Ukrainian Journal of Ecology

Ukrainian Journal of Ecology, 2017, 7(3), 134–138, doi: 10.15421/2017_61

ORIGINAL ARTICLE *UDC 595.78(94)*

The taxonomic status of *Cossus cossus afghanistanus* (Lepidoptera, Cossidae) from Afghanistan: insights from molecular and morphological data

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Submitted: 12.07.2017. Accepted: 15.08.2017

In our study we use a 658 bp fragment of the *COI* gene to analyze a taxon from Afghanistan usually treated in literature as *Cossus cossus afghanistanus* (Daniel, 1953). The previous conclusions on taxonomy and nomenclature were not supported by molecular data therefore the question of identity of this taxon has remained unverified. Phylogenetic analysis revealed *C. c. afghanistanus* to be strongly differentiated from nominotypical *Cossus cossus* (Linnaeus, 1758) (p-distance: 6.7% ± 1.5%). *Cossus c. afghanistanus* forms a distinct well-supported clade in ML and BI trees. This fact, together with prominent morphological differences (wing color and genitalia structure) shows that *C. c. afghanistanus* represent a separate species rather than a subspecies of *Cossus cossus*.
Key words: Lepidoptera, Cossidae, DNA barcode, Afghanistan.

Introduction

Cossus cossus afghanistanus (*Cossus cossus afghanistana* in original description) was described by Daniel (1953) as a separate subspecies on the basis of two male specimens from Central Afghanistan (Wardik (holotype) and Kabul (paratype)). Afghan specimens differ from those found in Central Europe in having light brown wing color. Wing pattern was considered as a major diagnostic character to distinguish between the two taxa (Daniel, 1953) (Figs. 1a, b).

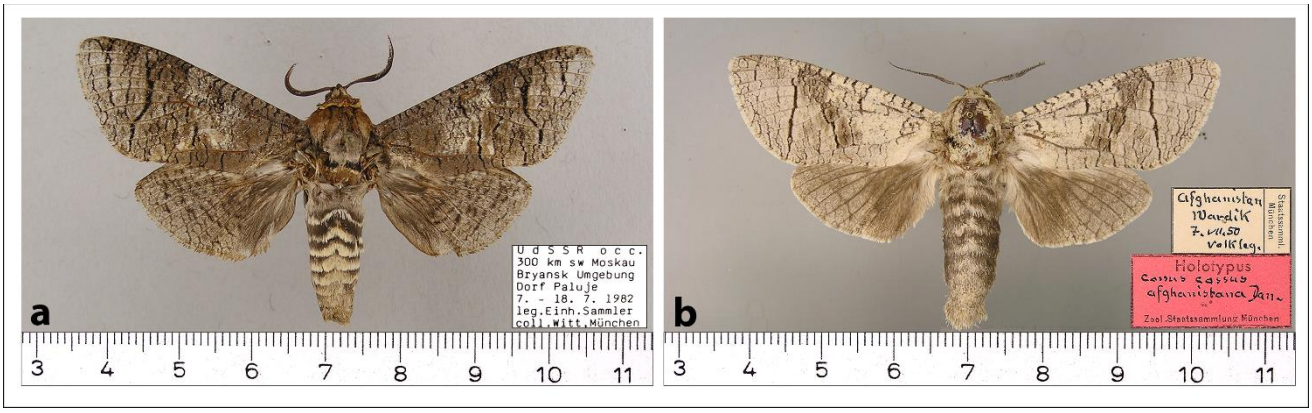

Figure 1. Males wing pattern of *Cossus cossus* (**a,** Bryansk, Russia) and *Cossus afghanistanus* (**b,** Afghanistan, Wardik; holotype,).

Recently, a morphological analysis showed that male genitalic structures of *C. c. afghanistanus* differ from those found in nominotypical *C. c. cossus* (Linnaeus, 1758) in having a smooth ridge on costal edge of valva, short processes of transtilla, and a slender phallus (Figs. 2a, b) (Yakovlev, 2009).

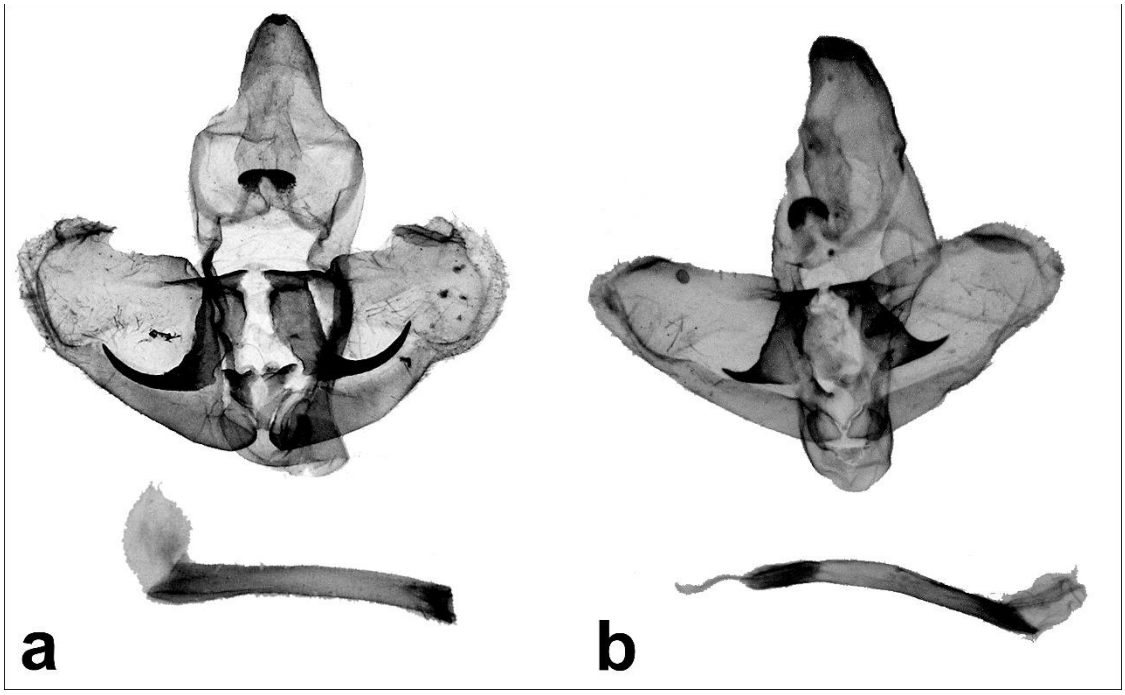

Figure 2. Male genitalia of *Cossus cossus* (**a,** Germany) and *Cossus afghanistanus* (**b,** Afghanistan, Panjir Valley).

Based on wing coloration and male genitalic structures, *C. c. afghanistanus* was treated by Yakovlev (2009) at the species level. *Cossus cossus afghanistanus* is known from the following specimens from Central Afghanistan (Yakovlev et al., 2015):
1 male (holotype), Afghanistan, Wardik, 7.VII.1950, Volk leg. (ZSM);
1 male, Kabul (ZMMU);
9 males, Afghanistan, Paghman, 30 km NW v. Kabul, 2100 m, 20-30.VII.1963, leg. Kasy & Vartian (MNHW);
2 males, Hindukush, bei Rukha, 2400 m, 21.V.1977, de Freina (MWM);
3 males, 30 km NW Kabul, 2500 m, 15.V.1965 (MWM);
1 male, Paghman, 3000 m, 06.VII.1973 (MWM);
2 males, Kabul prov., Tangi (MWM);
3 males, Khurd-Kabul (MWM);
2 males, Panjir Valley, Kotul e Shava, Astana, 2500 m, 2005 (MWM).
Cossus cossus has not been reported from Afghanistan, however this species is known from adjacent countries (large series of specimens are available from southern Kazakhstan, Kyrgyzstan, northwest China, Uzbekistan, and Tadzhikistan). Apparently, the material mentioned above can be assigned to subspecific taxon *C. c. tianshanus* Hua, Chou, Fang et Chen, 1990 (type locality: Manas, Xinjiang Uyghur Autonomous Region of northwestern China [90 km NWW Urumchi]).
It should be noted, that analyses based primarily on external morphological characters fail always clarify the phylogenetic position and identities of many Lepidoptera taxa (Hajibabaei et al., 2006; Lukhtanov et al., 2008; Dincă et al., 2013; Shapoval & Lukhtanov, 2016). *Cossus c. afghanistanus* has never been studied genetically, therefore its identification, taxonomic status and phylogenetic position have remained unconfirmed. Our paper provides first molecular analysis of this taxon.

Abbreviations used

MNHW	Museum Natural History (Wien, Austria);
MWM	Museum of Thomas Witt (Munich, Germany);
RYB	collection of Roman Yakovlev (Barnaul, Russia);
ZMMU	Zoological Museum at Moscow State University (Moscow, Russia);
ZSSM	Zoologische Staatssammlung München (Munich, Germany).

Material and methods

One specimen of *C. c. afghanistanus* (RYB) was collected by I. Pljushtch, O. Pak and Ju. Skryluik on 09.VI.2016 in Kabul (Afghanistan). The specimen (GenBank accession number MF596151) was processed at the Department of Karyosystematics of the Zoological Institute of the Russian Academy of Sciences. DNA extraction from a single leg removed from the specimen was accomplished using the QIAamp DNA Investigator Kit (Qiagen, Netherlands) following the manufacturer's protocol. Standard lepidopteran barcode primers (Hebert et al. 2004) were used for DNA amplification and resulted in a 658 bp fragment of the *mitochondrial cytochrome oxidase I* gene (*COI*). The PCR amplification was performed in a 50 µl reaction volume containing ca.

10-20 ng genomic DNA and 0.5 mM each of forward and reverse primer, 1 mM dNTPs, 10x PCR Buffer (0.01mM Tris-HCl, 0.05M KCl, 0.1% Triton X-100: pH 9.0), 1 unit Taq DNA Polymerase (Thermo Fisher Scientific, Lithuania), 5mM $MgCl_2$.
The temperature profile was as follows: initial denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 1 min with a final extension at 72°C for 10 min. Amplified fragment was purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Lithuania). Purification was carried out according to the manufacturer's protocol. The success of PCR amplification and purification was evaluated by electrophoresis of the product in 1% agarose gel. Purified PCR product was used for direct sequencing. Sequencing of the double-stranded product was carried out at the Research Resource Center for Molecular and Cell Technologies (St. Petersburg State University).
Representatives of nominotypical *C. cossus* were included in our analysis to clarify the taxonomic position and phylogenetic relationships of *C. c. afghanistanus*. We use obtained in the present study sequence of *Dyspessa salicicola* (Eversmann, 1848) from Azerbaijan (GenBank accession number MF596152) as an outgroup to root the phylogram. A complete list of specimens included in this study is given in Table 1.
A Bayesian approach and maximum-likelihood (ML) analyses were used for estimating the phylogeny. Bayesian analysis was performed using the software MrBayes 3.1.2 with the nucleotide substitution model GTR+G+I. jModelTest was used to determine optimal substitution models for Bayesian inference (BI) analysis (Posada, 2008). TRACER, version 1.4 was used for summarizing the results of the Bayesian phylogenetic analysis (http://beast.bio.ed.ac.uk/Tracer).
Maximum-likelihood (ML) analysis was performed using MEGA6 software (Tamura et al., 2013). We used non-parametric bootstrap values (Felsenstein, 1985) to estimate branch support on the reconstructed ML tree. Branch support was inferred from 1000 bootstrap replicates.

Table 1. List of studied material (29 specimens). (*) – sequence obtained in the present study. (**) – sequence obtained in the present study and used as an outgroup.

GenBank /BOLD Accession Number	Species	Locality
MF596152	*Dyspessa salicicola***	Azerbaijan
MF596151	*Cossus afghanistanus**	Afghanistan, Kabul
FBLMV612-09	*Cossus cossus*	Germany, Bavaria
GWORL447-09	*Cossus cossus*	Germany, Bavaria
PHLAG857-12	*Cossus cossus*	Germany, Bavaria
FBLMX240-11	*Cossus cossus*	Germany, Bavaria, Oberpfalz
ODOPE725-11	*Cossus cossus*	Germany, Bavaria
ODOPE724-11	*Cossus cossus*	Germany, Bavaria
GWOR4165-09	*Cossus cossus*	Germany, Bavaria
PHLAG864-12	*Cossus cossus*	Austria, Nord Tirol
PHLAG865-12	*Cossus cossus*	Austria, Nord Tirol
PHLAG866-12	*Cossus cossus*	Austria, Nord Tirol
LEATD168-13	*Cossus cossus*	Austria, Nord Tirol
LEATD248-13	*Cossus cossus*	Austria, Nord Tirol
PHLAI331-13	*Cossus cossus*	Austria, Nord Tirol
PHLAI332-13	*Cossus cossus*	Austria, Nord Tirol
PHLAI333-13	*Cossus cossus*	Austria, Nord Tirol
PHLAH053-12	*Cossus cossus*	Austria, Carinthia
PHLAG862-12	*Cossus cossus*	Austria, South Tirol
PHLAG872-12	*Cossus cossus*	Austria, Voralberg
LEFIA1067-10	*Cossus cossus*	Finland, Uusimaa
LEFIB066-10	*Cossus cossus*	Finland, Lapland
LEFID742-10	*Cossus cossus*	Finland, Aland Islands
PHLAH054-12	*Cossus cossus*	Romania
PHLAG859-12	*Cossus cossus*	Italy, Sicily
GWORZ171-10	*Cossus cossus*	Italy, Calabria
PHLAC470-10	*Cossus cossus*	Italy, South Tirol
PHLAG861-12	*Cossus cossus*	Italy, South Tirol
PHLAH357-12	*Cossus cossus*	Spain, Valenciana

Results and discussion

Phylogenetic analysis of the gene *COI* resulted in a consensus phylogram which indicated a high level of posterior probability and bootstrap values for the clades (Fig. 3). Both (BI and ML) analyses demonstrated that all the *Cossus* individuals formed two discrete, statistically supported clades. The first clade includes specimen of *C. c. afghanistanus*. The second clade is formed by the representatives of nominotypical *C. cossus*. Moreover, analysis of a dataset of 29 specimens recognized *C. c. afghanistanus* as a highly differentiated and strongly supported lineage with a basal position. *Cossus c. afghanistanus* differs from nominotypical *C. cossus* by numerous nucleotide substitutions (p-distance is 6.7% ± 1.5%).

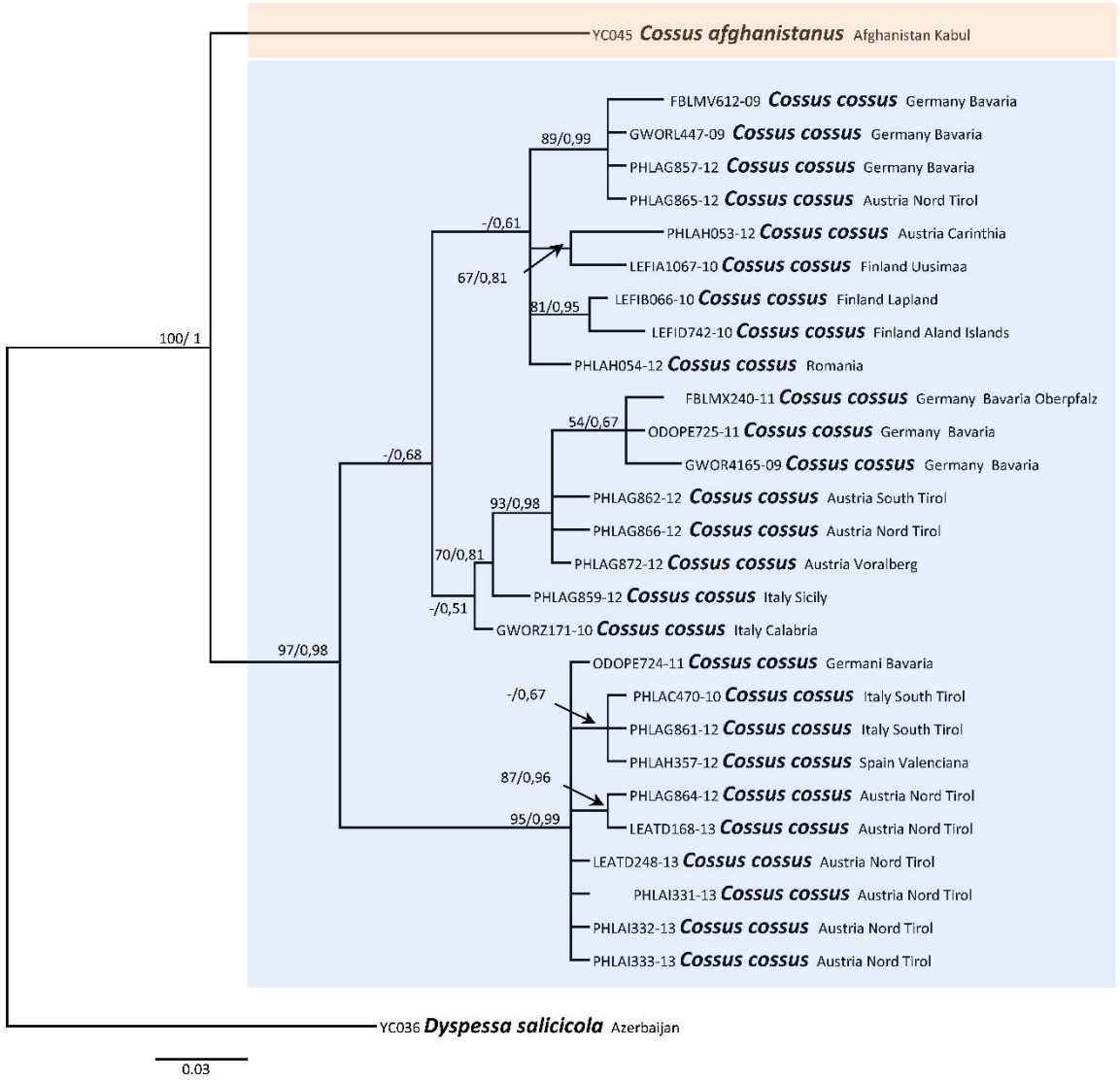

Figure 3. The Bayesian tree of *Cossus afghanistanus* and *Cossus cossus* based on analysis of the *cytochrome c oxidase subunit I* gene from 28 specimens. Numbers at nodes indicate ML bootstrap/ Bayesian posterior probability respectively, with non-matching clades using different analyses indicated by '–'. Scale bar = 0.3 substitutions per position. *Cossus afghanistanus* and *Cossus cossus* clusters highlighted in pink and blue respectively.

The *COI* gene as a part of mitochondrial DNA (mtDNA) is a widely accepted and frequently used marker in molecular and phylogenetic studies. mtDNA has numerous advantages, including lack of recombination due to uniparental inheritance and multicopy status in most cells (Avise, 2000). Furthermore, due to relatively higher substitution rates in the mitochondrial genome, mtDNA is generally less conserved than many nuclear genes, providing higher resolution for lower level phylogenies and species identification through DNA barcoding (Hebert et al., 2003). Nevertheless, several authors have pointed out that the phylogenetic studies, or species identification based on mtDNA alone, can be misleading as trees inferred from single markers sometimes display relationships that reflect the evolutionary history of individual genes rather than the species being studied (Ballard & Whitlock, 2004; Bensch et al., 2006). Moreover, mitochondrial introgression (Zakharov et al., 2009) and *Wolbachia* infection (Ritter et al., 2013) also can lead to erroneous phylogenetic reconstructions and species misidentification.

Despite these limitations, level of genetic distances can provide indirect evidence for conspecifity/non-conspecifity of species being studied. Two allopatric taxa can be considered as different species if the *COI* distance exceeds the "standard" 2.7–3.0% DNA-barcoding threshold (Lambert et al., 2005). Although this level is not an absolute threshold to distinguish between species, it was demonstrated that such a deep level (ca. 3%) of differentiation between *COI* barcodes is practically always associated with species level of the taxa compared (Hebert et al., 2003). Thus, considerable level of genetic distances can be used as a useful criterion while deciding on the taxonomic status of a group under analysis and inferring hypotheses about species borders (Lukhtanov, 2015). Furthermore, the presence of significant genetic distances between two taxa accompanied by solid morphological differences (e.g. in wing pattern, and/or in genitalia structure) can be considered as an additional independent evidence of the existence of two distinct species.

Cossus c. afghanistanus and *C. cossus* appear on our reconstruction as a two strongly differentiated monophyletic groups, and the p-distance between their *COI* barcodes (6.7% ± 1.5%) significantly exceeds the 2.7–3.0% DNA-barcoding threshold. Additionally, *C. c. afghanistanus* and nominotypical *C. cossus* differ in characters of wing coloration and male genitalic structure. Thus, in accordance with the criteria mentioned above they should be considered as a separate species.

Acknowledgements

The financial support for this study was provided by the grant from the Russian Science Foundation no. 14-14-00541 to the Zoological Institute of Russian Academy of Sciences. The work was partially performed using equipment of Centre for Molecular and Cell Technologies of St. Petersburg State University. The authors are grateful to Prof. Boris Kondratieff (Fort Colins) for language editing.

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Citation:
Shapoval, N.A., Yakovlev, R.V., Kuftina, G.N. (2017). The taxonomic status of Cossus cossus afghanistanus (Lepidoptera, Cossidae) from Afghanistan: insights from molecular and morphological data. *Ukrainian Journal of Ecology, 7*(3), 134–138.