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Variable performance of DNA barcoding and morphological characteristics for the identification of Arctic black-legged *Aedes* (Diptera: Culicidae), with a focus on the Punctor subgroup

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Abstract

Arctic ecosystems face increasing risks from vector-borne diseases due to climate-driven shifts in disease patterns and vector distribution. However, species identification challenges impact vector-borne disease surveillance, necessitates accurate identification. *Aedes* species are predominant among Arctic mosquitoes and pose health risks, with some species potentially carrying Jamestown Canyon and Snowshoe hare viruses. However, identifying *Aedes* species is challenging, especially under Arctic conditions and with complex adult traits. This study assessed the suitability of DNA barcoding (COI and ITS2 regions) and morphological characteristics for the identification of Arctic black-legged *Aedes*. It also aimed to evaluate the reliability of publicly available sequences. Our analysis focused on *Aedes impiger*, *Aedes nigripes*, and two species from the Punctor subgroup – *Aedes hexodontus* and *Aedes punctor*. In our study, the COI barcoding region distinguished *Ae. impiger* and *Ae. nigripes* but not within the species of the Punctor subgroup. In addition, the ITS2 barcoding region did not differentiate the species. When we evaluated GenBank and BOLD sequences, we found issues of under-representation and misidentifications, particularly within the Punctor subgroup. Based on these results, we recommend addressing identification difficulties, particularly within the Punctor subgroup, and advocate for more comprehensive morphological and molecular identification strategies. Integrating morphology and DNA barcoding holds promise for robust disease surveillance in Arctic regions, yet challenges persist, especially in complex species groups like the Punctor subgroup. Tackling these issues is pivotal to ensuring accurate vector status determination and reliable disease risk assessments in a rapidly changing Arctic ecosystem.

Key words

COI region, ITS2 region, *Aedes impiger*, *Aedes nigripes*, *Aedes hexodontus*, *Aedes punctor*

1. Introduction

Vector-borne diseases pose an increasing threat to Arctic wildlife and human populations, primarily driven by climate-induced alterations in disease transmission dynamics and the potential northward expansion of vector species (Pauchard et al. 2016; Waits et al. 2018; Ludwig et al. 2019). To anticipate health impacts on wildlife and humans, there is an urgent need to implement mosquito surveillance programs (Koltz and Culler 2021). However, the efficiency of this type of program relies directly on the accurate and precise identification of mosquito species. Identification can be challenging, especially in Arctic and subarctic regions, as most species belong to the black-legged *Aedes*, a group characterized by a significant overlap in morphological features among its members (Hocking et al. 1950; Vockeroth 1954; Steward 1968; Danks and Corbet 1973).

Aedes species comprise approximately 90% of all trapped Arctic mosquitoes (Snyman et al. 2023) and can be classified into two main divisions based on the morphology of the hind leg: white-banded tarsi (white-legged) and dark tarsi (black-legged) (Edwards 1932). In the North American Arctic, black-legged *Aedes* are more abundant than the white-legged *Aedes*, representing nearly all of the most common species (Danks 1981). However, the black-legged *Aedes* species also exhibit significant uniformity in adult morphological characters, leading to taxonomic complexities (Hocking et al. 1950; Beckel 1954; Vockeroth 1954; Steward 1968).

Traditional alpha taxonomy has been the primary method for differentiating among mosquito species, relying heavily on morphological apomorphies. This approach requires detailed morphological descriptions and/or keys coupled with skilled entomological technicians to accurately identify described species (Wood et al. 1979; Ward and Darsie 2005; Thielman and Hunter 2007). Furthermore, successful identification of adult specimens requires specimens to be in a good state (i.e. the preservation of scales and legs). During and after collection, the specimens can easily get damaged and delicate scales can easily rub off due to handling or inadequate storage (Beebe 2018). Morphological identification is further complicated by the existence of closely related sibling species within species complexes or larger groups with overlapping morphology (Beebe 2018). These complexities particularly impede the identification of Arctic mosquitoes. For instance, distinguishing female adults of the Punctor subgroup, here defined as comprising *Aedes punctor* Kirby, 1937 and *Aedes hexodontus* Dyar, 1916, within the black-legged Arctic species is typically not feasible without the fourth instar larval exuviae (Hocking et al. 1950; Steward 1968). In certain regions like Churchill (Manitoba, Canada), adult specimens of *Ae. punctor* cannot be differentiated from *Ae. hexodontus* (Beckel 1954). Likewise, females of the high Arctic species *Aedes impiger* Walker, 1948 and *Aedes nigripes* Zetterstedt, 1840 can only be distinguished based on the shape of their tarsal claws, but even this criterion requires careful evaluation (Vockeroth 1954; Danks and Corbet 1973). Furthermore, most studies concentrate on females, and there is a lack of research on the diagnostic features of male genitalia that could potentially help differentiate those species (Danks & Corbet 1973).

To overcome such challenges, a paradigm shift has occurred: rather than relying solely on morphological-based methods, a more integrative approach that includes molecular methods in the identification process, often termed DNA barcoding, is being used. This technique uses specific short DNA sequences from a standardized region of the genome to generate DNA barcodes suitable for species identification (Hebert et al. 2003). DNA barcoding empowers nontaxonomic experts who regularly handle large sample sizes to identify species (Wirta et al. 2016; Panda and Barik 2020). A portion of the mitochondrial DNA gene cytochrome c oxidase I (COI) is the most commonly used barcode region for animals (Hebert et al. 2003; Godfray 2007; Beebe 2018). The mitochondrial genome lacks introns and maternal inheritance makes it highly suitable for barcoding studies and is duly often employed in mosquito research (Folmer et al. 1994; Cywinska et al. 2006; Laurito et al. 2013). Otherbar codes, such as the second internal transcribed spacer (ITS2) in the nuclear ribosomal DNA, are also suitable for DNA barcoding and are used similarly to identify closely related animal species (Yao et al. 2010; Batovska et al. 2016; Beebe 2018). The ITS2 barcoding region is particularly useful due to its high interspecific polymorphism, which aids in describing relationships between mosquito species (Wilkerson et al. 2004; Marrelli et al. 2006; Walton et al. 2007; Sum et al. 2014; Ali et al. 2019; Hodge et al. 2021). Yet it is not without drawbacks: the internal transcribed spacers are often difficult to align and to reference sequences due to highly variable sections.

However, the adoption of such universal barcodes for a global bioidentification system has faced criticism (Meier et al. 2006; de Carvalho et al. 2007; Conflitti et al. 2012; Beebe 2018; Chaiphongpachara et al. 2022). Criticisms consist of the inability of barcoding regions to differentiate among all species, as well as issues associated with incorrect taxon name designation in publicly deposited sequences, including poor taxon coverage in public repositories (Nilsson et al. 2005; Bidartondo et al. 2008; Kang et al. 2010; Laurito et al. 2013). This poses challenges when relying on existing sequences to assign species names. Furthermore, at the time of writing, there is poor taxon coverage in public depositories for Arctic mosquitoes in major molecular depositories, such as GenBank (https://www.ncbi.nlm.nih.gov/genbank) and the Barcode of Life Data System (BOLD; https://www.boldsystems.org).

In this study, we focus on sequences from two barcoding regions, COI and ITS2, obtained from public databases and purpose-captured black-legged *Aedes* specimens from the North American Arctic. Our objectives were to (1) evaluate the suitability of barcoding regions as identification tools, (2) investigate discrepancies between the morphological and molecular identification of northern

Table 1. Names, sequences, and references of the primers used to amplify the COI and ITS2 gene sequences. The subscript of the target denotes the direction of the primer (F = forward, R = reverse).

Name (Target)	Sequence	Reference
LCOI490 (COI_F)	5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'	Folmer et al. 1994
HCO2198 (COI_R)	5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'	Folmer et al. 1994
5.8SF ($ITS2_F$)	5'-ATC ACT CGG CTC GTG GAT CG-3'	Djadid et al. 2007
28SR ($ITS2_R$)	5'-ATG CTT AAA TTT AGG GGG TAG TC-3'	Djadid et al. 2007

Table 2. List of the 26 black-legged *Aedes* species included in this study.

Black-legged *Aedes* species			
Ae. aboriginis	*Ae. decticus*	*Ae. nigripes*	*Ae. spencerii*
Ae. abserratus	*Ae. diantaeus*	*Ae. pionips*	*Ae. sticticus*
Ae. aurifer	*Ae. hendersoni*	*Ae. provocans*	*Ae. thibaulti*
Ae. cataphylla	*Ae. hexodontus*	*Ae. pullatus*	*Ae. triseriatus*
Ae. churchillensis	*Ae. impiger*	*Ae. punctor*	*Ae. trivittatus*
Ae. cinereus	*Ae. implicatus*	*Ae. rempeli*	
Ae. communis	*Ae. intrudens*	*Ae. schizopinax*	

black-legged *Aedes* species, and (3) assess the reliability of publicly available COI and ITS2 sequences data as a tool for species identification.

2. Methods

2.1. Mosquito collection and morphological identification

Live adult mosquitoes were captured at dusk in the summers of 2019 and 2020 using a sweep net. Sampling took place in the North American Arctic, namely in the United States of America (Toolik, Alaska: 64°54.91'N, 147°57.96'W) and in Canada (Cambridge Bay, Nunavut: 62°7.22'N, 105°2.7'W; Karrak Lake, Nunavut: 67°14.15'N, 100°15.42'W; Kuujjuaq, Nunavik, Québec: 58°7.63'N, 68°23.08'W). The mosquitoes collected in the field were placed in a labelled plastic container and frozen at –18°C until they were shipped to the Faculté de médecine vétérinaire of the Université de Montréal (Saint-Hyacinthe, Québec, Canada). In this study, morphological terminology follows that of Wood (1979). Female mosquitoes were identified in a chilled Petri dish using a stereomicroscope (Acuter, Model T1A) using external morphological characteristics and established taxonomic keys (Carpenter and LaCasse 1955; Wood et al. 1979; Thielman and Hunter 2007). The species terminology employed follows the classification outlined by Wilkerson et al. (2015). A leg of each specimen was removed and stored in RNA*later*® (Sigma-Aldrich), and the rest of the specimen was double-mounted on pins Photographs of each specimen were taken using a digital microscope (Keyence Canada Inc., Model VHX-71000). The specimens were then deposited as vouchers in the Ouellet-Robert's entomological collection of the Université de Montréal (http://qmor.umontreal.ca).

2.2. DNA extraction, amplification, and sequencing

Genomic DNA was obtained from one leg of each sample using the QIAGEN DNeasy Blood & Tissue Kit with slight modifications to the manufacturer's protocol. Each leg was macerated with a sterile micro pestle and digested in a thermomixer (1000 rpm at 56°C for 120 min). To increase the DNA yield, 50 µl of elution buffer was added to the center of the spin column on two separate occasions and spun down for a total final volume of 100 µl. The elution buffer was heated to approximately 37°C before adding it to the spin column. Invitrogen™ Platinum™ Taq DNA polymerase was used for amplification using specific primers (Table 1). Each 25 µl reaction consisted of 2.5 µl 10 × buffer, 1 µl $MgSO_4$, 0.5 µl of dNTP, forward and reverse primers, 0.1 µl high fidelity DNA polymerase, 10 µl DNA template and 9.9 µl ddH_2O. The following PCR conditions were used for amplification: initial denaturation at 94°C for 60 s, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 51°C (COI) / 55°C (ITS2) for 30 s, and extension at 68°C for 60 s. The final extension occurred at 68°C for 300 s. The amplified products were visualised on a 1.5% agarose gel and purified with the QIAGEN QIAquick PCR Purification Kit according to the manufacturer's protocol. Purified products were sent for Sanger sequencing at the National Research Council in Saskatoon (Saskatchewan, Canada). The forward and reverse sequences were assembled using the QIAGEN CLC Main Workbench. All the assembled sequences were uploaded to BOLD. GenBank accession numbers were obtained via BOLD.

The ingroup consisted of 26 species of North American black-legged *Aedes* (Table 2). Species were selected for this study based on two criteria: they needed to (1) have a distribution across North America (but not limited to the Arctic or subarctic regions) and (2) have DNA sequenc-

Table 3. Information on our own specimens of black-legged *Aedes*, including the sampling locations, the museum accession numbers, and the sequences accession numbers.

Specimens	Sampling locations	Collection #	BOLD # (COI; ITS2)	GenBank # (COI; ITS2)
Ae. hexodontus 1	Toolik (Alaska, USA)	QMOR74998	MOSQ020–23.COI; —	OR367027; —
Ae. hexodontus 2	Toolik (Alaska, USA)	QMOR74985	MOSQ009–23.COI; —	OR367031; —
Ae. hexodontus 3	Karrak Lake (Nunavut, CAN)	QMOR74999	MOSQ021–23.COI; —	OR367030; —
Ae. hexodontus 4	Toolik (Alaska, USA)	QMOR74983	MOSQ007–23.COI; —	OR367028; —
Ae. hexodontus 5	Toolik (Alaska, USA)	QMOR74984	MOSQ008–23.COI; —	OR367029; —
Ae. impiger 1	Karrak Lake (Nunavut, CAN)	QMOR74996	MOSQ018–23.COI; MOSQ018–23.ITS2	OR367035; OR367074
Ae. impiger 2	Karrak Lake (Nunavut, CAN)	QMOR74982	MOSQ006–23.COI; MOSQ006–23.ITS2	OR367036; OR367075
Ae. impiger 3	Cambridge Bay (Nunavut, CAN)	QMOR74980	MOSQ004–23.COI; —	OR367033; —
Ae. impiger 4	Cambridge Bay (Nunavut, CAN)	QMOR74981	MOSQ005–23.COI; —	OR367032; —
Ae. impiger 5	Karrak Lake (Nunavut, CAN)	QMOR74997	MOSQ019–23.COI; MOSQ019–23.ITS2	OR367034; OR367073
Ae. nigripes 1	Cambridge Bay (Nunavut, CAN)	QMOR74994	MOSQ016–23.COI; MOSQ016–23.ITS2	OR367041; OR367080
Ae. nigripes 2	Cambridge Bay (Nunavut, CAN)	QMOR74995	MOSQ017–23.COI; MOSQ017–23.ITS2	OR367037; OR367076
Ae. nigripes 3	Cambridge Bay (Nunavut, CAN)	QMOR74979	MOSQ003–23.COI; MOSQ003–23.ITS2	OR367040; OR367079
Ae. nigripes 4	Cambridge Bay (Nunavut, CAN)	QMOR74977	MOSQ001–23.COI; MOSQ001–23.ITS2	OR367038; OR367077
Ae. nigripes 5	Cambridge Bay (Nunavut, CAN)	QMOR74978	MOSQ002–23.COI MOSQ002–23.ITS2	OR367039; OR367078
Ae. punctor 1	Kuujjuaq (Nunavik, Québec, CAN)	QMOR75000	MOSQ022–23.COI; MOSQ022–23.ITS2	OR367043; OR367082
Ae. punctor 2	Toolik (Alaska, USA)	QMOR74986	MOSQ010–23.COI; MOSQ010–23.ITS2	OR367042; OR367081
Ae. punctor 3	Toolik (Alaska, USA)	QMOR74987	MOSQ011–23.COI; —	OR367045; —
Ae. punctor 4	Toolik (Alaska, USA)	QMOR74988	MOSQ012–23.COI; —	OR367044; —
Ae. punctor 5	Kuujjuaq (Nunavik, Québec, CAN)	QMOR75001	MOSQ023–23.COI; —	OR367046; —
Punctor subgroup 1	Kuujjuaq (Nunavik, Québec, CAN)	QMOR74991	MOSQ013–23.COI; —	OR367047; —
Punctor subgroup 2	Kuujjuaq (Nunavik, Québec, CAN)	QMOR74992	MOSQ014–23.COI; MOSQ014–23.ITS2	OR367050; OR367085
Punctor subgroup 3	Kuujjuaq (Nunavik, Québec, CAN)	QMOR74993	MOSQ015–23.COI; MOSQ015–23.ITS2	OR367049; OR367084
Punctor subgroup 4	Kuujjuaq (Nunavik, Québec, CAN)	QMOR75002	MOSQ024–23.COI; MOSQ024–23.ITS2	OR367048; OR367083
Punctor subgroup 5	Kuujjuaq (Nunavik, Québec, CAN)	QMOR75003	MOSQ025–23.COI; MOSQ025–23.ITS2	OR367051; OR367086

es of the COI and ITS2 barcoding genes available from captured female mosquitoes in BOLD. *Mansonia uniformis* Theobald, 1901 was used as the outgroup taxon. If a species met the selection criteria, up to five sequences were randomly selected and extracted from BOLD, and compiled into a FASTA file alongside the other sequences generated in this study.

2.3. Sequencing Alignment and Phylogenetic Analysis

Using the extracted sequences, three datasets were generated: one dataset containing COI sequences, one dataset containing ITS2 sequences, and one concatenated dataset regrouping COI sequences (partitioned as per codon

position and not partitioned), ITS2 sequences, and 28S sequences. All datasets were aligned using the online version of MAFFT7 (https://mafft.cbrc.jp/alignment/server) under default parameters. The aligned matrix was viewed, trimmed and edited using MEGA7 (Kumar et al. 2016). The most appropriate substitution model was determined for each dataset using the Akaike Information Criterion (AIC) in MEGA7, which was GTR+G+I for the COI dataset, and GTR+G for the ITS2 and concatenated datasets.

Phylogenetic analyses were conducted using Maximum Likelihood (ML) with RAxML (Stamatakis 2014) and Bayesian inference (BI) with MrBayes version 3 (Ronquist and Huelsenbeck 2003). For ML analysis, robustness of the tree was tested with 1000 bootstrapped datasets, with bootstrap support values shown on each node. For BI analysis, the Markov chain Monte Carlo (MCMC) simulation was run for one million generations (which resulted in an average standard deviation of split frequencies below 0.01). All phylogenetic trees were visualised using Figtree software version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree). Bootstrap values (B) of ML analysis higher than 70 and posterior probabilities (PP) of BI analysis higher than 0.95 were considered strong support values.

3. Results

3.1. Morphological characteristics of females

A total of 25 black-legged *Aedes* females were collected from five localities (Table 3) (Villeneuve 2023; see Table S1 for the vouchers' complete information). Morphologically, the specimens were in near-perfect condition and all specimens resembled the global description of females (Wood et al. 1979).

3.1.1. *Aedes impiger*

Tarsomeres dark scaled and without pale bands (*Ta*, Fig. 1**A**); patch of scales on the postprocoxal membrane (*PM*, Fig. 1**B**); numerous setae arising randomly from the posterodorsal half of the postpronotum (*PpS*, Fig. 1**C**); scutum with numerous scattered supra-alar setae, imparting a hairy appearance (*SaS*, Fig. 1**B**); scutum with a mix of yellow and brown scales (*S*, Fig. 1**E**); hind tarsal claw rather sharply bent beyond the long subbasal tooth, with the distal portion subparallel with the subbasal tooth (*Cl*, Fig. 1**D**).

3.1.2. *Aedes nigripes*

This species closely resembles *Ae. impiger* in the colour of the tarsomeres (*Ta*, Fig. 2**A**), scales of postprocoxal membrane (*PM*, Fig. 2**B**), setae of postpronotum (*PpS*, Fig. 2**C**), hairy appearance (*SaS*, Fig. 2**B**), and scales of scutum (*S*, Fig. 2**E**), but can be distinguished by the shape of the hind tarsal claw, which is moderately long and evenly curved beyond the subbasal tooth (*Cl*, Fig. 2**D**).

3.1.3. *Aedes hexodontus*

Tarsomeres dark-scaled and without pale bands (*Ta*, Fig. 3**A**); postpronotum with setae arising from an irregular row along the posterior margin (*PpS*, Fig. 3**C**); patch of scales on the postprocoxal membrane (*PM*, Fig. 3**B**); bare hypostigmal area (*HyA*, Fig. 3**B**); scales of katepisternum extending to the anterodorsal corner (*K*, Fig. 3**B**); mesepimeron completely covered with scales (*M*, Fig. 3**B**); scutum with uniform medium brown scales (*S*, Fig. 3**E**); probasisternum completely covered with pale scales (*Pb*, Fig. 4**A**), base of costa with an extended patch of pale scales (*C*, Fig. 4**D**); hind tarsal claw rather straight basally, curving moderately and uniformly beyond small subbasal tooth (*Cl*, Fig. 3**D**).

3.1.4. *Aedes punctor*

This species closely resembles *Ae. hexodontus* in the colour of the tarsomeres (*Ta*, Fig. 5**A**), postpronotum setae (*PpS*, Fig. 5**C**), postprocoxal membrane scales (*PM*, Fig. 5**B**); hypostigmal area (*HyA*, Fig. 5**B**), katepisternum (*K*, Fig. 5**B**), mesepimeron (*M*, Fig. 5**B**), scutum (*S*, Fig. 5**E**), and shape of the hind tarsal claw (*Cl*, Fig. 5**D**), but can be distinguished by the scattered pale scales on the probasisternum (*Pb*, Fig. 4**B**) and by two or three pale scales at the base of the costa (*C*, Fig. 4**E**).

Figure 5 should be placed at the end of the section 3.1.4 Aedes punctor; portrait; width of page.

3.1.5. Punctor subgroup's outlier

These specimens closely resemble *Ae. hexodontus* and *Ae. punctor* in the colour of the tarsomeres (*Ta*, Fig. 6**A**), postpronotum setae (*PpS*, Fig. 6**C**), scales of the postprocoxal membrane (*PM*, Fig. 6**B**); hypostigmal area (*HyA*, Fig. 6B), katepisternum (*K*, Fig. 6**B**), mesepimeron (*M*, Fig. 6**B**), scutum (*S*, Fig. 6**E**), and shape of the hind tarsal claw (*Cl*, Fig. 6**D**), but can be distinguished from *Ae. hexodontus* and *Ae. punctor* by the small patches of pale scales at the base of the costa (*C*, Fig. 4**F**) and probasisternum (*Pb*, Fig. 4**C**).

3.2. Phylogenetic analysis

The final aligned matrices used for analyses had a total length of 658 bp for the COI sequences and 381 bp for the ITS2 sequences, comprising 25 and 14 sequences, respectively. The sequences were deposited in BOLD under GenBank accession numbers OR367073–OR367086 for ITS2, OR367052–OR367072 for the putative 28S section amplified with the chosen primers and OR367027–OR367051 for COI ((Table 3; see Table S2 for all the sequences used to perform these analyses and see Figure S1 (trees) for the results of the BI and ML analyses of all datasets)).

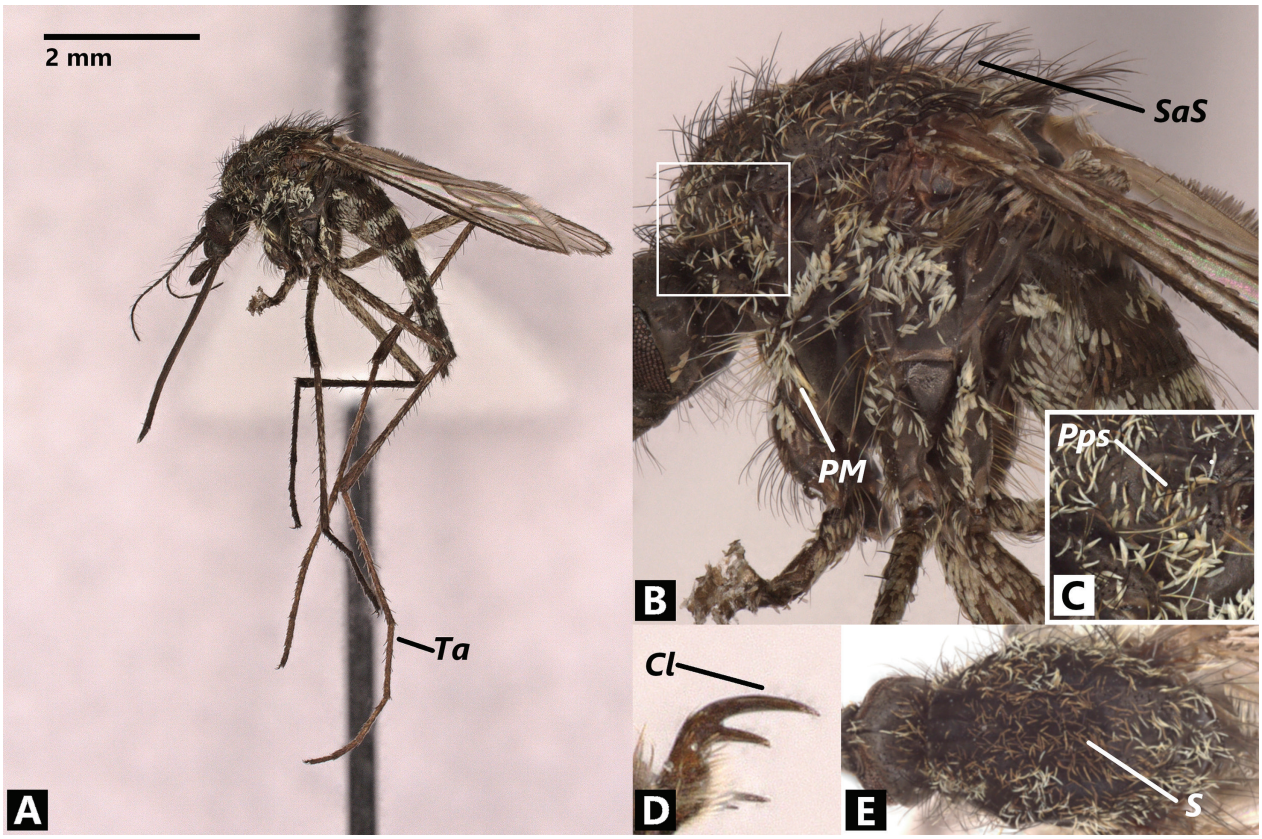

Figure 1. *Aedes impiger.* **A** lateral view of habitus, showing tarsomeres (*Ta*); **B** lateral view of thorax, showing postprocoxal membrane (*PM*) and supra-alar setae (*SaS*); **C** close-up of postpronotum (*PpS*); **D** close-up of hind tarsal claw (*Cl*); **E** dorsal view of scutum (*S*).

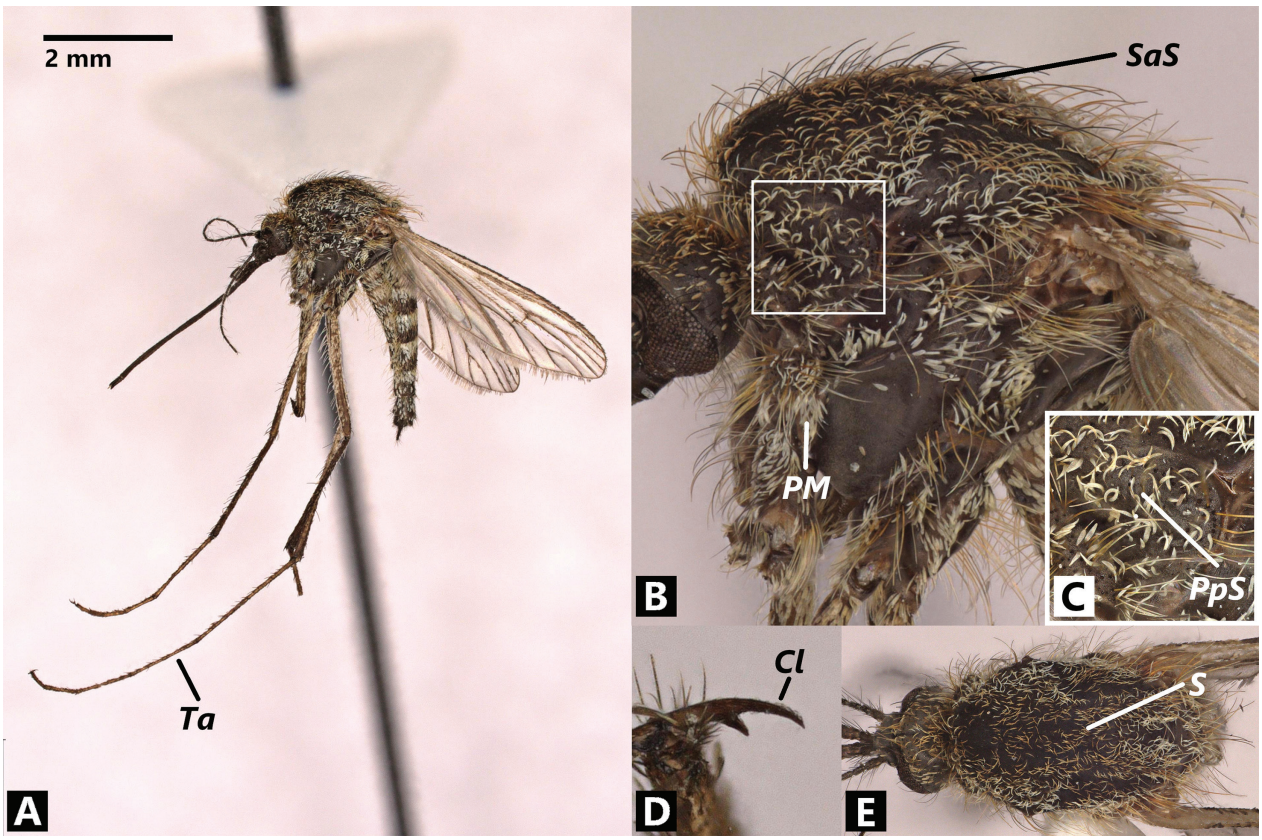

Figure 2. *Aedes nigripes.* **A** lateral view of habitus, showing tarsomeres (*Ta*); **B** lateral view of thorax, showing postprocoxal membrane (*PM*) and supra-alar setae (*SaS*); **C** close-up of postpronotum (*PpS*); **D** close-up of hind tarsal claw (*Cl*); **E** dorsal view of scutum (*S*).

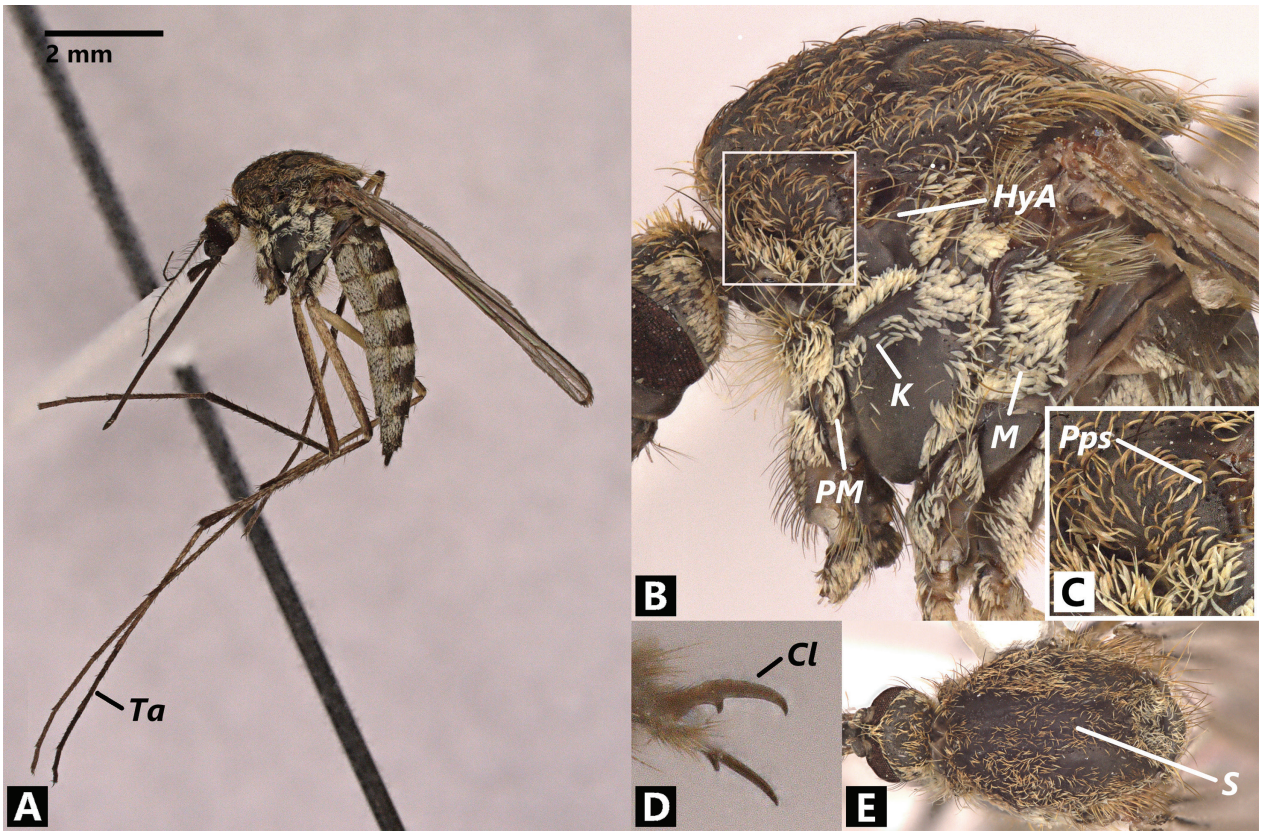

Figure 3. *Aedes hexodontus.* **A** lateral view of habitus, showing tarsomeres (*Ta*); **B** lateral view of thorax, showing postprocoxal membrane (*PM*), katepisternum (*K*), mesepimeron (*M*), and hypostigmal area (*HyA*); **C** close-up of postpronotum (*PpS*); **D** close-up of hind tarsal claw (*Cl*); **E** dorsal view of scutum (*S*).

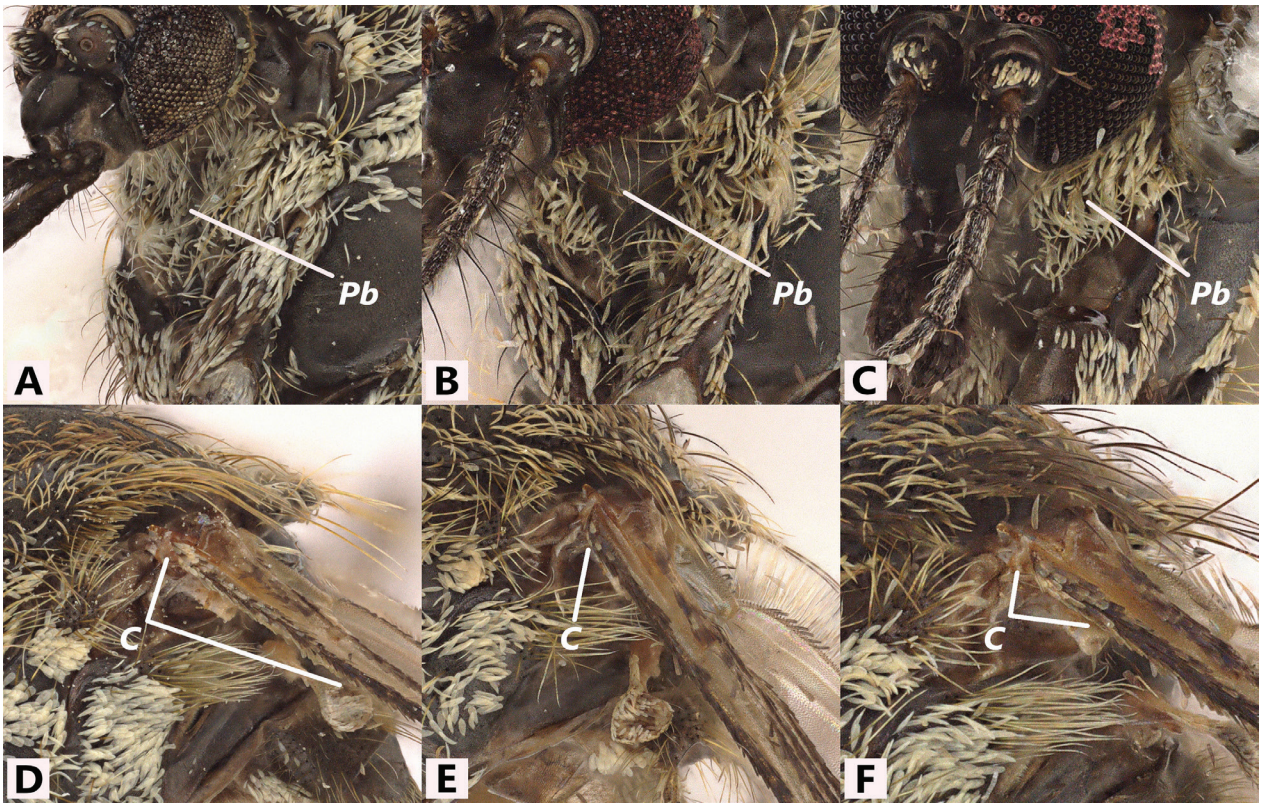

Figure 4. Close-up of *Aedes hexodontus*, *Aedes punctor* and Punctor subgroup's outlier. *Ae. hexodontus*: showing heavily scaled probasisternum (*Pb*; **A**) and extensive patch of white scales at base of costa (*C*; **D**); *Ae. punctor*: showing a few scattered scales on probasisternum (*Pb*; **B**) and a few pale scales at base of costa (*C*; **E**); Punctor subgroup's outlier: showing heavily scaled probasisternum (*Pb*; **C**) and small patch of scales at base of costa (*C*; **F**).

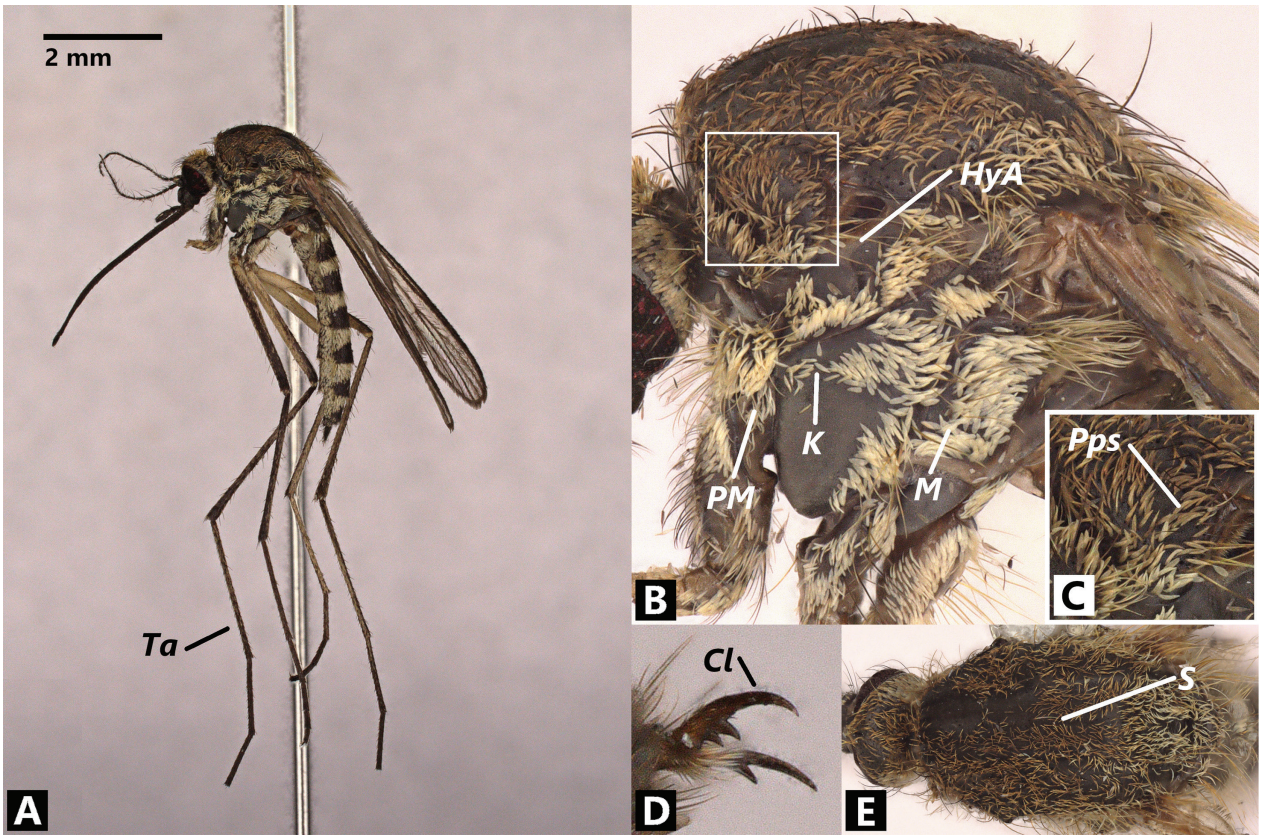

Figure 5. *Aedes punctor.* **A** lateral view of habitus, showing tarsomeres (*Ta*); **B** lateral view of thorax, showing postprocoxal membrane (*PM*), katepisternum (*K*), mesepimeron (*M*), and hypostigmal area (*HyA*); **C** close-up of postpronotum (*PpS*); **D** close-up of hind tarsal claw (*Cl*); **E** dorsal view of scutum (*S*).

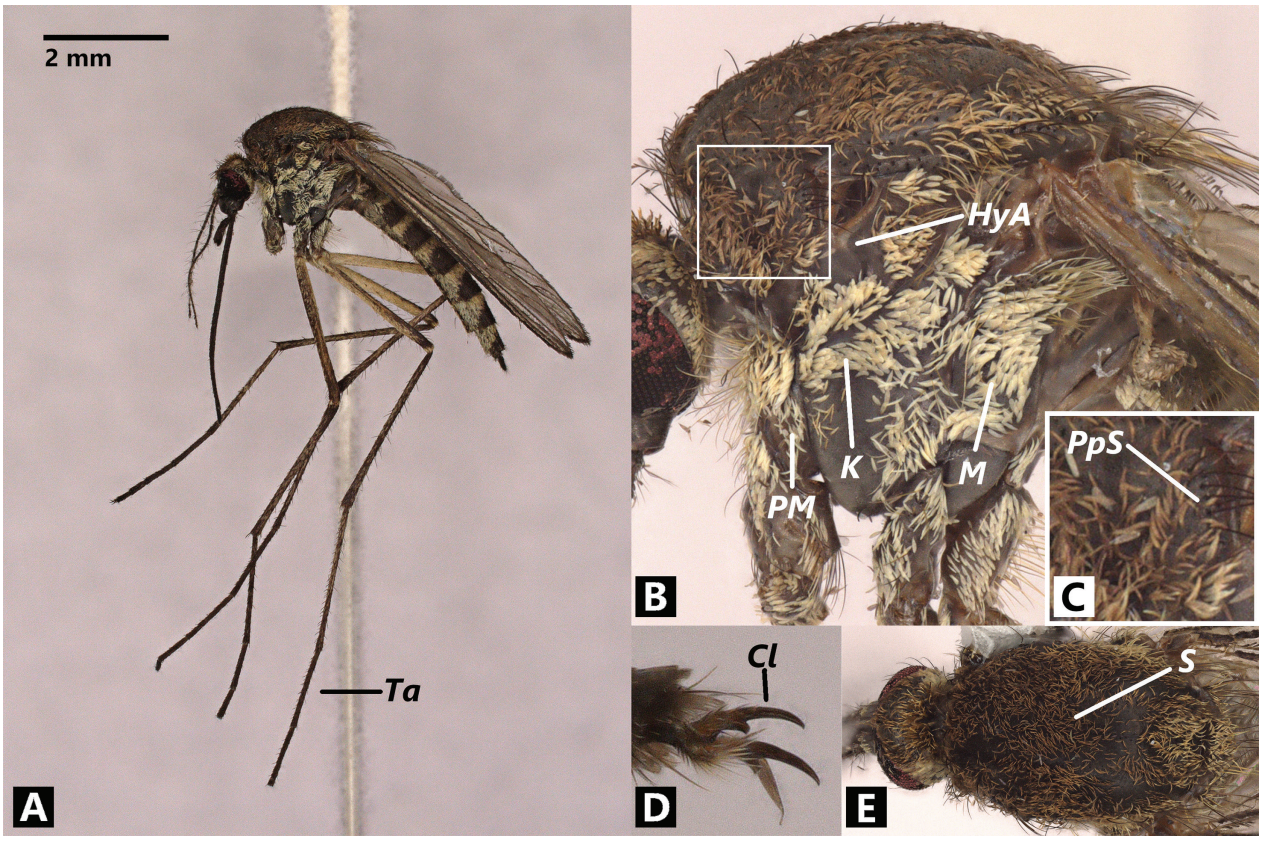

Figure 6. Punctor subgroup's outlier: **A** lateral view of habitus, showing tarsomeres (*Ta*); **B** lateral view of thorax, showing postprocoxal membrane (*PM*), katepisternum (*K*), mesepimeron (*M*), and hypostigmal area (*HyA*); **C** close-up of postpronotum (*PpS*); **D** close-up of hind tarsal claw (*Cl*); **E** dorsal view of scutum (*S*).

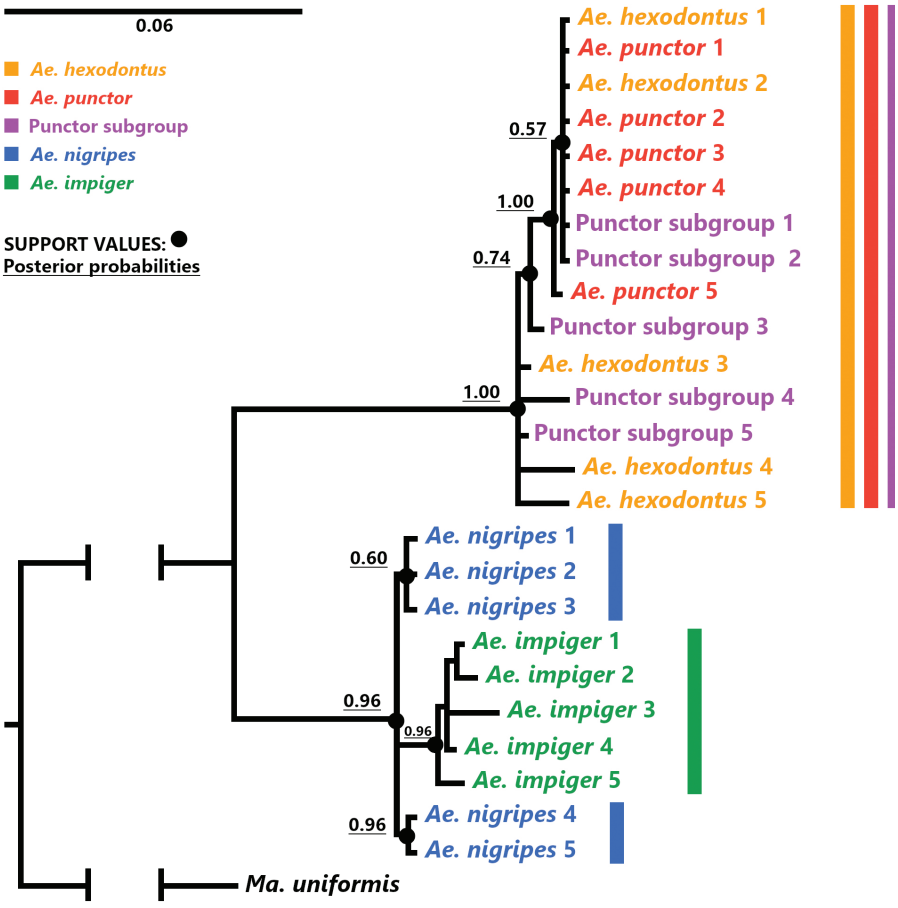

Figure 7. Phylogram from Bayesian inference analysis using COI, ITS2 and 28S partitioned concatenated dataset from current study. *Aedes impiger* (in green) forms monophyletic group, rendering *Aedes nigripes* (in blue) paraphyletic. *Aedes hexodontus* (in orange), *Aedes punctor* (in red) and Punctor subgroup's outliers (in magenta) formed polyphyletic group. *Mansonia uniformis* used as outgroup. Posterior probabilities (in bold, underlined) displayed as nodal support. Phylogram cropped for display purposes.

Analysis of concatenated COI, ITS2, and 28S gene regions generated three distinct morphology-based groups (Fig. 7): one monophyletic group (*Ae. impiger*), one paraphyletic group (*Ae. nigripes*), and one polyphyletic group (*Ae. punctor* and *Ae. hexodontus*). As expected, the molecular identification of the two isomorphic species, *Ae. punctor* and *Ae. hexodontus*, was not conclusive, further confirming the challenges associated with the Punctor subgroup.

Aedes hexodontus and *Ae. punctor* were recovered as a polyphyletic group in the BI and ML analyses on the concatenate dataset (Fig. 7: PP = 1.00), the ITS2 dataset (Fig. 8: B = 20, PP = 0.75), and the COI datasets, both partitioned (Fig. 9: B = 52, PP = 0.76) and unpartitioned (Fig. 9: B = 91, PP = 1.00).

Aedes impiger was recovered as a monophyletic group in the BI and ML analyses in the concatenated dataset (Fig. 7: PP = 0.96) and the COI datasets, both partitioned (Fig. 9: B = 38, PP = 0.80) and unpartitioned (Fig. 9: B = 73, PP = 0.98). The same results can be observed with *Ae. nigripes* for the BI and ML analyses on the COI datasets, both partitioned (Fig. 9: B = 61, PP = 0.95) and unpartitioned (Fig. 9: B = 62, PP = 0.87). Regarding the BI analysis on the concatenated dataset (Fig. 7: PP = 0.96 and PP = 0.60), *Ae. nigripes* was rendered paraphyletic. *Aedes impiger* and *Ae. nigripes* formed a polyphyly in the BI and ML analyses on the ITS2 dataset (Fig. 8: B = 73, PP = 0.52).

Concatenation of the gene sequences increased the power of our BI analysis for the Punctor subgroup (Fig. 7: PP = 1, vs Fig. 8: PP = 0.75 and Fig. 9: PP = 0.92), but not for *Ae. nigripes* (Fig. 9: PP = 0.97, vs Fig. 7: PP = 0.96 and 0.96) and *Ae. impiger* (Fig. 9: PP = 0.98, vs Fig. 7: PP = 0.96). Partitioning substantially decreased the support of our BI and ML analyses for the Punctor subgroup (Fig. 9: B = 91 vs 52, and PP = 1 vs 0.76) and *Ae. impiger* (Fig. 9: B = 73 vs 38, and PP = 0.98 vs 0.80), but slightly increased the support of our BI and ML analyses for *Ae. nigripes* (Fig. 9: B = 62 vs 61, and PP = 0.87 vs 0.95).

4. Discussion

4.1. The tarsal claw and the COI barcoding region can differentiate *Aedes impiger* and *Aedes nigripes*

Aedes impiger and *Aedes nigripes* are the only species of mosquitoes found in the high Arctic of North America (Vockeroth 1954; Wood et al. 1979) with *Ae. nigripes* being the dominant species north of the tree line (i.e. the highest latitude at which trees can grow) (Wood et al. 1979). These two species share striking morphological similarities throughout their life stages, necessitating the development of a specific key to aid in their identification (Danks and Corbet 1973). Although the female mosquitoes of these species exhibit a noticeable hairy appearance compared to other black-legged *Aedes* (Vockeroth

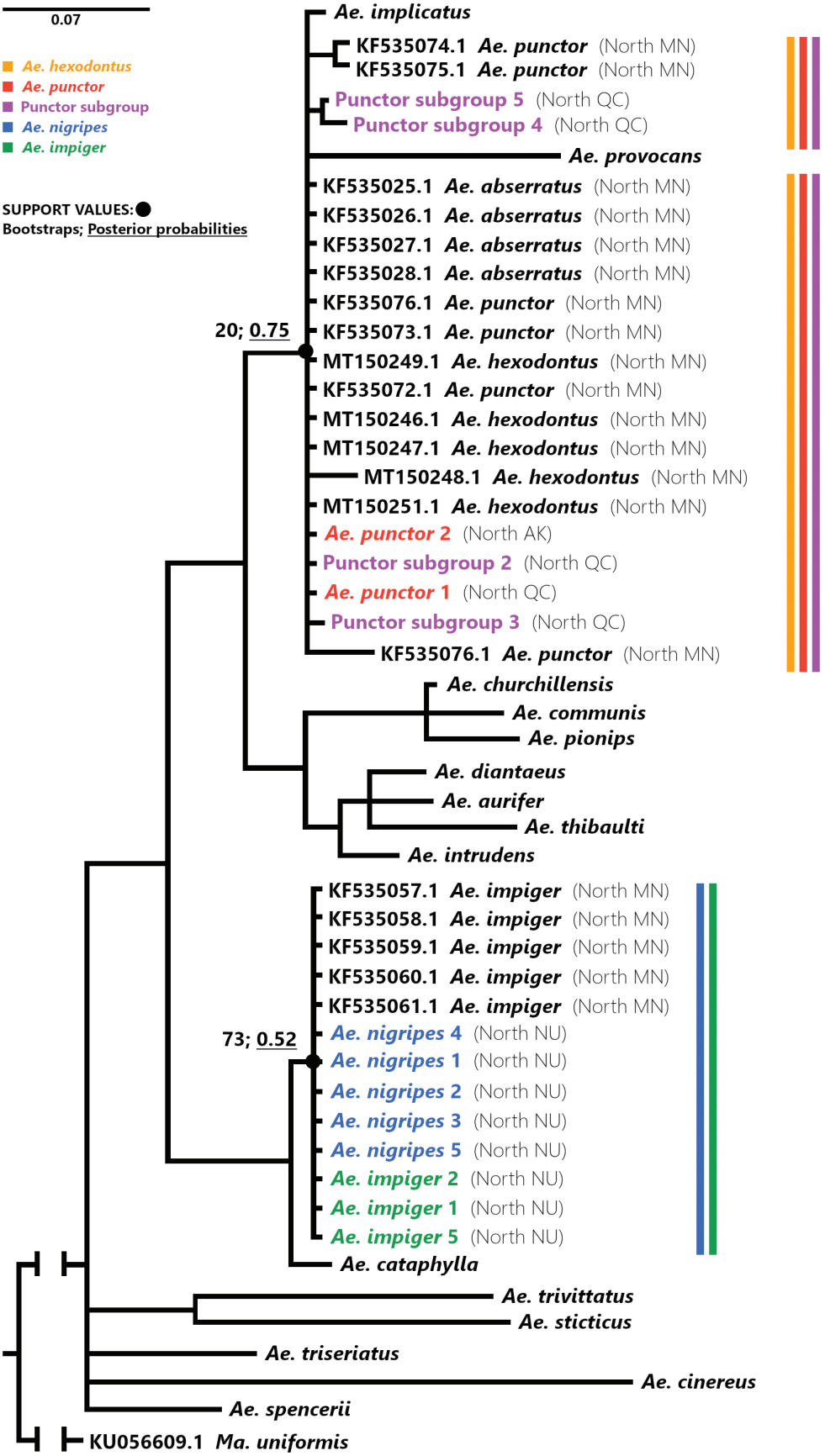

Figure 8. Simplified phylogram from Bayesian inference analysis using ITS2 dataset from current study, using public sequences (with their respective GeneBank number) and sequences from our own specimens (in colour). Results show two polyphyletic groups, regrouping *Aedes hexodontus* (in orange), *Aedes punctor* (in red) and Punctor subgroup's outliers (in magenta), as well as *Aedes impiger* (in green) and *Aedes nigripes* (in blue). *Mansonia uniformis* used as outgroup. Bootstrap values from Maximum likelihood analysis (in bold) and posterior probabilities from Bayesian inference analysis (in bold, underlined) displayed as support values. Sampling regions in parentheses: MN (Manitoba, CAN); QC (Québec, CAN); AK (Alaska, USA); NU (Nunavut, CAN).

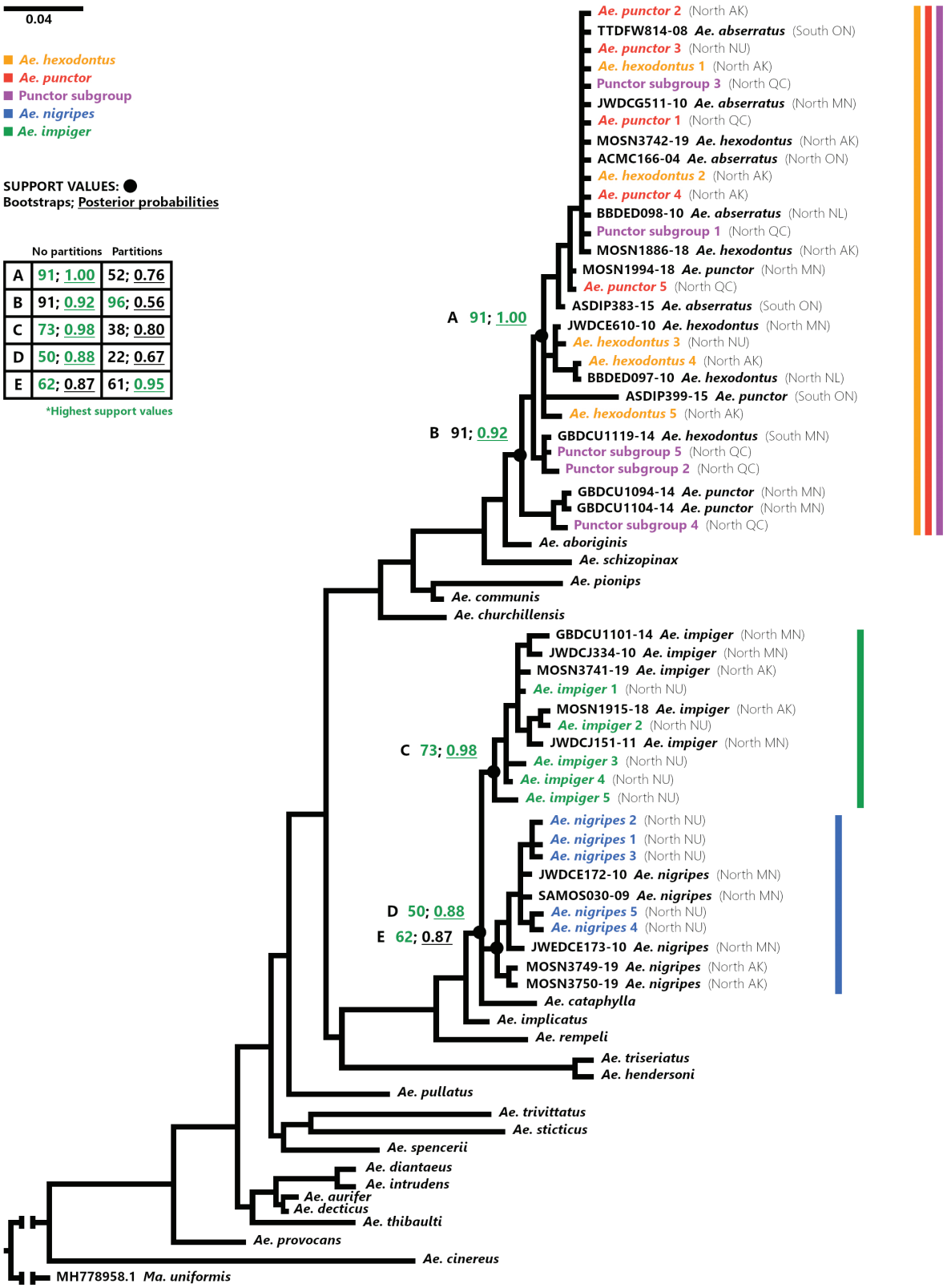

Figure 9. Simplified phylogram from Maximum Likelihood analysis using COI dataset from current study, using public sequences (with their respective GenBank number) and sequences from our own specimens (in colour). Sequences not partitioned per codon position. Results show one polyphyletic group, regrouping *Aedes hexodontus* (in orange), *Aedes punctor* (in red) and Punctor subgroup's outliers (in magenta), and two monophyletic groups, *Aedes impiger* (in green) and *Aedes nigripes* (in blue). *Mansonia uniformis* used as outgroup. Bootstrap values from Maximum likelihood analysis (in bold) and posterior probabilities from Bayesian inference analysis (in bold, underlined) displayed as support values. Five support values chosen (A to E, in yellow highlight) and compared with same dataset partitioned by codons. Table indicates superior performance of unpartitioned sequence data (higher support in green) in comparison to sequence data portioned per codon position. Sampling regions in parentheses: AK (Alaska, USA); ON (Ontario, CAN); NU (Nunavut, CAN); QC (Québec, CAN); MN (Manitoba, CAN); NL (Newfoundland and Labrador, CAN).

1954; Wood et al. 1979; Thielman and Hunter 2007), this criterion alone is insufficient to confirm accurate identification.

The arrangement of postpronotal setae serves to differentiate these two species from other black-legged *Aedes* mosquitoes (Carpenter and LaCasse 1955; Wood et al. 1979; Thielman and Hunter 2007). However, it can be quite challenging to observe this criterion since setae are frequently absent and scales on the postpronotum can conceal the setae attachment sites (Thielman and Hunter 2007). Consequently, this criterion is easily overlooked, especially by an untrained observer, leading to misidentification. This issue primarily affects *Ae. impiger*, as it inhabits subarctic environments alongside multiple other *Aedes* species (Wood et al. 1979; Ward and Darsie 2005). In situations where the arrangement of the postpronotum setae is challenging to assess, examination of the tarsal claw should confirm the identification of *Ae. impiger* since no other northern black-legged species of *Aedes* possesses claws with a similar shape (Knight 1951; Vockeroth 1954; Danks and Corbet 1973).

Our study represents one of the first attempts to assess the performance of the COI and ITS2 barcoding regions in identifying *Ae. impiger* and *Ae. nigripes*. The results of our analysis revealed that *Ae. impiger* and *Ae. nigripes* form two distinct monophyletic clades when using the COI barcoding region, thus supporting the use of this barcoding region as an effective means of identification for these two species. Since *Ae. nigripes* is rendered paraphyletic in the concatenated analysis, this may be a sign of species complexes or even cryptic species. Still, the number of samples would have to be considerably increased to explore those possibilities. However, when employing the ITS2 barcoding region, *Ae. impiger* and *Ae. nigripes* grouped together in a single polyphyly, indicating that this region is not reliable for distinguishing between the two species. Based on our findings, the COI, but not ITS2, barcoding region can be used for the identification of *Ae. impiger* and *Ae. nigripes*.

4.2. *Aedes hexodontus* and *Aedes punctor* are not reliably differentiated and may not warrant separate species status

The Punctor subgroup belongs to a distinct subdivision of the *Aedes communis* group (group G) of Edward's classification (Edwards 1932). It was first mentioned by Dyar and revised by Knight, primarily based on the morphology of male genitalia (Dyar 1922; Knight 1951). The species belonging to the Punctor subgroup are *Ae. punctor*, *Ae. hexodontus*, *Aedes aboriginis* Dyar, 1917, *Aedes abserratus* Felt and Young, 1904 and *Aedes punctodes* Kirby, 1837 (Dyar 1922). Among these species, *Ae. hexodontus* and *Ae. punctor* are the only ones with distributions that reach the Arctic (Wood et al. 1979; Ward and Darsie 2005).

In subarctic regions, the morphological characters usually used to differentiate between *Ae. hexodontus* and *Ae. punctor* become obscure. In *Ae. punctor*, the striped scutum starts fading and white scales can sometimes be observed at the base of the costa – in these cases, *Ae. punctor* is scarcely distinguishable from *Ae. hexodontus* (Wood et al. 1979; Ward and Darsie 2005).

To help differentiate between *Ae. hexodontus* and *Ae. punctor* morphologically, the number of scales on the probasisternum can be used. Typically, female *Ae. hexodontus* have a heavily scaled probasisternum, whereas female *Ae. punctor* have a bare probasisternum (Wood et al. 1979). However, even this morphological characteristic can be doubtful, as some individuals of *Ae. punctor* can also exhibit scattered pale scales on the probasisternum. In our case, the number of scales on the probasisternum proved to be a dubious criterion for identifying specimens of *Ae. punctor* and *Ae. hexodontus*. Specimens with a heavily scaled probasisternum did not fit into either group. These specimens exhibited morphological characteristics associated with both *Ae. punctor* (a few pale scales at the base of the costa) and *Ae. hexodontus* (a heavily scaled probasisternum), making it impossible to identify them beyond the Punctor subgroup. Gimnig (2000) reported comparable findings for *Ae. hexodontus* where the majority of the specimens displayed extensive scaling on the probasisternum. However, all of their *Ae. punctor* specimens had completely bare probasisternum (Gimnig 2000). It is worth noting that the specimens were collected exclusively from the West coast, and none of the sampling locations were in the Arctic or subarctic regions, which may explain the morphological differences. Gimnig (2000) concluded that in females, the presence of scales on the probasisternum and the number of white scales at the base of the costa did not provide a satisfactory separation of any member of the Punctor subgroup (Gimnig 2000). Due to the limited number of specimens in our study, we cannot confirm or refute this statement. The number of scales on the probasisternum and the number of white scales at the base of the costa have potential as a differentiating characteristic between the two species, but our findings indicate that these identification criteria cannot be universally applied to all the Punctor subgroup. There are two explanations for this discrepancy: either the number of scales on the probasisternum and at the base of the costa is not a reliable diagnostic feature, or the outlier specimens collected at the same sampling location (Kuujjuaq) are hybrids. Further analyses are necessary before confirming the inclusion of the number of scales on the probasisternum and at the base of the costa as definitive identification criteria for the Punctor subgroup.

Female morphology may not help to distinguish *Ae. hexodontus* and *Ae. punctor*, but it is possible to identify the species of the Punctor subgroup based on larval morphology (Knight 1951). The numbers and the length of the comb scales were first used as a criterion, since *Ae. hexodontus* have noticeably fewer and larger scales ($n<9$; >0.1 mm) than *Ae. punctor* ($n>10$; <0.08 mm) (Vockeroth 1954). However, it was later shown that there is a much greater overlap in the number of comb scales; both spe-

cies can have 5 to 12 comb scales (*hex* n = 4 to 12; *pun* n = 5 to 25) (Wood 1977). There is also an overlap in scale length in *Ae. hexodontus* larvae (as short as 0.089 mm) and *Ae. punctor* larvae (as long as 0.108 mm) (Gimnig 2000). However, other characteristics can be used, such as the shape of the cranial setae 5-C and 6-C, where *Ae. hexodontus* is double-branched and *Ae. punctor* is single-branched. However, these larval criteria are not useful as an identification tool for females captured in the field.

In addition to the morphological characteristics, the known geographic distribution could potentially help distinguish the two species. The distribution of *Ae. punctor* is practically confined to the boreal forest, seldom occurring to the south or north onto the tundra, whereas *Ae. hexodontus* is the dominant mosquito above the tree line (Wood et al. 1979). However, both species are present in subarctic locations. We also need to consider that, based on the difficulty of identification, the extent of their range is not truly known – specimens of *Ae. hexodontus* and *Ae. punctor* could have been confused with other members of the Punctor subgroup (Knight 1951; Carpenter and LaCasse 1955).

Our phylogenetic analysis showed that *Ae. hexodontus* and *Ae. punctor* form a single polyphyletic cluster when using the COI or ITS2 barcoding regions. A previous study on Canadian mosquitoes has also indicated the limitations of the COI barcoding region in delineating species within complex groups (Namin et al. 2014). For instance, a phylogenetic analysis of the *Aedes communis* complex using the COI barcoding region revealed comparable findings, with the complex appearing paraphyletic in relation to *Ae. abserratus* and *Aedes implicatus* Vockeroth, 1954 (Namin et al. 2014). These results underscore the need for expanded genetic and taxonomic sampling to accurately infer the phylogenetic relationships of the Punctor subgroup. Based on our findings, neither the COI nor the ITS2 barcoding regions can be used effectively for the identification of *Ae. hexodontus* and *Ae. punctor*.

Given these findings and the striking morphological similarities observed throughout the life stages of *Ae. hexodontus* and *Ae. punctor*, the question arises of whether they are truly distinct species. Some authors considered them two closely related species based mostly on larval morphology (Knight 1951; Vockeroth 1954), but these conclusions were reached using unreliable criteria for larval identification. A more recent study based on genetic and morphological comparison of *Ae. hexodontus*, *Ae. abserratus*, and *Ae. punctor* suggests three distinct species, but only in the southern part of their distribution (Gimnig 2000). This finding highlights the fact that, given their broad distribution, *Ae. hexodontus* and *Ae. punctor* might be separate species outside of the studied area. Overall, the question of whether *Ae. hexodontus* and *Ae. punctor* truly represent distinct species requires further investigation using comprehensive morphological and molecular approaches, especially for specimens in the northern part of their distribution.

4.3. Challenges with sequences on public databases: under-representation, inaccurate identifications, and overbinning

To what extent can we trust the publicly available DNA sequences attributed to the northern black-legged *Aedes* species? The difficulty of using morphology to distinguish among species could easily populate the molecular libraries with sequences bearing inaccurate species designations. Furthermore, we demonstrated that the COI and ITS2 barcoding regions do not effectively differentiate among *Ae. impiger*, *Ae. nigripes*, *Ae. hexodontus* and *Ae. punctor*. Therefore, the accuracy of the sequence identification might be questionable – especially for specimens that have not been morphologically pre-identified by a taxonomist familiar with the Punctor subgroup.

For example, due to challenges associated with accessing northern sampling locations, there are not many sequences of *Ae. nigripes* and *Ae. impiger* available online on GenBank (*nig* n = 103 ; *imp* n = 46) or BOLD (*nig* n = 150, *imp* n = 145). Accuracy of identification is also a challenge. On the BOLD database (Table 4; Table 5), both *Ae. impiger* and *Ae. nigripes* share the same Barcode Index Number (BIN; i.e., AAA3750). However, these two species account for only a fraction of the specimens associated with this BIN (9%); 86% are classified as unknown specimens, while 3% are identified as *Aedes cataphylla* Dyar, 1916, 1% as *Ae. implicatus*, and less than 1% as *Aedes niphadopsis* Dyar & Knab, 1918, *Aedes* sp., *Aedes leucomelas* Meigen, 1804, *Aedes ventrovittis* Dyar, 1916, *Ae. hexodontus*, *Aedes intrudens* Dyar, 1919, and *Aedes communis* DeGeer, 1776. This can be explained by misidentification of specimens at the species level. Therefore, it is not possible to use the associated BIN (AAA3750) for identifying *Ae. impiger* and *Ae. nigripes*, considering the diverse array of species linked to the same BIN. While our phylogenetic analyses indicate that *Ae. impiger* and *Ae. nigripes* form monophyletic clades when using the COI barcode, it is important to combine this identification method with other molecular and morphological traits.

Due to their wider geographic distribution, there are twice as many sequences for *Ae. hexodontus* and *Ae. punctor* on online databases such as GenBank (*hex* n = 201; *pun* n = 194) or BOLD (*hex* n = 327; *pun* n = 361). Unfortunately, the precision of BINs associated with the Punctor subgroup species is even more questionable (Table 4; Table 5). *Aedes hexodontus* and *Ae. punctor* are linked to seven and six different BINs, respectively, with three BINs shared between the two species. However, the majority of sequences associated with these species fall under BIN AAA3748, where *Ae. hexodontus* and *Ae. punctor* account for only one third of the specimens, with 58% classified as unknown specimens, 7% as *Ae. abserratus*, and less than 3% as *Ae. aboriginis*, *Aedes diantaeus* Howard, Dyar & Knab, 1913, *Aedes* sp., *Ae. ventrovittis*, *Ae. communis*, *Ae. nigripes*, *Aedes pionips* Dyar, 1919, *Aedes fitchii* Felt & Young, 1904, *Ae. intrudens*, and *Aedes decticus* Howard, Dyar & Knab, 1917. Given the numerous BINs associated with *Ae. hexodontus* and

Table 4. Summary of barcode indexing numbers (BINs) representing Arctic and sub-Arctic black-legged species (Accessed 18 Jul 2023). Total number of sequences assigned to each BIN (Total), total number of sequences with a species designation (Named seqs), total number of species designations associated with each BIN (Species) and the dominant designation(s) of each BIN (Designation) (arctic and subarctic black-legged species displayed in bold font). *Designated to *Ae. communis* complex

BIN	Total	Named seqs	Species	Designation
BOLD:AAA3748	1680 [1620 Public]	700	12	***Ae. punctor*** (280/1680); ***Ae. hexodontus*** (256/1680)
BOLD:AAA3750	3670 [3660 Public]	530	10	***Ae. nigripes*** (162/3670) ***Ae. impiger*** (152/3670) *Ae. cataphylla* (122/3670)
BOLD:AAA3751	885 [892 Public]	431	9	*Ae. communis* (376+20*/885)
BOLD:AAB6338	114 [111 Public]	83	3	*Ae. pionips* (80/114)
BOLD:AAT9839	43 [43 Public]	43	5	***Ae. punctor*** (29/43); ***Ae. hexodontus*** (8/43)
BOLD:AEF2604	50 [50 Public]	50	3	***Ae. hexodontus*** (48/50)
BOLD:AEF4724	6 [6 Public]	6	1	***Ae. hexodontus*** (6/6)
BOLD:AAA6148	606 [602 Public]	357	6	*Ae. communis* (339/606)
BOLD:AAC1238	160 [161 Public]	69	5	***Ae. punctor*** (45/160); *Ae. implicatus* (20/160)
BOLD:AEI5390	8 [8 Public]	4	1	***Ae. punctor*** (4/8)

Table 5. Arctic and subarctic black-legged *Aedes* species and associated BINs (Accessed 18 Jul 2023). Proportion of sequences with specific species designation/total number of sequences in BIN.

	BOLD designated Barcode Index Number (BIN)									
Species	**AAA3748**	**AAA3750**	**AAA3751**	**AAB6338**	**AAT9839**	**AEF2604**	**AEF4724**	**AAA6148**	**AAC1238**	**AEI5390**
Ae. nigripes	3/1680	162/3670								
Ae. impiger		152/3670								
Ae. hexodontus	256/1680	1/3670	1/885	1/114	29/43	48/50	6/6			
Ae. punctor	280/1680				8/43	1/50		6/606	45/160	4/8

Ae. punctor and the diversity of associated species, the use of BINs is futile. Northern specimens without clear distinguishing features such as a scutum distinctly striped with a bare probasisternum cannot be identified as *Ae. punctor* with certainty (Wood et al. 1979). Furthermore, the absence of accompanying morphological observations associated with public sequences creates uncertainty regarding the basis of identification of morphospecies. Therefore, we propose that public sequences labelled as *Ae. hexodontus* and *Ae. punctor* and identified through COI and ITS2 molecular barcodes, be regarded as the Punctor subgroup. This relabelling should only apply if the specimen meets at least one of the following criteria: (1) sampled near the tree line, or at any Arctic and subarctic locations, (2) no morphological identification prior to sequencing, (3) unstriped (or bare) scutum and/or probasisternum displaying scales.

4.4. Urgent needs for baselines on vectors and vector-borne diseases in the Arctic as the global hotspot of climate change

Accurate species identification is crucial for an effective arbovirus surveillance program, especially in northern locations where *Ae. hexodontus* and *Ae. punctor* frequently carry Jamestown Canyon virus (JCV) and Snowshoe hare virus (SSHV) (Iversen et al. 1973; McLean et al. 1977; Campbell et al. 1991; Hardy et al. 1993; Carson et al. 2017; Snyman et al. 2023). SSHV has also been isolated from *Ae. nigripes* (McLean and Lester 1984). Vector competence studies have also confirmed that both *Ae. hexodontus* and *Ae. punctor* can transmit one or both of these viruses (Boromisa and Grayson 1990; Heard et al. 1991; Kramer et al. 1993; Walker et al. 1993). Serological studies have shown that antibodies for both JCV and SSHV are present in wildlife and people in the Arctic (Zarnke et al. 1983; Walters et al. 1999; Miernyk et al. 2019; Buhler et al. 2023). Although human cases are rare, JCV and SSHV infections can cause symptoms ranging from fever and headache, to altered mental state ranging from confusion to coma, with or without additional signs of brain dysfunction, and death (Snyman et al. 2023). In the context of the Arctic, where the consequences of climate change are alarming, accurate species identification becomes especially significant (Hoberg and Brooks 2015). As warming trends persist, there is potential for additional vector and virus species to invade and survive in more northern regions, as well as an increase in abundance of existing species (Alto and Juliano 2001). These changes can disrupt the barriers that prevent the emergence of arboviruses by substantially affecting the population dynamics of viruses, mosquito vectors, and wild-

life hosts (Høye 2020; Koltz and Culler 2021). There is a need to monitor these changes due to the burden it could impose on northern communities (Villeneuve et al. 2021). However, to ensure an effective surveillance program, accurate vector species identification is indispensable, both to detect newly introduced species as well as to avoid mistaking newly detected species as newly introduced. Given that the Punctor subgroup constitutes a substantial portion of the mosquito population and is known to act as vectors for arboviruses, it becomes crucial to address the identification challenges associated with this subgroup.

In establishing an effective surveillance program, another crucial aspect is ensuring the reliability of the vector status of northern *Aedes* species. Most of our information is based on dated studies, which poses a problem, as their accuracy relies on the correct identification of the specimens. Before 1977, *Ae. hexodontus* and *Ae. punctor* were regarded as separate forms, namely the "normal" type and the "tundra" type variety, based on female and larval characteristics (Knight 1951). It was only after Wood (1977) re-examined Knight's specimens that he concluded the "tundra" type of *Ae. punctor* was, in fact, *Ae. hexodontus* (Wood 1977). Due to the challenges in identifying females of northern black-legged *Aedes*, we cannot be sure that both *Ae. hexodontus* and *Ae. punctor* are competent vectors for JCV and SSHV based on previous reports. Consequently, a considerable amount of work needs to be done to confirm the true Arctic vectors of JCV and SSHV and their transmission efficacy to better inform existing and future surveillance programs in the North.

5. Conclusion

Our study yielded several significant findings regarding the identification and taxonomy of *Aedes* spp. mosquitoes, including the Punctor subgroup. Firstly, we observed that the COI barcoding region can be utilized effectively to distinguish *Aedes impiger* and *Aedes nigripes*, in contrast to the ITS2 barcoding section. However, both barcoding sections are inadequate to differentiate species within the Punctor subgroup (*Aedes hexodontus* and *Aedes punctor*), as they group as a paraphyletic cluster. Moreover, we raised concerns about the precision of identifying *Ae. hexodontus* and *Ae. punctor* using molecular barcodes in public sequence databases, suggesting that their identification should not extend beyond the Punctor subgroup in many cases. Our results also revealed that the commonly used morphological characters in identification keys are not consistently reliable for distinguishing species within the Punctor subgroup.

These findings emphasize the urgent need to reassess the taxonomic status of species currently considered as members of the Punctor subgroup using an integrative approach. Such a revision should aim to include specimens beyond females, as male genitalia morphology may provide key features. Efforts should also be directed towards rearing adults from eggs in order to integrate morphological characteristics from all life stages. A molecular biogeographical approach that uses specimens from various biomes in North America, including boreal forests, taiga, tundra and mountainous regions, could prove invaluable on a lower taxonomic and/or hybridisation scale. Furthermore, additional molecular markers could be explored to determine their effectiveness in distinguishing between *Aedes hexodontus* and *Ae. punctor*. Finally, alternative methods to morphology and molecular barcodes such as near-infrared spectroscopy and/or MALDI-TOF are directions for future work as are a comparison of species delimiting approaches such as ASAP, GMYC, mPTP and BINs that have been proven useful in other taxonomic groups. Finally, microsatellites could prove a worthwhile method for investigating the degree of reproductive isolation.

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7. Competing interests

The authors have declared that no competing interests exist.

8. Author's contributions

Carol-Anne Villeneuve: Conceptualisation, data curation, formal analysis, investigation, project administration, resources, visualization, writing – original draft. — **Louwrens P. Snyman:** Conceptualisation, data curation, formal analysis, investigation, methodology, resources, validation, writing – original draft. — **Emily J. Jenkins:** funding acquisition, resources, writing – review & editing. — **Nicolas Lecomte:** funding acquisition, supervision, writing – review & editing. — **Isabelle Dusfour:** Conceptualisation, supervision, writing – review & editing. — **Patrick A. Leighton:** funding acquisition, supervision, writing – review & editing.

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Supplementary Material 1

Tables S1, S2

Authors: Villeneuve C-A, Louwrens SP, Jenkins EJ, Lecomte N, Dusfour I, Leighton PA (2024)
Data type: .xlsx
Explanation notes: Table S1. Vouchers info. Full information about the vouchers deposited in the entomological collection. — **Table S2.** Sequences info. All the sequences used in our analysis (3 datasets).

Link: https://doi.org/10.3897/asp.81.e111985.suppl1

Supplementary Material 2

Figure S1

Authors: Villeneuve C-A, Louwrens SP, Jenkins EJ, Lecomte N, Dusfour I, Leighton PA (2024)
Data type: .pdf
Explanation notes: Trees. Raw phylogenetic trees from all our ML and BI analyses.

Link: https://doi.org/10.3897/asp.81.e111985.suppl2