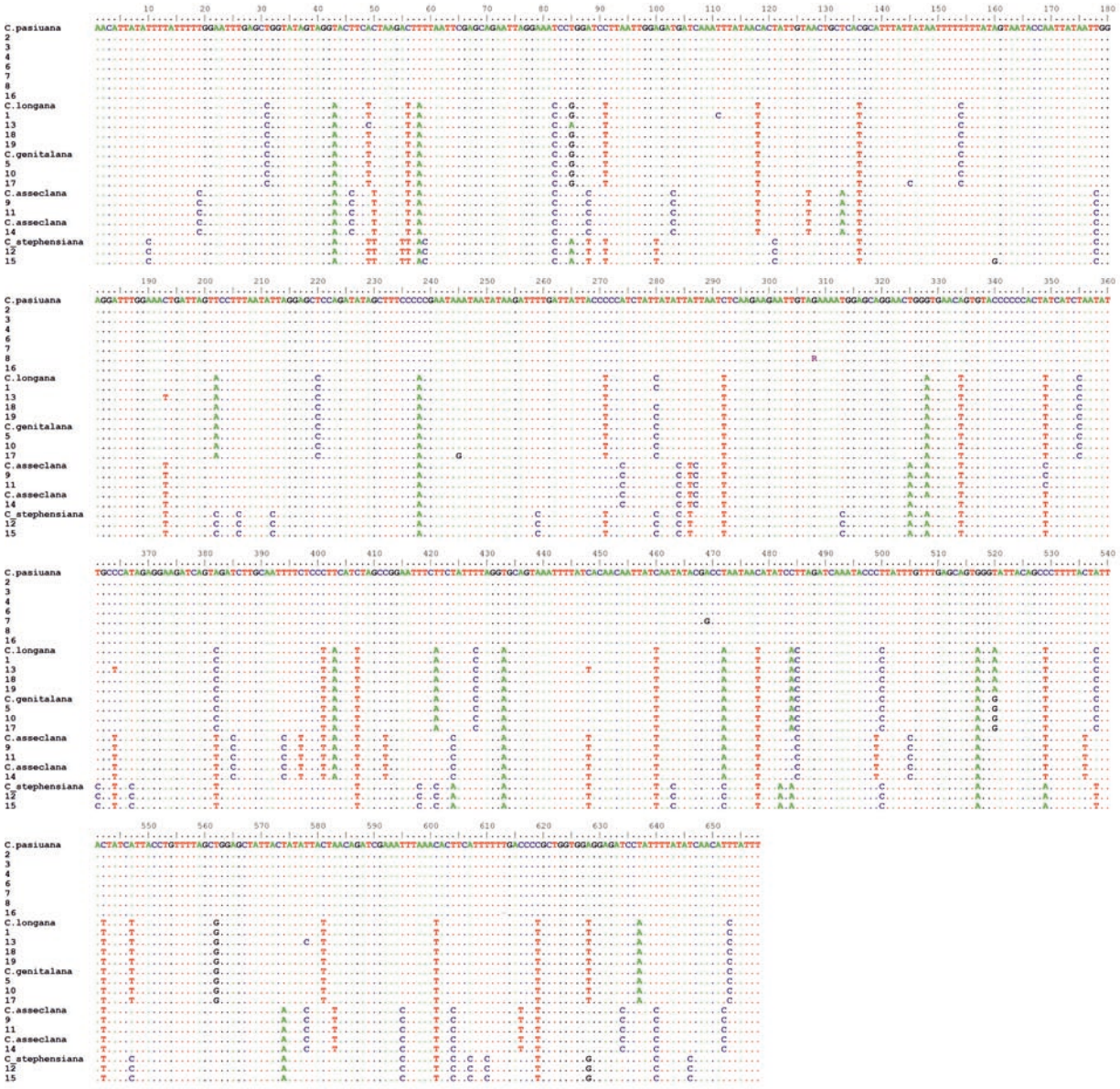

Fig. 2. The multiple sequence alignment of partial *mtCOI* gene of *Cnephasia* species. Analysis included sequences of individuals collected in Poland and the most similar sequences of *Cnephasia* species downloaded from Genbank (KX045015 *C. pasiuana*, JN286428 *C. longana*, KP253394 *C. genitalana*, GU706865 and GU706488 *C. asseclana*, GU706862 *C. stephensiana*). The sequences are grouped according to their similarity to known species

analyzed taxa. The specimens of *Cnephasia* collected in Poland were divided into two main clads inside of which several species groups were created (Fig. 3). In the first clad, individuals numbered 9, 11, 14 and 12, 15 were assigned. The first three 9, 11, 14 grouped with *C. asseclana*, whereas 12 and 15 showed short evolutionary distance to *C. stephensiana*. The remaining were grouped in the second clad, which seemed to be more phylogenetically differentiated. The individuals 2 – 4, 6 – 8 and 16 showed the highest genetic correlation with *C. pasiuana*. Specimen 13 was situated on a separate branch, and showed short evolutionary distance with 5, 10, 17, which in turn were clustered with representatives of *C. genitalana*. In this group, the separate taxon created by specimens 1, 18 and 19 were clustered together with *C. longana*.

Interestingly on the basis of the topology of the generated dendrogram the sequences of *C. pasiuana*, downloaded from Genbank, were genetically distant, thus the members were divided into two separate groups. It is noteworthy that in public databases the sequences of *C. pumicana* are lacking. In consideration of the fact that *C. pasiuana* and *C. pumicana* are morphologically difficult to distinguish, as well as the fact that they were previously synonymized (Langmaid *et al.* 2010), there is a far-reaching supposition that these records were mistakenly assigned. Furthermore, several specimens analyzed in this work were identified

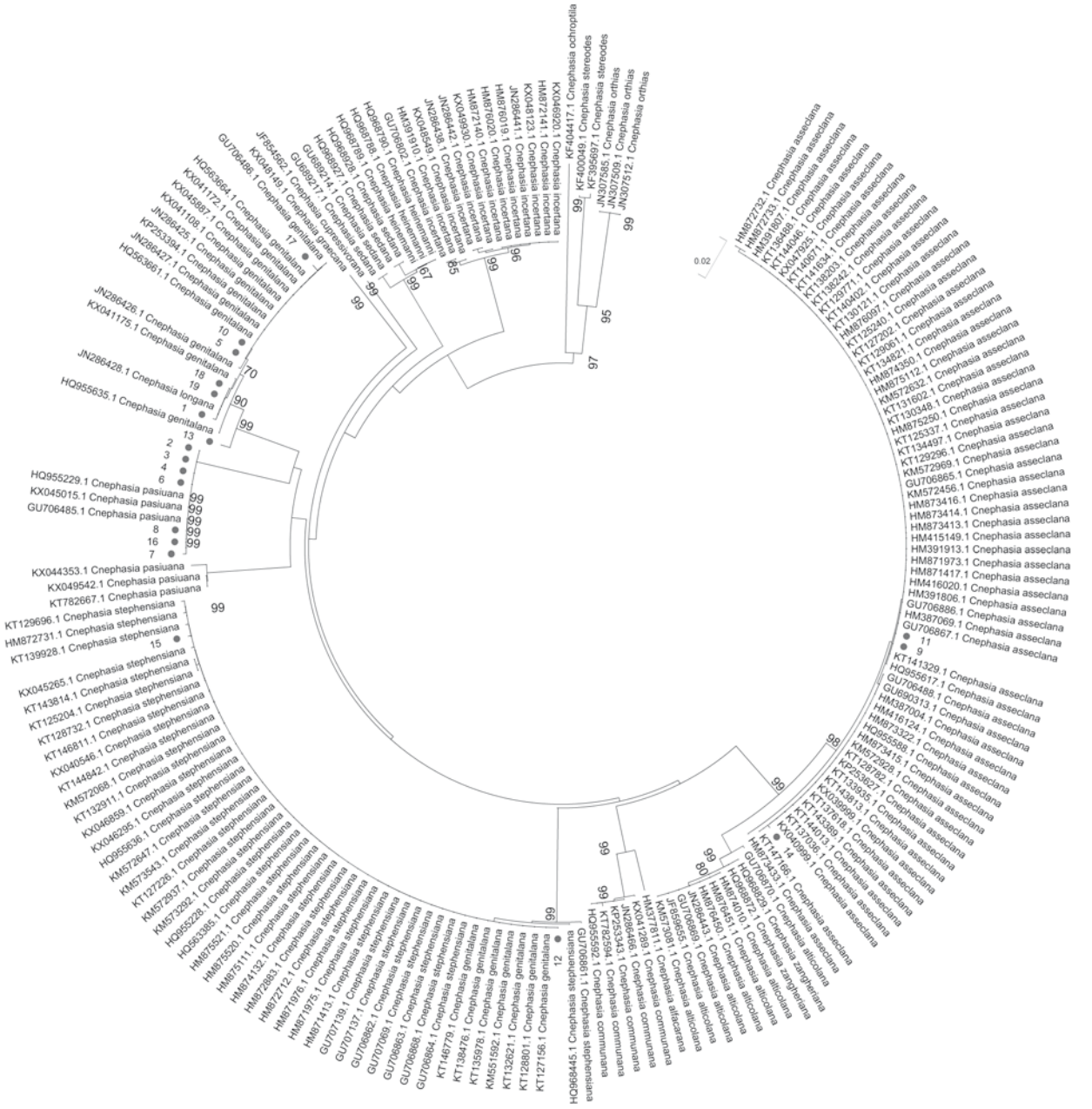

Fig. 3. Evolutionary relationships of specimens of *Cnephasia* collected in Poland. Tree generated in MEGAX using neighbor-joining method and 1,000 bootstrap values. The scale bar represents a genetic distance

morphometrically, according to Razowski (2002), as *C. pumicana,* but on the basis of phylogeny they were grouped with *C. pasiuana*. Regardless, in order to make proper morphological and molecular identification it is necessary to collect many more specimens of both species for further testing.

In conclusion, analyzed sequences of *mtCOI* of *Cnephasia* collected in Poland indicated interspecies genetic variability that might be helpful in their taxonomic classification. Due to the lack of molecular research regarding *Cnephasia* species, the obtained results are the basis for further development of rapid molecular approaches for species determination, such as real-time PCR with the use of TaqMan probes or restriction fragment length polymorphism PCR, that in turn might be a useful tool for effective pest control strategies.

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