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Improved Chironomid Barcode Database Enhances Identification of Water Mite Dietary Content

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Abstract: Chironomids are one of the most biodiverse and abundant members of freshwater ecosystems. They are a food source for many organisms, including fish and water mites. The accurate identification of chironomids is essential for many applications in ecological research, including determining which chironomid species are present in the diets of diverse predators. Larval and adult chironomids from diverse habitats, including lakes, rivers, inland gardens, coastal vegetation, and nearshore habitats of the Great Lakes, were collected from 2012 to 2019. After morphological identification of chironomids, DNA was extracted and cytochrome oxidase I (COI) barcodes were PCR amplified and sequenced. Here we describe an analysis of biodiverse adult and larval chironomids in the Great Lakes region of North America based on new collections to improve chironomid identification by curating a chironomid DNA barcode database, thereby expanding the diversity and taxonomic specificity of DNA reference libraries for the Chironomidae family. In addition to reporting many novel chironomid DNA barcodes, we demonstrate here the use of this chironomid COI barcode database to improve the identification of DNA barcodes of prey in the liquefied diets of water mites. The species identifications of the COI barcodes of chironomids ingested by *Lebertia davidcooki* and *L. quinquemaculosa* are more diverse for *L. davidcooki* and include *Parachironomus abortivus*, *Cryptochironomus ponderosus*. *Parachironomus tenuicaudatus*, *Glyptotendipes senilis*, *Dicrotendipes modestus*, *Chironomus riparius*, *Chironomus entis/plumosus*, *Chironomus maturus*, *Chironomus crassicaudatus*, *Endochironomus subtendens*, *Cricotopus sylvestris*, *Cricotopus festivellus*, *Orthocladius obumbratus*, *Tanypus punctipennis*, *Rheotanytarsus exiguus* gr., and *Paratanytarsus nr. bituberculatus*.

Keywords: non-biting midge; barcode gap; food web; *Lebertia*; Laurentian Great Lakes

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1. Introduction

Understanding trophic cascades of freshwater ecosystems can be extremely useful for managing aquatic habitats. Freshwater habitats are among the most threatened, and more research into their biodiversity and understanding the ecological interactions of organisms have been recommended [1]. Knowledge of prey is important to construct food web pathways of aquatic systems. We focus here on the chironomid prey of water mites.

Chironomidae (commonly referred to as chironomids, nonbiting flies, midges, or bloodworms) is an insect family whose aquatic larvae are an important constituent of freshwater systems. All stages of chironomid development, including eggs, larvae and adult flies, are used as food sources for various organisms [2]. The biomass of chironomids

is so great that, at times, they may be considered pests [3]. Chironomids have been used as biological indicators of aquatic health [4,5] and cultured as fish food [6].

Water mites are true aquatic arachnids that are ubiquitous and are considered the most biodiverse arachnid class [7]. Water mites belong to the suborder Parasitengona, and, as the name suggests, most water mites are parasitic as larvae [8]. They have been observed as parasitizing a wide array of aquatic hosts, and most of these associations are still not well understood [9]. Despite being "neglected" in freshwater research, water mites are also important predators with potentially significant predation effects on the variety of prey they consume, including crustaceans, ostracods, nematodes and aquatic Dipteran larvae, including chironomids and mosquitoes [10]. Water mite predation of chironomids can significantly reduce the standing crop of chironomids [11]. Since water mites digest their prey extra-orally [12,13], analysis of their diets cannot be accomplished by dissection and visualization of gut contents under a microscope. However, the DNA of ingested organisms, like chironomids, remains sufficiently intact so that their DNA sequences can be detected up to 24 h after ingestion [14]. Application of next-generation sequencing (NGS) to analyze DNA fragments of ingested prey in water mites freshly collected from the field revealed many chironomid taxa as prey items [15]. The identification of the species level of many of these prey organisms was difficult because many reference sequences in barcode databases were not identified for chironomids beyond generic or family-level classification. To resolve this difficulty and improve identifications of organisms in water mite diets, we used morphological identification and DNA barcodes to generate a more specific and broader curated database of chironomids that improved identifications.

Cytochrome oxidase I (COI) DNA barcodes are useful for characterizing biodiversity and studying diet [16,17]. Our previous work on chironomid COI barcodes resulted in the identification of several taxa of chironomids from the Lake Erie region [18]. DNA barcodes have been assisting with taxonomy since the development of metazoan primers called Folmer primers [19]. A combination of classical taxonomy and COI DNA barcodes helped us previously in multiple projects on biodiversity, invasive species detection, and clarification of cryptic species [18,20–22].

In this paper, we present an expanded, curated database of identified chironomid COI barcode sequences, including many novel chironomid DNA barcodes. In addition to exploring chironomid "barcode gaps" and the possible presence of cryptic species, we further demonstrate its application to improve the specificity of prey identification in water mites.

2. Materials and Methods

2.1. Sampling of Chironomids and Water Mites

We collected Chironomidae larvae and adults from sediment and aerial collections in the Laurentian Great Lakes region, focusing mainlyon Western Lake Erie, Southeast Michigan and several locations outside the Great Lakes watershed (Figure 1). Collection methods for samples from Lake Erie are described in Failla, Vasquez, Hudson, Fujimoto and Ram [18]. We collected sediments by Ponar grab, washed on a 500 µm sieve, and then stored in ethanol. Chironomid larvae from other sites were collected using either a Ponar grab or a circular 250 µm collecting net and then washed through a 250 µm sieve. Adult chironomid flies were collected from bushes and other structures—such as spider webs, surfaces of cars, boats, leaves and buildings—with 250 µm mesh sweep nets directly into vials containing either isopropanol or ethanol. We sampled for water mites from Blue Heron Lagoon, Detroit, MI, using a 250 µm circular net followed by washing on a 250 µm sieve and preservation in ethanol, as described in Vasquez, Mohiddin, Li, Bonnici, Gurdziel and Ram [15]. Specimens were transported to the laboratory for morphological identification.

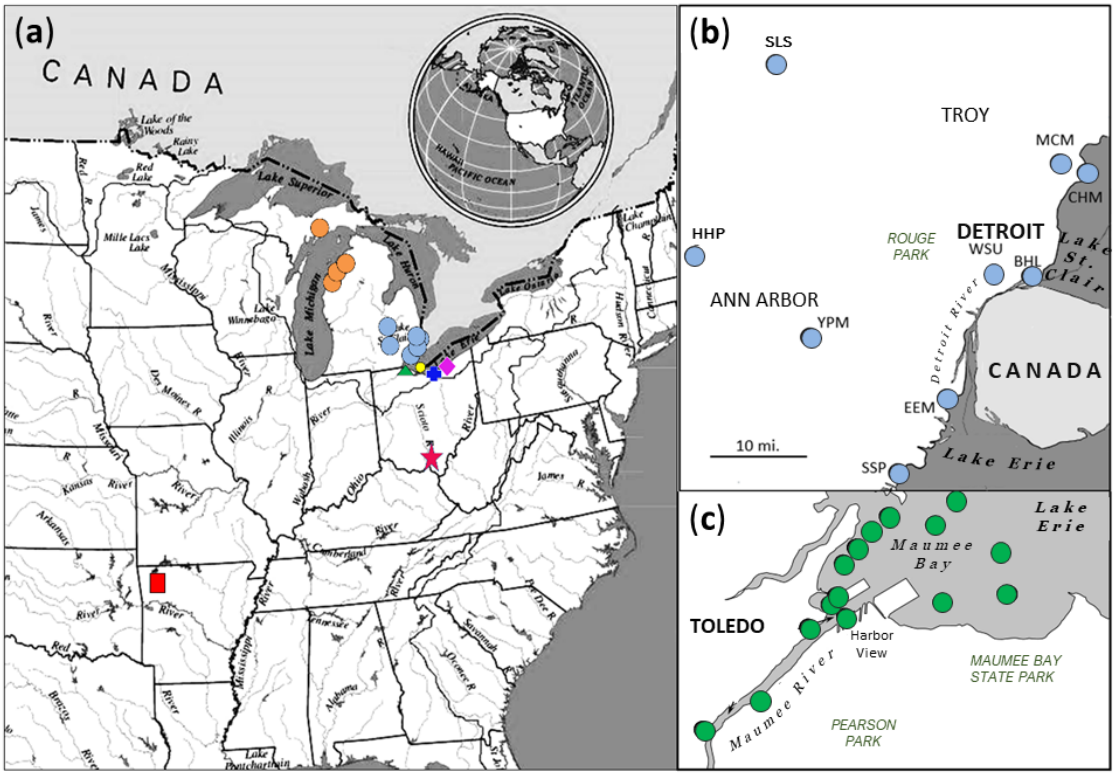

Figure 1. (**a**) Map depicting collection sites of chironomids across the Laurentian Great Lakes and North American rivers. (**b**) Inset showing detailed locations of collection sites throughout Southeastern Michigan. (**c**) Inset showing detailed locations of collection sites near Toledo Harbor, Ohio. Collection site latitudes and longitudes for the chironomids in the curated set are included in their GenBank accession annotations.

2.2. Morphological Identification of Chironomids and Water Mites

Taxonomic methods for the identification of larval and adult chironomids to species were the same as previously described [18]. The keys of Townes [23], Saether [24], Saether [25], Epler [26], Dendy and Sublette [27], Cranston et al. [28], Roback [29], and Heyn [30] were used. Water mite genera studied for this work were *Lebertia*, *Limnesia* and *Arrenurus* and were the same specimens described in Vasquez, Mohiddin, Li, Bonnici, Gurdziel and Ram [15].

2.3. DNA Extraction, Amplification, Sequencing

Adult and larval chironomid tissue samples from morphologically identified specimens were used for DNA extraction. Chironomid tissue was either sequenced by the Canadian Center for DNA Barcoding (CCDB; Biodiversity Institute of Ontario, University of Guelph, Ontario, Canada) as described in Failla, Vasquez, Hudson, Fujimoto and Ram [18] or was extracted for subsequent amplification and sequencing. DNA extraction employed the Qiagen DNeasy blood and tissue kit (Hilden, Germany) as described in Failla, Vasquez, Hudson, Fujimoto and Ram [18]. Tissues were put in lysis buffer in a 1.5 mL centrifuge tube, homogenized with a hand-held fitted pestle and treated with proteinase K enzyme for 3 h at 56 °C. Spin columns were then used to concentrate and purify DNA for subsequent PCR and sequencing. For water mites, a sterile minutien pin was used to puncture each water mite. The mites were then transferred to lysis buffer, where DNA was extracted using the Qiagen DNeasy kits similarly to the chironomid extraction—with the exception that the water mites were lysed overnight rather than homogenized—to more completely extract the DNA from the gut of the punctured water mite, as described in Vasquez, Mohiddin, Li, Bonnici, Gurdziel and Ram [15]. For chironomid specimens, DNA barcodes were generated by PCR as reported in Failla, Vasquez, Hudson, Fujimoto and Ram [18], amplifying the COI

gene (658 bases in length) with the Folmer primers [19]. The PCR products were sequenced using Sanger sequencing by Genewiz company (South Plainfield, NJ, USA). For water mites, COI barcodes were generated using Folmer primers to verify the mite identity and a second set of primers (modified mLEP and Folmer LCOI primers, which amplify insect sequences but not arachnids (and hence are able to amplify chironomid sequences but not the DNA from the water mite host)) [15]. The mLEP:FolmerLCOI primer set amplifies a somewhat shorter (332 bases) region of the COI gene than the Folmer primers and were modified with adapters for next-generation sequencing on a MiSeq V2 Illumina platform at the Michigan State University RTSF Genomics Core, as described by Vasquez, Mohiddin, Li, Bonnici, Gurdziel and Ram [15].

2.4. Bioinformatics of Chironomid Sequences

COI barcode sequences from chironomid specimens were assembled bidirectionally and trimmed to remove primer sequences using DNABaser (Heracle BioSoft SRL, Mioveni, Romania) and MEGA X, respectively [31]. DNABaser was used to determine sequence quality as described in Vasquez, Hudson, Fujimoto, Keeler, Armenio and Ram [20]. For graphical assistance in identifying clusters or unique branches of chironomid sequences, 631 sequences were displayed in a neighbor-joining tree using MEGA X, which generated the tree using the maximum composite likelihood method. We subsequently selected representative sequences from each branch to generate a curated set of sequences from branches that differed in sequence by no more than 3.5%. The branch distance of 3.5% was based on the previously described "barcode gap", below which chironomid sequences that differed by a smaller amount were always the same species when species identification was known [18]. Sequences within 3.5% were numbered sequentially for labelling and referencing purposes. Selected sequences were chosen to represent each branch, prioritizing sequence length, most specific taxon identification, and consensus in identification with the other members of the cluster. Generally, when a sequence from an identified adult was available, that sequence was chosen to represent the branch in the curated set, as identification to species level is usually more reliably accomplished in adults than in larval chironomids (see Appendix A for summarized methods).

2.5. Curation of Chironomid Sequences

Twenty-one specimens accounting for ~3% of the sequences and affecting approximately 20% of the branches (see Table S1) with different morphospecies identifications were present in a single cluster. The curation process involved making a rules-based decision as to whether a specific sequence should be excluded. The selection of which taxon would represent the branch was based on consensus among the other sequences in the cluster (e.g., a cluster that had three sequences identified as one species and one as a related species was represented as the first) or by comparison with GenBank or the Barcode of Life Database (BOLD) (i.e., if other sequences identified by reliable taxonomists were available and agreed with either identification, the consensus identification was used to represent the branch in the final version of the curated database). Discrepancies between database matches and morphological identifications were reviewed and decided in consultation with taxonomic evaluation and the taxonomic literature (see Table S1). In the case of one branch (*Chironomus entis/plumosus*), both identifications have been applied to the branch. Another branch in which two species appeared included *Dicrotendipes lucifer* and *D. simpsoni*. These two species have been described as members of a *D. lucifer* complex [32], and the branch has been given the name of the complex (*Dicrotendipes lucifer* agg.). The cause of these ambiguities could be several, including difficulty in determining morphospecies characters in closely related species, variability in the species, possible errors in labeling, sequencing, etc. Careful chain of custody methods were used. While errors or mistakes affecting approximately 3% of the sequences cannot be ruled out, other explanations, such as the presence of hybrids having the morphological characters of one species but mitochondria that are maternally inherited from another, are also possible.

Following this selection process, representative sequences were compiled and aligned. A "curated neighbor-joining tree" of the database of representative sequences was made in MEGA X. Sequence alignment was performed with CLUSTALW. The best-fit DNA substitution model was determined using the maximum composite likelihood method. The resulting phylogenetic trees were chosen from a heuristic search with a bootstrap value of 200 replicate iterations. The pairwise patristic distances between sequences used for the heuristic search were estimated with the Tamura–Nei model using the Neighbor-Join and BioNJ algorithms, with a discrete gamma distribution rate (5 categories (+G, parameter = 0.83) and invariable sites [31]. Representative sequences of each branch will be uploaded to GenBank [accession IDs will be provided upon acceptance of the manuscript].

Pairwise distance analysis matrices generated by MEGA X were used to generate histograms of pairwise distances among the 631 curated chironomid sequences. These histograms were examined for the presence of "barcode gaps" that might identify the distances which most reliably identified species and genera among chironomids (see Appendix A for the summarized method).

2.6. Identification of Water Mite Prey Using the Curated Chironomid Database

Chironomid sequences from high-throughput sequencing of water mite molecular gut contents from 16 *Lebertia quinquemaculosa*, 21 *Lebertia davidcooki*, 2 *Lebertia* sp., 1 *Arrenurus* sp., and 1 *Limnesia* sp. Specimens were compared to the sequences in the curated chironomid database. Chironomid sequences amplified by the mLEP:LCOI primer pair from each water mite were combined with 160 sequences representing all branches in the curated chironomid dataset and analyzed with MEGA X. For each of these combined datasets, MEGA X was used to generate a neighbor-joining tree that allowed for graphical taxa identification comparisons in which water mite diet sequences clustered together with sequences from the curated chironomid database. Pairwise distance matrices were generated for each of these combined datasets. Mite diet sequences that were <3.5% different from an identified database branch were putatively identified as having that taxonomic identity—i.e., to the species level if the matching branch of the curated database provided species-level identification, or to the genus level if the matching branch taxon was only identified to the genus level. Water mite diet sequences for which the closest curated sequence was >3.5% distant but <9.5% distant were identified only to the genus level even if the nearest pairwise match was at the species level. Subsequent reconsideration of these barcode gap boundaries in the Results indicates that these pairwise differences are reasonable for assigning genus and species to chironomid sequences. Previously, these mite diet chironomid sequences had been identified by family, genus, or species level only in relation to the existing GenBank chironomid sequences [15]. In the current paper, we, therefore, summarize quantitatively the improvements in taxonomic identification (from genus to species or from family to genus or species) by application of the new chironomid database, in comparison to what was previously available in GenBank. We also checked for additional identifications in the Barcode of Life Database.

3. Results

3.1. Chironomid Biodiversity and Barcode Gap Revealed by Morphology and DNA Barcodes

A total of 99 identified sequences were selected from the 631 identified chironomid sequences to represent each <3.5% similarity group for the curated database. Figure 2 shows the maximum composite likelihood tree constructed from the consensus set. Due to its large size of 99 major branches, this curated consensus tree is shown in 3 connected figures (Figure 2A–C). A total of 73 branch clusters were identified to species, while 26 were identified only to genus. A total of 42 branches were based on the sequence of a single identified specimen. Furthermore, 70 branches contained at least 1 sequence from a morphologically identified adult chironomid, and 29 branches were based on 2 or more morphologically identified adults.

As previously noted for the much smaller tree described in Failla, Vasquez, Hudson, Fujimoto and Ram [18], the clades of the curated database tree mostly show excellent congruence with previous morphological taxonomic classification at the family, subfamily, or tribe levels. Thus, Figure 2A comprises all Tanytarsini tribe (Chironominae) specimens (*Cladotanytarsus*, *Paratanytarsus*, *Tanytarsus*, *Rheotanytarsus*, and *Stempellina*). Greater than 90% of the branches in Figure 2B represent specimens of the Chironomini tribe of the Chironominae subfamily (*Axarus*, *Benthalia*, *Chironomus*, *Cladopelma*, *Cryptochironomus*, *Cryptotendipes*, *Glyptotendipes*, *Harnischia*, *Kiefferulus*, *Lobochironomus*, *Microchironumus*, *Parachironomus*, *Paracladopelma*, *Robackia*). Figure 2B also has several branches of *Dicrotendipes* (Chironominae) and all the Pseudochironomini tribe specimens (*Pseudochironomus*), as well as several remaining Chironomini tribe specimens (*Endochironomus*, *Polypedilum*, *Stictochironomus*, and *Tribelos*). Figure 2C has all the representatives of the subfamily Tanypodinae (*Ablabesmyia, Clinotanypus*, *Coelotanypus*, *Procladius*, and *Tanypus*) and >85% of the subfamily Orthocladinae (*Cricotopus*, *Eukieferriella*, *Hydrobaenus*, *Nanocladius*, *Orthocladius*, *Parakiefferella*, *Smittia*, and *Stilocladius*).

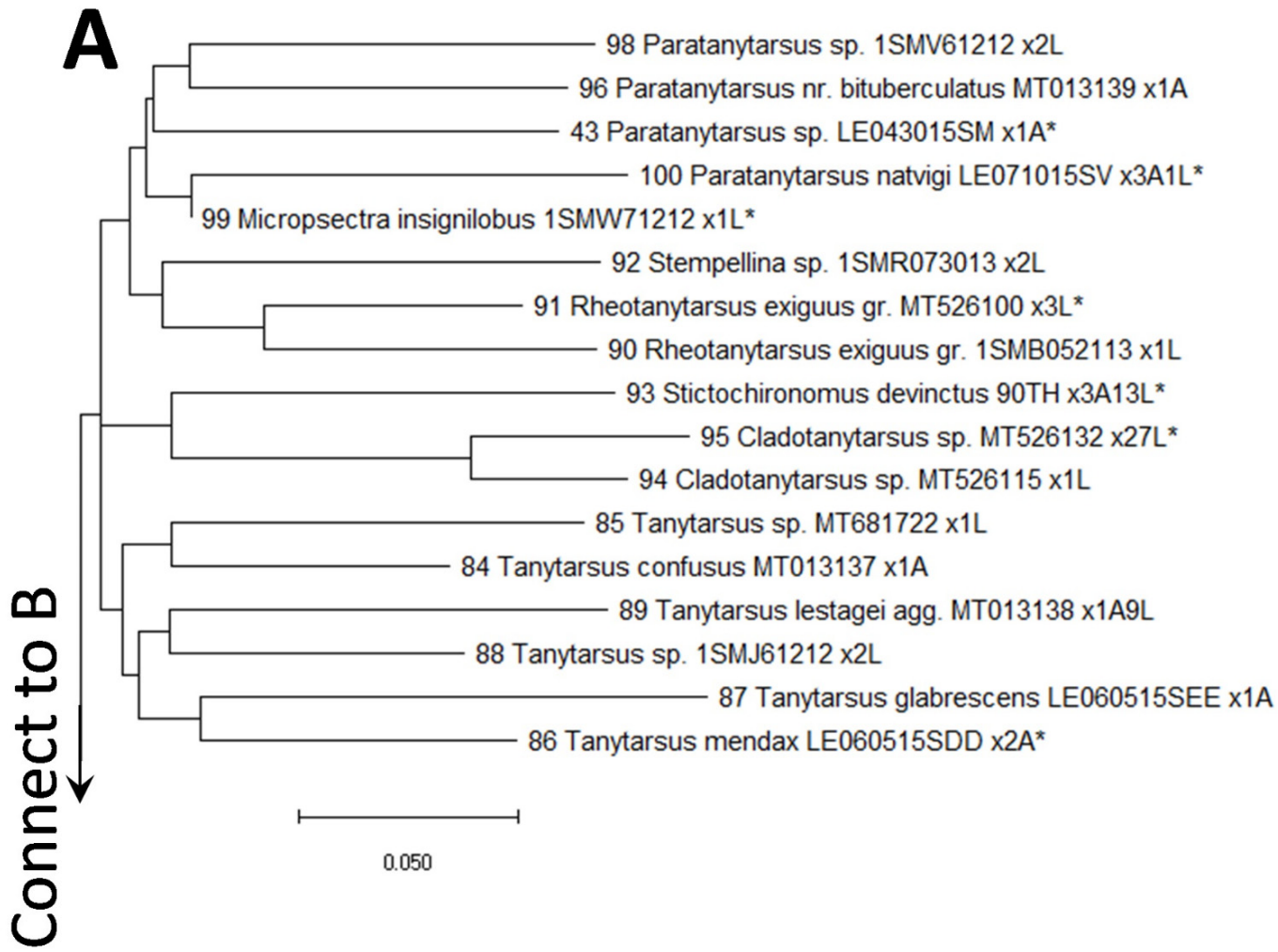

Figure 2. *Cont.*

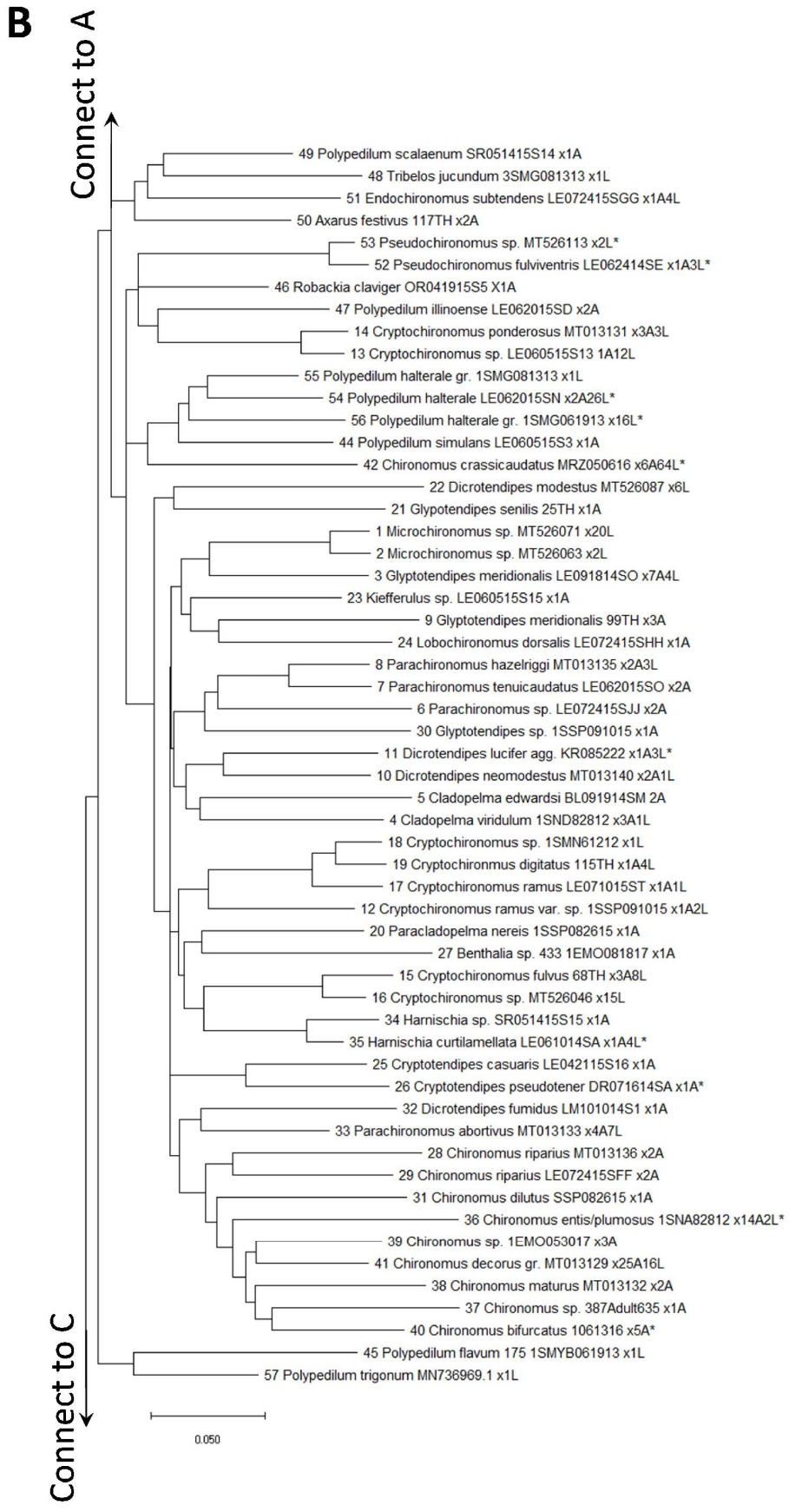

Figure 2. *Cont*.

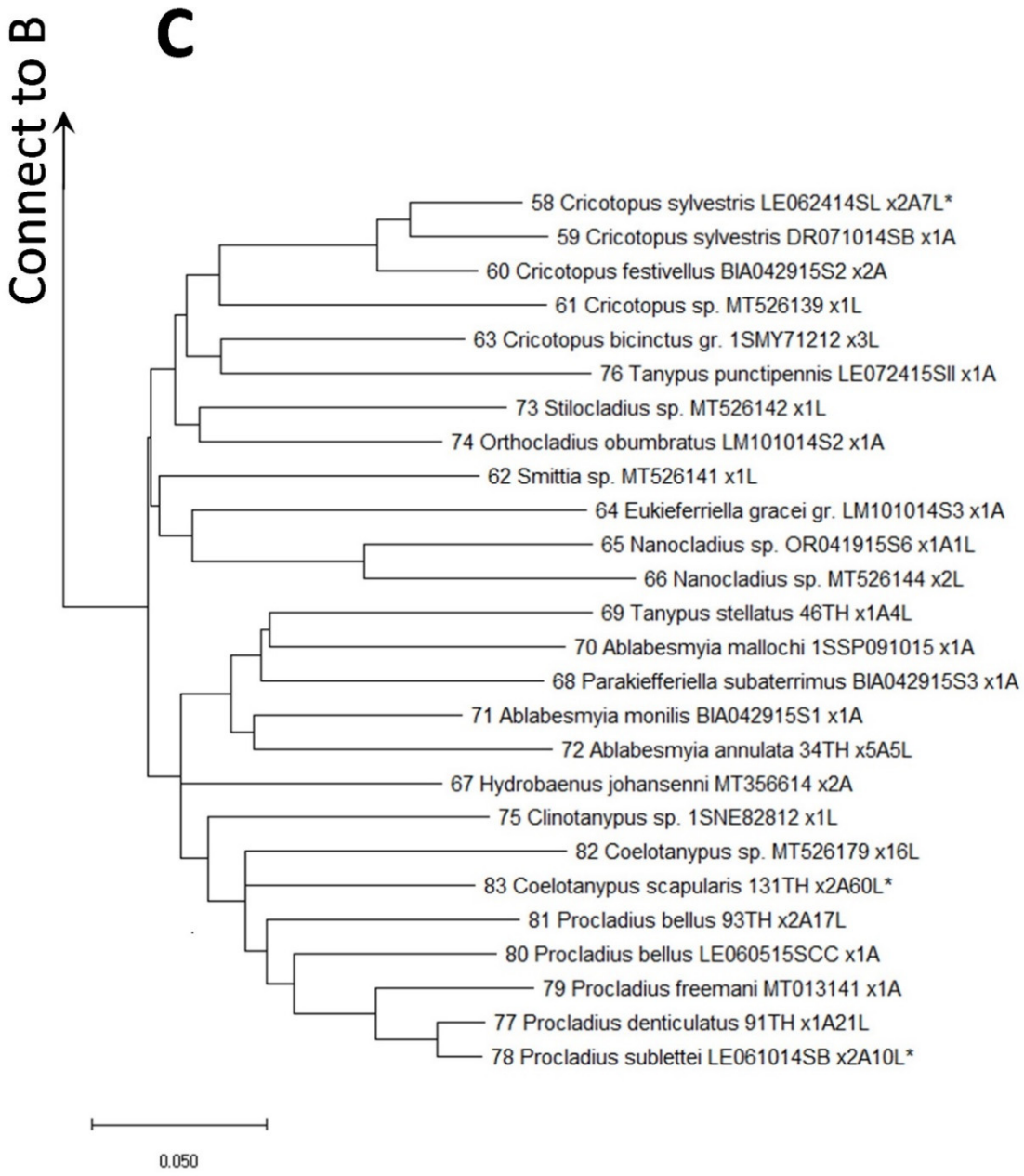

Figure 2. Curated consensus chironomid reference tree. The names on each branch represent the consensus of clusters of up to 71 specimens, taking into account criteria of sequence length, most specific taxon identification, and consistency with identification with the other members of the cluster. Due to the large size of the tree, the full tree is shown in three parts (**A**–**C**), of which the major constituents are (**A**) tribe Tanytarsini, (**B**) tribes Chironomini and Pseudochironomini, plus several *Dicrotendipes* spp. and (**C**) subfamilies Tanypodinae and Orthocladinae. See Figure S1 to for larger view of full tree seen in (**A**). Naming convention for each branch: Lab ID number, genus (XXX sp.) or species (XXX yyy), GenBank accession ID or RamLab ID (if not already uploaded), x number of adults (**A**) and number of larvae (L) used for branch consensus, * shown if a specimen has been removed or name revised due to non-consensus identification or other comment about the branch (see Table S1). The lines in the image at the left of A shows how the entire tree was split into three parts.

3.2. Pairwise Analysis of Distances between Curated Chironomid Sequences

A histogram of pairwise differences among the 631 identified curated sequences (i.e., after the removal of ~3% of non-consensus sequences) is shown in Figure 3. The analysis starts with a high number of pairs having small pairwise differences belonging to the same

species. The number of pairwise differences decreases to a relative minimum (i.e., not quite a definitive barcode "gap") at about 3.5% and rises to a small peak in pairs at around 4% that falls to a low level above 6%. This is followed by several peaks in the pairwise differences at around 8% and 10% pairwise differences before the differences arise in a continuum, more or less, with a broad peak at about 20–25% difference.

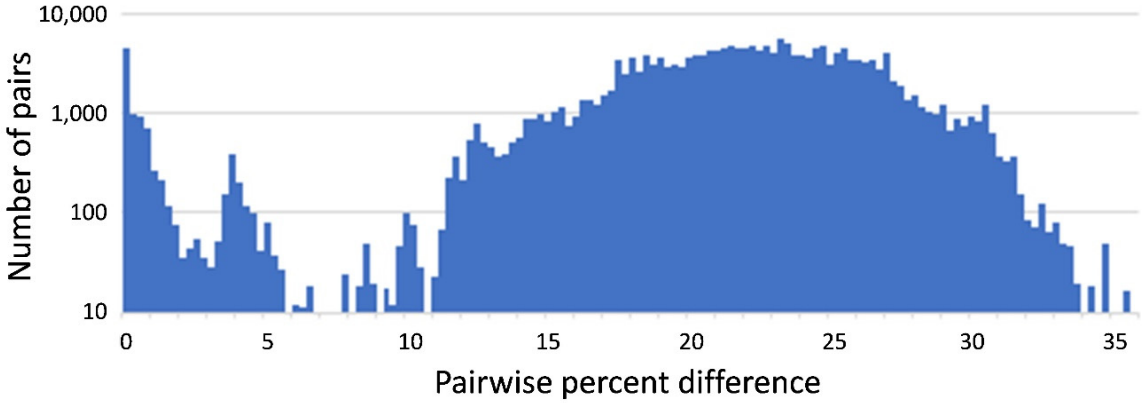

Figure 3. Histogram of pairwise similarities among the 631 chironomid sequences from the curated database (all specimens, minus 21 sequences known to be in error, listed in Table S1). The vertical axis is the number of pairwise observations, converted to percent pairwise difference and plotted on a semi-log scale for each 0.25% bin. The horizontal axis is the percent range of the matches.

With the exception of one, all pairs below 3.5% difference agreed in species when species was known (and, in any case, they always agreed in genus if identified only to sp.). The exceptional pair of species with less than a 3.5% difference is *Procladius denticulatus* and *P. sublettei*, which are separated in the curated tree by only 2.6%. The next closest pairwise difference between specimens identified to the species level was the pair *Cryptochironomus ramus* and *Cryptochironomus digitatus*, which differ in sequence by 5.8%.

In the range of 3.5% to 11%, all pairs agreed on the genus, with some pairs agreeing on the species as well. The lowest percent difference at which a pairwise difference occurred for 2 specimens differing in genus was at 11.0%; this occurred for the pairwise distance between *Parachironomus abortivus* and *Chironomus decorus*. Another example of a pair of genera with approximately this difference is *Coelotanypus scapularis* and *Procladius denticulatus*, differing by 11.1%.

Some pairs with identical morphospecies identification differed in sequence by more than 10% and could potentially represent cryptic species. These pairs include the following: *Glyptotendipes meridionalis* (represented by branches 3 and 9), differing by 15.2%; *Polypedilum halterale* (branches 54 and 56), 11.4%; *Rheotanytarsus exiguus* (branches 90 and 91), 11.8%; *Procladius bellus* (branches 80 and 81), 11.9%; and *Chironomus riparius* (branches 28 and 29), 13.5%.

3.3. Improved Identification of Water Mite Prey Using the Curated Chironomid Sequences Database

The curated chironomid sequence database was used in the present study both to confirm previous identifications of sequences in the water mite gut and, in many cases, to improve its specificity. An example is illustrated in Figure 4, in which the branches of a previously published neighbor-joining tree of dietary sequences in a specimen of *L. davidcooki* is paired with closely related sequences in the chironomid database [15]. In the original tree, half of the chironomid sequences were identified only to the genus level, and numerous other branches were identified only to family (Chironomidae) or subfamily (Orthocladinae, or Chironominae subfamilies) [15]. The species identities of some branches were confirmed (e.g., *Chironomus riparius* identities were supported by curated *Chironomus riparius* sequences that were 97–98% identical), while the genus of other branches was confirmed by a match better than 90.5% identity (e.g., KM995443.1 *Cricotopus* sp. at 91.3%

identity to *Cricotopus festivellus*). For other branches, the closest database sequence improved the identification from family to species (e.g., KR278055.1 Chironominae improved to *Endochironomus subtendens*, 97.6% identity match, highlighted in blue) or genus to species (e.g., KR276527.1 *Paratanytarsus* sp. was improved to *Paratanytarsus natvigi*, with 97.9% identity). One of the branches of KR955123.1 Chironominae was identified to genus by a 90.2% match to *Robackia claviger* (0.3% less than our usual "genus rule" of 90.5%).

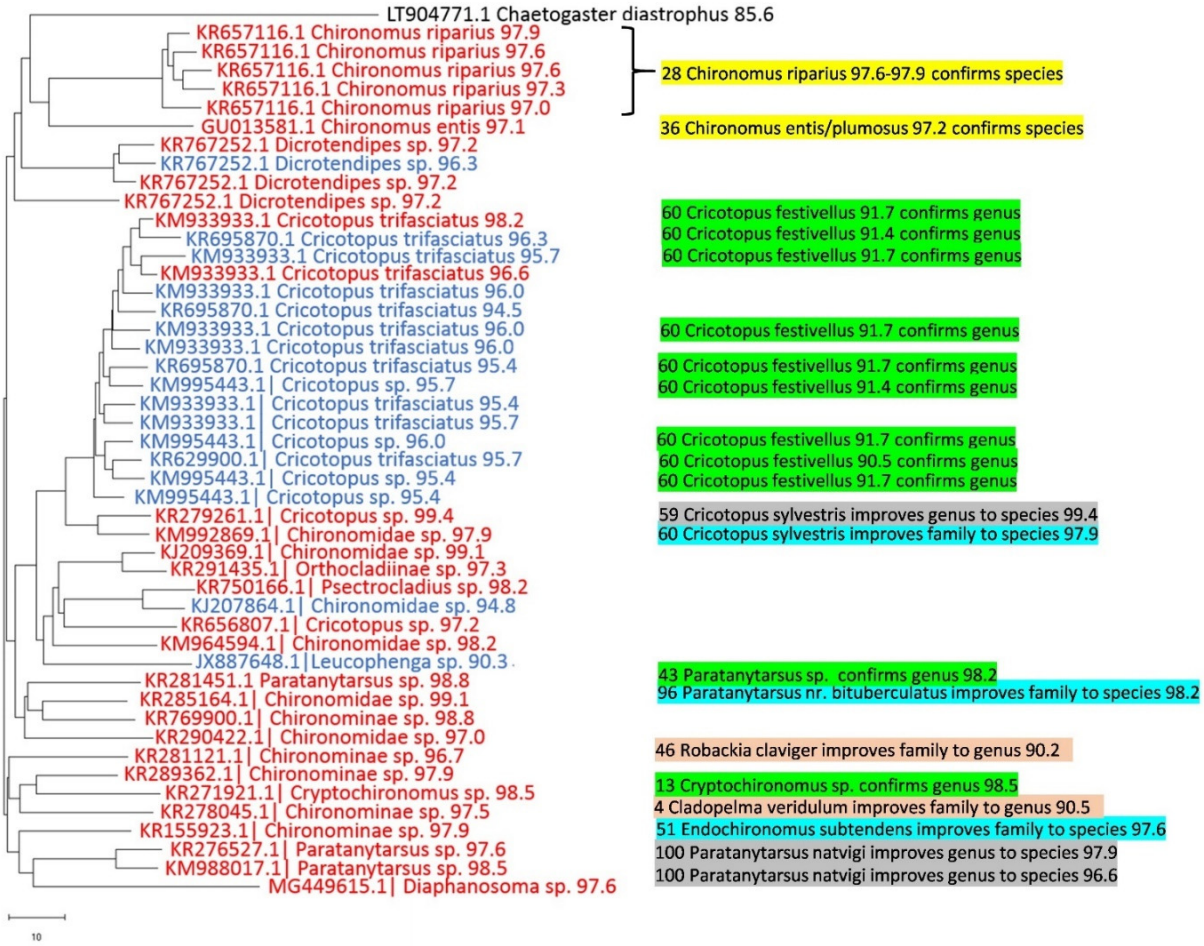

Figure 4. A representative example of confirmed and improved identification of water mite dietary content using the curated chironomid barcode database. The neighbor-joining tree (reproduced from Vasquez, Mohiddin, Li, Bonnici, Gurdziel and Ram [15]) aligns with the confirmations of identification highlighted in yellow for species and green for genus. Additionally, improvements are highlighted: grey for genus to species, blue for family to species and brown for family to genus. The curated sequence names are listed in the following format: Lab ID branch number, genus or species, percent similarity to the mite diet sequence.

In other water mites, similar improvements (family to species, genus to species, and family to genus) were observed, as well as various confirmations of genus or species. Figure 5 summarizes the number of members of the curated chironomid database that have confirmed or improved a previous identification of a dietary sequence in the set of 40 water mites whose diets were analyzed in this study. A total of 11 of the 99 branches of the curated chironomid database improved identifications to species that had previously been identified only to family (6, Table 1) or only to genus (5, Table 2). In addition, 13 members of the curated database improved family identifications in water mite diets to genus (Table 3).

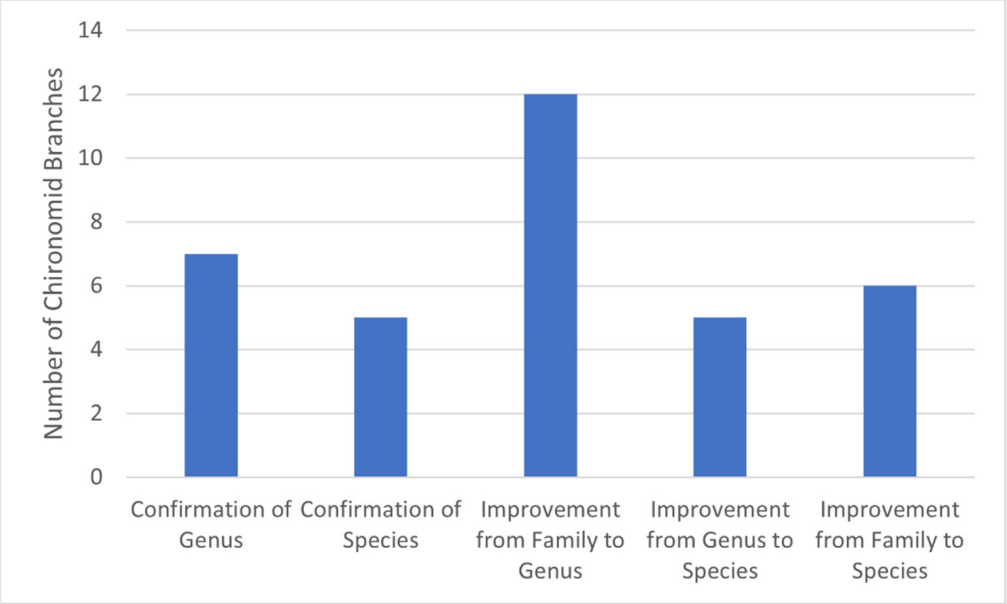

Figure 5. Summary of numbers of members of the curated chironomid database that either confirmed the identity of mite diet sequences or improved them from family to either genus or species or from genus to species.

Table 1. Summary of family to species improvements.

Previous GenBank Identification in Mite Diet	Improved Identification
Chironomidae	*Dicrotendipes modestus*
Chironomidae	*Parachironomus abortivus*
Chironominae	*Endochironomus subtendens*
Chironomidae	*Cricotopus festivellus*
Chironomidae	*Tanypus punctipennis*
Chironomidae	*Paratanytarsus nr. bituberculatus*

Table 2. Summary of genus to species Improvements.

Previous GenBank Identification in Mite Diet	Improved Identification
Parachironomus sp.	*Parachironomus tenuicaudatus*
Cricotopus sp. [1]	*Cricotopus sylvestris*
Cricotopus sp. [1]	*Cricotopus sylvestris*
Orthocladius sp.	*Orthocladius obumbratus*
Rheotanytarsus sp.	*Rheotanytarsus exiguus* gr.

[1] Two different branches identified as *Cricotopus* sp. in the mite diet correspond to two different branches in the curated tree identified as *C. sylvestris*, respectively.

Figure 6 summarizes that 38 of the 41 water mites had improvements in the identification of their dietary sequences. While a few water mites experienced improvements only of family to genus, 18 water mites experienced all 3 types of improvements. A total of 27 water mites had dietary constituents with improvements from family level identifications to species. Table 4 summarizes the dietary differences observed in the various water mite species that were the subject of this study, taking into account all of the improvements in the identification of sequences provided by the chironomid database. Among the specimens analyzed, the 21 specimens of *L. davidcooki* had by far the more diverse chironomid diet (33 different chironomid barcodes in its diet) compared to the 16 specimens of *L. quinquemaculosa* (17 chironomid barcodes) and the other species of water mites

in the table. Only one *Arrenurus*, two *Limnesia* and two unidentified specimens of *Lebertia* were analyzed.

Table 3. Summary of family to genus improvements.

Previous GenBank. Identification in Mite Diet	Improved Identification [1]
Chironominae	*Cladopelma veridulum*
Chironomidae	*Polypedilum simulans*
Chironominae	*Robackia claviger*
Chironomidae	*Ablabesmyia mallochi*
Chironomidae	*Ablabesmyia annulata*
Chironomidae	*Tanytarsus glabrescens*
Chironominae	*Chironomus* sp.
Chironomidae	*Cricotopus bicinctus* gr.
Chironomidae	*Polypedilum* cf. *halterale*
Chironomidae	*Polypedilum halterale* gr.
Chironominae	*Polypedilum trigonum*
Chironomidae	*Polypedilum scalaenum*

[1] Although the database provides species-level identification, the examples given are considered improvements only to genus because the distance was more than 3.5% and below 9.5%. Alternatively, if the database sequence was only identified to "sp.", the improvement was also only to genus.

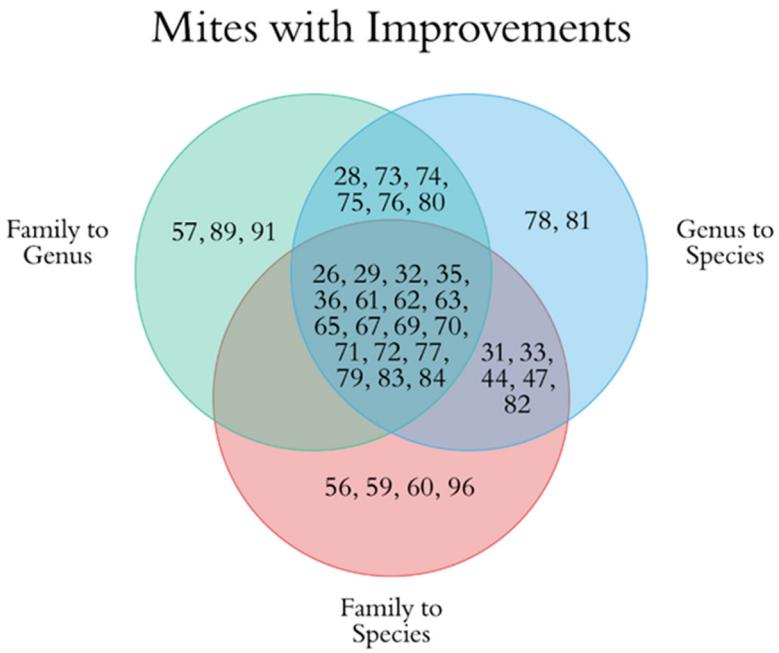

Figure 6. Water mite chironomid diet improvements from curated chironomid database. Improvements from family to genus (green circle), genus to species (blue circle) and family to species (red circle) are represented. The numbers in the diagram refer to specific mites in the water mite database for this project.

Table 4. Chironomids that were observed as prey in *Lebertia davidcooki*, *Lebertia quinquemaculosa*, *Lebertia* sp., *Limnesia* sp. and *Arrenurus* sp. indicated by check marks. Identifications that are between 3.5% and 9.5% pairwise difference from the mite diet sequence or that are identified as no better than to genus are marked with an asterisk.

Chironomids		Water Mite Species				
Chironomid Name	OTU Number	*Lebertia davidcooki*	*Lebertia quinquemaculosa*	*Lebertia* sp.	*Limnesia* sp.	*Arrenurus* sp.
Glyptotendipes meridionalis *	3	✓				

Table 4. *Cont.*

Chironomids		Water Mite Species				
Chironomid Name	**OTU Number**	***Lebertia davidcooki***	***Lebertia quinquemaculosa***	***Lebertia* sp.**	***Limnesia* sp.**	***Arrenurus* sp.**
Cladopelma veridulum *	4	✓	✓	✓	✓	✓
Parachironomus sp. *	6	✓	✓	✓		
Parachironomus tenuicaudatus	7	✓				
Parachironomus hazelriggi *	8	✓				
Cryptochironomus sp. *	13	✓	✓	✓		
Cryptochironomus ponderosus *	14	✓		✓		
Glypotendipes senilis	21	✓				
Dicrotendipes modestus	22	✓	✓			
Chironomus riparius	28	✓	✓	✓		✓
Chironomus riparius *	29	✓				
Parachironomus abortivus	33		✓			
Chironomus entis/plumosus	36	✓		✓		
Chironomus maturus	38	✓				✓
Chironomus sp. *	39	✓				
Chironomus crassicaudatus	42	✓	✓	✓		
Paratanytarsus sp. *	43	✓	✓	✓	✓	
Polypedilum simulans *	44	✓		✓		
Robackia claviger *	46	✓		✓		
Polypedilum illinoense *	47	✓				
Polypedilum scaleneum *	49	✓				
Endochironomus subtendens	51	✓	✓	✓		
*Polypedilum halterale**	54			✓		
Polypedilum halterale gr. *	55			✓		
Polypedilum trigonum *	57	✓	✓			
Cricotopus sylvestris	58	✓	✓	✓	✓	
Cricotopus sylvestris	59	✓	✓	✓	✓	
Cricotopus festivellus	60	✓	✓	✓	✓	
Cricotopus bicinctus gr. *	63	✓	✓			
Ablabesmyia mallochi *	70			✓		
Ablabesmyia annulate *	72			✓		
Orthocladius obumbratus	74	✓		✓		
Tanypus punctipennis	76	✓				
Coelotanypus sp. *	82	✓				
Tanytarsus glabrescens *	87	✓	✓			
Rheotanytarsus exiguous gr.	91	✓				
Paratanytarsus nr. *bituberculatus*	96	✓	✓	✓		
Paratanytarsus natvigi *	100	✓	✓	✓		✓

* Check marks that indicate chironomid taxa with asterisk were only identified to genus.

4. Discussion

Chironomids are a speciose dipteran found in many diverse aquatic habitats and are an important food source for multiple organisms, including water mites. A previous study on water mite prey DNA revealed that for *Lebertia* water mites, chironomids make up more than 80% of the diet content, but definitive identification of the majority of chironomid prey seen in the water mite diets was lacking [15]. Our current work developed an expanded chironomid reference sequence database by an intense multi-year sampling for chironomids to bioinformatically improve the identification of chironomids in water mite diets and to improve the study of chironomids in the environment in general. This work now contributes: (1) several new DNA COI barcodes for North American chironomid taxa, (2) insights into COI barcode gap parameters for future chironomid DNA barcoding work, (3) improved identification of chironomids found in the molecular gut contents of water mites, and (4) increased knowledge of chironomid genetic diversity in part of the Laurentian Great Lakes watershed.

4.1. New Barcodes

To generate the expanded chironomid database, we sampled further at our previously published chironomid collection sites: our paper by Failla et al. [18] was based on 2012 collections; the current paper includes a comparable number of specimens collected

in Toledo Harbor in 2013. We also collected at additional locations in Michigan, especially in the Detroit metropolitan area (Figure 1). These additional collections enabled the identification of specimens with novel DNA barcodes that had not previously been identified to the species level (and in many cases not appearing at all, even at genus identification) in either GenBank or BOLD databases. The novel species barcodes in this work include *Parachironomus abortivus*, *Endochironomus subtendens*, *Robackia claviger*, *Orthocladius obumbratus*, *Cryptochironomus ponderosus*, *Parachironomus hazelriggi*, *Paracladopelma nereis*, *Cryptochironomus ramus*, *Dicrotendipes fumidus*, *Cryptotendipes casuarius*, *Cryptotendipes pseudotener*, *Dicrotendipes neomodestus*, *Tribelos jucundum*, *Tanytarsus confuses*, *Pseudochironomus fulviventris*, *Polypedilum illinoense*, *Polypedilum trigonum*, *Rheotanytarsus exiguous*, *Eukiefferiella gracei*, *Parakiefferiella subaterrimus*, *Hydrobaenus johansenni*, *Procladius freeman*, and *Procladius sublettei*. *Harnischia curtilamellata, Tanypus punctipennis, Paratanytarsus nr. bituberculatus* and *Polypedilum flavum* were the first barcodes reported in North America for those species.

Among these new barcodes, we provide here additional information about *Robackia claviger*. This sequence is the first barcode for this genus and species in Genbank; however, several sequences of the same genus (*Robackia demeijerei*) are present in BOLD. Compared to the *R. demereijerei* sequences in BOLD, the *R. claviger* sequence differs by 6.5%, fully within the range expected to be considered belonging to the "same genus". A sequence in the diet of a specimen of *Lebertia davidcooki* that was originally identified from GenBank as Chironominae (i.e., only to family) was 90.2% identical to the *Robackia claviger* in our curated database, just slightly more distant than our usual requirement of 90.5% identity for assigning genus (however, see the discussion of barcode gaps below). *Robackia* has at least four recognized species as erected by Saether [33]. In the present study, *Robackia claviger* was sampled from the Ohio River at Shawnee State Park and has previously been found in lotic habitats in the southeastern United States [34]. In the Great Lakes region, *R. demereijerei* larvae were found in coarse sediments in Lake Michigan, and it was thought that their narrow head and tough outer body integuments allowed them to inhabit this embenthic habitat [35]. The *R. demereijerei* specimens in BOLD are from a lake in Sweden and Chequamegon Bay in Lake Superior. Due to the >3.5% distance from both *R. claviger* and *R. demereijerei*, we speculate that the species in the water mite diet may be either *R. pilicauda*, *R. aculeate*, or a new species since new species are still being described, such as *R. parallela sp. n.* from China [36].

4.2. Insights into "Barcode Gaps" in Chironomids

Figure 2 of Failla et al. [18] shows that pairwise differences >3.5% and <11% (but having very few pairs between 6% and 11%) were always of the same genus. Although the pairwise distance analysis in this paper of 631 chironomid sequences shows only a relative minimum at 3.5% and not a distinct "gap", that difference still seems to be a good delineation, at least among chironomids, of how far a pairwise distance could be and still be used reliably for species identification. We had only one exceptional species set below the 3.5% difference that did not follow this 3.5% parameter: two *Procladius* species (*denticulatus* and *sublettei*) were separated in the curated chironomid tree by only 2.6%. The next closest pairwise distance between specimens identified to the species level was the pair *Cryptochironomus ramus* and *Cryptochironomus digitatus,* which differed in sequence by 5.8%. The peak at 4% in the histogram of pairwise distances may also contain species differences. However, all of the pairs represented in this peak had at least one specimen that was identified only to genus (e.g., the distance between 13 *Cryptochironomus* sp. and 14 *Cryptochironomus ponderosus* is 3.8%, and the distance of 34 *Harnischia* sp. and 35 *Harnischia curtilamellata* is 4.6%). While these branches are clearly separate on the basis of sequence, it is unknown whether they represent different species. Additional species-level identifications of specimens that differ in sequence by 3.5–5.8% will be necessary to resolve whether differences beyond 3.5% (and how far?) are usually of the same species or not.

In the present study, we were conservative in assigning genus identifications based on sequence alone. We limited such assignments to differences of no greater than 9.5% except

in the case of *Robackia*, noted above, in which we assigned a tentative identification with a 9.8% pairwise distance. In fact, analysis of the pairwise data in the present study indicates that reliable genus identification extends out to greater distances. All of the pairs in the small pairwise peaks centered around 8% and 10% were between sequences of the same genus. We found that reliable genus identifications could be made up to 11% differences, beyond which pairs of different genera begin to be detected.

We also observed instances when specimens with identical morphospecies identifications differed greatly (i.e., defined as differences of >11%) from one another. These include *Glyptotendipes meridionalis*, *Polypedilum halterale, Rheotanytarsus exiguous*, *Procladius bellus*, and *Chironomus riparius*. Further study might reveal them as cryptic species. A review of the morphospecies characters of representative specimens of each cluster may reveal some new differentiating character by which animals in the two clusters can be distinguished.

From the above considerations and data, we, therefore, conclude that (a) assignment of sequences of operational taxonomic units to species can be done reliably up to a 3.5% pairwise difference, (b) assignment of genus can be done with confidence up to at least 9.5% and possibly more, and (c) identical morphospecies designations with greater than 11% difference in their sequence indicate the possible presence of cryptic species represented by one or both branches being compared.

4.3. Improved Water Mite Diet Identifications

Improving the identity of molecular water mite diet sequences enables us to better understand the diversity of chironomids in the diet contents and the trophic interactions of aquatic food webs in which water mites are embedded. For example, our curated chironomid database allowed us to identify the following species of chironomids as prey for water mites: *Parachironomus tenuicaudatus*, *Glyptotendipes senilis*, *Dicrotendipes modestus*, *Parachironomus abortivus*, *Chironomus riparius*, *Chironomus entis/plumosus*, *Chironomus maturus*, *Chironomus crassicaudatus*, *Endochironomus subtendens*, *Cricotopus sylvestris*, *Cricotopus festivellus*, *Orthocladius obumbratus*, *Tanypus punctipennis*, *Rheotanytarsus exiguous* gr., and *Paratanytarsus nr. bituberculatus*. Many of the barcode sequences of these prey species were previously known at best only to family.

Table 4, which lists the genera and species of chironomids that were found in the guts of *Lebertia quinquemaculosa*, *L. davidcooki*, *Lebertia* sp., *Limnesia* and *Arrenurus,* is expected to be a reliable list of taxa that the water mites have been ingesting. The greater richness of chironomid prey for *L. davidcooki* may indicate a dietary difference that is a result of niche partitioning. The dietary difference of the two species might also or alternatively be related to seasonal variation between collection dates for the two species, as described by Vasquez, Mohiddin, Li, Bonnici, Gurdziel and Ram [15].

In some cases, a dietary sequence of a water mite could be identified only to the genus, as in Table 3. This occurred in two ways: (1) BLAST comparisons to GenBank or to the curated database returned high identity (>96.5%) to sequences that were identified only to the genus in the database; or (2) the best match to the reference database was a <96.5% match to a known species or genus. Both types of genus identifications indicate inadequate species coverage in reference databases of the chironomids that *Lebertia* are ingesting.

While inadequate sampling effort may be part of the reason for incomplete species coverage of water mite diet sequences, another possibility is that water mites may be able to access chironomid habitats that our collecting and taxonomic methods have not yet encountered. Traditional sampling methods to capture chironomid larvae and adults are limited since chironomid larvae may inhabit unusual habitats, such as mined substrates like submerged wood [37], that may not be picked up by a ponar dredge and other collection methods used in this study. Chironomid larva of the genera *Cricotopus*, *Endochironomus*, *Glyptotendipes*, and *Parachironomus* are miners of substrates such as macrophytes, bryozoans and sponges [37] and were, nevertheless, found in the diets of the water mites studied. Water mites may be active predators seeking out and digging out chironomids from unusual habitats that human collectors may miss, as pointed out by Hudson many years ago [37].

In this regard, water mites seem to function as "DNA detectives", sometimes ingesting the DNA of rare or difficult to collect benthic microinvertebrates. This was also true for the DNA of oligochaetes that water mites ingested: the numerous oligochaete sequences associated with *L. quinquemaculosa* rarely matched any previously barcoded species or genus within 10% [15], suggesting that water mites are "discovering" species of organisms that collectors have not yet encountered or at least not yet bar-coded and submitted to a public database.

Specific Taxa Found in Water Mite Diets

Among the taxa that this study has newly identified in mite diets are *Tanypus punctipennis* and *Ablabesmyia*. *Tanypus punctipennis* is an example of a prey organism for which identification was improved from family to species, enabling a more specific analysis of trophic relationships. *Tanypus punctipennis* is a midge with distribution in all world regions except Australia [38]. *T. punctipennis* has a wide Palearctic distribution in temperate climates, consistent with its possible presence in the Great Lakes [39]. The late instar larvae of *T. punctipennis* are relatively small [40] compared to larger genera such as *Chironomus* and *Ablabesmyia annulata.* We speculate that the small size of *T. punctipennis* makes it a more suitable prey for the smaller *Lebertia davidcooki* water mite.

Some sequences in the *Lebertia* diet that were previously identified only to family (Chironomidae) were similar enough to *Ablabesmyia* (approximately 9% pairwise distance) to be identified with that genus. *Ablabesmyia* is a genus found worldwide, with over 90 identified species [41]. In the Americas, they are primarily found in the Nearctic region, including the Laurentian Great Lakes. *Ablabesmyia* is typically found on substrate in shallow littoral zones [42,43]. *A. monilis* has been reported from Northern Michigan and is found in muddy-bottomed lakes [44]. This is consistent with the Blue Heron Lagoon (Detroit, MI collection site) habitat, where the water mites studied in this work were obtained. *Ablabesmyia annulata* has the largest head capsule among 30 species of North American chironomids in which third instar larvae were compared [45]. *Ablabesmyia* is known to have a symbiotic relationship with freshwater mussels [46] and are predators of *Tubifex tubifex*, their own early instars, and other benthic macroinvertebrates [29,41,47,48]. *Ablabesmyia mallochi* is known to be more difficult to morphologically identify, so having an available barcode may assist in its identification in the future. Adults of *Ablabesmyia annulate,* on the other hand, are easily identifiable as they have anterior and posterior parapodia that are not darkened, three palpal segments, a long procercus, and a more quadrate head [49,50]. The larvae are just as easily differentiable from other species of *Ablabesmyia* [51]. *Ablabesmyia* DNA sequence was detected in only one water mite, suggesting that these genera of midges are rare in this habitat or that the species of water mites studied do not prefer *Ablabesmyia* sp. as prey potentially due to their larger larval size compared to other chironomids.

4.4. Need for Further Improvements of Knowledge of Chironomid Diversity

In our previous work on molecular barcodes of chironomids [18], we reported a tree with 45 larval operational taxonomic units and an additional adult barcode sequence not yet observed in larvae. That publication improved the species identification of the hitherto mostly genus-level identifications from 15.5% of the operational taxonomic units (OTUs) to more than 40% of the OTUs and reported sequences for 22 chironomid genera and 19 species. The present work expands the number of distinct chironomid OTUs from 46 to 99 and now includes barcodes from 39 genera and 60 species, a significant increase from previous studies.

However, in the curated database, several genera are identified only to genus level, either because they are for larval specimens for which species-level keys are not available or because the specimens were insufficiently intact to determine species. Of the 99 members of the curated chironomid barcode database, the following genera lack even one specimen identified to species: *Cladotanytarsus*, *Stempellina*, *Microchironomus*, *Kiefferulus*, *Benthalia*, *Smittia*, and *Clinotanypus*. We originally included *Nanocladius* in this list; however, a

sequence of *N. distinctus* from Sweden, updated on BOLD on 16 November 2021, is a 99.8% match to *Nanocladius* sp. MT526144. The other *Nanocladius* sp. sequence (65 *Nanocladius* sp. OR041915S6) in our curated database has no species-level match in either GenBank or BOLD; the closest species match on BOLD is *N. distinctus* at a pairwise distance of 11% and therefore is likely a different species. The recency of the *N. distinctus* record could suggest that this is an ongoing activity in several laboratories. The lack of species identifications of some OTUs of these genera emphasizes the need for more collecting, morphological identification and barcoding to attain more complete coverage of chironomids.

5. Conclusions and Future Considerations

The use of DNA molecular barcoding on chironomids has yielded significant advances in assisting with differentiating among multiple species of chironomids since specialized taxonomy in these aquatic organisms is not readily available [52]. DNA barcoding studies on chironomids combined with next-generation sequencing (NGS) techniques have been suggested as an efficient method to assess biodiversity and future environmental monitoring of these important aquatic invertebrates [53]. In addition to water mites, DNA barcoding has also been shown to be a useful tool in identifying the diet of other important aquatic organisms, such as fish [17]. Since chironomids are a significant part of fish diets, this curated database should assist diet analyses of fish as well.

Improvements in the species-level identifications of chironomids may enable investigations of the determinants of prey choice by water mites. Why, for example, is *Cladopelma* preyed upon by all five types of water mites studied and the various species of *Cricotopus* by four of the five types of water mites, in contrast to others that were detected as prey only in *Lebertia davidcooki* (notably, *Glyptotendipes*, *Tanypus*, *Coelotanypus*, and *Rheotanytarsus*)? Considering that the water mite diets matched five *Polypedilum* species only to genus level, what other species of *Polypedilum* might *Lebertia* be ingesting? Are there comparative features of the larvae of the four species of *Chironomus* (and two identified only to genus) that the various species of water mites ingested that would explain the different patterns of occurrence among the different water mite species? Identifying these organisms to the species level may enable future studies of differences in predator-prey dynamics in the laboratory and the field.

The methodology of our work does not differentiate whether the predator is feeding on the eggs, larvae, pupae or even the emerging adult stage of the prey. Some water mites feed on the larval stage of chironomids, although some other species of water mites are known to feed on dipteran eggs, including chironomids [10]. Follow-up experiments in the laboratory may be able to determine on which life stage(s) the predator is feeding.

Curation of barcoding data, as we have done here, is a critical step for using DNA barcodes in the future [54]. This paper demonstrates the use of DNA barcoding beyond simply biodiversity and biomonitoring analysis. The improved water mite diet information also sets the stage for future studies looking deeper into trophic interactions that require molecular analysis where morphological and observational data are not sufficient. While the advent of DNA barcoding and other genetic identification tools has brought advancement to identifying species, many more barcode sequences accompanied by careful morphospecies analyses are needed so that the sequences can be more usefully applied. Many taxonomic identifications were made decades ago, but with new technology and data, inconsistencies should be reviewed and updated (i.e., curated, as we have done). Expert taxonomists for aquatic invertebrate organisms are few and overburdened with material [55]; therefore, future advancements will require increased investment in the education and research of taxonomists who can combine morphological and molecular approaches to taxonomy whenever possible.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d14020065/s1, Table S1: Curation notes regarding the chironomid database: comments, corrections, and omitted sequences. Figure S1: 99 sequences in the Chironomid curated database, shown in a neighbor-joining tree (samedata as Figure 2 in the main text).

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Data Availability Statement: Sequence data generated from this work are openly available in the GenBank repository for nucleotide sequences (https://www.ncbi.nlm.nih.gov/genbank/ accessed on 10 January 2022).

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

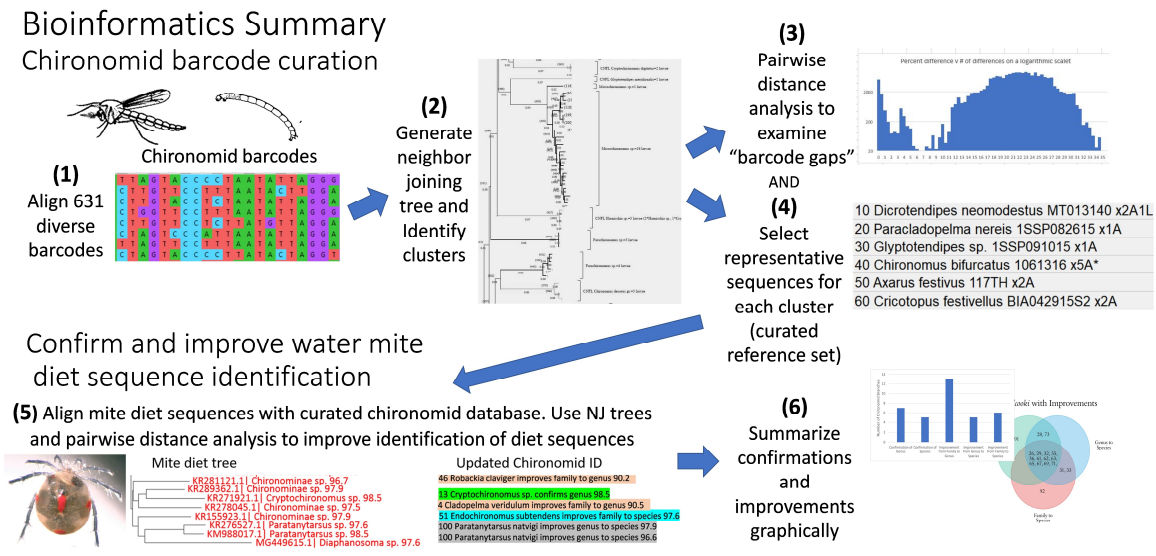

Figure A1. Graphical summary of methodology.

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