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Using terrestrial haematophagous leeches to enhance tropical biodiversity monitoring programmes in Bangladesh

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Abstract

1. Measuring mammal biodiversity in tropical rainforests is challenging, and methods that reduce effort while maximizing success are crucial for long-term monitoring programmes. Commonly used methods to assess mammal biodiversity may require substantial sampling effort to be effective. Genetic methods are a new and important sampling tool on the horizon, but obtaining sufficient DNA samples can be a challenge.
2. We evaluated the efficacy of using parasitic leeches *Haemadipsa* spp., as compared to camera trapping, to sample biodiversity. We collected 200 leeches from four forest patches in northeast Bangladesh, and identified recent vertebrate hosts using Sanger sequencing of the 16S rRNA gene extracted from each individual leech's blood meals. We then compared these data to species data from camera trapping conducted in the same forest patches.
3. Overall, 41.9% of sequenced leeches contained amplifiable non-human mammal DNA. Four days of collecting leeches led to the identification of 12 species, compared to 26 species identified in 1,334 camera trap nights.
4. *Synthesis and applications*. After assessing the cost, effort and power of each technique, there are pros and cons to both camera trapping and leech blood meal analysis. Camera trapping and leech collection appear to be complementary approaches. When used together, they may provide a more complete monitoring tool for mammal biodiversity in tropical rainforests. Managers should consider adding leech collection to their biodiversity monitoring toolkit, as improved information will allow managers to create more effective conservation programmes.

KEYWORDS

blood meal sequencing, camera trapping, DNA barcode, Haemadipsidae, leech, mammal biodiversity, mitochondrial DNA, non-invasive monitoring

1 | INTRODUCTION

Deforestation is a critical issue world-wide. Between 2000 and 2010, 400,000 km^2 of primary forest was lost. With forests supporting over half of terrestrial animal and plant species, this loss severely damages global biodiversity (Secretariat of the Convention on Biological Diversity, 2010). Asian species have been especially threatened by this loss, and in 2008, Asia and the Pacific reported the highest number of threatened species (Squires, 2013).

In order to create effective conservation strategies to combat these declines, we need baseline data, such as density, occupancy and abundance, along with monitoring programmes to assess success of conservation interventions and guide management decisions (Burton, 2012; Wong, Leader-Williams, & Linkie, 2013). Although mammals are a relatively well-studied taxa, knowledge gaps about species' distributions and taxonomy remain (Francis et al., 2010). This information is important to managers allocating limited conservation resources. However, monitoring mammal biodiversity is challenging, particularly when target species are rare, cryptic and highly mobile, as are many tropical species (Linkie, Dinata, Nugroho, & Haidir, 2007).

Common methods to assess mammal biodiversity (e.g. camera traps, track plates, genetic analyses of hair or faeces) can be difficult to implement; deploying cameras is time-consuming and expensive. While camera traps are effective at capturing most mammals, dependent on the camera position and bait used, an extensive survey of mammal biodiversity requires substantial effort. Finding scat samples can be challenging, require extensive survey effort, and not work well in the humid tropics. Moreover, rare and elusive species are difficult to capture, thus requiring substantial sampling effort (Tobler, Carrillo-Percastegui, Leite Pitman, Mares, & Powell, 2008).

Difficulty in gathering ecological information on tropical mammals has led the IUCN to list many species as "data deficient," a significant obstacle to conservation (Schipper et al., 2008; Schnell et al., 2012). To rectify data deficiencies, ecologists need an expanded set of tools to address current limitations. 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; Votýpka et al., 2015) and leeches (Schnell et al., 2012). 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 (PCR). As leeches have a wide prey base and are prevalent and easy to collect in tropical rainforests throughout Southeast Asia, this method could potentially provide previously inaccessible information regarding tropical biodiversity (Schnell et al., 2012).

Although leeches are a promising tool for sampling biodiversity, questions remain about their efficacy (Schnell et al., 2015). Understanding costs and benefits of different sampling methods is essential to creating effective study designs, especially for cost-limited vertebrate monitoring programmes (Lyra-Jorge, Ciocheti, Pivello, & Meirelles, 2008). In this study, we evaluated the efficacy of using terrestrial haematophagous leeches to estimate mammalian biodiversity in Bangladesh. Our objectives were to: (1) determine whether we can identify mammal species by sequencing leech blood meals, (2) determine whether leech size impacts amplification success rate of mammalian DNA, (3) compare performance of leech blood meal sequencing and camera trapping for detection of mammalian biodiversity, and (4) assess and compare costs and benefits of both methods.

2 | MATERIALS AND METHODS

2.1 | Study area

Bordering the Indo-Burma biodiversity hotspot, Bangladesh is a small country with diverse flora and fauna, but also a rapidly diminishing tropical rainforest (Chowdhury & Koike, 2010). This study was conducted in northeast Bangladesh, a once highly forested area containing tropical evergreen and mixed evergreen forests. Most of the area has been deforested for roads, plantations and agriculture; the remaining forest is contained in 10 fragmented forest patches (Islam et al., 2013; Quazi & Ticktin, 2016). These patches are located between 24°4′ and 24°21′N and 91°15′ and 91°7′E, and range from 10 to 100 km^2. They are predominantly bordered by industrial plantations or rural settlements (Bangladesh Forest Department, 2012). The Forest Department manages most of the patches as "reserve forest," indicating protected status with certain extraction activities permitted. The area also contains Satchari and Lawachara National Parks, and Rema-Kalenga Wildlife Sanctuary, where no extraction activities are allowed. The topography of the region is hilly, with elevations between 50 and 300 m above sea level. Patches consist of hill forest, shrubs and mixed bamboo forest, with many streams and swampy areas (Bangladesh Forest Department, 2012). Annual temperature ranges from 7 to 23°C and rainfall is 3,334 mm per year, with most occurring between May and September.

Four forest patches, Atora Hill Reserve Forest (AHRF, *c.* 100 km^2), Patharia Hill Reserve Forest (PHRF, *c.* 60 km^2), Rajkandi Reserve Forest (RRF, *c.* 62 km^2) and Tarap Hill Reserve Forest (THRF, *c.* 82 km^2) were selected for this study (Figures 1 and 2). AHRF and RRF are extensions of larger forest tracts in India that expand into Bhutan and Myanmar. PHRF connects with a larger forest in India, although a border fence and other development have reduced connectivity. THRF is the most isolated patch, and contains Rema-Kalenga wildlife sanctuary.

2.2 | Leech collection

Leeches were collected from the forest patches in October, 2015. We collected 50 leeches in each patch over four collection days, with one collection day per patch. At each patch, we picked five locations with previous camera trap data. We attempted to ᶜollect 10 leeches from each location, however if we were unable to find 10, we collected additional leeches at another nearby location to ensure we had 50 leeches per patch. We collected leeches by hand, using nitrile gloves to limit DNA contamination, and placed them into individual 1 mL test

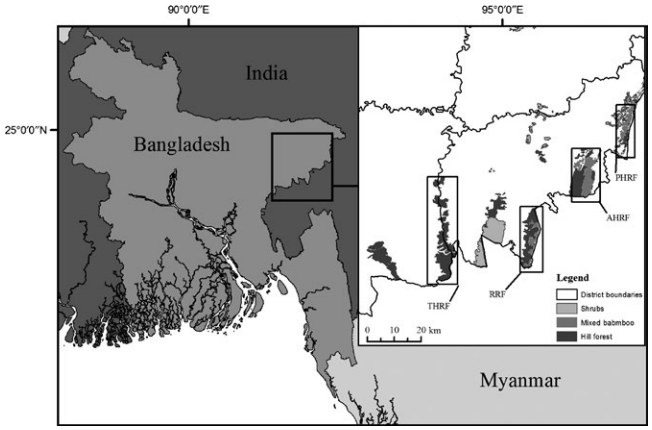

FIGURE 1 Map depicting four forest patches in northeast Bangladesh that were the focus of camera trap surveys from 2014 to 2015 and from which leeches were collected in 2015

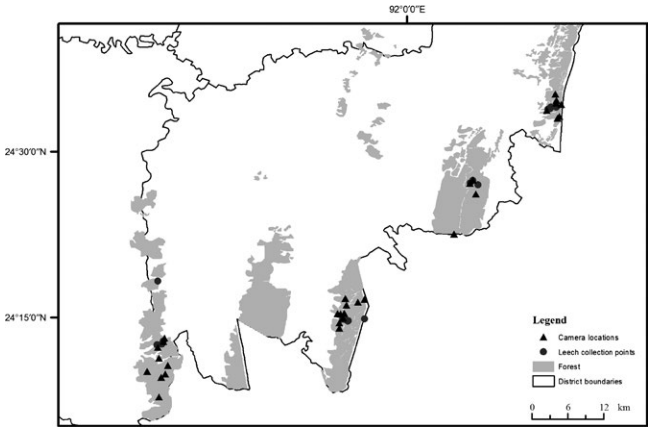

FIGURE 2 Map depicting camera trap locations (2014–2015) and leech collection points (2015) in four forest patches in northeast Bangladesh

tubes filled with RNAlater® to preserve DNA for extended periods of time without refrigeration.

2.3 | Leech processing and sequencing

We identified leech species based on external morphological characteristics. We measured leech length and width at the widest point using a micrometer on a compound microscope. This is not an accurate measure of leech size at collection, as they shrink in RNALater®; however, knowledge of relative sizes of successful leeches may provide a guideline for leech collection in the field.

To prepare leeches for sequencing, we removed a 2.5 mm leech segment (targeting the digestive tract) from just anterior of the rear sucker to the midpoint of the leech. This retains taxonomically important regions of the leeches, allowing for quality voucher specimens. We chopped segments into quarters and used a Qiagen DNeasy 96 Blood and Tissue Kit (Qiagen, Inc., Valencia, CA) to extract DNA following manufacturer's instructions with slight modifications to improve extraction quality. We performed extraction with longer incubation (overnight) and elution wait periods (20 min instead of 1 min) than specified. Samples were extracted in a laboratory with minimal contamination risk, as the only other vertebrates processed there are marine mammals.

After extraction, we performed PCR using 16Scp primers, which are known to amplify vertebrate DNA (Caragiulo, Dias-Freedman, Clark, Rabinowitz, & Amato, 2013; Chaves, Dias, & Pomilla, 2010; Wultsch et al., 2016). Expected amplicon length is 294 bp, although this may vary based on differences between species. The sequences were: forward primer 5′-CGAGGGCTTTACTGTCTCTT-3′, and reverse primer 5′-CCTATTGTCGATATGGACTCT-3′. We added 21.3 μl water, 10 μM forward primer, 10 μM reverse primer, 0.3 μl of BSA and 2 μl of template to illustra PuReTaq Ready-To-Go PCR Beads. The thermocycler profile was 94°C for 2 min, 10 cycles of 94°C for 15 s, 52.5°C for 15 s, 72°C for 45 s, 15 cycles of 94°C for 15 s, 52°C for 15 s, 72°C for 45 s, 20 cycles of 94°C for 15 s, 51.5°C for 15 s, 72°C for 45 s and a final cycle of 68°C for 20 min. Separate laboratories were used pre and post PCR.

We performed AMPure PCR purification, using a 2:1 ratio of AMPure to template to remove everything under 125 base pairs (Bekman Coulter). We used 20 μl of template and 40 μl of AMPure per well. Next, we performed cycle sequencing and ethanol precipitation (70% ethanol). We sequenced genes on an ABI 3730xl DNA Analyser (Applied Biosystems, Carlsbad, CA, USA) and compared sequences with the NCBI Nucleotide BLAST Database. This was done with the NCBI BLAST website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) searching the nr database with BLASTn.

For our analyses of BLAST results, we only included sequences with an *e*-value of less than e^{-30} to avoid low quality matches. We identified species using the top BLAST hit rather than a percent identity cutoff because we felt it was less subjective, as differences in inter- and intra-species similarities prevent determination of a universal cutoff (DeSalle, Egan, & Siddall, 2005). We indicated whether the top hit had at least a 1% better percent identity match (Table S1). This was assumed to be true if the first 100 matches were the same species. For the two blood meals where this was not the case, one was identified only to family, while the other was kept at species level since it is the only member of its genus in Bangladesh.

Several blood meals matched species that do not occur in Bangladesh. The true species in these blood meals are likely missing sequences in GenBank. We therefore reported these only to family but counted them as separate species in the analysis, as they were separate taxonomic units than those encountered in other leeches and may have been identifiable if we sequenced additional genes or bolstered the database by sequencing museum specimens. Our species identifications should be considered with caution, as we sequenced only one gene. However, because the exact identity of the species would not change our conclusions, we did not sequence additional genes.

2.4 | Camera trapping

Camera trapping was conducted between 1 May 2014 and 29 January 2015 using digital remote cameras (Bushnell Trophy Cam

HD, Overland Park, KS, USA) as part of a research project on felid conservation (H. A. Rahman, unpublished data). Cameras were set to operate continuously, using infrared photography at night, and were set to take two pictures when triggered with a 15 s delay before another photograph could be triggered. Date and time of photographs were automatically recorded. Camera trap locations were chosen, so that there was about 1.1 km between cameras, and cameras were placed within 200 m of the chosen trap location in areas where felids were most likely to travel, such as along trails. Twenty-seven cameras were deployed and moved periodically, so that there were 44 camera sites in total. Due to camera theft (11 units) and permanent malfunction (3 units), only 30 camera trap stations were effective: 4 in AHRF, 7 in PHRF, 10 in RRF and 9 in THRF. Cameras were placed approximately 25 cm above the ground, inside a theft proof box made of steel, and attached to a tree using a metal chain. They were checked every 15–20 days to change batteries and memory cards.

To maximize potential of capturing felids, scent lures were used at trap stations. Calvin Klein Obsession for Men (CK Obsession) was used at 36 trap stations, while chicken body parts were used at eight locations (Braczkowski & Watson, 2013). At stations using CK Obsession, cotton balls were sprayed with four to six sprays of cologne and placed inside plastic bottles on the ground or attached to a tree. At sites using chicken body parts, portions of chicken were placed in a plastic bag with holes that allowed scent to be released. Bags were placed on a tree at least 3 m above the ground to decrease chance of scavenging. At 26 locations, visual attractants in the form of chicken feathers were used. Chicken feathers attached to a wire were attached to a tree branch 25–30 cm above the ground. These were not used at sites where they might increase visibility of cameras to humans and increase chance of theft. All attractants were placed 2–3 m in front of cameras.

Number of trap nights for each camera was calculated as the number of days between deployment and retrieval. Each photograph of an animal was identified to species where possible. If photograph quality was too low for identification, we excluded the picture from analysis. Photographs of the same species taken within one hour of the first picture were considered one photographic event.

2.5 | Analyses

To compare efficacy of leech blood meal analysis and camera trapping as a biodiversity sampling technique, we used a Bayesian paired *t* test to determine differences in raw species richness (number of species identified) within patches and between the two methods. This test was implemented using the *bayes.t.test* function in the programme *BayesianFirstAid* (Bååth, 2014). Next, we constructed species accumulation curves using leech and camera trap data in R version 3.2.0 (R Core Team, 2016) using Kindt's exact method in the function *specaccum* of the *vegan* library (Oksanen et al., 2015). We then used function *specpool* in the *vegan* library to estimate overall species richness (observed and unobserved species) using both methods. We computed the estimates using Chao's method, because jackknife and bootstrapping tend to underestimate species richness if there are a high number of rare species, or too few samples, which is likely the case with the leeches (Chao, 1987).

To enable comparison of effort between methods, we used an iterative model to randomly sample camera locations to match the number of leech collection locations per forest patch. We then created a species accumulation curve using this subset of camera trap data. Within each of 5,000 iterations of this model, we extracted the number of trap nights needed to reach 12 species, the number found within our leech dataset. Finally, we created a dataset using a random sample of cameras, again matching the number of leech collection sites, and truncated it to the median number of trap nights needed to obtain 12 species as determined by the iterative model. We used this random, truncated, subset of data to create a final species accumulation curve representing an example of camera trapping at the same number of locations and reaching the same number of identified species as our leech collection efforts.

To evaluate the effect of leech size and species on DNA amplification success, we applied seven candidate logistic models in a Bayesian framework (Table 1). We ran models using leech species as a fixed effect, a random effect and not at all (leech species pooled). We ran the models in program *rstan* (Stan Development Team, 2016) using the package *rethinking* (McElreath, 2015) and used WAIC to select the best model.

Finally, we compared monetary and time costs of each method. Costs assume collaboration with an established genetics laboratory and that researchers are already in the country where data are collected. After determining that we needed an *c.* 13-fold increase in camera trap nights to progress from 12 to 26 identified species, we estimated the cost of collecting and analysing 13-fold more leeches (N = 2,600) using Sanger sequencing and Next-Generation sequencing (NGS).

3 | RESULTS

Two-hundred leeches were collected in situ, one leech was lost during transit, and eight leeches were maintained as unaltered voucher specimens, resulting in 191 leeches for genetic analysis (partially dissected vouchers retained). While one leech morphotype (n = 34) did not conform to any morphological species descriptions, most did: *Haemadipsa ornata* (n = 136), *Haemadipsa montevidicus* (n = 15) and *H. sylvestris* (n = 14). Molecular data would be helpful for confirming identifications, given the morphological difficulties found in this family (Borda & Siddall, 2011; Tessler et al., 2016). Overall, 41.9% (N = 80) of leeches contained amplifiable non-human mammal DNA, 10.5% (N = 20) contained human DNA, and 2.1% (N = 4) contained red junglefowl *Gallus gallus* DNA; the remainder (45.5%, N = 87) did not contain amplifiable vertebrate DNA. *Haemadipsa sylvestris* had the greatest percentage of amplifiable non-human mammal DNA with 58.3% (N = 7) of leeches successfully amplifying. Amplification success was lower for the remaining species, with 47.7% of *H. ornata* (N = 64), 8.3% of *H. montevidicus* (N = 1), and 24.2% of the unidentified species

TABLE 1 Candidate models and model comparison to evaluate effect of leech size and leech species on mammalian DNA amplification success from extracted blood meals. Models were implemented with vague normal priors ($M = 0$, $SD = 10$) for each coefficient

Model	Response	Link function	Intercept	Covariate	WAIC	Estimated effective number of parameters	Delta WAIC	Akaike weight	*SE* of Delta WAIC	Covariate parameter with 95% credible interval
1	Amplification success	Logit	Pooled	Leech length	256.6	2.1	3.5	0.09	5.43	0.78 (0.17–1.40)
2	Amplification success	Logit	Pooled	Leech width	257.4	2.1	4.3	0.06	6.03	3.03 (0.41–5.76)
3	Amplification success	Logit	Fixed species	Leech length	254.0	5.9	0.9	0.31	2.99	0.53 (−0.14 to 0.19)
4	Amplification success	Logit	Random species	Leech length	253.1	5.0	0	0.49	NA	0.56 (−0.11 to 1.21)
5	Amplification success	Logit	Pooled	Leech length × width	258.0	2.1	4.9	0.04	5.59	1.16 (0.15–2.21)
6	Amplification success	Logit	Pooled	Leech length÷width	261.9	2.0	8.8	0.01	6.84	0.18 (−0.22 to 0.57)
7	Amplification success	Logit	Pooled	None	260.7	1.0	7.6	0.01	7.24	N/A

(*N* = 8) containing amplifiable non-human mammal DNA. The percent of successful leeches varied by site, with 43.8% (*N* = 21) success from AHRF, 33.3% (*N* = 16) from PHRF, 39.6% (*N* = 19) from RRF and 51.1% (*N* = 24) from THRF.

We captured 863 independent mammal photographs in 1,334 camera trap nights. Twenty-six mammal species were identified from the photographs compared to 12 mammal species (nine identified species and three unknown species) identified in the leech blood meals. The Bayesian paired *t* test of species richness at each site estimated that camera traps found an average of 9.1 more species, but the credible interval (CI) was wide (95% CI: 2.0–16.3). Estimated total species richness was also higher using cameras (Table 2).

Across sites, in both leech blood meals and cameras, cows *Bos taurus* and pigs *Sus scrofa* were in the top three most frequently captured species (Figure 3). We captured greater rodent diversity on the cameras, but several were unable to be identified to species. The leeches failed to detect any felid species. When looking at the species composition at each site, results between cameras and leeches differed (Figure 4).

TABLE 2 Estimated mammal species richness in four forest patches in Bangladesh using camera traps (1,334 and 99 camera trap nights) and leeches (191). Estimates were calculated using Chao's method

Sampling method	Total species estimate	*SE*
Camera traps (1,134 trap nights)	29.12	3.66
Leeches	17.97	7.13
Camera trapping (99 trap nights)	17.94	6.42

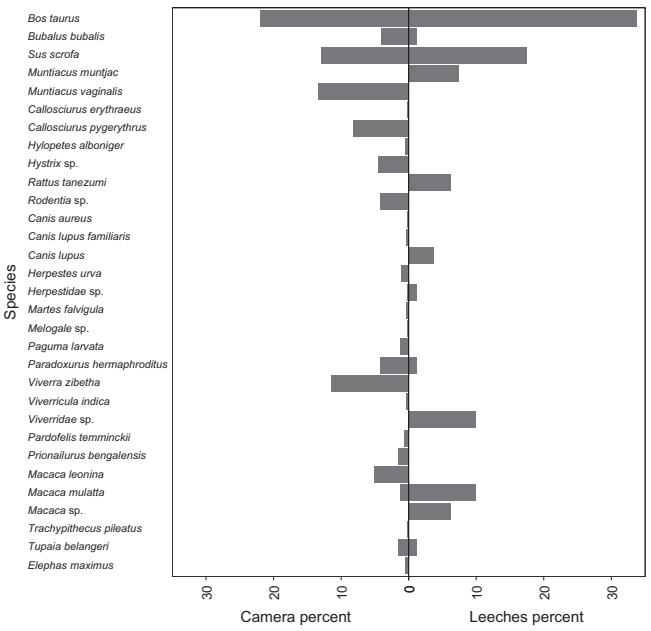

FIGURE 3 Non-human mammalian species composition of photographs from 1,334 camera trap nights (left) and blood meals from 80 leeches that successfully amplified non-human mammalian DNA (right)

The species accumulation curve made using all sequenced leeches did not reach its asymptote, and neither did the curve constructed from all camera trapping sites combined (Figure 5). Using the iteratively produced species accumulation curves, based on subsetting our camera trap data, a median of 99 (95% CI: 78–133) trap nights was required to reach 12 species, equivalent to 6.2 days of actual trapping using 16 cameras.

The best binomial model from our candidate set for predicting amplification success of non-human mammal DNA was model 4, which included a random effect for species. The length parameter in this model overlapped zero (Table 1, Figure 6). The model that included length but did not account for species did have a significant, non-zero parameter for leech length.

Total monetary costs for collecting and analysing 200 leeches using Sanger sequencing was $3,770 (Table 3). We estimated the costs of collecting and analysing 2,600 leeches using Sanger sequencing to be $35,975 and using NGS to be $5,770. Total monetary costs for collecting and analysing 1,334 nights of camera trapping

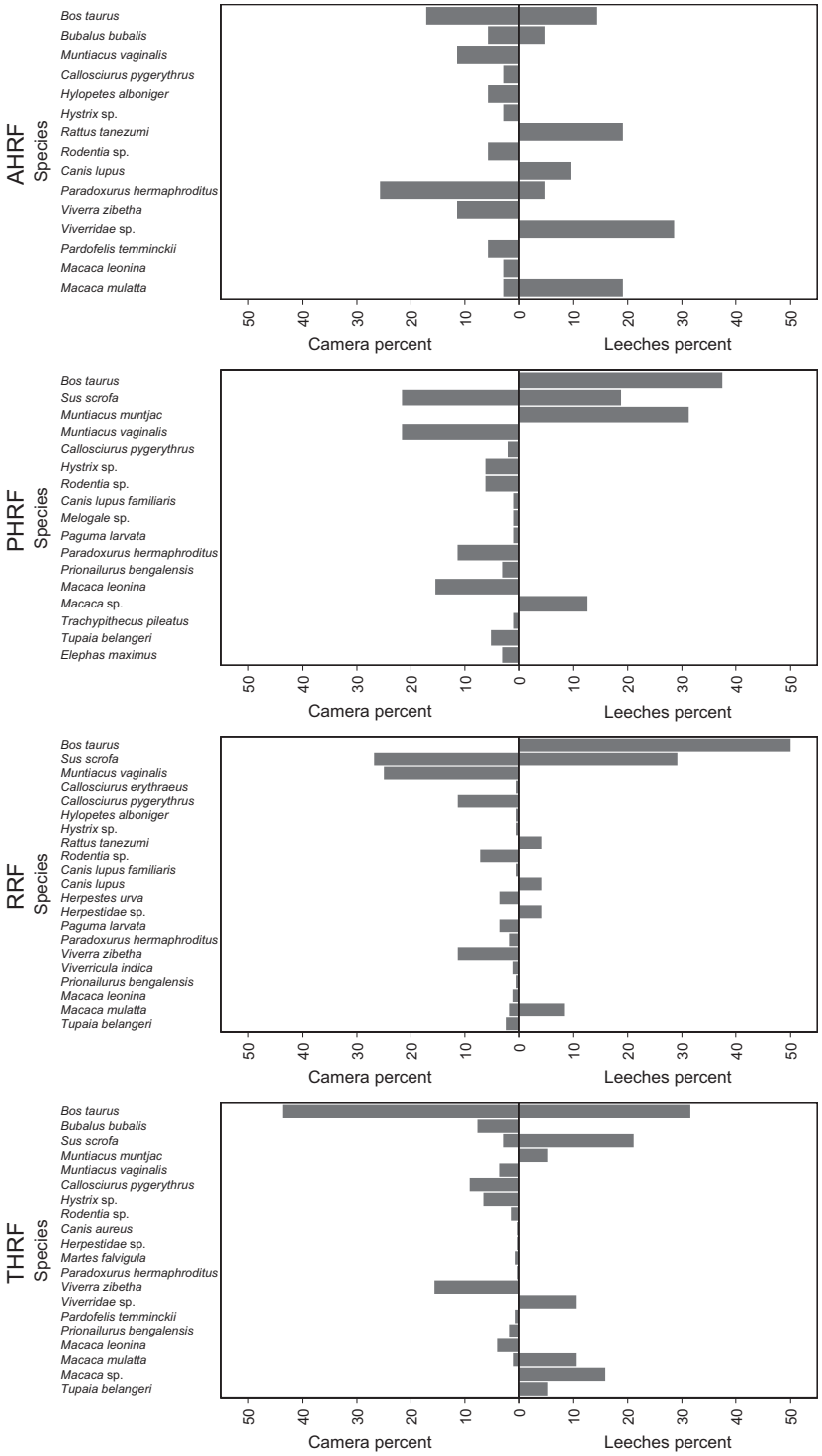

FIGURE 4 Non-human mammalian species composition found using camera trapping (left) and leech blood meals (right) at four sites in northeast Bangladesh (AHRF, Atora Hill Reserve Forest; PHRF, Patharia Hill Reserve Forest; RRF, Rajkandi Reserve Forest; THRF, Tarap Hill Reserve Forest)

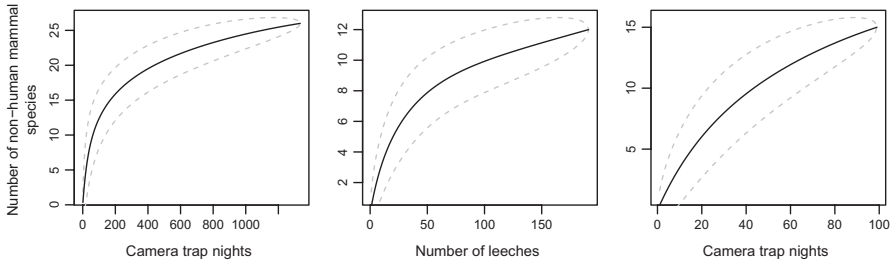

FIGURE 5 Left: Species accumulation curve constructed from 1,334 camera trap nights. Middle: Species accumulation curve constructed using 191 sequenced leeches. Right: Species accumulation curve constructed from 99 randomly selected camera trap nights from 16 randomly selected cameras. All data collected from four forest patches in Bangladesh

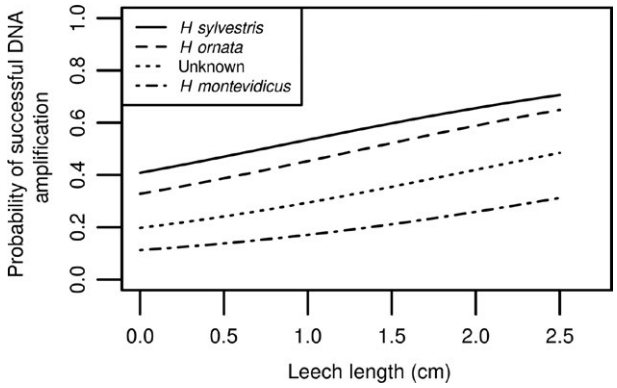

FIGURE 6 Predicted probability of successful non-human mammalian DNA amplification as a function of leech length and species. Predictions were based on the output of Model 4 described above

were $24,800 (Table 3). Time estimates for collecting and analysing 200 leeches and 99 camera trap nights were comparable, except for time differences required to obtain permits (3 months for leeches vs. 1 day for camera trapping).

4 | DISCUSSION

We collected leeches in Bangladesh after peak rainy season. While leeches were still prevalent and easy to collect, leeches may be a more efficient sampling method if field work is conducted during the rainy season. Even so, we found 12 mammal species in only 4 days of leech collection. Our collection points were also fairly close together and occurred in a single day in each patch. Increasing sample size, spatial range and/or collection time of leeches could improve results, as our species accumulation curve and total species richness estimate were similar to those for our subset of 99 camera trap nights (Figure 5, Table 2).

In addition to the mammal species reported above, 10.5% (N = 20) of leeches contained human DNA. We suspect that a large portion of these leeches fed on humans, because precautions were taken in the laboratory and field to prevent contamination, and humans were the most frequently captured species on the camera traps; however, we are unable to quantify potential contamination. Leeches may be a better sampling method in lower human density areas where leeches are less likely to feed on humans. Similarly, a large percent (34.6%) of our mammal records were cows, again an artefact of high human presence in the area. Readers should be cautious when interpreting results from cows and pigs, as they can be common contaminants (Leonard et al., 2007). While the presence/absence of pigs corresponded at all sites, cows did not, although leech collection and camera trapping were not conducted simultaneously. While we did not run PCR blanks, the lack of marine mammals (the only other mammals previously extracted in the laboratory) in our results and the fact that nearly half of our samples did not amplify give us greater confidence that our results are not due to contamination. The lack of amplification was not due to PCR inhibition or lack of quality DNA extractions, because we had nearly 100% amplification success for the leech COI gene from the same extractions.

Like camera traps, leeches may be biased towards ground-dwelling species, as we did not identify arboreal species in the blood meals (see also Tessler et al., 2018). We also did not find any felid species in the blood meals, but we did catch Asiatic golden cats *Pardofelis temminckii* and leopard cat *Prionailurus bengalensis* on the cameras. Conversely, we had a greater frequency of Rhesus macaques *Macaca mulatta* in the leeches than the cameras. We were also able to identify a rodent, *Rattus tanezumi*, which we did not identify on the cameras. Leeches may therefore improve identification success of small mammals, at least with a camera setup similar to ours. Overall, both camera traps and leeches were able to capture species with a range of sizes and life-history traits. Leeches may also be useful for identifying rare species. Using DNA analysis of leech blood meals, Schnell et al. (2012) confirmed the presence of the Annamite striped rabbit *Nesolagus timminsi*, which had never been caught in over 2,000 nights of camera trapping.

Further knowledge about life-history characteristics of leeches, such as host preferences and activity patterns, may improve our understanding of biases associated with the technique (Schnell et al., 2015; Tessler et al., 2018). Based on our top model, leech species may impact amplification success of non-human mammalian DNA, although our sample size for some species was small and we did not account for possible differences in amplification rate between patches. This model did not have a significant length parameter, but the model that included

TABLE 3 Cost breakdown of camera trapping (based on costs of a camera trap survey conducted 2014–2015) vs. the collection and blood meal sequencing of 191 leeches (based on work conducted 2015) vs. the estimated costs of Sanger sequencing and NGS of 2,600 leeches in four forest patches in northeast Bangladesh

Item	Cost for 10 months of camera trapping (USD)	Cost for 200 leeches (USD)	Estimated costs of Sanger sequencing 2,600 leeches (USD)	Estimated costs of NGS 2,600 leeches (USD)
Field assistant salary	2,700	200	990.00	990.00
Field guide salary	1,000	40	200	200
Travel	4,250	110	110	110
Lodging	1,000	80	400	400
Food	4,500	75	370	370
Miscellaneous field costs	1,170	400	600	600
30 Camera traps	6,000	NA	NA	NA
Taxes on supplies	3,000	305	305	305
Theft proof box	680	NA	NA	NA
Batteries	500	NA	NA	NA
Test tubes	NA	55	500	215
Sanger sequencing (includes PCR costs)	NA	1,890	26,500	NA
DNA extraction	NA	615	6,000	1,500[a]
Illumina MiSeq library prep for 4 samples	NA	NA	NA	80
Illumina MiSeq analysis	NA	NA	NA	1,000
Total	24,800	3,770	35,975	5,770

[a]This cost was estimated for pooling four leeches per extraction; number of leeches per pool probably depends on technique used.

only length did, possibly because leech species may vary in size. It may be difficult to identify leech species in the field, and while leech species vary around the world, collecting larger leeches may improve amplification success rate.

Overall, our non-human mammal amplification success rate was lower than that of Schnell et al. (2012, 41.9% compared to 84%). This may be due to different amplification techniques. AmpliTaq Gold, used by Schnell et al. (2012), has been found to be more specific and enhance PCR yields (Moretti, Koons, & Budowle, 1998). Another possibility is that our fragment sequence was longer than Schnell et al. (2012). While a smaller fragment might more readily amplify, it has reduced discrimination of amplicons. Additionally, leeches may have fed on non-mammalian prey. Our amplification success was also lower than studies of other haematophagous insect species (Gariepy, Lindsay, Ogden, & Gregory, 2012; Townzen, Brower, & Judd, 2008). Leeches can go several months without feeding, thus extracted DNA may be lower quality than DNA from an insect that has fed recently. It is also possible that collection time could affect amplification success rate, as leeches may be more likely to feed at different points in the rainy season. We do not think that our lower amplification success rate is due to the primers not working on prey species, as we used generic mammal primers.

We sequenced blood meals of individual leeches to compare against camera trap data. In the future, NGS will likely be the most common way to sequence blood meals. Pooling leeches may improve efficiency of sequencing and decrease costs of larger sample sizes dramatically (Schnell et al., 2015). NGS would also improve identification success if leeches contain multiple blood meals, as it can differentiate trace amounts of multiple DNA sequences, while the presence of multiple species' DNA in Sanger sequencing can create background noise and give low quality sequences (Logue et al., 2016). Also, species identification is limited by the databases with which sequences are compared. Missing species or inaccurate sequences in the database can lead to blood meal misidentification (Kent, 2009). In our study, two blood meal sequences most closely matched species that do not occur in Bangladesh. These false positives are a result of poor database coverage, which will continue to be a challenge for sampling tropical species until missing sequences are added to GenBank. As the goal of this study was to test the method rather than definitively determine biodiversity in our study area, we did not take additional steps to identify unknown species. Future studies should sequence multiple genes and obtain reference DNA from museum specimens to build the database to improve confidence in species identification. For several blood meals, the best BLAST match was *Muntiacus muntjac*. It is possible that these leeches fed on *M. vaginalis*, which was only recently upgraded from a subspecies of *M. muntjac* and does not have a sequence in GenBank (Groves, 2003). We also had a BLAST match with grey wolf *Canis lupus*. This is likely domestic dog *Canis lupus familiaris*, as grey wolves are

unlikely to occur in Bangladesh and it is impossible to differentiate between the subspecies using these methods.

Leeches may also be useful for other types of wildlife studies. Research on haematophagous insects suggests it may be possible to use leech blood meals to learn other useful information about host species, e.g. animal age, using gene expression (Kent, 2009), population estimates, using individual identification (Burkett-Cadena et al., 2010; Darbro, Dhondt, Vermeylen, & Harrington, 2007; Ligon et al., 2009; Martínez-de la Puente et al., 2015) or disease prevalence, using host immunoglobulins or viral or microbial DNA in blood meals (Baskova & Zavalova, 2001; Jasinskas, Jaworski, & Barbour, 2000; Wickramasekara, Bunikis, Wysocki, & Barbour, 2008).

Ultimately, both camera traps and leeches have benefits and drawbacks. Using leeches to sample biodiversity was cheaper but slightly less efficient to sample 12 non-human mammal species in the same number of collection locations. Most of the difference in time costs was spent obtaining an export permit for the leeches. Permit regulations are highly variable by country, and can affect the feasibility of using leeches. Anecdotally, we attempted to perform the same study in Sumatra, Indonesia, but were unable to obtain export permits. Conversely, it was possible to conduct camera trapping in that study site.

Collecting 200 leeches proved insufficient to sample biodiversity in our study area. However, we estimate that increasing the sample size to 2,600 leeches may provide equivalent results to 1,334 camera trap nights, although this remains to be tested. This would require only a slight increase in field effort as leeches were readily available in large numbers throughout each forest patch. Camera traps require several weeks to set up, months in the field to obtain sufficient data, and risk theft and malfunction. In contrast, leeches can be used to rapidly survey an area (although researchers should consider the ecological impact of over-collecting leeches). Using NGS on this increased sample size may save time and money, dependent on the desired spatial resolution (i.e. how data are partitioned into pools for analysis; Schnell et al., 2015). Ultimately, cost and effort of using leeches to monitor biodiversity will be context-specific, but could be less expensive and labour intensive. One drawback to the method is that leech blood meal analysis requires access to a laboratory and highly trained personnel with knowledge of degraded DNA analysis and species identification, whereas camera traps are easier to operate.

Monitoring programmes are desperately needed in the tropics, where biodiversity and threats to biodiversity are high and data are limited (Burton, 2012). In order to be effective and sustainable in the long term, monitoring programmes must be supported financially, politically and logistically (Lindenmayer, 1999). Using leech blood meals to monitor biodiversity is potentially cheaper and more efficient than camera trapping for large sample sizes. Even with as few as 200 leeches, we extended the species list from an extensive camera trapping effort. However, further studies are needed to determine whether larger leech sample sizes can detect comparable levels of species richness as camera trapping and what biases may be associated with the method. In this study, we presented some of the cost-performance trade-offs of camera trapping and leech blood meal sequencing, allowing managers to make more informed decisions about which technique to utilize. Camera trapping and other survey methods require independent corroboration, which can be provided rapidly and non-invasively by leech blood meal analysis. Managers should consider using the two methods together to improve the efficiency and capacity of monitoring programmes. Sustainable monitoring programmes and improved understanding of species diversity will allow researchers to create more effective conservation plans.

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AUTHORS' CONTRIBUTIONS

S.R.W., K.P.M., M.T., J.L.M., R.H. and M.E.S. conceived ideas and designed methodology. H.R. and M.M.F. collected data. S.R.W., M.T. and R.H. sequenced leeches. S.R.W., M.T. and K.P.M. analysed data. S.R.W. and K.P.M. led writing of the manuscript. All authors contributed critically to drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data are available from the Dyrad Digital Repository https://doi.org/10.5061/dryad.6224p (Weiskopf et al., 2018). Leech vouchers are stored in the American Museum of Natural History Ambrose Monell Cryo Collection (https://research.amnh.org/amcc/).

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SUPPORTING INFORMATION

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