A reference database for the DNA-based identification of North American Calliphorinae.

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Abstract: DNA-based species identifications are a robust tool for the forensic entomologist to identify immature or damaged specimens to species or genus taxonomic levels for further forensic analyses. However, much of that identification is dependent on the use of a reliable database that encompasses all species possible for a given geographic area. Here, we queried databases for DNA records of COI sequence data of 18 species of Calliphorinae present in North America to determine the applicability and reliability of DNA-based identifications, and to provide a resource for the community. Our results indicate that approximately 650bp of the standard DNA barcode is sufficient to classify most Calliphorinae species, however, some species the ~650bp COI barcode is insufficient to resolve to species for the North American Calliphorinae subfamily. We tested this reference dataset by selecting records that were deemed reliable (using the same criteria as establishing the reference dataset) to test the accuracy of the identifications based on DNA COI sequences and found that all test specimens were accurately identified except for the two paraphyletic species present in the dataset. We furthermore state what species can and cannot be reliably classified and provide guidance on using this database in future applications.

Keywords: Calliphorinae, DNA-based identification, COI Sequencing, cytochrome oxidase I, Calliphora, Cynomya, Bellardia, Cyanus, validation

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Introduction

Forensic entomological analyses are dependent on accurate identifications of the species in forensic cases. This identification plays an important role in the estimation of the minimum postmortem interval (PMI_{MIN}, if using development-based data) or the postmortem interval (PMI, if using succession-based reference data). Immature and some sister species taxa are difficult to identify morphologically, therefore, DNA-based identifications offer an additional avenue [1]. By sequencing a portion of the insects' genome (whether nuclear or mitochondrial) and making comparisons to reference data, an identification can be made. However, this method only works if there is reliable reference DNA dataset available, enough genetic differentiation among species, and the dataset encompasses all the relevant species that might be present in your location. Typically most of the challenge lies in separating evolutionarily close species that may not have enough time for the DNA locus to genetically differentiate; distinguishing between these species is especially important if their development rates or life histories differ enough to impact a forensic interpretation [2].

Initially, DNA-based species identifications using the *cytochrome oxidase subunit I* gene were deemed a robust method until the true

variation in mitochondrial DNA sequences at the *cytochrome oxidase subunit I* (COI) locus was thoroughly investigated. Soon, the addition of more taxa began to reveal the complicated utility of using COI sequence data for species identification due to close evolutionary relationships and hybridizations. One of the earliest examples was the inability to differentiate between *Lucilia cuprina* and *L. sericata* due to hybridizations and the presence of subspecies that remains mostly unresolved to this day [3-7]. Additional complicated evolutionary relationships include some species in Chrysomyinae [8, 9], and the non-forensically relevant Protocalliphora [10]. In Australia, many endemic species of Calliphorinae exists, their identifications via DNA-based methods are not possible [11, 12]. The key to the use of any DNA-based method is access to a locally relevant database of specimens that represent the population level variation present in the species, and their relationships to other species [13-16]. The less time since the most common ancestor between two species, the more difficult it is to both identify morphologically, and often, differentiate from other species with DNA. Therefore, if a species is misidentified and placed in a genetic database, then it is impossible to determine, when using said specimen, if it is due to misidentification, or if it is genetic variation present in the species. An example of such in which it was previously thought that the COI barcode could not differentiate two sympatric species of *Chrysomya*, and when more discriminating markers were used (amplified fragment length polymorphisms, AFLP), and a secondary review of identifications that included dissected male genitalia revealed the initial identifications were incorrect [17].

The purpose of this work was to curate a reliable and standardized dataset of COI sequence data for use in DNA-identifications of a subfamily of Calliphoridae, the Calliphorinae, and make clear when species can or cannot be reliably identified using this method. There is little data available on this subfamily, and it is forensically relevant across the United States and Canada. Currently, this subfamily has been thoroughly investigated in Australia [12], and South Korea [18] and a revision of the subfamily in North America was recently introduced [19]. Given large number of COI sequences currently available in genetic databases including GenBank [20] and BOLD [21], it is possible to select a subset of records that would encompass the overall intraspecific variation present and determine its utility for a DNA-based identification. Records for the reference database were chosen to represent the geographic distributions across the United States and Canada, with vouchered specimens and associated peer-reviewed publications that contain a record of the original identification key used.

Methods

In order to produce a reliable reference database of Calliphorinae species, an initial selection of 10 records from either GenBank or BOLD were selected for each Calliphorinae species with distributions in Canada and the United States based on [22] (Table 1). Records were preferred if they were published in peer-reviewed literature, contain voucher specimen records, and have at least 400bp of COI sequence. Effort was made to include as much diversity in sample selection as possible (i.e. not all samples were from the same sampling location or study), although that was not always possible. A single *Phormia regina* record was included as an outgroup.

Record Analysis and Selection

For the final selection of records, each record was selected and queried using BLASTn which returns hits according to sequence similarities, and the following data were recorded: (i) does BLASTn return correct species identification? To assess this, the top hit (according to e-value) and its % similarity to the query sequence was noted (if the query sequence was the top hit, then the next record was considered for correct species and % similarity noted); (ii), if the record was not the correct species, what species was it, and % similarity; (iii) when considering the top 10 hits, did all 10 result in the correct species identification?; (v) if they did not, what species and what proportions?; and, (vi) when sorting by percent identity, did any other records (different species) have 100% sequence similarity to the queried sequence? This

resulted in 155 individual BLAST results (including the outgroup *Phormia regina*) (Supplementary File 1). Records deemed inappropriate were then eliminated from the final reference dataset if they were not considered reliable based on proportion of sequence overlap and their BLAST results (discussed below, highlighted in Table 1).

Evaluation of the reference dataset based on COI Phylogenies

Sequences deemed appropriate and reliable were formatted into .fasta format and used to assess the reliability of the reference dataset for DNA-based identification using COI sequence data (sequence data available in Supplemental File 2). First, all species-specific sequences were parsed into haplotypes and intraspecific sequence similarities were calculated (Table 2). All sequences were aligned using Muscle Clustal Omega [59], and the alignment was imported into BioEdit v7.0.9.0 [60] and trimmed resulting in a final dataset that was 657 bp in length. The final sequence data corresponds to positions 1517-2174bp of the *Drosophila yakuba* COI gene. The aligned and trimmed data were exported as a .fasta file and imported into MEGA X v10.1.8 [61]. A neighbor-joining tree and a maximum likelihood phylogenetic analysis were done using default parameters with 100 bootstrap replicates [62]. The consensus bootstrap trees are reported with nodes with less than 50% support collapsed.

Validation

To assess the reliability of the reference dataset proposed (haplotype sequences), additional specimen records were obtained from BOLD systems database that contain similar criteria to the established criteria above (vouchered specimen, publication, reliable means of identification). Every validation sample was collected from a different location/different date from all those included in the reference dataset to avoid the possible use of a full sibling. These samples representing 12 species from Calliphorinae (and one outlier, *Lucilia sericata*) were then individually aligned to the reference haplotype sequences using Muscle Sequence alignment, and individually assessed using a distance-based approach for the phylogeny in Mega. In each case, if the unknown clustered with the appropriately identified species, the test was regarded as an accurate identification. Specimens used are indicated in Table 3.

Results

An initial set of 155 records were selected that encompassed 18 species (including one outgroup, *Phormia regina*) present in North America (Table 1) which included four genera (*Bellardia*, *Cyanus*, *Calliphora* and *Cynomya*). One record (*Calliphora vicina* JX402733.1) had to be reverse complemented to align properly. One record was eliminated because it contained a large run of N's in the middle of the sequence (*Cyanus elongatus*, MN411289.1), and a further nine records were eliminated from the phylogenetic analysis due to poor or no overlap across the COI fragment (see Table 1).

To evaluate the dataset as a reliable dataset that can be used to represent diversity of the taxa, each record was selected for quality control via BLAST. To investigate this, every sequence from Table 1 was queried and the BLAST results evaluated (see Supplementary File 1 for the full results). It is important to note that because the query sequence may be a part of the database, the query was not considered part of this data (~37% of the time the query sequence was the top hit based on e-value). The accuracy using GenBank when simply identifying the top hit as the correct identification was moderate (87.7%, Supplementary File 1), but should never be used for identification purposes. Furthermore, when sorting by percent similarity, more than 1/3 of the time there was an incorrect identification with 100% sequence similarity (Supplementary File 1). In the situations when the top hit was not a concordant species identification (ignoring those situations when just the genus name was the top hit, which was N = 9), there were 10 records that resulted in the discordant species identification based on the top hit only. Two of those records (*Aldrichina grahami* DQ328667.1 was identified

TABLE 1 — *List of NCBI/BOLD records used to develop the database of North American Calliphorinae species for DNA-based identifications.*

Species Name (synonyms)	GenBank Accession #	Location Collected	#base pairs sequenced	Method for Identification	Voucher Location (ID)	Citation
Calliphora vomitoria	KC617811.1	USA: Indiana	658	Not reported	U. S. Food and Drug Administration, Center for Veterinary Medicine	[23]
	KU874517.1	USA: Alaska	658	Identified by entomologists using taxa specific keys	University of Alaska Museum, Entomology (UAM: Ento: 97488)	[24]
	KU496696.1	USA: Alaska	658	Identified by entomologists using taxa specific keys	Kenai National Wildlife Refuge (KNWR-Ento-8776)	[24]
	KR753688.1	Canada: ON	576	Identified by a taxonomic expert	Biodiversity Institute of Ontario (BIOUG05706-E05)	[25]
	KX422302.1	USA: Arizona	658	[22, 26, 27]	Entomology, Washington St University (TLW214)	[19]
	JX438025.1	Portugal	658		Portugal: Lisbon, Campo Grande, Campus of the Faculty of Sciences, University of Lisbon	[28]
	JN257220.1	Germany	511	[27, 29, 30]		[13]
	KX161562.1	Spain	677	[14, 27, 31-38]		[39]
	KX161561.1	Spain	677	[27, 30, 40]		[28]
	KX161559.1	Spain	677	[27, 30, 40]		[28]
Calliphora livida	KX422305.1	USA: Arizona	658	[22, 26, 27]	Entomology, Washington St University (TLW213)	[19]
	KX422293.1	Canada:BC	658	[22, 26, 27]	Entomology, Washington St University (TLW146)	[19]
	KT119017.1	Canada: ON	658	Identified by a taxonomic expert	Biodiversity Institute of Ontario (BIOUG01347-H08)	[25]
	KR389410.1	Canada: NF	613		Biodiversity Institute of Ontario (BIOUG09399-B03)	
	MG114222.1	Canada: ON	561		Biodiversity Institute of Ontario (BIOUG21910-E05)	
	KM868452.1	Canada: ON	634		Biodiversity Institute of Ontario (BIOUG04289-D07)	
	KR385462.1	Canada: ON	582		Biodiversity Institute of Ontario (BIOUG08472-A12)	
	KR387710.1	Canada: ON	588		Biodiversity Institute of Ontario (BIOUG12596-G02)	
	KR398184.1	Canada: ON	555		Biodiversity Institute of Ontario (BIOUG08725-D12)	
	KR776879.1	Canada: ON	561	Assigned to order	Biodiversity Institute of Ontario (BIOUG05571-E07)	[25]
Cynomya cadaverina (*Cynomyopsis cadaverina*)	KT611134.1	Canada: ON	582	Identified to lowest taxonomic level possible	Biodiversity Institute of Ontario (BIOUG21776-C03)	[41]
	KU496711.1	USA: AK	658	Identified by entomologists using taxa specific keys	Kenai National Wildlife Refuge (KNWR-Ento-7218)	[24]
	KC617817.1	USA: WA	658		U. S. Food and Drug Administration, Center for Veterinary Medicine	[23]
	MG117533.1	Canada: ON	582		Biodiversity Institute of Ontario (BIOUG27396-F06)	
	KR394827.1	Canada: ON	584		Biodiversity Institute of Ontario (BIOUG08621-B05)	

Species Name (synonyms)	GenBank Accession #	Location Collected	#base pairs sequenced	Method for Identification	Voucher Location (ID)	Citation
	KR391438.1	Canada: NL	584		Biodiversity Institute of Ontario (BIOUG11684-G03)	
	KF030483.1[1]	USA: CA	296	[22, 42]	(SJSC:AN229)	[43]
	KU874770.1[2]	USA: AK	350	Identified by entomologists using taxa specific keys	University of Alaska Museum, Entomology (UAM: Ento: 97488)	[24]
	KC617818.1		658		Biodiversity Institute of Ontario (DNA vouchered only)	[23]
Calliphora coloradensis	KY435955.1	USA: OR	808	[22]	University of Cincinnati Biology Dept. (BF63Son)	[44]
	KX422282.1	USA: NM	658	[22, 26, 27]	Entomology, Washington St University (TLW212)	[19]
	KY435954.1	USA: NM	660	[22]	University of Cincinnati Biology Dept. (AZ01Son)	[44]
	KM861777.1	Canada: SK	636		Biodiversity Institute of Ontario (BIOUG03418-B04)	
	HQ945048.1	USA: CO	658		Biodiversity Institute of Ontario (BIOUG<CAN>:10BBDIP-0358)	
	MF764802.1	Canada: SK	582		Biodiversity Institute of Ontario (BIOUG20478-D05)	
	MF764499.1	Canada: SK	582		Biodiversity Institute of Ontario (BIOUG20478-B05)	
	JN263396.1	USA: WY	658		Entomology, Washington St University (TLW014)	
	JN263395.1	USA: OR	658		Entomology, Washington St University (TLW013)	
	JN263394.1	USA: NM	658		Entomology, Washington St University (TLW012)	
Calliphora latifrons (*Eucalliphora latifrons*, *Eucalliphora arta*, *E. lilaea*)	KR683434.1	Canada: BC	610	Identified by a taxonomic expert	Biodiversity Institute of Ontario (BIOUG12825-D09)	[25]
	MF762285.1	Canada: BC	579		Biodiversity Institute of Ontario (BIOUG22697-B02)	
	KX422290.1	USA: CO	658	[22, 26, 27]	Entomology, Washington St University (TLW216)	[19]
	KY435957.1[2]	USA: CA	559	[22]	University of Cincinnati Biology Dept. (AZ78Son)	[44]
	KM630458.1	Canada: AB	588		Biodiversity Institute of Ontario (BIOUG05064-G02)	
	AF295557.1		2300			[45]
	KM859779.1	Canada: AB	588		Biodiversity Institute of Ontario (BIOUG06621-A09)	
	KM570868.1[3]	Canada: AB	658		Biodiversity Institute of Ontario (08BBDIP-1186)	
	KF030480.1[2]	USA: CA	296	[22, 42]	(SJSC:AN2_3a)	[43]
	HQ945037.1	USA: AZ	658		Biodiversity Institute of Ontario (BIOUG<CAN>:10BBDIP-0347)	
Calliphora montana (*Acronesia montana*)	KR683051.1	Canada: MB	649	Identified by a taxonomic expert	Biodiversity Institute of Ontario (CHU06-FLY-080.1)	[25]
	KX422307.1	Canada: NS	449	[22, 26, 27]	Entomology, Washington St University (TLW209)	[19]
	KR667930.1	Canada: MB	658	Identified by a taxonomic expert	Biodiversity Institute of Ontario (10PROBE-15403)	[25]
	KR631290.1	Canada: MB	582	Identified by a taxonomic expert	Biodiversity Institute of Ontario (BIOUG17776-C02)	[25]
	KX422303.1	Canada: BC	658	[22, 26, 27]	Entomology, Washington St University (TLW205)	[19]

Species Name (synonyms)	GenBank Accession #	Location Collected	#base pairs sequenced	Method for Identification	Voucher Location (ID)	Citation
	KX422296.1	Canada: NL	658	[22, 26, 27]	Entomology, Washington St University (TLW208)	[19]
	KX422288.1	USA: AK	658	[22, 26, 27]	Entomology, Washington St University (TLW201)	[19]
	KX422291.1	Canada: BC	658	[22, 26, 27]	Entomology, Washington St University (TLW207)	[19]
	MF762162.1	Canada: YT	621		Biodiversity Institute of Ontario (BIOUG27506-E02)	
	JF877218.1	Canada: MB	658		Biodiversity Institute of Ontario (BIOUG<CAN>:10PROBE-13408)	
Calliphora terraenovae	KU873264.1	USA: AK	658	Identified by entomologists using taxa specific keys	University of Alaska Museum, Entomology (UAM: Ento: 234140)	[24]
	KR396838	Canada: NF	555		Biodiversity Institute of Ontario (BIOUG13523-E05)	
	KR671428	Canada: MB	641		Biodiversity Institute of Ontario (CHU05-FLY-180)	[25]
	KX422304	USA: AZ	658	[22, 26, 27]	Entomology, Washington St University (TLW217)	[19]
	KR667886	Canada: MB	600	Identified by a taxonomic expert	Biodiversity Institute of Ontario (CHU05-FLY-245)	[25]
	KF030470.1[2]	USA: CA	296	[22, 42]	SJSC:AN101	[43]
	KU874514.1	USA: AK	658	Identified by entomologists using taxa specific keys	University of Alaska Museum, Entomology (UAM: Ento: 97503)	[24]
	KX422298.1	USA: WA	633		Entomology, Washington St University (TLW145)	[19]
	KU873263.1	USA: AK	658	Identified by entomologists using taxa specific keys	University of Alaska Museum, Entomology (UAM: Ento: 230951)	[24]
	KU873258.1	USA: AK	658	Identified by entomologists using taxa specific keys	University of Alaska Museum, Entomology (UAM: Ento: 230930)	[24]
Calliphora vicina	KC617808.1	USA: NC	658	None reported	U. S. Food and Drug Administration, Center for Veterinary Medicine	[23]
	MK905397.1	USA: CA	551		North Carolina State University Population Health and Pathobiology (USNM:USNMENT01371066)	[46]
	JX402733.1[4]	Canada: ON	648		Center for Food Safety and Applied Nutrition, office of the FDA (USFDA CFSAN DI23-004)	
	KX422283.1	Canada: ON	658	[22, 26, 27]	Entomology, Washington St University (TLW211)	[19]
	JX438024.1	Portugal	658	[27, 30, 40]	(PMSp2.Cvic.1)	[28]
	JN257222.1	Germany	511	[27, 29, 30]		[13]
	KX161589.1	Spain	677	[14, 27, 31-38]	(P10E12)	[39]
	MN868831.1	Portugal	658		(INV00609)	[47]
	KR747439.1	Canada: ON	582	Identified by a taxonomic expert	Biodiversity Institute of Ontario (BIOUG05561-H08)	[25]
	KF918991.1	Belgium	1534		(NICC0350_11615002)	[48]
Calliphora grahami (*Aldrichina grahami*)	KX422286.1	USA: CA	658	[22, 26, 27]	Entomology, Washington St University (TLW215)	[19]
	FJ614831.1		649			

Species Name (synonyms)	GenBank Accession #	Location Collected	#base pairs sequenced	Method for Identification	Voucher Location (ID)	Citation
	DQ328667.1[2]		348			
	EU880180.1	South Korea	1539	[49]		[18]
	KY031809.1	China	659			
	KF030472.1[2]	USA: CA	296	[22, 42]	(SJSC:AN259)	[43]
	EU880182.1	South Korea	1539	[49]		[18]
	KY031810.1	China	658			
	KY031807.1	China	658			
Calliphora alaskensis (*Acronesia alaskensis*)	KR390547	Canada: QC	534		Biodiversity Institute of Ontario (BIOUG12717-D06)	
	JN263392	USA	658		Biodiversity Institute of Ontario (TLW008)	
	KR775633	Canada: AB	564	Identified by a taxonomic expert	Biodiversity Institute of Ontario (BIOUG16126-G10)	[25]
	KR946708	Canada: YT	606	Identified by a taxonomic expert	Biodiversity Institute of Ontario (BIOUG18816-A05)	[25]
	KX422300	Canada: QC	658	[22, 26, 27]	Entomology, Washington St University (TLW144)	[19]
	KX422287.1	Canada: BC	462	[22, 26, 27]	Entomology, Washington St University (TLW009)	[19]
	KY031765.1	China	658			
	KX422285.1	Canada: QC	658	[22, 26, 27]	Entomology, Washington St University (TLW143)	[19]
	MG112508.1	Canada: BC	576		Biodiversity Institute of Ontario (BIOUG24099-D07)	
	HM412265.1	Canada: NB	658		Biodiversity Institute of Ontario (BIOUG<CAN>:09BBEDI-0708)	
Calliphora aldrichia (*Acronesia aldrichia*)	KX422306	USA: CO	658	[22, 26, 27]	Entomology, Washington St University (TLW200)	[19]
	KX422308	Canada: BC	658	[22, 26, 27]	Entomology, Washington St University (TLW204)	[19]
	MG967864	USA: CO	658		Smithsonian Museum of Natural History (USNM:ENT:01443383)	
Calliphora loewi (*Calliphora mortica*)	KR621953.1	Canada: YT	552	Identified by a taxonomic expert	Biodiversity Institute of Ontario (BIOUG17355-B06)	[25]
	KX422299	Canada: AB	658	[22, 26, 27]	Entomology, Washington St University (TLW199)	[19]
	KU874513	USA: AK	658	Identified by entomologists using taxa specific keys	University of Alaska Museum, Entomology (UAM:Ento:97484)	[24]
	MG117929	Canada: NWT	546		Biodiversity Institute of Ontario (BIOUG22954-H06)	
	KR509319	Canada: NWT	555	Identified by a taxonomic expert	Biodiversity Institute of Ontario (BIOUG17053-G12)	[25]
	KU874512.1	USA: AK	658	Identified by entomologists using taxa specific keys	University of Alaska Museum, Entomology (UAM:Ento:97483)	[24]
	KX422294.1	USA: AK	658	[22, 26, 27]	Entomology, Washington St University (TLW218)	[19]
	KR945170.1	Canada: YT	555	Identified by a taxonomic expert	Biodiversity Institute of Ontario (BIOUG18816-A06)	[25]
	MF763863.1	Canada: BC	573		Biodiversity Institute of Ontario (BIOUG18066-G09)	

Species Name (synonyms)	GenBank Accession #	Location Collected	#base pairs sequenced	Method for Identification	Voucher Location (ID)	Citation
	MF757542	Canada: YT	573		Biodiversity Institute of Ontario (BIOUG26497-E11)	
Bellardia bayeri	None available					
Bellardia vulgaris	MG673776.1	Norway	658	BOLD Identification	(NIBIO 16OV-91)	[50]
	MG673783.1	Norway	658	BOLD Identification	(NIBIO 16OV-16)	[50]
	MG673791.1	Norway	658	BOLD Identification	(NIBIO 16OV-24)	[50]
	MG673798.1	Norway	658	BOLD Identification	(NIBIO 16OV-31)	[50]
	MG673801.1	Norway	658	BOLD Identification	(NIBIO 16OV-34)	[50]
	MG673843.1	Norway	658	BOLD Identification	(NIBIO 16OV-76)	[50]
	MG673846.1	Norway	658	BOLD Identification	(NIBIO 16OV-79)	[50]
	MG673850.1	Norway	658	BOLD Identification	(NIBIO 16OV-83)	[50]
	MG673851.1	Norway	658	BOLD Identification	(NIBIO 16OV-84)	[50]
	MG673858.1	Norway	658	BOLD Identification	(NIBIO 16OV-09)	[50]
Calliphora genarum (*Acronesia collini*, *Acronesia popoffana*)	MN683291.1	Canada: NT	654	BOLD Identification	Biodiversity Institute of Ontario (CHARS00224-C10)	[51]
	MN681774.1	Canada: NT	655	BOLD Identification	Biodiversity Institute of Ontario (CHARS00031-A07)	[51]
	MN681258.1	Canada: NT	655	BOLD Identification	Biodiversity Institute of Ontario (BIOUG45395-H02)	[51]
	MN680196.1	Canada: NT	654	BOLD Identification	Biodiversity Institute of Ontario (CHARS00262-E12)	[51]
	MN668659.1	Canada: NT	653	BOLD Identification	Biodiversity Institute of Ontario (BIOUG45395-C12)	[51]
	JN302812.1	Canada: MB	658		Biodiversity Institute of Ontario (BIOUG<CAN>:10PROBE-15595)	
	JF877738.1	Canada: MB	658		BIOUG<CAN>:10PROBE-14434	
	KU874511.1	USA: Alaska	576	Identified by entomologists using taxa specific keys	University of Alaska Museum, Entomology (UAM:Ento:20755)	[24]
	KX422295.1	Canada: NWT	407	[22, 26, 27]	Entomology, Washington St University (TLW220)	[19]
	MN677490.1	Canada: NWT	654		(CHARS00140-E04)	[52]
Calliphora stelviana (*Acronesia abina*, *A. anana*)	MF756025.1	Canada: NF	579		BIOUG18471-H09	
	MF763390.1	Canada: NF	576		BIOUG18471-C06	
	KX422289.1	Canada: YT	658	[22, 26, 27]	Entomology, Washington St University (TLW221)	[19]
	KR942663.1	Canada: NF	582	Identified by a taxonomic expert	BIOUG18469-A01	[25]
	KR944065.1	Canada: NF	594	Identified by a taxonomic expert	BIOUG18199-H05	[25]
	KR944534.1	Canada: NF	582	Identified by a taxonomic expert	BIOUG18199-B03	[25]
	KR944897.1	Canada: NF	594	Identified by a taxonomic expert	BIOUG18199-A09	[25]

Species Name (synonyms)	GenBank Accession #	Location Collected	#base pairs sequenced	Method for Identification	Voucher Location (ID)	Citation
	KR945130.1	Canada: NF	582	Identified by a taxonomic expert	BIOUG18469-A06	[25]
	KR946538.1	Canada: NF	582	Identified by a taxonomic expert	BIOUG18469-A03	[25]
	KR945473.1	Canada: NF	573	Identified by a taxonomic expert	BIOUG18795-A02	[25]
Cyanus elongatus	MN411289.1[5]	USA: ID	658		USNM:ENT:01443387	
	MG119903.1	Canada: YT	588		BIOUG25806-F01	
	MG118668.1	Canada: AB	594		BIOUG31116-C01	
	KM571239.1	Canada: MB	658	Identified by a taxonomic expert	08BBDIP-0017	[25]
	KM635707.1	Canada: MB	555	Identified by a taxonomic expert	BIOUG08959-C03	[25]
Cynomya mortuorum (*Cynomya hirta*)	KY031812.1	China	658		M34-1	
	FR719159.1	UK	1251	[27]	Ca4	[53]
	KF919018.1	Belgium	1534	[30, 36, 54-56]	NICC0468h_11620812	[48]
	KU373567.1	Greenland	658	[21]	BIOUG01013-B11	[57]
	KU373434.1	Greenland	658	[21]	BIOUG01914-B10	[57]
	KU374728.1	Greenland	658	[21]	za2009-10009	[57]
	KU874772.1	USA: AK	658	Identified by entomologists using taxa specific keys	University of Alaska Museum, Entomology (UAM:Ento:103699)	[24]
	KU874773.1	USA: AK	658	Identified by entomologists using taxa specific keys	University of Alaska Museum, Entomology (UAM:Ento:103698)	[24]
Phormia regina	AF295550	USA: CA	2303	[58]		[45]

as *Chrysomya rufifacies*, and *Aldrichina grahami* KF030472.1 was identified as *Sarcophaga peregrina*) did not have sufficient overlap with the dataset and were eliminated from the evolutionary analyses anyhow. Three *Calliphora aldrichia* (MG967864.1, KX422308.1, and KX422306.1) were identified as *Calliphora montana*, these species have yet to be reliably identified using DNA based methods, and their morphological identifications are equally troublesome (all three had 100% sequence similarity, see Figure 1) [19, 22]. One *Calliphora alaskensis* (KY031765.1) record's query returned *Calliphora sinensis* with 99.39% sequence similarity as the top hit. This specimen was collected in China, and is most likely misidentified as the distributions of *Ca. alaskensis* are not known outside of North America. Therefore, this record was removed from evolutionary analyses. Interestingly, one *Calliphora stelviana* record (KX422289.1) was identified as *Calliphora genarum*, and in fact, the nine subsequent records all point to different *Ca. genarum* records, albeit, the percent similarity averaged at 96.96%. Because of the low sequence similarity, this specimen record is unlikely to be *Ca. genarum*, however, it was odd that no *Ca. stelviana* were a part of the BLAST results. The sequence similarity between that record (KX422289.1) and the remaining *Ca. stelviana* specimens was 99.3%, and thus it was retained.

Another possible issue was a *Cynomya mortuorum* record (KF919018.1) returning a top hit as *Cynomya cadaverina* (AF295505.1) with 98.5% similarity. When taking this record (KF919018.1) and comparing it to other *Cynomya* records, it was 99.9% similar to *Cy.*

mortuorum. When selecting the *Cy. cadaverina* record (AF295505.1), it has 99.7% sequence similarity to *Cy. cadaverina* (vs. 98.4% to *Cy. mortuorum*). In this case even though the top BLAST hit was not a concordant species, this record is considered to be reliable and robust based on sequence similarities to all the other records for that species group.

here were two separate issues with the *Calliphora latifrons* selected as part of this dataset. *Calliphora latifrons* (AF295557.1) was discordantly identified as a *Calliphora loewi* (DQ345093.1) with 99.85% sequence similarity as the top BLAST hit, and a reciprocal BLASTn query using DQ345093.1 had 99.5% sequence similarity with *Ca. latifrons*, but only 94.6% sequence similarity with *Ca. loewi*. In this case, it is likely that the hit record (DQ345093.1) was misidentified, and therefore, the original record was retained as it was deemed to be accurate.

TABLE 2 — Haplotype table.

Species	Haplotype	Number	Accession numbers	Intraspecific sequence variation
Calliphora vomitoria	CavoA	5	KC617811.1, KU874517.1, KU496696.1, KR753688.1, KX422302.1	1.03%
	CavoB	2	JX438025.1, JN257220.1	
	CavoC	1	KX161559.1	
	CavoD	1	KX161561.1	
	CavoE	1	KX161562.1	
Calliphora livida	CaliA	5	KT119017.1, KR389410.1, KR385462.1, KR387710.1, KR776879.1	0.98%
	CaliB	1	KM868452.1	
	CaliC	1	MG114222.1	
	CaliD	1	KR398184.1	
	CaliE	1	KX422293.1	
	CaliF	1	KX422305.1	
Cynomya cadaverina	CycaA	4	KU496711.1, KC617817.1, MG117533.1, KC617818.1	1.14%
	CycaB	1	KU874770.1	
	CycaC	1	KR394827.1	
	CycaD	1	KR391438.1	
Calliphora coloradensis	CacoA	10	KX422282.1, KM861777.1, HQ945048.1, MF764802.1, MF764499.1, JN263396.1, JN263395.1, JN263394.1, KY435955.1, KY435954.1	N/A
Calliphora latifrons	CalaA	6	KR683434.1, MF762285.1, KX422290.1, KM630458.1, AF295557.1, HQ945037.1	0.17%
	CalaB	1	KM859779.1	
Calliphora montana	CamoA	10	JF877218.1, KX422296.1, KX422303.1, KR531290.1, KR667930.1, KR683051.1, MF762162.1, KX422292, KX422291.1, KX422288.1, KX422307.1	N/A
Calliphora terraenovae	CateA	7	KR396838.1, KR671428.1, KU873264.1, KX422304.1, KX422298.1, KU873263.1, KU873258.1	0.33%
	CateB	2	KU874514, KR396838	
	CateC	1	KR667886	
Calliphora vicina	CaviA	4	KX161598.1, JX438024.1, MN868831.1, KF918991.1	1.11%
	CaviB	2	KX422283.1, KR747439.1	
	CaviC	1	MK905397.1	
	CaviD	1	JX402733.1	
	CaviE	1	JN257222.1	
	CaviF	1	KC617808.1	
Calliphora grahami (Adrichina graham)	CagrA	5	KX422286.1, KY031809.1, EU880182.1, KY031810.1, KY031807.1	0.32%
	CagrB	1	FJ614831.1	
	CagrC	1	EU880180.1	

Species	Haplotype	Number	Accession numbers	Intraspecific sequence variation
Calliphora alaskensis	CaalA	4	KX422287.1, JN263392.1, KR775633.1, MG112508.1	1.21%
	CaalB	3	KX422300.1, KX422285.1, HM412265.1	
	CaalC	1	KR390547.1	
	CaalD	1	KY031765.1	
	CaalE	1	KR946708.1	
Calliphora aldrichia	CaadA	2	MG967864.1, KX422306.1	0.15%
	CaadB	1	KX422308.1	
Calliphora lowei	CaloA	7	KR621953.1, KU874513.1, KU874512.1, KX422294.1, KR945170.1, MF763863.1, MF757542.1	0.35%
	CaloB	1	KX422299.1	
	CaloC	1	MG117929.1	
	CaloD	1	KR509319.1	
Bellardia vulgaris	BevuA	8	MG673776.1, MG673783.1, MG673791.1, MG673798.1, MG673843.1, MG673850.1, MG673851.1, MG673858.1	0.15%
	BevuB	2	MG673801.1, MG673846.1	
Calliphora genarum	CageA	8	KX422295.1, MN683291.1, MN681258.1, MN680196.1, MN668659.1, JN302812.1, JF877738.1, MN677490.1	0.34%
	CageB	1	KU874511.1	
	CageC	1	MN681774.1	
Calliphora stelviana	CastA	9	MF756025.1, KR944065.1, KR944897.1, KR945473.1, MF763390.1, KR942663.1, KR944534.1, KR945130.1, KR946538.1	0.69%
	CastB	1	KX422289.1	
Cyanus elongatus	CyelA	2	KM571239.1, KM635707.1	0.23%
	CyelB	1	MG119903.1	
	CyelC	1	MG118668.1	
Cynomya mortuorum	CymoA	3	KF919018.1, KU373567.1, KU373434.1	0.30%
	CymoB	1	KU874772.1	
	CymoC	1	FR719159.1	
	CymoD	1	KY031812.1	
	CymoE	1	KU374728.1	
	CymoF	1	KU874773.1	
Phormia regina	PhreA	1	AF295550.1	N/A

An additional *Calliphora latifrons* (KM570868.1) was identified as *Ca. montana/Ca. aldrichia* with 100% sequence similarity. This record had 94.9% sequence similarity with *Ca. latifrons* and 99.9% sequence similarity with *Ca. montana*, therefore, this record was removed from the analysis it may not have been accurately identified originally, as 5% intraspecific variation is outside the bounds of known intraspecific variation thresholds (3%).

Lastly, there were a few additional results from the BLAST exercise. When all records of *Calliphora grahami* were queried in BLAST, located within the top 10 with 100% sequence similarity were three *Chrysomya rufifacies* hits (KY001824.1, KY001823.1 and KY001822.1). The manuscript that describes this work has identified these three records as *Aldrichina (Calliphora) grahami*, therefore, they are likely incorrectly labeled in Genbank [63]. Another concern was when *Calliphora coloradensis* (KY435955.1) was queried, an unusual *Lucilia cuprina* (JQ806999.1) was present in the top 10 with 99.38% sequence similarity. When this *Lu. cuprina* record was queried in BLAST, overwhelmingly the hits were of Calliphorinae species with very high sequence similarity. In this case, it is not possible to make an assessment on the reliability of the record identification (unpublished), and we did not consider the presence of this *Lucilia* record as a concerning discordant result.

Once all records were verified (Table 1), sequences representing each haplotype within species were then used to analyze for

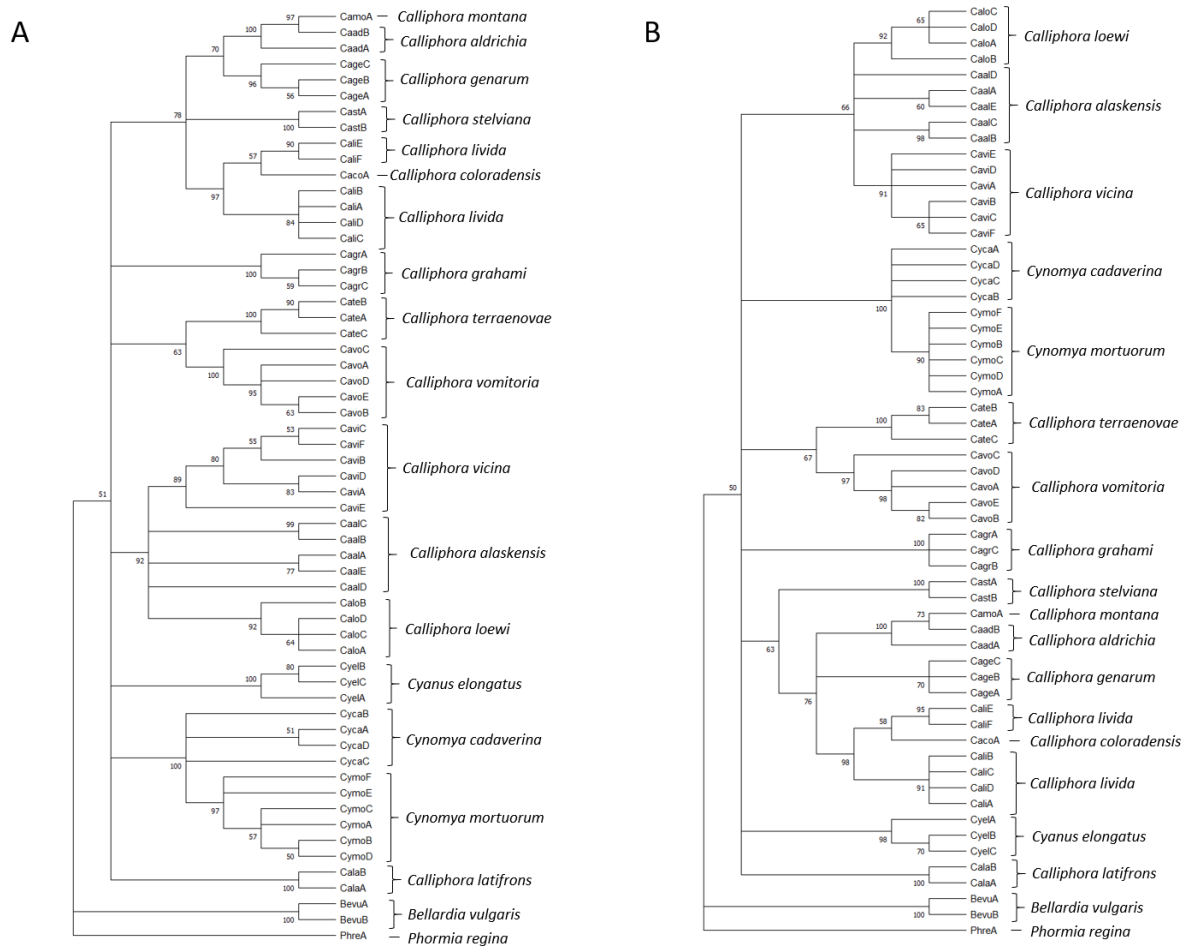

Figure 1. Evolutionary Analyses. A. Neighbor-joining distance tree with nodes containing >50% support shown. B. Maximum likelihood phylogenetic analysis with nodes with >50% support shown.

evolutionary relationships and test the hypothesis that the barcode sequence is sufficient to identify species in this subfamily of Calliphoridae. Of note, given no records exist for *Bellardia bayeri*, any DNA-based identification that would result within the *Bellardia* clade cannot be reliably identified to species, and would presumably only identify to genus, however, without specimens, this is speculation.

A distance-based neighbor-joining tree with bootstrap was made in order to compare to previous work [19] and is shown in Figure 1A. Because the tree collapsed any nodes that did not have >50% bootstrap support, many of relationships could not be resolved using the DNA barcode region alone, however, there is some support for some species identifications. For example, many of the species' clades have sufficient support, however, *Ca. alaskensis/Ca. loewi* and *Cy. cadaverina/Cy. mortuorum* did not have monophyletic relationships. Furthermore, *Ca. livida* is paraphyletic with *Ca. coloradensis*, and this result differs from [19]. The *Ca. aldrichia*/*Ca. montana* specimens cannot be resolved as *Ca. aldrichia* shares a haplotype with all of the *Ca. montana*. The species *Ca. montana* and *Ca. aldrichia* are thought of to have formed due to geographic isolation, with *Ca. montana* found primarily east of the Rocky Mountains and *Ca. aldrichia* to the West, however, their distributions overlap in parts of Canada, with specimens containing intermediate characters. Whitworth [22] concludes separating females is problematic, and based on the genetic data presented here

with only three DNA records for *Ca. aldrichia*, the barcode is not sufficient to separate out these species, given the special care given to revising the morphological characters for identification and their DNA sequences [19].

The bootstrap consensus maximum likelihood tree shown Figure 1B and details the evolutionary relationships among the species present in this group and does not differ significantly from the distance-based tree. As above, the overall evolutionary relationships of species are not well supported, however, most of the species are monophyletic and therefore likely to result in accurate identifications. There are two clades with paraphyletic relationships: *Calliphora livida* / *Calliphora alaskensis*; and *Calliphora montana / Calliphora aldrichia. Calliphora livida* and *Ca. alaskensis* both fall within a terminal category for their morphological identification per [22], with *Ca. alaskensis* having a broader distribution across the United States and Canada, widespread but rare, and typically only present at higher elevations, with notes that male genitalia will separate out the species. Both species are thought to be easily confused by *Ca. terraenovae*, however, our results appear to show this is an unlikely result of misidentification with *Ca. terraenovae*.

To test the reliability of the reference dataset, a validation was performed in which individual samples that were not a part of the original set were added to determine whether the identification was accurate. These samples were considered reliable samples based on the criteria outlined in the methods. In all cases, the identifications were correct except for those where the identification was predicted to fail – in non-monophyletic groups such as *C. montana* and *C. aldrichia* (Table 3, Supplemental file 4). An outlier (*L. sericata*) was also included. The aligned sequences that include the validation set are included as Supplemental File 5.

Discussion and Conclusion

As the barcode region of the mitochondrial DNA locus continues to generate data (for example, BOLD has 3,100 records for Calliphorinae species representing 70 species as of 4/26/2021), and thus reference databases are increasing as an exponential rate. The initial reaction to a DNA-based identification is to BLASTn the sequence to see what hits results. There is some value in this practice, as it is useful in hierarchical information such as family and possible genus, but it should not be used for species identification due to the possibility of variation in the barcode region. Specifically, certain geographic areas with overlapping distributions warrant caution, and with the changing climatic conditions, this could be exacerbated without our knowledge. The data collected here represents an available database that can be used for future identifications (data is available in Supplemental File 3, aligned haplotype sequences), however, there are species in which the standard DNA barcode is not sufficient for identification using DNA-based methods. In these cases, an analyst could elect to conclude that one of several species are possible as identifications.

Author Contributions

CJP conceived and designed the experiments, performed data analysis, and wrote the manuscript. DB and AB collected data, and AB performed analyses and contributed to writing. All authors edited the draft manuscripts.

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