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RESOURCE ARTICLE

The potential of aquatic bloodfeeding and nonbloodfeeding leeches as a tool for iDNA characterisation

Christina Lynggaard[1] | Alejandro Oceguera-Figueroa[2] | Sebastian Kvist[3,4] | M. Thomas P. Gilbert[1,5] | Kristine Bohmann[1]

[1]Section for Evolutionary Genomics, The Globe Institute, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen K, Denmark

[2]Laboratorio de Helmintología, Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Ciudad de México, México

[3]Department of Natural History, Royal Ontario Museum, Toronto, Ontario, Canada

[4]Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario, Canada

[5]University Museum, NTNU, Trondheim, Norway

Correspondence
Christina Lynggaard, Section for Evolutionary Genomics, The Globe Institute, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen K, Denmark.
Email: christina.lynggaard@sund.ku.dk

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Abstract

Leeches play important roles in food webs due to their abundance, diversity and feeding habits. Studies using invertebrate-derived DNA (iDNA) extracted from leech gut contents to target vertebrate DNA have focused on the Indo-Pacific region and mainly leveraged the leech family Haemadipsidae, composed of bloodfeeding terrestrial leeches, while predatory, fluid/tissue-feeding and aquatic bloodfeeding species have been largely disregarded. While there is some general knowledge regarding the taxonomic groups that leeches prefer to feed on, detailed taxonomic resolution is missing and, therefore, their potential use for monitoring animals is unknown. In this study, 116 leeches from 12 species (six families) and spanning the three feeding habits were collected in Mexico and Canada. We used DNA metabarcoding to investigate their diet and assess their potential use for biodiversity monitoring. We detected vertebrates from five orders including fish, turtles and birds in the diet of aquatic bloodfeeding leeches; eight invertebrate orders of annelids, arthropods and molluscs in leeches that feed on body fluids and tissues; and 10 orders of invertebrates belonging to Arthropoda and Annelida, as well as one vertebrate and one parasitic nematode, in predatory leeches. These results show the potential use of iDNA from aquatic bloodfeeding leeches for retrieving vertebrate taxa, and from predatory and fluid-feeding leeches for invertebrates. Our study provides information about the dietary range of freshwater leeches and one terrestrial leech and contributes proof-of-concept for the use of these leeches for animal monitoring, expanding our knowledge of the use of iDNA from leech gut contents to North America.

KEYWORDS
diet, iDNA, leeches, metabarcoding

1 | INTRODUCTION

Leeches (Hirudinea sensu Tessler, de Carle, et al., 2018) are found on all continents except Antarctica (Sket & Trontelj, 2008). There are around 900 known leech species (Magalhães et al., 2021) and these have varying and important roles in food webs; they can be ectoparasitic (or, on occasion, endoparasitic: Mann & Tyler, 1963), predatory, intermediate and final hosts for parasites (Sawyer, 1986), vectors of hemogregarine and trypanosome blood parasites (Barta & Desser, 1989; Siddall & Desser, 1991, 2001) and

serve as the primary diet for several fish species across the globe (e.g., Sawyer, 1986; Young & Spelling, 1986). Leeches are also found in terrestrial and marine habitats, yet most of the known leeches inhabit freshwater ecosystems (Sawyer, 1986; Sket & Trontelj, 2008). Their global distribution, coupled with their resilience to pollution have allowed leeches to become important indicator taxa for habitat quality in freshwater environments (Cortelezzi et al., 2018; Phillips et al., 2020).

Besides bloodfeeding (parasitism), the feeding habits of leeches range from macrophagy (i.e., predation of invertebrates) to liquidosomatophagy (i.e., feeding on internal fluids and soft tissues of invertebrates, mainly molluscs and oligochaetes) (Sawyer, 1986). While there is some general knowledge regarding the overall taxonomic groups that leeches prefer to feed on, more detailed taxonomic resolution of the diet of some species is still missing. For example, in predatory leeches, members of the freshwater families Erpobdellidae and Haemopidae are known to ingest oligochaetes, other invertebrates and even other hirudineans including members of their own species (Darabi & Malek, 2011; Kutschera & Wirtz, 2001; Simon & Barnes, 1996). However, information is lacking about preference to a specific taxonomic group and the prevalence of cannibalism. The paucity of dietary information extends to aquatic bloodfeeding leeches, such as those of the families Glossiphoniidae and Macrobdellidae, which are known to feed on blood of vertebrates, but whose specific dietary preferences are largely unknown. Whereas members of the genus *Placobdella* (family Glossiphoniidae) are primarily considered parasites of turtles (Sawyer, 1972), some species within the genus have been shown to feed on amphibians and birds and some will readily feed on humans (personal observation). Species of the genus *Haementeria* (family Glossiphoniidae) are known to be parasites of mammals but other vertebrate hosts have been recorded, indicating a flexible diet. One species in the family Macrobdellidae, *Macrobdella decora* (Say, 1824), has been found to feed on vertebrate blood, as well as on amphibian eggs (Trauth & Neal, 2004; Turbeville & Briggler, 2003). More information is needed to ascertain if this behaviour is opportunistic, or a preference. Similarly, members of the family Piscicolidae seem to show a preference for fish blood, including both Chondrichthyes and Osteichthyes; however, some isolated records of piscicolid leeches feeding on molluscs and crustaceans have been reported (López-Peraza et al., 2017; Nakano, 2017) indicating a broader dietary range. For terrestrial leeches (including members of the families Xerobdellidae, Cylicobdellidae, Americobdellidae and Haemopidae), detailed information is also lacking; for example, the diet of the Mexican terrestrial leech *Diestecostoma mexicana* (Baird, 1869) is still completely unknown. The major exception to this is the family Haemadipsidae, for which the diet is relatively well understood through studies on invertebrate-derived DNA, or iDNA, which aim to monitor vertebrates through DNA detection in leech bloodmeals (Drinkwater et al., 2020; Fahmy et al., 2019, 2020; Schnell et al., 2012, 2018; Nguyen et al., 2021; Siddall et al., 2019; Tessler et al., 2018; Weiskopf et al., 2018). As such, the dearth of data extends across the leech phylogeny, rather than being explicit to certain taxa. In this context, it is important to note that contemporary molecular phylogenetic studies find that bloodfeeding is the ancestral feeding mode in leeches (Siddall et al., 2016; Tessler, de Carle, et al., 2018) with several independent losses of the behaviour in only distantly related groups. The main driver of this assertion is the fact that members of both proboscis-bearing leeches (orders Oceanobdelliformes and Glossiphoniformes) at the base of the leech phylogeny, as well as nonproboscis-bearing species (order Hirudiniformes) in derived parts of the tree, exhibit bloodfeeding as the main feeding mode.

Traditional methods for studying leech diets have relied on direct observations of the leeches feeding on other animals (Darabi & Malek, 2011; Kutschera, 2003), or examining the gut contents (Sawyer, 2019; Toman & Dall, 1997). However, diet information can be difficult to obtain when working with free-living leeches that are not caught while feeding and that may have already partially digested the ingested food, or when dealing with bloodfeeding and fluid/tissue-feeding leech taxa as the diets will lack morphological characteristics. This has paved the way for studies of leech diets under experimental settings (Darabi & Malek, 2011; Gaudry et al., 2010). In recent years, the use of molecular methods has improved our knowledge on the role of leeches in trophic networks and has greatly increased our understanding of their diets. The emerging field of iDNA has therefore contributed to the knowledge of leech diets and leech-derived iDNA is now used as a complementary tool in studies of vertebrate community composition (Schnell et al., 2012; Ji et al., 2020). Such studies have focused almost exclusively on terrestrial bloodfeeding leeches of the family Haemadipsidae (order Hirudiniformes) and have been leveraged to detect vertebrates across their geographic distribution (see Borda et al., 2008), including Asia (Vietnam, Laos, Malaysia, Japan), Africa (Madagascar) and Oceania (Australia and Tasmania) (Abrams et al., 2019; Drinkwater et al., 2019; Fahmy et al., 2019; Ji et al., 2020; Morishima et al., 2020; Nguyen et al., 2021; Schnell et al., 2012, 2018; Tilker et al., 2020). If iDNA from leeches is to be used for biodiversity monitoring elsewhere in the world, the dietary range of members of all other families, including aquatic bloodfeeding, predatory and fluid/tissue-feeding species, will also need to be assessed. Only two previous iDNA studies have investigated iDNA from freshwater bloodfeeding leeches. The first study focused on a single leech species (*Haementeria acuecueyetzin* Oceguera-Figueroa, 2008) that could be inferred to actively feed on the Antillean manatee (*Trichechus manatus* Linnaeus, 1758) in Mexico, through sequencing of the leech bloodmeal (Pérez-Flores et al., 2016). The second study took a more all-encompassing approach to understanding the diet of the European medicinal leech (*Hirudo medicinalis* Linnaeus, 1758) and also leveraged this dietary information to pinpoint the geographic area where the leeches had been collected (Williams et al., 2020). In addition, Kvist et al., (2016) used gut-content-based DNA barcoding to identify the shark host for a specimen of the marine leech *Pontobdella macrothela* Schmarda, 1861 (family Piscicolidae) in order to infer that this species feeds on sharks. However, larger scale assessments of dietary taxa for most leeches, including also predatory and fluid/tissue-feeding forms, are still lacking, which prevents assessment of their potential as iDNA vertebrate monitoring tools. In this

study, we aimed to (i) provide insights into the dietary range of aquatic bloodfeeding, fluid/tissue-feeding, and predatory leeches collected in Mexico and Canada, in addition to elucidating the diet of a terrestrial leech *D. mexicana*; and (ii) assess the potential use of these leeches as biodiversity monitoring tools. To achieve this, we applied metabarcoding to DNA extracted from the gut contents of several freshwater and terrestrial leeches from six different families. The analysed leeches span all three feeding modes: bloodfeeding, predatory and fluid/tissue-feeding.

2 | MATERIALS AND METHODS

2.1 | Sample collection

A total of 116 leech specimens (Clitellata: Hirudinea) from the families Glossiphoniidae, Piscicolidae, Erpobdellidae, Macrobdellidae, Haemopidae and Xerobdellidae were collected in 2015 and 2018 in different localities in Mexico and Canada (Figure S1). Leech specimens were selected based on their phylogenetic position in order to include a broad swath of leech diversity. It is important to note that several of the families are only very distantly related to each other. In Mexico, leeches were collected from eight freshwater localities and one terrestrial forest habitat (Table 1, Supporting Information). In Canada, leeches were collected from two separate freshwater localities in Ontario. The collection methods varied for leeches caught in different habitats and with different feeding habits. Nonbloodfeeding freshwater leeches were hand collected from plants, logs and other submerged debris and the terrestrial leech, *Diestecostoma mexicana*, from under rocks on soil. Bloodfeeding freshwater leeches were collected directly from their host (in the case of *Myzobdella patzcuarensis* [Caballero, 1941] from fish found at the market), from under rocks immersed in water or by removing any attached leeches from exposed skin. Importantly, great care was taken to make sure that bloodfeeding leeches were collected before feeding on the collector's blood (but see Results for one case of human DNA inside of a single leech).

Prior to DNA extraction, leeches were identified using specialized literature (Klemm, 1985; Sawyer, 1986; Oceguera-Figueroa, 2020). Leeches were sterilised with a 10% bleach solution after both ends of the body were closed with tweezers to avoid bleach entering the leech and then rinsed with distilled water and stored individually in absolute ethanol in 2 ml Eppendorf or 15 ml Falcon tubes. Specimens were dissected under a stereomicroscope for the removal of the crop and intestine. However, due to their small size, for two individuals of *Helobdella temiscoensis* (Salas-Montiel et al., 2014), the crop and intestine could not be separated so the entire digestive tract was removed as one piece; for the remaining *Helobdella* species and two specimens of *Placobdella mexicana* from Hacienda Blanca, no dissection was possible also due to their small size, so the entire body was used for DNA extraction (Table 1). When dissecting *Erpobdella obscura*, larval nematodes were found encysted in the gastrointestinal tissue, that is, not in the intestine itself, and were therefore removed and stored in 70% ethanol for further analyses. Crops, entire digestive tracts and complete leeches were kept in absolute ethanol at −20°C until further DNA extraction.

2.2 | DNA metabarcoding

DNA was extracted using the spin-column protocol for animal tissues from the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions with slight modifications. First, the samples were subjected to three freeze-heat rounds of −80°C for 15 min and 50°C for 30 min prior to addition of Proteinase K. This was done in order to increase the rupture of bacterial membranes (Lever et al., 2015), in the anticipation that the DNA extracts might be used in future microbiome analyses. Second, to increase DNA yield, an incubation step of 37°C for 15 min was added after the addition of 100 µl of the elution buffer. A negative extraction control was included for every 20 samples.

The mlCOIintF (forward 5′-GGWACWGGWTGAACWGTWTAYCCYCC -3′) and jgHCO2198 (reverse 5′-TAIACYTCIGGRTGICCRAARAAYCA-3′) metabarcoding primer set was used to PCR amplify c. 313 base pairs of the mitochondrial cytochrome *c* oxidase subunit I (COI) barcode marker (Geller et al., 2013; Leray et al., 2013), as it is a universal eukaryote primer and expected to amplify both diet and host DNA, thereby enabling molecular verifications of the leech identities. To allow multiplexing, nucleotide tags were added to the 5′ ends of both forward and reverse primers (Binladen et al., 2007). Specifically, tags consisted of a total of 7–8 nucleotides of which six nucleotides were nucleotide tags and 1–2 nucleotides at the 5′ end of the tag were added to increase complexity on the flow cell during sequencing (De Barba et al., 2014).

Prior to the metabarcoding PCR amplifications, a dilution series (1:5 and 1:10) of a subset of the DNA extracts, and positive and negative controls, were screened using SYBR Green qPCR with the aim to determine optimal cycle number for the subsequent PCRs, screen for contamination in the negative controls and in the samples and calculate the maximum DNA template in which PCR inhibitors would not distort amplification. The 20 µl reaction consisted of 1 µl DNA template, 1 µl of SYBR Green/ROX solution (one part SYBR Green I nucleic acid gel stain [S7563] [Invitrogen], four parts ROX Reference Dye [12223– 012] [Invitrogen] and 2000 parts high-grade DMSO), 0.75 U AmpliTaq Gold, 1× Gold PCR Buffer and 2.5 mM $MgCl_2$ (all from Applied Biosystems), 0.6 µM each of 5′ nucleotide tagged forward and reverse primers, 0.2 mM dNTP mix (Invitrogen) and 0.5 mg/ml bovine serum albumin (BSA). The thermocycling profile was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 51°C for 30 s and 72°C for 60 s, followed by a dissociation curve. The amplification plots indicated that the use of a 1:5 diluted DNA extract and running 27 cycles was optimal across samples to be used in the following metabarcoding PCR amplification.

Tagged PCRs were subjected to three PCR replicates for each of the 116 DNA extracts, all negative extraction controls and two positive controls (DNA extracted from lion [*Panthera leo*] and giraffe [*Giraffa camelopardalis*]). Further, negative controls were included for every seven DNA extracts. PCR amplifications were performed

TABLE 1 Leeches collected in Mexico (MX) and Canada (CA) grouped by family, with information regarding their known diet, the environment in which they live, number of individuals collected, body part used for DNA extraction and the locality where they were collected

Suborder	Family	Species	Known diet	Habitat	No. individuals	Body part	Locality
Glossiphoniiformes	Glossiphoniidae	*Placobdella mexicana*	Bloodfeeding	Freshwater	7	Body	Hacienda Blanca, Querétaro, MX
					8	Body	La Vega, Jalisco, MX
		Haementeria officinalis	Bloodfeeding	Freshwater	4	Crop	Coroneo, Guanajuato, MX
		Helobdella elongata	Fluid/tissue-feeding	Freshwater	8	Body	La Vega, Jalisco, MX
		Helobdella adiastola	Fluid/tissue-feeding	Freshwater	4	Body	Hacienda Blanca, Querétaro, MX
					9	Body	La Vega, Jalisco, MX
					3	Body	Temixco, Morelos, MX
		Helobdella ocimulcensis	Fluid/tissue-feeding	Freshwater	3	Body	Hacienda Blanca, Querétaro, MX
					2	Body	Temixco, Morelos, MX
		Helobdella temiscoensis	Fluid/tissue-feeding	Freshwater	3	Body	Temixco, Morelos, MX
					2	Crop and intestine	Temixco, Morelos, MX
Oceanobdelliformes	Piscicolidae	*Myzobdella patzcuarensis*	Bloodfeeding	Freshwater	10	Body	Pátzcuaro, Michoacán, MX
Erpobdelliformes	Erpobdellidae	*Erpobdella ochoterenai*	Predatory	Freshwater	5	Crop	Los Dinamos, Mexico City, MX
					10	Crop	Pátzcuaro, Michoacán, MX
					11	Crop	La Vega, Jalisco, MX
		Erpobdella obscura	Predatory	Freshwater	5	Crop	Unnamed pond, Ontario, CA
Hirudiniformes	Macrobdellidae	*Macrobdella decora*	Bloodfeeding	Freshwater	4	Crop	Clear Lake, Ontario, CA
	Haemipidae	*Haemopis caballeroi*	Predatory	Freshwater	10	Crop	Temixco, Morelos, MX
	Xerobdellidae	*Diestecostoma mexicana*	Unkwnown	Terrestrial	8	Crop	Los Dinamos, Mexico City, MX

with nonmatching nucleotide tags (e.g., F1–R2, F1–R3, F1–R4…) to allow for more amplicons to be pooled together and reduce laboratory costs (Schnell et al., 2015). The 20 µl reactions were set up as described for the qPCR above but omitting SYBR Green/ROX and replacing the dissociation curve with a final extension time of 72°C for 7 min. Amplified PCR products were visualized on 2% agarose gels with GelRed against a 100 bp ladder. All negative controls appeared negative and all DNA extracts and positive controls showed successful amplification. PCR products of DNA extracts, including negative and positive controls carrying different nucleotide tag combinations, were pooled resulting in three amplicon pools: one pool per replicate.

Amplicon pools were purified with MagBio HiPrep beads (LabLife) using a 1.6x bead to amplicon pool ratio and eluted in 35 µl EB buffer (Qiagen). Purified amplicon pools were built into sequence libraries with the TagSteady protocol to avoid tag-jumping (Carøe & Bohmann, 2020). Libraries were purified with a 0.8× bead to library ratio and eluted in 30 µl EB buffer and qPCR quantified using the NEBNext Library Quant Kit for Illumina (New England BioLabs Inc.). Purified libraries were pooled equimolarly according to the qPCR results and sequenced at the GeoGenetics Sequencing Core, University of Copenhagen, Denmark. Libraries were sequenced using 250 bp paired-end reads on an Illumina MiSeq sequencing platform using v2 chemistry, aiming at 25,000 paired reads per PCR replicate.

2.3 | Data processing

Illumina adapters and low quality reads were removed and paired reads with a "minalignment" score of 50 and "minlength" score of 100 were merged using AdapterRemoval v2.2.2 (Schubert et al., 2016). Sequences were sorted within each amplicon library based on primer and tag sequences using Begum (https://github.com/shyamsg/Begum), allowing for two mismatches to primer sequences and no mismatches to tag sequences. Begum was further used to filter sequences across the three PCR replicates for each sample. Filtering parameters were set according to the sequenced negative and positive controls and sequences present in a minimum of two out of three PCR replicates and with a minimum of 25 copies were retained. Sequences were clustered into operational taxonomic units (OTUs) with 97% similarity using SUMACLUST (https://git.metabarcoding.org/obitools/sumaclust/wikis/home/). The LULU algorithm (Frøslev et al., 2017) was used with default settings to detect and remove erroneous OTUs.

Taxonomic assignment to the OTU sequences, including the leech and the gut contents, was performed using BLASTn against the NCBI nonredundant (nr) sequence database (www.ncbi.nlm.nih.gov/, 2020) and the output was imported into MEGAN Community Edition v6.12.7 (Huson et al., 2016) using a weighted LCA algorithm with 80% coverage, retaining only the hits within the 10% of the best score (top percent) and a using minimum score of 50 for the alignment (min score). Genus and species information was complemented with data retrieved from BOLD (Barcode of Life Database, http://www.boldsystems.org/). OTUs were assigned to species-level taxa if they had a percentage of identity higher than 99% to a reference sequence and matching to only one species. It should be noted that, during dissection, entire leeches were found in the crop of *Erpobdella* species, including individuals of their own species. Because of this, if detecting more than one OTU assigned to *Erpobdella*, OTUs with the highest number of reads were considered as sequences belonging to the predator and the remainder were considered gut contents. OTUs that could not be identified to any taxonomic level were discarded from further analyses.

3 | RESULTS

A total of 10,209,408 raw sequence reads were generated from the amplicon libraries. After filtering steps (Table S1), 3,824,526 reads were retained for downstream analyses and a total of 69 OTUs were detected in the analysed leech gut contents. None of the OTUs found in the positive or negative controls were found in the samples, indicating that there were no tag-jumps (Schnell et al., 2015) or cross contamination. In all cases, the morphological identification of the analysed leeches was confirmed by the DNA analysis. These leeches covered six families: Glossiphoniidae, Piscicolidae, Erpobdellidae, Macrobdellidae, Haemopidae and Xerobdellidae and, within these, eight genera. Of the 57 leeches, 21 represented bloodfeeding species, 18 were fluid/tissue-feeding (liquidosomatophagous) species and 18 were predatory (macrophagous) species. Bloodfeeding leeches are represented by four genera from three suborders (*sensu* Tessler, de Carle, et al., 2018): *Myzobdella* (suborder Oceanobdelliformes), *Macrobdella* (suborder Hirudiniformes) and *Haementeria* + Placobdella (suborder Glossiphoniformes). Predatory leeches are represented by three genera from two suborders: *Erpobdella* (suborder Erpobdelliformes), *Haemopis* (suborder Hirudiniformes) and *Diestecostoma* (suborder Hirudiniformes), each of these represents an independent transition from a bloodfeeding ancestor to predatory descendants (Borda & Siddall, 2004; Siddall et al., 2016; Tessler, de Carle, et al., 2018). Finally, fluid/tissue-feeding leeches included in this study represent a single genus, *Helobdella* (suborder Glossiphoniformes) that has an inferred bloodfeeding ancestor (Tessler, de Carle, et al., 2018). In addition to the identification of the leech taxa, both vertebrate and invertebrate DNA was found inside the various leeches.

3.1 | Animal taxa detected in leech gut content

From the 116 analysed leeches, gut content information was obtained from 57 (49.13%) leech individuals. A total of 69 taxa were detected in the leech intestines and crops and these spanned five phyla and 15 orders (Figure 1; Table 2). These taxa are all known to inhabit the geographical area where the leeches were collected (Supporting Information); for example, the drain fly *Psychoda alternata* (Say, 1824) was detected in a leech collected in a very polluted water body in Temixco, Mexico. In addition, the diet taxa varied

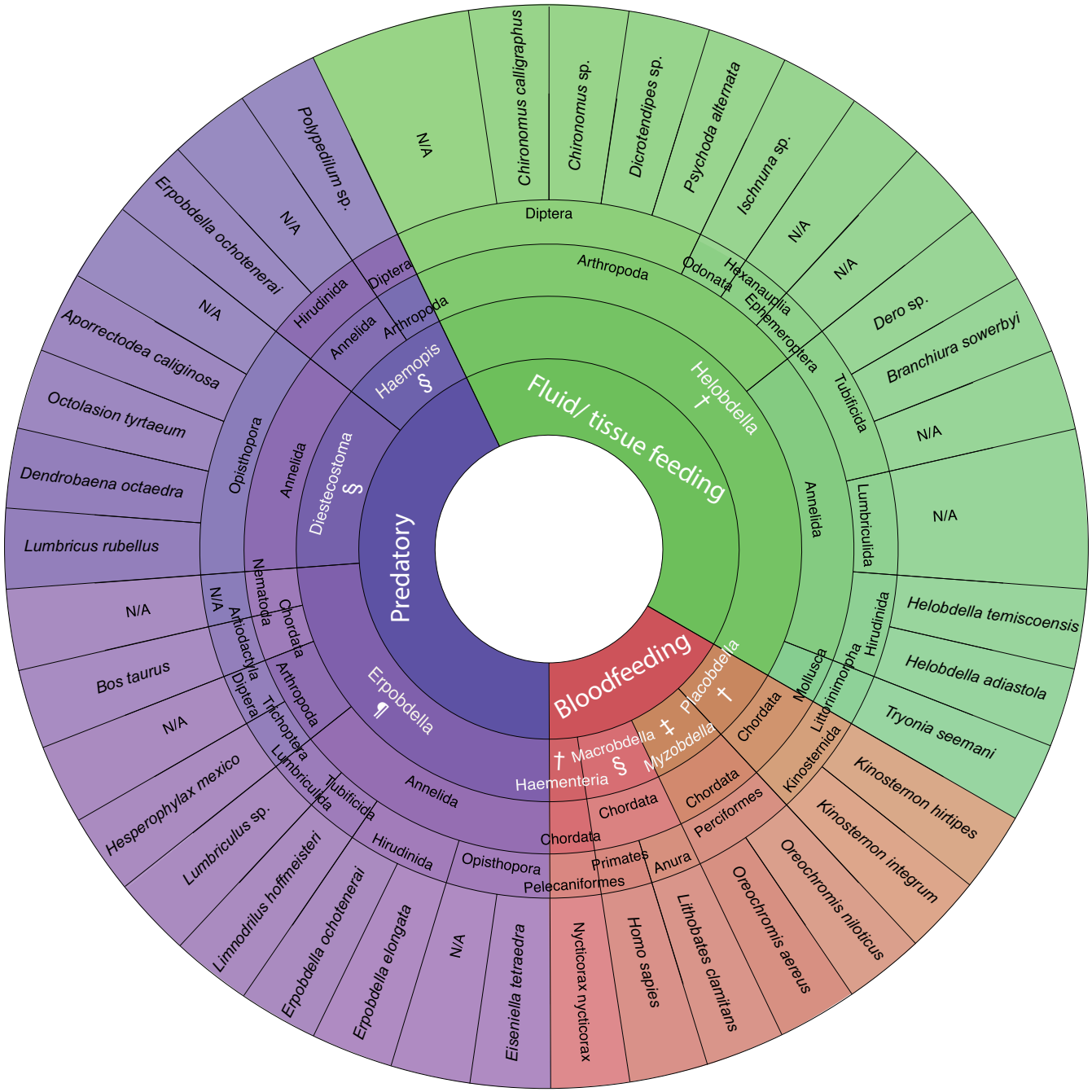

FIGURE 1 Taxa detected using DNA metabarcoding of the gut content of leeches collected in Mexico and Canada representing three feeding styles. Feeding habits of the leeches and their genus-level identifications are shown in white font, while detected dietary taxa are shown in black font. The suborder to which the leeches belong to is marked in †Glossiphoniiformes, ‡Oceanobdelliformes, § Hirudiniformes and ¶Erpobdelliformes. The taxonomic identification of the diet includes phylum-, order- and species-level assignment. N/A indicates that taxonomic information at order- or species-level could not be obtained. Data shown as presence and absence. Figure created using a Krona chart (Ondov et al., 2011)

greatly in body-size (from small chironomid midges to the large black-crowned night heron *Nycticorax nycticorax* [Linnaeus, 1758]), habitats (from terrestrial earthworms of the family Lumbricidae to aquatic crustaceans of the family Cyclopidae) and we also detected a parasitic nematode from the class Chromadorea; note that this taxon was not considered part of the diet since it was found encysted in the gastric tissues, but is considered part of the gut content. Out of the 68 detected ingested taxa (excluding the nematode), 24 could be assigned to species-level, whereas six could be assigned to genus-level, six to family-level and four to order-level taxa.

The taxa detected in the 21 bloodfeeding leeches exclusively belong to the phylum Chordata without any match to invertebrate DNA. The detected chordates present different lifestyles; semi-aquatic (such as mud turtles of the genus *Kinosternon*), volant (such

TABLE 2 Taxa detected in the digestive tract of leeches collected in Mexico (MX) and Canada (CA), including their taxonomic classification, common name, and ratio of detection versus number of individuals analysed. Leeches are separated by known feeding habits and locality

	Taxa detected						
Locality	Phylum	Class	Order	Family	Species	Common name	Number of leeches with detections
Bloodfeeding leech species							
Placobdella mexicana							
Hacienda Blanca, MX	Chordata	Testudines	Kinosternida	Kinosternonidae	*Kinosternon hirtipes*	Rough-footed mud turtle	6/7
La Vega, MX	Chordata	Testudines	Kinosternida	Kinosternonidae	*Kinosternon integrum*	Mexican mud turtle	2/8
Haementeria officinalis							
Coroneo, MX	Chordata	Aves	Pelecaniformes	Ardeidae	*Nycticorax nycticorax*	Black-crowned night-heron	1/4
Myzobdella patzcuarensis							
Pátzcuaro, MX	Chordata	Teleostei	Perciformes	Cichlidae	*Oreochromis niloticus*	Nile tilapia	8/10
					Oreochromis aereus	Blue tilapia	2/10
Macrobdella decora							
Ontario, CA	Chordata	Amphibia	Anura	Ranidae	*Lithobates clamitans*	Bronze frog	2/4
	Chordata	Mammalia	Primates	Hominidae	*Homo sapiens*	Human	1/4
Fluid/tissue-feeding leech species							
Helobdella adiastola							
La Vega, MX	Annelida	Clitellata	Lumbriculida	N/A	N/A	N/A	1/9
	Arthropoda	Insecta	Diptera	Chironomidae	*Chironomus calligraphus*	Chironomid midges	5/9
					Chironomus sp.	Chironomid midges	1/9
	Mollusca	Gastropoda	Littorinimorpha	Cochliopidae	*Tryonia seemani*	N/A	1/9
Hacienda Blanca, MX	Arthropoda	Insecta	Odonata	Coenagrionidae	*Ischnuna* sp.	N/A	1/4
			Diptera	Chironomidae	*Dicrotendipes* sp.	N/A	1/4
			Diptera	Chironomidae	N/A	N/A	1/4
		Hexanauplia	Cyclopoida	Cyclopidae	N/A	N/A	1/4
	Annelida	Clitellata	Lumbriculida	N/A	N/A	N/A	1/4
Temixco, MX	Arthropoda	Insecta	Diptera	Psychodidae	*Psychoda alternata*	Drain fly	1/3
				Culicidae	N/A	Mosquitoes	1/3
Helobdella elongata							
La Vega, MX	Annelida	Clitellata	Tubificida	Naididae	*Dero* sp.	N/A	1/8
					Branchiura sowerbyi	N/A	1/8
			N/A	N/A	N/A	N/A	2/8

(Continues)

TABLE 2 (Continued)

Locality	Taxa detected						Number of leeches with detections
	Phylum	Class	Order	Family	Species	Common name	
Helobdella socimulcensis							
Temixco, MX	Annelida	Clitellata	Hirudinida	Glossiphoniidae	*Helobdella temiscoensis*	N/A	1/2
	Arthropoda	Insecta	Ephemeroptera	Baetidae	N/A	Mayflies	1/2
Helobdella temiscoensis							
Temixco, MX	Annelida	Clitellata	Hirudinida	Glossiphoniidae	*Helobdella adiastola*	N/A	1/5
Predatory leech species							
Erpobdella ochotenerai							
Los Dinamos, MX	Arthropoda	Insecta	Trichoptera	Limnephilidae	*Hesperophylax mexico*	N/A	1/5
	Annelida	Clitellata	Tubificida	Naididae	*Limnodrilus hoffmeisteri*	Red worm	1/5
			Opisthopora	Lumbricidae	*Eiseniella tetraedra*	Squaretail worm	1/5
Pátzcuaro, MX	Annelida	Clitellata	Opisthopora	N/A	N/A	N/A	1/10
			Lumbriculida	Lumbriculidae	*Lumbriculus* sp.	N/A	1/10
La Vega, MX	Annelida	Clitellata	Hirudinida	Glossiphoniidae	*Helobdella elongata*	N/A	1/11
					Erpobdella ochotenerai	N/A	2/11
Erpobdella obscura							
Ontario, CA	Chordata	Mammalia	Artiodactyla	Bovidae	*Bos taurus*	Domestic cattle	1/5
	Nematoda[a]	Chromadorea[a]	N/A[a]	N/A[a]	N/A[a]	N/A[a]	3/5[a]
	Arthropoda	Insecta	Diptera	Chironomidae	N/A	N/A	1/5
Haemopis caballeroi							
Temixco, MX	Annelida	Clitellata	Hirudinida	Erpobdellidae	*Erpobdella ochoterenai*	N/A	1/10
				N/A	N/A	N/A	1/10
	Arthropoda	Insecta	Diptera	Chironomidae	*Polypedilum* sp.	Chironomid midge	1/10
Diestecostoma mexicana							
Dinamos, MX	Annelida	Clitellata	Opisthopora	Lumbricidae	*Lumbricus rubellus*	Red marsh worm	3/8
					Dendrobaena octaedra	Octagonal-tail worm	2/8
					Octolasion tyrtaeum	Woodland white worm	1/8
					Aporrectodea caliginosa	Grey worm	2/8
					N/A	N/A	1/8

[a]Parasite detected, that is, not part of the diet of the leech.

as the black-crowned night-heron *N. nycticorax*), amphibious (such as the bronze frog *Lithobates clamitans*: Latreille, 1801) and aquatic (such as tilapias from the genus *Oreochromis*). Human DNA was detected in only one individual of *Macrobdella decora* and it probably belongs to one of the collectors (SK), although it could be from another human present in the area. The taxa detected in the 18 fluid and tissue feeding leeches were invertebrates from the phyla Annelida, Arthropoda and Mollusca, with the former two phyla showing the highest number of taxa (8 each) (Figure 1). The identified annelids, the mollusc and the arthropods all have aquatic stages during their lifecycle. Finally, for the 18 predatory leeches, the taxa detected belong to the phyla Annelida, Arthropoda and Chordata; the detected annelid species could only be identified to the genus-level and has either an aquatic or terrestrial lifestyle depending on the species, the vertebrate taxon is terrestrial and the arthropods are aquatic in their larval stage (Figure 1; Table 2). Diet identification was successful for the terrestrial leech *Diestecostoma mexicana* as oligochaete DNA was detected. In addition to the detection of diet in the predatory species *Erpobdella obscura* (Verrill, 1872), a nematode from the family Chromadorea was detected in three individuals. In both fluid/tissue-feeding and predatory leeches, DNA from other leeches was detected, indicating that these are part of their diet.

3.2 | Detection rate of diet

Ingested taxa could be identified in all six included leech families and within leeches spanning the three feeding modes (Table 2). From the total number of analysed leech individuals known to be bloodfeeding (33), ingested taxa were detected in 21 (63.6%). Diet was detected in 53.3% (8 out of 15) of the specimens belonging to *Placobdella mexicana* Moore, 1898, 25% (1 out of 4) of the specimens of *Haementeria officinalis* de Filippi, 1849, 100% (10 out of 10) of the specimens of *Myzobdella patzcuarensis* and 50% (two out of four) of the specimens of *Macrobdella decora*.

In the 34 analysed fluid and tissue feeding individuals, ingested taxa were detected in 18 samples, corresponding to 52.9%. Of these, diet was detected in 68.7% (11 out of 16) of the specimens belonging to *Helobdella adiastola* (Ringuelet, 1972), 50% (4 out of 8) of the specimens of *H. elongata* (Castle, 1900), 40% (2 out of 5) of the specimens belonging to *H. socimulcensis* (Caballero, 1932) and 20% (1 out of 5) of the specimens of *H. temiscoensis*. For the 49 predatory individuals (41 known to be predatory and eight *D. mexicana* with previously unknown feeding preferences), ingested taxa were detected in 18 leeches (36.7%): 26.9% (7 out of 26) of the specimens belonging to *Erpobdella ochoterenai* (Caballero, 1932), 40% (2 out of 5) of specimens of *E. obscura*, 20% (2 out of 10) of specimens of *Haemopis caballeroi* (Richardson, 1971) and 87.5% (7 out of 8) of specimens belonging to *D. mexicana*.

The number of vertebrate taxa detected in each of the 21 bloodfeeding leeches ranged from one to two. The only leech in which two vertebrate taxa were detected was the bloodfeeding *M. decora* feeding on *Homo sapiens* (order Primates) and *Lithobates clamitans* (order Anura). For predatory and fluid/tissue-feeding leeches, the ingested taxa ranged from one to three per individual leech; in most of the leeches only a single taxon was detected. Two invertebrate taxa were detected in the macrophagous leeches *E. ochoterenai* feeding on *Eiseniella tetraedra* (Savigny, 1826) (order Lumbriculida) and *Hesperophylax mexico* (Parker & Wiggins, 1985) (order Trichoptera), in *H. caballeroi* feeding on *Polypedilum* sp. (order Diptera) and an unidentified clitellate taxon and *D. mexicana* feeding on *Dendrobaena octaedra* (Savigny, 1826) and *Lumbricus rubellus* Hoffmeister, 1843 (order Opistophora). The only leech species for which three invertebrate taxa were detected in the diet was the fluid/tissue-feeding *H. adiastola*, which was found to feed on invertebrates such as insects, oligochaets and crustaceans (Table 2).

4 | DISCUSSION

In this study, we used DNA metabarcoding of iDNA from leech gut contents to obtain diet information from a broad swath of leech diversity from Canada and Mexico. The leeches encompassed eight genera from six families and all three feeding modes (bloodfeeding, predatory and fluid/tissue-feeding) known to be utilised by leeches. The ingested taxa detected in these were aquatic and terrestrial vertebrates and invertebrates. In addition, our results show the high potential of metabarcoding using iDNA from aquatic bloodfeeding leeches to be used for vertebrate monitoring in areas with unknown animal diversity; note that the present study is the first large-scale, multispecies study to assess this potential in North American leeches. In the following, we discuss the dietary range of the collected leeches and use that information to further discuss the utility of iDNA from leech gut contents in monitoring and estimating animal diversity.

4.1 | Leech-ingested taxa

We identified the dietary range of aquatic leech groups for which detailed information is scarce, including the bloodfeeding leech species *Placobdella mexicana*, *Haementeria officinalis*, *Myzobdella patzcuarensis* and *Macrobdella decora*; the fluid/tissue-feeding leech species *Helobdella adiastola*, *H. elongata*, *H. socimulcensis* and *H. temiscoensis*; and the predatory leech species *Erpobdella ochotenrenai*, *E. obscura* and *Haemopis caballeroi*. In addition, dietary information was obtained from the terrestrial leech *Diestecostoma mexicana*, the diet of which was hitherto unknown. The low sample size for each leech species (from 4 to 26 individuals) and overall individuals per feeding habit (33–49) does not necessarily allow for reliable statistical analyses or for comprehensive conclusions regarding the diet of the collected leeches. Nevertheless, it does allow us to expand the knowledge about the dietary range in the different groups and to assess the potential of leech iDNA to be used for estimating biodiversity in a region. Briefly, when comparing the diversity of ingested taxa detected among the leeches with the three feeding habits,

aquatic bloodfeeding leeches had the least diverse diet (based solely on vertebrate blood), whereas the predatory leeches had the highest diversity with a diet based mostly on freshwater invertebrates (Table 2, Figure 1).

Previous dietary studies of both aquatic and terrestrial bloodfeeding leeches have suggested a heavily vertebrate-centric diet for the studied taxa (e.g., Drinkwater et al., 2020; Fahmy et al., 2019; Williams et al., 2020). As in Williams et al., (2020), we detected both aquatic and terrestrial vertebrate taxa in the analysed aquatic bloodfeeding leeches (Table 2; Figure 1). Unsurprisingly, this is in contrast to the well-studied, terrestrial, bloodfeeding haemadipsid leeches, in which besides for a snakehead murrel (Fahmny et al., 2020), no other aquatic vertebrates have so far been detected (Drinkwater et al., 2020, Drinkwater et al., 2019; Hanya et al., 2019; Schnell et al., 2012, 2018). Among the terrestrial vertebrates that we detected in the aquatic bloodfeeding leech species, we identified a single bird species, the black-crowned night heron, *Nycticorax nycticorax*. This bird species was detected in a leech belonging to the genus *Haementeria*, which is known to generally feed on mammals and reptiles (Charruau et al., 2020; Oceguera-Figueroa, 2012). As this bird was detected in only one individual leech, more sampling is needed to determine whether *H. officinalis* commonly feeds on birds. Bird DNA has also been detected using iDNA from leeches belonging to the family Haemadipsidae (Tessler, Weiskopf, et al., 2018). In addition, birds have previously been shown to be involved in passive and active dispersal of leeches (Davies et al., 1982; Nakano et al., 2020; Siddall et al., 2013) and our findings suggest that the black-crowned night heron may be involved in the dispersion of this leech along its broad geographical distribution in central Mexico. Other vertebrates detected in the diet of the aquatic bloodfeeding leeches included invasive aquatic species. In the diet of the aquatic bloodfeeding leech *M. patzcuarensis*, we detected the Nile tilapia and the Blue tilapia, both of which were introduced to the lake of Pátzcuaro for the purpose of breeding consumption fishes (Berlanga et al., 1997). *M. patzcuarensis* is known to be a parasite of fish and has previously been found to feed on both native and exotic fish species (López-Jiménez, 1985); this generalist pattern is further corroborated by our findings. Finally, both mammal and amphibian DNA were detected in the diet of the aquatic bloodfeeding *M. decora*. Despite the fact that metabarcoding cannot distinguish between life stages of prey (i.e., eggs or adults), our finding of frog DNA inside this leech, reinforces previous reports that it occasionally ingests frog eggs (Moore, 1953; Trauth & Neal, 2004; Turbeville & Briggler, 2003). Leeches with a diet consisting of body fluids and tissues (liquidosomatophagy) were found to feed exclusively on invertebrates (Figure 1). We found that members of the genus *Helobdella* feed on molluscs, arthropods (insects and crustaceans) and clitellates (oligochaetes and hirudineans) (Figure 1). While we observed molluscs at the three locations where *Helobdella* individuals were collected, only one mollusc taxon was detected in a single leech. The prevalence of insects in the leech diet was therefore surprisingly high based on previous observations, as it is thought that the fluid/tissue-feeding leeches feed mainly on molluscs and oligochaetes (Sawyer, 1986). Notably, two individuals of *Helobdella* were found to have fed on another congeneric hirudinean. This has been previously recorded as a rare behaviour in *Helobdella fusca* (Mathers, 1948), but our results indicate this may be more widespread than previously thought for the genus *Helobdella*, with *H. temiscoensis* and *H. socimulcensis* also presenting this behaviour. However, as this was only found in two individuals, sampling of more species is needed to fully understand this behaviour.

Predatory leeches were found feeding on invertebrates, which corroborates previous knowledge about the diet of these leeches (Sawyer, 1986). There was a clear preference for an annelid-based diet in the analysed leeches, as 13 out of the 17 taxa detected in the gut contents were annelids, including some hirudineans. The direct observation of the same species of leech inside the digestive tract during dissection supports this metabarcoding result. In addition, cattle DNA was also detected in one individual of the genus *Erpobdella*. Although Kutschera, (2003) previously recorded this leech feeding on tissues from dead decaying vertebrates, the detected cattle could potentially be a contamination by the bovine serum albumin (BSA) used for the PCR amplification. Therefore, analysis of more individuals is needed to clarify if this is a common behaviour in the species of the genus *Erpobdella*.

The diet of the terrestrial leech *D. mexicana* was previously unknown, but it was expected to be a bloodfeeder as it coexists with salamanders (Caballero, 1940). Our results indicate that this is not the case, as the eight individuals of *D. mexicana* were found to feed exclusively on oligochaetes, with a total of five taxa detected in their gut contents (Table 2, Figure 1). This is the first study to attempt to solve this issue and *D. mexicana* should now be viewed as predatory.

4.2 | Leeches for animal monitoring

Our results provide proof-of-concept that leeches exhibiting all three feeding modes, bloodfeeding, fluid/tissue-feeding and predatory, can provide information about the animals living in the same area and that iDNA from leech gut contents can be used in other geographical areas outside the Indo-Pacific region. Further, the detection of DNA from turtles, fish and birds, provides opportunities to use freshwater leeches as a monitoring tool. Given that iDNA from leech gut contents can be used as a tool to monitor for example, otherwise elusive and endangered vertebrates (Nguyen et al., 2021; Schnell et al., 2012), it is surprising that nonhaemadipsid leeches have been almost completely neglected for this purpose (but see Williams et al., 2020).

As in previous iDNA studies using leech gut contents (Schnell et al., 2018), we detected birds, reptiles and amphibians in the aquatic bloodfeeding leeches; note that reptile DNA has not been previously detected in aquatic leeches (Williams et al., 2020). However, with the exception of human DNA, we did not detect any mammals, despite this being a commonly targeted group for iDNA studies. Nevertheless and importantly, we did detect two fish species, both of which are known to be invasive species. Animal

monitoring is commonly used not only to detect the presence of rare species but also the presence of invasive species, as they may represent a serious challenge to the health of ecosystems (Ota et al., 2020). In aquatic ecosystems, invasive fish can have extreme negative consequences on biodiversity and therefore their monitoring is of vital importance (Trebitz et al., 2017). Using molecular methods to monitor fish minimizes the onus on taxonomic expertise, in contrast to using traditional methods such as fishing (Bessey et al., 2020). Through such an approach, environmental DNA (eDNA) from water is known to provide a complete overview of the aquatic communities (Cantera et al., 2019). Nevertheless, aquatic bloodfeeding leeches can be used to aid in monitoring studies of fish faunal changes in aquatic environments. In addition, future studies should investigate the role of these leeches in the transmission of diseases to endangered or commercially important fish species.

Whereas iDNA studies have traditionally targeted vertebrate DNA, our study shows that invertebrate DNA is equally viable. This is the case for predatory and fluid/tissue-feeding leeches. This information can be important as some invasive earthworms are known to be a menace to local flora and fauna as they ingest detritus and alter habitat quality of for example, salamanders (Ziemba et al., 2016). Predatory leeches such as those in the genera *Haemopis*, *Diestecostoma* and *Erpobdella*, can be used to monitor earthworms, especially in areas where invasive species are found. It is important to mention that even though a group of leeches is known to feed on a certain taxon, the diet is often more flexible. For example, although members of the genus *Helobdella* were herein found to only feed on invertebrates, other studies have found the leech to be a facultative parasite of amphibians (Tiberti & Gentilli, 2010; Zimić, 2015) and fish (Malek & McCallister, 1984). Therefore, if *Helobdella* is collected for iDNA studies, there is the potential for retrieving both invertebrate and vertebrate information.

The ease with which samples can be collected is important to take into account when using bloodfeeding organisms as biomonitoring tools. Terrestrial bloodfeeding leeches can be very abundant and are relatively easy to collect as they readily parasitize humans and will therefore actively hunt the collector (Schnell et al., 2012). Although found in less abundance, the collection of aquatic leeches is also relatively straightforward, even when targeting nonbloodfeeding species. Several species can be found under rocks, plants and debris submerged in water. In addition, if submerging the collector's legs at the same time, bloodfeeding leeches can also be collected from the exposed skin. Contrary to this, the terrestrial predatory leech *D. mexicana* was not easily collected and many hours were spent trying to find only a few individuals. Therefore, if the aim is to use a group of leeches for regular animal monitoring, an easily collected group should be chosen; bloodfeeding species if targeting vertebrates that live in or visit a specific waterbody, or nonbloodfeeding leeches if targeting small invertebrates. It is also important to ensure that the collection of leeches for iDNA studies will not endanger leech populations. In this study, none of the collected leeches are threatened and can therefore continue to be collected for diet analyses. Note here that the two commonly used European medicinal leech species, *Hirudo medicinalis* Linnaeus, 1758 and *Hirudo verbana* Carena, 1820 are both on the IUCN red list and should not be collected (see Williams et al., 2020).

Further studies focusing on animal diversity, especially in aquatic environments, should compare the use of other methods, for example, water or soil eDNA, camera traps, fishing and iDNA from leech gut contents, to clarify if these methods are comparable or if the taxa they detect are different and therefore provide complementary data. We hypothesize that, as seen in other iDNA studies (Abrams et al., 2019; Tilker et al., 2020; Weiskopf et al., 2018), the use of these leeches for animal monitoring will not provide a complete overview of the local fauna but will provide complementary information to other monitoring methods.

4.3 | Technical considerations

In this study, a universal primer set targeting a partial fragment of the COI barcode region (mlCOIintF/jgHCO2198) (Leray et al., 2013; Geller et al., 2013) was chosen to allow detection of diet across a broad taxonomic range of metazoans (Leray et al., 2013). In addition, this primer set can also provide information about possible metazoan endoparasites (Bohmann et al., 2018) and can simultaneously coamplify leech DNA, thereby providing DNA barcode-based host identification; another study using multiple primer sets have also detected vertebrate, parasite and bacterial DNA (Siddall et al., 2019). Nevertheless, this coamplification can cause lower detection rates of the diet, as the leech DNA can overpower the diet DNA in the leech gut contents. To ensure that the diet is also detected, the screening of samples using qPCR prior to tagged PCR is important. This screening provides information about the optimal cycle number to maximise the detection of taxa (Kelly et al., 2021), and the presence of PCR inhibitors which can cause stochasticity (Murray et al., 2015). Another possibility could be the use of taxa-specific primers to target only the diet.

Based on our results, future studies can easily gauge the targeted iDNA by employing more or less specific primer sets to amplify DNA for a chosen taxon. However, it is important to keep in mind that some leeches also feed on other hirudineans. In the present study, this was found for the genera *Erpobdella*, *Helobdella* and *Haemopis* and has also been recorded in other genera (Aminov, 2019). The ingestion of leeches from a different genus can be easily detected with molecular methods using primer sets that have been shown to scrutinize at species-levels, but the ingestion of a member of the same species may still pose issues. Unless direct observations of this have been made, it is difficult to assess if the DNA detected is from the leech or from the diet. As has been shown for arthropods (Elbrecht et al., 2018) and recently with rabbit DNA within iDNA of leeches (Nguyen et al., 2021), one way to overcome this is to determine if the leech and diet haplotypes are fully conserved; if a certain haplotype can be evinced from nongut tissue then separate haplotypes within the ingesta can infer species-level cannibalism.

Processing the leeches individually allowed us to have a better overview of the detection rate of animal DNA. With the exception of Hanya et al., (2019) who extracted and PCR amplified the DNA of leeches individually, most studies using DNA metabarcoding to target iDNA from leech gut contents have processed the leeches in pools of several individuals (Abrams et al., 2019; Fahmy et al., 2020; Williams et al., 2020). In addition, except for Hanya et al., (2019), Schnell et al., (2012) and Schnell et al., (2018), all previous studies have used several metabarcoding primer sets and, therefore, a direct comparison to our results is strenuous, as we processed the leeches individually and used only a single metabarcoding primer set. In addition, no other study has used metabarcoding to target iDNA from the gut contents of nonbloodfeeding leeches.

Nevertheless, in some ways, it is possible to compare our results to those of previous studies. Studies pooling several leeches have found between 0.5 to 1.6 ingested taxa per pool (Abrams et al., 2019; Axtner et al., 2019; Drinkwater et al., 2020; Schnell et al., 2018; Williams et al., 2020), whereas Hanya et al., (2019) and Schnell et al., (2012) detected 1–3 ingested taxa (including human) in 24.3% of their individual leeches. In the current study, ingested taxa were detected in more than 50% of the individually analysed bloodfeeding and fluid/tissue-feeding leeches, whereas the detection rate in the predatory leeches was 36.7%. Despite this, two ingested taxa were detected in three predatory leeches, while we only detected a single taxon per leech in the nonpredatory groups. Our higher detection rate can be due to the way the leeches were collected, as many of them we found under objects submerged in water which may indicate that they were not seeking food; however, this could be also be the result of the bloodfeeding *M. patzcuarensis* being collected directly from its host, thus increasing the probability of detecting fish DNA. Moreover, the processing of the samples can also contribute to the paucity of diversity in any individual leech. Hanya et al., (2019) reported variations in the detection of mammal species when the DNA extract also contained leech DNA and when using different primer sets. This could be another reason for the higher detection rate in our study, as we did not pool leeches; Fahmy et al., (2019) and Tessler, Weiskopf, et al., (2018) processed individual bloodfeeding terrestrial leeches coupled with Sanger sequencing and had a similar detection rate: 31%–43% and 34%, respectively. Likewise, the degradation rate of the ingested DNA can also play an important part in the diet detection. It is still unknown if DNA from the diet of fluid/tissue-feeding and predatory leeches is preserved for at least four months within the intestine, as it is in some bloodfeeding species (Schnell et al., 2012). Future studies should address this.

Finally, it is important to remember that the strategy employed by the present study is based on DNA barcodes and, thus, the lack of a robust and well-curated comparative database will negatively impact the potential for taxonomic identification of the obtained sequences. In the present study, 11 out of the 40 OTUs detected could not be identified to species level, therefore providing much less informative results. As databases become more complete, so will the taxonomic assignment of the detected taxa and, with this, our knowledge of the diet of this group of invertebrates will dramatically increase.

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CONFLICT OF INTEREST

The authors have no conflict of interests.

AUTHOR CONTRIBUTION

C.L., A.O.-F., K.B. and M.T.P.G. designed the study. C.L., A.O.-F. and S.K. collected the samples. C.L. generated and analysed the data. C.L. wrote the original manuscript, with input and revisions from all the coauthors. All authors contributed approved the final manuscript.

DATA AVAILABILITY STATEMENT

Sequence data have been made available online at the Electronic Research Data Archive (ERDA) at https://doi.org/10.17894/ucph.694e6b45-8f2c-417f-a95d-5cb372fc4e27

ORCID

Christina Lynggaard https://orcid.org/0000-0003-4451-6642
Kristine Bohmann https://orcid.org/0000-0001-7907-064X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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