


Article

# Back from the Past: DNA Barcodes and Morphology Support *Ablabesmyia americana* Fittkau as a Valid Species (Diptera: Chironomidae)

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**Abstract:** Short, standardized gene fragments for species identification (DNA barcodes) have proven effective in delineating closely-related insect species, and can be critical characters to include in taxonomic studies. This is also the case for the species-rich and widely distributed fly family Chironomidae (non-biting midges). Inspired by observed genetic differences in partial COI gene sequences between North American and European populations of the chironomid *Ablabesmyia monilis* sensu lato, we investigated whether or not the morphology of male and female adults supported the distinction of more than one species. Our results support that the junior synonym *Ablabesmyia americana* is a valid species separate from *A. monilis*, and that *A. monilis* sensu stricto is distributed both in the Palearctic region and in North America. We provide re-descriptions of all of the major life stages of *A. americana* and of the adult female of *A. monilis*.

**Keywords:** DNA barcodes; taxonomy; species delimitation; synonymy; Tanytopodinae

## 1. Introduction

Over the last ten years, molecular approaches to analyze species boundaries have seen rapid development. Genetic divergences in the partial cytochrome c oxidase subunit 1 (COI) sequences (so-called DNA barcodes [1]) have proven effective for species recognition in several animal groups, including non-biting midges belonging to the family Chironomidae (Insecta, Diptera) (e.g., [2–6]). In several cases, DNA barcodes have also aided the detection of presumably cryptic species, whose distinction a posteriori could be confirmed by both morphology and nuclear genetic markers (e.g., [7,8]). Furthermore, DNA barcodes have been shown suitable to associate life stages in the family Chironomidae [9–11].

*Ablabesmyia* is a large and ecologically diverse genus in the subfamily Tanytopodinae. About 90 species are known worldwide ([12] and citations within), many of them described from the Neotropical Region [13]. These small to medium size dipterans (1.00–6.00 mm) are found in a broad range of aquatic systems, from small streams and ponds to lakes and rivers. Larvae can live in freshwater sponge colonies [14]. The genus was erected by Johannsen [15] with *Tipula monilis* Linnaeus, 1758, as the type species. Historically *Ablabesmyia* had a wider concept, with most of its members placed in the *Pentaneura* group A of Edwards [16] and Johannsen [17]. Later, Freeman [18] and Roback [19] relegated *Ablabesmyia* to subgeneric status in *Pentaneura* (Philippi, 1865), while Fittkau [20] gave it full generic status. *Ablabesmyia* larvae are predators, and are recorded in many ecological studies (e.g., [21–24]). The genus is one of the most distinctive and well-defined genera within the tribe *Pentaneurini*, with adult males possessing a homologous complex of dorsomedial blades and lobes in their genitalia unique to the taxon [25,26].

The genus *Ablabesmyia* comprises four valid subgenera: *Ablabesmyia* Johannsen, *Asayia* Roback, *Karelia* Roback and *Sartaia* Roback. However, Oliveira, Silva and Gessner [13] reported inconsistencies in the establishment of these subgroups, and suggested at least one additional subgenus for *Ablabesmyia*, without promoting this taxonomic action. Identification of the adult *Ablabesmyia* is primarily based on features of the genitalia, as it is for many other members of Chironomidae. The morphological identification can be challenging, particularly for non-experts, and normally requires time-consuming dissections of the genitalia. In addition, some *Ablabesmyia* species lack diagnostic morphological traits in their adult life stage, and need associated larvae and pupae to be morphologically identifiable to species-level. Despite these obstacles, the taxonomy of *Ablabesmyia* has been investigated in multiple geographic regions, especially in the Neotropics (e.g., [13,14,27–31]), providing identification keys to species-level, as well as extensive reference collections. This makes *Ablabesmyia* a good model taxon to investigate the congruence between molecular and morphological traits.

*Ablabesmyia americana* was erected by Fittkau [20] as a species separate from *A. monilis* (Linnaeus, 1758) based on the inner features of the male genitalia, but without specifying the exact characters. Subsequently, Roback [25] compared North American with European specimens, and was unable to find any valid differences to justify the distinction of a separate species for the New World specimens, synonymizing *A. americana* with *A. monilis*. Through the sampling and DNA barcoding of *A. monilis* populations in North America and Europe, we discovered considerable genetic divergence across the northern Atlantic region. We were therefore inspired to investigate if this divergence could be supported by the morphology of larvae, pupae and adult life stages, and if the synonymy of *Ablabesmyia americana* and *A. monilis* can be supported.

## 2. Materials and Methods

The specimens included in this study were selected to represent taxa with a possible taxonomic conflict in the *Ablabesmyia* gr. *monilis* sensu Fittkau [20]. Fieldwork was conducted near and in the streams, rivers, fens, ponds and lakes of eastern, central and northern Norway, and Churchill, northern Canada (Manitoba). The material was initially collected for inventory studies and part of the International Barcode of Life initiative. Fourth-instar larvae and pupae were collected with hand nets, and adults sampled with sweep nets, emergence and Malaise traps. Specimens from Japan identified to *A. monilis* were kindly provided by Hiromi Niitsuma through Bohdan Bilyj. A small piece of tissue for DNA extraction was sampled under a stereomicroscope and sent to the Canadian Centre for DNA Barcoding at the University of Guelph (CCDB, [www.dnabarcoding.ca](http://www.dnabarcoding.ca)) for DNA isolation, polymerase chain reaction (PCR) and sequencing. The remnants of nearly all of the sampled specimens were then macerated in potassium hydroxide (KOH) and mounted on permanent microscope slides in Euparal for species identification with a compound microscope. Measurements were taken according to Epler [32]. Morphological terminology and abbreviations follow Roback [25] and Sæther [33], supplemented by Kowalyk [34] for larval cephalic setation (head hairiness) and Silva et al. [35,36] for larval terminology. One slide-mounted male and one slide mounted female specimen from the Johannsen collection out of Ithaca dated prior to his 1905 work were analyzed. Seven additional slides with larvae and two with females were also examined, but these either did not have their date or their locality written on the labels. Material from the Johannsen collection is deposited in the Cornell University Insect Collection, Ithaca, New York, USA (CUIC). Other examined material is deposited in the NTNU University Museum, Norwegian University of Science and Technology, Trondheim, Norway (NTNU-VM); or the Centre for Biodiversity Genomics, University of Guelph, Canada.

Sequence information along with an image and collateral information for each voucher specimen were entered into the Barcode of Life Data System (BOLD) [37]. The detailed specimen records and sequence information, including trace files, are available in BOLD through the dataset 'DS-ABLA, *Ablabesmyia americana* and *A. monilis*: A review' with doi: 10.5883/DS-ABLA. GenBank accessions are given in Table 1.

**Table 1.** List of analyzed specimens with associated sample localities, Specimen IDs and GenBank accessions.

Taxon	Country	Life Stage	BOLD ID	NTNU-VM	GB Accession
<i>Ablabesmyia americana</i>	Canada	Adult	10PROBE-11666		JF875917
<i>Ablabesmyia americana</i>	Canada	Adult	10PROBE-12218		JF876334
<i>Ablabesmyia americana</i>	Canada	Adult	10PROBE-12910		JF876913
<i>Ablabesmyia americana</i>	Canada	Adult	10PROBE-16464		KR655494
<i>Ablabesmyia americana</i>	Canada	Male adult	CHIR_CH10	201777	MK403766
<i>Ablabesmyia americana</i>	Canada	Male adult	CHIR_CH174	201778	MK403744
<i>Ablabesmyia americana</i>	Canada	Male adult	CHIR_CH228	201779	MK403740
<i>Ablabesmyia americana</i>	Canada	Male adult	CHIR_CH267	201780	MK403767
<i>Ablabesmyia americana</i>	Canada	Female adult	CHIR_CH268	201781	MK403747
<i>Ablabesmyia americana</i>	Canada	Male adult	CHIR_CH396	201782	MK403752
<i>Ablabesmyia americana</i>	Canada	Male adult	HLC-27231	201785	KR433631
<i>Ablabesmyia americana</i>	Canada	Female adult	HLC-27246	201786	KR433665
<i>Ablabesmyia americana</i>	Canada	Male adult	HLC-27252	201790	KR443910
<i>Ablabesmyia aspera</i>	Canada	Adult	10PROBE-10650		JF875206
<i>Ablabesmyia aspera</i>	Canada	Adult	10PROBE-11271		JF875757
<i>Ablabesmyia aspera</i>	Canada	Adult	10PROBE-12725		JF876793
<i>Ablabesmyia aspera</i>	Canada	Adult	10PROBE-12912		JF876915
<i>Ablabesmyia aspera</i>	Canada	Adult	10PROBE-13831		KR668901
<i>Ablabesmyia aspera</i>	Norway	Male adult	ATNA539	201788	MK403756
<i>Ablabesmyia aspera</i>	Norway	Male adult	CH-OSF172	143691	JN285991
<i>Ablabesmyia aspera</i>	Norway	Male adult	CH-OSF174	143693	MK403755
<i>Ablabesmyia aspera</i>	Canada	Male adult	CHIR_CH638	201783	MK403753
<i>Ablabesmyia aspera</i>	Canada	Male adult	HLC-27096	201784	KR435725
<i>Ablabesmyia aspera</i>	Norway	Female adult	SOE94	124714	HQ105015
<i>Ablabesmyia longistyla</i>	Norway	Male adult	TRD-CH185	200177	MK403739
<i>Ablabesmyia monilis</i>	Norway	Female adult	ATNA330	201789	HM421433
<i>Ablabesmyia monilis</i>	Norway	Female adult	ATNA538	201787	MK403749
<i>Ablabesmyia monilis</i>	Japan	Female adult	ES358	201791	MK403757
<i>Ablabesmyia monilis</i>	Japan	Male adult	ES360	201793	MK403737
<i>Ablabesmyia monilis</i>	Japan	Male adult	ES361	201794	MK403738
<i>Ablabesmyia monilis</i>	Norway	Male adult	Finnmark213	136311	JF870860
<i>Ablabesmyia monilis</i>	Norway	Male adult	Finnmark214	136312	JF870861
<i>Ablabesmyia monilis</i>	Norway	Male adult	Finnmark258	136355	JN285993
<i>Ablabesmyia monilis</i>	Norway	Male adult	Finnmark458	136554	JN286007
<i>Ablabesmyia monilis</i>	Norway	Male adult	Finnmark508	136603	JN286012
<i>Ablabesmyia monilis</i>	Norway	Male adult	Finnmark568	136663	JF870930
<i>Ablabesmyia monilis</i>	Norway	Male adult	Finnmark721	136814	JN286021
<i>Ablabesmyia monilis</i>	Norway	Male adult	NO 104	200861	MK403758
<i>Ablabesmyia monilis</i>	Norway	Female adult	NO 92	200850	MK403743
<i>Ablabesmyia monilis</i>	Norway	Male adult	RIN_CH13	148138	MK403765
<i>Ablabesmyia monilis</i>	Norway	Male adult	RIN_CH15	148140	MK403760
<i>Ablabesmyia monilis</i>	Norway	Male adult	RIN_CH29	148153	MK403748
<i>Ablabesmyia monilis</i>	Norway	Larva	RIN_CH30	148154	MK403741
<i>Ablabesmyia monilis</i>	Norway	Larva	RIN_CH32	148156	MK403754
<i>Ablabesmyia monilis</i>	Norway	Larva	RIN_CH34	148158	MK403764
<i>Ablabesmyia monilis</i>	Norway	Larva	RIN_CH50	148174	MK403759
<i>Ablabesmyia monilis</i>	Norway	Male adult	SOE10	124308	HQ105016
<i>Ablabesmyia monilis</i>	Norway	Male adult	SOE9	124307	HQ105017
<i>Ablabesmyia monilis</i>	Norway	Male adult	TRD-CH158	200150	MK403761
<i>Ablabesmyia monilis</i>	Norway	Female adult	TRD-CH221	200211	MK403750
<i>Ablabesmyia monilis</i>	Norway	Male adult	TRD-CH292	200278	MK403763
<i>Ablabesmyia monilis</i>	Norway	Male adult	TRD-CH308	200294	MK403762
<i>Ablabesmyia monilis</i>	Norway	Female adult	TRD-CH309	200295	MK403751
<i>Ablabesmyia monilis</i>	Norway	Male adult	TRD-CH325	200310	MK403742
<i>Ablabesmyia monilis</i>	Norway	Male pupa	TRD-CH427	200410	MK403745
<i>Ablabesmyia monilis</i>	Norway	Female adult	TRD-CH8	148261	MK403746

DNA extraction and amplification followed standard protocols at the CCDB and used the primers LepF1 + LepR1 [38], or a cocktail of the Lep and Folmer, et al. [39] primers (C\_LepFolF + C\_LepFolR) [40].

Polymerase chain reaction (PCR) products were purified for bidirectional sequencing with BigDye 3.1 termination (Applied Biosystems, Foster City, CA, USA) at the CCDB. Alignments were implemented on amino acid sequences, and refined using the Muscle algorithm in MEGA X [41], and were trivial, since no indels were present. Only sequences > 300 bp were included in the final alignment.

Maximum likelihood (ML) tree searches were performed using the Tamura 3-parameter with gamma correction for rate heterogeneity (T92+G). This model was determined to be the most suitable model of nucleotide substitution for our dataset using the hierarchical likelihood ratio test in MEGA X. Bootstrap analyses were conducted with 1000 pseudoreplicates [42]. Neighbor-joining tree searches and analyses of intra- and interspecific genetic divergences were conducted using the Kimura-2-parameter (K2P) model [43] for easier comparison with most published DNA barcode investigations. We used *Ablabesmyia aspera* (Roback, 1959) and *A. longistyla* Fittkau, 1962 as outgroups in the analysis, and rooted the resulting tree with *A. longistyla*, as this species is morphologically most divergent from *A. monilis* sensu lato. The Barcode Index Numbers [44] equivalent to genetic clusters in our resultant tree were analyzed in BOLD to investigate the documented distribution of each genetic lineage.

### 3. Results

Mitochondrial DNA COI (mtDNA COI) sequences were recovered from 56 specimens of four *Ablabesmyia* species (Table 1). Most of the aligned sequences were 658 base pairs long (94.6%) with 164 variable sites (24.9%), and included 137 sites (83.5%) being possibly parsimony informative. The majority of the variable sites were in the third codon-position (Table 2). The sequences were highly AT-biased, particularly in the third position, with a combined average AT-composition of 89.2% (Table 2). A hierarchical likelihood ratio test of aligned sequences in MEGA X returned the Tamura 3-parameter with the gamma correction for rate heterogeneity (T92+G) as the best model ( $-\ln L = 2435.952$ , BIC = 6045.744, AIC = 5096.616).

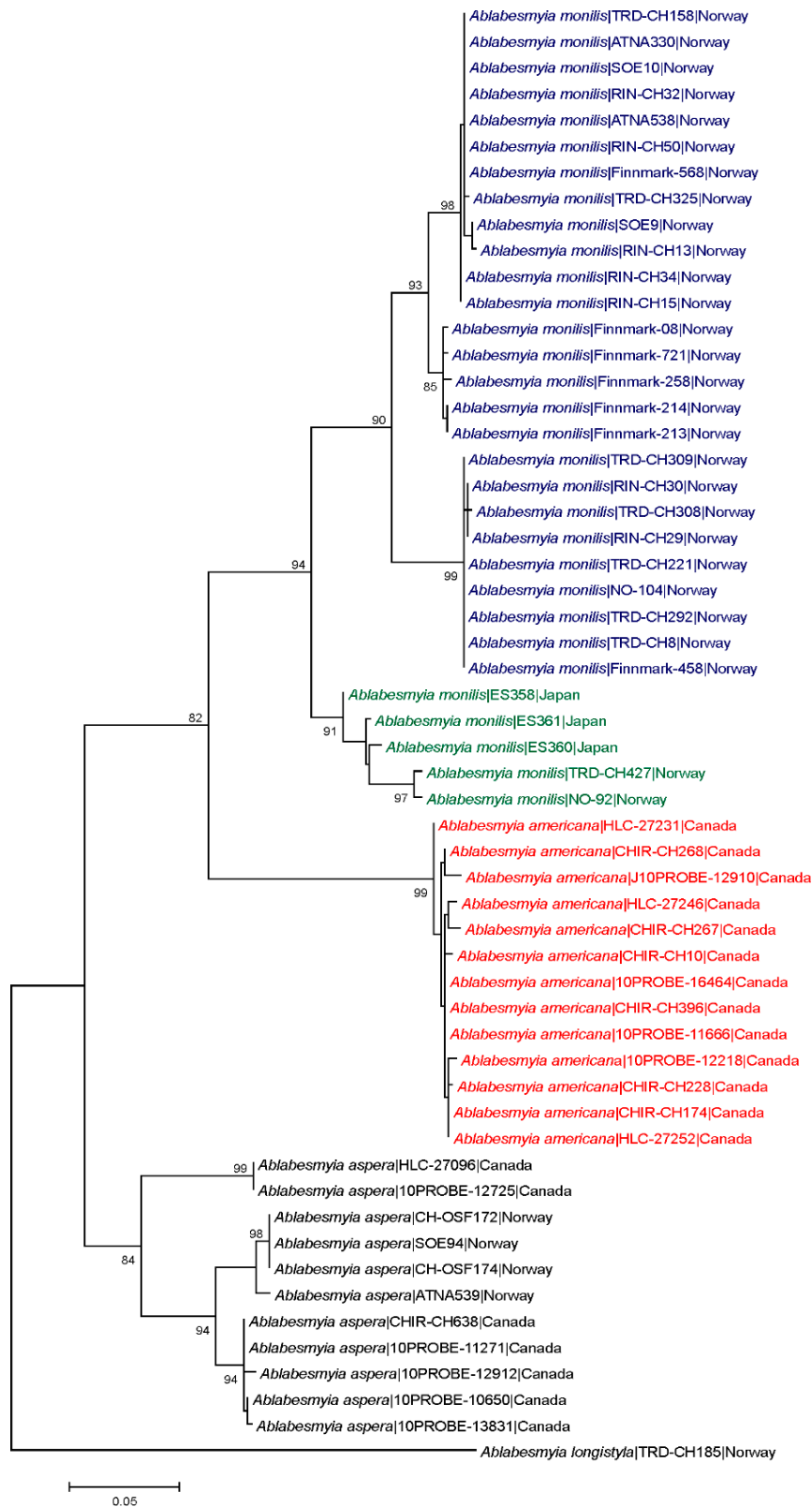
**Table 2.** Variable and informative sites, and average nucleotide composition in the aligned COI gene sequences.

Nucleotide Position	Variable Sites (%)	Informative Sites (%)	T (%)	C (%)	A (%)	G (%)	AT (%)	GC (%)
1st	15.2	13.9	27.2	16	26.4	30.4	53.6	46.4
2nd	1.9	0.0	43	27.4	13.6	16	56.6	43.4
3rd	82.9	86.1	47	8.4	42.2	2.4	89.2	10.8
All	24.9%	83.5%	39.1	17.3	27.4	16.2	66.5	33.5

Average intra- and interspecific K2P-distances for the examined *Ablabesmyia* species were 3.7% and 12.5%, respectively (Table 3). Maximum intraspecific divergence was found in *A. aspera* (8.0%). The lowest interspecific divergences were observed between *Ablabesmyia americana* and *A. monilis* (average 10.0%). Intraspecific and interspecific distances generated by the ML model yielded similar results (data not shown). There were no identical mtDNA COI sequences between species, and all species were distinguishable by genetic deviation and character state variances (Figure 1).

**Table 3.** Intra- and interspecific Kimura 2-parameter distances between *Ablabesmyia* species defined by morphology. N/A denotes species with only one specimen analyzed.

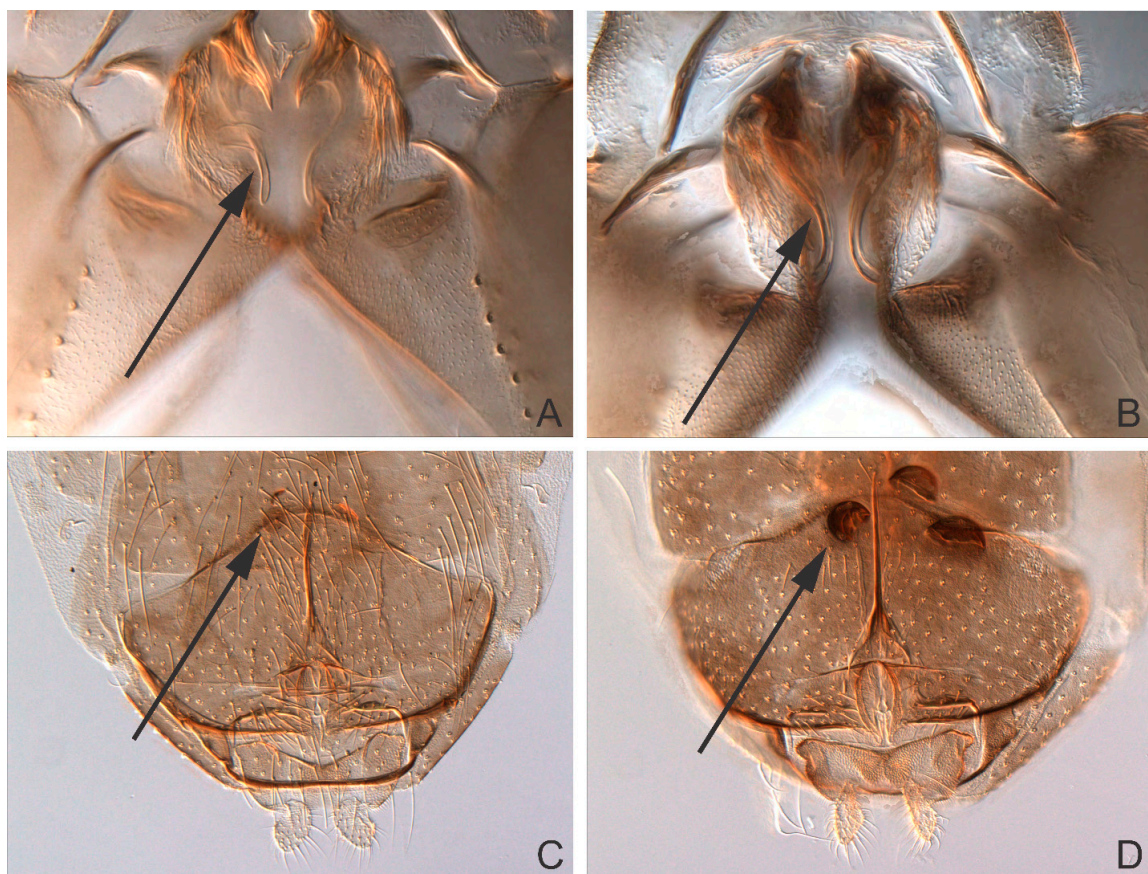
Species	Average Intraspecific K2-P Divergence	Maximum Intraspecific K2-P Divergence	K2-P Divergence to Nearest Neighbor	Average Interspecific K2-P Divergence
<i>Ablabesmyia aspera</i>	3.7%	8.0%	10.8%	12.6%
<i>Ablabesmyia americana</i>	0.6%	1.3%	10.0%	11.9%
<i>Ablabesmyia longistyla</i>	N/A	N/A	14.4%	16.2%
<i>Ablabesmyia monilis</i>	4.2%	8.6%	10.0%	12.5%



**Figure 1.** Maximum likelihood (ML) tree for species of *Ablabesmyia*, based on partial COI sequences (DNA barcodes) and using the Tamura 3-parameter model with gamma correction for rate heterogeneity. Numbers on branches are bootstrap values > 80%.



The ML tree shows large barcode divergence between all sampled *Ablabesmyia* species. *Ablabesmyia americana* and *A. monilis* comprised well-supported monophyletic clusters, completely concurring with the morphological identifications (Figure 1). For comparison, a Neighbor-joining tree was also generated using the K2P model, and produced identical trees (data not shown). Bootstrap support showed minor deviation between analyses, but were always 90% or higher for all species groups, except for the principal *Ablabesmyia aspera* group, which was endorsed by 84% of the bootstrap pseudoreplicates in the NJ-analysis. Nucleotide sequences from specimens recognized morphologically as *Ablabesmyia americana* and *A. monilis* differed by a minimum of 10.0%. The adult male of *Ablabesmyia americana* presents a shorter, less curved aedeagal blade, and slightly weaker, inferior volsella in the male hypopygium (Figure 2A,B), whereas *A. monilis* exhibits a longer, more strongly curved dorsal lobe, and more conspicuous inferior volsella. Regarding adult females, *Ablabesmyia americana* has less than one-fourth dark sclerotized seminal capsules as opposed to about one-half to two-thirds sclerotization in *A. monilis* (Figure 2C,D).



**Figure 2.** Male. (A) *Ablabesmyia americana*, (B) *Ablabesmyia monilis*: Aedeagal blade. Female. (C) *Ablabesmyia americana*, (D) *Ablabesmyia monilis*: Seminal capsules.

The specimens determined as *Ablabesmyia monilis* by morphology build at least two distinct barcode clusters (Figure 1). However, we found no evident morphological characters that aid separation the species in these groups. Specimens of *Ablabesmyia monilis* were collected in Japan and Norway (Table 1, Figure 1), but one of the clusters (BIN BOLD:ABY9333) includes 327 records from eastern and western Canada in BOLD. Finally, specimens of *Ablabesmyia aspera*, used as an outgroup in our analysis, were grouped into three separate barcode clusters (BINs BOLD:AAF3626, BOLD:AAF3627, BOLD:AAF3638). Specimens of *Ablabesmyia aspera* of these three clusters were collected in Canada and Norway (Table 1), and exhibited a clear geographical structuring of genetic diversity across the distribution range, with one of the group composed only by species from Norway (BIN BOLD:AAF3626).

### Taxonomy

*Ablabesmyia americana* Fittkau, 1962

*Ablabesmyia americana* Fittkau, 1962: 430 [20] (proposed new name for *A. monilis* Johannsen nec Linnaeus).

*Ablabesmyia americana* Sublette and Sublette, 1965: 148 [45] (taxonomic placement).

*Ablabesmyia americana* Roback, 1971: 375 [25] (proposed junior synonym of *Ablabesmyia monilis* Linnaeus).

Material examined. *Type material*: Lectotype (designated here, CUIC): Adult male with pupal and larval exuviae, USA, New York, Ithaca, Cascadilla Pond, 28.vii.1902, O.A.J. Lot 1507. 1 *Paralectotype*: Female with pupal and larval exuviae (CUIC), as previous except for Cascadilla Creek, 30.vii.1902, O.A.J. Lot 1508.

*Additional material*: Adult female (Specimen ID: HLC-27246, NTNU-VM 201786), CANADA, Manitoba, Churchill, Farnworth Lake, 10 Km SE Churchill, N 58.7°, W 94.05°, 21.vii.2007, leg. unknown. Adult female (Specimen ID: CHIR\_CH268, NTNU-VM 201781), as previous except for Ramsey Creek, Malaise trap, N 58.73054°, W 93.78007°, 13 m a.s.l., 15-17.vii.2006, leg. T. Ekrem and E. Stur. Larva (Specimen ID: BIOG03304-G12, CBG), as previous except for Ontario, Algonquin Provincial Park, stream by Leaf Lake Sign, Drainage stream in swamp, N 45.5517°, W 78.2696°, 350 m a.s.l., 26.vi.2011. leg. G. Martin and O. Zaheer. Larva (Specimen ID: BIOG01725-B09) as previous.

Diagnostic characters. *Ablabesmyia americana* differs from *Ablabesmyia monilis* by the following characters. Adult male: Hypopygium with short, moderately curved aedeagal blade and inconspicuous inferior volsella. Adult female: Genitalia with seminal capsules less than one-fourth dark sclerotized. Immature stages: Indistinguishable from most of the described species of *Ablabesmyia*.

*Adult female* ( $n = 2$ , except where otherwise stated)

Size. Total length 2.83–3.04 mm. Wing length 2.3–3.0 mm. Total length/wing length 1.09–1.24. Wing length/profemur length 2.72–2.93.

*General coloration*. Head pale brown with darker occipital margin; pedicel and antenna brown; maxillary palp pale brown. Thorax pale brown. Wing membrane transparent with dark spots. Femur with one brown band, tibia with three brown bands. Abdominal tergites and genitalia brown.

*Head*. Temporal setae 20–26, irregularly multiserial. Eye ratio 1.60–1.99. Tentorium 188–221  $\mu\text{m}$  long. Clypeus 157–163  $\mu\text{m}$  long, 96–111  $\mu\text{m}$  wide at largest part, bearing 30–32 setae. Cibarial pump 353–372  $\mu\text{m}$  long. Lengths of palpomeres 1–5 (in  $\mu\text{m}$ ): 65–68; 125–146; 175–198; 120–150; 212 (1). Antenna 690 (1)  $\mu\text{m}$  long, diameter of pedicel 98 (1)  $\mu\text{m}$ . AR 0.43 (1).

*Thorax*. Anteprenotals 7–8. Acrostichals 70–86, double staggered row which diverges posteriorly to join the dorsocentral row of setae; dorsocentrals 58–64, biserial anteriorly and uniserial posteriorly; prealars 32–44; supraalar 2. Anapleural suture ratio 0.65 (1). Scutellum with transverse row of 20–24 setae and group of numerous fine anterior setae. Scutal tubercle indistinct.

*Wing*. Width 0.81–0.96 mm. Costa 2.1–2.4 mm long. VR 0.86–0.94. WW 0.36–0.37. Brachiolum with 3–4 setae. Squama with 38–42 setae.

*Legs*. Foreleg: Width at apex of tibia 66–70  $\mu\text{m}$ , tibia with single, apical and pectinate spur 50 (1)  $\mu\text{m}$  long, with 9 (1) lateral teeth;  $ta_{1-3}$  with preapical pseudospurs. Mid leg: Width at apex of tibia 67–70  $\mu\text{m}$ , tibia with two apical spurs 36–37; 61–67  $\mu\text{m}$  long, with 4–6 lateral teeth;  $ta_{1-3}$  with preapical pseudospurs. Hind leg: Width at apex of tibia 65–71  $\mu\text{m}$ , tibia with two apical spurs 33–36; 70–71  $\mu\text{m}$  long, with 3–6 lateral teeth; comb not indistinct;  $ta_{1-3}$  with preapical pseudospurs. Claws slender, distally recurved and pointed and with large basal protuberance. Lengths and proportion of leg segments as in Table 4.

*Genitalia* (Figure 2C). Gonapophysis VIII elongated, 119–122  $\mu\text{m}$  long. Coxosternapodeme 91–102  $\mu\text{m}$  long. Postgenital plate broadly rounded. Cerci oval-quadrangle, 73 (1)  $\mu\text{m}$  long, 58 (1)  $\mu\text{m}$  wide, with 18–24 elongated setae. Labia with inconspicuous microtrichia. Notum 177–179  $\mu\text{m}$  long. Seminal capsules oblong, 56–58  $\mu\text{m}$  long, 51 (1)  $\mu\text{m}$  wide, less than one-fourth dark sclerotized, with conical-shaped necks. Length ratio SCa/No 0.31–0.32.

**Table 4.** Lengths (in  $\mu\text{m}$ ) and proportions of leg segments in *Ablabesmyia americana* Fittkau, female (n = 1–2).

Leg Position	fe	ti	ta <sub>1</sub>	ta <sub>2</sub>	ta <sub>3</sub>
p <sub>1</sub>	837	924	743	450	329
p <sub>2</sub>	985–1126	982–1125	778	508	329
p <sub>3</sub>	898–1075	1226–1381	1137	657	473
Leg Position	ta <sub>4</sub>	ta <sub>5</sub>	LR	BV	SV
p <sub>1</sub>	229	153	0.80	2.15	2.37
p <sub>2</sub>	227	168	0.69	2.46	2.89
p <sub>3</sub>	291	181	0.82	2.24	2.16

*Pupa* (n = 2, except where otherwise stated)

*Size.* Abdomen 3.6–3.8 mm long in male.

*General coloration.* Exuviae (pupal skins) mostly pale brown without any distinctive pattern; thoracic horn brown.

*Cephalothorax* (Figure 3A). Wing sheath smooth, 1.2–1.3 mm long. Thoracic horn 441–450  $\mu\text{m}$  long and 223–238  $\mu\text{m}$  wide (Figure 3A). THR 1.98–1.89. Respiratory atrium filling the lumen cavity, reticulation of respiratory atrium indistinct, lumen homogeneous, preapical papilla and plastron plate absent, aeropyle tube sinuate with single membranous lobe. External membrane with few spinules basally interconnected, forming scales. Basal lobe covered with small spinules. Thoracic comb with 11–13 conical tubercles (Figure 3A).

*Abdomen* (Figure 3B–D). Tergite I with scar 201–210  $\mu\text{m}$  long. Shagreen on tergites serially arranged in groups of 2–4 (Figure 3B), evenly distributed. Sternites I and II with sparse shagreen. Abdominal chaetotaxy as in Figure 3C. Abdominal segment VII with 4 LS-setae. A VIII with 5 LS-setae. Anal lobe 496–523  $\mu\text{m}$  long, 383–387  $\mu\text{m}$  wide (Figure 3D). ALR 1.28–1.36. Male genital sac not surpassing apex of anal lobe.

*4th instar larva* (n = 4, except where otherwise stated)

*General coloration.* Head yellow, postoccipital margin brown. Ligula pale yellow, with apex brown. Abdomen pale yellow. Procerus pale brown along anterior margin.

*Head* (Figure 4A). Length 787–899  $\mu\text{m}$ , 422–519  $\mu\text{m}$  wide; IC 0.51–0.59. Dorsally DP present, S5 and S8 postero-mesal to S7. Ventrally S9, S10 and SSm forming a right angle (Figure 4A).

*Antenna* (Figure 4B). Length 405–483 (3)  $\mu\text{m}$ , A<sub>1</sub> 346–410  $\mu\text{m}$  long, with ring organ located 0.51–0.62 from base, A<sub>2</sub> 54–63 (3)  $\mu\text{m}$  long. Peg sensilla very small. Blade and accessory blade extending somewhat beyond flagellum. AR 5.22–5.82.

*Maxilla* (Figure 4C). Maxillary palp with three segments, palpomere three reduced; palpomeres 1–2: 61–78; 44–46  $\mu\text{m}$  long; palpal formula 1+2; P<sub>1</sub>/P<sub>2</sub> 1.41–1.69 (3); A<sub>1</sub>/P<sub>1</sub> 4.98–6.14 (3); A<sub>2</sub>/P<sub>1</sub> 0.79–0.81. Ring organ located between palpomere 1 and 2.

*Mandible* (Figure 4D). Length 131–139  $\mu\text{m}$ . Large, bluntly rounded inner tooth, partly overlying seta subdentalis. Sensillum campaniformium located 0.68–0.79 from apex. AMD 2.48–3.08.

*Mentum and M appendage.* Dorsomentum sclerotized, without teeth. Labial vesicles oblong. Pseudoradula with fine granulation, not arranged in distinct longitudinal rows, 131 (3)  $\mu\text{m}$  long.

*Hypopharyngeal complex* (Figure 4E,F). Ligula with 5 teeth, 86–122  $\mu\text{m}$  long, 44–56  $\mu\text{m}$  wide at base; row of teeth concave, middle and inner teeth smaller in size, outer larger; inner teeth slightly curved outward (Figure 4E). IO 15–22, MO 17–21. Paraligula bifid, 45–52 (3)  $\mu\text{m}$  long, inner tooth 26–33 (3)  $\mu\text{m}$  long. Pecten hypopharyngis with 15–20 subequal teeth, corner tooth and middle teeth slightly broader than remainder (Figure 4F).

*Body* (Figure 4G). Without fringe of swim-setae. Procerus 96–132 (3)  $\mu\text{m}$  long, 31–44 (3)  $\mu\text{m}$  wide, with 7 anal setae 594–740 (3)  $\mu\text{m}$  long. L/W 2.86–3.19 (3). Anal tubules slender, 230 (1)  $\mu\text{m}$  long. Posterior parapod 391–456  $\mu\text{m}$  long. Claws simple (Figure 4G), some with small spines on inner and/or outer margin, 2 of smaller claws dark brown.

*Ablabesmyia monilis* (Linnaeus, 1758)



*Tipula monilis* Linnaeus 1758: 587 [46] (description of adults).

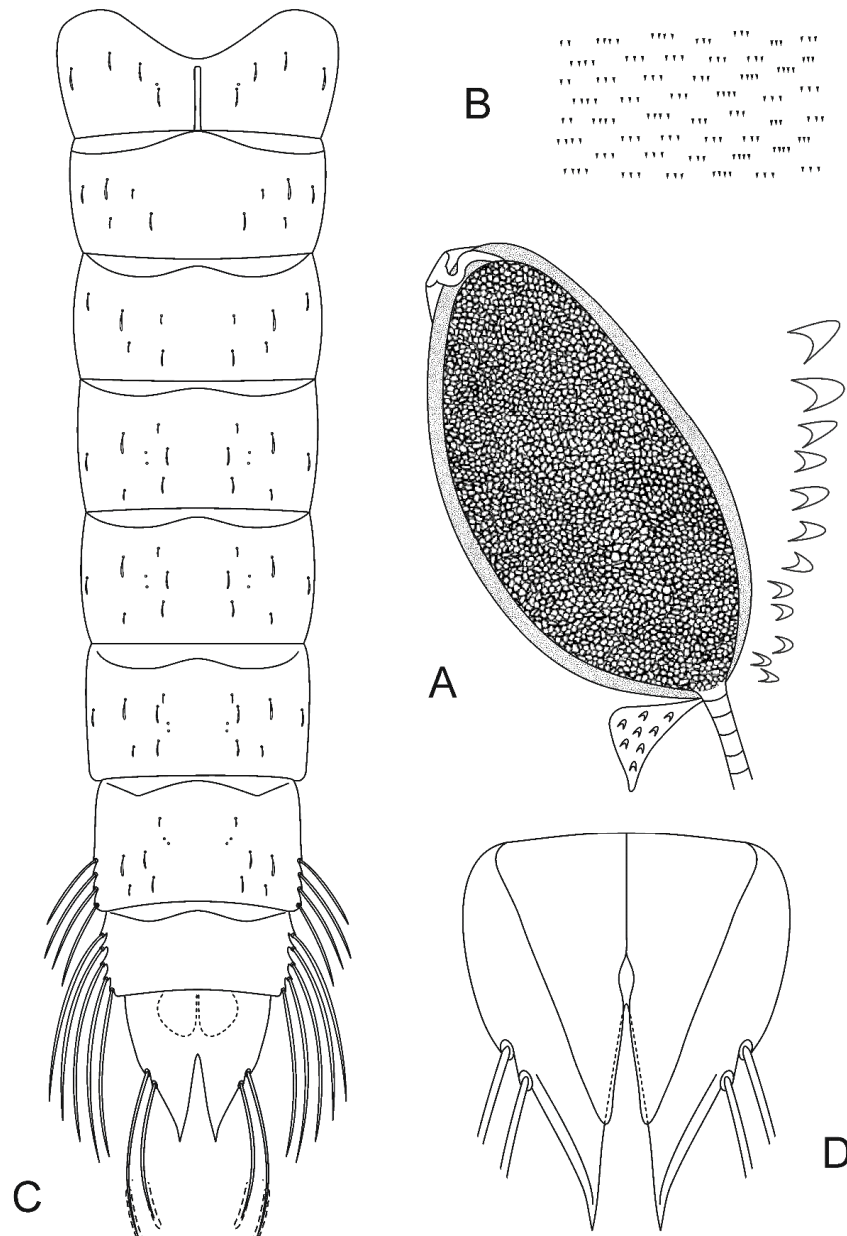
*Ablabesmyia monilis* Johannsen 1905: 142 [15] (description of adults and immature stages).

*Pentaneura monilis* Tokunaga 1937: 44 [15] (description of adults).

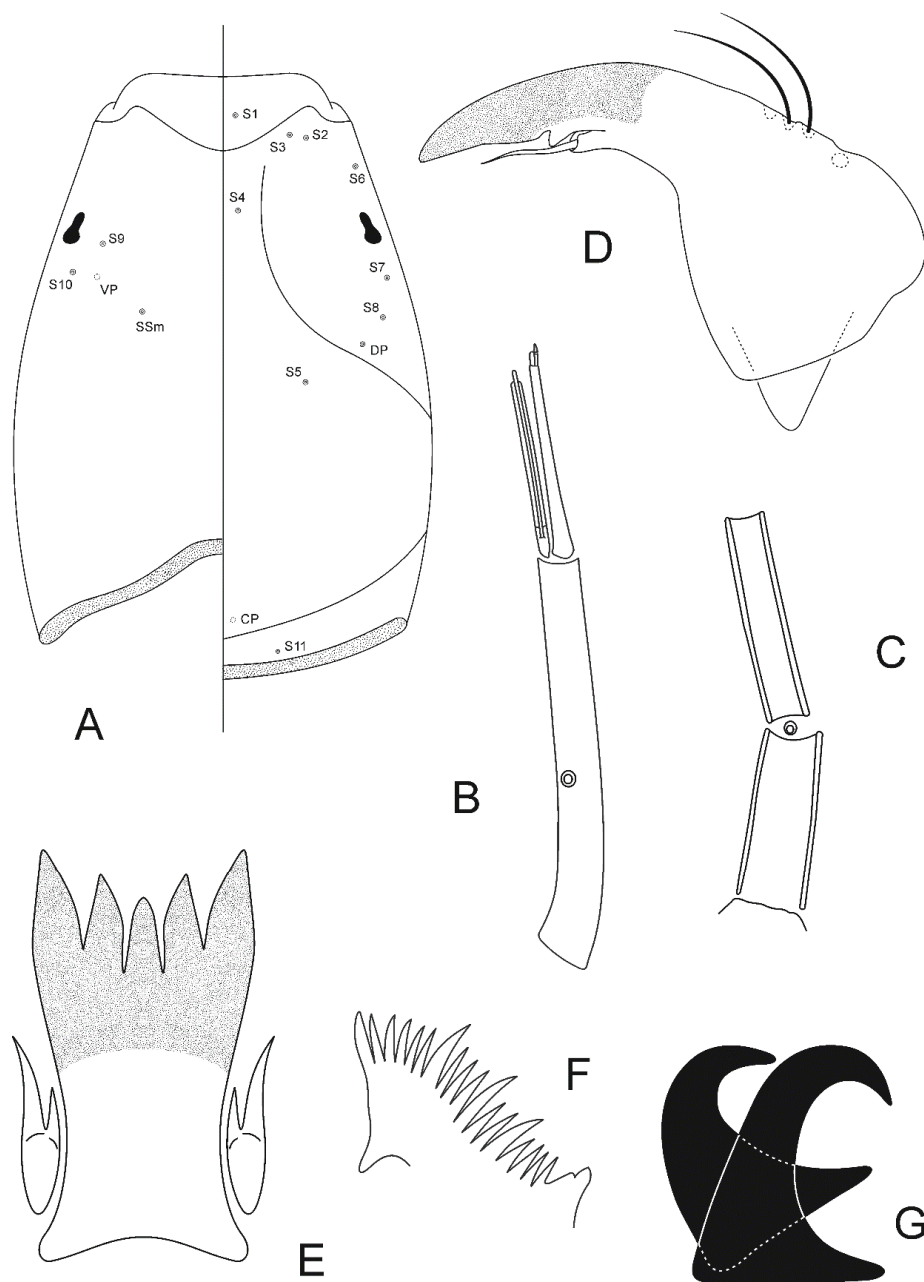
*Ablabesmyia monilis*: Fittkau 1962: 437 [20] (description of adults and immature stages).

*Ablabesmyia monilis* Roback 1971: 375 [25] (adult male description).

Material examined. Adult female: (Specimen ID: ATNA330, NTNU-VM 201789), NORWAY, Oppland, Rondane National Park, Skranglehaugen (P2), N 61.98141°, E 9.80480°, 1119 m a.s.l., 14–21.vii.2008, leg. T. Ekrem. Adult female: (Specimen ID: NO92, NTNU-VM 200850) as previous except for Trøndelag, Trondheim, Gjeddvatnet, N 63.38272°, E 10.60776°, 152 m a.s.l. 20.vi.2010, leg. J. K. Skei.



**Figure 3.** *Ablabesmyia americana* Fittkau. Pupa: (A) Thoracic horn with basal lobe and thoracic comb. (B) Shagreen on tergites. (C) Female abdominal segments with chaetotaxy, dorsal view. (D) Anal lobe and male genital sac, ventral view.



**Figure 4.** *Ablabesmyia americana* Fittkau. Larva: (A) Head with chaetotaxy. Left: Ventral view, right: Dorsal view. (B) Antenna. (C) Maxillary palp. (D) Mandible. (E) Ligula and paraligula. (F) Pecten hypopharyngis. (G) Dark claws of posterior parapod.

*Adult female* ( $n = 2$ , except where otherwise stated)

**Size.** Total length 3.63–3.77 mm. Wing length 2.7–3.3 mm. Total length/wing length 1.09–1.40. Wing length/profemur length 2.93 (1).

**General coloration.** Head pale brown with darker occipital margin; pedicel and antenna brown; maxillary palp pale brown. Thorax pale brown. Wing membrane transparent with dark spots. Femur with one brown band, tibia with three brown bands. Abdominal tergites and genitalia brown.

**Head.** Temporal setae 36–40, irregularly multiserial. Eye ratio 1.46–1.56. Tentorium 244–258  $\mu\text{m}$  long. Clypeus 179–183  $\mu\text{m}$  long, 130–139  $\mu\text{m}$  wide at largest part, bearing 50–56 setae. Cibarial pump 381–409  $\mu\text{m}$  long. Lengths of palpomeres 1–5 (in  $\mu\text{m}$ ): 69–74; 141–183; 178–235; 136–150; 258–268. Antenna 722–813  $\mu\text{m}$  long, diameter of pedicel 103–106  $\mu\text{m}$ . AR 0.40–0.41.

*Thorax.* Anteprenotals 11–15. Acrostichals 88–96, double staggered row which diverges posteriorly to join the dorsocentral row of setae; dorsocentrals 82–92, biserial anteriorly and uniserial posteriorly; prealars 36–50; supraalar 2. Anapleural suture ratio 0.65–0.67. Scutellum with transverse row of 18–22 setae and group of numerous fine anterior setae. Scutal tubercle indistinct.

*Wing.* Width 0.98–1.14 mm. Costa 2.6–3.3 mm long. VR 0.87–0.91. WW 0.34–0.37. Brachiolum with 2–3 setae. Squama with 46–78 setae.

*Legs.* Foreleg: Width at apex of tibia 91 (1)  $\mu\text{m}$ , tibia with single, apical and pectinate spur 72 (1)  $\mu\text{m}$  long, with 7 (1) lateral teeth;  $ta_{1-3}$  with preapical pseudospurs. Mid leg: Width at apex of tibia 71–86  $\mu\text{m}$ , tibia with two apical spurs 33–44; 68–93  $\mu\text{m}$  long, with 4–6 lateral teeth;  $ta_{1-3}$  with preapical pseudospurs. Hind leg: Width at apex of tibia 65–93  $\mu\text{m}$ , tibia with two apical spurs 44–47; 75–90  $\mu\text{m}$  long, with 4–6 lateral teeth; comb not indistinct;  $ta_{1-3}$  with preapical pseudospurs. Claws slender, distally recurved and pointed and with large basal protuberance. Lengths and proportion of leg segments as in Table 5.

*Genitalia* (Figure 2D). Gonapophysis VIII elongated, 118–130  $\mu\text{m}$  long. Coxosternapodeme 110–111  $\mu\text{m}$  long. Postgenital plate broadly rounded. Cerci oval-quadrangle, 68–73  $\mu\text{m}$  long, 62–63  $\mu\text{m}$  wide, with 20–24 elongated setae. Labia with inconspicuous microtrichia. Notum 202–206  $\mu\text{m}$  long. Seminal capsules oblong, 82–83  $\mu\text{m}$  long, 60–61  $\mu\text{m}$  wide, one-half to two-thirds sclerotized, with conical-shaped necks. Length ratio SCa/No 0.40–0.41.

**Table 5.** Lengths (in  $\mu\text{m}$ ) and proportions of leg segments for *Ablabesmyia monilis* (Linnaeus, 1758) (n = 1–2).

Leg Position	Fe	ti	ta <sub>1</sub>	ta <sub>2</sub>	ta <sub>3</sub>
p <sub>1</sub>	1134	1383	1079	661	480
p <sub>2</sub>	1114–1397	887–1413	556–1028	557–626	386–456
p <sub>3</sub>	1163–1174	1177–1246	876–1492	500–908	341–637
Leg Position	ta <sub>4</sub>	ta <sub>5</sub>	LR	BV	SV
p <sub>1</sub>	307	210	0.78	2.17	2.33
p <sub>2</sub>	261–297	177–196	0.63–0.73	1.85–2.44	2.73–3.60
p <sub>3</sub>	230–380	153–224	0.74–1.20	1.82–2.63	1.62–2.67

#### 4. Discussion

The analysis of partial COI sequences supports the separate species status of *Ablabesmyia americana* and *A. monilis*. Moreover, we could associate the species *A. aspera* and *A. longistyla* to molecular markers. The ML tree revealed that the majority of the analyzed species are distinctly separated by mtDNA COI sequences, and there are indications of morphologically cryptic lineages within *A. monilis* and *A. aspera*.

The presence of a barcode gap is a key concept in DNA barcoding [47]. When the amount of intraspecific genetic divergence is substantially smaller than the amount of interspecific genetic divergence, a barcode gap exists [48]. In our study, the pairwise interspecific genetic distances in the sampled *Ablabesmyia* species were clearly higher than the intraspecific divergences, demonstrating a barcode gap.

The average K2P distance values found for conspecific comparisons (3.7%) was discordant from those found in the literature, which range from 0.1% to 0.8% [49]. However, this disparity can be a result of cryptic diversity among our sampled species (see below). The mean cutoff value for species delimitation is likely higher in Chironomidae than in many other insect groups, and previous studies have found that morphological species fit poorly with Barcode Index Numbers [50]. For example, Lin, Stur and Ekrem [6] suggest a 4–5% threshold for genetically separate species of *Tanytarsus* (Chironominae), while Song, et al. [51] considered a 5–8% cutoff appropriate to discriminate species of *Polypedilum* (Chironominae). Herein, we refrain from suggesting an average threshold to delineate the species of *Ablabesmyia*. Yet any cutoff should be used with caution, since unusual deep intraspecific divergences can indicate cryptic species. Broader sampling throughout the distribution range of *A. monilis* would provide valuable data for the further analysis of the species boundary for this species. Nevertheless,

data currently in BOLD shows that one genetic lineage of *A. monilis* sensu stricto (BOLD:ABY9333) is widely distributed in western Canada. Other BINs within *A. monilis* have records from Europe (BOLD:AAU0774, BOLD:AAF3633), Japan (BOLD:ADB8891), and central Norway (BOLD:ACE5418).

*Ablabesmyia moniliformis* was established by Fittkau [20] on the basis of Japanese species treated as *Pentaneura monilis* (Linnaeus, 1758) by Tokunaga [52], which he considered different from the European species [53]. However, Fittkau proposed this new name without providing a diagnostic description, neither made he reference to one, nor designated a holotype. Yet the proposed name satisfies the provisions of Article 13.1.2 of the International Code of Zoological Nomenclature, and the name is available and considered a nomen dubium [29]. Moreover, it seems that the *Pentaneura monilis* Tokunaga [52] type series may, in fact, comprises two or perhaps three species, including *Ablabesmyia monilis*, *A. prorasha* Kobayashi and Kubota, 2002 and *A. jogancornua* Sasa and Okazawa, 1991 [29]. Furthermore, Tokunaga [52] did not describe nor illustrate the form of the aedeagal complex, a crucial feature for the diagnosis of the *Ablabesmyia* species. This has prevented the identification of these species unequivocally, and is likely the reasons that the name *moniliformis* has been overlooked (or disregarded) by Japanese peers [53]. Based on our results, there is evidence supporting a separate cluster of *A. monilis* including records from Japan and Norway. This could represent one of the species in Tokunaga's *Ablabesmyia monilis* specimens, and thus *A. moniliformis*. However, no morphological differences could be determined between these two barcode clusters. In addition, the material used by Tokunaga [52], deposited in the Kyushu University Museum, is deteriorated by the long-term storage in alcohol [29]. This prevents the comparison of our material with the *Ablabesmyia moniliformis* type series. The observed pattern could also be explained by the restricted geographical sampling of *A. monilis* throughout its distributional range. Therefore, additional material is needed to understand the variation between the specimens of *A. monilis* and closely-related species throughout their current distribution. Lastly, the female of *Ablabesmyia alba*, described by Chaudhuri, et al. [54] for India, resembles *A. monilis* by having seminal capsules with about one half to two thirds dark sclerotized, and may not be readily separable from this species. Further investigations using molecular data and morphological characters from all life stages will likely reveal the relationship of *A. alba* with *A. monilis*.

In addition to the divergences observed within *A. monilis*, there are two well-separated genetic lineages of *A. aspera* in Canada (BOLD:AAF3627 and BOLD:AAF3628), and a slightly divergent group with Norwegian specimens (BOLD:AAF3626), indicating cryptic species or divergence caused by geographical isolation in this group. *Ablabesmyia aspera* belongs to the *monilis* group sensu Roback, [19] and can be distinguished from all the species of that group by the longer, heavier aedeagal blades and the dorso-mesally approximated dorsal lobes, in natural position [19]. The species is widely distributed in Canada and the United States [25]. In Europe, *Ablabesmyia aspera* is only recorded from Norway [55].

DNA-aided species determination has become regular practice in various fields of study, including agriculture [56], bioengineering [57] and conservation biology [58]. Moreover, DNA barcodes are recognized as a powerful tool to separate species in a range of chironomid groups [5,6,10,11,51,59–62]. We found mtDNA COI valuable in exploring sequence diversity and distinguishing morphologically similar species within the genus *Ablabesmyia*. Furthermore, DNA barcodes indicate further cryptic species in *Ablabesmyia*, or deep intraspecific divergence caused by geographical isolation. We believe that broader geographical sampling and the inclusion of nuclear genes will strengthen our findings and provide additional evidence for the geographical structuring of genetic lineages in *Ablabesmyia*.

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