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eDNA from culture-independent *Hirudinaria bpling* Phillips 2012 (Annelida: Hirundinidae) as a tool for biodiversity assessment

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Abstract

Recent interest in the use of leeches as a source of eDNA by obtaining and analyzing traces of its last blood meal has proven noteworthy as a tool in screening biodiversity. As the method is fairly new and has relatively unexplored benefits, its assessment as a tool in such fields as ecology and biotechnology prompts further studies. This study was conducted to provide an alternative to collecting samples in poorly-explored areas or in areas where collection is very difficult, if not impossible. Using leeches collected from Angadanan, Isabela, DNA was extracted and cytochrome oxidase I (COI) gene was amplified using conventional PCR. Gene sequences from resulting amplicons were matched with similar sequences using BLASTn, where prey sequences were narrowed down to organisms with the highest match of no less than 85%. Highest score of similarities were obtained and species identified included *Cyprinus carpio* L. 1758, Homo sapiens L., 1758, *Bos taurus* L., 1758, *Bubalus bubalis carabanensis* L.S. Castillo, 1998, *Equus caballus* L., 1758, and *Canis familiaris* L., 1758. These results demonstrate that using leech-extracted blood meal may be a successful tool in screening vertebrate biodiversity.

Keywords: DNA analysis, biodiversity, leech, second-generation sequencing

Introduction

In recent times, great interest has developed in the application of environmental DNA (eDNA) as a non-invasive tool for obtaining information on biodiversity. eDNA refers to DNA that can be extracted from environmental samples without the need for the target organisms themselves. Preliminary studies have demonstrated that extraction and identification of DNA from an environmental sample has been effective for detecting and monitoring not only common species, but also those that are endangered, invasive, or elusive. It can recover information on organisms across a broad range of taxa with different sizes, and ecologies, and is a "potent tool for elucidating mechanistic insights in ecological and evolutionary processes" (Bohmann et al., 2014). The great demand for a more cost-effective, and strategic way of obtaining basic data on mammalian diversity and species occupancy justifies the need for further studies on DNA from micropredators.

One recent addition to the biodiversity monitoring toolkit is DNA extracted from carrion feeding or haematophagous insects (Calvignac-Spencer et al., 2013; Rovie-Ryan et al., 2013; Votypká et al., 2015), and leeches (Schnell et al., 2012). Using information from these ectoparasites is novel and may prove to be valuable in studies on biodiversity.

The invertebrates that have been used in iDNA (ingested DNA) studies are diverse, including mosquitoes, carrion and blow flies, midges, ticks, terrestrial and aquatic leeches. Schnell et al. (2012) found that 84% of collected leeches (N=25) contained mammalian DNA of sufficient quantity and quality to be extracted, and amplified by polymerase chain reaction or PCR. As these organisms inhabit temperate and tropical forests, and water bodies such as wetlands, ponds, rivers, and lakes, they have a wide spectrum of prey. Leeches assayed by Schnell et al. (2012, 2015), and Tessler et al. (2018) manifested bloodmeals from a wide range of mammalian orders (Artiodactyla, Carnivora, Lagomorpha, Rodentia, Scandentia, and Primates) with diverse behaviors, ecologies, and body sizes. These leeches are also easily collected as compared to other micropredators especially during wet seasons in tropical forests where bulk collection may be easily made. These leeches feed very sporadically at varying irregular time intervals, and are able to ingest large quantities of blood during one feeding event

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(Dickinson and Lent, 1984). The DNA from the blood of the host survives for at least four months after being ingested by the leech (Schnell et al., 2012). Given these advantages, bulk processing of leeches through simply digesting the entire leech, purifying the total DNA then subjecting it to metabarcoding PCRs combined with high throughput sequencing provides an efficient means to assess biodiversity of vertebrate species (Calvignac-Spencer et al., 2013; Schnell et al., 2012, 2015).

This study tests the use of eDNA in a remote area where identification of indigenous and newly discovered species as well as those thought to be extinct may be possible. One of the most beneficial aspects of this method is that the collection is cost-effective, rapid, and requires no special skills or equipment, allowing easy participation of those without scientific background.

Throughout tropical Asia, mammal populations are severely depleted by hunting, with remaining individuals wary of humans, and habitats are frequently dense, rugged, and humid, making detection of many species challenging (Schnell et al., 2012). The Philippines, known for having a very diverse set of fauna, hosts a number of extant and endangered species that have eluded sampling due to the difficulties of working in remote and less inhabited areas. Various species of leeches also abound in the humid forests of Southeast Asia, including the Philippines, which has one of the least known and most threatened mammal faunas in the world.

Hirudinaria bpling Phillips, also known as the Asian buffalo leech, is widespread throughout tropical South and Southeast Asia and may be found in the forests of the Philippines (Moore, 1938). It is distinct from *H. manillensis* (Lesson) in that it contains stripes of yellow and brown while the latter contains red and green (Phillips, 2012). *H. bpling* belongs to the family Hirudinidae which is comprised of mainly blood-sucking freshwater leeches (Tubtimon et al., 2014).

In this study, the aim was to extract and analyze the genetic material (DNA) of the hosts of *H. bpling*. From its gut contents, the host of these leeches will be determined by DNA extraction, sequencing and sequence alignment using bioinformatics tools. Ultimately, this technique of sequencing leech-derived eDNA may be shown to be a biodiversity screening tool.

Materials and Methods

Sampling site and collection of leeches

Twenty-five leeches were collected from San Roque creek, Angadanan, Isabela, Philippines during the month of August 2017. Leeches were randomly collected at the different parts of the creek after which the specimens were sustained by encapsulating them temporarily inside a plastic bottle with holes for air ventilation and partially filled with water from the place of collection. Specimens were transported to the laboratory, and were promptly transferred to a more stable bottle. Lastly, the leeches were placed in the refrigerator to relax prior to dissection.

Figure 1. *Hirudinaria bpling* Phillips, 2012 showing dorsal markings (*left*) and black ventral side (*right*).

Leech identification and authentication

Leech samples were sent to the National Museum and identified as *Hirudinaria bpling* Phillips 2012.

DNA extraction from collected blood

The leeches were cut open longitudinally with the use of a scalpel, and the expelled blood from its gut contents was collected in Petri dishes assigned per designated leech. DNA was extracted from each sample using the Wizard™ Genomic DNA Purification Kit (Promega), according to the manufacturer's protocol. Briefly, 300 µL of gut contents was mixed with 900 µL of cell lysis solution, incubated at room temperature for 10 min and centrifuged at 16,000xg for 20 s. The pellet was mixed with 300 µL nuclei lysis solution, vortexed and added with 100 µL protein precipitation solution, followed by centrifugation at 16,000xg for 3 min. The supernate was transferred to a new tube containing 300 µL isopropanol, mixed and centrifuged at 16,000xg for 1 min. The pellet was resuspended in 300 µL 70% ethanol, centrifuged at 16,000xg for 1 min, and the ethanol aspirated leaving the pellet to dry for ≈10 min before the addition of 100 µL DNA rehydration solution and storage at -20°C.

PCR amplification of extracted DNA

Tabunda et al. (2015) reported that mitochondrial cytochrome oxidase I (COI) was found to be more sensitive in detecting DNA even at low concentrations than cytochrome b,

and corroborated by Townzen et al. (2008), hence the use of the same marker for the present study.

For PCR amplification, 2 µl of the DNA sample was placed in a PCR tube together with 25 µl of 2X PCR Master Mix, 1 µl each of forward and reverse COI primer synthesized by AIT Biotech (Table 1), and 21 µl of nuclease-free water. The step cycle consisted of 95°C for 1 min; 35 cycles of 95°C for 30 s; 52°C for 50 s, 72°C for 1 min and a final extension of 72° C for 5 min. Amplicons were ran on 1.5% agarose gel with a 50 -base pair nd 100-base pair ladder.

Table 1. Primers used to identify leech bloodmeals

Primer Name	Location*	Primer Sequence
COI_long (f)	5934	AACCACAAAGACATTGG-CAC
COI_long (r)	6597	AAGAATCAGAA-TARGTGTTG

*Location based on human mtDNA (Townzen et al., 2008)

Sample equencing and Identification

Distinct bands in the gel were excised and the DNA was eluted with Amicon Ultrafree™-DA centrifugal units. Fifty µL of each sample were sent to Macrogen (Korea) for sequencing. The resulting DNA sequences were matched with entries in the database of homologous sequences contained in GenBank using the nucleotide basic local alignment search tool (BLASTn). This step displayed species that contained similar nucleotide sequences to the nucleotide sequences in question. Unknown bloodmeal sequences are considered putatively identified if they are > 95% identical (% nucleotide identities) to a sequence in GenBank for COI gene. When the bloodmeal sequence had a > 95% match to sequences of more than one species in GenBank (ambiguous matches), the geographical range, abundance, and similarity scores for the taxa producing significant alignments were used to aid in selection among alternative potential identifications (Townzen et al., 2008; http://www.ncbi. nlm.nih.gov/). The size of the bands produced by each sample on the gel was also used to cross-check the results to properly aid in species identification.

Results

Five out of twenty leeches showed bands of different sizes in the agarose gel (Figure 2). Purified DNA exhibited a DNA percentile match of 85% and above after being cross checked with the Basic Local Aligning Tool (BLAST), and NCBI GenBank base pairs reference. As shown in Table 2, an array of vertebrate species were identified, specifically from the Order Actinopterygii (Osteichthyes), and mammalian Orders Artiodactyla, Perissodactyla, Carnivora and Primates. On the contrary, the other leeches failed to yield clear sequences that can detect species that can be found in the sampling site. The presence of human DNA in most samples could have come from the volunteers who helped collect the leeches.

Discussion

Results showed that eDNA extracted from recent blood meal of leeches can be used to detect species and may be used as a tool for future screening of biodiversity and determining various species in a sampling area. Leeches were collected during the rainy season in the Philippines, which usually falls between June to September, while leeches were still common and easy to collect. Collection of leeches was done for one whole day with collection points chosen randomly and with close proximity from each other with only distances of 3-5 m apart. With this, the researchers were able to obtain a considerable proportion of leeches (25%) caught randomly in the sampling site which contained amplifiable fragments of DNA assignable to different vertebrates, with a few leeches providing multiple species of various animal classes. More accurately, in the process of randomly collecting 20 leeches only 5 allowed the recovery of DNA of one fish and four mammalian species. This may be due to the lack of the desired amount of

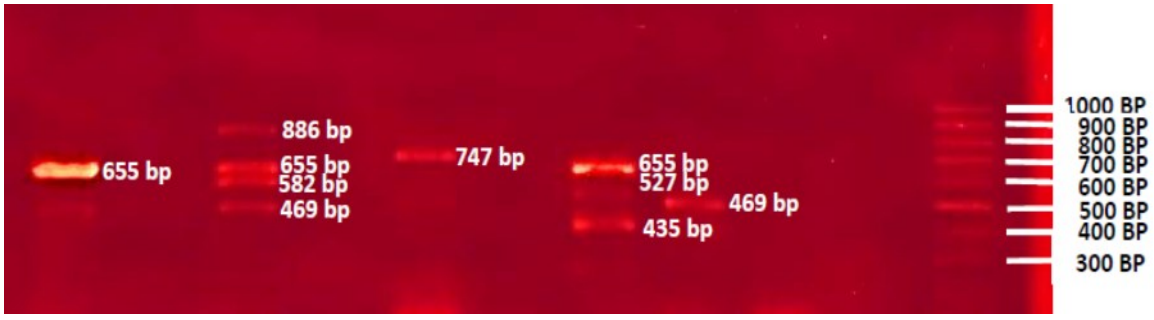

Figure 2. 1.5% Agarose gel electrophoresis resulting banding pattern of mtDNA amplified with COI_long primer pair. 50 bp DNA ladder

Table 2. Identified species from sequenced DNA

Leech	Band No.	Nucleotide length (bp)	Scientific Name	Common Name	% Match	Accession Number
1	1	655	*Cyprinus carpio*	Common carp	88%	KX145549.1
2	2	886	*Homo sapiens*	Human	91%	AB196726.1
	3	655	*Cyprinus carpio*	Common carp	88%	KX145549.1
	4	582	*Bos taurus*	Domestic cow	85%	HM102290.1
	5	469	*Homo sapiens*	Human	91%	DQ487095.1
3	6	747	*Bubalus bubalis carabanensis*	Carabao	98%	KF714291.1
4	7	655	*Cyprinus carpio*	Common carp	88%	KX145549.1
	8	527	*Equus caballus*	Horse	91%	JQ735459.1
	9	435	*Canis familiaris*	Domestic dog	89%	KF385957.1
5	10	469	*Homo sapiens*	Human	91%	DQ487095.1

blood meal of most of the obtained leech samples required for the protocol. The leeches may not have fed for a long time such that only traces of organismal DNA may have persisted that were not detectable using the DNA extraction procedure. This is due to the leeches' complex eating patterns where they may gorge themselves with blood at a time and may survive for long periods of time in between episodes of feeding (Lent and Dickinson, 1988). DNA from preyed-on species may survive up to four months in the gut of leeches, however, in some cases, these fragments may have exceeded the said time frame resulting in the expulsion and complete digestion of amplifiable traces of vertebrate DNA (Schnell et al., 2012). Designated wells containing multiple bands indicate how these leeches may have fed upon several organisms within the time span of the survival of said DNA sequences. However, as stated by Schnell et al. (2015), more recent blood meals possessed significantly higher levels than those previously fed on. Our results supported previous studies suggesting it may be possible to extract multiple DNA sequences from one leech.

The sampling area is also not heavily populated by humans and is rich in flora and fauna, despite the low number of species the leeches have fed on. Based on observations in the sampling location as well as from the results obtained, the location proved to be undisturbed by human intervention. Moreover, resulting human traces of DNA may have been obtained from locals who helped in collecting the leech samples.

Conclusion

The methodology employed in this research has successfully identified vertebrates from Myanmar, Australia and Madagascar (Siddall et al., 2019) making it a remarkable tool for alternatively assessing biodiversity in a specific sampling site. Firstly, the selected extraction kits and procedures administered in order to extract and analyze the said DNA samples were appropriate under existing laboratory conditions. Secondly, detected species sequences were able to align with organisms of various animal groups through the NCBI PubMed Genbank as a reference database. In some cases of ambiguous findings having a percentage of less than 95% DNA match, the range of potential hosts was narrowed down to organisms with the highest match of no less than 85%.

This research was not designed to find species which were thought to be extinct or new species of fauna. On the other hand, the researchers were presenting a novel way of exploring biodiversity in very remote, hard to access study areas where direct field collection could be very difficult. if not impractical. It is therefore recommended that in future studies, more leech samples should be collected and DNA samples from different sampling areas be analyzed in order to gather more data on a wider range of fauna, thereby increasing the chances of obtaining a higher percentage of amplifiable trace DNA. It would also be of great advantage to quantify the extracted DNA of samples by spectrophotometric means before having it sequenced.

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