



Clarification of the taxonomic status of the North American clerid *Thanasimus nubilus* stat. rev. (Coleoptera: Cleridae) by DNA barcodes and morphology

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Abstract

The North American checkered beetle (Cleridae) *Thanasimus nubilus* Klug, 1842 is currently listed as a subspecies of *Thanasimus undatulus* (Say, 1835). We test the species status of *T. nubilus* by investigation of DNA barcodes and morphological measurements of multiple specimens of each of the two taxa as well as of multiple specimens of two additional North American species of the genus. We use four species delimitation methods based on gene tree and clustering algorithms (barcode gap threshold clustering, Assemble Species by Automatic Partitioning [ASAP], and single and multi-rate Poisson Tree Processes [mPTP]). Evidence from mtDNA clearly supported treating these two subspecies as distinct species which was also supported by a morphometric analysis. We therefore propose *Thanasimus nubilus* Klug, 1842 **stat. rev.** to be reinstated as a valid species.

Key words

Thanasimus undatulus, integrative taxonomy, species delimitation, *cox1*

1. Introduction

The checkered beetles (Coleoptera: Cleridae) of the Nearctic region are relatively well-known taxonomically, although some species-rich genera (e.g. *Cymatodera*, *Enoclerus*, *Phyllobaenus*) need more revisionary work. Among them is the Holarctic genus *Thanasimus* Latreille, 1806, aka the ant beetles. *Thanasimus formicarius* (Liné, 1758) (originally in *Attelabus*), a Palearctic repre-

sentative, belongs to the first five beetles described by Linné (1758) within the family Cleridae, which Latreille (1806) made the type species of the genus *Thanasimus*. Their common name, ‘ant beetles’ is a misnomer, because it refers to their similarity to the aposematic coloration of the wasps known as Velvet Ants (Mutillidae). *Thanasimus* beetles prey on a wide range of scolytid beetles (Cur-

culionidae: Scolytinae) and therefore play an important role as pest control in forestry. They are mainly found on the true firs (*Abies*), true spruces (*Picea*) and Douglas fir (*Pseudotsuga*) where they are attracted to both scolytid pheromones and to volatiles produced by damaged host trees. However, despite being the subject of a wealth of publications on chemical responses to kairomones and other substances (Aukema and Raffa 2002; Bakke and Kvamme 1981; Costa and Reeve 2011; Erbilgin and Raffa 2001; Hellmund 2013; Herms et al. 1991; Poland, T.M.; Borden 1997; Zhou et al. 2001), the taxonomy is not satisfyingly established for all species.

Nearctic *Thanasimus* species comprise *T. dubius* (Fabricius, 1776), *T. repandus* Horn, 1871, *T. trifasciatus* (Say, 1825), *T. undatulus* (Say, 1835), and possibly the introduced *T. formicarius* (Linné, 1758). After *Thanasimus undatulus* underwent some taxonomic confusion as described below, it currently has two subspecies, *T. u. undatulus* and *T. u. nubilus*. Since specimens of the two subspecies have often been collected in the same locations (Webster et al. 2016), the subspecies status of *T. undatulus undatulus* and *T. undatulus nubilus* is an open question. *Thanasimus undatulus* was first described by Say (1835) as *Clerus undatulus*. Kirby (1837) gave a description of *T. undatulus nubilus* as *T. abdominalis*. Klug (1842) indicated that the epithet *abdominalis* was already used by Germar for the description of clerid specimens from East India and by Chevrolat for the description of Mexican clerid specimens. As such, Klug (1842) proposed *nubilus* as a replacement species name for the North American specimens. LeConte (1863) listed *T. undatulus* Say and *nubilus* Klug as valid species for North America and mentioned *T. abdominalis* Kirby “vix a praec. differt.” under *nubilus*. Horn (1876) indicated in a side sentence that *nubilus* is a variety of *undatulus*. Still, Wickham (1895) included *T. undatulus* Say and *nubilus* Klug in his work on the Cleridae of Ontario and Quebec. Wolcott

(1909) again indicated that *nubilus* Klug was a variety of *T. undatulus* Say. Also Schenkling (1910) indicated the var. *nubilus* Klug from North America under the species *T. undatulus* Say. From that point on, modern authors have considered *nubilus* Klug as a variety or subspecies of *T. undatulus* Say (Blackwelder and Blackwelder 1948; Bousquet et al. 2013; Chapman et al. 2025; Downie and Arnett 1996; Knoll 1951; van Dyke 1923; Wolcott 1947). However, consistent morphological differences exist between *nubilus* Klug and *T. undatulus* Say and their distributions overlap. Likewise, currently available molecular data (www.boldsystems.org, accessed 2025-08-06) of the two species show considerable differentiation which has never been properly investigated, which we set out to do.

We applied contemporary species delimitation methods on available and newly sequenced DNA barcode data of seven species of the genus *Thanasimus* including *T. undatulus* and *T. nubilus*. Evidence from morphometric measurements of body length of the two species complemented the results of DNA barcoding and allowed a conclusion on the species status of *T. nubilus*. Results from mtDNA gave hints to an interesting biogeographic history of the investigated species.

2. Material and Methods

2.1. Sampling

We analyzed DNA barcodes and/or morphology of a total of 274 adult *Thanasimus* specimens (DNA: 233, morphology: 49, DNA and morphology could be investigated for three of these individuals) of six ingroup and four outgroup species (Table S1; Fig. 1). We personally investigated or at least saw pictures of 153 specimens (DNA:

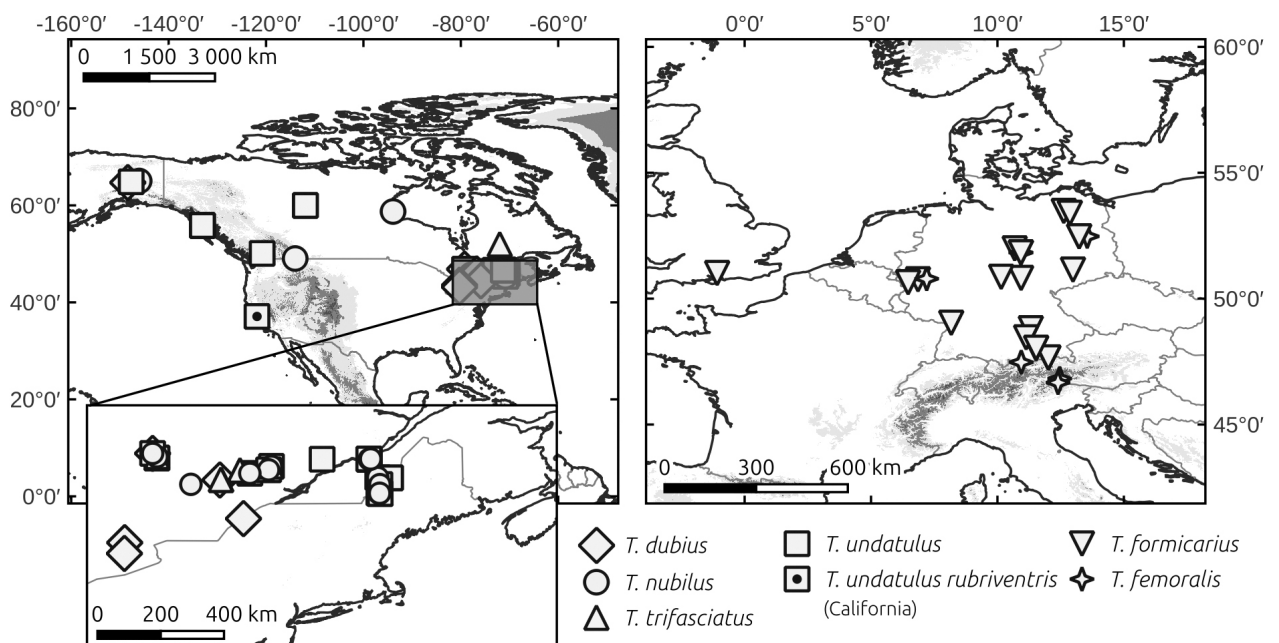


Figure 1. Geographic distribution of DNA samples.

107, morphology: 49, both: 3) from 89 locations. These 153 specimens were used in all reported analyses, however, a gene tree including all 233 DNA sequences was additionally estimated. Thirty-nine existing DNA barcodes were taken from Gerstmeier et al. (2019), 36 that had a photograph and had less than 2% missing data were downloaded from the BOLD (www.boldsystems.org, accessed 2025-08-06), and 119 additional public sequences determined to the genus *Thanasimus* were likewise downloaded from the BOLD, some of which were generated by Sikes et al. (2017). No records of *T. repandus* could be acquired. We newly sequenced 32 specimens that were collected as part of a work on the detection of invasive alien species with alpha-pinene and ethanol baited funnel traps (Lindgren 1983) by the Forest Pest Management Services from the Province of Québec's Ministère des Ressources naturelles et des Forêts from the months of May to September in 2020 and 2021. The collecting jars contained propylene glycol. After harvesting the samples, insects were visually identified and kept in ethanol at -20°C in 2 ml vials until further analysis. One specimen of the subspecies *T. undatulus rubriventris* was collected by hand in ethanol. For molecular work, *T. undatulus* (Say 1835) and *T. nubilus* (Klug 1842) were represented by 33 and 20 specimens, respectively. At eight localities, syntopic specimens from both *T. undatulus* and *T. nubilus* could be sampled (Table S1; Fig. 1). DNA barcodes of *Clerus mutillarius* (FBCOE355-12), *Opilo mollis* (FBCOF859-12), *Trichodes ornatus* (BBCA4236-12), like *Thanasimus* from the subfamily Clerinae, and *Tillus elongatus* (FBCOF1054-12) from Tillinae (Gunter et al. 2013) were downloaded from BOLD for use as an outgroup in phylogenetic analysis.

2.2. Molecular lab procedures

Samples analyzed at Université Laval were processed using the following protocol. For the extraction of genomic DNA, the abdomen was excised and its content was transferred into an Eppendorf tube containing 250 μL of proteinase K solution. The samples were incubated at 50°C for five hours. Approximately 80 μg of glycogen was added to each tube and then 125 μL of 6 M NaCl was added to the tubes. The samples were vortexed 2 min and then kept on ice for 10 min. Tubes were centrifuged at 16,000 g for 10 min at 4°C and the supernatant were transferred into 1 mL of cold 95% ethanol. Samples were then centrifuged at 16,000 g for 5 min at 4°C . The DNA/glycogen pellets were washed with 70% ethanol and re-centrifuged. Pellets were air dried and then resuspended in 12 μL of HPLC pure water. Two μL of DNA were used for each polymerase chain reaction (PCR). All samples were PCR amplified with the primers CLepFolF (5'-ATCAACCAATCATAAAGATATTGG-3') and CLepFoIR (5'-TAAACTTCTGGATGTCCAAAAATCA-3') (Gerstmeier et al. 2019) for the barcoding fragment (5' mitochondrial cytochrome oxidase subunit 1 – CO1). Amplifications were performed in 50 μL of solution containing 20 pmole of each primer, 1 μL of 10 mM dNTP mix, 5

μL of 10X Taq buffer, and 5 Units of Taq polymerase enzyme (from Bio Basic, Inc., ON, Canada). The PCR products (658 base pairs) were analyzed on a 2% agarose gel. For samples that did not show a PCR band, a nested PCR was performed with the primers FiveCOIP (5'-GGAACCTCTTTAAGACTATTA-3') and TreeCOIP (5'-AAATAAATGTTGATATAAAAATTGG-3') to obtain a smaller PCR product of 573 base pairs with one μL of the primary CLepFolF/ CLepFoIR PCR reaction. The reactions for all the PCR were 1 min at 94°C , 2 min at 54°C , and 2 min at 72°C for 35 cycles. The PCR products were sent to the Genomic Platform of the Centre de Recherche du CHU de Québec – Université Laval (Québec City, PQ, Canada) for Sanger sequencing. The PCR products were sequenced on both strands with the same PCR primers. The sequence data and trace files were uploaded to BOLD (Accession Numbers CLSPQ001-21–CLSPQ011-21).

From samples processed at the University of Salzburg (PLUS), genomic DNA was extracted from the middle-left leg using the Qiagen® (Hilden, Germany) Blood & Tissue kit, following the manufacturers protocol. The Qiagen® Multiplex PCR kit was used to amplify the same fragment as above, using 1.6 μL of each primer LCO1490-JJ (CHACWAAYCATAAAGATATYGG) and HCO2198-JJ (AWACTTCVGGRTGVCCAAARAATCA) (Astrin and Stüben 2008) with 1.5 μL undiluted DNA in 20 μL reaction mixes. We applied a touch down PCR program, reducing the annealing temperature by 1° per cycle during the first 15 cycles, starting at 55°C , and subsequent 25 cycles at 50°C annealing temperature and 90 s elongation time. Samples were sent to Eurofins Genomics (Ebersberg, Germany) for forward and reverse Sanger sequencing with the same primers. Forward and reverse sequences were assembled in Geneious Prime (Biomatters Ltd., Version 2021.1.1) and obvious mismatches between strands were manually corrected. The sequence data were uploaded to BOLD (Accession Numbers THANA001-25–THANA021-25).

2.3. Multiple sequence alignment and phylogenetic analyses

Sequences were aligned with MAFFT v. 7.520 (Katoh and Standley 2013). Remaining parts of primers were pruned from the alignments. We also assured that no stop codons occurred in the sequences. We used IQ-TREE (v. 2.1.3 Minh et al. 2020) to infer a gene tree for downstream analyses. The best fit substitution models and partitions (cox1-1: TN+F+I, cox1-2: HKY+F+I, cox1-3: TN+F+G4) were inferred with ModelFinder (Kalyaanamoorthy et al. 2017) based on initial partitions of 1st, 2nd, and 3rd codon positions. The algorithm was run ten times with the edge-linked partition model, allowing each partition to have its own substitution rate. The tree with the best likelihood was subsequently chosen. Branch support was assessed with 1000 Ultrafast Bootstrap (UFB) replicates (Hoang et al. 2018) which are interpreted as strong evidence when $\geq 95\%$. The tree was rooted with

Tillus elongatus in Dendroscope (v. 3.8.10 Huson and Scornavacca 2012), treating branch supports as such, and subsequently plotted with ggtree (version 3.13.0.001, Yu et al. 2017) in R (v. 4.3.1 R Core Team 2024). The same procedure was applied to the dataset including all *Thanasimus* sequences from the BOLD.

2.4. Molecular species delimitation

We used four methods to infer putative species based on molecular data (excluding outgroups): a simple distance threshold-based clustering, a more sophisticated recursive clustering method, and two gene-tree based partitioning method. Barcode gap (Meyer and Paulay 2005) threshold clustering used raw genetic distances calculated from the sequence data in a pairwise manner using ape (v. 5.7-1 Paradis and Schliep 2019). Local minima in the distribution of pairwise genetic distances were inferred using spider (v. 1.5.0 Brown et al. 2012). The smallest local minimum was then used as the threshold for clustering with spider (Meier et al. 2008).

Hierarchical clustering is also used in the ASAP algorithm (Linux version as downloaded on 2023-10-05; <https://bioinfo.mnhn.fr/abi/public/asap>; Puillandre et al. 2021). However, the final putative species entities are inferred without the need for a predefined distance threshold. Rather, an ASAP score, calculated from the probability that a partition constitutes a species and the width of the barcode gap, is assigned to all potential sets of partitions of sequences and tested for significance. The probability is derived from a comparison of the average pairwise distance of a partition with theoretical values obtained from a simulated panmictic species. The resulting partitions are then recursively tested for the presence of multiple putative species. We used the Kimura model of nucleotide substitution, which came closest to the model inferred by ModelFinder during the IQ-TREE analysis.

A different approach is used by mPTP (v. 0.2.5; Kapli et al. 2017), which is based on patristic distances from a gene tree. Assuming exponential distributions of branch lengths within putative species clusters as well as in-between species clusters, the optimal partitioning of specimens to putative species is determined by a maximum likelihood fit. Likelihood-ratio tests were applied among partitions at the significance level of 0.001. We applied the single-rate and the multi-rate Poisson tree process and report the results of both methods. Inferences were done on the gene tree that was inferred with IQ-TREE, after pruning the outgroup from the tree.

2.5. Morphometric measurements

Body length measurements were taken from 49 North American *Thanasimus* specimens (7 *T. dubius*, 6 *T. nubilus*, 5 *T. trifasciatus*, 31 *T. undatulus*). We measured total body length (from the apical clypeal margin to the elytral apices, L), maximum eye width from dorsal view (EyW), minimum distance between eyes (EyD), length

of pronotum (PL), width of pronotum at the broadest extreme (PW), length of elytra along the middle line (EL), and width of elytra at the broadest extreme (EW). Measurements were analyzed by principal component analysis using the prcomp-function from the base-R-package stats. Data were not scaled, i.e., analyses were based on the covariance matrix. Biplots were created with ggbiplot (Vu and Friendly 2024).

3. Results

Based on two independent lines of evidence, DNA barcoding and morphological analysis, we provide support for the reinstatement of *T. nubilus* as a species distinct from *T. undatulus*. Notable intraspecific genetic divergence was observed among *T. undatulus* populations sampled from California, western Canada, and Alaska (Fig. 1). Based on the combined findings from morphological traits and mtDNA analysis, we formally elevate *T. nubilus* **stat. rev.** to species status.

The mitochondrial *cox1*-gene tree showed monophyly of *T. nubilus* (UFB 100) and *T. undatulus* (UFB 99) and high mean interspecific genetic differentiation between the two species of 9.4% (8.2%–11.5%) (Figs 2, S1), including between syntopically sampled specimens. Intraspecific distances were distinctly lower in *T. nubilus* (mean: 1.6%, ranging from 0.0%–3.6%) but higher in *T. undatulus* (mean: 3.2%, ranging from 0.0%–9.6%). Interspecific genetic distances between *T. nubilus* and *T. undatulus* were comparable to cross-continental divergence with *T. formicarius* (*T. undatulus*: mean: 12.0%, ranging from 10.0%–14.6%; *T. nubilus*: mean: 10.7%, ranging from 9.6%–11.7%) and other well-established North American species (*T. trifasciatus*: mean: 11.6%, ranging from 11.1%–12.2%; *T. dubius*: mean: 9.7%, ranging from 8.7%–10.4%). All applied species delimitation algorithms identified the species boundary between *T. undatulus* and *T. nubilus* and all other valid *Thanasimus* species (Fig. 2). Except for *T. femoralis* and *T. trifasciatus*, all methods oversplit currently valid species. The barcode gap threshold tended to oversplit more compared to other algorithms. While *T. nubilus* was otherwise consistently recovered, the western lineage (Fig. 2) of *T. undatulus* was inferred as separate potential species with all methods.

In contrast to the molecular analyses, the morphological separation of the two species *T. undatulus* and *T. nubilus* was rather difficult. The body length allowed a relatively reliable differentiation (*T. nubilus*, mean: 7.90 mm, range: 6.25–9.33 mm; *T. undatulus*, mean: 6.68 mm, range: 5.08–8.83 mm; Fig. 3 inset). The base of the elytra and the pronotum of all *T. nubilus* examined were black, while the abdomen, antennae and legs were reddish brown. In *T. undatulus*, the pronotum was red (except for an apical black margin), the elytra were black with the base narrowly to more-or-less broadly red. Several *T. undatulus* from Alaska that nested in the west-

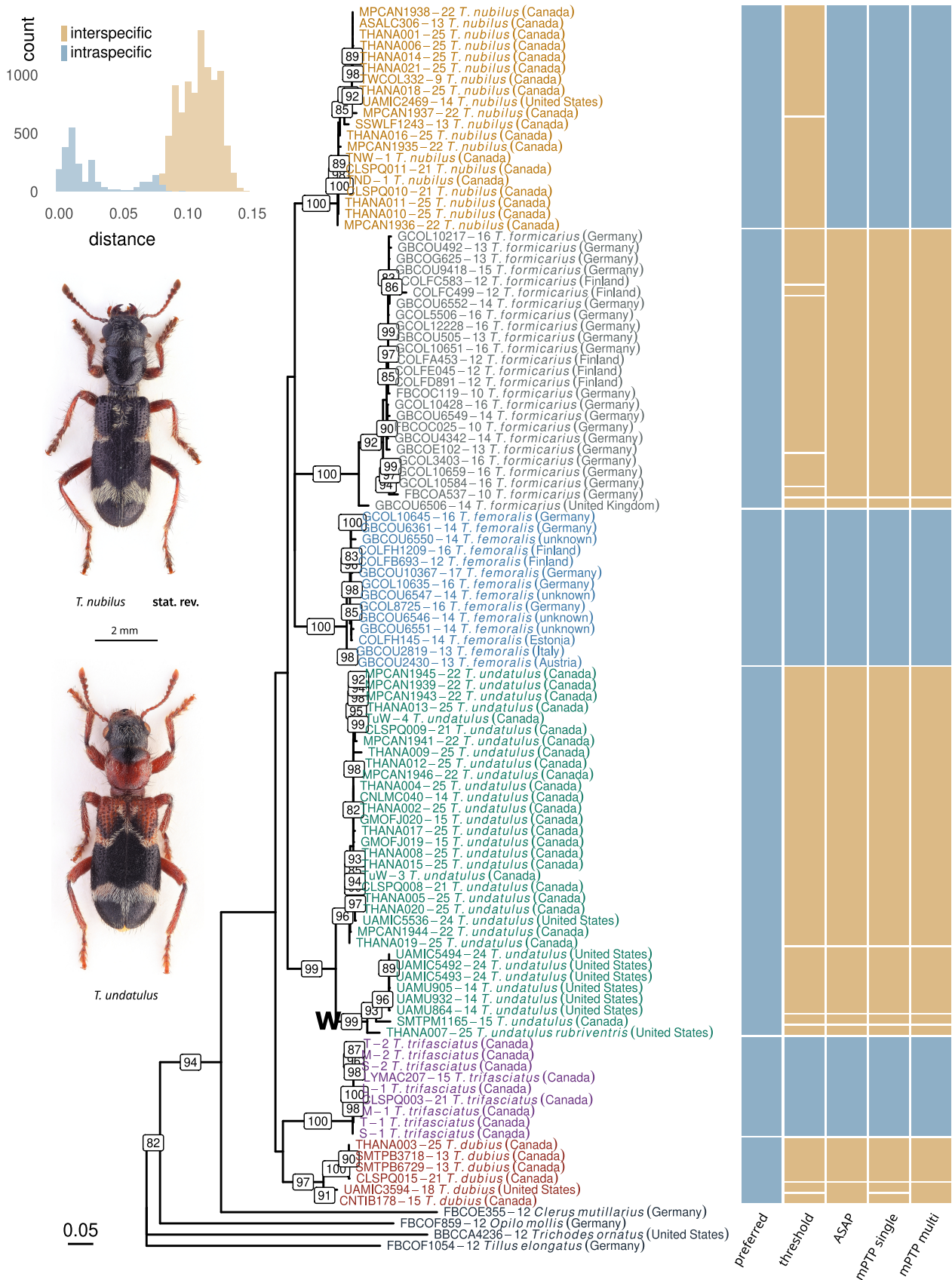


Figure 2. Maximum likelihood gene tree of six *Thanasimus* species, that was inferred from DNA barcodes with IQ-TREE. A bold “W” indicates the *T. undatulus*-lineage west of the Rocky Mountains (western lineage). The five columns to the right of the tree indicate delimitations of species as preferred based on total evidence and putative species inferred by four automated species delimitation methods based on DNA barcodes. Mismatches with morphology due to over- or under-splitting are highlighted in yellow. The histogram shows pairwise raw genetic distances of all specimens except outgroups. Ultrafast Bootstraps are displayed on nodes with > 80% support.

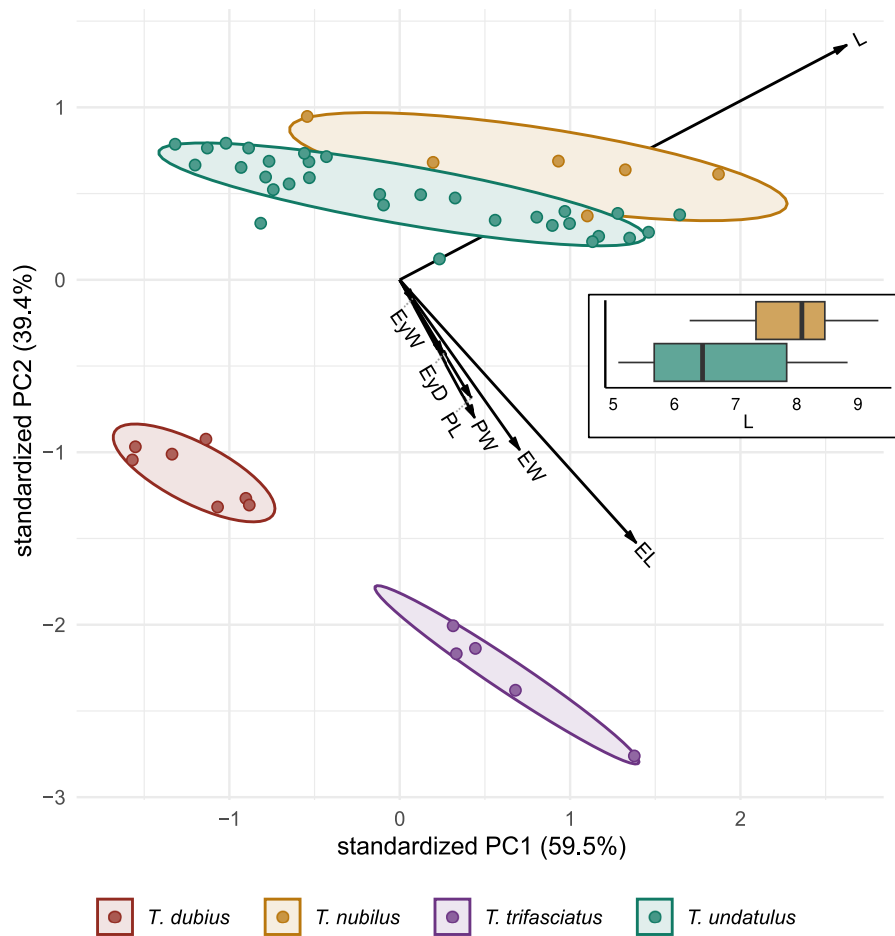


Figure 3. Morphological differentiation of four North American *Thanasimus* species as inferred by principal component analysis of seven body length measures. The biplot of principal component scores and loadings indicates that *T. nubilus* is mainly separated from *T. undatulus* by total body length (L). The inset shows color-coded boxplots of the total body length in mm of the two species. See the material and methods section for explanation of measurement acronyms.

ern clade (Fig. 2) only had the elytral humeri red and the pronotum entirely black. The elytra of both *T. nubilus* and *T. undatulus* bear two sinuate transverse bands of white pubescence with the anterior band extending along the suture (Fig. 2). Overall, the main driver of the separation of *T. nubilus* and *T. undatulus* was total body size (L). Finally, our multivariate measurements of 49 specimens resulted in clear separation of *T. dubius* and *T. trifasciatus* (Fig. 3).

4. Discussion

Although members of the family Cleridae exhibit a relatively distinct appearance and comprise a modest number of species, they continue to pose significant taxonomic challenges. These difficulties are largely attributable to the variability in external morphological traits and coloration, which can obscure species-level distinctions. Consequently, the integration of molecular data proves valuable for accurate species delimitation and identification. For example, a larval specimen that was included in the study (BOLD-Accession: SSGLC5295-15) was identified as *T. undatulus*. However, analysis of the DNA sequence data placed this larval specimen within the *nubilus*-clade (Fig. S1). While results based on the DNA barcoding data that were available for the current study must be interpreted

with caution, they clearly suggested different species in the case of *T. nubilus* and *T. undatulus*. Both species were monophyletic and not sister lineages in the *cox1*-gene tree, but this should be interpreted with caution, given the saturation of the *cox1*-gene at deeper time scales. However, the low intraspecific genetic variation in both species except for the western lineage (Fig. 2) of *T. undatulus*, in combination with the very clear interspecific differentiation of 20 syntopic specimens that were caught in the same trap at eight localities, suggested a lack of gene flow, and two separately evolving metapopulations (De Queiroz 2007). One of the delimitation methods we used, mPTP, is reported to have a tendency to overlump species (Jiang et al. 2024) and yet it supported the separation of *T. nubilus* from *T. undatulus* which we interpret as a conservative result. Due to the evidence of mtDNA and the weaker but present support by morphological characteristics, we therefore reinstate *Thanasimus nubilus* Klug, 1842 **stat. rev.** as a valid species.

Over the past two decades, DNA barcoding (Hebert et al. 2003) has emerged as a powerful and widely adopted tool in taxonomy. By comparing a short fragment of the mitochondrial cytochrome oxidase c subunit 1 gene (*cox1*) across and within species (Hebert et al. 2003), this method has often proven effective in distinguishing intra- and interspecific genetic variation (Čandek and Kuntner 2015), as demonstrated in European *Thanasimus* (Gerstmeier et al. 2019). While studies have highlighted challenges—such as limited interspecific variation and

elevated intraspecific divergence that may complicate species delineation (Eberle et al. 2016, 2019; Lukic et al. 2021; Meyer and Paulay 2005; Ranasinghe et al. 2022, 2023; Zhang and Bu 2022)—these insights have spurred methodological refinements and integrative approaches. Thanks to extensive reference databases, user-friendly protocols, and cost-effectiveness, DNA barcoding continues to provide valuable molecular data and often yields reliable estimates of species boundaries, especially when combined with species delimitation methods. To further enhance accuracy, the integration of complementary data from nuclear genes, morphology, and ecology remain essential (Ahrens et al. 2021; Zamani et al. 2022).

An exception from the very clear separation of intraspecific and interspecific genetic variation was found in specimens that stem west of the Rocky Mountains (western clade, Fig. 2), which showed a certain variability in their coloration. Increased intraspecific variation in these lineages might be a consequence of repeated glacial cycles, which caused isolation of small populations west of the Rocky Mountains from larger populations east of the mountain range (Schmitt 2020). The ice sheets repeatedly forced populations to small coastal glacial refugia (Schmitt 2020) where they could survive, although in diminished populations. Similar observations were found for the American black bear (*Ursus americanus*) and the nematode *Soboliphyme baturini* (Byun et al. 1997; Koehler et al. 2009; Schmitt 2020). Genetic drift alone might have caused the observed genetic differentiation since populations with small effective size (N_e) tend to accumulate substitutions more rapidly than large populations (Eberle et al. 2019; Galtier et al. 2009). Also, the morphological variation might reflect this effect. The genus *Thanasimus* appears to be a potentially interesting showcase of North American biogeography. Future studies should emphasize the use of nuclear DNA, preferably at a genomic scale. We need a more comprehensive geographic sampling of the populations west of the Rocky Mountains, which are partly difficult to collect (e.g. *T. undatulus* var. *rubriventris*), and also of Eurasian species to better understand the Holarctic biogeography of the genus.

5. Declarations

Authors' contributions. JE, ML, DS, and RG conceived the study; ML and DS collected the newly sequenced specimens; ML, DS, and JE generated DNA barcodes from them; RS and JE prepared and analyzed the data; JE, ML, DS, and RG drafted the manuscript; all authors proofread the final manuscript.

Competing interests. The authors have declared that no competing interests exist.

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Supplementary Material 1

Table S1

Authors: Eberle J, Schallegger R, Sikes DS, Lebel M, Gerstmeier R (2026)

Data type: .xlsx

Explanation notes: Details on all specimens in the study used for molecular analyses. IDs, localities, and further sampling information are given along with sequence origin. Equal letters in the syntopy column indicate pairs of syntopically collected *T. undatulus* and *T. nubilus*.

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Link: <https://doi.org/10.3897/asp.84.e176700.suppl1>

Supplementary Material 2

Figure S1

Authors: Eberle J, Schallegger R, Sikes DS, Lebel M, Gerstmeier R (2026)

Data type: .pdf

Explanation notes: Maximum likelihood gene tree of seven *Thanasimus* species, that was inferred with IQ-TREE from all DNA barcodes of the genus *Thanasimus* that were available at the BOLD. Ultrafast Bootstraps are displayed on nodes with > 80% support. Specimens CNCCH1946-12, NGSFT333-16, NGSFT3334-16, GMOFA013-21 all exhibit a black pronotum and base of the elytra and were thus identified to be *T. nubilus* in this study.

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