

***Quedius umbrinus*-complex (Coleoptera, Staphylinidae): updates and insights from nuclear gene data**

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Abstract

To assess the accuracy of the species composition of the *Q. umbrinus* complex proposed earlier by us (Salnitska and Solodovnikov 2021) using an integrative approach, we performed additional molecular analyses based on updated mitochondrial COI data and newly obtained nuclear wg data. Only DNA grade material was used in this study, as the combination of morphological and molecular data is necessary for effective species delimitation within this species complex. All phylogenetic analyses based on the separate COI and wg genes and the concatenated dataset confirmed the subdivision into four species: *Q. umbrinus*, *Q. pseudoumbrinus*, *Q. sigwalti* and *Q. volkeri*. The only discordance in the topologies between mitochondrial and nuclear genes was observed in the monophyly of *Q. umbrinus* complex which may be explained by conserved nature of nuclear gene. Most phylogenetic and species delimitation analyses revealed a significant subclade within *Q. pseudoumbrinus* comprising specimens from the Irkutsk Province, namely from the type locality of the already synonymized *Q. angaricus*. Even though the genetic distance of this subclade from other *Q. pseudoumbrinus* specimens reaches 4.1 %, we did not resurrect it due to the absence of the any distinct morphological characters.

Keywords

Coleoptera, *Quedius*, wingless, DNA-barcoding, species delimitation

Introduction

Quedius umbrinus Erichson, 1839 is a widely distributed West Palaearctic species characterized by the considerable morphological variability that led taxonomists to describe numerous closely related species (Sahlberg 1876; Last 1955; Coiffait 1967; Stourač 1998; Assing 2018 etc.) thereby forming a species complex.

The first serious effort to revise this complex group of species has been undertaken only in the last decade. Assing (2018), based on morphology only, synonymized 11 species within the *Q. umbrinus* complex, considering the differences described for each species as intraspecific variability. Later, Salnitska and Solodovnikov, 2021 revised the species complex using an integrative approach that combining morphological and molecular data, including deep analysis of the original descriptions. According to this research *Q. umbrinus* complex consists of four species *Q. sigwalti* Coiffait, 1972, *Q. umbrinus*, *Q. volkeri* Salnitska & Solodovnikov, 2021 and *Q. pseudoumbrinus* Lohse, 1958. Nevertheless, only *Q. sigwalti* is characterized by the presence of clear morphological characters, whereas others differentiated only by weak and subtle characters. Meanwhile, molecular data, particularly from the mitochondrial COI gene, show stronger differences and provide support for each species.

The mitochondrial gene COI is the most commonly used marker (“golden standard”) for barcoding since it shows strong congruence with morphology-based species delimitations and is widely used in taxonomic practice (Hendrich et al. 2015; Pentinsaari et al. 2019).

A number of species delimitation studies in Staphylinidae have been based on COI alone, in combination with morphology, with other mitochondrial or nuclear genes, or with both. Using COI alone, Chatzimanolis and Caterino (2007) examined the phylogeographic structure of *Sepedophilus castaneus*, Serri et al. (2016) tested the intraspecific genetic variation in Steninae. Using the barcoding fragment of COI in combination with morphology, Brunke et al. (2020a, b) explored species limits in the genus *Quedionuchus* and *Quedius*, Lee et al. (2020) in the genus *Coprophilus* and Hansen and Jenkins Shaw (2023) in the genus *Lobrathium*.

However, comprehensive species revisions nowadays require additional molecular data to validate and test COI based results. Usually, a combination of mitochondrial and nuclear gene fragments provides the most reliable results in species delimitation, particularly in Coleoptera and Staphylinidae (Song and Ahn 2014; Lee et al. 2020; Muñoz-Tobar and Caterino 2020; Yoo et al. 2021, 2022; Hansen and Jenkins Shaw 2023). Thus, for example, Song and Ahn (2014) assessed the accuracy of species delimitation and phylogenetic relationships within the *Aleochara fucicola* species complex using two mitochondrial (COI and COII) and three nuclear genes (CAD, EF1- α and Wg). Muñoz-Tobar and Caterino (2020) used the mitochondrial COI and the nuclear Wg genes to examine the concordance of morphological characters and geography with hypothesized species boundaries in the genus *Panabachia*.

The aim of this research was to evaluate the composition of *Q. umbrinus* complex, previously proposed based only on mitochondrial gene COI and morphological data (Salnitska and Solodovnikov 2021). This study was based on morphological and molecular data, including the mitochondrial gene COI and the additional nuclear gene wingless. The molecular dataset consisted of sequences obtained in previous research (Salnitska and Solodovnikov 2021), downloaded from GenBank and BOLD, and newly generated sequences obtained in this research. A representative sample of specimens covering the entire distribution area of *Q. umbrinus* complex was examined, including the westernmost specimens from Iceland and the easternmost from Irkutsk province of Russia. However, in this study only DNA grade material was used for the analysis, as the combination of morphological and molecular data is essential for effective species delimitation within *Q. umbrinus* complex, especially for distinguishing *Q. umbrinus* and *Q. pseudoumbrinus*. The molecular data were analyzed using phylogenetic and species delimitation methods, and genetic distance measurements, to assess the congruence among the results obtained by different methods. The structure of male genitalia was examined to delimit species identity within *Q. umbrinus* complex. This integrative approach allowed the validation of previously established species and the identification of a lineage encompassing specimens from the type locality of the previously synonymized *Q. angaricus*, which may represent a separate species.

Materials and methods

Specimen acquisition

Material for this study was examined from the collections of the following institutions: UTMN– University of Tyumen, Tyumen, Russia; NHMD Natural History Museum of Denmark at the University of Copenhagen that includes the Zoological Museum; ZIN– Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia. DNA sequences generated for this study have been submitted to GenBank. All geographic localities for the material in this study are shown in Fig. 1A, which was produced using QGIS 2.18.22 based on coordinates given on the labels or found by us when toponyms on the labels were recorded only verbally. In some cases, coordinates were found using online systems such as Google Maps. All examined material is listed in the Suppl. material 1: Table S1.

Examination of morphological characters

The material was examined under the Zeiss dissecting scopes Stemi 305 and Stereo Discovery V8. Genitalia was photographed with the Toups Camera UC-MOS05100KPA attached to Lomo MSP-2 ver. 2 scope. Photographs of the aedeagi were done from soft preparations of these structures in glycerin after dissecting.

Dissected aedeagi are kept in vials with 96% alcohol within the voucher specimens or in case of dry specimens permanently kept in glycerin-filled micro vials pinned under their respective specimens.

Taxon sampling for molecular analysis

The following sequences were examined in this study: 76 were taken from GenBank or BOLD (including all sequences of *Q. umbrinus* complex from Salnitska and Solodovnikov, 2021) and 77 were obtained in this research. Of them, 13 specimens were taken as outgroup for the following species: *Q. peregrinus* Gravenhorst, 1806, *Q. beesoni* Cameron, 1932, *Q. obliquieseriatus* Eppelsheim, 1889, *Q. capucinus* (Gravenhorst, 1806) and *Q. paraboops* Coiffait, 1975. DNA-grade specimens were used from material collected by authors during this research and earlier expeditions across Europe and Russia and some material obtained from colleagues (Suppl. material 1: Table S1).

Molecular work

The molecular work within the current study was performed in the Laboratory of Insect Systematics and Phylogenetics of the Institute of Environmental and Agricultural Biology (X-BIO), University of Tyumen (Tyumen, Russia) and the research Centre Chromas Core Facility of St. Petersburg State University (St. Petersburg, Russia).

DNA extraction, amplification and cleaning

DNA was extracted from the legs of each of the specimens, using Evrogen Extract DNA Blood and tissue Kit (Evrogen, Moscow, Russia, www.evrogen.ru), D-Tissues, animal tissues DNA isolation kit (Biolabmix, Novosibirsk, <https://biolabmix.ru/en/>) or the phenol-chloroform method. In the first two cases, DNA was extracted according to the manufacturer protocols or in the case of phenol chloroform according to the protocol used in Salnitska and Solodovnikov (2021). Extracted DNA was stored in Eppendorf tubes at -20 °C.

The conditions for obtaining COI amplicons and sequences were as follows. The pair of primers used for amplification was LCO1490 and HCO2198 (Folmer et al. 1994). The typical PCR was conducted using BioMaster HS-TaQ PCR (2×) (Biolabmix, Novosibirsk) in volume of 25 µL containing 1–2 µL genomic DNA and 23 µL mix containing 100 mM Tris-HCl (pH 8.5 at 25 °C) 100 mM KCl, 0.4 mM of each deoxynucleoside triphosphate, 4 mM MgCl₂, 0.06 U/µL Taq DNA polymerase, 0.2% Tween 20 and stabilizers of HS-TaQ DNA polymerase. Cycling parameters were as follows: initial denaturation 3min at 94°C, followed by 35 cycles at 30s at 94°C, 30s at 45–52°C (depending on the sample and reagent conditions), 1min at 72°C, and final elongation 5min at 72°C.

The amplification procedure for wg were performed using a nested reaction (Wild and Maddison 2008; Schomann and Solodovnikov 2017) with the abovementioned PCR kit. The first reaction with the external primers (Wg550F–WgAbRZ) consisted of an initial denaturation for 3 min at 94 °C, 35 cycles of: 30 s of denaturing at 94 °C, 30 s of primer annealing at 53 °C and 1.5 min of extension at 72 °C, followed by a 5 min final extension at 72°C. The product of the first pair is then used as a template for a reaction with the internal primers (Wg578F–WgAbR), consisting of the same temperature profile as the external primers.

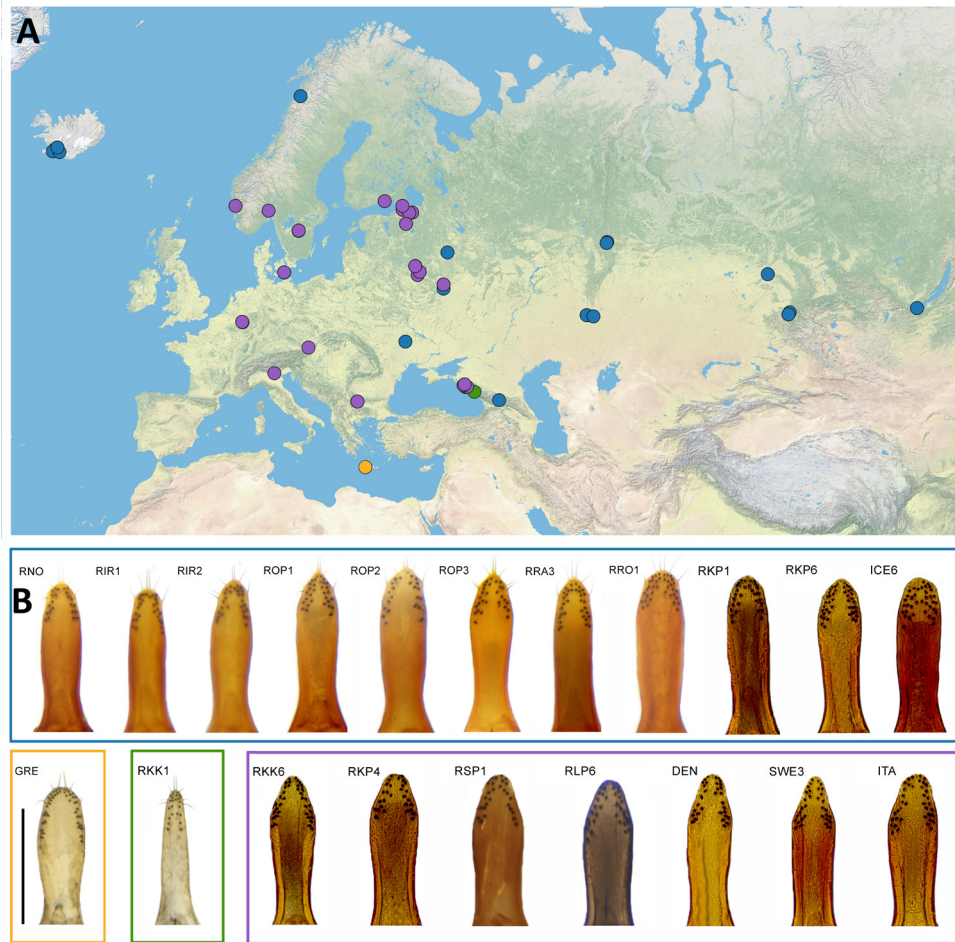


Figure 1. A. Distribution map of the *Q. umbrinus*-complex showing records for the specimens examined in this paper. Orange circle – *Q. sigwalti*, green – *Q. volkeri*, purple – *Q. umbrinus* and blue – *Q. pseudoumbrinus*. B. Variability of the paramere within species of the *Q. umbrinus*-complex, species are separated by frames coloured the same as the distribution circles. Scale bar – 1 mm.

Tagged primers with 13 bp tags (HM001-HM004, HM006-HM0010 and HL003-HL008) as described in Srivathsan et al. (2021) were used for the amplification of 23 samples (both COI and wg) (see Suppl. material 2: Table S2) sequenced by nanopore sequencing. In both cases the final PCR products were visualized in 1% agarose gel by electrophoresis and the gel was visualized with ethidium bromide under UV-illuminator apparatus to visualize the DNA bands.

The DNA cleaning was conducted using cleaning kit Cleanup S Cap (Evrogen, Moscow) according to the manufacturer's protocol.

Sequencing and preparing data for analyses

Sanger. The 54 cleaned products were sent for Sanger sequencing to Evrogen company (Moscow, Russia) and the rest of the products were sequenced on our own using nanopore sequencing technology (see below).

Sanger sequences were generated in both directions (with forward and reverse primers).

Library preparation, sequencing and processing of data got used nanopore sequencing technology. To prepare the DNA library, 1 µl of each of the purified PCR products was used. The DNA concentration of the resulting amplicon pool was measured using the Qubit™ 1X dsDNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific, USA). Followed by a DNA repair and end-prep, and adapter ligation and clean-up steps using the NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing (New England Biolabs, Germany), Native Barcoding Kit 24 V14 (Oxford Nanopore Technologies, UK) and AMPure XP Beads (Beckman Coulter, USA) according to the Oxford Nanopore Technologies protocols (ACDE_9163_v114_revAB_24Sep2025).

The DNA library was loaded onto the Flongle flow cell FLO-MIN114 (R10.4.1) connected to the MinION sequencer (Oxford Nanopore Technologies, UK) using the Flow Cell Priming Kit and Native Barcoding Kits 24 V14. Sequencing was run under the MinKNOW ver. 25.03.9 (Oxford Nanopore Technologies) with high-accuracy model for basecalling.

Two files were prepared for demultiplexing as input for ONTBarcoder 2.3 (Srivathsan et al. 2021). The input FastQ file was obtained by unzipping and merging all raw MinKNOW FastQ files using the bash commands in Linux terminal. The demultiplexing file was generated using Python script, where we imported two CSV files containing specimen metadata (specimen code, species name, locality) and the tag combination table. The resulting file followed the template from the ONTBarcoder GitHub repository https://github.com/asrivathsan/ONTbarcoder/blob/main/samples/MixedDipteraSubsample_demfile. All prepared files were imported into ONTBarcoder under MODE 1. Demultiplexing and consensus sequence generation were performed under default settings.

The DNA sequences obtained in this study have been also submitted to GenBank database (PZ510999-PZ511055, PZ384263-PZ384274, PZ432597-PZ432608).

Data editing

The sequence editing, alignment and check for the stop codons were carried out in MEGA XI (Tamura et al. 2021) using the ClustalW algorithm (Thompson et al. 1994) with the default settings.

Molecular phylogenetic analyses

The model selection under Bayesian Information Criterion (BIC) was performed using jModelTest (Darriba et al. 2012) on CIPRES (<https://www.phylo.org>) (Miller et al. 2010). The Bayesian Inference (BI) analysis was performed using MrBayes 3.2.7 (Ronquist et al. 2012) on local laptop. The analysis was run twice using 4 simultaneous chains for 2 000 000 to 5 000 000 generations (depending on the value of ASDSF, see below) with tree sampling each 1000 generations and discarding each 25 % as burn-in region. Convergence was checked manually for the following indicators: the stationary distribution plot of the log-likelihood values against a number of generations, the average standard deviation of split frequencies (ASDSF) (lower than 0.01), Potential Scale Reduction Factor (PSRF) (> 100) and Estimated Sample Size (EES) (1.0 almost per all parameters).

The Maximum Likelihood (ML) analysis was performed using IQ-tree (Wong et al. 2026) on CIPRES with the default setting except the selected by jModelTest model and an additional test of support was carried out with Ultrafast Bootstrap (UFB) (10 000 iterations).

The resulting trees were visualized and edited for illustrations using FigTree v1.4.4 (Rambaut 2018) and AdobePhotoshop CS5.

Species delimitation and haplotype network analyses

Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al. 2021) was performed on the Spart explorer web portal (<https://spartexplorer.mnhn.fr/delimitation>) with the default settings. In the analysis, both the Jukes-Cantor (JC69) and Kimura (K80) models for substitutions were used.

The updated Bayesian variant of PTP method (bPTP) was run on a web portal (<https://species.h-its.org/ptp/>) based on tree obtained by the Bayesian analysis mentioned above with the following options 500,000 generations, thinning every 1000 and 0.25 burn-in. The convergence was evaluated by visually checking the likelihood plot in the output files on the server.

The estimates of evolutionary divergence (p-distance) between sequences were computed in MEGA XI.

The haplotype network was generated in PopART (Leigh and Bryant 2015), to improve the accuracy, the outgroup was excluded from the analysis.

Results

Phylogeny of *Q. umbrinus*-complex

In total, 153 sequences were included to the final dataset: 82 COI and 71 wg sequences. The number of specimens represented by both COI and wg sequences amounted of 63 samples. The outgroup consisted of 6 sequences in COI dataset (*Q. capucinus*, *Q. peregrinus*, *Q. obliqueseriatus* and *Q. boops*) and 7 in wg dataset (*Q. capucinus*, *Q. peregrinus*, *Q. beelsoni*, *Q. obliqueseriatus* and *Q. boops*). The COI alignments consisted of 651 bp, whereas wg of 417 bp.

All species names provided according to species composition previously established by Salnitska and Solodovnikov (2021).

COI gene trees. The single partition evolutionary model selected by jModelTest for the COI dataset were HKY+G. The BI analysis successfully reached convergence after 2 million generations.

The trees obtained from both ML and BI analyses showed the same topologies (see Suppl. materials 3 & 4: Figs S1 & S2). The monophyly of *Q. umbrinus*-complex was highly supported (PP=100, UFB=99.4 and SH-aLRT=100) in both analyses as well as three clades bearing *Q. sigwalti* (PP=100, UFB=99.4 and SH-aLRT=100), *Q. umbrinus*+*Q. volkeri* (PP=73, UFB=67.4 and SH-aLRT=7) and *Q. pseudoumbrinus* (PP=100, UFB=93.6 and SH-aLRT=96). *Quedius volkeri* is cladded as sister to *Q. umbrinus* with relatively lower to other clades but still strong support (PP=100, UFB=99.4, SH-aLRT=99). The monophyly and unity of *Q. volkeri* clade is strong PP=100, UFB=100 and SH-aLRT=100. *Quedius umbrinus* clade comprised three high supported subclades representing local populations or general intraspecific variability. The *Quedius pseudoumbrinus* clade also comprised three highly supported subclades one of which (RIR1-RIR3, NO1) represented by the specimens from the type locality of the previously synonymized *Q. angaricus* (Listvyanka, Irkutsk Province) and a single specimen from Norway.

Wg gene trees. The BI and ML analyses were performed under the single partition evolutionary model K80+G for wg dataset. The BI analysis successfully reached convergence after 2 million generations.

The trees obtained from both ML and BI analyses showed almost identical topologies (see Suppl. materials 5 & 6: Figs S3 & S4). The monophyly of the in-group was not supported in both analyses and the clade comprising *Q. boops* and *Q. capucinus* was recovered as a sister to the taxa of *Q. umbrinus*-complex, but with the relatively low support – PP=59, UFB=81.8 and SH-aLRT=57. In both analyses *Q. umbrinus*-complex represented by two clades *Q. volkeri*+*Q. pseudoumbrinus* (PP=77, UFB=85.4 and SH-aLRT=76) and *Q. sigwalti*+*Q. umbrinus* (PP=99, UFB=91.9 and SH-aLRT=92). Both clades in both analyses were mostly unresolved and formed polytomies, except for the several subclades. The first subclade within *Q. volkeri*+*Q. pseudoumbrinus* clade comprised specimens from Irkutsk Province, Novosibirsk Province, Republic of Altai and Norway (PP=100, UFB=92.5 and SH-

aLRT=98). The second subclade within *Q. sigwalti*+*Q. umbrinus* clade comprised specimens from Krasnodar Territory (PP=97, UFB=84.7 and SH-aLRT=74).

Concatenated gene trees. The BI and ML analyses for the concatenated dataset were run under the respective evolutionary models mentioned above. The BI analysis successfully reached convergence after 5 million generations.

The trees obtained from both ML and BI analyses showed slightly different topologies (Fig. 2).

The monophyly of the ingroup was strongly supported (PP=100, UFB=99.4 and SH-aLRT=97) in both analyses. The BI topology comprised four clades corresponding to the four respective species *Q. sigwalti* (PP=100), *Q. volkeri* (PP=100), *Q. umbrinus* (PP=99) and *Q. pseudoumbrinus* (PP=100). While the ML comprised two clades uniting *Q. volkeri*+*Q. pseudoumbrinus* (UFB=83.4, SH-aLRT=71) and *Q. sigwalti*+*Q. umbrinus* (UFB=92.5, SH-aLRT=86). The rest subclades coincide except for subclade appears in ML (UFB=96.4, SH-aLRT=57) with the specimens from Irkutsk Province, Republic of Altai, Novosibirsk Province and Norway. The subclade with the specimens from Listvyanka plus specimen from Norway is highly supported in both analyses (PP=100, UFB=100 and SH-aLRT=100).

Species delimitation analyses

The ASAP analysis was conducted separately for COI and wg markers, whereas the PTP analysis was performed using the concatenated dataset.

The species delimitation analyses were congruent in recognizing *Q. sigwalti*, *Q. umbrinus* and *Q. umbrinus*+*Q. volkeri* as a distinct species-level lineages.

The ASAP analysis of the COI dataset revealed five partitions with the lowest ASAP scores of 1.50 under the Jukes-Cantor model and 2.50 under the K80 model. These partitions corresponded to *Q. sigwalti*, *Q. volkeri*, *Q. umbrinus*, a partial *Q. pseudoumbrinus* lineage including specimens from Irkutsk Province, Novosibirsk Province, the Altai Republic, and Norway, and the remaining specimens of *Q. pseudoumbrinus*.

The ASAP analysis of the wg dataset revealed five partitions with the lowest ASAP scores of 1.50 under the Jukes-Cantor model and 2.00 under the K80. These partitions corresponded to *Q. sigwalti*, *Q. volkeri*+*Q. pseudoumbrinus* (partially), a partial *Q. pseudoumbrinus* lineage including specimens from Irkutsk Province, Novosibirsk Province, the Altai Republic, and Norway, a partial *Q. umbrinus* lineage including specimens from Krasnodar Territory and the remaining specimens of *Q. umbrinus*.

The PTP analysis of the concatenated dataset showed the most fragmented pattern, recovering 13 groupings, including several represented by a single specimens, within the four lineages *Q. volkeri*, *Q. pseudoumbrinus*, *Q. sigwalti* and *Q. umbrinus* (Fig. 2).

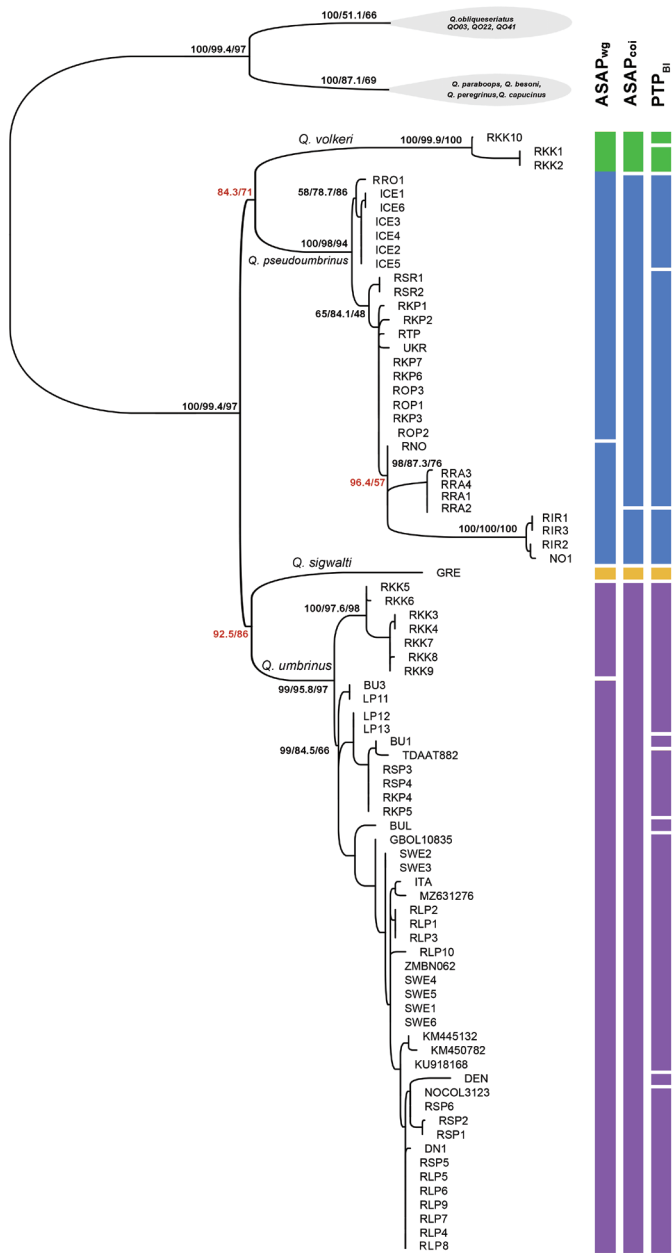


Figure 2. Phylogenetic tree obtained with Bayesian and Maximum Likelihood analyses of the concatenated COI+wg gene dataset, with support values. Posterior Probability (PP) ≥ 50 , Ultrafast Bootstrap (UFB) ≥ 50 and Shimodaira-Hasegawa-like approximate likelihood-ratio test (SH-aLRT) ≥ 40 , are given at the respective nodes. Support values are highlighted in red when the clade is not supported by the Bayesian analysis. Bars on the right side show clades resulting from species delimitation analyses as follows: ASAP (Assemble Species by Automatic Partitioning) based on COI and wg respectively and bPTPBI (the Bayesian variant of the Poisson tree process model based Bayesian tree).

Divergence and haplotype network analysis

The divergence (p-distance) between sequences within the *Q. umbrinus*-complex based on COI dataset does not exceed 8.1 %, within *Q. pseudoumbrinus* clade does not exceed 4.3 % and within *Q. umbrinus* does not exceed 2.9 % (see Suppl. material 7: Table S3). The divergence between sequences within the *Q. umbrinus*-complex based on wg dataset does not exceed 1.9 %, within *Q. pseudoumbrinus* clade does not exceed 1.7 % and within *Q. umbrinus* does not exceed 1.4 % (see Suppl. material 8: Table S4).

The haplotype analysis revealed 29 haplotypes in COI dataset (76 sequences) and 12 haplotypes in wg dataset (64 sequences) clustering into four distinct haplogroups corresponding to the four respective species. The COI network (Fig. 3) represented by a chain-like haplotype structure, with the number of substitutions between haplogroups ranging from 9 to 27. The wg network represented by a reticulated haplotype structure, with a lower number of substitutions between haplogroups, ranging from 1 to 3. In both cases, the haplotypes with the specimens from Irkutsk Province and Norway were distinct, although in the wg network they grouped together with specimens from the Altai Republic and Novosibirsk Province.

Morphology

The examination of the male genitalia of the newly examined specimens from the Republic of North Ossetia-Alania (RRO1), Orenburg Province (ROP1-ROP3), Irkutsk Province (IR1-IR3), the Altai Republic (RRA3, RRA4), and Norway revealed that they undoubtedly belong to *Q. pseudoumbrinus* (Fig. 2B). The specimen from Denmark belong to *Q. umbrinus*.

Discussion

The concept of the division of the *Q. umbrinus*-complex into four species, proposed by Salnitska and Solodonikov (2021), was generally supported by morphological and molecular data from the newly examined specimens and the nuclear wg gene analyzed in this study.

However, comparison between of the COI and wg topologies revealed a few significant discrepancies. The first, important mismatch concerned the monophyly of the *Q. umbrinus*-complex which was confirmed with high support by BI and ML analyses of both the COI and concatenated datasets. Meanwhile, the ingroup within the topology obtained based on wg dataset consists of *Q. umbrinus*-complex taxa together with *Q. boops* and *Q. capucinus* with the relatively lower support. This placement is highly controversial because neither species is closely related to *Q. umbrinus* and moreover *Q. capucinus* belongs to the distinct subgenus *Distichalius*. At the same time, the species potentially closer to *Q. umbrinus* – *Q. oblique-*

riatus clearly falls within the outgroup. Most likely, this discrepancy occurred due to the nature of nuclear genes, which are known to have lower resolution at the species and genus levels and are usually used in phylogenetic reconstructions at higher levels (Wild and Maddison 2008). The composition of the outgroup may also reflect the topology. However, the outgroup was compiled based on the limited wg sequences of *Quedius* available in GenBank and it was impossible to assemble a more representative sample including both closely and distantly related taxa.

The second mismatch concerned the relationships among the members of the *Q. umbrinus*-complex species. It differs depending on the gene and analysis and was represented the following combinations 1) *Q. sigwalti*, *Q. pseudoumbrinus*, *Q. umbrinus*+*Q. volkeri* (both wg trees), 2) *Q. pseudoumbrinus*+*Q. volkeri* and *Q. sigwalti*+*Q. umbrinus* (both COI trees and concatenated ML analysis), 3) *Q. sigwalti*, *Q. volkeri*, *Q. umbrinus*, *Q. pseudoumbrinus* (concatenated BI analysis). This indicates that both genes are sufficiently informative to delimit species lineages but insufficient for resolving relationships at the interspecific level. Thus, at present, it is impossible to reliably determine the relationships among the members of the *Q. umbrinus*-complex based solely on COI and wg. Additional relevant genes are needed for the more precise determination of the relationships within the complex.

The third mismatch concerned the groupings within clades. In most cases, these groupings were consistent and represented by evenly distributed specimens within a clade, reflecting genetic variability within species without strong geographic patterns. However, it is worth to mentioning the specimens examined in this research at first from Irkutsk Province and the Altai Republic. The groupings were as follows: specimens from Irkutsk Province+Norway and the Altai Republic+Novosibirsk Province (COI ML, COI BI, concatenated dataset BI) formed separate subclades or all specimens were split in one subclade (wg ML, wg BI, concatenated dataset ML) in one subclade. The separation between these groupings was also supported by the ASAP^{COI}, PTP and haplotype network analyses. Interestingly that the specimens from Irkutsk Province originated from the type locality of the previously synonymized *Q. angaricus* Coiffait, 1975 (Salnitska and Solodovnikov 2021). The genetic distance between the Irkutsk Province+Norway subclade from other specimens within *Q. pseudoumbrinus* clade reaches 4.1 % within COI and 1.7 % within wg, which is relatively high for the interspecific level. We were unable to find any significant differences in the external morphology or the structure of the aedeagus of the respective specimens. As stated in the original description of *Q. angaricus* (Coiffait, 1975), the specimens from Listvyanka are characterized by the absence of wings. However, this feature is not unique within the *Q. umbrinus*-complex and represents only an expression of wing polymorphism. Therefore, apparently *Q. umbrinus*, as the widely distributed West Palaearctic species, demonstrates a broad range of genetic variability. Nevertheless, specific morphological approaches, such as scanning electron microscopy or the examination of the endophallus structure, may shed light on this ambiguous case. At present, *Q. angaricus* should stay in synonymy with *Q. pseudoumbrinus*.

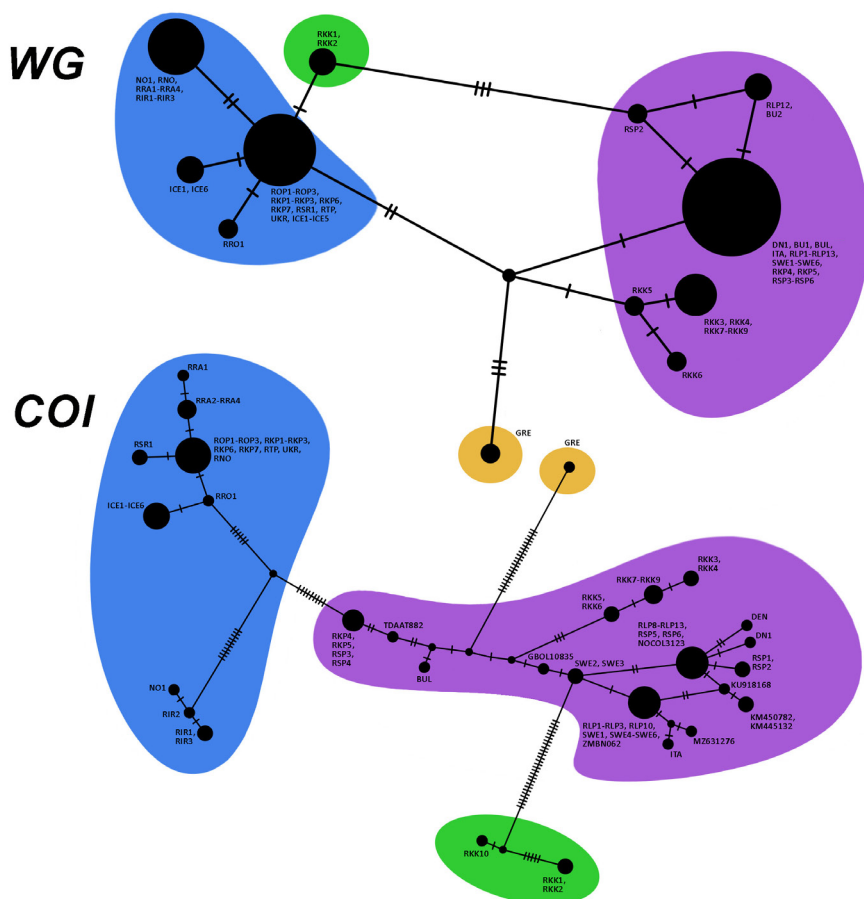


Figure 3. Haplotype network of *Q. umbrinus*-complex based on CO1 and wg gene obtained with TCS using PopArt. Each circle represents a unique haplotype found among the sequenced specimens of *Q. umbrinus*-complex.

The species delimitation analyses, and haplotype network almost unanimously supported the subdivision of the *Q. umbrinus*-complex into four species, except for the ASAP^{wg} analysis united which did not support *Q. volkeri* as a separate species. However, apparently the wg gene, as a more slowly evolving marker did not accumulate sufficient mutations in the case of the endemic species *Q. volkeri*.

The fact that the newly examined specimens from the eastern localities within the distribution range *Q. umbrinus*-complex (Russia: Orenburg, Irkutsk Province and the Altai Republic) belong to *Q. pseudoumbrinus* is highly predictable. So far *Q. pseudoumbrinus* has been known as the species with the widest distribution range within *Q. umbrinus*-complex, extending from Iceland through Europe to

Irkutsk Province and Middle Asia (Salnitska and Solodovnikov 2021, 2022). Two other specimens from the Republic of North Ossetia-Alania in Russia and Norway also belongs to *Q. pseudoumbrinus* and one from Denmark belongs to *Q. umbrinus* were found from the territories with the overlapping distribution of *Q. umbrinus* and *Q. pseudoumbrinus*. The morphology of the respective specimens fully conforms to the accepted combination of aedeagal characters for both species: parallel sides of paramere, tapering only near the apex, and an oval shaped apical portion in *Q. pseudoumbrinus* and distinctly converging sides of the paramere in the middle portion, along with a rhomboid apical portion, in *Q. umbrinus*.

The sequences from the GenBank and BOLD databases included in the COI dataset originating from Norway, Finland, France and Austria, fall within the *Q. umbrinus* clade, which matches their identifications in the databases. Based on the photo associated with the specimen IDs we were able to verify the species identity of the following specimens: MZ631276 from Finland, NOCLP3214-22 from Norway and KM445132.1 from Germany.

Conclusion

1. The nuclear wg gene is too conservative for the studying intra- and interspecific relationships within *Q. umbrinus*-complex on its own, but it may serve as a useful complement to a major markers such as the mitochondrial COI genes.
2. The analyses of all phylogenies obtained in this study support the subdivision of the *Q. umbrinus*-complex, as proposed by Salnitska and Solodovnikov (2021): *Q. sigwalti*, *Q. volkeri*, *Q. umbrinus* and *Q. pseudoumbrinus*. The mismatches between the topologies maybe explained by the nature of the genes or by data limitations and are insufficient to justify any taxonomic changes.
3. The subclade containing specimens from Irkutsk Province and Norway most likely represents a broad range of genetic variability within *Q. umbrinus* species. However, a more detailed morphological study is needed for accurate confirmation of this hypothesis.

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Supplementary material 1

Table S1. List of specimens examined in this paper, with the respective data: species names, codes, including those from GenBank, locality and other collecting details, preservation and depository information, and sequencing details

Author: Maria Salnitska, Angesom Gebremeskel

Data type: table

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Supplementary material 2

Table S2. Tags used for indexing specimens for nanopore sequencing

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Data type: table

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Supplementary material 3

Figure S1. Phylogenetic tree obtained with Maximum Likelihood analysis based on the COI dataset

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Supplementary material 4

Figure S2. Phylogenetic tree obtained with Bayesian analysis based on the COI dataset

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Data type: image

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Supplementary material 5

Figure S3. Phylogenetic tree obtained with Maximum Likelihood analysis based on the wg dataset

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Supplementary material 6

Figure S4. Phylogenetic tree obtained with Bayesian analysis based on the wg dataset

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Data type: image

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Supplementary material 7

Table S3. Genetic distance among all CO1 sequences within the *Q. umbrinus*-complex analyzed in this paper

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Data type: table

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Supplementary material 8

Table S4. Genetic distance among all wg sequences within the *Q. umbrinus*-complex analyzed in this paper

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Data type: table

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