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DNA Barcoding for Species Identification of Insect Skins: A Test on Chironomidae (Diptera) Pupal Exuviae

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Abstract

Chironomidae (Diptera) pupal exuviae samples are commonly used for biological monitoring of aquatic habitats. DNA barcoding has proved useful for species identification of chironomid life stages containing cellular tissue, but the barcoding success of chironomid pupal exuviae is unknown. We assessed whether standard DNA barcoding could be efficiently used for species identification of chironomid pupal exuviae when compared with morphological techniques and if there were differences in performance between temperate and tropical ecosystems, subfamilies, and tribes. PCR, sequence, and identification success differed significantly between geographic regions and taxonomic groups. For Norway, 27 out of 190 (14.2%) of pupal exuviae resulted in high-quality chironomid sequences that match species. For Costa Rica, 69 out of 190 (36.3%) Costa Rican pupal exuviae resulted in high-quality sequences, but none matched known species. Standard DNA barcoding of chironomid pupal exuviae had limited success in species identification of unknown specimens due to contaminations and lack of matching references in available barcode libraries, especially from Costa Rica. Therefore, we recommend future biodiversity studies that focus their efforts on understudied regions, to simultaneously use morphological and molecular identification techniques to identify all life stages of chironomids and populate the barcode reference library with identified sequences.

Key words: DNA barcodes, species identification, Costa Rica, Norway, bioassessment

Benthic macroinvertebrates are regularly used for biological monitoring of aquatic habitats, as they are common and widespread, with high species diversity and varying sensitivity to environmental disturbances (Rosenberg and Resh 1993, Resh 2007). Among benthic macroinvertebrates, the family Chironomidae (Diptera), commonly referred to as the non-biting midges or chironomids, is a species-rich aquatic insect group that is particularly sensitive to changes in water quality (Pinder 1986, Lindegaard 1995, Nicacio and Juen 2015). There are close to 1,300 chironomid species recorded from Europe (Sæther and Spies 2013), nearly 900 species described from the Neotropical region (Spies and Reiss 1996, Spies et al. 2009), and estimates that range up to 20,000 species worldwide (Ferrington 2008). Additionally, chironomids are usually the most abundant aquatic insect group in all types of freshwater with larval densities of many thousands per square meter (Anderson et al. 2013b) and also among the most widespread insects inhabiting terrestrial, semi-terrestrial, and aquatic environments in all geographical regions, including Antarctica (Ferrington 2008).

An efficient, low-cost, and easy-to-use method for assessing chironomid communities involves collections of pupal exuviae (Raunio and Muotka 2005, Wilson and Ruse 2005, Raunio et al. 2007, Kranzfelder et al. 2015), which is the exoskeleton shed by the adult as it emerges on the surface of the water. Some advantages offered by collections of pupal exuviae over larval sampling for biological monitoring and assessment are that: i) deep muddy rivers, canals and lakes, as well as riffles in shallow rivers, may be easily sampled, ii) a pupal exuviae sample integrates taxa from all kinds of microhabitats, iii) pupal exuviae collections are more time-efficient in resolving chironomid composition than standard dip-net sampling, and iv) identification to genus using available keys is relatively easy (Wilson and Ruse 2005, Bouchard and Ferrington 2011, Anderson and Ferrington 2012, Ferrington and Coffman 2014). A few disadvantages of collections of pupal exuviae over larval sampling are that: i) emergence is seasonally and diurnally variable, ii) pupal exuviae float passively on the water's surface, and therefore, it is not possible to know exactly from which area upstream or upwind of the sampling site the pupal exuviae originated, and iii) traditional morphological species identification requires rearing of adult males from larvae or pupae for life-stage association (Wilson and Ruse 2005). These constraints lead to widespread use of generic or higher taxonomic resolution for bioassessments with chironomids, which is problematic since species within a genus or family can display a broad range of sensitivities to various environmental stresses (Sweeney et al. 2011).

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DNA barcoding provides an alternative tool for species identification based on a short DNA sequence from a standardized genetic locus (Hebert et al. 2003). A partial region (658 bp) of the cytochrome c oxidase I (COI) gene has been useful for separating cryptic, small, or rare species (Hebert et al. 2004, Sinclair and Gresens 2008, Pauls et al. 2010, Sweeney et al. 2011, Anderson et al. 2013a, Jackson et al. 2014, Stur and Borkent 2014) and associating multiple life stages (Carew et al. 2005; Ekrem et al. 2007; Zhou et al. 2007, 2009; Stur and Ekrem 2011; Webb et al. 2012). Life-stage associations with molecular tools can be particularly valuable for chironomids, since the immature life stages (larvae and pupae) are difficult to separate morphologically to species, and can be difficult to rear, especially in Neotropical settings with relatively high natural water temperatures (Spies et al. 2009). Carew et al. (2005), Ekrem et al. (2007), Ekrem et al. (2010), Anderson et al. (2013a), and Lin et al. (2015) have found that DNA barcodes can be used to link different life stages of the same chironomid. Lin et al. (2015) found that DNA barcoding is generally effective for species identification in *Tanytarsus*, even for taxa that are sampled from multiple and large geographic areas.

Recently, non-destructive DNA isolation methods from chironomid pupal exuviae have been explored, since pupal exuviae yield DNA in the form of muscle tissue, hairs, and epithelial cells lining the foregut, hindgut and trachea that are left behind on the inner surface of the cuticle by the emerging adult (Nation 2008, Krosch and Cranston 2012, Kranzfelder et al. 2016). Krosch and Cranston (2012) obtained sequences from 27 out of 58 chironomid pupal exuviae, and more recently, Kranzfelder et al. (2016) successfully isolated genomic DNA from 61.2% of 570 sampled chironomid pupal exuviae. We determined that three DNA extraction kits, the NucleoSpin Tissue XS Kit, the DNeasy Blood and Tissue kit, and the QuickExtract DNA Extraction Solution, provided the best results in isolating DNA from single pupal exuviae (Kranzfelder et al. 2016). However, we did not study the success of chironomid pupal exuviae species delimitation using DNA barcodes, which is an important precursor for using DNA barcoding as part of bioassessment studies.

DNA barcoding has the potential to improve the use of chironomids in bioassessment studies of aquatic ecosystems by providing increased taxonomic resolution, improved accuracy and objectivity of data quality, and enhanced diagnostic ability of existing assessment tools (Pilgrim et al. 2011, Sweeney et al. 2011, Brodin et al. 2013, Carew et al. 2013, Stein et al. 2014). However, a collection of well-identified reference COI sequences in a DNA barcode library, such as the Barcode of Life Data Systems (BOLD, http://www.boldsystems.org/) or GenBank (https://www.ncbi.nlm.nih.gov/genbank/), is critical for identification through DNA barcoding (Ekrem et al. 2007). Currently, comprehensive DNA reference libraries for

certain taxonomic groups and geographic ranges of chironomids remain poorly developed.

In addition, Chironomidae pupal exuviae decomposition rates depend on biotic and abiotic environmental factors, such as microbial activity, nutrient concentration, temperature and turbulence. Specifically, higher microbial (i.e., bacteria and fungi) numbers, warmer waters, higher nutrient levels (i.e., nitrate and ammonia from untreated wastewaters), and higher turbulence lead to an increase in pupal exuviae decomposition rates (Kavanaugh et al. 2014). Additional abiotic factors in aquatic habitats, such as ultraviolet radiation, temperature, oxygen, pH, salinity, and substrate, can differ and impact DNA degradation (Barnes et al. 2014, Strickler et al. 2015, Eichmiller et al. 2016). For example, Strickler et al. (2015) found that aquatic habitats that are colder, more protected from solar radiation, and more alkaline have lower DNA degradation rates than those that are warmer, sunnier, and neutral or acidic. Based on these interacting abiotic and biotic environmental factors, we would expect chironomid pupal exuviae collected from tropical aquatic ecosystems to degrade faster than temperate systems. Also, decomposition rates of pupal exuviae are impacted by the degree of chitinization of the species as lightly chitinized exuviae sink faster (Kavanaugh et al. 2014). Therefore, we predict that taxonomic groups of pupal exuviae with heavier sclerotization would decompose slower than pupal exuviae with lighter sclerotization.

The aim of this present study was to assess whether standard DNA barcoding could be efficiently used for species identification of chironomid pupal exuviae when compared to morphological techniques. In addition, we wanted to identify factors that are significantly associated with barcode identification success by investigating if geographic regions (temperate vs. tropical) and taxonomic groups (subfamily and tribe) impact PCR amplification, COI sequencing, and species identification success.

Materials and Methods

Chironomid pupal exuviae were collected using drift nets (mesh size 250 μm) from multiple lentic and lotic aquatic systems in Norway and Costa Rica between August and December 2014 (Table 1). DNA extractions and polymerase chain reaction (PCR) amplifications were done on a total of 380 chironomid pupal exuviae at the NTNU University Museum. Based on results of Kranzfelder et al. (2016), DNA was extracted from 95 individuals using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and from 95 individuals using the QuickExtract DNA Extraction Solution (Epicentre, Eindhoven, The Netherlands) for a total of 190 individuals from both the Norway and Costa Rica material. See Kranzfelder et al. (2016) for a detailed description

Table 1. Locality information for chironomid pupal exuviae collections. All Norway and Costa Rica samples were collected in 2014

Sample site	Aquatic system	GPS	Sample date(s)
a. Norway			
Lianvatnet	Lake	N 63.403°, E 10.318°	Aug. 31
Nidelva	River	N 63.429°, E 10.379°	Sept. 5, 12, 17
b. Costa Rica			
Rio Grande	River	N 9.635°, W 82.678	Dec. 13
Quebrada Dos Aguas	Stream below a waterfall	N 9.631°, W 82.819°	Dec. 14
Río Negro	River	N 9.644°, W 82.732°	Dec. 15
Río Cocles	River	N 9.646°, W 82.735°	Dec. 15
Rio Punta Uva	River	N 9.636°, W 82.694°	Dec. 15
Rio Manzanillo	River	N 9.628°, W 82.677°	Dec. 15

of our chironomid pupal exuviae sample collection and preservation methodology and DNA extraction, PCR amplification, and sequencing methodology.

After DNA extraction, voucher specimens were dissected under a stereo microscope and slide mounted in Euparal on individual microscope slides. Finally, slide-mounted specimens were identified under a compound scope to morphospecies or the lowest taxonomic level possible using works by Brundin (1949), Fittkau (1962), Sæther (1981), Wiederholm (1986), Epler (1988), Wülker (1991), Langton (1991), Jacobsen (2008), Wiedenburg et al. (2009), and Anderson et al. (2013a). Some pupal exuviae were damaged (e.g., loss of thoracic horn or wrinkled and/or compressed abdomen) during DNA extraction, which made slide mounting and morphological identification difficult. Species that did not match Linnean names were given interim names (Table 2). Subfamilies and tribes were defined based on the results of Cranston et al. (2012). Slide-mounted voucher specimens are deposited at the NTNU University Museum Insect Collection (NTNU-VM, Norwegian material) or the University of Minnesota Insect Collection (UMSP, Costa Rican material).

DNA sequences were manually edited in Sequencher version 4.8 (Gene Codes Corp., Ann Arbor, MI, USA), checked for stop-codons and frame-shifts, and aligned by their amino acids using default MUSCLE options (Edgar 2004) in MEGA 6 (Tamura et al. 2013). Ambiguous base calls were given the appropriate International Union of Biochemistry ambiguity symbol. After trimming of uncertain bases at both ends, the aligned sequences were 616–630 bp long.

Metadata, photos, sequences, and trace-files are available in the Barcode of Life Data Systems (BOLD, www.boldsystems.org) through the dataset DS-TRPEX with doi: dx.doi.org/10.5883/DS-TRPEX and dataset DS-CRPEX with doi: dx.doi.org/10.5883/DS-CRPEX. Specimen data and GenBank accession numbers are given in Suppl. Tables 1 and 2 (online only).

The barcode sequence of each high-quality sequence (quality score of 80% or above in Sequencher version 4.8) was compared with every COI barcode record with a minimum sequence length of 500 bp in the BOLD Identification System (BOLD-IDS) (Ratnasingham and Hebert 2007) and GenBank's BLAST (Altschul et al. 1990). We accessed the databases on 7 April 2017.

The Fisher's exact test of independence was used to determine whether there were differences in PCR success, sequencing success, and identification success between geographic regions (Norway vs. Costa Rica). The chi-square test of independence with a post-hoc Bonferroni correction to determine whether there were differences in PCR success, sequencing success, and identification success between taxonomic groups (subfamily vs. subfamily and tribe vs. tribe) in the R software version 3.4.1 (R Development Core Team 2016). Only subfamilies with 10 or more specimens are included in significance tests, as groups with fewer representatives are considered less reliable. Success rates at these three stages (PCR, sequencing, and species identification) were compared with determine when and how failures occurred in the DNA barcoding pipeline. PCR success was indicated by the presence of a band for each sample on an agarose gel, sequence success by the presence of a high-quality sequence for each sample, and identification success by matching unknown sequences to known chironomid reference sequences with at least 95% similarity in the databases. We selected the 95% similarity criterion based on recommendations by Lin et al. (2015) that suggested a 4–5% threshold is appropriate to delineate closely related species in the chironomid genus *Tanytarsus*.

Table 2. List of species and number of pupal exuviae identified using morphology and DNA barcoding from Norway and Costa Rica

Species	Morphology	DNA barcoding
a. Norway		
Procladius (*Holotanypus*) *signatus* (Zetterstedt, 1850)	2	1
Rheopelopia maculipennis (Zetterstedt, 1838)	8	
Potthastia gaedii (Meigen, 1838)	2	
Prodiamesa olivacea (Meigen, 1818)	20	1
Corynoneura sp. 1PK	1	
Corynoneura sp. 15ES	1	1
Cricotopus sp. 1PK	12	
Cricotopus cf. *septentrionalis* Hirvenjoa, 1973	4	4
Cricotopus cf. *similis* Goetghebuer, 1921	20	
Eukiefferiella sp. 1PK	40	
Orthocladius sp. 1PK	2	1
Orthocladius (*Orthocladius*) *oblidens* (Walker, 1856)	18	1
Thienemanniella sp. 1PK	8	
Tvetenia calvescens (Edwards, 1929)	20	4
Chironomus sp. 1PK	2	2
Chironomus (*Chironomus*) cf. *tenuistylus* Brundin, 1949	5	3
Demicryptochironomus sp. 1PK	1	
Micropsectra sp. 1PK	11	
Micropsectra sp. 5ES	1	1
Micropsectra logani (Johannsen 1928)	8	8
Polypedilum sp. 1PK	2	
Tanytarsus aculeatus Brundin, 1949	2	
Total	190	27
b. Costa Rica		
Species	*N*	
Zavrelimyia sp. 1PK	1	
Corynoneura sp. 1PK	1	
Onconeura cf. *semifimbriata* Sæther, 1981	5	
Onconeura cf. *similispina* Wiedenburg et al. 2009	2	
Onconeura cf. *japi* Wiedenburg et al. 2009	2	
Thienemanniella sp. 1PK	1	
Cricotopus (*Isocladius*) sp. 1PK	139	
Cricotopus (*Cricotopus*) sp. 2PK	4	
Cricotopus (*Cricotopus*) sp. 3PK	2	
Cricotopus (*Cricotopus*) sp. 4PK	1	
Cricotopus (*Cricotopus*) sp. 5PK	1	
Cricotopus (*Isocladius*) sp. 6PK	2	
Cricotopus (*Nostococladius*) sp. 7PK	2	
Nanocladius (*Nanocladius*) sp. 1PK	2	
Nanocladius (*Nanocladius*) sp. 2PK	2	
Parametriocnemus sp. 1PK	14	
Apedilum cf. *elachistus* Townes, 1945	5	
Polypedilum sp. 1PK	1	
Polypedilum sp. 2PK	1	
Chironomini Genus A Jacobsen, 2008	1	
Tanytarsini #1PK	1	
Tanytarsini #2PK	1	
Total	190	0

Results

Morphological Identification

From the Norway pupal exuviae samples, we identified 22 species from 15 genera and eight tribes. Out of the 190 specimens, we identified 109 specimens (57.4%) to named, described species. However, we identified all 190 specimens (100%) to morphospecies, meaning that differences usually found to be diagnostic on the species level were observed without finding a match with described species. From the Costa Rica pupal exuviae samples, we identified 22 species from 12 genera and 5 tribes. Out of the 190 specimens, we identified 14 specimens (7.4%) to named, described species and identified the rest of the specimens to genus (173 specimens, 91.1%), and tribe (3 specimens, 1.6%). However, we identified all 190 specimens (100%) to morphospecies (Table 2).

Identification with DNA barcodes

Among the 190 specimens from Norway, 18 specimens (9.5%) failed PCR for COI and could not be sequenced. Of the remaining 172 specimens (90.5%) that had successful PCR, 145 specimens (76.3%) could not be identified as chironomids due to low-quality DNA sequences or non-target contamination, including a water flea (*Holopedium gibberum*) and water molds (*Aphanomyces* spp.). A total of 27 specimens (14.2%) resulted in high-quality chironomid DNA sequences, which clustered in 11 barcode index numbers (BINs) that closely approximate species of which seven were named, described species and four were undescribed species that matched the morphological names. Among the 190 specimens from Costa Rica, 42 specimens (22.1%) failed PCR for COI and could not be sequenced. Of the remaining 148 specimens (77.9%) that had successful PCR, 79 specimens (41.6%) could not be identified as chironomids due to low-quality DNA sequences or non-target contamination, including water molds (*Aphanomyces* spp.) and parasitic oomycetes (*Pythium* spp.). In total 69 specimens (36.3%) resulted in high-quality chironomid DNA sequences, which clustered in 10
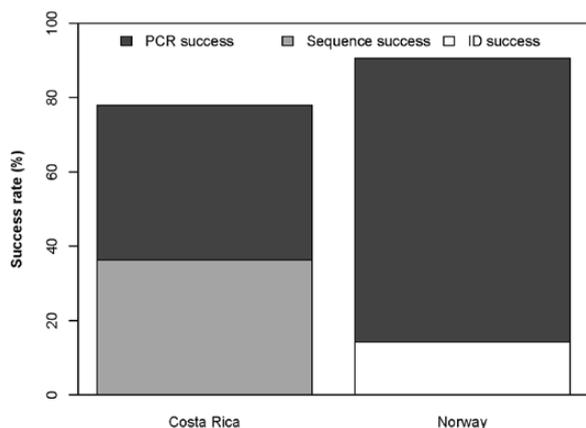
BINs that closely approximate species, but none of these sequences matched named, described species in BOLD (Table 2).

Factors Influencing Barcoding Success: Geographic Location

The 90.5% PCR success for the Norwegian pupal exuviae was significantly higher than the 77.9% success for the Costa Rican pupal exuviae ($P = 0.001$), but the 36.3% sequence success for Costa Rica was significantly higher than the 14.2% success for Norway ($P < 0.001$). However, 14.2% sequence success for Norwegian pupal exuviae was significantly higher than the 0.0% success for Costa Rican pupal exuviae ($P < 0.001$) (Fig. 1).

Factors Influencing Barcoding Success: Taxonomic Group

There was a significant difference in PCR success ($\chi^2(3) = 11.478$, $P = 0.009$), sequence success ($\chi^2(3) = 11.557$, $P = 0.009$), and identification success ($\chi^2(3) = 49.388$, $P < 0.001$) between subfamilies (Table 3). After Bonferroni corrections of the P values, there were no significant differences in subfamilies with regards to PCR success ($P > 0.008$). However, Chironominae pupal exuviae (43%) had significantly higher sequence success than Prodiamesinae (5%) ($P = 0.006$). Also, Chironominae (33%) pupal exuviae had significantly higher identification success than Orthocladiinae (4%) ($P < 0.001$) (Table 3).

There was a significant difference in PCR success ($P = 0.008$), sequence success ($P = 0.041$), and identification success between tribes ($P < 0.001$). After Bonferroni corrections of the P values, there were no significant differences in tribes with regards to PCR success and sequence success ($P > 0.002$). However, both Tanytarsini (38%) and Chironomini (28%) had significantly higher identification success than Orthocladiini (4%) ($P < 0.001$) (Table 4).

Discussion

Currently, species identification using morphology is more than twice as effective as standard DNA barcoding for chironomid pupal exuviae samples. For pupal exuviae samples from both Norway and Costa Rica, we identified 22 species using morphology, but 12 species were missing from the DNA barcoded samples. Similar to our results, Bista et al. (2017) found 10 genera that were identified morphologically, but were neither found in metabarcoding of eDNA or community DNA from samples with chironomid pupal exuviae. While PCR amplification success was high for chironomid pupal exuviae samples from both Costa Rica (77.9%) and Norway (90.5%), sequence and identification success was low. We speculate for both studies that morphological identifications are more effective than DNA barcoding because of either field non-target organism cross-contamination, low DNA quantities for certain taxa, or missing reference species/genera in the reference libraries. In our study, the presence of non-target species contaminant DNA in the chironomid pupal exuviae samples likely resulted in a reduction in sequencing success. Specifically, for the Norway samples, we believe that low barcoding success is related to

Fig. 1. Percent PCR, sequence, and identification (ID) success rate of Costa Rican versus Norwegian pupal exuviae. Norway sequence success was equal to identification success.

Table 3. Comparison of PCR, sequence, and identification success by subfamily for pooled samples from Norway and Costa Rica

Subfamily	No. of pupal exuviae	PCR success (%)	Sequence success (%)	Identification success (%)
Tanypodinae	11	7 (64)	2 (18)	1 (9)
Prodiamesinae	20	13 (65)	1 (5)	1 (5)
Orthocladiinae	305	259 (85)	75 (25)	11 (4)
Chironominae	42	39 (93)	18 (43)	14 (33)

Table 4. Comparison of PCR, sequence, and identification success by tribe for pooled samples from Norway and Costa Rica

Subfamily/Tribe	No. of pupal exuviae	PCR success (%)	Sequence success (%)	Identification success (%)
Pentaneurini	9	5 (56)	1 (11)	0 (0)
Procladiini	2	2 (100)	1 (50)	1 (50)
Prodiamesinae	20	13 (65)	1 (5)	1 (5)
Corynoneurini	20	15 (75)	4 (20)	1 (5)
Orthocladiini	285	244 (86)	71 (25)	10 (4)
Chironomini	18	15 (83)	8 (44)	5 (28)
Tanytarsini	24	24 (100)	10 (42)	9 (38)

Prodiamesinae is identified at the subfamily level, since there are no tribes within this subfamily.

cross-contamination that was most likely introduced in the field. For example, we found moderate-to-high densities of a species of water flea (*Holopedium gibberum*) floating on the surface of the water during fieldwork. Cells or free DNA from non-target organisms, like this water flea, could get stuck inside or attached to different parts of the pupal exuviae, contaminate the sample by competing with the low pupal exuviae DNA (Kranzfelder et al. 2016), and reduce the chances that the unknown sequences successfully match known chironomid sequences. In contrast, we did not observe high densities of non-target species floating on the surface of the water during fieldwork in our Costa Rican sample area. We speculate that biotic and abiotic environmental factors, including higher microbial (i.e., bacteria and fungi) numbers, warmer waters, and higher UV solar radiation (Kavanaugh et al. 2014), caused the DNA within the Costa Rican pupal exuviae samples to degrade faster than the Norwegian samples.

While the BINs or approximate number of species were the same for both Norway and Costa Rica, the sequencing and identification success rates were different when comparing the two geographic regions. In Norway, our pupal exuviae samples were collected near the Norwegian University of Science and Technology (NTNU) in Trondheim where chironomid barcode projects have been run for almost a decade as part of the Norwegian Barcode of Life (NorBOL). Therefore, it makes sense that all of our high-quality sequences matched known barcoded species. For Costa Rica, we identified all pupal exuviae to morphospecies; however, DNA barcoding did not improve species identifications. We were not able to identify any unknown barcode to known barcoded species, since the reference library lacked public barcode sequences with species identifications from Costa Rica. As of April 7, 2017, BOLD systems had 3,561 chironomid records for Norway representing 112 genera, 552 named species (Linnean and interim names), and 741 BINs. There are currently 631 chironomid species recorded from Norway (Artsdatabanken 2015) and an estimated 50 undescribed species. Costa Rica had 17,482 chironomid records in BOLD. There are currently 51 species recorded (Spies and Reiss 1996), but there are an estimated 1,000 undescribed species within the Dr. William P. Coffman Costa Rica collection at La Selva Biological Station (de la Rosa 2015) and perhaps 2,000 species in the country (C.L. de la Rosa, personal communication). Our results indicate that association of the Costa Rican specimens to named species suffers from the inadequacy of the barcode reference library for Chironomidae from this region. While many Costa Rican chironomids have been barcoded, most of these barcodes are not identified to species due to the lack of expertise in Central American chironomid taxonomy. Reliable species identification of chironomids with DNA barcoding requires the presence of named species in the reference library (Ekrem et al. 2007) and current reference libraries remain incomplete in terms of both species and geographic ranges.

In addition to differences in barcoding success related to geographic regions, we found significant differences in DNA barcoding success when comparing taxonomic groups (subfamilies and tribes). Subfamily Chironominae, and more specifically tribes Tanytarsini and Chironomini, had significantly higher sequence and identification success than subfamily Orthocladiinae. These results are not surprising since we have observed Tanytarsini and Chironomini specimens struggle to emerge as adults from their pupal skins during individual rearing. During emergence, these specimens possibly leave behind higher numbers of epithelial cells and other cell-based structures from the adult (the cuticle itself is extracellular) and meconial liquid (comprising the breakdown products of the immature stage hemoglobin) (P. Cranston, personal communication). The subfamily Chironominae tend to be darker and more sclerotized than other subfamilies, which would reduce their decomposition rate and increase the chances of trace DNA being available for DNA extraction. As a result, researchers that would like to identify chironomid pupal exuviae to species must consider the influence of environmental conditions in different geographic regions and the physical structures of various taxonomic groups on the preservation of DNA when designing their DNA barcoding studies.

DNA barcoding allows the inclusion of all life stages in biodiversity assessments (Ekrem et al. 2010) and collections of chironomid pupal exuviae are commonly used for biological monitoring of water quality (Kranzfelder et al. 2015). However, DNA barcoding of chironomid pupal exuviae alone is not currently effective for species identification of unknown specimens. Chironomid pupal exuviae samples have low quantities of DNA and are easily contaminated by non-target species DNA or degraded by biotic or abiotic environmental factors, like microbial activity, temperature, or UV-light. Also, public reference libraries, like BOLD, are not yet sufficiently populated by reference sequences with species names. For Neotropical biodiversity and bioassessment studies to take advantage of the benefits that DNA barcoding offers, inventories and taxonomic studies that identify chironomids using morphology should include registration of DNA barcodes. If such studies simultaneously use both morphological and molecular identification techniques to identify all life stages of chironomids (e.g., Drayson et al. 2015, Montagna et al. 2016), gaps in the reference library can be filled, life stages associated, and morphological characteristics evaluated simultaneously.

Supplementary data

Supplementary data are available at *Journal of Insect Science* online.

References Cited

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403–410.

Anderson, A. M., and Ferrington, L. C. 2012. Time-efficiency of sorting Chironomidae surface-floating pupal exuviae samples from urban trout streams in Northeast Minnesota, USA. Fauna Norv. 31: 19–23.

Anderson, A. M., E. Stur, and T. Ekrem. 2013a. Molecular and morphological methods reveal cryptic diversity and three new species of Nearctic *Micropsectra* (Diptera: Chironomidae). Freshw. Sci. 32: 892–921.

Anderson, T., T. Ekrem, and P. S. Cranston. 2013b. 1. The larvae of the Holarctic Chironomidae (Diptera) – introduction, pp. 7–12. *In* T. Andersen, P. S. Cranston and J. H. Eplers (eds.), *The larvae of Chironomidae (Diptera) of the Holarctic region – keys and diagnoses*. Insect Systematics and Evolution, Supplement Lund, Sweden.

Artsdatabanken 2015. Artsnavnebasen. Norsk taksonomisk database (http://www.artsportalen.artsdatabanken.no/).

Barnes, M. A., C. R. Turner, C. L. Jerde, M. A. Renshaw, W. L. Chadderton, and D. M. Lodge. 2014. Environmental conditions influence eDNA persistence in aquatic systems. Environ. Sci. Technol. 48: 1819–27.

Bista, I., G. R. Carvalho, K. Walsh, M. Seymour, M. Hajibabaei, D. Lallias, M. Christmas, and S. Creer. 2017. Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of lake ecosystem biodiversity. Nat. Commun. 8: 14087.

Bouchard, R. W. and L. C. Ferrington. 2011. The effects of subsampling and sampling frequency on the use of surface-floating pupal exuviae to measure Chironomidae (Diptera) communities in wadeable temperate streams. Environ. Monit. Assess. 181: 205–223.

Brodin, Y., G. Ejdung, J. Strandberg, and T. Lyrholm. 2013. Improving environmental and biodiversity monitoring in the Baltic Sea using DNA barcoding of Chironomidae (Diptera). Mol. Ecol. Resour. 13: 996–1004.

Brundin, L. 1949. Chironomiden und andere Bodentiere der südschwedischen Urgebirgsseen. Ein Beitrag zur Kenntnis der bodenfaunistischen Charakterzüge schwedischer oligotropher Seen. pp. 1–914. Reports from the Institute of Freshwater Research, Drottningholm.

Carew, M. E., V. Pettigrove, and A. A. Hoffmann. 2005. The utility of DNA markers in classical taxonomy: using cytochrome oxidase I markers to differentiate Australian *Cladopelma* (Diptera: Chironomidae) midges. Ann. Entomol. Soc. Am. 98: 587–594.

Carew, M., V. Pettigrove, L. Metzeling, and A. Hoffmann. 2013. Environmental monitoring using next generation sequencing: rapid identification of macroinvertebrate bioindicator species. Front. Zool. 10: 45.

Cranston, P. S., N. B. Hardy, and G. E. Morse. 2012. A dated molecular phylogeny for the Chironomidae (Diptera). Syst. Entomol. 37: 172–188.

De La Rosa, C. L. 2015. Chironomids: a personal journey. Chironomus Newsl. Chiron. Res. 28: 30–35.

Drayson, N., P. S. Cranston, and M. N. Krosch. 2015. Taxonomic review of the chironomid genus *Cricotopus* v.d. Wulp (Diptera: Chironomidae) from Australia: keys to males, females, pupae and larvae, description of ten new species and comments on *Paratrichocladius* Santos Abreu. Zootaxa 3919: 1–40.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32: 1792–1797.

Eichmiller, J. J., S. E. Best, and P. W. Sorensen. 2016. Effects of temperature and trophic state on degradation of environmental DNA in lake water. Environ. Sci. Technol. 50: 1859–67.

Ekrem, T., E. Willassen, and E. Stur. 2007. A comprehensive DNA sequence library is essential for identification with DNA barcodes. Mol. Phylogenet. Evol. 43: 530–542.

Ekrem, T., E. Stur, and P. D. N. Hebert. 2010. Females do count: documenting Chironomidae (Diptera) species diversity using DNA barcoding. Org. Divers. Evol. 10: 397–408.

Epler, J. H. 1988. A reconsideration of the genus *Apedilum* Townes, 1945 (Diptera: Chironomidae). Spixiana Suppl. 14: 105–116.

Ferrington, L. C. 2008. Global diversity of non-biting midges (Chironomidae; Insecta-Diptera) in freshwater, pp. 447–455. *In* E. V. Balian, C. Lévêque, H. Segers and K. Martenss (eds.), *Freshwater animal diversity assessment*. Springer Netherlands, The Netherlands.

Ferrington, L. C. and W. P. Coffman. 2014. Differential efficiencies of dip-net sampling versus sampling surface-floating pupal exuviae in a biodiversity survey of Chironomidae. Chironomus Newsl. Chiron. Res. 27: 31–40.

Fittkau, E. J. 1962. Die Tanypodinae (Diptera: Chironomidae). (Die Tribus Anatopyiini, Macropelopiinae and Pentaneurini). Abh. Larvalsyst. Insekten 6: 1–453.

Hebert, P. D. N., A. Cywinska, S. L. Ball, and J. R. Dewaard. 2003. Biological identifications through DNA barcodes. Proc. R. Soc. Lond. B Biol. Sci. 270: 313–321.

Hebert, P. D. N., E. H. Penton, J. M. Burns, D. H. Janzen, and W. Hallwachs. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proc. Natl. Acad. Sci. USA. 101: 14812–14817.

Jackson, J. K., J. M. Battle, B. P. White, E. M. Pilgrim, E. D. Stein, P. E. Miller, and B. W. Sweeney. 2014. Cryptic biodiversity in streams: a comparison of macroinvertebrate communities based on morphological and DNA barcode identifications. Freshw. Sci. 33: 312–324.

Jacobsen, R. E. 2008. A key to the pupal exuviae of the midges (Diptera: Chironomidae) of everglades National Park, Florida. *Scientific investigations report*. p. 119. U. S. Geological Survey, Fort Lauderdale, FL.

Kavanaugh, R. G., A. T. Egan, and L. C. Ferrington Jr. 2014. Factors affecting decomposition rates of chironomid (Diptera) pupal exuviae. Chironomus Newsl. Chiron. Res. 27: 16–24.

Kranzfelder, P., A. M. Anderson, A. T. Egan, J. E. Mazack, R. W. Bouchard, and L. C. Ferrington. 2015. Use of Chironomidae (Diptera) surface-floating pupal exuviae as a rapid bioassessment protocol for water bodies. J. Vis. Exp. 101: e52558.

Kranzfelder, P., T. Ekrem, and E. Stur. 2016. Trace DNA from insect skins: a comparison of five extraction protocols and direct PCR on chironomid pupal exuviae. Mol. Ecol. Resour. 16: 353–363.

Krosch, M. N., and P. S. Cranston. 2012. Non-destructive DNA extraction from Chironomidae, including of fragile pupal exuviae, extends analysable collections and enhances vouchering. Chironomus Newsl. Chiron. Res. 25.

Langton, P. H. 1991. *A key to pupal exuviae of West Palearctic Chironomidae*. P. H. Langton, Huntingdon, Cambridgeshire.

Lin, X., E. Stur, and T. Ekrem. 2015. Exploring genetic divergence in a species-Rich insect genus using 2790 DNA barcodes. PLoS One 10: e0138993.

Lindegaard, C. 1995. Classification of water-bodies and pollution, pp. 385–404. *In* P. D. Armitage, P. S. Cranston and L. C. V. Pinders (eds.), *The Chironomidae: biology and ecology of non-biting midges*. Springer Netherlands, Dordrecht.

Montagna, M., V. Mereghetti, V. Lencioni, and B. Rossaro. 2016. Integrated taxonomy and DNA barcoding of alpine midges (Diptera: Chironomidae). PLoS One 11: e0149673.

Nation, J. L. 2008. *Insect physiology and biochemistry*, *2nd ed.* Boca Raton (FL), Taylor and Francis.

Nicacio, G. and L. Juen. 2015. Chironomids as indicators in freshwater ecosystems: an assessment of the literature. Insect. Conserv. Divers 8: 393–403.

Pauls, S. U., R. J. Blahnik, X. Zhou, C. T. Wardwell, and R. W. Holzenthal. 2010. DNA barcode data confirm new species and reveal cryptic diversity in Chilean *Smicridea* (*Smicridea*) (Trichoptera: Hydropsychidae). J. North Am. Benthol. Soc. 29: 1058–1074.

Pilgrim, E. M., S. A. Jackson, S. Swenson, I. Turcsanyi, E. Friedman, L. Weigt, and M. J. Bagley. 2011. Incorporation of DNA barcoding into a large-scale biomonitoring program: opportunities and pitfalls. J. North Am. Benthol. Soc. 30: 217–231.

Pinder, L. C. V. 1986. Biology of freshwater Chironomidae. Annu. Rev. Entomol. 31: 1–23.

R Development Core Team 2016. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ratnasingham, S., and P. D. N. Hebert. 2007. BOLD: the barcode of life data system. Mol. Ecol. Notes 7: 355–364.

Raunio, J., and T. Muotka. 2005. The use of chironomid pupal exuviae in river biomonitoring: the importance of sampling strategy. Arch. Hydrobiol. 164: 529–545.

Raunio, J., R. Paavola, and T. Muotka. 2007. Effects of emergence phenology, taxa tolerances and taxonomic resolution on the use of the chironomid pupal exuvial technique in river biomonitoring. Freshw. Biol. 52: 165–176.

Resh, V. H. 2007. Which group is best? Attributes of different biological assemblages used in freshwater biomonitoring programs. Environ. Monit. Assess. 138: 131–138.

Rosenberg, D. M. and V. H. Resh. 1993. *Freshwater biomonitoring and benthic macroinvertebrates*. Chapman and Hall, New York, NY.

Sæther, O. A. 1981. Orthocladiinae (Diptera: Chironomidae) from the British West Indies, with descriptions of *Antillocladius* n.gen., *Lipurometriocnemus* n.gen., *Compterosmittia* n.gen. and *Diplosmittia* n.gen. Entomol. Scand. Suppl. 16: 1–46.

Sæther, O. A. and M. Spies. 2013. Fauna Europaea: Chironomidae. *In* P. Beck and T. Papes (eds.), *Fauna Europaea: Diptera: Nematocera*. Fauna Europaea 2.6.2 (https://fauna-eu.org).

Sinclair, C. S. and S. E. Gresens. 2008. Discrimination of *Cricotopus* species (Diptera: Chironomidae) by DNA barcoding. Bull. Entomol. Res. 98: 555–563.

Spies, M. and F. Reiss. 1996. Catalog and bibliography of Neotropical and Mexican Chironomidae (Insecta, Diptera). Spixiana Suppl. 22: 61–119.

Spies, M., T. Andersen, J. H. Epler, and C. N. Watson. 2009. Chironomidae (non-biting midges), pp. 437–480. *In* B. V. Browns (ed.) *Manual of Central American Diptera*. NRC Research Press, Ottawa.

Stein, E. D., B. P. White, R. D. Mazor, J. K. Jackson, J. M. Battle, P. E. Miller, E. M. Pilgrim, and B. W. Sweeney. 2014. Does DNA barcoding improve performance of traditional stream bioassessment metrics? Freshw. Sci. 33: 302–311.

Strickler, K. M., A. K. Fremier, and C. S. Goldberg. 2015. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biol. Conserv. 183: 85–92.

Stur, E. and A. Borkent. 2014. When DNA barcoding and morphology mesh: Ceratopogonidae diversity in Finnmark, Norway. ZooKeys 95–131.

Stur, E. and T. Ekrem. 2011. Exploring unknown life stages of Arctic Tanytarsini (Diptera: Chironomidae) with DNA barcoding. Zootaxa 2743: 27–39.

Sweeney, B. W., J. M. Battle, J. K. Jackson, and T. Dapkey. 2011. Can DNA barcodes of stream macroinvertebrates improve descriptions of community structure and water quality? J. North Am. Benthol. Soc. 30: 195–216.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. 30: 2725–2729.

Webb, J. M., L. M. Jacobus, D. H. Funk, X. Zhou, B. Kondratieff, C. J. Geraci, R. E. Dewalt, D. J. Baird, B. Richard, I. Phillips, et al. 2012. A DNA barcode library for North American Ephemeroptera: progress and prospects. PLoS One 7: e38063.

Wiedenburg, S., H. F. Mendes, M. Pepinelli, and S. Trivinho-Strixino. 2009. Review of the genus *Onconeura* Andersen et Sæther (Diptera: Chironomidae), with the description of four new species from Brazil. Zootaxa 2265: 1–26.

Wiederholm, T. 1986. *Chironomidae of the Holarctic region. Part 2. Pupae*, Entomologica Scandinavica Supplement 28, Sandby, Sweden, 482 pp.

Wilson, R. S. and L. P. Ruse. 2005. *A guide to the identification of genera of chironomid pupal exuviae occurring in Britain and Ireland (including common genera from Northern Europe) and their use in monitoring lotic and lentic fresh waters*. Freshwater Biological Association, Ambleside, Cumbria, UK.

Wülker, W. F. 1991. Chromosomal, morphological and biological differences between *Chironomus tenuistylus* Brundin and *C. longistylus* Goetghebuer (Diptera: Chironomidae) in Fennoscandia and the USA. Entomol. Scand. 22: 231–240.

Zhou, X., K. M. Kjer, and J. C. Morse. 2007. Associating larvae and adults of Chinese Hydropsychidae caddisflies (Insecta: Trichoptera) using DNA sequences. J. North Am. Benthol. Soc. 26: 719–742.

Zhou, X., S. J. Adamowicz, L. M., Jacobus, R. E. Dewalt, and P. D. Hebert. 2009. Towards a comprehensive barcode library for arctic life – Ephemeroptera, Plecoptera, and Trichoptera of Churchill, Manitoba, Canada. Front. Zool. 6: 1–9.